NMR ANALYSIS AND HYDROLYSIS STUDIES OF GLYCYRRHIZIC ACID, A MAJOR CONSTITUENT OF *GLYCYRRHIA GLABRA*

Venkata Sai Prakash Chaturvedula[a]*, Oliver Yu[b] and Guohong Mao[a]

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From the commercial extract of the roots of *Glycyrrhia glabra*, a triterpene glycoside was isolated which was characterized as 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronopyranosyl-(1→2)-β-*D*-glucuronide; also known as Glycyrrhizic acid or Glycyrrhizin. The complete ^{1}H and ^{13}C NMR assignments of Glycyrrhizin were achieved by the extensive 1D (^{1}H and ^{13}C), and 2D NMR (COSY, HMQC, and HMBC) as well as mass spectral data. Further, hydrolysis studies were performed on Glycyrrhizin to identify aglycone and sugar residues in its structure. Further, configuration of sugar moieties in the triterpene glycoside obtained during the course of acid hydrolysis studies were confirmed by preparing their corresponding thiocarbamoyl-thiazolidine carboxylate derivatives with L-cysteine methyl ester and *O*-tolyl isothiocyanate and in comparison of their retention times with standard sugars.

Corresponding Authors
Email: saipc@bluecal-ingredients.com
[a] Natural Ingredient Development, Blue California, 30111 Tomas, Rancho Santa Margarita, CA 92688, USA
[b] Wuxi NewWay Biotechnology, 401 North Xingyuan Road, Wuxi 210043, P.R. China.

Introduction

Glycyrrhiza glabra (*Fabaceae*) also known as Licorice is a well-known medicinal herb that grows in various parts of the world.[1] It is one of the oldest and widely used herbs known since several thousand years ago both in western and eastern countries. From the ancient medical history of Ayurveda, Licorice has been used both as a medicine and also as a flavoring agent to disguise the unpleasant flavor of other medications. 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.[2-3] Varies species of *Glycyrrhiza* family has been shown to have great antioxidant, free radical scavenging and anticonvulsant activities. In some countries Licorice root is used in food and tobacco products.[4] *G. glabra* root extract have been used for more than 60 years in Japan to treat chronic hepatitis, and also have therapeutic benefit against other viruses, expectorant, antitussive, mild laxative and anti-aging activities.[4-5] The main chemical constituents of G. *glabra* is composed of triterpene saponin, flavonoids, polysaccharides, pectin, simple sugars, amino acids, mineral salts and various other components. Its major bio-active constituent of root is a triterpenoid saponin, 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronopyranosyl-(1→2)-β-*D*-glucuronide (**1**); also known as glycyrrhizic acid or glycyrrhizin. Glycyrrhizic acid (GA) is the most studied active constituent of licorice 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.[6-7]

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*[8-11] and flavonoids from *Hovenia dulcis.*[12] 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 structural characterization of a triterpenoid saponin, glycyrrhizic acid (**1**) isolated from the commercial extract of the roots of *G. glabra*, which were achieved on the basis of 1D (^{1}H and ^{13}C) and 2D (COSY, HMQC 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. Further, configuration of sugar moieties in the triterpene glycoside **1** was confirmed by preparing their corresponding thiocarbamoyl-thiazolidine carboxylate derivative with L-cysteine methyl ester and *O*-tolyl isothiocyanate in comparison of their retention times with standard sugars.

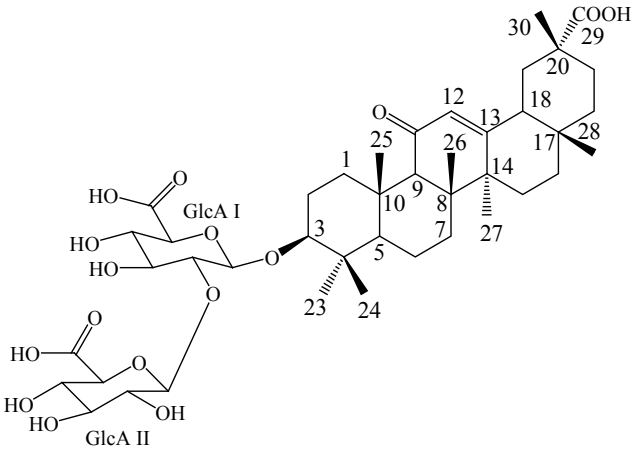

Figure 1. Structure of Glycyrrhizic acid (1)

EXPERIMENTAL

General Instrumentation Procedures

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 characterization of glycyrrhizin (1). NMR spectra were acquired on Bruker Advance 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 nanospray 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 by using the HPLC method as summarized below:

A binary solvent system comprising distilled water with 1% acetic acid (A) and 100 % acetonitrile (B) was used as mobile phase at a flow rate of 0.9 ml min^{-1} separations were performed using a linear gradient of increasing acetonitrile. Buffer B was increased from 15 % to 100 % in 25 min. The compounds were confirmed by the retention time and UV absorption at 254 nm.

