Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Fluorescent Probe for Detection of Cyanide Ion in Aqueous Medium: Cellular Uptake and Assay for β-Glucosidase and Hydroxynitrile Lyase

Hridesh Agarwalla,[1] Monalisa Gangopadhyay,[1] Dharmendar Kr. Sharma,[2] Santanu Kr. Basu,[3] Sameer Jadhav,[3] Arindam Chowdhury,*[2] Amitava Das*[1]

[1]Organic Chemistry Division, CSIR-National Chemical Laboratory, Pune 411008, India

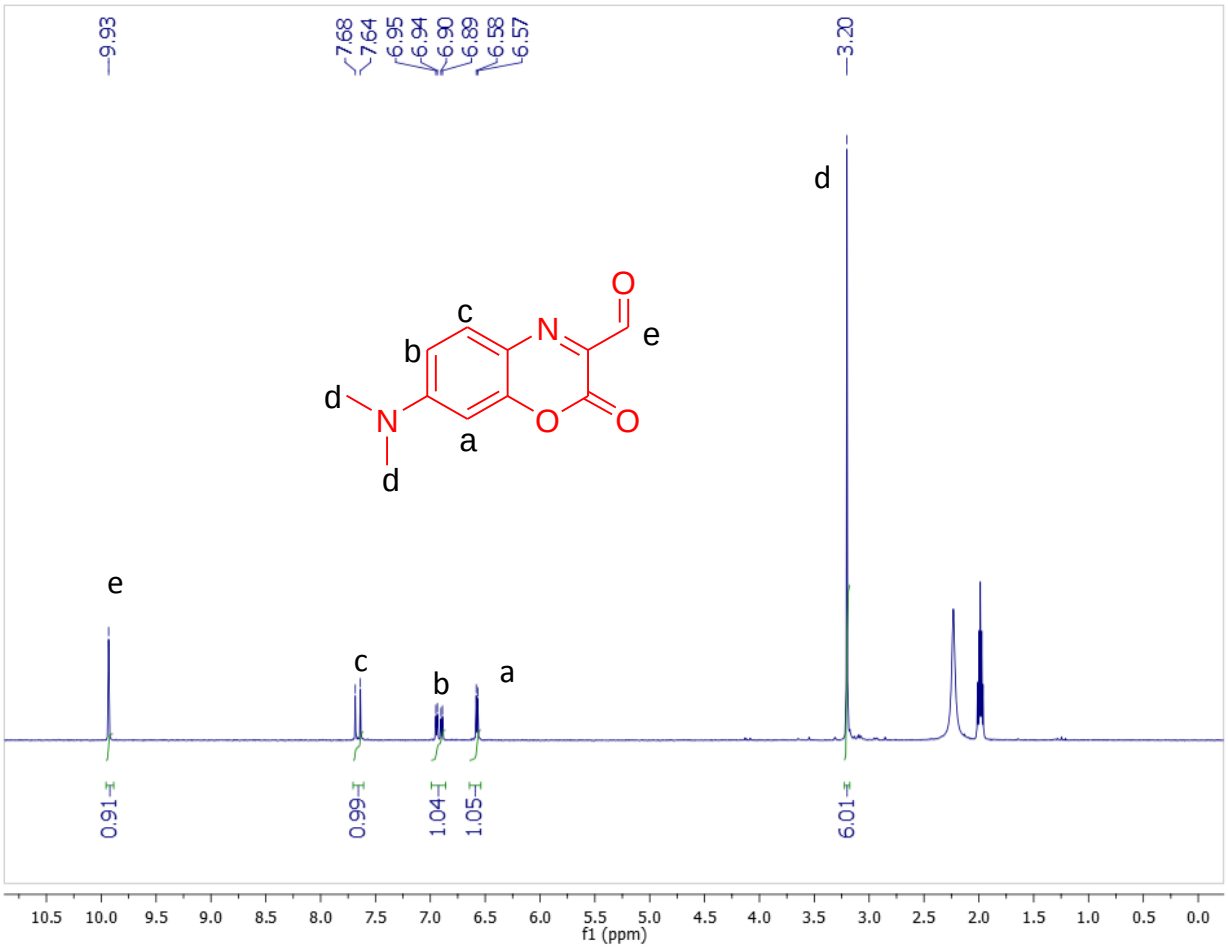
[2]Department of Chemistry and [3]Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India

* Corresponding authors

Synthesis of **1**:

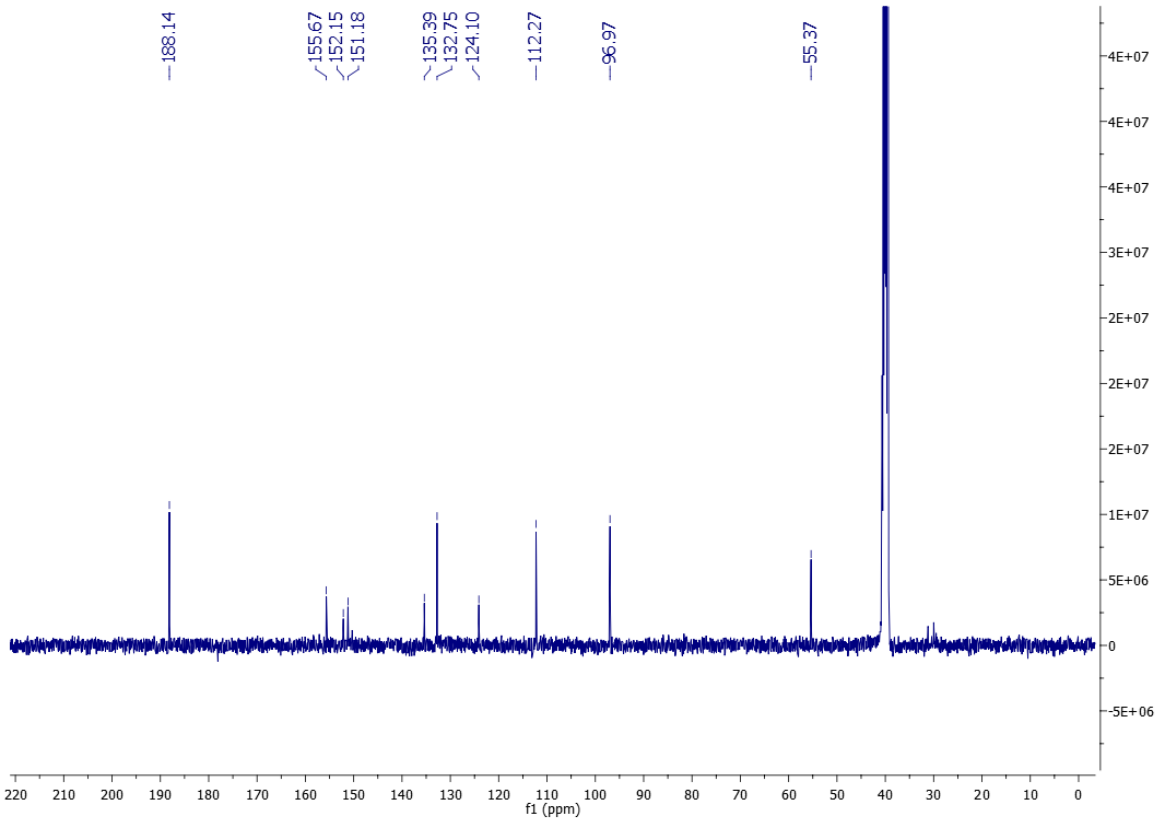
(7-(dimethylamino)-3-methyl-2H-benzo[b][1,4]oxazine-2-one was prepared by a similar method as previously reported.[25] (7-(dimethylamino)-3-methyl-2H-benzo[b][1,4]oxazine-2-one (500 mg, 2.2mmol) and selenium dioxide (333 mg , 3 mmol) was dissolved in 1,4-dioxan and heated at 75°C for overnight. Then filtered over celite-545, evaporated to dryness and purified by column chromatography using silica (100-200 mesh) as stationary phase and petether:ethylacetate (4:1) as eluent and further recrystalised from n-hexane to get pure product. (210 mg, 43 %). ^{1}H NMR (200 MHz, CD_3CN, 25 ^{o}C, TMS) 9.33 (s, $1H_e$,), 7.66 (d, $1H_c$, J = 8 Hz), 6.92 (dd, $1H_b$, J = 10 Hz, 2 Hz), 6.57 (d, $1H_a$, J =2 Hz), 3.2 (s, $6H_d$,). ^{13}C NMR (125 MHz, DMSO-d6, 25 ^{o}C, TMS) 55.37, 96.97, 112.27, 124.10, 132.75, 135.39, 151.18, 152.15, 155.67, 188.14. IR (KBr) ν_{max}/cm^{-1}: 2923, 2848, 1714, 1671, 1612, 1495, 1444, 1373. ESI-MS (*m/z*): 219.0764 $[M + H]^+$ Elemental analysis: $C_{11}H_{10}N_2O_3$ calculated C (60.55), H (4.62), N (12.84); found C (60.5), H (4.67), N (12.8).

