Fluorescent mimics of 5-hydroxytryptamine based on *N*-alkylated derivatives of 6-hydroxycarbostyril†

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Fluorescent probes based on a 6-hydroxycarbostyril core accumulate inside neurons and astroglia in the absence of a serotonin uptake inhibitor.

Since the development of the epifluorescent microscope nearly a century ago, fluorescent probes have played a central role in imaging and biotechnology applications.[1,2] In most, but not all cases, a fluorescent label is covalently appended to a recognition unit which imparts specificity for a target. Notable examples of this construct include fluorescently labeled antibodies used in immunohistochemical assays[3] and fluorescent fusion proteins which are invaluable reporters of protein expression or localization.[4] Fluorescent *analogs* of biomolecules offer an alternative strategy to fluorescently *labeled* biomolecules. By employing an inherently fluorescent structure that closely mimics the native molecule, it is possible to avoid additional steric bulk, changes in shape, or ionic character that some fluorophores impart. Fluorescent nucleobases have been successfully demonstrated in structural studies[5] and enzymatic assays[6] while fluorescent analogs of neuroactive compounds have been employed in binding assays[7] and as imaging agents of subcellular processes.[8]

We report the synthesis of a series of fluorescent probes designed to mimic 5-hydroxytryptamine (5HT, serotonin). 5HT is a classic neurotransmitter implicated in multiple emotional and behavioral disorders including depression. The serotonergic system is the subject of intense research aimed at understanding the basic mechanisms of disease and developing pharmaceutical interventions.[9] Therefore, new tools that enable detailed, molecular level investigations of the serotonergic system would be of great interest. Probes **1–4** (Fig. 1) were designed around a carbostyril core. Compared to structurally related coumarins, the carbostyril lactam enables *N*-alkylation as a convenient route for modification. While not identical to the indole of 5HT, 6-hydroxycarbostyril preserves a bicyclic structure and key hydroxy functionality while exhibiting attractive optical properties. The pendant ethylamine of 5HT is preserved in **1**, while **2** is *N*,*N*-dimethyl analog of **1**. Most ligands targeting neurotransmitter receptors and transporters possess aliphatic amines, but a few examples do not. Thus, we also wanted to explore the properties of carbostyrils lacking an ethylamine group with probes **3** and **4**.

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† Electronic supplementary information (ESI) available: Detailed description of synthesis, MS, HRMS, ^{1}H, ^{13}C, optical spectra, additional confocal images and bleaching experiments. See DOI: 10.1039/b917578d

6-Methoxycarbostyril, **5**, was synthesized as described by Fabian *et al.*[10] Reaction of **5** with 1.2 equiv. of 2-azidoethyl tosylate in DMF with K_2CO_3 as base produced intermediate azido compound **6**. Reduction with PMe_3 in wet THF afforded the aminoethyl carbostyril, **7**. Finally, removal of the methyl protecting group was achieved by reacting with 1.5 equiv. of thiophenol with K_2CO_3. The synthesis of analogs **2–4** followed a similar route: reaction of **5** with chloroethylamine followed by deprotection of the 6-hydroxy group produced **2**; **3** and **4** resulted from reaction of **5** with bromoethanol and bromopropylbenzene, respectively. In all cases the *N*-linked isomer was isolated.† All compounds are freely soluble in methanol; **1–3** are easily dissolved in PBS as well, while **4** is soluble only at dilute concentrations (<50 μM).

We investigated the optical properties of **1–4** in order to determine their suitability as fluorescent probes. Based on previous reports of related carbostyrils,[11,12] we anticipated that the phenol functionality would be sensitive to pH and would strongly influence the absorption and emission wavelengths. Absorption spectra were obtained in neutral, acidic and basic solutions. In DPBS (pH 7.2) **1–4** exhibit absorption maxima between 350 and 355 nm with two prominent shoulders at 340 nm and 370 nm (Fig. 2). Addition of acetic acid effected a hypsochromic shift (λ_{max} = 335 nm); the addition of triethylamine results in pronounced bathchromic (λ_{max} = 370 nm) and hyperchromic shifts. The linear

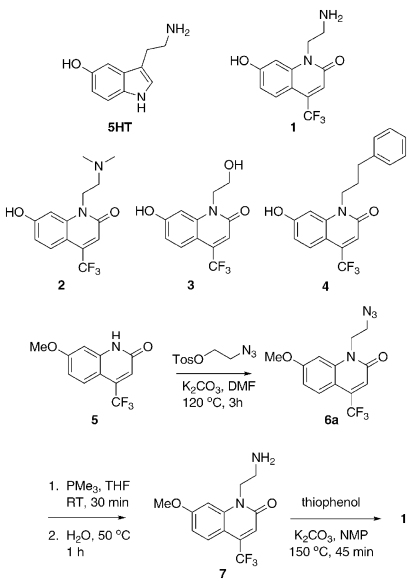

Fig. 1 Top, the structure of 5-hydroxytryptamine (5HT) and fluorescent mimics **1–4**. Bottom, the synthetic route to produce carbostyril probe **1**. Full experimental details for **1–4** are available in the ESI.†

