Microbial Metabolism. Part 12.[1] Isolation, Characterization and Bioactivity Evaluation of Eighteen Microbial Metabolites of 4′-Hydroxyflavanone

Julie Rakel Mikell,[a] Wimal Herath,[a] and Ikhlas Ahmad Khan*[,a,b]

[a] *National Center for Natural Products Research, The University of Mississippi; and* [b] *Department of Pharmacognosy, Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi; University, MS 38677, U.S.A.* Received November 13, 2010; accepted March 15, 2011; published online March 24, 2011

Fermentation of 4′-hydroxyflavanone (1) with fungal cultures, *Beauveria bassiana* (ATCC 13144 and ATCC 7159) yielded 6,3′,4′-trihydroxyflavanone (2), 3′,4′-dihydroxyflavanone 6-*O*-β-D-4-methoxyglucopyranoside (3), 4′-hydroxyflavanone 3′-sulfate (4), 6,4′-dihydroxyflavanone 3′-sulfate (5) and 4′-hydroxyflavanone 6-*O*-β-D-4-methoxyglucopyranoside (7). *B. bassiana* (ATCC 13144) and *B. bassiana* (ATCC 7159) in addition, gave one more metabolite each, namely, flavanone 4′-*O*-β-D-4-methoxyglucopyranoside (6) and 6,4′-dihydroxyflavanone (8) respectively. *Cunninghamella echinulata* (ATCC 9244) transformed 1 to 6,4′-dihydroxyflavanone (8), flavanone-4′-*O*-β-D-glucopyranoside (9), 3′-hydroxyflavanone 4′-sulfate (10), 3′,4′-dihydroxyflavanone (11) and 4′-hydroxyflavanone-3′-*O*-β-D-glucopyranoside (12). *Mucor ramannianus* (ATCC 9628) metabolized 1 to 2,4-*trans*-4′-hydroxyflavan-4-ol (13), 2,4-*cis*-4′-hydroxyflavan-4-ol (14), 2,4-*trans*-3′,4′-dihydroxyflavan-4-ol (15), 2,4-*cis*-3′,4′-dihydroxyflavan-4-ol (16), 2,4-*trans*-3′-hydroxy-4′-methoxyflavan-4-ol (17), flavanone 4′-*O*-α-D-6-deoxyallopyranoside (18) and 2,4-*cis*-4-hydroxyflavanone 4′-*O*-α-D-6-deoxyallopyranoside (19). Metabolites 13 and 14 were also produced by *Ramichloridium anceps* (ATCC 15672). The former was also produced by *C. echinulata*. Structures of the metabolic products were elucidated by means of spectroscopic data. None of the metabolites tested showed antibacterial, antifungal and antiprotozoal activities against selected organisms.

Key words flavonoid; microbial metabolism; *Beauveria bassiana*; *Cunninghamella echinulata*; *Mucor ramannianus*; *Ramichloridium anceps*

Flavonoids which constitute an important part of human diet are associated with many beneficial biological activities,[2] though some may exhibit mutagenic and/or prooxidant properties.[3] They are known to interact with the cytochrome P450 drug-metabolizing enzymes found in liver and other tissues including skin. The capability of flavonoids to induce biosynthesis of Cytochromes P450 (CYPs) and to modulate their activity could lead to loss of biological activity of a drug or to increase its concentration in the plasma resulting in an overdose.[3] They may also activate or inhibit CYPs involved in the process of chemical carcinogenesis. It has been observed that Flavonoids with hydroxyl groups tend to inhibit these enzymes, whereas those without seem to stimulate them.[4] The CYPs in turn can bring about transformation of flavonoids to metabolites which could interact with them and modify their activities. This underlines the importance of determining the metabolic pathways of flavonoids which are being consumed in measurable amounts by man. There are two major sites of flavonoid metabolism.[3] They are the liver and the colon microflora. Several *in vivo* and *in vitro* experiments were conducted to identify the mammalian metabolites of different classes of flavonoids. Based on these observations various suggestions were made with regards to their metabolic pathways. The results of one such experiment involving the metabolism of 16 naturally occurring flavonoids by rat liver microsomes suggested that the B-ring was the main structural moiety that was subjected to biotransformation.[5] The number and the position of the hydroxyl and methoxy groups in the B-ring seemed to determine the extent of metabolism. The non polar flavonoids were metabolized more extensively than the polar ones. The absence of hydroxyl groups in the B-ring or the presence of one such group at C-4′ position resulted in 3′ and 4′ dihydroxylated (catechol) products. It was suggested that the semiquinones or quinones that could result from the oxidation of catechol may be a source of mutagenic free radicals. However, it has been demonstrated that partial detoxification takes place by *O*-methylation of the catechol moiety.[5,6]

Flavanones, as compared to other flavonoids are less abundant in nature. Propolis, honey, citrus fruits and juices are their main dietary sources.[7] Flavanones are associated with many biological properties. The major constituent of citrus fruits, Naringenin for example was shown by *in vitro* experiments to have antioxidant, antiproliferative and estrogenic activities.[7] Since citrus fruits and juices are consumed by man in considerable amounts, investigations have been carried out to evaluate their metabolic parthways. In a transformation study with six flavanones by rat liver microsomes the major metabolic pathway observed was aromatic hydroxylation. However, several previously unreported metabolic pathways including the reduction of the keto group were also discovered.[7] As many as 43 urinary metabolites of flavanone in the rat were detected by a gas chromatographic mass spectrometric technique and the most prominent compounds among them were identified.[8] The main transformations observed were hydroxylation at the 3- or 6-position and the reduction of the keto group. However, only a little hydroxylation occurred in B-ring.[8]

