Notes

Biotransformation of Polymethoxylated Flavonoids: Access to Their 4′-*O*-Demethylated Metabolites

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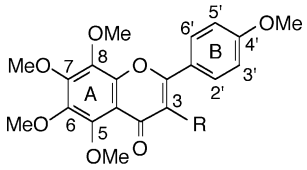
Regioselective *O*-demethylation of the flavones tangeretin (**1**) and 3-hydroxytangeretin (**6**) into their 4′-*O*-demethylated metabolites was performed by using an *Aspergillus niger* strain. This method serves as a straightforward alternative to multistep synthesis or semisynthesis. The microbial approach is complementary to the chemical procedure, which furnishes a 5-*O*-demethylated product. P450 inhibitors prevented the biotransformation of tangeretin (**1**). These results suggest that a P450 oxidation system might be involved in this *O*-demethylation and demonstrate a consequent similarity in both microbial and mammalian metabolism of polymethoxylated flavones.

Tangeretin (5,6,7,8,4′-pentamethoxyflavone) (**1**) is one of the most common citrus polymethoxylated flavones (PMFs), compounds that occur particularly in oranges, tangerines, and sour orange peel.[1] Several biological activities related to cancer have been shown for tangeretin (**1**), such as the ability to enhance gap junctional intercellular communication,[2] to counteract tumor promoter-induced inhibition of intercellular communication,[3] to induce cell-cycle G1 arrest,[4] and to inhibit cancer cell proliferation[5,6] and extracellular-signal-regulated kinase phosphorylation.[7] In addition PMFs, mainly tangeretin (**1**), were found to display hypolipidemic effects in vitro (in hamsters and human hepatoma HepG2 cells) and in vivo (in hamsters with experimental hypercholesterolemia).[1] Several studies has shown tangeretin (**1**) to be extensively metabolized.[1,8−10] In vitro biotransformation of this flavone by rat or human liver microsomes resulted mainly in the formation of 4′-hydroxy-5,6,7,8-tetramethoxyflavone (**2**).[8,9] This same compound was also the major metabolite identified from urine (after hydrolysis of glucuronides) and feces from rats,[10] while biotransformation of **1** by hamsters led to a mixture of unidentified dihydroxytrimethoxyflavones and monohydroxytetramethoxyflavones (as glucuronides and aglycons).[1] A previously formulated hypothesis that metabolites of tangeretin (**1**) might be directly responsible for its observed biological effects[1,10] led us to search for a facile preparative access to the main metabolite **2**.

Preparation of **2** could be planned by total synthesis according to methods described recently for tangeretin (**1**) or by semisynthesis starting from **1** itself. Multistep total synthetic procedures were not considered because of their excessive length related to the tetramethoxylated pattern of ring A (eight steps from 2′,4′,6′-trimethoxyacetophenone, 43% yield; 11 steps from 1,2,3-trimethoxybenzene, 12% yield).[11,12] Semisynthesis of **2** by a chemical regioselective demethylation at C-4′ of tangeretin (**1**) was no more promising, as it seemed very unlikely to occur according to the major studies of Horie et al. on the selective *O*-alkylation and dealkylation of flavonoids [i.e., (a) prior demethylation occurs always at the 5-methoxy group; (b) demethylation at C-4′ has not been reported from flavones with a 4′-monosubstituted B ring].[13] Access to compound **2** from tangeretin was therefore achieved by the following known four-step process[14] (Scheme 1): (a) alkaline degradation of tangeretin into 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone (**3**); (b) condensation of **3** with *p*-benzyloxybenzaldehyde to the chalcone **4**; (c) oxidative cyclization of **4** into a 4′-benzyloxyflavone (**5**); and (d) hydrogenolysis of **5**. The structure of **2** was confirmed by spectroscopic analysis and comparison with literature data.[15,16] The overall yield of this semisynthesic procedure was only 15%, lower than that previously described in the literature (36%).[14] This disappointing yield and the fact that this method implies successively a degradation then a synthesis of the flavone skeleton led us to turn to microbial biotransformation instead. As microorganisms are known in some cases to mimic animal or human metabolism of drugs and xenobiotics,[17] we thought that microbial transformation likely would be an alternative for a straightforward regioselective 4′-demethylation of **1**. Microbial *O*-demethylations of different flavonoid classes (chalcones, flavanones, flavones, isoflavones) have been already reported,[18,19] catalyzed mainly by fungi (*Cunninghamella elegans*, *Aspergillus niger*, *Aspergillus alliaceus*),[20−24] but also by a bacterium, *Eubacterium limosum*.[25]



