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]

Acknowledgment. This investigation was supported by NIH Grant CA-21659.

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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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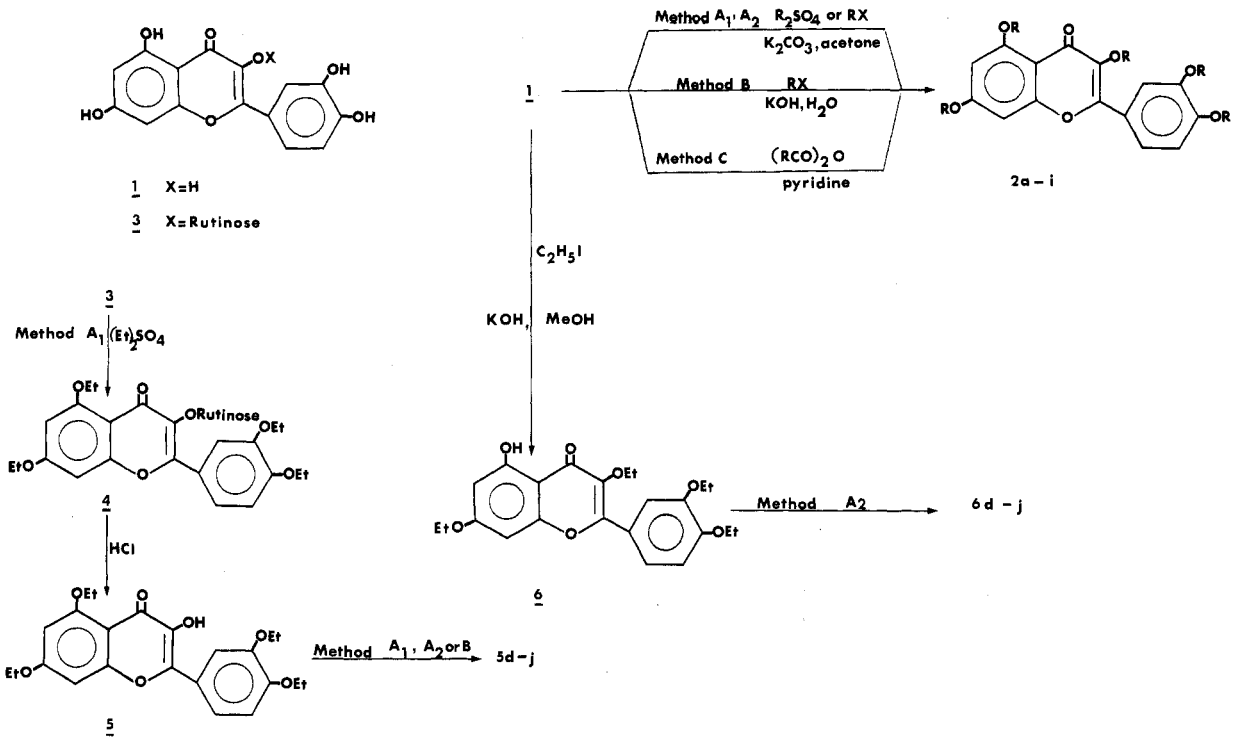
Scheme I



of lipophilic or hydrophilic variations in the five substituents around the quercetin moiety and then to study the importance of these variations in the 3- and 5-positions of quercetin. Indeed, modifications in these two positions, near the carbonyl group of the pyrone ring, are likely to induce the most important variations in the general electronic and lipophilic properties of the whole molecule. This strategy led us to the discovery of new potent and highly selective inhibitors of particulate cAMP phosphodiesterase activity.

Chemistry. The synthesis of 3,3′,4′,5,7-penta-O-substituted quercetin was carried out by standard procedures[13] as illustrated in Scheme I. Quercetin (1) was treated with various alkyl halides or sulfates either in dry acetone with potassium carbonate (method A) or in water with potassium hydroxide (method B) to give the corresponding penta-O-substituted derivatives **2a–f**. Acylation of 1 with appropriate acid anhydrides was performed in pyridine (method C) to give derivatives **2g–i**.

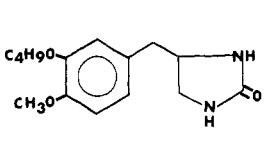
The synthetic route for the preparation of 3′,4′,5,7-tetra-*O*-ethylquercetin selectively substituted at the 3-position (**5**) is shown in Scheme I. Rutoside (**3**) used as starting material was first ethylated according to method A and then submitted to a mild hydrochloric acid hydrolysis to give the corresponding 3′,4′,5,7-tetra-*O*-ethylquercetin **5**. Derivatives **5d–j** were prepared as described in Scheme I.

