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Efficient asymmetric syntheses, determination of absolute configurations and biological activities of 1-[4,4-bis(4-fluorophenyl)butyl]-4-[2-hydroxy-3-(phenylamino)propyl]-piperazine as a novel potent dopamine uptake inhibitor in the central nervous system

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Abstract—An efficient asymmetric synthesis of the chiral *N*-(3-chloro-2-hydroxypropyl)anilines (**2a** and **2b**) was achieved through the regioselective ring-opening reaction of chiral epichlorohydrin with aniline. This was applied to an asymmetric synthesis of the enantiomers of 1-[4,4-bis(4-fluorophenyl)butyl]-4-[2-hydroxy-3-(phenylamino)propyl]piperazine **1** as a novel potent dopamine uptake inhibitor. Both enantiomers as trihydrochlorides, **4a**·3HCl and **4b**·3HCl, could be synthesized in good total yields and optical purities of 100% ee in three steps synthesis, respectively. The absolute configurations of **4a**·3HCl and **4b**·3HCl were determined using the modified Mosher's method with the related compounds, the intermediates (**2a** and **2b**) and the free bases (**4a** and **4b**). The analytical results indicated that **4a**·3HCl and **4b**·3HCl have the (*S*)- and (*R*)-configuration, respectively, and a series of reactions to provide them proceeded without the apparent influence on the stereochemistry at the chiral centers. In in vitro pharmacological evaluations, **4a**·3HCl and **4b**·3HCl showed potent dopamine transporter binding affinities, high dopamine, moderate serotonin, and weak norepinephrine uptake inhibitory activities, and **4a**·3HCl exhibited a more potent and selective dopamine uptake inhibition over the serotonin or norepinephrine uptake inhibition as compared with **4b**·3HCl. An ex vivo evaluation revealed that the oral administrations of both enantiomers at a dose of 30 mg/kg in rats displayed apparent dopamine uptake inhibitory activities and **4a**·3HCl had a stronger tendency to inhibit dopamine uptake compared with **4b**·3HCl.

1. Introduction

Dopamine (DA) is a neurotransmitter involved in many biological processes in the central nervous system (CNS) and the dopaminergic neurotransmission is terminated by a reuptake mechanism through the dopamine transporter (DAT) located in the dopaminergic nerve terminals.[1] Dopaminergic neurotransmission has been reported to be closely associated with the CNS disorders such as Parkinson's disease,[2] depression,[3] or cocaine abuse,[4,5] so the DAT has been suggested as one target for research into new treatments of these CNS disorders.

In our previous study,[6,7] we reported that novel diphenyl piperazine derivatives represented by 1-[4,4-bis(4-fluorophenyl)butyl]-4-[2-hydroxy-3-(phenylamino)propyl]piperazine **1** (Fig. 1) showed apparent binding affinities for

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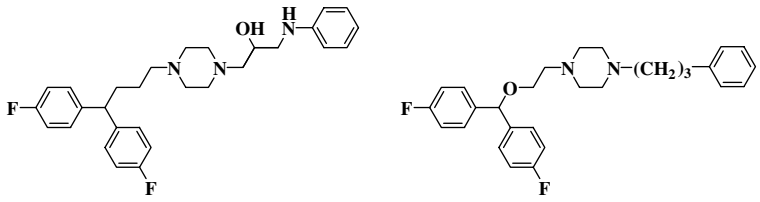


1 GBR12909

Figure 1.

the DAT in rat striatal membranes and some of them were approximately equivalent in activity to GBR12909[8] (Fig. 1) known as a potent DA uptake inhibitor. In addition, from a pharmacological study using in vivo brain microdialysis, compound **1** was found to increase extracellular DA levels in rat striatum significantly and dose dependently, which was much greater than that by GBR12909. These findings indicated that these novel diphenyl piperazine derivatives including compound **1** could serve as new DA uptake inhibitory molecules different from GBR12909 analogues. Moreover, it was suggested that these compounds might show great promise for the treatments of the CNS disorders caused by the depletion of DA or related to the DAT such as Parkinson's disease,[2] depression,[3] or cocaine addiction,[9] and also help to characterize biological and pharmacological profiles of the DAT.[10]

However, all of these compounds are racemates with respect to the asymmetric carbons in the β-amino-2-propanol moieties. Since it is generally accepted that enantiomers exhibit different biological properties,[11] it is very important and interesting to synthesize the enantiomers of these compounds, determine their absolute configurations, and compare their biological and pharmacological activities as a DA uptake inhibitor.

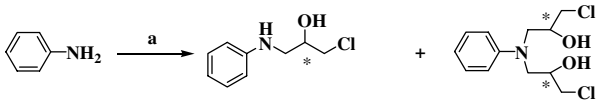
In this paper, we established an efficient asymmetric synthesis of the enantiomers of compound **1** as a representative of these novel diphenyl piperazine derivatives and determined their absolute configurations at the secondary alcohol moiety along with those of their important intermediates using the modified Mosher's method. As in vitro pharmacological evaluations, binding affinities for the DAT and uptake inhibitory activities for DA of both enantiomers were evaluated, and in order to evaluate the selectivity for DA uptake inhibition, their uptake inhibitory activities for serotonin (5-HT) and norepinephrine (NE) were also examined. Moreover, for a preliminary in vivo evaluation, ex vivo DA uptake inhibitory activities of both enantiomers were measured.

2. Results and discussion

2.1. Efficient synthesis of optically active 1-[4,4-bis(4-fluorophenyl)butyl]-4-[2-hydroxy-3-(phenylamino)propyl]piperazine

An efficient and scalable asymmetric synthesis of the enantiomers of compound **1** was required to obtain quantities sufficient for their biological and pharmacological studies. Since compound **1** has a β-phenylamino-2-propanol moiety containing an asymmetric carbon in its structure, it was considered that an efficient construction of this moiety in its optically active and pure form would play an important role in the asymmetric synthesis of the enantiomers of **1**. The ring opening of epoxides with amines is a simple and useful procedure for the synthesis of β-amino alcohols, and various procedures involving the direct reaction of epoxides with amines and the procedures using additives such as metal or metal catalyst have been reported.[12–18] Among them, we focused on the ring-opening reaction of epichlorohydrin with aniline for the efficient construction of the β-phenylamino-2-propanol moiety required for the subsequent reaction with 1-[4,4-bis(4-fluorophenyl)-butyl]piperazine, and selected commercially available chiral epichlorohydrin as the chiral synthon. However, all three carbons of epichlorohydrin have the potential to be attacked by amines depending on the reaction conditions, and this may cause a decrease in the yield and optical purity of the desirable chiral β-amino-2-propanol derivatives. Therefore, the ring-opening reaction of chiral epichlorohydrin with aniline was necessary to be regioselective and provide the optically pure product. So, we examined in detail this ring-opening reaction under neutral conditions without additives.

