O-Demethylation and Sulfation of 7-Methoxylated Flavanones by *Cunninghamella elegans*

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Metabolism of 7-*O*-methylnaringenin (sakuranetin) by *Cunninghamella elegans* NRRL 1392 yielded naringenin and naringenin-4′-sulfate. *C. elegans* also converted 5,3′,4′-trihydroxy-7-methoxyflavanone into eriodictyol-4′-sulfate. Furthermore, incubation of 5,4′-dihydroxy-7,3′-dimethoxyflavanone with the same fungus gave homoeriodictyol (5,7,4′-trihydroxy-3′-methoxyflavanone) and homoeriodicytol-7-sulfate. The structures of the new metabolites were established by spectral analysis including 2D-NMR, HR-ESI-FT-MS beside hydrolysis by acid.

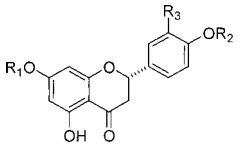
Key words 7-methoxyflavanone; sulfation; *Cunninghamella elegans*; NMR; ESI-MS

Interest to study microbial transformation of flavanones, beside the availability of relatively large quantities of 7-methoxylated flavanones (**1**, **4**, **6**) from a previous work[1,2] encouraged us to initiate the present study. Previous microbial transformation research indicated the capability of the filamentous fungus *Cunninghamella elegans* to specifically sulfate naringenin, a flavanone with cytotoxic potential,[3] at C-7 hydroxyl group.[4] The sulfation at the same position in the flavones chrysin and apigenin has also been documented, but longer incubation produced also 4′-sulfate conjugates.[5] Thus, it was deemed interesting to explore the nature of the microbial reaction when flavanones, methoxylatyed at C-7 are used as substrates for this microorganism. Phytochemical investigation of several plant families culminated in characterization of numerous naturally occurring flavone and flavonol sulfates.[6—8] Neverthless, sulfates of other minor flavonoid classes have not been described,[9] except for a recent report on the the isolation and characterization of torvanol, an antiviral C-4 sulfated isoflavonoid from the fruits of *Solanum torvum*.[10] However, there seems no reason why such compounds should not be found in nature.[11] Flavanones exhibited cytotoxic, inhibition of xanthine Oxidase enzyme, and superoxide scavenging activities.[12] The use of *C. elegans* to study the metabolism of these plant flavanones would give rise to a series of novel and potentially useful flavonoid sulfate derviatives, which can also be used as reference standards to check their production by synthetic means. Sulfates of numerous natural flavones showed antioxidant activity[13] and inhibited lens aldose reductase.[14] While a number of the microbially-produced flavanone sulfates showed antioxidant activity,[15] this particular class of compounds is yet to be explored for other bioactivities.

Results and Discussion

Sulfation of xenobiotics by mammalian systems is a well-established finding in the literature.[16] However, there are only a few reports on sulfation reactions carried out by microorganisms.[17] Demonstration of parallelism between mammalian and microbial systems, in phase II conjugation reactions is of considerable importance for metabolic drug investigations. Recent experiments established, that the filamentous fungus *Cunninghamella elegans* is able to sulfate naringenin (**2**), specifically at C-7 hydroxyl group.[4] Experiments with other flavonoids such as chrysin and apigenin provided a support for these results.[5]

The isolation of large amounts of flavanone aglycones, which were not commercially available, has given us an initiative to pursue such a reaction with other flavonoids. Initial screening experiments have unequivocally shown that *C. elegans* was the sole organism which converted compounds **1**, **4**, **6** to polar metabolites. Trials to extract these metabolites with AcOEt, without decomposition, failed. Thus *n*-BuOH was successfully used for the extraction. The acid liability of these metabolites to decomposition to the corresponding aglycones, as evidenced from the ^{1}H- and ^{13}C-NMR spectral data, suggested the sulfation of the substrates by the microorganism.[11] Thus, the polar metabolites **3**, **5**, **8** were subjected to acid hydrolysis[8] to provide the aglycones **2**, **9** and **7**, respectively. Furthermore, the aqueous layer left after extraction of the aglycones, gave positive test for sulfate with $BaCl_2$.



