Pure & Appl. Chem., Vol. 61, No. 3, pp. 505–508, 1989.
Printed in Great Britain.

Interspecies distribution and biogenetic origin of tetrodotoxin and its derivatives

Takeshi Yasumoto, Mari Yotsu, Amane Endo, Michio Murata and Hideo Naoki*

Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori, Sendai 980, Japan, *Sundory Institute for Bioorganic Research, Wakayamadai, Shimamoto-cho, Osaka 618, Japan

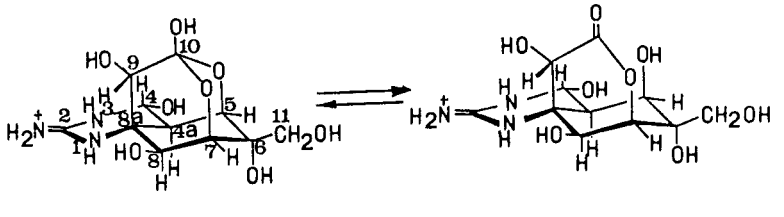
Abstract - Screening for tetrodotoxin among biota at low levels of the food chain led us to identification of bacteria as the primary source of the toxin. In parallel with the search for the biogenetic origin, we explored naturally occurring tetrodotoxin analogues which might shed light on the biogenetic pathways of the toxin. Isolation of 6-epitetrodotoxin and 11-deoxytetrodotoxin from the newt Cynops ensicauda suggested that tetrodotoxin biosynthesis involves an isoprenoid C_5 unit. Puffers contained 11-nortetrodotoxin-6(R)-ol, in addition to tetrodotoxin and the two analogues found in newts. Addition of trifluoroacetic acid-d to the NMR solvent resolved NMR signals and thus allowed us to assign for the first time all proton and carbon NMR signals of tetrodotoxin and its analogues.

INTRODUCTION

Tetrodotoxin (1, TTX) is a potent neurotoxin well known for its unique chemical structures and pharmacological properties. Nevertheless, its biogenetic origins as well as its biosynthetic or metabolic pathways have remained unknown. The toxin was first isolated from puffers (ref. 1-3), then from newt Taricha torosa (ref. 4), but neither of the animals seem to biosynthesize 1. Artificially raised puffers remained nontoxic, although they were capable of accumulating 1 through diets (ref. 5). Newts administered with radioactive precursors did not incorporate the precursors into 1 (ref. 6). After developing a sensitive and specific fluorometric HPLC analyzer, we traced the food chain back to the origin and identified bacteria as the primary source of 1. The analyzer was also used to explore natural TTX analogues in the hope of shedding light on the biogenetic pathways of this unique toxin. New analogues were isolated from newts and puffers, and structural determination of analogues were achieved mainly by NMR measurements.

THE ORIGIN OF TETRODOTOXIN

Herbivorous fished and crabs were screened for 1. Detection of low levels of 1 was fascilitated with HPLC analyzers, which separated 1 and its analogues on either a reversed phase column (ref. 7) or on an ion exchange gel column (ref. 8) and determined fluorophores derived from 1 or its analogues during post column reaction with sodium hydroxide. Parrot fishes Scarus gibbus and Ypsiscarus ovifrons, an angel fish Pomacanthus semicirculatus, and xanthid crabs Zosimus aeneus, Atergatis floridus, and A. intergerrimus contained 1 in variable amounts. A calcareous alga Jania sp., a common diet of the fishes and crabs, also contained 1. However, the content of 1 in the alga was so variable that 1 was presumed to have derived from symbiotic or epiphytic bacteria. The hypothesis was proven by detecting 1 and 4,9-anhydrotetrodotoxin (7, anhydroTTX) in culture broths of Alteromonas sp. isolated from the



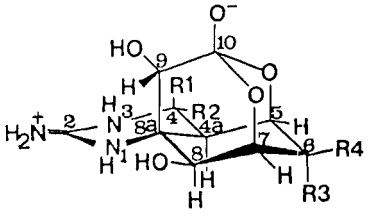
hemilactal (1a) lactone (1b)

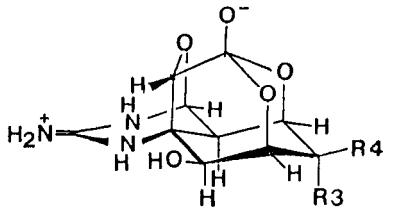
Fig. 1. Tautomerism of tetrodotoxin between hemilactal and lactone forms

alga. Identity of 1 and 7 produced by the bacteria was unambiguously established by chromatographic methods, fast atom bombardment mass spectrometry (FABMS), and degradation with sodium hydroxide to 2-amino-6-hydroxymethyl-8-hydroxylquinazoline (ref. 9, 10). Pseudomonas sp. isolated from the skin of the puffer Fugu poecilonotus also produced 1 and 7 (ref. 11).

