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First supramolecular sensors for phosphonate anions†

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Fluorescent tripodal anion sensors with a 1,3,5-triethylbenzene core display a turn-on fluorescence response to phosphonate and phosphate anions and may be used as optical sensors. The properties of the receptors and sensors as well as their anion binding behavior were investigated both in solution and in solid state. The turn-on fluorescence response can be leveraged in sensing of phosphate anions and, most importantly, hydrolysis products of the nerve gas sarin, isopropyl methylphosphonate (IMP), and methylphosphonate (MP). The fluorescence signal amplification in the presence of anions allows for application of these molecules in a sensor microarray suitable for high-throughput screening.

Introduction

Research efforts are currently focused towards the discovery of sensors capable of detecting phosphorus-related oxyanions. This is due to their ubiquity in the Nature, role in biological processes associated with phosphorylation, maintenance of phosphate homeostasis,[1] and balanced nutrition.[2] The industrial and environmental impact of phosphates is enormous due to their use in chemical industry as fire retardants, in waste water treatment,[3] cleaners, and fertilizers.[4,5] Similarly, phosphonates have a broad range of applications where they find utility as chelating agents, cleaners, scale inhibitors, and bleach stabilizers.[6] Phosphonates are also used in medicine including bone cancer treatments, antibiotics, and cure for diseases related to calcium metabolism and osteoporosis.[7] Importantly, nucleotide phosphonates are known to be powerful antiretroviral drugs.[8] The most noticeable examples of this family of phosphonates include cidofovir (HPMPC), adefovir (PMEA), and tenofovir (PMPA).[9] Finally, phosphonate anions are products of hydrolysis of warfare agents such as the nerve gas sarin (GB).[10] Typically, the focus is on direct detection of sarin and its vapours.[11] For example, Gale *et al.*[11c] have developed receptors for soman, Swager *et al.*[11d] have found indicators for chemical warefare derivatives whereas Rebek and Dale[11e] have tested a variety of sensors for nerve agents mimics.

However, nerve gasses such as sarin can be detected indirectly through their hydrolysis products, isopropyl methyl phosphonate (IMP) and methyl phosphonate (MP) (Fig. 1). The currently used methods for phosphonate detection utilize gas chromatography[12] and mass spectroscopy.[13,14] Whilst the research focused on these methods is currently underway, the development of new methods for IMP and MP detection is of great importance. Overall, both the natural and anthropogenic phosphates and phosphonates require monitoring in diverse setting and levels. A number of methods of phosphate levels determination are known.[15,18] However, unlike phosphates, phosphonates were mostly overlooked. To the best of our knowledge, there is no direct optical sensor capable of detecting phosphonates to date. Here, we present optical sensors capable of detecting both phosphates and phosphonates. This ability to discern different types of phosphorus related oxyanions is very important for the analysis of traces of IMP and MP in soil exposed to sarin contamination.

In the recent past, the research on artificial anion receptors and sensors has made a significant headway.[16] In this arena, we focused on the synthesis of chemosensor molecules utilizing both colorimetric[17] and fluorescence[18] output signals. A number of interesting receptors were designed by attaching anion-binding side-arms to the 1,3,5-triamino-methyl-2,4,6-triethylbenzene **1**.[19] For example, pyrrole,[18f,20a,c] amide,[20b,d] urea,[21] guanidinium,[22] and imidazolium[23] moieties yield various receptors

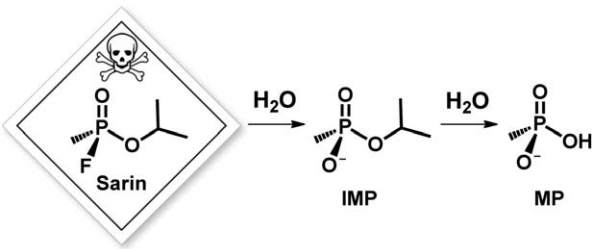

Fig. 1 Hydrolysis of sarin.

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and sensors with C_3-symmetry aimed at binding of anions with spherical or 3-fold symmetry such as phosphate, MP, or IMP.[24] In this study, we present the synthesis, properties, and anion sensing studies of tripodal receptors and sensors **2–8** that display a turn-on fluorimetric response for anions including the hydrolysis products of the nerve gas sarin (MP and IMP).

