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COMMUNICATION

Selective DCP detection with xanthene derivatives by carbonyl phosphorylation

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Kanhu Charan Behera[a,b], Bamaprasad Bag[a,*,†]

The Rhodamine derivatives(1-2) exhibited dual channel 'turn-on' photophysical signalling selectively with Diethyl chlorophosphate (DCP) among various organophosphates (OP), where the spiro-ring opening corresponds to an adduct formation through phosphorylation at their carbonyl O-$_{spiro}$ nucleophilic centre.

The vicious exploitation of chemical warfare nerve agents[1] against mankind has increasingly raised safety concerns. The nerve agents, particularly organophosphates, inflict inhibition effect on acetylcholine esterase leading to critical nervous system damage, thus considered to be highly toxic[2]. The usage of organophosphates as pesticides and herbicides inflict adverse effect[3] to the living organisms. The diethyl-chlorophosphate (DCP) is prominent among organophosphates due to its chemical reactivity based utilities; as a reagent for hydroxyl activation in chemical reactions, as intermediate in synthesis of various insecticides, less toxic in comparison to organophosphate based pesticides and as a nerve agent stimulant due to comparable reactivity. Therefore, selective detection and quantitive estimation of organophosphates and their degraded products, DCP in particular, has continued as a highly desirable and indispensable objective[4]. Various methodological explorations have been adapted for detection of DCP; however, implementation of such techniques has been jeopardized with one or other limitations of operational complexity, selectivity, sensitivity, cost-effectiveness, response time, portability, stability and throughput of real time monitoring. Analyte detection with molecular based probes where fluorescent and colorimetric modules as responsive signals have already been proven[5] its utility among alternative methodologies by rendering a viable and realistic solution to overcome such limitations in detection technology. In this context, adaptation of an appropriate methodology of suitable design of chemosensors plays a crucial role. The trending literature[6-9] highlights that analytical pathway of detection of organophosphates and their degraded products primarily follow an electrophilic phosphorylation depending upon on their molecular structures, where cleavage of phosphorus–halogen bond is facilitated by various nucleophilic reagents. Therefore, design of such molecular probes usually comprises of a nucleophilic receptor (as amino-, hydroxyl and oxime groups) which can selectively and sensitively recognize the targeted organophosphates through effective interaction and the recognition event is translated to the signaling subunit through perturbation of operative photophysical processes. Most of such 'signaling subunit - recognition site' ensembles for detection of DCP are reported to undergo phosphorylation at the hydroxyl[6]- or amino[7]- groups available on the receptor segment.

Xanthene dye based molecular ensembles have proven[10] to be advantageous as chemosensors utilizing their probe-analyte interaction driven structure-function correlation. Few such probes[8,9] were also been explored in detection of DCP. The methodological design of these reported probes incorporates a nucleophilic centre in form of a hydroxyl (-OH) group[8] as either hydroxamate, aliphatic and aromatic alcohols or, a substituted amino- functionality[9] attached to the spirolactam N-atom, which are primarily located at the substituents coupled to the signaling subunits. Their structural arrangements and associated mechanism of DCP mediated spiro-ring opening reasoned us to study on the role of substituents as an alternative approach of probe design for such phosphorylation. The xanthene derivatives **1**-**3** were chosen as molecular probes in this study (Fig. 1), where nucleophilic centres in their signaling unit is the basis of their design and their photophysical spectral responses towards various pesticides as the signal monitoring window. The structural aspect of their design is such that the amino-phosphorylation could be avoided though non-availability of amino- protons on the substituents at their hydrazide end through unsaturated coupling of substituents as imine linkage. Further, the substituents on Rhodamine-6G hydrazide derivative **1** does not contain –OH group, where as that in **2** contained an aromatic –OH group. On contrary, no hydroxyl group (–OH) was made available on the substituent attached to the fluorescein hydrazide derivative **3**, rather it is present on the xanthene ring. On

