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# GRAYANOSIDE D, A DITERPENE GLUCOSIDE FROM *LEUCOTHOE GRAYANA*

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**Key Word Index** *Leucothoe grayana*; Ericaceae; diterpenoid glucoside; grayanoside D; grayanotoxin-XV.

**Abstract**—A new diterpenoid glucoside, grayanoside D, has been isolated from *Leucothoe grayana*. Its structure was elucidated by chemical and spectroscopic means and by correlation with grayanotoxin-XV to be 3-*O*-(β-D-glucopyranosyl)-(10*S*)-dihydrograyanotoxin-XV.

## INTRODUCTION

In previous papers we reported the isolation of three diterpene glucosides, grayanosides A, B and C from *Leucothoe grayana* (Ericaceae) [1–3]. We now report the isolation of another new glycoside, grayanoside D.

## RESULTS AND DISCUSSION

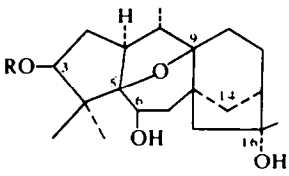
Grayanoside D (**1a**), a viscous syrup, was obtained from the *n*-BuOH soluble fraction of a MeOH extract of *Leucothoe grayana*. Its $^1$H NMR spectrum showed the presence of several hydroxyls, three tertiary methyls and one secondary methyl. Acetylation of **1a** gave a tetra-acetate (**1b**). Acid hydrolysis of **1a** yielded glucose, but its aglycone could not be obtained. Enzymatic hydrolysis of **1a** with naringinase gave a genuine aglycone (**2**), $C_{20}H_{32}O_4$, mp 201–203°. The $^{13}$C NMR and $^1$H NMR spectra revealed the presence of one secondary and three tertiary methyls, six methylenes, three methines, two quaternary carbons, two secondary alcohols and three quaternary carbons adjacent to oxygen. Assuming that **2** had a grayanane (A-nor-B-homo-*ent*-kaurane) skeleton, it must have an ether linkage. Since the $^1$H NMR spectrum of **2** showed no singlet signal around δ 4.50, it had no C-14 hydroxyl group in the grayanane skeleton [4].

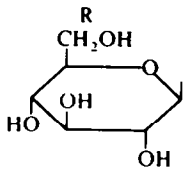
Of the eighteen grayanotoxins [grayanotoxin-I (G-I) to grayanotoxin-XVIII (G-XVIII)] previously isolated from *Leucothoe grayana*, only G-XV (**3**) has an ether linkage and no C-14 hydroxyl group. By analogy to these findings, the structure of **2** was deduced to be 10 (20) ξ-dihydro-G-XV. To verify this point, we planned to hydrogenate G-XV (**3**) in order to obtain **2**.

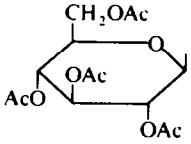
The yield of **3** from the plant was so poor that the structure determination was carried out by X-ray crystallographic analysis [5]. Fortunately, we had isolated G-XVIII (**4**) and its glucoside, grayanoside B (**5**), which also have no C-14 hydroxyl group [2]. Iwasa *et al.* [6, 7] reported that G-II (**6**) reacted with mercuric acetate to form an ether bond between C-5 and C-9 to give **7**. Therefore we attempted a chemical conversion of G-XVIII (**4**) into **3**. G-XVIII (**4**) was treated with mercuric acetate in THF–$H_2O$ to give a main product which showed an absorption at 243.5 nm in the UV and a molecular ion at *m/e* 318 in the MS. These data suggested that dehydration occurred in the course of the reaction to yield a conjugated diene (**8**) [7]. We then tried a similar conversion with grayanoside B (**5**) as the starting material, but this compound is a viscous syrup and practically insoluble in most organic solvents. Therefore its pentaacetate (**9**) was treated with mercuric acetate to yield the monodehydro product (**10**), mp 214–216°, which gave a monoacetate (**11**) by alkaline hydrolysis. Incubation of **11** with naringinase yielded an aglycone (**12**), which still had an acetyl group (δ 2.10, 3H, *s* in the $^1$H NMR spectrum). The aglycone (**12**) was refluxed in KOH–MeOH solution to yield a deacetylated compound (**3**), mp 235–237°, $C_{20}H_{30}O_4$. Acetylation of **12** with pyridine–$Ac_2O$ on a steam bath for 40 hr gave a peracetate (**13**). All spectral data of **13** were identical with those of triacetyl-G-XV cited in the literature [5]. In spite of small differences in the mp and $^1$H NMR data, the synthetic compound (**3**) was identified with natural G-XV by Si gel TLC, silanized Si gel TLC and IR. This demonstrates the chemical conversion of grayanoside B (**5**) into G-XV (**3**). Hamanaka *et al.* [5] assigned the $^1$H NMR signals at δ 3.69 (*br s*) and 3.84 (*br s*) to the C-20 methylene protons and those at 3.78 (*m*) and 5.12 (*q*) to the C-3 and C-6 protons [5]. In grayanotoxins, however, the C-20 methylene signals generally appear around 5.0 and those of the C-3 and C-6 protons at 3.5–4.5. Thus we conclude that the mp difference (mp 235–237° and mp 198–199°) between synthetic and natural G-XV can be attributable to the purity of the sample.