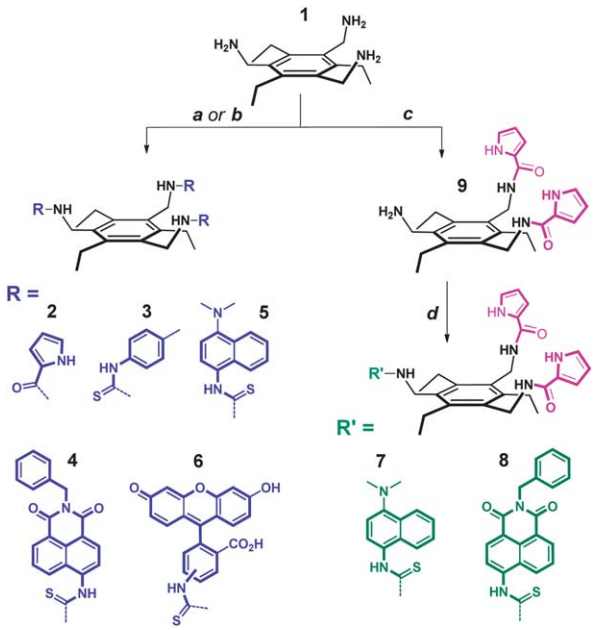
Results and discussion

The receptors and sensors **2**, **7**, and **8** were synthesized (Scheme 1) using Lieben-like reaction,[25] during which 3 equiv. of 2-(trichloroacetyl)pyrrole react with the benzylic amine **1** to yield amide **2**. Likewise, reaction of **1** with 2 equiv. of the pyrrole precursor gave intermediate **9**. This way, pyrrole-amides are obtained in one step from a commercially available reagent. Alternatively, triamine **1** can be reacted directly with 3–5 equiv. of an appropriate isothiocyanate precursor (ArNCS) to yield the thiourea receptors and sensors **3–6**. Reaction of the bis(pyrrole-amide) intermediate **9** with isothiocyanate yields bis(pyrrole-amide)thiourea sensors **7** and **8**.

Because of the alternate up-and-down arrangement of the ethyl and aminomethylene groups, **1** provides a preorganized scaffold comprising alternating up-and-down arrangement of the ethyl and amide substituents. However, we found that the arrangement of the substituents depends on the solvent or the presence of a template, which appear to control whether a chair-like or a cup-like structure is formed. With a cup-like shape three hydrogen-bond donor groups all face the same side of the aromatic ring, forming a cavity lined with hydrogen-bond donors.

First, the behaviour of the receptors **2** and **3** was studied in solution using NMR and spectrophotometric techniques, and in the solid state using X-ray crystallography. A combination of variable-temperature ^{1}H NMR (VT-NMR) measurements and single crystal X-ray studies confirmed a tendency toward self-association. The VT-NMR of **2** showed that the signals corresponding to the amide N–H are broadened and methylene resonances are split into two at low temperature (Fig. 2A), suggesting the existence of a conformation with two distinct environments for the substituents attached to the benzene core. The crystal structures of the free receptor **2** (Fig. 2B–D) show that the receptor adopts a chair-like structure stabilized by a network of intermolecular hydrogen bonds. Conformational behaviour similar to **2** is also observed in sensor **5**, which displays a similar behaviour (Fig. 3).

The cup-like conformation of the receptor **2** may be enforced by binding to an anion such as $H_2PO_4^-$, in which case VT-NMR experiments show no temperature-induced change in the spectra (see ESI†). This suggests that the conformation of the receptors and sensors is controlled by the intermolecular interactions and by the presence of a suitable anionic guest. Dilution experiments showed a continuous slight upfield shift in the pyrrole and amide NH resonances. The fact that the shift of the resonances did not reach a plateau suggests that a monomeric state was not obtained within the available concentration range. As mentioned above, the anions appear to act as templates that enforce the cup-like conformation. To diminish the effect of self-association, we decided to perform the qualitative titration by ^{1}H NMR and the



Scheme 1 Synthesis of receptors and sensors **2–8**. (a) ArNCS (3–5 mol. equiv.), THF, rt, 14–55 h; (b) 2-(trichloroacetyl)pyrrole (3 mol. equiv.), Et_3N, THF, rt, 24 h; (c) 2-(trichloroacetyl)pyrrole (2 mol. equiv.), Et_3N, THF, rt, 16 h; (d) ArNCS (1–2 mol. equiv.), THF or DMSO, rt, 15 min to 24 h.

