PERGAMON

Phytochemistry 57 (2001) 341–347

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Enzymatic halogenation of flavanones and flavones

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Received 26 October 2000; received in revised form 3 January 2001

Abstract

The whole cells and the chloroperoxidase enzyme of *Caldariomyces fumago* were capable of halogenating the flavanones, naringenin and hesperetin, at C-6 and C-8 in the presence of either Cl^- or Br^-. However, they did not act on other test flavones. The biohalogenated products of naringenin and hesperetin were isolated and found to be identical to those obtained from chemical reactions using molecular halogen and hypohalous acid.

Keywords: *Caldariomyces fumago*; Chloroperoxidase; Enzymatic halogenation; Flavanones; Flavones

1. Introduction

The use of microbial enzymes for the transformation of organic compounds has been employed as a powerful method in metabolism studies and in modern synthetic organic chemistry for decades (Smith and Rosazza, 1975; Faber, 1992). One of the enzymatic reactions that has been widely studied is a chloroperoxidase-catalyzed halogenation (Franssen, 1994). Chloroperoxidase from *Caldariomyces fumago* (CPO; EC 1.11.1.10) is a well-known enzyme, capable of halogenating a great variety of organic compounds such as β-ketoacids (Shaw and Hager, 1959), cyclic β-diketones (Hager et al., 1966), steroids (Levine et al., 1968), alkenes (Yamada et al., 1985), activated aromatic compounds (Wannstedt et al., 1990), and heterocylcic compounds (Franssen et al., 1987). The reaction mechanism of CPO involves the formation of a halogenium ion (X^+) or hypohalous acid (HOX) as an intermediate which can effect electrophilic substitution with electron-rich substrates (Yamada et al., 1985; Libby et al., 1992).

The enzymatic halogenation of several flavanones and flavones having electron-donating groups was investigated in this study. Flavonoids are very common as secondary metabolites found in nature with a great variety of structural forms and biological activities (Harborne, 1988). The vast literature related to flavonoid biotransformations is primarily concerned with oxidation, methylation and glucosidation (Ibrahim and Abul-Hajj, 1990a,b; Ibrahim et al., 1997). One study was found on the microbial chlorination of the isoflavone genistein by *Streptomyces griseus* and *S. plicatus* (Anyanwutaku et al., 1992). There are no previous reports on the chloroperoxidase-catalyzed halogenation of flavonoid compounds. Therefore, we examined the use of whole cells and CPO of *C. fumago* to brominate and chlorinate various flavanones and flavones. The enzymatic study was also compared with chemical halogenation using molecular halogen and hypohalous acid.

2. Results and discussion

The enzymatic bromination and chlorination of flavanones and flavones using *C. fumago* whole cells and CPO gave the results shown in Table 1. With Br^- and Cl^- present as halides, both *C. fumago* and CPO catalyzed the halogenation of the flavanones, naringenin and hesperetin, but were inactive toward the test flavones. The metabolic products were isolated and identified on the basis of NMR and mass spectral analyses. Mass spectra of halogenated compounds showed typical characteristics of isotope peak, suggesting a number of halogen atoms present in each metabolite.

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0031-9422/01/$ - see front matter
PII: S0031-9422(01)00075-9

Table 1
Reaction products from various flavanones and flavones by chloroperoxidase-catalyzed reaction

Substrate	Halide	Product	Yield (%)	
			C. fumago whole cells	$CPO/H_2O_2/X^-$
Flavone, flavonol,	Br^-	–	–	–
7-Hydroxyflavone,	Cl^-	–	–	–
5,7-Dihydroxyflavone,		–	–	–
Quercetin, 6-chloroflavone		–	–	–
Naringenin	Br^-	6,8-Dibromonaringenin (**1**)	2.4	9.3
		8-Bromonaringenin (**2**)	1.8	7.8
	Cl^-	6,8-Dichloronaringenin (**3**)	21	2.8
		6-Chloronaringenin (**4**)	7.6	1.9
Hesperetin	Br^-	6,8-Dibromohesperetin (**5**)	Not tested	8.3
		8-Bromohesperetin (**6**)	Not tested	2.2
	Cl^-	6,8-Dichlorohesperetin (**7**)	3.0	Trace
		6-Chlorohesperetin (**8**)	1.7	1.0

