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INHIBITION OF ALDOSE REDUCTASES FROM RAT AND BOVINE LENSES BY FLAVONOIDS

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Abstract—Thirty flavones, four isoflavones and thirteen coumarins were tested as inhibitors of lens aldose reductase, which is believed to participate in the initiation of cataract formation in diabetes. Many were found to be potent inhibitors, and the two most potent ones were axillarin (5,7,3′,4′-tetrahydroxy-3,6-dimethoxyflavone) and 6,3′,4′-trihydroxy-5,7,8-trimethoxyflavone (LARI 1). These two flavones inhibited aldose reductase purified from rat lens with IC_{50} values of 2.6×10^{-8} and 3.6×10^{-8} M respectively. They also inhibited aldose reductase purified from bovine lens with IC_{50} values of 1.8×10^{-7} M. The potencies of the two compounds were superior to those of all the previously reported inhibitors of aldose reductase. Inhibition of rat and bovine lens aldose reductases by the two compounds was of a non-competitive type with DL-glyceraldehyde as the variable substrate. Some flavones including axillarin and LARI 1 were found to be poorly or scarcely inhibitory against several adeninenucleotide-requiring enzymes, which are involved in glycolysis and other metabolic reactions. These results obtained show that the two flavones have some features which may be required in clinically useful drugs for diabetic patients. All the potent inhibitors of the compounds tested had a flavone skeleton, one (or two free) hydroxyl(s) in ring C, and more than three hydroxyls (free or methylated) in ring A. The possible relationships of structures to inhibitory potencies of the compounds tested are discussed.

Although insulin therapy or the use of oral hypoglycemic drugs has greatly reduced death from chronic complications of diabetes, the resulting longevity of diabetic patients has led to complications such as cataract, peripheral neuropathy and vascular disease particularly of the retina, kidney and heart. Increased activity of the sorbitol pathway of glucose metabolism has been implicated in the pathogenesis of these complications [1]. The sorbitol pathway contains two enzymes, aldose reductase (EC 1.1.1.21) and sorbitol dehydrogenase (EC 1.1.1.14). Since aldose reductase appears to initiate the events that lead to sugar cataract and other diabetic complications, aldose reductase inhibitors may be of value in the treatment of some of these complications [2].

In recent years, Varma and Kinoshita have reported that flavonoids are highly potent inhibitors of aldose reductase, and that the two most potent ones are quercitrin and quercitrin 2″-acetate [3]. More recently, sorbinil (*d*-6-fluoro-spiro-[chroman-4,4′-imidazolidine]-2′,5′-dione) has been shown to be a more potent inhibitor of rat lens aldose reductase (RLAR) than quercitrin [4] and also to be highly effective in the prevention of cataract formation in both diabetic and galactosemic animals [5, 6]. Both quercitrin and sorbinil, however, are not potent enough inhibitors in the ultimate use for prevention and/or amelioration of complications of diabetic patients.

In this paper, we present the results of a screening test for thirty-four flavonoids and thirteen coumarins as aldose reductase inhibitors and describe the possible relationships of structure to the inhibitory potencies of flavones. We also report the differences of RLAR and bovine lens aldose reductase (BLAR) in the susceptibility to inhibition by some flavones and the inhibitory effects of some flavones on adeninenucleotide-requiring enzymes other than aldose reductase.

MATERIALS AND METHODS

Materials. Rat lenses were removed from eyes of rats of the Wistar strain weighing 200–250 g. Bovine eyes were obtained from a local abattoir, and the lenses were removed and frozen until needed. $NADP^+$, NADPH, lactate dehydrogenase and glucose-6-phosphate dehydrogenase were obtained from the Oriental Yeast Co., Ltd. (Osaka, Japan). NAD^+, NADH, 2-phosphoenolpyruvate, glucose-6-phosphate, ATP, ADP and glutathione (oxidized form) were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). DL-Glyceraldehyde, quercitrin and pyridine 3-aldehyde were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). DEAE-Sephacel and Sephadex G-75 were from Pharmacia Fine Chemicals (Uppsala, Sweden). Mātrex gel red A was obtained from the Amicon Co. (Lexington, MA, U.S.A.). All other chemicals were of the highest grade commercially available.

Flavones [7–13], isoflavones [14–16] and coumarins [17–22] were isolated from various plants or

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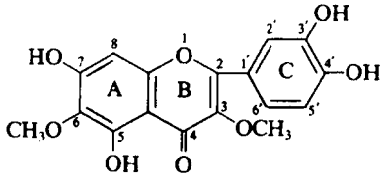
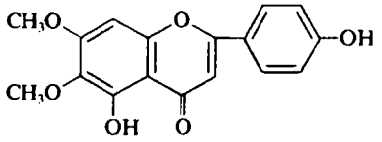
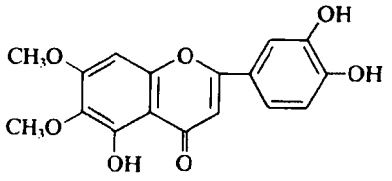
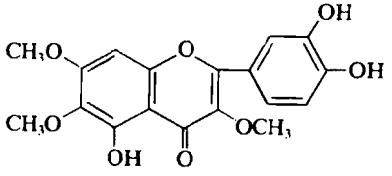
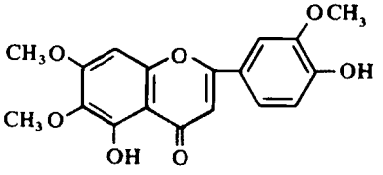
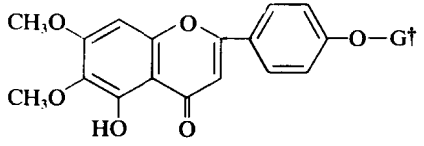
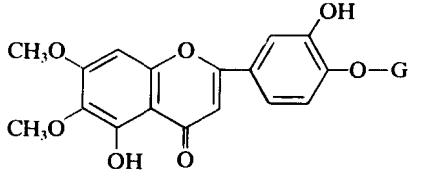
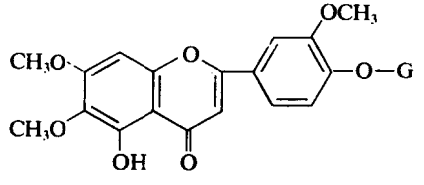
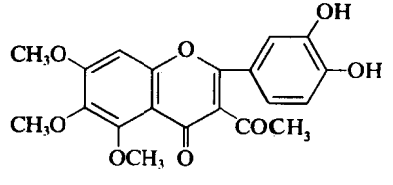
Table 1. Inhibition of lens aldose reductases by 5,6,7-trisubstituted flavone derivatives*