We believe that the $^1$H NMR spectral assignments in the literature [5] should be modified so that the signals at δ 5.07 (*d*) and 5.14 (*d*) are due to the vinylidene protons and those at 3.75 (*d*) and 3.93 (*m*) are assigned to the C-3 and C-6 protons.

G-XV (**3**), thus obtained, was hydrogenated to yield a dihydro derivative, which was identical in all respects with the aglycone of grayanoside D (**2**). Therefore it was confirmed that the structure of **2** was 10 (20)-dihydro-G-XV. However, at this stage the configuration of the C-10 methyl group, and the glucosidation pattern and position in the glucoside (**1a**) remained uncertain.



1a 

1b 

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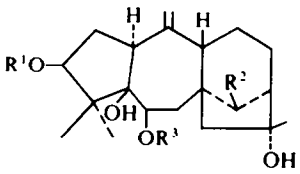
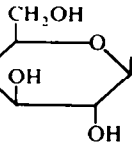
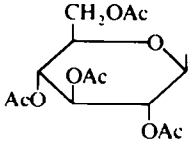
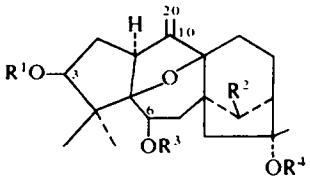
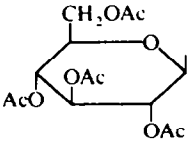
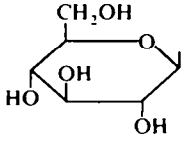
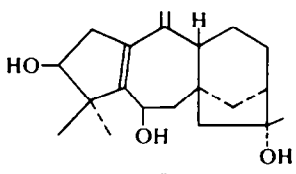


| | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| 4 |  | H | H |
| 5 | | H | H |
| 6 | H | OH | H |
| 9 |  | H | Ac |



| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| 3 | H | H | H | H |
| 7 | H | OH | H | H |
| 10 |  | H | Ac | H |
| 11 |  | H | Ac | H |
| 12 | H | H | Ac | H |
| 13 | Ac | H | Ac | Ac |



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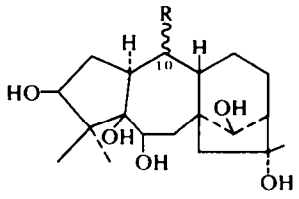

R

14 - - - Me

15 —— Me

The orientation of the C-10 methyl group was determined as follows. G-II (**6**) was hydrogenated to the two isomeric dihydro-compounds, **14** and **15** [8, 9]. The chemical shift and coupling constants of the C-10 methyl group of compounds **14** and **15** in the $^1$H NMR spectra ($C_5D_5N$) were $\delta$0.98 ($J$ = 6 Hz) and 1.45 ($J$ = 8 Hz), respectively, while those of **1a** and **2** were 0.96 ($J$ = 6 Hz) and 1.03 ($J$ = 6 Hz), respectively. Consequently, the C-10 methyl group of **2** must be $\alpha$-oriented, that is the (10*S*)-configuration.

In the $^1$H NMR spectra the anomeric proton of **1a** and **1b** was observed at $\delta$4.86, *d*, $J$ = 7 Hz and 4.45, *d*, $J$ = 7 Hz, respectively, so **1a** must be a $\beta$-D-glucoside. The glucosidation position was determined from the $^{13}$C NMR spectra. The $^{13}$C NMR signals were assigned by means of single-frequency off-resonance decoupling, selective proton decoupling and by comparing the spectra of several related compounds. In general, carbinyl carbon ($\alpha$-carbon) signals of aglycone alcohols are displaced by +5.5–10 ppm on glucosidation [10, 11]. The C-3 signal of the aglycone (**2**) appeared at $\delta$85.0, while that of the glucoside (**1a**) was observed at 91.1. Other carbinyl carbon signals scarcely shifted on glucosidation. Hence $\beta$-D-glucose must be attached at C-3 of the aglycone (**2**).