[a] Materials Chemistry Department, CSIR-Institute of Minerals and Materials Technology, P.O.: R.R.L., Bhubaneswar-751013, Odisha, India.
[b] co-affiliation: Department of Chemistry, Utkal University, Bhubaneswar-*751013, Odisha, India*
† Email: bpbag@immt.res.in, Tel: (+ 91) 674 237 9254, Fax: (+) 91 674 258 1637
Electronic Supplementary Information (ESI) available: [Detailed synthetic procedure, Characterization of 1-3 and their DP-adducts (crystallographic, ^{1}H, ^{13}C NMR, ESI-MS, FT-IR), absorption and emission spectral data, theoretical calculations]. See DOI: 10.1039/x0xx00000x

basis of present approach of probe design and with subsequent structure-function correlation, **1** is expected to have abridged response on interaction with organophosphates whereas **2** and **3** are arguably to be responsive through a preferential phosphorylation at respective hydroxyl sites. In this investigation on contrary, we report that **1** and **2** exhibited, and **3** failed, to exhibit selective detection of DCP as reflected through DCP mediated opening of spiro-ring which translated onto corresponding photophysical signals with dual channel chromogenic and fluorogenic signaling as monitoring window.

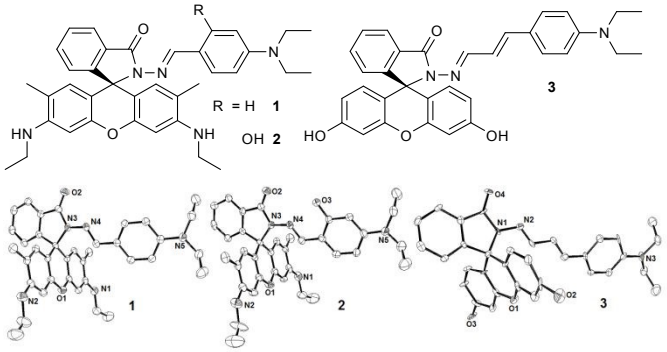

Fig. 1: Xanthene dye based compounds (**1**-**3**) used in this study and corresponding ORTEP diagram of their crystal structures. 40% ellipsoids, H-atoms are omitted for clarity.

The reaction of rhodamine-6G hydrazide with 4-diethylamino-benzaldehyde and 4-(diethylamino) salicylaldehyde resulted in **1** and **2**[11] respectively, similar reaction between fluorescein hydrazide and trans-(diethyl amino)-cinnamaldehyde resulted in **3.** These probes were characterized through ^{1}H-NMR, ^{13}C-NMR and ESI-MS spectroscopy(ESI). The molecular ion peak($[m/z]^+$) observed at 588.48$[M+1]^+$ and 604.50$[M+1]^+$, 532$[M+1]^+$, in ESI-MS spectra of **1**, **2** and **3** respectively have established their formation, also supported by carbonyl stretching frequencies(~1707 cm^{-1}) in their FT-IR spectra. The characteristic quaternary carbon peak of spirocyclic ring(C_{13}) in their ^{13}C-NMR spectra(δ ~ 65.6ppm, DMSO-d_6) and structural elucidation of their single crystals‡ confirm their existence in their spirocyclic form(Fig. 1). No significant peak was observed in 450-650nm region in their absorption or fluorescence spectra, rationalized to their spirocyclic structure.

In order to investigate guest-mediated photophysical signalling responses of **1** and **2**, various organophosphates were individually added to their solution in EtOH-H_2O (1:1 v/v, 0.1M PBS, pH 7.1) medium and to solution of **3** in DMSO. In this study, few organophosphates as well as non-organophosphate based pesticides were taken for comparison(ESI). Among all analytes added, **1** exhibited (Fig. 2a) an absorption peak with maximum at 533nm in selective presence of DCP ($\log\varepsilon_{[\mathbf{1}+DCP]}$ = 4.77) with concurrent colour change of the solution(colourless to pink). Similarly, excitation of **1** led to appearance of a fluorescence peak (λ_{em} = 553nm) in selective presence of DCP among all analytes under investigation(Fig. 2b). The observed dual mode colorimetric (colourless→pink, $\varepsilon_{[\mathbf{1}+DCP]}/\varepsilon_{\mathbf{1}}$=86) and fluorometric $((I_F/I_0)_{553}$=124, ϕ_F=0.611, τ_{av}=3.82ns(96.7%)) signal transduction in **1** is assignable to that of its DCP induced ring-opened conformation. **2** also exhibit similar absorbance ($\varepsilon_{[\mathbf{2}+DCP]}/\varepsilon_{\mathbf{2}}$=55) and fluorescence $((I_F/I_0)_{553}$=113, ϕ_F=0.547, τ_{av}=3.95ns(80%)) spectral amplification in selective presence of DCP among all analytes. Except DCP, other analytes failed to induce any such photophysical spectral transformation in both **1** and **2**.

