Preparation of 9-Hydroxy Grayanotoxin Derivatives and Their Acute Toxicity in Mice

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Novel 9α and 9β-hydroxy grayanotoxin II derivatives were prepared by photo-sensitized oxygenation of iso-grayanotoxin II and oxidation of grayanotoxin II tetraacetate with selenium dioxide respectively. The lethal dosage of 9α and 9β-hydroxy grayanotoxin II were lower than that of grayanotoxin II. In addition, the lethal dosage of 9β-hydroxy-dihydro grayanotoxin II was higher than that of dihydro grayanotoxin II.

Key words grayanotoxin II; 9-hydroxy grayanotoxins; acute toxicity; lethal dosage; X-ray analysis

Grayanotoxins (GTXs) are toxic compounds found in the leaves of some Ericaceae species.[1—4] GTXs have a unique tetracyclic diterpenoid carbon skeleton (grayanotoxane: A-nor-B-homo-kaurane skeleton), and several hydroxyl groups are located on the A, B, C, and D rings (Fig. 1). These toxic compounds exert a specific stimulatory action on membrane permeability to Na^+ ions in various excitable tissues.[5] To date, about 60 grayanotoxane compounds, GTXs, 1,5-*seco*-GTXs and their glucosides, have been isolated from various Ericaceae plants.[6] In a previous paper, we isolated the 9-hydroxy GTXs, asebotoxin VII (**1**), pieristoxin J (**2**) and pieristoxin K (**3**), from the leaves of *Pieris japonica* D. DON., and determined their structures by NMR spectroscopy.[7] However, a search of the literature revealed that are no reports on the toxicity 9-hydroxy GTXs derivatives, possibly due to the difficulty in obtaining them as natural products. In the present paper, we report the transformation of GTX-III to 9α- and 9β-hydroxy GTX derivatives (Chart 1), and investigate the acute toxicity of 9-hydroxy GTXs at various dosage level in mice. We also compared the toxicity of 9-hydroxy GTXs with that of natural GTX-II. 9α-Hydroxy GTX-II (**7**) was derived from GTX-III (**4**) in two steps. Dehydration of GTX-III (**4**) with *dil.* hydrochloric acid in methanol gave GTX-II (**5**) and iso-GTX-II (**6**).[8] Under UV light irradiation, photo-sensitized oxygenation of iso-GTX-II (**5**) in methanol gave two products, **7** and 3,5,6,9,14,16-hexahydroxy-grayanotoxa-$\Delta^{1(10)}$-ene (**8**). One of the products, **7**, showed an M^+ peak on MS at *m/z* 368 ($C_{20}H_{32}O_6$), and based on IR and ^{1}H-NMR

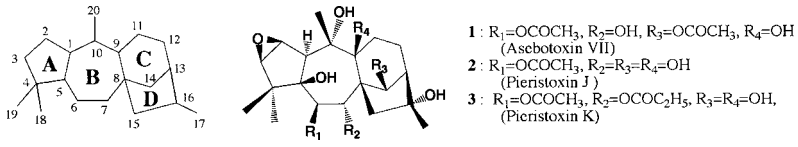
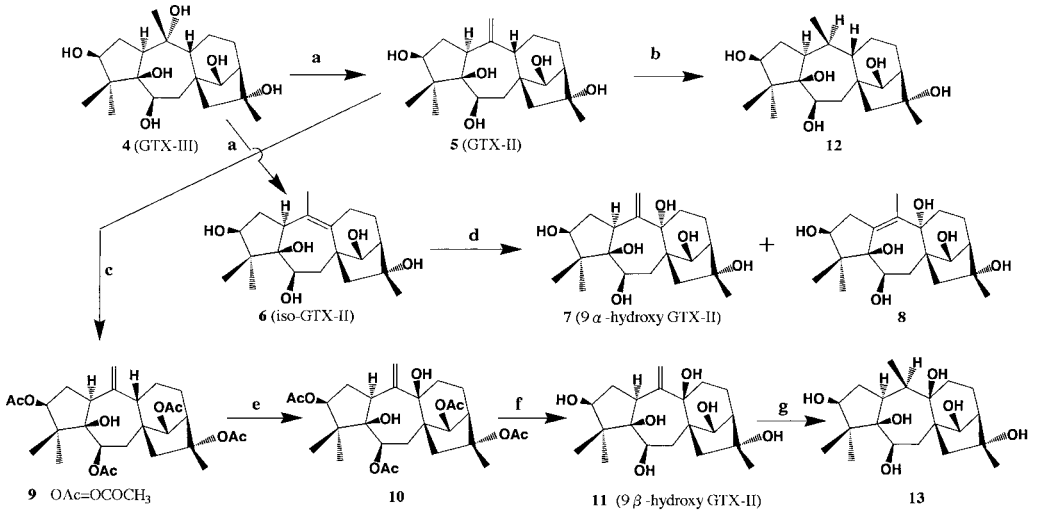


