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1-Hydroxy-5,11-dideoxytetrodotoxin, the First *N*-Hydroxy and Ring-Deoxy Derivative of Tetrodotoxin Found in the Newt *Taricha granulosa*

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Abstract: A new tetrodotoxin analogue, 1-hydroxy-5,11-dideoxytetrodotoxin, was isolated from the newt *Taricha granulosa*, and its structure was determined. It is the first example of an *N*-hydroxy and 5-deoxy derivative of tetrodotoxin found in nature, and its discovery may be highly significant with respect to the biosynthesis of tetrodotoxin and its toxigenesis in various tetrodotoxin-containing organisms.

Introduction

Tetrodotoxin (TTX) **1** is a potent neurotoxin, which is responsible for the fatal food poisoning caused by puffer fish. It is an important probe in neurophysiological experiments as a highly specific sodium-channel blocker and has been a subject of extensive chemical and pharmacological investigation.[1] The structure of TTX was elucidated simultaneously by an American and two Japanese groups in 1964.[2-4] The total synthesis was accomplished by Kishi and his co-workers in 1972.[5] In spite of this long history of TTX research, little is known about the biosynthetic origin of this unique molecule.

While TTX was first recognized as the puffer poison, Fuhrman, Mosher, and their co-workers proved that the toxin known in the Californian newt *Taricha torosa* is actually tetrodotoxin.[6] Since this revelation, TTX has been found in many other organisms,[7] and the distribution of this extraordinary structure in distantly related organisms has further mystified the biological and molecular source of this important compound. Although the recent studies point to bacteria as possible sources of the toxin,[8-12] many unanswered questions still remain regarding the true origin of this unique molecule.

In our earlier experiments,[13,14] the feeding of radioactive general metabolic precursors to the toxic newts and their symbiotic bacteria failed to effect the incorporation of radioactivity into tetrodotoxin.

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As an alternative approach, we have been searching for molecules which may provide clues for the biosynthetic pathway of TTX. In this paper, we repo.. the isolation of a new tetrodotoxin derivative **2**, which may cast new light on the biosynthesis of tetrodotoxin and its toxigenesis in many organisms.

Experimental Section

Material. The newts *Taricha granulosa* were collected in Oregon during springtime and sent to the laboratory in Rhode Island by Carolina Biological Supply Co. Upon arrival, the live newts were frozen in liquid nitrogen and stored at −20 °C before extraction. Two batches of the newts (1400 g, 112 bodies and 2450 g, 198 bodies) were used for this study.

Extraction and Purification. The animal bodies were minced and extracted in a homogenizer with a 3-fold volume of 0.1% acetic acid in methanol for 4 min, and the residue was reextracted twice with a 2-fold volume of the same solvent. The combined extracts were centrifuged (Du Pont, GSA rotor, 8000 rpm, 20 min), and the supernatant was concentrated to ca. 500 mL by a rotary evaporator, brought to 1000 mL by adding 1 N AcOH, and filtrated successively through Amicon XM100A (d 150 mm, MW 100000 cutoff) and PM 10 membranes (d 150 mm, MW 10000 cutoff).

The dialysate was evaporated to ca. 300 mL in order to eliminate acetic acid and loaded onto a Bio-Gel P-2 column (5.0 × 70 cm) after adjustment of the pH to 5.5 with a dilute NaOH solution. The column was first washed with 700 mL of water, and the toxin was eluted with 0.03–0.06 N AcOH. The same column chromatography was repeated. The toxin fractions were combined and concentrated, and the pH was adjusted to 6.5 with a dilute NaOH solution prior to loading onto a Bio-Rex 70 column (Bio-Rad Laboratory, H^+ form, −400 mesh, 2.8 × 55 cm).

The column was washed with a small amount of water, and fractions of 15 mL were collected. The toxin fractions eluted with 0.05 N AcOH were combined and further purified by Bio-Rex 70 column chromatography (1.8 × 56 cm, H^+ form, linear gradient elution with 0.03–0.06 N AcOH, 2 × 250 mL, followed by 0.06 and 0.5 N AcOH). Fractions of 5 mL were collected, and the toxin fractions were combined and lyophilized.

The toxin fraction was fractionated by using a Hitachi gel 3013-C column (H^+ form, 0.4 × 15 cm, 0.05 N AcOH). Fractions of 2 mL were collected. Further purification was performed by preparative HPLC using a HEMA-IEC BIO CM column (Alltech Assoc., 0.75 × 25 cm) with 0.05 N ammonium acetate (pH 5.75). The unknown TTX derivative fraction was collected and lyophilized three times by adding water to eliminate ammonium acetate. The preparative HPLC on the same column was performed with 0.1 N AcOH.

