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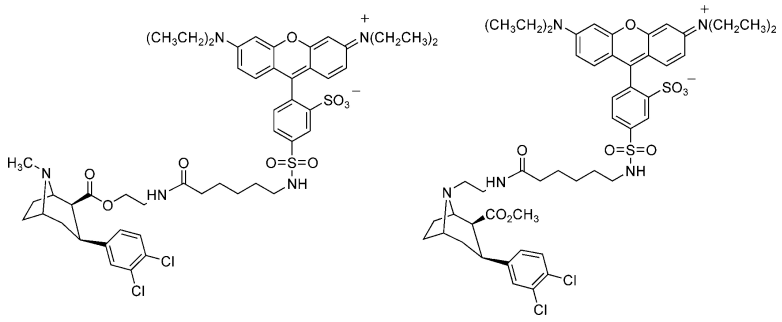

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Letters

Rhodamine-Labeled 2β-Carbomethoxy-3β-(3,4-dichlorophenyl)-tropane Analogues as High-Affinity Fluorescent Probes for the Dopamine Transporter

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Abstract: Novel fluorescent ligands were synthesized to identify a high-affinity probe that would enable visualization of the dopamine transporter (DAT) in living cells. Fluorescent tags were extended from the *N*- or 2-position of 2β-carbomethoxy-3β-(3,4-dichlorophenyl)tropane, using an ethylamino linker. The resulting 2-substituted (**5**) and N-substituted (**9**) rhodamine-labeled ligands provided the highest DAT binding affinities expressed in COS-7 cells (K_i = 27 and 18 nM, respectively) in the series. Visualization of the DAT with **5** and **9** was demonstrated by confocal fluorescence laser scanning microscopy in stably transfected HEK293 cells.

The use of fluorescence techniques to probe the structure and function of membrane proteins has proven to be extremely powerful. Numerous applications exist ranging from biophysical characterization to fluorescent imaging of membrane proteins in living cells.[1−4] Fluorescently labeled high-affinity ligands represent a class of widely applicable molecular tools. For example, fluorescent ligands can be used to visualize their target protein directly in living cells and to characterize the biophysical microenvironment of a binding site and its accessibility to the aqueous milieu upon which the location of the binding site in the protein structure may be inferred.[5−13] Previous studies using a cocaine analogue, which contained the fluorescent moiety nitrobenzoxadiazol (NBD), were conducted to explore the biophysical properties of the cocaine binding site in the rat serotonin transporter (rSERT).[6] This analogue could not be used for visualization of the SERT or the homologous DAT transporter in living cells most likely because of the low brightness of NBD (quantum yields for NBD derivatives in water are <0.1).[14] Thus, the low brightness of the analogue resulted in an unacceptably poor signal-to-noise ratio at the cell surface when using saturating concentrations of this ligand (Rasmussen and Gether, unpublished observation). More recently, cocaine−fluorophore conjugates have been reported that reproduce affinity constants of [^{3}H]cocaine and thus represent nonradioactive bioimaging tools with particular application toward the development of therapeutic antibodies against cocaine.[15]

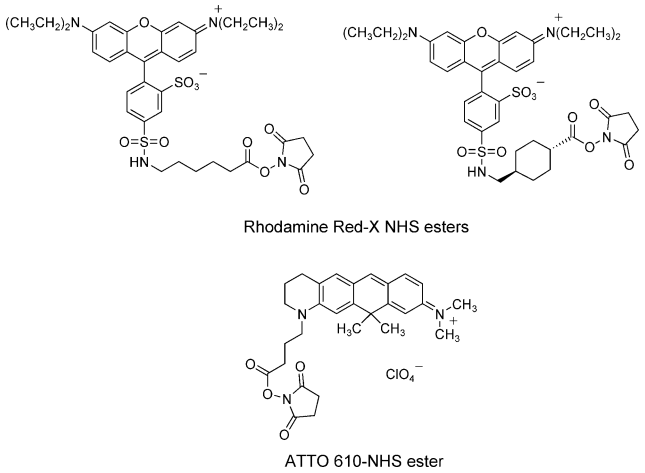

Figure 1. Fluorescent tags.

