Preparation of Glycyrrhetinic Acid Monoglucuronide by Selective Hydrolysis of Glycyrrhizic Acid via Biotransformation

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Abstract: **Objective** To search for the microorganisms which have the high selectivity of hydrolyzing glycyrrhizic acid (GL) into 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronide (GAMG) without glycyrrhetinic acid (GA) byproduct. **Methods** GL was biotransformed by *Aspergillus* sp., the products were separated by chromatography on reverse phase C_{18} column and semi-preparative HPLC, and their structures were elucidated on the basis of HR-ESI-MS, 1D NMR (^{1}H-NMR, ^{13}C-NMR, and NOESY) and 2D NMR (^{1}H-^{1}H COSY, HSQC, and HMBC) spectral analyses. **Results** *Aspergillus* sp. could partially hydrolyze GL into GAMG (**3**), along with two minor byproducts, 3-*O*-β-*D*-glucuronopyranosyl-18β-liquiritic acid (**1**) and 3-*O*-β-*D*-glucuronopyranosyl-24-hydroxy-18β-glycyrrhetinic acid (**2**). **Conclusion** *Aspergillus* sp. has the high selectivity of hydrolyzing GL into GAMG without GA byproduct and the yield of GAMG is about 60%. The complete assignments of ^{1}H-NMR and ^{13}C-NMR data for compounds **1** and **2** are reported for the first time.

Key words: *Aspergillus* sp.; biotransformation; 18β-glycyrrhetinic acid 3-*O*-β-*D*-glucuronide; glycyrrhizic acid; NMR
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Introduction

Glycyrrhizic acid [GL, 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronopyranosyl-(1→2)-β-*D*-glucuronide], a principle component of licorice extracts, has been used as a sweetener in food industry and as a traditional herbal ingredient with activities of antivirus, anti-inflammation, antitumor, and so on (Cinatl *et al*, 2003). The structure of GL contains one molecule of glycyrrhetinic acid (GA) and two molecules of glucuronic acid (Hennell *et al*, 2008), and it could be transformed into compound **3**, glycyrrhetinic acid monoglucuronide (GAMG, 18β-glycyrrhetinic acid-3-*O*-β-*D*-glucuronide) (Fig. 1) by removing one terminal glucuronic acid (Lu *et al*, 2006). The sweetness of GAMG as a sweetener is 1000-fold higher than that of sucrose and 5-fold higher than that of GL with low calorie (Mizutani *et al*, 1994). It was reported that GAMG had the similar (or stronger) pharmacological activities compared to GL, such as antitumor, antivirus, anti-allergic, and anti-inflammatory activities (Mizutani *et al*, 1998; Park *et al*, 2004; Ito *et al*, 1988). Previously, biotransformation was the main method to prepare GAMG. But few strain or enzyme could hydrolyze GL into GAMG without GA (Feng *et al*, 2006) or some strains could hydrolyze GL into GAMG and GA (Hala *et al*, 2011) or only GA, and the gene which could transform GL into GAMG was not clarified from all these reports. The best method to prepare industrial product GAMG is cloning the active gene. To search for the microorganisms which have the high selectivity to hydrolyze GL into GAMG, more than 200 strains were screened and one fungus was found which could transform GL into GAMG without GA. The investigation on the biotransformation of GL led to the isolation

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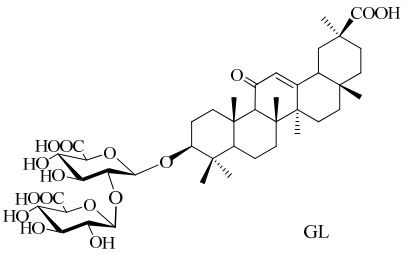
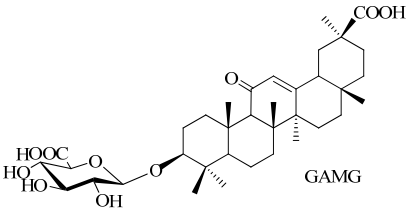
of GAMG (**3**), along with two minor derivatives of GAMG, 3-*O*-β-*D*-glucurono-pyranosyl-18β-liquiritic acid (**1**) and 3-*O*-β-*D*-glucurono-pyranosyl-24-hydroxy-18β-glycyrrhetinic acid (**2**). In the present paper, we described the purification and identification of the biotransformed products of GL. Compounds **1** and **2** had been reported previously but with incomplete NMR spectral data (Liu *et al*, 2001; Hayashi *et al*, 1992). The structures of compounds **1** and **2** were identified based on 1D NMR (^{1}H-NMR, ^{13}C-NMR, and NOESY) and 2D NMR (^{1}H-^{1}H COSY, HSQC, and HMBC), and the ^{1}H-NMR and ^{13}C-NMR data were completely assigned for the first time.