All toxin fractions were subjected to final purification by passing through small columns of Chelex-100 and Bio-Gel P-2 with 0.1 N AcOH.

Detection of Tetrodotoxin Derivatives. Mouse assay was carried out according to the standard method for TTX[15] using male CD-1 strain mice weighing about 20 g. Detection and identification of tetrodotoxin and its analogues were performed mainly by thin-layer chromatography (Whatman HP-KF with pyridine/ethyl acetate/acetic acid/water = 10:5:2:3). TTX derivatives were visualized under UV (305 nm) after spraying with 10% KOH in methanol and heating at 130 °C for 10 min. Toxins were monitored on preparative HPLC by a UV detector at 225 nm.

Optical rotations were measured in 0.05 N AcOH by using a microcell at 23 °C on a Rudolf Autopol-III polarimeter. All NMR spectra were taken in 4% CD_3COOD/D_2O on a Brucker instrument, 300-MHz for ^1H and 75.469 MHz for ^{13}C. FAB mass spectra of the toxin were taken in 5% AcOH by using glycerol as a matrix.

Results and Discussion

The newts used in this study were highly toxic. The crude extract from the first sample (1400 g) showed the toxicity of 1140000 mouse units or 814 mouse units/gram of the whole body, which amounts to approximately 2 mg of TTX equivalent per animal. Most of the toxicity was accounted for by TTX, which was obtained easily by reprecipitation from the eluate of Bio-Rex 70 column chromatography. The lyophilized toxic fraction was suspended in 0.5 N AcOH (3 mL), and a small amount of acetic acid was added to dissolve TTX completely. Then, 0.5 N ammonium hydroxide was added to the solution to bring the pH to 8.7 to precipitate TTX. A total of ca. 100 mg of almost pure TTX was obtained after this procedure.

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Table I. ^1H and ^{13}C NMR Chemical Shifts of 1(*N*)-Hydroxy-5,11-dideoxyTTX (**2**)[a]

	^1H (δ)		^{13}C (δ)
H-4	5.14 (d, *J* = 9.4 Hz)	C-2	158.9
H-4a	2.77 (ddd, *J* = 13.2, 9.3, & 3.9 Hz)	C-4	77.5
H-5α	2.14 (ddd, *J* = 15.6, 3.7, & 1.4 Hz)	C-4a	38.8
H-5β	1.38 (dd, *J* = 15.7 & 13.3 Hz)	C-5	34.3
H-7	4.46 (dd, *J* = 3.9 & 1.4 Hz)	C-6	69.8
H-8	4.58 (d, *J* = 3.9 Hz)	C-7	81.2
H-9	4.80 (s)	C-8	67.5
H-11	1.35 (s)	C-8a	74.1
		C-9	69.9
		C-10	176.6
		C-11	27.9

[a] The spectra were taken in 4% CD_3COOD/D_2O on a Bruker spectrometer, 300 MHz for ^1H and 75.469 MHz for ^{13}C.

Four distinctive components were detected by silica gel TLC in the eluates from Bio-Rex 70 column chromatography. These components (code-named A–D according to the elution order) were identified by comparing with the R_f values and ^1H NMR spectra of the authentic samples. The components A, B, C, and D were identical with TTX (**1**), 6-epiTTX (**3**), 11-deoxyTTX (**4**), and 4,9-anhydroTTX (**5**), respectively.

Three other components, which seemed to be TTX derivatives, were detected on silica gel TLC. One of the components, which showed the highest R_f values (0.82) of all was further purified by chromatography on a Hitachi gel 3013-C column. The compound was eluted from the column later than any other known TTX derivatives. The HEMA-CM (hydroxyethyl methacrylate/dimethylacrylate copolymer with carboxymethyl groups) column, which was, for the first time, tried for tetrodotoxin derivatives, proved to be highly effective in the final separation of the new derivative. After passing through small Chelex-100 and Bio-Gel P-2 columns to remove trace metal ions and impurities from the previous column chromatography, the new compound was finally obtained in pure form (1.2 and 0.23 mg from the two separate batches of newts, respectively). Two other minor components were not characterized completely due to their small sample sizes.

