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FULL ARTICLE

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Bacteriochlorin-bis(spermine) conjugate affords an effective photodynamic action to eradicate microorganisms

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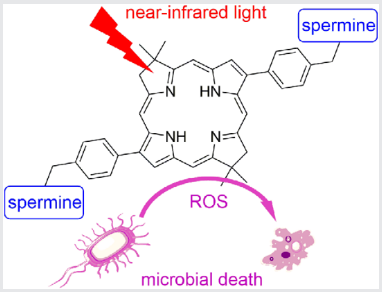
Abstract



A novel bacteriochlorin bearing two spermine units (**BCS**) was synthesized from 3,13-dibromo-8,8,18,18-tetramethylbacteriochlorin (**BC-Br^{3,13}**). The synthesis involved the Suzuki coupling of **BC-Br^{3,13}** to obtain a bacteriochlorin-dibenzaldehyde (**BCA**), which was subjected to reductive amination with spermine. The resulting bacteriochlorin **BCS** presents a strong near-infrared absorption band at 747 nm, emits at 750 nm with fluorescence quantum yield of 0.14, and generates singlet molecular oxygen, $O_2(^1\Delta_g)$, with a quantum yield of 0.27. Photokilling capacities mediated by **BCS** were evaluated in microbial cells. The viability of *Staphylococcus aureus* decreased 7 logs when cells were incubated with 1 μM **BCS** and irradiated for 15 minutes. Comparable photocytotoxic effect was obtained with *Escherichia coli*, when cells were treated for 30 minutes with visible light. **BCS** was also an effective photosensitizer to inactivate *Candida albicans*. In addition, this bacteriochlorin was able to eradicate bacteria at short incubation times. The structure of **BCS** contains eight basic amino groups that, when protonated in water, increase the binding to the cell envelope. In summary, the readily accessible bacteriochlorin **BCS** was highly effective at low concentrations as a broad-spectrum antimicrobial photosensitizer.

KEYWORDS
bacteria, bacteriochlorin, photodynamic inactivation, photokilling, photosensitizer

1 | INTRODUCTION

The development of resistance to traditional antibiotics denotes today one of the greatest threats to worldwide health [1]. Infections that are difficult to cure increase each day due to the decrease in the efficacy of antibiotics. It is projected that the appearance of bacterial resistance to antibiotics will be the cause of more than 700 000 deaths per year worldwide [2]. The new era of antibiotics stimulated pharmaceutical corporations to look for new medical strategies and technologies in order to battle infections. A proposed alternative therapeutic treatment is denoted by photodynamic inactivation (PDI) to eradicate microbial cells [3]. This new therapy consists in the administration of a phototherapeutic agent, which binds to microbes in a short time. Then, the aerobic irradiation of the infection leads to the generation of reactive oxygen species (ROS), which cause oxidative damage to the components of the cells.

These processes induce a loss of biological functionality leading to cell death. In general, ROS include the superoxide radical anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and oxygen-centered radicals of organic compounds (peroxyl, $ROO^{\bullet}$ and alkoxyl, $RO^{\bullet}$) together with other non-radical reactive compounds, such as hydrogen peroxide (H_2O_2) and singlet molecular oxygen, $O_2(^1\Delta_g)$ [4–7].

Although the possibility that bacterial cells can develop resistance to PDI treatments has been questioned, at present there is no convincing evidence that validates acquired microbial resistance for this therapy [8, 9]. Moreover, PDI is easy to implement, reliable, and has already been shown to be effective against viruses, protozoa, fungi and bacteria [10]. It is well documented that the Gram-positive pathogen *Staphylococcus aureus* has high ability to develop resistance against conventional antibiotics [11]. Therefore, this microorganism is a serious medical threat in hospitals. In this context, the Gram-negative organism *Escherichia coli*, not only causes severe nosocomial infections but also constitutes an important reservoir in animals and in the environment [12]. In addition, *Candida albicans* is the most recurrent disease-related yeast and antifungal resistance was detected in hospitals [13].

In recent years, several new photosensitizers have been developed for PDI and many of them proved to be effective for the eradication of microorganisms [3, 10]. In general, these compounds absorb intensely in the visible region and produce triplet excited states with good yields to generate ROS [14]. In addition, the presence of positive charges in the photosensitizer structure allows a better interaction with negative groups in the cell envelope, mainly in Gram-negative bacteria, which leads to an increased ability to inactivate cells [10]. For in vivo applications, an absorption band located in the phototherapeutic window (600-850 nm) may be necessary to obtain deep penetration of light in the tissue [15]. This is mainly necessary for the therapy of infected chronic wounds in deeper layers of the skin [16]. In this regard, a promising family of molecules for PDI is provided by bacteriochlorins, tetrahydroporphyrins containing alternating pyrrole and pyrroline rings [17]. Bacteriochlorins differ from other tetrapyrrole macrocycle derivatives because they have an intense long-wavelength absorption band (ε ~10^5 M^{-1} cm^{-1}), which is located in the near-infrared (NIR) region [18, 19]. The characteristics of bacteriochlorins often meet the demands of valuable phototherapeutic agents. Therefore, these macrocycles are appropriate structures for the development of new photosensitizers targeted to particular microorganisms and medical conditions. The NIR absorption band gives rise to the possibility of carrying out photochemical studies with light of lower energy and enables the capture of a large fraction of sunlight. In addition, bacteriochlorins absorb blue and green light, harvesting a wide range of visible radiation. Thus, bacteriochlorins bearing cationic groups were considered as promising phototherapeutic agents for the inactivation of bacterial infections [20–22].

The aim of the study described herein was to synthesize a novel bacteriochlorin that contains two spermine groups attached to the macrocycle at the *beta* positions. In this way, polar groups were added in synthetic accessible positions around the macrocycle structure to obtain a hydrophilic bacteriochlorin. Each spermine units contains four basic amine groups, which can acquire positive charges in aqueous media. Spectroscopic characteristics of this bacteriochlorin and its precursor were studied in *N*,*N*-dimethylformamide (DMF). Moreover, photosensitized production of $O_2(^1\Delta_g)$ mediated by both bacteriochlorins was determined in the same organic solvent. Finally, the photodynamic capacities mediated by bacteriochlorins were evaluated against two pathogenic bacteria, *S. aureus* and *E. coli*, and yeast, *C. albicans*.

2 | MATERIALS AND METHODS

2.1 | Synthesis

3,13-Dibromo-8,8,18,18-tetramethylbacteriochlorin (BC-Br^{3,13}). This compound was synthesized following a general route for bacteriochlorin synthesis [23–26]. Spectroscopic data agree with those previously reported [27].