1 R = H
6 R = OH
7 R = OCH_3

Accordingly, it was decided to study the biotransformation not only of **1** but also of its known 3-hydroxy (**6**) and 3-methoxy (**7**) analogues,[26] obtained by dimethyldioxirane oxidation of **1**[11] then methylation of **6**. To our knowledge, such a bioconversion has not been described for 3-flavonols, an important subclass of flavonoids, and we thought it would be interesting to evaluate the influence of the oxygenated group at C-3 upon demethylation. Miyazawa et al.

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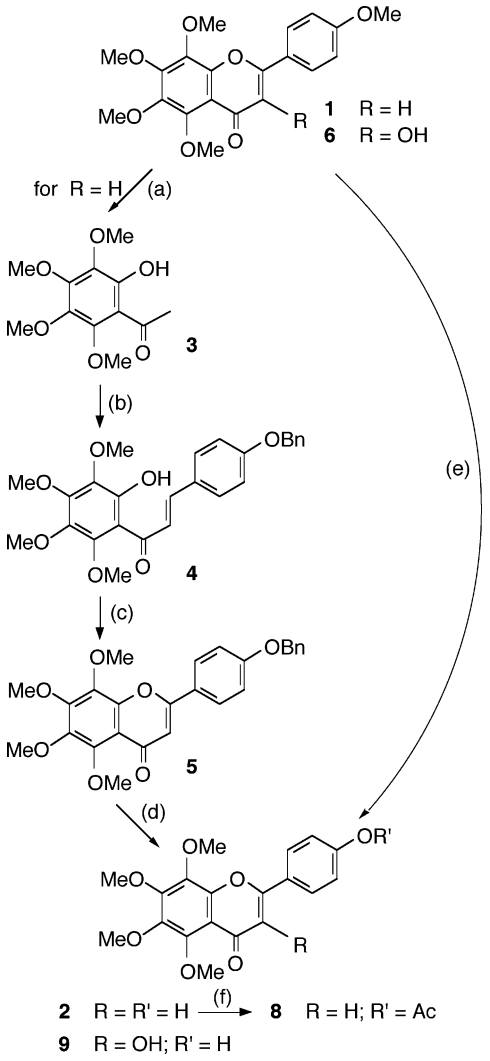
Scheme 1[a]



[a] (a) KOH, EtOH−H_2O, reflux, 5 h, 56%; (b) *p*-benzyloxybenzaldehyde, NaH, THF, reflux, 4 h, 51%; (c) I_2, pyridine, 120 °C, 9 h, 54%; (d) H_2, Pd−C 10%, DMF, rt, 95%; (e) *Aspergillus niger*; (f) Ac_2O, pyridine, rt, 3 h, 53%.

reported the regioselective 4′-*O*-demethylation of flavone[22] by *Aspergillus niger* IFO 4414. Then we investigated the biotransformation of tangeretin (**1**) and its 3-hydroxy (**6**) and 3-methoxy (**7**) derivatives by two *A. niger* strains. Microorganisms were cultured in usual culture medium for 60 h at 30 °C, and substrates were added in solutions of DMF to obtain a final concentration of 0.2 g/L. Incubations were performed under the same conditions, and the biotransformations were monitored by HPLC analysis. The first results obtained on an analytical scale (Table 1) showed that three substrates were metabolized by both strains of *A. niger* to give in each case a main metabolite. The ethyl acetate extracts were analyzed by liquid chromatography−mass spectrometry in the ESI positive mode. The products obtained by incubation of **1**, **6**, and **7** were more polar than the three substrates themselves and showed $[M + H]^+$ peaks at *m*/*z* 359, 375, and 389, respectively, corresponding to mono-demethylated products. However, contrary to tangeretin (**1**) and 3-hydroxytangeretin (**6**), 3-methoxytangeretin (**7**) was slowly and only partially metabolized by both strains of *A. niger*. Accordingly, the presence of a substituent at C-3 seems to have an effect on kinetics (**1** vs **6**) and rate (**1** and **6** vs **7**) of biotransformation. *A. niger* ATCC 9142, which exhibited the highest conversion of tangeretin (**1**), furnished another metabolite with a $[M + H]^+$ peak at *m*/*z* 345, corresponding to a di-demethylated product. This reached a maximal level of 10% after 96 h.

