Grayanane Diterpenoids from the Flowers of *Rhododendron molle* with Cytotoxic Activity against a *Spodoptera frugiperda* Cell Line

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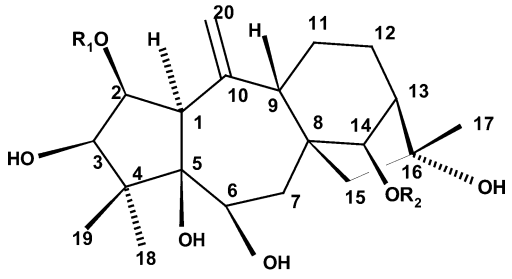
Two new grayanane diterpenoids, rhodomolins A (**1**) and B (**2**), together with two known diterpenoids, rhodomolleins I and rhodojaponin III, were isolated from the flowers of *Rhododendron molle*. The structures of **1** and **2** were elucidated on the basis of interpretation of spectroscopic data. All compounds were evaluated for cytotoxic activity against the *Spodoptera frugiperda* cell line Sf-9 and gave IC_{50} values in the range 12−80 μg/mL.

Rhododendron molle G. Don (Ericaceae), a well-known Chinese medicinal plant, is distributed widely in the southern regions of the People's Republic of China. It has long been used for insecticidal and medicinal purposes.[1] More than 20 diterpenoids, including rhodojaponins II, III, V, and VI, rhodomolleins I, II, III, IX, X, XI, XII, XIII, XIV, XV, XVI, XVII, XVIII, XIX, and XX, grayanotoxins II and III, and kalmanol, have been isolated from the fruits, flowers, and leaves of this plant.[2−10] Rhodojaponin III, a grayanane diterpenoid, has been demonstrated as the major insecticidal and medicinal component in this plant. It has been used successfully in the treatment of rheumatism, hypertension, and malaria in animal experiments.[11,12] It has been tested and shown to be a strong antifeedant, toxicant, or insect growth regulator against *Leptinotarsa decenlineata* (Say), *Spodoptera frugiperda* (J. E. Smith), *Tribolium confusum* (Jacquelin du Val), *Spodoptera litura* (Fab), *Liromyza sativae* (Blanchard), *Spodoptera exigua* (Hubner), and *Ostrinia furnacalis* (Guenea).[13−21] Although a number of other constituents have been identified from *R. molle*, their cytotoxic potency has not been reported.[13] The promising biological activity of rhodojaponin III encouraged us to continue investigating the insecticidal constituents and activity of *R. molle*. In the course of our study, two new grayanane diterpenoids, rhodomolins A (**1**) and B (**2**), were isolated along with two known diterpenoids,



1 R_1=Me R_2=H

2 R_1=H R_2=Ac

rhodomollein I[2] and rhodojaponin III.[6] The isolation and structural elucidation of **1** and **2** and the results of the cytotoxicity evaluation of these compounds are the subjects of this paper.

The MeOH percolate of powdered and dried *R. molle* flowers was fractionated with CH_2Cl_2 and EtOAc. The EtOAc-soluble fraction, which showed insecticidal activity, was subjected to repeated column chromatography on silica gel, Sephadex LH-20, and ODS to give two new diterpenoids, rhodomolins A (**1**) and B (**2**), along with the known rhodomellein I[2] and rhodojaponin III,[6] which were identified by comparison of their spectral data with literature values.

Rhodomolin A (**1**) was assigned a molecular formula of $C_{21}H_{34}O_6$ on the basis of the HRFABMS of a sodium adduct. NMR data (^{1}H,^{13}C, and DEPT) were consistent with the HRFABMS analysis. The ^{1}H NMR spectrum (Table 1) showed signals for three tertiary methyls (δ 1.34, 1.08, and 1.06), an olefinic methylene (δ 5.09 and 5.03), four oxygenated methines (δ 4.05, 4.00, 3.73, and 3.53), and a methoxyl group (δ 3.39). The ^{13}C NMR and DEPT spectra (Table 1) indicated the presence of three methyls (one oxygenated), five methylenes (one olefinic), seven methines (four oxygenated), and five quaternary carbons (two oxygenated and one olefinic). The ^{1}H−^{1}H COSY spectrum of **1** revealed spin-systems of −CHCH(OR)CH(OR)−, −CH(OR)CH_2−, and −$CHCH_2CH_2$CH(OR)−. These structural features were in accord with a $\Delta^{10(20)}$-grayanane diterpene with six oxygen-bearing carbons. Comparison of the ^{1}H and ^{13}C NMR data of **1** with those of rhodomollein I [2] showed that these compounds were very similar, except for the signals of the methoxyl group and the chemical shifts of H-2, C-2, C-3, and C-4. The signals of a methoxyl group were present in the ^{1}H and ^{13}C NMR spectra of **1** while absent in those of rhodomollein I. The C-2 signal of **1** (δ 94.8) was shifted downfield by 11.7 ppm in comparison with that of rhodomollein I (δ 83.1), while the signals of H-2 (δ 3.73), C-3 (δ 85.3), and C-4 (δ 49.2) of **1** were at higher field than those of rhodomollein I. All these data suggested that **1** is the 2-methyl ether of rhodomollein I. The placement of the methoxyl group at C-2 in **1** was supported by the HMBC spectrum (Table 1) in which the correlations between H-2 and that at C-2 were observed and the NOE interaction between MeO-2 and H-2 in the NOESY spectrum of **1** (Figure 1). The remaining carbons and protons were assigned by analysis of further ^{1}H, ^{13}C NMR, ^{1}H−^{1}H COSY, HMQC, and HMBC data and by comparison with the NMR spectral data of rhodomollein I.[2] Thus the structure of **1**

