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Aromatic Hydroxylation and Sulfation of 5-Hydroxyflavone by *Streptomyces fulvissimus*

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The conversion of 5-hydroxyflavone by various microorganisms was studied. Among them, *Streptomyces fulvissimus* was the sole microbe which produced a new polar metabolite from 5-hydroxyflavone in addition to 5,4′-dihydroxy- and 5,3′,4′-trihydroxyflavone. The structure of this polar metabolite was determined to be 5,4′-dihydroxyflavone-4′-sulfate on the basis of mass, infrared, and nuclear magnetic resonance spectroscopies. These results demonstrate that *S. fulvissimus* catalyzes sulfation at the 4′ position of 5,4′-dihydroxyflavone.

Flavonoids are ubiquitous in plants, and a normal human diet is likely to contain an appreciable amount of these natural products (estimated dietary intake is 1 g/day in the United States of America) (18, 20). Fortunately, the vast majority of flavonoids are nontoxic to humans and animals, and the known pharmacological activities are limited to a few substances of this group. However, recent studies have attributed several biological activities to the flavonoids, including anticancer (3, 24), antiviral (15), and estrogenic effects (16), and the general agreement is that flavonoids are biological response modifiers (17). In addition, several flavonoids were found to be mutagenic in the Ames test (1, 7, 23). Therefore, studies aimed at elucidating the metabolism of flavonoids are essential to our understanding of their actions in vivo, since some metabolites can be more toxic than the parent compound (5).

The use of microorganisms in the simulation of mammalian metabolic processes provides insight(s) into the mechanisms of action, toxicity, pharmacological activity (6, 21, 22), and metabolic pathway of drugs (19).

In this study, a unique microbial transformation product of 5-hydroxyflavone was isolated and the elucidation of its structure by spectroscopic techniques is reported.

MATERIALS AND METHODS

Equipment. Melting points were determined on a Fisher-Jones hot plate apparatus and are uncorrected. Infrared spectra were taken on a Nicolet 5DXCFT-IR spectrophotometer using KBr disks. Mass spectra were determined on a VG7070E-HF mass spectrometer. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were obtained on 300-MHz Nicolet NT-300-W3 and JEOL Fx-90Q FT-NMR spectrometers. UV spectra were taken on a Beckman DU8 spectrophotometer.

Microorganisms. Microorganisms were either purchased from the American Type Culture Collection or obtained from the U.S. Department of Agriculture, Northern Regional Research Laboratory, Peoria, Ill. Eleven genera (32 species) of microorganisms, including *Absidia* (2), *Aspergillus* (5), *Curvularia* (2), *Cunninghamella* (5), *Fusarium* (1), *Gliocladium* (1), *Penicillium* (3), *Rhizopus* (5), *Streptomyces* (4), *Thamnidium* (2), and *Trichoderma* (2) spp., were used.

Culture and fermentation screening procedures. Stock cultures were stored at 4°C and maintained on Sabouraud dextrose or potato dextrose agar (Difco Laboratories, Detroit, Mich.) slants. Biotransformation experiments were carried out as reported earlier (9) and were performed by shake culture techniques carried out by two-stage fermentation procedures in a medium consisting of 1% glucose, 1% glycerol, 0.5% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, and 0.5% NaCl. The medium was adjusted to pH 6 before sterilization by autoclaving at 121°C and 15-lb pressure for 15 min. After 72 h of incubation in the above-mentioned medium, 5 ml of stage I culture was used as inoculum for fresh stage II culture (50 ml per 250-ml flask). After 24 h of incubation of stage II culture, substrate was added (0.2 to 0.25 mg/ml) as a solution in dimethylformamide (200 mg/ml). Culture controls consisted of fermentation blanks in which organisms were grown under identical conditions but without substrate. Substrate controls consisted of sterile medium containing the same amount of substrate and incubated under the same conditions.

