

Synthesis and activity of conjugated nalidixic acid derivatives against biofilm-forming bacteria

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ABSTRACT

A series of novel conjugates of nalidixic acid with a marine natural product was designed and synthesized. Their antibacterial and antibiofilm activities were evaluated *in vitro* against Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria. Most of the compounds exhibited strong antibacterial activity against *S. aureus*, *E. coli*, and *P. aeruginosa* strains, except for the oxacillin-resistant *P. aeruginosa*. Among them, compound **2**, containing a decyl ammonium fragment, showed the highest antibacterial activity, with MIC values ranging from 2 to 8 µg/mL, superior to those of nalidixic acid. In addition, compound **2** inhibited biofilm formation by standard strains of *S. aureus*, *E. coli*, and *P. aeruginosa*, and by colistin-resistant *E. coli* by 98-100% at a concentration of 4 µg/mL.

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1. Introduction

Bacterial infections are one of the leading causes of death worldwide. Uncontrolled use of antibiotics has led to the emergence of multidrug-resistant bacteria, posing a major threat to human health.¹ Biofilm formation by bacteria contributes to antibiotic resistance and creates significant challenges for the treatment of infections.²⁻⁴ The most common bacteria associated with biofilms are *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*.⁵ Quinolones are antibacterial agents that are widely used to treat a variety of biofilm-related infections, including urinary and respiratory tract infections.⁶⁻⁹ The first quinolone drug to make it to the market was nalidixic acid, which has a 1,8-naphthyridine ring and is active mainly against Gram-negative bacteria.¹⁰ Although the use of nalidixic acid has been discontinued, several hybrid derivatives based on its structure have been developed, leading to improved antibacterial properties.¹¹⁻¹³ Marine natural compounds have been an important source of antibacterial agents for many decades.¹⁴⁻¹⁶ In particular, secondary brominated metabolites produced by marine sponges exhibit a wide range of therapeutic properties, including antibacterial activity.¹⁷ For example, bromotyrosine derivatives exhibit pronounced antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria and fungi.^{18,19} In addition, structural modification of these compounds with quaternary ammonium groups improves their efficiency as antimicrobial agents.²⁰⁻²² This study aimed to combine the favorable biological properties of a natural product with the antibacterial activity of nalidixic acid to develop conjugated derivatives effective against biofilm-forming bacteria. A series of quaternary ammonium salts based on nalidixic acid and bromotyrosine alkaloid methyl (3,5-dibromo-4-hydroxyphenyl)acetate²³ were synthesized and evaluated for their antibacterial and antibiofilm activities against *E. coli*, *S. aureus*, and *P. aeruginosa*, including antibiotic-resistant strains.

2. Results and Discussion

2.1. Chemistry

The target conjugates of nalidixic acid (**1-6**) were prepared by alkylation of 1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamides **IIa-b** with bromoalkoxy-substituted derivatives of (3,5-dibromo-4-hydroxyphenyl)acetate

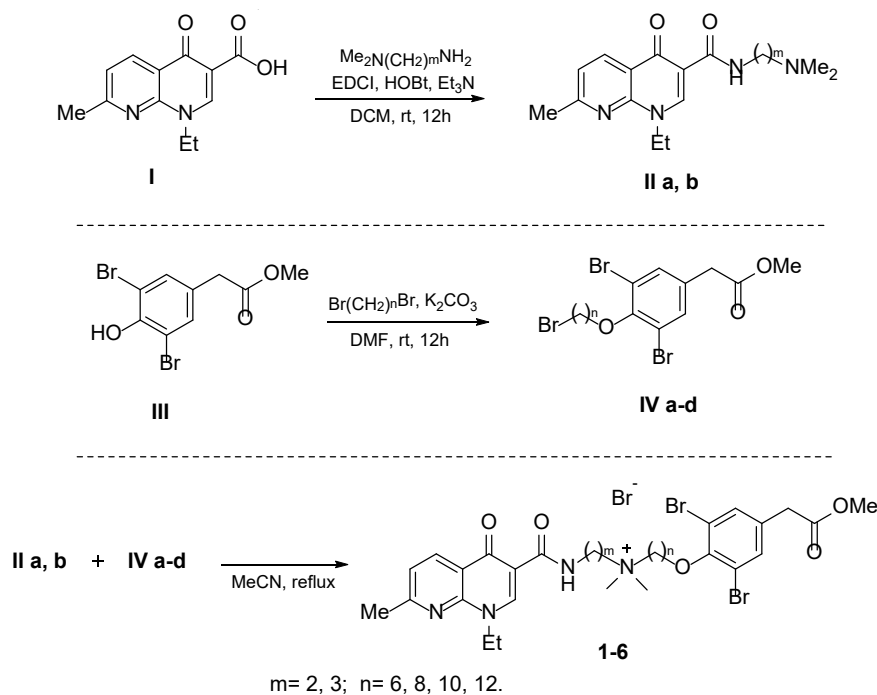
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IVa-d (Scheme 1). First, nalidixic acid **I** was condensed with *N,N*-dimethylethylenediamine or *N,N*-dimethyl-1,3-propanediamine under 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI)/1-hydroxybenzotriazole (HOBt) coupling conditions to produce key intermediate carboxamides **IIa** and **IIb**. Then, four alkoxy-substituted derivatives **IVa-d** were prepared from the synthetically available methyl (3,5-dibromo-4-hydroxyphenyl)acetate **III**, a natural product isolated from the alga *Symphycloadia latiuscula*.²³ Alkylation of **III** with dibromoalkanes was carried out in the presence of K_2CO_3 in *N,N*-dimethylformamide according to a previously described procedure.²⁴ Finally, conjugates **1-6** with an ammonium amphiphilic linker were prepared by boiling 1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamides **IIa** and **IIb** with bromoalkoxy-substituted derivatives **IVa-d** in acetonitrile for 12–18 h. The structures of the target conjugated nalidixic acid derivatives **1-6** are presented in Fig. 1.



