SYNTHESIS, HPLC ANALYSIS AND BIOLOGICAL ACTIVITY OF DYNAMIC POLYMYXIN

Martynov AV, Farber* BS., Sklyar NI.

Mechnikov Institute of Microbiology and Immunology, Kharkiv, Ukraine

***Noigel LLC, New-York, USA**

The peptide antibiotic polymyxin is one of the last line drugs in the fight against resistant forms of microorganisms[5]. Its mechanism action is associated with the destabilization of ion transport across the bacterial cell membrane. Polymyxin is an artificial porin and is a cyclic oligopeptide[1]. In its action mechanism, an excess positive charge formed by the residues of basic amino acids plays an important role. Due to this charge, polymyxin is adsorbed on the microbial wall, creates an artificial ion channel and depolarizes the cell membrane, respectively - the microbial cell dies[8]. Although polymyxin has been known as an antibiotic for more than 40 years, resistance to it in microorganisms has developed rather slowly. To date, the emergence of resistant strains has accelerated exponentially [4]. To overcome the bacterial resistance to polymyxin, we proposed to synthesize a dynamized supramolecular combinatorial polymyxin, simultaneously modified with succinic, maleic and acetic anhydrides [3]. With the calculated degree of modification, a mixture of more than 16,000 polymyxin derivatives is obtained, which interact in solution with each other to form complex supramolecular self-organizing structures and have the properties of adaptation to the microorganism. According to the theory of self-organizing structures (dissipative [2]), self-assembly of supramolecular structures occurs at the point of fluctuation - the site of action of the initial polymyxin on the microbial cell membrane[6]. Such a system behaves as a quasi-live, capable of adapting to the environment. To such a system, the development of resistance in microorganisms is almost impossible.

Thus, the aim of the work was to synthesize dynamic succinyl-maleinyl-acetyl-polymyxin, confirm the change in its structure using reverse-phase high-performance liquid chromatography (RP-HPLC), determine the MIC and MBC for the dynamic structure on the example of two strains of *Pseudomonas aeruginosa* - ATCC strain and polymyxin-resistant hospital strain.

Materials and methods

Chemicals and reagents
Polymyxin B sulfate (Sigma-Aldrich, USA), Acetonitrile (Merck, USA), chloric acid and lithium perchloride (Sigma-Aldrich, USA), Succinic anhydride (Merck, USA), Maleic anhydride (Medisca, USA), Acetic anhydride (Azot, Ukraine), nutrient media for microorganisms: dry Mueller-Hinton medium and nutrient agar (Biolife, Italy)

Synthesis the supramolecular combinatorial mixture of polymyxin (PPC)
164 µM polymyxin B (I) is dissolved in 10 ml of dioxane (CAS N 1404-26-8, Mr =1203.499 g / mol, n = 7) (I), 287 µM succinic anhydride (III), 287 µM acetic anhydride (II), 287 µM maleic anhydride are added, the solution is stirred and heated under reflux for 10 minutes. The solution was poured into ampoules and lyophilized to remove solvent and acetic acid. The combinatorial mixture (IVa-d) is used to obtain pharmaceutical compositions, study the structure, determine the biological activity. Figure 1 shows a synthesis scheme for combinatorial derivatives of polymyxin.
Calculations of the number of moles of modifiers are carried out according to the combinatorics formulas:

$$k= n (d+1)^{n-1} \quad (1)$$
$$m= (d+1)^{n}-1 \quad (2)$$

where m - is the number of different derivatives of molecules in the combinatorial mixture and the number of moles of polymyxin for the reaction; n- is the number of amino groups and hydroxyl groups available for modification in the structure of polymyxin (n = 7); k - is the number of moles of each modifier; d- is the number of modifiers (3 in our case – succinic anhydride, acetic anhydride and maleic anhydride).

Therefore, having only one initial polymyxin molecule and two modifiers after combinatorial synthesis, we obtain 16 384 combinatorial derivatives with different degrees of substitution, different positions of substituents and different permutations of the modifier residues, not just as a mixture, but as a difficult to separate modification (**n = 7**) . supramolecular mixture.

