Six New Andrographolide Metabolites in Rats

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Six new andrographolide metabolites M-5—M-10 were isolated from rat urine, feces, and the contents of the small intestine. Three (M-5—M-7) as sulfate ester compounds were identified as new compounds. The structures of these six metabolites were determined to be 14-deoxy-12(*R*)-sulfo andrographolide 3-sulfate (M-5), 14-deoxy-12(*S*)-sulfo andrographolide 3-sulfate (M-6), 14-sulfo isoandrographolide 3-sulfate (M-7), 14-deoxy-11,12-didehydroandrographolide (M-8), isoandrographolide (M-9), and 14-deoxy andrographolide (M-10), respectively, based on chemical evidence and spectroscopic analysis.

Key words andrographolide; metabolite in rats; sulfate ester; 14-deoxy-12(*R*)-sulfo andrographolide 3-sulfate; 14-deoxy-12(*S*)-sulfo andrographolide 3-sulfate; 14-sulfo isoandrographolide 3-sulfate

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), is one of the main active constituents of *Andrographis paniculate* (Burm) Nees, a famous traditional Chinese medicine. Andrographolide has many bioactivities, such as antiinflammatory and antimicrobial,[1,2] antiplatelet aggregation,[3,4] hepatoprotective,[5,6] and anti-human immunodeficiency virus (HIV) activity.[7] Andrographolide is widely used clinically with good results, but it did not show efficacy in 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. We isolated and analyzed the metabolites of andrographolide in blood, urine, bile, and the contents of the small intestine and stomach of rats. So far, we have identified 10 new metabolites. In this paper, six new metabolites are presented including their extraction, isolation, and identification.

Metabolite 5 (M-5), colorless powder, melting point >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 hydroxyl (3433 cm^{-1}), ester carbonyl (1744 cm^{-1}), carbon–carbon double bond (1643 cm^{-1}), and sulfonate (1205 cm^{-1}) groups in the molecule. In the high-resolution second ionization mass spectrum (HR-SI-MS), a quasimolecular peak $[M-H]^-$ at *m*/*z* 493.1205 (Calcd 493.1207) was seen, and thus the molecular formula of $C_{20}H_{30}O_{10}S_2$ was derived.

Wide variation in the chemical shifts at C_2, C_3, C_9, and C_{11}—C_{16} were observed, while the other carbons were almost the same when metabolite 5 was compared with the parent drug andrographolide, suggesting that the difference of metabolite 5 occurred at the side chain of lactone and ring A. The carbon signal of carbonyl shifted from 172.6 to 176.7 ppm, while the oxygen-linked carbon of C-15 shifted from 76.1 to 72.7 ppm. The changes in chemical shifts showed that the double bond at 12(13) of andrographolide changed to the 13(14) double bond, by which the double bond transferred from the outside to the inside of the lactone ring. In the heteronuclear multiple bond connectivity (HMBC) spectrum, the signal of H-15 (4.94, 2H) had peaks correlated with C-13 (131.3 ppm) and C-14 (151.7 ppm), indicating that a carbon–carbon double bond is located at 13(14). The singlet of H-14 (7.73, H, br s) had a cross-peak with 55.8 (C-12), and H-12 (3.97, H, br d, J=10.2 Hz) correlated with 131.3 (C-13), 151.7 (C-14), and 176.7 ppm (C-16). These correlated peaks showed that the sulfonate group is linked at C-12.

The carbon signals of metabolite 5 at ring A differed from those of andrographolide. The carbon signal of metabolite 5 at C-3 shifted far downfield, and C-2 had a moderately upfield shift compared with andrographolide. Combined with the HR-SI-MS results of metabolite 5, it can be concluded that sulfate esterification occurred at C-3.