The biobrominations of naringenin and hesperetin gave 6,8-dibromonaringenin (**1**), 8-bromonaringenin (**2**), 6,8-dibromohesperetin (**5**), and 8-bromohesperetin (**6**), respectively. The mass spectral analysis indicated the presence of two bromine atoms in **1** and **5** and one bromine atom in **2** and **6**. The ^1H NMR spectra of brominated compounds were similar to those of naringenin and hesperetin, except for the disappearance of two proton signals in **1** and **5** and one proton signal in **2** and **6** at the H-6 and/or H-8 regions (Tables 2 and 3). These data clearly supported that **1** and **5** were dibrominated products having two bromine atoms at positions 6 and 8. The position of the bromine atoms in **2** and **6** were identified by analysis of the ^{13}C NMR spectra. Relative to naringenin and hesperetin, the ^{13}C NMR spectra of **2** and **6** showed a new quaternary carbon signal around 88 ppm, while one of the two methine carbon peaks in the 96 ppm region disappeared (Table 4). This suggested that the bromine atom might be at either C-6 or C-8. HMBC data of **2** showed that the hydroxyl proton at 12.15 ppm (OH-5) had long range couplings to the methine carbon at 96.30 ppm, and two quaternary carbons at 102.97 and 162.97 ppm corresponding to C-6, C-4a, and C-5, respectively. The methine proton at 6.14 ppm (H-6) showed long range couplings with signals at 88.41 (C-8), 102.97 (C-4a), and 162.97 (C-5 and C-7) ppm. In addition, compounds **2** and **6** gave similar ^{13}C NMR spectral patterns and the carbon signals at C-7 and C-8a of **2** and **6** were shifted upfield by 4.5 ppm compared to those of naringenin and hesperetin. These results indicated that the bromine atom in **2** and **6** must be at C-8.

The enzymatic chlorinations of naringenin and hesperetin also gave four metabolic products, 6,8-dichloronaringenin (**3**), 6-chloronaringenin (**4**), 6,8-dichlorohesperetin (**7**) and 6-chlorohesperetin (**8**). The mass and ^1H NMR spectral analyses of **3** and **7** established the presence of two chlorine atoms at positions 6 and 8. The mass and ^1H NMR spectra of **4** and **8** indicated one chlorine atom at either position 6 or 8 (Tables 2 and 3). The ^{13}C NMR

Table 2
^1H NMR spectral data of naringenin and compounds **1–4** in $(CD_3)_2CO$

Position	Naringenin[a]	**1**[b]	**2**[b]	**3**[b]	**4**[a]
H-2	5.43, *dd* (3.0, 13.0)[c]	5.67, *dd* (3.1, 12.5)	5.58, *dd* (3.4, 12.5)	5.64, *dd* (2.8, 12.8)	5.45, *dd* (3.0, 12.9)
H-3ax	3.16, *dd* (13.1, 17.0)	3.32, *dd* (12.5, 17.1)	3.21, *dd* (12.5, 17.1)	3.31, *dd* (12.7, 17.2)	3.21, *dd* (12.9, 17.2)
H-3eq	2.71, *dd* (3.1, 17.3)	2.95, *dd* (3.1, 17.1)	2.85, *dd* (3.4, 17.1)	2.90, *dd* (3.1, 17.1)	2.74, *dd* (3.0, 17.4)
OH-5	12.17, *s*	12.92, *s*	12.15, *s*	12.81, *s*	12.74, *s*
H-6	5.94, *s*	–	6.14, *s*	–	–
H-8	5.94, *s*	–	–	–	6.13, *s*
H-2′	7.37, *d* (8.4)	7.43, *d* (8.2)	7.39, *d* (8.2)	7.43, *d* (8.9)	7.35, *d* (8.5)
H-3′	6.88, *d* (8.7)	6.92, *d* (8.6)	6.87, *d* (8.6)	6.91, *d* (8.9)	6.85, *d* (8.7)
H-5′	6.88, *d* (8.7)	6.92, *d* (8.6)	6.87, *d* (8.2)	6.91, *d* (8.9)	6.85, *d* (8.7)
H-6′	7.37, *d* (8.4)	7.43, *d* (8.2)	7.39, *d* (8.2)	7.43, *d* (8.9)	7.35, *d* (8.5)

[a] ^1H NMR spectrum taken at 270 MHz.
[b] ^1H NMR spectrum taken at 500 MHz.
[c] Coupling constants (Hz) in parentheses.