^{1}H NMR of **1**



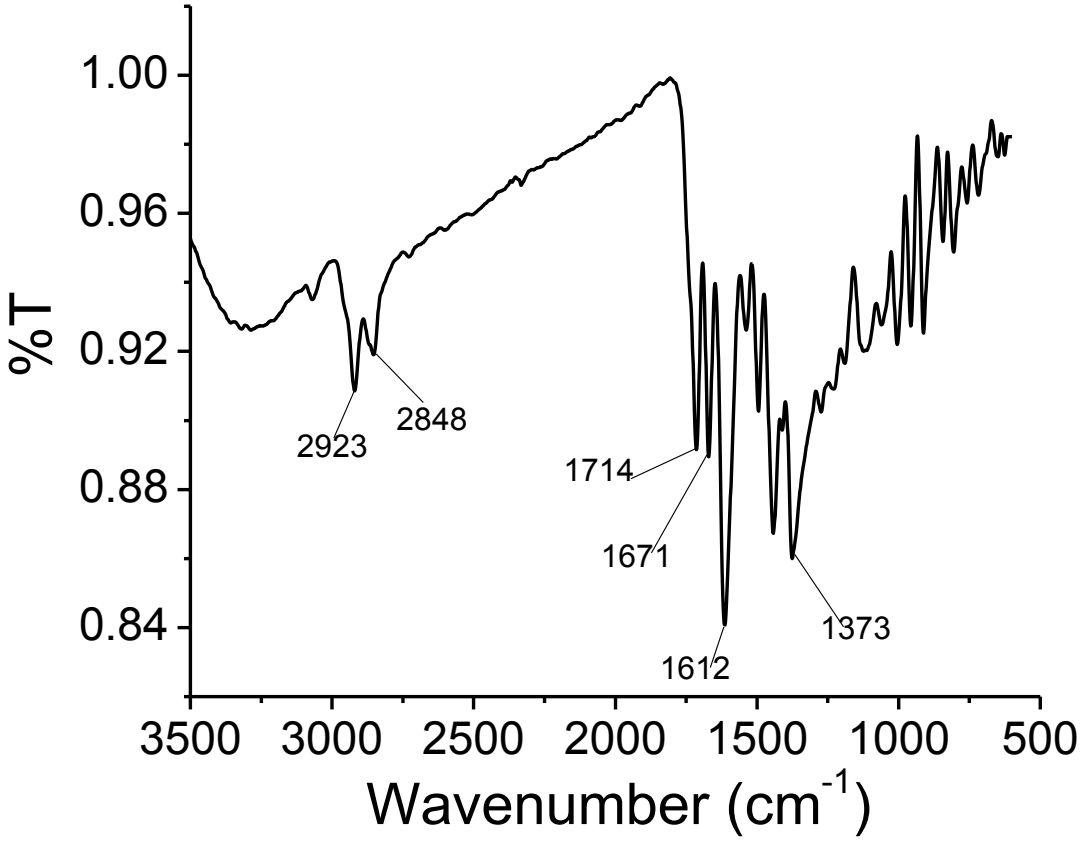
ESI figure 1. ^{1}H NMR spectra of **1** in CD_3CN.

^{13}C NMR of **1**



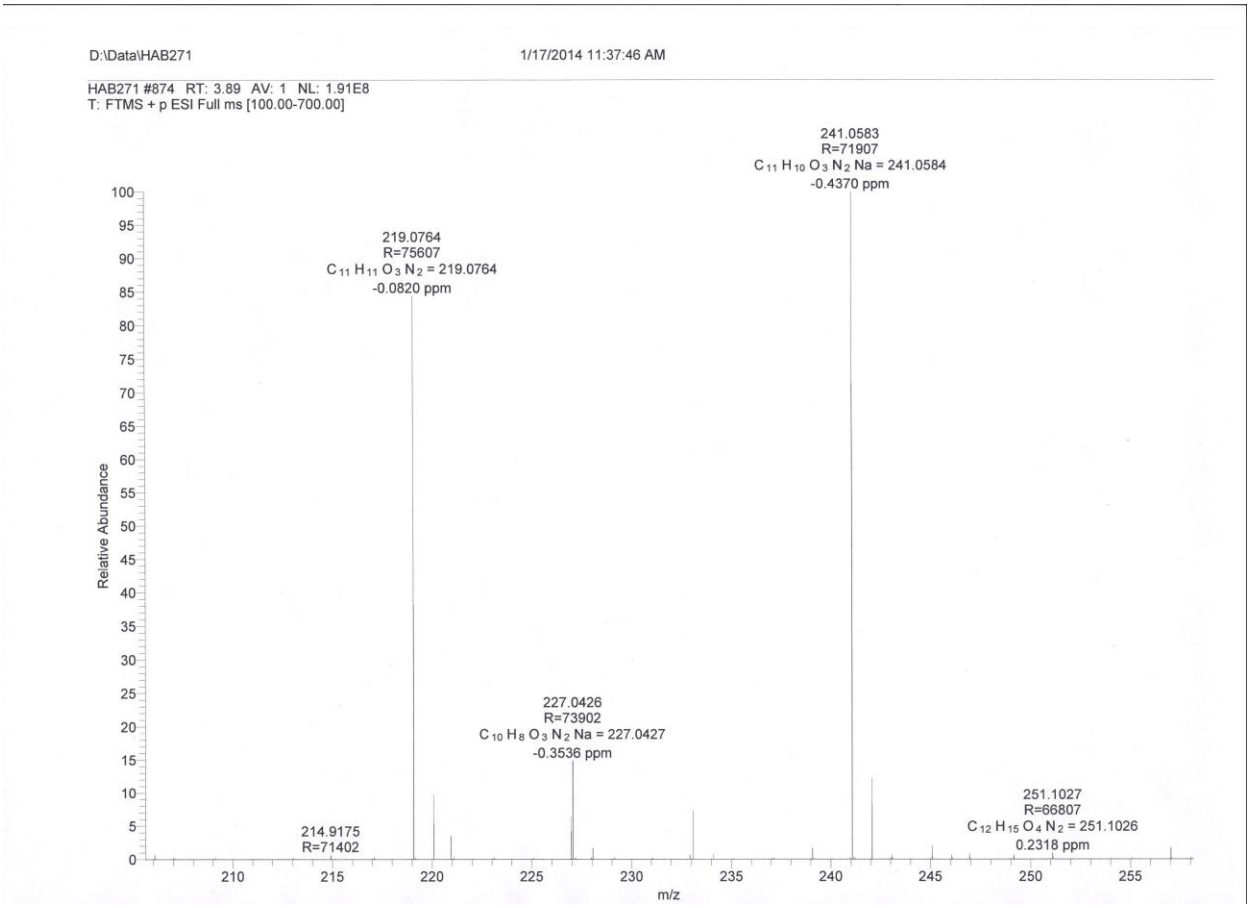
ESI Figure 2. ^{13}C NMR of **1** in DMSO-d_6.

FTIR spectra of **1**



ESI Figure 3. FTIR spectra of **1** recorded as KBr palette.

Mass spectra of **1**



ESI Figure 4. HRMS spectra of **1**

Experimental Section:

Materials and methods

3-(dimethylamino)phenol, hydrazine hydrate, ethyl pyruvate, selenium dioxide were purchased from Sigma-Aldrich and were used as received. Sodium salts of different anions and other reagents were used as received from S. D. Fine Chemical, India. Amygdalin, mandelonitrile, β-glucosidase and hydroxylnitrile lyase were purchased from Sigma-Aldrich and used as received. Dimethyl sulphoxide (DMSO) of HPLC grade was used for our studies. Deionized water was obtained from a Millipore water purification system. 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, ^{13}C NMR spectra were recorded using Bruker Avance-DPX 200/500 MHz FT NMR with trimethylsilane (TMS) as an internal standard. Electronic and steady state emission spectra were recorded with Perkin Elmer Lambda 950 UV-Vis spectrophotometer and PTI Quantam Master 400 spectrophotometer, respectively. ESI-MS measurements have been carried out on a Waters QTof-Micro instrument. Microanalyses (C, H, N) were performed using a Perkin-Elmer 4100 elemental analyzer.

