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Research Article

Synthesis of a New Zinc (II) Phthalocyanine from Vinamidinium Salt and the Investigation of its Aggregation Behaviour and Antimicrobial Potential

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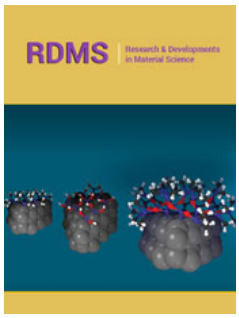

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Abstract

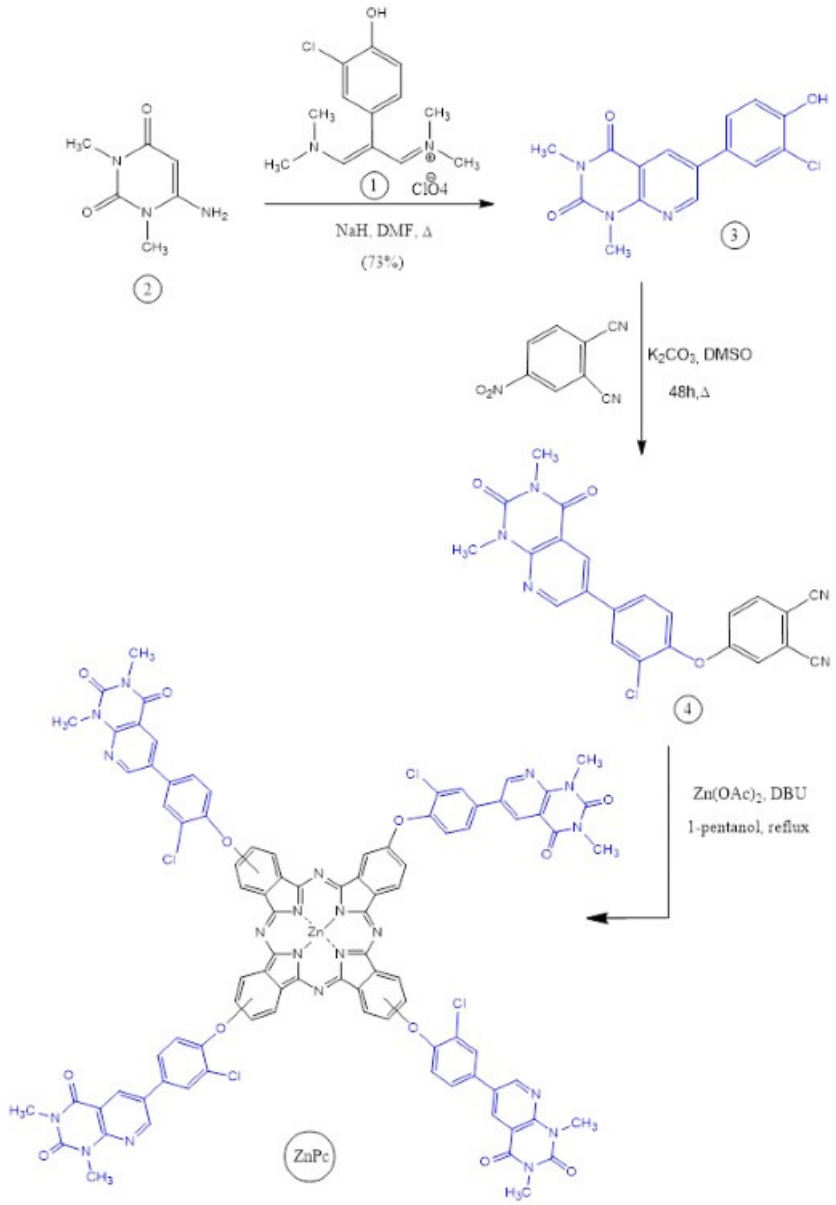
A novel and efficient synthesis of new zinc (II) phthalocyanine, starting from vinamidinium salt, was performed by a simple nucleophilic displacement reaction followed by a cyclotetramerization in presence of correspondent metal salt. The new component was purified and characterized via various spectroscopic methods including infrared IR, nuclear magnetic resonance 1H NMR, and UV-visible spectroscopy. Furthermore, the aggregation behaviour was also investigated in different organic solvents. Moreover, biological in vitro studies have shown that this component is effective versus different varieties of candida and bacteria.

