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Structure/Activity Studies of Flavonoids As Inhibitors of Cyclic AMP Phosphodiesterase and Relationship to Quantum Chemical Indices

JAMES E. FERRELL, JR., PETER D. G. CHANG SING, GILDA LOEW, ROY KING, JOAN M. MANSOUR, AND TAG E. MANSOUR

Department of Genetics and Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

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SUMMARY

FERRELL, J. E. JR., P. D. G. CHANG SING, G. LOEW, R. KING, J. M. MANSOUR, AND T. MANSOUR. Structure/activity studies of flavonoids as inhibitors of cyclic AMP phosphodiesterase and relationship to quantum chemical indices. *Mol. Pharmacol.* **16**: 556-568 (1979).

A series of 45 flavonoids and related compounds were tested as inhibitors of liver fluke cyclic nucleotide phosphodiesterase. Many were found to be potent inhibitors; seven were as potent or more potent than any inhibitor previously tested. The kinetics of six compounds spanning a wide range of activities were investigated and found to be competitive. The most potent inhibitors, cyanidin chloride and quercetin, had K_i values of 10 ± 3 μM and 13 ± 6 μM, respectively, approaching the K_m for cAMP (8 μM). Structure/activity studies showed that adding exocyclic substituents to the basic flavonoid skeleton affected activity only slightly, while changing the planarity of the heterocyclic ring greatly decreased activity. This observation, taken with the competitive kinetics, suggests that flavonoids compete with cAMP for a nucleotide binding site at which stacking occurs, perhaps similar to the binding sites of bovine pancreatic ribonuclease A and lobster glyceraldehyde-3-phosphate dehydrogenase. Quantum chemical calculations further suggest that the competition arises from the mimicking of the pyrimidine ring in cAMP by the pyranone ring of the flavonoids. If the flavonoids are comparably potent inhibitors of other phosphodiesterases, several reported pharmacological effects of the flavonoids might be explained.

INTRODUCTION

The flavonoids form a large class of natural products synthesized by ferns and flowering plants. They are important components of many preparations used in folk medicine and constitute up to 5% of the dry weight of some herbal teas (1). Flavonoids first interested pharmacologists when Szent-Györgi asserted that some possess vitamin-like properties (2). Since then numerous pharmacological effects have been attributed to various flavonoids (3). On a gross pharmacological level, some flavonoids and related compounds produce coronary vasodilation (4), spasmolysis (4), or anti-asthmatic activity (5). On a biochemical level, several flavonoids inhibit mast cell degranulation (6); others elevate levels of cAMP[1] and inhibit glycolysis in Erlich ascites tumor cells (7, 8).

[1] The abbreviations used are: cAMP, adenosine 3',5'-cyclic monophosphate; ATP, adenosine triphosphate; 5'-AMP, 5'-adenosine monophosphate; DMSO, dimethyl sulfoxide; PDE, cyclic nucleotide phosphodiesterase; P_i, inorganic phosphate; NaEDTA, sodium (ethylenedinitrilo)tetraacetate; IBMX, 3-isobutyl-1-methylxanthine; RNase, ribonuclease; NAD^+, nicotinamide-adenine dinucleotide; GAPDH, glyceraldehyde-

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The ability of flavonoids to inhibit one or more enzymes has been invoked to explain some of their observed biochemical and pharmacological effects. For example, there is a statistically significant correlation between the extent to which flavonoids inhibit rabbit skeletal muscle Ca^{2+}-dependent ATPase and rat cell histamine release.[2] Some of the effects, however, have not yet been explained, notably the reported β-adrenergic-like effects (4).

A simple hypothesis to account for these unexplained effects is that certain flavonoids affect one of the enzymes involved in regulating cAMP levels. Intracellular levels of cAMP are controlled by two enzymes: adenylate cyclase, which catalyzes its formation from ATP; and cyclic 3',5'-nucleotide phosphodiesterase, which catalyzes its degradation to 5'-AMP. Stimulation of the former or inhibition of the latter might produce the observed effects.

In the present study, the hypothesis that flavonoids inhibit phosphodiesterase is considered. This possibility is supported by the recent demonstration that one flavonoid, quercetin, decreases degradation of cAMP in Ehrlich ascites tumor cell homogenates (7). Further rationale for this hypothesis is provided by a survey of the enzymes which are known to be inhibited by flavonoids. It has been established that flavonoids inhibit isolated (Na^+, K^+) ATPases (9), mitochondrial ATPases (8, 10), a Ca^{2+} dependent ATPase (6), lens aldose reductase (11), malate dehydrogenase (12), glycerol dehydrogenase (13), glutamate decarboxylase (13), hexokinase (13), pancreatic ribonuclease (14), and catechol-O-methyl transferase (15). Like phosphodiesterase, most of these enzymes require the binding of adenine containing nucleotides and have a demonstrated or postulated dinucleotide fold at their nucleotide binding sites (16). Moreover, the chromophore of the affinity gels which bind to phosphodiesterases as well as kinases and dehydrogenases (17) is structurally related to the flavonoids.

The present investigation is a three phase study of the flavonoids as inhibitors of the phosphodiesterase obtained from the liver fluke, *Fasciola hepatica*. The role of cyclic AMP in the regulation of metabolism in the liver fluke has already been reported (18). This study is a further contribution to our understanding of the enzyme systems that regulate the levels of cyclic AMP in this parasite. In the first phase, 45 flavonoids and related compound were screened as inhibitors, and the structural features necessary for inhibition were determined. In the second phase, the kinetics of the inhibition were examined for six flavonoids spanning a wide range of activities. Finally, quantum chemical indices were calculated for several flavonoids and cAMP to investigate electronic aspects of the inhibition.