In this study, we set out to develop new fluorescent analogues of cocaine displaying high binding affinity for DAT and lower background staining than previously prepared ligands. Moreover, we wished to use fluorescent moieties with higher photostability and quantum yield than NBD, such as rhodamine derivatives that display quantum yields of 0.95−1.00.[16] Ideally, we would also like to obtain selectivity between SERT, DAT, and the norepinephrine transporter (NET) that also display high affinity for cocaine; however, for many purposes this might be of less importance, e.g., when studying systems where only one of the transporters is expressed. Structure−activity relationships derived from the 3-phenyltropane class of DAT inhibitors have shown that the 3β-3,4-diCl-phenyl ring substitution on the tropane ring gives optimal DAT affinity and that large functional groups attached to the tropane *N*- or the 2-position, via an extended linking chain, are predicted to be well tolerated.[17] Hence, we designed and synthesized compounds in which rhodamine red-X NHS ester or the novel fluorescent tag *N*-[*trans*-4(succinimidyloxycarbonyl)cyclohexylmethyl]sulforhodamine B acid amide, a rhodamine red-X NHS ester analogue, or ATTO 610-NHS ester ({1-[3-(2,5-dioxopyrrolidin-1-yloxycarbonyl)propyl]-11,11-dimethyl-2,3,4,11-tetrahydro-1*H*-naphtho[2,3*g*]quinolin-9-ylidene}dimethylammonium, Figure 1) was extended from either the *N*- or 2-position of 2β-carbomethoxy-3β-(3,4-dichlorophenyl)tropane (**1**), using an ethylamino linker and the amine-reactive fluorescent molecule derivatives.

In Scheme 1, **1** was prepared as previously reported[18] and de-esterified. Formation of the acid chloride fol-

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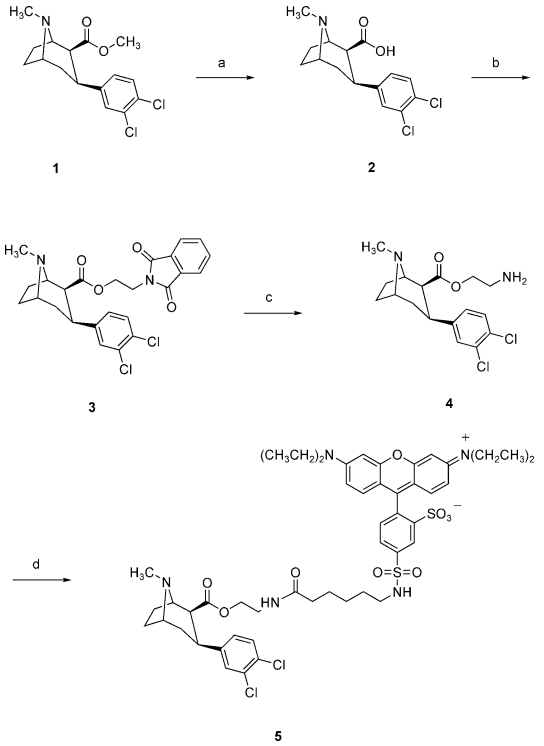
Scheme 1[a]



[a] Reagents and conditions: (a) 1,4-dioxane/H_2O, reflux, 17 h, 73%; (b) (i) $POCl_3$, room temp, 4 h, (ii) hydroxyethylphthalimide, CH_2Cl_2, Et_3N, room temp, 17 h, 74%, two steps; (c) hydrazine, ethanol, reflux, 1.5 h, 76%; (d) rhodamine red-X NHS ester, $(i\text{Pr})_2$NEt, DMF, 40−45 °C, 5 days, 43%.