4′-Hydroxyflavanone, a synthetic analogue of flavanones is associated with biological activities including, endothelium-independent full vasorelaxing efficacy on rat aortic rings,[9] aromatase inhibitor properties,[10] decreasing collegen concentration in human dermal fibroblasts[11] and moderate binding ability to the rat uterine estrogen receptor.[12] Its metabolic transformation by rat liver microsomes resulted in the formation of 3′,4′-dihydroxyflavanone, 4′-

* To whom correspondence should be addressed. e-mail: ikhan@olemiss.edu

hydroxyflavone, 6,4′-dihydroxyflavanone (tentative) and a chromone.[7] The formation of 3′,4′-dihydroxyflavanone as the major metabolite was consistent with the observation that the flavonoids with no hydroxyl groups in the B-ring or had one at 4′ position get converted to the corresponding catechol by microsomal enzymes.[5] Microorganisms are good predictors of mammalian drug metabolites with the ability to yield sufficient quantities of metabolites for structure analysis and further pharmacological evaluation.[13] In view of low yields of metabolites produced in previous experiments preventing complete structure elucidation as well as to obtain transformed products with enhanced biological properties, we subjected 4′-hydroxyflavanone to microbial transformation and obtained 18 transformed products. Production, isolation structure elucidation and some bioactivity studies of the metabolites are reported herein.

Results and Discussion

4′-Hydroxyflavanone (**1**) was screened using the standard two stage procedure with forty fungal strains to identify cultures that could give maximum yields of the metabolic products.[14] The results of the scale up studies with selected fungal cultures were summarized in Figs. 1 and 2. The total conversion of **1** to its metabolites was about 47%. Determination of the molecular formulae of the compounds was based on HR-ESI-MS data.

The known compounds, **8**,[15] **11**[16] and **13**[16] were identified as 6,4′-dihydroxyflavanone, 3′,4′-dihydroxyflavanone and 2,4-*trans*-4′-hydroxyflavan-4-ol (**13**) respectively, by comparison with published data. Compound **11** was detected previously as a rat liver microsomal incubation product of 4′-hydroxyflavanone and 3′-dihydroxyflavanone[7] as well as a microbial metabolite of flavanone.[16]

Compound **2** (143.1 mg, 28.6% and 14.9 mg, 2.98% yield by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144), respectively), a yellowish solid with a molecular formula, $C_{15}H_{12}O_5$ (*m/z* 273.0741 $[M+H]^+$) corresponded to a product of **1** with two more oxygen atoms present as a phenolic hydroxyl groups as determined by ^{1}H- and ^{13}C-NMR data. The ^{1}H-NMR spectrum of **2** was similar to that of **1** but showed a reduction of aromatic protons from 8 to 6 with A [δ_H 7.08 (1H, d, J=2.8 Hz), 7.00 dd (1H, J=9.2, 2.8 Hz) and 6.89 (1H, d, J=9.2 Hz)] and B [δ_H 6.88 (1H, br s) and 6.73 (2H, br s)] rings showing 1, 3, 4 aromatic substitution. The location of the hydroxyl group was determined by 2D-NMR experiments. It was identified as 6,3′,4′-trihydroxyflavanone (**2**).

Metabolite **3** (38.4 mg, 7.08% and 9.4 mg, 1.88% yield by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144), respectively) of **1** with a molecular mass of 448 was assigned a molecular formula $C_{22}H_{24}O_{10}$. Its spectroscopic data were similar to those of **2**. The difference observed was the pres-

