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Synthesis, characterization and biomolecule-binding properties of novel tetra-platinum(II)-thiopyridylporphyrins

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Abstract

The new complexes tetra-platinum(II)-thiopyridylporphyrin **3** and tetra-platinum(II)-thiopyridylporphyrinato Zn(II) **4** were obtained by coordination of the peripheral thiopyridyl units of the free-base 5,10,15,20-tetrakis[2,3,5,6-tetrafluoro-4-(4-pyridylsulfanyl)phenyl]porphyrin **1** or their corresponding zinc complex **2**, respectively, with four chloro(2,2'-bipyridine)platinum(II) $[Pt(bpy)Cl]^+$ units. Both compounds were characterized by several spectroscopic techniques demonstrating a particular behaviour in the emission spectra due to the absence or presence of zinc. The tetra-platinum(II)-thiopyridylporphyrins exhibited an increase of the emission quantum yield when compared with the starting thiopyridylporphyrins **1** and **2**. Spectroscopic studies of both platinum derivatives reveal the ability to interact unequivocally with DNA from calf thymus and DNA of low molecular weight from salmon sperm, and also with the most abundant protein in human blood plasma – human serum albumin (HSA). Herein, both tetra-platinum(II)-thiopyridylporphyrins **3** and **4** exhibit electrostatic surface binding with the negative phosphate groups of DNA. Remarkably to cationic-anionic binding with DNA, tetra-platinum(II)-thiopyridylporphyrinato zinc(II) demonstrates a particular

binding intercalation mode with DNA. Photophysical studies demonstrated that both porphyrins are photostable and able to generate singlet oxygen ($^1O_2$) after light irradiation. Exposure of pMT123 plasmid DNA to tetra-platinum(II)-thiopyridylporphyrins and irradiated with light leads to single-strand break formation as determined by the conversion of the supercoiled form of the plasmid (form I) into the nicked circular form (form II). The tetra-platinum(II)-thiopyridylporphyrinato Zn(II) demonstrates a particular intercalation binding mode with DNA and high ability to cleavage DNA after photo-excitation.

Keywords: Photodynamic Therapy (PDT), Porphyrins, Platinum(II) complexes, Supramolecular Chemistry, Human serum albumin (HSA), Deoxyribonucleic acid (DNA).

Introduction

The biological activity of platinum(II) complexes was discovered by Rosenberg and co-workers[1] showing the ability of cisplatin to interact non-covalently with nucleic acids. Indeed, the study regarding the interaction of platinum(II) and related metal complexes[2-5] with biomolecules has emerged noticeably,[6-8] pursuing not only the discovery of new antitumoral drugs, but also the understanding of specific aspects of metal containing proteins and nucleic acids.[9] Nowadays, there are several organometallic compounds based on platinum units (*e.g.* cisplatin and its structural congeners), which have a key role in several cancer treatment protocols. While the clinical application of platinum(II) based compounds is widespread,[10] their efficacy has been hampered by mechanisms related with cellular resistance (such as decreased uptake, increased inactivation by thiol-containing proteins and increased DNA repair). Therefore, it is necessary to develop new targeting platinum(II) based drugs. One way to achieve this goal has been the incorporation of porphyrin macrocycles into platinum(II) based complexes.[9,10]

Porphyrin macrocycles are highly conjugated aromatic systems with unequivocal optical properties accomplishing an extensive multiplicity of functions in natural and synthetic structures.[11-14] They have been used as photosensitizers in cancer photodynamic therapy (PDT),[15-17,27] microbial photodynamic inactivation,[18-22] chemical sensors owing to their emission and electrochemical features,[23-26] gene therapy,[18,27-29] efficient components of light harvesting systems[30-32] and also as a new generation of

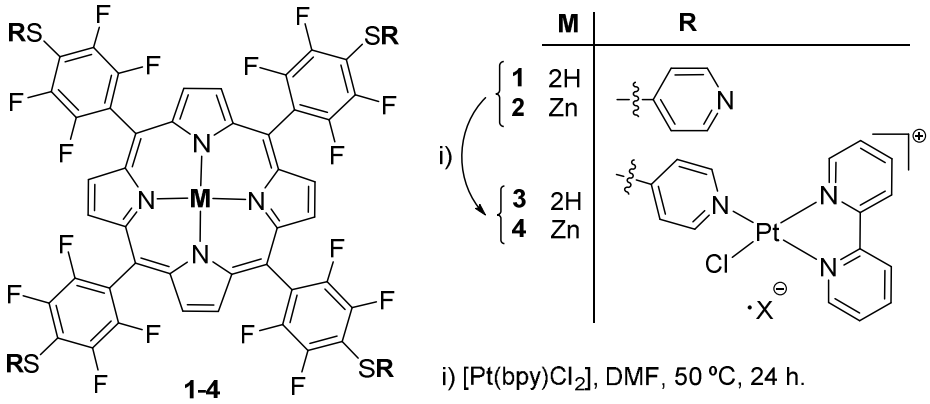
artificial enzymes in bio-mimetic chemistry.[33,34] Amongst porphyrin derivatives, cationic porphyrins are the most designed and studied compounds as DNA binding agents performing a class of versatile building blocks.[35-39] The negative charge of the backbone of nucleic acids promotes the interaction of DNA with cationic porphyrin derivatives. In the case of cationic porphyrins, there are several studies reporting the interaction of nucleic acids with tetrakis-(*N*-methylpyridinium-4-yl)porphyrin and its metal complexes.[35,40-45]

The binding ability of cationic porphyrins with DNA can be either intercalative, external, and in special circumstances with self-stacking, depending on the porphyrin charge distribution, absence/presence and type of the porphyrin central metal ion, and the peripheral substituent.[35,40,46] After photo-activation, porphyrins in the presence of molecular oxygen are able to generate reactive oxygen species (ROSs), such as singlet oxygen ($^1O_2$), thus inducing cell death.[47] DNA is a valuable target on the development of new compounds, since this biomolecule has important roles in ageing, tumours development and gene regulation.[9,48] The ability of porphyrins to interact with DNA and their capability to generate ROS has been related with DNA strand breaks and cell death mediated by cellular oxidative stress.[49]

Porphyrin-platinum(II) conjugates have been synthesized,[50-52] aiming to have on the same molecule platinum-containing groups[53] and porphyrin systems useful for cytostatic activity and photodynamic therapy promoted by the platinum and porphyrin parts, respectively.[9] Herein, we aimed to synthesize a new series of Por-Pt(II) complexes (**3** and **4**, Scheme 1) with potential ability to bind biomolecules, specially DNA. For that, the free-base 5,10,15,20-tetrakis[2,3,5,6-tetrafluoro-4-(4-pyridylsulfanyl)phenyl]porphyrin **1**[54] and the corresponding zinc complex **2** were functionalized with four $[Pt(bpy)Cl]^+$ moieties at the peripheral positions. The binding ability of porphyrin derivatives **1-4** with biomolecules, such as human serum albumin (HSA), DNA from salmon sperm (ssDNA) and DNA from calf-thymus (ctDNA) was studied by UV-Vis and emission spectroscopy. The spectroscopic properties of the resulting Pt(II)-porphyrinoid complexes **3** and **4**, and the interaction of porphyrin–DNA assemblies were analysed. By transient absorption spectroscopy, tetra-platinum(II)-thiopyridylporphyrin **3** has electrostatic surface binding with DNA, while tetra-platinum(II)-thiopyridylporphyrinato Zn(II) **4** has a particular intercalation binding mode with DNA. Additionally, we also demonstrate a high ability of porphyrin **4** to generate $^1O_2$ and to photocleavage pMT123 plamid DNA after light irradiation.

