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SCREENING AND CHARACTERISTIC OF REGULATORS OF ANTIBIOTIC BIOSYNTHESIS IN *STREPTOMYCES*

The aim *was to study the new regulators of antibiotic biosynthesis and sporulation in Streptomyces.* ***Methods*** *include thin layer chromatography, spectrophotometry and mass determination by HPLC/MS using ESI and APCI techniques.* ***Results.*** *Forty eight strains of streptomycetes isolating from soils of different regions of Ukraine were studied on the possibility to produce the regulators of antibiotic biosynthesis and morphogenesis by means of two test-systems – mutant strains of Streptomyces globisporus 1912-B2 and S. griseus 1439, losing the ability to synthesize the antibiotics landomycin E and streptomycin, correspondingly, as well to form the spores.*

Investigating streptomycetes were divided on three groups: 31 strains (62 %) restored the ability of both mutants to produce the antibiotics and spores, 17 strains (35 %) did not possessed above mentioned biological activity, and only one strain (2 %) produced regulator restoring antibiotic activity and morphogenesis in the strain 1439.

The individual compounds were isolated and purified from agar cultures of the strains of the first group K4 and TR144 by means of thin layer chromatography restoring antibiotic biosynthesis and morphogenesis in the test-strains 1912-B2 and 1439. Both compounds were characterized by the same Rt (2,34 min) and maximum of absorption (255 nm), and have molecular weights (m/z) 136 and 155, correspondingly.

Conclusions. *Two related compounds producing by different Streptomyses strains K4 and TR144 and restoring antibiotic biosynthesis and sporulation in test mutant strains were purified and some of their properties (Rf, maxima of absorption and m/z) were characterized.The next study of the isolated regulators by means of NMR will give the possibility to elucidate their molecular structures.*

K e y w o r d s: Streptomyces globisporus 1912-B2, S. griseus 1439, soil streptomycetes, transcriptional regulators.

The representatives of the genus *Streptomyces* belong to Gram-positive soil bacteria possessing the complex morphological organization and playing an important role in the fertility of soil. *Streptomyces* produce a large number of the secondary metabolites with a range of biological activities including over two-thirds of the clinically used antibiotics, different regulators, immunosuppressors, pigments, enzymes and other compounds [1, 2].

Adaptation and responding of *Streptomyces* to different environmental conditions provide the precise regulatory systems. Regulation of the antibiotic biosynthesis is realized on the different levels of the structural organization and cell differentiation by a large number of the genes products of which belong to the different families of regulatory proteins [3].

The main regulatory systems are presented by one– or two-component systems controlling a wide range of secondary metabolites in *Streptomyces* [4]. There is a large number of the transcriptional regulators binding DNA and activating or repressing the transcription of specific genes [5]. These regulators are presented by the different chemical compound giving possibility

their producers to form the aerial mycelia, secondary metabolites and adapt to environmental conditions [4, 5].

Present paper is dedicated the screening and characteristic of unknown low molecular weight extracellular compounds produced by freshly isolated strains of *Streptomyces* and like A-factor restoring antibiotic biosynthesis and sporulation in the mutant strains *Streptomyces globisporus* 1912-B2 and *Streptomyces griseus* 1439.

Materials and methods. *Strains and media.* Two mutant strains were used as the test system for screening of unknown regulators. The strain *S. globisporus* 1912-B2 is defective in biosynthesis of the transcriptional regulator *N*-methylphenylalanyl-dehydrobutyrine diketopiperazine (MDD), an A-factor mimic that restores antibiotic biosynthesis and morphogenesis in this strain and *S. griseus* 1439 [6]. The last strain is defective in biosynthesis of A-factor, which belongs to the family of γ-butyrolactones, small signaling molecules that establish communication between the neighboring hyphae of streptomycetes [7, 8].

The corn-soy medium was used for detection of streptomycetes producing the regulators (g/l): corn meal – 20,0, soy meal – 10,0, NaCl – 5,0, pH – 7,2, sterilization at 1,0 atmosphere during 30 min. The investigating streptomycetes were put on the fresh lawn of the cultures 1912-B2 and 1439 in Petri dish in the form of spots with diameter of 1,0 cm and the results were examined after 3-5 days of the cultures cultivation at 28° C as the appearance or absence of red zones of landomycin E or sporulation around the spots. The minimal solid medium was used for production and isolation of the regulators (g/l): asparagine – 1,0, glycine – 1,0, K_2HPO_4 – 0,5, $MgSO_4$ – 0,2, NaCl – 4,0, glycerol – 15,0, trace elements ($FeSO_4$, $CuCl_2$, $MnSO_4$, $CaCl_2$, 10,0 mg each) in 1,0 l distilled water, pH 7,2, with sterilization at 0,75 bar overpressure for 30 min.

