ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue



Cite this: *Chem. Commun.*, 2014, **50**, 5851

Received 19th March 2014,
Accepted 10th April 2014

DOI: 10.1039/c4cc02036g

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# A tetranaphthoimidazolium receptor as a fluorescent chemosensor for phytate†

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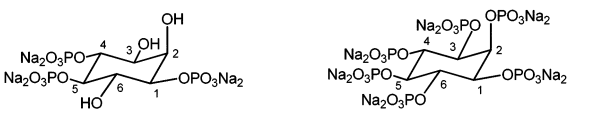
**A new tetranaphthoimidazolium receptor was synthesized and reported to be a selective fluorescent chemosensor for phytate, *myo*-inositol hexakisphosphate ($IP_6$). In a 100% aqueous solution at pH 7.4, chemosensor 1 showed a selective fluorescence enhancement for IP6 over $IP_3$, phosphates, pyrophosphates, AMP, ADP and ATP. An excimer emission at 465 nm linearly increases in the range of 300 nM to 1 μM with a detection limit of 2.28 × $10^{-7}$ M. In addition, first live cell imaging of $IP_6$ has been demonstrated by using a synthetic receptor.**

Inositol 1,4,5-trisphosphate ($IP_3$) is known to be an important second messenger in intracellular signal transduction processes, which can also control the cellular $Ca^{2+}$ concentration.[1] *myo*-Inositol hexakisphosphate (phytate, $IP_6$) is a fully phosphorylated form of inositol (Fig. 1), which is found in blood, urine, and intracellular fluids.[2] $IP_6$ has been regarded as an antinutrient due to its ability to chelate essential trace minerals, such as Fe, Zn, and Ca.[3] Recent studies have reported beneficial properties of $IP_6$, such as blood-glucose-lowering and lipid-lowering effects, antioxidative properties and anticancer activities.[4] In addition, a previous study suggested that phytic acid is a cofactor in DNA repair by nonhomologous end-joining.[5a] Another report using yeast mutants also suggested that intracellular phytic acid may be involved in *m*RNA export from the nucleus to the cytosol.[5b] However, the exact physiological roles of intracellular phytic acid are still unclear.[5c]




$IP_3$ : *myo*-inositol 1,4,5-trisphosphate  $IP_6$ : *myo*-inositol 1,2,3,4,5,6-hexaphosphate

Fig. 1 Chemical structures of $IP_3$ and $IP_6$ employed in this study.

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† Electronic supplementary information (ESI) available: Experimental details and supplementary figures and characterization of compounds. See DOI: 10.1039/c4cc02036g

Accordingly, various detection methods for the determination of $IP_6$ have been reported, such as refractive index HPLC analysis of phytate itself or of its hydrolysis products (inositol and phosphate),[6] flow injection-capillary zone electrophoresis,[7] and gas chromatography mass spectrometry.[8] However, these methods suffer from a time consuming sample pretreatment process or the use of advanced instruments. A fluorescence chemosensing approach certainly has advantages over these methods, in particular, the opportunity for *in vivo* imaging.[9]

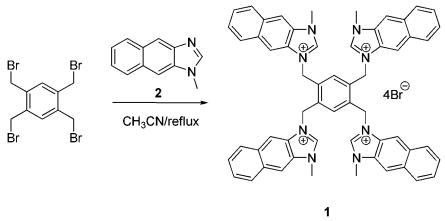
There have been some efforts to sense $IP_3$[10] and $IP_6$[11–13] *via* fluorescence changes. These methods were based on a ligand exchange in which metal ions were removed from metal complexes by IP6. Notably, the Ahn group reported a fluorogenic chemosensing ensemble for $IP_6$ using an eosine and $Cu^{2+}$ complex.[12] The Han group, on the other hand, reported a colorimetric sensing system for $IP_6$ using a combination of tris-Zn benzene derivative as the receptor unit and 11-mercaptoundecylphosphoric acid functionalized gold nanoparticles as the reporter unit.[13] Recently, Kubo and a coworker reported fluorescence sensing of $IP_6$ without using metal ions as the binding sites, in which an isothiouronium-attached polythiophene shows a selective fluorescence quenching effect with $IP_6$ at pH 5.5.[14]

Imidazolium-based receptors have been actively studied due to the unique ionic hydrogen bonding interactions between imidazolium $(C–H)^+$ groups and anions.[15] Specifically, fluorescent receptors bearing imidazoliums, naphthoimidazolium or bisbenzoimidazoliums have been recently utilized as selective fluorescent chemosensors for various anionic targets.[16]

As described above, previously reported examples utilized metal ion-complex systems. In most of these systems, metal ions are removed by $IP_6$ and thus cannot be easily applied to image $IP_6$ in the cell.

