MICROBIOLOGICAL TRANSFORMATION OF (±)-FLAVANONE AND (±)-ISOFLAVANONE[1]

ABDEL-RAHIM IBRAHIM[1] and YUSUF J. ABUL-HAJJ*

Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT.—The microbiological transformation of flavanone and isoflavanone was explored using a group of 80 microorganisms in the initial screening. Ten metabolites of flavanone were isolated and identified as 4'-hydroxyflavanone [3], 3',4'-dihydroxyflavanone [4], 3-hydroxyflavone [2], flavone [5], 2'-hydroxydihydrochalcone [7], 2',4-dihydroxydihydrochalcone [6], 2',3,4-trihydroxydihydrochalcone [8], 2',5'-dihydroxydihydrochalcone [9], 4'-hydroxyflavan-4α-ol [11], and 2'-hydroxydibenzoylmethane [10]. The isoflavanone metabolites were identified as isoflavone [15], 2-hydroxyisoflavanone [16], 4'-hydroxyisoflavanone [13], 6,4'-dihydroxyisoflavanone [17], and 3',4'-dihydroxyisoflavone [14]. The structures of the metabolites were established using spectroscopic techniques including ir, ms, uv, ^{1}H-nmr, and ^{13}C-nmr spectroscopy. Production of 4'-hydroxyflavanone, 3',4'-dihydroxyflavanone, and 2',4-dihydroxydihydrochalcone by 13 microorganisms was assayed using reversed-phase hplc.

Flavonoids are widespread plant natural products and are consumed in measurable amounts by humans. The estimated daily dietary intake is 1 g/day in the United States (1). While evaluation of most flavonoids indicated that these natural products are safe for human consumption, recent studies on flavonoids indicated the diversity of pharmacological and physiological effects of these ubiquitous plant natural products. One of the interesting findings about flavonoids is the influence they have on the incidence of cancer by modulating the cytochrome p-450 monooxygenase system (2) and inhibition of the effects of tumor promoters (3). However, flavonols such as quercetin were found to be mutagenic in Ames' test (4). Flavonoids may also influence the incidence of breast cancer by acting as antiestrogens (5) or as aromatase inhibitors (6).

The cleavage of flavonoids by intestinal microflora makes understanding of the mammalian metabolism of these natural products complicated. Indeed, more than 40 mammalian metabolites have been identified, many of which are hydroxylated at the 3 and 6 positions (7). In view of the fact that microbial systems can mimic metabolic patterns observed in mammals (8), we initiated studies on microbial metabolism of flavonoids. In previous studies, Udupa and co-workers (9–13) obtained several products of chalcones and dihydrochalcones with hydroxylations at 4' and 3',4'-positions as well as carbonyl reduction products (for numbering see Figure 1). Recent studies in our laboratories showed that most of the microbes investigated produced primarily ring-B hydroxylations at 4' and 3',4' positions of flavone and isoflavone (14). Additionally, some cleavage products of flavone but not of isoflavone were obtained. A sulfation prod-
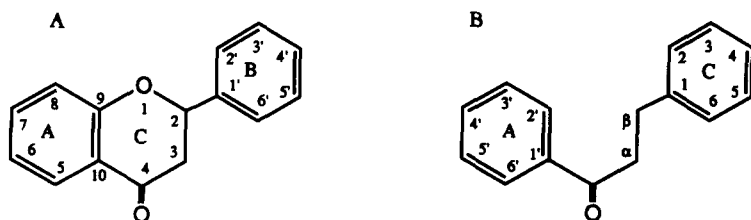

FIGURE 1. Structure and numbering of flavanone (A) and dihydrochalcone (B).

uct of 5-hydroxyflavone was also isolated (15), indicating that microorganisms are also capable of carrying out phase II reactions. In the present study, several microbial transformation products of flavanone and isoflavanone have been isolated, and the elucidation of their structures using spectroscopic techniques is reported.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on Fischer Jones hot plate apparatus and are uncorrected. Infrared spectra were obtained on a 5 DXC FT-IR spectrometer using KBr discs or neat techniques. Mass spectra were determined on AEIMS-30 and Finnigan 4000 mass spectrometers. ^{1}H-nmr and ^{13}C-nmr spectra were recorded on a 300 MHz Nicolet NT-300-W3 spectrometer operating at 300 and 78 MHz, respectively. Uv spectra were taken on Beckman Du 70 spectrophotometer. Optical rotation was measured in MeOH using a Perkin-Elmer 141 polarimeter.

HPLC ANALYSIS.—Hplc analysis was performed with a Beckman system consisting of a single 100 A pump and a single wavelength absorbance detector at 254 nm. A 5 μ C18 ODS (Altex Scientific, Berkeley, California) column (4.6 mm × 25 cm) was used, and the separation was achieved with MeOH-H$_2$O-HOAc (60:40:1) as the eluting solvent at a flow rate of 1 ml/min. The external standard method (16) was used for the percentage production of three flavanone metabolites: 4'-hydroxyflavanone [3], 3',4'-dihydroxyflavanone [4], and 2',4-dihydroxydihydrochalcone [6], which were produced by many cultures. Standard curves were obtained by adding known amounts of metabolites from DMF stock solution to 5 ml medium which was then extracted with 5 ml CHCl$_3$. After drying the CHCl$_3$ extract over anhydrous Na$_2$SO$_4$, the solvent was evaporated at 40°. The residue was dissolved in 1 ml MeOH, and 20 ml of MeOH solution was injected. Peak heights were plotted against concentration to give rise to standard curves. Quantification of metabolites was carried out by adding 2 ml of stage I culture to 23 ml of sterile medium. After incubation for 24 h, 5 mg substrate, dissolved in 0.25 ml DMF, was added (0.2 mg/ml), and fermentation continued for 5 days. The fifth-day culture was homogenized, and 5 ml of the homogenate was extracted with 5 ml CHCl$_3$. Drying and evaporation of the CHCl$_3$ gave a residue which was treated identically as in the preparation of the standard curve.

MICROORGANISMS.—Microorganisms were obtained from either American Type Culture Collection (ATCC) or Northern Regional Research Laboratories (NRRL). Other cultures were obtained from other colleagues. Twenty genera (80 species) of microorganisms, which include *Absidia* (3), *Aspergillus* (10), *Curvularia* (2), *Cunninghamella* (5), *Fusarium* (8), *Gliocladium* (4), *Gymnascella* (1), *Helicostylum* (3), *Manascus* (3), *Mycobacterium* (2), *Penicillium* (9), *Sepedonium* (2), *Streptomyces* (6), *Thamnidium* (2), *Trichocladium* (2), and *Trichoderma* (2), were used.

CULTURE AND FERMENTATION SCREENING PROCEDURES.—Fungal stock cultures were stored at 4° and maintained by subculturing on Sabourad dextrose agar or potato dextrose agar (Difco) slants. Biotransformation experiments were performed by shake culture technique. The two-stage fermentation procedure was utilized in a medium consisting of 1% glucose, 1% glycerol, 0.5% yeast extract, 0.5% peptone, 0.5% K$_2$PO$_4$, and 0.5% NaCl. The medium was adjusted to pH 6 before sterilization by autoclaving at 121° and 15 lb pressure for 15 min. After 72 h of incubation in the above medium, 5 ml stage I culture was used as inoculum for fresh stage II culture (50 ml/250 ml flask). After 24-h incubation of stage II, substrate was added (0.2–0.25 mg/ml) as a solution in DMF (200 mg/ml). Culture control of organism without substrate and substrate control consisting of substrate without organism were used. The fermentations were sampled by extraction of 4 ml of the culture with 4 ml of CHCl$_3$. The extracts were concentrated and spotted on tlc Si gel plates and developed using C$_6$H$_6$-EtOAc (4:1) or hexane-Et$_2$O (2:1). The tlc plates were dried and checked under uv light and then either exposed to NH$_3$ vapors or sprayed with 5% alcoholic AlCl$_3$ solution and rechecked under uv light.

