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Four new andrographolide metabolites in rats

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Abstract—Four new sulfonate metabolites of andrographolide, 14-deoxy-12(*R*)-sulfo andrographolide (Metabolite **1**), 14-deoxy-12(*S*)-sulfo andrographolide (Metabolite **2**), 14-deoxy-12(*R*)-sulfo-9(*S*)-andrographolide (Metabolite **3**) and 14-sulfo isoandrographolide (Metabolite **4**), were isolated from urine and feces in rats. Their structures were elucidated by chemical and spectroscopic analyses. These four metabolites were formed through a rare metabolic reaction and were all new compounds.
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1. Introduction

Andrographolide, chemically designated as 2(3*H*)-furanone, 3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5, 8a-dimethyl-2-methylene-1-napthalenyl] ethylidene] dihydro-4-hydroxy- (Fig. 1), was one of the main active constituents of *Andrographis paniculate* (Burm) Nees, a famous traditional Chinese medicine. Andrographolide has many types of bioactivity, such as anti-inflammatory, antimicrobial,[1,2] anti-platelet aggregation,[3,4] hepatoprotective,[5,6] and anti-HIV[7] activities. Andrographolide is widely used clinically with good results, but it does not show efficacy in the activity assays in vitro. Andrographolide has aroused the interest of many pharmacologists, and numerous experiments have been performed, although much remains to be clarified. A few investigations of the pharmacokinetics have been reported after oral administration of andrographolide.[8,9] However, to the best of our knowledge, there has been no report on its metabolites either in humans or animals. Thus, isolation and identification for the metabolites of andrographolide in vivo were attempted and 4 new metabolites were obtained. In the present paper, the extraction, isolation and identification of the 4 new metabolites are described in detail.

2. Results and discussion

The urine and feces of rats were collected in 48 h after a single oral administration of andrographolide at room temperature. The urine was extracted with ethyl acetate and *n*-butanol, respectively. Metabolite **1** and metabolite **2** were isolated from the butanol layer. The rat feces were extracted with methanol and partitioned with ethyl acetate and water-saturated *n*-butanol, respectively, and metabolite **3** and **4** were obtained from the water layer and butanol layer, respectively.

Metabolite **1**, colorless needle crystal, mp>300°C (decomposed), was positive for the Legal and Kedde reactions, suggesting the presence of an α,β-unsaturated lactone. The IR spectrum showed the presence of sulfonate group (1200.1 cm^{-1}). The molecular formula of $C_{20}H_{30}O_7S$ was thus drawn based on all ^1H NMR, ^{13}C NMR and IR data. The negative high-resolution SI-MS showed the quasi-molecular ion $[M-H]^-$ at *m/z* 413.1634 (calcd 413.1639), which confirmed the molecular formula further.

Wide variation in the chemical shifts at C_9, C_{11}–C_{16} were observed, while the other carbons were almost the same when metabolite **1** was compared with the parent drug-andrographolide, suggesting that the varieties of metabolite **1** occurred at the side-chain of lactone only, without any attack on ring A and B. The carbon signal of carbonyl (C-14) shifted from 172.6 to 177.2 ppm while the oxygen-linked carbon C-15 was from 76.1 to 73.3 ppm. The changes in chemical shifts showed that the carbon–carbon double bond at 12(13) of andrographolide changed to 13(14) double bond, by which the double bond transferred from outside to inside ring of the lactone. In HMBC spectra, the signal of H-15 (4.95, 2H) had peaks correlated with 132.5 (C-13) and 152.1 (C-14), indicating that a carbon–carbon double bond is located at 13(14). In UV spectrum, the maximal absorption of metabolite **1** was at 204 nm, which was different than that of andrographolide at 225 nm. The hypsochromic shift in UV spectrum suggested the change of conjugated system. On the other hand, the signal of H-14 (7.65, H, t, *J*=1.8 Hz) had a cross-peak with 56.8 (C-12),

