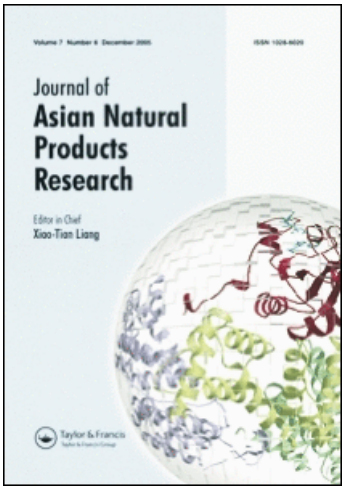

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Microbial transformation of asiatic acid by *Alternaria longipes*

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ORIGINAL ARTICLE

Microbial transformation of asiatic acid by *Alternaria longipes*

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Asiatic acid is a major pentacyclic triterpene isolated from *Centella asiatica*. It shows a variety of bioactivities. In order to obtain its derivatives, potentially useful for detailed pharmacological studies, the substrate was subjected to incubations with selected micro-organisms. In this work, asiatic acid was converted into three new compounds: 2α,3β,23,30-tetrahydroxyurs-12-ene-28-oic acid (**1**), 2α,3β,22β,23-tetrahydroxyurs-12-ene-28-oic acid (**2**), and 2α,3β,22β,23,30-pentahydroxyurs-12-ene-28-oic acid (**3**) by the fungus *Alternaria longipes AS* 3.2875. The structures of the three metabolites were determined by 1D and 2D NMR spectral data.

Keywords: asiatic acid; *Alternaria longipes*; microbial transformation; triterpene

1. Introduction

Asiatic acid is a naturally occurring pentacyclic triterpene found in many plants; however, one of the most widely reported sources of it is *Centella asiatica* [1]. Previous investigations have shown that it possessed anti-oxidative, anti-inflammatory, hepatoprotective, anti-Alzheimer's disease, and anti-depressant activities. Furthermore, it can also protect cardio-cerebral vascular and induce apoptosis of tumor cells such as human melanoma SK-MEL-2 cells [2–6]. Because of such properties, the preparation of derivatives of this compound for structure–activity relationship studies is valuable. However, because the structures of triterpenoids are complicated, the chemical procedures that can be used had some limitations. Now, microbial transformation is an important tool in the structural modification of organic compounds, especially for natural products, due to its significant region- and stereoselectivities [7,8]. There is no report on biotransformation of asiatic acid by fungi. Therefore, the objective of this work was to obtain new derivatives of asiatic acid by the fungus *Alternaria longipes AS* 3.2875, potentially useful for pharmacological studies.

2. Results and discussion

Asiatic acid (substrate) showed purity above 95% by HPLC/UV analysis. The ^{1}H NMR spectrum exhibited six methyl signals at δ 1.05 (9H, s, H-24, 26, 27), 1.18 (3H, s, H-25), 0.90 (3H, d, H-29), and 0.92 (3H, d, H-30), whereas the signal at δ 5.45 (1H, t, $J = 4.0$ Hz) was attributed to H-12 [9]. The ^{13}C NMR spectral data are shown in Table 1.

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Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data of compounds **1**–**3** and asiatic acid (pyridine-d_5, δ in ppm, J in Hz).