Keywords: Zinc (II) phthalocyanine; Vinamidinium salt; Aggregation behaviour; Antimicrobial activity

Introduction

Tetrabenzo [5,10,15,20] tetraazaporphyrins, commonly known as phthalocyanines, are a two-dimensional 18 π-electrons aromatic synthetic macrocycles analogue to the naturally occurring porphyrins. Thanks to their unequalled physical and chemical properties, they have attracted numerous interest [1,2]. Other than being an excellent green-blue dye stuffs widely used as inks, colouring plastics, metal surfaces, and even some fabrics [3], nowadays they are exploited in various fields such as semiconductors [4], liquid crystals [5], solar photovoltaic cells [6], chemical sensors [7], Photodynamic Therapy (PDT) of certain type of tumours [8,9] and multiple pharmacological activity [10,11].

Herein we report an innovative synthetic approach for the preparation of a new "peripherally tetrasubstituted" "symmetric" zinc (II) phthalocyanine via an uracil derivative vinamidinium salt [12], well recognized in organic [13] and biological [14] chemistry for its use in wide-ranging sectors like antibacterial [15], antitumoral [16], vasodilator [17], cardiotonic [18] and hepatoprotective [19] activities. The methodology we adopted for the synthesis of the macromolecule is shown in Scheme 1. The resulting compound was characterized by FT-IR, 1H NMR, and UV-visible spectroscopy showing both aggregated and non-aggregated behaviour in different organic solvents.



Scheme 1: Synthesis of the novel ZnPc.

Then it was evaluated pharmacologically against various gram positive and gram-negative bacteria, and many species of candida (fungus).

Results and Discussion

Synthesis

the synthesis of new ZnPc, tetra(2-chloro-4-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidin-6-yl)phénoxy) phthalocyanine was carried out following similar procedure to the standard one described originally by Tomoda [20,21].

Starting from the synthesis of the vinamidinium salt, the 2-(3-chloro-4-hydroxyphényl)-1(dimethylamino)-3-(dimethyliminio) propene perchlorate (1) via a Vilsmeier-Haak reaction realized according to literature [22,23] to obtain a yellowish solid in a 68% yield. The two-component condensation of 6-amino-1,3-dimethyluracil (2) and the vinamidinium salt (1), led to the desired 6-(3-chloro-4-hydroxyphenyl)-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine (3) in a good yield (73%). After that, a nucleophilic substitution of the nitro group in 4-nitrophthalonitrile by the phenol function of the compound (3) in a polar aprotic dry solvent (DMSO), employing potassium carbonate as a basic catalyst, gave the wanted phthalonitrile derivative, the 4-(2-chloro-4-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidin-6-yl)phenoxy)phthalonitrile (4) (87% yield), which led to the tetra-(2-chloro-4-(1,3-diméthyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidin-6-yl) phenoxy))phthalocyanine (ZnPc) after a self-condensation at reflux temperature in a high-boiling solvent (pentanol), with few drops of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and anhydrous $Zn(OAc)_2$ under a nitrogen atmosphere. The green ZnPc was washed vigorously with acetonitrile and acetone to remove any possible traces of unreacted starting materials. The green pure product was

finally obtained in a 60% yield.

The synthesis route employed to obtain the new dinitrile compound (4) and the novel derivatized zinc (II) phthalocyanine complex (ZnPc) is given in Scheme 1.

Investigation of the aggregation Behaviour of the ZnPc

The UV-visible spectroscopy is the best technique to study and determine the structural properties and aggregation behaviour of phthalocyanines [24]. The UV-vis spectra of the new phthalocyanine revealed two typical strong absorption regions.

