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Inhibitory Effect of Benzyl Oxazolecarbamate Analogues on Aldose Reductase

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Twelve kinds of benzyl oxazolecarbamate analogues were tested *in vitro* for inhibition of aldose reductases, which play a leading role in the etiology of diabetic complications such as cataract, retinopathy and neuropathy. The comparative study of various analogues substituted with a benzylcarbamate group at C-2, C-4 or C-5 of the oxazole skeleton showed that the benzylcarbamate group at the C-2 position was absolutely necessary for potent aldose reductase inhibitory activity. The group at C-4 or C-5 was ineffective. Introduction of an alkyl group at the C-4 position of benzyl 5-phenyl-2-oxazolecarbamate increased the inhibitory activity, and in particular, the 4-isopropyl analogue was found to be a potent inhibitor. The 50% inhibition concentration of benzyl 4-isopropyl-5-phenyl-2-oxazolecarbamate (IV) for aldose reductases Ia and Ib was about 3.5×10^{-7} M, whereas that of benzyl 5-phenyl-2-oxazolecarbamate (I) was about 1.5×10^{-5} M. Inhibition of rabbit lens aldose reductase by compound IV was of a non-competitive type with DL-glyceraldehyde as a substrate. Compound IV appears to be a specific inhibitor of rabbit lens aldose reductase, because it was found to have little inhibitory effect against several other enzymes.

Keywords——aldose reductase; lens aldose reductase; aldose reductase inhibitor; oxazole derivative; benzyl oxazolecarbamate

In diabetes mellitus, sorbitol accumulates in insulin-insensitive tissues such as lens,[1] nerve[2] and retina.[3] Elevated sorbitol levels result in a loss of osmotic integrity and cellular damage,[4] which are linked to development of some complications of chronic diabetes, *e.g.* cataracts, neuropathy and retinopathy. Excessive sorbitol is produced from glucose by aldose reductase through the polyol pathway. These findings suggest that potent aldose reductase inhibitors may be of value in the treatment and prevention of chronic diabetic complications. Recently, some aldose reductase inhibitors, for example sorbinil, tolrestat and ONO-2235, have been found useful for preventing or treating chronic complications of diabetes.[5] In addition, many heterocyclic compounds, *i.e.*, imidazolidine-2,4-diones,[6] thiazolidine-2,4-diones,[7] oxazolidine-2,4-diones,[8] and phthalazines[9] have been investigated as inhibitors of aldose reductase. We have previously studied the synthesis and the inhibitory activity of various oxazole derivatives, and found that a compound possessing phenyl and benzylcarbamate groups at C-5 and C-2, respectively, of the oxazole skeleton, *i.e.*, benzyl 5-phenyl-2-oxazolecarbamate (I), exhibited relatively strong inhibitory activity.[10] With the aim of developing stronger aldose reductase inhibitors, we have attempted to modify compound I, namely by introducing various alkyl groups at the C-4 position of the oxazole skeleton and by moving benzylcarbamate group to other positions of the oxazole skeleton of I. In this paper, we describe the results of screening tests of 12 kinds of benzyl oxazolecarbamate derivatives and we discuss the effect of the structural alterations on the inhibitory activity.

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Experimental

Materials——Aldose reductases Ia and Ib from rabbit lens were prepared by the method described in the previous paper.[11] Briefly, a 25—55% ammonium sulfate fraction was subjected to gel filtration on Sephadex G-200, followed by three column chromatographic steps, *i.e.*, affinity chromatography using Matrex gel orange A, gel filtration on Sephadex G-100 and chromatofocusing using polybuffer exchanger 94 and polybuffer 74. Aldose reductases Ia and Ib were purified about 800- and 1100-fold, respectively. Each of the purified enzymes was homogeneous on polyacrylamide gel electrophoresis. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Oriental Yeast Co. Sorbitol dehydrogenase, *myo*-inositol dehydrogenase and glutamate dehydrogenase were obtained from Sigma Chemical Co. Hexokinase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase and 6-phosphogluconate dehydrogenase were purchased from Oriental Yeast Co., and 20β-hydroxysteroid dehydrogenase was from Boehringer Mannheim GmbH.