3,3′,4′,7-Tetra-*O*-ethylquercetin selectively substituted at position 5 (**6**) was similarly prepared from quercetin (1). Tetra-O-ethylation of quercetin was obtained with stoichiometric amounts of ethyl iodide in alcoholic potassium hydroxide solution.

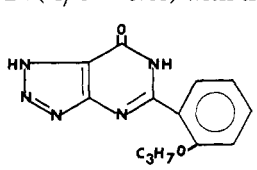
Biochemical Results and Discussion

Quercetin (1), which was shown to be a potent inhibitor of bovine heart and lung cyclic nucleotide phosphodiesterase activity[9,10] (I_{50} in 4–20 μM range), proved less effective toward the rat brain enzyme (Table II). In contrast with that reported for some flavonoids with enzyme from peripheral tissue,[10] quercetin appeared to be a selective inhibitor of cAMP hydrolysis in rat brain tissue, as illustrated by the A/G ratio shown in Table II.

The penta-O-substitution of the quercetin moiety induced drastic modifications in its inhibitory activity, which became either enhanced or totally abolished according to the nature and the size of the introduced substituent. Small alkyl substituents, such as methyl or ethyl, improved inhibition of cAMP hydrolysis and decreased inhibition of cGMP hydrolysis, which led to highly selective inhibitors of cAMP phosphodiesterase activity (compare A/G ratio for compounds **2a** and particularly **2b** to A/G ratio for quercetin). Furthermore, the selectivity for cAMP hydrolyzing activity is by far higher than that obtained with the reference compound Ro 20-1724 (A/G = 0.16) with the



Ro 20-1724



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cytosolic enzyme and in the same order of magnitude with the particulate enzyme. It must be emphasized that compound Ro 20-1724 is one of the most specific cAMP phosphodiesterase inhibitors currently used as a reference compound.[10] Derivatives with larger groups, such as propyl (**2c**), ethoxycarbonylmethyl (**2d**), propionyl (**2h**), and butyryl (**2i**), were entirely devoid of inhibitory activity toward cyclic nucleotide phosphodiesterase, both in cytosolic and in particulate fractions of the rat brain. Thus, increasing the size of the substituents on the quercetin molecule improved the binding to the cAMP hydrolyzing sites up to a limit value that ideally approximates a group with two carbon atoms, such as the ethyl group. Beyond this length, the steric hindrance of the substituents is likely involved in the important loss of inhibitory activity. cGMP hydrolyzing sites seemed to be even more sensitive to the molecule bulk, since any substituent larger than H reduced the potency of the quercetin derivatives to inhibit both

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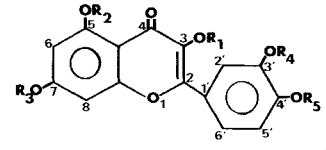
Table I. Physical Properties of Penta-O-substituted Analogues of Quercetin



