Biotransformation of Ursolic Acid by an Endophytic Fungus from Medicinal Plant *Huperzia serrata*

Shao-bin Fu,[a] Jun-shan Yang,[a] Jin-long Cui,[a] Xu Feng,[b] and Di-an Sun*,[a]

[a] *Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College; Beijing 100193, China: and* [b] *Navy Medical Research Institute; Shanghai 200433, China.*

Received April 17, 2011; accepted May 30, 2011; published online June 3, 2011

Endophytic fungi were used not only for their producing bioactive products but also for their ability to transform natural compounds. An endophytic fungus, isolated from medicinal plant *Huperzia serrata*, was identified as *Umbelopsis isabellina* based on the internal transcribed spacer of ribosomal DNA (rDNA-ITS) region. It was used to transform ursolic acid (1), a pentacyclic triterpene. Incubation of ursolic acid with *U. isabellina* afforded three products, 3β-hydroxy-urs-11-en-28,13-lactone (2), 3β,7β-dihydroxy-urs-11-en-28,13-lactone (3), 1β,3β-dihydroxy-urs-11-en-28,13-lactone (4). Although product 2 was a known compound, it was first obtained by microbial transformation. Products 3 and 4 were new compounds. The structural elucidation of the three compounds was achieved mainly by the 1D- and 2D-NMR, MS, IR data. The endophytic fungus *U. isabellina* can hydroxyate the C12–C13 double bond at position 13 of ursolic acid 1 and form a five-member lactone effectively. In the meantime, this fungus can also introduce the hydroxyl group at C-1 or C-7 of ursolic acid 1.

Key words biotransformation; ursolic acid; endophytic fungus; *Umbelopsis isabellina*; medicinal plant

Endophytes are bacterial or fungal microorganisms which colonize in the healthy plant tissue inter- and/or intracellularly without causing any apparent symptoms of disease.[1] Endophytes can produce bioactive substances, such as paclitaxel,[2,3] podophyllotoxin *etc.*[4,5] Endophytic fungi extensively metabolized 2-hydroxy-1,4-benzoxazin-3(2*H*)-one (HBOA) and transformed it to less toxic metabolites probable by their oxidase and reductases.[6] Thus, endophytes attracted more and more attention not only for producing many novel substances but also to transform the natural product.

In an attempt to find endophytes that might produce Huperzine A, a natural product isolated from the medicinal plant *Huperzia serrata* that was used for the treatment of Alzheimer's disease, we collected the medicinal plant *Huperzia serrata* and isolated over one hundred endophytes. Unfortunately, we were not able to find any of them that could produce Huperzine A.

Since microorganisms possess multi-enzyme systems which have significant regio- and stereo-selectivities, we hope to use these endophytes to transform some of the triterpenoids that is usually hard to achieve from chemical modification.[7]

Ursolic acid was a natural triterpenoid with many bioactivities, including anti-inflammatory activity,[8] anti-bacterial activity,[9] anti-virus activity.[10] Since ursolic acid has limited active sites for chemical modification, it's not easy to obtain large number of its derivatives for effective structure–activity relationship (SAR) study. In an attempt to modify ursolic acid, especially to obtain its hydroxylation products, we have used large number of microorganisms to transform this compound. In this article, we reported the microbial transformation ursolic acid by an endophytic fungus *U. isabellina* isolated from medicinal plant *H. serrata*.

Results and Discussion

Product **2** was obtained as white solid. Its molecular weight was determined as 454 according to ESI-MS data (*m*/*z*: 455 $[M+H]^+$, 931 $[2M+Na]^+$). It has a molecular weight that was two units less than that of the substrate **1**. Seven characteristic methyl group (two doublets and five singlets in the proton NMR) and proton signals of H-3 weren't changed compared to the ^{1}H-NMR spectrum of the substrate **1**. Two olefinic proton signals appeared at δ_H 5.94 (1H, br d, J=10.8 Hz) and δ_H 5.51 (1H, dd, J=4.8, 10.8 Hz). Correspondingly, two olefinic carbon signals at δ_C 134.1 and δ_C 130.0 were shown in its ^{13}C-NMR spectrum. Obviously, the double bond was not consistent with that in the substrate **1**. Moreover, there was another new oxygenated carbon at δ_C 90.0 in product **2**. The possibility is that the double bond was transferred from C12–C13 to C11–C12 with C-13 hydroxylated. The chemical shift of carbon-18 was also affected (downshifted from about δ_C 53.9 to δ_C 60.8). Deduced from the NMR data, there were two possible structures for this compound. The first one was that the 13-OH and 28-COOH were free. The second one was that there was a lactone formation between 13-OH and 28-COOH. Since its molecular

