JOURNAL OF NATURAL PRODUCTS

ARTICLE

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Ferruginenes A−C from *Rhododendron ferrugineum* and Their Cytotoxic Evaluation

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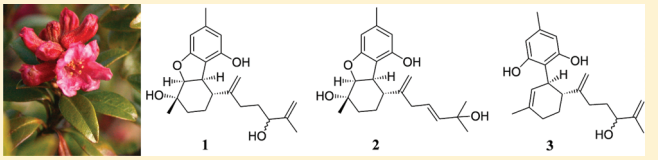
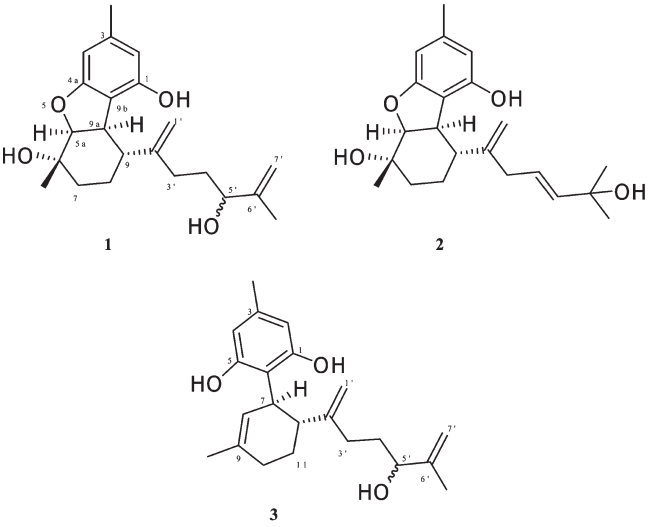
Supporting Information

ABSTRACT: Three new compounds, ferruginenes A (**1**) and B (**2**) and a mixture of C-5′(*R*) and C-5′(*S*) ferruginene C (**3**) diastereomers, have been isolated from a cytotoxic chloroform-soluble fraction of the leaves of *Rhododendron ferrugineum* together with 12 known compounds. The structures of these new compounds were elucidated by analyses of NMR spectroscopic and mass spectrometric data. Compounds **1**−**3** were tested for their cytotoxicity against three human cancer cell lines, namely, HL-60, HeLa-S3, and MCF-7.



Rhododendron ferrugineum L. (Ericaceae), an evergreen shrub that grows on acid soil just above the tree line in the Pyrenees and the Alps, is one of the few rhododendron species occurring in Europe.[1] An aqueous decoction of the leaves has been used in traditional medicine against ailments such as rheumatism, hypertension, neuralgia, and myalgia.[1,2] Previous phytochemical research led to the isolation of phloracetophenones,[3] phloracetophenone glucosides,[3] dihydroflavonol glycosides,[4] and flavonoid glycosides[5] from the flowers and the leaves of this species. *Rhododendron* species are known as toxic plants, causing intoxications in animals and poisoning in humans by contaminating honey. The latter is characterized by gastrointestinal tract symptoms, hypotension, and bradycardia and is attributed to the grayanotoxin polyhydroxylated diterpenes.[6] Although the toxicity of *R. ferrugineum* in animals has been reported, there are no data regarding its effects in humans. Recently, Louis and co-workers have shown that an aqueous extract of *R. ferrugineum* exerts no negative influence on cell activity.[5] However, a toxic effect of this species is feasible, at least in part due to the possible presence of grayanotoxins. On the basis of the toxicity of *Rhododendron* species, *R. ferrugineum* indicates a potential source of cytotoxic compounds. Hence, the present work is aimed at investigating the cytotoxicity of the nonpolar extract of *R. ferrugineum* and its components.

The cytotoxicity of the chloroform-soluble fraction of *R. ferrugineum* was demonstrated, and the bioassay-guided isolation and characterization of compounds responsible for the cytotoxicity of the plant conducted. We report herein the purification and structure elucidation of the new compounds **1**−**3**, as well as the isolation of 12 known compounds. Compounds **1**−**3** were evaluated for their cytotoxicity against a small panel of human cancer cell lines.