MATERIALS AND METHODS

Experimental. Liver fluke phosphodiesterase is present only as a high affinity form, with a K_m of 8 μM for cAMP. It is Mg^{2+}-dependent and Ca^{2+}-independent and is inhibited by quazodine, papaverine, and methylxanthines. The enzyme used in these experiments was obtained from a 2000 g supernatant, prepared as described previously (18).

Some of the forty-five compounds tested were obtained from commercial sources; others were generously provided by Dr. Martin Apple. The source of each compound is given in Tables 1-3 and the representative classes of structures to which they belong are shown in Figure 1. Because of the poor solubility of some of the compounds, a small quantity of each inhibitor was dissolved in DMSO just prior to running the assay and the resulting solution was quickly diluted in hot water. Aliquots of this solution were then added to the reaction mixture to yield a final flavonoid concentration of 5 μM to 1 mM and a DMSO

3-phosphate dehydrogenase; IEHT, iterative extended Hückel theory; CNDO/2, complete neglect of differential overlap; LEMO, lowest energy empty molecular orbital.

[2] Assuming a normal distribution, a straight line defined by the equation $Y = 33\% + 1.20\ X$, where Y and X are, respectively, histamine release and ATPase activity measured as percent of control, can be fit to the data reported by Fewtrell and Gomperts (6). The correlation is significant at the 1% level, with $r = 0.79$ (and thus $r^2 = 0.62$).

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TABLE 1

Inhibition of liver fluke cyclic nucleotide phosphodiesterase by 21 flavone derivatives

Trivial name[c]	Systematic name	Inhibition[a]
Quercetin[d]	3,3′,4′,5,7-Pentahydroxyflavone	90%
Kaempferol	3,4′,5,7-Tetrahydroxyflavone	85%
Flavonamine	3-Aminoflavone	85%
Flavone[d]	Flavone	80%
Rhamnetin	3,3′,4′,5-Tetrahydroxy-7-methoxyflavone	80%
—	3-Amino-3′,4′,7-trimethoxyflavone	80%
Fisetin ether	3,7-Dihydroxy-3′,4′-(methylenedioxy)-flavone	75%
Quercitrin	Quercetin-3-L-rhamnoside	75%
Chrysin	5,7-Dihydroxyflavone	75%
Morin[d]	2′,3,4′,5,7-Pentahydroxyflavone	70%
Myricetin	3,3′,4′,5,5′,7-Hexahydroxyflavone	70%
Robinetin	3,3′4′,5′,7-Pentahydroxyflavone	70%
—	3-Amino-4′,7-dimethoxyflavone	70%
—	3-Chloro-5,7-diacetoxyflavone	70%
Flavonol	3-Hydroxyflavone	65%
—	3-Hydroxylamino-3′,4′-dimethoxyflavone	65%
Fisetin	3,3′,4′,7-Tetrahydroxyflavone	65%
—	3-Amino-3′,4′-dimethoxyflavone	60%
Apiin	4′,5,7-Trihydroxyflavone-7-apiosylglucoside	50%
Dichloroquercetin	3,5,7-Trihydroxy-3′,4′-dichloroflavone	30%[b]
Dichlorofisetin	3,7-Dihydroxy-3′,4′-dichloroflavone	15%[b]

[a] Inhibition expressed relative to a 0.5% DMSO blank run simultaneously. Unless otherwise indicated, the reaction was run with an inhibitor concentration of 100 μM and a cAMP concentration of 2.478 μM.

[b] Inhibition measured in a saturated solution with an inhibitor concentration less than 25 μM.

[c] Unless otherwise indicated, compounds were generously provided by Dr. Martin Apple.

[d] Obtained from Aldrich.

concentration of 0.5%. This amount of DMSO did not measurably inhibit the phosphodiesterase; nevertheless, all enzyme activities were measured relative to 0.5% DMSO blanks run simultaneously.

TABLE 2

Inhibition of liver fluke cyclic nucleotide phosphodiesterase by 9 non-flavone flavonoids

Trivial name	Systematic name	Inhibition[a]
Cyanidine chloride	3,3′,4′,5,7-Pentahydroxyflavylium chloride	100%
Flavanone[c]	Flavanone	55%
—	4-Isonitrosoflavan	50%
Naringenin	4′,5,7-Trihydroxyflavanone	50%
—	3′,5-Dihydroxy-4′-methoxyflavanone	40%
Malvidin chloride	3,4′,5,7-Tetrahydroxy-3′,5′-dimethoxyflavylium chloride	15%
L-Catechin	L-3,3′,4′,5,7-Flavanpentol	15%
Fustin	3,3′,4′,7-Tetrahydroxyflavanone	0%
Leucocyanidin	3,3′,4,4′,5,7-Hexahydroxyflavan	—[b]

[a] See footnote [a], Table 1.

[b] Insoluble in DMSO.

[c] Obtained from Aldrich.

To assay the phosphodiesterase activity, a modified version of the method of Butcher and Sutherland was employed (19). This procedure consists of two reactions:

$$\text{cAMP} \xrightarrow{\text{PDE}} 5'\text{-AMP} \xrightarrow[\text{venom}]{\textit{Crotalus atrox}} \text{adenosine} + P_i,$$

and the resulting nucleoside was separated from unreacted nucleotide by anion exchange chromatography.

