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PHYSICOCHEMICAL PROPERTIES AND ANTIMICROBIAL ACTIVITY OF THE *STREPTOMYCES* sp. B19 ANTIBIOTIC

Streptomyces represent an important part of soil microbiota and play an important role in the decay of vegetation, the natural defenses of plants and nutrient cycling. These microorganisms synthesize various compounds, including antibiotics that have been successfully used in medicine, veterinary science and agriculture. Furthermore, they have the potential to help overcome the growing problem of antibiotic resistance in microorganisms. The **aim** of the present work was to search for soil streptomyces and to study their ability to synthesize antibiotics against pathogenic microorganisms, as well as the physicochemical properties of the antibiotics. **Methods.** Suspensions of soil were sowed on Čapek medium with nystatin in Petri dishes in order to identify individual colonies of streptomyces and reseed them on soybean-corn medium. The most active antibiotic producers among the isolated streptomyces were identified by their diffusion into agar medium S seeded with appropriate bacterial test cultures. The antibiotic was extracted from the streptomyces culture in two ways: with water and using a chloroform-acetone mixture (2:1), followed by evaporation of the mixture in a rotary evaporator at 80 °C and dissolution of the dry extract of the antibiotic in 40% ethanol. The pure antibiotic was isolated by thin-layer chromatography (TLC) of both organic and aqueous extracts, and its absorption spectrum was obtained by spectrophotometry. The molecular weight of the purified antibiotic was determined using a high-performance liquid chromatography with mass spectrometry detection (HPLC-MS) system Agilent 1260 Infinity II. **Results.** 33 strains of soil streptomyces were isolated, of which 17 strains (51.5%) showed antibacterial activity against 4 pathogenic bacteria — *Staphylococcus aureus* and three phytopathogens. Only 4 strains of streptomyces B8, B19, B2, and B28 showed high antibiotic activity, the diameters of the zones of absence of bacterial growth were above 20 mm. From the most active culture *Streptomyces* sp. B19, two antibiotics were extracted and purified with TLC. The absorption spectrum of the extracted antibiotics between 200 and 500 nm was determined spectrophotometrically. We found that an antibiotic with mass spectrum mass of 1077 remains on the starting line during organic phase chromatography of

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positively charged ions of an alkaline extract of the streptomycete culture. For the aqueous phase two compound with mass spectra of 585.5/773.5 and 1194/1301 are isolated. **Conclusions.** The presence of peptide bonds in the structure and the absorption spectrum of the antibiotic with a molecular mass of 1077 indicate its classification among the new glycopeptide antibiotics.

Keywords: soil streptomycetes, antibiotic activity, bacteria, thin layer chromatography, absorption spectra, mass spectra of antibiotics.

Soil remains an inexhaustible source of various microorganisms, among which the genus *Streptomyces* occupies one of the most important places in terms of its quantity and maintenance of soil fertility (Boone et al., 2001). In addition, streptomycetes are the producers of most of the known antibiotics used in medicine, veterinary medicine, and agriculture (Hopwood, 2007; Chater, 2016). The search for streptomycetes producing new antibiotics is an important problem of biological and medical science due to the spread of antibiotic-resistant microorganisms (Odoncor & Addo, 2011).

Materials and Methods. Soil samples were taken from the greenhouse of the D.K. Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine and dissolved in sterile distilled water in a proportion of 1.0: 10.0. The soil suspension was inoculated with a loop on Chapek agar in Petri dishes containing nystatin to inhibit fungal growth. The dishes were incubated in a thermostat at 28 °C for one week, and the grown colonies of streptomycetes were sown on a slanted soybean-corn medium (g/L): corn meal — 20.0, soy meal — 10.0, NaCl — 5.0, agar — 15.0, distilled water — 1.0 L; sterilization at 121 °C for 30.0 min for further studies.

The antibiotic activity of the isolated streptomycetes was tested by applying agar disks from lawns of 5-day cultures to the surface of meat-peptone agar inoculated with test cultures of bacteria. Gram-positive and Gram-negative bacteria were obtained from the Ukrainian Collection of Microorganisms UCM (2014). The antibiotic was extracted from the most active culture of *Streptomyces* sp. B19 using ethyl ace-

tate and filter paper. The lawns of the 5-day culture of *Streptomyces* on soybean-corn medium were cut into 1.0 × 1.0 × 1.0 cm cubes, covered with ethyl acetate in a flask and extracted for two days. The resulting antibiotic extract in ethyl acetate was dried in a rotary vacuum evaporator and dissolved in 40% ethanol.

