11-NORTETRODOTOXIN-6,6-DIOL and 11-NORTETRODOTOXIN-6-OL

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Abstract — Tetrodotoxin (1) is oxidized to 11-nortetrodotoxin-6,6-diol (2b) which is reduced to 11-nortetrodotoxin-6-ol (3); the gem-diol 2b has 20-40% and the reduction product 3 about 10% of the in vivo neurotoxicity of tetrodotoxin. The formation of the O-methyloxime of 2 has been reinvestigated.

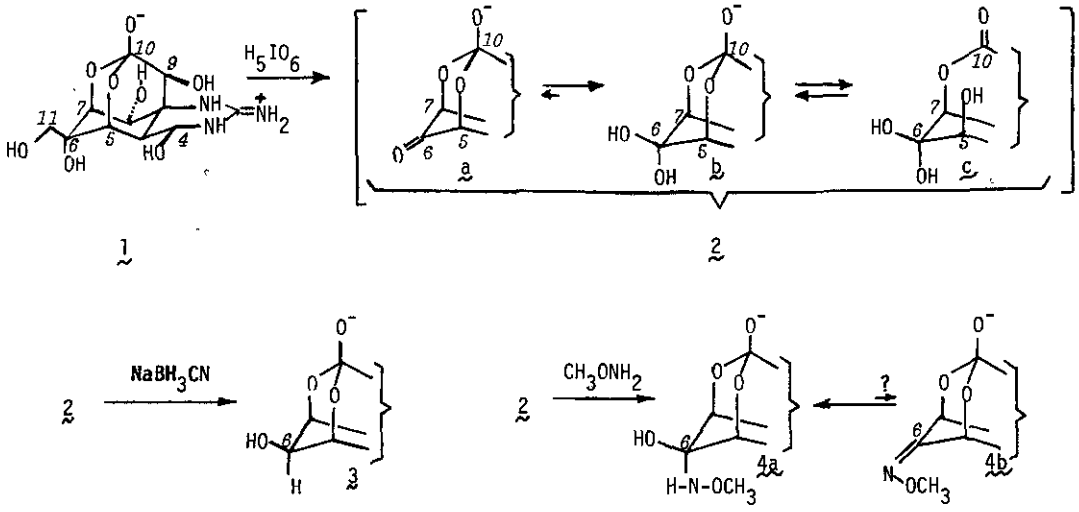
Dedication — This paper is dedicated to Professor Kyosuke Tsuda for his pioneering research on tetrodotoxin and his generous cooperation with us on our early studies on tarichatoxin-tetrodotoxin.

Structural modifications of tetrodotoxin (1) to give products which retain the sodium channel blocking activity of the parent compound hold the potential of being of great value in neurophysiological research.[3-5] However, earlier studies[6-8] indicated that no tetrodotoxin derivative showed activity approaching that of tetrodotoxin itself. The discovery of chiriquitoxin,[9-11] which is chemically and pharmacologically related to tetrodotoxin, brought the realization that tetrodotoxin could be structurally modified at positions 6 and 11 and still retain its unique channel blocking ability. The earliest such modification was the periodate oxidation of tetrodotoxin (1) reported by Tsuda et al.[6] and Goto et al.[12-13] to give the 6-keto derivative (2) named "nortetrodotoxin." These experiments gave formaldehyde, thus establishing the presence of a terminal 1,2-diol at positions 6-11 in tetrodotoxin, but the toxicity of the nortetrodotoxin (2), was either not reported,[6] or reported to show no toxicity.[12] We have undertaken a reinvestigation of this reaction.

Our preparations of nortetrodotoxin showed it to have about 20-40% of the toxicity (intraperitoneally, mouse) of tetrodotoxin itself. While our studies were in progress, Tsien et al.[14] reported the preparation of nortetrodotoxin and its effect on the action potentials of desheathed frog sciatic nerves and on the sodium currents of giant axons from Myxicola. They reported that potency was "several hundred fold less than tetrodotoxin." The nortetrodotoxin solution was treated with O-methylhydroxylamine with the expectation of forming an O-methyloxime (4b). They

reported that the solution was transiently active in blocking action potentials of neurons. Since we have found that nortetrodotoxin does block conduction in the frog sciatic nerve, at six times the concentration of tetrodotoxin in variance with these reports, it is difficult to interpret the results of Tsien et al.