The reaction mixtures contained 6 mM magnesium acetate, 25 mM Tris-HCl, (pH 7.5), 25 mM imidazole (pH 7.5), enough [8-^{14}C] cAMP to yield 10^4 cpm, 2 mM–50 mM non-labeled cAMP, 5 mM–1000 mM flavonoid or other inhibitor, 0.5% DMSO, and enough water to yield a total volume of 0.45 ml. The excess of Mg^{2+} ensured that the flavonoids could not affect enzyme activity by chelation. The imidazole activated the enzyme, and control experiments done in its absence suggested that flavonoids do not interfere with this activation. To begin the assay, the reaction mixtures were warmed in a shaking bath to 37° and the first reaction was initiated by the addition of 0.05 ml of the phosphodiesterase diluted in 0.25 M

TABLE 3

Inhibition of liver fluke cyclic nucleotide phosphodiesterase by 16 non-flavonoid compounds

Trivial name	Systematic name	Inhibition[a]
Xanthone[d]	9-Xanthenone	70%
IBMX[e]	3-Isobutyl-1-methylxanthine	65 ± 1%
Khellin[e]	4,9-Dimethoxy-7-methyl-5H-furo[3,2-g][1]benzopyran-5-one	55%
Methoxsalen[e]	6-Hydroxy-7-methoxy-5-benzofuranacrylic acid δ-lactone	45%
Trifluoperazine[f] hydrochloride	10-[3-(4-Methylpiperazin-1-yl)propyl]-2-trifluoromethylphenothiazine hydrochloride	45%[b]
Xanthene[d]	Xanthene	40%
—[g]	2-Ethylchromone	35%
—	2-Carboxychromone	30%
Menadione[d]	2-Methyl-1,4-naphthoquinone	30%
Dimethyl pyrone[d]	2,6-Dimethyl-4H-pyran-4-one	15%
Coumarin[d]	1,2-Benzopyrone	5%[c]
Pyrone[d]	4H-Pyran-4-one	0%
Chalcone[d]	1,3-Diphenyl-2-propen-1-one	0%
Anthrone[d]	9,10-Dihydro-9-oxoanthracene	0%[b]
—[d]	α-Naphthoflavone	0%[b]
—[d]	β-Naphthoflavone	0%[b]

[a] See footnote a, Table 1.
[b] Inhibition measured at an inhibitor concentration of 85 μM.
[c] Inhibition measured at an inhibitor concentration of 100 μM.
[d] Obtained from Aldrich.
[e] Obtained from Sigma.
[f] Obtained from Smith, Kline, & French.
[g] Obtained from K & K Labs Division, ICN.
[h] Inhibition measured in a saturated solution with an inhibitor concentration less than 25 μM.

sucrose to hydrolyze a maximum of 30% of the substrate in the subsequent 10 minute incubation. After this incubation, the reaction was stopped by adding 0.05 ml of 0.1 M NaEDTA. Immediately the second reaction was begun by adding 0.1 ml of *Crotalus atrox* venom, suspended at 5 mg/ml in 0.05 M Tris (pH 7.5). After another 10 minute incubation, the reaction mixtures were removed from the shaking bath, 0.35 ml of 0.1 mM adenosine was added to each, and 0.8 ml of the resulting mixtures were placed on anion exchange columns over scintillation vials. Once these mixtures ran through, the columns were eluted with 4 ml of 20 mM ammonium formate. Finally, 5 ml Instagel was added to each scintillation vial and the samples were counted for 10 minutes in a liquid scintillation spectrophotometer. For blanks the phosphodiesterase was added just after the NaEDTA and snake venom. A standard inhibitor, IBMX, was included in each set of assays as an extra control. Further information on this procedure can be found in a previous publication (18).

Theoretical. It is not obvious why the biochemistry of flavonoids should resemble the biochemistry of cAMP. Neither structural formulas nor molecular models reveal any clear similarities between the molecules. To determine if there might be more subtle electronic similarities between cAMP and the flavonoids, quantum mechanical calculations were carried out for eight flavonoids; cyanidin, quercetin, flavone, myricetin, morin, robinetin, fisetin, and flavanone; three related compounds: xanthone, xanthene and pyrone; and cAMP. These compounds were chosen because they allow investigation of the effects of exocyclic modifications retaining a flavone skeleton (flavone, quercetin, myricetin, morin, robinetin, and fisetin) as well as effects of modifying the skeleton (cyanidin versus quercetin; flavone versus flavanone, xanthone, xanthene, and pyrone). Two interrelated questions were addressed by these calculations. First, the possibility that the flavonoids might mimic qualitatively cAMP in their conformations, charge distributions, nucleophilicities, or electrophil-