RP-HPLC analysis of polymyxin derivatives
For the HPLC, a Milichrom A-02 microcolumn chromatograph in a gradient of acetonitrile (5-100%) / 0.1 M perchloric acid + 0.5 M lithium perchlorate was used. The combinatorial derivative in the chromatogram gave one clear broadened peak and was not separated into components, although the retention time differed from both the original polymyxin and its completely substituted derivatives. This testified to the fact that complex supramolecular structures were formed between different combinatorial derivatives (in our case, 16 383), which were not separated chromatographically. This combinatorial derivative (CPP) behaves similarly and when separated in a thin layer (acetonitrile: water) gives only one band, which does not coincide with any of the obtained derivatives. An attempt to use two-dimensional TLC in different conditions also did not allow us to separate the combinatorial derivative. This is a characteristic feature of the supramolecular structure in the combinatorial derivative (IVa-d).
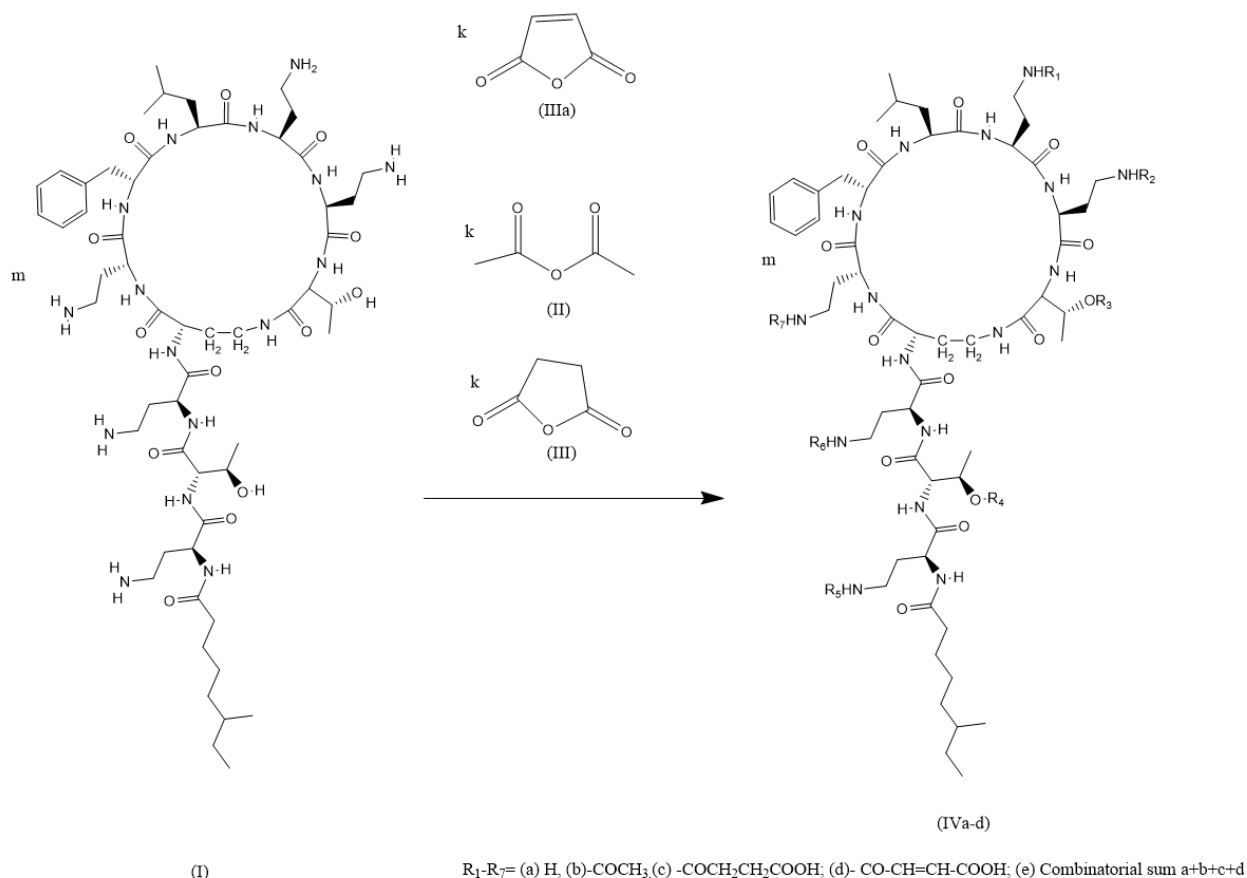
Fig.1. Scheme of combinatorial synthesis of polymyxin derivatives with the formation of supramolecular combinatorial derivative (IVa-d): polymyxin reacts with two modifying agents - succinic anhydride and acetic anhydride in the calculated proportions. In this case, a supramolecular structure of 16384 polymyxin derivatives is formed.