Lazdunski and coworkers[5,15-17] have also shown that nortetrodotoxin blocks sodium channels. They have used tritium and radioactive carbon tracer techniques to demonstrate the preparation (in 5% to 10% yields) of two photoactive nitroazidophenyl derivatives of nortetrodotoxin both of which showed reversible blocking of sodium channels of crab nerves in the dark and inreversible blocking after irradiation. There is also a report of the preparation of fluorescent labels for membrane sodium channel studies based on nortetrodotoxin.[19] In all of these studies "nortetrodotoxin" and the reported derivatives have been minimally characterized due to several factors: the severely limited amounts of tetrodotoxin available, the instability of nortetrodotoxin at pHs above 8 and below 5, its virtual insolubility in organic solvents and its existence in the diol (2b⇌2c) rather than keto form (2a).



The periodate oxidations of 1 to 2 were studied on a 1-mg scale in an NMR tube. The reaction could be followed by watching the disappearance of the sharp C-11 methyl signal (δ 4.1 ppm) of tetrodotoxin, the appearance of the methylene signal for formaldehyde ($CH_2(OH)_2$, δ 4.9 ppm) and the upfield shift of the C_{4a} proton signal from 2.3 to 2.2 ppm during the conversion of 1 to 2. The absence of tetrodotoxin in the product was insured by using a slight excess of periodate and was confirmed by NMR spectra. The final preparation was on a 12-mg scale.

Both proton (^1H) and carbon-13 (^{13}C) NMR signals are recorded in Table I. To our limit of detection, this product seemed to be present as the hydrate, 11-nortetrodotoxin-6,6-diol (2b⇌2c) since neither ^{13}C nor distinct IR signals for a ketone carbonyl were observed. Vacuum drying of a

TABLE I

NMR SPECTRA FOR NORTETRODOTOXIN[1]

Proton Assignment	Chemical Shift, δ, ppm[2] Proton D_2O	Proton D_2O-CD_3COOD	Carbon-13 D_2O	Proton Assignment
C_{4a}	2.14[3]	2.19[3]	43.7	C_{4a}
	2.24[3]	2.23[3]	45.9	
	3.91	3.91	54.1	
		3.93	59.1	C_{8a}
C_5	4.17	4.16	67.8	
C_7	4.21		69.0	
C_8	4.27	4.25	70.8	C_4
C_9		4.36	71.9	C_5
		4.55	72.2	C_6
		4.80	73.4	C_7
C_4	5.46[3]	5.46[3]	74.3	C_8
	5.48[3]	5.48[3]	75.7	C_9
			81.8	
			84.2	
			97.7	
			153.7	C_2
			155.0	
			109.8[4]	C_{10}
			174.1[4]	

1. The signals can be accounted for by assuming an equilibrium mixture lactone, 2c ⇌ hemilactal 2b, in solution.
2. Proton shifts, δ, relative to CHD_2COOD set at 2.03 ppm relative to TMS = 0; reference CHD_2COOD added to pure D_2O after spectrum taken. The ^{13}C shifts, δ, relative to DMSO set at 40.9 ppm with respect to TMS = 0.
3. Doublet with coupling of 9Hz. We assume that the downfield doublet for C_{4a} at 2.24 ppm corresponds to the lactone form 2c.
4. The signal at 109.8 ppm is assigned to C_{10} of the hemilactal form (2b) and that at 174.1 ppm to C_{10} of the lactone form (2c).