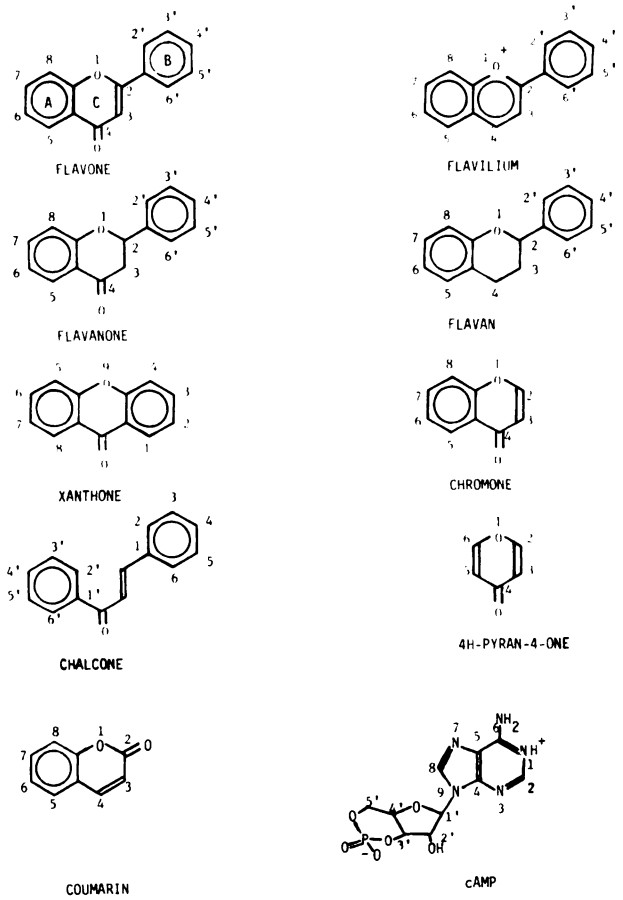


FIG. 1. *Structures and numbering schemes for nine flavonoid and flavonoid-like skeletons and cAMP*

icities was examined. Second, the possibility that the extent of similarity between the inhibitors and substrate might determine quantitatively the potency of the inhibitors was investigated.

The method chosen for these calculations was an all-valence electron semi-empirical linear combination of atomic orbitals self-consistent field theory, CNDO/2 (20). The CNDO/2 wave function was used for a Mulliken population analysis of the filled molecular orbitals to determine the net atomic charges. To measure the propensity of the molecules to act as nucleophiles in non-covalent complex formation, the energies and spatial distributions of the highest filled molecular orbitals were examined. These parameters are a first approximation to the expressions for the propensity of a molecule to act as an electron donor as formulated in second order perturbation theory (21). Similarly, the energies and spatial distributions of the lowest empty molecular orbitals, a first approximation to the reactivity of the molecules as electron acceptors, were examined to measure the propensity of the molecules to act as electrophiles. The CNDO/2 wave function was deorthogonalized to calculate these reactivity parameters (22).

To ensure that trends in the electronic structures of the inhibitors did not depend on the particular (CNDO/2) method chosen, calculations were made on seven of the inhibitors using another semi-empirical, all valence electron method called IEHT (23). In contrast to CNDO/2, which tends to over-localize charge on heavy atoms, IEHT tends to over-delocalize charge. Since the actual charges should lie somewhere be-

tween the IEHT and CNDO/2 charges, trends in the charge distributions common to both methods should be reliable.

Molecular geometries are needed as input data for the calculations. The geometry of cAMP was constructed by attaching the nucleoside portion of the crystal structure of 5′-methyleneadenosine-3′,5′-cyclic monophosphonate to the phosphate portion of the crystal structure of uridine-3′,5′-cyclic monophosphate (24). The zwitterion form of cAMP with N(1) protonated was used in accordance with the crystal structure of the former.

The bond lengths and angles for xanthone and xanthene were adapted from the crystal structure of a related compound (25). This crystal structure was also used as the basic flavonoid skeleton; however, the flavonoids have an additional degree of freedom in the orientation of the B-ring with respect to the chromone moiety. Thus, it was necessary to determine the minimum energy conformation for rotations about the C(2)-C(1′) bond. For this study, another all-valence electron semi-empirical quantum mechanical method, perturbative configuration interaction using localized orbitals (PCILO) (26) was used. This method was developed specifically for energy-conformation studies of large molecules and has been used with a great deal of success for this purpose. The conformation studies were carried out for three flavonoids: cyanidin, myricetin, and flavone. The last two compounds were chosen because they are the most and least substituted flavone derivatives and the conformational energetics of the other flavone compounds should resemble them; cyanidin was chosen because of the possibility that delocalization of its net charge might have a profound influence on its conformational energetics. The conformations used in the CNDO/2 and IEHT calculations were based on the results of the PCILO study.

RESULTS

A large number of flavonoids and related compounds were screened as inhibitors of phosphodiesterase by measuring their inhibitory effect at one concentration of the inhibitor (100 mM) and substrate (2.478 mM). The percent inhibition of these compounds was expressed relative to control assays run simultaneously in the presence of 0.5% DMSO. The results summarized in Tables 1-3 show that of 45 flavonoids tested, 19 had inhibitory potency comparable to IBMX, one of the most potent inhibitors known.

Kinetic studies. The kinetics of enzyme inhibition were examined for six of the inhibitors spanning a wide range of potencies. Lineweaver-Burk plots (27) were used to determine the nature of the inhibition, with least squares lines fitted to the data. For all six compounds the results were consistent with Michaelis-Menten kinetics. Within each compound there were no statistically significant differences in the maximal enzyme velocities (V_{max}) determined at each inhibitor concentration, which suggests that the inhibition is in all cases competitive. This conclusion was supported on the basis of Hofstee plots (not shown). The K_i values were determined from Dixon plots and Hofstee plots (not shown) as well as the Lineweaver-Burk (shown for cyanidin chloride [Fig. 2] and quercetin [Fig. 3]) with each method yielding approximately the same values. Average K_i values are summarized in Table 4.