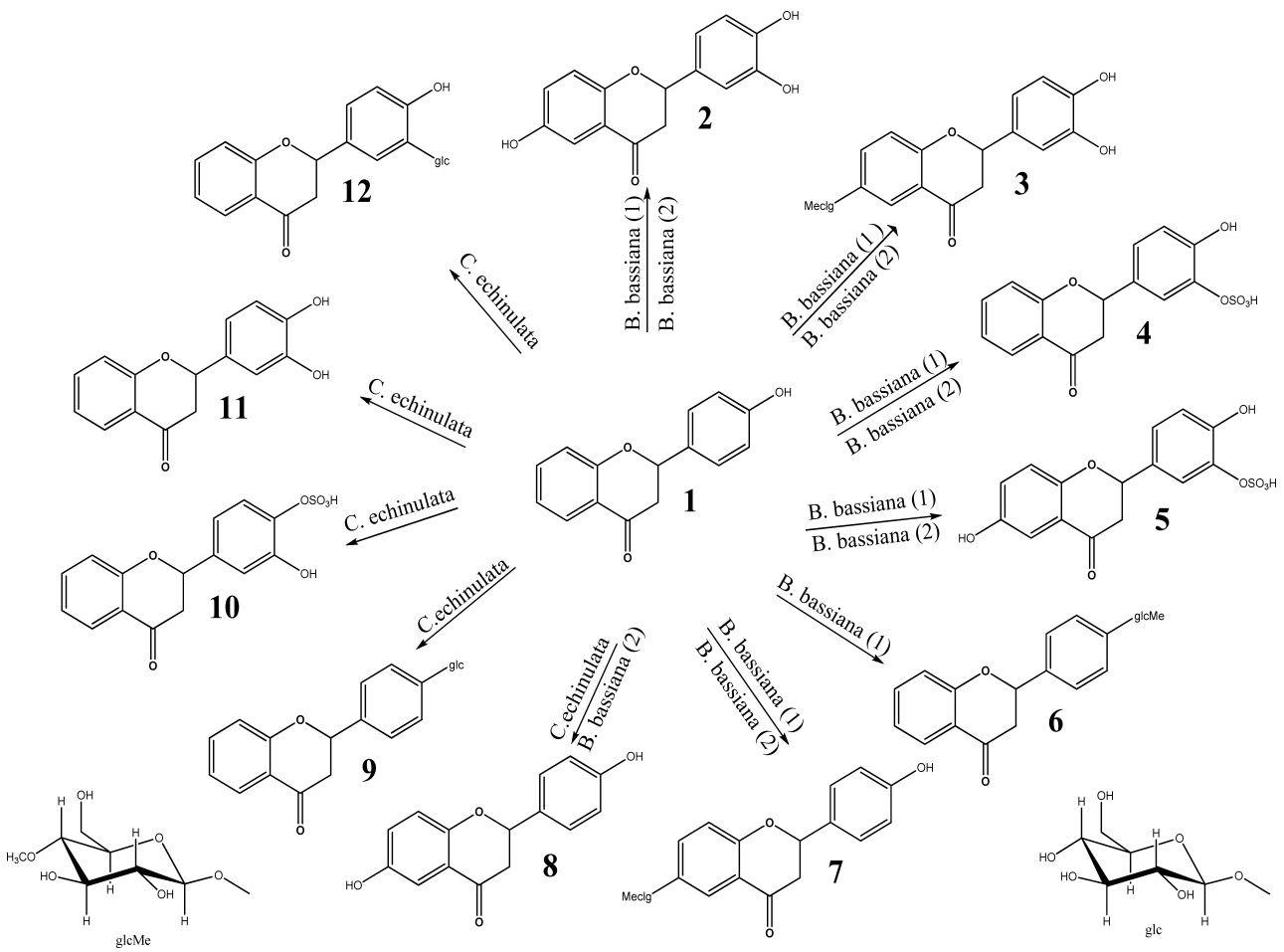

Fig. 1. Microbial Metabolites of 4′-Hydroxyflavanone

B. bassiana (**1**): (ATCC 13144), *B. bassiana* (**2**): (7159).

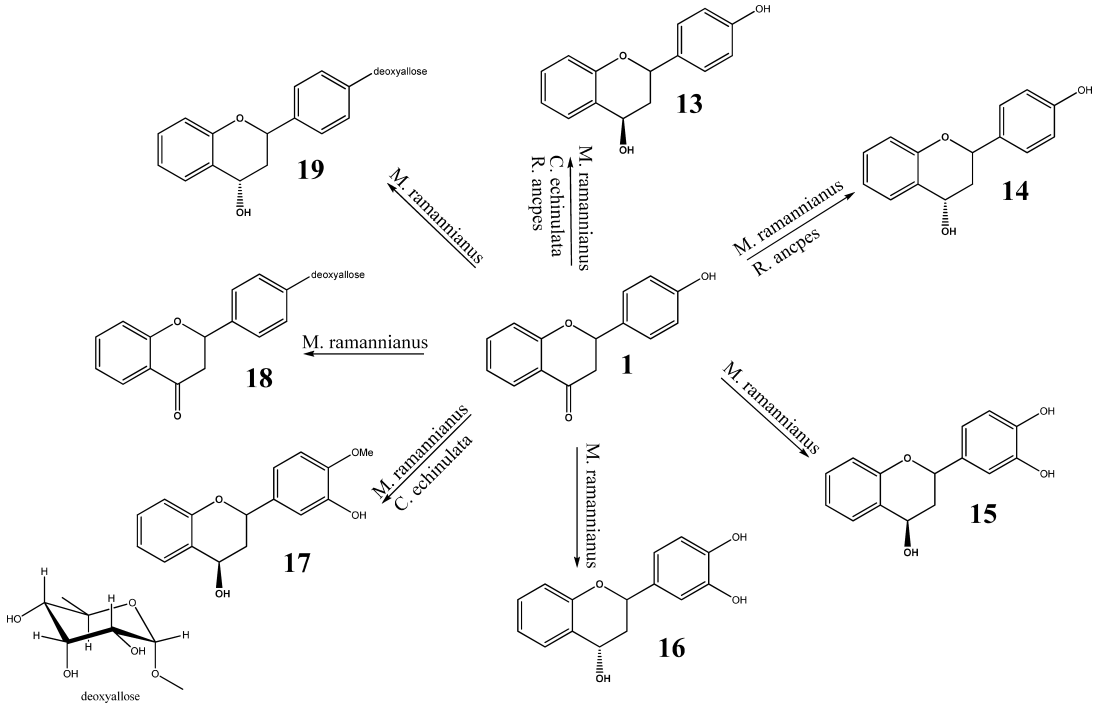

Fig. 2. Microbial Metabolites of 4′-Hydroxyflavanone

ence of an *O*-methyl-D-glucopyranosyl moiety with 176 mass units. The coupling constant of the anomeric proton at δ 4.77 (1H, d, J=7.6 Hz, H-1″) indicated a β-configuration of the glycosyl unit. The three bond correlation of the anomeric proton with the quaternary carbon at δ 152.2 (C-6) indicated *O*-glucosylation at C-6. The large coupling constants between H-2″/3″, H-3″/H-4″ and H-4″/5″ protons indicated *trans*-diaxial relationships. The downfield shift of C-4″ (δ 79.5) along with the HMBC correlations indicated that the *O*-methyl group was attached to C-4″. Thus, the metabolite **3** was characterized as 3′,4′-dihydroxyflavanone 6-*O*-β-D-4-methoxyglucopyranoside.