Similar DCP-induced spectral enhancements in 450-650nm region were not observed with **3**. However, addition of DCP to **3** showed (a) a decrease in intensity of its characteristics absorption at 405nm, and (b) increase in absorption of that at 345nm with an isobestic point at 360nm. This indicates that interaction of **3** with DCP, which failed to open its spiro-ring, still formed a non-fluorescent ($\phi_F \leq 0.001$ for **3**, **3**-DCP) adduct in a different coupling mode[12] that exhibited a signalling response in a different output channel. Such photophysical responses of **3** were observed to be selective with DCP among the analytes studied.

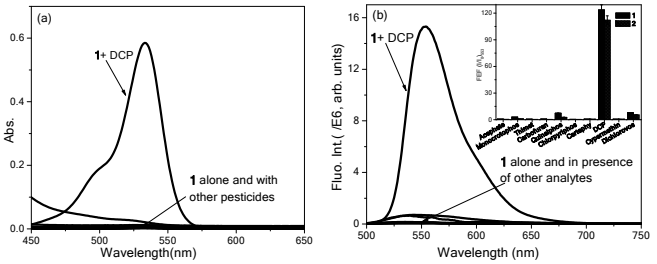

Fig. 2: (a) Absorption, (b) fluorescence spectra of **1** and (Inset b) FE factor in **1** and **2**(ESI) with various analytes in buffered EtOH (1:1 v/v, 0.1MPBS) medium. λ_{ex} (Fluo.)= 480nm [probe]= 10μM(abs.), 1μM(flu.), [analyte]= 10-50μM.

The plot of absorbance of **1** or **2** versus mole fractions of added DCP (Job's plot, λ_{obs} = 533nm) indicated to a 1:1 (probe: DCP) complexation stoichiometry(ESI). The absorption titration of **1**(10μM) with DCP in EtOH-H_2O(1:1 v/v, 0.1MPBS, pH = 7.1) revealed that its DCP-induced A_{533} absorption peak gradually increased (Fig. 3a) gradually addition of DCP and remained constant thereafter, with concurrence colour change in the solution. Similar trend in spectral enhancement against concentration of added DCP was observed in its fluorescence spectra (Fig. 3b). Similar spectral pattern were observed for **2** on titration with DCP. The association constants (K_a) for DCP complexation to **1** and **2** were determined for 1:1 stoichiometry through implicit functions of non-linear regression fit to the plot of absorption or fluorescence intensities against logarithm of concentrations of DCP added in buffered EtOH medium(ESI). The higher K_a, derived from fluorescence titration of **1**($\log K_a$= 10.58) and **2**($\log K_a$= 10.71) with DCP inferred to an effective probe-analyte interaction. The determined K_a from their absorption spectral titrations complement well to those obtained from fluorescence titrations, and are correlated with a factor [{$\log K_a$(fluo.)/ $\log K_a$(abs.)} = 1.27(in **1**) and 1.05(in **2**)] close to unity. The limit of detection of DCP with **1** and **2** were calculated to be 1.36μM and 26μM respectively(ESI), a concentration range adequate for their practical applications. Similarly, respective limit of quantification of **1** and **2** were estimated to be 4.53μM and 86.66μM.

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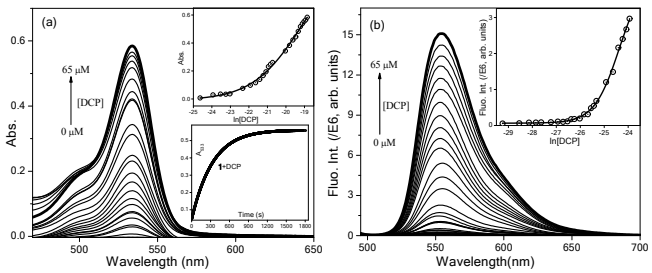

Fig. 3: (a) Absorption and (b) Fluorescence spectral pattern and (Inset, a and b) corresponding profiles of **1** as a function of added DCP in buffered EtOH (1:1 v/v, 0.1MPBS). (Inset a): Absorbance profile of **1** added with DCP(1:1) versus time (s). Abs.:[**1**] = 10 μM; λ_{obs} = 533nm; Flu.: [**1**] = 1 μM, λ_{ex} = 480nm, em./ex. b. p. = 5nm.