Fig. 1. Structures of Natural 9-Hydroxy-grayanotoxin Derivatives



Reagents: (**a**) HCl/MeOH; (**b**) PtO_2-H_2/MeOH; (**c**) Ac_2O/Py; (**d**) UV-O_2/MeOH; (**e**) SeO_2-H_2O_2/Dioxane; (**f**) 5%-KOH/MeOH; (**g**) PtO_2-H_2/MeOH

Chart 1

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spectra was believed to contain three tertiary methyl groups (δ: 1.10, 1.52, 1.61 ppm), and a vinylidene group (ν_{max} 1634 cm^{-1}, δ: 5.48 ppm, 2H.s). ^{13}C-NMR spectrum indicated three tertiary hydroxy groups at C_5, C_9 and C_{16} (δ: 83.6, 78.8, 81.4 ppm), and three secondary hydroxy groups at C_3, C_6 and C_{14} (δ: 81.6, 69.2, 81.4 ppm). The ^{1}H- and ^{13}C-NMR signals of **7** were assigned based on ^{13}C–^{1}H correlation spectroscopy (C–H COSY), distortionless enhancement by polarization transfer (DEPT) and comparison with spectra of known derivatives.

The other product, **8**, also showed an M^+ peak on MS at *m/z* 368 ($C_{20}H_{32}O_6$), and ^{1}H-NMR spectra indicated the presence of three tertiary methyl groups (δ: 1.13, 1.48, 1.79 ppm), and an olefinic methyl groups (δ: 1.94 ppm, C_{20}-H_3). The ^{13}C-NMR spectrum suggested three tertiary hydroxy groups at C_5, C_9 and C_{16} (δ: 80.5, 80.7, 82.0 ppm), and three secondary hydroxy groups at C_3, C_6 and C_{14} (δ: 80.9, 73.2, 85.7 ppm). We then studied the X-ray crystallography data for compound **7** in order to clarify the ring conformation around the C_9 atom. The stereo structure of the **7** was desired and is shown in Chart 1 and Fig. 2. GTX-II 3,6,14,16-tetraacetate (**9**) was prepared by acetylation of GTX-II (**5**) with acetic anhydride–pyridine by the usual procedure. Oxidation of the tetraacetate (**9**) in dioxane–water at room temperature using selenium dioxide and H_2O_2 as an oxidant gave 9β-hydroxy-GTX-II-3,6,14,16-tetra-*O*-acetate (**10**). Hydration of **10** with 5%-KOH gave 9β-hydroxy-GTX-II (**11**). The product, **11** ($C_{20}H_{32}O_6$, mp 215—216 °C) gave a ^{1}H-NMR spectrum that indicated three tertiary methyl groups (δ: 1.22, 1.50, 1.78 ppm), and a vinylidene group (δ: 5.39, 5.62 ppm). The ^{13}C-NMR spectrum indicated three tertiary hydroxy groups at C_5, C_9 and C_{16} (δ: 83.6, 78.4, 80.3 ppm), and three secondary hydroxy groups at C_3, C_6 and C_{14} (δ: 82.0, 74.0, 80.7 ppm). Hydrogenation of **11** with PtO_2–H_2 gave 9β-hydroxy-10α-dihydro GTX-II (**13**). The ^{1}H-NMR spectrum of **13** indicated three tertiary methyl groups and a secondary methyl groups (δ: 1.47 ppm, 3H, s, J=15.9 Hz). The ^{13}C-NMR spectrum suggested three tertiary hydroxy groups at C_5, C_{16} and C_9 (δ: 87.6, 78.8, 76.8 ppm), and three secondary hydroxy groups at C_3, C_6 and C_{14} (δ: 82.0, 73.9, 79.7 ppm).

