AD_____

SITE-SPECIFIC ANTAGONISTS TO TETRODOTOXIN AND SAXITOXIN

Annual Report

C. Y. KAO

May 1, 1988

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7094

State University of New York Downstate Medical Center
Brooklyn, NY 11203-9967

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS auth
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution ~~limited~~ to DoD Components only; premature dissemination, May 27, 1988.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION State University of New York Downstate Medical Center 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Brooklyn, New York 11203-9967	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable) SGRD-RMI-S	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-87-C-7094
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 62770A; PROJECT NO. 3M1-62770A871; TASK NO. AA; WORK UNIT ACCESSION NO. 395
11. TITLE (Include Security Classification) (U) Site-Specific Antagonists to Tetrodotoxin and Saxitoxin	
12. PERSONAL AUTHOR(S) C. Y. Kao	
13a. TYPE OF REPORT Annual	13b. TIME COVERED FROM 4/1/87 TO 3/31/88
14. DATE OF REPORT (Year, Month, Day) 1988 May 1	15. PAGE COUNT 12
16. SUPPLEMENTARY NOTATION (Continued from p. 1)	

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	03	
06	16	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Tetrodotoxin; Saxitoxin; Sodium-channel antagonist

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Towards the objective of developing site-specific antagonists to tetrodotoxin and saxitoxin chemical synthesis of simpler guanidine compounds are underway simultaneously as electrophysiological studies on newer natural and synthetic analogues of TTX on intact membranes. Synthetically, significant progress has been made in producing monosubstituted guanidines, which can then be coupled to sugars to form amino-monosaccharides. There are some structural resemblence between these compounds and the guanidinium end of the TTX molecule. Their biological activity will be tested at some future date when those compounds are fully characterized. On STX analogs work has been rather slow for lack of voucher standard material. However, as an essential first step in all that work, an HPLC analyzer has been completed for quantitative analysis of small amounts of STX and other PSP toxins. Electrophysiologically, 8 related dihydropyrimidine compounds have been found to have very little effect on interferring with the propagation of compound action potential in frog sciatic nerve fibers. 6,11 isopropylidene TTX and c-epi TTX have been found to affect the sodium channel, but at significantly lower potency than TTX. (KT)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian	22b. TELEPHONE (Include Area Code) 301-663-7325 22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

INTRODUCTION

Project goals. The objective of this project is to generate more knowledge about the specific chemical structure of the tetrodotoxin (TTX)/saxitoxin (STX) binding site on the protein molecule of the voltage-gated sodium channel of many excitable membranes. From such knowledge, site-specific antagonists to these toxins and/or other sodium-channel effectors can be developed rationally.

By design, this project has been progressing on two parallel tracks: (a) to expand and refine current knowledge of the structure-activity relations of TTX/STX analogues, and (b) to produce new synthetic compounds with partial structural resemblence to TTX and/or STX. On the first track, the work consists largely of electrophysiological studies of known or newly discovered natural analogues of TTX and/or STX, utilizing the voltage-clamp preparation to study specific ionic conductances. On the second track, colloborative work continues with long-time colleagues in attempts to produce synthetic compounds which have partial structural resemblence to TTX, which might mimick the actions of TTX. A concurrent objective in this direction is to appropriately derivatize TTX such that stable labelled TTX derivatives can be developed to aid in this and similar studies by others on the sodium-channel. The same goals hold for STX studies.

Background. Tetrodotoxin (TTX) and saxitoxin (STX) are important neurobiological tools because of their specific reaction with the voltage-gated sodium channel. They are very different in chemical structure, but their biological actions are virtually identical. On the basis of current knowledge of the structure-activity relations of these toxins and their respective analogues, stereospecific similarities have been recognized in each molecule (for summary, see 1). These groups are:

TTX	STX
1,2,3 guandinium	7,8,9 guanidinium
C-9, C-10 hydroxyl	C-12 hydroxyl (gem-diol)

These groups form the basic active portions of each toxin molecule. From this information, we attempt to fan out in search of refinements and other important details which may add to the success of this project.

WORK DONE IN PREVIOUS YEAR

Chemical Work.

This portion of the work is done by subcontract to Prof. H. S. Mosher of Stanford University on TTX, and subcontract to Dr. Gregory Boyer of State University of New York College of Environmental Science and Forestry, Syracuse, NY on STX. Accounts of their work will be reported in this section.

Prof. Takeshi Yasumoto of Tohoku University, Sendai, Japan has been studying independently natural analogues of TTX, but as friends, we have been studying the ionic-channel effects of these analogues with considerable interest. Prof. Yong Hae Kim of the Korea Advanced Institute of Science and Technology in Seoul, South Korea has been independently studying the chemistry of certain

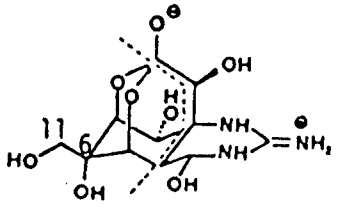
dihydropyrimidine compounds. We have assayed some of those compounds for possible sodium-channel effects. These studies will be reported under the section on Electrophysiological Studies.