Microbial transformation of 5-hydroxyflavone by *Streptomyces fulvissimus* NRRL B1453. Preparative scale fermentation of 5-hydroxyflavone (Indofine Chemical Company, Somerville, N.J.) by *S. fulvissimus* was carried out in 8 liters of medium held in 2-liter Erlenmeyer flasks (400 ml per flask). A total of 2 g of 5-hydroxyflavone (in 10 ml of dimethylformamide) was distributed evenly among the cultures. The cultures were incubated on a rotary shaker (250 rpm) at room temperature for 6 days. The cells were separated from the filtrate with cheesecloth. The pooled filtrate was extracted with chloroform, and the chloroform extract was dried and evaporated in vacuo (40°C) to leave a brown, oily residue (0.52 g) which, when chromatographed (thin-layer chromatography and column), showed no traces of flavonoid compound. The cells (290 g [wet weight]) were extracted with cold acetone and concentrated in vacuo to leave an aqueous suspension. The suspension was filtered, and the precipitate was washed with ethyl acetate and dried at 80°C to yield 360 mg of a yellow solid. Recrystallization from aqueous acetone provided the analytical sample, which was checked for purity on cellulose thin-layer chromatography. The R_f values of the metabolite were as follows: 0.52 in butanol-acetic acid-water (4:1:5) and 0.45 in isobutanol-acetic acid-water (6:1:2).

The purified metabolite had a melting point of >300°C. Infrared (KBr) spectra were 3360, 1652, 1621, 1591, 1577, 1505, 1488, 1423, 1262, 1239, 1210, 1050 (sulfonic acid), 862, 801 (s-o stretching), 755, 727, and 607 per cm. Anion fast atom bombardment mass spectrometry showed fragments at *m/e* 333 (M^+), 253, 249, 233, 197 (phenylacetylene sulfate),

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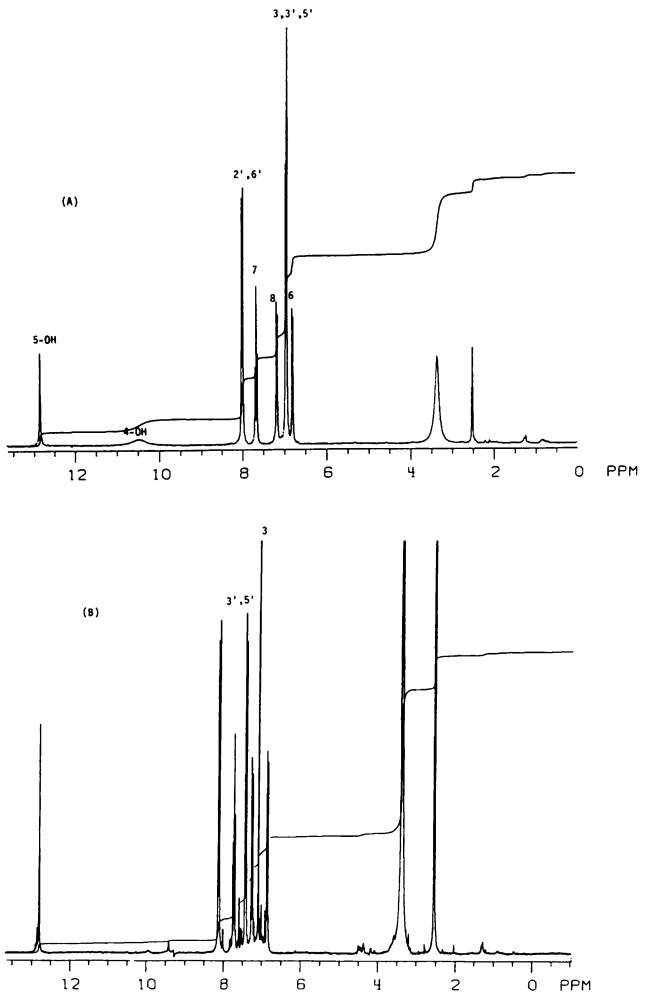

FIG. 1. ^{1}H NMR spectra (300 MHz in dimethyl sulfoxide d_6) of 5,4'-dihydroxyflavone (A) and unknown substance (B).