RESULTS AND DISCUSSION

The chloroform-soluble fraction of the leaves of *R. ferrugineum* showed cytotoxic activity for the HL-60 cell line at a concentration of 10 μg/mL after 24 h, decreasing cell viability to ∼1% (data not shown). Since the toxicity of *Rhododendron* species has been attributed to the presence of grayanane diterpenes, the contribution of grayanotoxins to the cytotoxicity of the chloroform-soluble fraction of *R. ferrugineum* was investigated. TLC

Received: October 27, 2010
Published: March 28, 2011

dx.doi.org/10.1021/np100778k | *J. Nat. Prod.* 2011, 74, 712–717

Table 1. NMR Spectroscopic Data for Compounds 1 and 2 in $CDCl_3$

	ferruginene A (**1**)				ferruginene B (**2**)			
position	δ_C	mult	δ_H (J in Hz)	HMBC	δ_C	mult	δ_H (J in Hz)	HMBC
1	151.87	C			151.82	C		
2	109.98	CH	6.23 (s)	1,4,CH_3-3	109.87	CH	6.25 (s)	1,4,CH_3-3
3	139.57	C			139.69	C		
4	103.80	CH	6.27 (s)	2,9b,CH_3-3	103.95	CH	6.27 (s)	2,9b,CH_3-3
4a	159.97	C			160.26	C		
5a	89.48	CH	4.10 (d, 5.6)	6,7,9,CH_3-6	89.45	CH	4.10 (d, 5.6)	6,7,9,CH_3-6
6	69.21	C			69.19	C		
7	34.93	CH_2	1.71−1.73 (m)	5a,CH_3-6	34.90	CH_2	1.68−1.72 (m)	5a,CH_3-6
8	26.92	CH_2	1.52−1.61 (m)		27.07	CH_2	1.58−1.63 (m)	
9	47.28	CH	1.80 (m)	5a	45.31	CH	1.83 (dt, 11.4, 11.4, 3.7)	1′,3′,5a
9a	42.67	CH	3.35 (dd, 10.8, 5.6)	4a,9b	42.35	CH	3.35 (dd, 11.4, 5.6)	4a,9b
9b	116.22	C			116.49	C		
1′	109.44	CH_2	5.21 (s)		110.37	CH_2	5.13 (s)	3′,9
			5.09 (s)	3′			5.24 (s)	3′,9
2′	157.44	C			157.12	C		
3′	32.49	CH_2	1.52−1.61 (m)	1′	40.73	CH_2	2.80 (dd, 14.7, 6.2)	1′,2′,4′,5′,9
							2.66 (dd, 14.7, 8.4)	1′,2′,4′,5′,9
4′	32.34	CH_2	1.52−1.61 (m)		123.02	CH	5.39 (m)	5′
5′	75.16	CH	4.02 (m)	7′,CH_3-6′	141.31	CH	5.55 (d, 15.5)	3′,4′,7′,6′,CH_3-6′
6′	147.15	C			70.26	C		
7′	111.27	CH_2	4.90 (s)		29.47	CH_3	1.14 (s)[a]	5′,6′,CH_3-6′
			4.81 (s)	5′,CH_3-6′				
CH_3-3	21.57	CH_3	2.24 (s)	2,3,4	21.50	CH_3	2.24 (s)	2,3,4
CH_3-6	28.17	CH_3	1.48 (s)	5a,6,7	28.16	CH_3	1.48 (s)	5a,6,7
CH_3-6′	17.46	CH_3	1.66 (s)	5′,6′,7′	28.96	CH_3	1.19 (s)[a]	5′,6′,7′

[a] Assignments are interchangeable.

analysis showed no detectable grayanotoxin derivatives (i.e., grayanotoxins I−IV, XIV, XVIII, grayanosides A and C, the aglycone of grayanoside C, and kalmitoxin I) in the purified nonpolar extract of the plant. Moreover, assessment of cell viability indicated no significant effect of the grayanotoxin derivatives for HL-60 cells at concentrations of 0.5−25 μM after 24 h (data not shown). Accordingly, it was not considered that grayanotoxins contributed to the cytotoxicity of the chloroform-soluble fraction. Instead, it is suggested that different chemical constituents were responsible for the observed activity. The chloroform-soluble fraction of *R. ferrugineum* was then subjected to bioassay-guided fractionation. Three new compounds, ferruginenes A (**1**), B (**2**), and C (**3**), the latter being obtained as a mixture of C-5′(*R*) and C-5′(*S*) diastereomers, were isolated together with 12 known compounds, namely, a mixture of ursolic and oleanolic acids, a mixture of uvaol and erythrodiol, corosolic acid, 23-hydroxyursolic acid, farrerol, scopoletin, orsellinic acid methyl ester, isoacetovanillone, and a mixture of *p*-anisic and benzoic acids.

