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Inhibition of Aldose Reductase by 3',4'-Dihydroxyflavones

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A series of 3',4'-dihydroxyflavones was assayed *in vitro* for inhibition of lens aldose reductase which has been implicated in the pathogenesis of sugar cataract. All the compounds tested were shown to be potent inhibitors of aldose reductase and 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone (lens aldose reductase inhibitor 4, LARI 4), the most potent one, inhibited the enzymes from rat and bovine lenses by 50% at 3.2×10^{-8} and 1.7×10^{-7} M, respectively. Inhibition of the enzyme by 3',4'-dihydroxyflavones was non-competitive with respect to both DL-glyceraldehyde and reduced form of nicotinamide adenine dinucleotide phosphate. The inhibitory activities of 3',4'-dihydroxyflavones against lens aldose reductase were pH-dependent, being maximal around pH 7.0.

Keywords—flavone; aldose reductase inhibitor; rat lens; bovine lens; aldose reductase; structure-activity relationship

Aldose reductase (EC 1.1.1.21) along with the coenzyme nicotinamide adenine dinucleotide phosphate (NADPH) catalyzes the reduction of aldose to alditol. This production of sugar alcohol has been implicated in the development of cataract in diabetes and galactosemia.¹⁾ That aldose reductase plays a significant role under abnormal conditions such as diabetes makes the inhibition of aldose reductase a selective potential treatment for diabetic cataract.

In recent years, flavonoids have been reported to be potent inhibitors of this enzyme.²⁾ Quercitrin (3,5,7,3',4'-pentahydroxyflavone-3-rhamnoside) orally administered to diabetic animals inhibited D-glucitol accumulation in the lens, resulting in the prevention of the cataractous processes.³⁾

More recently, we⁴⁾ have reported that axillarin (5,7,3',4'-tetrahydroxy-3,6-dimethoxyflavone) and LARI 1 (6,3',4'-trihydroxy-5,7,8-trimethoxyflavone) are the most potent inhibitors of aldose reductase among flavonoids tested so far and that they are at least 10 times more potent than quercitrin. We have also indicated that 3',4'-dihydroxyflavones, flavones having two hydroxyl groups in a catechol orientation in ring C, are more potent than the corresponding flavones having no hydroxyl, or having a monohydroxyl or methoxyl group in ring C. Hence, the purpose of the present study was to examine the inhibitory activities of over twenty 3',4'-dihydroxyflavones on rat lens aldose reductase (RLAR) and bovine lens aldose reductase (BLAR) as part of our search for more promising aldose reductase inhibitors.

Materials and Methods

Materials—All chemicals were of the highest grade commercially available. DL-Glyceraldehyde and quercitrin

were purchased from Nakarai Chemicals Ltd., Kyoto, Japan. NADPH was from Oriental Yeast Co., Ltd., Osaka, Japan. DEAE-Sephacel and Sephadex G-75 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Mätrex gel red A was purchased from Amicon Co., Lexington, MA, U.S.A. 3',4'-Dihydroxy-5,6,7-trisubstituted flavones,⁵⁾ 3',4'-dihydroxy-5,7,8-trisubstituted flavones,^{5c,6)} and 3',4'-dihydroxy-5,6,7,8-tetrasubstituted flavones⁷⁾ were isolated from various plants or chemically synthesized as reported previously.

Assay of Aldose Reductase Activity—Aldose reductase activity was assayed at 30 °C by following the decrease of absorbance at 340 nm accompanying the oxidation of NADPH. Unless otherwise stated the reaction mixture contained 0.1 M sodium phosphate buffer (pH 6.2, and optimum pH for the enzyme), 0.4 M ammonium sulfate, 10 mM DL-glyceraldehyde, 0.16 mM NADPH and the enzyme in a total volume of 1.0 ml. The reaction was initiated by the addition of the substrate. One unit of the enzyme was defined as the amount catalyzing the oxidation of 1 μ mol of NADPH per min under the assay conditions used. The effect of inhibitor on the enzyme activity was determined by including in the reaction mixture 10 μ l of inhibitor solution at various concentrations (10^{-6} – 10^{-3} M). Inhibitors were dissolved in propylene glycol to give a concentration of 10^{-3} M and diluted to the desired concentrations with the solvent. Propylene glycol at 1% inhibited the enzyme by only 5% or less and did not affect the inhibitory potency of flavones. The inhibitory potency determined just after mixing of the enzyme and a flavone was the same as that observed after preincubation of the reaction mixture (containing an inhibitor, but not the substrate) at 30 °C for 10 min. Hence, the enzyme activity in the presence of an inhibitor was assayed without any preincubation throughout this study.