no.	R_1	R_2	R_3	R_4	R_5	synth method	mp, °C	recrystn solvent	yield, %	R_f[a]	formula[b]
1	H	H	H	H	H					0.51	
2a	CH_3	CH_3	CH_3	CH_3	CH_3	A_1	151[c]	EtOH	72	0.34	$C_{20}H_{20}O_7$
2b	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5	A_1	117-119	EtOH/H_2O	78	0.67	$C_{25}H_{30}O_7$
2c	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	A_2	260	EtOH	20		$C_{30}H_{40}O_7$
2d	$CH_2COOC_2H_5$	$CH_2COOC_2H_5$	$CH_2COOC_2H_5$	$CH_2COOC_2H_5$	$CH_2COOC_2H_5$	A_2	92-93[d]	toluene/EtOH	65	0.56	$C_{35}H_{40}O_{17}$
2d′	CH_2COOH	CH_2COOH	CH_2COOH	CH_2COOH	CH_2COOH	*e*	210	EtOH	70	0	$C_{25}H_{20}O_{17}$
2e	CH_2CH_2OH	CH_2CH_2OH	CH_2CH_2OH	CH_2CH_2OH	CH_2CH_2OH	B	151-153[f]	EtOH	32	0	$C_{25}H_{30}O_{12}$
2f[g]	CH_2CH_2Br	CH_2CH_2Br	CH_2CH_2Br	CH_2CH_2Br	CH_2CH_2Br	*e*	186-188	EtOH	30	0.05	$C_{25}H_{25}Br_5O_7$
2g	$COCH_3$	$COCH_3$	$COCH_3$	$COCH_3$	$COCH_3$	C	198	EtOH	70	0.66	$C_{25}H_{20}C_{12}$
2h	COC_2H_5	COC_2H_5	COC_2H_5	COC_2H_5	COC_2H_5	C	129-130	EtOH	84	0.75	$C_{30}H_{30}O_{12}$
2i	$CO(CH_2)_2CH_3$	$CO(CH_2)_2CH_3$	$CO(CH_2)_2CH_3$	$CO(CH_2)_2CH_3$	$CO(CH_2)_2CH_3$	C	102	EtOH	82	0.78	$C_{35}H_{40}O_{12}$
5	H	C_2H_5	C_2H_5	C_2H_5	C_2H_5	*h*	183[i]	EtOH	39	0.67	$C_{23}H_{26}O_7$
5d	$CH_2COOC_2H_5$	C_2H_5	C_2H_5	C_2H_5	C_2H_5	A_2	124-125	EtOH	60	0.69	$C_{27}H_{32}O_9$
5d′	CH_2COOH	C_2H_5	C_2H_5	C_2H_5	C_2H_5	*h*	170-172	EtOH	80	0.03	$C_{25}H_{28}O_9$
5e	CH_2CH_2OH	C_2H_5	C_2H_5	C_2H_5	C_2H_5	B	180-184	EtOH	41	0.67	$C_{25}H_{30}O_8$
5j[j]	$(CH_2)_2N(C_2H_5)_2$	C_2H_5	C_2H_5	C_2H_5	C_2H_5	B	130-133	acetone	85	0.02	$C_{29}H_{40}ClO_7N$
6	C_2H_5	H	C_2H_5	C_2H_5	C_2H_5	*h*	116-120	acetone/H_2O	40	0.76	$C_{23}H_{26}O_7$
6d	C_2H_5	$CH_2COOC_2H_5$	C_2H_5	C_2H_5	C_2H_5	A_2	116-118	EtOH/CH_3OH	44	0.59	$C_{27}H_{32}O_9$
6d′	C_2H_5	CH_2COOH	C_2H_5	C_2H_5	C_2H_5	*h*	190-194	EtOH	90	0.02	$C_{25}H_{28}O_9$
6f	C_2H_5	CH_2CH_2Br	C_2H_5	C_2H_5	C_2H_5	A_2	118-120	EtOH	35	0.48	$C_{25}H_{29}BrO_7$
6j[j]	C_2H_5	$(CH_2)_2N(C_2H_5)_2$	C_2H_5	C_2H_5	C_2H_5	B	154	EtOH	50	0.018	$C_{29}H_{40}ClO_7N$

[a] R_f values were obtained with a solvent system of acetone/pentane (2:1). [b] All compounds were analyzed for C and H and checked within 0.4% of the theoretical values. [c] Literature[13] mp 150-151 °C. [d] Literature[29] mp 92-94 °C. [e] See Experimental Section. [f] Literature[30] mp 152-154 °C. [g] Compound 2f was prepared analogously to compounds in method A_2, except that the alkyl halide was replaced by dialkyl halide ($BrCH_2CH_2Br$) and acetone was replaced by DMF. [h] See Experimental Section. [i] Literature[31] mp 182-183 °C. [j] HCl.

Table II. Inhibition of cAMP and cGMP Phosphodiesterase from Rat Brain by Some Penta-O-substituted Analogues of Quercetin and Reference Compounds

	cytosolic phosphodiesterase			particulate phosphodiesterase		
	I_{50},[a] μM			I_{50},[a] μM		
no.	cAMP (A)	cGMP (G)	A/G	cAMP (A)	cGMP (G)	A/G
1	87	275	0.32	20	81.3	0.24
2a	6.3	>1 000	<0.008	10.2	96	0.10
2b	70	>10 000	<0.007	7.7	350	0.02
2c	I[b]	I		I	I	
2d	I	I		I	I	
2d'	I	I		I	I	
2e	470	2 800	0.17	69	1 300	0.05
2f	500	10 000	0.05	18	24.5	0.73
2g	355	I		126	275	0.46
2h	I	I		600	I	
2i	I	I		I	I	
5	I	I		I	I	
5d	I	I		I	I	
5d'	10	>1 000	<0.01	22	71	0.31
5e	I	I		I	I	
5j	37	47	0.79	20	126	0.16
6	I	I		I	I	
6d	80	>1 000	<0.08	8.3	700	0.01
6d'	20	63	0.32	16	48	0.32
6f	40	>1 000	<0.04	17	96	0.18
6j	35	58	0.60	10	80	0.11
		Reference Compounds[c]				
Ro 20-1724	1660	10 000	0.16	74.1	10 000	0.007
M&B 22 948	66.1	93.3	0.71	74.1	63.1	1.17

