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Liquid Chromatography/Mass Spectrometric Detection of Anatoxin-a, a Neurotoxin from Cyanobacteria

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ABSTRACT: Anatoxin-a (**1**) is the first neurotoxin obtained from a freshwater cyanobacterium, *Anbaena flos-aquae* and has a high toxicity (LD_{50}, i.p., mouse: 200 μg/kg). **1** has occurred limitedly in North America and northern Europe and no occurrence has been reported in Japan so far. Thermospray-liquid chromatography/mass spectrometry (TSP-LS/MS) was applied to the analysis of trace amount of **1** together with its degradation product (**2**) after the following optimizations: HPLC conditions, TSP operating conditions, slight modification of the reported clean-up method using a tandem cartridge system and introduction of acetyltropine as an internal standard. The established TSP-LC/MS is of great advantage to the conventional HPLC/UV and the detection limit was 500 pg. The method was used for trace analysis of **1** and **2** in laboratory strains and bloom samples and made possible the first detection of them in Japanese samples.

Several genera of fresh and brackish water cyanobacteria, *Microcystis, Oscillatoria, Anabaena, Aphanizomenon and Nodularia*, produce lethal toxins[1] Toxins from these genera include cyclic hepatotoxic hepta- or pentapeptides, and neurotoxic alkaloids. These heptapeptides and pentapeptide are named microcystins and nodularin, respectively, and show

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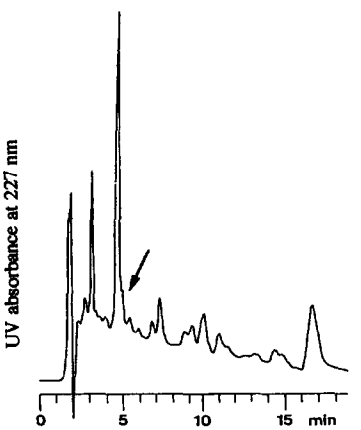
This paper is dedicated to Professor Carl Djerassi in honor of his 70 th birthday.

a tumor-promoting activity on rat liver with inhibition of protein phosphatases 1 and 2A.[2] Recently another hepatotoxin, cylindrospermopsin, was isolated from *Cylindrospermopsis raciborskii*[3] and its structure has been determined.[4]

Anatoxin-a (ANTX-a, **1**) is the first toxin obtained from a freshwater cyanobacterium, *Anabaena flos-aquae* to be chemically defined as the secondary amine, 2-acetyl-9-azabicyclo[4. 2. 1.]non-2-ene with the molecular weight of 165.[5] It is a potent nicotinic agonist which acts as a postsynaptic, depolarizing and neuromuscular blocking agent, and has a high toxicity (LD_{50}, i.p., mouse: 200 μg/kg).[6] **1** has occurred limitedly in North America and northern Europe and no occurrence has been reported in Japan so far. It is relatively unstable especially under basic conditions, and Stevens and Krieger studied stability of **1** under field conditions.[7] Additionally, it is known that *Anabaena flos-aquae* produces simultaneously a neurotoxin and hepatotoxins.[8,9] So a suitable analysis method has been required for the investigation of distribution and detoxification of **1**.

There have been several analysis methods for **1** such as GC,[10] GC/MS[11,12] and HPLC[13] and our group has also established an analysis system including solid-phase extraction and HPLC with UV detection.[14] As an example, Fig. 1 shows the high performance liquid chromatogram/UV (227 nm) of a toxic fraction of a culture strain by our analytical method. It is probably, however, difficult to accurately quantify the amount of **1** indicated by the arrow, because the amount contained in the toxic fraction is very limited and the fraction includes many contaminants. To analyze such samples a more sensitive and specific method has been required.



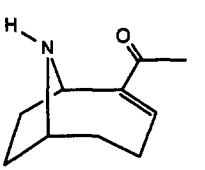


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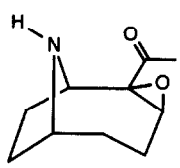
Fig. 1. High performance liquid chromatrogram of a toxic fraction of TAC210 (Arrow indicates anatoxin-a (**1**)).