Instrumentation
The HPLC equipment consisted of Milichrom A-02 HPLC system (Novsibirsk, RF). The LC column was ProntoSIL 120-5 C18 AQ (Bischoff Anal. GmbH, Germany) and obtained from Analitica LLC (Kharkiv, Ukraine). A gradient mobile phase was employed: phase A: [4M LiClO4 – 0,1M HClO4]: H$_2$O=1:19; and phase B: 100% CH$_3$CN were used. In all experiments, the columns were thermostated at 40 °C. Other conditions for separation also were used (see description under Figures). Multiwave UV detector was used for detection of fractions.

Sample preparation
10 mg of polymixin sample was diluted with 700 μL water and 300 μL acetonitrile and centrifuged for 3 min. The solvent was removed from the isolated polymixin fractions by rotary evaporation. The solid sample was dissolved in 200 μL of the mobile phase A, and 50 μL was injected into the LC injector.

Antimicrobial activity study in vitro

The antimicrobial activity of the compounds was studied in a collection of test strains of microorganisms obtained from the Institute of Microorganisms Museum and living culture museums of various laboratories of the IMI NAMS (Kharkov). The collection included the following multiresistant strains: bacteria - *Pseudomnas aeruginosa* IMI Res3 and *P. aeruginosa* ATCC 27853.
For the cultivation of bacteria, Mueller-Hinton broth (pH 7.2-7.4) was used. Antimicrobial activity was evaluated by the minimum inhibitory concentration (MIC) - the smallest amount of a substance that completely inhibited the growth of bacteria or fungi after cultivation. IPC was determined by the conventional method of serial dilutions with a coefficient of 2 in a liquid nutrient medium. For this purpose, the initial dilution of the test compound with a concentration of 50 μg / ml of culture medium (MH broth) was prepared. Subsequently, a sequential double dilution was carried out, as a result of which 25 ml were contained in 1 ml of culture medium; 12.5; 6.25; 3.12 μg/mL, etc.[7]
The reference standard was ethacridine lactate. This combinatorial mixture of derivatives IV behaves like a quasi-

fluid system - it adapts to the individual conditions of the body, preventing the emergence of resistance in bacteria. The results of studies of the antimicrobial and antifungal activity of derivatives of tannins are presented in table. 1.

Statistics
All experiments were repeated at least 7 times, the statistical hypothesis of differences between concentrations was confirmed for the original polymyxin between resistant and non-resistant strains. For statistical calculations, analysis of variance and Xi-square were used.

