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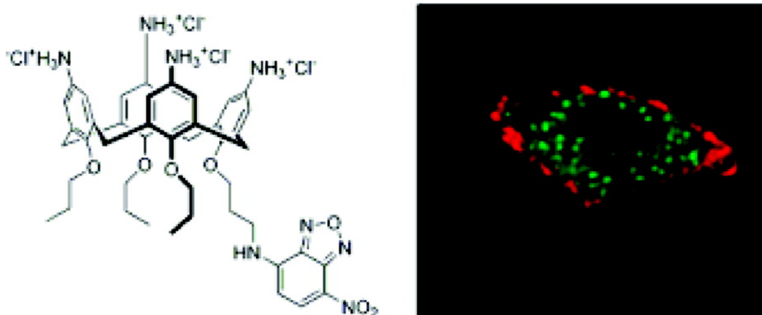

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Cellular Uptake of a Fluorescent Calix[4]arene Derivative

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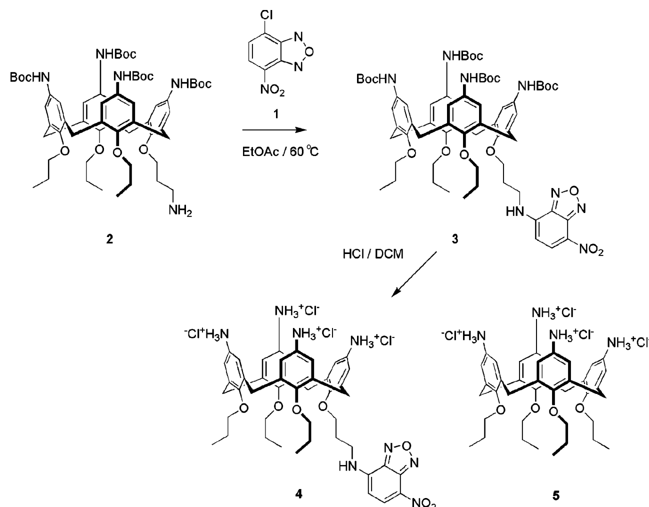
Calix[*n*]arenes[1] have found numerous applications in many fields including complexation of anions[2] and cations[3] for sensing and waste remediation and catalysis.[4] Recently, interest has focused on the use of calixarenes in biological systems. Proposed applications include their use as mimics of ion channels,[5] as enzyme mimics,[6] as agents for the surface recognition of proteins,[7] as platforms for magnetic resonance imaging agents,[8] as gene transfection vectors,[9] and drug delivery systems, including solid lipid nanoparticles[10] and antimicrobials.[11] While some limited cell viability,[12] immunogenicity,[13] and toxicity studies[7] have been performed for calix[4]-arene derivatives, to the best of our knowledge, no information on the cellular uptake processes and cellular fate for calix[4]arenes has been reported. The preparation of fluorescent derivatives of calix[4]arenes would enable ready investigation of uptake and localization in cellular organelles and provide useful information for researchers developing calix[4]arenes as drugs and as drug delivery systems. In fact the fluorescent probe could itself be considered to be a 'model drug'. 4-Chloro-7-nitrobenzofurazan (NBD chloride)[14] is a particularly attractive fluorescent probe. On conjugation it undergoes a change in fluorescence intensity from weak to strong, its photophysical properties make it suitable for use with a fluorescein isothiocyanate (FITC) filter, and its introduction is straightforward.

This choice of probe necessitated the preparation of a water-soluble calixarene bearing a single aliphatic amine functionality for attachment. Upper-rim tetraamino derivatives are particularly attractive as they are readily synthesized from their nitro precursors[15] and their salts are water-soluble. Additionally, due to their straightforward protection, as the Boc derivatives,[16] they are amenable to further chemical modification of the macrocyclic core at the lower-rim for preparation of asymmetrically functionalized derivatives incorporating a single fluorescent unit, which is important to avoid intermolecular quenching. The fluorescent probe **4** was prepared following a two-step synthesis from the known protected monofunctionalized derivative 5,11,17,23-tetra-Boc-amino-25,26,27-tripropoxy-28-aminopropoxycalix[4]arene **2**.[9a] The fluorescent marker was introduced by treatment of **2** with NBD chloride (EtOAc, 4 h, 60 °C),[17] which following purification by column chromatography over silica gel gave NBD derivative **3**. The upper-rim Boc amino protection was removed using HCl gas to give the water-soluble fluorescent calixarene derivative **4** for evaluation in cultured cells. Tetraamino calix[4]arene tetrapropyl ether **5**[18] was prepared as its hydrochloride salt for evaluation as a nonfluorescent water-soluble calixarene control for comparison in biological studies.

