Supporting Information

for

Rationally Designed Molecule for Removal of Cyanide from Human Blood Serum and Cytochrome c Oxidase

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Materials and Methods: Assay buffer, Enzyme dilution buffer, Dithiothretol solution, Cytochrome c, Cytochrome c oxidase, Dimethyl sulphoxide, mesitylene, oxindole, $AlCl_3$, Formaldehyde, HBr-AcOH, NaCN, TBACN

General note: ^{1}H and ^{13}C NMR spectra were recorded on JEOL 300 MHz, Bruker 500 MHz and 75 MHz, 125 MHz NMR spectrometer, respectively using $CDCl_3$ and DMSO-d_6 as a solvent. Chemical shifts are given in ppm with TMS as internal reference. *J* values are given in Hertz. Mass spectra were recorded on Bruker micrOTOF-Q II Mass spectrometer. Reactions were monitored by thin layer chromatography (TLC) on glass plates coated with silica gel GF-254. Column chromatography was performed with 60-120 mesh silica. IR and UV spectral data were recorded on FTIR (VARIAN 660 IR) and BIOTEK Synergy H1 Hybrid Reader instruments, respectively. HPLC was performed on Shimadhu LC-20 AD instrument. SEM images were recorded on ZEISS EVO LS10 scanning electron microscope.

UV-vis spectral studies:

Stock solutions of the compounds (10^{-3} M) were prepared in HPLC grade DMSO. Stock solution of CN (10^{-3} M) was prepared in distilled water. As per requirement, dilutions of the compound and cyanide solutions were made in ethanol/DMSO – water.

Equation for calculation of binding constants

Binding constants of compound-cyanide complex were calculated using Benesi-Hildebrand Equation.

$$1/ (A_f - A_{obs}) = 1/ (A_f - A_{fc}) + 1/ K (A_f - A_{fc}) [Ligand]$$

where A_f is absorbance of free host, A_{obs} is absorbance observed, A_{fc} is absorbance at saturation, K is binding constant.

Experiments with human blood serum. Human blood serum was taken with thanks from the local Medical College and used as provided. 1ml of the serum was diluted in 10 ml ultrapure water. A certain amount of NaCN/CuCN was added to this solution so that the

concentration of cyanide becomes 1 nM. Presence of 200 nM compound **3** in the serum solution turned its color dark red. For extraction of the new chemical entity which was formed by the reaction of compound **3** with cyanide, higher equivalents of the cyanide and compound were used. The dark red solution was extracted with ethyl acetate. The organic layer was evaporated under vacuum to get a solid mass which after analysis was found to be compound **7**.

Disposal of compound 7. Solution of compound **7** in ethanol – water (1:1) pH 9.5-10 was stirred at room temperature and the changes were monitored with HRMS.

NMR spectral studies: ^{1}H NMR spectra of pure compounds (0.35 mM) were recorded in $CDCl_3$ at probe temperature 25 ^{o}C. Deuterium exchange (using D_2O) was performed to identify the OH signals. Calculated amount of TBACN was stepwise added to the NMR tube and spectrum was recorded at each step.

Mass spectral studies: All the mass spectra were recorded on Bruker MicroTOF QII machine in +ve/-ve ESI mode with capillary voltage 4500 V and source temperature 180 ^{o}C. Solutions of 1 μM concentration of the compounds alone as well as in combination with CN^- were prepared in ACN-H_2O (3:7) and directly injected to the ion source of the spectrometer.

Cytochrome c oxidase assay

All the components of the assay kit were diluted as per the assay manual.[1] UV-vis absorption of cytochrome c at 550 nm changes with its oxidation state. This property was the basis for the assay. Cytochrome c is reduced with dithiothreitol and then oxidized by the cytochrome c oxidase (CcOX). The decrease in absorbance of ferrocytochrome c at 550 nm is caused by its oxidation to ferricytochrome c by cytochrome c oxidase. During present studies, first the solution of sodium cyanide was stepwise added to solution of cytochrome c oxidase in enzyme buffer. After each addition of cyanide to CcOX, it was added to solution of ferrocytochrome c (0.55 μM) in assay buffer and absorbance at 550 nm was measured.

Concentration of sodium cyanide, at which it completely blocks the activity of the enzyme, so that oxidation of ferrocytochrome c does not occur, was optimized as 2.8 μM. Next, solution of compound **3** was stepwise added to solution of cyanide bound cytochrome c oxidase. This solution was added to the solution of ferrocytochrome c in assay buffer and changes in absorbance at 550 nm were monitored. With the addition of 13 μM of compound **3** to cyanide bound cyt c oxidase and its subsequent addition to cyt c finished the absorbance at 550 nm. At this stage, compound **3** removed the cyanide from cyt c oxidase and its oxidizing activity was restored.

1. Cytochrome c Oxidase Assay kit, Sigma Aldrich, Product Code: CYTOCOX1

Time course for the oxidation of reduced cytochrome c catalyzed by the CcOX in the presence of 2.8 μM cyanide was measured at 550 nm and recovery of CcOX activity was calculated by using the following formula:

Calculation of CcOX activity:

CcOX activity (μmol red. cyt. c/min)= (ΔA/min X 1.1)/(vol of enzyme) X 21.84

where, $\Delta A/min = A/minute_{(sample)} - A/minute_{(blank)}$

1.1 = reaction volume

21.84 = $\Delta\varepsilon^{mM}$ between ferrocytochrome c and ferricytochrome c at 550 nm

Toxicity assay. The toxicity of the compound was checked by National Cancer Institute, Bethesda, USA as per their standard protocol (www.dtp.nci.nih.gov).

Scanning electron microscope images. The samples were vacuum dried and precoated with gold using Quorum Q150R ES coating machine. SEM images were recorded on ZEISS EVO LS10 scanning electron microscope.

UV-vis spectra showing selective and competitive interactions of compound 2 with CN^-

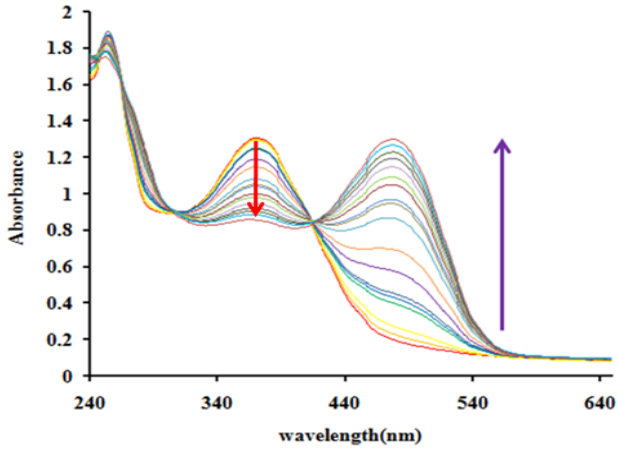


Figure S1. UV-vis absorption spectra of compound **2** (10 μM, ethanol-water, 1:1 v/v) upon incremental addition of CN^- (up to 30 equiv). No change in UV-vis spectrum was observed when more of CN^- was added.

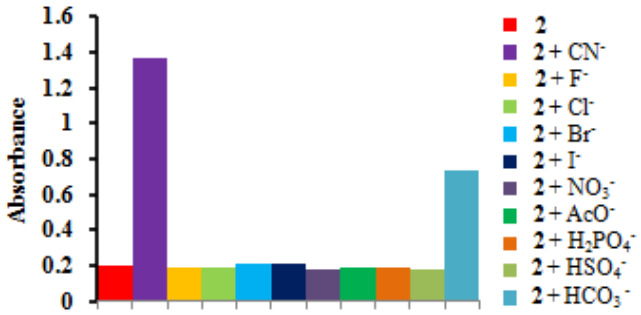


Figure S2. Selectivity of compound **2** for CN^-. Bar graph showing absorbance of compound **2** at 477 nm in presence of 30 equiv of various anions.

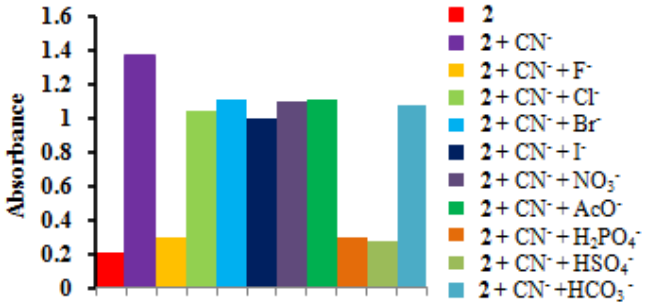


Figure S3. Competitive bonding of compound **2** with CN^-. Absorption intensity at 477 nm of solution of compound **2** (10 μM) in presence of CN^- and other anions.

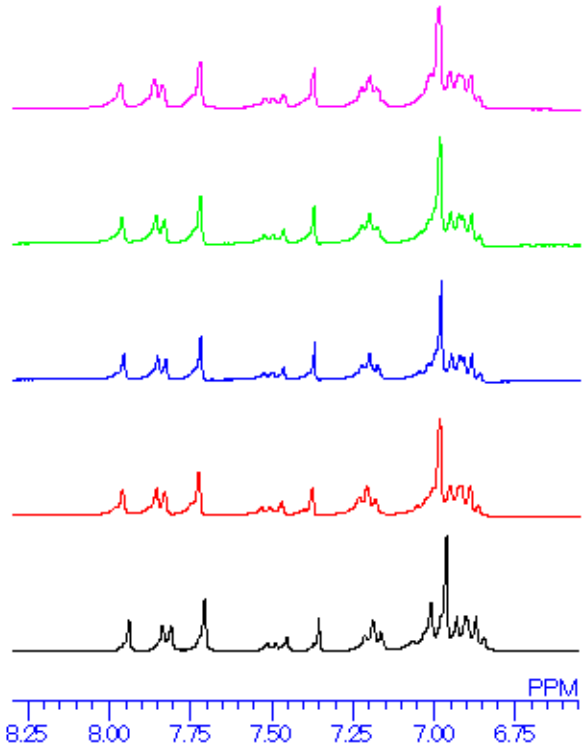


Figure S4. ^{1}H NMR spectrum of compound **2** (black trace) in presence of CN^- showing disappearance of OH peak at δ 6.98. **2**+0.2 equiv CN^- (red trace), **2**+0.4 equiv CN^- (blue trace), **2**+0.6 equiv CN^- (green trace), **2**+0.8 equiv CN^- (pink trace).

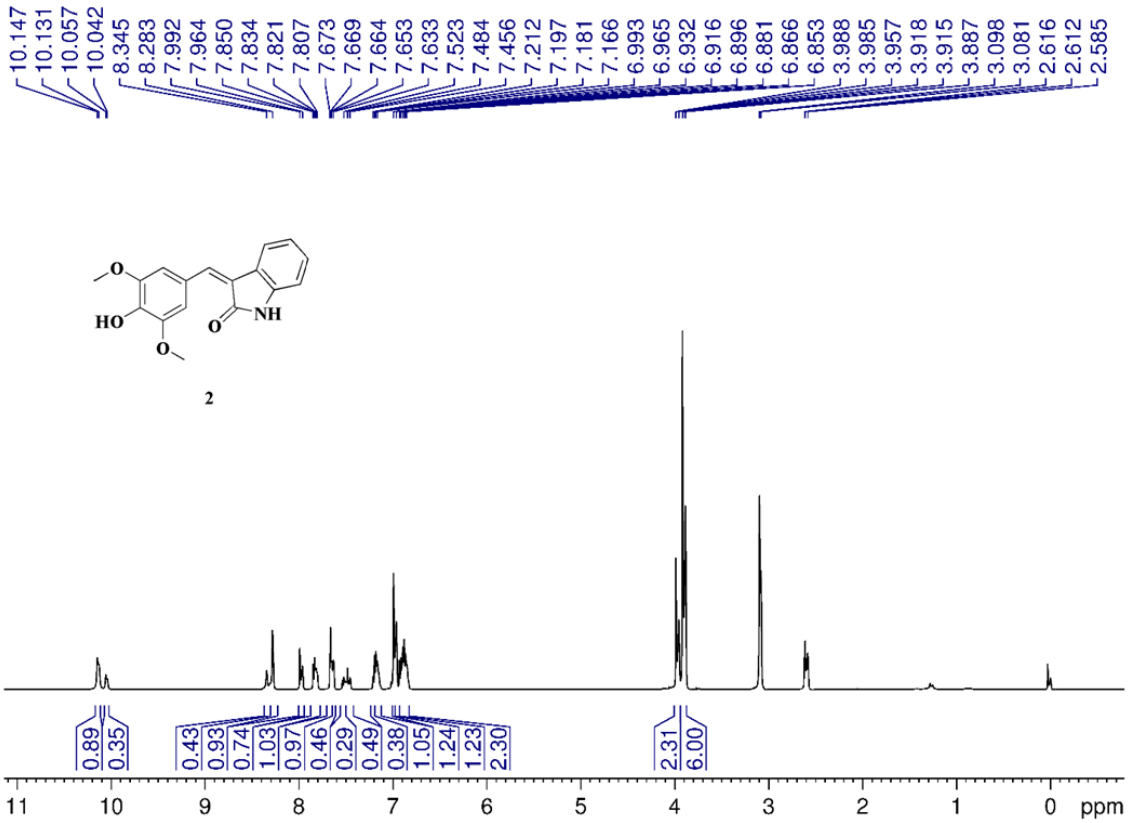


Figure S5. ^{1}H NMR spectrum of compound **2** ($CDCl_3$ + DMSO-d_6).

