Photodynamic Inactivation of *Staphylococcus auerus* by Novel Troponyl Methyl (Pyro)pheophorbides

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The emergence of antibiotic resistance among pathogenic bacteria has led to a major research effort to find the Photodynamic Therapy as an alternative antibacterial therapy.

Bacterial photodynamic inactivation (PDI) is based on the administration of a photosensitizer, which is preferentially accumulated in the microbial cells. The subsequent irradiation with visible light, in the presence of oxygen, specifically produces cell damages that inactivate the microorganisms.

PDI is known to be effective against viruses, bacteria, and fungi and therefore has been proposed to be used as a therapy for localized infections.[1,2]

It is known that Gram-positive bacteria are generally more susceptible to PDT as compared to Gram-negative species. This difference is explained by the structural differences in the cell walls.[3,4]

The novel candidates for PDI are based on methyl pheophorbide-a (MPa) and methyl pyropheophorbide-a (MPPa), isolated from alga *Spirulina pacifica*, which were linked with tropolone, seven membered cyclic compound by substitution on 3-position.

Tropolone, as the substituent of new photosensitizers in this study, has been found to have the following biological activities: antimicrobial activity, cytotoxic effects on various human and murine tumor cell lines, phytogrowth-inhibitory activity, insecticidal effect, metalloprotease inhibition, and antifungal activity on wood rotting fungi.[5,6]

Meanwhile, chlorin-based compounds show effective anticancer photodynamic activities.[7] Therefore, the combined compound of novel photosensitizers as PDT and the tropolones as antimicrobials were prepared to see the dual function activities.

In this paper, we wish to report the preliminary results of the research on Gram-positive bacterial cell *S. aureus* (strain KC1927) using troponyl methyl (pyro)pheophorbides.

Table 1. Newly synthesized photosensitizers

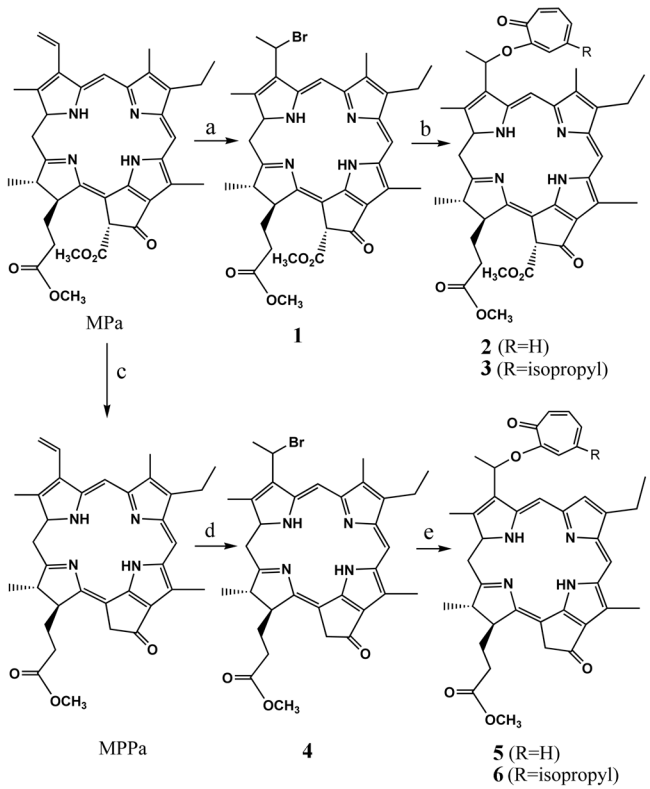
Name	Entry	Chemical formula	MW
B-1	MPa	$C_{36}H_{38}N_4O_5$	606.7
B-11	2	$C_{43}H_{44}N_4O_5$	728.85
B-12	3	$C_{46}H_{50}N_4O_7$	770.94
B-2	MPPa	$C_{34}H_{36}N_4O_3$	548.7
B-21	5	$C_{41}H_{42}N_4O_5$	670.81
B-22	6	$C_{44}H_{48}N_4O_5$	712.89

Experiments were carried out in order to determine the antibacterial photodynamic activities of newly synthesized troponyl methyl (pyro)pheophorbides.