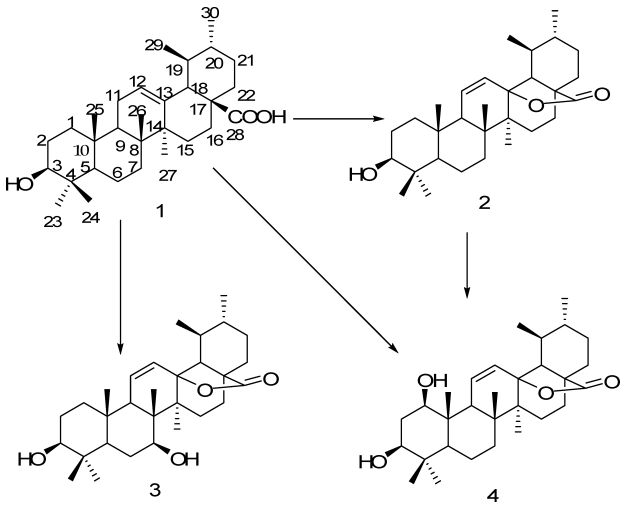

Fig. 1. Biotransformation of Ursolic Acid by Endophytic Fungus *U. isabellina* from *H. serrata*

* To whom correspondence should be addressed. e-mail: diansun@sina.com

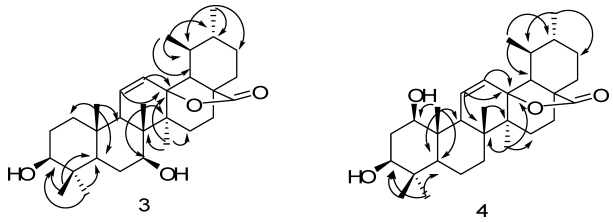

Fig. 2. Selected HMBC Correlations of Compounds **3** and **4**

weight was 454. It belongs to the second structure. Thus compound **2** was elucidated as 3b-hydroxy-urs-11-en-28,13-lactone. It was a known compound obtained from chemical modification.[11] Since there were no detailed NMR data reported for this compound, we reported its NMR data for further reference.

Product **3** was obtained as white power. It had the molecular formula of $C_{30}H_{46}O_4$, as evidenced by the HR-ESI-MS data (*m/z*: 469.3333 $[M-H]^+$, Calcd for $C_{30}H_{45}O_4$, 469.3312). It had one more oxygen atom and two less hydrogen atoms than the substrate **1**. It had one more oxygen atom than product **2**. In the ^{1}H-NMR spectrum, all seven methyl groups with the splitting pattern of ursan-type triterpene were appeared in the high field. There were two olefinic proton signals similar to those of compound **2**. From the APT (^{13}C-NMR) and HMQC spectra, two more hydroxyl groups were introduced. One carbon (δ_C 73.2) was connected with proton δ_H 4.25 (1H, dd, J=4.2, 10.8 Hz). The other carbon (δ_C 90.1) was a quaternary carbon. The correlations of H-27 (δ_H 1.40) and two olefinic protons with the carbon (δ_C 90.1) were observed in the HMBC spectra (Fig. 2). It was deduced that the double bond was at C-11–C-12. From the molecular weight 470, product **3** was supposed to have been formed an ester at C-28 with C-13 and thus lost one molecule water similar to product **2**. The carbon at δ_C 73.2 was assigned to C-7 based on its correlation with H-26 (δ_H 1.56) in the HMBC spectrum. The β-orientation of 7-OH was deduced from the coupling constants of H-7 (δ_H 4.25, 1H, dd, J=4.2, 10.8 Hz) in ^{1}H-NMR spectrum with H-7 at axial (a) position. The carbon with δ_C 61.2 was attributed to C-18 because of its correlation with H-29 (δ_H 1.05, 3H, d, J=6.6 Hz) in HMBC spectrum (Fig. 2). Thus, the structure of compound **3** was elucidated as 3β,7β-dihydroxy-urs-11-en-28,13-lactone. It was a new compound.

