Microbial Metabolism of Biologically Active Secondary Metabolites from *Nerium oleander* L.

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Ursolic acid (1) and kaempferol (3) are two major constituents of the Mediterranean plant *Nerium oleander* L. Microbial metabolism of (1) with *Aspergillus flavus* (ATCC 9170) resulted in the formation of 3-oxo-ursolic acid derivative, ursonic acid (2). On the other hand, *Cunninghamella blakesleeana* (ATCC 8688A) was able to convert (3) into kaempferol 3-*O*-β-D-glucopyranoside (4) as well as the new natural product kaempferol 4′-sulfate (5). Incubation of kaempferol with *Mucor ramannianus* (ATCC 9628) led to the isolation of one metabolite identified as kaempferol 4′-*O*-α-L-rhamnopyranoside (6). Transformation of kaempferol to the new compound kaempferol 7-*O*-β-D-4-*O*-methylglucopyranoside (7) and herbacetin 8-*O*-β-D-glucopyranoside (8) was observed after fermentation with *Beauveria bassiana* (ATCC 13144). Cytotoxic as well as antioxidant activities of the isolated metabolites were determined.

Key words kaempferol; *Nerium oleander*; microbial metabolism; ursolic acid

Nerium oleander L. is a medium-sized flowering evergreen tree of 2—5 m in height and is planted throughout the world tropical regions as garden and roadside trees. *N. oleander* was originally distributed in the Mediterranean region, subtropical Asia, and Indo-Pakistan subcontinent. This plant possesses cardiotonic, antibacterial, anticancer, and anti platelet aggregation activity and depresses the central nervous system.[1,2] In connection with these biological activities, many kinds of secondary metabolites have been reported. Ursolic acid (**1**), a pentacyclic triterpenoid acid of ursane type, occurs free or as glycosides and glycoside esters in *N. oleander* L. as well as medicinal herbs and various other plants.[3,4] This compound is of interest for pharmaceutical industry because of the wide spectrum of pharmaceutical activities. Antiviral,[5] antitumor and cytotoxic,[6] antibacterial,[7] hepatoprotective,[8] trypanocidal,[9] antitubercular,[10] antidiabetic,[11] antioxidant[12] and anti-inflammatory activities[13] have been reported for ursolic acid. Due to the former mentioned interesting bioactivities ursolic acid has been reported to be used as a drug and food additive.[14] It is known that pentacyclic triterpenoids especially the carboxylic acids are recalcitrant to biodegradation and biotransformation,[15] to the extent of being used as chemical markers for taxonomic studies due to their high stability.[16] The pentacyclic triterpenoid acid nature and high stability has stood as a barrier against any *in vivo* metabolic studies to determine the biological fate of ursolic acid. On the other hand previous incubation of **1** with *Nocardia* sp., NRRL 5646 resulted in the isolation of two metabolites, oleanolic acid and ursolic acid methyl ester.[17] Another class of compounds isolated in large quantities from genus *Nerium* is the flavonoids. Flavonoids account for approximately two thirds of the phenolics in our diet as well as a high percentage of the secondary metabolites in *N. oleander*.[18] Kaempferol is a flovonol type flavonoid with a wide spectrum of bioactivity. It has been isolated from *N. oleander* L. as well as a wide range of medicinal herbs.[19] Kaempferol represents one of the most potent antioxidant drugs isolated from natural sources.[20,21] Kaempferol was also found to possess antiatherogenic,[22] anti-inflammatory[23] as well as antidepressant activities.[24] An important factor in the evaluation of safety and efficacy of a drug is the knowledge of how the drug is metabolized. Metabolism is defined as simply the structural modification of a drug by enzymatic systems.[25] Traditionally, drug metabolism studies are conducted on small animal models, *in vitro* cell cultures, and *in vitro* enzyme systems. Microbial models may constitute an alternative to animal models as they can mimic the mammalian metabolism.[26] Microorganisms have been widely utilized as models of drug metabolism to predict the fate of xenobiotics in mammalian systems.[27] Since this method often gives sufficient quantities of metabolites, complete chemical structures and pharmacological activities could be detected. Also, they can be used as analytical standards to detect and characterize particular metabolites which could be present in very small amounts in biological fluids.[28] This methodology has the advantage of reducing the use of animals particularly in the early phases of drug development. Presently, it is in use with the same intention to obtain more active or less toxic substances and some selective conversions of compounds to useful derivatives.[26] In the present study, a panel of 41 microorganisms has been screened for the ability to exert a chemical modification on ursolic acid and kaempferol in a trial to predict their fate after mammalian metabolism.

Experimental

General Experimental Procedures 1D and 2D nuclear magnetic resonance (NMR) spectra were recorded in pyridine-d_5 (ursolic acid) and dimethyl sulfoxide (DMSO)-d_6 (kaempferol) on a Bruker Avance DPX-500 spectrometer and on a Varian AS 400 spectrometer using trimethyl silane (TMS) as the internal standard with chemical shifts expressed in δ and coupling constants (J) in Hz. InfraRed (IR) spectra were recorded on a Bruker Tensor 27 spectrophotometer. Ultra violet (UV) spectra were obtained on a Varian Cary 50 Bio UV–Visible spectrophotometer. Optical rotations were measured at ambient temperature using a Rudolph Research Analytical Autopol IV automatic polarimeter. High resolution electrospray ionization mass

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spectrometric (HR-ESI-MS) was obtained using a Bruker Bioapex FTMS in ESI mode. GCMS analyses were carried out on a ThermoQuest Trace 2000 GC.

