Two New Steroidal Saponins from "Gualou-xiebai-baijiu-tang" Consisting of *Fructus trichosanthis* and *Bulbus allii macrostemi*

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Two new steroidal saponins were isolated from the Chinese folk medicine called Gualou-xiebai-baijiu-tang. The structures were determined to be spirost 25(27)-ene-2β,3β-diol-3-*O*-β-D-glucopyranosyl(1→2)-β-D-galactopyranoside and 26-*O*-β-D-glucopyranosyl-22α-hydroxy-5β-furost-25(27)-ene-1β,3β,6β,26-tetraol-3-*O*-β-D-galactopyranoside, respectively, based on chemical evidences and spectral analysis.

Key words Gualou-xiebai-baijiu-tang; spirostanol saponin; furostanol saponin

Gualou-xiebai-baijiu-tang is a well-known classic preparation in traditional Chinese medicine. It originates from *Jingui Yaolüe*, a great pharmaceutical book written by the famous physician Zhang Zhongjing during the Han dynasty. The prescription consists of *Fructus trichosanthisi* and *Bulbus allii macrostemi* and is extracted with wine. In traditional Chinese medicine, this prescription is used to activate the blood circulatory system and eliminate stasis. It has been used to treat coronary heart disease and angina pectoris for several hundred years and has achieved good results in modern clinical practice.[1)] Research on the prescription has focused on its pharmacological activities and the chemical constituents of *Trichosanthes kirilowii* and *Allium macrostemon*. We have isolated 22 new steroidal saponins and several nitrogen-containing compounds from *A. macrostemon*.[2,3)] There have not yet been any reports on the constituents of the entire prescription. The main active constituents of this prescription, the chemical bases of its pharmacological actions, and how to elucidate their mechanisms using modern medical theories and allow people world wide to accept and understand it are the main unknowns. These questions are common to all traditional Chinese medicinal prescriptions and are crucial to the modernization of traditional Chinese medicine.

Our research was focused on answering the questions above. In our research, we investigate the prescription as a whole and try to determine the active principles using multi-bioactivity-guided isolation procedures. Then the active principles are tested for their pharmacological activities. Combining the theories of traditional Chinese medicine with modern medical methods, the material base and mechanism of actions of the prescription may be elucidated.[4)]

Guided by these concepts, we have researched the prescription of Gualou-xiebai-baijiu-tang. Many active constituents have been isolated from this prescription. In this paper, we report two new steroidal saponins isolated from the active part of this prescription.

Compound **1**, obtained as an amorphous powder, mp 265—266 °C (uncorrected), which was shown to have the molecular formula $C_{39}H_{62}O_{14}$ by high-resolution (HR) FAB-MS (negative mode) (m/z=753.408366 $[M-H]^-$, calculated 753.406132) in conjunction with the ^{13}C-NMR data (39 carbon signals). The IR spectrum showed a strong absorption at 3425.4 cm^{-1} due to hydroxyl groups and an olefinic group at 1629.0 cm^{-1}, but lacked the characteristic bands of the spirostanol rings. It was positive for the anisaldehyde and Molish reactions, but negative to Ehrlich reagent.

The ^{1}H-NMR spectrum of **1** in pyridine-d_5 displayed signals for two tertiary methyl groups at δ 0.78 (3H, s, Me-18) and 0.92 (3H, s, Me-19), a secondary methyl group at δ 1.06 (3H, d, J=8.0 Hz, Me-21), and two anomeric proton signals at δ 4.98 (1H, d, J=7.8 Hz) and 5.28 (1H, d, J=7.6 Hz), suggesting that **1** was a diglycoside.

The carbonyl signal located at δ 109.4 (C) in the ^{13}C-NMR spectrum was assigned to C-22 of the spirostanol skeleton. Two signals at δ 144.5 (=C<) and 108.7 (=CH_2) were a pair of olefinic carbons, which were assigned to the C-25 and C-27 positions, respectively.[5)] Apart from some signals due to the F-ring and C-3, most of the ^{13}C-NMR data of the aglycone part of compound **1** were at almost the same positions as for samogenin. These all indicate that the aglycone of **1** was 25(27)-ene samogenin.[2)] On acid hydrolysis of **1** with 1 M HCl in dioxane/H_2O (1 : 1), glucose and galactose were identified by cochromatography on high performance thin-layer chromatography (HPTLC) with authentic samples. Based on FAB-MS fragments, it can be concluded that the ratio of glucose and galactose was 1 to 1. The β-form anomeric configurations of the galactose and glucose were judged from their coupling constants of anomeric protons (J=7.8, 7.6 Hz, respectively).

