Макрогетероциклы
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DOI: 10.6060/mhc267029s

Novel Morpholine Substituted Chlorin p_6 Derivatives with Antimicrobial Activity

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The emergence of antibiotic-resistant bacterial strains is a global health challenge. The World Health Organization reports a growing trend of antimicrobial resistance. One approach to counteracting bacterial drug resistance involves the development of alternative methods for pathogen control, among which antimicrobial photodynamic therapy (aPDT) is a promising strategy. Various tetrapyrrole-based photosensitizers (PSs) are under investigation, with particular interest in those derived from natural chlorins. However, chlorin-based PSs used in clinical practice often exhibit insufficient efficacy against bacteria, necessitating the development of new molecular designs. In this work, we synthesized a series of photosensitizers based on a chlorin p_6 derivative functionalized with multiple morpholine residues at the macrocycle periphery. Their photophysical properties and photoinduced antimicrobial toxicity were evaluated in vitro against S. aureus and P. aeruginosa. Our results indicate that incorporating a morpholine moiety into the chlorin-based photosensitizer is a promising strategy for enhancing phototoxicity against both Gram-positive and Gram-negative pathogens.

Keywords: Chlorin p_6, morpholine, antibiotic resistance, antimicrobial photodynamic therapy, singlet oxygen generation.

Новые морфолин-замещённые производные хлорина p_6 с антимикробной активностью

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Появление новых антибиотикорезистентных штаммов бактерий является одной из глобальных проблем здоравоохранения. По данным Всемирной организации здравоохранения наблюдается тенденция роста антимикробной устойчивости. Одним из шагов к снижению устойчивости бактерий к действию лекарственных препаратов является разработка альтернативных методов борьбы с патогенами, среди которых многообещающим является антимикробная фотодинамическая терапия (АФДТ). На сегодняшний день разрабатываются различные тетрапиррольные фотосенсибилизаторы (ФС), особый интерес среди которых представляют ФС на основе природных хлоринов, однако применяемые в клинической практике хлориновые фо-

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тосенсибилизаторы обладают недостаточной эффективностью в отношении бактерий, что ставит перед исследователями задачу по разработке новых молекул. В настоящей работе были получены фотосенсибилизаторы на основе производного хлорина p_6, содержащие несколько остатков морфолина на периферии макроцикла, изучены их фотофизические свойства и фотоиндуцированная антимикробная токсичность in vitro в отношении бактерий S. aureus и P. aeruginosa. Показано, что введение морфолинового фрагмента в состав хлоринового фотосенсибилизатора является перспективной стратегией повышения фототоксичности в отношении грамположительных и грамотрицательных патогенов.

Ключевые слова: Хлорин p_6, морфолин, устойчивость к антибиотикам, антимикробная фотодинамическая терапия, генерация синглетного кислорода.

Introduction

The emergence of new antibiotic-resistant bacterial strains is a global public health challenge. According to the World Health Organization, there is a trend of increasing antimicrobial drug resistance. For instance, the resistance of *E. coli* to third-generation cephalosporins rose from 26.9 % in 2016 to 41 % in 2021, while methicillin resistance in *S. aureus* increased from 13.6% to 32%.[1] This problem is exacerbated by the ability of bacteria to form biofilms, which significantly complicates treatment and leads to the development of recurrent infections.[2] It is noted that each year, more than half of fatal cases of bacterial infections are associated with pathogen resistance to the drugs used.[3,4] According to statistical data,[4] in 2021, approximately 1.2 million people died due to bacterial resistance to antimicrobial agents. One approach to addressing this issue is the development of alternative antimicrobial therapies capable of preventing infection, replacing existing antibiotics, or enhancing their efficacy while reducing the likelihood of pathogen resistance development.

Over the past two decades, various methods to combat pathogens without relying on antibiotics have been actively developed. These include the design of antimicrobial peptides, the use of nanomaterials and nanoparticles, the application of monoclonal antibodies, bacteriophage therapy, phototherapy, the use of probiotics, and the development of vaccines.[5–9] Antimicrobial photodynamic therapy (aPDT) stands out as one of the most promising approaches to addressing the challenge of antibiotic resistance.[10] The essence of aPDT lies in the photoinduced excitation of a photosensitizer (PS), which leads to the generation of reactive oxygen species (ROS) and subsequent oxidative damage to bacterial cells.[11] This method offers several advantages: it enables localized treatment with minimized systemic side effects, is non-invasive, and exhibits a broad spectrum of antibacterial activity.[5,11–13]

Currently, various photosensitizers of both inorganic and organic origin are under development. Although inorganic photosensitizers possess suitable photophysical properties and high photostability, they are typically characterized by high cytotoxicity and low biocompatibility.[11] Organic photosensitizers generally lack these drawbacks. The literature describes examples of synthesizing organic photosensitizers based on BODIPY,[14] phthalocyanines,[15] porphyrins,[16] chlorins,[17] and synthetic bacterio-chlorins.[18] Tetrapyrrole compounds constitute a broad class of photosensitizers first proposed for use in photodynamic therapy (PDT).[19] Chlorin-based photosensitizers derived from natural sources are of particular interest within this group, offering advantages such as low cost of the starting material, high-intensity light absorption in the red spectral region, biocompatibility, and low dark toxicity.[19–21] However, the chlorin-based photosensitizers currently used in clinical practice still exhibit insufficient efficacy against pathogens, necessitating the development of novel compounds.[21–23]