Thin-layer chromatography (TLC) was carried out on aluminum-backed plates pre-coated with silica gel F_{254} (20×20 cm, 200 μm, 60 Å, Merck) and on glass-backed plates pre-coated with silica gel F_{254} (10×20 cm, 200 μm, 60 Å, Analtech) Visualization was accomplished by spraying with *p*-anisaldehyde spray reagent followed by heating. Flash silica gel (40—63 μm, 60 Å, Silicycle) and Sephadex LH20 were used for column chromatography.

Flash column liquid chromatography was performed (Biotage Horizon) and silica gel (KP-silTM 60 Å cartridge).

Organisms and Substrates Ursolic acid was obtained from SIGMA Co. (U.S.A.) and kaempferol was obtained from Chromadex Co., U.S.A.

A total of 41 microorganisms from the collection of the National Center for Natural Products Research, University of Mississippi, U.S.A. were used in the preliminary screening experiments to identify organisms capable of metabolizing ursolic acid and kaempferol.

Analytical Scale Fermentation Medium-α[29] consisting of (dextrose, 20 g; NaCl, 5 g; K_2HPO_4, 5 g; bacto-peptone (Difco Labs, Detroit, MI, U.S.A.), 5 g and yeast extract (Difco Labs), 5 g per liter of distilled water was used in all fermentation experiments based on a two-stage procedure.[30] Initial screening was performed in 125 ml Erlenmeyer flasks (125 ml) containing 25 ml medium-α. The media were autoclaved for 20 min at 121 °C and 15 psi before innoculation. The substrates were added in dimethylformamide (DMF) (5 mg/500 μl) solution to 24 h old stage II cultures. Incubation was carried out at room temperature for a period of 14 d on a rotary shaker (New Brunswick Model G10-21) at 100 rpm.

Monitoring of the samples was carried out at 7-d intervals following substrate addition. Each sample (5 ml) was extracted by shaking with 3×5 ml (EtOAc–$(Me)_2CHOH$), (8 : 2) and spun at 3000 for 5 min in a desk-top centrifuge. Each organic solvent extract from all samples was spotted on TLC plates and developed with $CHCl_3$–MeOH (9 : 1) and (95 : 5) in case of **1** and (8 : 2) and (8 : 3) in case of **3** as the solvent systems. Culture controls were run simultaneously with the above experiments.[29]

Preparative Scale Fermentation Quartet sets of five 2 l flasks, each containing 500 ml of medium-α were used for preparative scale fermentation of **1** with *Aspergillus flavus* (ATCC 9170) and kaempferol **3** with *Cunninghamella blakesleeana* (ATCC 8688A), *Mucor ramannianus* (ATCC 9628) and *Beauveria bassiana* (ATCC 13144) were prepared according to a two-stage procedure. Each flask in a set received 100 mg of substrate dispersed 10 ml of DMF for the preparative scale fermentation with each microorganism separately. Culture controls were run simultaneously with the above experiments.[29] Incubation was carried out at room temperature for a period of 14 d on a rotary shaker 100 rpm. The cultures of each microorganism were exhaustively extracted with (EtOAc : $(Me)_2CHOH$), (8 : 2) 3×500 ml for each culture flask separately. The solvent was evaporated to dryness under reduced pressure (at 40 °C) and the resulted extract of the collective flasks extracts for each microorganism was further dried over anhydrous Na_2SO_4.

Isolation and Purification of the Metabolites The resulting extract (493 mg) from fermentation of **1** with *Aspergillus flavus* (ATCC 9170) was chromatographed over silica gel 60 column (100 g, column ϕ3 cm) using hexanes gradually enriched with $CHCl_3$. Purification of metabolite **2** (50.2 mg) was achieved through repeated column and preparative thin layer chromatography ($CHCl_3$–MeOH), (95 : 5). For the microbial transformation of **3** by *Cunninghamella blakesleeana* (ATCC 8688A), the combined cultures extract (572 mg) was chromatographed over silica gel column (120 g, column ϕ3 cm) with $CHCl_3$ gradually enriched with MeOH until reaching pure MeOH. The fraction containing the metabolites was subjected to further purification using Sephadex LH-20 packed open glass columns (100 g, column ϕ2 cm) with pure MeOH as an eluent to yield two metabolites **4** (51.5 mg) and **5** (25 mg). The extract (667 mg) obtained from the fermentation of **3** with *Mucor ramannianus* (ATCC 9628) was subjected to flash chromatography (Biotage Horizon) over silica gel (KP-silTM 60 Å cartridge). A gradient MeOH in $CHCl_3$ was used as the solvent system. The fraction containing the metabolite was further purified using Sephadex LH-20 packed column (50 g, column ϕ1 cm) using MeOH as an eluent to yield one metabolite **6** (2 mg). On the other hand the extract (670 mg) resulted from microbial metabolism of **3** with *Beauveria bassiana* (ATCC 13144) yielded two metabolites **7** (56 mg) and **8** (6.7 mg). The extract was chromatographed over silica gel (150 g, column ϕ3 cm) with $CHCl_3$ gradually enriched with MeOH until reaching MeOH. The fraction containing the metabolites was subjected to further purification using different sizes of Sephadex LH-20 open glass columns with MeOH as an eluent.

