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Kinetic Study of Flavonoid Reactions with Stable Radicals

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The antiradical activities of some flavonols (kaempferol, quercetin, robinetin, quercetagetin, and myricetin), flavones (apigenin, baicalein, and luteolin), flavanones (naringenin and dihydroquercetin), and flavanols [(+)-catechin and (−)-epicatechin] were determined by measuring the reaction kinetics with 2,2-diphenyl-1-picrylhydrazyl (DPPH) and α,γ-bisdiphenylene-β-phenylallyl (BDPA) radicals. The reactions, which follow the mixed second-order rate law, were investigated under pseudo-first-order conditions by use of a large excess of flavonoids, and their stoichiometry was determined by spectrophotometric titration. The results confirm stoichiometric factors of 1, 2, and 3 for flavonoids with one, two, and three hydroxyl groups in the B-ring, respectively, excluding kaempferol, which, despite a single OH group in the B-ring, has a factor of 2, which is explained by the 3-OH group supporting the reaction with free radicals. Structure−activity considerations indicate for the present series of flavonoids the importance of multiple OH substitutions and conjugation. The logarithms of reaction rate constants with the OH, DPPH, and BDPA radicals correlate well with the reduction potential of the flavonoids.

KEYWORDS: Flavonoids; antiradical activity; reaction rate constants; DPPH and BDPA radicals; correlation with reduction potential; structure−activity relationship (SAR)

INTRODUCTION

Flavonoids are compounds found in fruits, vegetables, and certain beverages that have diverse biochemical (*1*) and antioxidant effects (*2*−*4*). Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyran rings, and most possess high antioxidant and free radical scavenging activityies (*5*, *6*). The antioxidant activity of flavonoids and their metabolites in vitro depends on the arrangements of functional groups about the nuclear structure. Dietary flavonoids differ in the arrangements of hydroxy, methoxy, and glycosidic groups and in the conjugation between the C- and B-rings. The protective effects of flavonoids in biological systems are ascribed to their capacity to transfer electrons to free radicals, chelate metals, and activate antioxidant enzymes. Here we are interested in the antiradical activity of some flavonols, flavones, flavanones, and flavan-3-ols (**Scheme 1**).

An imbalance between antioxidants and pro-oxidants results in oxidative stress, leading to cellular damage. Oxidative stress has been linked to cancer, atherosclerosis, ischemic injury, inflammation, and neurodegenerative diseases, among others (*7*). A well-known fact is that antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species, for example, the superoxide anion radicals, hydroxy radicals, and peroxyl radicals (*8*, *9*).

Free radical scavenging capacity is primarily attributed to the high reactivity of hydroxy substituents that participate in the following reaction (F-OH = flavonoid):

$$\text{F-OH} + \text{R}^{\bullet} \rightarrow \text{F-O}^{\bullet} + \text{RH} \qquad (1)$$

Hydroxy groups donate hydrogen and an electron to radicals, stabilizing them and giving rise to a relatively stable flavonoid radical (*10*). Due mainly to its simplicity, the most popular assay to determine radical-scavenging activities is the bleaching of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (*11*, *12*). Unfortunately, the method has never been standardized with respect to absolute and relative concentrations of substrates and the DPPH radical, the observation period, etc. Despite earlier suggestions of defining the inverse of IC_{50} values as "antioxidant reduction potential" (ARP) (*13*−*15*), most compilations list IC_{50} values (decrease of the initial DPPH concentration by 50%) (*16*−*19*), data that, if obtained in different laboratories, cannot be directly compared. In fact, the use of EC_{50}/IC_{50} has been severely criticized as it does not take into account the various kinetic behaviors and the authors define the inverse value as "antiradical efficiency" (AE) (*20*).

Although the generally accepted mechanism is the transfer of hydrogen atoms to the DPPH radical, this has only recently been verified by the identification of reaction products (*21*−*25*). Interestingly enough, the earlier study (*21*) reported only the formation of the B-ring *o*-quinones from (+)-catechin and (−)-epicatechin, whereas the later studies also discovered various dimers of the catechins (*23*, *24*), formed via intermediary quinone methides and phenolic coupling reactions (*26*).

