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Isolation and NMR Spectral Assignments of 18-Glycyrrhetinic acid-3-*O*-*D*-glucuronide and 18-Glycyrrhetinic acid

Venkata Sai Prakash Chaturvedula [1], Oliver Yu [2], and Guohong Mao [3]
[1,] *Natural Ingredient Development, Blue California, 30111 Tomas, Rancho Santa Margarita, CA 92688, USA*
[2,] *Wuxi New Way Biotechnology, 401 North Xingyuan Road, Wuxi 210043, P.R. China.*

ABSTRACT: *Two olenane triterpenes, 18β-glycyrrhetinic acid-3-O-β-D-glucuronide and 18β-glycyrrhetinic acid were obtained from the acid hydrolysis of 18β-glycyrrhetinic acid-3-O-β-D-glucuronopyranosyl-(1→2)-β-D-glucuronide (Glycyrrhizic acid or Glycyrrhizin), a triterpene glucuronide isolated from the commercial extract of the roots of Glycyrrhia glabra. The complete ^{1}H and ^{13}C NMR assignments of the two triterpenes 18β-glycyrrhetinic acid-3-O-β-D-glucuronide and 18β-glycyrrhetinic acid were achieved on the basis of extensive 1D and 2D NMR (^{1}H and ^{13}C, COSY, HSQC, and HMBC) as well as mass spectral data.*

KEYWORDS*: Glycyrrhiza glabra, Fabaceae, Glycyrrhizin, Triterpene glucuronide, Hydrolysis products, 1D and 2D NMR spectral data, Structure characterization*.

I. INTRODUCTION

The triterpenoid saponin, glycyrrhizic acid (glycyrrhizin) was the major bio-active constituent isolated from the roots of *Glycyrrhiza glabra* (Fabaceae), which is a sweet-tasting material and is about 50 times sweeter than sugar, making it a widely used as a sweetening additive in the food industry [1-3]. The structure of glycyrrhizin has been characterized as 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronopyranosyl-(1→2)-β-*D*-glucuronide (**1**) on the basis of NMR and mass spectral data as well as hydrolysis studies [4-5]. *G. glabra* also known as Licorice is a well-known medicinal herb that grows in various parts of the world, and is one of the oldest and widely used herbs known both in western and eastern countries since several thousand years ago [2-4]. Licorice has been used in the traditional system of medicine: the roots and rhizomes of *G. glabra* have various pharmaceuticals activities like antispasmodic, demulcent, pectoral, anti-inflammatory, antiulcer, expectorant, antimicrobial and anxiolytic activities as well as a flavoring agent to disguise the unpleasant flavor of other medications [3-4].

As a part of our continuing research to discover natural products, we have isolated several diterpene glycosides from the commercial extracts of the leaves of *S. rebaudiana* [6-9], flavonoids from *Hovenia dulcis* [10], and triterpenes from *Glycyrrhia glabra* [5]. The structures of the isolated compounds were characterized on the basis of extensive 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR as well as high resolution mass spectroscopic data and chemical modifications. In this paper, we are describing the isolation, and proton as well as carbon NMR spectral assignments of the two triterpenoids, 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronide (**2**) and 18β-glycyrrhetinic acid (**3**) that were obtained from the acid hydrolysis of the triterpene glycoside 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronopyranosyl-(1→2)-β-*D*-glucuronide (**1**) isolated from the commercial extract of the roots of *G. glabra*, The structures of compounds **2** and **3** were achieved on the basis of 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR and high resolution mass spectroscopic (MS) data, as well as by comparison of the physical and spectral data of reported in literature.

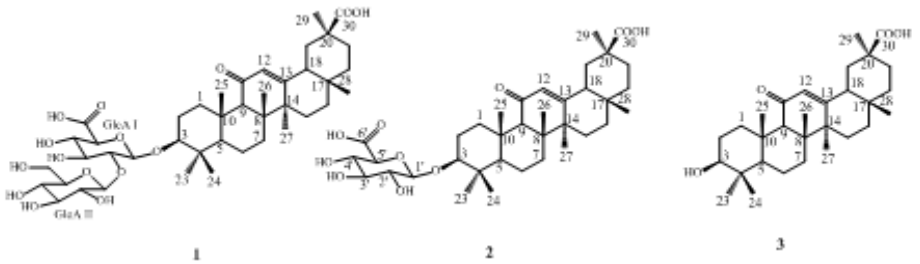

Figure 1: Structures of glycyrrhizic acid (**1**), 18β-glycyrrhetinic acid -3-*O*-β-glucuronide (**2**), and 18β-glycyrrhetinic acid (**3**)

