Toxicity of *Gonyaulax tamarensis* var. *excavata* Cells to the Brine Shrimp *Artemia salina* L.

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Received February 25, 1981, from the **Department of Biology, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104* and the *‡ National Marine Fisheries Service, Northeast Fisheries Center, Milford Laboratory, Milford, CT 06460.* Accepted for publication August 21, 1981.

Abstract □ Brine shrimp (*Artemia salina* L.) were exposed to concentrations of live *Gonyaulax tamarensis* var. *excavata* cells ranging from 0 to 13,400 cells/ml in unialgal culture. The shrimp ingested the dinoflagellate cells and an LD_{50} determination was made. The shrimp had a 72-hr LD_{50} of 1258 *G. tamarensis* cells/ml, which was calculated to correspond to 73,385 μg/kg of body weight. This figure can be compared with an oral LD_{50} value of 2100 μg/kg for the mouse. Since it requires one-tenth as much toxin to kill a mouse (LD_{50} approximately 0.04 mg) as to kill an *Artemia* (LD_{50} approximately 0.4 mg), the whole cell *G. tamarensis* bioassay is a poor alternative to the current mouse assay.

Keyphrases □ Paralytic shellfish poisoning—toxicity of *Gonyaulax tamarensis* var. *excavata* cells to brine shrimp □ Brine shrimp—toxicity of *Gonyaulax tamarensis* var. *excavata*, potential bioassay for paralytic shellfish poisoning □ Bioassay—potential, toxicity of *Gonyaulax tamarensis* var. *excavata* to brine shrimp, paralytic shellfish poisoning

Paralytic shellfish poisoning is a potentially fatal form of food poisoning caused by ingestion of shellfish that have been feeding on blooms of toxic dinoflagellates (1). Mollusks feeding on the dinoflagellates appear unaffected, the immunity apparently related to a lack of sensitivity of the nerves of bivalve mollusks to the action-potential blocking effects of the toxins (2). Isolated crayfish (*Procambarus clarkii*) (3), spider crab (*Maia squinado*) (4), and American lobster (*Homarus americanus*) (5) nerves have been found to be highly susceptible to the toxins. Controlled feeding of highly toxic clams to lobsters has, however, failed to demonstrate any toxicity to the intact animal (6).

The present study attempted to develop an inexpensive bioassay for paralytic shellfish poison to replace the costly mouse assay, and to determine the LD_{50} value of the gonyautoxins present in the Atlantic dinoflagellate *Gonyaulax tamarensis* var. *excavata*, to a crustacean (*Artemia salina* L.). This was a preliminary step in the investigation of the immunity mechanism of crustaceans to the toxins.

EXPERIMENTAL

Culture of Organisms and Confirmation of Dinoflagellate Toxicity—Experimental media for culture of *Gonyaulax* (*Gonyaulax* medium) were prepared from salt well water[1] and supplemented with 100 mg of KNO_3, 10 mg of K_2HPO_4, 1 mg of $FeCl_3$, and 0.05 mg of Na_2SiO_3/liter. The pH was adjusted to 8.6 with 1 *N* NaOH (7). This solution was sterilized by autoclaving for 15 min at 15 psi. Traces of cyanocobalamin and thiamine solutions which had been filtered through a sterile membrane filter unit were added to the medium aseptically (8).

The culture was initiated by inoculating 1 liter of medium with 1 ml of stock *Gonyaulax tamarensis* var. *excavata*, (Strain IB-72)[2] culture containing 20,000 cells/ml and was then incubated at 20° for 30 days in 3-liter Fernbach flasks. All cultures were incubated under continuous illumination in an environmental chamber. *G. tamarensis* cells in the culture were counted with a hemacytometer slide using appropriate dilutions to count 1-ml samples (6). The toxicity of this *G. tamarensis* strain was confirmed by mouse assay of an extract of the dinoflagellate. Three liters of 25-day *G. tamarensis* culture containing 2500 cells/ml were membrane filtered. The filter pads were then placed into 100 ml of 0.1 *N* HCl to lyse the cells. The clear yellow-green solution was refiltered to remove cellular debris, and the solution assayed using female white mice (9). *Dunaliella salina*[3] (a nontoxic marine phytoflagellate) was extracted and assayed as described. Brine shrimp (*Artemia salina* L.) eggs[4] were hatched and raised to the adult state (10) with *Dunaliella salina* as an additional food source.