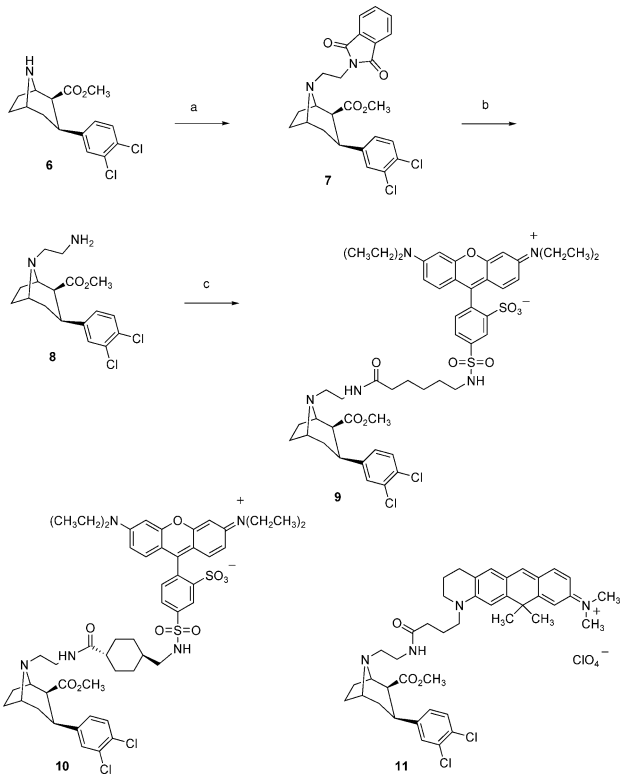
Table 1. Uptake Inhibition Data at hDAT, hNET, and hSERT[a]

compd	DAT K_i (nM) [SE interval]	NET K_i (nM) [SE interval]	SERT K_i (nM) [SE interval]
1	2.3 [1.3−3.9]	5.0 [4.3−5.7]	12 [8−18]
5	72 [45−116]	177 [157−199]	1490 [1340−1660]
9	62 [37−105]	194 [153−244]	392 [286−537]
10	436 [382−497]	627 [594−661]	705 [450−1440]
11	250 [198−318]	308 [268−354]	227 [197−261]

[a] K_i values (mean of $n = 3$ with SE interval) determined from uptake inhibition experiments in transfected COS-7 cells ([^{3}H]dopamine uptake for hDAT and hNET; [^{3}H]5-HT uptake for hSERT). The IC_{50} values used in the estimation of K_i values were determined from nonlinear regression analysis of uptake data and calculated from the mean of pIC_{50} values and the SE interval from the $pIC_{50} \pm$ SE. For determination of K_i values we used a K_M for [^{3}H]dopamine in DAT (1.1 μM [0.9−1.3]), NET (0.18 μM [0.16−0.2]), and [^{3}H]5-HT in SERT (1.1 μM [0.8−1.5]) determined in parallel. Data are the mean with SE intervals of three to six experiments performed in triplicate.

lowed by esterification with hydroxyethylphthalimide gave the amine-protected intermediate **3**. Deprotection with hydrazine was followed by reaction with the amine-reactive rhodamine red-X NHS ester (Molecular Probes). This reaction was very slow, and the yield of the final product **5** was not optimal (43%). In Scheme 2, **6**[18] was reacted with 2-bromoethylphthalimide to give intermediate **7**. Deprotection was followed by reaction of **8** with the rhodamine red-X NHS ester, which proceeded in 18 h and gave higher yields (87%) of the final product **9**. As the synthesis of the N-substituted product was more satisfactory, addition of the two novel fluorescent tags (Figure 1) was conducted likewise to give **10** and **11**. Isolation of the pure products was achieved by preparative thin-layer chromatography producing the conjugates as red (**5**, **9**, and **10**) or blue (**11**) solids.

