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Solvatochromic fluorescent cyanophenoxazine: design, synthesis, photophysical properties and fluorescence light-up sensing of ct-DNA†

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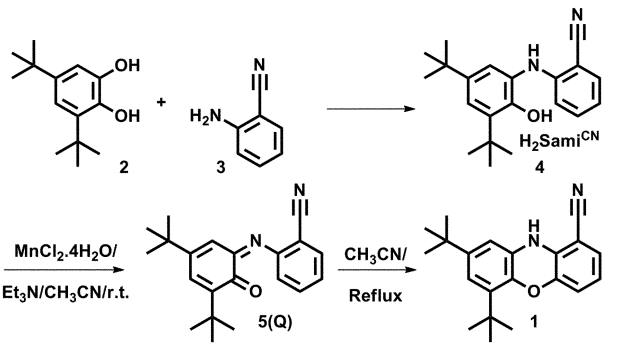
We report our new methodology for the Mn^{2+}-catalysed synthesis of donor–acceptor substituted classical fluorescent phenoxazine which showed high solvatochromic emission properties and ct-DNA sensing efficiency *via* a light-up fluorescence response.

The conjugation or complexation of a small organic fluorophore to a biomolecule can extract inner biomolecular information. To investigate chemical and biochemical phenomena, solvatochromic fluorophores are the best suited fluorescent probes because of their ability to sense small variations in dielectric constant within a biomolecular microenvironment.[1] Moreover, for biomolecular applications, long wavelength emission, especially emission in the visible region is highly desirable to discard misleading signals exerted by the auto fluorescence shown by biological macromolecules. In this note, the development of a small organic fluorophore, having strong absorption and long wavelength emission, is described.

Phenoxazines are known for their unexceptional absorbance characteristics that make them non-fluorescent molecules.[2] Fusion of a benzene ring onto the phenoxazine heterocycle does not enhance its fluorescence properties. On the other hand, donor and/or acceptor substituents can give rise to benzophenoxazine fluorophores.[2] Employing this concept, some benzophenoxazines have been generated. Among them very few substituted benzophenoxazines, *e.g.* Meldola's Blue 1, Nile Red 2, Nile Blue 3, are known to show fluorescence properties.[2,3] The available fluorescent benzophenoxazines are mainly based on the benzo[*a*]phenoxazine series and not any other ring fusion series. It is also noticeable that there is no report of simple planar tricyclic classical phenoxazines with fluorescent properties. Furthermore, the synthesis of phenoxazine dyes consists of economically less favorable high temperature condensation methods, and many of them are difficult to carry out in a simple reaction set up.[2e] Therefore, looking at the importance of fluorescent benzophenoxazine-based probes in biological research, there is a need to develop more useful fluorescent phenoxazines/benzophenoxazines which can be utilized as labels/probes of biomolecules *via* a simple but novel synthetic methodology. Hence, in our ongoing research into the design and synthesis of solvatochromic fluorophores,[4] we have considered phenoxazine dyes with donor–acceptor substituents as new potential solvatochromic fluorophores for investigating their possible use in biophysical applications. Herein, we present the synthesis and solvatochromic properties of a phenoxazine derivative, along with its efficient DNA sensing ability *via* a light-up fluorescence response.

Thus, reaction of 1 : 1 3,5-di-*tert*-butylcatechol and 2-aminobenzonitrile in hexane in the presence of Et_3N provided H_2Sami^{CN} (**4**, Scheme 1). When a 1 : 1 acetonitrile solution of H_2Sami^{CN} (**4**) and $MnCl_2{\cdot}4H_2O$ was refluxed in the presence of Et_3N, 69% **1** was isolated.[5] The reaction under room temperature (30 °C) provided **Q** (**5**) in 97% yield. Interestingly, using a catalytic amount of $MnCl_2{\cdot}4H_2O$ (4 mol%), **Q** (**5**) can be obtained in 98% yield. Under reflux, an acetonitrile solution of **Q** provided 96% **1**. It was found that there is no role of the metal salt in the formation of **1** from **Q** (**5**) under the above stated reaction conditions. The final structure of the probe **1** was characterised by NMR, IR, mass spectrometry and X-ray single crystallography.