As calixarenes have been proposed for a variety of biological applications, the imaging studies were designed to answer two major questions, how the calixarene enters the cell and how it is processed by the cell. Thus we focused on three main areas: the suitability of **4** for imaging cells, its use to determine the method of cell uptake and its localization, and an evaluation of its cytotoxicity at ideal imaging concentrations. Imaging was performed initially using a 150 mM solution of **4** in H_2O. Clear images of Chinese Hamster Ovary (CHO) cells were acquired (Figure S1, Supporting Information), and control studies using both **5** (the nonlabeled calixarene derivative) and NBD chloride indicated that imaging was a consequence of the NBD conjugate rather than of the calixarene aromatic core or free NBD chloride (Figure S2, Supporting Information). Uptake was rapid, with high quality images being seen after a 10 min incubation time, and no loss of imaging ability was observed over a 60 min time-course (Figure S1, Supporting Information). Acceptable cellular imaging was achieved on further decrease of the probe concentration to 15 mM well within the safe cellular concentration.

Scheme 1. Synthesis of Fluorescently Labeled Water-Soluble Calix[4]arene **4** and Structure of Unlabeled Control **5**



While treatment of cells with β-cyclodextrin leads to visible deformation of the cell surface,[19] this was not the case with the calixarene, although the exact nature of the uptake mechanism is still unclear. Competitive imaging studies with inhibitors of cell uptake by clathrin coated pits and lipid rafts[20] were undertaken to elucidate this mechanism. Images acquired (Figure S3a, Supporting Information) following coincubation of **4** with filipin,[21] a selective disrupter of caveolae and caveolae-like structures responsible for internalization, showed no inhibition of the uptake of **4**. This indicates that uptake is not based on caveolae-linked endocytosis or lipid-raft processes, where uptake occurs through the formation of invaginations into the membrane which are subsequently pinched off as endosomes into the inside of the cell. Equally, preincubation with sucrose[22] (Figure S3b, Supporting Information), a selective inhibitor of clathrin-coated pit endocytosis, or with β-cyclodextrin[23]

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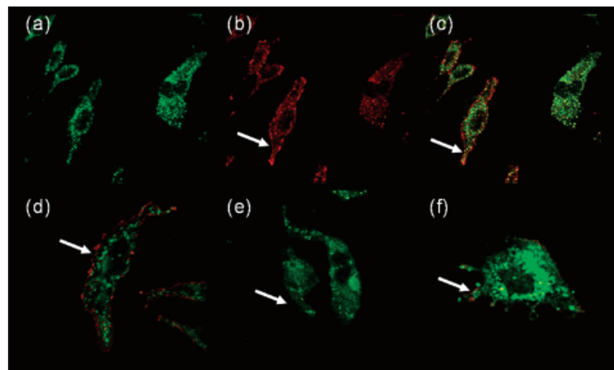

Figure 1. Colocalization studies of **4** (15 mM) and anti CCR5 receptor IgG (HEK/1/85a/7a) counterstain in CHO (a) **4**, (b) HEK/1/85a/7a, (c) overlay of **4** and HEK/1/85a/7a, and (d) preincubation with filipin, (e) sucrose, and (f) β-cyclodextrin. Arrows indicate receptor in membrane. Images taken after 10 min incubation using a Leica TCS SP2 UV Confocal Imaging system.

(Figure S3c, Supporting Information), which inhibits mainly caveolae, had no effect on uptake into cells demonstrating that **4** does not enter the cell by either of the two common pathways.

Visualization of localization within the cells was achieved using confocal microscopy with an anti-CCR5 receptor IgG counterstain.[24] This counterstaining shows that there is no accumulation of **4** within the cell membrane, where a high concentration of the IgG is located, and that **4** accumulates within the cytoplasm and is not taken up into the nucleus of the cell (Figure 1a−c). Additional colocalization studies, in the presence of uptake inhibitors, clearly demonstrate that the uptake of the calixarene conjugate is unaffected by inhibitors of clathrin-coated pits or lipid rafts (Figure 1d−f).

The use of calixarenes as biological probes requires low toxicity at concentrations appropriate for imaging. The viability of CHO cells and HL-60 cells in the presence of **4** and **5** were investigated using an MTS assay. Both were of low toxicity, comparable to that of PBS buffer over a range of concentrations (0.0015−15 mM) with IC_{50} = 82 mM for **4** and IC_{50} = 81 mM for **5** in CHO cells (Figure S4a, Supporting Information). In HL-60 cells the IC_{50} is about 10-fold lower (IC_{50} = 8 mM for **4** cf., IC_{50} = ~0.1 mM for β-cyclodextrin). The IC_{50} indicates the concentration at which 50% cell death is observed. Thus incorporation of the fluorescent marker in **4** does not affect overall cell viability. An additional coincubation study with cyclodextrin indicated that the IC_{50} of **4** was unaffected (Figure S4b, Supporting Information) by the presence of this cholesterol-sequestering macrocycle and that there was no synergistic toxicity due to disruption of the cell membrane.

In this preliminary study, the suitability and applicability of a fluorescent calixarene for cell imaging has been demonstrated clearly. The nature of the cellular uptake of the derivative has been probed and shown, in this case, to be a nonspecific process, not linked to either of the main endocytic pathways (clathrin coated pits and lipid rafts) which results in accumulation of the probe within the cell cytoplasm.

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Supporting Information Available: Full experimental procedures (Figures S1−S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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