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Isolation and Structure Determination of a Proteasome Inhibitory Metabolite from a Culture of *Scytonema hofmanni*

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Cyanobacteria, blue-green algae, are a rich source of bioactive secondary metabolites with many potential applications. The ubiquitin-proteasome proteolytic system plays an important role in selective protein degradation and regulates cellular events including apoptosis. Cancer cells are more sensitive to the proapoptotic effects of proteasome inhibition than normal cells. Thus, proteasome inhibitors can be potential anticancer agents. Cyanobacteria have been shown to be a rich source of highly effective inhibitors of proteases. A proteasome inhibitor was screened from an extract of the culture of *Scytonema hofmanni* on the basis of its inhibitory activity, which led to the isolation of nostodione A with an IC_{50} value of 50 μM. Its structure was determined by spectroscopic methods such as ^{1}H-NMR and ESI-MS spectral analyses.

Keywords: Cyanobacteria, proteasome inhibitor, *Scytonema hofmanni*, structure determination, nostodione A

Cyanobacteria, blue-green algae, are a rich source of secondary metabolites, such as alkaloids, phenolic dilactones, polyketides, macrolides, or peptides, each of which originate from different pathways and show a broad spectrum of biological activities [5, 12, 24]. Some of these metabolites are potent toxins [3], and initially caught the attention of the food and health industries [9]. Selective pharmacological activities have also been observed; for example, antiviral [22], antitumor [32], or immunosuppressive effects [34] were reported for a variety of these compounds, and some cyanobacterial metabolites were even introduced as molecular tools in protein phosphatase research [31].

Both marine and non-marine species have proven to be a rich source of diverse metabolites [2, 6, 7, 11, 13]. Of marine cyanobacteria, *Lyngbya majuscula* is one of the most prolific producers of secondary metabolites displaying significant structural diversity and biological activity, including antillatoxin [25], apratoxins [17, 18], and kalkitoxin [33], which are each potentially novel pharmaceuticals. Of the non-marine cyanobacteria, the freshwater cyanobacterium, *Scytonema hofmanni*, is known to be highly toxic toward other cyanobacteria and green algae [19]. It has been suggested that *Scytonema hofmanni* produces allelopathic substances like the chlorine-containing γ-lactone, allowing this slow-growing organism to compete with more prolific organisms [27]. Besides this, several depsipeptides with biological activities, especially protease inhibition, have been reported from this species [20, 21].

However, cyanobacterial cultures grow particularly slowly and have low biomass yields, which make it difficult to study these promising microorganisms. We herein employed a micro NMR tube with high-field NMR to overcome this barrier.

The promising biological role of the ubiquitin protease pathway (UPP) in cancer therapy has emerged recently. The ubiquitin-proteasome proteolytic system plays an important role in selective protein degradation and regulates cellular events, including cell-cycle progression, apoptosis, and inflammation [23]. Since proteasomes interact primarily with endogenous proteins, inhibition of the proteasome may block the signaling action of the transcription factor NF-κB and, thus, inhibit the completion of the cell cycle and the mitotic proliferation of cancerous cells, leading to cell death by apoptosis, and inhibition of angiogenesis and metastasis. The potential of specific proteasome inhibitors to function as anticancer agents is now of considerable interest in the drug discovery process [26]. Preclinical evaluation has shown that cancer cells are more sensitive to the proapoptotic effects of proteasome inhibition than normal cells [1].

Three major distinctive peptidase activities have been identified and characterized as chymotrypsin-like activity (cleaves after hydrophobic residues), trypsin-like activity

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(cleaves after basic residues), and peptidylglutaryl peptide hydrolyzing activity (cleaves after acidic residues). Unlike a typical protease, the 20S proteasome ensures that virtually all peptide bonds within a protein substrate are susceptible to cleavage by processing within several proteolytic chambers [30].

Cyanobacteria are a rich source of highly effective inhibitors of proteases [4, 8, 10, 15, 29]. As part of a project directed toward the discovery of anticancer agents from cyanobacteria, *Scytonema hofmanni* was isolated from Lake Michigan and cultured in the laboratory. The methylene chloride/methanol-soluble extract of this species was found to inhibit the chymotrypsin-like activity of the proteasome *in vitro*. Bioactivity-guided fractionation of this extract led to the isolation of nostodione A (**1**). Here, we report the isolation, structure determination, and proteasome inhibitory activity of compound **1**.