Scheme 1. Synthesis of key intermediates and conjugated nalidixic acid derivatives **1-6**.

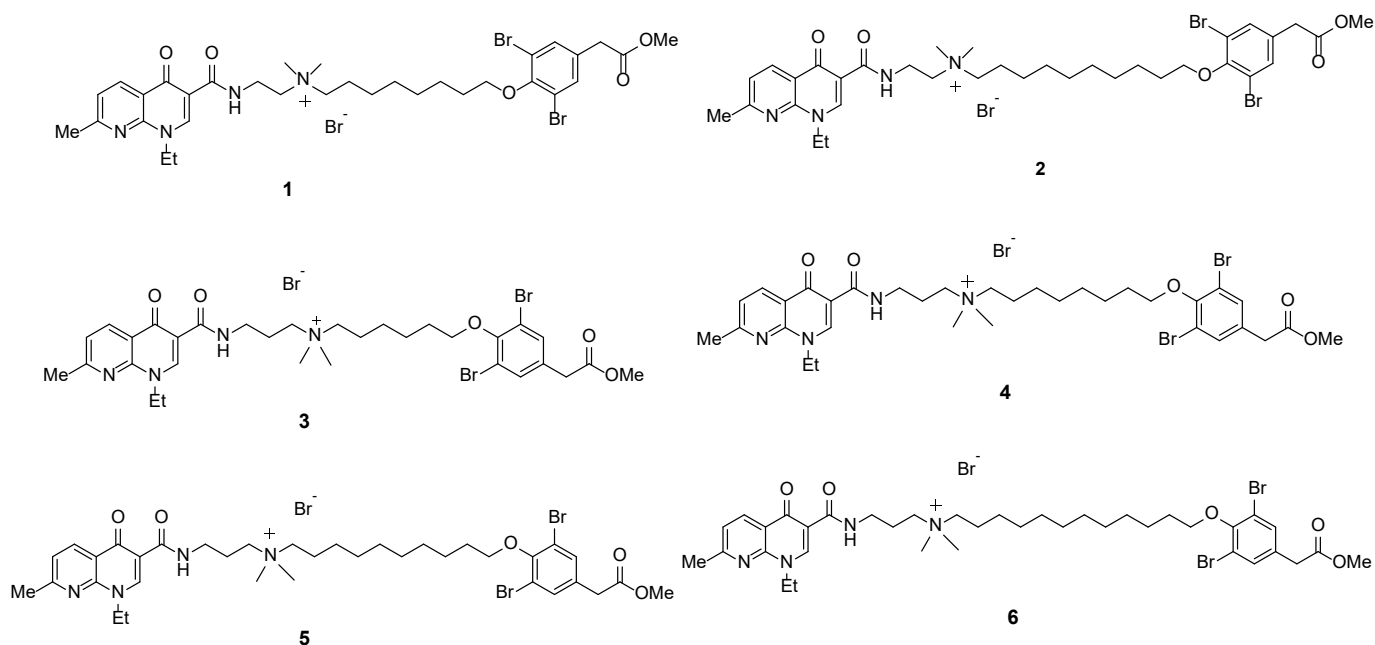


Fig. 1. Structures of the target conjugated nalidixic acid derivatives **1-6**.

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

2.2. Biology

2.2.1 Antibacterial activity

The synthesized compounds **1-6** were evaluated for their antibacterial activity against Gram-positive strains of *S. aureus* ATCC 25923 and colistin-resistant *S. aureus* (CRSA) and Gram-negative strains of *E. coli* ATCC 25922, colistin-resistant *E. coli* (CREC), *P. aeruginosa* PA01, and oxacillin-resistant *P. aeruginosa* (ORPA).

The obtained activity results were expressed as minimum inhibitory concentration (MIC, $\mu\text{g/mL}$) values (**Table 1**). All tested compounds **1-6**, except compound **3**, demonstrated similar levels of antibacterial activity against all bacterial cultures. *P. aeruginosa* ORPA isolate was the most insensitive strain, with all tested compounds showing MICs of more than 512 $\mu\text{g/mL}$.

The activity of compounds **1, 2, and 4-6** against other bacterial strains ranged from 2 to 8 $\mu\text{g/mL}$, which was superior to that of nalidixic acid. Among them, compound **2** with a decyl ammonium chain was the most potent against *E. coli* ATCC 25922 and *P. aeruginosa* PA01 (MIC=2 $\mu\text{g/mL}$). The activity of compound **3** was comparable to that of nalidixic acid, with MICs of 16-32 $\mu\text{g/mL}$.

Table 2. Antibacterial activity of the conjugated nalidixic acid derivatives **1-6** (MIC, $\mu\text{g/mL}$).

