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Enhancement of 5-Aminolevulinic Acid-Mediated Photodynamic Inactivation of *Proteus mirabilis* Using Phosphoric and Bisaminophosphinic Acids as Permeabilizing Agents

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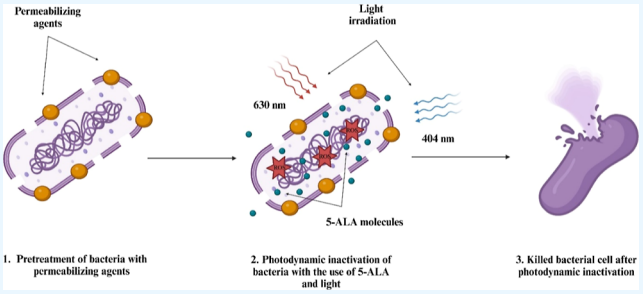
ABSTRACT: The purpose of this work was to examine the effect of phosphoric and bisaminophosphinic acids on the effectiveness of photoinactivation of *Proteus mirabilis* with 5-aminolevulinic acid (5-ALA) as a precursor of protoporphyrin IX. Two diode lasers λ = 404 nm (radiation intensity 26 mW cm^{-2}) and λ = 630 nm (radiation intensity 55 mW cm^{-2}) were used as sources of light. The most effective agent was (*R*)-(−)-1,1′-binaphthyl-2,2′-diylhydrogen phosphate, and a significant improvement in bactericidal effect of 5-ALA-aPDI was achieved by pretreating *P. mirabilis* with this compound at nontoxic concentrations of 0.368 mM. It was found that 15 min of blue light illumination was enough to achieve bacterial cell mortality of 99.999%. Photoelimination of this pathogen using red light was less effective, and the required killing effect (at least 99.99%) was not achieved until 45 min of exposure. The mechanism of increased pathogen destruction by the examined acids is multifaceted and includes not only the destabilization of the outer bacterial cell membrane by organophosphates but also an increase in the level of protoporphyrin IX in cells due to chelation of iron ions. Furthermore, a synergistic effect of intracellular photosensitizers and (*R*)-(−)-1,1′-binaphthyl-2,2′-diylhydrogen phosphate acting as an additional blue/red light-induced photosensitizer cannot be excluded.



1. INTRODUCTION

In recent decades, antibacterial photodynamic inactivation (aPDI) has become increasingly popular as a new approach to killing pathogens, independent of the existing status of antibiotic resistance. This technique is based on the treatment of pathogenic cells with a nontoxic dye [called photosensitizer (PS)] and subsequent irradiation with appropriate wavelength of visible light in the presence of molecular oxygen.[1] The combination of photosensitizer, light, and molecular oxygen leads to the absorption of a photon by the photosensitizer, reaching the excited singlet state. The charge is then transferred to surrounding substrates (type I mechanism), generating superoxide radicals ($O_2^{\bullet-}$), which can further react with itself to produce hydrogen peroxide (H_2O_2) and oxygen. Superoxide is also important in the production of the highly reactive free hydroxyl radical ($HO^{\bullet}$). The direct transfer of energy to molecular oxygen is also possible (type II mechanism), leading to the formation of singlet oxygen ($^1O_2$), which is considered the most important reactive form in the photodynamic process.[2] Various reactive oxygen species (ROS) generated during oxidative processes do not selectively destroy bacterial structures.[3] Cell envelopes are considered the main targets of photodestruction, and the fact that Gram-positive bacteria exhibit higher susceptibility to photoinactivation than Gram-negative cells confirms this assumption.[4] Photosensitizers can relatively easily penetrate the porous layer of peptidoglycan and lipoteichoic acid, and therefore, Gram-positive bacteria are easier to destroy under the influence of light irradiation. The complex structure of the envelope of Gram-negative bacteria is highly impermeable to many drugs because it consists of two membranes, an outer membrane and an inner membrane, surrounding a periplasmic space.[5] In particular, the outer membrane contributes to poor bactericide penetration due to the outer leaflet containing lipopolysaccharides (LPS). The tight packing of saturated lipid chains in adjacent LPS molecules and the polyanionic surface charge conferred by LPS oligosaccharides hinder the passive diffusion of small molecules.

In recent years, numerous studies have been reported on photodestruction of various Gram-negative pathogens,[6,7] but only a few studies have focused on photodynamic eradication of *Proteus mirabilis*, which is an important factor in catheter-associated urinary tract infections (CAUTIs), accounting for

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up to 40% of hospital-acquired infections worldwide.[8,9] It has already been shown that CAUTIs have already been shown to be often polymicrobial infections, but *P. mirabilis* remains a key pathogen of interest.[10,11] This microorganism may also complicate urinary tract infections by inducing the formation of urinary stones.[11−13] Furthermore, these bacteria are characterized by their ability to swarm on the surface of catheters and their strong use of urease as a virulence factor.[13,14]

It is important to emphasize that *P. mirabilis* is considered part of the normal microflora of the mammalian gastrointestinal tract[15] and is an excellent example of an opportunistic nosocomial pathogen.[16] In addition to CAUTIs, these bacilli have been shown to cause prostatitis, wound infections and burns, and sometimes respiratory tract infections, chronic purulent otitis media, eye infections (endophthalmitis), and meningitis.[17−20]

To date, only a few effective systems for destroying this pathogen under the influence of light have been described. It has been shown that a high level of mortality was achieved when photosensitizers such as polyethylenimine−chlorine PS(e6) conjugate,[21] C60 fullerene functionalized with three dimethylpyrrolidinium groups (BF6),[22] graphene-based materials [graphene oxide, graphene quantum dots, carbon quantum dots (CQD), and nitrogen-doped carbon quantum dots[23] were applied. Very interesting photosensitizing systems based on aminotetraphenylporphyrin with L-ornithine were described by Meng et al.[24] The wound healing effect in *P. mirabilis*-infected mouse wound models showed that accelerated wound healing through the bactericidal effect was possible.

The photoantimicrobial effects of cationic porphyrins coordinated with cisplatin derivatives on *P. mirabilis* were studied by Clerici et al.[25] The neutral porphyrins 3-H_2TPyP and 4-H_2TPyP and the tetra-cationic derivatives 3-*cis*-PtTPyP and 4-*cis*-PtTPyP were evaluated, and the best effectiveness in killing cells of this pathogen was **3-*cis*-PtTPyP** in combination with a white-light source.

The efficacy of photodynamic antimicrobial therapy for inactivating a variety of antibiotic-resistant clinical strains of diabetic foot ulcers was studied by Piksa et al.[26] The authors focused on aPDI based on Methylene Blue (MB) as a photosensitizer and organic light-emitting diodes. Light treatment of *P. mirabilis* was shown to result in a reduction in the number of viable bacteria by approximately 3 logarithmic units.

Recently, the antimicrobial activity of 5-aminolevulinic-mediated aPDI (5-ALA-aPDI) has been studied, and several reports have shown that this nonprotein amino acid can induce photodynamic effects on Gram-positive and Gram-negative bacteria.[27−29] This molecule is not a photosensitizer but is a precursor of all porphyrins in the heme metabolic pathway.[30] It is assumed that increased levels of endogenous metal-free porphyrins are excited by light, leading to energy transfer and the production of cytotoxic ROS, primarily singlet oxygen ($^1O_2$).[31,32]

Photodynamic therapy and 5-ALA-induced diagnostics have been reported since 1990 and were approved by the FDA in 2017 (5-ALA; Gleolan; photonamic GmbH and Co. KG).[33]

There are two known problems that limit the wide use of 5-ALA in the light-induced destruction of pathogens. It has been shown that under neutral and basic conditions, this compound can readily dimerize to give 2,5-dicarboxyethyl-3,6-dihydropyrazine (DHPY), which can spontaneously oxidize to 2,5-dicarboxyethylpyrazine.[34,35]

The second problem is the hydrophilic nature of 5-ALA that may limit its ability to penetrate through skin, thereby restricting the use of 5-ALA-aPDI to the treatment of superficial diseases.[36]

These limitations have been partially overcome by the synthesis of 5-aminolevulinic acid esters, and it should be emphasized that methyl ester (Metvix or MAL) has been approved for the treatment of some forms of cancer. This clinically approved prodrug has increased lipophilicity compared to 5-ALA alone, resulting in better penetration of cell envelopes and more efficient accumulation of PpIX by various cells.[37]

