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Proceeding Paper

Diversity Profile and Specific of Antifungal Peptaibols Biosynthesis Produced by *Emericellopsis* Fungi Derived from Soda and Saline Lakes

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Abstract: Alkaliphilic micromycetes are capable of synthesizing antibiotic substances that are active against opportunistic and clinically significant strains of microorganisms that cause various infections. One factor in this ability is their extreme habitat, which favors the production of specific secondary metabolites. There are more and more studies on the discovery of new peptaibols found in micromycetes isolated from marine, saline and soda sources and other extreme habitats. Our previous studies revealed lipopetaibols emericellipsins A-E, which are produced by the alkaliphilic micromycetes *E. alkalina*. EmiA was similar in its action to amphotericin B against resistant strains of pathogenic micromycetes that cause aspergillosis or cryptococcosis. Continuing our research, we focused on the diversity of antimicrobial peptides produced by *Emericellopsis* strains isolated from various soda and saline habitats.

In total, 38 alkaliphilic and alkalitolerant strains of the genus *Emericellopsis* (*E.alkalina*, *E.* cf. *maritima*, *E.* cf. *terricola*, *Emericellopsis* sp.) isolated from various extreme habitats and belonging to soil-ecological marine, terrestrial and soda soil ecological clade. As a result of screening, in addition to the target component of EmiA, we were also able to identify new compounds. Analysis of strains of *Emericellopsis* sp. (1KS17-1, 2KS17-1), belonging to marine and terrestrial clades from chloride soils, revealed another new form of EmiA devoid of hydroxyl (dEmiA). The biological activity of dEmiA against *Aspergillus niger* and *Candida albicans* with MICs of 4 and 2 μg/mL was consistent with that of EmiA. The target component of EmiA, in addition to biological activity, also showed a strong inhibitory effect on cell proliferation and viability of the HCT 116 cell line, depending on the dose and time, and induced apoptosis.

Keywords: alkaliphilic fungi; peptide; antifungal activity; antitumor activity; *Emericellopsis alkalina*; emericellipsin's complex

1. Introduction

Opportunistic and pathogenic fungal infections are one of the causes of high mortality which is about 1.5 million people in a year. Invasive mycoses, in particular invasive aspergillosis, are often opportunistic infections in diseases such as oncology, HIV, covid-19 and others.

Antimicrobial peptides are interesting as potential source of new antifungals. They are able to be synthesized by almost all living organisms [1–5].

Peptaibols, the largest group of peptaibiotics, are a class of linear peptides that have an acylated N-terminal group, a C-terminal amino acid and a high content of α-aminoisobutyric acid (Aib) - approximately 40% Aib in long peptaibols and from 14 to 56% for short peptaibols [6–14]. Peptaibols the dominant secondary metabolites of fungi among others, including mycoparasites fungi [8,10,11,15,16].

About 30 genera of micromycetes are known, mainly belonging to the order Hypocreales, which are promising for the production of peptaibols [3,5,17–21]. At the moment, most peptaibols have been found in fungi of the genus *Trichoderma*, and the most studied species are *T. viride*, *T. brevicompactum*, *T. virens*, *T. parceramosum*/*T. ghanense* and *T. longibrachiatum* [15,22–24]. Fungi of the genus *Emericellopsis* are also known as producers of the peptaibols zervamycins, bergofungins, emerimycins, and emericillipsins [6,7,17].

Fungi of the genus *Emericellopsis* are ubiquitous, including in the terrestrial and marine environment, and they are also one of the most important dominants on the shores of soda and salt lakes. According to recent studies of the phylogeny of multilocus genes, these species are placed in three ecological clades: terrestrial, marine, and soda soils [25-28]. Fungi of the genus *Emericellopsis* are capable of biosynthesis of peptaibols with antibacterial and antifungal activity: antiamebins I-XI from *E. salmosynnemata* and *E. synnematicola*, bergofungins A-D from *E. donezkii*, emerimycins II, III, IV. from *E. microspora* and *E. minima*, heptaibin from *Emericellopsis* sp., servamycin from *E. salmosynnemata*. Currently, according to the Norine database, a total of 32 peptides have been found, which were grouped into 5 families. In our previous studies, we managed to discover a new complex of peptaibols with antimicrobial activity, called Emericellipsins A-E [18,27–30]. The leader compound EmiA was able to inhibit clinical isolates of fungi of the genera *Aspergillus*, *Saccharomyces* and *Cryptococcus* which were resistant to fluconazole and amphotericin B [18].

