Nostodione A, a Novel Mitotic Spindle Poison* from a Blue-Green Alga *Nostoc commune*

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Nostodione A, Mitotic Spindle Poison, Blue-Green Alga, *Nostoc commune,* Sea-Urchin Eggs

A novel antimitotic compound named Nostodione A (Nd A) was isolated from a terrestrial blue-green alga *Nostoc commune.* Nostodione A disturbed the mitotic spindle formation of sea-urchin eggs and gave small spindles with low birefringence density. Nostodione A, however, had no phytotoxicity on the germination of a dicotyledonous plant *Medicago sativa.* Based on the spectral analysis and chemical degradation, the structure of Nostodione A was elucidated.

Introduction

Recently, blue-green algae (cyanobacteria) have got a lot of attention as a source of various bioactive compounds such as fungicides (Moon *et al.,* 1992), tumor promoters (Nishiwaki *et al.,* 1992), hepatotoxins (Sivonen *et al.,* 1992) and the like (Murakami *et al.,* 1991; Glombiza *et al.,* 1989). Blue-green algae have also been checked for their metabolism influenced by symbiotic or parasitic microorganisms. For example, lichens, symbiotic organisms consisting of algae and fungi, produce peculiar secondary metabolites called lichen substances (Shibata, 1976).

Nostoc commune is a blue-green alga which often grows on wet ground surface in jelly clumps consisting of polysaccharides. A few researches on the protective mechanisms of this alga against UV radiation have been conducted, and its UV-absorbing pigment was well studied (Scherer *et al.,* 1988). Our preliminary experiment of the methanolic extract of this alga showed a pronounced toxic effect on sea-urchin eggs, a dicotyledonous plant, *Medicago sativa* and on a fungus, *Cladosporium herbarum* (Kobayashi *et al.,* 1992). The methanolic extracts from algal samples collected in the different places exhibited diverse activity patterns toward the above organisms, suggesting that microorganisms residing in the jelly clumps may play an important role in the secondary metabolite production. As the first step to clarify the symbiotic relationship between the alga and microorganisms, we attempted to isolate the antimitotic principle from this alga and to determine its chemical structure.

Results and Discussion

Preliminary examination of the bioactive metabolites of Nostoc commune

Nostoc samples were collected at the campus of Okayama University (OK-1, OK-2), at Mino Park in Okayama City (Mino) and in Shiga Prefecture (Shiga-4). Each algal sample was dried and soaked in the methanol for 10 days. The methanol extracts thus obtained were used for an antimicrobial test against *Bacillus subtilis,* a spore germination inhibition test against *Cladosporium herbarum,* a cytotoxicity test against sea-urchin eggs *(Hemicentrotus pulcherrimus)* and for a seed germination inhibition test against *Medicago sativa.*

Table I indicates the minimum inhibitory concentration (MIC; μg/ml) of the each sample in 4 biological tests mentioned above. The meaning of MIC was described in the Experimental section. OK-1 showed a remarkable cytotoxicity against sea-urchin eggs and moderate inhibition activities in the other tests. On the other hand, Shiga-4 and

* In this paper, the term "mitotic spindle poison" was used for the compound which disturbed the formation of mitotic spindles of sea-urchin eggs.

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Table I. A variety of inhibitory activities* of methanol extracts of *Nostoc commune* collected from different places** in some biological tests.

Samples of *Nostoc*	Minimum inhibitory concentration [MIC; μg/ml] Test organisms			
	Bacillus subtilis	*Cladosporium herbarum*	Sea-urchin eggs	*Medicago sativa*
OK-1	160	640	12.5	20,000
OK-2	320	>2500	50	>40,000
Mino	>1250	1250	>50	>40,000
Shiga-4	>1250	>2500	>50	>40,000

* Method for each test and the meaning of minimum inhibitory concentration were described in the Experimental section.

** Samples of *Nostoc commune* were collected at the campus of Okayama University (OK-1, OK-2), at Mino Park in Okayama City (Mino) and in Shiga Prefecture (Shiga-4).

Mino had no significant activity. OK-2 had weak cytotoxicities against *Bacillus subtilis* and against sea-urchin eggs. The reason for the difference in activities among the samples was not clear but microorganisms such as bacteria, green algae and fungi residing in the jelly clumps may participate in the accumulation of active constituent(s).

