

New Diterpenoids from the Aerial Parts of *Salvia reuterana*

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Abstract

The genus *Salvia* is a valuable origin of structurally diverse terpenoids. In a project directed at structurally interesting bioactive metabolites from Iranian *Salvia* species, we studied *Salvia reuterana*. Two new labdane diterpene, 6 β , 14 α -dihydroxy-15-acetoxysclareol (1), and 14 α , 15-dihydroxy sclareol (2), were isolated from the aerial part of the plant. Their structures were established mainly by 1D and 2D NMR spectroscopic techniques, including ¹H-¹H COSY, HSQC and HMBC methods and HR-ESI-TOFMS spectral data. Compound 1 and 2 were tested for their inhibitory activity toward HeLa and MCF-7 cell lines. Our results showed that *S. reuterana* is a rich source of labdane diterpenoids. These compounds are rather rare in *Salvia* species, although they are frequently found in other genera of the *Lamiaceae*. *S. reuterana* is a new source of these diterpenoids.

Keyword: *Salvia reuterana*; Labdane diterpene; Structure elucidation; NMR; Cytotoxicity.

Introduction

Salvia, consisting of about 1000 species, is the largest genus in the family *Lamiaceae* and widely distributed in various regions of the world, Namely, the Mediterranean area, south Africa, central and south America, and southeast Asia (1). Plant of the genus *Salvia* have attracted much attention owing a variety of medicinal properties and biological activities, such as antibacterial, antioxidant, to antitumor, anti-diabetic and anti-inflammatory activities (1-3). The Iranian flora comprises 61 *Salvia* species, 17 of which are endemic (4). Some Iranian *Salvia* species have been investigated from a phytochemical viewpoint, and antibacterial, antiprotozoal, and anticancer compounds have been reported (5-12.). *Salvia reuterana* Boiss.

is an endemic species which grows in the highlands of central Iran (13). Its common name in Persian is “Mariam Goli Esfahani” (14), and the aerial parts of the plant are traditionally used as sedative and anxiolytic herbal medicine. In addition, the antibacterial, antioxidant, free radical scavenging and anti-anxiety properties of this herb have been proved in recent studies (15–19). In a previous work, we have reported the presence of six labdane diterpenoids from this plant (20). Further examination of the *n*-hexane extract of *S. reuterana* led to the isolation of two new other labdane diterpenoids (1, 2).

Experimental

General

HR-ESI-MS was carried out on a Bruker 147 microTOF-ESIMS system. NMR spectra were recorded on Bruker AVANCE III-500 spectrometer operating at 500.13 MHz for ¹H

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NMR and 125.77 MHz for ^{13}C NMR with TMS as an internal standard. Silica gel (70-230 and 230-400mesh) used for column chromatography and silica gel F₂₅₄ (20×20 cm) for TLC, were both supplied by the Merck company.

Plant material

The aerial parts of *Salvia reuterana* were collected from the northern hilly areas of Tehran in July 2011. A voucher specimen (MPH-1321) has been deposited in the herbarium of Medicinal Plants and Drugs Research Institute (MPH) of Shahid Behshti University, Tehran, Iran.

Extraction and Isolation

The air-dried aerial parts of *S. reuterana* (3.0 kg) was crushed and extracted with n-hexane (3 × 15 L) by maceration at room temperature. Extract was concentrated in *vacuo*, to afford 130g of a dark gummy residue. The residue was separated on a silica gel column (230-400 mesh, 850g) with a gradient of n-hexane-EtOAc (100/0 to 0/100) as eluent, followed by increasing concentration of MeOH (up to 25%) in EtOAc. On the basis of TLC analysis, fractions with similar composition were pooled to yield 27 combined fractions.