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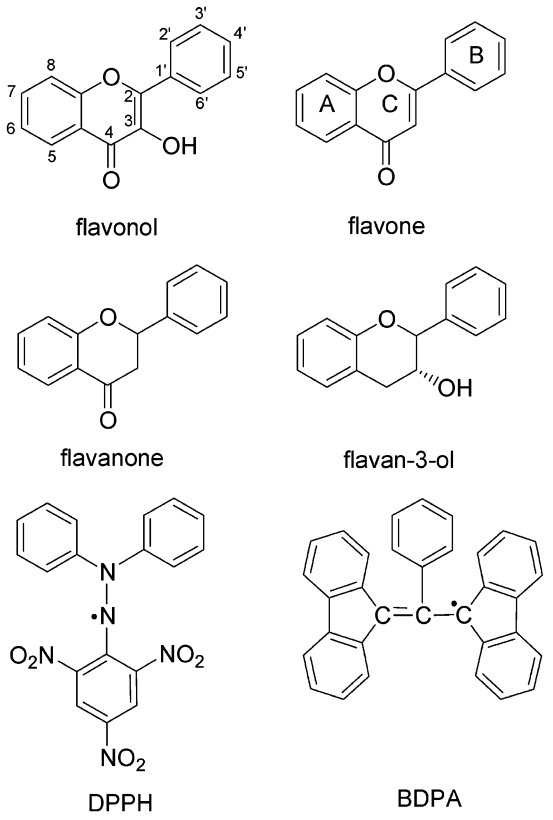
Scheme 1. Backbone Structures of Flavonoids Investigated and of the Stable Radicals



Yordanov (*27*) has thus far been the only one to attempt a quantification of the DPPH procedure, whereas kinetic studies to determine absolute rate constants of the hydrogen transfer to the DPPH radical have been carried out with flavones by Lindberg Madsen et al. (*28*) and Goupy et al. (*29*). In the present work we have measured the second-order rate constants for the reaction of some flavonoids with DPPH, comparing some of the data with those obtained with α,γ-bisdiphenylene-β-phenylallyl (BDPA) radicals.

MATERIALS AND METHODS

The flavonoids were of commercial origin (Fluka, Sigma, Extrasynthese) and were used without further purification. The stable free radicals DPPH and BDPA [complex with benzene (1:1)] were from Aldrich—the latter is no longer available. Methanol and 2-propanol (Kemika) were distilled as needed. Doubly distilled water, additionally purified by a passage through a Milli-Q system, was used.

Spectral and kinetic data were obtained using an HP Agilent 8453 diode array spectrophotometer and a Durrum D-110 stopped-flow instrument. The kinetics were followed at the absorption maximum of the DPPH (516 nm, $\log \epsilon = 3.985$) and BDPA (480 nm, $\log \epsilon = 4.323$) radicals. The data were collected under pseudo-first-order conditions by use of a large excess of flavonoids [$(1.8-5.6) \times 10^{-4}$ M] over the concentration of DPPH radicals [$(1.8-2.5) \times 10^{-5}$ M], whereas in the experiments with BDPA radicals the initial concentrations were of flavonoids [$(0.45-1.3) \times 10^{-3}$ M] and BDPA [$(3.8-8) \times 10^{-5}$ M]. We chose BDPA as another stable radical reacting with flavonoids, and its absorption in the visible region allowed us to measure the kinetics. The data were evaluated with the program for the first-order reaction on the UV−vis Chemstation.

In the stoichiometric titrations, the titrant flavonoid was added slowly to the DPPH solution until the equivalence point was reached with no further change in absorbance. The quantity of the flavonoid that is required to consume all of the DPPH radical is expressed as a ratio of the concentrations of both reactants ([F]/[DPPH]). We assume an overall margin of error in our experiments of ∼10%. Most of the reactions were carried out in methanol as solvent, although in several experiments a 1:1 mixture of 2-propanol/water was used.

