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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Inhibitory effect of propafenone derivatives on *Pseudomonas aeruginosa* biofilm and pyocyanin production

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SUMMARY

Introduction/Objective Biofilm and pyocyanin production are essential components of *Pseudomonas aeruginosa* virulence and antibiotic resistance.

Our objective was to examine inhibitory effect of synthetized propafenone derivatives 3-(2-Fluorophenyl)-1-(2- (2-hydroxy-3-propylamino-propoxy)-phenyl)-propan-1-one hydrochloride (5OF) and3-(2-Trifluoromethyl-phenyl)-1-(2-(2-hydroxy-3-propylamino-propoxy)-phenyl)-propan-1-one hydrochloride (5CF3) on biofilm and pyocyanin in *Pseudomonas aeruginosa* clinical strains.

Methods Effects were tested on nine clinical isolates and one control laboratory strain of *P. aeruginosa*. *In vitro* analysis of biofilm growing was performed by incubating bacteria (0.5 McFarland) with 5OF and 5CF3 (500–31.2 μg/ml) and measuring optical density (OD) at 570 nm. Bacteria in medium without compounds were positive control. Blank medium (an uninoculated medium without test compounds) was used as negative control. Pyocyanin production was estimated by OD at 520 nm, after bacteria incubated with 5CF3 and 5OF (250 and 500 μg/ml), treated with chloroform, and chloroform layer mixed with HCl.

Results A total of 500 μg/ml of 5OF and 5CF3 completely inhibited biofilm formation in 10/10 and 4/10 strains, respectively. A total of 250 μg/ml of 5OF and 5CF3 strongly inhibited biofilm formation in 7/10 strains, while inhibition with 125 μg/ml of 5OF and 5CF3 was moderate. Lower concentrations had almost no effect on biofilm production. Pyocyanin production was reduced to less than 40% of the control value in 6/9, and less than 50% of the control in 7/9 strains with 500 μg/ml of 5OF and 5CF3, respectively. At 250 μg/ml 5OF and 5CF3, most strains had pyocyanin production above 50% of the control value.

Conclusion Synthetized propafenone derivatives, 5OF and 5CF3, inhibited biofilms and pyocyanin production of *Pseudomonas aeruginosa* clinical strains. Presented results suggest that propafenone derivatives are potential lead-compounds for synthesis of novel antipseudomonal drugs.

Keywords: propafenone derivatives; *Pseudomonas aeruginosa;* biofilm; pyocyanin

INTRODUCTION

As an opportunistic human pathogen, *Pseudomonas aeruginosa* has evolved a number of immunoevasive strategies to impair host defense, including growing in biofilm [1, 2].

Biofilms are bacterial clusters encased in self-produced polymeric matrix attached to the epithelial surfaces or surface of medical implants. They are characterized by lower metabolic activity, increased synthesis of protective molecules, prolonged doubling time, and genetic diversity of bacterial cells, all together improving bacterial tolerance to antibiotics and survival in harsh conditions [3, 4]. Biofilm production in P*seudomonas aeruginosa* is a well-known causative agent of antibiotic resistant infections in humans, such as pneumonia, and infections in patients with bronchiectasis and cystic fibrosis [5, 6]. Due to resistance to phagocytosis and pronounced antibody response, those infections lead to chronic inflammation, often with a severe fatal outcome [7, 8]. Thus, there is an urgent need to develop new drugs for the treatment of *Pseudomonas aeruginosa* biofilm-associated infections. In addition, *Pseudomonas aeruginosa* pathogenicity is intimately linked to its ability to produce large variety of virulence factors, including phenazine, and most abundant pyocyanin [9, 10]. Pyocyanin is highly diffusible blue pigment, which can interact with molecular oxygen and stimulate generation of oxygen radicals**,** leading to redox disbalance, injury and death of host cells [11]. As virulence factor in chronic lung infection pyocyanin disrupts redox control, inhibits respiration in human cells, accelerates neutrophil apoptosis, therefore impairing host defense and favoring bacterial persistence [12, 13, 14].