2. Materials and Methods

Molecular studies to determine the species of strains were carried out using PCR amplification of spacer rDNA as described previously [31]. The resulting sequences were deposited in Gen-Bank [32]. A phylogenetic reconstruction was made which included 24 strains of *Emericellopsis*. The alignment was calculated using the MAFFT v. 7.429 [33] as well as when using the L-INS-I strategy. The most suitable substitution model for alignment was selected based on the Akaike Information Criterion (AIC), for this purpose the IQ-TREE web service was used [34].

The effect of pH on the growth rate of cultures was evaluated in triplicate using various variants of nutrient media based on citrate, phosphate and carbonate buffers. Such pH variants as 4, 5, 6, 7, 8, 9, 10 (± 0.2) were tested.

The preparation of extracts from the culture liquid (CL) as well as the isolation of peptaibol from the obtained extracts, was carried out using the methods described earlier [31].

In order to determine the molecular weights MALDI-TOF MS was used and the mass measurement accuracy is about 1 Da. The technique itself was described in detail in our study earlier [31].

The antimicrobial activity of the extract containing peptaibols was measured using the disk diffusion method. To this goal disks with a diameter of 6 mm which contained 40 μL of the test sample were placed on dishes with PDA (Sigma-Aldrich, St. Louis, MO, USA). Zones of inhibition were measured after 24 h at 28 °C. As a control the activity of amphotericin B (Sigma-Aldrich) was measured on the same plates. The value of the minimum inhibitory concentration (MIC) of each substance was determined in accordance with the recommendations of CLSI/NCCLS M27-A3 and M38-A2 [35,36]. The strains *Candida albicans* ATCC 14053 and *Aspergillus niger* ATCC 16404 were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA).

A colorectal carcinoma (HCT116) cell line (ATCC® CCL-247TM) was used for the real-time cell analyzer test. To this goal the cells were thawed and then passaged 2–3 times and cultured in a cell incubator at 5% CO_2 and 37 °C using DMEM nutrient medium with 10% fetal bovine serum. Real-time proliferation analysis was performed using the iCELLigence RTCA system. The density of the HCT116 cell suspension was 1×10^5 cells/mL. 300 μL of the original cell suspension was added to each well of the device, after 1 day the nutrient medium was changed to a new one that contained different concentrations of EmiA. The medium without peptide was used as a control. Cells were incubated for two days. The CI value was set by the RTCA software package based on the impedance signal.

3. Results

The 22 strains used in the work were previously identified by 6 loci (LSU and SSU rDNA, RPB2, TEF1-α, β-tub, and ITS region) as *E. alkalina* [27]. In addition, 16 strains of the genus *Emericellopsis* isolated over the period 2017–2018 were also studied. Thus, in total, 38 strains of fungi of the genus *Emericellopsis* isolated from the soils of the margins of various soda and salt lakes of the Kulunda steppe (Altai, Russia) were studied in the work. As a result of the research, 16 new added strains were grouped into 3 ecological clades: 10 strains of *E. alkalina* fell into the "soda soil" clade, 4 strains into the "marine" clade, and 2 strains ended up in the "terrestrial" clade. 2 strains from the marine clade were identified as *Emericellopsis* cf. *maritima*, 1 strain from the terrestrial clade was identified as *Emericellopsis* cf. *terricola*, some of the remaining strains were not identified among the known species and therefore they are marked as *Emericellopsis* sp. (Figure 1).