Spectrophotometric studies:

Deionised water and HPLC grade DMSO were used as solvent for spectroscopic studies. A stock solution of **1** (5.0×10^{-3} M) in DMSO was prepared and used for further studies. Another solution having 1.2 mM of CTAB (CMC for CTAB is 1 mM) in 10 mM aq. HEPES buffer (pH 7.2) was prepared. To a 5 ml of this 1.2 mM of CTAB solution, 20 μl of stock solution of the reagent **1** (5.0×10^{-3} M in DMSO) was added and the resulting aq. solution (aq. HEPES buffer:DMSO of 250:1, v/v; pH 7.2) having this reagent trapped inside the micellar structure of CTAB was used for all spectroscopic study. Different anions as their sodium salt were used for

studies and all spectra were recorded at room temperature. For systematic titration studies, CN^- was added in an incremental manner.

Animal cell studies:

Materials: Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), PBS without Ca(II) and Mg(II), trypsin-EDTA, penicillin-streptomycin, paraformaldehyde were obtained from HiMedia Laboratories, Mumbai, India.

Cell culture:

MDA-MB-231 human breast adenocarcinoma cells were obtained from the National Centre for Cell Science, Pune, India and were cultured in complete medium at 37°C under 5% CO_2 atmosphere. The complete medium was comprised of DMEM, supplemented with 10% FBS, 1% antibiotics. For the experiments, after trypsinization, cells were seeded on to glass bottomed petridishes at the density of 5000 cells per square cm and incubated for 24 hours.

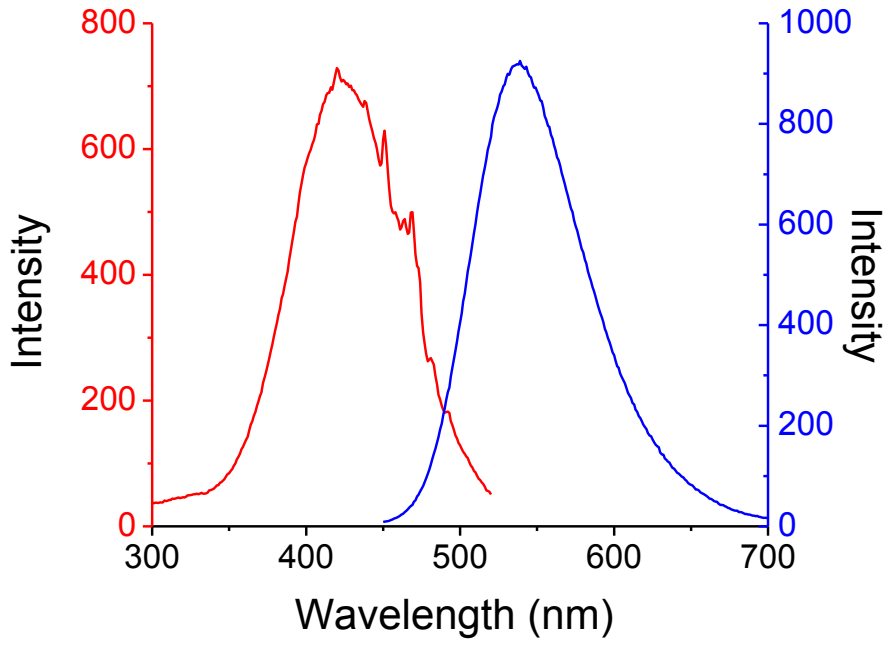
Epifluorescence Microscopy

For fluorescence intensity imaging, MDA-MB-231 cells were fixed with 4% paraformaldehyde for 15-20 minutes, followed by incubation of the cells with 20 μL of **1** (10^{-3} M) in 1 mL of PBS in two different Petri-dishes for 30 minutes at 37°C under 5% CO_2 atmosphere. After incubation, cells were washed twice with pre-warmed (37°C) phosphate-buffered saline (pH 7.4). For cyanide sensing these cells were incubated with 200 μM of CN^- for 15 minutes and then washed twice with PBS buffer, and visualized under inverted microscope (Olympus IX71) equipped with epifluorescence optics using a 10× phase-contrast objective, and a halogen lamp as a light source (Olympus FITC filter 488 - 525 nm). Images were taken with Q Imaging camera controlled by Image pro plus software.

Spatially resolved fluorescence spectroscopy:

A home-built objective-based epifluorescence/TIRF microscopy setup was used to perform intensity and spectrally-resolved imaging of Breast cancer cells, the details of which can be found in [S1] In brief, a 458 nm cw Ar^+ laser was used to illuminate the sample (~30 mm diameter) of fixed cells under TIRF mode through an inverted microscope (Nikon Eclipse 2000U). The emerging fluorescence was collected by the same objective and separated from the excitation beam by using 488 nm dichroic mirror and a long pass filter, and imaged using a CCD camera (DVC 1412AM). Intensity images were collected at 10 Hz with excitation powers low enough (200 W/cm^2) to minimize photobleaching. To obtain the fluorescence emission profiles from submicroscopic regions (~0.25 μm^2) in a high-throughput manner, the entire emission in absence of filters was collected through a combination of an adjustable narrow slit and transmission grating (70 l/mm, Optometrics) mounted in front of the CCD. Different microscopic regions within a cell as well as different cells in the ensemble were selected by moving the sample stage laterally while maintaining the focus. The dispersed emission spectra collected via the narrow vertically oriented slits were integrated along 5 pixels in vertical direction, and pixel to wavelength conversion was performed using several laser lines. All spectral data, arbitarily chosen from different subcellular regions or from various cells, were obtained at identical excitation powers (~200 W/cm^2) and 300 ms exposure time, and corrected for detector wavelength response. To construct statistically relevant distributions of emission peak positions and integrated intensities, more than 1000 spatially-resolved (0.25 μm^2) emission spectra were collected from ~15 cells in the absence and presence of cyanide, of which ~470 spectra were analyzed (for each) using Origin 8.0. All the measurements are carried out under ambient conditions at 295K.

Excitation and emission spectra of **1** with CN^-



ESI Figure 5. Excitation (λ_{Ems} of 535 nm) and emission (λ_{Ext} of 430 nm) spectra of **1** (20 μM) in presence of 3 equivalents of CN^-. Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB.

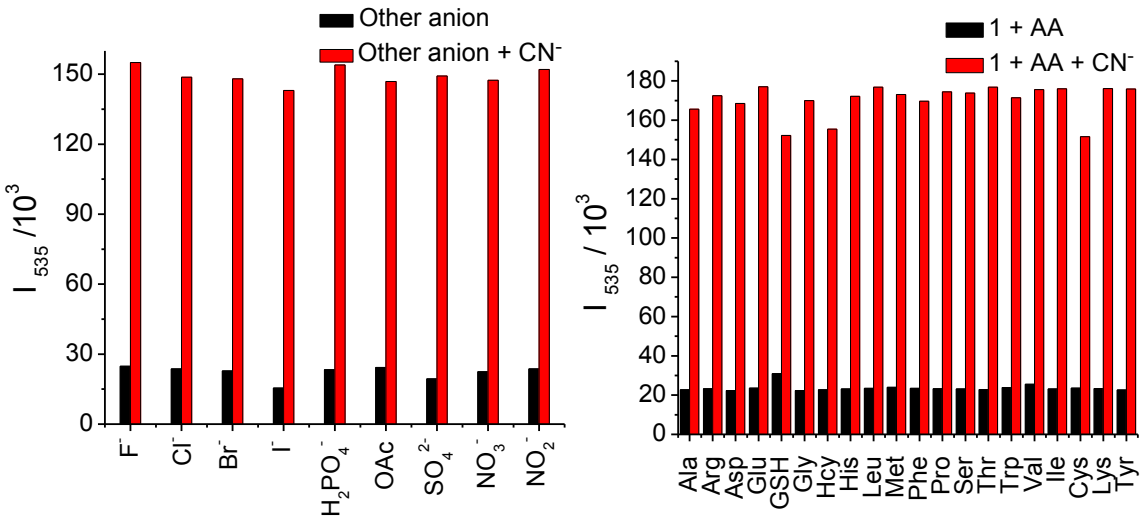
Quantum yield calculation:

The relative fluorescence quantum yields (Φ_f) were calculated using equation 1 in aq.-HEPES buffer-DMSO (250:1, pH 7.2) medium by using the integrated emission intensity of Coumarine-6 (Φ_f = 0.78 in ethanol) as a reference.

