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TOXIN PRODUCTION AND IMMUNOASSAY DEVELOPMENT

I. PALYTOXIN

ANNUAL REPORT

DOUGLAS C. VANN

MARCH 31, 1988

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LIST OF ABBREVIATIONS

-SH	SULFHYDRYL GROUP
2-IMT	2-IMINOTHIOLANE
AP BUFFER	ALKALINE PHOSPHATASE BUFFER (0.025M TRIS, 0.15M NaCl, 5 mM MgCl ₂)
B/B ₀	SAMPLE OD/NO INHIBITOR OD VALUES
BCA	BCA PROTEIN ASSAY KIT (PIERCE)
BSA	BOVINE SERUM ALBUMIN
BSA-PTM	BSA COUPLED WITH Palytoxin-maleimide hapten
BSA-PTX-SPDP	BSA COUPLED WITH Palytoxin SPDP hapten
BSA-SH	thiolated BSA
CIEIA	COMPETITIVE INHIBITION ENZYME IMMUNOASSAY
COTR	CONTRACTING OFFICER'S TECHNICAL REPRESENTATIVE
DMSO	DIMETHYLSULFOXIDE
DTDP	DITHIODIPYRIDINE
EDTA	ETHYLENEDIAMINETETRACETIC ACID
ELISA	ENZYME LINKED IMMUNOSORBENT ASSAY
EtOH	ETHANOL
GAM1g-AP	COMMERCIAL GOAT ANTI-MOUSE IMMUNOGLOBULIN-ALKALINE PHOSPHATASE CONJUGATE
GAR1g-AP	COMMERCIAL GOAT ANTI-RABBIT IMMUNOGLOBULIN-ALKALINE PHOSPHATASE CONJUGATE
KLH	KEYHOLE LIMPET HEMOCYANIN
KLH-FTM	KLH COUPLED WITH Palytoxin-maleimide hapten
KLH-PTX-SPDP	KLH COUPLED WITH Palytoxin-SPDP hapten
LD50	DOSE CAPABLE OF CAUSING 50% DEATH IN A GROUP OF ANIMALS OR POPULATION OF CELLS
MDD	MINIMUM DETECTABLE DOSE
nmr	NUCLEAR MAGNETIC RESONANCE
PBS	PHOSPHATE BUFFERED SALINE (0.05 M PHOSPHATE, 0.15 M NaCl)
PBS-T20	PBS CONTAINING 0.05% (V/V) TWEEN 20
PTM	PALYTOXIN-MALEIMIDE HAPTEN
PTX	PALYTOXIN
PTX-SPDP	PALYTOXIN-SPDP HAPTEN
SIGMA 104	p-NITROPHENYL PHOSPHATE, DISODIUM SALT
SPDP	SUCCINIMIDYL 3-(2-PYRIDYLDITHIO)PROPIONATE
sulfo-SMCC	SULFOSUCCINIMIDYL 4-(N-MALEIMIDOMETHYL)CYCLOHEXANE-1-CARBOXYLATE
supp. MEM	SUPPLEMENTED MEM



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STATEMENT OF THE PROBLEM UNDER STUDY

Palytoxin is an extremely toxic non-proteinaceous toxin first isolated from marine soft corals of the genus Palythoa. It is a membrane active agent whose mechanism of action is not fully understood. Palytoxin is also a novel type of tumor promoter. Until recently, there was no specific means of detecting and quantitating palytoxin and there was no known antidote.

This project was undertaken in response to the U.S. Army's need for research quantities of palytoxin and the need for a rapid, simple, and specific means to identify and quantitate palytoxin. In each of the first two years of this contract we will isolate and deliver to USAMRIID 100 mg of palytoxin. In addition, we are utilizing derivatives of palytoxin to produce immunogens which are being used to generate anti-palytoxin polyclonal and monoclonal antibodies. These antibodies are being incorporated into enzyme linked immunosorbent assays for palytoxin. The reagents and protocols needed for these immunoassays will be delivered to USAMRIID.

Immunosorbent assay

BACKGROUND

Palytoxin, Figure 1, is one of the most highly toxic non-proteinaceous substances known. It was first isolated from Palythoa toxica by Moore and Scheuer (ref 1). Depending on the species from which the palytoxin is isolated, the molecular weight varies slightly. Based on the gross structural determinations of Moore's group in Hawaii (ref 2) and Hirata's group in Japan (ref 3), palytoxin from Palythoa tuberculosa is known to have a molecular weight of 2678.5 daltons (ref 4).

The intravenous LD₅₀ of palytoxin in the rabbit and mouse is 33 ng/kg and 450 ng/kg, respectively (ref 5). Palytoxin is a fast acting toxin which causes neurological and vascular distress in experimental animals, followed by death due to congestive heart failure (ref 6). At the cellular level, palytoxin is known to cause hemolysis (ref 7), stimulate arachidonic acid metabolism (ref 8) and alter transmembrane ion flux (ref 9). It is also a non-phorbol ester type of tumor promoter in the two stage model of carcinogenesis on mouse skin (ref 10).

For many years, the only method available for identifying palytoxin involved the tedious procedures of bioassay and purification followed by characterization of the compound by u.v. and nmr spectroscopy (ref 11). Recently, HPLC methods have been reported (ref 12).

PALYTOXIN

m.w. = 2678.5 daltons

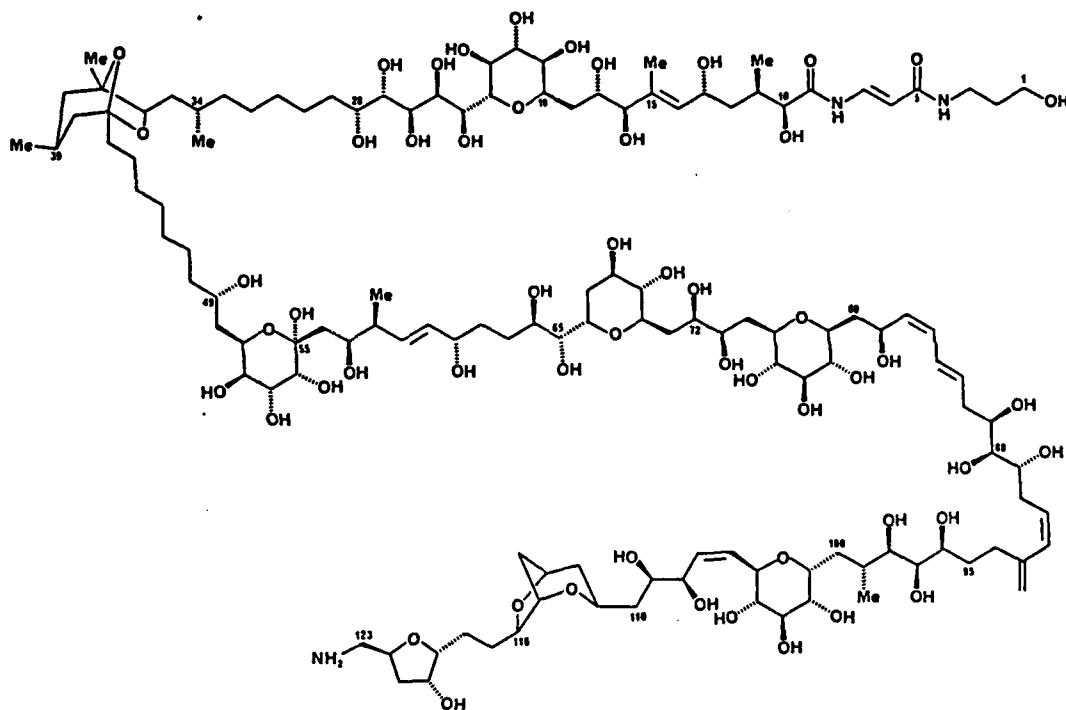


Figure 1. Palytoxin.

Since immunoassays commonly provide rapid, inexpensive, sensitive and highly selective methods for the detection and quantitation of a wide variety of drugs and other molecules of biomedical significance (ref 13) and standard procedures for the elicitation of the required antibodies are well known, it is logical to propose the application of these methodologies to palytoxin assay.

The potential for success in this project was strengthened by reports that antibodies and/or immunoassays for several other important marine and aquatic toxins have been produced (ref 14). In 1986 we generated rabbit antisera to palytoxin (unpublished information). More recently, Levine and associates (ref 15) reported the production of rabbit anti-palytoxin antibodies which were capable of neutralizing some of the biological properties of palytoxin.

RATIONALE

Palythoa tuberculosa is moderately abundant in Hawaii and can be collected locally as a source of palytoxin. Our staff is proficient in palytoxin isolation procedures. Prior research in our laboratory established reproducible methods for producing palytoxin immunogens. The immunology staff is currently optimizing the parameters of two different immunoassays which we are developing to detect and quantitate palytoxin in diverse matrices.