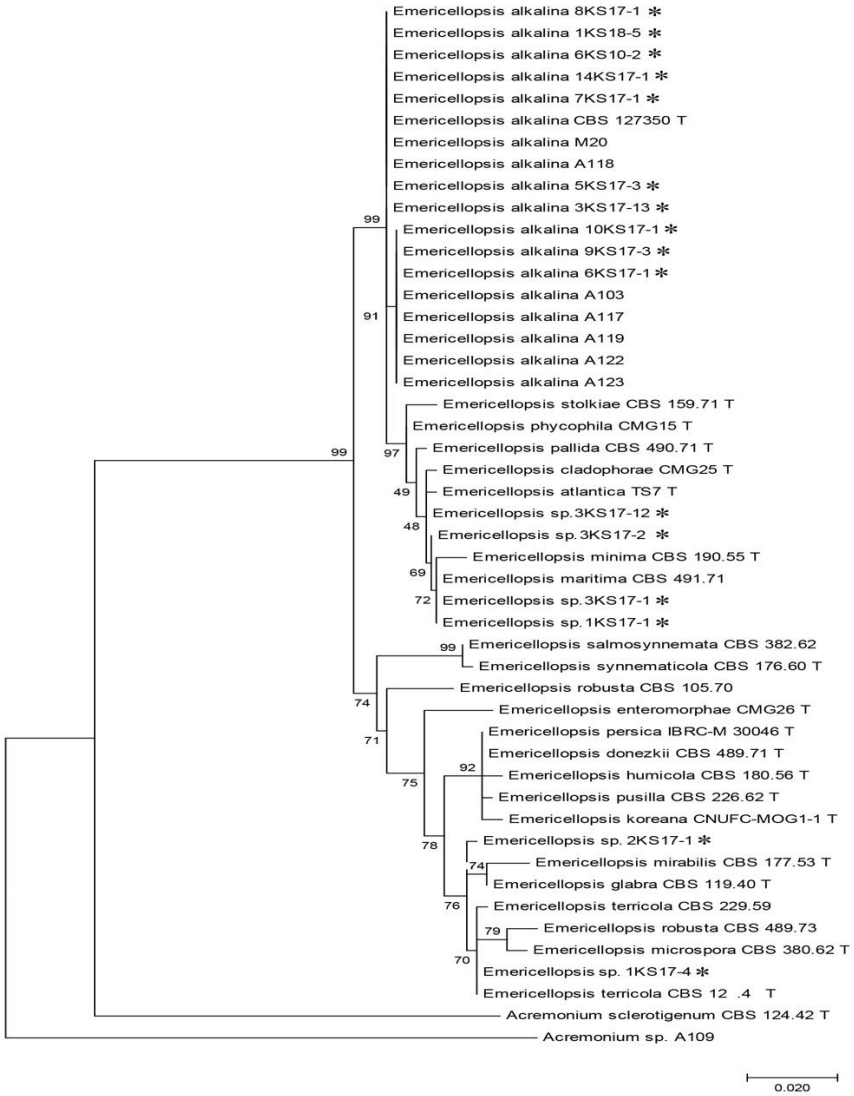

Figure 1. Maximum likelihood tree for the *Emericellopsis* genus based on partial sequences for ITS rDNA (including 5.8S rDNA) region. Branch lengths are proportional to the estimated number of nucleotide substitutions. *Emericellopsis* spp. and related species were clustered into a «Marine», «Soda soil» or «Terrestrial» clade. Taxa names of the isolates obtained in this study are highlighted in an asterisk.

32 strains of *E. alkalina* were able to grow at pH from 6 to 10 units, while the optimal value was 9 units. Thus, all studied strains of *E. alkalina* exhibited an alkaliphilic phenotype. Other strains were assigned to the alkalinoresistant phenotype. Thus, 4 strains from the marine clade showed optimum growth at pH 7 or 8, but practically did not grow at higher pH values. One strain from the terrestrial clade had a growth optimum at pH 8 and was able to grow at its higher values, another strain from the same clade showed a similar growth optimum at pH 7, but practically did not grow at higher values.

