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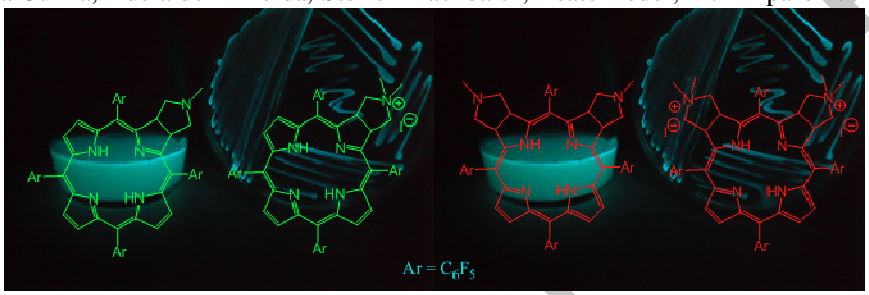

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Photodynamic inactivation of bioluminescent *Escherichia coli* by neutral and cationic pyrrolidine-fused chlorins and isobacteriochlorins

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ABSTRACT

Photodynamic inactivation of bioluminescent *E. coli* in the presence of cationic chlorin and isobacteriochlorin photosensitizers (PSs) obtained from 5,10,15,20-tetrakis(pentafluorophenyl)-porphyrin is described. The spectroscopic data for the neutral and cationic derivatives and their photophysical characterizations, especially fluorescence and singlet oxygen generation capacity are also reported. The results show that there is a direct relation between the inactivation efficiency and the increasing number of charges on the molecules. The combined effect of higher wavelength absorption and number of positive charges on the PS shows a 6.1 log reduction during the inactivation process. Overall this study shows that the cationic isobacteriochlorin has high potential to be used as PS for the inactivation of Gram (-) bacteria.

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Antimicrobial photodynamic inactivation (aPDI) represents a potential alternative methodology to inactivate microbial cells and has already shown to be effective *in vitro* against bacteria, fungi, viruses and protozoa.[1,2] The aPDI approach is based on the photodynamic therapy (PDT) concept that comprises the action of three components: a photosensitizer (PS), a light source and oxygen.[1,3] Porphyrins, chlorins, bacteriochlorins and isobacteriochlorins each have unique photophysical properties and can be used as PS.[4] Chlorins and isobacteriochlorins are distinct from the parent porphyrins due to the presence of reduced peripheral double bonds and this diminished symmetry leads to strong changes in the corresponding absorption spectrum.[5]

Bacteria have adopted a large variety of mechanisms to enhance their resistance to antibacterial drugs. These mechanisms include the thickening of their outer wall, encoding of new proteins which avoid the penetration of drugs, and onset of mutants deficient in those porin channels allowing the influx of externally added chemicals.[1] So the appearance of antibiotic resistance by pathogenic bacteria has led to the investigation of capable alternative methods with which mechanisms of resistance must not occur.[1,6,7] The aPDI represents a potential approach to inactivate pathogenic bacteria[3,8,9] and, as the main targets of this technique are cell wall structures and also membranes,[10] the PS does not need to enter the bacterial cell.[11] Therefore, the target bacteria have no possibility of developing resistance by stopping uptake, increasing export of the PS or raising metabolic detoxification.[1,3]

Our previous work using mono-, di-, tri- and tetra-cationic porphyrins for the inactivation of a Gram (+) bacterium (*Enterococcus faecalis*) and a Gram (-) bacterium (*Escherichia coli*) has shown that the number of positive charges, the charge distribution in the porphyrin molecule and the structure of the *meso*-substituents provoke different effects on the process of inactivation.[12] This study revealed that a porphyrin bearing three *meso*-(*N*-methylpyridinium) groups and a *meso*-lipophilic group (the pentafluorophenyl group); Tri-Py^{+}-Me-PF is an excellent PS for bacterial inactivation.[12] Subsequent studies using the same PS for the inactivation of microorganisms like *Vibrio fischeri* and recombinant *E. coli* and T4-like bacteriophages indicated no viability recovery or development of resistance mechanisms.[13,14] The possibility of using solar light in photodynamic inactivation against *V. fischeri* in aquaculture was also demonstrated by using the Tri-Py^{+}-Me-PF.[15]

