Agric. Biol. Chem., **46** (7), 1861～1864, 1982

Structures of Two Paralytic Shellfish Toxins, Gonyautoxins V and VI, Isolated from a Tropical Dinoflagellate, *Pyrodinium bahamense* var. *compressa*†

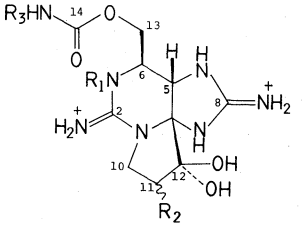
Takako HARADA, Yasukatsu OSHIMA and Takeshi YASUMOTO

Department of Food Chemistry, Faculty of Agriculture, Tohoku University, Tsutsumi-dori, Sendai 980, Japan

Received January 18, 1982

Two paralytic shellfish toxins, gonyautoxin V and gonyautoxin VI, isolated from a tropical dinoflagellate, *Pyrodinium bahamense* var. *compressa*, were identified respectively to be derivatives of saxitoxin and neosaxitoxin with a sulfonatocarbamoyl moiety.

Paralytic shellfish poisoning is one of the most notorious marine food poisonings prevalent in many parts of the world including Japanese coastal waters. The dinoflagellates *Protogonyaulax tamarensis* and *P. catenella* have been determined to be the cause in temperate and cold waters. The presence of potent neurotoxins of the saxitoxin family was recognized in both dinoflagellates and shellfish. According to Shimizu[1] at least 10 toxins have been recognized: gonyautoxins I～VIII ($GTX_{1\sim8}$), saxitoxin (STX) and neosaxitoxin (neoSTX). The chemical structures of seven of them have been elucidated (**1**～**7**).[1,2] Among the three toxins, $GTX_{5\sim7}$, that remain to be clarified, GTX_5 is rather common in both dinoflagellates and shellfish and has been known to have a low specific toxicity. On the other hand, previous records of GTX_6 are confined on Alaskan mussels.[1]



		R_1	R_2	R_3
(1)	STX	-H	-H	-H
(2)	neoSTX	-OH	-H	-H
(3)	GTX_1	-OH	-αOSO$_3^-$	-H
(4)	GTX_2	-H	-αOSO$_3^-$	-H
(5)	GTX_3	-H	-βOSO$_3^-$	-H
(6)	GTX_4	-OH	-βOSO$_3^-$	-H
(7)	GTX_8	-H	-βOSO$_3^-$	-SO$_3^-$
(8)	GTX_5	-H	-H	-SO$_3^-$
(9)	GTX_6	-OH	-H	-SO$_3^-$

In our recent study we presented for the first time evidence that a tropical dinoflagellate, *Pyrodinium bahamense* var. *compressa*, also produces paralytic shellfish toxins consisting of STX (**1**), neoSTX (**2**), GTX_5, GTX_6 and a new toxin tentatively coded PBT.[3] This finding prompted us to undertake a structural study on GTX_5, GTX_6 and PBT. In the present paper we report the chemical structures of GTX_5 and GTX_6.

Both GTX_5 and GTX_6 were isolated as very hygroscopic colorless solids from the *Pyrodinium* cells following the steps described in the previous paper.[3] The complete separation of the two toxins which had very close retention times was achieved by repeating Bio-Rex 70 chromatography with linear gradient elution with acetic acid. Homogeneity of both toxins was confirmed by TLC and electrophoresis.

PMR and CMR spectral data of GTX_5 are listed in Tables I and II in comparison with those of STX. The chemical shifts are essentially the same as those of STX, except for the

† Studies on Paralytic Shellfish Poisoning in Tropical Waters. Part IV.

TABLE I. COMPARISON OF PMR DATA OF SAXITOXIN, GONYAUTOXIN V, NEOSAXITOXIN AND GONYAUTOXIN VI

Chemical shifts are expressed in ppm downfield from TMS (internal standard, *t*-BuOH = 1.23 ppm). Coupling constants in parentheses are given in Hz.

Proton	STX	GTX_5	neoSTX	GTX_6
H- 5	4.77 (1)	4.76 (1)	4.83 (s)	4.83 (s)
H- 6	3.90 (9, 5, 1)	3.88 (9. 5, 1)	4.14 (6, 6)	4.13 (6, 6)
H-10α	3.81 (10)	3.81 (10)	3.78 (10)	3.78 (10)
H-10β	3.61 (10)	3.61 (10)	3.58 (10)	3.58 (10)
H-11	2.41	—[a]	—[a]	—[a]
H-13-pro-*S*	4.33 (11, 9)	4.42 (12, 9)	4.44 (11, 6)	4.52 (11, 6)
H-13-pro-*R*	4.07 (11, 5)	4.12 (12, 5)	4.21 (11, 6)	4.26 (11, 6)

[a] Unobserved due to deuterium replacement at C-11.