For this purpose a method combining HPLC and mass spectrometry (MS) using an appropriate interface would offer significant advantages. In fact we have successfully used Frit-fast atom bombardment (FAB) LC/MS for separation and identification of microcystins in cyanobacteria.[15] Thermospray (TSP) is a relatively older interface than recently developed ones such as electrospray, but has been widely used because whole effluent can be introduced into mass spectrometer. TSP-LC/MS would be expected to provide a sensitive quantification and specific detection of **1** in various samples. As pointed out by Stevens and Krieger[7] there are several degradation products of **1** and we have also isolated an oxidative degradation product (**2**) which has no neurotoxicity. Since **2** was considered to be important for a detoxification study, it was also analyzed by the established method. This paper describes an optimization of TSP-LC/MS operating conditions and analysis results of **1** and related compounds by the established method.

RESULTS AND DISCUSSION

Optimization of TSP-LC/MS operating conditions

It is well known that it is relatively difficult to obtain reproducible results by TSP-LC/MS. This may be mainly caused by its vacuum system and heating problems of the interface. To overcome these problems and to obtain an accurate analysis, an internal standard, acetyltropin (**3**) was introduced. Although it is impossible to detect **2** and **3** by HPLC with UV detection, they were investigated together with **1** under TSP-LC/MS conditions in the present study.



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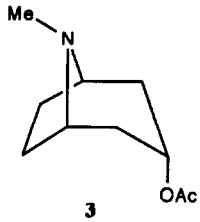

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We have established a HPLC method for analysis of **1**, in which a mobile phase, methanol:0.01M ammonium chloride=1:9, is used.[14] To apply this mobile phase to TSP-LC/MS , there are two problems: **1** shows a slight tailing and ammonium chloride is involatile. For a mobile phase to be satisfactory for use with TSP-LC/MS, the following conditions are required:

1. Some aqueous portion is reqiured.
2. Volatile inorganic salt such as ammonium acetate is added to aqueous portion.
3. Its concentration is desirable to be around 0.1M.[16]

According to these limitations, the HPLC conditions were optimized.

The retention power of **1** on a reversed phase column increased, whereas **1** showed considerable peak tailing with increasing concentration of ammonium acetate, leading to the conclusion that the practical concentration of ammonium acetate is 0.1M. Another factor, pH of the aqueous portion of mobile phase, strongly affected the resulting peak shapes of these three compounds, particularly **3**. The lower pH improved the peak tailing and retention behavior of these compounds, but the ionization efficiency of **2** was significantly decreased under acidic conditions. So the following HPLC conditions were finally established:

Column: Cosmosil 5 PH

Mobile phase: methanol:0.1M ammonium acetate (pH 5)=14:86

Flow rate: 1.2 mL/min

Reproducible analysis by TSP-LC/MS depends on control of heating in the interface. It was found that **1** vaporizes at the temperature of about 240°C, leading to the optimal conditions of heating the interface as shown in Experimental section. Since the target compounds show clearly the protonated molecules, $(M+H)^+$ in the TSP mass spectra, these ion species were selected as m/z values for selected ion monitoring (SIM).

The TSP-LC/MS was applied to the sample as shown in Fig. 1 to evaluate the optimized conditions. Figure 2 shows the TSP-LC/MS analysis data monitored at m/z 166 for **1**, m/z 182 for **2** and m/z 184 for **3**. In comparison with the HPLC chromatogram in Fig.1, **1** was surely and clearly detected by this method along with **2** and **3** which cannot be detected by HPLC (UV), suggesting that this method is applicable to quantification of trace amount of **1** and **2**. The relationship between the concentration of **1** and **2**, and peak area or peak height was evaluated four times on different days. More excellent linearity between the concentration of **1** ranging from 1 to 40 ng and peak heights was found with the correlation coefficient over 0.999 (Fig. 3). Almost same results were obtained in the case of **2**. The detection limits for both compounds were found to be about 500 pg.

