Synthesis and Evaluation of *N*-(Phenylalkyl)acetohydroxamic Acids as Potential Substrates for *N*-Arylhydroxamic Acid *N,O*-Acyltransferase

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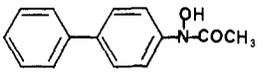
N-(4-Phenylcyclohexyl)acetohydroxamic acid and a series of *N*-(phenylalkyl)acetohydroxamic acids were synthesized and evaluated as substrates for partially purified rat and hamster hepatic arylhydroxamic acid *N,O*-acyltransferase systems (AHAT). The compounds were assayed for their abilities to function as acetyl donors in the AHAT-mediated transacetylation of 4-aminoazobenzene and for their abilities to participate in the AHAT-mediated conversion of *N*-arylhydroxylamines to electrophilic intermediates that form methylthio adducts upon reaction with *N*-acetylmethionine. None of the newly synthesized compounds displayed significant activity in either of the assays. The results of this study indicate that acetohydroxamic acids that have the nitrogen atom of the hydroxamic acid group attached directly to aliphatic or cycloalkyl groups are not likely to serve as substrates or inhibitors of AHAT.

Several published reports have indicated that the soluble enzyme system *N*-arylhydroxamic acid *N,O*-acyltransferase (AHAT) is rather widely distributed in mammalian tissues and that it is capable of converting a number of *N*-arylhydroxamic acids, such as *N*-hydroxy-4-acetamidobiphenyl (1), into electrophilic species that form covalently bound



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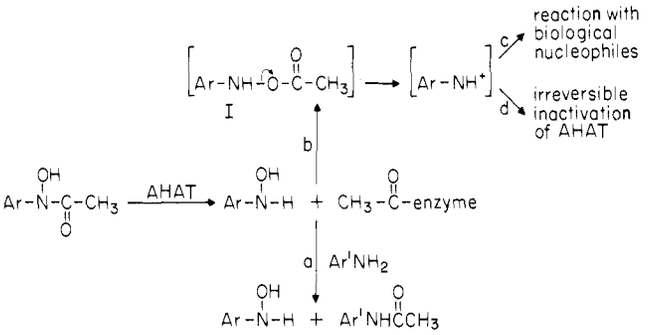
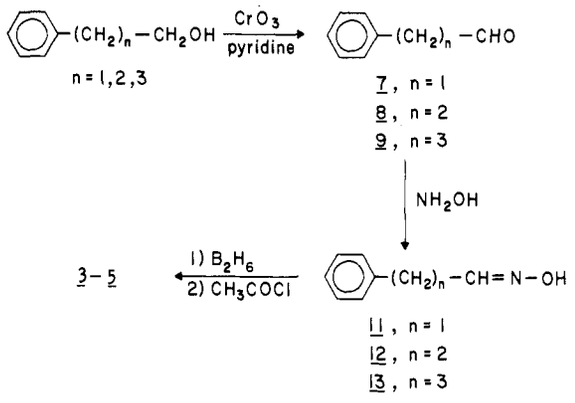
arylamine adducts with biological nucleophiles, including nucleophilic groups that are present on AHAT itself (Scheme I).[1-6] Of particular interest is the report that rabbit hepatic AHAT appears to be identical with the acetylcoenzyme A dependent *N*-acetyltransferase (EC 2.3.1.5) that is responsible for the metabolic *N*-acetylation of a variety of aromatic amines and related compounds.[6,7] The characteristics of the AHAT system and the potential toxicological implications of the AHAT-catalyzed bioactivation process have been recently reviewed.[8]

The central role that is played in chemical carcinogenesis and chemical toxicology by various bioactivation processes makes it imperative to gain a knowledge of the types of organic compounds that are subject to metabolic conversion to reactive electrophiles or that can participate in the activation of other compounds. Therefore, as part of a structure–activity investigation of AHAT-catalyzed bioactivations, a series of *N*-(phenylalkyl)acetohydroxamic acids (**2–5**, Table I), as well as *N*-(4-phenylcyclohexyl)-acetohydroxamic acid (**6**, Table I), were prepared and evaluated as substrates for partially purified rat and hamster hepatic AHAT preparations. The primary purpose of this study was to determine whether it is necessary for the nitrogen atom of the hydroxamic acid to be attached to an aromatic group in order for the compound to serve as a substrate for AHAT.

