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Structure Determination of Four Compounds Isolating from Rhizomes of *Rhodiola rosea* using NMR Spectrometer

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Abstract *Rhodiola rosea*, also known as gold root or rose root, is a perennial plant in the family of Crassulaceae. The rhizhome of *R. rosea* has been widely used as a hemostatic, tonic for burns and contusions in traditional Chinese medicine. Recent studies reported its strong antioxidant and adaptogenic properties. In this paper, we attempted to isolate compounds from the methanolic extract of *R. rosea* rhizomes. Four compounds including one new compound (**1**), two kaempferol glycosides (**3** and **4**) were isolated from chloroform and ethyl acetate soluble fraction of *R. rosea* extract. The structures of **1**~**4** including relative stereochemistry were determined by MS and NMR analysis.

Keywords *Rhodiola rosea*; constituents; flavonoid; 1D and 2D NMR

Introduction

Rhodiola species in the family of Crassulaceae grow wild in dry, high-altitude areas in continental Asia, Europe, and America.[1, 2] There are over 200 species from the genus *Rhodiola* of which at least 20 species are known to have variable medicinal effects.[1,3-5] Alpine Rhodiola has been widely used as traditional medicines for clinical treatments due to its strong antioxidant effects.

Rhodiola rosea L. is a succulent perennial flowering plant that has a rose-like fragrance. The rhizhome of *R. rosea* has been reported for its potent anxiolytic, antidepressant, neuroprotective and cognitive-enhancing properties and nonspecific "adaptogenic" effects.[6] Recently, the adaptogenic properties of *R. rosea* are well established according to the preclinical studies.[7-10] Phytochemical analysis revealed that *R. rosea* contain 5 distinct groups of active components; flavonoids, phenylpropanoids derivatives, phenylethanoid derivatives, monoterpenoid and phenolic acid.[11] Among which phenylpropanoids, rosavin and rosarin are considered to occur only in *R. rosea*.[1,12] Flavonoids appear to be the main constituents in *R. rosea* as mainly, i.e. rodiolin, rodionin, rodiosin.

In this paper, we report the isolation and structure determination of two linear chained compounds and two flavonoid glycosides from the methanolic extract of *R. rosea* rhizomes. Among them, compound **1** was identified as a new analog derived from **2**. The interpretation of 1D and 2D NMR spectra completely led to the NMR assignments for all isolated

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compounds.

Experimental Methods

General Experimental - All NMR spectra were measured on a Varian VNMRS 500 spectrometer using $CDCl_3$ for compounds **1** and **2** and CD_3OD for compounds **3** and **4** as solvent. The ESI mass spectra were acquired using an ABSCIEX QTRAP 3200 instrument. HPLC was performed using a Varian Prostar system with a 355 refractive index (RI) detector or Agilent 1200 Chemstation with DAD detector. The separation was performed using the YMC ODS-A column. All solvents were distilled prior to use.

Plant material - The rhizomes of *R. rosea* were collected from high-altitude region at Tibet in 2012. A voucher specimen (GNP-79) has been deposited in the laboratory of pharmacognosy, college of life sciences and natural resources, Gyeongnam National University of Science and Technology.

Extraction and Isolation - The dried *R. rosea* rhizomes (2.0 kg) were extracted three times with 80% methanol for 3 h each in an ultrasonic apparatus. Removal of the solvent in vacuo yielded a methanolic extract (737.4 g). The methanolic extract was then suspended in distilled water and partitioned successively with chloroform (20.55 g), ethyl acetate (112.32 g), and *n*-butanol (154.4 g). Chloroform soluble fraction was subjected to column chromatography on a silica gel column using mixtures of ethyl acetate−methanol of increasing polarity as eluents to give 26 fractions (C1 ~ 26). C5 was further subjected to column chromatography on a silica gel column using mixtures of *n*-hexane−ethyl acetate of increasing polarity as eluents to give 17 sub-fractions (C5 – 1 ~ 17). Compounds **1** and **2** were isolated from C5-8 through purification using HPLC (YMC Pack Pro C_{18}, 60% MeOH, 1.5 ml/min) with RI detector. Ethyl acetate soluble fraction was subjected to column chromatography on a silica gel column using mixtures of chloroform−methanol−water as eluents to give 27 fractions (E1 ~ 27). E16 was further subjected to column chromatography on a silica gel column using mixtures of *n*-hexane−ethyl acetate−methanol of increasing polarity as eluents to give 15 sub-fractions (E16 - 1 ~ 15). E16-6 was subjected to column chromatography on Sephadex LH-20 (MeOH) to give compounds **3** and **4**.

