Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 1749–1755

BIOORGANIC & MEDICINAL CHEMISTRY

Flavonols from *Heterotheca inuloides*: Tyrosinase Inhibitory Activity and Structural Criteria†

Isao Kubo,[a,*] Ikuyo Kinst-Hori,[a] Swapan K. Chaudhuri,[a] Yumi Kubo,[a] Yolanda Sánchez[b] and Tetsuya Ogura[b]

[a]*Department of Environmental Science, Policy and Management, University of California, Berkeley, CA 94720-3112, USA*
[b]*Departamento de Quimica, Universidad Autonoma de Guadalajara, Guadalajara, Jalisco, Mexico*

Received 29 October 1999; accepted 20 March 2000

Abstract—Tyrosinase inhibitory activity of flavonols, galangin, kaempferol and quercetin, was found to come from their ability to chelate copper in the enzyme. In contrast, the corresponding flavones, chrysin, apigenin and luteolin, did not chelate copper in the enzyme. The chelation mechanism seems to be specific to flavonols as long as the 3-hydroxyl group is free. Interestingly, flavonols affect the enzyme activity in different ways. For example, quercetin behaves as a cofactor and does not inhibit monophenolase activity. On the other hand, galangin inhibits monophenolase activity and does not act as a cofactor. Kaempferol neither acts as a cofactor nor inhibits monophenolase activity. However, these three flavonols are common to inhibit diphenolase activity by chelating copper in the enzyme.

Introduction

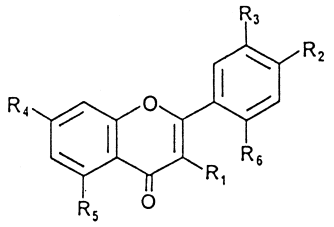
In a previous paper, we reported the characterization of quercetin (**1**) (structures, see Figure 1) as the principal tyrosinase inhibitor from the dried flower of *Heterotheca inuloides* Cass (Compositae), known as "arnica" in Mexico.[1] This common flavonol was also isolated as the principal tyrosinase inhibitor from the fresh flower of *Trixis michuacana* var *longifolia* (D. Dow) C. Anderson (Compositae), known as "huipate floreado" in Mexico. Both medicinal plants are used for similar purposes.[2] Quercetin was found to inhibit the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by mushroom tyrosinase with an ID_{50} of 22 μg/mL (0.07 mM). The inhibition kinetics analyzed by a Lineweaver–Burk plot established quercetin to be a competitive inhibitor with respect to this oxidation. Furthermore, a pre-incubation experiment of the enzyme in the presence of 0.07 mM of quercetin and in the absence of L-DOPA increased the inhibition activity from 45 to 77%. In contrast to quercetin, its 3-*O*-glycosides, isoquercitrin or quercetin 3-*O*-glucoside (**2**) and rutin or quercetin 3-*O*-rutinoside (**3**), were found to behave as neither inhibitors nor substrates[1] and hence the hydroxyl group at the 3-position relates to the activity.

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO),[3] contains a strongly coupled binuclear copper active site and functions both as a monophenolase and as an *o*-diphenolase.[4] In previous reports, tyrosinase was described to possess separate catalytic sites for these two oxidations, and yet another independent binding site for L-DOPA as a cofactor.[5–8] On the basis of the consideration of these previous reports,[1] we proposed that quercetin preferentially displaced L-DOPA from the active site of the cofactor because of its structural resemblance because most competitive inhibitors closely resemble, at least in part, the structure of the substrate. In the case of quercetin, a portion of the structure "**1a**" as shown in bold line is analogous to L-DOPA. Consequently, it may overlap the lock-and-key model but a bulky sugar moiety attached to the 3-hydroxyl group in the 3-*O*-glycoside analogues (**2**,**3**) may hinder their approach to the active site in the enzyme. However, all the interactions between tyrosinase and substrates are suggested to take place at the binuclear copper site.[4,9] The two different types of substrates, monophenol and *o*-diphenol, have been found to react with different oxidation states of the same coupled binuclear copper sites. This prompted us to reinvestigate the above inhibition mechanisms of quercetin and its congeners.

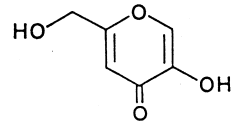
*Corresponding author. Tel.: + 1-510-643-6303; fax: + 1-510-643-0215; e-mail: ikubo@uclink4.berkeley.edu

†Dedicated to Prof. Dr. Takashi Kubota on the occasion of his 90th birthday.