	R_1	R_2	R_3
1	Me	H	H
2	H	H	H
3	H	SO_3H	H
4	Me	H	OH
5	H	SO_3H	OH
6	Me	H	OMe
7	H	H	OMe
8	SO_3H	H	OMe
9	H	H	OH

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The HR-ESI-MS of **3** displayed a molecular ion peak at *m/z* 351.0203 $[M-H]^+$ corresponding to $C_{15}H_{12}O_8S$, which supported the presence of sulfur as being the unusual heteroatom. The presence of a sulfate grouping at 1257, 1053 and 860 cm^{-1} (S=O stretching and deformation) in the IR spectrum of **3** substantiated the sulfation reaction. Inspection of the UV spectral data indicated the presence of 5,7-dihydroxyflavanone. This was demonstrated by a bathochromic shift of band II with NaOAc (38 nm)[18] and a bathochromic shift of band II with NaOMe (39 nm).[19] Also, in the $AlCl_3$ UV spectrum, a significant bathochromic shift indicated the presence of free C-5 hydroxyl group.[18] Observation of only a weak shoulder at 419 nm indicated the presence of an esterified hydroxyl group at C-4′. The foregoing results suggested that substrate **1** underwent C-7 demethylation followed by sulfation at C-4′ position. EI-MS exhibited mass fragments at *m/z* 272 $[M-sulfate]^+$ and at *m/z* 120 and 153 which are identical to those of naringenin (**2**).[4] Inspection of ^1H- and ^{13}C-NMR spectra of **3** revealed that all its proton and carbon signals except those belonging to ring B were identical to the corresponding signals of NMR spectra of naringenin (Tables 1, 2). The shift in proton and carbon signals of ring B was indicative of C-4′ sulfation of **3**.[4,20]

The foregoing data suggested that substrate **1** underwent C-7 demethylation followed by sulfation at C-4′. Thus structure **3** was established as naringenin-4′-sulfate. In addition to naringenin-4′-sulfate (**3**), the fermentation of 7-*O*-methylnaringenin (**1**) with *C. elegans* gave a metabolite **2**, which lacked the methyl group signal, indicating 7-*O*-demethylation. The identification of **2** as naringenin was confirmed by ^1H-, ^{13}C-NMR, EI-MS spectral analysis and by direct comparison with reference compound. It is noteworthy to mention that fermentation of naringenin with *C. elegans* produced naringenin-7-sulfate.[4] The difference in *Rf* values be-