METHODS

Collection. All collections were performed by our staff. Palythoa toxica was collected twice from the Hana, Maui tidepool originally described by Moore, Helfrich and Patterson (refs 1, 16). Since the tidepool is quite small and contains a very limited amount of P. toxica, the more abundant but less potent species, P. tuberculosa was sought. Two scouting and collecting trips to the south and north shores of Maui yielded only minimally promising amounts of P. tuberculosa. Previously known collecting sites on Molokai were definitely superior in both the quantity and quality of the soft coral. Arrangements were made with a local boat operator to transport parties of two to four divers to the north shore of Molokai between Kalaupapa Peninsula and Halawa Valley. Scuba gear was used to collect from vertical rock faces at depths of two to ten meters. One diver could harvest two to three kg. of soft coral per tank of air. After collection, the material was immediately placed in sturdy plastic bags and packed in ice. Usually one day would elapse before the samples could be delivered to the laboratory. Upon receipt in the laboratory the bags of coral were weighed, and stored frozen at -20° C. Material stored for as long as six months has produced adequate yields of palytoxin upon processing.

Isolation. Palytoxin isolation followed the procedure published by Moore (ref 16). A flow diagram of the procedure followed is presented in Figure 2. The isolated toxin was characterized by u.v. spectroscopy, ¹H and ¹³C nmr, and in vitro cytotoxicity when tested on murine lymphoid cells according to the protocol given in Appendix 1.

PALYTOXIN ISOLATION

PALYTHOA TUBERCULOSA

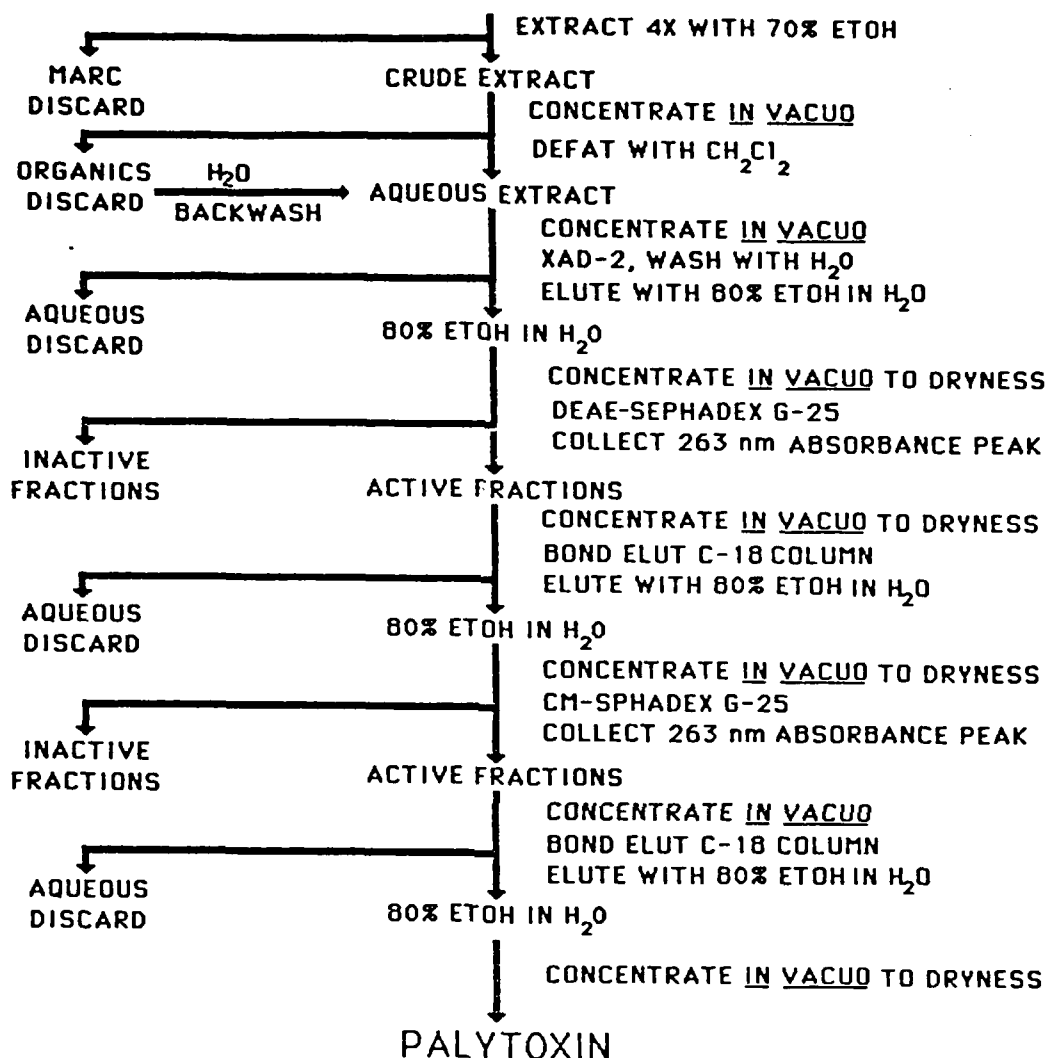
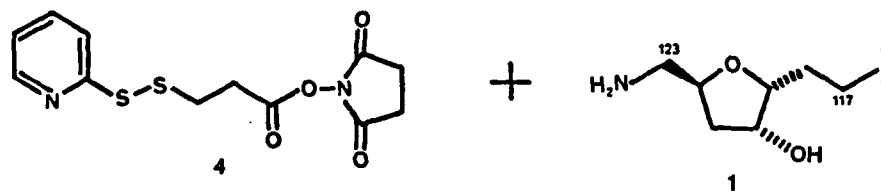


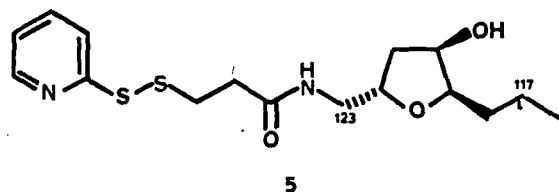
Figure 2. Palytoxin Isolation Flow Diagram.

Palytoxin Haptens. Palytoxin derivatives which could be readily conjugated to carrier proteins for use as immunogens or as antigens in immunoassays were produced following unpublished methods developed by us prior to the initiation of the present contract work. Briefly, palytoxin was treated with the commercially available (Pierce) bifunctional linkers, succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) as shown in Schemes 1 and 2, respectively. The resulting amide derivatives were characterized by ^1H nmr spectroscopy.

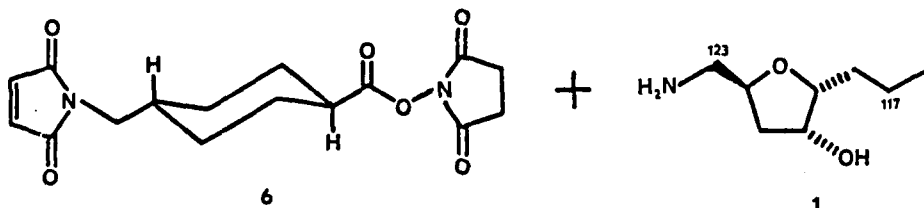
Scheme 1. Production of Palytoxin-SPDP Hapten.



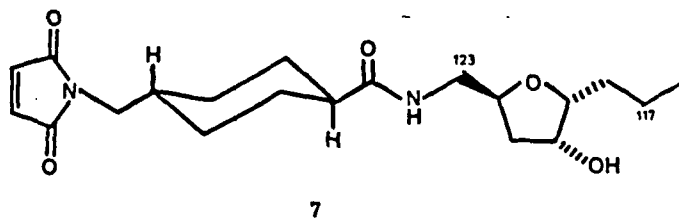
1. pH 7.5 phosphate buffer:
DMSO (19:1)
2. Room temp. 3.5 to 4 hours.
3. Extract excess linker with CH_2Cl_2 .
4. Chromatography on Sephadex CM-G25
 NaH_2PO_4 . 0.02M. pH - 4.6.



Scheme 2. Production of Palytoxin-Maleimide Hapten.



1. pH 7.5 phosphate buffer:
DMSO (19:1)
2. Methylene chloride extraction.
3. Chromatography on C-18 (BondElut),
 H_2O then 80% EtOH.
4. Chromatography on Sephadex CM-G25
 NaH_2PO_4 . 0.02M. pH - 4.6.



Hapten-Carrier Conjugation. Palytoxin-SPDP (PTX-SPDP) hapten will readily conjugate with thiolated proteins following the procedure of Carlson (ref 17). The protocol we used in conjugating PTX-SPDP to BSA is included as Appendix 2.

Palytoxin maleimide hapten (PTM) was conjugated to BSA by the procedure given in Appendix 3.

Palytoxin-protein conjugates were characterized by an indirect method for the estimation of the moles of palytoxin per mole of protein. Total protein concentration was determined using the BCA protein analysis obtained from Pierce Chemical Co. (see Appendix 5). Free thiol groups on the proteins were determined using the dithiodipyridine (DTDP) method (see Appendix 6). The decrease in free sulfhydryl groups of thiolated proteins after reaction with the palytoxin haptens provides a means of estimating the extent of palytoxin conjugation to the carrier.

Immunization of Rabbits. Rabbits were immunized according to standard procedures (ref 18). Primary immunization was with 400 to 500 ug KLH-PTX-SPDP in 1 ml phosphate buffered saline, pH 7.2, and 2 ml complete Freund's adjuvant. Secondary boosts utilizing 250 ug of immunogen in incomplete Freund's adjuvant were given one to four months later. All rabbit immunizations were divided among subscapular and intramuscular sites. Animals were bled by cardiac puncture under general anesthetic.