[a] The cyclic nucleotide phosphodiesterase assays were performed as described under Experimental Section. cAMP and cGMP substrate concentrations were 0.25 μM (in the range of the physiological ones). A = I_{50} values obtained with cAMP as substrate; G = I_{50} values obtained with cGMP as substrate. [b] For inactive compounds (I), no inhibition was observed up to 1 mM drug concentration. [c] Compound Ro 20-1724 was a generous gift from Dr. H. Gutman, Hoffmann La Roche & Cie. CH 4002 Bâle-Suisse. Compound M&B 22 948 was kindly supplied by Dr. R. Broad, May & Baker Ltd, Dagenham Essex RM 107×5.

cytosolic and particulate cGMP phosphodiesterase activity. The bromoethyl group (**2f**) appeared to be an exception to this rule, as indicated by the fourfold higher inhibitory potency of compound **2f** for the particulate enzyme as compared with quercetin. These observations are in good agreement with the hypothetical scheme proposed by Severin et al.[14] On the basis of inhibition studies with cAMP analogues, these authors assume the active site of phosphodiesterase to be located in a narrow slit that does not allow the approach of bulky molecules. However, the steric size of the substituent may not be the only factor that determines the effect of substitution on phosphodiesterase activity. Thus, among substituents with two carbon atoms, which approximates the optimal size for the inhibition of cAMP hydrolyzing activity, polar groups, such as carboxymethyl (**2d**), hydroxyethyl (**2e**), and bromoethyl (**2f**), or electron-withdrawing groups, such as acetyl (**2g**), drastically reduced the potency of the molecules to inhibit the cytosolic enzyme. In contrast, the particulate enzyme can accomodate such groups with only moderate loss in inhibitory activity with respect to the parent compound quercetin. Although any substitution proved unfavorable for the inhibition of cGMP phosphodiesterase activity, the cGMP hydrolyzing sites seemed to better tolerate polar or electron-withdrawing groups than lipophilic ones, especially with the particulate enzyme. From examination of the general lipophilicity or hydrophilicity of the whole molecules, it appears that penta-O-substituted derivatives more lipophilic than quercetin proved more active and especially far more specific toward cAMP hydrolysis than quercetin itself. On the other hand, water-soluble derivatives proved less active and less specific with respect to cAMP hydrolysis than the parent compound **1**. Such a decrease in the inhibitory potency of water-soluble bioflavonoids was frequently reported with other enzymes, such as pig heart mitochondrial ATPase,[15] or other biological systems.[8] Finally, it is noteworthy that all the active penta-O-substituted derivatives (**2**) of quercetin, except compound **2a**, exhibited a higher affinity for the particulate enzyme than for the cytosolic rat brain preparation.

However, the addition of the same substituent simultaneously into five positions of the quercetin molecule makes impossible the attribution of differences in phosphodiesterase inhibitory activity to the modification of a particular position or set of positions. So, inhibition studies with penta-O-substituted derivatives of quercetin selectively modified in a single position are likely to bring further information about structure–activity relationships. Since, in our biochemical experiments, penta-*O*-ethylquercetin (**2b**) proved the most specific toward cAMP hydrolysis from both cytosolic and particulate preparations and also proved the most pharmacologically active, we decided to study the influence of the selective substitution in position 3 or 5 of the basic flavonoid skeleton with substituted quercetin bearing four ethyl groups in the other positions.

Some substitution other than H at the position 3 of the 3′,4′,5,7-tetra-*O*-ethylquercetin molecule was apparently required for inhibition of either form of phosphodiesterase, as indicated by the lack of inhibitory activity of compound **5** (Table II). However, the influence of the substitution in this 3-position appeared rather variable. Thus, beside

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(15) A. Di Pietro, C. Godinot, M. L. Bouillant, and D. Gautheron, *Biochimie*, **57**, 959 (1975).

