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Synthesis, antibacterial, and antibiofilm activities of pulmonarin B analogues

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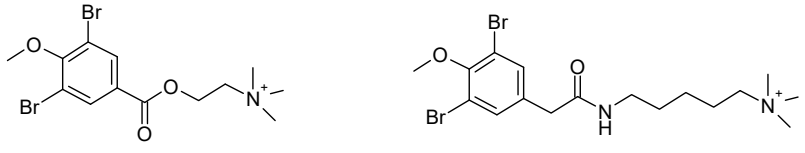
New analogues of pulmonarin B were synthesized from (3,5-dibromo-4-hydroxyphenyl)acetic acid derivatives, and their antibacterial and antibiofilm activities against *E. coli*, *S. aureus*, and *P. aeruginosa* were evaluated. The antibacterial activity of synthesized ammonium salts against the methicillin-resistant strain of *S. aureus* 222 depends on the length of the alkyl chain. Bis-quaternary ammonium salt **5** demonstrated better activity against *S. aureus* 222, *E. coli* 311, and *P. aeruginosa* 449 compared to mono-alkylated derivatives. Analogues of pulmonarin B **5** and **4d** reduced *S. aureus* 222 biofilm formation by 74.5% and 89.4%, respectively. In addition, compound **4c** turned out to be the most active against the biofilm formation of *P. aeruginosa* 449 (biomass decreased by 39.8%).

1. Introduction

Overuse of antibiotics has led to the emergence of antibiotic-resistant strains of bacteria, including *S. aureus*.[1] Most bacterial infections are biofilm-mediated.[2] Biofilms resistant to antibiotic therapy are the main cause of infections caused by clinically important pathogens *P. aeruginosa*, *E. coli,* and *S. aureus*.[3,4] Therefore, the development of anti-biofilm compounds that inhibit biofilm formation and prevent infection is necessary and relevant.

Marine natural products are considered a valuable source of bioactive compounds with potential antimicrobial activity.[5,6] In addition, marine-derived natural products have great diversity, providing new structures for the development of effective antibiofilm agents.[7,8] Marine sponges are a rich resource of biologically active metabolites, in particular, marine demosponges of the Verongiida order contain high concentrations of brominated alkaloids, which after induced bioconversion are enzymatically cleaved to dibromotyrosine derivatives.[9] Most of the selected brominated metabolites demonstrated moderate to high antimicrobial and antifouling activity.[10-12]



Pulmonarin A ***Pulmonarin B***

Fig. 1. Structures of pulmonarin A and B

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The dibromotyrosine derivatives pulmonarin A and B (**Fig. 1**), isolated from the ascidian *Synoicum pulmonaria*, showed antifouling effects directed against bacterial adhesion and growth and exhibited inhibitory activity against acetylcholinesterase (AChE). [13,14] However, synthetic derivatives of pulmonarine alkaloids showed high antiviral activity against *tobacco mosaic virus* and potent fungicidal activity. [15]

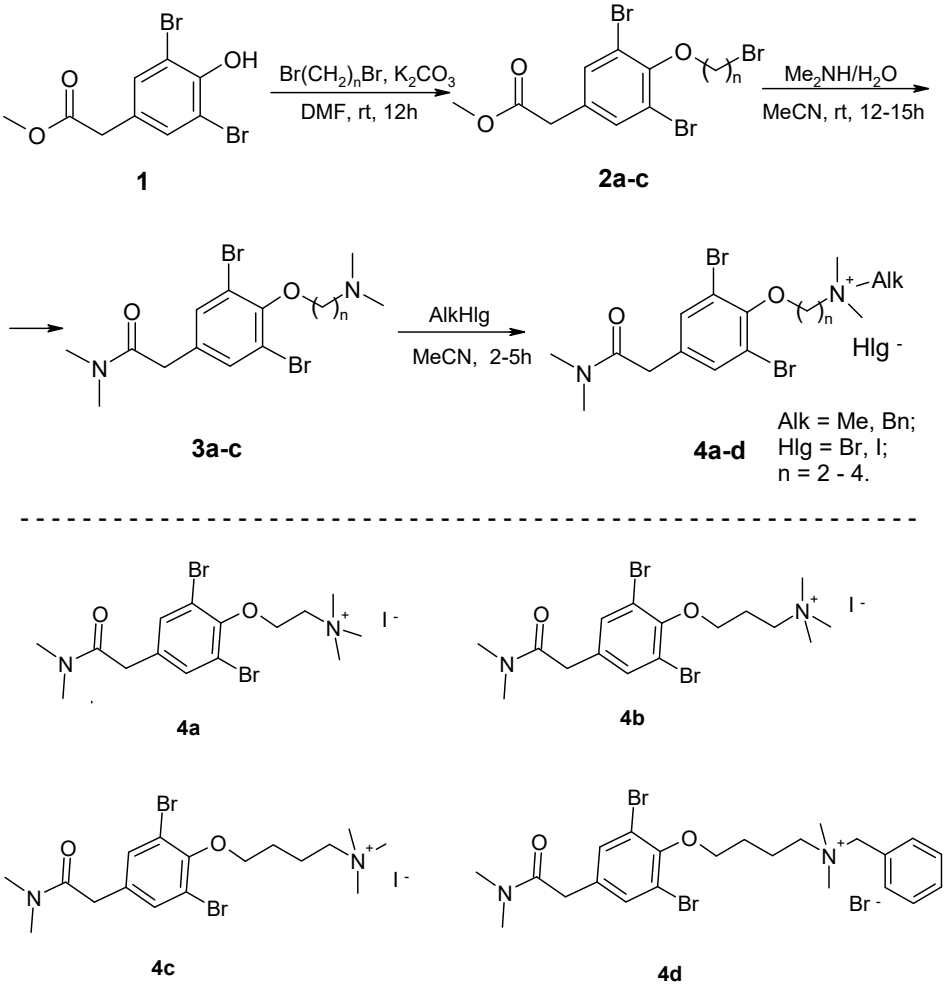
Compounds containing a quaternary ammonium group show good antimicrobial activity against gram-positive and gram-negative bacteria and fungi. [16,17] Quaternary ammonium salts as cationic surfactants have been widely used in disinfectants and antiseptics for many years. [18] Such compounds prevent the adhesion of microorganisms to various biotic and abiotic surfaces and cause the eradication of biofilms produced by pathogenic microorganisms. [19-21]