Compound **5** (59.5 mg, 11.9% and 9.6 mg, 1.92% yield by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144), respectively) showed a molecular ion peak at *m*/*z* 350.9835 $[M-H]^+$ consistant with the molecular formula $C_{15}H_{12}O_8S$. Comparison of this compound with **2** revealed significant similarity except for the presence of a sulfate moiety, the position of which was determined as C-3′ by correlation NMR spectra. It was further supported by the shielding of the signal due to the *ipso* carbon at C-3′ position by 4.4 ppm and the deshielding of C-2′ and C-4′ carbons *ortho* to and C-6′ carbon *para* to the sulfation site by values of 7.6, 3.9 and 5.7 ppm respectively, in the ^{13}C-NMR spectrum when compared with that of **2**.[17] The loss of 80 mass units together with 1250, 1019 and 883 absorptions observed in the MS and IR spectra respectively, gave further evidence to the presence of a sulfate group in the molecule. The forgoing data suggested 6,4′-dihydroxyflavanone 3′-sulfate as the structure of compound **5**.

Compound **4** (40.5 mg, 8.1% and 87.8 mg, 17.56% yield by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144), respectively) with the molecular formula $C_{15}H_{12}O_7S$ (*m*/*z* 335.0165 $[M-H]^+$) showed spectroscopic data in close agreement with those of **5** with the exception of the absence of a hydroxyl group in the molecule. ^{1}H-NMR coupling constants, NMR correlation spectra along with MS (*m*/*z* 255.1593; $[M-SO_4]^+$) and IR (1237, 1175, 827) data confirmed that structure as 4′-hydroxyflavanone 3′-sulfate.

Molecular formula $C_{22}H_{24}O_8$, based on the molecular ion peak at *m*/*z* 439.1358 $[M+Na]^+$ was assigned to metabolite **6** (43.7 mg, 8.74% yield by *B. bassiana* (ATCC 13144). It was found by spectroscopic data to be an *O*-methylated glucopyranosyl conjugate of **1**. The large coupling constants observed between H-2″/3″, H-3″/H-4″ and H-4″/5″ protons of the sugar moiety established their *trans*-diaxial relationships. HMBC correlations and the downfield shift of C-4″ (δ 79.7) detected in the ^{13}C-NMR spectrum indicated that the *O*-methyl group was attached to C-4″ carbon. *O*-glucosylation was shown to be at C-4′ by the three bond correlation of the anomeric proton with the carbon at δ 158.1 (C-4′) which in turn correlate similarly with H-2′ and H-6′ (δ 7.44). The large coupling constant of the anomeric proton at δ 4.89 (1H, d, J=8.0 Hz, H-1″) revealed the β-configuration of the glucosyl unit. The structure of **6** was thus, elucidated as flavanone 4′-*O*-β-D-4-methoxyglucopyranoside.

Compound **7** (9.7 mg, 1.94% and 101.4 mg, 20.28% yield by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144), respectively) with a molecular formula $C_{22}H_{24}O_9$ (*m*/*z* 455.1277 $[M+Na]^+$) was determined to be an *O*-methylated glucopyranosyl conjugate of **1**. The sugar moiety was identified as 4-*O*-methyl-D-glucopyranose by a detailed study of NMR data as in compound **6**. Three bond correlation of anomeric proton with carbon at δ 152.3 (C-6) and similar correlation of C-6 with H-8 (δ 7.00) indicated *O*-glucosylation at C-6. β-Configuration of the glucosyl unit was detrmined by the large coupling constant of the anomeric proton δ 4.79 (1H, d, J=7.2 Hz, H-1″). The structure of **7** was thus, established as 4′-hydroxyflavanone 6-*O*-β-D-4-methoxyglucopyranoside.

Metabolite **9** (129.3 mg, 25.86% yield) had a molecular formula $C_{21}H_{22}O_8$ derived from molecular ion peak at *m/z* 425.1228 $[M+Na]^+$. Its spectroscopic data revealed its close resemblance to those of **1**. The exception was the presence of D-glucopyranose group. The structure and the position of the sugar moiety were determined by correlation NMR data as in compound **6**. The upfield shift of the *ipso* and the downfield shift of the *ortho* carbon atoms together with the lowfield shift of the *para* carbon with a higher magnitude in the ^{13}C-NMR spectrum provided further evidence for the presence of the sugar unit at C-4′.[17] Its structure was determined as flavanone-4′-*O*-*β*-D-glucopyranoside (**9**).