Results and Discussion

Four compounds, including one new compound and two flavonoid glycosides, were obtained from chloroform and ethyl acetate soluble fraction of *R. rosea* extract (Figure 1). In fact, nonpolar compounds **1** and **2** from chloroform fraction were mixed which could not be further separated due to their very similar structures. At first glance the isolated compound could be considered as single purified one by the proton integration in the ^1H NMR spectrum shown in Figure 1, but careful analyses of the 1D and 2D NMR spectra turned out the existence of two similar compounds mixed with a similar rate (Figure 3).

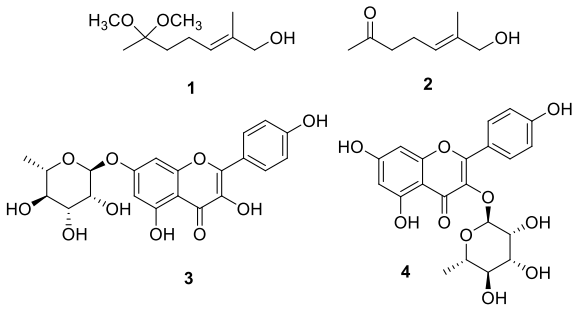

Figure 1. Structures of **1**~**4** isolated from Rhizomes of *Rhodiola rosea*

Compound **1** had the molecular formula $C_{10}H_{20}O_3$ on the basis of the pseudo molecular ion $[M + Na]^+$ at *m/z* 211 in the ESIMS following the interpretation of the 1D and 2D NMR spectra. Slightly intensive proton signals in the ^1H NMR spectrum were assigned to **1**: one oxygen-bearing signal at δ_H 3.91 and one olefinic proton at δ_H 5.40 (Table 1). Similarly, more intensive carbon signals in the ^{13}C NMR spectrum were also assigned to **1**: a methylene at δ_C 23.5, an oxomthylene at δ_C 68.8), and one olefinic double bond (δ_C 126.2 and 136.2). Based on these

signals, HSQC and sequential COSY correlations revealed the partial structure -CH_2CH_2CH- presented

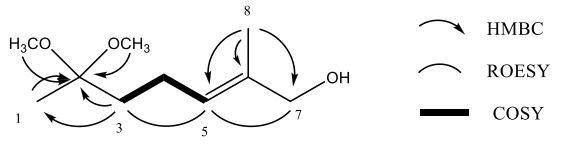

Figure 2. Key correlations of **1**

as bolds in Figure 2. The terminal methylene protons in the partial structure showed HMBC correlation with the characteristic quaternary carbon at δ_C 102.9 and the methyl carbon at δ_C 21.2.

Furthermore, the HMBC spectrum showed the correlation between the quaternary carbon and a large proton signal corresponding to two methoxy groups at δ_H 3.16 (Figure 2). On the other hand, the olefinic methyl proton at δ_H 1.65 had the HMBC correlations with the two olefinic carbons (δC 126.2 and 136.2) and the oxymethylene carbon, completing the planar structure of 1. One existing double bond was configured as E form by the ROE correlation between the two protons at δH 5.40 and 3.91. Therefore, **1** was defined to be (*E*)-6,6-dimethoxy-2-methylhept-2-en-1-ol, identified as a new compound.