3,13-Bis(4-formylphenyl)-8,8,18,18-tetramethylbacteriochlorin (BCA). A mixture of **BC-Br^{3,13}** (9.0 mg, 17 μmol, 10 mM), 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde (15 mg, 64.8 μmol), previously purified by bulb-to-bulb distillation, $Pd_2(dba)_3{\cdot}CHCl_3$ (5.0 mg, 4.8 μmol), 2-dicyclohexylphosphino-2′,6′-dimethoxybiphenyl (SPhos, 8.0 mg, 19 μmol) and K_2CO_3 (18.0 mg, 130 μmol) was placed into a 10 mL Schlenk flask, which was pump-purged three times with argon. Then, toluene/DMF (1.6 mL, [2:1]) was added and the mixture was stirred at 90°C for 24 hours. The solvents were evaporated under reduced pressure. The solid obtained was dissolved with dichloromethane (DCM, 50 mL). This organic mixture was washed with 25 mL of an aqueous saturated solution of $NaHCO_3$, dried with Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure and **BCA** was purified by flash column chromatography (silica gel, hexanes/DCM gradient/triethylamine 5%). The product was dried by high vacuum and washed with hexanes and methanol to afford 5.2 mg (53%) of **BCA** as a green solid. TLC analysis (silica gel, hexanes/DCM [1:1]) R_f 0.28. ^{1}H NMR ($CDCl_3$) δ [ppm]: −1.80 (br, 2H), 1.99 (s, 12H), 4.45 (s, 4H), 8.27 (d, J = 8.0 Hz, 4H), 8.39 (d, J = 8.0 Hz, 4H), 8.74 (s, 2H), 8.87 (s, 2H), 8.91 (s, 2H), 10.27 (s, 2H). MALDI-TOF-MS

[*m/z*]: 579.2 $[M + H]^+$, 564.3 $[M + H{-}CH_3]^+$. ESI-MS [*m/z*]: 579.2741 (579.2755 calculated for $[M + H]^+$, M = $C_{38}H_{34}N_4O_2$). UV-Vis ($CHCl_3$) λ_{abs} [nm]: 350, 376, 508, 750.

3,13-Bis[4-({[3-({4-[(3-aminopropyl)amino]butyl}amino) propyl]amino}methyl)phenyl]-8,8,18,18-tetramethylbacteriochlorin (BCS). A solution of **BCA** (2.8 mg, 4.8 μmol) in 0.5 mL of 1,2-dichloroethane (DCE)/methanol (MeOH) [4:1] was treated with spermine (*N*,*N*′-bis(3-aminopropyl)-1,4-diaminobutane, 9.8 mg, 48.4 μmol). The reaction mixture was stirred for 1 hour at room temperature. After that, the formation of the imine intermediate was monitored by MALDI-TOF-MS [*m/z*]: 947.6 $[M + H]^+$, 474.3 $[M + 2H]^{2+}$. ESI-MS [*m/z*]: 474.3456 (474.3466 calculated for $[M + 2H]^{2+}$, M = $C_{58}H_{82}N_{12}$). λ_{abs} ($CHCl_3$): 354, 373, 501, 740 nm. Then, $NaBH(OAc)_3$ (61.5 mg, 0.290 mmol) and glacial acetic acid (HOAc, 8.3 μL, 0.15 mmol) were added and the mixture was stirred for 24 hours. The crude reaction mixture was dried and diluted with DCM (50 mL). Afterward, a saturated solution of $NaHCO_3$ and NaCl in water was added (25 mL). The aqueous solution was separated and dried under vacuum. The organic product was redissolved in ethanol and filtered to obtain 1.2 mg (27%) of **BCS**. ^{1}H NMR ($CDCl_3$) δ [ppm]: −2.00 (br, 2H), 1.44-1.55 (m, 8H), 1.60-1.72 (m, 8H), 1.98 (s, 12H), 2.60-2.85 (m, 24 H), 4.03 (s, 4H), 4.45 (s, 4H), 7.70 (d, *J* = 7.6 Hz, 4H), 8.16 (d, *J* = 7.6 Hz, 4H), 8.71 (s, 2H), 8.78 (s, 2H), 8.92 (s, 2H). MALDI-TOF-MS [*m/z*]: 951.7 $[M + H]^+$, 476.4 $[M + 2H]^{2+}$. ESI-MS [*m/z*]: 951.7176 (951.7171 calculated for $[M + H]^+$, M = $C_{58}H_{86}N_{12}$). UV-Vis ($CHCl_3$) λ_{abs} [nm]: 352, 374, 500, 738.

2.2 | Spectroscopic studies

UV-visible absorption and fluorescence spectra were carried out as reported [7]. An excitation wavelength of 503 nm was used to acquire the emission spectra. 5,10,15,20-Tetrakis (4-methoxyphenyl)porphyrin (**TMP**) was used as a reference (Φ_F = 0.14) to determine the fluorescence quantum yield (Φ_F) of each bacteriochlorin in DMF [28]. All spectral measurements were performed at room temperature.

2.3 | Determination of $O_2(^1\Delta_g)$ production

The consumption of 9,10-dimethylanthracene (DMA, 35 μM) in the presence of photosensitizer (absorbance 0.1 at the irradiation wavelength) was studied in DMF (2 mL), irradiating the samples with light at λ_{irr} = 503 or 665 nm in 1 cm path length quartz cells [7]. The quantum yield of $O_2(^1\Delta_g)$ generation (Φ_Δ) was determined using **TMP** (Φ_Δ = 0.65) as a reference at 503 nm or methylene blue (**MB**) (Φ_Δ = 0.52) as a reference at 665 nm [28, 29].

2.4 | Microbial strain and preparation of cultures

The Gram-positive strain *S. aureus* ATCC 25923, Gram-negative *E. coli* EC7 and yeast *C. albicans* PC31 were used in this study [28, 30]. Cultivation of microorganisms and handling of cells to obtain ~10^8 colony forming units (CFU)/mL for bacteria and ~10^6 CFU/mL for yeast in phosphate-buffered saline (PBS, pH = 7.4) were achieved as reported [31]. Viable microbial cells were determined by serial dilutions 10-fold in PBS and using the spread plate technique after ~24 hours incubation at 37°C in the dark.

2.5 | Photoinactivation of microbial cells

Bacterial suspensions in PBS (1 mL, ~10^8 CFU/mL) were incubated with the corresponding bacteriochlorin at different concentrations for 30 minutes in the dark at 37°C [30, 32]. The bacteriochlorins were added from stock solutions in DMF (0.5 mM). Then, the cultures were exposed to visible light in a 96-well microtiter plate for different periods (5, 15 and 30 minutes, which correspond to a light fluence of 6, 18 and 36 J/cm^2, respectively). Photoinactivation of bacteria incubated with **BCS** for 5 minutes in the dark was performed as described above. PDI of *C. albicans* was performed as reported earlier [30, 31]. Control experiments and statistical analysis with microbial cells were carried out as previously indicated [7].