In this study, we report the synthesis of new quaternary ammonium salts, analogues of pulmonarin B, based on (3,5-dibromo-4-hydroxyphenyl)acetic acid derivatives, and evaluate their antibacterial and antibiofilm activities against *E. coli*, *S. aureus*, and *P. aeruginosa.*

2. Results and Discussion

2.1. Chemistry

Synthesis of the target analogues of pulmonarin B **4** was carried out according to **Scheme 1**. The starting material **1** was obtained by the esterification reaction of 3,5-dibromo-4-hydroxyphenylacetic acid and absolute methanol in the presence of concentrated sulfuric acid. [22] *O*-alkylation of compound **1** with dibromoalkanes in the presence of K_2CO_3 in DMF afforded alkoxy-substituted derivatives **2a-c** in high yields. Finally, the conversion of compounds **2a-c** to 3,5-dibromo-4-(dimethylamino)alkylphenyl-*N,N*-dimethylacetamides **3a-c** followed by alkylation gave the target quaternary ammonium salts **4a-d**.

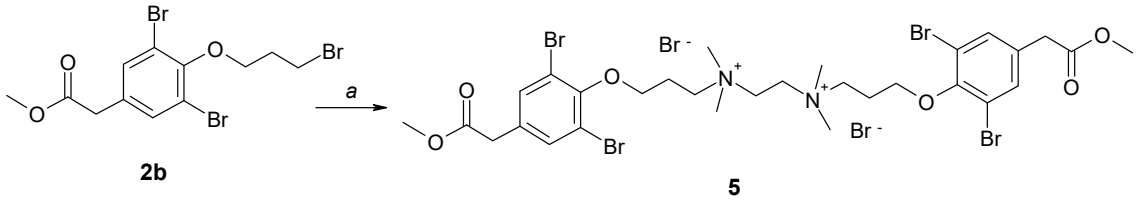


1 **2a-c** **3a-c** **4a-d**

4a **4b** **4c** **4d**

Scheme 1. Synthesis of pulmonarin B analogues **4a-d**

Considering the excellent antimicrobial activity of bis-quaternary ammonium salts, [23,24] for comparative purposes, a symmetrical bis-alkylated derivative of N,N,N′,N′-tetramethylethylenediamine (TMEDA) was synthesized (**Scheme 2**). The target bis-quaternary ammonium salt **5** was obtained by refluxing excess methyl [3,5-dibromo-4-(3-bromopropoxy)phenyl]acetate **2b** and TMEDA in acetonitrile.



2b **5**

a: $Me_2N(CH_2)_2NMe_2$, MeCN, reflux, 2h

Scheme 2. Synthesis of bis-quaternary ammonium salt **5**

The structures of the synthesized compounds were confirmed by the elemental analysis, IR, ^1H and ^{13}C NMR, and mass spectrometry.

2.2. Biology

2.2.1 Antibacterial activity

The results of the antibacterial activity of synthesized analogues of pulmonarin B **4a-d**, **5** against gram-positive and gram-negative microorganisms are shown in **Table 1**. Compound **5** was the most active against *S. aureus* 222, *E. coli* 311, and *P. aeruginosa* 449. Its MICs were 12.5, 100.0, and 50.0 μg/mL respectively. According to the obtained data, compound **4d** exhibited significant antimicrobial activity only against *S. aureus* 222 (MIC value was 25.0 μg/mL). Against gram-negative bacteria, MICs were >200.0 μg/mL. Compounds **4b** and **4c** showed a less pronounced antimicrobial activity: MICs against *S. aureus* 222 were 200.0 μg/mL, against *E. coli* 311 and *P. aeruginosa* 449 ≥ 200.0 μg/mL. Compound **4a** did not exhibit significant antimicrobial activity against tested microorganisms: MICs were >200.0 μg/mL.

Table. 1. Antibacterial activity (MIC, μg/mL) of new analogues pulmonarin B

Bacterial strain	MIC, μg/mL								
	4a	**4b**	**4c**	**4d**	**5**	**CIP**	**GEN**	**MEM**	**AZM**
S. aureus 222	≥ 200.0	200.0	200.0	25.0	12.5	0.25	–	–	1.0
E. coli 311	≥ 200.0	≥ 200.0	≥ 200.0	≥ 200.0	100.0	31.3	1.0	1.0	–
P. aeruginosa 449	≥ 200.0	≥ 200.0	≥ 200.0	≥ 200.0	50.0	0.5	0.25	4.0	–

Note. CIP – ciprofloxacin; MEM – meropenem; GEN – gentamycin; AZM – azithromycin; "–" – not recommended for susceptibility testing purposes. [25]

Antibacterial activity of new analogues pulmonarin B is less pronounced in comparison to ciprofloxacin (*S. aureus* 222, *E. coli* 311, *P. aeruginosa* 449), azithromycin (*S. aureus* 222), gentamicin and meropenem (*E. coli* 311, *P. aeruginosa* 449) activity.