Scheme 1 The synthetic route to **1**.

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† Electronic supplementary information (ESI) available: Experimental details, photophysical spectra and theoretical data. CCDC reference numbers 898030. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c3ra23463k

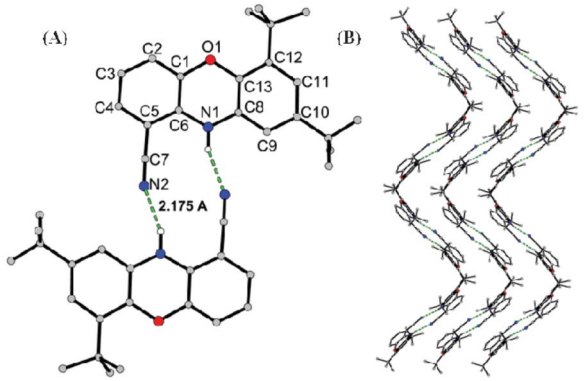

Fig. 1 (A) H-bonding between two individual molecules; (B) H-bonded molecules forming a helical layer (CCDC number for **1**: CCDC 898030).

Crystal structure inspection revealed that two adjacent molecules, connected to each other *via* two strong hydrogen bonds (2.175 Å), form a pair (Fig. 1A). Each pair is almost perpendicular to the previous pair. This results in helical layers with a 2.92 Å interlayer separation (Fig. 1B).

After obtaining the very pure compound in hand we next turned our attention to study its photophysical properties. Thus, the absorption spectra of **1** measured in the 250–700 nm region employing various solvent systems showed two absorption bands at ~309 and ~373 nm. The 373 nm band was assigned as an intramolecular charge transfer (ICT) band owing to the broad shape, intensity, and solvatochromicity (~16 nm) of the band (Fig. 2A).[6] The charge delocalisation from the donor secondary amine (–NH–) to the acceptor cyanide (–CN) substituent is possibly responsible for the polar ground state and ICT nature of the molecule which caused a red shift of the absorption maxima.

A fluorescence photophysical study revealed that the effect of the solvent polarity on the emission maxima is more pronounced than that on the absorption maxima of **1** (Fig. 2B). The fluorescence spectra showed a structureless broad band for the solvents chloroform, ethylacetate, MeOH and CH_3CN, while structured bands were observed for solvents like cyclohexane, toluene, DMSO. An increase in solvent polarity led to a large Stokes shift of the emission maxima for **1**. A simultaneous decrease in both the fluorescence intensity, and the quantum yield (Table 1) was also observed.

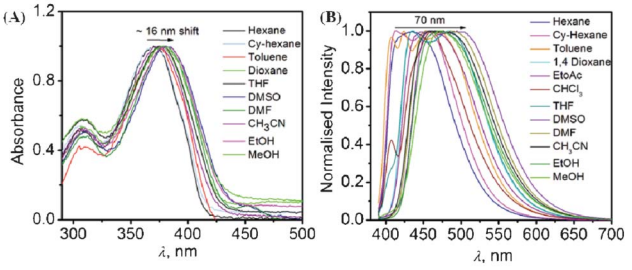

Fig. 2 (A) UV-Vis spectra; (B) normalized emission spectra in various solvents of the donor–acceptor substituted classical phenoxazine **1**.

Table 1 Summary of photophysical properties of **1**

		UV-Vis and fluorescence					
Solvents	Δf	λ_{max}^{abs}(nm)	λ_{max}^{fl}(nm)	Φ_f	τ_1	k_f	k_{nr}
Cyclohexane	0.00	309, 373	415, 457	0.72	—	—	—
Hexane	0.00	308, 373	437	0.78	3.9	1.9	0.5
Toluene	0.01	312, 377	426, 449, 475	0.79	—	—	—
Dioxane	0.02	312, 377	437, 485	0.68	—	—	—
$CHCl_3$	0.15	310, 393	460	0.31	4.6	0.7	1.5
EtOAc	0.20	311, 377	460	0.27	—	—	—
THF	0.21	310, 385	437, 486	0.36	—	—	—
DMSO	0.27	312, 384	460, 503	0.37	6.1	0.6	1.0
DMF	0.28	311, 382	455, 495	0.30	—	—	—
EtOH	0.29	311, 383	474	0.18	—	—	—
ACN	0.31	310, 375	473	0.39	—	—	—
MeOH	0.31	310, 379	477	0.15	3.4	0.4	2.5

