NOVEL VIOLET PIGMENT, NOSTOCINE A, AN EXTRACELLULAR METABOLITE FROM CYANOBACTERIUM *NOSTOC SPONGIAEFORME*

Kazumasa Hirata,[a] Hirofumi Nakagami,[a] Jun Takashina,[a] Taifo Mahmud,[a] Motomasa Kobayashi,[a] Yasuko In,[b] Toshimasa Ishida,[b] and Kazuhisa Miyamoto*[a]

[a]Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan
[b]Osaka University of Pharmaceutical Sciences, 4-20-1, Nasahara, Takatsuki, Osaka 569-11, Japan

Abstract - A freshwater cyanobacterium, *Nostoc spongiaeforme* TISTR 8169, produced and excreted a novel violet pigment, nostocine A (**1**), which had broad-spectrum of growth inhibitory activity. The chemical structure of **1** was determined as 6-methyl-1,3,6,7,8-pentaazabicyclo[4.3.0]nonan-1,3,5(7)-trien-9-one on the bases of physicochemical evidence and an X-ray crystallographic analysis.

Cyanobacteria are well known to produce extracellular metabolites with a wide variety of biological activities including toxins, antibiotics, fungicides, and antineoplastic agents.[1-5] However, there are very few studies on the cyanobacterial extracellular pigments.[6] We have isolated a novel violet pigment which was produced and excreted into a medium by *Nostoc spongiaeforme* TISTR 8169, a filamentous N_2-fixing cyanobacterium. The pigment named nostocine A (**1**) has an unique nitrogen-rich ring

structure and shows a growth inhibitory activity with broad-spectrum against various organisms and cultured cells. In this study, the chemical structure of **1** was elucidated on the bases of physicochemical properties and an X-ray crystallographic analysis.

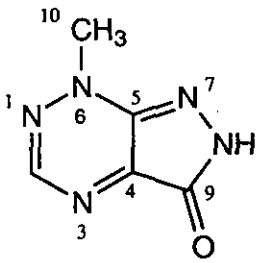
The violet pigment was adsorbed in charcoal from the culture medium (2 liter) of *Nostoc spongiaeforme* TISTR 8169 and reextracted with methanol. The methanol-soluble portion was purified by Sephadex LH-20 and reversed-phase hplc to give nostocine A (**1**) (10 mg). Nostocine A (**1**) was obtained as a violet powder and crystallized from aqueous methanol to give black needles of mp 171-172°C. The Elms of **1** gave a molecular ion peak at *m/z* 151, the composition of which was defined as $C_5H_5N_5O$ from the high-resolution ms analysis. The ir spectrum of **1** showed absorption bands assignable to amide (3482 and 1676 cm^{-1}) and imine (1627 cm^{-1}) groups, while the uv spectrum of **1** showed absorption maxima at 555 nm (ε = 900, br), 277 (3000, sh), and 239 (6400), which suggested the presence of a poly-conjugated chromophore in **1**.

The ^{1}H-nmr spectrum of **1** taken in CD_3OD exhibited a very simple signal pattern ascribable to one methyl proton at δ 4.02 (3H, s) and one olefinic proton at δ 8.74 (1H, s). On the other hand, the ^{1}H-nmr spectrum of **1** taken in $CDCl_3$ showed another new broad singlet signal at δ 9.57, which was presumably assignable to a secondary amine proton. The ^{13}C-nmr spectrum of **1** disclosed the presence of one methyl carbon (δc 42.3), one doublet olefinic carbon (δc 143.5), and three singlet olefinic carbons (δc 139.4, 155.4, and 161.8). In the HMBC experiment of **1**, the ^{1}H-^{13}C correlations were observed only between methyl proton and quaternary carbon at δc 139.4 and between olefinic proton and another quaternary carbon at δc 155.4. However, any further information was not available from its nmr analysis including 2D-nmr for making a complete identification of the chemical structure of **1**.

The reduction of **1** with sodium borohydride or lithium borohydride and the catalytic

hydrogenation of **1** with Pd/C, Pd black, or Pt black gave decolorized product, which was immediately reoxidized back to violet pigment on exposure to air.