A molecular ion of *m/z* 335.1140 $[M-H]^+$ displayed by compound **10** (63.1 mg, 12.62% yield) was consistant with the molecular formula, $C_{15}H_{12}O_7S$. The presence of peaks at 1273, 1050 and 845 in the IR spectrum along with the loss of 80 mass units in the MS spectrum indicated the presence of a sulfate group in the molecule. The spectral data were similar to those of metabolite **4** except for the position of the sulphate group. NMR correlation data together with the shielding of the *ipso* carbon at C-4′ position by 8.6 ppm and the deshielding of C-3′ and C-5′ carbons *ortho* to and C-1′ carbon *para* to the sulfation site by values of 8.3, 5.7 and 5.5 ppm respectively, in the ^{13}C-NMR spectrum compared to that of **4** suggested structure 3′-hydroxyflavanone-4′-sulfate for compound **10**.

The NMR data of compound **12** (56.0 mg, 11.2% yield) with the molecular formula, $C_{21}H_{22}O_9$ (417.0845 $[M-H]^+$) were in close resemblance to those of **11**[16] except for the presence of a D-glucopyranose moiety at C-3′. The structure and the position of the sugar moiety were determined by correlation NMR data. *β*-Configuration of the glucosyl unit was indicated by the large coupling constant of the anomeric proton at δ 4.70 (1H, d, J=7.2 Hz, H-1″). Its structure was elucidated as 4′-hydroxyflavanone-3′-*O*-*β*-D-glucopyranoside.

HR-ESI-MS (241.0566 $[M-H]^+$) established the molecular formula of **13** (63.4 mg, 12.7% yield by *R. anceps*) as $C_{15}H_{14}O_3$. It corresponded to a product of **1** with two additional hydrogen atoms. The spectral data were in agreement with those published for 2,4-*cis*-4′-hydroxyflavan-4-ol.[16]

Metabolite **14** (56.51 mg, 11.3% yield) (HR-ESI-MS (241.0557 $[M-H]^+$) was spectroscopically similar to compound **13**, with the exception of the appearance of the C-4 signal δ 4.92 as a quartet with J=12 Hz, as against a triplet signal at δ 4.62 (J=3.2 Hz) in the C-2, C-4 *cis* isomer (**13**) indicating it to be 2,4-*cis*-4′-hydroxyflavan-4-ol.

Metabolite **15** (37.5 mg, 7.5% yield) with the molecular formula, $C_{15}H_{14}O_4$ (257.0500 $[M-H]^+$) showed spectroscopic data similar to those of compound **11** with the exception of the reduction of the carbonyl group. A quartet signal at δ 5.02 (J=10.4 Hz) assigned to H-2 and a broad singlet at δ 4.60 suggested a 2,4-*trans* structure. It was thus, identified as, 2,4-*trans*-3′, 4′-dihydroxyflavan-4-ol.

Compound **16** (47.0 mg, 9.4% yield) exhibited a molecular ion of *m/z* 257.0515 $[M-H]^+$ which was consistant with the molecular formula, $C_{15}H_{14}O_4$ (257.0812 $[M-H]^+$). The product whose spectroscopic data were similar to those of the metabolite **11** except for the reduction of the carbonyl group in the former was identified by means of NMR data. The double doublet of 4-H at δ 4.90 in the ^1H-NMR and the replacement of the C-4 signal of **11** at δ 192.6 by the signal at δ 64.7 in the ^{13}C-NMR spectrum of **16** indicated the reduction of the carbonyl group. The double doublet at δ 5.01 (J=12.0, 1.6 Hz) assigned to H-2 and the double doublet at δ 4.90 (J=11.2, 6.4 Hz) assigned to H-4 indicated a 2,4-*cis* structure.[18] **16** was thus, identified as 2,4-*cis*-3′, 4′-dihydroxyflavan-4-ol.

Spectroscopic data of the metabolite **17** (2.8 mg, 0.56% yield), with the molecular formula, $C_{16}H_{16}O_4$ (*m/z* 271.1085 $[M-H]^+$) were similar those of compound **11** with the exceptions of a methoxy group attached to C-4′ as determined by NMR correlation data and the reduction of the carbonyl group. The coupling constants of the signals of H-2 and H-4 in the ^1H-NMR spectrum indicated that were in a *trans* arrangement. It was assigned the structure 2,4-*trans*-3′-hydroxy-4′-methoxyflavan-4-ol.

Metabolite **18** (10 mg, 2% yield), was assigned the molecular formula $C_{21}H_{22}O_7$ based on *m/z* 409.1473 $[M+Na]^+$. The ^1H-NMR spectral data of the compound was similar to those of **1**, except for the presence of glycosyl unit shown by the additional five carbon resanances observed at δ 67.9—98.6 in the ^{13}C-NMR spectrum. It was identified as D-6-deoxyallose by analysing the ^1H-, ^{13}C-NMR and their correlation data. They were in total agreement with those of the flavanoid glycosides obtained during microbial transformation studies carried out by us using the same organism, *M. ramannianus*.[19] HMBC correlations permitted the assignment of the sugar unit at C-4′. The structure of the metabolite **18** was determined as flavanone 4′-*O*-*α*-D-6-deoxyallopyranoside.