2.2.2 Antibiofilm activity

The ability of the synthesized compounds to disrupt the formation of biofilms on abiotic surfaces was investigated by simultaneously introducing compounds and microbial inoculum into the wells of microtiter plates. The obtained results are presented in **Fig.2**, **Fig. 3**, and **Fig. 4**.

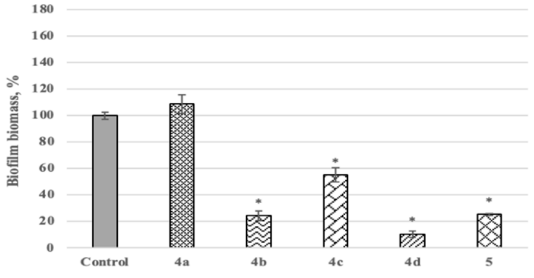


Fig. 2. The action of new analogues pulmonarin B on the *S. aureus* 222 biofilm formation. The value of the intact control is taken as 100%. *p<0.05 as compared to control

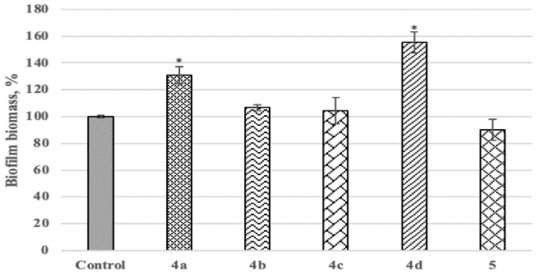


Fig. 3. The action of new analogues pulmonarin B on the *E. coli* 311 biofilm formation. The value of the intact control is taken as 100%. *p<0.05 as compared to control

It was shown (**Fig. 2**) that compound **4d** disrupted the biofilm formation of *S. aureus* 222 most actively (the biomass decreased by 89.4%, $p<0.05$). Compounds **4b** and **5** showed a significant inhibitory effect on the biofilm formation: the percentage of inhibition reached 75.6% and 74.5%, respectively ($p<0.05$). Compound **4c** also had an antibiofilm activity: under its action, the biomass of *S. aureus* biofilm was 44.8% less compared to the control ($p < 0.05$). In contrast, compound **4a** did not exhibit any inhibitory effect against *S. aureus* 222.

The results of experiments demonstrated that analogues of pulmonarin B **5** (**Fig. 3**) did not have a significant inhibitory effect on the formation of *E. coli* 311 biofilms: the biomass decreased by 9.9% compared to the control ($p>0.05$). Contrary, compound **4d** showed a significant inhibitory activity against *E. coli* 311: the biofilm biomass decreased by 55.3% compared to the control ($p<0.05$). Compound **4a** stimulated the formation of biofilms by *E. coli* 311. At the concentration of 25 μg/ml, a statistically significant increase in the biofilm biomass was observed (30.9%, $p<0.05$). Compounds **4b** and **4c** did not affect *E. coli* 311 biofilm formation: the biomass increased by 4.4 – 6.2 % compared to control ($p>0.05$).

When analogues of pulmonarin B acted on the formation of biofilms of *P. aeruginosa* 449, both the inhibitory and stimulating effects were noted (**Fig. 4**). Compound **4c** turned out to be the most active against the biofilm formation of *P. aeruginosa* 449: the biomass decreased by 39.8% ($p<0.05$). Compound **4b** exhibited lower antibiofilm activity with an inhibition percentage of 26.3% ($p<0.05$). On the other hand, compound **5** stimulated the biofilm formation: in this case, the biomass increased by 24.8%. Compounds **4a** and **4d** did not exhibit significant inhibitory activity against *P. aeruginosa* 449 biofilms: the biomass decreased by 2.2 – 6.4% compared to control ($p>0.05$).

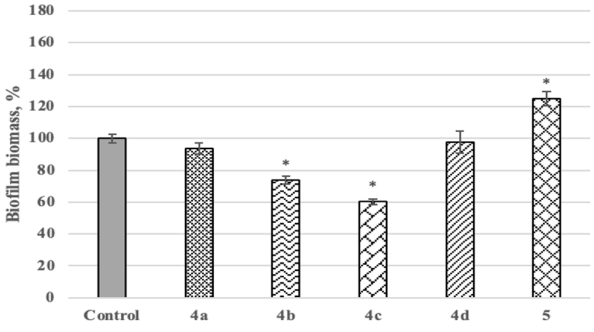


Fig. 4. The action of new analogues pulmonarin B on the *P. aeruginosa* 449 biofilm formation. The value of the intact control is taken as 100%. *$p < 0.05$ as compared to control

3. Conclusions

In this study, four pulmonarin B analogues and a bis-quaternary ammonium salt were synthesized and their antibacterial and antibiofilm activities against *S. aureus* 222, *E. coli* 311, and *P. aeruginosa* 449 were evaluated. The antibacterial activity of quaternary ammonium salts against *S. aureus* 222 increased with increasing length of the alkyl chain. The strongest antibacterial effect was observed for bis-quaternary ammonium salt **5** (against *S. aureus* 222 and *P. aeruginosa* 449) and compound **4d** (against *S. aureus* 222). Antibiofilm activity of pulmonarin B analogues is most pronounced against *S. aureus* 222 (biomass decreases by 74.5 - 89.4% for compounds **4b**, **4d,** and **5**). The formation of biofilms of *E. coli* 311 under the influence of compounds in subinhibitory concentrations is practically not disturbed. Compound **4c** is the most active against *P. aeruginosa* 449 (the biomass is 60.2%).

