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THE STRUCTURE OF TETRODOTOXIN

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TETRODOTOXIN is one of the most toxic compounds among the poisons having low molecular weight. It is obtained from ovaries of swellfish (Spheroides rubripes) and the intraperitoneal dose for the mouse is 0.01γ/g of the body weight* (1). Poisoning due to the toxin has long been a serious problem in Japan since swellfish is highly esteemed for the table. Since the end of last century many workers have investigated, but the toxin had not been obtained as a pure form until 1952, when Yokoo** (2) obtained the crystalline toxin and assigned the molecular formula $C_{12}H_{17}O_{10}N_3$. Kakisawa et al. (3) and Tsuda et al. (4), however, favored $C_{12}H_{19}O_9N_3$ for it. In this communication we wish to report the structure of tetrodotoxin.

From the elementary analyses and molecular weight determinations of the toxin and its derivatives the molecular formula of tetrodotoxin (I) was revised to $C_{11}H_{17}O_8N_3$ (5). The toxin (I) darkens above 220° without melt, exhibits no UV absorptions (end, $\varepsilon_{220\,m\mu}^{0.1nHCl}$ 75) and is a monoacidic base (pKa' 8.3); ν_{max}^{KBr} 1670, 1613, 1075 cm^{-1}. Permanganate oxidation afforded guanidine, which was characterized as a picrate, and hence it is evident that three nitrogen atoms in the toxin form a guanidine group, which corresponds to the pKa (8.3). It consumed two moles of periodate in 0.1n sulfuric acid solution at 5°, and, when the first one mole of the reagent was consumed, gave formaldehyde.

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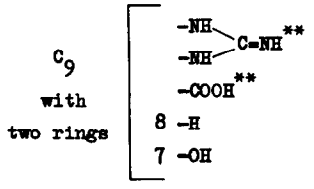
* Only the shellfish toxin is known to have the comparable toxicity: E. J. Schantz, Annals New York Acad. Sci. 90, 843 (1960).

** He used the name "spheroidine" for the toxin.

When heated with water, the toxin (I) was hydrolyzed to tetrodoic acid (II), $C_{11}H_{19}O_9N_3$ (Found: C, 39.14, 38.87; H, 5.96, 5.75; N, 12.33, 12.25. Calcd.: C, 39.17; H, 5.68; N, 12.46%), which was obtained as long needles in 45% yield. The acid (II) darkens above 260° without melt, exhibited an end absorption ($\varepsilon^{0.1nHCl}_{220\ m\mu}$ 410); ν^{KBr}_{max} 1693, 1633, 1590 cm^{-1}; and did not consume bromine, but two moles of periodate (see below). It is a zwitterionic compound having pKa's below 2.5 (COOH) and ca. 11.8 (guanidine). The existence of the carboxylate group was also demonstrated by the IR spectra; whereas the IR spectrum of (II) has a carboxylate band at 1590, (II) hydrochloride shows a band at 1720 cm^{-1} corresponding to a free carboxylic acid.

The negative Sakaguchi reaction of the acid (II) indicates that the guanidine group is not a monosubstituted one. The NMR spectrum of (II) hydrochloride taken in a deuterium oxide solution (Fig. 1) shows signals corresponding to eight protons that can not be exchanged by deuterium*. From these results and the molecular formula of (II), it is deduced that the acid (II) can be represented by (IIa), which contains a disubstituted guanidine, seven hydroxyl groups and consequently two rings. If a tri- or more substituted guanidine group and/or oxygen atoms other than hydroxyl groups were present, the number of non-exchangeable protons must exceed eight.



(IIa)

* Possibilities of overlapping of the proton signals with the strong DOH signal were eliminated by the addition of pyridine hydrochloride to the sample solution; the DOH signal was shifted to lower field, but no signal appeared in the region previously covered by the DOH signal.

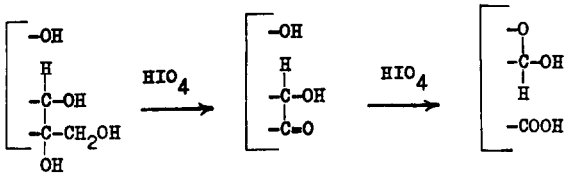
** These two groups exist as a zwitterion, but, for the sake of convenience, they are represented in this paper as shown. An N,N-disubstituted guanidine, instead of the N,N'-disubstituted one, is also possible.