sample in a pressed KBr window failed to develop ketone absorption in the IR. The hydrated ketone at C-6 can be explained by the presence of adjacent electron-withdrawing oxygen groups and relief of ring strain in going from sp^2 (2a) to sp^3 (2b) carbon. The ^{13}C signal at 97.7 ppm corresponds to that of the hydrated carbonyl at 99.0 ppm in saxitoxin[19] and 97.5 ppm in gonyautoxin II. It is also clear from both ^1H and ^{13}C NMR spectra (Table I) that nortetrodotoxin exists as a mixture of at least two forms in solution. We believe these to be 2c (^{13}C lactone carbonyl at 174.1 ppm) and 2b (^{13}C hemilactal carbon at 109.0 ppm). Inspections of molecular models indicate that the C-7 lactone (2c) should be more stable than the C-5 lactone. The x-ray structure of crystalline 4-O-methyl—6,11-0,0-isopropylidinetetrodotoxin hydrochloride, Gougoutas hydrochloride,[20] showed the C-7 lactone structure; in D_2O solution this compound existed as a hemilactal-lactone mixture. These conclusions correspond to those reached by Goto et al.[12,13] The ^1H NMR spectra of nortetrodotoxin changes in going from pure D_2O to D_2O-5% CD_3COOD, as shown in Table I. From these spectra we deduce that the hemilactal form (2b) predominates in pure D_2O and the lactone form 2c in dilute acid. We have been unable to obtain crystals of nortetrodotoxin; this may be due in part to the mixture of forms in solution.

Tetrodotoxin 12.8 mg (40.1 μmole, citrate free, Sankyo Co., Japan) suspended in 1.00 mL of water was dissolved by the addition of 50 μL of acetic acid and treated with 9.42 mg of H_5IO_6 (41.2 μmole) dissolved in 38 μL of water at 5°. After 12 hours at 5° a total of 27.8 μL of freshly distilled 57% HI was added in small portions at 5°. Each addition was immediately followed by CCl_4 extraction to remove I_2. The clear aqueous layer was frozen and lyophilized, redissolved and relyophilized to give 11.4 mg (93% yield) of a white solid which showed no residual tetrodotoxin by ^1H NMR (100.0% D_2O + 3% CD_3COOD). We were unable to obtain crystals of this product.

Toxicity by intraperitoneal (i.p.) injection in single female Swiss-Webster white mice varied from sample to sample, perhaps depending upon the pH of the solution and the time after preparation. Values ranged from 2,000 to 4,000 mouse units[21] per mg (MU/mg), approximately 1/4 to 1/2 as active as tetrodotoxin. A conventional toxicity study with groups of mice on one sample indicated an LD_{50} of 80 μg/Kg which is about 1/8th that of tetrodotoxin. The compound action potentials in desheathed sciatic nerves from Rana catesbiana were blocked in a manner similar to that of tetrodotoxin by concentrations about six times greater. Details of pharmacological studies on frog muscle and squid giant axions will be published elsewhere by C. Y. Kao.[22]

Reduction of nortetrodotoxin (2) with sodium cyanoborohydride in dilute acetic acid gave an amorphous 11-nortetrodotoxin-6-ol to which we assign the equatorial alcohol structure 3 based on NMR spectra (lack of observable couplings attributable to protons at C_5, C_6 and C_7). This substance had an in vivo toxicity (i.p. in Swiss-Webster white mice) of about 90% of 2 from which it comes.

Nortetrodotoxin (2.0 mg) was treated with a large excess of distilled methoxyamine (O-methylhydroxylamine, CH_3ONH_2, 1 μL, b.p. 49-50°) in D_2O (350 μL) and CD_3COOD (10 μL) at 5° for 48 hours. Its ^1H NMR differed from that of the starting material only in the presence of an O-methyl signal at 3.94 ppm., which corresponded to 60-70% of that expected for a 1:1 adduct, and the methyl signal of unreacted CH_3ONH_2 at 3.84 ppm. The reaction mixture was rapidly lyophilized to remove free CH_3ONH_2, and the residual white powder redissolved in D_2O-CD_3COOD. No additional C=N absorption appeared in the IR spectrum of this reaction product. It had a toxicity of 2300 MU/mg.[21] The 3.84 ppm signal was gone from the initially lyophylized product, but slowly reappeared on standing at the expense of the 3.94 ppm signal. Double lyophilization of this adduct resulted in the loss of the 3.94 ppm signal and a product whose NMR was indistinguishable from that of 2. We conclude that a toxic adduct, 4a, is formed in equilibrium with nortetrodotoxin (2), but that this adduct does not lose water to give the oxime, 4b.