Ferruginene A (**1**) was isolated as a brownish-yellow, amorphous solid, $[\alpha]^{20}_D$ +107.7 (*c* 0.052, MeOH). Analysis of its ^{1}H and ^{13}C NMR spectroscopic data revealed signals for 22 carbons and 28 protons (Table 1). The molecular formula of $C_{22}H_{30}O_4$ was determined by HRESIMS, which showed a deprotonated molecular ion at *m/z* 357.2079 $[M - H]^-$ (calcd for $C_{22}H_{29}O_4$ 357.2071). The carbon backbone of **1** was determined using COSY, HSQC, and HMBC NMR spectroscopy (Table 1). Thus, the structure of **1** is based on a 5a,6,7,8,9,9a-hexahydro-1,6-dibenzofuran ring system, which is substituted by a 5-hydroxy-6-methylhepta-1,6-dien-2-yl residue at position C-9. The relative configuration of the four chiral centers in the cyclohexane ring was determined by NOESY spectroscopy. Hence, two cross-peaks were observed from H-5a to H-9a and from H-1′ to H-9a, indicating the *cis* configuration of H-5a, H-9a, and the heptadienyl side chain at position C-9 of the cyclohexane ring. The vicinal coupling constants of protons H-5a, H-9a, and H-9 revealed the axial−equatorial arrangement of H-9a and H-5a ($J_{9a,5a}$ = 5.6 Hz) and the *trans*-diaxial relationship between H-9a and H-9 ($J_{9a,9}$ = 10.8 Hz). Finally, the lack of any NOESY correlation between H-9a and the methyl group at position C-6 indicated the *trans* configuration of these substituents in the cyclohexane ring. Due to the large interatomic distance between the chiral centers of the cyclohexane ring and the asymmetric carbon at position C-5′, it was not possible to elucidate the relative configuration of C-5′ by using NOESY data.

Ferruginene B (**2**), a brownish-yellow, amorphous solid, $[\alpha]^{20}_D$ +96.4 (*c* 0.044, MeOH), was found to possess the same molecular formula as **1**, $C_{22}H_{30}O_4$ (HRESIMS). The ^{13}C and ^{1}H NMR spectroscopic data of **2** were similar to those of **1** (Table 1), indicating that the carbon backbone of **2** is also based on a 5a,6,7,8,9,9a-hexahydro-1,6-dibenzofuran ring system. The constitution of the side chain at position C-9 of the cyclohexane ring is different, although both **1** and **2** have an allylic alcohol moiety. Structure determination by COSY, HSQC, and HMBC

dx.doi.org/10.1021/np100778k |*J. Nat. Prod.* 2011, 74, 712–717

Table 2. NMR Spectroscopic Data for Compound 3 in $CDCl_3$

position	δ_C[a]	δ_C[b]	mult	δ_H (J in Hz)	HMBC
1	153.98	153.98	C		
2	109.20[c]	109.20[c]	CH	6.12 (brs)	
3	137.96	138.00	C		
4	110.60[c]	110.60[c]	CH	6.12 (brs)	
5	153.98	153.98	C		
6	113.79	113.72	C		
7	37.88	38.09	CH	3.86 (d, 10.1)	
8	124.12	124.12	CH	5.55 (brs)	10,12,CH_3-9
9	140.02	140.05	C		
10	30.53	30.55	CH_2	2.25 (m)	
				2.08 (m	
11	29.08	29.19	CH_2	1.83 (m)	
				1.75 (m)	
12	45.23	45.10	CH	2.40 (m)	
1′	109.22	109.16	CH_2	4.73−4.80 (m)	12,3′
2′	153.56	153.56	C		
3′	30.21	31.16	CH_2	1.92−1.96 (m)	2′,4′
4′	32.49	32.63	CH_2	1.51−1.54 (m)	2′,3′,5′,6′
5′	75.21	75.77	CH	3.97 (t, 6.3)[a]	4′,6′,7′,CH_3-6′[a]
				3.92 (t, 6.3)[b]	6′,7[b]
6′	147.19	147.15	C		
7′	110.91	111.13	CH_2	4.86 (s)	5′,6′,CH_3-6′
				4.78 (s)	5′,6′,CH_3-6′
CH_3-3	21.03	21.03	CH_3	2.18 (s)	2,3,4
CH_3-9	23.65	23.65	CH_3	1.78 (s)	8,9,10
CH_3-6′	17.66	17.41	CH_3	1.65 (s)	5′,6′,7′
OH-5′	23.65	23.65	CH_3	5.96 (brs)	

