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PRODUCERS, BIOLOGY, SELECTION, AND GENE ENGINEERING

Isolation and Characterization of Toxic Cyanobacteria from Different Natural Sources

K. Bolatkhan[a, *], N. R. Akmukhanova[a], B. K. Zayadan[a, *], A. K. Sadvakasova[a], M. A. Sinetova[b], and D. A. Los[b]

[a] *Al-Farabi Kazakh National University, Almaty, 480078 Kazakhstan*
[b] *Timiryazev Institute for Plant Physiology, Russian Academy of Sciences, Moscow, 127276 Russia*
**e-mail: zbolatkhan@mail.ru*

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Abstract—We isolated seven algologically and five bacteriologically pure cultures of toxin-producing cyanobacteria from Turgen gorge (Kazakhstan), Karlovy Vary (Czech Republic), and Shar-Nuur Lake, Bayan Ulgiiregion (Mongolia) springs. According to the *Daphnia magna* test, *Desertifilum* sp. and *Nostoc* sp. strains were the most toxic in the test of isolated strains (complete death of all test organisms was detected after 48 h). These strains possessed the highest inhibitory effect on proliferation of the *HeLa* cancer cell line. The *Anabaena* sp. 35 and *Nostoc* sp. 4 strains were also high toxic. Model strains *Synechocystis* PCC 6803 and *Synechococcus elongates* PCC 7942, as well as the strain isolated in the present work, *Synechococcus* sp. 55, were less toxic. Mass spectrometry made it possible to assign cyanobacterial toxins to cyclic depsipeptides. Two cyclic depsipeptides, micropeptin T and oscillapeptin, were detected in *Desertifilum* sp. extracts. Cryptophycin and small amounts of cyclic depsipeptide micropeptin SD were detected in *Nostoc* sp. extract.

Keywords: biotoxins, cyanobacteria, cytotoxins, toxins
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INTRODUCTION

Cyanobacteria are model organisms used to study oxygenic photosynthesis, atmospheric nitrogen fixation, cell division, hydrogen production, and a number of fundamentally scientific and applied problems. These organisms are easily cultivated and are characterized by a high growth rate and metabolic plasticity [1, 2]. Cyanobacteria are able to utilize natural aromatic hydrocarbons and xenobiotics [3]. Cyanobacterial communities are used to remove oil contamination from water and soil, since they are able to utilize crude oil and alkanes as an energy source. Cyanobacteria also efficiently adsorb metals from water. Polysaccharides of cell envelopes of *Microcystis aeruginosa* and *Aphanothece haophytica* effectively adsorb heavy metals ions including copper, lead, and zinc [5].

To adapt to extreme conditions, cyanobacteria began to produce various secondary metabolites including cytotoxins and biotoxins. Cytotoxins affect separate cell functions, e.g., they inhibit some enzymes. Water contamination with cyanobacterial cytotoxins can lead to mass poisoning of people and animals associated with liver and kidney damages. Some cytotoxins kill bacterial and algal cells [6]. Cytotoxin activity is studied in mammalian cell cultures, including cancer cell cultures. Biotoxins are divided into two groups: hepatotoxic cyclic peptides, which cause the death of laboratory animals (mice) in 1–4 h, and neurotoxic alkaloids, which cause death in 2–30 min [7].

Although cyanobacterial toxins are extremely dangerous for people and animals, some of them can be used as components of medicines. For example, *Microcystis* strains produce eruginosins with unique amino acid residues (2-carboxy-6-hydroxy- Octahydro-indole) that inhibit serine proteases. Tricyclic depsipeptides from toxic strains of *Microcystis viridis* and *Lyngbia* sp. inhibit tyrosine kinase and elastase [8].

According to their chemical structure, cyanobacterial toxins are divided into three groups: peptides (cyclic and linear), alkaloids, and lipopolysaccharides [1]. The first and second groups are secondary metabolites, i.e. they are not involved in central metabolism. Representatives of the third group are structural components of the cell walls. In addition to neurotoxicity, toxins can possess immunotoxicity, embryotoxicity, and dermatotoxicity, as well as mutagenic and carcinogenic properties [9, 10].