The ring-opening reaction of (*S*)-(+)-epichlorohydrin with aniline was undertaken under various reaction conditions (Scheme 1), and the results are shown in Table 1. The treatment of (*S*)-(+)-epichlorohydrin with 1.0 equiv of aniline in ethanol (EtOH) at room temperature for 65 h produced the desirable chiral *N*-(3-chloro-2-hydroxypropyl)aniline **2a** in a 54% yield and 97.3% ee (entry 1), together with *N*,*N*-bis(3-chloro-2-hydroxypropyl)aniline **3a** in a 7% yield as a major by-product that was separable by silica gel column chromatography.



2a : from (S)-epichlorohydrin **3a : from (S)-epichlorohydrin**
2b : from (R)-epichlorohydrin

Scheme 1. Reagents and conditions: (a) (*S*)-(+)- or (*R*)-(−)-epichlorohydrin, EtOH.

Table 1. Preparations of chiral *N*-(3-chloro-2-hydroxypropyl)anilines **2a** and **2b**

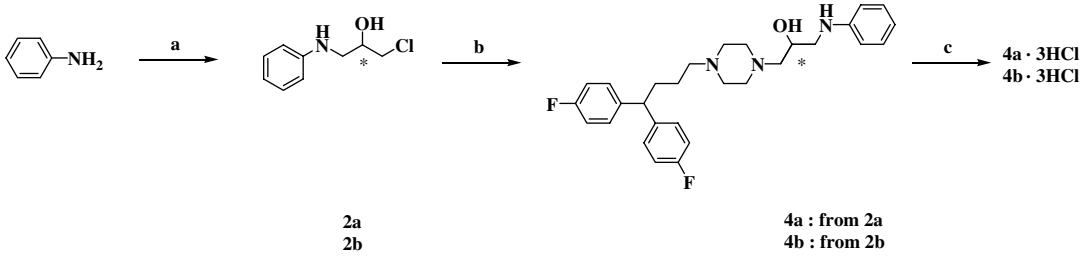
Entry	Epichlorohydrin (configuration)	Conditions				Product	Yield (%)	ee[a] (%)
		Aniline (equiv)	Solvent	Temp.	Time (h)			
1	(*S*)	1.0	EtOH	rt	65	**2a**	54	97.3
2	(*S*)	1.5	EtOH	rt	65	**2a**	60	97.8
3	(*S*)	2.0	EtOH	rt	65	**2a**	65	97.9
4	(*S*)	2.0	EtOH	40 °C	9	**2a**	72	96.9
5	(*S*)	2.0	EtOH	Reflux	2.5	**2a**	89	96.7
6	(*S*)	2.0	—	Reflux	6	**2a**	52	94.9
7	(*R*)	2.0	EtOH	Reflux	2.5	**2b**	89	96.9

[a] Enantiomeric excesses were determined by chiral HPLC analysis.

No remarkable decrease in the optical purity of **2a** was observed by chiral HPLC analysis, showing that the optical purity of **2a** was almost retained considering that of chiral epichlorohydrin (98.8% ee) as the starting material. To suppress the production of **3a** and increase that of **2a**, (*S*)-(+)-epichlorohydrin was treated with 1.5 or 2.0 equiv of aniline in EtOH at room temperature (entries 2 and 3). The use of excess aniline simultaneously induced an increase in **2a** production and suppression in **3a**, and the production of **3a** was reduced to a minimum (0.9% yield) when 2.0 equiv of aniline were used. Reaction temperature is closely related to reaction time and product yield and is an important factor for efficient synthesis. Therefore, we next examined the effect of the reaction temperature on this reaction (entries 4 and 5). The results showed that increasing the reaction temperature was effective in reducing the reaction time and increasing the yield of **2a** without the apparent influence on the regioselectivity and optical purity. Eventually, under reflux conditions, **2a** could be prepared in an 89% yield and 96.7% ee (entry 5). Since the ring-opening reaction of racemic epichlorohydrin with aniline in the absence of solvent has been reported,[12] a similar reaction using (*S*)-(+)-epichlorohydrin was attempted (entry 6). However, this reaction without solvent under heated conditions resulted in a significant decrease of the yield of **2a** with an increase of **3a** and a decrease of the optical purity of **2a** responsible for the production of **2b**. Using the same procedure as in entry 5, the ring-opening reaction of (*R*)-(−)-epichlorohydrin with aniline gave the other enantiomer **2b** in an 89% yield and 96.9% ee (entry 7). Thus, the above results indicate that the ring opening of chiral epichlorohydrin with aniline proceeded in a highly regioselective manner with high optical purity. Moreover, this ring-opening reaction of chiral epichlorohydrin was also found to be applicable to the asymmetric synthesis of β-amino- or β-thio-2-propanol derivatives using various aliphatic and aromatic amines or thiols (data not shown).

Next, we attempted to apply this regioselective ring-opening reaction of chiral epichlorohydrin with aniline to the asymmetric synthesis of both enantiomers of **1** (Scheme 2). As described above, the chiral *N*-(3-chloro-2-hydroxypropyl)anilines, **2a** and **2b**, as the important intermediates were prepared from chiral epichlorohydrins and aniline under reflux conditions in EtOH. The subsequent alkylation of 1-[4,4-bis(4-fluorophenyl)-butyl]piperazine with **2a** or **2b** in the presence of potassium carbonate and potassium iodide in EtOH under reflux gave the desirable diphenylbutyl piperazine derivatives **4a** or **4b** in 90% or 92% yield after purification by silica gel column chromatography, respectively, and both compounds showed an optical purity greater than 98.5% ee. These findings indicate that this alkylation proceeded in high yield and excellent optical purity, showing no occurrence of racemization in the β-amino-2-propanol moiety. Subsequently, the treatment of the resultant diphenyl piperazine derivatives **4a** and **4b** with hydrogen chloride in EtOH followed by recrystallization gave the corresponding trihydrochlorides, **4a**·3HCl and **4b**·3HCl, in high yields and optical purities of 100% ee, respectively.

Thus, the total yields of **4a**·3HCl and **4b**·3HCl after three steps synthesis starting from chiral epichlorohydrin were 66% and 77%, respectively. Furthermore, the efficient procedure to provide **4a**·3HCl and **4b**·3HCl established herein has also been found to be applicable to their asymmetric syntheses on a kilogram scale by a minor modification (data not shown). Both enantiomers of **1**



Scheme 2. Reagents and conditions: (a) (*S*)-(+)- or (*R*)-(−)-epichlorohydrin, EtOH, reflux, 89%; (b) 1-[4,4-bis(4-fluorophenyl)butyl]piperazine, K_2CO_3, KI, EtOH, reflux 90–92%; (c) 7.0 N HCl in EtOH, 82–94%.

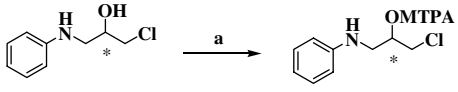
obtained as trihydrochlorides, **4a**·3HCl and **4b**·3HCl, were used for the pharmacological evaluations.

