Chem. Pharm. Bull. 12(11)1357~1374(1964)

UDC 547.933 : 597.54

189. Kyosuke Tsuda,*1 Susumu Ikuma, Masaaki Kawamura, Ryuji Tachikawa, Kiyoshi Sakai, Chihiro Tamura, and Osamu Amakasu*2 : Tetrodotoxin. VII.*3,*4 On the Structures of Tetrodotoxin and its Derivatives.

(*Institute of Applied Microbiology, University of Tokyo**1 *and Research Laboratories, Sankyo Co., Ltd.**2)

Tetrodotoxin (I), one of the most frightfully toxic compounds among the paralytic poisons having low molecular weight, has attracted the attention of chemists and biologists. It is found in the viscera, chiefly in the liver and ovaries of pufferfish, genus *Fugu*.

More recently tetrodotoxin was isolated also from the eggs and embryos of the Cailfornia newt, *Taricha torosa*, by H. S. Mosher, *et al.*[1]

Previously, we had obtained two quinazoline derivatives,*5,*6,[2,3] $C_9H_9O_2N_3$ (II) and $C_9H_9N_3$ (III) from tetrodotoxin (I) by alkaline and reductive degradations, which were established synthetically to be 2-amino-8-hydroxy-6-quinazoline methanol (II) and 2-amino-6-methylquinazoline (III) respectively.[2,3]

Subsequently we obtained a new well defined crystalline derivative, $C_{11}H_{17}O_8N_3H_2O$, from tetrodotoxin on refluxing with water and designated the anhydrous compound as tetrodonic acid (IV).*7 The elementary analysis of tetrodonic acid monohydrate provides the molecular formula, $C_{11}H_{19}O_9N_3$, but the formula changed to $C_{11}H_{17}O_8N_3$ losing a mole of water of crystallization on drying *in vacuo* at about 100° for thirty hours. Moreover, this anhydrous crystalline substance was very easily reconverted into the initial hydrate on treatment with water at room temperature. Accordingly it is certain that the molecular formula of tetrodonic acid is $C_{11}H_{17}O_8N_3$, and that it usually contains one mole of water of crystallization which is also confirmed by inspection of the nuclear magnetic resonance spectrum (*vide infra*). Chemical and X-ray analytical investigations on the structure of tetrodonic acid have been conducted and arrived at exactly the same conclusion. Tetrodonic acid does not show a sharp melting point and it is a zwitterionic compound

*1 Mukogaoka Yayoi-cho, Bunkyo-ku, Tokyo (津田恭介).

*2 Nishi-Shinagawa, Shinagawa-ku, Tokyo (生熊 晋, 河村正朗, 太刀川隆治, 酒井 浄, 田村千尋, 甘粕治).

*3 Part VI. K. Tsuda, S. Ikuma, M. Kawamura, R. Tachikawa, T. Miyadera : This Bulletin, **10**, 868 (1962).

*4 Presented at the 84th Annual Meeting of the Pharmaceutical Society of Japan, April, 7, 1964 (Tokyo University) and at the IUPAC Symposium on the Chemistry of Natural Products, April 13, 1964 (Kyoto). Published communications : This Bulletin, **11**, 1473 (1963), **12**, 634, 642 (1964).

*5 T. Goto, Y. Kishi and Y. Hirata also assigned the same structure to C_9-base (II) after we reported : Bull. Chem. Soc. Japan, **35**, 1045 (1962).

*6 R. B. Woodward, *et al.* also assigned the same structure to the C_9-base from its formation of Cu-chelate compound and from its nuclear magnetic resonance spectrum : Pure and Applied Chemistry, **9**, 49 (1964).

*7 K. Tsuda, C. Tamura, R. Tachikawa, K. Sakai, O. Amakasu, M. Kawamura, S. Ikuma : This Bulletin, **11**, 1473 (1963). T. Goto, Y. Kishi, S. Takahashi and Y. Hirata also obtained $C_{11}H_{19}O_9N_3$ compound from I and named it tetrodoic acid which should be identical with our tetrodonic acid : Tetrahedron Letters, No. 30, 2105 (1963).

1) M. S. Brown, H. S. Mosher : Science, **140**, 295 (1963); H. D. Buchward, L. Durham, H. G. Fisher, R. Harada, H. S. Mosher, C. Y. Kao, F. A. Fuhrman : Science, **143**, 475 (1964); H. S. Mosher, F. A. Fuhrman, H. D. Buchwald, H. G. Fisher : Science, **144**, 1100 (1964).

2) M. Kawamura : This Bulletin, **8**, 262 (1960); K. Tsuda, S. Ikuma, M. Kawamura, R. Tachikawa, Y. Baba, T. Miyadera : *Ibid.*, **10**, 247, 856 (1962).

3) K. Tsuda, S. Ikuma, M. Kawamura, R. Tachikawa, T. Miyadera : *Ibid.*, **10**, 865, 868 (1962).

having pKa's 2.9 (COOH) and 11.9 (guanidinium). That there is a guanidine group corresponding to the pKa', 11.9, is also evident from the observation that destructive oxidation of Ⅳ with permanganate afforded guanidine which was characterized as a picrate. A negative Sakaguchi reaction and positive Weber-test of Ⅳ suggest that the guanidine moiety exists disubstituted in that molecule.

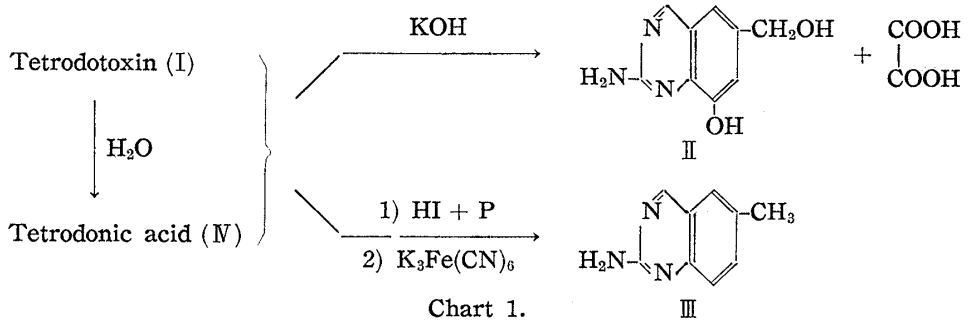


Chart 1.

Tetrodonic acid afforded 2-amino-8-hydroxy-6-quinazoline methanol (Ⅱ) and oxalic acid on alkali degradation, and 2-amino-6-methylquinazoline (Ⅲ) on reduction with hydroiodic acid and red phosphorus and subsequent potassium ferricyanide oxidation just as in the case of tetrodotoxin. Thus it could be supposed that tetrodonic acid has a perhydroquinazoline nucleus since both compounds, Ⅱ and Ⅲ, were obtained very easily and quantitatively. Moreover, from the molecular fromula of Ⅳ, the source of oxalic acid must exist in tetrodonic acid as an oxycarboxylic acid group, which is also assigned from its pKa' value and infrared spectrum, as shown in the following :

$$C_{11}H_{17}O_8N_3 - C_9H_9O_2N_3 = 4H_2O + C_2H_2O_3$$

Here, since $C_2H_2O_3$ does not correspond to oxalic acid but glyoxylic acid, this glyoxylic acid would be oxidized to oxalic acid $C_2H_2O_4$, through the reaction procedures. Accordingly, the source of glyoxylic acid must be a oxycarboxylic acid group not a ketocarboxylic acid group in the molecule of Ⅳ.

Table I. Nuclear Magnetic Resonance Spectrum of Tetrodonic Acid (Ⅳ) at 60 Mc. in Deuterium Oxide containing Sulfuric Acid

p.p.m.[a]		$I_{re.}$
2.92	doublet (J=5 c.p.s.)	1
3.67	dioxane (internal standard)	—
3.86	singlet	2
3.98	quartet (J=4.2 c.p.s., J'=1.1 c.p.s.)	1
4.13	〃 (J=5 c.p.s., J'=1.1 c.p.s.)	1
4.42	doublet (J=4.2 c.p.s.)	1
5.42	singlet	1
5.47	〃	1

a) Band positions given as downfield displacement in p.p.m. from external $(CH_3)_4Si$.

The nuclear magnetic resonance spectrum of Ⅳ is shown in Table I. Of particular importance is the fact that the acid (Ⅳ) contains eight C-protons from its nuclear magnetic resonance spectrum. It consumed two moles of sodium periodate in aqueous solution at 0~2° accompanied by the fromation of a dicarboxylic acid (Ⅴ),[*8] $C_{10}H_{15}O_9N_3NH_3$ (see Experimental), and after the first one mole of the reagent was consumed, formaldehyde was isolated quantitatively as the dimedon derivative. Thus the existence of

[*8] Ⅴ is considered to be the same compound as seconortetrododioic acid prepared by Hirata, *et al.* (cf. reference 5).

the group, $(C)_2$=C(OH)CH_2OH, is demonstrated and the signal at 3.86 p.p.m. can be assigned to hydroxymethyl protons of this group which corresponds to C_6-position from the formation of quinazoline derivatives, II and III. Furthermore, the hydroxy function of oxycarboxylic acid group must form an ether linkage with another available hydroxy function since no other carbon compound was lost except formaldehyde in the periodate oxidation which afforded the C_{10}-compound, V.

From the above observations it is deduced that tetrodonic acid consists of a disubstituted guanidine moiety, a oxycarboxylic acid group, a $(C)_2$=C(OH)CH_2OH group, five C-H bonds, three hydroxyl groups and a quaternary carbon.

Tetrodonic acid forms hydrochloride, $C_{11}H_{17}O_8N_3HCl$ (VI), and hydrobromide, $C_{11}H_{17}O_8N_3HBr$ (VII), respectively and the presence of a free carboxyl group in each salt is demonstrated in the infrared spectrum, by absorption band at 1724 $cm.^{-1}$ Free tetrodonic acid was recovered very easily from both salts on treatment with water. Acid (IV) was the first suitable crystalline derivative prepared for X-ray crystallographic analysis which was expected to furnish the most fruitful information on the structure of tetrodotoxin, since sufficient crystalline tetrodotoxin for taking the X-ray photographs hadn't been prepared as yet. In particular the hydrobromide, VII, is a more suitable derivative than the hydrochloride, VI, comparing the large scattering factor of bromine atom to the summation of those of other light atoms.

The crystal data were determined by Weissenberg and Precession technique. This crystalline bromide occurs as very thin colorless needles. The space group belongs to $P2_12_12_1$, and cell constants measured as standardized on those of sodium chloride, are :

$$a = 20.231\ \text{Å}$$
$$b = 10.590\ \text{Å}$$
$$c = 6.813\ \text{Å}$$
$$v = 1561\ \text{Å}^3$$

By the floatation method the density of this crystal was observed to be 1.821 $g./cm^3$. Molecular weight calculated from these data is 400.5, which is in a good agreement with that of molecular formula of VII, $C_{11}H_{17}O_8N_3HBr$, obtained from elementary analysis. The intensity data of reflections were collected by visual comparison with a standard intensity strip. To enhance the heavy atom vector, three different types of sharpened Patterson functions were computed. Bromine atom's vector peaks were easily determined. Phases based on this bromine atom were employed to compute a three dimensional Fourier function. Light atom coordinates were pursued from peaks of Fourier map. The interpretation of three dimensional Fourier synthesis from the calculation of the stucture factor with its nineteen light atoms and one heavy atom, gave the complete skeleton of the molecule with the assistance of some chemical information. At this stage Fourier map and its structure are shown in Figs. 1 and 2. No least square refinement was used as yet, but the R-factor reached 21%.

