Chapter 10

Instrumental Analysis of Tetrodotoxin

Manabu Asakawa, Yasuo Shida, Keisuke Miyazawa and Tamao Noguchi

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/48668

1. Introduction

Tetrodotoxin (TTX), a pufferfish ("fugu" in Japanese) toxin named after its order name Tetraodontiformes, is the toxic principle of puffer fish poisoning. This toxin ($C_{11}H_{17}N_3O_8$; a molecular weight of 319) is one of the most potent nonproteinaceous toxins as well as the best-known marine natural toxins (Figure 1). In Japan, pufferfish have been a traditional food for many years, and since people have become accustomed to eating them, cases of TTX poisoning are frequent. It poses a serious hazard to public health. These cases have occurred on a regular basis not only in Japan but also in Asia for a number of years, sporadically resulting in severe poisoning or even death. On the other hand, the Japanese are aware of the its toxicity and have devised methods to reduce TTX levels especially in the liver. However, TTX poisoning incidents continue to occur in Japan. Since there is no antidote for the toxin, patient mortality is very high. Judging from statistics provided by the Japanese Ministry of Health, Labour and Welfare, the number of deaths due to puffer poisoning has steadily declined, from more than 10 cases every year between 1960 and 1981 to less than 10 cases with low mortality every year since 1982, generally with low mortality. This decline is probably due to not only strict adherence to government regulations but also an increase in cultured puffer rather than a decrease in wild puffer. The toxicosis is characterized by the onset of symptoms in the victim. Treatment of the illness is mainly based on the symptoms of the patient. More fruitful treatment can be provided if the causative toxin is identified. In 1950, TTX was isolated for the first time as a crystalline prism from toxic pufferfish ovaries by Yokoo [1]. Its structure was elucidated by three groups in 1964 [2-4]. TTX is a powerful and specific sodium channel blocker [5]. When ingested by humans, it acts to block the sodium channels in the nerve cells and skeletal muscles, and to thereby block excitatory conduction, resulting in the occurrence of typical symptoms and signs such as respiratory paralysis and even death in severe cases. The lethal potency is 5000 to 6000 MU/mg. One MU (mouse unit) is defined as the amount of toxin required to kill a 20g male mouse within 30 min after intraperitoneal administration, and the minimum lethal dose (MLD) for humans is estimated to be approximately 10,000 MU

equivalent to 2 mg of pure TTX crystals [6]. Many derivatives of TTX have been found, although their toxicities vary widely. As seen in Figures 1, 2 TTX is a heterocyclic guanide compound whose chemical structure has been characterized. Various TTX derivatives from pufferfish and other TTX-bearing organisms have been identified to date as a result of recent progress in the instrumental analysis of TTX (Figure 3). In marine pufferfish species, toxicity is generally highest in the liver and ovary, whereas in brackish and freshwater species, toxicity is higher in the skin [7-13]. TTX was long believed to be present only in the pufferfish. In 1964, Mosher *et al.* detected TTX in California newt *Taricha torosa*, which was the first TTX-containing organism other than pufferfish [14]. Since then, the distribution of TTX has spread to animals other than pufferfish. The toxin has been detected in a tropical goby *Yongeichthys criniger* [15], atelopid frogs of Costa Rica *Atelopus* spp.[16], blue-ringed octopus *Hapalochlaena maculosus* [17], and several species of carnivorous gastropods such as trumpet shell *Charonia sauliae*[18], ivory shell *Babylonia japonica* [19], frog shell *Tutufa lissostoma* [20] as shown in Table 1. In addition, some species of starfish on which these gastropods prefer to feed also contain TTX [21]. The trumpet shell *Charonia sauliae* accumulates TTX by ingesting toxic starfish, supporting the hypothesis that the TTX of pufferfish is not endogenous, but is introduced via the food chain. The exact origin of TTX in the food chain, however, remains unknown. Because the ecologic environments of TTX-bearing animals apparently have no common factor other than being closely related to an aquatic system, bacteria (omnipresent organisms that commonly inhabit the aquatic system), are implicated as the primary source of TTX. Toxic crabs, flatworms, horseshoe crabs, ribbon worms and arrow worms were also added to the list of TTX-bearing animals. In Japan, TTX is assayed by the official method using mice [22]. It requires ddY strain male mice, but no special instrumentation. This method is simple and convenient but not so sufficiently accurate, and provides no information on the composition of the toxin, nor is it able to distinguish TTX from other neurotoxins such as paralytic shellfish poison (PSP). In addition, animal rights activists across the world are strongly opposed to bioassays using live animals, including mice. Thin layer chromatography (TLC) and electrophoresis are useful means for TTX detection, but they are not suitable for TTX determination. With this background, attempts have been made to develop analytical methods using high performance liquid chromatography (HPLC) in Japan. Detection and determination of TTX are essential not only for diagnosis and treatment purposes, but also for issuing quarantines and public awareness. Quantitative and/or qualitative detection of TTX in a sample is/are performed by a few instrumental analysis methods. The toxin has long been believed to occur exclusively in pufferfish. However, owing to the recent outstanding progress in instrumental methods for the analysis of TTX, its distribution and accumulation in various aquatic organisms have been established. In addition, this toxin has been detected in many other vertebrates and invertebrates. A few intestinal bacteria of TTX-bearing animals were found to produce TTX. This suggested that the accumulated TTX in these animals was being passed along the food web having been acquired from such TTX-producing bacteria. In this section, although there are many aspects of TTX research with respect to treatment and prevention, biologic distribution, sources, infestation mechanism, detection methods,

chemistry and pharmacology, the focus is to provide an overview of the instrumental analysis of TTX and present chromatographic methods for the isolation of TTX. Rapid and accurate analysis of a mixture and its analogs occurring in a variety of marine organisms is becoming increasingly important from the standpoint of public health, since food poisoning from the ingestion of these toxins is often fatal to a human. There are also increasing demands for chemical assays of TTX for the study of its biosynthetic and metabolic pathways, which remain unknown. In an attempt to protect consumers from TTX-intoxication, the mouse bioassay has historically been the universally applied tool to determine the toxicity level in monitoring programs. This bioassay, however, shows low precision and requires a continuous supply of mice of a specific size. These potential drawbacks and world-wide pressure to refrain from the unnecessary killing of live animals subsequently led the scientists to develop chemical-based alternatives to the mouse bioassay for TTX detection and quantification. In addition, the mouse assay can neither provide any information on toxin composition, nor distinguish TTX from other neurotoxins such as paralytic shellfish poison (PSP). A few marine animals have been found to contain both TTX and PSP simultaneously. Many detection methods for TTX have been developed. A few methods including the mouse bioassay, high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) are typically used to qualitatively and quantitatively detect TTX, but other methods including gas-chromatography-mass spectrometry (GC-MS), infrared (IR) spectrometry and nuclear magnetic resonance (^{1}H-NMR) spectrometry are often used to qualitatively detect TTX. Among them, HPLC and LC-MS are the most powerful and sensitive tool for qualitatively and quantitatively detecting TTX. In addition these methods, TTX can also be identified by thin-layer chromatography (TLC) or electrophoresis. Though these methods are not instrumental analysis, these methods are simpler and more practical. In this section, an attempt has been made to review the current information and the recent progress on instrumental analysis of TTX.

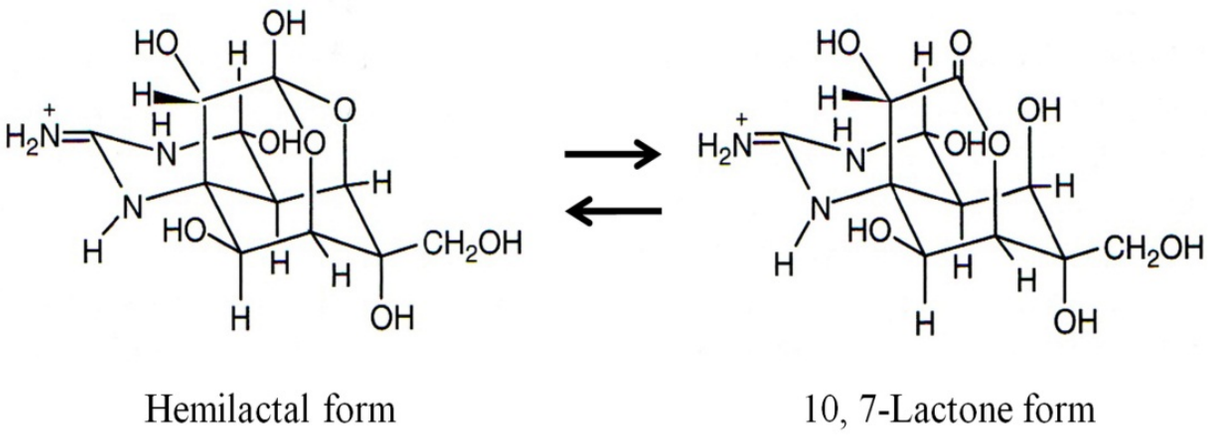
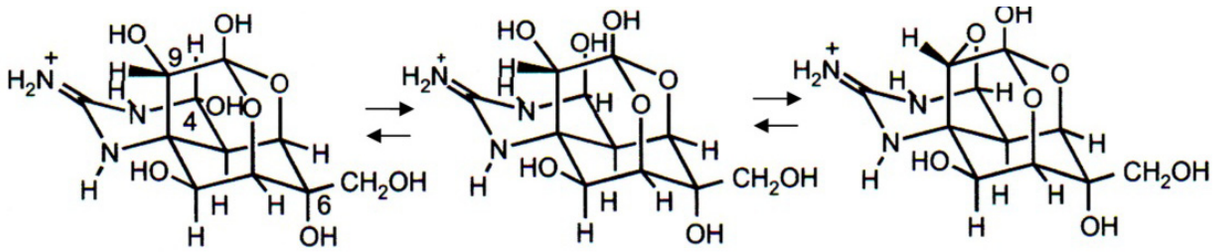


Figure 1. The tautomer of TTX

	Animals		Part
1	Platyhelminthes	Flatworms	
	Turbellaria	*Planocera* spp.	Whole body
2	Nemertinea	Ribbon worms	Whole body
		Lineus fuscoviridis	Whole body
		Tubulanus punctatus	Whole body
		Cerebratulus lacteus	Whole body
		Cephalothrix linearis	Whole body
3	Mollusca	*Charonia sauliae*	Digestive gland
	Gastropoda	*Babylonia japonica*	Digestive gland
		Tutufa lissostoma	Digestive gland
		Zeuxis siquijorensis	Digestive gland
		Niotha clathrata	Digestive gland
		Natica lineata	Whole body
		Rapana spp.	Digestive gland
		Cymatium echo	Digestive gland
		Pugilina ternotona	Digestive gland
	Cephalopoda	*Hapalochlaena maculosa*	Postsalivary gland
4	Annelida	*Pseudopotamilla occelata*	Whole body
	Polychaeta	*Lepidonotus helotypus*	Whole body
		Halosydna brevisetosa	Whole body
		Harmothoe imbricata	Whole body
5	Arthropoda	*Atergatis floridus*	Whole body
		Zosimus aeneus	Whole body
		Carcinoscorpius rotundicauda	Egg
6	Chaetognatha	Arrowworms	
		Eukrohonia hamata	Head
		Parasagitta spp.	Head
		Flaccisagitta spp.	Head
7	Echinodermata	Starfish	
		Astropecten polyacanthus	Whole body
		Astropecten latespinosus	Whole body
		Astropecten scoparius	Whole body
8	Vertebrate	*Takifugu* spp.	Skin, liver, ovary
	Pisces	*Yongeichthys criniger*	Skin, viscera, gonad
	Amphibia	*Taricha* spp.	Skin, egg, ovary, muscle, blood
		Notophthalmus spp.	Skin, egg, ovary
		Cynops spp.	Skin, egg, ovary, muscle, blood
		Triturus spp.	Skin, egg, ovary, muscle, blood
		Ambystoma sp.	Skin, egg, ovary, muscle
		Paramesotriton sp.	Skin, egg, ovary, muscle
		Polypedates sp.	Skin
		Atelopus spp.	Skin
		Colostethus spp.	Skin
9	Red Clacareous alga	*Jania* spp.	Whole body
10	Dinoflagellate	*Alexandrium tamarense*	Whole body

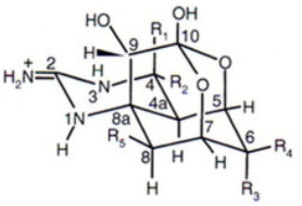
Table 1. Distribution of TTX in animals



TTX (MH$^+$ *m/z* 320) | 4-*epi*TTX (MH$^+$ *m/z* 320) | 4, 9-anhydroTTX (MH$^+$ *m/z* 302)

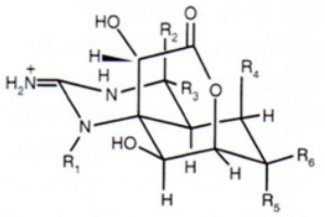
Figure 2. Chemically equilibrium of TTX, 4-*epi*TTX and 4, 9-anhydroTTX