2.2. Determination of absolute configuration using the modified Mosher's method

The absolute configurations of organic compounds can be determined by a few direct physical methods including X-ray crystallography, circular dichroism, or optical rotatory dispersion, but they have some limitations. On the other hand, there are popular indirect chemical methods such as Mosher's method, which can also predict the absolute configurations of organic substances. In 1973, Dale and Mosher proposed that a method using 2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) esters[19] could be used to determine the absolute configurations of many chiral alcohols and amines with various auxiliaries.[20,21] The robustness of the method was demonstrated by modifying the original Mosher's method to expand the range of adaptive compounds that allowed for the absolute configuration determination of many different organic compounds including natural products.[22–24]

The absolute configurations of four compounds, the important intermediates (**2a** and **2b**) and the free bases (**4a** and **4b**), containing the β-amino-2-propanol moiety were determined using the modified Mosher's method to elucidate those of **4a**·3HCl and **4b**·3HCl. The basic concept of Mosher's method as well as a configurational correlation model based on the method for elucidating the absolute configuration at the secondary carbinol stereogenic center are shown in Figure 2.[19,22] The (*R*)- or (*S*)-MTPA esters **5**–**12** were prepared from the corresponding secondary alcohols, **2a**, **2b**, **4a**, and **4b**, and (*S*)- or (*R*)-MTPA chloride in the presence of *N*,*N*-dimethylaminopyridine and triethylamine in dichloromethane (Schemes 3 and 4).[22,23] The ^{1}H NMR spectra obtained from the MTPA esters could be registered, and the chemical shifts for as many protons as possible with respect to the obtained MTPA esters could be assigned. The chemical shift differences ($\Delta\delta = \delta_S - \delta_R$) are calculated and summarized in Figures 3 and 4.

With respect to the chemical shift differences obtained from the **2a** MTPA esters, **5** and **6**, the $\Delta\delta$ values for the methylene protons adjacent to the chlorine atom located on the right side of the MTPA plane were all positive, while those for methylene protons adjacent to the aniline moiety and aromatic protons at the *ortho*-position located on the left side of the MTPA plane were all negative. When these results are applied to the configurational correlation model in Figure 2, **2a** is shown to have the (*S*)-configuration at the secondary carbinol stereogenic center. For the chemical shift differences



2a
2b

5 : from 2a and (R)-MTPA chloride
6 : from 2a and (S)-MTPA chloride
7 : from 2b and (R)-MTPA chloride
8 : from 2b and (S)-MTPA chloride

Scheme 3. Reagents and conditions: (a) (*S*)- or (*R*)-MTPA chloride, DMAP, Et_3N, CH_2Cl_2, room temperature, 90–94%.

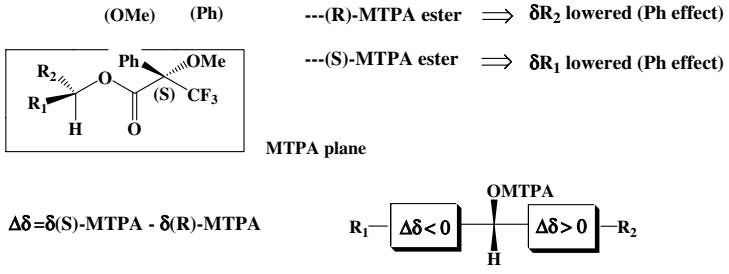
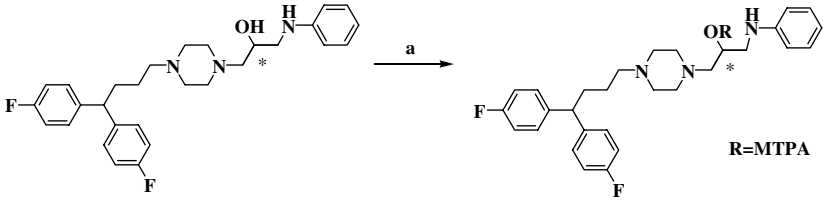


Figure 2. Preferred conformation for MTPA esters proposed by Mosher and a model to determine the absolute configuration of secondary alcohols.



4a
4b

9 : from 4a and (R)-MTPA chloride
10 : from 4a and (S)-MTPA chloride
11 : from 4b and (R)-MTPA chloride
12 : from 4b and (S)-MTPA chloride

Scheme 4. Reagents and conditions: (a) (*S*)- or (*R*)-MTPA chloride, DMAP, Et_3N, CH_2Cl_2, room temperature, 89–94%.

obtained from the **2b** MTPA esters, **7** and **8**, the signs of the $\Delta\delta$ values on both sides of the MTPA plane were opposite to those of the **2a** MTPA esters indicating that the absolute configuration of **2b** is the (*R*)-configuration.

In the case of the **4a** MTPA esters, **9** and **10**, the $\Delta\delta$ values for the methylene protons, amine proton, and aromatic protons at the *ortho*-position on the aniline moiety side located on the left side of the MTPA plane were all negative. On the other hand, the $\Delta\delta$ value for one methylene proton on the piperazine moiety side located on the right side of the MTPA plane was positive, and that for another methylene proton was almost zero, possibly because it was located near the MTPA plane. For the **4b** MTPA esters, **11** and **12**, the contrary chemical shift differences to those of the MTPA esters of **4a** were observed. These findings indicate that **4a** and **4b** have the (*S*)-configuration and (*R*)-configuration, respectively, and the final compounds, **4a**·3HCl and **4b**·3HCl, also have these corresponding configurations.

From the results described above, it is indicated that a series of reactions to provide **4a**·3HCl and **4b**·3HCl starting from the ring opening of chiral epichlorohydrin with aniline proceeded without the apparent influence on the stereochemistry at the chiral centers.

2.3. DAT binding studies

Both enantiomers, **4a**·3HCl and **4b**·3HCl, were evaluated for DAT binding affinity using [^{3}H]GBR12935 in rat striatal membranes,[25] and the results are shown in Table 2. Both compounds showed the same potent DAT binding affinities with IC_{50} values of 2.00 nM, which were equivalent in potency to the racemate **1** as a trihydrochloride. These results indicate that their absolute configurations have little influence on the binding affinity for the GBR12935 binding site at the DAT.

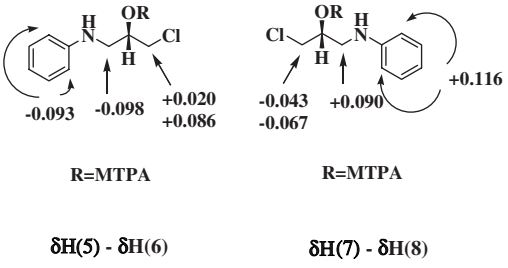


Figure 3. [1]H NMR chemical shift differences between (*S*)- and (*R*)-MTPA esters **5**, **6**, **7**, and **8** derived from **2a** and **2b**, respectively, expressed in ppm.

2.4. Uptake inhibitory activities for monoamines, DA, 5-HT, and NE

As described in the DAT binding study, both enantiomers possessed equipotent binding affinities for the DAT. Subsequently, to examine the difference in DA uptake inhibition, DA uptake inhibitory activities of **4a**·3HCl and **4b**·3HCl were measured using radiolabeled DA in rat striatal homogenates.[26] In addition, to evaluate the selectivity for DA uptake inhibition relative to other monoamine uptake inhibitions, 5-HT and NE uptake inhibitory activities with them were measured using the corresponding radiolabeled monoamines in rat cerebral cortex homogenates.[26] The results of these uptake inhibitory activities for DA, 5-HT, and NE are shown in Table 2.

