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RECENT PROGRESS IN MARINE TOXIN RESEARCH

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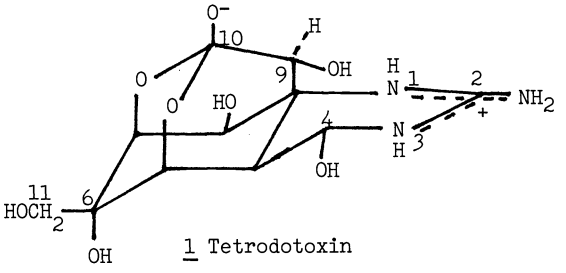
Abstract - A series of toxins isolated from the dinoflagellates, Gonyaulax spp., sheds new light on the structure of the receptors on excitable membranes. Biosynthetic origins of these toxins and tetrodotoxin, another sodium channel-specific marine toxin, are discussed. A new polyether type of toxin from the dinoflagellate, Gymnodinium breve, are also presented.

INTRODUCTION

Since the historic experiment with curare by Claude Bernard, the roles natural toxins have played in understanding the neuromuscular mechanisms cannot be overstated. Particularly in recent years, marine organisms have proved to be rich sources of unique toxins. First, the toxic component of puffer fish, tetrodotoxin, was found to prevent the formation of action potentials by selectively blocking the sodium ion influx into the excitable cells. Later the same activity was found with saxitoxin which was originally isolated from the toxic Alaska butter clam, Saxidomus giganteus (Ref. 1). Interestingly, the two toxins which have such a unique action were both first discovered in marine organisms.



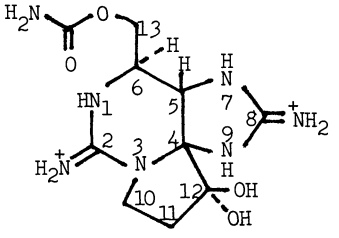
1 Tetrodotoxin

STRUCTURES AND ACTIVITY OF SAXITOXIN DERIVATIVES

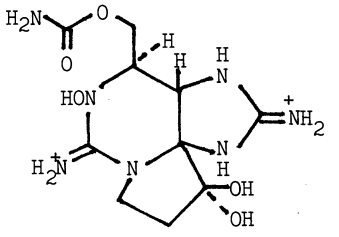
Since the discovery of the unique action of tetrodotoxin and saxitoxin, neurophysiologists have been studying the molecular interactions occurring between the toxins and toxin receptors located on excitable membranes. This is being investigated in order to enhance our knowledge of the excitable membranes, especially the sodium channel structure. Recently a series of new toxins has been isolated from the deleterious dinoflagellates, Gonyaulax spp. by our group and others, and their structures have been elucidated. These newly introduced molecules and further understanding of the fine structural features of saxitoxin have enabled us to make a new speculation on the toxin receptors.

At present, ten toxins related to saxitoxin are known. Structurally they can be divided into three groups: A) those without sulfate conjugation; B) those with sulfated 11-hydroxyl groups; and C) those with N-sulfate on the carbamoyl group.

A.

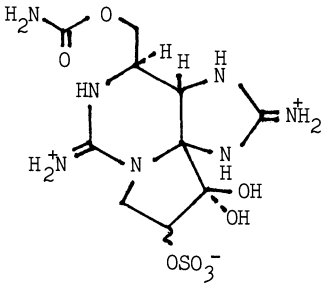


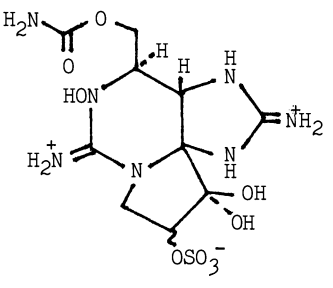
2 Saxitoxin



3 Neosaxitoxin

B.

