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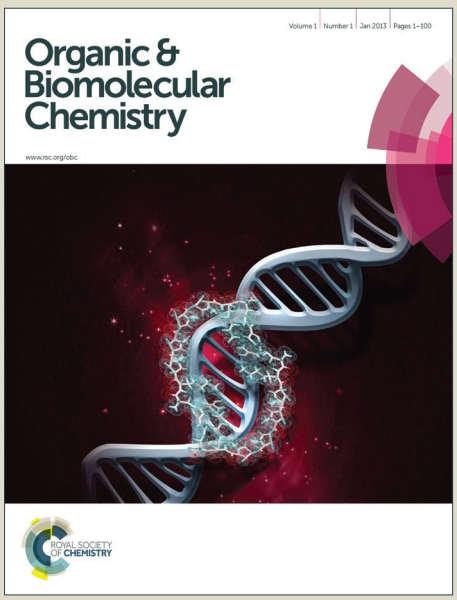

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COMMUNICATION

Synthesis of a β-CCT-Lanthanide Conjugate for Binding the Dopamine Transporter

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The development of a β-CCT-lanthanide conjugate that binds the dopamine transporter (DAT) with high affinity (K_d = 303 nM) is described. Contrast agents such as the one described herein could be used as molecular probes to directly study the binding of small molecules to receptors such as DAT via MRI, PET or SPECT.

Dopamine, because of its role in reinforcement learning, is one of the most significant neurotransmitters in the human brain. Disrupted levels of this biogenic amine have been linked to many psychological disorders and neurodegenerative diseases, such as depression, schizophrenia, and Parkinson's disease.[1] Additionally, amphetamine stimulants cause the release of increased amounts of dopamine, and addictive drugs such as cocaine compete with dopamine for binding sites on the dopamine transporter (DAT), thus preventing reuptake of the neurotransmitter. Molecular probes that could bind and/or image DAT could be used to quantify dopaminergic neurons and drug binding, thus allowing for better diagnostics and treatments of neurologic diseases.

Herein, we describe the synthesis of a molecular probe that binds to the dopamine receptor. The Gd(III)-complex of this probe can be used for in vitro binding studies without the hassle and danger associated with radiolabeled molecular probes. However, the probe could be easily used to chelate a transition metal radioisotope for subsequent in vivo studies employing either positron emission tomography (PET) or single photon emission computed tomography (SPECT).

The role of DAT in neurobiology has been extensively studied using histological techniques in post-mortem brain tissues.[1] Recently, imaging technology has augmented this work by allowing researchers to study the role of neurotransmitters in the living brain. The current state-of-the-art method uses radiolabeled ligands such as TRODAT-1™ and [^{123}I]IBZM to bind to DAT and dopamine receptors. The radiochemical signal from these agents is then detected by PET or SPECT.[1,2] Herein we report our initial efforts towards the development of a novel probe for studying the DAT in vitro that does not rely on radiolabels.

