ISOLATION OF SIDE-CHAIN SULFATED SAXITOXIN ANALOGS

THEIR SIGNIFICANCE IN INTERPRETATION OF THE MECHANISM OF ACTION

Y. Shimizu,* M. Kobayashi, A. Genenah and Y. Oshima
Department of Pharmacognosy and Environmental Health Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI 02881, U.S.A.

Abstract—A newly discovered toxin, gonyautoxin-VIII, and two previously reported toxins, gonyautoxin-V and -VI, have been found to have carbamoyl N-sulfate conjugation. The exceptionally low toxic nature of the derivatives seems to support the hypothesis that the toxins interact on the surface of excitable membranes.

Two marine toxins, saxitoxin (STX) (**1**) and tetrodotoxin (TTX) (**2**), have been noted for their unique action to selectively block the influx of Na ions acting from the outside of the excitable membrane. Because of this highly specific action, both toxins have been used as important tools in numerous neurophysiological experiments.[2,3] Concurrently researchers have been trying to find clues for the structure and action mechanism of Na channels by studying the interaction between the toxin molecules and the receptors. The recent discovery and structure elucidation of new toxins have enabled us to make more intelligent speculations on the action mechanism. This paper is intended to provide detailed accounts of the isolation and structure elucidation of N-sulfated toxins, which are extremely interesting because their weak toxicity.

In 1975 gonyautoxin (GTX)-I (**3**), gonyautoxin-II (**4**), and gonyautoxin-III (**5**) were isolated from the clams; *Mya arenaria* exposed to a *Gonyaulax tamarensis* bloom,[4,5] and subsequently their structures were established.[6–12] The toxins were the first examples of paralytic shellfish toxins other than long-known STX (**1**). The isolation procedure was based upon the rather selective adsorption of the toxins on an acrylamide gel, Bio-Gel P-2 followed by chromatography on a weakly acidic resin, Bio-Rex 70. In the earlier isolation work, we noticed small toxin peaks preceding GTX-I in Bio-Rex 70 chromatography. In 1977 four minor toxins GTX-IV (**6**), -V (**7**) and -VI (**8**) were isolated along with neosaxitoxin (neoSTX) (**9**).[13,14] Among them, GTX-IV was found to be the 11β-epimer of GTX-1, whose structure was established as 11α-hydroxyneosaxitoxin-11-O-sulfate.

GTX-V (**7**) and GTX-VI (**8**) have been found in most *Gonyaulax* cells and intoxicated shellfish. However, the amounts in the examined samples were usually very small except for *Gonyaulax* spp. and mussels collected at Owase Bay and Inland Sea, Japan.[15,16] Although only a very small amount was available, the compound (**7**) proved to be an intriguing substance. Upon H₂O₂-NaOH oxidation, GTX-V

(**7**) gave the same fluorescent purine propionic acid derivatives as those obtained by the oxidation of STX (**1**).[17] Its fluorescence yield relative to toxicity was, however, about ten times that of STX indicating that the toxin is only very weakly toxic. Later, its low specific toxicity was confirmed by comparison of its N-content and toxicity.[18] In electrophoresis the mobility of **7** relative to **1** was 0.32 to cathode indicating that its net positive charge was less than half of STXs.[19] The PMR spectrum of **7** was almost identical with that of STX (**1**) (Table 1). Thus, a sample of **7** in D₂O underwent a rapid deuterium exchange of C-11 hydrogens just as **1**,[20] and two proton signals corresponding to H-10 in **1** appeared in a clean AB pattern. The chemical shifts of all signals were close to those in **1** except H-13 signals which were slightly shifted to the up-field. On the basis of the above observations, we speculated that **7** is a conjugated form of saxitoxin. In view of the toxin's weak toxicity, the nature of the conjugation was of particular interest because we expected that it might provide an important key to consider the action mechanism of the toxins.

Meanwhile, during our biosynthetic study of the toxins produced by *Gonyaulax tamarensis*, we noticed the presence of a radioactivity peak with very weak toxicity in the early fraction of Bio-Gel P-2 column chromatography which was discarded in the past. The compound, named GTX-VIII, was easily purified by repeated chromatography on Bio-Gel P-2.