Results and Discussion

Acute Toxicity Thirty six mice of the ICR strain (body weight: 27.1—32.4 g) were injected intraperitoneally with a single dose of GTX-II (**5**), 9α-hydroxy-GTX-II (**7**), 9β-hydroxy-GTX-II (**11**), dihydro GTX-II (**12**) and 9β-hydroxy-dihydro-GTX-II (**13**), and toxic signs and mortality were recorded continuously for 2 h, then twice daily (in the morning and evening) for the following 2 d. Toxic signs consisted of sedation, ptosis and piloerection were observed immediately after dosing in all mice treated with **5**, **7**, **11**, **12** and **13**. Each three mice that received 10 and 100 mg/kg of GTX-II (**5**) died with dyspnea within 15 and 5 min after dosing respectively, following the appearance of severe toxic symptoms. Each three mice that received 40 and 100 mg/kg of 9β-hydroxy-GTX-II (**11**) also died within 15 min after dosing, but all mice that received 10 mg/kg of 9β-hydroxy-GTX-II (**11**) and dihydro GTX-II (**12**) recovered completely within 24 h after dosing. All three mice also survived even in the 100 mg/kg dosage group of 9α-hydroxy-GTX-II (**7**). Moderate sedation, ptosis and piloerection appeared for several min after the dosing were common toxic signs in surviving mice. Judging from the results of the present study (Table 2) the approximate value of LD_{50} was estimated to be comparable between **11** and **12** and apparently lower than that of GTX-II (**5**). Similarly, acute toxicity of 9β-hydroxy-GTX-II (**11**) and GTX-II (**5**) was comparable, although one animal received 10 mg/kg of 9β-hydroxy-GTX-II (**11**) was survived and dead

Table 1. ^{13}C-NMR Chemical Shifts (δ) of Grayanotoxin Derivatives[a)]

C. No.	**6**	**7**	**8**	**10**	**11**	**12**	**13**
1	44.4	42.7	139.8	47.6	48.9	39.4	42.5
2	37.0	38.6	42.2	40.8	41.3	35.8	37.3
3	80.5	81.6	80.9	81.4	82.0	83.3	82.0
4	48.8	50.6	51.4	51.1	51.7	51.4	50.8
5	85.1	83.6	85.0	89.5	86.3	86.8	87.6
6	67.6	69.2	73.2	76.5	74.0	77.6	73.9
7	41.2	35.3	42.5	33.2	34.8	43.0	34.8
8	56.7	56.6	55.0	54.5	57.5	51.9	57.8
9	124.4	78.8	80.7	78.8	78.4	52.3	76.8
10	137.4	152.8	137.5	150.9	153.7	37.8	39.1
11	26.2	31.8	33.8	32.8	33.1	24.7	32.2
12	26.7	23.6	24.2	23.4	23.5	25.9	22.8
13	53.6	53.1	52.3	49.9	55.1	55.4	53.9
14	88.1	81.4	85.7	79.9	80.7	80.3	79.7
15	58.7	49.4	55.9	52.7	55.4	59.2	54.7
16	82.2	81.4	82.0	82.6	80.3	79.2	78.8
17	24.0	24.9	20.0	19.6	23.4	23.5	23.0
18	24.3	24.6	22.9	25.3	23.5	23.2	22.6
19	17.7	18.9	24.9	19.2	20.4	19.6	19.1
20	20.4	112.4	18.4	118.1	116.6	19.6	14.3
				21.1			
				21.3			
				21.5			
–O–Ac				22.6			
				169.7			
				170.3			
				170.8			
				171.2			

a) Measured at 300 MHz in C_5D_5N using tetramethylsilane (TMS) as an internal standard.

Table 2. Relationship between Dosage Levels and Mortality and/or Toxic Symptoms[a)]

Dosage level (mg/kg)	**5**		**7**		**11**		**12**		**13**	
	Mortality	Toxic symptoms	Mortality	Toxic symptoms	Mortality	Toxic symptoms	Mortality	Toxic symptoms	Mortality	Toxic symptoms
10	3/3	++	0/3	±	0/3	+	0/3	+	2/3	++
40	—		—		3/3	++	3/3	++	—	
100	3/3	++	0/3	+	3/3	++	3/3	++	3/3	++
Lethal dose	<10 mg/kg		>100 mg/kg		>10 mg/kg		>10 mg/kg		<10 mg/kg	

a) Symptoms: ++, moderate; +, slight; —, negative.

animals survived much longer than those received 10 mg of GTX-II (**5**). Therefore, acute toxicity might be milder in order of 9α-hydroxy-GTX-II (**7**), 9β-hydroxy-GTX-II (**11**) and dihydro GTX-II (**12**), 9β-hydroxy-dihydro-GTX-II (**13**) and GTX-II (**5**). Based on these results, we can concluded that the transformation of GTX-II to 9β-dihydro- and 9α-hydroxy-GTX-II resulted slight and marked reduction of acute toxicity, respectively.

