PERGAMON

Phytochemistry 53 (2000) 209–212

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Sulfation of naringenin by *Cunninghamella elegans*

Abdel-Rahim S. Ibrahim

Department of Pharmacognosy, Faculty of Pharmacy, Tanta University, Tanta, Egypt

Received 27 April 1999; accepted 9 September 1999

Abstract

A new flavonoid sulfate, naringenin-7-sulfate, was obtained by fermentation of naringenin using the fungus *Cunninghamella elegans* NRRL 1392 in 23% yield. Structural elucidation of the metabolite was achieved using EIMS, UV, IR, 1D and 2D NMR spectroscopy beside acid and enzyme hydrolyses. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Naringenin; Sulfation; *Cunninghamella elegans*; NMR

1. Introduction

Flavonoids are ubiquitous plant natural products that possess many pharmacological actions (Cos et al., 1998; Constantinou, Mehta, Runyan, Rao, Vaughan & Moon, 1995). The interest in studying the effects of flavonoids is attributed to their presence in appreciable amount in a normal human diet (Pierpoint, 1986). Of these flavonoids, the sulfates caused an enhancement of the known flavonoid anti-oxidant activity (Yagi, Uemura, Okamura, Haraguchi, Imoto & Hashimoto, 1994) and an inhibition of lens aldose reductase (Haraguchi et al., 1996).

The use of microorganisms in studying metabolism of natural and synthetic drugs has been documented (Lin & Rosazza, 1998). Thus, many studies on microbial metabolism of flavonoids have been conducted (Ibrahim & Abul-Hajj, 1989, 1990a, 1990b, 1990c). Of these studies, the sulfation of 5-hydroxyflavone by *Streptomyces fulvissimus* (Ibrahim & Abul-Hajj, 1989) and glucosidation of the flavones of *Psiadia arabica* by *Cunninghamella elegans* (Ibrahim, Galal, Mossa & El-Feraly, 1997) are particularly interesting. The latter organism, namely *C. elegans*, was also the sole organism to convert naringenin, a flavanone which enhances cytotoxicity of tumor necrosis factor (Habtemariam, 1997), into a highly polar, unstable metabolite. The present study describes the isolation and identification of this polar metabolite.

2. Results and discussion

Screening 5,7,4′-trihydroxyflavanone (naringenin,**1**) with several microorganisms showed that only *C. elegans* NRRL 1392 was able to produce a highly polar metabolite (**2**). Trials to isolate this metabolite from large-scale incubations, using EtOAc as extraction solvent, were unsuccessful and only the substrate (**1**) could be recovered. This may be attributed to traces of HOAc in the EtOAc. However, the metabolite was remarkably stable when extracted with *n*-BuOH.

To determine the structure of this unknown substance, NMR spectroscopy was carried out, which showed no sugar signals in both ^{1}H- and ^{13}C-NMR spectra. Since *C. elegans* is known to convert phenolic hydrocarbons into their sulfate or glucuronide conjugates (Cerniglia, Freeman & Mitchum, 1982), the metabolite **2** was presumed to be a sulfate of naringenin (**1**). To confirm the presence of a sulfate group, the unknown metabolite was hydrolyzed by treatment with acid at room temperature to yield the aglycone, which was identified as naringenin (**1**). The aqueous fraction from acid hydrolysis was treated with $BaCl_2$, resulting in a white precipitate presumed to be $BaSO_4$. Further support for the presence of sulfate conjugate was obtained after treatment of **2** with *Helix pomatia* aryl sulfatase, which was also coincident with that of a substance formed from sulfate and naringenin. The presence of naringenin was also indicated by EIMS