Cleavage of tetrodoic acid (II) with one mole of periodic acid afforded formaldehyde and nortetrodoic acid (III), which consumed further one mole of the reagent and yielded seconortetrododioic acid (IV). The properties of the products are as follows.

Nortetrodoic acid (III): needles darken above 250° without melt, $C_{10}H_{15}O_8N_3 \cdot 1/2\ H_2O$ (Found: C, 38.81, 38.01; H, 5.35, 5.47; N, 13.60. Calcd.: C, 38.22; H, 5.13; N, 13.37%); pKa's below 3 (COOH) and above 11 (guanidine); end absorption ($\varepsilon^{H_2O}_{210\ m\mu}$ 2300, $\varepsilon^{0.1nHCl}_{210\ m\mu}$ 3000); one mole periodate is consumed for 3 hr at 4° in 0.1n sulfuric acid; Tollens and tetrazolium tests are positive; ν^{KBr}_{max} $1690_{sh?}$(C=O), 1670 and 1645 (guanidine, 1612 cm^{-1} (COO^-).

Seconortetrododioic acid (IV): prisms darken above 250° without melt, $C_{10}H_{15}O_9N_3$ (Found: C, 36.75, 37.00, 36.84; H, 4.96, 5.09, 4.94; N, 13.21, 13.05, 13.18, 13.15. Calcd.: C, 37.39; H, 4.71; N, 13.08%); pKa's below 2 (COOH), 3.3 (COOH), above 10.5 (guanidine); end absorption ($\varepsilon^{H_2O}_{210\ m\mu}$ 2500, $\varepsilon^{0.1nHCl}_{210\ m\mu}$ 2800); ν^{KBr}_{max} 1750 (COOH), 1675 and 1640 (guanidine), 1600 (COO^-); silver salt ν^{KBr}_{max} 1675 and 1635 (guanidine), 1610 and 1410 cm^{-1} (COO^-); no periodate consumption after 24 hr either in 0.1n sulfuric acid or at pH 4.4.

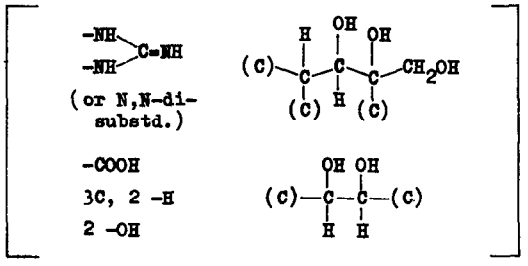
In the NMR spectrum of (IV) (Fig. 1), a doublet among the signals at 2.0 - 2.5 ppm, which were in the spectrum of (III), disappeared and a new doublet appeared at 1.50 ppm. The latter signal is assumed to be for a proton attached to a hemiacetal carbon atom, which was produced from a secondary alcoholic carbon by periodate cleavage with formation of a hemiacetal linkage. Thus, transformations of tetrodoic acid (II) to the seconordioic acid (IV) can be represented by the following sequence.



(IIb) (IIIb) (IVb)

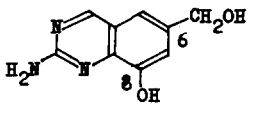
The presence of an α-ketol group in (III) was supported by the positive Tollens and tetrazolium tests. The hemiacetal proton in (IV) must be vicinal to the proton attached to a tertially carbon atom that does not link with oxygen or nitrogen atom, since the signal at 1.50 ppm is spin-coupled with a doublet at the highest field (3.75 ppm, J = 9 cps).

The acid (II) contains a disecondary vic-glycol grouping as demonstrated by the NMR signals at 2.05 and 2.45 ppm with a coupling constant J = 4 cps. The glycol grouping is retained also in the nor acid (III); the corresponding signals appeared at 2.09 and 2.50 ppm (J = 4 cps). Partial structure (IIc) for tetrodoic acid can, therefore, be considered to be established.

