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Cell permeable fluorescent receptor for detection of $H_2PO_4^-$ in aqueous solvent†

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A new colorimetric fluorescent receptor for $H_2PO_4^-$ is reported in this communication. The receptor can detect dihydrogen phosphates optically by developing a color change from yellow to green. Acute spectral responses to $H_2PO_4^-$ in HEPES buffer (DMSO–HEPES 1 : 9) have been observed. The selectivity zone in terms of pH of the receptor for $H_2PO_4^-$ is attributed to the fitness in the acidity (pK_a) of receptor with $H_2PO_4^-$. Hydrogen bonding plays the key role here which is confirmed by ^1H NMR titration. The receptor also has good potential for bio-imaging. The mode of interaction has also been established by *ab initio* calculation.

Introduction

The development of simple receptors for selective sensing of anionic substrates is an active area of research today.[1] Many chemosensors and receptors for metal ions and pH sensors have been developed.[2] But now there is an increasing interest in anion recognisation[3a–c] and anion sensing[3d,e] due to their intensive role in biology, pharmacy and environmental science.[4] Among the reported methods,[5] fluorescent receptors possess innate advantages over receptors of other types because of their high sensitivity, specificity, simplicity of implementation and fast response times, offering application methods not only for *in vitro* assays but also for *in vivo* imaging studies.[6]

Among the interest in biologically functional anions, phosphate and its derivatives are of the particular importance owing to their established role in transduction, energy storage, and gene construction in biological systems. Moreover phosphate is the key intermediate for many biochemical reactions and is the main component of biomolecules. It is a component of adenosine triphosphate (ATP), a fundamental energy source in living things and is deeply involved in DNA duplication and transcription.[7] Phosphate is also an essential ingredient of bone matrix and teeth. In addition, chemotherapeutic and antiviral drugs have phosphates as integral structural components. Furthermore, phosphates are industrially important components of both medicinal drugs and fertilizers. Pollution from phosphate and phosphorylated compounds is in part responsible for the eutrophication of natural water sources, leading to a dangerous increase in toxic algal blooms.[8] The adverse effect of excess phosphate in blood causes hyperphosphatemia, hyperparathyroidism, soft tissue calcification, kidney failure, cardiovascular complications resulting from the development of metastatic calcifications at the cardiac level, and increased morbidity and mortality.[9]

For the last few decades different types of fluorescent sensor such as neutral amide type sensor derived from urea, thiourea, imidazole, indole, amine, pyrrole and sulphonamide moieties with hydrogen bond donor functionalities[10] and cationic receptors derived from ammonium, guanidium, quinolinium, phosphonium protonated quinoxaline salts, with electrostatic interaction[11] between the anionic guest and cationic receptors have been used for anion sensing studies. More recently metal complexes have also been used for detection of anionic analyte.[12,2b]

Several synthetic receptors including metal complexes have been reported for sensing phosphate,[3c,13a–d] and pyrophosphate[13e,f] but only a few for dihydrogen phosphate have been reported.[3a,13g] However among them some receptors involve a fluorescence "turn-off" response as the readout mechanism.[13h] Limitations of the currently available "turn-on" phosphate sensor include lack of selectivity,[13i] low sensitivity and/or excitation profiles in the ultraviolet region.[13j] Recently Zn-complexes[13a] have been shown to have a tendency to sense dihydrogen phosphate but to prepare the probe in this way is time consuming and tedious and they are not practicable for intracellular imaging.

To overcome this problem we have designed and synthesized a new small Schiff-base compound that

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fluorometrically recognizes one of the most important anions dihydrogen phosphate by hydrogen bonding with bio-friendly visible region excitation and intracellular imaging.

Experimental

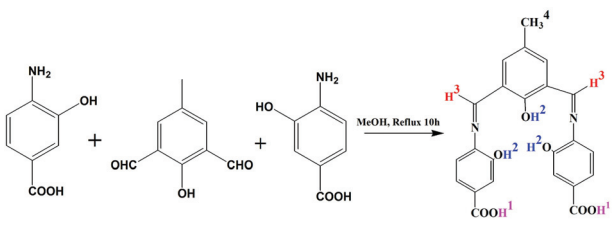
Instruments and reagents

The elemental analyses (C, H and N) were performed on a Perkin Elmer 2400 CHN elemental analyzer. Electronic absorption spectra were recorded on a JASCO UV-Vis spectrophotometer model V-570. IR spectra were recorded on a JASCO FTIR spectrophotometer (model: FTIR-H20) using KBr disks. ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer using TMS as an internal standard in DMSO-d_6. ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer using TMS as an internal standard in DMSO-d_6. Electron spray ionization (ESI) mass spectra were recorded on a Qtof Micro YA263 mass spectrometer. The measurement of pH was done with the help of a digital pH meter, Equip-Tronics (ER-614A) model. The fluorescence spectra of the titration of phosphate with the receptor were recorded using the fluorometer Hitachi-4500.