The first intensive feature observed, in the blue wavelength region around 360- 380nm, is the B-band, called also Soret peak [25], which originates from the transition from the deeper π levels to the LUMO transition.

The second feature, perceived at 600-700nm, is characteristic of the Q band assigned to the π-π* transition from the Highest Occupied Molecular Orbital (HOMO) to the Lowest Unoccupied Molecular Orbital (LUMO) of the Pc's ring [26,27].

The Zn(Pc) generated a typical UV-vis spectrum for non-aggregated phthalocyanines in DMF [28] (Figure 1), showing a sharp and intense Q-band in the red visible region located between 623 and 730nm. On the other hand, the B-bands around 360nm did not exhibit any unusual properties, and remain almost unchanged while increasing the concentration of the ZnPc. DMF being a coordinating solvent, it is able to bind axially to the Zn(Pc) macrocycles and thus reduce their aggregation tendency. The non-aggregated status in DMF as a solvent, is thought independent of the concentration.

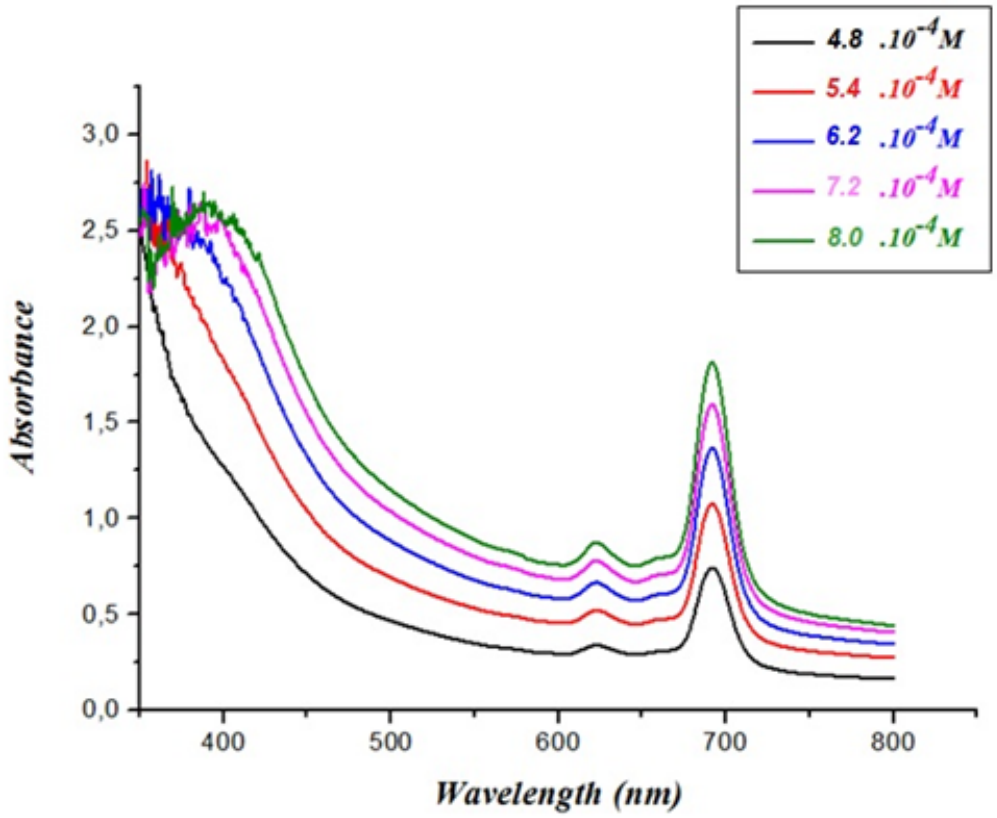


Figure 1: The non-aggregated behaviour of the ZnPc in DMF.

The Q-band absorption intensity increased proportionally to the rise of the concentration and still no apparition of any new bands due to aggregation species. The Beer-Lambert's law was obeyed for the ZnPc in the concentration ranging from 4×10^{-4}M to 8×10^{-4}M. The graph of the molar absorptivity versus the concentration of the phthalocyanine showed a constant value this range of concentration.