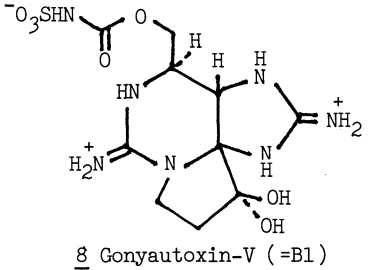


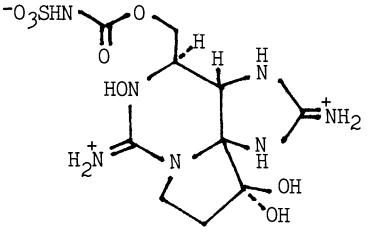


4 Gonyautoxin-II:(11α)
5 Gonyautoxin-III:(11β)

6 Gonyautoxin-I:(11α)
7 Gonyautoxin-IV:(11β)

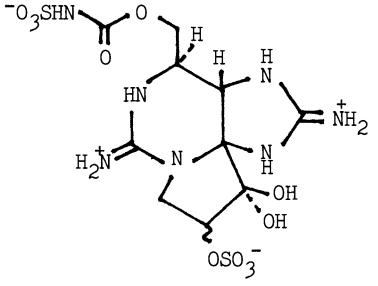
C.





8 Gonyautoxin-V (=B1)

9 Gonyautoxin-VI (=B2)



10 Gonyautoxin-VIII (=C2):(11β)
11 11-Epigonyautoxin-VIII (=C1):(11α)

TABLE 1. Specific and related mouse toxicity of Gonyaulax toxins.

	Compounds							
	2	3	4	5	6	7	8	10
Mouse units/μmol	2045	1038	793	2234	1638	673	354	280
Relative toxicity	1	0.50	0.39	1.09	0.80	0.33	0.17	0.14

Saxitoxin, 2, was the first structure elucidated in the series (Ref. 2). The structure of neosaxitoxin was tentatively assigned as 1(N)-hydroxysaxitoxin, 3 (Ref. 3). Gonyautoxin-II, 4 and gonyautoxin-III, 5 were first assigned as the epimeric isomers of 11-hydroxysaxitoxin (Ref. 4) but later found to be the 11-O-sulfate forms (Ref. 5). The structures of gonyautoxin-I and gonyautoxin-IV were assigned as the epimeric 11-hydroxyneosaxitoxin sulfates, 6 and 7 respectively (Ref. 6). A series of the carbamoyl-N-sulfated derivatives, 8-11 of the above mentioned toxins has recently been discovered. The first structure of the derivatives to be elucidated was gonyautoxin-VIII or C2, 10 (Ref. 7). Mild acid hydrolysis cleaves the N-sulfate group and thus 10 affords 5 in a quantitative yield. Two weakly toxic compounds, gonyautoxin-V and -VI afford saxitoxin and neosaxitoxin respectively upon hydrolysis. Now their structures were confirmed by synthesis as the carbamoyl-N-sulfates (Ref. 8).

The toxicity of these toxins are customarily expressed in mouse units as one mouse unit is defined as the minimum toxicity required to kill a mouse in 15 minutes. The mouse toxicity may not necessarily correlate with the toxins affinity or activity on the receptors because the bioavailability of individual toxins may vary and also because there is always the possibility of biotransformation occurring within the mouse's body. Nevertheless, the past data shows a fairly good correlation between the mouse toxicity and the action against the neuroaxons. Table 1 summarizes the mouse toxicity of the toxins measured by our group (Ref. 9).

Although there are some variations, the toxicity of 2-7 generally stays in the same order. The fact that the presence of a dissociable hydroxyl group at N-1 has little effect on the toxicity seems to support the earlier assumption that the imidazole guanidine is the active site of the molecule which is thought to plug the sodium channel (Ref. 10). An addition of the bulky and negatively charged α- or β-oriented O-sulfate group at C-11 also does not have much effect on the manifestation of the toxicity. The implication is that the plugging of the channels--if it takes place at all--must be done by a molecular penetration shallower than that proposed in the earlier model (Fig. 1).