Results and discussion
The combinatorial derivative in the chromatogram gave one clear broadened peak and was not separated into components, although the retention time differed from both the original polymyxin and its completely substituted derivatives. This testified to the fact that complex supramolecular structures were formed between different combinatorial derivatives (in our case, 16 383), which were not separated chromatographically. This combinatorial derivative (CPP) behaves similarly and when separated in a thin layer (acetonitrile: water) gives only one band, which does not coincide with any of the obtained derivatives. An attempt to use two-dimensional TLC in different conditions also did not allow us to separate the combinatorial derivative. This is a characteristic feature of the supramolecular structure in the combinatorial derivative (IVa-d).
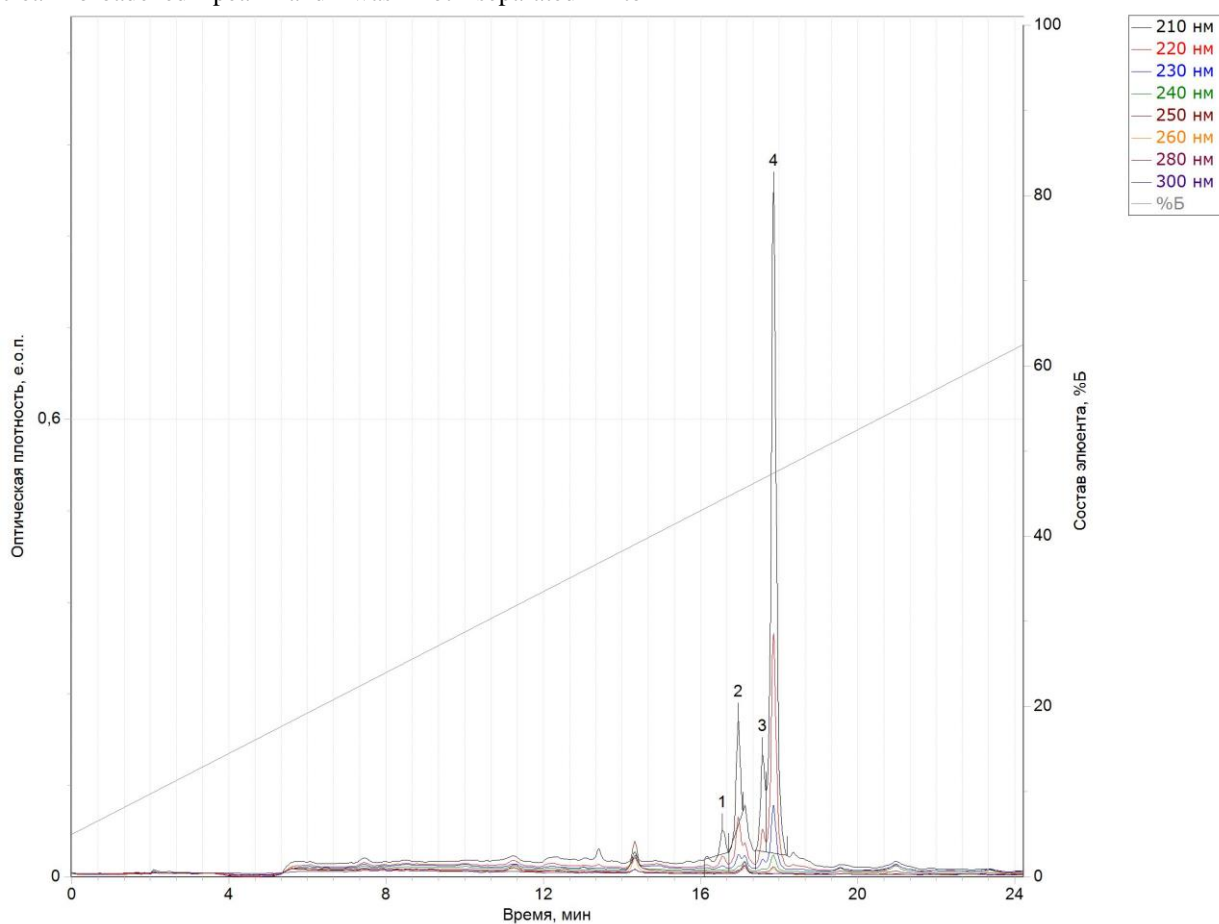