Preparative scale fermentations were conducted on 500–2000 mg substrates. Transformation continued for 3–5 days. The mycelium was filtered from the fermentation medium, and the filtrate was then extracted three times with an equal volume of CHCl$_3$. The mycelium was lyophilized and extracted with Me$_2$CO and Me$_2$CO extract concentrated and extracted with EtOAc. All extracts (CHCl$_3$ and EtOAc) were washed with H$_2$O and dried over anhydrous Na$_2$SO$_4$. Si gel cc was used for the isolation of the metabolites using a gradient of hexane/Et$_2$O. Flavanone was obtained from Aldrich Chemical Company and possessed physical and spectral data consistent with those reported in the literature.

FLAVANONE [1].—Mol wt 224; mp 76–78°; ir (KBr) 1690, 1606, 1574, 1452, 1369, 1324, 1304, 1228, 1146, 1112, 1067, 1025, 983, 911, 862, 967, 703, 491 cm^{-1}; ms m/z 224, 147, 120, 104, 92, 77, 63, 51, 40; ^{1}H nmr (CDCl$_3$) δ 7.293 (1H, d, H-5, J = 10 Hz), 7.43 (7H, unresolved multiplet, H-6 and H-8, B ring protons), 7.035 (1H, d of d, H-6), 5.46 (1H, q, H-2), 3.07 (1H, q, H-3 cis), 2.873 (1H, q, H-3 trans); ^{13}C nmr (CDCl$_3$) see Table 1.

TABLE 1. ^{13}C-nmr Chemical Shifts (δ) of Compounds **1**, **2**, **3**, and **4**.

Carbon	Compound			
	1	**2**	**3**	**4**
C-2	79.49	144.972	78.881	78.818
C-3	44.57	138.504	43.376	43.545
C-4	191.53	174.000	191.868	191.894
C-5	128.73	125.529	129.082	126.246
C-6	121.52	124.585	121.239	121.209
C-7	135.95	133.698	136.100	136.158
C-8	118.00	118.350	117.982	115.043
C-9	161.50	156.000	161.213	161.159
C-10	121.00	120.724	120.599	120.597
C-1′	138.82	131.138	126.082	129.662
C-2′	126.05	127.817	128.259	114.298
C-3′	128.73	128.660	115.133	145.105
C-4′	126.96	130.249	157.600	145.572
C-5′	128.73	128.660	115.133	115.290
C-6′	126.05	127.817	128.259	117.952

4′-HYDROXYFLAVANONE [**3**].—Produced from transformation of flavanone by *Streptomyces fulvissimus* NRRL B1453 in 10% yield as well as by other microorganisms (Table 5): mp (hexane/EtOAc) 192–193°; ir (KBr) 3156, 3029, 2882, 1670, 1603, 1518, 1465, 1377, 1314, 1279, 1230, 1181, 1152, 1117, 1068, 1019, 990, 836, 773, 506 cm^{-1}; ms (solid probe) m/z [M]$^+$ 240, 239, 223, 147, 121, 120, 107, 92, 91, 77, 65; ^{1}H nmr (DMSO-d_6) δ 9.596 (4′-OH), 7.77 (1H, d, H-5, J = 8 Hz), 7.559 (1H, t, H-7), 7.337 (2H, d, H-2′, H-6′, J = 9 Hz), 7.061 (2H, m, H-6, H-8), 6.795 (2H, d, H-3′, H-5′, J = 9 Hz), 5.51 (1H, q, H-2), 3.26 (1H, q, H-3 cis), 2.73 (1H, q, H-3 trans); ^{13}C nmr (DMSO) see Table 1; uv λ max 320, 251.5, 219.5 nm.

3′,4′-DIHYDROXYFLAVANONE [**4**].—Produced from transformation of flavanone by *Streptomyces fulvissimus* NRRL B1453 in 10% yield as well as by other organisms (Table 5): mp (EtOAc/hexane) 175–177°; ir (KBr) 3492, 3247, 1673, 1597, 1520, 1463, 1368, 1311, 1292, 1220, 1182, 1154, 1111, 1073, 954, 887, 816, 763 cm^{-1}; ms (direct probe) m/z 256, 255, 239, 147, 136, 121, 120, 92, 89, 77; ^{1}H nmr (DMSO-d_6) δ 9.2 (two hydroxy protons), 7.765 (1H, d, H-5, J = 8 Hz), 7.556 (1H, t, H-7), 7.056 (2H, m, H-6, H-8), 6.91 (1H, s, H-2′), 6.757 (2H, m, H-5′, H-6′), 5.454 (1H, q, H-2), 3.184 (1H, q, H-3 cis), 2.73 (1H, q, H-3 trans); ^{13}C nmr (DMSO) see Table 1.

3-HYDROXYFLAVONE [**2**].—This metabolite was obtained in 6% yield from transformation of flavanone by *Aspergillus niger* NRRL 599: mp (hexane/Et$_2$O) 170°; ir (KBr) 3213, 1628, 1607, 1562, 1481, 1471, 1417, 1351, 1308, 1287, 1212, 1131, 1078, 1036, 992, 902, 780, 759, 701, 688, 471, 437 cm^{-1}; ms (solid probe): m/z [M]$^+$ 238, 237, 210, 209, 181, 152, 105, 104, 118, 89, 77, 63, 50; ^{1}H nmr (CDCl$_3$) δ 8.267 (3H, d, H-5, H-2′, H-6′, J = 9.64 Hz), 7.707 (1H, t, H-7), 7.5 (5H, m, remaining aromatic protons), 7.11 (3-OH); ^{13}C nmr (CDCl$_3$) see Table 1; uv λ max 343, 305, 238, 204 nm.

2′,4-DIHYDROXYDIHYDROCHALCONE [**6**].—Produced from transformation of flavanone by *Streptomyces fulvissimus* NRRL B1453 in 1% yield as well as by several other microorganisms (Table 5): mp (hexane/Et$_2$O) 106°; ir (KBr) 3417, 1638, 1614, 1579, 1516, 1493, 1448, 1438, 1435, 1307, 1260, 1201, 980, 830, 762, 727, 657, 605 cm^{-1}; ms (solid probe) m/z [M]$^+$ 242, 224, 223, 207, 102, 107; ^{1}H nmr (CDCl$_3$) δ 12.328 (H-2′ hydroxy proton), 7.73 (1H, d, H-6′, J = 9 Hz), 7.45 (1H, t, H-4′), 7.1 (2H, d, H-2, H-6, J = 8 Hz), 6.98 (1H, d, H-3′, J = 9 Hz), 6.87 (1H, t, H-5′), 6.76 (2H, d, H-3, H-5, J = 8.5 Hz), 4.864 (1H, 4-OH), 3.28 (2H, t, H-α), 2.994 (2H, t, H-β); ^{13}C nmr (CDCl$_3$) see Table 2; uv λ max 324, 279, 251, 220 nm.

2′-HYDROXYDIHYDROCHALCONE [**7**].—Obtained from transformation of flavanone by *Penicillium chrysogenum* 10002 K in 30% yield as an oily liquid that could not be crystallized: ir (neat) 3036, 2931, 1637, 1609, 1581, 1496, 1455, 1363, 1300, 1145, 1032, 983, 759 cm^{-1}; ms m/z [M]$^+$ 226, 208, 207, 121, 105, 92, 65, 57, 39; ^{1}H nmr (CDCl$_3$) δ 12.88 (2′-OH), 7.718 (1H, d, H-6′, J = 9 Hz), 7.437 (1H, t, H-4′), 7.26 (5H, m, B-ring protons), 6.968 (1H, d, H-3′, J = 9 Hz), 6.855 (1H, t, H-5′), 3.309 (2H, t, H-α), 3.052 (2H, t, H-β); ^{13}C nmr (CDCl$_3$) see Table 2; uv λ max 325, 244, 222 nm.