penta-*O*-ethylquercetin (**2b**), the only two compounds that proved effective inhibitors of the enzyme were the water-soluble derivatives **5d′** (carboxymethyl) and **5j** (diethylaminoethyl, HCl). However, they appeared far less specific than penta-*O*-ethylquercetin (**2b**) itself toward cAMP hydrolysis by the particulate rat brain enzyme. Theoretical calculations by the CNDO/2 method have previously shown that the charge distribution of the pyrone ring resembles that of the pyrimidine ring of cAMP.[11] So, according to the authors, the 3-position of flavonoids corresponds to the N_1 position of cAMP. This comparison could also be extended to the N_1 position of cIMP and to the N_1 position of the xanthine nucleus. Several observations from other laboratories supported this finding. Meyer et al.[16] found various N_1-alkoxy derivatives of cAMP (methoxy, ethoxy, phenoxy, methyl) to be effective as inhibitors of rabbit lung and bovine heart phosphodiesterase with I_{50} values (35–150 μM) in the same range as those reported in Table II for the active position-3-modified derivatives **2b**, **5d′**, and **5j** (10–126 μM). On the other hand, Kramer et al.[17] reported that substitution other than H was required at position 1 of the xanthine nucleus but that substituents larger than methyl were rather unfavorable, since none of these compounds proved as effective as 1-methyl-3-isobutylxanthine in inhibiting pig coronary artery phosphodiesterase. Furthermore, Severin et al.[14] observed that some N_1-substituted cyclic AMP derivatives were only weak competitive inhibitors of a partially purified rat liver phosphodiesterase. Thus, the influence of the substitution at the 3-position of the flavonoid ring, as well as at the equivalent N_1 position of the xanthine nucleus and the pyrimidine ring of cyclic nucleotides, appears very complex. Obviously, the steric size of the groups is not the only factor involved, since the large and polar substituent diethylaminoethyl, HCl (**5j**), is well accepted by the cytosolic and the particulate brain enzymes. Both cAMP and cGMP hydrolyzing sites can accomodate polar substituents, which results in a loss of specificity, especially with the particulate brain enzyme. However, the inhibitory potency and/or the selectivity of an inhibitor may vary from one tissue to another. In particular, such variations were frequently observed by others with cIMP derivatives. Thus, cIMP itself, the resemblance of which with the flavonoid skeleton is even more straightforward, was found to be a potent inhibitor of the cytosolic cAMP phosphodiesterase from cat heart (I_{50} = 2 μM) and a weak inhibitor of this enzyme in the rat brain (I_{50} = 940 μM).[18] Similarly, cIMP proved more potent in inhibiting the beef heart phosphodiesterase than the rabbit lung enzyme.[19] Furthermore, it exhibited an opposite specificity in these two organs: a preferential inhibition of cGMP hydrolysis in the lung and a slightly preferential inhibition of cAMP hydrolysis in the heart.

Position 5 of the tetra-O-ethylated quercetin molecule also required some substitution other than H. With the exception of the 5-unsubstituted compound **6**, all the selectively 5-substituted 3,3′,4′,7-tetra-*O*-ethyl derivatives of quercetin proved powerful inhibitors of phosphodiesterase activity and especially of the particulate cAMP phosphodiesterase enzyme, as illustrated by their I_{50} values lower than 17 μM (Table II). Among these 3,3′,4′,7-tetra-*O*-ethylquercetins, compound **6d**, with an ethoxycarbonylmethyl group at position 5, and compound **6f**, with a bromoethyl group at the same position, exhibited a high selectivity with respect to cytosolic cyclic AMP hydrolysis, similar to that observed for the penta-*O*-ethylquercetin (**2b**); **6d** and **6f** also exhibited a lipophilicity quite similar to that shown by the latter compound. Water-soluble derivatives **6d′** (carboxymethyl) and **6j** (diethylaminoethyl, HCl) roughly presented the same inhibitory profile as their homologous compounds **5d′** and **5j**, a high inhibitory potency and a low selectivity. On the basis of the theoretical calculations proposed by Ferrell et al.[11] the 5-position of flavonoid derivatives should be compared to the N_7 position of the purine ring of cAMP and cIMp and to the N_7 position of the xanthine nucleus. Indeed, tubercidin cyclic phosphate, a 7-deaza isostere of cAMP, and two closely related compounds substituted in the 7-position by a nitrile or an amido group were all very good inhibitors of a "high K_m" cAMP phosphodiesterase from beef heart and rabbit lung, with I_{50} values in a 2–20 μM range.[20] Furthermore, some 7-substituted 1-methyl-3-isobutylxanthine derivatives showed a good inhibitory potency for pig coronary artery phosphodiesterase (I_{50} = 2–142 μM) with variable selectivity for either cAMP or cGMP hydrolyzing activities.[21] In this 5-position, the steric hindrance of the substituent does not seem to be involved, since large groups with more than two carbon atoms (**6d,j**) are effective inhibitors of the cytosolic and particulate rat brain enzymes. cAMP hydrolyzing sites tolerate quite well lipophilic and polar groups, while cGMP hydrolyzing sites better accomodate polar groups than lipophilic ones, especially in the cytosolic preparation, which finally results in a lack of selectivity for the water-soluble derivatives, as observed with the two other groups of flavonoid compounds **2a–i** and **5d–j**.