Brine Shrimp Assay—The experimental vessels for the study of *G. tamarensis* toxicity to brine shrimp were 125-ml Erlenmeyer flasks which had been loosely stoppered with nonabsorbent cotton and autoclaved at 15 psi for 15 min. The dinoflagellate source was a 29-day *G. tamarensis* culture with an original concentration of 13,400 cells/ml. The experiment was performed using five different dinoflagellate concentrations (13,400, 6700, 3400, 1300, and 135 cells/ml) and a control containing no cells. Each flask was prepared by removing a portion of the stock culture with a sterile pipet and placing the calculated volume containing the appropriate number of cells into an aliquot of diluent (sterile *Gonyaulax* medium) in one of the experimental flasks. Five brine shrimp were then added to each flask, and five flasks were used for each concentration of dinoflagellate cells. The experiment was repeated four times for a total of 100 shrimp at each concentration of cells. After restoppering the flask with its cotton plug, it was examined for cessation of *Artemia* swimming motions at intervals of 20 min, 1, 8, 24, and 72 hr.

Two controls for this portion of the experiment were sterile *Gonyaulax* medium to which no inoculum had been added, and medium from an active culture of *G. tamarensis* (13,400 cells/ml) from which the cells had been removed by membrane filtration. Bioassay data on *G. tamarensis* were used to calculate LD_{50} values by transforming the curve for percent mortality *versus* log dose of toxin (cells/ml) to a straight line by probit

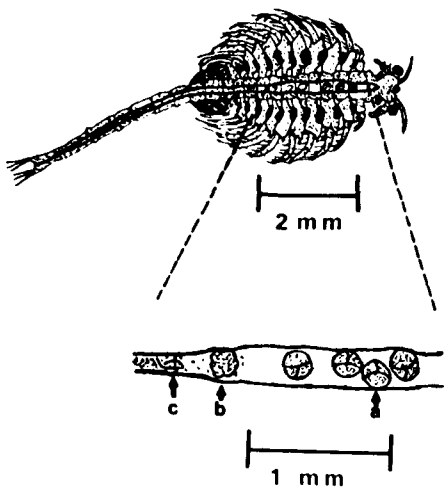

Figure 1—*Partially digested* Gonyaulax tamarensis *var.* excavata *within the digestive tract of a brine shrimp (*Artemia salina *L.). Top—view of* Artemia *with ingested dinoflagellate for size comparison. Bottom—Enlarged section of digestive tract showing phases of dinoflagellate degradation: (a) Intact* G. tamarensis *cells at beginning of tract, (b) cell further along in tract showing swelling and loss of cellular detail, and (c) cell fragments.*

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[2] Dr. Christopher Martin, University of Massachusetts Marine Station, Gloucester, MA 01930.
[3] Carolina Biological Supply Co., Burlington, NC 27215.
[4] Ward's of California, Monterey, CA 93940.

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Table I—Intraperitoneal Toxicity of Dinoflagellate and *Dunaliella salina* Control Extracts to Mice

Number of Mice	Toxin Source	Average Weight, g	Dilution	pH	Median Toxicity: Mouse, Unit	Median Toxicity: μg/ml	Median Toxicity: μg/100 g Meat
3	GTX ext[a]	10.6	1:5	2.0	1.008	1.09	218[b]
3	*Dunaliella* extract	10.9	1:1	2.0	0.0	0.0	000

[a] GTX ext = *G. tamarensis* cell extract. [b] Calculated theoretical values for purposes of comparison.

