Eur J Med Chem (1995) 30, 141–146
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Theoretical and experimental study of flavones as inhibitors of xanthine oxidase

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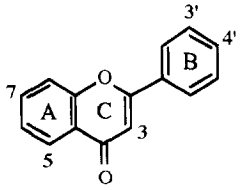
(Received 22 June 1994; accepted 11 October 1994)

Summary — Inhibitory activities of 19 polyhydroxylated and polymethoxylated flavones towards xanthine oxidase have been obtained by accurate measurements of the dissociation constants of enzyme–inhibitor complexes (K_{EI}). A topological description of the congeneric series has been adopted to derive quantitative structure–activity relationships (QSAR) with the use of multiple linear regression analysis. For interpretative purposes, molecular orbital calculations have also been performed. Inhibition appears to involve flavones as donors and the anion at the C-7 hydroxyl as the most active form in solution. Substituents in the 4*H*-1-benzopyran-4-one moiety can directly affect the availability of the C-7 anion in solution; substituents in the 2-phenyl moiety are probably involved in secondary local interactions with the enzyme.

flavones / xanthine oxidase / multiple linear regression analysis / QSAR / molecular orbital calculations

Introduction

Flavones (2-phenyl-4*H*-1-benzopyran-4-one derivatives) are a class of compounds widely present in nature. Their pharmacological and pharmaceutical functions



Flavone

have been reviewed by Havsteen [1]. More recently, flavones have been reported to be inhibitors of HIV-1 proteinase [2], hyaluronidase [3], prostaglandin endoperoxide synthase [4] and NAD(P)H-quinone acceptor oxidoreductase [5]. In addition, these compounds are known to inhibit xanthine oxidase [6–11], an enzyme which exists *in vivo* predominantly as an NAD+-dependent oxidase and converts hypoxanthine to xanthine and then to uric acid. A high production of uric acid leads to a high concentration of sodium urate in extracellular fluids ultimately causing gout. Xanthine oxidase can also be transformed into an oxygen-dependent oxidase during tissue ischemia. The activity of this form of the enzyme produces oxygen free radicals during riperfusion and causes severe tissue damage [12].

While the ability of flavones to inhibit xanthine oxidase is known, the problem of structure–activity relationships has so far been examined only at a qualitative level.

The main obstacle in the search for quantitative structure–activity relationships (QSAR) in flavones resides in the difficulties of identifying the chemical structures to which the activity should be ascribed. In fact, owing to the presence of several acidic hydroxyl substituents, flavones can be present in solution as complex mixtures consisting of both the undissociated species and several dissociated tautomeric species. For example, quercetin (3,5,7,3',4'-pentahydroxyflavone, compound **19F** in table I) can give rise to five anionic tautomeric forms in solution depending on which of the five hydroxyls is dissociated (fig 1). It follows that the overall inhibitory activity is the result of contributions coming from the different forms present in solution, each contribution depending on the abundance of that form and on its intrinsic activity, *ie* the activity that the particular form would have if it were present at 100%.

A true structure–activity relationship should consider the structure of each and every specific molecular form and its corresponding intrinsic activity. Since the individual molecular forms cannot be isolated, structural information cannot be directly obtained nor can intrinsic activities be measured.

On the other hand, the chemical formula of a flavone contains, albeit in a highly implicit language,

Table I. Substitution pattern, binary representation and inhibitory activities (pK_{EI}) of the 19 tested flavones.