Isolation and structure determination of an antimitotic metabolite

Dried samples of *Nostoc commune* (OK-1; 1.4 kg) were soaked in methanol for 10 days at room temperature. The filtered extract was evaporated to remove methanol. The aqueous concentrate was partitioned with ethyl acetate. Isolation of an active constituent was guided by a cytotoxicity test using sea-urchin eggs (Kobayashi *et al.*, 1988). A pronounced cytotoxicity was found in the ethyl acetate layer. Repeated column chromatography and recrystallization gave an active principle (9.6 mg). On the silica gel TLC, it was characterized as two orange spots with R_f 0.48, 0.53 (solvent system; isopropyl ether–ethyl acetate, 6:4). This principle gave two peaks when subjected to HPLC analysis (R_t = 15.2, R_t = 18.5; YMC packed column AQ313, eluent: 60% methanol in water, flow rate: 2 ml/min), and each component prepared by HPLC peak collection afforded an identical chromatographic pattern to that of the original sample suggesting that these components could be a pair of tautomers.

Positive color reaction of this compound toward reduced methylene-blue dye as well as the adduct formation with *ortho*-phenylenediamine was suggestive of the presence of an α-diketone group. The molecular formula, $C_{18}H_{11}NO_3$ (289.0740 calcd for $C_{18}H_{11}NO_3$, found 289.0736), was established to this dione by the high resolution MS. In the ^1H NMR spectrum, every peak was accompanied by a satellite peak, the ratio of the paired peaks being 3:1 (acetone-d_6). A pair of doublets (A_2B_2 splitting) appearing at δ 7.00 (δ 6.95: minor tautomer) and δ 7.76 (δ 8.17: minor tautomer) indicated the presence of 1,4-disubstituted benzene ring, and one counterpart of a pair of doublets was significantly deshielded (Δ 0.30 ppm) after acetylation. This supported that this compound possessed a *p*-hydroxyphenyl moiety. A set of aromatic protons at δ 7.33 (1H, dd, J = 7.5, 8.0 Hz), δ 7.40 (1H, dd, J = 8.0, 8.3 Hz), δ 7.62 (δ 7.58: minor tautomer) (1H, d, J = 8.3 Hz), and δ 7.92 (δ 7.85: minor tautomer) (1H, d, J = 7.5 Hz) were also assigned to 1,2-disubstituted benzene ring. A 1H singlet at δ 7.37 (δ 7.26: minor tautomer, acetone-d_6) was rationally attributed to an olefinic proton. In the acetylated form, a broad 1H singlet was observed at δ 9.11 (chloroform-*d*) and this signal was assigned to a proton (N–H) because of its disappearance by D_2O addition. In the NOE difference spectrum of the acetylated compound, saturation of the N–H proton caused an significant enhancement (*ca.* 10%) of the signal of a counterpart of the A_2B_2 splitting pattern. These spectral analyses enabled us to depict three possible structures, **1, 2,** and **3** for this diketone (Fig. 1). The UV spectrum [λ_{max} (MeOH) nm (ε): 278 (7500), 300 (9000), 382 (22,000)] of this compound was close to that of 3-indoleglyoxylic acid (Lebuhn *et al.*, 1993), suggesting that this compound could have an indole skeleton fused with α-diketone system. Furthermore, ozonolysis of this compound almost quantitatively afforded *p*-hydroxybenzaldehyde and this fragment was confirmed by HPLC and GC-MS (see Experimental). These results indicated that this compound had a novel structure and was named Nostodione A (**1,** Nd A).

In a solution, this compound is present in tautomeric forms as mentioned above. The most significant difference of chemical shifts among the all signals were seen in a counterpart of the A_2B_2

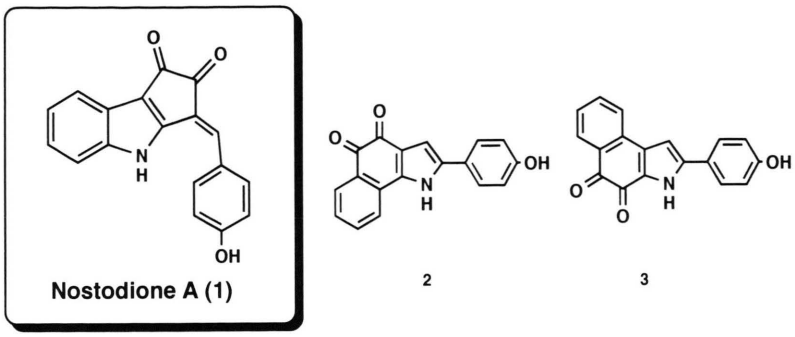

Fig. 1. The structure of the active compound (= No. 1). No. 2, 3 are possible alternatives which were ruled out from the spectral data.