GL



GAMG

Fig. 1 Structures of GL and GAMG

Materials and methods

Materials

Aspergillus sp. was isolated from soil collected in Kashi of Xinjiang Uygur Autonomous Region (China) by the laboratory of Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College (China), and this strain was saved in our laboratory. GL with the purity of 60% as a substrate was purchased from Tianjin Zhongyi Pharmaceutical Co., Ltd. (China).

Equipments

The HR-ESI-MS data were recorded on Synapt Q/TOF MS (Waters Corporation, USA). The NMR spectra were recorded on Varian^{UNITY} Inova 600 (600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR) in CD_3OD, and the chemical shifts were calculated with TMS as an internal standard. HPLC was performed on Waters 2695 Alliance system (Waters, USA) equipped with a Hanbon lichrospher C_{18} column (250 mm × 4.6 mm, 5 μm, Hanbon Sci & Tech, China), a semi-preparative column, and a Hanbon lichrospher C_{18} column (250 mm × 10.0 mm, 5 μm, Hanbon Sci & Tech, China); Thin layer chromatography (TLC) was performed on a pre-coated kiesel gel GF_{254} plate (0.2—0.25 mm, 200 mm × 100 mm, Qingdao Haiyang Chemical Group Co., Ltd., China) developed with $CHCl_3$-MeOH-H_2O (70:26:6), and the detection was achieved by 10% of H_2SO_4-EtOH (10:90) solution followed by heating.

Extraction and isolation

More than 200 strains (including bacteria, actinomycetes, and fungi) were cultivated with GL (6 mg) and shaken for 4 d at (28 ± 1) ℃ in modified yeast peptone glucose (YPG) medium containing yeast (0.4%), glucose (1.5%), KH_2PO_4 (0.1%), and $MgSO_4 \cdot 7H_2O$ (0.05%). Then the pH value was adjusted to 6.0. Each fermentation was extracted by *n*-butanol saturated with water. The transformed products were detected by TLC and ascertained by HPLC. At last, it was found that a fungus, tentatively named *Aspergillus* sp., had the activity of converting GL into GAMG.

GL (4 g) was cultured with *Aspergillus* sp. for 4 d in modified YPG medium and shaken at (28 ± 1) ℃. Then the biotransformed product was extracted with acetic ether to remove GL remained.

The extract was concentrated under the reduced pressure to give 1.488 g of residue (Fr. A). Then Fr. A (1.4 g) was chromatographed on reverse phase C_{18} column with a gradient mixture of CH_3OH-H_2O (83:17→86:14) to give compound **3** (905.6 mg) and Fr. A_1 (150.8 mg). Fr. A_1 was chromatographed by semi-preparative HPLC eluted with CH_3OH-H_2O (82:18) to yield compounds **1** (72.4 mg) and **2** (26.5 mg) (Fig. 2).

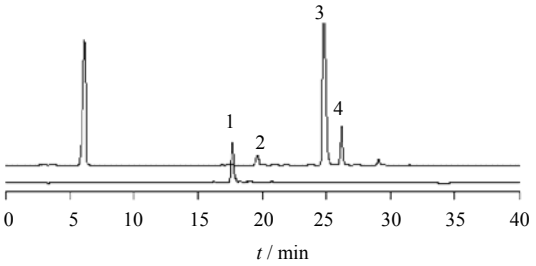


Fig. 2 HPLC chromatograms of GL and transformed products

1: GL 2: compound **2** 3: compound **3** 4: compound **1**

Results and discussion

After screening more than 200 strains including bacteria, actinobacteria, and fungi isolated from the soil, a strain named *Aspergillus* sp. was found and used to hydrolyze GL into GAMG without GA. To obtain enough GAMG, GL (4 g) was transformed by *Aspergillus* sp. into GAMG and two minor derivatives of GAMG were obtained.

The yield of GAMG from the transformation of GL was about 60%, and there was no GA in the biotransformation product. The yield would be increased by optimizing the conditions of reaction. The biotransformed products were chromatographed on reverse phase C_{18} column and further purified by semi-preparative HPLC to afford compounds **1** — **3**. Comparison of the physicochemical properties and ^{13}C-NMR data with those reported in the literature (Park *et al*, 2004) allowed us to identify compound **3** as GAMG. The structures of compounds **1** and **2** were elucidated by 1D NMR and 2D NMR in combination with MS data (Fig. 3).