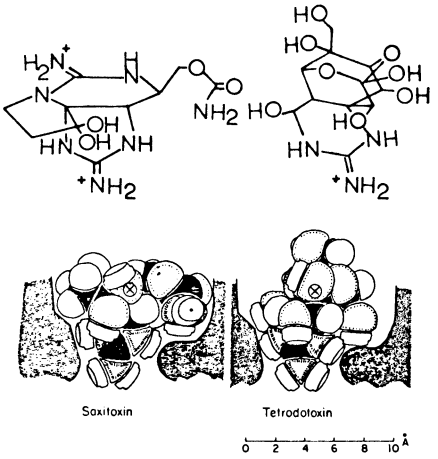
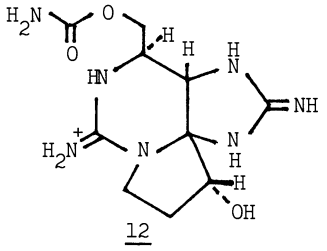


Fig. 1. Hypothetical picture of saxitoxin and tetrodotoxin on the receptors by Hille (Ref. 10).

Another important aspect is the drastic loss of toxicity accompanying the introduction of a sulfate group at the carbamoyl nitrogen, which will be discussed later.

An ion pair occurring between the guanidium group and an anionic site in the channel and hydrogen bondings were first proposed to be the binding force between the toxins and the channels (Ref. 11). After the hydrated-keto structure of saxitoxin was revealed by X-ray crystallography, the possibility of covalent bonds between the toxins and receptors by hemiketal or hemiaminoketal formation at C-12 has been also brought into consideration (Ref. 10). The corresponding chemically equivalent site in tetrodotoxin would be the hemiaminoacetal moiety at C-4. The idea seemed to have some strength because dihydrosaxitoxin was reported to lack toxicity (Ref. 12). Kao and Walker, however, have recently reported that a mixture of epimeric dihydrosaxitoxin isomers actually possesses significant activity against receptors (1/100 of 2) (Ref. 13). Our group was able to prepare two isomeric dihydrosaxitoxins and establish their stereochemistry (Ref. 14). In a collaborative work with Dr. Strichartz using these stereochemically pure specimens, it was demonstrated that 11α-OH-dihydrosaxitoxin, 12, has 1/10 and the 11β-isomer, 13, 1/1000 of the saxitoxin activity (Ref. 15).



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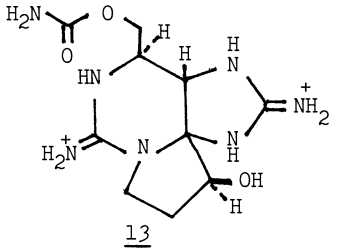

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The results indicate that 11α-hydroxyl group plays the crucial role in the binding, and it is probably augmented by 11β-hydroxyl group. Nmr studies of saxitoxin showed that at lower pH there is a considerable keto-form contribution (Ref. 14,16). However, if the above hypothesis is true, only the hydrated form should be active. Nmr studies also indicated that the conformation of saxitoxin is essentially the composite of 12 and 13 (Ref. 14).

A similar arrangement of functional groups can be found in the molecular structure of tetrodotoxin. Thus the hydroxyl groups at C-9 and C-10 correspond to 12α-OH and 12β-OH in saxitoxin respectively. It is important to note that except for this similarity in the relative locations of two hydroxyl groups and guanidium moiety, the shapes of the two molecules have no resemblance, rather they are almost antipodal in respect to the guanidium moieties.