Saxitoxin. This phase of the work has been progressing rather slowly, because we need to set up an HPLC system for specific chemical analysis of STX and other paralytic shellfish toxins. Dr. Boyer started his subcontract work in October 1987. He reports that the system is now functional, and on or around April 30, he received 0.1 mg of STX from Dr. Sherwood Hall of the FDA for standardization purposes. A calibration curve is enclosed as Figure 1, but it has not been calibrated against the primary STX standard yet.

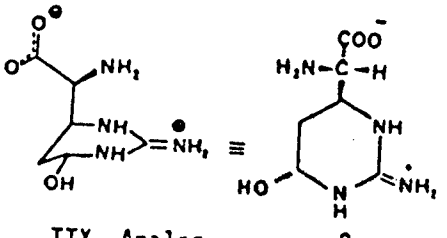
Because of the delay in obtaining the standard, and because of expected difficulties of obtaining any significant amount of STX or other PSP toxins for future work, Dr. Boyer is now embarking on an isolation of STX from some toxic shellfish which he collected in Canada in the summer of 1987. This isolation will further test his HPLC system, as well as provide some raw material for derivitization experiments.

For those experiments, our current focus is still on the C-13 group where the carbamoyl tail is attached. F. E. Koehn had great difficulties in introducing reactive groups on the decarbamoyl STX (2). However, Boyer feels that new attempts should be undertaken.

Tetrodotoxin. Under the subcontract, Prof. Mosher and his postdoctoral fellow, Dr. Keekyung Kim, attempted to make modifications of the TTX molecule in the area of the C-6 and/or C-11 positions, and on developing synthetic TTX analogues. The first part has turned out to be difficult and is progressing slowly. On the second part, they have had more success. The rationale is that certain simpler guanidine derivatives resembling the guanidinium end of the TTX molecule may



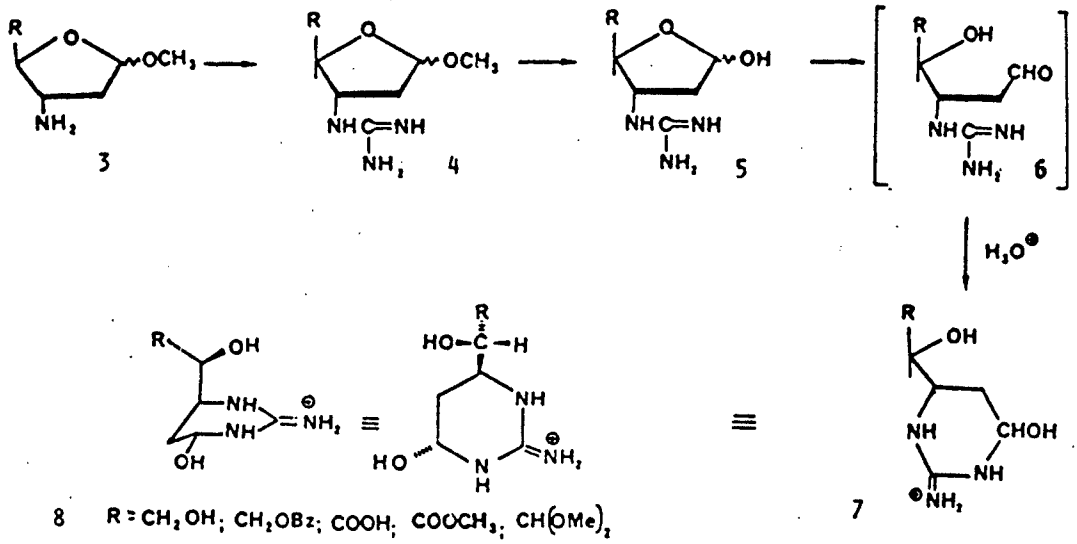
1 TTX



TTX Analog 2

possess the sodium-channel blocking activity. Such molecules would be easier to synthesize and to manipulate chemically than the complex and sensitive TTX molecule.

The basic synthetic approach is shown diagrammatically in the following:

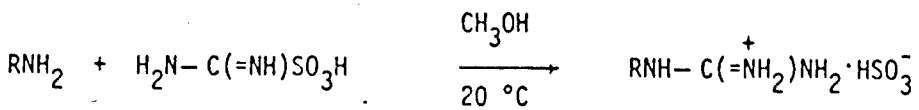


8 R = CH_2OH; CH_2OBz; COOH; $COOCH_3$; $CH(OMe)_2$

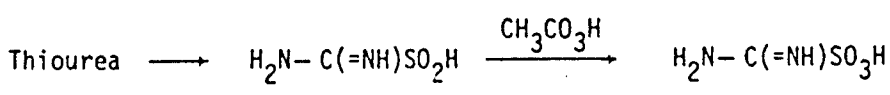
The sequence 3 — 4 — 5 — 6 — 7 represents deprotecting the anomeric position of 4 by acid hydrolysis which will liberate the potential aldehyde group of the carbohydrate derivative 5 — 6. The internal cyclization 6 — 7 should take place spontaneously to give the desired 7 which can be viewed as a simplified TTX analog such as 2.