The diversity of cyanobacteria in different habitats ensures the discovery of cyanobacterial secondary metabolites that can be used as novel medicines.

Abbreviations: HPLC—high-performance liquid chromatography, MSI—ionized molecules, *m/z*—mass-charge, DA—ultraviolet polychromatic detector.

The goal of the present work was to select cyanobacterial strains isolated from different habitats by the content of potentially toxic compounds and to isolate and characterize the toxins.

MATERIAL AND METHODS

Samples of water and cyanobacterial mats were collected during the summer season from the following springs: a hot spring in Turgen gorge (Almaty Region, Kazakhstan) with a water temperature of 55°C and a pH of 5.0; a hot spring in Karlovy Vary (Czech Republic) with a water temperature of 50°C and a pH of 6.5; and Shar-Nuur Lake (Bayan Ulgii region (Mongolia) with a water temperature of 35°C and a pH of 6.0. In total, 12 algological samples were collected. Cyanobacteria were identified with the "Manual for Systematization of Blue-Green Algae in USSR" [11]. Standard microbiological methods were used to isolate algologically pure cultures. Bacteria and fungi were removed from the natural associations with cyanobacteria by means of treatment on agarized medium with an antibiotic mixture containing gentamicin, penicillin, tetracycline, and nystatin in final concentrations of 5 to 50 μg/mL. The following mineral nutrient media were used: 04, Gromov medium no 6, Tamiya medium, Zarukk medium, and BG-11 [12].

Toxicity was evaluated using cyanobacterial biomass grown in liquid medium under optimal conditions and lyophilized with Telstar LyoQuest benchtop freeze dryer (Terrace, Spain). Cyanobacterial toxicity was studied in short (acute) *Daphnia magna* Straus tests. Mature, 3-day-old, medium-sized females without parthenogenetic embryos were used for the tests. The experiments were performed according to the following scheme: 20 mL of media containing 10.0, 1.0, and 0.1 mg/mL of lyophilized cyanobacterial biomass were placed in 100-mL flasks. The medium without biomass was used as the control [13].

Cytotoxicity was studied in the Laboratory of Biotechnology and Algae of the Institute of Microbiology (Trebon, Czech Republic). The sample of lyophilized cyanobacterial biomass (200 mg) was ground in a mortar for 2–3 min with the gradual addition of 6 mL of 70% methanol. The obtained mixture was incubated for 1 min at 22–24°C and centrifuged at 4000 *g* for 10 min. Supernatant was transferred to a flask and stored in the fridge until the start of the experiment. The M *HeLa* cell line was used to determine the cytotoxicity of cyanobacterial extracts. We cultivated cancer cells according to the common method [14]. Cell cultures with a cell density of 50000 cells/mL were placed into 96-well plates (100 μL in each well). Culture medium was removed after 24 of the incubation and replaced with medium containing different concentrations of methanol cyanobacterial extracts. Cells were incubated in the presence of extracts for 72 h; 70% methanol without extracts was used as a control.

Cyanobacterial extracts were fractioned and analyzed with an Agilent HP 1100 LCMC high-performance liquid chromatograph equipped with a mass-spectrometer (Agilent Technologies, Hewlett Packard, United States) [15]. Cyclic peptides were separated with a Zorbax XDB-C18 analytical column (4.6 × 150 mm, Agilent Technologies, United States). A methanol/water mixture was used as the mobile phase (30-min linear gradient from 30 to 100% methanol). The flow rate was 0.6. mL/min at 30°C. The volume of the extract sample was 20 μL. Peaks were registered with two sensors: an "ion-trap" mass-spectrometer and an ultraviolet polychromatic detector (PDA). Cyclic peptides were detected with a chromatograph at 230 nm (residence time 10–25 min). The mass to charge ratios (m/z) of ionized molecules (MSI) were determined by tandem mass spectrometry. The toxins were identified by means of comparison of the molecular mass (m/z) of the compounds and the output time of corresponded cyclic peptides.