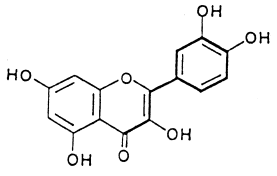
0968-0896/00/$ - see front matter
PII: S0968-0896(00)00102-4



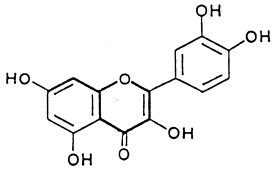
1 R_1=R_2=R_3=R_4=R_5=OH, R_6=H
2 R_1=OGlc, R_2=R_3=R_4=R_5=OH, R_6=H
3 R_1=OGlc^6-Rha, R_2=R_3=R_4=R_5=OH, R_6=H
4 R_1=R_2=R_4=R_5=OH, R_3=R_6=H
5 R_1=OGlc^2-Glc, R_2=R_4=R_5=OH, R_3=R_6=H
6 R_1=OGlc, R_2=R_4=R_5=OH, R_3=R_6=H
8 R_1=R_2=R_5=OH, R_4=OGlc^6-*p*-coumaroyl, R_3=R_6=H
9 R_1=R_2=R_5=OH, R_3=OCH_3, R_4=OGlc^6-*p*-coumaroyl, R_6=H
10 R_1=R_4=R_5=OH, R_2=R_3=R_6=H
11 R_1=OH, R_2=R_3=R_4=R_5=R_6=H
12 R_1=R_2=R_4=R_5=R_6=OH, R_3=H
13 R_1=R_2=R_3=R_5=OH, R_4=OCH_3, R_6=H



7



1a



1b

Figure 1. Chemical structures of flavonols (**1**–**6**, **8**–**13**) and kojic acid (**7**).

Results and Discussion

In addition to flavonols characterized from *H. inuloides*, a number of their congeners were also studied for comparison. It should be noted that some of the flavonoids tested are hardly soluble in the water based test solution. Kaempferol (**4**) is an example of this inferior solubility problem. The ID_{50} of kaempferol was obtained by using three concentrations at which this flavonol was soluble. The addition of 5.2 and 10.4 μg/mL of kaempferol to the assay system containing L-DOPA caused the inhibition of tyrosinase by 16 and 25%, respectively. The inhibition was linearly elevated to 34% when 20.8 μg/mL of kaempferol was added and then the ID_{50} was estimated to be 67 μg/mL (0.23 mM). The solubility problem prevented ID_{50} values of some flavonoids from being established unequivocally. The data needed for discussion are summarized in Table 1. The previous findings of differences between quercetin and its 3-*O*-glycosides on mushroom tyrosinase were also observed with kaempferol (**4**) and its 3-*O*-glycosides, kaempferol 3-*O*-sophoroside (**5**) and kaempferol 3-*O*-glucoside (**6**). Kaempferol inhibited the oxidation of L-DOPA catalyzed by mushroom tyrosinase, but its 3-*O*-glycoside derivatives (**5**,**6**) did not inhibit this oxidation up to 1 mg/mL. Similar to quercetin, the inhibition kinetics analyzed by a Lineweaver–Burk plot found that kaempferol is a competitive inhibitor for this oxidation. The same pre-incubation experiment resulted in increasing the inhibition from 24 to 58%. It should be noted that the pre-incubated enzyme was mostly *met*-tyrosinase, known as the resting form of the enzyme, indicating quercetin and kaempferol can chelate copper in the *met*-form of tyrosinase.

The lag time is known for the oxidation of monophenolic substrates such as L-tyrosine to L-DOPA. This lag time can be shortened or abolished by the presence of reducing agents (hydrogen donors), especially *o*-diphenols.[8,10] Judging from its chemical structure, quercetin (*o*-diphenol) can be an alternative cofactor to initiate this hydroxylase (monophenolase) activity but kaempferol (monophenol) cannot be. In fact, L-tyrosine was oxidized by the enzyme without the lag phase in the presence of quercetin, as shown in Figure 2. However, kaempferol did not suppress this lag time. Both quercetin and kaempferol inhibit *o*-diphenolase activity and it appears that quercetin activates monophenolase activity as a cofactor while kaempferol does not. As expected, quercetin 3-*O*-glycosides (**2**,**3**) also somewhat suppressed the lag phase as *o*-diphenols, though these 3-*O*-glycosides behave as neither inhibitors nor substrates. In our continuing search for tyrosinase inhibitors from plants, we have become aware that a variety of *o*-diphenols such as catechol, caffeic acid and chlorogenic acid also serve as cofactors. The fact that cofactors are reductants or proton donors indicates that they do not need specific binding sites. This definition of cofactors does not support the previously proposed lock-and-key concept. In contrast to quercetin, caffeic acid, catechol and chlorogenic acid were easily oxidized by the enzyme, indicating these *o*-diphenols behave as cofactors and substrates,[11,12] similar to L-DOPA. It appears that quercetin acts as a cofactor but not as a substrate.