The first step then is to develop a reaction which will accomplish the conversion of an amino carbohydrate 3 to a guanidino carbohydrate 4. Mosher and Kim found no report in the literature in which any amino carbohydrate has been converted to a guanidino carbohydrate, in spite of the fact that several ways are available for achieving this transformation with simple amines. Therefore, their first step was to develop a reaction for that tranfromation (3 — 4) which would use mild conditions.

A new synthesis of monosubstituted guanidines. The reaction of aminoiminomethanesulfonic acid (10, $AIMSO_3H$) with simple amines (9, NH_2) under mild conditions (20°C, CH_3OH solvents, a few hours) gives good yields of monosubstituted guanidines 11, when RNH_2 is a simple amine such as n-butylamine,

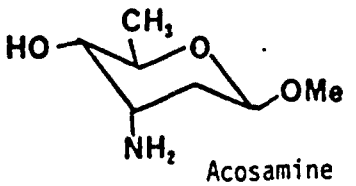


cyclohexylamine, etc. This reagent (10) is readily prepared by the peroxyacetic acid oxidation of the commercially available aminoiminomethanesulfinic acid (12, $AIMSO_2H$).



Details of this synthesis with many examples are contained in a manuscript already submitted for publication in the Journal of Organic Chemistry.

Continuing studies on the synthesis of Guanidino Monosaccherides. When the R group in the amine (9, RNH_2) of the guanidine synthesis is an amino carbohydrate, the reaction seems to proceed to about 20-30% and then stops. Their model compound for these studies has been acosamine. In many experiments they



Acosamine

have studied the effect of pH, solvent, reaction time, temperature, different methods of isolation, and the effects of addition of amines such as triethylamine and pyridine. They have still to find conditioons which give a satisfactory yield. They believe a major problem is decomposition of the $AIMSO_3H$ reagent in the presence of base and extended reaction times. The product from reagent 10 may

$$\underset{\underline{10}}{H_2N-C(=NH)SO_3H} \longrightarrow H_2SO_3 + H_2N-C\equiv N \longrightarrow (C_3H_6N_6)_n$$

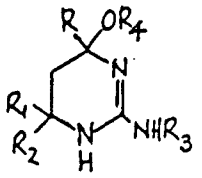
be a polymer, possibly of cyanamide. They are looking for conditions to minimize this decomposition and to isolate the desired guanidinio sugar produced from the reaction mixture containing such polymer.

Synthesis of Additional Amino-monosaccharides. In addition to the 3-aminocarbohydrate 3, Mosher and Kim have prepared two other protected 3-aminomonosaccharides which they will use for conversion to the corresponding guanidino derivatives when they have perfected the synthetic procedure represented by 3 — 4. These are: 3-amino-5-benzoyloxy-2,3-dideoxy-1,2-iso propylidien-arabinofuranose and 3-amino-1,2:5,6-diisopropylidien-3-deoxy-arabinofuranohexose. These starting material, when substituted for 3 in the sequence 3 — 4 — 5 — 8, should give analogs with additional substituents on the guanidino ring and the side chain R group.

Electrophysiological Studies.

Dihydropyrimidine compounds. Prof. Y. H. Kim of Seoul, South Korea had been synthesizing a series of dihydropyrimidine compounds. His interest in these compounds developed, in part, in the early 1970's while a postdoctoral fellow with Prof. H. S. Mosher. At that time, there were great difficulties in making any biologically active TTX derivatives to study structure-activity relations. So, Mosher, Fuhrman and I felt that it might be worthwhile to test some synthetic

cyclic guanidinium compounds with some resemblence to the guanidinium end of the TTX molecule. Some of those compounds were tested by constant-current studies on frog skeletal muscle fibers, and found to have some specificity for the sodium channel (3; 4). In part because of their very low potency, that work was not actively persued. Kim found the chemical questions of synthesizing those compounds to be challenging, and had continued with some of the studies. Shortly before the contract became active, he submitted 8 compound of the general structure:



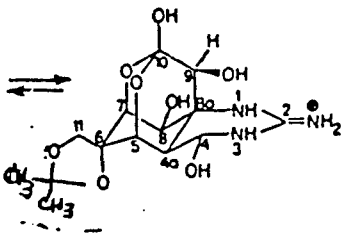
for testing for possible effect on electrical activity.