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Considering that ion channels are integral part of each living cell, which play a key role in cell division, proliferation, excitation, and apoptosis, modulators of ion channel activity have become important target molecules in medical chemistry [15]. Propiophenone is relatively simple compound commercially obtained from benzoic and propionic acid, it has channel-modulatory effect and serve as a precursor of numerous drugs (e.g. ephedrine, arylalkene) [16, 17, 18]. Propiophenone derivatives called propafenone are primarily known on their antiarrhythmic action, but they are also involved in treatment of many different diseases including lupus erythematosus, epilepsy, Alzheimer's disease, malaria, ebola, cancer [19–25]. In addition, recent studies have shown that analogs of propafenone exhibit antifungal activity [26]. Therefore, the molecule of propafenone has become a model of compounds used in multidrug-resistant studies [27].

Since data on antibacterial activity of propafenone derivatives are scarce, we decided to test potential antibacterial activity of 3-(2-Fluoro-phenyl)-1-(2- (2-hydroxy-3-propylamino-propoxy)-phenyl)-propan-1-one hydrochloride (5OF) and 3-(2-Trifluoromethyl-phenyl)-1-(2-(2-hydroxy-3-propylamino-propoxy)-phenyl)-propan-1-one hydrochloride (5CF3). Even more, because the influence of propafenone derivatives on the *Pseudomonas aeruginosa* biofilm and pyocyanin production has not yet been tested, we expanded our examinations on the influence of propafenone derivatives on expression of *Pseudomonas aeruginosa* virulence factors.

In the present study, we aimed to evaluate the inhibitory effects of ortho-fluorinated propafenone derivatives, which were synthetized in our laboratory, on biofilm and pyocyanin production in *Pseudomonas aeruginosa* clinical strains.

METHODS

Effect of ortho-fluorinated propafenone derivatives on the *Pseudomonas aeruginosa* biofilms

Test compounds

Ortho-fluorinated propafenone derivatives were synthesized at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Belgrade, Serbia: 5CF3: 3- (2-Trifluoromethyl-phenyl) -1- (2- (2-hydroxy-3-propylamino-propoxy) -phenyl) -propan-1-one hydrochloride and 5OF: 3- (2-Fluoro-phenyl) -1- (2- (2-hydroxy-3-propylamino-propoxy) -phenyl) -propan-1-one hydrochloride [28]. The structure of synthesized derivatives was spectrophotometrically analyzed at FT-IR spectrophotometer Nicolet iS10 (Thermo Fisher Scientific Inc., Waltham, MA, USA) [29].

The stock solutions of 5CF3 or 5OF (1 mg/ml) were prepared in 5% dimethyl sulfoxide (DMSO). The working solutions were prepared in trypticase soybean broth (TSB) with the addition of 1% glucose (Lab M Limited, Lancashire, UK) according to Knobloch et al. [30]. The concentrations of working solutions of 5CF3 or 5OF were 31.2, 62.5, 125, 250, and 500 μg/ml. In previous studies, we already investigated antimicrobial effect of tested compounds in the concentration range from 500 μg/ml to 62.5 μg/ml, and the best activity was observed for 250 μg/ml and 500 μg/ml of 5OF and 5CF3 [31].

Pseudomonas aeruginosa *clinical isolates*

The effects of tested compounds were investigated on nine clinical isolates obtained from urine (strains 1, 2, 5, 8, 9), ear swab (strains 3, 6, 7) or sputum (strain 4) and one laboratory control strain (ATCC 27853). Bacteria were stored at -70°C in Brain Heart Infusion Broth (Lab M Limited) until needed.

Culture medium

TSB and trypticase soybean agar (Lab M Limited) were used.

Analysis

Biofilm production and quantification were performed according to protocols described by Stepanović et al. [32]. Briefly, bacteria were resuspended in saline to the density of a 0.5 McFarland standard (~10^8 CFU/ml). In 96 microtiter plates, 180 μl of test compounds and 20 μl of bacterial suspension were added in triplicate. Bacteria incubated in medium without test compounds were used as positive control, while blank medium (uninoculated medium without test compounds) represented negative control. After incubation, which lasted 24h at 35°C, plates were washed in phosphate buffer (PBS, pH 7.2), dried, fixed with methanol, and stained with 2% crystal violet (Himedia, Mumbai, India). After washing, the color was extracted from bacteria with 96% ethanol. The OD was measured spectrophotometrically at 570 nm (ICN Flow Titertek Multiscan Plus, ICN, USA). Each experiment was repeated three times. To calculate the category of biofilm production, the optical density cut-off (ODc) was determined as three standard deviations above the mean OD of the negative control. According to the calculated results, all tested strains were categorized into four groups: $OD \leq ODc$ - category 0 (no biofilm production); $ODc < OD \leq 2 \times ODc$ - category 1 (weak biofilm production); $2 \times ODc < OD \leq 4 \times ODc$ - category 2 (moderate biofilm production), and $4 \times ODc < OD$ - category 3 (strong biofilm production).