3 | RESULTS AND DISCUSSION

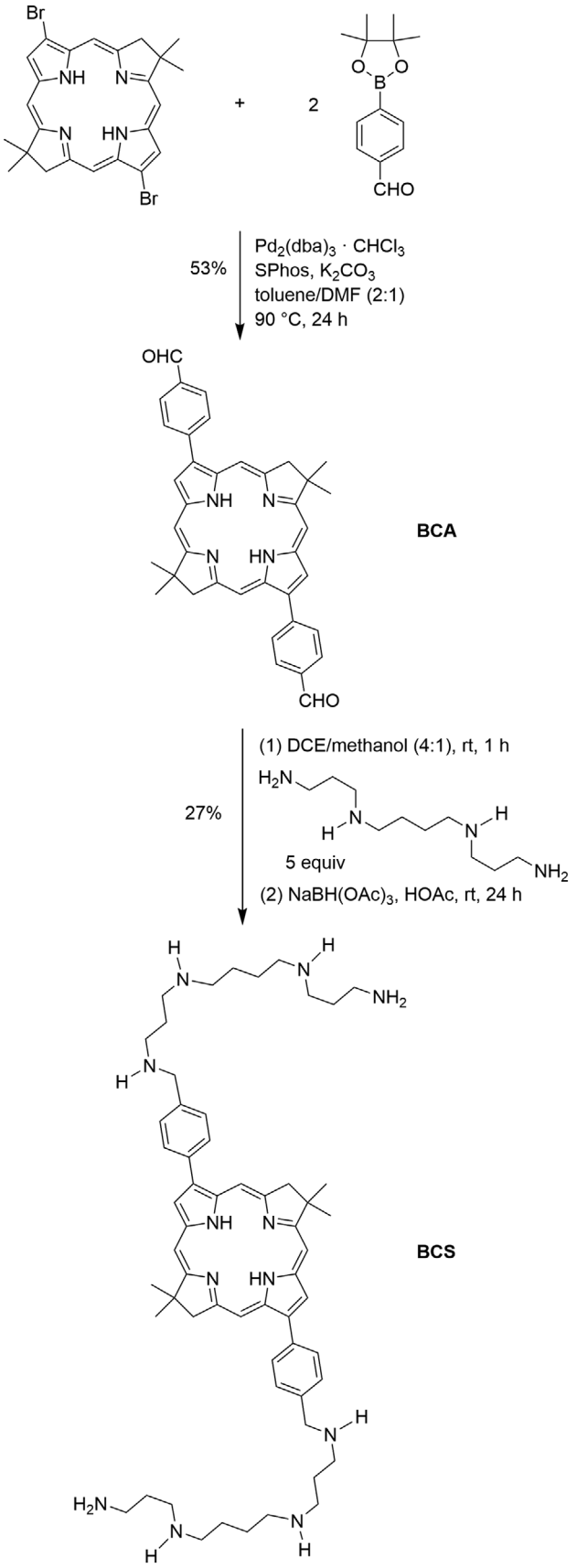
3.1 | Synthesis of BCS

Bacteriochlorin **BC-Br^{3,13}** was synthesized by the condensation of two molecules of 8-bromodihydrodipyrrin-acetal [27, 33]. The generic synthesis of dihydrodipyrrin-acetals entails five steps from a pyrrole: (a) formylation to give a pyrrole-2-carboxaldehyde, (b) nitro-aldol (Henry) condensation with nitromethane to give a nitrovinylpyrrole, (c) reduction to give the 2-(2-nitroethyl)pyrrole, (d) Michael addition with an α,β-unsaturated ketone-acetal to give a nitrohexanone-pyrrole, and (e) McMurry-type ring closure [25, 33]. Self-condensation of the dihydrodipyrrin-acetal in the presence of $BF_3{\cdot}OEt_2$ at modest concentration in CH_3CN typically provides a mixture of two bacteriochlorins and one tetradehydrocorrin, but the use of 140 mM $BF_3{\cdot}OEt_2$ and 18 mM dihydrodipyrrin-acetal typically affords the free base 5-unsubstituted bacteriochlorin as the dominant product. Several aspects of those reaction conditions were previously explored [19, 26].

Bacteriochlorin **BC-Br^{3,13}** has previously been examined as a scaffold for the synthesis of several bacteriochlorins by coupling reactions mediated by Pd, which enable introduction of diverse functionality including auxochromes at both the 3- and 13-positions [34]. Furthermore, this

bacteriochlorin is stable to possible oxidative processes owing to the geminal dimethyl substituent positioned in each pyrroline ring [33]. To install the desired functional groups on **BC-Br**[3,13], a late-stage diversification was performed. Thus, a Suzuki coupling was carried out mixing **BC-Br**[3,13] and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde in the presence of $Pd_2(dba)_3{\cdot}CHCl_3$, SPhos and K_2CO_3, affording the bacteriochlorin **BCA** in 53% yield (Scheme 1). The choice of the dioxaborolane derivative facilitated the purification process [35]. Finally, a solution of **BCA** in DCE/MeOH was treated with spermine (5 equiv per carboxaldehyde). The mixture was stirred for 1 hour at room temperature and then $NaBH(OAc)_3$ and glacial acetic acid were added to the sample containing the imine product. This procedure, previously described for the reductive amination of ketones and aldehydes [36], produced the bacteriochlorin-bis(spermine) conjugate **BCS** in 27% yield. The bacteriochlorins **BCA** and **BCS** were robust molecular structures. For example, each compound was stable upon standing on the benchtop in solution exposed to air, chromatography on silica, and dissolution in a variety of solvents (DMF, DCE, DCM, toluene, methanol, ethanol, PBS).

A variety of bacteriochlorins with appended polar motifs has been previously prepared. Mironov et al [37] synthesized a series of cationic bacteriochlorins (e.g., **BChl-1**, Chart 1) from natural bacteriochlorophyll *a* (*Bchl a*) via the intermediacy of bacteriochlorin *N*-aminocycloimide. These new compounds were synthesized as a means for fighting microbial contamination. On the other hand, Jiang et al [38] prepared two fully synthetic cationic bacteriochlorins, named **BC-1** and **BC-2**. Both bear four ammonium groups, but differ in that the former is more compact and has benzylammonium units, whereas the latter contains alkylammonium groups attached via ester moieties. Each bacteriochlorin in the set contains a common scaffold and was derived by Suzuki coupling with **BC-Br**[3,13]. In 2016, Yang et al [18] synthesized two bacteriochlorins (**BC-3** and **BC-4**) bearing cationic groups at the 3- and 13-positions of the macrocycle. The substituents of **BC-3** and **BC-4** have characteristics in common with cyanine dyes, which have terminal *N*-alkylated heterocycles at the ends of a polyene chain. In **BC-3** and **BC-4**, the bacteriochlorin constitutes the polyene chain and each quaternized nitrogen terminal unit (**BC-3**, *N*-ethylpyridinium units; **BC-4**, *N*-ethylquinolinium units) bears an intrinsic cationic charge. The new bacteriochlorin **BCS** synthesized herein contains two flexible spermine units, which afford conformationally mobile alkylamine groups. Considering the pK_A values of spermine [39], the acid-base equilibria of the amine groups should be shifted to the protonated form. Therefore, a high percentage of the amine groups can acquire positive charges by



SCHEME 1 Synthesis of **BCA** by Suzuki coupling and **BCS** by reductive amination