These results lay the foundation for further research and development of new antimicrobial and antibiofilm agents among synthetic analogues of natural compounds.

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4. Experimental

4.1. Materials and Methods

4.1.1. Chemistry

All reagents and solvents were commercially available and were used without further purification. IR spectra were recorded on Bruker Vertex 70 FT-IR spectrometer, equipped with a Harrick MVP2 diamond ATR device (KBr transmittance and ATR). ^{1}H and ^{13}C NMR spectra were acquired on Varian Unity INOVA 400 (400 MHz for ^{1}H nuclei) and Bruker Avance DRX-500 (500 and 125 MHz for ^{1}H and ^{13}C nuclei, respectively) instruments (TMS as internal reference)

in DMSO-d_6. ^{13}C NMR signals were assigned by using APT method. LCMS spectra were performed on Agilent 1100 Series HPLC equipped with diode array and Agilent LC/MSD SL mass selective detector, ionization method – chemical ionization at atmospheric pressure. Zorbax SB-C18 column was used, and gradient elution with 0.1% HCOOH in H_2O–MeCN was applied. Elemental analysis was performed at the Analytical laboratory of the V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry of the NAS of Ukraine. Melting points were determined on the Boetius hot stage apparatus.

Methyl (3,5-dibromo-4-hydroxyphenyl)acetate (**1**). Concentrated sulfuric acid (0.5 ml) was added to a solution of (3,5-dibromo-4-hydroxyphenyl)acetic acid **7** (10 mmol) in methanol (25 mL). The mixture was stirred at room temperature for 12 h and water (3 mL) was added. The resulting crystalline precipitate was filtered and dried. Yield 90%, colorless solid, mp 103-105 °C. IR (KBr): 3409 (OH), 3068-2849 (C-H, aromatic and aliphatic), 1726 (C=O) cm^{-1}.^{1}H NMR (400 MHz, DMSO-d_6): δ 3.61 (s, 3H, OCH_3), 3.64 (s, 2H, CH_2), 7.46 (s, 2H, 2CH), 9.85 (br s, 1H, OH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 37.9 (CH_2), 51.8 (OCH_3), 111.6, 128.8, 133.2, 149.5, 171.4. LCMS $[M+H]^+$: 325.0. Anal. Calcd. for $C_9H_8Br_2O_3$: C, 33.37; H, 2.49; Br, 49.33. Found: C, 33.42; H, 2.51; Br, 49.28.

Methyl [3,5-dibromo-4-bromoalkoxyphenyl]acetates **2a-c.** The corresponding dibromoalkane (15 mmol) was added to a suspension of ester **1** (5 mmol) and K_2CO_3 (10 mmol) in DMF (10 mL). The mixture was stirred at room temperature for 12 h and cold water (20 mL) was added. The suspension was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The remains of dibromoalkane were removed in a vacuum at 60-80 °C. Compound **2a** was purified by crystallization from hexane.

Methyl [3,5-dibromo-4-(2-bromoethoxy)phenyl]acetate (**2a**). Yield 70%, colorless solid, mp 49-51 °C. IR (KBr): 3016-2846 (C-H, aromatic and aliphatic), 1735 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.63 (s, 3H, OCH_3), 3.73 (s, 2H, CH_2), 3.84 (t, J = 5.2 Hz, 2H, CH_2), 4.28 (t, J = 5.2 Hz, 2H, CH_2), 7.61 (s, 2H, 2CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 31.1 (CH_2), 38.0 (CH_2), 51.7 (OCH_3), 72.6 (CH_2), 117.1, 134.0, 134.1, 150.7, 170.9. LCMS $[M+H]^+$: 432.2. Anal. Calcd. for $C_{11}H_{11}Br_3O_3$: C, 30.66; H, 2.57; Br, 55.63. Found: C, 30.62; H, 2.60; Br, 55.71.

Methyl [3,5-dibromo-4-(3-bromopropoxy)phenyl]acetate (**2b**). Yield 78%, yellowish oil. IR (ATR): 3021-2842 (C-H, aromatic and aliphatic), 1730 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.26-2.34 (m, 2H, CH_2), 3.61 (s, 3H, OCH_3), 3.70-3.75 (m, 4H, 2CH_2), 4.05 (t, J = 5.6 Hz, 2H, CH_2), 7.58 (s, 2H, 2CH). LCMS $[M+H]^+$: 446.2. Anal. Calcd. for $C_{12}H_{13}Br_3O_3$: C, 32.39; H, 2.94; Br, 53.87. Found: C, 30.33; H, 2.91; Br, 53.82.

Methyl [3,5-dibromo-4-(4-bromobutoxy)phenyl]acetate (**2c**). Yield 67%, yellowish oil. IR (ATR): 2950-2843 (C-H, aromatic and aliphatic), 1735 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.91-1.93 (m, 2H, CH_2), 2.05-2.09 (m, 2H, CH_2), 3.62-3.65 (m, 5H, OCH_3, CH_2), 3.72 (s, 2H, CH_2), 3.96 (t, J = 6.0 Hz, 2H, CH_2), 7.59 (s, 2H, 2CH). LCMS $[M+H]^+$: 460.0. Anal. Calcd. for $C_{13}H_{15}Br_3O_3$: C, 34.02; H, 3.29; Br, 52.23. Found: C, 34.13; H, 3.24; Br, 53.28.