Compound **4** was obtained as white amorphous power. Its molecular formula was determined as $C_{30}H_{46}O_4$ according to the HR-EI-MS data (*m/z*: 470.3365 $[M^+]$, Calcd for $C_{30}H_{46}O_4$, 470.3396). Compared to product **2**, the ^{1}H-NMR spectrum of **4** exhibited a new hydroxylated proton signals at δ_H 3.85 (1H, dd, J=4.2, 10.8 Hz) except for H-3 (δ_H 3.61, 1H, dd, J=5.6, 12.6 Hz). Correspondingly, the ^{13}C-NMR spectrum of **4** showed a new oxygenated carbon signals at δ_C 80.2. In the HMQC spectrum, the carbon at δ_C 80.2 was correlated with the proton at δ_H 3.85. The correlation between H-25 (δ_H 1.29) with the carbon at δ_C 80.2 in the HMBC spectrum (Fig. 2) clearly showed that it had a C-1 hydroxylation. The hydroxyl group at C-1 was β-orientation was based on the coupling constants and splitting pattern of H-1 (δ_H 3.85,1H, dd, J=4.2, 10.8 Hz). Similar to compounds **2** and **3**, it also had the double bond transferred from C12–C13 to C11–C12 and C-13 was hydroxylated. The correlations between both H-11 (δ_H 5.65, 1H, dd, J=2.4, 10.8 Hz) and H-12 (δ_H 7.89, 1H, d, J=10.8 Hz) with C-13 (δ_C 89.9) were conspicuous in HMBC spectrum (Fig. 2). The other two correlations between H-11 with C-8 (Δ_C 42.7) and H-12 with C-14 (Δ_C 42.9) were also observed from HMBC data. H-27, H-11 and H-12 were correlated with the hydroxylated C-13 (δ_C 89.9) in the HMBC spectrum which further confirmed the above deduction. Since compound **4** also has a molecular weight of 470 the same as product **3**, it was also supposed to have been formed a five-member lactone between C-28 and C-13 that was in the same way as product **2** and **3**. Based on the above evidences, the structure of product **4** was identified as 1β,3β-dihydroxy-urs-11-en-28,13-lactone. It was also a new compound.

Many endophytic fungi were isolated from medicinal plant *H. serrata* by other research groups with few of them were used to modify natural products. We investigated the ability of the endophytic filamentous *U. isabellina* to transform ursolic acid. In all three products, the double bond transferred from C12–C13 to C11–C12 with the formation of a lactone between C-28 and C-13 correspondingly. This strain may produce the transferase and esterase that can transfer the double bond in this type of structure. The endophytic fungus *U. isabellina* can also introduce hydyoxyl groups into C-1 and C-7 positions.

In conclusion, endophytic fungi were useful resource not only for their bioactivity of their metabolites but also for their biotransformation ability of natural products. Endophytic fungus *U. isabellina* transferred the double bond of ursolic acid **1** effectively, and introduced the hydroxyl groups at saturated carbon in the triterpenoid rings that was hard to achieve through chemical methods.

Experimental

General Mps were determined by the apparatus that was uncorrected. Optical rotations were measured using a Perkin-Elmer 341 apparatus at 589 nm and 20 °C. The IR spectra were measured on a FTIR-8400S infrared spectrometer with KBr pellets. NMR spectra were acquired with Bruker DRX-600 spectrometer operating at 600 MHz (for proton NMR) and 150 MHz (for carbon NMR) using TMS as internal standard. UV spectra were recorded on Shimadzu UV2550 spectrometer. High resolution mass (HR-EI-MS) spectra employed a monoisotopic mass spectrometer. High-resolution electrospray ionization mass spectra (HR-ESI-MS) and ESI-MS were obtained with a Thermo LTQ Orbitrap XL mass spectrometer. Sterilization was carried out in an YX-2800 autoclave. Aseptic operations were carried out in SW-CJ-ID laminar flow cabinet. Incubations were on a HZQ-X100 constant temperature shake incubator. Column chromatography was carried out on silica gel (100—200, 200—300, 300—400 mesh, Qingdao Oceanic Chemicals, China). TLC was performed on 0.25 mm thick silica prepared plates also from Qingdao Oceanic Chemicals, China. Spot visualizations were made by spraying with H_2SO_4/95% EtOH (9 : 1, v/v) followed by heating.