Scheme 2[a]



[a] Reagents and conditions: (a) 2-bromoethylphthalimide, K_2CO_3, DMF, 60 °C, 15 h, 93%; (b) hydrazine, ethanol, reflux, 1.5 h, 82%; (c) rhodamine red-X NHS ester, $(i\text{Pr})_2$NEt, DMF, 40−45 °C, 18 h, 87% **9** or the novel analogue of rhodamine red X-NHS ester, DMF, room temp, 24 h, 68% **10** or ATTO 610-NHS ester, $(i\text{Pr})_2$NEt, DMF, room temp, 24 h, 58% **11**.

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

compd	DAT K_i (nM) [SE interval]
1	0.2 [0.1−0.5]
5	27 [23−31]
9	18 [15−22]
11	50 [39−65]

[a] K_i values determined from competition binding experiments on intact COS-7 cells using [^{3}H]CFT (2β-carbomethoxy-3β-(4-fluorophenyl)tropane) as the radioligand. K_i values were calculated from nonlinear regression analysis of binding assays performed as described in Supporting Information. For determination of K_i values, we used a K_D for [^{3}H]CFT determined in parallel. Data are the mean of two to three experiments performed in triplicate.

To assess the binding affinities of **5** and **9**−**11** for the human dopamine transporter (hDAT) in comparison to the corresponding norepinephrine (hNET) and serotonin transporters (hSERT), we determined the potency by which they inhibited [^{3}H]dopamine uptake into COS-7 cells transiently expressing the hDAT or hNET and [^{3}H]5-HT uptake into COS-7 cells transiently expressing the hSERT (Table 1). Compounds **5** and **9** demonstrated the highest apparent binding affinities for hDAT (72 and 62 nM, respectively), whereas **10** and **11** displayed 4- to 6-fold lower apparent affinities than **5** and **9**. During the course of these studies, we detected that **10** and **11** appeared to be less stable than **5** and **9** and their lower binding affinities may have reflected some decomposition in solution. The affinities of all the fluorescent analogues were lower than those observed for the parent compound (**1**) having an apparent affinity of 2.3 nM. To

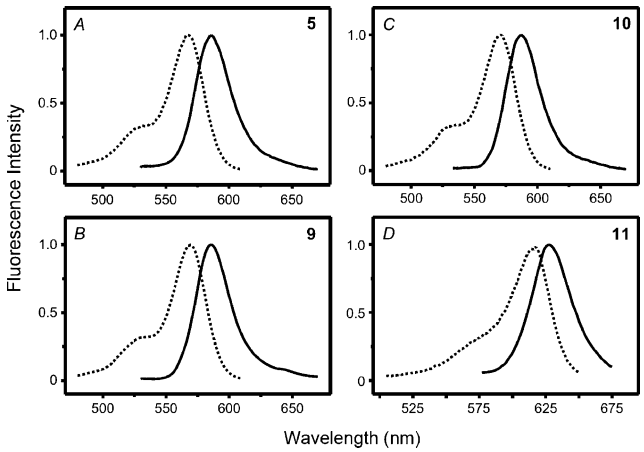


Figure 2. Excitation and emission spectra of (A) **5**, (B) **9**, (C) **10**, and (D) **11**. The excitation (dashed lines) and emission (solid lines) spectra were obtained as described in Supporting Information.