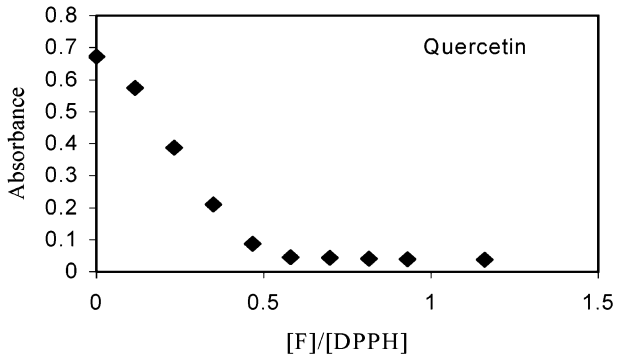


Figure 1. Spectrophotometric titration of DPPH with quercetin at 516 nm.

Table 1. Kinetic Data for the Reaction of the DPPH Radical with Flavonoids[a]

flavonoids	systematic name	k_F/L mol^{-1} s^{-1}
flavonols		
kaempferol	3,5,7,4′-tetrahydroxyflavonol	2.38×10^3
		7.0×10^2 (*28*)
quercetin	3,5,7,3′,4′-pentahydroxyflavonol	4.76×10^2
		6.2×10^2 [b]
		1.89×10^3 (*29*)
robinetin	3,7,3′,4′,5′-pentahydroxyflavonol	1.14×10^2
		1.4×10^2 [b]
quercetagetin	3,5,6,7,3′,4′-hexahydroxyflavonol	4.4×10^3
myricetin	3,5,7,3′,4′,5′-hexahydroxyflavonol	7.55×10^1
		1.3×10^2 [b]
flavones		
apigenin	5,7,4′-trihydroxyflavone	0.1
baicalein	5,6,7 -trihydroxyflavone	6.3×10^2
luteolin	5,7,3′,4′-tetrahydroxyflavone	2.29×10^3
flavanone		
naringenin	5,7,4′-trihydroxyflavanone	4.0
dihydroquercetin	3,5,7,3′,4′-pentahydroxyflavanone	2.2×10^2
flavanol		
(+)-catechin	3,5,7,3′,4′-flavanol	5.3×10^2
		1.09×10^3 (*29*)
(−)-epicatechin	3,5,7,3′,4′-flavanol	4.89×10^2
		1.12×10^3 (*29*)

[a] 25 °C, methanol solution. [b] In 1:1 H_2O/2-propanol (v/v).

RESULTS AND DISCUSSION

The DPPH radical scavenging method has been applied to the evaluation of the antiradical activity of numerous substances (*11*, *12*). As mentioned earlier, most of these papers presented the radical scavenging activity as EC_{50}.

The stoichiometry for the reaction of several flavonoids was determined by spectrophotometric titration at the wavelength of the maximum absorption of the DPPH radicals (*29*; see **Figure 1**). The ratios were 0.5 for (+)-catechin, quercetin, luteolin, and kaempferol, 0.3 for myricetin and robinetin, and 1 for naringenin. The results confirm the overall 1:1 stoichiometry for flavonoids with one hydroxy group in the B-ring, 1:2 stoichiometry for flavonoids with two hydroxy groups in the B-ring (but also including kaempferol), that is, values of $n = 1$, 2, and 3 for the stoichiometry factor, respectively.

The reaction of flavonoids and DPPH radicals followed the mixed second-order rate law (eq 2), yielding the values of k_F in **Table 1**.

$$-\mathrm{d[DPPH]}/\mathrm{d}t = -n\mathrm{d[F]}/\mathrm{d}t = k_F[\mathrm{DPPH}][\mathrm{F}] \qquad (2)$$

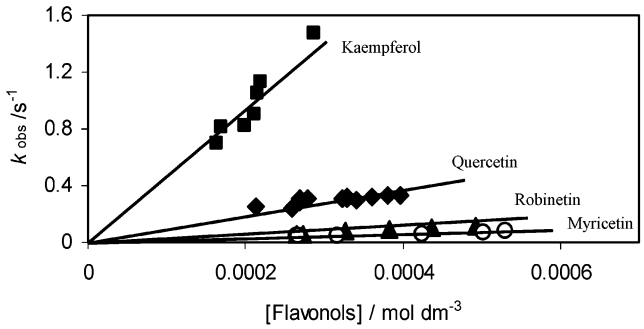


Figure 2. Plot of k_{obs} versus the concentrations of flavonols for the reaction with DPPH radicals.