Chemically, there are several possible positions to which the oxycarboxylic acid group can be attached, but of these attachment at C_{8a} provides excellent paths for the degradative reactions of IV to quinazoline derivatives of known structure.

Furthermore, structure (VII), shown in Fig. 2 is also supported by the following observations and considerations from the nuclear magnetic resonance spectrum*[9] of IV in acidic solution : i) there is only one highfield signal at 2.92 p.p.m. which corresponds to C_{4a}-proton, ii) the C_{4a}-proton at 2.92 p.p.m. and C_5-proton at 4.13 p.p.m. are *cis* to each other from the coupling constant, J=5 c.p.s., iii) C_4-proton at 5.47 p.p.m. and C_{4a}-proton are also mutually *cis* and the dihedral angle between both bonds is very nearly 90° because the coupling constant is less than 1 c.p.s., iv) the singlet at 3.86 p.p.m. can

*[9] Assignment of each signal in this nuclear magnetic resonance spectrum is confirmed using decoupling.

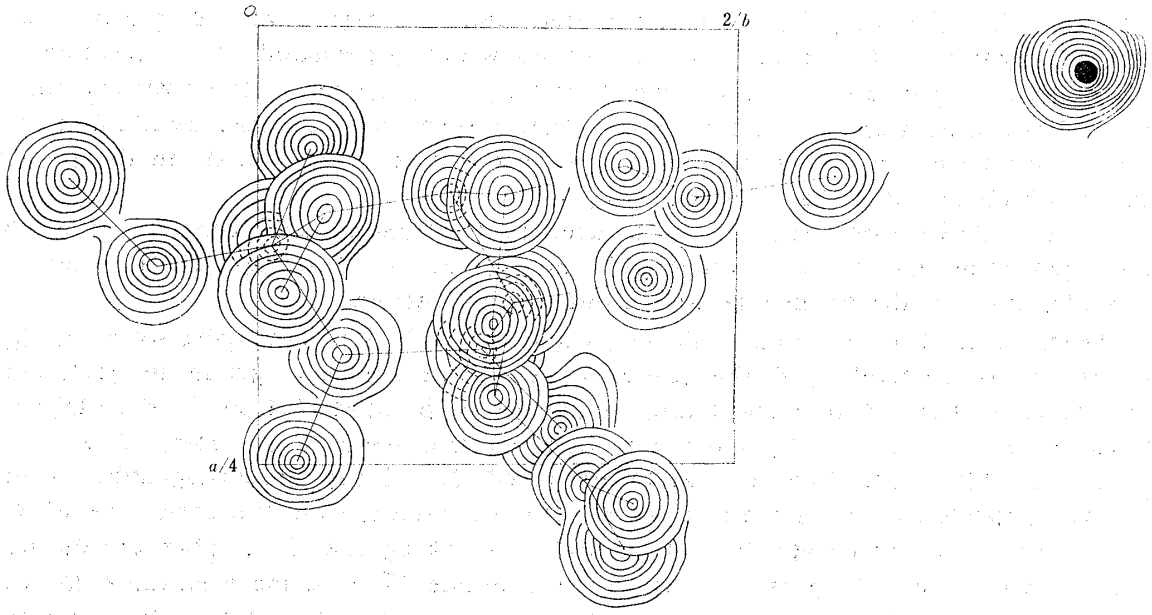


Fig. 1. Composite Fourier Map of Tetrodonic Acid Hydrobromide (Ⅶ) along *c*-Axis (Black Spot is Bromine Atom)

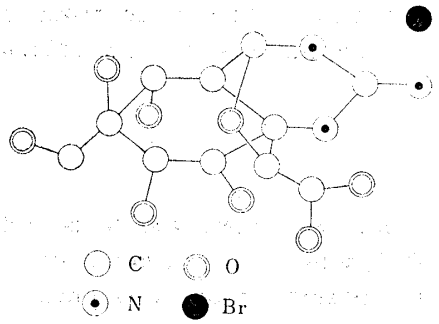
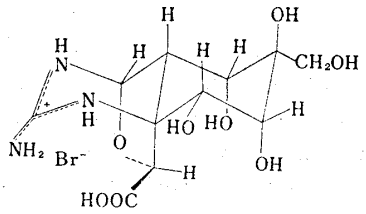
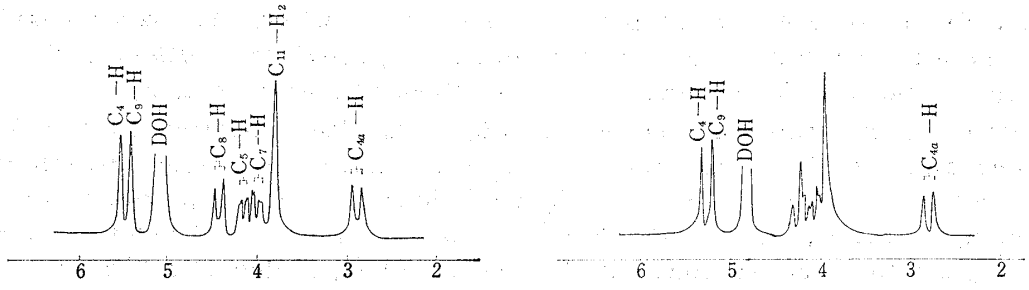


Fig. 2. Molecular Framework of Tetrodonic Acid Hydrobromide (Ⅶ)



Ⅶ : Tetrodonic acid hydrobromide

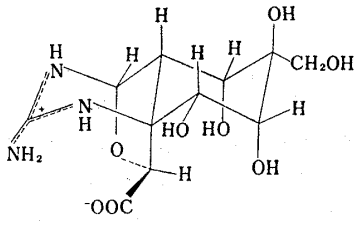
be assigned to C_{11}-methylene protons, v) that those signals at 3.98 p.p.m. and 4.42 p.p.m. which due to C_7- and C_8-protons respectively, are spin coupled with a coupling constant, J=4.2 c.p.s. indicates *cis* configuration for both protons, vi) equatorial C_5- and C_7-protons are also assigned from the fact that both protons in spectrum show long range spin-spin coupling, J=1.1 c.p.s., and that both signals are displaced to slightly higher field than



Tetrodonic Acid (Ⅳ) in D_2O-H_2SO_4 Tetrodonic Acid (Ⅳ) in D_2O-NaOH

Fig. 3. Nuclear Magnetic Resonance Spectra of Tetrodonic Acid (Ⅳ) in Acidic and Basic Solutions. (Band positions given as downfield displacement in p.p.m. from external $(CH_3)_4Si$)

that of the C_8-proton, vii) the nuclear magnetic resonance spectra of Ⅳ at both 60 Mc. and 100 Mc. indicate that the splitting of bands at 5.42 p.p.m. and 5.47 p.p.m. at lowfield which correspond to C_9- and C_4-protons respectively, is due to chemical shifts not to spin coupling, viii) each signal assigned to C_4- and C_9-protons in basic solution is also singlet at 5.16 p.p.m. and 5.23 p.p.m. as shown in Fig. 3, which indicates that tetrodonic acid itself contains the ether linkage between C_4 and C_9 as in Ⅶ.



Ⅳ : Tetrodonic acid

From the X-ray crystallographic analysis of Ⅶ and from the above chemical observations, the structure of tetrodonic acid is established as Ⅳ.

In addition to tetrodonic acid, Ⅳ, we obtained other derivatives*[10] of tetrodotoxin by means of the procedures summarized in Chart 2.

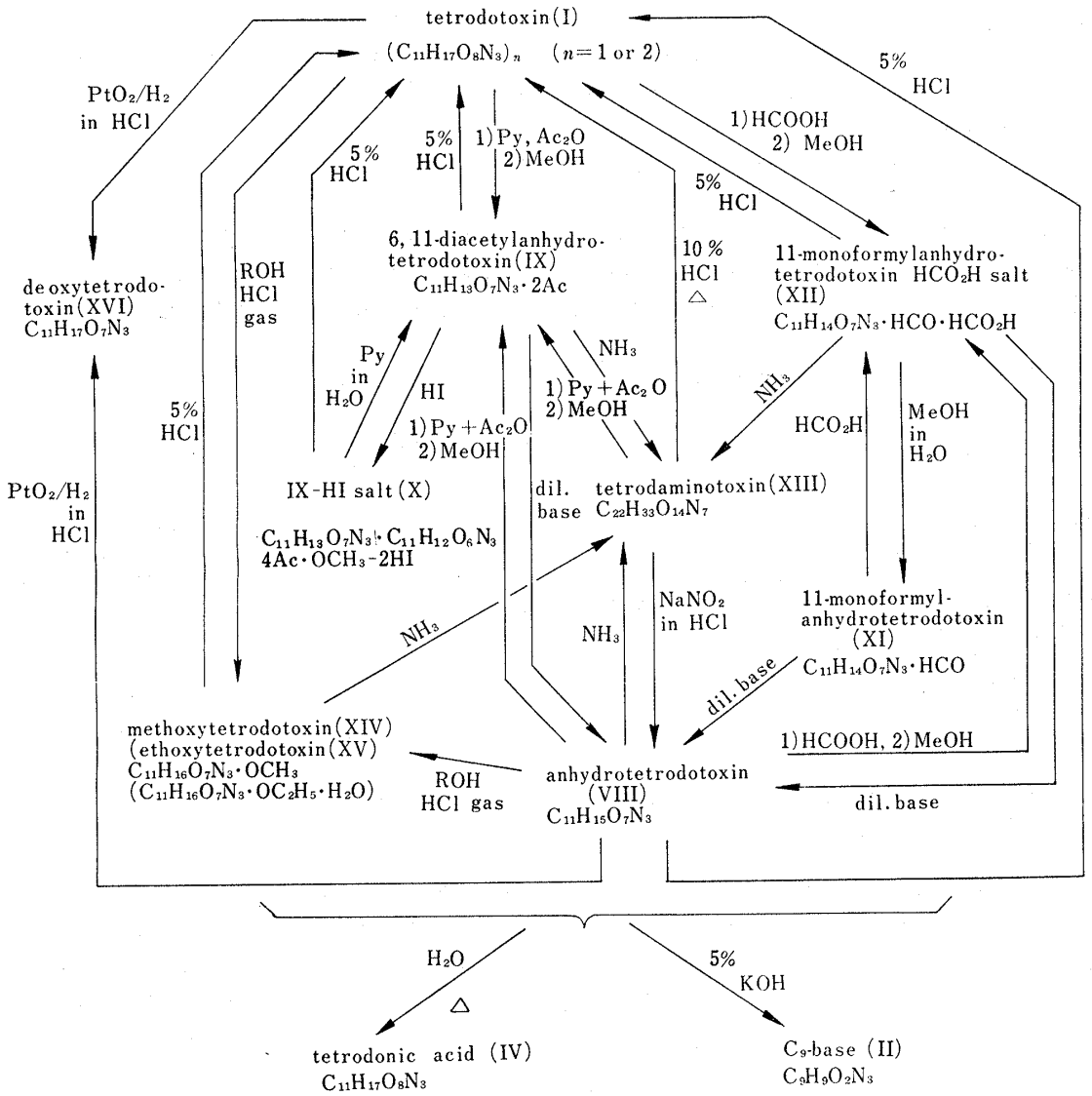


Chart 2.