Table 1. ^1H-NMR (500 MHz, δ, DMSO-d_6) Data for Compounds **1**—**8**

Position	**1**	**2**	**3**	**4**[a]	**5**	**6**[a]	**7**	**8**
2	5.46 dd (2.6, 12.0)	5.41 dd (2.7, 12.7)	5.52 dd (2.4, 12.7)	5.30 dd (3.2, 12.2)	5.46 dd (2.8, 11.0)	5.33 dd (3.0, 12.5)	5.40 dd (2.8, 12.8)	5.49 dd (2.5, 12.9)
3	2.70 *cis* dd (2.8, 18.0) 3.3 *trans* m	2.67 *cis* dd (2.86, 17.1) 3.2 *trans* m	2.75 *cis* dd (2.7, 17.1) 3.29 *trans* m	2.70 *cis* dd (2.8, 18) 3.20 *trans* m	2.74 *cis* dd (2.8, 17.1) 3.2 *trans* m	2.78 *cis* dd (2.4, 17) 3.1 *trans* dd (12.4, 19.2)	2.66 *cis* dd (2.8, 17.1) 3.22 *trans* m	2.76 *cis* dd (2.71, 17.1) 3.38 *trans* dd (13.0, 17.1)
4								
5								
6	6.08 d (2.0)	5.89 s	5.92 s	6.10 d (2.0)	5.90 s	6.06 d (2.2)	5.87 s	6.34 d (1.9)
7								
8	6.06 d (2.0)	5.89 s	5.93 s	6.10 d (2.0)	5.92 s	6.06 d (2.2)	5.87 s	6.38 d (1.9)
9								
10								
1′								
2′	7.31 d (8.4)	7.30 d (8.4)	7.44 d (8.3)	6.80 s	6.99 br s	6.94 br s	7.07 d (1.7)	7.12 br s
3′	6.78 d (8.4)	6.80 d (8.4)	7.24 d (8.3)					
4′								
5′	6.78 d (8.4)	6.80 d (8.4)	7.24 d (8.3)	6.80 d (8.0)	7.22 d (8.2)	6.95 d (8.8)	6.77 d (8.1)	6.82 d (8.1)
6′	7.31 d (8.4)	7.30 d (8.4)	7.44 d (8.3)	6.91 br d (8.0)	6.89 br d (8.2)	6.96 br d (8.8)	6.88 dd (8.1, 1.7)	6.93 dd (1.4, 8.1)
OCH_3	3.76 s			3.79 s		3.93 s, 3.80 s	3.87 s	3.80 s

The numbers in parentheses are *J* values in Hz. *a*) Taken in DMSO-d_6 at 300 MHz.

Table 2. ^{13}C-NMR (125 MHz, δ, DMSO-d_6) for Compounds **1**—**8**

Position	**1**	**2**	**3**	**4**[a]	**5**	**6**[a]	**7**	**8**
2	79.0	78.8	78.6	78.6	78.5	79.5	78.6	79.1
3	42.4	42.3	42.4	42.1	42.4	42.8	41.9	42.7
4	197.3	196.7	196.4	198.2	196.3	197.6	196.3	197.7
5	163.6	163.8	163.8	163.4	163.8	163.8	163.3	162.7
6	95.0	96.2	96.6	94.5	96.3	95.3	94.8	99.6
7	167.8	167.0	167.1	167.3	167.0	168.2	166.6	162.5
8	94.1	95.4	95.4	93.7	95.4	94.4	94.8	98.7
9	163.2	163.3	163.2	162.8	163.1	163.5	162.8	162.2
10	102.9	102.1	102.1	102.5	102.1	103.2	101.6	104.0
1′	129.1	129.2	133.1	129.2	135.6	129.9	129.2	129.6
2′	128.7	128.6	128.6	114.3	115.8	111.8	111.0	111.5
3′	115.5	115.6	120.8	145.7	149.4	148.2	147.4	147.9
4′	158.1	158.1	154.0	145.1	141.3	147.6	146.8	147.9
5′	115.5	115.6	120.8	115.2	123.1	115.8	115.0	115.6
6′	128.7	128.6	128.6	117.9	118.0	120.4	119.5	120.0
OCH_3	56.2			55.8		56.3, 56.5	55.5	56.1

a) Taken in DMSO-d_6 at 75 MHz.

tween C-4′ and C-7 sulfate conjugates of naringenin was very small and the differentiation between sulfation at C-4′ and C-7 was accomplished by inspection of the ^{1}H-, ^{13}C-NMR and UV spectra.