All reactions were carried out under nitrogen atmosphere while workup procedures were done in air. All the solvents utilized in this work were obtained from Merck with analytical reagent grade and have been used without further purification. Milli-Q 18 Ω water was used throughout all the experiments. 4-Amino-3-hydroxybenzoic acid and all reagents were purchased from Sigma-Aldrich and Merck, India respectively. 4-Methyl-2,6-diformylphenol was prepared according to literature procedures.[2b] The selectivity study of the receptor towards different anions was carried out using tetrabutylammonium salts of fluoride, chloride, bromide, iodide, acetate, dihydrogen phosphate and sodium salt of monohydrogen phosphate, sulphate, azide, thiocyanate, nitrate.

Synthesis of 4-methyl-2,6-bis((3-hydroxy-4-carboxyphenyl)-imino)phenol (1)

To synthesize (**1**) firstly 4-methyl-2,6-diformylphenol was synthesized starting from *p*-cresol by following a published procedure.[2b] Then 3-hydroxy-4-aminobenzoic acid (0.306 g; 2 mmol in 10 mL methanol) was added to the methanolic solution of 4-methyl-2,6-diformylphenol (0.164 g; 1 mmol in 25 mL) as shown in Scheme 1. Then the reaction mixture was refluxed at 80 °C for 12.0 h in nitrogen atmosphere. After the solution was cooled, the reaction mixture was filtered and a red colored crystalline solid (**1**) was obtained after evaporation with 90% yield. The purity was checked by ^{1}H NMR, ^{13}C NMR, ESI-MS and FTIR study (see ESI†).



Scheme 1 Synthetic scheme for **1**.

$C_{23}H_{18}N_2O_7$: Anal. Found: C, 63.59; H, 4.18; N, 6.45; Calc.: C, 63.67; H, 4.25; N, 6.52%. EI-MS: $[M + H]^+$, *m*/*z*, 435.04 $[M + H]^+$; IR (KBr, cm^{-1}): $\nu_{C=N}$, 1618, ν_{C-H}, 2858; ν_{O-H}, (phenolic –OH), 3366; ν_{O-H}, (carboxylic –OH) 2913. ^{1}H NMR (δ, ppm in DMSO-d_6, 400 MHz): 10.434 (s, 2H); 9.913 (br, 3H); 9.058 (s, 2H); 7.757–7.459 (m, 8H); 2.321 (s, 3H). ^{13}C NMR (400 MHz, δ, ppm in DMSO-d_6): 189.2, 168.0, 163.9, 162.5, 151.2, 150.9, 134.6, 132.6, 124.1, 123.0, 115.3, 112.9, 30.2. Yield: 90%.

Synthesis of compound 2 (1-dihydrogen phosphate compound)

The preparation of the solid complex of dihydrogen phosphate was carried out as follows (*viz.* Scheme S1†). 2 mL DMSO solution of $Bu_4N[H_2PO_4]$ (74.68 mg, 0.22 mmol) was added slowly to a stirred 8 mL DMSO solution of **1** (100 mg; 0.22 mmol) and stirred for 10.0 h. The orange–red colored reaction mixture was filtered and left to allow slow evaporation. After a few days, the red colored dihydrogen phosphate complex was obtained by filtration and dried *in vacuo* for performing the characterization.

$C_{23}H_{20}N_2PO_{11}$: Anal. Found: C, 52.38; H, 3.06; N, 5.31; Calc.: C, 52.43; H, 3.11; N, 5.37%. EI-MS: $[M + H]^+$, *m*/*z*, 435.04 $[M + H]^+$; IR (KBr, cm^{-1}): $\nu_{C=N}$, 1626, ν_{C-H}, 2862; ν_{O-H}, 3369 (phenolic –OH), ν_{O-H}, 2913 (carboxylic –OH), ν_{O-H}, 3052 (chelate compound) (see ESI†).

