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Original Article

A Report on Finding a New Peptide Aldehyde from Cyanobacterium *Nostoc sp.* Bahar M by LC-MS and Marfey's Analysis

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Abstract

Background: Cyanobacteria have a worldwide distribution in the terrestrial habitats, occurring predominantly on the surface of the soils, stones, rocks, and trees, practically in moist, neutral or alkaline aeries. The unique natural and bioactive compounds from cyanobacteria with various biological activities and an extensive range of chemical classes have a significant capability for expansion of the pharmaceuticals and other biomedical purposes.

Objectives: Regardless of the progresses in our knowledge on cyanobacteria, however, cyanobacteria are still viewed as an unexplored source of potential drugs. In this study presence of bioactive compounds among the cyanobacteria culture collection of Iran, where a wide variety of strains can be found, was investigated.

Material and Methods: We explored one *Nostoc* strain isolated from rice fields in Golestan province of northern Iran for searching for novel products. The chemical construction of the new bioactive compound was clarified by application of liquid chromatography-mass spectrometer (LC-MS) and Marfey's analysis of the degradation products.

Results: We found a novel peptide aldehyde compound from a hydrophilic extract of the *Nostoc sp.* Bahar_M, which is composed of the three subunits, 2-hydroxy-4-(4-hydroxyphenyl) butanoic acid (Hhpba), L-Ile, and L-argininal. According to the structural information, we predicted that the novel peptide-aldehyde compound probably to be trypsin inhibitors.

Conclusions: Results demonstrated that terrestrial cyanobacteria are a promissing resource of bioactive natural products.

Keywords: Bioactive Compounds; Cyanobacteria; *Nostoc*

1. Background

The *Nostoc* species produce a large number of pharmaceutical compounds with varying bioactivities (1).

The ecological implication of the *Nostoc* strains expands beyond their production, though, as many of these prokaryotes are able to adjust their territory due to the synthesis of the pharmaceutical products (2).

These compounds reveal various ranges of medicinal activities, together with unique cyclic and linear lipopeptides, fatty acids, alkaloids, and other organic chemicals (3). A huge amount of innovative antimicrobial mediators have been recognized from the genus *Nostoc* with cytotoxic (4), antifungal (5), antibacterial (6), immunosuppressive, enzyme inhibiting, and antiviral (7) activities.

2. Objective

Cyanobacteria which are considered as the good producers of bioactive products, produce a number of linear and cyclic peptide inhibitors of the serine proteases, like aeruginosin, spumigin, banyasin, cyanopeptolin, micropeptin, anabaenopeptin, kempopeptins, microginin, *Nostoc*arboline, and microviridin (8-16).

Such findings have signified cyanobacteria as a hopeful, but, still unknown natural source for holding lots of natural compounds valuable for the pharmaceutical manufacturing. However, the bioactive compounds of terrestrial cyanobacteria in Iran remain to be evaluated. Consequently, the major point of this study is the identification of the structure and bioactivity of a new peptide aldehyde compound, by using LC-MS and Marfey's.

3. Materials and Methods

3.1. Culture and Purification of Nostoc Strain

Soil samples were collected in September 2010 from five rice fields in Golestan province in northern Iran. To grow cyanobacteria, soil samples were transferred to the sterilized and sufficient quantities of the liquid $BG11_0$ media (17) without $NaNO_3$ and pH was maintained at 7.1. For providing a constant pH, CO_2 supply was constant; however, there may be a little variation in pH. Cultures were kept at 28°C for two weeks in a culture chamber provided with continuous artificial illumination with a light density of approximately 1500-2000 lux (18). Hormogonia were used for the purification and preparation of the uni-algal cultures. The *Nostoc* strain was regularly tested for the axenicity by microscopic examination as well as inoculation on an R2A (LAB163) medium for the bacterial colonies. The morphological observations were examined by the bright-field microscopy and use of phase-contrast illumination. The subsequent factors were chosen to explain the morphology of the strain and finally, the strain was identified according to (19). Finally, One strain of heterocystous cyanobacteria (*Nostoc sp.* Bahar_M), which was mainly found strain in the rice fields (20), was selected for molecular identification and estimation of the chemical analysis.

3.2. Chemical Analysis

3.2.1. 15N- Labeling Culture

Two different sets of methods were used for further structural characterization of the new peptide aldehyde compound. The first method screened the methanolic extracts of the *Nostoc* cells, and the second method was labeling the culture with 15N- urea. A new 15N-labeled peptide aldehyde compound was found as explained by (21). In this experiment, 15N- urea (98 + % 15N, ISOTEC, USA) and nitrogen-free argon (with 20.9 % O_2 and 0.45 % CO_2; quality5.7; AGA Gas Ab, Sweden) were used as nitrogen supply into the culture to avoid the nitrogen fixation through the air. To maximize the degree of labeling in new peptide aldehyde compound, *Nostoc* was consequently cultivated three times and the cells from the fourth cultivation were used in LC-MS analysis.

3.2.2. Preparation of Extracts for LC-MS Analysis

Nostoc sp. Bahar_M was grown in the Z8 liquid medium (22-24). The harvested biomasses were freeze-dried using Edwards lyophilizer. The extract for the sample analysis was prepared from 50 mg freeze-dried sample. The microtube containing the culture was supplemented with glass beads and the methanol and the cells were cracked automatically using a Fast Prep device (FP120, Bio101, Thermo Electron Corporation, Qbiogene, Inc., CA, USA). The homogenized combination was centrifuged and injected into LC-MS to identify the bioactive compounds of the strain. The Luna C8 (2) reverse phase column was used for separation and detection of the new compounds. The mobile phase A consisted of the formic acid (0.1 %) (Fluka, Sigma Aldrich, Steinheim, Germany) and the mobile phase B was consisted of the Isopropyl alcohol. The inoculation amount of each sample was 10 µL, respectively.