With respect to the DA uptake inhibitory activity, both enantiomers, **4a**·3HCl and **4b**·3HCl, showed potent activities with IC_{50} values of 2.6 and 6.7 nM, respectively. Thus, both enantiomers showed distinct DA uptake inhibitory activities and **4a**·3HCl with the (*S*)-configuration was 2.6-fold more active than **4b**·3HCl with the (*R*)-configuration. These results indicate that their absolute configurations apparently influence the DA uptake inhibitory activity, although both compounds show equipotent binding affinities for the DAT. Since [^{3}H]GBR12935 has been known to bind to a piperazine acceptor site in addition to the DAT,[27] the difference in the results between both enantiomers observed in the DAT binding affinity and DA uptake inhibitory activity may be due to the different assay conditions with the radioligands having the different character.

Concerning the 5-HT uptake inhibitory activity, **4a**·3HCl and **4b**·3HCl showed moderate activities with IC_{50} values of 214.5 and 481.0 nM, respectively.

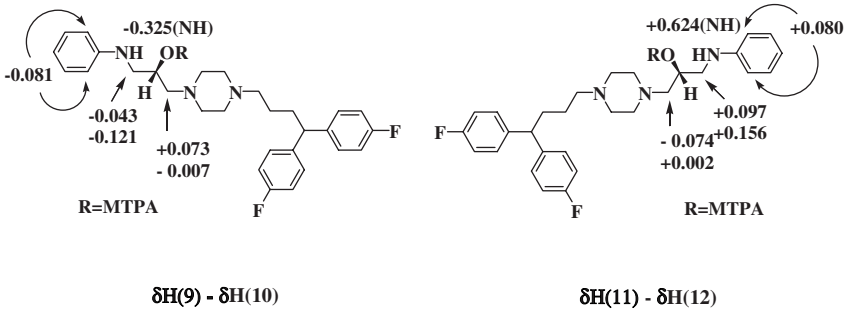


Figure 4. [1]H NMR chemical shift differences between (*S*)- and (*R*)-MTPA esters **9**, **10**, **11**, and **12** derived from **4a** and **4b**, respectively, expressed in ppm.

Table 2. DAT binding affinities and monoamine uptake inhibitory activities of the racemate and the enantiomers

Compd	Configuration	DAT binding affinity IC_{50} (nM)[a]	Monoamine uptake inhibitory activity IC_{50} (nM)[a]		
			[^{3}H]DA	[^{3}H]5-HT	[^{3}H]NE
1a·3HCl	(*R*,*S*)	2.00 ± 0.29	NT[b]	NT[b]	NT[b]
4a·3HCl	(*S*)	2.00 ± 0.10	2.6 ± 1.0	214.5 ± 10.8	1817.0 ± 883.9
4b·3HCl	(*R*)	2.00 ± 0.53	6.7 ± 2.7	481.0 ± 193.0	2058.4 ± 240.4

[a] IC_{50} values represent the concentrations inhibiting 50% of specific bindings and were calculated by nonlinear regression fitting. Each value represents the mean ± SE from three experiments conducted in duplicate.
[b] Not tested.

Compound **4a**·3HCl possessed an approximately 2.2-fold more potent 5-HT uptake inhibitory activity than **4b**·3HCl, which shows that their absolute configurations have an apparent influence on potency. In comparison between their DA and 5-HT uptake inhibitions, the selectivities for DA to 5-HT of **4a**·3HCl and **4b**·3HCl were 83- and 72-fold, respectively, showing that **4a**·3HCl has a more selective uptake inhibitory activity for DA over 5-HT compared with **4b**·3HCl.

With respect to the NE uptake inhibitory activity, **4a**·3HCl and **4b**·3HCl possessed weak activities with IC_{50} values of 1817.0 and 2058.4 nM, respectively, and the difference in the NE uptake inhibition between both enantiomers was slight. On the other hand, the ratios of uptake inhibition for NE to DA with **4a**·3HCl and **4b**·3HCl were 699 and 307, respectively, showing that the differences in uptake inhibitory activities of both compounds between DA and NE were greater than two orders of magnitude. Again, **4a**·3HCl possessed an approximately 2.3-fold more selective DA uptake inhibitory activity as compared with **4b**·3HCl.

The above results for monoamine uptake inhibitory activity indicated that both enantiomers possessed high DA, moderate 5-HT, and weak NE uptake inhibitory activities. Compound **4a**·3HCl was more potent in all three monoamine uptake inhibitory activities compared with **4b**·3HCl. Furthermore, **4a**·3HCl exhibited an approximately 80- or 700-fold selective DA uptake inhibitory activity over the 5-HT or NE uptake inhibitory activity, and these selectivities were greater compared with 70- or 300-fold selectivity of **4b**·3HCl. Thus, their absolute configurations were shown to have an apparent tendency to influence the DA, 5-HT, and NE uptake inhibitory activities with the selectivity for DA uptake inhibition. These results indicate that **4a**·3HCl with the (*S*)-configuration is preferable over **4b**·3HCl with the (*R*)-configuration for the DA uptake inhibitory activity and selectivity, which may be very useful for obtaining knowledge about the structure and biological and pharmacological profiles of the DAT. Furthermore, from an in vitro selectivity study by means of other 49 neurotransmitter receptors, ion channels, and transporters, **4a**·3HCl has also been found to display the highest affinity and selectivity for the DAT (data not shown).

2.5. Ex vivo DA uptake inhibitory activities

As described in the in vitro study, both enantiomers, **4a**·3HCl and **4b**·3HCl, exhibited notable binding affinities for the DAT, and potent and selective DA uptake inhibitory activities. Then, for a preliminary in vivo evaluation, we attempted to examine and compare the inhibition of DA uptake by both enantiomers in an ex vivo study. The changes in DA uptake inhibition in rat striatum for 6 h after the oral administrations of **4a**·3HCl and **4b**·3HCl at a dose of 30 mg/kg were measured, and the results are shown in Figure 5.

The oral administrations of **4a**·3HCl and **4b**·3HCl induced eminent DA uptake inhibitions. The inhibitions by both enantiomers reached maximum levels quickly, and maintained high inhibition levels for 6 h, although their activities gradually decreased. Compound **4a**·3HCl had a stronger tendency to inhibit DA uptake than **4b**·3HCl, exhibiting a significant difference at 2 and 3 h after following administrations, which may reflect their observed in vitro DA uptake inhibitory activities. However, the difference in the ex vivo DA uptake inhibitory activity between both compounds was not large compared with that in the in vitro DA uptake inhibitory activity, which appeared due to the differences in their pharmacokinetic properties such as absorption, metabolism, protein binding, or distribution through blood–brain barrier.