In order to analyze a trace amount of a desired compound from a complicated matrix, an effective clean-up method is highly required. We have established a clean-up method including a solid phase extraction with a reversed phase carboxylic acid cartridge for analysis of **1**.[14] It was found that the clean-up method can be fully applied to the analysis of **1** and **2** without any modification. The combination of TSP-LC/MS and clean-up method made possible a sensitive, specific and reproducible analysis of **1** and **2** as described later.

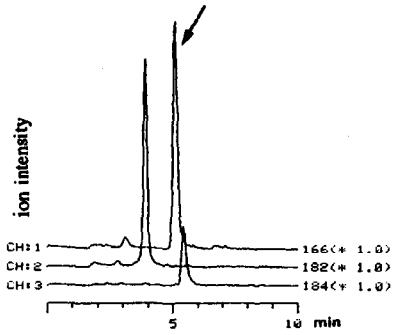


Fig. 2. Selected ion monitoring of **1**, **2**, and **3** at m/z 166, 182 and 184, respectively, under TSP-LC/MS conditions.

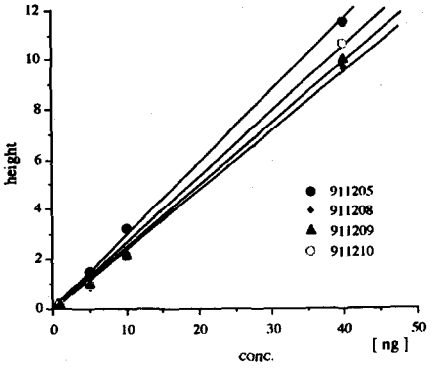


Fig. 3. Calibration curve for anatoxin-a (**1**) by TSP-LC/MS.

Application

The established method was applied to the analysis of **1** and **2** in various foreign and Japanese samples and the results obtained are summarized in Table 1. Sivonen *et al.* reported concentrations of **1** contained in Finnish bloom samples by the GC/MS method and their concentrations of several samples were estimated to range from 1450 to 4360 μg/g.[17] In the present study Finnish samples, four strains and one bloom sample, were analyzed by this method. These samples contain considerable amounts of **1**, which ranged from 1100 to 2700 μg/g. Additionally, the amounts of **2** could be simultaneously quantified and their amounts varied between one-tenth and one-twentieth of those of **1**.

It is well known that *Anabaena flos-aquae* NRC 44-1-S produces a large amount of **1**[18], and purified **1** was isolated from this strain.[5,14] Two cultivated cells of this strain, old and new, were analyzed by the LC/MS. Although the old cells showed the amount of 13 mg/g, that of the new ones was about 1.1 mg/g. In addition, the proportion of **2** in the new cells increased in comparison with that in the old ones. In fact the toxicity of the new cells by mouse bioassay was much reduced.

Table 1. Analysis data of anatoxin-a (**1**) and its degradation product (**2**) in various cyanobacteria by TSP-LC/MS

samples	species	**1**	**2**
Canadian and Finnish samples			
NRC 44-1-S (old)	*Anabaena flos-aquae*	13013	1941
NRC 44-1-S (new)	*Anabaena flos-aquae*	1119	697
Finland -1	*Anabaena flos-aquae*	1107	130
Finland-2	*Anabaena circinalis*	1396	189
Finland-3	*Aphanizomenon* sp.	1562	84
Finland-4	*Oscillatoria* sp.	2713	295
Finland-5	Bloom	2642	116
Japanese samples			
TAC210 (910320)	*Anabaena* sp.	2.1	2.6
TAC210 (910617)	*Anabaena* sp.	1.5	1.8
TAC210 (910703)	*Anabaena* sp.	0.6	0.8
TAC121 (910107)	*Microcystis aeruginosa*	52.4	5.7
TAC121 (910320)	*Microcystis aeruginosa*	38.9	2.2
TAC121 (910709)	*Microcystis aeruginosa*	0.4	0.9
TAC80	*Microcystis aeruginosa*	0.2	0.1
Lake Kasumi (910731)	Bloom	0.4	N.D.
Lake Kasumi (910912)	Bloom	0.4	N.D.