N°	Substitution pattern					pKEI	Binary variables							
	3	5	7	3'	4'		H3	H5	H7	H3'	H4'	M7	M3'	M4'
1F	-	-	-	-	-	-	0	0	0	0	0	0	0	0
2F	-	OH	-	-	-	-1.93	0	1	0	0	0	0	0	0
3F	-	-	OH	-	-	-0.66	0	0	1	0	0	0	0	0
4F	-	-	OH	-	OCH_3	-0.04	0	0	1	0	0	0	0	1
5F	-	OH	OH	-	-	0.39	0	1	1	0	0	0	0	0
6F	-	OH	OH	-	OCH_3	1.38	0	1	1	0	0	0	0	1
7F	-	OH	OH	-	OH	0.98	0	1	1	0	1	0	0	0
8F	-	-	OH	OH	OH	0.55	0	0	1	1	1	0	0	0
9F	-	OH	OH	OCH_3	OH	1.24	0	1	1	0	1	0	1	0
10F	OH	OH	OH	-	OCH_3	1.36	1	1	1	0	0	0	0	1
11F	-	OH	OH	OH	OCH_3	1.84	0	1	1	1	0	0	0	1
12F	OH	OH	OCH_3	OH	OCH_3	0.55	1	1	0	1	0	1	0	1
13F	OH	OH	OH	-	OH	1.06	1	1	1	0	1	0	0	0
14F	OH	OH	OH	OCH_3	OH	1.19	1	1	1	0	1	0	1	0
15F	-	OH	OH	OH	OH	1.02	0	1	1	1	1	0	0	0
16F	OH	-	OH	OH	OH	0.64	1	0	1	1	1	0	0	0
17F	OH	OH	OCH_3	OH	OH	-0.50	1	1	0	1	1	1	0	0
18F	OH	OH	OH	OH	OCH_3	2.40	1	1	1	1	0	0	0	1
19F	OH	OH	OH	OH	OH	1.19	1	1	1	1	1	0	0	0

the complete structural information needed to describe the overall properties of the compound, including inhibitory activity. In our congeneric series, the structural information consists of the definition of a fixed reference skeleton (the 2-phenyl-4*H*-1-benzopyran-4-one moiety) and of a variable substitution pattern (hydroxyl and methoxyl groups) in five selected sites. A proper account of the substitution pattern can then provide a complete and explicit representation of the structural variation in the congeneric series.

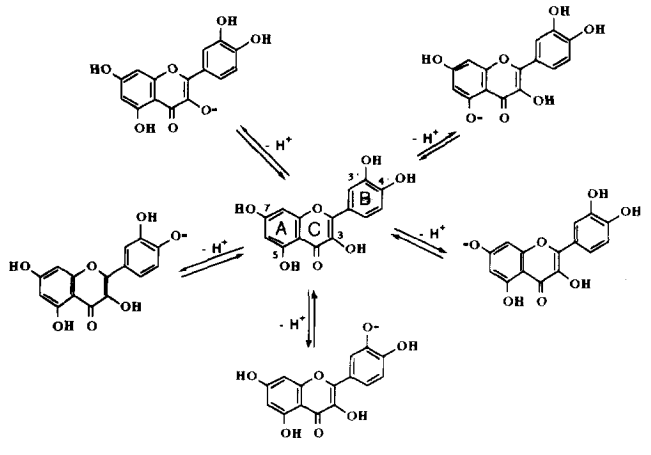


Fig 1. Dissociation equilibria of quercetin.

As regards the inhibitory activity of flavones, only a few of the compounds in table I had previously been tested as xanthine oxidase inhibitors. Biological assays have not even been performed in uniform manner [6, 8–11] and were therefore unsuitable for our purposes.

In this work we will report the dissociation constants of the enzyme–inhibitor complexes for 19 polyhydroxylated and polymethoxylated flavone inhibitors of xanthine oxidase. Moreover, the substitution pattern of the flavones will be encoded in topological binary variables to be used for developing QSAR models.

Multiple linear regression analysis will be carried out for highlighting both the basic requirements and the favourable combinations of substituents towards the inhibitory activity of flavones. In particular, the dissociated form most relevant to activity will be identified. This is an essential step for a more thorough

investigation of the relationship between the electronic structure of specific forms and their ability to inhibit the enzyme. To this end, the electronic features of a few different forms of flavones will also be calculated by means of the AM1 molecular orbital (MO) method and the MO results will then be combined with the statistical results in order to obtain satisfactory explanations.

Experimental protocols

Biological assays

The flavones reported in table I were purchased from Extrasynthese (Genay, France) at the highest level of purity. Xanthine and xanthine oxidase (from butter milk, 1.36 U/mg of protein) were purchased from Fluka. The flavones stock solutions were prepared in dimethylsulphoxide.