Table 3
^{1}H NMR spectal data of hesperetin and compounds **5–8** in $(CD_3)_2CO$

Position	Hesperetin[a]	**5**[b]	**6**[b]	**7**[a]	**8**[b]
H-2	5.43, *dd* (3.1, 12.6)[c]	5.65, *dd* (3.4, 12.2)	5.60, *dd* (3.2, 11.9)	5.63, *dd* (3.3, 12.2)	5.60, *dd* (3.1, 12.2)
H-3ax	3.15, *dd* (12.6, 17.2)	3.29, *dd* (12.1, 17.2)	3.22, *dd* (11.9, 17.1)	3.28, *dd* (12.5, 17.0)	3.24, *dd* (12.2, 17.1)
H-3eq	2.74, *dd* (3.0, 17.0)	2.97, *dd* (3.2, 17.2)	2.90, *dd* (3.4, 17.1)	2.92, *dd* (3.3, 17.1)	2.89, *dd* (3.4, 17.1)
OH-5	12.17, *s*	12.98, *s*	12.17, *s*	12.80, *s*	12.12, *s*
H-6	5.94, *d* (2.0)	–	6.16, *s*	–	–
H-8	5.96, *d* (2.1)	–	–	–	6.15, *s*
H-2′	7.04, *m*	7.09, *m*	7.09, *m*	7.08, *m*	7.09, *m*
OMe-4′	3.85, *s*	3.86, *s*	3.86, *s*	3.85, *s*	3.86, *s*
H-5′	6.97, *m*	7.00, *m*	6.99, *m*	6.97, *m*	7.00, *m*
H-6′	6.97, *m*	7.00, *m*	6.99, *m*	6.97, *m*	7.00, *m*

[a] ^{1}H NMR spectrum taken at 270 MHz.
[b] ^{1}H NMR spectrum taken at 500 MHz.
[c] Coupling constants (Hz) in parentheses.

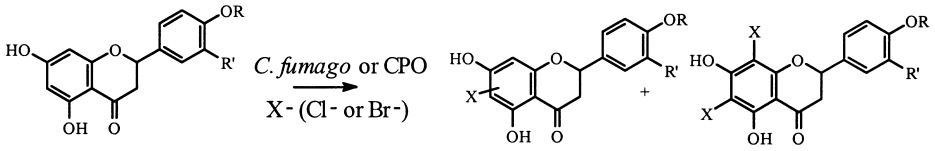
Table 4
^{13}C NMR spectral data of naringenin, hesperetin and compounds **2**, **4, 6** and **8** in $(CD_3)_2CO$

Position	Naringenin[a]	**2**[b]	**4**[a]	Hesperetin[a]	**6**[b]	**8**[b]
C-2	79.86 *d*	79.50 *d*	80.22 *d*	79.79 *d*	79.35 *d*	79.98 *d*
C-3	43.43 *t*	42.01 *t*	43.25 *t*	43.52 *t*	41.97 *t*	43.23 *t*
C-4	197.15 *s*	196.37 *s*	197.70 *s*	197.09 *s*	196.31 *s*	197.34 *s*
C-5	165.23 *s*	162.97 *s*	160.33 *s*	165.29 *s*	163.27 *s*[c]	160.27 *s*
C-6	96.81 *d*	96.30 *d*	101.02 *s*	96.87 *d*	96.46 *d*	101.10 *s*
C-7	167.30 *s*	162.97 *s*	162.20 *s*[c]	167.36 *s*	163.20 *s*[c]	162.03 *s*[c]
C-8	95.83 *d*	88.41 *s*	96.26 *d*	95.91 *d*	88.51 *s*	96.37 *d*
C-4a	103.19 *s*	102.97 *s*	103.52 *s*	103.28 *s*	103.02 *s*	103.25 *s*
C-8a	164.31 *s*	159.51 *s*	162.46 *s*[c]	164.28 *s*	159.39 *s*	163.10 *s*[c]
C-1′	130.73 *s*	129.48 *s*	130.48 *s*	132.84 *s*	131.49 *s*	132.51 *s*
C-2′	128.94 *d*	127.99 *d*	129.09 *d*	114.38 *d*	113.50 *d*	114.37 *d*
C-3′	116.15 *d*	115.35 *d*	116.25 *d*	147.61 *s*	146.59 *s*	147.58 *s*
C-4′	158.62 *s*	157.85 *s*	158.83 *s*	148.69 *s*	147.94 *s*	148.74 *s*
C-5′	116.15 *d*	115.35 *d*	116.25 *d*	112.38 *d*	111.54 *d*	112.30 *d*
C-6′	128.94 *d*	127.99 *d*	129.09 *d*	118.78 *d*	117.81 *d*	118.82 *d*
OMe-4′				56.37 *q*	55.98 *q*	56.31 *q*

