Biotransformation of Chrysin and Apigenin by *Cunninghamella elegans*

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Biotransformation of chrysin by *Cunninghamella elegans* NRRL 1392 produced apigenin, apigenin 7-sulfate, apigenin 7,4′-disulfate, and a new metabolite identified as chrysin 7-sulfate. On the other hand, fermentation of apigenin, using the same microorganism, yielded apigenin 7-sulfate and apigenin 7,4′-disulfate. The structures of the metabolites were established by spectral analysis, and acid and enzyme hydrolyses in addition to comparison with reference samples.

Key words biotransformation; chrysin; apigenin; *Cunninghamella elegans*

In a previous publication, the sulfation of the hydroxyl group at C-7 of naringenin by the filamentous fungus *Cunninghamella elegans* was reported.[1)] Metabolism of quercetin with this organism produced quercetin-4′-sulfate. The same results were obtained with flavanones, methoxylated at C-7.[2)] Preliminary studies of flavones such as kaempferol and morin as well as the flavanones hesperitin and dihydroquercetin showed that they were converted to polar metabolites by this fungus, which were hydrolyzed by mild acid at room temperature to the respective substrates. The potential of flavonoid sulfates as therapeutically useful agents,[3,4)] as standards to study flavonoid conjugation by mammals,[5)] and for comparison with natural products[6)] requires the preparation of flavonoids sulfated at specific sites.

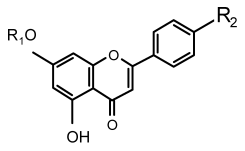
Chemical synthesis usually provides mixtures of flavonoid sulfates with sulfate groups randomly allocated[6)] and may be difficult to purify. Thus the use of microorganisms to introduce sufalte groups at specific positions of the flavonoid nucleus seems interesting. Many microbial enzymes were found to possess broad substrate specificity, yet they catalyze reactions with high degree of regio and stereospecificity.[7)]

In order to check the breadth of substrate specificity of *C. elegans* sulfating enzyme(s), two biologically active flavones: chrysin (5,7-dihydoxyflavone) and apigenin (5,7,4′-trihydroxyflavone) were subjected to metabolic studies using a culture of the same microorganism. Apigenin was found to possess anticarcinogenic,[8)] anti-tumor promoter,[9)] antioxidative, free radical scavenging and anti-inflammatory activities.[10)] Both chrysin and apigenin were found to be potent inhibitors of enzyme aromatase and can protect against breast cancer[11—13)] and were not mutagenic in the Ames test.[14—16)]

Previous mammalian metabolic studies of chrysin and apigenin in rat showed that they are conjugated at the C-7 hydroxyl group to produce ethereal sulfates and glucuronides, although no structures were provided, and that chrysin is hydroxylated to apigenin.[17)] Luteolin and apigenin were also obtained from incubation of apigenin and chrysin, respectively with rat liver microsomes.[18)] Only glucuronide and sulfate conjugates of apigenin were obtained from incubation with $HepG_2$ cells, which emphasizes the importance of phase II conjugation reactions in the metabolism of flavonoids.[19)] Fungi are eukaryotic organisms possessing similar metabolic machinery to those of mammals and the outcome of xenobiotic metabolism in both systems is expected to be closely related.[20)] Since microorganisms are known to hydroxylate flavonoids at the C-4′ position,[21)] an additional goal of this work was to convert chrysin to the far more expensive and important apigenin. No microbial biotransformation studies on chrysin or apigenin have previously been reported.

Results and Discussion

Microbial transformation studies, using *C. elegans*, have indicated the capability of this microorganism to simulate metabolic reactions of drugs performed by mammals,[22)] to sulfate organic molecules in general,[23)] and flavonoids in particular.[1)] The present study lends further support to these studies. Thus, the incubation of chrysin with *C. elegans* gave four metabolites (**2**—**5**), three of which are sulfated derivatives. In addition to apigenin (**2**), which was identified by UV, superimposable IR, EI-MS, ^{1}H-NMR and comparison with an authentic sample,[24)] metabolite **4** was obtained in 7% yield. Both acid and enzyme hydrolyses of this metabolite gave apigenin (CoTLC, MS and UV spectra) and sulfate as indicated by the $BaCl_2$ test. The EI-MS of **4** also showed peaks for apigenin at *m*/*z* 270 (M^+). Negative mode HR-ESI-MS displayed a pseudo molecular ion peak (a divalent anion) at *m*/*z* 213.97384 (mass=427.94768) (M−2H) corresponding to $C_{15}H_8O_{11}S_2$ and a fragment divalent ion at *m*/*z* 173.99708 (mass=347.99416) (M−2H−sulfate) which best fits the formula $C_{15}H_8O_8S$. Furthermore, the IR spectroscopy showed the regular sulfate bands at 1250, 1050 and 790 cm^{-1}. These results suggest that **4** is a sulfate conjugate of apigenin. The UV spectral data of this metabolite indicated that while hydroxyl groups at C-7 and C-4′ are blocked, the hydroxyl group at C-5 is free (NaOAc, NaOMe and $AlCl_3$ shifts, respectively). On the other hand, ^{1}H- and ^{13}C-NMR spectral data of **4** (see experimental) were identical to those of apigenin 7,4′-disulfate.[25,26)] The identification of the metabolite **4** as apigenin 7,4′-disulfate was confirmed by