In the nuclear Overhauser effect spectroscopy (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) is located in the side of C-1. H-12 had NOE 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. Moreover, 20-CH_3 had NOE correlations with 19-CH_2OH, H_{2a}, H_{6a}, H_{7a}, $H_{11\alpha}$, and $H_{11\beta}$. 19-CH_2OH had correlations with H_{2a} and H_{6a}. Thus, it was concluded that 20-CH_3 and 19-CH_2OH occurred at all axial orientations. The signal of H-3 had NOE effects with 18-CH_3, H_{1a}, and H_{5a}. Thus H-3 had an axial orientation. Based on the above analysis, the stereostructure of metabolite 5 could be determined, as shown in Fig. 1. From the above evidence, metabolite 5 was determined to be 14-deoxy-12(*R*)-sulfo andrographolide 3-sulfate. The full assignments of carbon and proton signals are summarized in Table 1.

Metabolite 6 (M-6), colorless powder, melting point >300 °C (decomposed), was positive for the Legal and Kedde reactions. In the IR spectrum, 1205 cm^{-1} was the characteristic absorption of the sulfonate group. In the electrospray ionization (ESI)-MS, the molecular weight was shown at *m*/*z* 494. The formula was determined to be $C_{20}H_{30}O_{10}S_2$ by combining the ^{1}H-, ^{13}C-NMR, and IR spectral data.

The carbon signals of metabolite 6 and metabolite 5 were very similar except for the signals at C_8, C_{12}, and C_{14}. Combined with the results reported in the literature,[10] it could be concluded that the difference is due to the C-12 *R*/*S* configuration. In the NOESY spectrum of metabolite 6, H-12 had peaks correlated with H-14, H-9, H-20, and H-11α, and also

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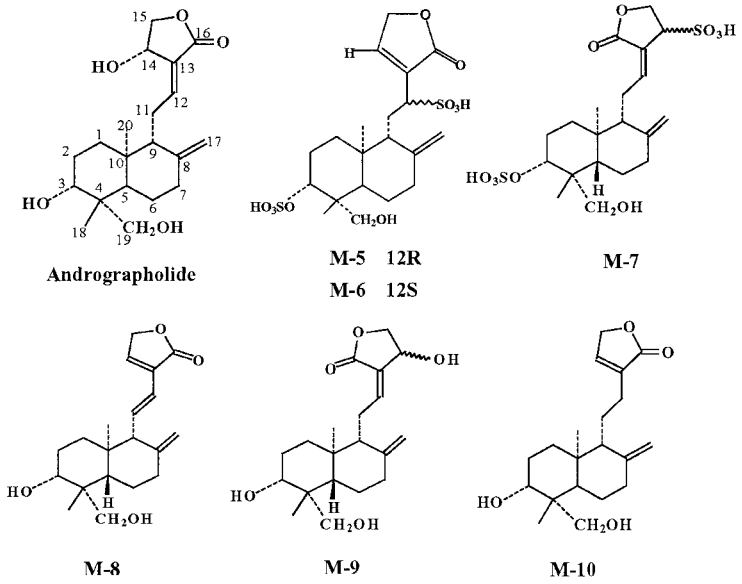