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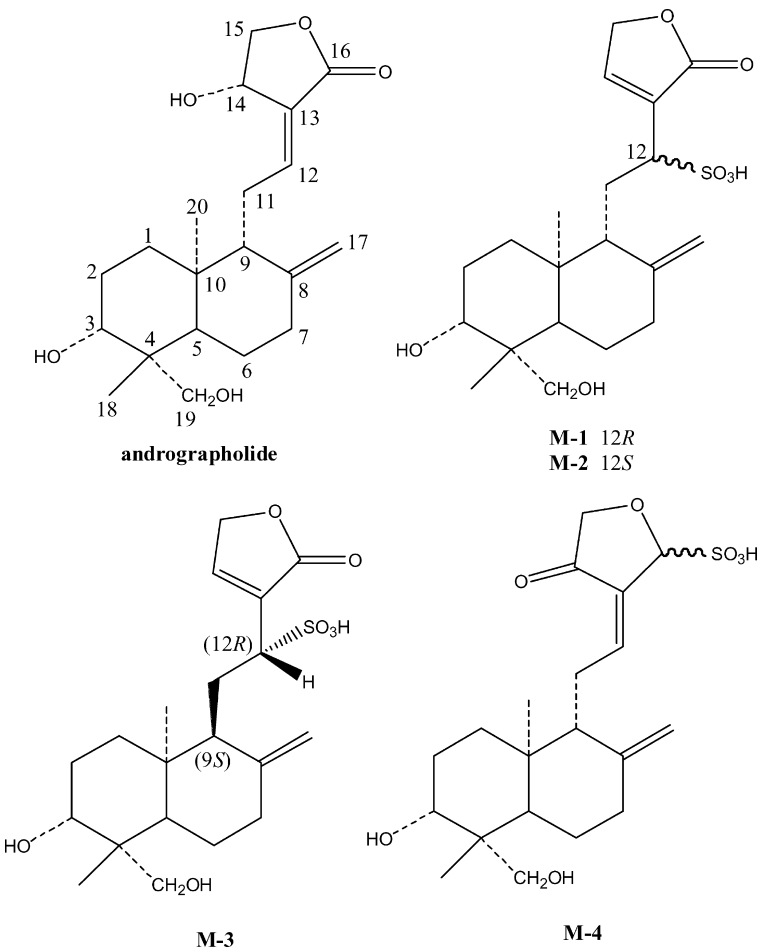

Figure 1. The structures of andrographolide and Metabolite **1** to Metabolite **4**.

and H-12 (3.92, H, dd, J=12.2, 1.8 Hz) correlated with 132.5 (C-13), 152.1 (C-14) and 177.2 (C-16). These correlated peaks showed that the sulfonate group linked at C-12.

In the NOESY spectrum, H-14 had obvious cross-peaks with the signals of H-1a, H-1e and H-9, suggesting that the double bond at 13(14) located in the side of C-1. H-12 had correlations with H-14, H-9 and H-11α, but had no NOE correlation with H-1a and H-1e. Therefore, the *R*-configuration of C-12 could be assumed. Combined with the finding at 20-CH_3 had NOE correlations with 19-CH_2OH, H_{2a}, H_{6a}, H_{7a}, $H_{11\alpha}$ and $H_{11\beta}$, and 19-CH_2OH had correlations with H_{2a} and H_{6a}, it is suggested that 20-CH_3 and 19-CH_2OH located at axial orientations. The signal of H-3 had NOE effects with 18-CH_3, H_{1a}, and H_{5a} revealed that H-3 located at axial orientation. Based on the above evidence, the stereostructure of metabolite **1** was elucidated to be 14-deoxy-12(*R*)-sulfo andrographolide. The full assignments of the signals are summarized in the Table 1.

Metabolite **2**, colorless needle crystal, was positive for the Legal and Kedde reactions. Negative high-resolution SI-MS showed the quasi-molecular $[M-H]^-$ at m/z 413.1634 (calcd 413.1639) corresponding to the molecular formula of $C_{20}H_{30}O_7S$.

Except for the signals at C_8, C_{12}, C_{14} and C_{16}, the carbon signals of metabolite **1** and metabolite **2** were very similar. Combined with results reported in the literature,[10] it could be concluded that the difference between metabolites **1** and **2** is due to the C-12 *R*/*S* configuration. The metabolite **2** was *S*-configuration while metabolite **1** was *R*-configuration. In the NOESY spectrum, H-12 had peaks correlated with H-14, H-9, H-20, and H-11α, and also had NOE correlations with H-1a and H-1e. In the NOESY spectrum of metabolite **1**, H-12 had no NOE effects with H-1a and H-1e. Thus the configuration of C-12 of metabolite **2** was *S*-configuration. Based on the above analysis, the structure of metabolite **2** was determined to be 14-deoxy-12(*S*)-sulfo andrographolide. The full assignments of the signals of proton and carbon were summarized in Table 1.

