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Synthesis and antibiofilm activity of novel 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides

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Aim. Synthesis of novel alkyl-substituted 4-oxo-1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides and evaluation of their antibiofilm activity *in vitro*. **Methods.** Organic synthesis, analytical and spectral methods, broth microilution method, biofilm formation on abiotic surface. **Results.** A simple and efficient method for the synthesis of new 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxylic acid derivatives was developed. The results of antibiofilm activity screening showed that among the synthesized alkyl-substituted 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides there are the compounds capable of disrupting the formation of biofilm of methicillin-resistant strain *S. aureus* 222, *E. coli* 311 and *P. aeruginosa* 449. Compound 6g is active against biofilms of *E. coli* 311, biomass decreases by 91.2 %, and against *S. aureus* 222 (reduction by 54.0 %). Compound 6d is active against biofilms of *P. aeruginosa* 449 and *S. aureus* 222 (reduction by 78.7 % and 50.2 %, respectively). **Conclusions.** A series of novel substituted 1-alkyl-4-oxo-1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides were synthesized. The activity of the synthesized pyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidines towards the *S. aureus* 222, *E. coli* 311 and *P. aeruginosa* 449 biofilm formation was investigated, and the compounds with pronounced antibiofilm activity were found.

Keywords: pyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidines, pyrido[1,2-*a*]pyrimidine-3-carbaldehydes, synthesis, antibiofilm activity.

Introduction

The introduction of penicillin and other antibiotics into clinical practice revolutionized modern medicine and saved the lives of millions of people [1]. However, almost simultaneously with the application of antimicrobial agents (AMAs), the antibiotic-resistant strains

were identified, which decline the effectiveness of antimicrobial therapy. A manifestation of antibiotic resistance is the formation of microbial biofilms, the structured communities of bacterial cells surrounded by a polymer matrix, attached to surfaces [2].

The biofilm development begins with the adhesion to the substrate and ends with the matrix rupture and the planktonic cell dissemination with the subsequent colonization of new abiotic or biotic surfaces. Biofilms contribute to approximately 80 % of diseases with a chronic, recurrent course, such as chronic wound infection, osteomyelitis, chronic rhinosinusitis, urinary tract pathology, *etc.* [3, 4]. The biofilm microorganisms are resistant to environmental stress factors, tolerant to antimicrobial therapy, ensured by a complex array of resistance mechanisms. The realization of resistance occurs through mechanisms inherent to both planktonic cells (antibiotic inactivation by enzymes, hyperactivity of efflux pumps, modification of target structure, reduction of the cell membrane permeability) and specific mechanisms, such as the presence of the matrix that prevents the AMAs penetration into the biofilm at a concentration sufficient to inhibit cells, the activation of Quorum Sensing (QS) systems, and the formation of persister cells with a slowed metabolism [5–7].

The complexity of the biofilm structure, its functional characteristics, the presence of a comprehensive resistance mechanism, and the insufficiency of pharmaceuticals with the antibiofilm action highlight the problem of developing the agents capable of affecting various stages of biofilm formation. The structural components of the biofilm, regulatory signaling systems (c-di-GMP, QS, non-coding sRNAs, eDNA), genes responsible for adhesion, extracellular matrix synthesis, and the transition from planktonic to biofilm state can be the main targets for the compounds with the antibiofilm activity [3].

Currently, in addition to research on the antibiofilm action of modern AMAs, there is an initiative to explore the potential of new substances to block the synthesis of virulence factors and specific matrix enzymes of biofilms [6, 8]. It has been established that DNAse, proteinase K, and trypsin affect the matrix eDNA, disrupting the matrix structure and density [9]. *N*-acetylcysteine and benzimidazole contribute to the biofilm breakdown and prevent the exopolysaccharide (EPS) formation [10, 11]. The compounds acting as chelators for bivalent cations (Ca2+, Mg2+) reduce the adhesion to surfaces and disturb the biofilm formation [12]. Nanoparticles of quercetin, silver, and furanone also disturb the film formation. The antibiofilm activity has been observed in the gallimanane diterpenoid derivatives of imidazole and indole, plant extracts, peptides, and polysaccharides derived from both synthetic and natural sources [13]. The mechanism of their action is associated with influencing Quorum Sensing systems and disrupting the transcription of specific genes essential for QS [14].

Recently, pyrido[1,2-*a*]pyrimidine derivatives have attracted the interest of researchers due to their importance and application in the synthesis of tricyclic condensed systems with a potential biological activity [15]. Among them, dipyridopyrimidines are the most studied scaffolds used as a source of bioactive compounds such as potent chitinase inhibitors for

pathogen and pest control [16, 17]. Additionally, iminodipyridinopyrimidine derivatives were identified as hepatitis C virus (HCV) inhibitors [18] and speckle-type POZ protein (SPOP) inhibitors against kidney cancer [19]. Moreover, sulfonyl-substituted 2-imino-1,2-dihydro-5*H*-dipyrido[1,2-*a*:2',3'-*d*]pyrimidin-5-ones can effectively inhibit the growth of certain cancer cell lines [20].

Pyridopyrrolopyrimidine-based compounds have recently been identified as SARS-CoV-2-M^{Pro} inhibitors [21].

In this work, the antimicrobial and antibiofilm activity of novel 1-alkyl-4-oxo-1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides against the *E. coli, S. aureus* or *P. aeruginosa* strains was evaluated.

