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6,11-Dideoxytetrodotoxin from the puffer fish, *Fugu pardalis*

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Abstract

The presence of an unknown dideoxy analog of tetrodotoxin was suggested on the liquid chromatography/electrospray ionization-mass spectrometry mass chromatogram of the ovaries of the puffer fish, *Fugu pardalis*, in single ion monitoring mode to detect at m/z 288. We succeeded to isolate this analog (approximately 0.4 mg) from 200 g of the ovaries and the structure was determined as 6,11-dideoxytetrodotoxin by spectroscopic methods (high resolution-fast atom bombardment-MS and NMR spectroscopy). The discovery of the new analog is highly significant with respect to the biosynthesis or metabolism of tetrodotoxin. We also roughly determined the value of IC_{50} (mice, intraperitoneal) for 6,11-dideoxytetrodotoxin as 420 μg/kg and thus it is 42 times less toxic than tetrodotoxin.

Keywords: Tetrodotoxin; NMR; Puffer fish; Structure determination; LC/MS

1. Introduction

We have found various analogs of tetrodotoxin (TTX, Fig. 1), such as, 6-*epi*TTX (Yasumoto et al., 1988), 11-norTTX-6(*R*)-ol (Endo et al., 1988), 11-norTTX-6(*S*)-ol (Yotsu-Yamashita et al., 1992), 11-deoxyTTX (Yasumoto et al., 1988), 5-deoxyTTX (Yotsu-Yamashita et al., 1999a), 5,6,11-trideoxyTTX (Yotsu-Yamashita et al., 1995), and 4-*S*-cysteinylTTX (4-CysTTX) (Yotsu-Yamashita et al., 2005) from puffer fish and/or newts, and proposed that these analogs might be involved in metabolism or biosynthetic pathway of TTX (Yasumoto and Yotsu-Yamashita, 1996; Yotsu-Yamashita, 2001). Kotaki and Shimizu (1993) also isolated 1-hydroxy-5,11-dideoxyTTX from the newt, *Taricha granulosa*. Recently, we reported distribution of some of these TTX analogs among tissues of the puffer fish, *Fugu pardalis* (Jang and Yotsu-Yamashita, 2006), quantified by liquid chromatography-fluorescent detection (LC-FLD) and hydrophilic interaction liquid chromatography-electrospray ionization/ mass spectrometry (HILIC-ESI/MS) (Nakagawa et al., 2006). As a result, 5,6,11-trideoxyTTX was found to be the major TTX analog in all tissues, especially in the ovaries. We report here the isolation and structure determination of the new analog of TTX, 6,11-dideoxyTTX (Fig. 1), and discuss its significance with respect to the biosynthesis or metabolism of TTX. We also roughly estimated IC_{50} value (mice, intraperitoneal) for 6,11-dideoxyTTX and compared it with those of TTX and other deoxy analogs.

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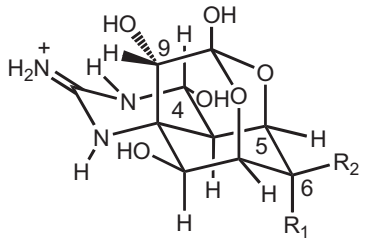
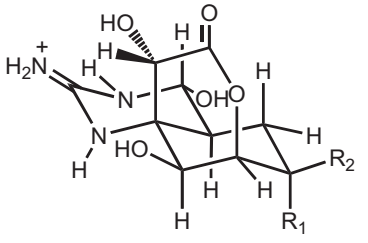


	R_1	R_2	MH^+ (m/z)
TTX	OH	$^{11}CH_2OH$	320
11-deoxyTTX	OH	$^{11}CH_3$	304
6,11-dideoxyTTX	H	$^{11}CH_3$	288



	R_1	R_2	MH^+ (m/z)
5-deoxyTTX	OH	$^{11}CH_2OH$	304
5,6,11-trideoxyTTX	H	$^{11}CH_3$	272

Fig. 1. The structures of TTX and its naturally occurring deoxy analogs found in puffer fish.

2. Materials and methods

2.1. Purification of 6,11-dideoxyTTX

The ovaries (200 g) obtained from *F. pardalis* captured in April 2002 in Miyagi Prefecture, Japan, were immediately frozen and kept below −20 °C until use. They were homogenized and extracted with two volume of 0.05 M acetic acid by heating in boiling water for 10 min, and centrifuged for 20 min at 5300*g* at 4 °C. The supernatant was filtrated under decompression. The filtrate was defatted with EtOAc, adjusted to pH 5.5, and loaded on an activated charcoal column (30 i.d. × 300 mm) equilibrated with H_2O. After the column was washed with H_2O, TTXs were eluted with acetic acid/EtOH/H_2O (1:25:74, v/v). The residue from the eluate was successively chromatographed on the weak cation exchange columns of Bio-Gel P2 (10 i.d. × 500 mm, 200–400 mesh, Bio Rad, Hercules, CA) and Hitachi gel 3011C (7.0 i.d. × 300 mm), all equilibrated with H_2O before use. Elution of 6,11-dideoxyTTX from the columns was monitored by LC/MS. 6,11-DideoxyTTX retained on the columns with H_2O was eluted with 0.5 M acetic acid and 0.15 M acetic acid from Bio-Gel P2 and Hitachi gel, respectively. To remove coexisting arginine, 6,11-dideoxyTTX was purified again on Hitachi gel 3013C with 5 mM ammonium formate pH 5.5, and then ammonium formate in the eluate was sublimated by lyophilization. Finally, pure 6,11-dideoxyTTX (approximately 0.4 mg) was obtained as a white amorphous solid, which was applied to fast atom bombardment (FAB)-MS and NMR analysis.

2.2. LC/MS

LC/MS was performed based on HILIC as we reported previously (Nakagawa et al., 2006; Jang and Yotsu-Yamashita, 2006). Six ions at m/z 272, 288, 290, 302, 304, and 320 corresponding to those $(M+H)^+$ ions of TTX analogs (Fig. 2) were detected in the selected ion monitoring (SIM) mode by API2000 mass spectrometer (Applied Biosystems MDS SCIEX, Foster City, CA) equipped with an ESI source.

2.3. NMR spectroscopy

The NMR spectra of 6,11-dideoxyTTX (approximately 0.4 mg) in 0.3 ml of CD_3COOD-D_2O (4:96, v/v) were obtained on a Varian Unity INOVA 600 spectrometer (Palo Alto, CA) at 20 °C.

2.4. Toxicity to mice

Three different doses of 6,11-dideoxyTTX (3.8, 7.5, and 15 μg) were intraperitoneally injected to two mice (ddY, male, 15–18 g) for each dose individually, and the mice were observed for 24 h, almost according to the official method for determination of the lethality of TTX to mice (Kodama and Sato, 2005). Due to the limited amount of 6,11-dideoxyTTX, we only tested two mice for each dose.

3. Results

3.1. Structure determination of 6,11-dideoxyTTX

The extract of the ovaries of *F. pardalis* with 0.05 M acetic acid showed an unknown peak for dideoxyTTX at 8.4 min on the SIM mass chromatogram detected at m/z 288 as shown in Fig. 2.