Eur J Med Chem (1996) 31, 693–699

A rational approach to the design of flavones as xanthine oxidase inhibitors

L Costantino, G Rastelli, A Albasini

Dipartimento di Scienze Farmaceutiche, Università di Modena, Via Campi 183, 41100 Modena, Italy

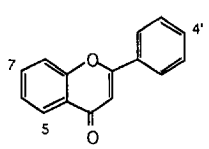
(Received 3 January 1996; accepted 11 March 1996)

Summary — In the light of previous QSAR studies on flavones as inhibitors of xanthine oxidase, we synthesized and tested a new series of 7-hydroxyflavones carrying a wide and balanced variety of substituents (π, σ_p) at the 4' position in order to explore the effect of substituents at this position on the xanthine oxidase inhibitory activity. The results of pK_a determinations show that the electronic effects of the substituents are not transferred to the hydroxyl at C7, previously found to be fundamental for activity. An excellent correlation is found between molar refractivity of the substituents and the inhibitory activity. These results, applied to the more active 5,7-dihydroxyflavones, allowed the design and synthesis of a very active inhibitor, with an IC_{50} in the nanomolar range. On interpretative grounds, C4' substituents of flavones are involved in dispersion interactions with the enzyme. The calculation of quantum chemical polarizabilities and solvent accessible surface areas suggests the existence of π–π stacking interactions with an aromatic aminoacidic residue of the enzyme.

substituted flavone / xanthine oxidase / proton dissociation constant / QSAR

Introduction

In a previous paper [1], a series of polyhydroxylated and polymethoxylated flavones (2-phenyl-4*H*-1-benzopyran-4-one derivatives) was tested for inhibition of xanthine oxidase, and statistical analysis was performed to derive quantitative structure–activity relationships. In order to interpret the statistical results, quantum chemical calculations of some selected electronic properties of flavones were also performed [1].



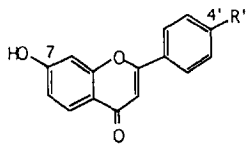
Despite the heterogeneity of the series considered, two important findings emerged. First, given that the polyhydroxylated flavones are in equilibrium between neutral and dissociated forms in solution, it was concluded that they are active in their dissociated (anionic) form; in particular, flavones were proposed to act as donors in the interaction with the enzyme by means of the anionic species that originate from proton dissociation of the C7-hydroxyl. Second, substituents at the C4' substitution site appeared to be mainly involved in local secondary interactions with the enzyme that do not require proton dissociation at the 2-phenyl moiety of the inhibitor [1].

In the present work, we determine the effects of substituents at the C4' substitution site on the activity of flavones by means of the design, synthesis and biological evaluation of a broad new series of C4'-substituted compounds. In order to limit the number of forms (neutral and anionic) present in solution, only the C7-hydroxyl, which is crucial to activity, is retained. C4' substituents were varied in order to explore maximum variations in the hydrophobic (Hansch's π) and electronic (Hammett's σ_p) constants of substituents (table I) [2]. In order to find out whether the C4' substituents are able to affect the acidity of the C7 hydroxyl, the dissociation constants of some selected flavones were examined.

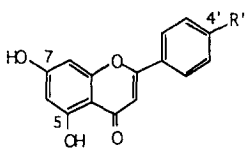
On the basis of the results obtained, some selected 5,7-dihydroxy-4'-substituted flavones were synthesized and tested, since the presence of an additional hydroxyl at C5 has been found to increase the inhibitory activity by about one order of magnitude [1].

The aim of the present work is to obtain new xanthine oxidase inhibitors with a reduced number of hydroxyl substituents and an improved inhibitory activity. The former aspect is particularly important in light of the very poor selectivity of natural polyhydroxylated flavones, such as quercetin (3,5,7,3',4'-

Table I. Substitution pattern, substituent descriptors and xanthine oxidase inhibitory activity of the flavones.



Compound	*R'*	π^a	$\sigma_p{}^a$	*IC_{50} M (95% CL)*
1	$-NH_2$	–1.23	–0.66	$1.95\ (1.73–2.20)\times10^{-5}$
2	-CN	–0.57	–0.66	$1.05\ (0.75–1.48)\times10^{-5}$
3	$-NO_2$	–0.28	0.78	$9.72\ (7.13–13.25)\times10^{-6}$
4	$-OCH_3$	–0.02	–0.27	$7.04\ (6.99–7.09)\times10^{-6}$
5	-H	0.00	0.00	$3.76\ (3.51–4.03)\times10^{-5}$
6	$-N(CH_3)_2$	0.18	–0.83	$2.04\ (1.82–2.28)\times10^{-6}$
7	$-OC_2H_5$	0.38	–0.24	$2.94\ (2.61–3.31)\times10^{-6}$
8	$-CH_3$	0.56	–0.17	$1.34\ (1.17–1.54)\times10^{-5}$
9	-Cl	0.71	0.23	$9.85\ (9.03–10.74)\times10^{-6}$
10	$-CF_3$	0.88	0.54	$1.35\ (1.29–1.41)\times10^{-5}$
11	$-C_2H_5$	1.02	–0.15	$5.32\ (4.81–5.89)\times10^{-6}$
12	$-OCF_3$	1.04	0.35	$6.80\ (5.53–8.36)\times10^{-6}$
13	$-O\text{-}n\text{-}C_4H_9$	1.55	–0.32	$1.41\ (1.08–1.84)\times10^{-6}$
14	$-n\text{-}C_3H_7$	1.55	–0.13	$3.57\ (3.25–3.91)\times10^{-6}$
15	$-C_6H_5$	1.96	–0.01	$3.47\ (2.97–4.05)\times10^{-7}$
16	$-t\text{-}C_4H_9$	1.98	–0.20	$2.68\ (2.16–3.34)\times10^{-6}$
17	$-n\text{-}C_4H_9$	2.13	–0.16	$1.84\ (1.50–2.24)\times10^{-6}$