Fig. 1. Structures of Metabolites 5 to 10

Table 1. Assignments of Carbon and Proton Signals of Metabolites 5, 6, and 7

No.	Carbon signals[a)]			Proton signals[a—d)]		
	M-5	M-6	M-7	M-5	M-6	M-7
1	38.0	37.5	38.0	1.76 (H, m) 1.11 (H, m)	2.35 (H, m) 1.86 (H, m)	1.84 (H, m) 1.25 (H, m)
2	25.5	25.7	25.5	1.85 (2H, m)	1.84 (2H, m)	1.80 (2H, m)
3	88.2	88.5	88.2	4.04 (H, dd, J=11.7, 4.1 Hz)	4.12 (H, dd, J=11.5, 3.4 Hz)	4.08 (H, dd, J=11.7, 3.8 Hz)
4	44.3	44.4	44.4			
5	56.8	56.8	56.7	1.25 (H, m)	1.25 (H, m)	1.38 (H, m)
6	25.7	25.8	25.8	2.39 (H, m) 2.23 (H, m)	2.48 (H, m) 2.24 (H, m)	2.20 (H, m) 1.86 (H, m)
7	39.1	39.0	38.9	2.36 (2H, m)	2.39 (2H, m)	2.43 (H, m) 2.00 (H, m)
8	148.1	151.5	148.6			
9	54.2	55.4	57.2	1.45 (H, m)	2.22 (H, m)	1.83 (H, m)
10	39.8	40.1	39.9			
11	27.4	28.2	26.4	2.35 (H, m) 2.14 (H, t, J=10.2 Hz)	2.46 (2H, m)	2.67 (2H, o)
12	55.8	56.7	148.6	3.97 (H, d, J=11.5 Hz)	3.76 (H, m)	6.90 (H, t, J=4.6 Hz)
13	131.3	132.5	124.2			
14	151.7	149.3	58.8	7.73 (H, br s)	7.68 (H, br s)	4.37 (H, br s)
15	72.7	72.6	69.4	4.94 (2H, o)	4.94 (2H, o)	4.80 (H, o) 4.50 (H, o)
16	176.7	176.4	173.2			
17	108.4	107.7	109.2	4.86 (2H, o)	4.78 (H, o) 4.35 (H, o)	4.90 (H, o) 4.70 (H, o)
18	23.1	23.1	23.1	1.15 (3H, s)	1.15 (3H, s)	1.17 (3H, s)
19	63.5	63.6	63.6	3.90 (H, d, J=11.8 Hz) 3.46 (H, d, J=11.8 Hz)	3.92 (H, d, J=12.0 Hz) 3.42 (H, d, J=12.0 Hz)	3.93 (H, d, J=11.8 Hz) 3.45 (H, d, J=11.8 Hz)
20	15.1	15.0	15.0	0.74 (3H, s)	0.66 (3H, s)	0.66 (3H, s)

Notes: *a*) All spectra were recorded on ARX 300, in CD_3OD. *b*) The carbon and proton signals were assigned unambiguously on ^{1}H-, ^{13}C-NMR, COSY, NOESY, and HMQC. *c*) m, multiple split. *d*) o, overlapped peaks.

had NOE correlations with H-1a and H-1e. In the NOESY spectrum of metabolite 5, H-12 had no correlation with H-1a and H-1e. Thus the configuration of C-12 of metabolite 6 was *S*. Based on the above analysis, the stereostructure of metabolite 6 was determined to be 14-deoxy-12(*S*)-sulfo andrographolide 3-sulfate. The full assignments of all signals are summarized in Table 1.

Metabolite 7 (M-7), obtained as a colorless powder, melting point >300 °C (decomposed), was positive for the Legal and Kedde reactions. There was absorbance of a sulfonate group at 1206 cm^{-1} in the IR spectrum. The molecular weight was shown at *m*/*z* 494 in the ESI-MS. Combined with its ^{1}H-, ^{13}C-NMR, and IR spectral data, the formula of metabolite 7 was determined to be $C_{20}H_{30}O_{10}S_2$.

Comparing the ^{13}C-NMR data of metabolite 7 with those of andrographolide, the signals of C-14 and C-15 of metabolite 7 shifted to a higher field and appeared at 58.8 and 69.4 ppm, respectively. The shift results showed the hydroxyl at C-14 of andrographolide was substituted with a sulfonate group. In the HMBC spectrum, the signal of H-15 (4.50 ppm) was correlated with 124.2 (C-13), 173.2 (C-16), and 58.8 ppm (C-14). The signal at 4.37 ppm (H-14) was correlated with C-13, C-12, and C-16. All these cross-peaks in the HMBC confirmed that the sulfonate group is linked at C-14.

