Transformation of Grayanotoxin III to 10-*epi*-Grayanotoxin III. Its X-Ray Crystallographic Analysis and Acute Toxicity in Mice

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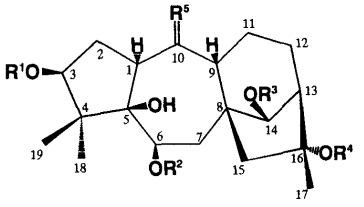
Grayanotoxin(GTX) III, a tetracyclic diterpenoid, is the main toxic component obtained from the leaves of *Leucothoe grayana* MAX. In this paper, we report the correlation between toxicity and C_{10} stereostructure of GTX-III. 10-*epi*-GTX-III was derived from GTX-III in four steps. Oxidation of the GTX-II-3,6,14,16-tetraacetate with formic acid/hydrogen peroxide in chloroform gave 10,20-epoxy derivative in high yield. Reduction of the 10,20-epoxyacetate with $LiAlH_4$ in tetrahydrofuran gave the two products, 10-*epi*-GTX-III and 10,20-epoxy-GTX-II. The absolute structure of the 10-*epi*-GTX-III was deduced from the results of X-ray crystallographic analysis. Dosage level of acute toxicity of 10-*epi*-GTX-III in mice was estimated at about half of natural GTX-III.

Key words grayanotoxin III; 10-*epi*-grayanotoxin III; acute toxicity; X-ray analysis

Grayanotoxin (GTX) III (**1**), a tetracyclic diterpenoid (A-nor-B-homokaurane skeleton), is one of the main toxic components obtained from the leaves of *Leucothoe grayana* MAX. (Ericaceae species).[1] These toxic components have a specific stimulatory action on membrane permeability to Na^+ ion in various excitable tissues.[2] Hikino *et al.*[3] reported the relationship between toxicity and chemical structures of GTXs but made no mention of a correlation between the toxicity and stereostructure of the C_{10}-hydroxy group. The object of this work was to compare the toxicity of 10-*epi*-GTX-III (**2**) with that of natural GTX-III (**1**) in mice. We report the transformation of GTX-III (**1**) to 10-*epi*-GTX-III (**2**) and acute toxicity of 10-*epi*-GTX-III at each dosage level in mice. The structure of 10-*epi*-GTX-III (**2**) was decided by the results of X-ray analysis and the complete assignment of ^{13}C-NMR spectrum of **2** using two dimensional-NMR C–H correlation spectroscopy (C–H COSY)).

10-*epi*-GTX-III (**2**) was derived from GTX-III (**1**) in four steps. Dehydration of GTX-III (**1**) with acetic acid in methanol gave GTX-II (**5**).[4] GTX-II was acetylated by usual methods to give GTX-II tetraacetate (**3**). Epoxidation of **3** with formic acid/hydrogen peroxide in chloroform gave 10,20-epoxy tetraacetate (**4**) in 81.5% yield. It was, however, difficult to obtain 10,20-epoxy-GTX-II (**6**) from GTX-II (**5**) directly using performic acid or *m*-chloroperbenzoic acid as the oxidant. Gasa *et al.*[5] reported that the oxidation of GTX-II (**5**) with hydrogen peroxide gave 10,20-epoxy GTX-II derivative (**6**). In this method, however, conc. hydrogen peroxide (90%) must be used and the yield of the reaction is also low. The infrared and ^{1}H-NMR data of 10,20-epoxyacetate (**4**) were identical with those of the authentic sample prepared by the acetylation of **6**, which was synthesized from GTX-II by Gasa's method.[5] Reduction of the 10,20-epoxytetraacetate (**4**) with lithium aluminum tetrahydride in tetrahydrofuran gave the two products, **2** and **6**. Product, **2**, $C_{20}H_{34}O_6$, MS *m/z*: 352 ($M^+ - H_2O$), was indicated by its ^{1}H- and ^{13}C-NMR spectra to contain a geminal methyl (δ_C 16.3, and 22.6 ppm), two tertiary methyls bearing α-hydroxy group (δ_H 1.34, 1.57 ppm, δ_C 24.3, and 26.4 ppm), and three tertiary carbinyl carbons (δ_C 65.5, 80.3, and 82.2 ppm). Based on the above evidences and the X-ray crystallographic analysis (Fig. 2), the structure of the 10-*epi*-GTX-III (**2**) was determined as shown in Fig. 1.