PII: S0031-9422(99)00487-2

Table 1
NMR data for naringenin (**1**) and metabolite **2** in MDSO-d_6

	δH		δC	
Position	**2**	**1**	**2**	**1**[a]
2	5.47, *dd*, (2.5, 12.7)	5.43, *dd*, (4.0, 13.0)	78.9(*d*)	79.0(*d*)
3	2.70 *cis*, *dd*, (2.8, 17.0) 3.20 *trans*, *m*	2.7 *cis*, *dd*, (2.8, 17.0) 3.20 *trans*, *m*	42.7(*t*)	42.6 (*t*)
4	–	–	197.7(*s*)	196.4(*s*)
5	–	–	162.8(*s*)[b]	163.7 (*s*)
6	6.27, *d*, (2.5)	5.88, *s*	99.8 (*d*)	96.4 (*d*)
7	–	–	162.6(*s*)[b]	166.9(*s*)
8	6.31, *d*, (2.5)	5.88, *s*	98.8(*d*)	95.5(*d*)
9	–	–	162.5(*s*)[b]	163.3(*s*)
10	–	–	104.1(*s*)	102.3(*s*)
1′	–	–	129.3(*s*)	129.4(*s*)
2′	7.30, *d*, (8.4)	7.32, *d*, (9.0)	128.7(*d*)	128.7(*d*)
3′	6.78, *d*, (8.4)	6.79, *d*, (9.0)	115.7(*d*)	115.7(*d*)
4′	–	–	158.2(*s*)	158.1(*s*)
5′	6.78, *d*, (8.4)	6.79, *d*, (9.0)	115.7(*d*)	115.7(*d*)
6′	7.30, *d*, (8.4)	7.32, *d*, (9.0)	128.7(*d*)	128.7(*d*)
5-OH	11.92, *s*	12.14, *s*	–	–
7-OH	–	10.82, *s*	–	–
4′-OH	9.50, *s*	9.60, *s*	–	–

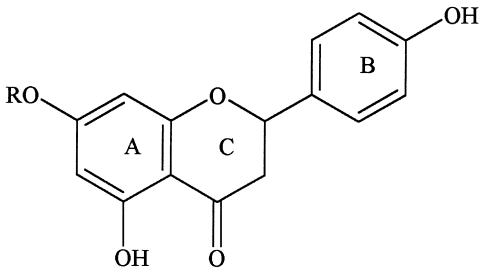
[a] Taken from the reference by Agrawal & Bansal, 1989 and included for comparison.
[b] Assignments can be permuted.

peak at 272(M- sulfate). On the other hand, infrared spectroscopy lent further support to the presence of sulfate moiety as it showed strong bands at 1030 cm^{-1} (C–O–S) and 1250 cm^{-1} (S═O).

In order to identify the site of attachment of the sulfate group, UV spectroscopy was carried out which clearly showed free 5 and 4′-hydroxyl groups ($AlCl_3$ and NaOMe shifts). However, the lack of shift with NaOAc indicated that C-7 hydroxyl is blocked. Reexamination of ^{13}C-NMR data of the unknown metabolite and those of **1** (Table 1) showed that while C-7 signal underwent an upfield shift by 4.3 ppm, C-6, C-8 and C-10 signals are shifted downfield by value of 3.4, 3.3 and 1.8 ppm, respectively. These shifts are consistent with a C-7 sulfated metabolite as the ipso carbon of a 7-sulfated flavonoid experiences an upfield shift while *ortho* and *para* related carbons are shifted downfield (Agrawal & Bansal, 1989). Further confirmation of the bonding position was obtained from ^{1}H-NMR spectra (Table 1). The phenolic hydroxyl groups at positions 5 and 4′, occuring at 12.14 and 9.60 ppm in naringenin, still occurred at 11.92 and 9.50 ppm, respectively in the sulfate conjugate, thus excluding substitution of these two positions. The remaining proton signals of the metabolite were similar to those of the substrate except for H-6 and H-8 signals which underwent downfield shift by 0.4 ppm. Such downshifts can be ascribed to the *ortho* effect of the sulfate moiety at C-7 (Op de, Dijoux, Cartier & Mariotte, 1998; Ibrahim & Abul-Hajj, 1989).

The results obtained from mass spectrometry, infrared spectroscopy, and both acid and enzyme hydrolyses clearly support the identification of the metabolite **2** as sulfate conjugate of naringenin, while the results obtained from UV, ^{1}H- and ^{13}C-NMR spectroscopies support sulfation at C-7 position. Thus, the metabolite **2** was identified as naringenin-7-sulfate.

Conjugation with sulfate represents one of the major mechanisms of detoxifying phenolics in animal tissues (William, 1964). In plants, sulfation of flavonoids was presumed to represent a way of inactivating harmful waste products and may play a role in transfer of sulfur from inorganic to organic state (Harborne, 1975). It is worthwhile to mention that while several sulfated flavones and flavonols are known to occur in plant, sulfated flavanones have yet to be described (Barron, Varin, Ibrahim, Harborne & William, 1988). However, sulfation in microbial systems is extremely rare and reports may be limited to sulfation of 5-hydroxyflavone by *Streptomyces fulvissimus* (Ibrahim & Abul-Hajj, 1989) and sulfate conjugation of phenolic hydrocarbons by *Cunninghamella elegans* (Cerniglia et al., 1982) mentioned earlier. Results obtained from this study showed unequivocally that *C. elegans* is capable of sulfation of flavonoids and demonstrates parallels between microbial and mammalian metabolism not only in phase I but also in phase II metabolism (Davis, 1988).