	1		**2**		**3**		Asiatic acid	
Position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	47.9	1.78, 2.30, m	47.9	2.27, m	47.9	1.80, 2.24, m	47.9	
2	68.8	4.20, d, $J = 10.0$	68.7	4.21, d, $J = 10.0$	68.9	4.20, d, $J = 10.0$	68.8	4.23, d, $J = 10.0$
3	78.2	4.20, d, $J = 10.0$	78.2	4.21, d, $J = 10.0$	78.3	4.19, d, $J = 10.0$	78.2	4.23, d, $J = 10.0$
4	43.6		43.6		43.6		43.6	
5	47.9	1.78, m	47.8	1.37, m	48.0	1.33, m	47.9	
6	18.5	1.62, m	18.6	1.98, 2.69, m	18.7	1.65, 2.73, m	18.5	
7	33.2	1.28, m	33.0	1.70, 1.35, m	33.0	1.33, 1.68, m	33.1	
8	40.0		40.2		40.2		40.0	
9	48.1	1.84, m	48.0	1.83, m	48.1	1.85, m	48.1	
10	38.3		38.3		38.3		38.3	
11	23.8	2.01, 2.02, m	23.8	1.99, m	23.8	1.98, m	23.8	
12	125.6	5.53, br s	125.5	5.43, br s	125.8	5.48, br s	125.6	5.45, t, $J = 4.0$
13	139.4		139.3		139.2		139.3	
14	42.6		43.2		43.2		42.6	
15	28.7	1.10, 1.12, m	28.2	1.21, 2.60, m	28.3	1.21, 2.64, m	28.6	
16	25.0	1.99, m	18.5	1.41, 1.70, m	18.5	1.33, 2.09, m	24.9	
17	48.0		54.1		54.1		48.0	
18	53.6	2.73, d, $J = 11.0$	54.5	2.64, d, $J = 11.0$	54.6	2.73, d, $J = 11.0$	53.5	
19	34.0	1.99, m	39.0	1.50, m	33.4	2.01, m	39.4	
20	47.5	1.28, m	37.6	1.21, m	45.7	1.45, m	39.4	
21	25.7	1.99, m	40.1	1.73, 1.98, m	34.9	2.48, 2.29, m	31.0	
22	37.5	1.99, 2.19, m	74.4	4.38, dd, $J = 4.0$, 12.0	74.9	4.20, dd, $J = 4.0$, 12.0	37.4	
23	66.5	3.70, 4.20, d, $J = 10.8$	66.4	3.72, 4.20, d, $J = 10.8$	66.9	3.72, 4.20, d, $J = 10.8$	66.5	3.72, 4.21, d, $J = 12.0$
24	14.4	1.05, s	14.4	1.05, s	14.4	1.06, s	14.4	1.05, s
25	17.5	1.05, s	17.5	1.04, s	17.5	1.05, s	17.5	1.18, s
26	17.6	1.05, s	17.6	1.10, s	17.6	1.12, s	17.5	1.05, s
27	23.9	1.16, s	24.2	1.16, s	24.3	1.20, s	23.9	1.05, s
28	179.9		179.9		178.8		179.9	
29	17.2	1.10, d, $J = 6.4$	17.3	0.95, d, $J = 6.4$	17.1	1.10, d, $J = 6.4$	17.5	0.90, d, $J = 6.4$
30	65.2	3.87, 3.96, d, $J = 8.8$	21.2	0.93, d, $J = 6.4$	64.9	3.95, 3.99, m	21.4	0.92, d, $J = 6.4$

Compound **1** was obtained as a white amorphous powder with mp 211–212°C. The IR spectrum showed the absorption bands at 3363.3 (—OH) and 1689.4 (C═C) cm^{-1}. It had a molecular formula $C_{30}H_{48}O_6$ established from its HR-ESI-MS at *m/z* 527.3333 $[M + Na]^+$. Compared to the substrate, the ^1H NMR spectrum showed two new signals at δ 3.87 and 3.96 integrating for one proton, respectively. Meanwhile, a methyl doublet signal was absent in **1**. Those differences suggested the hydroxylation of a methyl carbon, and this was confirmed by analysis of the ^{13}C NMR and DEPT spectra (Table 1), which showed 10 methylenes, instead of nine for the parent compound, as well as the disappearance of a methyl signal. Namely, the resonance of C-30 (a methyl group at δ 21.4 in asiatic acid) disappeared but another methylene signal appeared at δ 65.2 in **1**. The hydroxylation at C-30 was further authenticated by the paramagnetic shifts of the neighboring carbons C-20 (from δ 39.4 of the substrate to δ 47.5), while the signals of C-19 and C-21 shifted upfield by 5.4 and 5.3 ppm, respectively. It was also supported by the HMBC spectrum (see Figure 1), which exhibited correlations of H-30 with C-19 and C-21, as well as H-29 with C-18, C-19, and C-20. Thus, compound **1** was determined as 2α,3β,23,30-tetrahydroxyurs-12-ene-28-oic acid, which is a new compound.