On the basis of the above data, the structure of grayanoside D (**1a**) was concluded to be 3-*O*-($\beta$-D-glucopyranosyl)-(10*S*)-dihydrograyanotoxin-XV.

## EXPERIMENTAL

Mps were uncorr. $^1$H NMR spectra were measured at 100 MHz. $^{13}$C NMR spectra were measured at 25 MHz. The $\delta$ values are expressed in ppm downfield from TMS as an internal standard. MS (20 eV) were taken with a direct inlet.

*Extraction and isolation of* **1a**. Compound **1a** was obtained in the course of the isolation of grayanosides A, B and C [1–3]. Activated charcoal column chromatography of the *n*-BuOH extract afforded crude **1a** in the fraction eluted with 95–100% MeOH. Repeated chromatography by Si gel and silanized Si gel [eluent: $CHCl_3$–MeOH (8:2) and MeOH–$H_2O$ (1:1), respectively] gave pure **1a**.

*Grayanoside D* (**1a**). Viscous syrup. $[\alpha]_D^{23}$ − 6.52° (MeOH, $c$ = 2.30). IR $\nu_{max}^{KBr}$cm$^{-1}$: 3400, 1077, 1030. $^1$H NMR ($C_5D_5N$): $\delta$0.96 (3 H, *d*, $J$ = 6 Hz), 1.29, 1.46, 1.54 (each 3 H, *s*), 3.8–4.6 (many protons), 4.86 (1 H, *d*, $J$ = 7 Hz). $^{13}$C NMR ($C_5D_5N$): $\delta$14.3 (Me), 19.8 (Me), 24.3 (Me), 24.8 (Me), 25.8 (C-11 and C-12), 31.7 (−$CH_2$−), 36.4 (C-10), 40.3 (−$CH_2$−), 40.6 (−$CH_2$−), 46.2 (C-1 or C-13), 46.7 (C-4 or C-8), 47.8 (C-8 or C-4), 48.0 (C-13 or C-1), 51.9 (C-15), 63.0 (glucose C-6), 69.6 (C-6), 71.9 (glucose C-4), 75.8 (glucose C-2), 78.1 (glucose C-3 and C-5), 79.9 (C-16), 89.0 (C-5 or C-9), 91.1 (C-3), 93.4 (C-9 or C-5), 105.4 (glucose C-1).

*Tetraacetylgrayanoside D* (**1b**). Treatment of **1a** with $Ac_2O$–pyridine overnight at room temp. gave **1b**. Mp 105–108° ($Et_2O$). High resolution MS (70 eV, direct inlet): 666.3191, required for $C_{34}H_{50}O_{13}$: 666.3251. IR $\nu_{max}^{KBr}$cm$^{-1}$: 3375, 1755, 1230. $^1$H NMR($C_5D_5N$): $\delta$0.98 (3 H, *d*, $J$ = 6 Hz), 1.14, 1.38, 1.45 (each 3 H, *s*), 1.99, 2.01, 2.04, 2.07 (each 3 H, *s*), 4.45 (1 H, *d*, $J$ = 7 Hz). $^{13}$C NMR($C_5D_5N$): $\delta$169.2, 169.7, 170.1, 170.3 (acetyl CO).

*Acid hydrolysis of* **1a**. A soln of **1a** (6 mg) in dioxane (1 ml) and 5% $H_2SO_4$ (2 ml) was heated for 2.5 hr on a steam bath. The mixture was cooled, diluted with $H_2O$ (2 ml) and extracted with EtOAc. The EtOAc extract was evapd *in vacuo* to give a complex mixture. The aq. layer was treated with Amberlite CG-4B ($OH^-$) and evapd *in vacuo* to give the sugar moiety. The sugar was converted to its TMSi derivative and identification was made by comparison of $R_t$ of the authentic TMSi-D-glucose by GLC. GLC was performed at 175°, using FID on a stainless column (2 m × 3 mm) of 5% OV-1 on Chromosorb W(AW).

