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Synthesis of Unsymmetrical Water-Soluble Cationic Pyridinium Mesoporphyrinic Free-Base Porphyrins and Its Zn (II) Complex: Photophysical and Photocytotoxicity Evaluation

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Abstract : *To assess the properties of pyridinium substituted porphyrins, new meso-porphyrinic derivative namely 5-mono-(4-pyridinum)- 10,15,20-tris-(4-carboxymethylphenyl)porphyrin* ***MPyEt3*** *and 5-mono-(4-pyridinum)-10,15,20-tris-(4-carboxymethylphenyl)-21,23-Zn(II)*-porphine ***MPyEt3Zn*** *were synthesized in high yields. The porphyrin ester* ***PyEt3*** *was synthesized through a conventional method. Direct meso-substituted pyridyl derivatives of porphyrins prefer alkylation producing pyridinium ions for inducing water-solubility. The structures of the free-based porphyrin ligand and Zn(II) complex were confirmed through UV-Vis, emission,* ^{1}*H-NMR, and ESI HRMS spectral data. The spectral absorption and emission properties of the ligand and complex were studied in organic solvents of different polarities, and the influence of solvent polarity on the wavelengths of the absorbance and fluorescence band maxima is described. Changes in fluorescence intensity, emission peak location and overall fluorescence line-shapes were examined in variation with temperature between 7*^{o}*C to 97*^{o}*C of each 10*^{o}*C increments. The photophysical properties and photocytotoxicity evaluation of compounds* ***MPyEt3*** *and* ***MPyEt3Zn*** *with a view to assessing their possible use in the photodiagnosis and cancer photodynamic therapy using AGS and SW480 cell lines.*

Keywords: *Water-soluble meso-porphyrinic derivative, Zn(II) complex, MTT Assays, AGS cell line, Sw480 cell line.*

I. Introduction

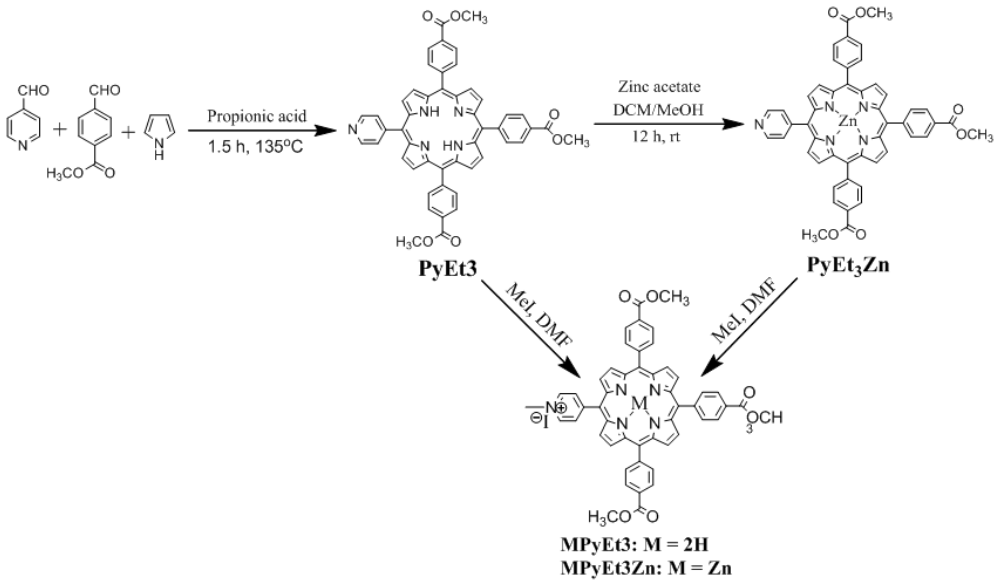
Photodynamic therapy (PDT) is an emerging treatment for different types of cancers.[1–4] It involves the inactivation of living cells by the combined action of light, a photoactive molecule called sensitizer and molecular oxygen.[1,5,6] A variety of sensitizers have been examined for their use in PDT. The role of photosensitizers (PSs) in the development of dyes has gained popularity due to their versatile applications in photobiology and photophysics. Designing of both free-base and metallated porphyrin macrocycles with specific functional groups offers the scope of generating PSs with ubiquitous involvement as agents in cancer photodynamic therapy (PDT).[7-10] Metalloporphyrins have attracted attention for many decades because of their importance in the field of biomedicine, especially in diagnosis and treatment of cancer[11-17] as well as in photodynamic antimicrobial therapy.[18-21] Free-base porphyrins and metalloporphyrins have also been investigated for use as fluorescent markers in cancer diagnosis because have appropriate fluorescence characteristics such as convenient emissions in the phototherapeutic window (~ 600 to 1100 nm), high singlet oxygen quantum yield, large stokes shifts, and reduced photolability.[14,22,23]

