Biosynthesis

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C5–C10 Directly Bonded Tetrodotoxin Analogues: Possible Biosynthetic Precursors of Tetrodotoxin From Newts**

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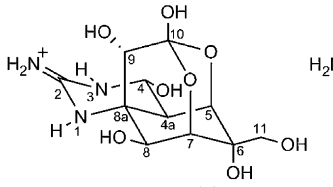
Abstract: *The identification of novel tetrodotoxin (TTX, **1**) analogues would significantly contribute to the elucidation of its biosynthetic pathway. In this study, the first C5–C10 directly bonded TTX analogues, 4,9-anhydro-10-hemiketal-5-deoxyTTX (**2**) and 4,9-anhydro-8-epi-10-hemiketal-5,6,11-trideoxyTTX (**3**), were found in the newt Cynops ensicauda popei by using a screening method involving HILIC-LC–MS/MS focused on the fragment ions of TTX analogues, and their structures were elucidated by spectroscopic methods. Compound **2** was detected in a wide range of newt species, and the **2** and TTX contents of 22 newt specimens were correlated ($r_s = 0.88$). Based on these results and its structural features, **2** was predicted to serve as a precursor of TTX that would be directly converted into 4,9-anhydroTTX (**4**) by Baeyer–Villiger-like oxidation or via 4,9-anhydro-5-deoxyTTX formed by cleavage of the C5–C10 bond. The bicyclic carbon skeletons of **2** and **3** suggested a possible monoterpene origin for TTX.*

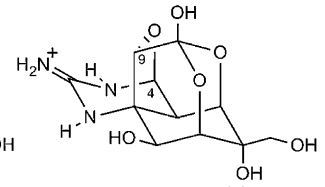
Tetrodotoxin (TTX, **1**), a potent and selective voltage-gated sodium channels blocker,[1] was isolated in 1909 as the toxic principle of pufferfish, and its structure was determined in 1964.[2] TTX occurs in a wide range of marine animals[3] (e.g., pufferfish, crabs, snails, etc.) and terrestrial animals[4] (e.g., newts and frogs). Although the stereoselective total synthesis of TTX has been achieved,[5] the biosynthesis of TTX is still a mystery. TTX production by many bacteria has been reported,[6] and pufferfish have been proven to possess the ability to accumulate TTX provided in the diet.[7] Therefore, TTX in marine animals is assumed to be produced by bacteria and then accumulated in these toxic animals through the food chain.[3b,8] However, the incorporation of isotope-labeled compounds into TTX and the determination of the corresponding gene clusters for TTX biosynthesis have been unsuccessful thus far.[9] For terrestrial toxic amphibians, less is known about the origin of TTX.[4]

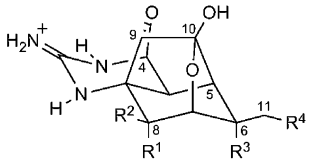
We approached this issue by searching for natural TTX analogues that might reflect the biosynthesis process and identified 6-*epi*TTX, 11-deoxyTTX, and 8-*epi*-5,6,11-trideoxyTTX and its 1-*N*-hydroxy analogues in newts;[10] 5-deoxyTTX, 6-deoxyTTX, 5,11-dideoxyTTX, 6,11-dideoxyTTX, and 5,6,11-trideoxyTTX and so on in pufferfish;[8,11] and chiriquitoxin[12] in frogs. Shimizu et al. also isolated 1-*N*-hydroxy-5,11-dideoxyTTX from newts.[13] For the analysis of these TTX analogues, we developed a comprehensive HILIC (hydrophilic interaction chromatography)-LC–MS/MS method and found that the aminoquinazoline derivatives were observed as the major fragment ions from these compounds (Figures S1, S2 in the Supporting Information).[11c,14]

We attempted structure-based screening to identify the biosynthetic precursors of TTX through HILIC-LC-MS/MS. Herein, we report the discovery of two novel TTX analogues (**2** and **3**; Scheme 1) from newts, and a proposed biosynthetic pathway of TTX based on their structures.

The extract from the Japanese toxic newt, *Cynops ensicauda popei,* exhibited an unknown peak corresponding to **2** on the HR-LC–MS chromatogram at *m*/*z* 286.1034. In the MS/MS spectrum of **2**, the characteristic fragment ion at *m*/*z* 162.0656 ($C_8H_8N_3O$) was detected (Figure S3). Compound **2**







	R^1	R^2	R^3	R^4
4,9-anhydro-10-hemiketal-5-deoxyTTX (**2**)	H	OH	OH	OH
4,9-anhydro-8-*epi*-10-hemiketal-5,6,11-trideoxyTTX (**3**)	OH	H	H	H

Scheme 1. Structures of tetrodotoxin and its analogues.