Compounds	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> CRSA	<i>E. coli</i> ATCC 25922	<i>E. coli</i> CREC	<i>P. aeruginosa</i> PA01	<i>P. aeruginosa</i> ORPA
1	4	8	8	4	4	>512
2	4	8	2	4	2	>512
3	16	32	16	16	32	>512
4	2	8	8	4	4	>512
5	4	8	4	4	4	>512
6	4	8	4	8	8	>512
Nalidixic acid	16	32	16	32	16	>512
Colistin	>128	>128	>128	>128	32	32
Oxacillin	≤ 0.06	64	≤ 0.06	32	≤ 0.06	>128

Antibiotics colistin and oxacillin were also tested for comparison. Colistin is a natural polypeptide antibiotic of last resort for the treatment of infections caused by Gram-negative bacteria with multiple-drug resistance. It had low efficiency against *S. aureus* and *E. coli* strains (MIC >128 $\mu\text{g/mL}$) but showed moderate activity (MIC=32 $\mu\text{g/mL}$) against both *P. aeruginosa* strains. Oxacillin demonstrated excellent activity (MIC ≤ 0.06 $\mu\text{g/mL}$) against all tested standard strains. However, its activity against drug-resistant *S. aureus* and *E. coli* (MICs of 32-64 $\mu\text{g/mL}$) was lower compared to the tested compounds **1-6**.

2.2.2 Antibiofilm activity

Compounds **1-6** were also analyzed for their ability to inhibit biofilm formation by six bacterial strains. The antibiofilm activity was determined at a MIC concentration of 4 $\mu\text{g/mL}$ (**Fig. 2**).

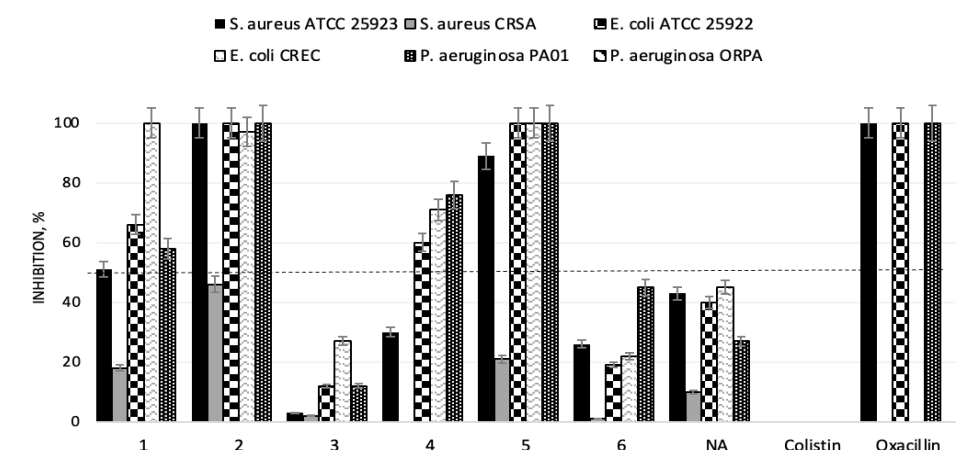


Fig. 2. Inhibition of bacterial biofilm formation by the studied compounds and antibiotics. The biofilm inhibition above 50% was considered strong and between 0 and 50% low. The statistical significance of $p < 0.05$.

Evaluation of antibiofilm activity indicated that the compounds **1, 2, and 5** demonstrated a strong inhibitory effect (51-100% of inhibition) on biofilm formation by *S. aureus* ATCC, *E. coli* ATCC, *P. aeruginosa* PA01, and *E. coli* CREC strains

and low antibiofilm activity (18-46% of inhibition) against *S. aureus* CRSA isolate. Among them, compound **2** was the best inhibitor of biofilm formation. Compound **4** showed strong antibiofilm activity (60-76% of inhibition) against *E. coli* ATCC, *P. aeruginosa* PA01, and *E. coli* CREC strains. Finally, compounds **3** and **6**, as well as nalidixic acid, were the least active. For comparison, all bacterial strains were resistant to colistin, and oxacillin was able to inhibit biofilm formation only by the standard strains of *S. aureus*, *E. coli* (ATCC), and *P. aeruginosa* (PA01). It is worth noting that all tested compounds and antibiotics had no inhibitory effect on biofilm formation by the *P. aeruginosa* ORPA isolate.

3. Conclusions

In this study, novel conjugated derivatives of nalidixic acid based on the marine natural product methyl (3,5-dibromo-4-hydroxyphenyl)acetate were synthesized and evaluated for their antibacterial and antibiofilm activities against *S. aureus*, *E. coli*, and *P. aeruginosa*, including antibiotic-resistant strains. The synthesized conjugates showed good antibacterial activity, with MIC values ranging from 2 to 32 µg/mL against *S. aureus*, *E. coli*, and *P. aeruginosa* strains, except for the oxacillin-resistant *P. aeruginosa*. Among them, compound **2**, with a decyl ammonium fragment, demonstrated the best antibacterial activity, with MIC values of 2-8 µg/mL, lower than those of nalidixic acid. In addition, compound **2** inhibited biofilm formation by *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *E. coli* CREC, and *P. aeruginosa* PA01 by 98-100% at a concentration of 4 µg/mL. These results highlight the potential for the development of efficient antibacterial and antibiofilm agents based on the conjugation of nalidixic acid with natural product derivatives.

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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. ¹H and ¹³C NMR spectra were acquired on Varian Unity INOVA 400 or Bruker Avance DRX-500 instruments (TMS as internal reference) in DMSO-*d*₆ or CDCl₃. 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 H₂O–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.