The data presented in Table 2 and Figures 1-2 show that the illumination of *S. aureus* cells which had been exposed to troponyl methyl (pyro)pheophorbide derivatives caused a considerable decrease cell survival, comparing to control conditions.

Our findings clearly demonstrate that newly synthesized troponyl methyl (pyro)pheophorbide derivatives (**2**, **3** and **5**, **6**) exhibit significant antimicrobial photodynamic activities against Gram-positive bacterium *S. aureus* activity at micromolar concentrations.

Kill rate of bacterial cells increased in proportion to the increase of the incubation time and were depending on



Scheme 1. Synthetic pathway of troponyl derivatives from MPPa and MPa. Reagents: a) HBr/AcOH, b) tropone/K_2CO_3/CH_2Cl_2, c) collidine/reflux, d) HBr/AcOH, e) tropone/K_2CO_3/CH_2Cl_2 R = H, isopropyl

Table 2. Cell killing results from the growth delay experiments of *S. aureus*
A. Methyl pheophorbide-a (MPa) and its derivatives

Irradiance time (min)	0.5 μM		1 μM		2.5 μM		5.0 μM		10.0 μM	
	CFU/mL	%	CFU/mL	%	CFU/mL	%	CFU/mL	%	CFU/mL	%
					Compound B-1					
0	10.2×10^5	100	9.8×10^5	100	9.6×10^5	100	9.4×10^5	100	8.3×10^5	100
15	9×10^5	88	8.1×10^5	83	7.6×10^5	79	6.4×10^5	68	4.5×10^5	54
30	3.2×10^5	31	3×10^5	30	1.5×10^5	15	1.2×10^5	13	0.9×10^5	11
					Compound B-11					
0	5.5×10^5	100	4.1×10^5	100	3.6×10^5	100	2.3×10^5	100	1.3×10^5	100
15	2.9×10^5	53	1.6×105	39	0.6×10^5	17	0.9×10^4	3.9	0.2×10^4	1.6
30	4.6×10^4	9	2.8×10^4	6.8	0.76×10^4	2.1	2.3×10^3	1	0.9×10^4	0.07
					Compound B-12					
0	1.9×10^5	100	1.4×10^5	100	1.1×10^5	100	0.8×10^5	100	0.37×10^5	100
15	7.9×10^4	42	5.2×10^4	37	0.9×10^4	8	0.2×10^3	2.5	0.1×10^3	0.27
30	4.9×10^4	3	1×10^3	0.71	0.69×10^3	0.6	0.72×10^2	0.09	0	0

B. Methyl pyropheophorbide-a (MPPa) and its derivatives

Irradiance time (min)	0.5 μM		1 μM		2.5 μM		5.0 μM		10.0 μM	
	CFU/mL	%	CFU/mL	%	CFU/mL	%	CFU/mL	%	CFU/mL	%
					Compound B-2					
0	9.5×10^5	100	9.1×10^5	100	8.1×10^5	100	6.5×10^5	100	6.1×10^5	100
15	8.9×10^5	94	8.2×10^5	90	6.5×10^5	80	4.0×10^5	62	1.2×10^5	20
30	6.9×10^5	73	3.6×10^5	40	2.7×10^5	33.3	1.1×10^5	17	0.3×10^5	5
					Compound B-21					
0	8.5×10^5	100	7.6×10^5	100	4.5×10^5	100	2.1×10^5	100	1.4×10^5	100
15	5.7×10^5	67	4.8×10^5	63	1.5×104	51	0.5×10^5	24	0.1×10^5	7.1
30	3.5×10^5	41	1.7×10^5	22	0.02×10^5	9.5	0.02×10^5	1	0	0
					Compound B-22					
0	8.0×10^5	100	7.8×10^5	100	6.0×10^5	100	6.5×10^5	100	0.3×10^5	100
15	4.6×10^5	57.5	1.3×10^5	16.6	0.3×10^5	5.76	0.01×10^5	0.28	0.5×10^3	1.6
30	1.3×10^5	16.3	0.3×10^5	3.8	0.01×10^5	0.19	0.26×10^3	0.07	0	0