NMR data of flavanone **19** (30.1 mg, 6.02% yield, $C_{21}H_{24}O_7$, 411.1529 $[M+Na]^+$) were in close agreement with those of metabolite **13**. However, there were additional resonances due to the presence of a sugar unit, which was established as D-6-deoxyallose as in compound **18**. In addition, the large $J_{4,3ax}$ value along with the downfield shift of the C-2 compared to the *trans*-flavan-4-ols in the ^{13}C-NMR spectrum indicated a 2,4-*cis* structure.[18] It was thus, identified as 2,4-*cis*-4-hydroxyflavanone 4′-*O*-*α*-D-6-deoxyallopyranoside (**19**).

Conclusion

In this study, extensive metabolism of 4′-hydroxyflavanone (**1**) by the fungal cultures, *B. bassiana*, *C. echinulata*, *M. ramannianus*, and *R. anceps* was observed with the formation of 18 compounds. Metabolites **2**—**5**, **10**—**12** and **15**—**17** suggested that the primarily conversion occurred in the B-ring with the generation of catechol (**2**—**3**, **11** and **15**, **16**) and the conjugated products, **4**, **5**, **10**, **12** and **17** showing metabolic pathways similar to those observed *in vitro* biotransformations of flavonoids by mammalian microsomes.[5] Further, the formation of C-4 carbonyl group reduction compounds, **13**—**17** and **19** along with the hydroxylation at 3′-position (**2**, **3**, **10**, **11** and **15**—**17**), at 6-position (**2**, **5** and **8**) and the conjugation products (**3**—**5** and **7**) also paralleled the mammalian metabolism of flavanone.[8] Present study underlined the importance of using microbial models to mimic mammalian metabolism of xenobiotics. The completely characterized microbial metabolites of **1** could thus, be utilized to confirm tentatively identified mammalian metabolites[5] due to lack of material and also to carry out further pharmacological studies. The formation of functional groups at carbon 6 position in this investigation is significant, as C-6 derivatives

of flavonoids are somewhat rare. This method therefore, could be of immense help in generating C-6 derivatives of flavanoids in synthetic medicinal and organic chemistry. Further, although the organisms showed similar patterns of phase I conversions, certain specificities were observed in the conjugation products. *B. bassiana* strains whilst introduced a 4-methoxyglucopyranosyl moiety, *C. echinulata* coupled the molecule with a glucopyranosyl unit. *M. ramannianus* on the other hand was able to convert some of the phase I metabolites to the respective 6-deoxyallopyranosides. In addition, only *M. ramannianus* was able to bring about reduction of the carbonyl group. Investigators have characterized ring cleavage products during the microbial transformation studies of flavanone.[16] However, several attempts made during the present investigation to detect and isolate cleavage products of **1** using available microbial cultures were unsuccessful indicating perhaps the necessity of specific microbial strains for conversion. Flavanones are associated with a variety of biological activities. However, none of the metabolites subjected to *in vitro* investigations carried out with the available tests, namely, antibacterial, antiviral and antihelmenthis, showed any activity.

Experimental

General Experimental Procedures UV spectra were measured on a Hewlett Packard 8452A diode array spectrometer. An ATI Mattson Genesis series FT-IR spectrophotometer was used to run IR spectra in $CHCl_3$. ^{1}H- and ^{13}C-NMR were acquired in $CDCl_3$ and DMSO-d_6 on a Varian Unity Inova 600 spectrometer unless otherwise stated. HR-ESI-MS data were obtained using a Bruker GioApex 3.0. Jasco DIP-370 digital polarimeter was used to measure optical rotations.

Substrate 4′-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 α.[20] Each compound was added separately in dimethylformamide (0.5 mg/ml) solution to 24 h old stage II cultures and incubated for 14 d on a rotary shaker (New Brunswick Model G10-21) at 100 rpm. Precoated Si gel 60 F_{254} TLC plates (E. Merck) and *p*-anisaldehyde spray reagent were used to monitor the progress of conversion. In preparative scale fermentations 500 mg of each substrate in dimethylformamide were distributed equally among five 2 l flasks, each containing 500 ml medium α. The combined culture filtrates were extracted with $(Me)_2CHCH_2OH$–EtOAc (2 : 9). The residues obtained by the evaporation of solvents were flash chromatographed using silica gel with $CHCl_3$ enriched with MeOH as the mobile phase. The fractions thus obtained were separated over sephadex LX-20 using MeOH as the eluent to isolate the metabolites. Culture and substrate controls were run along with the above experiments.[21]

Microbial transformation of **1** by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144) yielded metabolites, **2**—**5** and **7**. The latter in addition gave compound **6**.

6,3′,4′-Trihydroxyflavanone (**2**) was obtained as a yellowish solid (143.1 mg, 28.6% and 14.9 mg, 2.98% yield by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144), respectively). *Rf* 0.64 [MeOH–$CHCl_3$ (1 : 9)]; $[\alpha]_D^{27}$ 0.0 (c=0.06, MeOH). It was identified by means of spectroscopic data.

3′,4′-Dihydroxyflavanone 6-*O*-β-D-4-methoxyglucopyranoside (**3**) was a white solid (38.4 mg, 7.08% and 9.4 mg, 1.88% yield by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144), respectively) with a *Rf* 0.21 [MeOH–$CHCl_3$ (1 : 9)]; $[\alpha]_D^{27}$ −9.5 (c=0.32, MeOH).