[a] Major diastereomer (60%). [b] Minor diastereomer (40%). [c] Signal observed in HSQC spectrum.

spectroscopy revealed an *E*-configured double bond at positions C-4′ and C-5′ ($J_{4',5}$ = 15.5 Hz) and two methyl groups at the allylic carbon C-6′. These data indicated a rearrangement of the allylic alcohol from C-5′(OH)−C-6′=C-7′ in **1** to C-4′=C-5′−C-6′(OH) in **2**. NOESY spectroscopic studies and measurement of coupling constants ($J_{9a,9}$ = 10.4 Hz, $J_{9a,5a}$ = 5.6 Hz) indicated that **1** and **2** have the same relative configuration at the chiral centers in the cyclohexane ring.

Ferruginene C (**3**) was isolated as a brownish gum and proved to be a mixture of C-5′(*R*) and C-5′(*S*) diastereomers. It was assigned the molecular formula $C_{22}H_{30}O_3$, from the HRESIMS $[M - H]^-$ peak at *m*/*z* 341.2134 (calcd for $C_{22}H_{29}O_3$ 341.2122). The ^{13}C and ^1H NMR spectroscopic data of **3** were found to be similar to those reported for **1** (see Tables 1 and 2), but the constitution of the cyclohexane ring moiety was different. The ^{13}C NMR spectrum of **3** showed signals of an olefinic methine (C-8) and an olefinic quaternary carbon (C-9), while in the ^1H NMR spectrum, a signal was revealed for an olefinic methine proton (H-8) instead of the oxymethine proton present in **1**. These observations suggested that compound **3** has no ether bridge between C-4a and C-5a and no hydroxy group at C-6 as in ferruginenes A (**1**) and B (**2**). The carbon backbone of **3** was confirmed using COSY, HSQC, and HMBC spectroscopy (Table 2). A comparison with data of **1** (Table 1) indicated that the terpenoid side chain was equal in constitution, but that two diastereomers were evident. The proton shifts of the diastereomers of **3** were similar, with the exeption of shifts of H-5′ in major δ_H 3.97 (t, J = 6.3 Hz) and minor δ_H 3.92 (t, J = 6.3 Hz) diastereomers. Thus, the ratio of diastereomers (6:4) could be determined by ^1H NMR spectroscopy. NOESY spectroscopic studies and the measurement of coupling constants ($J_{7,12}$ = 10.1 Hz) indicated a *trans*-diaxial relationship between H-7 and H-12, similar to the results obtained for **1** and **2**.

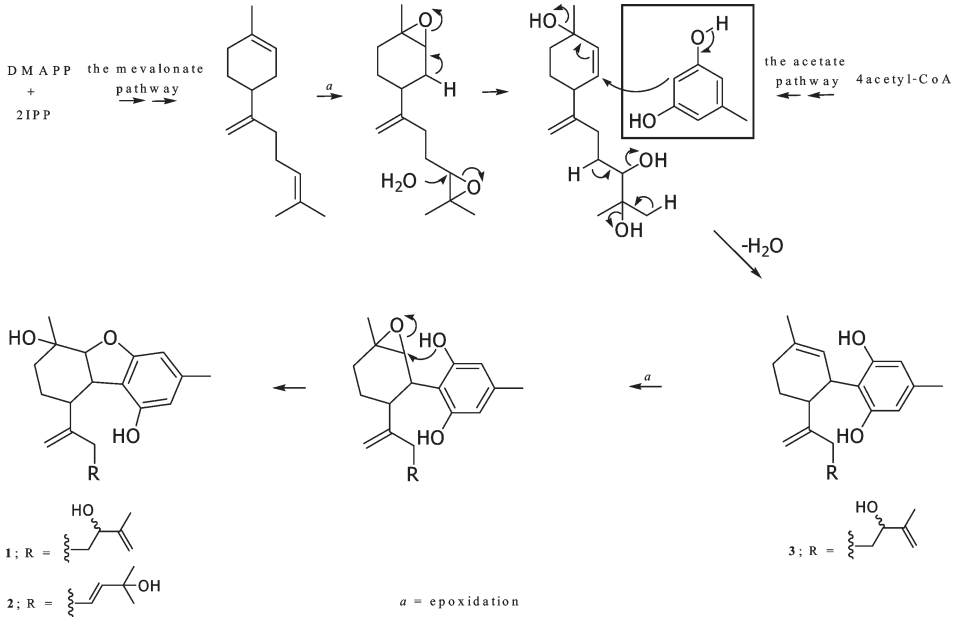
The co-occurrence and the same carbon skeleton of compounds **1** and **3** suggested that **1** was biosynthesized from **3** through epoxidation of the C-5a, C-6 double bond followed by cyclization from one of the phenolic groups to the epoxide ring of **3**. On the basis of this hypothesis, compound **1** should be presented as a mixture of C-5′(*R*) and C-5′(*S*) diastereomers. However, the ^1H and ^{13}C NMR spectroscopic data showed that only a single diastereomer of **1** was isolated. Its diastereomer at the position C-5′ might have been lost during the purification process due to a low amount contained by the fraction. A proposed biosynthetic pathway for the formation of compounds **1**, **2**, and **3** is shown in Scheme 1.