II. EXPERIMENTAL

General Instrumentation Methods

IR spectral data was acquired using a Perkin Elmer 400 Fourier Transform Infrared (FT-IR) Spectrometer with Universal attenuated total reflectance (UATR) polarization accessory. HPLC analysis was performed using a Dionex UPLC ultimate 3000 system (Sunnyvale, CA), including a quaternary pump, a temperature controlled column compartment, an auto sampler and a UV absorbance detector. Phenomenex Luna C18 reversed-phase with guard column, 150x4.6 mm, 3µm (100A) were used for the isolation and purification of glycyrrhizin (**1**). NMR spectra were acquired on Bruker Avance DRX 500 MHz or Varian INOVA 600 MHz instrument instruments using standard pulse sequences. The NMR spectra were performed in C_5D_5N; chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. MS and MS/MS data were generated with a Thermo LTQ-FTMS mass spectrometer (100,000 resolutions) equipped with a Nano spray ionization source. Samples were diluted with methanol and introduced via infusion using the onboard syringe.

Isolation and Characterization

Compound **1** was purified from the commercial aqueous alcoholic extract of *G. glabra* root as reported earlier [5].

Partial hydrolysis of 1 using Oxalic acid

Compound **1** (50 mg) was treated with 50 mg oxalic acid in 20 ml of 1:1 MeOH:water and the mixture was heated at 60^o C for 24 hours. The reaction mixture was extracted with ethyl acetate (EtOAc) (2 x 250 ml) to give an aqueous fraction containing sugars and an EtOAc fraction containing the partial hydrolyzed product. Concentration of the EtOAC fraction followed by purification using normal phase PTLC using the solvent system n-hexane/EtOAC (70:30) yielded a white which has been identified as 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronide (**2**) on the basis of NMR and Mass spectral data [11].

18β-glycyrrhetinic acid-3-*O*--*D*-glucuronide (2)

White powder; IR ν_{max}: 3318, 2960, 2905, 1678, 1530, 1150, 1054, 913 cm^{-1}; ^{1}H-NMR (600 MHz, C_5D_5N, δ ppm) and ^{13}C-NMR (150 MHz, C_5D_5N, δ ppm) spectroscopic data see Table 1; HRMS $(M+H)^+$ *m/z* 647.3782 (calcd. for $C_{36}H_{55}O_{10}$: 647.3795).

Acid hydrolysis of 1 using Sulfuric acid

To a solution of compound **1** (50 mg) in MeOH (25 ml) was added 100 ml of 10% H_2SO_4 in MeOH and the mixture was refluxed for 72 hours. The reaction mixture was then extracted with ethyl acetate (EtOAc) (2 x 250 ml) and concentration of the organic fraction yielded an aglycone, which has been identified as glycyrrhetinic acid (**3**) on the basis of spectral data and co-TLC with standard compound [12].

18β-glycyrrhetinic acid (3)

White powder; IR ν_{max}: 3315, 2956, 2908, 1676, 1524, 1155, 1053, 910 cm^{-1}; ^{1}H-NMR (600 MHz, C_5D_5N, δ ppm) and ^{13}C-NMR (150 MHz, C_5D_5N, δ ppm) spectroscopic data see Table 1; HRMS $(M+H)^+$ *m/z* 471.3462 (calcd. for $C_{30}H_{47}O_4$: 471.3474).

