Eleven Microbial Metabolites of 6-Hydroxyflavanone

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Received January 14, 2015; accepted May 6, 2015

In aerobic organisms oxygen is the electron acceptor in the electron transport system that generates energy as ATP. Although it is a diradical it does not cause considerable oxidative stress within the body due to spin restriction as it is in its triplet state. Most of the biological molecules are in the singlet state. However, free radicals generate (superoxide, O2−) when unpaired electrons get transferred directly to oxygen due to the leakage of electrons from the intermediate electron carriers of the mitochondrial electron transport chain. These free radicals are useful when they are involved in energy production, phagocytosis, intercellular signaling and regulation of cell growth. They are also useful in facilitating the synthesis of certain biologically important compounds. On the negative side, free radicals apart from modifying proteins, damage cell membranes and DNA which lead to heart disease, cancer, cataracts and cognitive dysfunction.

The body, however, has the ability to minimize the formation of free radicals and to repair molecules that were damaged by them (endogenous defense system). Due to limited efficiency of the defense mechanism aided by a physiopathological environment (air pollutants) an imbalance could occur between the production of reactive species and antioxidant defense (oxidative stress) leading to a potential damage to the tissues. The body therefore depends on dietary antioxidants such as vitamins C, E, A and carotenoids to minimize damage. It has been suggested that plant polyphenols like flavonoids which seem to have a variety of pharmacological properties of which many are attributed to their antioxidant character could also act as defense antioxidants. Since the daily intake of flavonoids by humans is estimated to be around 800mg, it is important to evaluate their efficacy. To date there are many unanswered questions with regards to efficacy and bioavailability of flavonoids.

Flavonoids should bind to target proteins to bring about biological effects. Evaluation of such processes are somewhat complicated specially with the formation of metabolites which bind to different sets of enzymes leading to conflicting results of in vitro and in vivo experiments. Small structural differences caused by functionalization could alter biological properties significantly. Thus, there is a need to analyze molecular targets to determine the risks and benefits of flavonoids for pharmaceutical applications. Lower metabolite levels in biological fluids are added limitations for such investigations. As such, only a few studies are reported on the interaction of flavonoids with defined cellular proteins. The ability of microorganism to mimic mammalian metabolism is suggested to be helpful in obtaining desired quantities of completely characterized metabolites (where appropriate) for complete biological evaluations and to use them as reference material.

The possible antioxidant capabilities together with the observation that hydroxyl or propionoxyl group located at the C-6 position of flavanone can have strong cytotoxic and apoptotic activities against cancer cells prompted us to carry out microbial transformation studies on 6-hydroxyflavanone prospectively to obtain metabolites for further investigations. The study yielded twelve metabolites whose structures were elucidated using spectral data.

Key words flavonoid; microbial metabolism; Cunninghamella blakesleeanal Aspergillus alliaceus; Beauveria bassiana; Mucor ramannianus
Results and Discussion

Forty fungal strains were used in the initial screening of 1. A standard two-stage procedure was used in all the transformation experiments. Selection of fungal cultures for scale up studies was based on the highest number of metabolites formed in good yield. The present work involving four selected strains yielded 11 metabolites (2–12) (Fig. 1). Compounds, flavanone 6-sulfate (3), 6-hydroxyflavanone 7-sulfate (4), 6,4'-dihydroxyflavanone (6), flavanone 6-O-β-D-glucopyranoside (7), 2,4-cis-6-hydroxyflavan-4-ol (8), 2,4-trans-6-hydroxyflavan-4-ol (9) have been reported in the literature. Compounds 3 and 9 however, had been identified only by means of MS data. We have therefore provided further spectroscopy evidence to support the structures. The structure elucidations of the new metabolites (2, 5, 10–12) were based on spectroscopic data as given below.

Molecular formula C_{15}H_{12}O_{6}, confirmed with the molecular ion peak at m/z 403.1391 [M+H]^{+} was assigned to metabolite 2 (1.7 mg, 0.34% yield). Its spectroscopic data revealed its close resemblance to those of 1 and was found to be its O-glucopyranosyl conjugate. The large coupling constants observed between H-2'/3', H-3'/4' and H-4'/5' protons of the sugar moiety established their trans-diaxial relationships. O-Glucosylation was shown to be at C-6 by the long-range heteronuclear multiple correlation (HMBC) three bond correlation of the anomeric proton with the carbon at δ_{H} 5.12 ppm (δ_{C} 7.02). The large coupling constant of the anomeric proton at δ_{H} 4.80 (1H, J=7.8 Hz, H-1') revealed the β-configuration of the glucosyl unit. The structure of 2 was thus, elucidated as flavanone 6-O-β-D-glucopyranoside.