Materials and Methods

Chemistry

^{1}H and ^{13}C NMR spectra were acquired on Varian Unity Plus 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, with TMS as internal standard. ^{13}C NMR signals were assigned by using APT method. LC-MS 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, m/z scan range from 80 to 1000. 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 Boetius hot stage apparatus. The reaction progress and purity of the obtained compounds were controlled by TLC on Silufol UV-254 plates using a 19:1 mixture of $CHCl_3$-MeOH as eluent.

The starting 2-chloro-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-3-carbaldehydes **1a-c** was prepared using the method [16, 22].

General procedure for the synthesis of substituted 4-oxo-1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxylic acids 4a-e. A mixture of 2-chloro-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-3-carbaldehyde **1a-c** (10 mmol), ethyl *N*-alkylglycinate (10 mmol) and triethylamine (20 mmol) in methanol (20 mL) was stirred at room temperature for 1 h. The precipitate was filtered, obtaining intermediate substituted aldehyde **2a-e**. A solution of sodium methoxide (10 mmol) in methanol (20 mL) was added to intermediates **2a-e**, stirred for 1 hour at room temperature, and water (10 mL) was added. The mixture was heated at 50–60 °C for 0.5–1 h, cooled to room temperature, and acidified with HCl (1:1) to pH < 7. The precipitate was filtered and washed with water.

*1-Methyl-4-oxo-1,4-dihydropyrido[1,2-a]pyrrolo[2,3-d]pyrimidine-2-carboxylic acid (**4a**)* Yield 62 %, mp 281–283 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 3.94 (s, 3H, CH_3), 7.07 (t, J = 7.2 Hz, 1H, CH), 7.28 (s, 1H, CH), 7.50 (d, J = 7.2 Hz, 1H, CH), 7.73 (t, J = 7.2 Hz, 1H, CH), 8.84 (d, J = 7.2 Hz, 1H, CH), 12.80 (br.s, 1H, OH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 30.6 (CH_3), 101.1 (C), 109.9 (CH), 113.0 (CH), 125.0 (CH), 126.4 (C), 126.8 (CH), 135.3 (CH), 148.4 (C), 149.3 (C), 154.1 (C), 161.9 (C). MS (CI): m/z 244.1 $[M+H]^+$. Calcd. for $C_{12}H_9N_3O_3$: C 59.26; H 3.73; N 17.28. Found: C 59.30; H 3.75; N 17.31.

4-Oxo-1-propyl-1,4-dihydropyrido[1,2-a]pyrrolo[2,3-d]pyrimidine-2-carboxylic acid

*(**4b**)*. Yield 53 %, mp 264–266 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 0.83 (t, J = 7.2 Hz, 3H, CH_3), 1.72–1.77 (m, 2H, CH_2), 4.52 (t, J = 7.2 Hz, 2H, CH_2), 7.11 (t, J = 7.2 Hz, 1H, CH), 7.38 (s, 1H, CH), 7.56 (d, J = 7.2 Hz, 1H, CH), 7.75 (t, J = 7.2 Hz, 1H, CH), 8.89 (d, J = 7.2 Hz, 1H, CH), 12.65 (br.s, 1H, OH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 10.9 (CH_3), 23.4 (CH_2), 44.3 (CH_2), 101.0 (C), 110.7 (CH), 113.1 (CH), 125.0 (CH), 125.3 (C), 126.8 (CH), 135.4 (CH), 148.5 (C), 149.4 (C), 154.2 (C), 161.7 (C). MS (CI): m/z 272.1 $[M+H]^+$. Calcd. for $C_{14}H_{13}N_3O_3$: C 61.99; H 4.83; N 15.49. Found: C 62.03; H 4.85; N 15.45.

*1,9-Dimethyl-4-oxo-1,4-dihydropyrido[1,2-a]pyrrolo[2,3-d]pyrimidine-2-carboxylic acid (**4c**)*. Yield 55 %, mp 269–271 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 2.41 (s, 3H, CH_3), 3.88 (s, 3H, CH_3), 6.93 (t, J = 7.2 Hz, 1H, CH), 7.21 (s, 1H, CH), 7.55 (d, J = 7.2 Hz, 1H, CH), 8.68 (d, J = 7.2 Hz, 1H, CH), 12.95 (br.s, 1H, OH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 17.8 (CH_3), 30.4 (CH_3), 100.7 (C), 110.0 (CH), 112.3 (CH), 124.8 (CH), 125.8 (C), 132.6 (C), 133.6 (CH), 147.9 (C), 148.7 (C), 154.3 (C), 161.9 (C). MS (CI): m/z 258.0 $[M+H]^+$. Calcd. for $C_{13}H_{11}N_3O_3$: C 60.70; H 4.31; N 16.33. Found: C 60.67; H 4.46; N 16.34.

*1-(3-Methoxypropyl)-9-methyl-4-oxo-1,4-dihydropyrido[1,2-a]pyrrolo[2,3-d]pyrimidine-2-carboxylic acid (**4d**)*. Yield 64 %, mp 215–217 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 1.96–1.99 (m, 2H, CH_2), 2.49 (s, 3H, CH_3), 3.17 (s, 3H, OCH_3), 3.31 (t, J = 7.0 Hz, 2H, CH_2), 4.63 (t, J = 7.0 Hz, 2H, CH_2), 7.01 (t, J = 7.0 Hz, 1H, CH), 7.34 (s, 1H, CH), 7.65 (d, J = 7.0 Hz, 1H, CH), 8.77 (d, J = 7.0 Hz, 1H, CH), 13.04 (br.s, 1H, OH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 17.7 (CH_3), 30.0 (CH_2), 41.2 (CH_2), 57.7 (OCH_3), 69.3 (CH_2), 100.7 (C), 110.6 (CH), 112.4 (CH), 124.9 (CH), 125.3 (C), 132.7 (C), 133.7 (CH), 148.1 (C), 148.9 (C), 154.4 (C), 161.7 (C). MS (CI): m/z 316.1 $[M+H]^+$. Calcd. for $C_{16}H_{17}N_3O_4$: C 60.94; H 5.43; N 13.33. Found: C 60.97; H 5.50; N 13.37.