Despite this scientific success, research is still being carried out that covers not only the synthesis of 5-ALA derivatives but also various nanomedicine strategies to improve the efficiency of transport of this drug into cells.[38,39] A subsequent strategy to improve the efficiency of 5-ALA delivery to cells is coadministration of 5-ALA with an adjuvant that damages the membrane of Gram-negative bacteria. The most commonly used are chelating agents (e.g., EDTA), which improve the permeability of the outer membrane by sequestering divalent cations that electrostatically cross-link LPS (i.e., Ca^{2+}, Mg^{2+}).[40,41]

The aim of this study was to demonstrate, for the first time, the possibility of enhancing the effectiveness of light-induced *P. mirabilis* inactivation by pretreating pathogenic cells with phosphoric or bisaminophosphinic acids as permeable adjuvants and then exposing these bacteria to visible light by using 5-ALA as a precursor of endogenous photosensitizers. The first group of permeabilizing compounds studied were aromatic esters of phosphoric acids that may interact with LPS and disrupt the fluidity of cell membranes. Their destabilizing effect on bacterial membranes may also be related to the presence of functional groups capable of potentially binding calcium[42] and magnesium[43] ions. The second group consisted of aminomethylphosphinic acids, originally designed and synthesized as small molecule inhibitors of bacterial urease. Their diffusibility into Gram-negative bacterial cells was confirmed in studies on mitigation of ureolysis in live *Helicobacter pylori* and *P. mirabilis*.[44,45]

2. RESULTS

2.1. Dark-Cytotoxicity Studies. In our initial experiments, it was vital to determine the dark toxicity of 5-ALA, phosphoric acids [designated for experimental purposes as (**1**) and (**2**)], and bisaminophosphinic acids [designated as (**3**−**7**)] toward *P. mirabilis*. Based on previous observations,[46,47] it appears that the dark toxicity of 5-ALA toward bacterial cells cannot be excluded, and therefore, it was justified to study the bactericidal effect of this amino acid without light induction. It was assumed that the optimal concentration of 5-ALA and the studied permeabilizers should be the highest concentration of these compounds that caused no more than 20% of bacterial cell death.

The effect of 5-ALA on the viability of the studied rods was examined at doses ranging from 1.25 to 10.0 mM, and the results obtained are collected in Table S1 (Supporting Information). 5-ALA was shown to be slightly toxic to bacteria at these concentrations, and the highest reduction in cell viability by 0.13 ± 0.01 to 0.14 ± 0.01 $\log_{10}$ units was detected for this amino acid at concentrations of 5.0−10.0 mM after 30

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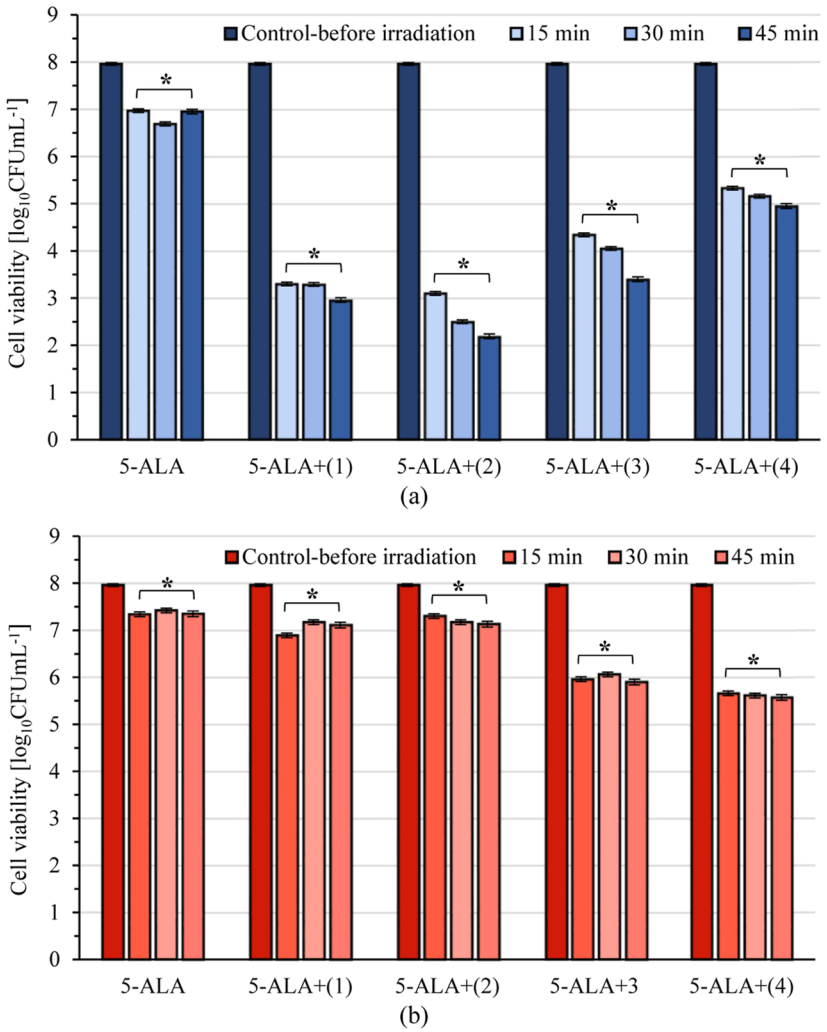

Figure 1. Effect of 5-ALA alone; 5-ALA + (**1**); 5-ALA + (**2**); 5-ALA + (**3**); 5-ALA + (**4**) on the viability of *P. mirabilis* suspension after 15, 30, and 45 min exposure to blue light (404 nm) (energy fluence was 23.5, 47, and 70 J cm^{-2}, respectively) (a); after 15, 30, and 45 min exposure to red light (630 nm) (energy fluence was 50, 100, and 150 J cm^{-2}, respectively) (b). Average ± SD of three independent experiments is shown. The values of $P < 0.05$ were considered significant.

min of incubation. 5-ALA was used at a concentration of 2.5 mM in all subsequent experiments.

The effect of phosphoric acids (**1**) and (**2**) on the viability of *P. mirabilis* is summarized in Table S2 (Supporting Information). The data obtained clearly indicated that the dark toxicity of the compounds examined was dependent on their concentration, and the studied phosphoric acids (**1**) and (**2**) met the established criterion at concentrations of 0.512 and 0.368 mM, respectively. Incubation of bacteria with these phosphoric acids extended to 24 h did not result in an increase in the mortality rate (data not shown). In all subsequent experiments, phosphoric acids were used at concentrations of 0.512 and 0.368 mM for (**1**) and (**2**), respectively.

The dark cytotoxicity of bisaminophosphinic acids (**3**–**7**) after 2 and 24 h of incubation with *P. mirabilis* was also estimated by a colony counting assay. It turned out that these compounds at concentrations higher than 1 mM influenced the growth of bacteria, and the number of living cells was observed to decrease by about 50% (data not shown). Insignificant dark toxicity toward bacteria was observed only for 1 mM acids (**3**) and (**4**), and the results obtained after 2 and 24 h of incubation are presented in Table S3 (Supporting Information). In all subsequent experiments, bisaminophosphinic acids (**3**) and (**4**) were used at a concentration of 1 mM.

Moreover, it was established that the effective enhancement of photoeradication of *P. mirabilis* by studied permeabilizers depended on the contact time of the bacteria with these acids, and in the case of (**1**) and (**2**), it was found that a 2 h pretreatment was sufficient to induce the assumed effect. Compounds (**3**) and (**4**) required longer contact with cells, and therefore, a pretreatment time of 24 h was used.

2.2. Effect of Extracellular 5-ALA Administration Time on the Level of Protoporphyrin IX. The important parameter that influenced the effectiveness of the light-induced mortality of *P. mirabilis* was the incubation time of the bacteria with exogenous 5-ALA (in the dark), resulting in the highest concentration of protoporphyrin IX in the cells. The effect of extracellular 5-ALA administration time on the level of protoporphyrin IX accumulated in bacterial cells was studied, and the results obtained are collected in Table S4 (Supporting Information). The highest concentration of PpIX detected in the bacterial lysates (6.1 ± 0.1 ng $cell^{-1} \times 10^{-5}$) was observed after 30 min of incubation of the *P. mirabilis* suspension with 5-ALA in the dark. In all further experiments, a 30 min

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incubation of bacterial cells with 5-ALA (preirradiation) was applied.

2.3. Phototoxicity Studies and Efficiency Enhancement. The effect of light from two laser diodes alone, 404 nm (output power 20 mW; radiation intensity 26 mW cm^{-2}) and 630 nm (output power 40 mW; radiation intensity 55 mW cm^{-2}), on the viability of *P. mirabilis*, was examined to determine whether the bacteria contained any endogenous photosensitizers activatable by light at 404 and 630 nm. A dose-dependent reduction in bacterial viability of 0.18−0.23 log_{10} units was observed after blue light irradiation. Bacterial mortality under the influence of red light was lower and did not exceed 0.17 log_{10} after 45 min of exposure of this pathogen to light (Supporting Information, Table S5).