3.2.3. PCR Amplification of the NOS Gene and Analysis

The coding sequence for the NOS gene were amplified by PCR using two oligonucleotide primers set NOSF and NOSR (25). After purification of the NOSF and NOSR fragments, sequencing was done using the Big Dye Terminator Cycle Sequencing kit and analyzed on the ABI 310 Genetic Analyser. The BLASTX search of the partial NOS genes of *Nostoc sp.* Bahar_M was used to discover similar sequences. The NOS gene sequence and reference sequences were aligned with CLUSTALW. The maximum likelihood trees were constructed by the MEGA version 7 using the Kimura two-parameter model. The robustness of the tree was estimated by performing 1000 bootstrap (**Fig. 1**).

3.2.4. Reduction of New Peptide-Aldehyde Compound

Freeze-dried biomass (3g) was extracted twice with methanol (120 mL). The extract was partitioned between water and dichloromethane according to the proportion of 1:1:1. The water layer was evaporated by a rotary evaporator and residues were dissolved in methanol. The obtained solution was incubated with NaBH4 to reduce the aldehyde version of the new peptide-aldehyde compound into alcohol version. The evaporated solution was dissolved in acetonitrile and analyzed with LC-MS (26).

3.2.5. Amino Acid Hydrolysis Analysis

The structure of central part of the peptide-aldehyde compound was still ambiguous, therefore partial amino acid hydrolysis of peptide-aldehyde compounds was performed by dissolving peptide in HCl and hydrolysis at 105 °C (**Fig. S1**) (26). The acid was diapered, and the residue solubilized in water. D-Leucine, L-Leucine, D-Isoleucine, Leupeptin, and L-Isoleucine (DAA-20, Sigma Aldrich, Germany) were used as a standard. The amino acid fragmentation products were analyzed with LC-MS software version 2.1.

4. Results

The analysis of methanol extract of the freeze-dried biomass of *Nostoc sp.* strain Bahar_M with LC-MS yielded, a new peptide-aldehyde compound m/z 450.2 (**Fig. 2**). The total ion chromatogram (TIC) and extracted ion chromatogram (EIC) showed that the protonated $[MH^+]$ is a new peptide aldehyde compound (450.2 m/z), $(M + H_2O + H^+)$ (468 m/z) and $(M + IPA + H^+)$ (510.2 m/z) as shown in **Figure 3.**

Figure 1. Consensus bootstrap tree on the basis of the maximum likelihood distances of the 41 amino acids long partial-length nos gene of *Nostoc sp.* Bahar M and the sequences that were taken from the GenBank. Numbers near nodes indicate bootstrap values for ML analyses (Bar = 0.06 mutations per amino acid position).

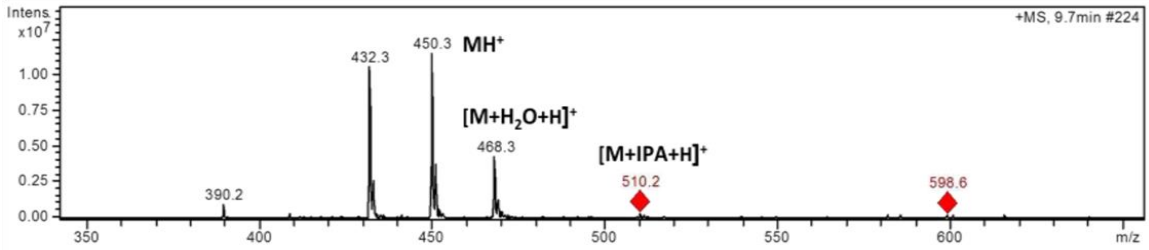

Figure 2. +MS fragmentation patterns of the protonated new peptide aldehyde compound (450.3 m/z), [M + H_2O + H^+] (468.3 m/z) and [M + IPA + H^+] (510.2 m/z). m/z= Mass-to-charge ratios, The intensity of the ion on the y-axis is given as counted ions per second (cps) and the mass-to-charge ratio (m/z) on the x-axis.