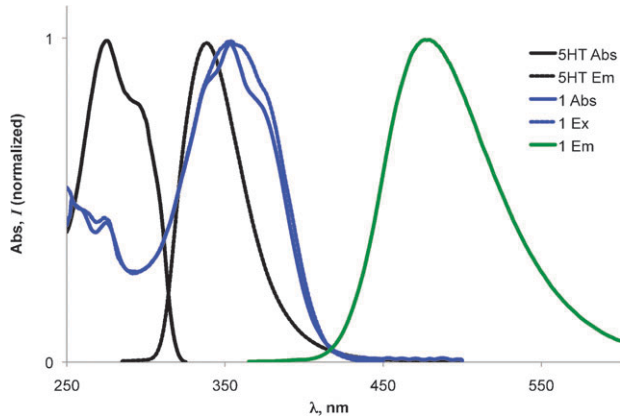

Fig. 2 Absorbance, excitation and emission spectra of **1** compared with 5HT.

combination of the absorption spectra with excess acid and excess base nearly perfectly overlaps with the spectrum obtained at pH 7.2 (Fig. S5, ESI†). Therefore, we conclude that near neutral pH, both protonated and unprotonated species exist at roughly equal concentrations.

The emission maxima for **1–4** were found to be *ca.* 480 nm in DPBS, representing a Stokes shift of 125 nm. The addition of excess base did not significantly shift the emission maxima. Thus, we can conclude that in neutral and basic solutions, **1–4** emit from a deprotonated excited state species. Emission in acidic solutions is dominated by the deprotonated species as well. In the case of **3** and **4** a small peak at 380 nm was observed, which is likely due to photoemission from the protonated form of these molecules. While 5HT itself is fluorescent, single photon excitation of 5HT requires specialized optics and a UV excitation source ($\lambda_{max,abs}$ = 277 nm). Furthermore, the autofluorescence of biological samples is also quite high in the UV region. The longer wavelength absorption of **1–4** avoids excitation of aromatic amino acid residues or nucleobases and the overall brightness of these probes enables their imaging in the presence of other endogenous fluorophores (see below). The excitation and emission wavelengths of **1–4** are comparable to the commonly employed probes DAPI and Hoechst 33 258 enabling the use of commercially available filter sets. In addition, the absorption spectrum possesses a broad shoulder extending past 400 nm which facilitates excitation by standard 405 nm diode lasers as well.

Mixed cell cultures of neurons and astroglia isolated from the brain stems of E14 chick embryos were exposed to solutions of **1–4**. Serotonergic cells are clustered in the raphe nucleus (RN) located along the midline of the brain stem; they project into other regions of the brain including the hypothalamus, the olfactory bulb, cortex and cerebellum.[9] We hypothesized that if **1–4** were effective as serotonin mimics, they would exhibit affinities for the serotonergic cells present in the RN. The inherent fluorescence of the carbostyril core enabled direct detection of their cellular uptake on a microwell plate reader (λ_{ex} = 355 nm, λ_{em} = 444 nm) (Fig. 3). A high emission response was observed for **1** and to a lesser extent, **4**, while **2** and **3** did not differ significantly from the controls (control A: cells alone, control B: wells treated with compound only, then rinsed). We anticipated that several modes of interaction are possible between the fluorescent probes and the cultured cells including specific interactions such as active transport *via* neurotransmitter transporters, or binding to neurotransmitter receptors, as well as non-specific interactions such as insertion into the cell membrane. Pre-treatment of the cell cultures with solutions of clomipramine, a serotonin reuptake inhibitor[13] (SRI), significantly reduced the uptake of **1** and **4**. This suggests that at least one mode of fluorophore–cell interaction involves serotonin transporters.

The lack of response of **2** relative to **1** is somewhat surprising as they differ only by two methyl groups. However, a survey of compounds that function as SRIs reveals that many bear secondary and tertiary aliphatic amines.[14] Specifically, SRIs citalopram, clomipramine and zimelidine possess tertiary dimethylamines suggesting that **2** may not be an effective substrate for serotonin transporters (SERT). The observed uptake of **4** was also unexpected as it lacks the amine functionality typical of most neuroactive compounds. However, several serotonin mimics bearing large aromatic appendages are known including roxindole, a $5HT_{1A}$ agonist, and BRL 15 572, a $5HT_{1D}$ partial agonist. More importantly, computed 3D molecular models of SERT (and related neurotransmitter transporters) reveal that the binding pocket and pore contain multiple aromatic residues.[15] It is plausible that π–π interactions may allow **4** to be a substrate for SERT. We are currently investigating the uptake of **1–4** against a broad range of SRIs, DRIs and NRIs in order to determine the exact mechanism of transport.