Xanthine oxidase activity was assayed spectrophotometrically in phosphate buffer (pH 7.6, ionic strength I = 0.1) at 20°C using a Perkin-Elmer UV/VIS λ15 spectrophotometer with a thermostated cuvette holder and the increase in uric acid (the product of the enzymatic reaction) concentration was evaluated at 295 nm. Kinetic studies were performed with at least three different concentrations of inhibitor in the presence of variable concentrations of xanthine (the substrate), three repetitions being performed at each concentration. The dissociation constants of the enzyme–inhibitor complexes (K_{EI}, μM) were determined from the slopes in double-reciprocal plots. A detailed description of the experimental protocol adopted for the biological assays is given in reference [7].

All the compounds under investigation showed mixed-type inhibition with respect to xanthine. The inhibitory activities reported in table I are expressed as $pK_{EI} = \log 1/K_{EI}$.

Multiple linear regression (MLR) analysis and molecular orbital calculations

MLR Analysis

Table I reports a topological description of the congeneric series of flavones made with the use of binary (0/1) variables. The possible hydroxyl and methoxyl substituents in their *n* positions are labelled as H*n* and M*n*, respectively, where *n* is restricted to the 3, 5, 7, 3' and 4' substitution sites of the reference skeleton.

MLR analyses were carried out with the automatized statistical package SPSS (version 5) on a personal computer.

The goals of the statistical analysis were to: i) identify the subset of variables most relevant to activity; ii) develop relationships between the inhibitory activity data and the set of independent structural parameters encoded in binary form; and iii) predict activities from the regression equation. The selection of the most significant variables was done with various procedures; the predictability of the model was also checked by the 'leave-one-out' validation test. The inactive compound **1F** was not used in statistics.

MO calculations

The AM1 [13] MO method was used to calculate the electronic descriptors of flavones. Geometries were completely optimized using the increased convergence criteria available in the AMPAC [14] program. While an extended set of MO descriptors of the neutral and dissociated forms is available; only highest occupied molecular orbital (HOMO) energies and coefficients will be discussed in the present work. (A complete description of the electronic features of the various forms is available upon request from the authors) Calculations were run on a Convex C-220 computer at the Centro Interdipartimentale di Calcolo Elettronico (CICAIA) of Modena, Italy.

Results and discussion

The results of the biological assays are reported in table I. These range from the inactive unsubstituted flavone (**1F**) to the highly active 3,5,7,3'-tetrahydroxy-4'-methoxyflavone (**18F**), spanning more than four orders of magnitude. From our biological results, compound **18F** turns out to be more than ten times as active as quercetin (**19F**), which is considered one of the most potent inhibitors of xanthine oxidase [8]; this suggests the possible use of compound **18F** as a therapeutic agent.

MLR analysis

The results of the MLR analysis are collected in table II. The independent variables H3, M7 and M3' were coherently excluded from the regression equation by all the three available selection procedures, *ie* forward, backward and stepwise procedures, when default thresholds were adopted (PIN ≤ 0.05, POUT ≥ 0.1). Likewise, the independent variables H7, H5 and M4' were found to be the most important whichever statistical index was chosen. The goodness of fit is documented by the values of the suitable parameters in table II and by the plot of figure 2. The predictability of the regression model was tested by the 'leave-one-out' validation procedure and found to be satisfactory; figure 3 reports the plot of experimental *versus* predicted activities. Comparison of the two plots shows that compounds **2F** and **18F**, which are at the extremes of the explored range of activity, contribute significantly to the quality of the regression model, but also that their exclusion does not prevent acceptable fits to be obtained (see also significance levels in table II).

Owing to the binary 0/1 code adopted for the independent variables, each regression coefficient in the statistical model directly represents (on the pK_{EI} scale) the substituent/position contribution to activity, whereas the regression constant (–2.69) represents the activity of the unsubstituted compound **1F**. Such a low value for **1F** agrees with the finding that activity could not be detected for this compound at the concentrations allowed by its solubility.

It is important to note that the regression coefficients of variables are all positive, so that any substitution in the reference structure (limited to OH and OMe substituents in the relevant 5, 7, 3' and 4' pos-

Table II. Multiple linear regression analysis*.

