Isolation and Partial Characterization of Toxins from the Dinoflagellate *Gymnodinium breve* Davis

M. ALAM *[1], **N. M. TRIEFF** *[x], **S. M. RAY** [‡], and **J. E. HUDSON** [§]

Abstract ☐ Three neurotoxins were isolated from unialgal cultures of the dinoflagellate *Gymnodinium breve* Davis. Of the three toxins, only one toxin (T_1) has hemolytic activity. The major toxin (T_2), in chromatographically pure form, appears to have a molecular weight of 725. The neurotoxin T_2 has no antiacetylcholinesterase activity.

Keyphrases ☐ *Gymnodinium breve* Davis—isolation and partial characterization of three neurotoxins ☐ Dinoflagellate (*Gymnodinium breve*)—isolation and partial characterization of three neurotoxins ☐ Toxins—isolation from *Gymnodinium breve*, partial characterization

The dinoflagellate *Gymnodinium breve* Davis has been reported to be responsible for the massive fish-kill associated with the so-called red-tide which occurs periodically along the west coast of Florida and the Gulf of Mexico. The dinoflagellate produces endotoxins which have been shown to be toxic to marine as well as to laboratory animals (1–4). A number of workers have reported the isolation and partial characterization of toxin(s) from *G. breve* (5–9). The physiological actions of the crude and partially purified toxin(s) have also been investigated (8, 10–12). Most reports about the purification indicate that the toxin(s) was contaminated with carotenoid(s). It is also significant that no two workers have reported the isolation of the same toxin from *G. breve* culture.

For the past few years this laboratory has been studying *G. breve* toxins and now wishes to report a modified method for the isolation of three toxins from the ether extract of *G. breve* culture. Some physical and physiological properties of the major toxin (T_2) are also reported.

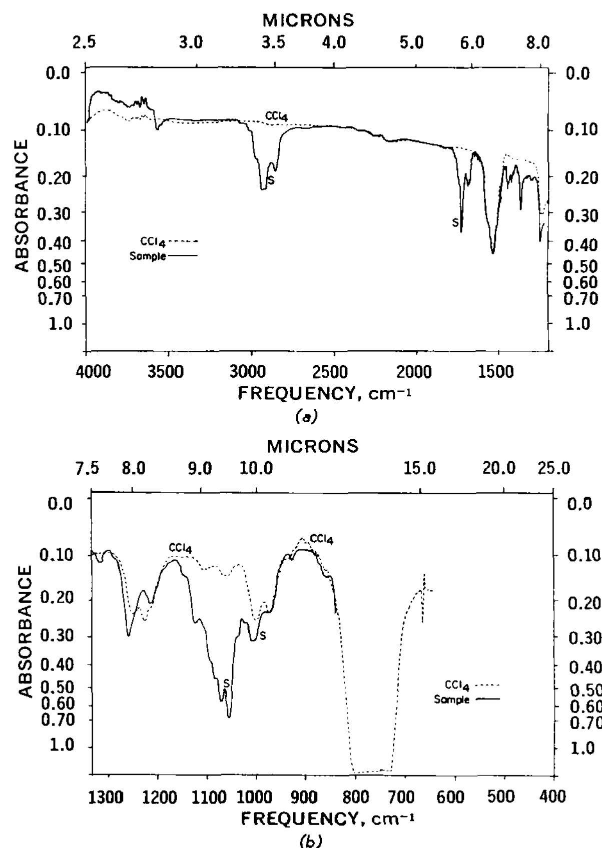

Figure 1—*IR spectrum of* G. breve *toxin T_2 in carbon tetrachloride. Concentration of T_2 is approximately 5 g %; 0.1-mm KBr cells were used with air as reference. The dotted line is the solvent; the solid line is the sample. Key:* (a), *frequency range of 4000–1200 cm^{-1}; and* (b), *frequency range of 1300–400 cm^{-1}.*

EXPERIMENTAL

G. breve[1] was cultured in modified NH-15 medium by the method of Gates and Wilson (13) and extracted by the method of Trieff *et al.* (7). The ether extract was evaporated to dryness *in vacuo*, and the residue was redissolved in a small volume of ether. Suspended particles were removed by centrifugation. The supernate was evaporated to dryness, and the residue was dissolved in 3 ml of benzene–ethyl acetate (2:1 v/v).