Acid Hydrolysis and Identification of Sugar The compounds **4**, **6**, **7** and **8** (1—2 mg) were refluxed with 0.5 N HCl (3 ml) for 2 h. The reaction mixture was diluted with water and extracted with $CHCl_3$. The water layer was dried under reduced pressure as well as under N_2 to give the monosaccharide. Identification of sugars was achieved *via* ^{1}H- and ^{13}C-NMR. The absolute configurations were determined through comparing the specific rotation and/or retention time using GCMS with an authentic sample as described by Hara *et al.*[31] The obtained sugars after the acid hydrolysis were dissolved in pyridine (1 ml) and 0.1 M L-cysteine methyl ester hydrochloride in pyridine (2 ml) was added. The mixture was heated at 60 °C for 1 h. An equal volume of Ac_2O was added with heating continued for another 1 h. Acetylated thiazolidine derivatives were subjected to GCMS analysis (Conditions: Column, JW DB-5, 30 m×0.25 mm, 0.25 μm; carrier gas He; injection temperature 280 °C, detection temperature 280 °C, column temperature; 150 °C (1 min), 10 °C/min to 250 °C (30 min). The configurations were determined by comparing their retention times with acetylated thiazolidine derivatives prepared in a similar way from standard sugars (Sigma–Aldrich) (t_R D-glucose 21.32 min, t_R L-glucose 22.68 min, t_R D-glactose 22.12 min, t_R L-rhamnose 15.64 min, t_R L-xylose 16.90 min t_R L-arabinose 16.11 min).

Assay for Cytotoxicity of the Isolated Metabolites Cytotoxicity was determined against a panel of mammalian cells that included two noncancer kidney cells (Vero and LLC-PK_{11}) and four solid tumor cells. (SK-MEL skin malignant melanoma, KB oral epidermal carcinoma, BT-549 breast ductal carcinoma and SK-OV3 ovary carcinoma) as described earlier.[30]

Assay for Antioxidant Activity of Isolated Metabolites Inhibition of ROS (reactive oxygen species) generation in HL-60 cells was determined by a flourimetric method as described earlier.[32] Cells were cultured in RPMI medium with 10% FBS and antibiotics at 37 °C in an atmosphere of 5% CO_2. For the assay cells were seeded (125000 cells/well) to the wells of 96-well plates. After treatment with different concentrations of the test compounds for 30 min, cells were stimulated with 100 ng/ml phorbol 12-myristate-13-acetate (PMA, Sigma) for 30 min. DCFH-DA (Molecular Probes, 5 μg/ml) was added and cells were further incubated for 15 min. Plates were read on a PolarStar with excitation wavelength of 485 nm and emission wavelength of 530 nm.

DCFH-DA is a non-fluorescent probe that diffuses into the cells, where cytoplasmic esterases hydrolyse the DCFH-DA to 2′,7′-dichlorofluorescin (DCFH). The ROS generated within HL-60 cells oxidize DCFH to the fluorescent dye 2′,7′-dichlorofluorescein (DCF). The ability of the metabolites to inhibit ROS-catalysed oxidation of DCFH is measured in comparison to PMA treated vehicle controls. Vitamin C was used as the positive control. In parallel to antioxidant activity, cytotoxicity to HL-60 cells was also determined as described earlier.[32]

Results and Discussion

Initial screening of **1** and **3** was carried out with 41 microorganisms using the standard two stage procedure.[29] Only *Aspergillus flavus* (ATCC 9170) showed the capability of transforming **1** to one less polar metabolite, on the other hand, three organisms showed the capability of transforming **3** into different and more polar metabolites. Based on the screening results *Aspergillus flavus* (ATCC 9170) was selected for scale up fermentation procedure of **1** to yield the 3-oxo derivative of ursolic acid (**2**). However, *Cunninghamella blakesleeana* (ATCC 8688A) was able to convert **3** into kaempferol 3-*O*-β-D-glucopyranoside (**4**) and kaempferol 4′-sulfate (**5**). Incubation of **3** with *Mucor ramannianus* (ATCC 9628) led to the isolation of metabolite (**4**) again as well as one minor metabolite identified as kaempferol 4′-*O*-α-L-rhamnopyranoside (**6**). Transformation of **3** to kaempferol 7-*O*-β-D-4-*O*-methylglucopyranoside (**7**) and herbacetin 8-*O*-β-D-glucopyranoside (**8**) was observed when fermented with *Beauveria bassiana* (ATCC 13144).

Metabolite **2**: (50.2 mg, 10.25% yield) isolated as a white amorphous powder, $[\alpha]_D^{25}$ +80° (c=0.5%, MeOH). Its HR-ESI-MS data showed an $[M+H]^+$: (m/z) 455.3698, when considered in conjugation with ^{1}H and ^{13}C this indicated a

molecular formula of $C_{30}H_{46}O_3$. IR (KBr) cm^{-1} absorption band at 1705 (saturated six-membered ring ketone band) together with the resonance at δ 216.6 at the ^{13}C-NMR spectrum indicated the presence of a carbonyl moiety in the structure. The ^{1}H-NMR spectrum (400 MHz, pyridine-d_5) of **2** displayed resonances at δ 0.89 (3H, s, H-25), 0.98 (3H, d, J=6.0 Hz, H-29), 1.02 (3H, d, J=8.8 Hz, H-30), 1.02 (3H, s, H-24), 1.05 (3H, s, H-26), 1.16 (3H, s, H-23) and 1.22 (3H, s, H-27).