2-[3,5-Dibromo-4-(dimethylaminoalkoxy)phenyl]-N,N-dimethylacetamides **3a-c**. A mixture of one of the compounds **2a-c** (2 mmol) and dimethylamine (40% aqueous solution) (10 mL) in MeCN (5 mL) was stirred at room temperature for 12-15 h. Water (20 mL) was added to the reaction mixture and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure.

2-{3,5-Dibromo-4-[2-(dimethylamino)ethoxy]phenyl}-N,N-dimethylacetamide (**3a**). Yield 68%, colorless oil. IR (ATR): 3025-2825 (C-H, aromatic and aliphatic), 1642 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 6H, $N(CH_3)_2$), 2.72 (t, J = 6.0 Hz, 2H, CH_2), 2.83 (s, 3H, CH_3), 3.02 (s, 3H, CH_3), 3.68 (s, 2H, CH_2), 4.00 (t, J = 6.0 Hz, 2H, CH_2), 7.50 (s, 2H, 2CH). LCMS $[M+H]^+$: 409.2. Anal. Calcd. for $C_{14}H_{20}Br_2N_2O_2$: C, 41.20; H, 4.94; Br, 39.16. Found: C, 41.23; H, 4.91; Br, 39.22.

2-{3,5-Dibromo-4-[3-(dimethylamino)propoxy]phenyl}-N,N-dimethylacetamide (**3b**). Yield 59%, colorless oil. IR (ATR): 3018-2851 (C-H, aromatic and aliphatic), 1635 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.90-1.94 (m, 2H, CH_2), 2.16 (s, 6H, $N(CH_3)_2$), 2.44 (t, J = 6.4 Hz, 2H, CH_2), 2.83 (s, 3H, CH_3), 3.02 (s, 3H, CH_3), 3.69 (s, 2H, CH_2), 3.97 (t, J = 6.4 Hz, 2H, CH_2), 7.50 (s, 2H, 2CH). LCMS $[M+H]^+$: 423.2. Anal. Calcd. for $C_{15}H_{22}Br_2N_2O_2$: C, 42.68; H, 5.25; Br, 37.85. Found: C, 42.63; H, 5.34; Br, 37.82.

2-{3,5-Dibromo-4-[4-(dimethylamino)butoxy]phenyl}-N,N-dimethylacetamide (**3c**). Yield 65%, colorless oil. IR (ATR): 3021-2834 (C-H, aromatic and aliphatic), 1638 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.60-1.63 (m, 2H, CH_2), 1.76-1.79 (m, 2H, CH_2), 2.14 (s, 6H, $N(CH_3)_2$), 2.29 (t, J = 6.8 Hz, 2H, CH_2), 2.82 (s, 3H, CH_3), 3.00 (s, 3H, CH_3), 3.67 (s, 2H, CH_2), 3.92 (t, J = 6.8 Hz, 2H, CH_2), 7.49 (s, 2H, 2CH). LCMS $[M+H]^+$: 437.0. Anal. Calcd. for $C_{16}H_{24}Br_2N_2O_2$: C, 44.06; H, 5.55; Br, 36.64. Found: C, 44.12; H, 5.50; Br, 36.71.

2-{2,6-Dibromo-4-[2-(dimethylamino)-2-oxoethyl]phenoxy}-N,N,N-trimethylalkanaminium **4a-d.** Methyl iodide or benzyl bromide (1.5 mmol) was added to a solution of one of the compounds **3a-c** (1 mmol) in MeCN (5 mL). The reaction mixture was stirred at room temperature for 3-5 h (for benzyl bromide, the mixture was refluxed for 2 h). The solvent was

removed under reduced pressure and acetone (5 mL) was added to the obtained residue. The resulting precipitate was filtered and dried.

2-{2,6-Dibromo-4-[2-(dimethylamino)-2-oxoethyl]phenoxy}-N,N,N-trimethylethanaminium iodide (**4a**). Yield 89%, white solid, mp 193-195 °C. IR (KBr): 3055-2910 (C-H, aromatic and aliphatic), 1646 (C=O) cm^{-1}.^{1}H NMR (400 MHz, DMSO-d_6): δ 2.82 (s, 3H, CH_3), 3.02 (s, 3H, CH_3), 3.28 (s, 9H, $N(CH_3)_3$), 3.72 (s, 2H, CH_2), 3.93 (t, J = 5.6 Hz, 2H, CH_2), 4.35 (t, J = 5.6 Hz, 2H, CH_2), 7.55 (s, 2H, 2CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 35.1 (CH_2), 36.9 (CH_3), 37.5 (CH_3), 53.4 ($N(CH_3)_3$), 64.5 (CH_2), 66.7 (CH_2), 116.5, 133.9 (CH), 136.7, 149.9, 169.3. LCMS $[M\text{-}I]^+$: 423.2. Anal. Calcd. for $C_{15}H_{23}Br_2IN_2O_2$: C, 32.75; H, 4.21; Br, 29.05; N, 5.09. Found: C, 32.79; H, 4.15; Br, 29.13; N, 5.00.