Fig. 2. RP-HPLC for initial polymyxin sulfate (I) (column ProntoSIL 120-5 C18 AQ A gradient mobile phase was employed: phase A: [4M LiClO4 – 0,1M HClO4]: H_2O=1:19; and phase B: 100% CH_3CN were used).
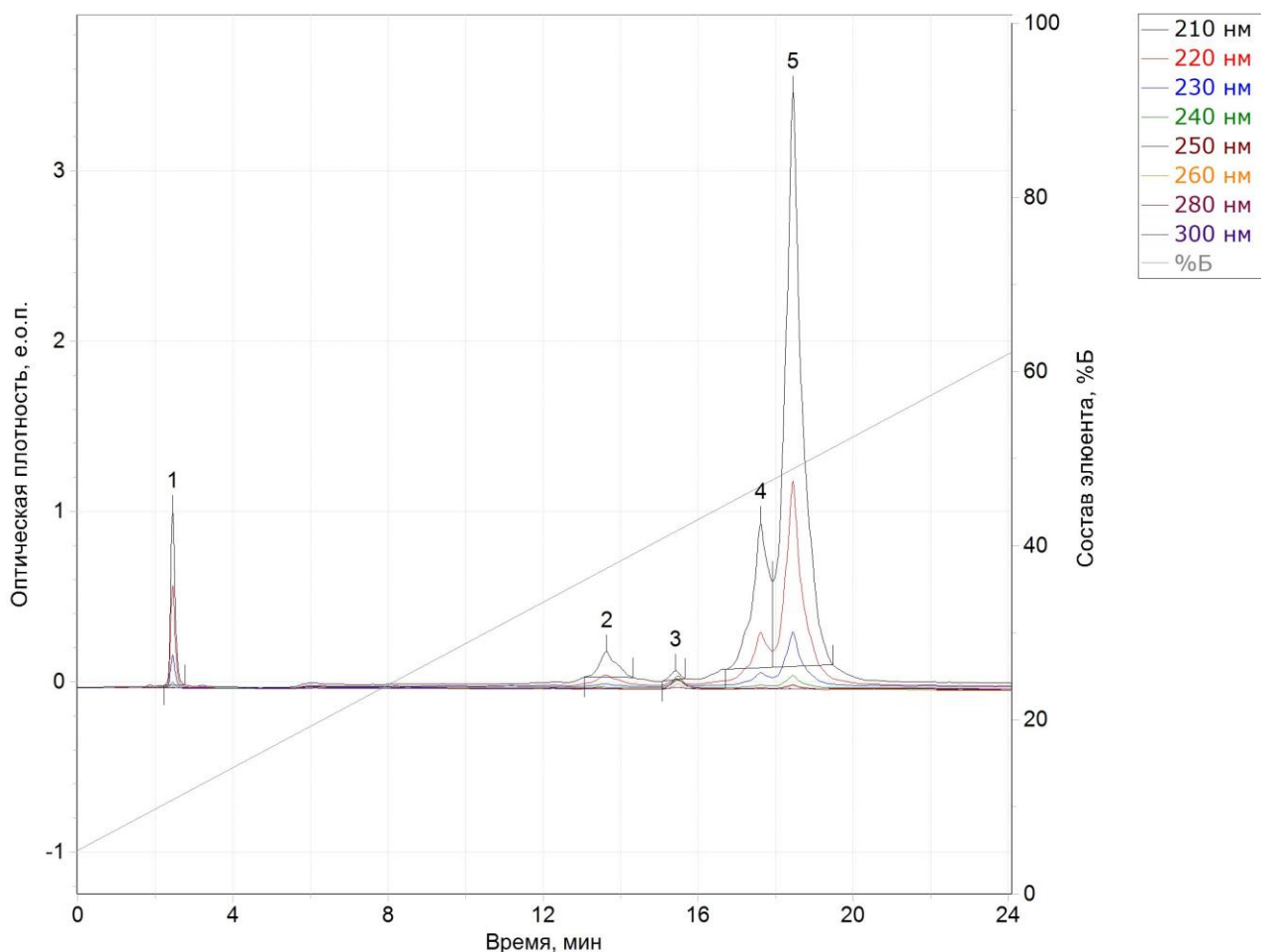
Fig. 3. RP-HPLC for combinatorial dynamic derivative (IV) (column ProntoSIL 120-5 C18 AQ A gradient mobile phase was employed: phase A: [4M LiClO4 – 0,1M HClO4]: H₂O=1:19; and phase B: 100% CH₃CN were used).

Polymyxin initially contains its own fragments in the sample. Pure polymyxin is several molecules of different sizes, which on the chromatogram give 4-5 absorption peaks with one dominant peak of the full cyclic peptide and three small fragments (Figure 2). After dynamization of the polymyxin structure, the absorption peaks of all components shift and expand. The 4th and 5th absorption peaks of a main components are slightly shifted to the hydrophobic region, which indicates the completion of the chemical reaction of structure modification. At the same time, complete separation into individual derivatives is not observed, probably due to the very similar molecular masses and charges of these derivatives and their large number (16383 components). In addition, the absorption intensity of all components also increased.