The above results seem to support the hypothesis that the toxins bind the outside surface of the sodium channel rather than the inside (Ref. 17). To explain the lack of interference of

bulky substituents at C-11 in saxitoxin, the best model seems to be a classical three-point interaction with the flat surface of the excitable membrane (Fig. 2). In this model, the saxitoxin molecule is slightly tilted, and the C-13 side chain spreads in close proximity

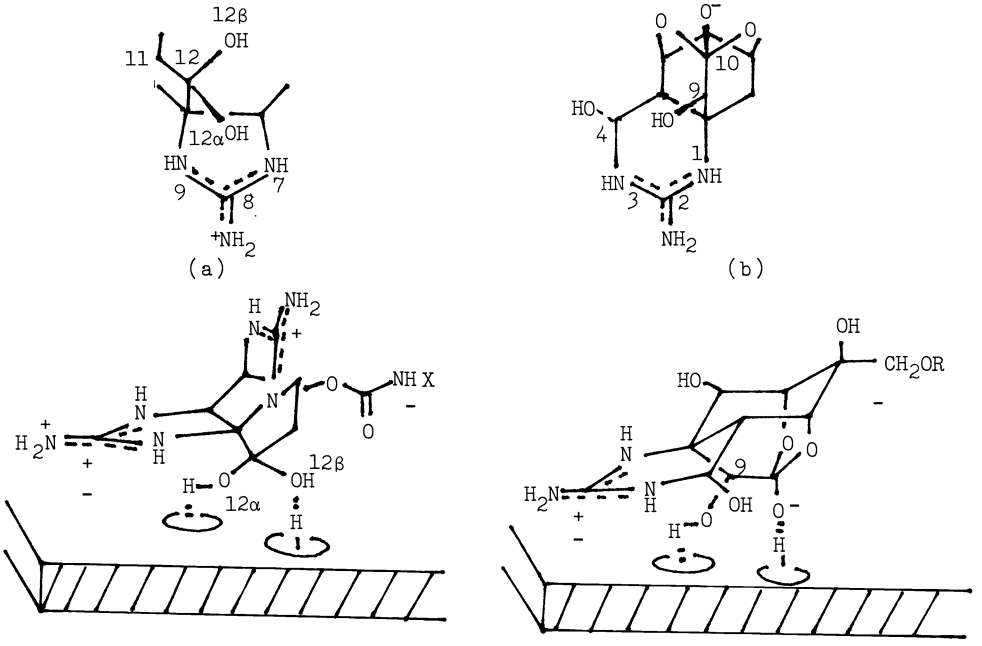
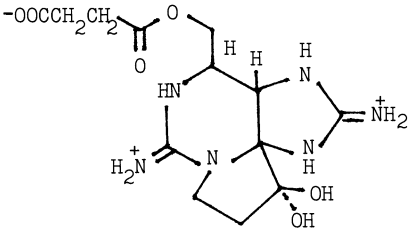


Fig. 2. Three points interaction models for saxitoxin (a) and tetrodotoxin derivatives (b).

to the membrane surface. This could account for the marked decrease in toxicity caused by the introduction of a sulfate group onto the side chain. Such negatively charged moieties interact repulsively with anionic sites which are hypothesized to be dispersed on the membrane. The model also can explain the secondary influence on the potassium channel demonstrated by chiriquitoxin, which presumably has a large substituent in place of C-11 carbinol in tetrodotoxin (Ref. 18). We also found decarbamoyl saxitoxin hemisuccinate, 14, almost nontoxic. Thus all these data suggest that the toxins work as lids rather than plugs to the sodium channel.



14

BIOSYNTHETIC ORIGINS OF SAXITOXIN ANALOGS AND TETRODOTOXIN

Although saxitoxin analogs have been found in diversified marine organisms, it is assumed that they are all synthesized by dinoflagellates de novo. However, the matter has not been completely settled. Our recent investigation of many Gonyaulax tamarensis strains from various locations has indicated tremendous variations in toxin productivity. For this reason and also in view of a capricious occurrence of saxitoxin analogs in the normally nontoxic freshwater blue-green, Aphanizomenon flos-aquae (Ref. 19), some people are proposing that endosymbiotic bacterium or a transferred plasmid is actually responsible for the toxin production.