1 R = H
2 R = -SO_3H

3. Experimental

3.1. General

Naringenin was obtained from Aldrich Chemical, Milwaukee, USA. IR spectra were recorded using a PYE Unicam infrared spectrophotometer. UV spectra were taken on a Shimadzu 1601 PC ultraviolet spectrophotometer and specific rotations were obtained on a Perkin–Elmer digital polarimeter model 241 MC. The

^{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. The chemical shift values are reported as ppm using tetramethylsilane (TMS) as internal standard and coupling constants are expressed in Hz. Electron ionization (EI) mass spectra were taken on Shimadzu QP5000 mass spectrometer.

3.2. Microorganisms and culture conditions

Microorganisms were obtained from either American Type Culture Collection (ATCC) or Northern Regional Research Laboratories (NRRL). Organisms were maintained on Sabouraud dextrose agar (Oxoid) slants at 4° and were used to inoculate the autoclaved culture medium. Twenty microorganisms were used for the preliminary screening as follows.

Aspergillus alliaceous NRRL 315, *A. flavips* ATCC 11013, *A. niger* NRRL 599, *A. niger* NRRL 2295, *A. ochraceous* NRRL 398, *A. ochraceous* NRRL 405, *Candida albicans*, Lab isolate, *Cunninghamella blakesleeana* MR 198, *C. echinulata* NRRL 1382 (ATCC 42616), *C. elegans* NRRL 1392 (ATCC 10028a), *Gymnascella citrina* NRRL 6050 (ATCC 16956), *Lindera pinnespora* NRRL 2237, *Penicillium chrysogenum* ATCC 10002, *P. chrysogenum* ATCC 10002-K, *P. purpureus* UI 193, *P. vermiculatum* NRRL 1009, *Rhizopus nigricans* NRRL 1477, *Rhodotorula rubra* NRRL y1592, *Saccharomyces cerevisae* (Baker's yeast) and *Streptomyces fulvissimus* NRRL 1453B.

3.3. Components of culture medium (Ibrahim et al., 1997)

All fermentation experiments were carried out in a medium of the following composition: 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 autoclaving at 121° for 15 min.

3.4. Cultivation of microorganisms (Ibrahim et al., 1997)

Cells of microorganisms were transferred from 2-week old slants into sterile culture medium and kept on a gyratory shaker for 72 h to give stage I culture. 5 ml of stage I cultures were used as inoculum for stage II cultures (50 ml per 250 ml flask). After 24 h incubation of stage II cultures, naringenin (**1**) was added as a solution in dimethylformamide (10 mg 0.25 ml^{-1}). Both substrate and organism controls were made. Fermentations were sampled by extracting 5 ml culture with 5 ml EtOAc or *n*-BuOH. After evaporation of the solvent, the residue was chromatographed on silica gel plates F254 using $CHCl_3$–MeOH (4:1) or EtOAc–MeCOEt–HCO_2H–H_2O (5:3:1:1) as solvent systems. Flavonoid spots were examined in UV light (254 nm), then sprayed with P-anisaldehyde, $AlCl_3$ or NH_3 solutions.

3.5. Fermentation of naringenin (1) with C. elegans

Naringenin [**1**, 600 mg], dissolved in 15 ml dimethylformamide, was evenly distributed among 60 flasks containing stage II cultures. Fermentation was stopped after 3 weeks, the cells were removed by filtration and the fermentation broth was extracted with an equal volume of *n*-BuOH. Repeated sephadex LH_{20} column chromatography of the butanolic residue (1.8 g), using MeOH as eluent, gave 140 mg of pure **2**; mp > 300°; $[\alpha]_D$ +21. UV λ_{max}^{MeOH} nm: 228, 281, 334 (*sh*); +$AlCl_3$: 223, 306, 390; + NaOAc: 280, 330 (*sh*); +NaOMe: 244, 324; EIMS *m*/*z* (rel. int.): 272 (M-80)$^+$ (12), 166 (7), 153 (19), 83 (27); IR (ν_{max}) (KBr)cm^{-1}: 1640 (C═O), 1250 (S═O), 1030 (C–O–S), 800 (S–O); ^{1}H- and ^{13}C-NMR (DMSO-d_6)δ (see Table 1).