Finally, all the penta-*O*-substituted derivatives of quercetin reported herein bear the same O-substituted 3′,4′-dihydroxyphenyl group at the 2-position of the pyrone ring. So they could be compared with 2-aryl-cAMP or -cIMP derivatives. Indeed, many of these 2-aryl-substituted cyclic nucleotides were described as potent inhibitors of cyclic nucleotide phosphodiesterase from various organs or tissues. Marumoto et al.[22] reported that 2-phenyl-, 2-benzyl-, and 2-phenoxy-cAMP were good inhibitors of rat brain cAMP phosphodiesterase, with I_{50} values in the 24–64 μM range. Similarly, Meyer et al.[23] found that among various alkyl, alkylthio, or phenyl substituents, the aromatic ones imparted the best inhibition ability upon the cAMP molecule toward dog heart phosphodiesterase (I_{50} in the micromolar range). The best of these inhibitors, 2-styryl-cAMP, exhibited a I_{50} of 0.42 μM. A slight decrease in the inhibitory potency was observed with other preparations from beef heart and rabbit lung.

Conclusions

Although it is difficult to extract general structure–activity relationships from the present results, some structural features that play an important role in the inhibitory

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properties of the substituted quercetin can be emphasized. With the rat brain enzyme, quercetin and its active penta-*O*-substituted analogues inhibit more selectively cAMP breakdown than cGMP, since all the A/G ratios shown in Table II are lower than 1. Substituents that increase the general lipophilicity of the molecules also enhance the inhibitory potency toward the particulate enzyme but, above all, drastically improve the selectivity of inhibition toward cAMP hydrolysis, both with the cytosolic and the particulate phosphodiesterase preparations. Compounds **2b** and **6d** are 100-fold more potent inhibitors of cAMP phosphodiesterase activity than of cGMP phosphodiesterase activity. The selectivity observed with quercetin under the same conditions is 30-fold smaller. With the cytosolic preparation, five compounds prove totally inactive toward cGMP hydrolysis, while they are potent inhibitors of cAMP phosphodiesterase activity. Nevertheless, the steric hindrance of the substituent rapidly opposes this improvement, and bulky substituents make the molecule inactive. On the other hand, water-soluble derivatives of quercetin prove either inactive or potently active, but with a low specificity. Our biochemical results further support the hypothesis that bioflavonoids could exhibit some affinity for the nucleotide binding sites of several enzymes involving stacking interactions between the base and the enzymatic protein. With regard to cyclic nucleotide phosphodiesterase inhibition, some similarities between the inhibitory potency of various cyclic nucleotide analogues and the penta-O-substituted quercetin derivatives described herein are in good agreement with the mimicking of the pyrimidine ring in cyclic nucleotides by the pyrone ring of the flavonoids proposed by Ferrell et al.[11] A last important point has to be stressed. Quercetin and its active penta-O-substituted derivatives inhibit more potently the particulate enzyme than the cytosolic preparation. The affinity of the most selective compounds for the membrane-bound cAMP phosphodiesterase is 10- to 30-fold higher than that observed for the cytosolic fraction. Such an inhibition of a particulate cyclic nucleotide phosphodiesterase by bioflavonoids has never been reported before. Since bioflavonoids are known to inhibit various membrane-bound enzymes and biological systems, our results on phosphodiesterases are consistent with the hypothesis that quercetin and related compounds exert a generalized effect at the level of the cell membrane.[24] Pharmacological properties and particularly "antifatigue properties" of these penta-O-substituted quercetin derivatives are presently under investigation. Their potential activities as psychotropic, antihypoxic, spasmolytic, phlebotonic, cerebral, or peripheral vasodilator agents will also be examined.

Experimental Section

Melting points were determined with a Kofler apparatus. Structures of the products were confirmed by NMR spectra obtained on a Brucker 80 spectrophotometer, and IR spectra were recorded on a Beckman Acculab 4 spectrophotometer. Data were consistent with assigned structures for all products. Elementary analyses were performed by the Service Central d'Analyse du CNRS (Division de Lyon) and are within 0.4% of the theoretical values. TLC was performed using fluorescent silica gel plates (Merck) with acetone–pentane (2:1) as solvent system. Components were visualized by UV fluorescence properties and by spraying with diazotized benzidine reagent. Representative procedures for the preparation of compounds **2a–i**, **5d–j**, and **6d–j** are illustrated in the following paragraphs. Capital letters designate the synthetic procedures as given in Scheme I and in Table I.