The molecular ion, m/z 318.9953 [M−H]^{-} of the metabolite 3 [206.7 mg, 41.3% produced by Cunninghamella blakesleleana (ATCC 8688A), and [2.2 mg, 0.44% by Aspergillus alliaceus (ATCC 10060)], was consistent with the molecular formula C_{15}H_{12}O_{5}S. The presence of peaks at 1249, 1050, and 817 in the IR spectrum along with the loss of 80 mass units in the MS spectrum indicated the presence of a sulfate group, the position of which was determined to be at C-6 by HMBC correlation data. It was further supported by the shielding effect of the group on the ipso carbon at C-6 position by 5.12 ppm and the deshielding of C-5 and C-7 carbons ortho to and C-9 para to the sulfation site by values of 7.49, 5.49, 3.15 ppm respectively in the 13C-NMR spectrum in comparison to that of 1. Thus, the structure flavanone 6-sulfate was assigned for compound 3.

Compound 5 (4.7 mg, 0.94% yield) with a molecular ion peak at m/z 335.0111 [M−H]^{-} indicated a molecular formula, C_{15}H_{12}O_{5}S. MS and IR data (m/z 255.0526 [M−HSO_{3}]^{-}, IR: 1267, 1050, 826), respectively, gave evidence to the presence of a sulfate group whose position was determined to be at C-6 by NMR data as in compound 3. Metabolite 5 showed spectroscopic data in close agreement with those of 3 except for the presence of a hydroxyl group at C-4' as determined by 1H- and 13C-NMR data. The B-ring protons indicated a para-substitution [δ_{H} 7.31 (2H, d, J=8.4 Hz), 6.76 (2H, d, J=8.4 Hz)]. The 13C-NMR spectrum revealed a highly deshielded carbon at δ 158.1 corresponding to C-4'. HMBC confirmed this assignment. Metabolite 5 was thus characterized as 4'-hydroxyflavanone 6-sulfate.
Compound 9 (34.1 mg, 6.82% yield) exhibited a molecular ion of m/z 241.0701 [M–H]− which was consistent with the molecular formula, C13H16O3. The product whose spectroscopic data were similar to those of 1 except for the reduction of the carbonyl group in the former was identified by means of NMR data. The double doublet of H-4 at δ 4.65 in the 1H-NMR and the replacement of the C-4 signal of 1 at δ 192.2 by the signal at δ 62.4 in the 13C-NMR spectrum of 9 indicated the reduction of the carbonyl group. The double doublet at δ 5.12 (J=12.0, 2.4Hz) assigned to H-2 and the double doublet at δ 4.65 (J=2.4, 2.4Hz) indicated a 2,4-trans-structure. 20

The NMR data of compound 10 displayed a molecular ion at m/z 353.0530 [M−H]+ which confirmed the molecular formula C16H18O5S. The B-ring protons indicated a para-substitution [δH: 7.25 (2H, d, J=9.0Hz), 6.78 (2H, d, J=9.0Hz)]. The 13C-NMR spectrum revealed a highly deshielded carbon at δ 156.9 corresponding to C-4'. The double doublet of H-4 at δ 5.15 in the 1H-NMR and the replacement of the C-4 signal of 1 at δ 192.2 by the signal at δ 58.8 in the 13C-NMR spectrum of 10 indicated the reduction of the carbonyl. A quartet signal at δH 5.09 (J=12.0, 2.4Hz) assigned to H-2 and the double doublet at δH 5.15 (J=2.4, 2.4Hz) assigned to H-4 indicates a 2,4-trans-structure. 21 MS and IR data (m/z 337.0406[M–SO3H]+, IR; 1222, 1034, 875) respectively, gave evidence to the presence of a sulfate group in the molecule. HMBC correlation data together with the deshielding of the ipso carbon at C-5 position by 28 ppm and the shielding of C-6 and C-10 carbons ortho to and C-8 para to the sulfation site by values of 9.2, 2.6, and 5.1 ppm respectively, in the 13C-NMR spectrum compared to that of 8 suggested a structure 2,4-trans-6,4'-dihydroxyflavan-4-ol 5-sulfate for compound 10.