Mice were also immunized in a standard fashion (ref 19). Primary innocula contained 100 ug of immunogen in approximately 0.3 ml complete Freund's adjuvant. Secondary innocula consisted of 50 ug of immunogen in incomplete Freund's adjuvant. All injections were administered intraperitoneally. 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 50 ug immunogen in 0.2 ml phosphate buffered saline, pH 7.2, by the intraperitoneal route, four days prior to sacrifice.

Hybridoma Generation and Monoclonal Antibody Production. Standard procedures (ref 19) were followed using P3X63Ag8.653 as the fusion partner.

Primary ELISA Screen For Polyclonal and Monoclonal Antibodies.

The protocol for the primary ELISA screen for rabbit or mouse antibodies to palytoxin is presented in Appendix 7.

Competitive ELISA (CIEIA) for the Detection and Quantitation of Palytoxin. The protocol for palytoxin CIEIA using rabbit antisera or immunoglobulin fractions is included as Appendix 8.

The protocol for palytoxin CIEIA using mouse monoclonal antibodies is included as Appendix 9.

Palytoxin Sandwich ELISA. The protocol for the sandwich ELISA for palytoxin is included as Appendix 10.

Data Analysis. Each data point was usually done in triplicate and the mean value calculated. Plates usually contained larger numbers of wells containing no inhibitor in order to give a better estimate of the maximum response (B_0). B/B_0 values were calculated as follows: the mean of a given set of replicates was divided by the mean of all the wells containing no inhibitor. For determination of unknowns, the Rodbard four parameter logistic function (ref 20), as found in the BioRad MacReader program for the Macintosh computer was used. Appropriate statistics were calculated as needed.

RESULTS

Collection. Two trips were made to Hana, Maui to collect Palythoa toxica. The first trip, on July 15, 1987, yielded 120 grams wet weight of polyps. On December 3, 1987 we made a second visit without harvesting any of the soft coral. This was because the growth in the tidepool was relatively depleted and we did not deem it wise to further reduce the standing crop of organisms. We do not know why the growth was sparse in December. Subjectively, it appeared to be a natural fluctuation in the coral growth. We will continue to monitor the stand of organisms in the tidepool and only collect when greater than one half of the available submerged substrate is covered with healthy appearing P. toxica.

The results of P. tuberculosa collection are summarized in Table 1.

TABLE 1. SUMMARY OF 1987 PALYTHOA TUBERCULOSA COLLECTIONS.

BAG NOS.	COLLECTION SITE	DATE	WEIGHT(KG)
1--10	Nuu Bay, Maui	6/12/87	33.37
11--15	Keawenui, Molokai	6/28/87	14.68
16--24	Okala Island, Molokai	6/29/87	27.86
25--31	Maliko, Maui	7/25/87	21.72
32--43	Okala Island, Molokai	8/2/87	40.42
44--58	Okala Island, Molokai	8/15/87	51.16
59--59	Okala Island, Molokai	9/5/87	38.81
TOTAL			225.97

Isolation. Five major palytoxin isolations were performed during the past year. The first used P. toxica and the remaining four used P. tuberculosa. The results of the isolation runs are summarized in Table 2. Prior to the five major isolation efforts indicated below, a series of small isolations attempts provided us with approximately 50 mg of palytoxin.

TABLE 2. SUMMARY OF MAJOR PALYTOXIN ISOLATION EXPERIMENTS.

DATE BEGUN	ORGANISM USED	BAG NUMBERS	PALTYHOA WEIGHT	PALYTOXIN YIELD (MG)
7/16/87	<u>P. toxica</u>	n.a. ^a	120 g	33.4
8/14/87	<u>P. tuberculosa</u>	20, 24	6.55 kg	3.8
9/2/87	<u>P. tuberculosa</u>	16-19	11.35 kg	15.89
12/11/87	<u>P. tuberculosa</u>	21-23, 34	12.46 kg	17.1
2/17/88	<u>P. tuberculosa</u>	32, 33, 36	9.83 kg	27.75
TOTAL				97.94

a. P. toxica was collected 7/15/87.

Each batch of palytoxin was characterized by u.v. spectroscopy and judged to be greater than 95% pure. In January, 1988 we pooled all the palytoxin we had on hand, characterized it by u.v. spectroscopy and set aside approximately 110 mg for delivery to USAMRIID. A major problem was encountered when we attempted to produce small batches of lyophilized palytoxin for delivery. When we attempted to package the palytoxin in 1 and 10 mg batches we found that we could not dry the samples and their containers well enough to obtain accurate weights. When we prepared a solution of palytoxin which was aliquoted in calculated volumes containing 1 and 10 mg, we could not control foaming during the freeze drying process. Therefore, after consultation with the COTR, we prepared two batches of 54 mg each which could be delivered in a 100 ml pear-shaped flask. Explicit handling instructions and information characterizing the product were included with each shipment. Shipments were made on January 18, 1988 and March 21, 1988. Safe receipt of both batches has been acknowledged by the COTR. The characterization information and the instructions for storage and preparation for use are included as Appendices 11 and 12.

Hapten-Carrier Conjugates. Approximately 10 mg of KLH-PTX-SPDP was prepared prior to commencing the present contract work. Several batches of BSA-PTX-SPDP were also prepared. These conjugates were utilized during the early phases of this project. Over time, additional material was needed and we began to prepare conjugates using PTM. The conjugates prepared during the past year are summarized in Table 3.

TABLE 3. SUMMARY OF PALYTOXIN-PROTEIN CONJUGATIONS.

DATE	CARRIER	PROTEIN		2-IMT ^a	HAPTEN	YIELD	CONJ.
		mg	mmol	mmol	mmol	mg	RATIO ^b
3/2/87	BSA	10 (5 mg after thiolation)	1.5×10^{-4}	7.3×10^{-4}	3.8×10^{-4} (PTX-SPDP)	4.4	1.9
9/29/87	BSA	5 (4 mg after thiolation)	7.6×10^{-5}	3.6×10^{-4}	3.0×10^{-4} (PTX-SPDP)	3.2	2.5
12/4/87	BSA	10 (5 mg after thiolation)	1.5×10^{-4}	7.3×10^{-4}	3.8×10^{-4} (PTM)	3.8	1.5
1/22/88	KLH	7 (3.7 mg after thiolation)	7×10^{-5}	3.5×10^{-3}	1.9×10^{-3} (PTM)	0.8 ^c	8.3

a. 2-IMT = 2-iminothiolane

b. CONJ. RATIO = mol PTX/mol PROTEIN

c. Technical loss resulted in low yield.

Production of Antibodies to Palytoxin. Rabbit HBG 007 was immunized and found to be producing anti-palytoxin antibodies prior to the commencement of our contract work. We have continued to use this rabbit as a source of useful antibodies during the past year.

Mice were immunized and three cycles of hybridoma production were carried out. Mouse monoclonal antibodies to palytoxin were selected first on the criterion of reactivity to BSA-PTX coating antigen (see Appendix 7) and second on the criterion of inhibition of the reaction with BSA-PTX by free

palytoxin. From three fusions, we obtained two useful anti-palytoxin monoclonal antibodies, 73D3 and 89F9. Hybridoma production results are summarized in Table 4.

TABLE 4. SUMMARY OF HYBRIDOMA PRODUCTION.

DATE	FUSION NUMBER	NUMBER OF CULTURES PLATED	NUMBER POSITIVE 1° SCREEN	NUMBER WITH USEFUL Ab	STABLE CLONES PRODUCED
3/2/87	1	258	9	0	NONE
6/1/87	2	265	38	3	73D3
8/14/87	3	465	12	1	89F9

Both 73D3 and 89F9 are IgG1-kappa. They show similar inhibition curves with free palytoxin (IC₅₀ values, the minimum detectable dose, and the slopes of the inhibition curves are indistinguishable). There is no evidence of additivity when the two antibodies are mixed. Although they are derived from immune cells arising in two different mice, there is no other way to distinguish these antibodies, so far. Both 73D3 and 89F9 have been successfully grown as immunoglobulin-producing ascites tumors and both antibodies have been purified to at least 95% purity.

Development of Rabbit Anti-palytoxin CIEIA. Rabbit HBG 007 was bled two weeks after the second booster immunization and the serum tested for the presence of palytoxin specific antibodies. The results of the primary screen for antibodies are shown in Figure 3.