Synthesis and characterization of peripheral Pt(II)complexes

Considering the remarkable photochemical and photophysical properties of porphyrins and excellent binding features of $[Pt(bpy)Cl]^+$ moieties with DNA, we envisage a simple access to obtain innovative Por-Pt(II)bipyridine complexes. The new complexes were synthesized *via* post-modification of tetra-substituted thiopyridyl porphyrins **1**[54] and **2**, playing with the number of equivalents of $[Pt(bpy)Cl]^+$ units. The reaction was carried out in dry *N,N'*-dimethylformamide (DMF) with a little excess of *cis*-dichloro(2,2′-bipyridine)platinum(II) $[Pt(bpy)Cl_2]$ *per* pyridyl group at 50 ºC for 24 h and under nitrogen atmosphere (Scheme 1).



Scheme 1 Structures of porphyrin derivatives $H_2TPPF_{16}(SPy)_4$ **1**, $ZnTPPF_{16}(SPy)_4$ **2**, $H_2TPPF_{16}(SPyPt)_4$ **3** and $ZnTPPF_{16}(SPyPt)_4$ **4**.

The reaction progress was monitored by UV-Vis absorption spectroscopy, showing the absorption band of the $[Pt(bpy)Cl]^+$ moiety at 290-400 nm and the porphyrin absorption Soret- and Q-bands at 410-419 nm and 504-633 nm, respectively. At the end of the reactions, the reaction mixtures were concentrated under reduced pressure until around 1 mL of DMF and then precipitated with a saturated aqueous solution of NH_4PF_6. After this, each solid product was filtrated and washed several times with water, followed by crystallization in pure acetonitrile (CH_3CN). The obtained derivatives **3** and **4** exhibited dark-purple colour and yields above 85%. These structures were characterized and confirmed by UV-Vis and emission spectroscopy (Figures SI 1-3), ^1H, COSY 2D ^1H-^1H and ^{19}F NMR (Figures SI 5-12) and HRMS-ESI mass spectrometry (Figures SI 13).

Experimental Section

Materials

The reagents and solvents were of analytical grade. The *cis*-dichloro(2,2'-bipyridine)platinum(II) complex was synthesized according to the literature.[55] Human Serum Albumin (HSA), DNA from salmon sperm (ssDNA) and DNA from calf thymus (ctDNA) were purchased from Sigma-Aldrich.

Physical measurements

^{1}H and ^{19}F NMR spectra were recorded on a *Bruker Avance-300* spectrometer at 300.13 and 282.38 MHz, respectively or on a *Bruker Avance-500* at 500 MHz for ^{1}H NMR. DMSO-d_6 was used as solvent and TMS as internal reference. The chemical shifts are expressed in δ (ppm) and the coupling constants (J) in Hz. Unequivocal ^{1}H assignments were made with aid of 2D COSY (^{1}H -^{1}H).

Absorption and emission spectra were recorded using a *Shimadzu UV-2501-PC* and *FluoroMax3* (excitation wavelengths at 420 nm, slit 2 nm, emission range 600–800 nm), respectively. Analytical TLC was carried out on pre-coated silica gel sheets (Merck, 60, 0.2 mm). HRMS-ESI were recorded on a APEXQe FT-ICR mass spectrometer (Bruker Daltonics, Billerica, MA).

Porphyrin interaction with DNA and HSA were performed by spectral measurements at room temperature in phosphate buffered saline (PBS) at pH 7.4. The DNA pair base concentrations of low molecular weight DNA from salmon sperm (ssDNA) and DNA from calf thymus (ctDNA) were determined by spectroscopy, using the molar extinction coefficients 6600 and 13100 $M^{-1}.cm^{-1}$ (per base pair) at 260 nm, respectively. Porphyrin solutions (2.0 μM) in PBS were titrated with increasing concentrations of ssDNA or ctDNA (ranging from 0.0 to 8.0 μM). The absorption spectra of porphyrins were acquired for the wavelength range of 300-900 nm.

The intrinsic binding constants of compounds **1-4** were calculated according to the decay of the absorption Soret-band using the following equation,[56,57] through a plot of [DNA] / ($\varepsilon_a - \varepsilon_f$) *versus* [DNA],

$$[DNA] / (\varepsilon_a - \varepsilon_f) = [DNA] / (\varepsilon_b - \varepsilon_f) + 1 / K_b \, . \, (\varepsilon_b - \varepsilon_f),$$

where [DNA] is the concentration of DNA in the base pairs, ε_a the extinction coefficient (A_{obs}/[porphyrin]), ε_b and ε_f are the extinction coefficients of free and fully bound forms, respectively. In plots of [DNA] / ($\varepsilon_a - \varepsilon_f$) *versus* [DNA], K_b is given by the ratio of the slope to the interception.

The emission spectra of the interaction of the porphyrin derivatives with DNA were acquired in the wavelength range at 600-800 nm upon excitation at 420 nm.

For the determination of porphyrin derivatives interaction with human serum albumin (HSA, Sigma-Aldrich), 2.0 mL of HSA solution (2.0 μM in PBS) was titrated with increasing concentrations of porphyrin derivatives (ranging from 0.0 to 8.0 μM). The emission spectra of the HSA´s tryptophan residues were acquired in the wavelength range of 300-450 nm upon excitation at 280 nm.

Photostability and $^1O_2$ generation

The photostability of the porphyrin derivatives was determined by measuring the absorbance at 415 nm (**1** and **3**) or 425 nm (**2** and **4**) before and after white light irradiation (400-800 nm) at a fluence rate of 50 mW.cm^{-2}.

For the determination of singlet oxygen production, solutions containing DPBF (25 μM) with or without porphyrin derivatives at 0.25 μM were prepared in DMF/H_2O (9:1 v/v) in a 1 x 1 cm quartz cuvette. The solutions were irradiated, at room temperature and under gentle magnetic stirring, with a LED array system emitting red light (λ > 600 nm) at a fluence rate of 12 mW.cm^{-2}. The breakdown of DPBF was monitored by measuring the decrease in absorbance at 415 nm at pre-established irradiation intervals.