Metabolite 11 (5.0mg, 1.0% yield) had a molecular formula of C16H16O6S confirmed with a molecular ion peak at m/z 297.1035 [M+Na]+. The NMR data suggested the presence of a propanol moiety showing signals at δC 75.1 and 70.8 with corresponding δH 4.23 (1H, dd, J=8.8, 4.8Hz, H-1) and 4.58 (1H, dd, J=7.2, 6.8Hz, H-3). 13C signal at δ 56.3 and 1-H1-NMR singlet at δ 3.02 along with correlation data indicated the presence of OMe functionality at position 1. Signals for 1,2,4-trisubstituted aromatic ring A were observed at δC 116.3 (C-3'), 114.8 (C-4') and 113.0 (C-6') with the respective proton peaks at δH 6.55 (1H, d, J=8.8Hz, H-3'), 6.43 (1H, dd, J=8.8, 2.8Hz, H-4') and 6.60 (1H, d, J=2.8Hz, H-6'). Signals for aromatic B ring were detected at δC 145.8 (C-1'), 126.0 (C-2', C-6'), 128.4 (C-3', C-5') and 127.2 (C-4') with the corresponding proton signals at δH 7.29 (2H, d, J=6.8Hz, H-2', 6'), δH 7.27 (2H, dd, J=6.8Hz, H-3', 5') and 7.22 (1H, d, J=6.8Hz, H-4'). Signals at δC 150.4 and 147.4 were assigned to hydroxylated carbons at C-5' and C-2' respectively. Signals due to methylene protons appeared at δ 1.97 (1H, ddd, J=13.6, 8.8, 6.8Hz) and δ 1.79 (1H, ddd, J=13.6, 7.2, 4.8Hz). HMBC measurements provided evidence for the above assignments. The above evidence suggested the structure for 11 as 1, 3-cis-1-methoxy-1-(2,5-dihydroxyphenyl)-3-phenylpropane.

Spectroscopic data of the metabolite 12 (4.4mg, 0.88% yield), with the molecular formula C16H18O4S (m/z 321.0491 [M–H]−) were similar to those of 3 with the exception of the reduction of the carbonyl group in the former as indicated by the NMR data. A quartet signal at δ 5.19 (J=12.0Hz) assigned to H-2 and a broad singlet at δ 4.72 suggested a 2,4-trans structure and was identified as 2,4-trans-flavan-4-ol 6-sulfate.

**Conclusion**

Health benefits of flavonoids are suggested to be due to their antioxidant properties.20 However, the actual role played by flavonoids in disease control is yet to be established.22 In this regard it is important to take into consideration the properties of the mammalian and gut microflora metabolites in evaluating the biological activities, as very little of the unchanged flavanones reach the tissues.23 Since it is shown that microorganisms are able to mimic mammalian metabolism it may be beneficial to obtain microbial metabolites as they can be obtained in sufficient quantities for structure elucidation and biological evaluation.23

In the present study, microbial conversions performed prospectively on the synthetic 6-hydroxyflavanone resulted in the formation of several metabolites. It is a compound with strong cytotoxic and apoptotic activities against cancer cells,24,25 as well as some antioxidant properties.26 Previous studies on 6-hydroxyflavanone to obtain microbial metabolites with improved antioxidant activity had yielded four metabolites.27 In another study the use of several strains of *Aspergillus niger* to convert 6-hydroxyflavanone resulted in the formation of two metabolites due to the C-4 carbonyl reduction in one and C2, C3 double bond formation in the other.28 Present work on the microbial transformation of 6-hydroxyflavanone with four fungal strains yielded 11 microbial metabolites (2–12) by hydroxylation, C-ring cleavage of the benzo[β]pyrene system, carbonyl group reduction and phase II conjugation. They were characterized using spectroscopic data. Metabolites (2, 5, 9–12) are described as new compounds. Viewing the reported data, it could be said that microbial transformation efficiency of a given compound could be improved by using a variety of microbial strains.18,19,27,21 and its metabolites showed no activity against available test such as antibacterial and antifungal assays. However, weak antileishmanial activity was observed for metabolite 11 when tested against *Leishmania donovani*.