Next, 5-ALA at a nontoxic concentration as a precursor of protoporphyrin IX (photosensitizer) in photodynamic treatment against *P. mirabilis* was used. Figure 1 shows the mean CFU mL^{-1} (log_{10}) and standard deviation (SD) of planktonic and biofilm cells after the different light/time treatments.

Blue light (404 nm) with an intensity of 26 mW cm^{-2} was used to induce a photobiocidal effect against the pathogenic bacteria studied. The highest mean reductions for 5-ALA mediated aPDI were 1.27 log_{10} corresponding to 47 J cm^{-2} energy fluence (30 min of blue light illumination) (Figure 1a). When red light (630 nm) was used in the experiments, the highest level of eradication of this pathogen was 0.62 log_{10} units, which was observed after irradiating the bacteria with a light dose of 16.5 J cm^{-2} (15 min of illumination) (Figure 1b).

As can be seen in Figure 1a, pretreatment of bacteria with phosphoric acids (**1**) or (**2**) (at nontoxic concentrations) greatly improved the photoinactivation of *P. mirabilis* as 15 min of blue light irradiation (23.5 J cm^{-2}) in the presence of 5-ALA resulting in a reduction in bacteria viability by 4.66 and 4.86 log_{10} units, respectively. Prolonged illumination time to 30−45 min (light dose of 47 and 70 J cm^{-2}) after pretreatment of cells with (**1**) caused a reduction in the viability of bacteria of 4.67 and 5.0 log_{10}, respectively.

Under the same experimental conditions but after pretreatment with (**2**), mortality rates of this pathogen were detected at the level of 5.46 and 5.77 log_{10} units.

A similar phenomenon of increased efficiency of aPDI was observed using two-step inactivation of *P. mirabilis* with red light irradiation (Figure 1b). Pretreatment of bacteria with (**1**) followed by illumination with red light showed a reduction in the viability of *P. mirabilis* by 1.07 log_{10} after 15 min of exposure to light (energy fluence was 50 J cm^{-2}). Significant photodestruction of planktonic cells was accomplished using (**2**) as a permeabilizing agent. The kill achieved under this experimental condition was light dose dependent when the exposure time was extended from 15 to 45 min. The viable cell count showed a reduction of 0.66, 0.79, and 0.83 log_{10}, after 15, 30, and 45 min of red light treatment, respectively.

An increase in the effectiveness of aPDI against *P. mirabilis* was also observed when bisaminophosphinic acids (**3**) or (**4**) were applied as permeabilizers. Photodynamic inactivation of the pathogen studied, but preceded by incubation of bacteria with (**3**), caused a significant reduction of planktonic cultures by 3.62, 3.91, and 4.56 log_{10} units after irradiation with a blue LED for 15, 30, and 45 min, respectively (Figure 1a). As expected, the use of red light was less effective in reducing the number of viable cells under the same experimental conditions. It was revealed that after 15, 30, and 45 min of irradiation of bacterial suspensions, the number of viable cells decreased by 2.0, 1.9, and 2.06 log_{10} units, respectively (Figure 1b).

Significant photoelimination of *P. mirabilis* was also achieved using (**4**) as a cell membrane damaging agent. The killing effect was also dependent on the light dose, and it was shown that the number of viable cells was reduced by 2.63, 2.8, and 3.01 log_{10} after 15, 30, and 45 min of blue light irradiation, respectively (Figure 1a). Under the same experimental conditions, the effectiveness of reducing the number of viable bacteria using the red LED light was lower (compared to the blue light) and amounted to 2.3, 2.35, and 2.39 log_{10} units (Figure 1b).

Determination of bacterial cell integrity was performed using a LIVE/DEAD test with SYTO9 and propidium iodide to confirm the photobiocidal activity of blue and red light against *P. mirabilis*. Figure 2 shows fluorescence microscopy images of

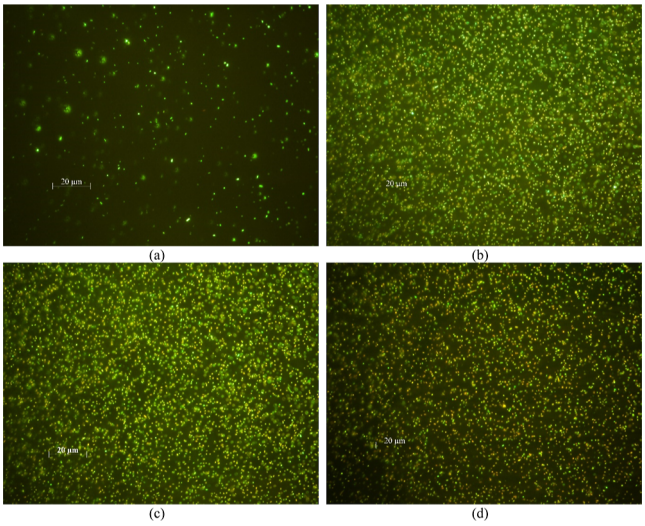

Figure 2. Fluorescence microscope images of *P. mirabilis* cells before (a) and after exposure to red light for 45 min (b); after exposure to blue light for 30 (c) and 45 min (d). The concentration of 5-ALA is 2.5 mM. Scale bar 20 μm.

the examined planktonic bacterial cells after treatment with LED light. Living bacterial cells with an intact cell membrane were observed as green structures (photo a). Bacterial cells with damaged cell membranes stained orange-red fluorescently[48] (photos b−d). As expected, the number of bacterial cells that absorbed propidium iodine increased with exposure time of the microorganism to a LED. The images obtained confirmed that treatment of bacteria with blue light (photo b) in the presence of 5-ALA as a precursor of endogenous photosensitizers (compared to red light; photos c, d) had a less impact on the integrity of the pathogen's cell membrane.

2.4. Studies on the Mechanism of Improving the Effectiveness of aPDI Based on 5-ALA against *P. mirabilis* by Phosphoric and Bisaminophosphinic Acids. *2.4.1. Effect of Phosphoric and Bisaminophosphinic Acids on the Permeability of the Cell Membrane.* The effect of (**1**), (**2**), (**3**), and (**4**) on the efficiency of the transport of MB to *P. mirabilis* cells was examined, and the results obtained are presented in Figure S1 (Supporting Information). The level of uptake after 30 min of cell incubation with this dye alone (without contact with the studied phosphoric or bisaminophosphinic acids) was approximately 4.5 ± 0.1 ng $cell^{-1}$ × 10^{-4}. After pretreatment of *P. mirabilis* with the phosphoric

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acids (**1**), (**2**), the efficiency of MB uptake by bacteria, under the same experimental conditions, increased by 80 ± 1% (up to approximately 8.1 ± 0.1 ng cell^{-1} × 10^{-4}) and by 116 ± 1% (up to approximately 9.7 ± 0.1 ng cell^{-1} × 10^{-4}), respectively.

A significant effect of increasing MB uptake by the bacteria studied was also observed after pretreatment of living cells with bisaminophosphinic acids (**3**), (**4**). MB uptake by bacteria was higher by 80 ± 1% (amounted to approximately 8.1 ± 0.1 ng cell^{-1} × 10^{-4}), and by 70 ± 1% (amounted to approximately 7.6 ± 0.1 ng cell^{-1} × 10^{-4}) compared to the control sample (without pretreatment with bisaminophosphinic acids).