So far, the development of cationic porphyrins for the inactivation of microorganisms has been mainly focused on the *meso* position.[16,17,18,19,20] Although this has led to highly efficient molecules for inactivation, the effect of greater absorption of incident light using chlorins or isobacteriochlorins and the number of charges correlation had been less addressed. A pyrrolidine fused chlorin PS with 5 positive charges was recently developed and tested against two antibiotic resistant bacterial strains, *Staphylococcus aureus* and *Pseudomonas aeruginosa* which showed a 7.0 log reduction after a few minutes of irradiation with red light.[21] Also, studies using cationic synthetic bacteriochlorins with 2, 4 and 6 charges have been reported against *S. aureus*, *E. coli*, and a fungal yeast *Candida albicans*.[22] The bacteriochlorin with 6 positive charge was able to photoinactivate *S. aureus* and *E. coli* more than 6 log at a low concentration (1.0 μM) after irradiation with 10 J cm^{-2} of 732 nm laser light.[22]

The development of new and better PSs with high absorption coefficients in the red region of the visible spectrum for the photodynamic inactivation of bacteria is in fact a challenging area.[9,23]

Herein we report the results obtained in the photodynamic inactivation of bioluminescent *E. coli*, a Gram (-) bacterium, in the presence of neutral and cationic pyrrolidine fused chlorins and isobacteriochlorins (**1-4**; Fig. 1) obtained from 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin (TPFPP) by 1,3-dipolar cycloadditions and cationization.[5]

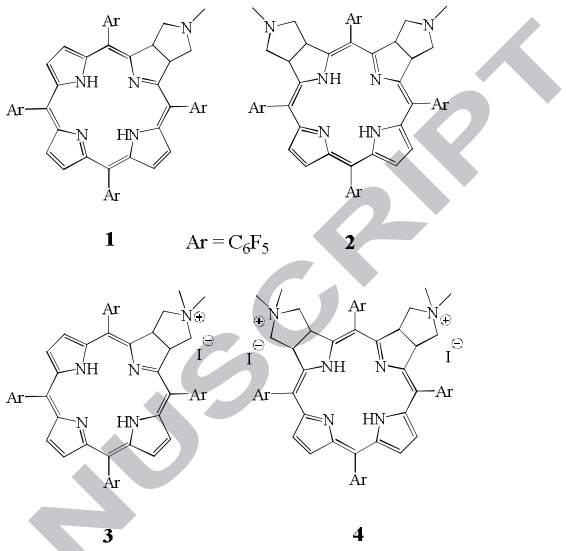

Figure 1- Structures of chlorin and isobacteriochlorin derivatives used in this study

The synthesis of the starting porphyrin, TPFPP, was carried out by condensation of pentafluorobenzaldehyde with pyrrole in a refluxing mixture of acetic acid and nitrobenzene.[24] The reaction of TPFPP with paraformaldehyde and *N*-methylglycine under nitrogen atmosphere for 12 h followed by column chromatography and preparative thin layer chromatography afforded pure chlorin **1**[25] and isobacteriochlorin **2**[26] (Figure 1). Both compounds were analysed by UV-vis, ^{1}H NMR, HRMS and compared with literature data.[5] Cationization of both derivatives **1** and **2** to afford **3**[27] and **4**[28] was carried out using an excess of methyl iodide and toluene as solvent at 40 °C for 24 h. The cationic derivatives were isolated by precipitation using diethyl ether (thoroughly washed with diethyl ether to remove unreacted methyl iodide). The filtered solids were dissolved in a mixture of chloroform/methanol and after solvent evaporation; the final crystallizations were carried out in chloroform/hexane mixtures.