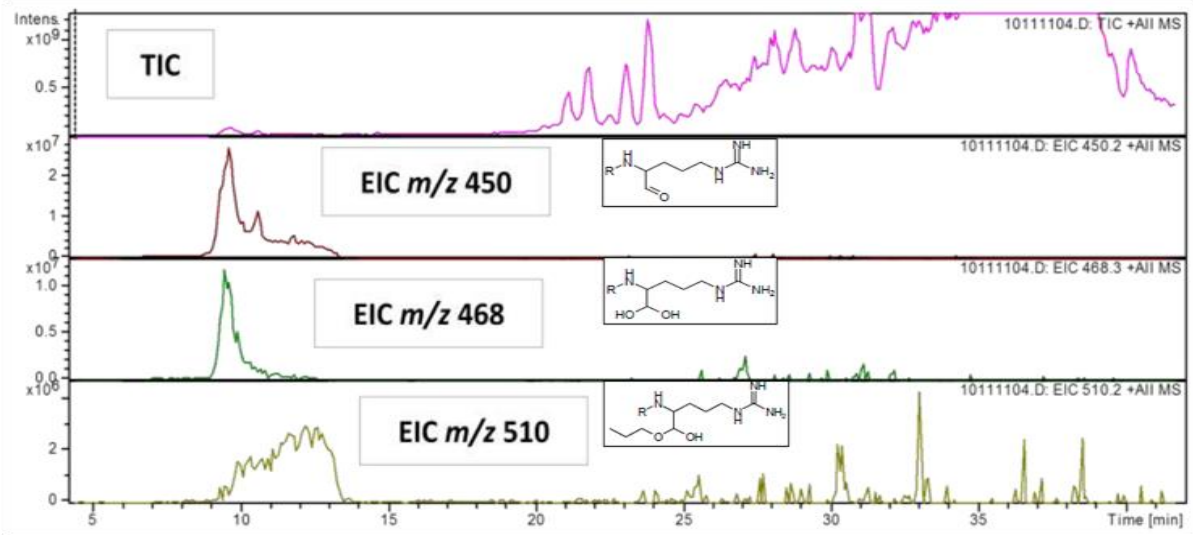

Figure 3. Total ion chromatogarm (TIC) and extracted ion chromatogarm (EIC) of the protonated [MH^+] new peptide aldehyde compound (450.2 m/z), [M + H_2O + H^+] (468 m/z) and [M + IPA + H^+] (510.2 m/z). The x-axis represents retention time (min), and the y-axis represents signal intensity. Intensity is measured in counts per second (cps).

N15 labeling experiment was performed to confirm the subunit structure of the new peptide aldehyde compound. By comparing the LC-MS results of the labeled with that of the unlabeled extract of the ASN biomass, a 5-Dalton shift was observed, and 5 nitrogen atoms confirmed the existence of Ile/Leu or Val (1 nitrogen) as well as argininal (4 nitrogens) (**Fig. 4**) (**Fig. S2**).

The MS_2 fragmentation patterns indicated a reduction of the protonated 15N-labeled new peptide aldehyde

compound (452 m/z) by $NaBH_4$ (Fig. 5) (Fig. S3). The chromatogram obtained by the Marfey's analysis and hydrolysis of amino acid from the original containing compounds indicated the presence of L-ILE amino acid. This result showed that the structure of the peptide aldehyde compound in comparison to spumigins and aeruginosins is a new peptide, respectively (Fig. 6 **and** 7).

The structure of the new peptide aldehyde compound showed an identical structure with that of spumigins, and aeruginosins, expect, in the middle amino acid (Fig. S1). Initially isolated from *Nodularia spumigena* AVI, spumingins are structurally analogous to the aeruginosins, while Choi is changed to the (2S, 4S)-4-methylproline (Fig. S4).

The results of the PCR amplification showed that a 358 bp fraction of the NOS gene was lucratively amplified from the PCR amplicon taken from *Nostoc sp.* strain Bahar_M. Moreover, the result of NCBI-BLAST search showed that amplified sequence from the *Nostoc sp.* Bahar_M (KT763390.1) is responsible for coding of a conserved hypothetical protein, therefore this sequence are not involved in the biosynthesis of the 4-methylproline. The maximum likelihood tree is shown in Fig. 1 and the partial NOS gene sequence has been deposited in the Data Bank of Japan (DDBJ) under the accession No. MG726068 and named as conserved hypothetical protein (*Nostoc sp.* Bahar_M).

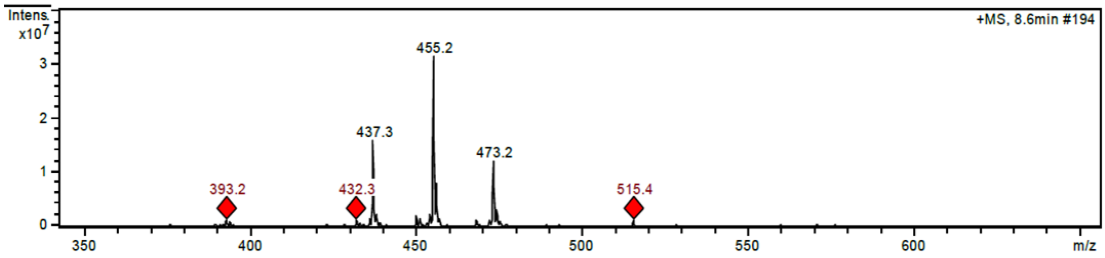

Figure 4. +MS fragmentation patterns of the protonated from 15N-labeled new peptide aldehyde compound (455 m/z), [M + H_2O + H^+] (473 m/z) and [M + IPA + H^+] (515 m/z). The +MS fragmentation patterns of the protonated (450.3 m/z), and the 15N-labeled new peptide aldehyde compound (455 m/z) showed an increased mass spectrum by 5 nitrogen atom. m/z= Mass-to-charge ratios, The intensity of the ion on the y-axis is given as counted ions per second (cps) and the mass-to-charge ratio (m/z) on the x-axis.