GTX-VIII (**10**) was the first toxin found to have negative net charge, its mobility relative to saxitoxin at pH 8.7 was −0.43.[9] Since the guanidium group in the five-membered ring of STX nucleus is only partially protonated around the pH employed (pKa 8.26),[21,22] it was speculated that the molecule has two negatively charged functional groups on STX skeleton to bring the negative net charge.

The PMR and CMR spectra of GTX-VIII (**10**) showed that the compound is very close to GTX-III (**5**). The signal pattern and chemical shifts were essentially identical with those observed with **5** (Table 1) except for small increments in chemical shifts for
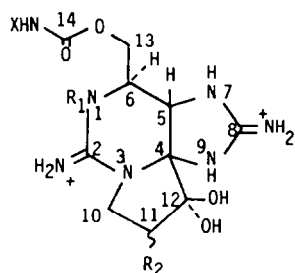
1   $R_1, R_2 = H$, $X = H$
3   $R_1 = OH$, $R_2 = \alpha\text{-}OSO_3^-$, $X = H$
4   $R_1 = H$, $R_2 = \alpha\text{-}OSO_3^-$, $X = H$
5   $R_1 = H$, $R_2 = \beta\text{-}OSO_3^-$, $X = H$
6   $R_1 = OH$, $R_2 = \beta\text{-}OSO_3^-$, $X = H$
7   $R_1 = H$, $R_2 = H$, $X = SO_3^-$
8   $R_1 = OH$, $R_2 = H$, $X = SO_3^-$
9   $R_1 = OH$, $R_2 = H$, $X = H$
10 $R_1 = H$, $R_2 = \beta\text{-}OSO_3^-$, $X = SO_3^-$
11 $R_1 = H$, $R_2 = \alpha\text{-}OSO_3^-$, $X = SO_3^-$
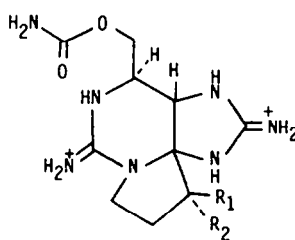
12 $R_1 = H$, $R_2 = OH$
13 $R_1 = OH$, $R_2 = H$

pKa 8.75

2a

2b
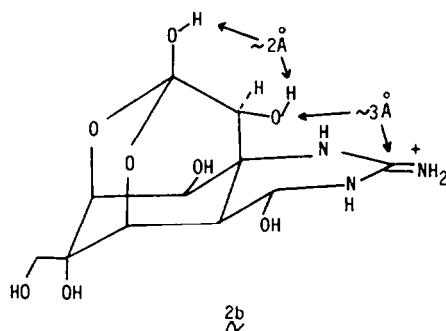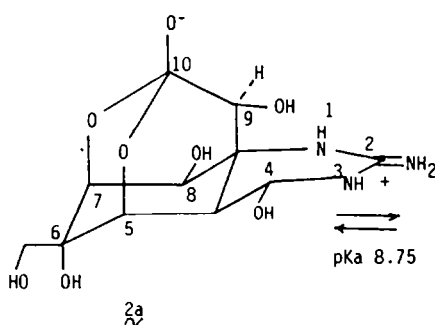
C-13 and attached protons: $\Delta\delta + 0.04$ for 13α-H, $+0.09$ for 13β-H and $+0.7$ ppm for C-13. The similarity of both compounds was further demonstrated by the fact that GTX-VIII (10) undergoes epimerization to form a *ca* 1:2 equilibrium mixture of GTX-VIII (10) and its epimer (11) under a slightly elevated pH. A similar equilibrium was noted for GTX-III (5) and its 11-epimer GTX-II (4).[6] Subsequently, 10 was found to give quantitatively 5 and one mole of sulfate upon mild acid hydrolysis. Similar treatment of an equilibrium mixture of 10 and its epimer (11) afforded a mixture of 5 and 4. Thus, GTX-VIII (10) is a sulfate derivative of GTX-III (5), and on the basis of NMR data, its linkage position was assigned at the side chain carbamoyl N, which was confirmed by X-ray crystallography.[23] Only very few sulfates on amide type nitrogens have been reported, and little is known about their chemical properties. A recent example is an antibiotic sulfated on the β-lactam N.[24] There are, however, a number of reports on N-phosphates including such important compounds as creatinine phosphate. Significantly, they also undergo facile acid hydrolysis. Confirmation of the structure of GTX-VIII as a carbamoyl N-sulfate led us to speculate that previously isolated GTX-V (7) and GTX-VI (8) are also the carbamoyl N-sulfates of STX (1) and neoSTX (9),

respectively and it was confirmed by the mild acid treatment of both compounds.