Substrate, Microorganism, and Culture Medium. Substrate The substrate UA (with purity >98%) was purchased from Changsha Staherb Natural Ingredients Co., Ltd., Changsha, China. Its structure was characterized by comparison its ^{1}H- and ^{13}C-NMR data with those reported in the literature.[12]

Microorganism Endophytes were isolated from healthy medicinal plant *H. serrata* collected from Guizhou, China with a method described by Strobel *et al.* with slightly modification.[13] Leaves and stems of the plant were washed under running water 2 h, and rinsed 5 times with sterile distilled water. The leaves were sterilized with 75% EtOH (v/v) for 1 min and 0.1% mercuric chloride (v/v) for 5 min. The stems sere sterilized with 0.1% mercuric chloride (v/v) for 8 min. Finally, the samples were rinsed six times again in sterile water and were cut into small sections using a sterile knife. The cut segments were placed on Petri dishes containing potato dextrose agar and were incubated at 28 °C in darkness. The Petri dishes with the last sterile water as the control incubated under the same conditions. Pure cultures were then transformed to potato dextrose agar (PDA) plates. The puri-

fied fungi were stored in the PDA slant at 4 °C.

Culture Media The preliminary screening experiment was carried out in liquid PDA medium (consisting of 200 g potatoes boiling for 20 min; dextrose, 20 g; peptone, 10 g; distilled H_2O, 1000 ml).

Identification of Endophytic Fungus The fungus was identified by sequencing the internal transcribed region of ribosome deoxyribonucleic acid (rDNA-ITS). The universal primers ITS1 OF (5′-AACTCGGCCATTTA-GAGGAAGT-3′) and ITS4 OF (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS regions from the DNA extract.[14] The polymerase chain reaction (PCR) reaction was performed with the following cycles: (1) 94 °C for 3 min; (2) 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and (3) 72 °C for 10 min.

Fermentation of Compound and Extraction of Metabolites. Preliminary Screening The preliminary screenings were carried out in 100 ml Erlenmeyer flasks containing 40 ml of liquid PDA medium. After incubation at 28 °C and 160 rpm in the shaker for 2 d, the substrate 0.5 mg (dissolved in ethanol) were added into the flaks for another 10 d and harvested. Substrate controls with the substrate but without fungi and culture controls with the fungi but without substrate were incubated at the same conditions described as above. The cultures were extracted with ethyl acetate for 40 min at ultrasonic condition. The extracts were evaporated under reduced pressure on a rotary evaporator (50 °C) and then checked by TLC plates. The controls with the substrate but without fungi and the controls with the fungi but without substrate were incubated at the same conditions described as above.

Preparative Fermentation The preparative fermentation was carried out according to standard two-stage fermentation. The seed cultures were grown in 250 ml flasks each containing 100 ml of medium. 2% of the seed cultures were added fifteen 1000 ml flask that contains 400 ml liquid PDA media and several grains of glass beads which can increase the oxygen. After 3 d, 800 mg substrate ursolic acid (suspended in ethanol : DMSO=1 : 1) were distributed evenly in fifteen flasks. The cultures were further incubated for 10 d and then harvested. Mycelia were filtered and were ultrasonic extracted with ethyl acetate 40 min (3 times). The aqueous layer was extracted with the equal volumes of the same solvent for 3 times. Both extracts were combined and evaporated under reduced pressure on a rotary evaporator (50 °C) and 490 mg crude extracts were obtained.