The ^{13}C-NMR spectrum (100 MHz, pyridine-d_5) of **2** showed resonances for 30 carbons differentiated by Distortionless Enhancement by polarization transfer (DEPT-135) experiment into 7 methyl, 9 metylene, 6 methine and 8 quaternary carbons suggesting ursane and not an oleanane skeleton. The spectrum revealed the presence of two oxygenated carbons at δ 180.3 and 218.6 indicating the carboxylic acid and the carbonyl moieties at C-3 and C-28 respectively. The ^{13}C-NMR shows two olefinic carbon resonances at δ 125.7 and 139.6, the latter disappears in the DEPT spectrum indicating one quaternary olefinic carbon. The above data strongly indicate the ursane—not oleanane—pentacyclic triterpenoid frame work with a carbonyl group. This assumption has further confirmed *via* the long-range heteronuclear multiple correlations (HMBC) of H_2-2/C-3 as well as H_3-23/C-3 and H_3-24/C-3. The positions of the seven methyl groups were confirmed *via* the HMBC correlations H_3-23/C-10, C-3; H_3-24/C-3; H_3-26/C-14, C-9; H_3-27/C-14; H_3-30/C-20. The ^{13}C-NMR chemical shifts for **1** and **2** were shown in Table 1; it is evident that the ^{1}H and ^{13}C chemical shifts data of **1** and **2** were similar with few points of difference. First, the appearance of a new peak resonating at δ 216.6 in the metabolite ^{13}C spectrum together with the disappearance of the CH–OH resonance which was supposed to appear at δ 78.4 as in case of ursolic acid. Assuming a carbonyl moiety at C-3 instead of the secondary alcohol may also explain the downfield shift of both C-2 (δ 28.4) and C-4 (δ 39.7) in case of **1** to δ 34.7 and δ 47.4 in **2**. These differences are rationalized by assuming oxidative effect of *Aspergillus flavus* (ATCC 9170) enzymes on the secondary alcohol group at C-3 of ursolic acid 3β-hydroxy-urs-12-en-28-oic acid to the corresponding ketone 3-oxo-urs-12-en-28-oic acid (ursonic acid) Fig. 1.

Table 1. ^{13}C-NMR Chemical Shifts of Ursolic Acid **1** and Metabolite **2** in Pyridine-d_6 (100 MHz)

No.	**1**	**2**
1	39.4	39.6
2	28.4	34.7
3	78.4	216.6
4	39.7	47.4
5	56.1	55.6
6	19.1	20.1
7	33.9	33.2
8	40.3	39.7
9	48.3	47.4
10	37.6	37.1
11	23.9	24.0
12	125.9	125.7
13	139.6	139.6
14	42.8	42.9
15	29.0	28.9
16	25.2	25.2
17	48.3	47.7
18	53.8	53.9
19	39.7	40.1
20	39.7	39.8
21	31.4	31.4
22	37.7	37.7
23	28.1	27.0
24	16.9	21.7
25	16.0	15.4
26	17.7	17.6
27	24.2	24.1
28	180.1	180.3
29	17.7	17.8
30	21.7	21.9

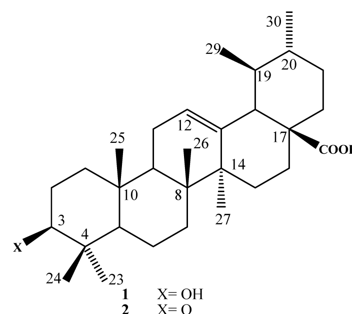

Fig. 1. Structures of Compounds **1** and **2**

Metabolite **4**: (51.5 mg, 9% yield) isolated as a yellowish white needles, $[\alpha]_D^{25}$ −54.4° (c=0.3%, MeOH). It was shown to have a molecular formula of $C_{21}H_{20}O_{11}$ by HR-ESI-MS (m/z 447.0808) $[M-H]^-$. IR ν_{max} (KBr) cm^{-1} showed strong absorption bands at 3406, 1750, 1657, 1360, 1210, and 1073. UV λ_{max} (MeOH) nm (log ε); 265 (4.1) and 350 (4.02). A comparison between the ^{13}C-NMR spectra of **4** and **3** revealed that the carbon resonances are almost identical, except for the additional six carbon resonances at δ: (61.4—78.1 and the anomeric carbon at δ 101.5), which indicated the presence of a glycosyl moiety. The shifts at C-2 and C-3 from δ 147.4 and 136.3 in case of **3** to δ 157.0 and 133.8 respectively as well as the correlation H-1″/C-3 observed in the HMBC spectrum permitted the assignment of the glycosyl group at C-3. The large coupling constant (J=7.2 Hz) between the (anomeric) proton H-1″ and H-2″ indicated the *trans*-diaxial relationship establishing a β-glycosidic linkage. On the basis of spectroscopic data in agreement with the published data.[33,34] The librated sugar after hydrolysis showed comparable specific rotation $[\alpha]_D^{25}$ +17.2° (c=0.1%, H_2O) with the standard D-glucose. The acetylated thiazolidine derivative of the librated sugar showed the same t_R with the standard D-glucose acetylated thiazolidine derivative (21.11 min). Metabolite **4** was assigned as kaempferol 3-*O*-β-D-glucopyranoside.

Metabolite **5**: (25 mg, 4.8% yield) isolated as yellow solid. Positive mode HR-ESI-MS showed a pseudo molecular ion at (m/z 367.0096 $[M+H]^+$ Calcd for $C_{15}H_9O_9S$+H: 367.0124). This was consistent with a formula of $C_{15}H_9O_9S$ which supported the presence of sulfur as being the unusual heteroatom in the structure. IR ν_{max} (KBr) cm^{-1} showed bands at 3800, 3733, 1625, 1600 as well as typical sulfate conjugates at 1050 (C–O–S) and 1250 (S=O). UV λ_{max} (MeOH) nm (log ε): 360 (4.02) and 270 (3.96); +NaOMe 405 (4.13), 278 (3.82); +NaOAC 384 (4.12), 275 (3.92); +$AlCl_3$ 420 (4.04), 270 (4.07) indicated the free hydroxyl groups at 3, 7 and 5, respectively.[35] Although the ^{1}H- and

^{13}C-NMR chemical shifts of **3** and **5** were almost identical, the ^{13}C-NMR spectrum showed prominent downfield chemical shift of C-4′ from δ_C 159.8 to δ_C 155.5 leading to assignment the position of sulfate group at C-4′. The position of sulfate group was further confirmed by HMBC between H-3′/C-4′ ($^2J_{CH}$), H-5′/C-4′ ($^2J_{CH}$), H-2′/C-4′ ($^3J_{CH}$) and H-6′/C-4′ ($^3J_{CH}$). Substitution of the sulfate group at C-4′ lead to the upfield chemical shift of H-3′ and H-5′ from δ_H 6.92 (d, 8.4) in **3** to δ_H 7.34 (d, 8.8) in compound **5**. ^{1}H- and ^{13}C-NMR data of metabolite **5** were shown in Tables 2 and 3. Based on the above mentioned data and detailed analysis of ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and HMBC correlations were found to be in complete agreement with the proposed structure for metabolite **5** which was assigned as kaempferol 4′-sulfate. This is the first report for this metabolite from natural source.