Purification, absorption spectra and m/z of the regulators. The minimal solid medium of the grown cultures of *Streptomyces* was cut into cubic pieces and extracted with chloroform-acetone (2 : 1) to obtain the unknown regulators. The crude extracts were evaporated to dryness using a rotary evaporator and the residues were dissolved in ethanol. The chemical compounds were separated by thin layer chromatography using Silica gel 60 F254 aluminum sheets of Merck Co and system of solutions benzene-ethyl acetate-acetone-ethanol (4 : 2 : 1 : 0,5). The individual stripes of the metabolites absorbing the UV light were removed from plates together with silica gel, dissolved in ethanol and the carrier was sedimented by centrifugation at 10000 rot/min. The spectra of absorption of the regulators were obtained by means of spectrophotometer Beckman DU-8. LC/MS was performed using a liquid chromatograph, Agilent Technologies 1200, with a single quadrupole detector, model G19, and a G1978A ion source in ESI mode was used.

The separations were performed on a Zorbax Hypersyl ODS column (12,0 x 125,0 mm^2, 3 μm) with hexane : ethanol (98 : 2) at flow rate of 0,17 ml min^{-1}, and with dual detection at 210 – 400 nm. The chromatograms were analyzed with the Chemstation software.

Results. Forty eight strains of *Streptomyces* isolating from soil samples of different regions of Ukraine were divided in 3 groups on the basis of their biological activity (Table). Thirty strains (62 %) restored antibiotic biosynthesis

Table

Restoration of antibiotic biosynthesis and sporulation in test-mutants *S. globisporus* 1912-B2 and *S. griseus* 1439 by agar culture blocks of streptomycetes

Origin of soil sample (strain code) and agar culture blocks of streptomycetes	*S. globisporus* 1912-B2	*S. griseus* 1439	Both test-strains	No activity
Brovary city, Kyiv region (Bx)	0	0	4	3
Dunayivci village, Chernigiv region (Ch)	0	0	9	0
Zhytomyr city and region (Zr)	0	0	3	0
"Askania – Nova" reserve (AN)	0	0	4	0
"Valley of narcissus", Zakarpats`ka				
Region (DL)	0	1	3	1
Zhytomyr region, store-house of mineral fertilizers (ZH)	0	0	4	2
Kyiv city and region (K)	0	0	1	2
Luhansk region, deposits of copper (M)	0	0	0	2
"Kamyana Mohyla" reserve (MK)	0	0	0	5
Rzhyshchiv, Kaniv city (RK)	0	0	1	1
Trypillya village, Kyiv region (TR)	0	0	1	1
Total	0	1	30	17

and sporulation in the both test-strains *S. globisporus* 1912-B2 and *S. griseus* 1439, seventeen strains (35 %) were not active in this relation and only one strain DL 195 produced unknown metabolite as regulator of streptomycin biosynthesis and morphogenesis in the strain 1439 (Table).

One can suppose that the strains of the first large group may produce different classes of diffusible signaling molecules as the transcriptional regulators like the gamma-butyrolactones, *N*-methyl-phenylalanyl-dehydrobutyrine diketo-piperazine or 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids which demonstrate the similar biological activity in both test-strains [6, 9]. The strains of the second group not produce above mentioned regulators which may be detected by the used test-strains. And only one strain of the third group produced an unknown regulator restoring biosynthesis of streptomycin and sporulation in the test-strain 1439.

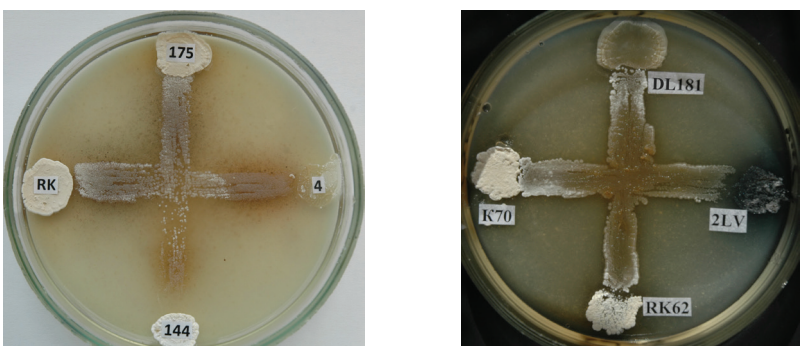

Fig. 1. Restoration of landomycin E biosynthesis (a, dark area)) and sporulation (b, white area) in the cultures of the test-strains *S. globisporus* 1912-B2 (a) and *S. griseus* 1439 (b) by the regulators of streptomycetes (spots on the periphery of the dishes) ZH175, K4, TR144, RK20-62 (a) and DL181, 2LV, RK20-62,K70) (b).

