A Highly Specific Aldose Reductase Inhibitor, Ethyl 1-Benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylate, and Its Congeners

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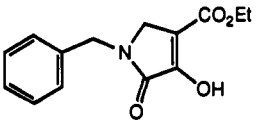
Ethyl 1-benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylate (1, EBPC) is a potent and specific inhibitor of aldose reductase. It was >4000× more potent in its inhibition of rat lens aldose reductase than the closely related rat or pig kidney aldehyde reductase, thus making it the most selective inhibitor of a NADPH-dependent carbonyl reductase identified to date. In agreement with this observation, it was found to be a highly potent inhibitor of aldose reductase from rat sciatic nerve with >98% inhibition at 1 μM, but it was practically devoid of activity against aldehyde reductases from rat liver and brain. Inhibition of aldose reductase was mixed type for glyceraldehyde (K_i = 8.0 × 10^{-8} M) and noncompetitive for NADPH (K_i = 1.70 × 10^{-8} M). Its potential as an in vitro tool to quantitate monomeric aldo/keto reductase activities in crude tissue extracts is presented. Structure–activity relationships emerging from synthetic modifications of EBPC are discussed. Several modifications were found to be active in vitro against aldose reductase from human placenta and in vivo in a rat model of diabetic complications, but none was more potent than EBPC.

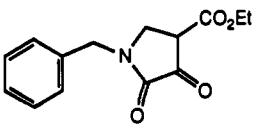
Aldose (EC 1.1.1.21) and aldehyde (EC 1.1.1.2) reductases are members of a class of enzymes called the monomeric NADPH-dependent carbonyl reductases.[1-3] These enzymes have broadly overlapping substrate specificity and in concert with sorbitol dehydrogenase metabolize glucose to sorbitol and fructose.[4,5] They are also capable of reducing a variety of aliphatic and aromatic aldehydes to their corresponding alcohols. Under diabetic conditions, the increased glucose flux through the sorbitol or polyol pathway results in abnormal intracellular accumulation of sorbitol in tissues that are insulin independent for glucose transport. There is mounting evidence that increased glucose flux through the sorbitol pathway resulting in excess accumulation of sorbitol may be involved in the etiology of late-onset diabetic complications, neuropathy, nephropathy, retinopathy, and cataracts.[6-9] Currently, sorbitol pathway inhibitors that have received attention in the clinic belong to two major chemical classes—spirohydantoins and carboxylic acids.[10] They include sorbinil, ponalrestat, and tolrestat. In vitro, these compounds inhibit both aldose and aldehyde reductase.[11] The need for specific inhibitors to elucidate the role of each enzyme in diabetic complications has already been expressed.[2c,12] Menadione bisulfite[13] is reported to be a selective aldose reductase inhibitor while phenobarbital[11d] and related derivatives[14] and valproic acid[14d,15] are reported to be selective aldehyde reductase inhibitors. These inhibitors have not been evaluated in the clinic for diabetic complications presumably because they are not sufficiently potent. A very recent publication[16] claims that ponalrestat, [3-(4-bromo-2-fluorobenzyl)-4-oxo-3,4-dihydrophthalazin-1-yl]acetic acid, is a selective inhibitor of aldose reductase. It is not yet known whether an inhibitor specific to aldose or aldehyde reductase would offer any advantages in the clinic over dual inhibitors. A few years ago, we set out to discover selective aldose/aldehyde reductase inhibitors of a novel chemical class, using rat lens aldose[3] and the recently characterized pig kidney[14b,17] aldehyde reductase. In this endeavor, we have found that the previously described ethyl 1-benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylate[18a,b] (EBPC) is a potent and extremely specific aldose reductase inhibitor. Herein we report enzymological studies with EBPC, its application as an in vitro tool to quantitate aldose and aldehyde reductase activities in target diabetic tissues, and structure–activity relationships emerging from medicinal chemical followup of EBPC.