(A) Hemilactal type

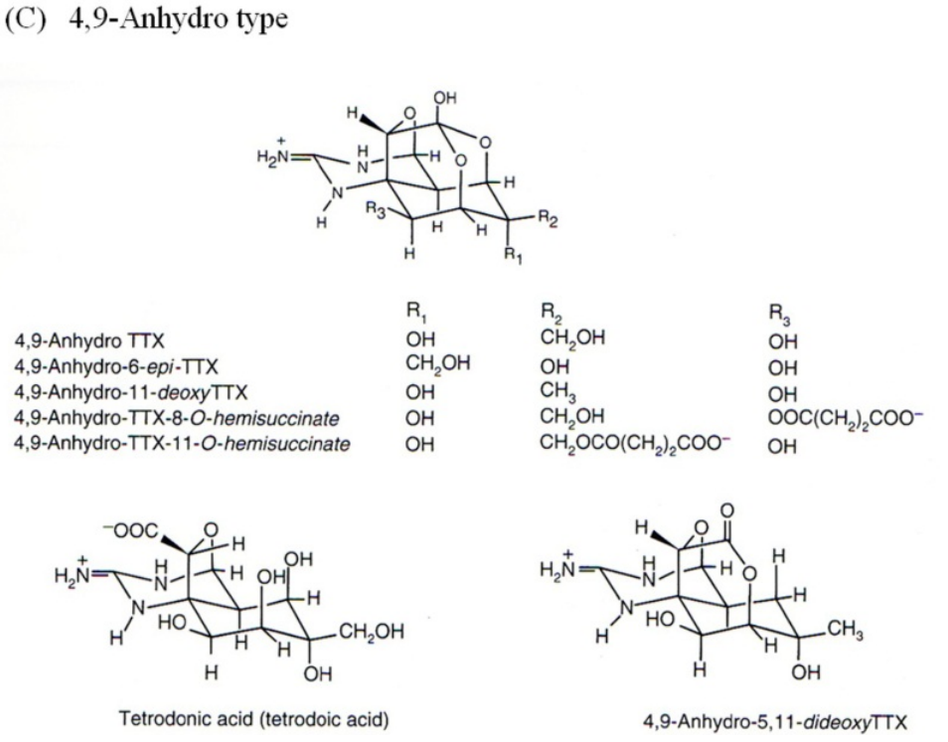


	R_1	R_2	R_3	R_4	R_5
TTX	H	OH	OH	CH_2OH	OH
4-*epi*TTX	OH	H	OH	CH_2OH	OH
6-*epi*TTX	H	OH	CH_2OH	OH	OH
11-*deoxy*TTX	H	OH	OH	CH_3	OH
6-*epi*-11-*deoxy*TTX	OH	H	OH	CH_3	OH
TTX-8-*O*-hemisuccinate	H	OH	OH	CH_2OH	$OOC(CH_2)_2COO^-$
				R *S*	
Chiriquitoxin	H	OH	OH	$CH(OH)CH(NH_3^+)COO^-$	OH
11-*nor*TTX-6(*S*)-ol	H	OH	OH	H	OH
11-*nor*TTX-6(*R*)-ol	H	OH	H	OH	OH
11-*nor*TTX-6,6-diol	H	OH	OH	OH	OH
11-*oxo*TTX	H	OH	OH	$CH(OH)_2$	OH
TTX-11-carboxylic acid	H	OH	OH	COO^-	OH

(B) Lactone type



	R_1	R_2	R_3	R_4	R_5	R_6
TTX (lactone)	H	H	OH	OH	OH	CH_2OH
6-*epi*TTX (lactone)	H	H	OH	OH	CH_2OH	OH
11-*deoxy*TTX (lactone)	H	H	OH	OH	OH	CH_3
11-*nor*TTX-6(*S*)-ol (lactone)	H	H	OH	OH	OH	H
11-*nor*TTX-6(*R*)-ol (lactone)	H	H	OH	OH	H	OH
11-*nor*TTX-6,6-diol (lactone)	H	H	OH	OH	OH	OH
5-*deoxy*TTX	H	H	OH	H	OH	CH_2OH
5,11-*dideoxy*TTX	H	H	OH	H	OH	CH_3
6-*epi*-5,11-*dideoxy*TTX	H	OH	H	H	OH	CH_3
1-hydroxy-5,11-*dideoxy*TTX	OH	H	OH	H	OH	CH_3
5,6,11-*trideoxy*TTX	H	H	OH	H	H	CH_3
4-*epi*-5,6,11-*trideoxy*TTX	H	OH	H	H	H	CH_3



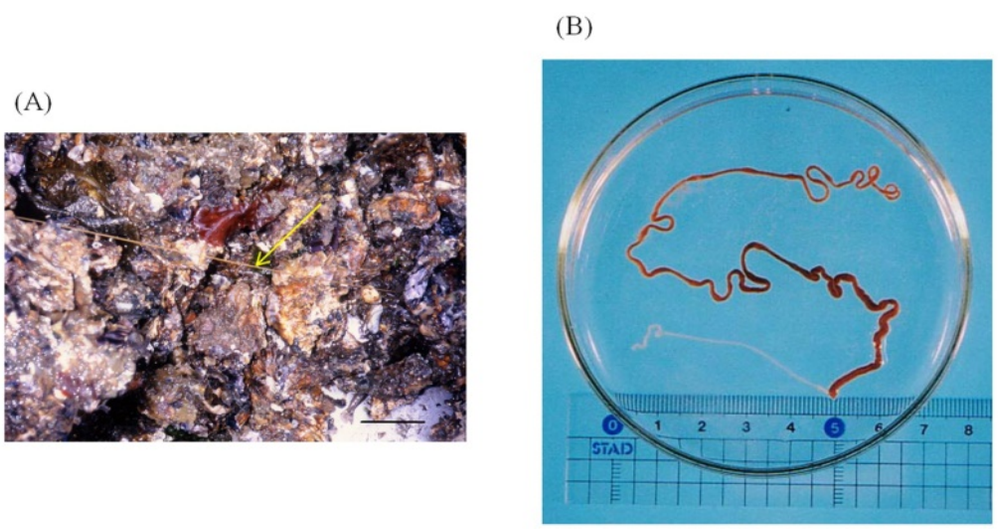
(A) Hemilactal type, (B) Lacton type, (C) 4,9-Anhydro type

Figure 3. The structure of three types of TTX analogues

2. Isolation of TTX crystals by column chromatography

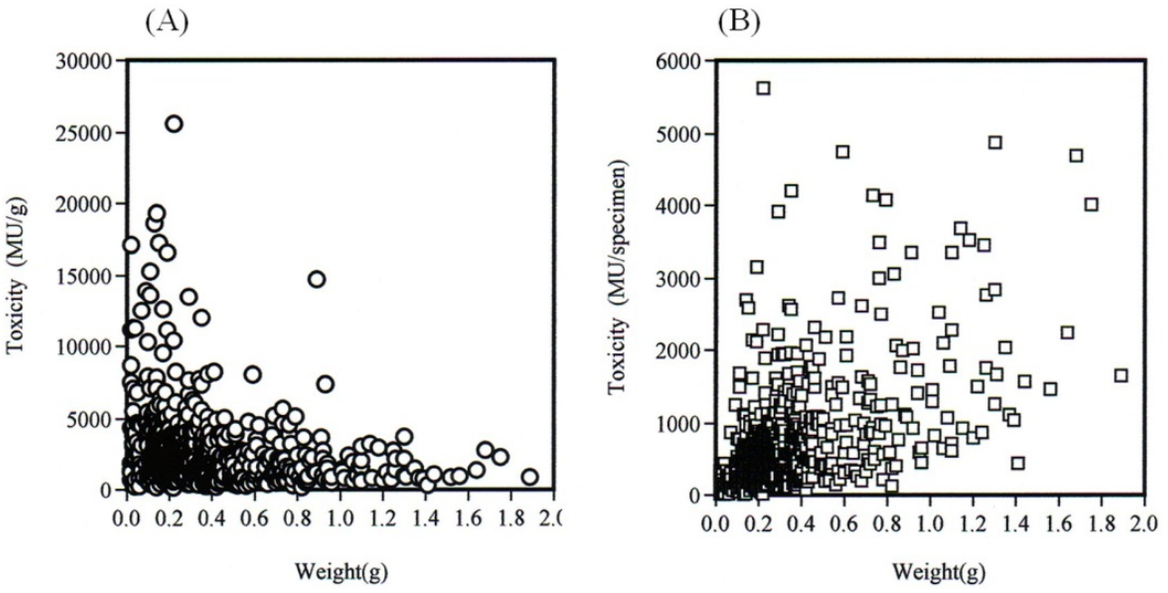
Of the TTX-bearing animals, our specimens of ribbon worms ("himomushi" in Japanese) adherent to the cultured oyster *Crassostrea gigas* hanging onto floating culture rafts, were found to be extremely toxic and to contain tetrodotoxin, during surveillance of the toxicity of various marine fouling organisms in Hiroshima Bay, Hiroshima Prefecture, which is one of the largest oyster culture areas in Japan [23]. In these analyses, the toxicity was examined on each ribbon worm specimen by the standard bioassay method for TTX. Ribbon worm specimens were collected in Hiroshima Bay between November and May from 1998 to 2005 approximately every two weeks during the harvest time for cultured oysters. A total of 764 specimens were collected, and assayed for toxicity. All specimens that were assayed throughout the season covered found to be toxic, and the toxicity scores ranged from 169 to 25,590 MU/g (Figures 4 and 5). The ratio of strongly toxic (more than 1,000 MU/g) specimens to the total number of specimens was 80%. Furthermore, the percentage of specimens possessing toxicity scores higher than 2,000 MU/g to the total was high (48%). The highest toxicity detected was 25,590 MU/g from a specimen collected on June25 (1999). The total toxicity for this sample was approximately calculated to be 5,631 MU, which is approximately equivalent to half of the minimum lethal dose of TTX in humans, which is reported to be 10,000 MU. The specimens of ribbon worms (390 g) obtained during the survey were semi-defrosted and homogenized with three volumes of 1% AcOH in 80% MeOH for 3 min, then centrifuged. This operation was repeated two more times. The supernatants (total toxicity; 2,897,000 MU) were combined, concentrated under reduced pressure, and defatted by shaking gently with approximately the same volume of chloroform several times. The aqueous layer (2,750,000 MU) was applied to an activated charcoal column and the

adsorbed toxin was eluted with 1% AcOH in 20% EtOH after washing the column with dist. H_2O. The water eluate (Fr.I) and the eluate with 1% AcOH in 20% EtOH (Fr.II) was isolated (Figure 6). The main toxic fraction (Fr.II) was evaporated to dryness *in vacuo*. The resulting residue (total toxicity 2,433,000 MU; specific toxicity 99 MU/mg) was dissolved in a small amount of water, and the pH was adjusted to 5.5 with 1N NaOH. This solution was applied to a Bio-Gel P-2 column (ϕ 3.5 × 100cm). The column was washed with 3000 ml of water and then developed with 2,000 ml of 0.03 M AcOH. The toxicity was detected exclusively in the 0.03 M AcOH fraction. This fraction was concentrated to dryness under reduced pressure and the residue (3,300 MU/mg) was dissolved in a small volume of water. The resulting solution was chromatographed on a Bio-Rex 70 (H^+ form, ϕ1.0 x 100cm) column using a linear gradient of H_2O and 0.03 M AcOH at the flow rate of 0.5 ml /min. The toxic fractions were monitored for TTX via the mouse bioassay and high performance liquid chromatography (HPLC) as described later. The main toxic fractions (fr. 85–100; Fr.I) and minor fractions (fr.50-84) were obtained, and rechromatographed in the same manner (Figure 7). The toxic fraction (Fr.I) thus obtained were freeze-dried, and then dissolved in 0.5 ml of 1% AcOH. Approximately 2.0 ml of MeOH and 5.0 ml of diethylether were added to this solution, and the mixture was stored in the refrigerator overnight. During storage, stratified plate-like crystals appeared (Figure. 8). The crystals were isolated by decantation, and recrystallized by method same as that described above. Approximately, Bio-Gel P-2 column chromatography was very effective, as the specific toxicity sharply increased from 99 to 3,300 MU/mg. After recrystallization, the specific toxicity of this toxin ultimately increased to 3,520 MU/mg. From the combined homogenates with the toxicity of roughly 7,400 MU/g, approximately 25 mg of the stratified plate-like crystalline TTX was obtained. Generally, the ribbon worm has a simple structure. Since pure crystals of TTX could be obtained from this highly toxic ribbon worm efficiently by the above-described series of chromatographies, the ribbon worm is a promising source of TTX for use as a reagent in the fields of medicine and pharmacology. Previously, authentic specimens of TTX were typically prepared from pufferfish ovaries for use as reference standards, as reported in [4].



(A): Arrow indicates the ribbon worm (scale bar = 10 cm) on the surface of the cultured oyster shell.
(B): Ribbon worm removed from the cultured oyster shell

Figure 4. Ribbon worms *Cephalothrix* sp. ("himomushi" in Japanese) adherent to the cultured oyster *Crassostrea gigas* hanging onto floating culture rafts in Hiroshima Bay.