We would like to note that the synthesis of the target compound EmiA for all studied strains of *E. alkalina* was possible only when grown under alkaline conditions. At the same time, EmiA could be detected both in extracts from the culture liquid and from the mycelium. A high content of lipopeptaibol EmiA was detected in 9 strains in mycelium extracts and in 5 strains in culture fluid extracts. The maximum amount was found in the culture fluid extract of the E101 type strain.

E. alkalina strains isolated from soda chloride-sulfate and chloride-sulfate soil types, as well as 2 strains from soda soils, are characterized by a high content of EmiA. In 2 strains, among other species of fungi of the genus *Emericellopsis*, the presence of EmiA was also detected, and they are characterized by a higher content of the target compound in the mycelium compared to the culture liquid. Among other *Emericellopsis* sp. no EmiA was found, and they also don´t have antifungal activity. We noted that strains with a high content of EmiA also synthesized EmiB-D homologues, while isolates from chloride and chloride-sulfate soils did not have them.

We would like to note the results of scientific interest in the analysis of 2 strains of *Emericellopsis* sp. isolated from chloride soils and belonging to the marine and terrestrial clades. Biautography revealed two zones of growth inhibition of the *A.niger* test strain with average Rf values: 0.2 and 0.55 in the CHCl3:MeOH = 3:1 system. When analyzing the initial fraction using HPLC, two peaks were detected on the chromatogram: 1 (tR = 37.3 min) and 2 (tR = 39.4 min) (Fig. 2a), each of which was collected separately and analyzed with using MALDI-TOF MS. Peak 1 had a molecular weight of 1050.7 Da, and peak 2 had a molecular weight of 1032.7 Da (Figs. 2b and 2c). The identical nature of the mass distribution of the fragments was observed, which indicates the same nature of both substances. We would also like to note that the difference between the masses of the two peaks correlates with the mass of the water molecule (18Da). The chemical structure of the AHMOA residue contains a hydroxyl located one carbon atom away from the amide bond and is possibly a good site for water abstraction. In this case, a double bond should be formed between the corresponding carbon atoms while maintaining the principle of valency. Therefore, the fragment with a molecular weight of 195 Da presumably refers to the residue of 2-amino-4-methyl-8-oxo-dec-6-enoic acid. Thus, the mass of 1050.7 Da is most likely related to EmiA because it corresponds to the calculated mass due to its structure, and the mass of 1032.7 Da to the dehydrated form of EmiA (dEmiA). dEmiA is a more hydrophobic analogue of EmiA.

