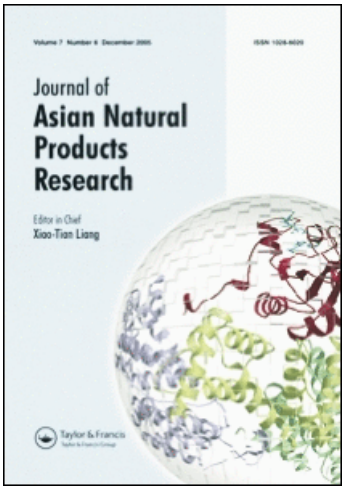

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A new minor triterpene saponin from kaixin-san prescription

Jiang-Yun Liu[a]; Sheng-Yuan Xiao[b]; Wei-Fen Shang[a]; Li-Zhen Xu[a]; Shi-Lin Yang[a]
[a] Institute of Medicinal Plants Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China [b] Analysis Center, Tsinghua University, Beijing, China

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A new minor triterpene saponin from kaixin-san prescription

JIANG-YUN LIU†, SHENG-YUAN XIAO‡, WEI-FEN SHANG†, LI-ZHEN XU†* and SHI-LIN YANG†

†Institute of Medicinal Plants Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100094, China
‡Analysis Center, Tsinghua University, Beijing 100084, China

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A new minor oleanane-type saponin, ginsenoside-R_{OA} (**1**), has been isolated from the extract of Kaixin-San, a prescription composed of *Panax ginseng* and other medicinal herbs, together with 15 known ginsenosides. The structure of **1** was established as 3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucuronopyranosyl oleanolic acid 28-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside by chemical and spectroscopic evidence. In addition, the presence of ginsenoside-R_{OA} in ginseng was confirmed by HPLC-ESI-MS.

Keywords: Kaixin-San; *Panax ginseng*; Araliaceae; Triterpene saponin; Ginsenoside-R_{OA}; Ginsenosides

1. Introduction

Alzheimer's disease (AD) is a severe central neurodegenerative epidemic disease, mostly occurring in aged people with the progressive loss of memory and learning abilities; there are no curative medicines yet. Though its mechanism is not clearly understood, it is popularly accepted that AD is caused by multi-cofactors, and traditional Chinese medicine (TCM) may have an advantage in any cure of AD. Kaixin-San, which first appeared in Qianjin-Fang (AD 650), is a famous fundamental prescription in China to cure dementia and other related diseases. It was made up of four medicinal herbs, *Panax ginseng*, *Polygala tenuifolia*, *Poria cocos* and *Acorus tatarinowii*, and many modern pharmacological works have proved its effects in learning and memory improving, nerve-protection and other related activities in different animal models [1–5].

Despite many studies on the chemistry of the four herbs, there has been no report on the constituents of the whole prescription till now. We are thus interested in investigating the bioactive constituents of this prescription. Our primary pharmacological work has proved the effects of one fraction of the Kaixin-San (KXS) in improving the abilities of learning and memory dysfunctions in mice [6]. We report here the isolation and structure elucidation

*Corresponding author. Tel.: + 86-10-62899705. Fax: + 86-10-62895086. E-mail: xulizhen2002@hotmail.com

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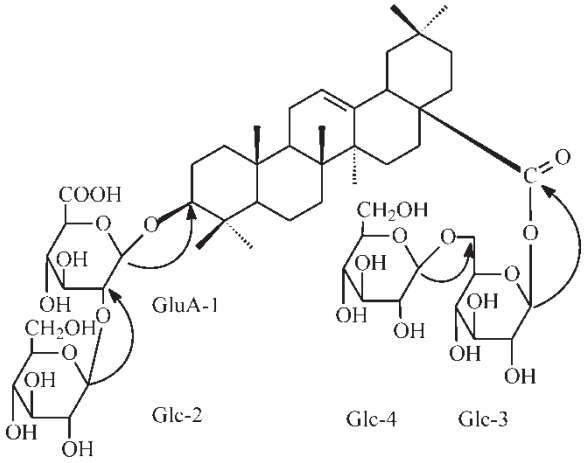

Figure 1. Structure and key HMBC correlations of compound **1**.

of a new oleanane-type saponin, ginsenoside-R_{OA} (**1**) (figure 1) from KXS, together with the 15 known ginsenosides, Ro, Ra_1, Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_3, Rg_5, Rh_1, notoginsenoside R_4, notoginsenoside R_2, and pseudogensinoside-RC_1. The presence of ginsenoside-R_{OA} in ginseng was confirmed by HPLC-ESI-MS detection.