Experimental

All melting points (mps) are uncorrected. IR spectra were measured with a Shimadzu IR-430 instrument. ^{1}H- and ^{13}C-NMR were measured on a Unity-300 (Varian Co.) spectrometer in $CDCl_3$ or pyridine-d_5, using tetramethylsilane (TMS) as an internal standard. The ^{1}H- and ^{13}C-NMR signals of each derivatives were assigned DEPT, ^{1}H–^{1}H correlation spectroscopy (H–H COSY), ^{13}C–^{1}H correlation spectroscopy (C–H COSY) and by comparison with spectra of known derivatives. HR mass spectra were obtained with a Nihondenshi Co. mass spectrometer (JMS-700T). ^{13}C-NMR data and assignment of GTX derivatives were shown in Table 1.

9α-Hydroxy GTX-II (7) and 3β,5β,6β,9α,14β,15α-Hexahydroxy-grayanotoxa-$\Delta^{1(10)}$-ene (8) Air was passed through a solution of iso-GTX-II (600 mg, 1.70 mmol) and Rose Bengal (2 mg) in methanol (10 ml) under irradiation by UV light at 25 °C for 1.5 h. The mixture was treated with 5%-KOH *soln.* and active carbon, and the solvent was evaporated to give 360 mg of products mixture, **7** and **8**. The mixture was purified by silica gel (Wakogel C-300) column chromatography. Gradient elution with ethyl acetate from chloroform-ethyl acetate gave **7** (110 mg, 18%) and **8** (85 mg, 14%). The crude **7** was recrystallized from hexane–ethyl acetate, mp 248—250 °C. EI-HR-MS *m/z*: 368.2191 (Calcd for $C_{20}H_{32}O_6$: 368.2191). IR (Nujol) cm^{-1}: 3362 (OH), 1634 (>C=CH_2), 1319, 1049, 1028, 927, 914; ^{1}H-NMR (pyridine-d_5) δ: 1.10 (3H, s, C_{18}-H_3), 1.52 (3H, s, C_{19}-H_3), 1.61 (3H, s, C_{17}-H_3), 2.30 (2H, m, C_2-H_2), 2.75 (1H, d, J=15.3 Hz, C_{15}-H), 2.91 (1H, dd, J=2.7, 15.6 Hz, C_7-H), 3.16 (1H, dd, J=6.0, 15.6 Hz, C_7-H), 3.95 (1H, m, C_3-H), 4.11 (1H, t, J=9.6 Hz, C_1-H), 4.71 (1H, m, C_6-H), 5.13 (1H, d, J=7.5 Hz, C_{14}-H), 5.48 (2H, d, J=6.6 Hz, C_{20}-H_2). ^{13}C-NMR data of **7** was shown in Table 1. The other product, **8**, was recrystallized from hexane–ethyl acetate, mp 136—138 °C; IR (Nujol) cm^{-1}: 3360 (OH), 1221, 1153, 1072, 1045, 997, 993. EI-HR-MS *m/z*: 368.2189 (Calcd for $C_{20}H_{32}O_6$: 368.2191). ^{1}H-NMR (pyridine-d_5) δ: 1.13 (3H, s, C_{18}-H_3), 1.48 (3H, s, C_{19}-H_3), 1.79 (3H, s, C_{17}-H_3), 1.94 (3H, s, C_{20}-H_3), 2.97 (2H, dd, J=3.3, 17.7 Hz, C_2-H_2), 3.00 (1H, m, C_7-H), 3.44 (1H, dd, J=7.2, 14.4 Hz, C_7-H), 3.93 (1H, d, J=4.5 Hz, C_3-H), 4.81 (1H, dd, J=7.2, 9.9 Hz, C_6-H), 4.87 (1H, s, C_{14}-H).

9β-Hydroxy-GTX-II-3,6,14,16-tetra-*O*-acetate (10) To a solution of GTX-II-tetraacetate (**9**) (500 mg) in 10 ml dioxane and 1 ml water, SeO_2 (40 mg) and 36%-H_2O_2 (1 ml) were added, and the solution was stirred at room temperature for 1 week. The mixture was concentrated and extracted with ethyl acetate. The combined ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to give oily products mixture. The products mixture was purified by silica gel (Wakogel C-300) column chromatography. Elution with hexane–ethyl acetate (4 : 3) and crystallization gave 160 mg (31%) of 9β-hydroxy-GTX-II-tetraacetate (**10**), mp 211—212 °C. IR (Nujol) cm^{-1}: 3467 (OH), 1744 (C=O), 1701 (C=O), 1256 (–O–CO), 1034, 965. ^{1}H-NMR (pyridine-d_5) δ: 1.07 (3H, s, C_{18}-H_3), 1.40 (3H, s, C_{19}-H_3), 2.00 (3H, s, C_{17}-H_3), 1.66, 1.99, 2.03, 2.40 (each 3H, s, –$COCH_3$×4), 2.10 (1H, m, C_7-H), 2.70 (C_7-H), 2.30 (1H, m, C_2-H), 2.90 (1H, m, C_2-H), 3.18 (1H, m, C_{13}-H), 3.34 (1H, m, C_1-H), 5.06 (1H, dd, J=5.1, 7.5 Hz, C_3-H), 5.15 (1H, dd, J=3.9, 11.6 Hz, C_6-H), 5.22 and 5.47 (each 1H, d, J=2.1, C_{20}-H_2), 5.36 (1H, m, C_{14}-H).

