Supporting information

A CN$^-$ Specific *Turn-On* Phosphorescent Probe with Probable Application for Enzymatic Assay and as an Imaging Reagent

Upendar Reddy G.,$^a$ Priyadip Das,$^a$ Sukdeb Saha,$^a$ Mithu Baidya,$^b$ Sudip K. Ghosh$^{b,*}$ and Amitava Das$^{a,*}$

$^a$Central Salt and Marine Chemicals Research Institute (CSIR), G.B. Marg, Bhavnagar, 364002, Gujarat, India; E-mail: amitava@csmcri.org;

$^b$Department of Biotechnology, Indian Institute of Technology (IIT), Kharagpur, 721302, West Bengal, India; E-mail: sudip@hijli.iitkgp.ernet.in;

Telephone number: +91 278 2567760 [6720]; Fax number: +91 278 2567562/6970
Table of Contents:

Materials and method                                S3
Synthetic scheme                                    S4
Synthesis of [Ir2(ppy)4Cl2]                         S4
Synthesis and characterisation of L1                S5
Synthesis and characterisation of L2                S5
Synthesis and characterisation of 1                 S5
Synthesis and characterisation of 1.Cu              S6
1H NMR spectra of L1                                S7
Mass spectra of L1                                  S8
IR spectra of L1                                    S9
1H NMR spectra of L2                                S10
Mass spectra of L2                                  S11
1H NMR spectra of 1                                 S12
Mass spectra of 1                                   S13
IR spectra of 1                                     S14
Mass spectra of 1.Cu                               S15
Absorbance spectra of 1 and 1.Cu                    S16
Fluorescence response of 1 with different Concentrations of Cu²⁺. S16
Benesi-Hildebrand plot for binding studies of Cu²⁺ towards S17
Luminescence response of 1.Cu towards varying [CN⁻] S18
Mass spectra of TBACN addition to 1.Cu              S18
Evaluation of Michaelis constant for the enzymatic reaction between Mandelonitrile (mndl) and hydroxynitrile lyase (HNL). S19-S20
Experimental Section.

Materials. 2-phenylpyridine (ppy), IrCl$_3$.3H$_2$O, di-(2-picoly) amine (dpa), Sodium cyanoborohydride (NaBH$_3$CN), 4,4'-dimethyl-2,2'-bipyridyl, mandelonitrile, hydroxynitrile lyase were obtained from Sigma Aldrich. Selenium dioxide was purchased from Across India. Glacial acetic acid, Cu(ClO$_4$)$_2$.H$_2$O, NaCl, NaBr, KI, KNO$_3$, NaCN, CH$_3$COONa.3H$_2$O, NaIO$_4$, Na$_2$SO$_3$, NaF, Na$_2$HPO$_4$.2H$_2$O, NaH$_2$PO$_4$.2H$_2$O were purchased from SD Fine Chemicals in India.

Analytical Methods:

FTIR spectra were recorded as KBr pellets in a cell fitted with a KBr window, using a Perkin-Elmer Spectra GX 2000 spectrometer. $^1$H NMR spectra were recorded on a Bruker 200 MHz FT NMR (Model: Avance-DPX 200) or on a Bruker 500 MHz FT NMR (Model: Avance-DPX 500) using CD$_3$CN, CDCl$_3$ and CD$_3$OD as the solvent and tetra methyl silane (TMS) as an internal standard. ESI-MS measurements were carried out on a Waters QTof-Micro instrument. Electronic spectra were recorded with a Shimadzu UV-3101 PC spectrophotometer; while fluorescence spectra were recorded using an Edinburgh instrument Xe 900 spectro fluorometer.