μg/g of lyophilized cell

As mentioned earlier, **1** has not been discovered so far in Japan. Three strains and two bloom samples from Japanese lakes were investigated by the method. Figure 4 shows the HPLC chromatogram with UV detection and TSP-LC/MS data of a culture strain, TAC80.

While it is impossible to quantify precisely amount of **1** (arrow) due to elevated base line by unresolved materials from the column in the chromatogram, the TSP-LC/MS can specifically provide the clear peaks for **1** and **2**. To our knowledge this is the first case of detection of **1** from a Japanese sample. Two strains, TAC210 and TAC121 were cultivated three times and their analysis results are also shown in Table 1. At first TAC121 produced about 50 μg/g, but only a trace amount of **1** was detected in the last cultivation. It is not understood why the cells lose their productivity of **1**. These results clearly show that the established method can be applied to analysis of **1** and its degradation products in any sample, even if they contain a trace amount of **1** and many contaminants.

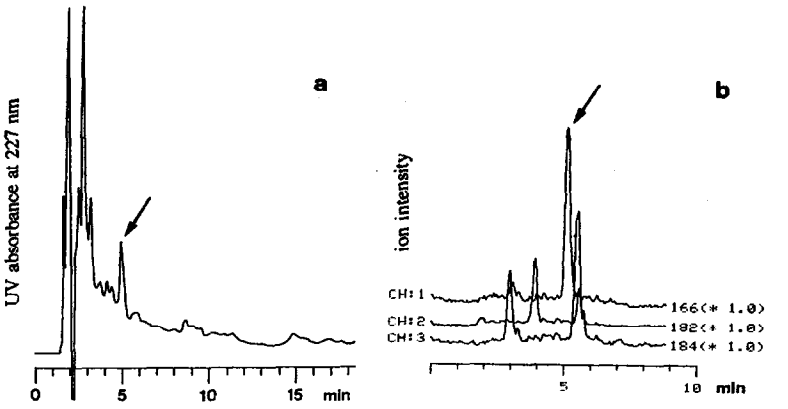


Fig. 4. High performance liquid chromatogram (a) and selected ion monitoring at m/z 166 and 182, 184 (b) of a toxic fraction from TAC80 (Arrows indicate anatoxin-a (**1**)).

In this field one of the most important works is to pursue a desired toxin and to know the distribution of the toxin under field conditions. It is found that **1** decomposes naturally by four pathways, dilution, adsorption, photolysis and non-photochemical degradation. The resulting degradation products have molecular weights of 165 (the same as **1**), 179 (**1** + 14) and 181 (**1** + 16).[7] Fig. 5 shows the TSP-LC/MS chromatogram of two samples monitored at m/z 166, 182 and 184. There is one peak at m/z 166 (a) and another peak at m/z 182 (b) in addition to **1** and **2** in the chromatograms, which might correspond to the degradation products mentioned above. The TSP-LC/MS should contribute to the study of detoxification of **1**.

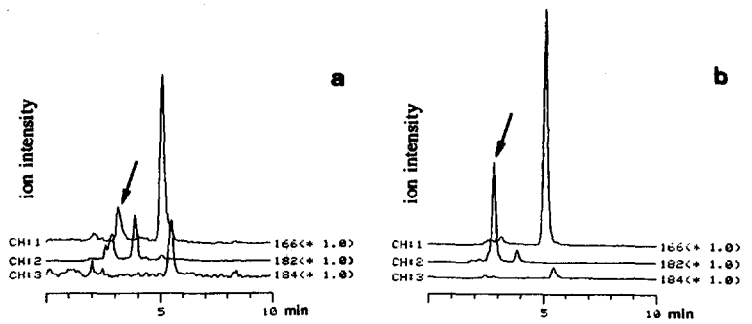
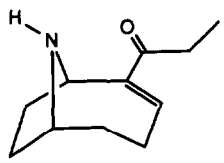


Fig. 5. Selected ion monitoring at m/z 166, 182 and 184 of toxic fractions of two samples.