General procedure for the synthesis of acetamides IIa and IIb. A mixture of nalidixic acid **I** (10.0 mmol), *N,N*-dimethylethylenediamine or *N,N*-dimethyl-1,3-propanediamine (12.0 mmol), HOBt (12.0 mmol), triethylamine (30.0 mmol) and EDCI (12.0 mmol) in dichloromethane was stirred at room temperature for 12 h. The reaction mixture was then diluted with water, neutralized with 3M HCl, and extracted with CH₂Cl₂. The organic layer was dried over Na₂SO₄ and concentrated in vacuo. The residue was treated with saturated NaHCO₃ solution and extracted with CH₂Cl₂. The solvent was dried over Na₂SO₄, concentrated in vacuo, and purified by crystallization from methanol.

N-[2-(Dimethylamino)ethyl]-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**IIa**). Yield 64%, white solid, m.p. 152-154 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.82 (s, 1H, NH), 8.90 (s, 1H, CH), 8.50 (d, *J* = 8.0 Hz, 1H, CH), 7.42 (d, *J* = 8.0 Hz, 1H, CH), 4.63 – 4.46 (m, 2H, CH₂), 3.49 – 3.39 (m, 2H, CH₂), 2.63 (s, 3H, CH₃), 2.40 (t, *J* = 5.9 Hz, 2H, CH₂), 2.18 (s, 6H, 2CH₃), 1.37 (t, *J* = 6.7 Hz, 3H, CH₃). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 176.1(C=O), 164.0 (C=O), 163.5 (C), 148.5 (C), 148.2 (CH), 136.3 (CH), 121.7 (CH), 120.1 (C), 112.6 (C), 58.7 (CH₂), 46.4 (CH₂), 45.6 (CH₃), 36.9(CH₂), 25.3 (CH₃), 15.4 (CH₃). LCMS [M+H]⁺: 303.0. Anal. Calcd. for C₁₆H₂₂N₄O₂: C, 63.56; H, 7.33; N, 18.53. Found: C, 63.59; H, 7.28; N, 18.57.

N-[3-(Dimethylamino)propyl]-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (**IIb**). Yield 55%, white solid, m.p. 115-117 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.80 (t, *J* = 5.2 Hz, 1H, NH), 8.91 (s, 1H, CH), 8.50 (d, *J* = 8.1 Hz, 1H, CH), 7.42 (d, *J* = 8.1 Hz, 1H, CH), 4.53 (q, *J* = 6.9 Hz, 2H, CH₂), 3.37 – 3.29 (m, 2H, CH₂), 2.63 (s, 3H, CH₃), 2.25 (t, *J* = 7.0 Hz, 2H, CH₂), 2.11 (s, 6H, 2CH₃), 1.73 – 1.55 (m, 2H, CH₂), 1.37 (t, *J* = 7.0 Hz, 3H, CH₃). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 176.3 (C=O), 164.1(C=O), 163.5 (C), 148.5 (C), 148.2 (CH), 136.3 (CH), 121.8 (CH), 120.0 (C), 112.6 (C), 57.2 (CH₂), 46.4 (CH₂), 45.6 (CH₃), 37.1 (CH₂), 27.8 (CH₂), 25.3 (CH₃), 15.4 (CH₃). LCMS [M+H]⁺: 317.0. Anal. Calcd. for C₁₇H₂₄N₄O₂: C, 64.53; H, 7.65; N, 17.71. Found: C, 64.48; H, 7.67; N, 17.79.

General procedure for the synthesis of bromoalkoxy-substituted derivatives IVa-d. A mixture of acetate **II** (5 mmol), dibromoalkane (15 mmol), and K_2CO_3 (15 mmol) in *N,N*-dimethylformamide (15 mL) was stirred at room temperature for 12 h. Cold water was added, and reaction mixture was extracted with CH_2Cl_2 . The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by column chromatography.

Methyl {3,5-dibromo-4-[(6-bromohexyl)oxy]phenyl}acetate (IVa). Yield 65%, yellowish oil. 1H NMR (400 MHz, $DMSO-d_6$) δ 7.53 (s, 2H, 2CH), 3.88 (t, $J = 6.5$ Hz, 2H, CH_2), 3.65 (s, 2H, CH_2), 3.58 (s, 3H, CH_3), 3.48 (t, $J = 6.5$ Hz, 2H, CH_2), 1.77 (dq, $J = 17.9, 6.7$ Hz, 4H, 2 CH_2), 1.53 – 1.37 (m, 4H, 2 CH_2). ^{13}C NMR (126 MHz, $DMSO-d_6$) δ 171.4 (C=O), 152.0 (C), 134.3 (CH), 134.0 (C), 117.7 (C), 73.4 (CH_2), 52.3 (CH_3), 38.6 (CH_2), 35.4 (CH_2), 32.7 (CH_2), 29.8 (CH_2), 27.8 (CH_2), 25.0 (CH_2). LCMS $[M+H]^+$: 488.0. Anal. Calcd. for $C_{15}H_{19}Br_3O_3$: C, 36.99; H, 3.93; Br, 49.22. Found: C, 36.43; H, 3.87; Br, 49.30.

