Tetrahedron Letters No.14, pp. 779-786, 1964. Pergamon Press Ltd.
Printed in Great Britain.

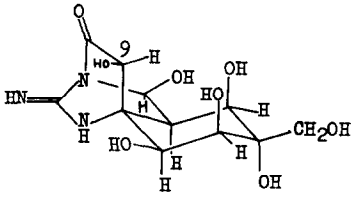
FURTHER STUDIES ON THE STRUCTURE OF TETRODOTOXIN

Toshio Goto, Yoshito Kishi, Sho Takahashi
and Yoshimasa Hirata

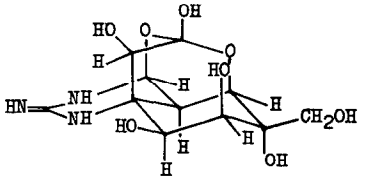
Chemical Institute, Faculty of Science
Nagoya University, Nagoya, Japan

(Received 20 February 1964)

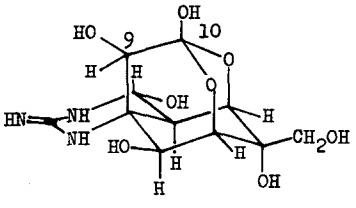
IN the previous communication (1), based on the X-ray crystallography (2) of bromoanhydrotetrodoic lactone (III) and on some chemical reactions, four possible formulas, Ia - Id, were deduced for tetrodotoxin. Among them, Ib and Id were excluded by the evidence obtained from the NMR and IR spectral analysis. The ortho-ester formula Ic was then eliminated since it did not seem to be able to account for the pKa value (8.76) of the toxin. However, the further evidence given in this communication makes Ic considered again.



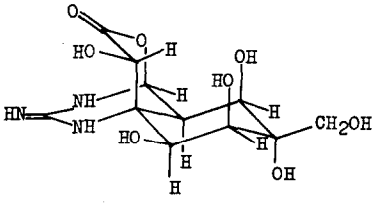
(Ia)



(Ib)

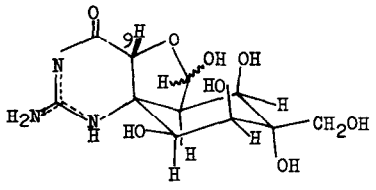


(Ic)

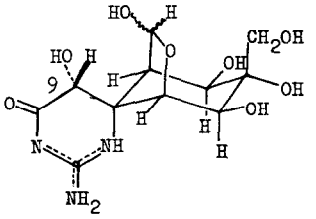


(Id)

Tsuda et al. (3) established the structure of "tetrodonsäure"* (IV) by the X-ray analysis, and also suggested, for possible structures of tetrodotoxin, the following three formulas, Ie - Ig; Ie being the C_9-epimer of Ia. They further suggested a possibility of a dimeric form without giving a definite formula.



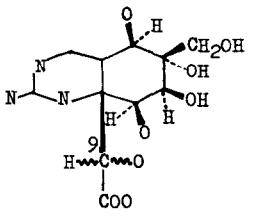
(If)



(Ig)

Configuration of C_9-position

The structures of the bromo lactone III and tetrodoic acid (IV) have the same framework and configurations shown in formula A, except the configuration at C_9. This means that the configuration must have been inverted during either reaction A, which afforded anhydrotetrodoic acid (II), or reaction B, by which tetrodoic acid (IV) was formed. That it was indeed inverted during the hydrolysis of the toxin to the acid IV (reaction B) is shown by the following experiment.

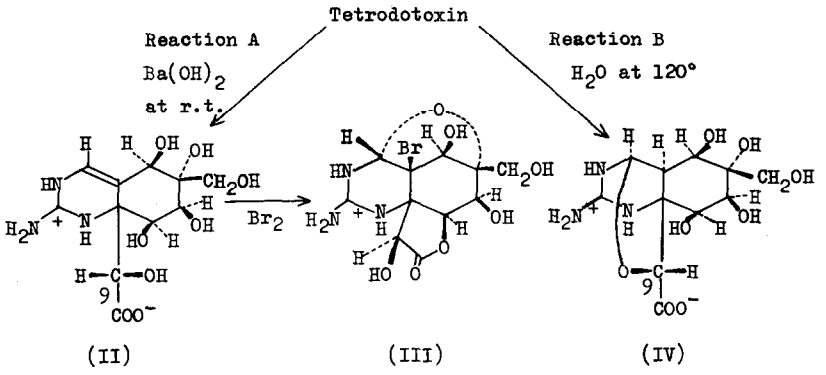


(A)

Both acids III and IV were prepared from the toxin in deuterium oxide and their NMR spectra were measured. Whereas the spectrum of the anhydro acid III was identical in all respects with that of the acid III obtained in water instead of deuterium oxide, in the spectrum (4) of the acid IV a signal at the lowest field, which can be assigned to the proton at C_9**, was completely disappeared. This suggests the

* We named it as tetrodoic acid (4).
** Assignments in ref. (4) must be changed to: 0.95 ppm = H_9; 1.03 = H_4.

possibility that the reaction B involves epimerization at C_9 through an enol intermediate.