The known compounds were identified on the basis of their spectroscopic data and by the comparison with values of the triterpenoids ursolic, oleanolic, corosolic, and 2,3-hydroxyursolic acids reported in the literature.[7−10] The first three of these compounds and erythrodiol are known secondary metabolites produced by *Rhododendron* species.[11−15] In addition, the isolation of 2,3-hydroxyursolic acid from the stem bark of *Cussonia bancoensis*[16,17] and the leaves of *Lagerstroemia speciosa*[18] has been reported, while farrerol and scopoletin were extracted from the leaves of *R. ferrugineum*[5] and the stems of *R. mucronulatum*,[19] respectively. Corosolic acid has been isolated previously from the leaves of *Prunus zippeliana*, *Eriobotrta japonica*, and *Tiarella polyphylla*.[20−22]

The isolated compounds **1**, **2**, and **3** (mixture of diastereomers) were tested for their cytotoxic activity against the HL-60, HeLa-S3, and MCF-7 cancer cell lines. All compounds gave IC_{50} values of >20 μM for all cell lines, with the exception of compound **3** in the HL-60 cell line (IC_{50} 13.7 μM). Compound **3** provided an IC_{50} value of >20 μM for the HEK-293 mammalian noncancerous cell line, thus suggesting a significant selectivity toward the HL-60 cell line. However, the difference in activity between the two cell lines could reside in a general stronger effect of the compound in suspension than in adherent cultures.

Ursolic, oleanolic, and corosolic acids have been reported to exhibit cytotoxic activity against various cell lines.[23−27] Ursolic acid induced apoptosis via the suppression of NF-kB-mediated activation of Bcl-2 in melanoma cells[28] and the activation of caspase-dependent and -independent pathways in prostate cancer cells.[29] Oleanolic acid caused intracellular calcium increase and apoptosis in human lung adenocarcinoma cells,[30] while corosolic acid triggered apoptosis through the mitochondrial pathway in HeLa cells.[31] Erythrodiol and 23-hydroxyursolic acid demonstrated antiproliferative and proapoptotic activity in human colon cancer and cervical squamous carcinoma cells, respectively.[32,33] The antiproliferative and cytotoxic effect of orsellinic acid methyl ester has been recently reported for K-562 human leukemia and HeLa cells.[34] Scopoletin indicated significant cytotoxic activity with the induction of apoptosis in several cancer cell lines.[35−37] In HL-60 cells, scopoletin caused programmed cell death via NF-kB activation and caspase-3 cleavage.[38] Thus, the cytotoxic activity of the extract for the HL-60 cells is due to a cumulative effect of the isolated antiproliferative substances including ursolic, 23-hydroxyursolic, oleanolic, and corosolic acids, orsellinic acid methyl ester,