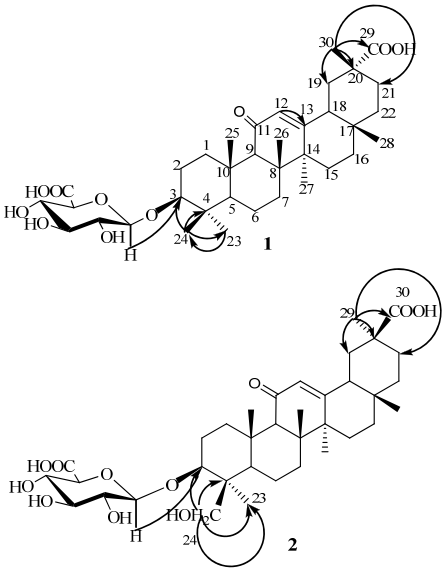


Fig. 3 Key HMBC correlations of compounds 1 and 2

Compound **1**: white amorphous powder. The HR-EI-MS spectrum showed the pseudomolecular ion at *m/z* 645.3630 $[M-H]^-$ (calc. for $C_{36}H_{53}O_{10}$ 645.3639), and a significant fragment was *m/z* 469.3328 $[M-H-176]^-$, indicating that a glucuronyl residue has been lost. It had the same formula as GAMG ($C_{36}H_{54}O_{10}$), but different remaining time in HPLC. The ^{1}H-NMR spectrum of compound **1** in CD_3OD showed seven methyl proton signals at δ_H 1.06 (s, H-23), 0.87 (s, H-24), 1.14 (s, H-25), 1.14 (s, H-26), 1.42 (s, H-27), 0.91 (s, H-28), and 1.23 (s, H-30), and a trisubstituted olefinic proton at δ_H 5.54. The ^{13}C-NMR spectrum showed two carboxyl groups at δ_C 182.1 (C-29) and 172.8 (C-6′), one carbonyl group at δ_C 202.6 (C-11), and two olefinic carbon signals at δ_C 129.1 (C-12) and 172.5 (C-13). The HMBC experiment showed long-range correlations between δ_H 1.06 (H-23) and δ_C 16.94 (C-24), 40.53 (C-4), 56.38 (C-5), and 90.70 (C-3); and between δ_H 1.23 (H-30) and δ_C 182.1 (C-29), 43.35 (C-20), 40.37 (C-19), and 30.26 (C-21). Comparison of the ^{13}C-NMR data of compound **1** with those of GAMG (Park *et al*, 2004), the difference existed in signals at δ_C 19.68 (C-30) and 182.1 (C-29) [signals of GAMG at 29.2 (C-29) and 180.4 (C-30)]. To determine the configuration of C-30, the NOESY spectrum was used. When H-28 was irradiated, the NOESY spectrum showed the correlation between H-28/H-30 and H-18, which indicated that the methyl at C-20 and the proton at C-18 were β orientations. In the HMBC, the long-range correlations between the anomeric proton signal δ_H 4.37 (H-1′) and δ_C 90.70 (C-3) indicated that the glucuronyl group was attached to C-3 (Fig. 2). On the basis of these data compound **1** was identified as 3-*O*-β-*D*-glucuronopyranosyl-18β-liquiritic acid (Liu *et al*, 2001).

Compound **2**: white amorphous powder. The HR-ESI-MS spectrum showed the deprotonated molecular ion at *m/z* 661.3589 $[M-H]^-$ (calcd. for $C_{36}H_{53}O_{11}$ 661.3588), two characteristic fragments at *m/z* 485.3282 $[M-H-176]^-$ and 467.3161 $[M-H-176-H_2O]^-$ which indicated there was a glucuronyl residue and a hydroxy group lost. The molecular formula $C_{36}H_{54}O_{11}$ was also supported by the ^{1}H-NMR and ^{13}C-NMR data. The ^{1}H-NMR spectrum in CD_3OD showed six methyl proton signals at δ_H 0.82 (s, H-28), 1.21 (s, H-23), 1.16 (s, H-29), 1.12 (s, H-26), 1.07 (s, H-25), and 1.41 (s, H-27), and a pair of coupled methylene proton signals at δ_H 4.02 (1H, d, J = 11.4 Hz) and 3.36 (1H, d, J = 11.4 Hz), indicative of the secondary alcoholic functionality. In the ^{1}H-NMR spectrum, the anomeric proton signal had been recognized at δ_H 4.45 (d, J = 7.8 Hz). Comparing the ^{13}C-NMR data of compound **2** with those of compound **1** and GAMG, a new signal at δ_C 64.00 and one