Methyl {3,5-dibromo-4-[(8-bromooctyl)oxy]phenyl}acetate (IVb). Yield 60%, yellowish oil. 1H NMR (400 MHz, $DMSO-d_6$) δ 7.56 (s, 2H, 2CH), 3.91 (t, $J = 6.3$ Hz, 2H, CH_2), 3.68 (s, 2H, CH_2), 3.61 (s, 3H, CH_3), 3.50 (t, $J = 6.7$ Hz, 2H, CH_2), 1.85 – 1.69 (m, 4H, 2 CH_2), 1.53 – 1.42 (m, 2H, CH_2), 1.42 – 1.24 (m, 6H, 3 CH_2). ^{13}C NMR (126 MHz, $DMSO-d_6$) δ 171.4 (C=O), 152.0 (C), 134.3 (CH), 134.0 (C), 117.7 (C), 73.6 (CH_2), 52.3 (CH_3), 38.6 (CH_2), 35.5 (CH_2), 32.7 (CH_2), 29.9 (CH_2), 29.1 (CH_2), 28.5 (CH_2), 27.9 (CH_2), 25.7 (CH_2). LCMS $[M+H]^+$: 516.2. Anal. Calcd. for $C_{17}H_{23}Br_3O_3$: C, 39.64; H, 4.50; Br, 46.54. Found: C, 39.68; H, 4.56; Br, 46.49.

Methyl {3,5-dibromo-4-[(10-bromodecyl)oxy]phenyl}acetate (IVc). Yield 67%, yellowish oil. 1H NMR (400 MHz, $DMSO-d_6$) δ 7.55 (s, 2H, 2CH), 3.89 (t, $J = 6.0$ Hz, 2H, CH_2), 3.67 (s, 2H, CH_2), 3.60 (s, 3H, CH_3), 3.47 (t, $J = 6.6$ Hz, 2H, CH_2), 1.84 – 1.69 (m, 4H, 2 CH_2), 1.52 – 1.40 (m, 2H, CH_2), 1.40 – 1.21 (m, 10H, 5 CH_2). ^{13}C NMR (101 MHz, $DMSO-d_6$) δ 171.4 (C=O), 151.9 (C), 134.3 (CH), 133.9 (C), 117.7 (C), 73.5 (CH_2), 52.3 (CH_3), 38.5 (CH_2), 35.5 (CH_2), 32.7 (CH_2), 29.9 (CH_2), 29.3 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 28.6 (CH_2), 27.9 (CH_2), 25.8 (CH_2). LCMS $[M+H]^+$: 544.0. Anal. Calcd. for $C_{19}H_{27}Br_3O_3$: C, 42.02; H, 5.01; Br, 44.13. Found: C, 39.97; H, 4.96; Br, 5.05.

Methyl {3,5-dibromo-4-[(12-bromododecyl)oxy]phenyl}acetate (IVd). Yield 56%, yellowish oil. 1H NMR (400 MHz, $DMSO-d_6$) δ 7.55 (s, 2H, 2CH), 3.90 (t, $J = 6.3$ Hz, 2H, CH_2), 3.68 (s, 2H, CH_2), 3.61 (s, 3H, CH_3), 3.48 (t, $J = 6.7$ Hz, 2H, CH_2), 1.76 (p, $J = 6.5$ Hz, 4H, 2 CH_2), 1.51 – 1.39 (m, 2H, CH_2), 1.38 – 1.17 (m, 14H, 7 CH_2). ^{13}C NMR (126 MHz, $DMSO-d_6$) δ 171.4 (C=O), 151.9 (C), 134.3 (CH), 134.0 (C), 117.7 (C), 73.6 (CH_2), 52.3 (CH_3), 38.6 (CH_2), 35.5 (CH_2), 32.7 (CH_2), 29.9 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 28.6 (CH_2), 28.0 (CH_2), 25.8 (CH_2). LCMS $[M+H]^+$: 572.0. Anal. Calcd. for $C_{21}H_{31}Br_3O_3$: C, 44.16; H, 5.47; Br, 41.97. Found: C, 44.13; H, 5.48; Br, 5.01.

General procedure for the synthesis of conjugated nalidixic acid derivatives 1-6. A mixture of one of compounds **IIa** or **IIb** (0.5 mmol) and the corresponding bromoalkoxy-substituted derivatives **IVa-d** (0.55 mmol) in MeCN (5 mL) was refluxed for 12-18 h. The solvent was removed in vacuo, and the oily residue was treated twice with hexane. The resulting solid was filtered and dried.