(A): Relationship between toxicity (MU/g) and body weight (g)
(B): Relationship between toxicity (MU/specimen) and body weight (g)

Figure 5. Toxicity of ribbon worm *Cephalothrix* sp. from Hiroshima Bay (1998-2005)

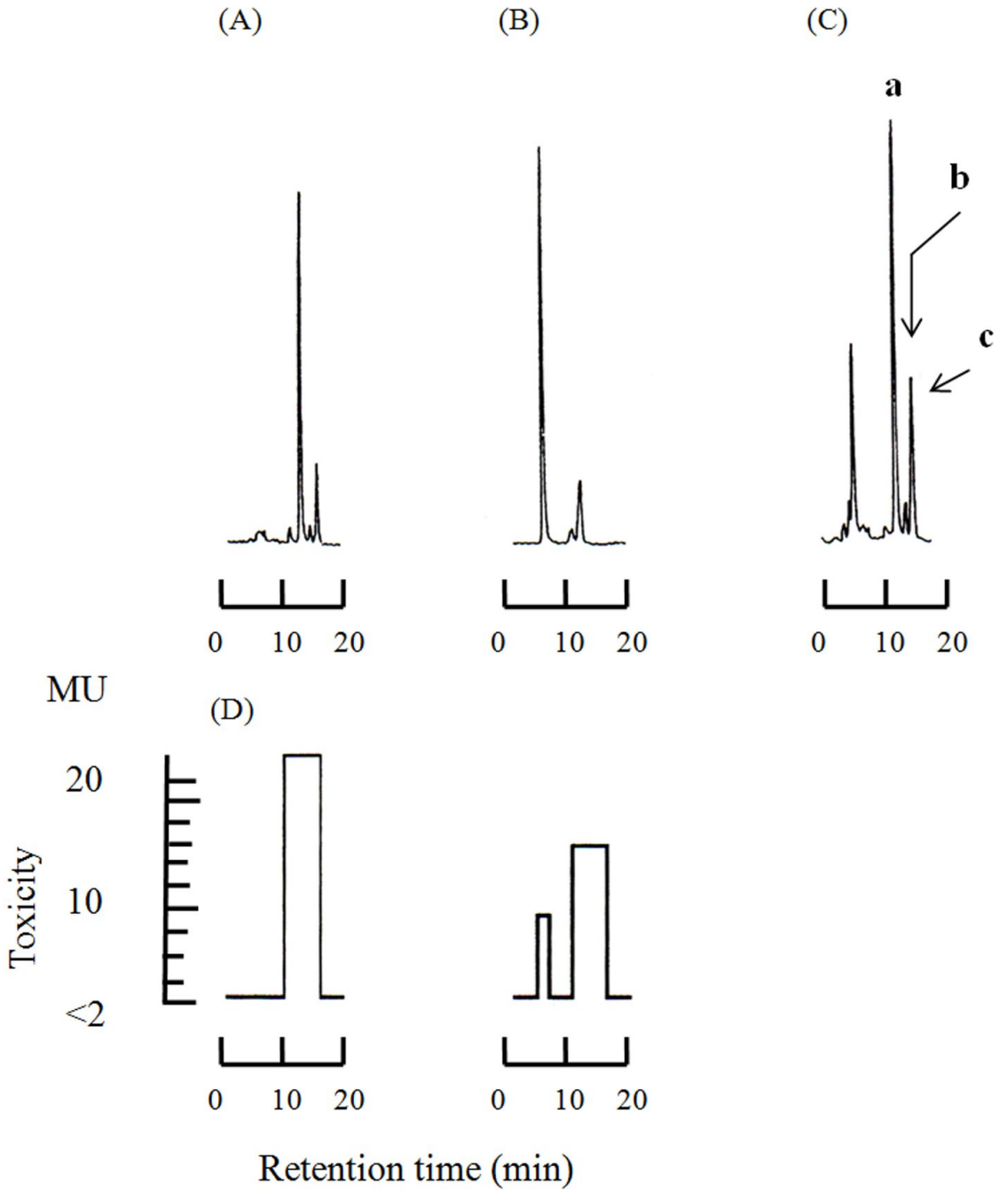
3. HPLC – Fluorescence detection

Rapid progress in TTX research, especially in intoxication mechanism of TTX-bearing organisms, is due to recent advancements in instrumental analysis. In particular, postcolumn-HPLC fluorescence detection (HPLC-FLD) methods expected to replace the conventional mouse bioassay, have been explored by many researchers for both qualitative and quantitative analysis of TTX and its analogs. HPLC techniques allow the separation and sensitive detection of individual TTX and its analogs irrespective of their number and group. Therefore, HPLC methods have opened up a new dimension in TTX analysis. However, the results obtained have to be comparable to those of the mouse bioassay. Additionally, accurate HPLC determination of the various TTX components in the samples is a necessity. Using these methods, the toxic principles produced peaks identical to those of authentic TTX and its derivatives. The HPLC- FLD method utilizes a computer controlled by a high pressure pump with a syringe loading sample injector or an autosampler system, a stainless steel column, a reaction pump for delivering reagents, and a fluoromonitor and chromato-recorder for calculation of the peak area. In this method, a strong alkali treatment is applied to TTX which produces a fluorescent compound with excitation and emission wavelengths of 384 and 505 nm, respectively. In this system, first, toxins are separated from the contaminants by a buffer solution on a reversed-phase column packed with C18 resin with an ion-pair reagent (sodium 1-heptanesulfonate; HSA). Then, the isolated toxins are mixed with NaOH, which converts them into fluorescent compounds that are then passed through a stainless steel tube (ϕ 0.25mm × 100cm) placed in an oven. Eventually, when the fluorescent compounds are passed through a fluoromonitor equipped with a lamp, the retention time of the toxin and fluorescence intensity are recorded. The treated toxins are

identified by comparing their retention times with those of authentic TTXs. For the quantitative analysis by HPLC, the detection limit of TTX is approximately 0.03μg, which is satisfactory for practical applications. To date, several continuous improvements have been made to detect TTX and its analogs under different HPLC conditions, and a number of advances in understanding the biochemistry of TTXs are the outcomes of these developments. Briefly, a few promising methodologies are described as follows. In the early 1980's, a fluorometric continuous TTX analyzer was constructed by combining HPLC and a post-column reaction with NaOH to monitor potentially harmful puffer toxins [24]. In this system, the toxin was first separated from contaminants on a column composed of a weak cation exchange gel with a 0.06 M citrate buffer solution (pH 4.0), and toxin concentrations above 8 MU/g were detected. However, because of the poor performance of the original system in separating and detecting TTX analogs, an improved analyzer was later constructed. Using HPLC-FLD, naturally occurring TTX analogs, 4-*epi*TTX, 4,9-anhydroTTX [2,3,4,25] 6-*epi*TTX [26], 11-deoxyTTX [26], 11-oxoTTX [27], 11-*nor*TTX-6(*R*)-ol [28], 11-*nor*TTX-6(*S*)-ol [29], 1-hydroxy-5,11-dideoxyTTX [30], 5,6,11-trideoxyTTX [31], 5-deoxyTTX [32], 4,9-anhydro-6-*epi*TTX, 4-*epi*-11-deoxy-TTX, and 4,9-anhydro-11-deoxyTTX isolated from puffer and newt specimens and/or frogs [33]. The separation of TTX and 6-*epi*TTX is considered as a major achievement for this improved analyzer. In addition to this, attempts were made to apply a post-column fluorescent-HPLC system for quantitative assay of TTX and its analog 6-*epi*TTX in newts from southern Germany [34]. A reversed-phase ion-pairing HPLC method, in which HAS is used as a counter ion has also been the system preferred by many researchers for fastest and most efficient analysis of TTX and its analogs. In this method, the detection reagent for TTX and related substances does not react with any PSP component if present in the contaminant sample. A reversed - phase HPLC system (Table 2) with slight modification in the method proposed by of Nagashima *et al.*[35] is commonly used to analyze toxin compositions of extremely toxic Japanese ribbon worms, the xanthid crab *Demania cultripes* from Cebu Island, Philippines(Figure 6,7 and 9) and TTX-producing bacteria[23, 36, 37].

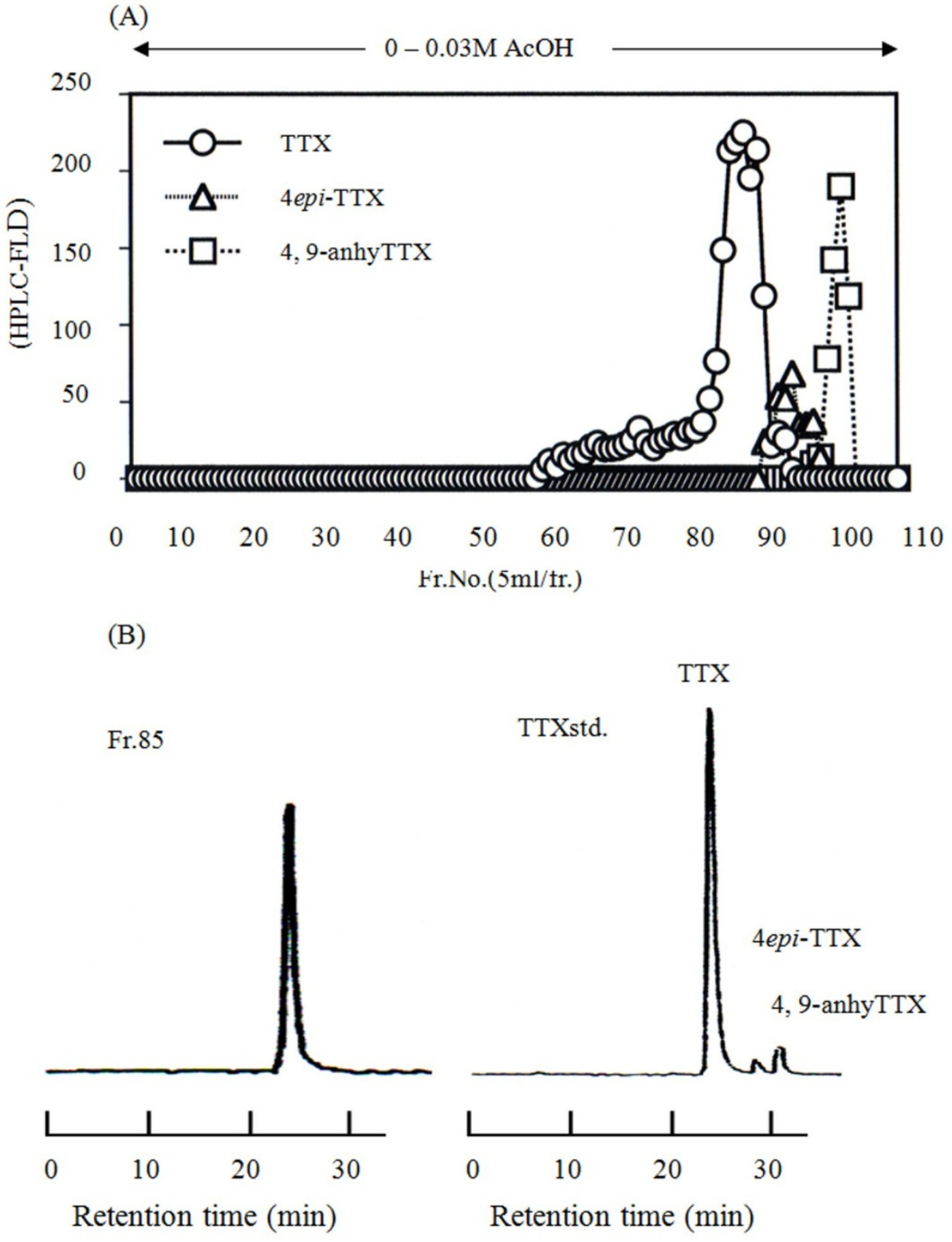
HPLC control system	JASCO - BORWIN/HSS-2000	
Column	LiChroCART 250-4 (Merck) (LiChrospher 100 RP-18e, 5μm)	Column size: 4 x 250 mm
Column temperature	30°C	CO-2067 plus (JASCO)
Mobile phase	60mM ammomium phosphate buffer (pH5.0) containing 10mM HAS and 2% CH_3CN	PU-2080 plus (JASCO) 0.5 ml/min
Reagent	3 M NaOH	MINICHEMI PUMP SP-D-2502 (Nihonseimitsukagaku) 0.5 ml/min
Reaction temperature	110°C	860 CO (JASCO)
Detection	Excitation 384nm, emission 505nm	JASCO FP-2025 plus

Table 2. Operating conditions of HPLC system for the analysis of TTX



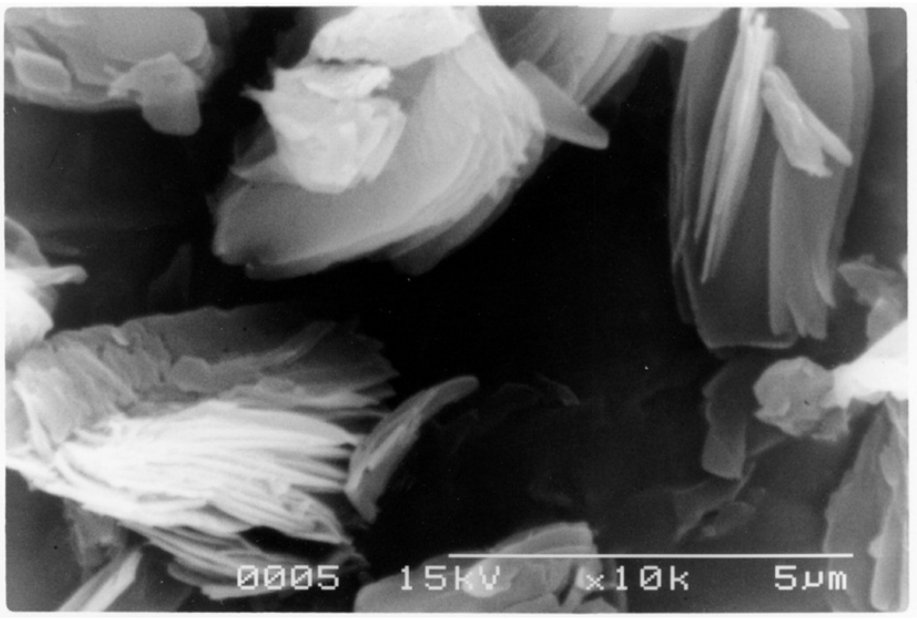
(A): Fraction II bound on activated charcoal column
(B): Fraction I unbound on activated charcoal column
(C): TTX standards; TDA (tetrodonic acid), TTX (tetrodotoxin), 4-*epi*TTX (4*epi*tetrodotoxin), 4, 9-anhyTTX (4,9-anhydrotetrodotoxin)
*HPLC-FLD: high performance liquid chromatography-fluorescence detection (HPLC-FLD)

Figure 6. HPLC-FLD * patterns (top) of fractions from the toxins contained in the ribbon worm *Cephalothrix* sp. in an activated charcoal column chromatography. The bottom patterns represent the distribution of toxicity in HPLC chromatograms, as estimated by mouse bioassay.