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was extracted from the whole body, excluding the viscera, of *C. e. popei* (65 g, wet weight) and was purified by continuous column chromatography. Owing to the similarity between the chromatographic properties of compound **2** and those of the known analogue 4,9-anhydroTTX (**4**),[2b–d] compound **2** (approximately 0.2 mg by ^{1}H NMR) was obtained as a mixture with **4** (**2**:**4** approximately 2:3, mol/mol). The molecular formula of **2** ($C_{11}H_{15}N_3O_6$) determined from the HR-ESI-MS peak at *m*/*z* 286.1036 ($[M+H]^+$, calcd. 286.1034, Figure S5) suggests that **2** is a novel monodeoxy and anhydro-type analogue of TTX. The structure elucidation of **2** and assignment of all of the ^{1}H and ^{13}C signals were achieved through COSY, TOCSY, HSQC, and HMBC experiments with a 600 MHz NMR instrument (Table 1, Figures S7–11). The proton chemical shifts of **2** demonstrated a pattern similar to those of **4**,[3a] except for H5 (Table S3). In the HSQC spectrum, C5 in **2** was identified at 48.0 ppm as a methine carbon, but the C5 carbons in the hemilactal TTX analogues were observed much further downfield (e.g., for C5 in **4**, δ_C = 72.2 ppm).[8b] These data suggest that **2** is a 5-deoxyTTX analogue. The HMBC spectrum of **2** clarifies the connectivities of the quaternary carbons at C2, C6, C8a, and C10 (Table 1). The HMBC correlations of C4/H9 and C9/H4 in **2** confirmed the C4–O–C9 linkage, and the correlations of C5/H9 and C10/H4a clearly specify that **2** possesses a 10-hemiketal structure (Figure 1), in which the ether linkage C5–O–C10 in TTX is replaced with a direct C5–C10 bond.

Table 1: NMR spectroscopic data for **2** in CD_3COOD/D_2O (4:96, *v*/*v*).

	4,9-anhydro-10-hemiketal-5-deoxyTTX (**2**)			
Position	δ_C, type	δ_H [*J* in Hz]	HMBC	NOE
2	156.4, C			
4	82.7, CH	5.23, s	2, 8a, 9	4a
4a	42.0, CH	2.82, s	8a, 9, 10	4, 5, 8
5	48.0, CH	2.21, s	4, 6, 7, 8a, 10, 11	4, 4a
6	80.2, C			
7	81.2, CH	4.19, s	5, 8a, 10	8
8	70.8, CH	4.42, s	8a, 9	4a, 7
8a	67.1, C			
9	90.0, CH	4.40, s	4, 4a, 5	
10	106.9, C			
11	66.4, CH_2	3.81, d (12.0)	6, 7	5, 7
		3.58, d (12.3)		5

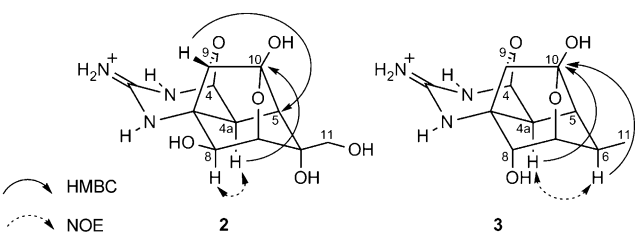

Figure 1. Correlation analysis of **2** and **3**. The key HMBC data for the C5–C10 bond and key NOEs are indicated.

The NOEs observed in the NOESY1D spectra of **2** confirmed the axial configuration of H8 (Figure 1, S12); irradiation at H4a enhanced the signal intensities of H4, H5 and H8; and irradiation at H8 enhanced the signal intensities of H4a and H7. The stereochemistry of C6 in **2** was found to be identical to that of TTX; irradiations at H4a and H8 demonstrated no enhancement of the signal intensity of H_2-11. Moreover, if **2** contained an axial substitution of the 11-hydroxymethyl group, an upfield shift of H_2-11 would be observed in comparison with 5-deoxyTTX[11a] [$\Delta\delta_H$ (5-deoxyTTX-**2**) are $\delta = -0.11$ and -0.07 ppm], as is observed in a similar comparison between 6-*epi*TTX and TTX at H_2-11 [$\Delta\delta_H$ (TTX–6-*epi*TTX)] are 0.28 and 0.30 ppm).[10a] These data resulted in the assignment of the novel TTX analogue as 4,9-anhydro-10-hemiketal-5-deoxyTTX (**2**).