9β-Hydroxy-GTX-II (11) To a solution of the tetraacetate (**10**) (350 mg) in methanol (10 ml), 5%-KOH *soln.* (5 ml) was added, and the solution was stirred at room temperature for 24 h. The mixture was neutralized with dil. HCl and extracted with ethyl acetate. The combined ethyl acetate layers were washed with water, and dried over anhydrous Na_2SO_4 and evaporated to give crude **11** (205 mg, 85%). The product, **11**, was recrystallized from ethyl acetate, mp 215—216 °C. IR (Nujol) cm^{-1}: 3200 (OH), 1323, 1088, 1051, 982, 938. EI-HR-MS *m/z*: 368.2192 (Calcd for $C_{20}H_{32}O_6$: 368.2191). ^{1}H-NMR (pyridine-d_5) δ: 1.22 (3H, s, C_{18}-H_3), 1.50 (3H, s, C_{17}-H_3), 1.78 (3H, s, C_{19}-H_3), 2.90 (1H, m, C_7-H), 2.80 (1H, m, C_2-H), 3.39 (1H, m, C_1-H), 3.89 (1H, t, J=6.0 Hz, C_3-H), 4.18 (1H, s, C_{14}-H), 4.54 (1H, m, C_6-H), 5.39 and 5.62 (each 1H, d, J=2.4 Hz, C_{20}-H_2).

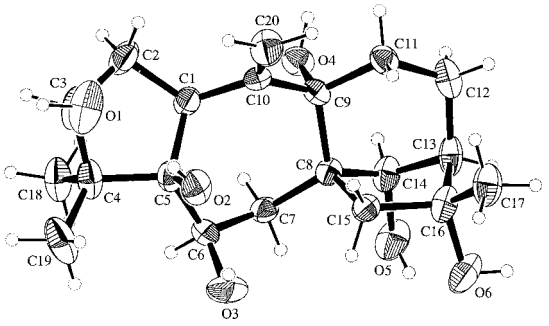

Fig. 2. A Perspective View of the Molecule of **7**

9β-Hydroxy-α-dihydro-GTX-II (13) A mixture of 9β-hydroxy-GTX-II (**11**) (200 mg) and PtO_2 (5 mg) in methanol (10 ml) was stirred at room temperature for 5 h. under H_2, and then filtered. The filtrate was concentrated under reduced pressure to leave white crystals. Which were crystallized from hexane–ethyl acetate to give 9β-hydroxy-α-dihydro-GTX-II (**13**) (180 mg, 90%), mp 250—265 °C. EI-HR-MS *m/z*: 370.2357 (Calcd for $C_{20}H_{34}O_6$: 370.2355). ^{1}H-NMR (pyridine-d_5) δ: 1.16 (3H, s, C_{18}-H_3), 1.47 (3H, s, C_{17}-H_3), 1.66 (3H, s, C_{19}-H_3), 1.47 (3H, d, J=15.9 Hz, C_{20}-H_3), 3.87 (1H, m, C_3-H), 4.27 (1H, s, C_{14}-H), 4.39 (1H, dd, J=6.9, 9.0 Hz, C_6-H).

X-Ray Crystallographic Analysis A crystal,[9] 9α-hydroxy-GTX-II (**7**), used for X-ray crystallographic analysis, was obtained by slow evaporation from an ethyl acetate solution at room temperature. X-ray diffraction data of the crystal were collected on a Rigaku AFC-5R diffractometer with graphite monochromated Cu$K\alpha$ radiation and a rotating anode generator. Of the 1739 reflections which were collected, 1716 were unique (R_{int}=0.000). The intensities of three representative reflections were measured after every 150 reflections. The structure was solved by direct methods using the SHELX97 program,[10] and all the computations were carried out on the teXan crystallographic soft ware package.[11] Final *R*-factors were R=0.056, R_W=0.111. The atomic scattering factors used for non-hydrogen atoms were taken from the International Table[12] and for hydrogen atoms from a reference.[13]

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