$$\Phi_f = \Phi_f' \, (I_{sample}/I_{std})(A_{std}/A_{sample})(\eta^2 sample/ \eta^2 std) \qquad \text{Eq. 1}$$

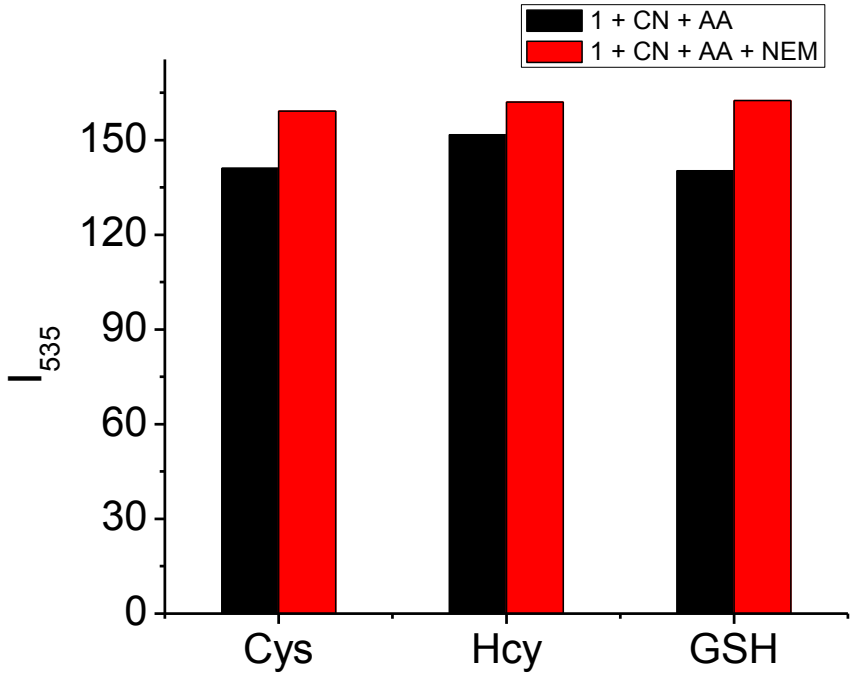
where, Φ_f' is the absolute quantum yield for the Coumarine-6, I_{sample} and I_{std} are the integrated emission intensities; A_{sample} and A_{std} are the absorbance at the excitation wavelength, and η_{sample} and η_{std} are the respective refractive indices.

Intereference study:



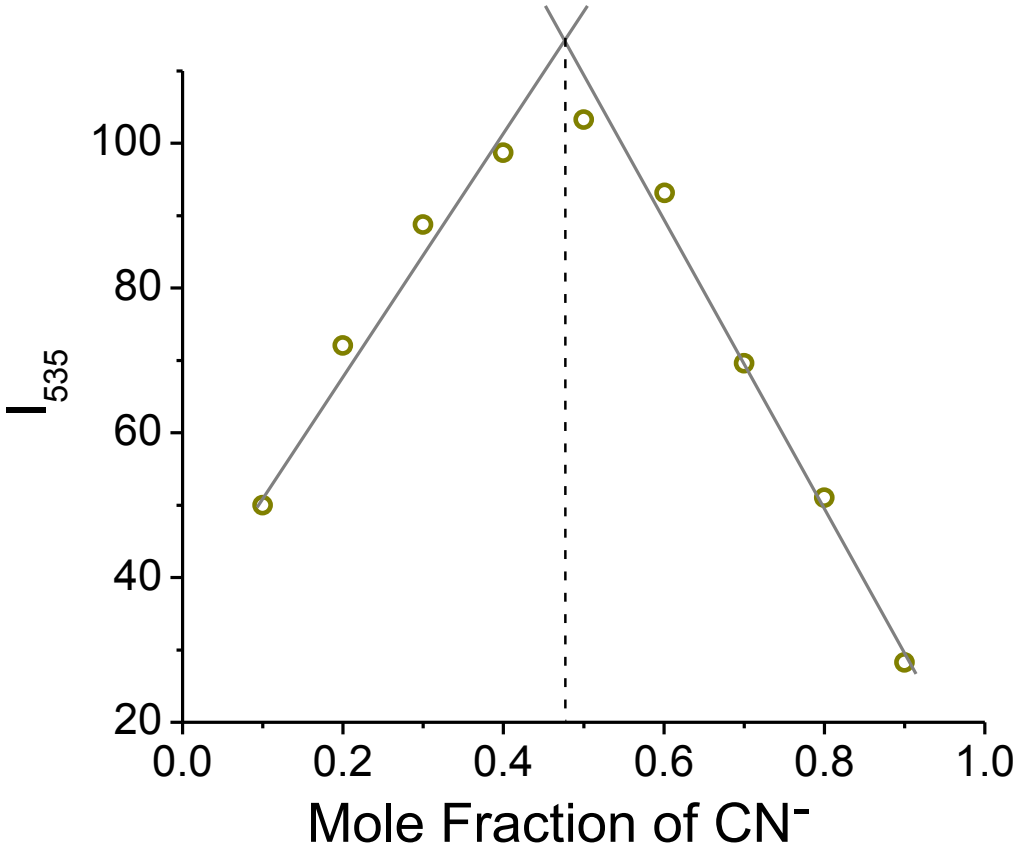
ESI Figure 6 : Variation of relative fluorescence intensity at 535 nm of **1** (20μM) in presence of 200 μM of competitive (a) anions F^-, Cl^-, Br^-, I^-, $H_2PO_4^-$, OAc^-, SO_4^-, NO_3^-, NO_2 (b) AA's and GSH in absence and presence of 100 μM of NaCN. Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB using λ_{Ext} of 430 nm.

Interference study with Cys, Hcy and GSH in absence and presence of NEM:



ESI Figure 7. Emission of **1** (20 μM) monitored at 535 nm with 5 equivalent of CN^-, 10 equivalent of Cys, Hcy, GSH in absence and presence of 50 equivalents of NEM. Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB using λ_{Ext} of 430 nm.

Jobs plot for reaction stoichiometry:



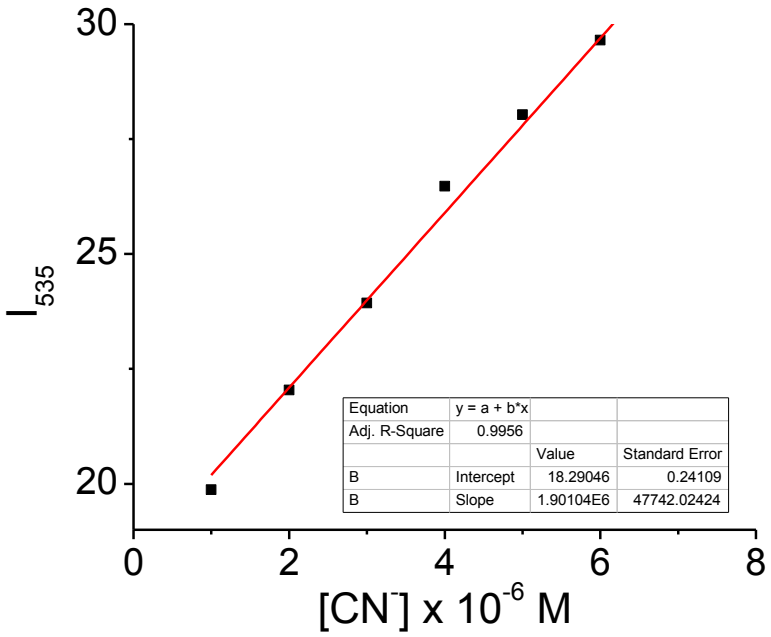
ESI Figure 8. Jobs plot analysis of reaction of **1** with CN^-. Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB using λ_{Ext} of 430 nm.

Lowest detection limit:

The detection limit of CN was calculated by following equation 2

DL= K. σ /s …..Equation 2

Where K= 2, σ is the standard deviation of blank measurement, s is the slope of intensity *vs.* [CN^-] plot.