Incubation of 7-*O*-Methyleriodictyol (**4**) with *C. elegans* produced the polar metabolite **5**, in a yield approaching 11%, in addition to a minor unidentified metabolite. Metabolite **5** was found to be a sulfate conjugate, based on acid hydrolysis and spectral data. EI-MS exhibited an ion peak at *m*/*z* 288 $(M-SO_4)^+$, which indicated loss of the methyl group. HR-ESI-MS showed a molecular ion peak at *m*/*z* 369.0278 $[M+H]^+$ consistent with a molecular formula $C_{15}H_{12}O_9S$. The loss of C-7 methyl group was evident from the ^{1}H- and ^{13}C-NMR spectral data, where signals at δ 3.79 and δ 55.8 corresponding to OMe-7 in the substrate are lacking (Tables 1, 2). The NMR data were also useful in determining the location of sulfate moiety, where the deshielding of C-3′ and C-5′ carbons, which are *ortho* to sulfation site compared to those of the substrate **4** was observed in the ^{13}C-NMR of **5** by 3.7 and 7.9 ppm, respectively. Furthermore, the pararelated carbon at C-1′ in **5** was deshielded by about 6 ppm, while the ipso carbon at C-4′ position was shielded by 3.8 ppm. The data mentioned herein supported sulfation of C-4′ hydroxyl group.[20] Further support was deriven from deshielding of H-5′ proton signal in the ^{1}H-NMR of **5** by 0.42 ppm compared to H-5′ signal in the substrate (**4**) spectrum. Concerning UV spectra, both NaOAc and $AlCl_3$ bathochromic shifts of bands II and I, respectively indicated that **5** was a 5,7-dihydroxylated flavanone.[18,19] On the other hand, the C-4′ hydroxyl group is not free due to lack of shift with NaOMe.

The metabolism of 5,4′-dihydroxy-7,3′-dimethoxyflavanone (persicogenin, **6**), gave a major metabolite **8** in a 27% yield, in addition to 1.5% of a minor metabolite **7**. Examination of the ^{1}H-NMR spectral data of the minor metabolite exhibited the presence of a single methoxyl group, which resonated at δ 3.87. These data suggested that one methyl group was lost. A closer look at the ^{13}C-NMR signals of **7** showed that they are identical to those reported for homoeriodictyol (5,7,4′-trihydroxy-3′-methoxyflavanone)[20] (Table 2). The foregoing data are consistent with the tendency of *C. elegans* to carry out C-7 demethylation, which was also observed in case of substrates **1**, **4**. The positive ion HR-ESI-MS spectrum of **8** displayed a pseudomolecular ion peak $[M+H]^+$ at *m*/*z* 383.0442 consistent with the molecular formula $C_{16}H_{14}O_9S$. Mild acid hydrolysis of **8** gave the aglycone **7** in addition to a sulfate ($BaCl_2$ precipitation reaction). These data suggested that **8** is a sulfate conjugate of homoeriodictyol, which was also displayed by the presence of S=O absorption bands at ν_{max} 1272, 1063, 801 cm^{-1} in the IR spectrum. Comparison of ^{13}C-NMR spectrum of **8** with that of homoeriodictyol, showed that while C-7 signal is shielded by about 4 ppm, C-6 and C-8 signals are deshielded by about 4.8 and 3.9 ppm, respectively (Table 2). This was consistent with homoeriodictyol-7-sulfate structure. Moreover, the UV spectral data provided additional support to this conclusion, as it showed free 5, 4′ positions ($AlCl_3$ and NaOMe shifts), while position **7** was shown to be blocked (lack of shift with NaOAc).

In conclusion, the fungus *C. elegans* NRRL1392 exhibited preference towards sulfation of the hydroxyl group located at C-7 of the flavonoid nucleus.[4,5] In case of 7-methoxylated flavanones, the fungus carries out a demethylation reaction as a prelude for incorporation of a sulfate grouping. On the other hand, incubation for longer time resulted in sulfation of the hydroxyl group at C-4′ position. Much less frequently, only the C-4′ sulfated flavanones could be characterized, which would be explained on the basis of the unstability of sulfate conjugates of the C-7 hydroxyl groups.

Sulfation is rarely encountered as metabolic pathway in the biosynthesis of plant secondary metabolites.[21] However, flavone, flavonol and more recently cardenolide sulfates have been isolated from higher plants.[21] Isolation of flavanone sulfates from angiospermae has not been reported yet, and utilization of the fungus *C. elegans* seems to be indespensible in preparation of such derivatives.