While in the microwell plate assay above, **1** showed differential affinities for cultured brainstem cells in the absence and presence of an SRI, we wanted to determine the fate of the fluorescent probe. Was it localized to the cell membrane, implying binding to transporters or receptors, or was it internalized, implying active transport into the cytosol? Neurons and astroglia isolated from E14 chick brainstems were grown on poly-L-lysine and laminin treated coverslips affixed to 35 mm culture dishes enabling imaging of live cells in culture media. Excitation was *via* a 405 nm diode laser; emission wavelengths were selected by adjusting the window of a prism to 450 through 600 nm. Cells were exposed to the probe solution for one minute, the media was then changed and additional images were captured. As is evident in Fig. 4, **1** bound to and illuminated most healthy cells. We obtained z-stacks of the cells (Fig. 4, bottom row) and found that **1** was indeed localized internally after only one minute of exposure. During the course of our imaging experiments, some photobleaching was observed. However, **1** was sufficiently stable to capture multiple images over 10 min without the use of antifade reagents or solutions.

Progress in understanding the molecular basis of neurological diseases and disorders will benefit from the development of tools that enable imaging of subcellular processes. Fluorescent analogs of neurotransmitters offer a complementary strategy to commercially available probes for imaging neurons and astroglia. For example, fluorescently tagged antibodies or fluorescent fusion proteins can specifically label receptors and transporters; they do not enable direct monitoring of neurotransmitter localization, transport or release. Additionally,

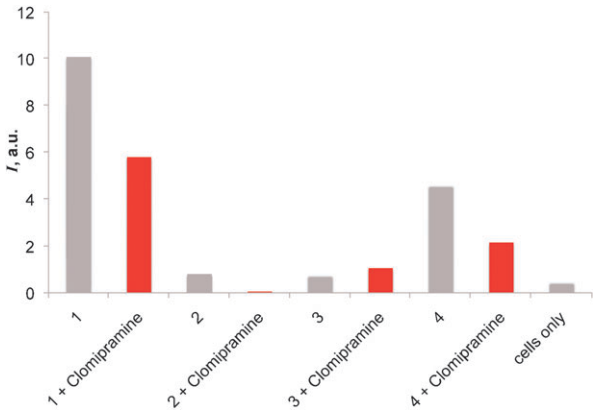

Fig. 3 A microwell plate assay using the inherent fluorescence of the carbostyril probes demonstrates the affinity of **1** and **4** for cultured neurons and astroglia isolated from E14 chick brainstems. Clomipramine, a serotonin reuptake inhibitor, reduces the response of both **1** and **4**.

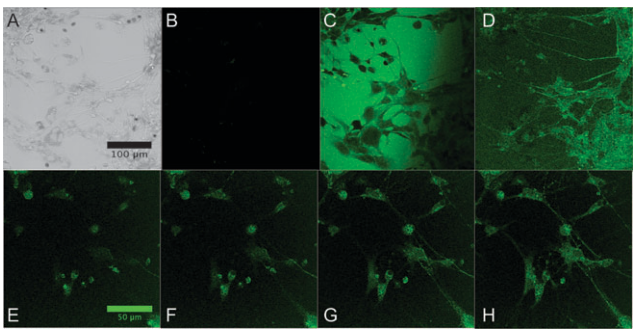

Fig. 4 E14 chick brainstem cells exposed to **1** rapidly uptake the fluorescent probe. Top row: A, phase contrast image showing the viewing area; B, control image before addition of **1** showing minimal cellular autofluorescence (laser intensity and gain were not altered during the acquisition of subsequent images); C, silhouetted cells as the fluorophore solution is added; D, 90 s after addition, virtually all healthy cells have accumulated the probe. Bottom row: E–H, selected z-stack sections (9 μm top to bottom) clearly demonstrating the internalization of the fluorophore. Complete z-stack and 3D rotations available in the ESI.†

histochemical techniques such as fluorogenic reactions of neuronal monoamines with formaldehyde can only be performed on fixed or dried tissue samples preluding their use in imaging of dynamic processes.[16] Fluorescently labeled neurotransmitters have been reported, yet they either are limited to reporting the presence of cell surface receptors[17] or exhibit activity markedly different from the natural substrate.[18]

We have synthesized a series of fluorescent analogs of 5HT as novel probes for the serotonergic system, and explored their optical properties and behavior *in vitro*. In screening their activity against cultured neurons and astroglia in the presence and absence of the SRI clomipramine, we have determined that two probes, **1** and **4**, may function as fluorescent analogs of 5HT. Confocal images of cells exposed to **1** reveal that this probe is localized to the interior of both astroglia and neurons. While these results implicate SERT as a possible pathway, we are currently working to determine the biomolecular machinery responsible for uptake.

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