Photocleavage of circular plasmid DNA

Buffered solutions of pMT123 plasmid DNA (1 μg) with the porphyrin derivatives (0, 1, 10 or 40 μM) were incubated in the dark for 1 h at room temperature. The mixtures were then photoirradiated for 1 h with a LED array system emitting light with two emission peaks at λ = 450 ± 20 nm and λ = 550 ± 50 nm (white light).[27] Immediately after the treatments, sample analysis was carried out by electrophoresis on

1% (w/v) agarose gel (containing ethidium bromide stain) at 50 V for 90 min (Figure SI 4).

Results and Discussion

Compounds **1**-**4** were fully characterized by UV-Vis, NMR spectroscopy and ESI-HRMS and elemental analysis. The ^{1}H NMR of compound **3** shows the internal proton resonances at high fields (-3.11 ppm), while at low fields are located the other proton resonances corresponding to the peripheral of the porphyrin. In case of the thiopyridyl proton the resonances appears as multiplets at 8.07–8.12 and 8.92–9.01 ppm for the *ortho*-H and at 8.50–8.55 and 8.60–8.66 ppm for the *meta*-H. On the other hand, the multiplet resonances around 7.84, 8.41, 8.58, 9.49 and 9.61 ppm are attributed to the $[Pt(bpy)Cl]^{+}$ moieties. The ^{1}H NMR of compound **4** shows the disappearance of the resonances at high fields when metallated with zinc(II) ion, and the same profile was observed for the proton resonances of the thiopyridylporphyrin groups and $[Pt(bpy)Cl]^{+}$ moieties.

Electronic absorption and emission assays

The electronic absorption spectra of the porphyrin Pt(II) complexes in acetonitrile consist of an envelope of superimposed absorption bands in the range of 300-700 nm, arising from the characteristic absorption properties of porphyrin and $Pt(bpy)Cl^{+}$ entities (Figure 1). $H_2TPPF_{16}(SPyPt)_4$ **3** and $ZnTPPF_{16}(SPyPt)_4$ **4** exhibited absorption bands at 307 nm (log ε = 4.68) and 308 nm (log ε = 4.62), respectively. Additionally, both complexes exhibited absorption bands at the wavelength range of 360-390 nm (broad shoulder) which can be ascribed to the Pt(dπ) → bpy(pπ*), while the peaks at 320 nm (log ε = 4.71 for **3**) and 321 nm (log ε = 4.67 for **4**) are associated to the bpyπ → π* transitions.[58]

The characteristic Soret-band is observed at 410 nm (log ε = 5.32), and the Q-bands at 504 ($Q_{y(1-0)}$), 536 ($Q_{y(0-0)}$), 580 ($Q_{x(1-0)}$) and 633 nm ($Q_{x(0-0)}$) for **3**. The corresponding absorption bands for **4** are observed at 308 (log ε = 4.62), 321, 396, and 419 nm (Soret-band, log ε = 5.27), 552 and 625 nm (Q-bands), respectively (Table 1). In spite of their similarities, it should be noted that the Soret-band is quite narrow for the free-base porphyrin **3**, when compared with the one of Zn(II) complex **4** (shoulder at

429 nm). This observation is consistent with a stronger electronic coupling between the Zn(II) porphyrin and the platinum(II) complexes moieties.

In the case of $H_2TPPF_{16}(SPyPt)_4$ **3**, the absence of the metal ion in the porphyrin core could result in a lower electronic interactions between the macrocycle core and peripheral groups.

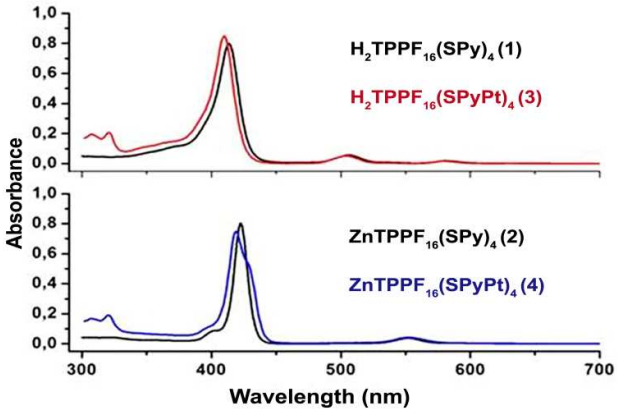


Fig. 1. Absorption spectra of porphyrin derivatives **1**-**4** in acetonitrile.

The spectroscopic experiments show the emission spectra of porphyrins **3** and **4** in CH_3CN (excitation wavelength at 420 nm) and the emission spectral data are given in Table 1. The spectra are shown in figure 2 and the emission quantum yield were estimated from the emission and absorption spectra of the reference 5,10,15,20-tetraphenylporphyrin (H_2TPP) by a comparative method..[21] The higher emission quantum yields of porphyrins **3** and **4**, comparing to their precursors **1** and **2**, respectively, must be related to their higher solubility in CH_3CN due to the bipyridin moieties and the PF_6^- contra ions.

The $Q_{(0,0)}$ and $Q_{(0,1)}$ emission bands of porphyrin derivatives are in the region of 500-700 nm, respectively. Compared with emission bands of H_2TPP (data not shown) in CH_3CN, the emission peaks of $H_2TPPF_{16}(SPyPt)_4$ **3** and $ZnTPPF_{16}(SPyPt)_4$ **4** shift to the red region by 2-5 nm showing transference of energy between the porphyrin ring and $[Pt(bpy)Cl]^+$ moieties. The insertion of the Zn(II) metal ion into the macrocycle resulted in a decrease of the emission intensity and quantum yield. The different values for the quantum yields may be a clue concerning the electronic coupling between $[Pt(bpy)Cl]^+$ unit and the porphyrin ring related with the presence or absence of the Zn(II) metal ion.

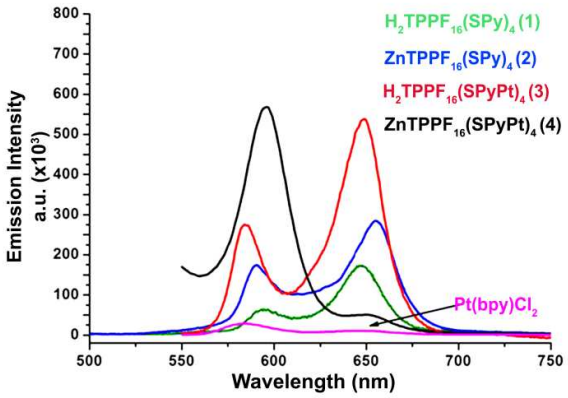


Fig. 2. Emission spectra of porphyrin derivatives **1-4** and [Pt(bpy)Cl_2] complex ($\lambda_{exc.}$= 420 nm) in acetonitrile.

Table 1. Electronic absorption and emission data of porphyrin derivatives **1-4** in acetonitrile.