Four of these compounds could not be dissolved in aqueous solvents to be compatible for study on the living nerve. The other four were tested on the desheathed frog sciatic nerve for effects on the compound action potential. This test was to serve as the first screening test to select any compound worth persuing on single cells.

Figure 2 summarize our observations. In a concentration up to 10 mM, they produced no effect on the amplitude or the conduction velocity of the compound action potentials. I felt that 10 mM was already a rather high concentration, as in diluting stock solutions in water, we had to make a 10% dilution of the salt contents of our saline solution. Our conclusion was that these compounds, although possessing some structural resemblence to the guanidinium end of the TTX molecule, did not possess the appropriate sodium-channel affecting properties we desired.

6,11 O-isopropylidiene TTX. Prof. Mosher and a postdoctoral fellow, Dr. Biqui Wu produced this compound in pure form and good yield, and thought it could serve as an appropriate intermediary to produce TTX derivatives suitable for labelled or covalent markers.

TTX (1)



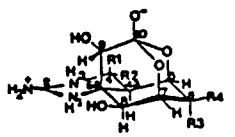
(2) 6,11-isopropylidine-TTX

We tested this compound on both the internally-perfused squid giant axon, and on the frog skeletal muscle fiber under voltage-clamped conditions. Figures 3 show the dose-response relation on the frog skeletal muscle fiber, where the ED_{50} was 578 nM, or about 130 less active than TTX. On the voltage-clamped internally

perfused squid giant axon, the ED_{50} was 73 nM, or about 14 times less active than TTX (Fig. 4). The nearly 10 fold difference in the two tissues was unexpected and surprising, but we have verified that it is not due to deterioration of the compound in solution.

The absolute potency of the material is not a very important issue. The voltage-clamp data from both the squid axon and the frog muscle show clearly that the compound has the specificity for sodium channel. From that point of view, it is worth continuing with this line of investigations, to see whether some derivatized compound could be made to label the TTX/STX receptor site.

6-epi TTX. This is a newly discovered natural analogue (5) in which the positions of the H and CH_2OH on C-6 are just inverted from those in TTX. Much



		R1	R2	R3	R4
1	TTX	H	OH	OH	CH_2OH
2	4-epiTTX	OH	H	OH	CH_2OH
4	6-epiTTX	H	OH	CH_2OH	OH
8	11-deoxyTTX	H	OH	OH	CH_3

effort was spent on carefully studying the actions of this compound. On the frog skeletal muscle fiber, it blocks the sodium channel as TTX, but the ED_{50} turns out to be 134 nM, or nearly 35 times less potent. The effects of 6-epi TTX are less reversible than those of TTX. Full recovery is possible, but usually not attained before 240 min (4 hrs) after washout. The half-time for recovery is about 60 min, contrasted with about 30 min for TTX.

Equally interesting is that in about half of the fibers tested, the activation of the potassium current is markedly slowed in the presence of 6-epi TTX. If the depolarizing step is long enough, then the steady-state potassium current is not different from that with TTX, or normally. However, if an insufficient duration is used, then the potassium current at the end of the pulse appears to be reduced. Considerable efforts were spent on further clarifying this phenomenon. What we know at this time is that the effect on the potassium current is not reversed by the application of high concentrations of TTX.

11-deoxyTTX. This is also one of the newly discovered natural analogues of TTX (5). Very early results suggest that half-reduction of the sodium current in frog muscle fibers can be attained at around 20 nM. Definitive results must await more experiments.

This work has not progessed as well as I had hoped, because of difficulties in the supply of frogs over the past winter. The muscle fibers used came from the common English or Irish frog, Rana temporaria, which exhibits very robust and stable potassium currents as well as good sodium currents. In contrast, the muscle fiber of the common American leopard frog, Rana pipiens, have gnerally smaller currents, with the potassium current especially feeble and fickle. The supply of Rana temporaria was very poor last winter apparently because of climatic reasons in Europe, and the amount of work we could accomplish was limited. Now, we have a further delay because spring frogs, just emerging from hibernation are always in very poor shape, and the muscle fibers have such large

leakage conductances that most experiments cannot last for more than 30 min. Because of the very limited supply of these rare natural analogues, we cannot afford to be losing them on unsuccessful experiments. However, summer frogs are expected within the next 2 weeks, and I expect to complete these studies before the fall.

Neosaxitoxin. This is STX with an -OH group on the N-1 position. From previous work of ours, it is generally thought that the active guanidinium function is in the 7,8,9 group. The 1,2,3 guanidinium group has such an alkaline pKa (over 11) that it is completely protonated within the physiological pH range of about 6 to 9. The role it plays in binding to the receptor site, and hence blocking the sodium channel, just could not be tested experimentally. In neoSTX, the N-1 -OH deprotonates with a pKa of 6.75, thereby providing a different route for assessing the role of the 1,2,3 guanidinium in receptor binding. P. N. Kao et al., (6) first reported that at pH 8.25, neoSTX was much weaker than might be expected from the protonation state of the 7,8,9 guanidinium group. We have re-examined this problem.