The new zinc phthalocyanine (ZnPc) is unfortunately soluble in few organic solvents only, like dimethylsulfoxide (DMSO), dimethylformamide (DMF), and partially soluble in tetrahydrofuran (THF), acetonitrile (MeCN) and dichloromethane (DCM). Only three of them were chosen to draw the spectral profile of the new phthalocyanine, namely the DMSO, DMF and THF because they ensure the best solubility of the product and thus allow to observe the Pc behaviour in different solvent parameters (solvatochromic parameters α, β and π*, dielectric constant ε and refractive index n). The UV-vis spectra of the new ZnPc in these organic solvents is presented in the next figure (Figure 2).

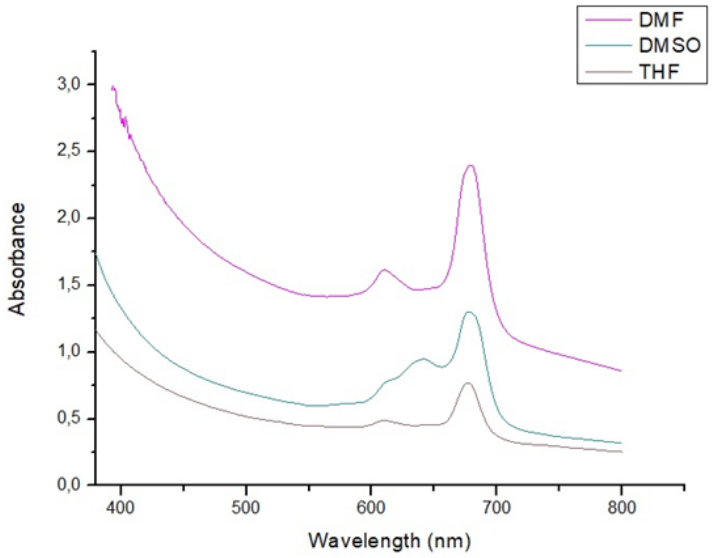


Figure 2: UV-vis spectra of the new ZnPc in different organic solvents (DMF, DMSO, THF).

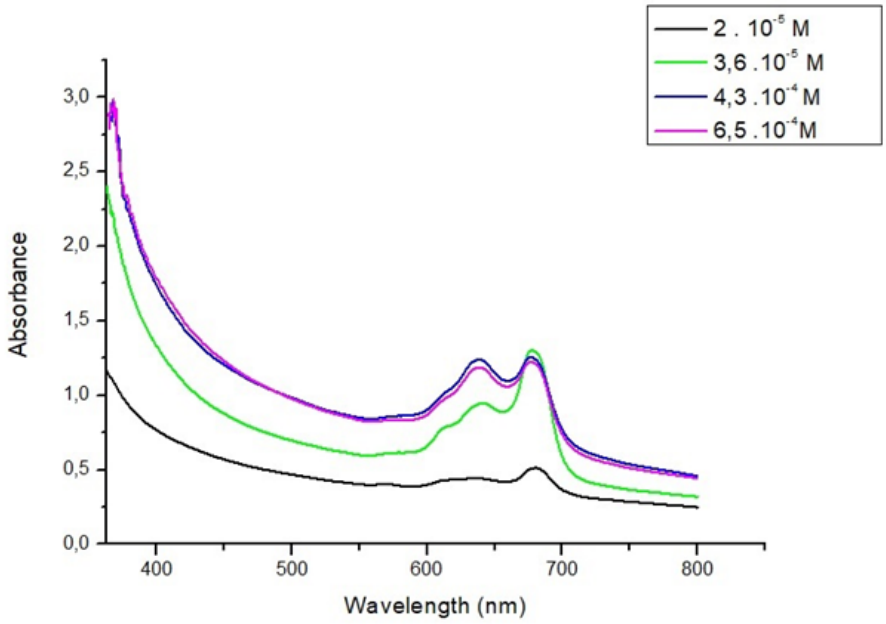


Figure 3: Aggregation behaviour of the ZnPc in DMSO.