Scheme I



Scheme II



Synthesis. The *N*-(phenylalkyl)acetohydroxamic acids **3–5** were prepared as outlined in Scheme II. The oxidation of the primary alcohols was accomplished by the chromium trioxide–pyridine complex method to produce the aldehydes **7–9**.[9] Compounds **7–9** were converted directly to their oxime derivatives **11–13** by a standard procedure.[10] Oximes **10** and **14** (see Experimental Section) were formed in the same manner from benzaldehyde and 4-phenylcyclohexanone, respectively.

The synthesis of the acetohydroxamic acids involved the diborane reduction of the oximes to the corresponding hydroxylamines according to the method reported by Feuer and co-workers.[11] The hydroxylamines were acetylated, without isolation or purification, to yield the acetohydroxamic acids listed in Table I (Scheme II). In order to obtain satisfactory yields of the acetohydroxamic

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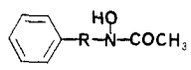
Table I. Physical Properties of Acetohydroxamic Acids



no.	R	mp,[a] °C	yield, %	formula
2	CH_2	123-124	45	$C_9H_{11}NO_2$
3	$(CH_2)_2$	46-48	30	$C_{10}H_{13}NO_2$
4	$(CH_2)_3$	70-71	62	$C_{11}H_{15}NO_2$
5	$(CH_2)_4$	oil	50	$C_{12}H_{17}NO_2$
6	c-C_6H_{10}	112-113	30[b]	$C_{14}H_{19}NO_2$

[a] Compounds 2-4 were recrystallized from petroleum ether (60-70 °C). Compound 5 was purified by column chromatography on silica gel (50 g, Brinkman 60); the column was eluted with $CHCl_3$. Compound 6 was recrystallized from ether. [b] Gas chromatographic analysis (column temperature 200 °C, detector temperature 220 °C) revealed two components in approximately equal concentrations. Retention times, 16.5 and 18 min.

acids, it was necessary to modify the workup procedure of the diborane reaction mixture so that the stability of the hydroxylamine intermediates was enhanced. In general, these modifications involved the use of glassware that was free of trace metals and the extraction of the hydroxylamines from phosphate buffer. Beckett and coworkers have described and recommended conditions that will minimize the decomposition and transformation of hydroxylamines.[12]

There are two possible diastereoisomeric forms of the cyclohexyl analogue **6**. It was not possible to separate the diastereomers by crystallization or by TLC. Gas chromatographic analysis of **6** indicated the presence of two components in approximately equal concentrations, and it was this diastereomeric mixture that was used in the enzymatic assays.

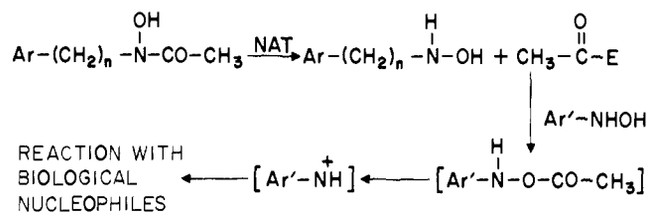
Results

Transacetylation Assay. As illustrated in Scheme I, the inclusion of an aromatic amine in the incubation mixture with AHAT and an *N*-arylhydroxamic acid results in the AHAT-catalyzed transacetylation of the aromatic amine. The abilities of hydroxamic acids **2–6** to function as acetyl donors in this process was measured by the spectrophotometric method of Booth, in which 4-aminoazobenzene is used as the aromatic amine that undergoes transacetylation.[13]

N-(4-Phenylbutyl)acetohydroxamic acid (**5**) exhibited a transacetylation rate of 0.73 (±0.06) nmol of 4-aminoazobenzene acetylated (mg of protein)$^{-1}$ min^{-1} (mean ± SD) (n = 3) with the partially purified rat hepatic AHAT preparation. The transacetylation rate of the prototype *N*-arylhydroxamic acid, *N*-hydroxy-4-acetamidobiphenyl (**1**), was 4.99 (±0.07) nmol (mg of protein)$^{-1}$ min^{-1} (mean ± SD) (n = 3). None of the other compounds shown in Table I had measurable transacetylation rates with either the rat or hamster hepatic preparations. With the hamster enzyme preparation, the rate of transacetylation by *N*-hydroxy-4-acetamidobiphenyl (**1**) was 9.14 (±0.86) nmol (mg of protein)$^{-1}$ min^{-1} (mean ± SD) (n = 3).