The sugar linkages were determined by comparison with the known compound, samogenin 3-*O*-β-D-glucopyranosyl(1→2)-β-D-galactopyranoside,[6)] which was isolated from bulbs of *A. macrostemon* several years ago. The ^{13}C-NMR spectra data of two compounds at the sugar moieties were very similar. Thus combined with the results of acid hydrolysis and spectroscopic evidence, the sugar moiety of **1** could be determined. By comparison of the ^{13}C-NMR spectral data of **1** with those of samogenin, the signal due to C-3 shifted to downfield by approximately +14.5 ppm, the signals due to C-2 and C-4 shifted to higher fields by −3.0 ppm and −1.7 ppm, respectively. According to the glycosylation shift value of 1,2-diol-type saponin proposed by Tanaka,[7)] the absolute stereochemistry of C-3 was deduced to be the *R*-configuration. All these indicated that the sugar moiety of **1** was linked at the C-3 position of 25(27)-ene samogenin. Furthermore, the characteristic ion peaks at m/z 755 $[M+H]^+$, 593 $[M+H-Glc]^+$, 431 $[M+H-Glc-Gal]^+$, and 413 $[Aglycone+H-H_2O]^+$ in the positive ion FAB-MS were also

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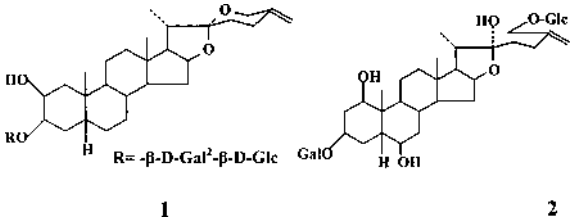

1 2

Fig. 1. Structures of Compounds **1** and **2**

confirmed to be linked to the sugar linkages.

Based on the above evidence, the structure of **1** was elucidated to be spirost 25(27)-ene-2β,3β-diol-3-*O*-β-D-glucopyranosyl(1→2)-β-D-galactopyranoside.

Compound **2** was obtained as an amorphous powder, mp 175—176 °C (uncorrected), which was shown to have the molecular formula $C_{39}H_{64}O_{16}$ by negative FAB-MS (*m/z* 787 $[M-H]^-$) in conjunction with the ^{13}C-NMR data (39 carbon signals). The IR spectrum showed a strong absorption at 3414.8 cm^{-1} due to hydroxyl groups and an olefinic group at 1631.9 cm^{-1}, but lacked the characteristic bands of the spirostanol ring. It was positive for the Molish, anisaldehyde, and Ehrlich reactions. These suggested that **2** is a furostanol saponin.

The ^{1}H-NMR spectrum of **2** in C_5D_5N showed two methyl signals at δ 0.92 (3H, s, Me-18) and 1.05 (3H, s, Me-19), one three-proton doublet at δ 1.60 (3H, d, J=6.9 Hz, Me-21), and two anomeric proton signals at δ 4.76 (1H, d, J=7.5 Hz, Gal-H-1) and 4.84 (1H, d, J=7.8 Hz, Glc-H-1), further suggesting that **2** is a furostanol steroidal diglycoside.

In the ^{13}C-NMR spectrum, the carbonyl signal at δ 110.2 (C) was assigned to C-22 of the furostanol skeleton. The ^{13}C-NMR data of C-22 suggested that a hydroxyl group instead of a methoxyl group was linked at C-22, as reported in according to the literature.[5] The signals at δ 147.1 (C) and 110.7 (CH_2) were a pair of olefinic carbons due to the C-25 and C-27 positions. Comparing the ^{13}C-NMR spectral data of the aglycone part with the known compound, which was isolated from *Allium tuberosum*,[8] it displayed similarity except for some variation at the A ring and C-22—C-27 positions. Combined with the molecular formula of **2**, the data suggest that the aglycone of **2** had an additional hydroxyl group compared with β-chologenin. The signals due to C-2 and C-19 shifted downfield by approximately +1.6 ppm and +2.2 ppm, respectively, while the signal due to C-3 shifted to a higher field by −5.2 ppm. Thus the free hydroxyl group can be assigned to the C-1 rather than to the C-2 position. The downfield shifts of C-2 and C-19 were due to the location at the β-position of the C-1 hydroxyl group, while an upfield shift of the C-3 signal was caused by the γ-*gauche* effect. Thus **2** was suggested to be a (5β)-25(27)-ene-1β,3β,6β-trihydroxyl furostanol saponin.