Table II—LD_{50} Values for *G. tamarensis* Cells to *Artemia salina* L. at 20 ± 2°

G. tamarensis Concentration, cells/ml	Number of Test Organisms	Number of Test Organisms Dead, hr: 1	8	24	48	72
13,400	100	0	3	38	69	87
6,700	100	0	3	28	69	81
3,400	100	0	0	16	42	61
1,300	100	0	2	16	48	62
135	100	0	1	4	13	39
0[a]	100	0	1	5	12	19
0[b]	100	0	0	1	1	2

	8 hr	24 hr	48 hr	72 hr
LD_{50} (cells/ml) from probit analysis	7.24×10^{10}	36308	5875	1258[c]
95% confidence limits	1.53×10^{12}	45709	9078	2058.7[c]
	3.43×10^{9}	28840	3802	703.6[c]
Slope of probit line	0.28069	0.9345	1.0145	0.8098
	±0.1576	±0.0538	±0.0464	±0.0151

[a] Control using inoculated *Gonyaulax* medium with cells removed by membrane filtration. This value was used in LD_{50} computations. [b] Control using sterile *Gonyaulax* medium. [c] Indicates value obtained by computer.

analysis (11), using a computer program written specifically for the purpose[5].

RESULTS AND DISCUSSION

Confirmation of Toxicity of *G. tamarensis* Culture—The solution obtained from extraction of the dinoflagellate was found to be toxic to mice (Table I). Concentration of toxin as determined by mouse assay was 109 μg/1000 ml of extract. This is 1 μg of saxitoxin equivalent/2294 cells. In the *Dunaliella* control solution, all mice survived.

Brine Shrimp Assay—The two control groups using *Gonyaulax* medium (uninoculated and inoculated with cells removed) gave negligible mortality and a pronounced mortality of 19%, respectively (Table II). This second mortality was taken into account when calculating the LD_{50}.

Mortality in the control group followed a pattern similar to that seen in the experimental group and was probably caused by toxins released into the medium by dinoflagellate cells (12). Brine shrimp added to *G. tamarensis* cells became intoxicated and died. Microscopic examination of the *Artemia* disclosed the presence of dinoflagellate cells within their digestive tracts (Fig. 1). The computer program used for statistical analysis of the data took into account the 19% control mortality (11). Thus, the rate at which *Artemia* were killed (according to this model) depended on the concentration of *G. tamarensis* cells rather than on the rate of dinoflagellate ingestion by the brine shrimp. Further studies on the rate of dinoflagellate ingestion should provide more accurate estimates of oral LD_{50}. Upon intoxication, the *Artemia* underwent a period of hyperactivity during which they evinced a series of contractions (anterior–posterior curling and uncurling motions). This lasted for 1–2 hr and then ceased. Soon after, the shrimp lost their ability to remain near the surface of the medium and sank to the bottom of the container. The vigor of swimming motions decreased gradually and the organism ceased all movements.

The LD_{50} for 72 hr was 1258 cells/ml in 50 ml of medium (Table II). This value was converted to a microgram per kilogram lethal dose by taking the value of 1 μg of toxin/2294 cells and multiplying it by the 20% toxin reduction expected between the 25th and 29th days of culture (13). The corrected value for toxin content per dinoflagellate cells is 1 μg of toxin/2868 cells. This figure yields a toxin content of 0.00035 μg/*G. tamarensis* cell. When this concentration is multiplied by the number of cells per lethal dose (Table II), it is found that the LD_{50} in terms of amount of toxin is 0.4403 mg/*Artemia*.