In previous papers, some flavonoids were described to chelate copper.[13,14] A portion of the structure "**1b**" (3-hydroxy-4-keto moiety) in quercetin, as shown in the bold line, is clearly analogous to kojic acid (**7**), a potent tyrosinase inhibitor. The inhibition exerted by kojic acid is well established to come from its ability to chelate copper in the enzyme.[15] In addition, quercetin was reported to inhibit copper-catalyzed oxidation of lard by forming a chelate with copper.[16] This supports the above conclusion that quercetin and kaempferol chelate copper in the enzyme. Thus, tyrosinase inhibitory activity of quercetin and kaempferol can be explained by assuming that they chelate copper in the enzyme. In the UV–visible spectra of quercetin and kaempferol, characteristic bathochromic shifts (368→430 nm and 362→404 nm) were observed by adding excess Cu^{2+}, respectively. Figure 3 shows the observed bathochromic shift of quercetin. Since the same bathochromic shift was not observed with kaempferol 3-*O*-glycosides (**5**,**6**), the shift was due to the chelate formation involving the

Table 1. Summary of tyrosinase inhibitory related activity of flavonoids and related compounds

Compounds tested	ID_{50} (mM)	Mode of inhibition[a]	Shift by Cu^{2+}	Cofactor activity[f]
Flavonols				
Quercetin	0.07	Competitive	368→430	+ +
Kaempferol	0.23	Competitive	362→404	
Galangin	♣[e]	n.t.[b]	355→410	
Rhamnetin	n.t.	n.t.	366→432	+ +
Morin	2.32	Competitive	#[d]	
Buddlenoid A	0.39[18]	n.t.	n.t.	n.t.
Buddlenoid B	0.44035[18]	n.t.	n.t.	n.t.
Isoquercitrin	Ω[c]	n.t.	352→382	±
Rutin	Ω	n.t.	352→382	+
Flavones				
Luteolin	0.19	Noncompetitive	348→398	+
Apigenin	♣	n.t.	340→400(sh)	
Chrysin	♣	n.t.	320→400(sh)	
Baicalein	0.29	♣	n.t.	±
Baicalin	Ω	n.t.	n.t.	
Luteolin 7-*O*-glucoside	0.50	Noncompetitive	346→396	+
Kojic acid	0.014	Mixed[27]	n.t.	n.t.
Caffeic acid	♣	n.t.	n.t.	+ +
Catechol	♣	n.t.	n.t.	+ +

[a]With respect to L-DOPA.
[b]n.t., Not tested.
[c]Ω Did not inhibit.
[d]# Did not shift.
[e]♣ Unable to establish.
[f]Tested at 0.01 mM.

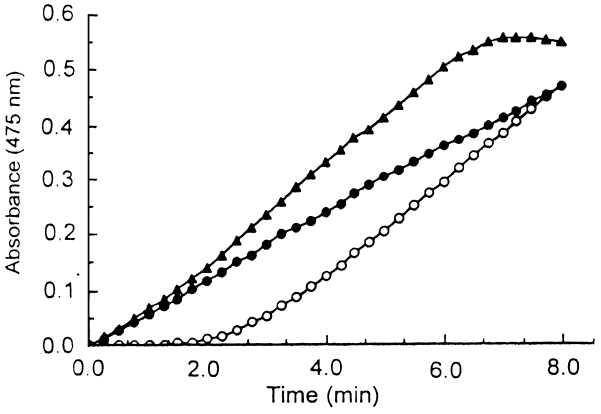


Figure 2. Cofactor activity of quercetin; the lag phase of (○) L-tyrosine (0.85 mM) was completely suppressed by (●) quercetin (0.01 mM), similar to (▲) L-DOPA (0.01 mM).