Effect of ortho-fluorinated propafenone derivatives on the *Pseudomonas aeruginosa* pyocyanin production

Test compounds

Ortho-fluorinated propafenone derivatives, 5CF3 and 5OF, were synthesized at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Belgrade, Serbia.

Working dilutions of 250 and 500 μg/ml 5CF3 and 5OF in 5% DMSO were prepared from the stock solution of 1 mg/ml in 5% DMSO. Working concentrations were chosen based on our results about 5CF3 and 5OF effect on biofilm formation, where concentrations of 250 μg/ml and 500 μg/ml appeared to have the strongest inhibitory effect.

Pseudomonas aeruginosa *clinical strains*

The effects of tested compounds were investigated on nine *Pseudomonas aeruginosa* clinical isolates. The sources of bacteria and storage conditions were the same as previously described in section Methods.

Culture medium

Mueller–Hinton broth for bacteria (Torlak, Belgrade, Serbia) was used (Figure 1).

Pyocyanin determination

Pyocyanin was determined as previously described by Glamočlija et al. [33]. Five milliliters of bacterial cultures in exponential phase of growth were incubated with test compounds for 24h at 37°C and then treated with 3 ml of chloroform. Separated chloroform layer was mixed with 1 ml of 0.2 M HCl. OD was measured at 520 nm [34]. Positive controls for each isolate were cultivated at the same conditions in medium without tested compounds. Values were expressed as a ratio $(OD_{520}/OD_{600}) \times 100$. Two experiments, each in triplicate, were performed. Results were calculated as the percent of the pyocyanin production compared to the positive control (expressed as 100% ± SD).

Statistical analysis

Obtained data were analyzed using statistical analysis software package – SPSS Statistics Version 18.0 for Windows (SPSS Inc., Chicago, USA) and Student's t-test [35].

RESULTS

Effect of ortho-fluorinated propafenone derivatives on the *Pseudomonas aeruginosa* biofilm formation

Ortho-fluorinated propafenone derivatives, 5OF and 5CF3, inhibited production of *Pseudomonas aeruginosa* biofilms. The intensity of inhibitory effects changed in concentration dependent manner, thus, higher drug concentrations lead to stronger inhibition. The highest inhibition occurred at concentration of 500 μg/ml of both compounds. When the 5OF and 5CF3 concentrations decreased to 250 μg/ml, 125 μg/ml, 62.5 μg/ml, or 31.2 μg/ml, the inhibitory effect was also decreased. In addition, there was a variance in different isolates sensitivity to particular drug concentration. Biofilm formation was completely inhibited by 500 μg/ml of 5OF and 5CF3 in 10/10 and 4/10 strains, respectively. In 7/10 strains, biofilm formation was strongly inhibited by 250 μg/ml 5OF and 5CF3, while inhibition with 125 μg/ml 5OF and 5CF3 was moderate. In the presence of lower 5OF and 5CF3 concentrations, 62.5 μg/ml and 31.2, 8/10 tested strains exerted strong biofilm production. Categories of biofilm production in different isolates and in the presence of various concentrations of tested compounds are presented in Table 1 and 2.

Table 1. *In vitro* effect of 5OF on the biofilm production of *Pseudomonas aeruginosa*

Parameters	5OF μg/ml					
	500	250	125	62.5	31.2	Positive control
Pseudomonas aeruginosa Strain number	Category of biofilm production					
1	0	1	2	2	2	2
2	0	1	1	3	3	3
3	0	0	1	2	2	2
4	0	1	1	3	3	3
5	0	1	2	3	3	3
6	0	1	2	3	3	3
7	0	2	2	3	3	3
8	0	1	2	3	3	3
9	0	1	1	2	3	3
ATCC 27853	0	0	2	3	3	3