Table 2. Assignments of Carbon and Proton Signals of Metabolites 8, 9, and 10

No.	Carbon signals			Proton signals[c—e]		
	M-8[a]	M-9[a]	M-10[b]	M-8[a]	M-9[a]	M-10[b]
1	39.5	38.1	36.5	1.83 (H, m) 1.34 (H, m)	1.85 (H, m) 1.37 (H, m)	1.66 (H, m) 1.14 (H, m)
2	28.9	29.0	28.0	1.75 (2H, m)	1.78 (2H, m)	1.60 (2H, m)
3	81.2	81.0	78.6	3.34 (H, m)	3.40 (H, m)	3.20 (H, o)
4	43.8	43.7	42.3			
5	55.8	56.5	54.6	1.35 (H, m)	1.34 (H, m)	1.12 (H, m)
6	24.4	25.3	24.0	1.81 (2H, m)	1.84 (2H, m)	2.23 (H, m) 1.99 (H, m)
7	37.8	39.1	38.0	2.34 (H, m) 2.03 (H, m)	2.38 (H, m) 2.00 (H, m)	2.33 (H, d, J=12.4 Hz) 1.85 (H, m)
8	150.1	149.1	147.8			
9	62.8	57.9	55.5	1.86 (H, m)	1.94 (H, m)	1.56 (H, m)
10	39.6	40.1	38.8			
11	136.5	24.8	21.7	6.85 (H, dd, J=15.8, 10.1 Hz)	2.87 (2H, t, J=6.9 Hz)	1.70 (H, m) 1.56 (H, m)
12	122.5	151.2	24.2	6.15 (H, d, J=15.8 Hz)	6.50 (H, t, J=7.0 Hz)	1.71 (H, m) 1.36 (H, m)
13	129.6	129.4	132.2			
14	146.6	69.9	147.0	7.43 (H, br t)	4.73 (H, dd, J=6.1, 2.9 Hz)	7.47 (H, s)
15	71.6	75.0	70.5	4.35 (2H, m)	4.40 (H, dd, J=9.9, 6.1 Hz) 4.05 (H, dd, J=9.9, 2.9 Hz)	4.81 (2H, o)
16	174.8	171.8	174.2			
17	109.1	108.6	106.7	4.75 (H, d, J=1.8 Hz) 4.81 (H, d, J=1.8 Hz)	4.82 (H, brs) 4.51 (H, br s)	4.81 (H, m) 4.59 (H, br s)
18	23.3	23.4	23.1	1.22 (3H, s)	1.20 (3H, s)	1.07 (3H, s)
19	65.0	65.0	62.7	4.13 (H, d, J=11.1 Hz) 3.38 (H, d, J=11.1 Hz)	4.10 (H, d, J=11.1 Hz) 3.35 (H, d, J=11.1 Hz)	3.82 (H, d, J=11.7 Hz) 3.22 (H, m)
20	16.3	15.6	14.9	0.84 (3H, s)	0.73 (3H, s)	0.60 (3H, s)

Notes: *a*) Recorded on BRUKER ARX 300, in CD_3OD. *b*) Recorded on BRUKER ARX 300, in DMSO-d_6. *c*) The carbon and proton signals were assigned unambiguously on ^{1}H-, ^{13}C-NMR, COSY, NOESY, HMQC. *d*) m, multiple split. *e*) o, overlapped peaks.

Therefore, the planar structure of metabolite 7 was established based on its HMBC spectra. Since the signal of H-14 had no correlation with H-1a and H-1e in the NOESY spectrum, the configuration of the double bond at 12(13) was diverted into the E form. Moreover, the carbon signals of metabolite 7 at ring A differed from those of andrographolide. The carbon signal at C-3 shifted downfield and C-2 had a moderate upfield shift. Combined with the MS data, it was concluded that sulfate esterification at C-3 occurred.

In the NOESY spectrum, the proton signal of H-14 had no NOE correlation with H-1a and H-1e, suggesting that the carbon–carbon double bond at 12(13) had taken the *E* form. Based on chemical and spectroscopic analyses, metabolite 7 was identified as 14-sulfo-isoandrographolide 3-sulfate. The full assignments of all signals are summarized in Table 1.