No.	Structure	Name	Inhibition (%)					
			RLAR			BLAR		
			10^{-5} M	10^{-6} M	10^{-7} M	10^{-5} M	10^{-6} M	10^{-7} M
1		5,7,3′,4′-Tetrahydroxy-3,6-dimethoxyflavone (Axillarin)	97	94	80	100	90	37
2		5,4′-Dihydroxy-6,7-dimethoxyflavone (Cirsimaritin)	76	49	12	79	38	7
3		5,3′,4′-Trihydroxy-6,7-dimethoxyflavone (Cirsiliol)	89	77	38	79	52	11
4		5,3′,4′-Trihydroxy-3,6,7-trimethoxyflavone (7-Methylaxillarin)	91	75	24	92	50	12
5		5,4′-Dihydroxy-6,7,3′-trimethoxyflavone (Cirsilineol)	61	0	0	53	17	0

6		5,4′-Dihydroxy-6,7-dimethoxyflavone 4′-glucoside (Cirsimaritin 4′-glucoside)	38	8	0	62	20	0
7		5,3′,4′-Trihydroxy-6,7-dimethoxyflavone 4′-glucoside (Cirsiliol 4′-glucoside)	53	25	11	61	24	0
8		5,4′-Dihydroxy-6,7,3′-trimethoxyflavone 4′-glucoside (Cirsilineol 4′-glucoside)	42	0	0	59	20	0
9		3-Acetyl-3′,4′-dihydroxy-5,6,7-trimethoxyflavone	52	8	0	44	15	0

* Assays were carried out as described in Materials and Methods. Each compound was tested 3–5 times, and the standard deviation of each value listed was less than 5%.

† G represents D-glucosyl residue.

chemically synthesized as reported previously. Because of poor water-solubility of many of the compounds, all compounds as well as quercitrin were dissolved in propylene glycol, which is a useful solvent of low toxicity for some water-insoluble drugs. Usually a 10^{-3} M solution was prepared and diluted to desired concentrations with propylene glycol. Aliquots of the diluted solution were added to the reaction mixture to yield compound concentrations of 10^{-8} M to 10^{-5} M and a propylene glycol concentration of 1%. Propylene glycol at 1% inhibited the activity of aldose reductase by only 5% or less, but did not affect the inhibitory potency of compounds; the inhibitory activities (84 and 30%) of quercitrin and sudachiin A at 10^{-5} M in the presence of 1% propylene glycol were similar to those (82 and 32%) in the absence of the solvent.

Assay of aldose reductase activity. Aldose reductase assays were conducted according to the procedure of Hayman and Kinoshita [23], except for the addition of ammonium sulfate instead of lithium sulfate to the reaction mixture. Assays were performed at 30° in 0.1 M sodium phosphate buffer (pH 6.2) containing 0.4 M ammonium sulfate, 10 mM DL-glyceraldehyde, 0.16 mM NADPH and the enzyme (0.010 to 0.016 units) in a total volume of 1.0 ml. The effects of inhibitors on the enzyme activity were determined by including in the reaction mixture 10 μl of each inhibitor solution at desired concentrations. The reference blank to correct for non-specific reduction of NADPH contained all of the above compounds except the substrate. The reaction was initiated by the addition of substrate, and the rate of NADPH oxidation was followed by recording the decrease in absorbance at 340 nm on a Gilford model 250 spectrophotometer.

Purification of lens aldose reductase. BLAR and RLAR were purified according to the method of Inagaki *et al.* [24]. Briefly, a 40–75% ammonium sulfate fraction was subjected to chromatography on DEAE-Sephacel, followed by two column chromatographic steps, i.e. affinity chromatography using Mātrex gel red A and gel filtration on Sephadex G-75. In this procedure, a key step was affinity chromatography on Mātrex gel red A. BLAR and RLAR adsorbed on this gel were eluted with 0.33 mM NADPH and a linear gradient of NaCl (0 to 1.0 M) respectively. BLAR and RLAR were purified over 12,000-fold (4.8 units/mg protein) and 380-fold (4.7 units/mg protein) respectively.

Preparation of crude rat lens aldose reductase (crude RLAR). The supernatant fraction of the homogenate of rat lenses was prepared according to the method of Kador and Sharpless [25] and used as crude RLAR for determining IC_{50} values of flavones.

Assay of other enzyme activities. All the enzyme assays were performed at 30°. The assays of hexokinase (EC 2.7.1.1), pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), glucose-6-phosphate dehydrogenase (EC 1.1.1.47), glutathione reductase (EC 1.6.4.2) and alcohol dehydrogenase (EC 1.1.1.1) were carried out according to the methods of Sharma *et al.* [26], Staal *et al.* [27], Stolzenbach [28], Cohen and Rosemeyer [29], Racker [30] and Dalziel [31] respectively. Aldehyde reductase (EC 1.1.1.2) was assayed by a slight modification of the method of Tulsiani and Touster [32]: the reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 6.4), 0.1 mM pyridine 3-aldehyde, 0.16 mM NADPH and the enzyme in a total volume of 1.0 ml. All operations for preparing crude enzymes were performed at 4°. Rat lenses were homogenized in 5 mM sodium phosphate buffer (pH 7.4) (0.4 ml/lens) containing 10 mM D-glucose and 1 mM 2-mercaptoethanol, and the homogenate was dialyzed against the same buffer for 3 hr and then centrifuged at 20,000 *g* for 30 min. The supernatant fraction thus obtained was used as a hexokinase preparation. The enzyme preparation for the assays of pyruvate kinase, lactate dehydrogenase and glucose-6-phosphate dehydrogenase was obtained from rat lenses by the same method as described above, with 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol. The supernatant fraction containing glutathione reductase was prepared from the homogenate of rat lenses with 5 mM sodium phosphate buffer (pH 7.4) by the same method as used for the preparation of the hexokinase sample. Rat brains were homogenized in 2 vol. of 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol. The homogenate was treated by the same method as described in the preparation of the hexokinase sample, and the supernatant fraction obtained was used as an aldehyde reductase preparation. Rat livers were homogenized in 5 vol. of 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol. The homogenate was then centrifuged at 20,000 *g* for 20 min, and the supernatant was fractionated with ammonium sulfate. A 50–75% fraction was dissolved in a small volume of 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol and was dialyzed against the same buffer. The solution thus obtained was used as an alcohol dehydrogenase preparation.

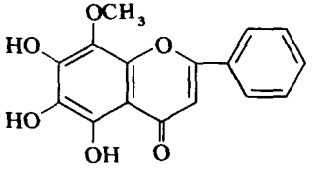
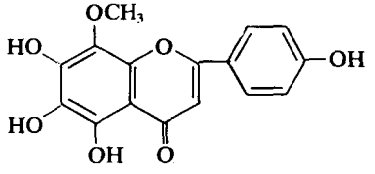
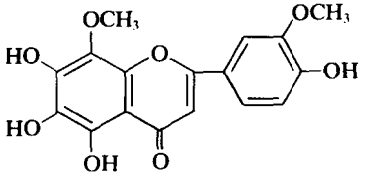
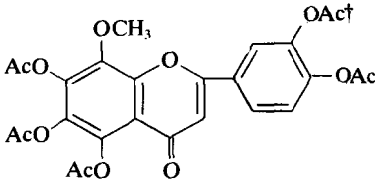
Determination of IC_{50}. The concentration of inhibitor needed to elicit 50% inhibition (IC_{50}) was determined by the method described in Ref. 33.