5OF – 3- (2-fluoro-phenyl) -1- (2- (2-hydroxy-3-propylamino-propoxy) -phenyl) -propan-1-one hydrochloride; Positive control – bacterial growth in medium without tested compound; 0 – no biofilm production; 1 – weak biofilm production; 2 – moderate biofilm production; 3 – strong biofilm production

Table 2. *In vitro* effect of 5CF3 on the biofilm production of *Pseudomonas aeruginosa*

Parameters	5CF3 μg/ml					
	500	250	125	62.5	31.2	Positive control
Pseudomonas aeruginosa Strain number	Category of biofilm production					
1	0	1	2	2	2	2
2	1	1	2	3	3	3
3	0	1	2	2	2	2
4	1	2	2	3	3	3
5	1	1	2	3	3	3
6	0	1	2	3	3	3
7	1	2	2	3	3	3
8	1	1	3	3	3	3
9	1	2	2	3	3	3
ATCC 27853	0	1	0	3	3	3

5CF3 – 3- (2-trifluoromethyl-phenyl) -1- (2- (2-hydroxy-3-propylamino-propoxy) -phenyl) -propan-1-one hydrochloride; Positive control – biofilm production in medium without tested compound; 0 – no biofilm production; 1 – weak biofilm production; 2 – moderate biofilm production; 3 – strong biofilm production

Effect of ortho-fluorinated propafenone derivatives on the *Pseudomonas aeruginosa* pyocyanin production

Ortho-fluorinated propafenone derivatives, 5CF3 and 5OF, inhibited production of pyocyanin in *Pseudomonas aeruginosa*. In the presence of 500 μg/ml 5OF or 5CF3

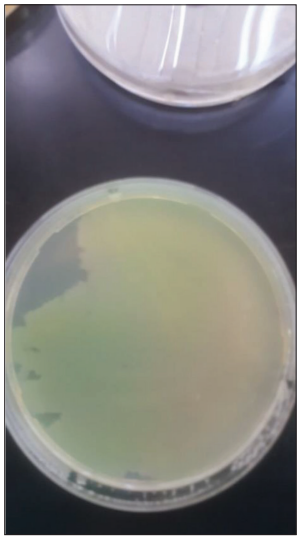

Figure 1. *Pseudomonas aeruginosa* growth on Mueller–Hinton agar

production of pyocyanin was reduced to less than 40% of the control value in 6/9 strains, and less than 50% of the control in 7/9 strains, respectively. In the presence of 250 μg/ml 5OF or 5CF3, most strains had pyocyanin production above 50% of the control value. The difference in the sensitivity to the tested compounds among various strains was also detected. Results of inhibitory action of 5OF and 5CF3 on the pyocyanin production in *Pseudomonas aeruginosa* are expressed as the percentage of the absorbance of positive controls (presented as 100% ± SD) (Table 3).

Table 3. *In vitro* effect of ortho-fluorinated propafenone derivatives 5OF and 5CF3 on the production of pyocyanin in *Pseudomonas aeruginosa* strains

Parameters	5OF μg/ml		5CF3 μg/ml	
	500	250	500	250
Pseudomonas aeruginosa Strain number	Pyocyanin production as% of positive control			
1	48.6	70.5	74	79.8
2	27.1	42	36.6	79.8
3	39.3	51.8	68.5	99.4
4	33.7	31.9	34.6	54.2
5	39.4	61.1	48.3	104.9
6	34.3	49.5	35.2	46.6
7	42.7	53.4	51	56.3
8	29.8	54.2	36.9	52.4
ATCC 27853	43.6	57.6	47.9	64

5OF – 3- (2-fluoro-phenyl) -1- (2- (2-hydroxy-3-propylamino-propoxy) -phenyl) -propan-1-one hydrochloride; 5CF3 – 3- (2-trifluoromethyl-phenyl) -1- (2- (2-hydroxy-3-propylamino-propoxy) -phenyl) -propan-1-one hydrochloride; Positive control – pyocyanin production of each isolate in the absence of the tested compounds (100%).

DISCUSSION

Antibiotic compounds that inhibit different virulence factor, such as enterotoxins, hemolysins, biofilm, or pigments, became the focus of the present research [36]. The resistance of *Pseudomonas aeruginosa* isolates to antimicrobial drugs is largely attributed to its ability to form a biofilm and produce bacterial pigment pyocyanin [37]. In this study, we used synthesized propafenone derivatives, 5CF3, and 5OF, to test inhibitory effect on *Pseudomonas aeruginosa* biofilm and pigment production.