In addition, an aqueous solution of the antibiotic was obtained by extracting the latter from the streptomycete culture on solid soybean-corn medium through soaking filter paper. Disks of filter paper were placed below the cultured medium to absorb the liquid from the culture. Once moistened, they were dried, and the operation was repeated until the culture no longer moistened the filter paper. The antibiotic was extracted from the filter paper with sterile distilled water. The antibiotic activity of the extracts was determined by adding 120 µL of the latter to the wells of medium S (g/L): peptone — 4.0, yeast extract — 4.0, K₂HPO₄ — 0.5, MgSO₄ — 0.25, agar — 15.0, distilled water — 1.0 L) in Petri dishes inoculated with the corresponding test cultures.

The purified antibiotic was obtained by thin-layer chromatography (TLC) using 20.0 × 20.0 aluminum plates from Merck KGaA (Silica gel 60 F254). The mobile phase was benzene-ethyl acetate-acetone-ethanol (4:2:1:0.5) for the organic extract and 1% ammonium bicarbonate and methanol (9:1) for the aqueous extract. Separate bands of substances, clearly visible in UV light, were scraped off the plates together with silica gel, dissolved in 40 % ethanol, and added to the wells of agar medium S inoculated with the corresponding test cultures. Ethanol solutions of the purified antibiotic were used to obtain the

absorption spectrum using a Specord 210+ spectrophotometer (Analytical Jena, USA). The presence of peptide bonds in the antibiotic structure was investigated by a specific biuret reaction (Anal Biochem, 2021).

The molecular mass of the purified antibiotic was determined using a high-performance liq-

Table 1. Antibacterial activity of streptomycetes

Streptomycetes	<i>S. aureus</i> B-904	<i>P. syringae</i> 8511	<i>C. michiganensis</i> 10	<i>X. campestris</i> 8003
B1	0	0	0	0
B2	14.0 *	22.0	24.0	26.0
B3	15.0	26.0	13.0	20.0
B4	0	0	0	0
B5	0	0	0	0
B6	12.0	10.0	13.0	14.0
B7	0	0	0	0
B8	26.0	26.0	24.0	23.0
B9	16.0	14.0	18.0	20.0
B10	0	0	0	0
B11	0	0	0	0
B12	14.0	20.0	22.0	24.0
B13	19.0	12.0	20.0	22.0
B14	0	0	0	0
B15	20.0	18.0	22.0	24.0
B16	0	0	0	0
B17	16.0	12.0	14.0	16.0
B18	0	0	0	0
B19	32.0	30.0	34.0	30.0
B20	0	0	0	0
B21	25.0	22.0	26.0	22.0
B22	0	0	0	0
B23	0	0	0	0
B24	26.0	20.0	20.0	23.0
B25	12.0	18.0	20.0	27.0
B26	0	0	0	0
B27	0	0	0	0
B28	24.0	25.0	25.0	24.0
B29	0	0	0	0
B30	0	0	0	0
B31	25.0	0	25.0	23.0
B32	0	0	0	0
B33	24.0	0	22.0	24.0

* Diameter of no-growth zones in test cultures, mm

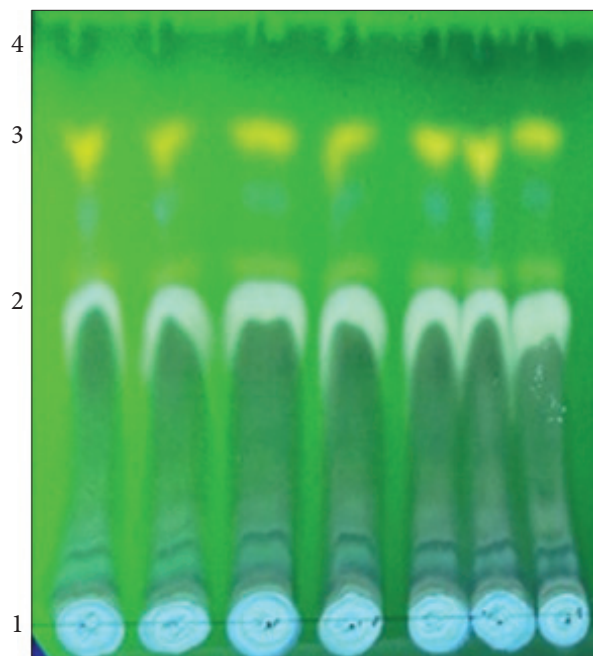


Fig. 1. Thin-layer chromatography of the ethyl acetate extract of streptomycete B19 culture

uid chromatography with mass spectrometry detection (HPLC-MS) system Agilent 1260 Infinity II (Jurgen, 2017). For this, the antibiotic was extracted from the gel powder on the TLC with an alkaline aqueous-alcoholic solution of 50% isopropanol. Mass spectra were recorded by electrospray ionization to m/z 1450 for both positive and negative ions (the mobile phase was sensitized with ammonium formate). The sample was injected directly into the ion source, without a chromatographic column.

