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PRINCIPAL INVESTIGATOR: Paul G. Grothaus, Ph.D.

CONTRACTING ORGANIZATION: Hawaii Biotechnology Group, Inc.
99-193 Aiea Heights Drive
Aiea, Hawaii 96701

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FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 85-23, Revised 1985).



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STATEMENT OF THE PROBLEM UNDER STUDY

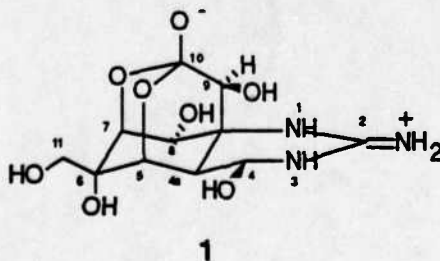
Tetrodotoxin (TTX) is an extremely potent low molecular weight neurotoxin found in a few widely divergent marine and amphibian animal species including puffer fish, gobies, salamanders, frogs, octopus, shellfish and starfish [1,2]. Following ingestion or invenomnation, humans may rapidly exhibit neurological symptoms leading to respiratory paralysis. Deaths occur in about 40% of the reported cases [2,3]. Current methods of detecting and quantitating TTX suffer from low sensitivity, the presence of interfering compounds or do not lend themselves to large scale screening.

This project was undertaken in response to the U.S. Army's need for a rapid, simple, and specific means to identify and quantitate TTX in a variety of biological matrices. Such an assay may also have commercial value in Japan where approximately 100 human fatalities occur annually due to the consumption of puffer fish containing high levels of TTX [4].

During the two years of this project, our overall goal was to develop a sensitive immunoassay for the detection of trace quantities of tetrodotoxin in complex biological matrices. Accomplishment of this goal required the synthesis of TTX immunogens, generation of anti-TTX polyclonal and monoclonal antibodies (MAbs), and incorporation of these antibodies into Enzyme Linked ImmunoSorbent Assays (ELISAs) for TTX. The reagents and protocols needed for these immunoassays have been delivered to USAMRIID.

BACKGROUND

In 1964, four groups independently confirmed that crystalline TTX has the complex structure 1 [5-7]. Tetrodotoxin possesses several unusual structural features. Chief among these is the



orthoester functionality, which Woodward aptly termed a "hemilactal" [5]. It has been shown that the hemilactal is the dominant form in aqueous solution but that it is in equilibrium with the 5-hydroxy- and 7-hydroxy- α -hydroxylactone tautomers [5]. The C.4 carbinolamine is unusually stable, presumably due to the loss of guanidinium resonance which would occur on formation of an imine at N.3 [8]. Tetrodotoxin is highly functionalized, indeed,

the number of heteroatoms is equal to the number of carbon atoms. Finally, the crystalline compound exists as a zwitterion.

As a result of this unique and very complex structure, TTX displays unusual and often unpredictable chemical behavior. It is unstable to base and soluble only in acidic media. This limited solubility imposes severe restrictions on the types of chemical reactions which can be used for the modification of TTX. The isolation, structural elucidation and total synthesis of TTX [9] are landmarks events in natural product chemistry.

In the mouse, TTX has an LD₅₀ of 8 µg/kg when administered intraperitoneally [3]. Tetrodotoxin acts by preventing the inward movement of sodium ions across nerve cell membranes, thereby blocking the conduction of nerve action potentials. As little as 1-10 nM concentrations of TTX in nerve and muscle tissue is sufficient to block these sodium currents [10].

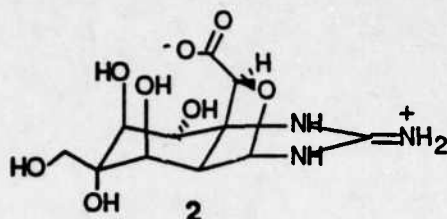
The standard method to assay for TTX is the mouse bioassay [11]. This is an expensive and labor-intensive method which suffers from low sensitivity and the inability to discriminate among toxins. An alternative and more sensitive bioassay employing the house fly [12] has not found wide acceptance due to its tediousness. A number of HPLC methods have appeared in recent years [13-17]. All of these HPLC methods require sophisticated equipment and time-consuming sample preparation procedures. Additionally, the most sensitive of these HPLC methods has a detection limit of only 440 ng/ml of TTX [16]. Bioreceptor assays utilizing crude brain membranes can detect less than a nanogram of TTX per milliliter of sample [18], however, the nature of this assay precludes its application to large scale screening.

Immunoassays are commonly used as rapid, inexpensive, sensitive and highly selective methods for the detection and quantitation of a wide variety of drugs and other molecules of biomedical significance. No such methods had been reported for TTX at the time this Phase II project was begun, undoubtedly due to the extremely difficult chemistry involved in preparing suitable TTX derivatives for conjugation to immunogenic carrier proteins. Two reports subsequently appeared near the end of the first year of this project.

Huot, Armstrong and Chanh reported the production of two anti-TTX MAbs [19]. They used an immunogen prepared by treating a mixture of TTX and keyhole limpet hemocyanin (KLH) with an excess of formaldehyde. The resulting antibodies were screened against a bovine serum albumin (BSA) conjugate prepared in an analogous manner. A total of 9,329 hybridomas prepared from the spleens of immunized mice were screened to find the two useful clones. Binding of these MAbs was only partially inhibited (48% and 25%) by free TTX at a concentration of 50 ng/ml.

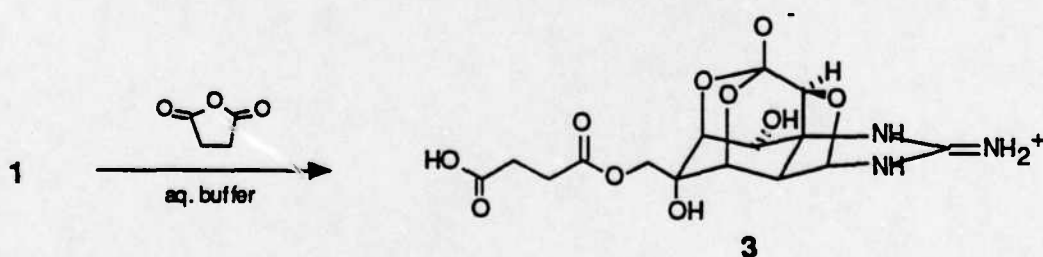
Watabe et al. [20] prepared an immunogen by treatment of tetrodonic acid (2), a naturally occurring, non-toxic analog of TTX, with BSA and formaldehyde. Underivatized TTX was used as the

coating antigen for screening hybridoma clones. No indication is given for the amount of TTX which binds to the ELISA plate; given the hydrophilic character of TTX, it seems unlikely that much TTX would bind to a standard microtiter plate. They report a MAb reactive to TTX at a concentration of 30 ng/ml.



Obviously, neither of these approaches has produced an immunoassay of the needed sensitivity. However they do indicate the potential of such an approach. The major problem to be solved is the synthesis of suitably functionalized TTX derivatives for the preparation of protein conjugates. Ideally, these derivatives would retain the overall structural shape and charge of the portion of the molecule available for interaction with antibodies.

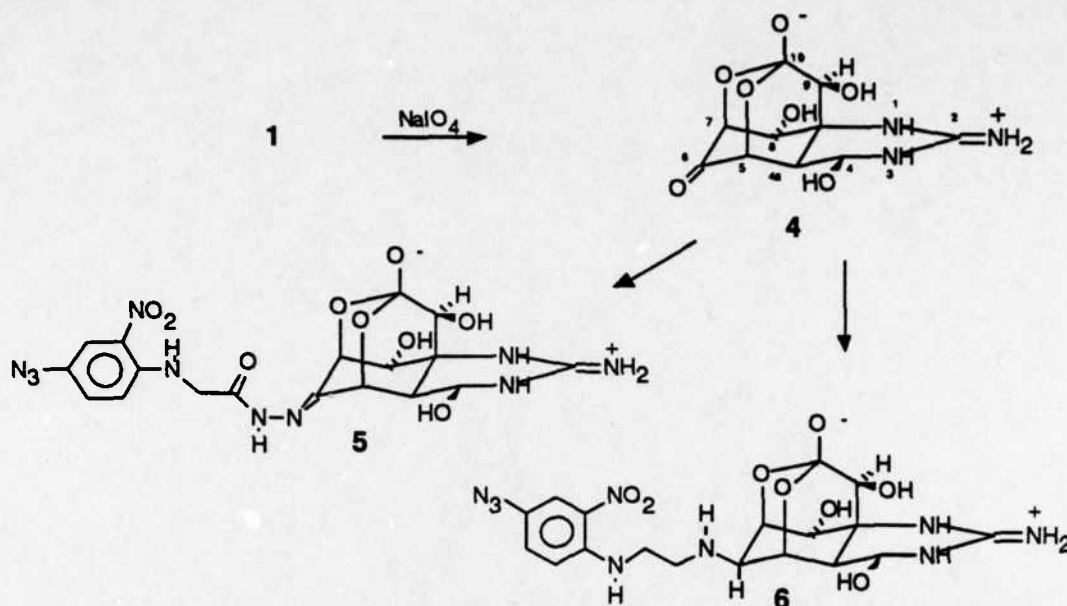
Although TTX has been the focus of intense synthetic effort for over 20 years, most of the reported TTX derivatives are produced in poor yields and are accompanied by undesired modifications of the basic tetrodotoxin framework. As a result, it is doubtful that any of these compounds would be useful as haptens for eliciting anti-TTX antibodies. Strong and Keana [21] attempted to produce the C.11 hemisuccinate ester of TTX. They obtained the hemisuccinate ester of anhydrotetrodotoxin (3) in approximately 3% yield. This compound was subject to slow hydrolysis at pH 7.2, apparently back to TTX.



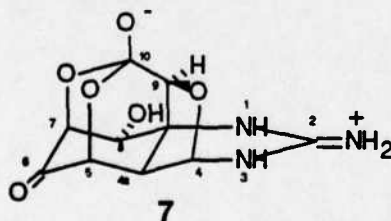
Guillory and co-workers [22] claimed to have esterified TTX at the C.11 hydroxy group with arylazido- β -alanine and carbodiimidazole. This product demonstrated sodium channel blocking activity but was never characterized spectroscopically. Other investigators attempted to repeat this synthesis but were unsuccessful [8].

Periodate oxidation of TTX to nortetrodotoxin (4) was first reported by Goto et al. [23]. Lazdunsky's group reported the preparation of hydrazone 5 and amine 6 from 4 [24]. The best yields obtained were less than 10% and the products were not

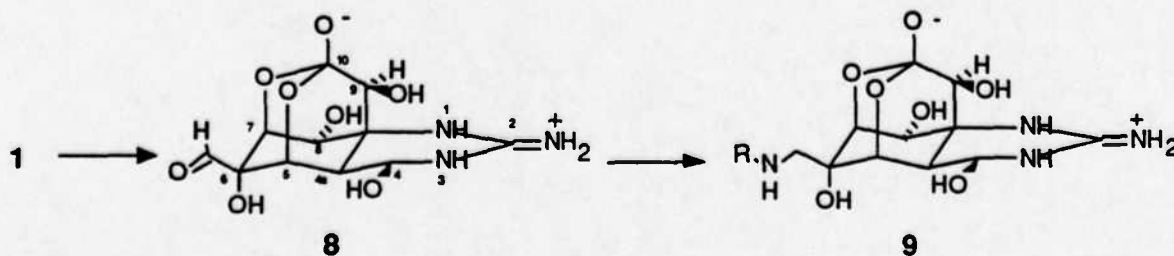
characterized by NMR. Angelides and co-workers [25] were able to produce the C.6 amine by reductive amination of NTTX with sodium cyanoborohydride. The yield of this compound was approximately 5%.



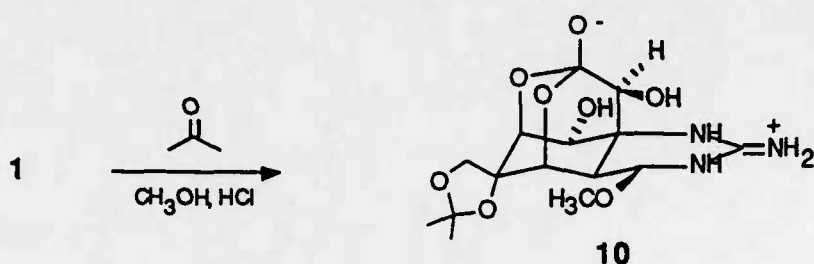
Mosher, Nachman and Pavelka [8,26,27] have recently developed high yield syntheses of both 4 and noranhydroTTX (7). Surprisingly, attempts to prepare derivatives of 4 and 7 by standard reactions of ketones were unsuccessful with the exception of reduction to the C.6 alcohol.



Lazdunsky et al. obtained the C.11 aldehyde (8) by Pfitzner-Moffat oxidation of TTX [28]. This compound was transformed to five amino derivatives (9) by reductive amination with radiolabeled amines. The yields were so small that no spectroscopic determination of the final structures was possible; the structures were inferred from the detection of incorporated radiolabel.



Woodward and Gougotas [5] reported the preparation and spectral characterization of the acetonide **10**. Mosher and Nachman [8] repeated this synthesis and determined that **10** was nontoxic. This crystalline derivative was used in X-ray diffraction studies which played a major role in the elucidation of the structure of TTX.



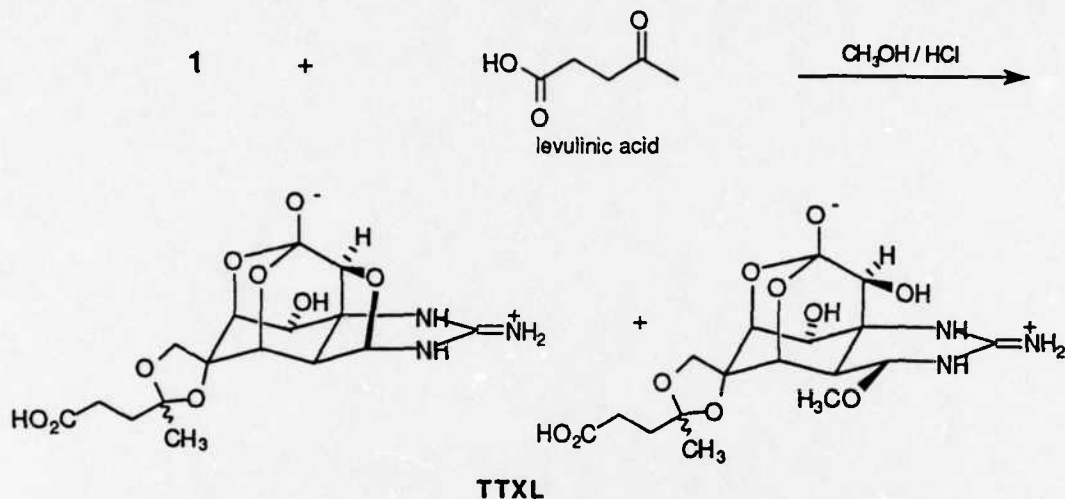
RATIONALE

Small molecules, such as TTX, are non-immunogenic in animals. Such molecules may be rendered immunogenic by covalent attachment to a macromolecule such as a protein. If the small molecule possesses an amino or carboxylic acid moiety, it can be directly coupled to glutamate or lysine residues of the protein via formation of an amide bond. Otherwise, the small molecule is first coupled with a linker molecule to give a derivative (called a hapten) possessing a protein-reactive functional group. To facilitate characterization and reproducibility of the conjugation reaction, it is normally preferable to use a structurally defined, homogeneous hapten which will conjugate in a predictable manner to the carrier protein. In addition, when the molecule of interest is a potent toxin, it must be made less toxic by chemical modification. The modified compound (hapten) must retain the overall structural features of the native toxin in order to elicit toxin-specific antibodies. For stimulating an antibody response against a toxic small molecule such as TTX, the initial objective would be to maximize the immunogenicity and minimize the toxicity of the resulting carrier-hapten conjugate.

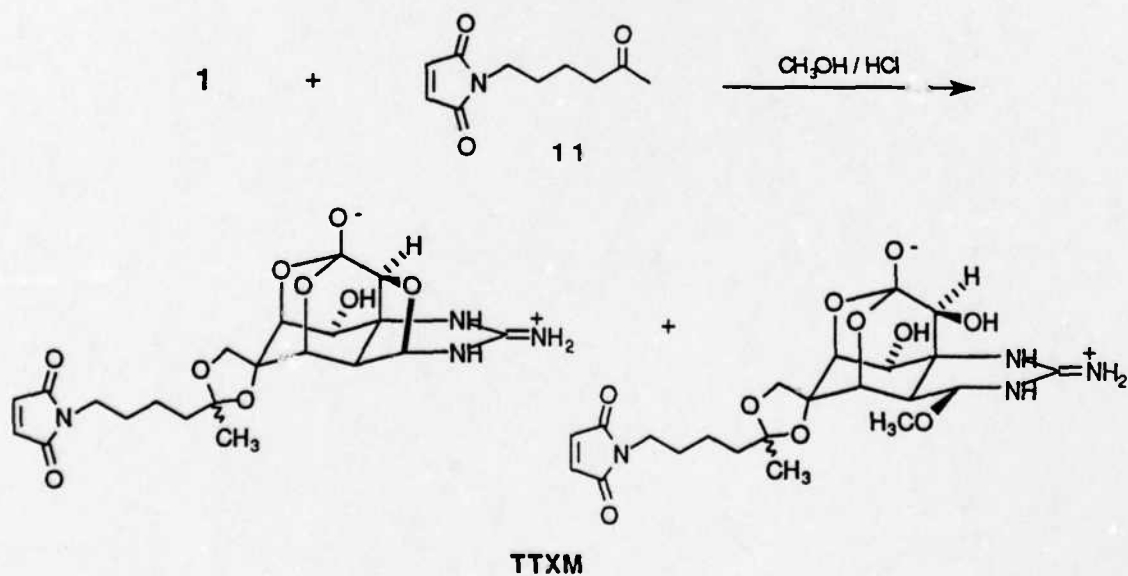
After thoroughly reviewing the reported reactions of TTX, we decided that our initial attempts to prepare TTX haptens would utilize the Woodward-Gougotas ketalization procedure [5,8]. In Phase I, we improved the yield of **10** by increasing the temperature and scrupulously drying the solvent. While the desired functional group transformation readily occurs, these conditions also cause unwanted modifications of the TTX framework at the C.4 position leading to both the 4-anhydro and 4-methoxy isopropylidene derivatives. We hypothesized that since the methyl group is relatively small, the 4-methoxy isomer would retain the basic shape of TTX and elicit antibodies which would cross-react with native TTX. Antibodies raised against the 4-anhydro form would most likely not cross-react with TTX, however, the antibodies produced would be useful for the detection of naturally occurring 4-anhydroTTX derivatives. Judicious selection of monoclonal

antibodies with the desired specificity was expected to enable the development of useful immunoassays.

In Phase I of this research, we reported the preparation of two novel derivatives of TTX, 6,11-(4'-carboxy-2-butanone)TTX ketal (TTXL) and 6,11-(6'-maleimidyl-2-hexanone)TTX ketal (TTXM), which we believed would be appropriate haptens for the elicitation of anti-TTX antibodies. These compounds were prepared by the same procedure as 10 with the substitution of levulinic acid and 6-maleimido-2-hexanone (11) for acetone. The syntheses are shown in Schemes 1 and 2.

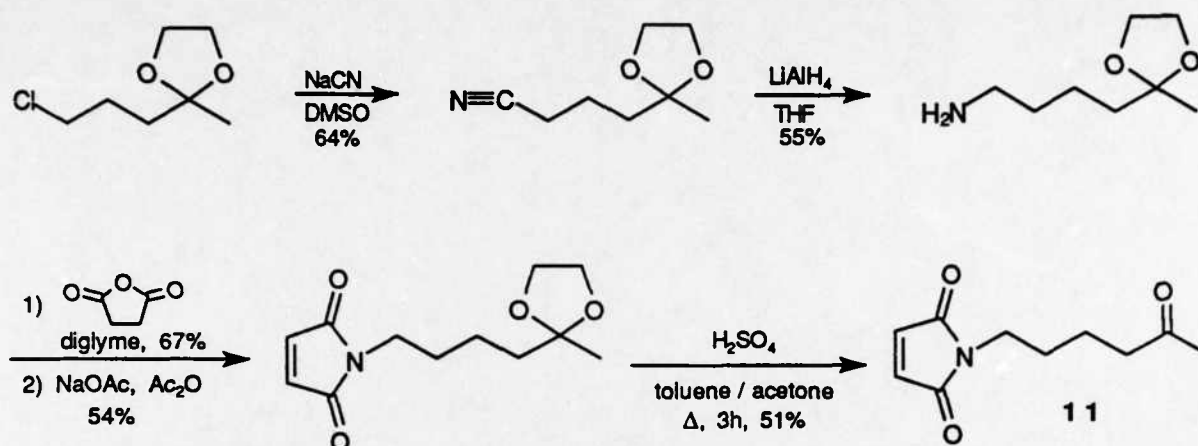


Scheme 1. Synthesis of TTXL



Scheme 2. Synthesis of TTXM

Both haptens are diastereomeric mixtures of two compounds, the 4-anhydro and the 4-methoxy analogs, thus each hapten is a complex mixture of 4 compounds. The synthesis of the new heterobifunctional linker **11** is presented in Scheme 3. Spectral data to support the structural assignments shown for TTXL, TTXM, and **11** was presented in both the Phase I final report [29] and Phase II proposal [30].