Under our experimental conditions the data show linear dependence of the pseudo-first-order rate constants on flavonoids concentration (**Figure 2**.)

The least-squares fits for the lines expressed by $y = ax + b$ yield the second-order rate constants from the slope (dimension L mol^{-1} s^{-1}). The lack of intercept denotes the stability of the DPPH radical. The rate constants observed for the reaction of DPPH radicals with various flavonoids are presented in **Table 1**. The scavenging activity of flavonoids toward radicals was found to vary depending on the group of flavonoids.

Although rate constants of only kaempferol and eriodictyol with DPPH were measured by Lindberg Madsen et al. (*28*) under similar pseudo-first-order conditions as we employed with (on average) a 10-fold excess of the flavones, the values obtained by Groupy et al. (*29*) are 2−3 times higher as consequence of using the stoichiometry factor in eq 2. The latter authors used a kinetic modeling approach assuming as initial reaction the reduction of the DPPH radical and formation of a flavonoid quinone—which in effect is a two-electron oxidation.

The rate constants for the reaction with BDPA radicals, although much lower than for DPPH, decrease in the order kaempferol (0.141 L mol^{-1} s^{-1}), luteolin (0.131 L mol^{-1} s^{-1}), quercetin (0.091 L mol^{-1} s^{-1}), and myricetin (0.026 L mol^{-1} s^{-1}) and thus follow the same trend as observed for the reaction of the flavonoids with the DPPH radical.

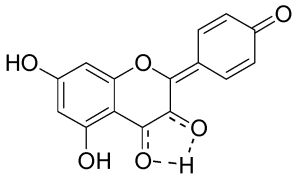
Some structure−activity relationship can been proposed from this series of the flavonoids, pointing out the importance of the structure of the B-ring and the influences from the rest of the molecule. As observed, the k_F values for the flavonols decrease in the order quercetagetin > kaempferol > quercetin > robinetin > myricetin. The flavonol quercetagetin, which contains a pyrogallol-like substitution in the A-ring (5,6,7-tri-OH) and an *o*-di-OH substitution in the B-ring, has the highest reactivity with DPPH. It seems that besides the well-established importance of the latter, multiple substitution on the A-ring is playing also an important role in hydrogen atom transfer reaction. However, kaempferol with one hydroxy group in the B-ring reacts more rapidly than quercetin with 3′,4′-di-OH substitution, which is explained by interaction with its 3-OH substituent (vide infra). Also, flavonols with a pyrogallol-like structure in the B-ring show low reactivity.

The rate constant of the flavone luteolin with 3′,4′-di-OH substitution in the B-ring and 5,7-di-OH substitution in the A-ring of 2.29 × 10^3 L mol^{-1} s^{-1} is almost as high as for kaempferol. It is worth noting that apigenin (K_F = 0.1 L mol^{-1} s^{-1}) with 4′-OH in the B-ring compared to luteolin reacts significantly slower. The rate of reaction of baicalein, which has no substitution in the B-ring, but a pyrogallol-like structure on the A-ring (5,6,7-tri-OH), is between those found for luteolin and apigenin.

The rate constants observed for (+)-dihydroquercetin (2.2 × 10^2 L mol^{-1} s^{-1}), quercetin (4.76 × 10^2 L mol^{-1} s^{-1}), and luteolin (2.29 × 10^3 L mol^{-1} s^{-1}) indicate different activities, which depend on their backbone structures. They all have 5,7-di-OH substitution in the A-ring and 3′,4′-di-OH substitution in the B-ring, but the former two have a 3-OH substitution at the C-ring and the latter two a C_2-C_3 double bond. The flavanone (+)-dihydroquercetin reacts 2 times more slowly than quercetin and luteolin reacts 5 times more rapidly than quercetin with DPPH radicals. These results reveal a strong (5-fold) effect of removing a 3-OH group and smaller (2-fold) effect of adding a C_2-C_3 double bond on reactivity increase.