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Table 1. ^{1}H and ^{13}C NMR Data and HMBC Correlations of Compounds **1** and **2** (in CD_3OD)[a]

	1			**2**		
position	δ_H	δ_C	HMBC[b]	δ_H	δ_C	HMBC[b]
1	2.78 d (7.6)	52.8 d	C-5, C-2, C-20, C-10	2.53 d (9.2)	51.2 d	C-4, C-6, C-5, C-2, C-20, C-10
2	3.73 dd (7.6, 1.6)	94.8 d	CH_3O, C-3, C-10	4.09 dd (9.2, 4.0)	82.9 d	C-1, C-3, C-10
3	3.53 d (1.6)	85.3 d	C-2, C-19, C-5	3.43 d (4.0)	88.3 d	C-19, C-2, C-5
4		49.2 s			56.5 s	
5		84.0 s			82.5 s	
6	4.05 dd (8.8, 2.4)	69.8 d	C-8	3.68 br d (8.8)	71.3 d	
7α	1.97 dd (14.8, 9.2)	41.4 t		1.96 dd (14.4, 10.4)	39.7 t	
7β	1.81 br d (14.8)		C-8, C-15, C-6, C-5, C-14, C-9	1.54 dd (14.4, 2.0)		C-5, C-6, C-8, C-14, C-15
8		50.4 s			49.0 s.	
9	1.97 t (4.2)	54.1 d	C-12, C-11, C-7, C-8	2.68 br t (8.4)	52.6 d	C-12, C-1, C-15, C-14, C-20, C-10
10		150.8 s			148.2 s	
11α	1.83 m	24.8 t	C-8	1.68 m	23.8 t	
11β	1.66 m			1.44 m		
12α	1.66 m	24.8 t	C-11	1.98 m	24.7 t	C-13, C14, C-16
12β	1.51 m			1.89 m		
13	2.76 m	54.1 d		2.08 d (5.6)	52.6 d	C-11, C-12, C-8, C-16
14	4.00 br s	81.3 d	C-15, C-16	5.18 br s	82.5 d	C-12, C-15, C-16, C=O
15α	2.02 d (13.6)	61.0 t		2.11 d (13.2)	62.0 t	
15β	1.98 d (13.6)		C-7, C-8, C-9, C-14, C-13, C-16	2.08 d (13.2)		C-7, C-8, C-16
16		82.7 s			80.4 s	
17	1.34 s	24.5 q	C-13, C-15, C-16	1.39 s	26.9 q	C-13, C-15, C-16
18	1.06 s	18.6 q	C-3, C-4, C-5	1.11 s	19.4 q	C-19, C-4, C-3, C-5
19	1.08 s	24.5 q	C-3, C-4, C-5	1.07 s	27.5 q	C-18, C-4, C-3, C-5
20α	5.03 br s	113.1 t	C-1	4.99 br s	114.5 t	C-1, C-9
20β	5.09 br s			5.07 br s		
OMe	3.39	58.1 q	C-2		21.3 q	
OAc				2.09 s	173.1 s	C=O

[a] ^{1}H at 400 MHz; ^{13}C at 100 MHz. Coupling constants (parentheses) are given in Hz. [b] ^{1}H signal correlating with ^{13}C resonance indicated.