General procedure for fluorescence and absorption study

The fluorescence properties of the sensor were investigated in HEPES buffer (100 mM) (DMSO–HEPES 1 : 9) co-solvent at 25 °C temperature. The pH of the solvent mixture was adjusted to ~7.2. We use such a polar co-solvent mixture to diminish the scope of intramolecular H-bonding of **1**. The selectivity study of **1** towards different anions was carried out with the previously mentioned anions in HEPES buffer (DMSO–HEPES 1 : 9) solution. During the selectivity study the anion concentration is 10 times more than that of receptor. The pH study was done in 100 mM HEPES buffer solution by adjusting pH with HCl or NaOH. An *in vivo* study was also completed at biological pH ~ 7.2. The absorption property of the receptor was studied in HEPES buffer (DMSO–HEPES 1 : 9) solution. The fluorescence quantum yields (Φ) were estimated by integrating the area under the fluorescence curves with the equation:

$$\Phi_{sample} = \frac{OD_{standard} \times A_{sample}}{OD_{sample} \times A_{standard}} \times \Phi_{standard}$$

where *A* is the area under the fluorescence spectral curve and OD is the optical density of the compound at the excitation wavelength. The standard used for the measurement of fluorescence quantum yield was anthracene (Φ = 0.29 in ethanol). The binding constant values were determined from the emission intensity data following the modified Benesi–Hildebrand

equation:[14,15]

$$1/\Delta F = 1/\Delta F_{max} + (1/K[C])(1/\Delta F_{max}), \Delta F = F_x - F_0, \Delta F_{max} = F_\infty - F_0$$

where F_0, F_x, and F_∞ are the emission intensities of the organic moiety considered in the absence of anion, at an intermediate anion concentration, and at a concentration of complete interaction, respectively, and where K is the binding constant and [C] is the anion concentration.

Geometry optimization

Trial geometries of the host and host–guest complex were constructed using Molden software.[16] Geometry optimization of the trial structures were performed by the *ab initio* method using the 6-31G* basis set (HF/6-31G*). The GAMESS[17] quantum mechanical package was used for geometry optimization. Analysis of the vibrational modes of the optimized geometries revealed that there was no negative frequency, which confirmed the optimized geometries as minimum energy structures.

Preparation of cells

Grains of *Tecoma stans* are collected by gently rupturing pollen sacs of fresh flower buds on a sterile Petri plate and suspending them in HEPES buffer (0.1 M, pH ~ 7.2). Debris of pollen sacs was removed by filtering the suspension through a thin layer of nonabsorbent cotton and then pollen was precipitated by centrifugation at 5000 rpm for 5 min. The supernatant was discarded and the pollen pellet was re-suspended in the same buffer containing the receptor (50 μM) and 0.01% Triton X100 as permeability enhancing agent. After 30 minutes of incubation the pollen grains were washed twice in HEPES buffer (0.1 M, pH ~ 7.2) by centrifugation as mentioned above.

Exponentially growing cells of *Candida albicans* (IMTECH No. 3018) in a yeast extract glucose broth medium (pH 6.0, incubation temperature, and 37 °C) were precipitated by centrifugation at 5000 rpm for 10 minutes and then washed twice by suspending them in HEPES buffer (0.1 M, pH ~ 7.2) followed by centrifugation at 5000 rpm for 10 minutes to remove trace of the growth medium used. Then cells were treated with the (5 μM) solution for 30 minutes. After incubation, the cells were again washed with the same HEPES buffer and **1** treated cells were mounted on a grease free glass slide.

The cells of bacterium used in the present experiment were *Bacillus* sp. which has long been used as an excellent bio-pesticide for controlling looper caterpillars at tea plantations. Bacterial cells from an exponentially growing overnight culture in nutrient broth medium were centrifuged at 6000 rpm for 10 minutes, the supernatant was discarded and bacterial pellets were washed using HEPES buffer (0.1 M, pH ~ 7.2) by centrifugation. Washed cells were suspended in the same buffer containing the **1** (5 μM) for 30 minutes and then centrifuged at 6000 rpm for 10 minutes and the supernatant was discarded.

Then all cells prepared above were photographed under high power magnification (100×) of a florescence microscope (LEICA DM 1000 LED) using UV filter. Cells without ligand were used as control.

Imaging system

An inverted fluorescence microscope (Leica DM 1000 LED) was used for cellular imaging. The microscope was equipped with a digital compact camera (Leica DFC 420C), an image processor (Leica Application Suite v3.3.0) and a mercury 50 watt lamp.