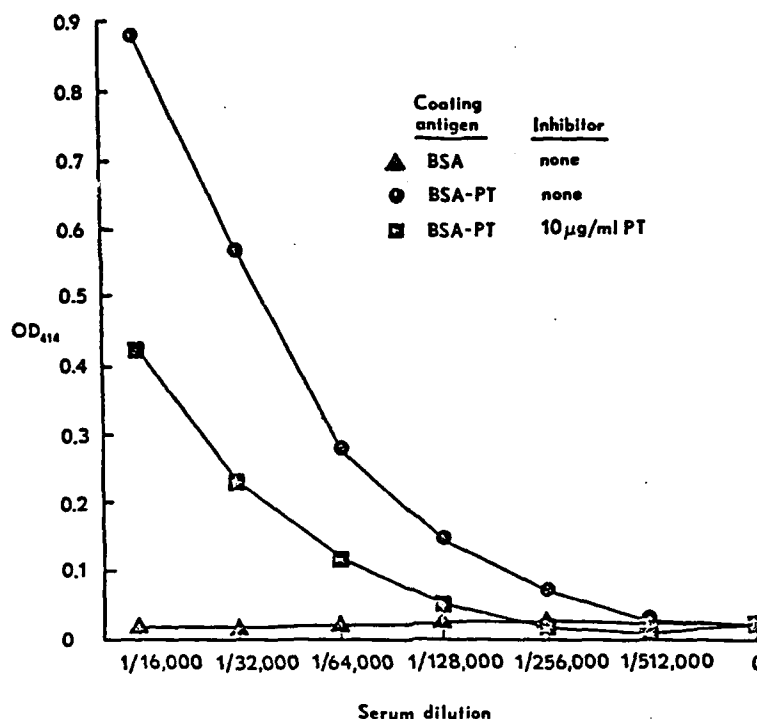


Figure 3. Primary ELISA Screen for Rabbit Antibodies to Palytoxin. The protocol given in Appendix 7 was followed. Rabbit 007, bleed 3 serum was subjected to serial dilution and tested on BSA or BSA-palytoxin in the presence or absence of free palytoxin. Absorbance at 414 nm was measured after one hour incubation at room temperature.

The presence of anti-palytoxin antibody is demonstrated by immunoglobulin binding to BSA-PTX-SPDP, but not BSA alone. The specificity of the reaction is further indicated by the fact that free palytoxin is able to inhibit significant amounts of the binding.

After selecting optimal concentrations of coating antigen (a saturating dose), rabbit anti-palytoxin antibody (approximately one half saturation), and goat anti-rabbit alkaline phosphatase conjugate (a dilution which gave and OD₄₁₄ value of 0.3 to 0.8 after one hour of incubation with substrate at room temperature), dilutions of palytoxin were tested to establish the range of inhibition obtainable. As shown in Figure 4, palytoxin inhibition decreased from about 50% to near zero over a range in palytoxin concentration of 10 µg/ml to 1 ng/ml.

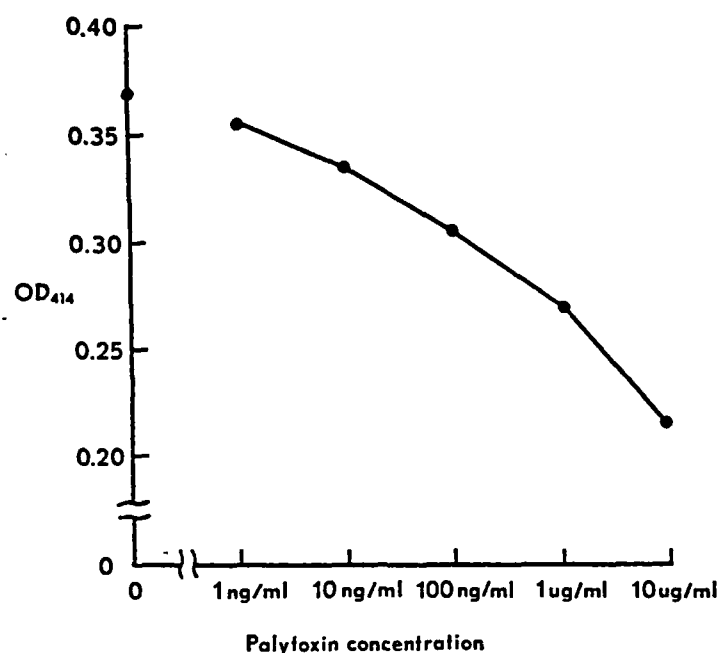


Figure 4. Titration of Palytoxin in Rabbit Anti-palytoxin CIEIA. The protocol given in Appendix 7 was followed. Rabbit anti-palytoxin antiserum HBG 007, bleed 4 diluted 1/32,000 was used. Palytoxin was diluted as indicated. OD₄₁₄ was determined after 45 minutes.

Although the slope of the inhibition curve was shallow and incomplete, it could be used for the determination of palytoxin in unknown samples. When a new batch of BSA-PTX-SPDP was prepared on March 12, 1987, full inhibition was seen over the same range of palytoxin concentrations (see Figure 5).

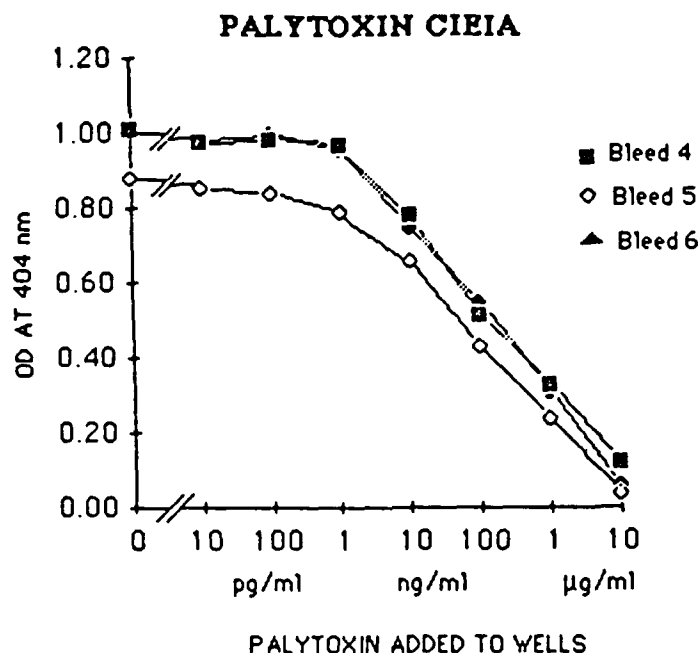


Figure 5. Titration of Palytoxin in Rabbit Anti-palytoxin CIEIA Using BSA-PTX-SPDP coating antigen prepared March 2, 1987. The protocol given in Appendix 7 was followed. Rabbit antisera were diluted 1/16,000.

The steeper slope of the inhibition curves observed in Figure 5 led to full inhibition at 10 mg/ml. This result was encouraging since it offered the possibility of more accurate measurements of palytoxin concentration in unknown samples. However, the completeness of inhibition at 10 mg/ml palytoxin concentration began to diminish over the succeeding months. Subsequently, it was determined that the age of the coating antigen had an effect on the slope of the inhibition curve, as shown in Table 5.

Table 5. Effect of the Age of the BSA-PTX-SPDP Coating Antigen on Inhibition of Rabbit Anti-palytoxin Antiserum HBG 007, Bleed 4 by Free Palytoxin.

DATE TESTED	BATCH OF COATING ANTIGEN	
	3/26/86 BSA-PTX-SPDP	3/2/87 BSA-PTX-SPDP
	B/B ₀ at 10 ug/ml Palytoxin ^a	
5/6/87	0.33	0.05
7/1/87	0.50	0.16
8/5/87	n.d. ^b	0.27

a. B/B₀ = OD₄₁₄ of sample/OD₄₁₄ of zero palytoxin control.

b. n.d. = not done.

BSA-PTM coating antigen behaved similarly to aged BSA-PTX-SPDP, as shown in Figure 6.

COMPARISON OF TWO COATING ANTIGENS

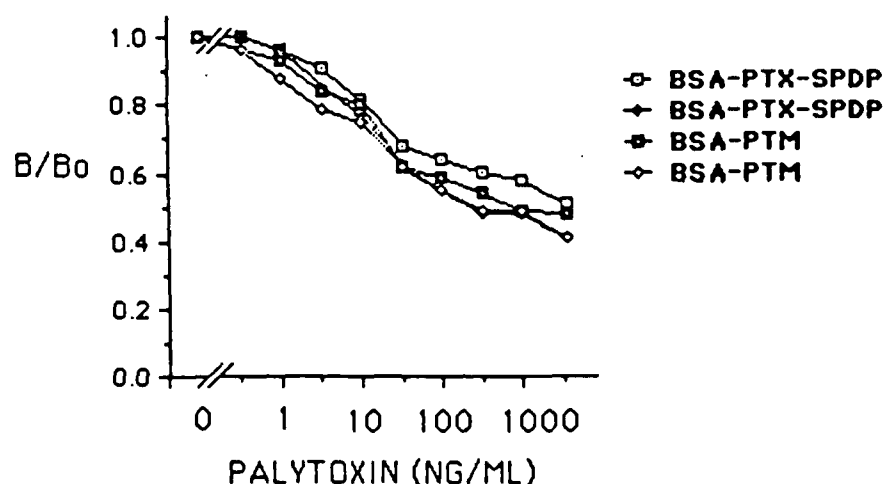


Figure 6. Comparison of Coating Antigens. BSA-PTX-SPDP and BSA-PTM were used to coat ELISA plate wells. The protocol given in Appendix 7 was followed. Key to symbols: squares - rabbit HBG 007, bleed 4, diluted 1/8000; diamonds - rabbit HBG 007, bleed 4, diluted 1/16000.

Development of Mouse Monoclonal Antibody Palytoxin CIEIA. Mouse monoclonal anti-palytoxin antibody 73D3 has been used extensively for the immunoassay of palytoxin according to the protocol given in Appendix 9. A representative standard curve of inhibition by free palytoxin is shown in Figure 7. Inhibition ranges from virtually 100% to zero in a palytoxin concentration range of 1 to 100 ng/ml. The inhibitor concentration resulting in 50% inhibition (IC_{50}) is 6.5 ng/ml.