This paper reports the synthesis of some new unsymmetrical *meso*-substituted water-soluble cationic pyridinum porphyrin ***MPyEt3*** and its Zn(II) complex ***MPyEt3Zn*** namely 5-*mono*-(4-pyridinum)- 10,15,20-*tris*-(4-carboxymethylphenyl)porphyrin (denoted as ***MPyEt3Zn*** scheme 1) and 5-*mono*-(4-pyridinum)-10,15,20-*tris*-(4-carboxymethylphenyl)-21,23-Zn(II)-porphine (denoted as ***MPyEt3Zn*** scheme 1). The photophysical properties and photocytotoxicity evaluation of compounds ***MPyEt3*** *and* ***MPyEt3Zn*** with a view to assessing their possible use in the photodiagnosis and cancer photodynamic therapy.

II. Results

2.1 Synthesis: Scheme 1 represents the synthetic route employed to arrive at the target compounds **MPyEt3** and **MPyEt3Zn**. Condensation of pyrrole, 4-pyridinecarboxaldehyde and methyl-4-formylbenzoate in propionic acid at 150 ^{o}C for 1.5 h resulted in the formation of **PyEt3** (Yield = 12%). The compound was purified and isolated using column chromatography. The porphyrin **PyEt3** was treated with zinc acetate in a

dichloromethane/methanol medium (9:1 v/v) for 12 h to obtain **PyEt3Zn** (Yields = 92%) as shown in Scheme 1. Alkylation of the compounds **PyEt3** and **PyEt3Zn** were achieved by treating with excess of methyl iodide in dimethylformamide for 24 h at room temperature to obtain **MPyEt3** and **MPyEt3Zn** with yields of **84%** and **82%** respectively. The free-base porphyrin and its corresponding Zn (II) complex were characterized by various spectroscopic techniques. The formation of alkylated products **MPyEt3** and **MPyEt3Zn** were confirmed through the distinct appearance of cationic methyl peak in ^{1}HNMR in the 3–4 ppm region. Furthermore, The HRMS (ESI) spectra confirms the formation of the desired products. The formation of the target compounds and their precursors was confirmed from the ESI-HRMS data.



Scheme1*. Reaction scheme of the synthesis of water-soluble porphyrin (**MPyEt3**) and its Zn(II) complex (**MPyEt3Zn**)*

2.2 Absorption spectra: The absorption spectra of the free base compound **MPyEt3** and its corresponding Zn complex **MPyEt3Zn** were acquired in dimethylformamide and H_2O at 10 µM concentration. The resulting spectral plot and absorption data are represented in **Fig. 1** and **Table 1**. The free base compounds **MPyEt3** exhibit a typical porphyrinoid spectra with a strong Soret band at 426 nm and 422 in DMF and H_2O respectively, and the lower symmetry of non-metallated porphyrin causes the exitations to resolve into x and y symmetric orbitals and four Q bands are observed in the spectrum lebelled $Q_x(0,0)$, $Q_x(1,0)$, $Q_y(0,0)$, and $Q_y(1,0)$ are 646 nm, 590 nm, 554 nm and 516 nm for compound **MPyEt3** in DMF and 639 nm, 587 nm, 549 nm, 512 nm for compound **MPyEt3** in H_2O respectively in order of the increasing energy. The obtained molecular electronic spectra of the zinc synthesized complex **MPyEt3Zn** are typical of metalloporohyrins.[24,25] It consist of the Soret band, as a result of the a_{1u} (π) → e_g (π*) transition at 436 nm and 433 nm in DMF and H_2O respectively, and two Q bands corresponding to the a_{2u} (π) → e_g (π*) transition at 559 nm, 607 nm in DMF and 561 nm, 609 nm in H_2O.