Preparation of Oxazole Derivatives——Among the benzyl oxazolecarbamate analogues shown in Table I, 2-carbamates (I—V, VII), 4-carbamates (VIII—XI) and 5-carbamate (XII) were prepared by the methods reported previously.[12]

Synthesis of Benzyl 2-Benzoxazolecarbamate (VI)——A solution of $NaNO_2$ (2.3 g) in water was added dropwise to a stirred mixture of a 2-benzoxazolecarbohydrazide[13] (5 g), conc. HCl (3.3 ml), acetic acid (83 ml) and benzene (83 ml) at 0—5 °C. The stirring was continued for 30 min. The reaction mixture was diluted with water, and extracted with benzene, then the extract was dried. Benzyl alcohol (9.2 g) was added, and the mixture was refluxed for 5 h. After removal of the solvent, the excess benzyl alcohol was removed by steam distillation. The product was recrystallized from dioxane to give VI (4.7 g, 62.4%) as colorless leaflets, mp 204—206 °C. IR ν_{max}^{Nujol} cm^{-1}: 1735 (CO). ^{1}H-NMR (DMSO-d_6): 3.37 (1H, br, NH), 5.32 (2H, s, CH_2), 7.3—7.7 (9H, m, Ar-H). *Anal.* Calcd for $C_{15}H_{12}N_2O_3$: C, 67.15; H, 4.51; N, 10.44. Found: C, 66.94; H, 4.70; N, 10.39.

Assay of Aldose Reductase Activity——The assay was performed at 25 °C in 0.1 M sodium phosphate buffer (pH 6.2) containing 0.3 M ammonium sulfate, 10 mM DL-glyceraldehyde, 0.15 mM NADPH and an appropriate amount of enzyme in a total volume of 3.0 ml. The effects of the oxazole derivatives on the enzyme activity were determined by adding 5 μl of test compound solution to the reaction mixture. The appropriate blank to correct for nonspecific oxidation of NADPH was prepared. The reaction was initiated by the addition of enzyme, and the rate of NADPH oxidation was followed by recording the decrease in absorbance at 340 nm on a Hitachi 557 dual-wavelength double-beam spectrophotometer equipped with a temperature-controlled cuvette chamber.

Preparation of Test Compound Solutions——Because of the poor solubility of the oxazole derivatives, all compounds were dissolved in dimethylsulfoxide. Dimethylsulfoxide at 0.15% in the final assay solution did not inhibit the activity of the enzymes by more than 5%.

Determination of IC_{50}——The concentration of inhibitor giving 50% inhibition of enzyme activity (IC_{50}) was estimated from the least-squares regression line of plots of the logarithm of inhibitor concentration *versus* remaining activity.

Results and Discussion

Inhibitory Effect of Benzyl Oxazolecarbamate Analogues on Rabbit Lens Aldose Reductase

The inhibitory activities of various analogues of benzyl oxazolecarbamate toward rabbit lens aldose reductases (Ia and Ib) and the IC_{50} values of some potent compounds are shown in Table I. Introduction of an alkyl group at the C-4 position of benzyl 5-phenyl-2-oxazolecarbamate (I) enhanced the inhibitory activity. The activities of 4-alkyl analogues of I increased in the following order: 4-*tert*-butyl < 4-methyl < 4-ethyl < 4-isopropyl. The 4-*tert*-butyl, 4-methyl, 4-ethyl and 4-isopropyl analogues at 10^{-6} M inhibited both aldose reductases by 20—25%, 30—40%, 50% and 65%, respectively, though I was ineffective at the same concentration. The IC_{50} value of benzyl 4-isopropyl-5-phenyl-2-oxazolecarbamate (IV) was decreased by two orders of magnitude over that of I. Although the IC_{50} values of I for the two enzymes were about 1.5×10^{-5} M, the values of IV were 3.5×10^{-7} M (for aldose reductase Ia) and 3.8×10^{-7} M (for aldose reductase Ib). The IC_{50} values of benzyl 4-methyl-5-phenyl-2-oxazolecarbamate (II) and benzyl 4-ethyl-5-phenyl-2-oxazolecarbamate (III) were about 2×10^{-6} and 8×10^{-7} M, respectively. The increase of inhibitory activity by the introduction of an alkyl group at the C-4 position may be due to the hydrophobic interaction between the 4-alkyl group and enzyme molecule. The introduction of a *tert*-butyl group at the C-4 position did not increase the inhibitory effect, in contrast to the case of the isopropyl group. The IC_{50}

