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Pyridinium-based flexible tripodal cleft: A case of fluorescence sensing of ATP and dihydrogen phosphate under different conditions and cell imaging

Kumares Ghosh, Debojyoti Taraftar, Asmita Samadder and Anisur Rahman Khuda-Bukhsh

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Pyridinium-based chemosensor 1 built on tris(aminomethyl)amine (tren) has been designed, synthesized and established as a chemosensor for ATP over ADP, AMP and a series of other anions in aqueous CH$_3$CN at pH 6.5. Compound 1 exhibits significant change in emission upon complexation of ATP. In CH$_3$CN, the sensor selectively binds H$_2$PO$_4^-$ and forms excimer with significant intensity. Furthermore, the intracellular ATP detection using 1 has been possible through fluorescent confocal imaging.

Introduction

Anions play key role in a variety of environmental and biological processes.$^1$ In particular, phosphate–based biomolecules such as ATP, ADP, AMP and related inorganic phosphates are considered to be important due to their ubiquitous presence in a range of life processes spanning from energy storage and signal transduction to gene construction.$^2$ ATP is associated with the transport of chemical energy within the cells for metabolism. Apart from its role in intracellular energy transfer, ATP is involved in DNA duplication and transcription.$^3$ Deficiency of ATP results in ischemia, Parkinson’s disease and hypoglycaemia.$^4$ Therefore, selective detection of ATP or inorganic phosphates released from the hydrolysis of the phosphate chain is considered to be important in anion recognition chemistry. In detection of these anions, use of fluorescent receptors draws attention due to the simplicity and high sensitivity of fluorescence technique. Considerable effort in this direction has already been given by different research groups in last few decades.$^5$ Inspite of reasonable progress, the use of organic cation in devising fluorescent receptors for nucleotides is less explored.$^6$ Survey of the literature reveals that metal-ligand complexes have been maximally used for phosphate and phosphate-based biomolecule recognition.$^7$ In this paper, we wish to report a simple tris(aminomethyl)amine (tren) coupled pyridinium-based tripodal chemosensor 1 that fluorimetrically selectively senses ATP over ADP, AMP and a series of other anions in CH$_3$CN/H$_2$O (1:1, v/v, pH = 6.5, 10 mM HEPES buffer). Furthermore, while the sensor was non responsive to different inorganic phosphates in CH$_3$CN/H$_2$O (1:1, v/v, pH = 6.5), it exhibited strong and selective interaction with H$_2$PO$_4^-$ in pure CH$_3$CN. The versatility of 3-aminopyridinium-based compounds in anion recognition is recently reviewed by us.$^8$ Careful scrutiny in this domain, reveals that pyridinium-based compounds is little explored in nucleotide binding although we, for the first time, reported dipodal$^6$ and tripodal$^6$ receptors for selective sensing of ATP and AMP, respectively. Compound 1 in the present report is the modification of our earlier reported tripod 2 that showed selective sensing of AMP in water.$^6$ The replacement of the butyryl amide in 2 by 1-naphthyl acetamide group results in compound 1 which selectively binds ATP in aqueous CH$_3$CN and thus makes the pyridinium motif-based tripod core versatile in nucleotide binding.

Scheme 1 describes the synthetic route to the synthesis of compound 1. The reaction of tris(2-aminoethyl)amine with chloroacetyl chloride in CHCl$_3$-H$_2$O (1:1, v/v) gave amide 3.$^9$ Amide 3 was next refluxed with pyridine amide which was obtained from the reaction of 3-aminopyridine with 1-naphthyl acetyl chloride 4, in dry CH$_3$CN-DMF (5:1, v/v) mixture solvent for 6 days to have pure trichloride salt 6. Subsequent anion...
exchange reaction of 6 with NH₄PF₆ in MeOH–H₂O gave the desired compound 1. All the compounds were characterized by usual spectroscopic techniques.