splitting pattern (δ 7.76, δ 8.17: minor tautomer). In acetylated Nd A, a series of minor peaks disappeared, suggesting that tautomerism no more occurred in acetyl Nd A. Based on these findings, it would be rationalized that each tautomer (**1a** and **1b**) is converted through a tautomeric intermediate like **1c** shown in Fig. 2. The major component was easily deduced to be **1a** since the chemical shifts of acetyl Nd A were almost identical to those of the major component, and the NOE was observed between N–H and the phenyl proton(s) in acetyl Nd A as aforementioned. Moreover, the calculation of the free energy of each tautomer suggested that **1a** was a preferable structure to **1b.**

The effect of Nostodione A on sea-urchin embryo development

A cytotoxicity test was performed with sea-urchin eggs and sperm *(Hemicentrotus pulcherrimus).* At the concentration of 2.5 μg/ml, Nd A arrested the first cleavage completely. At the lower concentration, 1.25 μg/ml, the embryonic development was blocked in the morula stage, *ca.* 12 h after fertilization. It has been known that most of respiratory inhibitors block the motility of the sperm of sea-urchin, and that the decrease of the fertility rate is primarily parallel to the sperm motility. Indeed, a typical respiratory inhibitor, 2,4-dinitrophenol, suppresses the fertility (Kobayashi *et al.,* 1986). However, the sperm treated with Nd A (20 μg/ml) still held fertility, suggesting that this compound has a different mode of operation from that of respiration inhibitors.

These findings suggested that Nd A could act on the mitotic spindle. Therefore, the mitotic spindle formation of the Nd A-treated eggs was followed under a polarization microscope as well as a fluorescence microscope. When 20 μg of Nd A was administered, the normal spindles were not seen at the metaphase but small spindles with low birefringence density appeared, and the following egg division was completely suppressed.

The Nd A-treated eggs were stained by DAPI (4′,6-diamidino-2-phenylindole-dihydrochloride) when the second cleavage was supposed to complete in the normal development (120 min after fertilization) and the ploidy was quantitatively evaluated by the fluorescence intensity of the stained chromosomes under a fluorescence microscope. Fig. 3 shows the relative fluorescence intensity of Nd A-treated eggs (n = 50) and the control eggs (the s-stage cells with diploid in the first cell cycle, n = 50). High incidence (*ca.* 80%) of octaploid cells appeared in the Nd A-treated

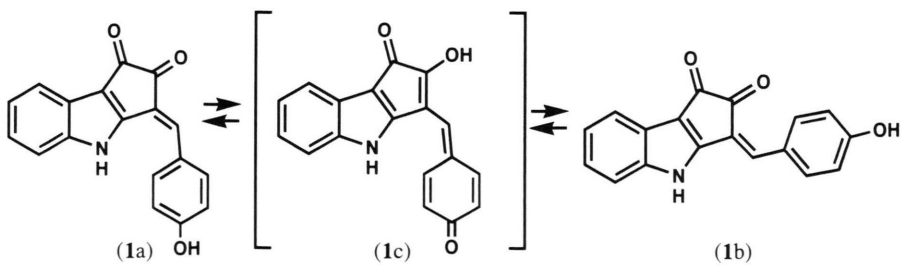

Fig. 2. The tautomerism of Nostodione A.

eggs. This result indicated that Nd A did not inhibit the DNA synthesis but suppressed the formation of mitotic spindle. This mode of operation was almost identical to that of a typical spindle poison, colcemid (Kobayashi *et al.*, 1986) (data not shown).

These findings gave a strong evidence that Nd A was a mitotic poison. Nostodione A, however, showed neither a seed germination inhibitory effect toward a dicotyledonous plant *(Medicago sativa)* nor an antifungal activity against *Cladosporium herbarum* (data not shown), suggesting that the methanolic extract should contain other bioactive components besides Nd A.

Nostodione A is not only the first compound found in blue-green algae but also possesses a very unique carbon skeleton, close to that of scytonemin (Proteau *et al.*, 1993).