Method A1. A solution of quercetin (2.0 g, 0.0066 mol) and dialkyl sulfate (0.08 mol) in dry acetone (300 mL) was heated to reflux for 21 h with K_2CO_3 (30 g, 0.22 mol). The reaction mixture was filtered, the carbonate was washed with hot acetone, and the acetone was evaporated to dryness under reduced pressure.

Method A2. The penta-O-substituted derivatives were prepared as described in method A1, except that dialkyl sulfate was replaced by an equivalent amount of alkyl halide.

Method B. Quercetin (2.0 g, 0.0066 mol) was dissolved in an aqueous solution of 2 N KOH (50 mL). Alkyl iodide was then rapidly added with stirring. The reaction mixture was heated at 80 °C for 20 h. The solution was adjusted to pH 4–5 with dilute hydrochloric acid and evaporated under reduced pressure. The residue was crystallized from EtOH.

Method C. Quercetin (6.0 g, 0.02 mol), acid anhydride (0.6 mol), and pyridine (6 mL) were heated to reflux for 5 h. Ice–water (100 g) was then added to the warm mixture. The resulting precipitate was filtered and washed with water.

3-Hydroxy-3′,4′,5,7-tetra-*O*-ethylquercetin (5). Compound 4 (8.0 g, 0.011 mol) was heated to reflux in 2 N HCl (800 mL) for 2 h. When the mixture cooled, a pink precipitate formed, which was separated by filtration and recrystallized from EtOH.

Carboxylic Acid Derivatives (2d′, 5d′, and 6d′). The carboxylic acid ester was heated to reflux in 6 N sulfuric acid (20 mL/mmol) for 1 h. When the reaction mixture cooled, a precipitate formed, which was separated by filtration and recrystallized.

3-[(Diethylamino)ethyl]-3′,4′,5,7-tetra-*O*-ethylquercetin Hydrochloride (5j). Compound **5** (2.5 g, 0.006 mol), 2-(diethylamino)ethyl chloride hydrochloride (1.03 g, 0.006 mol), and KOH (0.67 g, 0.012 mol) were dissolved in acetone (200 mL) and stirred under reflux for 12 h. The KCl that formed was filtered and washed with warm acetone. The solution was evaporated under reduced pressure. The residue was acidified with 10% HCl and then washed with Et_2O. The organic layers were separated. The aqueous layer was further washed with 10% NaOH and then extracted with benzene and $CHCl_3$. The organic layers were combined, dried on Na_2SO_4, and evaporated to a brown oil. The resulting oil was precipitated by addition of etheral HCl to give the hydrochloride (**5j**).

5-Hydroxy-3,3′,4′,7-tetra-*O*-ethylquercetin (6). A mixture of **1** (10 g, 0.033 mol), ethyl iodide (34.8 g, 0.2 mol), and KOH (9.3 g, 0.16 mol) in 200 mL of MeOH was stirred at reflux for 20 h. The solution was then poured into ice–water (1 L), extracted with benzene, dried (Na_2SO_4), and evaporated to dryness. The resulting oil was recrystallized from H_2O–acetone (1:1) to give a yellow solid.

5-[(Diethylamino)ethyl]-3,3′,4′,7-tetra-*O*-ethylquercetin Hydrochloride (6j). Compound **6f** (1.2 g, 0.02 mol) and diethylamine (1 mL, 0.09 mol) in benzene were stirred at reflux for 4 h. KOH (0.09 mol) in MeOH was added to the reaction mixture. Solvents and diethylamine were eliminated under reduced pressure. HCl (10%) and $CHCl_3$ were then added to the residue. The aqueous solution was made alkaline with 10% NaOH and then extracted with benzene. The organic layer was dried (Na_2SO_4) and evaporated. The resulting oil was precipitated by addition of etheral HCl to give the hydrochloride **6j**, which was very hygroscopic and did not give a satisfactory analysis.

Rat Brain Cyclic Nucleotide Phosphodiesterase Preparation. Crude cytosolic and particulate cyclic nucleotide phosphodiesterases from rat brain were prepared as previously described.[25,26] Briefly, rat brains (without cerebellum) were rapidly removed and homogenized in 10 vol (w/v) of 0.32 M sucrose, 5 mM Tris, HCl (pH 7.5), 1 mM $MgCl_2$, and 0.1 mM dithiothreitol. The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant solution was centrifuged at 105000*g* for 60 min. The resulting 105000*g* supernatant was dialyzed overnight against 20 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$ and 0.1 mM

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dithiothreitol. Aliquots of the dialyzed solution were frozen at −20 °C and used as the source of soluble phosphodiesterase. The 105000*g* pellet was resuspended in the original volume of sucrose buffer and rehomogenized. After a second 105000*g* centrifugation, the washed pellet was resuspended in a minimal volume of 20 mM Tris-HCl, pH 7.5, buffer containing 1 mM $MgCl_2$ and 0.1 mM dithiothreitol and dialyzed overnight against the same buffer. This preparation was kept frozen at −20 °C and used as the source of particulate enzyme.

