Chemistry of Natural Compounds, Vol. 36, No. 1, 2000

STRUCTURE—FUNCTION ANALYSIS OF THE RADIOPROTECTIVE AND ANTIOXIDANT ACTIVITY OF FLAVONOIDS

E. P. Kemertelidze, V. G. Tsitsishvili, M. D. Alaniya, and T. G. Sagareishvili

UDC 547.972

The recombination kinetics of a series of flavonoids with stable DPPH radicals are studied. Flavonols are the most reactive. Polarization of the carbonyl in flavones reduces the reactivity. Substitution on C-7 and the B ring has practically no effect on the reactivity. Glycosylation of C-3 reduces the reactivity if the sugar can form two intramolecular H-bonds. The reactivity of the flavonoid phenol hydroxyls, with the exception of the hydroxyl on C-5, is proportional to their number. The most promising antioxidants are lespedin, kaempferitrin, kaempferol, kaempferol-7-rhamnoside, kaempferol-3-robinobioside, and robinin.

Key words: flavonols, flavones, radioprotective and antioxidant activity analysis.

Ketocompounds in many instances are tested as radioprotective agents and antioxidants because their structural features can inhibit energy-transfer processes and migration of elementary particles during irradiation [1]. Ketones in which the carbonyl group is conjugated to aromatic rings activated by electron-donor substituents act as protectors. Either conjugation with two rings or strong activation of the rings is required. Therefore, flavonoid glycosides are being examined as potential radioprotectors. The mechanism of protective activity consists of the reaction of the keto group with metal ions and metal-containing enzymes. This stabilizes redox processes in irradiated cells [2].

Isolfavones and their derivatives have been found to be antioxidants. They suppress oxidative processes not in the aqueous but in the lipid phase of liposomes [3].

The interesting antioxidant activity of flavonoids has been reported in the literature [2-5]. The longevity of mice after irradiation is used as a biological test.

Screening requires time and money and can be avoided if adequate physicochemical methods are used. In this instance, the relative reactivity toward recombination of the studied compound with a stable radical, e.g., diphenylpicrylhydrazyl (DPPH), can be used to make predictions [5]. A compound that reacts quickly with DPPH is assumed to possess significant radioprotective or antioxidant activity.

In the present work, results are presented from a study of several flavonoids isolated at the Institute of Pharmacochemistry of the Georgian Academy of Sciences [6-8].

The experimental results (Table 1) show that the studied compounds can be arbitrarily divided into three groups. The first group contains compounds (Fig. 1) that recombine most quickly. At room temperature, T_e are several minutes. The corresponding constants exceed 500 ml/mole·sec. The glycosides of this group in general, and the 3-glycosides in particular, have greater recombination constants than the aglycones.

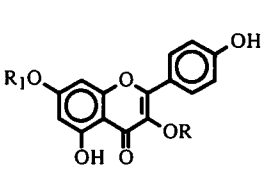
The second group includes compounds (Fig. 2) that recombine 4-7 times slower. The T_e values are tens of minutes. The recombination constants are less than 200 ml/mole·sec. This group includes compounds that are not glycosylated at C-3 such as apigenin and luteolin and their derivatives. It is noteworthy that the correlation between the recombination constant and the number of phenol groups is completely reliable. An estimate of the partial contribution to the recombination constant of a single phenol group averages 25 ml/μmole·sec.

I. G. Kutateladze Institute of Pharmacochemistry, Georgian Academy of Sciences, Tbilisi, fax (99532)-25-00-26.
Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 42-46, January-February, 2000. Original article submitted July 30, 1999.