Metabolite **6**: (2 mg, 0.3% yield) isolated as a pale yellow needles as a product of the metabolism of kaempferol by *Mucor ramannianus* (ATCC 9628). Negative mode HR-ESI-MS showed a quasi molecular ion at (*m/z* 431.1120 [M−H]$^-$). IR ν_{max} (KBr) cm^{-1} showed absorption bands at 3398, 1750, 1677, 1290, 1190, and 1069. UV λ_{max} (MeOH) nm (log ε) 275 (4.01) and 365 (4.04). The ^{1}H-NMR spectrum of **6** was close to that of **3** but showed some extra proton signals. These signals were consistent with those of rhamnose[36,37] and shown in Table 2. These proton signals were easily linked to the new carbon signals at δ 98.4, 70.1, 68.1, 72.0, 68.2 and 17.1, assigned to C-1″—6″ of rhamnose, respectively. The location of the sugar was assigned at 4′-position based on HMBC results where the anomeric proton of the sugar at δ 5.51 (d, J=3.0 Hz) is correlated with the carbon signal resonating at δ 159.0 (C-4′). The small coupling constant (J=3.0 Hz) between the (anomeric) proton H-1″ and H-2″ indicated α-glycosidic linkage. The librated sugar after hydrolysis showed comparable specific rotation $[\alpha]_D^{25}$ +4.4° (c=0.25%, H_2O) with standard L-rhamnose. The acetylated thiazolidine derivative of the librated sugar showed the same t_R with the standard L-rhamnose acetylated thiazolidine derivative (15.64 min). These results concluded the structure of **6** to be kaempferol 4′-*O*-α-L-rhamnopyranoside.

Metabolite **7**: (56 mg, 10.2% yield) isolated as an intense yellow amorphous solid; $[\alpha]_D^{25}$ −19.9° (c=0.5%, MeOH); as the first product of the metabolism of **3** by *Beauveria bassiana* (ATCC 13144). Its molecular formula was determined to be $C_{22}H_{22}O_{11}$ based on positive mode HR-ESI-MS data of (*m/z* 463.1313 [M+H]$^+$ Calcd for $C_{22}H_{22}O_{11}$–H: 463.1240). IR ν_{max} (KBr) cm^{-1} showed absorption bands at 3374, 2940 and 1617. UV λ_{max} (MeOH) nm (log ε) 365 (4.04) and 264 (3.98), +NaOMe 415 (4.03), 270 (4.01), +NaOAc 390 (3.98), 260 (4.09) indicating free 3, 4′ hydroxyl groups with a probability of oxygenation at C-7. Analysis of its ^{13}C-NMR spectrum with the help of DEPT-135 indicated that the observed 20 signals comprised one methyl, one methylene, nine methines and nine quaternary carbons. The ^{1}H-NMR spectrum of **7** was close to that of **3** but showed some extra proton signals. These signals are consistent with those of *O*-β-D-4-*O*-methylglucopyranoside[38,39] and shown in Table 2. Further evidence came from the ^{13}C-NMR spectrum which showed signals resonating at δ 73.9, 76.7, 79.4 and 76.3 for the oxymethine carbons, signals at δ 60.8 for an *O*-methyl carbon, δ 60.3 for oxymethylene carbon and at δ 100.1 for the anomeric carbon. The 7-*O*-gluco-

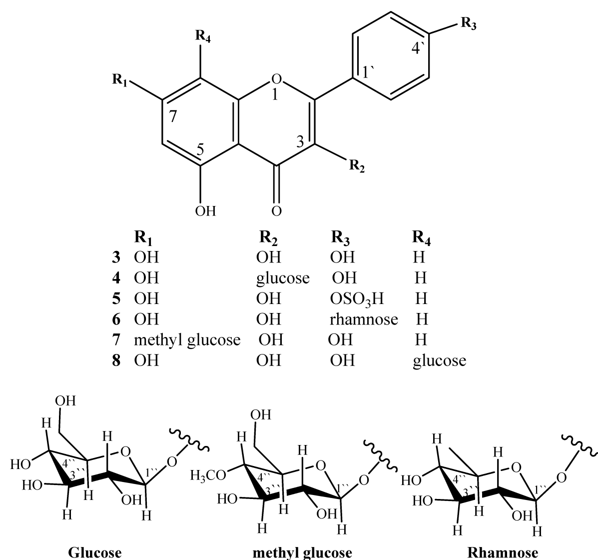

Fig. 2. Structures of Compounds **3**—**8**

Table 2. ^{1}H-NMR Data (δ) for Kaempferol and Its Microbial Metabolites in DMSO-d_6 (J in Hz)

