⁄@¡B⁄⁄⁄ ″K›n

§ ⁄ ¸ *Cunninghamella elegans* ´ · « ¤ ª ⁄ › ⁄ T « (ursolic acid) G» ˜ (3β-hydroxy-urs-12-en-28-oic acid) (**1**) ¥ ¡ ⊖¤A ´ _ı ″ ″ ¥ N ` ″ « ¡ A 4-hydroxy-3,4-*seco*-urs-12-ene-3,28-dioic acid (**2**) ⁄ ¸ 3-methoxy-4-hydroxy-3,4-*seco*-urs-12-ene-3,28-dioic acid (**3**) ¡ F ƒ ¥ 1 » P *Nocardia* sp. ¶ ¡ ƒ ¥ ¡ ⊖¤ ¥ ⁄ ″ ″ ″ ″ G» (**4**) ¡ F¤ ⁄ ⁄ ¥ N ˇ ″ 2 ⁄ ¸ 3 ‹ › ″ ƒ , ○ ″ ″ ⁄ ˘ ƒ X ″ « A ⁄ ´ ″ ″ ¥ N ˇ ″ « g ¥ ƒ U ″ ¥ œ ⁄ ¤ ″ R ¥ H ¡ C ¥

^ ` ¡ G L ¥ ″ « ´ · « ¡ A ⁄ T « ^ ¡ A ″ G» ¡ ˜ A ¥ N ´ ″ «

›^⁄ ″K›n

Microbial transformation of the pentacyclic triterpene ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) (**1**) was studied. Preparative scale biotransformation of **1** with *Cunninghamella elegans* resulted in the ring-A cleavage, which has been characterized as 4-hydroxy-3,4-*seco*-urs-12-ene-3,28-dioic acid (**2**) and 3-methyoxy-4-hydroxy-3,4-*seco*-urs-12-ene-3,28-dioic acid (**3**). Preparative scale biotransformation of **1** with *Nocardia* sp. afforded methyl ursolate (**4**). Metabolites **2** and **3** are the first reported. Metabolite structures were established on the basis of HRFABMS, 1D and 2D NMR spectral data.

Keywords: microbial transformations, triterpene, ursolic acid.

⁄G¡B‰t¥ »P¥ "″

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) (**1**), is a pentacyclic ursane type of triterpene that is widely distributed in nature. Ursolic acid has been shown to exhibit a variety of biological activities, including antiinflammatory,[1-5] hepatoprotection,[6,7] trypanodcidal,[8] Cox-2 inhibition,[9,10] DNA polymerase β inhibition,[11] anti-tumor,[12,13] anti-HIV,[14,15] anti-angiogenic activity,[16] antileukemic activity,[1,17] and antibacterial activity.[18,19]

Microorganisms have already been successfully applied for the selective oxygenation of organic compounds, especially the inactivated sites of hydrocarbons.[20] Utilizing microorganisms for studying the metabolism of natural and synthetic drugs has been documented.[21] The bridge ring system of the ursolic acid similar to steroid structure makes it a suitable substrate for the effect of structure on the regiochemistry of microbiological hydroxylation. Despite the diverse pharmacological actions and the potential of using ursolic acid for different indications, there is only one report about microbial metabolism of methyl ursolic acid with *Mucor plumbeus*.[22] Thus, in order to obtain novel and more-effective compounds, structural modification of **1** by microbial transformation was carried out. In this report, the identification of two new metabolites (**2** and **3**) and one known metabolite (**4**) are presented herein.

⁄T¡Bx ª¤s⁄Ł"k

Twenty microbial cultures including 11 genera (number of species): *Aspergillus* (2), *Bacillus* (2), *Beauveria* (2), *Cunninghamella* (3), *Gliocladium* (1), *Mortierella* (1), *Mucor* (2), *Mycorbacterium* (1), *Nocardia* (3), *Pseudomonas* (1), *Streptomyces* (2), were used for the preliminary screening. The screening experiment was performed by a two-stage fermentation procedure as described previously.[23] Three metabolites were reproducibly by *C. elegans* and *Nocardia* sp.. Preparative-scale bioconversion of **1** with *C. elegans* and *Nocardia* sp. was conducted as described previously.[23] After solvent extraction and evaporation *in vacuum*, the crude extracts were subjected to column chromatography over silica gel. By repeated silica gel column chromatography or HPLC purification, metabolites **2**-**4** were yielded.