Metabolite **3**, colorless needle crystal, was positive for the Legal and Kedde reactions. Negative high-resolution SI-MS analysis of metabolite **3** provided a molecular formula of $C_{20}H_{30}O_7S$ $[(M-H)^-]$ (found 413.1633, calcd 413.1639). The carbon signals of metabolite **3** at C-9, C-11 and C-13 varied obviously compared with the data of metabolite **1**, suggesting that metabolite **3** was another isomer of metabolite **1**. Combining with the literature,[10,11] the difference between metabolite **3** and **1** was the C-9 configuration. Meanwhile, the coupling constant of H-9 in the metabolite **3** was 9.3 Hz, while it was 11.6 Hz in the metabolite **1**. The varieties of coupling constants at H-9

Table 1. The full assignments of carbon and proton signals of Metabolite **1**, compound **A** and **2**

C#	Metabolite **1**			Compound **A**			Metabolite **2**		
	δ_C	δ_H	mult, *J* (Hz)	δ_C	δ_H	mult, *J* (Hz)	δ_C	δ_H	mult, *J* (Hz)
1α	38.9	1.02	m	39.1	1.83	m	37.9	1.05	m
1β		1.83	m		1.02	m		1.78	m
2	29.8	1.71	m	30.0	1.71	m	29.1	1.74	m
3	81.7	3.30	t, 9.8	81.9	3.31	t, 9.4	80.6	3.32	m
4	44.5			44.8			43.6		
5	57.2	1.10	dd, 12.6, 2.4	57.4	1.10	dd, 12.2, 2.1	56.2	1.10	dd, 12.6, 2.4
6α	26.1	1.28	qd, 12.6, 4.2	26.6	1.30	m	25.2	1.21	dd, 12.6, 3.8
6β		1.80	m		1.80	m		1.74	m
7α	40.1	1.86	m	40.0	1.85	m	39.2	1.78	m
7β		2.36	m		2.36	m		2.20	m
8	149.1			149.3			151.3		
9	55.3	1.38	d, 11.8	55.6	1.40	br. d, 11.5	54.3	1.42	d, 11.2
10	40.8			41.0			39.9		
11α	28.0	2.08	t, 12.6	28.3	2.10	t, 12.5	27.2	2.14	t, 12.2
11β		2.31	dd, 12.6, 11.4		2.32	m		2.42	t, 12.2
12	56.8	3.92	dd, 12.2, 1.8	57.4	3.94	brd, 11.8	56.2	4.07	d, 10.9
13	132.5			132.8			131.6		
14	152.1	7.65	t, 1.8	152.1	7.65	br, s	148.2	7.77	br, s
15	73.3	4.95	Overlapped	73.4	4.92	Overlapped	72.4	4.85	Overlapped
16	177.2			177.3			176.3		
17	109.2	4.87	Overlapped	109.5	4.87	Overlapped	108.5	4.94	m
18	24.2	1.12	s	24.3	1.16	s	23.4	1.15	s
19α	65.8	3.27	d, 11.4	65.9	3.28	d, 11.8	64.8	3.30	m
19β		4.05	d, 11.4		4.07	d, 11.1		4.07	d, 10.9
20	16.4	0.68	s	16.6	0.70	s	15.6	0.62	s

All spectra were recorded on INOVA 600, in CD_3OD, 600 MHz for ^1H and 150 MHz for ^{13}C. The carbon and proton signals were assigned unambiguously on ^1H NMR, ^{13}C NMR, COSY, TOCSY, NOESY, HMQC, HMBC. m: Multiple split; o: overlapped peaks.

confirmed the configuration conversion of C-9. In the NOESY spectrum, the signal of H-9e had obvious NOE correlations with H-17 and H-20, while these NOE correlations could not be found in the metabolite **1**. H-9e and H-7a had no NOE correlation in the metabolite **3**, while there were obvious correlations in the metabolite **1**. Based on the chemical and spectroscopic evidences, the metabolite **3** was determined to be 14-deoxy-12(*R*)-sulfo-9(*S*)-andrographolide. The full assignments of proton and carbon signals of metabolite **3** were summarized in Table 2.

Metabolite **4**, white amorphous powder, was positive for the Legal and Kedde reactions, suggesting it was an α,β-unsaturated lactone. The molecular formula of $C_{20}H_{30}O_7S$ was drawn from negative high-resolution SI-MS, which showed the quasi-molecular peak $[M-H]^-$ at *m*/*z* 413.1637 (calcd 413.1639). The maximal UV absorptive wavelength of metabolite **4** was 228.6 nm, which was very similar to andrographolide (λ_{max} 225 nm), suggesting that metabolite **4** maintained the conjugated system of andrographolide.