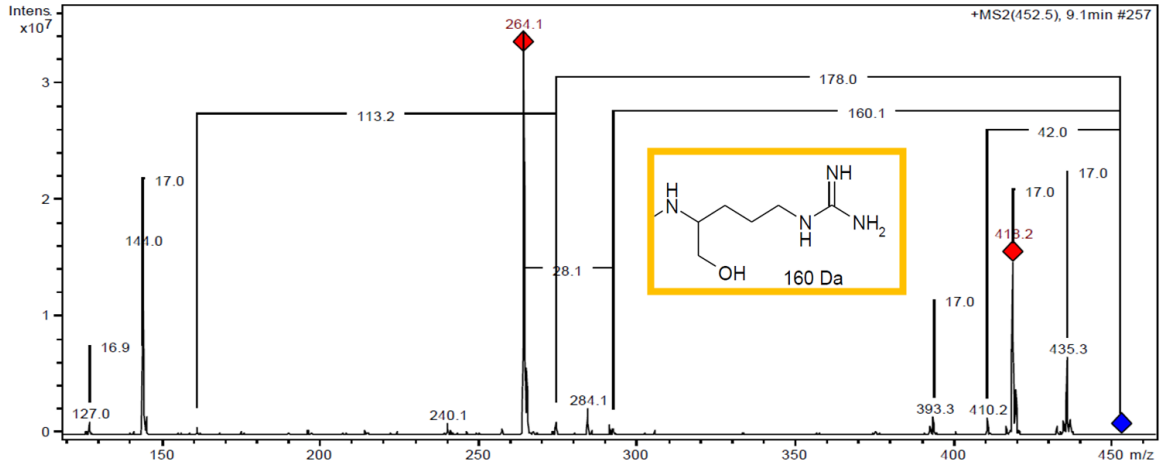

Figure 5. MS_2 fragmentation pattern of the protonated from of the 15N-labeled new peptide aldehyde compound (452 m/z) after reduction by NaBH4. m/z= Mass-to-charge ratios. The intensity of the ion on the y-axis is given as counted ions per second (cps) and the mass-to-charge ratio (m/z) on the x-axis.

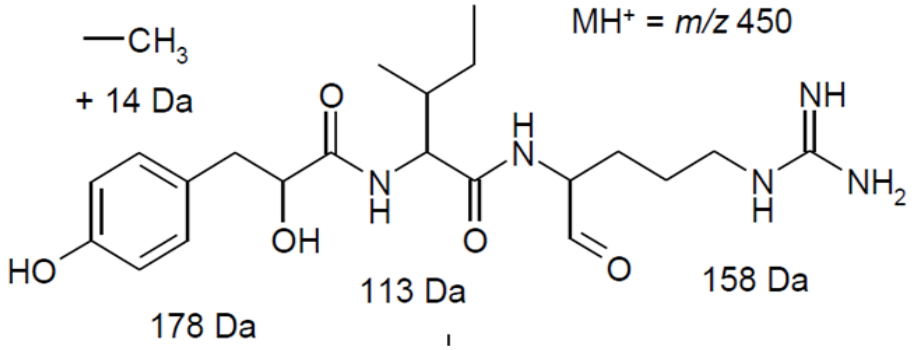

Figure 6. An overall structure prediction of the new peptide aldehyde compound by LC-MS. The predicted structure is composed of three units, HHPBA, L-IIe, and argininal/argininol.

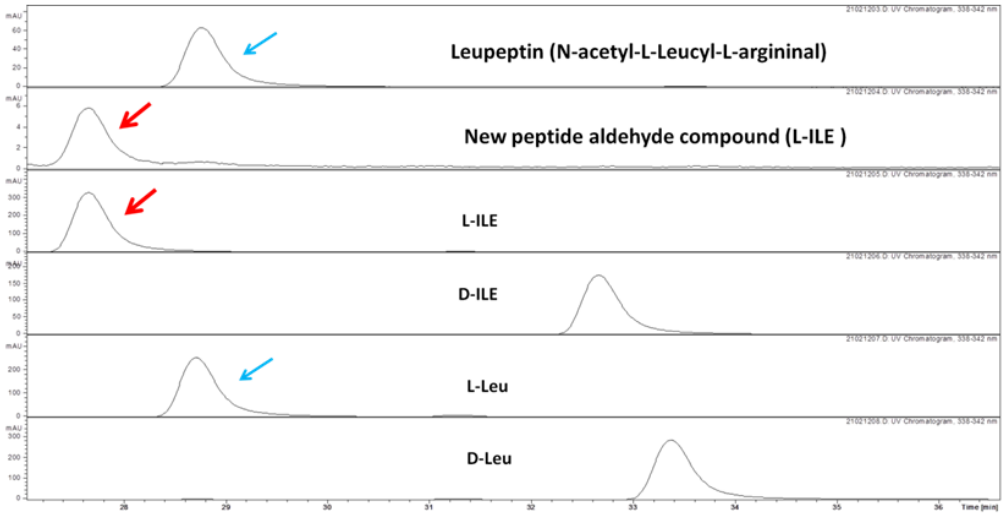

Figure 7. Marfey's analysis and hydrolysis of the amino acid indicate the presence of L-ILE amino acid in the new peptide aldehyde compound in comparison to Leupeptin which has L-Leu amino acid. The x-axis represents retention time (min) and the y-axis shows the peak area as expressed by absorbance units (mAU).

This is evidence that the structure of the new peptide aldehyde compound is new. Monophyletic origin of heterocystous cyanobacteria was supported by the phylogenetic tree and three well supported evolutionary lineages (cluster H1-H3) within heterocystous cyanobacteria were found. Unfortunately, not all of the relationships are well supported by the bootstrap values. However, the conserved hypothetical protein of the studied strain (*Nostoc sp.* Bahar_M) falls into the Cluster H3 (bootstrap value of 60% ML). This cluster was divided into two main subclusters (H3-1 and H3-2) (**Fig. 1**). Since aeruginosin and spumigin inhibitory effect on serine protease has already been proven by others (9), therefore, we considered the new peptide aldehyde compound might be a new protease inhibitor from *Nostoc sp.* Bahar_M.