PALYTOXIN MONOCLONAL CIEIA STD. CURVE

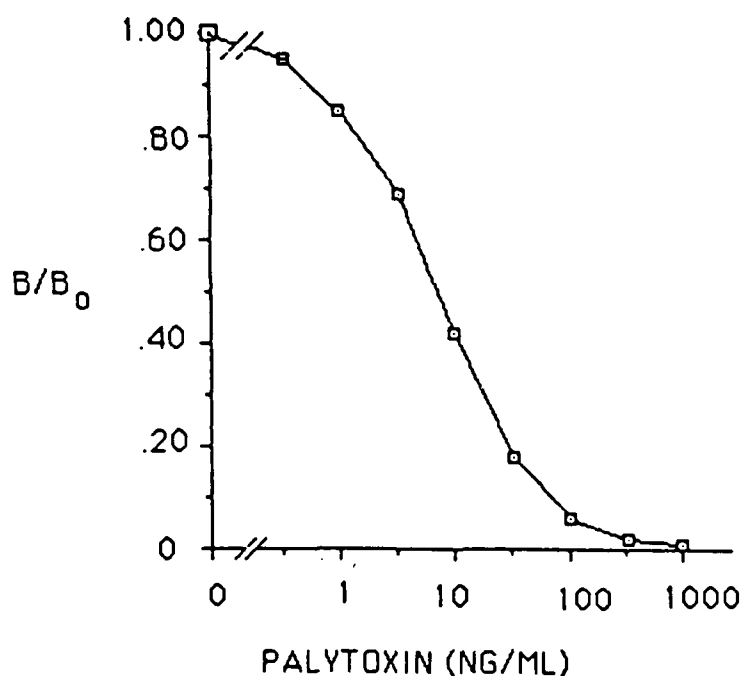


Figure 7. Titration of Palytoxin in Monoclonal Anti-palytoxin CIEIA. The protocol given in Appendix 9 was followed. Mouse monoclonal 73D3 culture medium supernatant diluted 1/64 was used. Palytoxin was diluted as indicated. OD_{414} was determined after one hour. B/B_0 is OD_{414} of sample divided by OD_{414} of zero palytoxin controls.

The minimum detectable dose (MDD) in this assay is the lowest concentration of palytoxin which had B/B_0 values significantly different from the zero palytoxin controls. By Student's unpaired t test, the MDD was 1 ng/ml with a p value of $.025 < p \leq .05$.

The reproducibility of the immunoassay can be assessed by measuring the constancy of IC₅₀ values obtained over a period of time. The results of 30 experiments conducted by two different investigators over a period of 72 days are plotted as a scattergram in Figure 8. The mean and upper and lower 95% confidence limits are shown. One experiment fell outside of the upper confidence limit by a great margin and another fell slightly above the upper confidence limit. By the criterion generated from the 95% confidence limits, results obtained on either of those days should be discarded.

IC₅₀ SCATTERGRAM

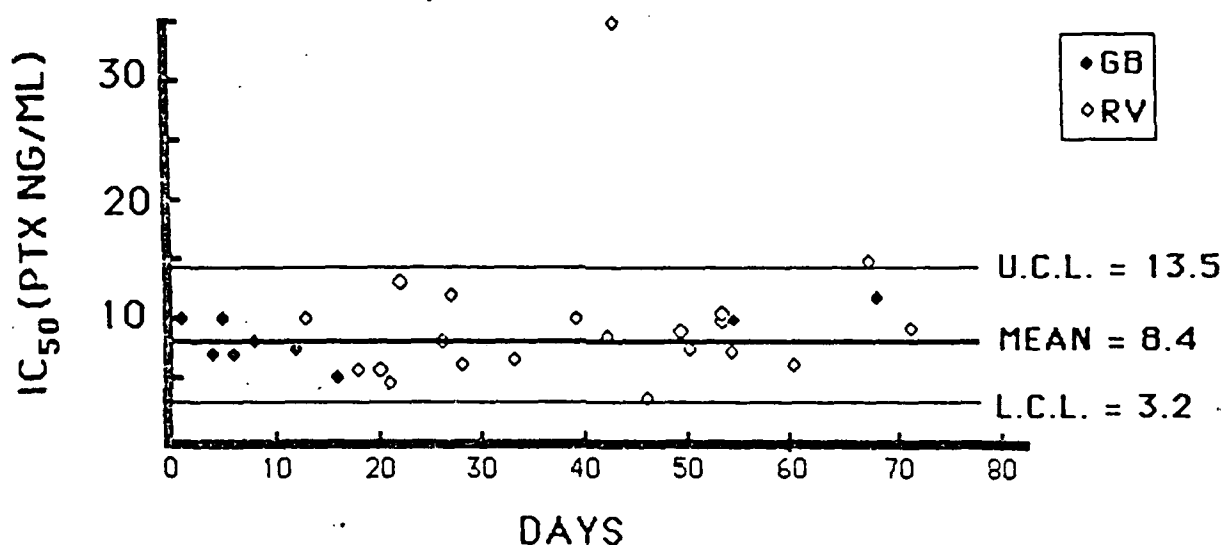


Figure 8. Scattergram of IC₅₀ Values. IC₅₀ values from 30 runs of the monoclonal anti-palytoxin CIEIA are plotted as a function of time. Monoclonal antibody 73D3 was used according the protocol given in Appendix 9. GB and RV are two investigators in our laboratory.

Test method accuracy can be assessed by spiking samples with known amounts of analyte and comparing recovery with input (ref 20). We prepared extracts from seven discrete colonies of *P. tuberculosa* collected at Kahe Point, Oahu in May, 1987. Each extract was spiked with an amount of palytoxin which would increase the palytoxin content of the diluted samples of the extract by 10 ng/ml. The untreated and spiked extracts were then analyzed by monoclonal CIEIA relative to a standard curve generated with purified palytoxin. The resulting estimates of palytoxin concentrations are presented in Table 6. The mean and the standard deviation of the percent recovery indicate the accuracy of the method.

Table 6. Palytoxin CIEIA Spike and Recovery Experiment.

SAMPLE	CRUDE EXTRACT ^a	SPIKED EXTRACT ^b	INCREMENT	PERCENT RECOVERY
	(ng/ml palytoxin) ^c			
1	41.8	52.8	11.0	110
2	2.7	13.6	10.9	109
3	30.5	46.4	15.9	159
4	49.0	58.4	9.4	94
5	15.3	23.2	7.9	79
6	1.9	8.6	6.7	67
7	3.5	13.5	10.0	100

MEAN = 102.7 ± 29.3

^a Extracts were prepared by extracting one unit (by weight) of *P. tuberculosis* with two units (by volume) of 70% ethanol in water for 24 hours.

^b Samples were spiked with palytoxin to provide an increment of 10 ng/ml in the palytoxin concentration of the spiked sample.

^c ng/ml of the diluted sample placed in the well.

As a test for matrix interference, a sample previously estimated to contain approximately 11.5 ug/ml palytoxin was selected. Serial doubling dilutions of the extract ranging from 1/500 to 1/2000 were prepared. Each dilution step was then subdivided into a series of aliquots. The aliquots were spiked with increasing amounts of palytoxin. Two to four replicate experiments were then conducted to determine the total palytoxin content of each tube by monoclonal anti-palytoxin CIEIA. The recovered palytoxin content of the samples placed in the well are shown in Figure 9.