¥|¡B †"G»P Q‰

Screening a rang of microorganisms incubated with ursolic acid (**1**) resulted in the use of *C. elegans* and *Nocardia* sp. to generate two new ring-A cleaved biotransformation products (**2** and **3**) along with one known compound (**4**). Incubation of **1** with *C. elegans* for 6 days led to the formation of compounds **2** and **3**. The ^{13}C NMR spectrum displayed resonances for 30 carbons, while the DEPT spectrum showed the presence of the seven methyl, nine methylene, seven methine, and five quaternary carbons. The tertiary carbinol carbon at C-3 was disappeared instead of one quaternary carbon at δ 74.8. The presence of two carboxyl groups in **2** was inferred by the ^{13}C NMR spectrum (δ 180.1 and 178.0). In the ^{1}H NMR spectrum of **2**, signals of seven methyl groups were observed showing that no methyl group in the original compound **1** was oxygenated. The HMQC and HMBC spectra showed no differences in ring-B to ring-E except for ring-A. Comparison of ^{13}C NMR spectra of **1** and **2** revealed that the CH_3-23 was shifted from δ 16.6 to 33.9 due to hydroxyl group at C-4. In the HMBC spectrum, δ 74.8 exhibited cross-peaks with δ 1.44 (CH_3-24), 1.48 (CH_3-23), and 1.68 (H-5). The chemical shift at δ 178.0 (C-3) showed correlation with δ 3.01 (H-1). Thus, it suggested **2** to be 3,4-*seco*-urs-12-ene-3,28-dioic acid derived from **1** via oxidative cleavage of the ring-A (Figure 1). On the basis of spectral evidence, metabolite **2** was assigned the structure

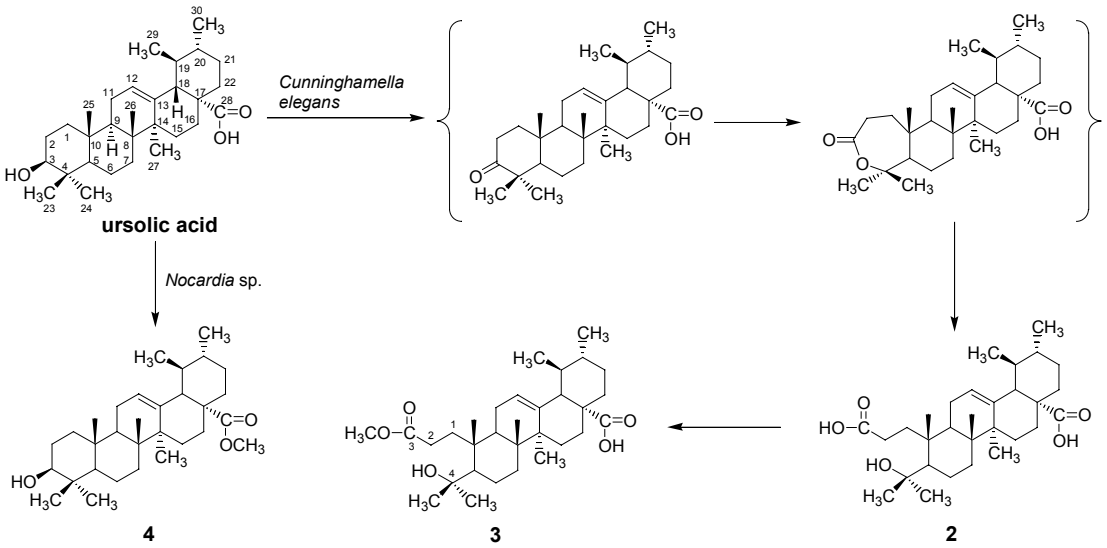

Figure 1. Structures of ursolic acid and its metabolites **2**-**4**.

4-hydroxy-3,4-*seco*-urs-12-ene-3,28-dioic acid, which has not been reported in the literature. Metabolite **3** was isolated as white powder. Its HRFABMS showed a quasi-molecular ion $[M + Na]^+$ at *m/z* 525.3559, indicating a molecular formular of $C_{31}H_{50}O_5Na$ (calcd 525.3556). In the DEPT spectrum, the tertiary carbinol carbon at C-3 was also disappeared instead of one quaternary carbon at δ 76.1. The ^1H- and ^{13}C NMR spectra showed close similarity to those of **2** and displayed additional proton and carbon signals for OCH_3 at δ 3.62 and 52.1, respectively. The methoxy moiety was confidently linked at C-3 basing on the HMBC correlation between the C-3 (δ 177.1) and OCH_3 (δ 3.62). Comparison of COSY, NOESY, DEPT, HMQC, and HMBC data with those of **1** and **2** indicates that metabolite **3** is determined as 3-methyoxy-4-hydroxy-3,4-*seco*-urs-12-ene-3,28-dioic acid.