3a	$-NO_2$	$1.14\ (1.07–1.21)\times10^{-6}$
4a	$-OCH_3$	$2.20\ (1.96–2.46)\times10^{-7}$
5a	-H	$1.60\ (1.41–1.82)\times10^{-6}$
15a	$-C_6H_5$	$1.36\ (1.19–1.55)\times10^{-8}$
16a	$-t\text{-}C_4H_9$	$9.08\ (8.50–9.70)\times10^{-8}$
Quercetin		$3.83\ (3.69–3.97)\times10^{-7}$

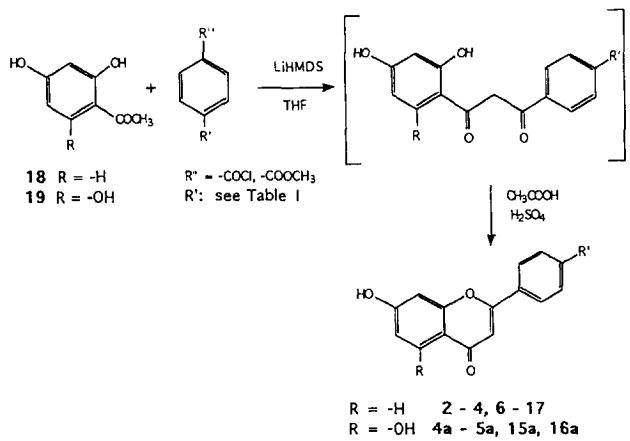
[a]From ref [2].

pentahydroxyflavone), which is very active not only as a xanthine oxidase inhibitor [1] but also towards a number of biological targets [3].

On interpretative grounds, the present findings will also afford insight into the interactions between flavones and the inhibitory binding site of xanthine oxidase.

Chemistry

Compounds **2–4**, **6–17**, **3a–5a**, **15a** and **16a** were prepared following the general procedure described in [4] (scheme 1). The hydroxylated acetophenones **18** and **19** were treated with enough lithium bis(trimethyl)-silyl amide (4 and 5 equiv respectively for **18** and **19**) to deprotonate all of the phenols as well as generating the lithium enolate of the ketone. The acid chlorides or, in the case of **6**, the ester [5] were added and the intermediate 1,3-diketones were then cyclized to yield the compounds in question. The synthesis of **3a** required the use of 2,4-bis(benzyloxy)-6-hydroxyacetophenone, prepared according to [6]; debenzylation was performed with BBr_3. Compound **1** was prepared by catalytic hydrogenation of **3**, according to [7].



Scheme 1.

Results and discussion

The results of the biological assays are reported in table I. All the C4'-substituted compounds display inhibitory activities higher than those of the unsubstituted (R' = H) reference compound **5**; this finding confirms the importance of the substitution at the position 4' of flavones for their interaction with xanthine oxidase.

Since the anionic form generated by the C7-hydroxyl is the main species responsible for activity, the first priority was to find out whether C4' substituents are able to affect the acidity of the C7-hydroxyl. Table II reports the dissociation constants (pK_a values) of some selected flavones bearing both electron-withdrawing and electron-donating substituents. The finding that these C4'-substituted compounds have pK_a values very similar to that of the unsubstituted reference compound **5** allows us to conclude that the observed increase in inhibitory activity is not due to an increase in concentration of the anionic form in solution. Compounds **1** (R' = NH_2) and **3** (R' = NO_2), chosen at the extremities of the σ_p

Table II. Proton dissociation constants (pK_a) of selected flavones.

Compound	*R'*	*pK_a (±SD[a]) (I = 0.1 M, 25 °C)*	*[Ionized/neutral] (pH 7.60)*
1	$-NH_2$	7.34 ± 0.06	1.82
2	-CN	7.06 ± 0.07	3.47
3	$-NO_2$	7.03 + 0.04	3.72
5	-H	7.19 ± 0.03	2.57
8	$-CH_3$	7.26 ± 0.03	2.19
5a	-H	7.12 ± 0.02	3.02

[a] SD: standard deviation.

scale, have pK_a values that differ by only 0.3 units. Even considering σ_p as a local descriptor of the electronic availability of substituents, a complete absence of correlation in the plot of σ_p versus –log IC_{50} is observed.