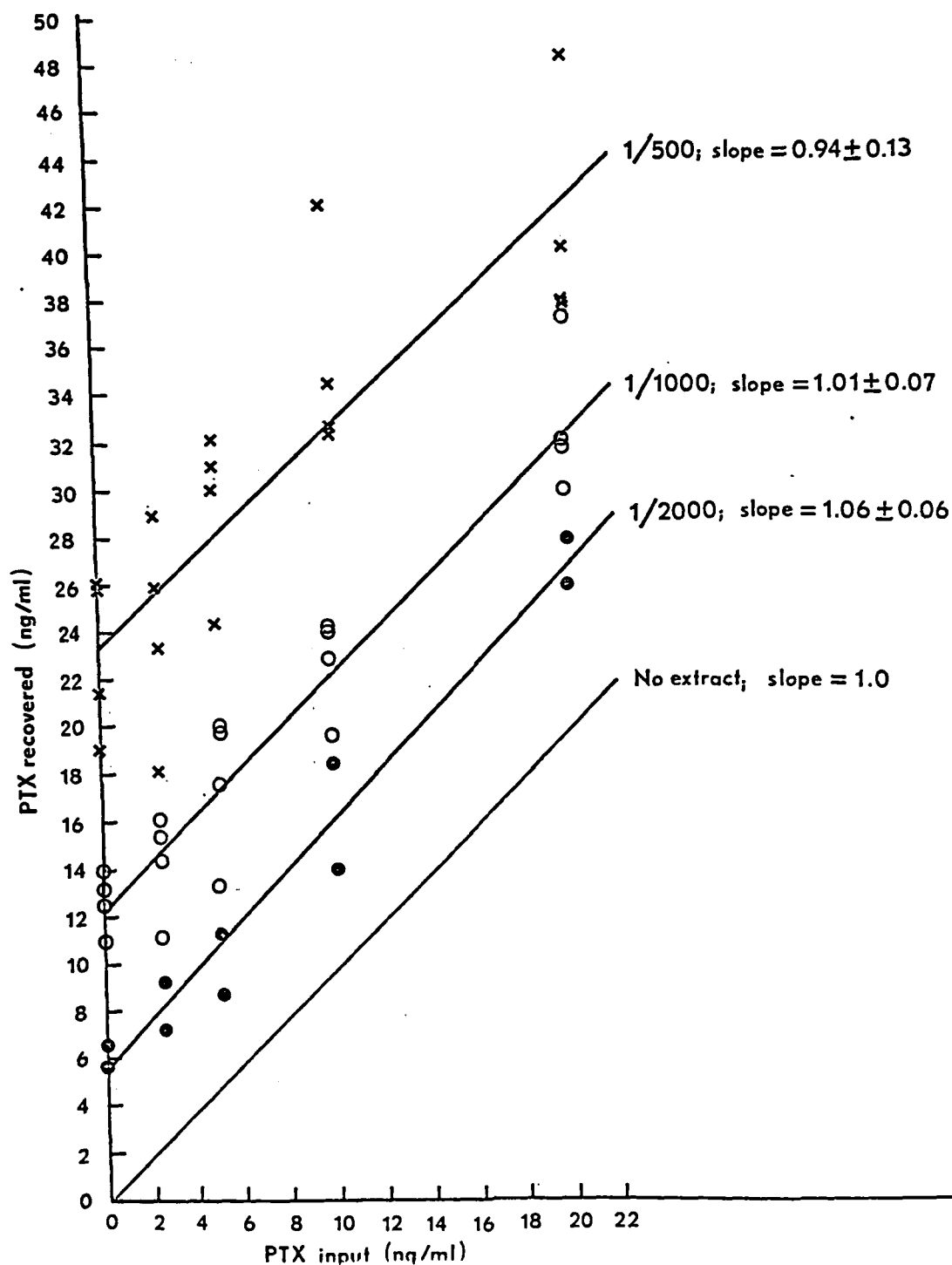


Figure 9. Recovery of Palytoxin in Varying Dilutions of a Palythoa Extract. Different dilutions of a single Palythoa extract were prepared and spiked with varying amounts of palytoxin. Data points from two to four experiments are shown along with calculated regression lines. The protocol presented in Appendix 9 was followed except that the incubation time for palytoxin and monoclonal anti-palytoxin antibody was four hours instead of the usual one hour.

Subsequently, another batch of extracts was prepared and tested for "parallelism" (ref 21). The results are shown in Figure 10. Tests run at different concentrations of extract should all give the same calculated value of analyte concentration in the original sample. This would show up in the plot as a horizontal line. Some extracts behaved in the expected manner and some did not.

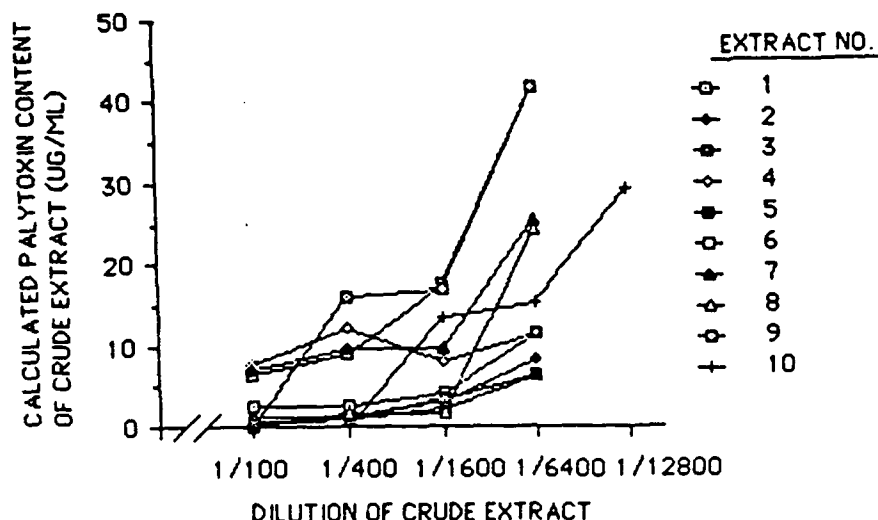


Figure 10. Test for Parallelism. Ten extracts were prepared and tested for palytoxin content by monoclonal CIEIA. The calculated value for the palytoxin content of the original samples are plotted.

Development of a "Sandwich" ELISA for palytoxin. Competitive inhibition immunoassays are less favorable for detecting small amounts of analyte than assays in which the amount of signal increases with increasing analyte. A sandwich or immunometric type of assay was therefore attempted. Partially purified antibody 73D3 was prepared from mouse ascites fluid by ammonium sulfate precipitation and used to coat 96 well ELISA plates. Palytoxin was then added, followed by rabbit anti-palytoxin antiserum. Bound rabbit antibodies were detected by addition of goat anti-rabbit immunoglobulin alkaline phosphatase conjugate and a suitable substrate. A positive reaction was observed. Each component of the system was then titrated to determine optimal concentrations. The procedure presented in Appendix 10 ensued. A typical standard curve showing the effect of increasing palytoxin content on the amount of signal observed is presented in Figure 11.

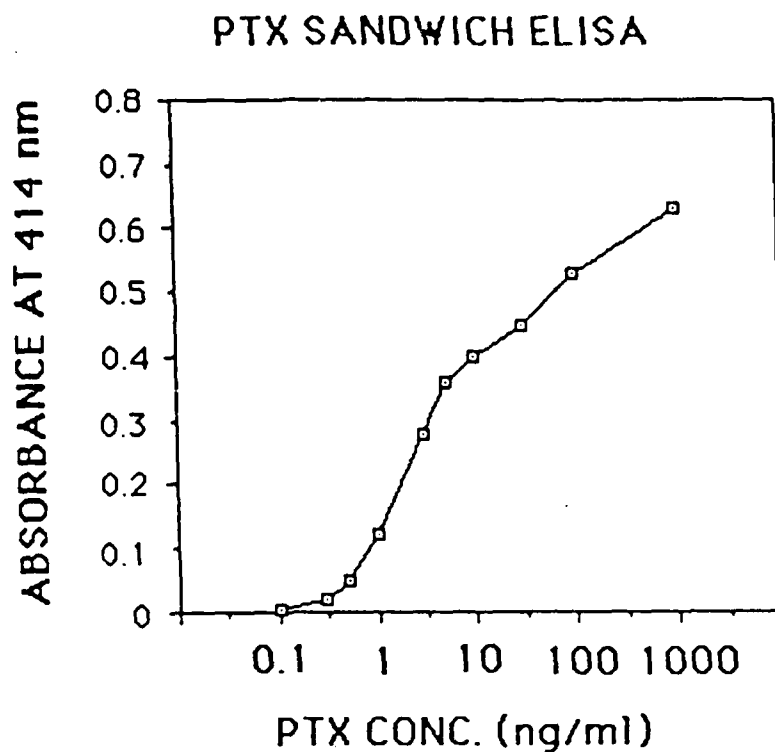


Figure 11. Palytoxin Sandwich Immunoassay Standard Curve. The procedure given in Appendix 10 was followed. Absorbance at 414 nm was measured after one hour.

The MDD for this experiment is 0.3 ng/ml ($.025 < p \leq .05$). The uneven upper portion of the curve is characteristic of 10 replications of this experiment. At this time we do not have an explanation for this irregular dose response relationship at high analyte concentrations. We are confident, however, that the lower portion of the curve is accurately able to measure palytoxin concentrations between 0.3 and 10 ng/ml.

DISCUSSION

The major milestones for the first year of this project were met.

Collection, isolation, and delivery of 100 mg of purified palytoxin were successful. Based on this year's experience, year two should proceed well in this area.

Procedures previously designed for the production of palytoxin haptens linked at the terminal amino group enabled the production of two types of hapten-protein conjugates. The SPDP hapten couples to proteins via disulfide bridges and the maleimide hapten forms a thioether linkage. Disulfide bridges can be broken by reducing agents. The thioether linkage should be much more stable. Although we have had early success with PTX-SPDP, we now intend to focus primarily on maleimide containing haptens. BSA, KLH, alkaline phosphatase and horseradish peroxidase conjugates have all been produced (data was not shown for the two enzyme conjugates since they were not extensively characterized or tested).

Although the method of characterizing palytoxin-protein conjugates is indirect, the results we obtained appear adequate to allow a reasonable level of standardization and reproducibility of the product.

The rabbit antiserum CIEIA produced so far is not the method of choice. The dose response curves for palytoxin detection are very shallow and are variable in terms of degree of inhibition at a moderately high concentrations of palytoxin. Although evidence was obtained that the age of the coating antigen had an affect of the observable inhibition, no explanation for the phenomenon is proposed. Thioether as well as disulfide linked conjugates showed the phenomenon. However, this effect was not observed with either of the monoclonal antibody based immunoassays.