RESULTS AND DISCUSSION

Isolation of Algologically and Bacteriologically Pure Cyanobacterial Cultures

The maximal number of cyanobacterial species (16) was found in the hot spring in Turgen gorge, which can be explained by the wide range of water temperatures (28–45°C) and weak stream. At 40°C the microbial mats were bright emerald and representatives of the genera *Synechocystis*, *Synechococcus*, *Gloeocapsa*, *Anabaena*, and *Phormidium* were detected.

Twelve cyanobacteria were isolated from the hot spring in Karlovy Vary. They were represented mainly by the species of the orders *Chroococcales*, *Nostocales*, and *Oscillatoriales* (which was predominant).

Ten cyanobacteria were found in Shar-Nuur Lake. Cyanobacteria of the order *Oscillatoriales* were predominant, whereas representatives of the genera *Nostoc* and *Anabaena* were detected more rarely.

Then, seven enrichment cultures were established from the samples of these three ecosystems, and algologically pure cultures were isolated by serial passages (Table 1).

At the next stage, algologically pure cultures were purified to remove bacterial satellites, since all cultures contained corresponding microbiota. The satellite microbiota of the isolated cyanobacteria was mainly represented by gram-negative and gram-positive bacteria, as well as by yeasts and molds. The purification of isolated cultures is a complicated, time-consuming process, since cyanobacteria and bacteria form close ecological relationships [16]. Slime envelopes of cyanobacteria are the nutrient source and habitat for other microorganisms.

To purify the cultures, a mixture of broad-spectrum antibiotics (inhibiting gram-negative and gram-positive bacteria, as well as fungi) were used, since the

Table 1. Isolated, algologically and bacteriologically pure cyanobacterial cultures

Culture designation	Genus	Territory of isolation	Isolation of acteriologically pure cultures
SP-35	*Anabaena*	Mongolia	+
SP-55	*Synechococcus*	Turgenskii spring	+
SP-4	*Nostoc*	Turgenskii spring	+
SP-45	*Nostoc*	Karlovy Vary	+
SP-D	*Desertifilum*	Mongolia	+
SP-3	*Merismopedia*	Karlovy Vary	–
SP-6	*Spirulina*	Karlovy Vary	–

Table 2. Lethality (%) of *Daphnia magna* test-culture in the presence of different concentrations of lyophilized cyanobacterial biomass

Culture	Duration of testing, h											
	1			6			24			48		
	Concentration of lyophilized cyanobacteria cells, mg/mL											
	0.1	1	10	0.1	1	10	0.1	1	10	0.1	1	10
Se-7942*	0	0	0	0	0	6	0	2	9	0	2	12
S-6803*	0	0	0	0	2	4	0	2	5	0	4	11
SP-55	0	0	0	0	0	3	0	1	10	0	4	16
SP-4	8	20	31	12	25	50	17	54	69	20	71	84
SP-35	9	15	27	10	21	45	14	47	65	14	54	80
SP-45	10	27	32	14	30	56	28	82	94	47	95	100
SP-D	14	30	39	17	36	67	32	83	97	45	98	100

* Se-7942 is the *Synechococcus elongatus* PCC 7942 control strain; S-6803 is the *Synechcystis* sp. PCC 6803 control strain.

satellite microorganisms differed in theirs reaction to pure antibiotics. Fungicidal antibiotic nystatin was used in all variants as the antifungal agent. Antibiotic treatment allow the purification of five axenic cultures, whereas two cultures (SP-3 and SP-6) were not purified of the satellite bacteria. An ithe ncrease in antibiotic content inhibited not only bacteria but also cyanobacterial cultures.