Incubation of **1** with *Nocardia* sp. yielded metabolite **4**. Metabolite **4** was obtained as white needles after recrystallization with ethyl acetate. The HRFABMS mass spectrum gave a molecular ion peak $[M + H]^+$ at *m/z* 471.3837 (calcd 471.3838), corresponding to the molecular formula $C_{31}H_{51}O_3$. The striking difference between **1** and **4** was the additional proton and carbon signals for OCH_3 at δ 3.72 and 51.5, respectively. In ^{13}C NMR spectrum, the chemical shift at C-28 was shifted from δ 179.9 to 177.8 due to OCH_3 group. The methoxy moiety was confidently linked at C-28 basing on the HMBC correlation between the C-28 (δ 177.8) and OCH_3 (δ 3.72). Thus, by comparison spectra data with literature,[22] metabolite **4** was established as methyl ursolate.

In conclusion, oxidative ring cleavage and methylation were found in this study of the transformation of ursolic acid with *C. elegans* and *Nocardia* sp.. The microbial oxidative cleavage of ring-A to yield 4-hydroxy-3,4-*seco*-3-oic acid has been reported in the biotransformation of betulonic acid by *Chaetomium longirostre* IFO 9873,[24] eburicoic acid by *Glomerella fusarioides*,[25] and oleanonic acid by *C. longirostre* RF-1095.[26] However, metabolites **2** and **3** are the first reported from the biotransformation of ursolic acid (**1**). The mechanism of ring-cleavage has been suggested an enzymic controlled Baeyer-Villiger type reaction with the 3-ketone as intermediate.[26] The ring-A cleavage of ursolic acid might be occurred by oxidation of 3-hydroxyl group to 3-ketone first, and then re-oxidization to the intermediate lactone, followed by hydrolysis to yield **2**. Thereafter, metabolite **2** was selectively methylated at C-3 to yield **3**. On the other hand, *Nocardia* sp. could transform ursolic acid to the one major metabolite, methyl ursolate. These isolated metabolites will be used as reference standards for study on the mammalian metabolism of ursolic acid.

Table 1. ^{13}CNMR assignments for ursolic acid (**1**) and metabolites **2-4** (C_5D_5N, δ values in ppm)

Carbon	**1**	**2**	**3***	**4**
1	39.1	35.5	35.5	39.1
2	28.2	30.0	30.0	28.2
3	78.2	178.0	177.1	78.2
4	39.4	74.8	76.1	39.4
5	55.9	52.1	53.1	55.9
6	18.8	22.9	23.3	18.8
7	33.6	33.0	33.7	33.5
8	40.0	39.9	40.6	39.9
9	48.0	39.3	40.4	48.0
10	37.3	23.5	42.1	37.3
11	23.7	126.2	24.1	23.6
12	125.7	138.9	127.2	125.9
13	139.3	138.9	139.4	138.8
14	42.5	43.2	43.8	42.4
15	28.7	28.8	29.2	28.4
16	24.9	24.9	25.3	24.6
17	48.1	48.2	49.2	48.4
18	53.6	53.6	54.4	53.4
19	39.5	39.6	40.6	39.2
20	39.4	39.4	40.5	39.3
21	31.1	31.1	31.8	30.8
22	37.5	37.5	38.1	37.1
23	16.6	33.9	32.9	16.6
24	28.8	28.4	28.3	28.8
25	15.7	20.4	20.5	15.7
26	17.5	17.8	17.7	17.4
27	23.9	23.7	23.8	23.9
28	179.9	180.1	181.7	177.8
29	21.4	21.4	21.5	21.3
30	17.5	17.7	17.7	17.2
OCH_3			52.1	51.5

* in CD_3OD

⁄›¡B›p ef¤‴Gf ß

Microbial metabolism studies have been used as inexpensive *in vitro* model systems to predict mammalian metabolism or to increase the efficacy of a drug through metabolic activation.[21] Ring-A cleavage of ursolic acid by microorganism has not been reported. In some isolated minor metabolites, the tertiary carbinol carbon at C-3 was also disappeared instead of one quaternary carbon in the DEPT spectra. It suggests that these metabolites also have ring-A cleaved skeleton. The structural elucidation is still in progress. Ring-A cleaved analogues of triterpenes might show significant biological activities.[24,27] Thus, the isolated metabolites will be further tested for anticancer and antibacterial activities.

⁄»¡B ƒ ⁄ ˜m

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