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Design and Development of Fluorescence-Capable Novel Pyrazine-Based Polycyclic Heteroaromatics for Cellular Bioimaging

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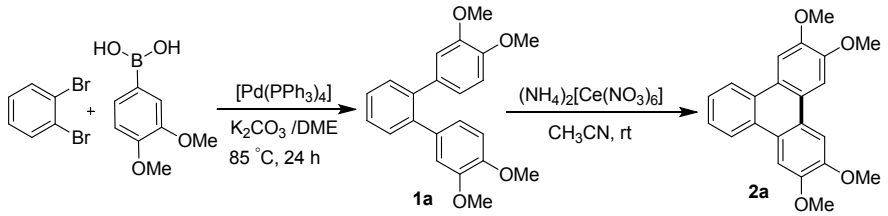
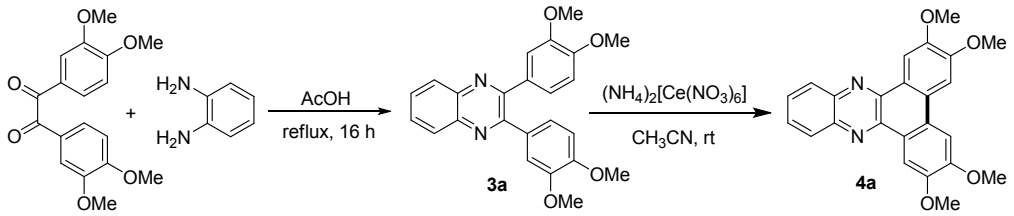
1. Synthesis of polycyclic aromatics/heteroarmatics

1.1 Synthesis of 2,3,6,7-tetramethoxytriphenylene2a.[1,2]



o-terphenyl **1a** was prepared by very well-known Suzuki-Miyaura coupling reaction.[1] According to the reported literature procedure[2], *o*-terphenyl **1a** (1.0 equiv) was taken in dry acetonitrile and subjected to oxidative-coupling with ceric(IV) ammonium nitrate (2.0 equiv) under nitrogen gas atmosphere. The reaction mixture was stirred at room temperature and then work up and purified to afford cyclized product **2a**.

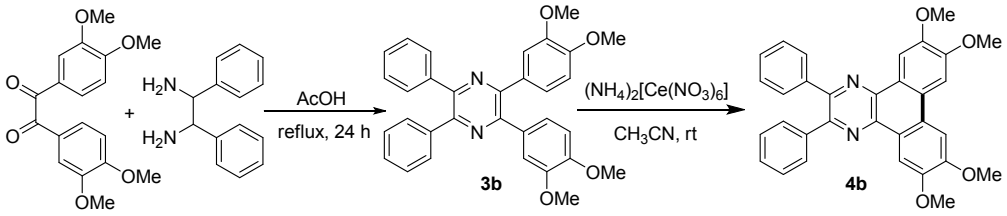
1.2 Synthesis of 2,3,6,7-tetramethoxydibenzo[*a*,*c*]phenazine (4a).[2,3]



Compound **3a** was synthesized according to the reported literature procedure.[3] 1,2-bis(3,4-dimethoxyphenyl)ethane-1,2-dione (1.0 equiv) and benzene-1,2-diamine (1.2 equiv) was taken in acetic acid as a solvent and the resulting reaction mixture was refluxed for 16 h. The reaction mixture was cooled to room temperature, as this water was added and extracted with dichloromethane. The resulting solution was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography over silica gel with ethylacetate and hexane as eluent to afford the desired product **3a** as yellow solid in 81 % yield. ^{1}H NMR was matching to the reported literature.[2] Further, **3a** (1.0 equiv) was taken in dry acetonitrile and subjected to oxidative-coupling with ceric(IV) ammonium nitrate (2.0 equiv) under nitrogen gas atmosphere. The reaction mixture was stirred at room temperature and then work up and purified according to the literature procedure[2] afforded yellow solid **4a**. ^{1}H NMR [300 MHz, $CDCl_{3(\text{Figure S4})}$]: δ 8.68 (s, 2H), 8.27 (dd, *J* = 6.4, 3.4 Hz, 2H), 7.81 (dd, *J* = 6.5, 3.4 Hz, 2H), 7.54 (s, 2H), 4.18 (s, 6H), 4.11 (s, 6H). ^{13}C NMR [75 MHz, $CDCl_{3\ (\text{Figure S5})}$]: δ 151.6, 149.2, 141.6, 141.5, 129.2, 129.1, 126.3, 123.7, 106.9, 103.7, 56.2, 56.1. IR (KBr) cm^{-1} :

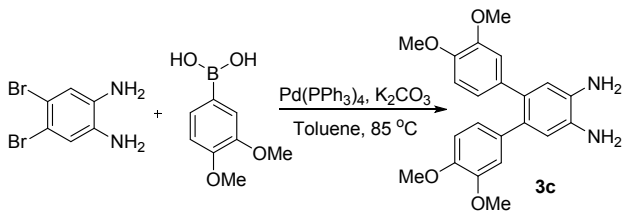
3445, 2925, 2360, 1604, 1510, 1450, 1260, 1142, 1023, 861, 780; HRMS ESI: [M+H]+ , Calcd. for $C_{24}H_{21}N_2O_4$ 401.1496; found 401.1505. (**4a**)