In order to produce and unambiguously identify their major metabolites, substrates **1** and **6** were incubated on preparative scale in a pH 7 phosphate buffer. In order to avoid the formation of the di-demethylated product, **1** was incubated with *A. niger* ATCC 984199. The major product was purified by chromatography and crystallization and was then isolated in 71% yield. Its structure was proved to be identical to compound **2** obtained chemically. The *O*-demethylation at C-4′ was confirmed by the ^1H NMR spectrum of its acetate (**8**),[15] which displayed the H-3′,5′ and H-2′,6′ signals deshielded relative to **2**. In the same manner but using the more active microorganism, **6** was incubated with *A. niger* ATCC 9142, which led to the product (**9**) in 60% yield. The structure of **9**, hitherto unreported, was established by mass spectrometry and comparison of the ^1H and ^{13}C NMR spectra of **6** and **9** (particularly significant was loss of the characteristic shielded signal of the 4′-methoxyl group in the ^{13}C NMR spectrum).[26]

The *O*-demethylation reactions are often the result of the cytochrome P450-dependent hydroxylation of the methyl group to give the corresponding hemiacetal followed by its hydrolysis. In mammals, cytochrome P450 1A2 is known to be involved in *O*-demethylation of tangeretin (**1**).[9] In bacteria and fungi, direct demonstration of the involvement of cytochrome P450 enzymes in biotransformation is rare because it is difficult to obtain active cell-free extracts, especially when catalytic activity needs a multicomponent system. However, indirect evidence could be obtained by the use of cytochrome P450 inhibitors. The effects of four known P450 inhibitors (menadione, miconazole, metapyrone, and SKF 525A) on the *O*-demethylation of tangeretin (**1**) by *A. niger* ATCC9142 cells were examined by adding each inhibitor (0.5 mM) to the incubation medium. SFK 525A and menadione were potent inhibitors that completely prevented demethylation, while metyrapone and miconazole partially abolished this activity (Table 2). Our results agree with previous studies that have described the use of these P450 inhibitors in microbial biotransformations, such as the inhibition by menadione and miconazole of hydroxylation of adamantane by *Streptomyces griseoplanus*[27] and the inhibition by metyrapone of demethylation of thebaine derivatives by *Cunninghamella echinulata*.[28] However, while SKF 525A inhibited the 4′-*O*-demethylation activity of *A. niger* on tangeretin at the final concentration of 0.5 mM, it has been shown[21] that the 3′-*O*-demethylation of chalcone and flavanone by *A. alliaceus* was blocked only with a high concentration (5 mM) of SKF 525A.

In conclusion, the present results demonstrate that the demethylation of tangeretin (**1**) by *A. niger* involves a cytochrome P450 and confirm the regioselectivity at C-4′ already reported for nobiletin[22] and sinesetin.[29] It is noteworthy that *A. alliaceus* showed a C-3′ position regioselectivity in the *O*-demethylation of flavanone[21] and that the 3′- and 4′-*O*-demethylation activities of

Table 1. Biotransformation of Tangeretin and Derivatives by *A. niger* Strains

substrate	microorganism	incubation time (h)	conversion (%)	product retention times (ratio)	
				mono-demethylated	di-demethylated
1	*A. n* ATCC 9142	48	98	10.25 min (85)	5.7 min (15)
	A. n ATCC 984199	96	95	10.25 min (>95)	<2
6	*A. n* ATCC 9142	96	98	14.8 min (>95)	<2
	A. n ATCC 984199	96	80	14.8 min (>95)	<2
7	*A. n* ATCC 9142	120	42	13.3 min (>95)	<2
	A. n ATCC 984199	120	39	13.23 min (>95)	<2

Table 2. Effect of P450 Inhibitors on Demethylation of Tangeretin by *A. niger*

P450 inhibitor	relative activity (%)
none	100
metyrapone	40
miconazole	10
SKF 525A	0
menadione	0

Aspergillus strains were not similarly inhibited by SKF 525A. Therefore, it can be assumed that two different isoforms of cytochrome P450 are involved. Finally, this contribution to the flavonoid field is an additional example showing the usefulness of microbial bioconversion as a straightforward alternative to chemical synthesis.