Column Chromatography—The *G. breve* extract in benzene–ethyl acetate (2:1 v/v) was placed on a column (7.5 × 7.5 cm), which was prepared by packing with a slurry of silicic acid[2] in benzene–ethyl acetate (2:1 v/v). The column was developed with benzene–ethyl acetate (2:1 v/v, 360 ml, fractions 1–9) followed by benzene–ethyl acetate (1:1 v/v, 360 ml, fractions 10–18) and finally with methanol (200 ml, fractions 19–23). In all, 23 fractions (40 ml each) were collected from column chromatography.

Each fraction was concentrated to 3–4 ml *in vacuo* on a rotating evaporator and spotted on a TLC plate to check its purity. On the basis of TLC in Solvent Systems I and II, fractions were combined to give a total of nine fractions. All column chromatography was carried out in the dark to avoid photochemical transformation.

TLC—Glass plates (20 × 20 cm) coated with silicic acid[3] (300 and 500 μm thickness) were used for qualitative and preparative work, respectively. Plates were activated for 0.5 hr at 110° just before use. TLC was carried out in the dark for the reason mentioned before. The solvent system used was benzene–ethyl acetate (2:1 v/v, Solvent System I; 1:1 v/v, Solvent System II). Location reagents used were 50% sulfuric acid, 1% aqueous potassium permanganate, and Dragendorff's reagent[4].

Combined fractions 7 and 8 were diluted with an equal volume of benzene and lyophilized and worked up to yield T_1 and T_2, a mixture of the two toxins being present in both combined fractions. The light-yellow residue in each case was dissolved in 2 ml of benzene and spotted on preparative TLC plates. The plates were developed in Solvent System II. After drying, the end portions of

[1] The unialgal starter culture was obtained from the culture collection of Dr. W. B. Wilson, Moody College of Marine Sciences and Maritime Resources, Texas A & M University, Galveston, TX 77550
[2] SilicAR 7GF for TLC, Mallinckrodt Chemical Works, St. Louis, Mo.
[3] Silica gel for TLC, D-5, Camag Inc., New Berlin, Wis.
[4] Dragendorff's reagent type QSR-D, Quantum Industries, Fairfield, N.J.

Table I—Weight, R_f Values, Toxicity, and Physiological Properties of the Toxins from *G. breve* Davis

	Fraction Number											
	Crude	1	2	3	4	5	6	7	8	9	T_1	T_2
Column chromatography eluting solvent		Benzene–ethyl acetate (2:1 v/v)					Benzene–ethyl acetate (1:1 v/v)			Methanol		
Weight of toxin(s), mg[a]	353.7	168.0	25.0	4.3	5.46	6.0	25.4	5.07	16.6	97.1	2.3	4.3
Phosphorus, % of weight	0.025	0.010	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.062	0.00	0.00
$R_f \times 100$ in Solvent System I	—	96, 90 84, 71	90, 84 71	84, 71	71, 60	71, 60 43	43, 25 12	11, 08 05	08, 05	<05	08, 05	08, 05
$R_f \times 100$ in Solvent System II	—	—	—	—	—	92, 85 74	85, 74 70	60, 50 42, 35	42, 35 26, 08 04, 00	26, 20	42	35
Antiacetylcholinesterase activity	(−)[c]	(−)	(−)	(−)	(−)	(−)	(−)	—[d]	—[d]	(−)	(−)	(−)
Hemolytic activity[b], H_{50}, µg	100	(−)	(−)	(−)	(−)	(−)	(−)	—[d]	—[d]	(−)	25	(−)
Mouse toxicity, LD_{100}, mg/kg	5.9	>10	>10	>10	>10	>10	>10	0.30	0.49	0.20	0.30	0.25