NeoSTX is equipotent with STX at pH 7.8. At pH 7.4, it is 1.4 times more potent, but this difference is apparently insufficient to be recognized in the usual bioassay method of determining potency. At lower pH's, neoSTX becomes increasing more potent than STX: at 7.25, it is 1.9 times more potent, and at 6.5, it is 3.5 times more potent than STX. At pH 8.25, where there are more protonated fraction of the 7,8,9 guanidinium in neoSTX than in STX (and where one expect neoSTX to be about 1.2 times more potent than STX), neoSTX is only 0.5 as potent as STX.

Changes of pH on the living membrane are always liable to be complicated by alterations in the surface negative charges which can in turn affect the local concnetrations of cationic toxin molecules. To understand the above findings, we also examined the potencies of STX (with 2+ charges) and TTX (with 1+ charge) at the same pH ranges. Between pH 6.5 to 8.25, the potencies of STX and TTX follow closely the protonation status of the active guanidinium group. This observation shows that any alterations in the surface negative charges are too small to affect toxin binding.

Therefore, the conclusion about neoSTX is that the protonation state of the N-1 -OH plays a very imortant role in its binding to the receptor site. We interpret the results to mean that the N-1 functions come rather close to an anionic site ($-COO^-$) in the channel protein, such that it is charge-repelled when the N-1 is deprotonated to $-NO^-$, and hydrogen-bonded when it is protonated as -NOH. Our interpretation of the data differs markedly from Strichartz observations of higher potency of neoSTX based on the compound action potential (7). Strichartz concluded that in neoSTX, the gem-diol on C-12 more readily formed some covalent bond with the receptor site than the same groups in STX. The problem with that interpretation is that neosaxitoxinol in which the C-12 group are in an alcoholic configuration (and cannot form covalent bonds) possess intrinsic activity (8).

Our idea of an additional binding site for the N-1 functions is being persued further with the hope of utilizing this binding site as a possible anchor for tethering site-specific antagonists.

REFERENCES

1. Kao, C. Y., 1986. Structure-activity relations of tetrodotoxin, saxitoxin and analogues. p. in Tetrodotoxin, Saxitoxin and the Molecular Biology of the Sodium Channel, ed. by C. Y. Kao and S. R. Levinson. Annals of the New York Academy of Sciences, vol. 479.

2. Koehn, F. E., 1983. A chemical investigation of saxitoxin and derivatives. Ph.D. thesis, University of Wisconsin, Madison, WI., p. 88-89.

3. Spiegelstein, M. Y., and Kao, C. Y., 1971. Interference with excitation by 5-benzoyl-2-iminohexahydropyrimidine HCl. Journal of Pharmacology and Experimental Therapeutics. 177:34-39.

4. Goldberg, P. B., and Kao, C. Y., 1973. Interference with excitation by some guanidinium compounds. Journal of Pharmacology and Experimental Therapeutics. 186:569-578.

5. Yasumoto, T., Yotsu, M., and Murata, M., 1988. New tetrodotoxin analogues from the newt Cynops. Journal of the American Chemical Society 110:2344-2345.

6. Kao, P. N., James-Kracke, M. R., and Kao, C. Y., 1983. The active guanidinium group of saxitoxin and neosaxitoxin identified by the effects of pH on their activities on squid axon. Pflügers Archiv. 398:198-203.

7. Strichartz, G. R., 1984. Structural determinants of the affinity of saxitoxin for neuronal sodium channels. Journal of General Physiology. 84:281-305.

8. Wichmann, C. F., 1981. Characterization of dinoflagellate neurotoxins. Ph. D. thesis, University of Wisconsin, Madison, WI., p.102.