RESULTS

Inhibition of aldose reductase by flavonoids and coumarins. All of the flavonoids and coumarins used in the present study have not yet been tested for inhibitory activity against aldose reductase. The percentages of inhibition of RLAR and BLAR by flavonoids and coumarins are summarized in Tables 1–7. All of the flavones tested have more than three free or methylated hydroxyls in ring A. The flavone derivatives were grouped in Tables 1–4 primarily according to the degree of hydroxylation in ring A and to that of methylation of hydroxyl groups. Homogeneously purified RLAR and BLAR were employed in the screening test for inhibitory activity unless otherwise stated.

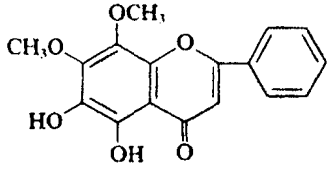
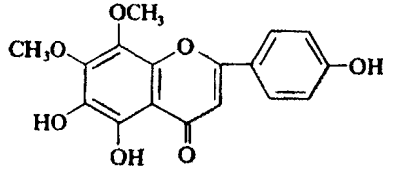
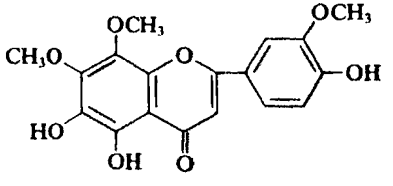
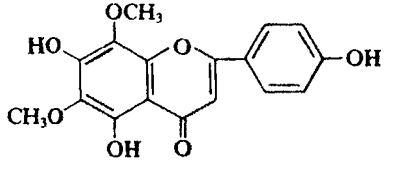
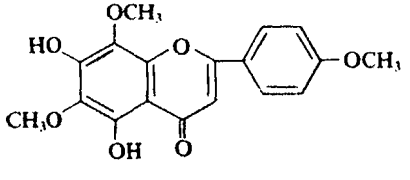
The inhibitory activities of 5,6,7-trisubstituted flavone derivatives are summarized in Table 1. Axillarin (No. 1, 5,7,3′,4′-tetrahydroxy-3,6-dimethoxyflavone) [8] was one of the two most potent inhibitors among the flavonoids and coumarins tested. It inhibited RLAR and BLAR by 80 and 37% at 10^{-7} M respectively. The addition of a methoxyl

Table 2. Inhibition of lens aldose reductases by 8-methoxy-5,6,7-trihydroxyflavone derivatives*

No.	Structure	Name	Inhibition (%)					
			RLAR			BLAR		
			10^{-5} M	10^{-6} M	10^{-7} M	10^{-5} M	10^{-6} M	10^{-7} M
10		5,6,7-Trihydroxy-8-methoxyflavone	53	14	0	61	19	0
11		5,6,7,4′-Tetrahydroxy-8-methoxyflavone	85	49	11	77	45	0
12		5,6,7,4′-Tetrahydroxy-8,3′-dimethoxyflavone	85	52	0	86	40	0
13		5,6,7,3′,4′-Penta-acetoxy-8-methoxy-flavone	93	73	30	95	67	15

* Details of the experiments were the same as described in the legend to Table 1.
† Ac represents an acetyl group.

Table 3. Inhibition of lens aldose reductases by 5,6-dihydroxy-7,8-dimethoxyflavone derivatives and 5,7-dihydroxy-6,8-dimethoxyflavone derivatives*

No.	Structure	Name	Inhibition (%)					
			RLAR			BLAR		
			10^{-5} M	10^{-6} M	10^{-7} M	10^{-5} M	10^{-6} M	10^{-7} M
14		5,6-Dihydroxy-7,8-dimethoxyflavone	54	6	0	40	16	0
15		5,6,4′-Trihydroxy-7,8-dimethoxyflavone	85	52	20	82	42	0
16		5,6,4′-Trihydroxy-7,8,3′-trimethoxyflavone	33	16	0	46	11	0
17		5,7,4′-Trihydroxy-6,8-dimethoxyflavone (Demethoxy sudachitin)	88	64	23	89	62	9
18		5,7-Dihydroxy-6,8,4′-trimethoxyflavone (Nevadensin)	56	12	0	51	15	0

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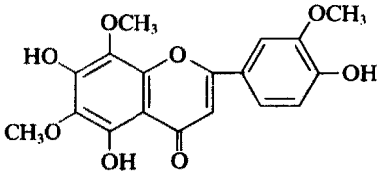
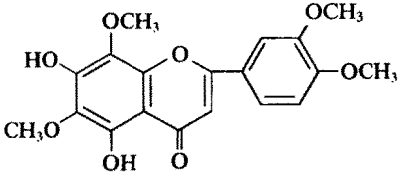
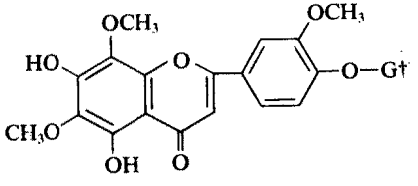
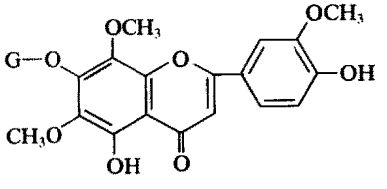
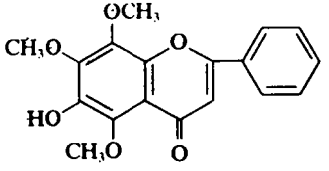
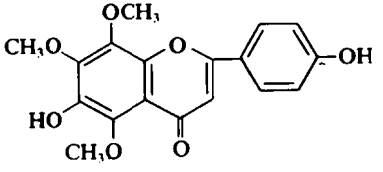
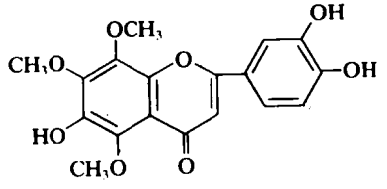
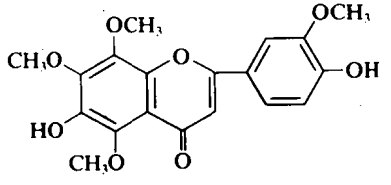
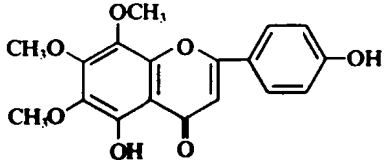
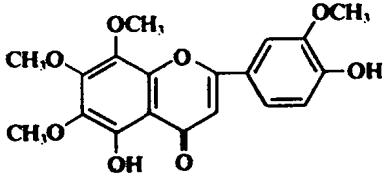
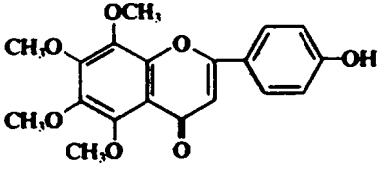
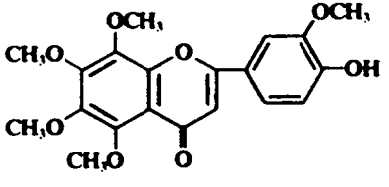
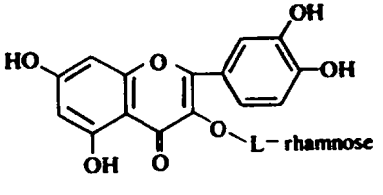
19		5,7,4′-Trihydroxy-6,8,3′-trimethoxy-flavone (Sudachitin)	66	19	0	56	19	0
20		5,7-Dihydroxy-6,8,3′,4′-tetramethoxy-flavone (Hymenoxin)	47	9	0	41	12	0
21		5,7,4′-Trihydroxy-6,8,3′-trimethoxy-flavone 4′-glucoside (Sudachiin A)	30	0	0	39	12	0
22		5,7,4′-Trihydroxy-6,8,3′-trimethoxy-flavone 7-glucoside (Sudachitin 7-glucoside)	27	0	0	20	7	0