Better results can be obtained by plotting the hydrophobic constants of substituents (π) versus the values of –log IC_{50} (fig 1); a trend can now be recognized in which the more hydrophobic substituents have higher values of –log IC_{50}. However, the correlation is rather low (regression coefficient r = 0.69, Fisher's F-test F = 13.5), and a certain split between purely hydrocarbon substituents and substituents containing heteroatoms can be observed. Therefore, the classical hydrophobic effect encoded into the partition coefficient between water and octanol (π) reflects the preference for non-polar substituents in our series, but is not able to represent the type of molecular interactions with the enzyme; this is because π mainly represents the cavity repulsion effects and the rearrangement of water around the solute, but does not reflect the attractive dispersion interactions with the enzyme.

One molecular descriptor frequently interpreted as reflecting drug–receptor dispersion interactions is molecular refractivity (MR) [8]. The values of MR for the substituents are reported in table III. An excellent correlation between MR and –log IC_{50} is found (fig 2, r = 0.96, F = 187.3): the higher the MR values, the higher the inhibitory activity. This correlation suggests that C4'-substituents are probably involved in dispersion interactions with the enzyme. On interpretative grounds, MR does not have a purely steric character but, being proportional to the polarizability of the molecule, it also contains an electronic component. This ambiguity prevents a distinction being drawn between steric and electronic aspects of the

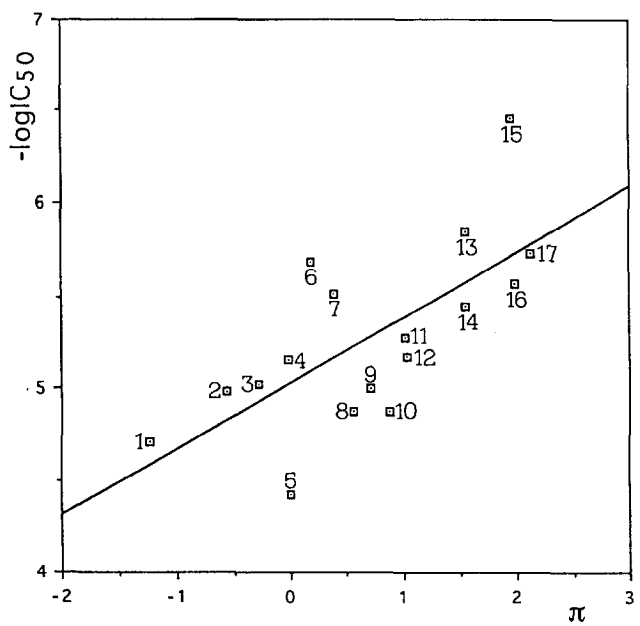


Fig 1. Plot of xanthine oxidase inhibitory activity (–log IC_{50}) versus π of substituents of 7-hydroxyflavones **1–17**. Regression equation: –log IC_{50} = 0.36π + 5.03; r = 0.69, F = 13.5.

Table III. Molecular refractivities (MR) of the substituents, calculated polarizabilities (α) and solvent accessible surface area (sasA) of flavones.

Compound	*R'*	*MR[a]*	*α (Å^3)*	*sasA (Å^2)*
1	$-NH_2$	5.42	24.46	247.7
2	-CN	6.33	26.04	254.9
3	$-NO_2$	7.36	25.96	256.4
4	$-OCH_3$	7.87	25.04	263.2
5	-H	1.03	22.62	236.2
6	$-N(CH_3)_2$	15.55	27.10	282.7
7	$-OC_2H_5$	12.47	26.30	284.3
8	$-CH_3$	5.65	24.11	254.4
9	-Cl	6.03	24.47	253.2
10	$-CF_3$	5.02	24.43	263.9
11	$-C_2H_5$	10.30	25.18	271.9
12	$-OCF_3$	7.86	25.15	272.8
13	$-O-n-C_4H_9$	21.66	28.55	321.6
14	$-n-C_3H_7$	14.96	26.41	290.1
15	$-C_6H_5$	25.36	31.71	311.2
16	$-t-C_4H_9$	19.62	27.36	298.1
17	$-n-C_4H_9$	19.69	27.50	308.4
3a			26.35	260.9
4a			25.49	267.6
5a			23.05	240.8
15a			32.15	315.8
16a			27.80	302.8

[a]From ref [2].