The fluorescence and singlet oxygen quantum yields of neutral and cationic chlorins and isobacteriochlorins obtained in dimethylformamide (DMF) are listed in Table 1. The methods for steady state absorption and fluorescence, as well as time resolved singlet oxygen luminescence detection are described.[29,30,31] Tetraphenylporphyrin (TPP) was used as a standard. The UV-vis spectra of the neutral and cationic chlorin **1** and **3** show the Soret band maximum at ~ 404 nm and the characteristic Q band at 650 nm, while the isobacteriochlorins **2** and **4** show the Soret band maximum between 382-408 nm and the characteristic Q band at 588 nm (Fig. 2). The fluorescence spectra (λ_{exc} = 532 nm) have two maxima: in case of chlorins **1** at 660 and 725 nm and **3** at 650 nm and 725 nm, while the isobacteriochlorins **2** and **4** show bands at 600, 650 nm and 610, 660 nm, respectively (Fig. 3).

It is known that the optical properties of tissues (absorption and scattering of light) are wavelength dependent, in particular in the blue region, and the chromophores present in tissues show

absorption at wavelengths shorter than 600 nm. On account of these reasons, it is important to have PSs which are able to absorb in the so-called "optical window" where the effective tissue penetration of light is maximized (Fig. 2). The fact that the emissive properties of PS, considering the emission range (600-725 nm), could be used in diagnostic imaging should also be pointed out. It is a known fact that fluorine atoms in aromatic rings lead to an increase of fluorescence quantum yields.[32] It is evident from the fluorescence spectra (Fig. 3 and Table 1) that the quantum yield of the cationic PSs (**3** and **4**) is slightly lower when compared to the neutral PSs (**1** and **2**). However, the results indicate that most of the excited molecules in singlet state cross over to the triplet state by inter system crossing (ISC), resulting in higher singlet oxygen yields (Table 1).

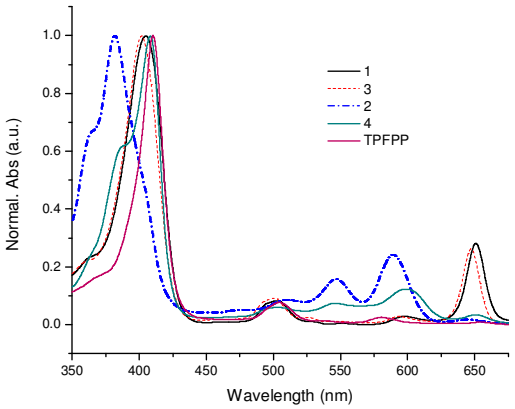

Figure 2- Normalized absorption spectra of compounds **1-4** and starting porphyrin TPFPP in DMF

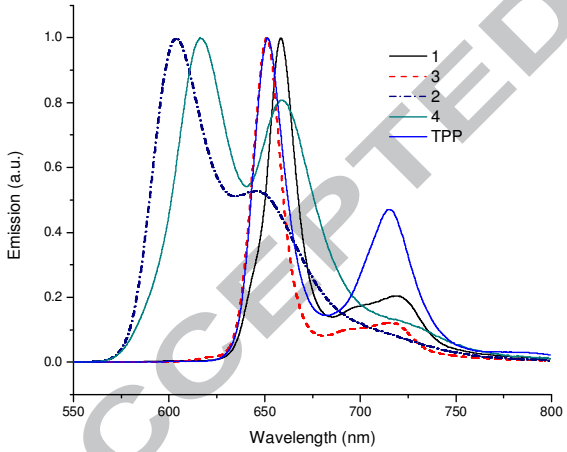

Figure 3- Normalized fluorescence spectra of compounds **1-4** and the standard TPP in DMF

Table 1-Photophysical properties of compounds**1-4** in DMF

Compound	Fluorescence quantum yield[#] (Φ_{fl})±0.05	Singlet oxygen quantum yield[§] (Φ_{Δ})±0.05
1	0.16	0.41
2	0.10	0.49
3	0.13	0.71
4	0.08	0.62
TPP	0.11	0.65