The new compound **2** was obtained as a white amorphous powder, $[\alpha]_D$ 23 °C +14.47 ± 2.37 (*c* = 0.3, 0.05 N AcOH), and showed solubility and chromatographic behavior considerably different from those of the other TTX derivatives. It dissolved in dilute AcOH more readily than TTX and gave the highest R_f value on TLC (R_f relative to that of TTX = 1.5, Whatman HP-KF, pyridine/EtOAc/AcOH/H_2O, 10:5:2:3) of all the other known TTX derivatives and revealed a fluorescent spot on TLC only after KOH spraying and prolonged heating at 130 °C or higher temperature. Thus, it is very likely that the compound could not be detected previously by the conventional HPLC system for TTX derivatives, which used a post-column KOH-fluorescence detector with a brief heating.[16] In our chromatographic separation, the compound was monitored by UV absorption at 225 nm. The compound **2** was found to have a molecular formula of $C_{11}H_{17}N_3O_7$ (FAB MS, MH^+, calcd *m/z* 304.1144, found *m/z* 304.1141).

The ^1H, ^1H–^1H COSY, and ^{13}C NMR spectra[17] showed that this compound has a structure of 5,11-dideoxyTTX. The 5-methine hydrogen signal is missing in the spectra of **2**, and, instead, there is an ABX signal due to methylene protons, which has a connectivity to the C-4 hydrogen. The presence of W-coupling between 7-H and one of the methylene hydrogens, 5α-H, indicates the same conformational arrangement of the six-membered ring as in the other TTX derivatives. The hydroxymethyl group signal at C-11 is missing and replaced with a methyl signal (δ 1.35). The

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(17) The NMR spectra of different batches of chromatographically pure samples always showed signals of a small amount of an isomeric compound, probably due to the 4-epi or open lactone form as in the case of most TTX derivatives.

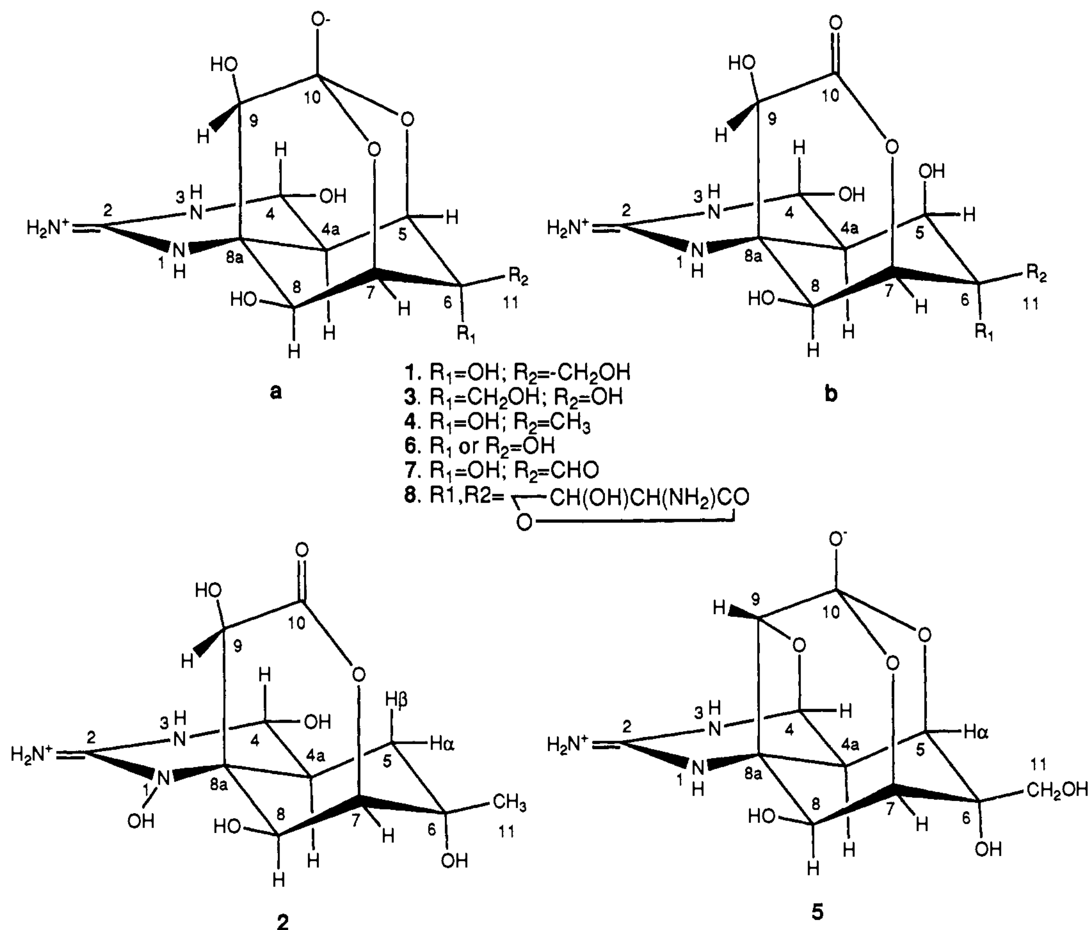
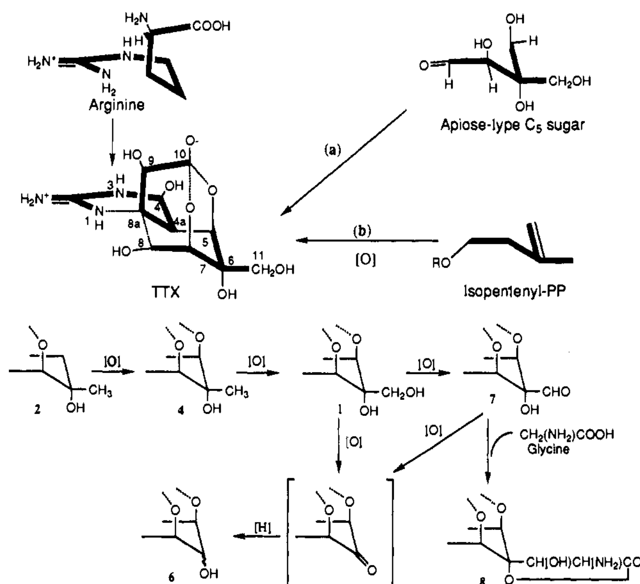