The response time is a crucial parameter in describing effectiveness of signalling probes. The rate of spiro-ring opening in **1** and **2** on interaction with DCP were determined from their absorption spectral responses(λ_{obs}= 533nm) as a function of time that followed a kinetics of first order ($A = A_0 e^{-kt}$). The rate of DCP-mediated spiro-ring opening in **1** was found to be to $0.001s^{-1}$, inferring to a faster response time of signalling. Interestingly, the A_{533} absorption in **2** upon addition of DCP first increased ($k_1 = 0.20s^{-1}$) and then decreased ($k_{-1} = 0.002s^{-1}$) before resulting in steady spectra till saturation, indicative of a different coordination patterns of adduct formation in **1** and **2** respectively. It is worth mentioning that in in-situ **3**-DCP adduct, where DCP did not open the spiro-ring but resulted in quenching of its A_{405} transition inferring to a different mode of its coordination to **3**, the spectral decay profile resulted in a rate constant of $0.036s^{-1}$.

In general, the rhodamine based probes are susceptible to raise false positive signals in analyte detection through proton mediated spiro-ring opening, therefore, the photophysical spectral profile of **1** and **2** and those of their corresponding DCP adducts were studied in varying pH(ESI). The spectral amplifications in both **1** and **2** were observed at lower pH (2-5 pH range) attributed to their spirocyclic ring opening under acidic environment. Their spectral pattern alone and in presence of DCP at 6-10pH inferred to their stability in DCP-mediated enhanced signals, without interference of protons. The control experiments with **1** and **2** by addition of Trifluoroacetic acid (TFA) in varied proportions in buffered EtOH overruled the proton mediated interference on their DCP selectivity (ESI).

The influence of other analytes on DCP selectivity of **1** was assessed by measuring absorbance at 553nm of solutions containing (a) **1** and individual pesticides followed by addition of DCP and (b) **1** and DCP followed by addition of those analytes. Addition of DCP to individual solutions containing **1** and other analytes exhibited a strong absorption peak(A_{533}) in each case to a comparable extent of that observed for **1** in presence of DCP alone(ESI). On contrary, addition of any of the interfering analytes to the solution containing **1** and DCP exhibited none or negligible changes to its DCP-induced absorption(A_{533}), which ascertained that the detection of DCP is not affected in presence of these analytes investigated here. The fluorescence spectra also inferred to similar signaling pattern. The DCP induced phosphorylation at carbonyl site in **1** and **2** may be argued for a cross-selectivity of compounds containing similar electrophilic centres. Therefore, the absorption spectral responses of both **1** and **2** were measured with p-toluene sulfonyl chloride (TsCl) and benzoyl chloride(BzCl). Addition of TsCl and BzCl to **1** (ε_{1+TsCl} =1154, ε_{1+BzCl} =5695 $dm^3mol^{-1}cm^{-1}$) or **2** (ε_{2+TsCl} =10489, ε_{2+BzCl} =29894 $dm^3mol^{-1}cm^{-1}$) were observed to undergo electrophilic tosylation/benzoylation and show spiro-ring opened turn on signalling, however, the extent of absorption spectral enhancements with TsCl and BzCl are significantly lower in comparison to those on addition of DCP(ε_{1+DCP} =58634, ε_{2+DCP} =97525 $dm^3mol^{-1}cm^{-1}$) under similar experimental conditions(ESI). These control experiments not only supported the DCP-mediated electrophilic phosphorylation at carbonyl end of **1** and **2**, but also ascertained their selectivity towards DCP over other interfering electrophilic compounds(TsCl and BzCl). Therefore, these probes are suitable enough to detect DCP selectivity among interfering structurally related organophosphates and other electrophilic species. The effective detection of DCP with **1** was demonstrated in solutions of soil samples through bare-eye observation with paper-strip/TLC plate methodology, where interaction of **1** (and **2** also) with DCP led to significant colour change (ESI) for visual detection.