The other product, **6**, $C_{20}H_{32}O_6 \cdot H_2O$, mp 183—185 °C, MS *m/z*: 350 ($M^+ - H_2O$), was indicated by its ^{1}H-NMR (C_5D_5N, $+D_2O$) and ^{13}C-NMR to contain three methyls, three tertiary carbinyl carbons, and an epoxy group. Based on the above physical and spectral data, the structure of **6** was decided as shown in Fig. 1. The IR and NMR spectral data of **6** were identical of an authentic sample of 10,20-epoxy-GTX-II derived from GTX-II (**5**) by Gasa's method.[5] Because **6** crystallizes with one molecule of

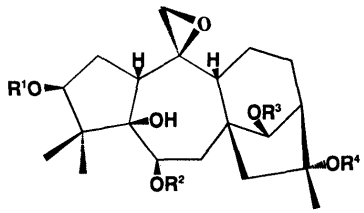


1: $R^1 = R^2 = R^3 = R^4 = H$, $R^5 = \alpha$-CH$_3$, β-OH

2: $R^1 = R^2 = R^3 = R^4 = H$, $R^5 = \alpha$-OH, β-CH$_3$

3: $R^1 = R^2 = R^3 = R^4 = COCH_3$, $R^5 = CH_2$

5: $R^1 = R^2 = R^3 = R^4 = H$, $R^5 = CH_2$



4: $R^1 = R^2 = R^3 = R^4 = COCH_3$

6: $R^1 = R^2 = R^3 = R^4 = H$

Fig. 1. Structures of GTX Derivatives

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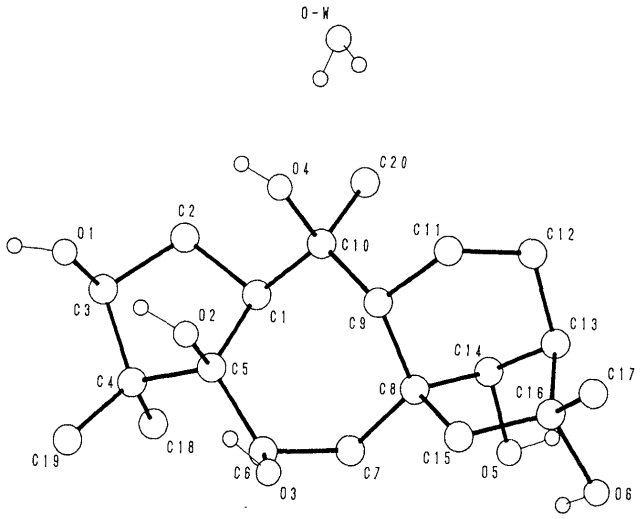

Fig. 2. PLUTO Drawing of 10-*epi*-GTX-III (**2**) by the Atomic Numbering System

water, the melting point of **6** would be lower than that of the authentic sample of 10,20-epoxy-GTX-II (lit.[5] mp 199.5—201 °C). The product, **6**, is regarded as a deacetylated product of 10,20-epoxytetraacetate (**4**).

Results and Discussion

Acute Toxicity Eighteen male mice of ICR strain, average body weight 33.0 ± 3.2 g, were injected intraperitoneally with a single dose of GTX-III (**1**) and 10-*epi*-GTX-III (**2**) and toxic symptoms and mortality were recorded continuously for 2 h, then once in the evening and morning for the following 2 d. Toxic symptoms consisted of salivation, dyspnea and ataxic gait with hindleg paralysis were observed in all GTX-III (**1**) treated mice immediately after dosing, but disappeared within 2 h in those treated with less than 20 mg/kg. Two mice receiving 40 mg/kg of GTX-III (**1**) and two receiving 80 mg/kg of 10-*epi*-GTX-III (**2**) died within 10 min after dosing following the appearance of severe symptoms. No toxic symptom was observed in the 5 or 10 mg/kg dosage groups of 10-*epi*-GTX-III (**2**), but milder toxic symptoms similar to those of GTX-III (**1**) were noted in animals dosed at 20 mg/kg or more. From these results dosage level of acute toxicity of 10-*epi*-GTX-III (**2**) in mice was estimated at about half of GTX-III (Table 1), however, the LD_{50} value of GTX-III was much higher than that previously reported.[3] Masutani and co-workers[6] reported that the essential groups in the GTX molecule for the biological activity were 3β-OH or 2β,3β-epoxy group, 5β-OH, 6β-OH and 10β-methyl groups in frog skeletal muscle. The closely related hydroxy groups 3β-OH, 5β-OH and 6β-OH with toxicity were all positioned axial like and on the same plane in 10-*epi*-GTX-III (**2**). 10β-OH in 10-*epi*-GTX-III (**2**), however, was positioned axial like instead of 10β-methyl in natural GTX-III (**1**). It can be concluded that the presence of 10β-OH (axial hydroxy group) reduces the acute toxicity in mice.