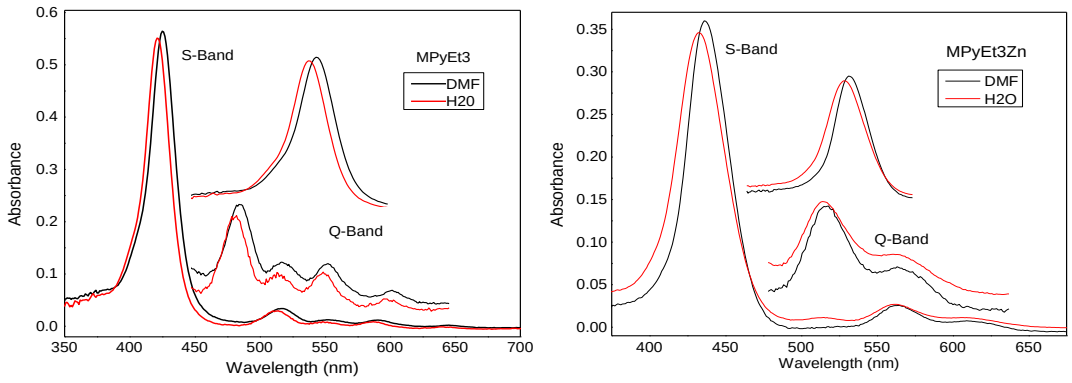


Fig. 1 Absorption spectra of water-soluble compounds **MPyEt3** and **MPyEt3Zn** in DMF and H_20 at 10µM solution.

2.3 Emission spectra: The emission spectra of the synthesized free-base compounds **MPyEt3** and its Zn (II) complex **MPyEt3Zn** is shown in **Fig. 2**. The spectral data was recorded at the excitation wavelength of their Soret band at 423 nm, and 428 nm for the free-base ligands and the zinc complexes, respectively using a 10 μM solution of the compounds in DMF and H_2O at different temperature in the range from 7°C to 97°C, with 10 °C increment. The results have been tabulated in **Table 1**. The fluorescence spectral data of free-base compound **MPyEt3** shows two bands located in the 660−721 nm both in DMF and H_2O. The Zn(II) complex **MPyEt3Zn** shows two bands located in the 617−666 nm both in DMF and H_2O which is blue shifted as compared to its free-base precursor. In case of solvent DMF, with increase in temperature intensity of the emission spectra decreases but in case of solvent H_2O the reverse result obtained.

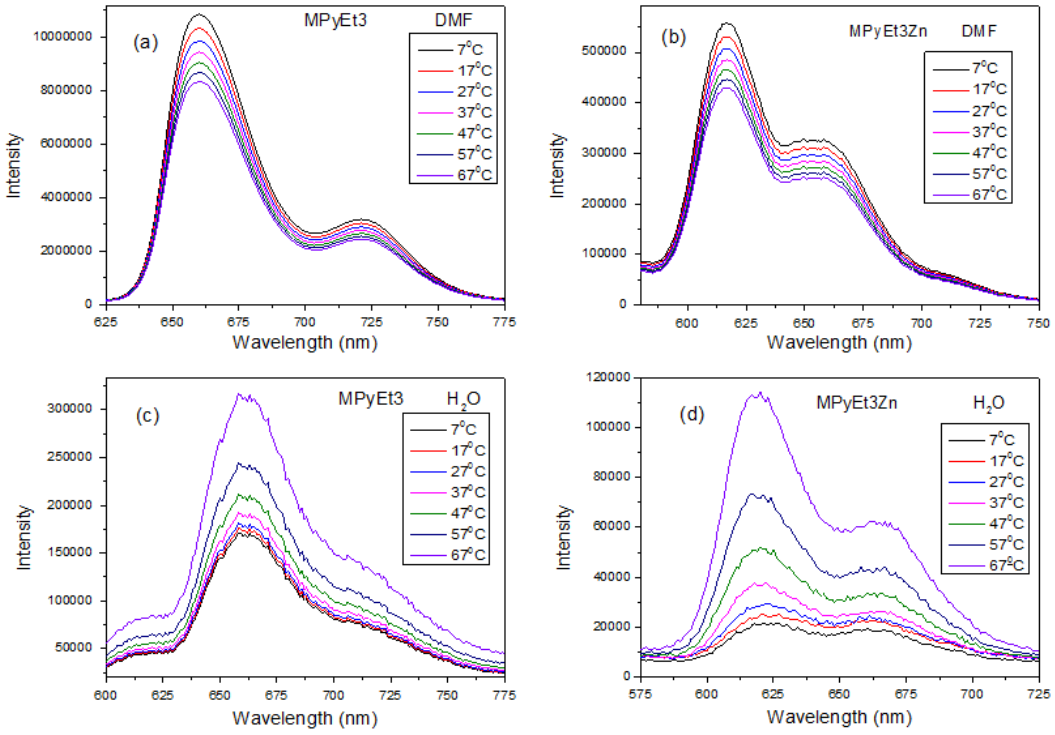


Fig.2 Emission spectra of **MPyEt3 (a)** in DMF at 10μM solution, (c) in H_2O at 10μM solution; Emission spectra of **MPyEt3 Zn (b)** in DMF at 10μM solution and (d) in H_2O at 10μM solution.