Several strategies exist to enhance the antimicrobial activity of chlorin derivatives. These include developing photosensitizer delivery systems,[24] synthesizing cationic compounds,[25] and modifying the chlorin macrocycle by attaching bioactive molecules to its periphery.[26,27] Morpholine and its derivatives represent one of the most important structural motifs in the design of bioactive molecules due to their attractive physicochemical, biological, and metabolic properties.[28] Substituted morpholines exhibit a broad spectrum of biological activities, including antibacterial, antifungal, and anti-inflammatory effects.[29–31] Various antimicrobial agents with improved pharmacokinetic profiles that incorporate a morpholine ring are currently developed and actively used in clinical practice, such as Finafloxacin[28] and Linezolid.[32] In the former, the morpholine moiety enhances antibiotic activity at low p*H* values,[28] while in the latter, it improves the pharmacokinetic profile and reduces the toxicity of oxazolidinone-class drugs.[33] Ahumada-Santos Y.P. and colleagues[34] synthesized a series of *N*-alkylmorpholines and evaluated their antibacterial activity in comparison with Linezolid. They demonstrated that increasing the alkyl chain length to 12 and 13 methylene units leads to enhanced activity, with a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 3.9 μg/mL, exceeding the efficacy of the commercial drug. Molecular docking confirmed the importance of the morpholine ring for forming stacking interactions with the 50S ribosomal protein of *S. aureus* at positions Lys54, Arg58, Asn91, and Phe57. Harmandar K. *et al.*[16] synthesized AB_3-type *meso*-arylporphyrin derivatives containing substituted morpholine residues, which exhibited antimicrobial activity, predominantly against Gram-positive bacteria. Various morpholine-containing chlorins[35] and bacteriochlorins[36] have also been developed, wherein one of the pyrrole rings is replaced by a substituted morpholine residue. Such modification improves the photosensitiser's spectral properties and increases the efficiency of reactive oxygen species generation.

Earlier, a method for introducing a morpholine moiety into the structure of a natural chlorin to enhance the photosensitizer's hydrophilicity was described. For instance,

the work[37] reported the opening of the exocycle E of methylpheophorbide *a* under the action of secondary amines, including morpholine, yielding a trimethyl ester derivative of chlorin e_6 containing one morpholine residue at the 13-position of the macrocycle. Building upon these findings, the present study develops novel approaches for incorporating multiple morpholine fragments onto the periphery of the chlorin macrocycle. This modification is expected to improve both the efficacy of antimicrobial PDT and the pharmacokinetic profile of the resulting photosensitizers . Within this framework, chlorin p_6 derivatives bearing multiple morpholine residues at various macrocycle positions were synthesized. Their photophysical properties and antimicrobial activity against both Gram-positive and Gram-negative bacteria were investigated.

Experimental

Materials

tert-Butyl-(2-aminoethyl)-carbamate (**1**), purpurin 18, and the trimethyl ester of 3-devinyl-3-carboxychlorin p_6 were obtained according to previously described procedures.[25,38,39] Solvents were purified using standard methods. Reaction progress was monitored by analytical thin-layer chromatography (TLC) on Kieselgel 60 F_{254} plates (Merck). Chromatographic purification of the obtained compounds was performed on columns packed with Kieselgel 60 40/63 μm silica gel (Merck) and on preparative TLC plates coated with Kieselgel 430 40/60 μm silica gel (Merck). ^{1}H NMR spectra were recorded on a Bruker DPX-300 spectrometer operating at 300 MHz at 25 °C using $CDCl_3$ as the solvent. Signals were assigned using the residual proton signal of chloroform as an internal reference. High-resolution mass spectra (HRMS) were recorded on an Orbitrap Elite mass spectrometer (Thermo Scientific). ESI-MS spectra were acquired on a Bruker micrOTOF II mass spectrometer using electrospray ionization (ESI). Electronic absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer. Fluorescence emission spectra were recorded on a Cary Eclipse spectrofluorimeter (Agilent Technologies) in $CHCl_3$. The fluorescence quantum yield (Φ_F) was calculated relative to a standard – the trimethyl ester of chlorin e_6 (absolute Φ_F=0.25 in CH_2Cl_2). The quantum yield of singlet oxygen generation (Φ_Δ) was determined using 5,6,11,12-tetraphenyltetracene (Rubrene) as a trap and a light source based on a red light-emitting diode ($\lambda = 680 \pm 20$ nm, Ps = 16 mW/cm^2). Microorganisms were grown using Mueller-Hinton broth. For biological studies, an LED-based irradiator was used as the light source ($\lambda = 660 \pm 10$ nm, Ps = 7 mW/cm^2). To measure the optical density of bacterial suspensions, iMark photometer was used. Biosan microspin 12 centrifuge was used to separate cell cultures from the liquid medium.