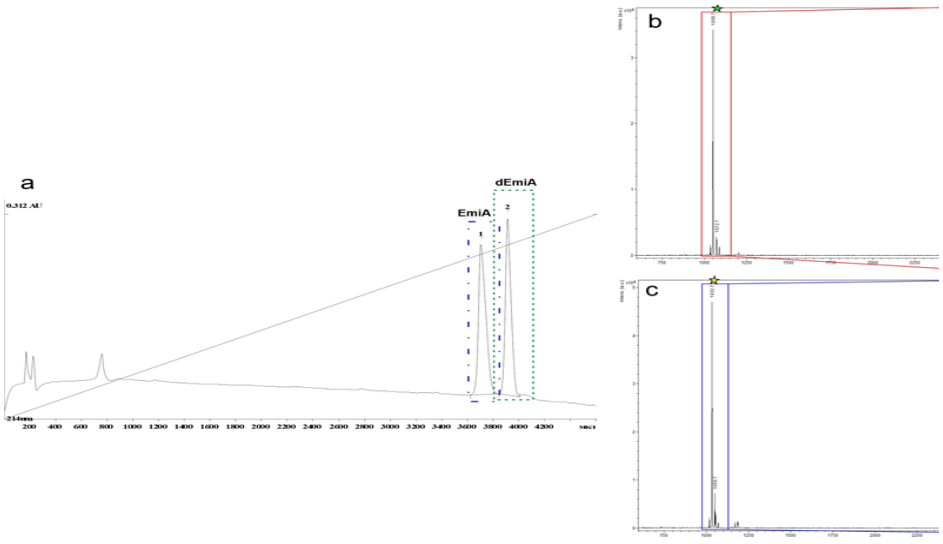

Figure 2. a. The Chromatogram of analyzed sample with HPLC method is presented. There only two peaks 1 and 2 were visualized with retention times 37.4 min and 39.3 min, correspondingly (on the chromatogram values are presented in volume units). The names of probably compounds are marked upper the corresponding peaks. Both peaks were picked out with dotted rectangles colored in blue and green and were related to the corresponding MALDI-TOF MS specters. **b.** The MALDI-TOF MS specter of chromatographic fraction related to peak with retention time of 37.4 min. **c.** The MALDI-TOF MS specter of chromatographic fraction related to peak with retention time of 39.3 min. In the red rectangles the zoomed specters of masses are represented, and the major peaks

related to the masses of 1050.7 Da and 1032.7 Da, which correlate with masses of EmiA and dEmiA are marked with green and with yellow star, correspondingly.

As we have shown earlier, the target compound EmiA had biological activity against the micromycetes *Aspergillus niger* ATCC 16404 and *Candida albicans* ATCC 14053, while the MIC values were 4 and 2 μg/mL, respectively [18, 29]. In our experiments with the above strains, dEmiA showed exactly the same activity (Table 1). Thus, it can be assumed that the single hydroxyl group of the EmiA does not have a decisive influence on the mechanism of action of our antimicrobial compound. Perhaps dEmiA is a premature form of EmiA.

Table 1. Minimum Inhibitory Concentrations (MIC) of the EmiA and dEmiA against fungi, μg/mL.

Minimal Inhibitory Concentration (MIC, μg/mL)				
	Compound			
Strain	**EmiA**	**dEmiA**	**AmpB**	**FZ**
Aspergillus niger ATCC 16404	4	4	1	2
Candida albicans ATCC 14053	2	2	0.25	4

AmpB—amphotericin B; FZ—fluconazole.

The target component of EmiA, in addition to biological activity, also showed a strong inhibitory effect on cell proliferation and viability of the HCT 116 cell line, depending on the dose and time, and induced apoptosis. At the same time differences in cell proliferation were observed under the influence of increasing concentrations of EmiA with a change in CI. After the addition of the peptide during the incubation period, the CI value changed depending on the value of the amount of the added peptide: in the control, from 0.4 to 1.3; at 0.25 μg/mL-from 0.4 to 1.1; at 1.0 μg/mL-from 0.4 to 0.7; at 4.0 μg/mL from 0.4 to 0.6; at 16.0 μg/mL-from 0.4 to 0.5 (Figure 3).

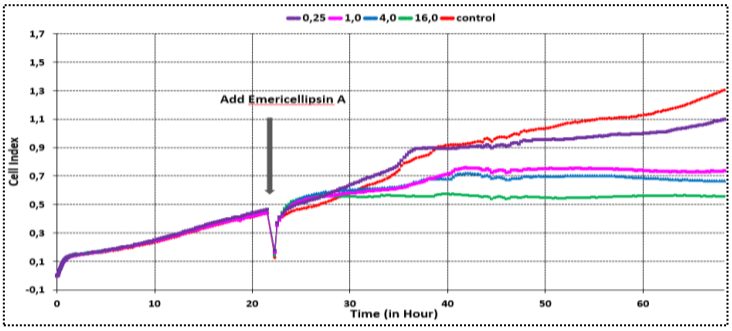

Figure 3. Cell index of the HCT116 cell line at different concentrations of Emericellipsin A peptide: 0.25 μg/mL; 1.0 μg/mL; 4.0 μg/mL; 16.0 μg/mL.

4. Conclusion

Our results show that lipopeptaibols EmiA, produced by the alkaliphilic micromycete *E. alkalina*, are an alternative antibiotic used in medical practice, active against resistant pathogens of aspergillosis. In terms of biological activity, EmiA is similar to amphotericin B. At the same time, EmiA is not able to replace doxorubicin as a chemotherapeutic agent, but it can be used for palliative treatment of cancer patients whose course of the disease is complicated by aspergillosis.

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Conflicts of Interest: The author declares no conflict of interest

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