Scheme 1. Proposed Biosynthetic Pathway for Compounds 1, 2, and 3



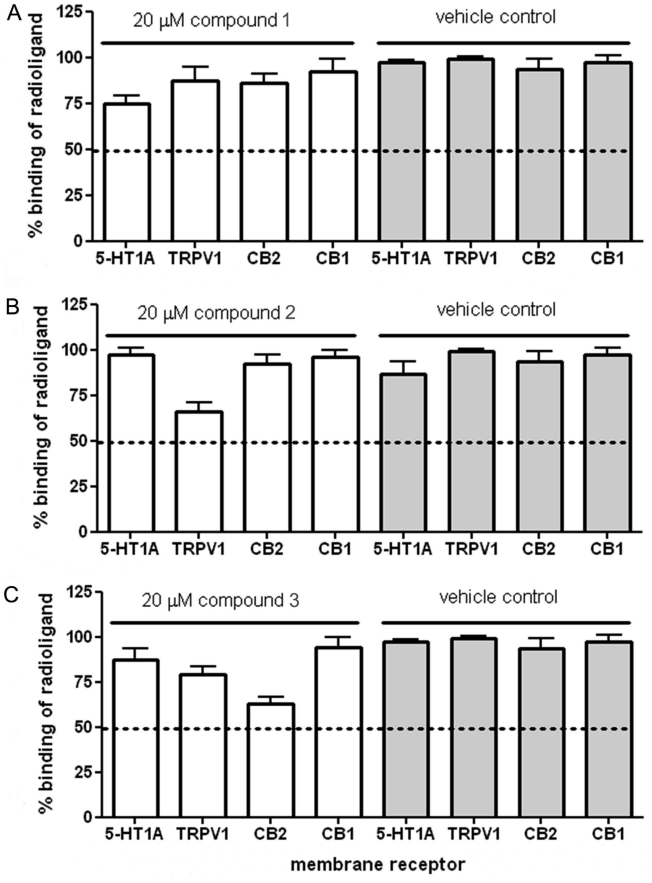


Figure 1. Displacement of selective radioligands from different membrane proteins (serotonin $5HT_{1A}$ receptor, transient receptor potential vanilloid-1 (TRPV1), cannabinoid type-2 receptor (CB_2), cannabinoid type-1 receptor (CB_1)) by compounds (A) **1**, (B) **2**, and (C) **3**. Data represent mean values ± SEM of three independent experiments.

erythrodiol, and scopoletin. In addition, benzoic acid showed genotoxic effects in human peripherial blood lymphocytes[39] and frequent mitotic and chromosomal aberrations in the root tips of *Allium sativum*,[40] hence, pointing to the genotoxic potential of the extract.

Compounds **1**−**3** share structural similarities with the class of cannabinoids. Consequently, their interaction was investigated with receptors that are targeted by endo-, phyto-, and synthetic cannabinoids. Neither of the compounds showed significant affinity to the major radioligand CB_1, CB_2, $5\text{-}HT_{1A}$, and TRPV1 receptor binding site at a concentration of 20 μM (Figure 1). However, compound **2** and the mixture of **3** indicated the tendency to interact weakly with the TRPV1 and the CB_2 receptor, respectively.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined with a Perkin-Elmer digital polarimeter. UV−visible spectra were measured on a Beckman DU 640 spectrophotometer. NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 500 spectrometer at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR). HRESIMS were obtained on an ESI-Qq-TOF mass spectrometer (micrOTOF-Q II, Bruker Daltonics). For all compounds, the given sum formula was ranked #1 by the SmartFormula algorithm based on the mass accuracy and True Isotope Pattern analysis. Positive- and negative-ion mode $ESIMS^n$ were obtained on a 3D-ion trap mass spectrometer (HCT, Bruker Daltonics). For the cell viability assay, a Vi-Cell cell viability analyzer was used. Grayanotoxin III was purchased from Enzo Life Sciences GmbH (Lorrach, Germany). All other grayanotoxin derivatives were kindly provided by Prof. Raymond W. Doskotch.[41]

Plant Material. The leaves of *R. ferrugineum* were collected from Arlberg, Austria, in July 2008 and identified by Dr. Anton Russell, Department of Systematic and Evolutionary Botany, University of Vienna. The voucher specimen was deposited at the Herbarium of the Department of Pharmacognosy, University of Vienna (acronym WUP, s.n., http://sciweb.nybg.org/science2/IndexHerbariorum.asp). The plant material was dried at room temperature and then powdered.

dx.doi.org/10.1021/np100778k |*J. Nat. Prod.* 2011, 74, 712–717

Extraction and Isolation. The leaves of *R. ferrugineum* were extracted according to the method described by Wall et al.[42] Briefly, the air-dried, powdered leaves (1 kg) were extracted with MeOH (1 L for 100 g) to obtain a crude MeOH extract, which was then dissolved in MeOH−H_2O and defatted by partition with petroleum ether. Next, the MeOH−H_2O layer was diluted with H_2O and further partitioned with $CHCl_3$. The collected $CHCl_3$ layer was washed with 1% aqueous NaCl and then evaporated under reduced pressure to give a crude chloroform-soluble fraction (283 g).