Results and discussion

Design and synthesis

The design of synthetic receptors in anion sensing is the most vital work. One of the important interactions between sensor and anion is related to hydrogen bonding. Keeping this in mind the receptor (**1**) was synthesized. The receptor 4-methyl-2,6-bis((3-hydroxy-4-carboxyphenyl)imino) phenol was synthesized by a facile one step condensation of 3-hydroxy-4-aminobenzoic acid and 4-methyl-2,6-diformylphenol. It was characterized by analytical data: ^{1}H NMR, ^{13}C NMR, ESI-mass spectra and IR study (ESI, Fig. S1–S5†). The spectroscopic ESI-mass spectra (Fig. S4†), IR study (Fig. S5†) and also the ^{1}H NMR titration data also support the formation of the **1**–H_2PO_4 (**2**) assembly in solid state.

Absorption study

To get an excellent anion sensing the photophysical property of the sensor was investigated in protic solvent (DMSO : buffer 9 : 1). The receptor was yellow color (λ_{max} = 440 nm) in the above medium. Addition of dihydrogen phosphate to the yellow colored receptor solution causes instantaneous development of green color (λ_{max} = 525 nm) (Fig. 1). The peak at 440 nm in the absorption spectra gradually decreases with addition of $H_2PO_4^-$ salt and a new peak generates with an 85 nm red shift at 525 nm (Fig. 2) through an isobestic point at 464 nm. This suggested a hydrogen bond mediated strong interaction of $H_2PO_4^-$ with the receptor (Scheme S1†). This red

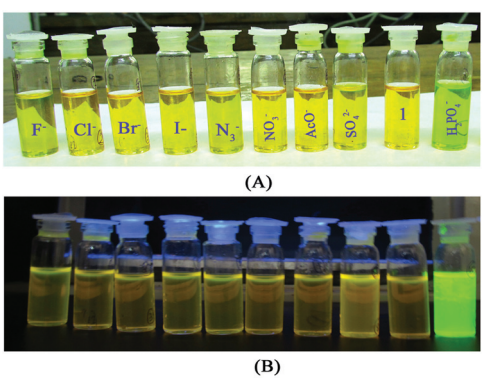


Fig. 1 Photographic images of the receptor in the presence of different anions (A) visual color change and (B) fluorescence color change. The concentration of anions is 100 times greater than the receptor.

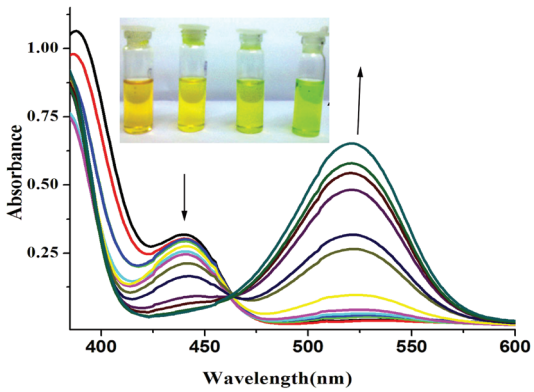


Fig. 2 Absorption spectra of 6.25 μM of **1** in the presence of 0, 2.50, 5.0, 7.5, 8.75, 10.0, 12.5, 20.0, 25.0, 50.0, 75.0, 125.0, 200.0 μM of $H_2PO_4^-$ in HEPES buffer (DMSO–HEPES 1 : 9, v/v) at 25 °C (inset is the image of UV-Vis titration).

shift induces a colour change from yellow to green after addition of dihydrogen phosphate which can be recognized by the naked eye. This receptor is capable of optically sensing changes in $H_2PO_4^-$ concentration at a level of 10^{-4} mol dm^{-3}.

Emission study

The signaling mechanism operating here is through intramolecular H-bonding (IHB) of three hydrogen atoms with three oxygen atoms (–OH···O) which is also a negative factor which weakens the fluorescence intensity of the sensor. But in presence of the competitive anions the scope of the IHB decreases and intermolecular H-bonding between anion and sensor dramatically enhances the emission intensity of the receptor Scheme S1.†

On excitation at 480 nm the receptor gives emission at 550 nm in the fluorescence spectrum in DMSO : HEPES buffer 9 : 1 (Fig. 3). The change of fluorescence intensity of **1** in the presence 10 equiv. amounts of different anions was studied and the result has been summarized in Fig. 4. Here only $H_2PO_4^-$ gives a high signal but other anions HPO_4^{2-}, F^-, Cl^-, Br^-, I^-, N_3^-, HSO_4^-, NO_3^-, AcO^- remain entirely silent (taking Bu_4N^+ salts for halogens and Na^+ salts for the others). This result demonstrated that receptor **1** exhibited high selectivity towards $H_2PO_4^-$ in such a polar solvent mixture. This selectivity observed is due to the charge density of monobasic anions. From Fig. 5 it was observed that detection of $H_2PO_4^-$ was not affected by the other mentioned anions. In the presence of various anions the fluorescence intensity enhancement of **1** is almost the same as that shown by $H_2PO_4^-$ alone. The addition of $H_2PO_4^-$ offers a dramatic change in emission intensity of **1** with a more than 30 fold increase in quantum yield from **1** ($\Phi_1 = 4.51 \times 10^{-3}$ and $\Phi_{complex} = 1.4 \times 10^{-1}$) and also in emission spectra. The emission intensity increases with gradual addition of the $H_2PO_4^-$ and is recorded in Fig. 6. The pK_a value of dihydrogen phosphate is ~7.2 which means that protonation of the guest occurs above 7.2. The plot of emission intensity *vs.* $H_2PO_4^-$ concentration (Fig. 7) indicates that the fluorescence intensity increases with increasing analyte concentration. There is a good linearity of fluorescence intensity