τ_1 is in ns; k_f and k_{nr} are $\times 10^{-8}$ s

The fluorophore **1** is solvatochromic as evident by the red shift of the maxima (~40–70 nm) with the increase of solvent polarity ($\lambda_{max,\ cyclohexane}$ 415 nm, and $\lambda_{max,\ DMSO}$ 503 nm). The red shift and signal broadening of the spectra indicated that the conjugation is well extended between the acceptor –CN moiety and the donor –NH– moiety through the internal aromatic benzene ring system. Though the quantum yields decrease gradually as the solvent polarity increases, the intensity of the emission in polar protic solvents is sufficiently high enough for biological applications. Protic solvents, like methanol, are known to induce fluorescence quenching through non-radiative pathways *via* hydrogen bonding.[6] Thus, the fluorescence quantum yield in methanol was low probably because of protic solvent–solute interactions. These properties indicated that **1** has charge transfer (CT) character. The conjugation between the –NH– moiety as an electron donor and the –CN group as an electron acceptor plays an important role in the change in dipole during excitation and makes fluorophore **1** solvatochromic.

A reasonably high slope of $\tilde{\nu}_{max}^{fl}$ *vs.* Δf plot indicated again that the fluorescence states of **1** are of ICT character (ESI†).[7] Time dependent DFT calculations also showed a more significant electron redistribution of the emissive state of **1** and rationalized the explanation of the ICT origin and the solvent polarity dependency of the fluorophores' emission (ESI†).[8] To understand the fluorescence behaviour more precisely, we have measured the fluorescence lifetime in different solvents. It was found that the decay followed a single exponential fitting. Thus, it was clear that the effect of polarity is very similar to what was observed for the fluorescence quantum yield, *i.e.* increasing the polarity of the solvent has led to a shortening of the fluorescence lifetime, and the lifetime is sensitive to H-bonding. In protic polar solvents, like methanol, the fluorescence lifetime of **1** decreases dramatically. A comparison between the decrease in rate constant of the fluorescence emission (k_r) and an increase in rate constant of radiationless deactivation (k_{nr}) points out that the dependence of the fluorescence quantum yield of **1** on the nature of the solvent is mainly dictated by the changes in the rate of radiationless deactivation (ESI†).[6]

The encouraging solvatochromic fluorescence properties shown by **1** and the importance of benzophenoxazine derivatives as labels for several biomolecules has motivated us to examine and explore the possible sensing of biological microenvironments. As an example Nile blue was found to interact with ct-DNA but with a decrease in fluorescence.[2h] Therefore, we have studied the interaction behavior of our probe with calf-thymus DNA (ct-DNA), an easily available biomolecule with wide applications, by spectroscopic means in an aqueous phosphate buffer (pH 7.0).[9] Thus, the gradual addition of ct-DNA showed a negligible effect on the change in absorption maxima and intensity of fluorophore **1** located at 375 nm (ESI†).

The fluorescence titration experiment showed that the emission intensity (λ_{em} = 470 nm) of the probe upon gradual addition of ct-DNA was significantly enhanced upon excitation at 345 nm. The emission reached a maximum at 1 : 1 probe : ct-DNA concentration. This observation clearly indicated the well-defined binding of the probe with ct-DNA (Fig. 3A).[10] Beyond this ratio fluorescence quenching was observed that might be due to the association of more DNA to the probe's surrounding and thus it is likely that the radiationless channel opens up. Further studies of the mechanism of fluorescence quenching are under investigation. The emission response represented the possible binding of the probes along the groove side. The association constant of the probe with ct-DNA was also determined by a Benesi–Hildebrand plot (ESI†) which was found to be $1.4 \times 10^4\ M^{-1}$ with a free energy of binding of -5.6 kcal mol^{-1}.