The destabilizing properties of phosphoric and bisaminophosphinic acids toward the membranes of *P. mirabilis* PCM 543 was detected in nondiffusible 1-*N*-phenylnaphthylamine (NPN) uptake assay with EDTA as the reference compound. In order to differentiate between the effect of chelation of Mg^{2+} in LPS and possible membrane intercalation of the alkyl and aryl hydrophobic moieties, parallel assays were conducted in the presence of an excess of magnesium ions (Table 1). After

Table 1. Fluorescence Intensity Values Obtained in the NPN Uptake Assay after Pretreatment of Bacterial Cells with the Phosphoric and Bisaminophosphinic Acids for 2 or 24 h

Sample	Pretreatment time [h]	
	2 h	24 h
	Fluorescence intensity (mean ± SD)	
control (cells)	561 ± 42	
cells after preincubation with (**1**)	814 ± 62	not determined
cells after preincubation with (**2**)	1443 ± 54	
cells after preincubation with (**1**) + $MgCl_2$	681 ± 47	
cells after preincubation with (**2**) + $MgCl_2$	1048 ± 115	
cells after preincubation with EDTA	733 ± 21	
cells after preincubation with EDTA + $MgCl_2$	594 ± 71	
control (cells)	not determined	536 ± 17
cells after preincubation with (**3**)		812 ± 52
cells after preincubation with (**4**)		785 ± 21
cells after preincubation with (**3**) + $MgCl_2$		631 ± 13
cells after preincubation with (**4**) + $MgCl_2$		659 ± 23
cells after preincubation with EDTA		742 ± 39
cells after preincubation with EDTA + $MgCl_2$		575 ± 38

24 h incubation with permeabilizing compounds (**3**) and (**4**), the NPN fluorescence in cells separated from the incubation mixture was 152 ± 14% and 146 ± 8% of the untreated control, respectively. For comparison, the fluorescence observed upon incubation with 1 mM EDTA as a reference permeabilizing agent was 139 ± 11% of the control.

When 5 times excess of divalent magnesium cations was present in the incubation mixtures, the NPN fluorescence enhancement after treatment with compounds (**3**) and (**4**) was reduced to 118 ± 6% and 123 ± 8%, respectively, while the effect of EDTA was totally abolished. A similar result was obtained after incubation of *P. mirabilis* cells with phosphoric acid (**1**) that caused NPN uptake increase equal to 145 ± 22%, and 121 ± 17% when magnesium ions were present.

The most profound membrane penetration with NPN was observed after 2 h cells exposure to compound (**2**)—the fluorescence was 257 ± 29% of the untreated control, moreover, despite the presence of 5 mM $MgCl_2$, the enhancement was still evident as it amounted to 186 ± 34%.

2.4.2. Effect of Phosphoric and Bisaminophosphinic Acids on the Cell Surface Charge. To investigate whether the surface charge could change as a result of the accumulation of (**1**), (**2**), (**3**), and (**4**) on the membrane of the *P. mirabilis* membrane, ζ potential measurements were carried out. The results obtained (Figure S2; Supporting Information) showed that in the absence of the organophosphorus compounds, the *P. mirabilis* cells had a negative potential (−31.5 ± 0.5 mV). After incubation of bacteria with (**1**) and (**2**) (at nontoxic concentrations), the surface charge values were −28.2 ± 0.4 and −31.3 ± 0.3 mV, respectively. It was observed that after incubation of bacteria with (**3**) and (**4**), the ζ potential value shifted to −27.3 ± 0.3 and −29.6 ± 0.5 mV, respectively.

2.4.3. Effect of Phosphoric and Bisaminophosphinic Acids on the Accumulation of Protoporphyrin IX in P. mirabilis Cells. The estimation of the concentration of accumulated PpIX in cells of *P. mirabilis* was carried out according to the procedure described in subsection: Quantification of Protoporphyrin IX in the Bacterial Cell Lysates. The results obtained are shown in Figure S3a,b (Supporting Information). The concentration of PpIX in *P. mirabilis* cell lysates without an external source of 5-ALA was estimated at 1.9 ± 0.18 ng cell^{-1} × 10^{-5}. It was observed that in lysates obtained from cells preincubated with phosphoric acids, the concentration of protoporphyrin IX increased by 42−53% compared to lysates obtained from bacteria without preincubation with these compounds and amounted to 2.7 ± 0.2 ng cell^{-1} × 10^{-5} and 2.9 ± 0.06 ng cell^{-1} × 10^{-5} for (**1**) and (**2**), respectively. A slight increase in the concentration of protoporphyrin IX was also observed in the case of preincubation of bacteria with (**4**) and it was 2.4 ± 0.39 ng cell^{-1} × 10^{-5} (Figure S3a; Supporting Information). As seen in Figure S3b, the concentration of PpIX was 5.8 ± 0.15 ng cell^{-1} × 10^{-5} in lysates obtained from bacterial cells incubated with 5-ALA alone. Slightly higher protoporphyrin IX concentrations were observed in lysates prepared from cells incubated with exogenous 5-ALA preceded by contact with the tested phosphoric acids and were 5.9 ± 0.34 ng cell^{-1} × 10^{-5} and 6.3 ± 0.09 ng cell^{-1} × 10^{-5} for lysates obtained from cells after contact with (**1**) or (**2**), respectively. The concentration of PpIX in cell lysates obtained from cells preincubated with (**3**) and (**4**) decreased and amounted to 3.8 ± 0.36 ng cell^{-1} × 10^{-5} and 4.4 ± 0.2 ng cell^{-1} × 10^{-5}.

2.5. Studies on the Photosensitizing Properties of the Phosphoric Acids. This part of our experiment was preceded by recording the UV−vis spectra of the phosphoric acids (see Figure S4a,b; Supporting Information). Diphenylphosphoric acid (**1**) and (*R*)-(−)-1,1′-binaphthyl-2,2′-diylhydrogenphosphate (**2**) were characterized by wide absorption bands, which may suggest the possibility of their excitation with blue and red light. Therefore, in the next part of our experiments, the studied acids were used as photosensitizers for photoeradication of *P. mirabilis*. It was shown that after 15 min of blue (404 nm) or red light (630 nm) irradiation in the presence of (**1**), a reduction in CFU of approximately 0.46 ±

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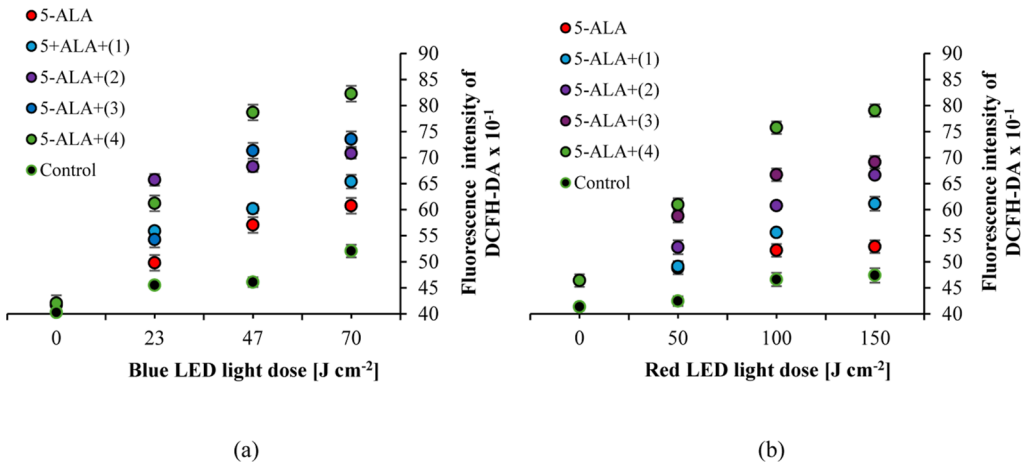
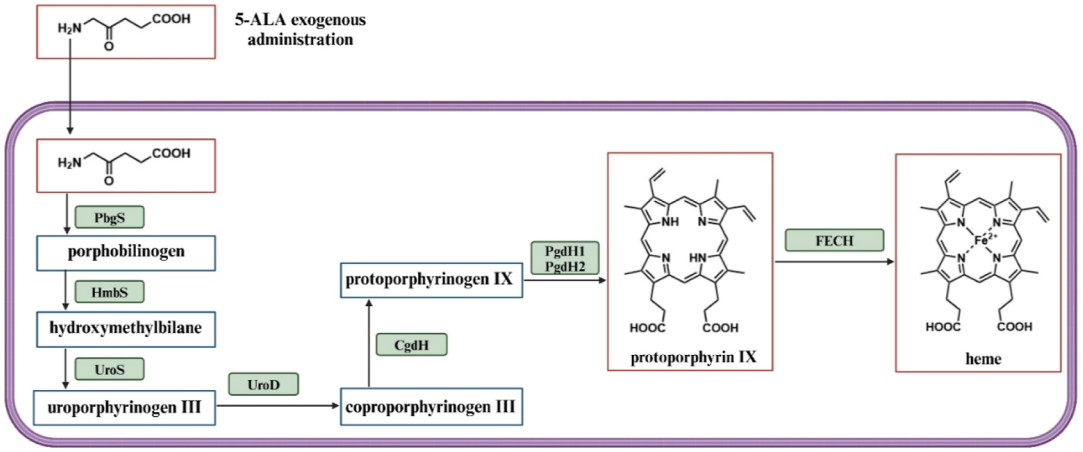


Figure 3. Effectiveness of increasing the oxidation rate of the bacterial cells (oxidative stress) by 5-ALA alone; 5-ALA administrated after pretreatment with (**1**), (**2**), (**3**), and (**4**) and blue (a) or red (b) light irradiation. Dichlorodihydrofluorescein diacetate (DCFH-DA) was used as a fluorescent probe. Average ± SD of three independent experiments is shown.