139, 137, 135 (M^+-phenylacetylene sulfate). ^{1}H NMR (dimethyl sulfoxide-d_6) δ 6.83 (1H, d, C-6), 7.06 (1H, s, C-3), 7.23 (1H, d, C-8), 7.39 (2H, d, C-3', C-5'), 7.70 (1H, t, C-7), 8.09 (2H, d, C-2', C-6'), 12.76 (1H, s, 5-OH proton). ^{13}C NMR (dimethyl sulfoxide) δ 183.108 (C-4), 164.236 (C-2), 159.837 (C-4'), 157.208 (C-5), 155.871 (C-9), 135.826 (C-7), 127.90 (C-2', C-6'), 124.530 (C-1), 120.023 (C-3', C-5'), 110.909 (C-6), 110.027 (C-10), 107.525 (C-8), 104.559 (C-3). UV spectrum: λ_{max} CH_3OH 305, 272, 212, 206 nm. ^{31}P NMR showed no phosphate signal.

Hydrolysis of 5,4'-dihydroxyflavone-4'-sulfate. (i) Acid hydrolysis. Ten milligrams of the metabolite was dissolved in 25 ml of 0.1 N HCl and refluxed in a round-bottomed flask for 2 h as described by Cabrera et al. (2). These conditions result in complete hydrolysis of the conjugate. After cooling to room temperature, the hydrolysate was extracted with chloroform. The recrystallized material showed data identical to those of 5,4'-dihydroxyflavone. $BaCl_2$ was added to the aqueous solution, resulting in the formation of a white precipitate.

(ii) Enzyme hydrolysis. A sample of 5 mg of metabolite was incubated with sulfatase from *Helix pomatia* (Sigma Chemical Co., St. Louis, Mo.) in 5 ml of 0.1 M acetate buffer (pH 5) at 37°C (10). Complete hydrolysis was observed after 4 h of incubation. The mixture was then extracted with chloroform, and the organic phase was concentrated and used for thin-layer chromatography analysis, which showed a compound having the same melting point as and R_f values similar to those of 5,4'-dihydroxyflavone. During the enzymic incubation, the appearance of turbidity was observed, presumably due to precipitation of 5,4'-dihydroxyflavone. Again, addition of $BaCl_2$ to the aqueous layer resulted in the formation of a white precipitate.

TABLE 1. ^{1}H NMR and ^{13}C NMR chemical shifts (ppm) of 5,4'-dihydroxyflavone and 5,4'-dihydroxyflavone-4'-sulfate

Position	Values for:			
	δH		δc	
	5,4'-Dihydroxyflavone	5,4'-Hydroxyflavone-4'-sulfate	5,4'-Dihydroxyflavone	5,4'-Hydroxyflavone-4'-sulfate
2			164.674	164.236
3	6.92	7.06	103.337	104.559
4			182.942	183.108
5			159.836	157.208
6	6.78	6.83	110.843	110.909
7	7.60	7.70	135.665	135.826
8	7.14	7.23	107.364	107.525
9			155.777	155.871
10			109.913	110.027
1'			120.917	124.530
2', 6'	7.97	8.09	128.776	127.9
3', 5'	6.96	7.40	116.037	120.023
4'			161.528	159.837
5-OH	12.82	12.76		
4'-OH	10.50			

RESULTS AND DISCUSSION

Screening of 5-hydroxyflavone with several microorganisms showed the formation of several metabolites, which were produced in relatively small amounts. However, incubations with *S. fulvissimus* showed complete transformation of 5-hydroxyflavone within 48 h. The initial metabolites isolated after 48 h were identified as 5,4'-dihydroxyflavone and 5,3',4'-trihydroxyflavone (results will be reported elsewhere). Longer incubations resulted in a decrease in the amount of 5,4'-dihydroxyflavone which was associated with an increase in the formation of a metabolite that was very polar. This metabolite was found to be present primarily in the cells and not in the fermentation broth. Fortunately, extraction of the metabolite with acetone gave an almost pure product which could be recrystallized from acetone.