TABLE 2. ^{13}C-nmr Chemical Shifts (δ) of Compounds **6–10**.

Carbon	Compound				
	6	**7**	**8**	**9**	**10**
C-α	40.28	39.994	40.186	40.097	92.357
C-β	29.244	29.989	29.455	29.941	177.554
C-1	132.761	140.642	141.95	140.587	119.343
C-2	129.473	128.53	115.432	128.325	126.881
C-3	115.37	128.314	133.56	128.561	128.849
C-4	153.952	126.243	143.651	126.29	128.849
C-5	115.37	128.314	115.432	128.561	128.849
C-6	129.473	128.53	120.6	128.325	126.881
C-1′	119.239	119.207	119.224	118.801	128.562
C-2′	162.346	162.387	162.235	156.648	162.538
C-3′	118.495	118.494	119.009	119.368	118.888
C-4′	136.303	136.25	136.37	124.8	135.89
C-5′	118.898	118.834	118.462	147.294	119.145
C-6′	129.801	129.737	129.865	114.624	132.
C=O	205.552	205.237	205.699	204.712	195.729

2′,3,4-TRIHYDROXYDIHYDROCHALCONE [**8**].—Produced from transformation of flavanone by *As. niger* X172 in 3% yield: mp (hexane/Et$_2$O) 148–150°, ir (KBr) 3481, 3346, 1643, 1602, 1519, 1492, 1448, 1357, 1336, 1306, 1282, 1268, 1260, 1201, 1186, 1160, 1109, 986, 949, 824, 828, 776, 756, 659, 485 cm^{-1}; ms (direct probe) *m/z* [M]$^+$ 258, 240, 223, 136, 123, 121, 93, 77, 65; ^{1}H nmr (CDCl$_3$) δ 12.341 (2′-OH), 7.72 (1H, H-6′, d, *J* = 9.12 Hz), 7.452 (1H, t, H-4′), 6.97 (1H, d, H-3′, *J* = 7.97 Hz), 6.873 (1H, t, H-5′), 6.77 (1H, s, H-2), 6.776 (1H, d, H-6), 6.644 (1H, d, H-5, *J* = 9.97 Hz), 5.82 (broad signal for 3-OH and 4-OH), 3.25 (2H, t, H-α), 2.93 (2H, t, H-β); ^{13}C nmr (CDCl$_3$) see Table 2; uv λ max 324, 284, 251, 221 nm.

2′,5′-DIHYDROXYDIHYDROCHALCONE [**9**].—Produced from transformation of flavanone by *As. niger* NRRL 599 in 1% yield: mp (hexane/Et$_2$O) 110°; ir (KBr) 3392, 1644, 1626, 1592, 1496, 1483, 1451, 1300, 1287, 1242, 1177, 996, 931, 824, 792, 787, 612 cm^{-1}; ms (solid probe) *m/z* [M]$^+$ 242, 223, 147, 137, 109, 91, 77, 65, 52, 41, 28; ^{1}H-nmr (CDCl$_3$) δ 11.851 (2′-OH), 7.331–7.169 (6H, m), 7 (1H, d, H-4′, *J* = 9 Hz), 6.88 (1H, d, H-3′, *J* = 9 Hz), 3.267 (2H, t, H-α), 3.055 (2H, t, H-β); ^{13}C nmr (CDCl$_3$) see Table 2; uv λ max 263, 256, 225, 209 nm.

2′-HYDROXYDIBENZOYLMETHANE [**10**].—Obtained from transformation of flavanone by *As. niger* X172 in 8% yield: mp (hexane) 118–120°; ir (KBr) 1617, 1607, 1584, 1573, 1489, 1332, 1302, 1244, 1194, 1180, 1150, 1025, 893, 758, 731, 620 cm^{-1}; ms (solid probe) *m/z* [M]$^+$ 240, 223, 163, 121, 120, 105, 92, 77, 65, 51, 40, 28; ^{1}H nmr (CDCl$_3$) δ 12.106 (2′-OH), 7.95 (2H, d, H-2, H-6, *J* = 7.32), 7.79 (1H, d, H-6′, *J* = 7.42 Hz), 7.569 (4H, m, H-3, H-5, H-4, H-5′), 7.031 (1H, d, H-3′, *J* = 8.31 Hz), 6.929 (1H, t, H-4′), 6.849 (2H, s, methylene protons); ^{13}C nmr (CDCl$_3$) see Table 2; uv λ max 363, 251, 206.5 nm.

4′-HYDROXYFLAVAN-4α-OL [**11**].—Produced from transformation of flavanone by *Absidia blackes-leeana* NRRL 1306 in 2% yield: mp (hexane/EtOAc) 216–217°; [α]D −40.8, ir (KBr) 3437, 3180, 1609, 1519, 1486, 1463, 1240, 1227, 1206, 1115, 1010, 828, 755 cm^{-1}; cims *m/z* [M]$^+$ 242, 224, 120, 107; ^{1}H nmr (Me$_2$CO-*d$_6$*) δ 7.325 (3H, H-5, H-2′, H-6′), 7.155 (1H, t, H-7), 6.85 (4H, H-3′, H-5′, H-6, H-8), 5.2 (1H, q, H-2, *J* = 15 Hz), 4.76 (1H, t, H-4, *J* = 5 Hz), 2.15 (2H, m, H-3); ^{13}C nmr (Me$_2$CO) see Table 3; uv λ max 275.5, 223.5 nm.

FLAVONE [**5**].—Produced from transformation of flavanone by *As. niger* NRRL 599 in trace amount: mp (hexane) 95–96°; ir (CHCl$_3$) 1644, 1567, 1370 cm^{-1}; ms (solid probe) *m/z* [M]$^+$ 222, 149, 165, 120, 102, 97, 92, 64, 50; uv λ max 294, 250, 206 nm; ms, ir, and uv data identical to standard flavone.

SYNTHESIS OF ISOFLAVANONE.—To a solution of isoflavone (14) in MeOH (1 g/100 ml), NaBH$_4$ (2 g) was added, and reactants were left for 15 min. The mixture was concentrated, diluted with H$_2$O, and extracted with CHCl$_3$. Evaporation of CHCl$_3$ followed by dissolving the residue in Me$_2$CO and oxidation with Jones reagent (33) gave isoflavanone in more than 90% yield. Pure isoflavanone was obtained by chromatography on Si gel using hexane-Et$_2$O (95:5) as an eluent: mp (hexane) 75–76°; ir (KBr) 1686,

TABLE 3.   ¹H- and ¹³C-nmr Spectral Data of Flavanone [1] and 4'-Hydroxyflavan-4α-ol [11].

Atom	¹H-nmr		¹³C-nmr	
	1	**11**	**1**	**11**
2	5.46 (q)	5.2 (q, J = 15 Hz)	79.49	73.65
3	3.07 (cis, q) 2.873 (trans, q)	2.15 (m)	44.57	39.54
4	—	4.76 (t, J = 5 Hz)	191.53	63.75
5	7.293 (d)	7.325 (m)	128.73	131.446
6	7.035 (d of d)	6.85 (m)	121.52	120.829
7		7.155 (t)	135.95	129.752
8		6.85 (m)	118.00	117.441
9			161.50	155.861
10			121.00	125.551
1'			138.82	133.233
2'		7.325 (m)	126.05	128.507
3'		6.85 (m)	128.73	115.94
4'		—	126.96	158.001
5'		6.85 (m)	128.73	115.94
6'		7.325 (m)	126.05	128.507

1600, 1584, 1488, 1456, 1315, 1260, 1200, 1135, 992, 845, 752, 704 cm⁻¹; ms (direct inlet) m/z 224, 120, 104, 92, 77, 78, 63, 51, 39; ¹H nmr (CDCl₃) δ 7.974 (1H, d, H-5, J = 9.54 Hz), 7.516 (1H, t, H-7), 7.320 (5H, m, B-ring protons), 7.046 (2H, m, H-6, H-8), 4.681 (2H, d, H-2 protons, J = 7.71 Hz), 4.008 (1H, t, H-3); ¹³C nmr (CDCl₃) 192.099 (C-4), 161.559 (C-9), 136.008 (C-7), 134.991 (C-1'), 129.148 (C-3', C-5'), 128.858 (C-2', C-6'), 127.751, 128.571 (C-5, C-4'), 121.579 (C-6), 121.03 (C-10), 117.838 (C-8), 71.48 (C-2), 52.317 (C-3); uv λ max 321.5, 251.5, 215.5 nm.