Reversibility of Inhibition of Aldose Reductase—One ml of a solution containing 0.1 M sodium phosphate buffer (pH 6.2), 10^{-7} M flavone (3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone (LARI 4, 26) or axillarin (8)), and BLAR (30 munits) was dialyzed at 4 °C for 20 h against two changes of 500 ml of 0.1 M sodium phosphate buffer (pH 6.2) containing 0.1 mM mercaptoethanol. Thereafter, an aliquot (0.4 ml) of the impermeate was subjected to assay of the enzyme activity. The same procedure was applied to another solution (as a control) containing phosphate buffer and the enzyme, but not a flavone.

Purification of Aldose Reductase from Rat and Bovine Lenses—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 lenses were removed and frozen until needed. RLAR and BLAR were purified according to the method of Inagaki *et al.*⁸⁾ 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 Mätrex gel red A and gel filtration on Sephadex G-75. RLAR and BLAR were purified over 380- and 12000-fold, respectively.

Determination of IC_{50} —The concentration of an inhibitor needed to elicit 50% inhibition (IC_{50}) was determined by the method described in ref. 9. In this paper, IC_{50} values of inhibitors are expressed as their final concentrations in the reaction mixture.

Results

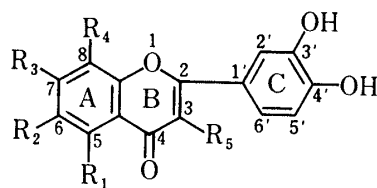
Inhibitory Activities of Flavones against Aldose Reductase

IC_{50} values of 3',4'-dihydroxyflavones against RLAR and BLAR are shown in Table I. Flavones were classed into three groups (1–11, 12–20, and 21–26) primarily according to the degree of substitution in ring A.

1) 3',4'-Dihydroxy-5,6,7-trisubstituted Flavones (1–11)—Methylation of the hydroxyl group at C_5 enhanced the inhibitory activity as shown by 6,3',4'-trihydroxy-5,7-dimethoxyflavone (4), 3',4'-dihydroxy-5,6,7-trimethoxyflavone (5), and 6,3',4'-trihydroxy-3,5,7-trimethoxyflavone (10), which are more potent inhibitors than pedalitin (2), cirsiolol (3), and 5,6,3',4'-tetrahydroxy-3,7-dimethoxyflavone (7), respectively. 3',4'-Dihydroxy-3,5,6,7-tetramethoxyflavone (11), however, had almost the same potency as the corresponding phenolic compound (9, 7-methylaxillarin). Methylation of the hydroxyl group at C_6 increased or little affected the inhibitory potency. We⁴⁾ previously reported that the free hydroxyl group at C_7 in 5,6,7-trisubstituted flavones is favorable for the inhibitory activity (axillarin (8) > 7-methylaxillarin (9)). This was confirmed in the present study by the fact that 5,6,7,3',4'-pentahydroxyflavone (1) and 5,6,7,3',4'-pentahydroxy-3-methoxyflavone (6) were more potent inhibitors than pedalitin (2) and compound (7), respectively. Methoxylation at C_3 decreased or little affected the potency.