Scheme 3. Synthesis of maleimide linker **11**.

Our specific objectives at the beginning of this Phase II project were:

- 1) Immediately prepare fresh TTXM and TTXL.
- 2) Prepare KLH conjugates of both haptens.
- 3) Immunize mice with KLH-TTXM and KLH-TTXL.
- 4) Prepare BSA conjugates of both haptens and use as coating antigens for Enzyme Linked ImmunoSorbent Assays (ELISAs) to screen antisera.
- 5) While awaiting immunology results, separate and characterize TTXM and TTXL isomers.
- 6) Synthesize radiolabeled derivatives of **11** and levulinic acid to facilitate determination of hapten-protein conjugation ratios.
- 7) If high titer antisera were obtained, proceed with fusions and screen for monoclonal antibodies with a high affinity for TTX.
- 8) Use MAbs to develop Competitive Inhibition Enzyme ImmunoAssays (CIEIAs) for TTX.
- 9) Refine, characterize, and validate the most promising assay.
- 10) If no antibodies with the desired specificity were found, investigate the synthesis of alternative defined haptens.

Due to unsatisfactory results with this original approach, our specific objectives were ammended late in the first year of the project:

- 1) Synthesize and chemically characterize one or more alternative defined TTX haptens.
- 2) Prepare conjugates of these haptens with KLH and BSA.
- 3) Conjugate TTX to KLH and BSA via formaldehyde condensation (an undefined hapten or "shotgun" approach).
- 4) Immunize rabbits with the KLH-hapten conjugates and screen the antisera against the BSA-hapten conjugates.
- 5) If rabbit antisera, whose reactivity with BSA-hapten conjugate is inhibitable by TTX, were produced, develop polyclonal-based immunoassays for the detection of TTX.
- 6) Immunize mice with the KLH-hapten conjugates and screen the antisera against the BSA-hapten conjugates.
- 7) If antisera reactive with BSA-hapten conjugate were produced, proceed with fusions and screen for monoclonal antibodies with a high affinity for TTX.
- 8) Use MAbs to develop CIEIAs for TTX.
- 9) Refine, characterize, and validate the most promising assay(s), whether polyclonal or monoclonal based.
- 10) Deliver the reagents and protocols needed for these immunoassays to USAMRIID.

MATERIALS AND METHODS

Source of TTX: Citrate-free tetrodotoxin (200 mg) was obtained from Calbiochem.

Chemicals: All chemicals and biochemicals were purchased from reputable and reliable commercial sources.

Synthesis of TTXL and TTXM haptens: Fresh batches of the TTXL and TTXM haptens were prepared via the experimental procedures are presented in Appendix A.

Hapten characterization: ^1H -NMR spectra were obtained on the NT-300 spectrometer at the University of Hawaii. Dr. Walter Niemczura provided technical assistance in obtaining and interpreting the spectra.

Analytical and preparative HPLC was performed with a Gilson binary gradient instrument using conditions cited in the Results section of this report.

TLC analysis was obtained under the conditions reported by Mosher et al. [3]. Visualization was achieved by spraying the plates with aqueous KOH, heating and viewing under UV light.

Preparation of TTX-Protein Conjugates: Detailed procedures for the preparation of KLH-TTXM, KLH-TTXL, BSA-TTXM and BSA-TTXL are presented in Appendices B-E.

Tetrodotoxin-protein conjugates were characterized by an indirect method for the estimation of the moles of TTX per mole of protein. Total protein concentration was determined using the BCA protein analysis obtained from Pierce Chemical Co. (Appendix F). Free thiol groups on the proteins were measured using the dithiodipyridine (DTDP) method (Appendix G). The decrease in free sulfhydryl groups of thiolated proteins after reaction with the TTXM hapten provides a means of estimating the extent of TTX conjugation to the carrier. Similarly, free amino groups were determined by the method of Snyder and Sobocinski (Appendix H) to estimate the extent of TTX-protein conjugation following reaction with TTXL.

KLH-TTXF and BSA-TTXF were produced as described in appendices I and J of this report, respectively.

Immunization of Animals: During Year 1 of this project, mice were immunized using standard procedures [31-33]. Primary inocula contained 20 µg of immunogen in approximately 0.2 ml RIBI R-700 MPL+TDM emulsion. All injections were administered intraperitoneally (i.p.). Mice were bled from their tail vein to obtain sera for testing their response to immunization. Mice to be used as spleen cell donors for hybridoma production were given 20 µg immunogen in 0.2 ml phosphate buffered saline, pH 7.2, by the intraperitoneal route, four days prior to sacrifice.

During Year 2, modified immunization schedules were employed using the KLH-hapten conjugates as follows:

A) KLH-TTXM: BALB/c male mice immunized with KLH-TTXM received a priming immunization of 20-50 µg of conjugate in complete Freund's adjuvant, followed by biweekly booster immunizations of 10-25 µg in incomplete Freund's adjuvant. Animals were monitored for appearance of a serum antibody response to BSA-TTXM. One of these animals was used as a spleen donor for hybridoma production and received an i.p. boost of 10-25 µg in incomplete Freund's adjuvant four days prior to fusion.

B) KLH-TTXL: BALB/c female mice immunized with KLH-TTXL received a priming immunization of 20-50 µg conjugate in complete Freund's adjuvant, followed by three weekly booster immunizations of 10-25 µg in RIBI adjuvant, and then five weekly booster immunizations of 10-25 µg conjugate in PBS. Animals were monitored for appearance of a serum antibody response to BSA-TTXL and BSA-TTXM.

C) KLH-TTXF: BALB/c female mice immunized with alum precipitated KLH-TTXF (Appendix I) received a priming dose of 20-50 µg of conjugate in complete Freund's adjuvant, administered to multiple subcutaneous sites, followed one week later by a subcutaneous booster immunization in incomplete Freund's adjuvant of 20-50 µg conjugate. Thereafter, these animals received weekly booster immunizations of 10-25 µg conjugate, administered i.p., in PBS. Animals were monitored for appearance of a serum antibody response to BSA-TTXF. Those animals selected to be spleen donors

for hybridoma production received daily i.p. boosts of 10-25 μ g in PBS each of the four days prior to fusion.

Female New Zealand White rabbits immunized with alum precipitated KLH-TTXF animals received a priming injection of 63 μ g of conjugate in complete Freund's adjuvant, administered to multiple sites, followed by biweekly booster injections of 50 μ g conjugate in incomplete Freund's adjuvant, also at multiple sites. During weeks 9-13, injections were administered weekly. Animals were monitored for appearance of a serum antibody response to BSA-TTXF.

Primary Indirect ELISA Screen for Antibodies Against Tetradotoxin: The protocol for this ELISA system is given in Appendix K.

Hybridoma generation and monoclonal antibody production: Murine hybridomas were produced using standard procedures [31-33] with the fusion partner, P3X63Ag8.653. The resulting hybridomas were screened for antibody production, as described in appendix K. Hybridomas producing monoclonal antibodies reactive with TTX-F-BSA were tested for inhibition of this reactivity by 200 ng/ml unconjugated TTX. Those cultures showing significant TTX-specific inhibition of solid-phase binding were cloned by limiting dilution. Stable antibody producing subclones were expanded and cryopreserved, as follows. Primary and subcloned monoclonal hybridoma cultures containing $1-10 \times 10^6$ cells were pelleted by centrifugation and then suspended in 10% (v/v) dimethylsulfoxide in fetal bovine serum. These suspensions were cooled under controlled conditions and stored in liquid nitrogen.

Production of Ascitic Fluid: BALB/c mice were injected intraperitoneally with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane) or Freund's Incomplete Adjuvant (FIA) [34]. After 7 to 10 days in the case of pristane-primed mice, and 24 hours in the case of FIA-primed mice, each animal was injected intraperitoneally with 5×10^6 recloned T20G10 hybridoma cells. Mice were then observed for abdominal swelling due to ascitic fluid accumulation. Ascitic fluid was tapped by insertion of an 18G, 1.5 inch hypodermic needle into the swollen abdomen, and collected in a sterile tube. Cells were removed from pooled fluid by centrifugation, the clarified fluid was stored, after testing by ELISA, at -20°C .

Affinity Purification of Antibodies: Affinity purification of IgG antibodies from mouse ascitic fluid using Protein A chromatography was performed using the protocol in Appendix L.

Alkaline Phosphatase Conjugates: Alkaline Phosphatase (AP) was conjugated to Protein A-purified T20G10 using the procedure detailed in Appendix M. Methods for determination of total protein concentration and free sulfhydryl groups are given in Appendices F and G respectively.

Enzyme Immunoassays for Tetrodotoxin Detection: Protocols for the indirect competitive inhibition enzyme immunoassay (CIEIA) and the direct CIEIA using AP-T20G10 are given in Appendices N and O respectively.

Data analysis: All samples tested by ELISA and CIEIA were run in triplicate and the mean result of each set of replicates was calculated.

The end point titer of antibody preparations tested in the ELISA system was noted, together with the dilution that resulted in an OD₄₁₄ of about 0.5 one hour after substrate addition. A working dilution of half this dilution was employed for each antibody preparation in the indirect CIEIA system.

For CIEIAs, standard curves were constructed for each experiment using a set of known TTX concentrations.

B/B₀ values were calculated by dividing the mean of a given set of replicates containing TTX inhibitor by the mean of all the wells containing no inhibitor. CIEIA results in each experiment were calculated from a standard curve as the TTX concentration corresponding to a B/B₀ value of either 0.5 (a concentration causing 50% inhibition - IC₅₀) or 0.8 (a concentration causing 20% inhibition - IC₂₀).

Appropriate statistics were calculated as needed.

RESULTS

Preparation of TTXM and TTXL

New batches of TTXM and TTXL were produced according to the procedures developed in Phase I (Appendix A). The ¹H-NMR spectra of the resulting product mixtures essentially matched our earlier data, however, the isomer ratios varied slightly between different batches. These reactions are run under thermodynamic conditions, thus, the reaction is reversible and the product ratio reflects relative product stability. Apparently, equilibration of these systems is a slow process and true equilibrium had not been achieved before the reactions were stopped. The first batches of haptens were used, without further characterization, to prepare carrier-hapten conjugates so that the immunology work could be initiated immediately.

Additional batches of material were prepared as needed for chemical characterization studies.

Carrier-hapten conjugations

TTXM was conjugated to both KLH and BSA using the procedure in Appendices B and D. The hapten/carrier ratios were estimated to be ~10:1 TTX:KLH and ~8:1 TTX:BSA based upon 2,2'-dithio-dipyridine

(DTDP) titration of free thiol groups in the conjugate compared to the carrier protein (see Appendix G).

Conjugation of TTXL to BSA proved to be a difficult and unreliable procedure, often resulting in extensive protein cross-linking. This insoluble material was unsuitable for use as an ELISA coating antigen. After several variations of reaction conditions were attempted, a soluble BSA-TTXL conjugate was obtained (see Appendix E). The hapten/carrier ratio was too low for estimation, however trinitrobenzenesulfonic acid (TNBS) titration indicated a slight decrease in the number of available amino groups (see Appendix H). Further evidence for successful formation of this conjugate was provided by the observed cross-reactivity of sera, from mice immunized with KLH-TTXM, with this coating antigen.

Similar problems were encountered with the conjugation of TTXL to KLH. Every procedure which was tried resulted in extensive protein cross-linking. No soluble protein could be detected in the reaction products. The procedure of Bauminger [35] was finally used to produce KLH-TTX (see Appendix C), which was used without further characterization due to its insolubility.

Production of Antisera with TTXM and TTXL Haptens

Table 1 presents the initial immunization schedules which were utilized to produce mouse antisera with KLH-TTXM and KLH-TTXL. Mice are identified by group numbers (Mn indicates that KLH-TTXM was the immunogen and Ln indicates that KLH-TTXL was used).

Table 1. Initial Immunization Schedule for Mice.

Group	# of mice	11/23/88	12/13/88	12/19/88	1/3/89	3/20/89	4/19/89	5/8/89
M1	1	√ ¹	√	√ ²				
M2	6	√ ³	√		√ ⁴			
M3	3		√ ⁵		√			
L1	6					√	√	√

1. Initially immunized at end of the Phase I project.
2. Sacrificed for fusion on 12/23/88.
3. Three mice died prior to next boost.
4. Two mice sacrificed on 1/6/89; other mouse died prior to fusion.
5. One mouse died prior to next boost.

Sera from test bleeds were assayed by ELISA for reactivity against both TTX-BSA conjugates and BSA. Sera from mice immunized with KLH-TTXM exhibited high titer responses against BSA-TTXM coating antigen, low titer response against BSA-TTXL coating antigen, and no activity against BSA coating antigen (control). Figure 1 presents a typical titration curve for test sera obtained from these mice. Unfortunately, the anti-BSA-TTXM activity was not inhibitable in a CIEIA system by free TTX. Sera from animals immunized with KLH-TTXL exhibited extremely low titer ELISA

reactivity against both BSA-TTXL and BSA-TTXM coating antigens. The anti-TTX-hapten activity of these sera was not inhibitable by free TTX in a CIEIA system.

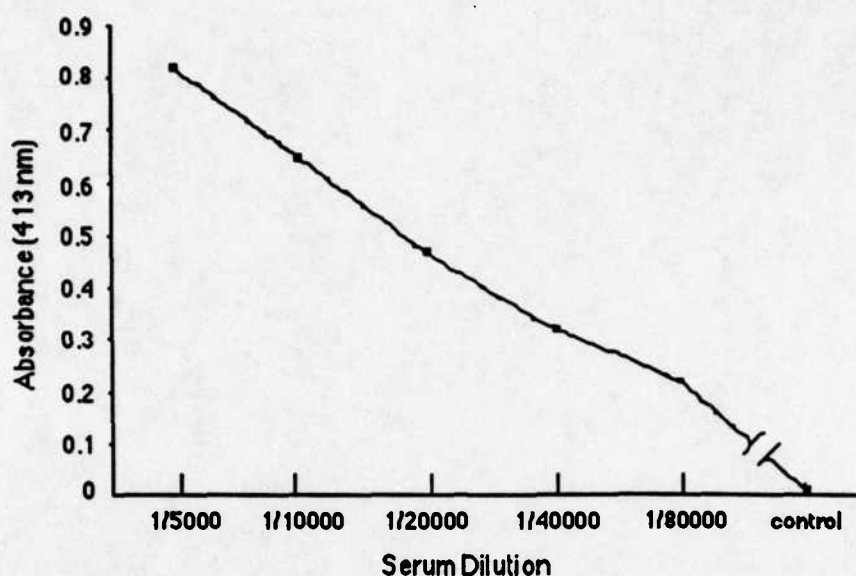


Figure 1. Antibody response to BSA-TTXM. Mouse serum was diluted and tested in ELISA using BSA-TTXM coating antigen. There was no reaction with BSA coating antigen (control).

The high titer polyclonal response to the coating antigens indicates that a large number of lymphocytes were induced to secrete antibodies against the KLH-TTX immunogens. However, the reactivity of these polyclonal sera was not inhibitable by free TTX. There appeared to be four possible explanations for these observations:

- 1) The proportion of antibodies in the polyclonal sera cross-reactive with free TTX may have been so small as to be undetectable in a CIEIA.
- 2) The haptens are mixtures of anhydroTTX and 4-methoxy-TTX derivatives. These haptens may not be structurally similar enough to elicit antibodies cross-reactive with native TTX.
- 3) The ketal linkage of the haptens may not have survived the conjugation conditions and therefore antibodies may have been raised to a linker immunogen rather than the TTX derivatives.
- 4) The heterogeneous nature of TTXM and TTXL (4 isomers each) would have given rise to equally heterogeneous BSA conjugates. Such a coating antigen may have made detection of weakly positive antibodies difficult. This would also have been a factor in Huot's and Watabe's work and may explain their inability to find higher affinity antibodies.

All of these alternative explanations argued for the synthesis of alternative haptens which more closely resembled TTX itself. Efforts to synthesize additional haptens commenced during the third quarter of the first year of this project. However, since the probability of the successful synthesis of new haptens was uncertain, it was decided to proceed with hybridoma production from the mice immunized with TTXM on the chance that a monoclonal antibody of the desired specificity could be identified.

Hybridoma Production and Screening: Two fusions were performed with spleens from KLH-TTXM immunized mice with the intention of producing anti-TTX monoclonal antibodies (Table 2). The single surviving mouse from Phase I of this project (group M1) was used for the first fusion. The surviving two mice from group M2 were used for the second fusion.

Table 2. Hybridomas Produced from Mice Immunized with KLH-TTXM and KLH-TTXL

Fusion	Date	Number of Wells	Primary Screen ^a Positives	Secondary Screen ^b Positives
1	12/23/88	252	28	0
2	1/ 6/89	378	30	0

a. Primary screen fusion 1: undiluted supernatants were tested for ELISA reactivity with BSA-TTXM.

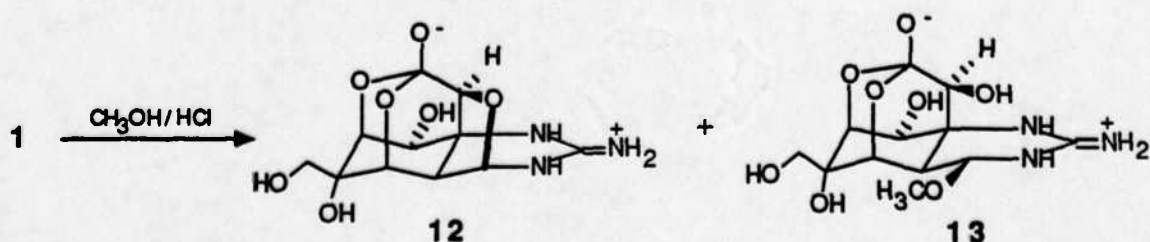
Primary screen fusion 2: supernatants were diluted 1/10 before testing for ELISA reactivity with BSA-TTXM.

b. Secondary screens: supernatants were tested to determine if 200 ng/ml TTX would inhibit binding to BSA-TTXM.

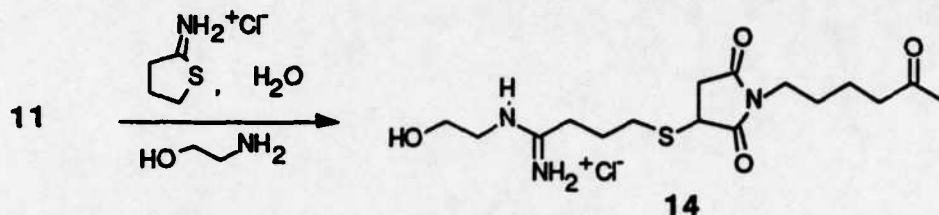
The data in Table 2 indicate that though 58 TTX hapten-positive hybridomas were detected in the primary ELISA screen, none of these were inhibitable by free TTX in a CIEIA system.

Specificity testing: It was decided to determine whether the existing haptens had the wrong structure for eliciting TTX-specific antibodies or if the ketal linkage of the haptens was unstable. These experiments required testing 4-anhydroTTX, 4-methoxyTTX and linker 11 as inhibitors in the CIEIA.

A mixture of 4-anhydroTTX (12) and 4-methoxyTTX (13) was prepared using the same conditions as for the synthesis of the haptens (no linker was added). The ¹H-NMR of the minor component matched the reported data [8] for 12 and the major isomer was consistent with structure 13.

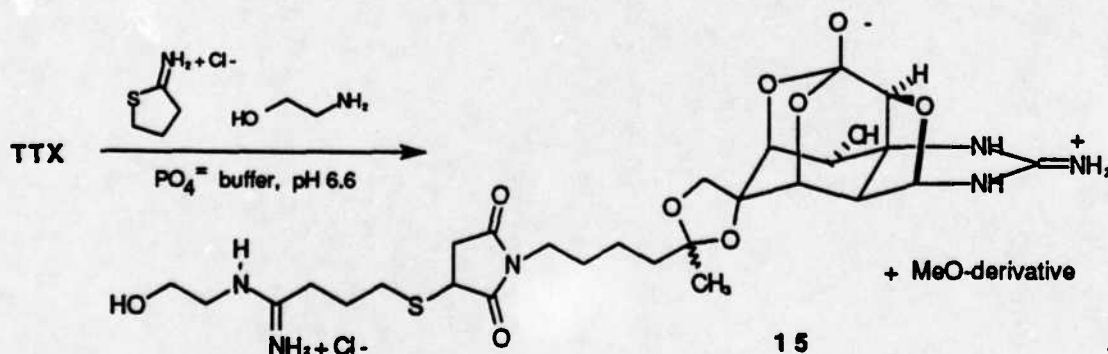


Linker **11** could not be used directly in inhibition studies because the proteins present in the assay medium (BSA and antibodies) would react irreversibly via their sulfhydryl groups with the maleimide moiety. A blocked maleimide linker (**14**) was prepared by the substitution of ethanolamine for the carrier protein under the maleimide conjugation conditions.



