ACS Medicinal Chemistry Letters

Letter

pubs.acs.org/acsmedchemlett

Novel and High Affinity Fluorescent Ligands for the Serotonin Transporter Based on (*S*)-Citalopram

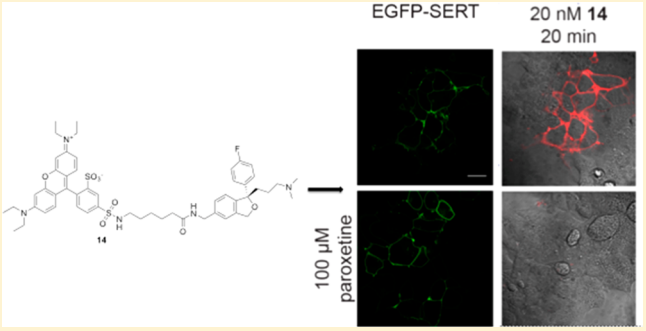
Vivek Kumar,[†] Troels Rahbek-Clemmensen,[‡] Christian B. Billesbølle,[‡] Trine Nygaard Jorgensen,[‡] Ulrik Gether,[‡] and Amy Hauck Newman*[,†]

[†]Medicinal Chemistry Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse-Intramural Research Program, National Institutes of Health, 333 Cassell Drive, Baltimore, Maryland 21224, United States

[‡]Molecular Neuropharmacology Laboratory and Center for Pharmacogenomics, Department of Neuroscience and Pharmacology, The Faculty of Health and Medical Sciences, University of Copenhagen, DK-2200 Copenhagen N, Denmark

Supporting Information

ABSTRACT: Novel rhodamine-labeled ligands, based on (*S*)-citalopram, were synthesized and evaluated for uptake inhibition at the human serotonin, dopamine, and norepinephrine transporters (hSERT, hDAT, and hNET, respectively) and for binding at SERT, in transiently transfected COS7 cells. Compound **14** demonstrated high affinity binding and selectivity for SERT (K_i = 3 nM). Visualization of SERT, using confocal laser scanning microscopy, validated compound **14** as a novel tool for studying SERT expression and distribution in living cells.



KEYWORDS: *Fluorescent ligand, SERT, citalopram, rhodamine*

The serotonin transporter (SERT) is a member of the Neurotransmitter:Sodium Symporter (NSS) family of transporters and regulates serotonin signaling and homeostasis by mediating reuptake of extracellular serotonin.[1] The important classes of selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) that are prescribed for the treatment of anxiety, and major depressive disorders work through inhibition of serotonin reuptake via SERT. Nevertheless, only a rudimentary understanding of the molecular and cellular mechanisms governing native SERT expression, distribution, and trafficking exists currently. Hence, further investigation is critical for understanding the pathophysiology of these disorders, the effects of chronic drug use, and how to improve long-term treatment.[2]

One approach to studying native protein function in live cells or brain tissue is to use fluorescently labeled small molecules.[3,4] Fluorescent ligands that have high affinity and selectivity for their target protein can retain pharmacological properties of the parent molecule and allow concomitant localization and real time monitoring of cellular processes, including distribution, trafficking, sequestering, and postendocytotic sorting.[4,5] Thus, with the technological advances in fluorescent microscopy, these small molecules can serve as powerful tools for investigating CNS target−ligand interactions and the systems in which they reside and function.

The tropane-based fluorescent ligand, **JHC1-64**, as well as related analogues that have fluorophores other than rhodamine, have proven highly useful in studying DAT in transfected heterologous cell systems as well as monitoring endogenous DAT in cultured dopaminergic neurons.[5−8] However, the lack of selectivity across monoamine transporters and relatively low affinity for SERT decreases the utility of this fluorescent ligand for SERT trafficking studies. Recently, we reported our first attempt at a SERT selective fluorescent ligand **ZP 455**, using (±)-citalopram as the parent compound (Figure 1).[9] While **ZP 455** demonstrated moderately high affinity and selectivity for SERT (K_i = 225 nM) over DAT and NET in HEK293 cells, labeling of SERT in neurons was weak and characterized by

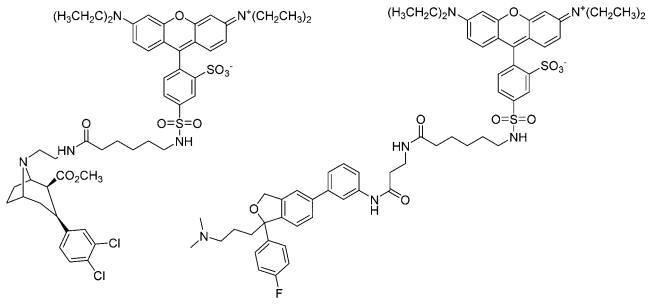


Figure 1. JHC1-64 and **ZP 455** (compound **8** in Zhang et al.[9]).