Scheme 1. Metabolic Pathway of Conversion of 5-ALA to Protoporphyrin IX in Gram-Negative Bacterial Cells[a]



[a]Key abbreviations are boxed. PbgS = porphobilinogen synthase; HmbS = hydroxymethylbilane synthase; UroS = uroporphyrinogen III synthase; UroD = uroporphyrinogen III decarboxylase; CgdH = coproporphyrinogen III dehydrogenase; PgdH1 = protoporphyrinogen IX dehydrogenase 1; PgdH2 = protoporphyrinogen IX dehydrogenase 2; FECH = ferrochelatase.

0.03 to 0.44 ± 0.02 $\log_{10}$ was achieved, which was about 65−66% death (Figure S5a; Supporting Information). Longer exposure to light (30−45 min) did not increase the mortality of *P. mirabilis*. A similar phenomenon was observed for (**2**) as a photosensitizer. Bacterial cell mortality after 15 min of exposure to red light did not exceed 0.35 ± 0.05 $\log_{10}$, which means approximately 52% death (Figure S5b; Supporting Information). When blue light was used in the experiments, the observed cell mortality depended on the time of exposure of the pathogen to light, and after 45 min, it was approximately 55%.

2.6. Alterations of the Inner Bacterial Cell. To study the effect of preincubation of bacteria with the examined acids on changes in the internal bacterial cell after exogenous administration of 5-ALA, a simple total cellular ROS detection protocol based on dichlorodihydrofluorescein diacetate (DCFH-DA) as a fluorescent probe was used (Figure 3a,b). As can be seen, the fluorescence intensity of 2′,7′-dichlorofluorescein (DCF) detected in bacterial cells before incubation with the compounds tested remained at a similar level and did not exceed the value of 530 and 480 after 45 min of blue and red light irradiation, respectively.

Incubation of *P. mirabilis* with 5-ALA alone and then irradiated with LED light induced significant conversion of DCFH-DA to DCF. For example, the fluorescence intensity of DCF formed in cells after a 45 min exposure to blue LED light in the presence of 5-ALA alone reached the value of 600, which indicates an increase in the intensity of formation of this compound by 25%. A significant increase in DCF fluorescence intensity was observed after preincubation of bacteria with (**3**) (by 45%) or (**4**) (by 70%) and exogenous administration of 5-ALA, followed by irradiation with blue light at a dose of 70 J cm^{-2}. A similar effect of increasing the fluorescence intensity of DCF (but slightly lower compared with the exposure of cells to blue light) was observed when the cells of the tested pathogen were treated with red light (630 nm). It should be noted that pretreatment of bacterial cells with phosphoric or bisamino-phosphinic acids before exogenous administration of 5-ALA, and subsequent light irradiation (regardless of the wavelength used), significantly increased the DCF fluorescence intensity.

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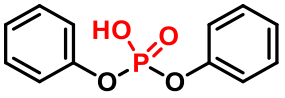
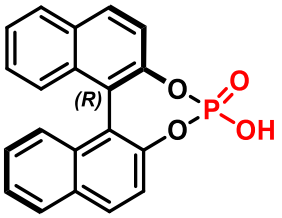
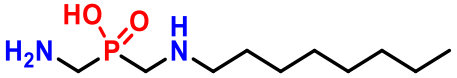
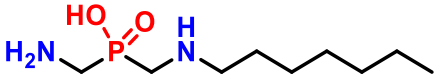
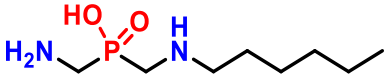
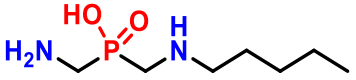
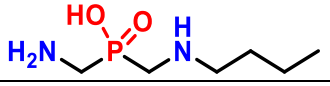
Table 2. List of the Studied Phosphoric and Bisaminophosphinic Acids

No.	Compound	Name
(1)		diphenylphosphoric acid
(2)		(*R*)-(−)-1,1′-binaphthyl-2,2′-diylhydrogenphosphate
(3)		aminomethyl(*N*-n-octylaminomethyl)phosphinic acid
(4)		aminomethyl(*N*-n-heptylaminomethyl)phosphinic acid
(5)		aminomethyl(*N*-n-hexylaminomethyl)phosphinic acid
(6)		aminomethyl-(*N*-n-pentylaminomethyl)phosphinic acid
(7)		aminomethyl(*N*-n-butylaminomethyl)phosphinic acid

3. DISCUSSION

Most studies on 5-ALA as a precursor of endogenous photosensitizers described in the literature concerned the use of this amino acid in various types of photodynamic diagnosis (5-ALA-PDD) or photodynamic therapy (5-ALA-PDT) of cancers.[49,50] Our interest was focused on the possibility of using this nonproteinogenic amino acid for phototoxic destruction of *P. mirabilis*. It is widely accepted that the effectiveness of aPDI based on 5-ALA as a photosensitizer precursor in the light-induced inactivation of Gram-negative bacteria depends on the intracellular concentration of protoporphyrin IX (PpIX).[29] Unfortunately, protoporphyrin IX in metabolically active bacterial cells is readily converted to heme by ferrochelatase (Scheme 1), which limits its use in the photodestruction of microorganisms. It is known that maintaining a constant level of porphyrins in the cell can be achieved by exogenous administration of 5-ALA.[29]

The new strategy described in this work aimed at maintaining a high level of protoporphyrin IX in cells, which resulted in improved efficiency of light-induced eradication of *P. mirabilis*, and consisted of a two-step protocol that included initial damage to the bacterial cell membrane using permeabilizing adjuvants and then exposure of bacteria to light after exogenous delivery of 5-ALA as a precursor of intracellular photosensitizers. For this purpose, phosphoric and bisaminophosphinic acids (Table 2) were applied as potential permeabilizers to help overcome the barrier of the *P. mirabilis* cell envelope.

Based on literature data showing the necessary properties of organic compounds as bacterial membrane destabilizers,[50,51] aromatic esters of phosphoric acids (**1**) and (**2**) were selected as those capable of meeting the required criteria. Structures that destabilize biological membranes should be small and stable molecules and have the ability to interact with lipopolysaccharides (LPS) and disrupt fluidity or charge, which is crucial for interactions with associated proteins.[52,53] Furthermore, the lipophilicity of hydrophobic organic compounds, attributed to alkyl chains or aromatic rings, serves as a mechanism for destabilizing outer membrane interactions, thus facilitating the passage of hydrophobic molecules through such barriers. Moreover, the studied compounds contain functional groups (Figure 4a) capable of potentially binding calcium and magnesium ions.

Introducing a neutral acid could compete with interactions of the parent hydrophilic portion of the membrane, destabilizing its structure through protonation reversal and resulting LPS charge disruption (Figure 4b). Furthermore, phosphoric acids exhibit properties conducive to hydrogen bond formation, serving as both donors and acceptors of bonding.[54] We hypothesize that these properties may induce interactions with associated proteins, leading to destabilization of the system.

Specifically, bis(amino)phosphinic acids, which contain additional basic functionalities in the form of amino groups, can assist in chelating divalent ions and participate in hydrogen bond formation. Additionally, because of the presence of alkyl substituents, these compounds may facilitate penetration through the cell membranes. In diarylphosphorothioic acids, in addition to binding calcium and magnesium ions and participating in hydrogen bonding interactions, aryl groups not only increase lipophilicity but also improve the absorption of electromagnetic radiation (Figure S4a,b; Supporting Information). It is worth noting that the phosphoric acid group may also be responsible for the interactions.

