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Tetrodotoxin Constituents in the Vietnamese Sea Pufferfish *Takifugu oblongus*

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Abstract:
From the Takifugu oblongus viscera collected in Vietnamese sea, three compounds were isolated. By means of spectroscopic methods including NMR and MS, theire structures were identified as tetrodotoxin (1), 6-epitetrodotoxin (2), and 5-deoxytetrodotoxin (3). This is the first time 5-deoxytetrodotoxin was found in Takifugu oblongus.

Keywords: *Takifugu oblongus, tetrodotoxin, 6-epitetrodotoxin, 5-deoxytetrodotoxin*

1. Introduction

Tetrodotoxin (TTX)has been known as a potent neurotoxin which causes death in humans upon ingestion of only 1-2 mg. This compound is capable to block excitability through its selective inhibition of the sodium-carrying system without affecting the potassium-carrying system[1].Due to the highaffinity and specificity on blocking voltage-gated sodium channels, TTX is expected to exhibit analgesic properties [2].Recently, TTX is being investigated as a possible treatment for cancer-associated pain. Early clinical trials demonstrate significant pain relief in some patients [3]. Puffer fish is the most recognizable living organism that contains tetrodotoxin (TTX) and its analogues. *Takifugu oblongus* is one of the most commonpuffer fish found in Vietnamese sea (Figure 1).

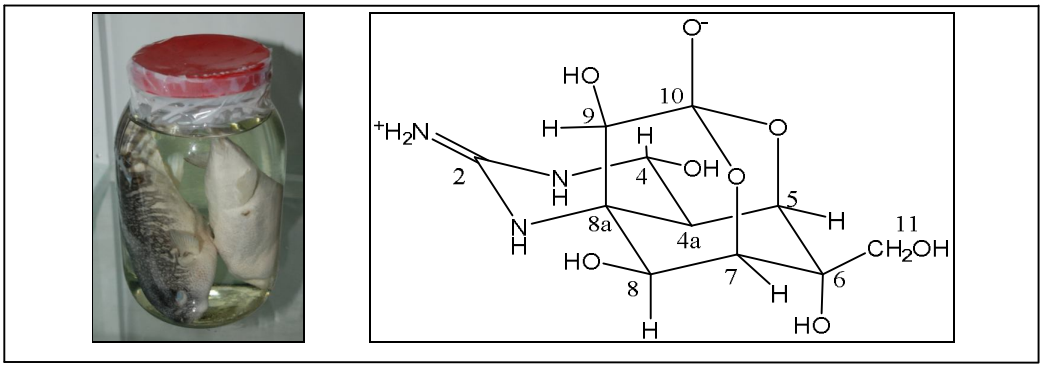

Figure 1: Takifugu oblongus and the structure of TTX

Various TTX and its analogs have been detected in *T. oblongus* organsby LC-MS analysis[4-6].Notably, Diener et al. found the presence of TTX, 4-epi TTX, anhydro TTX, 5,6,11-trideoxy TTX, TTX-11-carboxylic acid, and 11-deoxyTTX. The authors also revealed that TTX was the major analogue in liver, muscle and skin whereas 5, 6, 11-trideoxyTTX was the major analogue in ovaries of *T. oblongus* [4].TTX, 4-epiTTX and 4,6-anhTTX were also detected in*T. oblongus*collected in Vietnamese sea [5].However, very few compounds were isolated in pure form from this fish.According to the Hwang's method, TTX (94.3% purity) was purified from the liver of *T. oblongus*[6].The present paper described the isolation and structural identification of three compounds from the viscera of *T. oblongus* collected in Vietnamese sea.

2. Materials and Methods

2.1. General Experimental Procedures

NMR experiments were carried out on a Bruker AM500 FT-NMR spectrometer and a Varian 500-MHz NMR spectrometer using $CF_3COOD-D_2O$ (1:99, v/v) and $CD_3COOD-D_2O$ (4:96, v/v) as solvents. Mass spectra were recorded on an API Q-STAR PULSAR I of Applied Biosystem.

2.2. Animal Materials

The puffer fish *Takifugu oblongus* were collected in Vung Tausea, Vietnam during Jun2012 and identified by Dr. Dao Viet Ha, Institute of Oceanography, Vietnam Academy of Science and Technology. The voucher specimens were deposited at National Institute of Drug Quality Control. The viscera were collected, frozen and stored below −15 °C until use.

2.3. Extraction and Isolation

The*T.oblongus*viscera(3 kg) were homogenized extracted with 0.2 M acetic acid by heating for 20 min in boiling water. After defatted with dichloromethane,the extract was neutralized to pH 6.5 by 1MNaOH and loaded on an activated charcoal column pre-equilibrated with water. After the column was thoroughly washed with water, TTXs were eluted with 2% acetic acid in 50% aqueous ethanol. The eluate was concentrated and successively subjected to cation exchange columns of Bio-Gel P-2 and Bio-Rex 70 pre-equilibrated with water. The elution using a gradient of 0.1 to 0.5 M acetic acid allows to obtain compound **1** (650 μg), **2** (310 μg) and **3** (450 μg).

Tetrodotoxin (**1**): ^{1}H-NMR (500 MHz, $CF_3COOD-D_2O$) and^{13}C-NMR (125 MHz, $CF_3COOD-D_2O$): see Table 1.HR-ESI-MS: *m/z*320.1087 $[M + H]^+$ (calcd. for $C_{11}H_{18}N_3O_8$, 320.1094).