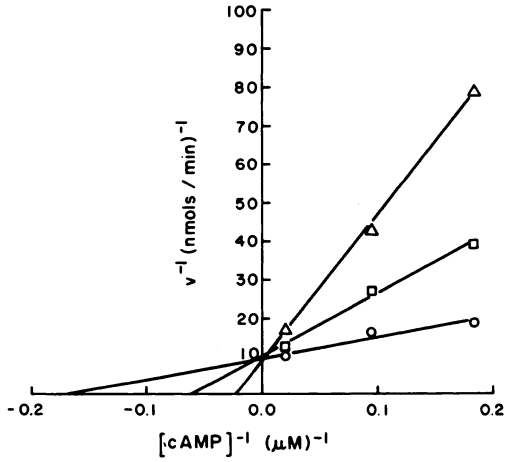


FIG. 2. *Lineweaver-Burk plot showing the inhibition of liver fluke phosphodiesterase by cyanidin chloride*

Lines were drawn by least square fits with $r > 0.99$ for each line. The enzyme used was a 2000 *g* supernatant diluted 1:11 and the inhibitor concentrations were 50 μM (△), 25 μM (□), and 0 μM (○).

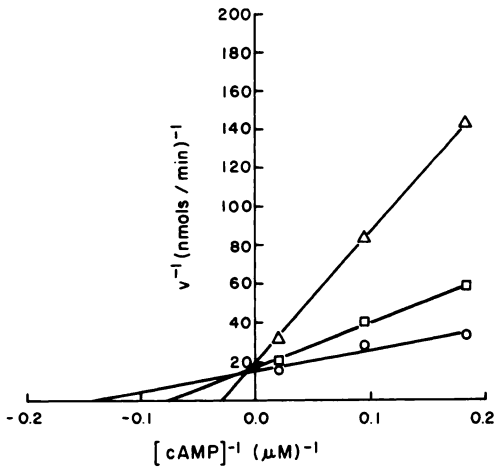


FIG. 3. *Lineweaver-Burk plot showing the inhibition of liver fluke phosphodiesterase by quercetin*

Lines were drawn by least square fits with $r = 0.95$ (△), $r = 1.00$ (□), and $r = 0.96$ (○). The enzyme was a 2000 *g* supernatant diluted 1:20, and the inhibitor concentrations were 50 μM (△), 25 μM (□), and 0 μM (○).

TABLE 4

K_i values obtained for seven competitive inhibitors of liver fluke cyclic nucleotide phosphodiesterase.

Trivial name	K_i
Cyanidin chloride	10 ± 3 μM
Quercetin	13 ± 6 μM
Flavone	23 ± 5 μM
Morin	48 ± 10 μM
Isobutylmethylxanthine (IBMX)	55 μM
Flavanone	100 ± 20 μM
L-Catechin	1200 ± 200 μM

Structure/activity studies. These studies involved two different types of alterations of the simple flavonoid, flavone: exocyclic substitutions, and replacement or modification of the A, B and C rings (Fig. 1). Flavone was found to have a K_i value of 23 ± 5 μM, which makes it as potent as any compound previously tested on this enzyme (18). Twenty other compounds retaining the same skeleton but with varying exocyclic groups were tested (Table 1). The results show that a wide range of substituents produce only minor changes in the measured inhibitory potencies. With the exception of apiin, which has a bulky disaccharide substituted at the 7-position, all of these modifications resulted in compounds whose expected K_i values would be within a range of 15–75 μM. The variations within this range appear to be idiosyncratic. For example, fisetin differs from quercetin only by the absence of a 5-hydroxyl group, and they represent the most and one of the least active compounds retaining the flavone skeleton. However, robinetin and myricetin, which differ by the same 5-hydroxyl group, are equally active. A summary of the exocyclic modifications can be found in Table 5.

A limited number of modifications of the A ring were tested. Table 6 summarizes

TABLE 5

Effects of exocyclic modifications retaining a common skeleton on potency as phosphodiesterase inhibitors

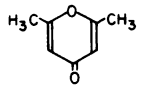
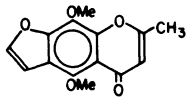
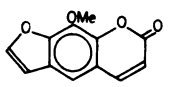
Exocyclic modification	Change in activity
3 – H → OH, Cl, NH_2, NHOH, rhamnose	N.S.[a]
5 – H → OH	N.S.
7 – H → OH	N.S.
→ apiose-glucose disaccharide	decreased
2′ – H → OH	N.S.
3′ – H → OH, OCH_3	N.S.
4′ – H → OH, OCH_3	N.S.
3′ – H, 4′ – H → 3′-OCH_2O-4′	N.S.
5′ – H → OH	N.S.
s′ – H, 5′ – H → 3′,5′-diOCH_3	decreased

[a] Refer to tables 1, 2, and 3 for quantitative comparisons.

N.S. means not significant.

TABLE 6

Effects of modifications of the A ring on potency as phosphodiesterase inhibitors.

Structure	Compound	Inhibition[a]
	Pyrone	15%
	Dimethyl pyrone	0%
	Khellin	55%
	Methoxsalen	45%

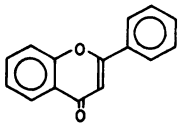
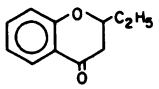
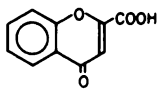
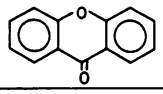
[a] See Table 1, footnote a.

these results. The presence of the A ring appears to contribute to activity, as pyrone and dimethyl pyrone which lack the ring are less active than 2-ethylchromone, the closest analogue retaining it. No compounds eliminating the A ring but preserving the B ring were tested. The two compounds which modify the A ring by attaching a 6,7-furano ring were moderately active, but exact analogues lacking this addition could not be found for comparison.