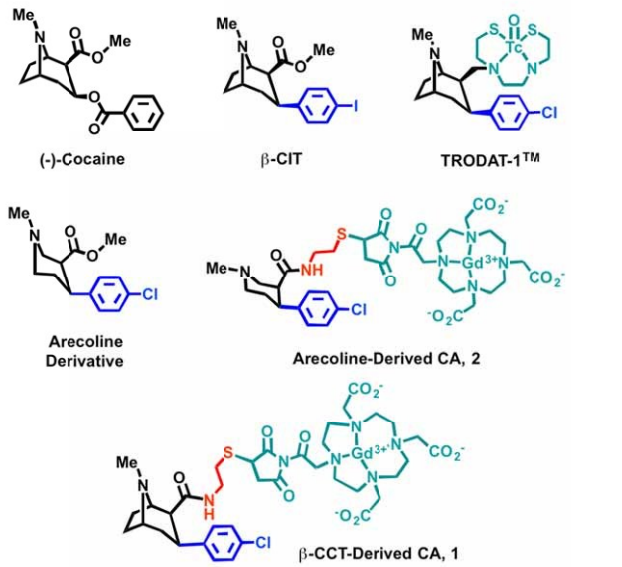
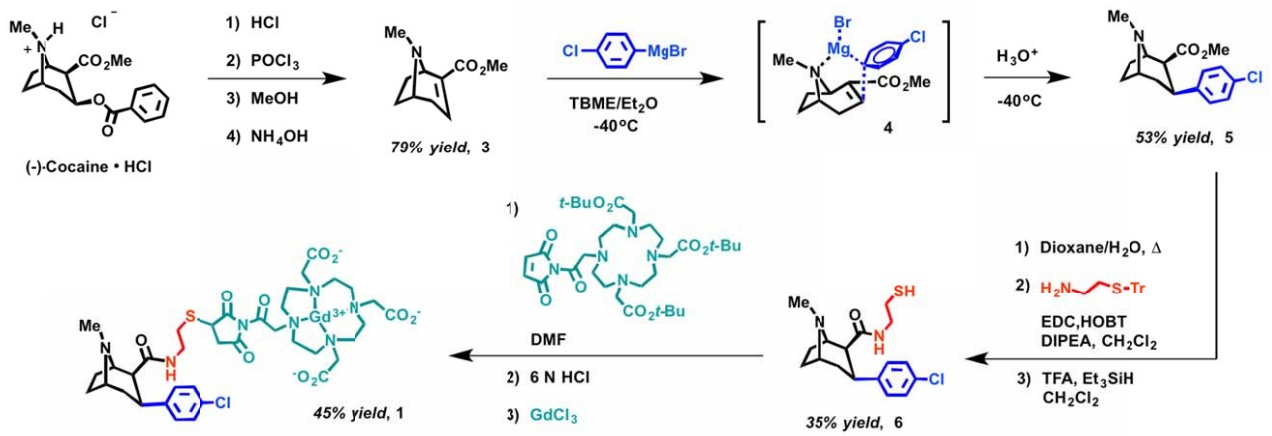


Figure 1 Common ligands for DAT and two proposed dynamic contrast agents (CAs).

In essence, our hypothesis was to develop a molecular probe by conjoining a selective DAT ligand to a lanthanide ion, namely gadolinium(III). Lanthanide ions are often complexed by cyclen- for



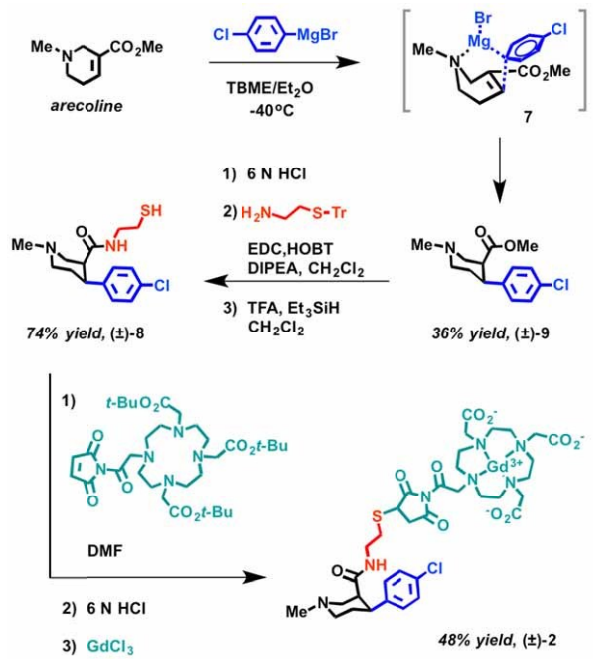
Scheme 1 Synthesis of β-CCT-Derived CA, **1**. (TBME = *tert*-butyl methyl ether, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, HOBT = hydroxybenzotriazole, DIPEA = diisopropyl ethylamine, TFA = trifluoroacetic acid.

derived ligands such as DO3A (1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid). The resulting complexes are stable and may be used biomedical applications. For example, gadolinium(III)-DO3A complexes are used as contrast agents for magnetic resonance imaging.[3,4] The cyclen-derived ligands serve as a means to prevent heavy metal toxicity in patients. Additionally, near infrared probes are often synthesized by ligating Eu(III), Dy(III) or Tb(III) ions to the DO3A moiety. Similar lanthanide-DO3A complexes can also be used as molecular probes that can be detected by chemical exchange saturation transfer (CEST) NMR. DO3A is also one of the most common ligands for the radionuclides gallium-67 and indium-111, which are increasingly popular nuclei for molecular imaging due to their long half-life (3.3 and 2.8 days, respectively) and their ready availability in North America.