2-HYDROXYISOFLAVANONE [16].—Produced from transformation of isoflavanone by *As. niger* X172 in 12% yield: mp (hexane/Et₂O) 96°; ir (KBr) 3402, 1679, 1607, 1477, 1467, 1457, 1449, 1349, 1307, 1259, 1224, 1130, 1097, 1037, 1021, 950, 758, 748, 697, 629 cm⁻¹; ms (solid probe) m/z [M]⁺ 240, 212, 121, 120, 105, 92, 77, 65, 28; ¹H nmr (CDCl₃): δ 7.936 (1H, d, H-5, J = 8.07 Hz), 7.353–7.384 (3H, m), 7.065 (1H, t, H-6), 6.961 (1H, d, H-8, J = 8.26 Hz), 4.855 (1H, d, H-2, J = 11.66 Hz), 4.47 (1H, H-3, J = 11.45 Hz); ¹³C nmr (CDCl₃) 194.501 (C-4), 161.51 (C-9), 138.471 (C-1'), 136.80 (C-7), 128.762 (C-3', C-5'), 128.693 (C-2', C-6'), 127.582 (C-2), 125.92 (C-5, C-4'), 121.974 (C-6), 119.149 (C-10), 118.04 (C-8), 73.76 (C-3); uv λ max 322, 252, 219 nm.

4'-HYDROXYISOFLAVANONE [13].—Obtained from transformation of isoflavanone using *Ab. blackesleeana* NRRL 1306 in 7% yield: mp (hexane/Et₂O) 140°; ir (KBr) 3442, 3344, 1677, 1602, 1514, 1477, 1463, 1444, 1328, 1288, 1236, 1212, 1202, 755, 622 cm⁻¹; ms (direct probe) m/z [M]⁺ 240, 121, 120, 107, 91, 77, 65, 51, 39; ¹H nmr (CDCl₃) δ 7.953 (1H, d, H-5, J = 9.67 Hz), 7.504 (1H, t, H-7), 7.095 (2H, d, H-2', H-6'), 6.748 (2H, d, H-3', H-5', J = 8.5 Hz), 7.03 (2H, m, H-8, H-6), 5.523 (4'-OH), 4.6 (2H, m, H-2), 3.3 (1H, t, H-3); ¹³C nmr (CDCl₃) 192.642 (C-4), 161.564 (C-9), 155.2 (C-4'), 136.025 (C-7), 129.777 (C-2', C-6'), 126.943 (C-1'), 121.547 (C-6), 120.939 (C-10), 117.816 (C-8), 115.793 (C-3', C-5'), 71.553 (C-2), 51.524 (C-3), 127.782 (C-5).

6,4'-DIHYDROXYISOFLAVANONE [17].—Produced from transformation of isoflavanone by *Ab. blackesleeana* NRRL 1306 in 2% yield: mp (hexane/Et₂O) 204–206°; ir 3369, 1678, 1518, 1497, 1465, 1208, 1162, 750 cm⁻¹; ms m/z [M]⁺ 256, 150, 137, 136, 120, 107, 92, 80, 77, 65, 52, 39, 27; ¹H nmr (CDCl₃ + drops of CD₃OD) δ 7.262 (1H, d, H-5, J = 3.3 Hz), 7.077 (2H, d, H-2', H-6', J = 8.7 Hz), 7.025 (1H, not well resolved from H-2', H-6' signal), 6.804 (2H, d, H-3', H-5', J = 8.1 Hz), 6.894 (1H, d, H-8, J = 8.1 Hz), 4.5 (2H, d, H-2), 3.8 (1H, t, H-3); ¹³C nmr (Me₂CO-d_6) δ 192.722 (C-4), 157.587 (C-4'), 156.09 (C-9), 152.466 (C-6), 130.532 (C-2', C-6'), 127.612 (C-5), 124.959 (C-7), 122.2 (C-1'), 119.562 (C-3', C-5'), 116.145 (C-8), 111.64 (C-10), 72.392 (C-2).

ISOFLAVONE [15].—Obtained from transformation of isoflavanone by *As. niger* NRRL 599 in 12% yield: mp (hexane) 130–131°; ir (KBr) 1639.5, 1617, 1467, 1381, 1357, 1288, 761 cm⁻¹; uv λ max: 306, 243, 210 nm; hplc, tlc, ir, uv identical to those of a synthetic sample of isoflavone; ¹H nmr (CDCl₃) δ 8.382 (1H, d, H-5, J = 7.97 Hz), 8.032 (1H, s, H-2), 7.693 (1H, t, H-7), 7.576 (2H, d), 7.45 (5H, unresolved multiplet).

3',4'-DIHYDROXYISOFLAVONE [14].—Produced from transformation of isoflavanone by *As. niger* X172 in 5% yield: mp (hexane/EtOAc) 190–191°, ir (KBr) 1626, 1618, 1588, 1487, 1304, 753 cm^{-1}; ms (direct probe) m/z [M]$^+$ 254, 225, 197, 181, 134, 121, 118, 104, 92, 88, 78, 65, 62, 50, 39; ^{1}H nmr (Me$_2$CO-d_6) 8.256 (1H, s, H-2), 8.203 (1H, d, H-5, J = 9.65 Hz), 7.787 (1H, t, H-7), 7.585 (1H, d, H-8, J = 8.5 Hz), 7.504 (1H, t, H-6), 7.195 (1H, s, H-2'), 6.974 (1H, d, H-6', J = 8.1 Hz), 6.869 (1H, d, H-5', J = 8.15 Hz).

RESULTS AND DISCUSSION

Flavanone and isoflavanone were initially screened for biotransformation using a group of microorganisms comprising 20 genera (80 species). Six of these organisms were found to be efficient in biotransformations of the two substrates and were used in large-scale preparation of the metabolites.