	$B \pm se$[a]	β[b]	t[c]	$Sig\ t$[d]	$Bval$[e]
Variables in the equation					
H7	1.88 ± 0.23	0.720	8.10	0.0000	1.69 ± 2.14
M4'	1.22 ± 0.26	0.589	4.69	0.0005	1.06 ± 1.52
H5	1.06 ± 0.20	0.452	5.40	0.0002	0.76 ± 1.14
H4'	0.71 ± 0.25	0.366	2.80	0.0161	0.56 ± 0.87
H3'	0.55 ± 0.18	0.280	3.04	0.0103	0.37 ± 0.62
Const	−2.69 ± 0.30		9.04	0.0000	−2.36 ± 2.88
Variables out of the equation					
H3		0.123	1.40	0.1879	
M7		0.208	1.41	0.1872	
M3'		0.139	1.54	0.1519	

[a]Regression coefficients ± estimated standard errors; [b]standardized regression coefficients; [c]Student's t; [d]significance level of t; [e]partial regression coefficient interval in the 'leave-one-out' validation test. **Parameters of the regression*: $N = 18$; $s = 0.328$, standard error of the regression; $R^2 = 0.93$, multiple correlation coefficient; $F = 30.46$, Fisher F-test; R^2 adj = 0.90, adjusted R^2; Sig $F = 0.0000$, significance level of F. *Predictability from 'leave-one-out' validation*: $N = 18$; $s = 0.458$, standard error of the validation series; $R^2 = 0.80$, multiple correlation coefficient; $F = 65.26$ Fisher F-test; pK_{EI} exp = (0.94 ± 0.12)pK_{EI} pred + (0.03 ± 0.14).

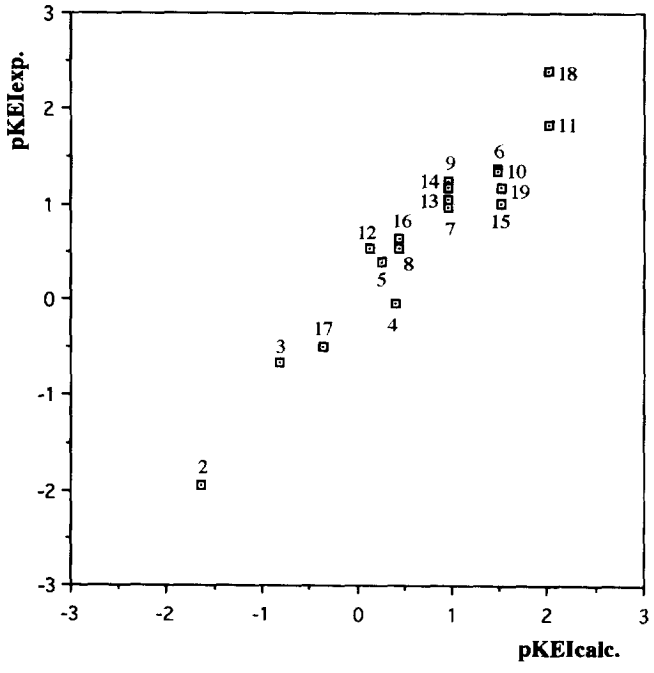


Fig 2. Plot of experimental inhibitory activity *versus* calculated activity (regression equation).