Metabolite 8 (M-8), colorless needle-like crystals (methanol), melting point 204—205 °C, was positive for the Legal and Kedde reactions. The signal at δ 174.8 ppm belonged to an unsaturated ester carbonyl carbon (C-16) in the lowest field in the ^{13}C-NMR spectrum. There were six sp^2 carbon signals from 100 to 160 ppm, suggesting there were three carbon–carbon double bonds in the molecule. The broad triple peaks at δ 7.43 (H-14) in the ^{1}H-NMR spectrum were the β-proton signals of an α,β-unsaturated-γ-lactone. The signals of δ 6.85 (H-11, H, dd, J=15.8, 10.1 Hz) and δ 6.15 (H-12, H, d, J=15.8 Hz) were a pair of *trans*-coupling olefinic signals and assigned to position H-11 and H-12, respectively. The signal of δ 4.75 (H, d, J=1.8 Hz) and 4.86 (H, d, J=1.8 Hz) were a pair of olefinic protons at C-17. From above analyses, metabolite 8 was a product of andrographolide which occurred as a rearrangement after dehydration at position 14. Therefore metabolite 8 was established to be 14-deoxy-11,12-didehydroandrographolide.[11] The assignments of the signals of metabolite 8 are listed in Table 2.

Metabolite 9 (M-9) was obtained as colorless sheet crystals (methanol) and its melting point was 210—211 °C. It was positive for the Legal and Kedde reactions. In the ESI-MS (+), it showed a quasimolecular ion peak $[M+H]^+$ at m/z 351. The molecular formula was concluded to be $C_{20}H_{30}O_5$ in combination with the ^{1}H- and ^{13}C-NMR spectral data. The ^{13}C-NMR spectral data of metabolite 9 showed no difference in the chemical shifts compared with andrographolide except those from C-12 to C-16. The C-12 signal of metabolite 9 shifted downfield by +1.9 ppm from 149.3 to 151.2 ppm, while C-13 shifted to a higher field by about −0.4 ppm. There was a +3.2 ppm downfield shift of C-14 in metabolite 9 compared with that of andrographolide. From the above analyses, the chemical shift changes between metabolite 9 and andrographolide were focused from C-12 to C-16, although these two compounds had the same formula and molecular weight. Therefore metabolite 9 and andrographolide are a pair of *cis-trans* isomers at the 12(13) sites. In NOESY spectrum, there was no correlated signal between H-14 and H-1a, and H-1e, further confirming our assumption. Through above chemical and spectroscopic approaches, as well as the results reported in literature,[11] metabolite 9 was identified as isoandrographolide. The full assignments of NMR signals are listed in Table 2.

Metabolite 10, obtained as a white powder (methanol), melting point 176—178 °C, was positive for the Legal and Kedde reactions, which indicated it is also an α,β-unsaturated lactone. The high resolution secondary ion (HR-SI)-MS showed a quasimolecular peak $[M-H]^-$ at m/z 333.2071

(Calcd 333.2071) corresponding to the formula $C_{20}H_{29}O_4$.

Compared with andrographolide, there were no difference in the A and B rings of metabolite 10. However, differences between the two compounds occurred at C-11 to C-16. The signal of C-15 shifted upfield by −3.9 ppm, from 74.4 ppm in andrographolide to 70.5 ppm in metabolite 10. The ester carbonyl and C-13 shifted downfield by +4.2 and +3.2 ppm, respectively. These shift results indicate that metabolite 10 is the product of andrographolide after dehydration at position 14 and rearrangement of the carbon–carbon double bonds, which was confirmed by its HR-SI-MS. The carbon and proton signals could be assigned based on ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H-detected heteronuclear multipe quantum coherence (HMQC) data. Combined with chemical and spectroscopic evidence and previous results,[11] metabolite 10 was identified as 14-deoxy andrographolide. The signal assignments are listed in Table 2.