Figure 1. Calibration Curve for Saxitoxin Analyzer

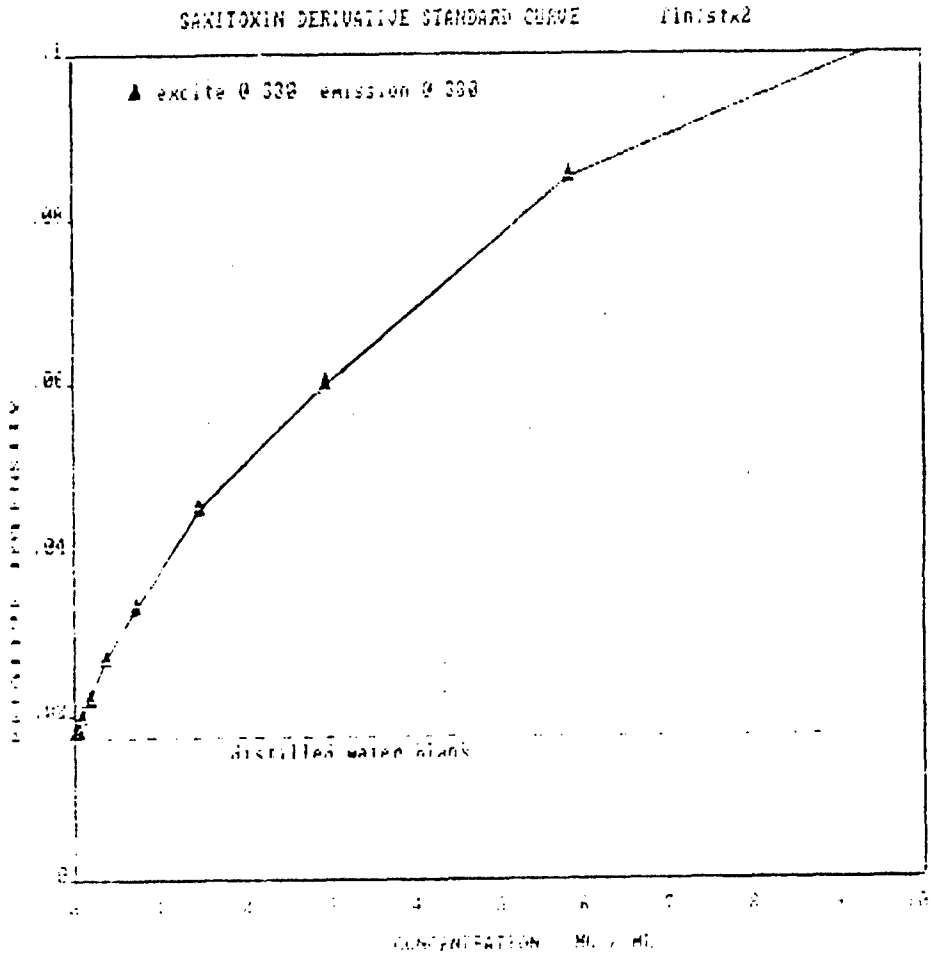
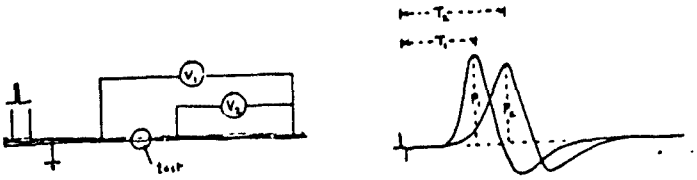
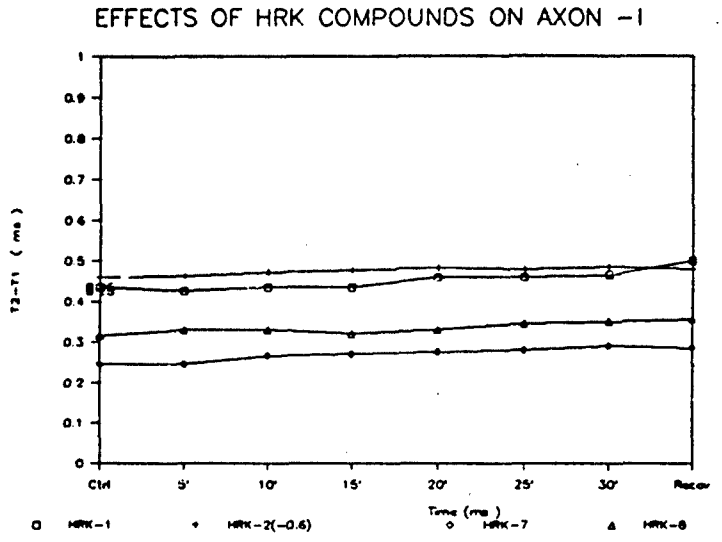


Figure 2. Inaction of dihydropyrimidine compounds on action potential of frog nerve.





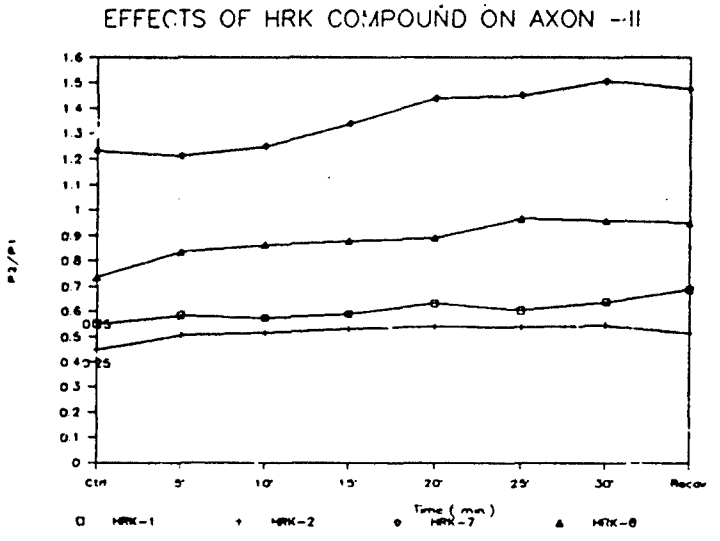
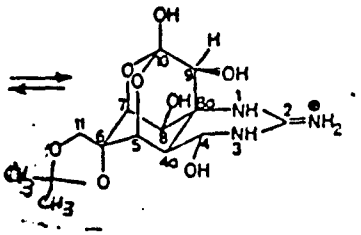


Figure 3. Action of 6, 11 isopropylidene TTX on frog muscle fiber.