*[10] Hirata, *et al.* also obtained anhydroepitetrodotoxin and aminodeoxytetrodotoxin which would be identical respectively with our anhydrotetrodotoxin (Ⅷ) and tetrodaminotoxin (XIII). T. Goto, Y. Kishi, S. Takahashi, Y. Hirata : Tetrahedron Letters, No. 14, 779 (1964).

Among these derivatives, the molecular weight of monoformylanhydrotetrodotoxin formic acid salt (XII) was determined by X-ray crystallographic methods. Oscillation, Weissenberg and Precession methods were used for obtaining the crystal data. The cell constants are :

$$a = 15.67\ \text{Å}$$
$$b = 11.03\ \text{Å}$$
$$c = 8.94\ \text{Å}$$

The space group belongs to $P2_12_12_1$ from the extinction rule and the observed density of this crystal was determined by the floatation method to be 1.68 g./cm^3. Therefore, the molecular weight of XII should be 390, as four molecules were in the unit cell and from elementary analysis the molecular formula of XII was determined as $C_{11}H_{15}O_7N_3HCO\cdot HCOOH$. The diacetate hydroiodide (X) was also established to be a C_{11}-compound by X-ray method and elementary analysis.

The analytical value of pure tetrodotoxin*[11] prepared from the crystalline 11-monoformylanhydrotetrodotoxin formic acid salt (XII) or 6,11-diacetylanhydrotetrodotoxin (IX) which were proved to be C_{11}-compounds by X-ray crystallographic methods and elementary analysis, shows a good agreement with $(C_{11}H_{17}O_8N_3)_{n=1\text{ or }2}$*[12] and makes revision of the C_{12}-formula proposed previously[4] necessary.

Tetrodotoxin is the most powerful poisonous substance known, except for certain bacterial toxins but its derivative, tetrodonic acid has not toxicity refering to mice. The toxicities of tetrodotoxin derivatives are summarized in Table II, in which any correlation between toxicities and structures is not immediately evident. Further studies on physiological activities of these tetrodotoxin derivatives are now actively in progress.

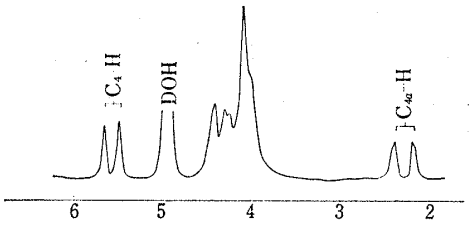
Table II. Minimum Lethal Dose of Tetrodotoxin Derivatives[a]

Derivative	Minimum Lethal Dose (μg./kg.)	
Tetrodotoxin (I)	8.22[b]	intravenously
Anhydrotetrodotoxin (VIII)	4,140[b]	〃
6,11-Diacetylanhydrotetrodotoxin (IX)	> 50,000	intraperitonearly
11-Monoformylanhydrotetrodotoxin HCOOH salt (XII)	ca. 3,000	〃
Tetrodaminotoxin (XIII)	841[b]	intravenously
Methoxytetrodotoxin (XIV)	341	〃
Ethoxytetrodotoxin (XV)	692	intraperitonearly
Deoxytetrodotoxin (XVI)	84.5	intravenously
Tetrodonic acid (IV)	⩾ 300,000	〃

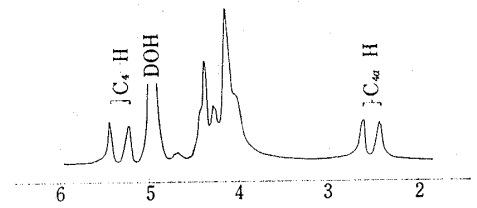
a) Minimum lethal dose refers to mongrel mouse, administered in a pH 5 buffer solution.
b) LD_{50} in mongrel mouse.

All tetrodotoxin derivatives except deoxytetrodotoxin (XVI) afforded tetrodonic acid on refluxing with water in good yields. However, the infrared spectra and pKa′ values indicate that there is no free carboxyl groups in these molecules. They are clearly not lactone derivatives also since their infrared spectra do not contain bands assignable to such a group. Accordingly, the carboxyl group could either be involved in formation of

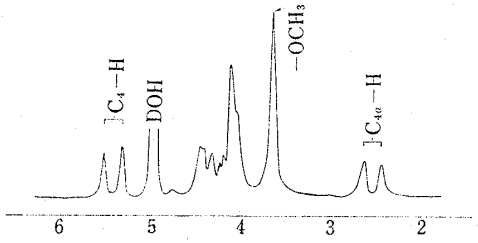
*11 The crude tetrodotoxin usually contains a small amount of anhydrotetrodotoxin.
*12 Hirata, *et al.* also assigned $C_{11}H_{17}O_8N_3$ formula to tetrodotoxin by means of titration and osmometer methods. T. Goto, S. Takahashi Y. Kishi Y. Hirata : Bull. Chem. Soc. Japan, **37**, 283 (1964).
4) A. Yokoo : Proc. Japan Acad., **28**, 200 (1952); J. Chem. Soc. Japan, **71**, 590 (1950); H. Kakisawa, Y. Okumura, Y. Hirata : *Ibid.* **80**, 1483 (1959); K. Tsuda, M. Kawamura, R. Hayatsu : This Bulletin, **8**, 257 (1960).



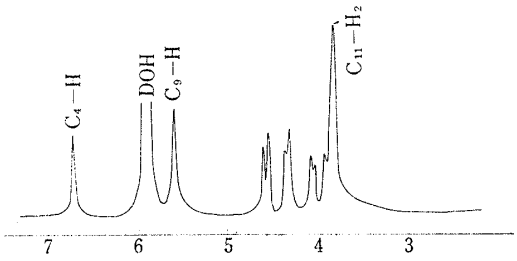
Tetrodotoxin (I) in CD_3COOD-D_2O



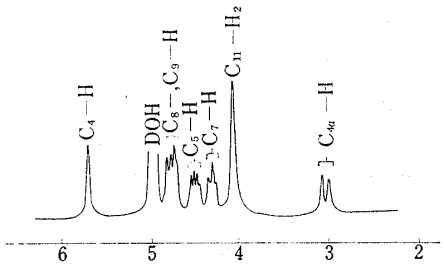
Tetrodaminotoxin (XIII) in CD_3COOD-D_2O



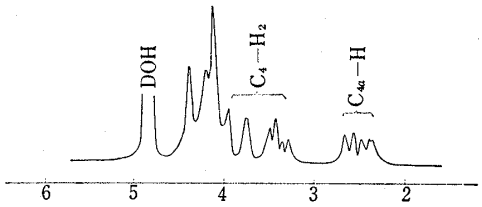
Methoxytetrodotoxin (XIV) in CD_3COOD-D_2O



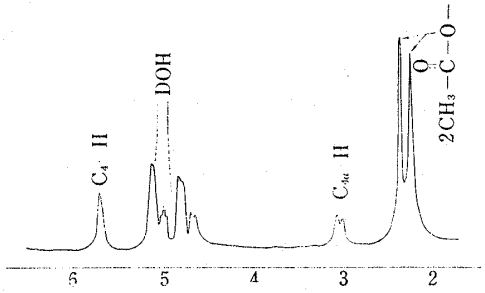
Anhydrotetrodoic Acid (Hirata) in CD_3COOD-D_2O



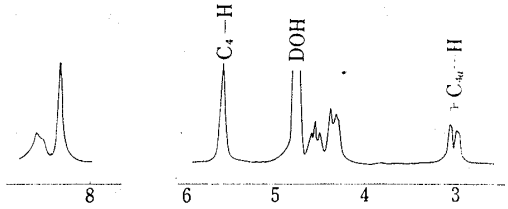
Anhydrotetrodotoxin (VIII) in CD_3COOD-D_2O



Desoxytetrodotoxin (XVI) in CD_3COOD-D_2O



6,11-Diacetylanhydrotetrodotoxin (X) in CD_3COOD-D_2O



11-Monoformylanhydrotetrodotoxin HCOOH Salt (XII) in D_2O

Fig. 4. Nuclear Magnetic Resonance Spectra of Tetrodotoxin and its Derivatives
(Band positions given as downfield displacement in p.p.m. from external $(CH_3)_4Si$)

a lactam*[13] or *ortho*-ester with available nitrogen or oxygen atoms in tetrodotoxin and its derivatives except for V. The nuclear magnetic resonance spectra of I and its derivatives are shown in Fig. 4.

The spectrum of tetrodotoxin which was recovered from anhydrotetrodotoxin (VIII) by treatment with 5% deuteriumchloride in deuterium oxide solution, is identical with that

*[13] Previously, we presumed three possible lactam structures for tetrodotoxin from only its pKa′ value but the further studies in this paper necessitate the exclusion of lactam forms for I. K. Tsuda, C, Tamura, R. Tachikawa, K. Sakai, O. Amakasu, M. Kawamura, S. Ikuma : This Bulletin, **11**, 1473 (1963).

of natural tetrodotoxin in 10% deuteroacetic acid solution. In the nuclear magnetic resonance spectrum of Ⅳ which was prepared from I or Ⅷ by refluxing with deuterium oxide instead of water, the signal assigned to $C_{4\alpha}$-proton didn't vanish but the signal corresponding to C_9-proton disappeared. Consequently, in tetrodotoxin and anhydrotetrodotoxin, $C_{4\alpha}$-aldehyde bond $C_{8\alpha}$-guanidine bond are *trans* to each other as well as in tetrodonic acid.

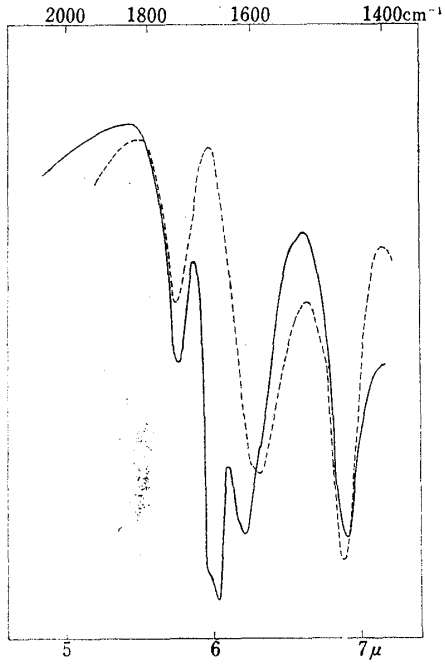


Fig. 5. Infrared Spectra of Tetrodotoxin HCl Salt and D_2O-treated Tetrodotoxin HCl Salt (Nujol)

——— Tetrodotoxin HCl salt
– – – – – D_2O-treated tetrodotoxin HCl salt

On the other hand, anhydrotetrodoic acid prepared by us according to Hirata's method[5] using barium deuteroxide in deuterium oxide solution, shows the same nuclear magnetic resonance pattern as authentic anhydrotetrodoic acid, which demonstrates that the C_9-position was not deuterated.