More than one pathway can be postulated for the biosynthesis of the unique structure. Feeding experiments using G. tamarensis cultures have proved to be very difficult. Among several putative precursors, such compounds as [1,2-^{13}C]-acetate, [1-^{13}C]-arginine, [1-^{13}C]-ornithine and [1-^{13}C]-histidine were only incorporated in random manners. The basically nonheterotrophic organism is highly selective in utilization of exogenous organic material.

Thus the above precursors probably never reached the compartmentalized synthesis sites in intact forms.

Feeding of [2-^{13}C]-glycine resulted in the enrichment of all the carbons in gonyautoxin-II, 4, but in this case, C-11 and C-12 were enriched significantly higher than the rest of the carbons (Fig. 3). The pathway shown in Fig. 1 can provide a plausible explanation for this rather unexpected enrichment of the neighboring carbons from the single labelled precursor. Glycine was first introduced into the TCA cycle via the glyoxalate pathway. In the TCA cycle, the symmetry of molecule will be lost in succinate, and compounds such as glutamate or arginine will come to carry the labelling on C-3 and C-4, which will be C-12 and C-11 respectively in the postulated synthetic scheme.

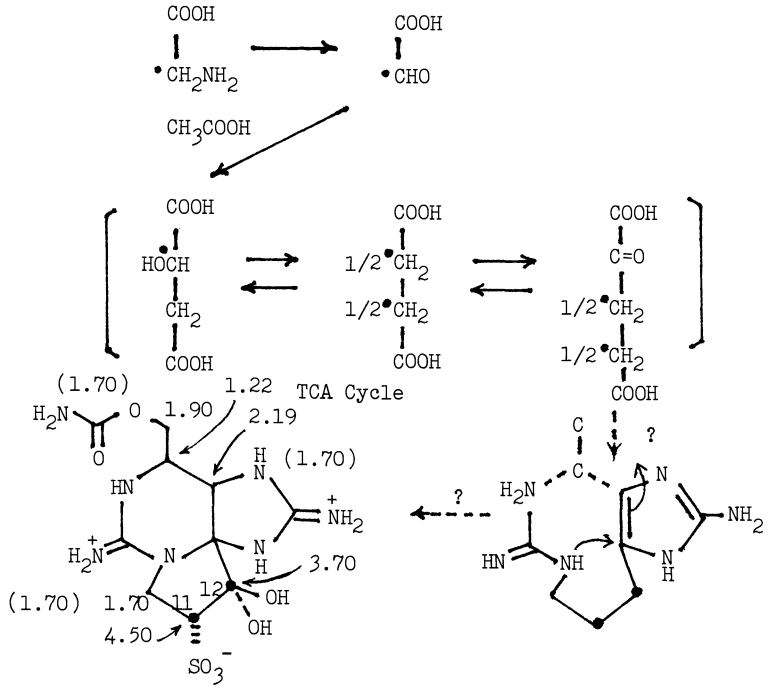


Fig. 3 ^{13}C-Enrichment pattern of gonyautoxin-II, 4, derived from the [2-^{13}C]-glycine fed Gonyaulax tamarensis culture, and a possible pathway leading to the enrichment at C-11 and C-12. The numbers depict atom excess % calculated from the relative peak intensities in the spectra of the enriched and unenriched samples based on an average enrichment of 2.2 atom excess %.

Feeding of [guanido-^{14}C]-arginine resulted in ca. one-third of the incorporated radioactivity found with the carbamoyl carbon of 5, which was confirmed by converting it to 1 by the reductive cleavage of sulfate followed by hydrolysis to carbon dioxide and decarbamoyl-saxitoxin.