* Details of the experiments were the same as described in the legend to Table 1.
† G represents D-glucosyl residue.

Table 4. Inhibition of lens aldose reductases by derivatives of 6-hydroxy-5,6,8-trimethoxyflavone, 5-hydroxy-6,7,8-trimethoxyflavone, and 5,6,7,8-tetramethoxyflavone*

No.	Structure	Name	Inhibition (%)					
			RLAR			BLAR		
			10^{-5} M	10^{-6} M	10^{-7} M	10^{-5} M	10^{-6} M	10^{-7} M
23		6-Hydroxy-5,7,8-trimethoxyflavone	32	0	0	40	8	0
24		6,4′-Dihydroxy-5,7,8-trimethoxyflavone	92	74	36	95	70	18
25		6,3′,4′-Trihydroxy-5,7,8-trimethoxyflavone (LARI 1)	96	92	73	100	91	35
26		6,4′-Dihydroxy-5,7,8,3′-tetramethoxyflavone	58	22	6	63	19	0

27		5,4'-Dihydroxy-6,7,8-trimethoxyflavone	81	61	25	81	53	8
28		5,4'-Dihydroxy-6,7,8,3'-tetramethoxyflavone (7-Methylsudachitin)	40	15	0	56	19	0
29		4'-Hydroxy-5,6,7,8-tetramethoxyflavone (LARI 2)	91	81	44	94	80	24
30		4'-Hydroxy-5,6,7,8,3'-pentamethoxyflavone	47	14	0	54	16	0
		3,5,7,3',4'-Penta-hydroxyflavone-3-rhamnoside (Quercitrin)	84	56	38	66	32	0

* Details of the experiments were the same as described in the legend to Table 1.

group to ring B at C_3, however, was without any significant effect, since the inhibitory activity of 7-methylaxillarin (No. 4) was similar to that of cirsiliol (No. 3). That the free hydroxyl group at C_7 is essential for the activity was suggested by 7-methylaxillarin (No. 4), which was a less potent inhibitor than axillarin (No. 1) itself. It was reported by Varma and Kinoshita [3] that the addition of a second hydroxyl group to give a catechol orientation in ring C enhanced the inhibitory activity. This was confirmed in the present study by the fact that cirsiliol (No. 3) was a more potent inhibitor than cirsimaritin (No. 2, 5,4′-dihydroxy-6,7-dimethoxyflavone). In addition, Varma and Kinoshita [3] reported that the free hydroxyl group in ring C is more conductive to the inhibitory activity as compared to the methoxyl group. This was also confirmed by cirsilineol 4′-glucoside (No. 8), which was less active than cirsiliol 4′-glucoside (No. 7). Furthermore, the glucosylation of the 4′-OH was found to diminish the inhibitory activity: cirsimaritin 4′-glucoside (No. 6), for example, was less active than the aglycone cirsimaritin (No. 2).

The inhibitory activities of 5,6,7,8-tetrasubstituted flavone derivatives and quercitrin, one of the earlier most potent inhibitors of aldose reductase, against RLAR and BLAR are summarized in Tables 2–4. Some of the flavones listed in Tables 1–4 were significantly more potent than quercitrin. Of the 5,6,7,8-tetrasubstituted flavone derivatives, 6,3′,4′-trihydroxy-5,7,8-trimethoxyflavone (No. 25)* named LARI 1 (meaning lens aldose reductase inhibitor 1) was the most potent inhibitor. This compound at 10^{-7} M inhibited RLAR and BLAR by 73 and 35%, respectively, indicating that its inhibitory activity was at the same level as that of axillarin (No. 1).

It was impossible to draw a conclusion about the effect of the degree of methylation of hydroxyl group(s) in ring A of 5,6,7,8-tetrasubstituted flavone derivatives (Tables 2–4). In a series of 4′-hydroxy-5,6,7,8-tetrasubstituted flavone derivatives, for instance, no relationship was observed between the degree or position of methylation of hydroxyl group(s) in ring A and the inhibitory activity. 5,6,7,4′-Tetrahydroxy-8-methoxyflavone (No. 11) was similar in potency as an inhibitor to 5,6,4′-trihydroxy-7,8-dimethoxyflavone (No. 15) and 5,4′-dihydroxy-6,7,8-trimethoxyflavone (No. 27), whereas the inhibitory activities of both 6,4′-dihydroxy-5,7,8-trimethoxyflavone (No. 24) and 4′-hydroxy-5,6,7,8-tetramethoxyflavone (No. 29, LARI 2)* were superior to those of the above three compounds. In addition, 5,7,4′-trihydroxy-6,8-dimethoxyflavone (No. 17) possessed an activity intermediate between those of the former three compounds and the latter two compounds.

That the free hydroxyl group at C_7 in 5,6,7-trisubstituted flavone derivatives enhances the inhibitory activity applied to two 5,6,7,8-tetrasubstituted flavone derivatives, 5,6,7,4′-tetrahydroxy-8,3′-dimethoxyflavone (No. 12) vs 5,6,4′-trihydroxy-7,8,3′-trimethoxyflavone (No. 16). This phenomenon, however, did not occur for two pairs of 5,6,7,8-tetrasubstituted flavones, 5,6,7-trihydroxy-8-methoxyflavone (No. 10) vs 5,6-dihydroxy-7,8-dimethoxyflavone (No. 14), and 5,6,7,4′-tetrahydroxy-8-methoxyflavone (No. 11) vs 5,6,4′-trihydroxy-7,8-dimethoxyflavone (No. 15), in which the methylation of the hydroxyl group at C_7 was without any effect.