We were unable to obtain crystalline derivatives such as a picrate, picrolonate, hydrazone, 2,4-dinitrophenylhydrazone or hydrochloride. Other attempted derivatizations of nortetrodotoxin on a 1-mg scale were unsuccessful. These included reaction with methyl glycinate followed by treatment with $NaCNBH_3$, cyanohydrin formation, phenylhydrazone formation and ketal formation (CH_3OH, $CH_3C(OCH_3)_2CH_3$, CF_3COOD).

SUMMARY

The demonstrated pharmacological activity of 11-nortetrodotoxin-6,6-diol (2b) and 11-nortetrodotoxin-6-ol (3) substantiate the results of Lazdunsky showing that tetrodotoxin can be modified in the 6-position to give products which retain the activity of the parent compound. Although derivatives can be made starting from nortetrodotoxin, it has proven to be a poor intermediate for elaborating further modified structures in reasonable yields, presumably because the 6,6-diol is much more stable than the 6-keto form. We are continuing other avenues for the preparation of such derivatives which may be more amenable to the desired chemical modifications.

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REFERENCES

1. Taken in part from the Ph.D. thesis of L. A. P., Stanford University, 1980.
2. H. S. Mosher, F. A. Fuhrman, H. D. Buchwald and H. G. Fisher, Science, 1964, 144, 1100.
3. F. A. Fuhrman, Scientific American, 1967, 217, 61.
4. M. H. Evans, Int. Rev. Neurobiol., 1972, 15, 83.
5. R. Chicheportiche, M. Balerna, A. Lombet, C. Romey, and M. Lazdunski, J. Biol. Chem., 1979, 254, 1552.
6. K. Tsuda, S. Ikuma, M. Kawamura, R. Tachikawa, K. Sakai, C. Tamura, and O. Amakasu, Chem. Pharm. Bull. (Tokyo), 1964, 12, 1357.
7. T. Deguchi, Jap. J. Pharmacol., 1967, 17, 267.
8. T. Narahashi, J. W. Moore, and R. N. Poston, Science, 1967, 156, 976. Enough tetrodotoxin was found by NMR in the most active derivative to account for its toxicity.
9. Y. H. Kim, G. B. Brown, H. S. Mosher, and F. A. Fuhrman, Science, 1975, 189, 151.
10. L. A. Pavelka, Y. H. Kim, and H. S. Mosher, Toxicon., 1977, 15, 135.
11. C. Y. Kao, P. N. Yeo, M. D. Goldfinger, F. A. Fuhrman, and H. S. Mosher, J. Pharmacol. Exp. Therap., 1981, 217, 416.
12. T. Goto, Y. Kishi, S. Takahashi, and Y. Hirata, Tetrahedron Lett., 1964, 779.
13. T. Goto, Y. Kishi, S. Takahashi, and Y. Hirata, Tetrahedron, 1965, 21, 2059.
14. R. Y. Tsien, D. P. L. Green, S. R. Levinson, B. Rudy, and J. K. M. Sanders, Proc Roy. Soc. Ser. B., 1975, 191, 555.
15. R. Chicheportiche, M. Balerna, A. Lombet, G. Romey, and M. Lazdunski, Eur. J. Biochem., 1980 104, 617; J. Biol. Chem., 1979, 254, 1552.
16. A. Lombet, J. F. Renaud, R. Chicheportiche, and M. Lazdunski, Biochemistry, 1981, 20, 1279.
17. M. Lazdunski et al., in "Neurotransmitters and Their Receptors" eds. V. Z. Littauer, Y. Dudoi, I. Silman, V.I. Teichberg and Z. Vogel, John Wiley and Sons, N.Y. 1980, pp.511-530.
18. N. Ross and K. J. Angelides, Abstract National Meeting, Am. Chem. Soc., Div. Biol. Chem., BIOL 95 (Aug. 1980).
19. R. S. Rogers and H. Rappoport, J. Am. Chem. Soc., 1980, 102, 7335.
20. R. B. Woodward, Pure Appl. Chem., 1964, 9, 49.
21. A mouse unit, MU, is that amount of substance injected intraperitoneally which will kill a 20g. Swiss-Webster female white mouse in 10 minutes. Mice which did not die in 10 minutes survived; for tetrodotoxin, mice that did not die in 20 minutes survived.
22. Pharmacology studies on 11-nortetrodotoxin-6,6-diol will be published by C. Y. Kao.

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