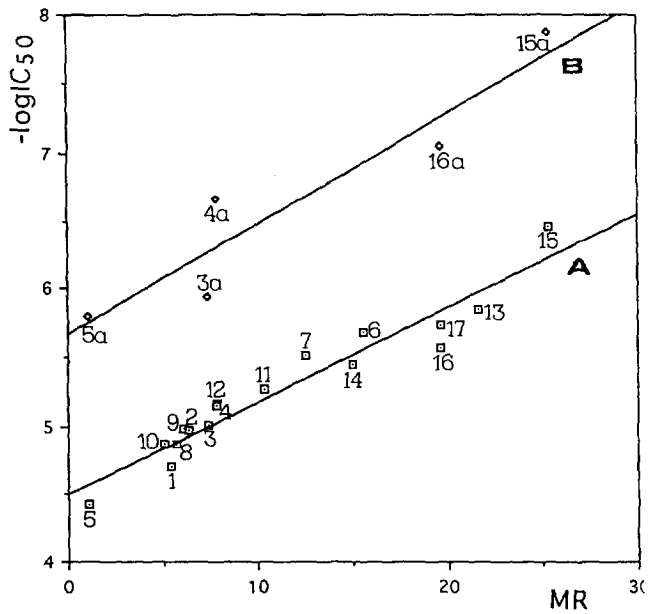


Fig 2. Plot of xanthine oxidase inhibitory activity (–log IC_{50}) versus MR of substituents of 7-hydroxyflavones **1–17** (**A**) and of 5,7-dihydroxyflavones **3a–5a**, **15a** and **16a** (**B**). Regression equations: (**A**) –log IC_{50} = 0.07 MR + 4.51, r = 0.96, F = 187.3; (**B**) –log IC_{50} = 0.08 MR + 5.67, r = 0.95, F = 26.0.

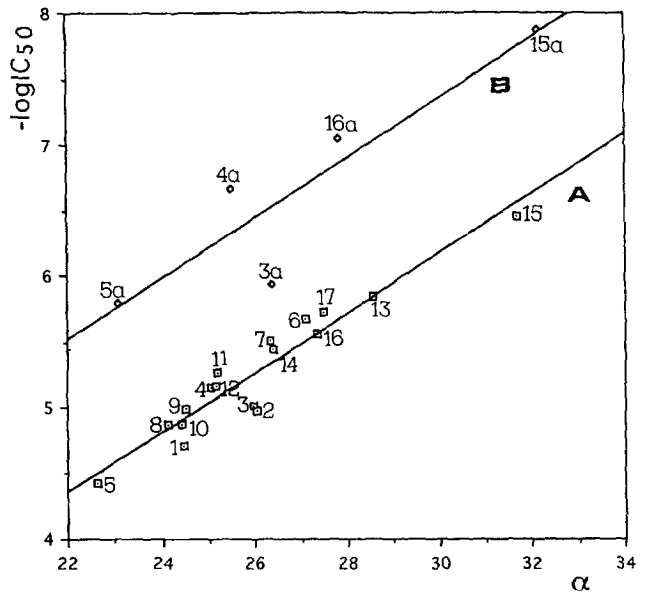


Fig 3. Plot of xanthine oxidase inhibitory activity (–log IC_{50}) versus the calculated polarizabilities (α) of 7-hydroxyflavones **1–17** (**A**) and 5,7-dihydroxyflavones **3a–5a**, **15a** and **16a** (**B**). Regression equations: (**A**) –log IC_{50} = 0.23α – 0.62, r = 0.95, F = 148.6; (**B**) –log IC_{50} = 0.23α + 0.49, r = 0.91, F = 14.8.

molecular interactions. In order to gain insight into this problem, the polarizabilities [9] of the flavones in table I were calculated with quantum chemical methods. There is an excellent correlation between the calculated polarizabilities and the inhibitory activity (fig 3); this correlation, due to the proportionality between MR and polarizability, resembles that of figure 2. An attempt to distinguish the 'pure' molecular-size part from the electronic part was made by computing the solvent accessible surface area (sasA) of the flavones; this surface is that traced out by the surface of a probe sphere (water) rolling over the van der Waals surface of the molecule [10]. Interestingly, a satisfactory correlation between sasA and polarizability is found (fig 4), with the exception of the phenyl-substituted compound **15**. The phenyl ring, in fact, has a much higher polarizability than is expected from its sasA value. In turn, sasA is correlated with –log IC_{50} value, again with the exception of the phenyl derivative. Therefore, the molecular-size part is determinant for activity, but is not able to account for the peculiarity of the phenyl derivative. We propose that the phenyl ring interacts with an aromatic residue of the enzyme binding site (a phenylalanine, a tyrosine or a tryptophan) by means of π–π stacking interactions.

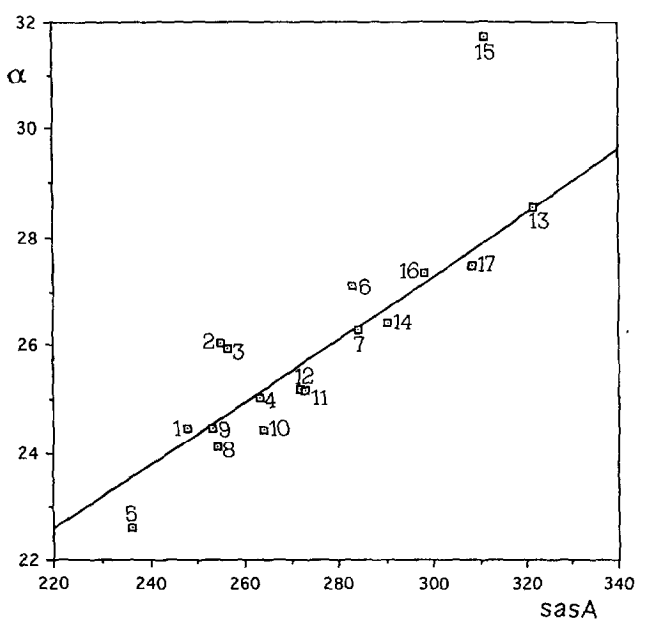


Fig 4. Plot of the calculated polarizability (α) versus the solvent accessible surface area (sasA) of 7-hydroxyflavones **1–17**. Regression equation: α = 0.06 sasA + 9.75 (compound **15** excluded), r = 0.90, F = 59.9.