No.	**3**$^{a)}$	**4**$^{a)}$	**5**$^{a)}$	**6**$^{b)}$	**7**$^{a)}$	**8**$^{a)}$
5-OH	12.47	12.60	12.47	12.40	12.61	12.12
6	6.19 (br s)	6.21 (br s)	6.19 (br s)	6.15 (d, 1.8)	6.40 (br s)	5.97 (br s)
8	6.43 (br s)	6.44 (br s)	6.45 (br s)	6.41 (d, 1.2)	6.77 (br s)	
2′	8.04 (d, 8.8)	8.02 (d, 8.4)	8.09 (d, 8.8)	8.10 (d, 9.0)	8.05 (d, 8.7)	8.26 (d, 8.2)
3′	6.92 (d, 8.4)	6.88 (d, 8.8)	7.34 (d, 8.8)	7.17 (d, 9.0)	6.92 (d, 8.4)	6.90 (d, 8.6)
5′	6.92 (d, 8.4)	6.88 (d, 8.8)	7.34 (d, 8.8)	7.17 (d, 9.0)	6.92 (d, 8.4)	6.90 (d, 8.6)
6′	8.04 (d, 8.8)	8.02 (d, 8.4)	8.09 (d, 8.8)	8.10 (d, 9.0)	8.05	8.26 (d, 8.2)
1″		5.45 (d, 7.2)		5.51 (d, 3.0)	5.07 (d, 7.6)	4.46 (d,7.6)
2″		3.27 m		3.77 (br s)	3.26 (dd, 8.0, 8.0)	3.26 m
3″		3.49 m		3.77 (br s)	3.03 (dd, 8.8, 8.8)	3.44 (br d)
4″		3.54 m		3.55 (br s)	3.46 (dd, 8.8, 8.8)	3.57 m
5″		3.49 m		3.83 m	3.03 (dd, 8.8, 8.8)	3.44 m
6″		3.69 m			3.65 (d, 8.8)	3.69 m
OCH_3					3.44 3H, s	
CH_3				1.02 (d, 6)		

a) 400 MHz, *b*) 500 MHz.

sylation was deduced from the H-1″/C-7 correlation observed in the HMBC spectrum. The β-configuration of the sugar moiety was consistent with the coupling constant of the anomeric proton (δ 5.07, d, J=7.6 Hz).[40] *trans*-Diaxial relationships were suggested between H-2″/3″, H-3″/4″, H-4″/5″ protons, as large coupling constants, ($J_{2''/3''}$=8.0 Hz, $J_{3''/4''}$=8.8 Hz, $J_{4''/5''}$=8.8 Hz) were observed between the respective pairs of protons. The sugar part was further confirmed by comparing the ^{13}C-NMR resonances with those reported for the corresponding carbons in a similar *O*-β-D-4-*O*-methylglucopyranoside.[39] The remaining signals of the NMR spectra confirmed the structure of the aglycone moiety. The assignment of signals of compound **7** was based on COSY, HMQC and HMBC spectra. The configuration of the sugar moiety was assigned by comparison of the physical data, ^{1}H- and ^{13}C-NMR of librated sugar after hydrolysis with synthetic β-D-4-*O*-methylglucopyranoside as reported in the literature in which the optical rotation [−28.8° (c=0.5, acetone)] was in good agreement with those of known synthetic β-D-4-*O*-methylglucopyranoside [−28.4° (c=0.52, acetone)].[41] Consequently, metabolite **7** was identified as the new compound kaempferol 7-*O*-β-D-4-*O*-methylglucopyranoside. It has previously been shown that *Beauveria bassiana* can specifically produce rare 4-*O*-methyl-D-glucose-conjugated derivatives of phenols.[42,43]

Metabolite **8**: (6.7 mg, 1.2% yield) whitish yellow amorphous solid; $[\alpha]_D^{25}$ +46.9° (c=0.1%, MeOH); isolated as the second metabolite of **3** by *Beauveria bassiana* ATCC 13144. Negative mode HR-ESI-MS showed a quasi molecular ion at (m/z 463.0752 [M−H]$^-$). IR ν_{max} (KBr) cm^{-1} showed strong absorption bands at 3400, 3465, 1667 and 1279. UV λ_{max} (MeOH) nm (log ε) 370 (4.01) and 270 (4.00), +NaOMe 420 (4.03), 270 (4.04), +NaOAc 399 (3.78), 275 (4.11), +$AlCl_3$ 424 (3, 78), 270 (4.09) and no change upon addition of HCl, indicating free 3, 4′, 5 and 7 hydroxyl groups.[35] The ^{1}H-NMR spectrum of **8** showed a prominent deviation from that of **3** which was the disappearance of one of the H-6 or H-8 protons resonating around δ 6.19 and δ 6.43 in the substrate and its former mentioned metabolites. Another noticeable deviation from the ^{1}H-NMR spectrum of kaempferol was the appearance of additional six proton signals resonating from δ 3.26 to δ 4.46 indicating a glycosyl moiety in the structure. Similarly the ^{13}C-NMR encountered prominent changes, the downfield shift of C-8 from δ 94.1 in **3** to δ 127.1 in **8** indicating the probability of hydroxylation at that position. The additional five carbon resonances at the aliphatic region δ (61.2—77.8) further confirmed the presence of a glycosyl moiety. These additional signals in both ^{13}C- and ^{1}H-NMR spectra of the metabolite were consistent with those of glucose[33,34] and shown in Tables 2 and 3, respectively. The methine carbon resonating at δ 108.3 showed a one bond correlation (HMQC) with the proton at δ 5.97 which in turn had a three bond correlation (HMBC) with the quaternary carbon at δ 104.6 (C-10) permitting its identification as C-6. The anomeric carbon resonating at δ 108.3 showed a one bond correlation (HMQC) with the anomeric proton at δ 4.46 which in turn had a three bond correlation (HMBC) with the methine carbon at δ 127.1 (C-8). These data suggested *O*-glucosylation at the newly introduced hydroxyl group at C-8 kaempferol. The large coupling constant (J=7.6 Hz) between the (anomeric) proton H-1″ and H-2″ indicated the *trans*-diaxial relationship establishing a β-glycosodic linkage. The librated sugar after hydrolysis showed comparable specific rotation $[\alpha]_D^{25}$ +17.2° (c=0.1%, H_2O) with the standard D-glucose. The acetylated thiazolidine derivative of the librated sugar showed the same t_R with the standard D-glucose acetylated thiazolidine derivative (21.11 min). Based on the above mentioned spectroscopic data compound **8** was identified as herbacetin 8-*O*-β-D-glucopyranoside.