UV-Vis and Fluorescence study

1.0 x 10$^{-4}$ M solution of the 1 and 1.Cu in aq.-HEPES buffer-CH$_3$CN (98: 2; 99.6: 0.4(v/v); pH 7.6) medium was prepared and stored in dark. This solution was used for all spectroscopic studies after appropriate dilution. The effective ratio for the aq.-HEPES buffer-CH$_3$CN in the final solution was 99.6: 0.4. 1.0 x 10$^{-3}$ M solutions of inorganic salt of respective anions were prepared in HEPES buffer (pH = 7.6). Solution of the compound 1 and 1.Cu was further diluted for spectroscopic titrations, and the effective final concentration of the solution of compound 1 and 1.Cu used for the fluorescence study was 2.0 x 10$^{-5}$ M, while the final analyte concentration during emission spectral scanning was 8.0 x 10$^{-4}$ M. For emission spectral titration effective [CN$^-$] was varied between (0 to 6.0 x 10$^{-4}$ M), while maintaining [1.Cu] as 2.0 x 10$^{-5}$ M. For the evaluation of the formation constant of 1.Cu, emission spectral titration was carried out using effective [Cu$^{2+}$] between (0 to 1.4 x 10$^{-4}$ M), while maintaining [1] unchanged as 2.0 x 10$^{-5}$ M. All emission studies were performed in aq.-HEPES buffer-CH$_3$CN (99.6: 0.4(v/v); pH 7.6) medium using $\lambda_{\text{ext}} = 380$ nm, $\lambda_{\text{Mon}} = 583$ nm.
and a slit width of 2.5 nm. The relative fluorescence quantum yields (\(\phi_f\)) were estimated using equation 1 in aq.-HEPES buffer-CH\(_3\)CN (99.6 : 0.4(v/v); pH 7.6) medium by using the integrated emission intensity of Ru(bpy)\(_3\)\(^{2+}\) (\(\phi_f = 0.042\) in aqueous at RT) as a reference:\(^1\)

\[
\phi_f = \phi'_f \left( \frac{I_{\text{sample}}}{I_{\text{std}}} \right) \left( \frac{A_{\text{std}}}{A_{\text{sample}}} \right) \left( \frac{\eta_{\text{sample}}^2}{\eta_{\text{std}}^2} \right)
\]

where, \(\phi'_f\) is the absolute quantum yield for the Ru(bpy)\(_3\)\(^{2+}\), used as reference; \(I_{\text{sample}}\) and \(I_{\text{std}}\) are the integrated emission intensities; \(A_{\text{sample}}\) and \(A_{\text{std}}\) are the absorbances at the excitation wavelength, and \(\eta_{\text{sample}}\) and \(\eta_{\text{std}}\) are the respective refractive indices.

Scheme 1: Methodologies that were adopted for synthesis of \(L_1\), \(L_2\), \(\text{Ir}_2(\text{ppy})_4(\text{Cl})_2\), 1 and 1.Cu.

**Synthesis of \([\text{Ir}_2(\text{ppy})_4\text{Cl}_2]\)\(^2\)**

A mixture of 2-methoxy ethanol and water (3: 1, v/v) was added to round bottom flask containing IrCl\(_3\).H\(_2\)O (0.5 g, 1.58 mmol) and 2-phenyl pyridine (0.612 g, 3.95 mmol). The mixture was refluxed for 36 h. After cooling to room temperature, yellow solid precipitated. This was filtered to give crude cyclometallated Iridium(III) Chloro-bridged dimer ((ppy)\(_2\)Ir(\(\mu\)-Cl)\(_2\)Ir(ppy)\(_2\)), which was further washed with cold methanol and water for further purification and then dried in a desiccator. Yield: 0.6 gm, 33%. This was used for further reactions.

**Synthesis of 4'-Methyl-2,2'-bipyridyline-4-Carbaldehyde (L\(_1\))\(^3\)**

The compound 4,4'-dimethyl-2,2'-bipyridyl (1.8 g, 9.78 mmol) and SeO\(_2\) (1.1 g, 9.9 mmol) were added to 1,4-dioxane (50 mL) and the mixture was heated at reflux under an inert atmosphere for 24 h. The solution was filtered while hot to remove precipitated metallic selenium. Then this filtrate was cooled to room temperature and allowed to stand for 1 h,
while a yellow precipitate appeared. This was filtered again to remove this pale yellow precipitate. Then, the final filtrate was evaporated to dryness. The solid mass was redissolved in ~ 300 mL of ethyl acetate and was treated with aqueous Na₂CO₃ (0.1 M, 20 mL) to remove any carboxylic acid derivative that could have generated (during the oxidation reaction by SeO₂) and remained in this solution. The ethyl acetate layer was separated and further treated with 0.2 M aqueous solution of NaHSO₃ (3 x 30 mL). Three aqueous extracts were combined and pH was adjusted to ~ 9 by the adding aq. solution Na₂CO₃. Then the desired compound was extracted into the dichloromethane (3 x 30 mL) layer. The dichloromethane extracts were combined and evaporated to give 4'-methyl-2,2'-bipyridyl-4-carbaldehyde. Yield: 0.6 g, (40%).