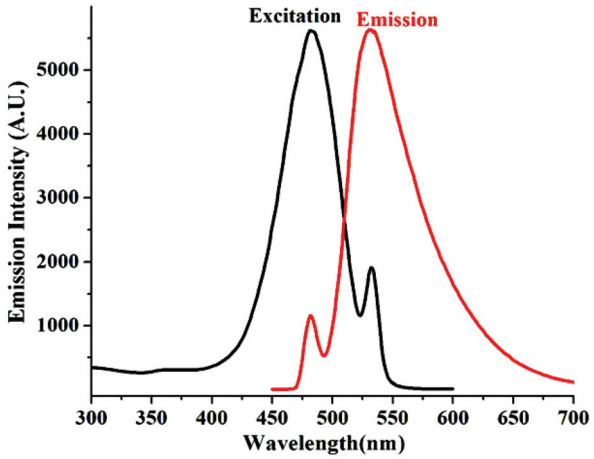


Fig. 3 Excitation and emission spectra of 12.5 μM of **1** in HEPES buffer (DMSO–HEPES 1 : 9) at 25 °C.

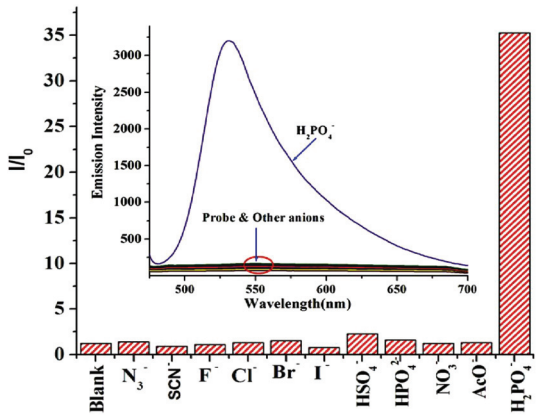


Fig. 4 Emission enhancement spectra of **1** (10 μM) in the presence of different anions (100 μM) in HEPES buffer (DMSO–HEPES 1 : 9) at 25 °C, λ_{ex} = 480 nm.

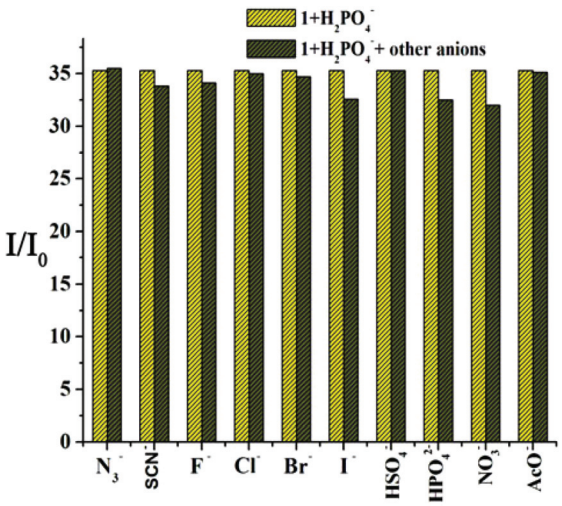


Fig. 5 Interference plot of different anions (100 μM) in the presence of **1** (10 μM) and $[H_2PO_4^-]$ = 100 μM in HEPES buffer (DMSO–HEPES 1 : 9) at 25 °C.