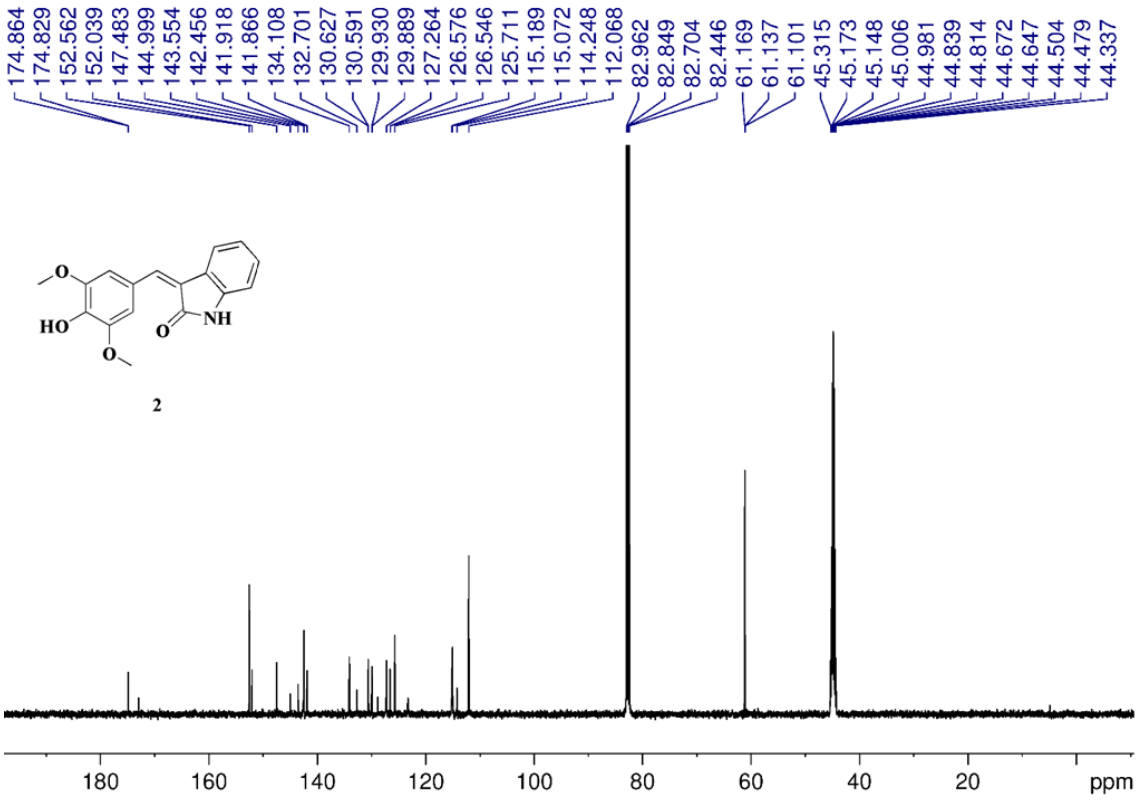


Figure S6. ^{13}C NMR spectrum of compound **2** ($CDCl_3$ + DMSO-d_6).

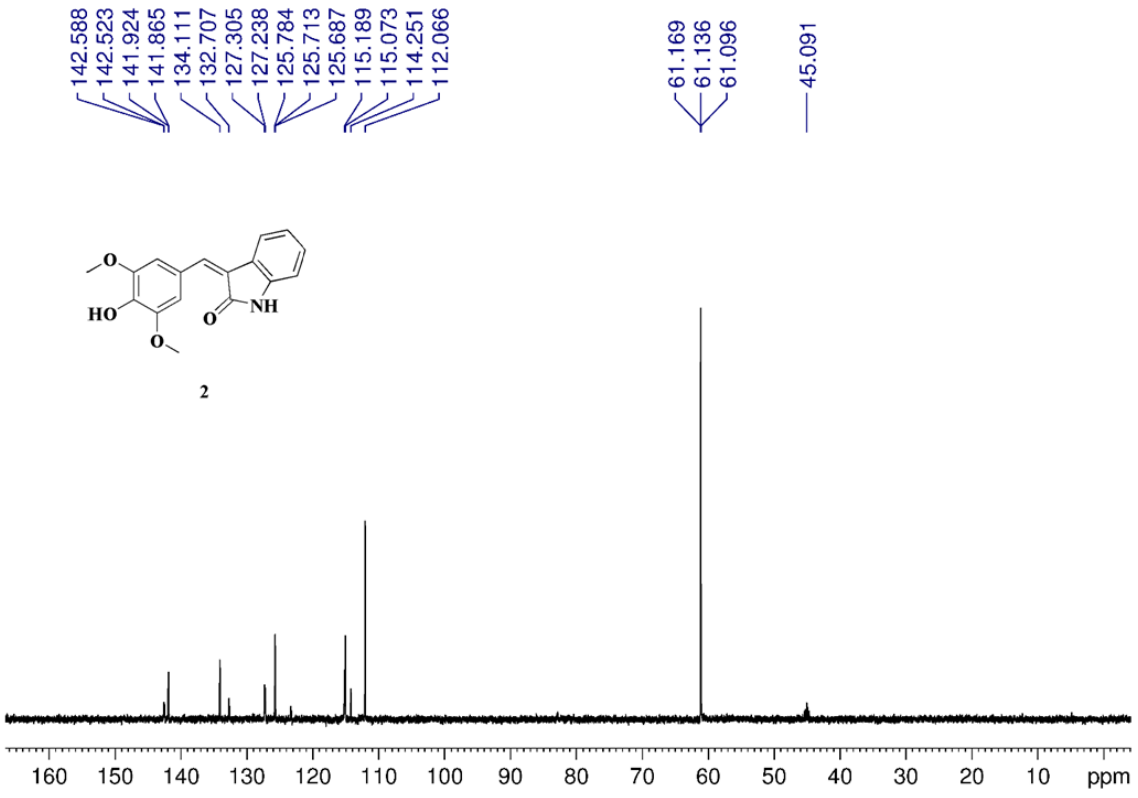


Figure S7. DEPT-135 spectrum of compound **2** ($CDCl_3$ + DMSO-d_6).

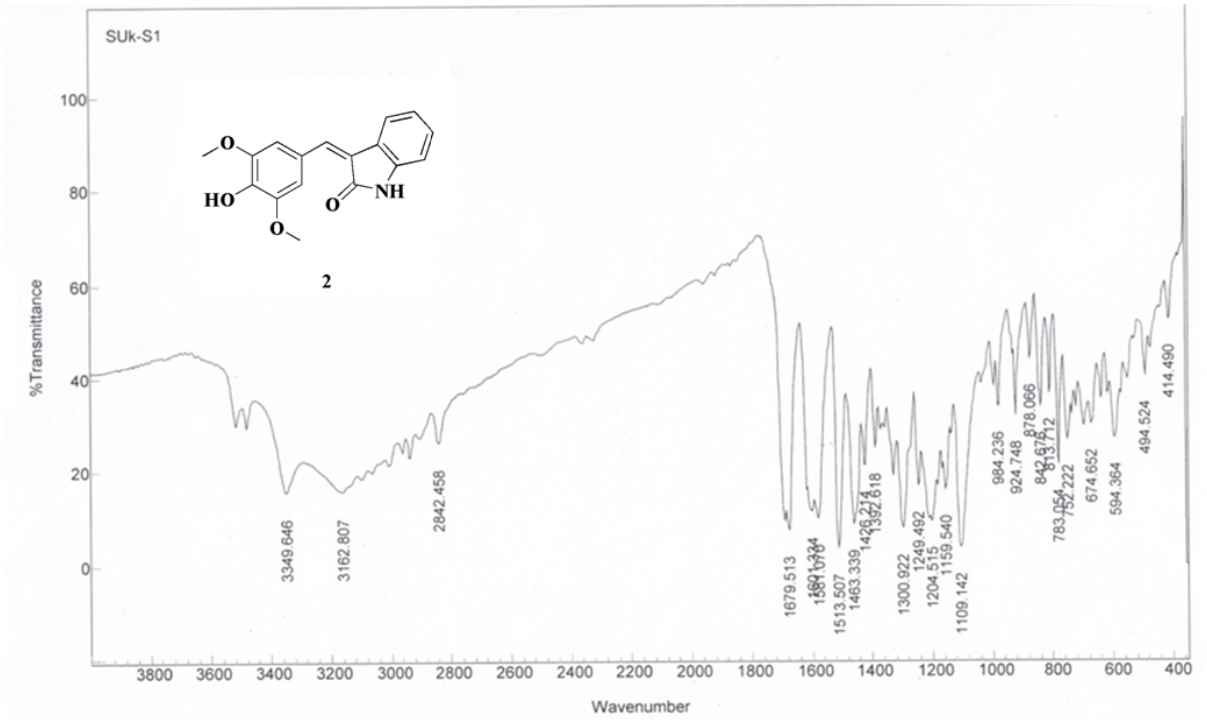


Figure S8. IR spectrum of compound **2**.

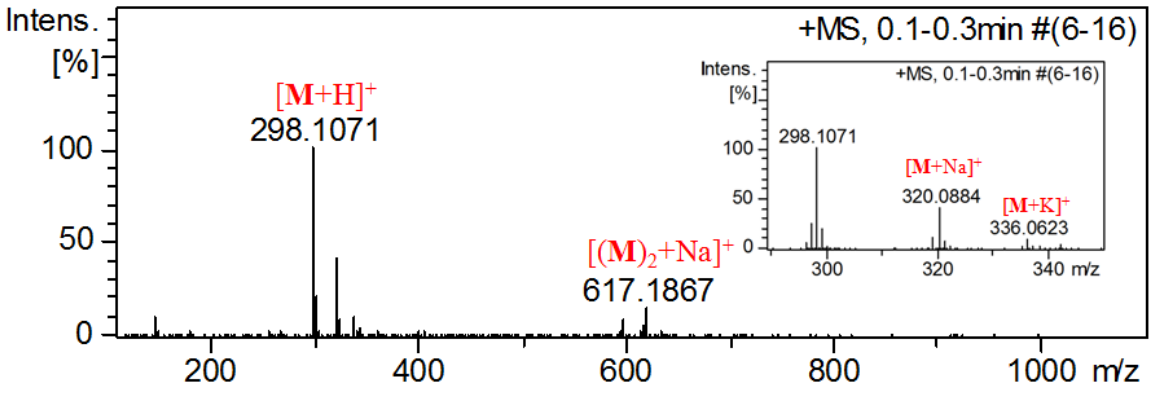


Figure S9. High resolution mass spectrum of compound **2** with *m/z* 298.1071 (calcd *m/z* 298.1074, $[M+H]^+$).

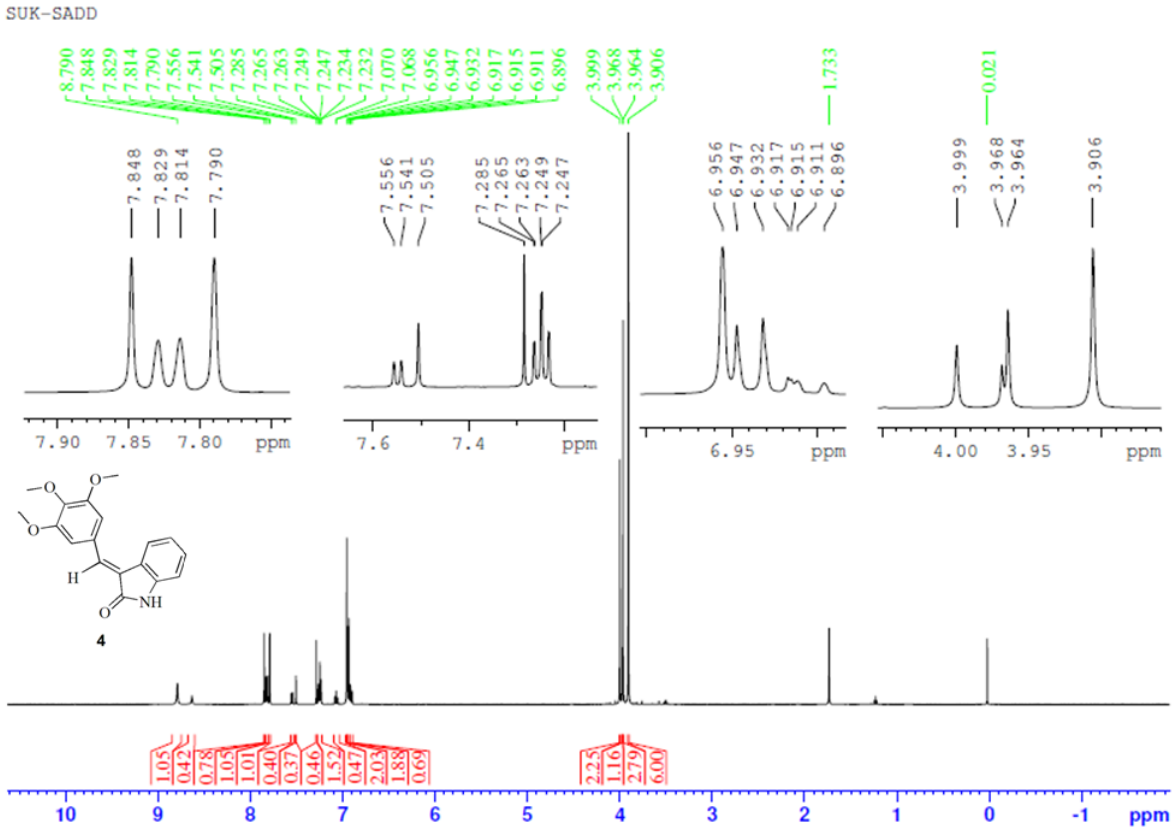
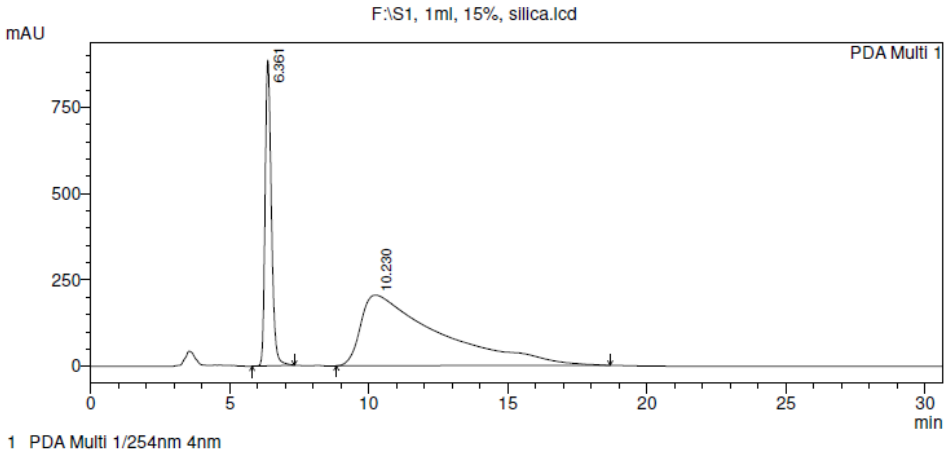