The results of the B ring variations are summarized in Table 7. Replacing the ring with a 2-ethyl group decreased the single point % inhibition from 80% to 35% and a 2-carboxyl group decreased it further to 30%. Replacing the 2-phenyl group with a fused 2,3-benzo ring system, however, had little effect on activity. The resulting tricyclic compound is xanthone, the prototype for another large class of natural products with interesting pharmacological properties (5, 28).

TABLE 7

Effects of modifications of the B ring on potency as phosphodiesterase inhibitors

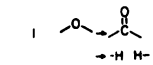
Structure	Compound	Inhibition[a]
	Flavone	80%
	2-Ethylchromone	35%
	2-Carboxychromone	30%
	Xanthone	70%

[a] See Table 1, footnote a.

The C ring modifications are summarized in Table 8. Replacing the 1-oxygen with a carbonyl group had little effect on the potency. Accordingly, menadione (vitamin K_3) had nearly as much activity as the closely related 2-ethylchromone. However, eliminating the oxygen and opening the C ring by converting flavone to chalcone resulted in an inactive compound. Saturation of the 2 = 3 double bond, which disrupts the planarity and conjugation of the ring, produced a large decrease in potency, as can be seen from the activities of flavanone, naringenin, 3′,5-dihydroxy-4′-methoxyflavanone, and fustin (dihydrofisetin). Subsequent replacement of the planar 4-carbonyl group of flavanone with a planar isonitrosyl group did not further reduce the activity. The modification of the 4-position involved in converting the flavone quercetin to the flavylium compound cyanidin chloride, which entails replacing the oxo group with a proton, creating a net positive charge but preserving the C ring planarity, produced a small increase in activity. However, saturation of the 4-carbon position decreased activity, as seen by the low activity of *l*-catechin with respect to quercetin and the low activity of xanthene with respect to xanthone. The phenothiazine derivative trifluoperazine was less active than its closest analogue, xanthone, but it is uncertain whether this effect is due to C ring variation or the influence of the large exocyclic substituents. The unsaturated lactone derivative methoxsalen had comparable activity

TABLE 8

Effects of modifications of the C ring on potency as phosphodiesterase inhibitors

C ring modification	Change in activity	Based on
	N.S.	Menadione vs. 2-ethylchromone
	no activity	Flavone vs. chalcone
2 = 3 → 2 − 3	decreased	Flavanones vs. flavones
4 = O → 4 = NOH	N.S.	4-Isonitrosoflavan vs. flavanone
→ 4 − H	N.S.	Cyanidin chloride vs. quercetin
→ 4 − H_2	N.S.	L-Catechin vs. flavanones; Xanthene vs. xanthone
Pyranone → lactone	N.S.	Methoxsalen vs. khellin
	or decreased	Coumarin vs. 2-ethylchromone

to its closest pyranone analogue khellin; however, the prototype lactone, coumarin, had less activity than 2-ethylchromone.

Quantum chemical studies. The PCILO conformation studies showed that the minimum energy conformer of cyanidin has the B-ring in the plane of the A and C rings for maximum delocalization of the net charge. The barrier for rotation is steep as shown in Fig. 4. In contrast, flavone and myricetin both have low energy conformers which resemble biphenyl, with their B-rings skewed and low barriers to rotation (1 ± 1 kcal/mol). It is expected that the other flavonoids would exhibit similar conformational energetics. It should be noted that the asymmetry in the energy curves shown in figure 4 arises mainly as an artifact of the PCILO method, which uses completely localized bond orbitals as a first approximation to the electronic structure and would disappear if the average of two Kekulé structures were used.

Based on the PCILO results, electronic structures of the flavonoids were calculated with the B ring rotated 45° with respect to the A and C rings. In addition, the planar conformers of cyanidin and quercetin were calculated. As can be seen from Tables 9 and 10, the B ring rotation has little effect on the charge distribution, with the atomic charges becoming only slightly more delocalized in the planar conformers.

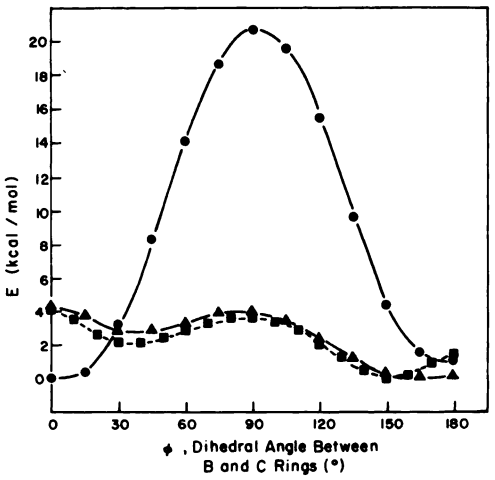


FIG. 4. *Energy as a function of B ring rotation angle for flavone* (△), *myricetin* (□), *and cyanidin* (○)

Zero energy is taken as the lowest energy conformer for each compound. The curve for flavone should be symmetrical about $\phi = 90°$; the asymmetry is an artifact of the PCILO method. The cyanidin and myricetin curves would probably be nearly symmetrical.