Experimental

7-Methoxyflavanones (**1**, **4**, **6**) were purified from *Rhus retinorrhea*.[1,2] Infrared spectra were recorded with an ATI mattson genesis series fourier transform (FT-IR) spectrophotometer. Ultraviolet spectra were obtained on a Shimadzu 1601 PC and Hewlett Packard 8452A diode array spectrophotometers. Optical rotations were recorded at ambient temperature using JASCO, DIP 370 digital polarimeter. 1D- and 2D-NMR spectra were obtained on a Bruker Avance DRX500 spectrometer. EI-MS was measured on Shimadzu QP5000 mass spectrometer, while HR-ESI-FT-MS were taken on a Bruker Bioapex FT-MS in ESI mode. TLC was performed using glass supported silica gel plates (0.25-mm layer, F_{254}, Merck). For column chromatography, Sephadex LH 20 columns, Pharmacia were used.

Microorganisms and Culture, Conditions The microorganisms, used in this study, were purchased from two sources: American Type Culture Collection (ATCC) and National Center for Agricultural Utilization Research (NCAUR). Sabouraud dextrose agar (Oxoid) slants were used to subculture the battery of microorganisms every three months and kept at 4 °C. Two-week old slants were used to inoculate sterilized culture medium and initiate stage I culture. Several microorganisms were used in the initial screening as previously reported.[22]

Components of the Culture Medium[23] The Components of the culture medium, used in all fermentation experiments, are 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[24] Cells from two week-old slants were used to inoculate the autoclaved medium and kept on a gyratory shaker for 72 h to give stage I culture. An inoculum of 5 ml of stage I culture was used to inoculate stage II cultures (50 ml per 250-ml flasks). After incubation for 24 h, substrates **1**, **4** or **6** were added separately as a solution in dimethylformamide (10 mg, 0.25 ml^{-1}). The substrate and organism controls were made simultaneously. Fermentations were analyzed periodically by extraction of culture samples with an equal volume of AcOEt or *n*-BuOH. After evaporation of the solvent, the residue was chromatographed on silica gel plates (E. Merck, F_{254}) using $CHCl_3$–MeOH (4 : 1) beside other solvent systems. Flavonoid spots were inspected under the UV light (254, 365 nm) before and after spraying with $AlCl_3$, NH_3 or *p*-anisaldehyde reagents.

Fermentation of Sakuranetin (1) with *C. elegans* 7-Methylnaringenin [**1**, 700 mg], dissolved in 17.5 ml of dimetylformamide, was equally distributed among 70 flasks containing stage II cultures of the fungus. After 10 d, the fermentation was stopped and the cells were removed by filtration. The fermentation broth, Which contained the metabolite, was extracted with an equal volume of *n*-butanol. The *n*-BuOH extract was concentrated at 50 °C, under reduced pressure to give 1.9 g of a brownish residue, which was repeatedly chromatographed on sephadex LH 20 columns using MeOH as an eluent to give 60.0 mg of naringenin (**2**) and 110 mg of narigenin-4′-sulfate (**3**).

Naringenin (**2**): EI-MS (rel. int.) 272 (M^+) (25), 254 (4), 153 (49), 120 (30), ^{1}H- and ^{13}C-NMR (DMSO-d_6) δ (see Tables 1, 2, respectively). All these data are identical to those reported for naringenin.[19,20] The metabolite was also directly compared with an authentic sample of naringenin (TLC, mp).

Naringenin-4′-sulfate (**3**): mp >300 °C; $[\alpha]_D^{25}$ −45° (*c*=0.020; MeOH); UV λ_{max} (MeOH) nm: 289, 325 (sh), +NaOMe: 290 (sh), 325; +NaOAc: 287 (sh), 325; +NaOAc/H_3BO_3: 288, 322; +$AlCl_3$: 310, 380; EI-MS *m*/*z* (%rel. int): 272 (50) $(M-80)^+$, 179 (32), 153 (76), 120 (45); HR-ESI-MS:

351.0203 (M−H), using the negative mode (Calcd: 351.0180); IR (ν_{max}) (film) cm^{-1}: 1644 (C=O), 1505, 1257 (S=O), 1053 (C–O–S), 1017, 860; ^{1}H- and ^{13}C-NMR: See Tables 1 and 2.