To determine the structure of this unknown substance, fast atom bombardment mass spectrometry was carried out. The molecular ion peak of the unknown substance was observed at *m/e* 333, and the molecular weight was coincident with that of a substance formed from SO_3 and 5,4'-dihyroxyflavone. To confirm the presence of a sulfate group, the unknown metabolite was hydrolyzed by acid to yield the aglycone, which was identified as 5,4'-dihydroxyflavone. The aqueous fraction from acid hydrolysis was treated with $BaCl_2$, resulting in a white precipitate which was presumed to be $BaSO_4$. Further support for the presence of a sulfate conjugate was obtained after treatment of the unknown metabolite with *Helix pomatia* sulfatase. The formation of the aglycone identified as 5,4'-dihydroxyflavone as well as the formation of a white precipitate after the addition of

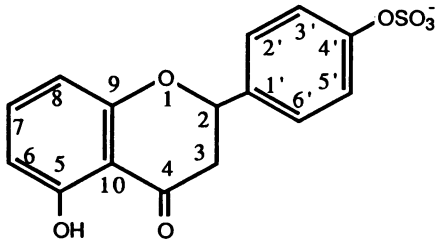

FIG. 2. Chemical structure of the unknown substance.

$BaCl_2$ to the aqueous layer strongly supports the presence of a sulfate group in the unknown metabolite.

Infrared spectroscopy of the metabolite showed an additional peak at 1,052 per cm which was due to the presence of a sulfonic acid moiety. In the ^{13}C NMR spectrum of the unknown substance shown in Table 1, downshifts in the C-3′ and C-5′ carbons (4 ppm) and an upfield shift of about 2 ppm for the C-4′ signal are indicated. These results are consistent with those for other flavonoid sulfates reported earlier by Cabrera et al. (2). For further confirmation of the bonding position of the aglycone, ^1H NMR spectroscopy was carried out (Fig. 1; Table 1). The proton signals at the 3′ and 5′ positions of 5,4′-dihydroxyflavone appeared at 6.95 ppm, while the signals for the 3′,5′ position of the sulfate conjugate were shifted to 7.40 ppm. The phenolic hydroxyl group at position 5 appearing at 12.82 ppm in 5,4′-dihydroxyflavone still occurred at 12.76 ppm in the sulfate conjugate, while the proton of the phenolic hydroxyl group at the 4′ position, observed at 10.50 ppm in 5,4′-dihydroxyflavone, disappeared in the sulfate conjugate.

The results obtained from mass spectrometry, infrared spectroscopy, and both acid and sulfatase hydrolyses clearly support the identification of the sulfate conjugate as 5,4′-dihydroxyflavone, while the results obtained from both ^{13}C NMR and ^1H NMR spectroscopies support sulfation at the C-4′ position. Thus, the unknown polar metabolite obtained from incubations of 5-hydroxyflavone was identified as 5,4′-dihydroxyflavone-4′-sulfate (Fig. 2).

Conjugation of natural products (including flavonoids) and xenobiotics as either sulfates or glucuronides is a well-documented metabolic pathway in mammalian species (11, 12). Furthermore, sulfation of flavonoids has been shown to occur in plant systems (13, 14). However, while glycosylation by microorganisms is not a common pathway (8), sulfation by microbial systems is extremely rare. Recent studies by Cerniglia et al. (4) showed that *Cunninghamella elegans* has the ability to form glucuronide and sulfate conjugates of phenolic hydrocarbons. The results obtained from this study showed unequivocally that *S. fulvissimus* is capable of carrying out sulfation reactions. These results are particularly interesting because this is one of the few reports which demonstrates parallels between microbial and mammalian systems not only in phase 1 but also in phase 2 metabolism. Although conjugation reactions in mammals are known to be important in facilitating detoxification and excretion of xenobiotic compounds, the importance of conjugation in microbial metabolism is poorly understood and additional studies are needed to fully elucidate this pathway of xenobiotic metabolism in microbial systems.

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