1.3 Synthesis of 6,7,10,11-tetramethoxy-2,3-diphenyldibenzo[*f*,*h*]quinoxaline (4b).[2,3]



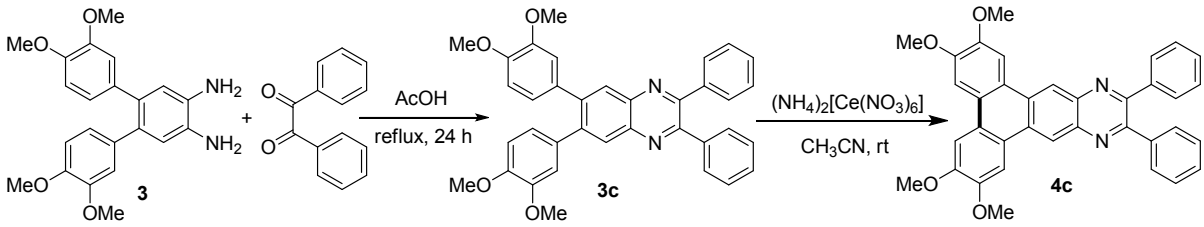
Compound **3b** was synthesized according to the literature procedure.[3] 1,2-bis(3,4-dimethoxyphenyl)ethane-1,2-dione (1.0 equiv) and 1,2-diphenylethane-1,2-diamine (1.2 equiv) was taken in acetic acid as a solvent and the resulting reaction mixture was refluxed for 24 h. The reaction mixture was cooled to room temperature, to this water was added and extracted with dichloromethane. The resulting solution was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography over silica gel with ethylacetate and hexane as eluent to afford the desired product **3b** as yellow solid in 85 % yield. ^{1}H NMR was matching to the reported literature.[2] Further, **3b** (1.0 equiv) was taken in dry acetonitrile and subjected to oxidative-coupling with ceric(IV) ammonium nitrate (2.0 equiv) under nitrogen gas atmosphere. The reaction mixture was stirred at room temperature and then work up and purified according to the literature procedure[2] afforded yellow solid **4b**. ^{1}H NMR [300 MHz, $CDCl_3$ (Figure S6)]: δ 8.70 (s, 2H), 7.75 (s, 2H), 7.65-7.68 (m, 4H), 7.37-7.38 (m, 6H), 4.15 (s, 6H), 4.14 (s, 6H). ^{13}C NMR [75 MHz, $CDCl_3$ (Figure S7)]: δ 151.3, 150.3, 149.3, 139.9, 138.0, 130.2, 128.5, 128.4, 126.0, 123.8, 106.3, 103.7, 56.2. IR (KBr) cm^{-1}: 3436, 2926, 2360, 1602, 1512, 1450, 1345, 1254, 1165, 1022, 858, 766. HRMS ESI: [M+H]+ , Calcd. for $C_{32}H_{27}N_2O_4$ 503.1965; found 503.1911.

1.4 Preparation of 3,3'',4,4''-tetramethoxy-[1,1':2',1''-terphenyl]-4',5'-diamine (3).[3]



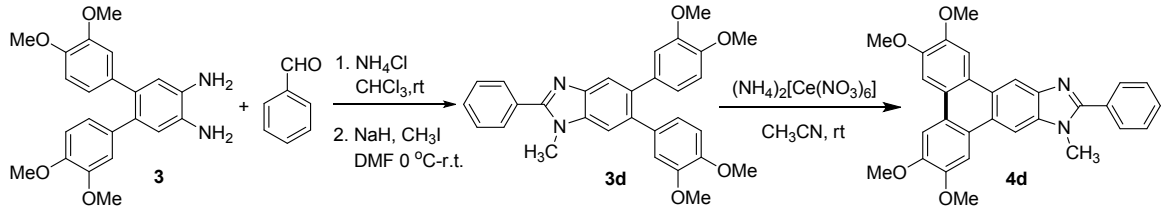
4, 5-dibromo-1, 2-phenylenediamine (1.0 g, 3.76 mmol), 3,4-dimethoxyphenylboronic acid (1.7 g, 9.4 mmol) and $Pd(PPh_3)_4$ (0.11 g, 0.094 mmol) were taken in 60 mL toluene. To this 15 ml of 2M K_2CO_3 aqueous solution was added. The resulting suspension was degassed and heated at 85 ^oC for 24 h under N_2 atmosphere. After that, the reaction mixture was cooled to room temperature, to this water was added and the compound was extracted with ethylacetate, dried over Na_2SO_4. The solvent was concentrated under reduced pressure and compound was purified by column chromatography on silica gel with EtOAc and hexane (9:1) as eluent to afford compound **3** as brown solid, yield 50%.

1.5 Synthesis of 2,3,6,7-tetramethoxy-11,12-diphenylphenanthro[9,10-*g*]quinoxaline (4c).[2,3]



Benzil (1.0 equiv.) and **3** (1.2 equiv.) was taken in acetic acid as a solvent and the resulting reaction mixture was refluxed for 24 h. The reaction mixture was cooled to room temperature, as this water was added and extracted with dichloromethane. The resulting solution was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography over silica gel with ethylacetate and hexane as eluent to afford the desired product **3c** as yellow solid in 85 % yield. Thereafter, **3c** (1.0 equiv) was taken in dry acetonitrile and subjected to oxidative-coupling with ceric(IV) ammonium nitrate (2.0 equiv) under nitrogen gas atmosphere. The reaction mixture was stirred at room temperature and then work up and purified according to the literature procedure[2] afforded yellow solid **4c**. ^1H NMR [300 MHz, $CDCl_{3\ (Figure\ S8)}$]: δ 9.17 (s, 2H), 8.11 (s, 2H), 7.72 (s, 2H), 7.56-7.59 (m, 4H), 7.37- 7.39 (m, 6H), 4.12 (s, 12H). ^{13}C NMR [75 MHz, $CDCl_{3\ (Figure\ S9)}$]: δ 154.1, 150.4, 149.2, 139.4, 139.1, 131.6, 130.0, 129.1, 128.4, 124.7, 123.1, 122.2, 106.1, 104.7, 56.2. IR (KBr, cm^{-1}): 3461, 2927, 2362, 1618, 1518, 1414, 1326, 1266, 1165, 1044, 841, 770. HRMS ESI: [M+H]+ , Calcd. for C36H29N2O4 553.2122; found 553.2123.