Compound	Absorption bands / λ, nm (log ε)	Emission bands/λ, nm[a)]	Φ_f[b)]
1	415 (5.48); 507 (4.34); 581 (3.87) and 633 (3.59)	594, 647	0.060
2	422 (5.29); 552 (4.06) and 587 (2.59)	593, 647	0.030
3	307 (4.68); 320 (4.71); 360 (4.51); 410 (5.32); 504 (4.10); 536 (3.14); 580 (3.60) and 633 (2.59)	634,698	0.300
4	308 (4.62); 321 (4.67); 396 (4.42); 419 (5.27); 552 (4.03) and 625 (2.58)	646, 702	0.200

[a)]excited at 420 nm; [b)]using H_2TPP as reference in DMF (Φ_F = 0.11).[54]

Photostability and generation of singlet oxygen ($^1O_2$)

To evaluate the potentialities of porphyrin derivatives **3** and **4** to induce DNA strand breaks by a mechanism dependent on their ability to generate ROS, mainly $^1O_2$, their photostability and ability to generate $^1O_2$ upon photoexcitation were determined. The photostability of **3** and **4** was studied by monitoring the decrease of the absorbance of their Soret-bands, after different times of white light irradiation (400-800 nm) delivered by an illumination system at a fluence rate of 50 mW.cm^{-2}.[15,39] In PBS solutions both compounds at 1.5 μM showed high photostability over the investigated irradiation period (30 min; Table SI 1).

The ability to generate $^1O_2$ by **3** and **4** in DMF:H_2O (9:1 v/v) was determined by a chemical method using 1,3-diphenylisobenzofuran (DPBF) as $^1O_2$ scavenger.[15,39] Porphyrin derivatives **1** and **2** were used as references for **3** and **4**, respectively. Compounds **1-4** at 0.25 μM were able to photo-oxidize DPBF at 25 μM (Figure 3). The porphyrins **3** and **4** demonstrated to be potent generators of $^1O_2$ and at 0.25 μM these dyes decompose 46.8% and 72.0% of DPBF, respectively, after 20 min of light

irradiation. Both dyes have shown higher ability to photo-oxidize DPBF when compared to the corresponding references **1** and **2**. The ability of these derivatives to photo-oxidize DPBF decreases in the order $ZnTPPF_{16}(SPyPt)_4$ **4** > $ZnTPPF_{16}(SPy)_4$ **2** > $H_2TPPF_{16}(SPyPt)_4$ **3** > $H_2TPPF_{16}(SPy)_4$ **1**. The high photostability and ability to generate $^1O_2$ of **3** and **4** after being exposed to light and oxygen, allowed us to envisage them as potential PSs.

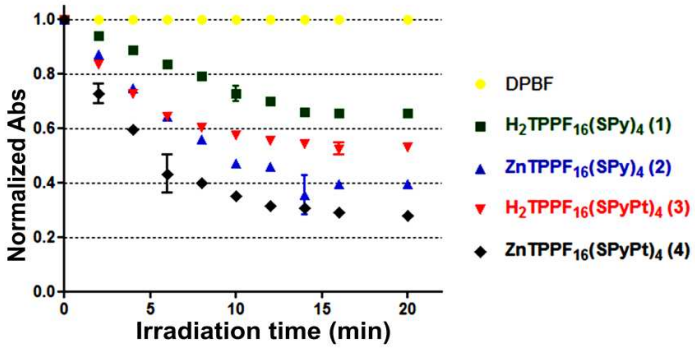


Fig. 3. Photo-oxidation of DPBF (25 μM) in DMF/H_2O (9:1, v/v) with or without porphyrin derivatives **1**-**4** at 0.25 μM, after red light irradiation (LEDs array system) at a potency of 12 $mW.cm^{-2}$. The DPBF absorbance was recorded at 415 nm.

Spectral properties of the interaction of the porphyrins with biomolecules

Interactions with DNA

The interaction of **3** and **4** with nucleic acids was studied by UV-Vis and emission spectroscopy, since the interaction of the platinum(II) porphyrins with DNA and ability to cleavage this biomolecule upon photo-activation, opens a high potential for their application as photosensitizers in cancer photodynamic therapy.[59] It is commonly accepted that platinum based drugs are able to promote cytotoxicity by targeting and inducing damages in DNA.[60] Additionally, the positive charged derivatives **3** and **4** should promote an electrostatic surface interaction with the negative charged phosphate groups on DNA.[37]

To compare the binding affinity of cationic porphyrins **3** and **4** with the corresponding non-cationic precursors, additional studies with the corresponding porphyrin precursors **1** and **2** were also performed. A series of DNA titrations were carried out using solutions of porphyrins at constant concentration (2.0 μM in aqueous buffered solution) with increasing concentrations of DNA of low molecular weight from salmon sperm (ssDNA) and DNA from calf thymus (ctDNA). As an example, figure 4

exhibits the overall changes in the absorption spectra of $ZnTPPF_{16}(SPyPt)_4$ **4** with both DNA. In the absence of DNA, compound **4** has a higher-energy of the absorption Soret-band around 400 nm. The other DNA titration experiments with compounds **1**-**3** are presented in supplementary information section (Figures SI 2).

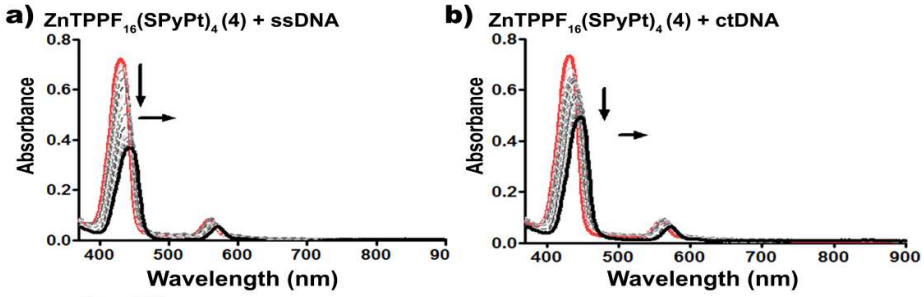


Fig. 4. Electronic UV-Vis absorption spectra of $ZnTPPF_{16}(SPyPt)_4$ **4** (2.0 μM) with increasing a) ssDNA and b) ctDNA concentrations ranging from 0.0 to 8.0 μM in PBS.

A general trend of hypochromism (the decrease in the absorbance of the Soret-band) for all the porphyrins after addition of ssDNA or ctDNA solutions was observed. The observed porphyrin hypochromicity on the UV-Vis spectra (Table 2) after addition of solutions with increasing concentrations of DNA decreases in the order $ZnTPPF_{16}(SPyPt)_4$ **4** > $H_2TPPF_{16}(SPyPt)_4$ **3** > $ZnTPPF_{16}(SPy)_4$ **2** > $H_2TPPF_{16}(SPy)_4$ **1**. Even though non-cationic compounds **1** and **2** lack the potential for cationic-anionic electrostatic binding with the DNA phosphate groups, these porphyrins demonstrated a general trend of hypochromicity of Soret-band absorption that was lower than for complexes **3** and **4**. In fact, previous studies have reported that non-cationic porphyrins have activity against carcinogenic DNA replication.[61] The obtained hypochromicity values for porphyrins **1** and **2** (around 20%) led us to suppose that these derivatives bind to DNA by non-classical modes involving most probably the partial insertion of pyridyl ring between adjacent base pairs on DNA. The partial interaction *via* pyridine ring into the base pairs of DNA has been described for hexa-aza macrocyclic copper(II) complexes.[62]

To further clarify the DNA-binding, the intrinsic binding constants of compounds **1**-**4** were previously calculated as described in experimental section and summarized in Table 2.