Results and Discussion

Compound **2** was isolated as a colorless powder and its positive mode of ESI Time of Flight (TOF) mass spectrum indicated an $[M+H]^+$ ion at *m/z* 647.3782 which was in good agreement with the molecular formula $C_{36}H_{54}O_{10}$; the chemical composition was further supported by the ^{13}C NMR spectral data. Liebermann-Burchard reaction indicated compound **2** is having a terpenoid skeleton [13], as in **1**. The ^{1}H NMR spectra of compound **2** showed the presence of seven methyl sinlgets at δ 0.79, 1.06, 1.23, 1.29, 1.38, 1.41 and 1.45, and a signal corresponding to the H-3 of the oxymethine proton which was appeared as a doublet of doublets at δ 3.35. The ^{1}H NMR spectra of compound **2** also showed the presence of a singlet at δ 5.96 corresponding to the presence of a olefinic proton of a trisubstituted double bond. Further, the ^{13}C NMR spectral data of **2** showed the presence of a carbonyl group resonating at δ 200.0 which showedan HMBC correlation to the trisubstituted olefinic proton resonating at δ 5.96 indicated the presence of an α,β-unstaurated carbonyl group in compound **2**. The above spectral data of **2** suggested the presence of an oleanane triterpene skeleton having a hydroxyl group at C-3 position with a double bond at C-12/C-13 and seven methyl groups. Also, the ^{1}H NMR spectrum of **2** showed an anomeric proton at δ 5.43 as doublets indicating the presence of a sugar unit in its structure. The presence of four secondary hydroxyl groups together with anomeric carbon and a carboxylic carbonyl group in the ^{13}C NMR spectral data of **2** suggested the presence of glucuronic acid as the sugar unit in its structure. The large coupling constant observed for the anomeric proton of the glucuronic acid moiety at δ 5.43 (d, *J*=8.1 Hz), suggested its β-orientation as reported earlier for glycyrrhizin. The ^{1}H and ^{13}C NMR values for all the protons and carbons were assigned on the basis of COSY, HMQC and HMBC correlations and were given in Table 1.

Table 1. ^{1}H and ^{13}C NMR chemical shift values for compounds **2** and **3** recorded in C_5D_5N [a-c].

Position	2		3	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	0.97 m, 3.08 dt (*J* = 7.6, 8.9)	39.7	0.96 m, 3.24 dt (*J* = 7.8, 9.1)	38.9
2	1.78 m, 2.06 m	27.0	1.76 m, 2.04 m	29.0
3	3.35 dd (*J* = 5.7, 12.1)	89.2	3.52 dd (*J* = 5.1, 12.4)	78.4
4	-	40.3	-	40.3
5	0.84 m	55.7	0.90 m	55.8
6	1.44 m, 1.72 m	18.0	1.47 m, 1.74 m	18.5
7	1.48 m, 1.74 m	33.2	1.49 m, 1.76 m	33.5
8	-	43.7	-	44.0
9	2.47 s	62.5	2.57 s	62.7
10	-	37.8	-	38.2
11	-	200.0	-	200.2
12	5.96 s	129.0	6.04 s	129.2
13	-	172.9	-	170.2
14	-	46.0	-	46.1
15	1.26 m, 2.15 m	27.0	1.26 m, 2.17 m	27.2
16	1.06 m, 2.17 m	27.2	1.05 m, 2.18 m	27.4
17	-	32.5	-	32.7
18	2.18 m	49.1	2.20 m	49.3
19	1.52 m, 2.37 m	42.1	1.47 m, 2.31 m	42.2
20	-	44.5	-	44.6
21	1.55 m, 2.12 m	32.0	1.50 m, 2.10 m	32.0
22	1.32 m, 1.76 m	38.8	1.31 m, 1.74 m	38.9
23	1.23 s	28.6	1.16 s	28.7
24	0.79 s	17.0	0.83 s	17.1
25	1.29 s	17.2	1.30 s	17.4
26	1.38 s	19.2	1.37 s	19.4
27	1.41 s	24.0	1.38 s	24.0
28	1.06 s	29.1	1.11 s	29.2
29	1.45 s	29.2	1.43 s	29.3
30		179.5		179.6
1′	5.43 d (*J* = 8.1)	107.5		
2′	4.12 dd (*J* = 7.8, 8.4)	78.5		
3′	4.04 dd (*J* = 8.1, 8.4)	78.2		
4′	4.23 t (*J* = 8.1)	75.9		
5′	4.46 t (*J* = 8.4)	73.8		
6′		170.1		

[a] assignments made on the basis of COSY, HSQC and HMBC correlations; [b] Chemical shift values are in δ (ppm); [c] Coupling constants are in Hz.