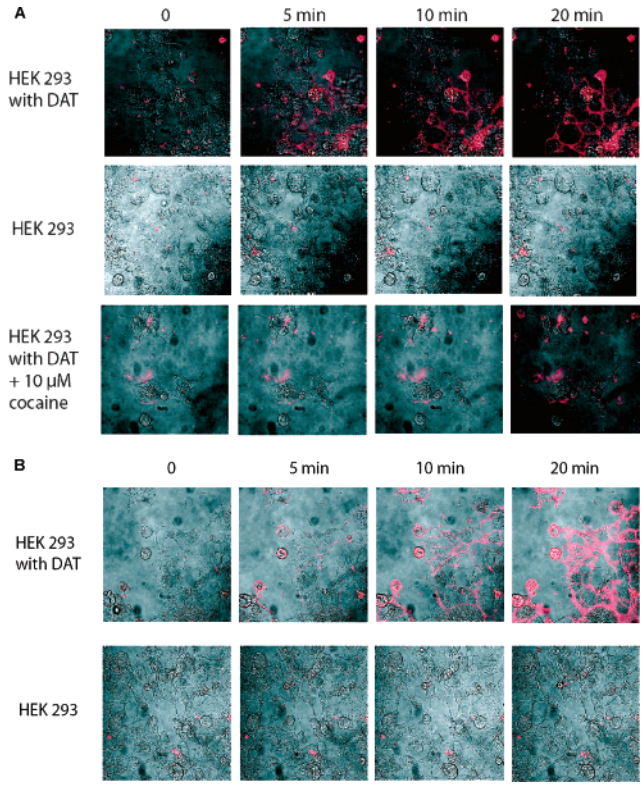


Figure 3. Visualization of DAT in HEK293 cells expressing DAT by confocal laser scanning microscopy. Cells expressing hDAT, or nontransfected controls cells, were incubated with 25 nM **5** (A) or 10 nM **9** (B) for the indicated times. All experiments were performed at room temperature in PBS buffer containing 5 mM D-glucose.

further evaluate the affinities of the compounds for hDAT, we also performed a competition binding assay using the cocaine analogue [^{3}H]CFT (2β-carbomethoxy-3β-(4-fluorophenyl)tropane) as the radioligand (Table 2). This showed the same rank order of potencies as that observed in the uptake assay; however, the estimated affinities were somewhat higher (Table 2). Altogether the uptake and binding data demonstrated that although addition of the fluorescent moieties decreased binding affinities at the DAT, compared to the parent ligand **1**, compounds **5** and **9** still demonstrated affinities for the DAT that made them useful as fluorescent labels. Compounds **5** and **9** also showed relatively high apparent affinities for the hNET (~2-fold lower than for the hDAT), whereas the affinity of **5** for the hSERT was ~20-fold lower and for **9** ~4-fold lower than that observed for hDAT.

The fluorescent properties of **5** and **9**–**11** were determined as shown in Figure 2. For compounds **5**, **9**, and **10** the excitation maxima were 567, 570, and 570 nm, respectively. The emission maxima for **5**, **9**, and **10** were 586, 585, and 587 nm, respectively. For **11** the excitation and emission maxima were 618 and 631 nm, respectively.

On the basis of their chemical stability, binding, and fluorescent profiles, **5** and **9** were further evaluated for their capability to label the hDAT by use of confocal laser scanning microscopy. HEK293 cells stably expressing the hDAT, or nontransfected controls cells, were incubated with 25 nM **5** or 10 nM **9** for the indicated times in Figure 3. With both fluorescent reagents, DAT on the cell surface could be visualized within minutes and with increasing intensity over time, reaching maximum staining after approximately 20 min. This was not observed in HEK293 cells devoid of DAT. An additional experiment was conducted in which pretreatment with 10 μM cocaine of the HEK293 cells expressing hDAT protected the DAT from binding **5** because only nonspecific fluorescence was observed. Importantly, parallel experiments with COS7 transiently expressing hDAT showed similar results, with **5** and **9** displaying labeling that could be blocked with cocaine and dopamine (data not shown).

In summary, novel fluorescent ligands for the DAT have been discovered with high DAT binding affinity and appropriate fluorescence for visualization using confocal microscopy. Compound **9** demonstrated the best DAT fluoroprobe profile overall and has thus been selected for future studies. The possibility of using this ligand for the direct labeling of the DAT in living neuronal cells represents a new and important approach for understanding cellular targeting and trafficking.

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Supporting Information Available: Experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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