Synthesis

*N¹,N¹-Bis[2-(N-morpholino)ethyl]ethane-1,2-diamine (**3**).* A mixture of *tert*-butyl(2-aminoethyl)carbamate **(1)** (0.500 g, 3.121 mmol), *N*-(2-chloroethyl)morpholine hydrochloride (**2**) (1.452 g, 7.803 mmol), KI (1.036 g, 6.241 mmol), and K_2CO_3 (1.725 g, 12.482 mmol) in acetonitrile (15 mL) was heated under reflux with stirring for 6 h. The reaction progress was monitored by TLC. Upon completion, the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate, washed with water and a saturated aqueous NaCl solution, and extracted with ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (eluent CH_2Cl_2/MeOH/NH_4OH, 200/10/1, v/v/v). Yield: 41% (0.490 g). ^{1}H NMR (DMSO-d_6) δ_H ppm: 6.78 (t, J = 4.9 Hz, 1H, 1-NH), 3.56 (t, J = 4.7 Hz, 8H, 7-CH_2), 2.96 (q, J = 5.9 Hz, 2H, 2-CH_2), 2.53 (t, J = 6.9 Hz, 4H, 4-CH_2), 2.45 (t, J = 6.3 Hz, 2H, 3-CH_2), 2.41–2.22 (m, 12H, 5,6-CH_2), 1.37 (s, 9H, 9-CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ_C ppm: 155.5, 77.5, 66.1, 56.1, 53.5, 51.8, 50.7, 28.3. HRMS (ESI$^+$), *m/z*: 387.2971 $[M+H]^+$ calculated for $C_{19}H_{39}N_4O_4$ 387.2967.

General procedure for the synthesis of chlorin-morpholine conjugates. To a solution of the corresponding chlorin p_6 ester (1 eq.) in DMF (1 mL per 20 mg of chlorin) was added a solution of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1.4 eq.) and triethylamine (1.4 eq.) in DMF (0.4 mL per 7 mg of the ester). The carboxyl group was activated by stirring the mixture for 45 min at room temperature under an argon atmosphere.

For the removal of the Boc-protecting group from the amine component, compound **3** (1.2 eq.) was treated with a 10% solution of trifluoroacetic acid in dichloromethane (1 mL per 11 mg of **3**). The reaction was stirred for 30 min at room temperature. Subsequently, the reaction mixture was concentrated under reduced pressure, redissolved in dichloromethane (1 mL per 7 mg of the initial mass), and concentrated again (this cycle was repeated twice). The residue was then dried under a vacuum from a membrane pump for 10 min.

To a solution of the obtained N^1,N^1-bis(2-morpholinoethyl)-ethane-1,2-diamine (**4**) in DMF (1 mL per 22 mg) was added Et_3N (4 eq. relative to **4**). This mixture was then added to the activated chlorin derivative. The reaction was stirred for 15 h at room temperature under an argon atmosphere. The solvent was removed under reduced pressure. The crude mixture was dissolved in dichloromethane, washed once with water, and extracted with dichloromethane (except for compound **11**). The combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The product was purified by preparative thin-layer chromatography (PTLC) (eluent CH_2Cl_2/MeOH/NH_4OH 100/10/1, v/v/; 150/10/1, v/v/v for compound **11**).

Starting from compound **6** (30 mg, 0.049 mmol), compound **7** was obtained in 81% yield (35 mg). ^{1}H NMR ($CDCl_3$) δ_H ppm: 9.69 (s, 1H, 10-H), 9.45 (s, 1H, 5-H), 8.68 (s, 1H, 20-H), 7.96 (dd, J = 17.8, 11.5 Hz, 1H, 3-CH), 6.33–6.27 (dd, J = 17.8, 1.5 Hz, 1H, 3-CH_2(a)), 6.18 (s, 1H, 17-NH), 6.14–6.10 (dd, J = 11.5, 1.5 Hz, 1H, 3-CH_2(b)), 5.17 (m, 1H, 17-H), 4.48 (q, J = 6.8 Hz, 1H, 18-H), 4.24 (s, 3H, 13-CH_3), 4.18 (s, 3H, 15-CH_3), 3.72 (m, 2H, 8-CH_2), 3.65 (s, 3H, 2-CH_3), 3.40 (m, 11H, 12-CH_3, 9^2, $9'^2$, 9^4, $9'^4$-CH_2 Mor), 3.22 (s, 3H, 7-CH_3), 3.09 (m, 2H, 17^5-CH_2), 2.39 (m, 8H, 17^1-CH_2(a), 17^2-CH_2(a), 17^6-CH_2, 17^8-CH_2, $17^{8'}$-CH_2), 2.13 (m, 14H, 17^1-CH_2(b), 17^2-CH_2(b), 17^9-CH_2, $17^{9'}$-CH_2, 9^1, $9'^1$, 9^5, $9'^5$-CH_2 Mor), 1.85 (d, J = 7.2 Hz, 3H, 18-CH_3), 1.68 (t, J = 7.7 Hz, 3H, 8-CH_3), –0.86 (bs, 1H, 21-NH), -0.99 (bs, 1H, 23-NH). HRMS (ESI$^+$), *m/z*: 877.4982 $[M-H]^-$ calculated for $C_{49}H_{66}N_8O_7$ 877.501; 440.2603 $[M+2H]^{2+}$ calculated 440.2600. UV/VIS ($CHCl_3$) λ_{max} nm ($\varepsilon \times M^{-1}$ cm^{-1}): 402 (110.6); 500 (9.74); 531 (5.74); 615 (4.82); 670 (35.9).