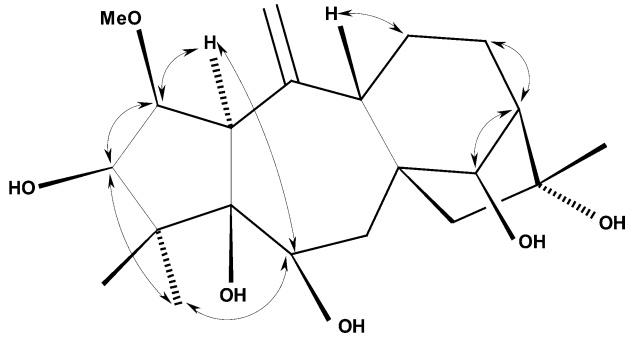

Figure 1. Selected NOESY correlations for **1**.

was characterized as 3β,5β,6β,14β,16α-pentahydroxy-2β-methoxygrayan-10(20)-ene. This is the first example of a grayanane diterpene methyl ether.

Rhodomolin B (**2**) was assigned the molecular formula $C_{22}H_{34}O_7$ on the basis of the HRFABMS of a sodium adduct. NMR data (^{1}H, ^{13}C, and DEPT) were consistent with the HRFABMS analysis. The IR spectrum of **2** showed absorptions for hydroxyl groups, an ester carbonyl group, and an olefinic bond. The ^{1}H NMR spectrum (Table 1) showed signals for three tertiary methyls (δ 1.39, 1.11, and 1.08), an acetyl methyl (δ 2.09), an olefinic methylene (δ 5.07 and 4.99), and four oxygenated methines (δ 5.18, 4.09, 3.68, and 3.43). The ^{13}C NMR and DEPT spectra (Table 1) revealed, apart from the acetyl group (δ 21.3, 173.1), 20 carbons, including three methyls, four methylenes (one olefinic), eight methines (four oxygenated and one olefinic), and five quaternary carbons (one oxygenated and two olefinic). The ^{1}H−^{1}H COSY spectrum of **2** revealed the structural fragments −CHCH(OH)CH(OH)−, −CH(OH)CH_2−, and −CH-CH_2CH_2CH−, in accord with the proton sequence from H-1 to H-3, H-6 to H-7, and H-9 to H-14, respectively. The above spectral data suggested that **2** was a $\Delta^{10(20)}$-grayanane diterpene with six oxygen-bearing carbons (C-2, C-3, C-5, C-6, C-14, and C-16). Comparison of ^{1}H and ^{13}C NMR spectral data of **2** with those of rhodomellin I (Table 1) showed that both compounds were quite similar except for the data for H-14 and C-14.[2] The H-14 signal was shifted downfield (δ 5.18) in **2** compared with that in rhodomellin I (δ 4.11). The C-14 signal of **2** (δ 82.5) was also shifted downfield by 1.8 ppm in comparison with C-14 in rhodomellin I (δ 80.7). Furthermore, in the HMBC spectrum of **2** (Table 1), the carbonyl carbon signal at δ 173.1 correlated with the H-14 signals at δ 5.18, and the proton signal of the acetyl methyl at δ 2.09 in the NOESY spectrum of **2** correlated with the H-14 signal. On the basis of all these spectral data, **2** was established as 2β,3β,5β,6β,16α-pentahydroxy-14-acetylgrayan-10(20)-ene.

The results of the cytotoxicity assay have demonstrated that rhodomolins A (**1**) and B (**2**) possesses cytotoxic activity against the *Spodoptera frugiperda* cell line Sf-9. The IC_{50} (inhibition median concentration) values were 37.8, 25.6, and 80.4 μg/mL for rhodomolins A (**1**) and B (**2**) and rhodomollein I against Sf-9 48 h after treatment (Table 2). In addition, rhodojaponin III and azadirachtin, a well-known botanical insecticidal ingredient isolated *Azadirachta indica* A. Juss, also displayed significantly cytotoxicity toward Sf-9 with IC_{50} values of 12.6 and 20.3 μg/mL, respectively (Table 2).

Experimental Section

General Experimental Procedures. The optical rotations were obtained on a SchMDF Haensch Poloptronic HNQWS polarimeter with MeOH as solvent. IR spectra were recorded in KBr with a WQF-410 FT-IR spectrophotometer. ^{1}H (400 MHz,CD_3OD), ^{13}C (100 MHz, CD_3OD), and 2D NMR spectra were recorded using a Bruker DRX-400 instrument. Chemical shifts are reported in ppm (δ) with solvent (CD_3OD) signals used as internal standards (the signal of CD_3OD at δ 3.30 for the ^{1}H NMR data and a triplet centered at δ 49.0 for the ^{13}C NMR data). EIMS were measured with a Micromass Platform

Table 2. Cytotoxicity of Compounds **1**, **2**, Rodomellin I, Rhodojaponin III, and Azadirachtin against the *S. frugiperda* Sf-9 Cell Line

	1	**2**	rhodomollein I	rhodojaponin III	azadirachtin[a]
IC_{50} (μg/mL)	37.8 ± 1.4	25.6 ± 0.7	80.4 ± 3.2	12.6 ± 1.1	20.3 ± 1.2

[a] Positive control substance.