Scytonema hofmanni was collected from Lake Michigan and cultured in three 1-l Erlenmeyer flasks, each containing 350 ml of media BG-11, under continuous illumination with fluorescent lamps at 29 μmol m^{-2} s^{-1}. The strain was harvested after 5 weeks and separated by centrifugation. Filtration was used as an alternative harvest method for samples containing enclosed air that did not pellet well during the centrifugation. Harvested cells were lyophilized and kept in a freezer at −70°C until extraction. The lyophilized cells were extracted with CH_2Cl_2/MeOH (1:1) by maceration and then dried. The dried extract showed potent proteasome inhibition activity. The commercially available 20S Proteasome Assay Kit (BIOMOL International LP) was used for determining proteasome inhibition. The assay buffer was added to the blank and control wells, and dilutions of the positive control were added to the inhibitor wells. Dilutions of the test substances (extract, fractions, or pure isolates) were prepared in proteasome assay buffer and added to the appropriate wells. The enriched proteasome fraction was diluted to a final assay concentration of 50 μg/ml using assay buffer. This dilution was then added to each well, and then the plate was preincubated for 10 min at 37°C to allow inhibitor/enzyme interactions. The enzyme reaction was started by adding Suc-LLVY-AMC substrate to a final concentration of 10 μM. The chymotrypsin-like proteasome activity was determined by measuring the generation of free AMC using a fluorescent plate reader capable of excitation at a wavelength of 360 nm and detection of emitted light at 460 nm. A potent proteasome inhibitor from natural resources, epoximicin, was used as the positive control (IC_{50} 7.5 μM).

The crude extract (280 mg) obtained from three 3-l Erlenmyer flasks was subjected to silica gel column chromatography using a $CHCl_3$-MeOH solvent to afford 15 fractions. A 9.5-mg portion of the fractions eluted with 100:5 $CHCl_3$-MeOH was further separated by reversed-phase HPLC (20% CH_3CN in H_2O for 10 min and then 20 to 100% CH_3CN in H_2O for 30 min) to afford two different peak collections. However, the ESI-MS and ^{1}H-NMR spectra for these two collections were exactly the same. The molecular ion peaks of each peak collection have the same pseudomolecular ion peak at *m/z* 288 $[M-H]^-$ on the negative mode of ESI-MS. When each peak collection was subjected to reversed-phase HPLC, using the same conditions as above, they showed an identical chromatographic pattern to that of the original sample (R_t=12 min, R_t=13 min, C18-packed column, eluent; 20% CH_3CN in H_2O for 10 min, 20% CH_3CN in H_2O 100% CH_3CN for 30 min; flow rate 2 ml/min). Even though several attempts to separate the two by different conditions of HPLC analysis were made, these produced the same results as those of the original sample. Therefore, we assume that these peak collections represent a pair of tautomers. This hypothesis was confirmed by comparison with the literature [14].

Nostodione A (1): orange solid; UV λmax^{MeOH} (log ε): 383 (4.24), 300 (3.89), 280 (3.78) nm; ^{1}H NMR (400 MHz, acetone-d_6): major tautomer, δ 7.90 (1H, d, *J*=8.0 Hz), δ 7.86 (2H, d, *J*=8.0 Hz), δ 7.60 (1H, d, *J*=8.0 Hz), δ 7.37 (1H, d, *J*=7.8, 8.0 Hz), δ 7.33 (1H, s), δ 7.32 (1H, dd, *J*= 7.8, 8.0 Hz), 7.05 (2H, d, *J*=8.0 Hz); minor tautomer, δ 8.18 (2H, d, *J*=8.0 Hz), δ 7.84 (1H, d, *J*=8.0 Hz), δ 7.59 (1H, d, *J*=8.0 Hz), δ 7.37 (1H, dd, *J*=7.8, 8.0 Hz), δ 7.32 (1H, dd, *J*=7.8, 8.0 Hz), δ 7.28 (1H, s), δ 6.94 (2H, d, *J*= 8.0 Hz); ESI-MS observed *m/z* 288.4 $[M-H]^+$.