The relaxation rate of a given Gdl(III)-containing contrast agent, as described by the Solomon-Bloembergen-Morgan theory, may be modulated by the rate of molecular rotation, τ_R.[5] In general, the slower a molecule rotates in solution, the more pronounced its relaxation rate will be. Thus, the binding of a relatively small contrast agent to a macromolecular target is characterized by an increase in observed spin-latice relaxation rate, r_1.[6,7]

We hypothesized that a CA that selectively binds DAT could be developed by conjugating cocaine analogs to ligated Gd(III) ions. These single molecule contrast agents should produce a quantifiable relaxation rate increase via τ_R-modulation upon binding to the transmembrane protein DAT. Cocaine and many of its synthetic analogs have proven abilities to successfully cross the blood-brain-barrier (BBB) and to bind DAT. Specifically, the tropane (–)-2β-carbomethoxy-3β-(4-chlorophenyl)tropane (β-CCT, also known as RTI-31) and the arecoline derivative shown in Figure 1 selectively bind the dopamine transporter 100x and 30x better than natural (–)-cocaine, respectively.[8] We planned to take advantage of this phenomenon to selectively direct a Gd(III) ion to the DAT using contrast agents **1** and **2** (Figure 1).

The synthesis of the prototype CAs, **1** and **2**, was accomplished via addition of Grignard nucleophiles to the α,β-unsaturated methyl esters of ecgonidine (**3**) and arecoline (Schemes 1 and 2).[9] We hypothesized that the regioselectivity of the 1,4,-conjugate addition of the *para*-chlorophenyl Grignard reagent could be enhanced by addition of catalytic or stoichiometric Cu(I) salts (**3** → **5**). Interestingly, we found no significant improvement in the yield of the reaction upon the addition of catalytic or stoichiometric amounts of Cu(I) salts such as CuBr and CuCN. The most important factors for controlling the regiochemistry of the addition were solvent and temperature. The origin of the selectivity favoring the 1,4-addition even in the absence of Cu(I) salts is likely the result of an intermediate in which the amine of **3** coordinates with the Grignard reagent's cation to direct the addition to the β-carbon of the α,β-unsaturated ester (**4**).[9a]



Scheme 2 Synthesis of Arecoline–Derived CA, **2**

The coupling of the carboxylic acid derivative of **5** with trityl-protected 2-amino-ethanethiol failed to proceed to completion, even when strong coupling agents like PyBOP (benzotriazol-1-yl-

oxytripyrrolidinophosphonium hexafluorophosphate) or HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) were used (Scheme 1). However, the starting material (**5**) was easily recovered and resubmitted to the reaction conditions, eventually leading to usable quantities of **6**, following deprotection of the trityl group. Finally, conjugation of the thiol to a maleimide-containing DO3A ligand, deprotection of the carboxylic acids and insertion of Gd(III) provided the desired CA (**1**).

The arecoline-derived CA (**2**) was synthesized in an analogous manner (Scheme 2). As observed for the synthesis of the phenylptrophane, **5**, the addition of a phenyl Grignard reagent to the α,β-unsaturated ester of arecoline resulted in 1,4-addition, presumably via a cyclic intermediate (**7**). The installation of the 2-amino-ethanethiol linker and subsequent conjugation with the maleimide-containing DO3A ligand, followed by deprotection and metalation with Gd(III) provided the CA (**2**).

To analyze the ability of **1** and **2** to enhance their observed relaxation rate upon binding to DAT, we stably transfected HEK293 cells with a plasmid vector driving expression of the DAT, and we quantified their ability to bind cocaine analogues via orthodox radioligand binding studies (Figure 2A and B). We then used these cells for relaxometry and binding studies.