Nitta, *et al.*[6] determined by X-ray crystallographic analysis the structure of bromoanhydrotetrodoic lactone hydrobromide, in which the configuration at C_9 in inverted with respect to that of tetrodonic acid (Ⅳ). From the above experiments with deuterium reagents we can conclude that the epimerization at C_9 occurred in Ⅳ through enolization during the course of the reaction whereas the Hirata's anhydrotetrodoic acid retains the same configuration at C_9 as that of tetrodotoxin.

Moreover, lactam forms for tetrodotoxin and anhydrotetrodotoxin (Ⅷ) are excluded from the following evidence. i) The absorption band at 1660 cm^{-1} observed in infrared spectrum of amorphous tetrodotoxin hydrochloride disappeared on deuteration indicating that tetrodotoxin would have no lactam group (see Fig. 5). ii) The molecular formula of Ⅷ, $C_{11}H_{15}O_7N_3$, the dehydrated form of tetrodotoxin, $(C_{11}H_{17}O_8N_3)_{n=1 \text{ or } 2}$, shows that Ⅷ has a double bond, an epoxide or an ether linkage which does not exist in I. That both hydroxymethyl and hydroxyl groups at C_6 in tetrodotoxin or Ⅷ are free as in tetrodonic acid, is demonstrated by the formation of one mole formaldehyde on the periodate oxidation of I or Ⅷ in each case. The formation of ether linkage is impossible in some lactam forms and even if the only possible extra ether linkage in anhydrotetrodoxin existed between C_9 and C_5 or C_7, it is very difficult to account for the results of periodate oxidation according to the following considerations. The presence of a double bond in Ⅷ is eliminated from the lack of ultraviolet absorption and since eight C-protons are assignable in the nuclear magnetic resonance spectra of both Ⅷ and I, which was recovered from Ⅷ on treatment with 5% deuterium chloride in deuterium oxide, as well as in that of tetrodonic acid. That anhydrotetrodotoxin has no epoxide group is a matter of course from the results of periodate oxidation. Whereas two or three moles of sodium periodate is consumed very

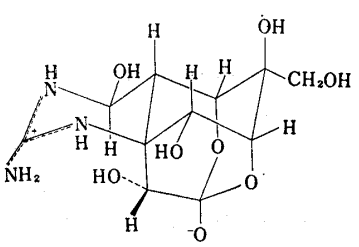
5) T. Goto, S. Takahashi, Y. Kishi, Y. Hirata : Tetrahedron Letters, No. 30, 2115 (1963). They also tried the same experiments as we on the configurations at C_9-position. (see *10).

6) Y. Tomiie, A. Furusaki, K. Kasami, N. Yasuoka, K. Miyake, M. Haisa, I. Nitta : Tetrahedron Letters, No. 30, 2101 (1963). By X-ray method the absolute configuration of bromoanhydrotetrodoic lactone hydrobromide (XXII) was established as an antipode to the formula (XXII) and presented at IUPAC Symposium on the Chemistry of Natural Products, April 13, 1964 (Kyoto).

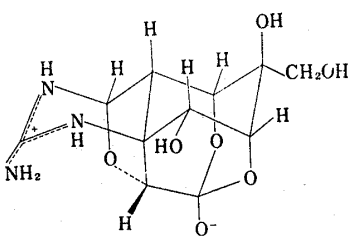
easily in the oxidation of tetrodotoxin, anhydrotetrodotoxin requires many hours to consume the second mole of reagent under the same conditions as tetrodotoxin. This leads us to suppose that the existence of a extra ether linkage in Ⅷ prevents the second step oxidation with periodate. From results of periodate oxidation, the possibility of lactam forms for I and Ⅷ should be eliminated and consequently both tetrodotoxin and anhydrotetrodotoxin are *ortho*-ester compounds. Further support is evidenced from the solvent effect in their dissociations and from mechanistic considerations of the reactions shown in Chart 2.

The pKa′ values of tetrodotoxin were 8.84 in water and 9.54 in 60% aqueous ethanol respectively. This solvent effect indicates that this pKa′ value should correspoud to a weakly acidic function which would be more reasonable for the hydroxyl group at C_{10} in the *ortho*-ester structures, rather than for basic groups which was presumed previously*[13] to be a lactam of the guanidine moiety. The same solvent effect was also on pKa′ values of anhydrotetrodotoxin, 7.92 in water and 9.02 in 60% aqueous ethanol, observed. These solvent effects were also pointed out previously by Hirata, *et al.*[10] and Woodward, *et al.*[6]

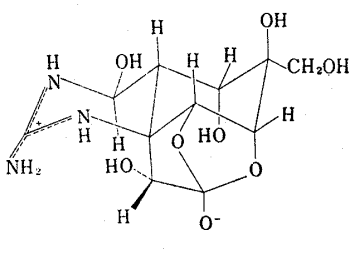
There are three possible *ortho*-ester forms for both tetrodotoxin and anhydrotetrodotoxin, XVⅡa, b, XVⅢa, b, and XIXa, b.



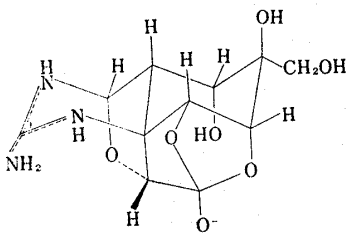
XVⅡa



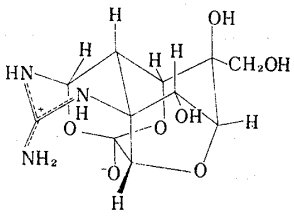
XVⅡb



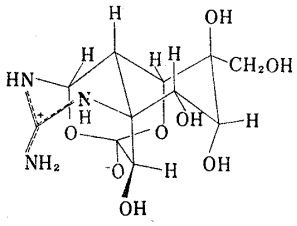
XVⅢa



XVⅢb



XIXa



XIXb

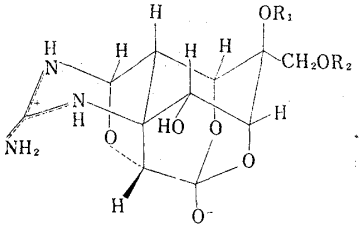
As tetrodotoxin and its derivatives are easily transformed into each other as shown in Chart 2, they should possess the same *ortho*-ester skeleton.

Subsequently we can note that the derivatives of tetrodotoxin except XVI can be classified according to the nuclear magnetic resonance spectra shown in Fig. 4 into two groups, one of which includes tetrodotoxin (I), methoxytetrodotoxin (XIV) and tetrodaminotoxin (XⅢ), and the other consisting of anhydrotetrodotoxin (Ⅷ), 6,11-diacetylanhydrotetrodotoxin (Ⅸ), its hydroiodide (X), 11-monoformylanhydrotetrodotoxin (Ⅺ) and its formic acid salt (Ⅻ). In each of the former spectra, the downfield signal corresponding to C_4-proton is a doublet spin coupled to the highfield signal of the C_{4a}-proton with a large coupling constant, J=ca. 10 c.p.s. This suggests that the dihedral angle between C_4-H and C_{4a}-H bonds is ca. 0° or 180°. However, in that of the later, the downfield signal assigned to C_4-proton is a singlet indicating that the dihedral angle between C_4-H and C_{4a}-H bonds is very nearly 90°.

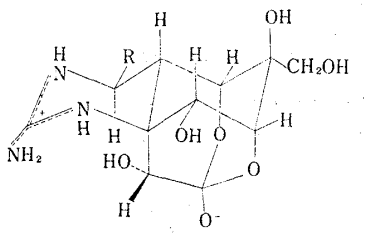
Accordingly, it can be concluded that tetrodotoxin and anhydrotetrodotoxin in which an ether linkage is present between C_4 and C_9, should be epimeric with each other at

C_4-position in the same *ortho*-ester skeleton, XVII (a, b) with assistance of the observations on the above mentioned periodate oxidation.

The displacement of the signal corresponding to C_{11}-protons to lower fields by about 1.0 and 0.7 p.p.m. in the nuclear magnetic resonance spectra of the diacetate (IX) and the formate (XII) respectively as compared with that of anhydrotetrodotoxin, indicates that in both derivatives the C_{11}-hydroxymethyl is acylated. However, the position of the another acetyl group in IX couldn't be assigned definitely by chemical methods though it was confirmed by X-ray analysis to be attached at the tertiary C_6-hydroxyl group.



VIII : $R_1=R_2=H$, Anhydrotetrodotoxin
IX : $R_1=R_2=Ac$, 6,11-Diacetylanhydrotetrodotoxin
XI : $R_1=H$, $R_2=HCO$, 11-Monoformylanhydrotetrodotoxin



I : R=OH, Tetrodotoxin
XIV : R=OMe, Methoxytetrodotoxin
XV : R=OEt, Ethoxytetrodotoxin
XVI : R=H, Desoxytetrodotoxin

Methoxy-, ethoxytetrodotoxin and deoxytetrodotoxin can be represented by the formulas (XIV, XV, and XVI) respectively from the following observations : i) methoxytetrodotoxin was changed into tetrodotoxin by treatment with a dilute mineral acid and to tetrodaminotoxin by action of aqueous ammonia, ii) the nuclear magnetic resonance spectrum of methoxytetrodotoxin is very similar to that of tetrodotoxin as shown in Fig. 4, iii) the alkoxyl groups in methoxy- and ethoxytetrodotoxin are rather stable on neutralization with dilute aqueous ammonia at room temperature, whereas the methoxyl group at C_{10}-position in the diacetate hydroiodide (X) (*vide infra*) was easily hydrolysed by the action of dilute aqueous pyridine or ammonia, iv) the nuclear magnetic resonance spectrum of deoxytetrodotoxin shows that the aldehyde carbon, C_4, was reduced catalytically.