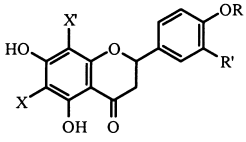
[a] ^{13}C NMR spectrum taken at 67.9 MHz.
[b] ^{13}C NMR spectrum taken at 125 MHz.
[c] Assignments could be reversed.

spectra of **4** and **8** showed a quaternary carbon and a methine carbon signals in the C-6 and C-8 regions at lower field compared to those of their precursors (Table 4). The structural assignment of **4** as 6-chloronaringenin was supported by a COLOC experiment. The hydroxy proton at δ 12.74 (OH-5) had long range couplings to the quaternary signals at 101.02, 103.52, and 160.33 ppm thus corresponding to C-6, C-4a, and C-5, respectively. Since the ^{13}C NMR spectral pattern of **8** was similar to that of **4,** the chlorine atom in **8** was placed at C-6. This was supported by the appearance of carbon signals at C-5 and C-7 of **4** and **8** at higher field by about 5 ppm compared to those of naringenin and hesperetin.

It was noted that, when the CPO enzyme was employed to halogenate naringenin and hesperetin, brominated products were obtained in better yields than chlorinated ones (Table 1). This was explainable since the halogenation of CPO is electrophilic and the reactivity of halogenium ions increases in the following order: Cl^+, Br^+ and I^+ (Yamada et al., 1985). However, when *C. fumago* whole cells were used, yields of chlorinated metabolites were higher. This might be due to the weak growth of *C. fumago* in the medium containing KBr. Overall, the halogenated products were obtained in low yields together with starting material. The halogenation of the substrates may proceed in competition with the halogenation of CPO itself at tyrosine residues until the enzyme is no longer active (Wannstedt et al., 1990). Furthermore, since the enzymatic reactions were carried out in suspension, the uptake of the substrates for biotransformation may be poor.



naringenin; R = R' = H
hesperetin; R = Me, R' = OH



6,8-dibromonaringenin (**1**); R = R' = H, X = X' = Br
8-bromonaringenin (**2**); R = R' = H, X = H, X' = Br
6,8-dichloronaringenin (**3**); R = R' = H, X = X' = Cl
6-chloronaringenin (**4**); R = R' = H, X = Cl, X' = H
6,8-dibromohesperetin (**5**); R = Me, R' = OH, X = X' = Br
8-bromohesperetin (**6**); R = Me, R' = OH, X = H, X' = Br
6,8-dichlorohesperetin (**7**); R = Me, R' = OH, X = X' = Cl
6-chlorohesperetin (**8**); R = Me, R' = OH, X = Cl, X' = H

The flavone-type compounds, flavone, flavonol, 7-hydroxyflavone, 5,7-dihydroxyflavone, quercetin, and 6-chloroflavone, were not suitable as substrates of CPO. The aromatic rings of these compounds possessing a strongly conjugated system were quite stable, and were thus inert to the enzymatic reaction. The relevant flavanones were reactive since the conjugation in the flavanone system was disrupted by removing the 2,3-double bond. The biomimetic reactions of naringenin and hesperetin with chemical halogenating agents were examined. Their reaction products were identical to those obtained from the enzymatic reactions. The halogenations of naringenin and hesperetin are electrophilic and occurred at the C-6 and C-8 positions on the A-rings. With the two hydroxy groups at C-5 and C-7, the A-rings of naringenin and hesperetin are considerably more nucleophilic and hence reactive towards electrophilic halogenating species than their B-rings.

3. Experimental

3.1. General experimental procedures

^{1}H NMR spectra were recorded on an IBM AF-270 and a JNM-A500 spectrometer. ^{13}C NMR spectra were recorded on an IBM AF-270 at 67.9 MHz, a JNM-A500 and a Bruker AM-500 at 125 MHz. $(CD_3)_2CO$ was used as solvent. MS was performed using a VG 70 250S mass spectrometer and HP 5989B MS engine at 70 eV. FTIR spectra were obtained using a Magna-IR^{TM} spectrometer 750, using KBr plates. Optical rotations were measured with a Perkin Elmer 241 photoelectric polarimeter.