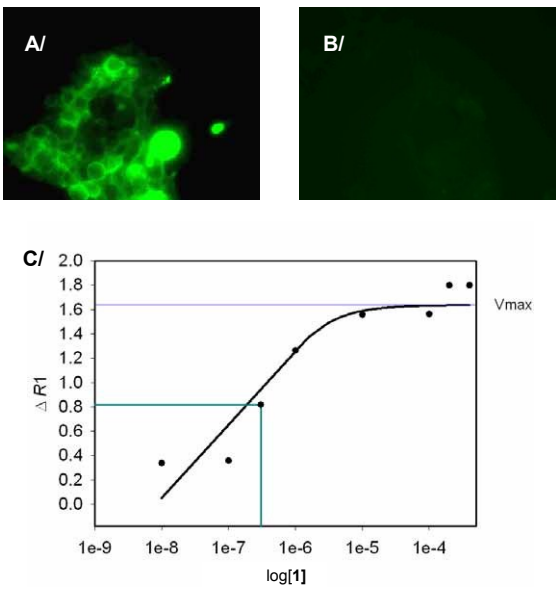


Figure 2 A/ Immufluorescence of HEK293 cell line stably expressing DAT or **B/** untransfected HEK293 cells stained with an anti-DAT primary antibody and FITC labeled secondary. **C/** Binding of **1** to DAT as measured by relaxometry. $\Delta R1 = R1_{obs} - R1_{free}$, where $R1_{free}$ was measured before DAT was added. (Green lines show K_d). 250,000 DAT-transfected cells (0.55 nmol DAT) were added as a solution in 50 uL of buffer.

Solutions of CAs **1** and **2** in PBS buffer (95:5 D_2O/H_2O) were placed in NMR tubes and inversion-recovery experiments were performed in a 7T (300 MHz) NMR spectrometer. Longitudinal relaxation times (T_1) were analyzed as a function of probe concentrations in the presence and absence of the DAT-transfected HEK293 cells. Sufficient quantities of the DAT-cells were used to saturate the CAs. The dilution of the protonated water with deuterated water allowed for a lengthening of the observed relaxation time, thus facilitating the detection. The observed spin-lattice relaxivity, $r1_{free}$, of **2** at 300 MHz and 25 °C in PBS buffer was 6.32 $mM^{-1}s^{-1}$, and this value increases by 38% ($r1_{obs}$ = 8.71 $mM^{-1}s^{-1}$) upon binding to the DAT-transfected HEK293 cells. Relaxivity amplification resulting from non-specific binding of **1** to non-transfected HEK293 cells was not observed. No significant change in relaxivity was observed with the arecoline-derived probe **2**, which is indicative of a failure to bind the DAT.[10]

The relaxation data in the presence and absence of DAT constitutes a straightforward dose-response experiment, which can be used to measure the affinity of the CA for the protein (Figure 2C). From this data, we calculated the binding constant for **1**, K_d, as 303 ± 138 nM, which is comparable to that of natural cocaine.[11]

It is unlikely that a molecular probe such as **1** could be used for in vivo studies of DAT via MRI due to the inherent insensitivity of magnetic resonance techniques and the nanomolar conentrations DAT in vivo. The indium-111 or copper-64 derivatives of **1**, however, of these compounds could be easily prepared via an analogous method and employed for in vivo SPECT and PET imaging studies, respectively. While large polar molecules like **1** often do not cross the BBB, the transition metal-tropane conjugate, TRODAT-1™ (see Figure 1 for structure), does enter the brain in sufficient quantities that enable SPECT imaging. This provides precedent that neutrally charged, metal-containing cocaine analogs can be transported across the BBB, presumably via a passive diffusion mechanism. However, in the event that a probe similar to **1** does not cross the BBB, it could still be used for in vitro imaging or for in vivo imaging studies with knockout mice that have permeable BBBs[12] or with healthy animals in combination with high-intensity focused ultrasound.[13] Additionally, numerous methods for transporting both drugs and imaging agents across the BBB are currently being developed and could be employed to deliver **1**, or variants bearing radionuclei, to the dopaminergic regions of the brain.[14,15]

In conclusion, we have synthesized a β-CCT-DO3A conjugate, **1**, that bound the DAT with an affinity comparable to that of cocaine. The affordable and readily synthesized arecoline-derived probe, **2**, failed to bind the DAT. When ligated to Gd(III), **1** behaved as a dynamic contrast agent, whose longitudinal relaxation rate increased dramatically upon binding the DAT due to a slowing of the rate of molecular rotation, τ_R. While indium-111 and copper-64 derivatives of **2** may be suitable for in vivo SPECT and PET imaging, the CA described herein is suitable for in vitro studies of the DAT.

Notes and references

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