Fluorescence and singlet quantum yields were calculated by comparison with TPP as standard by excitation at λ = 532 nm; [#]OD for all samples at 532 nm = 0.02. [§] OD for all samples at 532 nm = 0.2

The photodynamic inactivation potential of compounds **1-4** was investigated using the bacterial bioluminescent method to monitor in real time the bacterial activity of the recombinant bioluminescent *E. coli*. The Gram (-) *E. coli* was stored at 4 ºC in triptic soy agar (TSA, Merck) supplemented with 50 mg mL^{-1} of ampicillin (Amp) and 34 mg mL^{-1} of chloramphenicol (Cm). Before each assay, the strain was grown for 20 hours at 25 ºC in 30 mL of triptic soy broth (TSB, Merck) supplemented with Amp and Cm. An aliquot of this culture (100 µL) was aseptically transferred to 30 mL of fresh TSB medium with Amp and Cm and grown overnight at 25 ºC to reach an optical density (O.D.600) of ~ 1.3, corresponding to ~10^8 cells mL^{-1}.[33]

Bacterial suspensions were prepared from the overnight bacterial culture (~10^8 cells mL^{-1}) by ten-fold dilution in phosphate buffered saline (PBS), pH 7.4, to a concentration of ~ 10^7 CFU mL^{-1} which corresponds approximately to 10^5 RLU mL^{-1}.[33] Bacterial suspensions were aseptically distributed in 100 mL acid-washed, sterilised glass beakers and the PS was added from the stock solution (500 µM in DMSO) to achieve final concentrations of: 5.0 µM and 20 µM of chlorin **1** and isobacteriochlorin **2**, respectively, and 5.0 µM and 20 µM of the corresponding cationic derivatives **3** and **4** (total volume was 10 mL per beaker). The samples were protected from light with aluminium foil and incubated for 10 min in the dark under 100 rpm stirring (23-26 °C) to promote the PS binding to *E. coli* cells. Then, the mixtures were exposed to white light for 180 min (total light dose of 42.3 J cm^{-2}). Sample aliquots were collected at time 0 and after 30 (7.2), 60 (14.4), 90 (21.6), 120 (28.8), 150 (36) and 180 min (42.3 J cm^{-2}) of light exposure (total light dose) and the bioluminescence signal was measured in the luminometer (TD-20/20 Luminometer, Turner Designs, Inc., USA). Light and dark controls were carried out simultaneously. In the light control no PS was added to the bacterial suspension, but the beaker was exposed to the same irradiation protocol. In the dark control, the PS at the highest concentration used (5.0 µM for **1** or 20 µM for **2**-**4**) was added to the beaker containing the bacterial suspension and it was covered with aluminium foil. All experiments were performed in triplicate and the results are averaged.

Bacterial inactivation was estimated by exposing bioluminescent *E. coli* in laboratory conditions to white light (PAR radiation, 13 lamps OSRAM 21 of 18 W each one, 380–700 nm) with an irradiance of 4.0 mW cm^{-2} (measured with a radiometer LI-COR Model LI-250). As the recombinant bioluminescent *E. coli* emits light at temperatures below 30 °C,[34] the beakers were placed on a tray with a water bath in order to maintain the samples at a constant temperature (23-26 °C).

The inactivation kinetics of bioluminescent *E. coli* by derivatives **1-4** show that all PSs tested did not exhibit dark toxicity for the bacterial strain (dark control) at the highest concentration used (Fig. 4 and 5). In addition, direct exposure of this bacterial strain to light, in the absence of PSs (light control), also did not cause any cytotoxic effect. In these controls ~5 log RLU mL^{-1} were maintained during all experiments. This means that any decrease obtained in cell viability after photodynamic inactivation of the treated samples was due to the photosensitizing effect of the PSs.