Chemicals, all test samples, flavone, flavonol, 7-hydroxyflavone, 5,7-dihydroxyflavone, quercetin, 6-chloroflavone, naringenin, and hesperetin, were obtained from Sigma. Bromine and CPO were also purchased from Sigma. Sodium hypochlorite was obtained from Carlo. Other chemicals used were reagent grade.

3.2. Micro-organism

Caldariomyces fumago ATCC 11925 was obtained from the American Type Culture Collection. Live cultures were maintained by subculturing on suitable slants at room temperature or storage at 4°C in tightly capped culture tubes.

3.3. Enzymatic halogenation of flavanones and flavones by C. fumago whole cells

A two-stage fermentation technique was employed. Stage I and stage II cultures were prepared by the following method. The surface growth from *C. fumago* slants on mycophil agar (BBL) was suspended in 5 ml volumes of normal saline solution. One milliliter of the suspension was transferred into 100 ml of stage I medium in 500 ml erlenmeyer flasks. The stage I cultures were incubated at ambient temperature (20°C) on a rotary shaker at 250 rpm for 48–72 h. Approximately 10 ml volumes of the actively growing stage I cultures were transferred to 100 ml of fresh medium in 500 ml erlenmeyer flasks. All fermentation experiments were carried out in a Czapek-yeast- KCl or Czapek-yeast-KBr medium, consisting of glucose, 30 g; $NaNO_3$, 3.0 g; K_2SO_4, 1.0 g; $MgSO_4$, 0.5 g; KCl or KBr, 0.5 g; $(Fe)_2(SO_4)_3$, 0.01 g; yeast extract, 3.3 g in 1 l H_2O. Media were sterilized by autoclaving at 121°C, 15 lb/in^2 for 15 min.

For initial screening experiments, 10 mg of each sample as a solution in DMF (50 mg/ml) was added and suspended into each flask of 24 h stage II cultures. The culture was further incubated and monitored. Ten milliliters of the cultures were sampled daily and extracted

with 10 ml of $CHCl_3$, twice. The extracts were concentrated to dryness and redissolved in 0.25 ml of 95% EtOH. The solutions were subjected to silica gel TLC and developed with toluene-Me_2CO (10:1) and/or CH_3Cl-MeOH (40:1). TLC plates exposed to UV light and Fast Blue BB spray reagent were analyzed. Control cultures of organism without substrate and controls consisting of substrate in medium without organism were performed along with sample cultures.

Preparative scale fermentations were conducted on 150–190 mg of substrates, using a two-stage shaken culture fermentation as described above. The stage II cultures with substrate were incubated for 24–48 h before being harvested. The whole culture broths were exhaustively extracted twice with an equal volume of $CHCl_3$. The $CHCl_3$ extracts were dried over Na_2SO_4, evaporated and purified.

3.4. Enzymatic halogenation of flavanones and flavones by CPO (Sigma)

The reaction conditions were modified from the method described by Yamada et al. (1985). For initial screening, the enzymatic reaction mixture contained 10 ml of 0.1 M potassium phosphate buffer (pH 2.75), 100 μmol of KBr or KCl, 25–50 units of CPO, and 20 μmol of a substrate. All substrates were insoluble in water, and had to be first dissolved in 70 μl of warm EtOH, then poured and suspended in the reaction mixture. The reaction was initiated by slowly adding 100 μmol of H_2O_2 and continued for 60 min at room temperature with constant stirring. The control test was performed without the enzyme. The reaction was monitored by TLC as above.

Preparative scale halogenation was conducted on 0.8 mmol of substrates. Each substrate was dissolved in 3 ml of warm EtOH, then poured into the reaction mixture. The reaction mixture consisted of 400 ml of 0.1 M potassium phosphate buffer (pH 2.75), 4 mmol of KBr or KCl, and 1000 units of CPO. H_2O_2 (4 mmol) was then slowly added. After 60 min, the reaction mixture was extracted with $CHCl_3$ (200 ml×3). The extracts were concentrated to dryness and followed by a series of chromatographic methods for the isolation of the halogenated products.