The second group of effective cell membrane destabilizers studied was aminomethylphosphinic acids (**3**) and (**4**). The

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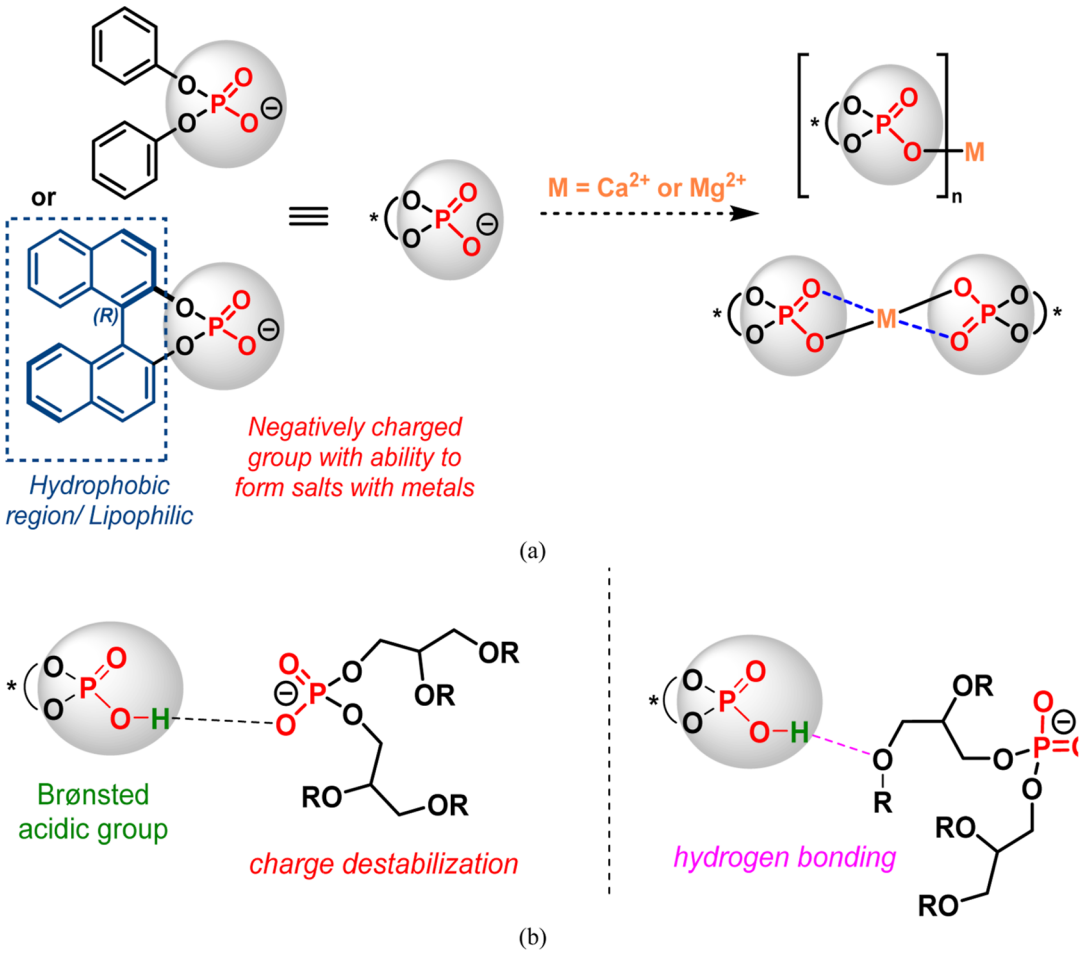

Figure 4. Potential properties of phosphoric acids to bind the calcium and magnesium cations (a); phosphoric acids as hydrogen-source and hydrogen-bond donors (b).

presence of the P−C−N scaffold in these biologically active compounds ensures their hydrolytic stability, resistance to microbiological degradation and low toxicity. Monosubstituted bis(aminomethyl)phosphinic acids containing linear alkyl chains, the *n*-heptyl derivative (**4**) and the *n*-octyl derivative (**3**), combine the characteristics of positively charged detergents and efficient chelators.

It should be noticed that the photobiocidal activity of endogenous photosensitizers[29]—mainly protoporphyrin IX (without exogenous administration of 5-ALA) is possible; therefore, it was also necessary to determine the bactericidal effect of diode light itself on *P. mirabilis* cells. It was previously demonstrated that the absorption spectra of PpIX in the UV−vis range are characterized by an intense Soret band centered around 401−402 nm and four weaker Q bands in the visible range (502, 537, 573, and 628 nm),[29] and therefore, LED lights with wavelengths of 404 and 630 nm were used in all photokilling experiments. The bacteria were irradiated with blue light at energy doses of 23.5, 47, and 70 J cm^{-2}, and it was found that under these experimental conditions, the reduction in bacterial viability was not greater than 42 ± 2%. Considering that various photosensitizers such as protoporphyrin, coproporphyrin, and uroporphyrin, which are intermediates in heme biosynthesis, can accumulate in the cytoplasmic matrix,[29] the biocidal effect of blue light seems obvious. However, it should be emphasized that the exact location of these porphyrins in the bacterial cell has not yet been fully revealed. It was also shown that red light (630 nm) was slightly less effective in photodestruction of these pathogens, and irradiation of the bacterial suspension for 45 min was required to kill only 32 ± 2% of cells (the light dose was 150 J cm^{-2}).

Our results clearly indicated that the irradiation of bacteria with blue light after exogenous administration of a nontoxic 5-ALA concentration resulted in a high effectiveness of aPDI, and mortality of pathogens after the application of a light dose of 47 J cm^{-2} was 94.6 ± 2%. Photoeradication of *P. mirabilis* using red light was less efficient, and no satisfactory bactericidal effect was achieved until 45 min of illumination (the mortality rate did not exceed 79 ± 2%).

A significant improvement in the bactericidal effect of 5-ALA-aPDI was obtained by pretreating *P. mirabilis* with phosphoric acids (**1**) and (**2**) at nontoxic concentrations, and it was found that a blue light dose of 23.5 J cm^{-2} was sufficient to achieve cell mortality of 99.998% and 99.999%, respectively. Photoelimination of this pathogen using red light was less effective, and the required killing effect (at least 99.99%) was not achieved until 45 min of exposure.

As mentioned above, bisaminophosphinic acids (**3**) and (**4**) needed a significantly extended pretreatment time in the dark (up to 24 h) with bacteria to effectively increase the efficiency of photoinactivation of the studied pathogen. A light dose-dependent increase in the killing efficiency of *P. mirabilis* was observed, and after 45 min of exposure of bacteria in the presence of 5-ALA [after preincubation with (**3**)] to blue light

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(a light dose was 70 J cm^{-2}), cell mortality exceeded 99.997%. Significant worse pathogen eradication was found under red light illumination, and a dose of 150 J cm^{-2} caused the destruction of bacterial cells at the level of 99.13%. Photoeradication of bacterial cells after pretreatment with (**4**) was significantly less effective, and the required mortality effect (at least 99.99%) was not achieved until 45 min of exposure to blue/red light.

Three analytical techniques were used to confirm the assumed mechanism of bacterial membrane permeabilization by the examined phosphoric and biosaminophosphinic acids. The increased efficiency of MB uptake by bacteria after preincubation with (**1**), (**2**), (**3**) and (**4**) confirmed that the increase in the efficiency of aPDI may result from the cell membrane permeabilizing effect of these acids.

The destabilizing properties of (**1**), (**2**), (**3**), and (**4**) toward the membranes of *P. mirabilis* was also studied using 1-*N*-phenylnaphthylamine (NPN) uptake assay with EDTA as the reference control. It is well known that an intact outer bacterial membrane constitutes a permeability barrier and excludes hydrophobic substances such as NPN, but when this structure is damaged, it is possible for NPN to enter the phospholipid layer, causing distinct fluorescence. Our data showed that quantitative and highly reproducible levels of NPN uptake was observed after pretreatment *P. mirabilis* with (**1**), (**2**), (**3**), and (**4**). A 5-fold excess of divalent magnesium cations in the reaction mixtures resulted in a decrease in the NPN fluorescence intensity, but no complete abolition of the NPN fluorescence enhancement was observed, which indicates that chelation was not the only mechanism for increasing the permeability of *P. mirabilis* membranes by the phosphoric and bisaminophosphinic acids studied.

The charge of the surface of the bacteria has often been described by the ζ potential, an electrochemical property of the cell surface, which represents the potential in the double shear plane of the electrical double layer that encompasses a cell in solution.[55] Halder et al.[56] proved that alteration of ζ potential may be correlated with improved membrane permeability. Changes in the surface charge of *P. mirabilis* cells have been estimated under the influence of phosphoric and bisaminophosphinic acids, and it seems justified to assume that diphenylphosphoric acid (**1**) and aminomethyl(*N*-*n*-octylaminomethyl)phosphinic acid (**3**) can increase the permeability of the bacterial membranes.