Starting from compound **8** (30 mg, 0.049 mmol), compound **9** was obtained in 61% yield (36 mg). ^{1}H NMR ($CDCl_3$) δ_H ppm: 9.79 (s, 1H, 10-H), 9.62 (s, 1H, 5-H), 8.72 (s, 1H, 20-H), 8.01 (s, 1H, 3-NH), 5.18 (m, 1H, 17-H), 4.42 (q, J = 6.7 Hz, 1H, 18-H), 4.24 (s, 3H, 13-CH_3), 4.18 (s, 3H, 15-CH_3), 3.96 (m, 2H, 3^3-CH_2), 3.62 (m, 5H, 2-CH_3, 8-CH_2), 3.57 (s, 3H, 12-CH_3), 3.54 (s, 3H, 17-CH_3), 3.38 (m, 8H, 7^2, $7'^2$, 7^4, $7'^4$-CH_2 Mor), 3.19 (s, 3H, 7-CH_3), 3.07 (m, 2H, 3^4-CH_2), 2.86 (m, 4H, 3^6-CH_2, $3^{6'}$-CH_2), 2.54 (m, 4H, 3^7-CH_2, $3^{7'}$-CH_2), 2.38 (m, 10H, 17^1-CH_2(a), 17^2-CH_2(a), 7^1, $7'^1$, 7^5, $7'^5$-CH_2 Mor), 2.14 (m, 2H, 17^1-CH_2(b), 17^2-CH_2(b)), 1.84 (d, J = 7.1 Hz, 3H, 18-CH_3), 1.64 (t, J = 7.6 Hz, 3H, 8-CH_3), -1.00 (bs, 1H, 21-NH), –1.34 (bs, 1H, 23-NH). MS ESI$^+$, *m/z*: 911.4 $[M+H]^+$ calculated for $C_{49}H_{66}N_8O_9$ 911.5. UV/VIS ($CHCl_3$) λ_{max} nm ($\varepsilon \times M^{-1}$ cm^{-1}): 400 (105.9); 500 (9.53); 531 (5.25); 619 (4.78); 674 (36.4).

Note: All reagent quantities were doubled for the synthesis of compound **11**. Starting from compound **10** (39 mg, 0.063 mmol), compound **11** was obtained in 27% yield (20 mg). ^{1}H NMR ($CDCl_3$) δ_H ppm: 9.82 (s, 1H, 10-H), 9.72 (s, 1H, 5-H), 8.75 (s, 1H, 20-H), 8.00 (bs, 1H, 3-NH), 6.77 (bs, 1H, 17-NH), 5.14 (m, 1H, 17-H), 4.50 (m, 1H, 18-H), 4.23 (s, 3H, 13-CH_3), 4.18 (s, 3H, 15-CH_3), 3.95 (m, 2H, 3^3-CH_2), 3.72 (m, 2H, 8-CH_2), 3.64 (s, 3H, 2-CH_3), 3.56 (s, 3H, 12-CH_3), 3.43–3.36 (m, 20H, 3^4-CH_2, 17^5-CH_2, 7^2, $7'^2$, 7^4, $7'^4$, 9^2, $9'^2$, 9^4, $9'^4$-CH_2 Mor), 3.25 (s, 3H, 7-CH_3), 3.07 (m, 6H, 3^6, $3^{6'}$, 17^6-CH_2), 2.87 (m, 4H, 17^8, $17^{8'}$-CH_2)), 2.56 (m, 8H, 3^7, $3^{7'}$,17^9, $17^{9'}$-CH_2), 2.43–2.32 (m, 20H, 17^1-CH_2, 17^2-CH_2, 7^1, $7'^1$, 7^5, $7'^5$, 9^1, $9'^1$, 9^5, $9'^5$-CH_2 Mor), 1.84 (m, 3H, 18-CH_3), 1.69 (t, J = 7.7 Hz, 3H, 8-CH_3), –0.95 (bs, 1H, 21-NH), –1.25 (bs, 1H, 23-NH). MS ESI$^+$, *m/z*: 1165,7 $[M+H]^+$ calculated for $C_{62}H_{92}N_{12}O_{10}$ 1165.7. UV/VIS (acetone) λ_{max} nm ($\varepsilon \times M^{-1}$ cm^{-1}): 396 (68.8); 497 (6.60); 527 (2.92); 614 (2.92); 669 (26.2).

General procedure for obtaining micellar forms. A solution of the photosensitizer (PS) in CH_2Cl_2 (1 mL per 1 mg of compound) was added to a 4% (w/v) solution of Kolliphor® ELP in phosphate-buffered saline (PBS) (1 mL per 1 mg of compound). The resulting mixture was purged with a stream of argon while heating on a water bath at 41 °C. The concentration of the final solutions was determined spectrophotometrically. Conjugate **11** was dissolved directly in a 4% (w/v) solution of Kolliphor® ELP in PBS under sonication.

Photoinduced toxicity study

To evaluate the antimicrobial activity, reference strains of the Gram-positive bacterium *S. aureus* ATCC 25923 and the Gram-negative bacterium *P. aeruginosa* ATCC 27853 were used. Aliquots (600 μL) of microbial suspensions at a concentration of 10^9 CFU/mL were placed into centrifuge tubes. Solutions of compounds **7**, **9**, and **11** were added to the tubes to achieve final photosensitizer concentrations of 3.125, 6.25, 12.5, 25, and 50 μM, respectively. The cells were incubated with these solutions for 30 min. Subsequently, the suspensions were centrifuged, and the supernatant was removed. The pellets were resuspended in 600 μL of PBS. The centrifugation and washing procedure was repeated twice. Finally, the pellet was resuspended in 600 μL of Mueller-Hinton broth.

The resulting suspensions (100 μL aliquots) were transferred into a 96-well microplate. One set of wells was then irradiated with red light (λ = 660 nm, Ps = 10 mW/cm^2) to achieve a light dose of 10 J/cm^2, while another set was kept in the dark. The viability of the microorganisms was determined photometrically by measuring the optical density (λ = 600 nm) in each well after incubating the microplates in the dark at 37 °C for 24 h.