Considerable changes in the absorption profile of the ZnPc are noticed due the variation of the polarity of the solvent. The ZnPc was found both aggregated and non-aggregated in the three chosen solvents. It obviously has high aggregation tendency in DMSO while monomeric species were dominant in both DMF and THF. The aggregation takes place due to the interaction between the 18 π electrons system and is usually described as a coplanar association of the Pc's rings progressing from the monomer form to dimer and higher-order complex forms [29]. This phenomenon depends of various factors, such as the concentration of the Pc, the nature of the solvent, the substituents of the Pc, the metal ion and even the temperature [30], and it can be detected with electronic spectroscopy [31]. Moreover, several types of aggregates can be displayed, mostly the H-aggregates and the J-Aggregates whether on solid support surfaces or in solutions. And these two types can be distinguished by the characteristic bands they generate. A broad blue shifted absorbance characterizes the H-aggregates, while a tight, red shifted absorbance band, relative to the monomeric dye, in the visible region distinguish the J-aggregate ones. Generally, aggregation causes a decrease of the Q-band intensity indicative for a monomeric species as shown in this case with the DMSO as solvent (Figure 3) and correspondingly, a new broader and blue shifted band around 635nm is observed. This shift to lower wavelengths is caused usually by H-type aggregates. The linear curve observed

while increasing concentrations proves that only one aggregated form is present, and it is probably a dimer. The Beer-Lambert's law was obeyed for the ZnPc in the concentration ranging from 2×10^{-4}M to 6.5×10^{-6}M.

Antibacterial activity

In order to study the efficiency of the synthetised Pc against microorganisms, we have chosen two types of bacteria: gram (+) *staphylococcus aureus* (ATCC 6538), and *Bacillus subtilis* (ATCC 6633) and Gram (-) Escherichia coli (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 9027) and assayed their antimicrobial potential using the disk-diffusion following the usual protocols reported in the literature [32].

We analysed this antibacterial activity in the presence and in the absence of irradiation with a 675nm red LED light [33] in order to compare the effect of the new phthalocyanine on bacteria in both cases. To perform this essay, a bacterial suspension with a cell density of 10^6 CFU mL^{-1} was prepared in a Brain Heart Infusion BHI Broth in the first place. After that, on a Mueller-Hinton (M.H) broth [34], previously sterilized discs were plated on the microbial mat than soaked with 10µl solution of the Pc solubilized in DMSO. After incubation for 24h at 37°, in the dark and with a 675nm red LED light, the inhibition zones were recorded in millimetre in both methods showing far different results.

Gentamicine (30µg/disk) was used as positive control to prove the non-resistance of the bacterial strains to antibiotics., whereas 15µL DMSO was used as a negative control to be certain that no bactericidal or bacteriostatic effects will occur due to the solvent.

A non-peripherally substituted Zinc II phthalocyanine ZnPcH was assayed simultaneously as a reference to judge the efficiency of the new substituted Zinc II phthalocyanine compared to a non-substituted one. All experiments were realized in duplicate.

Tests in the absence of irradiation were negative for most strains except for *E.coli*, which surprisingly showed increasing diameters of inhibition zones while diluting the main solution of C=1mg/ml concentration as it went along. Results are shown in the next table (Table 1).

Table 1: effect of the ZnPc on *E.coli* in the absence of irradiation.

	Inhibition zone (mm) *E.Coli* after 24h incubation in the dark
ZnPc4UC	0,6
ZnPc4U $C_{1/10}$	0,9
ZnPc4U $C_{1/100}$	1

Furthermore, in the presence of a 675nm red light, the ZnPc reacted positively to all bacteria without exception, proving the hoped-for antimicrobial potency of the ZnPc.

The results of ZnPc activity against bacterial strains in the presence of irradiation are shown in the following table (Table 2).

Table 2: Comparison of the effect of the ZnPcH and ZnPc on different bacterial strains.