Figure S10a. ^{1}H NMR spectrum of compound **4.**

==== Shimadzu LCsolution Analysis Report ====



PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
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2	10.230	40289582	204652	74.356	18.775
Total		54184521	1090004	100.000	100.000

Figure S10b. HPLC chromatogram of compound **4** in 15% IPA and 85% hexane.

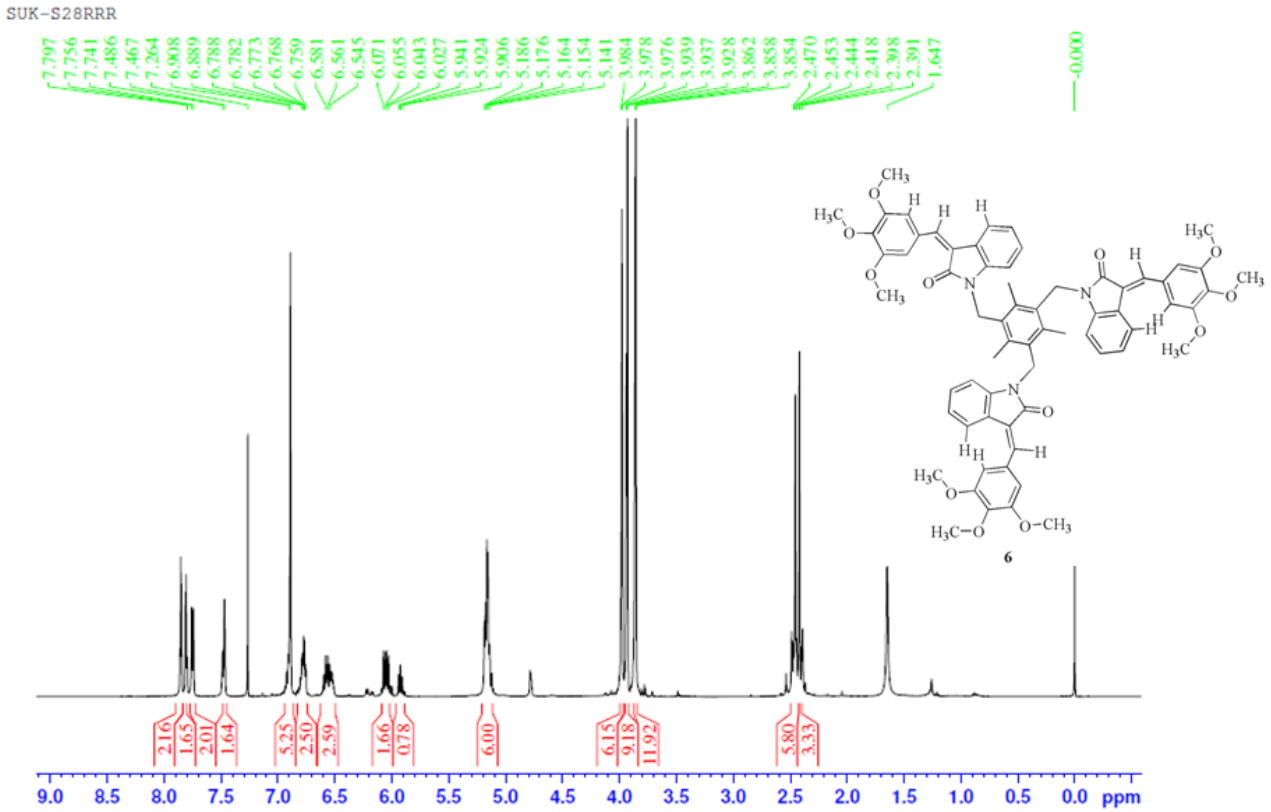


Figure S11. ^{1}H NMR spectrum of compound **6**.

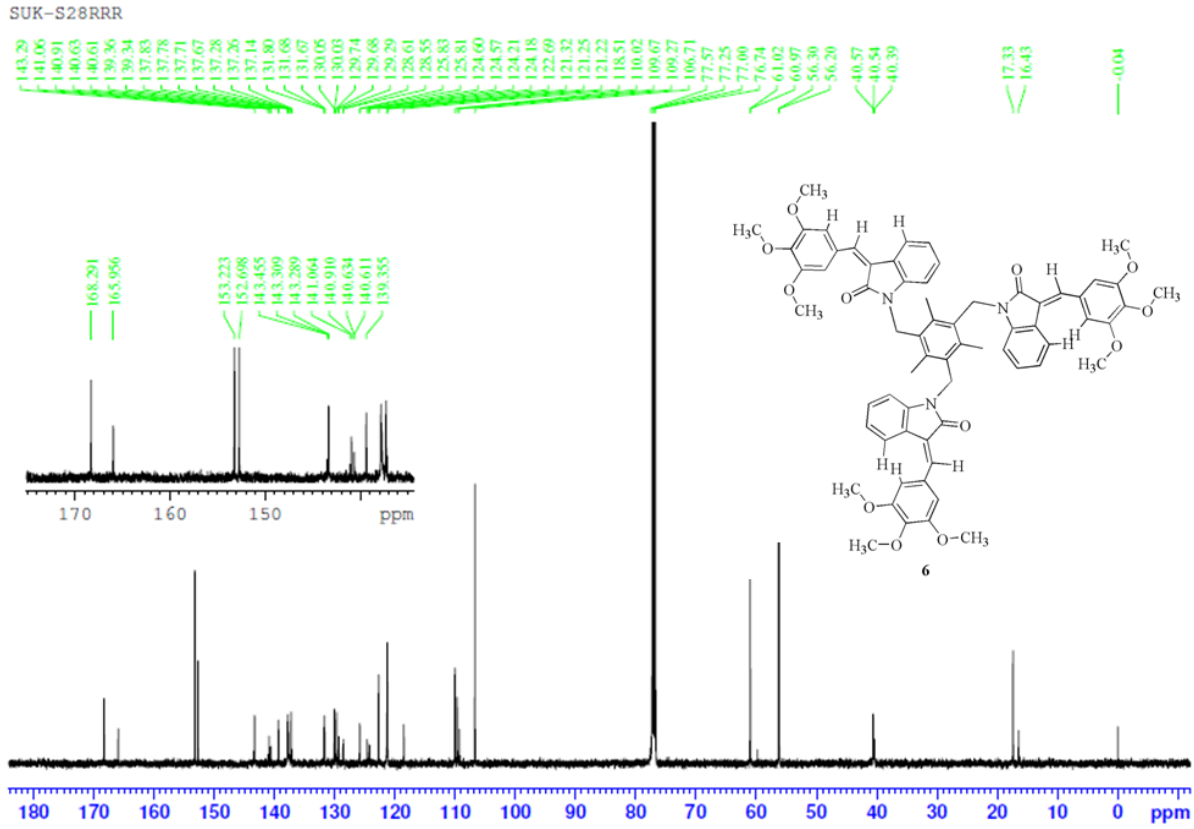


Figure S12. ^{13}C NMR spectrum of compound **6**.

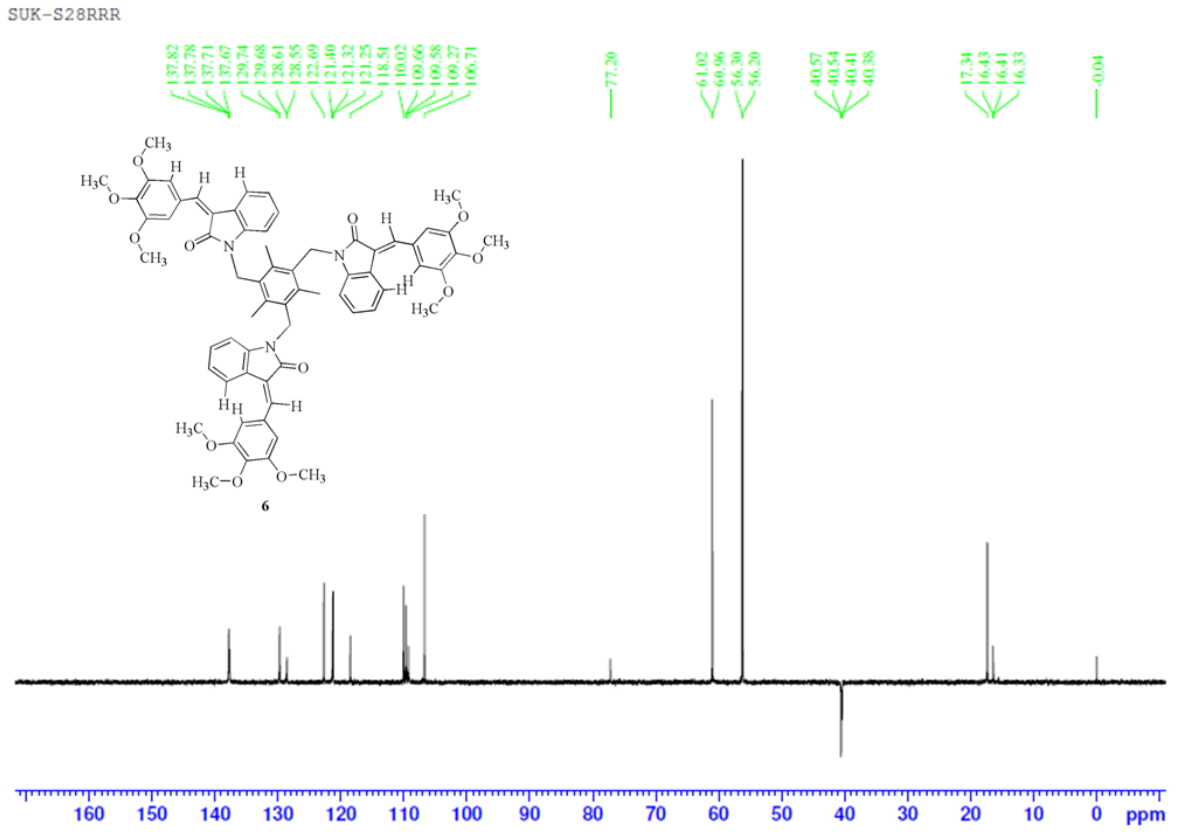
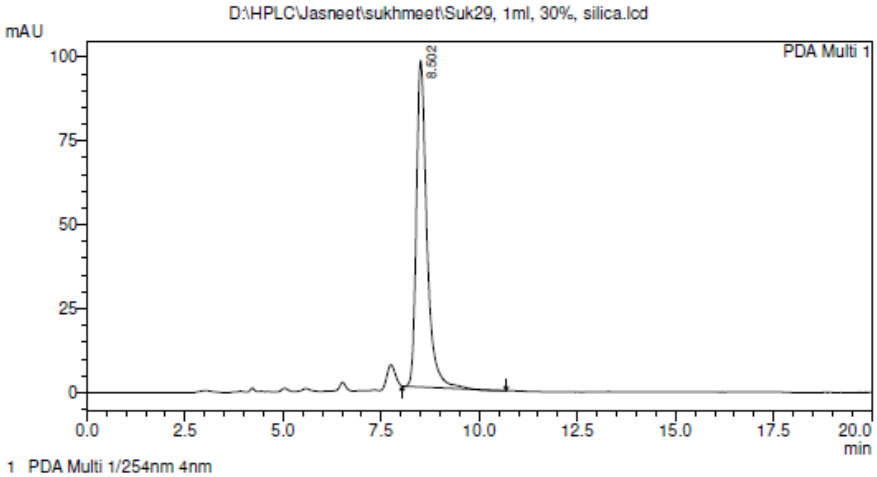


Figure S13. DEPT-135 NMR spectrum of compound **6**.

==== Shimadzu LCsolution Analysis Report ====



1 PDA Multi 1/254nm 4nm

PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	8.502	1814119	97377	100.000	100.000
Total		1814119	97377	100.000	100.000

Figure S14a. HPLC chromatogram of compound **3** in 30% IPA and 70% hexane.