TTX

(1)



(2) 6,11-isopropylidine-TTX

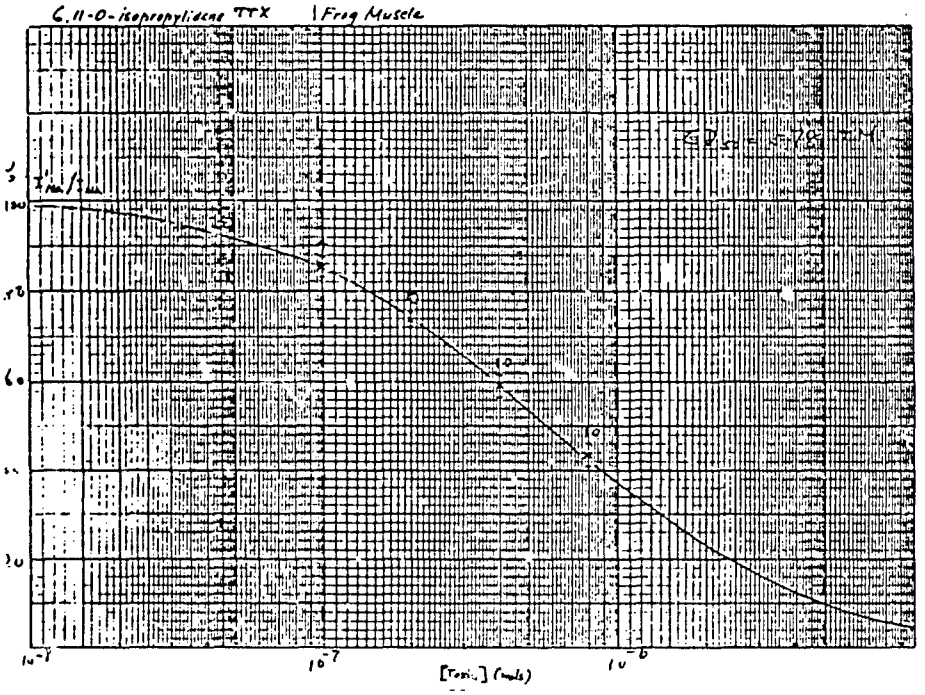
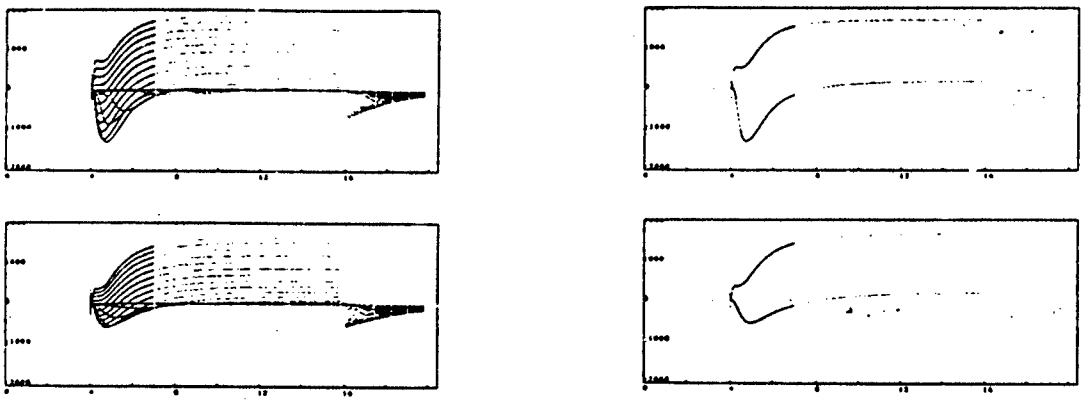
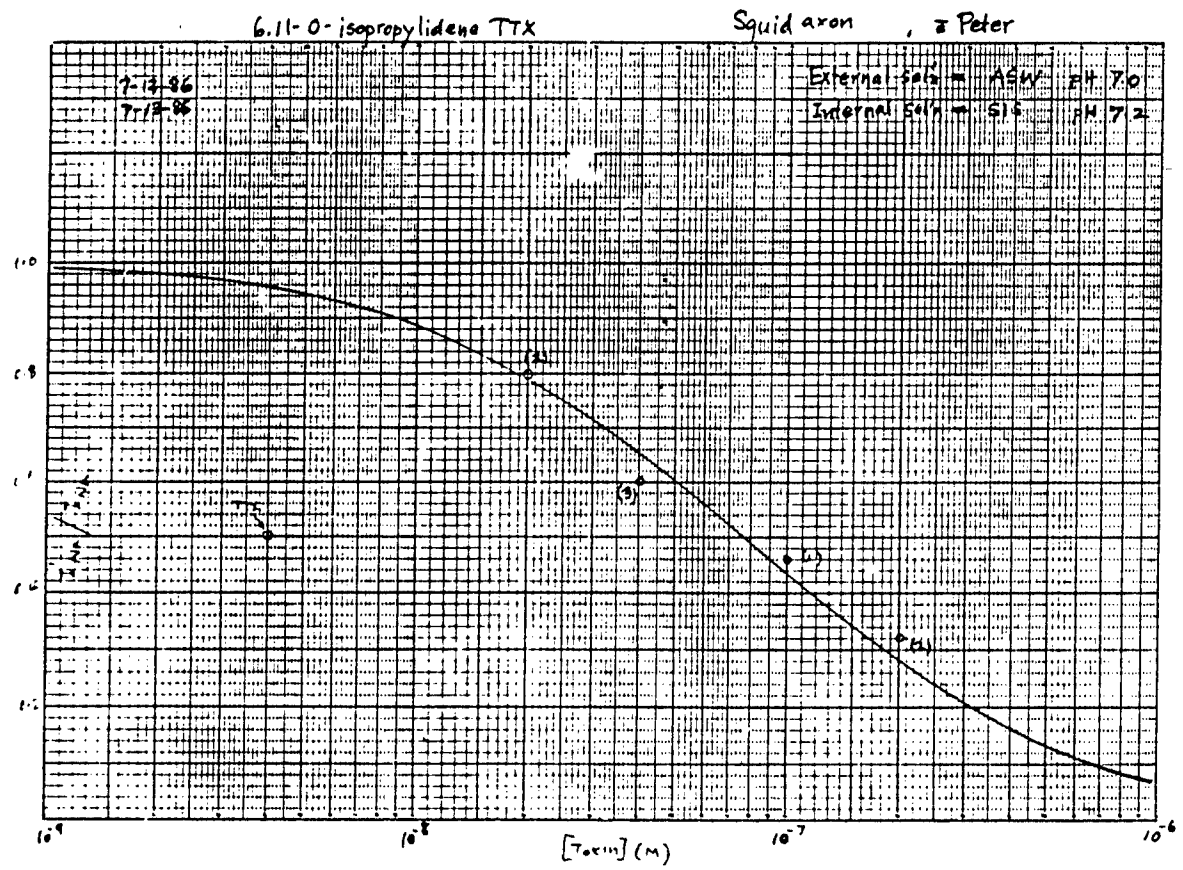