The obtained axenic cultures belonged to the following genera: SP-35—*Anabaena*, SP-4 and SP-45—*Nostoc*, SP-55—*Synechococcus*, SP-6—*Spirulina*, SP-3—*Merismopedia*, and *SP-D—Desertifilum* (Table 1).

Study of the Toxicity of Isolated Cyanobacterial Strains with the Daphnia Magna Test-System

Biotesting with daphnia is widely used to evaluate the quality of natural waters [12]. Death of 50% of the crustaceans in comparison to the control after 24 and 48 h of incubation indicates acute toxicity. The death of crustaceans was evaluated by immobility (immobilization). The daphnia fell to the bottom of the glass; swimming movements were absent and did not recommence after a weak touch by the water jet and swinging of the glass.

D. magna death was low during the first hours of incubation in the presence of the studied concentration of lyophilized biomass of all cyanobacteria with the exception of the SP-D (*Desertifilum* sp.) and SP-45 (*Nostoc* sp.) cultures. In the presence of 1–10 mg/mL of biomass, the movements of the crustaceans significantly changed, which can be explained by the behavioral reaction to the toxicants. In experiments with SP-D (*Desertifilum* sp.) and SP-45 (*Nostoc* sp.) cultures, mortality in the presence of 1 mg/mL of biomass comprised 82–83% after 24 h. An increase in the cyanobacterial biomass concentration up to 10 mg/mL led to 100% mortality of the daphnia. In experiments with control cultures Se-7942 (*Synechocystis* PCC 6803) and S-6803 (*Synechococcus elongates* PCC 7942), as well as with the SP-55 (*Synechococcus* sp.) isolated in the present work, changes in the crustacean survivability were insignificant after 1 and 6 h. The death of 11–16% of the daphnia was detected after 48 h in the presence of 10 mg/mL of the biomass (Table 2).

According to the 4-point Stroganov scale [18] cyanobacterial cultures were arranged in the following order:

4 points—highly toxic strains SP-D (*Desertifilum* sp.) and SP-45 (*Nostoc* sp.); their extracts caused complete death of the test organisms in 48 h;

3 points—toxic strains SP-35 (*Anabaena* sp.) and SP-4 (*Nostoc* sp.); their extracts caused death of 80–84% of the test organisms in 48 h;

1 point—slightly toxic strains Se-7942 (*Synechocystis* PCC 6803), S-6803 (*Synechococcus elongates* PCC 7942),and SP-55 (*Synechococcus* sp.); their