The monoclonal antibody CIEIA has excellent characteristics in terms of the limit of detection, the slope of the titration curve, and reproducibility over time. Spiking experiments with crude extracts showed recovery not significantly different from expected. The accuracy is about $\pm 50\%$, which is about normal for many immunoassay systems. In the example presented in Figure 9, matrix interference was not detected when different dilutions of a crude extract were spiked with known amounts of purified palytoxin. The slopes of the lines were consistent with the expected value of 1.0 and the increments between extracts of different dilutions conformed to predicted behavior. However, as shown in Figure 10, we have observed non-ideal behavior with other extracts. We are currently attempting to determine if

this result is caused by matrix interference, bias in the assay, or other effects (ref 21).

The principle underlying a sandwich ELISA requires that antibodies detect two distinct epitopes on the analyte molecule. Monoclonal antibody 73D3 was partially purified to increase the efficiency of plate coating and used as an immunoextraction antibody. The polyclonal rabbit anti-palytoxin was used as the second antibody. Detection of the antibody-antigen-antibody sandwich was achieved with commercially available goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate. Sandwich immunoassays are considered to be highly specific for an intact antigen since a positive signal can only result when two specific binding events have taken place.

It is noteworthy that two antibodies are able to recognize and simultaneously bind to a molecule the size of palytoxin. To date, we have not found any other example of a double antibody sandwich immunoassay for a molecule this small. However, we are aware that Valentine and Green (ref 22) were able to produce exquisite electron micrographs of the simultaneous binding of two antibodies to a divalent antigen of molecular weight of 476 daltons. Since the same palytoxin immunogen, prepared via the palytoxin amino group, was used to elicit all of our currently available antibodies, we know that at least two separate epitopes exist distal to the amino terminus. It is possible that through hapten formation at alternative conjugation sites, more epitopes could be defined. This possibility offers a rationale for our projected search for haptens linked at primary or secondary hydroxyl groups on palytoxin.

In the coming year the sandwich ELISA will be further characterized and tested. We anticipate delivery of this system by the end of the year.

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¹⁴C-LEUCINE INCORPORATION CYTOTOXICITY ASSAY FOR PALYTOXIN

NOTE: Steps 1-7 require aseptic technique.

1. Culture murine T-cell leukemia, EL-4, in Dulbecco's minimal essential medium supplemented with 10% (v.v) calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate (supp. MEM).
2. Harvest EL-4 cells by centrifugation at 200 x g for 7 minutes. Resuspend in supp. MEM (leucine free) at 2×10^6 cells/ml.
3. Dilute purified palytoxin standard preparation to 50 to 0.5 pg/ml in supp. MEM. Dilute palytoxin "unknowns".
4. To each well of a sterile 96-well tissue culture tray, add 50 ul of the EL-4 suspension. Then add 50 ul of diluted palytoxin standards, diluted palytoxin unknowns or medium controls to appropriate wells.
5. Incubate for 18 hours in 5% CO₂/95% humidified air at 37° C.
6. Add 50 ul supp. MEM containing 0.1 uCi ¹⁴C-leucine to each well.
7. Incubate for 2 hours in 5% CO₂/95% humidified air at 37° C.
8. Harvest contents of wells onto glass fiber filters usin MA Bioproducts MiniMash unit. Chase with 50 ml water then 50 ml 95% ethanol.
9. Dry filters at 50° C under vacuum for at least one hour.
10. Place disks in scintillation cocktail and count radioactivity.
11. Plot results relative to plain medium controls.

BSA-PTX-SPDP CONJUGATION

June 3, 1987

1. Add 1 ml of 10 mg/ml BSA to 1 ml of 0.1 mg/ml 2-iminothiolane, both in 25 mM borate buffer, pH 9.0 (5-fold molar excess of 2-IMT to BSA). React 1 hr. at room temp, with continuous stirring.
2. Apply mixture to Sephadex G-25 column, equilibrated with 50 mM sodium phosphate buffer, pH 6.6, 1 mM EDTA. Elute with same buffer. Collect 16-drop fractions while monitoring OD₂₅₄ on Uvicord III. Read OD₂₈₀ of fractions on Beckman DU-7. Pool fractions having OD >0.5.
3. Do 2-DTDP assay for free sulfhydryl groups and BCA assay for protein content of sample. There should be around 1-2 mol -SH per mole of BSA.
4. Mix 5 mg of BSA-SH with a 5-fold molar excess of PTX-SPDP. React overnight at 40° C with continuous stirring.
5. Pre-rinse Centricon 30 unit with 2 ml phosphate buffer, pH 6.6 to remove glycerine and azide. Spin down sample 5X, bringing volume back up to 2 ml each time with phosphate buffer pH 6.6. Scan first filtrate for pyridine-2-thione release (OD₃₄₀). Calculate mole pyridine-2-thione released per mole of BSA (should be about 1-2).
6. Do 2-DTDP and BCA assays on sample. Should indicate <1 mole -SH per mole BSA.
7. Sterilize BSA-PTX-SPDP using a Centrex 22 and store at 40° C.

Ref.: Expts. JKU 5-16, -17, -18.

BSA-PALYTOXIN MALEIMIDE CONJUGATION

March 22, 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-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. palytoxin maleimide hapten (PTM)

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 PTM, relative to the net BSA thiolation. React for 1 hr. at room temperature, with stirring.
5. Dialyze the BSA-PTM against 500 ml phosphate buffer, changing buffer 4X over 4 days.
6. Transfer BSA-PTM 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 palytoxin conjugation to BSA.

KLH-PALYTOXIN MALEIMIDE CONJUGATION

March 22, 1988

MATERIALS

1. 25 mM borate buffer, pH 9.0
2. 0.1 M sodium phosphate buffer, pH 6.6, 1 mM EDTA
3. KLH, dialyzed in 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. palytoxin maleimide hapten (PTM)

METHOD

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.
2. 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.
3. Do DTDP and BCA assays, calculate KLH concentration and mole -SH per mole KLH.
4. Mix KLH-SH with a 5-fold molar excess of PTM, relative to the net KLH thiolation. React for 1 hr. at room temperature, with stirring.
5. Dialyze the KLH-PTM against 500 ml phosphate buffer, changing buffer 4X over 4 days.
6. Transfer KLH-PTM 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 palytoxin conjugation to KLH.

BCA PROTEIN ASSAY
(Pierce Chemical Co. Protocol)

MATERIALS:

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

METHOD:

1. Make up assay samples in duplicate. To separate tubes containing 100, 80, 60, 40, 20 and 0 ul buffer, add 0, 20, 40, 60, 80, and 100 ul protein standard at 1 mg/ml. Add 100 ul samples 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 temp in tap water bath. Read OD₅₆₂.

DETERMINATION OF -SH GROUPS

(Grasseti, D.R. and J.F. Murray, 1967. Arch. Biochem. Biophys. 119: 41-49.)

MATERIALS

1. 2×10^{-3} M 2,2'dithiodipyridine (DTDP). Weigh out 10 mg DTDP into an Eppendorf tube, add 100 μ l DMF, 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 2×10^{-3} M DTDP, mix.
2. Incubate samples 15 min at room temp. Read OD₃₄₃ using tube containing 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.)

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

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 separating the protein from the low m.w. 2-thiopyridine by centricon ultrafiltration would work. Using alkaline phosphatase or bovine serum albumin, this assay seems to give reliable results for protein concentrations of around 0.2 to 0.8 mg/ml.

PRIMARY SCREEN FOR ANTIBODIES TO PALYTOXIN

1. Coat with BSA-PTX-SPDP or BSA-PTM (@....* ug/ml) in PBS pH 7.0.
100 ul/well. 1 hr.
2. Wash 3X with PBS-T20.
3. Block with 1% BSA in PBS pH 7.0. 200 ul/well 1 hr.
4. Wash 3X with PBS-T20.
5. Add serum or culture medium being tested for anti-PT antibody
diluted in 1% BSA in PBS pH 7.0. 100 ul/well. 1 hr.
6. Wash 3X with PBS-T20.
7. Add goat anti-rabbit Ig-Alkaline phosphatase conjugate (or anti-
mouse Ig conjugate, as appropriate) diluted in 1% BSA in PBS pH 7.0.
100 ul/well. 1 hr.
8. Wash 4X with PBS-T20.
9. Add alkaline phosphatase substrate (per plate: 25 ml alkaline
phosphatase substrate buffer, pH 9.5 + 5 Sigma 104 tablets). 200 ul/well.
10. Read on dual wavelength absorbance mode (reference wave length
- 690 nm; measurement wavelength - 414 nm).

*COATING ANTIGEN CONCENTRATION MUST BE DETERMINED IN ADVANCE
FOR EACH BATCH. NORMALLY, TWICE THE MINIMUM SATURATING
CONCENTRATION IS USED.

PALYTOXIN CIEIA (RABBIT ANTISERA)

June 2, 1987/Ammended 9/20/97

	<u>TIME</u>
1. Coat with BSA-PT (2 ug/well in carbonate buffer, pH 7.0). 100 ul/well.	1 hr.
2. Wash 3X with PBS-T20.	
3. Block with 1% BSA in PBS. 200 ul/well.	1 hr.
4. Wash 2X with PBS-T20.	
5. Add PT dilutions (in PBS containing 1% BSA), then add anti-PT antibody dilutions (in 0.05 % BSA). 50 ul each per well.	1 hr.
6. Wash 3X with PBS-T20.	
7. Add GARIg-AP (diluted in PBS-T20).	1 hr.
8. Wash 4X with TBS-T20.	
9. Add AP substrate (Sigma 104 in AP buffer). 200 ul/well.	Variable
10. Read on Multiskan dual mode with filters 8/1.	