3.5. Isolation procedures

3.5.1. Halogenated products 1–4 of naringenin formed by C. fumago whole cells

From the microbial bromination of naringenin (180 mg) with *C. fumago* in Czapek-yeast-KBr, the dried $CHCl_3$ extract was subjected to silica gel PF_{254} chromatography using toluene–Me_2CO (10:1) as eluant. Naringenin (58 mg) was recovered. Flavonoid-containing fractions (18 mg) were subjected to prep. TLC using a silica gel 60 F_{254} plate at 0.25 mm thickness. The chromatogram was developed three times, using toluene–Me_2CO (10:1) for the first development, then, $CHCl_3$–MeOH (40:1) for the last two. The plate was visualized under UV light at 254 nm. Two major bands were observed. The compound from each band was extracted from silica gel using Me_2CO. 6,8-Dibromonaringenin (**1**) (4.3 mg) and 8-bromonaringenin (**2**) (3.2 mg) were isolated.

The microbial chlorination of naringenin (190 mg) was performed with *C. fumago* in Czapek-yeast-KCl. The dried $CHCl_3$ extract was applied to a silica gel PF_{254} column with toluene–Me_2CO (12:1) giving 6,8-dichloronaringenin (**3**) (40 mg) and 6-chloronaringenin (**4**) (14.5 mg).

3.5.2. Halogenated products 1–4 of naringenin formed by CPO (Sigma)

Each preparative scale bromination and chlorination was conducted on 217 mg of naringenin according to the method described above. The dried extract of each reaction mixture was subjected to silica gel 60 GF_{254} column chromatography with toluene–Me_2CO (12:1) as eluant. 6,8-Dibromonaringenin (**1**) (20.2 mg), 8-bromonaringenin (**2**) (16.9 mg), and naringenin (126 mg) were isolated from the dried extract of the enzymatic bromination. 6,8-Dichloronaringenin (**3**) (6 mg), 6-chloronaringenin (**4**) (4.2 mg), and naringenin (135 mg) were isolated from the dried extract of the enzymatic chlorination mixture.

*3.5.2.1. 6,8-Dibromonaringenin (**1**).* An amorphous powder; $[\alpha]_D^{20}$ 0° (MeOH; *c* 0.1); λ_{max}^{MeOH} nm (log ε): 224 (4.1), 331 (4.1); ν_{max} (KBr) cm^{-1}: 3350 (OH), 1625 (>C=O); ^1H NMR cf. Table 2; EIMS *m/z* (rel. int.): 432 $[M+4]^+$ (7), 430 $[M+2]^+$ (18), 428 $[M]^+$ (9), 311 $[(M+2)\text{-}120+H]^+$ (29), 120 [M-308]$^+$ (100).

*3.5.2.2. 8-Bromonaringenin (**2**).* An amorphous powder; $[\alpha]_D^{20}$ 0° (MeOH; *c* 0.1); λ_{max}^{MeOH} nm (log ε): 224 (4.3), 291 (4.0), 329 (4.0); ν_{max} (KBr) cm^{-1}: 3325 (OH), 1635 (>C=O); ^1H NMR cf. Table 2; ^{13}C NMR cf. Table 4; EIMS *m/z* (rel. int.): 352 $[M+2]^+$ (69), 350 $[M]^+$ (71), 233 $[(M+2)\text{-}120+H]^+$ (15), 231 $[M\text{-}120+H]^+$ (11), 120 [M-230]$^+$ (100).

*3.5.2.3. 6,8-Dichloronaringenin (**3**).* An amorphous powder; $[\alpha]_D^{20}$ 0° (MeOH; *c* 0.1); λ_{max}^{MeOH} nm (log ε): 223 (3.7), 253 (3.3), 331 (3.7); ν_{max} (KBr) cm^{-1}: 3350 (OH), 1619 (>C=O); ^1H NMR cf. Table 2; EIMS *m/z* (rel. int.): 344 $[M+4]^+$ (4), 342 $[M+2]^+$ (19), 340 $[M]^+$ (35), 221 $[M\text{-}120+H]^+$ (43), 120 [M-220]$^+$ (100).