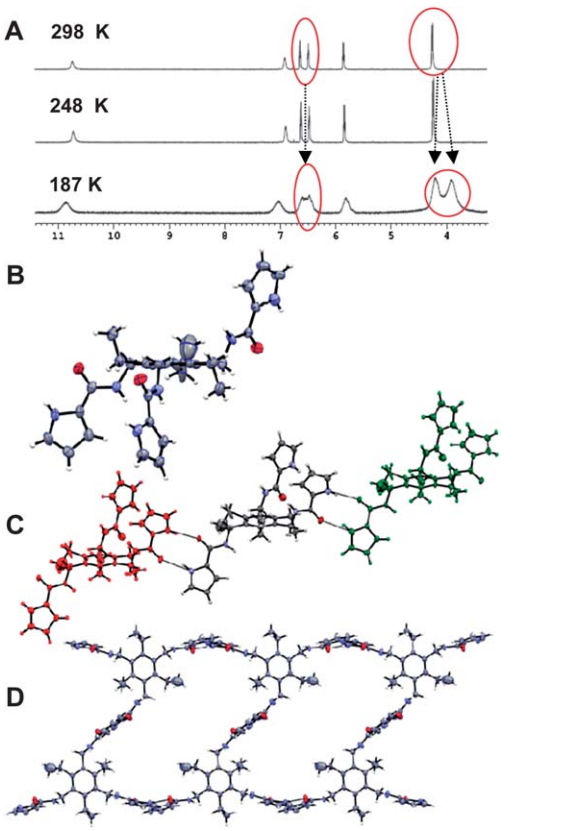


Fig. 2 VT-NMR of the receptor **2** (A) shows splitting of the substituent resonances due to the two different environments in CD_3CN : acetone-d_6 : DMSO-d_6 = 10 : 1 : 0.5. The up-up-down arrangement of the substituents in receptor **2** (B) is presumably due to the establishment of intermolecular hydrogen bonds (C and D).

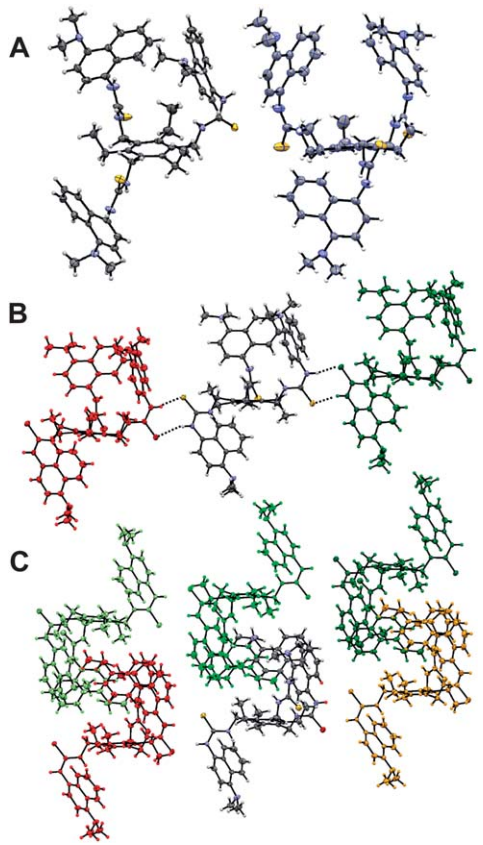


Fig. 3 The up-up-down arrangement of the substituents in sensor **3** (A), intermolecular hydrogen bonds (B) and packing arrangement (C).