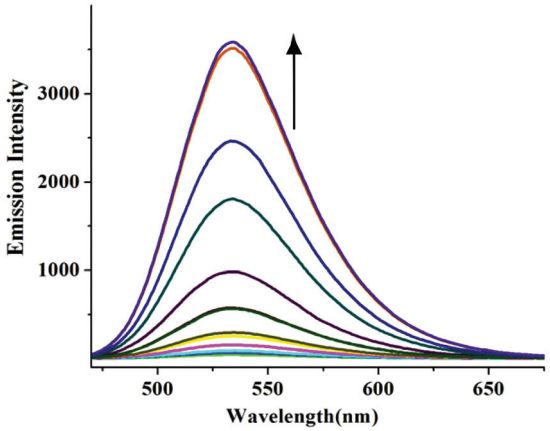


Fig. 6 Emission spectra of 6.25 μM of **1** in the presence of 0, 0.63, 1.25, 1.88, 2.50, 3.75, 5.0, 6.25, 7.5, 8.75, 10.0, 12.5, 20.0, 25.0, 50.0, 125.0 μM of $H_2PO_4^-$ in HEPES buffer (DMSO–HEPES 1 : 9, v/v) at 25 °C.

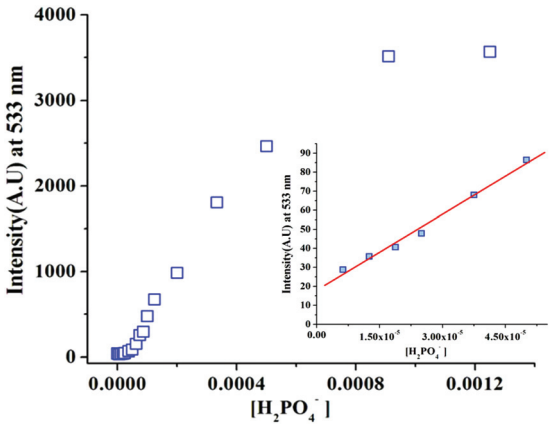


Fig. 7 Fluorescence intensity as a function of $[H_2PO_4^-]$ concentration. Inset: linearity up to 4.5×10^{-5} M.

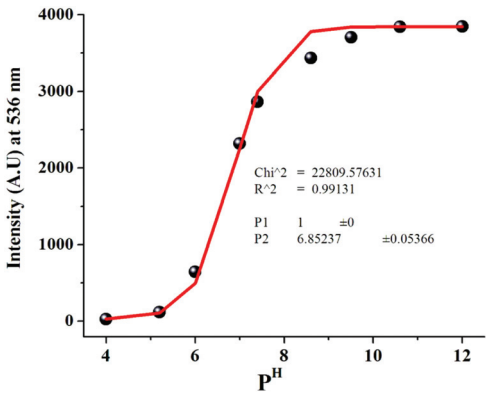


Fig. 8 Fluorescence response to pH of **1** in 100 mM HEPES buffer at 25 °C, pK_a = 6.85237 ± 0.05366.

of **1** as a function of $[H_2PO_4^-]$ concentration in the low region up to 4.5×10^{-5} M. The pK_a value of our receptor is 6.85 (Fig. 8). This value suggests strong H-bonding of the host–guest near neutral pH. The stoichiometry of the receptor–

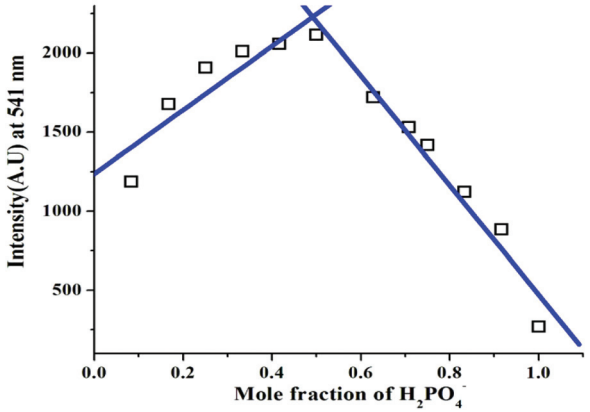


Fig. 9 Jobs plot showing maxima at 1 : 1 (**1** + $H_2PO_4^-$ = 100 μM).

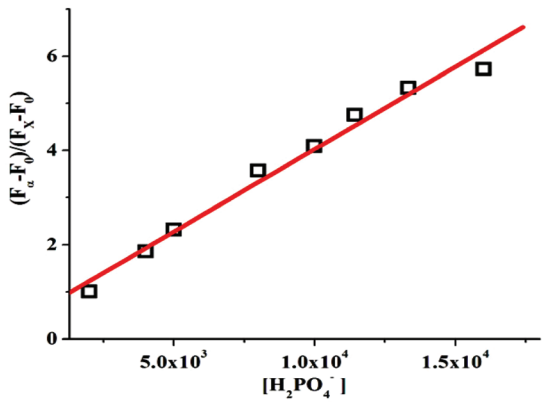


Fig. 10 Double reciprocal plot for **1**–$H_2PO_4^-$ assembly (k_a = 0.2 × 10^4 M^{-1}) in the lower concentration region of $H_2PO_4^-$ in HEPES buffer (DMSO–HEPES 1 : 9, v/v) at 25 °C.