Experiments were carried out in order to determine whether compounds of the type shown in Table I could inhibit the AHAT-catalyzed transacetylation activity of *N*-hydroxy-4-acetamidobiphenyl (**1**). When 2 mM *N*-cyclohexyl analogue, **6** was included in an incubation mixture containing AHAT, 4-aminoazobenzene, and 1 mM

Scheme III



N-hydroxy-4-acetamidobiphenyl (**1**), the transacetylation rate was depressed only 10%. Concentrations of 0.5–1.0 mM **6** had no effect on the AHAT-catalyzed transacetylation of 4-aminoazobenzene by **1**.

Electrophile Generation Assay. The bioactivation of *N*-arylhydroxamic acids by AHAT results in the production of a resonance-stabilized *N*-arylnitrenium ion that reacts with nucleophilic species (Scheme I). Since compounds **2–6** are *N*-alkyl- and *N*-cycloalkylhydroxamic acids, they are not capable of being transformed into stable electrophiles by the pathway depicted in Scheme I and would not, therefore, be expected to exhibit a significant extent of reaction with nucleophiles. On the other hand, if compounds **2–6** were capable of forming the acylenzyme intermediate, it is conceivable that an *N*-arylhydroxylamine could be converted to an *N*-acetoxyarylamine that would undergo heterolytic breakdown to the expected *N*-arylnitrenium ion as depicted in Scheme III. Such a process is made more plausible by the finding of Bartsch and co-workers that added hydroxylamines enhanced the extent of AHAT-catalyzed methylthio adduct formation when *N*-acetylmethionine was used as a nucleophilic trapping agent.[3]

The partially purified hamster hepatic AHAT preparation was used in these experiments because the hamster liver preparation contains a higher level of AHAT activity than rat liver preparations. When *N*-hydroxy-4-acetamidobiphenyl (**1**) (1 mM) was incubated with 0.1 mM *N*-hydroxy-4-aminobiphenyl and 10 mM *N*-acetylmethionine in the presence of hamster hepatic AHAT, the rate of methylthio adduct formation was 120 ± 19.3 nmol (mg of protein)$^{-1}$ 30 min^{-1} (mean ± SD) (n = 3). None of the compounds **2–6** exhibited more than 1% of the activity of *N*-hydroxy-4-acetamidobiphenyl in this assay.

Experiments were also conducted in order to determine whether compounds **3** and **6** would inhibit methylthio adduct formation when they were included in incubation mixtures in which *N*-hydroxy-4-acetamidobiphenyl (1 mM) was used as the substrate. Compound **3** in concentrations of 1 and 2 mM caused 8 and 17% reductions, respectively, in methylthio adduct formation. The *N*-cyclohexyl analogue **6** in concentrations of 1 and 2 mM produced 13 and 23% reductions in product formation, respectively.

Discussion

The potential importance of *N,O*-acyltransferases (AHAT) in the bioactivation of toxic and carcinogenic hydroxamic acids has prompted investigation of the structural characteristics of these substances that enhance or diminish the likelihood that they will be converted to reactive electrophiles or that they will function as acyl donors in the AHAT-catalyzed activation of arylhydroxylamines. It has been reported that only limited structural variations in the acyl group of *N*-arylhydroxamic acids are permissable without loss of substrate activity.[14,15]

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Recently, it was found that replacement of the *p*-phenyl ring of *N*-hydroxy-4-acetamidobiphenyl (1) with a variety of hydrophobic alkyl, alkenyl, and cycloalklyl groups allowed the compounds to function as effective substrates for partially purified AHAT preparations.[16]

In the present investigation, compounds **2–6** (Table I) were prepared in which the *p*-phenyl ring of *N*-hydroxy-4-acetamidobiphenyl was retained, but the phenyl ring bearing the hydroxamic acid function was replaced with *n*-alkyl groups and with a cyclohexyl group. Compounds **2–6** failed to serve as effective substrates for AHAT as determined by 4-aminoazobenzene transacetylation activity or by participation as acetyl donors in the conversion of *N*-arylhydroxylamines to electrophilic species capable of forming methylthio adducts as a result of reaction with *N*-acetylmethionine. These results indicate that the attachment of the N atom of hydroxamic acids to alkyl or cycloalkyl groups drastically compromises the ability of the compounds to participate in reaction processes that are catalyzed by AHAT.