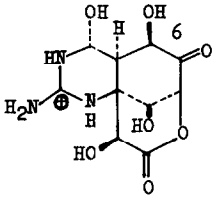
Dimeric form of tetrodotoxin

The possibility that tetrodotoxin exists in a dimeric form (C_{22}) in solution is excluded by the following evidence. Although a titration curve obtained from the toxin purified by the precipitation method (5) was somewhat irregular, purified tetrodotoxin through its picrate (5) gave a typical titration curve for a monobasic acid or a monoacidic base. Thus, the pKa values obtained from several points in the titration curve fell within a range of 8.76 ± 0.01. Theoretically, two pKa values of a dibasic acid or a diacidic base must differ from each other at least ΔpKa = 0.6 unit (6).

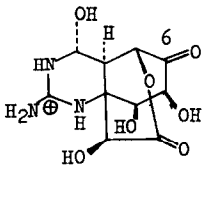
Evidence suggesting the ortho-ester structure Ic

When treated with one mole of periodic acid in 0.1 N sulfuric acid at 0°, the toxin gave, beside formaldehyde, amorphous nortetrodotoxin sulfate (V) in good yield, which exhibits positive Sakaguchi and tetrazolium tests. That V does not contain the starting material was shown by injection of V to mice; no toxicity was observed. Incidentally, tetrodotoxin is stable in 0.1 N sulfuric acid at room temperature. It is evident from a band at 1745 (KBr) and at 1750 cm^{-1} (in dimethylsulfoxide) in its IR spectra that the nor compound V has a carbonyl group. It consumed 1 mole of sodium hydroxide at pH 8 - 9, and the product showed a carboxylate band at 1600 cm^{-1} and no band

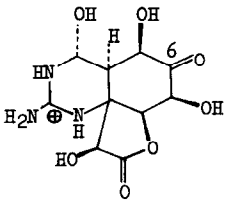
between 1700-1800 cm^{-1}. Back titration gave a pKa 3.35, corresponding to a free carboxyl group. This suggests the presence of a lactone group. Three possible formulas, Va - Vc, can therefore be written for the nor compound V.



(Va)



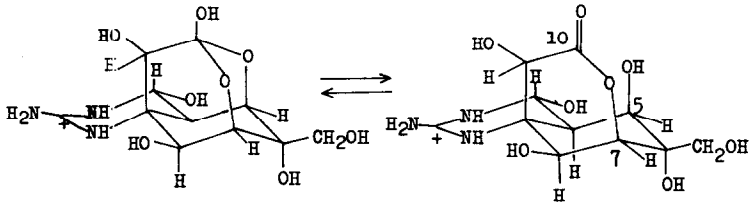
(Vb)



(Vc)

The carbonyl streching band of the γ-lactone group of the bromo lactone III, which corresponds to the lactone of formula Vc, appears at 1800 (KBr) and 1795 (in dimethylsulfoxide). An amorphous product, obtained from the acid IV by refluxing it with 3 N hydrochloric acid, also shows a γ-lactone band at 1800 cm^{-1} (KBr). Thus, the carbonyl band (1745 cm^{-1}) of the nor lactone V can be assigned to the δ -lactone group in Va or Vb. Since the nor carboxylate obtained from V exhibits no ketonic carbonyl band in its IR spectrum, the carbonyl group at the 6-position probably exists in a hydrate form.

Whereas tetrodotoxin itself shows no carbonyl band between 1700-1800 cm^{-1} (KBr), its salts exhibit a weak band at 1747-1750 cm^{-1} in dimethylsulfoxide solution (sulfate, hydrobromide, and picrate) and at 1730-1745 in a KBr disc (amorphous sulfate and hydrobromide). These phenomena may be explained in terms of tautomerization between the ortho-ester Ic and the lactone Ih or Ii; Ii being the (10 → 5)lac-



(Ic) (Ih)

tone isomeric with Ih. Tetrodotoxin and its crystalline hydrobromide, $C_{11}H_{17}O_8N_3 \cdot HBr$*, show no carbonyl absorption (KBr) suggesting that they exist completely in the ortho-ester form Ic.