Table 1. Antibacterial activity of supramolecular combinatorial derivatives of antibiotics based on MIC, μg / ml

	Strains of microorganisms *, MIC, μg/mL	
Derivatives	*P.aeruginosa* IMI res3	*P.aeruginosa* ATCC 27853
IV	3,12	3,12
I	>500**	6,25**
Ethacridine lactates	125	125

Notes: - - does not have activity in a dose of up to 500 μg/mL; * - the initial unmodified derivatives of antibiotics did not affect the growth of these strains even at doses higher than 500 μg/mL. **Differences statistically differences in 7 iteration (P<0.005)

Table 2. Antibacterial activity of supramolecular combinatorial derivatives of antibiotics based on MBC, μg / ml

	Strains of microorganisms *, MIC, μg/mL	
Derivatives	*P.aeruginosa* IMI res3	*P.aeruginosa* ATCC 27853
IV	6,25	6,25
I	>500**	12,5**
Ethacridine lactates	125	125

Notes: - - does not have activity in a dose of up to 500 μg/mL; * - the initial unmodified derivatives of antibiotics did not affect the growth of these strains even at doses higher than 500 μg/mL. **Differences statistically differences in 7 iteration (P<0.005)

As can be seen from the tables 1 and 2, the maximum antimicrobial activity (MIC and MBC) against all strains of *P.aeruginosa* was shown by all combinatorial derivatives IV, in contrast to their unmodified derivative I, which initially did not have antimicrobial activity against resistant strain *P.aeruginosa* IMI res3 . MIC derivative IV for resistance strain *P. aeruginosa* IMI res3 was 3,12 μg/mL, whereas MIC initial substance I was >500 μg/mL. Wherein, MIC between IV and I are not different and were 3,12 μg/mL. MBC for IV was high than MIC and for both strains strain its was 6,25 μg/mL. For initial I, resistance strain practically not killed (MBC>500 μg/mL) and for standard strain *P.aeruginosa* ATCC 27853 MBC was 12,5 μg/mL.

Conclusion
1. For the first time, a dynamic supramolecular succinyl-maleinyl-acetyl-polymyxin (IV) was synthesized, its containing 16383 minor components of various derivatives.
2. It has been shown that the synthesized dynamic polymyxin (IV) is more hydrophobic than the original polymyxin (I) molecule.
3. MIC (IV) for both the resistant strain of *P. aeruginosa* and for the standard strain *P.aeruginosa* ATCC 27853 was 3.12 μg/mL, while the original (I) was active only against *P.aeruginosa* ATCC 27853 and amounted to 6, 25 μg/mL
4. MBC (IV) for both experimental strains of *P. aeruginosa* coincided and amounted to 6.25 μg/mL, while MBC (I) was 12.5 μg/mL, and the original polymyxin (I) was ineffective for the resistant strain *P. aeruginosa*.

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coefficient of 2 in a liquid nutrient medium. For this purpose, the initial dilution of the test compound with a concentration of 50 μg / ml of culture medium (MH broth) was prepared. Subsequently, a sequential double dilution was carried out, as a result of which 25 ml were contained in 1 ml of culture medium; 12.5; 6.25; 3.12 μg/mL, etc. **Results and discussion.** After dynamization of the polymyxin structure, the absorption peaks of all components shift and expand. The 4th and 5th absorption peaks of a main components are slightly shifted to the hydrophobic region, which indicates the completion of the chemical reaction of structure modification. At the same time, complete separation into individual derivatives is not observed, probably due to the very similar molecular masses and charges of these derivatives and their large number (16383 components). **Conclusion**. For the first time, a dynamic supramolecular succinyl-maleinyl-acetyl-polymyxin (IV) was synthesized, its containing 16383 minor components of various derivatives. It has been shown that the synthesized dynamic polymyxin (IV) is more hydrophobic than the original polymyxin (I) molecule. MIC (IV) for both the resistant strain of P. aeruginosa and for the standard strain *P.aeruginosa* ATCC 27853 was 3.12 μg/mL, while the original (I) was active only against *P.aeruginosa* ATCC 27853 and amounted to 6, 25 μg/mL MBC (IV) for both experimental strains of *P. aeruginosa* coincided and amounted to 6.25 μg/mL, while MBC (I) was 12.5 μg/mL, and the original polymyxin (I) was ineffective for the resistant strain *P. aeruginosa*
Keywords: polymyxin, derivatives, multiresistans, *P.aeruginosa*, dynamic drugs, dissipative structutres

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