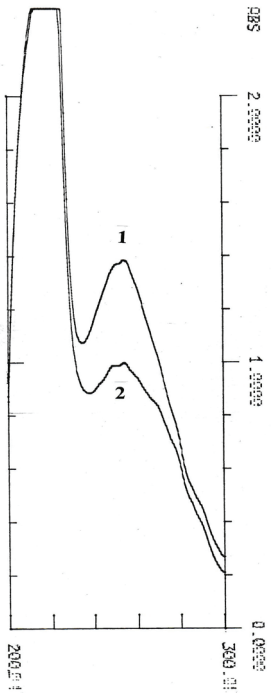

Fig. 2. Maxima ob absorption of the purified regulators dissolved in the ethanol. 1 – regulator of the strain K4, 2 – regulator of the strain TR144.

Restoration of antibiotic biosynthesis and sporulation in the test-strains 1912-B2 and 1339 by the metabolites of the investigating strains is shown on the Fig. 1. Two strains K4 and TR 144 were chosen for the next study of their unknown regulators.

Thin layer chromatography of the both purified compounds revealed the same maxima absorption at 223 and 255 nm (Fig. 2). Molecular mass of these regulators was studied by means of the liquid chromatograph Agilent

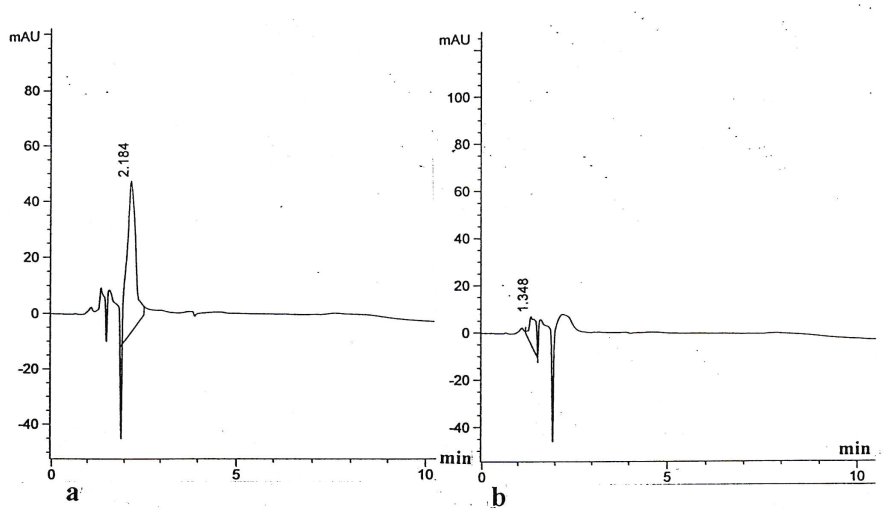

Fig. 3. HPLC of the regulators of the strains K4 (a) and TR144 (b): DAD1; Sig. 220,4; MS/GRAD.