Thus, the above results combined with the in vitro DA uptake inhibitory activity and selectivity indicate that

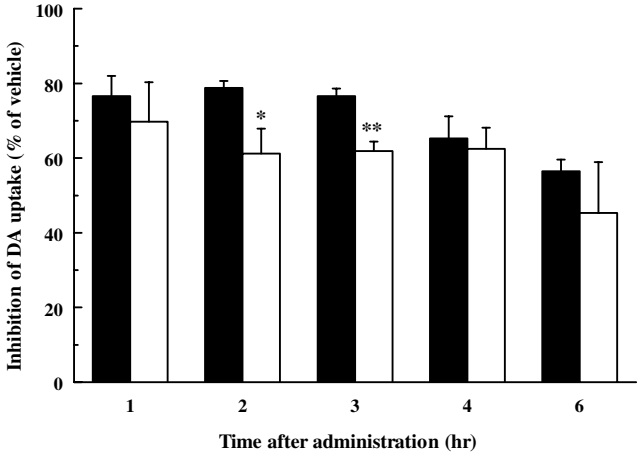


Figure 5. Effects of **4a**·3HCl and **4b**·3HCl on the DA uptake in the rat striatal synaptosomes ex vivo. Compound **4a**·3HCl (■: 30 mg/kg), **4b**·3HCl (□: 30 mg/kg), or vehicle (saline) was administered orally, and the uptake of 100 nM [^{3}H]DA was measured for 5 min. The results are expressed as % inhibition of specific uptake in vehicle control. Each value represents the mean with SEM of four rats. *$p < 0.05$, **$p < 0.01$: significantly different from **4a**·3HCl (*t*-test).

both enantiomers are potent and selective DA uptake inhibitors in the CNS.

3. Conclusion

We established an efficient asymmetric synthesis of the chiral *N*-(3-chloro-2-hydoroxypropyl)anilines (**2a** and **2b**) through the regioselective ring-opening reaction of chiral epichlorohydrin with aniline and applied this reaction to an asymmetric synthesis of the enantiomers of **1** as a novel potent dopamine uptake inhibitor. Both enantiomers of **1** as trihydrochlorides, **4a**·3HCl and **4b**·3HCl, could be synthesized in good total yields and optical purities of 100% ee in three steps synthesis, respectively. The absolute configurations of **4a**·3HCl and **4b**·3HCl were determined using the modified Mosher's method with the related compounds, the intermediates (**2a** and **2b**) and the free bases (**4a** and **4b**). The analytical results indicated that **4a**·3HCl and **4b**·3HCl have the (*S*)- and (*R*)-configuration, respectively, and a series of reactions to provide them proceeded without the apparent influence on the stereochemistry at the chiral centers. In in vitro pharmacological evaluations, both enantiomers possessed high DA uptake inhibitory activities and **4a**·3HCl exhibited a 2.6-fold more potent DA uptake inhibitory activity compared with **4b**·3HCl, although both compounds showed equipotent DAT binding affinities. In comparison between their DA, 5-HT, and NE uptake inhibitory activities, both enantiomers showed notably selective DA uptake inhibitions over the 5-HT or NE uptake inhibition, and **4a**·3HCl was more selective than **4b**·3HCl. In an ex vivo evaluation, the oral administrations of both enantiomers at a dose of 30 mg/kg in rats displayed apparent DA uptake inhibitions, with **4a**·3HCl having a stronger tendency to inhibit DA uptake compared with **4b**·3HCl. Thus, all the above data suggest that **4a**·3HCl and **4b**·3HCl have excellent potential as effective DA uptake inhibitors for the treatment of diseases in the CNS such as Parkinson's disease, depression, or cocaine addiction, and also useful tools for the characterization of the structure and biological and pharmacological profiles of the DAT. And now, we conduct these studies using animal models.

4. Experimental

All melting points were determined using a Büchi micromelting point apparatus without correction. IR spectra were measured with a Nicolet FT-IR 205 spectrometer. ^{1}H NMR spectra were recorded on a JEOL GSX spectrometer (270 MHz). Chemical shifts were reported in ppm (δ) values, based on tetramethylsilane as an internal standard. The following abbreviations were used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, dt = double triplet, br s = broad singlet. MS spectra were recorded using a JEOL SX-102 mass spectrometer. Optical rotations were measured on a JASCO DIP-370 apparatus. Elemental analyses were performed by Yanaco CHN CORDER MT-5 (C, H, N) and Flask Combustion (Cl). Column chromatography were performed on silica gel (BW-200, Fuji Silisia Chemical, Ltd, 100–200 mesh).

4.1. Determination of optical purity

HPLC analyses on optical purity of the intermediates, **2a** and **2b**, the free bases, **4a** and **4b**, and their corresponding trihydrochlorides, **4a**·3HCl and **4b**·3HCl, were performed on a Daisel Chiralcel OD-R column (4.6 mm i.d. × 250 mm) using a JASCO system equipped with a UV detector [eluent (0.5 M $NaClO_4$, $HClO_4$: pH 2.0)/CH_3CN = 70:30; flow rate, 0.8 mL/min; UV at 245 nm; temperature, 24 °C]. Their optical purities were calculated based on the peak area ratios of (*R*)- and (*S*)-isomers.

4.1.1. (*S*)-(−)-*N*-(3-Chloro-2-hydroxypropyl)aniline (2a) and (*S*)-*N*,*N*-bis(3-chloro-2-hydroxypropyl)aniline (3a). These compounds were prepared under various conditions as described in Table 1. Herein, the preparation under an optimum condition (entry 5) is described. A mixture of (*S*)-(+)-epichlorohydrin (1.92 g, 20.8 mmol) and aniline (3.87 g, 41.6 mmol) in EtOH (21 mL) was heated under reflux for 2.5 h. The mixture was cooled to room temperature and concentrated in vacuo. The residue was separated by silica gel column chromatography ($CHCl_3$ as an eluent) to give **2a** (3.43 g, 89%, 96.7% ee) as white crystals and **3a** (0.05 g, 0.9%) as a colorless oil.

Compound **2a**. Mp 35–37 °C. IR (KBr) cm^{-1}: 3264, 1606, 1242. ^{1}H NMR ($CDCl_3$) δ 3.24 (1H, dd, J = 7.8, 14.0 Hz), 3.39 (1H, dd, J = 7.8, 14.0 Hz), 3.62–3.72 (2H, m), 3.98–4.11 (1H, m), 6.64 (2H, d, J = 7.8 Hz), 6.73 (1H, t, J = 7.8 Hz), 7.16 (2H, t, J = 7.8 Hz). HRFAB-MS calcd for $C_9H_{13}ClNO$ $[M+H]^+$: 186.0686. Found: 186.0684. $[\alpha]_D^{25}$ −11.0 (c 1.01, $CHCl_3$).

Compound **3a**. ^{1}H NMR ($CDCl_3$) δ 3.24–3.42 (4H, m), 3.63–3.79 (4H, m), 3.98–4.13 (2H, m), 6.61–7.24 (5H, m). FAB-MS m/z 278 $[M+H]^+$.