Table 3. ^{13}C-NMR Data (δ) for Kaempferol and Its Microbial Metabolites in DMSO-d_6

No.	3[a]	4[a]	5[a]	6[b]	7[a]	8[a]
2	147.4	157.0	146.6	146.7	148.1	146.0
3	136.3	133.8	137.2	136.3	136.7	135.5
4	176.5	178.1	176.7	176.5	176.5	175.5
5	161.3	161.8	161.3	161.4	161.0	161.8
6	98.8	99.3	99.0	99.0	99.3	100.7
7	164.5	164.8	165.2	164.5	164.5	157.1
8	94.1	94.3	94.3	94.2	94.9	127.1
9	156.8	156.9	157.0	156.9	156.4	149.0
10	103.7	104.6	103.6	103.6	105.3	104.6
1′	122.3	121.5	126.1	124.9	122.1	122.9
2′	130.1	131.5	129.2	129.8	130.3	130.5
3′	116.1	115.8	120.5	117.1	116.1	115.9
4′	159.8	160.6	155.5	159.0	160.0	159.5
5′	116.1	115.8	120.5	117.1	116.1	115.9
6′	130.1	131.5	129.2	129.8	130.3	130.5
1″		101.5		98.4	100.1	108.3
2″		74.8		70.1	73.9	74.8
3″		77.0		68.1	76.7	77.0
4″		70.5		72.0	79.4	69.7
5″		78.1		68.2	76.3	77.8
6″		61.4			60.8	61.2
OCH_3					60.3	
CH_3				17.1		

a) 100 MHz, *b*) 125 MHz.

Cytotoxicity of the Isolated Metabolites In our studies none of the isolated metabolites (of both ursolic acid and kaempferol) have shown any cytotoxicity up to 25 μg/ml against the tested cell lines. However, it has been reported that carbonylation of ursolic acid at the C-3 position enhances its cytotoxicity[44] and ursonic acid was found to be more active against a series of human cancer cell lines than the parent ursolic acid.[45,46] This finding puts an emphasis on the fact that drug transformation during its metabolism takes place resulting in the formation of either less toxic and highly polar substances or more active metabolites.[26]

Antioxidant Activity of the Isolated Metabolites As shown in Table 4, ursolic acid (**1**) and its metabolite (**2**) inhibited ROS generation in HL-60 cells with an IC_{50} of 12 and 25 μg/ml, respectively indicating that **2** was less active than **1**. The antioxidant effects of both **1** and **2** were also associated with their cytotoxicity in HL-60 cells.

Kaempferol **3** exhibited a potent antioxidant effect with IC_{50} of 0.21 μg/ml without showing any cytotoxic effect up to a highest concentration of 31.25 μg/ml. All the four metabolites of **3** also showed antioxidant properties (Table 4). Kaempferol sulfate (**5**) was relatively more potent (IC_{50} 0.15 mg/ml) than the parent compound (**3**). The antioxidant activity of kaempferol 7-*O*-β-D-4-*O*-methylglucopyranoside (**7**) was much higher (39 fold) than kaempferol 3-*O*-β-D-glucopyranoside (**4**). Herbacetin 8-*O*-β-D-glucopyranoside (**8**)

Table 4. Antioxidant Activity and Cytotoxicity to HL-60 Cells

Compound	Antioxidant activity IC_{50} (μg/ml)	Cytotoxicity IC_{50} (μg/ml)
1	12	17
2	25	26
3	0.21	NC
4	18	NC
5	0.15	NC
7	0.46	NC
8	1.7	NC
Vitamin C	0.6	NT
Doxorubicin	NT	0.25

NC: no cytotoxicity, NT: not tested.

was much less active than **3**, **5** and **7**.

In conclusion, there were four types of reactions through microbial biotransformation in this report, *i.e.*, oxidation, glycosylation, hydroxylation as well as sulfate conjugation. This study proved that microbial transformation was a useful way to increase the diversity of the natural products as well as the conversion of abundant prototypes to rare products.