In addition to being ineffective substrates for AHAT, compounds **3** and **6** were very weak inhibitors of either the transacetylation activity or methylthio adduct formation activity of *N*-hydroxy-4-acetamidobiphenyl. Although the mechanism of the modest amount of inhibition produced by **3** and **6** is not known, this result, considered together with the lack of effectiveness as substrates for AHAT, may indicate poor affinity for the enzyme.

Experimental Section

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra were obtained with a Perkin-Elmer 281 recording spectrophotometer. NMR spectra were obtained with Varian T-60 and A-60D spectrometers; the samples were dissolved in $CDCl_3$ with tetramethylsilane as the internal reference standard. Mass spectra were obtained with an Associated Eletronic Industries (AEI) MS 30 mass spectrometer in the University of Minnesota Department of Chemistry Mass Spectrometry Laboratory; samples were introduced by direct inlet, and spectra were run in the electron-impact mode. For each compound, the IR, NMR, and mass spectra were consistent with the assigned structure. Analytical TLC was carried out with plastic-backed plates (Eastman 13181 silica gel with fluorescent indicator, no. 6060). Elemental analyses were performed by Midwest Microlab Inc., Indianapolis, IN, and were within 0.4% of theoretical values. The yields, physical properties, and recrystallization solvents for compounds **2–6** are listed in Table I. Gas chromatographic analysis of **6** was carried out on a Varian Aerograph 2100 with a 6-ft OV-1 3% Chrom. W-80 column.

General Method for Synthesis of Aldehydes 7–9. Anhydrous pyridine was prepared by distillation of reagent-grade pyridine from barium oxide; the distilled product was stored over 4A molecular sieves. Methylene chloride was purified by washing with concentrated H_2SO_4 and then with H_2O and saturated NaCl, followed by distillation; the distilled product was stored over 4A molecular sieves.

3-Phenylpropanal (8). Chromium trioxide (36 g, 0.36 mol) was added in portions to a stirred solution of 56.9 g (0.72 mol) of pyridine in 500 mL of CH_2Cl_2. The mixture was stirred for 15 min at room temperature. A solution of 3-phenylpropanol (8.17 g, 0.06 mol) in 25 mL of CH_2Cl_2 was added in one portion, and the mixture was stirred for an additional 15 min at room temperature. The solvent was decanted, and the residue was washed with several portions of ether. The combined organic solutions were washed with five 200-mL portions of 5% NaOH, 300 mL of 5% HCl, 300 mL of 5% $NaHCO_3$, and 300 mL of saturated NaCl and were dried ($MgSO_4$). Evaporation of the solvent in vacuo afforded **8** (6.8 g, 83%) as a liquid material, which was used directly in the next reaction without further purification. TLC of **8** (silica gel) in several solvent systems ($CHCl_3$–EtOAc, 4:1; C_6H_{14}–Et_2O–CH_2Cl_2, 5:2:3; $CHCl_3$) indicated the presence of a single component. The same method was used to prepare 2-phenylethanal (**7**) in 66% yield from 2-phenylethanol, and 4-phenylbutanal (**9**) was obtained in 90% yield from 4-phenylbutanol.

General Method for the Synthesis of Oximes (10–14). The oximes were prepared according to method of Shriner, Fuson, and Curtin.[10] A minimum volume of CH_3OH was used to dissolve the aldehyde or ketone.

Benzaldoxime (10): yield 81%; recrystallized from Et_2O, mp 35 °C (lit.[10] mp 35 °C).

2-Phenylethanal oxime (11): yield 65%; recrystallized from Et_2O, mp 83–86 °C. Anal. (C_8H_9NO) C, H; N: calcd, 10.36; found, 9.84.