4.1.2. (*R*)-(+)-*N*-(3-Chloro-2-hydroxypropyl)aniline (2b). This compound was prepared from (*R*)-(−)-epichlorohydrin and aniline in 89% yield and 96.9% ee according to the procedure described for **2a**. White crystals. Mp 35–37 °C. IR (KBr) cm^{-1}: 3264, 1606, 1242. ^{1}H NMR ($CDCl_3$) δ 3.24 (1H, dd, J = 7.8, 14.0 Hz), 3.39 (1H, dd, J = 7.8, 14.0 Hz), 3.62–3.72 (2H, m), 3.98–4.11 (1H, m), 6.64 (2H, d, J = 7.8 Hz), 6.73 (1H, t, J = 7.8 Hz), 7.16 (2H, t, J = 7.8 Hz). HRFAB-MS calcd for $C_9H_{13}ClNO_3$ $[M+H]^+$: 186.0686. Found: 186.0682. $[\alpha]_D^{25}$ +10.0 (c 0.98, $CHCl_3$).

4.1.3. (*S*)-(+)-1-[4,4-Bis(4-fluorophenyl)butyl]-4-[2-hydroxy-3-(phenylamino)propyl]piperazine (4a). A mixture of **2a** (1.00 g, 5.39 mmol), 1-[4,4-bis(4-fluorophenyl)butyl]piperazine (1.78 g, 5.39 mmol), potassium

carbonate (0.89 g, 6.44 mmol), and potassium iodide (0.09 g, 0.542 mmol) in EtOH (20 mL) was heated under reflux for 4 h. After removal of the insoluble materials by filtration, the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3/CH_3OH = 99:1$ as an eluent) to give **4a** (2.33 g, 90%, 98.7% ee) as white crystals. Mp 99–100 °C. IR (KBr) cm^{-1}: 3329, 1603, 1222. ^1H NMR ($CDCl_3$) δ 1.48–1.59 (2H, m), 1.98–2.10 (2H, m), 2.28–2.64 (10H, m), 2.65–2.72 (2H, m), 3.06 (1H, dd, $J = 5.9$, 12.9 Hz), 3.25 (1H, dd, $J = 4.1$, 12.9 Hz), 3.86 (1H, t, $J = 7.8$ Hz), 3.89–4.02 (1H, m), 6.63 (2H, d, $J = 7.6$ Hz), 6.92–7.00 (4H, m), 7.01 (1H, t, $J = 7.6$ Hz), 7.23–7.34 (6H, m). HRFAB-MS calcd for $C_{29}H_{36}F_2N_3O$ $[M+H]^+$: 480.2826. Found: 480.2830. $[\alpha]_D^{25}$ +12.2 (*c* 1.02, $CHCl_3$).

4.1.4. (*R*)-(−)-1-[4,4-Bis(4-fluorophenyl)butyl]-4-[2-hydroxy-3-(phenylamino)propyl]piperazine (4b). This compound was prepared from **2b** and 1-[4,4-bis(4-fluorophenyl)butyl]piperazine in 92% yield and 98.8% ee according to the procedure described for **4a**. White crystals. Mp 99–100 °C. IR (KBr) cm^{-1}: 3329, 1603, 1222. ^1H NMR ($CDCl_3$) δ 1.48–1.59 (2H, m), 1.98–2.10 (2H, m), 2.28–2.64 (10H, m), 2.65–2.72 (2H, m), 3.06 (1H, dd, $J = 5.9$, 12.9 Hz), 3.25 (1H, dd, $J = 4.1$, 12.9 Hz), 3.86 (1H, t, $J = 7.8$ Hz), 3.89–4.02 (1H, m), 6.63 (2H, d, $J = 7.6$ Hz), 6.92–7.00 (4H, m), 7.01 (1H, t, $J = 7.6$ Hz), 7.23–7.34 (6H, m). HRFAB-MS calcd for $C_{29}H_{36}F_2N_3O$ $[M+H]^+$: 480.2826. Found: 480.2835. $[\alpha]_D^{25}$ −12.5 (*c* 1.03, $CHCl_3$).

4.1.5. (*S*)-(−)-1-[4,4-Bis(4-fluorophenyl)butyl]-4-[2-hydroxy-3-(phenylamino)propyl]piperazine trihydrochloride (4a·3HCl). A solution of 7.0 N HCl/EtOH (2.0 mL) was added dropwise to a solution of **4a** (2.00 g, 4.17 mmol) in EtOH (10 mL) under ice bath cooling and the mixture was stirred at room temperature for 1 h. The resultant precipitates were collected by filtration and recrystallized from EtOH to give **4a**·3HCl (2.26 g, 82%, 100% ee) as white crystals. Mp 232–233 °C. IR (KBr) cm^{-1}: 3318, 1602, 1508, 1223. ^1H NMR (DMSO-d_6) δ 1.52–1.68 (2H, m), 2.03–2.09 (2H, m), 2.24–2.94 (12H, m), 3.11–3.32 (2H, m), 4.02 (1H, t, $J = 7.8$ Hz), 4.19–4.32 (1H, m), 6.83 (2H, d, $J = 7.3$ Hz), 7.09–7.34 (7H, m), 7.43–7.71 (4H, m). Anal. Calcd for $C_{29}H_{35}F_2N_3O \cdot 3HCl$: C, 59.14; H, 6.50; N, 7.13; Cl, 18.06. Found: C, 59.41; H, 6.55; N, 7.08; Cl, 17.99. $[\alpha]_D^{25}$ −7.6 (*c* 0.99, CH_3OH).

4.1.6. (*R*)-(+)-1-[4,4-Bis(4-fluorophenyl)butyl]-4-[2-hydroxy-3-(phenylamino)propyl]piperazine trihydrochloride (4b·3HCl). This compound was prepared from **4b** in 94% yield and 100% ee according to the procedure described for **4a**·3HCl. White crystals. Mp 232–233 °C. IR (KBr) cm^{-1}: 3318, 1602, 1508, 1223. ^1H NMR (DMSO-d_6) δ 1.52–1.68 (2H, m), 2.04–2.09 (2H, m), 2.24–2.94 (12H, m), 3.12–3.32 (2H, m), 4.02 (1H, t, $J = 7.8$ Hz), 4.19–4.32 (1H, m), 6.83 (2H, d, $J = 7.3$ Hz), 7.09–7.34 (7H, m), 7.43–7.71 (4H, m). Anal. Calcd for $C_{29}H_{35}F_2N_3O \cdot 3HCl$: C, 59.14; H, 6.50; N, 7.13; Cl, 18.06. Found: 59.21; H, 6.52; N, 7.11; Cl, 18.01. $[\alpha]_D^{25}$ +8.1 (*c* 1.02, CH_3OH).

4.2. General procedure for syntheses of MTPA esters[22,23]

A mixture of each secondary alcohol (**2a**, **2b**, **4a**, or **4b**), *N*,*N*-dimethylaminopyridine (1.2 equiv), triethylamine (3.0 equiv), and (*R*)- or (*S*)-MTPA chloride (1.2 equiv) in dichloromethane was stirred at room temperature under a nitrogen atmosphere. After removal of the solvent, the residue was purified by silica gel column chromatography ($CHCl_3/CH_3OH = 99:1$ as an eluent) to obtain the corresponding MTPA esters.