Using the above mentioned HPLC method, collected the peak eluting at t_R 12.023 min over several rounds of injection of crude extract; dried the corresponding solution under nitrogen yielded a pure compound, which was characterized as **1**.

18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronopyranosyl-(1→2)-β-*D*-glucuronide (Glycyrrhizic acid, 1)

White powder; IR ν_{max}: 3312, 2965, 1722, 1055, 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* 823.4138 (calcd. for $C_{42}H_{63}O_{16}$: 823.4116); $(M+Na)^+$ *m/z* 845.3954 (calcd. for $C_{42}H_{62}O_{16}Na$: 845.3936).

Acid hydrolysis of 1

To a solution of compound **1** (5 mg) in MeOH (3 ml) was added 25 ml of 10 % H_2SO_4 and the mixture was refluxed for 72 hours. The reaction mixture was then extracted with ethyl acetate (EtOAc) (2 x 25 ml) to give an aqueous fraction containing sugars and an EtOAc fraction containing the aglycone part, which has been identified as glycyrrhetinic acid on the basis of comparison with the spectral data of the standard and co-TLC.[6-7] The aqueous phase was concentrated and compared with standard sugars using the TLC systems EtOAc/*n*-butanol/water (2:7:1) and CH_2Cl_2/MeOH/water (10:6:1);[13-15] the sugar was identified as glucose.

Determination of sugar configuration in 1

Compound **1** (3 mg) was hydrolyzed with 1 M HCl (10 mL) for 1.5 h. After cooling, the mixture was passed through an Amberlite IRA400 column and the eluate was lyophilized. The residue was dissolved in pyridine (5 mL) and heated with L-cysteine methyl ester HCl (15 mg) at 60 ºC for 1.5 h, and then *O*-tolyl isothiocyanate (100 µL) was added to the mixture and heated at 60 ºC for an additional 1.5 h. The reaction mixture was analyzed by HPLC: column Phenomenex Luna C18, 150 x 4.6 mm (5 u); 25 % acetonitrile-0.2 % TFA water, 1 mL min^{-1}; UV detection at 250 nm. The sugar was identified as D-glucuronic acid (t_R, 18.34 min) [authentic samples, D-glucuronic acid (t_R, 18.17) and L-glucuronic acid (t_R, 17.64 min)].[16]

Results and Discussion

Compound **1** 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* 823.4138 together with $[M+Na]^+$ adduct ion at *m/z* 849.3954, respectively; which were in good agreement with the molecular formula $C_{42}H_{62}O_{16}$. The chemical composition of **1** was further supported by the ^{13}C NMR spectral data.

The ^{1}H NMR spectra of compound **1** showed the presence of seven methyl sinlgets at δ 0.79, 1.05, 1.21, 1.27, 1.37, 1.42 and 1.44. Liebermann-Burchard reaction indicated compound **1** is having a terpenoid skeleton.[17-18] The signal corresponding to the H-3 of the oxymethine proton in the terpene moiety of **1** was appeared as a doublet of doublets at δ 3.37. Compound **1** also showed a proton at δ 5.98 as a singlet suggesting the presence of a trisubstituted olefinic bond. Further, the down field shift value of the trisubstituted olefinic proton indicated the presence of a carbonyl group at C-11 position, which was supported by the carbonyl group resonating at δ 200.1. The above spectral data supported the presence of oleanane triterpene skeleton having a hydroxyl group at C-3 position with a double bond at C-12/C-13 with seven methyl groups.