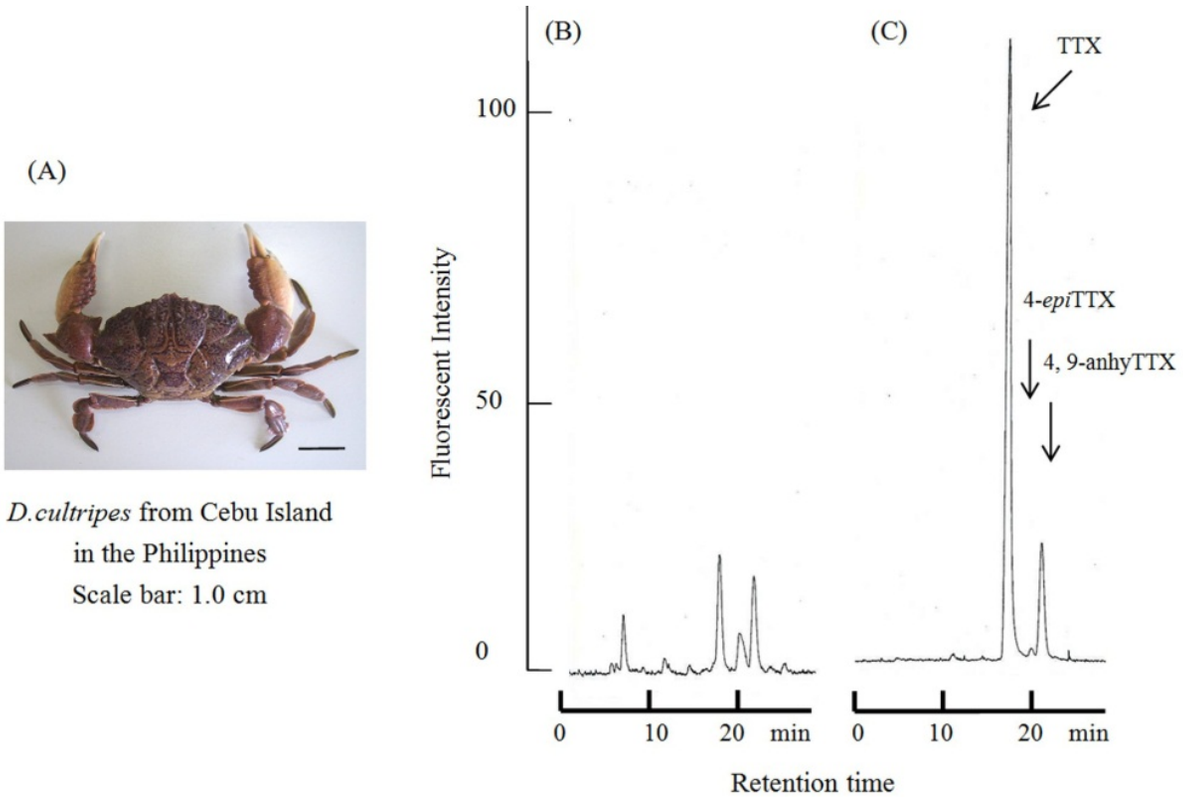
(A): Elution diagram
(B): Toxin compositions contained in fraction fr. 85 by HPLC-FLD analysis
fr.85(left), TTX standards(right)

Figure 7. Elution profile of the ribbon worm *Cephalothrix* sp. toxin from a Bio-Rex 70 column with linear gradient from 0 to 0.03M AcOH



scale bar = 5 μm

Figure 8. Scanning electron micrograph of crystalline toxin isolated from the ribbon worm *Cephalothrix* sp. from Hiroshima Bay.



(A): *Demania cultripes*
(B): HPLC-FID analysis of the toxin contained in the viscera of *D.cultripes*
(C): TTX standards

Figure 9. HPLC-FLD analysis of TTX in the viscera of toxic crab *Demania cultripes* from Cebu Island, in the Philippines

4. Mass spectrometry

4.1 Gas-Chromatography-Mass Spectrometry

Gas chromatography (GC) and mass spectrometry (MS) form an effective combination for chemical analysis. GC-MS analysis is an indirect method to detect TTX in a crude extract which is difficult to purify in other advanced analysis methods [33]. In this method, TTX and its derivatives are dissolved in 2 ml of 3 M NaOH and heated in a boiling water bath for 30 min. After cooling to room temperature, the alkaline solution of decomposed compounds is adjusted to pH 4.0 with 1N HCl and the resulting mixture is chromatographed on a Sep-Pak C18 cartridge (Waters). After washing with H_2O first and then 10% MeOH, 100% MeOH fraction were collected and evaporated to dryness *in vacuo*. To the resulting residue, a mixture of *N*, *O*-bis acetamide, trimethylchlorosilane and pyridine (2: 1: 1) is added to generate trimethylsilyl (TMS) ''C9-base'' compounds (Figure 10). The derivatives are then placed in a Hewlett Packard gas chromatograph (HP-5890-II) equipped with a mass spectrometer (AutoSpec, Micromass Inc., UK). A column (ϕ 0.25 mm × 250 cm) of UB-5 (GL Sci., Japan) is used, and the column temperature is increased from 180 to 250°C at the rate of 5 or 8°C/min. The flow rate of inlet helium carrier gas is maintained at 20 ml/min. The ionizing voltage is generally maintained at 70 eV with the ion source temperature at 200°C. Scanning was performed in the mass range of *m*/*z* 40–600 at 3s intervals. The total ion chromatogram (TIC) and the fragment ion chromatogram (FIC) were selectively monitored. The TMS derivative of 2-amino-6-hydroxymethyl-8-hydroxyquinazoline (C9 base), prepared using gastropods and ribbon worms from Hiroshima Bay by the procedure described previously, was analyzed by GC-MS [18, 38, 39]. Recently, the isolation and characterization of bacteria from the copepod *Pseudocaligus fugu* ectoparasitie on the panther puffer T*akifugu pardalis* with an emphasis on TTX was reported [40]. The mass spectrum of the peak showed typical ions at *m*/*z* = 407 and 392, which correspond to M^+ and $(M\text{-}CH_3)^+$ of C9-base-$(TMS)_3$, respectively. Sharp fragment ions appear at *m*/*z* = 407 (parent peak), 392 (base peak) and 376, indicating the presence of quinazoline skeleton in the toxin (Figure 11). It is noteworthy that each peak of selected ion monitored at *m*/*z* = 376, 392 and 407 appears at the same retention time. In the selected ion-monitored mass chromatogram of the TMS derivatives of alkali-hydrolyzed from crystals prepared from ribbon worm in Hiroshima Bay, mass fragment ion peaks at *m*/*z* 376, 392 and 407, which are characteristic of the quinazoline skeleton (C9 base), appeared at retention times (8.33 and 8.34 min.), almost the same as those from the TMS-C9 base derived from authentic TTX (Figure 12). Screening of tetrodotoxin in pufferfish using GC-MS was reported [41]. Sensitive analysis of TTX in human plasma by solid-phase extraction and GC-MS was reported [42].

4.2. Fast atom bombardment mass spectrometry

Fast atom bombardment mass spectrometry (FAB-MS) is a direct method for the qualitative confirmation of TTX. The analysis was performed on a JEOL JMX DX-300 mass spectrometer [43]. Xenon is used to provide the primary beam of atoms, the acceleration voltage of the primary ion being 3 kV. Scanning is repeated within a mass range of *m*/*z* = 100-500. In this analysis, approximately 0.1 mg of TTX and glycerol are placed as the matrix on the sample