$H_2PO_4^-$ assembly was established as 1 : 1 from Job's plot analysis (Fig. 9). To observe the binding interaction the binding constant value has been determined from the modified Benesi–Hildebrand equation and the K_a value is 0.2 × 10^4 M^{-1} for $H_2PO_4^-$ binding (Fig. 10). The effects of pH on the fluorescence property of **1** also investigated. The receptor exhibits high fluorescence intensity in the basic region since the phenolic –OH groups of **1** remain as O^- in the basic region with no possibility of IHB. Therefore, **1** senses the anions with high intensity in the basic region (Fig. 11). It is noteworthy to mention that the fluorescence emission of **1** is also high in the basic region in the presence of $H_2PO_4^-$. The detection limit was determined from the fluorescence titration data[18] (Fig. 12) and we could detect $H_2PO_4^-$ in the limits of 3.5×10^{-6} M which is significantly low.

In order to know more about the pattern of interaction between receptor **1** and tetrabutylammonium phosphate, ^1H NMR titration was performed in DMSO-d_6 solvent. As shown in Fig. 13 the broad phenolic protons (H^2) peak (equivalent to 3H) vanish and become a sharp peak (equivalent to 1H) after addition of $H_2PO_4^-$, indicating the hydrogen bonding interactions between **1** and $H_2PO_4^-$. This signal of one H^2 proton

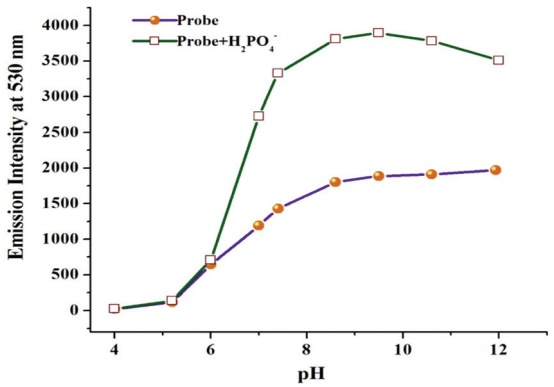


Fig. 11 Emission response of probe 10 μM of **1** in the absence and presence of $H_2PO_4^-$ (1 equivalent) at different pH in HEPES buffer (DMSO–HEPES buffer: 1 : 9).

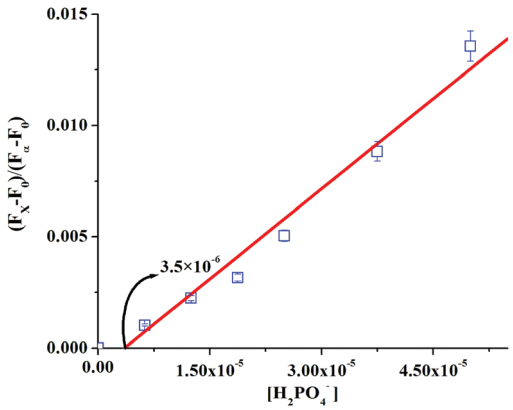


Fig. 12 Detection limit of $H_2PO_4^-$ in HEPES buffer (DMSO–HEPES 1 : 9, v/v).

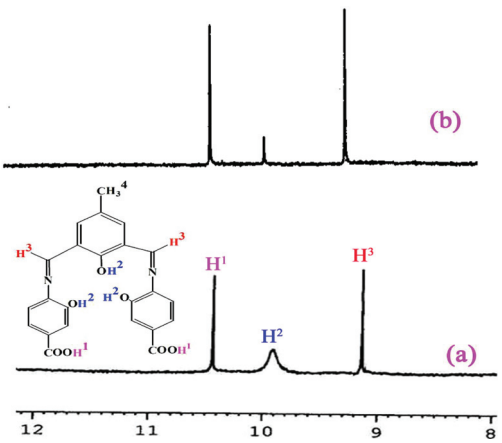


Fig. 13 Partial ^{1}H NMR titration (DMSO-d_6, 400 MHz) (a) of **1**; (*conc.* = 1.16 × 10^{-3} M) (b) **1** + 1.0 equivalent of tetrabutylammonium dihydrogen phosphate.

remains intact in Fig. 13b because it is involved in intramolecular H-bonding.