2.4 Determination of Fluorescence quenching efficiency in DMF and H_2O: From the temperature variation emission spectra of water-soluble compounds **MPyEt3**, and **MPyEt3Zn**. It was established that, in organic solvent DMF, the temperature rise is making destructive contribution to the fluorescence yield.[13] With the growth of temperature, the frequency and energy of molecular collision in solution, as well as the amplitude of internal molecular vibrations, increases, leading to an increase in nonradioactive relaxation of the excited levels, and thus fluorescence quenching efficiency increases as shown in **Fig. 3(a)**. In case of water, organic molecules tend to form associated dimer, trimer, and so forth, where fluorescence quantum yield is much lower than that in the individual molecules. At sufficient high concentration, the fluorescence spectrum is formed as a superposition of the spectra of individual molecules and their associates. Some associates divided into separate molecules with increasing temperature that is accompanied by a relative increase in fluorescence intensity. The fluorescence quenching efficiency of the compound **MPyEt3** and **MPyEt3Zn** decreases with increasing temperature in case of aqueous solvent H_2O shown in **Fig. 3(b).**

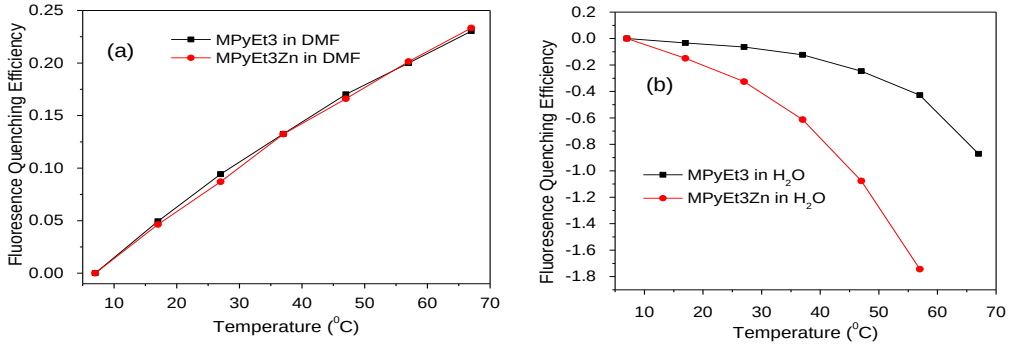


Fig.3 Fluorescence quenching efficiency of (a) **MPyEt3** and **MPyEt3Zn** in DMF at 10μM solution (b) **MPyEt3** and **MPyEt3Zn** in H_2O at 10μM solution

Table 1. Absorption and emission data of compounds **MPyEt3** and **MPyEt3Zn** at 10 μM solution.

Solvents	**Absorption**					**Emission**		**Stoke Shift**
	λmax (nm)					λmax (nm)		Q(0,0)-B(0,0)
	B(0,0)	Qy(1,0)	Qy(0,0)	Qx(1,0)	Qx(0,0)	Q(0,0)	Q(0,1)	B(0,0)/ Qx(0,0)
5,10,15-tris-(4-methylbenzoate)-20-mono-(4-pyridinum) porphyrin (MPyEt3)								
DMF	426	516	553	590	644	660	721	234
H2O	422	512	549	587	639	662	713	240
5,10,15-tris-(4-methylbenzoate)-20-mono-(4-pyridinum) Zinc porphyrin (MPyEt3Zn)								
DMF	436	--	563	610	--	617	657	183
H2O	433	--	561	609	--	620	666	187

2.5 Fluorescence lifetime and fluorescence quantum yields: The fluorescence lifetimes of free-base compounds **MPyEt3** and Zn (II) complexes **MPyEt3Zn** in DMF were measured with the time-correlated single photon counting technique, and the results are displayed in **Fig. 4**. The samples in DMF were excited at 425 nm (Soret band), and the fluorescence lifetimes were recorded at the maximum of the corresponding fluorescence Q(0,1) band. All the free-base fluorescence transients were fitted with a mono-exponential decay function for better χ^2 value and obtain lifetimes 10.86 ns of χ^2 value 1.071 for compound **MPyEt3**, 2.14 ns of χ^2 value 1.023 for Zn (II) compound **MPyEt3Zn**. For free-base compound **MPyEt3** the fluorescence quantum yield is ϕ_f = 0.091 and for Zn (II) compound **MPyEt3Zn** the fluorescence quantum yield is ϕ_f = 0.028.