1.6 Synthesis of 2,3,6,7-tetramethoxy-10-methyl-11-phenyl-10H-triphenyleno[2,3-d]imidazole (4d).[2,4]



According to the reported literature procedure[4], benzaldehyde (1.0 equiv) was added to a stirred solution of 3,3'',4,4''-tetramethoxy-[1,1':2',1''-terphenyl]-4',5'-diamine **3** (1.0 equiv), and ammonium chloride (4.0 equiv) in chloroform for five minutes at room temperature. The resulting reaction mixture was stirred at room temperature for 4 h. The compound was extracted with dichloromethane and the organic layer washed with H_2O (10 ml). The compound was purified by column chromatography on silica gel with EtOAc and hexane (8:2) as eluent. Further, the compound was subjected for *N*-methylation afforded white solid **3d**. ^{1}H NMR was matching to the reported literature.[2] Further, **3d** (1.0 equiv) was taken in dry acetonitrile and subjected to oxidative-coupling with ceric(IV) ammonium nitrate (2.0 equiv) under nitrogen gas atmosphere. The reaction mixture was stirred at room temperature and then work up and purified according to the literature procedure[2] afforded solid **4d**. ^{1}H NMR [300 MHz, DMSO-$d_{6\,(\text{Figure S10})}$]: δ 9.06 (s, 1H), 8.83 (s, 1H), 8.31 (s, 1H), 8.24 (s, 1H), 8.0 (d, *J* = 6.0 Hz, 4H), 7.63 (d, *J* = 6.0 Hz, 3H), 4.13 (s, 3H), 4.1 (s, 3H), 4.07-4.05 (m, 9H). ^{13}C NMR [75 MHz, DMSO-$d_{6\,(\text{Figure S11})}$]: δ 155.4, 149.1, 148.8, 148.8, 148.8, 142.2, 136.8, 130.2, 129.8, 129.3, 128.7, 125.2, 124.8, 123.9, 123.6, 123.0, 122.5, 112.5, 106.1, 105.4, 103.5, 56.0, 55.9, 55.9, 55.7, 32.2. IR (KBr, cm^{-1}): 3460, 2920, 2361, 1615, 1525, 1412, 1265, 1165, 1350, 868, 745. HRMS ESI: [M+H]+ , Calcd. for $C_{30}H_{27}N_2O_4$ 479.1965; found 479.1965.

2. UV-vis Absorption and Emission spectra

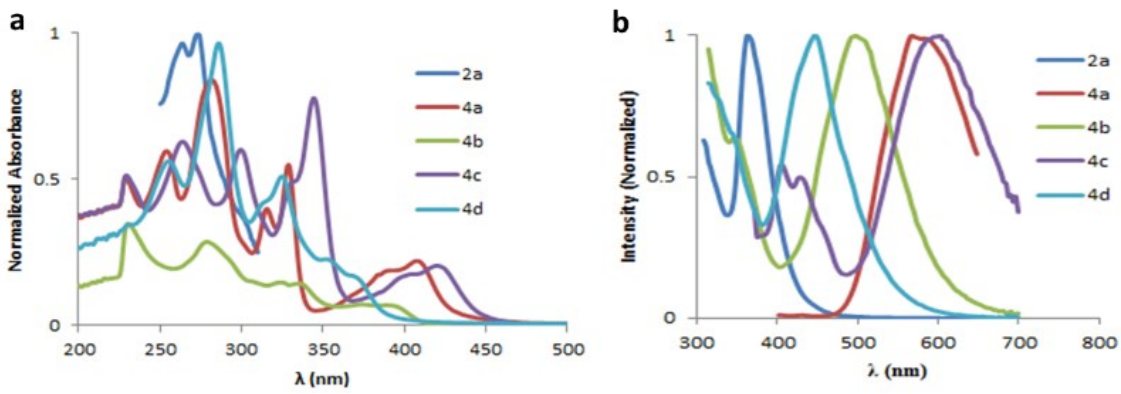


Figure S1. (a) UV-vis spectra (**2a**, **4a**-**d**) (b) Fluorescence spectra of **2a** (λ_{ex} = 273 nm), **4a** (λ_{ex} = 282 nm), **4b** (λ_{ex} = 280 nm), **4c** (λ_{ex} = 345 nm) and **4d** (λ_{ex} = 286 nm) in DCM with concentration 10^{-6} M.

3. Quantum yield estimation:

Relative Fluorescence quantum yield (QY) of compound **4a** was calculated using Rhodamine B, having quantum yield of 0.7, as a reference. To estimate QY simply using single-point method, following formula was used:

$$QY_s = QY_r \times (A_r/A_s) \times (E_s/E_r) \times (n_s/n_r)^2$$

where:

Subscript 'r' refer to reference and 's' to test sample.

A = Absorbance of solution

E = Integrated fluorescence intensity of emitted light

n = refractive index of solution

Briefly, absorbance and fluorescence spectra of freshly prepared rhodamine B in DCM was recorded under similar conditions as the absorbance and fluorescence emission spectra of compound **4a** from figure 3. Integrated fluorescence intensities were calculated by estimating area under the curve of fluorescence emission spectra.

4. pH dependence of fluorescence emission: Compounds **4a**-**c** were dissolved in DMSO and titrated with organic acid trifluoroacetic acid to obtain a range of different pH (8.5-2.5) solutions. Fluorescence emission spectra and absolute values of fluorescence intensity for all the compounds at different pH solution was recorded on a multiplate reader at their respective excitation wavelengths.

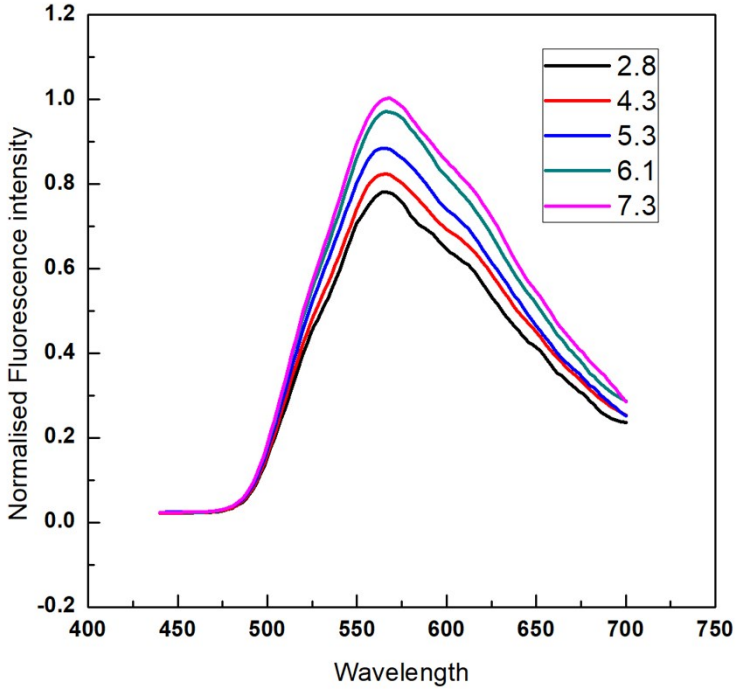


Figure S2. Fluorescence spectra of compound **4a** (concentration 10^{-6} M in DMSO) at various pH (7.3-2.8), λex = 412 nm.