The intrinsic binding constants are comparable to that of *meso*-tetra-[(PtbpyCl)-pyridyl]porphyrins,[9] indicating that $H_2TPPF_{16}(SPyPt)_4$ **3** and $ZnTPPF_{16}(SPyPt)_4$ **4** can

bind more tightly to ctDNA and/or ssDNA, following the decreasing order of ctDNA(K_b): **4** > **2** > **3** > **1**; and ssDNA (K_b): **4** > **3** > **2** > **1**.

Table 2. Data for the interaction of compounds **1-4** with ssDNA, ctDNA and HSA. Binding constant (K_b) and number of binding sites (n) of porphyrins **1-4**.

	Parameter	1	3	2	4
ssDNA	Hypochromicity (H, %)[a]	19.0	28.0	23.0	49.0
	Red shift (Δλ, nm)[b]	0	0	0	15.0
	Quenching (Q, %)[c]	22.0	40.0	24.0	36.0
	K_b (M^{-1})[d]× 10^5	4.25 (± 0.06)	14.5 (± 0.17)	5.96 (± 0.03)	62.8 (± 0.03)
ctDNA	Hypochromicity (H, %)[a]	19.0	27.0	23.0	33.0
	Red shift (Δλ, nm)[b]	0	0	0	16.0
	Quenching (Q, %)[c]	36.0	44.0	39.0	40.0
	K_b (M^{-1})[d]×10^5	3.98 (± 0.08)	7.29 (± 0.48)	12.0 (± 0.63)	76.7 (± 0.08)
HSA	K_b (M^{-1})[e]	1.18×10^4	6.97×10^7	2.87×10^3	1.07×10^7
	n	0.88	1.40	0.67	1.27

[a]**H (%) = (Abs initial$_{Soret\text{-}band}$ - Abs final$_{Soret\text{-}band}$)/(Abs initial$_{Soret\text{-}band}$) × 100;** [b]**Δλ (nm) = $\lambda_{final\ Soret\text{-}band}$ - $\lambda_{initial\ Soret\text{-}band}$;** [c]**Q (%) = (Max. initial emission - Max. final emission) / (Max. initial emission) × 100;** [d]**Binding constant (K_b).**[54,55]

The hypochromicity effect for $H_2TPPF_{16}(SPyPt)_4$ **3** was 1.50 higher than the corresponding non-cationic precursor **1**. An even greater hypochromicity for $ZnTPPF_{16}(SPyPt)_4$ **4** was observed in parallel with a 15.0 or 16.0 nm red shift (bathochromic shift) in the absorbance of the Soret-band after addition of ssDNA or ctDNA, respectively (Figure 4). Bathochromic shifts have been described as an indicative of the intercalation to the double stranded oligonucleotides π-systems stacking (between the C and G nucleobases).[63] The isosbestic point observed in figure 4 suggests the formation of a well-defined porphyrin **4**-DNA complex stabilized by intercalation and also by cationic-anionic electrostatic interaction between porphyrin and the phosphate groups located on DNA[64] (Table 2).

The effect of the addition of ctDNA and ssDNA on the porphyrins **1-4** was also monitored by emission spectroscopy (Figures 5 and SI 3). Here, the porphyrin solutions were titrated with increasing concentrations of DNA, where the porphyrin derivatives emit between 600-800 nm after excitation at 420 nm, and the emission bands remain constant but the intensity decreases (emission quenching, Table 2). The observed emission quenching of the porphyrin derivatives after addition of DNA solutions (Figure 5) decreases in the order $ZnTPPF_{16}(SPyPt)_4$ **4** ≈ $H_2TPPF_{16}(SPyPt)_4$ **3** > $ZnTPPF_{16}(SPy)_4$ **2** ≈ $H_2TPPF_{16}(SPy)_4$ **1**.

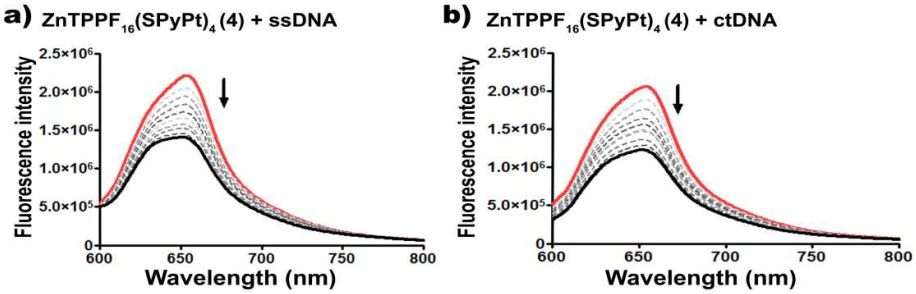


Fig. 5. Emission spectra of $ZnTPPF_{16}(SPyPt)_4$ **4** (2 μM) with increasing a) ssDNA and b) ctDNA concentrations ranging from 0.0 to 8.0 μM in PBS (λ_{exc} = 420 nm).

The largest hypochromicity (without any bathochromic shift) and the observed emission quenching for compound **3** after addition of DNA demonstrate that this compound has a non-intercalation mode with DNA. Therefore the major mode of interaction between porphyrin **3** and DNA seems to be a simple electrostatic surface binding between the positive charged porphyrin and the negative phosphate groups of DNA (Figure 6).