An aliquot (24 g) of the chloroform-soluble fraction that showed cytotoxic activity against HL-60 cancer cells was subjected to vacuum-liquid chromatography (VLC) ($CHCl_3$−MeOH−H_2O, 70:22:3.5), to obtain seven main fractions, A1 to A7. Based on the observed cytotoxic activity, the active fraction A1 (0.9 g) was selected and purified by column chromatography (CC) on silica gel (CH_2Cl_2−EtOAc, 100:0 to 0:100, and then EtOAc−MeOH, 100:0 to 80:20), and three main subfractions, A1.1, A1.2, and A1.3, were collected. A mixture of compounds uvaol and erythrodiol (colorless powder; 10.8 mg) was obtained from subfraction A1.1 by CC on silica gel (CH_2Cl_2−EtOAc, 100:0 to 80:20) followed by Sephadex LH-20 (CH_2Cl_2−MeOH, 20:80). Subfraction A1.2 was first fractionated over a Sephadex LH-20 column (CH_2Cl_2−MeOH, 20:80), and the collected fraction was further purified by CC on silica gel (CH_2Cl_2−EtOAc, 100:0 to 80:20), resulting in a 1:1 mixture of ursolic and oleanolic acids (colorless powder; 64 mg) and the pure compounds **1** (brownish-yellow, amorphous solid; 4.4 mg) and **2** (brownish-yellow, amorphous solid; 5.8 mg). Subfraction A1.3 was fractionated on a Sephadex LH-20 column (CH_2Cl_2−MeOH, 20:80), and the collected fraction was further purified by silica gel CC to yield a mixture of ursolic and oleanolic acids (70 mg). Active fraction A2 (18 g) was separated using VLC (CH_2Cl_2−EtOAc, 100:0 to 0:100, and then EtOAc−MeOH, 100:0 to 80:20), and three main subfractions, A2.1, A2.2 (5 g; mostly contained a mixture of ursolic and oleanolic acids), and A2.3, were obtained. Subfraction A2.1 (2 g) was separated by CC on a Sephadex LH-20 (CH_2Cl_2−MeOH, 20:80) to provide three subfractions, A2.1.1, A2.1.2, and A2.1.3. The mixture of **3** diastereomers (brownish gum; 60 mg) and the mixture of uvaol and erythrodiol (27 mg) were isolated from subfraction A2.1.1 using silica gel (CH_2Cl_2−EtOAc, 100:0 to 0:100) followed by reversed-phase C_{18} CC (MeOH−H_2O, 95:5). Orsellinic acid methyl ester (light yellow, amorphous solid; 160 mg), scopoletin (light yellow powder; 2 mg), farrerol (yellow powder; 7 mg), isoacetovanillone (yellow powder; 8 mg), and a mixture of *p*-anisic and benzoic acids (light yellow, amorphous solid; 30 mg) were obtained from subfraction A2.1.2 using CC on silica gel (hexane−CH_2Cl_2, 30:70, to CH_2Cl_2−EtOAc, 100:0 to 60:40). Subfraction A2.1.3 was fractionated by silica gel CC (hexane−CH_2Cl_2, 30:70, to CH_2Cl_2−EtOAc, 100:0 to 60:40) to yield orsellinic acid methyl ester (5 mg) and farrerol (61 mg). Corosolic acid (white, amorphous solid; 14 mg) and 23-hydroxyursolic acid (white, amorphous solid; 4.5 mg) were isolated from subfraction A2.3 (1.9 g) by passage over silica gel ($CHCl_3$−MeOH−H_2O, 90:3.5:0.2) followed by reversed-phase C_{18} CC (MeOH−H_2O, 95:5).

TLC analysis for the comparison of the chloroform-soluble fraction of *R. ferrugineum* with the grayanotoxin derivatives (100 μg each) used silica gel ($CHCl_3$−MeOH−H_2O, 70:22:3.5) and vanillin−H_2SO_4 reagent for detection, heated at 105 °C. Plates were visualized under white or UV (λ = 365 nm) light. The R_f values of the standards were 78.7 (grayanotoxin I), 76 (grayanotoxin II, XIV, XVIII), 60 (grayanotoxin III), 81.3 (grayanotoxin IV), 48 (grayanoside A), 42 (grayanoside C), 73.3 (the aglycone of grayanoside C), and 64 (kalmitoxin I).