The interaction of **1** with DCP was studied by comparing ^{1}H-NMR spectra of **1** before and after addition of stoichiometric amount of DCP (in DMSO-d_6). The DCP induced down-field shifts of aromatic protons on xanthene core of **1** and that of its imino-proton inferred to a phosphorylated adduct formation. The quaternary carbon(C_9) peak at 65.29ppm in ^{13}C NMR (DMSO-d_6) of **1** corresponding to spirocyclic conformation disappeared for in-situ **1**-DCP complex. This confirmed the xanthene spiro-ring opening during **1**-DCP interaction. The ESI-MS analysis with DCP, revealed to phosphorylation reaction with the loss of HCl, and formation of diethyl phosphate derivative of **1** in a 1:1 (probe: DCP) stoichiometry. Further, the FT-IR peak corresponding to spiro-cyclic carbonyl stretch of **1** was not observed in **1**-DCP. The imino C=N stretch of its amide form, P=O stretch and P-O-C stretch frequencies were observed (ESI), which ascertained the formation of diethyl phosphate derivatized **1** in its ring-opened conformation. The spectroscopic observations supported to a DCP-induced spiro-ring opening in **1** (Fig. 4) and therefore, complemented to that observed through photophysical spectral responses for DCP detection.

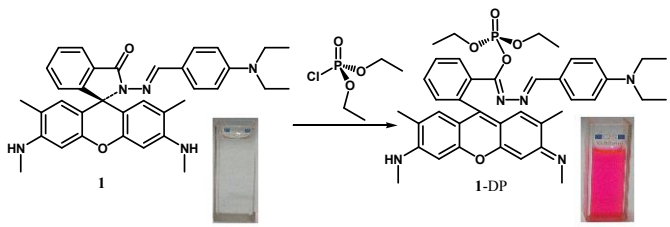

Fig. 4: Mechanism of phosphorylation in formation of **1**-DP and observed colour change in solution of **1**.

The energy minimized structures of the probes and the analytes were compared in order to understand the probe's preferential adduct formation with DCP over various analytes used in this study(ESI). The energies of HOMO and LUMO in **1** in ground state were calculated to be -4.79eV and -1.29eV respectively, estimating the energy of HOMO–LUMO transition in **1** to 3.49 eV. The HOMO–LUMO energy gap in the ground state corresponds to absorption transitions, therefore, calculated that at 354nm in **1**. Similarly the

HOMO(-4.77eV)–LUMO(-1.20eV) energy gap(3.56eV) corresponds to an absorption transition at 347nm in **2**. The estimated absorption transitions in both **1** and **2** are in good agreement with the observed experimental absorption peaks (±25 nm). Absence of any HOMO → LUMO transition in the 400–600 nm region in the elucidated structures of **1** and **2** also inferred to their existence of rhodamine's spirocyclic conformation. In the diethyl phosphate appended **1** (**1**-DP), the energy of HOMO (-4.60eV) was found to be slightly increased and that of LUMO (-1.73 eV) was more stabilized in comparison to those corresponding in **1**, due to enhanced conjugation in its ring-opened conformation in **1**-DP adduct. As a result, the HOMO-LUMO energy gap(2.86 eV) in **1**-DP adduct corresponds to a red-shifted absorption transition at 433nm. The formation of diethyl phosphate derivative of ring-opened **1** was evidenced from the spectroscopic evidences. It is assumed that the reaction of **1** as Lewis base with DCP as Lewis acid undergoes reorganization prior to formation of the **1**-DP adduct where the P-O bond formation compensate highly their combined reorganization energies. The stability of newly formed P-O bond in **1**-DP depends predominantly on interacting parameters of the two involved reactants, **1** and DCP. The stabilized energy of formation in the energy-minimized structure calculation of **1**-DP ascertained that as well. In this context, the chemical potential and HOMO-LUMO energy gap of the interacting molecules may be taken as the index of chemical reactivity and subsequent stability of adduct formed. A correlation of various parameters observed(ESI) for **1** and the analytes, despite presumed to follow a pre-organizational approach prior to react for a new adduct formation, predicted reaction preferences of **1** towards DCP. (a)The HOMO-LUMO energy gap in DCP(8.06eV) was observed to be higher than that of other analytes where as that of **1**(3.49eV) was lower in comparison to all analytes studied. (b) The difference in calculated chemical potentials between **1**(3.04 eV) and DCP(4.29 eV) was found to be higher than those in cases of other analytes. (c) The ground state dipole moment (μ_g) was estimated to be higher in DCP(4.64D) than other analytes and lower than that in **1**(4.98D), which correlates to a higher affinity of DCP for adduct formation representing through a lower difference in μ_g between **1** and DCP over that between **1** and other analytes. (d) The Mulliken charge over P-atom in DCP(1.1811e) was calculated to be higher than that over P-atoms in other organophosphate analytes, in correlation to the charge over spiro-cyclic carbonyl O-atom(-0.5789e) in **1**. Although a detailed investigation would verify these observations for a comprehension of reaction mechanism and despite the fact that mechanistic behaviors of the molecules explained with the gas phase theoretical calculations would diverge with the processes in solutions, the preliminary correlations support the probe's preferences towards DCP as observed through their photophysical signaling responses.