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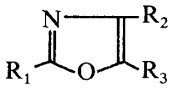
TABLE I. Inhibition of Rabbit Lens Aldose Reductases by Benzyl Oxazolecarbamate Analogues



Compd. No.	R_1	R_2	R_3	Enzyme	Inhibition (%) Concentration (μM) 0.1	0.5	1	5	10	50	IC_{50} (μM)
I	–NHCOOCH$_2$–Ph	–H	–Ph	Ia	—	—	2	26	40	72	16.3
				Ib	—	—	0	23	43	78	14.8
II	–NHCOOCH$_2$–Ph	–CH_3	–Ph	Ia	1	24	33	61	71	—	2.5
				Ib	5	31	41	65	71	—	2.0
III	–NHCOOCH$_2$–Ph	–CH_2CH_3	–Ph	Ia	17	42	52	73	—	—	0.84
				Ib	19	46	56	76	—	—	0.68
IV	–NHCOOCH$_2$–Ph	–$CH(CH_3)_2$	–Ph	Ia	31	54	65	80	—	—	0.35
				Ib	23	54	64	82	—	—	0.38
V	–NHCOOCH$_2$–Ph	–$C(CH_3)_3$	–Ph	Ia	—	17	26	43	49	62	12.9
				Ib	—	9	20	43	52	66	10.6
VI	–NHCOOCH$_2$–Ph	–CH=CH–CH=CH–		Ia	—	—	—	—	0	0	—
				Ib	—	—	—	—	0	0	—
VII	–NHCOOCH$_2$–Ph	–CH_3	–CH_3	Ia	—	—	—	—	0	6	—
				Ib	—	—	—	—	0	3	—
VIII	–CH_3	–NHCOOCH$_2$–Ph	–CH_3	Ia	—	—	—	—	0	0	—
				Ib	—	—	—	—	0	0	—
IX	–CH_3	–NHCOOCH$_2$–Ph	–Ph	Ia	—	—	—	—	0	5	—
				Ib	—	—	—	—	0	3	—
X	–Ph	–NHCOOCH$_2$–Ph	–H	Ia	—	—	—	—	0	0	—
				Ib	—	—	—	—	0	0	—
XI	–Ph	–NHCOOCH$_2$–Ph	–CH_3	Ia	—	—	0	1	4	13	—
				Ib	—	—	0	0	5	10	—
XII	–Ph	–CH_3	–NHCOOCH$_2$–Ph	Ia	—	—	0	6	8	19	—
				Ib	—	—	0	5	9	16	—

values of benzyl 4-*tert*-butyl-5-phenyl-2-oxazolecarbamate (V) were about $1.1—1.3 \times 10^{-5}$ M for the two enzymes, being slightly lower than those of I. The inability of the *tert*-butyl group to cause enhancement may be attributed to steric hindrance, as the *tert*-butyl group is bulkier than a methyl, ethyl or isopropyl group.

The activity of the 5-methyl derivative (VII) of benzyl 4-methyl-2-oxazolecarbamate was very weak as compared with that of the 5-phenyl derivative (II). This finding supported the result obtained in the previous report[10] that the presence of a phenyl group at C-5 was necessary for potent inhibitory activity of oxazole derivatives. A compound with a condensed ring (conjugated butadiene) between C-4 and C-5, *i.e.*, benzyl 2-benzoxazolecarbamate (VI) was completely inactive as an aldose reductase inhibitor at the concentration tested. This finding provides strong support for the suggestion in the previous paper[10] that the inhibitory activity of oxazole derivatives is inherent in the oxazole structure.

The inhibitory effects of various oxazole derivatives having a benzylcarbamate group at C-4 or C-5 of the oxazole skeleton on aldose reductase activity were evaluated. The percentages of inhibition of the enzymes by these compounds at 5×10^{-5} M to 1×10^{-6} M are summarized in Table I. Analogues with the benzylcarbamate group at C-4 of the oxazole skeleton (VIII, IX, X and XI) were ineffective or only slightly effective at 10^{-5} M. Introduction of the

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benzylcarbamate group at the C-5 position was also disadvantageous; compare the inhibitory activities of benzyl 4-methyl-5-phenyl-2-oxazolecarbamate (II) and benzyl 2-phenyl-4-methyl-5-oxazolecarbamate (XII). Thus, although the benzylcarbamate group at the C-2 position was necessary for potent inhibitory activity as described previously,[10] its presence at C-4 or C-5 was ineffective. These results suggest that the oxazole skeleton takes a definite orientation in relative to the enzyme molecule.