The spin systems for sugars were assigned on the basis of spectroscopic evidence obtained by ^{1}H–^{1}H-correlation spectroscopy (COSY) and ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) experiments. Based on the ^{1}H–^{1}H-COSY spectrum, the signals of protons of the two sugars can be assigned. The sugar linkages of **2** were determined on the basis of the heteronuclear multiple bond connectivity (HMBC) spectrum. Long-range couplings ($^3J_{H,C}$) were observed between a proton signal at δ 4.84 (Glc-H-1) and a carbon signal at δ 72.0 (C-26), whereas a proton signal at δ 4.76 (Gal-H-1′) had a cross peak with the carbon signal at δ 74.9 (C-3) in the HMBC spectrum. All these indicate that the two sugar moieties of **2** were linked at C-3 and C-26 of the aglycone, respectively. Moreover, the characteristic ion peaks at *m/z* 787 $[M-H]^-$, 626 $[M-Glc]^-$, and 463 $[Aglycone-H]^-$ in the negative FAB-MS were also confirmed to be due to the sugar linkages.

Table 1. Assignment of Carbon Signals of Compounds **1** and **2**[a]

No.	**1**	Samogenin	**2**	No.	**1**	**2**
1	40.2	39.2	72.2	C3 Gal-1	103.4	104.7
2	67.2	70.2	28.3	2	81.8	71.5
3	82.0	67.5	74.9	3	75.2	74.9
4	31.8	33.5	27.6	4	69.8	71.5
5	36.6	36.1	34.1	5	77.0	75.2
6	26.3	26.3	66.8	6	62.0	63.7
7	26.8	26.8	43.1	Glc-1	106.3	
8	35.6	35.7	35.0	2	75.2	
9	40.6	41.5	41.6	3	78.6	
10	37.1	37.0	41.0	4	71.7	
11	21.3	21.3	21.2	5	78.0	
12	40.8	40.4	40.0	6	62.8	
13	41.4	40.9	43.1			
14	56.3	56.5	55.9	26-Glc-1		103.7
15	32.1	32.1	32.3	2		73.7
16	81.6	81.1	81.0	3		78.3
17	63.1	63.1	64.5	4		71.5
18	16.6	16.6	16.6	5		78.3
19	23.9	24.1	19.8	6		62.5
20	41.9	42.0	41.6			
21	15.0	14.9	16.2			
22	109.4	109.1	110.2			
23	33.3	31.8	35.5			
24	29.0	29.2	28.3			
25	144.5	30.6	147.1			
26	65.0	66.8	72.0			
27	108.7	17.3	110.7			

a) All spectra were recorded on a 500-MHz NMR spectrometer. The signals of carbon were unambiguously assigned using distortionless enhancement by polarization transfer (DEPT), HMQC, COSY, and HMBC.

From the above evidence, the structure of **2** was established as 26-*O*-β-D-glucopyranosyl-22α-hydroxy-5β-furost-25(27)-ene-1β,3β,6β,26-tetraol-3-*O*-β-D-galactopyrnoside.

Experimental

General Experimental Procedure Melting points were measured on a Yanaco micromelting point apparatus (uncorrected). NMR spectra were recorded at 500 MHz for ^{1}H- and 125 MHz for ^{13}C-NMR in C_5D_5N at room temperature, using tetramethylsilane (TMS) as an internal standard. IR spectra were determined with a Bruker infrared spectrometer. FAB-MS and HR-FAB-MS were measured on a VG Autospec 300 Mass Spectrometer.

Column chromatography was carried out on silica gel (200—300 mesh, Qingdao Marine Chemical Industry, Qingdao, China), ODS (ODS-SS-1020T, Senshu Scientific Co., Ltd, Japan) and Sephadex(r) LH-20 (Pharmacia, Uppsala, Sweden). TLC analyses were carried out on precoated Merck Kieselgel 60 F_{254} (Merck, Germany) with $CHCl_3$–MeOH–H_2O (65 : 35 : 8) as developing solvents for saponins, and $CHCl_3$–MeOH (9 : 1) for sapogenins on which both spots were detected by spraying with 10% H_2SO_4-ethanol solution followed by heating at 115 °C.

Preparative HPLC was carried out on an LC-10 liquid chromatograph (Japan Analytical Industry Co., Ltd.) equipped with an RI detector, using an Econosphere C_{18} column (22 mm×250 mm, Alltech, U.S.A.): mobile phase, MeOH–H_2O; flow rate, 6.0 ml/min; an range of detector, 32 or 16.

Plant Materials *T. kirilowii* and *A. macrostemon* were purchased from the Liaoning Provincial Station of medicinal materials, Shenyang, P.R. China, in July 1999, and identified by Professor Qishi Sun, Department of

Pharmacognosy, Shenyang Pharmaceutical University, Shenyang, P.R. China. The voucher specimens are deposited in the Department of Natural Product Chemistry, Shenyang Pharmaceutical University.