3-Phenylpropanal oxime (12): yield 80%; recrystallized from Et_2O, mp 92–94 °C. Anal. ($C_9H_{11}NO$) C, H, N.

4-Phenylbutanal oxime (13): yield 80%; recrystallized from petroleum ether (bp 30–60 °C), mp 42–45 °C. Anal. ($C_{10}H_{13}NO$) C, H, N.

4-Phenylcyclohexanone oxime (14): yield 80%; recrystallized from petroleum ether (bp 60–70 °C), mp 112–113 °C (lit.[17] mp 112 °C).

General Method of Synthesis of Acetohydroxamic Acids (2–6). All glassware was soaked overnight in 50% HNO_3, washed with distilled water, dried overnight at 120 °C, and flushed with N_2 while being cooled to room temperature. The oxime (0.03 mol) was dissolved in 10 mL of dry THF and was cooled to 0 °C under a N_2 atmosphere. To the oxime solution was added 48 mL of a 1 M solution of diborane in THF; the addition was made by means of a syringe at such a rate that the temperature did not exceed 5 °C. The reaction mixture was stirred for 4 h (12 h for the reduction of **10**) at room temperature under static N_2 pressure. The solvent was removed in vacuo, and 10 mL of 20% HCl was added from a syringe at such a rate that the temperature did not exceed 5 °C. The resulting mixture was heated under reflux for 1 h, cooled to 0 °C, and neutralized with 10% NaOH. Phosphate buffer (20 mL, pH 7.4) was added, and the mixture was rapidly extracted with three 50-mL portions of Et_2O. To the combined Et_2O extracts was added saturated $NaHCO_3$ solution (20 mL), and the resulting mixture was cooled in an ice–methanol bath. Freshly distilled acetyl chloride (0.026 mol) dissolved in 50 mL of ether was added dropwise over a period of 1 h to the stirred mixture. After the mixture was stirred for an additional 15 min, the Et_2O was evaporated in vacuo, and 20 mL of saturated $NaHCO_3$ was added. The mixture was stirred for 1 h in an ice–methanol bath and filtered, and the filtrate was extracted with Et_2O. The combined ether portions were extracted with three 50-mL portions of cold 1% NaOH. The aqueous base was acidified (pH 5) with concentrated HCl and extracted with three 50-mL portions of Et_2O, which were combined and dried ($MgSO_4$). The acetohydroxamic acids were further purified as indicated in Table I.

Enzymatic Studies. Male, golden, Syrian hamsters were purchased from Charles River (Wilmington, MA) and male Sprague–Dawley rats from Bio-Lab (White Bear lake, MN). Ultracentrifugation was performed on a Beckman L5-65 ultracentrifuge and low-spin centrifugation on a Beckman J-21B or a J2-21 centrifuge. A Beckman 24 or 24/25 spectrophotometer was used. Incubations were performed in a Dubnoff shaker bath. Dithiothreitol (DTT), methionine, and Grade III NAD^+ were obtained from Sigma Chemical Co. *N*-Acetyl-L-[*methyl*-^{14}C]-methionine (0.2–0.3 mCi/mmol) was prepared from L-[*methyl*-^{14}C]methionine (New England Nuclear Corp.) and acetic anhydride according to the procedure of Wheeler and Ingersoll.[18] The preparation and properties of *N*-hydroxy-4-acetamidobiphenyl (1) and 4-biphenylhydroxylamine have been reported.[16]

Tissue Preparation. The details of the tissue preparation have been recently reported.[15,16]

Enzyme Preparation. Arylhydroxamic acid *N*,*O*-acyltransferase (AHAT) was partially purified (2- to 3-fold) from hamster or rat liver cytosol (25% solution of 105000*g* supernatant)

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by ammonium sulfate fractionation as described by King.[1]

The enzyme pellets obtained from the fractionation procedure were stored at −70 °C and were reconstituted in enough 0.05 M sodium pyrophosphate buffer (pH 7.0, 1 mM DTT) to give approximately 30 mg/mL of protein. Protein concentration was measured by the method of Lowry et al. with bovine serum albumin as the standard.[19]

AAB Transacylation Assay. The details of this assay were recently described.[15] Reactions were started by addition of substrates (hydroxamic acid/4-aminoazobenzene) dissolved in 0.1 mL of 95% ethanol and were carried out at 37 °C in air for 5–20 min.