Preparative incubation of **1** with *S. fulvissimus* gave 4'-hydroxyflavanone [**3**], 3',4'-dihydroxyflavanone [**4**], and 2',4-dihydroxydihydrochalcone {**6**} (Figure 2). A band at 3156 cm^{-1} in the ir spectrum and a display of a molecular ion at m/z 240 in the ms indicated that compound **3** is a monohydroxylated flavanone metabolite. The two retro-Diels-Alder fragments at 120 and 121 indicated hydroxylation in ring B. In ^{1}H nmr, the upfield region of the spectrum showed signals at 5.51, 3.26, and 2.73 ppm, which
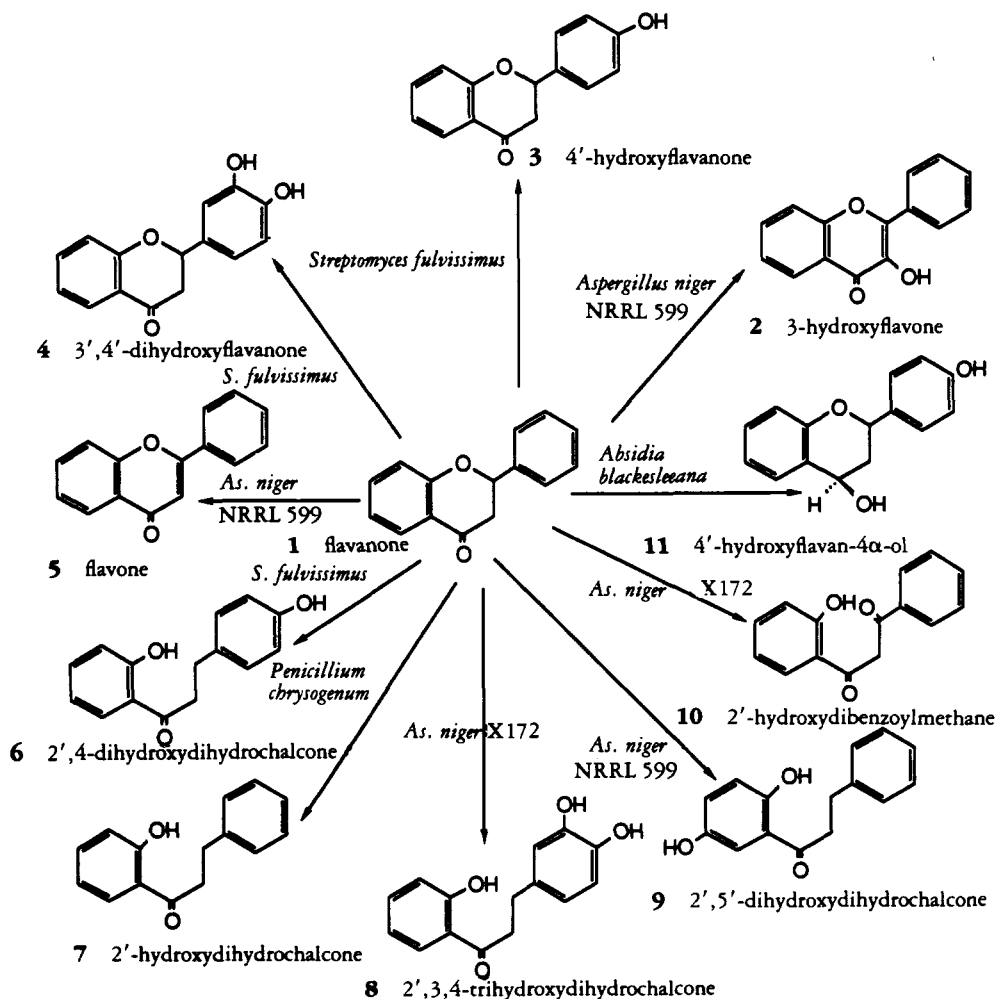

FIGURE 2. Products of microbial transformation of flavanone.

is typical of flavanone-type structures. In the aromatic region, the spectrum of the compound was different from that of flavanone, showing an A_2B_2 pattern of ring B protons. The mass spectrum, ^1H nmr, and ir, as well as melting point, were identical to those reported for 4'-hydroxyflavanone (10). Furthermore, ^{13}C nmr of the metabolite (Table 1) was different in the C-4' signal which was shifted downfield while the C-3',C-5' signal was shifted upfield. The symmetry observed in the C-3',C-5' signal on the one hand and the C-2',C-6' signal on the other confirmed hydroxylation at the C-4' position.

The instability of compound 4 on tlc and in solution suggested a catechol structure. The ir spectrum showed bands at 3492 and 3247 cm^{-1} for hydroxy group(s). A molecular ion at 256 and two retro-Diels-Alder fragments at 136 and 121 indicated ring-B hydroxylation. ^1H nmr also showed a typical flavanone system in the upfield region of the spectrum. Ring-B protons displayed a singlet at 6.91 ppm and two proton multiplets at 6.76 ppm. Melting point, mass spectral data, and ^1H-nmr data were identical to those reported for this compound (12). Furthermore, comparison of the ^{13}C-nmr spectrum of the metabolite to that of flavanone showed differences only in B-ring carbon signals, consistent with a catechol structure at C-3',C-4' (Table 1).

^1H nmr of compound 6 showed two triplets in the upfield region of the spectrum, indicating the presence of two methylenes which are presumably formed by cleavage of ring C of flavanone. The sharp singlet at 12.33 ppm indicated a phenolic proton, which is hydrogen-bonded and was assigned to 2'-OH hydroxyl (17). Symmetry observed (A_2B_2 system) can only be obtained by C-4 hydroxylation. ^{13}C nmr also confirmed the presence of two methylenes (Table 2). The downfield shift of C-4 and the upfield shift of the C-3,C-5 signal compared to flavanone and 2'-hydroxydihydrochalcone indicated hydroxylation at C-4. The increase by 18 mass units of the molecular ion is in support of the dihydrochalcone type structure suggested by the ^1H- and ^{13}C-nmr spectra. The other fragments in ms at m/z 121 and 108 are consistent with a dihydrochalcone hydroxylated at C-4 and C-2' (18). Mp, ms, and ^1H-nmr data were identical to those reported for 2',4-dihydroxydihydrochalcone (10).

Large-scale incubations of flavanone with *P. chrysogenum* 10002K afforded 2'-hydroxydihydrochalcone [7] as an oily compound in 30% yield (Figure 2). The molecular ion at m/z 226 indicated addition of two hydrogens to the substrate. The two retro-Diels-Alder fragments at 121 and 105 suggest that no hydroxy groups were introduced into the substrate. However, the presence of intramolecularly hydrogen-bonded hydroxy proton was supported both by ir band at 3050 cm^{-1} and ^1H-nmr signal at 12.88 ppm. This signal was subsequently assigned to 2'-OH formed by cleavage of ring C of flavanone. The dihydrochalcone nature was indicated by the two methylene triplets at 3.31 and 3.1 ppm. While uv, ir, ms, and ^1H nmr were identical to those reported for this compound in the literature (11), ^{13}C nmr also showed methylene carbons at 29.9 and 39.9 ppm. The C-β of the metabolite 7 corresponding to C-2 of the flavanone has been shielded from 79.49 to 29.9 ppm, which is consistent with the lack of the deshielding influence of the ether function.

Preparative incubations of flavanone [1] with *As. niger* NRRL 599 gave 3-hydroxyflavone [2], 2',5'-dihydroxydihydrochalcone [9], and traces of flavone [5] (Figure 2). A spot of compound 2 on tlc gave intense blue fluorescence with $AlCl_3$ solution. This finding and uv maxima at 343, 305, and 238 are similar to those of 3-hydroxyflavone obtained by transformation of flavanone by *Gibberella fujikuroi* (14). Ir bands at 3213 and 1623 cm^{-1} indicated hydroxylation and dehydrogenation, respectively. The molecular ion at m/z 238 in ms also indicated that the introduction of hydroxy group into the substrate was associated with dehydrogenation. Other fragments in ms at 181 $[M - 1 - CO - CO]^+$ and 118 $[C_6H_5 - CHO]^+$ were also consistent with a flavonol-type structure (19). In ^1H nmr, the disappearance of flavanone signals at δ 5.46 and

3.00 suggested the desaturation of C-2–C-3 bond. However, no C-3 singlet character-
istic of flavones could be observed. Thus, ^{1}H nmr also suggests dehydrogenation and
hydroxylation of C-3 of the substrate. Further confirmation of the flavonol nature of the
metabolite came from ^{13}C nmr, which showed deshielding of C-2 (from 79.5 to 144)
and C-3 (44.57 to 138). Dehydrogenation was also suggested by the shift of the car-
bonyl signal from 191.53 to 177.92 ppm.