3-{2,6-Dibromo-4-[2-(dimethylamino)-2-oxoethyl]phenoxy}-N,N,N-trimethylpropan-1-aminium iodide (**4b**). Yield 81%, white solid, mp 173-175 °C. IR (KBr): 3002-2880 (C-H, aromatic and aliphatic), 1639 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25-2.27 (m, 2H, CH_2), 2.82 (s, 3H, CH_3), 3.02 (s, 3H, CH_3), 3.14 (s, 9H, $N(CH_3)_3$), 3.62-3.70 (m, 4H, $2CH_2$), 4.02 (t, J = 5.2 Hz, 2H, CH_2), 7.52 (s, 2H, 2CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.5 (CH_2), 35.1 (CH_2), 37.0 (CH_3), 37.4 (CH_3), 52.3 ($N(CH_3)_3$), 63.1 (CH_2), 70.0 (CH_2), 116.8, 133.9 (CH), 136.2, 150.3, 169.5. LCMS $[M\text{-}I]^+$: 437.2. Anal. Calcd. for $C_{16}H_{25}Br_2IN_2O_2$: C, 34.07; H, 4.47; Br, 28.33; N, 4.97. Found: C, 34.99; H, 4.43; Br, 28.17; N, 5.02.

4-{2,6-Dibromo-4-[2-(dimethylamino)-2-oxoethyl]phenoxy}-N,N,N-trimethylbutan-1-aminium iodide (**4c**). Yield 68%, white solid, mp 145-147 °C. IR (KBr): 3031-2882 (C-H, aromatic and aliphatic), 1623 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.71-1.83 (m, 2H, CH_2), 1.89-1.97 (m, 2H, CH_2), 2.82 (s, 3H, CH_3), 3.02 (s, 3H, CH_3), 3.08 (s, 9H, $N(CH_3)_3$), 3.37-3.41 (m, 2H, CH_2), 3.69 (s, 2H, CH_2), 3.99 (t, J = 5.6 Hz, 2H, CH_2), 7.51 (s, 2H, 2CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 19.2 (CH_2), 26.4 (CH_2), 35.1 (CH_2), 37.0 (CH_3), 37.4 (CH_3), 52.2 ($N(CH_3)_3$), 65.1 (CH_2), 72.3 (CH_2), 116.9, 133.9 (CH), 135.7, 150.9, 169.5. LCMS $[M\text{-}I]^+$: 451.0. Anal. Calcd. for $C_{17}H_{27}Br_2IN_2O_2$: C, 35.32; H, 4.71; Br, 27.64; N, 4.85. Found: C, 35.39; H, 4.64; Br, 27.57; N, 4.79.

N-Benzyl-4-{2,6-dibromo-4-[2-(dimethylamino)-2-oxoethyl]phenoxy}-N,N-dimethylbutan-1-aminium bromide (**4d**). Yield 73%, white solid, mp 188-190 °C. IR (KBr): 3008-2927 (C-H, aromatic and aliphatic), 1652 (C=O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.81-1.87 (m, 2H, CH_2), 2.02-2.08 (m, 2H, CH_2), 2.82 (s, 3H, CH_3), 3.00 (s, 9H, $(CH_3)_2$, CH_3), 3.38-3.41 (m, 2H, CH_2), 3.69 (s, 2H, CH_2), 3.99 (t, J = 5.6 Hz, 2H, CH_2), 4.59 (s, 2H, CH_2), 7.50-7.61 (m, 7H, 2CH, ArH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 18.8 (CH_2), 26.5 (CH_2), 35.1 (CH_2), 37.0 (CH_3), 37.4 (CH_3), 49.1 ($N(CH_3)_3$), 63.1 (CH_2), 66.5 (CH_2), 72.3 (CH_2), 116.9, 128.1, 128.9, 130.3, 132.9, 133.9 (CH), 135.8, 150.7, 169.3. LCMS $[M\text{-}Br]^+$: 527.2. Anal. Calcd. for $C_{23}H_{31}Br_3N_2O_2$: C, 45.49; H, 5.15; Br, 39.48; N, 4.61. Found: C, 45.43; H, 5.19; Br, 39.40; N, 4.69.

N,N'-Bis{3-[2,6-dibromo-4-(2-methoxy-2-oxoethyl)phenoxy]propyl}-N,N,N',N'-tetramethylethane-1,2-diaminium dibromide (**5**). A mixture of compound **2b** (0.65 mmol) and TMEDA (0.3 mmol) in MeCN (5 mL) was refluxed for 2 h. The solvent was removed under reduced pressure and 2-propanol (5 mL) was added to the obtained residue. The resulting precipitate was filtered and dried. Yield 69%, white solid, mp 185-187 °C. IR (KBr): 3009-2880 (C-H, aromatic and aliphatic), 1737 (C=O) cm^{-1}.^{1}H NMR (400 MHz, DMSO-d_6): δ 2.33-2.36 (m, 4H, $2CH_2$), 3.26 (s, 12H, $4CH_3$), 3.62 (s, 6H, $2OCH_3$), 3.73-3.77 (m, 8H, $4CH_2$), 4.04-4.08 (m, 8H, $4CH_2$), 7.62 (s, 4H, 4CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.2 (CH_2), 38.0 (CH_2), 50.5 (CH_3), 51.9 (CH_3), 55.1 (CH_2), 62.5 (CH_2), 66.9 (CH_2), 117.1, 133.9 (CH), 134.2, 150.8, 171.0. LCMS $[M\text{-}2Br]^{2+}$: 423.2. Anal. Calcd. for $C_{30}H_{42}Br_6N_2O_6$: C, 35.81; H, 4.21; Br, 47.65; N, 2.78. Found: C, 35.75; H, 4.16; Br, 47.69; N, 2.67.