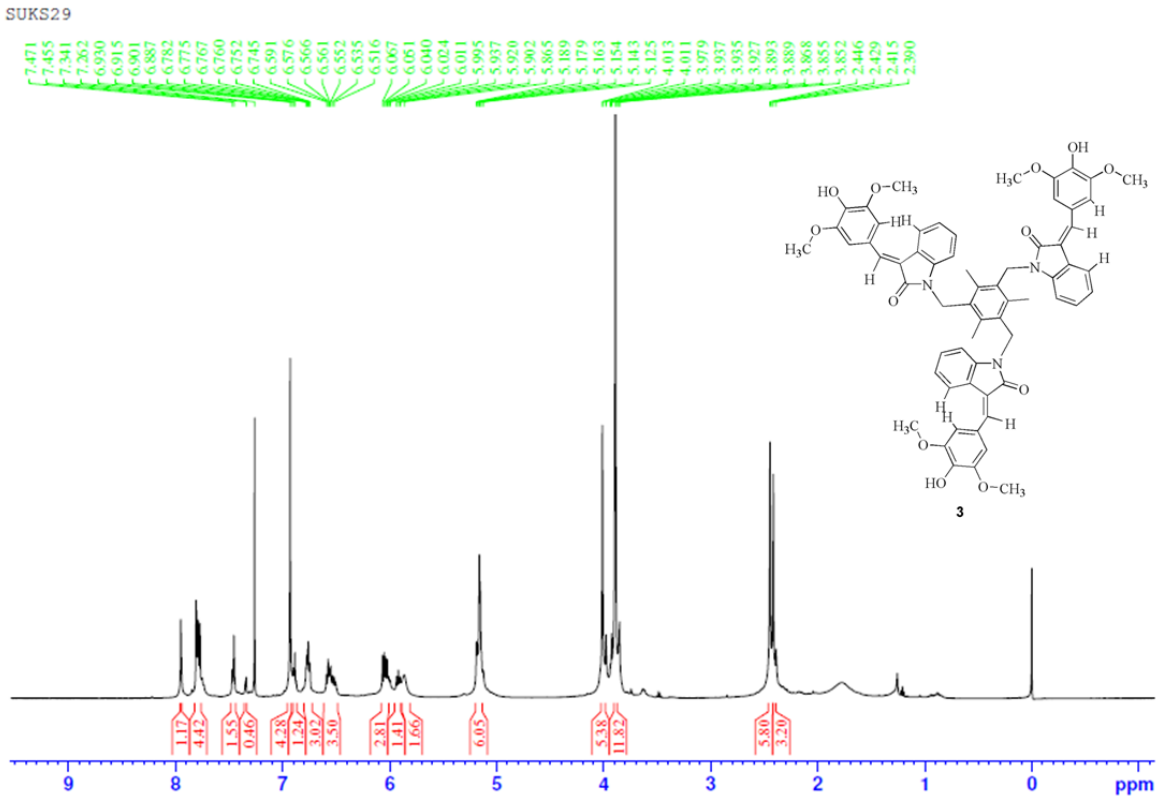


Figure S14b. ^{1}H NMR spectrum of compound **3** ($CDCl_3$ as solvent)

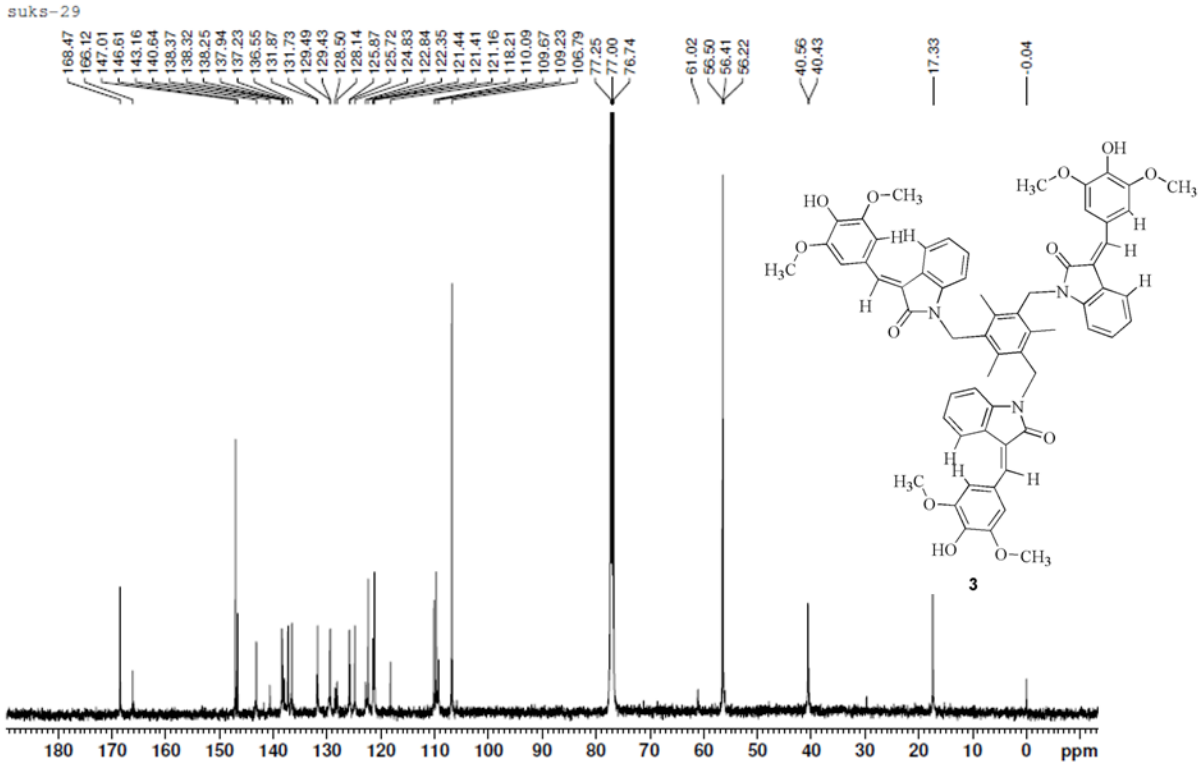


Figure S14c. ^{13}C NMR spectrum of compound **3** ($CDCl_3$ as solvent)

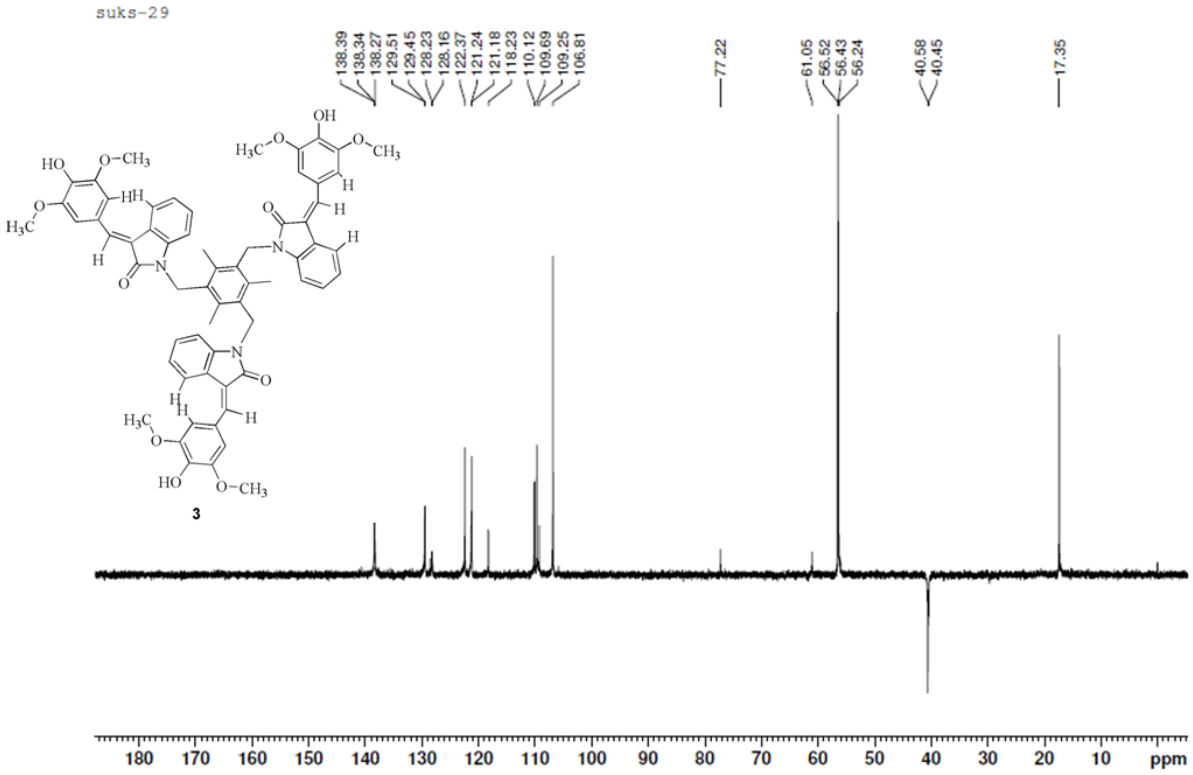


Figure S14d. DEPT-135 NMR spectrum of compound **3** ($CDCl_3$ as solvent)

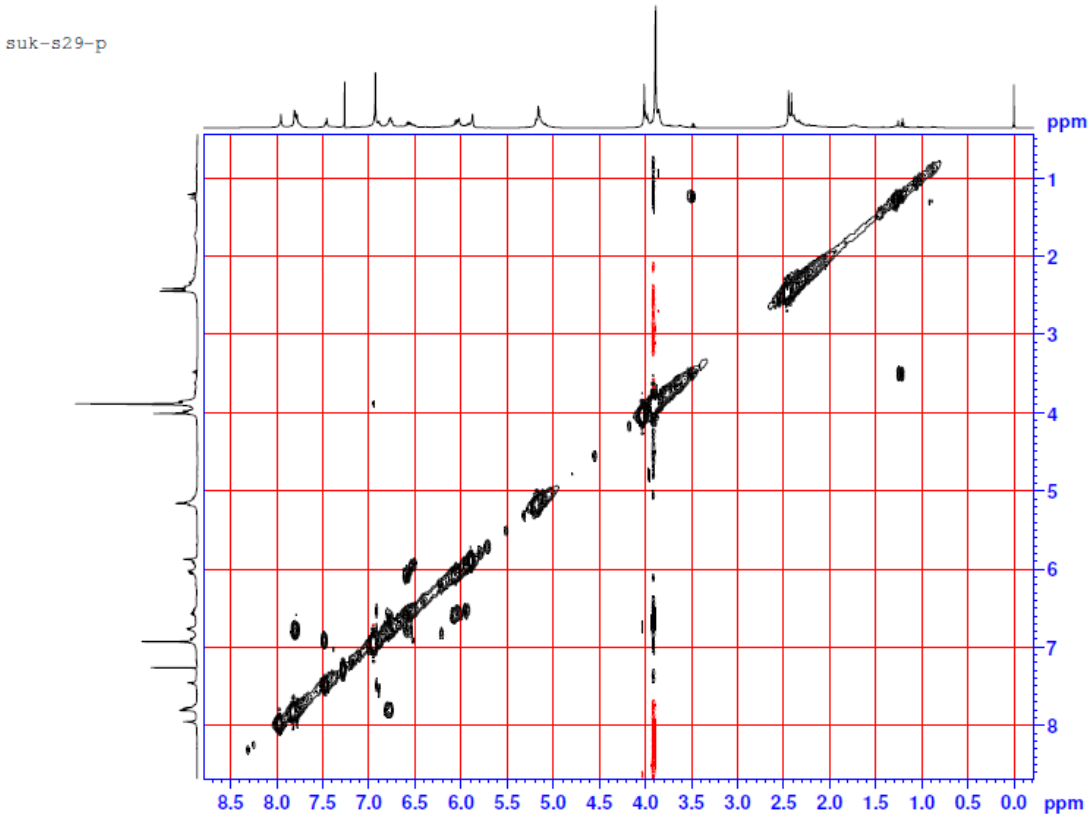


Figure S14e. ^{1}H-^{1}H COSY NMR spectrum of compound **3** ($CDCl_3$ as solvent).