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References

1) Huq M. M., Jabbar A., Rashid M. A., Hasan C. M., *Fitoterapia*, **70**, 5—9 (1999).
2) Burrows G. E., Tyrl R. J., "Toxic Plants of North America," Iowa State University Press, Ames, 2001, pp. 78—82.
3) Le M. J., Pourrat H., *Ann. Pharm. Fr.*, **10**, 349—351 (1952).
4) Paris R. R., Duret S., *Bull. Soc. Botan. Fr.*, **119**, 531—542 (1972).
5) Chiang L., Ng L. T., Cheng P. W., Chiang W., Chun C., *Clin. Exp. Pharmacol. Physiol.*, **32**, 811—816 (2005).
6) Ma C., Cai S., Cui J., Wang R., Tu P., Hattori M., Daneshtalab M., *Eur. J. Med. Chem.*, **40**, 582—589 (2005).
7) Zhang P., Li X., Li N., Xu J., Li Z. L., Wang Y., Wang J., *Arch. Pharmacal. Res.*, **28**, 889—891 (2005).
8) Xiong X., Chen W., Xiao X., Cui J. X., *Zhongcaoyao*, **36**, 1207—1209 (2005).
9) Taketa A., Gnoatto S., Gosmann G., Pires V. S., Schenkel F., Guillaume D., *J. Nat. Prod.*, **67**, 1697—1700 (2004).
10) Wachter G., Valcic S., Flagg M., Franzblau S., Montenegro G., Suarez E., Timmermann B. N., *Phytomedicine*, **6**, 341—345 (1999).
11) Jung S., Ha Y., Shim E., Choi S., Jin J., Yun-Choi H., Lee J., *Biochem. J.*, **403**, 243—250 (2007).
12) Hung C., Yen G., *J. Agric. Food Chem.*, **50**, 2993—2997 (2002).
13) Deepak M., Handa S., *Phytother. Res.*, **14**, 463—465 (2000).
14) Beindorff C., Cain F. W., Pierce J., Schmid U., Schweitzer E., Van Straalen J., *Eur. Pat. Appl.*, **2001**, 9 (2001).
15) Singh A., Sharma O. P., Dawra R. K., Kanwar S. S., Mahato S. B., *Biodegradation*, **10**, 373—381 (1999).
16) Mallavadhani U. V., Panda A., Rao Y. R., *Phytochemistry*, **49**, 901—951 (1998).
17) Zhang J., Zhi-Hong Y., Bo-Yang C., Geoffrey A., Qiu S., *Tetrahedron Lett.*, **46**, 2337—2340 (2005).
18) De Mejia G., Castano-Tostado E., Loarca-Pina G., *Mutat. Res.*, **441**, 1—9 (1999).
19) Wagner H., Luck R., *Naturwissenschaften*, **42**, 607 (1955).
20) Park J., Rho H., Kim D., Chang., *J. Agric. Food Chem.*, **54**, 2951—2956 (2006).
21) Rao Y., Geethangili M., Fang S., Tzeng Y., *Food Chem. Toxicol.*, **45**, 1770—1776 (2007).
22) Tu Y., Lian T. W., Yen J., Chen Z., Wu M., *J. Agric. Food Chem.*, **55**, 9969—9976 (2007).
23) Parveen Z., Deng Y., Saeed M., Dai R., Ahamad W., Yu Y., *Yakugaku Zasshi*, **127**, 1275—1279 (2007).
24) Hosseinzadeh H., Motamedshariaty V., Hadizadeh F., *Pharmacologyonline*, **2**, 367—370 (2007).
25) Srisilam K., Ciddi V., *Biotechnol. Adv.*, **21**, 3—39 (2003).
26) Venisetty R., Ciddi V., *Curr. Pharm. Biotech.*, **4**, 153—167 (2003).
27) Rosazza P. N., Duffel M. W., "Alkaloids: Chemistry and Pharmacology," Vol. 27, Chap. 4, ed. by Brossi A., Academic Press, New York, 1986, pp. 391—392.
28) Davis P. J., "Antibiotics and Microbial Transformations," ed. by Lamba S. S., Walker C. A., CRC, Boca Raton, Florida, 1987, pp. 47—70.
29) Abourashed E. A., Khan I. A., *Chem. Pharm. Bull.*, **48**, 1996—1998 (2000).
30) Abbas F. A., Almassarrany S., Khan S., Al-Howarreeny T., Moussa G., Abourashed E., *Nat. Prod. Res.*, **21**, 383—391 (2007).
31) Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **35**, 501—506 (1987).
32) Reddy M., Gupta S., Jacob M., Khan S., Ferreira D., *Planta Med.*, **73**, 461—467 (2007).
33) Ziyan L., Jinghua Y., Yunsong W., Rong H., Liang H., *Zhongcaoyao*, **37**, 826—829 (2006).
34) Jin Y., Wei X. N., Shi Y. P., *Helv. Chim. Acta*, **90**, 776—782 (2007).
35) Mabry T. J., Markham K. R., Thomas M. B., "The Systematic Identification of Flavonoids," ed. by Mabry T. J., Springer-Verlag, New York, 1970, pp. 41—61.
36) Dianpeng L., Ikeda T., Matsuoka N., Nohara T., Zhang H., Sakamoto T., Nonaka G., *Chem. Pharm. Bull.*, **54**, 1425—1428 (2006).
37) Yoshikawa M., Wang T., Morikawa T., Xie H., Matsuda H., *Chem. Pharm. Bull.*, **55**, 1308—1315 (2007).
38) Zhan J., Gunatilaka A., Leslie A., *J. Nat. Prod.*, **69**, 1525—1527 (2006).
39) Herath M., Mikelle J. R., Hale A., Ferreira D., Khan I. A., *Chem. Pharm. Bull.*, **54**, 320—324 (2006).
40) Markham K. R., Geiger H., "The Flavonoids: Advances in Research Since 1986," Chap. 10, ed. by Harbone J. B., Chapman and Hall, London, 1993, pp. 441—493.
41) Gallant M., Link J. T., Danishefsky S., *J. Org. Chem.*, **58**, 343—349 (1993).
42) Vigne B., Archelas A., Fourneron J. D., Furstoss R., *Tetrahedron*, **42**, 2451—2456 (1986).
43) Vigne B., Archelas A., Furstoss R., *Tetrahedron*, **47**, 1447—1458 (1991).
44) Akihisa T., Yasukawa K., "Studies in Natural Products Chemistry," ed. by Rahman A.,Vol. 25, Part F, Elsevier, Amsterdam, 2001, p. 57.
45) Ryu S., Choi S., Lee. C., Ahn J., *Arch. Pharm. Res.*, **17**, 375—377 (1994).
44) Min B., Kim Y., Lee S., Jung H., Lee J., Na M., Lee C., Lee J., Bae K., *Arch. Pharm. Res.*, **23**, 155—158 (2000).
46) Huang R., Wang W., Kuo Y., Lin Y., *Chin. Pharm. J.*, **53**, 179—184 (2001).