TABLE II. COMPARISON OF CMR DATA OF SAXITOXIN AND GONYAUTOXIN V

Chemical shifts in ppm downfield from TMS (internal standard, dioxan = 67.4 ppm).

Carbon	STX	GTX_5
C- 4	82.6	82.6 (s)
C- 5	57.1	57.2 (d)
C- 6	53.2	53.0 (d)
C-10	42.8	42.9 (t)
C-11	—[a]	—[a]
C-12	98.6	98.6 (s)
C-13	63.3	64.0 (t)
C- 2	156.2[b]	156.1 (s)
C- 8	158.1[b]	158.1 (s)
C-14	159.1[b]	154.1 (s)

[a] Unobserved due to deuterium replacement at C-11.
[b] Assignment of C-2, 8, 14 was made according to the data of Rogers and Rapoport.[8]

slight difference in the proton and carbon chemical shifts for C-13 and C-14: $\Delta\delta$ +0.09 ppm for H-13-pro-*S* and +0.05 ppm for H-13-pro-*R*, and +0.7 ppm for C-13 and −5.0 ppm for C-14. These differences correspond well with those observed between GTX_3 (**5**) and GTX_8 (**7**).[2] GTX_5 was converted to STX in a dilute hydrochloric acid solution even at room temperature. One normal hydrochloric acid sufficed for complete conversion in 5 min at 100°C. While the specific toxicity of GTX_5 was the very low level of 136 MU/μmol, acid treatment increased it by about 15 times. Colorimetric sulfate determination and bioassaying of STX in the acid hydrolyzate confirmed that 1.0 mol of GTX_5 produced 1.0 mol of free sulfate and 1.1 mol of STX. This result was also supported by elemental analysis of GTX_5. Although the extremely hygroscopic nature of the specimen prevented an accurate molecular formula from being obtained, nitrogen and sulfur were confirmed to be present in a ratio of 7.2:1. Thus it is obvious that GTX_5 is a derivatives of STX with an additional sulfate moiety. The IR spectrum of GTX_5 showed prominent absorptions at 630, 1040 and 1230 cm^{-1}, indicating a N-sulfonate moiety.[4] The chemical shift trend for C-13, C-14 and 13 protons support that the possible conjugation site for sulfate is the carbamato-nitrogen. The vulnerability of GTX_5 to acid is also in accordance with the observed properties of GTX_8 (**7**) which contains a sulfonato-carbamoyl function.[2] Thus all the data unequivocally support the structure of (**8**) for GTX_5.

GTX_6 likewise was converted to neoSTX on heating in dilute hydrochloric acid, resulting in an increase of toxicity of approximately 15 times. Since the specific toxicity of neoSTX was 1617 MU/μmol, that of GTX_6 was assumed to be 108 MU/μmol. PMR spectral data of GTX_6 are given in Table I together with those of neoSTX. The chemical shifts are essentially the same as those of neoSTX with only noticeable changes for H-13: $\Delta\delta$ +0.08

ppm for H-13-pro-*S* and +0.05 ppm for H-13-pro-*R*. On acid treatment, 1.0 mol of GTX_6 generated 1.0 mol of neoSTX and 0.9 mol of free sulfate, indicating that GTX_6 is a neoSTX sulfonate. From the complete agreement of the chemical shifts of the protons between GTX_6 and neoSTX except for the protons attached to C-13, it is deduced that GTX_6 is a neoSTX derivative with a sulfonatocarbamoyl function (**9**).

Occurrence of a sulfonatocarbamoyl moiety in the paralytic shellfish toxin family was first demonstrated in GTX_8 (**7**).[2] The vulnerability of this moiety during the bioassay procedure which employs dilute acid and possibly during the metabolic pathway in the human body may add new problems to the evaluation of the safety standards for shellfish infested with GTX_5, GTX_6 and GTX_8 since they are easily convertible to far more potent toxins. Our previous observation of the greater abundance of STX in bivalves than in the causative dinoflagellate shows that such conversion actually takes place in the animal tissue.[3]

Hall *et al.*[5] reported the presence of two components coded B_1 and B_2 in *P. tamarensis* which were easily converted to STX and neoSTX respectively upon heating with hydrochloric acid. They may correspond to GTX_5 and GTX_6, respectively.

EXPERIMENTAL

Instruments. NMR spectra were taken with a JEOL FX-100 spectrometer. For measurement of PMR a D_2O solution containing *t*-BuOH was used. The strong water band at around 4.8 ppm was removed by PRFT techniques. CMR spectra were measured in a D_2O solution containing dioxane. IR spectra were taken with a JASCO A-202 spectrometer and the colorimetric determination was done on a HITACHI model 100-10 spectrophotometer.