Extraction and Isolation The prescription (25 kg, *T. kirilowii* 15 kg and *A. macrostemon* 10 kg) was extracted three times with 60% ethanol at room temperature. The combined 60% ethanol extracts were evaporated under reduced pressure and successfully partitioned with ethyl acetate and *n*-butanol. The *n*-butanol layer (448 g) was subjected to Diaion (D101) chromatography and gradually eluted with H_2O and 10%, 30%, 50%, and ethanol. The bioactive parts of the 30% ethanol eluate (40.13 g) and ethanol eluate (50.34 g) were subjected to chromatography on silica gel using a $CHCl_3$–MeOH system. Further separation using Diaion HP-20, repeated ODS opening column, and HPLC (Rp18) with the H_2O/MeOH system, was performed to obtain compound **2** from 30% ethanol and **1** from the ethanol part, respectively.

Compound **1**: mp 265—266 °C (uncorrected), colorless amorphous powder was positive for the Molish and anisaldehyde reactions and negative for the Ehrlich reagent. IR (KBr) cm^{-1}: 3425.4, 2927.3, 1629.0, 1384.1, 1075.5. ^{1}H-NMR (500 MHz, ppm, in C_5D_5N): 0.78, 0.92 (3H each, all s, 18,19-H_3), 1.06 (3H, d, J=8.0 Hz, 21-H), 2.24 (H, o, 24-Ha), 2.72 (H, o, 24-He), 4.02 (H, d, J=11.6 Hz, 26-Ha), 4.30 (H, m, 2-H), 4.45 (H, d, J=11.6 Hz, 26-He), 4.46 (H, m, 3-H), 4.60 (H, m, 16-H), 4.66 (H, t, J=8.0 Hz, Gal-2-H), 4.78 (H, s, 27-Ha), 4.82 (H, s, 27-Hb), 4.98 (H, d, J=7.8 Hz, Gal-1-H), 5.28 (H, d, J=7.6 Hz, Glc-1-H). ^{13}C-NMR (125 MHz, ppm, in C_5D_5N): see Table 1. FAB-MS (positive) m/z: 755 $[M+H]^+$, 593 $[M+H-Glc]^+$, 431 $[M+H-Glc-Gal]^+$, and 413 $[aglycone+H-H_2O]^+$.

Compound **2**: mp 175—176 °C (uncorrected), colorless amorphous powder, was positive for the Molish, anisaldehyde, and Ehrlich reactions. IR (KBr) cm^{-1}: 3414.8, 2931.1, 1631.9, 1452.3, 1076.6, 609.6. ^{1}H-NMR (500 MHz, ppm, in C_5D_5N): 0.92, 1.05 (3H each, all s, 18,19-H_3), 1.60 (3H, d, J=6.9 Hz, 21-H), 3.98 (H, m, 3-H), 4.34 (H, d, J=10.2 Hz, 26-Ha), 4.38 (H, m), 4.50 (H, d, J=10.2 Hz, 26-He), 4.56 (H, m, 16-H), 4.60 (H, d, J=7.2 Hz, 1-H), 4.76 (1H, d, J=7.5 Hz, Gal-1-H), 4.84 (1H, d, J=7.8 Hz, Glc-1-H), 4.96 (H, s, 27-Ha), 5.06 (H, s, 27-Hb). ^{13}C-NMR (125 MHz, ppm, in C_5D_5N): see Table 1. FAB-MS (negative) m/z: 787 $[M-H]^-$, 626 $[M-H-Glc]^-$, and 463 $[aglycone-H]^-$.

Acid Hydrolysis of Saponins Compounds **1** and **2** (2 mg each) were heated with 1 M HCl in dioxane/H_2O at room temperature for 8 h. The reaction mixture was neutralized with 1 M NaOH and filtered. The filtrate was extracted with $CHCl_3$ and H_2O. The water layer was concentrated, and galactose and xylose were identified by HPTLC with authentic samples.

Bioassay Systems Rabbit platelet aggregation Test: Fresh blood was drawn from rabbit ears. Platelet-rich plasma (PRP) and Platelet-poor plasma (PPP) were prepared at room temperature by centrifuging the blood. Platelet aggregation was measured turbidimetrically with an aggregation aggregometer. PPP was used to represent 100% aggregation. PRP 0.5 ml was pipetted into 1-ml siliconized glass cuvettes and warmed to 37 °C for 1 min with stirring. A sample of 50% aqueous MeOH solution was then added and allowed to react for 4 min, followed by the addition of adenosine 5′-diphosphate (ADP) 50 μl (2 μM). The aggregation rates of the samples were calculated in relation to the vehicle control.[9]

Discussion

To ascertain the sources of compounds **1** and **2**, we carried out Rp-HPLC analyses. Based on HPLC analyses, it can be concluded that compounds **1** and **2** originated from *T. kirilowii* and *A. macrostemon*, respectively.

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