The hydroiodide of 6,11-diacetylanhydrotetrodotoxin (X) prepared from the free base, is highly suitable for X-ray crystallographic analysis for establishing the structure of tetrodotoxin, because the free base was recovered from this hydroiode by action one drop of pyridine in aqueous or methanolic solution and tetrodotoxin itself was obtained directly from X by treatment with 5% hydrochloric acid. The hydroiodide (X) was obtained as a single crystal and was of convenient size for taking photographs. The nuclear magnetic resonance spectrum of X is very similar to that of 6,11-diacetylanhydrotetrodotoxin except that there is a signal corresponding to methyl of methanol or methoxyl having a relative area corresponding to 1.5 protons in the spectrum of X. Crystals obtained by recrystallization with ethanol instead of methanol, shows a signal assignable to ethyl of ethanol or ethoxyl in the nuclear magnetic resonance spectrum having a relative area due to 2.5 protons. Thus the hydroiodide evidently contains 0.5 mole solvent of crystllization. These crystals occur as thin colorless needles having the space group $P2_1$-C_2^2, and cell constants are :

$$a = 19.34\ \text{Å}$$
$$b = 14.63\ \text{Å}$$
$$c = 7.18\ \text{Å}$$
$$\beta = 82.6^\circ$$

The density observed by floatation method is 1.69 g./cm^3 and thus molecular weight of this hydroiodide was calculated as 513 or 1026. Intensity data was collected by Weissenberg

technique and viewing as compared with a standard calibrated strip. General Patterson function for total reflections was computed and from this three dimensional site of iodine atoms determined. Fourier calculations were made from phases of those two iodine positions. Fortunately, as there are two heavy atoms in the assymmetric unit phase ambiguity would be eliminated comparing with $P2_1$ for one heavy atom. From six times of structure factor and Fourier calculation the light atom positions were determined. A composite Fouier map gives a well resolved molecular structure of the atoms. The molecular structure of this 6,11-diacetylanhydrotetrodotoxin hydroiodide was related to tetrodonic acid, however, the stereographic Fourier map were not as same. There is some ambiguity about the methoxyl group at C_{10} but X-ray crystallographic analysis and chemical evidence for the structure of this salt, X, lead to the same conclusion, a C_5-C_{10}-C_7 *ortho*-ester form, independently. The Fourier map and structure are shown in Figs. 6 and 7.

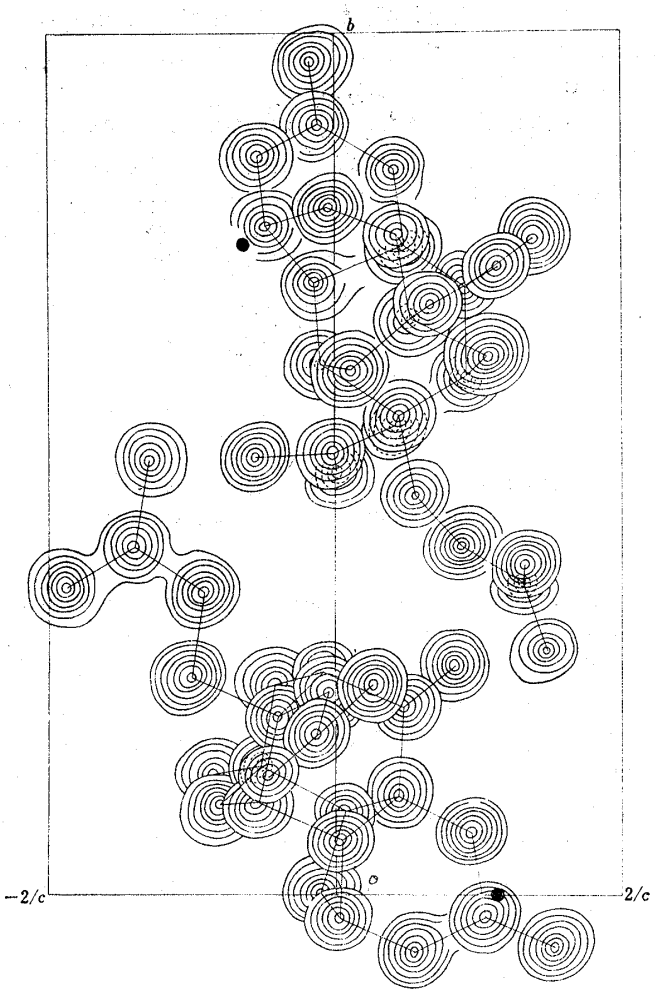


Fig. 6. Composite Fourier Map of 6,11-Diacetylanhydrotetrodoxin Hydroiodide (X) along *a*-Axis (Black Spots are Iodine Atoms)

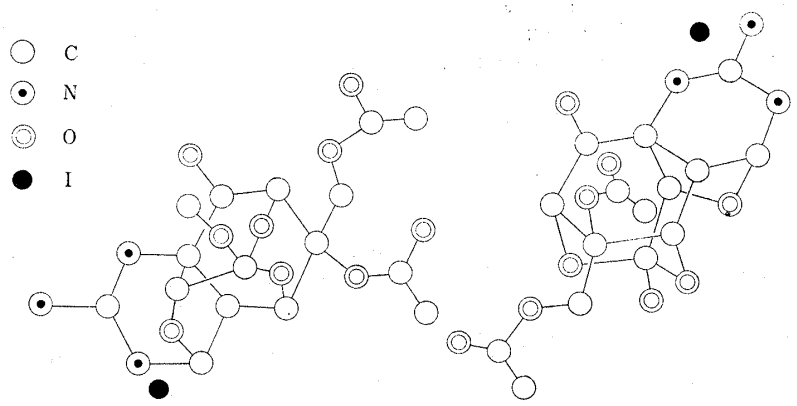
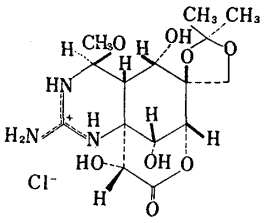


Fig. 7. Molecular Framework of 6,11-Diacetylanhydrotetrodotoxin Hydroiodide (X)

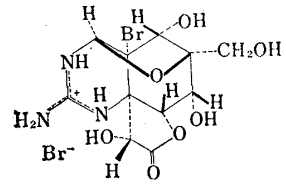
Tetrodotoxin doesn't show a definite melting point and darkens gradually above 220°. Many derivatives can be prepared as shown in Chart 2 and if the molecular formula of tetrodotoxin would be $(C_{11}H_{17}O_8N_3)_{n=1}$ its structure must be represented formula (I) from the accumulated evidence.

Woodward, *et al.*[*6] assigned the same structure for I based upon the X-ray crystallographic analysis of O-methyl-O′,O″-isopropylidenetetrodotoxin hydrochloride (XXI) and some chemical evidence.

Hirata, *et al.*[*10] also deduced the same structure for I from chemical observations and the X-ray crystallographic structure determination of bromoanhydrotetrodoic lactone hydrobromide (XXII) which was established by Nitta, *et al.*[*6]



XXI : O-Methyl-O′,O″-isopropylidenetetrodotoxin Hydrochloride (Woodward)



XXII : Bromoanhydrotetrodoic Lactone Hydrobromide (Nitta)

However, there are some analytical observations suggesting that tetrodotoxin might be a C_{22}-compound. The analytical values of tetrodaminotoxin which was prepared from anhydrotetrodotoxin, 6,11-diacetylanhydrotetrodotoxin or methoxytetrodotoxin by action of aqueous ammonia, were in better agreement with the formula, $C_{22}H_{33}O_{14}N_7$, than with $C_{11}H_{18}O_7N_4H_2O$ (cf. Experimental); thus suggesting that tetrodaminotoxin[*14] (XIII) was formed from one mole ammonia and two moles of anhydrotetrodotoxin. Quantitative determinations of nitrogen in tetrodotoxin, its derivatives and in tetrodaminotoxin by the Kjeldahl method also predicted that type of structure. In order to distinguish the newly introduced nitrogen from those of the guanidine moiety, we prepared tetrodaminotoxin

Table III.

Compound	Weight of compd. (mg.)	Nitrogen by titrat. (mg.)	1 mole equiv. Nitrogen (mg.)
1) Tetrodonic acid (IV) $C_{11}H_{17}O_8N_3H_2O$	119.09	5.06	14.33
2) Anhydrotetrodotoxin (VIII) $C_{11}H_{15}O_7N_3$	105.64	5.33	15.20
3) Tetrodotoxin (I) $(C_{11}H_{17}O_8N_3)_{1 \text{ or } 2}$	117.11	5.05	13.78 (or 27.56)
4) N^{15}-Tetrodotoxin (I) $(C_{11}H_{17}O_8N_3)_{1 \text{ or } 2}$	100.41	4.86	15.45 (or 30.90)
5) N^{15}-Tetrodaminotoxin (XIII) $C_{22}H_{33}O_{14}N_7$	122.75	8.62	43.55
6) N^{15}-Tetrodaminotoxin (XIII) $C_{22}H_{33}O_{14}N_7$	180.21	12.51	43.05

Table IV.

Compound	Weight of compd. (mg.)	Nitrogen by titrat. (mg.)	N^{15}-Atom-%	Weight of N^{15} (mg.)
4) N^{15}-Tetrodotoxin (I)	100.41	4.86	0.467	0.024
5) N^{15}-Tetrodaminotoxin (XIII)	122.75	8.62	25.1	2.277
* (N^{15} Value calcd. from $C_{22}H_{33}O_{14}N_7$)				(2.019)
* (N^{15} Value calcd. from $C_{11}H_{18}O_7N_4H_2O$)				(3.715)
6) N^{15}-Tetrodaminotoxin (XIII)	180.21	12.51	24.8	3.266
* (N^{15} Value calcd. from $C_{22}H_{33}O_{14}N_7$)				(2.964)
* (N^{15} Value calcd. from $C_{11}H_{18}O_7N_4H_2O$)				(5.455)

*14 It was found that the tetrodaminotoxin is dimorphic, the second form shows in IR ν_{max}^{Nujol} cm^{-1} : 1667, 1614 and 1228 bands.

containing N^{15} (N^{15}-tetrodaminotoxin) from 6,11-diacetylanhydrotetrodotoxin by treatment with aqueous ammonia containing 68% excess of N^{15}. Subsequently the concentration of N^{15} was measured by mass spectrometry and the results are summarized in Tables Ⅲ and Ⅳ.

Table Ⅲ shows that the observed values for nitrogen of the guanidine moiety in tetrodotoxin and its derivatives, correspond approximately to one nitrogen atom calculated for C_{11}-formula or two nitrogens calculated for C_{22}-formula and about three nitrogen atoms for tetrodaminotoxin. Table Ⅳ demonstrates the results of N^{15} measurement by mass spectrometry. That no exchange of N^{15} with nitrogens of the guanidine moiety in Ⅸ occured during the reaction is shown by observed N^{15} concentration value in N^{15}-tetrodotoxin prepared from N^{15}-tetrodaminotoxin which was almost the same as that of the natural nitrogen. The observed N^{15} concentrations of N^{15}-tetrodaminotoxin are closer to those calculated for $C_{22}H_{33}O_{14}N_7$ than those for $C_{11}H_{18}O_7N_4H_2O$. These results suggest that tetrodaminotoxin would be a C_{22}-compound. The sharp signal assigned the C_4-proton in nuclear magnetic resonance spectrum of XIII and the high yield of anhydrotetrodotoxin from the reaction of XIII with sodium nitrite in hydrochloric acid excludes any possibility that XIII is a mixture consisting of amino-derivative and anhydrotetrodotoxin or tetrodotoxin with the same ratio.