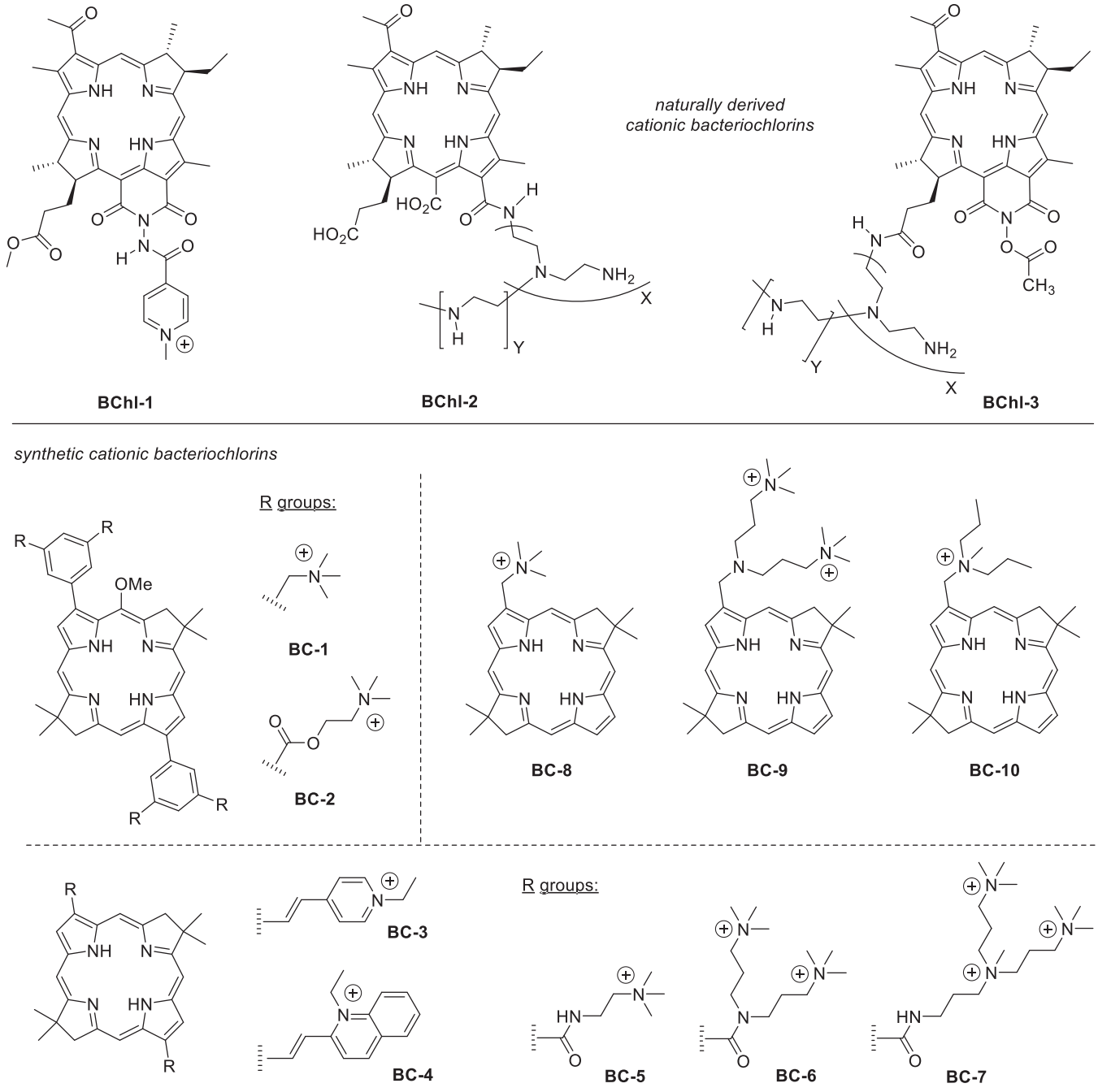


CHART 1 Cationic bacteriochlorins for PDI studies (counter ions omitted for clarity)

protonation in water, increasing the interaction with the cell envelope [30, 32]. Moreover, it was previously demonstrated that photosensitizers with basic amine groups are as effective as those with intrinsic positive charges to inactivate microorganisms [40].

3.2 | Absorption and fluorescence spectroscopic properties

The absorption spectra of the synthetic bacteriochlorins, **BCA** and **BCS**, in DMF are shown in Figure 1A. For comparison purposes, spectra were normalized to the maximum intensity of the $Q_y(0,0)$ band, which is the prominent, longest-wavelength feature in the spectrum. The assignment of the bands from lowest to highest energy (Q_y, Q_x, B_x, B_y) follows the standard convention [41]. The absorption spectra of the bacteriochlorins exhibit general features expected for this genre of macrocycle in three spectral domains. These include an intense NIR Q_y band (670-820 nm), weaker visible Q_x band (500-580 nm), and the strong near-ultraviolet (NUV) B_x and B_y features (360-400 nm), also known as the Soret bands [19]. Typically, a weaker (1,0) satellite feature

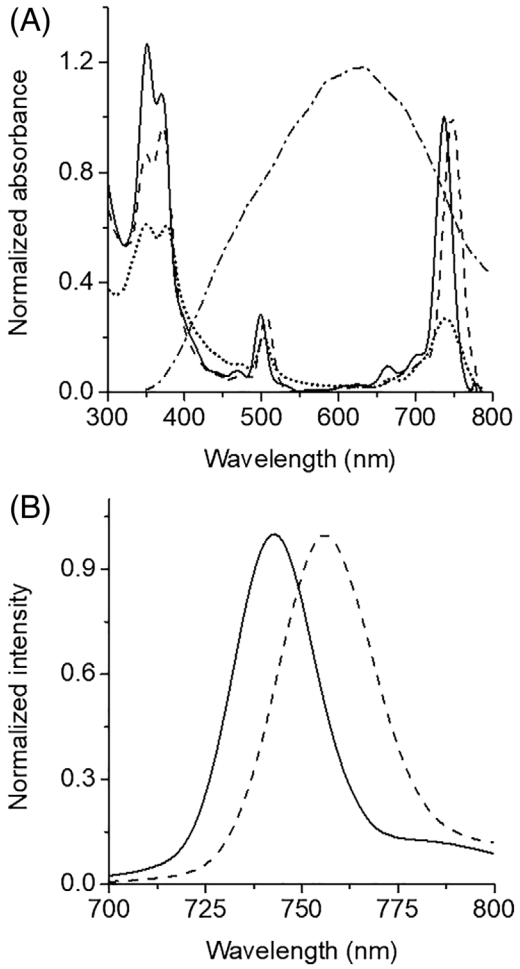


FIGURE 1 A, Absorption spectra of bacteriochlorins **BCA** (dashed line) and **BCS** (solid line) normalized at the Q_y bands in DMF, **BCS** (dotted line) 2.5 μM in PBS and emission spectrum of the light source (dash-dotted line, intensity a.u.). B, Fluorescence emission spectra of bacteriochlorins **BCA** (dashed line) and **BCS** (solid line) in DMF ($\lambda_{exc} = 503$ nm)

can be resolved to higher energy than each (0,0) origin band. There is considerable overlap of the B_y and B_x origin and vibronic components in the NUV. The spectroscopic data of the bacteriochlorins are specified in Table 1. Sharp absorption Q_y bands, with the full-width-at-half-maximum (FWHM) of 28 and 22 nm, were obtained for **BCA** and **BCS**, respectively. These data are indicative that the photosensitizers are mainly non-aggregated in DMF (Figure 1A). In addition, both bacteriochlorins exhibited a light green appearance in dilute solution in DMF. In general, the UV-visible absorption spectra of bacteriochlorins showed a strong dependence on the substituents at the 3- and 13-positions [38]. Thus, for **BCA** the absorption maxima of the $Q_y(0,0)$ and $Q_x(0,0)$ bands were at 747 and 506 nm, respectively. For **BCS** the analogous bands were at 737 and 499 nm, respectively.