More than 50 species of actinomycete and bacteria were isolated from the jelly clumps of this alga collected in different places, but none of the pure cultures of the microorganisms produced Nd A. After massive isolation efforts, the pure culture of *Nostoc commune* was established for the first time. (The methods for establishing the *Nostoc commune* pure culture will be reported elsewhere.) However, any trace amount of Nd A was not found in the pure culture. It remains uncertain whether or not some symbiotic relationship is definitely required or what organism can produce this active compound.

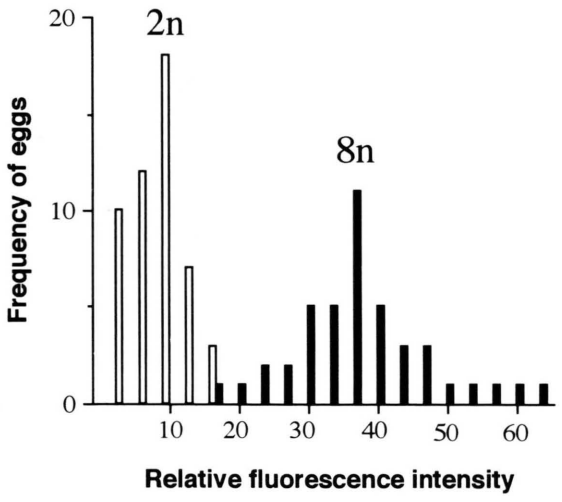

Fig. 3. The ploidy of Nostodione A-treated sea-urchin eggs. The control eggs (diploid; 2n) were stained with DAPI and fixed with PIPES stabilizing medium at 25 min after fertilization. The Nostodione A-treated eggs were stained and fixed in the same way at 120 min after fertilization. The fluorescence intensity of the stained chromosomes were separately measured in 50 eggs and shown in a histogram. The white and black columns indicate the frequency of control eggs (n = 50) and Nostodione A-treated eggs (n = 50) respectively. Most of the Nostodione A-treated eggs (*ca.* 80%) were regarded as octaploid (8n) by comparison with the control. See Experimental.

Experimental

Analytical methods

^{1}H NMR spectra were recorded with a Varian VXR-500 instrument. Mass spectra were recorded with a JEOL JMS-D300 and a Hewlett-Packard 5971 mass spectrometer with 5890 gas chromatograph. IR spectra were measured with a Nicolet 710 FT-IR, and UV spectra were measured with a JASCO Ubest-30 spectrometer. The free energy calculation of **1a** and **1b** was performed by Chem 3D plus program (Cambridge Scientific Computing, Inc.) with Macintosh Color Classic II computer (Apple Computer Inc.). The fluorescence intensity of the stained chromosomes was measured by Olympus BH2-QRFL fluorescence microscope with OSP-1 photometer.

Antimicrobial test against Bacillus subtilis and Cladosporium herbarum (Kobayashi *et al.*, 1993)

Antimicrobial tests against bacteria and fungi were performed by the 2-fold dilution method. Bacteria were precultured in 10 ml of a nutrient-broth medium for 12 h at 27 °C on a shaker, and then diluted 100-fold with the same medium. Fungi were inoculated into 10 ml of a potato-molt extract–sucrose agar medium, and incubated at 27 °C for 7 days to form a well-expanded fungal mat with spores. These spores were collected by filtration and suspended in 50 ml of a medium (0.2% glucose, 0.1% yeast extract, 0.1% citric acid, and 0.37% $Na_2HPO_4 \cdot 12H_2O$). The liquid cultures of bacteria or the spore suspension of fungi containing the various concentrations of test materials (1 μg/ml ~ 2500 μg/ml) were placed in the wells of a 96-well microplate and incubated at 27 °C for 24 h. The growth of bacteria was evaluated by the degree of turbidity of the culture with naked eye, and the spore germination was examined under a

microscope. The MIC values were determined by the comparison with the control.

Seed germination inhibition test for Medicago sativa

Various amounts of the samples (1 ~ 12 mg) was applied to paper disks (18 mm O.D.) as methanol solutions and dried *in vacuo*. The paper disks were transferred to small beakers (20 mm O.D.) and 200 µl of sterilized H_2O was added. Seven seeds of *Medicago sativa*, soaked for 12 h in sterilized H_2O, were then placed on each disk. After 58 h incubation at 25 °C, the seed germination was checked and the MIC was determined by the comparison with the control.