Chemistry

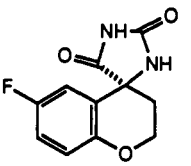
The target *N*-substituted-3-hydroxy-2(5*H*)-oxopyrrole-4-carboxylates **5a** were prepared according to literature procedure[18] starting from appropriate alkyl- or aralkyl-



1 (EBPC)



1a



2 (Sorbinil)

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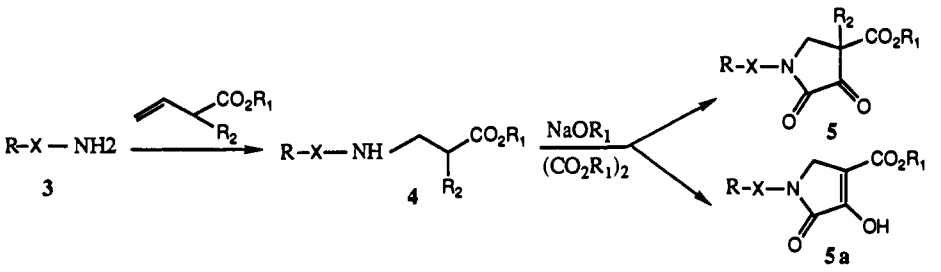
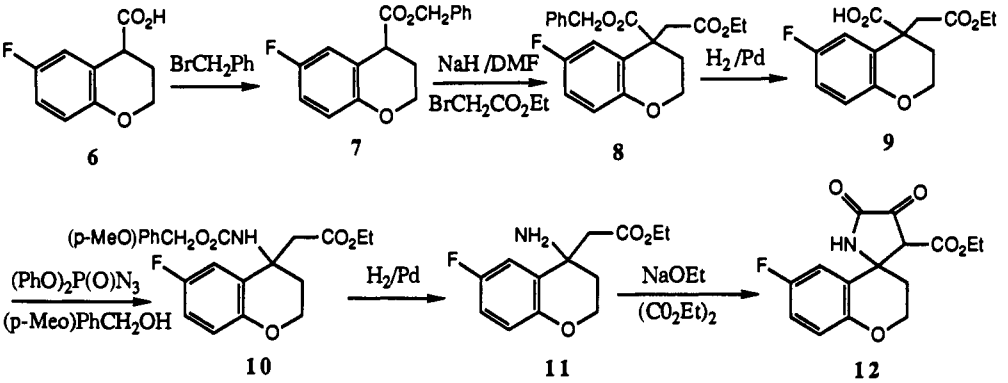
Scheme I



Scheme II



Scheme III

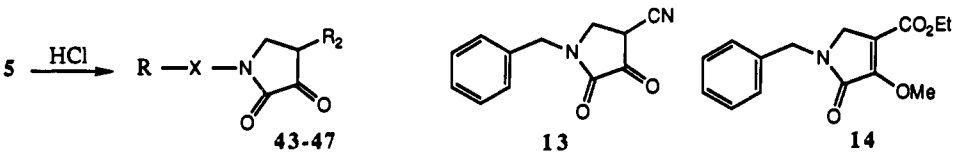
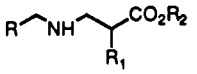


Table I. Physicochemical Data for Substituted Aminopropionates



compd	R	R_1	R_2	formula	bp, °C/mm
15	2-pyridyl	H	Et	$C_{11}H_{16}N_2O_2$	110–118/0.3
16	4-pyridyl	H	Et	$C_{11}H_{16}N_2O_2$	124–131/0.5
17	2-thienyl	H	Et	$C_{10}H_{15}NO_2S$	118–126/3.0
18	2-thienyl	H	*t*-Bu	$C_{10}H_{19}NO_2S$	112–116/0.35
19	1-naphthyl	H	Et	$C_{16}H_{19}NO_2$	159–162/0.5
20	phenyl	SMe	Et	$C_{13}H_{19}NO_2S$	117–124/17

amines **3**. The synthetic sequence employed is shown in Scheme I. The substituted aminopropionates **4** prepared early in the program were purified by distillation (Table I), but with experience it became expedient to use the crude products directly in the cyclization step. The preparation of the spirocylic compound **12** is depicted in Scheme II. The chromanecarboxylic acid **6**[19] was converted to the corresponding benzyl ester **7**, which was alkylated with ethyl bromoacetate to obtain **8**. The benzyl ester in **8** was deprotected, and the resulting acid **9** was subjected to a modified Curtius reaction[20] to obtain **10**. Hydrogenolysis of **10** gave the key intermediate **11** whose base-promoted condensation with diethyl oxalate led to **12**. Pyrrolidinediones **43–47** were prepared according to Scheme III. The nitrile **13** (mp 182–185 °C) was obtained according to Scheme I with benzylamine as the starting