4.1.2. Bacterial strains

S. aureus strain 222, *P. aeruginosa* strain 449, and *E. coli* strain 311 were isolated from patients with purulent inflammatory diseases. The isolates were identified by morphological and biochemical conventional laboratory methods. They were maintained in trypticase soy broth (TSB) supplemented with 15% glycerol and stored at –20 °C. The antibiotic susceptibility testing was performed by the disk diffusion method in accordance with recommendations of the European Committee on Antimicrobial Susceptibility Testing, except the susceptibility to vancomycin determined by the serial dilution method with determination of minimum inhibitory concentration (MIC). [25, 26] The strain *S. aureus 222* was resistant to oxacillin, cefoxitin and susceptible to vancomycin and clindamycin. It was identified as *MRSA* by molecular methods on the ground of the *mecA* gene expression. *P. aeruginosa* 449 was resistant to cefepime and susceptible to ciprofloxacin, meropenem, aztreonam, and amikacin. *E. coli* 311 was resistant to amikacin, norfloxacin, cefoperazone, ciprofloxacin and susceptible to gentamicin.

4.1.3. Minimum Inhibitory Concentration (MIC) determination

The antibacterial activity of the synthesized compounds was tested by the twofold serial dilution method [26] against gram-positive (*S. aureus* 222) and gram-negative (*E coli* 311, *P. aeruginosa* 449) bacteria. Inoculum density was 1-2×10^5 CFU/mL culture media. The 96-well microtiter plates with bacterial cultures were incubated at 35-37 °C for 18-24 h. Mueller-Hinton broth was used for the minimal inhibitory concentration (MIC) determination. The lowest compound

concentration inhibiting microbial growth was considered as the MIC. Meropenem, ciprofloxacin, gentamicin, and azithromycin were used as reference preparations: Meropenem (Meropenem, manufactured by Sigma-Aldrich, Co., USA, series LRAD4417); Ciprofloxacin (Ciprofloxacin, manufactured by Sigma-Aldrich, Co., USA, series LRAD2244); Gentamicin (Gentamicin Sulfate, manufactured by Sigma-Aldrich, Co., USA, series LRAD6098); Azithromycin (Azithromycin, manufactured by Sigma-Aldrich, Co., USA, series LRAC6480). All assays were performed in triplicate.

4.1.4. Biofilm assay

Antibiofilm activity of new pulmonarin B analogs was tested on polystyrene microplates as described by O'Toole. [27] Compound **5** was studied at the subinhibitory concentration (0.5 MIC) **(Table 1)**. The activity of **4d** against *S. aureus* was studied at the subinhibitory concentration (0.5 MIC), against *P. aeruginosa* and *E. coli*− at the concentration of 25.0 μg/mL. The antibiofilm activity of compounds **4a**, **4b**, and **4c** was studied at a concentration of 25.0 μg/mL. When evaluating the compound's effect on the biofilm formation, its solution and inoculum (an overnight bacterial culture diluted with fresh TSB by 1:100, final OD_{600}=0.055±0.001) were applied to wells simultaneously. Thereafter, microtiter plates were incubated for 24 h at 37 °C. To determine the biofilm biomass, the content of plates was removed, the wells were washed three times with distilled water, and 0.1% solution of gentian violet was added and incubated for 10-15 min. To detect biofilm, the dye was extracted with ethanol (15 min). Optical density was measured by Adsorbance Microplate Reader ELx × 800 (BioTek, USA) at a wavelength of 630 nm. Intact cultures of microorganisms grown under the same conditions without the compound addition were served as a control.

Statistical analyses for the biofilm assay were made using the nonparametric Kruskal-Wallis H-test. A p-value of <0.05 was considered as significant. All experiments were repeated in triplicate.

References

1 Vivas R., Barbosa A.A.T., Dolabela S.S., and Jain S. (**2019**) Multidrug-Resistant Bacteria and Alternative Methods to Control Them: An Overview. *Microb. Drug Resist.*, 25(6) 890-908.

2 Srinivasan R., Santhakumari S., Poonguzhali P., Geetha M., Dyavaiah M., and Xiangmin L. (**2021**) Bacterial Biofilm Inhibition: A Focused Review on Recent Therapeutic Strategies for Combating the Biofilm Mediated Infections. *Front. Microbiol.,* 12 676458.

3 Verderosa A.D., Totsika M., and Fairfull-Smith K.E. (**2019**) Bacterial Biofilm Eradication Agents: A Current Review. *Front. Chem.,* 28(7) 824-841.

4 Nadar S., Khan T., Patching S.G., and Omri A. (**2022**) Development of Antibiofilm Therapeutics Strategies to Overcome Antimicrobial Drug Resistance. *Microorganisms*, 10(2) 303-331.

5 Choudhary A., Naughton L.M., Montánchez I., Dobson A.D.W., and Rai D.K. (**2017**) Current Status and Future Prospects of Marine Natural Products (MNPs) as Antimicrobials. *Mar. Drugs,* 15(9) 272-314.

6 Wang K.L., Dou Z.R., Gong G.F., Li H.F., Jiang B., and Xu Y. (**2022**) Anti-Larval and Anti-Algal Natural Products from Marine Microorganisms as Sources of Anti-Biofilm Agents. *Mar. Drugs*, 20(2) 90-115.

7 Lahiri D., Nag M., Dey A., Sarkar T., Pati S., Nirmal N.P., Ray R.R., Upadhye V.J., Pandit S., Moovendhan M., and Kavisri M. (**2023**) Marine bioactive compounds as antibiofilm agent: a metabolomic approach. *Arch. Microbiol*., 205(1) 54.

8 Deng Y., Liu Y., Li J., Wang X., He S., Yan X., Shi Y., Zhang W., and Ding L. (**2022**) Marine natural products and their synthetic analogs as promising antibiofilm agents for antibiotics discovery and development. *Eur. J. Med. Chem.,* 239 114513.