The increase in the inhibitory activity of LARI 1 (No. 25) as compared to 6,4′-dihydroxy-5,7,8-trimethoxyflavone (No. 24) was due to the addition of a second hydroxyl group in catechol orientation to ring C as stated in an earlier example (No. 2 vs No. 3). The presence of a hydroxyl group at the 4′-position in ring C enhanced the inhibitory activity, as already suggested by Varma and Kinoshita [3]: 5,6,7,4′-tetrahydroxy-8-methoxyflavone (No. 11), 5,6,4′-trihydroxy-7,8-dimethoxyflavone (No. 15), and 6,4′-dihydroxy-5,7,8-trimethoxyflavone (No. 24) were more potent than their respective 4′-dehydroxy compounds (No. 10, No. 14 and No. 23). The decrease in the inhibitory activity, stated in an earlier example (No. 7 vs No. 8), by methylation of a hydroxyl group in ring C was again shown, in that nevadensin (No. 18), hymenoxin (No. 20) and 6,4′-dihydroxy-5,7,8,3′-tetramethoxyflavone (No. 26) were less potent inhibitors than the corresponding phenolic compounds (No. 17, No. 19 and No. 25).

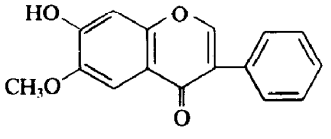
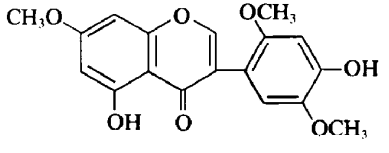
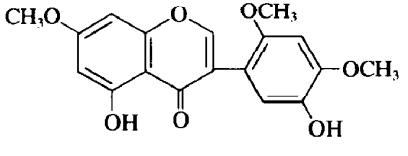
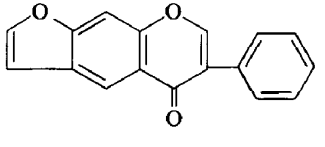
The glucosylation at the 7-OH reduced the inhibitory activity as demonstrated, in that sudachitin 7-glucoside (No. 22) was less potent than the parent aglycone sudachitin (No. 19). The decrease in inhibitory activity by glucosylation of the 4′-OH was again shown by the results with 5,6,7,8-tetrasubstituted flavone derivatives; sudachiin A (No. 21) was less active than the aglycone sudachitin (No. 19). Furthermore, the addition of a methoxyl group to ring A at C_8 little affected the inhibitory activity: the activities of cirsimaritin (No. 2) and cirsilineol (No. 5) were similar to those of 5,4′-dihydroxy-6,7,8-trimethoxyflavone (No. 27) and 7-methylsudachitin (No. 28) respectively.

Some of the isoflavones and coumarins tested were also found to inhibit lens aldose reductase, although they were far less potent than flavones (Tables 5–7). Even the most potent compound, 4-hydroxy-6,7-methylenedioxycoumarin (No. 42) [17], was at least one order of magnitude less active than esculetin (6,7-dihydroxycoumarin), which has been reported to inhibit RLAR by 53% at 10^{-6} M [3]. The decreased activity of isoflavones and coumarins may be due to the position of the ring C and carbonyl group of the benzopyran ring system respectively.

The IC_{50} values (inhibitor concentrations necessary for 50% inhibition of activity) of twenty-one highly potent flavones which inhibited aldose reductase by more than about 50% at 10^{-5} M were estimated and listed in Table 8. The IC_{50} of quercitrin was also estimated to compare with those of the twenty-one flavones. A crude preparation of RLAR, as well as purified RLAR, was used as a test enzyme, because almost all the previous papers of other investigators have dealt with the IC_{50} values of crude (or partially purified) RLAR, but not of purified RLAR. The susceptibility to inhibition by the flavones listed in Table 8 was not different between crude and purified

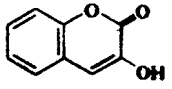
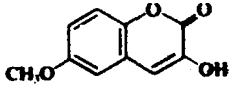
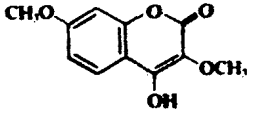
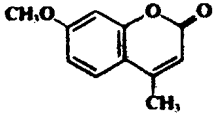
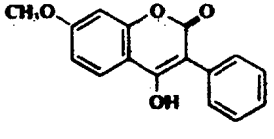
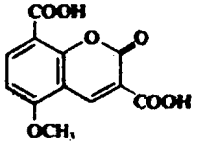
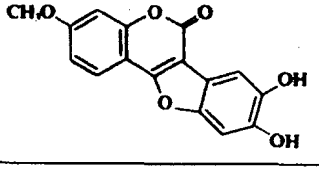
* T. Horie and M. Nakayama, unpublished results.

Table 5. Inhibition of lens aldose reductases by isoflavone derivatives*

No.	Structure	Name	Inhibition (%)					
			RLAR			BLAR		
			10^{-5} M	10^{-6} M	10^{-7} M	10^{-5} M	10^{-6} M	10^{-7} M
31		7-Hydroxy-6-methoxy-isoflavone	0	0	0	0	0	0
32		5,4'-Dihydroxy-7,2',5'-trimethoxy-isoflavone	0	0	0	6	0	0
33		5,5'-Dihydroxy-7,2',4'-trimethoxyisoflavone	20	0	0	7	0	0
34		Furo[3',2':,6,7]iso-flavone	18	0	0	0	0	0

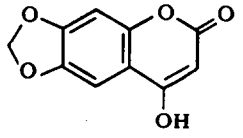
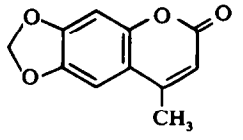
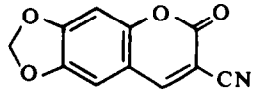
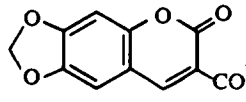
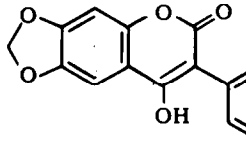
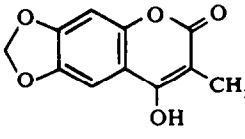
* Details of the experiments were the same as described in the legend to Table 1.

Table 6. Inhibition of lens aldose reductases by coumarin derivatives*

No.	Structure	Name	Inhibition (%)					
			RLAR			BLAR		
			10^{-5} M	10^{-6} M	10^{-7} M	10^{-5} M	10^{-6} M	10^{-7} M
35		3-Hydroxycoumarin	11	0	0	10	0	0
36		3-Hydroxy-6-methoxy-coumarin	15	0	0	15	0	0
37		4-Hydroxy-3,7-dimethoxy-coumarin	9	0	0	15	0	0
38		7-Methoxy-4-methyl-coumarin	7	0	0	7	0	0
39		4-Hydroxy-7-methoxy-3-phenylcoumarin	24	0	0	14	0	0
40		3,8-Dicarboxy-5-methoxycoumarin	11	0	0	37	0	0
41		8,9-Dihydroxy-3-methoxy-coumestan	27	0	0	41	0	0

* Details of the experiments were the same as described in the legend to Table 1.