The bathochromic shift value resulting from the interaction between porphyrin **4** and DNA suggests that, in addition to electrostatic interaction, this porphyrin has probably intercalation with DNA (Figure 6). According to the observed differences between platinum(II) complexes **3** and **4** it is expected some interaction between the phosphate groups of DNA and the Zn(II) centre of compound **4**. The interaction involving phosphate groups on DNA and Zn(II) on complex **4** promotes somehow the insertion of porphyrin **4** into the DNA major and/or minor grooves. Previous studies have reported that the Zn(II) ion into the porphyrin macrocycle can promote interaction with DNA by electrostatic and/or coordination binding with phosphate groups of DNA.[4]

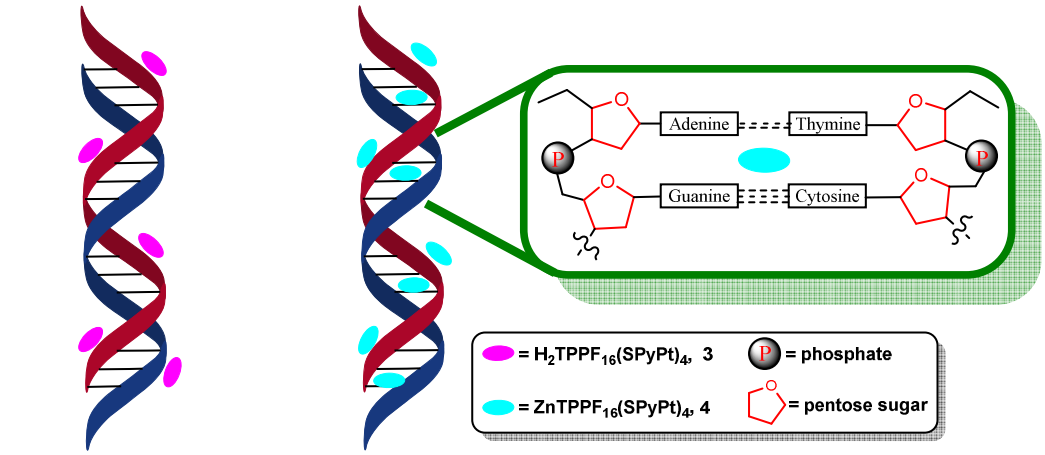


Fig. 6. Proposed possible binding modes of $H_2TPPF_{16}(SPyPt)_4$ **3** and $ZnTPPF_{16}(SPyPt)_4$ **4** with DNA.

Interaction with human serum albumin (HSA) by emission assays

Knowing that HSA is able to bind anticancer drugs and to deliver them to the target organs, the interaction of porphyrins **1-4** with the abundant plasma protein HSA was studied by emission quenching of tryptophan residues in HSA solutions, after increasing addition of derivatives **1-4** at concentrations from 0.0 to 8.0 μM in PBS with < 1.0% v/v DMSO.[65,66] HSA demonstrates a characteristic emission maximum band at 335 nm after excitation at 280 nm. The effects of DMSO on HSA emission quenching were tested, since the stock solutions of the porphyrins were prepared in this organic solvent. Over a concentration range of 0.0-1.0% v/v, DMSO did not quench HSA emission (data not shown). The addition of porphyrin to HSA led to emission quenching of tryptophan residues (Figure SI 1 and Table 2), which for porphyrins **1** and **2**, when compared respectively with porphyrin derivatives **3** and **4**, was lower. The binding constant (K_b) and the number of binding sites (n) of compounds **1-4** were determined as described in the literature (Figure SI 1).[66] The K_b values of the porphyrins decrease in the order $H_2TPPF_{16}(SPyPt)_4$ **3** > $ZnTPPF_{16}(SPyPt)_4$ **4** > $H_2TPPF_{16}(SPy)_4$ **1** > $ZnTPPF_{16}(SPy)_4$ **2** (Table 2). The number of binding sites indicates that there is only one binding site for the porphyrins close to the tryptophan residues of HSA (Table 2). The architecture of the porphyrin derivatives have a high flexibility comparatively with the porphyrins synthesized by Toma and co-workers[9] owing the presence of the thiopyridyl-$[Pt(bpy)Cl]^+$ moieties in the *para*-positions of the porphyrin, which promotes a high binding constant.

Photocleavage of plasmid DNA

The ability to generate $^1O_2$ of the porphyrin derivatives **3** and **4** (Figure 3) and to interact with DNA (Table 2) prompted us to test their ability to photodamage DNA. Herein, the DNA cleavage activities of porphyrins **1-4** were determined by their effectiveness in converting circular supercoiled DNA (form I) to circular relaxed DNA (form II). Porphyrin derivatives **1-4** (1-40 μM) and pMT123 plasmid DNA (1 μg) were incubated at room temperature for 1 h. After incubation of porphyrins with DNA, the samples were irradiated using a LED array system with white light for 1 h. As a first

approach, the effects of DMSO on DNA cleavage were tested, since the stock solutions of the porphyrin derivatives were prepared in this solvent. DMSO (up to 0.2%) did not have any effect on the DNA cleavage (Figure SI 4). No cleavage of DNA was observed, if compounds **1**-**4** were mixed with DNA without illumination (Figure SI 4). In the presence of light, porphyrin derivatives **1** and **2** were not able to cleave DNA (Figure SI 4). Otherwise, after photo-excitation, the porphyrin derivatives **3** and **4** at the concentration of 40 μM were able to convert supercoiled DNA plasmid (form I) into circular relaxed form (form II).

Comparing with porphyrin **3**, their corresponding zinc complex **4** had the strongest ability to cleave DNA. Therefore, the high ability of tetra-platinum(II)-thiopyridylporphyrinato Zn(II) **4** to cleave DNA can be related with its particular intercalation binding mode (Table 2) and high ability to generate $^1O_2$ (Figure 3).

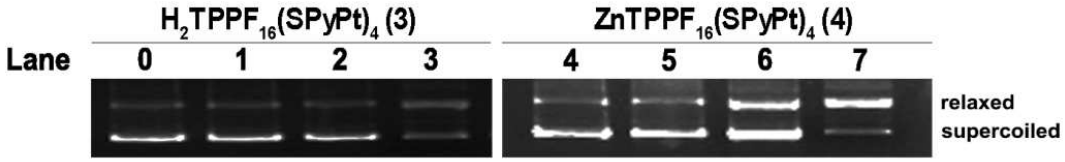


Fig. 7. Agarose gel electrophoresis (1%) of supercoiled pMT123 plasmid DNA (1 μg) photosensitized with porphyrin derivatives **3** and **4** at different concentrations (0, 1, 10 and 40 μM) in 3 mM Tris–HCl, 0.3 mM EDTA, pH 8.0. Lane 0 and 4: DNA+irradiation; lane 1: DNA+$H_2TPPF_{16}(SPyPt)_4$ **3** (1 μM)+irradiation; lane 2: DNA+$H_2TPPF_{16}(SPyPt)_4$ **3** (10 μM)+irradiation; lane 3: DNA+$H_2TPPF_{16}(SPyPt)_4$ **3** (40 μM)+irradiation; lane 5: DNA+$ZnTPPF_{16}(SPyPt)_4$ **4** (1 μM)+irradiation; lane 6: DNA+$ZnTPPF_{16}(SPyPt)_4$ **4** (10 μM)+irradiation; lane 7: DNA+$ZnTPPF_{16}(SPyPt)_4$ **4** (40 μM)+irradiation.