Recently, Skulberg *et al.* reported the isolation and structure determination of homoanatoxin-a (**4**) from *Oscillatoria formosa*, whose biological activity is very similar to that of **1**.[19] Although the TSP-LC/MS was applicable to a screening of **4** without the standard sample, no **4** was detected by the SIM of m/z 180 in all samples of the present study.



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The present TSP-LC/MS method has good accuracy and sensitivity, and should be applicable to any anatoxin-a containing sample. It should contibute to routine analysis and detoxicification studies in this field. A simultaneous determination of anatoxin-a and microcystins in various samples by a combination of this method and the usual HPLC will be reported elsewhere.

EXPERIMENTAL SECTION

Materials

The following strain and bloom samples were used for analysis: *Anabaena flos-aquae*, NRC-44-1[18] (cultivation in 1988 and 1991); Finland-1; *Anabaena circinalis*, Finland-2;

Anabaena sp.; TAC 210, *Aphanizomenon* sp., Finland-3; *Oscillatoria* sp., Finland-4; *Microcystis aeruginosa*, TAC 80; TAC 121; One Finnish bloom sample, Finland-5; Japanese bloom samples were collected in Lake Kasumigaura in July 31 and September 12, 1991. All chemicals used were of analytical grade.

Methods

HPLC

A high performance liquid chromatograph equipped with a constant flow pump (Hitachi 655, Tokyo, Japan) and a UV detector (Hitachi 655A) were used. HPLC separations were accomplished under reversed phase isocratic conditions with a Cosmosil 5C18-P (150 x 4.6 mm, Nacalai Tesque, Kyoto Japan) or a Cosmosil 5PH (150 x 4.6 mm) column, which organo silane with phenyl group is bonded to silica gel, and mobile phase of methanol:0.1M ammonium acetate (pH 3 or 4 adjusted with TFA) = 1:9. Flow rate was 1 mL/min and UV (227 nm) was used for detection.

LC/MS

LC/MS was carried out under the following conditions: LC: pump, Shimadzu (Kyoto, Japan) LC-9A; detector, Shimazdu SPD-6A; Column, Cosmosil 5PH (150 x 4.6 mm); mobile phase, methanol:0.1M ammonium acetate (adjusted pH 5 with TFA) = 14:86; flow rate, 1.2 mL/min; detection, UV (227 nm). MS: mass spectrometer, Shimadzu LC/MS-QP 1000; SIM, m/z 166, 182 and 184; interface, Shimadzu TSP-100; control temperature, 155-160°C; tip temperature, 230-238°C; block temperature, 250-270°C; vapor temperature, 240-255°C; tip heater temperature, 250-265°C.

Extraction and clean-up

Lyophilized cells (100 mg) were extracted three times with 10 mL of 0.05M acetic acid for 30 min while stirring. The extract was centrifuged at 4000 rpm for 15 min and the supernatant was adjusted to pH 10 with 7% ammonium hydroxide. It was applied to a preconditioned ODS silica gel cartridge (Baker, Phillipsburgh, NJ, U.S.A.), which was then washed with 10 mL of water, followed by 10 mL of water:methanol (9:1). The toxic fraction was eluted with 20 mL of methanol and evaporated to dryness. The residue was dissolved in 10 mL of water and was subjected to cation exchange on organosilan bonded to silica gel (Baker 10 COOH). The cartridge was rinsed with 10 mL of water, followed by 10 mL of water and the desired toxic fraction was eluted with 20 mL of 0.01% TFA:methanol. The fraction was evaporated and the resulting residue was dissolved in methanol. A sample solution with an appropriate concentration was injected into HPLC or LC/MS.

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