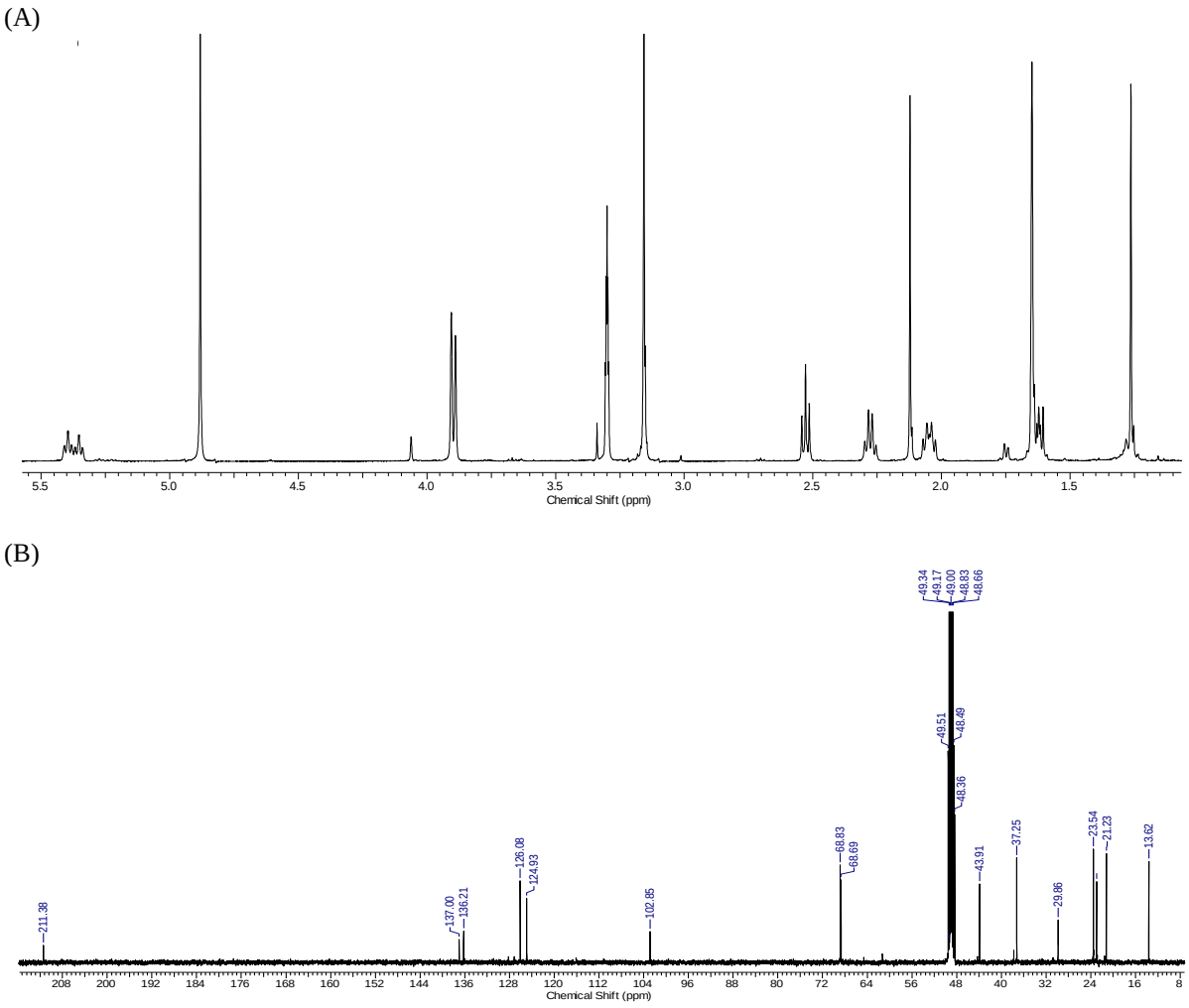

Figure 3. ^{1}H NMR (A) and ^{13}C NMR (B) spectra of mixture of compounds **1** and **2**

