Confirmation of the configuration of two glucuronic acid units in glycyrrhizic acid
(Received October 22, 2014)
(Accepted December 15, 2014)

Yusai Ito a, b, Kyoko Ishizuki a, Naoki Sugimoto a, Tsukiko Tada a, Takumi Akiyama a,
Kyoko Sato a, Hiroshi Akiyama a, Yukihiro Goda a

a) National Institute of Health Sciences
b) Kyoritsu Women’s University

Abstract
Glycyrrhizic acid (GA), a triterpenoid saponin containing two glucuronic acid (GlcA1 and GlcA2) units, is found in the roots of Glycyrrhiza plants, and has been widely used as a natural sweetener for foods as well as a natural medicine. Purified GA is commercially available from various manufacturers as an analytical standard or a biochemical reagent. While producers describe the configurations of GlcA1 and GlcA2 as α and β-forms, respectively, reports of the structural elucidation of GA have proposed that both GlcA units are β-form. To clarify this point, commercial GA from various sources was analyzed by 1D and 2D NMR studies. Results confirmed that the actual configuration of both GlcA units in GA is β-form.

Keywords: glycyrrhizic acid, glucuronic acid, natural sweetener

I Introduction
Glycyrrhizic acid (GA) is a triterpenoid saponin found in Glycyrrhiza plants such as Glycyrrhiza glabra (licorice), G. inflata, and G. uralensis. Since GA is 30–50 times sweeter than sucrose, root extracts of Glycyrrhiza (known as licorice root extract) have been used as a natural sweetener for foods. In addition, it has been extensively reported that GA has several pharmacological activities, including anti-inflammatory, immunomodulatory, anti-ulcer, and anti-tumorigenic effects. Moreover, licorice root is a well-known oriental and occidental herbal medicine that is frequently prescribed for the treatment of various diseases. Purified GA is commercially available, and is utilized as an authentic standard in natural medicines and as a research agent for the investigation of biochemical and pharmaceutical activities.

GA is composed of a triterpenoid aglycone, glycyrrhetinic acid (GLA), and two glucuronic acid units (GlcA1 and GlcA2). The two GlcA units are connected via a 1 → 2 glycoside linkage (Fig. 1) and the GlcA1 connects to position 3 of the aglycone GLA via a glycoside linkage. With respect to the configuration of the two GlcA units, recent papers dealing with GA prepared from Glycyrrhiza plants proposed that both were β-form. However, we noted that the labels of commercial GA and GA salts indicate the configurations of GlcA1 and GlcA2 as α-form and β-form, respectively. In our investigation of commercial reagent labeling, all commercial GA and GA salts were labeled as containing the α-form configuration of GlcA1 in their product catalogues. The origin of the labeling might be an authentic database such as the
Chemical Abstract Service (CAS) that states that the structure of GlcA1 is α-form, e.g., GA (CAS Registry Number: 1405-86-3), GA ammonium salt (CAS Registry Number: 53956-04-0), and GA dipotassium salt hydrate (CAS Registry Number: 68797-35-3). These inconsistencies create confusion in analytical and biochemical investigations using commercial GA reagents as authentic standards.

In order to clarify the present situation, we present the correct structural determination of commercial GA reagents purchased from various distributors using high-resolution NMR analysis.

II Material and Methods

Reagents: Two GA reagents and five GA ammonium salt reagents were purchased from seven sources as follows: glycyrrhizic acid standard (Wako Pure Chemical Industries, Ltd., Osaka, Japan), glycyrrhizic acid (Tokyo Chemical Industry co., Ltd., Tokyo Japan), glycyrrhizic acid monoammonium salt (Sigma-Aldrich, MO, USA), glycyrrhizic acid monoammonium salt standard (Kanto Chemical Co., Inc., Tokyo, Japan), glycyrrhizic acid ammonium salt, trihydrate (LKT Labs, Inc., MN, USA), glycyrrhizic acid ammonium salt (ChromaDex, Inc., CA, USA), and glycyrrhizic acid monoammonium salt (Acros Organics, Geel, Belgium). Commercial catalogues of these sources noted the configurations of GlcA1 and GlcA2 in all reagents as α-form and β-form, respectively.