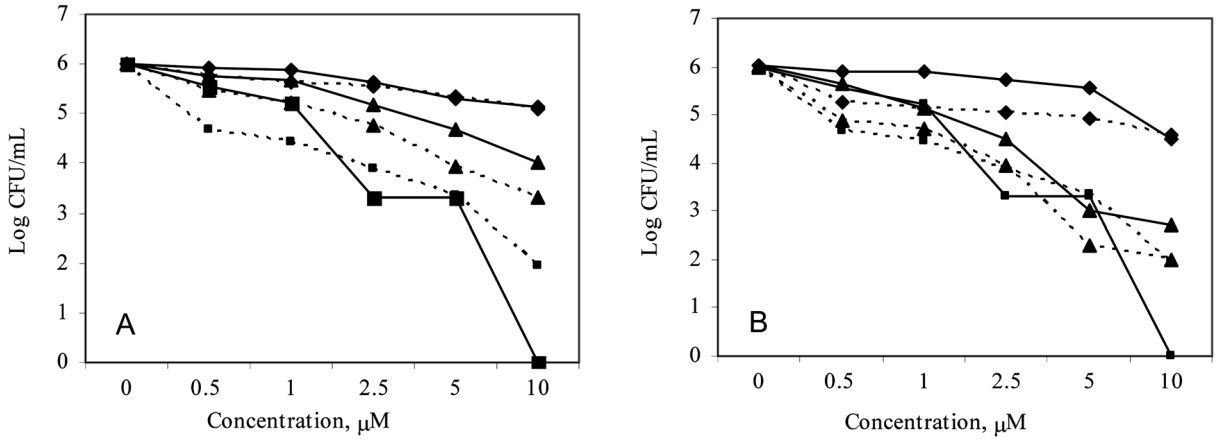


Figure 1. Survival curves of *S. aureus* incubated with various concentrations of newly synthesized B-1 series (A) and B-2 series (B) compounds and exposed to LED for 0, 15 min, and 30 min. A. Dotted line (B11) and ◆- no light, ▲- 15 min, ■- 30 min; black line (B12) and ◆- no light, ▲- 15 min, ■- 30 min. B. Dotted line (B21) and ◆- no light, ▲- 15 min, ■- 30 min; black line (B22) and ◆- no light, ▲- 15 min, ■- 30 min. Results are the mean values of at least three experiments.

chemical structure of the photosensitizing agents.

In the absence of irradiation, incubation with photosensitizers at concentrations of 5-10 μM for 10 min at dark caused a slight decrease in number of CFU/mL, showing antibacterial activity of tropolone itself. As a result we see the dual function activities of photosensitizers linked with