4′-Hydroxyflavanone 3′-sulfate (**4**) was isolated as a brownish solid (40.5 mg, 8.1% and 87.8 mg, 17.56% yield by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144), respectively). *Rf* 0.24 [MeOH–$CHCl_3$ (3 : 17)]; $[\alpha]_D^{27}$ 3.5 (c=0.63, MeOH).

6,4′-Dihydroxyflavanone 3′-sulfate (**5**) was purified as a brownish solid (59.5 mg, 11.9% and 9.6 mg, 1.92% yield by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144), respectively). *Rf* 0.17 [MeOH–$CHCl_3$ (3 : 17)]; $[\alpha]_D^{27}$ −14.4 (c=0.06, MeOH).

Flavanone 4′-*O*-β-D-methylglucopyranoside (**6**), obtained as a yellowish solid (43.7 mg, 8.74% yield by *B. bassiana* (ATCC 13144) showed a *Rf* value of 0.61 [MeOH–$CHCl_3$ (1 : 9)]; $[\alpha]_D^{27}$ −17.6 (c=0.06, MeOH).

4′-Hydroxyflavanone 6-*O*-β-D-4-methoxyglucopyranoside (**7**) was isolated as a white solid (9.7 mg, 1.94% and 101.4 mg, 20.28% yield by *B. bassiana* (ATCC 7159) and *B. bassiana* (ATCC 13144), respectively). *Rf* 0.31 [MeOH–$CHCl_3$ (1 : 9)]; $[\alpha]_D^{27}$ −16 (c=0.06, MeOH).

Microbial transformation of **1** by *C. echinulata* (ATCC 9244) resulted in the formation of six metabolites, **8**—**13**. Compound **8** was also given by *B. bassiana* (ATCC 7159).

6,4′-Dihydroxyflavanone (**8**), a yellowish solid (38.7 mg, 7.74% and 1.0 mg, 0.2% yield by *C. echinulata* (ATCC 9244) and *B. bassiana* (ATCC 7159), respectively) with a *Rf* value of 0.26 [MeOH–$CHCl_3$ (1 : 19)]; $[\alpha]_D^{27}$ 0.0 (c=0.43, MeOH) was identified by comparison with published data.[15]

Flavanone-4′-*O*-β-D-glucopyranoside (**9**) was obtained as a white solid (129.3 mg, 25.80% yield). *Rf* 0.29 [MeOH–$CHCl_3$ (1 : 9)]; $[\alpha]_D^{27}$ 0.00 (c=0.10, MeOH).

3′-Hydroxyflavanone 4′-sulfate (**10**) was isolated as a brownish solid (63.1 mg, 12.62% yield). *Rf* 0.31 [MeOH–$CHCl_3$ (1 : 4)]; $[\alpha]_D^{27}$ −3.9 (c=0.25, MeOH).

3′,4′-Dihydroxyflavanone (**11**), purified as a yellowish white solid (8.3 mg, 1.66% yield). *Rf* 0.28 [MeOH–$CHCl_3$ (1 : 19)]; $[\alpha]_D^{27}$ −11.7 (c= 0.54, MeOH) was identified with the aid of literature data.[15]

4′-Hydroxyflavanone-3′-*O*-β-D-glucopyranoside (**12**). (56.0 mg, 11.2% yield). *Rf* 0.27 [MeOH–$CHCl_3$ (1 : 9)]; $[\alpha]_D^{27}$ 2.3 (c=0.18, MeOH).

Microbial transformation of **1** by *Mucor ramannianus* (ATCC 9628) yielded seven compounds, **13**—**19**. Metabolites **13** and **14** were also produced by *Ramichloridium anceps* (ATCC 15672). They were isolated as white solids.

2,4-*trans*-4′-Hydroxyflavan-4-ol (**13**): (63.4 mg, 12.7% yield by *R. anceps*). *Rf* 0.27 [MeOH–$CHCl_3$ (1 : 19)]; $[\alpha]_D^{27}$ 18.4 (c=0.31, MeOH).

2,4-*cis*-4′-Hydroxyflavan-4-ol (**14**): (56.51 mg, 11.3% yield by *R. anceps*). *Rf* 0.27 [MeOH–$CHCl_3$ (1 : 19)]; $[\alpha]_D^{27}$ 1.1 (c=0.28, MeOH).

2,4-*trans*-3′,4′-Dihydroxyflavan-4-ol (**15**): (37.5 mg, 7.5% yield). *Rf* 0.40 [MeOH–$CHCl_3$ (1 : 9)]; $[\alpha]_D^{27}$ −11.4 (c=0.31, MeOH).

2,4-*cis*-3′,4′-Dihydroxyflavan-4-ol (**16**): (47.0 mg, 9.4% yield). *Rf* 0.40 [MeOH–$CHCl_3$ (1 : 9)]; $[\alpha]_D^{27}$ 6.5 (c=0.31, MeOH).

2,4-*cis*-3′-hydroxy-4′-Methoxyflavan-4-ol (**17**): (2.8 mg, 0.56% yield). *Rf* 0.60 [MeOH–$CHCl_3$ (1 : 19)]; $[\alpha]_D^{27}$ −1.5 (c=0.33, MeOH).

Flavanone 4′-*O*-α-D-6-deoxyallopyranoside (**18**): (10 mg, 2% yield). *Rf* 0.36 [MeOH–$CHCl_3$ (1 : 9)]; $[\alpha]_D^{27}$ −24.0 (c=0.06, MeOH).