Received: February 21, 2014
Accepted: March 21, 2014
Published: March 27, 2014

rapid dissociation of the ligand, possibly due to its relatively low affinity for SERT.

In the present study, using recently developed SAR[10] and chiral separation of (±)-citalopram to give the pure eutomer, (*S*)-citalopram, we developed novel C-1 and C-5 substituted rhodamine labeled ligands with high binding affinity and SERT selectivity.

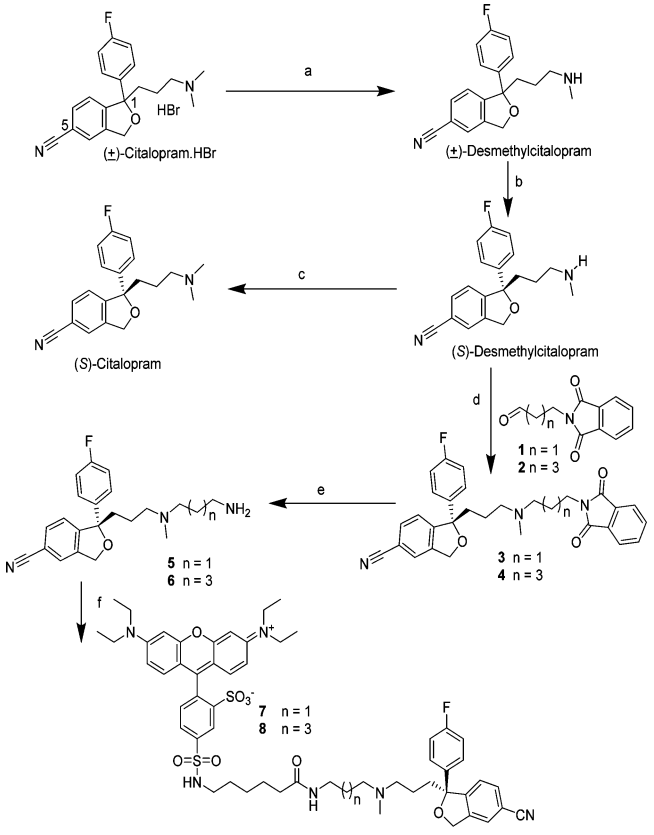
Scheme 1 outlines the synthetic strategy used for the synthesis of C-1 based fluorescent citalopram analogues **7** and **8**. The HBr salt of (±)-citalopram was neutralized and demethylated to afford desmethylcitalopram, as previously described.[10] The (*S*)-enantiomer was separated using (+)-DPTTA ((+)-*O*,*O*′-di-*p*-toluoyl-D-tartaric acid) as the chiral resolving agent with modifications of a previously reported procedure.[11] (*S*)-Desmethylcitalopram was further *N*-alkylated and reduced in the presence of $NaBH(OAc)_3$ to give intermediates **3** and **4**.[12] Deprotection of compounds **3** and **4** using hydrazine gave the free amines **5** and **6**, respectively. Rhodamine-NHS ester was coupled to the free amine intermediate **5** and **6** under basic conditions to afford the final products **7** and **8**. (*S*)-Desmethylcitalopram was *N*-alkylated to afford (*S*)-citalopram according to the published procedure.[13]

To carry out the synthesis of the C-5 based fluorescent ligand **12**, (*S*)-citalopram was oxidized in the presence of an aluminum−nickel catalyst in formic acid to its aldehyde intermediate **9** (Scheme 2).[10] Compound **9** underwent nucleophilic substitution with cyanoacetic acid to afford intermediate **10**, which upon reduction provided compound **11**.[14,15] Rhodamine-NHS ester was coupled to **11** under basic conditions to afford compound **12**. To synthesize compound **14**, (*S*)-citalopram was reduced to intermediate **13**[10] and coupled with rhodamine-NHS ester.[9]