*1,7-Dimethyl-4-oxo-1,4-dihydropyrido[1,2-a]pyrrolo[2,3-d]pyrimidine-2-carboxylic acid (**4e**)*. Yield 59 %, mp 243–245 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 2.35 (s, 3H, CH_3), 3.78 (s, 3H, CH_3), 6.88 (s, 1H, CH), 7.50 (d, J = 7.6 Hz, 1H, CH), 7.67 (d, J = 7.6 Hz, 1H, CH), 8.82 (s, 1H, CH), 12.97 (br.s, 1H, OH). MS (CI): m/z 258.0 $[M+H]^+$. Calcd. for $C_{13}H_{11}N_3O_3$: C 60.70; H 4.31; N 16.33. Found: C 60.66; H 4.27; N 16.30.

General procedure for the synthesis of 1-alkyl-4-oxo-1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides 6a-h. A suspension of carboxylic acids **4a-e** (2 mmol) in thionyl chloride (5 mL) was refluxed for 1–3 h. The solvent was evaporated to dryness, and a solution of the appropriate amine (2 mmol) and triethylamine (3 mmol) in acetonitrile (10 mL) was added to the residue. The reaction mixture was refluxed for 1–3 h and cooled to room temperature. The precipitate was filtered and washed with water. The product was recrystallized from MeOH or MeOH-DMF.

*N-Allyl-1-methyl-4oxo-1,4-dihydropyrido[1,2-a]pyrrolo[2,3-d]pyrimidine-2-carboxamide (**6a**)*. Yield 89 %, mp 231–233 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 3.89–3.91 (m, 2H, CH_2), 3.95 (s, 3H, CH_3), 5.10 (d, J = 10.4 Hz, 1H) and 5.21 (d, J = 16.8 Hz, 1H, CH_2), 5.86–5.96 (m, 1H, CH), 7.09 (t, J =

7.6 Hz, 1H, CH), 7.44 (s, 1H, CH), 7.54 (d, *J* = 7.6 Hz, 1H, CH), 7.72 (t, *J* = 7.6 Hz, 1H, CH), 8.68 (t, *J* = 7.6 Hz, 1H, NH), 8.88 (d, *J* = 7.6 Hz, 1H, CH); ^{13}C NMR (125 MHz, DMSO-d_6) d: 30.5 (CH_3), 41.0 (CH_2), 100.8 (C), 105.4 (CH), 112.8 (CH), 115.2 (CH_2), 124.9 (CH), 126.5 (CH), 129.0 (C), 134.7 (CH), 135.4 (CH), 147.9 (C), 148.9 (C), 154.1 (C), 160.5 (C). MS (CI): m/z 283.1 $[M+H]^+$. Calcd. for $C_{15}H_{14}N_4O_2$: C 63.82; H 5.00; N 19.85. Found: C 63.87; H 4.96; N 19.81.

*N,N-Diethyl-1-methyl-4oxo-1,4-dihydropyrido[1,2-a]pyrrolo[2,3-d]pyrimidine-2-carboxamide (**6b**)*. Yield 77 %, mp 110–112 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 1.17 (t, *J* = 6.8 Hz, 6H, $2CH_3$), 3.34 (q, *J* = 6.8 Hz, 4H, $2CH_2$), 3.72 (s, 3H, NCH_3), 6.85 (s, 1H, CH), 7.10 (t, *J* = 7.2 Hz, 1H, CH), 7.56 (d, *J* = 7.2 Hz, 1H, CH), 7.73 (t, *J* = 7.2 Hz, 1H, CH), 8.92 (d, *J* = 7.2 Hz, 1H, CH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 13.5 ($2CH_3$), 29.7 (CH_3), 39.5 ($2CH_2$), 100.5 (C), 101.7 (CH), 112.9 (CH), 124.9 (CH), 126.7 (CH), 130.2 (C), 134.6 (CH), 147.4 (C), 148.0 (C), 153.9 (C), 161.7 (C). MS (CI): m/z 299.1 $[M+H]^+$. Calcd. for $C_{16}H_{18}N_4O_2$: C 64.41; H 6.08; N 18.78. Found: C 64.37; H 6.12; N 18.83.

*1-Methyl-2-(pyrrolidin-1-ylcarbonyl) pyrido[1,2-a]pyrrolo[2,3-d]pyrimidin-4(1H)-one (**6c**)*. Yield 85 %, mp 164–166 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 1.86–1.90 (m, 4H, $2CH_2$), 3.42–3.52 (m, 2H, CH_2), 3.70–3.73 (m, 2H, CH_2), 3.85 (s, 3H, NCH_3), 7.11–7.13 (m, 2H, 2CH), 7.58 (d, *J* = 8.0 Hz, 1H, CH), 7.75 (t, *J* = 8.0 Hz, 1H, CH), 8.93 (d, *J* = 8.0 Hz, 1H, CH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 23.8 (CH_2), 25.9 (CH_2), 30.4 (CH_3), 46.2 (CH_2), 48.9 (CH_2) 100.6 (C), 105.0 (CH), 113.0 (CH), 124.9 (CH), 126.8 (CH), 129.9 (C), 134.9 (CH), 147.8 (C), 148.4 (C), 153.9 (C), 160.2 (C). MS (CI): m/z 297.2 $[M+H]^+$. Calcd. for $C_{16}H_{16}N_4O_2$: C 64.85; H 5.44; N 18.91. Found: C 64.78; H 5.50; N 18.96.