**ESI-Ms (m/z):** Calculated for C₁₂H₁₀N₂O: 198.08, Observed: 199.10 [M + H⁺]; ¹H NMR [200 MHz, CDCl₃; δ (ppm)] 10.19 (1H, s, -CHO); 8.90 (1H, d, J = 4.8 Hz, H⁵ (bpy)); 8.83 (1H, s, H³ (bpy)); 8.58 (1H, d, J = 4.6 Hz, H⁶ (bpy)); 8.28 (1H, s, H⁵ (bpy)); 7.72 (1H, d, J = 4.2 Hz, H⁶ (bpy)); 7.20 (1H, d, J = 4.4 Hz, H⁵ (bpy)); 2.47 (3H, s, (bpy-CH₃)). IR (KBr) [γ/cm⁻¹]: 1705 (s, -CHO).

**Synthesis of (L₂):**

L₁ (0.4 g, 2.08 mmol) and di-(2-picolyl) amine (0.413 g, 2.08 mmol) were dissolved in fresh methanol (35 mL). A catalytic amount of (two drops) of glacial acetic acid was added to the solution, which was refluxed for half an hour. The reaction mixture was allowed to cool to room temperature using an ice bath, and then to this cold solution NaBH₃CN (0.262 g, 416 mmol) was slowly added with rapid stirring. The ice bath was then removed and the reaction mixture was stirred overnight at room temperature. After that, the reaction mixture was treated with saturated aqueous sodium carbonate solution and subsequent extraction using dichloromethane was performed. The organic layer was recovered, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The crude product was subjected to neutral alumina gel gravity chromatography using ethyl acetate: hexane (2: 3, v/v) as eluent. Major fraction was collected and dried under vacuum, which afforded a sticky oil solid. Yield: 0.396 g (50%).

**ESI-Ms (m/z):** Calculated for C₂₄H₂₃N₅: 381, Observed: 382 [M + H⁺]; ¹H NMR [200 MHz, CD₃OD; δ (ppm)]. 8.43 (1H, d, J = 5.2 Hz, H⁶ (bpy)); 8.39 (1H, d, J = 5.2 Hz, H⁶ (bpy)); 8.32 (2H, d, J = 4.8 Hz, H⁴, H⁶ (pyridyl rings of dpa)); 8.19 (1H, s, H³ (bpy)); 7.98 (1H, s, H³ (bpy)); 7.73-7.58 (4H, m, H⁴, H³, H⁵ and H⁵ (pyridyl rings of dpa)); 7.40 (1H, d, J = 5.0 Hz, H⁵ (bpy)); 7.18-7.12 (3H, m, H³, H³, H⁵ (pyridyl rings of dpa) and H⁵ (bpy)); 3.74 (4H, s, -CH₂ (dpa)); 3.70 (2H, s, -CH₂ (bpy)); 2.34 (3H, s, -CH₃ (bpy)).
Synthesis of the [Ir(ppy)2L2]PF6 (1):
A mixture of [Ir2(ppy)4Cl2] (0.3 g, 0.28 mmol) and L2 (0.245 g, 0.644 mmol) in methanol-dichloromethane (60 mL; 1: 3 (v/v)) was refluxed in dark for 4 h. After cooling to ambient temperature, solution volume was reduced to ~ 45 mL under vacuum. To this concentrated solution a methanol solution (5 mL) of NH4PF6 (0.2 gm) was added with stirring. The solution was stirred for 30 min. A precipitate appeared and the solution was filtered. Solid was dissolved in dichloromethane and was purified by column chromatography using neutral alumina gel column. Eventually, the desired product was eluted with dichloromethane-methanol (9:1, v/v). Yield: 0.238 gm, (94 %). ESI-Ms (m/z): Calculated for C46H39IrN7P F6: 1027, Observed 882 [M- PF6]+; 1H NMR [500 MHz, CD3CN, δ (ppm)]: 8.66 (1H, s, H3 (bpy)); 8.48 (2H, d, J = 4.0 Hz, (H6, H6' (pyridyl rings of dpa)); 8.40 (1H, s, H3' (bpy)); 8.06 (2H, t, J = 7.5 Hz, (H4', H4'' (pyridyl rings of dpa)); 7.86-7.77 (6H, m, (H5, H5' (pyridyl rings of dpa), H4, H4' (pyridine of ppy), H3, H3' (phenyl of ppy)); 7.65 (2H, t, J = 7.5 Hz, H3, H3' (pyridine of ppy)); 7.60 (1H, d, J = 5.5 Hz, H6 (bpy)); 7.55-7.51 (3H, m, H3, H3' (phenyl of ppy), 1H, H1 (bpy)); 7.48 (1H, d, J = 5.0 Hz, H6 (bpy)); 7.33 (1H, d, J = 5.0 Hz, H5 (bpy)); 7.16 (2H, t, J = 6 Hz, H3, H3' pyridine of ppy)); 7.06-7.00 (4H, m, H4, H4' (pyridine of ppy), H4', H4'' (phenyl of ppy)); 6.91 (2H, t, J = 7.5 Hz, H5, H5' (phenyl of ppy)); 6.27 (2H, d, J = 7.5 Hz, H3, H3' (pyridyl rings of dpa)); 3.95(2H, s, -CH2 (bpy)); 3.88 (4H, s, -CH2 (dpa)); 2.59 (3H, s, -CH3 (bpy)); IR (KBr, γ/cm⁻¹): 840(s, PF6). 