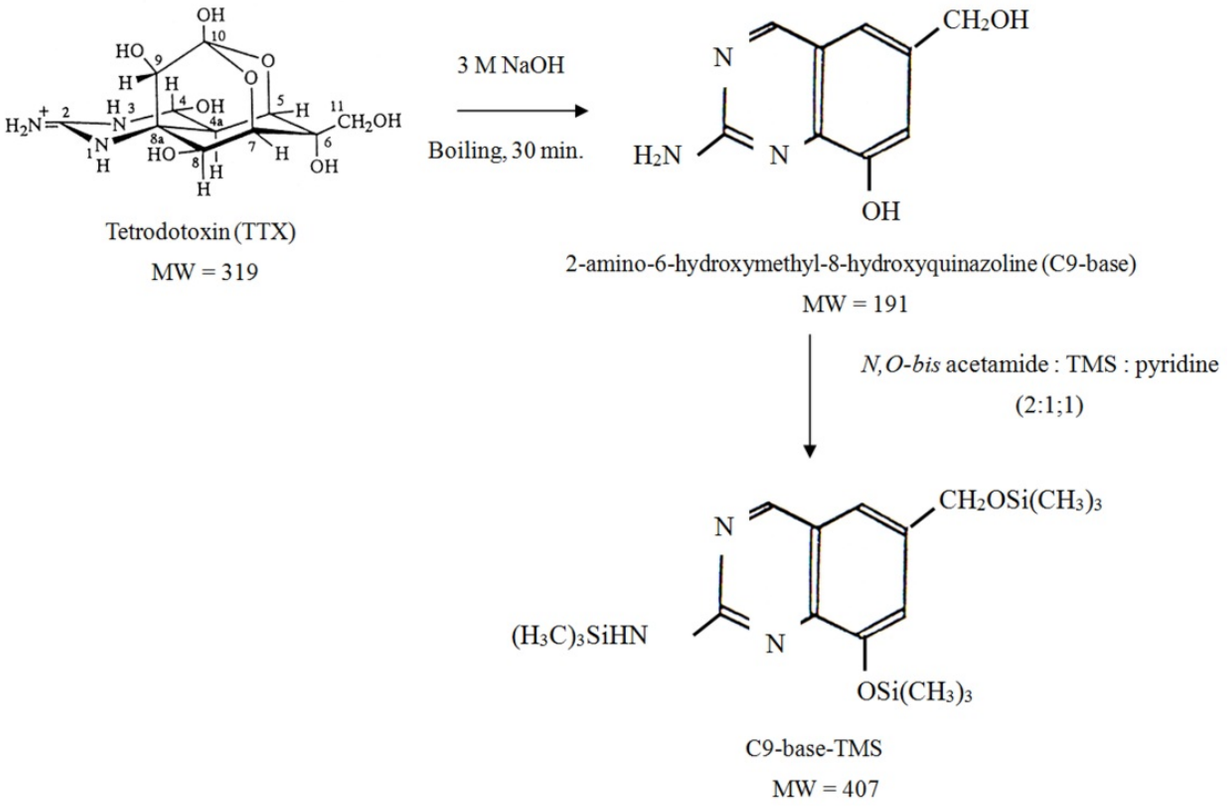


Figure 10. Reaction pathways from TTX to C9-base-TMS

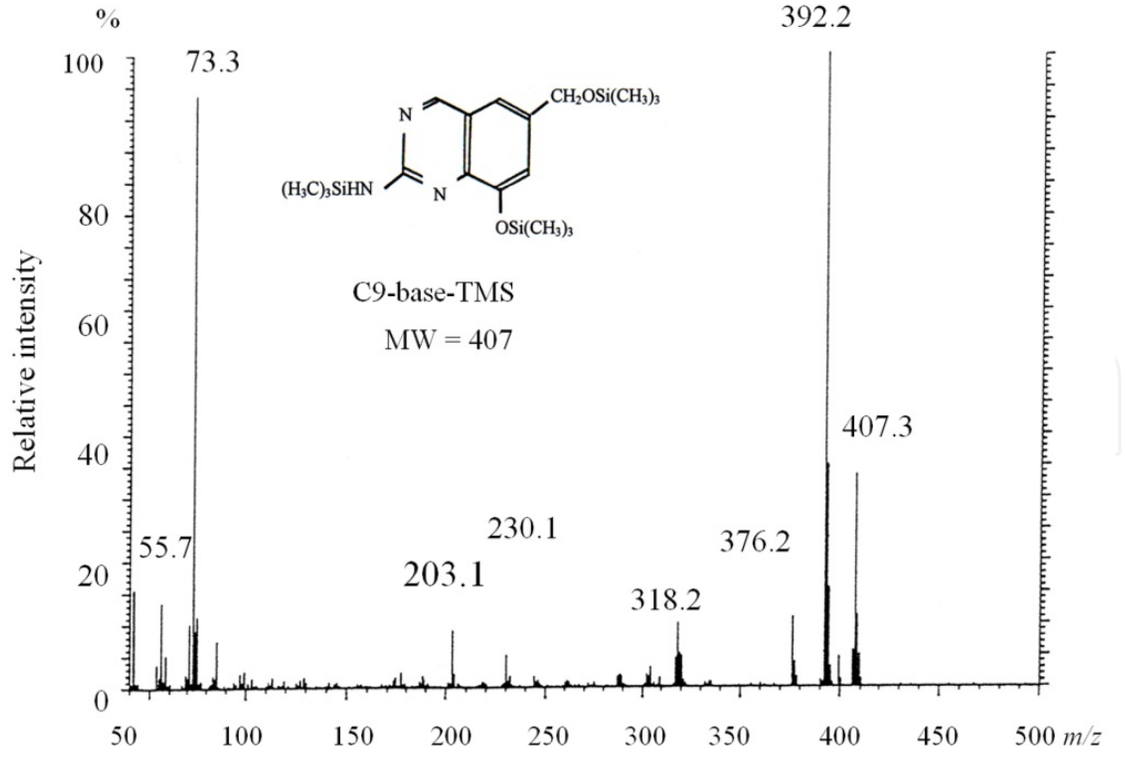
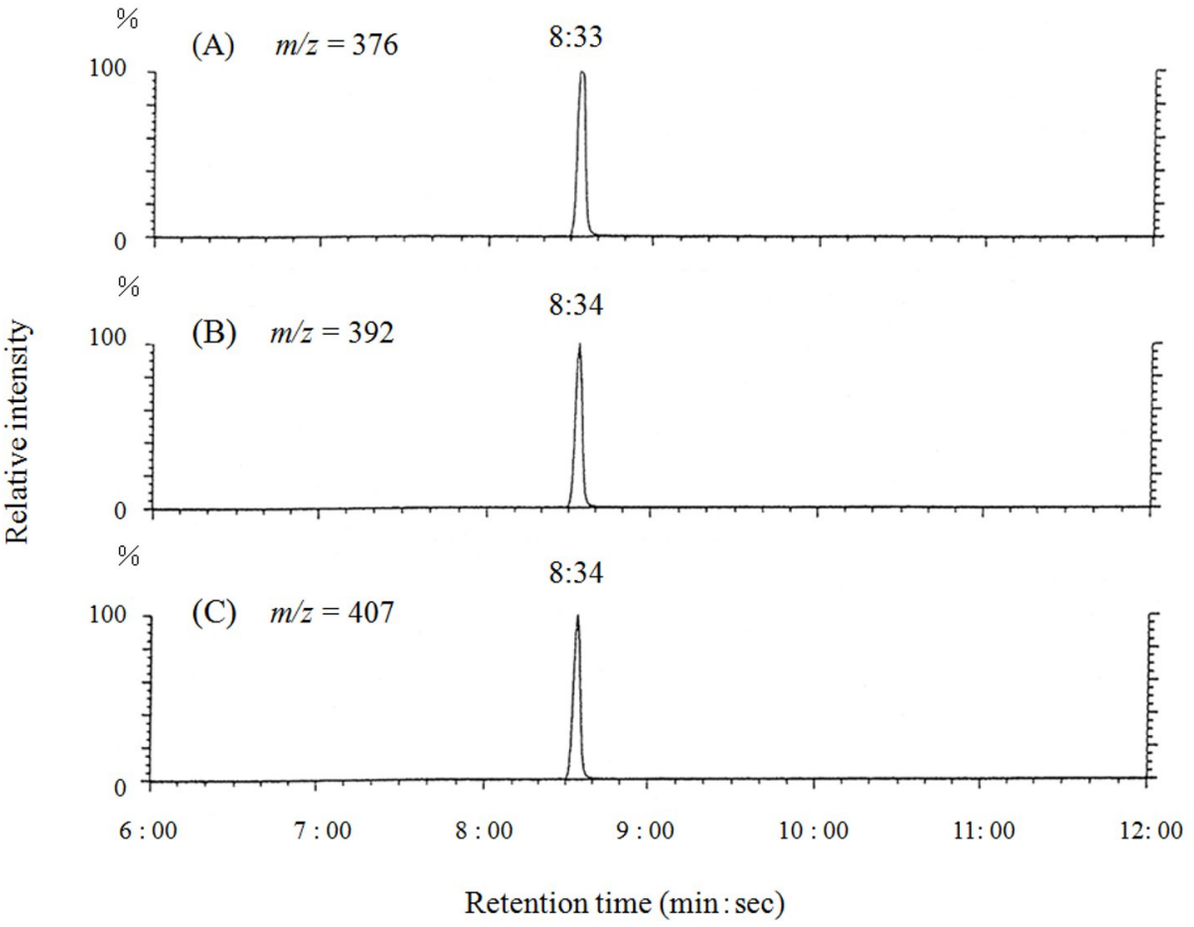


Figure 11. Mass spectrum of the trimethylsilyl (TMS) derivatives of alkali-hydrolyzed toxin from the ribbon worm *Cephalothrix* sp. from Hiroshima Bay.



(A) $m/z = 392$ (B) $m/z = 407$ (C) $m/z = 376$

Figure 12. Selected ion-monitored (SIM) mass chromatograms of the trimethylsilyl (TMS) derivatives of alkali-hydrolyzed toxin from the ribbon worm *Cephalothrix* sp. from Hiroshima Bay.

stage of the mass spectrometer, mixed well, and placed in the ion chamber of the spectrometer. Then, both positive and negative mass spectra of TTX are measured. TTX shows $(M+H)^+$ and $(M+H-H_2O)^+$ ion peaks at m/z 320 and 302, respectively, in the positive mass spectrum, and an $(M-H)^-$ peak at m/z 318 in the negative spectrum (Figure 13). Secondary ion mass spectrometry (SIMS) performed with a Hitachi M-80B mass spectrometer gave essentially the same result as that obtained by FABMS. An extensively purified sample is required for the successful application of this method. Nagashima *et al*. developed a method to detect TTX by TLC-FAB-MS, in which the limit for detection TTX was approximately 0.1 μgTTX. [44]. TTX was also detected clearly by cellulose acetate membrane electrophoresis/FAB-MS, along with selected ion-monitored chromatograms of a mixture of TTX, anhydroTTX, and tetrodonic acid (TDA).

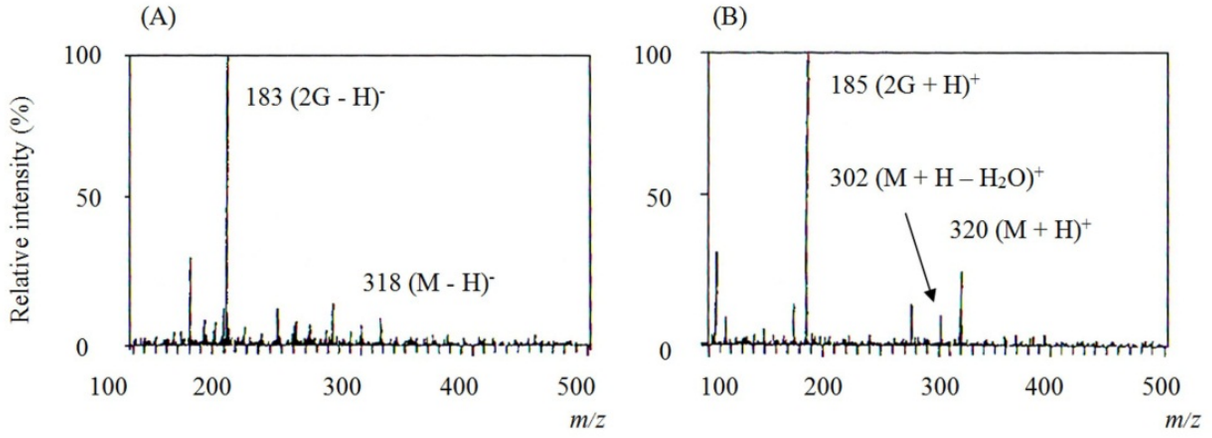


Figure 13. Positive (right) and negative (left) FAB mass spectra of TTX [41].

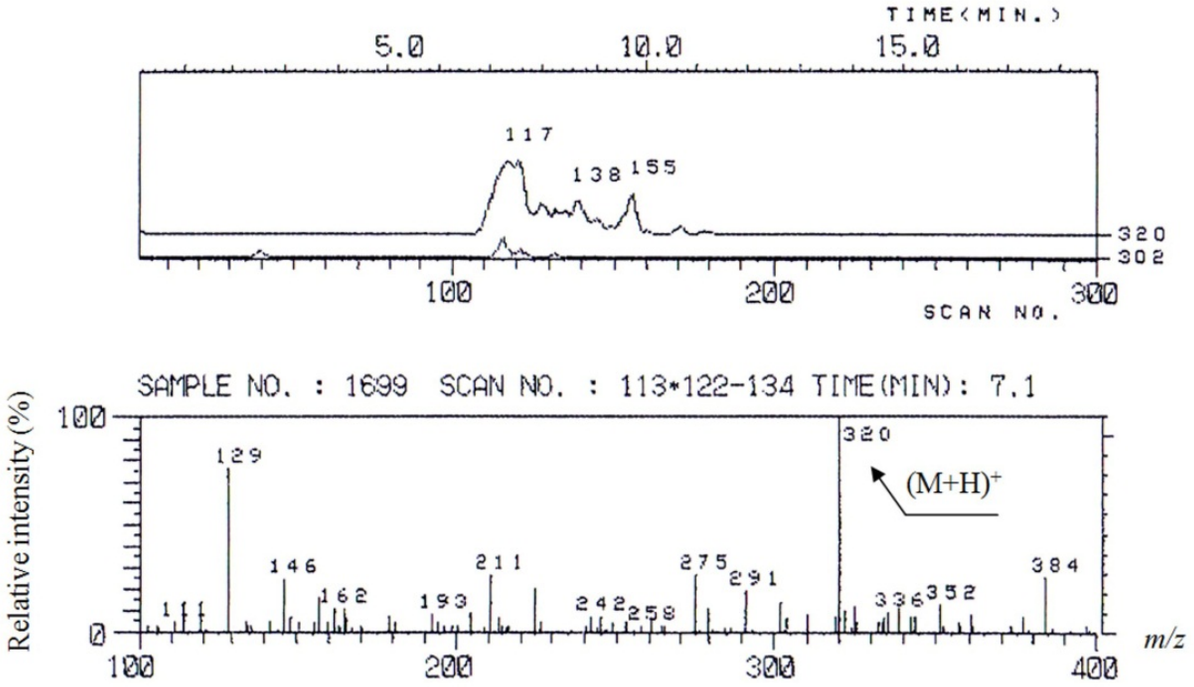
4.3. Liquid chromatography mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) is developed to detect TTX with considerable accuracy [45]. The major disadvantage of LC-FLD is the large difference in the structure-dependent fluorescence intensities of the analogs. In particular, the fluorescence intensities of 5-deoxyTTX and 11-deoxyTTX are approximately 1/20 and less than 1/100 of that of TTX, respectively, while those of 6-*epi*TTX and 11-norTTX-6(*R*)-ol are approximately 20-fold and 10-fold greater than that of TTX, respectively [46]. LC-MS could solve this problem, if sufficient separation and high ionization intensities could be achieved. In this method, combined HPLC-MS is performed using a Hitachi M-1000 system coupled to a mass spectrometer. The HPLC system is equipped with an ODS (ϕ 1.5 × 150 mm) column. MeOH or acetonitrile (50%, flow rate 70 μl/min) is used as the mobile phase. The effluent from the column is split to provide flow to the ion-spray interface. Brackish water puffer toxins were analyzed by LC-MS [10, 11]. An example of LC-MS of a toxin purified from ribbon worms from Hiroshima Bay is shown in Figure 14. In the MS, a protonated molecular ion peak (M+H)+ appeared at *m/z* = 320 showing a molecular weight for the toxin(319) in good accordance with that of TTX. Tsuruda *et al.*, detected TTX, 4-*epi*TTX, 4, 9-anhydroTTX, 6-*epi*TTX and 4, 9-anhydro-6-*epi*TTX from toxin secreted by newts *Cynops pyrrhogaster* on being subjected to "handling stimulus" [47].