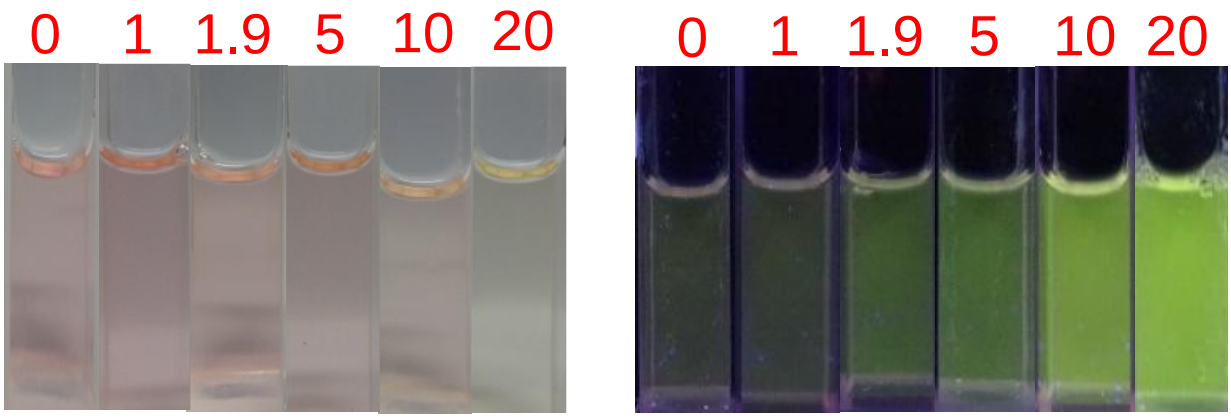


ESI Figure 9: Calibration curve for determining lowest detection limit. Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB using λ_{Ext} of 430 nm.

Here σ is found to be 0.2719 and slope form the graph is 1.90×10^6

DL calculated is 2.86×10^{-7} M.

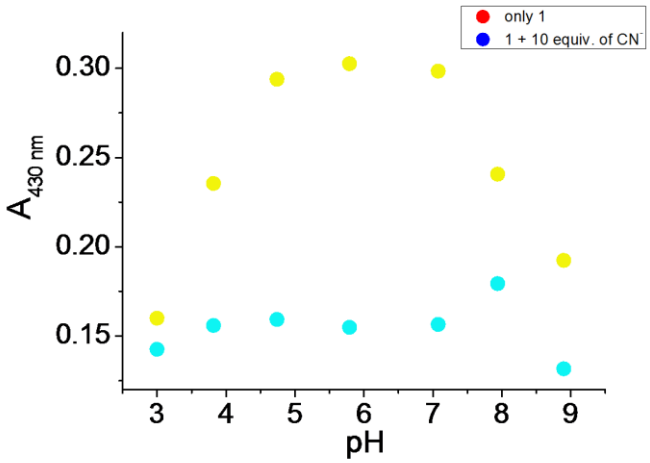
Visual detection of CN



ESI Figure 10. Visually detectable change in solution colour and luminescence for reagent **1** in absence and presence of varying [CN^-]. 1.9 μM concentrations is the threshold limit for portable and drinking water according to WHO norms. Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB. For luminescence photograph, a hand held Uv lamp (365nm) was used for illumination.

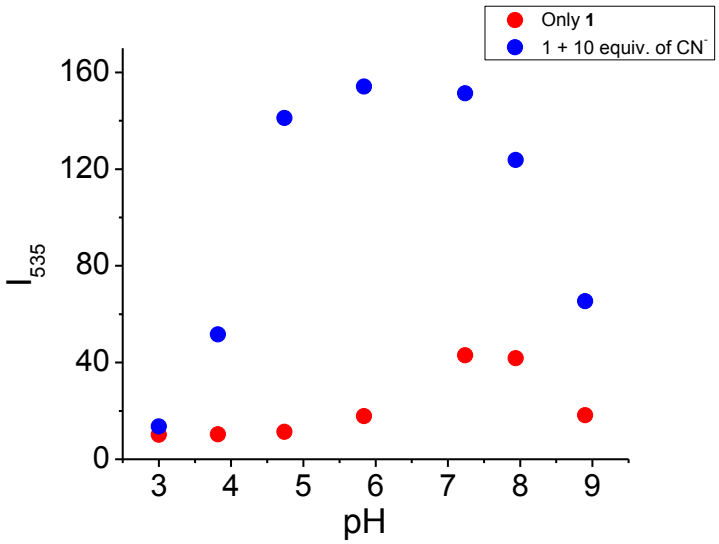
pH dependent study:

Uv-Vis study:



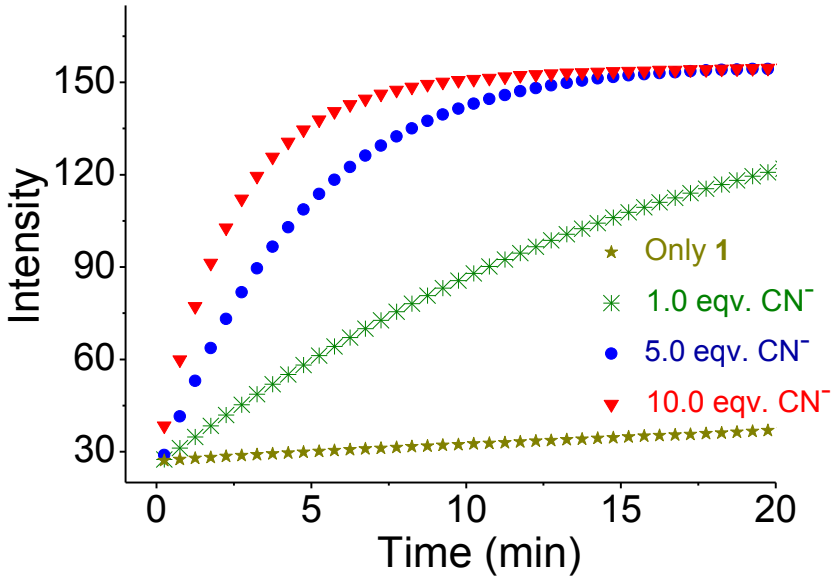
ESI Figure 11. pH dependent absorbance of **1** monitored at 430 nm in absence and presence of 10 equivalent of CN^-. Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB.

Fluorescence study



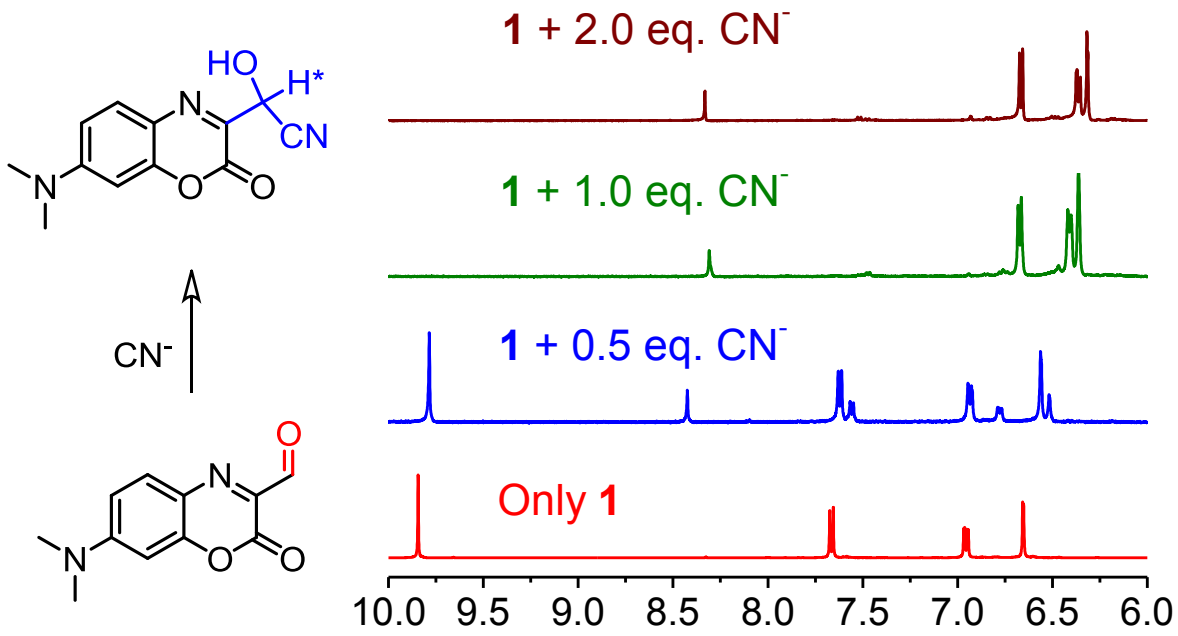
ESI Figure 12. pH dependent emission of **1** monitored at 535 nm in absence and presence of 10 equivalent of CN^-. Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB using λ_{Ext} of 430 nm.