This would explain the peculiarity of the phenyl derivative, which is the only aromatic substituent present in our series that is able to take advantage of its very high polarizability to enhance the attractive dispersion interactions with one of these aromatic aminoacidic residues.

Finally, we report on the activity of a restricted number of 5,7-dihydroxy derivatives bearing the most interesting C4'-substituents developed here, since the presence of an additional C5-hydroxyl was found to increase the inhibitory activity by about one order of magnitude [1] without introducing new dissociation equilibria (pK_a = 11.6) [11]. The results reported in table I confirm the higher inhibitory activity of the 5,7-dihydroxyflavones with respect to the corresponding 7-hydroxyflavones. Compound **15a**, bearing the phenyl substituent at C4', is a very potent inhibitor of xanthine oxidase; it is about one order of magnitude more active than quercetin (table I), which has long been considered a potent inhibitor of this enzyme [1, 12], but with the undesirable property of inhibiting many other enzymes [3].

It is worth noting that the inclusion of the 5,7-dihydroxy derivatives in the plot of figure 2 (–log IC_{50} versus MR) shows that 5,7- and 7-derivatives have coherent trends, the former compounds being shifted only about one order of magnitude up the activity scale. The increase in activity is not attributable to a variation in the acidity of the C7-hydroxyl, as was previously hypothesized [1] on the basis of literature pK_a values [11, 13], since 5,7-dihydroxyflavone has a very similar pK_a value to that of 7-hydroxyflavone (table II). Neither is it due to an increase in the polarizability of the molecule, because the 5,7-dihydroxy derivatives show a parallel trend to that of the 7-hydroxyflavones, also in the plot of –log IC_{50} versus the calculated polarizability (fig 3). These results indicate that 5,7-dihydroxy and 7-hydroxy derivatives exhibit a similar type of binding to xanthine oxidase; they probably enter the binding site cavity oriented in a similar way to ensure a strong electrostatic interaction of the negatively charged oxygen of the C7-hydroxyl, and place the 2-phenyl moiety in the proper position to enhance dispersion interactions with the enzyme. In this context, the C5-hydroxyl could probably hydrogen bond to one residue of xanthine oxidase.

Experimental section

Organic synthesis

Reagent-grade chemicals were used without purification. UV-vis spectra were obtained with a Perkin-Elmer spectrophotometer mod λ16 equipped with a thermostatted cuvette holder. Melting points were determined on a Buchi 510 melting point apparatus and are uncorrected. ^{1}H-NMR (400 MHz) and ^{13}C-NMR spectra (100 MHz) were recorded on a Bruker AMX 400 (Centro Interdipartimentale grandi Strumenti, Modena University) in DMSO-d_6. Chemical shifts are reported in ppm from tetramethylsilane as internal standard. J values are given in Hz. The following abbreviations are used to designate the multiplicity of individual signals: s = singlet; d = doublet, dd = two doublets and m = multiplet. Microanalyses were carried out in the Microanalysis Laboratory of the Dipartimento di Scienze Farmaceutiche, Modena University. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. Mass spectra were recorded on a Finnigan MAT SSQ 710. TLC was performed on precoated silica-gel F254 plates (Merck). Silica-gel (Merck; 70-230 mesh) was used for column chromatography. Quercetin was purchased from Fluka; 7-hydroxyflavone (**5**) was purchased from Roth.

*General procedure for the synthesis of 7-hydroxyflavones **1–4** and **6–17***

A solution of lithium bis(trimethyl)silyl amide (LiHMDS) in tetrahydrofuran (1 M, 40 mmol) was added to a well-stirred solution of 2,4-dihydroxyacetophenone **18** (10 mmol) in THF under a nitrogen atmosphere at –78 °C over 15 min. The reaction mixture was stirred at –78 °C for 3 h and a solution of the approriate benzoyl chloride (10 mmol) or, in the case of **6**, of methyl-4-dimethylamminobenzoate (10 mmol), in THF (20 mL) was added over 10 min. Stirring was continued for 1 h at –78 °C and at room temperature for 4 h (for 24 h in the case of **6**). Then the reaction mixture was poured into a mixture of ice water (250 g) and HCl (10 mL). It was extracted with $CHCl_3$ (3 × 25 mL) (in the case of **6** the reaction mixture was treated with 4% NaOH up to pH 5) and the combined extracts were dried (Na_2SO_4). The solvent was evaporated and the residue mixed with glacial acetic acid (50 mL) and H_2SO_4 (0.25 mL) and heated at 100 °C for 1 h. Water was added (in the case of **6**, diethyl ether) and the product was filtered, washed with water (diethyl ether for **6**) and crystallized from acetone/petrol ether bp 40–60 °C. In the case of **6**, the crude product was dissolved in methanol (100 mL), treated with water (50 mL) and brought to pH 5 with a solution of 4% $NaHCO_3$. The product was filtered and crystallized from acetone/petrol ether bp 40–60 °C (table IV).