The pKa value of tetrodotoxin in water is 8.76 and in 50% aqueous ethanol it is 9.4. This solvent effect indicates, contrary to the previous assignment (4), that the toxin is a monobasic acid rather than a monoacidic base (7); the C_{10}-hydroxyl group of the ortho-ester structure Ic would account for the acidic property. The similar solvent effect is also observed with anhydroepi- (XI), diacetylanhydroepi- (X), and aminodesoxy-tetrodotoxin (XII) (vide infra). β-alacreatinine has pKa 7.05 both in water and in 50% ethanol.

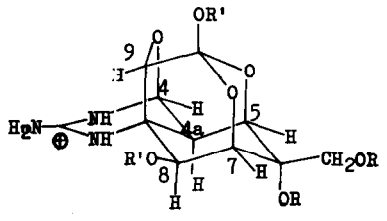
Treatment of tetrodotoxin with acetic anhydride and p-toluenesulfonic acid afforded amorphous tetraacetylanhydroepitetrodotoxin p-toluenesulfonate (VIII)*, $C_{11}H_{11}O_7N_3(COCH_3)_4 \cdot C_7H_8O_3S \cdot H_2O$, m.p. 162-164°, which on further acetylation with acetic anhydride and pyridine gave crystalline pentaacetylanhydroepitetrodotoxin p-toluenesulfonate (IX)*, $C_{11}H_{10}O_7N_3(COCH_3)_5 \cdot C_7H_8O_3S$, m.p. 208-210°; pKa' 5.1 (H_2O), 4.9 (50% EtOH) (5). The tetraacetate VIII and the pentaacetate IX were hydrolyzed with a limited amount of aqueous ammonia or trimethylamine to diacetylanhydroepitetrodotoxin (X), $C_{11}H_{13}O_7N_3(COCH_3)_2 \cdot H_2O$**; pKa' 7.7 ($H_2O$), 8.2 (50% EtOH); and with an excess aqueous ammonia to anhydroepitetrodotoxin (XI), $C_{11}H_{15}O_7N_3$**; pKa of this compound will be discussed later. On treatment with a large excess of ammonia, the four compounds, VIII - XI, were converted to aminodesoxytetrodotoxin (XII), $C_{11}H_{18}O_7N_4 \cdot 1/2H_2O$**; pKa' 3.95 and 8.58 ($H_2O$), 3.86 and 9.30 (50% EtOH).

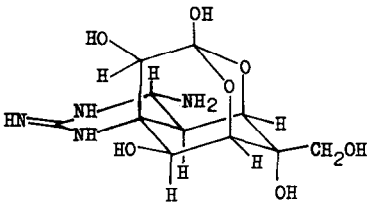
In 1 N sulfuric acid anhydroepitetrodotoxin (XI) is slowly epimerized to tetrodotoxin (I). Thus, in the acidic solution, both the toxin I and anhydroepi compound XI resulted an aquilibrium mixture containing I and XI in ca. 4:1 ratio (NMR). "Tetrodotoxin" purified by the precipitation method (5) contains the epimer XI, which can be removed through its picrate (5).

---

* The compounds VIII and IX were reported as tetraacetyltetrodotoxin p-toluenesulfonate and pentaacetylanhydrotetrodotoxin p-toluenesulfonate, respectively, in ref. 5.

** Satisfactory analyses were obtained.





(XII)

(VIII) R=R'=Ac

(IX) R=R'=Ac
(An additional Ac on guanidine)

(X) R=Ac, R'=H

(XI) R=R'=H

The following observations are reasonably explained by the structures VIII - XII derived from the ortho-ester Ic.

TABLE 1. NMR spectra at 60 Mc

	Pentaacetate (IX)*	Diacetate (X)**	Anhydroepi (XI)**	Aminodesoxy (XII)**
H_4	5.66	0.95	0.93	1.07
H_{4a}	2.97	3.53	3.52	3.72
H_5	5.10	~1.57	2.32	
H_7	4.83	2.00	2.13	
H_8	5.58	1.72	1.83	
H_9	5.0	1.87	1.90	2.52
$-CH_2O-$	5.03	1.57	2.52	2.47
$-COCH_3$	2.10 (9H)	4.38 (3H)		
	2.17 (3H)	4.45 (3H)		
	2.27 (3H)			
$J_{4,4a}$	~0	~0	~0	11
$J_{4a,5}$	3	3	3	< 2
$J_{7,8}$	2.2	2.2	2-3	