Antimicrobials are generally dedicated to kill bacteria (bactericidal) or to inhibited bacterial growth (bacteriostatic). However, mostly due to frequent chromosomal mutations, *Pseudomonas aeruginosa* appeared to be extremely adaptive and acquired resistance to many antibiotics such as carbapenems, penicillins and cephalosporins. Recent efforts to develop novel class of anti-pseudomonas agents moved their focus to *Pseudomonas aeruginosa* physiology and collective behavior of bacterial population [38]. Therefore, biofilm formation and its modulation became a subject of our research interest. Our results have shown that propafenone derivative 5OF and 5CF3 significantly reduced biofilm production in all tested isolates of *Pseudomonas aeruginosa.* Previous study on propafenone compounds reported antimicrobial effect due to inhibition of ubiquitous bacterial multidrug efflux pumps [39]. Thus, by channel-blocking propafenone may decrease drug resistance and positively influence clinical outcome of *Pseudomonas aeruginosa* infections [40]. On the other hand, to the best of our knowledge, this study revealed identification of ortho-fluorinated propafenone derivatives as efficient agents that inhibit *Pseudomonas aeruginosa* biofilm formation for the first time. The inhibitory effects of both 5OF and 5CF3 were found. Numerous external factors affect biofilm formation by *Pseudomonas aeruginosa*. In addition, the type of tissue has strong impact on biofilm formation, and researchers commonly test biofilm formation of *Pseudomonas aeruginosa* from a variety of clinical sources [41]. In our study, various clinical strains showed differences in sensitivity to tested compounds, but those variations were not connected to specific bacterial source (urine, ear swab, sputum). However, we tested only nine clinical isolates (5 – urine, 3 – ear swab, 1 – sputum) and for such a small number of samples statistical data processing is not relevant.

The highest tested dose of both compounds (500 μg/ml) was the most efficient, reducing bacterial growth to the highest extent. However, when the concentration of test agents decreased bacterial growth recovered. In the present study, 5OF was more effective in reducing bacterial growth compared to 5CF3. This could be explained by higher binding affinity to bacterial transport porin in a case of monofluorinated propafenone derivatives (such as 5OF), compared to trifluoromethyl derivative (5CF3), as found in our docking studies (data not shown) [31]. Namely, biofilm formation depends on the presence of an extracellular matrix, which is a mixture of polysaccharides, proteins, and nucleic acids [extracellular DNA (eDNA)]. Matrix polysaccharides (alginate and lipopolysaccharides), which are synthetized in bacterial cytoplasm**,** bind to membrane transporters to be extruded out of the cell [42]. It was found that both fluorinated derivatives tested in this study briefly occupied key substrate-specific sites in the bacterial porin (Arg124). This discovery might be associated with interruption of the transport of carbohydrate compounds involved in synthesis of biofilm [43].

The blue pigment pyocyanin, chemical derivative of phenazine, is one of the most important virulence factors in *Pseudomonas aeruginosa* [44]. Pyocyanin is toxic for respiratory epithelium, it acts on the cell structure and function, disrupts normal expression of genes involved in efflux pumps, redox homeostasis and iron acquisition in human cells [45, 46, 47]. Thus, control of pyocyanin production may be a mechanism to reduce bacterial pathogenicity. Results of our study have shown that both 5OF and 5CF3 inhibited production of pyocyanin in all tested *Pseudomonas aeruginosa* isolates. The inhibitory effect was concentration dependent, higher concentrations caused stronger inhibition, while inhibitory effect decreased with lower drugs concentration. Literature survey on other drugs suggests that ortho-fluorinated propafenone derivative 5OF had significantly stronger inhibitory effect on the production of pyocyanin in *Pseudomonas aeruginosa* strains than commercial antibiotics ampicillin or streptomycin. Namely, we have shown that lower concentrations of 5OF, 500μg/ml, and 250 μg/ml, exerted same or even enhanced inhibitory effect compared to commercial antibiotics when applied in 2–4 times higher concentration (1 mg/ml) [33, 48]. Similarly, the concentrations of 5CF3, which led to pyocyanin reduction, were within the same range as concentration of standard drugs. The observed propafenone-induced pyocyanin inhibition could be discussed in a view of recent results on pyocyanin impact on eDNA and biofilm formation [11]. It was found that pyocyanin decreases eDNA content within the *Pseudomonas aeruginosa* biofilm. Since eDNA promotes bacterial adhesion and cellular aggregation, depletion of eDNA can reduce biofilm strength and disturb protection of bacterial cells against antibiotics. Based on the aforementioned, it was assumed that reduction of pyocyanin production as detected in our study could be a model of propafenone derivatives action against *Pseudomonas aeruginosa* pathogenicity and infection.