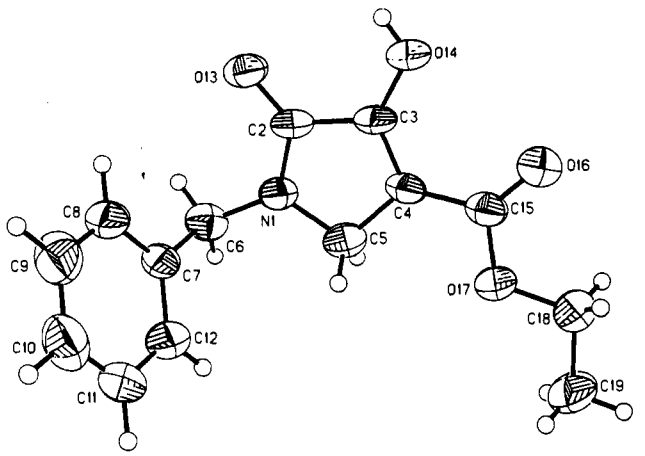


Figure 1.

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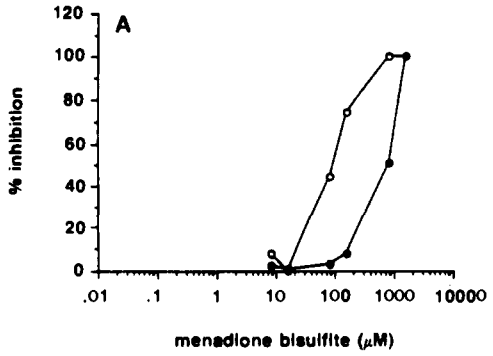

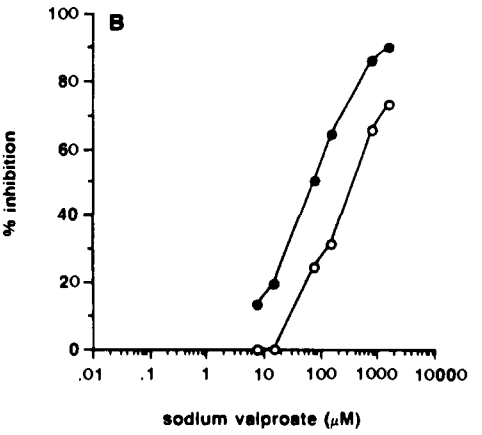

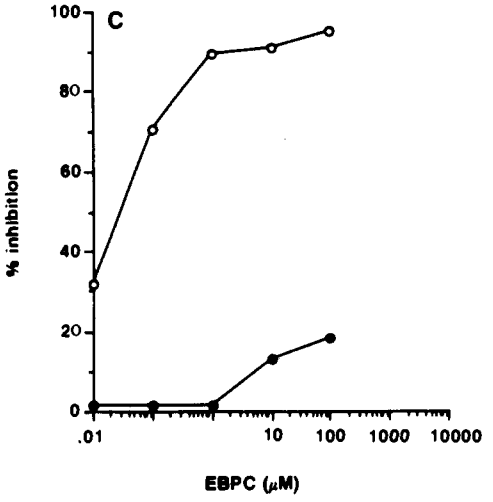

Inhibitor	IC_{50} (M) aldose reductase	IC_{50} (M) aldehyde reductase	Selectivity: IC_{50} aldehyde/ IC_{50} aldose
menadione bisulfite	8.2×10^{-5}	4.9×10^{-4}	6
sodium valproate	3.8×10^{-4}	8.9×10^{-5}	0.2 *
EBPC	4.7×10^{-8}	2% at 10^{-4}	>4000

*Corresponds to 5-fold selectivity for aldehyde reductase as discussed in the text

Figure 2. Specificity of menadione bisulfite (A), sodium valproate (B), and EBPC (C) for aldose (○) and aldehyde (●) reductase. Purified rat lens aldose reductase and pig kidney aldehyde reductase were incubated in the presence of the indicated concentrations of inhibitors. Data are presented as the mean of quadruplicate determinations; the standard deviation is no greater than ±4%. IC_{50} values were calculated by a log linear regression analysis.

material and with use of acrylonitrile in place of acrylate. The preparation of **14** has been described previously.[18] All compounds listed in Tables IV and V gave a red color reaction with ferric chloride, which is characteristic of enolic compounds. On the basis of a single-crystal X-ray analysis of EBPC which supports the enolic structure (Figure 1),[21] compounds in Table IV are represented in the enolic form rather than in the dioxo form.