4.4. Electrospray ionization – Time of flight – Mass spectrometry

Electrospray ionization time of flight mass spectrometry (ESI-TOF-MS) is applicable to many fields including the analysis of proteins, natural extracts, synthetic mixtures and medical drugs. ESI-TOF-MS is a valuable technique for identification of TTX, although it is not widely used to date in marine toxin determinations. In this analysis, a portion of purified TTX (less than 0.05 mg) is dissolved in a small amount of 1% AcOH, and the resulting solution is added to 50% aqueous MeOH. ESI-TOF-MS is run on a Micromass Q-TOF mass spectrometer. TTX in a tree frog *Polypedates* sp. extract has been successfully evaluated by ESI-TOF-MS analysis [48]. As shown in the spectrum of the toxin (Figure 15),

the protonated molecular ion peak (M + H)+ appeared at *m/z* = 320.1103, suggesting the molecular weight of the toxin to be 319.1025 which agrees well with that of authentic TTX ($C_{11}H_{17}N_3O_8$ = 319.1016).



(A): Mass chromatogram of the ribbon worm toxin
(B): Mass spectrum of the ribbon worm toxin

Figure 14. LC-MS of the toxin from the ribbon worm *Cephalothrix* sp. from Hiroshima Bay.

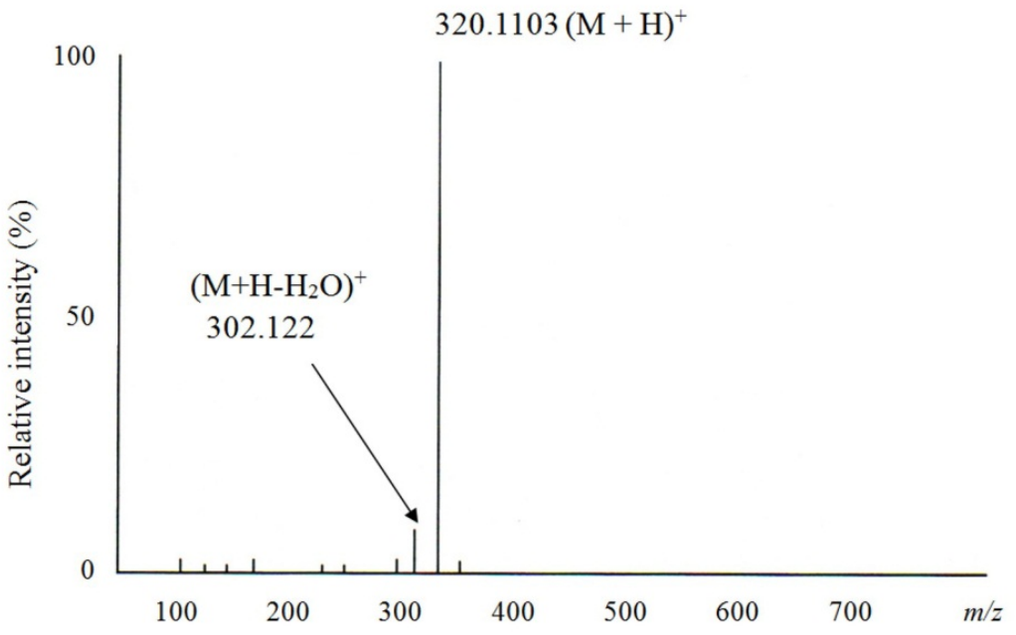
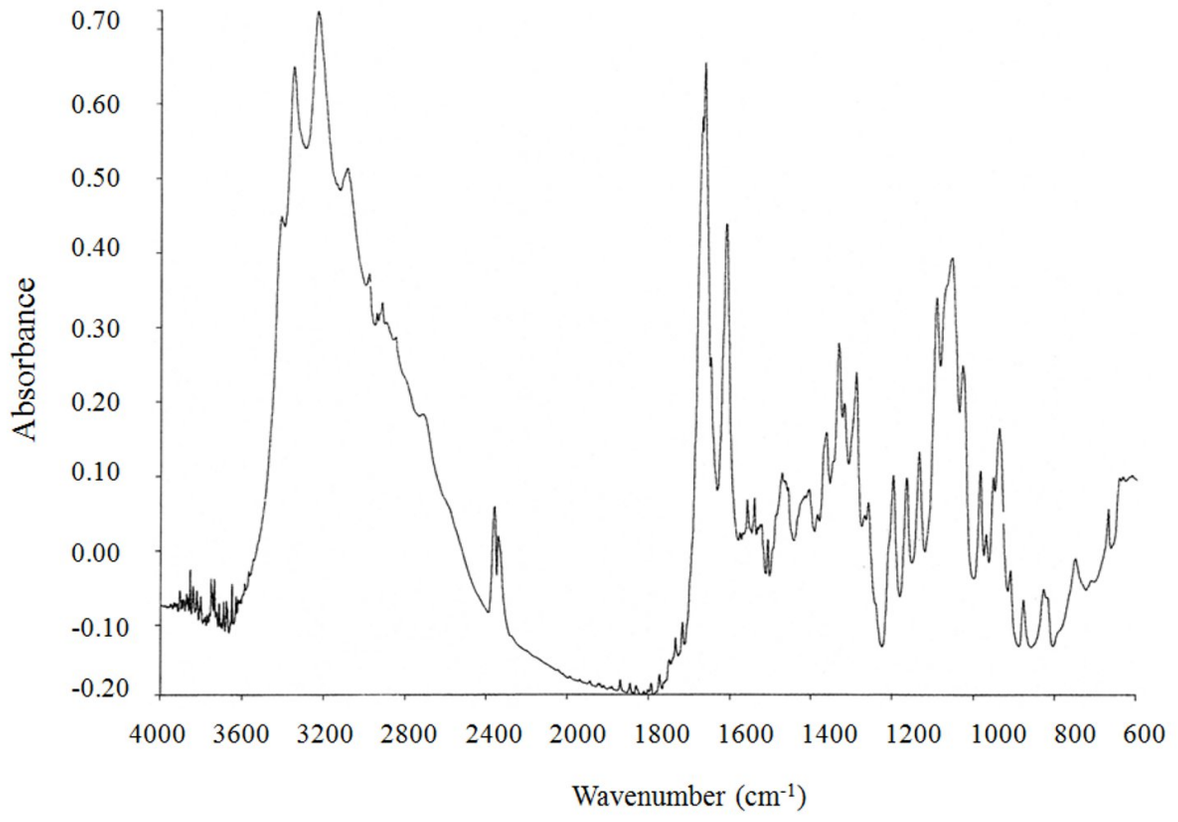


Figure 15. ESI-TOF/MS analysis of a frog *Polypedates* sp. toxin [46].

5. Infrared (IR) spectrometry

IR spectrometry is the analytical technique for the determination of functional groups in TTX. Although the IR spectrum is presumed to be complex, it is a helpful tool to identify TTX. IR-spectra of KBr pellet were acquired using IR spectrophotometer, which was used by Onoue *et al.* for determination the IR spectrum of pufferfish toxin [49]. On the other hand, Tsuda *et al*. reported the IR spectrum of a TTX-HCl salt by the "Nujol" method [2]. Here we introduce another method as mentioned below. A part of TTX crystals purified from the specimens of ribbon worms were placed on a small KBr plate, and the IR spectrum was acquired using a FT-IR spectrometer (Perkin Elmer, Spectrum 2000) equipped with FT-IR microscope. As shown in Figure 16, absorption bands at 3353, 3235, 1666, 1612 and 1076 cm^{-1} were observed in the spectrum of this crystal. This spectrum was indistinguishable from that of TTX reported previously, showing characteristic absorptions for the functional groups OH, guanidium, and COO^- [2]. The absorption near 2400 cm^{-1} was derived from the existence of CO_2 in the air. The absorption around 1800 cm^{-1} and in the range of 3,600 – 4,000 cm^{-1} was derived from H_2O in the air. Although the spectrum appears to be complex, it is a helpful tool for identification of TTX.

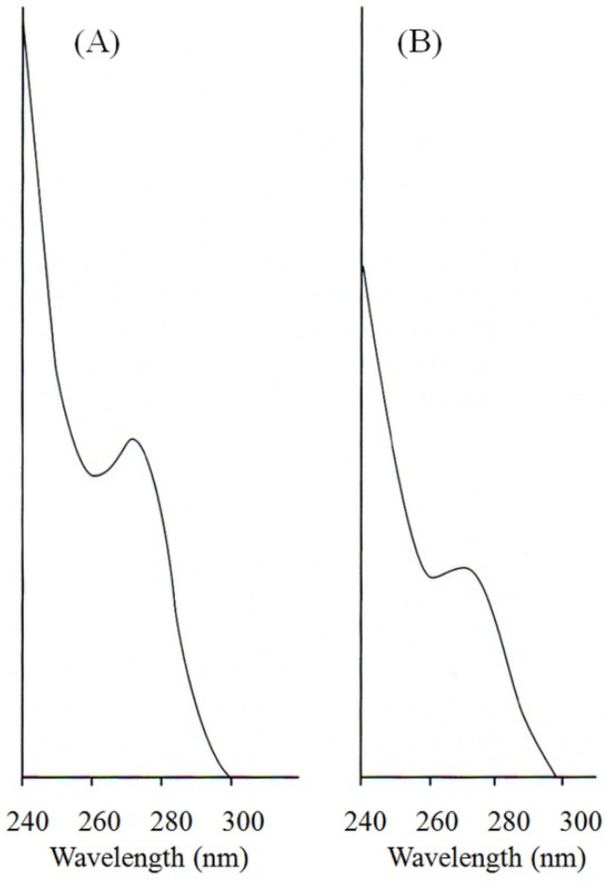


IR spectrum was taken on a FT-IR spectrometer (Perkin Elmer, Spectrum 2000) equipped with FT-IR microscope.

Figure 16. IR spectrum of the toxin isolated from the ribbon worm *Cephalothrix* sp. from Hiroshima Bay.

6. Ultraviolet (UV) spectroscopy

In UV spectroscopy, TTX is generally determined by irradiating a crude toxin with UV light. A small amount of TTX is dissolved in 2 ml of 2 M NaOH and heated in a boiling water bath for 45 min. After cooling to room temperature, the UV spectrum of the solution is examined for characteristic absorptions, associated with C9-base, 2-amino-6-hydroxymethyl-8-hydroxyquinazoline, possibly formed from TTX and/or related substances, if present. In the analysis, the UVspectrum of the alkali decomposed compounds of TTX appears as a shoulder at near 276 nm, indicating the formation of C9-base specific to TTX or related substances (Figure 17). Saito *et al.* used this method in experiments analyzing TTX and its derivatives in toxic and nontoxic pufferfish [50].



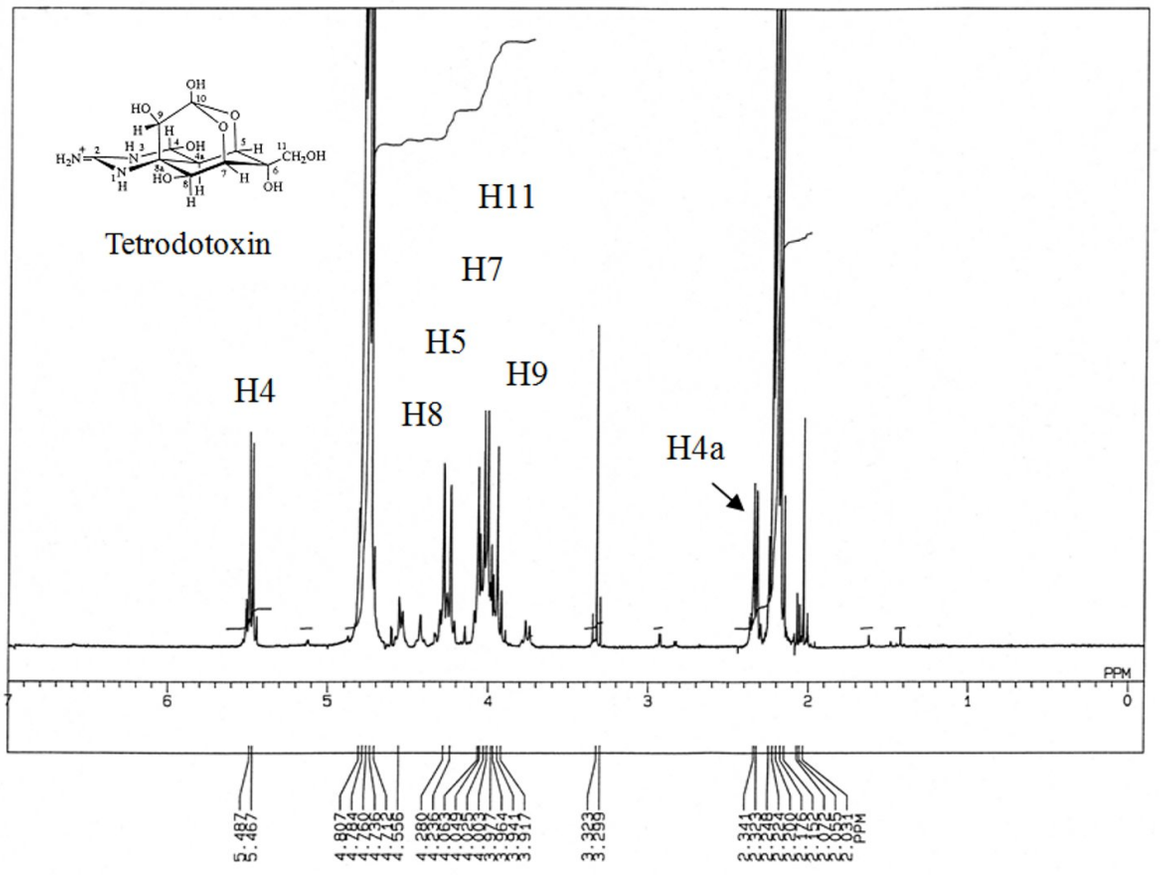
(A): TTX fraction from non toxic species of pufferfish *L. wheeleri*
(B): Authentic TTX

Figure 17. UV absorption spectra of the alkaline hydrolyzates of TTX fraction from non toxic species of pufferfish ("shirosabafugu" in Japanese) *Lagocephalus wheeleri* [48].