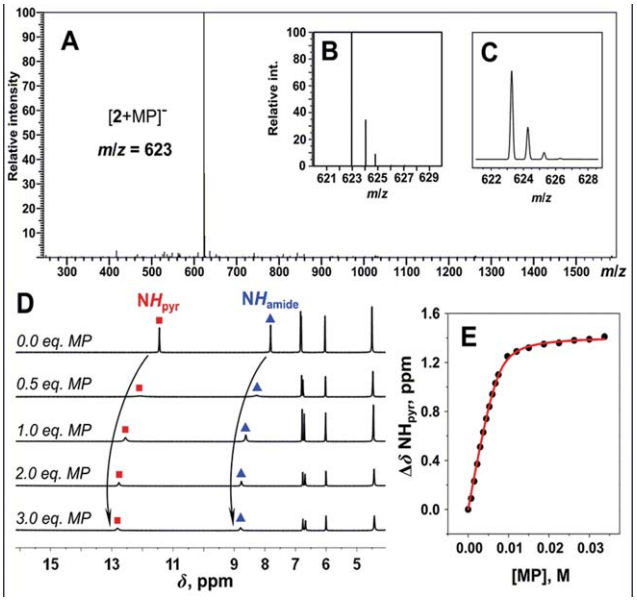


Fig. 4 Panel A: The ESI MS spectrum of the complex of **2** and MP; B: expansion of the ESI spectrum of **2**·MP peak; C: calculated isotope pattern for $C_{31}H_{40}N_6OP^-$; D: ^1H NMR (500 MHz) titration of **2** upon the addition of MP in DMSO-d_6; E: titration isotherm of the pyrrole NH peak corresponding to the MP-induced chemical shift.

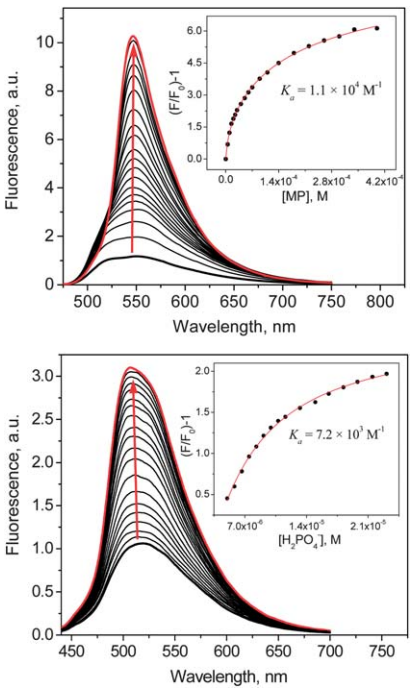


Fig. 5 Fluorescence intensity amplification of **6** λ_{ex} = 450 nm (top) and **4** λ_{ex} = 400 nm (bottom) upon addition of MP and dihydrogen phosphate anions, respectively in DMSO.

quantitative experiments by fluorescence intensity measurements, as these are carried out at μM concentration (while the ^1H NMR requires mM concentration).

The first confirmation of the anion-binding properties of **2–8** was obtained by MALDI-TOF MS or ESI MS. For example, Fig. 4 shows ESI spectrum of the of **2**·MP complex suggesting a 1 : 1 binding stoichiometry (A) and the progress of NMR titrations of **2** with MP (D).

Importantly, the receptors and sensors **2–8** are not deprotonated by the anions at low anion concentration in 10% water–DMSO-d_6, and the stoichiometry of the receptor and sensor-anion complexes established by Job's method is 1 : 1 (see ESI†). A qualitative measure of the bonding strength is the change in chemical shift of the resonances directly involved in hydrogen bonding. Here, the pyrrole NH resonances undergo a dramatic downfield shift (from 10.8 to 14.7, 13.6, and 12.9 for F^-, $H_2PO_4^-$, and MP, respectively). Similarly, the pyrrole-amide NH is shifted downfield from 7.8 to 9.8, 8.6, and 8.8 for F^-, $H_2PO_4^-$, and MP, respectively. The relative order of affinities observed for anions was MP ≈ $H_2PO_4^-$ > F^- > $CH_3CO_2^-$ > Cl^-.