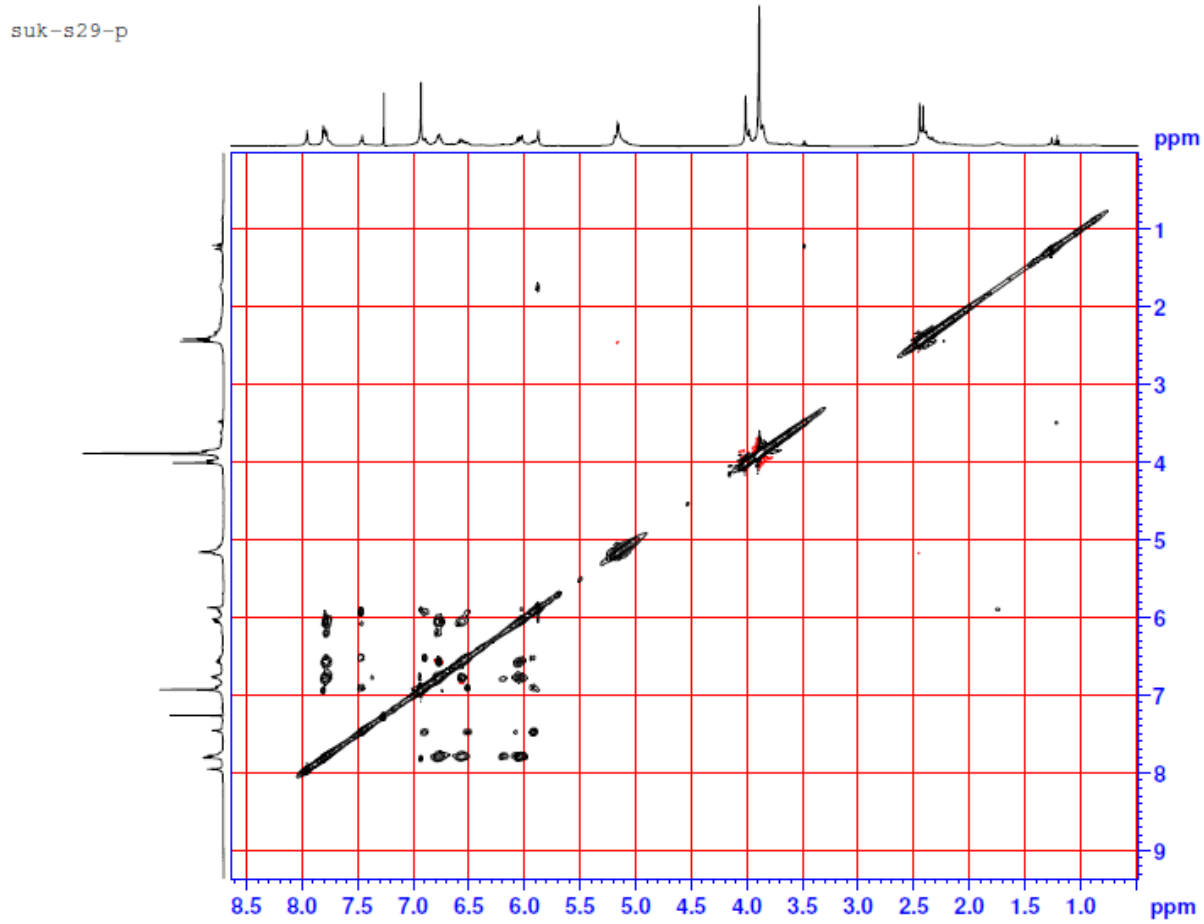


Figure S14f. ^{1}H-^{1}H TOCSY NMR spectrum of compound **3** ($CDCl_3$ as solvent).

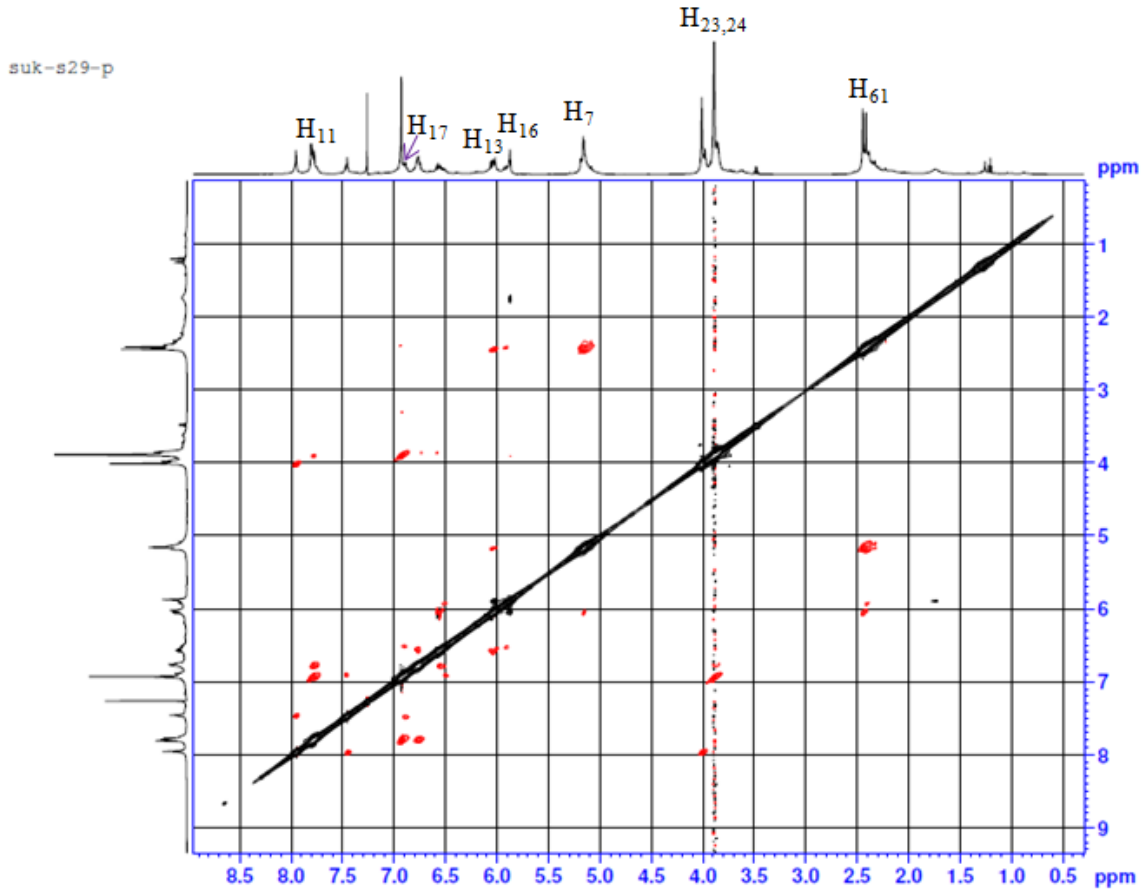


Figure S14g. ^{1}H-^{1}H ROESY NMR spectrum of compound **3** ($CDCl_3$ as solvent). For clarity reasons, peaks are partially labelled.

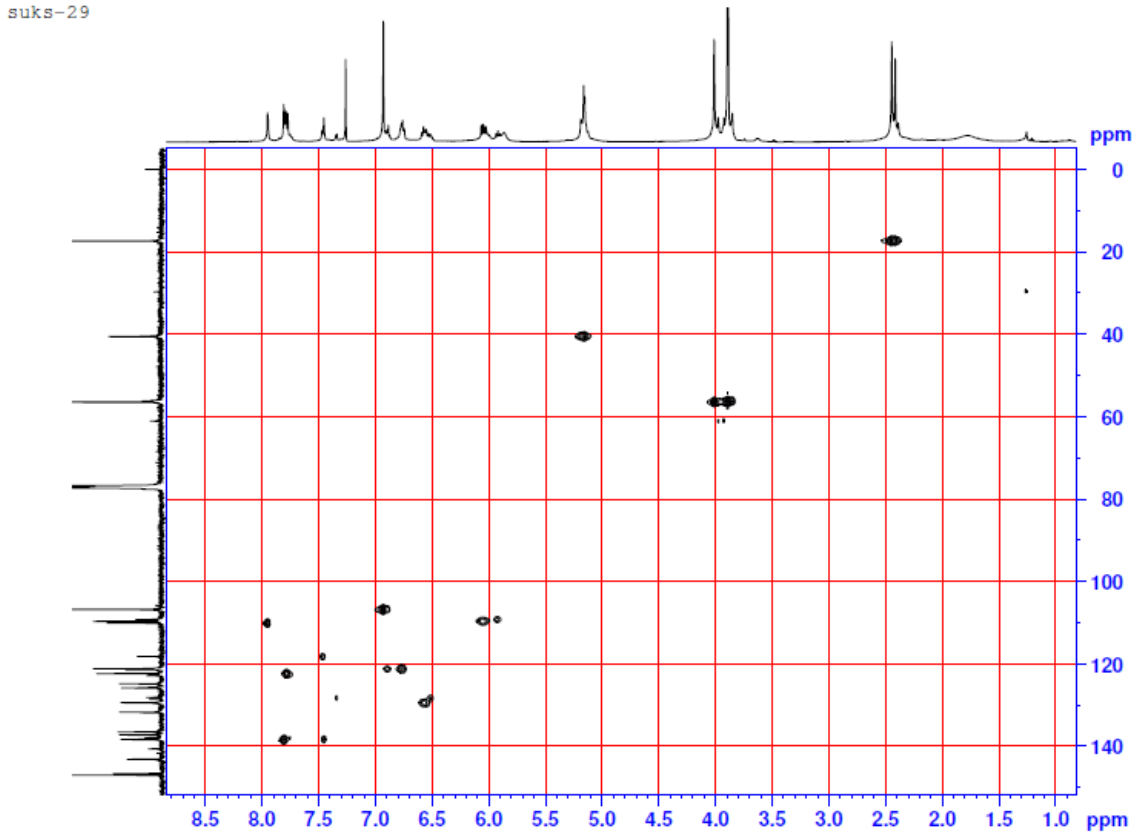


Figure S14h. ^{1}H-^{13}C HSQC NMR spectrum of compound **3** ($CDCl_3$ as solvent).

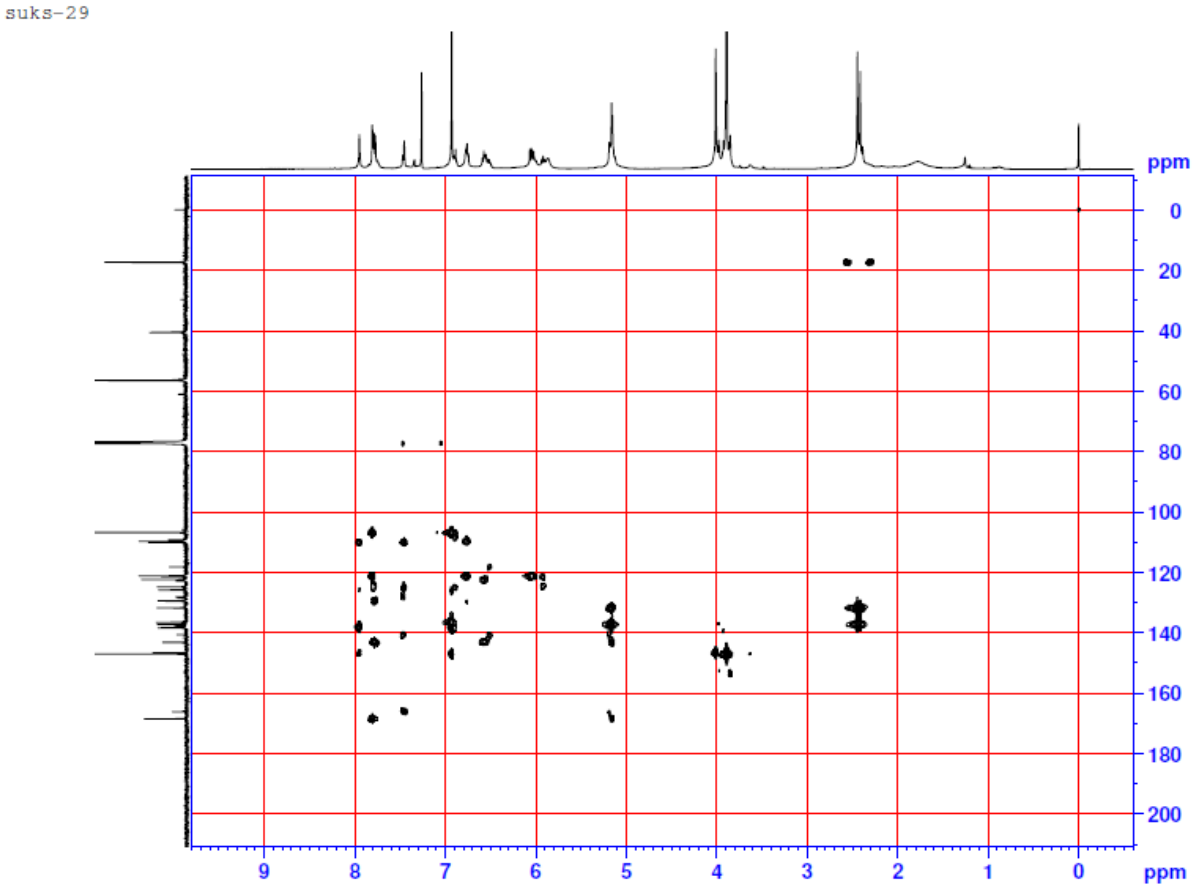


Figure S14i. ^{1}H-^{13}C HMBC NMR spectrum of compound **3** ($CDCl_3$ as solvent).