7. Proton nuclear magnetic resonance (^{1}H-NMR) spectrometry

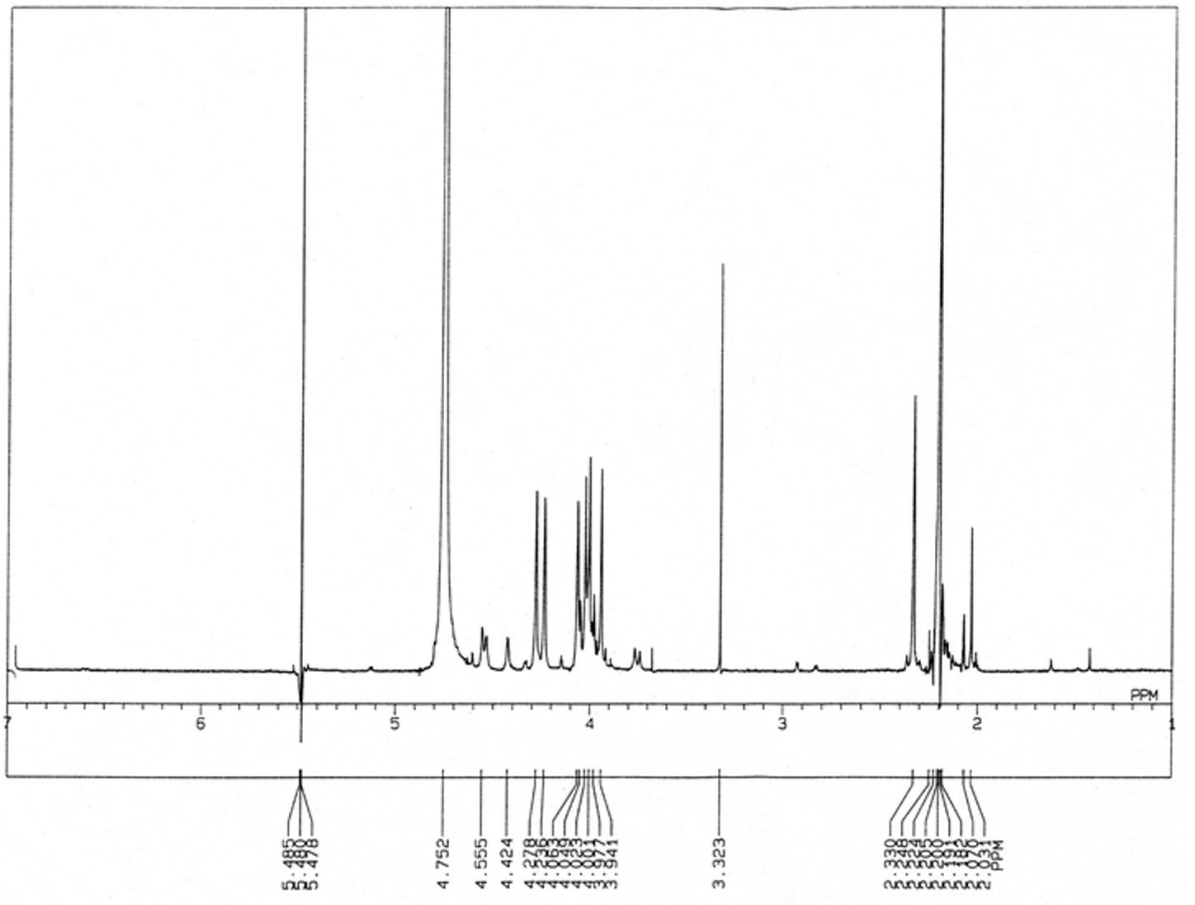
^{1}H-NMR has played an important role as a complementary method to determine the absolute configuration of TTX. To date, many derivatives of TTX have been isolated, and their ^{1}H-NMR data have been reported by various investigators. In a typical ^{1}H-NMR

analysis, 5 mg of TTX crystals have been dissolved in 0.5 ml of 1% CD_3COOD in D_2O, and placed in a test tube. Figure 18 shows the ^{1}H-NMR spectrum obtained with a 500 MHz JEOL JNM-500 spectrometer, using the methyl group protons of acetone as the internal standard [39]. The ^{1}H-NMR spectrum exhibited a singlet at 2.20 ppm (CH_3COCH_3), a doublet centered at 2.33 ppm (J =10.0 Hz), a large proton peak at 4.76 ppm (HDO) and a doublet centered at 5.48 ppm (J=10.0 Hz). The pair of doublets around 2.33 and 5.48 ppm, which are the hallmarks of TTX and are assigned to H-4a and H-4, respectively, have been confirmed to be coupled with each other by double irradiation (Figure 19). These results agree well with the corresponding data of TTX. The signals at 4.24, 4.06, 4.28, 3.94, 4.00 and 4.02 ppm are assigned toH-5, H-7, H-8, H-9 and H-11, respectively (Figure 20). A toxin isolated from the horseshoe crab *Carcinoscorpius rotundicauda* from Bangladesh was analyzed by HPLC-FLD, TLC, electrophoresis and ^{1}H-NMR, and was identified as TTX [51]. Identification of a neurotoxin from the blue-ringed octopus, brackish water pufferfish, marine pufferfish and so on as TTX via this method were reported [9, 10, 11, 17].



Five milligrams of the ribbon worm toxin was dissolved in 0.5ml of 1% CD_3COOD in D_2O and measured for ^{1}H-NMR spectrum measured on a JEOL JNM-500NMR spectrometer, using acetone as the internal standard.

Figure 18. ^{1}H-NMR spectrum of the toxin isolated from the ribbon worm *Cephalothrix* sp. from Hiroshima Bay.



Five milligrams of the ribbon worm toxin was dissolved in 0.5ml of 1% CD_3COOD in D_2O and measured for ^1H-NMR spectrum measured on a JEOL JNM-500NMR spectrometer, using acetone as the internal standard.

Figure 19. ^1H-NMR spectrum of the toxin isolated from the ribbon worm *Cephalothrix* sp. from Hiroshima Bay by means of irradiation at C_4-H.

H	Ribbon worm toxin
4a	2.33
4	5.48
5	4.24
7	4.06
8	4.28
9	3.94
11	4
	4.02

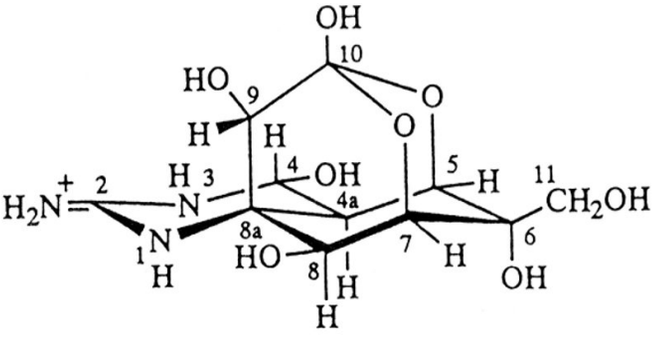


Figure 20. ^1H-NMR spectral data of the toxin isolated from the ribbon worm *Cephalothrix* sp. from Hiroshima Bay, along with the structure of TTX.

8. Thin-layer chromatography

TLC is a very commonly used technique in synthetic chemistry for identifying compounds, determining their purity. In TLC analysis, TTX is spotted onto a silica gel 60 precoated plate (Merck). The plate is developed in two different solvent systems of pyridine-ethyl acetate-AcOH -water (15:5:3:4) and 3-BuOH-AcOH-water (2:1:1) in a sealed chamber. The solvent rises by capillary action and an ascending chromatographic separation is obtained. The plate is then sprayed with 10% KOH followed by heating at 100℃ for 10 minutes. The toxin is visualized as a yellow fluorescent spot under UV light (365nm). In TLC analysis, the Rf values of TTX are around 0.71(0.65) and 0.50, respectively [10, 51]. It is also possible to detect TTX on the TLC plate using the Weber reagent that gives pink spot of the toxin. The detection limit is about 2 μg of TTX (10 MU). TLC is a useful technique in those laboratories where HPLC and other costly analytical systems are not available.

9. Electrophoresis

Electrophoresis is a relatively simple and rapid method with high resolution detection of polar compounds like TTX [10, 11, 51]. When 1 μl of TTX (10 MU, corresponding to 2 μg) is applied onto a 5 x 18 cm cellulose acetate membrane (Chemetron, Milano), the ion molecules of TTX move toward the cathode with a mobility (Rm) clearly smaller than that of authentic of STX. The analysis is performed for 30 minutes in an electrolytic buffer solution of 0.08 M Tris-HCl (pH8.7), under the influence of an applied electric field with a constant current of 0.8 mA/cm width. The toxin is visualized in the same manner as described for TLC.

10. Capillary isotachophoresis

Capillary isotachophoresis proved to be a very effective technique for the analyses of organic acids, carbohydrates, drugs and amino acids. It is a rapid and accurate detection technique for TTX [52]. It is performed using a cationic system, as TTX exists as cation under acidic and neutral conditions. Conditions for capillary isotachophoresis composed of a leading electrolyte of 5 mM potassium acetate (pH6.0), containing 0.2% Triton X-100 and 0.5 volume of dioxane, and a terminating electrolyte of 10mM β-alanine adjusted to pH 4.5 with acetic acid. When TTX is applied to isotachophoretic analyzer (Shimadzu IR-2A) equipped with a potential gradient 0.32, it is eventually monitored by the detector. The quantitative detection limit by this method is about 0.25μg of TTX. It was possible to quantify TTX content of contaminated puffer extracts without any pretreatment.

11. Conclusion

In an attempt to protect consumers from TTX-intoxication, the mouse bioassay has historically been the most universally applied tool to determine the toxicity level in monitoring programs. This bioassay, however, shows low precision and requires a continuous supply of mice of a specific size. These potential drawbacks and world-wide pressure to refrain from the unnecessary killing of live animals subsequently led scientists to

develop alternative chemical methods to the mouse bioassay for TTX detection and quantification. TTX levels in pufferfish are normally estimated using the mouse bioassay. However, this assay and other techniques such as TLC, electrophoresis, LC, spectrophotometry, and the enzyme-linked immunosorbent assay (ELISA) pose ethical concerns, are not specific and lack sensitivity and precision at low concentrations. HPLC-FLID and LC-MS/GC-MS are sensitive techniques for identification of TTX. However, due to the complexity of sample matrices and insolubility of TTX in organic solvents, HPLC-FLD and LC-MS (or LC-MS/MS) are more preferred methods than GC-MS. MS spectrometry is a powerful technique that also has an important future for the analysis of marine toxins. In addition to high sensitivity and selectivity, MS can provide structural information useful for the confirmation of toxin identity and the identification of new toxins. The drawback of LC-MS and LC-MS/MS analyses is that they involve the use of expensive instruments, which require higher maintenance compared to GC-MS. Nevertheless, for routine analysis of TTXs, HPLC-FID and LC-MS (LC-MS/MS) are expected to replace the conventional mouse bioassay.

Author details

Manabu Asakawa* and Keisuke Miyazawa
Department of Bioresource Science and Technology, Department of Bioresource Science and Technology, Graduate School of Biosphere Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima, Hiroshima, Japan

Yasuo Shida
Clean Energy Research Center, University of Yamanashi, Takeda, Kofu, Yamanashi, Japan

Tamao Noguchi
Tokyo Healthcare University, Setagaya, Tokyo, Japan

Acknowledgement

This work was supported by a Grant-in-Aid for Scientific Research (C) (2) (No.11660205), (C)(No.18580204), (C)(No.20580220) and (B)(No.20380110) from Japan Society for the Promotion of Science. We are grateful to Dr.Hiroshi Kajihara (Hokkaido University) for his helpful advice on ribbon worms.

12. References

[1] Yokoo, A. (1950) Chemical studies on pufferfish toxin (3) – separation of spheroidine. *Nippon KagakuZasshi*, 71:590-592.