5. Discussion

During the past few years numerous innovative and miscellaneous secondary natural combined with application in pharmaceutics and biological activities (e.g. antibiotic, enzymes, antiviral, anticancer, antifungal, anti-inflammatory mediator, and protease inhibitors) have been found in cyanobacteria which obviously has made cyanobacteria to have a precious potential for extracting new and varied natural compounds for drug and could be evaluated as a major source for drugs (27). Experiments have signified the occurrence of non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) secondary metabolite pathways in a diversity of cyanobacteria (28-33). The extent of the synthesis of natural products has aided cyanobacteria species survival in many competitive ecological niches (34). For all of these reasons, researchers try to find novel and pharmacologically active cyanobacterial metabolites (35-39).

Numerous evaluations have illustrated the multiple pharmaceutical and biological compounds isolated from marine cyanobacteria. However, freshwater and terrestrial cyanobacteria have also contributed molecules with noteworthy biological activities (30, 31).

4-mPro is an uncommon amino acid, which has been found in a small number of bioactive compounds identified from cyanobacteria. The 4-mPro is a special non-proteinogenic amino acid, in which the methyl group is connected to the second carbon of the side chain. In cyanobacteria, it was discovered in nostopeptolide A and *Nostoc*ycleopeptide A (40, 41). However, both of these compounds showed no bioactivity after the discovery. The biosynthesis of the 4-mPro has been revealed by Luesch et al., 2003 (42). A zinc-dependent long-chain dehydrogenase and a Δ1-pyrroline-5-carboxylic acid (P5C) reductase, which are coded by genes NOSE and NOSF in *Nostoc sp.* GSV 224, convert L-Leu into 4-mPro. Later on, 4-mPro biosynthetic genes were also found in the biosynthetic gene cluster of *Nostoc*ycleopeptin A in *Nostoc sp.* ATCC 53789, nostopeptolide A in *Nostoc sp.* GSV 224, nostopeptolide in *Nostoc punctiforme* PCC 73102 and spumigin E in *Nodularia spumigena* CCY9414 (**Fig. S4**) (9, 25, 43-45). The gene clusters of 4-mPro containing-compounds in cyanobacteria show that they have conserved distribution with an open reading frame (orf) and one ATP-binding cassette transporter (ABC transporter). Therefore, it is possible to make use of methylproline genes to screen new natural products from cyanobacteria with PCR (25). In this research, the result of the NCBI-BLAST search showed that the amplified sequence is not involved in the biosynthesis of

4-methylproline and its only responsible for coding a conserved hypothetical protein. This is evidence that the structure of the new peptide aldehyde compound is new.

Up to now, numerous strains of the genus *Nostoc* have been verified to be producer of the natural product. Moreover, numerous depsipeptides with pharmaceutical activities, chiefly protease inhibitors have been described from *Nostoc* species (46, 47). Furthermore, nostodione A formerly extracted from *Nostoc commune* was also extracted from this strain and nostodione A is recognized to be proteasome inhibitor with an IC50 value of 50 μM (48). It is probable that the protease inhibitors created by *Nostoc* species act as anti-grazing compounds, however, further research is essential to validate this hypothesis (29, 30).

Moreover, cyanobacteria produce a plethora of serine protease inhibitors with a broad range of chemical structures (47). Since protease compounds are are involved in a diverse pharmaceutical application, and several proteases are classified as drug targets (48, 49), the detection of the novel protease compounds is essential to the expansion of the pharmacological tools as well as prospective therapeutics impacts. For instance, cancer cells are more sensitive to the pro-apoptotic effects of the proteasome inhibition than normal cells. Thus, proteasome inhibitors can be potential anticancer agents. Protease inhibitors can be produced by both toxic cyanobacterial strains (e.g., those that produce hepatotoxins or neurotoxins) and nontoxic cyanobacterial strains of *Microcystic*, *Anabaena*, *Planktothrix*/*Oscillatoria* and *Nostoc* (50).

Quite a lot of protease inhibitors extracted from cyanaobacteria have been studied including aeruginopeptins, anabaenopeptilides, cyanopeptins, micropeptins, nostopeptins, oscillapeptins, miroviridins, aeruginosins, microcins, anabaenopeptins, oscillamides, banyasin A, largamides A-H, lyngbyastatins 4-7, planktocyclin, kempopeptins A and B, nostodione A and others (13, 48, 50-52).

Additionally, a number of protease inhibitors may also find function in medicine for curing of stroke, coronary artery occlusions, and pulmonary emphysema. For instance, inhibitors of the serine protease, thrombin, could be used to control blood clot formation in these diseases. Thrombin works by slicing a peptide fragment from fibrinogen which then guides to the production of fibrin, a key element of the blood clots. In the same way, angiotensin-switching enzyme inhibitors are being expanded as anti-hypertensive mediator. Protease inhibitors are also used in the treatment of HIV infections (53, 54). These inhibitors include linear and cyclic peptides, as well as depsipeptides and have been isolated mostly from Microcystis and Oscillatoria strains (55).

As a member of the protease family, trypsin has been revealed to play a significant role in a diversity of cancers or brain development. The trypsin-like protease (or called trypsinogen) has been found in many types of carcinomas, such as ovarian neoplasm, pancreatic cancer, lung neoplasm and colorectal cancers (55). The high level of tumor-associated trypsinogen 2 is able to cause an increased rate of tumors occurrence (56, 57). In the brain, trypsin IV has shown to have wide distribution. Through the activation of PAR (protease-activated receptors)-1 or PAR-2, trypsin IV could perform neuroprotection from toxic insults in the brain (58). In addition, trypsin IV was also proposed to contribute to the neurogenic inflammation and pain by inducing PAR-2-dependent hyperalgesia to thermal and mechanical stimuli (58). Therefore, trypsin or trypsin-like protease could be used as a good target for designing new drugs, and trypsin inhibitors will be one of the ideal drug leads. Here we have identified a new trypsin inhibitor, nostoginosin. These findings increased the diversity of the bioactive secondary metabolites characterized from cyanobacteria and provide new leads for drug research.