Compared the ^{13}C NMR data of metabolite **4** with andrographolide, the signals of C-14 and C-15 of metabolite **4** greatly shifted to high field and appeared at 59.9 and 70.0 ppm, respectively, suggesting the hydroxyl at C-14 of andrographolide was substituted by a sulfonate group. In the HMBC spectrum, the signal of H-15 (δ 4.72) had correlations with C-13 (δ 125.2), C-16 (δ 173.6) and C-14 (δ 59.9). The signal at δ 4.36 (H-14) was correlated with C-13, C-12 (δ 149.4) and C-16. All these correlations in the HMBC revealed that the sulfonate group linked at C-14. Therefore, the planar structure of metabolite **4** was corroborated by HMBC spectrum. The signal of H-14 had no NOE effect with H-1a and H-1e in the NOESY spectrum, suggesting the configuration of double bond at 12(13) was diverted into the *E*-form. Meanwhile, the conclusion coincided with the relative document.[11] From the above

Table 2. The full assignments of carbon and proton signals of metabolite **3** and **4**

C#	Metabolite 3			Metabolite 4		
	δ_C	δ_H	mult, *J* (Hz)	δ_C	δ_H	mult, *J* (Hz)
1α	38.1	1.04	m	38.7	1.34	m
1β		1.85	m		1.78	m
2	29.0	1.76	m	29.8	1.74	m
3	80.8	3.34	m	81.3	3.38	t, 7.8
4	43.7			44.5		
5	56.1	1.12	Overlapped	57.2	1.31	m
6α	25.3	1.23	m	26.1	1.82	m
6β		1.81	Overlapped		1.82	m
7α	39.3	1.90	m	39.8	2.01	m
7β		2.39	m		2.36	m
8	148.4			150.1		
9	54.4	1.42	d, 9.3	58.0	1.83	m
10	40.0			40.8		
11α	26.9	2.12	t, 12.8	28.0	2.42	m
11β		2.31	Overlapped		2.86	dt, 17.4, 1.0
12	56.1	3.96	d, 11.6	149.4	6.76	td, 4.8, 1.8
13	131.4			125.2		
14	151.9	7.77	br, s	59.9	4.36	br, s
15	72.7	4.96	m	70.0	4.43	dd, 10.2, 7.8
					4.72	dd, 10.2, 1.2
16	176.6			173.6		
17	108.5	4.84	m	109.4	4.36	br, s
					4.81	m
18	23.4	1.16	s	24.2	1.18	s
19α	64.9	3.29	m	65.8	3.32	d, 11.4
19β		4.07	d, 11.1		4.08	d, 11.4
20	15.6	0.70	s	16.5	0.71	s

All spectra were recorded on ARX 300, in CD_3OD. The carbon and proton signals were assigned unambiguously on ^1H NMR, ^{13}C NMR, COSY, NOESY, HMQC spectra. m: Multiple split; o: overlapped peaks.

chemical evidence and spectroscopic analysis, the structure of metabolite **4** was elucidated to be 14-sulfo isoandrographolide. The full assignments of proton and carbon signals are listed in Table 2.

There were rare reports that carbon could be linked with sulfonate in the metabolic reactions. To confirm the structure of metabolite **1**, we synthesized compound **A** with known method.[12] The ^{1}H and ^{13}C NMR of compound **A** and metabolite **1** were quite identical and thus suggested us that metabolite **1** and compound **A** have the similar planar structures. The proton and carbon signal of compound **A** were listed in Table 1.

The carbon–carbon double bond can easily undergo addition reactions with nucleophiles. The β-carbon (C-12 of andrographolide) of α,β-unsaturated carbonyl was attacked by sulphur atom with lone-paired electrons, followed by the dehydrating reaction of hydroxyl group (14-hydroxyl of andrographolide) at position 14, and a new carbon–carbon double bond was formed at 14(13), and formed sulfonic compounds, metabolite **1**. This was a rare metabolic reaction. It may be the main metabolic pathway of andrographolide in rats and somehow could explain why it could be easily eliminated from body. As to the sulfonic acid group nature of the polar metabolites, it could be sulphate, cysteine or glutathione in vivo. However, it was more reasonable to consider that the sulfonic group was derived directly from sulphate.[13,14]

3. Experimental

3.1. General methods

Melting points were determined on a Yanaco MP-3 micro-melting point apparatus. IR spectra were determined on a Bruker IFS 55 spectrometer in KBr pellets. UV spectra were measured on a Shimadzu UV-2201 spectrometer. ESI-MS spectra were recorded on a Finnigan MAT LCQ mass spectrometer. Negative high-resolution SI-MS spectra were recorded on a Bruker second ionization mass spectrometer. NMR spectra were measured on INOVA-600 or Bruker ARX-300 spectrometers.