4.2.1. (*S*)-MTPA ester (5) from 2a and (*R*)-MTPA chloride. A colorless oil. Yield 93%. IR (KBr) cm^{-1}: 1752, 1604, 1510, 1170. ^1H NMR ($CDCl_3$) δ 3.44 (2H, d, $J = 5.9$ Hz), 3.63 (3H, s), 3.77 (1H, dd, $J = 5.4$, 12.4 Hz), 3.84 (1H, dd, $J = 4.3$, 12.4 Hz), 5.39–5.48 (1H, m), 6.54 (2H, d, $J = 7.6$ Hz), 6.76 (1H, t, $J = 7.3$ Hz), 7.18 (2H, t, $J = 7.3$ Hz), 7.37 (3H, d, $J = 7.3$ Hz), 7.56 (2H, d, $J = 7.8$ Hz). HRFAB-MS calcd for $C_{19}H_{20}ClF_3NO_3$ $[M+H]^+$: 402.1084. Found: 402.1087. $[\alpha]_D^{25}$ −68.0 (*c* 1.01, $CHCl_3$).

4.2.2. (*R*)-MTPA ester (6) from 2a and (*S*)-MTPA chloride. A colorless oil. Yield 94%. IR (KBr) cm^{-1}: 1752, 1604, 1510, 1170. ^1H NMR ($CDCl_3$) δ 3.52 (3H, s), 3.54 (2H, d, $J = 7.3$ Hz), 3.75 (2H, dd, $J = 1.4$, 5.1 Hz), 5.37–5.47 (1H, m), 6.64 (2H, d, $J = 7.8$ Hz), 6.77 (1H, t, $J = 7.3$ Hz), 7.20 (2H, t, $J = 7.3$ Hz), 7.39 (3H, d, $J = 7.3$ Hz), 7.54 (2H, d, $J = 7.8$ Hz). HRFAB-MS calcd for $C_{19}H_{20}ClF_3NO_3$ $[M+H]^+$: 402.1084. Found: 402.1092. $[\alpha]_D^{25}$ +5.3 (*c* 1.02, $CHCl_3$).

4.2.3. (*S*)-MTPA ester (7) from 2b and (*R*)-MTPA chloride. A colorless oil. Yield 90%. IR (KBr) cm^{-1}: 1750, 1604, 1509, 1171. ^1H NMR ($CDCl_3$) δ 3.52 (3H, s), 3.53 (2H, d, $J = 5.5$ Hz), 3.73 (2H, dd, $J = 1.5$, 5.5 Hz), 5.36–5.47 (1H, m), 6.63 (2H, d, $J = 7.9$ Hz), 6.76 (1H, t, $J = 7.3$ Hz), 7.11–7.32 (2H, m), 7.33–7.51 (3H, m), 7.53–7.61 (2H, m). HRFAB-MS calcd for $C_{19}H_{20}ClF_3NO_3$ $[M+H]^+$: 402.1084. Found: 402.1082. $[\alpha]_D^{25}$ −5.8 (*c* 0.98, $CHCl_3$).

4.2.4. (*R*)-MTPA ester (8) from 2b and (*S*)-MTPA chloride. A colorless oil. Yield 91%. IR (KBr) cm^{-1}: 1751, 1604, 1509, 1170. ^1H NMR ($CDCl_3$) δ 3.44 (2H, d, $J = 5.9$ Hz), 3.63 (3H, s), 3.77 (1H, dd, $J = 5.4$, 12.4 Hz), 3.80 (1H, dd, $J = 4.3$, 12.4 Hz), 5.38–5.49 (1H, m), 6.51 (2H, dd, $J = 1.0$, 8.9 Hz), 6.76 (1H, dt, $J = 1.0$, 7.4 Hz), 7.18 (2H, t, $J = 7.3$ Hz), 7.37 (3H, d, $J = 7.3$ Hz), 7.56 (2H, d, $J = 7.8$ Hz). HRFAB-MS calcd for $C_{19}H_{20}ClF_3NO_3$ $[M+H]^+$: 402.1084. Found: 402.1079. $[\alpha]_D^{25}$ +66.7 (*c* 1.03, $CHCl_3$).

4.2.5. (*S*)-MTPA ester (9) from 4a and (*R*)-MTPA chloride. A colorless oil. Yield 90%. IR (KBr) cm^{-1}: 1749, 1603, 1507, 1224, 1160. ^1H NMR ($CDCl_3$) δ 1.42–1.53 (2H, m), 1.93–2.11 (2H, m), 2.32–2.64 (10H, m), 2.49 (1H, dd, $J = 4.6$, 13.5 Hz), 2.68 (1H, dd, $J = 8.4$,

13.5 Hz), 3.23 (1H, dd, $J = 6.5$, 13.8 Hz), 3.40 (1H, dd, $J = 4.6$, 13.8 Hz), 3.69 (3H, s), 3.72 (1H, br s), 3.86 (1H, t, $J = 8.1$ Hz), 5.43–5.51 (1H, m), 6.52 (2H, d, $J = 7.6$ Hz), 6.71 (1H, t, $J = 8.1$ Hz), 6.91–7.03 (3H, m), 7.12–7.24 (7H, m), 7.34–7.41 (3H, m), 7.53–7.61 (2H, m). HRFAB-MS calcd for $C_{39}H_{43}F_5N_3O_3$ $[M+H]^+$: 696.3225. Found: 696.3223. $[\alpha]_D^{25}$ −19.5 (*c* 1.01, $CHCl_3$).

4.2.6. (*R*)-MTPA ester (10) from 4a and (*S*)-MTPA chloride. A colorless oil. Yield 94%. IR (KBr) cm^{-1}: 1748, 1604, 1508, 1224, 1160. ^1H NMR ($CDCl_3$) δ 1.38–1.51 (2H, m), 1.89–2.13 (2H, m), 2.22–2.73 (10H, m), 2.50 (1H, dd, $J = 5.4$, 13.5 Hz), 2.61 (1H, dd, $J = 7.0$, 13.5 Hz), 3.35 (1H, dd, $J = 4.1$, 9.7 Hz), 3.44 (1H, dd, $J = 4.9$, 9.7 Hz), 3.57 (3H, s), 3.86 (1H, t, $J = 7.8$ Hz), 4.04 (1H, br s), 5.21–5.43 (1H, m), 6.60 (2H, d, $J = 7.3$ Hz), 6.73 (1H, t, $J = 7.3$ Hz), 6.92–7.03 (3H, m), 7.13–7.20 (7H, m), 7.34–7.42 (3H, m), 7.53–7.64 (2H, m). HRFAB-MS calcd for $C_{39}H_{43}F_5N_3O_3$ $[M+H]^+$: 696.3225. Found: 696.3231. $[\alpha]_D^{25}$ +15.2 (*c* 0.99, $CHCl_3$).