2) 3',4'-Dihydroxy-5,7,8-trisubstituted Flavones (12–20)—It appears that, as a whole, this series of flavones is similar in inhibitory activity to the corresponding 3',4'-dihydroxy-5,6,7-trisubstituted flavones. Methylation of the hydroxyl group at either C_5 or C_7 enhanced

TABLE I. Aldose Reductase Inhibitory Activity of 3',4'-Dihydroxyflavones



	R ₁	R ₂	R ₃	R ₄	R ₅	IC ₅₀ (M) × 10 ⁷	
						RLAR	BLAR
1	OH	OH	OH	H	H	2.0	4.6
2	OH	OH	OMe	H	H	3.0	16
3 ^{a)}	OH	OMe	OMe	H	H	2.2	12
4	OMe	OH	OMe	H	H	1.4	8.0
5	OMe	OMe	OMe	H	H	0.90	5.8
6	OH	OH	OH	H	OMe	0.58	3.7
7	OH	OH	OMe	H	OMe	8.0	21
8 ^{a)}	OH	OMe	OH	H	OMe	0.30	1.8
9 ^{a)}	OH	OMe	OMe	H	OMe	3.5	8.5
10	OMe	OH	OMe	H	OMe	3.0	16
11	OMe	OMe	OMe	H	OMe	2.7	17
12	OH	H	OH	OMe	H	2.8	14
13	OH	H	OMe	OH	H	2.3	8.8
14	OH	H	OMe	OMe	H	0.78	3.7
15	OMe	H	OMe	OH	H	1.6	12
16	OMe	H	OH	OMe	H	0.74	3.1
17	OMe	H	OMe	OMe	H	0.45	2.7
18	OH	H	OH	OMe	OMe	2.4	5.2
19	OMe	H	OH	OMe	OMe	2.8	4.5
20	OMe	H	OMe	OMe	OMe	1.7	5.4
21	OH	OH	OH	OMe	H	1.2	4.2
22	OH	OH	OMe	OMe	H	0.65	3.0
23	OH	OMe	OH	CH ₂ -Ph	H	0.34	1.9
24	OH	OMe	OMe	OMe	H	0.39	2.4
25 ^{a)}	OMe	OH	OMe	OMe	H	0.34	1.9
26 (LARI 4)	OMe	OMe	OMe	OMe	H	0.32	1.7
Quercitrin ^{a)}	OH	H	OH	H	<i>O</i> -L-Rham. ^{b)}	2.9	21

a) Compounds 3 (cirsiol), 8 (axillarlin), 9 (7-methylaxillarlin), and 25 (LARI 1) as well as quercitrin were assayed previously⁴⁾ and tested as references again in this study.

b) *O*-L-Rhamnosyl.

or little affected the inhibitory potency. On the other hand, methylation of the hydroxyl group at C₈ clearly increased the inhibitory activity: 5,3',4'-trihydroxy-7,8-dimethoxyflavone (14) and 3',4'-dihydroxy-5,7,8-trimethoxyflavone (17) were superior in potency to 5,8,3',4'-tetrahydroxy-7-methoxyflavone (13) and 8,3',4'-trihydroxy-5,7-dimethoxyflavone (15), respectively. Methoxylation at C₃ decreased or did not affect the inhibitory activity.

3) **3',4'-Dihydroxy-5,6,7,8-tetrasubstituted Flavones (21—26)**—No definite conclusion was obtained on the effect of methylation of hydroxyl group(s) in ring A of this series of flavones. It appears, however, that the inhibitory activity of 3',4'-dihydroxy-5,6,7,8-tetra-substituted flavones tends to be augmented along with increasing degree of methylation of hydroxyl group(s) in ring A. This is exemplified by the findings that 5,6,3',4'-tetrahydroxy-7,8-dimethoxyflavone (22), LARI 1 (25), and LARI 4 (26) were more potent than 5,6,7,3',4'-pentahydroxy-8-methoxyflavone (21), compound (22), and 5,3',4'-trihydroxy-6,7,8-tri-

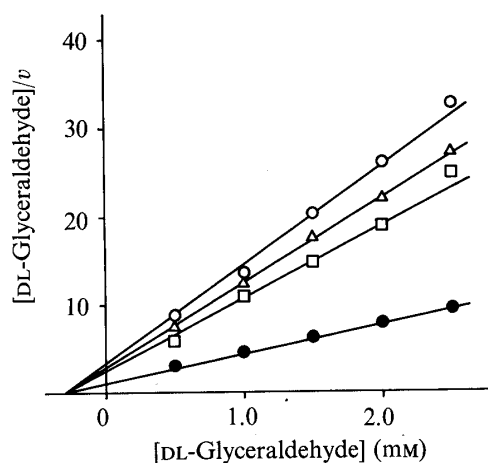


Fig. 1. Hanes-Woolf Plots of $[\text{DL-Glycerinaldehyde}]/v$ against $[\text{DL-Glycerinaldehyde}]$