3.2. Animal material

Wistar rats, male, 300±10 g, were provided by The Experimental Animal Center, Shenyang Pharmaceutical University (Shenyang, China), and the 2nd Clinical Hospital of China Medical University (Shenyang, China).

3.3. Extraction and isolation

The urine and feces of rats were collected through metabolic cages in 48 h after single oral administration of 120 mg/kg andrographolide at room temperature. The urine was extracted with ethyl acetate and *n*-butanol, respectively. The butanol layer was subjected to Diaion HP-20 and eluted with H_2O/MeOH stepwisely. 30% MeOH elution was further subjected to ODS, Sephadex LH-20 repeatedly and 101.1 mg metabolite **1** and 23.1 mg metabolite **2** were obtained. The feces were extracted with methanol at refluxing temperature for 2 h. The extraction was partitioned with ethyl acetate and *n*-butanol, respectively. The water layer was subjected to D101 column chromatography, them followed with ODS, C_8/MPLC, Sephadex LH-20 repeatedly and got metabolite **3** (81.8 mg). The butanol layer was subjected to Diaion HP-20 and eluted with H_2O/MeOH. The 50% MeOH part was further purified through ODS/MPLC and prepared HPLC and thus got metabolite **4** (10.0 mg).

3.3.1. Metabolite 1. Colorless needle crystal, mp>300°C (decomposed), $C_{20}H_{30}O_7S$, Legal and Kedde reactions: positive. UV (MeOH) λ_{max} 204.4 nm; IR (KBr) ν_{max} 3420, 2937, 1758, 1643, 1447, 1200, 1083, 1038, 859, 623 cm^{-1}; HRSI-MS *m/z* $[M-H]^-$ 413.1634 (calcd. 413.1639); EI-MS *m/z*: 69, 95, 107, 119 (BpI), 131, 149, 175, 188, 256, 284, 296, 314, 332, 343, 374, 386.

3.3.2. Metabolite 2. Colorless needle crystal, mp>300°C (decomposed), $C_{20}H_{30}O_7S$, Legal and Kedde reactions: positive. UV (MeOH) λ_{max} 202.2; IR (KBr) ν_{max} 3422, 2936, 1757, 1644, 1446, 1201, 1085, 1042, 895, 622 cm^{-1}; HRSI-MS *m/z* $[M-H]^-$ 413.1634 (calcd. 413.1639); EI-MS *m/z* 64, 81, 93, 107, 119, 133 (BpI), 149, 161, 177, 191, 205, 217, 231, 243, 257, 269, 286, 302, 314, 328, 354, 354, 374.

3.3.3. Metabolite 3. Colorless needle crystal, mp>300°C (decomposed), $C_{20}H_{30}O_7S$, Legal and Kedde reactions: positive. UV (MeOH) λ_{max} 203.4; IR (KBr) ν_{max} 3422, 2940, 1746, 1643, 1446, 1201, 1084, 1040, 921, 623 cm^{-1}; HRSI-MS *m/z* $[M-H]^-$ 413.1632 (calcd. 413.1639); EI-MS *m/z* 67, 93, 121 (BpI), 133, 145, 159, 175, 191, 217, 231, 243, 287, 302, 314, 326, 352, 378, 412.

3.3.4. Metabolite 4. White powder, mp>300°C (decomposed), $C_{20}H_{30}O_7S$, Legal and Kedde reactions: positive. UV (MeOH) λ_{max} 228.6; IR (KBr) ν_{max} 3424, 2932, 1745, 1644, 1454, 1384, 1204, 1039, 710, 602 cm^{-1}; HRSI-MS *m/z* $[M-H]^-$ 413.1637 (calcd. 413.1639).

3.4. Synthesis of compound A (Metabolite 1 reference)

Andrographolide (1.0 g) was dissolved in 15 mL of 95% (v/v) ethanol on heating at 50°C (solution 1). To 4 mL 1 M Na_2SO_3, 4.8 mL 2% H_2SO_4 (w/w) and 8 mL water were added (solution 2). Solution 1 was poured into solution 2 and refluxed for 30 min. The pH value of the reaction solution was adjusted to 6–7 by adding 2% H_2SO_4 (w/w) solution and evaporated to dryness. Addition of 20 mL water to resolve the residue was followed by extraction with the same volume chloroform for three times. The water layer was dried, dissolved in 10 mL methanol and filtrated. The filtrate was then evaporated to dryness and gave 0.6 g product at yield 50.7%.

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