6-epiTetrodotoxin (**2**): ^{1}H-NMR (500 MHz, $CD_3COOD-D_2O$) and^{13}C-NMR (125 MHz, $CD_3COOD-D_2O$): see Table 1. HR-ESI-MS: *m/z*320.1087 $[M + H]^+$ (calcd. for $C_{11}H_{18}N_3O_8$, 320.1094).

5-deozyTetrodotoxin (**3**): ^{1}H-NMR (500 MHz, $CD_3COOD-D_2O$) and^{13}C-NMR (125 MHz, $CD_3COOD-D_2O$): see Table 1. HR-ESI-MS: *m/z*305.1211 $[M + H]^+$ (calcd. for $C_{11}H_{19}N_3O_7$,305.1217).

3. Results and Discussion

The HR-ESI-MS spectrum of **1**revealed the ion pic at *m/z*320.1087 $[M + H]^+$corresponding to the molecular $C_{11}H_{18}N_3O_8$. In the ^{1}H-NMR spectrum of **1**, three proton doubletsat2.25 (1H, d, *J* = 9.5 Hz, H-4a), 3.93 (2H, d, *J* = 11.0 Hz, H-11), and 5.40 (1H, d, *J* = 9.5 Hz) together with four singlets at 3.85 (1H, s, H-9), 3.88 (1H, s, H-7),4.15 (1H, s, H-5), 4.19 (1H, s, H-8) were observed. The ^{13}C-NMR and DEPT spectra of **1**showed the presence of eleven signals including a methylene, six methine and three quaternary carbon atom groups.The methylene signal appeared at 63.0 suggesting that this is an oxymethylene moiety. The signal at 108.3 was attributed to the hemilactal group characteristic for tetrodotoxin skeleton. By comparing the obtained NMR data with those reported in literature for tetrodotoxin analogs [7],compound **1**was identified as tetrodotoxin (Fig 2).

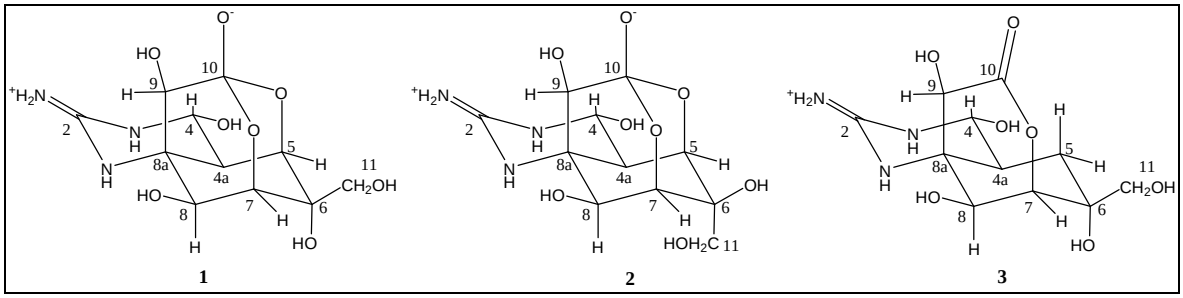

*Figure 2: Structure of compounds **1-3** isolated from T. oblongus viscera*

Compound **2**possess the same molecular weight with **1** based on similar HR-ESI-MS data.The ^{1}H and ^{13}C-NMR spectra of **2**(Table 1) were very identical to those of **1** except for the remarkable downfield shift of C-7 (88.1 in comparison with 77.1). This suggested the change in the orientation of the hydroxyl and oxymethylene groups at C-7. Thus **2**was assigned to be 6-epitetrodotoxin [7].

The HR-ESI-MS spectrum of **3** revealed the ion pic at *m/z*305.1211 $[M + H]^+$corresponding to the molecular $C_{11}H_{19}N_3O_7$. In the ^{13}C-NMR and DEPT spectra of **3**, a carboxylic group at 176.6and a methylene group at 29,7were recognized. This data suggested that a deoxylation occurred and the hemilactal structure changed to carboxylic group.By comparing the NMR values of **3**with those reported, **3** wasidentified as 5-deoxytetrodotoxinpreviously isolated from puffer fish *Fugu poecilonotus* [8].Thus compound **3** was detected for the first time in *Takifugu oblongus.*

C	TTX (1)		6-epiTTX(2)		5-deoxyTTX(3)	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
2	-	154.1	-	156.1	-	156.4
4	5.40 (1H, d, 9.5)	72.6	5.47(1H, d, 9.5)	74.8	5.22 (1H, d, 9.4)	77.6
4a	2.25 (1H, d, 9.5)	38.1	2.02(1H, s)	40.2	2.30 (1H, m)	42.5
5	4.15 (1H, s)	71.3	4.32(1H, s)	74.9	2.01 and 1.25(each 1H, m)	29,7
6	-	68.9	-	72.3	-	77.0
7	3.88 (1H, s)	77.1	4.10(1H, s)	82.1	4.62 (1H, s)	82.6
8	4.19 (1H, s)	70.2	4.15(1H, s)	73.1	4.43 (1H, s)	71.3
8a	-	57.2	-	59.2	-	61.4
9	3.85 (1H, s)	68.3	4.02(1H, s)	70.5	4.65 (1H, s)	72.7
10	-	108.3	-	110.4	-	176.6
11	3.94, 3.91(2H, d, 11.0)	63.0	3.75 (2H, d, 11.0)	65.2	3.51(1H, d, 11.0) 3.72(1H, d, 11.0)	67.0

*Table 1: NMR data of compounds **1-3***

4. Acknowledgement

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