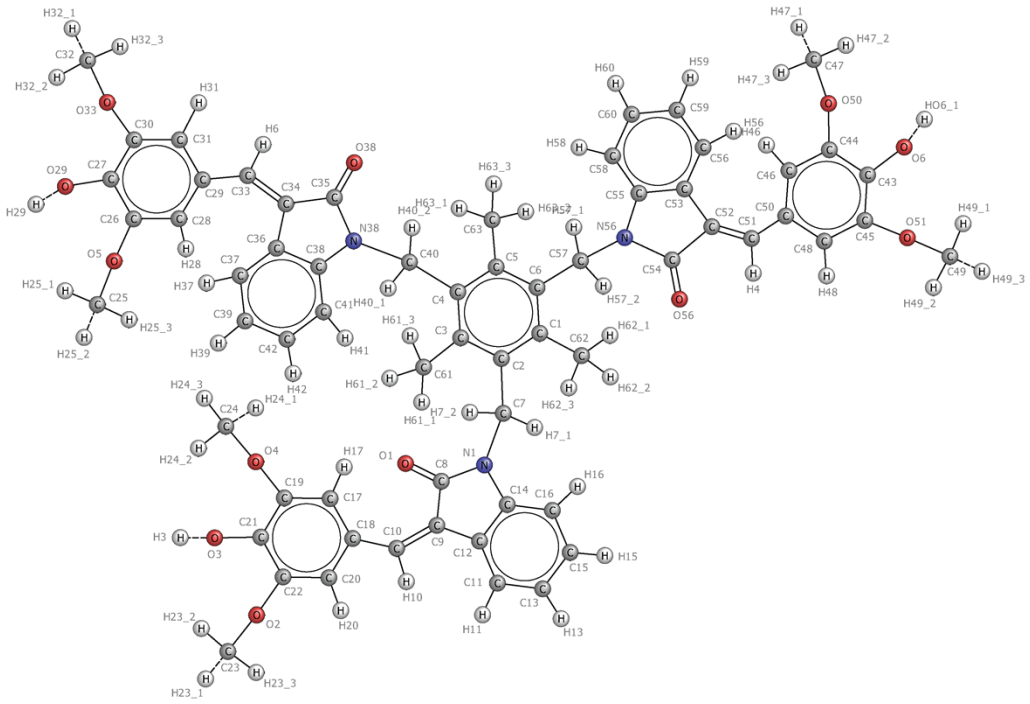


Figure S14j. Structure of compound **3** obtained with the help of different NMR experiments.

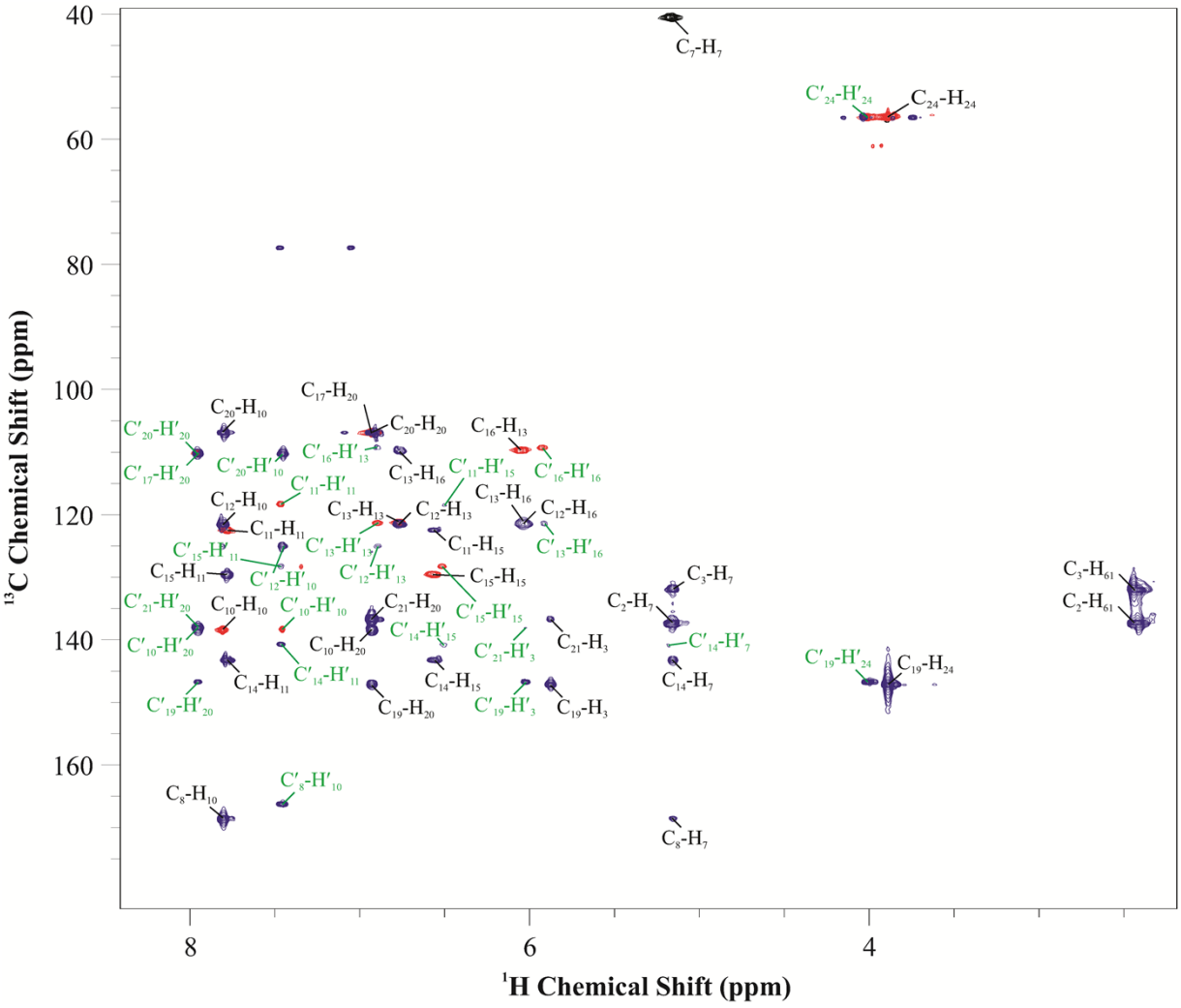


Figure S14k.Overlap of ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC NMR spectrum of compound **3** ($CDCl_3$ as a solvent).

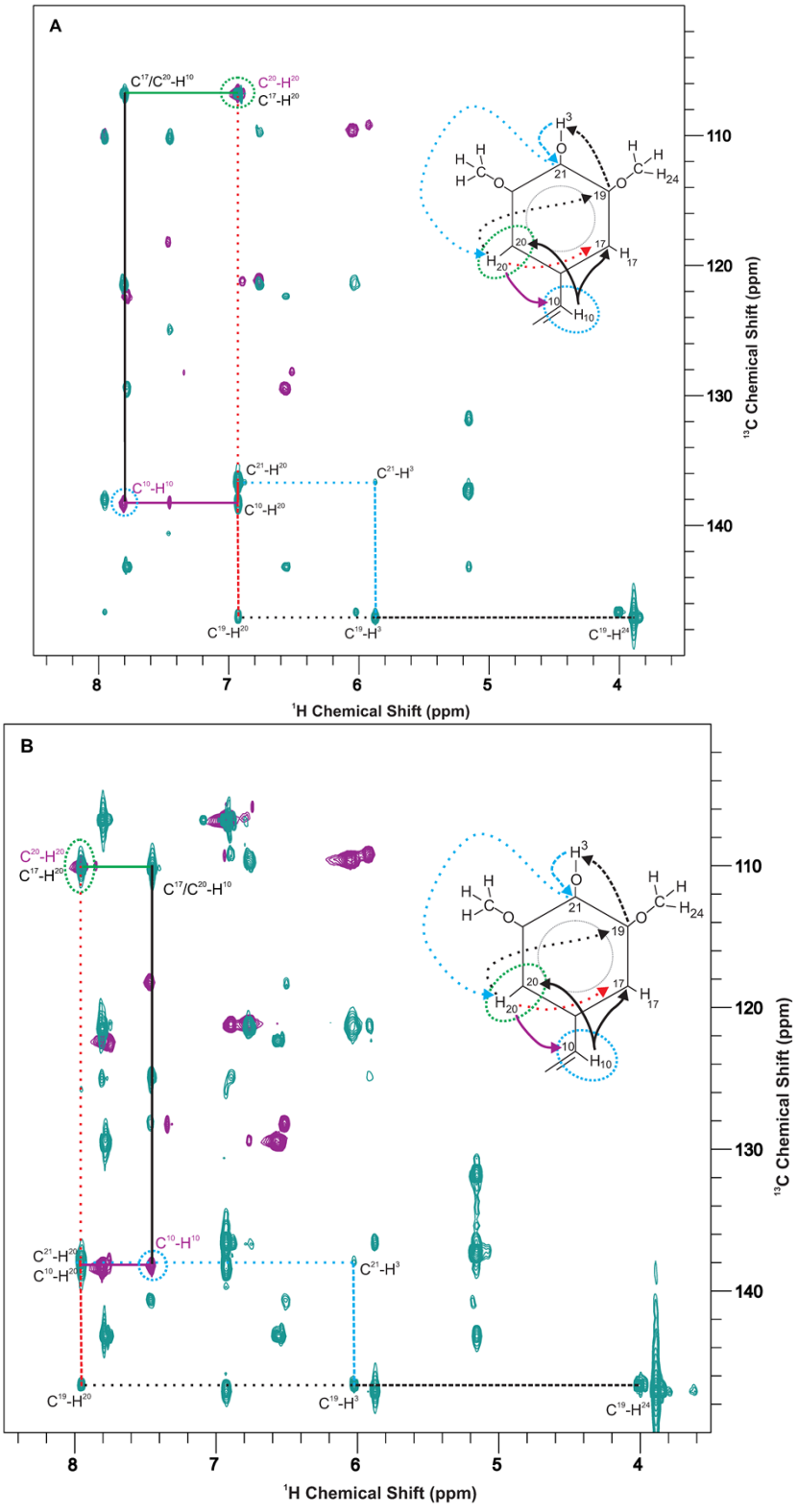


Figure S14l. Overlay of HSQC (Magenta coloured cross-peaks) and HMBC (Cyan coloured cross-peaks) spectra. HMBC correlations between are shown using different types of straight lines as follows; C^{19}-H^{3} (dash, black); C^{19}-H^{20} (dot, black); C^{10}-H^{20} (dash, orange); C^{21}-H^{20} (solid, orange); C^{17}-H^{20} (dot, orange); C^{17}/C^{20}-H^{20} (solid, green); C^{10}-H^{10} (solid, black); C^{21}-H^{3} (dash, sky blue); C^{21}-H^{20} (dot, sky blue). HSQC correlations are highlighted using dotted circles; C^{10}-H^{10} (sky blue); C^{20}-H^{20} (green). These correlations are also show on stick model of molecule (shown in inset) using the same line styles and colour schemes. Figure A and B show respectively the correlations for the A/B and C fragments of compound 3 (Scheme 1, main text).

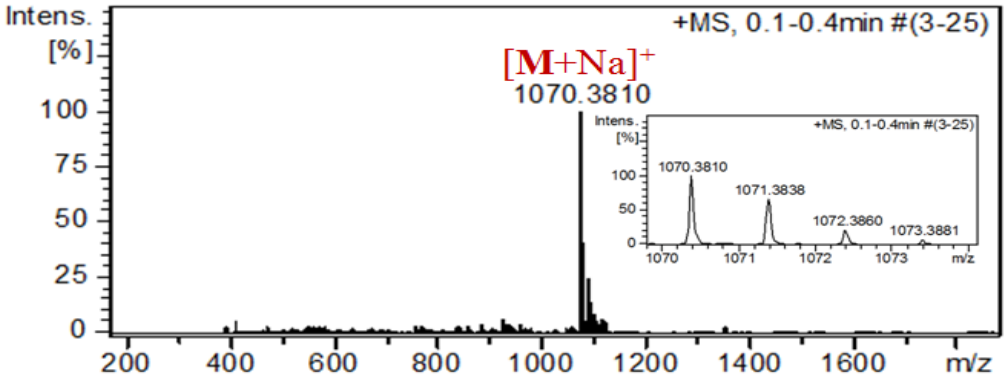


Figure S14m. High resolution mass spectrum of compound **3** in positive ESI mode (calcd *m/z* 1070.3798, $[M+Na]^+$).

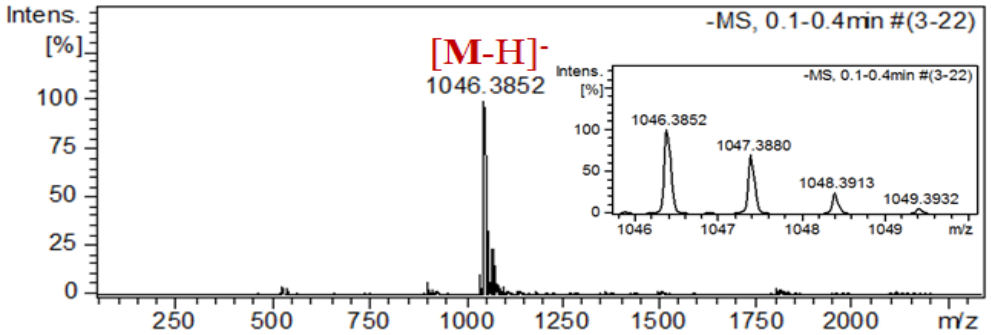


Figure S14n. High resolution mass spectrum of compound **3** in negative ESI mode (calcd *m/z* 1046.3856, $[M-H]^-$).