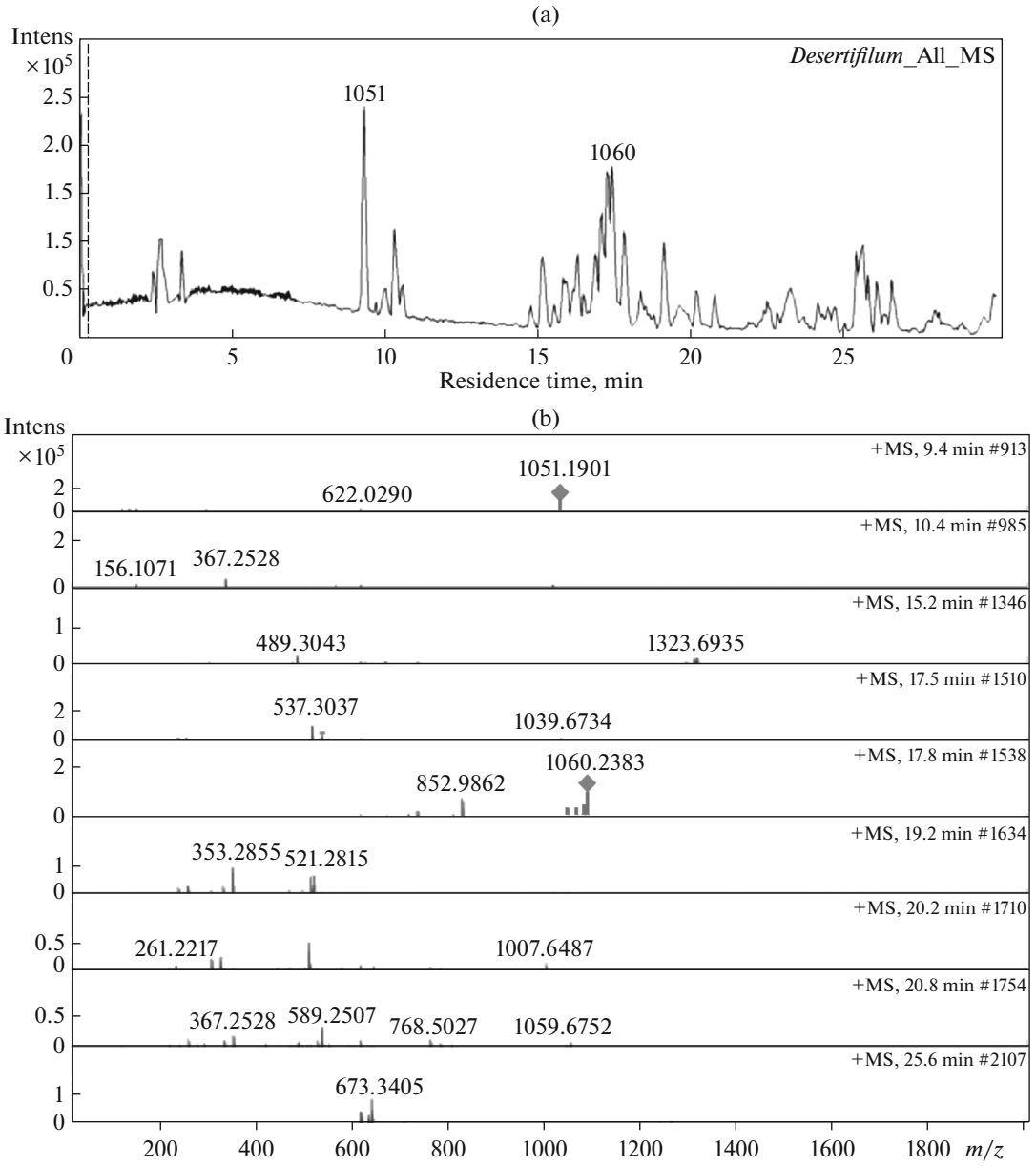

Fig. 1. HPLC of extracts of lyophilized SP-D (*Desertifilum* sp.) biomass (a) and their fragmentation in liquid chromatograph–mass-spectrograph (b). Figures indicate the *m/z* of the toxins: 1051—micropeptin T; 1060—oscillapeptin.

extracts caused death of 11—16% of the test organisms in 48 h.

Thus, SP-D (*Desertifilum* sp.) and SP-45 (*Nostoc* sp.) were the most toxic of the isolated strains for the test-organism *D. magna*, which is very sensitive to cyanotoxins [19, 20].

Study of the Toxicity of the Isolated Cyanobacterial Strains with HeLa Cancer Cell Test-System

We also evaluated the toxicity of the methanol extracts from cyanobacteria with the use of the *HeLa* cancer cell line (Table 3). The cytotoxicity of extracts from the cells of the SP-35 (*Anabaena* sp.) and SP-4 (*Nostoc* sp.) strains did not exceed 50% (Table 3). The maximum effect (43–45% dead cells) was detected in the presence of 5 μg/mL of the extracts from these strains.