In the current study, we synthesized a new tetranaphthoimidazolium receptor **1**, which shows a selective fluorescence enhancement with $IP_6$ in a 100% aqueous system at pH 7.4.

Naphthoimidazoliums maintain the unique properties of imidazolium, such as ionic hydrogen bonding interactions, and they are inherently fluorescent, so there is no need to introduce additional fluorophores.




Scheme 1 Synthesis of tetranaphthoimidazolium receptor **1**.

For the synthesis of fluorescent chemosensor **1**, 1-methyl-1*H*-naphtho[2,3-*d*]imidazole **2** was first synthesized according to the reported procedure.[17] A mixture of **2** and 1,2,4,5-tetrakis(bromomethyl)benzene in acetonitrile afforded **1** in an 80% yield (Scheme 1). Compound **1** was fully characterized by high-resolution FAB mass spectroscopy, $^{1}$H NMR, and $^{13}$C NMR spectroscopy (ESI†).

The selectivity of **1** was tested with sodium salts of $F^-$, $Cl^-$, $CH_3CO_2^-$, $HPO_4^{2-}$, $ClO_4^-$, $NO_3^-$, $HSO_4^-$, pyrophosphate (PPi), AMP, ADP, ATP, $IP_3$, and $IP_6$ (1 equiv.) in HEPES buffer (0.02 M, pH 7.4). A relatively small monomeric emission at 370 nm and large excimer emission at 465 nm were observed in the fluorescence spectrum, as shown in Fig. 2. Among these various anionic analysts, only $IP_6$ showed a selective fluorescence enhancement at 465 nm. On the other hand, simple anions such as, PPi, AMP, ADP and ATP did not induce any significant fluorescence change (Fig. 3). Fig. S6 (ESI†) demonstrates the fluorescence titrations of **1** (5 μM) in HEPES buffer (0.02 M, pH 7.4) upon adding 0–1 equiv. of $IP_6$. A Job plot showed 1 : 1 stoichiometry between **1** and $IP_6$, as shown in Fig. 2b. An excimer emission at 465 nm linearly increases in the range of 300 nM to 1 μM with a detection limit of $2.28 \times 10^{-7}$ M (Fig. S7, ESI†).

The partial $^{1}$H NMR spectra of **1** with $IP_6$ in DMSO-$d_6$–$D_2O$ (9 : 1, v/v) are presented in Fig. 4. An imidazolium C-2 proton ($H_a$) appears as a small signal as a result of exchange with $D_2O$ because of the acidic nature of this proton. However, a downfield shift of this proton could be clearly observed upon the addition of $IP_6$. Benzylic hydrogens also displayed a slight downfield shift. These changes can be attributed to the possible hydrogen bonding interactions between these protons and phosphate groups of $IP_6$. On the other hand, there were upfield shifts for aromatic protons, which are probably due to the excimer formations between two naphthoimidazolium groups.

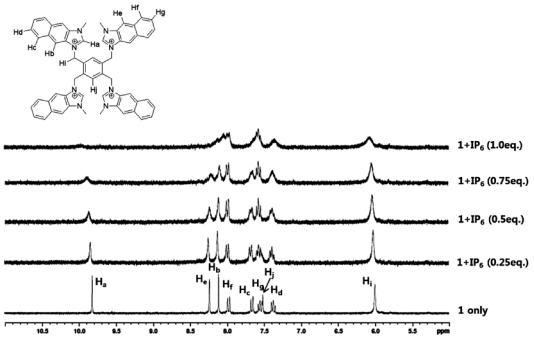



Fig. 4 Synthesis of tetranaphthoimidazolium receptor **1** in DMSO-$d_6$–$D_2O$ (9 : 1, v/v).