Results and Discussion

EBPC, menadione bisulfite, valproic acid, and clinically important sorbitol pathway inhibitors, epalrestat, ponalrestat, tolrestat, sorbinil, and alconil[10] were tested against rat lens aldose and pig kidney aldehyde reductase. As is common in studies with these enzymes,[2c] 3-pyridinecarboxaldehyde was used as the substrate. In our hands (Figure 2A), menadione bisulfite showed a 6-fold selectivity for aldose ($IC_{50} = 8.2 \times 10^{-5}$ M) over aldehyde reductase ($IC_{50} = 4.9 \times 10^{-4}$ M). Valproic acid was also a weak inhibitor of both aldose ($IC_{50} = 3.8 \times 10^{-4}$ M) and aldehyde reductase ($IC_{50} = 8.9 \times 10^{-5}$ M) but showed a 5-fold selectivity for aldehyde reductase (reciprocal of 0.2 in Figure 2B).

EBPC, at 1 μM, inhibited >90% of aldose reductase activity without significantly inhibiting aldehyde reductase. Separation of inhibitory activity against the two enzymes occurred at lower concentrations and was maintained throughout the concentration range tested. The IC_{50}s were 4.7×10^{-8} and $> 10^{-4}$ M against aldose and aldehyde reductase, respectively (Figure 2C). From these data, it is readily seen that EBPC is more than 4000-fold selective for inhibition of aldose reductase, which establishes it as the most selective inhibitor of a NADPH-dependent carbonyl reductase identified to date.

Initial velocity analyses using the rat lens enzyme indicated that EBPC was a mixed type inhibitor (Figure 3A) with respect to glyceraldehyde ($K_i = 8.0 \times 10^{-8}$ M) and a noncompetitive inhibitor (Figure 3B) with respect to NADPH ($K_i = 1.7 \times 10^{-8}$ M). The ability to EBPC to distinguish between aldose and aldehyde reductase in crude tissue extracts was initially examined with simulated extracts. Four mixtures of pure rat lens aldose and rat kidney aldehyde reductase were prepared in proportions ranging from 72% to 13% aldehyde reductase activity and 28% to 87% aldose reductase activity. These mixtures were representative of the range of aldose and aldehyde reductase activities reported for diabetic target tissues, with predominantly aldehyde reductase in the kidney (mixture 1) and aldose reductase in the lens (mixture 4). The four mixtures were then assayed for aldehyde/aldose reductase activity in the absence and presence of EBPC (1×10^{-6} M); at this concentration EBPC completely inhibits aldose reductase activity without significantly suppressing aldehyde reductase activity (Figure 2C). The results, presented in Figure 4, show that the enzyme activity measured in the presence of EBPC and the actual added aldehyde reductase activity was not statistically different for the mixtures. Aldose reductase activities, calculated by the mathematical difference in the activities

(21) We thank Dr. J. Bordner of Pfizer X-Ray Laboratory for the analysis.