Table 3. Cytotoxicity (%) of extracts from lyophilized cyanobacterial biomass to the *HeLa* tumor cell line

Culture	Concentration of lyophilized cyanobacterial cells extracts, μg/mL						
	0.05	0.1	0.2	0.5	1	2	5
SP-45	68	75	76	76	80	82	92
SP-35	35	37	38	39	42	42	45
SP-4	32	36	36	40	40	41	43
SP-D	82	83	89	89	89	90	97

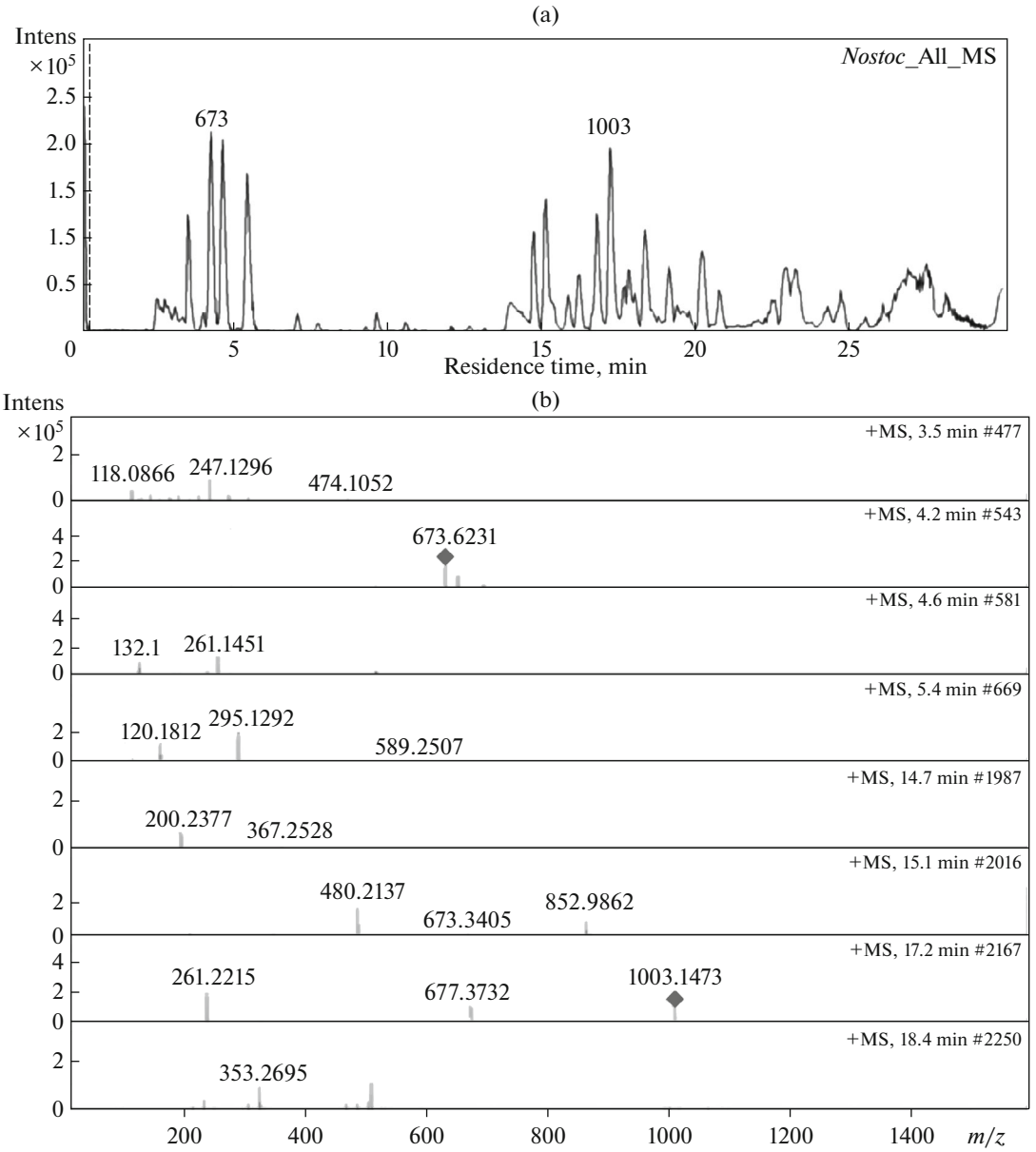

Fig. 2. HPLC of extracts of lyophilized SP-45 (*Nostoc* sp.) biomass (a) and their fragmentation in liquid chromatograph–mass-spectrograph (b). Figures indicate the *m/z* of the toxins: 673—cryptophycin; 1003—micropeptin SD.