	Inhibition Zone (mm)	
Phthalocyanine Bacteria	*ZnPcH*	*ZnPc*
Escherichia coli (E. coli) (gram -)	*9.8*	*13.1*
Pseudomonas aeruginosa (gram -)	*6.6*	*11.8*
Bacillus subtilis (gram +)	*9.7*	*12.9*
Staphylococcus aureus (gram +)	*7*	*11.3*

By examining these different results, we can conclude that the new zinc (II) phthalocyanine has a good inhibitory activity in the absence and the presence of irradiation. Thus, we can deduce that not only the ZnPc can play the role of a photosensitizer like most phthalocyanines, but it can also act as a bactericidal or bacteriostatic agent, only other more advanced studies can decide.

The outstanding antibacterial activity of the new substituted zinc phthalocyanine is visibly attributed to the substituents, as their unsubstituted counterpart ZnPcH has a much less significant activity. This provides irrefutable proof that this new zinc phthalocyanine does indeed act as a photosensitizer under the right conditions [35].

Antifungal activity

Nowadays, fungal infections are becoming more and more common. The candida type, especially candida albicans the most virulent candida yeast, causes the majority of systemic infections [36]. Unfortunately, these yeasts are considerably acquiring resistance to different antifungals, which poses serious problems to establish successful chemotherapy methods [37]. Alternative treatment strategies, including photodynamic therapy, are needed for controlling these new drug resistant pathogenic strains. In the present work, we evaluate the antifungal activities of the new zinc (II) phthalocyanine on *Candida albicans* and two non-albicans species *Candida albicans* and *Candida Krusei*. All fungus were obtained from the Pasteur Institute of Tunis collections. The Minimum Inhibitory Concentration (MIC) values of compounds were determined using the microplate assay [38]. Results revealed high inhibition values of the tested compound against fungal and argue for its use in large variety of therapeutic applications.

The antifungal activity of ZnPc 4U was tested against three strains of Candida: (*Candida albicans, Candida glabrata*, and *Candida krusei*) according to the Flat-bottomed microdilution plates method described by Santos and Hamdan [39]. Single colonies of Candida were grown the previous day of the screening assay incubated for 24 hours at 30 °C. Colonies were then scraped with an inoculation loop, diluted in YPD liquid and their optical densities were fixed. 100µl of the final volume are taken and added to a falcon tube containing already 10ml of YPD liquid.

The Minimum Inhibitory Concentration (MIC) values of the Zinc (II) phthalocyanine were determined by broth microdilution

method following the European Committee for Antimicrobial Susceptibility Testing (EUCAST) criteria [40]. The phthalocyanine were added to the first wells of the microplates and serial dilutions were performed to obtain a decreasing concentration range (10-100µM). Micro plates were incubated afterwards, covered overnight at 37 °C and the growth was monitored by optical density, measurement using a microplate reader at 600nm. The lowest concentrations, of the compound that inhibited the growth of Candida by 50% was determined later as MIC values [39].

Results shown in the next table (Table 3) reveal clear variation of the growth inhibition, depending mainly on the concentration of the phthalocyanine. The same tests were performed on a non-substituted zinc phthalocyanine ZnPcH in order to prove the role of substitution in increasing the antifungal potential of phthalocyanines. All assays were done in duplicate, and the values presented are the averages of the two determinations performed.

Table 3: Comparison of the effect of the ZnPcH and ZnPc on three strains of Candida (*Candida albicans*, *Candida glabrata*, and *Candida krusei*).