Conclusions

Two new platinum(II) porphyrin conjugates were synthesized, characterised and their spectroscopic properties revealed that these compounds have an interesting affinity behaviour with HSA, ctDNA and ssDNA, demonstrating at the same time distinct binding modes with these biomolecules. Herein, the tetra-platinum(II)-thiopyridylporphyrin **3** revealed the typical electrostatic surface binding with DNA. In addition to cationic-anionic binding with DNA, tetra-platinum(II)-thiopyridylporphyrinato Zn(II) **4** also demonstrated a particular intercalation binding mode, high ability to generate singlet oxygen and to cleavage DNA after photo-

433 excitation. Further studies are warranted to elucidate the possible interaction mechanism
434 of **4** with DNA as well as to study its ability to induce photodamages in cellular DNA.
435 A strong pattern of interaction between DNA and the porphyrin derivative could be a
436 potential methodology to treat cancer cells using PDT.
437
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449 **References**

1. B. Rosenberg, L. Vancamp, J. E. Trosko and V. H. Mansour, *Nature*, 1969, **222**, 385.
2. A. Koiti and H. E. Toma, *J. Photchem. Photobiol. A: Chem.*, 1994, **83**, 245.
3. D. M. Tomazela, F. C. Gozzo, I. Mayer, F. E. Engelmann, K. Araki, H. E. Toma and M. N. Eberlin, *J. Mass Spectrom*., 2004, **39**, 1161.
4. K. Araki, C. A. Silva, H. E. Toma, L. H. Catalani, M. H. G. Medeiros and P. D. Mascio, *J. Inorg. Biochem.*, 2000, **78**, 269.
5. H. E. Toma and K. Araki, Exploring the Supramolecular Coordination, Chemistry-Based Approach for Nanotechnology in Progress in Inorganic Chemistry, K. D. Karlin, Ed., John Wiley & Sons, Hoboken, 2009, vol. 56 (379–485).
6. F. Dufrasne and M. Galanski, *Curr. Pharm. Des.*, 2007, **13**, 1.
7. I. Kostova, Recent Patents on Anti-Cancer Drug Discovery, 2006, **1**, 1.
8. I. Manet, F. Manoli, M. P. Donzello, E. Viola, A. Masi, G. Andreano, G. Ricciardi, A. Rosa, L. Cellai, C. Ercolani and S.Monti, *Inorg. Chem*., 2013, **52**, 321.
9. J. A. Naue, S. H. Toma, J. A. Bonacin, K. Araki and H. E. Toma, *J. Inorg. Biochem.*, 2009, **103**, 182.
10. S. Y.-P. Ho, C. F. Au-Yeungand and K. K. W. To, *Med. Res. Rev*., 2003, **23**, 633.
11. J. A. S. Cavaleiro, J. P. C. Tomé and M. A. F. Faustino, Synthesis of Glycoporphyrins in Heterocycles from Carbohydrate Precursors, Springer, Berlin, 2007, vol. 7 (179–248).
12. K. M. Kadish, K. M. Smith and R. Guilard, Eds. The Porphyrin Handbook. Academic Press: New York, 2000, vol. 1–10.
13. R. Bonnett, Ed., Chemical Aspects of Photodynamic Therapy, Gordon and Breach Science Publishers, Amsterdam, 2000 (39–56).
14. L. M. O. Lourenço, J. P. C. Tomé, M. R. M. Domingues, P. Domingues, P. J. Costa, V. Felix, M. G. P. M. S. Neves and J. A. S. Cavaleiro, *Rapid Commun. Mass Spectrom*., 2009, **23**, 3478.
15. S. Silva, P. M. R. Pereira, P. Silva, F. A. A. Paz, M. A. F. Faustino, J. A. S. Cavaleiro and J. P. C. Tomé, *Chem. Commun.*, 2012, **48**, 3608.

17. E. Secret, M. Maynadier, A. Gallud, M. Gary-Bobo, A. Chaix, E. Belamie, P. Maillard, M. J. Sailor, M. Garcia, J.-O. Durandad and F. Cunin, *Chem. Commun*., 2013, **49**, 4202.
18. A. Almeida, Â. Cunha, M. A. F. Faustino, A. C. Tomé and M. G. P. M. S. Neves, Photodynamic Inactivation of Microbial Pathogens, Medical and Environmental Applications in Porphyrins as Antimicrobial Photosensitizing Agents, M. R. Hamblin and G., Jori, Eds., RSC Publishing: Cambridge, 2011 (83–160).
19. D. C. S. Costa, M. C. Gomes, M. A. F. Faustino, M. G. P. M. S. Neves, Â. Cunha, J. A. S. Cavaleiro, A. Almeida and J. P. C. Tomé, *Photochem. Photobiol. Sci.*, 2012, **11**, 1905.
20. E. Alves, M. A. F. Faustino, J. P. C. Tomé, M. G. P. M. S. Neves, A. C. Tomé, J. A. S. Cavaleiro, Â. Cunha, N. C. M. Gomes and A. Almeida, *Bioorganic & Med. Chem.*, 2013, **21**, 4311.
21. M. C. Gomes, S. Silva, M. A. F. Faustino, M. G. P. M. S. Neves, J. A. S. Cavaleiro, A. Almeida, Â. Cunha and J. P. C. Tomé, *Photochem. Photobiol. Sci.*, 2013, **12**, 262.
22. J. Almeida, J. P. C. Tomé, M. G. P. M. S. Neves, A. C. Tomé, J. A. S. Cavaleiro, Â. Cunha, L. Costa, M. A. F. Faustino and A. Almeida, *Photochem. Photobiol. Sic.*, 2014, **13**, 626.
23. J. M. M. Rodrigues, A. S. F. Farinha, P. V. Muteto, S. M. Woranovicz-Barreira, F. A. A. Paz, M. G. P. M. S. Neves, J. A. S. Cavaleiro, A. C. Tomé, M. T. S. R. Gomes, J. L. Sessler and J. P. C. Tomé, *Chem. Commun*., 2014, **50**, 1359.
24. N. M. M. Moura, C. Núñez, M. A. F. Faustino, J. A. S. Cavaleiro, M. G. P. M. S. Neves, J. L. Capelo and C. Lodeiro, *J. Mater. Chem. C*, 2014, **2**, 4772.
25. Nuno M. M. Moura, Cristina Nuñez, Sérgio M. Santos, Maria. A. F. Faustino, José A. S. Cavaleiro, Filipe A. A. Paz, Maria G. P. M. S. Neves, José. L. Capelo and Carlos Lodeiro, *Chem. Eur. J.*, 2014, **20**, 6684.
26. L. Lvova, P. Galloni, B. Floris, I. Lundström, R. Paolesse and C. D. Natale, *Sensors*, 2013, **13**, 5841.
27. P. M. R. Pereira, J. J. Carvalho, S. Silva, J. A. S. Cavaleiro, R. Schneider, R. Fernandes and J. P. C. Tomé, *Org. Biomol. Chem.*, 2014, **12**, 1804.