Synthesis of 1.Cu
The compound 1 (0.1 g, 0.097 mmol) was dissolved in 15 mL methanol, to this Cu(ClO4)2. H2O was added. Solution colour was changed immediately. The reaction mixture was stirred for 4 h and then transferred into a beaker. The solution was allowed to evaporate slowly and to reduce its volume to half. A precipitate was formed at the bottom of beaker. This was collected by filtration and this residue was washed thoroughly with cold water. This was then dried in a disector. Dry solid was pure enough for further studies. Yield 0.095 g. ESI-Ms (m/z) calculated for C46H39CuIrN7PF6.H2O: 963, observed: 963 [M3+].

References :
**SI Figure 1: **$^1$H NMR spectra of $L_1$ in CDCl$_3$ medium.
Mass spectra of L₁

SI Figure 2: ESI- mass spectra of L₁.
**IR spectra of L₁**

SI Figure 3: FTIR spectra of L₁ as KBR pellet.
SI Figure 4: $^1$H NMR spectra of L$_2$ in CD$_3$OD medium.
Mass spectra of $L_2$

SI Figure 5: ESI- mass spectra of $L_2$. 
1H NMR spectra of 1

SI Figure 6: 1H NMR spectra of 1 in CD3CN medium.
Mass spectra of 1

SI Figure 7: ESI- mass spectra of 1.
IR spectra of 1

SI Figure 8: FTIR spectra of 1 as KBR pellet.
**Mass spectra of 1.Cu**

SI Figure 9: ESI- mass spectra of 1.Cu.
Absorbance spectra of 1 and 1.Cu

SI Figure 10. Absorbance spectra of 1 (2.0 x 10^{-5} M) and 1.Cu (2 x 10^{-5} M) were performed in 10 Mm HEPES buffer (pH 7.6) medium.

Fluorescence response of 1 with different Concentrations of Cu.