1 R_1=R_2=H
2 R_1=H, R_2=OH
3 R_1=-SO_3H, R_2=H
4 R_1=-SO_3H, R_2=-OSO_3H
5 R_1=-SO_3H, R_2=OH

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direct comparison with authentic sample. Furthermore, the fermentation of **1** with *C. elegans* afforded metabolite **5** in 3% yield, which was identical to a reference sample of apigenin 7-sulfate (CoTLC, UV and IR spectra). Negative mode HR-ESI-MS showed a quasi molecular ion at *m/z* 349.00112 (M−H) consistent with a formula $C_{15}H_9O_8S$ which supported the presence of sulfur as being the unusual heteroatom. The ^{1}H-NMR spectral data of **5** were also identical to those reported for this compound.[25] Both enzyme and acid hydrolyses of **5** gave apigenin (TLC, UV, MS) and sulfate (barium chloride precipitation reaction).

Beside apigenin 7-sulfate and apigenin 7,4′-disulfate as chrysin sulfated metabolites, the incubation of chrysin (**1**) with *C. elegans* resulted in the formation of a polar metabolite (**3**). Both acid and enzyme hydrolyses were carried out, which yielded chrysin (CoTLC, UV, MS and NMR spectral data) and sulfate ($BaCl_2$ precipitation). While the EI-MS showed mass fragments identical to those of chrysin, the anion HR-ESI-MS spectrum of this compound displayed a pseudo molecular ion peak (M−H) at *m/z* 333.00612 consistent with formula $C_{15}H_9O_7S$. The UV spectral data of **3** implied that the hydroxyl group at C-7 is blocked (lack of shift with NaOAc), while the hydroxyl group at C-5 was found to be free due the large bathochromic shift with $AlCl_3$.[27] Furthermore, infrared spectroscopy of **3** showed bands typical of a sulfate conjugate at 1250 (S=O), 1050 (C–O–S) and 790 (S–O) cm^{-1}. Examination of the ^{1}H-NMR spectrum of this metabolite showed that while the protons of ring B in the metabolite (positions 2′, 3′, 4′, 5′, 6′) and H-3 possessed the same chemical shift as their counterparts in chrysin, positions 6, 8 underwent downfield shifts of 0.38 and 0.53, respectively (Table 1). These proton NMR downshifts support the sulfation of a hydroxyl group at C-7 and the identification of **3** as chrysin 7-sulfate.[25] It is worthwhile to mention that this flavonoid sulfate was not previously characterized from natural sources.

The metabolism of apigenin (**2**) by *C. elegans* produced a major metabolite (12%) in addition to 7% of another metabolite. Examination of the major metabolite showed that it is indistinguishable from the reference sample of apigenin 7,4′-disulfate (CoTLC, UV, IR and NMR signals), which was previously characterized as chrysin metabolite. On the other hand, the minor metabolite was similar to an authentic sample of apigenin 7-sulfate (**5**) as indicated by CoTLC and UV spectral analysis[27] as well as NMR signals.[25] It is interesting to note that **5** was the major metabolite in the early stages of the fermentation (TLC screen). The feeding of amentoflavone (3′,8″-biapigenin) to the stage II culture of *C. elegans* did not produce any metabolites, which can be attributed to its large molecular size.