Figure 4. Action of 6, 11 isopropylidene TTX on squid axon





DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MD 21702-5012

REPLY TO
ATTENTION OF:

SGRD-RMI-S (70-1y)

ERRATA AD-B124 671 7 APR 1994

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-HDS/William Bush, Cameron Station, Building 5, Alexandria, VA 22304-6145

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research, Development, Acquisition and Logistics Command (USAMRDALC)(Provisional), has reexamined the need for the limited distribution statement on the technical reports for Contract No. DAMD17-87-C-7094. Request the limited distribution statement for ADB124671, ADB134376, ADB145447, and ADB156671 be changed to "Approved for public release; distribution unlimited." Copies of these reports should be released to the National Technical Information Service.

2. Point of contact for this request is Mrs. Judy Pawlus, DSN 343-7322.

151, 20/14

AD-B124 671 ERRATA

Pawlus Rinehart for
CAREY O. LEVERETT
LTC, MS
Deputy Chief of Staff for
Information Management

A/O 10 May 94, Done

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MD 21702-5012

REPLY TO
ATTENTION OF:

SGRD-RMI-S (70-1y)

ERRATA

7 APR 1994

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-HDS/William Bush, Cameron Station, Building 5, Alexandria, VA 22304-6145

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research, Development, Acquisition and Logistics Command (USAMRDALC)(Provisional), has reexamined the need for the limited distribution statement on the technical reports for Contract No. DAMD17-87-C-7094. Request the limited distribution statement for ADB124671, [redacted], [redacted]7, and [redacted] be changed to "Approved for public release; distribution unlimited." Copies of these reports should be released to the National Technical Information Service.

2. Point of contact for this request is Mrs. Judy Pawlus, DSN 343-7322.

151, 20/14

ERRATA - AD-B124 871

Pauline Pinehart for
CAREY O. LEVERETT
LTC, MS
Deputy Chief of Staff for
Information Management

A/O 10 May 94, Done