The thermal melting behavior of ct-DNA in the presence of the probe indicated no destabilization of the ct-DNA, suggesting the probe as a possible groove binder (ESI†).[10] From a dye displacement study we observed no significant change in the fluorescence of an ethidium bromide containing EB–ct-DNA complex upon addition of **1** in different concentrations. This feature indicated that the probe was not interacting with ct-DNA as an intercalator but possibly as a groove binder (ESI†).[11] On the contrary, a decrease in fluorescence intensity of Hoechst 33258 in a Hoechst 33258–ct-DNA complex upon gradual addition of **1** suggested that **1** was most likely a groove binder of ct-DNA.[12] A negligible change in fluorescence anisotropy/polarisation also supported the groove binding of the probe (Fig. 3B).[13] The groove binding of the probe was further supported by the generation of a positive induced CD spectrum appearing at ~305 nm upon the binding of **1** to ct-DNA suggesting a minor groove binding mode which is also supported by macromodel optimized geometry calculations (Fig. 3C and ESI†).[14] Therefore, it is clear from the above facts that **1** senses ct-DNA with a light-up fluorescence response. The low fluorescence intensity of the probes in phosphate buffer in the absence of ct-DNA is not due to the insolubility of the probe but may be attributed to the radiationless channel assisted by the intermolecular hydrogen bonding that is present in aqueous solution.[6c,d] However, as the probe binds more and more along the groove of ct-DNA the nonradiative channels are possibly blocked to a greater extent and are less effective, ultimately leading to an enhancement of the fluorescence signal. Therefore, compared with other phenoxazine dyes such as Nile blue,[2h] our probe has the merit of sensing ct-DNA with a light-up fluorescence response.

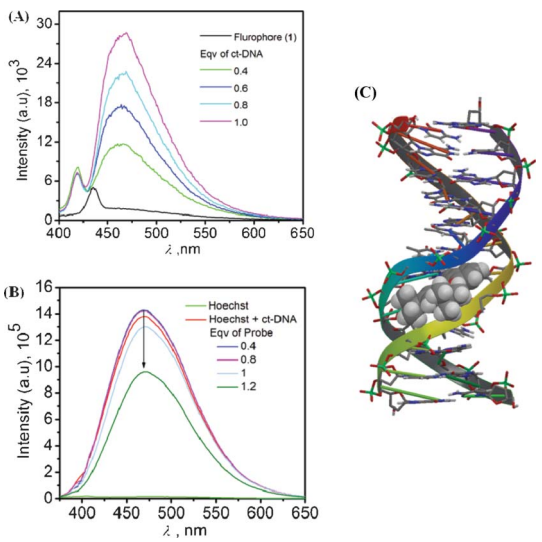

Fig. 3 (A) Fluorescence titration of probe **1** with various concentrations of ct-DNA ([probe] = 50 μM; phosphate buffer, pH 7.0, r.t., λ_{ex} = 345 nm). (B) Emission spectra of Hoechst (light green line at bottom), Hoechst–ct-DNA complex and Hoechst–ct-DNA complex titrated with various concentrations of probe **1**. (C) Amber* energy minimized geometry of the probe with model DNA, showing the minor groove binding of the probe. The DNA sequence was 5′-d(*CP*GP*CP*GP*AP*AP*TP*TP*CP*GP*CP*G)-3′, (PDB Id: 1DNH).

In conclusion, we have developed a metal catalysed quinonenamine (**Q**) formation, which subsequently undergoes electrocyclisation followed by aromatization, giving a route to a new classical fluorescent phenoxazine. The phenoxazine showed solvatochromic emission properties. The large to moderate Stokes shift in the emission maxima might make **1** a very effective probe for the analysis of biological microenvironments and labelling of biomolecules utilising the –NH– functionality. We have also shown that the probe **1** is able to sense ct-DNA *via* generation of an enhanced fluorescence signal. The synthesis and exploration of more phenoxazine dyes is our current research focus.

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