EI 200 GC/MS instrument at 70 eV by direct inlet. HRFABMS were recorded on a VG Auto Spec-3000 mass spectrometer in the positive-ion mode using glycerol as the matrix. For column chromatography, silica gel 60 (100−200 mesh, Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China), ODS (Developsit C_{18}), and Sephadex LH-20 (Pharmacia Biotech) were used. TLC (Kieselgel 60 GF_{254}, Merck) was performed on precoated plates with detection effected by UV light (254 nm), I_2 vapor, and concentrated sulfuric acid, respectively.

Plant Material. Flowers of *Rhododendron molle* G. Don were collected in Maba Town, Qujiang County, Guangdong Province, People's Republic of China, in April 2000, and identified by one of the authors (M.H.). A voucher specimen (No. 10201) of *R. molle* is growing in the insecticidal plant garden of the South China Agricultural University.

Extraction and Isolation. A 3.1 kg sample of the air-dried flowers of *R. molle* were ground and extracted by percolation with MeOH (3 × 10 L) at room temperature. The MeOH extracts were concentrated in vacuo to obtain a deep black syrup (280 g). This extract was suspended in H_2O and sequentially extracted three times each with CH_2Cl_2 and ethyl acetate. The combined EtOAc extracts (38 g) were chromatographed on a silica gel column (100−200 mesh) and utilizing a $CHCl_3$−MeOH gradient to afford 38 fractions. Fractions A1−A5 obtained with $CHCl_3$−MeOH (90:10) were combined after monitoring by TLC, and fraction A3 was further separated by a silica gel 60 column using $CHCl_3$−MeOH (95:5) as eluent to give five further fractions, B1−B5. Fraction B1 was separated on an ODS column with MeOH−H_2O (4:6) and purified on Sephadex LH-20 with absolute MeOH as eluent to yield rhodomolin A (**1**, 10.8 mg). Using the same method, fraction B3 was separated on an ODS column with MeOH−H_2O (4:6) to yield rhodomollein I [2] (11.2 mg) and with MeOH−H_2O (3:7) to yield rhodomolin B (**2**, 10.8 mg). Fraction B2 was further separated by a silica gel 60 column to give rhodojaponin III[6] (1.85 g).

Rhodomolin A (1): colorless oil; $[\alpha]^{24}_D$ −13.1° (*c* 0.11, MeOH); IR (KBr) ν_{max} 3384 (br, OH), 2937, 1446, 1373 (m, CH, CH_2, CH_3), 1255, 1095, 883, 802 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD), see Tables 1 and 2; EIMS *m/z* 382 (M^+, 3), 364 (5), 346 (11), 314 (32),119 (91); HRFABMS *m/z* 405.2243 $[M + Na]^+$ (calcd for $C_{21}H_{34}O_6Na$, 405.2253).

Rhodomolin B (2): colorless crystal; mp 282−284 °C; $[\alpha]^{24}_D$ −9.0° (*c* 0.17, MeOH); IR (KBr) ν_{max} 3407 (br, OH), 2939, 1710, 1450, 1377 (m, CH, CH_2, CH_3), 1249, 1037, 887, 810 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD), see Table 1; EIMS *m/z* 410 (M^+, 3), 374 (3), 314 (40), 290 (45), 248 (50), 230 (70), 212 (48), 119 (85), 69 (68); HRFABMS *m/z* 433.2210 $[M + Na]^+$ (calcd for $C_{22}H_{34}O_7Na$, 433.2202).

Cytotoxicity Assay. A *S. frugiperda* cell line Sf-9 was kindly provided by Institute of Entomology, Zhongshan University (Guangzhou, 510275, People's Republic of China) and propagated in Grace medium (Gibco Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Laboratories). Cells in the logarithmic phase were cultured at a density of 1×10^4 cells/0.1 mL per well in a 96-well plate. The cells were exposed to various concentrations of each test compound for 48 h. The microculture tetrazolium assay was used to evaluate the effects on cell growth, as described previously.[22] The IC_{50} value resulting from 50% inhibition of cell growth was calculated graphically in a comparison with the control.

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