Results. Out of 33 streptomycetes freshly isolated from soil samples, only strain *Streptomyces* sp. B19 formed test microorganism's no-growth zones with a diameter of 30–34 mm around the blocks of culture of streptomycetes (Table 1).

For further studies, we selected strain B19, which showed the greatest antagonistic activity against gram-positive and gram-negative microorganisms.

The unknown antibiotic of strain B19 was detected and separated from the other com-

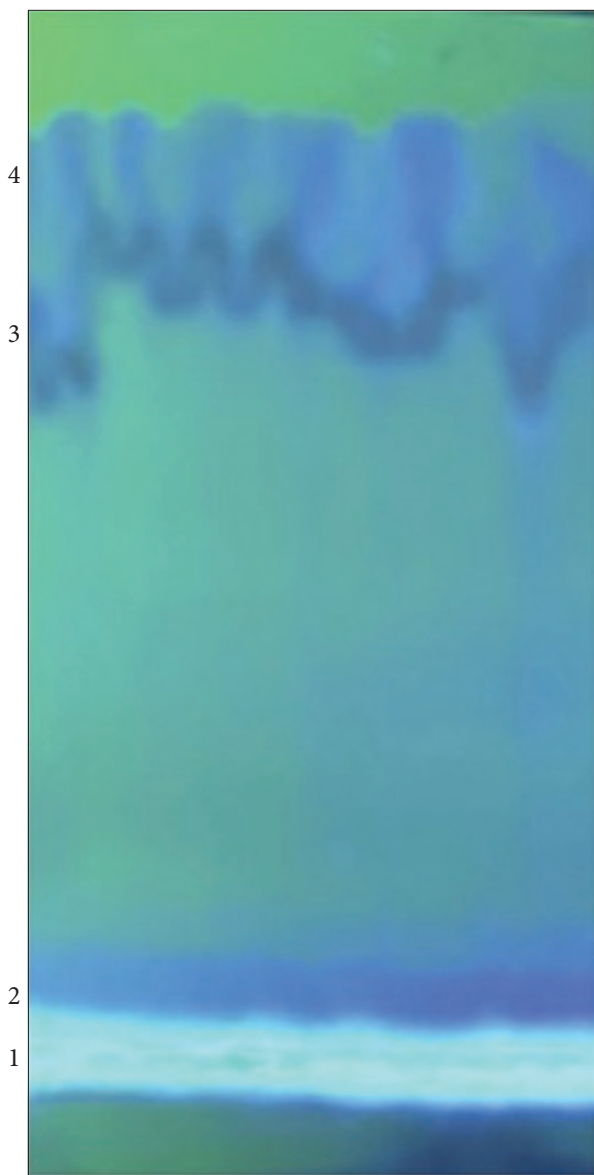


Fig. 2. Thin-layer chromatography of the aqueous extract of streptomycete B19 culture

pounds by thin-layer chromatography of streptomycete culture extracts on Merck aluminum plates. Fig. 1 shows the TLC of the organic ethyl acetate extract of strain B19 under UV light, where the individual bands of metabolites are marked with numbers.

The bands were scraped off the aluminum plates together with the gel, dissolved in 40 %

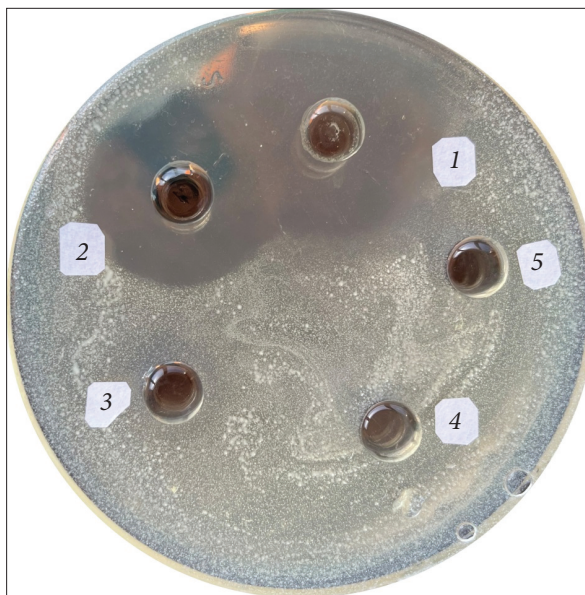


Fig. 3. Zones of no-growth of *Staphylococcus aureus* around wells 1 and 2 with antibiotic solutions from TLC bands 1 and 2 in Fig. 2