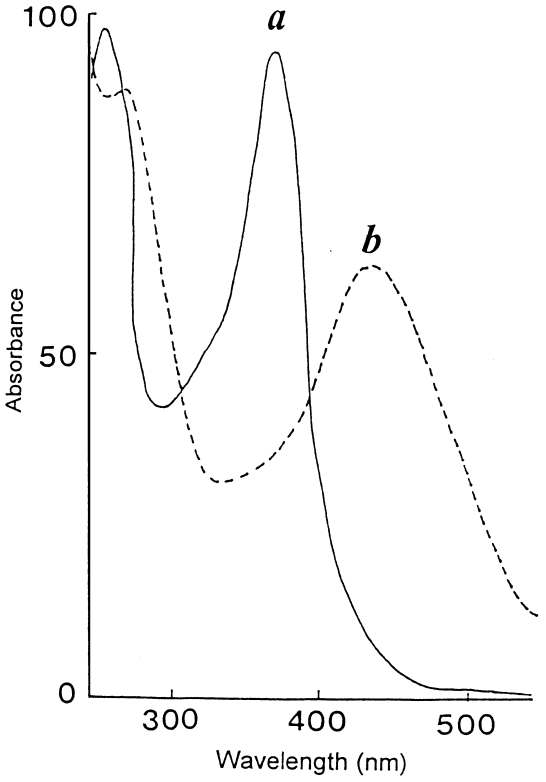


Figure 3. UV–vis spectrum of quercetin (0.05 mM), (***a***) without and (***b***) with $CuSO_4$ (0.125 mM).

3-hydroxyl and 4-carbonyl groups.[17] It should be noted that the formation of the 3-hydroxy-4-keto chelate is favored over the 5-hydroxy-4-keto chelate in flavonols.[14] A similar bathochromic shift was also observed with quercetin 3-*O*-glycosides (**2**,**3**) (352→ 382 nm) by adding excess Cu^{2+}. This shift is probably due to chelation with the vicinal 3′,4′-dihydroxy groups since the shift was not observed with kaempferol 3-*O*-glycosides (**5**,**6**). Interestingly, the above mentioned shift was not observed by adding excess Mg^{2+} or Ca^{2+} in the absorption spectra of quercetin and kaempferol, and their tyrosinase inhibitory activity was not diminished by adding these divalent cations. It seems that Cu^{2+} has the right size to form the chelation but the other divalent cations, Mg^{2+} and Ca^{2+}, do not. This copper chelation mechanism can be further supported by the observation of the noticeable shift when flavonols — such as quercetin and kaempferol — were incubated with the enzyme. For example, the same bathochromic shift was not observed in the absorption spectrum of quercetin and tyrosinase complex, but the characteristic shift to short wavelengths was observed as shown in Figure 4. It seems that flavonols form the chelation with copper in the enzyme and the resulting complex can no

longer keep its molecules flat, but rather twisted. This rules out the possibility that flavonols irreversibly inactivate the enzyme by removing copper from the active site of the enzyme.

Since the partial structure "**1b**" which is responsible for the ability to form chelation can be found only in flavonols, it appears very likely that the copper chelation is the main inhibition mechanism of flavonols as long as their 3-hydroxyl group is free. In other words, flavonols inhibit the oxidation of L-DOPA catalyzed by mushroom tyrosinase as copper chelators. As mentioned above, the 3-*O*-glycosides (**2**,**3**,**5**,**6**) behave as neither inhibitors nor substrates. In addition, we have previously reported the two flavonol glycosides, buddlenoids A (**8**) and B (**9**), in which the sugar moiety is located at the 7-position and does not block their chelate formation site in the molecules. These flavonols inhibited the oxidation of L-DOPA catalyzed by mushroom tyrosinase.[18]