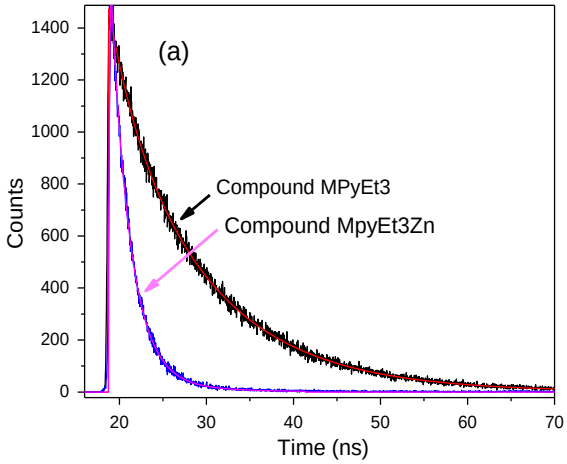


Fig. 4 Fluorescence decay profile of compounds **MPyEt3** and **MPyEt3Zn** in DMF at 10 μM solution

2.6 Cytotoxicity Studies of compound MPyEt3and MPyEt3Zn in AGS and SW480 Cell lines: Photocytotoxicity of porphyrin and metalloporphyrin derivatives have been studied extensively during the past years. The *in vitro* photodynamic activity of compound **MPyEt3** and **MPyEt3Zn** were evaluated against two different cell lines, namely, human gastric cancer cell line AGS and Human colon adenocarcinoma cell line SW480. The cytotoxicity of compound **MPyEt3** and **MPyEt3Zn** in these cell lines were investigated both in the presence and absence of light using MTT assay as shown in **Figure 5**. For the analysis a stock solution of **MPyEt3** and **MPyEt3Zn** were prepared in DMSO and diluted to appropriate concentrations with the culture medium. The cells, after being rinsed with phosphate buffered saline (PBS), were incubated with different concentration of **MPyEt3** and **MPyEt3Zn** along with AGS cells in Dulbecco's Modified Eagle Medium (DMEM) solution and separately with SW480 cells which were cultured in RPMI medium for 24 h at 37°C (with 5% CO_2) before being illuminated at an ambient light. Percentage of cell viability and growth inhibition were determined by means of the colorimetric assay called MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Approximately 1×10^4 cells were seeded in two 96 well cluster plate and allowed to reach the exponential phase of growth and then compound **MPyEt3** and **MPyEt3Zn** are added and analyzed for cytotoxicity. Cytotoxic studies revealed that compounds **MPyEt3** and **MPyEt3Zn** are essentially noncytotoxic in the absence of light but, under light irradiation (36W white light, 30 min), both exhibit high photocytotoxicity. With SW480 cell line, the free-base porphyrin **MPyEt3** and its Zn (II) complex **MPyEt3Zn** exhibited an IC_{50} value of 11.53 μM and 8.81 μM respectively, however with AGS cell line, the same compounds **MPyEt3** and **MPyEt3Zn exhibited** IC_{50} values of 15 μM and 10.41 μM respectively. Our

observations suggest that **MPyEt3Zn** shows better photocytotoxic effects towards adenocarcinomas over the other epithelial cancer cell lines studied.