Interesting results were obtained regarding the influence of phosphoric and bisaminophosphinic acids on the level of accumulated PpIX in bacterial cells. An increase in the concentration of protoporphyrin IX (by approximately 30−50%) was found in lysates obtained from bacterial cells preincubated with phosphoric acids (**1**), (**2**), and bisaminophosphinic acid (**3**) (without exogenous 5-ALA) compared to lysates obtained from bacteria without preliminary incubation with these compounds. A similar phenomenon of increasing the level of PpIX accumulated in bacterial cells was also observed in the case of extracellular administration of 5-ALA. Interestingly, the most effective compound that increased the intracellular PpIX concentration was (**2**). Previous studies have shown that accumulation of PpIX in cells is not only influenced by the 5-ALA-PpIX conversion ratio but also is related to ferrochelatase-mediated insertion of ferrous iron into the porphyrin macrocycle to form a heme molecule. Differences in iron content in cells have been shown to influence the level of accumulated PpIX.[57] Thus, some researchers have used iron chelators to increase PpIX levels by reducing the amount of iron available in cells.[57,58] It should be noted that selective accumulation of PpIX may also be the result of low ferrochelatase activity.[59] It appears possible that (**2**) has the ability to chelate iron ions and, thus, increase the level of PpIX in cells.

Photoeradication studies of *P. mirabilis* using (**1**) and (**2**) as photosensitizers have clearly shown that these compounds can be excited by light of an appropriate wavelength (mainly blue), which results in the destruction of bacterial pathogens. It seems that the increased efficiency of the photoelimination of bacteria may be the result of the synergistic effect of 5-ALA + diphenylphosphoric acid or 5-ALA + (*R*)-(−)-1,1′-binaphthyl-2,2′-diylhydrogen phosphate (see: studies on the synergistic effect; Supporting Information).

The DCFH-DA fluorometric assay is a fluorescent probe for direct measurement of the redox state.[60] As previously shown, DCFH-DA penetrates the cell membrane and is deacetylated by intracellular esterases. These reactions produce 2′,7′-dichlorodihydrofluorescein (DCFH), which reacts with intracellular hydrogen peroxide or other oxidizing ROS to form 2′,7′-dichlorofluorescein (DCF), which is strongly fluorescent at excitation and emission wavelengths of 485 and 520 nm, respectively. The results obtained clearly showed an increased concentration of oxidizing compounds inside bacterial cells after exposure to light. These data should be interpreted as the induced conversion of DCFH-DA to DCF resulting from the increased concentration of oxidizing compounds inside bacterial cells under specific experimental conditions (Figure S6; Supporting Information). The accumulation of ROS in bacteria suggests a breakdown of the cell's defense mechanisms, which results in its destruction.

4. CONCLUSIONS

In conclusion, we have observed that the efficiency of light-induced photoeradication of the Gram-negative rods *P. mirabilis* can be significantly enhanced using adjuvants—phosphoric or bisaminophosphinic acids, resulting in planktonic cell mortality above 99.99%. Our two-step protocol involved an initial pretreatment of the cells with adjuvants, followed by exposure of the bacteria to light after exogenous delivery of 5-ALA, serving as a precursor of intracellular photosensitizers. Interestingly, the most effective agent was found to be (*R*)-(−)-1,1′-binaphthyl-2,2′-diylhydrogen phosphate (**2**). The mechanism underpinning the increased pathogen destruction in our protocol is multifaceted, involving not only the destabilization of the outer bacterial cell membrane but also an increase in the level of protoporphyrin IX in cells due to the chelation of iron ions. Furthermore, we cannot rule out the potential synergistic effect of intracellular photosensitizers and (*R*)-(−)-1,1′-binaphthyl-2,2′-diylhydrogen phosphate (**2**) acting as an additional photosensitizer induced by blue/red light. Overall, the results obtained contributed to the current efforts to develop effective methods to combat Gram-negative bacterial pathogens.

5. EXPERIMENTAL SECTION

5.1. Reagents. All reagents were purchased from Sigma-Aldrich (Poland). The 5-aminolevulinic acid (5-ALA) powder was dissolved in deionized water and stored at −20 °C avoiding access to light. Phosphoric acids (**1**−**2**) (Table 2) were obtained from commercial suppliers and dissolved in

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dimethyl sulfoxide. Bisaminophosphinic acids (**3**−**7**) (Table 2) were originally designed and synthesized as described in refs 44 and 45 and dissolved in deionized water. All solutions were sterilized by filtration through 0.22 μm pore membranes (Millex-HP syringe-driven filter unit, Millipore).

5.2. Microorganism and Culture Conditions. 2 mL of Mueller−Hinton broth (BTL, Poland) was inoculated with one colony of *P. mirabilis* PCM543 and left for overnight incubation. Then, the bacterial culture was centrifuged at 500*g* for 5 min, and the supernatant was removed. The remaining pellet was suspended in sterile deionized water and diluted to achieve a McFarland standard of 0.5, which corresponds to approximately 1.0−1.8 × 10^8 CFU mL^{-1} (colony-forming units per 1 mL).

5.3. Light Source. All bacterial photoelimination experiments were conducted with the use of single-mode diode lasers with a peak power wavelength of 404 nm (output power 20 mW; radiation intensity 26 mW cm^{-2}) and 630 nm (output power 40 mW; radiation intensity 55 mW cm^{-2}).

5.4. Dark Cytotoxicity of 5-ALA, Phosphoric Acid, and Bisaminophosphinic Acids. 150 μL of the standardized suspension of *P. mirabilis* was transferred to each well of a black sterile microtiter plate (Thermo Fisher Scientific, USA). Subsequently, to obtain final 5-ALA concentrations of 1.25, 2.5, 5.0, and 10.0 mM, 150 μL of the appropriate 5-ALA water solution was added to each well likewise. The prepared suspensions were shifted to incubation on a rotary shaker at 37 °C for 30, 120, and 240 min. Incubation was carried out in the dark.

The effect of diphenylphosphoric acid (**1**) and (*R*)-(−)-1,1′-binaphthyl-2,2′-diylhydrogen phosphate (**2**) on the viability of *P. mirabilis* was tested as follows: 270 μL of standardized bacterial suspension was mixed with 30 μL of appropriate concentration of phosphoric acids to give final compounds concentrations of 0.512, 1.024, and 2.046 mM and 0.368, 0.735, and 1.472 mM for (**1**) and (**2**), respectively. The dark cytotoxic effect of bisaminophosphinic acids (compounds **3**−**7**) was tested as described above with the difference that the final compound concentration was in the range 1−2 mM. All samples were incubated at 37 °C for 2 and 24 h in the dark. The drop plate technique[61] was used to assess the biocidal effects of 5-ALA, phosphoric, and bisaminophosphinic acids on bacterial viability. Bacterial viability was evaluated as (1) reduction in CFU unit ($\log_{10}$ CFU mL^{-1}) calculated as $R = \log_{10} N_0 - \log_{10} N$ and (2) percent reduction (%) in bacterial viability calculated as $R = (N_0 - N) \times 100/N_0$, where N_0 and N are the numbers of CFU before incubation with 5-ALA, phosphoric acid, and bisaminophosphinic acids and remaining in suspension after contact with the compounds examined. The experiments were carried out in triplicates.

5.5. Quantification of Protoporphyrin IX in the Bacterial Cell Lysates. *P. mirabilis* suspensions, standardized to a concentration of 1.5 × 10^8 CFU mL^{-1}, were treated with 2.5 mM 5-ALA water solution for 30, 60, 120, 180, 240, and 300 min and 24 h. Then, the samples were centrifuged for 4 min at 500*g*, and supernatant was harvested. The remaining bacterial pellet was resuspended in 100 μL of lysis buffer containing 40 mM Tris, 20 mM acetate, 1 mM EDTA, and 1% sodium dodecyl sulfate were thoroughly mixed and kept for 10 min in an ice bath. The samples were then centrifuged again, the supernatant was removed, and the pellet was resuspended in sterile deionized water. The fluorescence intensity of the tested samples was then determined at 410 nm excitation and 631 nm emission. Protoporphyrin IX concentrations in bacterial cell lysated were calculated using a standard curve for protoporphyrin IX.

The effect of phosphoric (**1**), (**2**) and bisaminophosphinic acids (**3**), (**4**) on the concentration of protoporphyrin IX in bacterial lysates was examined according to the method described above, except that the quantification of protoporphyrin IX was preceded by incubation of bacteria with nontoxic concentration of (**1**), (**2**) for 2 h and (**3**), (**4**) for 24 h. The quantity of protoporphyrin IX in bacterial cells without external administration of 5-ALA was determined using the procedure described above, with the exception that the cells were not treated with 5-ALA prior to the lysates. All experiments were repeated 3 times.