To obtain an insight into the binding modes and fluorescence behaviors of **1** with $IP_3$ and $IP_6$, we carried out density functional theory (DFT) and time-dependent DFT (TDDFT) calculations with the M06-2x functional using a suite of Gaussian 09 programs.[18] The optimized structures of **1**, **1** + $IP_3$ and **1** + $IP_6$ are shown in Fig. 5. Compound **1** is well stacked with the naphthoimidazolium moieties at a distance of about 4.8 Å, which can lead to intramolecular excimer formation. The phosphate groups of $IP_3$/$IP_6$ and the $(C–H)^+$ of the imidazolium moieties as well as hydrogen atoms in the alkyl side-chain of

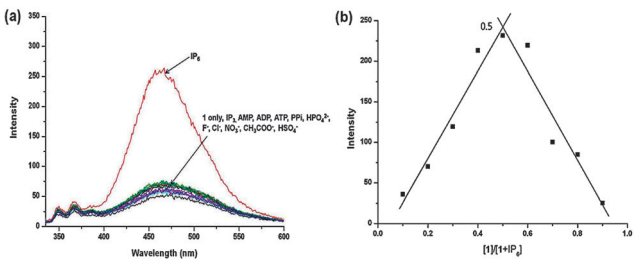



Fig. 2 (a) Fluorescence spectra of **1** (5 μM) in HEPES buffer (0.02 M, pH 7.4) upon addition of sodium salts of $F^-$, $Cl^-$, $CH_3CO_2^-$, $HPO_4^{2-}$, $NO_3^-$, $HSO_4^-$, PPi, AMP, ADP, ATP, $IP_3$ and $IP_6$ (1 equiv.). (b) Job's plot of **1** with $IP_6$ in HEPES buffer (0.02 M, pH 7.4).

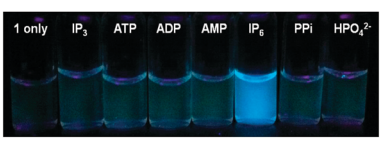



Fig. 3 Fluorescence changes of **1** (5 μM) with sodium salts of $HPO_4^{2-}$, PPi, AMP, ADP, ATP, $IP_3$ and $IP_6$ (1 equiv.) in HEPES buffer (0.02 M, pH 7.4).

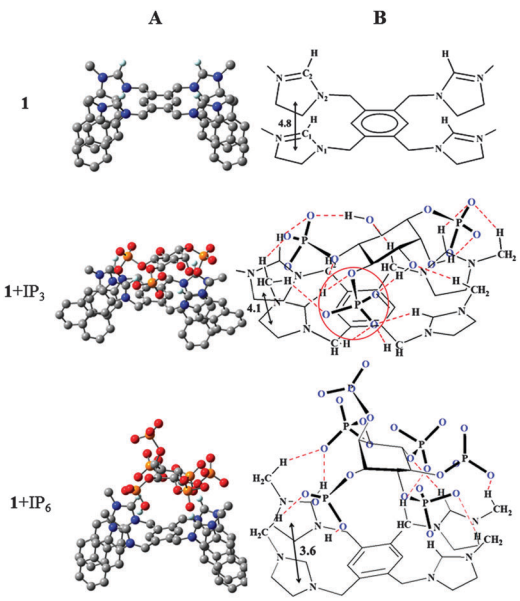



Fig. 5 Calculated structures and schematics for binding modes for the complexes of **1** with $IP_3$ and $IP_6$. Hydrogen atoms were omitted for clarity and dashed red lines indicate interactions of the phosphate with hydrogen atoms (<2.5 Å).

the imidazolium moieties are involved in interacting with $IP_3$ and $IP_6$. The π-stacking distance between naphthoimidazolium moieties was shortened to 4.1 Å and 3.6 Å for **1** + $IP_3$ and **1** + $IP_6$, respectively. In binding with $IP_6$, the naphthoimidazolium moieties are closer to each other than in binding with $IP_3$. In addition, the interplanar dihedral angle ($C_1$–$N_1$–$N_2$–$C_2$) of **1** + $IP_3$ is calculated to be 34° due to the space for motions, while it remains almost stacked with the dihedral angle of 4° in **1** + $IP_6$ due to the limited space as a result of the strong interactions with six phosphates. This structural feature is consistent with NMR experimental data, and responsible for the stronger fluorescence of **1** in binding with $IP_6$ than $IP_3$.