*COATING ANTIGEN USED AND CONCENTRATION MAY VARY WITH
EXPERIMENTAL PROTOCOL.

Ref.: Expt JKU 5-36.

PALYTOXIN CIEIA (MOUSE MoAb)

June 2, 1987/Ammended 9/20/97

	<u>TIME</u>
1. Coat with BSA-PT (2 ug/well in carbonate buffer, pH 7.0).* 100 ul/well.	1 hr.
2. Wash 3X with PBS-T20.	
3. Block with 1% BSA in PBS. 200 ul/well.	1 hr.
4. Wash 2X with PBS-T20.	
5. Add PT dilutions (in PBS containing 1% BSA), then add anti-PT antibody dilutions (in 0.05 % BSA). 50 ul each per well.	1 hr.
6. Wash 3X with PBS-T20.	
7. Add GAMIg-AP (diluted in PBS-T20).	1 hr.
8. Wash 4X with TBS-T20.	
9. Add AP substrate (Sigma 104 in AP buffer). 200 ul/well.	Variable
10. Read on Multiskan dual mode with filters 8/1.	

*COATING ANTIGEN USED AND CONCENTRATION MAY VARY WITH
EXPERIMENTAL PROTOCOL.

Ref.: Expt JKU 5-36.

PALYTOXIN SANDWICH ELISA

March 22, 1988

1. Coat wells with 73D3.2.1 (10 ug/ml in pH 7.0 PBS), 100 ul per well. 1 hr.
2. Wash 3X with PBS-Tween 20.
3. Block with 200 ul 1 % BSA in pH 7.0 PBS. 1 hr.
4. Wash 3X with PBS-Tween 20.
5. Add PTX standards, extracts, etc. (diluted in 0.5 mM $\text{Na}_2\text{B}_4\text{O}_7$ in 1 % BSA in PBS), 100 ul per well. 1 hr.
6. Wash 3X with PBS-Tween 20.
7. Add rabbit anti-PT antibody (Rabbit 007, Bleed 5, 1/3000 dilution in pH 7.0 PBS with 1 % BSA), 100 ul per well. 1 hr.
8. Wash 5X with PBS-Tween 20.
9. Add goat anti-rabbit Ig-alkaline phosphatase conjugate (1/4500 in pH 7.0 PBS with 1% BSA), 100 ul per well. 1 hr.
10. Wash 4X with TBS-Tween 20.
11. Add substrate (per plate: 25 ml alkaline phosphatase substrate buffer, pH 9.5 + 5 Sigma 104 tablets), 200 ul per well. 1 hr.
12. Read on dual wavelength absorbance mode (reference wave length = 690 nm; measurement wavelength = 414 nm). Variable

PALYTOXIN STORAGE AND PREPARATION FOR USE

1. STORAGE:

- STORE BULK SAMPLE DRY AT -20° C UNDER ARGON (NITROGEN COULD BE USED).

2. FOR USE:

- DISSOLVE CONTENTS OF FLASK IN 1.0 ML WATER.
- OBTAIN A DESIRED ALIQUOT (e.g. 20 μ L).
- DILUTE TO 1 MG/ML IN 50% ETHANOL:WATER (e.g. bring to 1.0 ml).
- STORE THIS WORKING STOCK TIGHTLY CAPPED AT -20° C.
- RETURN MAIN STOCK TO DRYNESS (SEE ITEM NO. 4, BELOW).

3. WORKING STOCK:

- ON THE DAY OF USE, CHARACTERIZE AND QUANTITATE BY U.V. SPECTROSCOPY.
- DILUTE AN ALIQUOT 1:50 IN WATER TO YIELD APPROX 0.02 MG/ML.
- SCAN O.D. OF THE DILUTED ALIQUOT FROM 190 TO 300 NM.
- CALCULATE PALYTOXIN CONCENTRATION USING $\epsilon_{263} = 23,600$ (REF.: MOORE, 1985--SEE BELOW).
- (OD₂₆₃ OF THE DILUTED ALIQUOT SHOULD BE ABOUT 0.15 TO 0.20)
- WHAT TO LOOK FOR:
 - REFER TO ATTACHED U.V. ABSORPTION SPECTRUM.
 - PEAKS AT 233 AND 263 NM SHOULD BE IN A RATIO OF APPROXIMATELY 1.6 TO 1.
 - ABSORPTION MINIMUM AT 200-210 NM SHOULD BE ABOUT EQUAL TO OR LESS THAN THE ABSORPTION MINIMUM AT 250 NM.
 - EXCESSIVE INCREASES IN END ABSORBANCE AT WAVELENGTHS BELOW 200 NM MAY INDICATE DEGRADATION OVER TIME.

4. RETURN MAIN STOCK TO DRYNESS

- FREEZE CONTENTS OF FLASK IN DRY ICE/ACETONE BATH--KEEP THE PALYTOXIN SOLUTION CONFINED TO THE BOTTOM OF THE FLASK.
- MAINTAIN FROZEN AT -100 to -200 C USING NaCl/WET ICE BATH.
- LYOPHILIZE USING A PUMP CAPABLE OF PULLING LESS THAN 0.006 ATM. (4.56 mm Hg) AND DRY ICE/ACETONE TRAPS.
- THIS PROCEDURE MAY TAKE UP TO THREE DAYS.
- WHEN DRY, RELEASE VACUUM, CAP UNDER ARGON AND STORE AT -20° C.

5. REFERENCE: MOORE, R.E., IN PROGRESS IN THE CHEMISTRY OF ORGANIC NATURAL PRODUCTS, VOL. 48, EDITED BY W. HERZ, H. GRISBACH, G.W. KIRBY AND CH. TAMM, SPRINGER-VERLAG, NEW YORK 1985, PP. 81-202.

DETERMINATION OF PALYTOXIN CONTENT

CALCULATIONS:

DILUTION FACTOR (DF) = 1/0.00019

OD₂₆₃ = 0.1792CONCENTRATION (MG/ML) = OD₂₆₃ X $\frac{M.W.}{\epsilon_{263}}$ X DF= 0.1792 X $\frac{2679 \text{ MG/M MOL}}{23,600 \text{ ML/M MOL}}$ X 1/0.00019

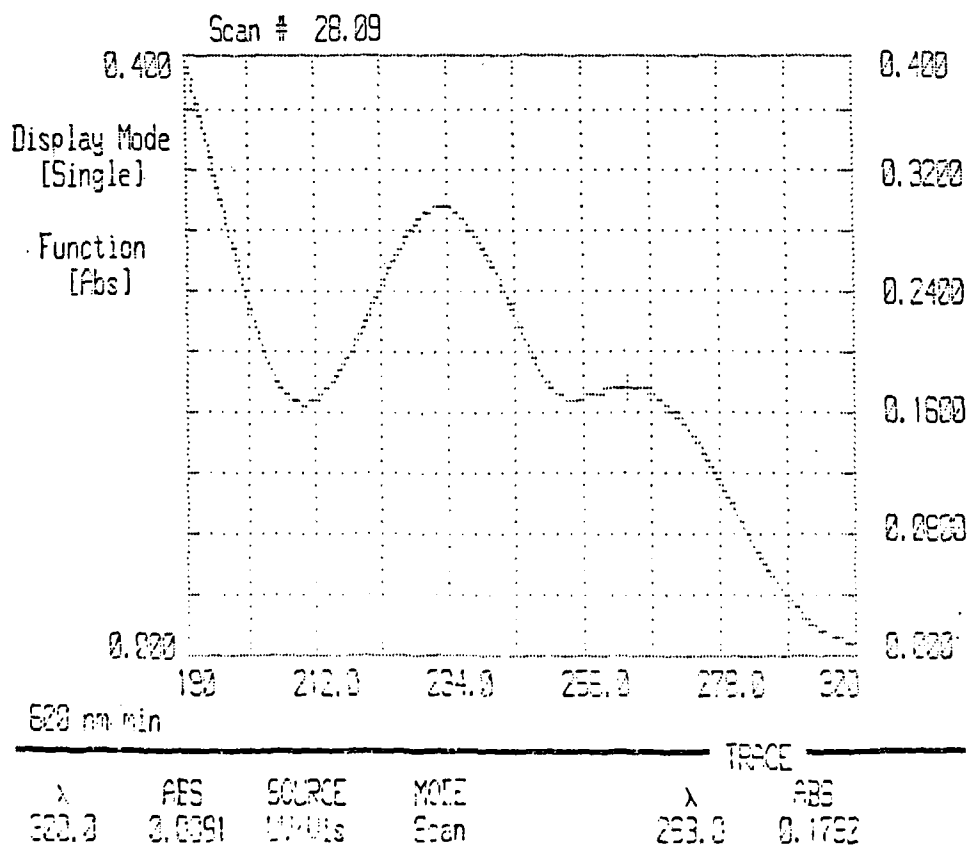
= 108 MG/ML

VOLUME USED = 0.5 ML

AMOUNT PALYTOXIN = 54 MG

DATE: 1/14/88

SIGNATURE: _____



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