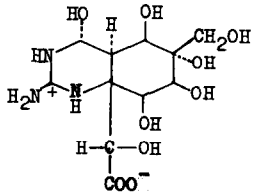
* $CDCl_3$ solution; ppm from internal tetramethylsilane

** D_2O (H_2SO_4) solution; ppm from external benzene

The configuration at C_4 can be deduced from $J_{4,4a}$. That $\Delta\delta$(H_4 - $-CH_2O-$) of the pentaacetate IX is nearly identical with that of the diacetate X indicates the absence of an acetoxyl group at C_4 of the pentaacetate IX. It is also deduced from the chemical shifts that one of the hydroxyl groups at C_7 and C_8 of IX is acetylated.

In the tetraacetate VIII, one of the acetoxyl groups (attributable to the C_{10}-acetoxyl group) was hydrolyzed very easily at pH 6.5-7.5, and the product (triacetate, not isolated) was shown to have a pKa' 7.8. Back titration of the solution gave a pKa' 4.74, corresponding to one mole of acetic acid. The pentaacetate IX has a pKa' 5.1 in water and 4.9 in 50% ethanol, indicating the presence of an acetyl group on the guanidine moiety.

Anhydroepitetrodotoxin (XI) is stable in dilute alkali and exhibits pKa's 7.95 and 11.5. This result clearly indicates that XI is a zwitterionic compound; the lower and the higher pKa's are corresponding to the C_{10}-hydroxyl and the guanidine group, respectively. Tetrodotoxin, however, decomposes with alkali. When an excess sodium hydroxide solution was added into an acidic solution of the toxin, a clear solution was obtained. The solution has no toxicity against mice and exhibits two pKa's 2.3 and ca. 10.8*, and no inflection in the titration curve was observed at pH ~8.7. Neutralization of the alkaline solution to pH 9 causes gradual precipitation of the toxin, suggesting a reversible formation of an acid VII from the toxin. When the alkaline solution was allowed to stand at room temperature, the intensity of its UV absorption at 293 mμ gradually increased owing to the formation of anhydrotetrodoic acid (II). The difference between the stability of the toxin and anhydroepi compound XI towards alkali may be attributable to the ether linkage between C_4 and C_9 in XI.



(VII)

The authors are indebted to Professors I. Nitta and Y. Tomiie, Kwansei-gakuin University, for helpful discussions during the course

* The pKa's were obtained by titration with 0.5 N HCl of a solution of 4 mg. of tetrodotoxin in 1 ml. of 0.02 N HCl, immediately after the addition of 2 ml. of 0.1 N KOH. All pKa measurements were done using Radiometer titrator type TTTlc with an automatic recorder.

of this investigation. Thanks are due to the Fujisawa Pharmaceutical Co., Ltd. for collecting ovaries of swellfish and to the Public Health Service, National Institutes of Health, U. S. A., for support of this work under Research Grant RG-7969.

REFERENCES

1. T. Goto, S. Takahashi, Y. Kishi, Y. Hirata, Y. Tomiie and I. Nitta, Tetrahedron Letters 2115 (1963).
2. Y. Tomiie, A. Furusaki, K. Kasami, N. Yasuoka, K. Miyake, M. Haisa and I. Nitta, Tetrahedron Letters 2101 (1963).
3. K. Tsuda, C. Tamura, R. Tachikawa, K. Sakai, O. Amakasu, M. Kawamura and S. Ikuma, Chem. Pharm. Bull. (Japan) 11, 1473 (1963).
4. T. Goto, Y. Kishi, S. Takahashi and Y. Hirata, Tetrahedron Letters 2105 (1963); addition to reference (6b) in this paper: K. Tsuda, S. Ikuma, M. Kawamura, R. Tachikawa, I.Baba, and T. Miyadera, Chem. Pharm. Bull. (Japan) 10, 247 (1962). The structure of the C_9-base was first established by Tsuda et al. by synthesis (6b). Later, we reported the same structure which was deduced by physical methods (6a).
5. T. Goto, S. Takahashi, Y. Kishi and Y. Hirata, Bull. Chem. Soc. Japan 37, No. 2 (1964).
6. H. C. Brown, D. H. McDaniel and O. Häfliger, Dissociation Constants in E. A. Braude and F. C. Nachod, Determination of Organic Structures by Physical Methods p. 623. Academic Press Inc., New York (1955).
7. Reference 6, p. 617.