Time dependent emission change:



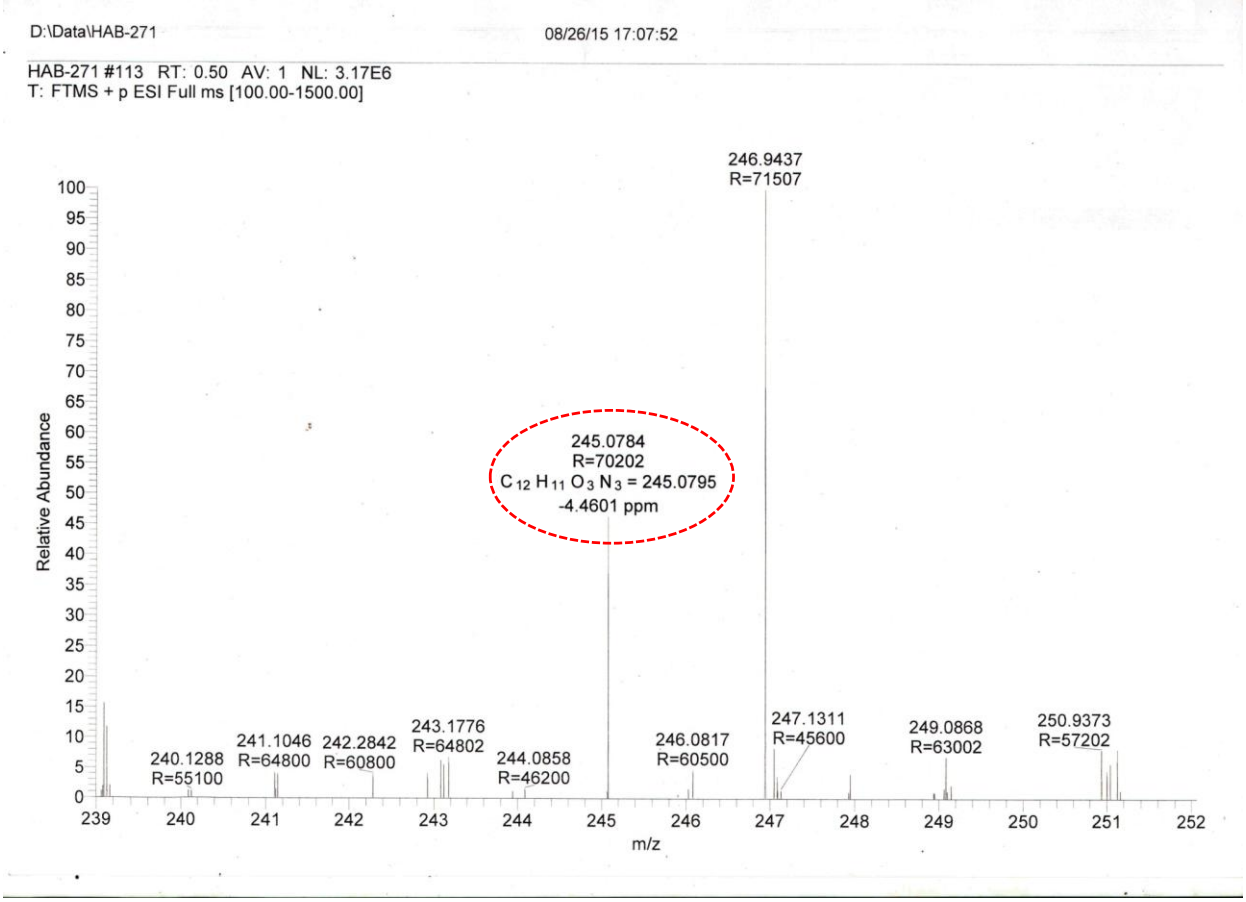
ESI Fig 13: Change in fluorescence of 1 (20 μM) at 535 nm on excitation at 430 nm in absence and presence of different concentration of CN^-. Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB using λ_{Ext} of 430 nm.

^{1}H NMR study:



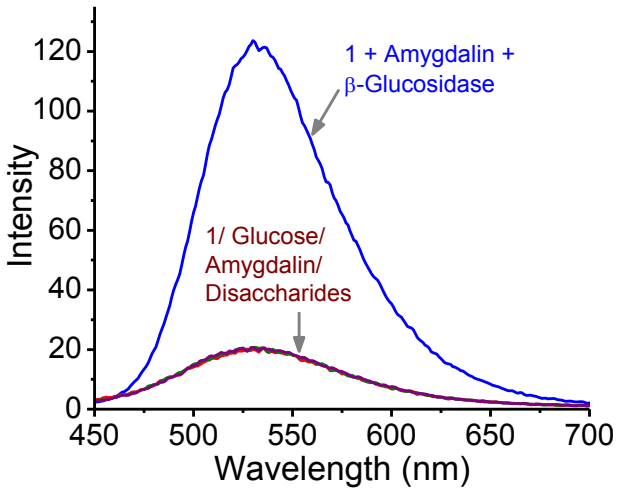
ESI Figure 14 : Partial ^{1}H NMR spectra of **1** were recorded in DMSO-d_6 in absence and presence of different mole equivalents of TBACN. Plausible reaction of **1** with CN^- for the corresponding cyanohydrins formation was also shown.

Mass spectra of **1** with CN^-



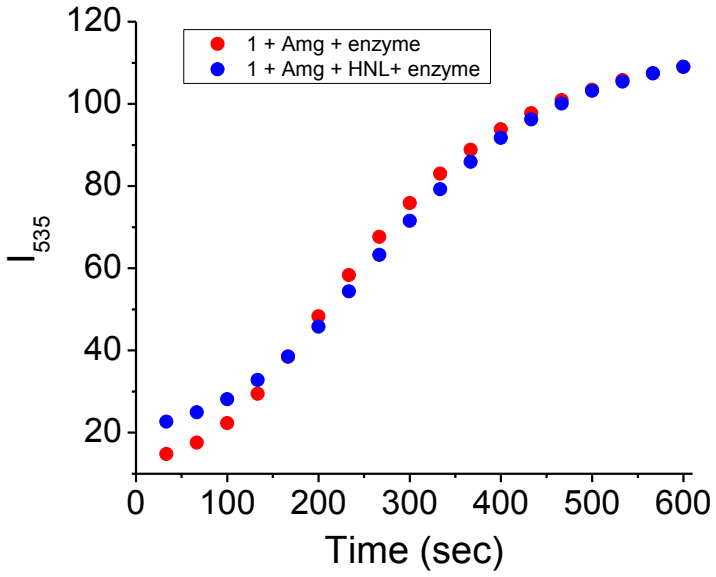
ESI Figure 15. ESI-Ms spectra of **1** with **CN^-.**