Cytotoxicity test for sea-urchin embryo (Sato *et al.*, 1989; Kobayashi *et al.*, 1989)

Sexually mature sea-urchins *(Hemicentrotus pulcherrimus)* were collected during the breeding season (January ~ March) from the coastal water near Ushimado Marine Laboratory in Okayama Prefecture. Eggs and sperm were obtained by KCl shedding. The sperm shaded from the genital papilla was collected with the glass capillary (dry sperm) and stored in the refrigerator (4 °C). The eggs were agitated and left for 3 min. The eggs near the surface and the bottom were removed by decantation and suction. This process promised better fertilization and synchronous development of the eggs. Throughout this experiment, more than 90% of fertilization rate was obtained. Eggs and sperm thus obtained were used for following experiments.

The eggs (*ca.* 4×10^3 eggs) was inseminated in the sperm suspension (1 ml, *ca.* 1000× dilution of the dry sperm). 5 min after fertilization, the fertilized eggs were placed in the sea water containing the samples (1.25 ~ 120 µg/ml). The following development of the eggs in the test solution was checked under an ordinary light microscope until the eggs in the control developed to the swimming blastula (24 h after fertilization). Up to this stage, the development of the eggs treated at the concentrations higher than MIC were blocked.

Evaluation of Nostodione A as a mitotic spindle poison

In the first cell cycle of the normal eggs the metaphase was 65–70 min and the first cleavage occurred at 85–90 min after fertilization in the normal development.

The fertilized eggs were treated with Nd A (20 µg/ml) at 35 min (shortly after the S-stage) and left for 45 min, and the eggs collected were added to the PIPES stabilizing medium (10 mM PIPES, 5 mM EGTA, 1 mM $MgSO_4$, 2 M glycerol, 1% Nonidet P-40; pH 6.9). The stabilized eggs were subjected to the microscopic analysis, and the shape of the mitotic spindles was compared with that of the control eggs at the metaphase using a polarizing microscope.

The DNA content was determined by DAPI-staining method (Kobayashi *et al.*, 1989). The fertilized eggs (*ca.* 4×10^3 eggs) were placed in 1 ml of the sea water in a small beaker which contained 5 µg of Nd A (5 µg/ml) at 25 min after fertilization and incubated for 95 min at 18 °C. After incubation, 5 µl of DAPI solution (1 mg/ml sea water) was added to the beaker. The treated eggs were then fixed with PIPES stabilizing medium and the fluorescence of the stained chromosomes of 50 eggs was quantitatively measured under a fluorescence microscope. The ploidy of the Nd A-treated eggs was determined on the bases of the fluorescence intensity of the control cells with diploid (n = 50: 25 min after fertilization).

Treatment of sperm with Nostodione A

5 µl of the dry sperm was suspended in 1 ml of sea water which contained Nd A (20 µg/ml) and left for 5 min. 50 µl of the sperm suspension was added to *ca.* 2×10^3 of the unfertilized eggs. Fertility was checked by observing the elevation of fertilization membrane under a microscope.

Extraction and isolation of Nostodione A

Nostoc commune (1.4 kg), collected on the campus of Okayama University, was soaked in 18 l of MeOH for 10 days at room temperature, and then filtered. The aqueous concentrate afforded after removing methanol *in vacuo* was partitioned with ethyl acetate (1 l X 2). The organic phase was concentrated to give a greenish residue (4.4 g). This extract was chromatographed on a gel filtration column (Sephadex LH-20, 100 g), eluting stepwise with 100 ml each of 0, 25, 50, 70, 80, 90 and 100% MeOH in H_2O. A marked cytotoxic activity was found in 90% MeOH eluate (600 mg), which was

further purified on a silica gel column (Wako Gel C-200, 150 g), eluting stepwise with 0, 20, 40, 50, 60, 80 and 100% EtOAc in benzene. 60% EtOAc eluate was concentrated to give amorphous powder (51 mg), which was then dissolved in MeOH (5 ml), and recrystallized in a refrigerator. Precipitates were collected by filtration and rinsed with *n*-hexane and H_2O. Nd A was obtained as an orange powder (9.6 mg, dec. 320 °C).