Enzyme Assay. Cyclic nucleotide phosphodiesterase activity was assayed as previously described,[27] following a modified method based on the original procedure of Thompson et al.[28] Substrate cAMP and cGMP concentration was 0.25 μM. All the test compounds were solubilized in dimethyl sulfoxide (Me_2SO) and brought to the adequate concentration with the incubation buffer. Final Me_2SO concentration did not exceed 5%. This Me_2SO amount slightly inhibited cyclic nucleotide phosphodiesterase activity but did not modify the percent inhibition found with water-soluble drugs. Test compounds and Me_2SO were examined to ensure that they did not interfere either with the nucleotidase step of the assay or with adenosine and guanosine recoveries. I_{50}'s were calculated by interpolating three to four values of inhibition, ranging from 30 to 80%, against the logarithm of inhibitor concentrations. All the assays were carried out at 30 °C and performed in triplicate at enzyme dilutions that gave 10–15% hydrolysis of substrate in the absence of inhibitor.

"Low K_m's" of the cytosolic and the particulate phosphodiesterase preparations were determined with cAMP and cGMP concentrations ranging from 0.25 to 5 μM. With the cytosolic preparation, the apparent low K_m's were 2–4 μM for cAMP and 3–5 μM for cGMP. With the particulate preparation, low K_m's values were 3–4 and 20–22 μM for cAMP and cGMP as substrate, respectively.

Penta-*O*-ethylquercetin (**2b**), one of the more selective quercetin analogues, was found to be a competitive inhibitor of both the cytosolic and the particulate cAMP phosphodiesterase with K_i of 200 and 15 μM, respectively.

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Importance of the Aromatic Ring in Adrenergic Amines. 7. Comparison of the Stereoselectivity of Norepinephrine *N*-Methyltransferase for Aromatic vs. Nonaromatic Substrates and Inhibitors[1a,b]

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Some nonaromatic analogues of amphetamine and α-methylbenzylamine were prepared and evaluated as competitive inhibitors of norepinephine *N*-methyltransferase (NMT). All of the nonaromatic analogues were significantly more active than their aromatic counterparts [K_i for amphetamine = 740 μM; K_i for 1-cyclooctyl-2-aminopropane = 86 μM]. In order to determine if the aliphatic ring of these analogues bound to the same binding site as the phenyl ring of amphetamine and α-methylbenzylamine, the stereoselectivity of NMT toward the different compounds was determined. Stereochemical requirements for aromatic and nonaromatic inhibitors were similar (in all cases the *S* isomer was more potent at inhibiting NMT). The stereochemical preference expressed for phenylethanolamine substrates and corresponding nonaromatic analogues was also found to be the same; however, as the lipophilicity of the nonaromatic ethanolamine analogues was increased, a loss in both stereoselectivity and substrate activity occurred. The results presented here are consistent with an aromatic ring binding site that is part of, or bordered by, a large hydrophobic area. The larger, more hydrophobic nonaromatic phenylethanolamine derivatives are drawn into the hydrophobic area, which reduces side-chain hydroxy interactions necessary for substrate activity.

The last step in the biosynthetic pathway of epinephrine is the N-methylation of norepinephrine, which is catalyzed by the enzyme norepinephrine *N*-methyltransferase (EC 2.1.1.28), usually abbreviated as NMT (also called phenylethanolamine *N*-methyltransferase, PNMT). *S*-Adenosyl-L-methionine (AdoMet) serves as the methyl donor in the reaction. Although NMT is primarily localized in the adrenal medulla, in 1974 Hokfelt et al.[2] employed immunohistochemical techniques to demonstrate the presence of NMT-containing cell bodies within the brainstem and hypothalamus; this observation led to the conclusion that epinephrine was utilized as a neurotransmitter. The fact that some of the NMT-containing nuclei within the brainstem had previously been thought to participate in the central control of blood pressure led several investigators to compare the NMT activity in the brainstem of spontaneously hypertensive rats (SHR) with normotensive controls; it was found that NMT activity was

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