TABLE 1 Spectroscopic and photodynamic properties of bacteriochlorins

Photosensitizer	BCA	BCS
B_x abs (nm)[a]	372	370
B_x abs (nm)[b]	—	376
Q_x abs (nm)[a]	506	499
Q_y (FWHM)[c] abs (nm)[a]	747 (28)	737 (22)
Q_y (FWHM) abs (nm)[b]	—	740 (~80)
I_{Q_y}/I_{B_x} [d]	1.04	0.92
Q_y em (nm)[a]	756	743
ε_{Q_y} (M^{-1} cm^{-1})	1.30×10^5	1.29×10^5
Φ_F^{DMF}	0.20 ± 0.02	0.14 ± 0.01
k_{obs}^{DMF} (s^{-1})	$(1.48 \pm 0.01) \times 10^{-4}$	$(1.10 \pm 0.01) \times 10^{-4}$
Φ_Δ^{DMFe}	0.36 ± 0.02	0.27 ± 0.02

[a]*N*,*N*-dimethylformamide (DMF).
[b]Phosphate-buffered saline (PBS).
[c]Full-width at half-maximum (FWHM) in nm.
[d]Ratio of the intensities of the Q_y and B bands.
[e]Using **TMP** as a reference $k_{obs}^{DMF} = (2.64 \pm 0.02) \times 10^{-4}$ and $\Phi_\Delta^{DMF} = 0.65$ from Reference [28].

The absorption profile for **BCS** in standard aqueous PBS (pH 7.4) (Figure 1A) showed an apparent increase in baseline starting at 600 nm and increasing into the NUV region, as may be expected for light scattering due to the formation of small aggregates. The extent of scattering was comparatively less for the same compound in DMF. Furthermore, the Q_y FWHM increases from 28 nm in DMF to 55 nm in PBS. This partial aggregation of **BCS** can preclude the photophysical properties of this photosensitizer in aqueous medium [19]. However, the main photodynamic action that leads to microbial death is induced by the photosensitizer that binds to the cells [42, 43]. Data for **BCA** in PBS is not given due to minimal solubility of this bacteriochlorin in aqueous media.

The fluorescence characteristics of the synthetic bacteriochlorins were determined in DMF. The emission spectra of **BCA** and **BCS** are shown in Figure 1B. In both cases, the fluorescence band mirrors the long-wavelength absorption band, exhibiting a strong $Q_y(0,0)$ transition and a much weaker, almost negligible $Q_y(0,1)$ transition. The fluorescence spectral properties are shown in Table 1. The Stokes shift between the absorption and emission origin transitions is 9 and 6 nm for **BCA** and **BCS**, respectively. A small Stokes shift for **BCS** indicated that the spectroscopic energies are similar to the relaxed energies of the lowest singlet excited state S_1. This is in accordance with the planar and rigid structure of the tetrapyrolic macrocycle [18]. Therefore, only

a minor geometric relaxation takes place in the first excited state. Moreover, the fluorescence quantum yield (Φ_F) of each bacteriochlorin was determined in DMF (Table 1). The Φ_F values for **BCA** and **BCS** agree with values for analogous bacteriochlorin derivatives [37, 41, 44].

3.3 | Photosensitized generation of $O_2(^1\Delta_g)$

Photodecomposition of DMA induced by bacteriochlorins was compared in DMF under aerobic conditions. The solutions were irradiated at 503 nm because at that wavelength both bacteriochlorins and **TMP** present absorption bands. The photodecomposition of DMA was examined by the decrease of the absorbance at λ_{max} = 379 nm (Figure S1). Figure S1 shows that the absorbance of the **BCS** did not decrease after 18 minutes of irradiation with monochromatic light (λ = 503 nm), indicating high photostability under this experimental condition. Figure 2 provides characteristic plots that describe the consumption of DMA as the photooxidation reaction proceeds. The observed rate constant (k_{obs}^{DMA}) values for DMA consumption were calculated from the decrease in the absorption of DMA at 379 nm, according to Figure 2. Bacteriochlorins efficiently sensitized the decomposition of DMA; however, the photooxidation rates of DMA mediated by both bacteriochlorins were lower in comparison with that of **TMP**.

The quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) of these bacteriochlorins was calculated from the kinetic data of DMA decomposition. This compound quenches $O_2(^1\Delta_g)$ by chemical reaction [45]. The values of Φ_Δ were determined by comparing the k_{obs}^{DMA} for the bacteriochlorin with that for the reference (**TMP**) from the plots shown in Figure 2. Comparable values of Φ_Δ were found for **BCA** and **BCS** (Table 1), which are similar to those reported before for free base bacteriochlorins in solution [46]. In addition, $O_2(^1\Delta_g)$ production of **BCS** was evaluated irradiating the sample at 665 nm and using **MB** as reference (Figure S2). Under this condition, a similar value of Φ_Δ (0.26 ± 0.02) was obtained with red light. Previously, Drogat et al [47] synthesized two polyaminated bacteriochlorins (**BChl-2**, **BChl-3**, Chart 1) from the *BChl a* derived from *Rhodobacter sphaeroides*. These compounds produced Φ_Δ values between 0.14 and 0.18 in ethanol.

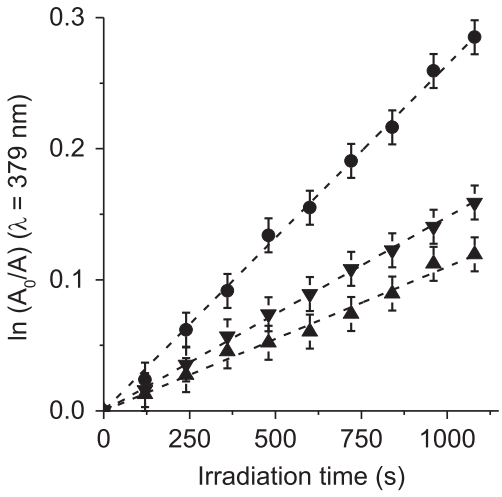


FIGURE 2 First-order plots for the photodecomposition of DMA (35 μM) photosensitized by **BCA** (▼), **BCS** (▲) and **TMP** (●) in DMF (λ_{irr} = 503 nm)

3.4 | PDI of microorganisms

The photodynamic action induced by **BCA** and **BCS** was assessed for in vitro inactivation of *S. aureus* cells. This Gram-positive bacterium is a commensal microorganism on human skin and anterior nares [48]. *S. aureus* strain has been reported as the main human pathogen causing skin and tissue infections, pneumonia, septicemia and diverse associated infections [11]. Due to adaptation to different antibiotics, this organism becomes multidrug-resistant leaving few therapeutic options for its treatment [49].