Compound **2** had the molecular formula $C_8H_{14}O_2$ on the basis of the pseudo molecular ion $[M + Na]^+$ at *m/z* 165 in the ESIMS and the unassigned carbons in the ^{13}C NMR spectrum. All unassigned signals in the ^1H and ^{13}C NMR spectrum for the compound from the chloroform fraction corresponded to **2** (Table 1). Compared to **1**, compound **2** was featured by the presence of carbonyl group in replacement of two methoxy groups. The IR spectrum of **2** also supported the presence of a carbonyl group from an absorption band at 1710 cm^{-1}. The HMBC correlation of methyl singlet at δ_H 2.12 (H-1) with the carbonyl carbon at δ_C 211.4 showed the possession of an acetyl group.[13]

Table 1. ^1H and ^{13}C NMR spectral data for compounds **1** and **2** in CDCl3 recorded at 500 MHz and 125 MHz

	1		**2**	
no	δ_H	δ_C	δ_H	δ_C
1	1.26, s	21.2	1.12, s	29.9
2		102.9		211.4
3	1.62, t (8.3)	37.3	2.53, t (7.3)	43.9
4	2.05, m	23.5	2.28, q (7.3)	23.0
5	5.40	126.2	5.35, dq (7.3, 1.2)	124.9
6		136.2		137.0
7	3.91, s	68.8	3.89, s	68.7
8	1.65, s	13.6	1.65, s	13.6
$-OCH_3$	3.16			

Compound **3** had the molecular formula $C_{21}H_{20}O_{10}$ on the basis of the pseudo molecular ion $[M + H]^+$ at *m/z* 433 in the ESIMS. In the ^{13}C NMR spectrum, fifteen carbons were observed including twelve aromatic, two olefin and one carbonyl carbons (Table 2). The ^1H NMR resonances were assigned to aromatic proton signals at δ_H 6.42 (1H, d, J = 2.0 Hz) and 6.74 (1H, d, J = 2.0 Hz), and A_2X_2-type aromatic proton signals at δ_H 6.91 (2H, d, J = 8.8 Hz) and 8.09 (2H, d, J = 8.8 Hz)]. Also, anomeric proton and carbon signals at δ_H 5.55 (1H, d, J = 1.5 Hz) and δ_C 99.8 were observed. Along with above data, interpretation of the 1D and 2D NMR spectra determined compound **3** to be kaempferol glycoside. The assignment of sugar in **3** was evidently established from the proton coupling constants and the NOESY analysis (Figure 4). Anomeric proton H-1" had coupling constant of 1.5 Hz, which suggested α-orientation of sugar. Coupling constants of successive well-resolved protons was afforded as the methyl pentoses rhamnose, occurring in L-form in nature: H-2" (dd, J = 3.4, 1.5 Hz), H-3" (dd, J = 3.4, 9.5 Hz), and H-4" (t, J = 9.5 Hz). Additional NOESY experiment, showing the correlations between H-1"/H-2" H-2"/H-3", and H-3"/H-6', supported L-rhamnose. The HMBC correlation between the anomeric proton and the carbon at δ_C 163.3 indicated the attachment of the sugar to C-7 of kaempferol moiety. Thus, **3** was determined as the well-known kaempferol-7-*O*-α-L-rhamnopyranoside.[14]