The display of a molecular ion at m/z 242 and ion fragments at 105 and 137 by com-
pound **9** suggested A-ring dihydroxylated dihydrochalcone. Further support of dihy-
drochalcone nature of this metabolite came from the two methylene signals at 3.3 and
3.1 ppm observed in ^{1}H nmr. Comparing ^{1}H nmr of **9** to that of 2'-hydroxydihy-
drochalcone [**7**], the AB doublet of C-3',C-4' and shielding of the C-4' proton from
7.45 to 7.00 ppm are consistent with C-5' hydroxylation. Table 2 also shows differ-
ences in the resonance peaks of C-4', C-5', and C-6' signals for **9** compared to 2'-hy-
droxydihydrochalcone [**7**].

2'-Hydroxydibenzoylmethane [**10**] and 2',3,4-trihydroxydihydrochalcone [**8**]
were obtained from preparative incubations of **1** with *As. niger* X172 (Figure 2). A
molecular ion of 240 displayed by compound **10** indicated hydroxylation of the sub-
strate. The generation of a phenolic hydroxy function was surmised from the [1]H-nmr
signal at 12.11 ppm as well as from the ir band at 3460 cm^{-1}. Mass spectrum also
showed fragment ions at m/z 105 and 121 for benzoyl and hydroxybenzoyl groups as
well as fragment ion at 163 resulting from loss of phenyl group from the molecular ion.
Other fragment ions at m/z 69 due to O=C-CH-C=O and 77 (C$_6$H$_5$) were also reported
for 2'-hydroxydibenzoylmethane (11). The downfield position of the C-2 methylene
proton singlet in the ^{1}H nmr is in close agreement with the deshielding influence of two
adjacent carbonyls. Moreover, the presence of a carbonyl in the metabolite in a position
analogous to C-2 of flavanone was deduced from the downfield shift of the H-2,H-6 sig-
nal from 7.4 to 7.95 ppm. The data, including mp, uv, ir, ms, and ^{1}H nmr, were iden-
tical to those reported for 2'-hydroxydibenzoylmethane in the literature (11). Further-
more, ^{13}C nmr showed signals for two carbonyls at 195.7 and 177.6 ppm. The latter
signal was ascribed to the analogous position occupied by C-2 of flavanone. The pres-
ence of such a carbonyl led to a downfield shift of the signal due to the methylene group
of the metabolite from 44.6 to 92.4 ppm. It has been reported (11) that the formation
of this metabolite takes place through isomerization of the initially formed 2-hydroxy-
flavanone.

The dihydrochalcone nature of compound **8** was suggested by the methylene sig-
nals in both ^{1}H nmr and ^{13}C nmr. A molecular ion at m/z 258 as well as the two retro-
Diels-Alder fragments at 121 and 137 indicated the introduction of two hydroxy
groups in ring B. The catechol nature of this metabolite was first suspected from darkening
of a spot on tlc when left exposed to light. When the ^{1}H nmr of this metabolite was
compared to that of 2'-hydroxydihydrochalcone, differences in H-2, H-5, and H-6 sig-
nals became clear. C-3 hydroxylation has led to a singlet signal for H-2 which was also
shifted upfield. The two AB doublets of H-5 and H-6 at 6.776 and 6.644 ppm is also
consistent with C-4 hydroxylation. Comparison of the ^{13}C nmr of compound **8** to that
of 2'-hydroxydihydrochalcone [**7**] indicated downfield shift in the C-3 and C-4 signals
(Table 2). Taken together, the structure that was assigned to compound **8** is 2',3,4-tri-
hydroxydihydrochalcone.

The formation of dihydrochalcones by microbial systems might have taken place by
isomerization of flavanone to the corresponding chalcones followed by reduction to di-
hydrochalcones. Although several chalcone metabolites could be detected on tlc, we
were unable to isolate any in sufficient amounts for full structural elucidation. How-
ever, other investigators were able to isolate chalcones such as 2'-hydroxychalcone as

metabolites of flavanone by *G. fujikuroi* (9). In plant systems, chalcone-flavanone isomerase is an enzyme capable of converting flavanones to chalcones and vice versa (20).

In addition to 4′-hydroxyflavanone and 2′,4-dihydroxydihydrochalcone, large scale fermentation of flavanone by *Ab. blackesleeana* NRRL 1306 gave rise to an optically active metabolite **11**, [α]D −40.8 (Figure 2). While ir absorption showed the disappearance of the carbonyl absorption (band at 1690 cm^{-1} in flavanone), the two bands at 3437 and 3180 cm^{-1} indicated reduction of the carbonyl and the introduction of hydroxyl groups. Differences in the chemical shifts of C-ring protons became clear when the spectra of compound **11** and flavanone were compared (Table 3). A quartet signal at 5.2 ppm ($J = 15$ Hz) assigned to H-2 and a triplet signal 4.76 ppm ($J = 5$ Hz) assigned to H-4 indicated that H-2 and H-4 are trans and that H-4 occupies a quasiequatorial position (21). In the enantiomer having C-2, C-4 cis, the H-4 signal is a quartet with $J = 16$ Hz (21). The specific rotation of −40.8 is consistent with a C-2, C-4 trans flavanol structure. All together, these data suggest that the reduction of the carbonyl proceeded stereospecifically. The A_2B_2 pattern of ring-B protons and the upfield shift of the H-3′,H-5′ signal (Table 3) indicated hydroxylation at C-4′. A molecular ion in cims at m/z 242 indicated that the reduction of the carbonyl is associated with hydroxylation. Other fragment ions at m/z 120, 121 (2 RDA), 224, 223, 131 in eims were all consistent with the flavan-4-ol structure (22). The spectral data showed the disappearance of the carbonyl signal at 191 ppm and appearance of a signal at 63.8 ppm assigned to C-4.

Throughout this investigation as well as other studies the carbonyl reduction of flavanone was the only reaction that proceeded with enantioselectivity as shown from measurement of optical rotation of commercial flavanone, flavanone recovered from different fermentations, and the metabolites that may possess optical activity (Table 4). The corresponding reduction of the carbonyl in other substrates such as flavone, isoflavone, and isoflavanone could not be demonstrated. This reduction reaction might have been achieved by a highly specific alcohol dehydrogenase (23), a hydroxysteroid dehydrogenase, or a flavonoid oxidoreductase.

Large-scale incubations of isoflavanone with *As. niger* X172 led to the formation of 2-hydroxyisoflavanone [**16**] and 3′,4′-dihydroxyisoflavone [**14**] (Figure 3). Ir band at 3402 cm^{-1} and molecular ion at m/z 240 indicated that compound **16** is a hydroxylated metabolite of isoflavanone. The two retro-Diels-Alder fragments at m/z 121 and 120 indicated hydroxylation of C-2, C-3, or ring B. However, the low intensity of the molecular ion suggests hydroxylation of an aliphatic carbon (24). ^{1}H-nmr data revealed

TABLE 4. Specific Rotation of Recovered Flavanone and its Microbial Products.

| Compound	Microorganism	Specific rotation
Commercial flavanone	—	—
Flavanone [1][a]	*Aspergillus niger* NRRL 599	+8
Flavanone [1][a]	*As. niger* X172	—
Flavanone [1][a]	*Penicillium purpu* U-1 193	−0.6
Flavanone [1][a]	*Absidia blackesleeana* NRRL 1306	+7.2
4′-Hydroxyflavanone [3]	*Streptomyces fulvissimus* NRRL B1453	+1.6
3′,4′-Dihydroxyflavanone [4]	*S. fulvissimus* NRRL B1453	+1.6
3′,4′-Dihydroxyflavanone [4]	*As. niger* X172	+16.4
4′-Hydroxyflavan-4α-ol [11]	*P. purpu* U-1 193	−25
4′-Hydroxyflavan-4α-ol [11]	*Ab. blackesleeana* NRRL 1306	−40.8