![Scheme 1](image)

**Scheme 1.** (i) Chloroacetyl chloride, K₂CO₃, CHCl₃–H₂O, stir 4h; (ii) oxalyl chloride, dry CH₂Cl₂, DMF (cat.), stir 8h; (iii) 3-aminopyridine, Et₃N, dry CH₂Cl₂, stir 3h; (iv) 3, CH₂CN, reflux, 4 days; (v) NH₄PF₆, MeOH–water, stirring for 1/2 h.

### Results and discussion

The anion recognition potential of compound 1 was evaluated by fluorescence, UV-vis and NMR spectroscopic techniques. In fluorescence, excitation of 1 in CH₃CN/H₂O (1:1, v/v, pH = 6.5, 10 mM HEPES buffer) at 290 nm introduced the emission at 386 nm. Upon interaction with some biologically relevant phosphate2 buffer; (b) change in fluorescence intensity of oxalyl chloride, dry CH₂Cl₂, DMF (cat.), stir 8h; (iii) 3-aminopyridine, Et₃N, dry CH₂Cl₂, stir 3h; (iv) 3, CH₂CN, reflux, 4 days; (v) NH₄PF₆, MeOH–water, stirring for 1/2 h.

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In this case, only F⁻ ion was observed to interfere to some extent although F⁻ induced change in emission of 1 in CH₃CN/H₂O (1:1, v/v, pH = 6.5, 10 mM HEPES buffer) was considerably small (ESI, Fig. 2S).

The linearity in Benesi-Hilderband plot in Fig. 3a corresponds to a 1:1 stoichiometric interaction between receptor 1 and ATP. This was also true for ADP (ESI, Fig. 3S). The association constant values were calculated from fluorescence titration data and they were found to be 1.16 x 10⁹ M⁻¹ and 1.10 x 10⁹ M⁻¹ for ATP and ADP, respectively. The detection limit for ATP was determined to be 8.44 x 10⁻⁶ M (ESI, Fig. 3S).

![Fig 1.](image)

**Fig 1.** (a) Change in emission ratio of 1 (c = 2.5 x 10⁻⁵ M) in the presence of 20 equiv. amounts of the sodium salts of various anions in CH₃CN–H₂O (1 : 1, v/v, pH = 6.5, 10 mM HEPES buffer); (b) Change in emission of 1 (c = 2.5 x 10⁻⁵ M) in CH₃CN–H₂O (1 : 1, v/v, pH = 6.5, 10 mM HEPES buffer) upon gradual addition of 30 equiv. of ATP (c = 1 x 10⁻⁵ M) (λexc = 290 nm).

![Fig 2.](image)

**Fig 2.** (a) Change in fluorescence ratio of 1 (c = 2.5 x 10⁻⁵ M) in absence and presence of 20 equiv. amounts of ATP in presence of sodium salts of various anions in CH₃CN–H₂O (1 : 1, v/v, pH = 6.5, 10 mM HEPES buffer); (b) change in fluorescence intensity of 1 in CH₃CN–H₂O (1 : 1, v/v, pH = 6.5, 10 mM HEPES buffer) upon addition of 20 equiv. amount of ATP in presence of other anions considered in the present study (λexc = 290 nm).

![Fig 3.](image)

**Fig 3.** Benesi-Hilderband plots for receptor 1 (c = 2.5 x 10⁻⁵ M) with (a) ATP and (b) ADP ([ATP] = [ADP] = 1 x 10⁻⁵ M) at 390 nm.

In UV-vis spectroscopy, the triiodal receptor 1 in CH₃CN/H₂O (1:1, v/v, pH = 6.5, 10 mM HEPES buffer) showed absorptions at 252 nm and 285 nm. During interaction with ATP, the band at 285 nm which was attributed to the absorption of naphthalene underwent minor change although the absorption at 252 nm was significant. This was true for ADP and AMP also and thus the distinction of these three nucleotides by 1 was not possible in the ground state (ESI, Fig. 4S). For other anions the change in absorption of the two peaks (252 nm and 285 nm) was negligible and thereby suggested the mere interaction of the receptor in the ground state (ESI, Fig. 4S).