3.6. Acid hydrolysis (Mann, Tofern, Kaloga & Eich, 1999)

5 mg of **2** was dissolved in 10 ml MeOH and mixed with 25 ml 3% HCl at room temperature. After evaporation of MeOH, the aglycone (**1**) was extracted with Et_2O and analyzed by TLC on silica gel with CH_2Cl_2–Me_2CO–HCO_2H (76:16:8). Sulfate was detected in the concentrated aqueous layer by giving white precipitate with $BaCl_2$.

3.7. Enzyme hydrolysis (Hackett & Griffith, 1982)

5 mg of **2** was incubated with aryl sulfatase from the snail, *Helix pomatia* (Sigma) in 5 ml of 0.1 M acetate buffer (pH = 5) at 37°. Complete hydrolysis was observed after 3 h of incubation. The mixture was then extracted with Et_2O and the concentrated organic phase was analyzed by TLC, which showed a compound identical with naringenin (mp, R_f and NMR signals). Again, addition of $BaCl_2$ to concentrated aqueous layer resulted in formation of white precipitate.

Acknowledgements

The author is grateful to Dr. Charles, D. Hufford, School of Pharmacy, University of Mississippi, USA, for the NMR spectra.

References

Agrawal, P. K., & Bansal, M. C. (1989). In P. Agrawal, *Carbon-13 NMR of Flavonoids* (pp. 149–150). Berlin: Elsevier.

Barron, D., Varin, L., Ibrahim, R. K., Harborne, J. B., & William, C. A. (1988). *Phytochemistry*, *27*, 2375.

Cerniglia, C. E., Freeman, J. P., & Mitchum, R. K. (1982). *Appl. Environ. Microbiol*, *43*, 1070.

Constantinou, A., Mehta, R., Runyan, C., Rao, K., Vaughan, A., & Moon, R. (1995). *J. Nat. Prod*, *58*, 217.

Cos, P., Ying, L., Calomme, M., Hu, J. P., Cimanga, K., Poel, B. V., Pieters, L., Vlietinck, A. J., & Berghe, D. V. (1998). *J. Nat. Prod*, *61*, 71.

Davis, P. J. (1988). *Devel. Indust. Microbiol*, *29*, 197.

Habtemariam, S. (1997). *J. Nat. Prod*, *60*, 775.

Hackett, A. M., & Griffith, L. A. (1982). *Xenobiotica*, *12*, 447.

Haraguchi, H., Ohmi, I., Sakai, S., Fukuda, A., Toihara, Y., Fujimoto, T., Okamura, N., & Yagi, A. (1996). *J. Nat. Prod*, *59*, 443.

Harborne, J. (1975). *Phytochemistry*, *14*, 1147.

Ibrahim, A.-R. S., & Abul-Hajj, Y. J. (1989). *Appl. Environ. Microbiol*, *55*, 3140.

Ibrahim, A.-R. S., & Abul-Hajj, Y. J. (1990a). *J. Nat. Prod*, *53*, 644.

Ibrahim, A.-R. S., & Abul-Hajj, Y. J. (1990b). *J. Nat. Prod*, *53*, 1471.

Ibrahim, A.-R. S., & Abul-Hajj, Y. J. (1990c). *Xenobiotica*, *20*, 363.

Ibrahim, A.-R. S., Galal, A. M., Mossa, J., & El-Feraly, F. S. (1997). *Phytochemistry*, *46*, 1193.

Lin, S. T., & Rosazza, J. P. (1998). *J. Nat. Prod*, *61*, 922.

Mann, P., Tofern, B., Kaloga, M., & Eich, E. (1999). *Phytochemistry*, *50*, 267.

Op de, B., Dijoux, M. G., Cartier, G., & Mariotte, A. M. (1998). *Phytochemistry*, *47*, 1171.

Pierpoint, W. S. (1986). In V. Cody, E. Middleton, & J. Harborne, *Plant flavonoids in biology and medicine*. New York: Alan R. Liss. Inc.

William, R. T. (1964). In J. B. Harborne, *Biochemistry of phenolic compounds* (p. 205). London: Academic Press.

Yagi, A., Uemura, T., Okamura, N., Haraguchi, H., Imoto, T., & Hashimoto, K. (1994). *Phytochemistry*, *35*, 885.