Meanwhile, in 1978 Oshima and Yasumoto reported the presence of toxins whose toxicity are enhanced by acid treatment.[25] Subsequently, Hall *et al.* reported the presence of four latent toxins denoted B_1, B_2, C_1 and C_2 in an Alaskan strain of *Protogonyaulax* sp.[26] In an independent study, C_1 and C_2 were proven to be 11 and 10.[23] Furthermore, the same group confirmed by partial synthesis that B_1 and B_2 are the carbamoyl N-sulfate derivatives of neoSTX and STX or GTX-VI (8) and GTX-V (7), respectively.[27] Recently, a Japanese group has also reported the identical structures for GTX-V and -VI, working with samples isolated from the tropical dinoflagellate, *Pyrodinium bahamense* var. *compressa.*[28]

DISCUSSION

Interest in the structure-activity relationship has led to extensive studies of pharmacological properties of gonyautoxins and neosaxitoxin. The results can be summarized as follows: (1) saxitoxin, gonyautoxin-I, -II, -III, -IV and neosaxitoxin possess the toxicity or blocking ability of the same order, (2) gonyautoxin-V, -VI and -VIII have remarkably less activity (1/10 or less). The results indicate that the

Table 1. Comparison of ^{1}H NMR chemical shifts of gonyautoxin-V (GTX-V), saxitoxin (STX), gonyautoxin-VIII (GTX-VIII) and gonyautoxin-III (GTX-III)

C-H No.	δ^* (J=Hz)			
	GTX-V (2)	STX (1)	GTX-VIII (10)	GTX-III (5)
5	4.61 (s)	4.77 (d,J=1)	4.78 (s)	4.79 (d,J=1)
6	3.86 (m)	3.87 (d,d,d,J=9,5,1)	3.81 (d,d,J=9.5,5)	3.79 (d,d,d,J=10,6,1)
10	3.62 (d,J=10)	3.57 (d,J=10)	3.54 (d,d,J=10.6,8)	3.55 (d,d,J=11,7)
	3.81 (d,J=10)	3.85 (d,J=10)	4.12 (d,d,J=10.6,8)	4.14 (d,d,J=11,9)
11	exchanged	exchanged	4.91 (t,J=8)	4.93 (d,d,J=9,7)
12				
13 α	4.13 (d,d,J=10.2,4,6)	4.05 (d,d,J=11,5)	4.11 (d,d,J=12,5)	4.02 (d,d,J=12,10)
β	4.40 (d,J=10.2)	4.27 (d,d,J=11,9)	4.32 (d,d,J=12,9.5)	4.28 (J=12,6)

*Measured by a 270 MHz Bruker Instrument.

δValues from DSS in D_2O. The values are quite different from those reported by Wichmann, et al.[23] and Koehn, et al.,[27] who used $CHCl_3$ as internal standard. Actually the $CHCl_3$ signal in D_2O appears around δ7.67, and if this solvent effect correction ca. 0.40 ppm is incorporated, both values seem to come close.

introduction of an dissociable group on the 6-membered ring guanidine have little effect to the activity. Also, the introduction of a bulky charged O-sulfate group at C-11 in either α- or β-orientation has little or no effect on the activity. On the other hand, the introduction of sulfate group on the side-chain remarkably diminishes the activity.