X-Ray Crystallographic Analysis X-Ray analysis showed that the crystal asymmetric unit contained one water molecule other than **2**. This molecule contributes to the an intermolecular network through hydrogen bonds in the crystal. Direct intermolecular hydrogen bonding also occurs between O6 atom and O1 atom at $(1+x, y, z)$.

All the other intermolecular distances for non-hydrogen atoms are usual van der Waals distances.

Table 1. Relationship between Dosage Levels and Mortality and/or Toxic Symptoms

Dosage level mg/kg (vol. ml/10 g)	GTX-III (**1**)		10-*epi*-GTX-III (**2**)	
	Mortality	Toxic symptoms	Mortality	Toxic symptoms
5 (0.05)	0/2	+	0/2	−
10 (0.1)	0/2	+	0/2	−
20 (0.2)	0/2	+	0/2	±
40 (0.4)	2/2	+	0/2	±
80 (0.8)			2/2	+

Experimental

All melting points (mp) are uncorrected. IR spectra were measured with a Shimadzu IR-430 instrument. ^{1}H- and ^{13}C-NMR spectra 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 **4**, **2** and **6** were assigned by H–H COSY, C–H COSY and by comparison with spectra of known derivatives. The substrate, GTX-II 3,6,14,16-tetraacetate (**3**), was obtained by acetylation of GTX-II (**5**) with acetic anhydride–pyridine by the usual procedure.[4]

10,20-Epoxy-GTX-II Tetraacetate (4) To a solution of GTX-II tetraacetate (**3**) (440 mg, 0.85 mmol) and 30% H_2O_2 (3 ml) in 10 ml of $CHCl_3$, 90% HCOOH (3 ml) was added and the solution was stirred at room temperature for 6 h. The organic layer was washed with $NaHCO_3$ aq. solution. The combined $CHCl_3$ layers were dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to give crystalline residue. Recrystallization from hexane–ethyl acetate gave 370 mg (81.5%) of 10,20-epoxy GTX tetraacetate (**4**) as colorless prisms, mp 171—173 °C. IR (Nujol) cm^{-1}: 3389 (OH), 1736 (C=O), 1260, 1244, 1160, 1102, 1036. ^{1}H-NMR (C_5D_5N) δ: 1.04 (3H, s, C_{18}-H_3), 1.30 (3H, s, C_{19}-H_3), 1.59 (3H, s, C_{17}-H_3), 1.99, 2.02, 2.04, 2.32 (each 3H, s, OCOC$\underline{H}_3 \times 4$), 2.05 (1H, m, C_{20}-H), 2.60 (1H, m, C_{20}-H), 4.96 (1H, dd, J=4.0, 7.2 Hz, C_3-H), 5.25 (1H, dd, J=3.4, 10.5 Hz, C_6-H), 5.45 (1H, s, C_{14}-H). ^{13}C-NMR (C_5D_5N) δ: 18.6 (q, C_{19}), 20.3 (t, C_{11}), 21.0 (q, OCO$\underline{C}H_3$), 21.3 (q, OCO$\underline{C}H_3$), 21.5 (q, C_{17}), 21.5 (q, OCO$\underline{C}H_3$), 22.5 (q, OCO$\underline{C}H_3$), 24.1 (t, C_{12}), 24.6 (q, C_{18}), 32.0 (t, C_2), 36.3 (t, C_{20}), 42.0 (t, C_7), 47.9 (s, C_4), 49.3 (d, C_1), 49.5 (d, C_9), 50.8 (s, C_8), 51.1 (d, C_{13}), 58.2 (t, C_{15}), 59.5 (s, C_{10}), 75.0 (d, C_6), 79.7 (d, C_{14}), 82.3 (d, C_3), 84.1 (s, C_{16}), 89.2 (s, C_5), 169.8, 170.3, 170.5, and 171.2 (each s, O$\underline{C}OCH_3 \times 4$). MS *m/z*: 536 ($M^+$), 518 ($M^+ - H_2O$), 476 ($M^+ - CH_3COOH$), 458 ($518 - CH_3COOH$), 446, 416, 398, 368, 356, 338, 326, 296, 278, 266. *Anal.* Calcd for $C_{28}H_{40}O_{10}$: C, 62.67; H, 7.51. Found: C, 62.47; H, 7.51.