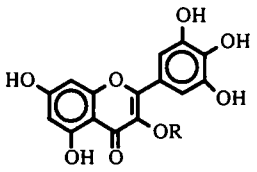
0009-3130/00/3601-0054$25.00

TABLE 1. Characteristic Times T_e and Recombination Constants K of Flavonoids

Compound	T_e, min	K, ml/mole·sec	Relative uncertainty, %
1a. Lespedin	3.8	720	16.67
1b. Kaempferitrin	3.8	740	16.22
2a. Kaempferol	4.0	630	15.87
3a. Kaempferol-7-rhamnoside	4.6	650	15.38
3b. Kaempferol-3-robinobioside	4.4	700	17.14
3c. Robinin	4.4	670	17.91
4a. Miricetin	4.3	620	16.13
5a. Luteolin	14.5	180	22.22
5b. Cinaroside	21.5	150	16.67
6a. Apigenin	16.5	150	20
6b. Kosmosiin	24	120	16.67
6c. Apigenin 7-glucuronide	29	100	20
6d. Roifolin	30	100	20
4b. Miricitrin	60	50	20
2b. Trifolin	100	30	20
7a. Isorhamnetin -3-glucoside	120	25	20
7b. Astragalegoside	110	25	20
7c. Isoastragalegoside	150	20	25
8a. Isoquercitrin	160	20	25
8b. Hyperin	150	20	25
9. Biohanin A-7-glucoside	150	20	25
10. Liquiritigenin	125	20	25

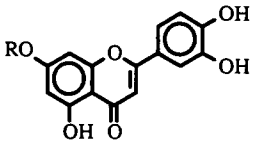


1a: R=R_1=Rha (pyranose)
1b: R=R_1=Rha (furanose)
2a: R=R_1=H
3a: R=H, R_1=Rha
3b: R=Gal(6→1)Rha, R_1=H
3c: R=Gal(6→ 1)Rha, R_1=Rha

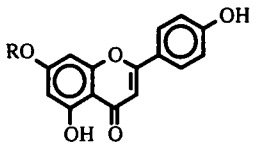


4a

Fig. 1. Structural formulas of the first group of compounds.



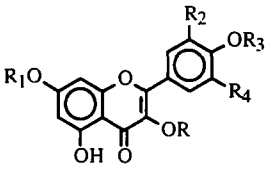
5a: R=H
5b: R=Glu

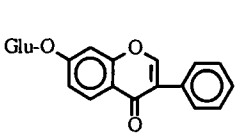


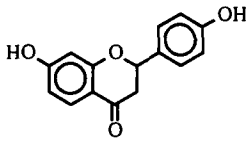
6a: R=H
6b: R=Glu
6c: R=$C_6H_9O_6$
6d: R=Glu(2→1)Rha

Fig. 2. Structural formulas of the second group of compounds.

Slowly recombining compounds fall in the third group (Fig. 3). For these, T_e values are of the order of an hour and greater. The recombination constants are less than 50 ml/mole·sec.







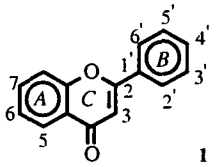
2b: R=Gal, R_1=R_2=R_3=R_4=H
4b: R=Rha, R_1=R_3=H, R_2=R_4=OH
7a: R=Glc, R_1=R_3=R_4=H, R_2=OCH_3
7b: R=R_3=Glc, R_2=OCH_3, R_1=R_4=H
7c: R=R_1=Glc, R_2=OCH_3, R_3=R_4=H
8a: R=Glc, R_1=R_3=R_4=H, R_2=OH
8b: R=Gal, R_1=R_3=R_4=H, R_2=OH

9 **10**

Fig. 3. Structural formulas of the third group of compounds.

The results as a whole do not contradict the hypothesis [2] that the keto groups in these molecules that is conjugated to aromatic rings activated by electron donors are responsible for the protective effect. Furthermore, namely those compounds for which the maximal biological effect was observed [2] exhibit the fastest recombination rate with DPPH. Apparently the low reactivity of compounds in the third group is due to steric shielding of the carbonyl group by substituents located on C-3. This result and data for the aglycones, which differ in degree of hydroxlation of C-3, agree with previous conclusions [4]. However, the discrepancy in the data for the glycosides is still important. The answer to this question can be found through a detailed examination of the structure and certain spectral characteristics of the studied compounds.