NMR study: 1D (1H and 13C) and 2D NMR (1H-1H COSY, 1H-13C HMQC, 1H-13C HMBC, and NOESY experiments) spectra of GA were recorded on JEOL ECA instruments (600 MHz) in CD3OD as the solvent at 25°C. The methyl proton signal at 3.30 ppm in CD3OD was referenced on the NMR measurement.

III Results

Seven commercial GA or GA ammonium salts were analyzed using NMR in this study. The commercial catalogues supplied by seven sources indicated the configuration of GlcA1 in GA as α-form, and that of GlcA2 as β-form. To confirm the configuration of GlcA units in GA, the standard GA reagent purchased from Wako Pure Chemical Industries was first analyzed using NMR. The chemical shifts (δh and δc) of GA were recorded in CD3OD at 25°C and all signals were assigned by the analysis of 1D (1H and 13C) and 2D NMR experiments (1H-1H COSY, 1H-13C HMQC, 1H-13C HMBC, and NOESY experiments). The 13C NMR spectrum of GA showed 42 signals, including a typical ketone signal (δc 202.7) corresponding to C11 on GLA and three carboxyl signals (δc 180.4, 172.6 and 172.1) corresponding to C30, C6' and C6", respectively (Table 1). The aglycone GLA is known to be an 18β-H-oleanane-type compound (18β-GLA)9. The 18α-epimer

<table>
<thead>
<tr>
<th>position</th>
<th>δH (multiplet, JHz)</th>
<th>δC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1'</td>
<td>4.51 (d, 7.5)</td>
<td>105.3</td>
</tr>
<tr>
<td>1&quot;</td>
<td>4.62 (d, 7.7)</td>
<td>106.3</td>
</tr>
<tr>
<td>2'</td>
<td>3.50 (t, 9.5)</td>
<td>84.0</td>
</tr>
<tr>
<td>2&quot;</td>
<td>3.28 (m)</td>
<td>76.3</td>
</tr>
<tr>
<td>3'</td>
<td>3.58 (t, 9.8)</td>
<td>77.1</td>
</tr>
<tr>
<td>3&quot;</td>
<td>3.38 (t, 9.8)</td>
<td>77.7</td>
</tr>
<tr>
<td>4'</td>
<td>3.54 (t, 9.8)</td>
<td>72.9</td>
</tr>
<tr>
<td>4&quot;</td>
<td>3.52 (t, 9.5)</td>
<td>73.1</td>
</tr>
<tr>
<td>5'</td>
<td>3.74 (d, 9.7)</td>
<td>77.6</td>
</tr>
<tr>
<td>5&quot;</td>
<td>3.74 (d, 9.7)</td>
<td>77.6</td>
</tr>
<tr>
<td>6'</td>
<td>172.6</td>
<td></td>
</tr>
<tr>
<td>6&quot;</td>
<td>172.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Chemical shifts of glycyrrhizic acid (GA) in CD3OD
of GLA was previously prepared from 18β-GLA and its derivatives were intensively synthesized and investigated for various pharmaceutical activities, including anticancer activity. The isomerization from 18β-epimer to 18α-epimer proceeded under an alkaline condition, which implies a possibility of contamination of 18α-isomer in GA reagents. Therefore, this study attempted to confirm the entire structure of GA, including GLA, using NMR.

To confirm the aglycone GLA structure, the detailed structure of GA was analyzed by 2D NMR experiments. The $^1$H-$^1$C HMQ experiment correlated all proton signals with the corresponding 21 carbon atoms. As shown in Fig. 2, the cross peaks observed in the COSY experiment gave five spin-spin systems (H1-H3, H5-H7, H15-H16, H18-H19, and H21-H22). The $^1$H-$^1$C HMBC correlations of seven singlet methyl signals (H23, H24, H25, H26, H27, H28, and H29) allowed the connection of these methyl groups to adjacent quaternary carbons (C4, C8, C10, C14, C17, and C20). Subsequently, the quaternary carbons were connected to the spin-spin systems by the HMBC correlations to yield two six-membered rings, namely the A and E rings (Fig. 2). The HMBC correlations from a singlet methine signal (H9) to C8, C10, and C11 revealed a bridge connection of two quaternary carbons of C8 and C10 via C9 to yield B rings, and further indicated the ketone (C11) to be located adjacent to C9 (Fig. 2). The HMBC correlations from a singlet olefin signal (H12) to the ketone C11 and to a quaternary olefinic carbon (C13) constructed the connection from C11 to C13. This connection was further extended to the quaternary carbon (C14) by the HMBC correlation of the methyl signal (C27) to C13 to yield a 6/6/6 ring system, namely the A, B, and C rings. The HMBC correlation from H12 to C18 showed a connection between C13 and C18 to make ring D. Finally, a carboxylic acid (C30) was connected to the quaternary carbon (C20) on the E ring by the HMBC correlations from the adjacent methyl signal (H29) to C30. Thus, the planar structure of GA was confirmed to be a triterpene structure containing five rings (Fig. 2).