ethanol, and added in 120.0 μL to the wells of the medium in Petri dishes inoculated with the corresponding test bacterial cultures. The antibiotic activity of individual metabolites was tested after incubation of the plates in a thermostat. Of 4 bands observed on the TLC under UV light (Fig. 2), only the compound of band 1 on the starting line caused a delay in the growth of *Staphylococcus aureus*. A thin-layer chromatogram of the aqueous extract of the strain B19 culture with the mobile phase of a 1.0 % aqueous solution of ammonium bicarbonate and methanol (9:1) is shown in Fig. 2.

Of 4 bands, only bands 1 and 2 showed antibiotic activity against *Staphylococcus aureus* (Fig. 3). It can be assumed that the culture of strain B19 synthesizes two related and high-molecular-mass compounds that exhibit antibiotic activity. The absorption spectrum of compound 1 dissolved in 70 % ethanol was recorded using a spectrophotometer.

Fig. 4 shows the UV light absorption curve of the unknown antibiotic in the range of 200–500 nm.

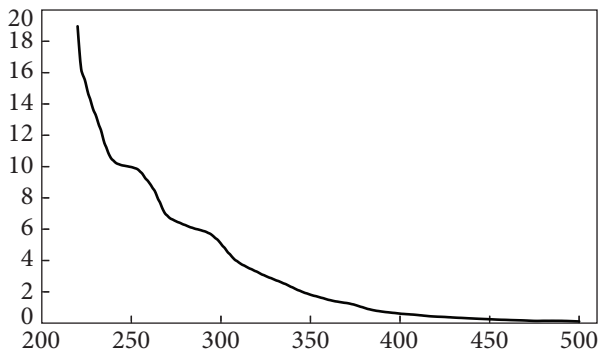


Fig. 4. Absorption spectrum of the antibiotic of streptomycete B 19 (area 1 of TLC, Fig. 2) axes

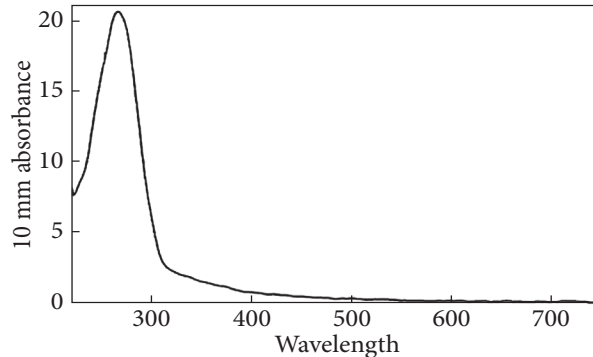


Fig. 5. Absorption spectrum of the antibiotic of streptomycete B 19 (area 2 of TLC, Fig. 2)

Microbiology sample 1 basic (no column) | MS1 + Scan ESI (rt: 0.095 min) Frag 90 V Gain = 1.0 | microbiology s