Positive controls consisted of test strains subjected to the same conditions, including light irradiation, but without incubation with the photosensitizers. Negative controls consisted of test strains that were neither exposed to light nor incubated with the photosensitizers. All experiments were performed in triplicate.

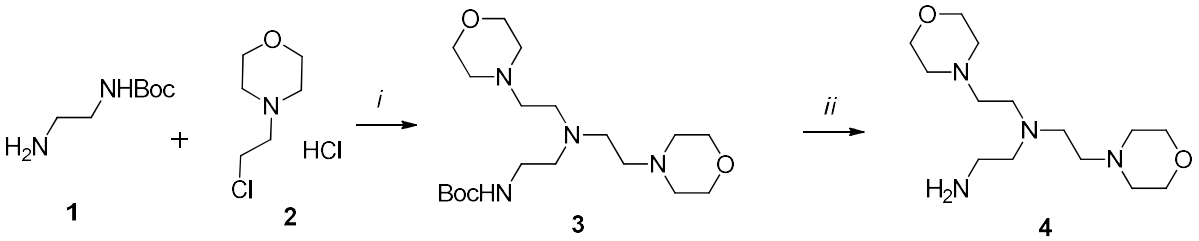
Results and Discussion

To synthesize chlorin-morpholine conjugates, the initial stage of the work involved the preparation of amino-amide derivatives of pheophorbide *a* according to previously described procedures,[40,41] followed by alkylation of the terminal amino group of the chlorin with *N*-(2-chloroethyl)morpholine. However, upon implementing this approach, it was found that under the alkylation conditions used, only one morpholine residue was introduced onto the macrocycle periphery. Further attempts to extend the reaction primarily increased the formation of undesired poly-alkylation products at the nitrogen atoms of the terminal amino group. To overcome this limitation, we modified the synthetic strategy and prepared compound 4, which contains two morpholine residues and a free amino group for subsequent conjugation with the chlorin molecule. Compound **4** was synthesized via alkylation of 1-[(*tert*-butoxycarbonyl)amino]-2-aminoethane (**1**) with an excess of the morpholine-containing derivative **2**, followed by removal of the Boc protecting group, as outlined in Scheme 1.

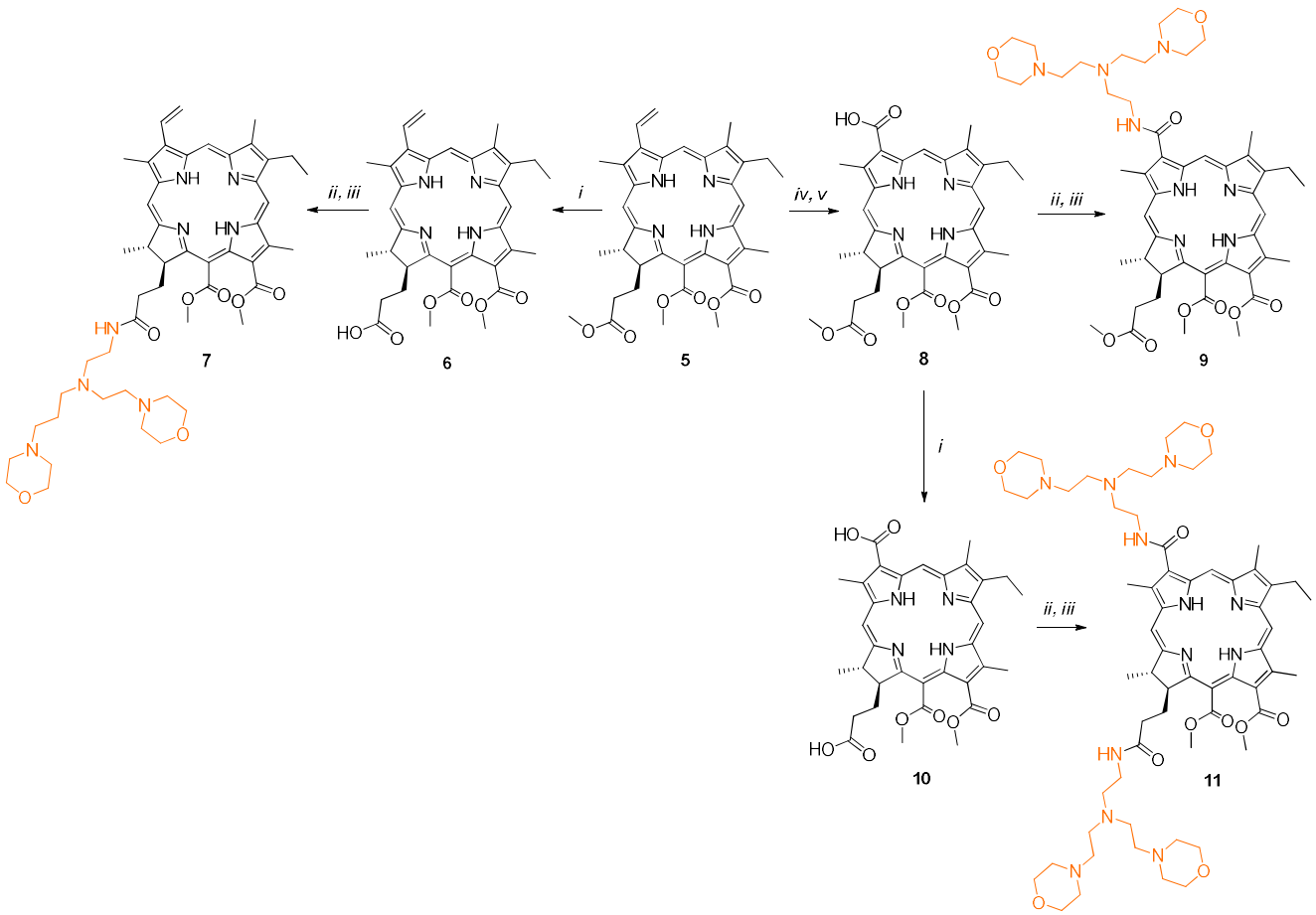
The structure of compound **3** was confirmed by NMR spectroscopy and mass spectrometry. The ^{1}H and ^{13}C NMR spectra displayed characteristic signals corresponding to the protons of the morpholine ring, the *tert*-butoxycarbonyl (Boc) protecting group, and the methylene linkers (Figures S1, S2, *Supporting Information*). The mass spectrum of compound 3 showed a molecular ion peak at an *m/z* value consistent with the calculated mass (Figure S3).