The biosynthetic origin of tetrodotoxin remains a mystery. The toxin occurs in puffer fish, gobies, newts, an octopus, a frog and more recently in a marine snail. Also even within identical species, the toxicity levels vary greatly. This rather whimsical pattern of the toxin occurrence raises a question about the true origin of the toxin. Hirata stated "it is interesting whether the toxin is produced by the fish itself or not, and how it is biosynthesized" (Ref. 20).

Our initial attempt to study the biosynthesis of tetrodotoxin using the Atlantic puffer, Spheroides maculatus was unsuccessful, because this reportedly toxic species captured in the northern waters proved to be nontoxic. Then we turned to the toxic newts, Taricha torosa and T. granulosa. The animals used for the experiments were highly toxic (1-2 mg of toxin per animal) throughout the captivity period.

The first objective was to feed very universal precursors to see if the de novo synthesis of the toxin takes place in the newts. The feedings were first done by injection, and later by oral and external administration to check for the possibility of synthesis by the intestinal or skin flora. In all cases, we observed significant incorporation of radioactivity into many metabolites such as amino acids and sterols but found absolutely no radioactivity associated with tetrodotoxin (Table 2).

TABLE 2. Radioactivities in the fractions from the newts fed by labelled precursors.

Precursor	No. of Newt	Species*1 (Feeding Method)	Precursor (μCi) and feeding period (days)	Radioactivity (cpm/mmole) Cholesterol	Amine and Amino acid	Tetrodotoxin
Acetate-[2-^{14}C] (35.9 mCi/mmole)	2	T.t (injection)	9.44 (6)	172,000	1.7×10^5	0
	2	T.t (oral feeding)	9.44 (13)	34,300	8.9×10^5	0
	2	T.t (injection)	9.44 (13)	29,600	1.1×10^5	0
	1	T.g (soaking)	9.44 (28)	16,500	4.5×10^6	0
	2	T.g (soaking)	51.92 (86)	29,500	1.5×10^7	0
Citrulline-[ureido-^{14}C] (53 mCi/mmole)	2	T.t (injection)	10 (17-29)	28,400	6.3×10^6	0
	1	T.g (injection)	5 (86)	3,200	2.6×10^6	0
	1	T.g (injection)	5 (105)	1,700	8.2×10^5	0
	1	T.g (soaking)	20 (33)	6,140	1.1×10^5	0
Arginine-[guanido-^{14}C] (23.01 mCi/mmole)	2	T.g (injection)	10 (50)	NA	NA	0
Glucose-D-[^{14}C(U)] (2.2 mCi/mmole)	3	T.g (injection)	15 (26)	NA	NA	0

*1 T.t = Taricha torosa T.g = Taricha granulosa

The experiment was repeated with the newts injected with gonadotropin, but the results were also negative.

The apparent lack of de novo synthesis of the toxin by the newts can be interpreted in many ways: 1) the toxin is synthesized during only a very limited developmental stage and its turnover is very slow; 2) the toxin is synthesized only under certain stressed conditions as a self-defense mechanism; 3) the toxin or its key precursor comes from a special dietary source; 4) the toxins stored by the newt is synthesized by a symbiotic microorganism, but in captivity, the conditions are not suitable for the organism or toxin production; 5) the precursors cannot reach the synthesis site, etc. Our results have a remarkable resemblance to those observed in the biosynthetic study of another potent neurotoxin, batrachotoxin, in the frog, Phyllobates aurotaenia (Ref. 21). The frog failed to synthesize the toxin from [1-^{14}C]-acetate, and the F1 frogs reared in captivity completely lacked the toxin. Interestingly, the puffer fish, Fugu rubripes rubripes commercially grown in Japan are completely devoid of toxicity when they are hatched from eggs and hand raised. Furthermore the feeding of pure tetrodotoxin to nontoxic fish did not result in the toxin being accumulated in the bodies (Ref. 22).

At present our knowledge about the origin of toxic compounds in marine organisms is still very limited.