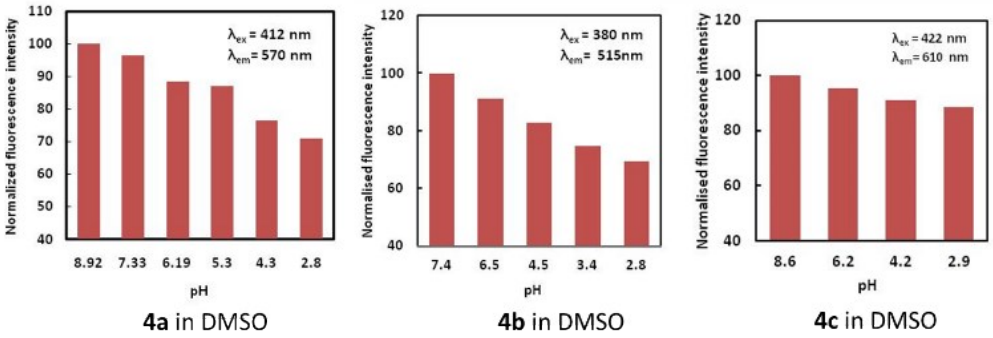


Figure S3: Effect of pH on fluorescence emission of compound **4a**-**c**.

5. Cell viability Assay: To evaluate toxicity of compounds **4a**-**c** on live cells, mitochondrial activity of ovarian cancer (Hela) cell line after exposure to various concentration of these dyes were examined. Cells were cultured in DMEM supplemented with 10% FBS under 5% CO_2. Then 1×10^4 cells/well were seeded in 200 µl culture medium in a 96 well plate. After overnight incubation of seeded cells, media was replaced with 200 µl compounds (**4a**-**c**) of various concentrations (7-40 µM) dispersed in DMEM. Each concentration of each compound was

added in triplicate and incubated for 24 h. After 24 h of culture, media was removed, and cells were washed with DPBS. MTT (0.5 mg/mL) in DMEM was added to the wells and incubated for another hour and half. At this point, formation of purple formazan crystals was clearly observed in the control wells. The media was carefully removed, and the formazan crystals were solubilized in 200ul DMSO. Absorbance was recorded at 550 nm using iMarkMicroplate Absorbance Reader (BIORAD). The data was plotted with standard deviation (from three sets of independent experiments) as error bars.

6. Cellular uptake: 5×10^4 cells/well were seeded in 1ml culture medium in a transparent 24 well plate and incubated overnight. Media was then replaced with 500 µl of compounds **4a** (0, 3 15µM) dispersed in DMEM and incubated for another 3-4h. After incubation, the medium was removed and cells were washed three times with DPBS. Cells were further treated with 300 µl of Tween-20 in PBS for 30s to remove any surface-bound molecules. To remove surfactant completely, cells were washed with PBS three times again; then imaged under bright field and a blue excitation filter under a fluorescence microscope. To further quantify the cellular uptake, cells were lysed by dissolving into DMSO and fluorescence of cell lysate was measured using iMarkMicroplate Absorbance Reader (BIORAD) and plotted as bars with standard deviation (from three sets of independent experiments) as error bars.

7. References

1. (*a*) A. Suzuki, *Chem. Commun.,* 2005, 4759; (*b*) J. Hassan, M. Sevignon, C. Gozzi, E. Schulz and M. Lemaire, *Chem. Rev.,* 2002, **102**, 1359.
2. V. Gupta, S. K. Pandey and R. P. Singh *Org. Biomol. Chem.,* 2018*,* **16***,* 7134.
3. B. Gao, M. Wang, Y. Cheng, L. Wang, X. Jing and F. Wang, *J. Am. Chem. Soc.,* 2008, **130**, 8297.
4. D. Nannapaneni, A. V. Gupta, M. Reddy and R. C. Sarva, *Journal of Young Pharmacists* 2010, **2**, 273.