In the inhibition experiments, the inhibitor, *N*-(4-phenylcyclohexyl)acetohydroxamic acid (**6**; 0.5–2 mM final concentration), was added simultaneously with *N*-hydroxy-4-acetamidobiphenyl (1; 1.0 mM final concentration); the incubation time was 10 min.

Electrophile Generation Assay. The production of electrophiles was measured by the procedure of Bartsch et al.[2,3] Incubation flasks contained 41 μmol of sodium phosphate buffer, pH 6.8, 0.8 μmol of NAD^+, 10 μmol of *N*-acetyl-L-[*methyl*-^{14}C]-methionine, 1 mg of partially purified hamster liver enzyme, 1 μmol of substrate [in 0.05 mL of Me_2SO–95% ethanol (1:4)], 1 μmol of 4-biphenylhydroxylamine (in 0.05 mL of 95% ethanol), and sufficient water to give a final volume of 1.0 mL.

Reactions were initiated by addition of substrates and were carried out at 37 °C in air for 30 min. The reaction mixtures were worked up, and the methylthio adducts were quantified as described previously.[15]

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Pentasubstituted Quercetin Analogues as Selective Inhibitors of Particulate 3′:5′-Cyclic-AMP Phosphodiesterase from Rat Brain

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Some penta-O-substituted analogues of quercetin were synthesized and tested for the inhibition of cytosolic and particulate rat brain cyclic AMP and cyclic GMP phosphodiesterase activities. Ten of these compounds are potent and highly selective inhibitors of cAMP hydrolysis with respect to cGMP hydrolysis. They inhibit more potently the particulate enzyme than the cytosolic preparation. The highest selectivity was observed with penta-*O*-ethylquercetin and analogue **6d**, which proved to be more selective and more potent inhibitors than the reference compound Ro 20-1724. Some structure–activity relationships are discussed.

Bioflavonoids, such as quercetin and related compounds, are known to exhibit various pharmacological effects, including smooth-muscle relaxation,[1] antiasthmatic activity,[2] or inhibition of histamine-induced gastric secretion.[3] On a biochemical level, they have been shown to inhibit the activity of several enzymes involved in energy-conversion reactions, such as mitochondrial ATPase,[4] (Na^+,K^+)-ATPase,[5] or (Ca^{2+},Mg^{2+})ATPase.[6] Furthermore, quercetin interacts with the adenosine cyclic 3′,5′-phosphate (cAMP) system in a great variety of cell types, including Ehrlich ascites tumor cells[7] or cultured cells of central nervous system (CNS) origin.[8] Several teams have also shown that bioflavonoids are potent inhibitors of cytosolic cyclic nucleotide phosphodiesterases from various mammalian organs (bovine heart,[9] bovine lung[10]) and from the liver fluke, *Fasciola hepatica*.[11] Studies already reported were performed with cytosolic phosphodiesterase from nonneural tissue and concerned only naturally occurring bioflavonoids with hydroxy or glycosyl substituents variously distributed on the basic flavonoid skeleton. Although such compounds proved potent inhibitors of cytosolic enzymes in cell-free preparations, most of them probably did not penetrate into the cell but only interacted with membrane lipids, as it was recently reported.[8] So their potential efficiency as therapeutic agents and especially as centrally active agents is somewhat restricted. The introduction of lipophilic exocyclic substituents might facilitate the entry of such compounds into the cell or increase their affinity for the plasma membrane and improve their biochemical and pharmacological properties. Indeed, penta-*O*-ethylquercetin exhibits original antifatigue properties and significantly improves the swimming capacity in mice.[12] So, on the basis of this assumption, we synthesized various O-substituted quercetin analogues and evaluated their potency as inhibitors of cytosolic and particulate rat brain cyclic nucleotide phosphodiesterase. Among the various naturally occurring flavonoid skeletons, we selected the flavonol structure characterized by the planarity of its heterocyclic ring and the presence of an hydroxy group at the 3-position. Flavonols were found to be the most efficacious for both ATPases and cyclic nucleotide phosphodiesterases inhibition[9,10] (inhibition constants in the micromolar range). Our aim was, at first, to investigate the influence

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