8-[2,6-Dibromo-4-(2-methoxy-2-oxoethyl)phenoxy]-N-(2-[(1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridin-3-yl)carbonyl]amino)ethyl)-N,N-dimethyloctan-1-aminium bromide (1). Yield 78%, white solid, m.p. 172-174 °C. 1H NMR (500 MHz, $DMSO-d_6$) δ 10.05 (t, $J = 6.0$ Hz, 1H, NH), 9.00 (s, 1H, CH), 8.55 (d, $J = 8.1$ Hz, 1H, CH), 7.57 (s, 2H, 2CH), 7.47 (d, $J = 8.1$ Hz, 1H, CH), 4.65 – 4.50 (m, 2H, CH_2), 3.86 – 3.75 (m, 4H, 2 CH_2), 3.70 (s, 2H, CH_2), 3.62 (s, 3H, CH_3), 3.50 (t, $J = 6.0$ Hz, 2H, CH_2), 3.38 – 3.30 (m, 2H, CH_2), 3.10 (s, 6H, 2 CH_3), 2.63 (s, 3H, CH_3), 1.70 – 1.58 (m, 4H, CH_2), 1.37 (t, $J = 6.0$ Hz, 3H, CH_3), 1.35 – 1.26 (m, 2H, CH_2), 1.26 – 1.16 (m, 6H, 3 CH_2). ^{13}C NMR (126 MHz, $DMSO-d_6$) δ 176.2 (C=O), 171.5 (C=O), 164.9 (C=O), 163.8 (C), 151.9 (C), 148.5 (CH), 148.5 (C), 136.3 (CH), 134.3 (CH), 134.1 (C), 122.0 (C), 120.1 (C), 117.6 (CH), 111.9 (C), 73.4 (CH_2), 63.5 (CH_2), 61.6 (CH_2), 52.3 (CH_3), 51.3 (CH_3), 46.5 (CH_2), 38.5 (CH_2), 33.4 (CH_2), 29.8 (CH_2), 29.0 (CH_3), 28.9 (CH_2), 26.2 (CH_2), 25.6 (CH_2), 25.2 (CH_2), 22.3 (CH_2), 15.5 (CH_3). LCMS $[M-Br]^+$: 737.2. Anal. Calcd. for $C_{33}H_{45}Br_3N_4O_5$: C, 48.49; H, 5.55; Br, 29.32. Found: C, 48.52; H, 5.51; Br, 29.35.

10-[2,6-Dibromo-4-(2-methoxy-2-oxoethyl)phenoxy]-N-(2-[(1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridin-3-yl)carbonyl]amino)ethyl)-N,N-dimethyldecan-1-aminium bromide (2). Yield 71%, white solid, m.p. 153-155 °C. 1H NMR (500 MHz, $DMSO-d_6$) δ 10.05 (s, 1H, NH), 9.00 (s, 1H, CH), 8.55 (d, $J = 8.1$ Hz, 1H, CH), 7.57 (s, 2H, 2CH), 7.49 (d, $J = 8.1$ Hz, 1H, CH), 4.65 – 4.53 (m, 2H, CH_2), 3.89 (t, $J = 6.1$ Hz, 2H, CH_2), 3.82 – 3.74 (m, 2H, CH_2), 3.70 (s, 2H, CH_2), 3.62 (s, 3H, CH_3), 3.55 – 3.47 (m, 2H, CH_2), 3.36 – 3.28 (m, 2H, CH_2), 3.09 (s, 6H, 2 CH_3), 2.66 (s, 3H, CH_3), 1.75 – 1.68 (m, 2H, CH_2), 1.67 – 1.60 (m, 2H, CH_2), 1.41 – 1.34 (m, 5H), 1.21 – 1.14 (m, 6H, 3 CH_2), 1.13 – 1.06 (m, 4H, 2 CH_2). ^{13}C NMR (126 MHz, $DMSO-d_6$) δ 176.2 (C=O), 171.5 (C=O), 164.9 (C=O), 163.8 (C), 151.9 (C), 148.6 (C), 148.5 (CH), 136.3 (CH), 134.3 (CH), 134.1 (C), 122.0 (C), 120.1 (C), 117.6 (CH), 112.0 (C), 73.6 (CH_2), 63.5 (CH_2), 61.6 (CH_2), 52.3 (CH_3), 51.3 (CH_3), 46.5 (CH_2), 40.5 (CH_2), 38.5 (CH_2), 33.4 (CH_2), 29.9 (CH_2), 29.2 (CH_3), 29.1 (CH_2), 29.0 (CH_2), 26.2 (CH_2), 25.7 (CH_2), 25.3 (CH_2), 22.3 (CH_2), 15.5 (CH_3). LCMS $[M-Br]^+$: 765.2. Anal. Calcd. for $C_{35}H_{49}Br_3N_4O_5$: C, 49.72; H, 5.84; Br, 28.35. Found: C, 49.74; H, 5.79; Br, 28.38.

6-[2,6-Dibromo-4-(2-methoxy-2-oxoethyl)phenoxy]-N-(3-[(1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridin-3-yl)carbonyl]amino)propyl)-N,N-dimethylhexan-1-aminium bromide (3). Yield 62%, white solid, m.p. 176-178 °C. 1H NMR

(400 MHz, DMSO-*d*₆) δ 9.92 (s, 1H, NH), 8.97 (s, 1H, CH), 8.51 (d, *J* = 8.2 Hz, 1H, CH), 7.56 (s, 2H, 2 CH), 7.42 (d, *J* = 8.5 Hz, 1H, CH), 4.65 – 4.46 (m, 2H, CH₂), 3.92 – 3.77 (m, 2H, CH₂), 3.69 (s, 2H, CH₂), 3.62 (s, 3H, CH₃), 3.35 – 3.21 (m, 4H, 2CH₂), 3.03 (s, 6H, 2CH₃), 2.63 (s, 3H, CH₃), 2.06 – 1.88 (m, 2H, CH₂), 1.84 – 1.58 (m, 4H, 2CH₂), 1.56 – 1.40 (m, 2H, CH₂), 1.43 – 1.24 (m, 5H, CH₂, CH₃). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 176.2 (C=O), 171.5 (C=O), 164.6 (C=O), 163.6 (C), 151.8 (C), 148.5 (CH), 148.3 (C), 136.2 (CH), 134.3 (CH), 134.1 (C), 121.9 (C), 120.0 (C), 117.6 (CH), 112.4 (C), 73.3 (CH₂), 63.2 (CH₂), 61.2 (CH₂), 52.3 (CH₃), 50.7 (CH₃), 46.4 (CH₂), 38.5 (CH₂), 36.0 (CH₂), 29.6 (CH₃), 26.0 (CH₂), 25.4 (CH₂), 25.3 (CH₂), 23.3 (CH₂), 22.1 (CH₂), 15.4 (CH₃). LCMS [M-Br]⁺: 723.0. Anal. Calcd. for C₃₂H₄₃Br₃N₄O₅: C, 47.84; H, 5.39; Br, 29.84. Found: C, 47.79; H, 5.41; Br, 29.89.