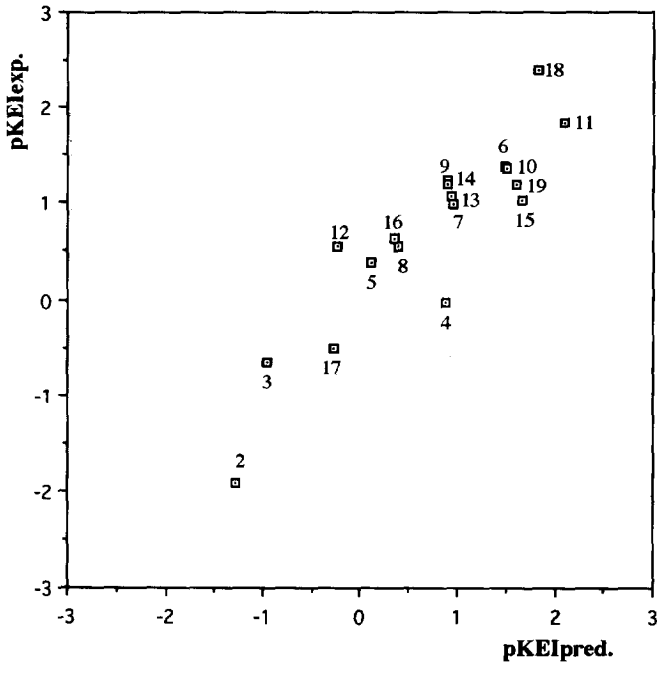


Fig 3. Plot of experimental inhibitory activity *versus* predicted activity ('leave-one-out' validation test).

Table III. pK_a values of mono- and dihydroxyflavones[a].

	pK_a	*Reference*
F(3)	9.6	15
F(5)	11.6	16
F(7)	8.12	17
F(3')	9.75	18
F(4')	9.14	17
F(5,7)	7.30	17
F(3,7)	8.30	17

[a]The numbers in parentheses indicate the position of the hydroxyl substituents.

itions) increases the inhibitory activity of the compounds.

Interpretation of the MLR equation

Let us now try to interpret the multiple regression equation, by using the available information on mono- and dihydroxylated flavones not included in table I.

The presence of a hydroxyl in C-7 is the highest determinant of activity. Inspection of the available pK_a of monohydroxyflavones [15–18] in table III reveals that position 7 bears the most acidic hydroxyl (pK_a = 8.12). This correspondence strongly suggests a connection between inhibitory activity and availability of anionic forms. Accordingly, hydroxyls at the less acidic 3, 5, 3' and 4' positions show lower contributions to activity. Later on, however, on the basis of frontier orbital arguments, we will maintain that anions at positions different from C-7 ought to be scarcely active even if they were present in solution.

The main reason why a hydroxyl in C-5 can effectively enhance activity must be completely different: indeed, its very high pK_a (pK_a = 11.6) is not consistent with an appreciable presence of its anion. Most probably, the inductive effect of the C-5 hydroxyl is the cause of the increased acidity of the C-7 hydroxyl (pK_a = 7.3 of the 5,7-dihydroxyflavone in table III) with subsequent increased concentration of the anion at the pH of the biological assay. No pK_a lowering is observed in 3,7-dihydroxyflavone (pK_a = 8.3), in agreement with the minor role of H3 in the regression analysis and, from a chemical point of view, with the unlikely interaction of the C-3 hydroxyl with the distant C-7 hydroxyl. The acidity of the C-4' hydroxyl (pK_a = 9.14 in 4'-hydroxyflavone) is consistent with about 3% of anion in solution. However, the finding that the C-4' methoxyl is more effective for activity than the C-4' hydroxyl leads us to conclude that the 4' substitution site is probably involved in effects that do not concern dissociation. Finally, the C-3' site has little relevance on activity both on account of the high pK_a of the hydroxyl group and of the uninfluential effect exherted by a methoxyl group in this position.

Support to the above interpretation of the regression equation can be obtained with the use of arguments in the field of theoretical organic chemistry. In the formation of enzyme–inhibitor complexes, anionic and neutral forms show very different behaviour both in the recognition step, where long-range electrostatic interactions dominate, and in the subsequent binding step, where short-range charge-transfer interactions largely contribute to the stabilization of the complex. As regards the binding step, in particular, the proposed importance of the anionic form suggests that the inhibitors act as donors in the charge-transfer mechanism. The donor properties are well represented, according to the frontier orbital theory, by the coefficients of the HOMO and by its energy, the former being able to signal the molecular regions of possible interactions, the latter the aptitude for interaction.