All four novel rhodamine analogues (**7**, **8**, **12**, and **14**) were evaluated for inhibition of serotonin or dopamine uptake at their respective transporters (e.g., hSERT, hDAT, and hNET) and compared to **JHC1-64** in transiently transfected COS7 cells using [^{3}H]5-HT for SERT and [^{3}H]dopamine for NET and DAT. On the basis of these assays, compounds **12** and **14** showed the highest apparent affinities at SERT suggesting that substitution at the C-5 position of (*S*)-citalopram resulted in the most well-tolerated analogues at SERT, and the affinities were not significantly influenced by the difference in the linker length between the fluorophore and the parent compound (Table 1). None of the four new fluorescent analogues had any inhibitory effect on the uptake of [^{3}H]dopamine at DAT or NET, at a concentration of 10 μM, in contrast to **JHC1-64**.

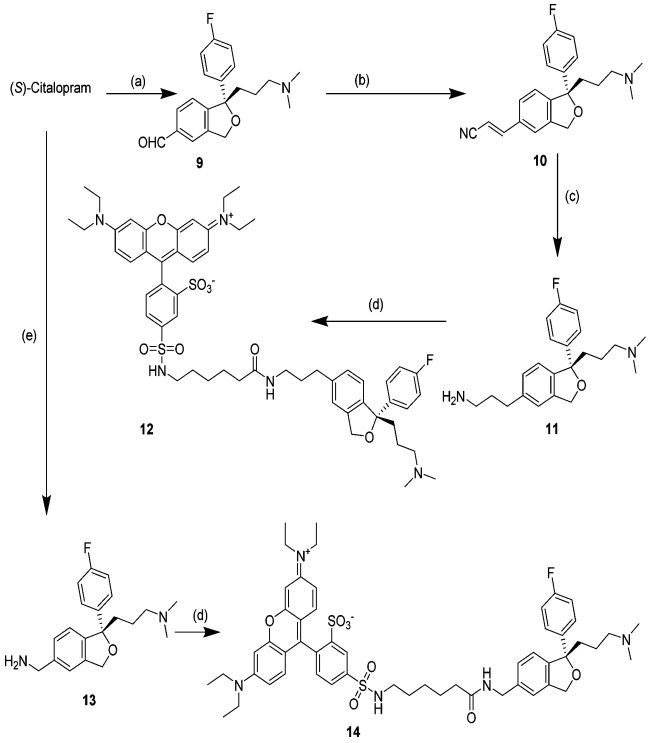
Next, we determined the affinities of the (*S*)-citalopram-based analogues in competition binding assays using [^{3}H](*S*)-citalopram as the radioligand. Because the compounds were highly SERT specific according to the uptake inhibition assay data, only hSERT binding affinities were assessed (Table 2). All four compounds showed moderate to high affinities for SERT; however, compound **14** displayed an affinity similar to that of

Scheme 1. Synthesis of SERT Fluorescent Compounds 7 and 8[a]



[a]Reagents and conditions: (a) (i) NH_4OH (ii) ACE-Cl, DCE, 18 h; (b) (+)-DPTTA, MeOH; (c) formaldehyde, formic acid, H_2O, reflux, 5 h; (d) $NaBH(OAc)_3$, AcOH, DCE, RT, 12 h; (e) hydrazine, EtOH, reflux, 3 h; (f) Rhodamine red-X NHS ester, (*i*-pr)$_2$ethylamine, DMF, 40−45 °C, 24 h.

Scheme 2. Synthesis of SERT Fluorescent Compounds 12 and 14[a]



[a]Reagents and conditions: (a) Ni-Al alloy, formic acid, reflux, 5 h; (b) cyanoacetic acid, morpholine, DMF, reflux, 5 h; (c) $NaBH_4$, $CoCl_2$, MeOH, 0 °C to RT, 16 h; (d) rhodamine-NHS ester, (*i*-pr)$_2$ethylamine, DMF, 40−45 °C, 24 h; (e) LAH, THF, 5 h.