8. ^{1}H and ^{13}C Spectra Compounds 4a-d

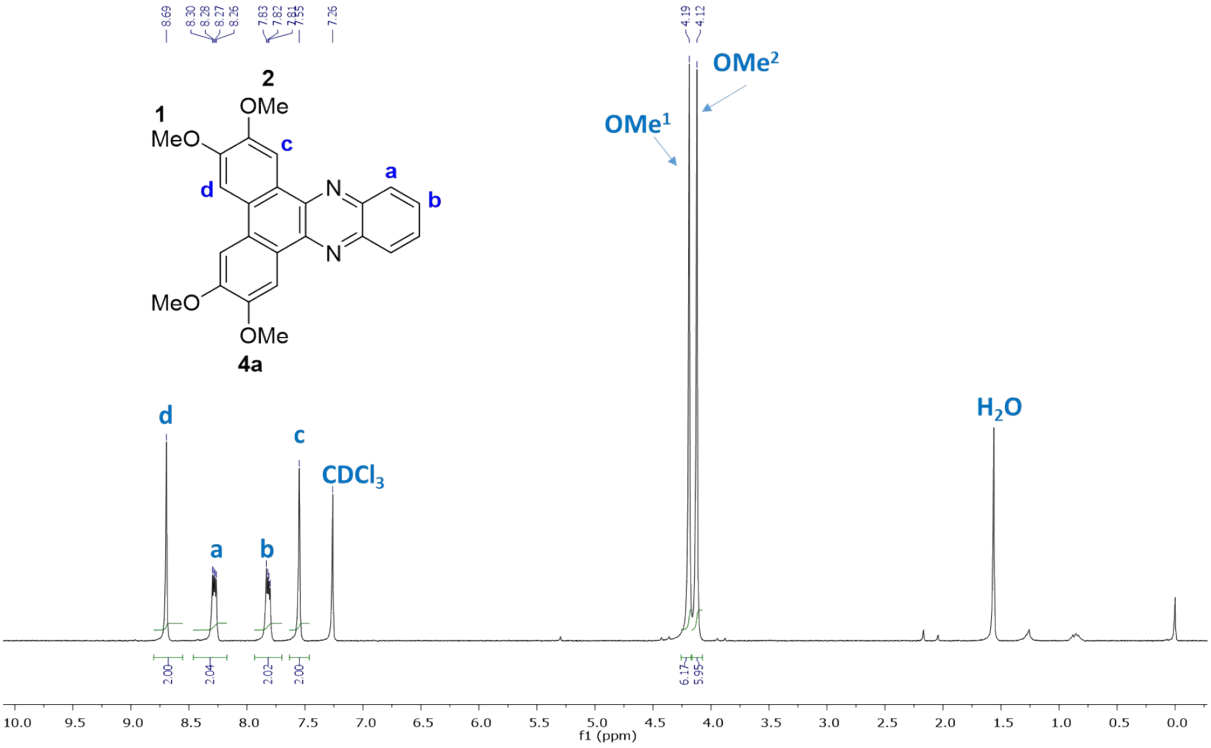


Figure S4: ^{1}H NMR of compound **4a** in $CDCl_3$.

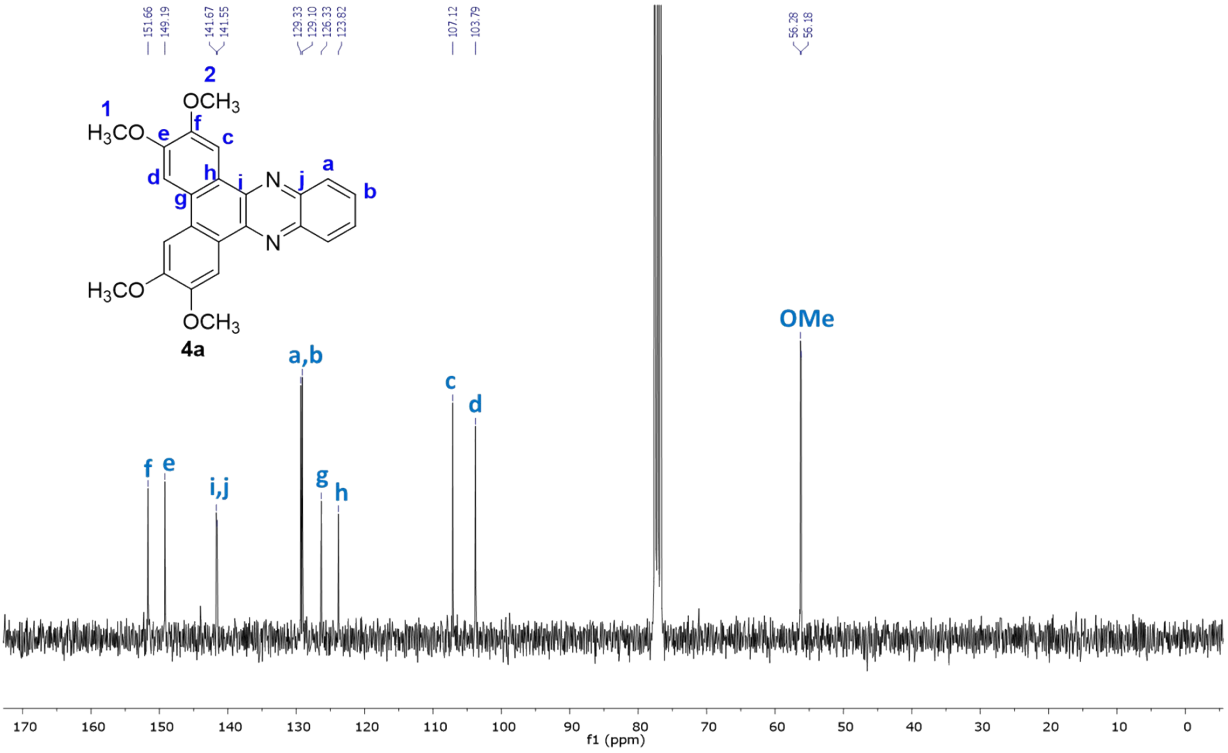


Figure S5: ^{13}C NMR of compound **4a** in $CDCl_3$.