CONCLUSION

The results of the study suggest that synthetized ortho-fluorinated propafenone derivatives inhibit biofilm and pyocyanin production in *Pseudomonas aeruginosa* clinical strains. Presented results suggest that propafenone derivatives could be considered as potential lead-compounds for synthesis of novel antipseudomonal drugs.

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This study was done in accordance with the institutional standards of the Committee on Ethics.

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Conflict of interest: None declared.

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Инхибиторни ефекат пропафенонских деривата на продукцију биофилма и пиоцијанина код бактерије *Pseudomonas aeruginosa*

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САЖЕТАК

Увод/Циљ Производња биофилма и пиоцијанина је важан фактор вируленције и антибиотске резистенције бактерије *Pseudomonas aeruginosa.*

Циљ рада је био да се испита инхибиторни ефекат синтетисаних пропафенонских деривата, 3-(2-флуоро-фенил)-1-[2-(2-хидрокси-3-пропиламино-пропокси)-фенил]-пропан-1-он-хидрохлорид) (5*OF*) и 3-(2-трифлуорометилфенил)-1-[2-(2-хидрокси-3-пропиламино-пропокси)-фенил]-пропан-1-он-хидрохлорид) (5*CF*3), на продукцију биофилма и пиоцијанина код клиничких изолата бактерије *Pseudomonas aeruginosa.*

Методе Ефекат пропафенонских деривата испитан је на девет клиничких изолата и једном стандардном сојe бактерије *P. aeruginosa.* Утицај на продукцију биофилма испитан је *in vitro,* инкубацијом бактерија (0,5 по Макфарланду) са 5*OF* и 5*CF*3 (500–31,2 *μg/ml*), и мерењем оптичке густине на 570 *nm*. Бактерије у медијуму без испитиваних једињења су биле позитивна контрола, а сам медијум негативна контрола. Производени пиоцијанин, који је одређиван мерењем оптичке густине на 520 *nm*, на коинкубације бактерија са 5*CF*3 или 5*OF* (250 и 500 *μg/ml*), третиран је хлороформом и мешањем хлороформског слоја са *HCl*.

Резултати При концентрацији од 500 *μg/ml* 5*OF* је довео до потпуне инхибиције продукције биофилма код свих испитиваних сојева (10/10). Инхибиција биофилма са 500 *μg/ml* 5*CF*3 била је потпуна код 4/10 сојева. При концентрацији 5*OF* и 5*CF*3 од 250 *μg/ml* продукција биофилма код већине испитаних изолата била је слаба, док је при концентрацији 125 *μg/ml* 5*OF* односно 5*CF*3 продукција била умерена. Ниже концентрације 5*OF* и 5*CF*3 нису имале инхибиторни ефекат на формирање биофилма. У присуству 500 *μg/ml* 5*OF* у 6/10 испитиваних сојева продукција пиоцијанина пала је на мање од 40% у односу на контролну вредност. Иста концентрација (500 *μg/ml*) 5*CF*3 снизила је продукцију пиоцијанина на мање од 50% од контроле у 7/9 сојева. При концентрацији 250 *μg/ml* 5*OF* или 5*CF*3 већина сојева продуковала је пиоцијанин изнад 50% у односу на позитивну контролу.

Закључак Синтетисани пропафенонски деривати, 5*OF* и 5*CF*3, инхибирају продукцију биофилма и пиоцијанина код клиничких сојева бактерије *Pseudomonas aeruginosa.* Добијени резултати указују на то да пропафенонски деривати представљају могућа полазна једињења за синтезу нових антипсеудомонасних агенаса.

Кључне речи: пропафенонски деривати; *Pseudomonas aeruginosa;* биофилм; пиоцијанин