The recombination rate with DPPH was studied for flavonoids (**1**):



1

Molecular models indicate that the *AC* bicyclic structure is practically planar. The C-2–C-1′ bond that is situated in this same plane is included in the conjugation. Hindrance to the reorientation of ring *B* relative to *AC* is expected. However, steric hindrance to the positioning of ring *B* in the plane of *AC* exists owing to the interaction of H-3 (or the substituent R) and H-2′ (H-6′ or the substituent). Thus, the electronic conjugation of rings *AC* and *B* is only partial, which increases the probability that ring *B* can reorient. The substituents on C-2′, which can be an OH group and a sugar, are especially interesting. The models show that the presence of such substituents makes full rotation of ring *B* impossible. The orientation of ring *B* relative to the plane of *AC* in any instance is determined mainly by the interaction with H-2′ (or H-6′) and the oxygen atom on C-3. The minimal dihedral angle between the planes of rings *AC* and *B* is 15°.

Molecular models indicate that an intramolecular H-bond can form between the carbonyl group and the OH group on C-5. The H-bonding pattern of flavonoid glycosides can be rather complicated, especially if C-3 is glycosylated. In this instance, the carbonyl oxygen atom can participate in the formation of H-bonds to the OH oxygen atom on C-2″ of the sugar. If C-6″ and C-3′ (or C-5′) have OH groups and C-2″ and C-5″ have the required configuration, it is geometrically possible to form another intramolecular OH-bond. Ring *B* is oriented at the minimal angle possible if a H-bond between the OH group of ring *B* and the OH group of the sugar forms or two intramolecular H-bonds form. Obviously oligosaccharide substitution on C-3 can complicate the intramolecular H-bonding pattern even more.

The geometrical aspects of glycosylation at C-5 differs considerably from that at C-3 because two H-bonds cannot form and interaction with ring *B* is impossible. The interaction of a carbonyl or OH on C-7 and the OH on C-6″ is definitely interesting. However, we did not study any compounds with this feature.

If C-7 is glycosylated, the carbonyl oxygen atom does not form intramolecular H-bonds. Only a H-bond involving the OH group on C-5 and C-6″ is possible. In this instance the carbonyl group is sterically unshielded.

The probability of steric shielding of the carbonyl, the participation of the phenol OH groups in intramolecular H-bonds,

and the conjugation of ring *B* to the carbonyl can be estimated by examining molecular models.

In addition to an examination of the geometric structure of the flavonoids, the ^{13}C NMR spectra are also interesting. It is known [9] that a linear correlation exists between the wavelength of the n-π^* electronic transition and the chemical shifts (CS) of the carbonyl carbon atom in carbonyl compounds. This can be extended to flavonoids, which have been rather extensively studied by NMR [10].

The apigenin (**6a**) derivatives have a narrow range of the carbonyl CSs (from 181.7 to 181.9 ppm). However, it should be noted that most existing data refer to compounds that are glycosylated at C-7. Glycosylation of luteolin (**5a**) at C-7 and on ring *B* decreases the CS of the carbonyl by 1.0-1.2 ppm, i.e., the range of CSs in this instance is exceedingly narrow. The effect is significant for luteolin-5-O-glucoside. Compared with the aglycone, the carbonyl CS decreases by 6 ppm. This effect in sign and magnitude is comparable to introducing an OH at C-3. This effect is satisfactorily explained quantitatively by the lack of a H-bond. For flavonoid derivatives glycosylated at C-3 (kaempferol, quercetin, miricetin, and their derivatives at C-3), glycosylation at any position increases the CS of the carbonyl by less than 2 ppm.

These data suggest that, with the exception of the C-5 position, glycosylation of flavonoids has practically no effect on the π-electronic density of the carbonyl. According to IR spectroscopy [11], the carbonyl in flavonoids has an absorption maximum at comparatively low frequencies. The decrease in frequency can be described by a particular increment scheme that is not additive. In particular, hydroxylation at C-5 precludes all other effects.

Despite the apparent "insensitivity" of IR and ^{13}C NMR spectroscopies, the data suggest certain conclusions. The substituent effect on the reaction rate can be examined in three ways [12]: 1) The substituents can significantly change the ionization potential and electron affinity of the starting molecule and, as a result, the polarity of the corresponding reaction. This will cause a fundamental change in the potential surface describing the reaction; 2) The substituents determine the symmetry of a given molecule and, therefore, the ability to intersect two diabatic surfaces; 3) The substituents control the density of various potential-energy surfaces.