[a]Recovered.
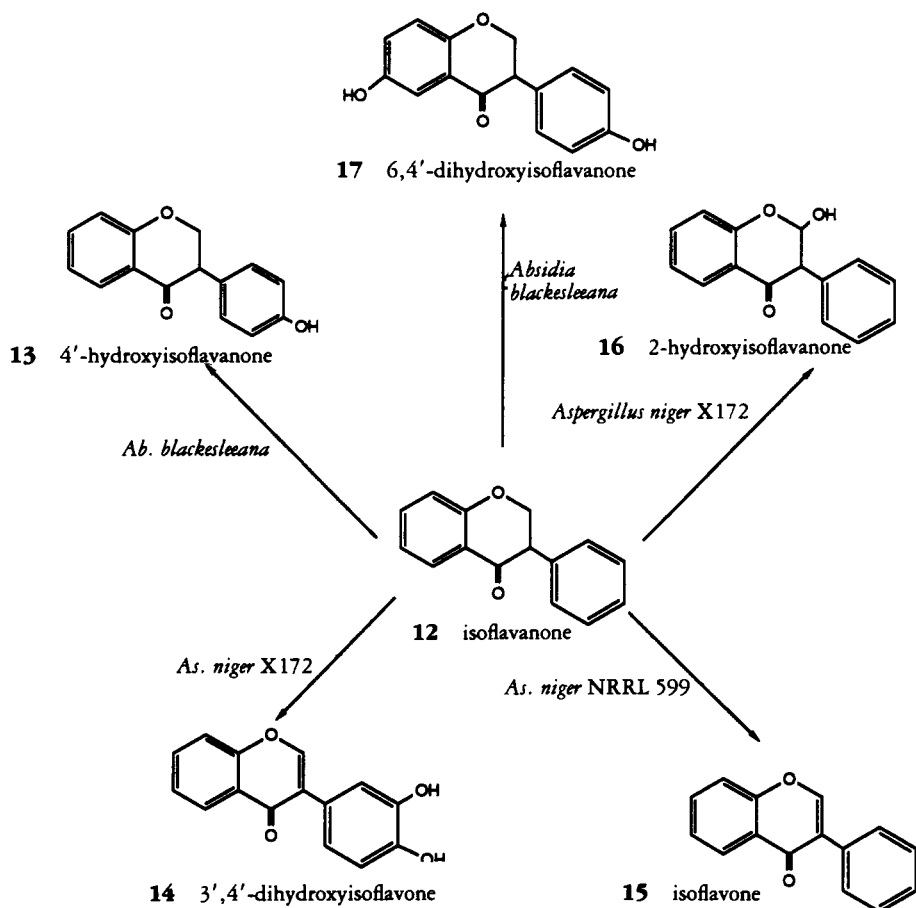
FIGURE 3. Products of microbial transformation of isoflavanone.

that the H-2 signal is integrated for only one proton and that the H-3 signal is changed from a triplet to a doublet, which is consistent with C-2 hydroxylation. The aromatic protons displayed a similar pattern to that of the substrate. Furthermore, the hydroxy proton signal at 4.165 ppm confirmed the aliphatic nature of the hydroxy group. In the ^{13}C nmr, the C-2 signal was shifted downfield from 71.5 to 127.6 ppm. The C-3 signal is also shifted downfield by about 20 ppm. These results are consistent with the fact that hydroxylation results in deshielding of α and β carbons in aliphatic systems (25).

Ab. blackesleeana NRRL 1306 metabolized isoflavanone [12] to 4'-hydroxyiso-flavanone [13] and 6,4'-dihydroxyisoflavanone [17] (Figure 3). The identification of compound 13 as 4'-hydroxyisoflavanone was based on the same observations outlined in assigning the structure of 4'-hydroxyflavanone [3], including ms, ir, and ^{1}H nmr. Furthermore, ring-B hydroxylation was confirmed by ^{13}C nmr. The downfield shift of C-4' signal from 128.6 to 155.2 ppm, the upfield shift of C-3', C-5' signal from 129.1 to 115.8 ppm, and the upfield shift of C-1' signal from 135 to 126.9 ppm are consistent with C-4' hydroxylation.

Compound 17 was obtained as a minor metabolite (2% yield). Both the ir band at 3370 cm^{-1} and the molecular ion peak at *m/z* 256 can be explained by dihydroxylation of the substrate. The two retro-Diels-Alder fragments at *m/z* 136 and 120 indicate hydroxylation of ring A and ring B. Comparison of ^{1}H nmr of 17 to that of isoflavanone showed an upfield shift of H-5, H-7 signals consistent with hydroxylation of C-6. The

TABLE 5.   Percent Yield Microbial Transformation Products Formed from Flavanone.[a]

Microorganism	Compound		
	3	4	6
Rhizopus. sp. NRRL 2234	6.7	2.8	—
Aspergillus ochraceous NRRL 405	41.3	0.5	—
Gymnascella citrina NRRL 6050	12.1	2.2	29.4
Aspergillus alliaceous NRRL 315	27.7	1.6	20.7
Gongronella butleri NRRL 1307	25.4	6.0	43.6
Cunninghamella blackesleeana ATCC 86880 A . . .	1.6	0.5	7.4
Helicostylus periformi QM 6954	18.6	8.1	—
Aspergillus niger X172	2.7	14.6	—
Manascus purpureus NRRL 2879	28.2	2.2	34.4
Linderina pinnispora NRRL 2237	25.4	4.0	5.2
Penicillium purpu U-1 193	1.0	1.0	—
Curvularia lunata NRRL 2178	—	0.5	7.0
Absidia blackesleeana NRRL 1306	60.0	2.2	11.6
Streptomyces fulvissimus NRRL B1453	10.0	10.0	1.0

[a]Quantification was obtained by hplc as described in the Experimental section.

C-5 proton showed a doublet with $J = 3.3$ Hz indicating meta coupling with C-7 (confirmed by nOe experiments). The fact that the H-3′, H-5′ signal is shielded and the observed symmetry of ring-B protons indicate hydroxylation at C-4′. ^{13}C-nmr also supported hydroxylation at C-4′, as the appropriate shifts in C-4′, C-5′, C-3′, and C-1′ were observed. On the other hand, a downfield shift of C-6 signal and upfield shift of C-5 and C-7 confirms hydroxylation at C-6.

With the exception of trace amounts (15 mg) of 6,4′-dihydroxyisoflavanone, all hydroxylation products of flavonoid-type nucleus obtained in this study and in a previous study (8) were ring-B and ring-C hydroxylation products. Based on the well-illustrated importance of the polar groups such as carbonyls and alcohols as sites for binding to the microbial hydroxylase (26–28), we propose that the oxygen functions (ether and carbonyl) of the chromanone ring system of the flavonoid nucleus bind to hydrophilic sites on the enzyme surface and expose ring B and the 2,3 positions of ring C to the hydroxylating site of the enzyme (Figure 4). While ring-B hydroxylations at 4′ and 3′,4′ positions were common to all systems, hydroxylations at C-2 and C-3 were observed only in substrates having a saturated C-2–C-3 bond (flavanone and isoflavanone).

The broad substrate specificity of ring B hydroxylase, which encompasses both flavones and isoflavones, can be explained by flipping the entire chromone ring system in the binding site of the enzyme, thus making ring B of isoflavone in close proximity to the hydroxyating site of the enzyme. The same model, when used to explain the lack of enantioselectivity in the biotransformation of flavanone or isoflavanone, showed that the two stereoisomers of flavanone are exposed differently to the hydroxylating site of the enzyme. However, the actual lack of enantioselectivity observed in these studies suggests that a conformational change takes place in the nonplanar ring C of flavanone such that the 4′ position of the two stereoisomers is equally accessible to the enzyme hydroxylating site. This observation is further supported by examination of molecular models which show that the 4′-positions of the *R* and *S* enantiomers of flavanone are almost superimposable.