*3.5.2.4. 6-Chloronaringenin (**4**).* An amorphous powder; $[\alpha]_D^{20}$ 0° (MeOH; *c* 0.1); λ_{max}^{MeOH} nm (log ε): 223 (3.6), 247 (3.1), 325 (3.5); ν_{max} (KBr) cm^{-1}: 3340 (OH), 1637 (>C=O); ^1H NMR cf. Table 2; ^{13}C NMR cf. Table 4; EIMS *m/z* (rel. int.): 308 $[M+2]^+$ (22), 306 $[M]^+$ (68), 189 $[(M+2)\text{-}120+H]^+$ (26), 187 $[M\text{-}120+H]^+$ (81), 120 [M-186]$^+$ (100).

*3.5.3. Halogenated products **5**–**8** of hesperetin formed by CPO (Sigma)*

Preparative scale bromination and chlorination were performed on 240 mg and 120 mg of hesperetin, respectively, according to the method described above. The dried extract of each reaction mixture was chromatographed over silica gel 60 GF_{254} with toluene–Me_2CO (15:1). 6,8-Dibromohesperetin (**5**) (20 mg), 8-bromohesperetin (**6**) (5.1 mg), and hesperetin (120.8 mg) were isolated from the dried extract of the enzymatic bromination mixture. 6-Chlorohesperetin (**8**) (1.2 mg) and hesperetin (82 mg) were isolated from the dried extract of the enzymatic chlorination mixture. Trace amounts of 6,8-dichlorohesperetin (**7**) were observed on a silica gel TLC plate.

*3.5.4. Chlorinated products **7** and **8** of hesperetin formed by C. fumago whole cells*

One hundred and ninety milligrams of hesperetin were biotransformed with *C. fumago* in Czapek-yeast-KCl. The dried $CHCl_3$ extract was chromatographed over silica gel 60 (230–400 mesh) with toluene–Me_2CO (12:1). Hesperetin (64 mg) was recovered. Prep. TLC of flavonoid-containing fractions (23 mg) was performed on a silica gel 60 F_{254} plate at 0.25 mm thickness using $CHCl_3$:MeOH (40:1). Two metabolic products, 6,8-dichlorohesperetin (**7**) (5.7 mg) and 6-chlorohesperetin (**8**) (3.2 mg), were isolated.

*3.5.4.1. 6,8-Dibromohesperetin (**5**).* An amorphous powder; $[\alpha]_D^{20}$ 0° (MeOH; c 0.1); λ_{max}^{MeOH} nm (log ε): 230 (4.1), 332 (4.0); ν_{max} (KBr) cm^{-1}: 3410 (OH), 1635 (>C=O); ^1H NMR cf. Table 3; EIMS *m/z* (rel. int.): 462 $[M+4]^+$ (28), 460 $[M+2]^+$ (51), 458 $[M]^+$ (27), 150 $[M\text{-}308]^+$ (100).

*3.5.4.2. 8-Bromohesperetin (**6**).* An amorphous powder; $[\alpha]_D^{20}$ 0° (MeOH; c 0.1); λ_{max}^{MeOH} nm (log ε): 230 (4.1), 294 (4.0), 330 (4.0); ν_{max} (KBr) cm^{-1}: 3400 (OH), 1635 (>C=O); ^1H NMR cf. Table 3; ^{13}C NMR cf. Table 4; EIMS *m/z* (rel. int.): 382 $[M+2]^+$ (84), 380 $[M]^+$ (86), 233 $[(M+2)\text{-}150+H]^+$ (13), 150 $[M\text{-}230]^+$ (84).

*3.5.4.3. 6,8-Dichlorohesperetin (**7**).* An amorphous powder; $[\alpha]_D^{20}$ 0° (MeOH; c 0.1); λ_{max}^{MeOH} nm (log ε): 231 (4.1), 287 (3.5), 332 (4.2); ν_{max} (KBr) cm^{-1}: 3400 (OH), 1635 (>C=O); ^1H NMR cf. Table 3; EIMS *m/z* (rel. int.): 374 $[M+4]^+$ (2), 372 $[M+2]^+$ (12), 370 $[M]^+$ (21), 220 $[M\text{-}150]^+$ (7), 150 $[M\text{-}220]^+$ (100).