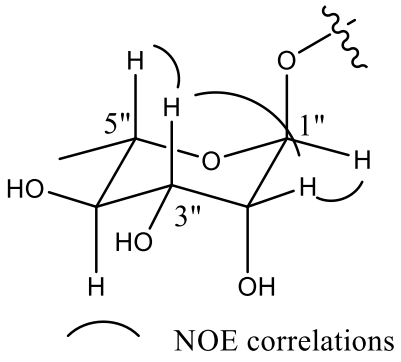

Figure 4. NOE correlations of **3**

Compound **4** had the same molecular formula as **3** on the basis of the ESIMS. The 1D and 2D NMR experiments revealed that **4** is also an kaempferol glycoside (Table 2). In comparison with **3**, all carbon chemical shifts of the two compounds are similar, while the proton chemical shifts are different with the exception of C-3' and C-5'. In particular, H-4" and H-5" within the sugar was overlapped as a multiplet at δ_H 3.32, which shows the difference with the well-separated corresponding signals in **3**. This observation could suggest the different configuration of C-4" or C-5" from the case of **3**, but the coupling constant of H-3" (dd, J = 3.4, 8.3 Hz) and NOE correlation of H-3"/H-5" allowed to assign the sugar as the same L-rhamnose. The HMBC correlation between the anomeric proton and the carbon at δ_C 136.2 indicated the attachment of the sugar to C-3 of Kaempferol moiety. Thus, **4** was determined as the well-known kaempferol-3-*O*-α-L-rhamnopyranoside. This structure rationalizes the chemical shifts of H-4" and 5", different from those of **3**. The placement of L-rhamnose in 4 is spatially close to carbonyl group which affect the chemical shift anisotropy on H-4" and 5".[15]

Table 2. ^{1}H and ^{13}C NMR spectral data for compounds **3** and **4** in $CDCl_3$ recorded at 500 MHz and 125 MHz

	3		**4**	
no	δ_H	δ_C	δ_H	δ_C
1				
2		148.7		158.5
3		137.6		136.2
4		177.5		179.6
5		162.3		163.2
6	6.42 (d, 2.0)	99.9	6.20, d (2.2)	99.8
7		163.3		165.9
8	6.74 (d, 2.0)	95.3	6.39, d (2.0)	94.7
9		157.8		159.3
10		106.2		105.9
1'		123.5		122.6
2'	8.09, d (8.8)	130.8	7.76, d (8.8)	131.9
3'	6.91, d (8.8)	116.4	6.92, d (8.8)	116.5
4'	6.91, d (8.8)	160.8	6.92, d (8.8)	161.6
5'	8.09, d (8.8)	116.4	7.76, d (8.8)	116.5
6'		130.8		131.9
1"	5.55, d (1.5)	99.8	5.37, d (1.5)	103.5
2"	4.01, dd (3.4, 1.5)	71.7	4.21, dd (3.4, 1.5)	71.9
3"	3.83, dd (9.5, 3.4)	72.1	3.70, dd (8.3, 3.4)	72.1
4"	3.47, t (9.5)	72.1	3.32, m	73.2
5"	3.60, dq (9.5, 6.1)	71.2	3.32, m	72.0
6"	1.24, d (6.1)	18.2	0.89, d (5.9)	17.7