NATURAL ANALOGUES OF TETRODOTOXIN

Our initial attempt to investigate the biosynthetic route for 1 using the TTX-producing bacteria was hampered because of the lowered toxin productivity of the organism after generations of culture. In parallel with the effort to potentiate the toxin production of the bacteria, we explored natural analogues of 1 specifically in newts and puffers. Eight tetrodotoxin analogues were isolated. The newt Cynops ensicauda (3.5 kg) collected in Okinawa, Japan, were extracted with 0.1 N acetic acid and the extracts were chromatographed successively on columns of charcoal, BioGel P-2, BioRex 70, and Hitachi ion exchange gel 3011C. Separation of the analogues was monitored by the fluorometric analyzers and by thin layer chromatography. The structural determination of the analogues was achieved through NMR and FABMS measurements. NMR spectra of 1 measured in deuterium oxide with deuterated acetic acid showed poor resolution of ^{1}H and ^{13}C signals due to the tautomerism between hemilactal and lactone forms (Fig. 1). Addition of trifluoroacetic acid-d to the solvent markedly improved the resolution of signals in the NMR spectra. The ^{1}H NMR spectrum of 1 revealed doubled sets of signals, conforming to the tautomerism between hemilactal and lactone forms. The tautomer giving rise to the signals with high intensity was assigned to a hemilactal form (1a) and the other to a lactone form (1b). Assignment of all ^{1}H and ^{13}C signals in the NMR spectra of 1 was achieved by ^{1}H-^{1}H and ^{13}C-^{1}H COSY measurements (Table 1). The structures of the analogues shown in Fig. 2 were determined by comparing NMR data with those of 1.





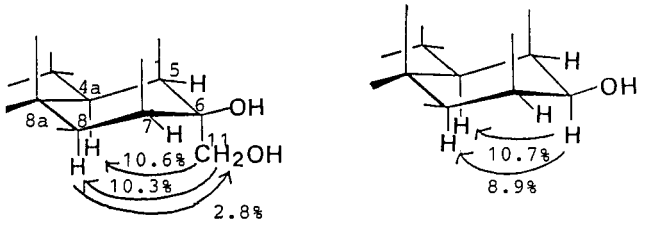
	R1	R2	R3	R4
1 TTX	H	OH	OH	CH_2OH
2 6-epiTTX	H	OH	CH_2OH	OH
3 11-deoxyTTX	H	OH	OH	CH_3
4 11-norTTX-6(R)-ol	H	OH	H	OH
5 4-epiTTX	OH	H	OH	CH_2OH
6 11-deoxy-4-epiTTX	OH	H	OH	CH_3

	R3	R4
7 4,9-anhydroTTX	OH	CH_2OH
8 4,9-anhydro-6-epiTTX	CH_2OH	OH
9 4,9-anhydro-11-deoxyTTX	OH	CH_3

Fig. 2. Structures of tetrodotoxin and its analogues isolated from newts and puffers

The molecular formula of 6-epitetrodotoxin (2, 6-epiTTX) was established by high resolution FABMS. Comparison of ^{13}C and ^{1}H signals of 1 and 2 is shown in Table 1. Assignments of signals were derived from ^{1}H-^{1}H and ^{13}C-^{1}H COSY measurements. Signals in 2 due to H-4a, H-8, H-11, C-4a, C-5, C-6, and C-7 were significantly shifted from the corresponding signals of 1, supporting the 6-epi assignment. ^{1}H-^{1}H COSY of 2 showed couplings between H-4/H-4a, H-4a/H-5, H-5/H-7 (W-type), and H-7/H-8, analogous with 1. The coupling patterns also agreed with those of 1. Thus structural change at C-4a, C-5, C-7, and C-8 was ruled out. The streochemistry at C-8a and C-9 in 2 was presumed to be the same as those in 1, because 1 was convertible to a 4,9-anhydro derivative in the presence of acid. The axial substitution of C-11 was confirmed by NOE measurements and difference spectra; irradiation at 3.74 (CH_2-11) enhanced signal intensities of H-4a and H-8 of 2 (Fig. 3).

The molecular formula of 11-deoxytetrodotoxin (3, 11-deoxyTTX) was established by high resolution FABMS. ^{1}H-^{1}H COSY of 3 showed that the coupling patterns, including a W-type coupling between H-4a and H-9, are essentially the same as those of 1 (Table 1). ^{1}H-^{1}H and ^{13}C-^{1}H COSY spectra of 3 indicated that the CH_2-11 signals in 1 were replaced by a methyl signal (Table 1). Comparison of ^{13}C NMR spectra of 3 and 1 further supported reduction at C-11. A signal assignable to C-6 was shifted upfield, and those of C-5 and C-7 were shifted downfield, while other signals of 3 agreed with those of 1 (Table 1). Methyl-11 was assigned equatorial conformation because no NOE was observed between H-4a and CH_3-11. Both 2 and 3



6-epiTTX 11-norTTX-6(R)-ol

Fig. 3. NOE measurements of 6-epitetrodotoxin (2) and 11-nortetrodotoxin-6(R)-ol (4) 360 MHz, 1% CF_3COOD, 4% CD_3COOD/D_2O

exist as hemilactal-lactone tautomers. Other analogues (5-9) were found to be derivable from 1, 2, or 3. Thus structures of all the tetrodotoxin analogues isolated from the newts were unambiguously established (ref. 12).