Quantitative estimation of the anion sensing ability of sensors was obtained by fluorescence titration by anions (as tetrabutyl-ammonium salts) in DMSO (Fig. 5). All sensors were excited at the isosbestic point of their absorption or at the point where no change in absorption is observed. To obtain a structure–binding relationship, simple anions such as fluoride, chloride, acetate, dihydrogen phosphate, and pyrophosphate were tested along with both aliphatic and aromatic phosphonates. Thus, we used methyl phosphonate (MP), isopropyl ester of methyl phosphonate (IMP) and phenyl phosphonate (PP). Due to a lack of fluorescence label, receptors **2** and **3** were not used for fluorescence investigation. The sensors **4–8** are not

particularly fluorescent in DMSO. While this may be surprising in light of the fact that these sensors comprise known fluorescent labels such as fluorescein and naphthalimide. We believe that the low fluorescence in the resting state is due to deactivation by the random intramolecular collisional quenching with the neighbouring side-arms, as well as rotational/vibrational quenching. The respective fluorescence quantum yields are follows: **4** ($\Phi_{fl} = 0.10$), **5** ($\Phi_{fl} = 0.33$), **6** ($\Phi_{fl} = 0.18$), **7** ($\Phi_{fl} = 0.40$), **8** ($\Phi_{fl} = 0.12$).

Importantly, the fluorescence intensity dramatically increases in the presence of a strongly bound anions such as dihydrogen phosphate, MP, IMP or fluoride.[26] These anions enforce a stable structure of the sensor–anion complex, which dramatically suppresses the degree of freedom of the fluorescent side-arms. As a result, the excited state deactivation is suppressed resulting in a dramatic increase in fluorescence, and a turn-on signalling of the anion is observed. Increase in fluorescence was used to calculate the association constants (Table 1).

The results suggest that sensors **4–8** have a strong response towards $H_2PO_4^-$ over other anions in the order: $H_2PO_4^- \geq$ MP > IMP > PP ≈ $HPPi^{3-}$ (Table 1). Sensor **8** exhibits specific affinity only towards dihydrogenphosphate and fluoride anions. A somewhat similar response was also observed with sensor **4** which was found to bind phosphonates as well. This behaviour is presumably due to the steric demand imposed by the naphthalimide fluorescent labels in **4** and **8**. Increasing the number of fluorescent labels (sensors **4–6** *vs.* **7**, **8**) did not result in a dramatic change in overall affinity for anions. On the other hand, one can see a clearer trend between different anions: in general, small spherical anions such as chloride, fluoride are not bound too well, presumably because they require the receptor arms to come too close together. However, C_3-symmetrical anions such as phosphate and methyl phosphonate fit nicely into the binding cavity and display the highest binding affinities. Introduction of a phenyl group into the phosphonate (MP *vs.* PP) slightly decreased the binding constants of the sensors. This is attributed to the steric bulk of the phenyl group. Similar reasoning may be used when comparing MP and IMP; however a different pattern of hydrogen bonding may have an effect.

Furthermore, some insight into anion binding was obtained by MS spectroscopy and theoretical calculations: First, the MS spectra and Job plot experiments in solution showed that compounds **2–8** form 1 : 1 complexes with anions. This was also indirectly confirmed by DFT B3LYP/6-31G* calculations. Fig. 6 shows the energy-minimized structure of the **2**·$H_2PO_4^-$ complex. However, the only X-ray-quality crystal we were able to grow was that of receptor **3** with dihydrogen phosphate (Fig. 7). To our surprise, the X-ray structure revealed a hydrogen-bonded $H_2PO_4^-$ trimer localized in the shallow bowl of the receptor. This is quite unusual, and we tend to attribute this conflicting behaviour to the demand of solid state, rather than this to be reflecting the behaviour in solution. However, we were not able to grow a crystal of a 1 : 1 complex.

Table 1 Binding constants (M^{-1})[a] for sensors **4–8** in DMSO for selected anions

Sensor					
Anion	**4**	**5**	**6**	**7**	**8**
F^-	5.6×10^3	ND[b]	8.4×10^4	ND[b]	1.1×10^4
Cl^-	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
$CH_3CO_2^-$	ND[b]	1.6×10^4	3.6×10^4	2.1×10^3	ND[b]
$H_2PO_4^-$	7.2×10^3	4.3×10^5	3.1×10^4	1.4×10^4	1.0×10^4
$HPPi^{3-}$	ND[b]	9.8×10^4	2.0×10^4	5.1×10^3	ND[b]
MP	8.4×10^3	4.1×10^4	1.1×10^4	3.1×10^4	ND[b]
IMP	3.7×10^3	7.8×10^3	1.3×10^4	2.9×10^4	ND[b]
PP	4.2×10^3	2.2×10^4	1.7×10^3	7.7×10^3	ND[b]