Table 7. Inhibition of lens aldose reductases by 7,8-methylenedioxycoumarin derivatives*

No.	Structure	Name	Inhibition (%)					
			RLAR			BLAR		
			10^{-5} M	10^{-6} M	10^{-7} M	10^{-5} M	10^{-6} M	10^{-7} M
42		4-Hydroxy-6,7-methylenedioxy-coumarin	51	10	0	47	19	0
43		6,7-Methylenedioxy-4-methylcoumarin	0	0	0	10	0	0
44		3-Cyano-6,7-methylene-dioxycoumarin	15	0	0	0	0	0
45		6,7-Methylenedioxy-coumarin-3-carboxylic acid	15	0	0	0	0	0
46		4-Hydroxy-6,7-methylenedioxy-3-phenylcoumarin	17	0	0	10	0	0
47		4-Hydroxy-6,7-methylenedioxy-3-methylcoumarin	0	0	0	12	0	0

* Details of the experiments were the same as described in the legend to Table 1.

RLAR. BLAR appeared to be less susceptible than both crude and purified RLAR. Judging from the data in Tables 1–7, however, the inhibitory activities of cirsimaritin 4′-glucoside (No. 6) and cirsilineol 4′-glucoside (No. 8) against BLAR were greater than those against RLAR. Against purified RLAR eight flavones were found to be more potent than quercitrin with an IC_{50} of 4.9×10^{-7} M. On the other hand, against purified BLAR thirteen flavones were more potent than quercitrin with an IC_{50} of 3.3×10^{-6} M. Axillarin (No. 1) and LARI 1 (No. 25) which were the two most potent inhibitors were 18 times, 14–19 times, and 6–7 times more potent than quercitrin against BLAR, purified RLAR and crude RLAR respectively.

Kinetic studies were performed with axillarin (No. 1), LARI 1 (No. 25), LARI 2 (No. 29) and quercitrin to determine their inhibitor constants (K_i) and the type of inhibition by flavones. The Hanes–Woolf plots of [DL-glyceraldehyde]/v vs [DL-glyceraldehyde] with axillarin (5×10^{-8} M) and quercitrin (10^{-6} M) as inhibitors are shown in Fig. 1. Not only these two compounds but also the other two compounds (No. 25 at 5×10^{-8} M and No. 29 at 10^{-7} M, not shown in Fig. 1) were found to be non-competitive inhibitors against both RLAR and BLAR. The K_i values of the four flavones are summarized in Table 9. These values agreed well with the IC_{50} values in Table 8.

Inhibition of some enzymes other than aldose reductase by flavones. Flavonoids have been shown to affect a variety of metabolic reactions [34–40]. To study the specificity of the inhibition of aldose reductase by the three most potent flavones (No. 1, No. 25 and No. 29) as well as quercitrin, the inhibitory effects of these four flavones on seven adeninenucleotide-requiring enzymes, i.e. hexokinase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, glutathione reductase, alcohol dehydrogenase and aldehyde reductase, were investigated. The results obtained

Table 8. IC_{50} values of flavones against crude RLAR, RLAR and BLAR and potency ratios of crude versus purified RLAR and BLAR versus RLAR*

Compound No.	IC_{50} (M) × 10^6			IC_{50} crude RLAR / IC_{50} RLAR	IC_{50} BLAR / IC_{50} RLAR
	Crude RLAR	RLAR	BLAR		
1 (Axillarin)	0.052	0.026	0.18	2.0	6.9
25 (LARI 1)	0.042	0.036	0.18	1.2	5.0
29 (LARI 2)	0.16	0.16	0.29	1.0	1.8
3	0.34	0.12	1.1	2.8	9.2
24	0.27	0.25	0.42	1.1	1.7
13	0.24	0.30	0.47	0.8	1.6
4	0.52	0.35	0.85	1.5	2.4
17	0.41	0.41	0.58	1.0	1.5
Quercitrin	0.31	0.49	3.3	0.6	6.7
27	0.42	0.54	0.83	0.8	1.5
15	0.86	0.85	1.5	1.0	1.8
12	1.1	1.2	2.8	0.9	2.3
11	1.4	1.2	1.6	1.2	1.3
2	1.8	1.4	1.8	1.3	1.3
19	5.4	4.5	6.8	1.2	1.5
26	7.4	6.3	5.0	1.2	1.8
5	14.5	6.7	8.7	2.2	1.2
18	12.0	7.2	9.1	1.7	1.3
10	6.8	8.2	5.4	0.8	0.7
7	10.0	8.2	4.9	1.2	0.6
14	15.0	8.4	25.5	1.8	3.0
9	17.0	8.9	16.0	1.9	1.8

* The assay of crude RLAR was carried out according to the method of Kador and Sharpless. [25]. The IC_{50} values were determined by the method of Kador *et al.* [33] as described in Materials and Methods.

are summarized in Table 10. All the enzymes except for aldehyde reductase were not remarkably inhibited by the flavones at 10^{-5} M. On the other hand, aldehyde reductase, an enzyme with many properties similar to aldose reductase, was significantly inhibited by all of the four compounds. The IC_{50} values of axillarin (No. 1), LARI 1 (No. 25), LARI 2 (No. 29) and quercitrin against rat brain aldehyde reductase were 3.9×10^{-7} M, 2.3×10^{-7} M, 1.5×10^{-6} M and 3.2×10^{-5} M respectively.

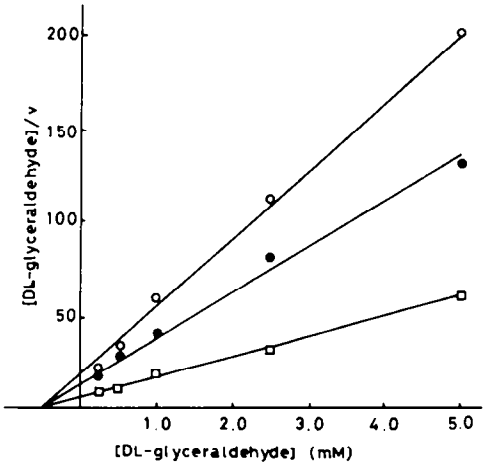


Fig. 1. Hanes–Woolf plots of [DL-glyceraldehyde]/v against [DL-glyceraldehyde]. Key: (□—□) control, (○—○) in the presence of 5×10^{-8} M axillarin, and (●—●) in the presence of 10^{-6} M quercitrin. The velocity of aldose reductase reaction was measured for 2 min at each substrate concentration, in the presence and absence of inhibitors. The reactions were carried out as described in Materials and Methods except that the DL-glyceraldehyde concentration was varied.

DISCUSSION

Currently, an active search for aldose reductase inhibitors is being conducted. Several compounds, with diverse structure, including flavonoids have been reported previously to have inhibitory potency against the enzyme [3, 25, 41–44]. The present study was also conducted in search of more potent flavonoids and coumarins which may possibly be useful for the prevention of cataract formation in diabetic patients. Thirteen of the thirty flavones tested inhibited both RLAR and BLAR by about 50% at concentrations as low as 10^{-6} M or 10^{-7} M (Tables 1–4), whereas all of the isoflavones and coumarins tested were far less potent than the flavones (Tables 5–7).