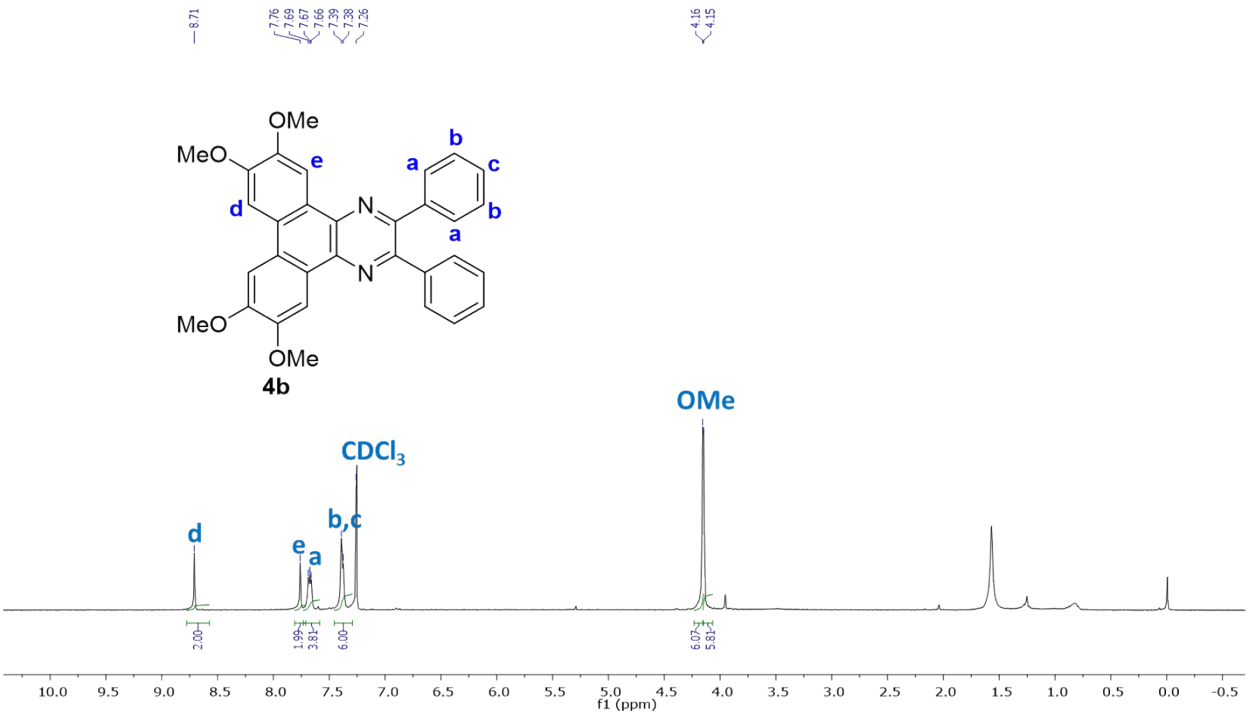


Figure S6: ^{1}H NMR of compound **4b** in $CDCl_3$.

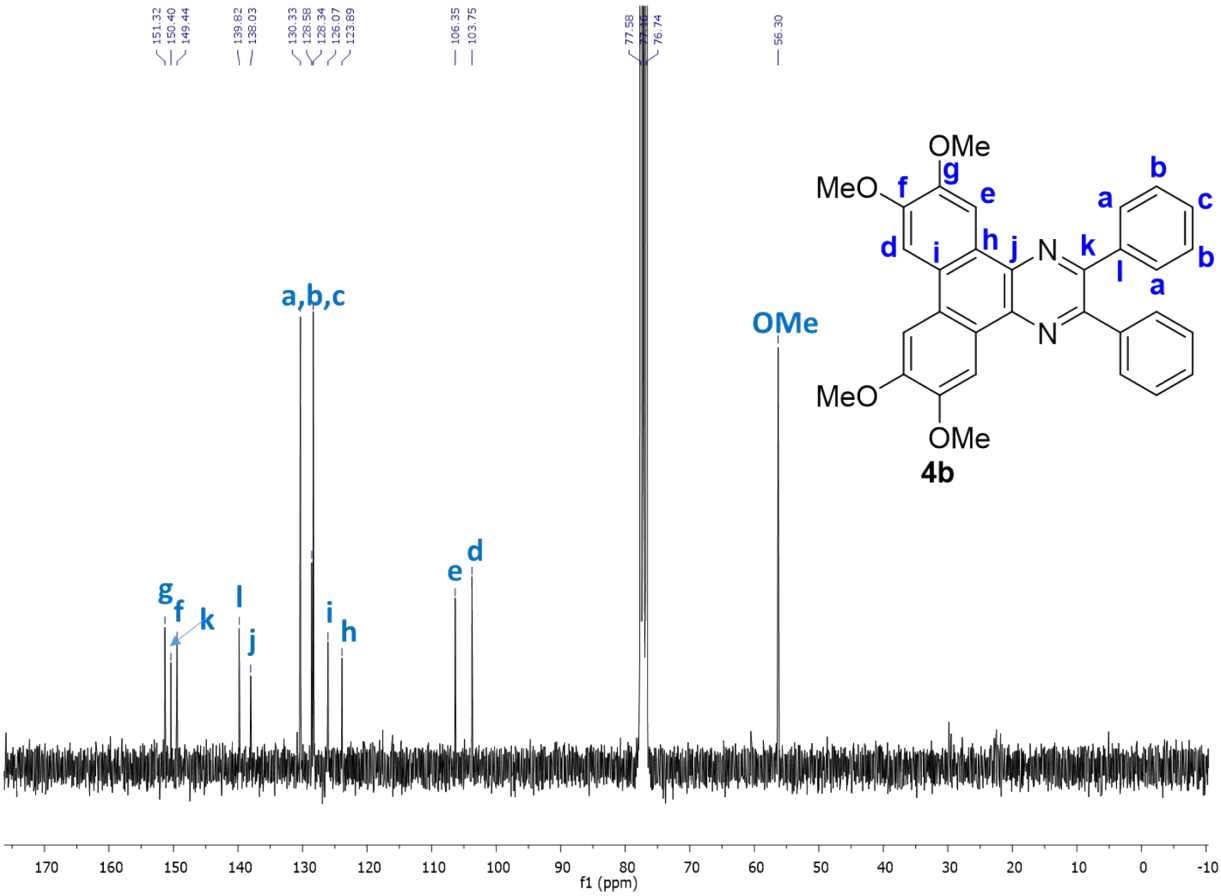


Figure S7: ^{13}C NMR of compound **4b** in $CDCl_3$.