In order to confirm the chemical structure of **1**, we attempted to prepare a crystal suitable for X-ray crystallographic analysis. The crystal of **1** from aqueous methanol was not good for this purpose. The derivatization (*e.g.* *N*-acylation and *N*-methylation) of **1** have been failed. Therefore, we tried to prepare a crystal of the salt of **1** by adding one equivalent of several acids. Fortunately, by the crystallization from acetonitrile-aqueous sulfuric acid, **1** gave a fragile single crystal, which was then subjected to X-ray crystallographic analysis.



nostocine A (**1**)

Consequently, the chemical structure of nostocine A (**1**) has been determined to be 6-methyl-1,3,6,7,8-pentaazabicyclo[4.3.0]-nonan-1,3,5(7)-trien-9-one and its perspective drawing is shown in Figure 1.

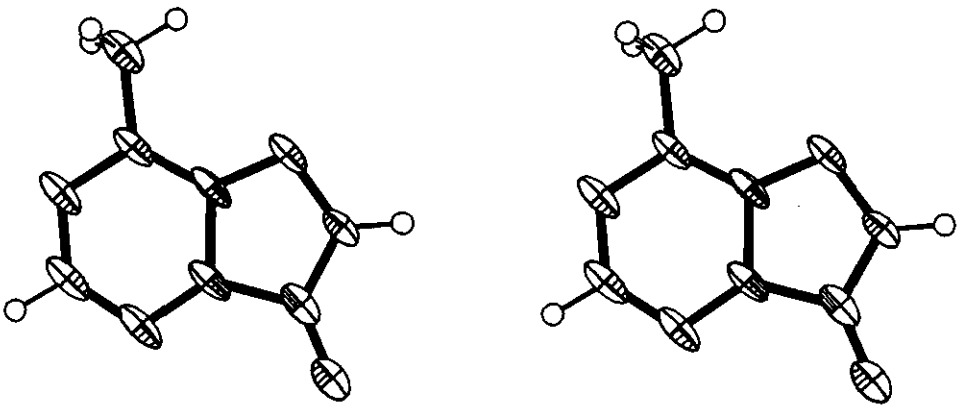


Figure 1 Perspective drawing of nostocine A (**1**)

It is noteworthy that the heterocyclic skeleton of nostocine A (**1**) is very rare and the first example isolated from cyanobacteria. So far, pseudoiodine[7] and reumycin,[8] the only two compounds related to nostocine A (**1**) have been found from bacteria and actinomycetes. It is known that cyanobacteria produce a wide variety of intracellular pigment (*e.g.* carotenoids and xanthophylls) as light-shielding properties. However, there is few information about biological role of extracellular pigment produced by cyanobacteria. Some bioactive compounds were produced and excreted from freshwater algae including cyanobacteria.[9-11] It has been suggested that a role of these compounds in nature may be an allelopathic effect. In our preliminary study on the biological activities of nostocine A (**1**), a growth inhibitory activity towards microorganisms, algae, cultured plant tissues, and established animal cell lines was observed (detailed results will be reported elsewhere). The result suggest that **1** may have also allelopathic effect like antibiotic and algicide to keep own species in its natural inhabiting environment.

EXPERIMENTAL

The ir spectrum was obtained with a Horiba FT-200 spectrophotometer. The ^{1}H- and ^{13}C-nmr were measured with JNM-EX270 (270MHz) and JNM-LA400 (400MHz) spectrometers, respectively. The uv spectrum was obtained with a Hitachi 330 spectrometer. The EI-ms and high-resolution ms were recorded on a JMS-DX303 and a JNM-A400 mass spectrometer, respectively. Melting point was determined on a Yanagimoto micro-melting point apparatus and recorded as read.