Control Experiments with Amygdalin and β-glucosidase



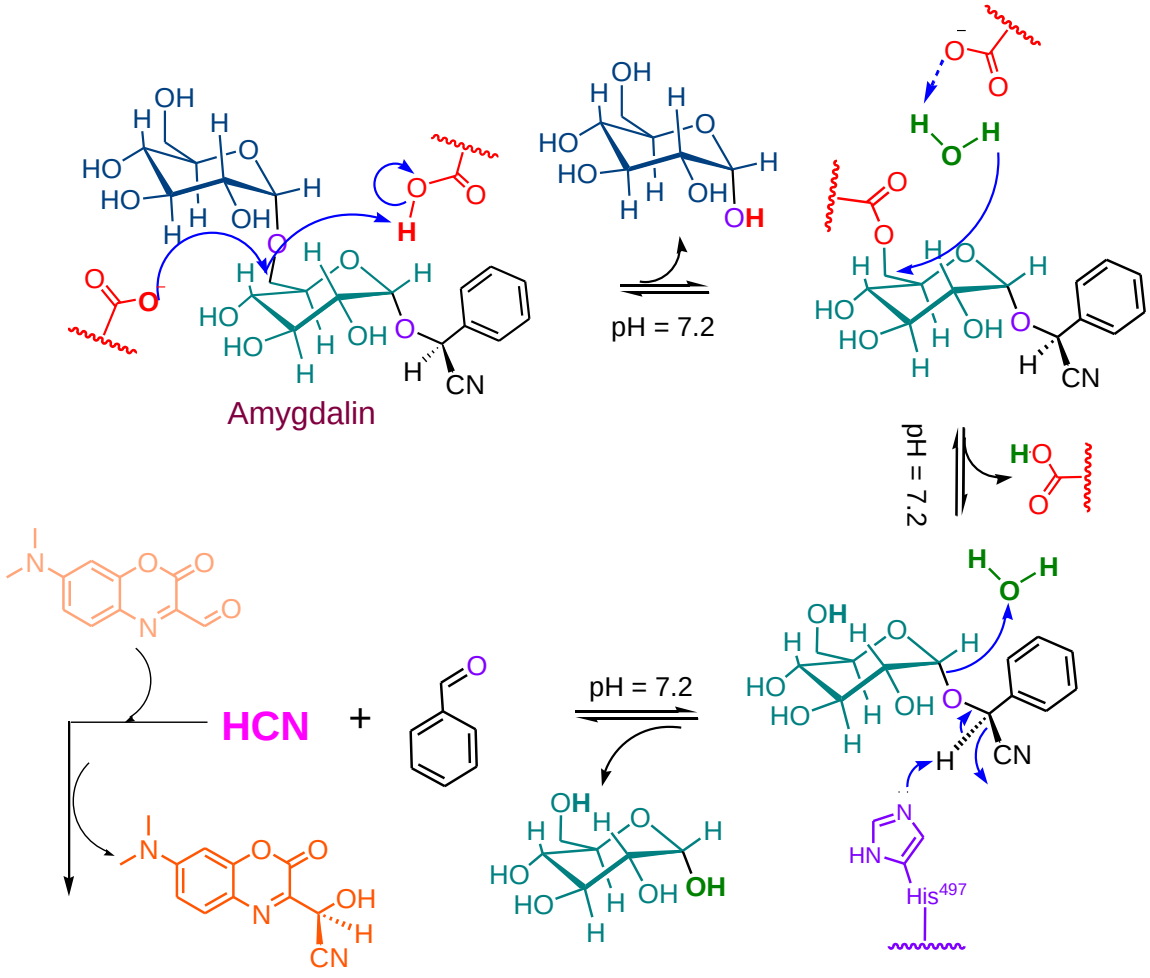
ESI Figure 16: Luminescence spectra of **1** (20μM) recorded with amygdalin (1 mM), glucose (1 mM) and disaccharides (maltose & lactose) in aq. HEPES buffer-DMSO (250:1, v/v; pH = 7.2) medium having 1.2 mM CTAB in absence and presence of β-glucosidase (0.0176 UN/ml) (using λ_{Ext} = 430 nm).

Interference of β-glucosidase with HNL



ESI Figure 17. Time dependent emission change at 535 nm of **1** (20 μM) with amygdalin (1mM) and β-glucosidase (0.0105 UN/ml) in absence and presence of HNL (3 UN/ml). Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB using λ_{Ext} of 430 nm.

Mechanism of enzymatic reaction of hydrolysis of amygdalin by β-glucosidase

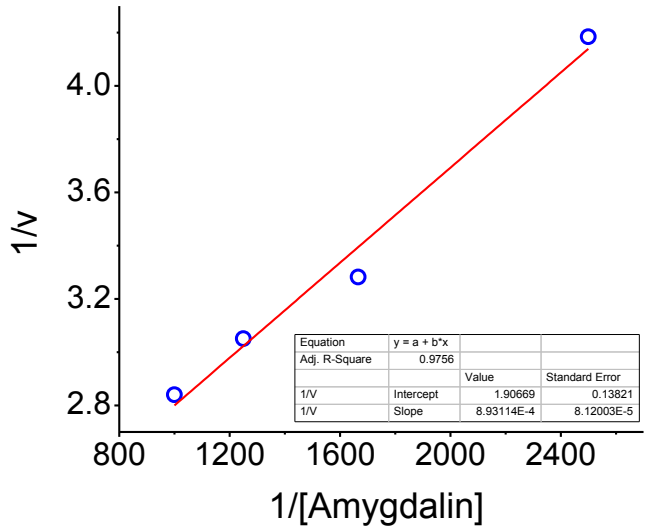


ESI scheme 1: schematic representation of hydrolysis of amygdalin by b-glucosidase and reaction of liberated HCN with probe 1.

Michaelis constant for β-glucosidase:

Michaelis constant was evaluated by using series of enzyme assay by varying amygdalin concentration from (0.4 - 1 mM) with fixed enzyme concentration of 0.0140 UN/ml. Initial rates were evaluated from the plot of Log [Ft-F0] vs time (in sec), where F_t is the luminescence intensity for **1** at 535 nm (λ_{ext} = 430 nm) at time t and F_0 is the initial luminescence intensity. Linear plots were obtained for amygdalin concentration (0.4 - 1 mM).

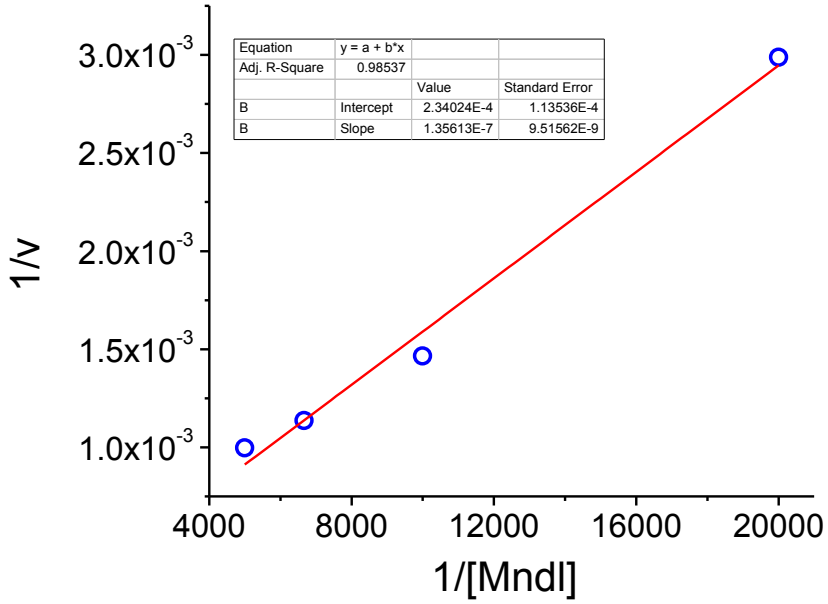
A plot of 1/v vs. 1/[S] would give intercept of $1/k_2[E]_0$ and slope of $Km/k_2[E]_0$. Thus, {slope/intercept} would result K_m.



ESI Figure 18: (1/v) vs 1/[amygdalin] plot.

Michaelis constant for HNL

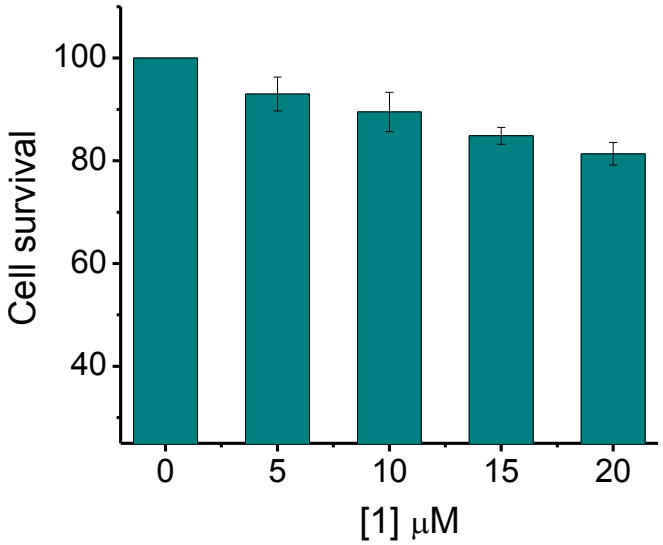
For calculating Michaelis constant of HNL, varying concentration of mandelonitrile (5×10^{-5} – 2×10^{-4} M) was used with fixed HNL concentration of 3 UN/ml. Initial rates were evaluated from the linear plot of Log [Ft-F0] vs time (in sec), where F_t is the luminescence intensity for **1** at 535 nm (λ_{ext} = 430 nm) at time t and F_0 is the initial luminescence intensity. A plot of 1/v vs. 1/[S] would give intercept of $1/k_2[E]_0$ and slope of $Km/k_2[E]_0$. Thus, {slope/intercept} would result K_m.



ESI Figure 19: (1/v) vs 1/[Mandelonitrile] plot.

MTT assay of **1**

To determine cell cytotoxicity of **1** on MDA-MB231 cell, MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellowtetrazole) assay was performed. In a 96 well plate cells (5000) were seeded and cultured in a 37°C incubator supplied with 5% CO_2. Cells were maintained in DMEM medium, supplemented with 10% FBS and antibiotics. Thereafter cells were treated with different concentration of **1** for 24 h, and then further treated with 0.5µg/µl of MTT reagent. Cells were incubated for 4 h at 37°C and then later 100 µl of Isopropyl Alcohol was added to each well. The optical density was measured at 570 nm using Multiskan Go (Thermo Scientific) to find the concentration of the cell inhibition.