*Enzymatic hydrolysis of* **1a**. To a soln of **1a** (70 mg) dissolved in HOAc–NaOAc buffer (pH 4.1, 5 ml) was added crude naringinase 'SANKYO' (70 mg) and the mixture incubated at 37°. After 34 hr, 70 mg of naringinase was added and further incubated for 24 hr. The soln was extracted with EtOAc and purified by Si gel PLC [eluent: $CHCl_3$–MeOH (9:1)] to give the aglycone (**2**) (35 mg). Mp 201–203° (EtOAc). (Found: C, 71.29; H, 9.48. Calc. for $C_{20}H_{32}O_4$: C, 71.39, H, 9.59%). IR $\nu_{max}^{KBr}$cm$^{-1}$: 3485, 3325, 1040. $^1$H NMR($C_5D_5N$): $\delta$1.03 (3 H, *d*, $J$ = 6 Hz), 1.05, 1.48, 1.60 (each 3 H, *s*), 3.70 (1 H, *m*), 3.87 (1 H, *m*). $^{13}$C NMR($C_5D_5N$): $\delta$15.2 (Me), 19.5 (Me), 23.6 (Me), 24.3 (Me), 25.7 (C-11 or C-12), 25.8 (C-12 or C-11), 32.3 (−$CH_2$−), 37.3 (C-10), 40.3 (−$CH_2$−), 40.5 (−$CH_2$−), 46.2 (C-1 or C-13), 46.7 (C-4 or C-8), 48.1 (C-8 or C-4), 48.7 (C-13 or C-1), 51.6 (C-15), 68.6 (C-6), 79.7 (C-16), 85.0 (C-3), 88.8 (C-5 or C-9), 95.9 (C-9 or C-5). MS *m/e* 336 ($M^+$), 318 ($M^+$−$H_2O$), 300 ($M^+$−2$H_2O$).

*Mercuric acetate treatment of* G-XVIII (**4**). To a stirred soln of **4** (47 mg) in THF (2 ml) and $H_2O$ (2 ml) was added Hg $(OAc)_2$ (50 mg) and the mixture was allowed to stand 9 hr at room temp. The ppt. was filtered off and the filtrate was evapd *in vacuo*. $H_2O$ was added to the residue and the mixture extracted with EtOAc. The EtOAc layer was purified by Si gel prep. TLC ($CHCl_3$–MeOH, 9:1) and silanized Si gel prep. TLC (MeOH–$H_2O$, 1:1), giving the diene **8** (11 mg). Mp 232° (EtOAc). (Found: C, 70.97; H, 9.82. Calc. for $C_{20}H_{30}O_3$. MeCOOEt: C, 70.90; H, 9.42%).

*Mercuric acetate treatment of* **9**. Compound **9** (200 mg) was treated with $Hg(OAc)_2$ (200 mg) in THF (2 ml) and $H_2O$ (2 ml) according to the procedure described above and the product (**10**) was worked up in the same way. Mp 214–216° (EtOAc). [Found: C, 61.25; H, 7.14. Calc. for $C_{36}H_{50}O_{14}$: C, 61.17; H, 7.13%). IR $\nu_{max}^{KBr}$cm$^{-1}$: 3455, 1745, 1710, 1240. $^1$H NMR ($CDCl_3$): $\delta$0.83, 1.07, 1.37 (each 3 H, *s*), 1.98–2.08 (15 H, 5 × OAc), 4.44 (1 H, *d*, $J$ = 8 Hz).

*Hydrolysis of* **10**. Compound **10** (180 mg) was added to a 5% KOH–MeOH soln (2 ml) and the mixture left 30 min at room temp. An equal vol. of $CHCl_3$ was added and the mixture chromatographed on a Si gel column ($CHCl_3$–MeOH, 1:1) to remove the inorganic compounds 137 mg of amorphous solid (**11**) was obtained. $^1$H NMR ($C_5D_5N$): $\delta$0.97, 1.46, 1.49 (each 3 H, *s*), 2.01 (3 H, *s*), 4.74 (1 H, *d*, $J$ = 7 Hz), 4.98, 5.04 (each 1 H, *s*).