In summary, **1** was shown to exhibit selective and sensitive 'turn-on' signaling in presence of DCP among various organophosphates, through their signature spectral features corresponding to DCP-induced spiro-ring opening. **2** also exhibited similar DCP-induced photophysical signalling responses whereas **3** did not show such spectral enhancements with DCP. The study also revealed to formation of diethyl phosphate derivatives of the probes (**1** and **2**) through phosphorylation at carbonyl end on their spirolactam in their spiro ring-opened conformation. On a comparison to phosphorylation at hydroxyl- or amino- nucleophilic centres on the substituents attached, the phosphorylation on xanthene based signalling subunit endorses the methodology of probe design for DCP detection on rhodamine platform.

Conflicts of interest

There are no conflicts to declare.

Notes and references

‡ Crystallographic data: The CCDC no. for **1**, **2** and **3** are 1963360, 1963358 and 1978815 respectively.

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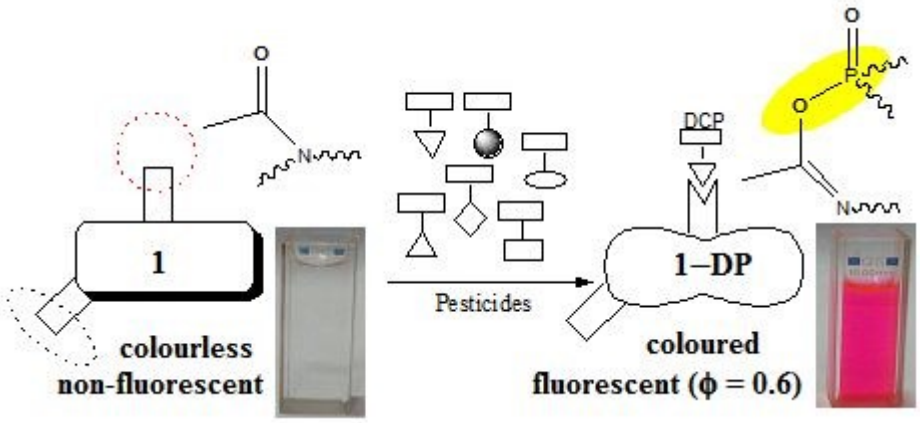
Selective DCP detection with xanthene derivatives by carbonyl phosphorylation

Kanhu Charan Behera[a, b] and Bamaprasad Bag[a,*]

[a] Materials Chemistry Department, CSIR-Institute of Minerals and Materials Technology, P.O.: R.R.L., Bhubaneswar-751013, Odisha, India. Fax: (+) 91 674 258 1637; Tel: (+ 91) 674 237 9254, Email: *bpbag@immt.res.in*
[b] Present affiliation: Department of Chemistry, Utkal University, P.O.: Bhubaneswar-751013, Odisha, India

Graphical Abstract



Dual mode signalling for DCP detection with rhodamine-6G derivatives through phosphorylation at spirocyclic carbonyl end.