Strain	Concentration (µg/ml)	10	12,5	20	25	40	50	80	100
	Growth inhibition (%)								
Candida glabrata	ZnPcH	94,5	93	83,6	87,1	33,7	29,8	2,8	3,4
	ZnPc	91,7	90,3	83,6	83,5	21,7	0	0	0
Candida krusei	ZnPcH	100	100	72.8	83	17	21.8	6.1	5.8
	ZnPc	100	100	66.2	76	9.3	0	0	0
Candida albicans	ZnPcH	100	96.4	95.12	84.5	59.5	57.1	14.6	5.6
	ZnPc	91.8	90.8	83.8	83.08	23.08	0	0	0

The substituted zinc phthalocyanine ZnPc shows significantly higher antifungal activity than the non-substituted zinc phthalocyanine ZnPcH. Inhibitory effect of the tested substituted phthalocyanine ZnPc, at the concentration of 50µg/ml, on the growth of inhibition was by 100% against the three candida species. This effect was less important for concentrations ranged between 10 and 40g/l. In conclusion, The higher the concentration, the greater the antimicrobial activity. The MICs values of ZnPc and ZnPcH tested for Candida krusei, Candida glabrata and Candida albicans strains are summarized in the following table (Table 4).

Table 4: MICs values of ZnPc and ZnPcH tested for *Candida albicans*, *Candida glabrata*, and *Candida krusei*.

	MICs (µg/ml)	
	ZnPcH	ZnPc
Candida albicans	56.3	34.7
Candida glabrata	36,1	32,8
Candida krusei	35,5	32,2

Studies treating the antifungal potential of zinc phthalocyanines are few and far between, mostly using the PDT dynamic phototherapy method which involves the ability of phthalocyanines to act as photosensitizers. Mainly because the assessing of the Minimum Inhibitory Concentration (MIC) for pathogenic fungi remains a technically challenging test. Insufficient standardization of the various parameters that are applied and that can influence the determination of the MIC value, such as inoculum preparation, incubation time and temperature, is often the basis of the problems that arise [41,42].

Experimental

Dimethylsulfoxide (DMSO), N,N-dimethylformamide (DMF), tetrahydrofuran (THF), dichloromethane (DCM), chloroform ($CHCl_3$), methanol (MeOH), ethanol (EtOH), acetone, phosphoryl chloride ($POCl_3$), n-hexane, 1-pentanol ($C_5H_{11}OH$), 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU), sodium hydride (NaH), 6-amino-1,3-dimethyluracil, ammonium chloride (NH_4Cl), potassium carbonate (K_2CO_3) and zinc acetate ($Zn(OAc)_2$) were used as received from the manufacturer (Aldrich chemicals and Fisher Scientific). Yet most solvent were dried over 4Å molecular sieves before being used. 4-nitrophtalonitrile was synthesized in our laboratory starting with the phthalimide as reported in literature [43].

Every single reaction was carried out under dry nitrogen atmosphere. NMR spectral data were recorded on a Bruker 300MHz and FT-IR spectra on a Perkin-Elmer BX FT-IR system spectrometer by dispersing sample in KBr pellets. UV-Visible absorption spectra were obtained by a Cary 2300 spectrophotometer. Melting points were measured with an Electrothermal Digital Melting Point Apparatus.

2-(3-chloro-4-hydroxyphényl)-1-(dimethylamino)-3-(dimethyliminio)propene perchlorate (1)

This vinamidinium salt was prepared through Vilsmeier-Haak reaction according to literature [22,23] starting from the 3-chloro-4-hydroxyphenylacetic acid resulting in a yellowish solid in a 68% yield, mp 190 °C. ^{1}H NMR (300 MHz; DMSO-d6): δ, ppm - 2.51 (s, 6H), 3.23 (s, 6H), 6.98-7.08 (m, 2H), 7.29 (d, J = 3Hz, 1H), 7.66 (s, 2H), 10.57 (s, 1H); ^{13}C NMR (75MHz; DMSO-d6): δ, ppm - 43.9, 48.5, 103.8, 116.3, 119.6, 123.6, 131.8, 133.0, 153.4, 163.2.