In the absence of eighth methyl group and the appearance of a carbonyl group resonating at δ 179.5 in the ^{13}C NMR spectral data of **2** suggested the presence of an acid functional group similar to **1**. The presence of the carboxylic acid group was identified at C-30 position by the key HMBC correlations as shown in Figure 2. Based on the results from 1D and 2D NMR spectral data, it was concluded that the structure of **2** has a triterpene aglycone moiety with an α,β-unsaturated carbonyl group, a carboxylic acid group, seven methyl singlets and one β-D-glucuronyl unit. Thus, the structure of **2** was assigned as the known compound 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronide; NMR and mass spectral data are consistent to the reported literature values [11].

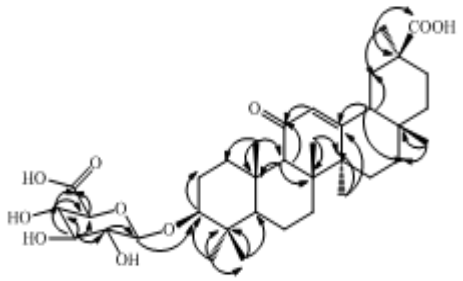

Figure 2: Key HMBC correlations of 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronide (**2**)

Compound **3** was also isolated as a colorless powder and its molecular formula has been deduced as $C_{30}H_{46}O_4$ from the positive mode of ESI TOF mass spectral data which indicated an $[M+H]^+$ ion at *m/z* 471.3462 and this composition was further supported by the ^{13}C NMR spectral data. Compound **3** also showed a positive Liebermann-Burchard reaction indicating a terpenoid skeleton [13], as in **1** and **2**. The ^1H NMR spectra of compound **3** also showed the presence of seven methyl sinlgets at δ 0.83, 1.11, 1.16, 1.30, 1.37, 1.38 and 1.43; a signal corresponding to the H-3 of the oxymethine proton which was appeared as a doublet of doublets at δ 3.52; and a singlet at δ 6.04 corresponding to the presence of a olefinic proton of an α,β-unstaurated carbonyl group as in **2**. In the absence of any anomeric proton together with appearance of 30 carbons in the ^{13}C NMR spectral data suggested the presence of an oleanane triterpene skeleton having a hydroxyl group at C-3 position with a double bond at C-12/C-13 and seven methyl groups in its structure as in **2**. The ^1H and ^{13}C NMR values for all the protons and carbons were assigned on the basis of COSY, HSQC and HMBC correlations and were given in Table 1. In the absence of eighth methyl group and the appearance of a carbonyl group resonating at δ 179.6 in the ^{13}C NMR spectral data of **3** as in **1** and **2** suggested the presence of an acid functional group at C-30 position. Based on the results from 1D and 2D NMR spectral data, it was concluded that the structure of **3** has a triterpene aglycone moiety with an α,β-unsaturated carbonyl group, a carboxylic acid group, seven methyl singlets. Thus, the structure of **3** was assigned as the known compound 18β-glycyrrhetinic acid supported further by the key HMBC correlations as shown in Figure 3 as well by comparison of the spectral data to the reported from the literature [12].

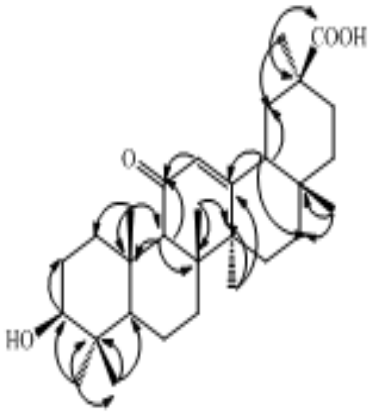

Figure 3: Key HMBC correlations of 18β-glycyrrhetinic acid (**3**)

III. CONCLUSIONS

We are herewith reporting the isolation and complete ^1H and ^{13}C NMR spectral assignments for the two oleanane triterpenes 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronide (**2**) and 18β-glycyrrhetinic acid (**3**) which were isolated from the hydrolysis studies of the triterpene glycoside 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronopyranosyl-(1→2)-β-*D*-glucuronide (**1**) which was isolated from the commercial extract of the roots of *Glycyrrhia glabra*. The proton and carbon assignments for compounds **2** and **3** were made on the basis of the extensive 1D and 2D NMR (^1H and ^{13}C, COSY, HSQC, and HMBC) as well as mass spectral data.

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