2. Results and discussion

Ginsenoside-R_{OA} (**1**) was obtained as an amorphous white powder, mp 182–184°C (MeOH–H_2O), $[\alpha]_D^{20}$ −5.8 ($c = 0.087$, pyridine), and gave positive result for the Liebermann-Burchard test. The positive-ion HR-FABMS gave a quasi-molecular ion peak at *m/z* 1163.5272, corresponding to the molecular formula of $C_{54}H_{85}O_{24}Na_2$. Negative ESI-MS gave a quasi-molecular ion peak at *m/z* 1117 $[M - H]^-$; ESI-MS/MS1117 gave a fragment at *m/z* 793 ($M - 162 \times 2$), indicating the potential presence of a terminal bihexose. The ^1H NMR spectrum of **1** showed the signals of seven methyl groups (δ 1.23, 1.21, 1.05, 1.05, 0.86, 0.85 and 0.81), and an olefinic proton (δ 5.37, 1H, t-like). Moreover, the ^{13}C NMR spectrum revealed seven methyl groups (δ 15.3, 16.5, 17.2, 23.4, 25.8, 28.0 and 32.9), and a pair of olefinic carbons (δ 122.8, 143.9). These data suggest that the aglycone of **1** is a triterpene. The tetrasaccharide nature of **1** is manifested by its ^1H NMR [δ 6.23 (1H, d, $J = 7.5$), 5.32 (1H, d, $J = 7.5$), 5.00 (1H, d, $J = 8.0$), 4.84 (1H, d, $J = 6.5$ Hz)] and ^{13}C NMR (δ 95.4, 104.7, 104.9, 105.5) data. On acid hydrolysis, using high-performance thin-layer chromatography (HPTLC) [7], **1** gave the same spots of glucose and glucuronic acid as those of ginsenoside Ro. Comparison of the ^{13}C NMR data of **1** with that of ginsenoside Ro methyl ester (**2**) (table 1) [8] revealed that **1** has a similar spectra to **2**, except that C-6 of one glucose at δ 69.0 was shifted down-field for 6.8 ppm compared with that of **2**, and additional signals of a hexose (Glc-4) in **1** indicate that Glc-4 is attached to C-6 of the glucose in **2**. The final identification of all NMR signals were carried out by ^1H–^1H COSY, HMQC, TOCSY and HMBC experiments, taking the assignment of **2** as a reference (table 1). The signal of C-6 at δ 69.0 is assigned as the C_6 of Glc-3, indicating that Glc-4 is attached to the C-6 of Glc-3. In the HMBC spectrum (figure 1), the cross peaks between H-1 (δ 4.84) of

Table 1. ^{13}C NMR data of compound **1** and ginsenoside Ro methyl ester (**2**) (125 MHz) and ^{1}H NMR data of compound **1** (500 MHz, δ in ppm, J in Hz).