Comparing the bioluminescence values obtained in the experiments, an obvious difference in the photoinactivation (PI) patterns of the four derivatives is observed (Fig. 4 and 5). The cationic isobacteriochlorin **4** even at the lower concentration tested (5.0 µM) is much more effective to photoinactivate the

Gram (-) bacterium *E.coli,* than the other tested PSs (**1**, **2** and **3**) (P < 0.05, ANOVA).[35] At this concentration PS **4** caused bioluminescence reduction of more than 5.0 log while chlorins **1**, **3** (5.0 μM) caused only a 0.57 and 0.77 log decrease in light emission, respectively, after a total light dose of 43.2 J cm^{-2} (Fig. 4 and 5). When the concentration of cationic chlorin **3** is increased four times to 20 μM, no changes are observed on the PI pattern of bioluminescent *E. coli*. In contrast, with regard to cationic isobacteriochlorin **4**, the effect of increasing concentration (20 μM) results in an increment in the PI of the bacteria (bioluminescence reduction of 6.1 log) after a light dose of 36 J cm^{-2}. On the other hand, the neutral isobacteriochlorin **2** at higher concentration (20 μM) caused only a 0.24 log decrease in light emission after 43.2 J cm^{-2}.

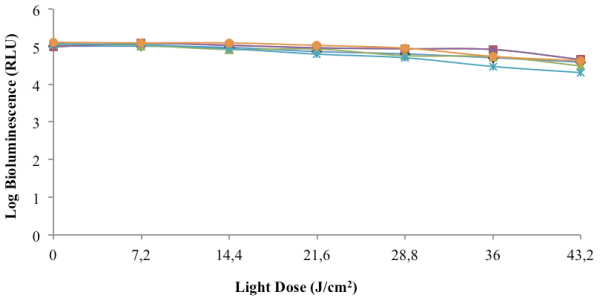

Figure 4: Bioluminescence monitoring of *E. coli* treated with chlorin derivatives **1** and **3** after exposed to different light doses of white light (380–700 nm) at an irradiance of 4.0 mW cm^{-2}; ◆ *Light control;* ■ *neutral chlorin* **1** (5.0 μM) *dark control;* × *cationic chlorin* **3** (20 μM) *dark control;* ▲ *neutral chlorin* **1**(5.0 μM); ✳ *cationic chlorin* **3** (5 μM); ● *cationic chlorin* **3** (20 μM); Error bars indicate the standard deviation. Lines just combine the points. Small bars are overlapped by the symbols.

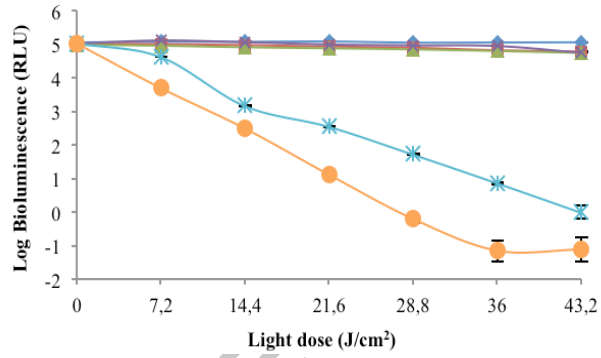

Figure 5: Bioluminescence monitoring of *E. coli* treated with isobacteriochlorin derivatives **2** and **4** after exposed to different light doses of white light (380–700 nm) at an irradiance of 4.0 mW cm^{-2}; ◆ *Light control;* ■ *neutral isobacteriochlorin* **2** (20 μM) *dark control;* ▲ *neutral isobacteriochlorin* **2** (20 μM); ×*cationic isobacteriochlorin* **4** (20 μM) *dark control;* ✳ *cationic isobacteriochlorin* **4** (5.0 μM); ● *cationic isobacteriochlorin* **4** (20 μM); Error bars indicate the standard deviation. Lines just combine the points. Small bars are overlapped by the symbols.