Figure 1. Structure of tetrodotoxin derivatives isolated from newts, puffer fish, and a frog.

chemical shift of 7-H indicates that the compound is in the lactone form as the TTX lactone **1b** and the 6-hydroxyl group is α-axial. However, there are large differences in the carbon chemical shifts of C-8 and C-8a and those of other TTX derivatives.[18] For example, the chemical shift of C-8 in **2** (δ 67.5) is at higher field (Δ −3.8 ppm) compared to δ 71.3 in 11-deoxyTTX lactone **4b**, whereas, the C-8a signal shows a large downfield shift (Δ +14.4 ppm, δ 74.1 in **2** and δ 59.7 in **4b**). These shifts can be explained nicely by γ- and β-effects caused by the introduction of a hydroxyl group at the 1(*N*)-position of the guanidine group. Similar chemical shift changes were observed with the *N*-hydroxyl substitution of guanidine in neosaxitoxin[19] and an anatoxin-a(s) derivative.[20] In the case of neosaxitoxin, the introduction of a hydroxyl group on the 1(*N*)-guanidine nitrogen resulted in a shift, Δ +11.2 ppm for the β-carbon and Δ −2.2 ppm for the γ-carbon. A modest downfield shift was seen with the proton signal of H-8 (Δ +0.12 ppm), which also corresponds to the shift observed with the β-hydrogen in neosaxitoxin (Δ +0.16 ppm). The *N*-hydroxyl structure is further supported by the fact that the molecular formula obtained by the FAB mass spectrum indicates the presence of an additional oxygen atom in the molecule. It can be placed only on the guanidinium group, unless it is a peroxide structure, which is very unlikely in view of the stability of the compound.

A number of tetrodotoxin derivatives have been reported in newts and other organisms. Among them, such compounds as 4-epiTTX, 6-epiTTX, and anhydroTTX are isomers or chemical equivalents of TTX, and they may not be so significant in order to consider the biosynthesis of TTX. On the other hand, Yasumoto

Scheme I. Hypothetical Biosynthetic Pathways of Tetrodotoxin (TTX) Derivatives



and his co-workers have reported 11-deoxyTTX (**4**) in puffer fish[18] and newts[21] and 11-norTTX-6-ols (**6**) and 11-oxoTTX (**7**) in puffer fish.[22] Also, his group demonstrated that chiriquitoxin found in the frog *Aterops chiriquiensis*[23] is an 11-carboxyaminomethyl

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derivative of TTX (**8**).[24] These derivatives were considered to be compounds at different stages of progressive oxidation or their derivatives as in the case of chiriquitoxin. While the structural variations found in the past are limited to the 6,11-position, i.e. the branching portion, the newly discovered compound **2** is the first TTX derivative which lacks an oxygen function on the ring. It seems to indicate that TTX is indeed formed by the stepwise oxidation of an alicyclic system as suggested by Yasumoto et al. (pathways b in Scheme I)[18] and excludes one of the earlier speculations[14] that TTX is formed by the condensation of arginine and a branched sugar (pathway a in Scheme I).