	^{13}C NMR				*^{13}C NMR*		
No	***1***	***2***	*^{1}H NMR of **1***	*No*	***1***	***2***	*^{1}H NMR of **1***
1	38.4	38.9	0.77 (m),1.30 (t-like)	C-3			
2	26.2	26.0	1.86 (overlap), 2.38 (overlap)	GlcA-1			
3	89.1	89.3	3.28 (br d)	1	104.7	105.4	4.84 (d, 6.5)
4	39.2	39.6	–	2	82.2	82.6	4.25 (m)
5	55.6	55.7	0.66 (br d, 11.5Hz)	3	77.6	77.6	4.25 (m)
6	18.2	18.5	1.28 (overlap),1.39 (m)	4	73.7	72.9	4.45 (br d, 10.5)
7	32.9	33.2	1.06 (overlap),1.28 (overlap)	5	75.3	76.8	4.56 (t-like, 9)
8	39.6	39.9	–	6	175.9	170.5	–
9	47.7	48.1	1.52 (t-like)	Glc-2			
10	36.6	37.0	–	1	105.5	106.0	5.32 (d, 7.5)
11	23.1	23.4	1.82 (m),1.86 (overlap)	2	76.7	77.1	4.07 (m)
12	122.8	123.0	5.37 (t-like)	3[a]	78.0	78.0	4.15 (m)
13	143.9	144.1	–	4	71.6	71.7	4.23 (m)
14	41.8	42.2	–	5	78.1	78.4	3.87 (m)
15	28.0	28.3	1.12 (t-like), 2.28 (overlap)	6	62.3	62.7	4.39 (br d, 12)
							4.45 (br d, 12)
16	23.5	23.9	1.97 (m), 2.04 (t-like)	C-28			
17	46.8	47.0	–	Glc-3			
18	41.3	41.8	3.15 (dd, 13.5Hz)	1	95.4	95.8	6.23 (d, 7.5)
19	46.0	46.3	1.22 (overlap),1.72 (t-like)	2	73.4	74.2	4.09 (m)
20	30.5	30.8	–	3[a]	77.9	78.9	4.18 (m)
21[a]	33.7	34.0	1.32 (m),1.85 (overlap)	4	71.3	71.1	4.23 (m)
22[a]	32.7	32.4	1.10 (overlap), 1.75 (m)	5	77.4	79.4	4.06 (m)
23	28.0	28.1	1.23 (3H, s)	6	69.0	62.2	4.30 (m)
							4.66 (d, 10.0)
24	16.5	16.7	1.05 (3H, s)	Glc-4			
25	15.3	15.5	0.81 (3H, s)	1	104.9		5.00 (d, 8.0)
26	17.2	17.5	1.05 (3H, s)	2	74.7		3.96 (br t, 8.5)
27	25.8	26.1	1.21 (3H, s)	3a	78.0		4.16 (m)
28	176.3	176.4	–	4	71.1		4.18 (m)
29	32.9	33.7	0.86 (3H, s)	5	78.2		3.84 (m)
30	23.4	23.2	0.85 (3H, s)	6	62.2		4.30 (m)
							4.39 (br d, 12)

Note: Data were measured in pyridine-d_5 with droplet of water for **1**; **2** in pyridine-d_5.
[a] may be exchanged in the same column.

GluA-1 and C-3 (δ 89.1) of the aglycone, H-1 (δ 5.32) of Glc-2 and C-2 (δ 82.2) of GlcA-1, H-1 (δ 6.23) of Glc-3 and C-28 (δ 176.3) of the aglycone, and H-1 (δ 5.00) of Glc-4 and C-6 (δ 69.0) of Glc-3 were easily observed, and confirmed the structure of **1**. The β-configurations of all four sugars could be determined by their coupling constants of the anomeric protons and ^{13}C NMR data. Thus, the structure of **1** was established as 3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucuronopyranosyl oleanolic acid 28-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside, which is a new compound and is named ginsenoside-R_{OA}.

Ginsenoside-R_{OA} was isolated from KXS and, according to its biogenesis, it should be obtained from *Panax ginseng*. To confirm this, we checked the presence of **1** in a ginseng sample using HPLC-ESI-MS. Ginsenoside-R_{OA} could be detected at $t_R = 32.4$ min with m/z 1118.7 (figure 2), thus directly proving the presence of ginsenoside-R_{OA} in ginseng.

Besides ginsenoside-R_{OA}, 15 known ginsenosides, Ro, Ra_1, Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_3, Rg_5, Rh_1, pseudogensinoside-RC_1, notoginsenoside R_4 and notoginsenoside R_2, were also obtained from KXS, and their structures identified by comparing the NMR data and