[2] Tsuda, K., Ikuma, S., Kawamura, M., Tachikawa, R., Sakai, K., Tamura, C., Amakasu, D. (1964) Tetrodotoxin. VII. On the structures of tetrodotoxin and its derivatives. *Chem.Pharm. Bull.* 12:1357-1374.

[3] Woodward, R.B. (1964) The structure of tetrodotoxin. *Pure Appl. Chem.* 9:49-74.

* Corresponding Author

[4] Goto, T., Kishi, Y., Takahashi, S., Hirata, Y. (1965) Tetrodotoxin. *Tetrahedron* 21:2059-2088.
[5] Narahashi, T. (2001) Pharmacology of tetrodotoxin. *J. Toxicol.-Toxin Rev.* 20:67-84
[6] Noguchi, T., Ebesu, J.S.M. (2001) Puffer poisoning: epidemiology and treatment. *J. Toxicol.-Toxin Rev*. 20: 1-10.
[7] Hashimoto, Y. (1979) Marine Toxins and Other Bioactive Marine Metabolites. Japan Scientific Societies Press, Tokyo, pp.70-91.
[8] Khora, S.S., Isa, J., Yasumoto, T. (1991) Toxicity of puffers from Okinawa, Japan, *Nippon Suisan Gakkaishi*, 57:163-167.
[9] Mahmud, Y., Tanu, M.B, Takatani, T., Asayama, E., Arakawa. O., Noguchi, T. (2001) *Chelonodon patoca*, a highly toxic marine puffer in Japan. *J. Nat Toxins*, 10:69-74.
[10] Mahmud, Y., Yamamori, K., Noguchi, T. (1999) Occurrence of TTX in a brackishwater puffer "Midorifugu" *Tetraodon nigroviridis*, collected from Thailand. *J.Food Hyg. Soc.*, 40:363-367.
[11] Mahmud, Y., Yamamori, K., Noguchi, T. (1999) Toxicity and tetrodotoxin as the toxic principle of a brackishwater puffer *Tetraodon steindachneri*, collected from Thailand, *J.Food Hyg. Soc.*, 40:391-395.
[12] Hwang, D.F, Kao, C.Y., Yang, H.C., Jeng, S.S., Noguchi, T., Hashimoto, K. (1992) Toxicity of puffer in Taiwan, *Nippon Suisan Gakkaishi*, 58:1541-1547.
[13] Mahmud, Y., Tanu, M.B., Noguchi, T. (1999) First occurrence of a food poisoning incident due to ingestion of *Takifugu oblongus*, along with a toxicological report on three marine puffer species in Bangladesh. *J.Food Hyg. Soc.*, 40:473-480.
[14] Mosher,H.S., Fuhrman,F.A., Buchwald,H.G., Fischer, H.G. (1964) Tarichatoxin - tetrodotoxin, a potent neurotoxin. *Science* 144: 1100-1110.
[15] Noguchi, T., Kao, H., Hashimoto, Y. (1971) Toxicity of the goby, *Gobius criniger*. *Bull. Jpn. Soc.Sci.Fish.*, *37:642*-647.
[16] Kim, Y.H., Brown,G.B., Mosher, H.S.,Furman, F.A. (1975) Tetrodotoxin : occurrence in Atelopid frogs of Costa Rica. *Science* 189:151-152.
[17] Sheumack, D.D., Howden, M.E.H., Spence, I., Quinn, R.J. (1978) Maculotoxin: a neurotoxin from the venom glands of the octopus *Hapalochlaena maculosa* identified as tetrodotoxin. *Science* 199:188-189.
[18] Narita,H., Noguchi, T., Maruyama,J, Ueda,Y., Hashimoto, K., Watanabe, Y., Hida,K. (1981) Occurrence of tetrodotoxin in a trumpet shell "boshubora" , *Charonia sauliae*. *Bull. Japan. Soc. Sci. Fish*., 47:935-941.
[19] Noguchi,T., Maruyama,J., Ueda,Y., Hashimoto,K. Harada,T. (1981) Occurrence of tetrodotoxin in the Japanese ivory shell *Babylonia japonica*. *Bull. Jpn. Soc.Sci.Fish.*, 47:909-913.
[20] Noguchi, T., Maruyama, J., Narita, H., Hashimoto, K. (1984) Occurrence of tetrodotoxin in the gastropod mollusk *Tutufa lissostoma* (frog shell). *Toxicon* 22:219-226.
[21] Noguchi, T. Narita, H. Maruyama, J. Hashimoto, K. (1982) Tetrodotoxin in the starfish *Astropecten polyacanthus*, in association with toxification of a trumpet shell, "boshubora", *Charonia sauliae*. *Bull. Japan. Soc. Sci. Fish*. 48, 1173-1177.
[22] Kawabata, T., 1978. In: Ministry of Health and Welfare of Japan, (Ed.), Assay Method for Tetrodotoxin, Food Hygiene Examination Manual, vol. 2. Japan Food Hygiene Association, Tokyo, pp. 232–239.

[23] Asakawa, M., Toyoshima, T., Shida, Y., Noguchi, T., Miyazawa, K.(2000) Paralytic toxins in a ribbon worm *Cephalothrix* species (Nemertean) adherent to cultured oysters in HiroshimaBay, Hiroshima Prefecture, Japan. *Toxicon* 38, 763–773.

[24] Yasumoto, T., Nakamura, M., Oshima, Y., Takahata, J. (1982) Construction of a continuous tetrodotoxin analyzer. *Bull.Jpn.Soc.Sci.Fish.*, 48:1481-1483.

[25] Nakamura, M., Yasumoto, T. (1985) Tetrodotoxin derivatives in puffer fish. *Toxicon* 23:271-276.

[26] Yasumoto, T., Yotsu, M., Murata, M., Naoki, H. (1988) New tetrodotoxin analogues from the newt *Cynops ensicauda*. *J.Am.Chem. Soc.*, 110:2344-2345.

[27] Khora, S. S., Yasumoto, T. (1989) Isolation of 11-oxotetrodotoxin from the puffer *Arothron nigropunctatus*. *Tetrahedron Lett.* 30: 4393-4394.

[28] Endo, A., Khora, S.S., Murata, M., Naoki, H., Yasumoto, T. (1988) Isolation of 11-nortetrodotoxin-6(R)-ol and other tetrodotoxin derivatives from the puffer *Fugu niphobles*. *Tetrahedron Lett.* 29: 4127-4128.

[29] Yotsu, M., Hayashi,Y., Khora, S.S., Sato, S., Yasumoto, T. (1992) Isolation and structural assignment of 11-nortetrodotoxin-6(S)-ol from the puffer *Arothron nigropunctatus*. *Biosci. Biotech. Biochem.* 56: 370-371.

[30] Kotaki, Y., Shimizu, Y. (1993) 1-hydoxy-5, 11-dideoxytetrodotoxin, the first N-hydroxy and ring-deoxy derivative of tetrodotoxin found in the newt *Taricha granulosa*. *J.Am.Chem.Soc.* 115: 827-830.

[31] Yotsu-Yamashita, M., Yamagishi, Y. Yasumoto, T. (1995) 5, 6, 11-trideoxytetrodotoxin from the puffer fish *Fugu poecilonotus*. *Tetrahedron Lett.* 36: 9329-9332.

[32] Yotsu-Yamashita, M., Schimmele, B., Yasumoto, T. (1999) Isolation and structural assignment of 5-deoxytetrodotoxin from the puffer fish *Fugu poecilonotus*. *Biosci. Biotech. Biochem.* 63: 961-963.

[33] Yotsu, M., Endo, A., Yasumoto, T. (1989) An improved tetrodotoxin analyzer. *Agric. Biol. Chem.*, 53: 893-895.

[34] Yotsu-Yamashita, M., Meds, D., Kwet, A., Schneider, M. (2007). Tetrodotoxin and its analog 6-*epi*tetrodotoxin in newts (*Triturus* spp.; Urodela, Salamandridae) from southern Germany. *Toxicon* 50: 306-309.

[35] Nagashima, Y., Maruyama, J., Noguchi, T., Hashimoto, K. (1987) Analysis of paralytic shellfish poison and tetrodotoxin by ion-pairing high performance liquid chromatography. *Nippon Suisan Gakkaishi*, 53:819-823.

[36] Asakawa,M., Delan,G.G., Tsuruda,S., Shimomura,M., Shida,Y., Taniyama,S., Quilantang, M.B., Shindo,J. (2010) Toxicity assessment of the xanthid crab *Demania cultripes* from Cebu Island, Philippines. *J.Toxicology*., Article ID172367.

[37] Noguchi, T., Jeon, J.K., Arakawa,O., Sugita,H., Deguchi,Y., Shida, Y., Hashimoto, K. (1986) Occurrence of tetrodotoxin and anhydrotetrodotoxin in *Vibrio* sp. isolated from the intestines of xanthid crab, *Atergatis floridus*. *J.Biochemistry*, 99:311-314.

[38] Narita, H., Noguchi, T., Maruyama,J., Nara, M., Hashimoto, K. (1984) Occurrence of a tetrodotoxin-associated substances in a gastropod, "Hanamushirogai" *Zeuxis siquijorensis*. *Bull.Jpn.Soc.Sci.Fish,* 50:85-88.

[39] Asakawa, M., Toyoshima,T., Ito, K., Bessho, K., Yamaguchi, C., Tsunetsugu, S., Shida, Y., Kajihara, H., Mawatari, S. F., Noguchi, T., Miyazawa, K.(2003) Paralytic toxicity in the ribbon worm Cephalothrix species (Nemertea) in Hiroshima Bay, Hiroshima Prefecture, Japan and the isolation of tetrodotoxin as a main component of its toxins. *Toxicon*, 41:747-753.

[40] Maran,B.A,V., Iwamoto, E., Okuda, J., Matsuda, S., Taniyama, S., Shida,Y., Asakawa, M., Ohtsuka, S., Nakai, T., Boxshall G.A. (2007) Isolation and characterization of bacteria from the copepod *Pseudocaligus fugu* ectoparasitic on the panther puffer T*akifugu pardalis* with the emphasis on TTX. *Toxicon*, 50:779-790.

[41] Man,C.N., Noor, N.M., Harn, G.L., Lajis, R. Mohamad, S. (2010) Screening of tetrodotoxin in puffers using gas chromatography-mass spectrometry. *J.Chrom.A*, 1217:7455-7459.

[42] Kurono,S., Hattori,H., Suzuki,O., Yamada,T., Seno, H. (2001) Sensitive analysis of tetrodotoxin in human plasma by solid-phase extraction and gas chromatography/mass spectrometry. *Anal.Letts.*, 34:2439-2446.

[43] Noguchi, T., Ali, A.E., Arakawa, O., Miyazawa, K., Kanoh, S., Shida, Y., Nishio, S., Hashimoto, K. (1991) Tetrodonic acid-like substance: a possible precursor of tetrodotoxin. *Toxicon*, 29:845-855.

[44] Nagashima,Y., Nishio, S., Noguchi, T., Arakawa,O., Kanoh,S., Hashimoto,K. (1988) Dtection of tetrodotoxin by thin-layer chromatography/fast atom bombardment mass spectrometry. *Anal.Biochem*. 175:258-262.

[45] Shida,Y., Arakawa, O., Onoue, Y. and Noguchi,T. (1998) LC/MS of marine toxin-1. In "Proceedings of the 46th Annual Conference on Mass Spectrometry,"

[46] Shoji, Y., Yamashita-Yotsu, M., Miyazawa, T., Yasumoyto,T. (2001) Electrospray ionization mass spectrometry of tetrodotoxin and its analogs: liquid chromatography/mass spectrometry, tandem mass spectrometry, and liquid chromatography/tandem mass spectrometry. *Analy. Bioch.*, 290: 10-17.

[47] Tsuruda,K., Arakawa, O., Kawatsu, K., Hamano, Y.,Takatani, T. and Noguchi, T. (2002) Secretory glands of tetrodotoxin in the skin of the Japanese newt *Cynops pyrrhogaster*. *Toxicon* 40:131-136.

[48] Tanu, M.B., Mahmud, Y., Tsuruda,K., Arakawa,O., Noguchi, T. (2001) Occurrence of tetrodotoxin in the skin of a rhacophoridid frog *Polypedates* sp. from Bangladesh. *Toxicon*, 39:937-941.

[49] Onoue,Y., Noguchi, T., Hashimoto,K. (1984) Tetrodotoxin determination method. *In* "Seafood Toxin" (E.P.Ragelis, ed.) pp.345-355. American Chemical Society, Washington, DC.

[50] Saito, T., Kanoh,S., Noguchi,T., Shida,Y., Hashimoto, K. (1987) Occurrence of anhydrotetrodotoxin in several nontoxic species of puffer. *In* "Progress in venom and toxin research" (P.Gopalakrishnakone and C.K.Tan, ed.) pp.314-324. National University of Singapore, Singapore.

[51] Tanu, M.B., Noguchi, T. (1999) Tetrodotoxin as a toxic principal in the horseshoe crab *Carcinoscorpius rotundicauda* collected from Bangladesh. *J.Food Hyg. Soc.*, 40:426-430.

[52] Shimada, K., Ohtsuru, M., Yamaguchi, T., Nigota, K. (1983) Determination of tetrodotoxin by capillary isotachophoresis. *J.Food Sci.*, 48:665-667.