4.2.7. (*S*)-MTPA ester (11) from 4b and (*R*)-MTPA chloride. A colorless oil. Yield 89%. IR (KBr) cm^{-1}: 1748, 1604, 1508, 1225, 1159. ^1H NMR ($CDCl_3$) δ 1.32–1.51 (2H, m), 1.92–2.13 (2H, m), 2.33–2.64 (10H, m), 2.49 (1H, dd, $J = 5.4$, 13.3 Hz), 2.61 (1H, dd, $J = 6.4$, 13.3 Hz), 3.39 (1H, dd, $J = 7.9$, 13.9 Hz), 3.50 (1H, dd, $J = 5.0$, 13.3 Hz), 3.52 (3H, s), 3.89 (1H, t, $J = 7.6$ Hz), 4.04 (1H, br s), 5.32–5.54 (1H, m), 6.60 (2H, d, $J = 7.6$ Hz), 6.73 (1H, t, $J = 7.6$ Hz), 6.92–7.03 (3H, m), 7.14–7.21 (7H, m), 7.43–7.51 (3H, m), 7.64–7.72 (2H, m). HRFAB-MS calcd for $C_{39}H_{43}F_5N_3O_3$ $[M+H]^+$: 696.3225. Found: 696.3229. $[\alpha]_D^{25}$ −14.2 (*c* 1.03, $CHCl_3$).

4.2.8. (*R*)-MTPA ester (12) from 4b and (*S*)-MTPA chloride. A colorless oil. Yield 89%. IR (KBr) cm^{-1}: 1748, 1604, 1507, 1225. ^1H NMR ($CDCl_3$) δ 1.33–1.48 (2H, m), 1.92–2.13 (2H, m), 2.27–2.54 (8H, m), 2.49 (1H, dd, $J = 4.3$, 13.2 Hz), 2.62–2.84 (2H, m), 2.68 (1H, dd, $J = 8.4$, 13.2 Hz), 3.24 (1H, dd, $J = 5.9$, 12.4 Hz), 3.41 (1H, dd, $J = 4.9$, 12.4 Hz), 3.42 (1H, br s), 3.60 (3H, s), 3.72 (1H, t, $J = 6.5$ Hz), 5.42–5.63 (1H, m), 6.52 (2H, d, $J = 7.3$ Hz), 6.71 (1H, t, $J = 7.3$ Hz), 6.93–7.02 (3H, m), 7.14–7.23 (7H, m), 7.38–7.54 (3H, m), 7.61–7.73 (2H, m). HRFAB-MS calcd for $C_{39}H_{43}F_5N_3O_3$ $[M+H]^+$: 696.3225. Found: 696.3218. $[\alpha]_D^{25}$ +21.4 (*c* 1.02, $CHCl_3$).

4.3. DAT binding studies

Male Wistar rats (6–7 weeks) were used for the DAT binding studies. Binding assay for the DAT was determined according to the published procedure.[25] Briefly, the rat striatal membranes were incubated with [^{3}H]GBR12935 (1 nM final concentration) and test compounds (final concentration range: 10^{-11}–10^{-5} M), which were diluted with dimethyl sulfoxide (final dimethyl sulfoxide concentration was less than 0.1%), for 60 min at 4 °C in 50 mM Tris–citrate (pH 7.4) buffer containing 120 mM NaCl and 4 mM $MgCl_2$. [^{3}H]GBR12935 (53 Ci/mmol) was purchased from Du Pont-NEN (Boston, MA, USA). The assay was terminated by filtration through Whatman GB/F glass fiber filtermats, presoaked with 0.1% bovine serum albumin solution, with a Brandel Cell Harvester (Gaithersberg, MD, USA). Filters were assayed for radioactivity with Packard Tris-Carb Liquid Scintillation Counter (Meriden, CT, USA) in 4 mL Aquasol-2.

4.4. [^{3}H]DA, [^{3}H]5-HT, and [^{3}H]NE uptake assays

Male Wistar rats (6–7 weeks) were used for the monoamine uptake assays. The [^{3}H]DA, [^{3}H]5-HT, and [^{3}H]NE uptake assays were proceeded according to the published procedure.[26] Briefly, synaptosomes were prepared by homogenization of rat striatum (for [^{3}H]DA uptake assay) or cerebral cortex (for [^{3}H]5-HT and [^{3}H]NE uptake assays). The uptake assays were initiated by the addition of 100 μL of [^{3}H]-monoamines to polystyrene tubes prefilled with 890 μL of synaptosomes in Krebs–Henseleit buffer, which contained 121 mM NaCl, 25 mM $NaHCO_3$, 11.1 mM glucose, 4.7 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 130 μM EDTA·2Na, 10 μM nialamide, 0.2% ascorbic acid, and 10 μL of test compounds (final concentration range: 10^{-11}–10^{-5} M), which were diluted with dimethyl sulfoxide (final dimethyl sulfoxide concentration was less than 0.1%), and they were incubated for 5 min at 37 °C. After they were cooled in an ice bath, 100 μL of [^{3}H]-monoamines (100 nM final concentration) were added and they were incubated for 5 min (for [^{3}H]DA uptake assay) or 10 min (for [^{3}H]5-HT and [^{3}H]NE uptake assays), respectively. The assays were terminated by filtration through Whatman GB/F glass fiber filtermats, presoaked with saline, with a Brandel Cell Harvester (Gaithersberg, MD, USA). Filters were assayed for radioactivity with Packard Tris-Carb Liquid Scintillation Counter (Meriden, CT, USA) in 10 mL Aquasol-2.

4.5. Ex vivo DA uptake inhibition studies

Male Sprague-Dawley rats were fasted overnight. Test compounds at 30 mg/kg or vehicle (saline) were administered orally. At 1, 2, 3, 4, and 6 h after the administrations, rats were decapitated. Their brains were removed to an ice-cooled dish for dissection of the striatum and they were weighted. The tissue was homogenized in 20 vol of ice-cold 0.32 M sucrose with a Teflon/glass homogenizer. Homogenates were centrifuged at 700*g* for 10 min, with retention of the supernatant followed by centrifugation at 17,500*g* for 20 min at 4 °C. The resulting pellet was re-suspended in 0.32 M sucrose, and then ice-cold Krebs–Henseleit buffer (121 mM NaCl, 25 mM $NaHCO_3$, 11.1 mM glucose, 4.7 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 130 μM EDTA·2Na, 10 μM nialamide, 0.2% ascorbic acid) bubbled with 95% O_2 and 5% CO_2 was added to prepare synaptosomes. DA uptake experiments were conducted in assay tubes containing 900 μL of synaptosomes and 100 μL of [^{3}H]DA (100 nM final

concentration) for 5 min at 37 °C. Uptake was terminated after 5 min by rapid filtration through a Brandel Cell Harvester (Gaithersberg, MD, USA) and the mixture was rinsed with 3×5 mL of ice-cold Krebs–Henseleit buffer through Whatman GB/F glass fiber filters that had been presoaked in saline for at least 1 h. Filter mats were allowed to air dry and then placed in scintillation vials containing 10 mL Aquasol-2. Radioactivity was determined by Packard Tris-Carb Liquid Scintillation Counter (Meriden, CT, USA). Specific uptake was determined by subtracting the accumulation at 4 °C from that at 37 °C. The results are expressed as % inhibition of specific uptake in vehicle control. Each value represents the mean with SEM of four rats.

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