Table 9. Inhibitor constants (K_i) for flavones with RLAR and BLAR*

No.	Name	K_i value (M)	
		RLAR	BLAR
1	Axillarin	2.2×10^{-8}	2.3×10^{-7}
25	LARI 1	2.6×10^{-8}	2.0×10^{-7}
29	LARI 2	1.1×10^{-7}	3.6×10^{-7}
	Quercitrin	7.8×10^{-7}	1.9×10^{-6}

* K_i values were obtained from the Hanes–Woolf plots of [DL-glyceraldehyde]/v against [DL-glyceraldehyde] as described in the legend to Fig. 1.

Table 10. Inhibition of various enzyme activities by flavones*

Enzyme	Inhibition (%)			
	Compound			
	Axillarin	LARI 1	LARI 2	Quercitrin
Hexokinase	0	0	0	0
Pyruvate kinase	23	0	0	9
Lactate dehydrogenase	0	0	0	0
Glucose-6-phosphate dehydrogenase	9	0	19	7
Glutathione reductase	9	18	9	14
Alcohol dehydrogenase	0	0	0	0
Aldehyde reductase	87	85	74	32

* Hexokinase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase and glutathione reductase were prepared from rat lens, alcohol dehydrogenase from rat liver, and aldehyde reductase from rat brain as described in Materials and Methods. Glucose-6-phosphate dehydrogenase was contaminated by an appreciable amount of 6-phosphogluconate dehydrogenase. Assays were carried out in a reaction system with or without an inhibitor at 10^{-5} M as described in Materials and Methods. Each value is the mean of two experiments.

Since many of the IC_{50} values of various compounds reported hitherto have been determined with crude RLAR, we used here the IC_{50} values against crude RLAR to compare with those reported by other investigators. The inhibitory potency ($IC_{50} = 3.1 \times 10^{-7}$ M) of quercitrin against crude RLAR (Table 8) estimated in the present study was at the same level as that ($IC_{50} = 6.1 \times 10^{-7}$ M) reported by Kador *et al.* [4]. Axillarin (No. 1, $IC_{50} = 5.2 \times 10^{-8}$ M) and LARI 1 (No. 25, $IC_{50} = 4.2 \times 10^{-8}$ M) were found to be at least 6 times more potent than quercitrin, when compared to the inhibitory activity against crude RLAR (Table 8). Varma and Kinoshita [3] reported that the IC_{50} values of quercitrin and quercitrin 2″-acetate against crude RLAR were 10^{-7} M and 4×10^{-8} M respectively. Later, their group [4] reported a much larger value (6.1×10^{-7} M) for the IC_{50} of quercitrin against the enzyme. Since it seems that the latter IC_{50} value of quercitrin is more reliable than the former, quercitrin 2″-acetate may possess an IC_{50} value of about 2.4×10^{-7} M. Axillarin and LARI 1 were thus believed to be about 5 times more potent than quercitrin 2″-acetate, the best aldose reductase inhibitor of the flavonoids previously tested. Recently, it has been reported that sorbinil, a hydantoin derivative, is a highly potent aldose reductase inhibitor both *in vitro* [4, 5] and *in vivo* [5, 6]. The IC_{50} values of this compound against crude RLAR and partially purified BLAR have been reported to be 7×10^{-8} M [4] and 5×10^{-7} M [5], respectively, so that axillarin and LARI 1 were estimated to be 1.3 to 2.8 times more potent than sorbinil. These two flavone derivatives (No. 1 and No. 25) were thus found to be the most potent inhibitors of aldose reductase known so far.

Quercitrin has been shown to non-competitively inhibit RLAR [3]. More recently, however, it has been reported that the enzyme is uncompetitively inhibited by quercitrin [33]. This discrepancy may be due to a characteristic of many uncompetitive inhibitors which display non-competitive inhibition at low concentrations and then switch to uncompetitive inhibition at higher concentrations. In our experiments, the three most potent flavones (No. 1, No. 25 and No. 29) as well as quercitrin were found to act as non-competitive inhibitors of RLAR at each concentration used.

Kador *et al.* [4, 33] have reported significant differences in the susceptibilities of aldose reductases from rat lens, human placenta and human lens to inhibition by various aldose reductase inhibitors. They have pointed out that the evaluation of aldose reductase inhibitors for potential clinical use may require the use of human aldose reductase from the appropriate target tissue. Because of the difficulty in obtaining human lenses, however, earlier workers have used aldose reductases from animal lenses in the survey of potent inhibitors against human lens aldose reductase. For the same reason, we also used aldose reductases from animal lenses in the present study. We compared the susceptibility of RLAR to inhibition by flavones with that of BLAR. BLAR was found to be less susceptible than RLAR to inhibition by almost all of the flavones tested, although there were some fluctuations in ratios of the potency against BLAR to that against RLAR.

Varma and Kinoshita [3] have indicated the possible relationships of structure to the inhibitory potencies of flavonoids. In the present study, we could confirm some of the structure/activity relationships revealed by them. Some additional relationships were inferred as follows: (1) addition of a methoxyl group to ring B at C_3 did not affect the potency; (2) in 5,6,7-trisubstituted flavone derivatives, methylation of the 7-OH led to a decrease in the inhibitory activity, although this phenomenon did not always apply to 5,6,7,8-tetrasubstituted flavone derivatives; (3) both 4′-*O*-glucosylation and 7-*O*-glucosylation were detrimental to the inhibitory potency; (4) addition of a methoxyl group to ring A at C_8 was without effect. No definite conclusion, however, was reached concerning the effect of methylation of hydroxyl(s) in ring A on the inhibitory activity. The structure/activity relationships obtained

by us and others seemed to hint that 5,7-dimethylaxillarin, 3-demethylaxillarin, or 3-demethyl-3-*O*-L-rhamnosylaxillarin, if available, may be more potent as an aldose reductase inhibitor than axillarin itself. Derivatization of axillarin to 5,7-dimethylaxillarin may also be advantageous to stability, because polyphenols are known to be generally susceptible to oxidation of hydroxyl groups by oxygen.

Aldose reductase is one of adeninenucleotide-requiring enzymes, since it requires NADPH as a coenzyme. Some adeninenucleotide-requiring enzymes, e.g. cyclic AMP phosphodiesterase [37], aldehyde reductase [38], ATPase [39, 40] and hexokinase [39], have been reported to be inhibited by flavones. A systematic study on inhibition of adeninenucleotide-requiring enzymes by flavones, however, has not been conducted. We tested, in the present study, the inhibitory activities of four flavones (No. 1, No. 25, No. 29 and quercitrin) against seven adenine nucleotide-requiring enzymes (hexokinase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, glutathione reductase, alcohol dehydrogenase and aldehyde reductase). As shown in Table 10, only aldehyde reductase was inhibited to a large extent by all of the four flavones. The inhibitory activity of each of these flavones against aldehyde reductase, however, was one order of magnitude lower than that against aldose reductase as judged by the IC_{50} values of these flavones against the two enzymes. The six adeninenucleotide-requiring enzymes other than aldehyde reductase were not remarkably affected by the flavones at 10^{-5} M. These results suggest that the four flavones highly selectively inhibit only aldose reductase. If this is the case, this feature may make these flavones promising as clinically useful drugs.