The starting material for the synthesis of the chlorin component **7** was purpurin 18, which was obtained from chlorophyll *a* isolated from the biomass of the microalga *Spirulina platensis* according to a previously described procedure.[37] Alkaline hydrolysis of the anhydride ring of purpurin 18, yielding chlorin p_6, followed by methylation with diazomethane, afforded the trimethyl ester of chlorin p_6 (**5**). To deprotect the carboxylic acid group at the 17^3-position, compound **5** was subjected to acid hydrolysis using a solution of HCl in tetrahydrofuran at room temperature. Under these conditions, regioselective hydrolysis of the methyl ester of the propionic acid side chain occurs, while preserving the aromatic methyl ester groups at the 13- and 15-positions[42] (Scheme 2).

The synthesis of conjugate **7** was accomplished using the activated ester method. The free carboxylic acid group of the 13,15-dimethyl ester of chlorin p_6 (**6**) was activated with HBTU in DMF. Subsequently, the morpholine derivative **4** was added to the activated chlorin intermediate.



Scheme 1. Synthesis of compound **4**.Reagents and conditions: i) K_2CO_3, KI, CH_3CN, Δ, 6 h; ii) CF_3COOH, CH_2Cl_2, 20 °C, 45 min.



Scheme 2. Synthesis of chlorin-morpholine conjugates. Reagents and conditions: i) HCl/H_2O, THF, 20 °C; ii) HBTU, Et_3N, DMF, Ar, 45 min; iii) trifluoroacetate salt of N^1,N^1-bis[2-(N-morpholino)ethyl]ethane-1,2-di-amine, Et_3N, DMF; iv) $NaIO_4$, OsO_4, THF/H_2O; v) $NaClO_2$, NH_2SO_3H, DMSO, H_2/THF.

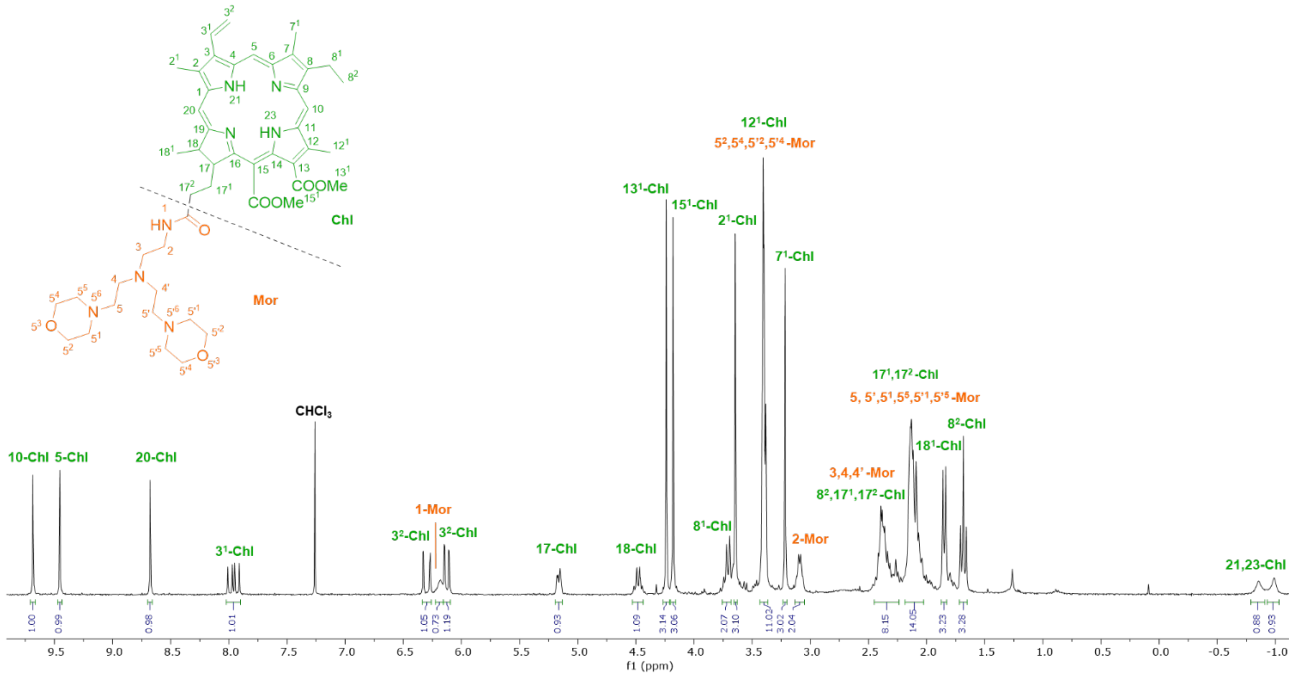


Figure 1. ^{1}H NMR spectrum of compound **7**.