Fermentation of 5,3′,4′-Trihydroxy-7-methoxyflavanone (4) 7-Methyleriodictyol (**4**, 305 mg) was dissolved in 7.5 ml DMF and equally distributed among 30 flasks containing stage II cultures of *C. elegans.* After an incubation period of 7 d, cells were removed by filtration over cheese cloth and the broth which contained the metabolite was extracted with an equal volume of *n*-BuOH and processed as described previously to give 900 mg of an amber colored semisolid residue. Sephadex LH 20 column chromatography of the butanolic residue gave 34 mg of eriodictyol-4′-sulfate (**5**) beside 14 mg of an unidentified minor product.

Eriodictyol-4′-sulfate: mp >300 °C; $[\alpha]_D^{25}$ −23° (c=0.024, MeOH); UV λ_{max} (MeOH) nm: 216, 290, 328 (sh); +$AlCl_3$: 218, 308, 378; +NaOMe: 216, 232, 284, 327 (sh) decomposition; +NaOAc: 302, 390. EI-MS *m*/*z* (%rel. int.): 288 $(M-80)^+$ (9), 166 (12), 153 (21), 136 (10); HR-ESI-MS: 369.0278 $(M+H)^+$ (Calcd: 369.0275), 289.0716 $(M-80+H)^+$; IR (ν_{max}) (KBr) cm^{-1}: 1640 (C=O), 1507, 1340, 1343, 1272 (S=O), 1185, 1162, 1048 (C–O–S), 804 (S–O); ^{1}H- and ^{13}C-NMR (DMSO-d_6): See Tables 1, 2.

Fermentation of 5,4′-Dihydroxy-7,3′-dimethoxyflavanone with *C. elegans* 7-Methylhomoeriodictyol [**6**, 550 mg], dissolved in 14 ml DMF, was equally distributed among 56 Flasks containing stage II culture of *C. elegans.* After incubation on a gyratory shaker for two weeks, the fermentation was stopped, cells filtered and the broth extracted with an equal volume of *n*-BuOH. Sephadex LH 20 column chromatography of the extract (2 g) gave 150 mg of homoeriodictyol-7-sulfate (**8**) and 8 mg of homoeriodictyol (**7**).

Homoeriodictyol-7-sulfate (**8**): mp >300 °C; $[\alpha]_D^{25}$ −2.7° (c=0.046, MeOH); UV λ_{max} (MeOH) nm: 218, 308, 378; +NaOMe: 214, 282, 337; +$AlCl_3$: 302, 390; +NaOAc: 218, 308, 378; EI-MS *m*/*z* (%rel. int.): 302 $(M-80)^+$ (55), 166 (22), 153 (61), 150 (56); HR-ESI-MS: 383.0442 $(M+H)^+$ (Calcd: 383.0431); IR (ν_{max}) (KBr) cm^{-1}: 1639 (C=O), 1272 (S=O), 1063 (C=O), 801 (S–O); ^{1}H- and ^{13}C-NMR: See Tables 1, 2.

Homoeriodictyol (**7**): ^{1}H- and ^{13}C-NMR data: See Tables 1, 2, respectively. All the NMR data were identical to those reported for homoeriodictyol.[19,20]

Acid Hydrolysis of 3, 5 and 8[8] 5 Mg of **3**, **5**, **8** were separately dissolved in 10 ml MeOH and mixed with 25 ml 3% HCl at room temperature. After evaporation of MeOH, under reduced pressure, $CHCl_3$ was used to extract the aglycones **2**, **9**, **7**, respectively. After evaporation of the solvent, the residue was analyzed by direct comparison with reference samples by TLC and spectroscopic measurements. The sulfate remaining in the aqueous layer was detected by giving white precipitate with $BaCl_2$.

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