Flavonoids are ubiquitously distributed in the plant kingdom. Many preparations of plants containing flavonoids are used as folk medicines. Since flavonoids which are also present commonly in food are relatively non-toxic, the present finding that axillarin and LARI 1 are the most potent aldose reductase inhibitors known so far suggests that they may be useful in preventing or delaying the onset of diabetic cataract. The *in vivo* study of inhibition of aldose reductase by these two flavones is now in progress.

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REFERENCES

1. K. H. Gabbay, *New Engl. J. Med.* **288**, 831 (1973).
2. J. H. Kinoshita, S. D. Varma and H. N. Fukui, *Jap. J. Ophthal.* **20**, 399 (1976).
3. S. D. Varma and J. H. Kinoshita, *Biochem. Pharmac.* **25**, 2505 (1976).
4. P. F. Kador, J. H. Kinoshita, W. H. Tung and L. T. Chylack, Jr., *Invest. Ophthal.* **19**, 980 (1980).
5. M. J. Peterson, R. Sarges, C. E. Aldinger and D. P. MacDonald, *Adv. exp. Med. Biol.* **119**, 347 (1979).
6. S. Fukushi, L. O. Merola and J. H. Kinoshita, *Invest. Ophthal.* **19**, 313 (1980).
7. K. Fukui, T. Matsumoto and T. Kinoshita, *Bull. chem. Soc. Japan* **37**, 662 (1964).
8. K. Fukui, M. Nakayama and T. Horie, *Bull. chem. Soc. Japan* **42**, 1649 (1969).
9. T. Horie, M. Nakayama, K. Kase, K. Fukui and M. Nakayama, *J. chem. Soc. Japan* 2400 (1974).
10. T. Horie, M. Nakayama, S. Hayashi, M. Tsukayama and M. Masumura, *Heterocycles* **10**, 53 (1978).
11. M. Nakayama, S. Eguchi, S. Hayashi, T. Horie, M. Suzuki, K. Harada, N. Takeda and A. Tatematsu, *Mass Spectros.* **27**, 53 (1979).
12. T. Horie, H. Kourai, M. Nakayama, M. Tsukayama and M. Masumura, *J. chem. Soc. Japan* 1397 (1980).
13. T. Horie and M. Nakayama, *Phytochemistry* **20**, 337 (1981).
14. K. Fukui and T. Matsumoto, *J. Sci. Hiroshima Univ.* (Ser. A-II) **28**, 47 (1964).
15. K. Fukui and M. Nakayama, *J. chem. Soc. Japan* **85**, 444 (1964).
16. M. Tsukayama, T. Horie, Y. Yamashita, M. Masumura and M. Nakayama, *Heterocycles* **14**, 1283 (1980).
17. K. Fukui and M. Nakayama, *Bull. chem. Soc. Japan* **35**, 1321 (1962).
18. K. Fukui and M. Nakayama, *J. Sci. Hiroshima Univ.* (Ser. A-II) **26**, 131 (1963).
19. K. Fukui and M. Nakayama, *Bull. chem. Soc. Japan* **37**, 300 (1964).
20. K. Fukui, M. Nakayama, H. Tsuge and K. Tsuzuki, *Experientia* **24**, 536 (1968).
21. M. Nakayama, *J. Sci. Hiroshima Univ.* (Ser. A-II) **33**, 205 (1969).
22. M. Nakayama, S. Eguchi, A. Matsuo, S. Hayashi, S. Hishida and Y. Kato, *Mass Spectros.* **20**, 89 (1972).
23. S. Hayman and J. H. Kinoshita, *J. biol. Chem.* **240**, 877 (1965).
24. K. Inagaki, I. Miwa and J. Okuda, *Archs Biochem. Biophys.* **216**, 337 (1982).
25. P. F. Kador and N. E. Sharpless, *Biophys. Chem.* **8**, 81 (1978).
26. C. Sharma, R. Manjeshwar and S. Weinhouse, *J. biol. Chem.* **238**, 3840 (1963).
27. G. E. J. Staal, J. F. Koster and C. Veeger, in *Methods in Enzymology* (Ed. W. A. Wood), Vol. 42, p. 182. Academic Press, New York (1975).
28. F. Stolzenbach, in *Methods in Enzymology* (Ed. W. A. Wood), Vol. 9, p. 278. Academic Press, New York (1965).
29. P. Cohen and M. A. Rosemeyer, *Eur. J. Biochem.* **8**, 1 (1969).
30. E. Racker, *J. biol. Chem.* **217**, 855 (1955).
31. K. Dalziel, *Acta chem. scand.* **11**, 397 (1957).
32. D. R. P. Tulsiani and O. Touster, *J. biol. Chem.* **252**, 2545 (1977).
33. P. F. Kador, L. O. Merola and J. H. Kinoshita, *Docum. Ophthal. Proc. Series* **18**, 117 (1979).
34. Von J. Dittmann, H. D. Herrmann and H. Palleske, *Arzneimittel-Forsch.* **21**, 1999 (1971).
35. Y. Graziani, J. Winikoff and R. Chayoth, *Biochim. biophys. Acta* **497**, 499 (1977).
36. E. M. Suolinna, D. R. Lang and E. Racker, *J. natn. Cancer Inst.* **53**, 1515 (1974).
37. J. E. Ferrell, Jr., P. D. G. Chang Sing, G. Loew, R. King, J. M. Mansour and T. E. Mansour, *Molec. Pharmac.* **16**, 556 (1979).
38. S. R. Whittle and A. J. Turner, *Biochem. Pharmac.* **30**, 1191 (1981).
39. Y. Graziani, *Biochim. biophys. Acta* **460**, 364 (1977).
40. C. Carpenedo, C. Bortignon, A. Bruni and R. Santi, *Biochem. Pharmac.* **18**, 1495 (1969).
41. J. C. Hutton, P. J. Schofield, J. F. Williams and F. C. Hollows, *Biochem. Pharmac.* **23**, 2991 (1974).
42. D. Dvornik, N. Simard-Duquesne, M. Krami, K. Sestanj, K. H. Gabbay, J. H. Kinoshita, S. D. Varma and L. O. Merola, *Science* **182**, 1146 (1973).
43. S. D. Varma, I. Mikuni and J. H. Kinoshita, *Science* **188**, 1215 (1975).
44. F. Fauran, C. Feniou, J. Mosser and G. Prat, *Eur. J. med. Chem.* **13**, 503 (1978).