Both CNDO/2 and IEHT show that the charge distribution of the pyrone ring of flavone resembles that of the pyrimidine ring of cAMP when oriented as shown in Table 9. The substitution of a hydroxyl group at the 3 position disrupts this resemblance slightly by withdrawing electrons from this position, but does not change the charge distribution of the rest of the ring significantly. More distant substituents had little effect on the C ring charge distribution. For example, a comparison of fisetin to quercetin or robinetin to myricetin shows that a 5-hydroxyl group does not change greatly the electronic structure of the C ring. Similarly, a comparison of quercetin, morin, and myricetin shows very little effect of B ring substituents on the electronic structure of the C ring. A comparison of quercetin to cyanidin shows that the flavone and flavylium C rings have very similar electronic structures. In fact, although the usual valence bond representation of cyanidin shows O(1) bearing a formal positive charge, the calculations show that it is instead slightly negative, just as in its analogue quercetin and the other flavonoids. A comparison of the electronic structures of pyrone to flavone shows that removing the A and B rings alters the charge distribution of the C ring surprisingly little. Thus, although the presence of a C ring which resembles the pyrimidine ring of cAMP seems necessary for potency, it is not sufficient.

No similarities in nucleophilicity parameters were found between cAMP and the flavonoids. However there was some similarity in electrophilicity parameters. The lowest empty molecular orbitals for both cAMP and the inhibitors as calculated by CNDO/2 are π-type orbitals with significant contributions from the pyrimidine ring atoms of cAMP or the C-ring atoms of the inhibitors. The low lying empty orbitals for the six inhibitors calculated by IEHT are similar in nature, lending credence to the CNDO/2 results.

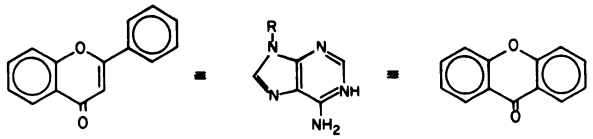
TABLE 9

Similarities between electronic properties of phosphodiesterase inhibitors and cAMP as calculated by CNDO/2 method

Compound	Inhibition[a]	Net atomic charges[b]						Total C ring	% C ring in LEMO[c]
		O(1)	C(2)	C(3)	C(4)	C(9)	C(10)		
cAMP	K_m = 8 μM	−.14	+.23	−.12	+.33	+.22	−.05	+.47	83%
Cyanidin 0°[d]	100%	−.13	+.23	+.08	+.11	+.24	−.09	+.44	67%
45°		−.13	+.24	+.08	+.12	+.24	−.10	+.45	71%
Quercetin 0°	90%	−.18	+.14	+.06	+.23	+.23	−.16	+.32	47%
45°		−.18	+.15	+.05	+.23	+.23	−.16	+.32	49%
Flavone	80%	−.19	+.21	−.14	+.24	+.18	−.06	+.24	44%
Myricetin	70%	−.18	+.14	+.05	+.23	+.23	−.16	+.31	49%
Morin	70%	−.20	+.16	+.03	+.25	+.22	−.15	+.31	44%
Robinetin	70%	−.18	+.14	+.06	+.21	+.20	−.09	+.34	45%
Xanthone	70%	−.19	+.19	−.07	+.23	+.19	−.07	+.28	37%
Fisetin	65%	−.18	+.14	+.05	+.21	+.20	−.09	+.33	45%
Flavanone	55%	−.23	+.19	−.04	+.23	+.19	−.08	+.26	41%
Xanthene	40%	−.21	+.15	−.02	+.04	+.15	−.02	+.09	27%
Pyrone	15%	−.13	+.16	−.10	+.24	+.16	−.10	+.23	83%

[a] See Table 1, footnote a.

[b] Charges determined by Mulliken population analysis and expressed in fractions of an electron. Atom positions correspond to flavone numbering (Fig. 1). Corresponding orientations for cAMP and xanthone are shown below:



For example, N(3) in cAMP and O(9) in xanthone are listed in the O(1) column.

[c] The percentage of the LEMO which is contributed by the C ring atoms, as calculated by Mulliken population analysis.

[d] Dihedral angle between A and C rings (taken as 45° for other flavonoids).

TABLE 10

Similarities between electronic properties of phosphodiesterase inhibitors and cAMP as calculated by IEHT method

Compound	Inhibition[a]	Net atomic charge[b] on						Total C ring	% C ring in LEMO[c]
		O(1)	C(2)	C(3)	C(4)	C(9)	C(10)		
cAMP[d]	K_m = 8 μM	−.21	+.10	−.05	+.14	+.10	+.06	+.14	NA
Quercetin 0°[e]	90%	−.26	+.11	+.11	+.10	+.10	+.03	+.19	38%
45°		−.26	+.12	+.11	+.10	+.10	+.04	+.21	42%
Flavone	80%	−.26	+.12	.00	+.09	+.10	+.03	+.08	39%
Morin	70%	−.25	+.13	+.11	+.12	+.11	+.04	+.26	42%
Xanthone	70%	−.26	+.01	+.03	+.10	+.10	+.03	+.01	31%
Flavanone	55%	−.34	+.09	−.02	+.10	+.08	+.02	−.07	34%
Xanthene	40%	−.32	+.06	+.01	−.04	+.06	+.01	−.22	47%

[a] See Table 1, footnote a.

[b] See Table 9, footnote b.

[c] See Table 9, footnote c.

[d] Because of convergence problems, the net charges on cAMP are only estimated ± 0.03.

[e] See Table 9, footnote d.