Experimental Section

General Experimental Procedures. Melting points were determined with a micro-Koffler apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Bruker AC-200 (200 MHz) or Bruker AM-400 (400 MHz) NMR spectrometers; NOESY experiments and the ^{1}H−^{1}H (COSY) and ^{1}H−^{13}C (HMQC and HMBC) spectra were performed with a Bruker AM-400 instrument. ESIMS were registered on a Navigator Aqa thermoquest with a ES source (MeOH, flow rate of 5 μL/min) (70 eV). HPLC analysis was performed on a Gilson HPLC interfaced to a computer using Gilson Unipoint software. The system involved pumps 305 and 306, gradient dynamic mixer 811B, and autoinjector 234, and the detector was a Shimadzu-SDP6A model. A C_{18} column was maintened in an oven (Shimadzu CTO-10A model) at 40 °C and eluted with a gradient solvent system: 100% (A) 70% NH_4OAc 1 mM/30% CH_3CN to 100% (B) 40% NH_4OAc 1 mM/60% CH_3CN over 21 min. Compound detection was carried out at 235 and 360 nm, and sample volumes were 20 μL. LC-MS data were obtained with a Surveyor-LCQ Advantage mass spectrometer, using the same column and solvent at a flow rate of 0.5 μL/min and UV detection. The mass spectrometer was in the ESI positive mode, using a 4 kV capillary tube voltage and an inlet temperature of 275 °C. Flash chromatography was performed with silica gel 60 (9385 Merck), and preparative TLC, with 60 F 254 silica gel (5715 Merck).

Analytical Experiments. Microbial cultures were maintained on agar slants containing (per liter) yeast extract (Difco) 5 g, Bacto-Peptone (Difco) 5 g, malt extract (Difco) 5 g, glucose 20 g, and Bacto-agar (Difco) 20 g and were stored at 4 °C. Liquid culture media containing (per L) glucose 16 g, yeast extract 4 g, malt extract 10 g, and soybean peptones (Organotechnie) 5 g were sterilized without glucose at 120 °C for 20 min. Microorganisms were cultivated in 5 mL of medium in a 25 mL Erlenmeyer flask at 30 °C for 65 h in an orbital shaker (200 rpm). After 65 h, substrates were added in solution in DMF (0.5% v/v), and the rates of their disappearance were followed for 96 h. For tangeretin (**1**), samples (0.5 mL of medium and some pellets) were withdrawn, methanol (0.5 mL) was added, and, after centrifugation, supernatants were analyzed by HPLC (the retention time of **1** was 21 min). For compounds **6** and **7**, samples (0.5 mL of medium and some pellets) were withdrawn and extracted with CH_2Cl_2 (1 mL). After centrifugation, the organic phases were evaporated, methanol (0.5 mL) and aqueous supernatant were used to dissolve the organic extracts, and the solutions were filtered over Celite and analyzed by HPLC (the retention times of **6** and **7** were 24.87 and 22.56 min, respectively). After a 96 h incubation, ethyl acetate (4 mL) was added to the cell suspensions, and the mixtures were stirred and centrifuged. The organic phases were evaporated and the extracts dissolved in methanol for LC-MS analysis [the starting compounds **1**, **6**, and **7** displayed $[M + H]^+$ molecular ions (retention time) at *m*/*z* 373 (20.0 min), 389 (22.7 min), and 403 (21.4 min), respectively].