SI Figure 11. Emission spectral responses of 1 (2.0 x 10^{-5} M) towards varying [Cu(ClO_4)_2] (0 to 1.4 x 10^{-4} M) in aq.-HEPES buffer-CH_3CN (99.6 : 0.4(v/v); pH 7.6) medium using \( \lambda_{\text{ext}} = 380 \) nm and \( \lambda_{\text{Mon}} = 583 \) nm. Slit width 2.5 nm. 10 mM HEPES buffer solution was use for maintaining solution pH.
SI Figure 12. Benesi-Hildebrand plot of 1 (2 x 10^{-5} M) for varying [Cu(II)] (0 to 1.4 x 10^{-4} M) \( \lambda_{\text{ext}} = 380 \) and \( \lambda_{\text{Mon}} = 583 \) nm. Good linear fit confirms the 1:1 binding stoichiometry. in aq.-HEPES buffer-CH\textsubscript{3}CN (99.6: 0.4(v/v); pH 7.6) medium using \( \lambda_{\text{ext}} = 380 \) nm and \( \lambda_{\text{Mon}} = 583 \) nm. Slit width 2.5 nm. 10 mM HEPES buffer solution was use for maintaining solution pH.
Luminescence response of 1.Cu towards varying [CN−]

SI Figure 13. Luminescence response of 1.Cu (2.0 x 10^{-5} M) towards varying [CN−] (0 to 6.0 x 10^{-4} M). Studies were performed in aq.-HEPES buffer-CH₃CN (99.6: 0.4 (v/v); pH 7.6) using λ_{ext} = 380 nm and λ_{Mon} = 583 nm. Slit width 2.5 nm. 10 mM HEPES buffer solution was use for maintaining solution pH.

Mass spectra of TBACN addition to 1.Cu

SI Figure 14. ESI mass spectra of 1.Cu in presence of TBACN.
Evaluation of Michaelis Constant of the enzymatic reaction for the generation of cyanide from the mandelonitrile (mdnl) using hydroxynitrile lyase (HNL).

Liberated cyanide could demetalate 1.Cu to generate 1 with subsequent enhancement in emission at ~ 583 nm, i.e., the switch on emission response. Please note that relative quantum yield for 1.Cu was much lower than that for 1.

Our studies reveal that the displacement of Cu(II) from 1.Cu for the generation of 1 and Cu(CN)₅²⁻ is an instantaneous process and thus the reaction rate is fast. In step 1, generation of HCN at pH 6.5 is the slow step or rate determining step.

As mentioned in the above scheme decomposition of the {mdnl.HNL} adducts into corresponding acetaldehyde and HCN in Step-1 is the rate determining step with overall rate constant of k.

For the above reaction shown in step 1, the rate of the reaction (v) is as follows:

$$v = k_2[mndl.HNL] = \frac{k_2[HNL]_0[mndl]}{K_m + [mdnl]}$$

where $K_m = (k_{-1} + k_2) / k_1$

$$\frac{1}{v} = \frac{K_m}{k_2[HNL]_0[mndl]} + \frac{1}{1/k_2[HNL]_0}$$

Overall rate constant (v) for a certain [mdnl] with [HNL] = 30 µL, [1.Cu] = 2.0 x 10⁻⁵ M and pH = 6.5 (10 mM HEPES buffer-CH₃CN (99.6: 0.4(v/v)) solution) was evaluated from the plot of Log [Fₜ-F₀] vs. time (in sec), where Fₜ is the luminescence intensity for 1 at 583 nm ($\lambda_{ext} = 380$ nm) at time t and F₀ is the initial luminescence intensity of 1.Cu at 570 nm. Kinetics of the individual reaction was studied for initial 60 % of the total reaction (based on the change in luminescence intensity at 570 nm). A reasonably linear plot was obtained for v vs. [mdnl] for [mdnl] varying from 1.5 x 10⁻⁴ M to 5 x 10⁻⁴ M. Beyond the concentration of
5.0 x 10^{-4} M, a deviation from linearity was observed due to the saturation effect and thus, were not considered for the $1/v$ vs. $1/\text{[mndl]}$ for the evaluation of $K_m$. Thus, a plot of $1/v$ vs. $1/\text{[mndl]}$ would give intercept of $1/k_2[HNL]_0$ and slope of $K_m/k_2[HNL]_0$. Thus, \{slope/intercept\} would result $K_m (9.99 \times 10^{-4})$.

SI Figure 15: $1/v$ vs. $1/\text{[mndl]}$ plot, while $[HNL] = 30 \mu$L, $[\text{[Cu]}] = 2.0 \times 10^{-5} M$; pH was maintained at 6.5 with 10 mM HEPES buffer-CH$_3$CN (99.6: 0.4(v/v)) solution having temperature of 22°C.