This amidinium salt could not be readily purified by conventional normal or reversed phase chromatography. Attempted conversion of **14** to the neutral amidine by neutralization with Na_2CO_3 was unsuccessful, the molecule underwent a base catalyzed fragmentation to regenerate **11**. This problem was overcome by a modified procedure. Cold, saturated aqueous NaHCO_3 was added to a biphasic mixture of an aqueous solution of **14** and CH_2Cl_2 in a separatory funnel, followed by immediate shaking for ten minutes. This allowed extraction of the product before it could undergo base-catalyzed decomposition in the aqueous phase. TLC and $^1\text{H-NMR}$ proved the absence of **11**. Following silica gel purification, the amidine was converted back to the pure salt **14** by treatment with methanolic HCl (to increase water-solubility).

The same reaction was attempted with TTXM for two reasons. We wished to have a suitably blocked TTXM derivative (**15**) to test as an inhibitor of antibody binding to BSA-TTXM. We also hoped to spectroscopically determine whether the ketal linkage of TTXM was stable to the conjugation conditions.



$^1\text{H-NMR}$ of the product confirmed the disappearance of the maleimide double bond, the presence of a methyl ether, and resonances similar to those observed in the spectra of **14** were present. Additionally, the C.4 and C.4a protons of both isomers of TTXM were still present. If the ketal linkage had been hydrolyzed, the product would be a mixture of anhydroTTX, 4-methoxyTTX and **14**.

These data may be analyzed by comparing the results in each of the columns:

- The data in Columns 1 & 2 show a promising indication that some antibodies have been elicited which react with BSA-TTXM but not native BSA.
- The data in Column 3 shows that the reactions with BSA-TTXM are not inhibited by free TTX.
- The data in Column 4 shows that the reactions with BSA-TTXM are not inhibited by a mixture of anhydroTTX and 4-methoxyTTX.
- The data in Column 5 shows that the blocked linker will slightly inhibit the reaction of some of the antibodies with BSA-TTXM.
- The data in Column 6 shows that only the mouse serum and one of the monoclonal antibodies reacted with BSA-TTXL.
- The data in Column 7 shows that there are some antibodies which react with BSA coupled with the linker only.
- The data in Column 8 shows that there is slight inhibition of reaction with the BSA-M by compound 14.

These data did not provide a definitive answer about the specificity of our antibodies. There was no evidence of TTX-specific reactivity. There was an indication of antibodies specific for 11, indicating the possibility that the ketal linkage of the hapten was hydrolyzed to some extent either during conjugation or in vivo. However, the observations that there were antibodies which reacted with BSA-TTXM and not with BSA-M and that the mouse serum reacts with BSA-TTXL argues that antibodies against something other than KLH and 11 are present.

Later experiments demonstrated very slight inhibition of the reaction of sera from mice immunized with KLH-TTXM against the BSA-TTXL coating antigen by TTX. Following this observation, hybridoma culture supernatants from earlier fusions that were done with mice that were immunized with KLH-TTXM were retested in a CIEIA with BSA-TTXL coating antigen and TTX inhibitor. Three clones which reacted with BSA-TTXL and exhibited some inhibition with free TTX were identified. One of these clones, T5B10, could not be recovered from liquid nitrogen storage. Data from the remaining two clones is summarized in Table 4.

Three clones were derived from each line and tested under what were believed to be optimal CIEIA conditions. Unfortunately, the reproducibility of the immunoassay with these clones was poor. Experiments performed with the same materials on different days give wide variations in response and degree of inhibition. The erratic results consisted of high %CV values (ca. 20-30%) for the concentration of TTX. The anti-TTX responses of both lines of subclones decreased as testing progressed. We were forced to

conclude that the T1C11 and T11D4 subclones were not useful for development of a TTX immunoassay.

Table 4. Reactivity/Inhibition of T11D4 and T1C11 Supernatants.

Supernatant	Reaction on BSA-TTXM	Reaction on BSA-TTXL
T11D4-original	+++ no inhibition by free TTX	+ slightly inhibitable by free TTX
T1C11-original	+ no inhibition by free TTX	+++ peculiar response; reaction appeared to increase slightly in the presence of TTX.
T11D4-regrown	+++ no inhibition by free TTX	+/- very weak response. not tested for inhibition
T1C11-regrown	+ no inhibition by free TTX	+++ partially inhibitable, erratic results, could have been detecting TTX down to 30 ng/ml

As an additional comparison, the formaldehyde hapten (TTXF) and BSA conjugate (BSA-TTXF) were prepared via the procedure of Huot and coworkers [19]. Antisera, raised with KLH-TTXM and KLH-TTXL, exhibited no reactivity with the BSA-TTXF coating antigen. KLH-TTXF was also prepared and antisera from six mice, which were immunized with this material, were screened for cross-reactivity to BSA-TTXM. We observed weak responses one month after the onset of a weekly immunization schedule. The anti-BSA-TTXF response of antisera from mice being immunized with KLH-TTXF was demonstrated to be slightly inhibitable by TTX.

In July 1989, a weekly immunization schedule, with KLH-TTXM and KLH-TTXL, was adopted in response to these disappointing results. It was hoped that this more intensive schedule would stimulate production of higher titer, higher affinity antisera in the animals. Unfortunately, this was unsuccessful, none of the mice immunized with KLH-TTXM and KLH-TTXL on this schedule exhibited inhibitable antibody responses.

Separation and characterization of TTXM and TTXL: To address the issue of coating antigen heterogeneity, and to complete the chemical characterization of TTXM and TTXL, separation of the hapten isomers was investigated. Successful purification of the hapten isomers would have enabled the preparation of homogeneous coating antigen which could have increased the chance of identifying TTX-specific Mabs from the earlier hybridoma fusions. A number of HPLC methods for the analysis of TTX samples have been reported [13-17]. Several of these methods were evaluated in our laboratory to determine their suitability for the separation of TTXM and TTXL isomers. A major difficulty is the detection of TTX derivatives since TTX lacks any significant absorption above ~ 215 nm. Most analytical methods employ alkaline degradation in a post-column reactor, followed by fluorometric detection.

Obviously, this detection method is useless for preparative work. Some of the reported methods use UV detection at 195 - 230 nm, however, the sensitivity of these methods is very low compared to fluorometric detection.

The reported methods with the longest retention times for TTX all use mobile phases in the pH range of 2.4 - 3.5. TLC experiments confirmed that the ketal linkage of TTXM and TTXL are readily hydrolyzed at these pH's.

After extensive experimentation, it was found that the ion-pairing technique of Yasumoto et al. [16] resolved the hapten mixtures into several peaks (Figure 2). However the retention times which were observed were less than half of the reported values for TTX under these conditions. The method used a C18 reversed-phase column with an isocratic mobile phase of acetonitrile/(0.003 N heptafluorobutyric acid in 0.005 N NH_4OAc).

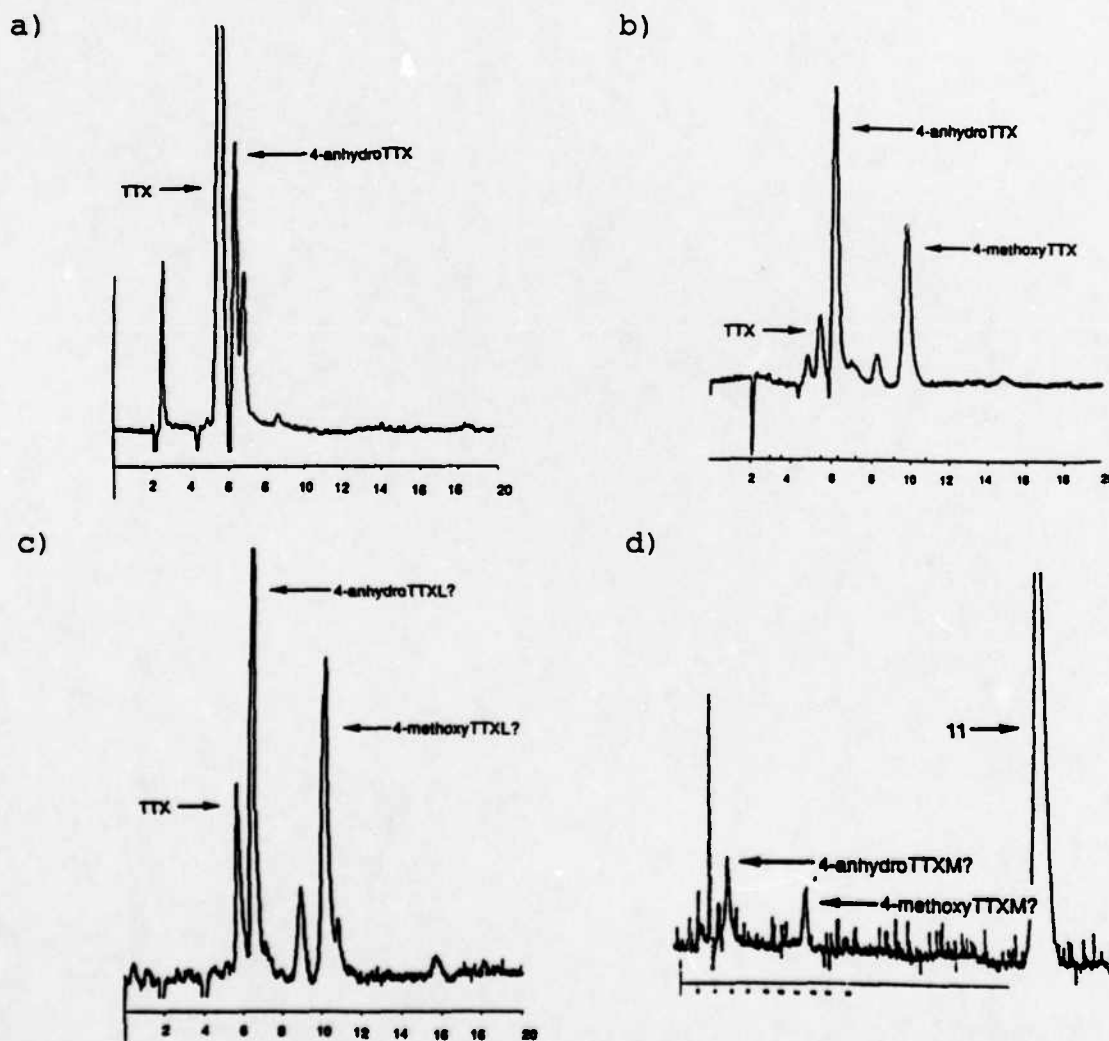


Figure 2. HPLC Chromatograms of a) TTX; b) mixture of 4-anhydro-TTX and 4-methoxyTTX; c) TTXL; and d) TTXM. C18 column; $\text{AcCN}/(0.005 \text{ N HFBA in } 0.005 \text{ N } \text{NH}_4\text{OAc})$; 1.0 ml/min.; detection - a)-c) UV, 205 nm, d) UV, 280 nm.

The acetonitrile concentration ranged from 1-11% depending upon which compounds are being chromatographed. UV detection at 206 nm gave good sensitivity for TTX, anhydroTTX, 4-methoxyTTX and the TTXL isomers. Linker 11 and TTXM isomers could be readily observed at 206 and 280 nm, however TTX was not detectable at 280 nm.

A sample of TTXM, which had been prepared at the beginning of Phase II and stored at -20 °C under argon, was shown by this method to have undergone extensive hydrolysis of the ketal linkage. In addition, the peak corresponding to 11 increased appreciably at the expense of the hapten peaks for an HPLC standard of freshly prepared TTXM which was stored in the elution buffer at 4 °C for 24 hours. These observations increased our suspicion that the hapten may be hydrolyzing, to some extent, during conjugation with the carrier proteins or following immunization.

Preparative HPLC separations of the hapten isomers and the anhydroTTX/4-methoxyTTX mixture were attempted using this system. Efforts to obtain good ¹H-NMR data on the purified samples were not successful. Either irreversible binding to the column resulted in poor recovery of the compounds or they decomposed during lyophilization of the buffer salt and HFBA. Separation of the hapten isomers was also attempted on charcoal and various ion-exchange columns. These methods either gave no separation or resulted in loss of the sample.

Change in strategy

At this point it was clear that use of the existing TTXM and TTXL haptens would not enable the accomplishment of our ultimate objective, the development of a useful immunoassay for TTX. The obvious approach was to synthesize alternative haptens which more closely resembled TTX itself, conjugate these new haptens, and proceed with antibody development. Both the literature and our experience with TTX chemistry in Phase I and II of this project indicated that the time required to produce new haptens could not be predicted. In light of this, the decision was made to continue immunizations with the undefined hapten (TTXF) prepared via Huot et al.'s procedure [19] in parallel with the synthesis of new defined haptens.

Alternate approaches to TTX haptens

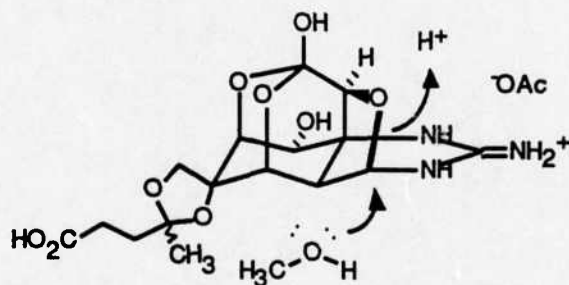
Our goal for alternative hapten synthesis was to develop stable haptens which were modified only at the C.6 and/or C.11 positions of TTX. The Phase II proposal included alternative strategies for hapten synthesis based upon Angelides' reductive amination of nortetrodotoxin [25]. Upon reexamination of the reported data, this approach was discarded due to the extremely low yields (~5%) and the lack of any spectral evidence to prove the structure. Instead, seven new strategies were proposed. One of these was abandoned after further literature investigation and the remaining six were attempted. Since our syntheses of TTXM and TTXL

proceeded in high yield, our first four approaches were attempts to modify this synthesis to obtain a useful TTX hapten.

In addition, two strategies for derivitization of TTX at the guanidinium group were proposed and investigated.

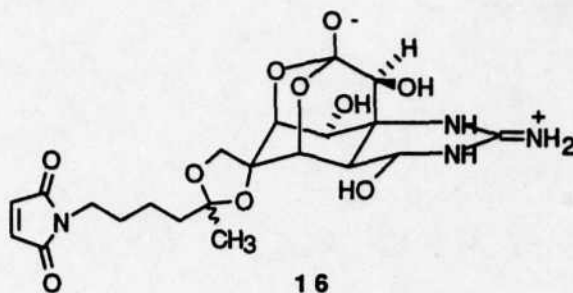
Opening of the anhydroTTX hapten: Mosher and Nachman reported the equilibration of 4-anhydroTTX and TTX in dilute HCl [26]. Since $^1\text{H-NMR}$'s of TTXM and TTXL had been obtained in 10% d_4 -acetic acid/ D_2O without hydrolysis of the ketal, we thought that it might be possible to open the anhydroTTXM under their conditions. Unfortunately, $^1\text{H-NMR}$ of solutions of both hapten mixtures in 5% $\text{DCl}/\text{D}_2\text{O}$ revealed that hydrolysis of the ketal occurs at a comparable rate to opening of the anhydro bridge.

Substitution of a bulky alcoholic solvent: Our second approach was based on the following analysis for the formation of 4-methoxyTTX analogs. Since 4-methoxyTTXL has the same stereochemistry at C.4 as TTX, it must arise via an $\text{S}_{\text{N}}2$ attack of methanol on the anhydroTTXL (or on anhydroTTX prior to ketalization). We hypothesized that this reaction would be suppressed by the substitution of a bulkier alcohol for the methanol.

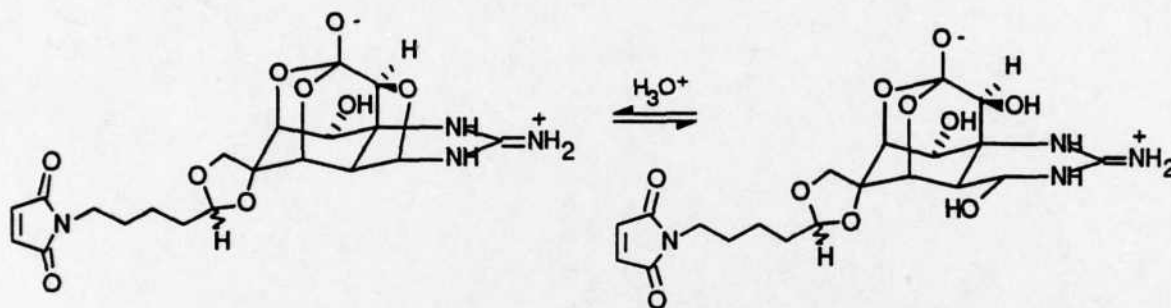


t-Butanol could not be used as the solvent for this reaction due to solubility limitations. Unfortunately, when the reaction was attempted in isopropanol, a mixture of the diastereomers of anhydroTTXL and 4-isopropoxyTTXL was obtained.

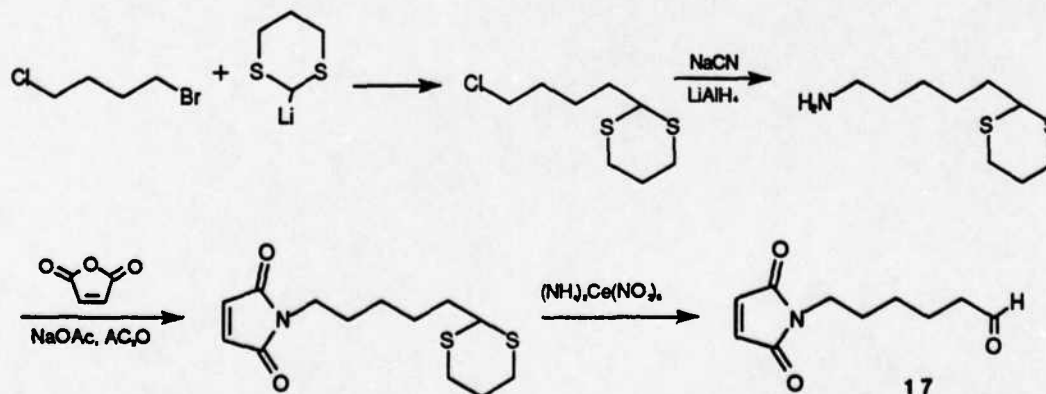
Formation of ketal/acetal hapten under non-nucleophilic conditions: We reasoned that if we could effect ketalization of TTX with **11** in a non-nucleophilic solvent, that only ketal with the native (**16**) or the anhydroTTX structure could form. This would prevent formation of the 4-methoxy analogs and result in a simpler, more easily characterized hapten mixture (2 isomers). TTX is insoluble in anything other than dilute acid, however, lyophilization of TTX from dilute aqueous trifluoroacetic acid gives TTX trifluoroacetate. Unlike native TTX or its other salts, this material is soluble in DMSO, DMF and water (pH 7.0) [27].



A variation of this approach was based on the fact that acetals are more resistant to hydrolysis than are ketals. This is a consequence of the secondary carbonium ion intermediate involved in hydrolysis of an acetal. Thus, we reasoned that, an acetal version of TTXM should be more resistant to hydrolysis and therefore amenable to acid-catalyzed equilibration of the anhydro form to the native C.4 substitution pattern.

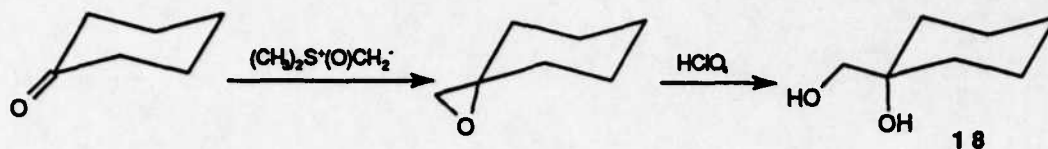


The required linker, 6-maleimidohexanal (**17**) was synthesized as shown below. Some difficulty was experienced in optimizing the final step, deprotection of the thioacetal group. A variety of standard conditions resulted in degradation and/or polymer formation. The aldehyde was finally obtained in 84% yield by the use of ceric ammonium nitrate.



Since several initial attempts to effect the ketalization of TTX with **11** in DMSO failed and we could find no reports of ketal formation under these conditions, we decided it was prudent to study this transformation in a simpler model system. 1-Hydroxy-

methylcyclohexanol (**18**) was prepared in a straightforward manner via treatment of cyclohexanone with dimethyloxosulfonium methylide followed by acid-catalyzed hydrolysis of the resulting epoxide.