Preparative Experiments. Demethyltangeretin (2) from Tangeretin (1). *A. niger* ATCC 984199 was cultivated in 250 mL of culture medium as described above. Then biomass was harvested by filtration and resuspended in 0.02 M phosphate buffer at pH 7 (250 mL). Tangeretin (**1**, 50 mg) was added in DMF (1 mL), and incubation was performed at 30 °C for 29 h on an orbital shaker (200 rpm). Culture medium was collected, saturated with NaCl, and extracted three times with CH_2Cl_2 (200 mL). The biomass was washed with MeOH (100 mL), the solvent was concentrated, and the residue was dissolved with CH_2Cl_2. The CH_2Cl_2 extract was dried ($MgSO_4$) and evaporated under reduced pressure. The extract was purified successively by flash chromatography (silica gel, CH_2Cl_2−MeOH, 95:5), crystallization with CH_2Cl_2−cyclohexane, and then preparative thin-layer chromatography (silica gel, cyclohexane−acetone, 2:1) to give pure 4′-hydroxy-5,6,7,8-tetramethoxyflavone (**2**) (mp 197−199 °C; lit.[15] 197−199 °C; 34 mg, 71%): ^{1}H NMR ($CDCl_3$) δ 3.93, 3.94, 4.02, 4.11 (12H, 4s, 4OC*H*$_3$), 6.62 (1H, s, H-3), 7.05 (2H, d, J = 9 Hz, H-3′ and 5′), 7.82 (2H, d, J = 9 Hz, H-2′ and 6′). Compound **2** (15 mg, 0.04 mmol) in the mixture pyridine−Ac_2O, 6:4 (1 mL) was left at room temperature for 3 h. A standard workup of the reaction mixture, then crystallization with MeOH, gave a pure acetate (**8**) (mp 153−154 °C; lit.[15] 154 °C) in 53% yield: ^{1}H NMR ($CDCl_3$) δ 2.36 (3H, s, COC*H*$_3$), 3.96 (6H, s, 2OC*H*$_3$), 4.01, 4.10 (6H, 2s, 2OC*H*$_3$), 6.65 (1H, s, H-3), 7.27 (2H, d, J = 9 Hz, H-3′ and 5′), 7.96 (2H, d, J = 9 Hz, H-2′ and 6′).

3,4′-Dihydroxy-5,6,7,8-tetramethoxyflavone (9) from 3-Hydroxy-5,6,7,8,4′-pentamethoxyflavone (6). *A. niger* ATCC 9142 was cultivated in 1 L of culture medium, and the incubation was performed as described above except that the biomass was suspended in 0.5 L of buffer. Compound **6** (138 mg) was added in solution in DMF (3 mL). The product was extracted as described above except that the biomass was washed with CH_2Cl_2. Purification of the dry residue by flash chromatography (silica gel, CH_2Cl_2−MeOH, 96:4), then crystallization from MeOH, gave pure compound **9** (80 mg, 60%). Compound **6**: ^{1}H NMR ($CDCl_3$) δ 3.89 (OC*H*$_3$-4′), 3.96 (OC*H*$_3$-6), 3.99 (OC*H*$_3$-5), 4.03 (OC*H*$_3$-8), 4.12 (OC*H*$_3$-7), 7.06 (2H, d, J = 9 Hz, H-3′ and 5′), 8.25 (2H, d, J = 9 Hz, H-2′ and 6′); ^{13}C NMR δ 55.0 (OMe-4′), 61.5−62.0 (OCH_3-5,6,7,8), 113.8 (C-3′ and 5′), 128.8 (C-2′ and 6′), 137.1 (OCH_3-8), 142.8 (OCH_3-6), 147.0 (OCH_3-5), 151.0 (OCH_3-7). Compound **9**: mp 216−218 °C; ^{1}H NMR ($CDCl_3$) δ 3.95 (3H, s, OC*H*$_3$-6), 3.99 (3H, s, OC*H*$_3$-5), 4.03 (3H, s, OC*H*$_3$-8), 4.11 (3H, s, OC*H*$_3$-7), 7.01 (2H, d, J = 9 Hz, H-3′ and 5′), 8.20 (2H, d, J = 9 Hz, H-2′ and 6′); ^{13}C NMR ($CDCl_3$) δ 61.5−62.0 (OCH_3-5,6,7,8), 115.9 (C-3′ and 5′), 129.5 (C-2′ and 6′), 137.2 (OCH_3-8), 143.2 (OCH_3-6), 147.1 (OCH_3-5), 151.7 (OCH_3-7); HRESIMS *m*/*z* 397.0908 (calcd for $C_{19}H_{18}O_8Na$, 397.0899).

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