Subsequently, the stereochemistry of GLA was elucidated from the NOESY correlations and coupling constants (Fig. 3). The NOESY correlations around the A and B rings (H1a/ H3, H3/H5, H1a/H5, H1a/H9, and H5/H9) indicated axial orientations of all these protons, which means that the hydroxyl group at C3 has a β-orientation. The equatorial position of the C23 methyl group was confirmed by a NOESY correlation observed between H3 and H23. The large coupling constant ($J_{H3,H23} = 13.5$ Hz) indicated an axial orientation at C18 on the E ring. The NOESY correlations between H18 and H28 showed the cis-form of H18 and the methyl group (C28). In addition to these data, the NOESY correlations between H12 and H18 indicated the δ-orientation of H18. The NOESY correlation between H29 and H16 showed that the carboxylic acid (C30) had an axial orientation, as with H18. These results confirmed that the aglycone of GA was 18β-glycyrrhetinic acid (18β-GLA).

To confirm the structure of the glucuronic acids (GlcA1 and GlcA2), the structure of GlcA1 and GlcA2 were analyzed by 2D NMR experiments and from the $J$ values from their $^1$H NMR spectra. The spin-spin systems starting from the anomeric proton (H1') at δH 4.51 to H5' at δH 3.76 in the GlcA1 unit was assigned by the COSY experiment (Fig. 2). The presence of a carboxylic acid of GlcA1 was confirmed by the HMBC correlation from H-5' to a carboxyl carbon (C6') at δC 172.6 (Table I). The signal of H1' was split into a doublet and showed a coupling constant ($J_{H1,H2}$) of 7.5 Hz (Table I). Generally, anomeric configurations are assigned from the magnitude of $J_{1,2}$ with values of 7-9 Hz for the diaxial coupling associated with a β-anomers, while 2-4 Hz is indicative of the equatorial-axial coupling of α-anomers. Furthermore, the coupling constants of other oxymethin protons, such as $J_{H2/H3}$, $J_{H3/H4}$, and $J_{H4/H5}$, were observed to be around 9.5 Hz (Table I). These values were sufficiently large to assure axial orientations for the five protons (H1' ~ H5'), allowing a chair form of a pyranosyl ring of GlcA1 (Fig. 3). This interpretation was also supported by the NOESY.

![Fig. 2. Key correlations of $^1$H-$^1$H COSY (bold lines) and $^1$H-$^1$C HMBC (arrows) of GA.](image)

![Fig. 3. Structure of GA and NOESY correlations.](image)
correlations for H1'/H3'; H1'/H5'; H3'/H5'; and H2'/H4' (Fig. 3). Thus, the glycosidic bond at C1' in the glucurono-pyranosyl structure of GlcA1 is oriented equatorially and this result definitely indicated that GlcA1 is β-form, not α-form. The glycosidic linkage from the anomeric proton to the aglycone GLA was confirmed by the HMBC correlations from H1' to C3 (Fig. 2). The structure of glucuronic acid, GlcA2, was also confirmed in a similar manner to GlcA1. An anomeric proton (H1") observed at δH 4.62 also had a large coupling constant (7.7 Hz), confirming the β-form of GlcA2, as with GlcA1 (Fig. 4). The inter-glycosidic linkage was confirmed to be a β1 → 2 link by the HMBC correlation from H1" to C2' at δC 84.0 of GlcA1 (Fig. 2). The chirality of GlcA units were determined to be both β-form, because previous studies indicated that both units were α-form and furthermore the presence of L-GlcA has not been reported from any natural sources. Based on the foregoing evidence, we concluded that the structure of GA is 3β-hydroxy-11-oxo-18β-hydroxy-11-oxo-18PH-olcan-12-en-30-oic acid 3-0-[β-0-glucuronopyranosyl-(1 → 2)-β-o-glucuronopyranoside].