*General procedure for the synthesis of 5,7-dihydroxyflavones **3a-5a**, **15a** and **16a***

The synthesis of compounds **4a–5a, 15a** and **16a** was performed starting from anhydrous 2,4,6-trihydroxyacetophenone **19** and five equivalents of LiHMDS. In this case, however, it was necessary to partially purify the intermediate 1,3-diketone by means of column chromatography (mobile phase cyclohexane/ethyl acetate 7.5:2.5 v/v, $R_f \approx 0.3$) before the treatment with H_2SO_4/CH_3COOH (table IV). For the synthesis of **3a**, starting from 1.00 g (2.87 mmol) of 2,4-bis(benzyloxy)-6-hydroxyacetophenone [6] and LiHMDS (8.61 mmol), 0.77 g (56%) of crude 5,7-bis(benzyloxy)-4'-nitroflavone were obtained. Mp 220–222 °C, ^{1}H-NMR: 5.37 (4H, s, CH_2), 6.63 (1H, d, J = 2.09, H-6), 7.05 (1H, d, J = 2.09, H-8), 7.35 (1H, s, H-3), 7.52 (10H, m, ArH), 8.48 (4H, m, ArH); mass spectrum m/z: 479 (M^+). This product (0.30 g, 0.63 mmol) was dissolved in CH_2Cl_2 (35 mL) under a nitrogen athmosphere, cooled to 0 °C and a solution of BBr_3 (1 M in CH_2Cl_2, 4.38 mL, 4.38 mmol) was slowly added over 10 min. The reaction mixture was stirred at room temperature for 24 h, cooled again to 0 °C and ice was added (5 g); the solid thus obtained was filtered, washed with water, chromatographed (cyclohexane/ethyl acetate, 8:2 v/v) and finally crystallized from acetone to yield **3a** (0.10 g, 53%) (table IV).

Table IV. Yields, physicochemical properties and ^{1}H-NMR data for compounds **1–5**, **6–17**, **3a–5a**, **15a** and **16a**.

Compound	*Mp (°C)*	*Formula*	*Anal*	*Yield (%)*	*^{1}H-NMR*[a]
1	> 300[b]	$C_{15}H_{11}NO_3$	C, H, N	6	6.02 (2H, br s, NH), 6.67 (1H, s, H-3), 6.77 (2H, m, H-3', H-5'), 7.81 (2H, m, H-2', H6')
2	291–293	$C_{16}H_9NO_3$	C, H, N	63	7.16 (1H, s, H-3), 8.13 (2H, m, H-3', H-5'), 8.35 (2H, m, H-2', H-6')
3	> 300[b]	$C_{15}H_9NO_5$	C, H, N	50	7.20 (1H, s, H-3), 8.45 (4H, m, ArH)
4	260-263[b]	$C_{16}H_{12}O_4$	C, H	73	3.95 (3H, s, OCH_3), 6.89 (1H, s, H-3), 7.20 (2H, m, H-3', H-5'), 8.11 (2H, m, H-2', H-6')
6	277–279	$C_{17}H_{15}NO_3$	C, H, N	66	3.12 (6H, s, CH_3), 6.75 (1H, s, H-3), 6.90 (2H, m, H-3', H-5'), 7.98 (2H, m, H-2', H-6')
7	235	$C_{17}H_{14}O_4$	C, H	90	1.45 (3H, t), 4.22 (2H, q), 6.87 (1H, s, H-3), 7.17 (2H, m, H-3', H-5'), 8.09 (2H, m, H-2', H-6')
8	270–272	$C_{16}H_{12}O_3$	C, H	72	2.49 (3H, s), 6.94 (1H, s, H-3), 7.48 (2H, m, H-3', H-5'), 8.05 (2H, m, H-2', H-6')
9	268–270[b]	$C_{15}H_9ClO_3$	C, H	68	7.02 (1H, s, H-3), 7.71 (2H, m, H-3', H-5'), 8.17 (2H, m, H-2', H-6')
10	230–233	$C_{16}H_9F_3O_3$	[d]	75	7.13 (1H, s, H-3), 8.19 (2H, m, H-3', H5'), 8.38 (2H, m, H-2', H-6')
11	205–207	$C_{17}H_{14}O_3$	C, H	79	1.32 (3H, t), 2.79 (2H, q), 6.94 (1H, s, H-3), 7.50 (2H, m, H-3', H-5'), 8.07 (2H, m, H-2', H-6')
12	191–193	$C_{16}H_9F_3O_4$	[d]	84	7.03 (1H, s, H-3), 7.64 (2H, m, H-3', H-5'), 8.29(2H, m, H-2', H-6')
13	193–195	$C_{19}H_{18}O_4$	C, H	90	1.03 (3H, t), 1.54 (2H, m), 1.81 (2H, m), 4.13 (2H, m), 6.87 (1H, s, H-3), 7.17 (2H, m, H-3', H-5'), 8.08 (2H, m, H-2', H-6')
14	220–222	$C_{18}H_{16}O_3$	C, H	74	1.00 (3H, t), 1.72 (2H, m), 2.73 (2H, t), 6.94 (1H, s, H-3), 7.48 (2H, m, H-3', H-5'), 8.06 (2H, m, H-2', H-6')
15	310–313	$C_{21}H_{14}O_3$	C, H	85	7.05 (1H, s, H-3), 7.53 (1H, m, H-4''), 7.61 (2H, m, H-3'', H-5''), 7.87 (2H, m, H-2'', H-6''), 7.97 (2H, m, H-3', H-5'), 8.25 (2H, m, H-2', H-6')
16	270	$C_{19}H_{18}O_3$	C, H	88	1.42 (9H, s), 6.94 (1H, s, H-3), 7.67 (2H, m, H-3', H-5'), 8.07 (2H, m, H-2', H-6')
17	158–160	$C_{19}H_{18}O_3$	C, H	92	0.99 (3H, t), 1.41 (2H, m), 1.67 (2H, m), 2.75 (2H, t), 6.93 (1H, s, H-3), 7.47 (2H, m, H-3', H-5'), 8.05 (2H, m, H-2', H-6')
3a	301–303[b]	$C_{15}H_9NO_6$	C, H, N	30[c]	7.26 (1H, s, H-3), 8.45 (4H, m, ArH)
4a	258–259[b]	$C_{16}H_{12}O_5$	C, H	28	3.96 (3H, s, OCH_3), 6.96 (1H, s, H-3)
5a	290[b]	$C_{15}H_{10}O_4$	C, H	15	7.06 (1H, s, H-3), 7.68 (3H, m, H-3', H-4', H-5'), 8.16 (2H, m, H-2', H-6')
15a	> 300	$C_{21}H_{14}O_4$	C, H	10	7.14 (1H, s, H-3), 7.54 (1H, m, H-4''), 7.62 (2H, m, H-3'', H-5''), 7.88 (2H, m, H-2'', H-6'', 7.98 (2H, m, H-3', H-5'), 8.27 (2H, H-2', H-6')
16a	254–256	$C_{19}H_{18}O_4$	C, H	5	1.42 (9H, s, CH_3), 7.02 (1H, s, H-3)