The examined spectroscopic data suggest that the first effect to a first approximation is constant for the examined compounds, which are hydroxylated at C-5. Thus, the effect of hydroxylation on the reactivity of the carbonyl (but not the phenols) may be related only to steric factors, which determine the availability of this site.

Kaempferol with pyranose (**1a**) and furanose (**1b**) sugars has the fastest recombination rate with DPPH for the 3,7-dirhamnosides. This is consistent with the literature [2]. The sugar of 3-glycosides of the first group is typically either a 6-deoxysugar or a 6-disaccharide. However, the 3-glucosides and 3-galactosides of these same aglycones fall in the third group.

As already mentioned, the change of reactivity for the second group is determined by the number of phenol groups. In biological experiments [4], luteolin, which has three OH groups that do not participate in H-bonding, exhibits the greatest activity compared with kaempferol (two similar OH groups) and slightly reduced activity compared with quercetin (three OH groups). This suggests that the antioxidant activity [4] is mainly due to the properties of flavonoids as phenols whereas our data for recombination with DPPH are determined mainly by the keto groups in the flavonoids. Obviously there is no appropriate basis for comparing these results. It can only be noted that there will be a certain agreement supported by the correlation between the reactivity and number of OH groups that was discussed above and also by the data for compounds in the third group if the applicability of the conclusion about the inactivation of molecules by substituting phenols by sugars that was drawn previously [4] is limited to processes involving phenol OH groups.

The third group includes 3-glucosides, isoflavonoid **9**, and liquiritigenin **10**, which has a saturated C-2–C-3 bond. The structures of the last two compounds clearly indicate that the keto group has low reactivity. The phenol groups are involved in the slow recombination. The low reactivity of the 3-glycosides, the keto groups of which have a polarizability analogous in nature to that of the first group of compounds, is explained only by steric shielding of the keto group. With the exception of miricitrin (**4b**), all remaining compounds contain glucose and galactose as the sugars. Two intramolecular H-bonds can be expected to form in them. These sterically shield the keto group and prevent its interaction with the radical.

The high reactivity of analogous compounds of the first group, which have no oxygen atom on C-6″ and, therefore, cannot form two H-bonds, becomes understandable based on this explanation. Furthermore, the important role of the number and location of the oxygen-containing substituents on ring *B* becomes obvious. All 3-glycosides of the first group are kaempferol derivatives with only one OH group in the position *para* to C-4′. This makes it impossible for them to form two H-bonds regardless of the nature of the sugar on C-3. A second H-bond involving the oxygen atom of the rhamnose and the OH of ring *B* can form for miricitrin. The comparatively high reactivity of the third group can be explained by the large number of phenol OH groups.

Placing a substituent on C-7, which is distant from the keto group, has no effect on the recombination rate. Apparently the diffusion stage of the interaction of flavonoids with DPPH is insignificant in this model system. This feature can be assigned to a certain shortcoming of the prediction method used because the diffusion stage in an actual biological system must play a definite role.

The following conclusions can be made based upon the results:

A comparison of the recombination kinetics of a series of compounds with the stable radical DPPH provides a test for preliminary quantitative estimation of their radioprotective and antioxidant properties. For flavonoids, this test reveals mainly the reactivity of the carbonyl. The phenolic properties are much less evident.

Flavonols are the most reactive. The polarization of the carbonyl in flavones reduces the reactivity. Placing a substituent on C-7 and ring *B* has practically no effect on the reactivity. Glycosylating C-3 sharply reduces the reactivity if the sugar is sterically capable of forming two intramolecular H-bonds. The reactivity of the phenols, with the exception of a hydroxy group on C-5, is proportional to their number.

Lespedin, kaempferitrin, kaempferol, kaempferol-7-rhamnoside, kaempferol-3-robinoside, and robinin are the most promising compounds for application. Apigenin, apigenin 7-glucuronide, kosmosiin, roifolin, luteolin, and cinaroside are promising as reactive phenols.