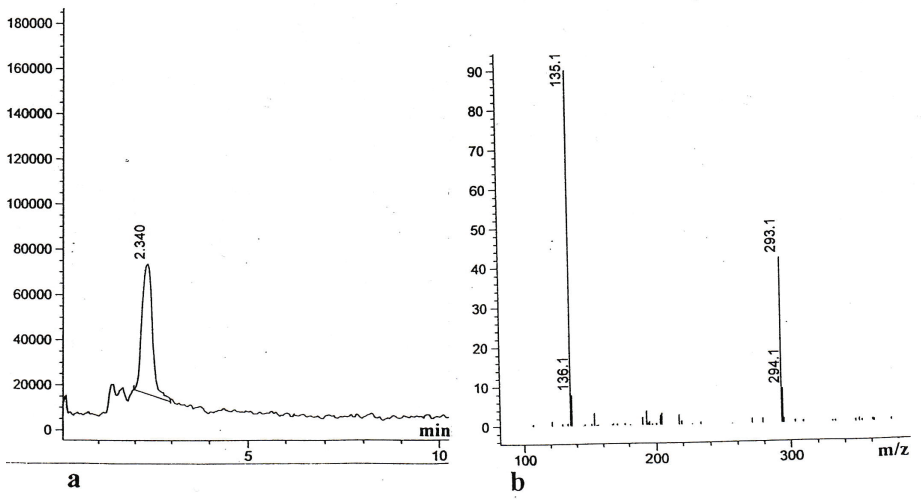

Fig. 4. HPLC/MS of the regulator of the strain K4: a) MSD2. MM-APCI, Scan., Frag.; b) MSD2, time 2,349, MM-APCI, Scan.

Technologies 1200. The retention time (Rt) of both compounds was similar and presented by one peak at 2,184 and 1,348 min, accordingly (Fig. 3).

The molecular mass of the regulators was studied in the different conditions of simultaneous electrospray (ESI) and atmospheric pressure chemical ionization (APCI) using DAD (matrix) or MSD (mass) detectors. MM-APSI scanning showed similar Rt of both regulators (2,34 min) and slightly different

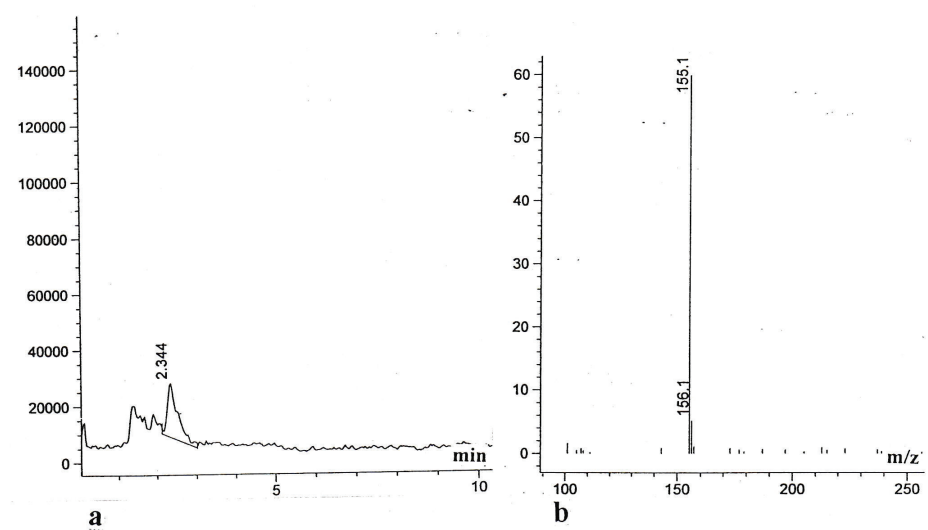

Fig. 5. HPLC/MS of the regulator of the strain TR 144: a) MSD2, MM-APCI, Scan., Frag.; b) MSD2, time 2,349, MM-APCI, Scan.

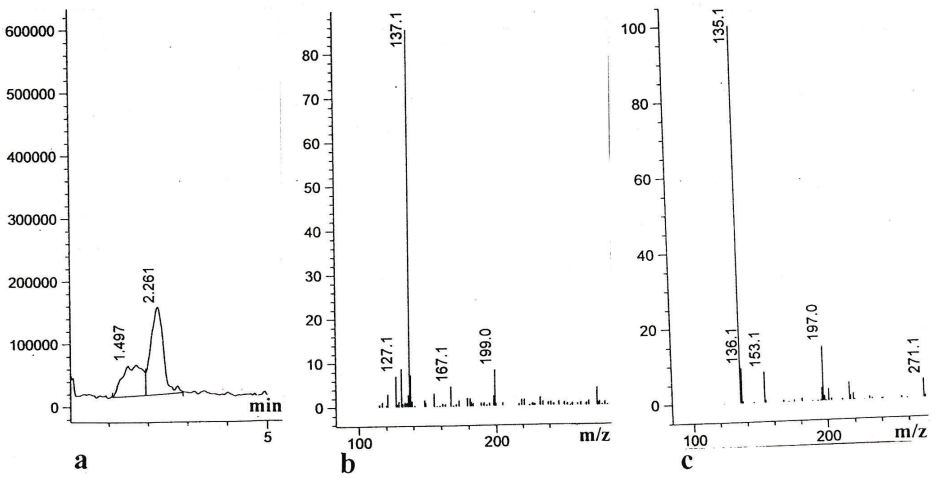

Fig. 6. HPLC/MS of the regulator of the strain K4: a) MSD1, MM-ES, Scan., Frag. 70 "ES-"; b) MSD2, time 1,499, MM-ES, Scan., Frag. 70 "ES-".

m/z (135,1 and 155,1, correspondingly) (Fig. 4, 5), whereas MM-ES scanning revealed the same index of m/z (135,1) of K4 compound (Fig. 6).