In order to be acquainted with the anion binding behaviour of 1 in non-aqueous solvent, we investigated the interaction of 1 with the anions such as F-, Cl-, Br-, I-, NO₃⁻, AcO-, ClO₄⁻, HSO₄⁻, HP₂O₇²⁻ and H₂PO₄⁻ (taken as their tetrabutylammonium salt) in pure CH₃CN (ESI, Fig. 5S). Among these anions, only H₂PO₄⁻ perturbed the emission of 1 markedly by showing excimer emission at 455 nm. We believe that this excimer emission at 455
nm which is retained in the midst of other anions (ESI, Fig. 6S), results in from the complexity induced pulling of the pendant naphthalenes closely. This observation is in accordance with the findings observed by us\(^6\) and other researchers.\(^1\)

Thus, this unique feature of emission can be considered as the diagnostic one for discrimination of H\(_3PO_4^–\) from the other anions examined in CH\(_2CN\). In UV–vis study, upon gradual addition of H\(_3PO_4^–\), the absorption of I was changed irregularly (ESI, Fig. 7S). In contrast, gradual addition of F\(^–\) ion initially did not bring any marked change in absorption spectra. But upon increasing the concentration of F\(^–\), the absorption at 252 nm was decreased followed by an appearance of a new absorption band at ~300 nm. Such ratiometric response introduced an isoemissive point at 284 nm. This suggests a strong ground state interaction of the association constant

These findings observed by us\(^\) from addition of 2 equiv. amounts of H\(_2PO_4^–\) to the solution of I.

The detection limit for H\(_3PO_4^–\) was determined to be 1.46 x 10\(^–6\) M (ESI, Fig. 9S).\(^10\)

Prior to identify the interacting protons of I in the complexation, we recorded NOESY spectrum of I in d\(_6\)-DMSO (Fig. 4). We did not record the NMR spectra in CD\(_3)CN due to appearance of non homogeneity of the solution of I in NMR concentration range. As can be seen from Fig. 4, naphthalene appended pyridinium motifs in the tripod are disposed in spread out conformation by exhibiting some correlations in the individual strand. In the presence of equivalent amount of H\(_3PO_4^–\) in d\(_6\)-DMSO some correlations of protons in each pod as shown in Fig. 4 were destroyed (ESI, Fig. 10S). Even no correlation among the pendant naphthalenes was observed. This finding suggests that the hydrogen bonding interaction of H\(_3PO_4^–\) occurs into the core involving pyridinium sites in such a way that the naphthalene rings from the different arms are pulled weakly without showing any characteristic NOESY correlation which depends on the distance of the nuclei. The details along this direction including the change in \(^1\)H-NMR of I during complexation of H\(_3PO_4^–\) can be found in the supporting information (ESI, Fig. 11S).

ATP amplified prominent change of optical behaviour of receptor I in fluorescence was understood from \(^1\)H NMR spectral change. In this experiment receptor I was taken in d\(_6\)-DMSO and to this solution ATP (dissolved in D\(_2\)O) was added in equivalent amount. In the presence of equiv. amount of ATP, amide protons of types ‘o’ and ‘d’ underwent downfield chemical shifts by 0.74 ppm and 0.45 ppm, respectively. On keeping the solution for ~15 mins, the exchangeable amide protons were not observed due to presence of D\(_2\)O in the mixture. The pyridinium ring protons (types ‘b’ and ‘c’) moved downfield (Fig 5). These results indicated that the pyridinium sites in I are involved in complexation of the phosphate chain. The downfield chemical shifts of the methylene protons of types ‘n’ and ‘m’ by 0.57 ppm and 0.27 ppm, respectively also suggested their participation in hydrogen bonding with the phosphate chain. Furthermore, during interaction the naphthyl ring protons (i,j,k and l types) suffered upfield chemical shift by 0.11 ppm and the adenine ring protons moved upfield by 0.001-0.03 ppm. These changes in chemical shift values may be ascribed to the stacking of adenine ring with the naphthyl unit (Fig. 6). Analysis of the NOESY spectrum reveals that in I,ATP complex, there is a cross-peak between the signal of adenine ring proton (δ 8.70 ppm) and methylene protons of type ‘m’ (Fig. 7). This observation means that adenine is located closely to naphthalene moiety in the suggested mode shown in Fig. 6 for which naphthalene-adenine-naphthalene π-stacking interaction exists in solution and allows the formation of exciplex at 390 nm (see Fig. 1b) in fluorescence.