2,4-*cis*-4-Hydroxyflavanone 4′-*O*-α-D-6-deoxyallopyranoside (**19**): (30.1 mg, 6.02% yield). *Rf* 0.24 [MeOH–$CHCl_3$ (1 : 9)]; $[\alpha]_D^{27}$ −87.3 (c=0.22, MeOH).

Structures of the metabolic products were elucidated by means of spectroscopic data.

Biological Activity Evaluation of biological activities of selected metabolites was carried out at NCNPR in the School of Pharmacy of The University of Mississippi.[22] Metabolites, **3**, **8**—**12** and **15**—**19** showed no antibacterial activity against *Staphylococcus aureus*, methicillin-rsistant *S. aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeroginosa* and *Micobacterium intracellulare*. The positive control used was Ciprofloxacin. The same metabolites when tested against the fungal strains, *Candida albicans*, *C. glabra*, *C. kusei*, *C. neoformans* and *Aspergillus fumigatus* also gave negative results. Amphotericin B was used as the positive control. Similar results were obtained when compounds **1**, **4**—**7** and **14** were screened against *C. albicans*, *A. fumigatus*, *C. neoformans*, *E. coli*, *P. aeroginosa*, MRSA and *M. intracellulare*. No antileishmanial activity was observed with compounds **3**, **8**—**12** and **15**—**19** when tested against *Leishmania donovani*. The standard drug, pentamidine was the positive control used. *In vitro* antimalarial activity was conducted using chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. The compounds used were **1**—**19**.

Supplementary Material Spectroscopic data of metabolites **2**—**19** are available as supplementary material.

Acknowledgements The authors thank Mr. Frank Wiggers for assistance in obtaining 2D-NMR spectra and Dr. Bharathi Avula for conducting HR-ESI-MS analysis. This work was supported, in part, by the United States Department of Agriculture, Agricultural Research Specific Cooperative Agreement No. 58-6408-2-00009.

References

1) Herath W., Khan I. A., *Chem. Pharm. Bull.*, **58**, 562—564 (2010).
2) Stipcevis T., Piljac J., Berghe D. V., *Plant Foods Hum. Nutr.*, **61**, 29—34 (2006).
3) Hodek P., Trefil P., Stiborová, *Chem. Biol. Interact.*, **139**, 1—21 (2002).
4) Li Y., Wang E., Patten C. J., Chen L., Yang C. S., *Drug Metab. Dispos.*, **32**, 387—397 (1994).
5) Nielsen S. E., Breinholt V., Justesen U., Cornett C., Dragsted L. O., *Xenobiotica*, **28**, 389—401 (1998).
6) Zhu B. T., Ezell E. L., Liehr J. G., *J. Biol. Chem.*, **269**, 292—299 (1994).
7) Nikolic D., van Breemen R. B., *Drug Metab. Dispos.*, **32**, 387—397 (2004).
8) Buset H., Scheline R. R., *Biol. Mass Spectrom.*, **6**, 212—220 (1979).
9) Calderone V., Chericoni S., Martinelli C., Testai L., Nardi A., Morelli I., Brechi M. C., Martinotti E., *Naunyn Schmiedebergs Arch. Pharmacol.*, **370**, 290—298 (2004).
10) Ibrahim A., Abul-Hajj Y. J., *J. Steroid Biochem. Mol. Biol.*, **37**, 257—260 (1990).
11) Stipcevic T., Piljac J., Berghe D. V., *Plant Foods Hum. Nutr.*, **61**, 29—34 (2006).
12) Branham W. S., Dial S. L., Moland C. L., Hass B. S., Blair R. M., Fang H., Shi L., Tong W., Perkins R. G., Sheehan D. M., *J. Nutr.*, **132**, 658—664 (2002).
13) Davis P. J., "Antibiotics and Microbial Transformations," ed. by Lamba S. S., Walker C. A., CRC, Boca Raton, Florida, 1987, pp. 47—70.
14) Elmarakby S. A., Clark A. M., Baker J. K., Hufford C. D., *J. Pharm. Sci.*, **75**, 614—618 (1986).
15) Lim S. S., Jung S. H., Ji J., Shin K. H., Keum S. R., *J. Pharm. Pharmacol.* **53**, 653—668 (2001).
16) Ibrahin A., Abul-Hajj Y. J., *J. Nat. Prod.*, **53**, 644—656 (1990).
17) Agrawal P. K., Bansal M. C., "Carbon-13 NMR of Flavonoids," Chap. 6, ed. by Agrawal P. K., Elsevier, Amsterdam, 1989, pp. 168—171, 286—293.
18) Pouget C., Fagnere C., Basly J., Leveque H., Chulia A., *Tetrahedron*, **56**, 6047—6052 (2000).
19) Herath W., Mikell J. R., Hale A. L., Ferreira D., Khan I. A., *Chem. Pharm. Bull.*, **56**, 418—422 (2008).
20) Herath W., Mikell J. R., Ferreira D., Khan I. A., *Chem. Pharm. Bull.*, **51**, 646—648 (2003).
21) Jurgens T. M., Hufford C. D., Clark A. M., *Xenobiotica*, **22**, 569—577 (1992).
22) Jain M., Khan S. I., Tekwani B. L., Jacob M. R., Singh S., Singh P. P., Jain R., *Bioorg. Med. Chem.*, **13**, 4458—4466 (2005).