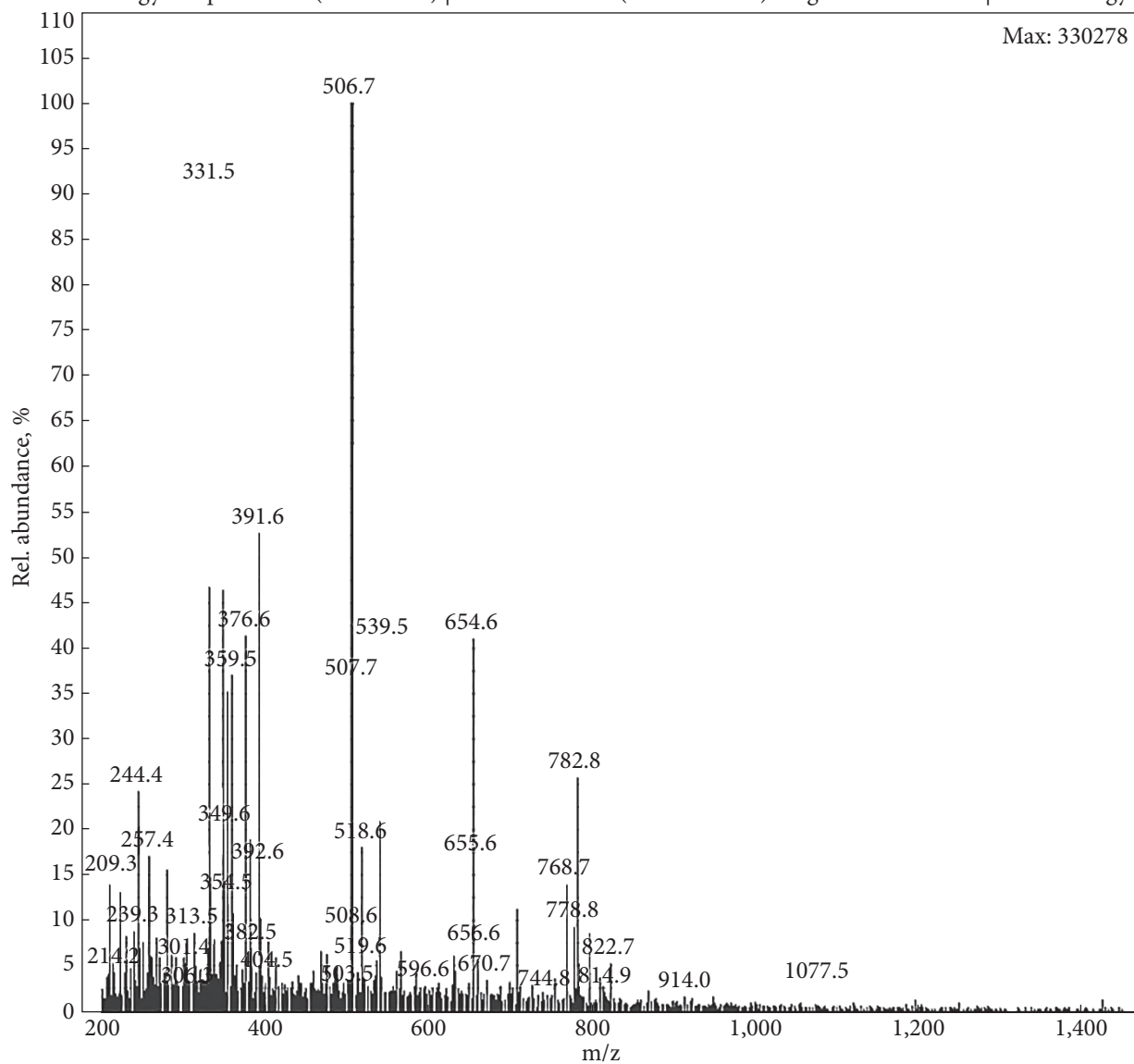


Fig. 6. Electrospray ionization mass spectra of positively charged ions of alkaline extract of TLC band 1 (Fig. 2)

The UV light absorption spectrum of compound 2, measured in the same way, differs from that of compound 1 (Fig. 5).

The presence of peptides in the structure of the studied antibiotic was detected by a specific biuret reaction. The addition of 2 mL of antibiotic solution to 8 mL of blue biuret reagent caused the mixture to turn purple due to the formation of a copper sulfate chelate complex after 30 minutes of observation. Thus, the biuret reaction indicates the presence of peptide bonds in the structure of the antibiotic under study.

To determine the mass spectrum of antibiotic of the thin-layer chromatogram (Fig. 2), band 1 was dissolved in three water-alcohol extracts: 50% isopropanol and 50% water (neutral extract), the same with a drop of hydrochloric acid (acidic extract), and the same with a drop of ammonia (alkaline extract). Mass spectra were recorded in the electrospray ionization mode up to m/Z 1450 of both positive and negative ions (the mobile phase into which the sample was introduced was sensitized with ammonium formate). The sample input method was direct, without a chromatographic column (the entire sample enters the ion source).

It was found that the antibiotics in both bands were only extracted in an alkaline medium.

Fig. 6 shows an overview of the mass spectrum of positively charged ions of the alkaline extract of antibiotic remaining on the starting line of TLC (band 1).

The largest masses, which presumably correspond to a single-charged ion, are 1075.9 and 1077.5. These may be adducts with proton or ammonium. The ion present in the mass spectrum with a mass of 539.5 (exactly half) probably corresponds to a two-charged ion, i.e., a molec-

ular mass of 1077; the $[M+H]^+$ ion has a m/z of 1078, and the $[M+2H]^{2+}$ ion has a m/z of 539.5. The other ions may correspond to more complex adducts ($[2M+3H]^{n+}$, etc.) or other substances.