*2-(Morpholin-4-ylcarbonyl)-1-propylpyrido[1,2-a]pyrrolo[2,3-d]pyrimidin-4(1H)-one (**6d**)*. Yield 87 %, mp 175–177 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 0.78 (t, *J* = 7.2 Hz, 3H, CH_3), 1.69–1.74 (m, 2H, CH_2), 3.64–3.68 (m, 8H, $4CH_2$), 4.30 (t, *J* = 7.2 Hz, 2H, CH_2), 6.95 (s, 1H, CH), 7.09 (t, *J* = 7.2 Hz, 1H, CH), 7.57 (d, *J* = 7.2 Hz, 1H, CH), 7.73 (t, *J* = 7.2 Hz, 1H, CH), 8.92 (d, *J* = 7.2 Hz, 1H, CH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 11.0 (CH_3), 23.3 (CH_2), 40.9 ($2CH_2$), 43.9 (CH_2), 66.2 ($2CH_2$), 100.7 (C), 104.5 (CH), 113.0 (CH), 125.1 (CH), 126.8 (CH), 128.0 (C), 134.9 (CH), 147.8 (C), 148.4 (C), 154.1 (C), 161.3 (C). MS (CI): m/z 341.1 $[M+H]^+$. Calcd. for $C_{18}H_{20}N_4O_3$: C 63.52; H 5.92; N 16.46. Found: C 63.54; H 4.88; N 16.43.

*1,9-Dimethyl-4-oxo-N-pyridin-3-yl-1,4-dihydropyrido[1,2-a]pyrrolo[2,3-d]pyrimidine-2-carboxamide (**6e**)*. Yield 80 %, mp 330–332 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 2.57 (s, 3H, CH_3), 4.06 (s, 3H, CH_3), 7.06 (t, *J* = 7.2 Hz, 1H, CH), 7.40 (t, *J* = 7.2 Hz, 1H, CH), 7.68 (d, *J* = 7.2 Hz, 1H, CH), 7.76 (s, 1H, CH), 8.18 (d, *J* = 7.2 Hz, 1H, CH), 8.31 (d, *J* = 7.2 Hz, 1H, CH), 8.85 (d, *J* = 7.2 Hz, 1H, CH), 8.95 (s, 1H, CH), 10.42 (s, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 17.9 (CH_3), 30.7 (CH_3), 100.6 (C), 107.4 (CH), 112.5 (CH), 123.5 (CH), 124.9 (CH), 126.9 (CH), 128.2 (C), 132.7 (C), 133.6 (CH), 135.7 (C), 141.7 (CH), 144.4 (CH), 148.0 (C), 148.8 (C), 154.6 (C), 159.7 (C). MS (CI): m/z 334.2

$[M+H]^+$. Calcd. for $C_{18}H_{15}N_5O_2$: C 64.86; H 4.54; N 21.01. Found: C 64.89; H 4.52; N 21.06.

*1,9-Dimethyl-2-(piperidin-1-ylcarbonyl) pyrido[1,2-a]pyrrolo[2,3-d]pyrimidin-4(1H)-one (**6f**)*. Yield 84 %, mp 184–186 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 1.56–1.65 (m, 6H, $3CH_2$), 2.55 (s, 3H, CH_3), 3.59–3.63 (m, 4H, $2CH_2$), 3.78 (s, 3H, CH_3), 6.84 (s, 1H, CH), 7.02 (t, J = 7.2 Hz, 1H, CH), 7.64 (d, J = 7.2 Hz, 1H, CH), 8.83 (d, J = 7.2 Hz, 1H, CH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 17.8 (CH_3), 24.0 (CH_2), 25.8 ($2CH_2$), 29.7 (CH_3), 40.8 ($2CH_2$), 100.3 (C), 102.8 (CH), 112.3 (CH), 124.8 (CH), 129.4 (C), 132.6 (C), 133.0 (CH), 147.2 (C), 147.8 (C), 154.4 (C), 160.9 (C). MS (CI): m/z 325.1 $[M+H]^+$. Calcd. for $C_{18}H_{20}N_4O_2$: C 66.65; H 6.21; N 17.27. Found: C 66.59; H 6.19; N 17.30.

*1-(3-Methoxypropyl)-9-methyl-N-(3-morpholin-4-ylpropyl)-4-oxo-1,4-dihydropyrido[1,2-a]pyrrolo[2,3-d]pyrimidine-2-carboxamide (**6g**)*. Yield 75 %, mp 103–105 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 1.69 (t, J = 6.6 Hz, 2H, CH_2), 1.95 (t, J = 6.6 Hz, 2H, CH_2), 2.23–2.41 (m, 6H, $3CH_2$), 2.60 (s, 3H, CH_3), 3.16 (s, 3H, OCH_3), 3.40–4.45 (m, 4H, $2CH_2$), 3.51–3.65 (m, 4H, $2CH_2$), 4.64 (t, J = 6.8 Hz, 2H, CH_2), 6.99 (t, J = 6.8 Hz, 1H, CH), 7.37 (s, 1H, CH), 7.63 (d, J = 6.8 Hz, 1H, CH), 8.51(t, J = 6.8 Hz, 1H, NH), 8.81 (d, J = 6.8 Hz, 1H, CH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 17.8 (CH_3), 26.0 (CH_2), 30.1 (CH_2), 37.1 (CH_2), 42.0 ($2CH_2$), 53.3 ($2CH_2$), 55.9 (CH_2), 57.5 (CH_3), 66.2 ($2CH_2$), 69.5 (CH_2), 100.3 (C), 105.3 (CH), 112.3 (CH), 124.8 (CH), 128.9 (C), 132.7 (CH), 133.1 (C), 147.5 (C), 148.3 (C), 154.3 (C), 160.7 (C). MS (CI): m/z 442.1 $[M+H]^+$. Calcd. for $C_{23}H_{31}N_5O_4$: C 62.57; H 7.08; N 15.86. Found: C 62.52; H 7.14; N 15.91.