In order to further support the above copper chelation mechanism, galangin (**10**), a simpler flavonol, was also assayed. This flavonol is hardly soluble in the water based test media so its ID_{50} value could not be measured unequivocally for the precise comparison. Therefore, an attempt to test 3-hydroxyflavon (**11**), the simplest compound in this series, was given up though it was reported to show a similar bathochromic shift.[14] Nevertheless, galangin seemed to show almost comparative inhibitory activity with kaempferol at the concentration (less than 0.05 mM) at which it was soluble. This is consistent with its bathochromic shift (355→410 nm) by adding excess Cu^{2+} and the characteristic shift to short wavelength by incubation with the enzyme. In our earlier study, the inhibition mechanism of the above mentioned buddlenoids A (**8**) and B (**9**) could not be established because of their limited availability.[18] It appears most likely that these flavonol derivatives are now considered as copper chelators and as competitive inhibitors. Two additional related flavonols, morin (**12**) and rhamnetin (**13**), were also studied. Rhamnetin showed a characteristic bathochromic shift (366→432 nm) by adding excess Cu^{2+} and also shifted by incubation with the enzyme as expected. In addition, rhamnetin activates monophenolase activity as a cofactor, similar to quercetin. However, morin neither showed the characteristic bathochromic shift by adding excess Cu^{2+} nor activated monophenolase activity as a cofactor. The shift in the absorption spectrum by incubation with the enzyme was similar to those observed with quercetin and kaempferol. More importantly, morin inhibited the oxidation of L-DOPA catalyzed by mushroom tyrosinase with an ID_{50} of 700 μg/mL (2.32 mM), which is about 30-fold less than that of quercetin. The difference in tyrosinase inhibitory activity with quercetin and morin originates from their substituent effects in the B-ring. This can be explained by an intramolecular hydrogen bond between 3- and 2′-hydroxyl groups which interferes with the chelate formation with copper in the enzyme involving the 3-hydroxyl and 4-carbonyl groups, indicating inferior inhibitory activity of morin.

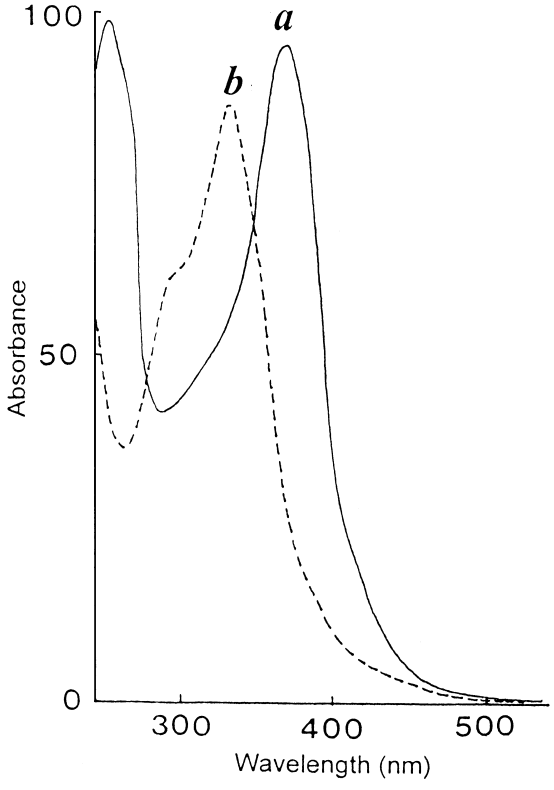
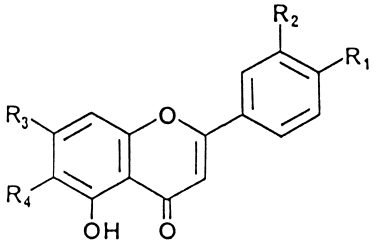


Figure 4. UV–vis spectrum of quercetin (0.05 mM), (***a***) without and (***b***) with tyrosinase (46 units/mL).

Although the dried flower of *H. inuloides* contains only flavonols and their 3-*O*-glycosides, the current study was extended to other types of flavonoids for comparison. The results will be reported in detail elsewhere, but some highlights are presented here. We first assayed three structurally related flavones, chrysin (**14**), apigenin (**15**) and luteolin (**16**), as well as baicalein (**17**) (Fig. 5). As expected from their structural similarity with galangin and kaempferol, the low solubility of **14** and **15** in the water based test medium did not permit us to assay them at appropriate concentrations to establish their ID_{50} values unequivocally. In the absorption spectra, chrysin (**14**) and apigenin (**15**) were found to shift by adding excess Cu^{2+} — presumably both from 5-hydroxy-



14 R_1=R_2=R_4=H, R_3=OH
15 R_1=R_3=OH, R_2= R_4=H
16 R_1=R_2=R_3= OH, R_4=H
17 R_1=R_2=H, R_3=R_4=OH
18 R_1=R_2=OH, R_3=OGlc, R_4=H
19 R_1=R_2=H, R_3=OGlc, R_4=OH

Figure 5. Chemical structures of flavones (**14**–**19**).