Since the discovery of the selective blocking of Na channels with STX and TTX, a number of research groups have been trying to elucidate the binding mechanism of the toxins. One pioneering and well-quoted model is Hille's plugging model.[29,30] According to the model, the Na channel is 3 Å × 5 Å hole in the membrane, through which monohydrated Na ions go during the first phase of excitation. The C-8 guanidium group of STX is placed into the channel, and the rest of the molecules sit on the wider opening of the channel. The finding that the substitution on guanidium group in the 6-membered ring does not alter the activity supports the assumption that the imidazolidine guanidine is indeed the critical moiety in STX molecule. However, the model has a difficulty in explaining the non-influential nature of O-sulfate substitution at C-11. Such a bulky group would certainly create a large steric hindrance. Especially in the 11α-configuration (GTX-I and GTX-II) the O-sulfate group would unavoidably extend toward the channel wall, along side the guanidinium group.

The chemical nature of toxin-binding to the receptor site has been also a subject of intense discussion. Hille is the first who suggested that the guanidinium charge is probably countered with an anionic site such as carboxylate located inside the channel, and several negative atoms at the basin of the channel opening favor H-bondings with the toxin molecules.[30] When the structure of STX with hydrated ketone was revealed by X-ray crystallography,[31] an attractive alternative for the H-bondings was hemiketal or hemiaminoketal formation with OH or NH₂ group at the channel site.[30] A similar function in TTX molecule is the hemiacetal at C-4,[32] although the location of C-4 relative to the guanidium group is quite different from that of C-12 in STX molecule. The hypothesis seemed to have strength because dihydro-saxitoxin, in which hemiketal formation is impossible, was considered to lack the activity.[33] Unfortunately neither stereochemical purity nor stereochemistry of the tested dihydrosaxitoxin was established. To determine whether hemiketal formation or H-bonding participates in the binding, it was felt imperative to test two isomers with un-equivocal stereochemistry.

Highly purified stereoisomeric dihydrosaxitoxins have been prepared[22] and examined for their binding ability. The results have shown that the 12α-isomer (12) actually possess significant activity (ca 1/10 of STXs), while the 12β-isomer (13) is much less potent (ca 1/100 of 12).[34] Kao and Walker also got a similar finding in an experiment using a mixture of 12 and 13 of undetermined ratio.[35]

The X-ray crystallography and PMR study of STX showed that N-3, C-4, C-10 and C-11 form a plane while the hydrated C-12 is out of the plane.[22,31] The PMR coupling data show that the solution conformation of the 5-membered ring in 12 and 13, is also the same as that in STX.[22] Therefore, the molecule of hydrated STX can be considered as a com-

posite of 12α- and 12β-dihydro STX. Thus, the above results strongly suggest that the H-bonding by the 12α-OH group is crucial in the manifestation of the activity, which is further augmented by an additional bonding by the 12β-OH group.

Significantly, one can find an almost identical arrangement of guanidium group and two OH groups in the TTX molecule; if the orthoester O is protonated, the 9- and 10-OH group in TTX correspond to 12α- and 12β-OH group in STX respectively, and their relative locations to the guanidium group is very close to those in STX molecule (see 9a and 9b). All these observations seem to support Kao and Walker's recent proposal that the toxin molecules bind on the surface of the membrane close to the orifice of the sodium channel.[35] Actually, one can draw a classic three points binding model in which the toxin molecule interact with the receptor by an ion-pair between guanidium group and an anionic site, and H bonding by two OH group ca 2 Å apart (Fig. 1).[16] This new model is convenient to explain some previous observations. It has been known that around pH 7–9 the activity of both STX and TTX decreases as pH increases.[32,36] In the case of STX, it can be easily explained by the deprotonation of the critical guanidium group which is now known to have an unusually low pKa value (8.26).[21,22] In the case of TTX, however, the guanidium group is always protonated in the pH range. If both 9- and 10-OH groups are important H-bond donors, this enhancement of activity can be now attributed to the protonation of the orthoester O whose pKa is described as 8.76.[37] The model also can explain the lack of activity in anhydrotetrodotoxin, in which 9-OH group is no longer available due to the intramolecular acetal formation.[3] The complete lack of activity in anhydrotetrodotoxin might be difficult to explain by the plugging mechanism because the location of 9-oxygen is not so different even after the intramolecular acetal formation. If we place gonyautoxin molecules by the three points interaction on the plane surface, neither 11α-O-sulfate nor 11β-O-sulfate seems to meet major steric hindrance.