5.6. Effect of Light on the Viability of *P. mirabilis*. 300 μL of the standardized suspension of *P. mirabilis* was added to the wells of the black sterile microtiter plate (Thermo Fisher Scientific) and exposed to diode laser lights (λ_1 = 404 nm or λ_2 = 630 nm) for 15, 30, and 45 min. After irradiation, 100 μL of the bacterial suspension was collected, and serial dilutions were made. 10 μL aliquots of each dilution were seeded on Mueller−Hinton agar using the drop plate method.[61] The seeded plates were incubated overnight at 37 °C in the dark. The influence of light on bacterial viability was determined using the approach given in Subsection: Dark 5.4. Samples without irradiation served as an overall control to estimate the initial number of bacteria in suspensions.

5.7. Bactericidal Effect of aPDI Using 5-ALA (5-ALA-aPDI) as a Precursor of Protoporphyrin IX. 300 μL aliquots of the standardized suspensions of *P. mirabilis* (see Subsection Microorganism and Culture Conditions) were preincubated for 30 min (in the dark) with 5-ALA at a final concentration of 2.5 mM in wells of in a flat bottom black microtiter plate (Thermo Fisher Scientific). Two diode laser lights (λ_1 = 404 nm or λ_2 = 630 nm) were used for illumination of the samples (for 15, 30, and 45 min). The samples without irradiation were an overall control. The drop plate method[61] was used to determine the number of surviving bacterial cells (CFU mL^{-1}). The photomortality of bacterial cells was defined as a $\log_{10}$ unit reduction (or mortality expressed as %) estimated by comparing the initial bacterial size to that following exposition to light in the presence of 5-ALA. The experiments were carried out in triplicates.

5.8. Studies on the Effect of Phosphoric and Bisaminophosphinic Acids on the Effectiveness of 5-ALA-aPDI. Diphenylphosphoric acid (**1**) (at a nontoxic concentration of 0.512 mM) and (*R*)-(−)-1,1′-binaphthyl-2,2′-diylhydrogen phosphate (**2**) (at a nontoxic concentration of 0.368 mM) were examined for their effect on the efficacy of 5-ALA-aPDI. Briefly, standardized *P. mirabilis* cultures were initially treated with these chemicals for 2 h at 37 °C in the dark. Following that, 2.5 mM 5-ALA was added to the bacterial samples, and all suspensions were incubated for an additional 30 min. Instead of the compounds tested, sterile deionized water was added to the sample and served as a control. Bacterial suspensions were then illuminated for 15, 30, and 45 min with the use of two laser lights (λ_1 = 404 nm or λ_2 = 630 nm). The drop plate technique[61] was used to determine the colony forming units per 1 mL. Samples obtained after preincubation with the studied chemicals (without illumination) served as an overall control to estimate the initial number of bacteria.

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The effect of the tested bisaminophosphinic acids (**3**), (**4**), (**5**), (**6**), and (**7**) on the efficacy of 5-ALA-aPDI against *P. mirabilis* cultures was performed as described above, with one modification that the standardized suspensions of bacteria were treated with these compounds at a concentration of 1 mM for 2 or 24 h (37 °C, in the dark). The experiments were conducted in triplicates.

5.9. Determination of Bacterial Cell Integrity. The viability of *P. mirabilis* was examined using the LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, USA). The Olympus 60BX light microscope (USA) with appropriate excitation/emission filter cubes (460−490 nm excitation bandpass filter, >515 nm long pass emission filter) was used for observation of bacterial cells.

5.10. Studies on the Effect of Phosphoric and Bisaminophosphinic Acids on Bacterial Cell Membrane Permeability. Phosphoric (**1**), (**2**) or bisaminophosphinic acids (**3**), (**4**) were added to standardized suspensions of *P. mirabilis* and incubated for 2 or 24 h, respectively. The control consisted of samples not treated with studied compounds. The assessment of the influence of phosphoric or bisaminophosphinic acids on the permeability of bacterial cells was carried out using the following techniques:

*5.10.1. **Technique 1**. Uptake of Methylene Blue.* 1 mL of a standardized suspension of *P. mirabilis* was incubated in the dark with MB (a final dye concentration was 15 mg L^{-1}) for 30 min. After that, the samples were centrifuged for 10 min at 10,000 rpm. Centrifugation allows sedimentation of cells along with the bound dye. Subsequently, pellets were washed with PBS buffer to eliminate nonadherent dye. Cell-bound MB was extracted with 1 mL of methanol at room temperature for 1 h. Then, each sample was centrifuged again (10,000 rpm, 10 min) to extract the adherent dye from the bacterial cells. The quantity of MB taken up by bacteria was measured spectrophotometrically at the maximal MB absorption wavelength of 660 nm (Shimadzu UV-1650PC).

The % uptake was calculated using the following formula

$$\text{Uptake percentage of MB} = \frac{\text{amount of MB in dissolved pellet}}{\text{the total amount of MB added}} \times 100\%$$

The effect of phosphoric (**1**), (**2**) or bisaminophosphinic (**3**), (**4**) acids (at nontoxic concentrations) on the efficiency of MB uptake by bacteria was determined according to the method described above, with the difference that the cells were pretreated for 2 h with (**1**) and (**2**) or 24 h with (**3**) and (**4**). All experiments were carried out in triplicate.

*5.10.2. **Technique 2**. NPN Probe Uptake Assay.* The uptake of the fluorescent probe NPN (1-*N*-phenylnaphtylamine) was carried out following the protocol of Helander and Mattila-Sandholm with slight modifications.[62] 1 mL of aliquots of overnight *P. mirabilis* were centrifuged at 500*g* for 5 min, and the supernatants were discarded. The pellets were resuspended in 1 mL of 5 mM HEPES with the addition of the tested compounds (at final concentrations of 0.512 and 0.368 mM for (**1**) and (**2**), respectively, and 1 mM for (**3**) and (**4**)). The samples with (**1**) or (**2**) were then incubated for 2 h and with (**3**) or (**4**) for 24 h at 37 °C on an incubator shaker New Brunswick Scientific Innova 40 at 121 rpm. Samples incubated without (**1**−**4**) were general controls. Parallel assays were performed in the presence of 5 mM $MgCl_2$ and tested compounds (at appropriate concentrations) or 1 mM EDTA. After incubation, the samples were centrifuged (5 min/500*g*), and the supernatants were discarded. The pellets were resuspended in 1 mL of 5 mM HEPES. 150 μL of cell suspension were then transferred to the white flat bottom NUNC (USA) plate walls. 50 μL of 160 μM NPN solution in 5 mM HEPES was added to each well, and the samples were incubated for another 30 min. After incubation the fluorescence intensities were measured using a SpectraMax Gemini spectrofluorometer by Molecular Devices equipped with Softmax Pro software (excitation wavelength: 350 nm; emission wavelength: 400 nm). The results were expressed as percent of fluorescence intensity enhancement in the presence of tested compounds with uncertainty calculated as a total differential of results of the three repetitions of compared assays.

*5.10.3. **Technique 3**. Determination of the ζ Potential.* For the ζ potential studies, *P. mirabilis* suspensions were prepared in HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) at a concentration of 10^7 CFU mL^{-1}. Compounds (**1**), (**2**), (**3**), and (**4**) were added to the bacterial suspensions at nontoxic concentrations. Samples with (**1**) or (**2**) were incubated for 2 h and with (**3**) or (**4**) for 24 h. Both treated and untreated suspensions were then transferred into zeta cells and allowed to equilibrate at 25 °C for 15 min. The measurements were taken using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). ζ potential was calculated from the mean of 15 measurements. All experiments were conducted in triplicate.

5.11. Studies on the Effect of 5-ALA-aPDI on Changes in the Interior of a Bacterial Cell. The standardized bacterial suspensions (see Subsection 2.2) were treated with 50 μM solution of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) before and after illumination. Subsequently, the formation of ROS was identified using a SpectraMax Gemini spectrophotometer and SoftMax Pro5 software (excitation at 485 nm and emission at 530 nm). The studies were performed in triplicates.

5.12. Statistical Analysis. The data are reported as the mean ± SD from triplicate experiments. The difference between the two means was analyzed with a two-tailed unpaired Student test. The $P < 0.05$ values were deemed significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c07696.

> Studies on dark cytotoxicity of 5-ALA, phosphoric acids, and bisaminophosphinic acids, studies on the synergistic effect, effect of 5-ALA on the viability of *P. mirabilis*, effect of phosphoric acids, correlation of protoporphyrin IX concentration, effect of light of two laser diodes wavelengths, efficiency of Methylene Blue uptake, effect of pretreatment of bacteria, absorption spectra, and changes in fluorescence intensity (PDF)

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Notes

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