*N,N-Diethyl-1,7-dimethyl-4-oxo-1,4-dihydropyrido[1,2-a]pyrrolo[2,3-d]pyrimidine-2-carboxamide (**6h**)*. Yield 79 %, mp 105–107 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) d: 1.18 (t, J = 6.8 Hz, 6H, $2CH_3$), 2.36 (s, 3H, CH_3), 3.48 (q, J = 6.8 Hz, 4H, $2CH_2$), 3.72 (s, 3H, CH_3), 6.84 (s, 1H, CH), 7.52 (d, J = 8.8 Hz, 1H, CH), 7.63 (d, J = 8.8 Hz, 1H, CH), 8.74 (s, 1H, CH). ^{13}C NMR (125 MHz, DMSO-d_6) d: 13.8 ($2CH_3$), 17.6 (CH_3), 29.7 (CH_3), 39.8 ($2CH_2$), 100.6 (C), 101.6 (CH), 122.4 (CH), 123.5 (C), 124.5 (CH), 130.0 (C), 137.6 (CH), 146.5 (C), 148.1 (C), 153.9 (C), 161.8 (C). MS (CI): m/z 313.1 $[M+H]^+$. Calcd. for $C_{17}H_{20}N_4O_2$: C 65.37; H 6.45; N 17.94. Found: C 65.43; H 6.46; N 17.99.

Antimicrobial activity

Bacterial strains. *S. aureus* strain 222, *E. coli* strain 311 and *P. aeruginosa* strain 449 were isolated from patients with the 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 to recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), except susceptibility to vancomycin determined by serial dilution method [23]. Minimum inhibitory concentration (MIC) was determined by the serial broth microdilution method according to [24]. *S. aureus* 222 was identified as MRSA by molecular methods on the ground of the

mecA gene expression. The strain was resistant to oxacillin, cefoxitin and susceptible to vancomycin and clindamycin. *E. coli* 311 was resistant to amikacin, norfloxacin, cefoperazone, ciprofloxacin and susceptible to gentamicin. *P. aeruginosa* 449 was resistant to cefepime; susceptible to ciprofloxacin, meropenem, aztreonam, and amikacin.

Biofilm assay. The antibiofilm activity of compounds **6a**, **6d, 6g**, and **6h** was studied at subinhibitory concentration (sub-MIC) 25.0 mg/L (MIC > 200.0 mg/L) in polystyrene microtiter plates as described by O'Toole [25]. The antibiofilm activity of compound **6b** against *S. aureus* was studied at concentration 37.5 mg/L (0.5 MIC), *E. coli* and *P. aeruginosa* — 25.0 mg/L (MIC > 200.0 mg/L) (Table 1). 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 $OD_{600} = 0.055 \pm 0.001$) were applied to wells simultaneously. Thereafter, the 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, 0.1 % solution of gentian violet was added and incubated for 10–15 min. To detect a 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. The intact cultures of microorganisms grown under the same conditions without the compound adding were served as a control.

Statistical analysis for the biofilm assay was made using the nonparametric Kruskal-Wallis H-test. A p-value of <0.05 was considered as significant. STATISTICA, version 10.0 (StatSoft, USA) was used for the data analysis. All experiments were repeated in triplicate.

Results and Discussion

The synthesis of target 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides **6** from substituted carboxylic acids **4** was implemented using carbaldehydes **1** as a starting material according to Fig. 1, 2. The 2-chloro-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-3-carbaldehydes **1a-c** were prepared in good yields, according to the previously reported method [16, 22]. In the presence of triethylamine, carbaldehydes **1a-c** were reacted with ethyl *N*-alkylglycinate in methanol to produce the pyrido[1,2-*a*]pyrimidine intermediates **2a-e** that cyclized via the addition of the sodium methoxide to form a tricyclic system, 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine derivatives **3a-e** (Fig. 1). Alkaline hydrolysis of esters **3a-e** gave substituted 1-alkyl-4-oxo-1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxylic acids **4a-e**. It should be noted that the examples of synthesis of fused pyrrole derivatives were previously described in [26, 27].

The target 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamide derivatives **6** were synthesized via a two-step process as shown in Fig. 2. First, treating compounds **4a-e** with thionyl chloride gave 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carbonyl chlorides **5a-e**. The interaction of intermediates **5a-e** with amines in acetonitrile using triethylamine as a base led to the formation of the corresponding 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamide de-