[a] Average of 15 column chromatography fractionations. [b] Concentration of the toxin (in micrograms) that will produce 50% hemolysis of rabbit erythrocytes under the experimental conditions (16). [c] (−) stands for a negative value in either antiacetylcholinesterase or hemolytic activity. [d] Was not determined because fractions 7 and 8 had both toxins T_1 and T_2.

the plates were sprayed with aqueous potassium permanganate and with water. The regions reacting positive with potassium permanganate [yellow spot on a pink background, R_f 0.42 (T_1) and 0.35 (T_2)] and water [dense spot on a translucent background, R_f 0.42 (T_1) and 0.35 (T_2)] were scraped off and eluted with benzene–ethyl acetate (1:1 v/v). The eluate was concentrated *in vacuo,* diluted with benzene, and lyophilized. The procedure was repeated until the eluate gave a single compact spot on a TLC plate.

Physiological and Physical Testing[5]—Phosphorus was determined by the method of Mason *et al.* (14) and Bartlett (15). The effect of crude and purified toxins and fractions 1–6 and 9 on human blood serum cholinesterase activity was determined by a spectrophotometric method[6]. Hemolytic activities of the crude and purified toxins T_1 and T_2 and fractions 1–6 and 9 were determined by the method of Reich *et al.* (16). Toxicities to the mice of various fractions and crude and purified toxins T_1 and T_2 were determined by the method of Trieff *et al.* (7).

Acid hydrolysis of toxin T_2 (9.0 mg) was carried out by refluxing toxin T_2 in 50 ml of 2 *N* hydrochloric acid in methanol at 70° for 9 hr. After hydrolysis, the methanol was removed by distillation and the hydrolysate was partitioned between equal volumes of water and ether. The ether layer was separated and evaporated to dryness *in vacuo.* The residue was spotted on a TLC plate, which was then developed in Solvent System II.

RESULTS AND DISCUSSION

Table I shows the R_f values and weights of combined fractions 1–9. Only fractions 7, 8, and 9 were found toxic to mice[7]. Fractions 7 and 8, by repeated preparative TLC, gave toxins T_1 and T_2 with R_f values of 0.42 and 0.35, respectively. Toxin T_3, contained primarily in fraction 9, was purified by column chromatography on silicic acid[2] and was eluted with 5% methanol in chloroform. Toxin T_3 was contaminated with small amounts of pigment(s) and was not further characterized at this time.

All three toxins were toxic to mice and appear to be neurotoxic in nature. The major toxin (T_2), a white amorphous powder, showed a molecular ion at *m/e* 725 in its mass spectrum. The elemental analysis[5] of toxin T_2 showed the following: C, 68.2; H, 8.1; N, 0.72; and O, 22.6%. The IR spectrum of toxin T_2 (Fig. 1) had bands at 3000 (shoulder, CH unsaturated), 2895 (CH saturated), 1740 and 1710 (carbonyl), and a strong absorption band between 1120 and 1025 (C—O—C, tertiary amine) cm^{-1}. The absence of any band in the 3700–3200-cm^{-1} region indicates the absence of hydroxy and primary or secondary amino groups. The UV spectrum of the toxin T_2 had absorption maxima at 260 ($\epsilon M = 5.0 \times 10^3$), 267, and 270 ($\epsilon M = 3.8 \times 10^3$) nm.