Since the culture was unialgal but not axenic, decomposition of *Artemia* proceeded rapidly after death, and attempts to obtain shrimp weights were futile unless performed immediately after death. An average *Artemia* weight of 6 mg was used in determining microgram per kilogram LD_{50} values (14). Thus, the toxicity of gonyautoxins to *Artemia salina* L. is ~73,385 μg/kg of body weight. This figure is almost 150 times larger than the oral LD_{50} of 500 μg/kg for man, and 18 times larger than the 4000 μg/kg for the most resistant recorded mammal, a monkey (15). Mice, which are the current assay organisms and are somewhat closer in size to *Artemia* than either of the aforementioned animals, have an oral LD_{50} value of 2100 μg/kg of body weight (15). Thus, one-tenth as much toxin is required to kill a mouse as to kill a brine shrimp (LD_{50} 0.04 mg and 0.4 mg/animal, respectively). This fact, coupled with the 72 hr duration of the test (compared to the 15 min required for the Association of Official Analytical Chemists mouse assay) make the whole cell assay of *G. tamarensis* by *Artemia* a poor alternative to the mouse assay for purposes of routine monitoring of shellfish toxicity.

Attempts to assay both homogenized cells and shellfish extract which had been proven toxic by mouse assay failed due to rapid fungal overgrowth of the test solution. *Artemia* subjected to overgrown extract died rapidly in the presence or absence of paralytic shellfish poison. Concomitant administration of an antifungal agent not only added another variable to the experiment, but also did not sufficiently control fungal growth over the 72 hr required for the test.

The cause of death in cases of saxitoxin intoxication is paralysis of muscular contraction by inhibition of nerve conduction (blockade of Na^+ influx) resulting in a peripheral paralysis of respiratory muscles (16), as opposed to a nonspecific cytolytic factor which physically destroys gill surfaces (as is the case with *Gymnodinium breve* toxin) (13). Absorption of the gonyautoxins is necessary for their activity, detoxification occurring in either the absorptive or metabolic phase of digestion.

Organic extraction procedures similar to those employed for brine shrimp assay of ciguatera toxins (17) were beyond the scope of this study but will be examined in future experiments.

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REFERENCES

(1) B. W. Halstead and D. A. Courville, "Posionous and Venomous Marine Animals of the World," vol. 1, U.S. Government Printing Office, Washington, D.C., 1965, p. 157.

(2) B. M. Twarog, T. Hidaka, and H. Yamaguchi, *Toxicon*, **10**, 273 (1972).

(3) J. S. D'Arrigo, *J. Membrane Biol.*, **29**, 231 (1976).

(4) P. F. Baker and K. A. Rubinson, *Nature (London)*, **257**, 412 (1975).

(5) J. Baumgold, *J. Neurochem.*, **34**, 327 (1980).

(6) C. M. Yentsch and W. Balch, *Environ. Lett.*, **9**, 249 (1975).
(7) E. J. Schantz, J. M. Lynch, G. Vaynada, K. Matsumoto, and H. Rapoport, *Biochemistry*, **5**, 1191 (1966).
(8) S. H. Hutner and J. J. A. McLaughlin, *Sci. Am.*, **199**, 92 (1958).
(9) "Official Methods of Analysis," 12th ed., Association of Official Analytical Chemists, Washington, D.C., 1975, p. 319.
(10) A. S. Michael, C. G. Thompson, and M. Abramovitz, *Science*, **123**, 463 (1956).
(11) D. J. Finney, "Probit Analysis," 2nd ed., University Press, Cambridge, England, 1962.
(12) A. Prakash, *J. Fish. Res. Board Can.*, **24**, 1589 (1967).
(13) A. W. White and L. Maranda, *ibid.*, **35**, 397 (1978).
(14) N. M. Trieff, M. McShan, D. Grajcer, and M. Alam, *Texas Rep. Biol. Med.*, **31**, 409 (1973).
(15) E. F. McFarren, M. L. Schafer, J. E. Campbell, K. H. Lewis, E. T. Jensen, and E. J. Schantz, *Proc. Natl. Shellfish Assoc.*, **47**, 114 (1956).
(16) M. H. Evans, *Br. J. Exp. Pathol.*, **46**, 245 (1965).
(17) H. R. Granade, P. C. Cheng, and N. J. Doorenbos, *J. Pharm. Sci.*, **65**, 1414 (1976).

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1,4-Bis(4-guanylphenylethyl)benzenes as Potential Antitrypanosomal Agents

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Received July 13, 1981, from the *Department of Chemistry, Georgia State University, Atlanta, GA 30303.* Accepted for publication August 14, 1981.