Further attempts have been made to understand the biological application of the compound I for detection of ATP under cellular condition in HEK293T cell line (Human Embryonic Kidney cells with T antigen of SV40) using confocal microscopy.
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Fig 6. Probable binding mode of 1 with ATP.

Fig 7. NOESY spectrum of \( \text{LATP (d}_6\text{DMSO/D}_2\text{O, 400 MHz)} \) (c = 1.83 \( \times \) 10\(^{-3} \) M).

(Fig. 8). Microscopic images revealed a significant increase in the blue fluorescence in HEK293T cells incubated with both ATP and compound 1 (J) when compared to cells treated with only compound 1 (F) and/or normal untreated cells (B). This finding suggests that the compound 1 is permeable through cellular membrane and is able to sense the presence of intracellular ATP. In MTT assay, there was no significant difference in percentage of viable cells of receptor treated cells when compared to that of solvent treated series of cell sets (ESI, Fig. 12S). This indicates that the receptor is non cytotoxic in nature.

Conclusion

We have thus reported a pyridinium motif coupled \textit{tren}-based tripodal receptor 1 which in aqueous CH\(_3\)CN selectively recognizes ATP over ADP, AMP, other inorganic phosphates and non-phosphate containing anions based on fluorescence. In pure CH\(_3\)CN, receptor 1 recognizes tetrabutylammonium dihydrogenphosphate flurometrically by exhibiting diagnostic excimer emission. The recognition takes place through hydrogen bonding and charge-charge interactions at the pyridinium sites. Our previous report on receptor 2 as well as the present observation of 1 firmly establishes that the tripodal core of 1,

Fig 8. Confocal microscopy of different sets of normal untreated and experimental sets of HEK293T cells: (A) Differential interference contrast (DIC) image and (B) Fluorescing image of untreated control cells, (C) Merge images of A + B, (D) Intensity profile of B (Scale: X axis= Length (\( \mu \text{m} \)) [0.32 \( \mu \text{m/Pixel} \), Y axis = Intensity/Pixel), (E) DIC image and (F) Fluorescing image of cells treated with 1 (10 \( \mu \text{M} \)) for 1 h at 37\(^\circ\)C, (G) Merge images of E + F, (H) Intensity profile of F (Scale: X axis= Length (\( \mu \text{m} \)) [0.32\( \mu \text{m/Pixel} \), Y axis = Intensity/Pixel), (I) DIC image and (J) Fluorescing image of cells which were pre incubated with 50 \( \mu \text{M} \) ATP and then treated with 1 (10 \( \mu \text{M} \)) for 30 min at 37\(^\circ\)C, (K) Merge images of I + J, (L) Intensity profile of one of the representative fluorescing cells in J (Scale: X axis= Length (\( \mu \text{m} \)) [0.32\( \mu \text{m/Pixel} \), Y axis = Intensity/Pixel).