So, it is possible to conclude that the both regulators produced by the strains K4 and TR 144 are presented by related chemical compounds with similar molecular mass. Next study of these regulators by means of NMR will give more exact reply on this question.

Discussion. Streptomycetes are the more complicated bacteria producing the substrate and aerial mycelia and spores, and possessing numerous regulation systems in comparison with the representatives of Eubactetria [5].

In this report two unknown and very similar transcriptional regulators were isolated from soil strains K4 and TR144 of *Streptomyces* and some of their properties were characterized. Both regulators like A-factor restored biosynthesis of landomycin E and streptomycin in the defective mutant strains *S. globisporus* 1912-B2 and *S. griseus* 1439, correspondingly and sporulation of cultures.

Early we described the new transcriptional regulator *N*-methylphenylalanyl-dehydrobutyrine diketopiperazine (MDD), an A-factor mimic that restores antibiotic biosythesis and morphogenesis in *Streptomyces* [6].

Comparison of some characteristics of A-factor, MDD and two unknown regulators showed their differance. Maximum spectra of absorption of A-factor, MDD and unknown regulators are 256, 245 and 255 nm, respectively, and their molecular mass (m/z) are 242, 244 and 137, accordingly.

The small molecular mass of the investigating regulators is more similar to the furan derivatives (monoterpenes) which are known as the members furan signaling systems [9]. Only NMR spectroscopy will help us to clarify the molecular structure of the new regulators.

Special attention deserves the unknown regulator of the strain DL195 restoring biosynthesis of streptomycin and sporulation only in the strain 1439. It may present a new regulator investigation of which will be continued in future.

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ПОШУК І ХАРАКТЕРИСТИКА РЕГУЛЯТОРІВ БІОСИНТЕЗУ АНТИБІОТИКІВ У СТРЕПТОМІЦЕТІВ

Р е з ю м е

Метою було дослідження нових регуляторів біосинтезу антибіотиків і споруляції у стрептоміцетів. **Методи** включали тонкошарову хроматографію, спектрофотометрію і визначення маси за допомогою HPLC/MS, використовуючи ESI і APCI методи.

Результати. Сорок вісім штамів стрептоміцетів, ізольованих із ґрунтів різних місцевостей України, перевірені на здатність продукувати регулятори біосинтезу антибіотиків і морфогенезу за допомогою двох тест-систем – мутантних штамів *Streptomyces globisporus* 1912-Б2 і *S. griseus* 1439, які втратили здатність синтезувати антибіотики ландоміцин Е і стрептоміцин відповідно, а також утворювати спори.

Досліджувані стрептоміцети розділені на три групи: 31 штам (62 %) відновлював здатність обох мутантів продукувати антибіотики і спори, 17 штамів (35 %) не проявляли згаданої біологічної активності і лише один штам (2 %) утворював регулятор, який відновлював антибіотичну активність і морфогенез у штаму 1439.

Із агаризованих культур штамів першої групи К4 і ТР144 виділено і очищено за допомогою тонкошарової хроматографії індивідуальні сполуки, які відновлювали біосинтез антибіотиків і морфогенез у тест-штамів 1912-Б2 і 1439. Обидві сполуки характеризуються однаковими показниками Rt (2,34 хв) і максимумами поглинання (255 нм) і мають молекулярні ваги (m/z) 135 і 155 відповідно.

Висновки. Дві споріднені сполуки, які продукуються штамами К4 і ТР144 різних стрептоміцетів і відновлюють біосинтез антибіотиків і споруляцію у тестових мутантних штамів, були очищені і деякі їхні властивості (Rf, максимуми поглинання і m/z) були охарактеризовані. Наступне дослідження виділених регуляторів за допомогою ЯМР дасть можливість встановити їхню молекулярну структуру.

К л ю ч о в і с л о в а: *Streptomyces globisporus* 1912-В2, *S. griseus* 1439, ґрунтові стрептоміцети, транскрипційні регулятори.