Fractions 22, 23 and 24 were combined (2g) and subjected to silica gel column chromatography (70-230 mesh), eluted with CHCl_3 - Me_2CO -MeOH (77:13:10) to give six subfraction (C_1 - C_6). Subfraction C_2 was rechromatographed over silica gel (70-230), eluted with CHCl_3 -MeOH (1:1), to afford three subfraction (C_{21} - C_{23}). Subfraction C_{21} was recrystallized from CHCl_3 to afford compound **1** (3.5mg). Fraction 25 (3g) was applied to a silica gel column chromatography (70-230, mesh), eluted with Me_2CO -hexane (2:1) to separate eight subfraction (f_1 - f_8). Subfraction f_2 was recrystallized from hexane to afford compound **2** (8mg).

6 β , 14 α -dihydroxy-15-acetoxysclareol (1): White powder; $[\alpha]_{\text{D}}^{25} = -9.6$ (c 0.2, CHCl_3); for ^1H and ^{13}C NMR data, see Table 1; HR-ESI-TOFMS m/z 423.2738 $[\text{M} + \text{Na}]^+$, calcd for 400.2802).

14 α , 15-dihydroxysclareol (2): White powder; $[\alpha]_{\text{D}}^{25} = -7.1$ (c 0.2, CHCl_3); for ^1H and ^{13}C NMR data, see Table 1; HR-ESI-TOFMS m/z 365.2712

$[\text{M} + \text{Na}]^+$, calcd for 342.5107).

Cytotoxicity assay

The human epithelioid cervix carcinoma (Hela) and human breast adenocarcinoma (MCF-7) cell lines were purchased from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran (Tehran, Iran), and maintained in DMEM medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. These cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2 . Compounds 1-3 were dissolved in DMSO to make a stock of 1mg/mL and further diluted to final concentrations of 10-100 $\mu\text{g}/\text{mL}$ with the serum free culture medium.

Cell viability assay

Cell viability was determined using the MTT assay. Briefly, 2.5×10^4 cells were seeded in 96-well plates at 37 °C with 5% CO_2 for overnight incubation and treated with appropriate concentrations of compounds 1-3 for 24 h. The cells were then incubated with a serum-free medium containing MTT at a final concentration of 0.5 mg/mL for 4 h. The dark formazan crystals formed were dissolved in DMSO and the absorbance was measured at 570 nm.

Results and Discussion

Compound **1** was obtained as a white amorphous powder. Its HI-ESI-TOFMS exhibited a molecular ion peak at m/z 423.2738 $[\text{M} + \text{Na}]^+$, agreed with the molecular formula $\text{C}_{22}\text{H}_{40}\text{O}_6$. The molecular formula accounted for three degrees of unsaturation. The ^{13}C NMR spectrum (Table 1), showed 22 carbon resonances which were analyzed from the DEPT-HSQC spectra to consist of six methyls, seven methylenes, four methines and four quaternary carbons. Two carbon signals at δ_{C} 73.1 and 73.4 indicated the presence of oxygen bearing sp^3 quaternary carbons. Another oxygenated carbons at δ_{C} 75.1 and 67.6 accounted for the carbons carrying two hydroxyl groups with the carbinolic methine protons coming at δ_{H} 3.55 (1H, dd, $J = 2.5, 8.5$ Hz) and δ_{H} 4.39 (1H, brd, $J = 2.6$ Hz), respectively. The signal at δ_{C} 66.2

Table 1. ^1H and ^{13}C NMR data of compounds 1 and 2 in $\text{CDCl}_3/\text{CD}_3\text{OD}$ (500 MHz for δ_{H} ; 125 MHz for δ_{C}).