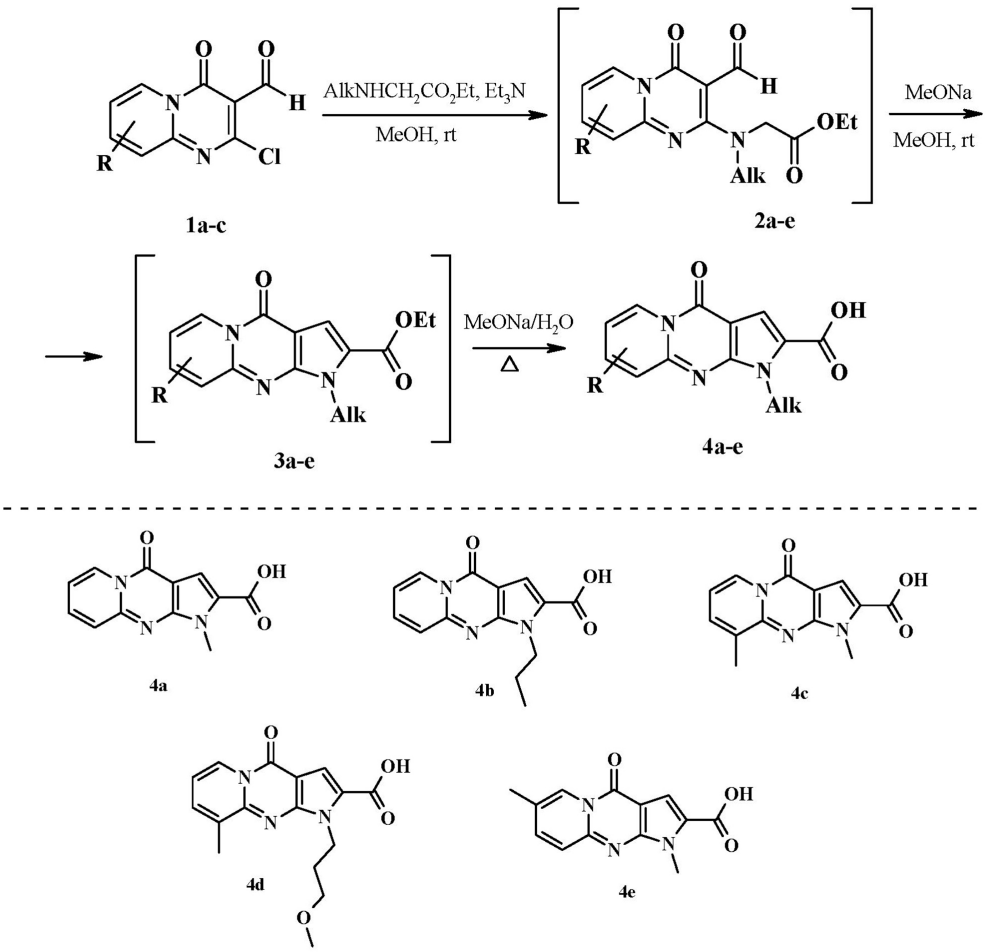

Fig. 1. Synthesis of substituted 4-oxo-1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxylic acids **4a-e.**

rivatives **6a-h** in yields ranging from 75 % to 89 %.

Antimicrobial activity

The antimicrobial activity of novel 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides against *P. aeruginosa* 449, *S. aureus* 222 and *E. coli* 311 is shown in the Table 1.

As seen from the results of experiments, novel 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides **6a-6h** do not exhibit a significant antimicrobial activity against the gram-negative microorganisms (*P. aeruginosa* 449 and *E. coli* 311). Their MICs are > 200.0 mg/L (Table 1). Against meticillin-resistant *S. aureus* 222, only **6b** exhibits the antimicrobial activity: MIC is 75.0 mg/L. MICs of other tested compounds are > 200.0 mg/L.