Compound **2** was obtained as a white amorphous powder with mp 270–271°C. The IR spectrum showed the absorption bands at 3367.5 (—OH) and 1691.3 (C═C) cm^{-1}. Its molecular formula was determined as $C_{30}H_{48}O_6$ from its HR-ESI-MS at *m/z* 503.3375 $[M - H]^-$. The ^1H NMR spectrum of the metabolite was similar to that of the substrate displaying six characteristic methyl groups. However, it showed a new signal at δ 4.38 integrating for one proton, indicating the possible hydroxylation at the methylene group. It was confirmed by the analysis of the ^{13}C NMR and DEPT spectra (Table 1), which showed nine methylenes, instead of 10 for the parent compound. The resonance of C-22 (a methylene group at δ 37.4) in the substrate was absent, but another methine signal was displayed at δ 74.4 in **2**. The hydroxylation at C-22 was further confirmed by the paramagnetic shifts of the neighboring carbons C-21 (from δ 31.0 of substrate to δ 40.1) and C-17 (from δ 48.0 of the substrate to δ 54.1). It was also supported by the HMBC spectrum (see Figure 1),

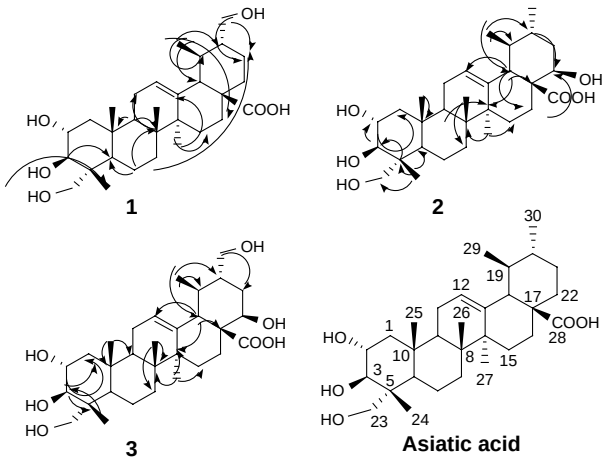

Figure 1. Key HMBC correlations of compounds **1**–**3** and the structure of asiatic acid.

which exhibited correlations of H-16 and H-21 with C-22. The stereochemistry at C-22 was deduced from the multiplicity of the corresponding C—H signal at δ 4.38 in the ^{1}H NMR spectrum. It was a double doublet ($J = 4.0$, 12.0 Hz) signal consistent with an axial (α) position for that proton. Therefore, the biotransformation product was 2α,3β,22β,23-tetrahydroxyurs-12-ene-28-oic acid, which is a new compound.

Compound **3** was obtained as a white amorphous powder with mp 231–232°C. The IR spectrum showed the absorption bands at 3340.9 (—OH) and 1716.7 (C=C) cm^{-1}. Its molecular formula, $C_{30}H_{48}O_7$, was determined according to HR-ESI-MS at *m/z* 519.3339 $[M - H]^-$. Comparing the ^{1}H NMR spectrum of the metabolite **3** with that of compound **2**, it has been found that two new signals were displayed at δ 3.95 and 3.99 integrating for one proton, respectively. However, a methyl doublet signal was absent in **3**. These were confirmed by the analysis of the ^{13}C NMR and DEPT spectra (Table 1), which showed only five methyl signals, instead of six. The methyl signal of C-30 at δ 21.4 in compound **2** disappeared, but a new methylene signal at δ 64.9 was exhibited. The hydroxylation at C-30 was further authenticated by the paramagnetic shifts of the neighboring carbons C-20 (from δ 37.6 of compound **2** to δ 45.7), while the signals of C-19 and C-21 shifted upfield to δ 33.4 and 34.9, respectively. It was also proved by the HMBC spectrum (see Figure 1), which exhibited the correlation of H-30 with C-21. As with compound **2**, the stereochemistry at C-22 was deduced from the multiplicity of the corresponding C—H signal in the ^{1}H NMR spectrum at δ 4.20. It was a double doublet ($J = 4.0$, 12.0 Hz) peak consistent with an axial (α) position for that proton. Therefore, the biotransformation product was 2α,3β,22β,23,30-pentahydroxyurs-12-ene-28-oic acid, which is a new compound.