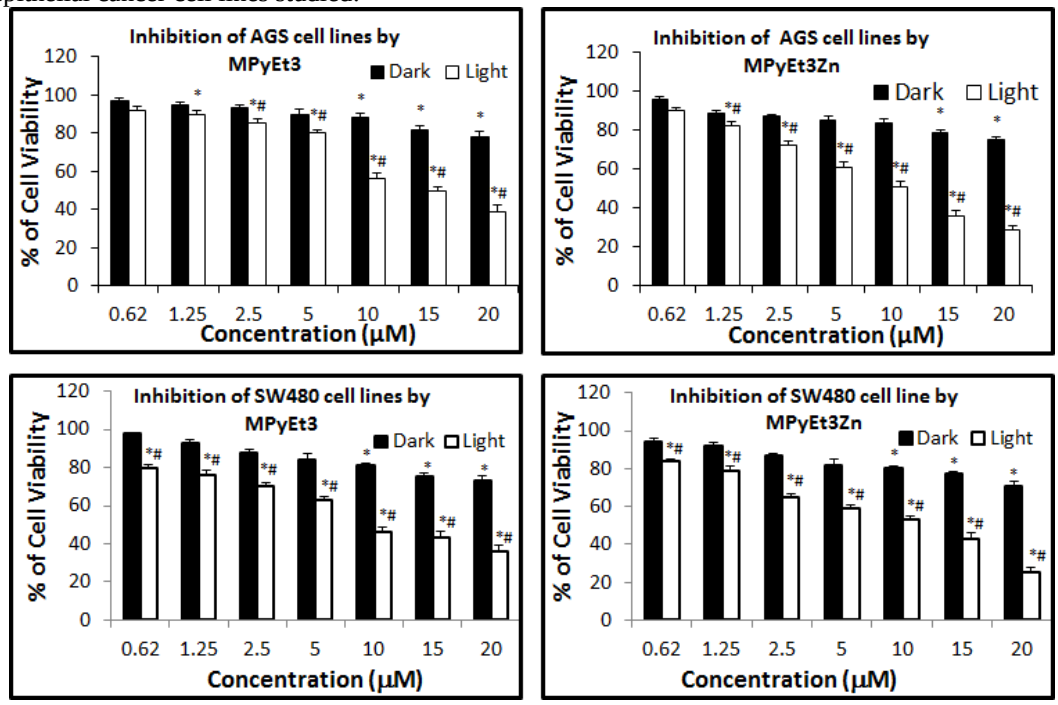


Fig.5 Inhibition of AGS and SW480 cell line by Compound **MPyEt3** and **MPyEt3Zn** at different concentrations in dark and under light, compared to control (cell line without treatment). There is a gradual decrease in cell viability with increase in concentration of the compound. However, in case of cell lines given treatment in dark, the viability of the cell line was found to be significantly decreased at the concentration of 10 μM and higher (denoted as *). When light was given, the viability was greatly reduced compared to the control the concentration of 1.25 μM and higher (denoted as *) as well as with the same dose of the compound but under dark at the concentration of 2.5 μM and higher (denoted as $^{\#}$). Data represent Mean ± SEM, n = 4, *$p \leq 0.05$ as compared to control of the respective groups (light/dark), $^{\#}p \leq 0.05$ as compared to treatment under dark for the same dose of the compound.

III. Conclusion

In conclusion, water-soluble cationic pyridinum derivatives of porphyrin **MPyEt3** and its Zn (II) complex **MPyEt3Zn** have been synthesized and characterized. The PDT applications of **MPyEt3** and **MPyEt3Zn** were investigated. Photophysical studies of the molecules have been conducted which shows promising singlet fluorescence lifetimes and quantum yields. *In vitro* analysis of **MPyEt3** and **MPyEt3Zn** with cancer cell lines (AGS and SW480) showed promising results. Upon illumination, both **MPyEt3** and **MPyEt3Zn** exhibited higher photocytotoxicity towards SW480 cell line as compared to AGS cell line. The photocytotoxic effects of the Zn(II) complex **MPyEt3Zn** on either of the cell lines-AGS or SW480 was higher than that of the free-base porphyrin **MPyEt3.**

IV. Experimental

4.1 Materials and Methods

4.1.1 Materials: Pyridine-4-carboxaldehyde, Methyl-4-formylbenzoate, propionic acid, dimethylformamide and methanol (Sigma Aldrich), zinc acetate, dicholomethane and hexane (Merck), were used without further purification. Pyrrole(Sigma Aldrich) was freshly distilled before use. Silica gel (70-230 mm mesh, & 100–200 mm mesh, Merck) were employed for column chromatography. For the microwave-assisted synthesis, a Uwave-1000 microwave reactor (Sineo Microwave Chemistry Technology Co. Ltd. Shanghai, China) was used.

4.1.2 Optical Spectroscopy: UV-Visible absorption spectra were measured in a PerkinElmer LAMBDA 750 UV/Vis/NIR spectrophotometer taking the sample in 1 cm well-stoppered quartz cuvette (λ_{max}in nm) at different concentration using DMF of spectroscopic grade as the solvent. Emission spectra were recorded on Fluoromax-4 spectroflouorometer (Horiba Scientific) at different concentration using DMF (spectroscopic grade, Sigma Aldrich) and H_2O (biology grade, Himedia) as the preferred solvent.