Properties of active compound

Nostodione A, 320 °C (dec.); EI-MS *m/z* (rel. int.): 289 [M^+] (35.8), 261 (29.9), 233 (22.3), 204 (21.6); HR-EI-MS *m/z* [M^+]: calcd for $C_{18}H_{11}NO_3$: 289.0740, found: 289.0736; UV λ_{max} (MeOH) nm (ε): 278 (7500), 300 (9000), 382 (25,000); IR ν_{max} (KBr) cm^{-1}: 3450, 2980, 2942, 1670, 1588, 1505, 1455, 1286, 1277; NMR δH (500 MHz, acetone-d_6): major tautomer, 7.00 (2H, d, J = 8.6 Hz), 7.37 (1H, s), 7.33 (1H, dd, J = 7.5, 8.0 Hz), 7.40 (1H, dd, J = 8.0, 8.3 Hz), 7.62 (1H, d, J = 8.3 Hz), 7.92 (1H, d, J = 7.5 Hz), 7.76 (2H, d, J = 8.6 Hz); minor tautomer, 6.95 (2H, d, J = 8.7 Hz), 7.26 (1H, s), 7.33 (1H, dd, J = 7.5, 8.0 Hz), 7.40 (1H, dd, J = 8.0, 8.3 Hz), 7.58 (1H, d, J = 8.3 Hz), 7.85 (1H, d, J = 7.5 Hz), 8.17 (2H, d, J = 8.7 Hz).

Acetylation of Nostodione A

Nd A (2.0 mg) was added to the solution containing *p*-TsOH (1 mg) and acetic anhydride (2 ml). After heating at 50 °C for 3 h, the reaction was stopped by adding MeOH (4 ml) and concentrated *in vacuo*. The solids were washed with aqueous $NaHCO_3$ and recovered using EtOAc (3×10 ml). The EtOAc concentrate was subjected to silica gel chromatography (Wako Gel C-300, benzene:EtOAc, 8:2). Acetyl Nd A (2.4 mg) was obtained as a yellow solid. EI-MS *m/z* (rel. int.): 331 [M^+] (26.6), 289 (78.1), 261 (100.0), 233 (49.8), 204 (37.6); UV λ_{max} (MeOH) nm (ε): 270 (66,800), 360 (12,000); IR ν_{max} (KBr) cm^{-1}: 3443, 2922, 2886, 1730, 1695, 1687, 1653, 1638, 1500, 1459, 1363, 1199; NMR δH (500 MHz, acetone-d_6): 2.34 (3H, s), 7.30 (2H, d, J = 8.6 Hz), 7.37 (1H, t, J = 8.0 Hz), 7.43 (1H, dd, J = 8.0, 7.4 Hz), 7.43 (1H, s), 7.59 (1H, d, J = 8.0 Hz), 7.89 (2H, d, J = 8.5 Hz), 7.95 (1H, d, J = 7.4 Hz).

Reaction of ortho-phenylenediamine with Nostodione A

To the benzene solution containing *ortho*-phenylenediamine (2 mg) and an acid catalyst (glacial AcOH, 1 ml) was added Nd A solution (2 mg of Nd A in 1 ml of benzene). After heating at 60 °C for 3 h the reaction mixture was cooled and concentrated *in vacuo*. On a silica gel column (Wako Gel C-200, benzene–EtOAc–isopropyl ether, 8:2:1) was chromatographed the reaction mixture to afford an adduct as a yellow solid (410 μg). EI-MS *m/z* (rel. int.): 369 [M^+] (100.0), 360 (44.1), 344 (5.9); UV λ_{max} (EtOH) nm (ε): 285 (4430), 353 (5750), IR ν_{max} (KBr) cm^{-1}: 3429, 2960, 2926, 2851, 1657 (w), 1639 (w), 1624 (w), 1601, 1579, 1262, 1160, 1075; NMR δH (500 MHz, $CDCl_3$): 7.05 (2H, d, J = 8.6 Hz), 7.27–7.32 (2H, m), 7.38 (1H, dd, J = 9.1, 2.1 Hz), 7.44 (1H, s), 7.55 (1H, ddd, J = 6.8, 6.8, 1.5 Hz), 7.63 (1H, ddd, J = 6.7, 6.8, 1.7 Hz), 7.68 (2H, d, J = 8.6 Hz), 8.00 (1H, dd, J = 6.8, 1.4 Hz), 8.06 (1H, dd, J = 9.1, 1.2 Hz), 8.19 (1H, dd, J = 6.7, 1.5 Hz), 8.57 (1H, s).