4-keto chelation. However, they did not shift by incubation with the enzyme, indicating they do not chelate copper in the enzyme. It seems that hydroxylation at the 5 and 7 positions of their A-ring has little influence on the tyrosinase inhibitory activity, similar to their effect on antioxidant activity.[19] On the other hand, luteolin (**16**) showed the bathochromic shift (348→398 nm) by adding excess Cu^{2+}, presumably due to the chelation with vicinal 3′,4′ -dihydroxy groups. It slightly shifted to the short wavelength by incubation with the enzyme, but not significantly compared to those observed with flavonols. Although the precise explanation of tyrosinase inhibitory activity of luteolin has not yet been established, this flavone very likely disrupts the tertiary structure of the enzyme through intermolecular hydrogen bonding as a noncompetitive inhibitor and reduces the affinity of the substrates with the enzyme. However, the alternative possibility that luteolin positions over the binuclear active site as a substrate analogue and inhibits the enzyme activity[20,21] cannot be entirely ruled out. Similar to quercetin, luteolin shortened the lag time as a cofactor but did not diminish it completely. It can be concluded that flavones do not chelate copper in the enzyme. Luteolin 7-*O*-glucoside (**18**), lacking the 3-hydroxyl group, also showed inhibitory activity with respect to the oxidation of L-DOPA catalyzed by mushroom tyrosinase. Its ID_{50} has been established as 223 μg/mL (0.50 mM) for this oxidation. As expected from its catechol moiety in the B-ring, luteolin 7-*O*-glucoside activated monophenolase activity as a cofactor and L-tyrosine was oxidized by the enzyme without the lag phase in the presence of catalytic amount of this flavone glucoside.

Interestingly, baicalein (**16**) inhibited the oxidation of L-DOPA catalyzed by mushroom tyrosinase with an ID_{50} of 77 μg/mL (0.29 mM), which is almost comparable with that of kaempferol. It should be noted that the assay was carried out in air-saturated aqueous solutions and hence, after several minutes, dopachrome formation reached a plateau as all the available oxygen in the cuvette is consumed. As shown in Figure 6, the difference of "**c**" indirectly demonstrates the amount of oxygen in the cuvette used for the oxidation of baicalein. In contrast to the flavones mentioned above, baicalein was a rare flavone being oxidized as a substrate. The oxidation products were presumably derived via *o*-quinone judging by formation of a yellow color. Because of this enzymatically oxidizable nature, its inhibition mechanism could not be established and the copper chelation is not the inhibition mechanism of this flavone. In addition, baicalein also activated monophenolase activity as a cofactor but did not completely diminish the lag phase. Being electron richer than ring-A, the B-ring of flavonoids is usually an apparent target of tyrosinase, but baicalein, which is unsubstituted in the B-ring, was still being oxidized. Baicalin or baicalein 7-*O*-glucoside (**19**) behaved as neither a substrate nor inhibitor.

The enzymatic oxidation of L-tyrosine to melanin synthesis is of considerable importance since melanin has many functions and alterations in melanin synthesis occur in many disease states. Tyrosinase inhibitors have become increasingly important in medicinal and cosmetic products but a few antimelanogenic reagents, such as monobenzone and hydroquinone, are currently useful. Since flavonols such as quercetin and kaempferol occur widely in many edible plants[22] and exhibit various biological activities, their tyrosinase inhibitory activity is worthy of further study. For example, the browning process in most foods has two components: enzymatic and non-enzymatic oxidation.[23] Hence, potent antioxidant and tyrosinase inhibitory activity of flavonols may render them excellent antibrowning agents. Flavonols are a rare example of tyrosinase inhibitors with antioxidant activity. Accumulation of the knowledge of the regulatory control of melanogenesis on a molecular basis may provide a more rational and scientific approach to design safe and effective tyrosinase control agents.

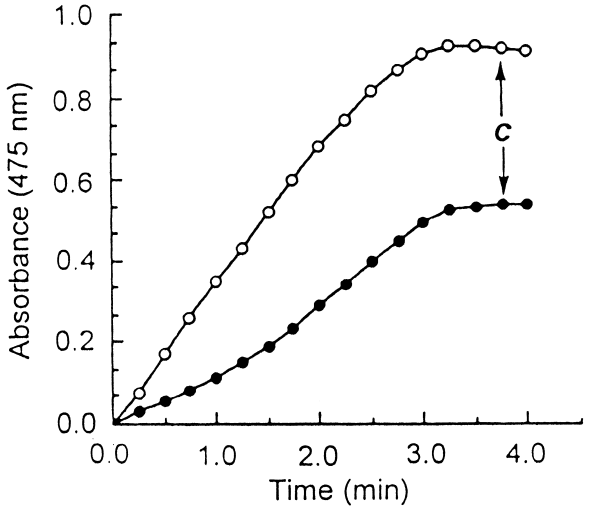


Figure 6. Inhibitory effect on the oxidation of L-DOPA catalyzed by mushroom tyrosinase, (○) without and (●) with baicalein (0.29 mM).

