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

Lee A. Pavelka, F. A. Fuhrman and Harry S. Mosher
Department of Chemistry, Stanford University
Stanford, California 94305 U.S.A.

Abstract — Tetrodotoxin (1) is oxidized to 11-nortetrodotoxin-6,6-diol (2a) which is reduced to 11-nortetrodotoxin-6-ol (3); the gem-diol 2a has 20-40% and the reduction product 2 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 chiriqitoxin, 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 (intra-peritoneally, 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 Myxiclea. 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 irreversible 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 (\(\text{C}_{13} \equiv \text{C}_{16}\)) rather than keto form (\(\text{C}_{12}\)).

The periodate oxidations of \(\text{l}\) to \(\text{z}\) 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 (\(\delta 4.1 \text{ ppm}\)) of tetrodotoxin, the appearance of the methylene signal for formaldehyde (\(\text{CH}_2(\text{OH})_2\), \(\delta 4.9 \text{ ppm}\)) and the upfield shift of the \(\text{C}_{4a}\) proton signal from 2.3 to 2.2 ppm during the conversion of \(\text{l}\) to \(\text{z}\). 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 (\(^{1}\text{H}\)) and carbon-13 (\(^{13}\text{C}\)) 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 (\(\text{C}_{13} \equiv \text{C}_{16}\)) since neither \(^{13}\text{C}\) nor distinct IR signals for a ketone carbonyl were observed. Vacuum drying of a
### TABLE I

**NMR SPECTRA FOR NORTETRODOTOXIN**

<table>
<thead>
<tr>
<th>Chemical Shift, δ, ppm</th>
<th>Proton Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proton</strong></td>
<td><strong>D₂O</strong></td>
</tr>
<tr>
<td>C₄α</td>
<td>2.14³</td>
</tr>
<tr>
<td>C₅</td>
<td>2.24³</td>
</tr>
<tr>
<td>C₇</td>
<td>3.91</td>
</tr>
<tr>
<td>C₈</td>
<td>4.17</td>
</tr>
<tr>
<td>C₉</td>
<td>4.21</td>
</tr>
<tr>
<td>C₈</td>
<td>4.27</td>
</tr>
<tr>
<td>C₉</td>
<td>4.36</td>
</tr>
<tr>
<td>C₆</td>
<td>4.55</td>
</tr>
<tr>
<td>C₇</td>
<td>4.80</td>
</tr>
<tr>
<td>C₈</td>
<td>5.46³</td>
</tr>
<tr>
<td>C₉</td>
<td>5.48³</td>
</tr>
</tbody>
</table>

1. The signals can be accounted for by assuming an equilibrium mixture lactone, 2c ↔ hemilactal 2b, in solution.
2. Proton shifts, δ, relative to CHD₂COOD set at 2.03 ppm relative to TMS = 0; reference CHD₂COOD added to pure D₂O after spectrum taken. The ¹³C 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 C₄α at 2.24 ppm corresponds to the lactone form 2c.
4. The signal at 109.8 ppm is assigned to C₁₀ of the hemilactal form (2b) and that at 174.1 ppm to C₁₀ 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 \( \text{sp}^2 \) (\( \text{sp}^3 \)) to \( \text{sp}^3 \) (\( \text{sp}^2 \)) carbon. The \( ^{13}\text{C} \) 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 \(^1\text{H} \) and \( ^{13}\text{C} \) NMR spectra (Table I) that nortetrodotoxin exists as a mixture of at least two forms in solution. We believe these to be \( \text{sp}^3 \text{lactone carbonyl at } 174.1 \text{ ppm} \) and \( \text{sp}^2 \text{hemilactal carbon at } 109.0 \text{ ppm} \). Inspections of molecular models indicate that the C-7 lactone (\( \text{sp}^3 \)) should be more stable than the C-5 lactone. The x-ray structure of crystalline 4-O-methyl-6,11-0-isopropylidinetetrodotoxin hydrochloride, Gougautaxhydrochloride,\(^{20} \) 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 \(^1\text{H} \) 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 (\( \text{sp}^2 \)) predominates in pure \( D_2O \) and the lactone form (\( \text{sp}^3 \)) 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 \text{umole, citrate free, Sankyo Co., Japan}) suspended in 1.00 mL of water was dissolved by the addition of 50 \text{uL} of acetic acid and treated with 9.42 mg of \( H_3IO_6 \) (41.2 \text{umole}) dissolved in 38 \text{uL} of water at 5\(^0 \). After 12 hours at 5\(^0 \) a total of 27.8 \text{uL} of freshly distilled 57\% HI was added in small portions at 5\(^0 \). Each addition was immediately followed by \( \text{CCl}_4 \) 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 \(^1\text{H} \) 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 Swisa-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\(^{21} \text{per mg (MU/mg)} \), 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 \text{ug/Kg} which is about 1/8th that of tetrodotoxin. The compound action potentials in desheathed sciatic nerves from \textit{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 axons will be published elsewhere by C. Y. Kao.\(^{22} \)

Reduction of nortetrodotoxin (\( \text{Z} \)) with sodium cyanoborohydride in dilute acetic acid gave an amorphous 11-nortetrodotoxin-6-ol to which we assign the equatorial alcohol structure (\( \text{Z} \)) 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 (0-methyl-hydroxylamine, CH$_3$ONH$_2$, 1 µL, b.p. 49-50') in D$_2$O (350 µL) and CD$_3$COOD (10 µL) at 5° for 48 hours. Its $^1$H 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$_3$ONH$_2$ at 3.84 ppm. The reaction mixture was rapidly lyophilized to remove free CH$_3$ONH$_2$, and the residual white powder redissolved in D$_2$O-CD$_3$COOD. No additional C=N absorption appeared in the IR spectrum of this reaction product. It had a toxicity of 2300 µg/mg. The 3.84 ppm signal was gone from the initially lyophilized 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 methylglycinate followed by treatment with NaCNBH$_3$, cyanohydrin formation, phenylhydrazone formation and ketal formation (CH$_3$OH, CH$_3$C(OCH$_3$)$_2$CH$_3$, CF$_3$COOD).

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.

ACKNOWLEDGMENTS

We are grateful to the National Institutes of Health Grants #NS 14345 and HL 22201 for support of these studies.
REFERENCES


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.


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-nortetradotoxin-6,6-diol will be published by C. Y. Kao.

Received, 26th August, 1981