The newly discovered series of carbamoyl sulfated toxins seems to further support the new binding model. They are all characterized by extremely low toxicity, about 1/10 or less of the original compounds. This low activity is intrinsic and in binding
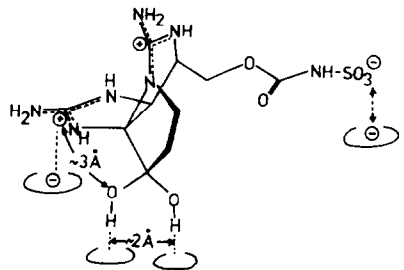

Fig. 1. Illustrative representation of interaction between saxitoxin molecule and excitable membrane. Sulfated side-chain is shown to demonstrate the possible electrostatic repulsion by an anionic site on the membrane.

study using brain membranes, GTX-VIII was found to have only 1/10 of STX activity.[38] Actually it was first considered that the side-chain is unimportant for the revelation of activity as exemplified by the toxicity of decarbamoylsaxitoxin which is only slightly less than that of STX. The side-chain of STX is known to extend outwards in both crystalline and solution state far away from the active C-8 guanidium moiety.[22,31] However, in the new model in the same conformation, the side-chain extends closely over the membrane surface, and the negatively charged sulfate group interacts repulsively with anionic sites known to exist on the membrane.

EXPERIMENTAL

TLC was run on Whatman LHP-K plates without fluorescence using the solvent system, Pyridine–EtOAc–water–AcOH (15:10:4:3) or on Silica gel 60 plates using pyridine–EtOAc–water–AcOH (75:25:15:30). Toxin spots were detected under long-wave UV after spraying 1% H_2O_2 and heating at 130° for 10 min. PMR spectra were obtained with a Bruker 270 MHz instrument in FT mode. CMR spectrum was obtained on a model CFT-20 Varian instrument. Mouse assays were done according to the AOAC method.[39]

Gonyautoxin-V (7)

Gonyaulax tamarensis (Ipswich strain)[6] cells were cultured in Guillard F medium (50 l.) in five 15 l. carboys under fluorescent illumination without aeration. After 5 weeks, when the cell numbers reached 6×10^3/ml, the cells were harvested by continuous centrifugation and extracted as previously reported.[13] The toxin extract (total 48,000 mouse units) was adjusted to pH 5.8 and placed on a Bio-Gel P-2 column (4×100 cm). The column was washed thoroughly with distilled water until all salts were eluted. The adsorbed toxin fraction was then eluted with 0.03 N AcOH. The eluate was concentrated in vacuo and then lyophilized. The residue was redissolved in water (2 ml), adjusted to pH 5.8, and charged to a Bio-Rex 70 column (acidic from 400 mesh, 1.7×190 cm). The column was then eluted with an AcOH gradient soln (0–0.01 N, 2,000 ml total). The GTX-V (7) was eluted overlapped with GTX-IV (6) and GTX-I (3) followed by GTX-III (5) and GTX-II (4). Further elution with 1N AcOH afforded fractions containing neoSTX (9) and STX (1).

The GTX-V (7) containing fractions were combined and lyophilized. The residue was dissolved in H_2O (2 ml) and charged to a Bio-Rex 70 column (acidic form, 0.6×190 cm). The column was then eluted with an AcOH gradient (0–0.03 N AcOH, 1000 ml). The fractions # 119–121 (5 ml each) were combined and rechromatographed by the same system. Fractions # 62–70 (4.5 ml each) were combined and rechromatographed on a Bio-Rex column (0.6×90 cm) using an AcOH gradient (0–0.015N, 500 ml total). The fractions # 55–65 (4.5 ml each), which showed only a GTX-V spot on TLC were combined and lyophilized. The toxin sample which was assayed as 238 mouse units afforded 66.56 μg of N in elemental analysis. The specific toxicity was estimated as 354 mouse units/μmol. The NMR sample was dissolved in 2 ml of D_2O (98%), lyophilized and redissolved in 100% D_2O (0.3 ml). Table 1 for the results.