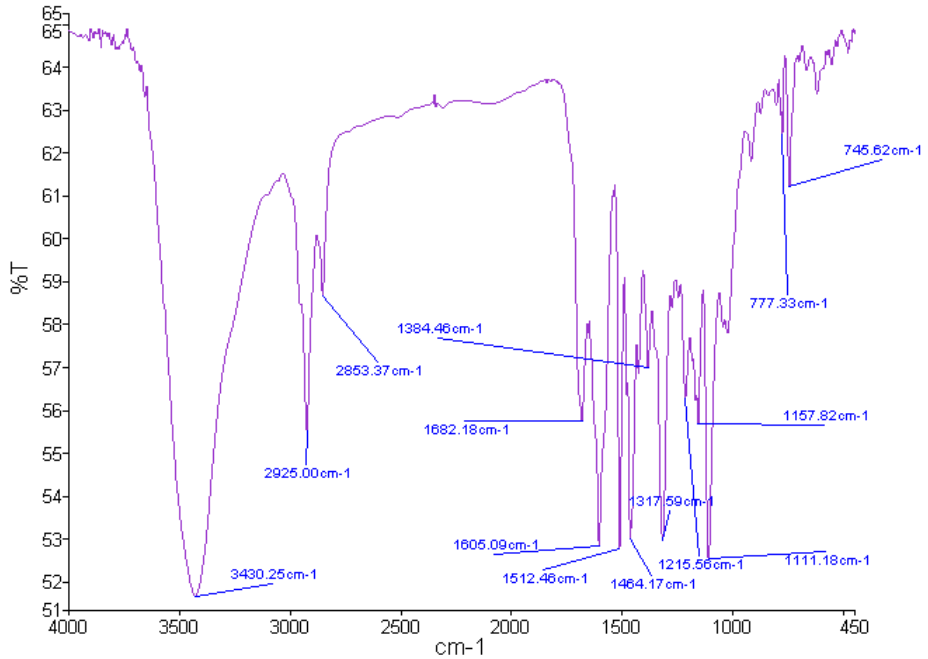


Figure S14o. IR spectrum of compound **3.**

Table S1. Composition of tap water and ultrapure water used in the present studies.

Physical parameter	**Water sample – I (ultrapure de-ionized)***	**Water sample – II (tap water)**
pH	7.1±0.1	7.2±0.1
TDS (mg/L)	<0.1	400±10
TSS (mg/L)	NIL	NIL
Cond. (µs)	2.9±0.1	240
Hardness (mg/L)	15±1	270
Calcium (mg/L)	NIL	60±5
Magnesium (mg/L)	NIL	30±5
Iron (mg/L)	NIL	0.02±0.01
Chlorides (mg/L)	NIL	14±2

*purchased from Sigma-Aldrich

Table S2. Addition of various salts to solution of compound **3** in ultrapure water.

Salt (1 mM)	Color change with	
	Compd 3	Compd 4
$Ca(HCO_3)_2$	No	No
$Mg(HCO_3)_2$	No	No
$CaCl_2$	No	No
$MgCl_2$	No	No
NaCN	orange	orange

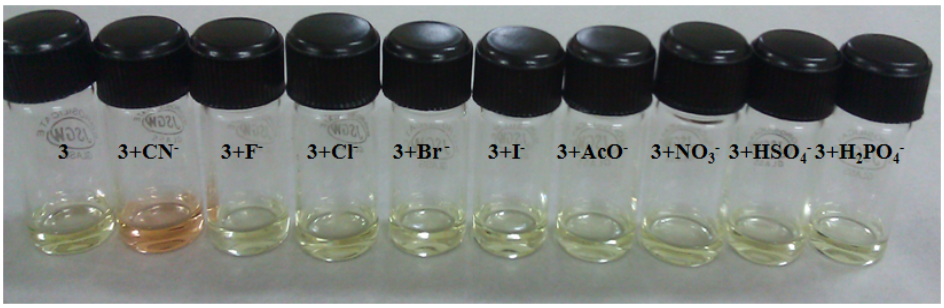


Figure S15. Colour change of compound **3** (1 μM, DMSO-water,1:1 v/v) in presence of anions: CN^-, F^-, Cl^-, Br^-, I^-, AcO^-, NO_3^-, HSO_4^-, $H_2PO_4^-$.

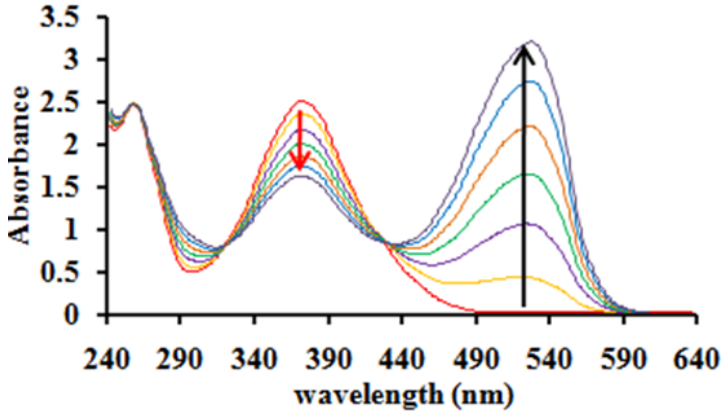


Figure S16. UV-Vis spectral change in 1 μM solution of compound **3** in $CHCl_3$ using TBACN as source of CN^-.

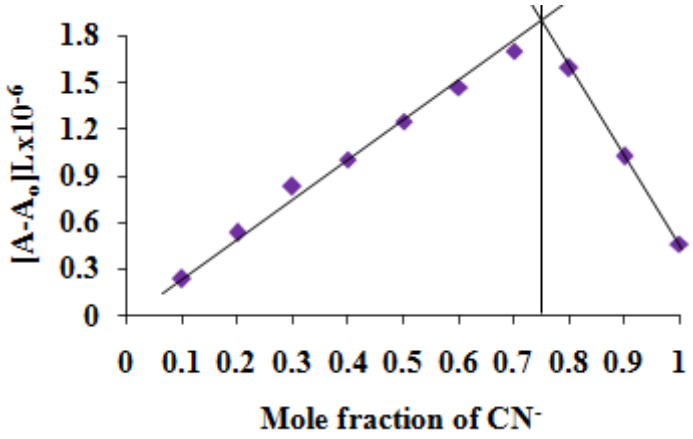


Figure S17. Job's plot for compound **3** with CN^- in DMSO-water (1:1, v/v), monitored at 501 nm.

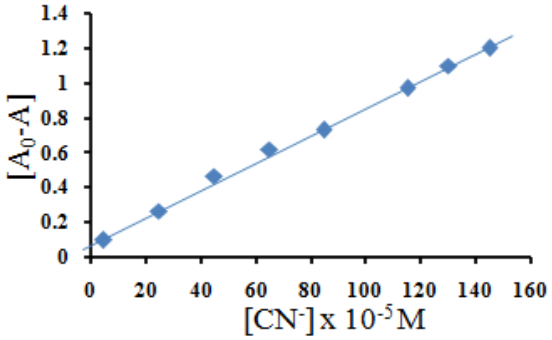


Figure S18. Linear plot of change in absorbance at 387 nm Vs [CN^-] for determination of detection limit of CN^- by compound **3**.

Calculations: Detection limit is obtained by using following formula:

DL (detection limit) = (3 X S. D.)/Slope

where S. D.(Standard Deviation)= 0.00025
Slope= 0.00779

Ref: Goswami, S.; Das, S.; Aich, K.; Sarkar, D.; Kumar, T.M.; Quahc, C. K.; Func, H.K. CHEF induced highly selective and sensitive turn-on fluorogenic and colorimetric sensor for Fe^{3+}. *Dalton Trans*. **2013**, *42*, 15113-15119.

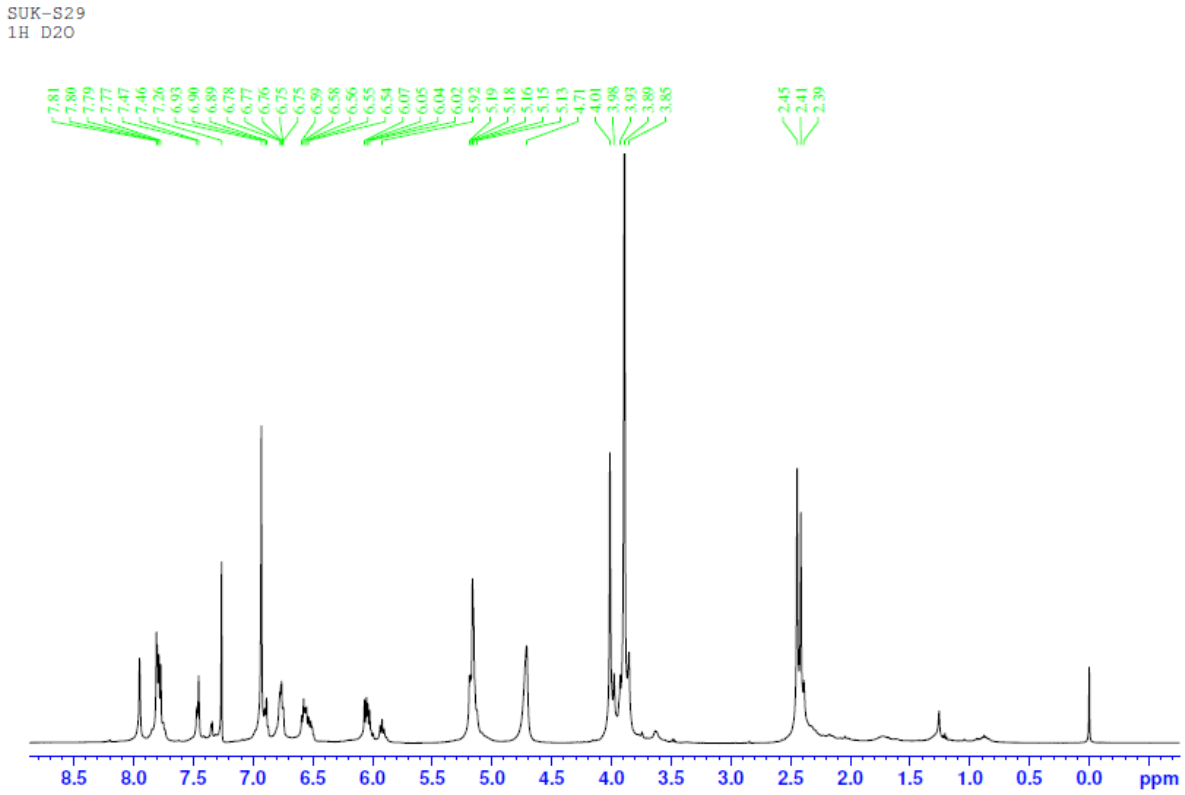


Figure 19a. D_2O exchange ^1H NMR spectrum of compound **3**

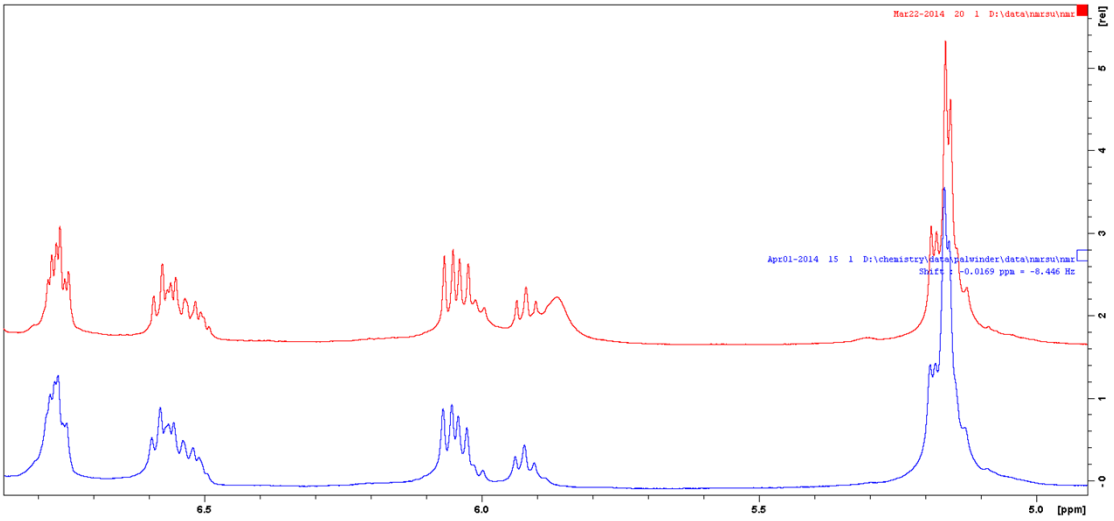


Figure S19b. Comparison of ^1H NMR spectra of compound **3** without D_2O (red trace) and with D_2O (blue trace).