Purification procedures. The dinoflagellate, *Pyrodinium bahamense* var. *compressa*, was collected at Palau Island in May 1981. Toxins were extracted with 75% ethanol (pH 3.8) three times. The extracts were concentrated under reduced pressure and washed with chloroform. The aqueous phase was freed from chloroform under vacuum, adjusted to pH 5.5, and treated successively with activated charcoal, Bio-Gel P-2 (Bio-Rad Laboratories), and Bio-Rex 70 (Bio-Rad Laboratories, H^+ form). GTX_5 and GTX_6 were separated on a Bio-Rex 70 column (0.5 × 100 cm) by linear gradient elution with acetic acid (0 ~ 0.02 N). From 709 g of the dinoflagellate cells 76 mg of GTX_5 and 8 mg of GTX_6 were obtained.

TLC and electrophoresis. TLC was performed on precoated Silica gel 60 plates (Merck) with pyridine–ethyl acetate–acetic acid–water (75 : 35 : 15 : 30). Electrophoresis was carried out on cellulose acetate strips (Separax, Fuji Photo Film Co.) in 0.083 M Tris-hydrochloric acid buffer (pH 8.7) at 0.56 mA/cm for 18 min. Toxins were detected under UV light (365 nm) after spraying with 1% hydrogen peroxide and heating at 130°C for 5 min. GTX_5, STX and neoSTX isolated from *P. tamarensis* were used as standards.

Bioassay. The mouse assay was carried out according to the method described in A.O.A.C.[6] Male mice of the ddY strain weighing from 18 to 20 g were used.

Elemental analysis. Owing to the extremely labile nature of the toxins, lyophilized samples were subjected to analysis without further dehydration by heating. *Anal.* Found for GTX_5 acetate: C, 31.54; H, 4.95; N, 20.10; S, 6.46%. *Calcd.* for $C_{10}H_{18}O_7N_7S \cdot 1\ CH_3COOH \cdot 2\ H_2O$: C, 30.24; H, 5.51; N, 20.58; S, 6.73%. NeoSTX diacetate showed a nitrogen content of 22.24%. This value was used in the determination of the specific toxicity.

Determination of sulfate. The toxins were hydrolyzed in sealed tubes with 1 ml of 0.05 N hydrochloric acid in a boiling water-bath for 30 min. The free sulphate in 0.2 ml of the hydrolyzate was determined by the benzidine method.[7] Solutions containing 10, 20, 30, 40 and 50 μgSO_4/ml in 0.05 N hydrochloric acid were used as standard solutions. 7.92×10^{-2} μmol of GTX_5 produced 8.40×10^{-2} μmol of STX and 8.31×10^{-2} μmol of free sulphate. 6.40×10^{-2} μmol of GTX_6 generated 6.40×10^{-2} μmol of neoSTX and 5.59×10^{-2} μmol free sulphate.

IR spectrometry. The spectra were taken with KBr pellets. GTX_5 was compared with STX as the acetate form. The spectrum of GTX_5 resembled that of STX but was distinguishable by having absorptions at 630, 1040 and 1230 cm^{-1}, characteristic of N-SO_3^-. The absorptions at around 3200 and 1700 cm^{-1} due to a guanidino group [NH_2–C(=NH)–NH–] were present in both spectra.

Acknowledgment. The present study was supported by a Grant-in-Aid from the Toyota Foundation.

REFERENCES

1) Y. Shimizu, *Kagaku to Seibutsu*, **18**, 792 (1980).
2) M. Kobayashi and Y. Shimizu, *J. Chem. Soc. Chem.*

Commun., **1981**, 827.
3) T. Harada, Y. Oshima, H. Kamiya and T. Yasumoto, *Bull Jpn. Soc. Sci. Fish.*, **48**, 821 (1982).
4) A. Imada, K. Kitano, K. Kinoshita, M. Muroi and M. Asai, *Nature*, **289**, 590 (1981).
5) S. Hall, P. B. Reichardt and R. A. Neve', *Biochem. Biophys. Res. Commun.*, **97**, 649 (1980).
6) W. Horwitz (ed.), "Official Methods of Analysis of the Association of Official Analytical Chemists," 13th Ed., A.O.A.C., Washington, D.C., 1980, pp. 298～299.
7) C. A. Antonopoulos, *Acta Chem. Scand.*, **16**, 1521 (1962).
8) R. S. Rogers and H. Rapoport, *J. Am. Chem. Soc.*, **102**, 7335 (1980).