position	1		2	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1 α	41.6	0.85 ^b	40.0	0.88 ^b
1 β		1.52 ^b		1.53 ^b
2 α	18.3	1.60 ^b	18.4	1.53 ^b
2 β		1.33 ^b		1.35 ^b
3 α	44.1	1.05 ^b	41.8	1.07 ^b
3 β		1.22 ^b		1.28 ^b
4	33.4		32.9	
5 α	56.7	0.83 ^b	56.7	0.83 ^b
6 α	67.6	4.39 brd (2.6)	20.6	1.57 ^b
6 β				1.18 ^b
7 α	51.3	1.55 ^b	43.8	1.34 ^b
7 β		1.91 dd (13.5, 2.0)		1.75 dt (12.5, 3.0)
8	73.4		74.5	
9 α	62.1	1.03 ^b	62.3	1.00 t (3.5)
10	38.9		39.2	
11 α	17.9	1.49	18.0	1.46 ^b
11 β		1.28		1.26 ^b
12 α	40.9	1.60 ^b	40.8	1.60 ^b
12 β		1.41 ^b		1.38 ^b
13	73.1		73.8	
14 β	75.1	3.55 dd (8.5, 2.5)	77.8	3.39 dd (7.5, 3.5)
15a	66.2	3.96 dd (11.5, 8.5)	62.5	3.50 dd (11.5, 7.5)
15b		4.22 dd (11.5, 2.5)		3.65 dd (11.5, 3.5)
16	22.6	1.10 s	22.0	1.08 s
17	24.8	1.32 s	23.6	1.09 s
18	32.8	0.89 s	33.4	0.79 s
19	24.2	1.10 s	21.5	0.71 s
20	16.1	1.08 s	14.9	0.73 s
CH ₃ CO	19.8	1.99 s		
CH ₃ CO	171.3			

^a δ values were extracted from COSY, HSQC and HMBC experiments.

^bOverlapping signals.

accounted for amethylenic carbon attached to an acetoxy group, with the corresponding methylene protons coming at 3.96, 4.22 each as a doublet of doublet. According to the degree of unsaturation the molecule was bicyclic and appeared to be a labdane diterpenoid (21). The ^1H and ^{13}C

NMR spectrum displayed features similar to those of 14 α -hydroxy-15-acetoxysclareol, isolated previously from this plant by us (20). However, the ^{13}C NMR spectrum of **1** showed the presence of an additional methine at δ_{C} 67.6 instead of the methylene group (C-6). Hence,

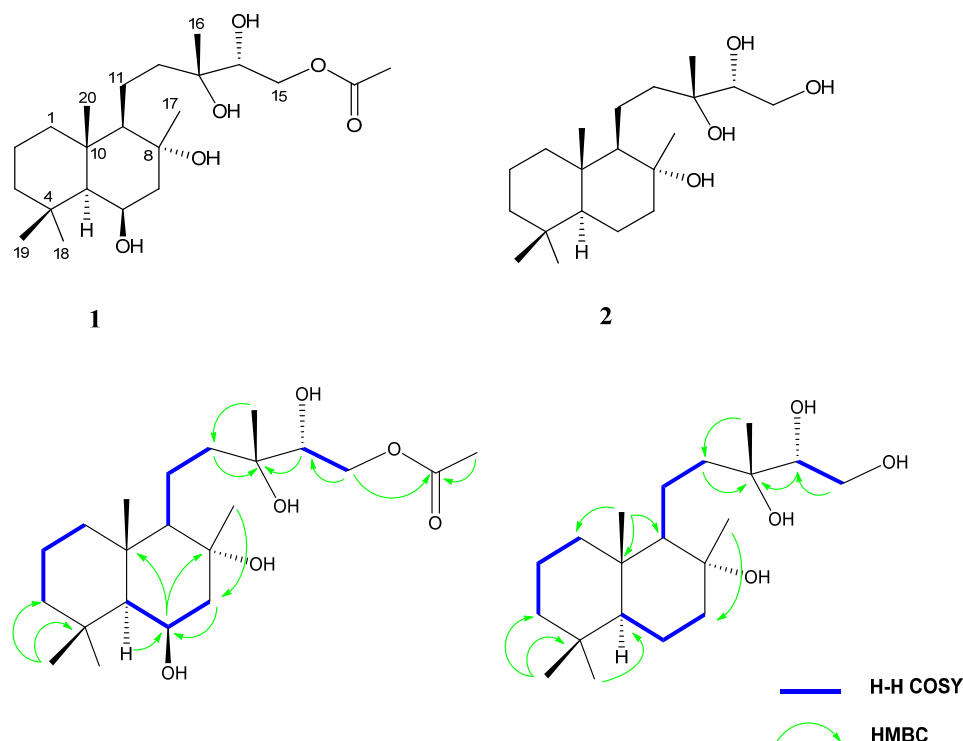


Figure 1. Key H–H COSY and HMBC connectivities of compounds **1** (left) and **2** (right).