*3.5.4.4. 6-Chlorohesperetin (**8**).* An amorphous powder; $[\alpha]_D^{20}$ 0° (MeOH; *c* 0.1); λ_{max}^{MeOH} nm (log ε): 231 (4.3), 287 (4.0), 329 (4.2); ν_{max} (KBr) cm^{-1}: 3400 (OH), 1635 (>C=O); ^1H NMR cf. Table 3; ^{13}C NMR cf. Table 4; EIMS *m/z* (rel. int.): 338 $[M+2]^+$ (12), 336 $[M]^+$ (38), 187 $[M\text{-}150+H]^+$ (7), 150 $[M\text{-}186]^+$ (100).

3.6. Chemical reaction with molecular halogen and hypohalous acid

3.6.1. Chemical bromination of naringenin

The biomimetic reaction of naringenin with bromine was examined under conditions similar to the chloroperoxidase reaction. The reaction mixture consisted of 7.8 ml of 1 M phosphate buffer (pH 2.75), 1.5 mmol of H_2O_2, 0.76 mmol of KBr, 20 ml of H_2O, and 10 ml of EtOH–Me_2CO (1:1). Naringenin (100 mg; 0.37 mmol) dissolved in 4.0 ml of EtOH–Me_2CO (1:1) was added to the reaction mixture and thoroughly mixed. Bromine solution (0.76 mmol of Br_2 in 400 μl of EtOH) was then slowly added at room temperature with mild stirring. After 45 min, the reaction mixture was extracted with $CHCl_3$ (25 ml×2). The combined extract was dried over Na_2SO_4 and evaporated to dryness. The residue was subjected to silica gel GF_{254} chromatography with toluene–Me_2CO (12:1) as eluant to give 6,8-dibromonaringenin (**1**) (26 mg) and 8-bromonaringenin (**2**) (17.0 mg).

3.6.2. Chemical bromination of hesperetin

Hesperetin (200 mg; 0.66 mmol) was dissolved in 8.0 ml of EtOH–Me_2CO (1:1), then poured into the reaction mixture. The reaction mixture contained 14 ml of 1 M phosphate buffer (pH 2.75), 2.6 mmol of H_2O_2, 1.3 mmol of KBr, 40 ml of H_2O, and 20 ml of EtOH-Me_2CO (1:1). The reaction was started by slowly adding bromine solution (1.3 mmol of Br_2 in 700 μl of EtOH). After 45 min, the reaction mixture was extracted with $CHCl_3$ (45 ml×2). The combined extract was dried over Na_2SO_4 and evaporated to dryness. The residue was applied to silica gel GF_{254} with toluene–Me_2CO (15:1) as eluant to afford 6,8-dibromohesperetin (**5**) (23.8 mg) and 8-bromonaringenin (**6**) (26.0 mg).

3.6.3. Chemical chlorination of naringenin

The reaction mixture consisted of 14 ml of 1 M phosphate buffer (pH 2.75), 40 ml of H_2O, 20 ml of EtOH–Me_2CO (1:1), and 200 mg of naringenin dissolved in 8.0 ml of EtOH–Me_2CO (1:1). To the reaction mixture was added dropwise 3.75 ml of sodium hypochlorite solution, then, 3.75 ml of HOAc. After stirring the mixture for 1 h at room temperature, the mixture was extracted with $CHCl_3$ (45 ml×2). The combined extract was dried over Na_2SO_4 and evaporated to dryness. The residue was subjected to silica gel GF_{254} chromatography with toluene–Me_2CO (12:1) as eluant. 6-Chloronaringenin (**4**) (6 mg) was isolated. Trace amounts of 6,8-dichloronaringenin (**3**) were observed by silica gel TLC plate.

3.6.4. Chemical chlorination of hesperetin

The biomimetic chlorination of hesperetin (200 mg) was performed under similar conditions as above. The dried residue was applied to silica gel GF_{254} with toluene-

Me_2CO (15:1) as eluant, yielding 6-chlorohesperetin (**8**) (9 mg). Trace amounts of 6,8-dichlorohesperetin (**7**) were observed by silica gel TLC plate.

Acknowledgements

This work was supported by grants from Graduate School Research Alumni, The Ohio State University, and National Science and Technology Development Agency, Thailand. We wish to thank Dr. Raymond W. Doskotch, College of Pharmacy, The Ohio State University, for his helpful suggestions and for the help with HMBC and COLOC experiments. Thanks are also given to Dr. David Chang, the Ohio State University chemical instrumentation, Miss Panit Wedkanjana and Mr. Witoon Ngow, Faculty of Science, Silpakorn University, for providing MS analysis.

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