Isolation of the Metabolites The crude residue (490 mg) was first purified by column chromatography on silica gel (200—300 mesh, 21 g) eluted with a stepwise petroleum ether/acetone from 100 : 1 to 1 : 1. Two fractions were obtained: fraction A (101 mg), B (60 mg). Fraction A was further purified by column chromatography on silica gel (300—400 mesh, 7 g) eluted with a stepwise petroleum ether/acetone from 10 : 1 to 8 : 1 to afford metabolite **2** (5 mg), and eluted with a stepwise dichloromethane /methanol from 90 : 1 to 60 : 1 to afford metabolite **3** (4 mg). Fraction B was further purified by column chromatography on silica gel (100—200 mesh, 12 g) eluted with a stepwise trichloromethane/methanol from 3 : 1 to 1 : 1 to afford metabolite **4** (7 mg).

Product **2**: White solid, mp 236—237 °C, ESI-MS: *m*/*z*: 455 $[M+H]^+$, 931 $[2M+Na]^+$, ^{1}H-NMR (C_5D_5N): 0.77 (3H, s), 0.89 (3H, s), 0.97—0.98 (6H, o), 1.04 (3H, (3H, s), 0.92 (3H, s), 1.14 (3H, s), 3.20 (m), 5.51 (1H, dd, J=10.8, 4.8 Hz, 11-H), 5.94 (1H, d, J=10.8 Hz, 12-H); ^{13}C-NMR (C_5D_5N): 38.5 (C-1), 23.5 (C-2), 78.3 (C-3), 39.9 (C-4), 55.4 (C-5), 18.3 (C-6), 31.9 (C-7), 42.4 (C-8), 53.7 (C-9), 37.0 (C-10), 130.0 (C-11), 134.1 (C-12), 89.8 (C-13), 42.6 (C-14), 28.2 (C-15), 26.2 (C-16), 45.5 (C-17), 60.8 (C-18), 38.9 (C-19), 40.7 (C-20), 21.3 (C-21), 32.3 (C-22), 28.7 (C-23), 16.2 (C-24), 16.5 (C-25), 19.5 (C-26), 18.6 (C-27), 179.8 (C-28), 18.5 (C-29), 19.7 (C-30).

Product **3**: White solid, mp 269—270 °C, $[\alpha]_D^{20}$ +5.3° (c=0.75, MeOH), UV λ_{max} (MeOH): 203 nm; IR (KBr) cm^{-1}: 3446, 3421, 2951, 2925, 2869, 2856, 1759, 1457, 1387, 1219. HR-ESI-MS: *m*/*z*: 469.3333 $[M+H]^+$, (Calcd for $C_{30}H_{45}O_4$, 469.3312). ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR (150 MHz, pyridine-d_5) see Table 1.

Product **4**: White solid, mp 274—277 °C, $[\alpha]_D^{20}$ +70.6° (c=0.11, MeOH), UV λ_{max} (MeOH): 205 nm; IR (KBr) cm^{-1}: 3546, 3437, 2969, 2951, 2933, 2868, 1757, 1467, 1387, 1360, 1220. HR-EI-MS: *m*/*z*: 470.3365 $[M]^+$, (Calcd for $C_{30}H_{46}O_4$, 470.3396). ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR (150 MHz, pyridine-d_5) see Table 1.

Acknowledgements This work is financially supported by the National Nature Science Foundation of China (Project No. 20772158) and the New Drug Development Project of China (Project No. 2009ZX09301-003).

Table 1. ^{1}H- (600 MHz) and ^{13}C-NMR (150 MHz) Data of Compounds **3** and **4** (Pyridine-d_5)