dx.doi.org/10.1021/ml5000806 | ACS Med. Chem. Lett. 2014, 5, 696−699

Table 1. Uptake Inhibition at hSERT, hDAT, and hNET Expressed in COS7 Cells[a]

compd	SERT K_i (nM) [SE interval]	DAT K_i (nM) [SE interval]	NET K_i (nM) [SE interval]
(*S*)-citalopram	7.4 [3.0−19]	IA	IA
7	140 [110−180]	IA	IA
8	250 [130−450]	IA	IA
12	37 [22−61]	IA	IA
14	34 [19−69]	IA	IA
JHC1-64	94 [72−120]	20 [8−51]	91 [73−110]

[a]For determination of K_i values, IC_{50} values were determined as described in the Supporting Information from nonlinear regression analysis of uptake inhibition assays on transfected COS7 cells using 2,5,6-[^{3}H]-dopamine for DAT and NET or 5-hydroxy[^{3}H]tryptamine for SERT. The K_M values for 2,5,6-[^{3}H]-dopamine or 5-hydroxy[^{3}H]-tryptamine were determined in parallel and used for calculating indicated K_i values. The SE interval for each K_i value is indicated and was calculated from pK_I ± SE. Data are means [SE interval] of three to four repeated measurements performed in triplicate. IA = inactive at a conc. of 10 μM.

Table 2. hSERT Affinities of the Fluorescent Analogues Assessed by Competition Binding[a]

compd	SERT K_i or K_d (nM) [SE interval]
(*S*)-citalopram	2.6 [1.8−3.8]
7	11 [10−13]
8	180 [160−190]
12	11 [6−19]
14	3.0 [2.9−3.1]

[a]For determination of K_i values, IC_{50} values were determined as described in the Supporting Information from nonlinear regression analysis of competition binding assays using [^{3}H]-*S*-citalopram (2 nM) as the radioligand. The K_d value for [^{3}H]-*S*-citalopram was determined in parallel and used for calculating indicated K_i values. The SE interval for each K_i value is indicated and was calculated from pK_I ± SE. Data are means [SE interval] of three to four repeated measurements performed in triplicate.

the parent compound, (*S*)-citalopram; ∼4-fold higher affinity than compounds **7** and **12** and 60-fold higher affinity than compound **8** (Table 2). The finding that compound **14** binds with low nanomolar affinity to SERT, and thus with an affinity comparable to the parent compound, is remarkable considering the size of the rhodamine appended to the C-5 position and suggests that it does not interfere with critical binding interactions at the SERT binding site. Our previous fluorescently tagged citalopram ligand, **ZP 455**, also had rhodamine appended in the C-5 position; however, its SERT binding affinity was ∼75-fold lower than for compound **14**. In addition to being racemic, **ZP 455** possessed an aniline moiety attached to the C-5 position, and this may have contributed to the poorer solubility and binding kinetics of **ZP 455** compared to the present set of compounds.

The fluorescent properties for the four compounds **7**, **8**, **12**, and **14** were determined on a fluorescence spectrometer (Figure 2). The compounds had an excitation maximum of 571, 570, 572, and 572 nm, respectively, and all four analogues had an emission maximum of 589 nm. Thus, the different linker lengths or position of substitution had no effect on their fluorescent properties.

To assess whether it was possible to selectively label and visualize SERT in live cells with compound **14**, we used HEK293 cells expressing SERT tagged at the N-terminus with

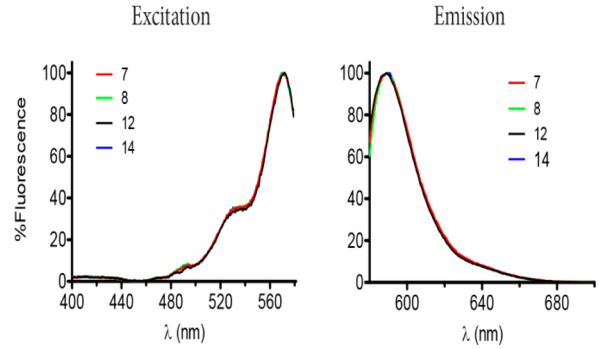


Figure 2. Excitation and emission data for compounds **7**, **8**, **12**, and **14**. Data are shown as percent of maximum fluorescence and obtained as described in the Supporting Information.

enhanced green fluorescent protein (EGFP-SERT). The cells were incubated with 20 nM **14** and imaged by confocal laser scanning microscopy after 0, 1, 5, 10, or 20 min. As seen in Figure 3, we observed a gradual increase in the fluorescent