9 Peng J., Li J., and Hamann M.T. (**2005**). The marine bromotyrosine derivatives. *Alkaloids. Chemistry Biology*, 61 59-262.

10 Binnewerg B., Schubert M., Voronkina A., Muzychka L., Wysokowski M., Petrenko I., Djurović M., Kovalchuk V.,Tsurkan M., Martinovic R., Bechmann N., Fursov A., Ivanenko V.N., Tabachnick K.R., Smolii O.B., Joseph Y., Giovine M., Bornstein S.R., Stelling A.L., Tunger A., and Ehrlich H. (**2020**) Marine biomaterials: Biomimetic and pharmacological potential of cultivated *Aplysina aerophoba* marine demosponge. *Materials Science and Engineering: C.,* 109 110566.

11 Muzychka L., Voronkina A., Kovalchuk V., Smolii O.B., Wysokowski M., Petrenko I., Youssef D.T.A., Ehrlich I., and Ehrlich H. (**2021**) Marine biomimetics: bromotyrosines loaded chitinous skeleton as source of antibacterial agents. *Applied Physics A.,* 127 15-26.

12 Tintillier F., Moriou C., Petek S., Fauchon M., Hellio C., Saulnier D., Ekins M., Hooper J.N.A., Al-Mourabit A., and Debitus C. (**2020**) Quorum Sensing Inhibitory and Antifouling Activities of New Bromotyrosine Metabolites from the Polynesian Sponge Pseudoceratina n. sp. *Mar. Drugs,* 18(5) 272-288.

13 Tadesse M., Svenson J., Sepčić K , Trembleau L., Engqvist M., Andersen J.H., Jaspars M., Stensvåg K., and Haug T. (**2014**) Isolation and synthesis of pulmonarins A and B, acetylcholinesterase inhibitors from the colonial ascidian *Synoicum pulmonaria*. *J. Nat. Prod.,* 77(2) 364-369.

14 Trepos R., Cervin G., Hellio C., Pavia H., Stensen W., Stensvåg K., and Svenson J. (**2014**). Antifouling Compounds from the Sub-Arctic Ascidian Synoicum pulmonaria: Synoxazolidinones A and C, Pulmonarins A and B, and Synthetic Analogues. *J. Nat. Prod.,* 77(9) 2105-2113.

15 `Zhang M., Ding X., Kang J., Gao Y., Wang Z., and Wang Q. (**2020**) Marine Natural Product for Pesticide Candidate: Pulmonarin Alkaloids as Novel Antiviral and Anti-Phytopathogenic-Fungus Agents. *J. Agric. Food Chem.,* 68(41) 11350-11357.
16 Kwaśniewska D., Chen Y.-L., and Wieczorek D. (**2020**) Biological Activity of Quaternary Ammonium Salts and Their Derivatives. *Pathogens,* 9(6) 459-470.
17 Zhou Z., Zhou S., Zhang X., Zeng S., Xu Y., Nie W., Zhou Y., Xu T., and Chen P. (**2023**) Quaternary Ammonium Salts: Insights into Synthesis and New Directions in Antibacterial Applications. *Bioconjugate Chem.,* 34(2) 302-325.
18 Nadagouda M.N., Vijayasarathy P., Sin A., Nam H, Khan S., Parambath J.B.M., Mohamed A.A., and Han C. (**2022**) Antimicrobial activity of quaternary ammonium salts: structure-activity relationship. *Med. Chem. Res.,* 31 1663-1678.
19 Kula N., Lamch Ł., Futoma-Kołoch B., Wilk K. A., and Obłąk E. (**2022**) The effectiveness of newly synthesized quaternary ammonium salts differing in chain length and type of counterion against priority human pathogens. *Sci. Rep.,* 12 21799-21816.
20 Obłąk E., Futoma-Kołoch B., and Wieczyńska A. (**2021**) Biological activity of quaternary ammonium salts and resistance of microorganisms to these compounds. *World J. Microbiol. Biotechnol.,* 37(2) 22-32.
21 Dan W., Gao J., Qi X., Wang J., and Dai J. (**2022**) Antibacterial quaternary ammonium agents: Chemical diversity and biological mechanism. *Eur. J. Med. Chem.,* 243 114765.
22 Shestak O.P., Novikov V.L., Ivanova E.P., and Gorshkova N.M. (**2001**) Synthesis and Antimicrobial Activity of [3,5-Dibromo(dichloro)-1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl]acetic Acids and Their Derivatives. *Pharm. Chem. J.,* 35 366-369.
23 Black J.W., Jennings M.C., Azarewicz J., Paniak T.J., Grenier M.C., Wuest W.M., and Minbiole K.P.C. (**2014**). TMEDA-derived biscationic amphiphiles: An economical preparation of potent antibacterial agents. *Bioorg. Med. Chem. Lett.,* 24(1), 99-102.
24 Obłąk E., Piecuch A., Guz-Regner K., and Dworniczek E. (**2014**) Antibacterial activity of gemini quaternary ammonium salts. *FEMS Microbiology Lett.*, 350(2) 190-198.
25 ESCMID - European Society of Clinical Microbiology and Infectious Diseases **2008**. (2023b, January 2). eucast: Clinical breakpoints and dosing of antibiotics. Retrieved from https://www.eucast.org/clinical_breakpoints
26 Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices Part 1: Broth microdilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases. (2022, October 25). Retrieved from https://www.iso.org/standard/70464.html
27 O'Toole G. A. (**2011**) Microtiter Dish Biofilm Formation Assay. *J. Vis. Exp.,* 47 2437-2438.