3. Experimental

3.1 General experimental procedure

Melting points were determined with the Electrothermal melting point apparatus and are uncorrected. The IR spectrum were recorded with a Nicolet Avatar spectrometer. NMR spectra were recorded in pyridine-d_5 with a Bruker Avance III 400 spectrometer. HR-ESI-MS was detected on a Bruker APEX IV FT-MS spectrometer. Silica gel (200–300 mesh; Qingdao Haiyang Chemical Company, Qingdao, China) was used for column chromatography. Other normal reagents were purchased from Beijing Chemical Corporation (Beijing, China). Semi-HPLC (Alltech 426 HPLC Pump and LINEAR UVis 200 Detector) and Agilent Zorbax SB-C_{18} column (Agilent Technologies, Inc., Santa Clara, CA, USA) were used for purification.

3.2 Chemicals and micro-organisms

Asiatic acid was purchased from Nanjing Zelang Medical Technological Co. Ltd (Nanjing, China). All micro-organisms screened in our experiments were obtained from the China General Microbiology Culture Center (Beijing, China). Stock cultures of the fungi were stored on potato dextrose agar slants at 4°C.

3.3 Biotransformation procedures

Screening scale biotransformation of asiatic acid was carried out in 250 ml Erlenmeyer flasks containing 80 ml of medium. The flasks were placed in a rotary shaker operating at 180 rpm at 25°C. The substrate was dissolved in methanol to reach a concentration of 20 mg/ml. After 48 h of pre-culture, 2 mg of asiatic acid was added into each flask and these flasks were maintained under fermentation conditions for 4 days. Culture controls consisted of fermentation blanks in which micro-organisms were grown for 48 h, and then fed with the same amount of methanol

without containing the substrate. Substrate controls contained the sterile medium with the same amount of the substrate and were incubated under the above conditions.

Preparative scale biotransformation of asiatic acid was carried out in 1000 ml Erlenmeyer flasks containing 350 ml of medium by *A. longipes* (Ellis et Everhart). A substrate of 20 mg with 1 ml methanol was added to each flask containing the pre-culture medium. In total, 200 mg of the substrate were used. Incubation conditions were the same as described above.

3.4 Extraction and isolation

The culture was filtered and the filtrate was extracted with the same volume of EtOAc three times. The organic phase was collected and concentrated *in vacuo*. The residue (460 mg) was applied to a silica gel column and eluted with chloroform–methanol (in a gradient from 20:1 to 1:1), which yielded fractions 1–3. Fraction 2 (100 mg) was purified by an ODS column (MeOH–H_2O: 20–100%) and semi-preparative HPLC (MeOH–H_2O: 58%) to give compounds **1** (10 mg) and **2** (22 mg). Fraction 3 (15 mg) was purified by a silica gel column repeatedly to afford compound **3** (5 mg).

*3.4.1 Compound **1***

White amorphous powder (10 mg), mp: 211–212°C. IR (KBr) ν_{max} (cm^{-1}): 3363.3, 1689.4 cm^{-1}; ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS *m/z*: 527.3333 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_6Na$, 527.3343).

*3.4.2 Compound **2***

White amorphous powder (22 mg), mp: 270–271°C. IR (KBr) ν_{max} (cm^{-1}): 3367.5, 1691.3 cm^{-1}; ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS *m/z*: 503.3375 $[M - H]^-$ (calcd for $C_{30}H_{47}O_6$, 503.3378).

*3.4.3 Compound **3***

White amorphous powder (5 mg), mp: 231–232°C. IR (KBr) ν_{max} (cm^{-1}): 3340.9, 1716.7 cm^{-1}; ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS *m/z*: 519.3339 $[M - H]^-$ (calcd for $C_{30}H_{47}O_7$, 519.3329).

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