4.1.3 Fluorescence Lifetime Measurements:
Fluorescence life times were measured using a time-correlated-single-photon counting (TCSPC) spectrophotometer (Horiba JobinYovin) with full width at half maximum (FWHM) ca.300 ps, repetition rate 1 MHz and the resolution was 28 ps per channel. The excitation wavelengths have been chosen to be 425nm and emission wavelength chosen was the wavelength corresponding to the Q(0,0) band in the emission spectra from **Table 1** respectivelyand MCP-PMT as a detector. The emission from the samples was collected at a right angle to the direction of the excitation beam maintaining magic-angle polarization (54.7^o). The data have been fitted to mono-exponential functions after deconvolution of the instrument response function by an iterative reconvolution technique using IBH DAS 6.2 data analysis software in which reduced χ^2 serves as a parameter for goodness of fit.

4.1.4 Quantum yields calculations: The fluorescence quantum yields (ϕ_f) of compounds **MPyEt3** and **MPyEt3Zn** were estimated from the emission and absorption spectra by a comparative method using the following equation,

$$\phi_f = \{[F(sample)][A(standard)][\mu_S]^2/[F(standard)][A(sample)][\mu_{Std}]^2\}\phi_f\,(standard) \qquad (1)$$

where [*F*(sample)] and [*F*(standard)] are the integrated fluorescence intensities of the compounds (3 and 4) and the standard, [*A*(sample)] and [*A*(standard)] the absorbances of compounds (**MPyEt3** and **MPyEt3Zn**) and the standard at the excitation wavelength, μ_S is the refractive index of the solvent in which the sample is soluble, μ_{Std} is the refractive index of the solvent in which the reference is soluble and ϕ_f (standard) the quantum yield of the standard sample. Free-base tetraphenylporphyrin (H2TPP, ϕ_f = 0.11 in toluene) was used as the standard for free-base porphyrin **MPyEt3**, and Zinc(II) tetraphenylporphyrin (ZnTPP) was used as the standard (ϕ_f = 0.033 in toluene) for the Zn^{2+} derivative **MPyEt3Zn**.

4.1.5 Cell Lines and Culture Conditions: Human gastric cancer cell lines, EBV negative AGS was maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum in 5% CO_2humidified atmosphere at 37°C. Human colon cancer cell line, SW 480, was grown in RPMI 1640 (Gibco, Carlsbad, CA) and the same condition was also maintained for it.

4.1.5 ^{1}H-NMR and Mass Spectra: The ^{1}H nuclear magnetic resonance spectra were recorded either on Bruker (400 MHz) or Bruker Advance II (400 MHz) in $CDCl_3$ or DMSO-d_6 (chemical shift inδ) solution with TMS as internal standard and the mass spectra were recorded using instruments like micrOTOF-Q II or Agilent 6530 Accurate-Mass Q-TOF (version Q-TOF B.05.00, B5042.1).

4.2 Preparation

4.2.1 Methyl 4-[5-mono-(4-pyridinyl)-10,15,20-tris-porphyrinyl] benzoate ester (PyEt3): In a 500 mL round bottom flask equipped with a magnetic stirrer, propionic acid (180 mL) was added and heated for 10 min at 110^oC. Methyl-4-formylbenzoate (7.71 g, 0.046 mol, 3.2 eq), pyridine-4-carboxaldehyde (4.34 mL, 0.021 mol, 1.5 eq) and pyrrole (4 mL, 0.057 mol, 4 eq) were added. The reaction mixture was protected from light. After stirring at 150^oC for 90 min the excess propionic acid was removed completely on distillation. Neutralization of the crude product was achieved by using 0.5 M ammonia solution and the solution was filtered. The residue isolated was first washed with copious amounts of water followed by cold diethyl ether. The dark crude product was loaded in a silica gel column and eluted with 0.5% Et_3N in 2-5% methanol in dichloromethane as the eluent. The porphyrin fractions were concentrated and then separated on a second silica gel column with 1% methanol in dichloromethane to yield 1.5 g (12%) of the target compound **MPyEt3**. ^{1}H–NMR (400 MHz, $CDCl_3$, 300 K), δ-8.97 (d, *J* = 6.4 Hz, 2H), 8.84 (d, *J* = 4.8 Hz, 2H), 8.82 (s, 4H), 8.81 (d, *J* = 4.8 Hz, 2H), 8.14 (d, *J* = 5.6 Hz, 2H), 8.46 (d, *J* = 8.4 Hz, 6H), 8.30 (d, *J* = 8.4 Hz, 2Hl), 4.16 (s, 9H), –2.83 (s, 2H). HRMS (ESI) Calclutated for $C_{49}H_{35}N_5O_6$, $[M+H]^+$ 791.2096 (experimental), 791.2059 (calclulated).