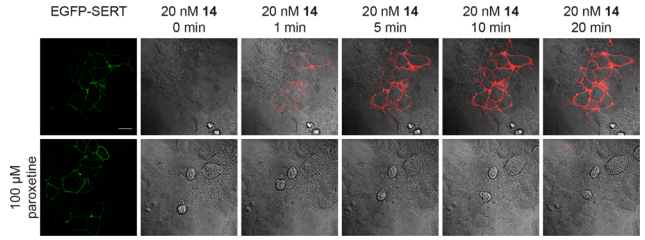


Figure 3. Compound **14** binds specifically to EGFP-SERT in a time-dependent manner in HEK293 cells. Visualization of SERT with compound **14** using confocal live imaging of HEK293 cells transiently expressing EGFP-SERT. Cells were incubated with 20 nM **14** for 0, 1, 5, 10, or 20 min ± 100 μM paroxetine at room temperature. Left panels show fluorescent EGFP signal (EGFP-SERT). Remaining panels show the fluorescent rhodamine signal. Lower panels show imaging of cells coincubated with an excess of paroxetine (100 μM). The imaging was performed as described in the Supporting Information. The fluorescent rhodamine images are merged with a transmission image in all panels. The experiment shown is representative of three identical experiments.

rhodamine signal that reached maximum after ∼10 min and overlapped with the fluorescent EGFP signal in the plasma membrane of EGFP-SERT expressing cells. Importantly, we did not see any labeling of cells without EGFP signal, and moreover, coincubation with an excess of the high affinity SERT inhibitor paroxetine completely blocked labeling of cells with compound **14** (Figure 3).

In summary, we report herein novel fluorescent ligands based on (*S*)-citalopram with modifications at the C-1 and C-5 positions. We discovered that unlike the tropane-based **JHC1-64** that binds to DAT, NET, and SERT, these (*S*)-citalopram-based fluorescent ligands are highly specific for SERT without any binding to DAT or NET. Moreover, modification at the C-5 position was particularly well tolerated at SERT, and binding affinity was not adversely influenced by the change in linker length between the fluorophore and (*S*)-citalopram. Compound **14** demonstrated the highest affinity and selectivity for SERT with K_i = 3.0 nM, which is comparable to the parent compound (K_i =2.6 nM), indicating that the rhodamine does not interfere with binding of the pharmacophore to the SERT binding site. Application of confocal microscopy demonstrated

dx.doi.org/10.1021/ml5000806 | ACS Med. Chem. Lett. 2014, 5, 696−699

furthermore that compound **14** can be used to specifically visualize SERT in the plasma membrane of live cells. Taken together, the combination of compound **14**'s high affinity and selectivity for SERT and the strong signal-to-noise ratio seen in confocal imaging illustrates how well-suited this unique fluorescent tool should be in further studies of SERT expression, localization, and trafficking directly in living cells.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details for the synthesis and purification of the compounds and their in vitro pharmacological characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(A.H.N.) Tel: +1-443-740-2887. Fax: +1-443-740-2111. E-mail: anewman@intra.nida.nih.gov.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This research was funded by the NIDA Intramural Research Program and by the National Institute of Health Grants P01 DA 12408 (to U.G.), the Danish Medical Research Council (to U.G.), and the Lundbeck Foundation Center for Biomembranes in Nanomedicine (to U.G.). V.K. was supported by an NIH Visiting Fellowship.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

A special thanks is given to Dr. Amina Woods for the MALDI-TOF analysis of all the final compounds. We thank Anette Dencker Kaas for excellent technical assistance.

■ ABBREVIATIONS

SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitors; DAT, dopamine transporter; NET, norepinephrine transporter; LAH, lithium aluminum hydride; EGFP, enhanced green fluorescent protein; RT, room temperature

■ REFERENCES

(1) Kristensen, A. S.; Andersen, J.; Jørgensen, T. N.; Sørensen, L.; Eriksen, J.; Loland, C. J.; Strømgaard, K.; Gether, U. SLC6 Neurotransmitter transporters: structure, function, and regulation. *Pharmacol. Rev.* **2011**, *63*, 585−640.