Dehydrogenation of isoflavanone to isoflavone [15] and flavanone to flavone [5] by *As. niger* NRRL 599 was deduced from mp, ms, ir, and uv as well as ^{1}H nmr of 15 and 5, respectively, which were identical to a synthetic sample of isoflavone (14) and a stan-
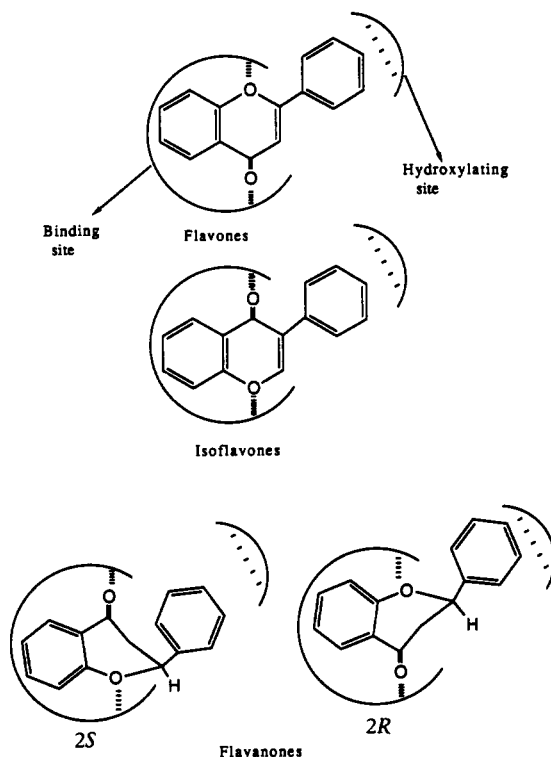
FIGURE 4.   Proposed model for the interaction of flavonoid nucleus with hydroxylase.

dard sample of flavone. It is interesting to report that 3',4'-dihydroxyisoflavanone [4] could be obtained only in trace amounts sufficient for ms from transformation of isoflavanone by *As. niger* X172, and the majority of the catechol metabolite was obtained in the dehydrogenated form. The properties of this metabolite were identical to those of 3',4'-dihydroxyisoflavone [14] that we reported earlier (14).

The fact that dehydrogenation of flavanone, isoflavanone, and chromanone (unpublished results) was achieved by the same organism suggests broad substrate specificity for this type of reaction. The formation of 3-hydroxyflavone as the main metabolite in the course of dehydrogenation of flavanone and the observation that 2-hydroxyisoflavanone was formed in the dehydrogenation of isoflavanone to isoflavone are reminiscent to dehydrogenation of flavonoids by plant systems (29–31). Plant dehydrogenases were shown to convert flavanones to flavones and flavonols by an enzyme system that utilizes 2-oxoglutarate, Fe^{+2}, and NADPH and was classified as a 2-oxoglutarate requiring dioxygenase as both 2-hydroxyflavanones and 2,3-dihydroxyflavanones were shown to be intermediates in this conversion (30). On the other hand, dehydrogenation of isoflavanones was found to proceed via a mono-oxygenase enzyme system (29). However, one cannot rule out the possible involvement of a flavoprotein similar to 1,2-dehydrogenase of *Syptomyxa affinis* (32). Knowledge of the exact mechanism of dehydrogenation must await additional studies on the cofactor requirement for this enzyme system.

ACKNOWLEDGMENTS

LITERATURE CITED

1. J. Kuhnau, *World Rev. Nutr. Diet.*, **24**, 117 (1976).
2. M.-T. Huang, E.F. Johnson, U. Muller-Eberhard, D.R. Koop, M.J. Coon, and A.H. Conney, *J. Biol. Chem.*, **256**, 10897 (1981).
3. H. Nishino, M. Nagao, H. Fujiki, and T. Sugimura, *Cancer Lett.*, **21**, 1 (1983).
4. B.N. Ames, *Science*, **221**, 1256 (1983).
5. B.Y. Yang and N.R. Adams, *J. Endocrinol.*, **85**, 291 (1980).
6. J.T. Kellis and L.E. Vickery, *Science*, **225**, 1032 (1984).
7. V.P. Das, K.N. Scott, and J.H. Duncan, *Biochem. J.*, **136**, 903 (1973).
8. J.P. Rosazza and R.V. Smith, *Adv. Appl. Microbiol.*, **25**, 169 (1979).
9. S.R. Udupa, A. Banerji, and M.S. Chadha, *Tetrahedron*, **25**, 5415 (1969).
10. S.R. Udupa, A. Banerji, and M.S. Chadha, *Tetrahedron Lett.*, **37**, 4003 (1969).
11. S.R. Udupa and M.S. Chadha, *Indian J. Biochem. Biophys.*, **9**, 141 (1972).
12. S.R. Udupa and M.S. Chadha, *Indian J. Biochem. Biophys.*, **15**, 828 (1978).
13. S.R. Udupa and M.S. Chadha, *Indian J. Biochem. Biophys.*, **18**, 142 (1981).
14. A.S. Ibrahim and Y.J. Abul-Hajj, *Xenobiotica*, in press.
15. A.S. Ibrahim and Y.J. Abul-Hajj, *Appl. Environ. Microbiol.*, **55**, 3140 (1989).
16. R.V. Smith and J.T. Stewart, "Textbook of Biopharmaceutic Analysis," Lea & Febiger, Philadelphia, 1981, p. 107.
17. J.R. Dyer, "Applications Of Absorption Spectroscopy of Organic Compounds," Prentice-Hall, Englewood Cliffs, New Jersey, 1965, p. 90.
18. W.A. Bohm and C.W. Glennie, *Phytochemistry*, **8**, 905 (1969).
19. B.A. Brady, W.I. O'Sullivan, and A.M. Duffield, *Org. Mass Spectrom.*, **6**, 199 (1972).
20. W. Heller, B. Eigen-Buller, S.E. Gardiner, K.H. Knobloch, U.M. Matern, J. Ebel, and K. Hahlbrock, *Plant Physiol.*, **64**, 371 (1979).
21. B.J. Bolger, A. Hirwe, K.G. Marathe, E.M.N. Philbin, M.A. Vickars, and C.P. Lillya, *Tetrahedron*, **22**, 621 (1966).
22. S.E. Drewes, *J. Chem. Soc. C*, 1140 (1968).
23. G.L. Lamiere, in: "Enzymes as Catalysts in Organic Synthesis." Ed. by M.P. Schneider, D. Reidel, Dordrecht, Boston, Lancaster, Tokyo, 1985, pp. 19–34.
24. J.W. Clark-Lewis, *Aust. J. Chem.*, **21**, 3025 (1968).
25. J.B. Stothers, in: "Carbon-13 NMR Spectroscopy." Academic Press, New York, 1972.
26. D.R. Brannon, F.W. Parrish, P.J. Wiley, and L. Long, *J. Org. Chem.*, **32**, 1521 (1966).
27. G.S. Fonken, M.E. Herr, H.C. Murry, and L.M. Reineke, *J. Am. Chem. Soc.*, **89**, 672 (1967).
28. A.M. Browne, W.A. Denny, E.R. Jones, A. Kasal, and G.D. Meakins, *J. Chem. Soc., Perkin Trans. 1*, 1493 (1973).
29. G. Kochs and H. Grisebach, *Eur. J. Biochem.*, **155**, 311 (1986).
30. L. Britsch, W. Heller, and H. Grisebach, *Z. Naturforsch.*, **36C**, 742 (1981).
31. E. Wong and J.M. Wilson, *Phytochemistry*, **11**, 875 (1972).
32. Y.J. Abul-Hajj, *J. Biol. Chem.*, **247**, 686 (1972).
33. K. Bowden, J.M. Heilbron, E.R.H. Jones, and B.C.L. Weedon, *J. Chem. Soc.*, 39 (1946).