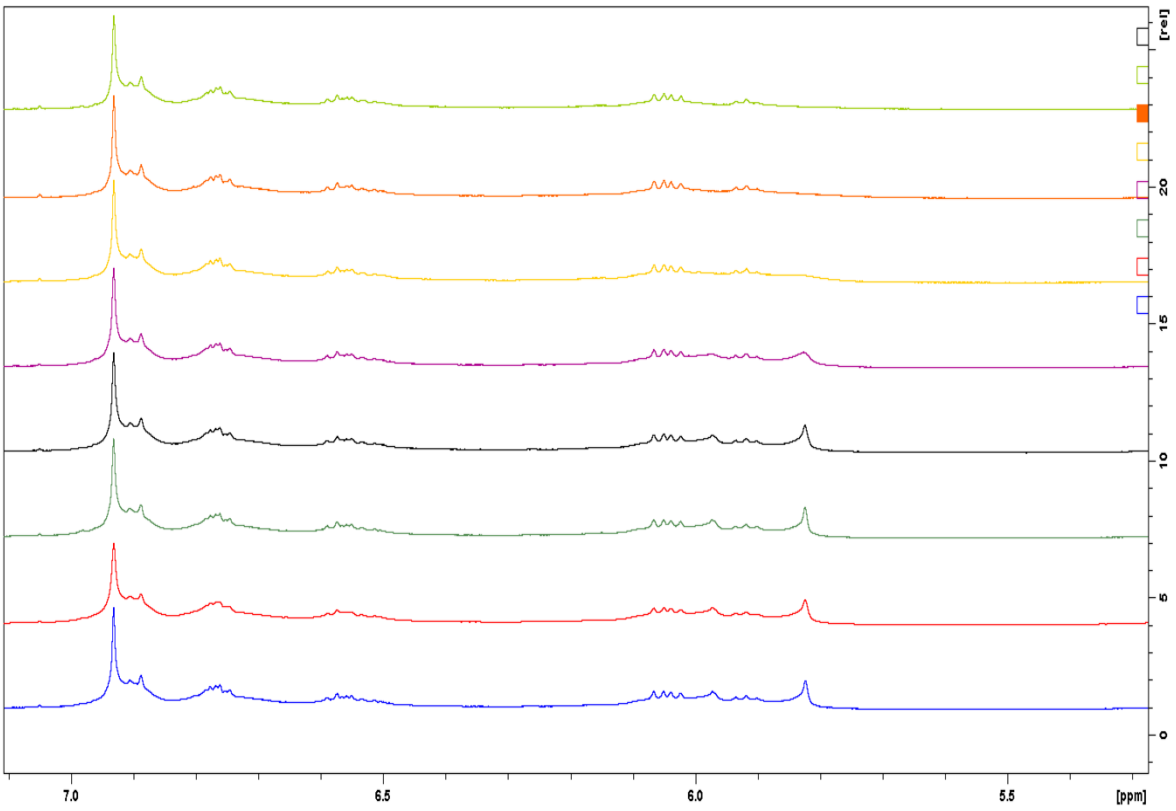


Figure S20a. Parts of the ^{1}H NMR spectrum of compound **3**. From bottom to upward : (a) pure compound; (b) with 0.001 eq. CN; (c) 0.003 eq. CN; (d) 0.005 eq. CN; (e) 0.01 eq. CN; (f) 0.02 eq. CN; (g) 0.04 eq. CN; (h) 0.06 eq. CN.

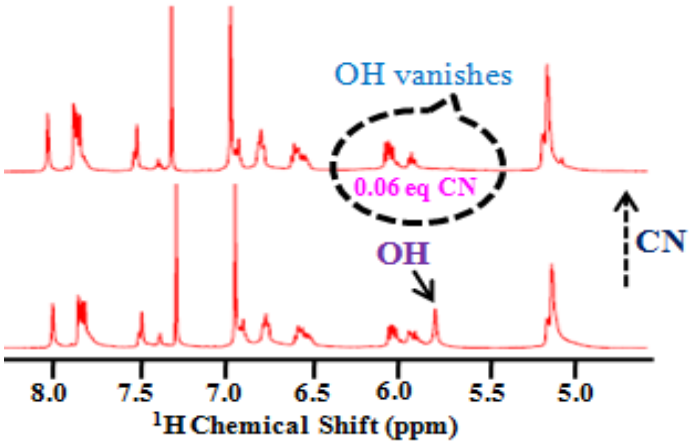


Figure 20b. Parts of ^{1}H NMR spectra of compound **3**: pure compound (lower trace) and in presence of CN^{-} (upper trace).

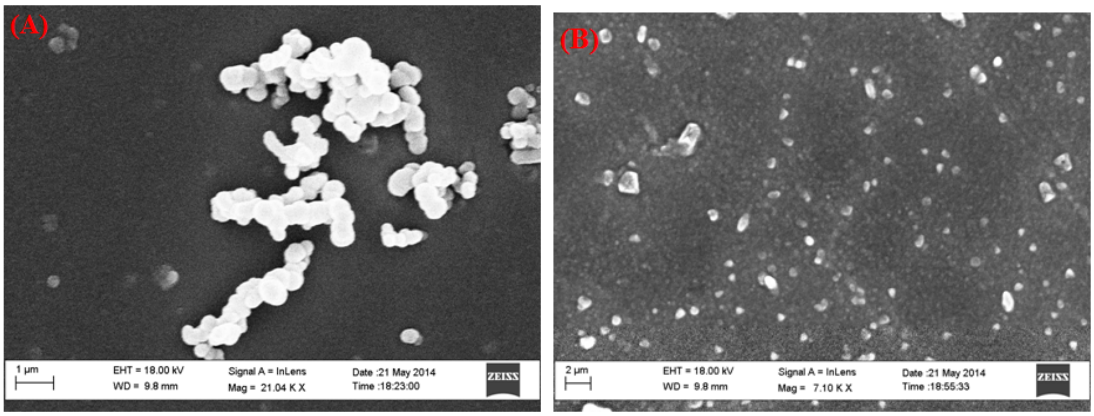
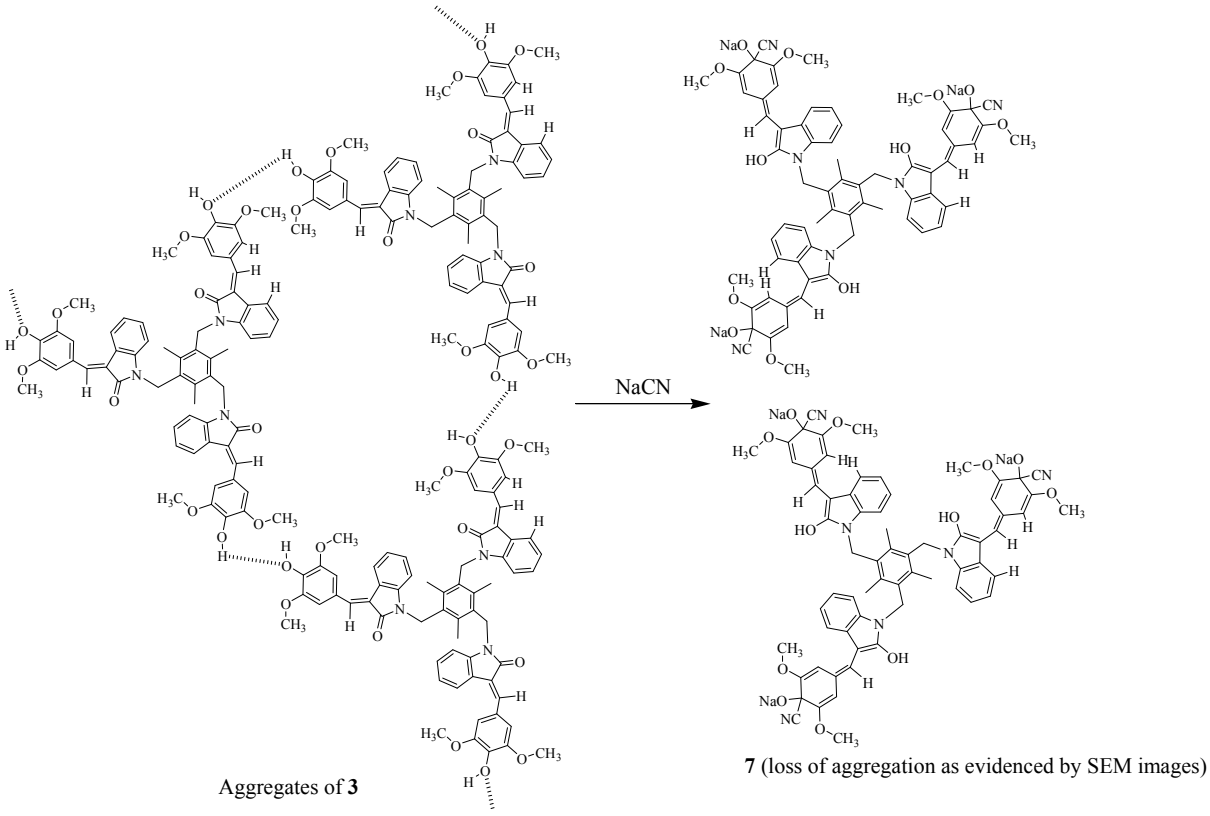
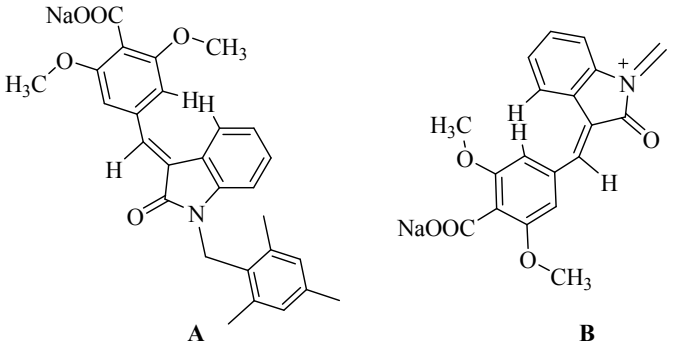


Figure S21. SEM images (A) Compound **3**: (B) Compound **7.**



Scheme S1



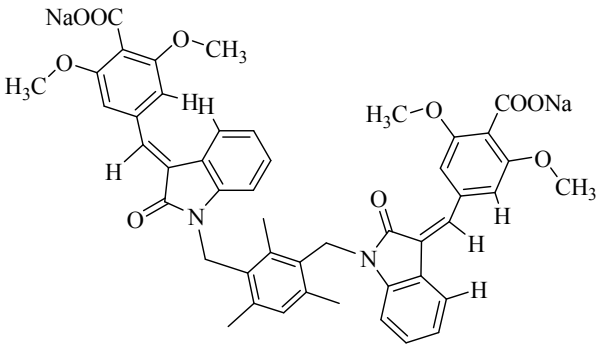


Chart S1

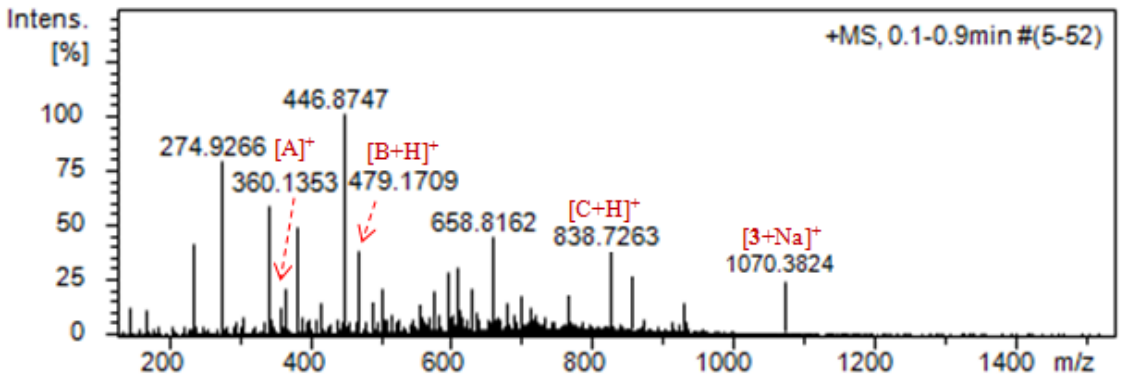


Figure S22. Mass spectrum showing breakdown of compound **7**/**8** to smaller components. For various species, refer Chart S1. Amongst these, fragment B (chart S1) is an analogue of trimethoprim (anti-bacterial drug) and possibly be metabolized safely. With the loss of water from the phenolic carbon, aromatization was also observed.

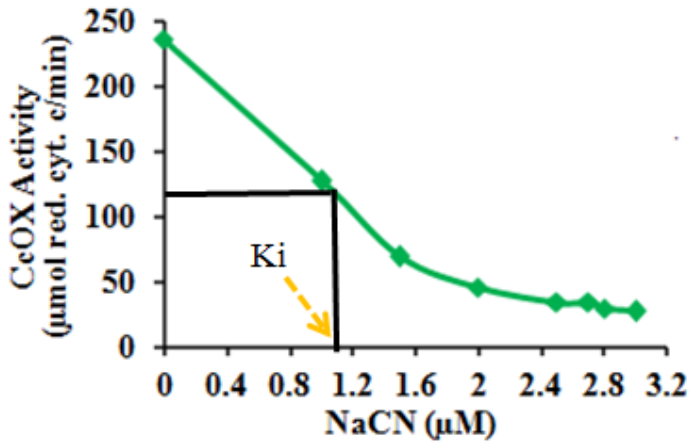


Figure S23. Inhibition constant (K_i) of cyanide for Cyt c oxidase.

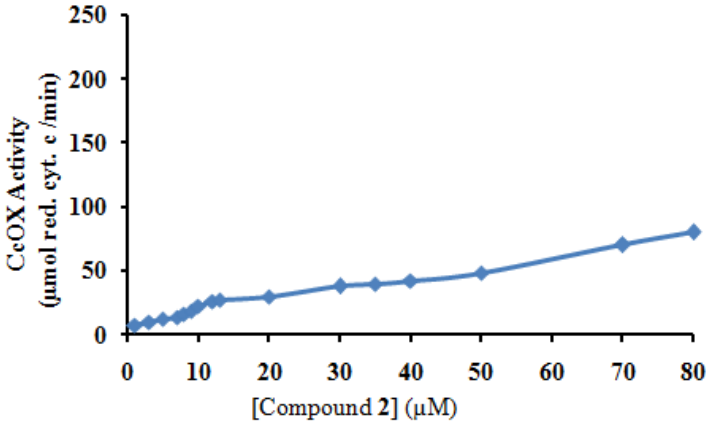


Figure S24. Recovery of cyanide bound CcOX activity in the presence of compound **2.**