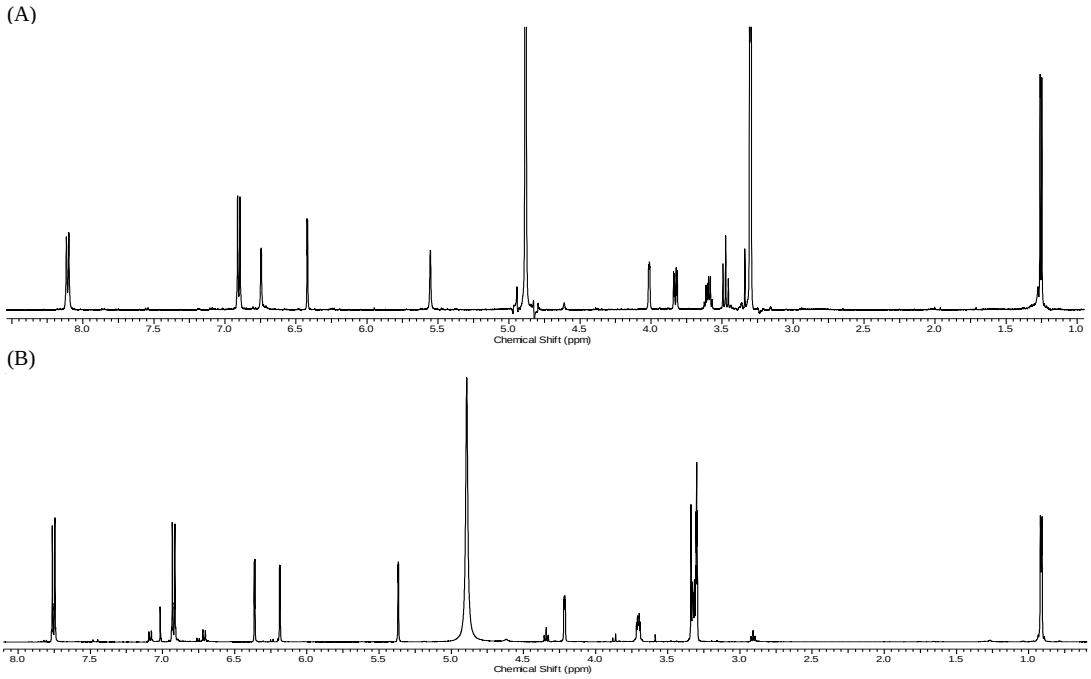

Figure 5. ^{1}H NMR spectra of compounds **3** (A) and **4** (B).

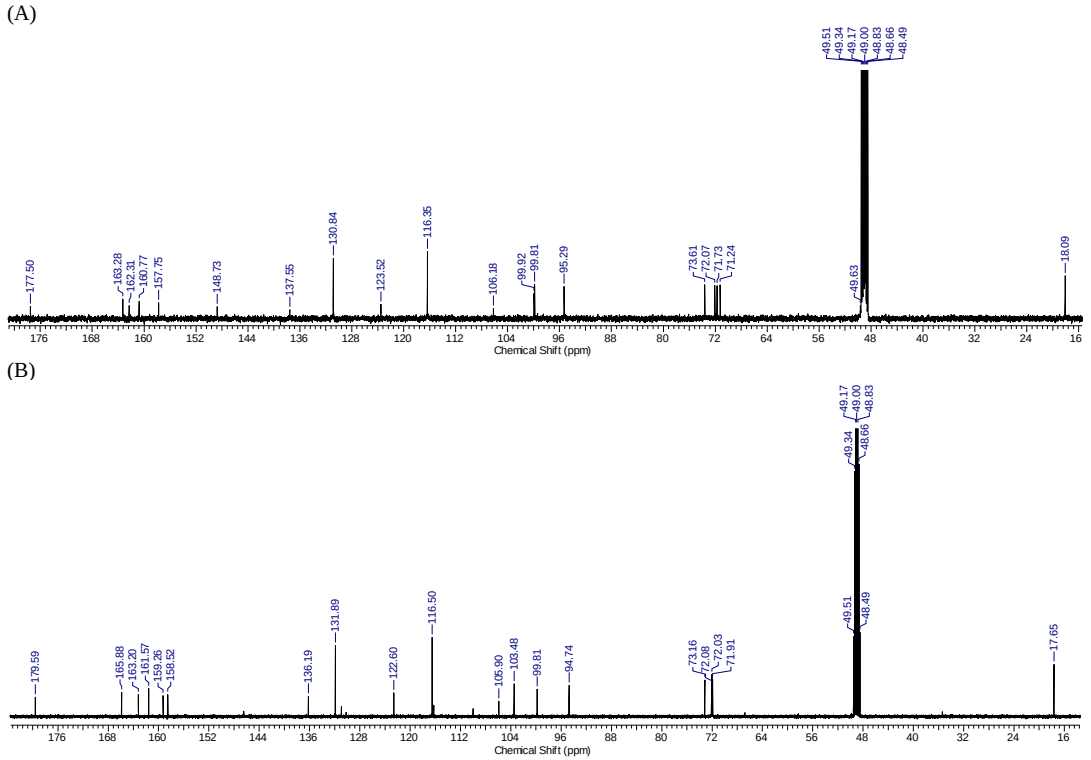

Figure 6. ^{13}C NMR spectra of compounds **3** (A) and **4** (B)

Acknowledgements

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