8-[2,6-Dibromo-4-(2-methoxy-2-oxoethyl)phenoxy]-*N*-(3-[(1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridin-3-yl)carbonyl]amino)propyl)-*N,N*-dimethyloctan-1-aminium bromide (4). Yield 68%, white solid, m.p. 165-167 °C. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.93 (s, 1H, NH), 8.97 (s, 1H, CH), 8.54 (d, *J* = 8.1 Hz, 1H, CH), 7.57 (s, 2H, 2CH), 7.47 (d, *J* = 8.2 Hz, 1H, CH), 4.63 – 4.51 (m, 2H, CH₂), 3.95 – 3.81 (m, 2H, CH₂), 3.70 (s, 2H, CH₂), 3.62 (s, 3H, CH₃), 3.34 – 3.27 (m, 2H, CH₂), 3.29 – 3.19 (m, 2H, CH₂), 3.01 (s, 6H, 2CH₃), 2.64 (s, 3H, CH₃), 2.06 – 1.89 (m, 2H, CH₂), 1.76 – 1.68 (m, 2H, CH₂), 1.67 – 1.57 (m, 2H, CH₂), 1.44 – 1.35 (m, 5H), 1.33 – 1.23 (m, 6H, 3CH₂). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 176.2 (C=O), 171.5 (C=O), 164.5 (C=O), 163.6 (C), 151.8 (C), 148.5 (CH), 148.3 (C), 136.2 (CH), 134.3 (CH), 134.0 (C), 121.9 (C), 120.0 (C), 117.6 (CH), 112.3 (C), 73.5 (CH₂), 63.0 (CH₂), 61.1 (CH₂), 52.3 (CH₃), 50.6 (CH₃), 46.4 (CH₂), 38.4 (CH₂), 35.9 (CH₂), 29.8 (CH₃), 28.9 (CH₂), 28.8 (CH₂), 26.1 (CH₂), 25.6 (CH₂), 25.2 (CH₂), 23.3 (CH₂), 22.0 (CH₂), 15.4 (CH₂). LCMS [M-Br]⁺: 751.2. Anal. Calcd. for C₃₄H₄₇Br₃N₄O₅: C, 49.11; H, 5.70; Br, 28.83. Found: C, 49.15; H, 5.73; Br, 28.79.

10-[2,6-Dibromo-4-(2-methoxy-2-oxoethyl)phenoxy]-*N*-(3-[(1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridin-3-yl)carbonyl]amino)propyl)-*N,N*-dimethyldecan-1-aminium bromide (5). Yield 65%, white solid, m.p. 161-163 °C. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.93 (t, *J* = 5.9 Hz, 1H, NH), 8.98 (s, 1H, CH), 8.54 (d, *J* = 8.1 Hz, 1H, CH), 7.57 (s, 2H, 2CH), 7.49 (d, *J* = 8.2 Hz, 1H, CH), 4.71 – 4.46 (m, 2H, CH₂), 3.89 (t, *J* = 6.3 Hz, 2H, CH₂), 3.70 (s, 2H, CH₂), 3.62 (s, 3H, CH₃), 3.35 – 3.26 (m, 2H, CH₂), 3.28 – 3.15 (m, 2H, CH₂), 3.00 (s, 6H, 2 CH₃), 2.66 (s, 3H, CH₃), 2.02 – 1.90 (m, 2H, CH₂), 1.79 – 1.69 (m, 2H, CH₂), 1.64 – 1.56 (m, 2H, CH₂), 1.47 – 1.35 (m, 5H), 1.31 – 1.15 (m, 8H, 4CH₂). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 176.2 (C=O), 171.5 (C=O), 164.5 (C=O), 163.7 (C), 151.9 (C), 148.5 (CH), 148.3 (C), 136.2 (CH), 134.3 (CH), 134.0 (C), 121.9 (C), 120.0 (C), 117.6 (CH), 112.3 (C), 73.5 (CH₂), 63.1 (CH₂), 61.1 (CH₂), 52.3 (CH₃), 50.7 (CH₃), 46.4 (CH₂), 38.4 (CH₂), 35.9 (CH₂), 29.8 (CH₃), 29.2 (CH₂), 29.1 (CH₂), 29.1 (CH₂), 28.9 (CH₂), 26.1 (CH₂), 25.7 (CH₂), 25.3 (CH₂), 23.3 (CH₂), 22.1 (CH₂), 15.4 (CH₃). LCMS [M-Br]⁺: 779.0. Anal. Calcd. for C₃₆H₅₁Br₃N₄O₅: C, 50.31; H, 5.98; Br, 27.89. Found: C, 50.34; H, 5.92; Br, 27.85.