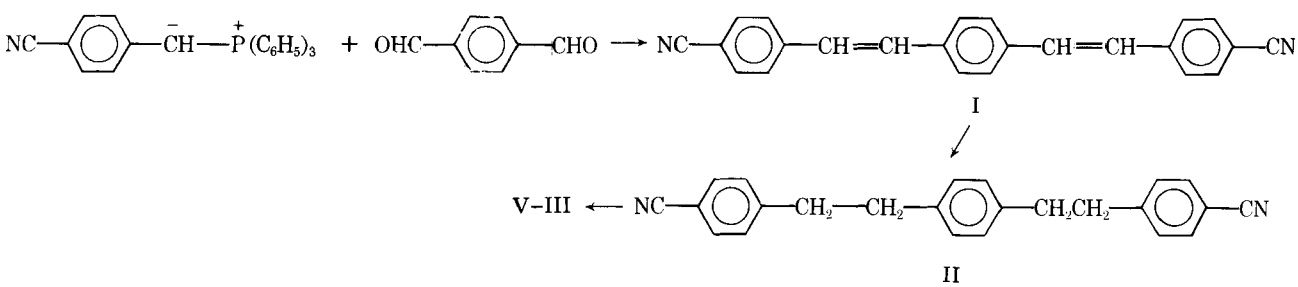
Abstract □ A series of 1,4-bis(4-guanylphenylethyl)benzenes, including masked amidines in which the guanyl function is incorporated into a heterocyclic ring, were prepared for screening as potential antitrypanosomal agents. Some of these compounds were active against *Trypanosoma rhodesiense* in mice. The diamidines were prepared by standard methods from 1,4-bis(4-cyanophenylethyl)benzene which was obtained from 1,4-bis(4-cyanostyryl)benzene by diimide reduction. The latter compound was prepared by the Wittig reaction between 4-cyanobenzylphosphonium ylide and terephthaldicarboxaldehyde.

Keyphrases □ Antitrypanosomal agents—potential, 1,4-bis(4-guanylphenylethyl)benzenes □ *Trypanosoma rhodesiense*—1,4-bis(4-guanylphenylethyl)benzenes, activity as antitrypanosomal agents □ Structure–activity relationships—1,4-bis(4-guanylphenylethyl)benzenes as potential antitrypanosomal agents

Aryl diamidines have been known to be useful antitrypanosomal agents for many years (1, 2). The aryl diamidines reported to exhibit antitrypanosomal activity fall into two general, apparently arbitrary, sets: those that have their guanyl functions separated by approximately 12Å and those that are separated by approximately 20Å (3). It is not known if this observation has any significance regarding the interaction of these compounds with their bioreceptor(s). A number of quite active aryl diamidines have been reported which fall into the category of the 12Å set (3–5) and this report describes efforts to synthesize compounds that fall into the 20Å class. Pentamidine (1), congocidin (1), and the terephthanilide amidines (6) are notable examples of the antitrypanosomal compounds that fall into the latter structural class. While pentamidine and the terephthanilide amidines have similar separation of the guanyl functions, they differ in that terephthanilide amidines probably exist in a planar conformation, whereas this is unlikely for pentamidine. The present study attempted to determine the effect on antitrypanosomal activity and binding to the bioreceptor for the terephthanilide amidine types which are conformationally more flexible. To test this point, 1,4-bis(4-guanylphenylethyl)benzene was synthesized along with related compounds in which the conformationally rigid carboxamido groups of the terephthanilide amidines were replaced by the conformationally flexible $-CH_2CH_2-$ units.

RESULTS AND DISCUSSION

The synthesis of the target diamidines was achieved by employing a conventional synthetic approach (Scheme I). The first step involves a Wittig reaction between the 4-cyanobenzylphosphonium ylide and terephthaldicarboxaldehyde to yield a bis-1,4-(4-cyanostyryl)benzene (I), the stereochemistry of which was not determined. The bis styryl



Scheme I

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