The present study demonstrates that it is possible to photoinactivate bioluminescent *E. coli,* a Gram (-) bacterium, using a cationic isobacteriochlorin as PS. The bioluminescent indicator strain was obtained by transformation of *E. coli* strain with the *lux* operon (*luxCDABE*) from the marine bacterium *V. fischeri* that emits light continuously without the addition of exogenous substrates.[36] The utilization of these stable bioluminescent bacteria enables the progress of the PI process to be followed with real time results.[33]

According to our results, we can point out that the bacterial photoinactivation varies with the number of positive charges present in the PS. It is well known that cationic porphyrin derivatives induce direct PI of Gram (+) and also of Gram (-) bacteria. This type of porphyrin derivatives allows the inactivation of Gram (-) cells because the positive charge on the PS molecule promotes a tight electrostatic interaction with negatively charged sites on the outer surface of the bacterial cells, which enhances the efficiency of the PI process[8, 16]

In this study only the two charged cationic isobacteriochlorin **4** was an effective PS against the bioluminescent *E. coli*, reaching the limit of detection (~6.1 log reduction) after a light dose of 36 J cm^{-2} for the highest concentration tested. The bioluminescence reduction after photodynamic inactivation for the maximum concentration was non-significant for the other compounds tested (**1**, **2** and **3**) and was similar to the results obtained for the controls (light and dark) (P > 0.05, ANOVA). Although some studies indicate that the number of charges does not affect the activity of PS against both bacterial Gram types[37] other studies demonstrate precisely the opposite[12] showing that with the increment in number of positive charges the cell killing increases. Our results suggest that the PS bearing 2 positive charges in beta position (**4**) affects the process of photoinactivation and is the only PS tested that has an effective action against bioluminescent *E. coli*. The particular efficiency of this cationic isobacteriochlorin may not be associated only with the production of singlet oxygen (Φ_Δ = 0.62; Table 1), but also with the location of the charges in the molecule.[21] In fact, the asymmetrical distribution of the charges on the macrocycle periphery can increase the amphiphilic character of the compounds.[10,12] In order to confirm this feature, the amphiphilic nature of the compounds was calculated using Molinspiration WebME Editor 3.81.[38] Compound **4** shows a logP value below 5 (4.82) while compounds **1-3** show values in the range 8.93-9.88 which is in agreement with the higher amphiphilicity character of the charged isobacteriochlorin **4**. Altogether, it is demonstrated that the number of positive charges in the PS is a determinant factor to be considered in the design of new PS for the photoinactivation of Gram (-) bacteria.

The *E. coli* photoinactivation profile by cationic isobacteriochlorin **4** shows that, using the adequate PS, the aPDI can be used for clinical applications. A previous study of the influence of light parameters and irradiation sources showed that the effectiveness of aPDI also depends on the light source, fluence rate and total light dose.[39] Taking into account the photophysical properties of **4**, if activated by an appropriate light source, it can be considered to treat deeper infections.

The results obtained in this work confirm that the trend of inactivation efficiency proceeds with the increasing number of charges on the PS, as reported in our earlier studies. The combined effect of higher wavelength absorption and number of positive charges in the isobacteriochlorin **4** leads to 6.1 log reduction in the inactivation process. Overall this study showed that cationic isobacteriochlorin PSs have high potential to be used for photodynamic inactivation of Gram (-) bacteria.

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25 Chlorin**1** (65% yield); ^{1}H NMR ($CDCl_3$): δ – 1.82 (s, 2H, NH), 2.18 (s, 3H, N-CH_3), 2.52–2.56 (m, 2H, pyrrolidine-H), 3.14 (t, *J* = 8.1 Hz, 2H, pyrrolidine-H), 5.25 (t, *J* = 5.0 Hz, 2H, β-H, reduced pyrrole), 8.39 (d, *J* = 4.9, 2H, β-H), 8.48 (s, 2H, β-H), 8.71 (d, *J* = 4.9, 2H, β-H). UV-vis (DMF) λ_{max} (log ε) 405 (5.19), 503 (4.18), 599 (3.71), 651 (4.65) nm; HRMS (ESI): *m/z* calcd for $C_{47}H_{18}F_{20}N_5$ $[M+H]^+$: 1032.12374; found: 1032.12321.