References

1. Dembitsky VM, Rezanka T. Metabolites produced by nitrogen-fixing Nostoc species. *Folia Microbiol (Praha)*. 2005;**50**(5):363-391. doi: 10.1007/BF02931419 pmid: 16475497
2. Ehrenreich IM, Waterbury JB, Webb EA. Distribution and diversity of natural product genes in marine and freshwater cyanobacterial cultures and genomes. *Appl Environ Microbiol*. 2005;**71**(11):7401-7413. doi: 10.1128/AEM.71.11.7401-7413.2005 pmid: 16269782
3. Dittmann E, Neilan BA, Borner T. Molecular biology of peptide and polyketide biosynthesis in cyanobacteria. *Appl Microbiol Biotechnol*. 2001;**57**(4):467-473. doi: 10.1007/s002530100810 pmid: 11764765
4. Bui HT, Jansen R, Pham HT, Mundt S. Carbamidocyclophanes A-E, chlorinated paracyclophanes with cytotoxic and antibiotic activity from the Vietnamese cyanobacterium Nostoc sp. *J Nat Prod*. 2007;**70**(4):499-503. doi: 10.1021/np060324m pmid: 17311455
5. Kajiyama S-i, Kanzaki H, Kawazu K, Kobayashi A. Nostofungicidine, an antifungal lipopeptide from the field-grown terrestrial blue-green alga Nostoc commune. *Tetrahedron Lett*. 1998;**39**(22):3737-3740. doi: 10.1016/s0040-4039(98)00573-5
6. Jaki B, Heilmann J, Sticher O. New antibacterial metabolites from the cyanobacterium Nostoc commune(EAWAG 122b). *J Nat Prod*. 2000;**63**(9):1283-1285. doi: 10.1021/np000033s pmid: 11000038
7. Kanekiyo K, Lee JB, Hayashi K, Takenaka H, Hayakawa Y, Endo S, et al. Isolation of an antiviral polysaccharide, nostoflan, from a terrestrial cyanobacterium, Nostoc flagelliforme. *J Nat Prod*. 2005;**68**(7):1037-1041. doi: 10.1021/np050056c pmid: 16038544
8. Murakami M, Ishida K, Okino T, Okita Y, Matsuda H, Yamaguchi K. Aeruginosins 98-A and B, trypsin inhibitors from the blue-green alga Microcystis aeruginosa (NIES-98). *Tetrahedron Lett*.