Ferruginene A (1): brownish-yellow, amorphous solid; $[\alpha]^{20}_D$ +107.7 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.22), 231 sh. (3.77) nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m*/*z* 357.3 $[M - H]^-$; ESIMS^2 (357.3 →) *m*/*z* 357.1 (12), 339.1 (90), 299.1 (24), 287.1 (100), 269.1 (18), 148.9 (29); ESIMS^3 (357.3 → 287.1 →) *m*/*z* 269.0 (100), 229.0 (64), 216.9 (24), 186.9 (16), 148.9 (81); HRESIMS *m*/*z* 357.2079 $[M - H]^-$ (calcd for $C_{22}H_{29}O_4$, 357.2071).

Ferruginene B (2): brownish-yellow, amorphous solid; $[\alpha]^{20}_D$ +96.4 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.08), sh. 230 (3.56) nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m*/*z* 357.3 $[M - H]^-$; ESIMS^2 (357.3 →) *m*/*z* 339.1 (77), 299.1 (100), 287.1 (15), 281.1 (23), 148.9 (26); ESIMS^3 (357.3 → 299.1 →) *m*/*z* 299.0 (12), 281.0 (100), 257.0 (13), 241.0 (45), 229.0 (19), 148.9 (61), 134.9 (11); HRESIMS *m*/*z* 357.2070 $[M - H]^-$ (calcd for $C_{22}H_{29}O_4$, 357.2071).

Ferruginene C (3): brown gum; $[\alpha]^{20}_D$ −183.0 (*c* 0.067, MeOH); UV (MeOH) λ_{max} (log ε) 209 (4.11), 234 sh (3.62) nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m*/*z* 341.2 $[M - H]^-$; ESIMS^2 (341.2 →) *m*/*z* 341.1 (14), 271.0 (100), 269.0 (20), 241.0 (12), 188.9 (15), 122.9 (40); HRESIMS *m*/*z* 341.2134 $[M - H]^-$ (calcd for $C_{22}H_{29}O_3$, 341.2122).

Cell Culture. HL-60 (human promyelocytic leukemia), HeLa-S3 (human cervix carcinoma), MCF-7 (breast adenocarcinoma), and HEK-293 (human embryonic kidney) cells were obtained from the American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 (HL-60 and HeLa-S3) or DMEM (MCF-7 and HEK-293) medium supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2.

Tryptan Blue Cell Viability Assay. HL-60, HeLa-S3, and HEK-293 cells were seeded in 24-well plates (0.1 × 106 cells/mL) and grown for 24 h, and MCF-7 cells were cultured in 24-well plates (0.2 × 106 cells/mL) for 48 h. Then, cells were incubated with solvent (0.3% DMSO) or the test compounds for 24 h. HL-60 cells in suspension and HeLa-S3, MCF-7, and HEK-293 trypsinized cells were stained with Tryptan blue (0.4%) and counted with a cell viability analyzer. IC_{50} values were calculated using the data obtained from three independent experiments and GraphPad Prism software version 4.03.

Radioligand Displacement Assay. The radioligand displacement assay was performed as previously described.[43,44]

■ ASSOCIATED CONTENT

Supporting Information. ^{1}H and ^{13}C NMR and HRMS spectra of compounds **1**−**3**. This information is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

P.S. acknowledges the Austrian Federal Ministry for Science and Research in the framework of ASEA UNINET for financial support during postdoctoral research within the program "Technology Grants Southeast Asia". The authors wish to thank A. Russell and G. Cordt for their help with the collection of the plant material, J. Winkler (Department of Medicinal Chemistry, University of Vienna) for the optical rotation and CD measurements, J. König and M. Köberl (Department of Nutritional Sciences, University of Vienna) for facilitating the measurements of the HRESI mass spectra, and J. Gertsch (Institute of Biochemistry and Molecular Medicine, University of Bern) for the membrane receptor testing. We thank R. W. Doskotch (Division of Medicinal Chemistry and

Pharmacognosy, The Ohio State University) for kindly supplying the grayanotoxin derivatives. We also wish to thank M. Eisenbauer (Institute of Cancer Research, Medical University of Vienna) for kindly providing the HeLa-S3 cell lines and M. Anzelmo for preparing the extracts.

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