The structure of the resulting product **7** was confirmed by NMR spectroscopy (Figure 1) and high-resolution mass spectrometry (Figure S4). Signal assignments for the morpholine and chlorin macrocycle fragments were accomplished using two-dimensional NMR spectroscopy (Figure S5). The high-resolution mass spectrum displayed molecular ion peaks consistent with the calculated values for compound **7**.

Our previously published studies demonstrated a significant influence of the substitution site on the biological activity of photosensitizers.[43] To establish a structure–activity relationship within the series of morpholine-containing chlorin photosensitizers, we synthesized a chlorin p_6 derivative bearing morpholine residues at the 3-position of the chlorin macrocycle (Scheme 2). This chemical modification was achieved by initially oxidizing the vinyl group in pyrrole ring A to a carboxylic acid group, following our previously optimized two-step procedure.[39]

The target conjugate **9** was prepared using a procedure analogous to that described above for compound **7** (Scheme 2). Product **9** was characterized by a combination of physicochemical analytical methods, including ^{1}H NMR spectroscopy (Figure S6), two-dimensional {^{1}H,^{1}H} COSY NMR spectroscopy (Figure S7), and mass spectrometry (Figure S8).

As mentioned earlier, a significant drawback of many existing photosensitizers is their hydrophobicity. To enhance the hydrophilicity of the morpholine-chlorin conjugates, we synthesised a derivative bearing four morpholine residues at the 3- and 17-positions. For this purpose, compound **8** was subjected to hydrolysis using HCl in THF, affording compound **10** with carboxylic acid groups at the 3- and 17-positions of the chlorin macrocycle (Scheme 2).

The target conjugate **11** was prepared analogously to the synthesis of compound **7** (Scheme 2). The ^{1}H NMR spectrum of chlorin **11** exhibits signals corresponding to the protons of the morpholine residues and methylene groups (Figure S9). The mass spectrum of compound **11** contains a molecular ion matching the calculated value (Figure S10).

Thus, we have synthesized a series of chlorin p_6-based photosensitizers containing multiple morpholine residues on the macrocycle periphery.

To evaluate the therapeutic potential of the obtained photosensitizers for antimicrobial photodynamic therapy (aPDT), their photophysical properties were investigated. The spectral characteristics and fluorescence quantum yields are presented in Table 1. The spectral profiles and fluorescence quantum yield values of all synthesised compounds indicate that the morpholine-substituted conjugates are suitable for studying antimicrobial effects both *in vitro* and *in vivo*.[44–46]

An important parameter for assessing photosensitiser efficacy is the singlet oxygen quantum yield (Φ_Δ). We performed an indirect measurement of $^1O_2$ generation by monitoring the decrease in absorption intensity of a photochemical trap, for which rubrene was selected. Chlorin e_6 trimethyl ester (Che_6 TME) was used as the reference standard photosensitizer. Measurements were carried out using a modified procedure[47] with a monochromatic diode light source at a power density of 16 ± 2 mW/cm^2.

The synthesized morpholine-containing chlorin p_6 derivatives possess sufficiently high singlet oxygen quantum yields (Φ_Δ) for effective aPDT. The most efficient compound was **7**, with a Φ_Δ value of 0.55, slightly exceeding that of the standard photosensitizer. For conjugates **9** and **11**, a decrease in $^1O_2$ generation was observed, attributed to the introduction of an electron-withdrawing amide substituent at the 3-position of the chlorin macrocycle (Table 1).

Table 1. Photophysical characteristics of compounds **7**, **9**, **11** in CH_2Cl_2.

Compound	λ_{max}, nm	ε, M^{-1}cm^{-1}	Φ_F	Φ_Δ
Che_6 TME	667	35000	0.25	0.5
7	670	36000	0.34	0.55
9	675	36000	0.27	0.32
11	669	26000	0.29	0.20

Assessment of Antimicrobial Photoinduced Cytotoxicity

The primary assessment of the photoinduced antimicrobial cytotoxicity of the synthesized morpholine-substituted chlorins **7**, **9**, and **11** was evaluated against Gram-