The newly introduced nitrogen function in XIII should be located at C_4 from the following observations : tetrodaminotoxin afforded i) anhydrotetrodotoxin, the C_4-epimer of tetrodotoxin, on treatment with sodium nitrite in 10% hydrochloric acid, ii) tetrodotoxin on heating with a mineral acid, iii) tetrodonic acid on refluxing with water. Moreover, the nuclear magnetic resonance spectra of both I and XIII showed very similar patterns as shown in Fig. 4 except that the signal corresponding to the C_4-proton of XIII is displaced slightly upfield with respect to that of I.

Therefore, in consideration of the above analytical results only, tetrodaminotoxin (XIII) might have a possibility to be a dimeric structure in which two molecules are connected at C-4 position by imino ether linkage.

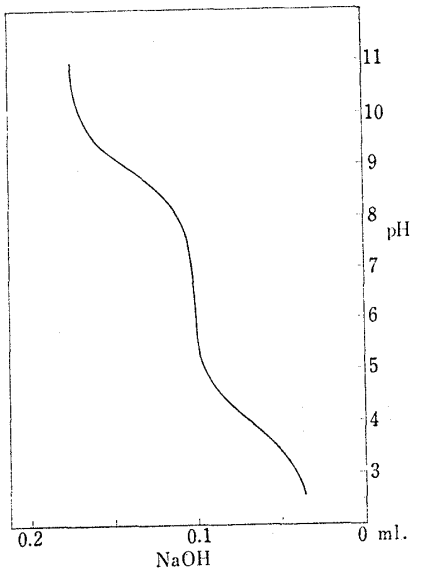


Fig. 8. Titration Curve of Tetrodaminotoxin (XIII)

3.379 mg. of Tetrodaminotoxin (XIII) in 0.5 ml. of 0.1*N* HCl was titrated with 0.0979*N* NaOH. The pKa' values were calculated from the observed values.

The nuclear magnetic resonance spectrum (see Fig. 4), infrared spectrum and chemical behavior of tetrodotoxin are very similar to those of tetrodaminotoxin. Furthermore, the similarity of I and XIII were strongly supported by the interplanar spacings calculated from the X-ray diffraction angles : comparison of these data shows quite a good correspondence not only in the interplanar spacings but also in these intensities as compared with those of the other derivatives, indicating that the lattice constants and the atomic co-ordinates are similar as shown in Table V.

Therefore, the above powder X-ray diffraction data and the quantitative determinations of N^{15} in tetrodaminotoxin remain also some possibility that tetrodotoxin itself might exist as a dimeric form in the free crystalline state.

In the other hand, the titration of tetrodaminotoxin shows two dissociations at pKa'=4.08 and 8.97, as shown in Fig. 8 and approximately one mole of sodium hydroxide was consumed at each

Table V. Powder X-ray Diffraction Data of Tetrodotoxin and its Derivatives[a)]

Tetrodotoxin		Tetrodaminotoxin-A[b)]		Tetrodaminotoxin-B[b)]		Anhydrotetrodotoxin-A[c)]		Anhydrotetrodotoxin-B[c)]		Methoxytetrodotoxin		Deoxytetrodotoxin	
d(Å)	I/I_1	d(Å)	I/I_1	d(Å)	I/I_1	d(Å)	I/I_1	d(Å)	I/I_1	d(Å)	I/I_1	d(Å)	I/I_1
7.31	7	7.26	8	7.43	95	8.12	2	7.14	100	9.40	14	7.62	100
6.28	100	6.24	100	6.37	30	7.38	100	5.94	36	8.34	20	6.60	82
5.99	22	5.99	18	6.02	54	6.86	3	5.44	5	7.49	92	6.46	71
5.68	26	5.72	18	5.43	54	6.42	17	5.13	5	6.70	90	6.10	14
5.54	36	5.54	18	5.06	4	6.11	31	4.62	10	6.23	100	5.90	16
5.22	28	5.16	11	4.82	28	5.87	2	3.77	3	5.94	18	5.64	4
4.93	3	4.87	1	4.46	100	5.57	3	3.43	43	5.75	19	5.09	26
4.75	36	4.72	32	4.19	8	5.28	1	3.19	2	5.53	8	4.77	16
4.65	7	4.65	2	3.96	5	5.10	1	2.88	2	5.30	15	4.69	16
4.42	11	4.40	7	3.88	25	4.72	13	2.54	1	5.12	14	4.48	1
3.85	1	3.83	2	3.73	11	4.31	2	2.43	2	4.98	9	3.95	2
3.68	6	3.62	5	3.49	7	4.19	1	2.36	2	4.82	17	3.90	3
3.52	8	3.59	5	3.32	3	3.92	1	2.31	1	4.67	12	3.80	22
3.35	14	3.35	7	3.18	8	3.80	3	1.80	2	4.33	6	3.73	12
3.27	57	3.27	13	3.09	13	3.69	3	1.73	1	4.23	5	3.63	30
3.13	3	3.13	3	2.95	6	3.59	2			3.86	38	3.47	20
3.03	36	3.06	20	2.91	3	3.49	45			3.69	26	3.38	2
2.98	3	2.95	3	2.81	4	3.23	4			3.53	7	3.30	4
2.92	4	2.94	4	2.67	7	3.10	2			3.49	35	3.23	18
2.85	1	2.90	1	2.62	6	3.05	1			3.38	18	3.17	7
2.76	10	2.76	3	2.44	11	2.98	2			3.32	32	3.12	10
2.73	7	2.71	2	2.37	5	2.88	3			3.29	27	2.99	3
2.60	4	2.62	1	2.35	4	2.82	5			2.95	14	2.94	9
2.56	3	2.56	1	2.28	6	2.77	1			2.65	14	2.85	5
2.41	1	2.46	1	2.24	2	2.59	1			2.40	6	2.79	6
2.38	4	2.42	3	2.12	3	2.55	3					2.68	5
2.35	11	2.40	8	2.05	5	2.45	3					2.62	2
2.27	4	2.30	5	1.89	3	2.39	3					2.54	6
2.15	4	2.17	3	1.88	3	2.33	2					2.52	6
2.13	1	2.13	1			2.23	1					2.48	13
2.06	1	2.09	1			1.81	2					2.39	3
2.04	1	2.06	1			1.74	1					2.37	2
1.98	3	1.98	1									2.28	4
1.97	4	1.96	2									2.22	2
1.73	1	1.77	2									2.20	2
1.66	2	1.66	1									2.16	3
1.56	2	1.56	1									2.14	3
												2.00	2
												1.93	2

a) A mixture of tetrodotoxin and anhydrotetrodotoxin shows a pattern consisted of those of both components. which is very different one from that of tetrodotoxin.

b) We named tetrodaminotoxin-A to one of two forms of tetrodaminotoxin, which shows in IR ν_{max}^{Nujol} cm^{-1}: 1679, 1623, and 1228 bands and tetrodaminotoxin-B to the another which shows in IR ν_{max}^{Nujol} cm^{-1}: 1667, 1614, and 1228 band.

c) See Experimental.

dissociation point.*[15] This result clearly shows that tetrodaminotoxin (XIII) is a C_{11}-compound.

*[15] Prof. R.B. Woodward pointed out the same facts in his private communication and the authors are grateful to him for his kindness. T. Goto, S. Takahashi, Y. Kishi and Y. Hirata also described the same observations in Tetrahedron Letters, No. 27, 1831 (1964). R.B. Woodward, *et al.* and Y. Hirata, *et al.* proposed the C_{11}-formula, I, for tetrodotoxin in aqueous solution from the observations that tetrodotoxin gave a typical titration curve of a monobasic acid. (see *6 and *10).

This ambiguity menthioned above will required further studies on the determination of the definite molecular size of free tetrodotoxin and tetrodaminotoxin.

Experimental*16

Tetrodonic Acid (IV) from Tetrodotoxin (I)——When 1 g. of tetrodotoxin refluxed with about 300 ml. of H_2O for 15~20 hr., the toxin gradually dissolved, and the solution became clear. Then the solution was filtered and condensed to about 50 ml. at the usual pressure. On cooling the crystalline tetrodonic acid precipitated and the acid was recrystallized from H_2O. The yield was about 600~620 mg. This was a hydrated form of tetrodonic acid, $C_{11}H_{17}O_8N_3$. Tetrodonic acid, $[\alpha]_D^{29}$ +10.1° (2% HCl), is a zwitterionic compound having pKa's 2.9 (COOH) and 11.9 (guanidinium). IR ν_{max}^{Nujol} cm^{-1} : 1690, 1576 (guanidine); 1594, 1416 (COO^-). *Anal.* Calcd. for $C_{11}H_{17}O_8N_3H_2O$: C, 39.17; H, 5.64; N, 12.64; O, 42.73. Found : C, 39.21; H, 5.64; N, 12.13; O, 42.94. When the hydrated form of tetrodonic acid was dried *in vacuo* at about 100° for 20~30 hr., water free tetrodonic acid was obtained. *Anal.* Calcd. for $C_{11}H_{17}O_8N_3$: C, 41.38; H, 5.33; N, 13.17; O, 40.12. Found : C, 41.42; H, 5.38; N, 12.99; O, 40.28. The hydrated form of tetrodonic acid ($C_{11}H_{17}O_8N_3H_2O$) was regenerated from anhydrous tetrodonic acid by treatment with H_2O for 2 or 3 hr. at room temperature.

C_9-Base (II) from Tetrodonic Acid (IV)——120 mg. of IV in about 20 ml. of 5% KOH solution was heated at 90~95° for 2 hr. The solution gradually became yellow and finally it turned dark brown. After cooling, the reaction mixture was neutralized to pH 6 with 5% HCl and extracted with butanol three or four times. The extract was dried over Na_2SO_4 and the solvent was evaporated *in vacuo*. The residue was sublimated *in vacuo* and subsequently recrystallized from H_2O. The product, m.p. 201°, was identified with 2-amino-8-hydroxy-6-quinazolinemethanol by IR absorption and UV absorption spectra. Also a mixed melting point with authentic sample showed no depressions.

2-Amino-6-methylquinazoline (III) from Tetrodonic Acid (IV)——355 mg. of IV was mixed with 180 mg. of powdered phosphorus and 100 ml. of 40% HI, and the mixture refluxed for about 2 hr. After cooling to room temperature, the reaction mixture was diluted with three times of H_2O. After filtration and neutralization with 10% NaOH solution afforded the powder product, which was washed with H_2O and dried. Without further purification, to this powder product suspended on 3 ml. of 30% KOH solution, 5 g. of $K_3Fe(CN)_6$ was added and the mixture was stirred at room temperature for 1 hr. Subsequently the reaction mixture was extracted with $CHCl_3$. The extract was washed with H_2O, dried over Na_2SO_4 and the solvent was evaporated, then the crude product was obtained. It was purified through a column of Al_2O_3. The yellow crystalline product, m.p. 232~233° was obtained and showed a close agreement with 2-amino-6-methylquinazoline in IR and UV spectra. A mixed melting point with authentic sample showed no depressions also. The yield was 52 mg.