The unsaturated nature of the toxin is evident from its reaction with aqueous potassium permanganate and from its IR spectrum. The ether-soluble hydrolytic product of the toxin T_2 gave a single spot (R_f 0.67) in Solvent System II. The IR spectrum of the hydrolytic product has a strong absorption at 3300 (O—H or N—H groups) and a weak absorption at 1750 (C=O group) cm^{-1}. On the basis of the IR spectrum of the ether-soluble hydrolytic product, it is evident that of the total oxygens in toxin T_2, at least two are present in a lactone ring or in an ester bond.

It may be noted from Table II that the present toxin T_2 is similar to the toxin T_2 of Spiegelstein *et al.* (9) and substance II of Martin and Chatterjee (6) in its hemolytic activity. The present toxin T_2 is different from the toxin T_2 of Spiegelstein *et al.* (9) in antiacetylcholinesterase activity but is similar to substance II of Martin and Chatterjee (6), who also reported the absence of antiacetylcholinesterase activity in their major toxin. However, the toxin T_2 now reported differs from the substance II of Martin and Chatterjee (6) in that it has no phosphorus. The physicochemical parameters of this toxin T_2 appear to be similar, although not identical, to those of ciguatoxin as described by Scheuer *et al.* (17), *i.e.,* IR bands at 3390 and 1742 cm^{-1}, UV absorption at 270 nm, no phosphorus, and Dragendorff positive. It is possible that certain species of marine life (grouper fish, moray eel, *etc.*) may take on toxins from a blue-green algae similar to *G. breve* toxins as has been previously postulated (18, 19) either directly through the gills or indirectly in the food chain. Toxins such as T_2 may thus be transformed by metabolic processes into ciguatoxin.

CONCLUSIONS

By a combined method of column chromatography and TLC,

Table II—Physiological Properties of *G. breve* Toxins

	Substance II of Ref. 6	T_2 of Ref. 9	Toxin T_2 of Present Study
Hemolytic activity	Not given	(−)	(−)
Antiacetylcholinesterase activity	(−)	(+)	(−)
Phosphorus	(+)	Not given	(−)
Nitrogen	(−)	Not given	(+)

[5] Visible and UV spectra were recorded in methanol solution (c 86.5 × 10^{-5} g %) on a Perkin-Elmer Coleman 124 spectrophotometer. The IR spectrum was recorded in carbon tetrachloride (c 0.05 g/ml) on a Perkin-Elmer 337 grating IR spectrophotometer. A mass spectrum was recorded on a CEC21-110B double-focusing instrument; the temperature was 380° and resolution was 8000 M/ΔM. Elemental analysis was performed by Huffman Laboratories, Inc., Wheat Ridge, Colo.

[6] See Sigma Technical Bulletin No. 420, Sigma Chemical Co., St. Louis, Mo.

[7] An LD_{100} of greater than 10 mg/kg was considered nontoxic.

three neurotoxins were isolated from unialgal cultures of the dinoflagellate *G. breve* Davis (the red-tide organism). Of the three toxins as assayed in mice, only one (T_1) has hemolytic activity. None of the toxins possesses antiacetylcholinesterase activity. The major toxin (T_2) appears to have a molecular weight of 725. This toxin is unsaturated and has no hydroxy groups, but it possesses a nitrogen (Dragendorff positive). The toxin has at least two oxygens in a lactone ring or in an ester bond. The physicochemical and toxicological properties of the toxin T_2 were contrasted with toxins isolated by other workers. Ciguatoxin may be a metabolic product of the major toxin T_2 or a closely related substance from blue-green algae.

REFERENCES

(1) S. M. Ray and W. B. Wilson, *Fish. Bull. 123*, **57**, 469(1957).

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

(3) J. J. Spikes, S. M. Ray, D. V. Aldrich, and J. B. Nash, *Toxicon*, **5**, 171(1968).

(4) A. M. Sievers, *J. Protozool.*, **16**, 401(1969).

(5) M. J. Cummins, A. A. Stevens, B. E. Huntley, W. F. Hill, and J. E. Higgins, in "Drugs from the Sea," H. D. Fredenthal, Ed., Marine Technology Society, Washington, D.C., 1968, p. 213.