Experimental

General experimental methods

All the procedures used were the same as previously described,[18,24] except NMR data which were recorded in C_5D_5N and CD_3OD. UV–visible (240–540 nm) spectra were recorded in 0.067 M phosphate buffer (pH 6.8) by a Hitachi 100-80 spectrophotometer. The bathochromic shift of flavonols (0.03–0.05 mM) was monitored by adding 0.125 mM of $CuSO_4$.

Plant materials

The fresh flowers of *T. michuacana* var *longifolia* were collected near Guadalajara and initial classification was done by Prof. J. A. Lomeli, School of Biology, Universidad Autonoma de Guadalajara, where a voucher specimen is deposited (GUADA #24575). Subsequently, this identification was confirmed by Dr. B. L. Turner, University of Texas. This yellow flower was extracted with MeOH at ambient temperature. Quercetin and its

3-*O*-glycosides were isolated from this flower extract in quantities by repeated CC (SiO_2) and identified by comparison with their authentic samples.

Chemicals

Quercetin and kaempferol, and their 3-*O*-glucosides, and luteolin and its 7-*O*-glucosides, baicalein and its 7-*O*-glucoside, rhamnetin and catechin were from our previous study[1] and generous gifts from Drs. M. Kozuka, M. Takasaki and K. Ishiguro. 3-Hydroxyflavon was provided by Prof. T. Kamikawa. Rutin, chrysin, apigenin and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Galangin, morin, caffeic acid, catechol, chlorogenic acid, L-DOPA and $MgSO_4$ ($7H_2O$) were obtained from Aldrich Chemical Co. (Milwaukee, WI). $CaSO_4$ ($2H_2O$) and $CuSO_4$ ($5H_2O$) were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Enzyme assay. The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO). Although mushroom tyrosinase differs somewhat from other sources, this fungal source was used for the present experiment due to its ready availability. It should be noted that the commercial tyrosinase was reported to contain numerous proteins besides tyrosinase[25] but was used without purification. Since the mode of inhibition depends on the structure of both the substrate and inhibitor, L-DOPA was used as the substrate in this experiment, unless otherwise specified. Therefore, inhibitors discussed in this paper are inhibitors of diphenolase activity of mushroom tyrosinase. The samples were first dissolved in DMSO and used in the experiments at 30 times dilution. All the samples tested were preliminarily assayed at 167 μg/mL. It should be noted, however, that several flavonoids tested, such as kaempferol, galangin, apigenin and chrysin, are hardly soluble in the water based test solution at this concentration. The enzyme activity was monitored by dopachrome formation at 475 nm up to the appropriate time (usually not exceeding 10 min). Although tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, the assay was carried out in air-saturated solutions.

All the samples were first dissolved in DMSO and used for the actual experiment at 30 times dilution. The assay was performed as previously described with slight modifications.[26] First, 1 mL of 2.5 mM L-DOPA or L-tyrosine solution was mixed with 1.8 mL of 0.1 M phosphate buffer (pH 6.8), and incubated at 25 °C for 10 min. Then, 0.1 mL of the sample solution and 0.1 mL of the aqueous solution of mushroom tyrosinase (138 units) was added to the mixture to immediately measure the initial rate of linear increase in optical density at 475 nm, on the basis of the formation of dopachrome. The extent of inhibition by the addition of samples is expressed as the percentage necessary for 50% inhibition (ID_{50}).

The pre-incubation mixture consisted of 1.8 mL of 0.1 M phosphate buffer (pH 6.8), 0.6 mL of water, 0.1 mL of the sample solution and 0.1 mL of the aqueous solution of mushroom tyrosinase (138 units). The mixture was pre-incubated at 25 °C for 5 min. Then, 0.4 mL of 6.3 mM L-DOPA was added and the reaction was monitored at 475 nm for 2 min.

For the measurement of the UV–vis (240–540 nm) spectra if flavonols can chelate copper in the enzyme, the mixture consisting of 1.8 mL of 0.1 M phosphate buffer (pH 6.8), 1.0 mL of water, 0.1 mL of the sample (0.05 mM) solution and 0.1 mL of the aqueous solution of the mushroom tyrosinase (138 units) was incubated at 25 °C for 30 min, and then the spectra were recorded.