These results and those previously reported[1,2] led to the conclusion that *C. elegans* has a preference towards sulfation of the hydroxyl group at C-7 of the flavonoid nucleus. However, longer incubation produces metabolites sulfated at both C-7 and C-4′ hydroxyl groups. The broad substrate specificity of this fungus to introduce sulfate groups to flavones and flavanones at specific sites can be exploited in the chemical synthesis of these compounds. It is worthwhile to mention that both conjugation of C-7 and hydroxylation of the C-4′ position in apigenin and chrysin, respectively, were also encountered in rat after oral administration,[17] and in *in vitro* studies of apigenin using rat liver microsomes[18] and $HepG_2$ hepatic cells.[19] Thus one more piece of evidence supporting the concept of microbial models of mammalian drug metabolism[22,28] could be obtained. This study may also provide means for the biotechnological production of apigenin and a number of its sulfate conjugates. It is interesting to note that flavonoid 7-sulfates are more easily obtained by chemical synthesis and are of more common natural occurrence in plants than the rest of the flavonoid sulfates.[25]

Experimental

General Chrysin and apigenin were obtained from Janssen and Sigma Chemical, respectively. Amentoflavone was purified from *Chrozophora oblongifolia*.[29] IR spectra were recorded on PYE Unicam and Bruker FT infrared spectrophotometers. UV spectra were obtained on a Shimadzu 160A ultraviolet spectrophotometer. Both ^{1}H- and ^{13}C-NMR spectra were obtained in DMSO-d_6 on a Bruker DRX-500 NMR spectrometer operating at 500 and 125 MHz, respectively. Some proton NMR spectra were obtained using a Varian 300 MHz spectrometer. The chemical shift values are reported as ppm using tetramethylsilane (TMS) as an internal standard and coupling constants are expressed in Hz. Electron ionization (EI) mass spectra were taken on a Shimadzu QP5000 mass spectrometer. Negative mode high resolution mass spectra (HR-FT-ESI-MS) were obtained on a Joel JMS-SX mass spectrometer.

Microorganisms and Culture Conditions Microorganisms were obtained from either American Type Culture Collection (ATCC) or Northern Regional Research Laboratories (NRRL). Organisms were subcultured quarterly on Sabouraud dextrose agar (Oxoid) slants and kept at 4 °C. Two-week old slants were used to inoculate the autoclaved culture medium. Twenty strains of microorganisms were used in the initial screening as previously reported.[1]

Components of the Culture Medium[30] All fermentation experiments were performed using a medium consisting of 10 g dextrose, 10 ml glycerol, 5 g yeast extract, 5 g peptone, 5 g K_2HPO_4, 5 g NaCl, and 1000 ml distilled water. The pH was adjusted to 6.0 before sterilization at 121 °C for 15 min.

Cultivation of Microorganisms[30] Cells were transferred from 2 week-old slants into sterile liquid medium and kept on a gyratory shaker for 72 h to give the stage 1 culture. Five milliliters of stage I cultures was used to inoculate stage 2 cultures (50 ml per 250-ml flask). After incubation for 24 h, chrysin (**1**) or apigenin (**2**) was added as a solution in dimethylformamide (10 mg 0.25 ml^{-1}). At the same time, both substrate and organism controls were made by omitting the organism or the substrate, respectively. Fermentations were sampled by extraction of culture specimens with an equal volume of AcOEt or *n*-BuOH. After evaporation of the solvent, the residue was chromatographed on silica gel plates (E. Merck, F254) using solvent system A: $CHCl_3$–MeOH (4 : 1), solvent system B: AcOEt–MeCOEt–HCO_2H–H_2O (5 : 3 : 1 : 1), solvent system C: AcOEt–pyridine–H_2O–MeOH (16 : 4 : 2 : 1) and solvent system D: benzene–pyridine–HCO_2H–MeOH (36 : 9 : 5 : 5).[27] Flavonoid spots were examined under UV light (254, 365 nm) before and after spraying with $AlCl_3$, NH_3 or P-anisaldehyde spray reagents. The *Rf* values of the isolated metabolites are given in Table 2.

Fermentation of Chrysin (1) with *C. elegans* Chrysin [**1**, 540 mg] dissolved in 14 ml dimethylformamide; was evenly distributed among 54 250-ml flasks each containing 50 ml stage 2 cultures. After 3 weeks of incubation, the fermentation was stopped and the cells were removed by filtration. The fermentation broth was extracted with an equal volume of *n*-BuOH. Chromatography of the butanolic extract on a Sephadex LH_{20} column, using MeOH as the eluent, produced 200 mg of a methanol soluble fraction. Further elution with MeOH–H_2O (4 : 1) gave 40 mg of apigenin 7,4′-disulfate (**4**). On the other hand, repeated chromatography of the methanol soluble fraction using Sephadex LH_{20} as well as flash silica gel columns yielded 20 mg of apigenin (**2**), 12 mg of apigenin 7-sulfate (**5**) and 20 mg of chrysin 7-sulfate (**3**). The flash silica column was basically used to purify apigenin (**2**).