1995;**36**(16):2785-2788. doi: 10.1016/0040-4039(95)00396-t
9. Fewer DP, Jokela J, Rouhiainen L, Wahlsten M, Koskenniemi K, Stal LJ, et al. The non-ribosomal assembly and frequent occurrence of the protease inhibitors spumigins in the bloom-forming cyanobacterium Nodularia spumigena. *Mol Microbiol*. 2009;**73**(5):924-937. doi: 10.1111/j.1365-2958.2009.06816.x pmid: 19691450
10. Pluotno A, Carmeli S. Banyasin A and banyasides A and B, three novel modified peptides from a water bloom of the cyanobacterium Nostoc sp. *Tetrahedron*. 2005;**61**(3):575-583. doi: 10.1016/j.tet.2004.11.016
11. von Elert E, Oberer L, Merkel P, Huhn T, Blom JF. Cyanopeptolin 954, a chlorine-containing chymotrypsin inhibitor of Microcystis aeruginosa NIVA Cya 43. *J Nat Prod*. 2005;**68**(9):1324-1327. doi: 10.1021/np050079r pmid: 16180807
12. Zafrir-Ilan E, Carmeli S. Eight novel serine proteases inhibitors from a water bloom of the cyanobacterium Microcystis sp. *Tetrahedron*. 2010;**66**(47):9194-9202. doi: 10.1016/j.tet.2010.09.067
13. Taori K, Paul VJ, Luesch H. Kempopeptins A and B, serine protease inhibitors with different selectivity profiles from a marine cyanobacterium, Lyngbya sp. *J Nat Prod*. 2008;**71**(9):1625-1629. doi: 10.1021/np8002172 pmid: 18693761
14. Reshef V, Carmeli S. Protease inhibitors from a water bloom of the cyanobacterium Microcystis aeruginosa. *Tetrahedron*. 2001;**57**(14):2885-2894. doi: 10.1016/s0040-4020(01)00141-7
15. Becher PG, Baumann HI, Gademann K, Jüttner F. The cyanobacterial alkaloid nostocarboline: an inhibitor of acetylcholinesterase and trypsin. *J Appl Phycol*. 2008;**21**(1):103-110. doi: 10.1007/s10811-008-9335-3
16. Reshef V, Carmeli S. New microviridins from a water bloom of the cyanobacterium Microcystis aeruginosa. *Tetrahedron*. 2006;**62**(31):7361-7369. doi: 10.1016/j.tet.2006.05.028
17. Rippka R, Stanier RY, Deruelles J, Herdman M, Waterbury JB. Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. *Microbiology*. 1979;**111**(1):1-61. doi: 10.1099/00221287-111-1-1
18. Kaushik BD. Laboratory methods for blue-green algae. New Delhi: Associated Publishing co.; 1987.
19. Desikachary TV. Cyanophyta. New Delhi: Indian council of Agricultural Research; 1959.
20. Nowruzi B, Khavari-Nejad R-A, Nejadsattari T, Sivonen K, Fewer D. A proposal for the unification of two cyanobacterial strains of Nostoc as the same species. *Rostaniha*. 2017;**17**(2):161-172.
21. Leikoski N, Fewer DP, Jokela J, Wahlsten M, Rouhiainen L, Sivonen K. Highly diverse cyanobactins in strains of the genus Anabaena. *Appl Environ Microbiol*. 2010;**76**(3):701-709. doi: 10.1128/AEM.01061-09 pmid: 20008171
22. Staub R. Ernährungsphysiologisch-autökologische Untersuchungen an der planktischen BlaualgeOscillatoria rubescens DC. *Schweizerische Zeitschrift für Hydrologie*. 1961;**23**(1):82-198. doi: 10.1007/bf02505618
23. Kotai J. Instructions for preparation of modified nutrient solution Z8 for algae. *Norwegian Inst Water Res*. 1972;**11**(69):5.
24. Niva A. Estimation of algal growth potential. *Norwegian Inst Water Res*. 1976;**11**(82):2-25.
25. Hoffmann D, Hevel JM, Moore RE, Moore BS. Sequence analysis and biochemical characterization of the nostopeptolide A biosynthetic gene cluster from Nostoc sp. GSV224. *Gene*. 2003;**311**:171-180. doi: 10.1016/S0378-1119(03)00587-0 pmid: 12853152
26. Jokela J, Herfindal L, Wahlsten M, Permi P, Selheim F, Vasconcelos V, et al. A novel cyanobacterial nostocyclopeptide is a potent antitoxin against microcystins. *Chembiochem*. 2010;**11**(11):1594-1599. doi: 10.1002/cbic.201000179 pmid: 20575133
27. Nowruzi B, Haghighat S, Fahimi H, Mohammadi E. Nostoc cyanobacteria species: a new and rich source of novel bioactive compounds with pharmaceutical potential. *J Pharm Health Serv Res*. 2018;**9**(1):5-12. doi: 10.1111/jphs.12202
28. B. N, Khavari-Nejad RA, Sivonen K, Kazemi B, Najafi F, Nejadsattari T. A gene expression study on strains of Nostoc (Cyanobacteria) revealing antimicrobial activity under mixotrophic conditions. *African J Biotech*. 2012;**11**(51):11296-11308. doi: 10.5897/ajb11.4129
29. Chlipala GE, Mo S, Orjala J. Chemodiversity in freshwater and terrestrial cyanobacteria - a source for drug discovery. *Curr Drug Targets*. 2011;**12**(11):1654-1673. doi: 10.2174/138945011798109455 pmid: 21561419
30. Burja AM, Banaigs B, Abou-Mansour E, Grant Burgess J, Wright PC. Marine cyanobacteria—a prolific source of natural products. *Tetrahedron*. 2001;**57**(46):9347-9377. doi: 10.1016/s0040-4020(01)00931-0
31. Tan LT. Bioactive natural products from marine cyanobacteria for drug discovery. *Phytochemistry*. 2007;**68**(7):954-979. doi: 10.1016/j.phytochem.2007.01.012 pmid: 17336349
32. Matthew S, Ratnayake R, Becerro MA, Ritson-Williams R, Paul VJ, Luesch H. Intramolecular modulation of serine protease inhibitor activity in a marine cyanobacterium with antifeedant properties. *Mar Drugs*. 2010;**8**(6):1803-1816. doi: 10.3390/md8061803 pmid: 20631871
33. Tooming-Klunderud A, Rohrlack T, Shalchian-Tabrizi K, Kristensen T, Jakobsen KS. Structural analysis of a non-ribosomal halogenated cyclic peptide and its putative operon from Microcystis: implications for evolution of cyanopeptolins. *Microbiology*. 2007;**153**(Pt 5):1382-1393. doi: 10.1099/mic.0.2006/001123-0 pmid: 17464052
34. Kalaitzis JA, Lauro FM, Neilan BA. Mining cyanobacterial genomes for genes encoding complex biosynthetic pathways. *Nat Prod Rep*. 2009;**26**(11):1447-1465. doi: 10.1039/b817074f pmid: 19844640
35. Jaspars M, Lawton LA. Cyanobacteria - a novel source of pharmaceuticals. *Curr Opin Drug Discov Devel*. 1998;**1**(1):77-84. pmid: 19649793
36. Ahmadi Moghadam A, Nowruzi B. A new report of n fixation by two species of cyanobacteria. *Iran J Sci Technol*. 2008;**32**(2):147-151.
37. Singh S, Kate BN, Banerjee UC. Bioactive compounds from cyanobacteria and microalgae: an overview. *Crit*

Rev Biotechnol. 2005;**25**(3):73-95. doi: 10.1080/07388550500248498 pmid: 16294828