The assay of RLAR was performed in the presence and absence of an inhibitor ($5 \times 10^{-8} \text{ M}$) as described in Materials and Methods except that the DL-glycerinaldehyde concentration was varied. (●) no inhibitor, (Δ) 8-benzyl-5,7,3',4'-tetrahydroxy-6-methoxyflavone (23), (□) LARI 1 (25), (○) LARI 4 (26).

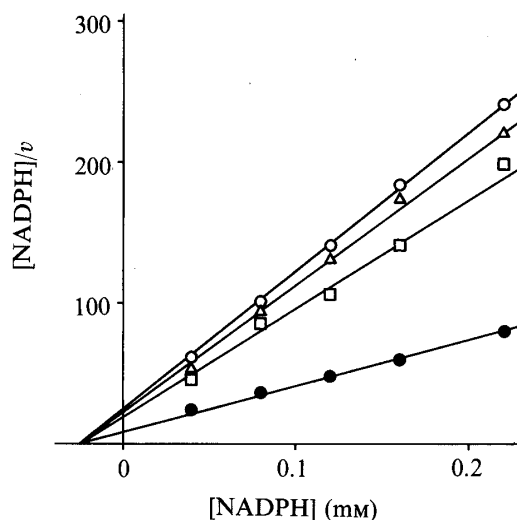


Fig. 2. Hanes-Woolf Plots of $[\text{NADPH}]/v$ against $[\text{NADPH}]$

The assay of RLAR was performed in the presence and absence of an inhibitor ($5 \times 10^{-8} \text{ M}$) as described in Materials and Methods except that the NADPH concentration was varied. (●) no inhibitor, (Δ) 8-benzyl-5,7,3',4'-tetrahydroxy-6-methoxyflavone (23), (□) LARI 1 (25), (○) LARI 4 (26).

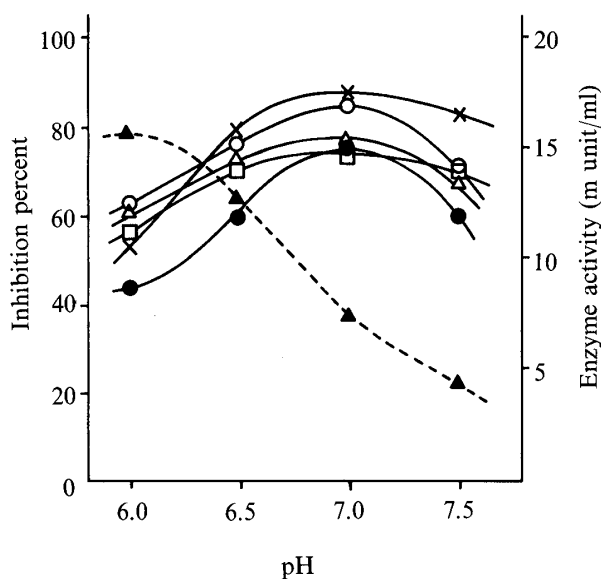


Fig. 3. Dependence on pH of the Inhibitory Activity of Flavones against RLAR

The assay of RLAR was performed in the presence and absence of an inhibitor ($5 \times 10^{-8} \text{ M}$) as described in Materials and Methods. (x) axillarin (8), (●) 3',4'-dihydroxy-5,7,8-trimethoxyflavone (17), (Δ) 8-benzyl-5,7,3',4'-tetrahydroxy-6-methoxyflavone (23), (□) LARI 1 (25), (○) LARI 4 (26). Aldose reductase activity in the absence of an inhibitor is shown by a broken line and expressed as m unit/ml of the reaction mixture. Values of inhibition percent were calculated as percentages of the rate of enzyme activity at various pH values in the absence of inhibitor. Each point is the mean of 2 experiments.

methoxyflavone (24), respectively. It should be noted that 8-benzyl-5,7,3',4'-tetrahydroxy-6-methoxyflavone (23), possessing a bulky substituent at C_8 , is rather potent.

