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 promising 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* carboline, 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 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). Homogenia were used for the purification and preparation of the unialgal 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$_2$; quality 5.7; 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$_2$O + 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 + H₂O + 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 chromatogram (TIC) and extracted ion chromatogram (EIC) of the protonated [MH⁺] new peptide aldehyde compound (450.2 m/z), [M + H₂O + 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 MS₂ 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, except, 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+O + 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-Ile, and argininal/argininol.
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) (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-proteogenic 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 Nostocycleopeptide 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 Nostocycleopeptin 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 Microcystis, Anabaena, Planktothrix/Oscillatoria and Nostoc \(^{50}\).

Quite a lot of protease inhibitors extracted from cyanobacteria have been studied including aeruginopeptins, anabaenopeptilides, cyanopeptins, micropeptins, nostopeptins, oscillapeptins, miroviridins, aeruginosins, microcins, anabaenopeptins, oscillamides, banyasin A, largamides A, oscillapetins, miroviridins, aeruginosins, microcins, anabaenopeptins, oscillamides, banyasin A, largamides A-H, lyngbyatins 4-7, planktoycydin, kempeptines 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.

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