To clarify the configurations and H-bonding features of the guest and guest–host complex *ab initio* calculations were

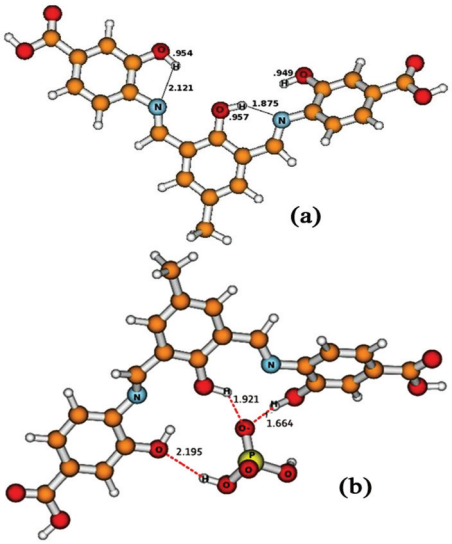


Fig. 14 Optimised geometry of (a) **1** and (b) **1**–phosphate assembly by *ab initio* method.

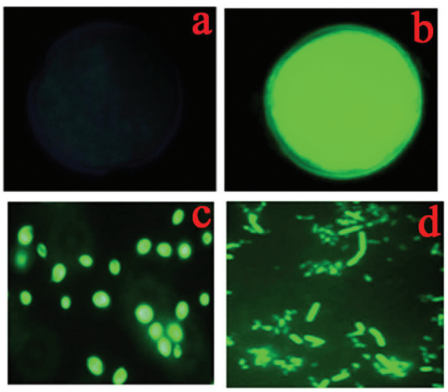


Fig. 15 Fluorescence image of (a) pollen grains of *Tecoma stanswi* without any receptor addition. (b) Pollen grains were incubated with 5 μM **1** for 30 minutes. (c) *Candida albicans* cells were incubated with 5 μM **1** for 30 minutes. (d) *Bacillus* sp. cells strained with 5 μM receptor after 30 min incubation. All the samples were excited at ~480 nm with emission *ca.* ~530 nm by using [40×] objective.

performed. The energy of HOMO and LUMO of receptor and receptor–$H_2PO_4^-$ complex are −8.09 eV, 1.21 eV, −5.53 eV, 3.74 eV. The difference in energies are 9.30 eV and 9.27 eV for **1** and **2**. This shows that these two compounds are equally stabilized and it demonstrates the facile conversion of **1** to $H_2PO_4^-$ complexation (Fig. 14). This is why the red shift was observed in the UV-Vis spectra due to the addition of $H_2PO_4^-$.

Due to the significant interaction of the receptors with the dihydrogen phosphates in both organic and aqueous organic systems, we planned to investigate their interactions with living cells. A cell consists of a higher order complex entity of dihydrogen phosphate ions. Consequently the receptor will be a good staining agent to visualize cells by interaction with *in vivo* dihydrogen phosphates. For this study three types of cells *viz.* pollen grains of *Tecoma stans*, *Bacillus* sp., *Candida albicans* were taken. Apart from the pollen the other two cells were not

visualised (black) through the fluorescence microscope. Only pollen has a slight blue fluorescence at the edge of the cell under the fluorescence microscope. But after incubation with 5 μM **1** for only 30 minutes all the cells sparkled with green fluorescence (Fig. 15). The distribution of the probe within the cells was observed by fluorescence microscopy following excitation at ~480 nm. These results indicate that **1** is an efficient staining agent and we can monitor the intracellular dihydrogen phosphate.

Conclusion

In conclusion, a colorimetric and fluorescent dual receptor for $H_2PO_4^-$ was synthesized by a facile one-step process. The formulation and detailed structural characterisations have been established using physico-chemical and spectroscopic tools. The mode of interaction was verified by ^1H NMR titration and theoretical calculation. This water soluble compound is a potent probe for $H_2PO_4^-$ in biological applications as both wavelengths (λ_{ex} = 480 nm and λ_{em} = 530 nm) are not in the UV region but in the bio-friendly visible region. The fluorescence intensities are also almost unaffected by the biologically relevant anions and the tolerable limit of different interfering anions is significantly high (up to 10 equivalent). There is a good linearity between fluorescence intensity of **1** and $[H_2PO_4^-]$ at low region and detection limit is 3.5×10^{-6} M. The study of the distribution of the probe in the living cells showed that the receptor (**1**) is significantly efficient to detect dihydrogen phosphate *in vitro* in aqueous medium at biological pH by developing a good image and has potential for bio-medical applications.

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