6-(3-chloro-4-hydroxyphenyl)-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]-pyrimidine (3)

6-amino-1,3-dimethyluracil (1 mmol), vinamidinum salt (1 mmol), and sodium hydride (2.2 mmol) washed twice with hexane previously, were mixed in DMF and stirred at 95 °C under nitrogen

atmosphere for 16h. The crude product was precipitated by adding a saturated NH_4Cl solution to the mixture after being cooled to room temperature. Then purified by column chromatography on silica gel (hexane/EtOAc 8:2) to give a white solid in a 73% yield, mp 200 °C. ^{1}H NMR (300MHz; DMSO-d6): δ, ppm - 2.50 (s, 3H), 3.63 (s, 3H), 7.44-7.53 (m, 1H), 7.94 (d, J = 12Hz, 1H), 8.16-8.22 (m, 2H), 8.66 (s, 1H), 9.15 (s, 1H). ^{13}C NMR (75 MHz; DMSO-d6): δ, ppm - 28.0, 29.4, 109.6, 111.0, 117.5, 125.4, 126.8, 128.3, 129.7, 133.4, 150.3, 151.3, 151.5, 153.5, 153.6.

4-(2-chloro-4-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidin-6-yl)phenoxy) phtalonitrile (4)

4-nitrophthalonitrile (11 mmol) and compound (3) (12 mmol) were mixed in 10 ml of DMSO under nitrogen atmosphere and stirred at 95° for 24h. Dry potassium carbonate K_2CO_3 (1.89mmol) was added gradually during 2 hours from the beginning. After cooling the mixture, iced water was added and a white product precipitated, then collected by filtration and washed twice with acetone. the obtained product was purified by chromatography over a silica gel column using hexane and ethyl acetate (ratio 1:4) as eluents resulting in a white powder in an 87% yield. ^{1}H NMR (300 MHz; DMSO-d6): δ, ppm - 3,52 (s, 3H), 3,85 (s, 3H), 6.92 (d, J =3Hz, 1H), 7.03-7.07 (dd, J_1 = 9Hz, J_2 = 3Hz, 1H), 7.23-7.26 (dd, J_1 = 9Hz, J_2 = 3Hz ,1H), 7.46 (d, J = 6Hz, 1H), 7.56-7.64 (m, 3H), 7.98 (d, J = 9Hz, 1H). ^{13}C NMR (75 MHz; DMSO-d6): δ, ppm - 55.6, 56.0, 107.6, 108.0, 115.2, 115.8, 116.3, 121.7, 122.0, 123.2, 128.5, 129.1, 130.4, 132.8, 133.1, 136.0, 137.4, 140.6, 151.4, 153.1, 154.1, 160.8, 163.3. FT-IR: U_{max}, cm^{-1} 3041 (Ar-CH), 2231-2233 (CN), 1659 (C=O).

Tétra-(4-(2-chloro-4-(1,3-diméthyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]-pyrimidin-6-yl)phénoxy)) phthalocyanine (ZnPc)

A mixture of 4-(2-chloro-4-(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidin-6-yl)phenoxy)phtalonitrile (4) (0.50g, 1.12mmol), anhydrous

zinc acetate (0.102g, 0.56 mmol) and DBU (0.4mL, 2.68 mmol), in 2ml of dry 1-pentanol, was stirred and heated at reflux temperature under nitrogen atmosphere for 16h. After cooling the mixture in an ice bath, methanol and water were added dropwise into it. A green product precipitated, filtered and vigorously washed twice with acetonitrile and acetone to remove any possible traces of unreacted starting materials. The crude powder was finally dried under vacuum. The green-coloured final product is obtained in a 35% yield. ^{1}H NMR (300MHz; DMSO-d6): δ, ppm - 6.73- 8.01 (32H, Ar-H), 3.53- 3.91 (24H, CH_3). UV-vis (DMF): λ_{max}, nm (logε) 678 (2.39), 609 (1.30), 393 (2.79). FT-IR: U_{max}, cm^{-1} 2927 (Ar-CH), 1656 (C=O).

Conclusion

The present work describes the efficient synthesis and the characterization of a new zinc II phthalocyanine starting from a vinamidinium salt. The aggregated and non-aggregated behaviour were investigated, and the novel phthalocyanine showed dimeric behaviour for studied concentration ranges in DMSO unlike the monomeric form that happens to be dominant in other organic solvents. Furthermore, the antimicrobial activities were explored against various bacteria and fungus showing spectacular results.

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