Data for the reaction of **11** and **17** with **18** are summarized in Table 5. As shown by experiments 2 & 3, *p*-toluenesulfonic acid monohydrate (*p*-TSA·H₂O) is not an effective catalyst for this reaction even in the presence of activated molecular sieves. Drying the acid by repeated azeotropic distillation of benzene followed by heating at 50 °C under vacuum produced an active catalyst (experiments 4 & 9). Neither these conditions nor the standard conditions (experiments 1 & 8) drove the reaction to completion. The extent of the reactions were estimated from the ¹H-NMR of the crude products. From our earlier work, we knew that TTX reacts quantitatively under the standard conditions and failed to react at all with DMSO/catalytic *p*-TSA·H₂O. Experiments 5 & 10 demonstrated that use of a full equivalent of *p*-TSA in DMSO gave results as good as or slightly better than the standard conditions. Use of the Lewis acid, boron trifluoride, gave inferior results (experiment 6, 7, 11 & 12). The aldehyde linker **17** proved to be unstable to prolonged reaction under any of these conditions.

Treatment of TTX with **11** under the *p*-TSA (1 equiv.)/DMSO conditions failed to promote any reaction. Only unchanged TTX was recovered. Variation of the time, temperature, and stoichiometry of the reaction yielded the same result.

Based on the model study, we decided to attempt formation of the 6,11-(6'-maleimidylhexanal)TTX acetal under both the MeOH/HCl and *p*-TSA (1 equiv.)/DMSO conditions. The expected products of the first reaction were (by analogy with the ketal) the corresponding acetals (**21** & **22**) of anhydroTTX and 4-methoxyTTX.

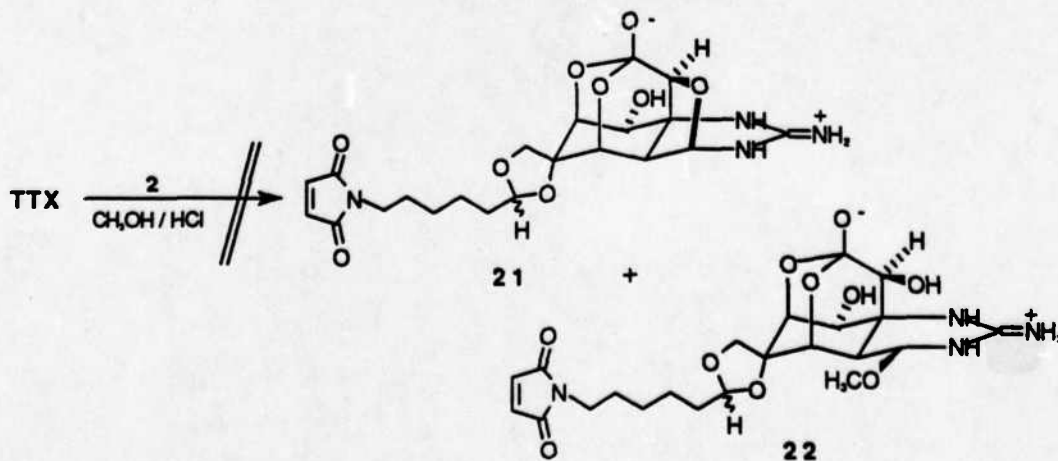
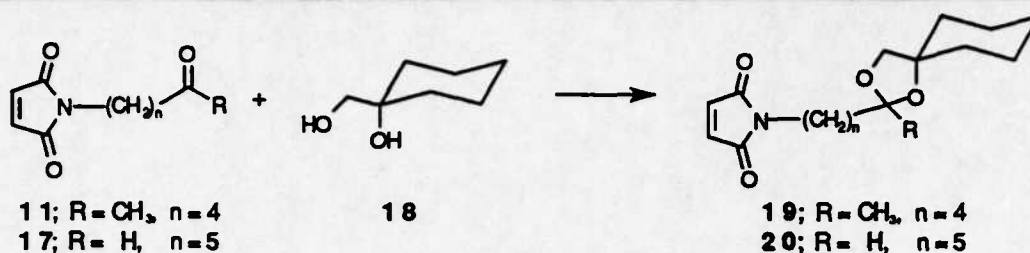
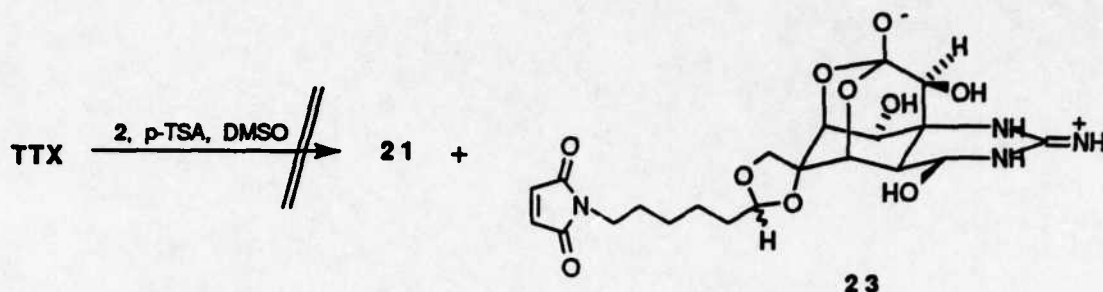


Table 5. Model Ketalization/Acetalization Studies

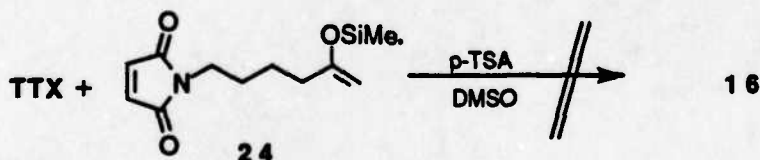
Experiment	Reagent	Conditions	Result
1	11	CH ₃ OH/HCl 50 °C, 72 h	~ 80% conversion, 19 was isolated and structure confirmed by NMR; dimethylketal of 1 formed as minor product.
2	11	DMSO/cat.p-TSA·H ₂ O 50 °C, 72 h	no reaction
3	11	DMSO/cat.p-TSA·H ₂ O/ 4A mol. sieves 50 °C, 72 h	no reaction
4	11	DMSO/cat.p-TSA (anhyd.) 50 °C, 72 h	slow formation of 19, mostly starting material after 3 days
5	11	DMSO/1 eq.p-TSA (anhyd.) 50 °C, 72 h	reached equilibrium after 24 h, ~ 90% conversion to 19
6	11	DMSO/cat.BF ₃ ·Et ₂ O 50 °C, 72 h	slow formation of 19, mostly starting material after 3 days
7	11	DMSO/1 eq. BF ₃ ·Et ₂ O 50 °C, 12 h	formation of 19 as well as several by-products; 19 subsequently decomposes
8	17	CH ₃ OH/HCl 50 °C, 72 h	~ 80% conversion, 20 was isolated and structure confirmed by NMR; Additional 17 added at 24 h intervals due to decomposition of the aldehyde.
9	17	DMSO/cat.p-TSA (anhyd.) 50 °C, 72 h	slow formation of 20, mostly starting material after 3 days; Additional 17 added at 24 h intervals due to decomposition of the aldehyde.
10	17	DMSO/1 eq.p-TSA (anhyd.) 50 °C, 72 h	reached equilibrium after 24 h, ~ 90% conversion to 20; Additional 17 added at 24 h intervals due to decomposition of the aldehyde.
11	17	DMSO/cat. BF ₃ ·Et ₂ O 50 °C, 72 h	formation of 20 as well as several by-products, 20 subsequently decomposes
12	17	DMSO/1 eq. BF ₃ ·Et ₂ O 50 °C, 12 h	formation of 20 as well as several by-products, 20 subsequently decomposes

The non-nucleophilic conditions were expected to give (20) and possibly the desired TTX acetal (22).



Surprisingly, both reactions produced identical products. There is no evidence for formation of the 4-methoxy derivative (21) in the first reaction. The major product appears to be an anhydroTTX derivative. The expected signals for the maleimide protons (δ 6.5) and the acetal proton (δ 4.9) are not present in the $^1\text{H-NMR}$. Major portions of the $^1\text{H-NMR}$ spectrum are obscured by signals from impurities. Attempted purification by high voltage paper electrophoresis and by HPLC gave poor recoveries of TTX-derived material, the spectra of which could not be fully interpreted. The lack of both the maleimide and the acetal proton absorptions proved that it was definitely not the desired acetal.

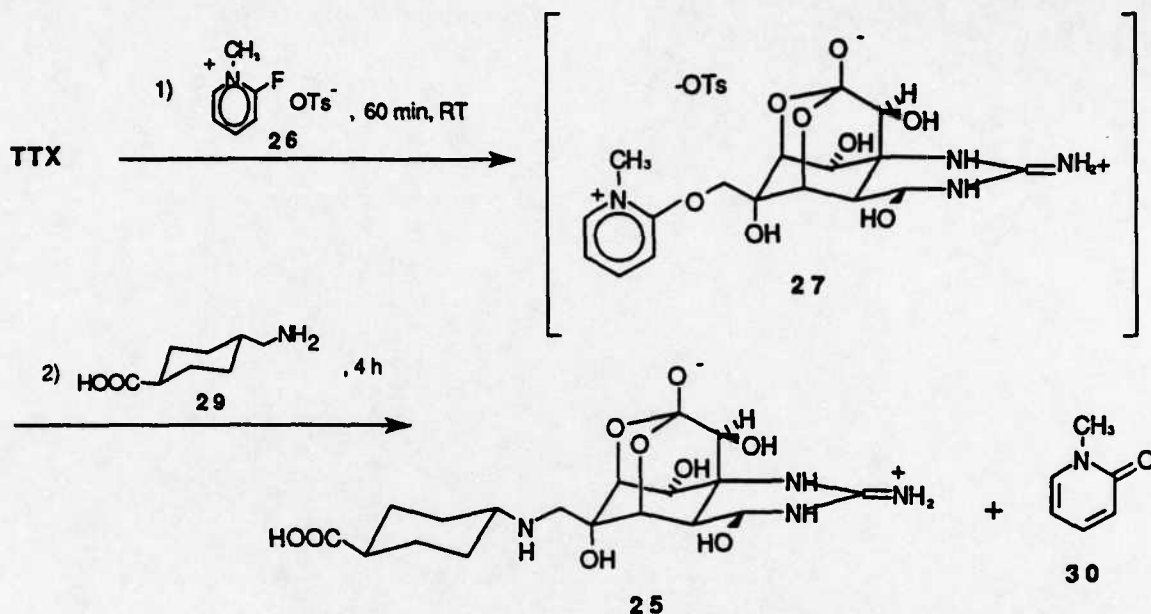
Trimethylsilyl enol ethers are known to react with diols to form ketals faster than the parent carbonyl compounds. There are known examples of the silyl enol ethers undergoing this reaction when the parent ketone was unreactive [36]. Therefore, the silylenol ether (24) of 11 was prepared via treatment of 1 with trimethylsilyl trifluoromethane-sulfonate and triethylamine. The reactions of 24 with TTX in DMSO with catalytic and stoichiometric *p*-TSA and catalytic $\text{BF}_3 \cdot \text{Et}_2\text{O}$ were investigated. None of these conditions resulted in formation of the desired ketal (16). The analogous reaction was not attempted with 17.



Based on these poor results, all further efforts to develop ketal or acetal based haptens of TTX were discontinued. As a result of this decision, preliminary efforts to synthesize radiolabeled analogs of 11 and levulinic acid were also abandoned.

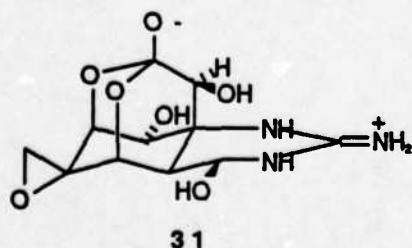
Activation by 2-fluoro-N-Methylpyridinium tosylate: An attempt was made to prepare a new, non-ketal based TTX hapten (25) via activation of TTX with 2-fluoro-N-Methylpyridinium tosylate (26) [37,38]. The initial attempt was done in water which had

been adjusted to pH 6.0. A mixture of TTX, tranexamic acid (29) and N-methyl-2-pyridone (30) was recovered. Apparently, hydrolysis of 26 is faster than the reaction with TTX or the tranexamic acid was added too early. Carboxylic acids react readily with 26 and the resulting products are rapidly cleaved in water.



The reaction was repeated in NH_4OAc buffer. There appeared to possibly be a small amount of 25 in the crude product. Evidence for this was the doubling of signals from both TTX and 29. The bulk of the mixture appears to be unreacted starting materials (TTX, 26 and 29). Attempted purification of this mixture failed. Subsequent attempts to repeat this result failed as well.

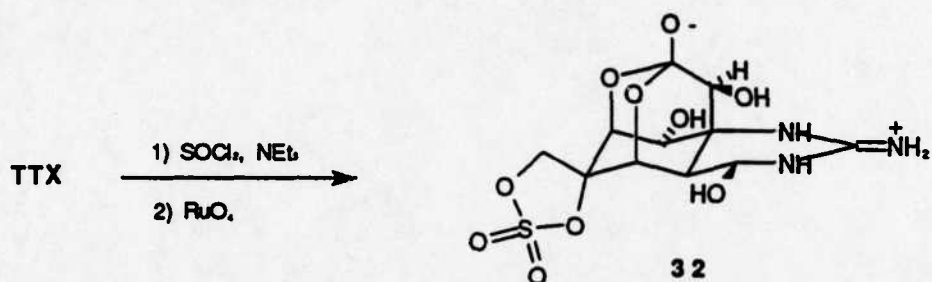
An obvious problem with this approach is the likely formation of the epoxide (31) via nucleophilic attack of the adjacent tertiary hydroxyl. If the epoxide had formed, the C.11 methylene singlet should have changed into an AB pattern. There was no such evidence for the formation of epoxide. In the event that 31 was isolated, it should have been possible to convert to a hapten via opening of the epoxide ring with an appropriately functionalized heterobifunctional reagent.



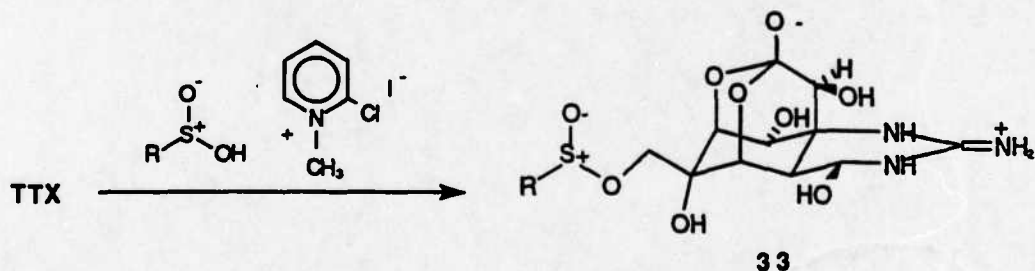
This reaction was attempted under several different conditions, in both anhydrous media and various aqueous buffers.

Only unreacted TTX was ever identified in the reaction products, no formation of 25, 27, or 31 was ever detected.

Cyclic sulfate/Sulfinic ester: On a similar scheme, Sharpless et al. have recently shown that cyclic sulfates are more reactive intermediates than epoxides and can be opened with a variety of nitrogen and sulfur nucleophiles [39,40]. Cyclic sulfates are prepared in a two step process. Treatment of a diol with thionyl chloride gives the unstable cyclic sulfite which is oxidized with catalytic ruthenium oxide in water. The major obstacle to investigation of this approach was to find an appropriate reaction solvent for the thionyl chloride reaction.



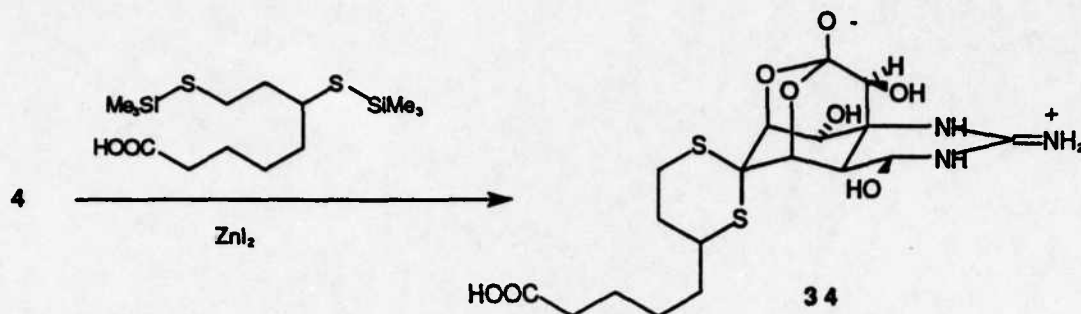
Alternatively, if the cyclic sulfate of TTX (32) could not be prepared, we thought that sulfinic ester derivatives (33) might have been accessible as shown [41].



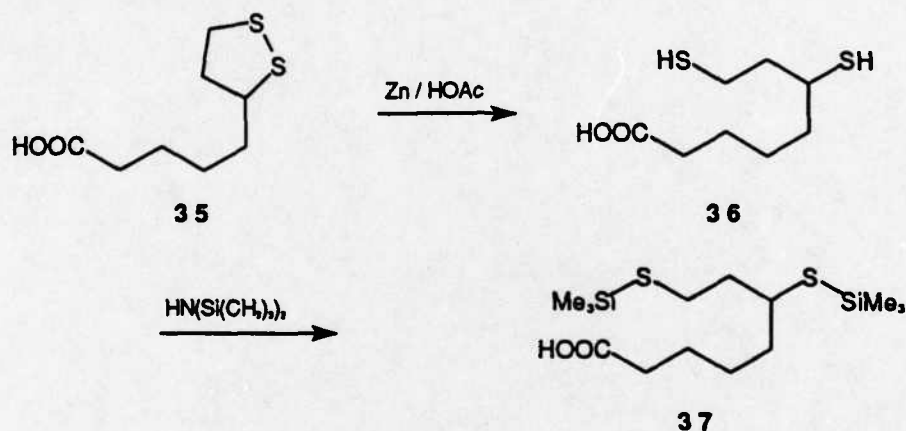
After an intensive review of the literature, we concluded that the solvents in which TTX is soluble were incompatible with the reagents needed for both of these transformations. We did not feel that it would be fruitful to commit any of our scarce supply of TTX to reactions with such uncertain outcomes. As a result, neither of these approaches proceeded past the planning stage.

Thioketalization of nortetradotoxin: Another proposed hapten was a thioketal of nor-TTX (4). Mosher and coworkers have developed a high-yield synthesis of 4, however, they also discovered that this ketone is not very reactive and failed to undergo ketalization with alcohols [8,26,27]. However, a number of unreactive ketones have been shown to react smoothly with 1,3-propanedithiobis(trimethylsilane) in the presence of trace amounts of Lewis acid [42] to form the corresponding thioketals. A TTX thioketal hapten such as 34 would be stable to hydrolysis,

possibly enabling the conversion of anhydro derivatives to the native form.

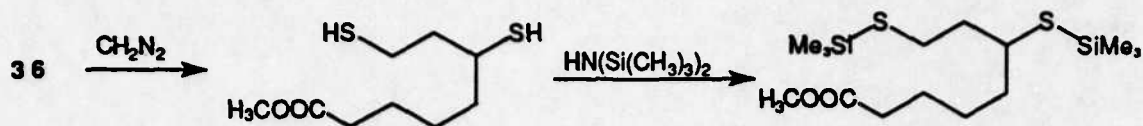


Thioctic acid (35) was reduced to 6,8-dimercaptooctanoic acid (disulfhydoctic acid, 36). Treatment of 36 with hexamethyldisilazane gave trimethylsilyl 6,8-dithiobis(trimethylsilane)octanoate (37).



No reaction was observed upon attempted thioketalization of a model ketone, cyclohexanone, with 37 and catalytic ZnI_2 in CH_2Cl_2 (standard conditions), acetonitrile or DMSO (polar solvents, e.g. DMSO or DMF are needed to solubilize 4). Addition of more catalyst failed to induce reaction. This was a surprising result since cyclohexanone has been reported to undergo thioketalization with 1,3-propanedithiobis(trimethylsilane) [42]. We hypothesized that the silyl ester was cleaved under the reaction conditions and the resulting carboxylate complexed with the ZnI_2 , effectively removing the catalyst and preventing the desired reaction.

To test this hypothesis, preparation of methyl 6,8-dithiobis(trimethylsilane)octanoate (38) was undertaken. Methyl 6,8-dimercaptooctanoate (39), was obtained by esterification of 36 with diazomethane. Surprisingly, treatment of 39 with hexamethyldisilazane failed to produce 38.

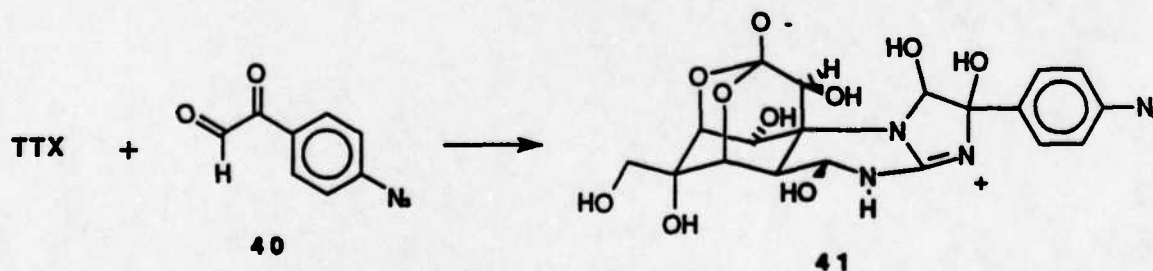


After several weeks of effort, it was discovered that **39** undergoes very facile oxidation to the corresponding disulfide upon exposure to air during workup. The sequence was repeated with extra precautions to avoid oxidation and **38** was finally successfully prepared, as evidenced by the successful thioketalization of cyclohexanone with this reagent.