Apigenin (**2**): *Rf* values: 0.84, 0.96, 0.88, 0.65 in solvent systems A, B, C, D, respectively.

Apigenin 7-Sulfate (**5**): *Rf* values: 0.24, 0.88, 0.49, 0.24 in solvent systems A, B, C, D; HR-ESI-MS: 349.00112 (M−H) (Calcd for $C_{15}H_9O_8S$: 349.00181, Δ=0.69).

Chrysin 7-Sulfate (**3**): *Rf* values: 0.36, 0.86, 0.51, 0.40 in solvent systems A, B, C, D, respectively; UV λ_{max} (MeOH) nm: 269, 300 (sh), 337 (sh); +NaOMe: 241, 277, 372; +$AlCl_3$: 249, 283, 322, 383; +$AlCl_3$/HCl: 215,

282, 321, 384; +NaOAc: 270, 329; EI-MS *m/z* (rel. int.): 254 $(M-80)^+$(100), 226 (26), 152 (25), 124 (29), 102 (9), 96 (15), 81 (4), 82 (5), 80 (1); HR-ESI-MS: 333.00612 (M−H) (Calcd for $C_{15}H_9O_7S$: 333.00690, Δ=0.77); IR (ν_{max}) (KBr) cm^{-1}: 1640 (C=O), 1610, 1250 (S=O), 1050 (C–O–S), 790 (S–O). ^{1}H-NMR (DMSO-d_6) δ: 6.59 (1H, s, H-6), 7.04 (1H, s, H-8), 6.99 (1H, s, H-3), 7.58 (3H, m, H-3′, 4′, 5′), 8.08 (2H, d, J=7.2 Hz, H-2′, 6′).

Apigenin 7,4′-Disulfate (**4**): *Rf* values: 0.05, 0.61, 0.20, 0.12 in solvent systems A, B, C, D, respectively; HR-ESI-MS: 427.94768 (M−2H) (Calcd for $C_{15}H_8O_{11}S_2$: 427.95080, Δ=3.12); 347.99416 (M−2H−sulfate) (Calcd for $C_{15}H_8O_8S$: 347.99399, Δ=0.18).

Fermentation of Apigenin (2) with *C. elegans* Apigenin [**2**, 40 mg] was incubated with stage 2 cultures of *C. elegans* as explained previously. After 8 d, the fermentation was stopped, cells were removed by filtration and the broth was extracted with an equal volume of *n*-BuOH. The butanolic residue (30 mg) was chromatographed on a short Sephadex LH_{20} column to give 5 mg of apigenin 7,4′-disulfate (**4**) in addition to 3 mg of apigenin 7-sulfate (**5**).

Acid Hydrolysis of 3, 4 and 5[31] One milligram samples of **3**, **4** and **5** were separately dissolved in 2 ml MeOH and mixed with 5 ml HCl at room temperature. After evaporation of MeOH, under reduced pressure, the aglycone (**1**) or (**2**) was extracted with $CHCl_3$ and ether, respectively. They were analyzed by TLC using solvent systems $CHCl_3$–MeOH (6 : 1) and AcOEt–toluene–formic acid (45 : 55 : 5) as well as UV spectroscopy. On the other hand, sulfate was detected in the concentrated aqueous layer based on the white precipitate produced with $BaCl_2$.

Enzyme Hydrolysis of 3, 4 and 5[32] One milligram of **3**, **4** or **5** was separately dissolved in 1 ml 0.1 M acetate buffer (pH=5) and incubated with aryl sulfatase from *Helix pomatia* (Sigma) at 37 °C. Complete hydrolysis was observed to take place after 3—5 h of incubation. The mixture was then extracted with $CHCl_3$ or ether and the concentrated organic phase was analyzed by TLC using the same solvent systems as indicated under acid hydrolysis in addition to UV spectroscopy, which showed that the aglycone of **3** is chrysin, while that of **4** and **5** was identical to apigenin. Furthermore, the addition of $BaCl_2$ to the concentrated aqueous layer gave a white precipitate presumably due to $BaSO_4$.

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