the methylene was replaced by an oxygenated methine. The signals of C-7 (δ_c 51.3) and C-5 (δ_c 56.7) were paramagnetically shifted ($\Delta\delta$ +7.3 and +0.7 ppm, respectively) in comparison to those of 14 α -hydroxy-15-acetoxysclareol. Also the resonances of neighboring H-7 α (δ_H 1.55), H-7 β (δ_H 1.91), and H-5 α (δ_H 0.83) were shifted ($\Delta\delta$ = +0.2, +0.15, and -0.03 ppm, respectively). HMBC correlations between H-5 α , H-7 α with C-6, and between H-6, C-8, and C-10 (Figure 1) confirmed the location of the hydroxyl group. Diagnostic COSY correlations were observed

between H-6 and H-7 α and H-7 β , and between H-6 and H-5. The relative configuration of the hydroxyl group at C-6 was determined as β based on the magnitude of the vicinal coupling constants of the H-6 resonance (δ_H 4.39, brd, J = 2.6 Hz). NOESY contacts of H-6 with H-5 and H-7 α were observed. The ^1H chemical shifts of CH₃-17, CH₃-19 and CH₃-20 (δ_H 1.32, 1.10 and 1.08, respectively) appeared downfield (δ_H 0.21, 0.38, and 0.35) relative to those of 14 α -hydroxy-15-acetoxysclareol. These differences were in agreement with an axial orientation of the

Table 2. Cytotoxic activities of compounds **1** and **2** and against two human tumor cell lines.

Compound	Hela (IC ₅₀ μM) ^a	MCF-7 (IC ₅₀ μM)
1	184.61 \pm 1.67	108.34 \pm 2.07
2	218.47 \pm 2.77	164.07 \pm 3.09
Paclitaxel ^b	0.004	0.033

^aData are expressed as mean \pm standard deviation (n = 3).

^bPositive control.

hydroxyl group at C-6. Thus, the structure of **1** was established as 6 β ,14 α -dihydroxy-15-acetoxysclareol.

Compound **2** was obtained as a white powder. The HR-ESI-TOFMS showed a peak at m/z 365.2712 [M+Na]⁺, in agreement with the elemental formula C₂₀H₃₃O₄, which accounted for three degrees of unsaturation. The ¹H and ¹³C NMR data (Table 1) strongly resembled those of **1**, indicating that the two compounds were structurally related. Inspection of the ¹H and ¹³C NMR spectra showed the lack of the signals belongs to the acetoxy group in compound **2**. Also, the methine signals at 67.6 ppm (δ_H 4.39) was replaced by a methylene group (δ_C 20.6 and δ_H 1.57, 1.18) in **2**. Thus, compound **2** was established as 14 α , 15- dihydroxy sclareol.

Compounds **1** and **2** were evaluated for their *in vitro* cytotoxic activity against Hela (human epitheloid cervix carcinoma) and MCF-7 (human breast adeno-carcinoma) cell lines. The results of the cytotoxicity studies were indicated in Table 2.

Conclusion

Our results showed that *S. reuterana* is a rich source of labdane diterpenoids. The most abundant diterpenoids in the genus are abietanes and rearranged abietanes. Labdane diterpenoids are rather rare in *Salvia* species although they are frequently found in other genera of the *Lamiaceae*. *S. reuterana* is a new source of these diterpenoids.

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