Cultivation and Isolation *Nostoc spongiaeforme* TISTR 8169 was a gift from Dr. A. Mahakhant of the Thailand Institute of Scientific and Technological Research (TISTR). It was cultivated in 1 liter culture bottle at 30°C under continuous illumination with 10 W/m^2 white fluorescent light in a modified No.18 medium at pH 7.5

aerated by air mixed with 1% CO_2 at 30 ml/min. The medium contained 70 mg NaCl, 380 mg $MgSO_4 \cdot 7H_2O$, 106 mg $CaCl_2 \cdot H_2O$, 10 mg $Fe_2(SO_4)_3 \cdot nH_2O$, 27 mg EDTA · 2Na, 600 mg K_2HPO_4, 3 mg H_3BO_3, 2 mg $MnSO_4 \cdot 4H_2O$, 8 mg $Na_2MoO_4 \cdot 2H_2O$, 0.3 mg $ZnSO_4 \cdot 7H_2O$, 0.08 mg $CuSO_4 \cdot 5H_2O$, and 0.04 mg $CoCl_2 \cdot 6H_2O$ in 1 liter. After removal of grown cells by filtration in the stationary phase, the culture medium (2 liter) was mixed with activated charcoal powder (Darco G-60) (10 g). The charcoal was collected by filtration and the adsorbed compounds were eluted with methanol and concentrated under reduced pressure. The methanol-extract was dissolved in ethanol and applied to gel-filtration column (15 mm x 500 mm, Sephadex LH-20, Pharmacia). Elution was carried out with ethanol at 30ml/h and violet-colored fractions were combined and concentrated to give crude pigment fraction (12 mg). The pigment fraction was further purified by a hplc system [column, Capcell Pak C18 (6 mm x 150 mm, Shiseido Co. LTD., Japan); mobile phase, 50% acetonitrile in water; flow rate, 1 ml/min; detection, 239 nm] to give nostocine A (**1**) (10 mg).

Physical Data for Nostocine A (1) Nostocine A (**1**), a violet powder or black needles of mp 171-172 °C (MeOH). EI-ms *m/z* : 151 (M^+). High-resolution EIms *m/z* : Calcd for $C_5H_5N_5O$: 151.0494. Found : 151.0493. Ir ν_{max} (KBr) cm^{-1}: 3482, 2463, 1676, 1627. Uv λ_{max} (MeOH) nm: 239 (6400), 277 (ε=3000, sh), 555 [900, br (540∼570 nm)]. ^{1}H-Nmr (400 MHz, CD_3OD) δ: 4.02 (3H, s, 10-H_3), 8.74 (1H, s, 2-H). ^{13}C-Nmr (100 MHz, CD_3OD) δc: 42.3 (C-10), 139.4 (C-5), 143.5 (C-2), 155.4 (C-4), 161.8 (C-9).

Crystallographic Data for the Crystalline of Nostocine A (1) X-Ray measurements were made on Rigaku AFC7R diffractometer with graphite monochromated Cu Kα radiation (λ=1.5418 ±) and a 12 kW rotating anode generator.

Unit-cell dimensions were determined by a least-squares refinement using the setting angles of 20 carefully centered reflections in the range of $20^{\circ}<\Theta<40^{\circ}$. The weak X-ray reflectional intensities ($Fo<3\sigma(Fo)$) were rescanned to ensure good counting statistics. The stationary background counts were recorded on each side of the reflections. Four standard reflections were monitored for every 100 reflection intervals and showed no significant time dependence. The intensities were corrected for Lorentz and polarization effects, but not for absorption.

The structure was solved by direct method with MULTAN87 program,[12] and refined by the full matrix least-squares method with anisotropic temperature factors for non-hydrogen atoms using the CRYSTAN GM program package.[13] All hydrogen atoms were calculated based on the stereochemical requirement, and included only for the calculation of structure factors. All numerical calculations were carried out at the Computation Center, Osaka University of Pharmaceutical Sciences.

Crystal Data and Data Collection of 1 Formula : $C_5H_5N_5O \cdot H_2O$, Mr = 169.14. Orthorhombic, a (Å) = 6.863 (2), b (Å) = 16.631 (2), c (Å) = 6.385 (2), V(Å^3) = 728.7 (2). Space group $P2_12_12_1$, Z = 4, Dx = 1.542 g $\cdot$ cm^{-3} μ(Cu-Kα)(cm^{-1}) = 10.11. Crystal size (mm^3): 0.3 x 0.1 x 0.5. No. of data with ($Fo<3\sigma(Fo)$) 528. No. of variables = 114. R_F = 0.057. R_{wF} = 0.079. Goodness of fit = 0.953.

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