Kinetics of Inhibition of Aldose Reductase

The inhibition of aldose reductase by flavones was found to be completely reversible from the dialysis experiments. Kinetic studies of the reversible inhibition were thus conducted to determine the nature of the inhibition of lens aldose reductase by flavones. The effect of varying the DL-glycerinaldehyde concentration on the inhibition of RLAR and BLAR by flavones ($5 \times 10^{-8} \text{ M}$) was investigated by graphing the kinetic data as Hanes-Woolf plots. These compounds exhibited non-competitive inhibition for both RLAR (Fig. 1) and BLAR (data not shown). The inhibitor constant (K_i), for example, of LARI 4 (26) for RLAR was

determined graphically to be 6×10^{-8} M. The effect of varying the NADPH concentration on the inhibition by flavones was also investigated. Inhibition of RLAR (Fig. 2) and BLAR (data not shown) by flavones was also of a non-competitive type with respect to NADPH.

Effect of pH on Inhibition of Aldose Reductase

It seems to be of value to determine whether or not the inhibition of aldose reductase by flavone derivatives is influenced by pH, because the intracellular pH is variable under various conditions. The effect of pH on the inhibition of RLAR by five 3',4'-dihydroxyflavones including LARI 4 is indicated in Fig. 3. The inhibitory activities of these flavones tested were all pH-dependent, being maximal around pH 7.0 when the pH was varied within a range of 6.0 to 7.5.

Discussion

Several classes of compounds of diverse structure are known to inhibit the enzyme aldose reductase,^{1,2a,4)} including a large number of flavonoids, e.g. quercitrin and myricitrin.^{1a,b)} These studies prompted us to investigate the inhibitory activities of various 3',4'-dihydroxyflavones. Of the 3',4'-dihydroxyflavones tested in this study, 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone (LARI 4, **26**) was the most potent inhibitor. Its activity was comparable to those of axillarin (**8**) and LARI 1 (**25**) (the most potent flavones previously reported).⁴⁾

Varma and Kinoshita^{1b)} indicated some possible relationships of structure to the inhibitory potencies of flavonoids, and we⁴⁾ recently reported some additional structure-activity relationships of flavonoids. The present work demonstrates the effect of methylation of hydroxyl groups in ring A of 3',4'-dihydroxyflavones. Our conclusions are as follows: (1) methylation of the hydroxyl group at either C₅ or C₆ increases or does not affect the inhibitory activity, (2) methylation of the 7-OH enhances the inhibitory activity of 3',4'-dihydroxy-5,7,8-trisubstituted and 3',4'-dihydroxy-5,6,7,8-tetrasubstituted flavones, and (3) methylation of the 8-OH potentiates the inhibitory activity. We⁴⁾ previously reported that methoxylation at C₃ did not affect the inhibitory potency. However, we found in this study that methoxylation at C₃ decreased the potency in 6 of 8 cases.

We¹⁰⁾ also reported previously that the inhibition of lens aldose reductase by hydantoin derivatives is pH-dependent, and suggested that the inhibition by 1-substituted hydantoins and by 5-substituted hydantoins is due to the non-ionized and ionized forms, respectively. The experiment on the effect of pH on aldose reductase inhibition by flavones revealed that the maximal inhibition occurs around pH 7.0 (Fig. 3). The pH-inhibition curve of LARI 4 (**26**) does not seem to be explainable in terms of pH-dependent structural change of this compound, in contrast to the case of hydantoin derivatives, because the pH-inhibition relation shows a bell-shaped curve and moreover the pK value at 30 °C (assay temperature) of LARI 4 (**26**) is supposed to be higher than 9.5 (the pK value of pyrocatechol at 20 °C is 9.85¹¹⁾). A possible explanation for the pH-dependency of aldose reductase inhibition by LARI 4 (**26**) is that the inhibitory potency of this flavone (non-ionized form) correlates with net electric charges of aldose reductase. This explanation appears to be applicable to four other flavones, because their pH-inhibition curves are similar to that of LARI 4 (**26**).

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