We intended to try again to produce the corresponding acid, bis(trimethylsilyl)-6,8-dimercaptooctanoic acid, and verify its structure by reaction with cyclohexanone. For reasons discussed below, this synthesis was not completed.

Guanidinium Based TTX Haptens: The last two approaches were based upon derivatives of the guanidinium moiety of TTX. The only reported reaction of this group is acetylation (following peracetylation of the hydroxy groups) [8]. Mosher and Nachman failed in their attempts to selectively trifluoroacetylate the guanidinium group [8,26]. α -Dicarbonyl compounds, such as glyoxal and cyclohexadione, have been used to selectively mask arginine and guanosine residues in biochemical studies [43,44]. The products of this reaction are bishemiaminals (or biscarbinolamines) which are reasonably stable in neutral or acidic media.

We decided to investigate the reaction of TTX with a commercially available arginine-specific photoaffinity label, 4-azidophenylglyoxal (APG, **40**) [45]. Conjugation of the adduct **39** would be achieved via photolytic decomposition of the azido moiety to the very reactive nitrene. While immunogenic conjugates have been prepared by such nitrene linkages, the nonspecific nature of this reaction often results in poor yields and uncharacterizable conjugates [46].



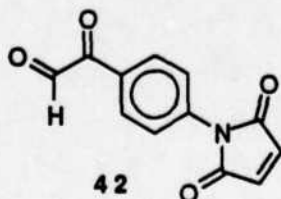
TTX was solubilized in D_2O by the dropwise addition of trifluoroacetic acid and treated with 1.1 equivalents of **40**. The reaction was kept at room temperature in the dark and monitored by 1H -NMR. No reaction had occurred after 1 day. After another day at RT, the mixture was kept at 50 °C overnight. The 1H -NMR was not fully interpretable but there appeared to be 5-10% of a new

compound. Excess **40** was removed by extraction with CH_2Cl_2 and the sample lyophilized. TLC of the reconstituted sample failed to detect any residual **40**. The UV spectrum indicated the presence of an aromatic ring in the product. $^1\text{H-NMR}$ confirmed that the sample was mostly TTX but indicated the presence of minor compounds which appear to be derived from TTX and APG, presumably these are TTX-APG adduct **41** and its regio- and stereoisomers (16 possible isomers).

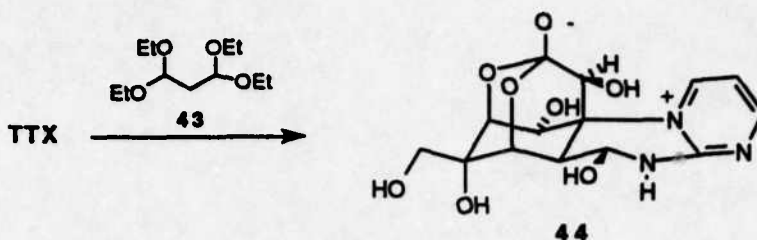
On the basis of this evidence, it could not be conclusively determined whether **41** had been formed. It was decided to proceed with a fresh preparation and immediately conjugate the product to KLH and BSA by photolytic decomposition to the nitrene.

A 10 mg sample of TTX was treated with TFA and **40** in H_2O at 50°C for 24 hours. The solution was cooled to RT and extracted with CH_2Cl_2 . The aqueous solution was divided into two portions. Following lyophilization, one portion was mixed with BSA at a molar ratio of 1:1 (TTX-APG:BSA), and the other with KLH at a molar ratio of 10:1 (TTX-APG:KLH). Both samples were exposed to long wavelength UV light (366 nm) for 3 hours to allow conjugation to occur. Microtiter plates were coated with each conjugate, then treated with AP-T20G10 followed by AP substrate (see Direct CIEIA section). Slight specific color development was observed, indicating that at least some conjugation of TTX to the protein had occurred. This in turn indicated that synthesis of **41**, in low yield, had been successful.

Two BALB/c and 2 Swiss-Webster mice were immunized with the KLH-TTX-APG conjugate. After 3 booster injections with this material in Freund's Incomplete Adjuvant, none of the mice had responded to the immunogen, as judged by the ELISA reactivity of their sera against BSA-TTX-APG. This approach was unsuccessful in stimulating production of antibodies against TTX. If any indication of antibody production had been observed, it was our intention to prepare a new glyoxalate linker, 4-maleimidylphenyl-glyoxalate (**42**), so as to permit the production of a more readily characterizable BSA conjugate for use as the coating antigen.



Another reagent which has been used to specifically block arginine residues is malonaldehyde. The product of the reaction is a stable pyrimidine ring [47]. Treatment of a D_2O solution of TTX with the bis(diethyl)-acetal of malonaldehyde (**43**) in the presence of TFA at 50°C for 2 days produced a new TTX derivative containing a heteroaromatic ring. Presumably this product is a mixture of **44** and its regioisomer.



Our plan, at this point, had been to develop a new malonaldehyde derivative substituted at C.2 with an appropriate protein-reactive functional group. Before either this approach or the thioketal could be further investigated, the decision was made that there was insufficient time remaining in the project to hyperimmunize mice and produce MABs against another TTX hapten, even if one were to be produced. In addition, development of an immunoassay using the TTXF hapten was progressing well, therefore, all efforts to develop new, characterized haptens were terminated in early June, 1990.

Production of Murine Antisera with KLH-TTXF

As reported above, a weekly immunization schedule had been adopted in response to unsatisfactory ELISA results with the original KLH-TTXM and KLH-TTXL immunogens. Since this approach failed to induce higher titer anti-TTX responses in the mice, when the stock supply of KLH-TTXM was depleted, one of these mice (MA-1 from group M3) was switched over to immunizations with KLH-TTXF. The immunization schedule of this mouse is shown in Table 6.

Table 6. Mouse MA-1 Immunization Schedule:

KLH-TTXM		KLH-TTXF	
1°	12/3/88	5°	8/17/89
2°	1/3/89	6°	8/25/89
3°	7/21/89	7°	8/31/89
4°	7/28/89	8°	9/7/89
		9°	9/8/89
		10°	9/9/89
		11°	9/10/89

The anti-BSA-TTXM response of antisera from mouse MA-1 (the KLH-TTXM/KLH-TTXF mouse) was shown to be significantly inhibited by free TTX. In addition, the anti-BSA-TTXF response of antisera from the mice being immunized with KLH-TTXF was demonstrated to be slightly inhibited by TTX. It was decided to commence hybridoma production with the spleens and/or lymph node cells taken from these mice.

Monoclonal Antibody Production and Characterization

On each of the preceding 4 days before fusion, the mice received consecutive injections with the appropriate immunogen in PBS. This procedure was reported to enhance the frequency of antigen-specific monoclonal antibody production [48]. On 9/13/89, the spleen of mouse MA-1 was taken for hybridoma production.

Preliminary ELISA screenings of the hybridoma supernatants demonstrated 72 wells (out of 356 total wells plated) with activity against the maleimide coating antigen (BSA-TTXM). Out of these, just 5 wells showed partial inhibition (up to 50% inhibition at 100 ng/ml) to free TTX. The hybridoma cells contained in the 5 wells were re-cloned and evaluated. None of these exhibited sufficient inhibition by TTX to warrant further work.

The mice which had been immunized with KLH-TTXF were used to prepare hybridomas and screened for anti-TTX monoclonal antibodies. Based on Huot's reports, we were not optimistic about finding any useful antibodies. A summary of hybridoma results is presented in Table 7.

Table 7. Hybridoma Summary

Fusion	Immunogen	Date	# wells plated	Primary screen positive	Secondary screen positive
3	KLH-TTXM/KLH-TTXF	9/13/90	356	72	5 ^a
4	KLH-TTXM	9/13/90	508	62	0
5	KLH-TTXF	10/17/89	672	33	1 ^b
6	KLH-TTXF	11/20/89	1478	45	0

a. no inhibition by TTX following re-cloning and further evaluation

b. produced MAbT20G10

Hybridoma T20G10 was produced in fusion 5, and produces an IgG₁,k monoclonal antibody reactive with TTX. The affinity of this antibody has been estimated to be 1.2×10^8 L/M, using a solid phase ELISA method described by Beatty et al [49].

T20G10 has been subcloned four times, thus far, to provide a stock of cultures cryopreserved in liquid nitrogen (Table 8).

Table 8. Cryopreserved Cultures of T20G10 Hybridoma Currently on Deposit at HBG

Clone	Number of Cryopreserved Cultures	Cloning Efficiency ¹
T20G10 ²	1	
T20G10.1	2	2/31
T20G10.2 ³	1	
T20G10.1.1	7	1/89
T20G10.1.1.1	3	30/32
T20G10.1.1.2	4	
T20G10.1.1.3	2	
T20G10.1.1.4	2	
T20G10.1.1.1.1	6	47/47
T20G10.1.1.1.2	5	

1. secreting clones/tested clones

2. uncloned

3. ceased secreting upon expansion

Production of T20G10 Ascitic Fluid

In January, 1990, a total of 14 pristane-primed BALB/c mice were injected with hybridoma cells from clone T20G10. Only one of these mice actually produced ascitic fluid, and this mouse produced only 6 ml. Ten more BALB/c mice were therefore primed by intraperitoneal injection of 0.5 ml Freund's Incomplete Adjuvant [34], then injected intraperitoneally 24 hours later with 5×10^6 recloned T20G10 hybridoma cells per mouse. By twelve days after injection of cells, all ten mice had produced ascites, and 40 ml of ascitic fluid has been collected. This ascitic fluid had an end point titre of 1 in 20480 in an indirect ELISA system using microtiter plates coated with BSA-TTXF and Alkaline Phosphatase labelled goat anti mouse IgG/IgM conjugate.

Approximately 5 mg of this monoclonal antibody was purified from 3.5 ml of T20G10 ascitic fluid by discontinuous pH gradient elution from Protein A - Sepharose [50]. The Specific Activities in ELISA of ascitic fluid and purified MAb were calculated. Purification of MAb by this method resulted in a 10-20 fold increase in ELISA Specific Activity, with a 13% yield of purified protein to total protein in ascitic fluid.

Delivery of T20G10 Ascitic Fluid and Hybridoma Cells to USARIID

In September 1990, a further 13 mice, primed with Freund's Incomplete Adjuvant, were injected intraperitoneally with T20G10 hybridoma cells. Ascitic fluid with an ELISA titer of 1 in 12,500 was collected from these mice. A total of 55 ml of fluid has been shipped to Dr. John Hewetson at Fort Detrick for evaluation of its TTX neutralizing activity in his system.

Hybridoma T20G10 was cloned to ensure a monoclonal hybridoma cell line, and 4 vials of these cells were also sent to Dr. Hewetson.

Indirect CIEIA System using T20G10 MAb

In a limited number of tests performed with an indirect CIEIA system, using microtiter plates coated with BSA-TTXF and Alkaline Phosphatase labelled goat anti mouse IgG/IgM conjugate, a 1 in 1000 dilution of the ascitic fluid detected free TTX at an IC_{50} of 35-90 ng/ml and an IC_{20} of 12-30 ng/ml. Figure 3 shows the standard curve for T20G10 MAb in the Indirect CIEIA system.

Direct CIEIA using Alkaline Phosphatase-T20G10 Conjugates

The use in EIA systems of analyte specific monoclonal or polyclonal antibodies directly conjugated to enzyme has many advantages. By avoiding the need for a species specific enzyme labelled antibody conjugate, it reduces the number of steps by one,

resulting in a proportional reduction in total assay time and reduces the assay cost in terms of both reagents and time.

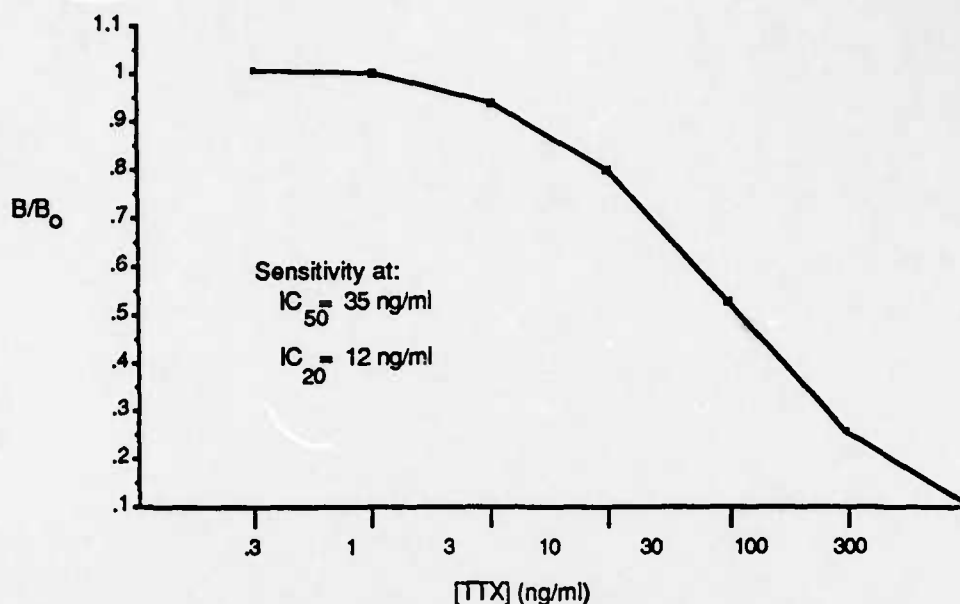


Figure 3. Indirect CIEIA: Standard Curve for Tetrodotoxin Detection.

Preparation of AP-T20G10 Conjugates: During Year 2 of this project, considerable effort was expended on investigating methods for preparing alkaline phosphatase-MAb conjugates. The one step and two step glutaraldehyde methods [51,52] did not prove to be suitable for MAb conjugates, so an appropriate maleimide conjugation method was sought.

Two alkaline phosphatase conjugates were therefore prepared from Protein A - Sepharose purified T20G10 MAb using the thiolating agent *S*-acetylmercaptosuccinic anhydride (SAMSA) and the heterobifunctional coupling agent succinimidyl 4-(*N*-maleimido-methyl)-cyclohexane-1-carboxylate (SMCC) [53]. Ratios of SMCC to AP of 25:1 and 5:1 were used with the aim of producing a conjugate with the best activity possible. A large amount of protein precipitation occurred in both preparations during the conjugation step, suggesting that the incubation time stated in the published method was too long for this antibody. In spite of this denaturation, both conjugates exhibited acceptable levels of activity in ELISAs (reactive at dilutions down to 1 in 4000 - 1 in 8000), and enabled similar levels of sensitivity for tetrodotoxin detection in CIEIAs when tested initially. A stability trial was therefore set up on both conjugates stored at 4 °C (see section on conjugate stability trials).

Unfortunately, the SMCC/SAMSA method has not proved to be a reliable method for preparation of AP-T20G10 conjugates. During the past year, 6 conjugations have produced only 3 AP-T20G10 conjugates that are usable in ELISA systems. This poor success

rate is thought to have been due to several factors. One factor seemed to be the supplier of AP enzyme. Another was low reproducibility in the number of -SH groups introduced into T20G10 immunoglobulins by treatment with SAMSA; substituting a molar ratio of SAMSA to immunoglobulin of 25:1 for the 100:1 published in the original method [53] seemed to solve this problem.

Direct CIEIA using AP-T20G10 conjugates: In the direct CIEIA system, each conjugate (at a working dilution of 1 in 500) reproducibly detected 5-10 ng/ml of tetrodotoxin. Thus, we had developed a direct CIEIA using an AP-MAb conjugate, that exhibited sensitivity superior to the indirect CIEIA (that employs MAb followed by AP-anti mouse Ig), and had a total test time of one hour (30%) less. Figure 4 shows the standard curve for AP-T20G10 MAb conjugate in the direct CIEIA system.

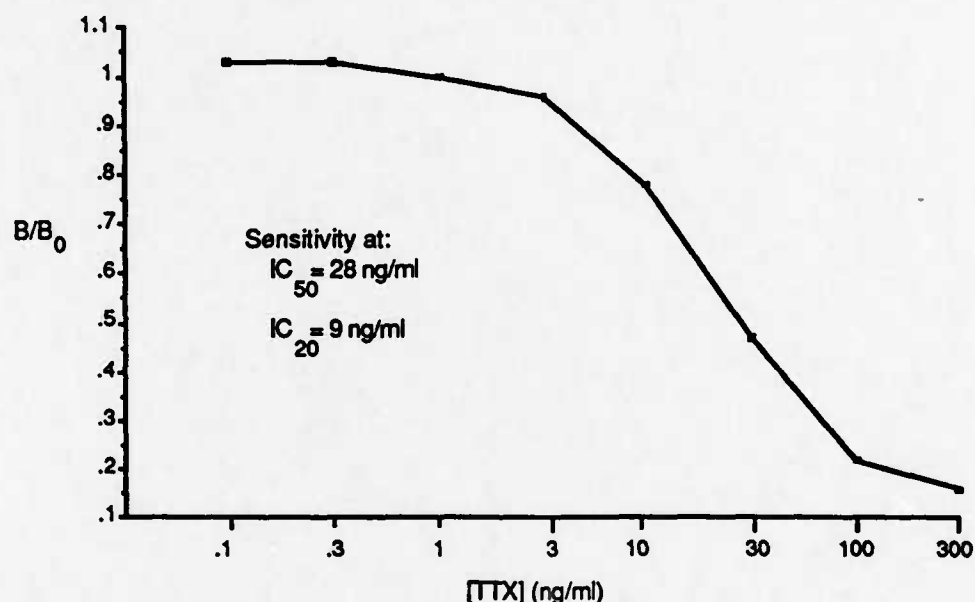


Figure 4. Direct CIEIA with AP-MAb Conjugate: Standard Curve for Tetrodotoxin Detection.

Continued optimization of the direct CIEIA system enabled increases in its sensitivity, finally resulting in a TTX detection limit at an IC_{50} of 8-10 ng/ml and an IC_{20} of 2-3 ng/ml (Figure 5).

Production of Rabbit Polyclonal Antiserum against TTX

A new batch of KLH-TTXF was prepared for hyperimmunization of rabbits. Three rabbits were given priming immunizations on July 25, 1990 and booster injections every 2 weeks thereafter.

By October 1990, only one of the these rabbits exhibited a slight reaction in ELISA against BSA-TTXF; all three exhibited significant reactivity against BSA. A modified immunization schedule, that had been successfully used to hyperimmunize mice with KLH-TTXF, was therefore adopted.

Injections were administered each week instead of every two weeks, and the concentration of TTX-F-KLH in each injection was doubled to 63 μ g per rabbit. By mid-October, the ELISA titers of rabbits #1, #2 and #3 had reached 6400, 100, and 1600, when rabbit #1 died suddenly. Immunization of the remaining two rabbits was continued until early November, when both rabbits were sacrificed and exsanguinated. Serum from this final bleed from rabbit #2 had an insignificant titer, while serum from rabbit #3 had an ELISA titer of 1 in 25,000.

Serum from the final bleed from rabbit #3 was used to develop an indirect CIEIA system. The TTX-detection limit of this system was compared with that of the direct CIEIA using AP-T20G10. Figure 5 shows that whereas the direct CIEIA with AP-T20G10 detects TTX with an IC_{20} (detection limit) of about 3 ng/ml, the indirect system with rabbit #3 serum exhibited an IC_{20} for TTX of approximately 300 ng/ml.