In six other commercial GA or GA ammonium salt reagents, the configurations of the two GlcA units were both β-form, as observed with the GA from Wako (Fig. 4). Two anomeric protons of GlcA1 and GlcA2 showed identical coupling constants in each GA or GA ammonium salt, although there were slight differences in chemical shifts between GA and GA ammonium salts (Fig. 4).

![Fig. 4. 1H NMR spectra (600 MHz) around anomeric proton signals of GlcA1 and GlcA2 of seven GA reagents in CD3OD](image)

Five spectra (a ~ e) are GA ammonium salts, while the remaining two spectra (f and g) are GA.

### IV Discussion

The present NMR studies demonstrated that GlcA1 and GlcA2 were both β-form in the seven GA and GA ammonium salt reagents purchased from seven sources. These results clearly indicated that the supplier information on GA structure is incorrect. We have little information as to why this misinterpretation of the GA structure has occurred. In 1950, Lythgoe and Trippett first identified two hexuronic acids in GA as GlcA units[15]. Furthermore, they proposed that the stereochemistry of GlcA units was one β-link as the internal glycosidic bond and the other as an α-link; this was determined by comparison of [α]D values between a permethylate derivative of GlcA units prepared from GA and authentic glycosidic compounds. This interpretation was generally accepted, and the α-configuration of GlcA in GA was taken as correct. After approximately 40 years, however, Khalilov et al. revealed the configuration of GlcA1 to be β-form based on an 13C-NMR study of GA purified from natural sources[12]. Report of the revised structure was subsequently followed by an advanced NMR study on GA[13].

During research on natural products in *Glycyrrhiza* plants, GA was often isolated as a by-product and its structure was elucidated[1-8]. These reports also showed that the GlcA1 configuration of the isolated GA is β-form. Furthermore, the X-ray crystal structure of GA dipotassium salt was recently analyzed to evaluate the coordination system of potassium ions to GA, which subsequently indicated GlcA1 to be β-form[14, 15]. Accordingly, recent research has determined the GlcA1 configuration in GA to be β-form. Nevertheless, commercial reagent catalogues and chemical databases, including package inserts for drugs, designated GlcA1 in GA as α-configuration, which has led to confusion in the research areas of analytical chemistry and biochemistry.

GA is one of the most well known and successful natural sweeteners, and is also used as a phytomedicine. Moreover, numerous biochemical and chemical studies dealing with GA have been reported. Yet, surprisingly, its incorrect structure continues to be used in commercial catalogue product information. Furthermore, since the incorrect catalogue information is likely to be recognized as the standard structure of the compound, this might lead to misinterpretation of research results. We believe that this study definitively clarifies this misinformation, and we urge the rapid revision of the incorrect structure of GA in commercial catalogues and other literature.

### V References

1) Ming, L. J., Yin, A. C.: Therapeutic effects of glycyrrhizin


論文

グリチルリチン酸に含まれるグルコン酸の立体化学の確認
(2014年10月22日受付)
(2014年12月15日受理)

伊藤裕才 a,b）、石附京子 a）、杉本直樹 a）、多田敦子 a）、秋山卓美 a）、佐藤恭子 a）、篠山 浩 a）、合田幸広 a)

a) 国立医薬品食品衛生研究所
b) 共立女子大学

キーワード: グリチルリチン酸、グルコン酸、天然甘味料

概要

グリチルリチン酸 (GA) は 2 つのグルコン酸 (GlcA1 と GlcA2) を含むトリルペル型サボニンである。GA はカンゾウ属 (Glycyrrhiza) の植物の根に含まれている。生薬として、また食品に添加する天然甘味料として長く用いられてきた。精製された GA は分析用の標準品または生化学試薬として複数の試薬会社から入手が可能である。試薬会社のカタログには、GlcA1 の立体化学はα型で、一方の GlcA2 はβ型と記されている。Chemical Abstract においても、GlcA1 は α 型で GlcA2 は β 型とされている。しかしながら近年の研究では、2 つの GlcA はともに β 型との報告が続いている。この混乱を解決すべく、複数の試薬会社から高純度に精製された GA または GA 塩の試薬を入手し、1次元および2次元 NMR によって構造を詳細に解析した。その結果、2 つの GlcA はともに β 型であることが確認された。