The presence of two sugar units in its structure was supported by the ^{1}H NMR spectrum of **1** which showed the anomeric protons at δ 5.08, and 5.47 as doublets. Acid hydrolysis of **1** with 10 % H_2SO_4 afforded glycyrrhetinic acid[6-7] and glucuronic acid[13-15] which were identified by direct comparison with authentic samples by co-TLC. The stereochemistry of the sugar was identified as D- glucuronic acid by preparing its corresponding thiocarbamoyl-thiazolidine carboxylate derivatives with L-cysteine methyl ester and *O*-tolyl isothiocyanate, and in comparison of their retention times with the standard sugars as described in the literature.[16] The large coupling constants observed for the

two anomeric protons of the glucose moieties at δ 5.08 (d, *J*=8.1 Hz), and 5.47 (d, *J*=8.4 Hz), suggested their β-orientation as reported earlier.[19-21] 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 Glycyrrhizic acid (**1**) recorded in C_5D_5N.[a-c]

Position	^{1}H NMR	^{13}C NMR
1	0.95 m, 3.05 dd (*J* = 8.1, 9.6)	39.9
2	1.75 m, 2.04 m	27.1
3	3.37 dd (*J* = 5.4, 11.6)	89.6
4	-	40.5
5	0.74 m	55.9
6	1.46 m, 1.68 m	18.1
7	1.48 m, 1.72 m	33.4
8	-	43.9
9	2.46 s	62.6
10	-	37.7
11	-	200.1
12	5.98 s	129.2
13	-	172.9
14	-	46.1
15	1.24 m, 2.12 m	27.2
16	1.08 m, 2.15 m	27.3
17	-	32.7
18	2.14 m	49.2
19	1.55 m, 2.34 m	42.2
20	-	44.6
21	1.53 m, 2.08 m	32.1
22	1.30 m, 1.73 m	38.9
23	1.21 s	28.6
24	0.79 s	17.2
25	1.27 s	17.3
26	1.37 s	19.3
27	1.42 s	24.1
28	1.05 s	29.2
29		179.7
30	1.44 s	29.3
Glucuronic Acid I (GlcA I)		
1′	5.47 d (*J* = 8.4)	107.5
2′	4.68 dd (*J* = 8.2, 9.4)	85.1
3′	4.32 dd (*J* = 8.1, 9.6)	78.0
4′	4.45 t (*J* = 9.4)	78.9
5′	4.66 t (*J* = 9.6)	78.2
6′		73.8
Glucuronic Acid II (GlcA II)		
1″	5.08 d (*J* = 8.1)	105.6
2″	4.64 dd (*J* = 8.4, 9.6)	78.3
3″	4.30 dd (*J* = 8.2, 9.1)	78.1
4″	4.38 t (*J* = 9.6)	77.3
5″	4.64 t (*J* = 9.4)	73.5
6″		172.6

[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 179.7 from the ^{13}C NMR spectral data of **1** suggested the presence of an acid functional group. The presence of the carboxylic acid group was identified at C-29 position by the key COSY and HMBC correlations as shown in Figure 2.

Based on the results from NMR spectral data and hydrolysis experiments, it was concluded that the structure of **1** has a oleanane triterpene aglycone moiety with an α,β-unsaturated carbonyl group, a carboxylic acid group, seven methyl singlets and two β-D-glucuronyl units. Thus, the structure of **1** was assigned as the known compound glycyrrhizic acid. The physical and spectral data are consistent to the reported literature values of glycyrrhizic acid.[6-7]

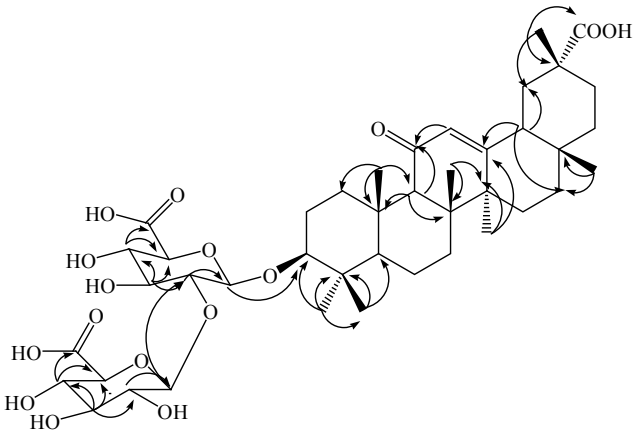

Figure 2. Key HMBC correlations of Glycyrrhizic acid (**1**)

Conclusions

We are herewith reporting the isolation and complete ^{1}H and ^{13}C NMR spectral assignments for 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronopyranosyl-(1→2)-β-*D*-glucuronide (Glycyrrhizic acid, **1**) that were made on the basis of extensive 1D and 2D NMR spectral data as well as high resolution mass spectral data.

Further, acid hydrolysis of **1** furnished D-glucuronic acid suggesting the presence of only one sugar unit and its configuration was confirmed for the first time by preparing its corresponding thiocarbamoyl-thiazolidine carboxylate derivatives with L-cysteine methyl ester and *O*-tolyl isothiocyanate.

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