disappeared methyl proton signal suggested there was a hydroxylated secondary carbon. The HMBC experiment showed long-range correlations between the anomeric proton signal δ_H 4.45 (H-1′) and δ_C 90.37 (C-3), indicated that the glucuronyl group was attached to C-3. The long-range correlations between δ_H 4.02 and δ_C 23.06 (C-23), 45.13 (C-4), 56.73 (C-5), and 90.37 (C-3) confirmed that the hydroxyl group was attached to C-24 (Fig. 3). The detailed analyses of the ^{1}H-NMR and ^{13}C-NMR spectra with the aid of ^{1}H-^{1}H COSY, HSQC, and HMBC spectra of compounds **1**—**3** were summarized in Table 1. C-18 at 49.9 indicated H-18 was β orientation (Khalilov *et al*, 1991). In NOESY spectrum, when H-23 was irradiated, the NOESY

Table 1 ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) data for compounds 1 and 2 in CD_3OD and compound 3 in pyridine-d_5 (δ, J in Hz)

No.	Compound **1**		Compound **2**		Compound **3**
	δ_C	δ_H	δ_C	δ_H	δ_C
1	40.2	2.67 (brd, 13.8), 1.01 (m)	39.9	2.68 (brd, 13.8m), 1.00 (m)	39.5
2	27.0	1.84 (m), 1.75 (m)	27.1	1.85 (m), 1.82 (m)	26.9
3	90.7	3.18 (dd, 4.8, 12)	90.4	3.41 (dd, 6.6, 10.2)	88.8
4	40.5	—	45.1	—	40.0
5	56.4	0.80 (m)	56.7	0.94 (m)	55.3
6	18.4	1.45 (m), 1.62 (brd, 13.2)	18.8	1.70 (m), 1.44 (m)	17.8
7	33.8	1.73 (m), 1.43 (m)	33.9	1.72 (m), 1.45 (m)	33.0
8	46.8	—	46.7	—	43.5
9	63.1	2.46 (s)	62.8	2.45 (s)	62.1
10	38.1	—	37.7	—	37.3
11	202.6	—	202.4	—	199.5
12	129.1	5.54 (s)	128.9	5.57 (s)	128.7
13	172.5	—	172.8	—	172.8
14	44.7	—	44.6	—	45.6
15	27.4	1.87(m), 1.25 (m)	27.6	1.87 (m), 1.24 (m)	26.7
16	27.4	2.16 (td, 13.2, 4.2), 1.02 (m)	27.4	2.14 (td, 12.6, 4.2), 1.03 (m)	26.8
17	33.4	—	33.0	—	32.2
18	47.9	2.21 (m)	49.9	2.19 (dd, 3.0, 13.2)	48.8
19	40.4	2.25 (m), 1.34 (brd, 12.6)	42.4	1.83 (m), 1.69 (m)	41.7
20	43.3	—	44.9	—	44.1
21	30.3	1.89 (m), 1.48 (m)	32.0	1.93 (m), 1.39 (o)	31.6
22	36.4	1.55 (td, 14.4, 4.2), 1.39 (m)	39.0	1.40 (o), 1.37 (m)	38.5
23	28.4	1.06 (s)	23.1	1.21 (s)	28.2
24	17.0	0.87 (s)	64.0	4.02 (d, 11.4), 3.36 (d, 11.4)	16.8
25	17.0	1.14 (s)	16.7	1.07 (s)	17.0
26	19.3	1.14 (s)	19.1	1.12 (s)	18.8
27	23.9	1.42 (s)	23.8	1.41 (s)	23.6
28	28.9	0.91 (s)	29.2	0.82 (s)	28.7
29	182.1	—	28.7	1.16 (s)	28.8
30	19.7	1.23 (s)	180.4	—	179.2
GlcA					
1′	107.0	4.37 (d, 7.8)	106.2	4.45 (d, 7.8)	107.3
2′	75.3	3.24 (dd, 7.8, 9.0)	75.2	3.20 (dd, 7.8, 9.0)	78.2
3′	77.7	3.35 (dd, 9.0, 9.6)	77.7	3.37 (m)	78.0
4′	73.2	3.50 (t, 9.6)	73.3	3.50 (dd, 9.0, 9.0)	75.6
5′	76.5	3.75 (d, 9.6)	76.1	3.76 (d, 10.2)	73.5
6′	172.8	—	1 72.8	—	169.6

correlations between H-23/H-3 and H-5 were observed, but no correlation between H-23 and H-25, indicated that C-23 was α-configuration and C-24 was β-configuration. On the basis of these data, compound **2** was identified as 3-*O*-β-*D*-glucuronopyranosyl-24-hydroxy-18β-glycyrrhetinic acid (Hayashi *et al*, 1992).

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