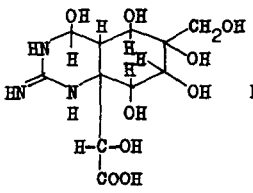


(IIc)

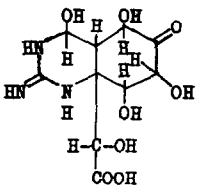
Tetrodoic acid (II), as well as tetrodotoxin (I), can be transformed into the C_9-base (V)(6) by heating with an aqueous barium hydroxide solution. This coupled with the fact that the acid (II) contains two rings constructed from carbon and nitrogen atoms suggests that the acid (II) has a perhydroquinazoline nucleus having a hydroxymethyl group at the C_6-position. To accomodate the partial formula (IIc) in the perhydroquinazoline nucleus, only formula (IId) is possible. Then, the nor acid (III) and the seconordioic acid (IV) must be represented by formulas (IIId) and (IVd), respectively.



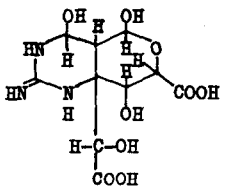
(V)



(IId)



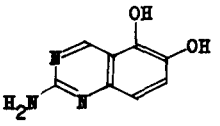
(IIId)



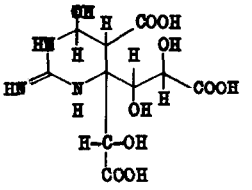
(IVd)

These formulas are consistent with the following observations. (a) Kawamura (6b) reported that oxalic acid was produced along with the C_9-base in the alkaline degradation of the toxin (I). Though oxalic acid was not isolated owing to the small amount of the sample, it is safe to assume that the alkaline degradation of the acid (II) also affords oxalic acid, since the reaction of (I) is considered to proceed through the acid (II). In the formula (IId), there is a two-carbon chain attached to the quinazoline nucleus and this accounts for the production of oxalic acid. (b) When refluxed with 1n hydrochloric acid, nortetrodoic acid (III) afforded yellow needles exhibiting $\lambda_{max}^{0.1nHCl}$ 310 (ε 7050), 265 (19100), 228 mμ (13900); $\lambda_{max}^{0.1nNaOH}$ 309 (12300), 228 mμ (13400)*; pKa's 4.9, 8.0; NMR (in D_2O, ppm from external benzene) −0.05 (1H, d, J=9 cps), −0.68 (1H, d, J=9cps), −2.50 (1H, s); and its elementary analysis indicated the empirical formula $C_8H_7O_2N_3 \cdot HCl \cdot H_2O$ (Found: C, 41.52; H, 4.50; N, 17.25, 17.26%). It is probably 2-amino-5,6-dihydroxyquinazoline (VI), the formation of which from the nor acid (III) is easily understandable. (c) Seconortetrodoidic acid (IV) consumed ca. 1 mole of bromine in water in the presence of strontium carbonate and yielded a crystalline product that is considered to be a strontium salt of seconortetrodotrioic acid (VII), $C_{10}H_{13}O_{10}N_3Sr$ (Found: Sr, 17. Calcd: Sr, 20%); ν_{max}^{KBr} 1660 (guanidine), 1600 and 1400 cm^{-1} (COO^-).

* After acidification, the solution showed a different spectrum from that recorded above; λ(0.1nHCl) 298 (11400), 220 mμ (9200).

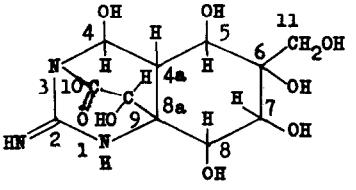


(VI)



(VII)

Tetrodotoxin (I) has no acidic function and its guanidine group shows unusually low pKa value (8.3). This suggests that the carboxyl group and the guanidine link together to form a cyclic acylguanidine moiety. Since only the nitrogen atom at 3-position can form a 6-membered ring, the structure of the toxin (I) must be represented by (Ia).



(Ia)

It seems probable that the carbonyl stretching band of (I) overlaps with the guanidine band at 1670 cm^{-1}, since an amorphous sulfate of (I) shows a carbonyl band at 1730 cm^{-1}.*

The NMR spectra (Fig. 1) provide evidence for the following structural features. (a) H_7 and H_8 are cis to each other since in the spectra of (II) and (III), those signals corresponding to the protons are spin-coupled with a coupling constant J = 4 cps. (b) Both H_5 and H_7 in (II) and (III) may be equatorial, for they show long range spin-spin coupling (J = ca. 1 cps, not resolved well). (c) The cis-configuration of H_{4a} and H_5 is evident from the coupling constants, which

* A model compound, β-alacreatinine, exhibits ν (KBr) 1670, 1620, and the hydrochloride ν (KBr) 1730, 1700 cm^{-1}.