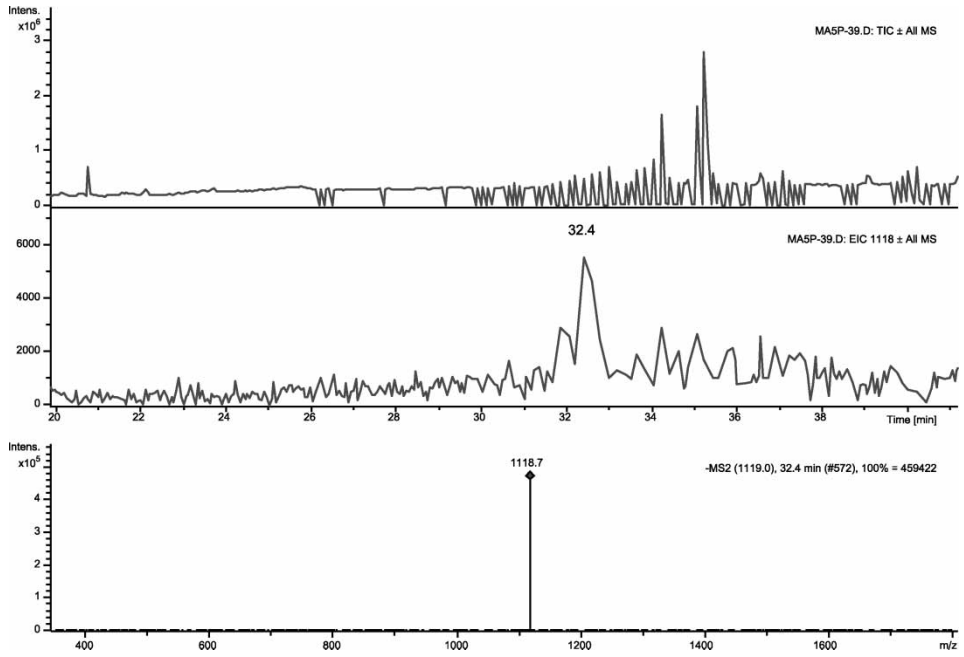

Figure 2. HPLC-ESI-MS spectrum of compound **1** in a ginseng sample (MA5P-39). TIC = total-ion chromatogram; EIC = extracted-ion chromatogram. A negative-ion peak at *m*/*z* 1118.7 could be detected at t_R = 32.4 min. It is a minor peak in TIC, but could be clearly observed in EIC at the same t_R.

physical properties with known compounds. Among them, pseudogensinoside-RC_1 (from *Panax pseudogensing* [9]) and notoginsenoside R_2 (from *Panax notogensing* [10]) were also obtained (not previously reported) from *Panax ginseng* according to its biogenesis.

3. Experimental

3.1 General experimental procedures

Melting points (uncorrected) were measured on a Fisher-Johns apparatus and are uncorrected. Optical rotations were obtained on a Perkin-Elmer 341 polarimeter. NMR spectra were recorded on a Bruker AM-500 (500 MHz) spectrometer. FABMS were obtained on a Zabspec E spectrometer; ESI-MS/MS and HPLC-MS detection was performed with an Agilent 1100, series no. LC-MSd-Trap instrument. A phenomenex Prodigy ODS column (no. 3, 5 μm, 250 × 4.6 mm) was used. For column chromatography, silica gel (200–300 mesh, Qingdao Haiyang), ODS (45–75 μm, Alltech) and Sephadex LH-20 (Pharmacia) were used. TLC and HPTLC (silica gel GF_{254}, Qingdao Haiyang) detection were obtained by spraying with 10% H_2SO_4 and heating.

3.2 Plant materials

The radix of *Panax ginseng* C. A. Mey were collected from Fusong, Jilin province. The radix of *Polygala tenuifolia* Wild. (produced in Shanxi province) and the thalli of *Poria cocos* (Schw.) Wolf. (produced in Yunnan province) were purchased from Anguo, Hebei province.

The rhizoma of *Acorus tatarinowii* Schott. were purchased from the Tongrentang Company in Beijing. All four herbs were identified by Professor Shou-Quan Lin, Institute of Medicinal Plants Development, Chinese Academy of Medical Sciences and Peking Union Medical College.

Four voucher specimens (Kaixin-San 0201–0204) have been deposited in the New Drug Research and Development Center of Institute of Medicinal Plant Development, PUMC and CAMS.