26 Isobacteriochlorin **2** (10% yield); ^{1}H NMR ($CDCl_3$): δ 2.81 (br s, 2H; NH); 3.25 (s, 6H, 2 × CH_3), 3.26-3.35, 3.67-3.84, 3.90-3.96 (3m, 8H, pyrrolidine-H), 5.27-5.33 (m, 4H, H-2,3,7,8), 7.40 (d, 2H, *J* = 4.6 Hz, β-H), 7.85 (d, 2H, *J* = 4.6 Hz, β-H). UV-vis (DMF) λ_{max} (log ε) 382 (5.01), 510 (3.96), 546 (4.22), 588 (4.40), 645 (3.29) nm; HRMS (ESI): *m/z* calcd for $C_{50}H_{26}F_{20}N_6[M^{2+}]$: 545.09443; found: 545.09393.

27 Cationic chlorin **3** (77% yield); ^{1}H NMR ($CDCl_3$+CD_3OD): δ -1.98 (s, 2H, NH),3.00 (s, 3H, CH_3),3.56-361 (m, 2H, pyrrolidine-H),3.66 (s, 3H, CH_3),4.74-4.80 (m, 2H, pyrrolidine-H), 5.93-5.96 (m, 2H, H – 2, 3), 8.41 (d, 2H, *J* = 4.9 Hz, β-H), 8.54 (s, 2H, H-12, 13), 8.79 (d, 2H, *J* = 4.9 Hz, β-H). UV-vis (DMF) λ_{max} (log ε) 402 (5.23), 500 (4.19), 525 (3.64), 594 (3.69), 647 (4.64) nm; HRMS (ESI): *m/z* calcd for $C_{48}H_{20}F_{20}N_5I$ $[M-I]^{+\bullet}$: 1046.13939; found: 1046.13883.

28 Cationic isobacteriochlorin **4** (75% yield); ^{1}H NMR ($(CD_3)_2CO$): δ 3.42 (s, 6H, 2 × CH_3), 3.49 (s, 6H, 2 × CH_3), 3.81-3.89 (m, 4H, pyrrolidine-H), 4.43-4.50 and 4.56-4.63 (2m, 4H, pyrrolidine-H), 5.54-5.65 (m, 4H, H-2, 3, 7, 8), 7.70 (d, 2H, *J* = 4.5 Hz, β-H), 8.17 (d, 2H, *J* = 4.5 Hz, β-H).UV-vis (DMF) λ_{max} (log ε) 408 (5.08), 503 (3.88), 546 (3.97), 599 (4.19), 650 (3.65) nm; HRMS (ESI): *m/z* calcd for $C_{52}H_{32}F_{20}N_6I_2$ $[M-2I]^{2+}$: 559.10972; found: 559.10967.

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Table 2: Drug-likeness property/Lipinski's 'rule of five' parameters calculated for compounds **1-4**.[a]

Compound	Molecular weight	miLog*P*	*n*-ROTB	*n*-ON acceptors	*n*-ONNH donors	*n* violations	Volume	TPSA
1	1031.648	9.875	4	5	2	2	719.108	60.604
2	1088.744	9.753	4	6	2	2	777.454	63.842
3	1046.683[b]	8.933	4	5	2	2	739.104	57.366
4	1118.814[b]	4.82	4	6	2	1	817.445	57.366

(a) n-ROTB, number of rotatable bonds; n-ON, number of hydrogen acceptors; n-OHNH, number of hydrogen bond donors; TPSA, topological polar surface area; n violations, number of violations according to the Lipinski 'rule of five'; (b) molecular weight and other properties calculated without counter ion mass.