In the case of the mass spectra of the aqueous extracts, samples 1 and 2 are similar. Unlike sample 1, sample 2 contains almost no high masses. In the alkaline extract of sample 2, there are two different compounds with masses of 585.5/773.5 and 1194/1301.

Discussion. In this study, two antibiotics produced by the *S. sp.* B19 culture were identified using TLC. The first antibiotic (sample 1, Fig. 2) has a retention factor (R_f) of zero and therefore remains at the starting line, while the second antibiotic (sample 2) only separates from the first by a small distance and stays close by. This indicates a large mass of antibiotics, which was confirmed by high-performance liquid chromatography with mass spectrometry detection (HPLC-MS). The first antibiotic has a mass of 1077, while the second one — significantly lower mass.

The specific biuret reaction revealed the presence of peptide bonds in the structure of the first antibiotic, allowing it to be classified among such peptide antibiotics as bacitracin, polymyxin B, nisin, gramicidin, and others. However, the absorption spectrum of the antibiotic turned out to be most similar to the spectrum of the complex tricyclic glycopeptide vancomycin, used to treat gram-positive bacterial infections caused by methicillin-resistant *S. aureus* (Hadwiger et al., 2012). Therefore, it can be assumed that the studied antibiotic, based on the absorption spectrum and large mass, belongs to the group of glycopeptide antibiotics and may have a new structure that can be investigated using NMR methods.

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Стрептоміцети становлять важливу частину мікробіоти ґрунту, яка відіграє важливу роль в утилізації рослинних залишків, захисті рослин і кругообігу речовин. Ці мікроорганізми синтезують багато різних сполук, включаючи антибіотики, які успішно використовуються в медицині, ветеринарії та сільському господарстві. Вони можуть використовуватися для успішного подолання проблеми резистентності мікроорганізмів до антибіотиків. **Метою** даної роботи були пошук ґрунтових стрептоміцетів і дослідження їх здатності синтезувати антибіотики проти патогенних мікроорганізмів, а також з'ясування фізико-хімічних властивостей останніх. **Методи**. Висіви суспензії ґрунту на середовище Чапека з ністатином в чашках Петрі з метою ідентифікації окремих колоній стрептоміцетів і пересіви останніх на соєво-кукурудзяне середовище. Ідентифікація найбільш активних продуцентів антибіотиків серед ізольованих стрептоміцетів відбувалася шляхом їх дифузії в агаризоване середовище S, засіяне відповідними тест-культурами бактерій. Екстракція антибіотиків із культур стрептоміцетів сумішшю хлороформу і ацетону (2 : 1) відбувалася випаровуванням суміші в роторному випарникові в умовах вакууму і нагрівання до 80°С з подальшим розчиненням сухого екстракту антибіотика в 40% етанолі. Чистий антибіотик виділяли за допомогою тонкошарової хроматографії (ТШХ) і одержання спектрів абсорбції спектрофотометрією. Його молекулярну масу визначали високоефективною рідинною хроматографією з виявленням маси спектрометрією за допомогою системи Agilent 1260 Infinity II. **Результати**. Виділено 33 штами стрептоміцетів, із яких 17 штамів (51.5 %) виявили антибактеріальну активність проти чотирьох патогенних бактерій — *Staphylococcus aureus* і трьох фітопатогенів. Тільки 4 штами стрептоміцетів B8, B19, B21 і B28 мали високу антибіотичну активність: діаметр зони відсутності росту бактерій становив більше 20 мм. Із найбільш активної культури *Streptomyces* sp. B19 екстраговано і очищено за допомогою ТШХ три антибіотики, спектри абсорбції яких визначено спектрофотометрією від 200 до 500 нм. Антибіотик із мас-спектром 1077 залишався на лінії старту впродовж хроматографії позитивно заряджених іонів лужного екстракту культури стрептоміцета, а два антибіотики з мас-спектрами 585.5/773.5 і 1194/1301, можливо, представляють дві інші сполуки. **Висновки**. Запропоновано гіпотезу про можливе віднесення досліджуваного антибіотика до групи глікопептидних антибіотиків з новою структурою.

Ключові слова: ґрунтові стрептоміцети, антибіотична активність, бактерії, тонкошарова хроматографія, спектри абсорбції, мас-спектри антибіотиків.