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ПОИСК И ХАРАКТЕРИСТИКА РЕГУЛЯТОРОВ БИОСИНТЕЗА АНТИБИОТИКОВ У СТРЕПТОМИЦЕТОВ

Р е з ю м е

Целью работы было изучение новых регуляторов биосинтеза антибиотиков и споруляции у стрептомицетов. **Методы** исследования включали тонкослойную хромато-графию, спектрофотометрию и определение массы с помощью HPLC/MS, используя ESI и APCI методы.

Результаты. Сорок восемь штаммов стрептомицетов, изолированных из почв разных областей Украины, проверены на способность продуцировать регуляторы биосинтеза антибиотиков и морфогенеза с помощью двух тест-систем – мутантных

штаммов *Streptomyces globisporus* 1912-Б2 и *S. griseus* 1439, потерявших способность синтезировать антибиотики ландомицин Е и стрептомицин соответственно, а также образовывать споры.

Исследованные стрептомицеты разделены на три группы: 31 штамм (62 %) восстанавливал способность обоих мутантов продуцировать антибиотики и споры, 17 штаммов (35 %) не проявляли указанной биологической активности и только один штамм (2 %) образовывал регулятор, восстанавливающий антибиотическую активность и морфогенез у штамма 1439.

Из агаризованных культур штаммов первой группы К4 и ТР144 выделены и очищены с помощью тонкослойной хроматографии индивидуальные соединения, восстанавливающие биосинтез антибиотиков и морфогенез у тест-штаммов 1912-Б2 и 1439. Оба вещества характеризуются одинаковыми показателями Rt (2,34 мин) и максимумами поглощения (255 нм) и имеют молекулярные массы (m/z) 135 и 155 соответственно.

Выводи. Два родственных соединения, продуцируемые штаммами К4 и ТР144 разных стрептомицетов и восстанавливающие биосинтез антибиотиков и спорулянцию у тестовых мутантных штаммов, были очищены и некоторые их свойства (Rf, максимумы поглощения и m/z) были охарактеризованы. Последующее исследование выделенных регуляторов с помощью ЯМР даст возможность установить их молекулярную структуру.

К л ю ч е в ы е с л о в а: *Streptomyces globisporus* 1912-B2, *S. griseus* 1439, почвенные стрептомицеты, транскрипционные регуляторы.

1. *Baltz R.H.* Molecular engineering approaches to peptide, polyketide and other antibiotics // Nature Biotechnol. – 2006. – **24**, № 12. – P. 1533–1540.
2. *Hopwood D.A*. Soil to genomics: the *Streptomyces* chromosome // Annu. Rev. Genetics. – 2006. – **40**. – P. 1–23.
3. *Bibb M.J. , Hesketh A. Analyzing the regulation of antibiotic production in* streptomycetes // Methods Enzymol. – 2009. – **458**. – P. 93-116.
4. *Rodriguez H., Rico S., Diaz M., Santamaria R.* Two component systems in *Streptomyces*: key regulators of antibiotic complex pathways // Microbial Cell Factories. – 2013. – **12**, № 1. – P. 1–10.
5. *Romero-Rodriguez A., Robledo-Casados I., Sanchez S.* An overview on transcriptional regulators in Streptomyces // Biochim. Biophys. Acta. – 2015. – **1849**. – P. 1017–1039.
6. *Matselyukh B., Mohammadipanah F., Laatsch H., Rohr J., Efremenkova O., Khilya V. N*-methylphenylalanyl-dehydrobutyrine diketopiperazine, an A-factor mimic that restores antibiotic biosynthesis and morphogenesis in *Streptomyces globisporus* 1912-B2 and *Streptomyces griseus* 1439 // J. Antibiot. – 2015. – **68**. – P. 9–14.
7. *Kleiner E.M., Pliner S.A., Soifer V.S., Onoprienko V.V., Balashova T.A., Ropzynov B.V., Khokhlov A.S.* The structure of A-factor, a bioregulator from *Streptomyces griseus* // Bioorganicheskaya Chimiya (Russian). – 1976. – **2**, № 8. – P. 1142-1147.
8. *Takano E.* γ-Butyrolactones: *Streptomyces* signaling molecules regulating antibiotic production and differentiation // Curr. Opin. Microbiol. – 2006. – **9**. – P. 287-794.
9. *Sidda J.D., Corre C.* Gamma-butyrolactone and furan signaling systems in *Streptomyces* // Methods Enzymol. – 2012. – **517**. – P. 71-87.

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