Another 10-hemiketal analogue of TTX, 4,9-anhydro-8-*epi*-10-hemiketal-5,6,11-trideoxyTTX (**3**, approximately 0.08 mg, by ^{1}H NMR), was obtained from the same newt. The molecular formula ($C_{11}H_{15}N_3O_4$) was determined from the HR-ESI-MS peak at *m*/*z* 254.1135 ($[M+H]^+$, calcd. 254.1135, Figure S6). Similar to the fragment ions from 5,6,11-trideoxyTTX,[11c] charactaristic quinazoline derivatives (*m*/*z* 162.1026 and 133.0760) were detected from **3** (Figure S4). Compound **3** was suggested to be a 4,9-anhydro-5,6,11-trideoxy analogue of TTX on the basis of its molecular formula and NMR data (Table S2, Figures S13–16). In the HSQC spectrum of **3**, C5 was observed at $\delta = 50.6$ ppm as a methine carbon similar to that of **2**, in contrast with previously isolated 5-deoxyTTX analogues, in which the C5 carbons were methylenes.[11a,c,e] Conclusive evidence for the C5–C10 bond was obtained from the cross peaks of C10/H4a and C10/H6 observed in the HMBC spectrum of **3** (Figure 1). The equatorial configuration of the H8 of **3** was suggested by the observed NOEs; irradiation at H6 of **3** only enhanced the signal intensity of H4a and not that of H8 (Figure 1, Figure S17), whereas in 5,6,11-trideoxyTTX, positive NOEs were observed between H6/H8 and H4a/H8 on the NOESY spectrum.[11e] The configuration of the 11-methyl group in **3** was indicated to be equatorial by the irradiation results for the H4a of **3**, which demonstrated no enhancement of the signal intensity of H_3-11 but did induce enhancement of the H4, H5 and H6 signals. Based on these results, compound **3** was determined to be 4,9-anhydro-8-*epi*-10-hemiketal-5,6,11-trideoxyTTX (**3**).

Compounds **2** and **3** are the first reported C5–C10 directly bonded TTX analogues. To explore the biosynthetic significance of **2** and **3**, we investigated the distribution of these compounds among 22 specimens of toxic newts across six species in four genera (*Cynops*, *Paramesotriton*, *Taricha*, *Notophthalmus*) from the United States, Japan, and China by using HR-LC–MS. All of the 22 specimens contained TTX (2.4–240 $\mu g\,g^{-1}$; Figure 2, Table S1), and **2** (< 0.052–12 $\mu g\,g^{-1}$) was also detected in all of the specimens except for one specimen of *C. pyrrhogaster*, most likely owing to the low TTX content (2.4 $\mu g\,g^{-1}$) of this sample. Interestingly, a strong correlation between the concentrations of TTX and compound **2** in the 22 newts was suggested by a Spearman's correlation coefficient value (r_s) of 0.88 ($P < 0.01$; Figure 2). The wide distribution of **2** in the newts and the high

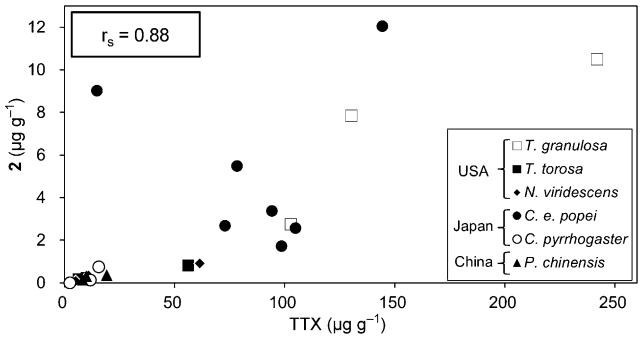

Figure 2. The TTX and **2** contents of 22 newt specimens. The data points represent the means of three measurements, with a coefficient of variation $<15\%$ for all samples. r_s indicates the Spearman's correlation coefficient value.

correlation between the TTX and **2** concentrations strongly suggest that **2** is a biosynthetic precursor of TTX.[15] In contrast to compound **2**, compound **3** might be a shunt product because **3** was detected only in some specimens of *C. e. popei*, and 8-*epi* analogues were minor compounds in the newts.[10b] Furthermore, other analogues such as 6-*epi*TTX and 11-deoxyTTX were absent in the newts of the specific genera evaluated in this and a previous study.[16]

