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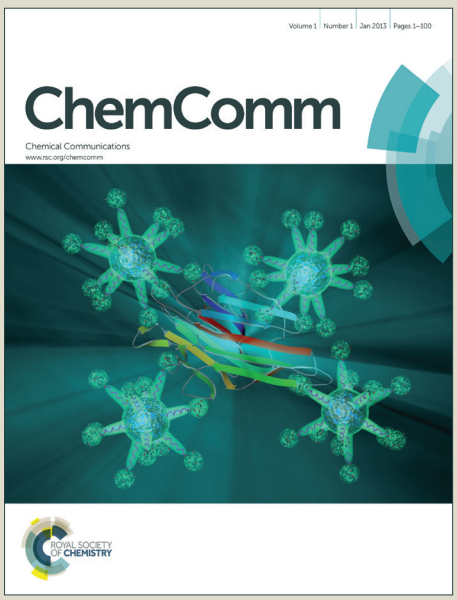

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ARTICLE TYPE

A Tetranaphthoimidazolium Receptor as a Fluorescence Chemosensor for Phytate†

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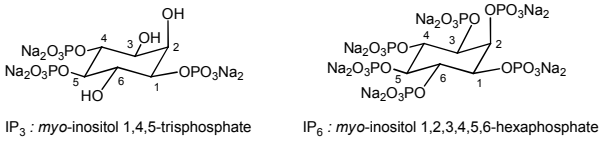
A new tetranaphthoimidazolium receptor was synthesized and reported as a selective fluorescent chemosensor for phytate, *myo*-inositol hexakisphosphate (IP_6). In a 100% aqueous solution at pH 7.4, chemosensor 1 showed a selective fluorescence enhancement for IP_6 over IP_3, phosphate, pyrophosphate, 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 as 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 report 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



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.

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]

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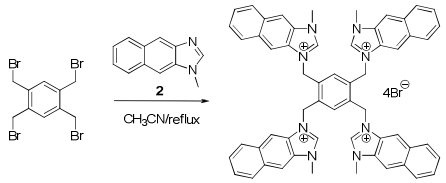
Accordingly, various detection methods for the determination of IP_6 have been reported, such as refractive index HPLC analysis for phytate itself or of its hydrolysis products (inositol and phosphate),[6] flow injection-capillary zone electrophoresis,[7] and gas chromatography mass spectrometry.[8] However, these methods suffer from a time consuming sample pretreatment process or advanced instruments. A fluorescent 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 fluorescent changes. These methods were based on a ligand exchange in which metal ions were removed from metal complexes by IP_6. Notably, the Ahn group reported a fluorogenic chemosensing ensemble for IP_6 using an eosine and Cu^{2+} complex.[12] 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 coworker reported fluorescence sensing of IP_6 without using metal ions as the binding sites, in which an isothiouronium-attached polythiophene showes a selective fluorescence quenching effect with IP_6 at pH 5.5.[14]



Scheme 1. Synthesis of tetranaphtoimidazolium receptor **1**.

Imidazolium-based receptors have been actively studied due to the unique ionic hydrogen bonding interactions between imidazolium $(C\text{-}H)^+$ 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 were 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.

For the synthesis of fluorescent chemosensor **1**, 1-methyl-1H-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** at an 80% yield (Scheme 1). Compound **1** was fully characterized by high-resolution FAB mass spectroscopy, ^{1}H NMR, and ^{13}C NMR spectroscopy (Supporting Information).

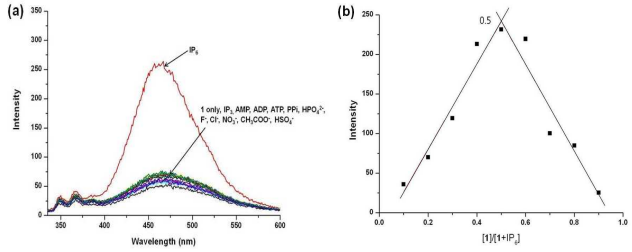


Fig. 2 (a) Fluorescent spectra of **1** (5 μM) in HEPES buffer (0.02 M, pH 7.4) upon addition of sodium salts of of F^-, Cl^-, $CH_3CO_2^-$, HPO_4^{2-}, NO_3^-, HSO_4^-, PPi, AMP, ADP, ATP, IP_3 and IP_6 (1 equiv.). (b) Job's plot of **1** with IP_6 in HEPES buffer (0.02 M, pH 7.4).

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. S6 demonstrates the fluorescence titrations of **1** (5 μM) in HEPES buffer (0.02 M, pH 7.4) upon adding of 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 2.28×10^{-7} M (Fig. S7).