12-[2,6-Dibromo-4-(2-methoxy-2-oxoethyl)phenoxy]-*N*-(3-[(1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridin-3-yl)carbonyl]amino)propyl)-*N,N*-dimethyldodecan-1-aminium bromide (6). Yield 57%, yellowish oil. ¹H NMR (400 MHz, CDCl₃) δ 10.16 (s, 1H, NH), 8.95 (s, 1H, CH), 8.60 (d, *J* = 8.3 Hz, 1H, CH), 7.42 (s, 2H, 2CH), 7.29 (d, *J* = 8.3 Hz, 1H, CH), 4.67 – 4.51 (m, 2H, CH₂), 4.06 – 3.91 (m, 2H, CH₂), 3.71 (s, 2H, CH₂), 3.68 – 3.57 (m, 5H), 3.57 – 3.47 (m, 4H, CH₂), 3.38 (s, 6H, 2 CH₃), 2.70 (s, 3H, CH₃), 2.54 – 2.37 (m, 4H, 2CH₂), 2.21 – 2.08 (m, 2H, CH₂), 1.90 – 1.80 (m, 2H, CH₂), 1.75 – 1.66 (m, 2H, CH₂), 1.58 – 1.45 (m, 6H, 3CH₂), 1.42 – 1.18 (m, 9H, 3CH₂, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 176.7 (C=O), 171.0 (C=O), 165.6 (C=O), 163.7 (C), 152.6 (C), 148.4 (CH), 147.5 (C), 136.1 (CH), 133.4 (CH), 121.6 (C), 119.9 (C), 118.2 (CH), 111.9 (C), 109.9 (C), 73.5 (CH₂), 64.1 (CH₂), 61.8 (CH₂), 52.3 (CH₃), 51.5 (CH₃), 47.1 (CH₂), 39.5 (CH₂), 36.0 (CH₂), 30.0 (CH₂), 29.4 (CH₃), 29.4 (CH₂), 29.3 (CH₂), 29.3 (CH₂), 29.2 (CH₂), 26.9 (CH₂), 26.2 (CH₂), 25.8 (CH₂), 25.2 (CH₂), 23.6 (CH₂), 22.7 (CH₂), 15.3 (CH₃). LCMS [M-Br]⁺: 807.2. Anal. Calcd. for C₃₈H₅₅Br₃N₄O₅: C, 51.42; H, 6.25; Br, 27.01. Found: C, 51.45; H, 6.20; Br, 27.05.

4.1.2. Minimum inhibitory concentration (MIC) determination

The antibacterial activity of synthesized compounds was tested against Gram-positive *S. aureus* ATCC 25923 and colistin-resistant *S. aureus* (CRSA) and against Gram-negative *E. coli* ATCC 25922, colistin-resistant *E. coli* (CREC), *P. aeruginosa* PA01, and oxacillin-resistant *P. aeruginosa* (ORPA) bacterial strains by the broth dilution microplate method.²⁵ All microbial cultures were obtained from the Collection of the Museum of Microorganism Cultures of the Shupyk National Healthcare University of Ukraine. All bacterial strains were grown for 24 h at 37 °C in Mueller-Hinton agar. The inoculum suspension 1 × 10⁸ CFU/mL (to McFarland standard) was further diluted to give the final inoculum density of 5 × 10⁵ CFU/mL. Serial two-fold dilutions in Mueller-Hinton broth were performed in sterile 96-well microplates at concentrations ranging from 512 µg/mL to 0.0625 µg/mL. The incubation time of the bacterial inoculum with the corresponding compound dilution was 24 h at 37 °C. The bacterial growth rate in the microplate wells was registered using a Microplate Reader MR-96A at a wavelength of 630 nm. Measurement identifiers obtained for wells containing bacterial inoculum were taken as a positive control for the experiment, and identifiers obtained for wells containing tested compounds in culture medium (without bacterial inoculum) were taken as a negative control. Testing was performed in triplicate.

4.1.3. Inhibition of biofilm formation

Antibiofilm activity of the synthesized compounds was determined as described by O'Toole.²⁶ Bacterial strains were grown overnight for 24 h at 37 °C in Mueller-Hinton agar. The inoculum by 0.5 McFarland (1.5×10^8 CFU/mL) was prepared from an overnight culture in Mueller-Hinton broth supplemented with 2% glucose. The cultures were diluted (final concentration of 5×10^7 CFU/mL) in a sterile flat-bottomed 96-well polystyrene microtiter plate (Greiner Bio-One GmbH, Germany). Incubation time of the tested compounds with the bacterial inoculum in the microplate wells was 24 h at 37 °C. The antibiofilm activity of the compounds was determined at a concentration of 4.0 µg/mL under the optimal MIC value. The biofilm formation of the bacteria was quantified using a crystal violet stain.

Optical density (OD) of each well was measured by Microplate Reader MR-96A at a wavelength of 630 nm. Biofilm inhibition was scored from 0 to 100%. Values from 0 to 50% indicated low antibiofilm activity, and above 50% indicated strong antibiofilm activity. Results from at least three separate replicates were averaged. The percentage of biofilm inhibition was determined by the formula:

$$\% \text{ Inhibition} = [(OD_{\text{negative control}} - OD_{\text{experiment sample}}) / OD_{\text{negative control}}] \times 100$$

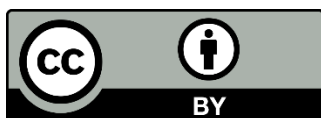
Bacterial biofilm growth measured in wells without the addition of the tested compounds (sterile medium with inoculum) served as a negative control.

Statistical analyses of the obtained results were made using MS Excel for Windows and Statistica 12.0. A p-value of <0.05 was considered as significant.

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