EXPERIMENTAL

The recombination kinetics of a series of flavonoids (for structural formulas, see Figs. 1-3) with 2,2-diphenyl-1-picrylhydrazide (DPPH) was studied using the literature method [5]. The recombination rate was measured using the decrease of optical density of an alcohol solution of DPPH at 520 nm (recorded on a Spekord M-40 spectrophotometer) that occurred when the DPPH radical was destroyed.

Owing to the comparatively low solubility of the studied compounds, we used solutions of flavonoid glycosides [(3-4)·10^{-7} M] and DPPH [(4-6)·10^{-8} M]. It has been reported [5] that DPPH readily reacts with free radicals and dehydrogenates compounds with labile H atoms, including phenols. Therefore, the reaction of DPPH with phenol and alcohol OH groups was considered during the experimental determination of the keto reactivity. Model experiments in alcohol solutions of glucose and rhamnose showed that the reaction with DPPH occurs exceedingly slowly. The observed change of absorption starts only after about 100 h and is 25% decreased after 240 h.

A qualitative comparison of the reactivity of keto and phenol OH groups suggests the following (see Table 1). The recombination rate of aglycones with a OH on C-3 (kaempferol and miricetin) is much greater than that of DPPH reacting with apigenin and luteolin, which do not have a phenol OH on C-3. Apparently the dehydrogenation of the OH on C-3 is not involved in the recombination because several derivatives glycosylated at C-3 (**1a**, **1b**, **3b**, and **3c**) also have fast recombination rates.

The T_e values listed in Table 1 were determined as the time for the optical density of the solution to decrease $e = 2.71828$ times and were obtained from at least three experiments at a constant concentration C of a given flavonoid. The T_e values and the recombination constant $K = (T_eC)^{-1}$ were determined by least-squares analysis of the kinetic plots.

The compounds were identified using IR spectroscopy (Zeiss UR-20 spectrophotometer) and ^{13}C NMR (Tesla BS-587.A Fourier spectrometer).

REFERENCES

1. G. W. Plumb, K. R. Price, M. T. Garcia-Conesa, and G. Williamson, in: *Polyphenol Communications. XIX International Conference on Polyphenols*, **1**, 35 (1998).
2. G. G. Vatulina, S. A. Bol'shakova, T. N. Tuzhilkova, A. I. Bokova, B. N. Maksudova, A. G. Khaitbaeva, and S. Z. Mukhametzhanov, *Khim.-Farm. Zh.*, No. 5, 552 (1985).
3. V. G. Pivovarenko, A. V. Tuganova, L. F. Osinskaya, and Yu. D. Kholodova, *Khim.-Farm. Zh.*, No. 3, 14 (1997).
4. A. D. Gordienko, N. F. Komissarenko, V. V. Levchenko, and Ya. I. Khadzhai, *Khim.-Farm. Zh.*, No. 1, 121 (1988).

5. T. V. Pochinok, M. L. Tarakhovskii, V. A. Portnyagina, M. F. Denisova, V. A. Vonsyatskii, A. N. Aleksandrova, and V. A. Mel'nichuk, *Khim.-Farm. Zh.*, No. 5, 565 (1985).
6. T. G. Sagareishvili, M. D. Alaniya, and E. P. Kemertelidze, *Khim. Prir. Soedin.*, 647 (1983); 119 (1990).
7. T. G. Sagareishvili and M. D. Alaniya, *Izv. Akad. Nauk GSSR, Ser. Khim.*, **16**, No. 2, 155 (1990).
8. K. G. Shalashvili, *Khim. Prir. Soedin.*, 666 (1974).
9. G. C. Levy and G. L. Nelson, *Carbon-13 Nuclear Magnetic Resonance for Organic Chemists*, Wiley-Interscience, New York (1972).
10. J. B. Harborne and T. J. Mabry, *The Flavonoids: Advances in Research*, Chapman & Hall Ltd., New York and London (1982), p. 19.
11. I. P. Kovalev and E. V. Titov, *IR Absorption Spectra of Certain Groups of Naturally Occurring Compounds. Atlas* [in Russian], Khar'kov State Univ., Khar'kov (1966), p. 14.
12. N. Epiotis, *Structural Theory of Organic Chemistry*, Mir, Moscow (1984).