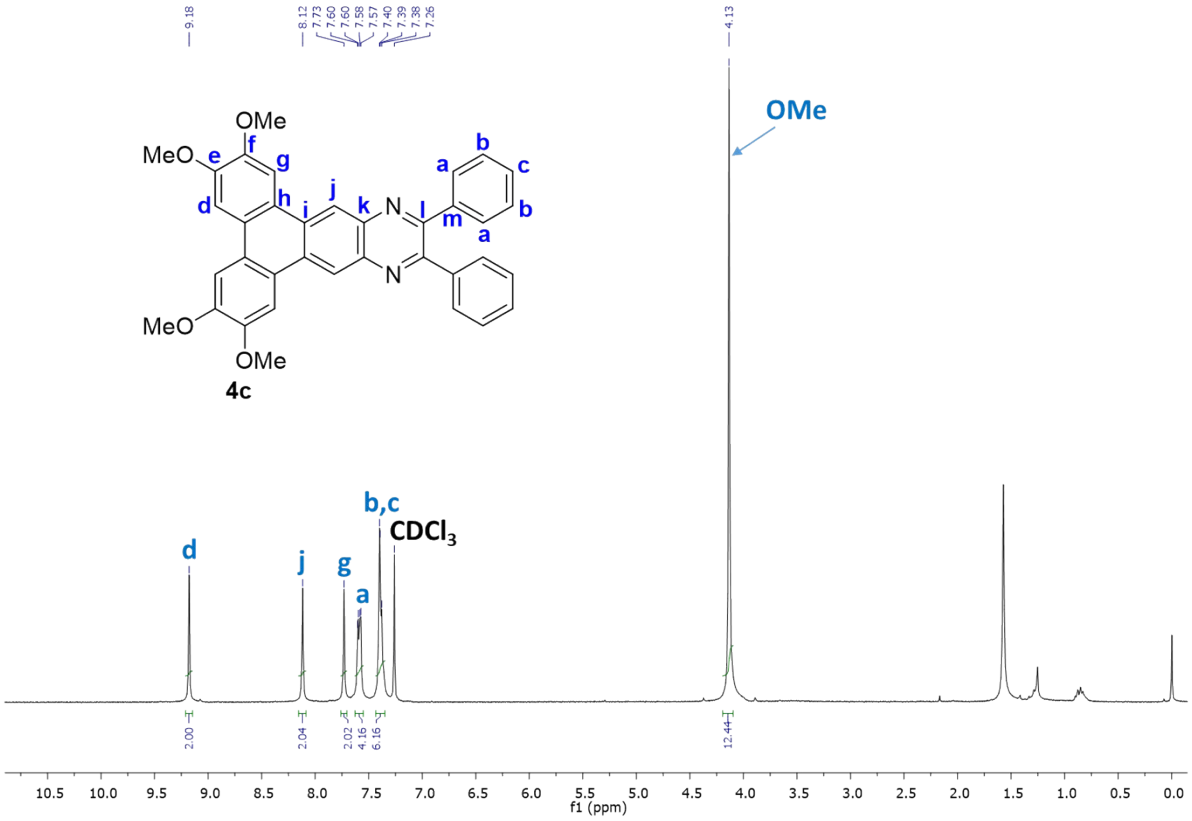


Figure S8: ^{1}H NMR of compound **4c** in $CDCl_3$

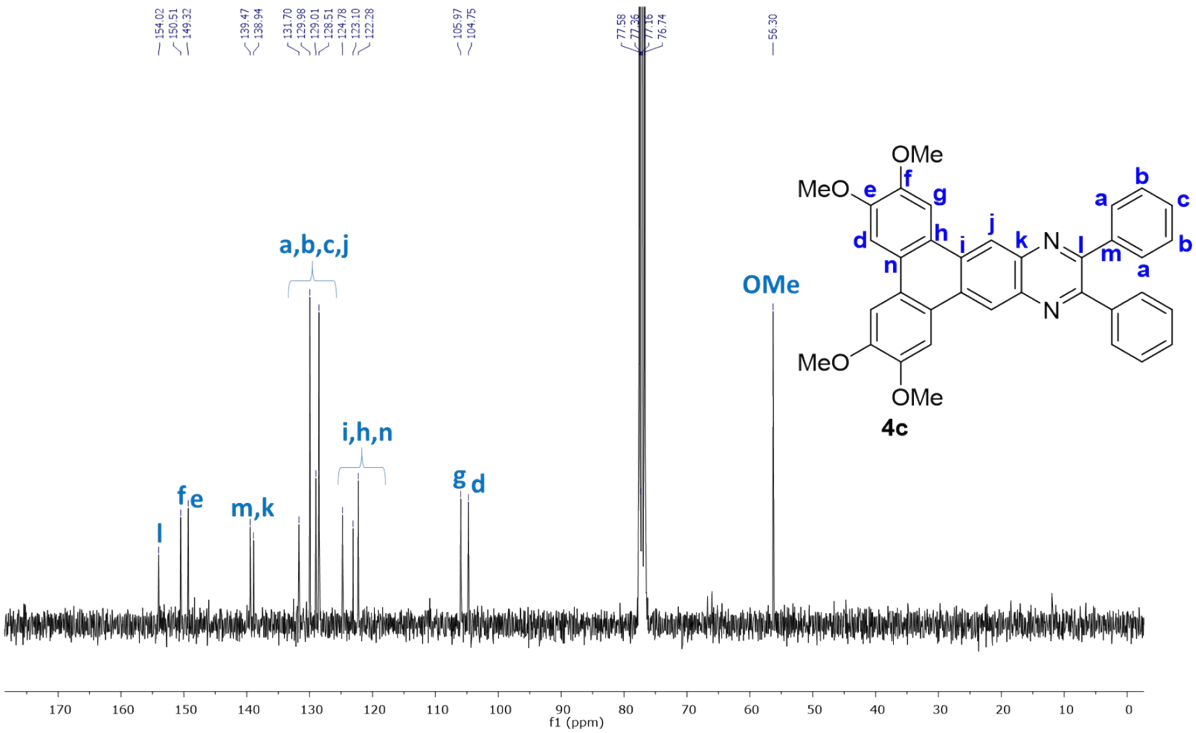


Figure S9: ^{13}C NMR of compound **4c** in $CDCl_3$.