[a]Common protons (compounds **1–4**, **6–17**): 6.97–7.06 (1H, dd, H-6, J = 8.7, J = 2.3), 7.03–7.13 (1H, d, H-8, J = 2.3), 7.93–8.00 (1H, d, H-5, J = 8.7), 10.73–10.99 (1H, s, 7-OH); common protons (compounds **3a–5a**, **15a**, **16a**): 6.30–6.35 (1H, d, H-6, J = 2.07), 6.60–6.66 (1H, d, H-8, J = 2.07), 10.92–11.09 (1H, s, 7-OH), 12.76–13.01 (1H, s, 5-OH). [b]**1**: mp 342–343 °C [7], **3**: mp 308–309 °C [7], **4**: mp 264 °C [14], **9**: mp 238–239 °C [15], **3a**: mp 304–305 °C [7], **4a**: mp 260–261 °C [4], **5a**: mp 290 °C [16]. [c]Overall yield, starting from 2,4-bis(benzyloxy)-6-hydroxyacetophenone. [d]**10**: Calculated for $C_{16}H_9F_3O_3$: C% 62.75, H% 2.96; found C% 59.32, H% 3.57. ^{13}C-NMR: 176.26 (C4), 162.94 (C7), 160.19 (C2), 157.47 (C9), 135.26 (Cl'), 131.07 (C4', q, J = 32), 127.02 (C2'), 126.56 (C5), 125.52 (C3', q, J = 3.7), 123.81 (CF_3, q, J = 272.4), 116.14 (C10), 115.27 (C6), 108.12 (C3), 102.55 (C8). Mass spectrum *m/z* 306 (M^+, 90), 278 (100%), 237 (4), 136 (16), 108 (20). The assignments of ^{13}C-NMR spectra were made according to [14]. **12**: Calculated for $C_{16}H_9F_3O_4$: C, 59.64, H, 2.82; found C 57.55, H, 3.18. ^{13}C-NMR: 176.42 (C4), 163.22 (C7), 160.71 (C2), 157.63 (C9), 150.51 (C4', q, J = 28.7), 130.59 (C1'), 128.60 (C2'), 126.73 (C5), 121.40 (C3', J 2.9), 118.79 (CF_3, the only signal of the expected quartet clearly detectable), 116.20 (C10), 115.29 (C6), 107.40 (C3), 102.68 (C8). Mass spectrum *m/z* 322 (M^+, 100%), 294 (60), 237 (10), 225 (21), 197 (16), 136 (30), 108 (18).

Determination of the proton dissociation constants (pK_a)

The compounds being studied were dissolved in DMSO at a concentration of 3.4 mM. Samples (15 μL) of this solution were each added to 3 mL of buffer at constant ionic strength [17] (I = 0.1 M) and with pH increasing each time in increments of 0.25 units; UV-vis spectra were recorded immediately at 25 ± 0.2 °C. In order to avoid the occurrence of precipitation phenomena, flavone concentrations were kept as low as possible (≈ 16 μM). After recording each spectrum, the pH of the solution was measured with a combined electrode (Orion SA 520) calibrated with buffers at pH 4.01, 7.00 and 10.01. The numerical values of the proton dissociation constants of the compounds under study were evaluated from the change in absorbance at the maximum λ of the undissociated forms according to the equation [18]:

$$pK_a(F) = -\log([F^-]/[F])\cdot a_H+ = -\log\frac{[A-A_{(F)}]}{[A_{(F^-)}-A]}\cdot a_H+$$

where $A_{(F)}$ and $A_{(F^-)}$ refers to the absorbances of the flavone in the neutral and anionic forms respectively; A is the absorbance at intermediate pH; a_H+ is the activity of the hydronium ion. The relative amounts of dissociated and undissociated flavone under the conditions of the enzymatic assay (I = 0.1 M, 25 °C, pH 7.60) were calculated by means of the Henderson–Hasselbach equation [19] (table II).