[a] The 1 : 1 stoichiometry was confirmed using Job's method. The K_as were calculated based on the change in fluorescence maxima upon addition of each analyte. Anions were used in the form of tetrabutylammonium salts. All errors were <14%. For more details see ESI.† [b] Binding constants cannot be determined due to the insufficient change in fluorescence response.

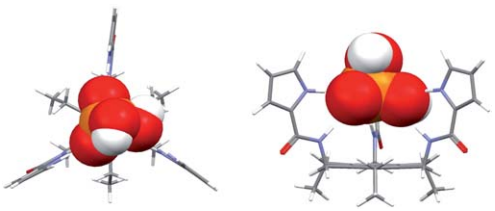


Fig. 6 DFT B3LYP/6-31G* calculation: energy minimized structure of the **2**·$H_2PO_4^-$ complex.

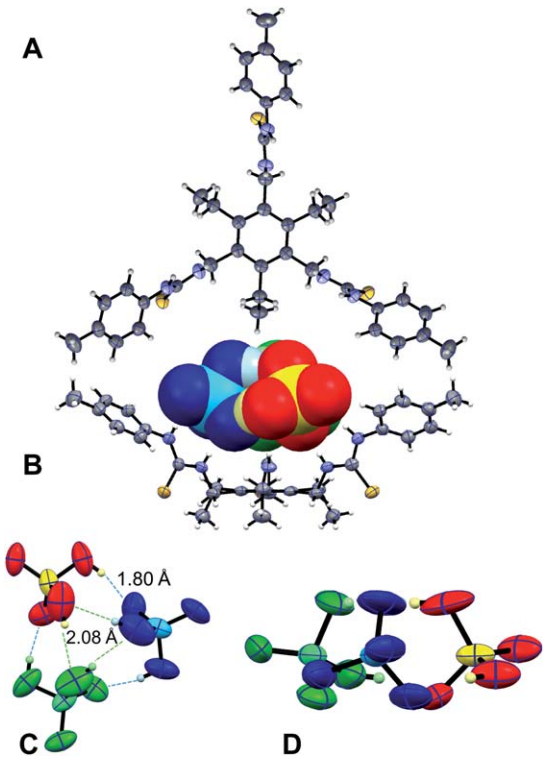


Fig. 7 X-ray structure of receptor **3** with three dihydrogen phosphate anion. Top view of the receptor (A), side view of the sensor–anion complex (B), top view of the hydrogen bonded phosphate anions within the receptor cavity (C), and a side-view of the bound phosphates (D).

From the potential practical perspective it is important that the described sensors may be incorporated in a simple microarray for anion sensing in the presence of water. While the sensors **4–8** are not water soluble, they tolerate well 20% of water in DMSO and the water does not interfere significantly with the fluorescence.

The ability of the sensors to distinguish MP and IMP from other simple anions such as fluoride, acetate, dihydrogen phosphate, dihydrogen pyrophosphate was confirmed in a qualitative assay. Here, the sensors were dissolved in DMSO and dispensed into conventional 1536-well microplates. The analytes were added in the form of their aqueous solutions and the fluorescence intensity recorded using a standard plate reader. This is important because water is a likely solvent for a number of anions including the products of sarin hydrolysis (IMP, MP), which would be extracted from contaminated soil.[27]

The unique fluorescence responses from each well were analyzed and evaluated by the statistical multivariate analysis method – linear discriminant analysis (LDA).[28] Using a leave-one out cross-validation routine, LDA can evaluate discriminatory power of the sensors array. Despite the fact that all of the sensors respond to the presence of anions by increase in fluorescence, the degree of the change in the fluorescence intensity for each anion is unique and generates enough difference that the pattern recognition analyses can differentiate between the samples (Fig. 8A).