An increase in the concentrations of extracts from SP-D (*Desertifilum* sp.) and SP-45 (*Nostoc* sp.) led to an increase in cytotoxicity. In the presence of 5 μg/mL of the extracts, cancer cell death comprised 92–97%. It corresponded to the literature data demonstrating that representatives of the orders *Oscillatoriales* (including the genus *Desertifilum*) and *Nostocales* are able to produce cyclic depsipeptides [20, 21]. For example, cryptophycins isolated from *Nostoc* sp are promising candidates for the development of antitumor medicals [22].

Thus, extracts from the isolated natural cyanobacterial strains differed in cytotoxicity to the *HeLa* cancer cells, which can be explained by the differences in the composition and amounts of the toxins in the studied cultures. SP-D (*Desertifilum* sp.) and SP-45 (*Nostoc* sp.) cell extracts possessed the highest inhibiting activity.

Identification of Toxins Produced by Cyanobacterial Cultures Desertifilum sp. and Nostoc sp.

As mentioned above, cyanobacterial toxins are divided into the three groups according to their chemical structure: peptides (cyclic and linear), alkaloids, and lipopolysaccharides [23].

Identification of the compounds contained in methanol extracts from lyophilized cyanobacterial cells detected at least two cyclic depsipeptides, micropeptin T and oscillapeptin C (Fig. 1), in SP-D (*Desert-*

ifilum sp.) extract and revealed the predominance of micropeptin T with an *m/z* of 1051. This widespread toxin, which was isolated for the first time from the fresh-water cyanobacterium *Microcystis aeruginosa* [24], inhibits chymotrypsin. Micropeptins were also found in planktonic fresh-water species belonging to the genera *Anabaena*, *Anabaenopsis*, *Microcystis*, *Nostoc*, and *Oscillatoria* [20]. Oscillapeptin C (*m/z* of 1060) also belongs to the cyclic depsipeptides.

The extract from the lyophilized biomass of the culture SP-45 (*Nostoc* sp.) contained cryptophycin corresponding to the peak 673 *m/z*, as well as low amounts of the cyclic depsipeptid, micropeptin SD with an *m/z* of 1003 [24, 25] (Fig. 2). Cyclic depsipeptides 2-amino-6-hydroxy-2-Piperidone were detected in toxic and nontoxic strains of the genera *Microcystis*, *Oscillatoria*, *Anabaena*, and *Nostoc*. Some compounds of this class are not toxins but inhibit serine proteases and tyrosine kinases [24, 25].

Thus, the toxins detected in biomass extracts of the strains SP-D (*Desertifilum* sp.) and SP-45 (*Nostoc* sp.) belong to the cyclic depsipeptides, which also include anabenopeptylides, micropeptins, microcystilides, oscilopeptins, cyanopeptolines, eruginopeptins, etc. [26]. Cyclic depsipeptides or cryptophycin [26, 27] are strong antitumor and antifungal depsipeptides that were extracted from cyanobacteria belonging to the family *Nostocaceae*.

Thus, in the present work, we isolated five axenic strains of cyanobacteria. Methanol extracts from the strains *Desertifilum* sp. SP-D and *Nostoc* sp. SP-45 were the most toxic of the obtained methanol extracts to both used test-systems, *Daphnia magna* crustaceans and *HeLa* cancer cells. HPLC of cyanobacterial extracts revealed the presence of cyclic depsipeptides, micropeptin T, and oscillapeptin in *Desertifilum* sp., as well as cryptophycin and micropeptin SD in the *Nostoc* sp.

The work confirmed the importance of and prospects for the study of natural cyanobacterial strains due to their significance for biotechnology and medicine.

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