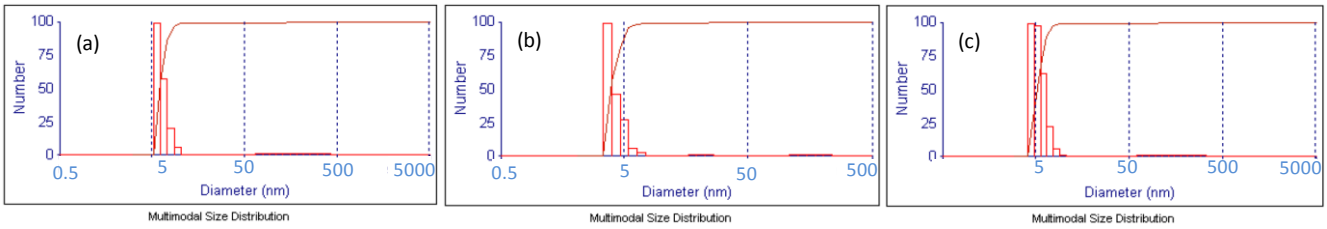


ESI Figure 20: Cell survival with different concentration of **1**.

References:

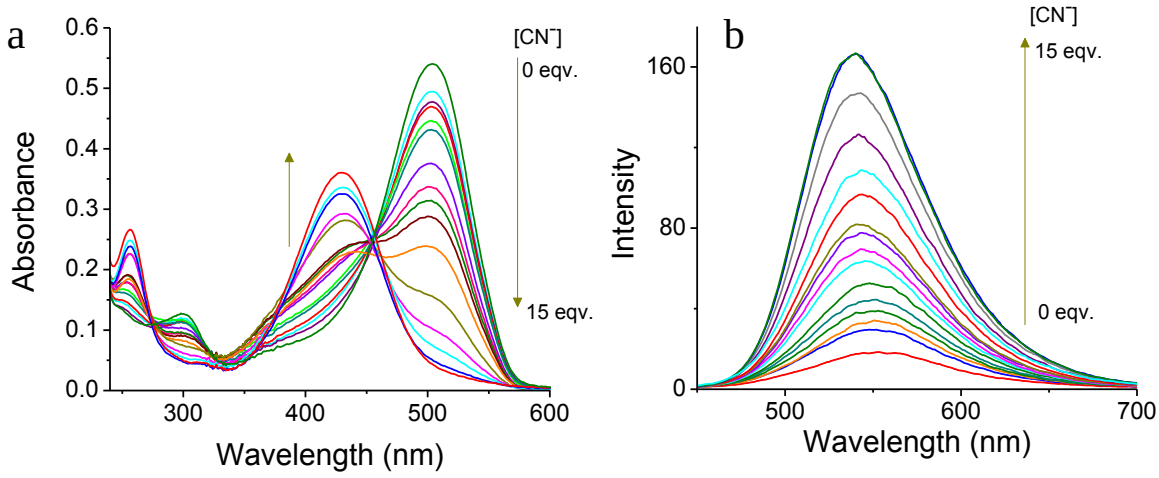
S1: Das, S.; Sharma, D. K.; Chakrabarty, S.; Chowdhury, A.; Gupta S. S. *Langmuir*, **2015**, *31*, 3402−3412.

DLS Study:



ESI Figure 21: Dynamic light scattering study of (a) CTAB (1.2 mM) solution, (b) for solution with **1** (20 mM) in CTAB (1.2 mM) , and (c) **1** (20 mM) in CTAB (1.2 mM) + CN^- (200 mM).

Interaction with Hydrogen sulfite:



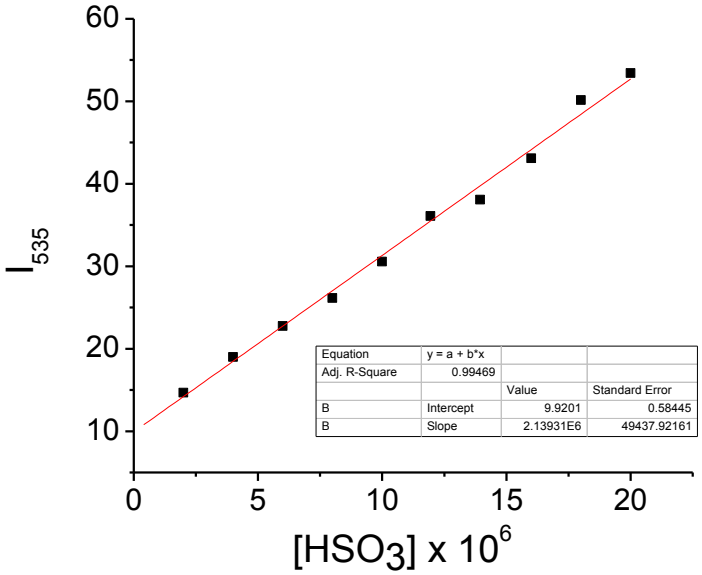
ESI Figure 22: Changes in (a) absorption and (b) emission spectra of **1** (20μM) in presence of 0-15 mole equivalents of $NaHSO_3$. Measurements were performed in 10 mM aq. HEPES-DMSO (250:1, v/v; pH 7.2) having 1.2 mM CTAB using λ_{Ext} of 430 nm.

Lowest detection limit of hydrogen sulfite:

The detection limit of HSO_3^- was calculated by following following equation 2

DL= K. σ /s …..Equation 2

Where K= 2, σ is the standard deviation of blank measurement, s is the slope of intensity *vs.* [CN^-] plot.

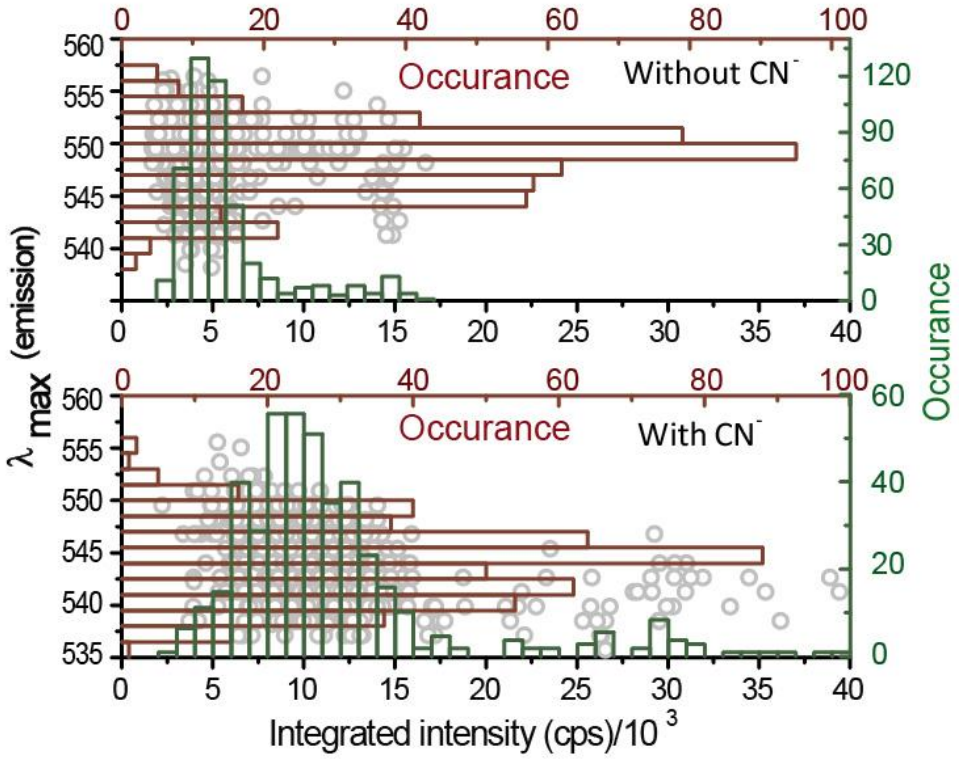


ESI Figure 23: Calibration curve for lowest detection limit.

Here σ is found to be 1.209 and slope form the graph is 2.13×10^6

DL calculated is 1.13×10^{-6} M.

Scatter Plot from TIRF images



ESI Figure 24: Scatter plots of emission spectral maxima against integrated intensity (circles) along with respective frequency distributions (bars) in absence and presence of cyanide species depicting the variation in sensing efficiency in cellular environments