Potassium Permanganate Oxidation of Tetrodonic Acid (IV)——To 1 g. of IV in 500 ml. of 0.5% KOH solution, 30 g. of potassium permanganate was added and the mixture was stirred at 50~60° for about 40 hr. The resulted MnO_2 was removed by filtration and the filtrate evaporated to dryness *in vacuo*. The residue was treated with EtOH containing 10% AcOH. An ethanolic solution of picric acid was added to this solution of the residue, then the picrate was precipitated. The IR of this picrate, purified by recrystallization from EtOH, agreed well with authentic guanidine picrate.

Sodium Periodate Oxidation of Tetrodonic Acid (IV)——One mole equivalent of sodium periodate was added to 43.1 mg. of IV in H_2O and the mixture was allowed to stand at 0~5° for 2 or 3 hr. The ethanolic dimedon solution was added to the mixture, then the dimedon derivative of formaldehyde, m.p. 186~187°, was obtained. It was identified with authentic sample by comparison of IR spectra. A mixed melting point showed no depressions also. The yield was 24.0 mg. (81.5% of theoretical). To 304 mg. of tetrodonic acid in 75 ml. of H_2O, 2.5 moles of sodium periodate was added and the mixture was allowed to stand at 0~2° for 3 hr. Then the solution and subsequently H_2O were passed through Ammberlite IR-120 to remove acidic substances. After that, aqueous ammonia was passed through and the eluted solution was evaporated *in vacuo* below 50°. The aqueous solution of the residue was passed through the column filled with a mixture of 1.5 g. of Celite-503 and 1.5 g. of charcoal (Darco, G-60) and the solvent was evaporated *in vacuo* below 50°. Recrystallization of the residual powder from H_2O-MeOH afforded 150 mg. of the prismatic crystals (V). IR ν_{max}^{Nujol} cm^{-1} : 1678, 1595 (br,), 1408, 1059, 1022. *Anal.* Calcd. for $C_{10}H_{15}O_9N_3NH_3$: C, 35.50; H, 5.36; N, 16.56; O, 42.57. Found : C, 35.81; H, 5.47; N, 16.59, 16.42; O, 42.27.

Tetrodonic Acid Hydrochloride (VI) and Hydrobromide (VII)——80 mg. of IV was dissolved in 0.7 ml. of 10% HCl and 4 ml. of MeOH was added to this solution. After filtration, Et_2O was gradually added

*16 All melting points are uncorrected. All pKa' measurements were done using Potentiometer titrator type E 336 (Metrohm) with an automatic recorder. All nuclear magnetic resonance spectra were measured using Varian Spectrometer A-60.

to the filtrate. After allowing to stand for 2 or 3 hr. at room temperature, the hydrochloride precipitated gradually as a beautiful needles. IR ν_{max}^{Nujol} cm^{-1} : 1665, 1576 (guanidinium), 1724 (COOH). *Anal.* Calcd. for $C_{11}H_{17}O_8N_3HCl$: C, 37.13; H, 5.06; N, 11.81; O, 36.01; Cl, 9.98. Found : C, 37.01; H, 5.18; N, 11.86; O, 35.91; Cl, 9.56. Tetrodonic acid hydrobromide was obtained by the same procedures. IR ν_{max}^{Nujol} cm^{-1} : 1665, 1576 (guanidinium), 1724 (COOH). *Anal.* Calcd. for $C_{11}H_{17}O_8N_3HBr$: C, 33.00; H, 4.50; N, 10.50; O, 32.00; Br, 20.00. Found : C, 32.96; H, 4.52; N, 10.60; O, 32.11; Br, 19.86.

6,11-Diacetylanhydrotetrodotoxin (IX)——500 mg. of I was dissolved into 10 ml. of 20% AcOH, and the solvent was evaporated *in vacuo* at 40~50°. The residual acetic acid salt of I which was dried completely under the high vacuum, was dissolved in 30 ml. of pyridine and 20 ml. of Ac_2O was added to this pyridine solution under ice-water cooling. The mixture was allowed to stand for 48 hr. at 0~5° and after pouring into about 1 litre of Petr. ether, the oily precipitate which was separated by decantation, was washed two or three times with hexane. The benzene solution of the oily product was washed with a small amount of H_2O and dried over Na_2SO_4. The solvent was evaporated *in vacuo*, then the residue was dissolved in 30 ml. of MeOH. After treatment with charcoal, it was filtered and allowed to stand for 2 or 3 days at room temperature. Small needles of IX was obtained and the yield was 152~195 mg. IR ν_{max}^{Nujol} cm^{-1} : 1747, 1676, 1604, 1232~1258. *Anal.* Calcd. for $C_{11}H_{13}O_7N_3(CH_3CO)_2$: C, 46.75; H, 4.93; N, 10.91; O, 37.40. Found : C, 46.88; H, 5.31; N, 10.76; O, 37.22.

6,11-Diacetylanhydrotetrodotoxin Hydroiodide (X)——40% HI solution of 100 mg. of IX was diluted with a proper amount of MeOH and filtered. Peroxide free Et_2O was gradually added to this solution, then X was obtained. It was recrystallized from MeOH-Et_2O. *Anal.* Calcd. for $C_{11}H_{13}O_7N_3C_{11}H_{12}O_6N_3$-$(CH_3CO)_4OCH_3 \cdot 2HI$: C, 35.78; H, 4.07; N, 8.08; O, 27.68; I, 24.39. Found : C, 35.12; H, 4.05; N, 7.68; O, 26.25; I, 22.91. When a drop of pyridine was added to the aqueous solution of this hydroiodide, IX was recovered in approximately quantitative yield.

11-Monoformylanhydrotetrodotoxin Formic Acid Salt (XII)——2 g. of tetrodotoxin was dissolved in 70 ml. of 99.8% formic acid and heated at 82~85° for about 2 hr. After evaporation of the solvent *in vacuo*, the residue was dissolved in 50 ml. of MeOH, treated with charcoal and filtered. A small amount of Et_2O was added to the filtrate until the solution became slightly cloudy and allowed to stand at room temperature. After a few hr. XII precipitated. It was recrystallized from a small amount of formic acid and MeOH-Et_2O. The yield was 1.602 g. IR ν_{max}^{Nujol} cm^{-1} : 1720, 1670, 1580, 1149. *Anal.* Calcd. for $C_{11}H_{14}O_7N_3HCOHCOOH$: C, 41.60; H, 4.57; N, 11.20; O, 42.63. Found : C, 41. 63; H, 4. 67; N, 11. 01; O, 42, 63.

11-Monoformylanhydrotetrodotoxin (XI)——To 260 mg. of XII in 8 ml. of H_2O, 40 ml. of MeOH was added, then XI was obtained. The yield was 192 mg. IR ν_{max}^{Nujol} cm^{-1} : 1725, 1680, 1152. *Anal.* Calcd. for $C_{11}H_{14}O_7N_3HCO$: C, 43.77; H, 4.59; N, 12.76; O, 38.88. Found : C, 42.57; H, 5.05; N, 12.54; O, 39.04. When proper amounts of Et_2O was added to 2 ml. methanolic solution of 32 mg. of XI containing a small amount of formic acid, XII was recovered. The yield was 25 mg.

Anhydrotetrodotoxin (VIII) from 11-Monoformylanhydrotetrodotoxin Formic Acid Salt (XII)——300 mg. of XII was dissolved in H_2O and a small amount of dilute base such as aqueous ammonia, aqueous monomethylamine, NaOH, Na_2CO_3 or $NaHCO_3$ was added in this solution. Then the crystalline product immediately precipitated. This precipitate was anhydrotetrodotoxin and there were two forms (A) and (B). The yield was approximately 245 mg. Depending on the conditions of recrystallization or formation, A or B was produced in each case, for instance, when 5~6% aqueous ammonia was added to 2~3% AcOH solution of VIII form (A) was obtained.

VIII-A, IR ν_{max}^{Nujol} cm^{-1} : 1670, 1586, 1105, 1079, 1057, 922. VIII-B, IR ν_{max}^{Nujol} cm^{-1} : 1667, 1598, 1100, 1064, 926. *Anal.* Calcd. for $C_{11}H_{15}O_7N_3$: C, 43.85; H, 5.02; N, 13.95; O, 37.18. Found : Form-A, C, 43.50; H, 5.33; N, 13.74; O, 36.96; Form-B, C, 43.40; H, 5.32; N, 13.51; O, 37.85. Anhydrotetrodotoxin was also obtained by refluxing a methanolic solution of XII in a good yield and from XI by the same procedure.

Anhydrotetrodotoxin (VIII) from 6,11-Diacetylanhydrotetrodotoxin (IX)——120 mg. of IX was suspended in 50 ml. of 0.2% NaOH solution and stirred at room temperature, and the diacetate dissolved little by little into the solutoin. When the solution became clear, it was filtered and allowed to stand at room temperature for a few hours. A beautiful crystalline anhydrotetrodotoxin precipitated gradually and the yield was 46~51 mg.

Acetic Acid Salt of Anhydrotetrodotoxin (VIII)——50 mg. of anhydrotetrodotoxin A or B was dissolved in 3 ml. of glacial AcOH and the solution was allowed to stand for about 2 hr. Then the same anhydrotetrodotoxin acetic acid salt precipitated from either VIII-A or VIII-B. IR ν_{max}^{Nujol} cm^{-1} : 1678, 1585, 1335, 976. *Anal.* Calcd. for $C_{11}H_{15}O_7N_3CH_3COOH$: C, 43.21; H, 5.30; N, 11.63; O, 39.86. Found : C, 43.46; H, 5.35; N, 11.26; O, 40.43.

Formic Acid Salt of Anhydrotetrodotoxin (VIII)——To 3 ml. methanolic solution of 50 mg. of VIII-A or VIII-B containing a small amount of formic acid, the proper amount of Et_2O was added, then the same anhydrotetrodotoxin formic acid salt was obtained from either VIII-A or VIII-B. IR ν_{max}^{Nujol} cm^{-1} : 1673,

1614, 1590, 1337, 1000. *Anal.* Calcd. for $C_{11}H_{15}O_7N_3HCOOH$: C, 41.50; H, 4.93; N, 12.10; O, 41.47. Found : C, 41.69; H, 5.02; N, 12.04; O, 41.70.

6,11-Diacetylanhydrotetrodotoxin (IX) from Anhydrotetrodotoxin (VIII)——From 120 mg. of Ⅷ, the diacetate (Ⅸ) was obtained in the same way as from tetrodotoxin. The yield was 107 mg.

11-Monoformylanhydrotetrodotoxin Formic Acid Salt (XII) from Anhydrotetrodotoxin (VIII)——From Ⅷ, Ⅻ was obtained by the same procedures as for tetrodotoxin.

Methoxytetrodotoxin (XIV) from Tetrodotoxin (I)——200 mg. of tetrodotoxin was dissolved in abs. MeOH containing 16% HCl and the mixture was allowed to stand at room temperature overnight. After evaporation of the solvent *in vacuo*, the residue was dissolved in 15 ml. of H_2O and neutralized with aqueous ammonia under cooling. Subsequently crystalline (XIV) precipitated. The yield was 151 mg. IR ν_{max}^{Nujol} cm^{-1} : 1665, 1610, 1187～1070. *Anal.* Calcd. for $C_{11}H_{16}O_7N_3OCH_3$: C, 43.24; H, 5.75; N, 12.61; O, 38.40. Found : C, 43.36; H, 5.97; N, 12.57; O, 38.09.