Experimental

General Experimental Procedure Melting points were determined on a Yanaco MP-3 micro-melting point apparatus (uncorrected). IR spectra were recorded on a Bruker IFS 55 spectrometer in KBr pellets. UV spectra were measured on a Shimadzu UV-2200 spectrometer, ESI-MS spectra on a Finnigan MAT LCQ mass spectrometer, and HR-SI-MS on a Bruker second ionization mass spectrometer. NMR spectra were measured on INOVA-600 or Bruker ARX-300 spectrometers.

Silica gel for column chromatography (200—300 mesh), and silica gel G_{60} and GF_{254} for thin-layer chromatography were products of Qingdao Marine Chemical Factory. Normal-phase and reverse-phase preparatory TLC as performed using products from Merck Company. D101 and Diaion HP-20 were purchased from Tianjing and Rensui, respectively. Sephadex® LH-20, ODS, and C8 were the products of Pharmacia Company.

Male Wistar rats, weighing 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).

Extraction and Isolation The urine and feces of rats were collected through metabolic cages 48 h after single oral administration 120 mg/kg andrographolide in room temperature. The feces were refluxed with methanol for 2 h. The extract was partitioned with ethyl acetate and *n*-butanol, respectively. The ethyl acetate layer was further isolated through silica gel column chromatography eluted with cyclohexane/ethyl acetate (2/1) to obtain metabolite 10 (5.0 mg). The water layer was subjected to D101 column chromatography, followed by repeated ODS, C_8/MPLC, and Sephadex LH-20 column chromatography to obtain metabolite 5 (67.1 mg), 6 (23.0 mg), and 7 (10.0 mg).

The small intestine of the rats was removed 1 h after single oral administration 120 mg/kg andrographolide. The intestine was minced and extracted with methanol for 30 min by sonication, filtered, condensed to dryness, and then dissolved in water. It was extracted with ethyl acetate three times at the same volume. The EtOAc layer was labeled as AIS-E, and AIS-E was isolated by preparatory TLC and silica gel column chromatography to obtain metabolite 8 (15.0 mg) and metabolite 9 (2.0 mg), respectively.

Metabolite 5: Colorless powder, mp >300 °C (decomposed), $C_{20}H_{30}O_{10}S_2$. Legal and Kedde reactions: positive. UV (MeOH) [nm]: 205.1. IR (KBr) cm^{-1}: 3433, 2947, 1744, 1643, 1205, 1055, 969, 919, 623. HRSI-MS *m*/*z*: $[M-H]^-$ 493.1205 (Calcd 493.1207).

Metabolite 6: Colorless powder, mp >300 °C (decomposed), $C_{20}H_{30}O_{10}S_2$. Legal and Kedde reactions: positive. UV (MeOH) [nm]: 205.4. IR (KBr) cm^{-1}: 3435, 1744, 1642, 1205, 1058, 603.

Metabolite 7: Colorless powder, mp >300 °C (decomposed), $C_{20}H_{30}O_{10}S_2$. Legal and Kedde reactions: positive. UV (MeOH) [nm]: 229.0. IR (KBr) cm^{-1}: 3437, 1740, 1636, 1206, 1050, 623.

Metabolite 8: Colorless powder (MeOH), mp 204—205 °C, $C_{20}H_{28}O_4$. Legal and Kedde Reactions: positive.

Metabolite 9: Colorless plate needle-like crystals (MeOH), mp 204—205 °C, $C_{20}H_{30}O_5$. Legal and Kedde reactions: Positive. ESI-MS (+) *m*/*z*: 351 $[M+H]^+$.

Metabolite 10: White powder (MeOH), mp 176—178 °C, $C_{20}H_{30}O_4$. Legal and Kedde reactions: positive. HR-SI-MS (*m*/*z*): $[M-H]^-$ 333.2071 (Calcd 333.2071).

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