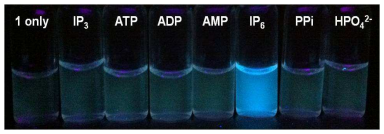


Fig. 3 Fluorescence changes of **1** (5 μM) with sodium salts of HPO_4^{2-}, PPi, AMP, ADP, ATP, IP_3 and IP_6 (1 equiv.) in HEPES buffer (0.02 M,

The partial ^{1}H NMR spectra of **1** with IP_6 in DMSO-d_6-D_2O (9:1, v/v) is presented in Fig. 4. Imidazolium C-2 proton (H_a) appears as a small signal 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 slight

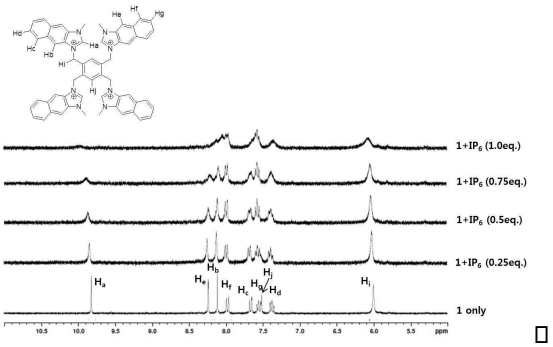


Fig. 4 Synthesis of tetranaphtoimidazolium receptor **1** in DMSO-d_6-D_2O (9:1, v/v).

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.

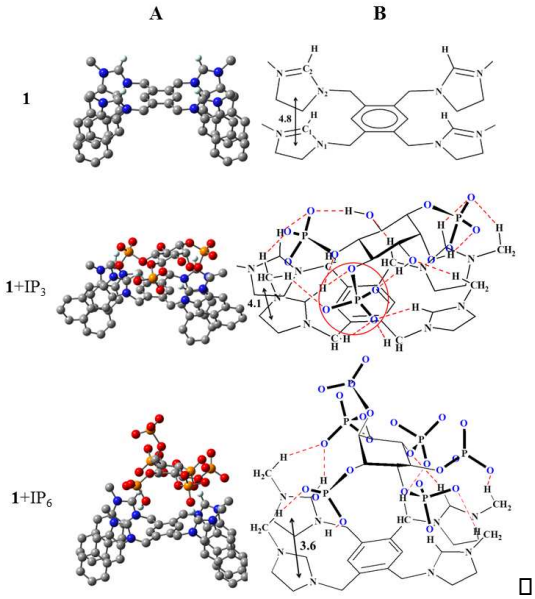


Fig. 5 Calculated structures and schematics for binding modes for the complexes of **1** with IP_3 and IP_6. Hydrogen atoms were omitted for clarity and dashed red lines indicate interactions of the phosphate with hydrogen atoms (>2.5 Å).

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 distances 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 the imidazolium moieties are involved in interacting with IP_3 and IP_6. The π-stacking distance between naphthoimidazolium moieties was shortened to be 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 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 keeps almost stacking with the dihedral angle of 4° in **1**+IP_6 due to the limited space through 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 property of receptor upon addition of IP_6, TDDFT calculations were performed. The important orbital transitions to the excitation and the corresponding orbital shapes were shown in Fig. S8. 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 experiment. Whereas in **1**+IP_6, HOMO-3→LUMO+8 and HOMO-4→LUMO+6 transitions where the electrons in one naphthoimidazolium group might interact with holes in another one resulting excimer emission.

Finally, probe **1** was further applied for live cell imaging. Fluorescence images of HeLa cells (adenocarcinoma) and WI38 VA-13 subclone 2RA cells (normal) labeled are explained in Fig. 6 and Fig. S9. It can be seen 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

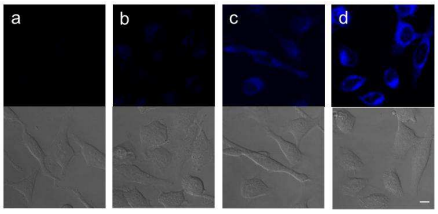


Fig. 6 Confocal fluorescence images of **1** in HeLa cells. (a) no **1** (b) 30 μM **1** after 30 mins. (c) 30 μM **1**, 5 μM Phytic acid (IP_6) after 30 mins. (d) 30 μM **1**, 50 μM Phytic acid (IP_6) after 30 mins. Lower images: bright field. ex 405/em BP 420-480 nm, scale bar: 10 μm.

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 50 μM of **1**, cell viability was more than 99% compared to those without **1** treatment (Fig. S10). 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, phosphate, pyrophosphate, 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.

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A Tetranaphthoimidazolium Receptor as a Fluorescence Chemosensor for Phytate

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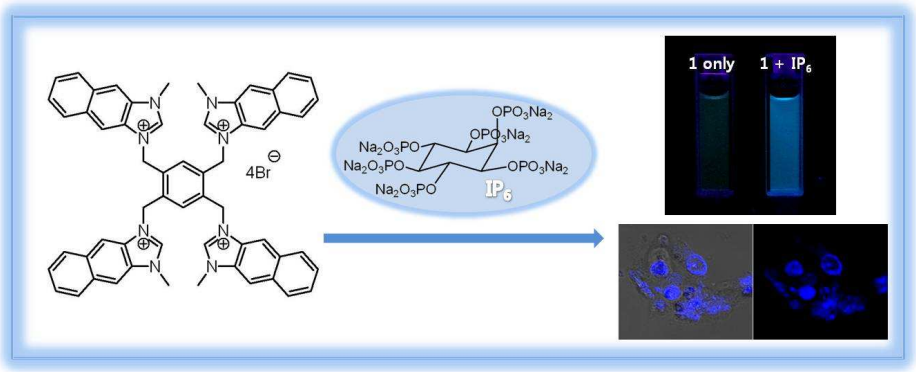

A new tetranaphthoimidazolium receptor showed a selective fluorescence enhancement with phytate, *myo*-inositol hexakisphosphate (IP_6) in 100 % aqueous solution at pH 7.4.