First, *S. aureus* cells were treated with both photosensitizers at a concentration of 5 and 10 μM for 30 minutes in the dark at 37°C. The cell suspensions were subsequently irradiated with visible light. Figure 3 shows the survival of *S. aureus* cells after different irradiation periods. Control experiments indicated that the viability of *S. aureus* was not affected by irradiation alone or by dark incubation with **BCA** for 30 minutes. Therefore, the *S. aureus* killing observed after irradiation of the cells incubated with **BCA**

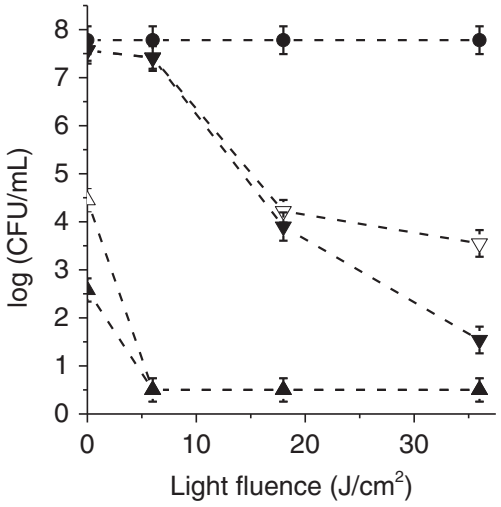


FIGURE 3 Survival curves of *Staphylococcus aureus* cells (~10^8 CFU/mL) incubated with 5 μM **BCA** (▽), 10 μM **BCA** (▼), 5 μM **BCS** (△) and 10 μM **BCS** (▲) for 30 minutes at 37°C in the dark and irradiated for different periods. Cells untreated with the photosensitizer and irradiated (●)

was mediated by the photosensitization activity of the agent. As shown in Figure 3, *S. aureus* cells were rapidly photoinactivated when the cultures treated with **BCA** were exposed to visible light. Bacterial suspensions treated with 5 μM **BCA** and irradiated for 30 minutes produced 4 logs decrease in viability, while 10 μM of this compound exhibited a photosensitizing activity of 6 log units. Similar results were previously obtained for the photoinactivation of *S. aureus* cells treated with 5 μM **TMP** after a light fluence of 27 J/cm^2 [30]. On the other hand, Figure 3 shows that bacterial suspension treated with 5 and 10 μM **BCS** in the dark produced 3 and 5 logs decrease in the viability, respectively. After irradiation for 5 minutes, no colony formation was detected when the cultures were treated with 5 or 10 μM **BCS**. Therefore, PDI mediated by **BCS** appears to be significantly more effective than by **BCA**, even though at these concentrations **BCS** was toxic in the dark.

To avoid the cytotoxicity of **BCS** in the dark and to observe the photodynamic effect, lower **BCS** concentrations (0.5 and 1 μM) were used for PDI assays with *S. aureus* (Figure 4). Under these conditions, **BCS** was not toxic in the dark, and the photoinactivation of *S. aureus* cells depended on **BCS** concentrations and irradiation times. In addition, no toxicity was determined for *S. aureus* cells treated with 1 μM spermine either in the dark (results not shown) or under irradiation with visible light. As can be observed in Figure 4, an inactivation of 6 logs was found for cells treated with 0.5 μM **BCS** and 30 minutes irradiation, while at 1 μM no colony formation was found. Also, **BCS** showed an effective photodynamic effect at short irradiation times. Therefore, photoinactivation of *S. aureus* induced by **BCS** was higher than by its non-cationic bacteriochlorin counterpart **BCA**.

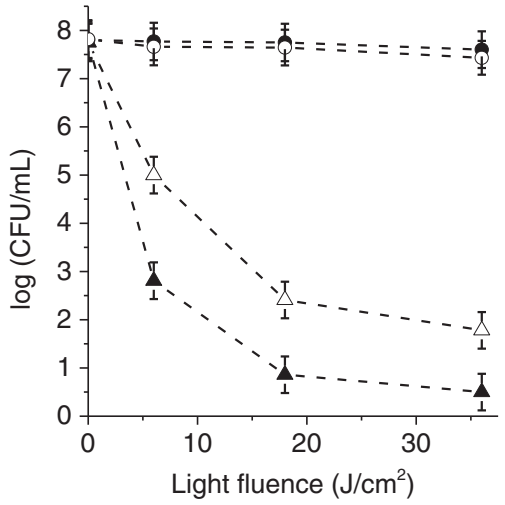


FIGURE 4 Survival curves of *Staphylococcus aureus* cells (~10^8 CFU/mL) incubated with **BCS** at concentrations of 0.5 μM (Δ) and 1 μM (▲) for 30 minutes at 37°C in the dark and irradiated for different periods. Cells untreated with **BCS** and irradiated (●) and cells treated with 1 μM spermine and irradiated (○)

Direct comparisons of the photoinactivation capacity of **BCS** with other photosensitizers previously reported is not always possible due mainly to different molecular structures, strains of bacteria and irradiation arrangements. In 2010, the PDI mediated by four bacteriochlorins, containing 2, 4, or 6 quaternized ammonium groups or 2 basic amine groups, was investigated against *S. aureus* [21]. Bis-cationic bacteriochlorin **BC-5** (Chart 1) was an effective photosensitizer to inactivate *S. aureus*, killing 7 logs at 0.1 μM after illumination with 10 J/cm^2 of 732 nm laser light. Less effective than bis-cationic bacteriochlorin were the tetrakis-cationic bacteriochlorin (**BC-6**, <6 log) and the hexakis-cationic bacteriochlorin (**BC-7**, <6 logs). The syntheses of three mono-substituted cationic bacteriochlorins (**BC-8-10**) also were reported [50]. The hypothesis that cationic quaternary ammonium groups in each compound would engender activity as antimicrobial photosensitizers was tried against a diversity of pathogenic microorganisms that include *S. aureus* [22]. These bacteriochlorins were highly effective against the Gram-positive bacteria, producing 5-6 logs of killing at 0.1 μM and 10 J/cm^2 of 700-850 nm light. Mono-cationic bacteriochlorin **BC-8** was better than **BC-9** and **BC-10** against *S. aureus*. Comparing with our results, even when different strains of *S. aureus* and irradiation systems are used, similar photoinactivation (5 logs) was obtained for microbial cells treated with 1 μM **BCS** and 5 minutes irradiation (6 J/cm^2, Figure 4).