Three species of puffers, Fugu niphobles, F. pardalis and F. poecilonotus, contained 11-nortetrodotoxin-6(R)-ol (4) in addition to 1, 2, and 3. The molecular formula of 4 was deduced from FABMS; $C_{10}H_{15}N_3O_7$, MH^+, m/z 290. The ^1H NMR spectrum indicated that 4 exists in tautomeric hemilactal and lactone forms in a ratio 3:1. The tautomerism, the presence of coupled signals of H-4a (δ 1.84, d, 9.2 Hz) and H-4 (δ 5.33, d, 9.1 Hz), and W-type couplings between H-4a/H-9 and H-5/H-7 supported skeletal resemblance between 4 and 1. In the ^1H NMR spectrum of 4, CH_2-11 signals of 1 was replaced by an oxymethine signal (δ 3.73, t, 1.7 Hz) which was coupled with H-5 and H-7. The axial configuration of H-6 was confirmed by the NOE decoupled difference spectrum (Fig. 3). The present study is the first to show the natural occurrence of 4, although 4 had been prepared from 1 previously (ref. 13).

Table 1. NMR spectral data of TTX, 6-epiTTX and 11-deoxyTTX.

	TTX* hemilactal C	H (mul.J)	lactone C	H (mul.J)	6-epiTTX** hemilactal C	H (mul.J)	lactone C	H (mul.J)	11-deoxyTTX** hemilactal C	H (mul.J)	lactone C	H (mul.J)
2	156.6		155.9		156.5		155.8		156.4	-		
4	75.1	5.50 (d 9.4)	74.8	5.50 (d 9.4)	75.1	5.55 (d 9.4)	75.1	5.55 (d 8.9)	75.0	5.49 (d 9.4)	74.8	5.51 (d 9.6)
4a	40.7	2.35 (d 9.5)	46.5	2.35 (d 9.5)	41.8	2.01 (d 9.0)	46.9	2.13 (d 9.0)	40.5	2.37 (d 9.4)	46.2	2.37 (d 9.4)
5	73.8	4.25 (br s)	69.2	4.03	75.4	4.30 (d 1.6)	68.4	4.03 (br s)	77.5	4.08 (br s)	72.0	3.87 (br s)
6	71.5	-			72.8		77.0		69.1	-		
7	79.7	4.08 (t 1.8)	82.5	4.55 (br s)	82.0	4.08 (br s)	85.5	4.62 (br s)	83.6	3.91 (t 1.6)	86.8	4.35 (t 2.0)
8	72.8	4.30 (d 1.5)	71.5	4.44 (br s)	72.9	4.17 (br s)	71.7	4.26 (br s)	72.6	4.30 (d 1.6)	71.3	4.46 (d 2.3)
8a	59.7		60.4		59.6		60.1		59.1		59.8	
9	70.9	3.96 (s)	74.0	4.57 (s)	70.8	4.00 (s)	73.7	4.59 (s)	70.8	3.94 (s)	73.9	4.55 (s)
10	110.8		176.1		110.7		175.8		110.6		175.4	
11	65.5	4.02 (d 12.6) 4.04 (d 12.6)	65.2	3.77 (d 12.6) 4.01 (d 12.6)	65.1	3.74 (s)	66.2	3.68 (d 14.0) 3.69 (d 14.0)	25.1	1.64 (s)	24.5	1.53 (s)

^{13}C NMR:75.5 MHz, $^{13}CD_3COOD$=22.4ppm (GN-300).
^1H NMR:* 360 MHz (NT360), ** 300 MHz (GN-300), CHD_2COOD=2.06ppm, J in Hz
Solvent:* 1% CF_3COOD, 4% CD_3COOD / D_2O, ** 4% CD_3COOD / D_2O.

DISTRIBUTION OF TETRODOTOXIN ANALOGUES AMONG NEWTS AND PUFFERS

The newts C. ensicauda, C. pyrrhogaster, Taricha granulosa, and Triturus alpestris contained 1, 2, and 3; Triturus vulgaris and Notophthalmus viridescens 1 and 2; Triturus oregon 1 and 3; and Paramesotriton hongkongensis 1. A salamander Ambystoma tigrinum contained 1 and 3. In C. ensicauda the relative ratio of 1, 2, and 3 was variable among tissues. The highest proportion of 1 was observed in the skin, while 2 was the major component in eggs. The presence of 4 was not confirmed in newts. In all three species of puffer tested, F. niphobles, F. pardalis, and F. poecilonotus, 2, 3, and 4 were present as minor components. Fig. 4 shows a chromatogram of representative analogues on the analyzer used for screening.