4.2.2 Methyl 4-[5-mono-(4-pyridinyl)-10,15,20-tris-porphyrinyl] benzoate ester-21,23-Zn(II)-porphine (PyEt3Zn): In a 25 mL amber color round bottom flask, compound **PyEt3 (**250 mg, 0.316 mmol) was dissolved in 9 mL dichloromethane which was then followed by the addition of a 1mL saturated methanol solution of zinc acetate dehydrate. The reaction mixture was stirred at room temperature for 12 h under light protection. The Greenish purple colored product **PyEt3Zn** with yield 92% was obtained which was purified using water, diethyl ether and then vacuum dried. ^{1}H–NMR (400 MHz, $CDCl_3$, 300 K), δ-8.92(d, *J* = 6.4 Hz, 2H), 8.81 (d, *J* = 4.8 Hz, 2H), 8.78 (s, 4H), 8.80 (d, *J* = 4.8 Hz, 2H), 8.13 (d, *J* = 5.6 Hz, 2H), 8.42 (d, *J* = 8.4 Hz, 6H), 8.28 (d, *J* = 8.4 Hz, 2H), 4.15 (s, 9H). HRMS (ESI) Calclutated for $C_{49}H_{33}N_5O_6Zn$, $[M+H]^+$ 852.1803 (experimental), 852.1811 (calclulated).

4.2.3 Methyl 4-[5-mono-(4-pyridinium)-10,15,20-tris-porphyrinyl] benzoate ester (MPyEt3): In a 25 mL amber colour round bottom flask, compound **PyEt3** (150 mg, 0.189 mmol) was dissolved in 10 mL dimethylformamide which was then followed by the addition of 1 mL methyl iodide (excess). This was then

allowed to stir 12 h at 50°C followed by the extraction of the product by direct distillation method and the compound was purified by washing with diethyl ether, dichloromethane and $CHCl_3$ and dried the product purging argon gas. The violet colored product **MPyEt3** was obtained with yield 84%. ^{1}H-NMR (400 MHz, DMSO-d_6, 300 K), δ = 9.293 (d, *J* = 6.8 Hz, 2H), 9.24 (d, *J* = 4.4 Hz, 2H), 9.01 (s, 4H), 8.92 (d, *J* = 4 Hz; 2H), 8.01 (d, *J* = 6.8 Hz, 2H), 8.83 (d, *J* = 4.8 Hz, 4H), 8.32 (d, *J* = 8.0 Hz, 4H), 8.76 (d, *J* = 8 Hz, 2H), 8.24 (d, *J* = 8.0 Hz, 4H), 4.56 (s, 9H), 3.914 (s, 9H), –3.18 (s, 2H). HRMS (ESI) Calclutated for $C_{50}H_{38}N_5O_6^+$, (m/z) 805.2659 (experimental), 805.2676 (calclulated).

4.2.4 Methyl 4-[5-mono-(4-pyridinium)-10,15,20-tris-porphyrinyl] benzoate ester-21,23-Zn(II)-porphine (MPyEt3Zn): According to the procedure for synthesis of **MPyEt3**, compound **PyEt3Zn** (50 mg, 0.058 mmol) was used as starting compound. The greenish purple color product **MPyEt3Zn** was obtained with yield 82%. ^{1}H-NMR (400 MHz, DMSO-d_6, 300 K), δ = 9.39 (d, *J* = 5.6 Hz, 2H), 9.06 (s, 4H), 8.98 (d, *J* = 4.8 Hz, 2H), 8.92 (d, *J* = 4.8 Hz, 2H), 8.89 (d, *J* = 4.8 Hz, 2H), 8.43 (d, *J* = 8.4 Hz, 6H), 8.33 (d, *J* = 8.4 Hz, 6H), 4.06 (s, 9H), 4.70 (s, 9H). HRMS (ESI) Calclutated for $C_{50}H_{36}N_5O_6Zn^+$, (m/z) 866.2059 (experimental), 866.2016 (calclulated).

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