The action of novel 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides

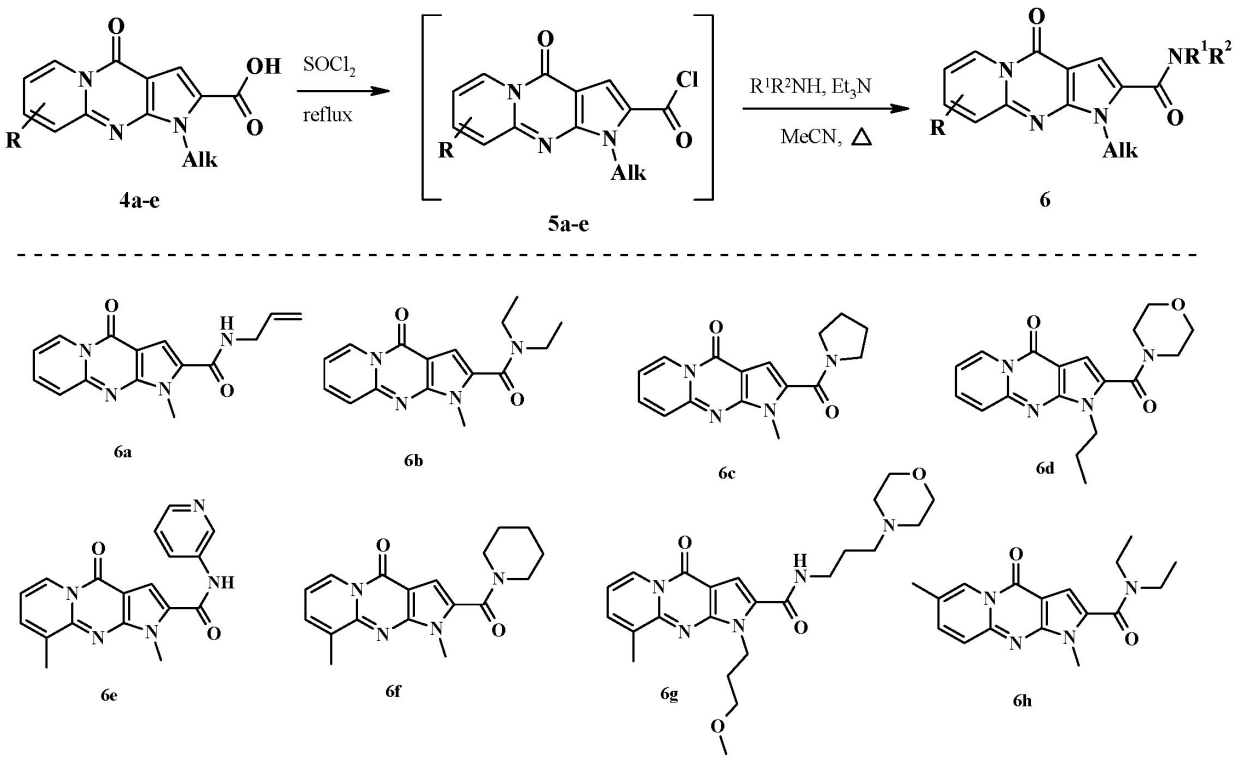

Fig. 2. Synthesis of 1-alkyl-4-oxo-1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides **6a-h.**

Table 1. **Antimicrobial activity (MIC, mg/L) of novel 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides**

Bacterial strain	MIC, mg/L				
	6a	6b	6d	6g	6h
***S. aureus* 222**	> 200.0	75.0	> 200.0	> 200.0	> 200.0
***E. coli* 311**	> 200.0	> 200.0	> 200.0	> 200.0	> 200.0
***P. aeruginosa* 449**	> 200.0	> 200.0	> 200.0	> 200.0	> 200.0

on the *S. aureus* 222, *E. coli* 311 and *P. aeruginosa* 449 biofilm formation is shown in Fig. 3.

According to the obtained data **(**Fig. 3A**)**, compound **6b** exhibits the most pronounced inhibitory effect on the biofilms formation by *S. aureus* 222: the biomass decreases by 62.6 % under the action of sub-inhibitory concentration ($p < 0.05$). Compounds **6d** and **6g**, on the other hand, demonstrate a less pronounced effect at sub-MIC, when the biomass decreases by 50.2 % and 54.0 %, respectively, as compared to control ($p < 0.05$). No antibiofilm effect is observed for compound **6a**. In

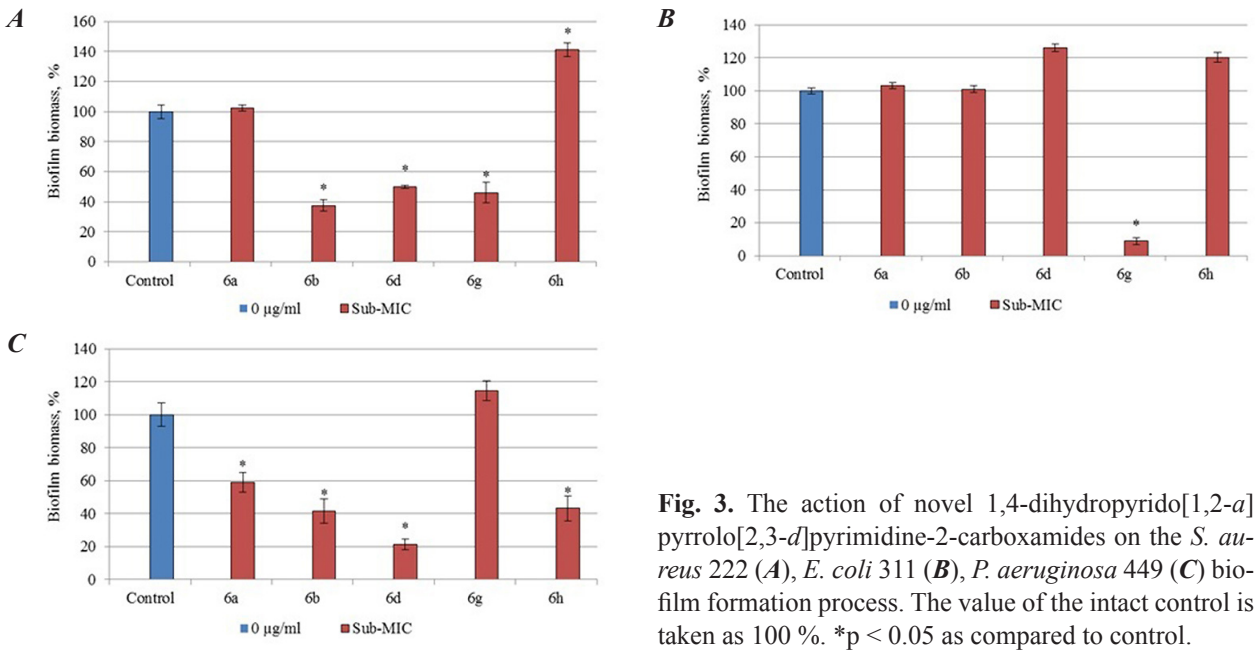

Fig. 3. The action of novel 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides on the *S. aureus* 222 (***A***), *E. coli* 311 (***B***), *P. aeruginosa* 449 (***C***) biofilm formation process. The value of the intact control is taken as 100 %. *$p < 0.05$ as compared to control.

contrast, compound **6h** stimulates the biofilm formation by 41.3 % as compared to control ($p<0.05$).

The investigation of the impact of novel 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides on *E. coli* 311 **(**Fig. 3B**)** reveals that compound **6g** exhibits the highest level of activity. Under the action of sub-inhibitory concentration, the biomass decreases by 91.2 % compared to control ($p<0.05$). Compound **6d**, conversely, stimulates the biofilms formation by *E. coli* 311 at 25 mg/L: an increase in the biofilm biomass is by 26.2 %. On other hand, compounds **6a, 6b,** and **6h** do not display an antibiofilm effect.