38. Sivonen K, Börner T. Bioactive compounds produced by cyanobacteria; The cyanobacteria: molecular biology, genomics and evolution. Norfolk: Caister academic press; 2008. 159-197 p.
39. Gademann K, Portmann C. Secondary Metabolites from Cyanobacteria: Complex Structures and Powerful Bioactivities. *Curr Org Chem.* 2008;**12**(4):326-341. doi: 10.2174/138527208783743750
40. Golakoti T, Yoshida WY, Chaganty S, Moore RE. Isolation and Structures of Nostopeptolides A1, A2 and A3 from the Cyanobacterium Nostoc sp. GSV224. *Tetrahedron.* 2000;**56**(46):9093-9102. doi: 10.1016/s0040-4020(00)00764-x
41. Golakoti T, Yoshida WY, Chaganty S, Moore RE. Isolation and structure determination of nostocyclopeptides A1 and A2 from the terrestrial cyanobacterium Nostoc sp. ATCC53789. *J Nat Prod.* 2001;**64**(1):54-59. doi: 10.1021/np000316k pmid: 11170666
42. Luesch H, Hoffmann D, Hevel JM, Becker JE, Golakoti T, Moore RE. Biosynthesis of 4-methylproline in cyanobacteria: cloning of nosE and nosF genes and biochemical characterization of the encoded dehydrogenase and reductase activities. *J Org Chem.* 2003;**68**(1):83-91. doi: 10.1021/jo026479q pmid: 12515465
43. Becker JE, Moore RE, Moore BS. Cloning, sequencing, and biochemical characterization of the nostocyclopeptide biosynthetic gene cluster: molecular basis for imine macrocyclization. *Gene.* 2004;**325**:35-42. doi: 10.1016/j.gene.2003.09.034 pmid: 14697508
44. Hunsucker SW, Klage K, Slaughter SM, Potts M, Helm RF. A preliminary investigation of the Nostoc punctiforme proteome. *Biochem Biophys Res Commun.* 2004;**317**(4):1121-1127. doi: 10.1016/j.bbrc.2004.03.173 pmid: 15094385
45. Fewer DP, Jokela J, Paukku E, Osterholm J, Wahlsten M, Permi P, et al. New structural variants of aeruginosin produced by the toxic bloom forming cyanobacterium Nodularia spumigena. *PLoS One.* 2013;**8**(9):e73618. doi: 10.1371/journal.pone.0073618 pmid: 24040002
46. Liu L, Jokela J, Wahlsten M, Nowruzi B, Permi P, Zhang YZ, et al. Nostosins, Trypsin Inhibitors Isolated from the Terrestrial Cyanobacterium Nostoc sp. Strain FSN. *J Nat Prod.* 2014;**77**(8):1784-1790. doi: 10.1021/np500106w pmid: 25069058
47. Liu L, Jokela J, Herfindal L, Wahlsten M, Sinkkonen J, Permi P, et al. 4-Methylproline guided natural product discovery: co-occurrence of 4-hydroxy- and 4-methylprolines in nostoweipeptins and nostopeptolides. *ACS Chem Biol.* 2014;**9**(11):2646-2655. doi: 10.1021/cb500436p pmid: 25203327
48. Shim SH, Chlipala G, Orjala J. Isolation and structure determination of a proteasome inhibitory metabolite from a culture of Scytonema hofmanni. *J Microbiol Biotechnol.* 2008;**18**(10):1655-1658. pmid: 18955814
49. Turk B. Targeting proteases: successes, failures and future prospects. *Nat Rev Drug Discov.* 2006;**5**(9):785-799. doi: 10.1038/nrd2092 pmid: 16955069
50. Smith JL, Boyer GL, Zimba PV. A review of cyanobacterial odorous and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture.* 2008;**280**(1-4):5-20. doi: 10.1016/j.aquaculture.2008.05.007
51. Welker M, von Dohren H. Cyanobacterial peptides - nature's own combinatorial biosynthesis. *FEMS Microbiol Rev.* 2006;**30**(4):530-563. doi: 10.1111/j.1574-6976.2006.00022.x pmid: 16774586
52. Baumann HI, Keller S, Wolter FE, Nicholson GJ, Jung G, Sussmuth RD, et al. Planktocyclin, a cyclooctapeptide protease inhibitor produced by the freshwater cyanobacterium Planktothrix rubescens. *J Nat Prod.* 2007;**70**(10):1611-1615. doi: 10.1021/np0700873 pmid: 17935298
53. Richman DD. HIV therapeutics. *Science.* 1996;**272**(5270):1886-1888. doi: 10.1126/science.272.5270.1886 pmid: 8658159
54. Mohammadian M, Farzampanah L, Behtash-oskouie A, Majdi S, Mohseni G, Imandar M, et al. A biosensor for detect nitrite (NO2-) and hydroxylamine (nh2oh) by using of hydroxylamine oxidase and modified electrode with ZnO nanoparticles. *Int J Electrochem Sci.* 2013;**8**(9):11215-11227.
55. Borowitzka MA. Pharmaceuticals and agrochemicals from microalgae. In: Cohen Z, editor. Chemicals from microalgae: Taylor and Francis Ltd; 1999. p. 313-352.
56. Koivunen E, Itkonen O, Halila H, Stenman UH. Cyst fluid of ovarian cancer patients contains high concentrations of trypsinogen-2. *Cancer Res.* 1990;**50**(8):2375-2378. pmid: 2180568
57. Nyberg P, Ylipalosaari M, Sorsa T, Salo T. Trypsins and their role in carcinoma growth. *Exp Cell Res.* 2006;**312**(8):1219-1228. doi: 10.1016/j.yexcr.2005.12.024 pmid: 16457812
58. Wang Y, Luo W, Reiser G. Trypsin and trypsin-like proteases in the brain: proteolysis and cellular functions. *Cell Mol Life Sci.* 2008;**65**(2):237-252. doi: 10.1007/s00018-007-7288-3 pmid: 17965832