Figure 4 shows some molecular diagrams of HOMOs of neutral and anionic forms of flavones. The active anions in C-7 of polysubstituted flavones show

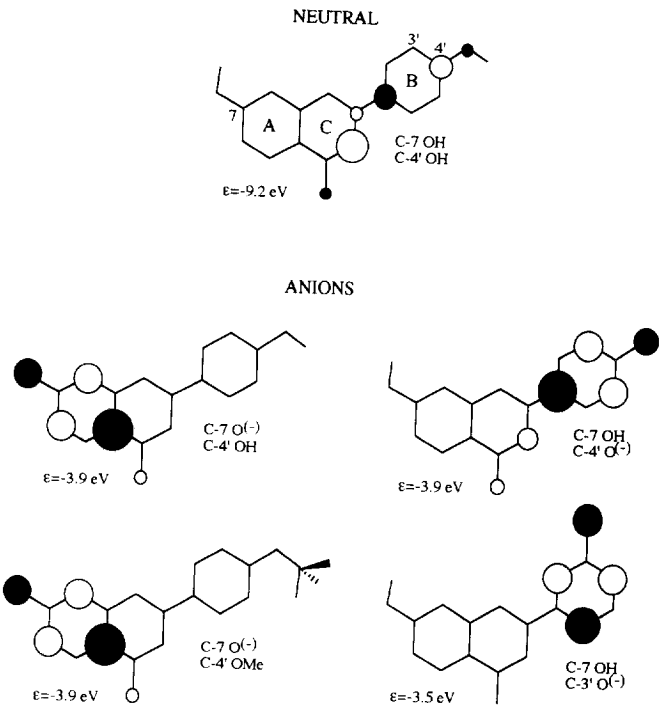


Fig 4. Molecular diagrams of HOMOs of neutral and anionic flavones. Circled area represents MO coefficient magnitude, black (white) denotes positive (negative) sign.

HOMOs localized on the fragment A, whereas anions in C-4' and C-3' have their HOMOs mainly localized on the fragment B (fig 4). This clear-cut distinction places the anions in two qualitatively different and unrelated classes of donors, unable, most likely, to both be effective toward the same acceptor (the enzyme). In particular, one can maintain that anions in C-4' and C-3' ought to be poorly active, since anions in C-7 are certainly active. For this argument, however, it is important to note that the pattern of the HOMO is necessarily related to the pattern of the molecular electrostatic potential (MEP), so that the two classes of anions are also well differentiated by their MEPs. It follows that active and poorly active anions might exhibit their electronic differences either in the recognition step or in the binding step (and even in both steps). Evidently, HOMO and MEP results alone cannot solve the mechanistic aspects involved in inhibition. Nevertheless, they nicely depict the differences in electronic features that are relevant to the interpretation of the QSAR results.

Once the above reservations are settled, the comparison of HOMOs can also be used to explain why neutral flavones are much less active than C-7 anions. In this case, different HOMO patterns are coupled with much higher ionization potentials (lower HOMO energies) for the neutral than for the anionic forms (fig 4).

Finally, the HOMO coefficients and energies of C-7 anions, either with hydroxyl or methoxyl (or none) substituents in C-4', are almost identical. This finding helps in concluding that electronic effects are not transmitted from the B to the AC fragment and that the increased activity due to the C-4' hydroxyl/methoxyl substitution can be ascribed to local secondary effects.

Conclusion

Statistical analysis combined with chemical information led us to explain the trend of the measured biological activity of a set of flavones as inhibitors of xanthine oxidase and to propose the following temporary conclusions about the structure–activity relationships.

Flavones act as donors in their interaction with the enzyme. Anionic forms have much lower ionization potentials (higher HOMO energies) and are more effective donors than neutral forms. Anions at the C-7 hydroxyl show very high activity, whereas anions at the C-4' or C-3' hydroxyls, having a completely different HOMO structure, are not suitable for the same interactions with the enzyme and cannot be highly active.

The availability of the active anion in solution is guaranteed by the low pK_a of the C-7 hydroxyl compared to hydroxyls in the other positions. In this context, a hydroxyl in C-5 enhances activity because it is able to lower the pK_a of the C-7 hydroxyl *via* inductive effects.

The C-4' substitution site appears to be mainly involved in local secondary interactions with the enzyme. However, this remark, as well as any role of substituents at the 2-phenyl moiety, needs to be further validated.

Acknowledgments

Financial support from the CNR (grant 92.00189.CT.03) is gratefully acknowledged.

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