On the other hand, *E. coli* microbial cells are nearly ubiquitous in the human gastrointestinal tract [12]. This Gram-negative bacterium was selected for the assays because it has been reported to be a common cause of diarrheagenic illness globally. Also, it is the most frequent cause of urinary tract infections and a leading reason of bacteremia and neonatal meningitis [51]. Diarrheal diseases are a serious public health problem and a significant motive of morbidity and mortality in children, particularly in developing countries [52]. Enlarged antibiotic resistance of *E. coli* cells has produced an increase in morbidity, mortality and considerable health expenses [53].

E. coli cells were treated with **BCS** at a concentration of 0.5 and 1 μM for 30 minutes in the dark at 37°C. Then, the cell suspensions were irradiated for different periods (5, 15 and 30 minutes). Figure 5 shows the survival of bacterial cells treated with **BCS**. Before the illumination, cell suspensions of *E. coli* were treated with the bacteriochlorin for 30 minutes in the dark. Control experiments established that the viability of *E. coli* was not modified by irradiation or by incubation with **BCS** in the dark for 30 minutes. Moreover, no toxicity was detected for *E. coli* cells treated with 1 μM spermine in the dark or irradiated for 30 minutes.

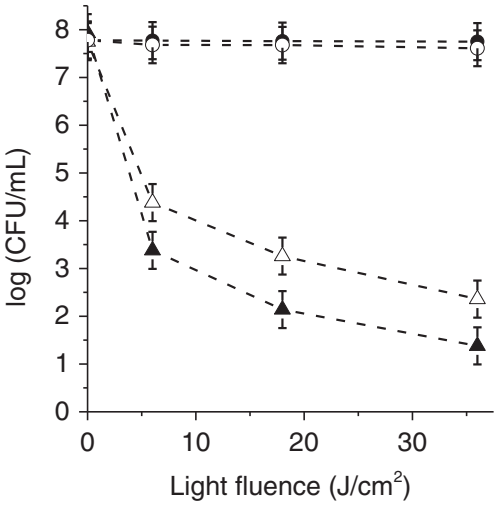


FIGURE 5 Survival curves of *Escherichia coli* cells (~10^8 CFU/mL) incubated with **BCS** at concentrations of 0.5 μM (Δ) and 1 μM (▲) for 30 minutes at 37°C in the dark and irradiated for different periods. Cells untreated with **BCS** and irradiated (●) and cells treated with 1 μM spermine and irradiated (○)

Consequently, the cell inactivation observed in *E. coli* cells treated with **BCS** after irradiation was mediated by the photosensitization activity of the bacteriochlorin. Bacterial suspensions incubated with 0.5 μM **BCS** and irradiated for 30 minutes produced 5.4 logs decrease in viability, whereas 1 μM of this compound exhibited a photosensitizing activity of 6.5 log units. These results indicate greater than 99.9999% of cellular inactivation.

In previous studies, a bis-cationic bacteriochlorin **BC-5** was evaluated against *E. coli* [21]. This photosensitizer produced an inactivation of 1 log at 1 μM, 3 logs at 10 μM and complete eradication at 100 μM. The tetrakis-cationic bacteriochlorin **BC-6** was more photoactive, inactivating 4 logs at 1 μM, 6 logs at 10 μM and killing the cells at 100 μM. The hexakis-cationic bacteriochlorin **BC-7** was even more effective, killing 1.5 logs at 0.100 μM and eradicating the *E. coli* cells at 1 μM after 10 J/cm^2 of 732 nm laser light. Also, three synthetic mono-substituted cationic bacteriochlorins (**BC-8**, **BC-9**, and **BC-10**) were evaluated as photosensitizers against *E. coli* [22]. The di-cationic compound **BC-9** gave a total eradication at 1 μM and 10 J/cm^2 of 700-850 nm light. In contrast, mono-cationic **BC-8** and **BC-10** were less effective for inactivating *E. coli*, requiring 5 μM to give eradication with **BC-8** and 5 logs of killing with **BC-10**. However, a low photoinactivation (<1.5 log) was found using 0.5 μM of these bacteriochlorins. In our case, 1 μM **BCS** caused 5 logs decrease in *E. coli* survival after 5 minutes irradiation (6 J/cm^2, Figure 5). Moreover, under similar experimental conditions and using the same bacterial strain, **BCS** was considerably more effective at inactivating *E. coli* than chlorin or porphyrin derivatives that contain four basic amino groups in the periphery of the macrocycle [30]. For Gram-negative bacteria a photosensitizer with positive charges is needed to bind to and penetrate the outer membrane, which contains negatively charged lipopolysaccharides [54].

In addition, photokilling ability of **BCS** was evaluated in a typical yeast, represented by *C. albicans*, to determinate a broader spectrum of activity of this photosensitizer [30, 31]. For *C. albicans* incubated with **BCS**, significant reductions in cell survival were obtained with respect to the control at all irradiated times (Figure 6). The photodynamic action yielded 3 logs decrease in the cell viability when *C. albicans* suspensions were treated with 0.5 μM **BCS** after 15 minutes irradiation. A high decrease of 4 logs in *C. albicans* survival was detected after 30 minutes irradiation. Using this fluence, a complete eradication of yeast cells was found for cultures incubated with 1 μM **BCS**. In previous studies, it was found that cationic bacteriochlorins **BC-5**, **BC-6** and **BC-7** were poorly effective to photoinactive *C. albicans* [21]. A low phototoxicity was found with the bis-cationic bacteriochlorin **BC-5**, with 1 to 2 logs of killing at 10-100 μM, while the tetrakis-cationic bacteriochlorin **BC-6** and the hexakis-cationic bacteriochlorin **BC-7** did not show any PDI effect. In particular, *C. albicans* was eradicated by **BC-10** at 1 μM, while **BC-8** required 2 μM to give the same eradication, and **BC-9** only gave 5 logs of cell killing at 5 μM [22].

Therefore, both the presence of positive charges and the amphiphilic character appear to play an important role in the molecular structure of effective photosensitizers to eradicate microorganisms [32, 55]. In this sense, spermine is a polyamine that is found as a polycation in a biological medium, converting **BCS** into an effective photosensitizer to kill microorganisms.