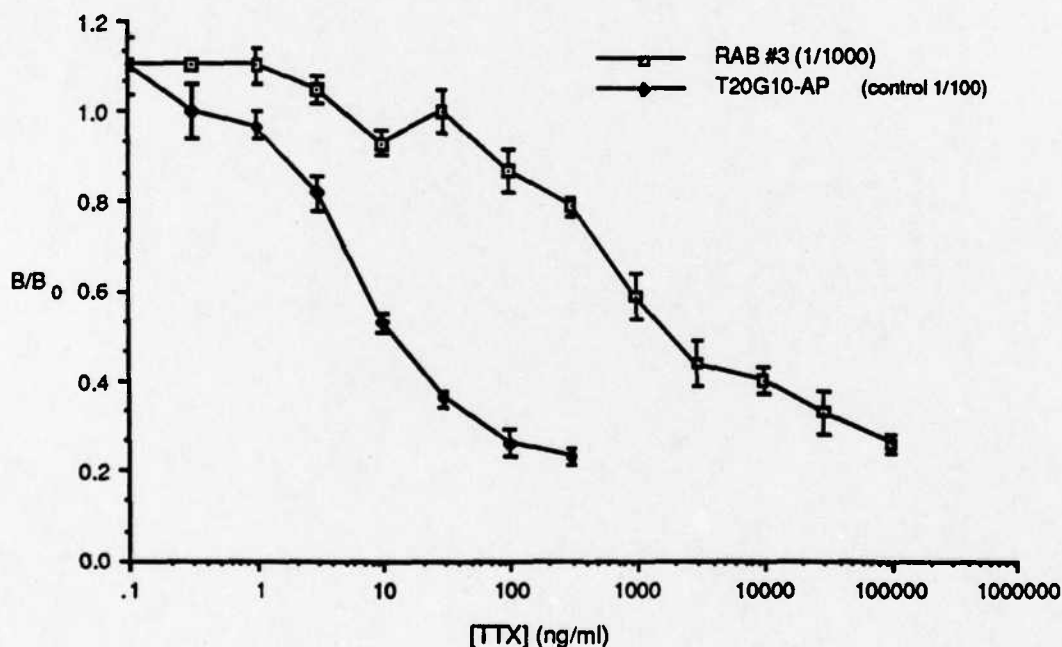


Figure 5. Comparison between Standard Curves for TTX CIEIAs using rabbit #3 Serum and AP-T20G10.

Stability Trials on TTX CIEIA Reagents

Stability of AP-T20G10 Conjugates: Several studies on the stability of AP-T20G10 conjugates to storage were set up during the two years of this Phase II project, and the results are summarized in Figures 6-8. These figures show the mean optical density value obtained from duplicate tests on the conjugate in the standard ELISA system (Appendix O), using the working dilution established for that conjugate when the stability trial was originally set up. Conjugates that gave optical density readings between 0.2 and 0.8,

when tested at their working dilution in the standard ELISA system, were considered satisfactory.

The original liquid AP-T20G10 conjugate, (prepared February 9, 1990 and stored in 0.01 M Tris-HCl buffer, pH 6.8 containing 0.1 M NaCl, 1 mM MgCl₂, 0.1 mM ZnCl₂, 0.5 g/l NaN₃ and 1% BSA) exhibited no significant drop in activity after 329 days storage at 4 °C (Figure 6).

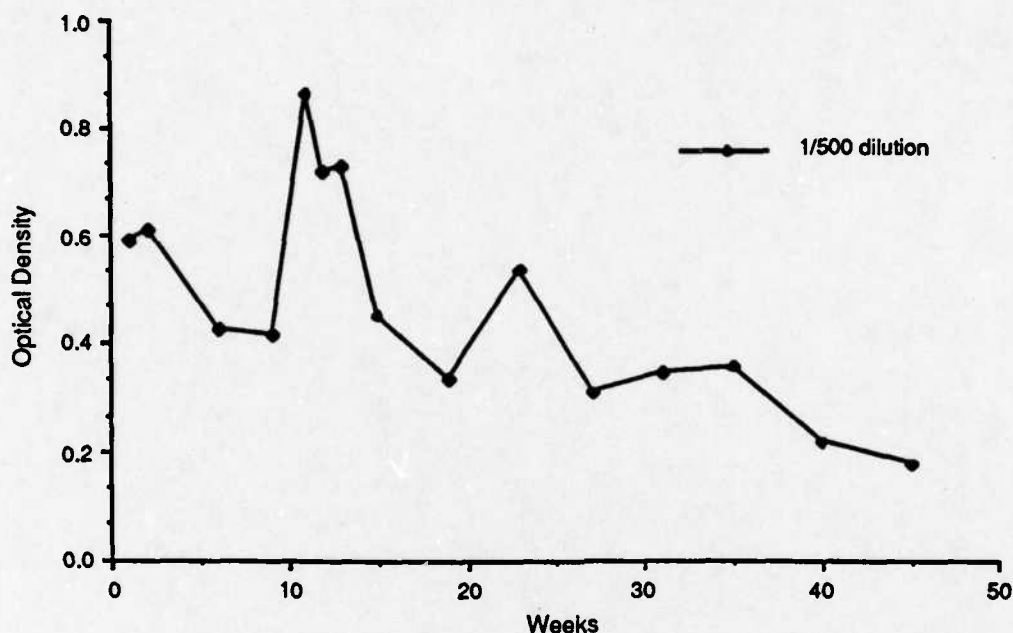


Figure 6. Stability of T20G10-Alkaline Phosphatase Conjugate Stored at 4 °C in 1% BSA.

Another AP-T20G10 conjugate was prepared in June 1990, and both liquid and lyophilized aliquots were stored at -20 °C, 4 °C and 37 °C. One set of liquid conjugate aliquots at -20 °C and 37 °C contained 50% glycerol as a stabilizing agent. Another set of liquid conjugate aliquots containing no added glycerol, was placed at 4 °C, as a control for the lyophilized samples.

The data in Figure 7 show that activity of the liquid conjugate stored at 37 °C dropped steadily, until after 24 weeks it became unacceptable. After this time period, this conjugate failed to perform satisfactorily even when tested at lower dilutions. The liquid conjugate stored in the freezer also exhibited a steady drop in activity, and after 24 weeks its working dilution had dropped from 1 in 1000 to 1 in 200. Liquid conjugate, stored without glycerol, still exhibited acceptable activity after 12 weeks storage at 4 °C (Figure 8).

Lyophilization of conjugate resulted in a very slight drop in ELISA activity (Figure 8). Lyophilized conjugate, however, exhibited no

further significant drop in activity after storage for 12 weeks at either 4 °C or 37 °C (Figure 8).

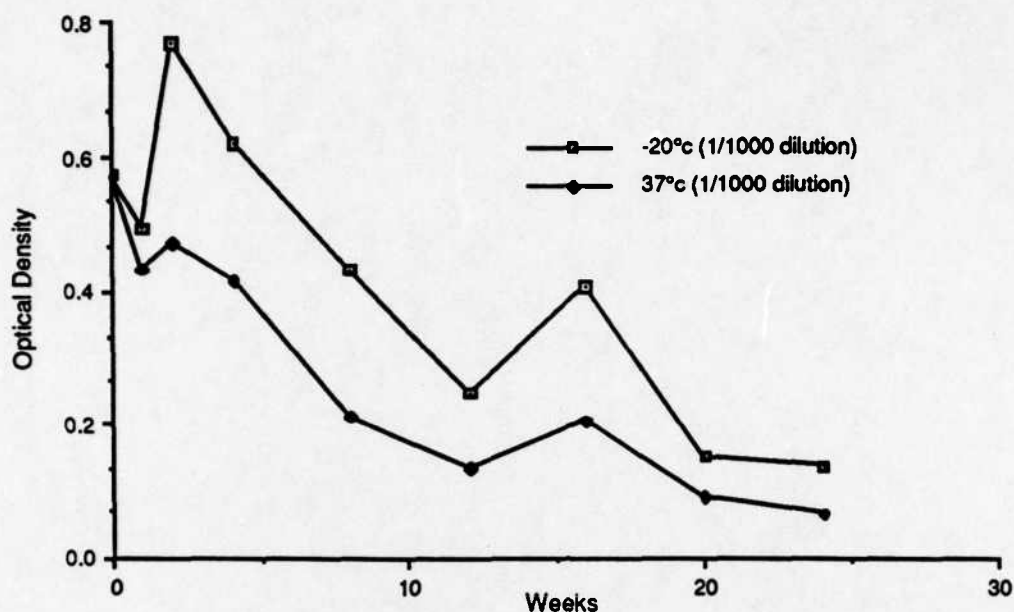


Figure 7. Stability of T20G10-Alkaline Phosphatase Conjugate Stored at -20 °C and 37 °C in 50% Glycerol.

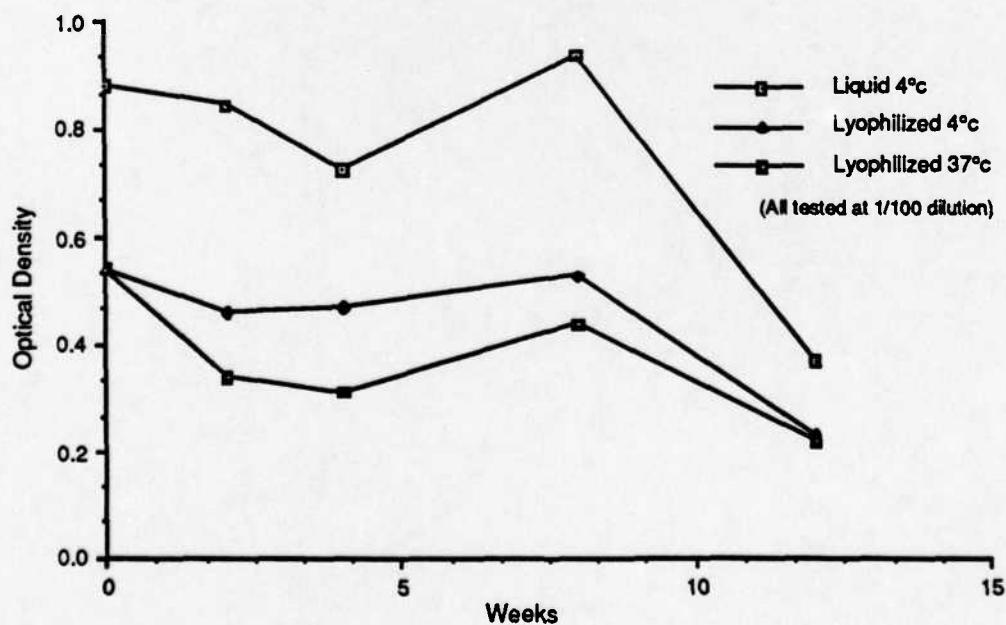


Figure 8. Stability of T20G10-Alkaline Phosphatase Conjugate Stored as Solution in 1% BSA and Lyophilized Powder from 1% BSA Solution.

Stability of ELISA Coating Antigen: Data on the TTX-F-BSA coating antigen indicates no drop in activity after storage at 4 °C for 12 months.

Validation of Direct CIEIA System

Reactivity with TTX-Congeners: The cross-reactivity of the TTX direct CIEIA with other available TTX congeners was investigated. Tetrodonic acid (2) was successfully synthesized and purified in our laboratory according to the method of Tsuda [7]. The TTX direct CIEIA did not cross react with this TTX congener. In addition, samples of anhydroTTX (12) and tetrodonic acid were supplied to us by Professor Takeshi Yasumoto of Tohoku University (Japan) and tested for cross-reactivity in the TTX direct CIEIA. While the IC₅₀ for TTX in this assay is 5-10 ng/ml, the IC₅₀s for these samples of 12 and 2 were 300 ng/ml and >>10,000 ng/ml respectively. By comparison, the indirect CIEIA using rabbit #3 serum detects 12 at a similar level to TTX, whereas 2 was not detected by this system at concentrations below 1000 ng/ml (data not shown). This indicates that HBG's direct CIEIA is effectively TTX-specific.

Evaluation of TTX CIEIA System with TTX-spiked Human Plasma Samples: The use of pooled human serum or plasma spiked with TTX as sample in the direct CIEIA initially resulted in a high degree of matrix interference. This problem was overcome by increasing the AP-T20G10 conjugate concentration, which enabled detection of similar TTX levels in plasma to those detectable in buffer.

Further human plasma specimens were obtained from 7 volunteers at HBG. Individual samples were spiked with TTX and tested in the TTX direct CIEIA. While the IC₅₀ of the CIEIA for detecting TTX in buffer in this particular experiment was about 3 ng/ml, the IC₅₀ with different individual human plasma samples ranged from 0.5 to 70 ng/ml. The IC₂₀ for detecting TTX in buffer in this experiment was approximately 0.15 ng/ml, and the IC₂₀ with different plasma samples ranged from this level to 4 ng/ml.

Correlation of TTX Direct CIEIA System with the Mouse Bioassay and HPLC: The TTX CIEIA was validated by direct comparison with the mouse bioassay and an HPLC method. Our intention was to utilize the official Japanese mouse bioassay protocol [11], however, this protocol required the use of the ddY strain of mice, a strain only obtainable from Japan. Extensive investigations revealed that the University of Hawaii LAS could obtain strain ddY mice for us, however, due to administrative procedures required to satisfy State of Hawaii animal importation restrictions, these mice would not arrive until after the completion date for the 3 month extension of the TTX project. The decision was therefore made to use Mosher's bioassay method [8], which utilizes Swiss-Webster mice.

The most sensitive HPLC assay for TTX, reported to date, is that of Professor Takeshi Yasumoto of Tohoku University (Japan)

[16]. This is the same method which we attempted to adapt for the separation of the TTXM and TTXL haptens. Professor Yasumoto offered to perform the HPLC measurements in his laboratory, since HBG's HPLC apparatus lacked the required fluorometric detector and post-column reactor. His offer to collaborate was accepted.

Two large porcupinefish (*Diodon hystrix*), caught off Makapu'u Point, O'ahu, were obtained from Sea Life Park. The porcupinefish is closely related to the pufferfish and is known to be tetrodotoxic [54]. The livers were removed from these fish and samples prepared for analysis via the method of Yasumoto et al. [16] (see Figure 9). Since relatively few cases of pufferfish poisoning have been reported in Hawaii and the toxicity of species is known to vary widely with the geographic location [54,55], we could not be confident that these fish contained appreciable amounts of TTX. Therefore, both fish extracts were divided into halves and half of each spiked with pure TTX. A set of six TTX standards was prepared by serial dilution of a pure TTX solution.

Spiked and unspiked samples were split into 3 aliquots and lyophilized. One aliquot of each was sent to Dr. Yasumoto for HPLC analysis. The second and third aliquots were tested by mouse bioassay and CIEIA respectively. All samples were reconstituted in 0.1 N acetic acid prior to assaying.

Table 9 summarizes the data from all three assays. This data is presented in graphical form in Figures 10-14. Dr. Yasumoto experienced difficulty in reconstituting the spiked fish extract samples, no such problems were observed at HBG. The spiked fish extracts gave anomalous results in all three assays, therefore, the data was plotted both with and without this data to establish the degree of correlation between the assays.

Table 9. Correlation of TTX CIEIA with Mouse Bioassay and HPLC.

Sample #	[TTX] $\mu\text{g/ml}$ as determined by				
	weight	CIEIA		Mouse Bioassay	HPLC
1	30.00	27.00	± 2.70	34.83 ± 7.45	31.79
2	10.00	6.80	± 0.38	13.65 ± 2.90	10.39
3	3.30	1.90	± 0.15	5.15 ± 0.31	3.98
4	1.10	0.54	± 0.07	0.91 ± 0.50	2.45
5	0.37	0.30	± 0.05	---	0.34
6	0.12	0.10	± 0.002	---	0.28
7 (fish 1)	?	<0.008		n.d.	<0.04*
8 (fish 1, spiked)	? + 25.00	40.32	± 2.82	30.92 ± 5.14	16.96*
9 (fish 2)	?	<0.008		n.d.	<0.04*
10 (fish 2, spiked)	? + 25.00	32.90	± 1.97	31.05 ± 2.70	17.70*

n.d. = not detected

* no other natural TTX analogs were detected

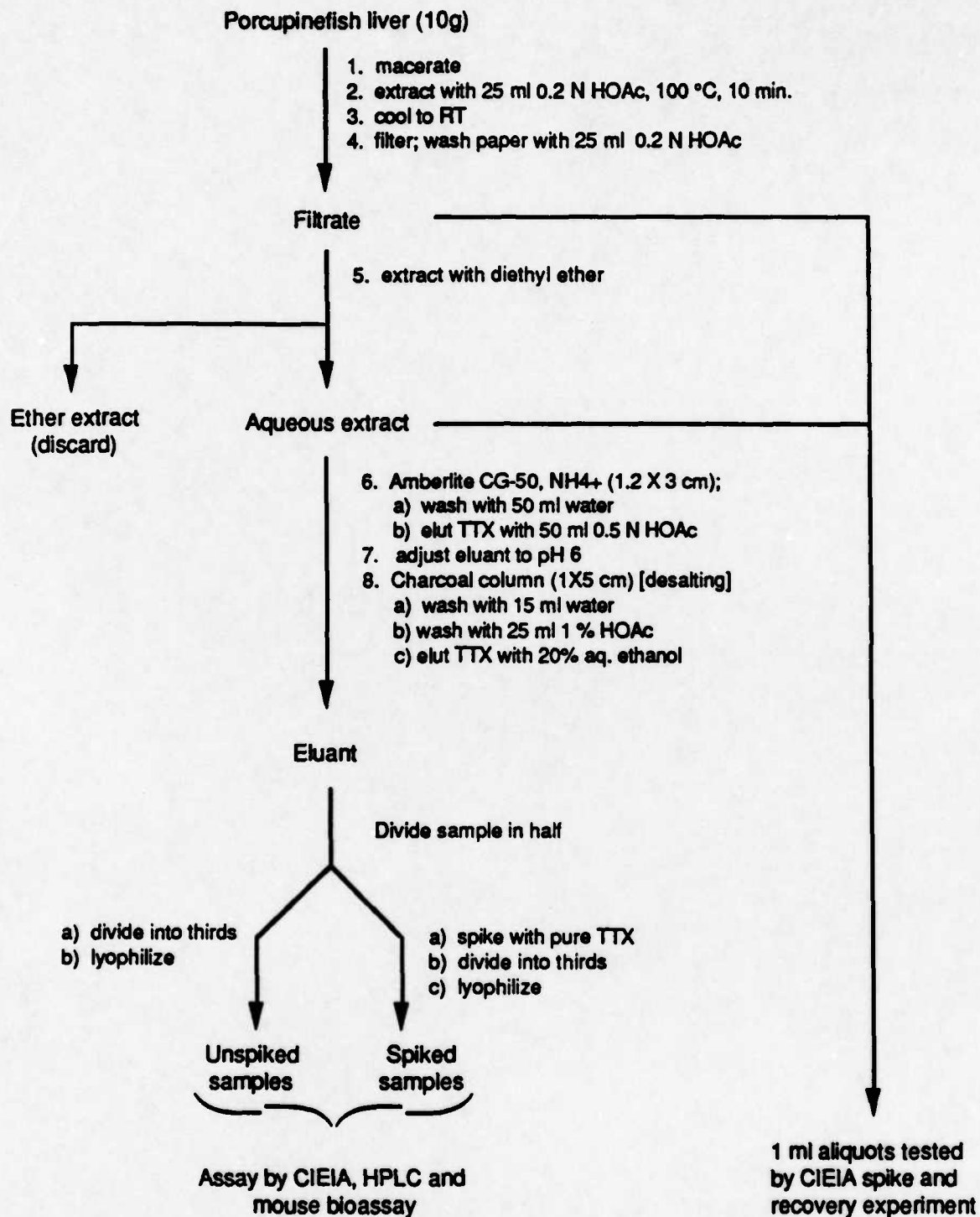


Figure 9. Preparation of Porcupinefish liver Extracts for TTX Determination.

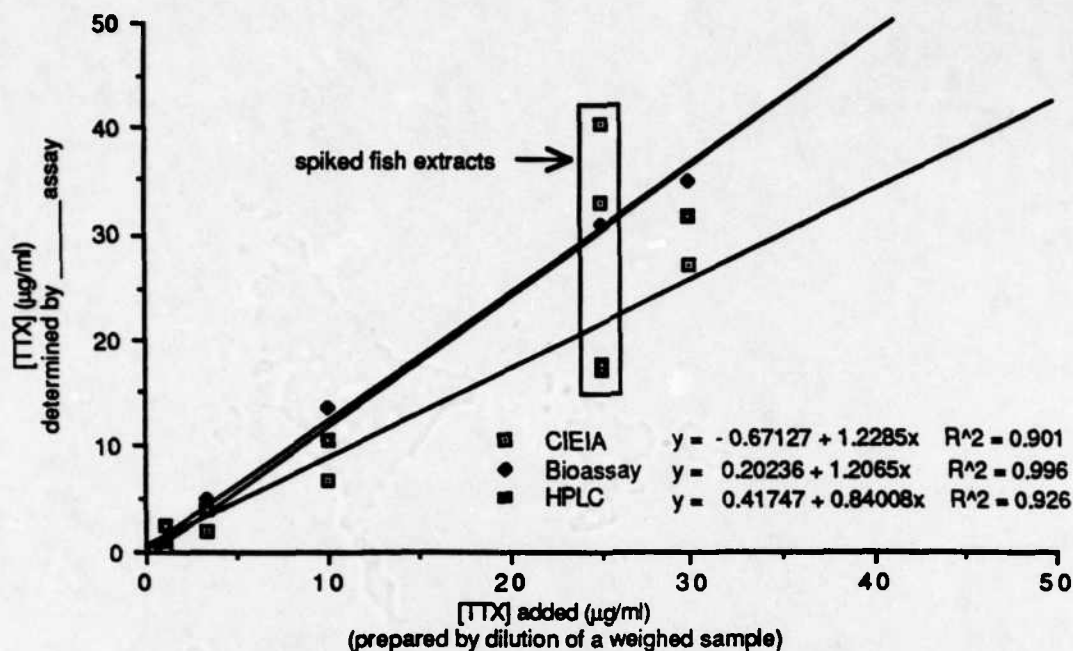


Figure 10. Correlation of CIEIA, HPLC, and Mouse Bioassay with TTX Standards and Spiked Fish Extracts.