Looking at the structures of 6-hydroxyflavanone and the metabolites it may appear that they may not exhibit the desired hydrogen donating antioxidant activity.29 The ArO radical when formed with the donation of a hydrogen atom is likely to be less stable as the saturated C ring breaks up the resonance of the aromatic rings.29,30 As suggested by kinetic experiments the formation and stability of aryloxy (ArO) radical of a flavonoid is related to its chemical structure.30 For maximum cellular antioxidant activity, catechol structure in the B-ring, 4-oxo group in conjugation with C-2, C-3 double bond and the presence of 3- and 5-OH groups are required to stabilize the ArO radical by resonance.31–33 However, considering the low bioavailability of flavonoids in vivo, 6-hydroxyflavanone and metabolites rather than acting as direct scavengers of free radicals may function as modulators of protein/enzyme functions, receptor activities and intracellular cell signalling.34

Although a considerable number of investigations have been carried out on the biological activities of flavanones, little attention has been paid to the circulating metabolites when proposing respective mechanisms based on the findings.35 The present study which was initiated to identify microbial metabolites is expected to contribute towards the evaluation of
the in vivo bioactivities of 6-hydroxyflavanone in mammals.

Experimental

**General Experimental Procedures** UV spectra were measured on a Hewlett Packard 8452 A diode array spectrometer. A PerkinElmer, Inc. Spectrum 100 FT-IR Spectrophotometer was used to obtain IR spectra. 1H-NMR and 13C-NMR were acquired in CD3OD and DMSO-d6 on a Varian Unity Inova 600 spectrometer unless otherwise stated. High-resolution-electrospray ionization-mass spectrometry (HR-ESI-MS) data were obtained using a Brucker GioApex 3.0 or an Agilent 1100 SL. Jasco DIP-370 digital polarimeter was used to measure optical rotations.

**Substrate** 6-Hydroxyflavanone (1) was purchased from Aldrich Co. (Milwaukee, Wisconsin, U.S.A.) and its authenticity was confirmed by NMR data.

**Organisms and Metabolism** Organisms capable of converting the flavonoid, 1 to their metabolites were selected by screening forty culture samples from the microbial collection of The National Center for Natural Products Research of The University of Mississippi. Screening experiments were carried out by the usual two-stage procedure in 125 mL Erlenmeyer flasks containing 25 mL medium. Each compound was added separately in dimethylformamide (0.5 mg/mL) solution to 24h old stage II cultures and incubated for 14 d on a rotary shaker (New Brunswick Model G10-21) at 100 rpm. Progress of conversion is monitored by UV visualization at 254 nm followed by p-anisaldehyde spray reagent detection on precoated Si gel 60 F254 TLC plates (E. Merck). In preparative scale fermentation 500 mg of each substrate in dimethylformamide was distributed equally among five 2 L flasks, each scaled fermentation 500 mg of each substrate in dimethylformamide containing 25 mL medium . Each culture filtrate was enriched with MeOH and DMSO-OD and used to measure optical rotations.

**Microbial Transformation of 1 by C. blakesleeana** (ATCC 8688A) resulted in the formation of three metabolites (2, 3, 4). Compound 3 was also given by A. alliaceus (ATCC 10060).

6-O-β-D-glucopyranoside (2) was obtained as a white solid (1.7 mg, 0.34% yield). Rf 0.40 [MeOH–CHCl3 (3:17)], greenish spot with p-anisaldehyde; [α]D27 0.57 (c=0.07, MeOH).

Flavonone 6-sulfate (3), a solid (20.67 mg, 41.3% yield) yielded by A. alliaceus (ATCC 8688A), 2.2 mg, 0.44% yielded by A. alliaceus] Rf 0.39 [MeOH–CHCl3 (1:3)] brownish spot with p-anisaldehyde; [α]D24 2.4 (c=0.33, MeOH). It was identified by means of spectroscopic data.