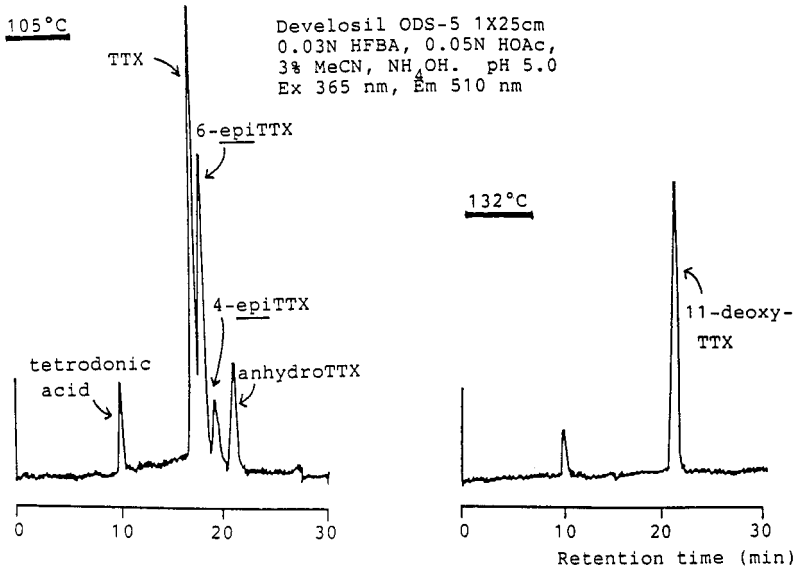


Fig. 4. A chromatogram for tetrodotoxin and its analogues occurring in the newt Cynops ensicauda taken on a fluorometric analyzer equipped with a reversed phase column. 11-Deoxytetrodotoxin was detected at an elevated temperature.

Biosynthesis of 1 supposedly involves arginine and a C_5 unit derived from either amino acids, isoprenoids, shikimates, or branched sugars (ref. 6). The wide distribution of 2, and 3 renders branched sugars unlikely precursors, and the shikimate pathways does not seem plausible because it rarely yields 1,2,4-trialkylcyclohexanes. An isoprenoid unit is favored because it possesses both an sp^2 carbon oxidizable to either 1 or 2 and a methyl that remains in 3. A possible biogenetic route for 4 might involve decarboxylation of derivatives of 1 or 2, which possess carboxylic acid groups at C-11. The presence of such hypothetic intermediates, however, remains to be confirmed.

REFERENCES

1. K. Tsuda, S. Ikuma, M. Kawamura, R. Tachikawa, K. Sakai, C. Tamura and O. Amakasu, Chem. Pharm. Bull. 12, 1357-1374 (1964).
2. R.B. Woodward, Pure Appl. Chem. 9, 49-74 (1964).
3. T. Goto, Y. Kishi, S. Takahashi and Y. Hirata, Tetrohedron, 21, 2059-2088 (1965).
4. H.S. Mosher and F.A. Fuhrman, Science, 144, 1100-1110 (1964).
5. T. Matsui, S. Hamano and S. Konosu, Nippon Suisan Gakkaishi, 47, 535-537 (1981).
6. Y. Shimizu and M. Kobayashi, Chem. Pharm. Bull. 31, 3625-3631 (1983).
7. T. Yasumoto and T. Michishita, Agric. Biol. Chem. 49, 3077-3080 (1985).
8. T. Yasumoto, M. Nakamura, Y. Oshima and J. Takahata, Nippon Suisan Gakkaishi, 48, 1481-1483 (1982).
9. T. Yasumoto, D. Yasumura, M. Yotsu, T. Michishita, A. Endo and Y. Kotaki, Agric. Biol. Chem. 50, 793-795 (1986).
10. T. Yasumoto, H. Nagai, D. Yasumura, T. Michishita, A. Endo, M. Yotsu and Y. Kotaki, Ann. N.Y. Acad. Sci. 479, 44-51 (1986).
11. M. Yotsu, T. Yamazaki, Y. Meguro, A. Endo, M. Murata, H. Naoki and T. Yasumoto, Toxicon, 25, 225-228 (1987).
12. T. Yasumoto, M. Yotsu, M. Murata and H. Naoki, J. Am. Chem. Soc. 110, 2344-2345 (1988).
13. L.A. Pavelka, F.A. Fuhrman and H.S. Mosher, Heterocycles, 17, 225-230 (1982).