It is shown (Fig. 3C) that compound **6a** influencing the *P. aeruginosa* biofilm formation decreases the biofilm biomass significantly by 40.9 %, **6b** — by 58.5 %, **6d** — by 78.7 %, **6h** — by 56.8 % at sub-MIC concentration. Compound **6g**, conversely, stimulates the biofilms formation by *P. aeruginosa* 449 at sub-MIC, when the biomass increases by 14.4 %, but these changes are not statistically significant ($p> 0.05$).

Considering the biofilm role in the development of inflectional processes, the bacterial resistance and the ineffectiveness of antimicrobial therapy, the synthesis and screening of new compounds with antibacterial and antibiofilm activities remain relevant. According to the data obtained, the novel 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides do not exhibit significant antimicrobial activity against planktonic cells of both gram-positive and gram-negative bacteria. However, in the sub-MICs they are capable of disrupting the biofilm formation by methicillin-resistant strain *S. aureus* 222, *E. coli* 311, and *P. aeruginosa* 449. Compound **6b** showed

the most pronounced antibiofilm activity against *S. aureus* 222. Compounds **6d** and **6g** are also active against biofilms of *S. aureus* 222 (reduction by 50.2 % and 54.0 % respectively). Compound **6g** turned out to be the most active against the biofilm formation of *E. coli* 311 (biomass decreased by 91.2 %). Compounds **6a**, **6b**, **6d**, and **6h** showed a reduction in biofilm formation of *P. aeruginosa* 449 by 40.9–78.7 %.

In the experiments at sub-inhibitory concentrations, 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides showed not only the inhibition but also stimulation of the biofilm formation, particularly under the action of compound **6h** on MRSA (an increase in biofilm biomass by 41.3 %). Such an effect is, as a rule, nonspecific and independent of the chemical class of compounds, their mechanism of action, or the type of microorganism. An increase in biofilm biomass is observed under the influence of various groups of antimicrobial agents and substances that do not exhibit inhibitory effects on gram-positive and gram-negative bacteria [28]. The mechanism of stimulation of the biofilm formation is not fully understood, but it may be associated with a protective response of the bacterial cells to adverse conditions. Under the influence of sub-MIC concentrations of compounds, the expression of genes regulating cell autolysis, release of extracellular DNA (eDNA) and enhancement of biofilm formation may increase in bacteria. Additionally, various substances at low concentrations can act as signaling molecules and directly or indirectly induce the transcriptional changes in genes responsible for adhesion and/or increased production of biofilm matrix components. They may also increase the production of secondary messengers (c-di-GMP, ppGpp) or induce a protective response to stress in bacteria [28].

Conclusions

An efficient method for the synthesis of new of 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxylic acid derivatives based on 2-chloro-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-3-carbaldehydes and ethyl *N*-alkylglycinates was developed. The investigation of antibiofilm activity indicates that novel 1,4-dihydropyrido[1,2-*a*]pyrrolo[2,3-*d*]pyrimidine-2-carboxamides can disrupt the biofilm formation of methicillin-resistant strain *S. aureus*, *E. coli* and *P. aeruginosa.* Compounds **6b**, **6d**, and **6g** showed the most pronounced antibiofilm activity against *S. aureus* 222, compound **6g —** against *E. coli* 311. Compounds **6a**, **6b**, **6d**, and **6h** demonstrated reduced biofilm formation of *P. aeruginosa* 449.

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Синтез та антибіоплівкова активність нових 1,4-дигідропіридо[1,2-*a*]піроло[2,3-*d*]піримідин-2-карбоксамідів

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Мета. Синтез нових алкілзаміщених 4-оксо-1,4-дигідропіридо[1,2-*a*]піроло[2,3-*d*]піримідин-2-карбоксамідів та оцінка їх антибіоплівкової активності в умовах *in vitro*. **Методи.** Органічний синтез, аналітичні та спектральні методи, мікрометод серійних розведень, формування біоплівок на абіотичній поверхні. **Результати.** Розроблено простий та ефективний метод синтезу нових похідних 1,4-дигідропіридо[1,2-*a*]піроло[2,3-*d*]піримідин-2-карбонової кислоти. Результати скринінгу антибіоплівкової активності показали, що серед синтезованих алкілзаміщених 1,4-дигідропіридо[1,2-*a*]піроло[2,3-*d*]піримідин-2-карбоксамідів виявлено сполуки, здатні порушувати плівкоутворення метицилінрезистентного штаму *S. aureus* 222, *E. coli* 311 та *P. aeruginosa* 449. Сполука **6g** є активною щодо біоплівок *E. coli* 311, біомаса зменшується на 91.2 % та щодо *S. aureus* 222 (зменшення на 54.0 %), **6d** — щодо біоплівок *P. aeruginosa* 449 та *S. aureus* 222 (зменшення на 78.7 % та 50.2 % відповідно). **Висновки.** Синтезовано ряд нових заміщених 1-алкіл-4-оксо-1,4-дигідропіридо[1,2-*a*]піроло[2,3-*d*]піримідин-2-карбоксамідів. Досліджено активність синтезованих піридо[1,2-*a*]піроло[2,3-*d*]піримідинів на формування біоплівок *S. aureus* 222, *E. coli* 311 та *P. aeruginosa* 449 та виявлено сполуки з виразною антибіоплівковою активністю.

Ключові слова: піридо[1,2-a]піроло[2,3-d]піримідини, піридо[1,2-*a*]піримідин-3-карбальдегіди, синтез, антибіоплівкова активність.

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