Another important aspect of compound **2** is the possible implication of the *N*-hydroxy compounds' participation in the toxigenesis of certain organisms. *N*-Hydroxy derivatives play an important role in the toxigenesis of other guanidine-containing toxins.[25,26] Those compounds have been shown to lose the hydroxy group reductively in biological systems. A typical example is the conversion of neosaxitoxin to saxitoxin by shellfish or bacteria.[26,27] Recently many strains of bacteria including several types of strains of marine bacteria have been reported to produce TTX. They include *Vibrio* spp., *Aeromonas* spp., *Alteromaonas* spp., *Photobacterium* sp., *Escherichia coli*, *Bacillus* sp., *Moraxella* spp., *Pseudomonas* spp., *Listonella* sp., and *Acinetobacter* sp.[8–11,28] These rather astounding reports on the production of TTX by a wide variety of bacteria ranging from Gram-negative and Gram-positive bacteria to actinomycetes have led to the speculation that there are rather ubiquitous precursors of TTX, which can be easily transformed to TTX by subtle structural modifications. In this regard, the finding that marine sediments contain appreciable amounts of TTX may be very significant.[29]

In our earlier experiments,[13,14] we fed various putative precursors to the newts *T. torosa* and *T. granulosa* under various conditions. All the experiments gave negative results. Attempts were made to incorporate highly radioactive acetate as a general metabolic precursor. We also tried [*guanido*-^{14}C]arginine, because, in our studies of saxitoxin biosynthesis, we learned that the guanidine group of arginine could be a universal source of guanidine group.[30] However, in both cases, when exhaustively purified, the isolated TTX samples were completely devoid of radioactivity, despite the fact that other general metabolites such as cholesterol and amino acids isolated in the experiments were significantly labeled. We also carried out similar experiments with Gram-negative bacteria isolated from the intestinal flora of the newts, which seemed to produce a tetrodotoxin-like substance.[12,14] However, again, the tetrodotoxin fraction isolated and purified by adding pure tetrodotoxin did not carry any radioactivity. All these results seemed to suggest the absence of de novo synthesis of TTX in the organisms, although, in our experiments, the newts sustained a high level of toxicity for 1–2 years and, moreover, there is evidence that they were constantly excreting small amounts of TTX in captivity. All these contradictory observations may be understandable, if there are compounds in the newts which can be easily transformed to TTX. *N*-Hydroxyl derivatives or deoxy compounds such as **2** certainly qualify as such precursors.

Conclusion

Despite numerous efforts over the years, the biosynthesis of TTX remains a mystery. All conventional approaches to the biosynthetic studies, including feeding experiments with the alleged TTX-producing organisms, have so far failed. Only some speculations have been made from the structural features of TTX and its derivatives. Of these compounds, the newly discovered compound, which lacks two oxygen functions on the carbon skeleton, seems to be the most informative with regard to the molecular origin of this most noted natural product. As to the biological origin of the alleged precursors of TTX, the question still remains unsolved. However, given the fact that low levels of TTX are widely found in a variety of organisms, they have to be derived from some very common sources and converted to TTX or its precursors in the organisms.

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Supplementary Material Available: ^{13}C, ^{13}C DEPT, ^{1}H, and ^{1}H–^{1}H COSY spectra of compound **2** (16 pages). Ordering information is given on any current masthead page.

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Neighboring Group Participation in Lewis Acid-Promoted [3 + 4] and [3 + 5] Annulations. The Synthesis of Oxabicyclo[3.*n*.1]alkan-3-ones

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Abstract: Lewis acids are employed as catalysts in the annulation of 1,4- and 1,5-dicarbonyl dielectrophiles with bis(trimethylsilyl) enol ethers of β-diketones and β-keto esters. A variety of 2-(alkoxycarbonyl)-*m*-oxabicyclo[3.*n*.1]alkan-3-ones can be constructed by this process in which two new carbon–carbon bonds are generated. Unusually high regiocontrol is observed, and good to excellent stereochemical control can be achieved at virtually every position on the new carbocycles. Intramolecular neighboring group participation is proposed to explain the unusually high selectivities attained in the annulation reaction.

With increasing frequency, molecules possessing seven-[2] and eight-membered-ring[3] substructural units are being discovered and evaluated for their potential use as therapeutic agents. Unlike the syntheses of five- and six-membered rings, wherein a variety

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