Xanthine oxidase inhibitory assay

Xanthine was purchased from Fluka; xanthine oxidase (XO) (EC 1.2.3.2) (from buttermilk, 1.36 U/mg protein) was from Boehringer. The inhibitors under study were dissolved in dimethylsulfoxide (DMSO). At the concentrations used (0.2% v/v), DMSO has no effect on XO activity; however, the assays were always conducted at the same DMSO concentration. XO activity was assayed spectrophotometrically in air-saturated phosphate buffer pH 7.60, I = 0.1 M, at 25 ± 0.2 °C. The increase in uric acid (the product of the enzymatic reaction) concentration was evaluated at 295 nm [20]. The concentration of the substrate xanthine was 21 μM and sufficient enzyme was added to obtain an average reaction rate for the control reaction of 0.035 ± 0.002 ΔA/min. IC_{50} values (the concentration required to produce 50% inhibition of the enzyme-catalyzed reaction) were determined from least-squares analyses of the linear portion of log dose–inhibition curves. Each curve was generated using at least three concentrations of inhibitor, producing an inhibition between 20 and 80% with four replicates at each concentration. The 95% confidence limits (95% CL) were calculated from T values for n–2, where n is the total number of determinations [19] (table I).

Molecular modelling

The PM3 [21] molecular orbital method was used to calculate the polarizabilities (α) [9] of flavones (table III). Geometries were completely optimized using the increased convergence criteria available in the Mopac [22] program. All flavones were calculated in their dissociated (anionic) form at the C-7 hydroxyl, according to the proposed mechanism of action of these inhibitors. The solvent-accessible surface areas (sasA) were calculated with the Midas package [10] (table III). Calculations were run on a Convex C-220 computer; graphical display and computation of solvent accessible surface areas were performed on a Personal Iris workstation at the Centro Interdipartimentale di Calcolo Elettronico of Modena.

Acknowledgment

Financial support from CNR (grant No 94.01429.CT03) is gratefully acknowledged.

References

1 Rastelli G, Costantino L, Albasini A (1995) *Eur J Med Chem* 30, 141–146
2 Hansch C, Leo A (1979) *Substituent Constants for Correlation Analysis in Chemistry and Biology*. Wiley, New York
3 Middleton E, Kandaswami C (1994) In: *The Flavonoids. Advances in Research Since 1986* (Harborne JB, ed), Chapman and Hall, London, 619–652
4 Cushman M, Nagarathnam D (1990) *Tetrahedron Lett* 31, 6497–6500
5 Nagarathnam D, Cushman M (1991) *J Org Chem* 56, 4884–4887
6 Tsukayama M, Fujimoto K, Horie T, Masumura M, Nakayama M (1985) *Bull Chem Soc Jpn* 58, 136–141
7 Cushman M, Nagarathnam D, Burg DL, Geahlen RL (1991) *J Med Chem* 34, 798–806
8 Franke R (1984) *Theoretical Drug Design Methods*. Elsevier, Amsterdam
9 Kurtz HA, Stewart JJP, Dieter KM (1990) *J Comp Chem* 11, 82–87
10 Ferrin TE, Huang CC, Jarvis LE, Langridge R (1988) *J Mol Graphics* 6, 13–27
11 Tyukavkina NA, Pogodaeva NN (1972) *Khim Prir Soedin* 2, 173–176
12 Bindoli A, Valente M, Cavallini L (1985) *Pharmacol Res Commun* 17, 831–839
13 Tyukavkina NA, Pogodaeva NN (1975) *Khim Prir Soedin* 11, 708–711
14 Gaydou EM, Bianchini JP (1978) *Bull Soc Chim France* 1–2, II43–II47
15 Ferlux (1969) British patent 1,140,800; *Chem Abstr* (1969) 70, 87577k
16 Cushman M, Nagarathnam D, Geahlen RL (1991) *J Nat Prod* 54, 1345–1352
17 Miller GL, Golder RH (1950) *Arch Biochem* 29, 420–423
18 Jaffé HH, Orchin M (1965) *Theory and Applications of Ultraviolet Spectroscopy*. Wiley, New York
19 Tallarida RJ, Murray RB (1987) *Manual of Pharmacologic Calculations with Computer Programs*, Second edition. Springer Verlag. New York
20 Costantino L, Rastelli G, Albasini A (1992) *Int J Pharm* 86, 17–23
21 Stewart J J P (1989) *J Comput Chem* 10, 209–220
22 Stewart JJP, MOPAC 6.0, QCPE 455