Ethoxytetrodotoxin (XV) from Tetrodotoxin (I)——120 mg. of tetrodotoxin and abs. EtOH containing 22.4% HCl also afforded 94 mg. of ethoxytetrodotoxin by the same treatment as in the case of XIV. IR ν_{max}^{Nujol} cm^{-1} : 1676, 1610, 1088. *Anal.* Calcd. for $C_{11}H_{16}O_7N_3OC_2H_5$: C, 40.73; H, 6.57; N, 10.96; O, 41.74. Found : C, 41.15; H, 6.16; N, 11.72; O, 41.57.

Methoxy- (XIV) or Ethoxytetrodotoxin (XV) from Anhydrotetrodotoxin (VIII)——Anhydrotetrodotoxin was converted into XIV or XV by the same procedures as the case of I.

Deoxytetrodotoxin (XVI) from Anhydrotetrodotoxin (VIII) or Tetrodotoxin (I)——320 mg. of Ⅷ was dissolved in 20 ml. of 5% HCl and 1.2 g. of platinum oxide was added to this solution. After catalytic reduction for about 40 hr., the solution was filtered to remove the catalyst and neutralized with dilute aqueous ammonia. Beautifully crystalline deoxytetrodotoxin was obtained and it was recrystallized from ca. 5% HCl and dilute aqueous ammonia. The yield was 275 mg. IR ν_{max}^{Nujol} cm^{-1} : 1659, 1623, 1073. *Anal.* Calcd. for $C_{11}H_{17}O_7N_3$: C, 43.56; H, 5.65; N, 13.86; O, 36.93. Found : C, 43.51; H, 6.01; N, 13.48; O, 36.71. Identical deoxytetrodotoxin (XVI) was obtained from I by the same method.

Tetrodaminotoxin (XIII) from the Diacetate (IX), Anhydrotetrodotoxin (VIII), the Formate (XII) or Methoxytetrodotoxin(XIV)——100 mg. of Ⅸ was suspended in a large excess of above 8% aqueous ammonia and the mixture was stirred at room temperature until specific bands (IR) of anhydrotetrodotoxin disappeared. A total of about 3 or 4 days was required. Then the insoluble, powdery product was separated by suction filtration. Recrystallization of this powdered product using 3% AcOH and dilute base gave crystalline tetrodaminotoxin. The yield was 87 mg. IR spectrum and NMR spectrum of this XIII shows no traces of anhydrotetrodotoxin (Ⅷ). IR ν_{max}^{Nujol} cm^{-1} : 1679, 1623, 1228. *Anal.* Calcd. for $C_{22}H_{33}O_{14}N_7$: C, 42.65; H, 5.33; N, 15.83; O, 36.19. Found : C, 42.41, 42.00, 41.99; H, 5.82, 5.84, 5.56; N, 16.27, 16.08, 16.20; O, 36.15, 35.75, 36.00.

From Ⅷ, Ⅻ or XIV, tetrodaminotoxin was also obtained by the same procedure. It was found that XIII had another dimorphic form-B. IR ν_{max}^{Nujol} cm^{-1} : 1667, 1614, 1228. *Anal.* Calcd. for $C_{22}H_{33}O_{14}N_7$: C, 42.65; H, 5.33; N, 15.83; O, 36.19. Found : C, 41.89; H, 5.81; N, 16.12; O, 35.86.

Anhydrotetrodotoxin (VIII) from Tetrodaminotoxin (XIII)——120 mg. of XIII was dissolved in 10 ml. of 10% HCl and little by little 500 mg. of $NaNO_2$ was added to this solution. After adding all of the reagent, the reaction mixture was allowed to stand for a few minutes and filtered. Neutralization with aqueous ammonia afforded the crystalline anhydrotetrodotoxin. The yield was 73 mg. Tetrodotoxin does not react under the same conditions.

Tetrodotoxin (I) from Tetrodaminotoxin (XIII)——85 mg. of XIII was dissolved in 10 ml. of ca. 10% HCl and the solution was heated at 90～95° on the steam bath for about 2 hr. After cooling to room temperature, the solution was allowed to stand overnight, then neutralized with aqueous ammonia. I was recovered. The yield was 11 mg.

6,11-Diacetylanhydrotetrodotoxin (IX) from Tetrodaminotoxin (XIII)——The diacetate (Ⅸ) was obtained from 145 mg. of XIII in the same manner as for I. The yield was 12 mg.

Refluxing Deoxytetrodotoxin (XVI) with Water——An aqueous solution 100 mg. of XVI was refluxed for several hours and the starting material was recovered unchanged.

Tetrodonic Acid (IV) from Anhydrotetrodotoxin (VIII), the Diacetate (IX), the Formate (XI), (XII), Tetrodaminotoxin (XIII) or Methoxytetrodotoxin (XIV)——When Ⅷ, Ⅸ, Ⅺ, Ⅻ, XIII or XIV was refluxed with H_2O for a suitable length of time, tetrodonic acid (Ⅳ) was obtained in the same manner as in the case of tetrodotoxin.

C_9-Base (II) from Some Tetrodotoxin Derivatives——From anhydrotetrodotoxin (Ⅷ), the diacetate (Ⅸ), the formate (Ⅺ), (Ⅻ), tetrodaminotoxin (XIII) or methoxytetrodotoxin (XIV), C_9-base (Ⅱ) (2-amino-8-hydroxy-6-quinazolinemethanol) was obtained by the same procedure as for tetrodotoxin.

Tetrodotoxin (I) from Anhydrotetrodotoxin (VIII), the Diacetate (IX), its Hydroiodide (X), the Formate (XI), (XII) or Methoxytetrodotoxin (XIV)——After a solution of 120 mg. of Ⅷ in 10 ml. of 5% HCl stood overnight, the solution was neutralized with dilute aqueous ammonia, and tetrodotoxin was recovered. The yield was 94 mg. Compounds (Ⅸ), (X), (Ⅺ), (Ⅻ) and (XIV) were also converted into tetrodotoxin by the same procedure. *Anal.* Calcd. for $(C_{11}H_{17}O_8N_3)_{n=1 \text{ or } 2}$: C, 41.38; H, 5.33; N, 13.17; O, 40.12.

Found : C, 41.14, 41.16, 40.88; H, 5.46, 5.52, 5.57; N, 13.16, 13.02, 13.23; O, 39.85, 39.59. When 5% DCl in D_2O was used instead of 5% HCl, anhydrotetrodotoxin (Ⅷ) was also converted into tetrodotoxin which showed the same NMR spectrum as ordinary tetrodotoxin in CD_3COOD-D_2O solution.

Tetrodonic Acid (IV) with Deuterium at C_9-Position from Tetrodotoxin (I) or Anhydrotetrodotoxin (VIII)——Refluxing 300 mg. of I with 50 ml. of D_2O, afforded 182 mg. of Ⅳ which was lacked the signal corresponding to the C_9-proton in the NMR spectrum. Ⅷ also gave Ⅳ deuterated at C_9-position by the same treatment. On the contrary, tetrodonic acid prepared from tetrodotoxin by usual method, was not deuterated at C_9-position on further boiling for 3 hr. with D_2O.

Anhydrotetrodoic Acid from Tetrodotoxin (I) by Action of Barium Deuteroxide in Deuterium Oxide——500 mg. of I was suspended on 60 ml. of 5% $Ba(OD)_2$ in D_2O and the mixture was stirred at room temperature. When the solution became approximately clear, carbon dioxide was passed through the solution, and the resulting precipitate was removed by filtration. The filtrate was concentrated to about 10 ml. and a suitable amount of tetrahydrofuran was added to the solution. Then 245 mg. of anhydrotetrodoic acid was obtained and in the NMR spectrum the signal corresponding to the C_9-proton was observed at 5.49 p.p.m. from $(CH_3)_4Si$.

Periodate Oxidation of Tetrodotoxin (I) and Anhydrotetrodotoxin (VIII)——To several 5 ml. buffer solutions (CH_3COOH and CH_3COONa, pH 4～4.5) which contained from 17 mg. to 25 mg. of tetrodotoxin (I) or anhydrotetrodotoxin (Ⅷ), exactly 15 ml. of sodium periodate solutions which contained 3.803 mg. of sodium periodate per each 1 ml. solution, were added respectively under cooling with ice-water. After standing at 2～4° for some hours the quantity of consumed sodium periodate in each sample was measured iodimetrically. That is, 5 ml. of 0.1*N* sodium arsenite was added to the solution and after standing for about 15 min., two drops of 20% KI and starch were also added. Then the mixture was titrated with 0.01*N* iodine reagent. Blank tests were also made and the results were as follows.

hr.	moles of consumed sodium periodate		hr.	moles of consumed sodium periodate	
	tetrodotoxin	anhydrotetrodotoxin		tetrodotoxin	anhydrotetrodotoxin
2	2.042	1.17	10	—	1.44
4	2.86	1.18	20	3.39	1.71
5	3.01	—	45	—	1.94
6	2.98	1.38	73	—	2.10
8	2.99	1.39			

After titration, 2 ml. of buffer solution (CH_3COOH and CH_3COONa, pH 4～4.5) and 2 ml. of 3% dimedon in EtOH were added to each of the first solutions respectively, which had stood for 2 hr. at 2～4° after adding sodium periodate to the buffer solutions of tetrodotoxin and anhydrotetrodotoxin, and the mixture were allowed to stand overnight. The crystalline dimedon derivatives of formaldehyde, m.p. 186～187°, which were confirmed through comparison with authentic sample in their IR spectra and mixed melting point, were obtained from both tetrodotoxin (I) and anhydrotetrodotoxin (Ⅷ). The blank test was negative.

The authors are grateful to M. Matsui, Managing Director of Research Laboratories, Sankyo Co. Ltd., for his encouragement throughout this work, to S. Suzuki and K. Fujita of Manufacturing Section 1 of Shinagawa Plant, Sankyo Co. Ltd., for extraction of tetrodotoxin, to Dr. Y. Sasada, Institute for Protein Research, Osaka University, for helpful advice on X-ray crystallographic analysis, to Dr. K. Takeda, Shionogi Research Laboratories for NMR spectral measurements and to Prof. S. Takahashi of Tokyo University and Dr. O. Kurihara, The Institute of Physical and Chemical Research, for N^{15}-measurements by mass spectrometry. The authors also wish to express their deep gratitude to Prof. V. Prelog, E.T.H. (Zurich) and other members of our Laboratories for their discussion and to co-workers for physical and analytical measurements.

Summary

The structures of both tetrodonic acid hydrobromide and 6,11-diacetylanhydrotetrodotoxin hydroiodide were established by chemical and X-ray crystallographical research. Based upon structures of both these salts and upon other chemical information, we determined that tetrodotoxin and anhydrotetrodotoxin have zwitterionic hemilactal structures.

(Received August 8, 1964)