To investigate the fluorescence properties of the receptor upon addition of $IP_6$, TDDFT calculations were performed. The important orbital transitions to the excitation and the corresponding orbital shapes are shown in Fig. S8 (ESI†). The major transition of **1** comes from HOMO → LUMO + 3 and HOMO − 1 → LUMO + 2 transitions. Though these orbitals are localized in four naphthoimidazolium groups, the on-site transition is likely to be dominated considering the weak fluorescence observed in the experiment. Whereas in **1** + $IP_6$, HOMO − 3 → LUMO + 8 and HOMO − 4 → LUMO + 6 transitions occur where the electrons in one naphthoimidazolium group might interact with holes in another one resulting in excimer emission.

Finally, probe **1** was further applied for live cell imaging. Fluorescence images of labeled HeLa cells (adenocarcinoma) and WI38 VA-13 subclone 2RA cells (normal) are shown in Fig. 6 and Fig. S9 (ESI†). Faint fluorescence is observed in the labeled cells; however, incubation with phytic acid (5 and 50 μM) induced strong fluorescence (Fig. 6). Probe **1** successfully passed through the live cell membrane and was possibly distributed in the cytoplasm and nuclei of cells. To identify the cytotoxic effect of **1**, HeLa cells were seeded in a 24-well plate. The cells were incubated with 0, 1, 5, and 50 μM **1** for 24 h at 37 °C, and cell viability was determined by counting live cells. When the cells were treated with **1** at a concentration of 50 μM, cell viability was more than 99% compared to those without probe **1** treatment (Fig. S10, ESI†). These results indicated that **1** is nontoxic and may play a role as a bio-probe for intracellular phytic acid, which has very useful applications in bioimaging assays.

In conclusion, we report a new tetranaphthoimidazolium receptor **1** as the fluorescent chemosensor for phytate, *myo*-inositol hexakisphosphate ($IP_6$), in 100% aqueous solution at pH 7.4. The fluorescent receptor **1** displayed a selective fluorescence enhancement with $IP_6$. The other simple anions, phosphates, pyrophosphates, AMP, ADP, ATP and $IP_3$ did not induce any significant fluorescence change. The possible binding modes and fluorescence changes are also explained by theoretical calculations. We further showed the first successful *in vivo* imaging of $IP_6$ in cells by using a relatively simple naphthoimidazolium-based fluorescent probe. By using this relatively simple receptor, we could obtain reasonable selectivity for $IP_6$ in 100% aqueous solution at pH 7.4.

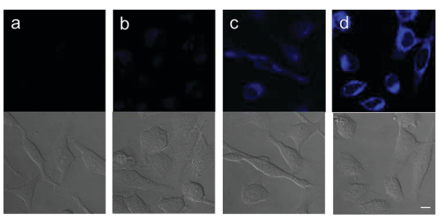



Fig. 6 Confocal fluorescence images of **1** in HeLa cells. (a) No **1** (b) 30 μM **1** after 30 min. (c) 30 μM **1**, 5 μM phytic acid ($IP_6$) after 30 min. (d) 30 μM **1**, 50 μM phytic acid ($IP_6$) after 30 min. Lower images: bright field. ex 405/em BP 420–480 nm, scale bar: 10 μm.

This research was financially supported by a grant from the National Creative Research Initiative programs of the National Research Foundation of Korea (NRF) funded by the Korean government (MSIP) (No. 2012R1A3A2048814). The work at the Sungkyunkwan University was supported by the NRF grant (2007-0056343) funded by MEST. JYL acknowledges the support from KISTI supercomputing center through the strategic support program for the supercomputing application research (No. KSC-2013-C2-027).

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