GYMNODINIUM BREVE (=PTYCHODISCUS BREVIS) TOXINS

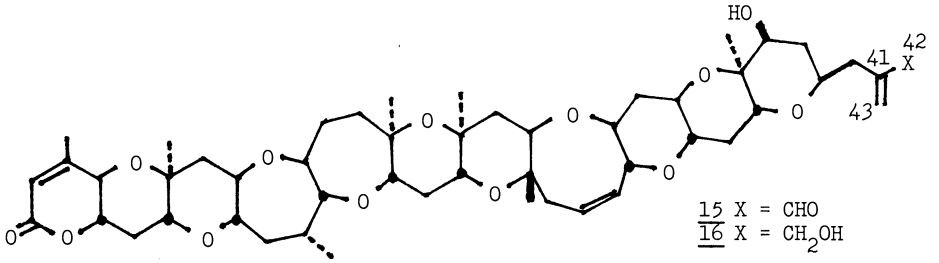
Gymnodinium breve is the dinoflagellate which is associated with massive fish kills in the Gulf of Mexico. It also causes the so-called neurotoxic shellfish poisonings as well as respiratory disorders when inhaled in an aerosol form. A large number of researchers have worked on its toxic components, in some cases, with conflicting results.

Our group has isolated four pure compounds, GB-M, GB-1, GB-2 and GB-3 (in the elution order) from the cultured cells. Two of them (GB-1 and GB-2 were obtained in crystalline form.

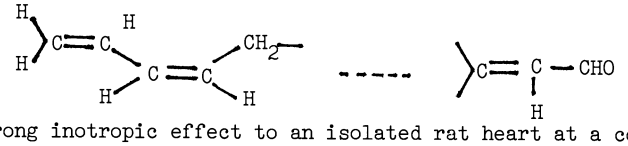
GB-2 toxin, prisms from ethanol or isopropyl ether, mp 300° (decomp), which was reported to have such functional groups as a conjugated aldehyde, several methyl groups and cyclic ethers (Ref. 23), has been identified with brevetoxin-B, whose unique X-ray determined structure, 15, was recently reported (Ref. 24). Since GB-2 was identified with Baden, et al.

T_{34} toxins (Ref. 25) and brevetoxin B with Risk, et al. T_{47} (Ref. 24), all these toxins should be identical despite the earlier report of the nonidentity of GB-2 (=T_2) and T_{47} (Ref. 26).

GB-3 has a α-methylene carbinol, and its structure 16, was speculated from the nmr data. Final confirmation of the structure was done by the reduction of 13 to 16 with $NaB(CN)H_3$.



GB-1,needles from acetonitrile, mp. 198°, has an α-methylene aldehyde, and is expected to have a structure very close to brevetoxin-B. The fourth compound GB-M is weakly ichthytoxic and seems to have a structure considerably different from those of GB-I, -II, and -III as seen in the following partial structures obtained from the nmr decoupling studies.



GB-2 was found to have a strong inotropic effect to an isolated rat heart at a concentration as low as 10^{-8} mol. The action is very similar to that of ouabain and has been speculated to have ATPase inhibitory action. Baden, et al. (Ref. 27) reported that their T_{34} toxin is cytotoxic at a concentration of 2 μg/ml, but this toxicity does not parallel the ichthyotoxicity, thereby indicating the possible involvement of more than one pharmacological action. One intriguing feature is the unprecedented linear polycyclic ether structure which very likely penetrates into the membranes. Unquestionably, we have a series of a new type of toxin which have potential of being very important tools in the study of receptor and membrane structures.

Acknowledgment - The work described in this paper was done in collaboration with dedicated colleagues, Drs. M. Kobayashi, A. Genenah, Y. Uchio, and Mr.H. N. Chou supported by NIH grants, GM-28754 and GM-24425, which are gratefully acknowledged. Thanks are also due to Dr. Reichardt for the preliminary information on B1 and B2 structures.

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