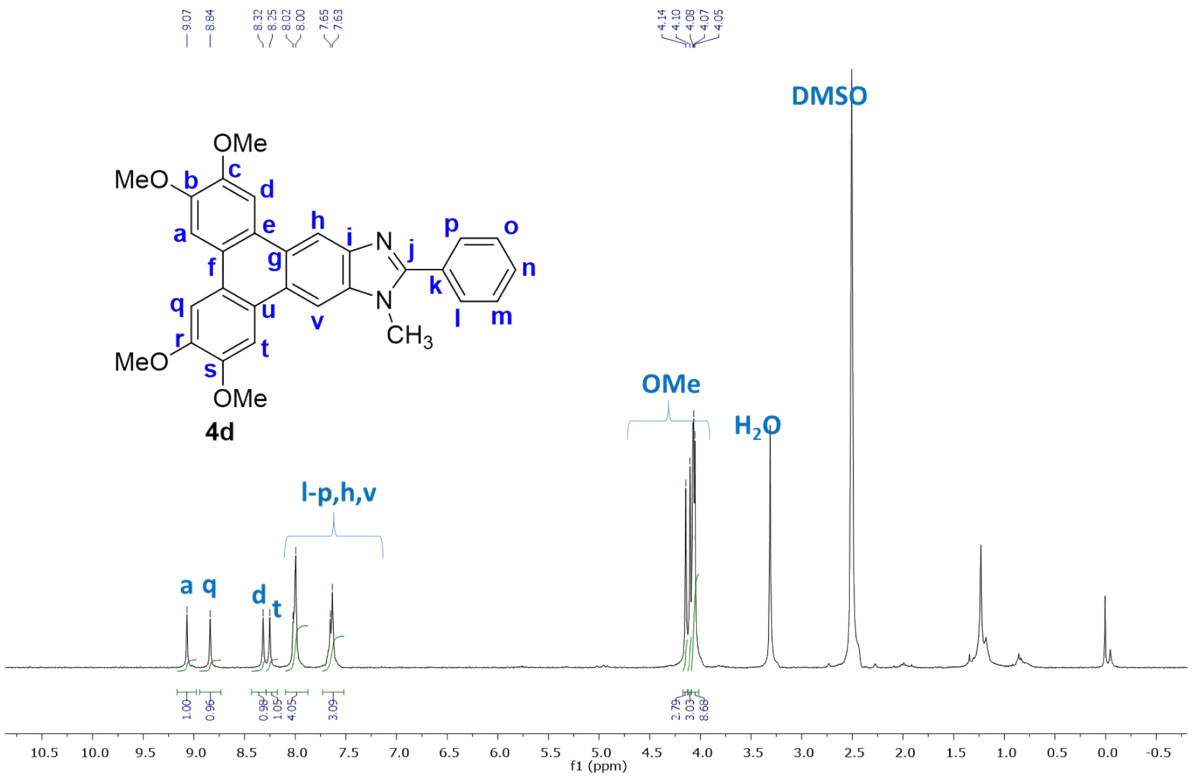


Figure S10: ^{1}H NMR of compound **4d** in DMSO-d_6.

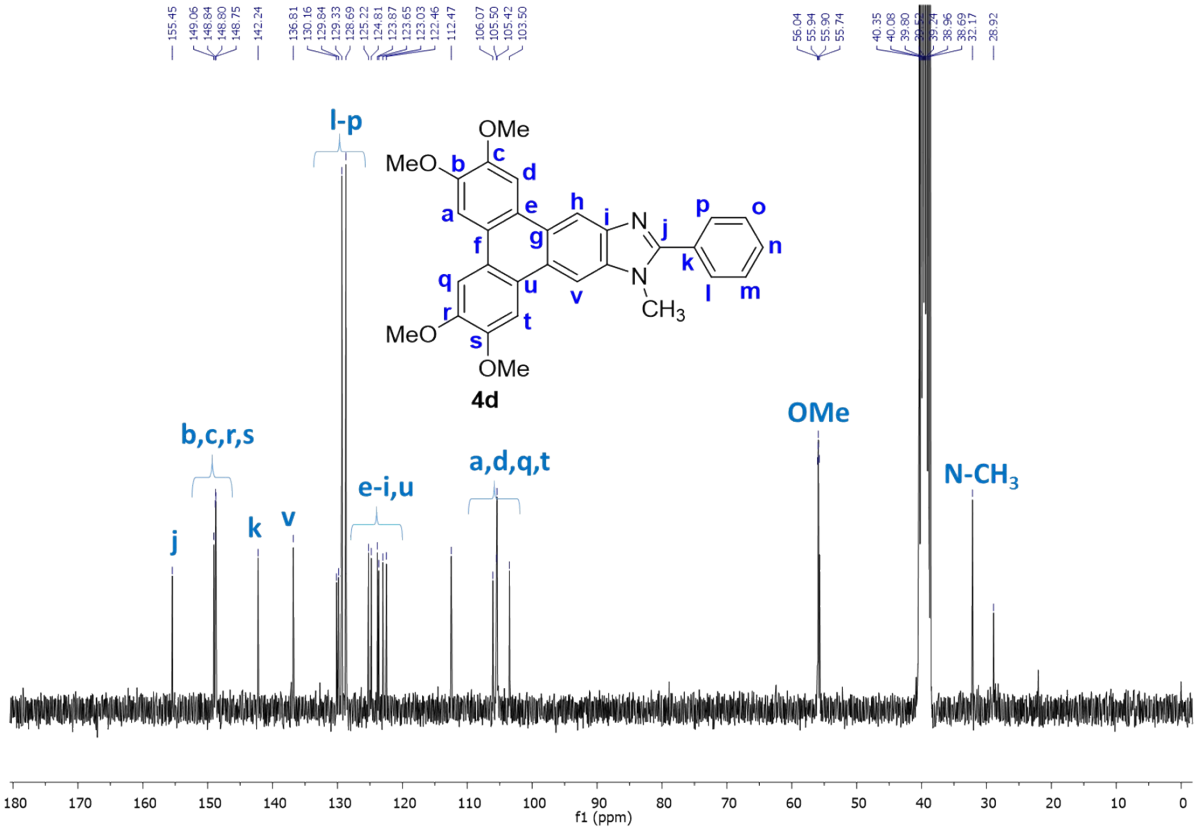


Figure S11: ^{13}C NMR of compound **4d** in DMSO-d_6.