$LiAlH_4$ Reduction of 10,20-Epoxy-GTX-II Tetraacetate (4) To a solution of epoxide (**4**) (200 mg, 0.37 mmol) in tetrahydrofuran (5 ml), $LiAlH_4$ (100 mg, 2.6 mmol) was added, and the solution was refluxed for 14 h. Excess of reagent was decomposed with ethyl acetate, and the solution was acidified with dilute hydrochloric acid and extracted with ethyl acetate. The solvent was evaporated off and the residue was chromatographed on silica gel using $CHCl_3$ as an eluent to give crude **2**, which was recrystallized from ethyl acetate to give **2** (100 mg, 72.5%) as prisms, mp 210—212 °C. IR (Nujol) cm^{-1}: 3200—3500 (OH), 1091, 1063, 1032. ^{1}H-NMR (C_5D_5N) δ: 0.75 (3H, s, C_{18}-H_3), 1.22 (3H, s, C_{19}-H_3), 1.34 (3H, s, C_{20}-H_3), 1.57 (3H, s, C_{17}-H_3), 3.89 (1H, dd, J=6.0, 7.0 Hz, C_3-H), 4.33 (1H, m, C_6-H), 4.33 (1H, s, C_{14}-H). ^{13}C-NMR (C_5D_5N) δ: 16.3 (q, C_{19}), 20.3 (t, C_{11}), 22.6 (q, C_{18}), 24.3 (q, C_{17}), 26.4 (q, C_{20}), 26.9 (t, C_{12}), 34.3 (t, C_2), 42.8 (t, C_7), 49.3 (t, C_1), 50.8 (s, C_4), 51.7 (s, C_8), 53.1 (d, C_9), 54.4 (d, C_{13}), 62.2 (t, C_{15}), 66.5 (d, C_6), 77.1 (s, C_{10}), 80.3 (d, C_3), 81.1 (s, C_{16}), 82.2 (d, C_{14}), 86.0 (s, C_5). MS *m/z*: 352 ($M^+ - H_2O$), 334 ($352 - H_2O$), 316 ($334 - H_2O$), 298 ($316 - H_2O$), 283, 273, 255. *Anal.* Calcd for $C_{20}H_{34}O_6$: C, 64.84; H, 9.25. Found: C, 64.88; H, 9.44.

Elution with $CHCl_3$–MeOH (9:1) gave crude **6**, which was recrystallized from ethyl acetate to give 30 mg (22.7%) of 10,20-epoxy-GTX-II

(**6**), mp 183—185 °C (lit.[5] mp 199.5—201 °C, anhydrous crystal). IR (Nujol) cm^{-1}: 3300—3500 (OH), 1224, 1134, 1060, 1032, 1012, 988, 934, 852. ^{1}H-NMR (C_5D_5N) δ: 1.00 (3H, s, C_{18}-H_3), 1.45 (3H, s, C_{17}-H_3), 1.55 (3H, s, C_{19}-H_3), 2.7—3.1 (2H, m, C_{20}-H_2), 3.88 (1H, br s, C_3-H), 4.20 (1H, s, C_{14}-H), 4.44 (1H, m, C_6-H). ^{13}C-NMR (C_5D_5N) δ: 18.6 (q, C_{19}), 19.8 (t, C_{11}), 22.8 (q, C_{18}), 23.8 (q, C_{17}), 26.4 (t, C_{12}), 33.1 (t, C_2), 43.3 (t, C_7), 46.5 (d, C_1), 46.6 (d, C_9), 48.1 (t, C_{20}), 51.0 (s, C_4), 51.6 (s, C_8), 55.2 (d, C_{13}), 58.2 (s, C_{10}), 60.3 (t, C_{15}), 71.1 (d, C_6), 80.2 (s, C_{16}), 81.3 (d, C_3), 81.7 (d, C_{14}), 85.5 (s, C_5). MS *m/z*: 350 ($M^+ - 2H_2O$), 332 ($350 - H_2O$), 314 ($332 - H_2O$), 296 ($314 - H_2O$), 286, 268. *Anal.* Calcd for $C_{20}H_{32}O_6 \cdot H_2O$: C, 62.15; H, 8.80. Found: C, 62.30; H, 8.91.

X-Ray Crystallographic Analysis A crystal, 10-*epi*-GTX-III (**2**), used for X-ray crystallographic analysis was obtained by slow evaporation from ethyl acetate solution at room temperature. X-Ray diffraction data of the crystal was collected on a Rigaku AFC-5R diffractometer at 20 °C. Integrated intensities of a total of 1719 reflections were measured by the θ–2θ scan method using a monitor count technique.

The intensities of symmetry-related reflections were then averaged to give independent reflection data ($R_{int} = 0.028$). The structure was solved by the direct method using the SHELX86 program[7] and refined by the full-matrix least-squares procedure using the FMLS program.[8] The atomic scattering factors used for non-hydrogen atoms were taken from the International Table[9] and for hydrogen atoms from reference.[10] The absolute configuration was assigned on the basis of internal comparison with that of GTX-III (**1**).

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