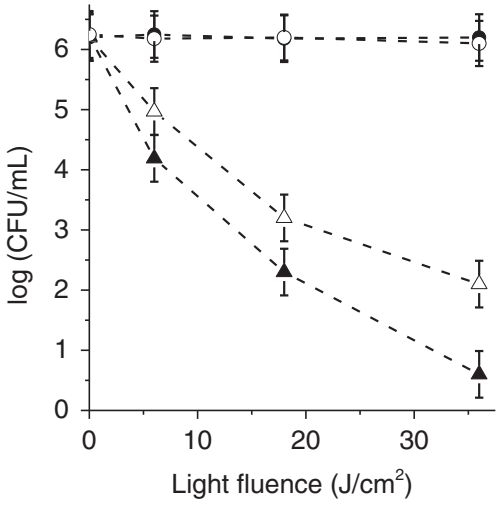


FIGURE 6 Survival curves of *Candida albicans* cells (~10^6 CFU/mL) incubated with **BCS** at concentrations of 0.5 μM (Δ) and 1 μM (▲) for 30 minutes at 37°C in the dark and irradiated for different periods. Cells untreated with **BCS** and irradiated (●) and cells treated with 1 μM spermine and irradiated (○)

3.5 | Dependence of PDI with incubation time

One of the fundamental concepts in PDI is to inactivate microorganisms using a low concentration of the phototherapeutic agent and also a low light fluence. Furthermore, a fast uptake of the photosensitizer is another of the principles to take into account to produce selective damage in microbial cells without affecting healthy tissue. Therefore, we investigated the effect of a short incubation time (5 minutes) on the cell survival of bacteria using different **BCS** concentrations (0.5 and 1 μM). Figure 7 shows the photoinactivation curves for *S. aureus* incubated with **BCS** for 5 minutes after different light fluences. Under these conditions, the photokilling of *S. aureus* cells treated with **BCS** for 5 minutes (Figure 7) was slightly lower than those found using 30 minutes of incubation (Figure 4). In addition, the photoinactivation of this Gram-positive bacterium was very similar after 30 minutes irradiation at two incubation times. On the other hand, survival curves of *E. coli* cells incubated with **BCS** for 5 minutes are shown in Figure 8. In contrast to *S. aureus*, a large difference was observed between 5 (Figure 8) and 30 minutes (Figure 5) incubation times. Photoinactivation considerably decreases in *E. coli* cells at the shorter irradiation times, although it remains high when 30 minutes of irradiation was used.

The differences observed in the efficacy of the PDI with the variation in the incubation time were previously investigated for cationic bacteriochlorins [22]. The greatest difference in cell death at different incubation times was found in Gram-negative *E. coli* and almost no modification with Gram-positive *S. aureus*. Moreover, upon comparison of both bacteria, *S. aureus* was more susceptible than *E. coli* to the photodynamic effect induced by **BCS**. It is well known

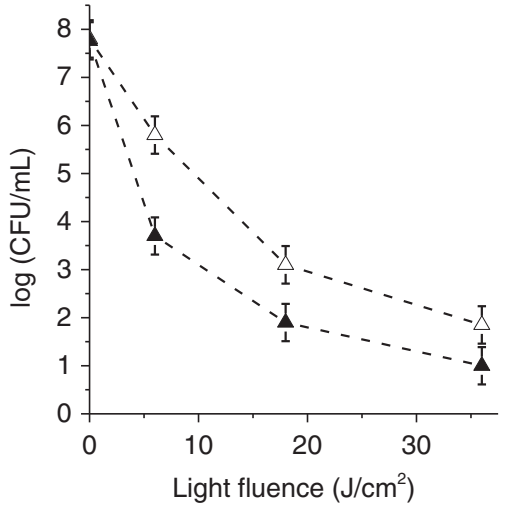


FIGURE 7 Survival curves of *Staphylococcus aureus* cells (~10^8 CFU/mL) incubated with 0.5 μM (Δ) and 1 μM (▲) **BCS** for 5 minutes in the dark at 37°C and irradiated for different periods

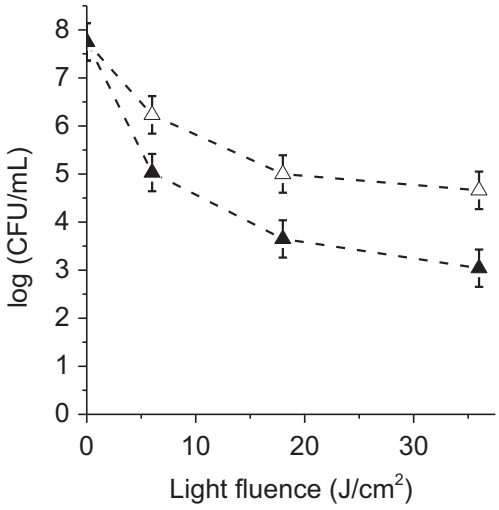


FIGURE 8 Survival curves of *Escherichia coli* cells (~10^8 CFU/mL) incubated with 0.5 μM (Δ) and 1 μM (▲) **BCS** for 5 minutes in the dark at 37°C and irradiated for different periods

that it is easier to inactivate Gram-positive than Gram-negative bacteria by PDI [56]. The outer membrane of Gram-negative bacteria forms an effective permeability barrier between the cell and the surrounding medium, tending to restrict the binding and penetration of many photosensitizer structures. Thus, for antimicrobial photosensitizers the presence of cationic groups is an important factor for a broad-spectrum antimicrobial effect. Therefore, Gram-positive bacteria are relatively porous cells for different agents and it is possible that photosensitizers diffuse through the cell wall relatively fast. In contrast, the mechanism of penetration of cationic photosensitizers into Gram-negative bacteria is different from those that depend on diffusion. Cationic photosensitizers can remove the divalent cations that hold the anionic lipopolysaccharide structures in the outer membrane. These electrostatic interactions disrupt the permeability barrier that is characteristic of Gram-negative bacteria [42]. This disruption process is expected to be slower than the process of diffusion into Gram-positive bacteria. In addition, it is possible that during the irradiation process a greater binding of the photosensitizer to the cells takes place. This uptake can mainly occur as the cell envelope becomes damaged by the photodynamic action. It is even conceivable that a redistribution of the photosensitizer also occurs in the cellular components [43].

4 | CONCLUSIONS

A new bacteriochlorin **BCS** substituted with two spermine groups was synthesized from **BC-Br**[3,13] by (a) a Suzuki coupling reaction to obtain **BCA** and (b) a reductive amination at two sites with spermine. The presence of multiple basic

groups of amines in the bacteriochlorin macrocycle may allow a better interaction of the phototherapeutic agent with the cell envelope of the bacterium. **BCS** showed a high absorption in the NIR spectral region that is accompanied by fluorescence emission. Moreover, **BCS** functioned as a photosensitizer to produce $O_2(^1\Delta_g)$ in DMF. **BCS** was evaluated as a novel photosensitizer with potential application in the photoinactivation of *S. aureus* and *E. coli*. Studies in vitro of PDI indicated that the photocytotoxic effect was higher for **BCS** than for **BCA**. **BCS** was able to eradicate (>7 logs killing) both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. This bacteriochlorin was also effective to kill *C. albicans*. The differences observed in the efficacy of the PDI with the variation in the incubation time indicated that the BCS was still effective in short treatment times. Although this photosensitizer is formally neutral, the eight basic amino groups in the spermine units can each acquire a positive charge in a biological medium. In addition, the mobility of such cationic groups spaced by aliphatic chains allows a strong interaction of the bacteriochlorin with the bacterial cell envelope. Therefore, **BCS** is a favorable molecular architecture as a phototherapeutic agent with applications in bacterial cell photoinactivation. **BCS** has high efficiency as a broad-spectrum antimicrobial photosensitizer. This molecular design of a phototherapeutic agent augurs very well for possible future clinical applications.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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