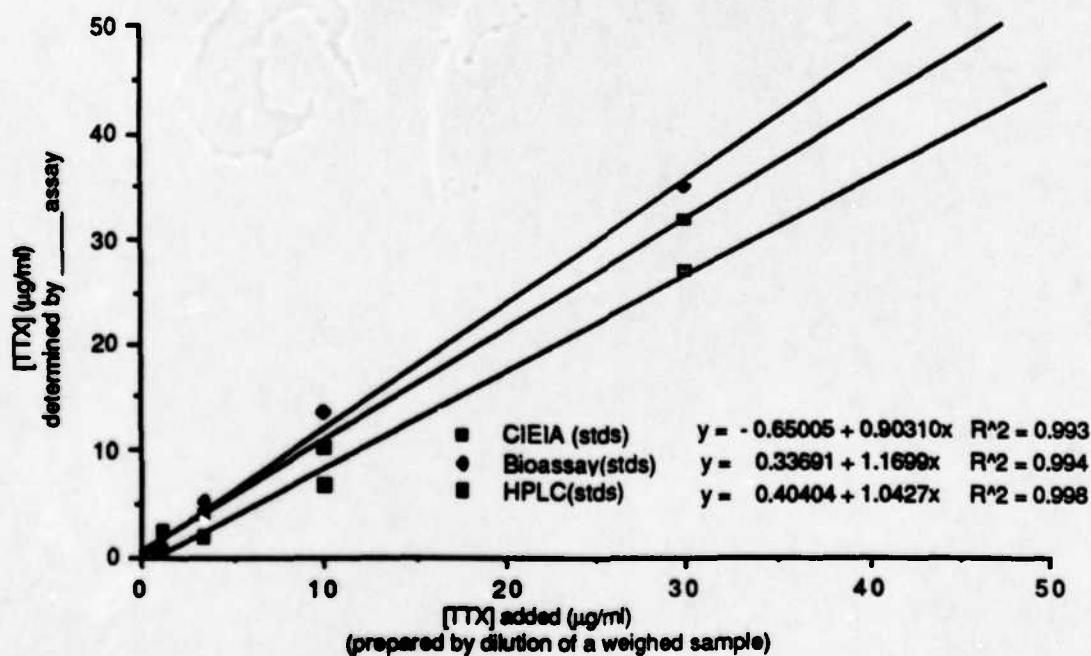


Figure 11. Correlation of CIEIA, HPLC, and Mouse Bioassay with TTX Standards.

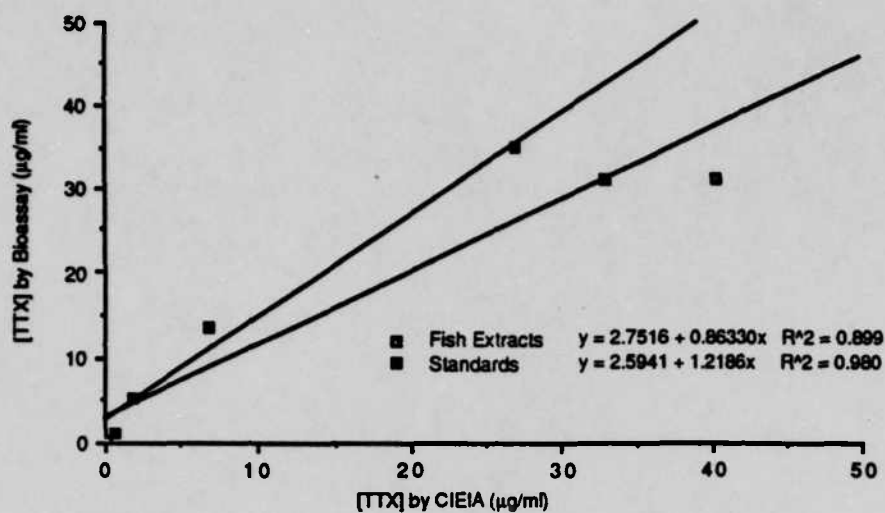


Figure 12. Correlation of CIEIA with Mouse Bioassay.

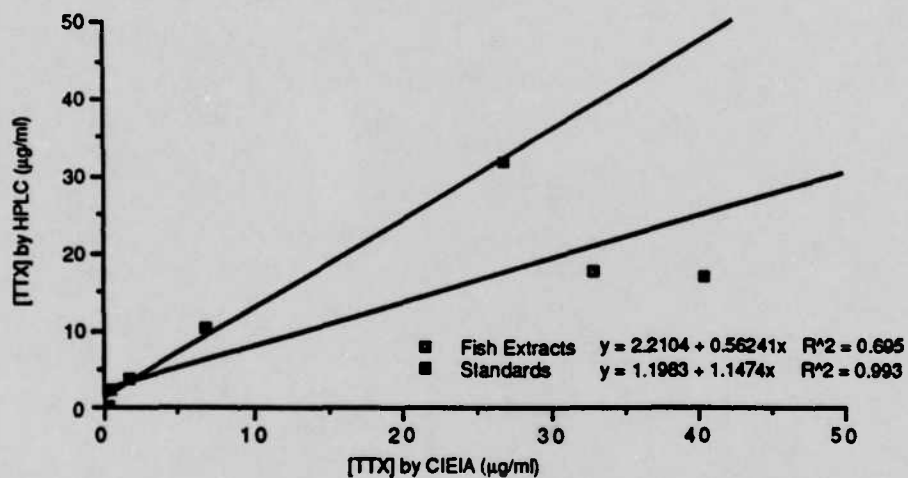


Figure 13. Correlation of CIEIA with HPLC.

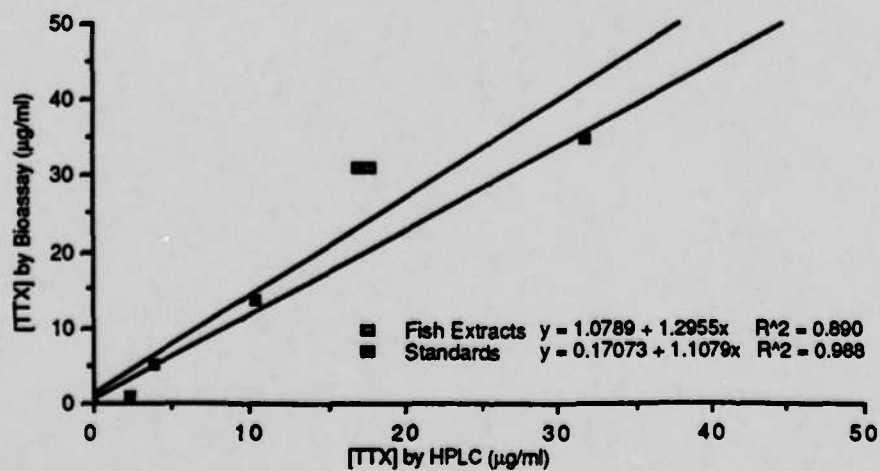


Figure 14. Correlation of HPLC with Mouse Bioassay.

An aliquot of the crude fish liver extract and an aliquot of the defatted extract (diethyl ether extraction) were saved and tested by CIEIA. Although no TTX was detected in either sample, a spike of 10 mg was fully recovered from each sample. This demonstrates that the time consuming sample preparation required for HPLC and mouse bioassay, is not necessary for the CIEIA.

Development of a Rapid Qualitative Immunoassay System for TTX Detection

A rapid, qualitative immunoassay system was developed using AP-T20G10 conjugate and BSA-TTXF coated affinity membranes (Autobloc™, Amicon, Beverly, MA) in the AbSolute™ device (Totem Diagnostics, Inc., Richmond, B.C., Canada). Membranes were "spotted" in the center with 0.5 µl of BSA-TTXF at a concentration of 12 mg/ml, and allowed to dry for 1 hour at room temperature. 0.5 ml of PBS containing 0.25% BSA, to which 20 µl of a 1 in 12.5 dilution of AP-T20G10 had been added, was mixed with 0.5 ml of TTX diluted in PBS containing 0.25% BSA in a test tube, and allowed to incubate at room temperature for 1 minute. The mixture was then drawn into a 1 ml disposable plastic syringe and passed through the membrane of the device. The membrane allowed to incubate for 2 minutes and then washed by passing 5 drops of PBS through it. Finally, 160 µl (4 drops) of BCIP/NBT substrate (Protoblot System, Promega Corp., Madison, WI) was dropped onto the membrane. The color reaction spot on the membrane was allowed to develop for 3 minutes, when the intensity of color was recorded. As this was an inhibition system, the appearance of a colored spot indicated a negative result, whereas the absence of color indicated a positive result.

Two devices were used for each TTX determination, one being the "test" device, and the other a "negative" control in which a colored spot developed on the membrane. Using a total assay time of 6 minutes, the prototype device detected TTX in PBS containing 0.25% BSA down to a concentration of 30 ng/ml.

DISCUSSION

The immunology results obtained during Year I of this project were very confusing. We observed some slight cross-reactivity of antisera elicited using KLH-TTXL and KLH-TTXF with the BSA-TTXM coating antigen. This implied that all three haptens had some structural features in common. We obviously had antibodies which could cross react to a small degree with TTX, as evidenced by the inhibitable binding of antibodies raised against KLH-TTXM with BSA-TTXL coating antigen. It is not clear why these antibodies exhibited such variability in binding nor why they were not inhibitable by TTX, when bound to BSA-TTXM coating antigen. One possible explanation is that the antibody binding site recognized both the TTX and maleimide linker components of the hapten and that the binding contribution of the linker portion was very strong. Thus, free TTX was not a good competitive inhibitor of antibody

binding to BSA-TTXM. Since only the binding contribution of the TTX moiety was present when screening anti-TTXM antibodies against BSA-TTXL, free TTX could compete more effectively.

During the third quarter of Year 1, we began to investigate the synthesis of additional TTX haptens. We decided against the originally proposed alternatives based upon reductive amination of nortetrodotoxin, due to the low yields reported by Angelides [25]. Instead, we investigated several new strategies. None of our proposed syntheses of alternative defined haptens succeeded. As a backup approach, we reinvestigated Huot's formaldehyde approach. Using this structurally undefined immunogen, both polyclonal and monoclonal antibodies against TTX were produced. We were extremely fortunate to identify and recover a high affinity MAb (T20G10) for TTX and this antibody preparation was used to develop a rapid, sensitive, direct CIEIA for TTX detection and quantitation.

The direct CIEIA detects TTX in buffer or biological matrices at concentrations in the 1-3 ng/ml range, which is significantly more sensitive than the immunoassays reported by Huot's and Watabe's groups. Some variability in assay sensitivity was observed with the different biological matrices examined. For example, the IC₂₀ detection limit for TTX in individual spiked plasma from different human volunteers ranged from approximately 0.15 ng/ml to 4 ng/ml, depending on which volunteer the plasma sample came from (data not shown). This variability is probably due to matrix interference which differs between plasma samples. The exact nature of the interfering species has not been defined.

Excellent correlation between the CIEIA, mouse bioassay and HPLC was observed for a set of TTX standards. This correlation was not as good when the fish liver extracts were measured. Both the CIEIA and mouse bioassay over-estimated the amount of TTX spike which had been added to the extracts. This could be due to interfering substances in the matrix or, in the case of the mouse bioassay, greater sensitivity to TTX of the Swiss-Webster mice compared to the standard ddY mice. In contrast, the values determined by HPLC were far lower than the amount of TTX spike. Dr. Yasumoto reported that his group experienced some difficulty in reconstituting these samples. Incomplete dissolution and/or decomposition of the TTX during shipment to Japan could explain these low values. Complete interpretation of this data is complicated by the fact that the porcupinefish contained no natural TTX. It is not clear whether the sample matrix produced from a toxin-negative fish is comparable to that from a toxin-containing fish. It would have been preferable to have obtained naturally toxic fish from Japan for this experiment. Due to a number of logistical problems, this was not possible.

Although further testing will be necessary to confirm the utility of the immunoassay for determining TTX content of pufferfish tissues, we have demonstrated that minimal sample preparation is required for the CIEIA. Additionally, it is preferable that the assay slightly overestimate rather than

underestimate the TTX concentration if the assay is intended to screen for TTX contamination in food samples.

Successful development of a rapid "field test" format for the TTX CIEIA warrants comment. Development of such a system was discussed with Dr. John Hewetson during a visit by James Raybould to Fort Detrick in March 1990. Dr Hewetson indicated that this "field test" could be up to 10-fold less sensitive than the microtiter plate CIEIA system provided that it had a total test time of 4-6 minutes. The membrane device system developed by HBG operates as a CIEIA, and therefore requires the use of two membrane devices for every TTX determination, one being the "test" device, and the other a "negative" control in which a colored spot develops on the membrane. Using a total assay time of 6 minutes, the prototype device detects TTX, in PBS containing 0.25% BSA, down to a concentration of 30 ng/ml.

The stability data on BSA-TTXF coating antigen and AP-T20G10 conjugates generated during Year 2 of this project is impressive. The BSA-TTXF coating antigen appeared to be stable to storage at 4 °C for at least one year. AP-T20G10 conjugates seemed to exhibit acceptable stability when stored at low temperatures. It is surprising that conjugates stored in buffer containing 1% BSA were more stable to prolonged storage (46 weeks at 4 °C) than those stored in 50% glycerol, since many commercial companies use 50% glycerol for preservation of enzyme-labelled conjugates. More extensive stability studies would be required to confirm these preliminary observations. The stability of lyophilized AP-T20G10 conjugates is encouraging. Lyophilized aliquots stored at either 4 °C or 37 °C seemed to retain their activity, even after 12 weeks (though activity was slightly reduced after this time - see Figure 8). This is particularly significant in the case of aliquots stored at 37 °C to obtain accelerated stability data. Addition of "bulking" agents, like sugars, prior to lyophilization, would probably improve this stability even more, and might reduce the initial decrease in activity resulting from lyophilization.

The final results obtained during this project confirm that, although we did not succeed in synthesizing well characterized TTX haptens, we did achieve our overall goal. A rapid, sensitive, direct CIEIA for TTX detection and quantitation was developed. The accuracy of this immunoassay compares very favorably with the existing alternatives and the sensitivity is far superior to any competing assay. Finally, this immunoassay is significantly more cost-effective as it requires reduced sample workup, assay time and investment in equipment than either HPLC or the mouse bioassay.

PERSONNEL

The following individuals, all of whom are present or former members of HBG's Immunology and Chemistry groups, contributed to the success of this project.

Immunology

Douglas C. Vann, Ph.D. - directed initial conjugations, immunizations and antisera screening.

Lawrence K. Inouye, Ph.D. - responsible for revised immunization schedules and reinvestigation of formaldehyde coupled immunogens.

T.J.G. Raybould, Ph.D. - directed immunoassay development using T20G10 MAb and T20G10-AP conjugates.

Gary S. Bignami, M.S. - responsible for hybridoma fusions, MAb production, and reinvestigation of formaldehyde coupled immunogens. Identified and recovered T20G10.

Samantha Simpson, B.S. - assisted in all phases of immunology work.

Jilanne B. Byrnes, B.S. - assisted in all phases of immunology work.

Chemistry

Paul G. Grothaus, Ph.D., Principal Investigator - directed overall project; primary responsibility for linker and hapten synthesis, purification and characterization.

Carolyn B. Lazo, B.S. - responsible for linker and hapten synthesis, purification and characterization.

Special acknowledgement to Professors Harry S. Mosher (Stanford University) and Marcus A. Tius (University of Hawaii) for consultation regarding hapten synthesis and Professor Takeshi Yasumoto and his co-workers (Tohoku University) for HPLC analyses and advice on validation of the TTX immunoassay.

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LIST OF ABBREVIATIONS AND ACRONYMS

APG	4-azidophenylglyoxal
BSA	Bovine serum albumin
%CV	Percent coefficient of variation
DCC	Dicyclohexylcarbodiimide
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DTDP	2,2'dithiodipyridine
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
HBG	Hawaii Biotechnology Group, Inc.
HPLC	High performance liquid chromatography
IC50	Concentration of analyte capable of causing 50% inhibition
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KLH	Keyhole limpet hemocyanin
LD50	Dose of toxin capable of causing 50% death
MAb	Monoclonal antibody
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate buffered saline
Sulfo-NHS	N-hydroxy-sulfosuccinimide
p-TSA	p-toluenesulfonic acid
TBS	Tris buffered saline
TNBS	2,4,6 trinitrobenzenesulfonic acid
TTX	Tetrodotoxin
TTXF	Tetrodotoxin formaldehyde hapten
TTXL	Tetrodotoxin levulinic acid hapten
TTXM	Tetrodotoxin maleimide hapten
UV	Ultraviolet

APPENDIX A.**Synthesis of Haptens**

6,11-(4'-Carboxy-2-butanone)TTX Ketal (**TTXL**)
and

6,11-(6'-Maleimidyl-2-hexanone)TTX Ketal (**TTXM**)

TTXM:

Tetrodotoxin (10 mg, 0.0312 mmol) was dissolved in anhydrous 3% methanolic HCl (0.8 ml) at room temperature under an argon atmosphere. 6-Maleimido-2-hexanone **11** (84 mg, 0.43 mmol, 14 equivalents) was added. The vial was sealed tightly and shaken at 42 °C in an incubator for 3 days. The vial was cooled to room temperature and the volatile components removed in vacuo. The residue was triturated with dichloromethane (4 X 0.5 ml), the remaining residue was dissolved in water and lyophilized. The sample was dissolved in 10% *d*₄-acetic acid/D₂O for NMR analysis.

TTXL:

Tetrodotoxin (10 mg, 0.0312 mmol) was dissolved in anhydrous 3% methanolic HCl (0.8 ml) at room temperature under an argon atmosphere. Levulinic acid (50 µl, 56.7 mg, 16 equivalents) was added. The vial was sealed tightly and shaken at 42 °C in an incubator for 3 days. The vial was cooled to room temperature and the volatile components removed in vacuo. The residue was triturated with 4:1 dichloromethane/methanol (4 X 0.5 ml), the remaining residue was dissolved in water and lyophilized. The sample was dissolved in 10% *d*₄-acetic acid/D₂O for NMR analysis.

APPENDIX B.**KLH-TTXM Conjugation**

November, 1988

Materials:

1. 25 mM borate buffer, pH 9.0
2. 0.05 M sodium phosphate buffer, pH 6.6, 1 mM EDTA
3. KLH, applied to a G-25 Sephadex column and eluted with borate buffer
4. 2-iminothiolane (2- IMT)
5. Sephadex G-25 column equilibrated with at least 2 bed volumes phosphate buffer
6. dithiodipyridine (DTDP) and BCA assay reagents
7. tetrodotoxin maleimide hapten (TTXM)

Methods:

1. Measure OD₂₈₀ of diluted KLH aliquot to estimate KLH concentration. (Extinction coefficient = $2.02 \text{ (mg/ml)}^{-1}$.)
2. Mix KLH with a 50-fold molar excess of 2-IMT, in borate buffer. React for 1 hr. at room temperature, with stirring.
3. Apply thiolated KLH (KLH-SH) to G-25 column. Elute with phosphate buffer collecting 16-20 drop fractions. Read OD₂₈₀ of fractions, pool desired fractions into weighed tube.
4. Do DTDP and BCA assays, calculate KLH concentration and mole -SH per mole KLH.
5. Mix KLH-SH with a 5-fold molar excess of TTXM, relative to the net KLH thiolation. React for 1 hr. at room temperature, with stirring.
6. Dialyze the KLH-TTXM against 500 ml phosphate buffer, changing buffer 4X over 4 days.
7. Transfer KLH-TTXM to weighed tube. Do DTDP and BCA assays. Calculate KLH concentration and moles -SH per mole KLH. The decrease in measureable sulfhydryls per KLH molecule is an indirect estimate of the degree of tetrodotoxin conjugation to KLH.

APPENDIX C.**KLH-TTXL Conjugation**

March, 1989

Materials:

1. 0.05 M sodium phosphate buffer, pH 7.0
2. KLH, eluted with phosphate buffer off a G-25 Sephadex column
3. phosphate buffered saline, pH 7.0 (PBS)
4. dimethylformamide (DMF)
5. dicyclohexylcarbodiimide (DCC)
6. N-hydroxysuccinimide (NHS)
7. amino group determination and BCA protein assay reagents
8. tetrodotoxin levulinic acid hapten (TTXL)

Methods:

1. In a reactovial, add the TTXL, DCC, and NHS in the following molar ratio 1 : 1 : 2.
2. Slowly add in the DMF (note: not to exceed 5% of the final reaction volume).
3. Incubate 2 hours at room temperature with stirring.
4. Transfer the activated hapten to a reactovial containing a 200-fold molar excess of KLH in phosphate buffer.
5. Incubate overnight at 4 °C with stirring, keeping reaction protected from the light.
6. Dialyze product against 500 ml PBS, changing the buffer 4X over 4 days.
7. Do the amino group determination and BCA protein assay to determine the effectiveness of the conjugation.