However, if we compare the structure and the rate constants for the kaempferol, naringenin, and apigenin—all with a single 4′-OH substitution in the B-ring, which is considered to yield low activity—we observe that kaempferol has considerable affinity toward radicals, indicating that here the 3-OH group at the C-ring supports the reaction with free radicals. The 3-OH substitution is thought to increase the stability of the flavonoid radical in this group of flavonoids because the torsion angle of the B-ring with respect to the rest of the molecule strongly influences free radical scavenging ability. Flavonols and flavanols with a 3-OH substitution are planar, whereas flavones and flavanones, lacking this feature, are slightly twisted (*30*, *31*).

The observed enhanced activity and stoichiometry factor of 2 for kaempferol can also be rationalized by the existence of a *p*-quinone methide structure upon the hydrogen atom abstraction by the DPPH radical



analogous to recent suggestions by Rietjens and colleagues for quercetin (*32*−*34*).

The abstraction of a hydrogen atom from flavonoids by radicals proceeds probably through a polar transition state. The reaction rates of flavonoids obtained in 2-propanol/water (1:1) solutions were slightly higher than those obtained in methanol, in agreement with the polarity of the solutions (*35*).

Skibstedt and co-workers were the first to correlate reduction potentials of flavonoids with their antioxidant potential (*28*, *36*). As shown in **Figure 3**, we also note a linear correlation between the reduction potentials of flavonoids and the logarithm of the rate constants for the reaction with DPPH radicals. The flavone luteolin and the flavanol (+)-catechin have reducing strengths similar to that of flavonol kaempferol, which shows good antiradical activity (*37*, *38*). Similar correlations were found for reactions with other radicals as different as OH (*10*) and BDPA, which indicates the same mechanism. In fact, because all three radicals are oxidative species, that is, they remove a hydrogen atom (or H^+/e^-) from the flavonoid hydroxyl groups, a correlation with the respective reduction potential should be expected.

In kinetic investigations one often observes empirical correlations of rate constants with other parameters, for example, Hammett or Marcus values. These so-called linear free energy relationships (LFER) are usually depicted as semilogarithmic plots, log $k = m(E) + b$. Both the slopes and the ordinate intercepts are specific for each correlation. In our case the reasonable similarity of the slopes (OH, log $k = 3.78x + 8.87$; DPPH, log $k = 5.84x + 1.21$; BDPA, log $k = 3.28x - 1.84$)

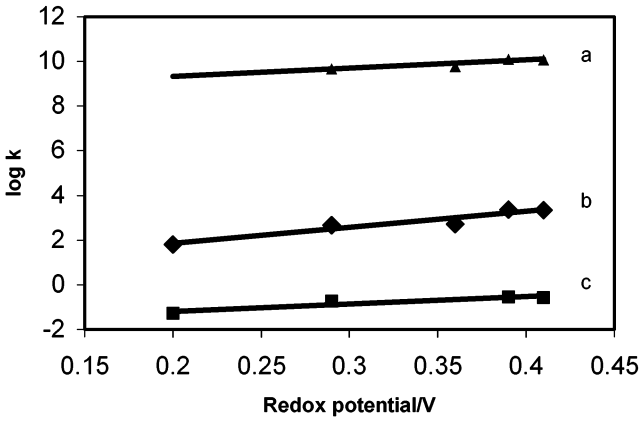


Figure 3. Plot of the logarithm of the rate constants for the reaction of flavonoids with OH (**a**; data from ref *10*), DPPH (**b**), and BDPA (**c**) radicals (this work) versus the reduction potential *E′* (V vs NHE) of the flavonoids [the flavonoids plotted are, from left to right, myricetin (0.2), quercetin (0.29), (+)-catechin (0.36), kaempferol (0.39), and luteolin (0.41) (*36*)].

indicates similar mechanisms, whereas the ordinate intercept reflects the geometric constraints of the DPPH and BDPA radicals as opposed to the diffusion-controlled reaction of the OH radical.

In conclusion, the relationship between structure and radical scavenging property against stable free radicals of several flavonoids has been shown. The results of the present kinetic study demonstrate that the structure−effect relationship is not simple but suggest that some flavonoids with specific structures react very rapidly indeed with free radicals.

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