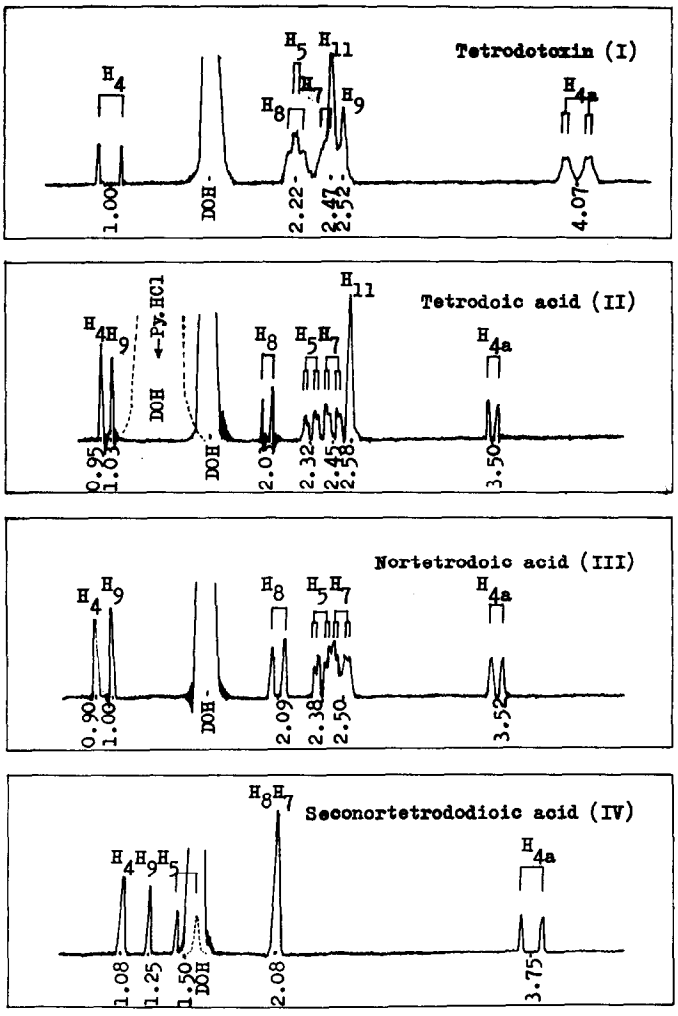
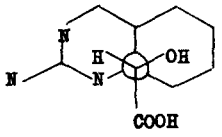
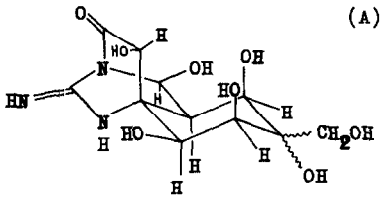


FIG. 1. NMR spectra at 60 Mc in D_2O containing HCl
(Ppm from external C_6H_6)

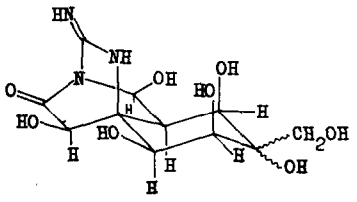
are ca. 2 cps in (I) (not resolved) and 4 cps in both (II) and (III). In (IV), however, they have a trans-diaxial configuration (J = 9 cps). (d) H_4 and H_{4a} is considered to be cis to each other since in (I) their coupling constant is 9 cps* and in (II), (III), and (IV) their coupling constants seem to be less than 1 cps. (e) The signal of H_9 was displaced strongly to lower field when the amide bond was hydrolyzed (I ⟶ II, III, and IV). The shift is probably due to anisotropic shielding factors of guanidine group. In the favored conformation (A) having C_9-configuration as shown, the proton (H_9) comes above the plane of the guanidine group, which may deshield the proton. From these facts the stereochemistry of the toxin (I) may be written as (Ib) or (Ic).



(A)



(Ib)



(Ic)

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* cis-Protons in bicyclo(2,2,2)octane system have a coupling constant J = 8-9 cps, while a coupling constant of trans-protons is 2-3 cps; K. Tori, Y. Takano, K. Kitahonoki and T. Nakagawa, Abstr. 2nd NMR Symposium 67 (1962)(Tokyo, Japan).

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