...comprised of pyridinium motifs, is a unique platform of sensing nucleotides. While the absence of aromatic surface onto the pyridinium motif in 2 directs the binding of AMP in pure water, the presence of naphthalenes in 2 modifies the cleft to sense ATP over ADP and AMP in aqueous CH\(_3\)CN. This versatility of the pyridinium motif-based tripodal core will definitely inspire chemists to enquire new findings in molecular recognition.

Experimental

Syntheses

\( \text{N,N',N''-(2,2',2''-Nitrilotris(ethane-2,1-diyl))tris(2 chloroaacetamide)} \) (3):\(^{26} \) To a stirred solution of tris-(2-aminoethyl) amine (0.6 g, 4.10mmol) in CHCl\(_3\) (30 mL), chloroacetyl chloride (1.53 g, 13.54 mmol) was added dropwise. Then water (10 mL) was added to the reaction mixture followed by the addition of K\(_2\)CO\(_3\) (1.87 g, 13.54 mmol). Catalytic amount of tetrabutylammonium hydrogensulphate was added to the reaction mixture and stirred for 2h. After completion of the
reaction, as monitored by TLC, organic layer was separated and dried over anhydrous Na$_2$SO$_4$. The solution was concentrated on a rotary evaporator and purified by silica gel column chromatography using 1% CH$_3$OH in CHCl$_3$ as eluent to afford pure compound 3 (1.40 g, 90%), mp 102°C; $^1$H NMR (CDCl$_3$, 400MHz) δ 7.05 (br s, 3H), 4.08 (s, 6H), 3.38 (q, 6H, J = 4 Hz), 2.65 (t, 6H, J = 4 Hz); FTIR (KBr, cm$^{-1}$) 3515, 3273, 2961, 1677, 1605, 1562.

2-(Naphthalen-1-yl)acetyl chloride (4): To a stirred solution of 1-naphthalic acid (0.700 g, 3.76 mmol) in dry CH$_2$Cl$_2$ (20 mL), oxalyl chloride (0.365 mL, 4.51 mmol) was added followed by addition of one drop of dry DMF. After stirring at room temperature for 8 h under nitrogen atmosphere, excess oxalyl chloride was completely removed under vacuum to give acid chloride 2 (0.720 g, yield 93%). This was directly used in the next step without any purification.

2-(Naphthalen-1-yl)-N-(pyridin-3-yl)acetamide (5): To a stirred solution of 3-aminopyridine (0.390 g, 4.14 mmol) in dry CH$_2$Cl$_2$ (3 x 40 mL). Organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. The crude product was purified by chromatography using 50% ethyl acetate in petroleum ether to give 5 in 88% yield (0.800 g); m.p. 125°C; $^1$H NMR (400 MHz, DMSO$_d_6$) δ 8.27–8.24 (m, 2H), 8.02–7.99 (m, 2H), 7.92–7.87 (m, 2H), 7.59–7.55 (m, 2H), 7.53–7.49 (m, 2H), 7.20–7.17 (m, 2H), 4.20 (s, 2H); $^{13}$C NMR (100 MHz, DMSO$_d_6$) δ 169.9, 145.1, 141.1, 134.6, 134.0, 133.0, 130.4, 123.9, 122.6, 119.5, 118.4, 117.9, 117.0, 126.3, 125.6, 123.6, 123.5, 42.33; FTIR (KBr, cm$^{-1}$) 3281, 3122, 2850, 1742, 1658, 1320, 1303, 1289, 1288, 1247, 1234, 1234, 1165, 940, 1481 cm$^{-1}$.