APPENDIX D.**BSA-TTXM Conjugation**

March, 1988

Materials:

1. 25 mM borate buffer, pH 9.0
2. 0.1 M sodium phosphate buffer, pH 6.6, 1 mM EDTA
3. BSA
4. 2-iminethiolane (2-IMT)
5. Sephadex G-25 column equilibrated with at least 2 bed volumes phosphate buffer
6. dithiodipyridine (DTDP) and BCA assay reagents
7. tetrodotoxin maleimide hapten (TTXM)

Method:

1. Mix BSA with a 50-fold molar excess of 2-IMT, in borate buffer. React for 1 hr. at room temperature, with stirring.
2. Apply thiolated BSA (BSA-SH) to G-25 column. Elute with phosphate buffer collecting 16-20 drop fractions. Read OD₂₈₀ of fractions, pool desired fractions into weighed tube.
3. Do DTDP and BCA assays, calculate BSA concentration and mole -SH per mole BSA.
4. Mix BSA-SH with a 5-fold molar excess of TTXM, relative to the netBSA thiolation. React for 1 hr. at room temperature, with stirring.
5. Dialyze the BSA-TTXM against 500 ml phosphate buffer, changing buffer 4X over 4 days.
6. Transfer BSA-TTXM to weighed tube. Do DTDP and BCA assays. Calculate BSA concentration and moles -SH per mole BSA. The decrease in measurable sulfhydryls per BSA molecule is an indirect estimate of the degree of tetrodotoxin conjugation to BSA.

APPENDIX E.**BSA-TTXL Conjugation**

February, 1989

Materials:

1. 0.01 M sodium phosphate buffer, pH 7.0
2. BSA
3. N-hydroxy-sulfosuccinimide (Sulfo-NHS)
4. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI)
5. Sephadex G-25 column equilibrated with at least two bed volumes of phosphate buffer.
6. amino group determination and BCA protein assay reagents
7. tetrodotoxin levulinic acid hapten (TTXL)

Methods:

1. To a reactivial, add BSA, TTXL, Sulfo-NHS, and EDCI, in the following molar ratio, 0.1:1.0:1.7:30.0.
2. Incubate 1 hour at room temperature with stirring, keeping the reaction protected from the light.
3. Apply the reaction mixture to a G-25 Sephadex column. Elute with phosphate buffer collecting 15 drop fractions. Read OD₂₈₀ of the fractions and pool desired fractions.
4. Do amino group determination and BCA protein assay to determine the result of the conjugation.

APPENDIX F**BCA Protein Assay
(Pierce Chemical Co. Protocol)****Materials:**

1. BCA working reagents: 50 parts Reagent A + 1 part Reagent B.
2. Buffer for diluting standards and samples.
3. Protein standard: BSA at 1 mg/ml.

Method:

1. Make up assay samples in duplicate. To separate tubes containing 100, 80, 60, 40, 20, and 0 μ l buffer to separate tubes at dilutions estimated to be in range of 0.2 to 0.8 mg/ml.
2. Add 2.0 ml BCA working reagent to each tube. Mix. Incubate 30 min. in 37° C water bath. Cool tubes to room temperature in tap water bath. Measure OD₅₆₂.

APPENDIX G**Determination of Sulfhydryl Groups (DTDP Assay)**

Adapted from: Grassetti, DR and JF Murray Arch. Biochem. Biophys.
1967, 119, 41-49

Materials:

1. 2 mM 2,2'-dithiopyridine (DTDP). Weigh out 10 mg of DTDP into an Eppendorf tube, add 100 μ l DMF to dissolve. Add this to 22.6 ml phosphate buffer, pH 7.
2. Phosphate buffer, pH 7 (Can use PBS or 0.05 M phosphate, pH 6.6, 1 mM EDTA).

Methods:

1. Make up samples in duplicate, diluted in phosphate buffer. To separate tubes containing 0.5 ml buffer, native proteins, thiolated proteins or conjugated proteins, add 0.5 ml 2 mM DTDP, mix.
2. Incubate samples 15 min. at room temperature. Read OD₃₄₃ using "buffer and DTDP only" as blank.
3. Calculate [-SH] in samples using the following formula:
Molar extinction coefficient of 2-thiopyridine at 343 nm = 7060
Equation 1. Calculation of free sulfhydryl groups

$$[-SH] = 2 \times \frac{OD_{343}}{7060 \text{ M}^{-1}}$$

Note: This assay will not give reliable results with peroxidase or KLH, both of which have significant optical absorbance in the region of 343 nm. A method involving separation of protein from the low m.w. 2-thiopyridine by Centricon (Amicon) ultrafiltration can be used. Using alkaline phosphatase or BSA, this assay gives reasonably reliable results for protein concentrations of 0.2 to 0.8 mg/ml.

APPENDIX H.**Amino Group Determination**

Adapted from: Snyder, S.L. and P.Z. Sobocinski, *Anal. Biochem.* 1975, 64, 284-288.

Materials:

1. 0.01 M sodium tetraborate buffer, pH 9.2.
2. 0.03M 2,4,6 trinitrobenzenesulfonic acid (TNBS). Weigh out 9 mg TNBS into an Eppendorf tube. Add 1 ml d.i. H₂O. Mix.
3. Protein standard at 1 mg/ml.

Methods:

1. Make up assay samples in duplicate, diluted in borate buffer. To separate tubes containing 1.00, 0.98, 0.96, 0.94, 0.92, and 0.90 ml borate buffer, add 0, 20, 40, 60, 80, and 100 μ l of 1 mg/ml protein standard. To separate tubes containing 0.90 ml borate buffer, add 100 μ l conjugated protein at an estimated concentration of around 1 mg/ml.
2. Add 25 μ l 0.03 M TNBS to each tube. Mix. Incubate 30 min at room temp. Read OD₄₂₀.

APPENDIX I

Preparation of KLH-TTXF and Alum precipitation

Adapted from: Huot, R.I., et al., *J. Clin. Invest.* **1989**, 83, 1821-1826. and Chu, F.S.; Fan, T.S.L. *J. Assoc. Off. Anal. Chem.* **1985**, 68, 13-16.

Materials:

1. 1 M Sodium Acetate buffer, pH 7.4
2. Tetrodotoxin (stock solution of 1 mg/ml in 1 M Sodium Acetate buffer, pH 7.4)
3. Keyhole limpet hemocyanin (stock solution 34.9 mg/ml)
4. 37% formaldehyde
5. amber glass vial
6. vortex mixer
7. dialysis tubing
8. 0.01 M Sodium phosphate, pH 7, 0.15 M sodium chloride (PBS)
9. centrifuge capable of 1000 x g.
10. 10% (w/v) aqueous aluminum potassium sulfate $\text{AlK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$
11. 1 N NaOH

Method:

1. Add dropwise to amber glass vial, in order, while stirring on vortex mixer:
 - 300 μl TTX (1 mg/ml)
 - 700 μl 1 M sodium acetate buffer, pH 7.4
 - 121 μl KLH
 - 60 μl 37% formaldehyde
2. Place reaction mixture in 37°C shaker and incubate for 3 days.
3. Following incubation, the reaction mixture/conjugate should be dialyzed against PBS for three days at 4°C with four 1 L changes of PBS.
4. An estimate of conjugate concentration is determined spectrophotometrically at 280 nm using a mg/ml extinction of 2.02.
5. After dialysis, the conjugate should be alum precipitated, as follows:
 - Add:
 - 800 μl 10% (w/v) $\text{AlK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$
 - ~500 μl 1 N NaOH, being careful to maintain the pH within the range of 3.5-8 during the process, as monitored by pH paper.
5. The resulting precipitate should be washed six times by centrifugation (approximately five minutes at 1000 x g at 4°C) with 15 volumes of ice cold PBS.
6. Adjust concentration of slurry with adjuvant or PBS to provide appropriate immunizing dose.

APPENDIX J**Preparation of BSA-TTXF Coating Antigen**

Adapted from: Huot, R.I., et al., *J. Clin. Invest.* **1989**, *83*, 1821-1826.

Materials:

1. 1 M Sodium Acetate buffer, pH 7.4
2. Tetrodotoxin (stock solution of 1 mg/ml in 1 M Sodium Acetate buffer, pH 7.4)
3. Bovine Serum Albumin (stock solution 33.6 mg/ml)
4. 37% formaldehyde
5. amber glass vial
6. vortex mixer
7. dialysis tubing
8. 0.01 M Sodium phosphate, pH 7, 0.15 M sodium chloride (PBS)
9. centrifuge capable of 1000 x g.

Method:

1. Add dropwise to amber glass vial, in order, while stirring on vortex mixer:
 - 700 μ l TTX (1 mg/ml)
 - 300 μ l 1 M sodium acetate buffer, pH 7.4
 - 179 μ l BSA (at 33.6 mg/ml)
 - 41 μ l 37% formaldehyde
2. Place reaction mixture in 37°C shaker and incubate for 3 days.
3. Following incubation, the reaction mixture/conjugate should be dialyzed against PBS for three days at 4°C with four 1 L changes of PBS.
4. An estimate of conjugate concentration is determined spectrophotometrically at 280 nm using a mg/ml extinction of 0.667.

APPENDIX K**Primary Indirect ELISA Screen for
Antibodies to Tetrodotoxin**

March, 1990

Materials:

1. Immulon 2 microtiter plates (Dynatech)
2. Coating antigen (BSA-TTXM, BSA-TTXL, or BSA-TTXF)
3. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl (PBS)
4. PBS containing 0.05% (v/v) Tween-20 (PBS-T)
5. 50 mM Tris HCl, pH 7.0, 0.15 M NaCl, 0.05% (v/v) Tween 20 (TBS-T)
6. Bovine Serum Albumin (BSA)
7. Alkaline Phosphatase goat anti-rabbit Ig conjugate (or alkaline phosphatase goat anti-mouse Ig conjugate for monoclonal antibody screen)
8. p-nitrophenylphosphate (pNPP) (Sigma 104, 5 mg tablets)
9. Alkaline Phosphatase substrate buffer:
 - 25 mM Trizma base, pH 9.5
 - 0.15 M NaCl
 - 5 mM MgCl₂
 - 0.02% (w/v) NaN₃

Methods:

1. Coat Immulon 2 microtiter plates with BSA-TTXM, BSA-TTXL, or BSA-TTXF, 100 µl/well, at ** µg/ml in PBS for 1 h. at room temperature.
 - ** coating antigen concentration must be determined in advance for each batch. Normally, twice the minimum saturating concentration is used.
2. Wash three times with PBS-T.
3. Block wells of microtiter plate with 200 µl/well of 1% BSA in PBS for 1 h. at room temperature.
4. Wash three times with PBS-T.
5. Add 100 µl/well sample being tested for anti-TTX antibody, titrated in 1% BSA in PBS. Incubate for 1 h. at room temperature.
6. Wash three times with PBS-T.
7. Add 100 µl/well goat alkaline phosphatase goat anti-rabbit Ig conjugate (or alkaline phosphatase goat anti-mouse Ig conjugate for monoclonal antibody screen), diluted to appropriate concentration with 1% BSA in PBS. Incubate 1 h. at room temperature.
8. Wash four times with TBS-T.
9. Add 200 µl/well 1 mg/ml pNPP diluted in alkaline phosphatase substrate buffer, pH 9.5.

10. Read on dual wavelength absorbance mode (reference wavelength = 690 nm = filter 8 on Titertek; sample wavelength = 414 nm = filter 3 on Titertek).

APPENDIX L

**Affinity Purification of Murine Monoclonal Antibody
from Ascitic Fluid by Discontinuous pH Gradient
Elution from Protein A-Sepharose**

March, 1990

Materials:

1. Staphylococcal Protein A-Sepharose-CL-4B (Pharmacia)
2. Econocolumn (Biorad)
3. Buffer #1: 1.45 M glycine-NaOH, 3 M NaCl, pH 8.9
4. Buffers #2-5: 0.04 M sodium citrate, 0.02 M NaCl, pH 6.0, 5.0, 4.0, and 3.2, respectively.
5. Neutralizing buffer: 0.5 M sodium phosphate, pH 7.7
6. UV monitor
7. Fraction collector
8. Ultrafiltration cells and filters
9. Ascitic fluid containing monoclonal antibody of interest.

Method:

1. Fully hydrate and equilibrate Protein A-Sepharose with Buffer 1 in a glass Econocolumn at 4 °C.
2. Dilute ascites fluid in 3 parts Buffer 1 and apply to column at a flow rate of 1-5 ml/min..
3. Wash column with Buffer 1 until entire unbound peak is eluted.
4. Collect run-through and successive protein peaks by fraction collector or by pooling an entire buffer elution.
5. Elute bound material successively with Buffers 3, 4, and 5, into 1/4 volume neutralizing buffer.
6. Alternatively bound material may be eluted in one step using buffer 4 only.
[Collecting individual buffer fractions as in step 5 provides a better purification of monoclonal antibody from non-specific antibody.]
7. Eluted fractions or pools may be assayed for antigen-specific monoclonal antibody by ELISA.
8. Concentrate appropriate eluates using ultrafiltration to 1-5 mg/ml. Store at -20 °C.
9. Antibody concentration may be estimated by measuring OD₂₈₀. A mouse IgG solution at 1 mg/ml = 1.44 Absorbance units (OD₂₈₀).

APPENDIX M**Alkaline Phosphatase-T20G10 Conjugation**

March, 1990

Materials:

1. Alkaline Phosphatase (AP) (Scripps Laboratories Inc)
2. Sulfo-SMCC: Sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce)
3. 50 mM sodium borate buffer, pH 7.6, 1 mM MgCl₂, 0.1 mM ZnCl₂
4. 1.0 ml Sephadex G-50 (fine) spin column
5. Dialysis tubing
6. 0.1 M Tris-HCl, pH 7.0, 1 mM MgCl₂, 0.1 mM ZnCl₂ (Buffer T)
7. T20G10.1.1.1. anti-tetrodotoxin monoclonal antibody (protein A purified)
8. 0.1 M sodium phosphate buffer, pH 6.5
9. *S*-acetylmercapto-succinic anhydride (SAMSA)
10. Dimethylformamide (DMF)
11. 0.1 M EDTA
12. 0.1 M Tris-HCl, pH 7.0
13. 1 M hydroxylamine-HCl, pH 6.5
14. 0.1 M sodium phosphate buffer, pH 6.0
15. Centricon 30 microconcentrators (Amicon)
16. DTDP assay reagents
17. 10 x 1 cm Sephadex G-200 column
18. 10 mM Tris-HCl buffer, pH 6.8 containing 0.1 M NaCl, 1 mM MgCl₂, 0.1 mM ZnCl₂ and 0.5 g/l NaN₃ (Eluting buffer)
19. IEC benchtop centrifuge

Method:

1. Dialyze AP against borate buffer at room temperature with two buffer changes.
2. React the AP with a 25 fold molar excess of sulfo-SMCC in borate buffer for 30 min. at 30°C.
3. Apply 100 µl aliquots of the AP-MCC to 1 ml Sephadex G-50 (fine) spin columns equilibrated with Buffer T. Centrifuge 2 min.
4. Collect AP-MCC filtrates and concentrate on a Centricon 30 equilibrated with buffer T.
5. Concentrate the protein A purified T20G10 and exchange the buffer with 0.1 M sodium phosphate, pH 6.5 using a Centricon 30 equilibrated with the same buffer. Centrifuge 20 min. at 3000 x g and collect retentate. Estimate protein concentration by measuring OD₂₈₀.
6. React the T20G10 with a 100 fold molar excess of SAMSA to thiolate the antibody. Incubate 30 min. at room temperature with stirring. Add 0.02 ml 0.1 M EDTA, 0.1 ml 0.1 M Tris-HCl, and 0.1 ml hydroxylamine-HCl. Incubate 4 min at 30°C.

7. Transfer thiolated T20G10 to a Centricon 30 equilibrated with pH 6 phosphate buffer, fill to 2 ml with pH 6 phosphate buffer, and centrifuge 20 min. at 3000 x g to remove unreacted SAMSA. Collect retentate in tared tube.
8. Perform the DTDP assay (Appendix D) on the thiolated T20G10 to determine the mole -SH to mole antibody ratio.
9. React thiolated T20G10 with a 5 fold molar excess of AP-MCC. Incubate overnight at 4°C with stirring.
10. Transfer the T20G10-AP reaction mix to an Eppendorf tube and microcentrifuge for 5 min. at top speed.
11. Apply the supernatant to a Sephadex G-200 column equilibrated with Eluting Buffer and elute 60 drop fractions, monitoring the absorbance at 280 nm.
12. Test selected fractions by ELISA and pool positive fractions.

APPENDIX N

Indirect CIEIA for Tetrodotoxin

March, 1990

Materials:

1. Immulon 2 microtiter plates (Dynatech)
2. BSA-TTXF coating antigen
3. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl (PBS)
4. PBS containing 0.05% (v/v) Tween-20 (PBS-T)
5. PBS containing 1% BSA (PBS-BSA)
6. 50 mM Tris HCl, pH 7.0, 0.15 M NaCl, 0.05% (v/v) Tween 20 (TBS-T)
7. Bovine Serum Albumin (BSA)
8. Alkaline Phosphatase goat anti-mouse Ig conjugate
9. p-nitrophenylphosphate (pNPP) (Sigma 104, 5 mg tablets)
10. Alkaline Phosphatase substrate buffer:
 - 25 mM Trizma base, pH 9.5
 - 0.15 M NaCl
 - 5 mM MgCl₂
 - 0.02% (w/v) NaN₃
11. Protein A-Sepharose purified T20G10 anti-tetrodotoxin monoclonal antibody or ascites fluid containing T20G10
12. Tetrodotoxin standard (stored in 5% aqueous acetic acid)

Methods:

1. Coat Immulon 2 microtiter plates with BSA-TTXF, 100 µl/well, at ** µg/ml in PBS for 1 h. at room temperature.
 - ** coating antigen concentration must be determined in advance for each batch. Normally, twice the minimum saturating concentration is used.
2. Wash three times with PBS-T.
3. Block wells of microtiter plate with 200 µl/well of 1% BSA in PBS for 1 h. at room temperature.
4. Wash three times with PBS-T.
5. Add 50 µl/well sample containing free TTX and 50 µl/well optimally diluted T20G10 anti-TTX murine monoclonal antibody, diluted in PBS-BSA. Incubate for 1 h. at room temperature.
6. Wash three times with PBS-T.
7. Add 100 µl/well Alkaline Phosphatase goat anti-mouse Ig conjugate, diluted to appropriate concentration in PBS-BSA. Incubate 1 h. at room temperature.
8. Wash four times with TBS-T.
9. Add 200 µl/well 1 mg/ml pNPP diluted in alkaline phosphatase substrate buffer, pH 9.5.
10. Read on dual wavelength absorbance mode (reference wavelength = 690 nm = filter 8 on Titertek; sample wavelength = 414 nm = filter 3 on Titertek).

APPENDIX O**Direct CIEIA for Tetrodotoxin using
AP-T20G10 Monoclonal Antibody**

March, 1990

Materials:

1. Immulon 2 microtiter plates (Dynatech)
2. BSA-TTXF coating antigen
3. 50 mM Sodium Phosphate, pH 7.0, 0.15 M NaCl (PBS)
4. PBS containing 1% BSA (PBS-BSA)
5. PBS containing 0.05% (v/v) Tween-20 (PBS-T)
6. 50 mM Tris HCl, pH 7.0, 0.15 M NaCl, 0.05% (v/v) Tween 20 (TBS-T)
7. Bovine Serum Albumin (BSA)
8. p-nitrophenylphosphate (pNPP) (Sigma 104, 5 mg tablets)
9. Alkaline Phosphatase substrate buffer:
 - 25 mM Trizma base, pH 9.5
 - 0.15 M NaCl
 - 5 mM MgCl₂
 - 0.02% (w/v) NaN₃
10. Protein A-Sepharose purified T20G10 Monoclonal Antibody, conjugated to Alkaline Phosphatase (AP-T20G10)
11. Tetrodotoxin standard (stored in 5% aqueous acetic acid)

Methods:

1. Coat Immulon 2 microtiter plates with BSA-TTXF, 100 µl/well, at
** µg/ml in PBS for 1 h. at room temperature.
 - ** coating antigen concentration must be determined in advance for each batch. Normally, twice the minimum saturating concentration is used.
2. Wash three times with PBS-T.
3. Block wells of microtiter plate with 200 µl/well of 1% BSA in PBS for 1 h. at room temperature.
4. Wash three times with PBS-T.
5. Add 50 µl/well samples containing free TTX and 50 µl/well optimally diluted AP-T20G10 MAb, diluted in PBS-BSA. Incubate for 1 h. at room temperature.
6. Wash four times with TBS-T.
7. Add 200 µl/well 1 mg/ml pNPP diluted in alkaline phosphatase substrate buffer, pH 9.5.
8. Read on dual wavelength absorbance mode (reference wavelength = 690 nm = filter 8 on Titertek; sample wavelength = 414 nm = filter 3 on Titertek).

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