The LDA showed excellent separation of the data clusters strongly suggesting that the five sensors are capable of distinguishing between six aqueous anions and a control (total of seven analytes), thus overcoming the 1 : 1 stoichiometry limitations posed by the lock-and-key principle (Fig. 8A).[29] It is important to mention that the size of the array can be effectively reduced while maintaining the discriminatory capacity. Fig. 8B illustrates the LDA utilizing only 2 sensors (**5** and **6**), 100% accurate classification of all 6 analytes and a control suggests that the reduction of the number of the sensors did not affect the discriminatory performance of the array.

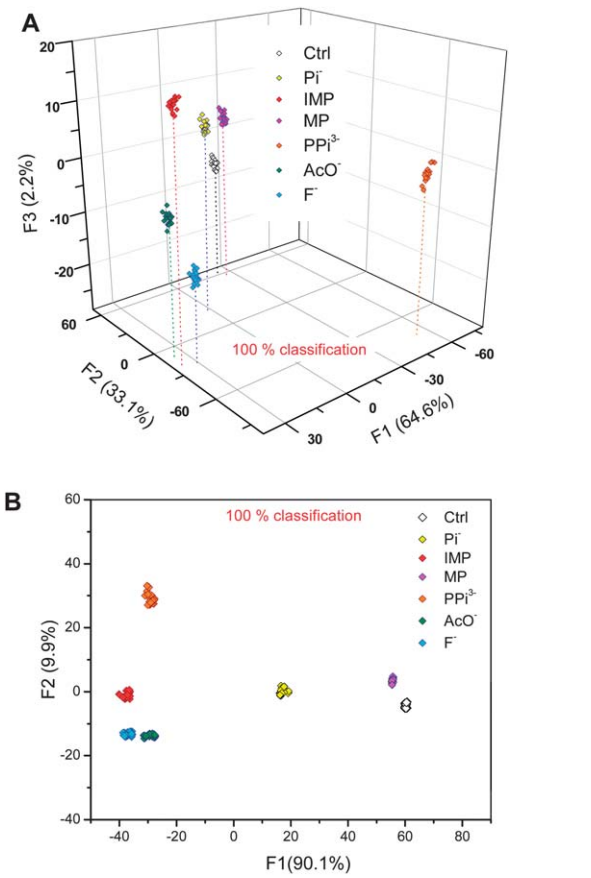


Fig. 8 Panel A: the linear discriminant analysis (LDA) graphical output of qualitative assay which shows canonical score plots for the first three canonical factors. B: LDA of qualitative assay using only sensors **5** and **6**.

Conclusions

The synthesis and characterization of a series of tripodal fluorescent receptors and sensors capable of detecting different classes of anions was successfully accomplished. Compounds **2–8** were designed to posses a cup-like binding cavity decorated with hydrogen bond donor, thioureas and/or pyrrole-2-yl amides. Structural elucidation using NMR and X-ray measurements showed that in the resting state the receptors adopt alternate conformation, in which the substituents on the central benzene moiety do not show up–down alternating arrangement of the substituents. This unexpected conformation behaviour is attributed to intermolecular interactions and larger than expected conformational freedom of the substituent of the central benzene. However, the presence of anions results in a change in conformation displaying the up–down conformation as evidenced by the combination of NMR, X-ray experiments, and DFT calculation. The crystal structure of $\mathbf{3}\cdot 3H_2PO_4^-$ exemplifies the design principle as it shows the cavity of the tris–thiourea binding a cluster of three dihydrogen phosphate anions held together by hydrogen bonds.

The anion binding of **2–8** was also verified by mass spectroscopy techniques and NMR spectroscopy. These receptors and sensors do not show deprotonation while the MS spectra show stable anion–receptor complexes. Most importantly, the fluorimetric titrations of sensors **4–8** with a variety of anions including aliphatic and aromatic phosphonates displayed a significant turn-on response with up to a 10-fold signal increase. Finally, the ability of the sensors to distinguish between different classes of anions was demonstrated in a simple cross-reactive microarray. This attractive feature can be applied to sensing of phosphonates, products of hydrolysis of the nerve gas sarin, suggesting that such a method could be used in forensic identification of a potential nerve gas use. To the best of our knowledge, this study shows the first optical sensors for phosphonates and nerve gas hydrolysed products.

Acknowledgements

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