Receptor 1: To a solution of tris amide 3 (0.150 g, 0.399 mmol) in CH$_3$CN (10 mL), compound 5 (0.419 g, 1.60 mmol) in CH$_3$CN (25 mL) was added. The reaction mixture was refluxed with stirring for 4 days under nitrogen atmosphere. On cooling the reaction mixture, precipitate appeared. The precipitate was filtered off and washed with CH$_3$CN for several times to have pure trichloride salt 6 (0.374 g, 80.7%). The pure trichloride salt 5 (0.100 g, 0.07 mmol) was dissolved in 2 mL hot MeOH, and NH$_4$PF$_6$ (0.053 g, 0.32 mmol) was added in one portion. After the reaction mixture for 20 min, water was added to precipitate the compound. Repeated washing of the precipitate with water and diethyl ether afforded the desired salt 1 in 82% yields (0.102 g), mp. 210°C; $^1$H NMR (400 MHz, DMSO$_d_6$) δ 11.37 (s, 3H), 9.35 (s, 3H), 8.57 (s, 3H), 8.39 (brs, 6H), 8.04–7.86 (m, 12H), 7.52–7.49 (m, 12H), 5.31 (s, 6H), 4.26 (s, 6H), 3.39 (6H overlapped with the signal for d$_6$-DMSO water), 3.12 (brm, 6H); $^{13}$C NMR (100 MHz, DMSO$_d_6$) δ 170.5, 164.1, 140.7, 138.4, 135.9, 134.4, 133.2, 131.8, 128.4, 128.4, 127.6, 126.2, 125.7, 125.4, 124.0, 61.9, 52.5, 38.7; 37.3 (one carbon in aromatic region is unresolved); FTIR (KBr, cm$^{-1}$) 3399, 3098, 1688, 1594, 1553, 1508, 1458; HRMS (TOF MS ES$^+$) calc for C$_{24}$H$_{16}$N$_{10}$O$_{6}$·2PF$_6$: 1345.4210 (M - PF$_6$); found: 1345.4215 (M - PF$_6$).

Cell culture reagents:
MTT [3-(4, 5-Dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide], and DMSO were of analytical grade and they were procured from Sigma-Aldrich Inc. (St-Louis, MO, USA); Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and antibiotics, namely, penicillin, streptomycin, and neomycin (PSN) were purchased from Gibco BRL (Grand Island, NY, USA). All organic solvents used were of high performance liquid chromatography grade.

Cell culture procedure:
HEK293T cells (Human embryonic kidney cells with T antigen of SV40), were obtained from National Centre for Cell Science, Pune, India. Cells were grown in 5% carbon dioxide, at 37°C in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% foetal bovine serum and 1% antibiotic (PSN). For experimental studies, the cells were allowed to grow to 70–80% confluence, after which they were harvested in ice-cold buffer saline (PBS) and plated at desired density. The cells were allowed to re-equilibrate for 24 h before any treatment.

Cytotoxicity assessment of receptor in living cells:
To check whether the receptor render any cytotoxicity to the cells, the MTT assay was conducted by following the standard technique of Mossman. Different concentrations of the receptor, ranging from 1µl through 5µl of stock solution, were added to the cultured cells (10$^6$ cells/ml) in 96-well micro plates and incubated for 24h. A set of cells, not exposed to any of the concentrations of the receptors, were kept as untreated control. At the termination of incubation period, MTT was added into each well. After 4 h, the formazons crystals thus formed were dissolved with dimethyl sulfoxide (DMSO) and the absorbance of the solution was measured at 595 nm using ELISA reader (Thermo scientific, Multiskan ELISA, USA). The percentage of cell survival was calculated as: (mean experimental absorbance/mean control absorbance) × 100%.

Evaluation of potential efficacy of the receptor to detect the presence of ATP in cultured cell line:
At first the cells were washed with phosphate buffered saline (PBS). They were then incubated in 20 µl of ATP solution (50µM). After 30 min of incubation at 37°C, the cells were again washed in PBS and then further treated with 30µl of receptor stock solution (10µM) made in PBS (maintained at pH 6.5) and kept for another 30 min at 37°C. Washing excess of receptor with PBS the cells were then photographed under Andor Spinning Disk Confocal Microscope and evaluated against untreated control set of cells. The fluorescence intensity was quantified using the Analysis software, NIS Elements AR Version 4.00.

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References