Hydrolysis of gonyautoxin-V (7)

A small aliquot (ca 50 μg) of 7 was dissolved in 0.01 N HCl (0.2 ml) and heated in a sealed vial for 40 min at 80°. The soln was lyophilized and checked by TLC and benzidine-thymol test for sulfate. TLC showed a single spot corresponding to STX (1) and the sulfate test gave a positive result.

Hydrolysis of gonyautoxin-VI (8)

A small aliquot (ca 20 μg) of 8 previously obtained from mussels from Alaska (described as UKN, TLC Rf value 0.56, relative electrophoretic mobility to STX at pH 8.5, 0.08)[40] was treated as described above. TLC examination showed a spot corresponding to neoSTX (9). The hydrolysate also gave a positive sulfate test.

Gonyautoxin-VIII (10)

(a) Combined cell extracts of G. tamarensis (total toxicity 196,600 mouse units) were defatted with $CHCl_3$, concentrated to 60 ml, adjusted to pH 5.7 with a 10% NaOH aq, and charged to a Bio-Gel P-2 column (4×100 cm). The column was eluted with H_2O (1600 ml) followed by 0.03 N AcOH (2000 ml) at a flow speed of 1.6 ml/min. Fractions # 49–57 (each 40 ml fraction) contained GTX-VIII (10) (total toxicity, 4472 mouse units), and fractions # 75–80 contained a mixture of GTX-I-V, neoSTX and STX (total toxicity 168,000 mouse units). The GTX-VIII fraction, which showed a single spot on TLC by H_2O_2 spraying, was once more chromatographed on Bio-Gel P-2. However, upon standing at slightly high pH or upon storing the lyophilized sample, the sample turned to an equilibrium mixture of 10 and 11, which was no longer separated by simple Bio-Gel P-2 chromatography. The PMR and CMR samples were prepared after exchange of active hydrogens by D_2O treatment and lyophilization. CMR: δ (in D_2O) ppm: 47.7 (C-10), 53.0 (C-6), 57.5 (C-5), 64.0 (C-13), 76.0 (C-11), 81.9 (C-4), 97.5 (C-12), 154.3, 156.1 and 158.3 (C-2, -8, -14). PMR: See Table 1.

(b) The culture of G. tamarensis (3×10 l) was fed with 15 μCi of [guanido-^{14}C]-arginine 6 days after inoculation. After 22 days the cells were harvested and processed as described above. A total of 1201 mouse unit equiv GTX-VIII (10) had a radioactivity of 11,524 cpm. GTX-III (4) isolated at the same time had a radioactivity of 12,348 cpm. After conversion of 4 to 1 by reductive cleavage of sulfate 10, the toxicity and radioactivity of 10 and 1 were compared. GTX-VIII (10) was shown to have 1/7.3 of STX (1) toxicity on 280 mouse units/mol. Values of 500 mouse units/mg for 10 and 36 mouse units/mg for its epimer (11) were reported by Wichman et al.[23]

Hydrolysis of gonyautoxin-VIII (10)

(a) Aliquots of 10 in (1) H_2O, (2) 0.01 HCl and (3) 0.1 N AcOH were kept at 37° and examined by TLC. After three days, (1) gave an additional spot (epi-GTX-VIII (11)), (2) gave GTX-III (4), and 4 was detected in (3).

(b) 10 (215 mouse units, 0.75 μmol) was treated in 1 ml of 0.1 N HCl in a sealed vial at 80–85° for 30 min. After the reduction, aliquots were taken and submitted to inorganic phosphate analysis (Fiske–Subbarow). The results were negative. However, the addition of 1% $BaCl_2$ soln to the mixture gave a ppt. After evaporation the mixture was analyzed by chromatography. On Bio-Rex 70 chromatography the hydrolysis product gave the same retention time as 4. TLC showed a single spot corresponding to 4.

Quantitative analysis of sulfate in gonyautoxin-VIII (10)

A soln (1.36 μmol) of 10 in 0.05N HCl was heated in a sealed vial at 80–85° for 40 min. After cooling, one half of the mixture was evaporated, dissolved in 1 ml of 25% HCOOH and used for sulfate quantitation according to Antonopoulos.[41] The result showed that 1.4 μmol or 1.03 mol equiv of sulfate was released. Under the same condition, GTX-III (4) did not release any sulfate.

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