Kinetics of Inhibition by Benzyl 4-Isopropyl-5-phenyl-2-oxazolecarbamate

Kinetic studies were conducted with benzyl 4-isopropyl-5-phenyl-2-oxazolecarbamate (IV), one of the most potent compounds of the series surveyed, in order to determine the inhibition constant (K_i) and the type of inhibition. The enzyme activities were assayed with DL-glyceraldehyde as a substrate. The Lineweaver–Burk plots for compound IV as an inhibitor of aldose reductase Ib are shown in Fig. 1. Compound IV was found to be a non-competitive inhibitor. It was also found to be a non-competitive inhibitor of the enzyme Ia. The K_i values of enzymes Ia and Ib for IV were 2.9×10^{-7} and 3.4×10^{-7} M, respectively. Many other inhibitors of aldose reductase, *e.g.*, alrestatin (1,3-dioxo-1*H*-benz[de]isoquinoline-2,3(*H*) acetic acid; AY-22284),[14] quercitrin,[14-16] axillarin (5,7,3′,4′-tetrahydroxy-3,6-dimethoxyflavone),[16] and 1-(phenylsulfonyl)hydantoin,[6b] also inhibit lens aldose reductase noncompetitively.

Inhibition of Some Enzymes Other than Aldose Reductase by Benzyl 4-Isopropyl-5-phenyl-2-oxazolecarbamate

To study the specificity of the inhibition of aldose reductase by benzyl 4-isopropyl-5-phenyl-2-oxazolecarbamate (IV), the inhibitory effects of the compound on hexokinase and several kinds of pyridine nucleotide-requiring enzymes (sorbitol dehydrogenase, 20β-hydroxysteroid dehydrogenase, *myo*-inositol dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase) were investigated. The results are shown in Table II. These enzymes were all unaffected or only slightly inhibited by 1.67×10^{-6} M IV, though aldose reductases Ia and Ib were inhibited by 75 and 73%, respectively. Thus, the inhibitory function of compound IV appears to be specific for aldose reductase.

TABLE II. Inhibitory Effect of Benzyl 4-Isopropyl-5-phenyl-2-oxazolecarbamate on Various Enzymes

Enzymes	Inhibition (%)
Hexokinase	1
20β-Hydroxysteroid dehydrogenase	0
Sorbitol dehydrogenase	8
Glucose 6-phosphate dehydrogenase	0
myo-Inositol dehydrogenase	0
Lactate dehydrogenase	2
Glutamate dehydrogenase	0
6-Phosphogluconate dehydrogenase	2
Rabbit lens aldose reductase Ia	75
Rabbit lens aldose reductase Ib	73

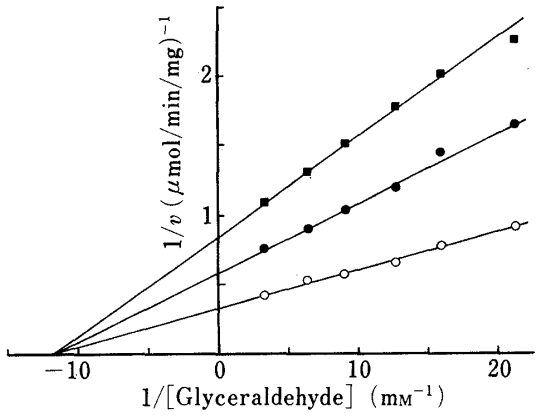

Fig. 1. Inhibition of Rabbit Lens Aldose Reductase Ib by Benzyl 4-Isopropyl-5-phenyl-2-oxazolecarbamate

Enzyme activity was measured at each substrate concentration in the presence (—●—, —■—) and absence (—○—) of inhibitor. DL-Glyceraldehyde was used as a substrate. Concentrations of the inhibitor were 2.8×10^{-7} (—●—) and 5.6×10^{-7} M (—■—).

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