No.	**3**		**4**	
	δ_H	δ_C	δ_H	δ_C
1	1.86 (o), 0.96 (m)	38.8	3.85 (dd, 4.2, 10.8)	80.2
2	1.92 (o)	28.7	2.35 (o), 2.39 (o)	39.7
3	3.49 (m)	78.2	3.61 (dd, 5.6, 12.6)	75.9
4	—	39.7	—	0.1
5	1.05 (m)	53.3	0.84 (o)	53.0
6	2.08 (o)	30.0	1.67 (o), 1.71 (o)	18.3
7	4.25 (dd, 4.2, 10.8)	73.2	1.13 (o), 1.44 (o)	31.9
8	—	43.8	—	42.7
9	2.06 (o)	53.4	2.49 (o)	54.9
10	—	37.1	—	43.2
11	5.75 (dd, 1.2, 10.2)	130.3	5.65 (dd, 2.4, 10.8)	128.2
12	6.05 (br d, 10.8)	133.3	7.89 (d, 10.8)	139.4
13	—	90.1	—	89.9
14	—	48.0	—	42.9
15	1.25 (o)	30.5	1.81 (o), 1.19 (o)	26.4
16	1.41 (o), 2.22 (td, 4.8, 12.6)	23.8	2.15 (o),1.38 (o)	23.6
17	—	45.6	—	45.6
18	1.71 (d, 12.0)	61.2	1.65 (d, 12.6)	60.7
19	1.83 (o)	39.6	1.81 (m)	38.6
20	0.78 (o)	40.8	0.75 (o)	40.7
21	1.42 (o)	31.4	1.43 (o)	31.4
22	1.88 (o)	32.4	1.85 (o), 1.90 (o)	32.3
23	1.25 (s)	29.9	1.25 (o)	28.8
24	1.04 (o)	16.3	1.10 (s)	16.1
25	0.98 (s)	15.5	1.29 (o)	15.5
26	1.56 (s)	15.5	1.36 (s)	20.2
27	1.40 (o)	17.0	1.29 (o)	16.7
28	—	180.0	—	179.9
29	1.05 (d, 6.6)	18.3	0.99 (d, 6.0)	18.1
30	0.88 (o)	18.4	0.86 (d, 6.0)	19.5

Chemical shifts: ppm; coupling constants: Hz; Mult, multiplicity: s, singlet; d, doublet; t, triplet; o, overlap. Assignments were based on ^{1}H-NMR, ^{13}C-NMR, APT, HMQC and HMBC experiments.

References

1) Wilson D., *Oikos*, **73**, 274—276 (1995).
2) Shrestha K., Strobel G. A., Shrivastava S. P., Gewali M. B., *Planta Med.*, **67**, 374—376 (2001).
3) Chakravarthi B. V., Das P., Surendranath K., Karande A. A., Jayabaskaran C., *J. Biosci.*, **33**, 259—267 (2008).
4) Puri S. C., Nazir A., Chawla R., Arora R., Riyaz-Ul-Hasan S., Amna T., Ahmed B., Verma V., Singh S., Sagar R., Sharma A., Kumar R., Rakesh K. S., Ghulam N. Q., *J. Biotechnol.*, **122**, 494—510 (2006).
5) Kour A., Shawl A. S., Rehman S., Sultan P., Parvaitz H. Q., Qazi P. H., Suden P., Khajuria R. K., Verma V., *World J. Microbiol. Biotechnol.*, **24**, 1115—1121 (2006).
6) Wu S. H., Chen Y. W., Qin S., Huang R., *J. Basic Microbiol.*, **48**, 140—142 (2008).
7) Feng X., Zou Z. M., Fu S. B., Sun L. Z., Su Z. H., Sun D. A., *J. Mol. Catal., B Enzym.*, **66**, 219—223 (2010).
8) Baricevic D., Sosa S., Della Loggia R., Tubaro A., Simonovska B., Krasna A., Zupancic A., *J. Ethnopharmacol.*, **75**, 125—132 (2001).
9) Fontanay S., Grare M., Mayer J., Finance C., Duval R. E., *J. Ethnopharmacol.*, **120**, 272—276 (2008).
10) Novotný L., Vachálková A., Biggs D., *Neoplasma*, **48**, 241—246 (2001).
11) Mezzeti T., Orzalesi G., Bellavita V., *Planta Med.*, **20**, 244—252 (1971).
12) Ju J. H., Zhou L., Lin G., Liu D., Wang L. W., Yang J. S., *Chin. Pharm. J.*, **38**, 752—757 (2003).
13) Strobel G., Yang X., Sears J., Kramer R., Sidhu R. S., Hess W. M., *Microbiology*, **142**, 435—440 (1996).
14) Taylor D. L., McCormick M. K., *New Phytol.*, **177**, 1020—1033 (2008).