In the ^{1}H-NMR spectrum, exactly two sets of signals appeared. The ratio of the two tautomers proved to be 3:1 in acetone-d_6 based on integration of each signal in the ^{1}H-NMR spectrum. Two correlating doublets at δ 7.86 (δ 8.18: minor tautomer) and δ 7.05 (δ 6.94: minor tautomer) showed an A_2B_2 aromatic proton system, indicating the presence of a 1,4-disubstituted benzene moiety that was presumed to be a *p*-hydroxyphenyl group owing to their chemical shifts and ^{1}H-^{1}H COSY spectrum. In addition, an ABXY aromatic proton system was observed at δ 7.90 (1H, d, *J*=8.0 Hz), δ 7.37 (1H, dd, *J*=7.8, 8.0 Hz), δ 7.32 (1H, dd, *J*=7.8, 8.0 Hz), and δ 7.60 (1H, d, *J*=8.0 Hz), which were assigned to be a 1,2-disubstituted benzene ring on the basis of the ^{1}H-^{1}H COSY spectrum as well as the splitting pattern. A singlet at δ 7.33 (δ 7.28: minor tautomer) was also assigned to be an olefinic proton. The UV spectrum of this compound was characteristic, with the λ_{max} at 280, 300, and 383 nm, and this was very close to that of 3-indoleglycolic acid. This suggests that this compound has an indole moiety fused with an α-diketone system [16]. On the basis of the above results, as well as a comparison with the literature [14], the compound was determined to be nostodione A (**1**), as shown in Fig. 1.

Nostodione A is known to exist as a tautomer in solution [14]. The most significant difference in chemical shifts appeared in the 1,4-disubstituted moiety. Kobayashi *et al.* [14] suggested that each tautomer (1A and 1B) is converted

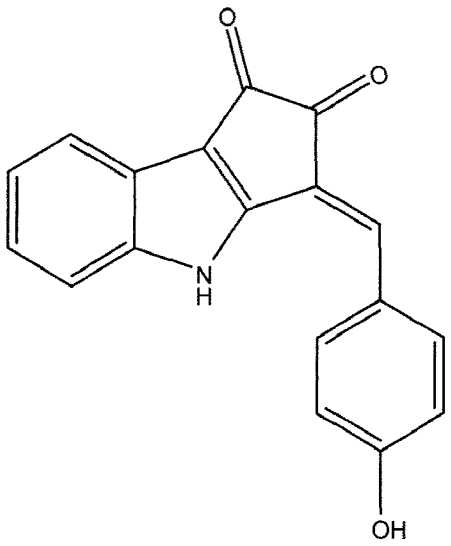

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Fig. 1. Chemical structure of nostodione A (**1**).

through an intermediate (1C), as shown in Fig. 2. Although we could not obtain the NOESY spectrum as cited in the literature [14] because of the limited amount of sample, we propose that **1A** is predominant on the basis of the energy minimization study as well as comparison of the NMR data with the literature [14].

Compound **1** has been previously obtained by ozonolysis of the cyanobacteria sunscreen pigment, scytonemin [28], except that **1** was reported to be isolated from *Nostoc commune*. Both nostodione A (**1**) and scytonemin are proposed to be biogenetically derived from condensation of tryptophan and phenylpropanoid-derived subunits.

To the best of our knowledge, this is the first report of the isolation of nostodione A from *Scytonema hofmanni*, even though much chemical investigation of that cyanobacterium has been carried out. More interestingly, this is the second report on the isolation of nostodione A in nature.

Nostodione A (**1**) showed a moderate activity in the proteasome inhibitory assay with an IC_{50} value of 50 μM.

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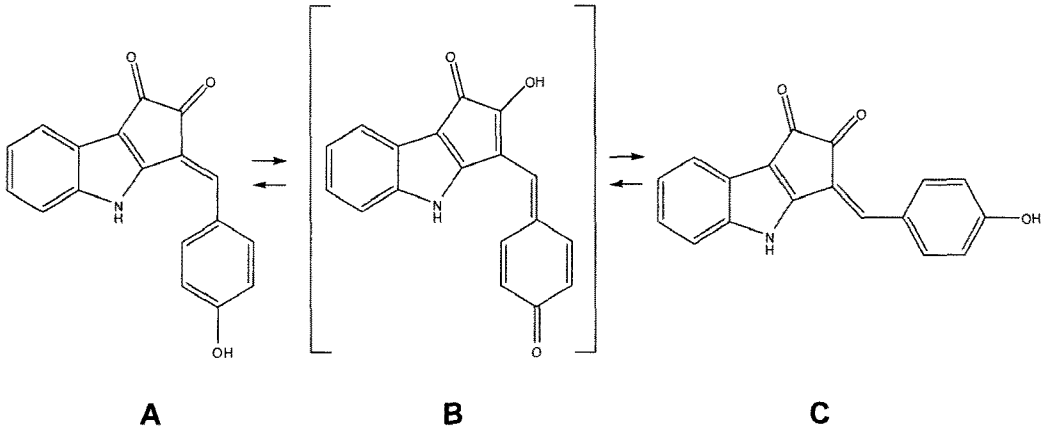

Fig. 2. The tautomerism of nostodione A.

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