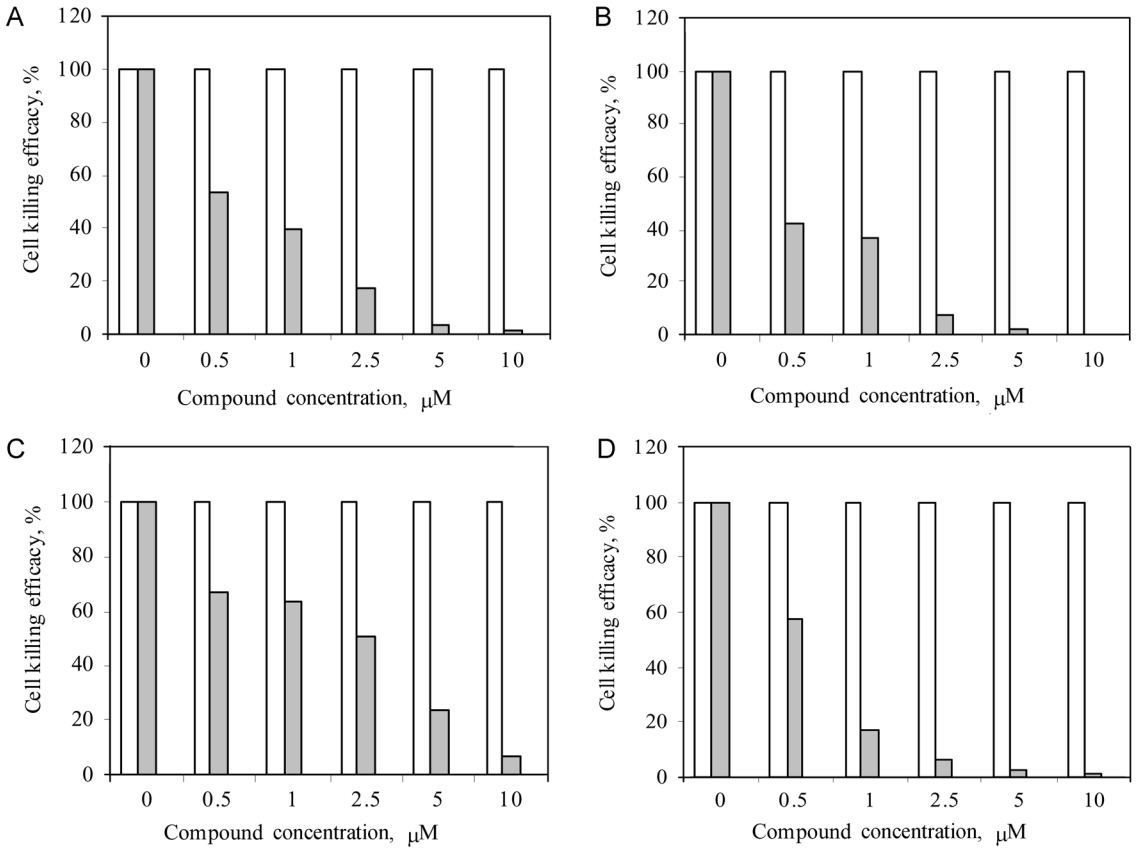


Figure 2. Cell killing efficacy of photosensitizers (A-B11, B-B12, C-B21, D-B22) against *S. aureus* after 10 min of incubation. Open bars, *S. aureus* cells after incubation with different concentrations without illumination; grey bars, *S. aureus* cells after incubation with different concentrations followed by illumination for 15 min.

tropolones over 5-10 μM concentration.

Pyropheophorbide derivatives are relatively better than pheophorbide derivatives and 30 min is enough to destroy bacterial cells completely less than 10.0 μM. Also isopropyl derivatives showed better inhibition than free tropolone, which may suggest more lipophilicity give better penetration for better inhibition.

The preliminary results presented indicate the promising activity of troponyl methyl (pyro)pheophorbide and its derivatives as potential candidates for antimicrobial photodynamic inactivation.

At present, we are extending our antimicrobial photodynamic studies to other group of photosensitizers and the photoinactivation efficiency on various Gram-negative and Gram-positive bacteria.

Experimental Section

General procedure for the reaction of bromo-chlorin (1,4[8]) with tropolone. For each reaction, we prepared troponyl methyl pheophorbide-a (**2**, **3**) and troponyl methyl pyropheophorbide-a. (**5**, **6**) The bromo-chlorin (1.3 mmol) and tropolone (1.5 mmol) were dissolved in dry dichloromethane (2 mL) with K_2CO_3 (3.0 mmol) and stirred for 12 h in the dark. The reaction mixture was partitioned with dichloromethane and water. The obtained organic phase was washed with 1 N HCl solution and brine, and dried over Na_2SO_4. The crude products were purified by short column chromatography over silica gel using 5% methanol in dichloromethane as the eluent to give the title compounds 30-50% yield.