6-Hydroxyflavanone 7-sulfate (4), a brownish solid (1.5 mg, 0.30% yield). Rf 0.14 [MeOH–CHCl3 (2:8)] brownish spot with p-anisaldehyde; [α]D24 -5.8 (c=0.15, MeOH).

6,4'-Dihydroxyflavonan (6) was obtained as a yellowish solid (1.3 mg, 0.26% yielded by A. alliaceus (ATCC 10060), and 2.2 mg, 0.44% yielded by B. bassiana (ATCC 7159)] with a Rf 0.26 MeOH–CHCl3 (1:19) yellowish spot with p-anisaldehyde; [α]D24 0.0 (c=0.19, MeOH) was identified by comparison with published data.12

**Microbial Transformation of 1 by B. bassiana** (ATCC 7159) yielded metabolites (6,7).

6-O-β-D-4-thioglucoopyranoside (7) was obtained as a white solid (187.9 mg, 37.6% yield) Rf 0.39 [MeOH–CHCl3 (1:9)] greenish spot with p-anisaldehyde; [α]D24 -34.8 (c=0.29, MeOH).

**Microbial Transformation of 1 by M. ramannianus** (ATCC 9628) resulted in the formation of 5 metabolites (8, 9, 10, 11, 12).

2-cis-6-Hydroxyflavanone (8): (1.9 mg, 0.38% yield). Rf 0.61 [MeOH–CHCl3 (1:9)] yellowish spot with p-anisaldehyde; [α]D24 0.0 (c=0.29, MeOH).

2,4-trans-6-Hydroxyflavanone (9): (34.1 mg, 6.8% yield). Rf 0.61 [MeOH–CHCl3 (1:9)] yellowish spot with p-anisaldehyde; [α]D24 4.3 (c=0.27, MeOH).

2,4-trans-6,4'-Dihydroxyflavanone 5-sulfate (10): (46.2 mg, 9.2% yield). Rf 0.34 [MeOH–CHCl3 (1:3)] brownish spot with p-anisaldehyde; [α]D24 1.8 (c=0.21, MeOH).

1,3-cis-1-Methoxy-1-(2,5-dihydroxyphenyl)-3-hydroxy-3-phenylpropene (11): (4.5 mg, 0.9% yield). Rf 0.43 [MeOH–CHCl3 (1:9)] yellowish spot with p-anisaldehyde; [α]D24 -2.5 (c=0.16, MeOH).

2,4-trans-Flavan-4-ol 6-sulfate (12): (4.4 mg, 0.88% yield). Rf 0.18 [MeOH–CHCl3 (1:4)] brownish spot with p-anisaldehyde; [α]D24 -2.7 (c=0.29, MeOH).

**Biological Activity** Evaluation of biological activities of selected metabolites was carried out at NCNPR in the School of Pharmacy of The University of Mississippi.25 Metabolites of 6-hydroxyflavanone showed no antibacterial activity against Staphylococcus aureus, methicillin-resistant S. aureus (MRSA), Escherichia coli, Pseudomonas aeruginosa and Microbacterium intracellulare. The positive control used was Ciprofloxacin. The metabolites when tested against the fungal strains, Candida albicans, C. glabrata, C. kusei, C. neoforans and Aspergillus fumigatus also gave negative results. Amphotericin B was used as the positive control. The antimalarial activity of metabolites of 6-hydroxyflavanone was determined in vitro using chloroquine-sensitive and chloroquine-resistant strains of Plasmodium falciparum. No activity was seen with any of the 12 metabolites of 1. Chloroquine and artemisinin were used as positive controls. Weak antileishmanial activity was observed for metabolites 11 when tested against Leishmania donovani. All other metabolites gave negative results. The standard drug, pentamidine was the positive control used.

**Acknowledgments** The authors thank Dr. Bharathi Avula for conducting HR-ESI-MS analysis and Ms. Amber Hale for her assistance in the maintenance and extraction of the cultures. This work was supported, in part by the United States Department of Agriculture, Agricultural Research Specific Cooperative Agreement No. 58-6408-00009.

**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this
article contains supplementary materials. Spectroscopic data of metabolites (2, 3, 5, 9–12) are available as supplementary material.

References

17) Kostrzewa-Susłow E., Dmochowska-Gladysz J., Biolańska A., Ciu-