DISCUSSION

Many factors could affect the potency of flavonoids as phosphodiesterase inhibitors. For example, since the nucleotide binding sites of several enzymes with dinucleotide folds are hydrophobic pockets (16), it might be thought that the most potent flavonoid inhibitors of phosphodiesterase would be highly water insoluble. Our results, however, show no connection between solubility and activity; for example, the two best inhibitors were one of the most soluble (cyanidin chloride) and one of the least soluble (quercetin) flavonoids tested.

The competitive nature of the kinetics suggests that the flavonoids might compete with cAMP for the same binding site in the fluke phosphodiesterase. This hypothesis implies that the potency of the flavonoids depends on the affinity of the compound for this binding site. The structure/activity relations reported in the previous section will be examined in light of this hypothesis.

Some nucleotide binding sites involve edge-on interactions between the base and the enzyme. For example, a model proposed for the ATP binding site of frog actomyosin involves three such interactions (29). Our results are not consistent with this sort of binding in fluke phosphodiesterase. Many of the modifications which had large effects on the physical and chemical characteristics of the edge of the flavone molecules had little effect on the activity of the compound.

Other nucleotide binding sites involve stacking interactions between the base and enzyme. Stacking occurs in the binding of cytidine monophosphates to bovine pancreatic ribonuclease A (RNase A) (30) and in the binding of NAD+ to lobster GAPDH (16). We hypothesize that the fluke phosphodiesterase nucleotide binding site involves stacking and $\pi - \pi$ interactions similar to those in RNase A and GAPDH. Support for this assertion is provided by a comparison of the structure/activity relations for the flavonoids as inhibitors of RNase A and fluke phosphodiesterase. As shown in Table 11, saturating the flavonoid C ring dramatically decreased potency in both enzymes. Saturation would interfere with stacking both by steric hindrance and by disruption of the π-electron system that could stabilize a stacked complex.

TABLE 11

Similarities between the structure/activity relationships for flavonoids and related compounds as inhibitors of fluke phosphodiesterase and bovine ribonuclease

Compound	Phosphodiesterase inhibition[a]	RNase inhibition[b]
Cyanidin chloride	100%	100% (77%)
Quercetin	90%	100% (19%)
Kaempferol	85%	84% (0%)
Chrysin	75%	15%
Fisetin	55%	55%
Flavanone	55%	0%[c]
Naringenin	40%	0%
Malvidin chloride	15%	22%
L-Catechin	15%	0%
Coumarin	5%[d]	0%
Fustin	0%	0%

[a] See Table 1, footnote a.

[b] Inhibitor concentration = 1 mM; numbers in parentheses determined at 100 μM. See reference 14.

[c] L-isomer tested on RNase.

[d] Inhibitor concentration = 200 μM.

The calculated electronic properties suggest an explanation of how the flavonoids can mimic cAMP in a stacking interaction. There is a resemblance between the charge distributions of the pyranone rings of the inhibitors and the pyrimidine ring of cAMP. In addition there is a resemblance between the substrate and the inhibitors in their propensity to accept electrons in a π-orbital. These similarities are not altered by the addition of exocyclic substituents to the A and B rings, or by converting the flavone C ring to a flavylium C ring. Finally, it should be noted that the most active inhibitor, cyanidin, is the only flavonoid predicted to have a totally planar lowest energy conformer. These observations provide a rationalization for why flavonoids compete with cAMP, but not a quantitative explanation of the observed structure/activity relationships. It is thus likely that factors beyond these electronic properties help determine the potency of the inhibitors.

To test whether stacking occurs and whether the pyranone and pyrimidine rings are oriented similarly at the binding site, several lines of investigation can be envi-

sioned. For example, a variety of spectroscopic techniques including fluorescence and NMR spectroscopy could elucidate the nature of the environment in which the substrate and inhibitors bind. In addition, photoaffinity analogues of cAMP and the flavonoids could help determine which amino acids are involved in the binding and how they are oriented with respect to the ligands. And of course there is always the possibility that the structures of such complexes might be solved by crystallography.

If the flavonoids are mimics of cAMP, then it is possible that the previously reported pharmacological effects of flavonoids where similar structure/activity relations were noted result from their actions on phosphodiesterase. It would be of interest to investigate the role of this enzyme in flavonoid-induced coronary vasodilation (4) and spasmolysis (4). Although these systems are phylogenetically distant from the fluke, preliminary experiments in this laboratory have shown that at least one flavonoid, morin, is a potent inhibitor of slime mold (*Physarum polycephalum*) and beef heart phosphodiesterases as well as fluke phosphodiesterase. These experiments, together with the demonstration that quercetin decreases cAMP degradation in Ehrlich ascites tumor cell homogenates (7), suggest that the flavonoids inhibit a broad spectrum of phosphodiesterases.

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Note added in proof: Since preparation of this manuscript, reports have been published showing flavonoids to be potent inhibitors of beef heart phosphodiesterase (Beretz, A., Anton, R., and J. C. Stoclet. Flavonoid compounds are potent inhibitors of cyclic AMP phosphodiesterase. *Experientia 34:* 1054–1055, 1978.) and of bovine lung phosphodiesterase (Ruckstuhl, M., Beretz, A., Anton, R., and Y. Landry. Flavonoids are selective cyclic GMP phosphodiesterase inhibitors. *Biochem. Pharmacol. 28:* 535–538, 1979.). The structure/activity relationship observed in the former differed from the present report only in that the presence of a 3-hydroxyl group appeared important for activity.

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