3.3 Extraction and isolation

The dried pulverized radix *ginseng* (6 kg), *Poria* (6 kg), radix *Polygala* (4 kg) and rhizoma *Acorus tatarinowii* (4 kg) were mixed together according to the prescription ratio of Kaixin-San. The total material were extracted with 70% EtOH (4 ×) under reflux. The EtOH extract was chromatographed on a resin column, eluted with H_2O–EtOH gradiently to give 4 fractions. Fraction 3 (KXS), which showed antiamnesia bioactivity, was then chromatographed on a silica-gel column, eluted with $CHCl_3$–MeOH–H_2O (90:10:1 → 0:100:0, gradient), to give 150 fractions. Fractions 146–149 were chromatographed on a silica-gel column, eluted with $CHCl_3$–MeOH–H_2O (68:32:5), and then subjected repeatedly to an ODS column (50% MeOH), purified with Sephadex LH-20 to give compound **1** (12 mg). Through similar ways, the other 15 known ginsenosides, Rh_1 and Rg_5 (from fractions 57–64), Rg_3 and notoginsenoside R_2, Rg_1, Rf and pseudogensinoside-RC_1 (from fractions 74–82), Re and Rd (from fractions 90–92), Rc, Rb_2 and Rb_1 (from fractions 127–130), Ro, Ra_1 and notoginsenoside R_4 (from fractions 146–149) were also isolated from KXS.

Compound **1** is a white amorphous powder, mp 182–184°C (MeOH–H_2O), $[\alpha]_D^{20}$ − 5.8 ($c = 0.087$, pyridine), and gave positive result to the Liebermann–Burchard test. On acid hydrolysis using high-performance thin-layer chromatography (HPTLC) [7], **1** gave the same spots of glucose and glucuronic acid as those of ginsenoside Ro. FABMS: *m/z*1163 $[M + 2Na-H]^+$, 1001, 839, 793, 677, 500; negative ESI-MS: *m/z* 1117 $[M - H]^-$, ESI-MS/MS1117: *m/z* 793, ESI-MS/MS/MS793: *m/z* 587, 569, 523, 453; HR-FABMS gave a quasi-molecular at *m/z* 1163.5272 ($[M + 2Na - H]^+$, calcd for $C_{54}H_{85}O_{24}Na_2$, 1163.5226). ^1H and ^{13}C NMR data (py-d_5, 500 and 125 MHz respectively), see table 1.

4. Hplc-esi-ms detection

Pulverized roots (202.1 mg) of *Panax ginseng* (MA5P-39) were suspended in MeOH for (5 ml) 6 h, and then extracted under ultrasound for 10 min, maintained for 2 h and then extracted under ultrasound for 10 min again. After filtration with a 0.45 μm mini-filter, the filtrate was subjected directly to HPLC. A phenomenex Prodigy ODS column (5 μm, 250 × 4.6 mm) was used, the mobile phase was composed of 0.5% (w/w) NH_4Ac solution (pH 7–7.5, adjusted by NH_4OH) (A) and MeCN (B), with a gradient elution of B (%): 0 → 0 (10 min) → 20 (20 min) → 50 (50 min) → 100 (60 min). The flow rate was 0.8 ml min^{-1}, and the injection volume was 20 μl each time. Ginsenoside-R_{OA} could be detected at $t_R = 32.4$ min with *m/z* 1118.7 (figure 2).

4.1 Pharmacological experiments: results of kxs

As shown earlier [6], KXS from a Kaixin-San prescription could improve both learning ability and memory in the mice water maze and passive avoidance performance models induced by scopolamine hydrobromide. Our continuing pharmacological work has proved its effectiveness in the mice water maze model induced by cerebral ischemia-reperfusion. In the mice water maze model induced by scopolamine, KXS showed activities against the elevation of acetylcholinesterase (AchE) activity and decreasing of choline acetyltransferase (ChAT) activity in mice brain. KXS could also reverse the decrease in superoxide dismutase (SOD) activity in mice whole brain induced by cerebral ischemia-reperfusion and scopolamine models. These results showed that KXS could improve the abilities of learning and memory dysfunctions in mice models through different pathways, such as ameliorating the central cholinergic neuronal system dysfunction and improving the antioxidation damage level in mice brain. The detailed experiments will be reported in another separately.

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