Ozonolysis of Nostodione A

Nd A (1 mg) was dissolved in methanol (200 μl) and cooled in a dry ice-acetone to which was added 100 μl of methanol saturated with ozone vapor at −78 °C. After 10 min the ozonide was treated with triphenylphosphine (50 mg). The identification of the product was established by HPLC (R_t = 5.6 min, column: YMC AQ313, eluent: 50% MeOH in water, flow rate: 2 ml/min) and GC-MS analyses using *p*-hydroxybenzaldehyde as an authentic sample. Authentic sample: *m/z* (rel. int.): 121 [M^+–H] (100), 93 (41), 65 (37), 39 (25), degradation sample: *m/z* (rel. int.): 121 [M^+–H] (100), 93 (39), 65 (33), 39 (23).

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Glombitza K.-W. and Koch M. (1989), in: Secondary Metabolites of Pharmaceutical Potential (R.-C. Cresswell, T.-A.-V. Ress and N. Shah, eds.), Longman Scientific and Technical, Burnt Mill, Harlow, Essex, Great Britain, pp. 161–238.

Kobayashi A., Akiyama K. and Kawazu K. (1993), A pterocarpan, (+)-2-hydroxypysatin from *Pisum sativum*. Phytochemistry **32,** 77–78.

Kobayashi A., Inawaka K., Kanzaki H. and Kawazu K. (1992), Abstracts of papers, the Annual Meeting of the Japan Society for Bioscience, Biotechnology and Agrochemistry, Tokyo, p. 58.

Kobayashi A., Ooe K. and Kawazu K. (1989), A new γ-dihydropyrone from *Streptomyces* sp. as a microtubule association inhibitor toward pronuclear fusion in sea-urchin eggs. Agric. Biol. Chem. **53,** 889–891.

Kobayashi A., Yata S. and Kawazu K. (1988), A new fungal naphthoquinone which stimulates the production of antifungal compounds in alfalfa callus. Agric. Biol. Chem. **52,** 3223–3227.

Kobayashi A. (1986), Screening for cell proliferation inhibitors using sea-urchin embryo assay system and their chemical structures. Nippon Nogeikagaku Kaishi **60,** 725–735.

Lebuhn M. and Hartmann A. (1993), Method for the determination of indole-3-acetic acid and related compounds of L-tryptophan catabolism in soils. J. Chromatogr. **629,** 255–266.

Moon S.-S., Chen J.-L., Moore R.-E. and Patterson G.-M.-L. (1992), Calophysin, a fungicidal cyclic decapeptide from the terrestrial blue-green alga *Calothrix fusca*. J. Org. Chem. **57,** 1097–1103.

Murakami M., Matsuda H., Makabe K. and Yamaguchi K. (1991), Oscillariolide, a novel macroride from a blue-green alga *Oscillatoria* sp. Tetrahedron Lett. **32,** 2391–2394.

Nishiwaki R.-M., Ohta T., Nishiwaki S., Sugamura M., Kohyama K., Ishikawa T., Carmichael W.-W. and Fujiki H. (1992), Liver tumor promotion by cyanobacterial cyclic peptide toxin Microcystin-LR. J. Cancer Res. Clin. Oncol. **118,** 420.

Proteau P.-J., Gerwick W.-H., Garcia-Pichel F. and Castenholz R. (1993), Abstracts of Papers, 34th Annual Meeting of the American Society of Pharmacognosy, San Diego, July, p. 6.

Sato H., Kobayashi A. and Itoh T.-J. (1989), Molecular basis of physical and chemical probes for spindle assembly. Cell Structure and Function **14,** 1–34.

Scherer S., Chen T.-W. and Böger P. (1988), New UV-A/B protecting pigment in the terrestrial cyanobacterium *Nostoc commune*. Plant Physiol. **88,** 1055–1057.

Shibata S. (1976), Lichen substances as the metabolites of lichen microbionts. Nova Acta Leopold. Suppl. **7,** 103–116.

Sivonen K., Namokoshi M., Evans W.-R., Fardig M., Carmichael W.-W. and Rinehart K.-L. (1992), Three new Microcystins, cyclic heptapeptide hepatotoxins, from *Nostoc* sp. strain 152. Chem. Res. Toxicol. **5,** 464–469.