Acknowledgements

We are grateful to Dr. M. Kozuka, Dr. M. Takasaki, Dr. K. Ishiguro and Prof. T. Kamikawa for providing us various authentic flavonoids, and Dr. D. N. Pelaez, Prof. J. A. Lomeli and Dr. B. L. Turner for identifying the plant specimens.

References

1. Kubo, I.; Kinst-Hori, I.; Ishiguro, K.; Chaudhuri, S. K.; Sanchez, Y.; Ogura, T. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1443.
2. Matinez, M. In *Catalogo de Nombres Vulgares y Cientificos de Plantas Mexicanas*; Fondo de Cultura Economica: Mexico, 1984; pp 145–146.
3. Mayer, A. M. *Phytochemistry* **1987**, *26*, 11.
4. Sánchez-Ferrer, A.; Rodríguez-López, J. N.; García-Cánovas, F.; García-Carmona, F. *Biochim. Biophys. Acta* **1995**, *1247*, 1.
5. Duckworth, H. W.; Coleman, J. E. *J. Biol. Chem.* **1970**, *245*, 1613.
6. Jolley, R. L., Jr.; Evans, L. H.; Makino, N.; Mason, S. H. *J. Biol. Chem.* **1974**, *249*, 335.
7. Gutteridge, S.; Robb, D. *Eur. J. Biochem.* **1975**, *54*, 107.
8. Hearing, V. J.; Ekel, T. M. *Biochem. J.* **1976**, *157*, 549.
9. Whitaker, J. R.; Lee, C. Y. In *Enzymatic Browning and its Prevention*; Lee, C. Y., Whitaker, J. R., Eds.; ACS Symposium Series 600, American Chemical Society: Washington, DC, 1995, pp 2–22.
10. Ros, J. R.; Rodríguez-López, J. N.; García-Cánovas, F. *Biochim. Biophys. Acta* **1993**, *1163*, 303.
11. Murata, M.; Kurokami, C.; Homma, S. *Biosci. Biotech. Biochem.* **1992**, *56*, 1705.
12. Oszmianski, J.; Lee, C. Y. *J. Agric. Food Chem.* **1990**, *38*, 1202.
13. Thompson, M.; Williams, C. R.; Elliot, G. E. P. *Anal. Chim. Acta* **1976**, *85*, 375.
14. Hudson, B. J. F.; Lewis, J. I. *Food Chem.* **1983**, *10*, 47.
15. Kahn, V.; Lindner, P.; Zakin, V. *J. Food Biochem.* **1995**, *18*, 253.
16. Mahgoub, S. E. O.; Hudson, B. J. F. *Food Chem.* **1985**, *16*, 97.
17. Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The Systematic Identification of Flavonoids*. Springer-Verlag: New York, 1970.
18. Kubo, I.; Yokokawa, Y. *Phytochemistry* **1992**, *31*, 1075.
19. Pratt, D. E.; Hudson, B. J. F. In *Food Antioxidants*; Hudson, B. J. F., Ed.; Elsevier Applied Science: London, 1990; pp 171–192.

20. Wilcox, D. E.; Porras, A. G.; Hwang, Y. T.; Lerch, K.; Winkler, M. E.; Solomon, E. I. *J. Am. Chem. Soc.* **1985**, *107*, 4015.
21. Winkler, M. E.; Lerch, K.; Solomon, E. I. *J. Am. Chem. Soc.* **1981**, *103*, 7001.
22. Crozier, A.; Lean, M. E.; McDonald, M. S.; Black, C. *J. Agric. Food Chem.* **1997**, *45*, 590.
23. Friedman, M. *J. Agric. Food Chem.* **1996**, *44*, 631.
24. Kubo, I.; Yokokawa, Y.; Kinst-Hori, I. *J. Nat. Prod.* **1995**, *58*, 739.
25. Kumar, M.; Flurkey, W. H. *Phytochemistry* **1991**, *30*, 3899.
26. Masamoto, Y.; Iida, S.; Kubo, M. *Planta Med.* **1980**, *40*, 361.
27. Chen, J. S.; Wei, C. I.; Rolle, R. S.; Otwell, W. S.; Balaban, M. O.; Marshall, M. R. *J. Agric. Food Chem.* **1991**, *39*, 1396.