(6) D. F. Martin and A. B. Chatterjee, *Fish. Bull.*, **68**, 433(1970).

(7) N. M. Trieff, J. J. Spikes, S. M. Ray, and J. B. Nash, in "Toxins of Animal and Plant Origin," vol. 2, A. DeVries and E. Kochva, Eds., Gordon and Breach, London, England, 1972, p. 557.

(8) J. J. Sasner, M. Ikawa, F. Thurberg, and M. Alam, *Toxicon*, **10**, 163(1972).

(9). M. Y. Spiegelstein, Z. Paster, and B. C. Abbott, *ibid.*, **11**, 85(1973).

(10) J. J. Sasner, Ph.D. thesis, University of California-Los Angeles, 1965, p. 94.

(11) Z. Paster and B. C. Abbott, *Toxicon*, **7**, 245(1969).

(12) B. C. Abbott and Z. Paster, in "Toxins of Animal and Plant Origin," vol. 2, A. DeVries and E. Kochva, Eds., Gordon and Breach, London, England, 1972, p. 761.

(13) E. J. Gates and W. B. Wilson, *Limnol. Oceanogr.*, **5**, 171(1960).

(14) W. T. Mason, R. S. Fager, and E. W. Abrahamson, *Biochim. Biophys. Acta*, **306**, 67(1973).

(15) G. R. Bartlett, *J. Biol. Chem.*, **234**, 466(1959).

(16) K. Reich, E. Bergmann, and M. Kidron, *Toxicon*, **3**, 33(1965).

(17) P. J. Scheuer, W. Takahashi, J. Tsutsumi, and T. Yoshida, *Science*, **155**, 1267(1967).

(18) J. E. Randall, *Bull. Mar. Sci. Gulf Carib.*, **8**, 236(1958).

(19) F. E. Russell, in "Venomous and Poisonous Marine Animals," vol. 3, Sir F. S. Russell, Ed., Academic Press, London, England, 1965, p. 314.

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Present address: Department of Pharmacognosy, College of Pharmacy, University of Rhode Island, Kingston, RI 02881

ˣ To whom inquiries should be directed.

Determination of Sigma and Pi Constants of Quinolinium Acid Derivatives

FOTIOS M. PLAKOGIANNIS

Abstract □ The apparent partition coefficients of 10 quinoline monocarboxylic acid derivatives in a chloroform–water system were determined. From these data, Hammett's σ and Hansch's π values were calculated.

Keyphrases □ Quinolinium acid derivatives—partition coefficients in chloroform–water system, sigma and pi constants □ Partition coefficients—10 quinoline monocarboxylic acid derivatives in chloroform–water system, sigma and pi constants □ Sigma constants—quinolinium acid derivatives □ Pi constants—quinolinium acid derivatives

By using Hammett's (1) equation (Eq. 1), considerable information concerning the effects of substituents on the reaction of given groups in the benzene series is available:

$$\log k - \log k_0 = \sigma\rho \qquad \text{(Eq. 1)}$$

where k is the rate or equilibrium constant for reactions of the substituents, k_0 is the corresponding rate or equilibrium constant of the unsubstituted compound, σ measures a change in electron density produced by a substituent, and ρ measures the susceptibility of the reaction in question to changes in electron density. The application of the Hammett equation has been relatively limited in heterocyclic compounds due to the presence of heteroatoms which themselves are capable of producing changes in electron density (2–8).

Furthermore, Hansch defined π as:

$$\pi = \log P_x - \log P_H \qquad \text{(Eq. 2)}$$

where P_H is the partition coefficient of a parent compound, and P_x is the partition coefficient of a derivative. It was shown by Hansch *et al.* (9–11) that a substituent constant, π, patterned after the Hammett σ constant, was useful in evaluating the lipohydrophilic character of a molecule upon which biological activity is dependent.