We and Shimizu et al. previously proposed that TTX is biosynthetically constructed from arginine and isoprene; or a combination of guanidine, a C_5 sugar, and isoprene; based on the structures of the natural TTX analogues 6-*epi*TTX, 11-deoxyTTX, and 1-*N*-hydroxy-5,11-dideoxyTTX in newts and 5,6,11-trideoxyTTX in pufferfish.[10a,11e,13] However, the bicyclic carbon skeletons of **2** and **3** consist of ten carbons (C_{10} unit in Figure 3), thus suggesting a monoterpene as another possible candidate for the biosynthetic precursor of TTX. In that case, a guanidine group might be directly introduced to the C_{10} unit or formed through stepwise amination and amidination. Furthermore, the final stage of the biosynthesis of TTX could be predicted based on the structures of **2** and **3** (Figure 3). In our experiments, the 4,9-ethers in **2** and **3** were not hydrolyzed under the same conditions in which the 4,9-ether in **4** was hydrolyzed to form TTX (5% TFA/H_2O (*v*/*v*) at 37 °C overnight).[2b–d] Therefore, if **2** is a precursor of TTX, the C5–C10 bond in **2** might be first oxidized to C5–O–C10 to form **4**, most likely through a Baeyer–Villiger-like oxidation, and then the 4,9-ether bond in **4** would be hydrolyzed to form TTX (Figure 3). Kono et al. reported that **4** was hydrolyzed to TTX even under biological conditions,[17] thus supporting this hypothesis. Although the 10-ketone form of **2** (Figure 3) was not observed by ^{1}H NMR spectroscopy measured in CD_3COOD/D_2O (4:96, *v*/*v*) at 20 °C, Baeyer–Villiger monooxygenase, which is widely distributed in bacteria,[18] is an enzyme that could plausibly catalyze the C5–C10 oxidation. As another possibility, **4** could also be formed from **2** via 4,9-anhydro-5-deoxyTTX[11a] because cleavage of the C5–C10 bond is also predictable from the existence of 8-*epi*-5,6,11-trideoxyTTX (**5**),[10b] which might be formed from **3** via 4,9-anhydro-8-*epi*-5,6,11-trideoxyTTX (**6**)[10b] (Figure 3). Other 5-deoxy-10,7-lactone analogues such as 1-*N*-hydroxy-5,11-dideoxyTTX[13] and 1-*N*-hydroxy-8-*epi*-5,6,11-trideoxyTTX[10b] have also been reported. Interestingly, neither **2** nor **3** were detected in TTX-containing marine animals. This disparity raises the new question of whether the biosynthetic routes of TTX are similar in newts and marine animals. We are endeavoring to collect more experimental data to answer to this question.

In conclusion, two novel TTX analogues suggested the possibility of a monoterpene as the biosynthetic origin of TTX. Baeyer–Villiger monooxygenase, or a specific enzyme that cleaves the C5–C10 bond in **2** and **3**, may be a key enzyme that could enable the identification of the biosynthetic gene clusters of TTX from bacterial metagenomes.

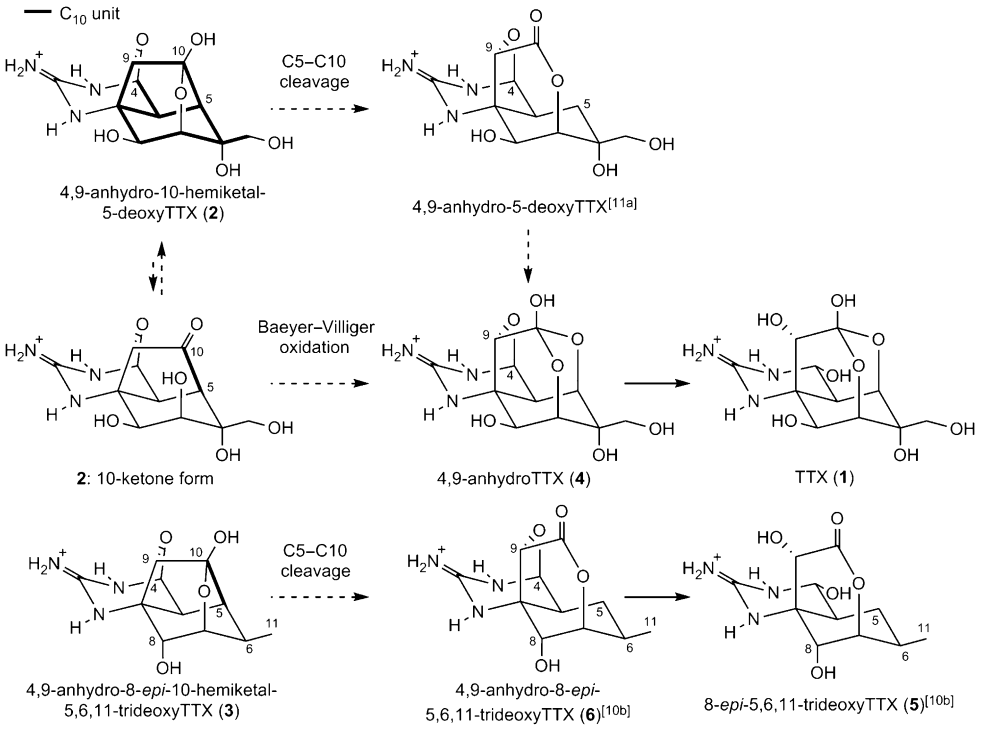

Figure 3. A proposed biosynthetic pathways of TTX (**1**), and 8-*epi*-5,6,11-trideoxyTTX (**5**) based on the structures of **2** and **3**.

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