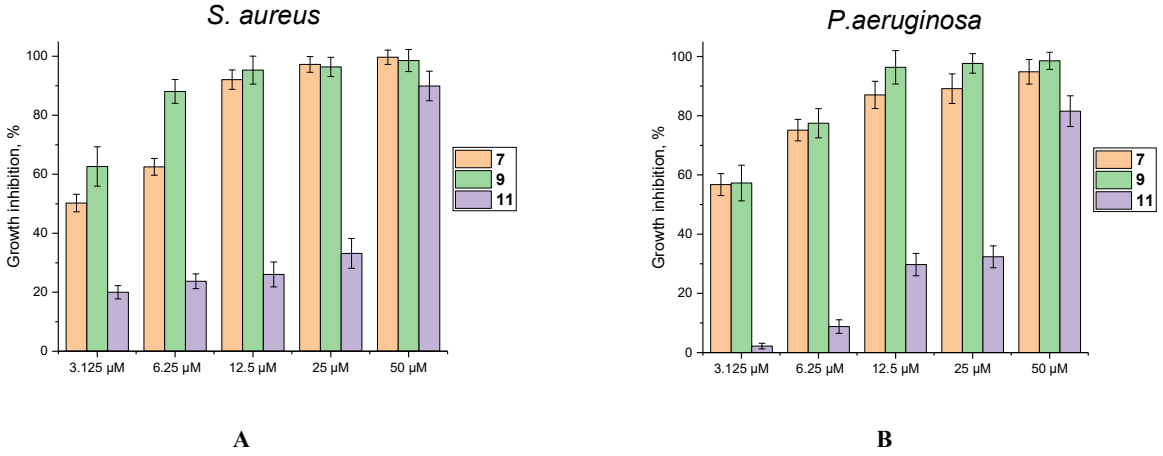


Figure 2. Photoinactivation of Gram-positive *S. aureus* (A) and Gram-negative *P. aeruginosa* (B) following incubation with photosensitizers **7**, **9**, and **11**.

positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria. For the studies, water-dispersible forms of photosensitizers **7** and **9** were prepared as emulsions in a 4% (w/v) solution of Kolliphor® ELP according to our previously described procedure.[48] Compound **11**, possessing greater hydrophilicity, was dissolved directly in the Kolliphor® ELP solution. Bacterial cells were incubated with photosensitizer solutions for 30 min at concentrations of 3.125, 6.25, 12.5, 25, and 50 μM, followed by washing to remove residual pigment. Subsequently, the cells were irradiated with red light ($\lambda = 660 \pm 10$ nm; Ps = 10 mW/cm^2) to achieve a total light dose of 10 J/cm^2. Control experiments utilized the same bacterial strains, which were irradiated without prior incubation with the photosensitizers.

Light irradiation of cells pre-incubated with the synthesized photosensitizers resulted in a concentration-dependent inhibition of bacterial growth. Conjugates **7** and **9**, each bearing two morpholine residues, exhibited greater photoinduced cytotoxicity across the entire concentration range compared to derivative **11**, which contains four morpholine residues (Figure 2). Notably, compound **11** displayed significant activity only at a concentration of 50 μM. For compound **9**, consistent photoinactivation of Gram-positive bacteria was observed at concentrations of 6.25 μM and above, while for Gram-negative pathogens, the effective concentration started at 12.5 μM. In contrast, photosensitizer **7** demonstrated a phototoxic effect at a concentration of 12.5 μM against both bacterial types. Although the singlet oxygen quantum yield for compound **7** is higher, the obtained data indicate greater activity for conjugate **9**. This discrepancy may be attributed to differences in the photosensitizers' ability to penetrate the bacterial cell wall. It is also important to note that the *in vitro* results are consistent with previously established structure–activity relationships for chlorin photosensitizers containing substituents on pyrrole ring A.[43]

Conclusions

In this study, a series of novel photosensitizers based on chlorin p_6 and functionalized with multiple morpholine residues at the macrocycle periphery was synthesized. The structures of the target compounds were confirmed by NMR spectroscopy and high-resolution mass spectrometry. Preliminary photoinduced antimicrobial activity of the synthesized compounds was evaluated against reference strains of Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria.

In vitro investigations demonstrated that incorporating a morpholine moiety into the chlorin-based photosensitiser is a promising strategy for enhancing its phototoxicity in the context of antimicrobial photodynamic therapy (aPDT). Notably, photosensitizers bearing two morpholine residues exhibited the highest activity. The introduction of a larger number of morpholine substituents led to a decrease in key photophysical parameters and, consequently, to a reduction in photoinduced cytotoxicity. This decline in efficacy may also be attributed to an excessive increase in molecular polarity, which hinders efficient penetration through the bacterial cell wall. Our findings identify PS **9** as a promising lead compound for further development. Thus, based on this compound, it is possible to obtain a polycationic derivative, which will potentially have a greater ability to penetrate into bacterial cells. However, definitive confirmation of its therapeutic potential and safety profile requires an expanded set of physicochemical and biological studies, including both *in vitro* and *in vivo* models.

Acknowledgements. The work was carried out with the support of the Ministry of Science and Higher Education of the Russian Federation (FSFZ-2024-0013).

This work was performed using the equipment of the Shared Science and Training Center for Collective Use of RTU MIREA and was supported by the Ministry of Science and Higher Education of the Russian Federation under Agreement No. 075-15-2025-548 (dated 18.06.2025).

Author Contribution. Margarita A. Shagabaeva: investigation; writing – original draft; visualization. Nikita V. Suvorov: conceptualization; project administration; funding acquisition. Alexey N. Noev: investigation; visualization. Viktoria V. Shchelkova: investigation; formal analysis. Alexander A. Popov: investigation. Sergey I. Tikhonov: investigation. Viktor A. Pogorilyy: writing – review and editing. Nadezhda V. Konovalova: investigation; writing – review and editing. Yuri L. Vasil'ev: methodology; resources. Mikhail A. Grin: supervision.

Supporting Information

^{1}H NMR spectra, mass spectra of compounds **3**, **7**, **9**, **11**, ^{13}C NMR spectrum of compound **3**, {^{1}H,^{1}H} COSY spectra for compounds **7** and **9**, and absorption spectra for compounds **7**, **9**, **11** are provided in the Supporting Information.

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Received 01.02.2026
Accepted 02.03.2026