(2) Benmansour, S.; Cecchi, M.; Morilak, D. A.; Gerhardt, G. A.; Javors, M. A.; Gould, G. G.; Frazer, A. Effects of chronic antidepressant treatments on serotonin transporter function, density, and mRNA level. *J. Neurosci.* **1999**, *19*, 10494−10501.

(3) Baindur, N.; Triggle, D. J. Concepts and progress in the development and utilization of receptor-specific fluorescent ligands. *Med. Res. Rev.* **1994**, *14*, 591−664.

(4) Joubert, J.; Dyk, S. V.; Malan, S. F. Small molecule fluorescent ligands as central nervous system imaging probes. Joubert et al. *Mini-Rev Med. Chem.* **2013**, *13*, 682−696.

(5) Eriksen, J.; Bjørn-Yoshimoto, W. E.; Jørgensen, T. N.; Newman, A. H.; Gether, U. *J. Biol. Chem.* **2010**, *28*, 27289−27301.

(6) Cha, J. H.; Zou, M. F.; Adkins, E. M.; Rasmussen, S. G. F.; Loland, C. J.; Schoenenberger, B.; Gether, U.; Newman, A. H. Rhodamine-labeled 2*b*-carbomethoxy-3*b*-(3,4-dichlorophenyl)-tropane analogues as high-affinity fluorescent probes for the dopamine transporter. *J. Med. Chem.* **2005**, *48*, 7513−7516.

(7) Eriksen, J.; Rasmussen, S. G. F.; Rasmussen, T. N.; Vaegter, C. B.; Cha, J. H.; Zou, M. F.; Newman, A. H.; Gether, U. Visualization of dopamine transporter trafficking in live neurons by use of fluorescent cocaine analogs. *J. Neurosci.* **2009**, *29*, 6794−6808.

(8) Rickhag, M.; Hansen, F. H.; Sørensen, G.; Strandfelt, K. N.; Andresen, B.; Gotfryd, K.; Madsen, K. L.; Klewe, I. V.; Ammendrup-Johnsen, I.; Eriksen, J.; Newman, A. H.; Füchtbauer, E. M.; Gomeza, J.; Woldbye, D. P.; Wörtwein, G.; Gether, U. *Nat. Commun.* **2013**, *4*, 1580.

(9) Zhang, P.; Jørgensen, T. N.; Loland, C. J.; Newman, A. H. A rhodamine-labeled citalopram analogue as a high-affinity fluorescent probe for the serotonin transporter. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 323−326.

(10) Banala, A. K.; Zhang, P.; Plenge, P.; Cyriac, G.; Kopajtic, T.; Katz, J. L.; Loland, C. J.; Newman, A. H. Design and synthesis of 1-(3-(dimethylamino)propyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile (citalopram) analogues as novel probes for the serotonin transporter S1 and S2 binding sites. *J. Med. Chem.* **2013**, *56*, 9709−9724.

(11) Kaushik, V. K.; Mohammed, U. K.; Bobbali, N. R.; Srinivasan, R. K.; Dandala, R.; Meenakshisunderam, S. Process for the preparation of Escitalopram. Eur. Pat. Appl. 2017271A1, 2009.

(12) Xiao, X.; Antony, A.; Kohlhagen, G.; Pommier, Y.; Cushman, M. Design, synthesis, and biological evaluation of cytotoxic 11-aminoalkenylindenoisoquinoline and 11- diaminoalkenylindenoisoquinoline topoisomerase I inhibitors. *Bioorg. Med. Chem.* **2004**, *12*, 5147−5160.

(13) Elati, C. R.; Kolla, N.; Vankawala, P. J.; Gangula, S.; Chalamala, S.; Sundaram, V.; Bhattacharya, A.; Vurimidi, H.; Mathad, V. T. Substrate modification approach to achieve efficient resolution: didesmethylcitalopram: a key intermediate for escitalopram. *Org. Process Res. Dev.* **2007**, *11*, 289−292.

(14) Masuda, K.; Ito, Y.; Horiguchi, M.; Fujita, H. Studies on the solvent dependence of the carbamic acid formation from -(1-naphthyl)alkylamines and carbon dioxide. *Tetrahedron* **2005**, *61*, 213−229.

(15) Gautier, F.-M.; Jones, S.; Li, X.; Martin, S. J. Scope of the organocatalysed asymmetric reductive amination of ketones with trichlorosilane. *Org. Biomol. Chem.* **2011**, *9*, 7860−7868.