28. M. J. Shieh, C. L. Peng, P. J. Lou, C. H. Chiu, T. Y. Tsai, C. Y. Hsu, C. Y. Yeh and P. S. Lai, *J. Control. Release*, 2008, **7**, 200.
29. D. Ma, Y. Zhao, X.-Y. Zhou, Q.-M. Lin, Y. Zhang, J.-T. Lin and W. Xue, *Macromol. Biosci*., 2013, **13**, 1221.
30. M. V. Martínez-Díaz, G. de la Torre and T., Torres, *Chem. Commun*., 2010, **46**, 7090.
31. A. M. V. M. Pereira, A. Hausmann, J. P. C. Tomé, O. Trukhina, M. Urbani, M. G. P. M. S. Neves, J. A. S. Cavaleiro, D. M. Guldi and T. Torres, *Chem. Eur. J.*, 2012, **18**, 3210.
32. I. Natori, S. Natori, A. Kanasashi, K. Tsuchiya and K. Ogino, *J. Polym. Sci., Part B: Polym. Phys.*, 2013, **51**, 368.
33. H. Wei and E. Wang, *Chem. Soc. Rev*., 2013, **42**, 6060.
34. J. H. Kim, S. H. Lee, J. S. Lee, M. Lee and C. B. Park, *Chem. Commun*., 2011, **47**, 10227.
35. C. I. V. Ramos, M. G. Santana-Marques and J. P. C. Tomé, *J. Mass. Spectrom*., 2012, **47**, 173.
36. S. C. Karunakaran, D. Ramaiah, I. Schulz and B. Epe, *Photochem. Photobiol.*, 2013, **89**, 1100.
37. D. Kumar, B. A. Mishra, K. P. C. Shekar, A. Kumar, K. Akamatsu, R. Kurihara and T. Ito, *Org. Biomol. Chem*., 2013, **11**, 6675.
38. G. Sargsyan, A. A. Schatz, J. Kubelka and M. Balaz, *Chem. Commun*., 2013, **49**, 1020.
39. E.M. P. Silva, C. I. V. Ramos, P. M. R. Pereira, F. Giuntini, M. A. F. Faustino, J. P. C. Tomé, A. C. Tomé, A. M. S. Silva, M. G. Santana-Marques, M. G. P. M. S. Neves and J. A. S. Cavaleiro, *J. Porphyrins Phthalocyanines*, 2012, **16**, 101.
40. R. J. Fiel, *J. Biomol. Struct.Dyn*., 1989, **6**, 1259.
41. G. Raner, B. Ward and J. C. Dabrowiak, *J. Coord. Chem*., 1988, **19**, 17.
42. U. Sehlstedt, S. K. Kim, P. Carter, J. Goodisman, J. F. Vollano, B. Nordén and J. C. Dabrowiak, *Biochemistry*, 1994, **33**, 417.
43. P. Lugo-Ponce and D. R. McMillin, *Coord. Chem. Rev*., 2000, **208**, 169.
44. G. B. Rowland, K. Barnett, J. I. DuPont, G. Akurathi, V. H. Le and E. A. Lewis, *Bioorg. Med. Chem.*, 2013, **21**, 7515.

45. P. Zhao, J. Li, L.-J. Yang, J.-Z. Lu, H.-M. Guo, L.-M. Ma and B.-H. Ou, *J. Coord. Chem.*, 2013, **66**, 4220.
46. G. Mező, L. Herényi, J. Habdas, Z. Majer, B. Myśliwa-Kurdziel, K. Tóth and G. Csík, *Biophys. Chem.*, 2011, **155**, 36.
47. E. Alves, M. A. F. Faustino, J. P. C. Tomé, M. G. P. M. S. Neves, A. C. Tomé, J. A. S. Cavaleiro, Â. Cunha, N. C. M. Gomes and A. Almeida, *Bioorg. Med. Chem*., 2013, **21**, 4311.
48. H. Guo, X. Pan, R. Mao, X. Zhang, L. Wang, X. Lu, J. Chang, J.-T. Guo, S. Passic, F. C. Krebs, B. Wigdahl, T. K. Warren, C. J. Retterer, S. Bavari, X. Xu, A. Cuconati and T. M. Block, *Antimicrob. Agents Chemother.*, 2011, **55**, 478.
49. P. Duez, M. Hanocq and J. Dubois, *Carcinogenesis*, 2001, **22**, 771.
50. C. M. Drain and J.-M. Lehn, *J. Chem. Soc., Chem. Commun.*, 1994, 2313.
51. C. M. Drain, F. Nifiatis, A. Vasenko and J. D. Batteas, *Angew. Chem. Int. Ed*., 1998, **37**, 2344.
52. E. Iengo, B. Milani, E. Zangrando, S. Geremia and E. Alessio, *Angew. Chem. Int. Ed*., 2000, **39**, 1096.
53. Z. Xu and S. Swavey, *Inorg. Chem. Commun*., 2011, **14**, 882.
54. M. C. Gomes, S. M. Woranovicz-Barreira, M. A. F. Faustino, R. Fernandes, M. G. P. M. S. Neves, A. C. Tomé, N. C. M. Gomes, A. Almeida, J. A. S. Cavaleiro, Â. Cunha and J. P. C. Tomé, *Photochem. Photobiol. Sci*., 2011, **10**, 1735.
55. G. Morgan and F. H. Burstall, *J. Chem. Soc*., 1934, 965.
56. J. Kang, H. Wu, X. Lu, Y. Wang and L. Zhou, *Spectrochim. Acta, Part A*, 2005, **61**, 2041.
57. M. S. Ibrahim, I. S. Shehatta and A. A. Al-Nayeli, *J. Pharm. Biomed. Anal*., 2002, **28**, 217.
58. D. Collison, F. E. Mabbs, E. J. L. McInnes, K. J. Taylor, A. J. Welch and L. J. Yellowlees, *J. Chem. Soc. Dalton Trans*., 1996, 329.
59. H. Brunner and N. Gruber, *Inorg. Chim. Acta*, 2004, **357**, 4423.
60. S. Ahmad, *Chem. Biodivers.*, 2010, 7, 543.
61. E. Barragán, B. Gordillo, G. Vargas and L. Velazco, *Appl. Organometal. Chem*., 2004, **18**, 311.

62. J. Liu, T. Zhang, T. Lu, L. Qu, H. Zhou, Q. Zhang and L. Ji, *J. Inorg. Biochem.*, 2002, **91**, 269.
63. J. E. N. Dolatabadi, *Int. J. Biol. Macromol*., 2011, **48**, 227.
64. L. A. Lipscomb, F. X. Zhou, S. R. Presnell, R. J. Woo, M. E. Peek, R. R. Plaskon and L. D. Williams, *Biochemistry*, 1996, **35**, 2818.
65. S. Silva, P. M. R. Pereira, P. Silva, F. A. A. Paz, M. A. F. Faustino, J. A. S. Cavaleiro and J. P. C. Tomé, *Chem. Commun.*, 2012, **48**, 3608.
66. Y.-Z. Zhang, B. Zhou, X. P. Zhang, P. Huang, C. H. Li, Y. Liu, *J. Hazard Mater.*, 2009, **163**, 1345.

Synthesis, characterization and biomolecule-binding properties of novel tetra-platinum(II)-thiopyridylporphyrins

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