*Hydrolysis of* **11** *with naringinase*. To a soln of **11** (78 mg) dissolved in HOAc–NaOAc (pH 4.1, 5 ml) was added crude naringinase 'SANKYO' (100 mg) and the mixture incubated at 37°. After 9 hr 80 mg of the enzyme was added and the incubation was continued another 5 days. The soln was extracted with EtOAc and purified by Si gel prep. TLC ($CHCl_3$–MeOH, 19:1) to give the aglycone (**12**) (20 mg). IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3500, 3425, 1735, 1655, 1245. $^1$H NMR ($CDCl_3$): $\delta$0.82, 1.21, 1.38, 2.10 (each 3 H, *s*), 3.59 (1 H, *dd*, $J$ = 4 and 12 Hz), 4.89 (1 H, *dd*, $J$ = 2 and 4 Hz), 5.07, 5.16 (each 1 H, *d*, $J$ = 2 Hz). MS *m/e* 376 ($M^+$), 358 $M^+$−$H_2C$), 316 ($M^+$−HOAc).

*Hydrolysis of* **12** *with alkali*. A soln of **12** (35 mg) in 5% KOH–MeOH (3 ml) was refluxed for 1 hr, diluted with $H_2O$ (3 ml) and extracted with EtOAc. CC on Si gel ($CHCl_3$–MeOH, 9:1) gave **3** (25 mg). Mp 235–237° (EtOAc). (lit. mp 198–199° [5]). Found: C, 72.05; H, 8.99. Calc. for $C_{20}H_{30}O_4$: C, 71.82; H, 9.04%). IR $\nu_{max}^{KBr}$cm$^{-1}$: 3495, 3385, 3310, 1657, 887. $^1$H NMR ($C_5D_5N$): $\delta$1.01, 1.48, 1.62 (each 3 H, *s*), 3.75 (1 H, *d*, $J$ = 6 Hz), 3.93 (1 H, *m*), 5.07, 5.14 (each 1 H, *d*, $J$ = 2 Hz). Compound **3** was identified with the authentic G-XV by Si gel TLC ($CHCl_3$–MeOH, 9:1), silanized Si gel TLC (MeOH–$H_2O$, 1:1) and IR.

*Acetylation of* **3**. Treatment of **3** (14 mg) with $Ac_2O$ (1 ml) and pyridine (1 ml) for 40 hr at 80–90° gave a triacetate. It was purified by Si gel prep. TLC ($C_6H_6$–EtOAc, 9:1) to yield an amorphous solid (**13**) (10 mg). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1730, 1250. $^1H$ NMR ($CDCl_3$): $\delta$ 0.88, 1.10, 1.62 (each 3 H, *s*), 1.95, 1.97, 2.10 (each 3 H, *s*), 4.75–4.85 (2 H, *m*), 4.95, 5.07 (each 1 H, *d*, $J$ = 2 Hz). MS *m/e* 400 ($M^+$–HOAc), 340 ($M^+$ − 2HOAc), 280 ($M^+$ − HOAc). The IR spectrum was nearly identical with that of the authentic triacetyl-G-XV.

*Hydrogenation of* **3**. Compound **3** (45 mg) was dissolved in 10 ml of EtOH and hydrogenated with 40 mg of $PtO_2$ catalyst until no more $H_2$ was absorbed. The catalyst was removed by filtration and the solvent was evapd *in vacuo* to leave 44 mg of dihydro derivative. Mp 202–205° (EtOAc). Mmp (with **2**) 202–205°. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3485, 3325, 1040; identical to the spectrum of **2**. $^{13}C$ NMR ($C_5D_5N$): $\delta$ 15.3, 19.5, 23.7, 24.4, 25.8, 25.8, 32.3, 37.3, 40.3, 40.6, 46.2, 46.7, 48.1, 48.7, 51.6, 68.6, 79.7, 85.0, 88.8, 95.9.

*Hydrogenation of* G-II (**6**). Hydrogenation of **6** was carried out in the same way as described in the literature [8, 9] to give **14** and **15**. $^1H$ NMR ($C_5D_5N$) of **14**: $\delta$ 0.85, 1.32, 1.51 (each 3 H, *s*), 0.98 (1 H, *d*, $J$ = 6 Hz), 3.84 (1 H, *dd*, $J$ = 2 and 7 Hz), 4.30 (1 H, *s*), 4.34 (1 H, *m*). $^1H$ NMR ($C_5D_5N$) of **15**: $\delta$ 1.15, 1.49, 1.61 (each 3 H, *s*), 1.45 (1 H, *d*, $J$ = 8 Hz), 3.82 (1 H, *d*, $J$ = 4 Hz), 4.20 (1 H, *s*), 4.34 (1 H, *m*).

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