Selected data, for **2**: UV-vis (CH_2Cl_2) λ_{max} = 664 (relative intensity, 0.70), 606 (0.12), 536 (0.15), 504 (1.15), 410 (1.81) nm; ^{1}H NMR (300 MHz. $CDCl_3$): δ 9.52, 9.38, 8.55 (each 1H, s, meso-H), 7.58-7.43 (5H, m, tropolone-H), 6.75, 6.24 (1H, s, 132-H), 4.44, 4.19 (2H, m, 17, 18-H), 3.85, 3.64, 3.57, 3.53, 3.37 (each 3H, s, Me+OMe), 3.64 (2H, m, 81-H), 2.80, 2.59 (4H, m, 171, 172-H), 2.24 (3H, d, J = 6.4 Hz, 32-Me), 1.79 (3H, d, J = 7.2 Hz,18-Me), 1.67 (3H, t, J = 7.6 Hz, 82-Me), 0.48-1.63 (2H, br s, NH).

For **3**: UV-vis (CH_2Cl_2) λ_{max} = 663.7 (relative intensity, 0.62), 606 (0.09), 536 (0.11), 506 (0.14), 410 (1.62); ^{1}H NMR (300 MHz, $CDCl_3$), δ: 9.51, 9.38, 8.56 (each, 1H, s, meso-H), 7.84-7.62 (4H, m, β-thujaplicin-H), 6.85, 6.27 (1H, s, 132-H), 4.46, 4.19 (2H, m, 17, 18-H), 3.84, 3.66, 3.54, 3.37 (each 3H, s, Me+OMe), 3.81, 3.64 (2H, m, 81-H), 2.85, 2.62 (4H, m, 171, 172-H), 2.17, (3H, d, J = 6.4 Hz, 32-Me), 1.79 (3H, d, J = 7.2 Hz, 18-Me), 1.68 (3H, t, J = 7.6 Hz, 82-Me), 1.34 (d, 6H, J = 6.8 Hz), 0.32-1.49 (2H, br s, NH).

Selected data, for **5**: UV-vis (CH_2Cl_2) λ_{max} = 663.4 (relative intensity, 0.79), 606 (0.14), 535 (0.17), 509 (0,18), 409 (2.04) nm; ^{1}H NMR (300 MHz, $CDCl_3$); δ 9.52, 9.38, 8.55

(each 1H, s, meso-H), 7.58-7.41 (5H, m, tropolone-H), 6.75, 6.24 (1H, s, 132-H), 4.44, 4.19 (2H, m, 17, 18-H), 3.64, 3.57, 3.53, 3.37 (each 3H, s, Me+OMe), 3.64 (2H, m, 81-H), 2.80, 2.59 (4H, m, 171, 172-H), 2.24, (3H, d, J = 6.4 Hz, 32-Me), 1.67 (3H, d, J = 7.2 Hz, 18-Me), 0.48-1.63 (2H, br s, NH).

For **6**: UV-vis (CH_2Cl_2) λ_{max}: 664.8 (relative intensity, 0.66), 606 (0.78), 535 (0.77), 505 (0.86), 410 (0.94) nm; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.60, 9.40, 8.54 (each 1H, s, meso-H), 7.99-7.89 (4H, m, β-thujaplicin-H), 6.75, 6.24 (1H, s, 132-H), 4.44, 4.27 (2H, m, 17, 18-H), 3.64, 3.59, 3.43, 3.37 (each 3H, s, Me+OMe), 3.84 (2H, m, 81H), 2.90, 2.65 (4H, m, 171, 172H), 2.25 (3H, d, J = 6.4 Hz, 32-Me) 1.78 (3H, d, J = 7.2 Hz, 18-Me), 1.68 (3H, t, J = 7.6 Hz, 82-Me), 1.31 (d, 6H, J = 6.8 Hz), 0.48-1.70 (2H, br, NH).

Antimicrobial activity. The cytotoxic action of troponyl methyl (pyro)pheophorbide derivatives on bacterial cells was assessed after 10 min of incubation in the dark. The irradiance was performed by a LED light source UFPh-630/675-01-BIOSPEC (BioSpec, Russia) for 15 and 30 min on the plate containing the microbial cell suspension at the top of 20 cm.

Survival fractions for the PDI experiments were expressed as the ratios of CFU from cultures treated with both light and compound to CFU measured for cultures treated with a compound or a light.[9,10,11]

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