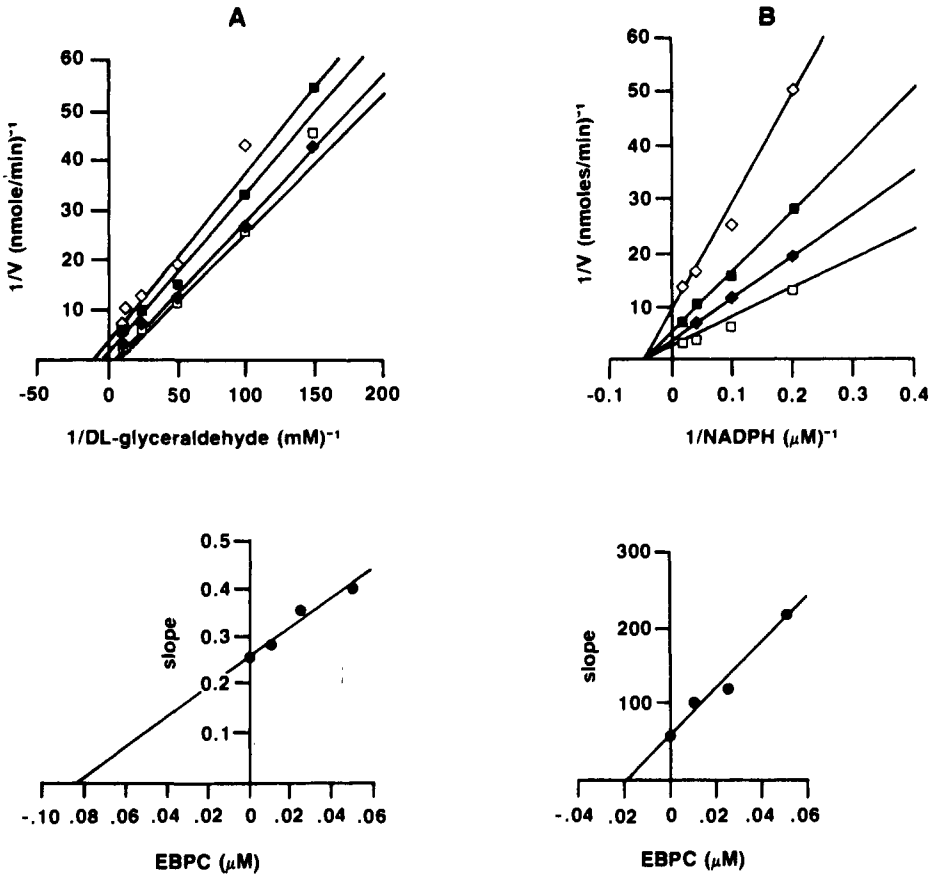

Figure 3. Mixed type inhibition for glyceraldehyde (A) and noncompetitive inhibition for NADPH (B) by EBPC at 0 (□), 1×10^{-8} M (◆), 2.5×10^{-8} M (■), and 5×10^{-8} M (◇) concentrations. Secondary plots of the slopes as a function of EBPC concentration.

assayed in the presence and absence of 1 μM EBPC, were not statistically different from the actual enzyme activities for the mixtures.

Further evidence of the specificity of EBPC for aldose reductase was provided by examining rat tissues in which the NADPH-dependent carbonyl reductases have been localized. Immunohistochemical and enzymological methods have localized aldose reductase in the renal inner medulla, lens, and sciatic nerve and aldehyde reductase in the renal cortex.[12b,22] Renal outer medulla, liver,[22a,f] and brain[22k,23] contain a mixture of both. Enriched preparations of aldehyde/aldose reductases were prepared from rat renal cortex, inner and outer medulla, liver, brain, and lens, and the intrinsic activity of EBPC was determined (Figure 5) with 3-pyridinecarboxaldehyde as the substrate. EBPC was a potent inhibitor of the renal inner medullary ($IC_{50} = 2.8 \times 10^{-8}$ M) and the lens ($IC_{50} = 4.7 \times 10^{-8}$ M) preparations. However, it was a weak inhibitor of the renal cortical ($IC_{50} = 4.8 \times 10^{-4}$ M), the outer medullary ($IC_{50} = 2.1 \times 10^{-4}$ M), liver (9% at 10^{-4} M), and brain (0% at 10^{-4} M) preparations. The IC_{50} for the whole kidney preparation is consistent with the predominant contribution of aldehyde reductase from renal cortex and outer medulla, which constitute the bulk of the kidney. Crude extracts of rat sciatic nerve, when assayed with 3-pyridinecarboxaldehyde ($n = 3$), yielded 1.23 ± 0.28 nmol/min aldose reductase activity. At a concentration of 1 μM, EBPC inhibited 98% of this activity (0.03 nmol/min). These data are consistent with selectivity of EBPC for aldose reductase and with distribution of the enzymes in target tissues.

EBPC has also been shown to discriminate between aldose and aldehyde reductase activities using endogenous (glucose) and artificial (3-pyridinecarboxaldehyde and glyceraldehyde) substrates (see Table II). NADPH oxidation in the presence of EBPC was previously shown to inhibit aldose reductase and permit measurement of only

Table II. Inhibition of Aldose and Aldehyde Reductases by EBPC Using Various Substrates (nmol of NADPH/10 min)[a]

	aldose reductase (rat lens)		aldehyde reductase (pig kidney)	
substrate	control	1 μM EBPC	control	1 μM EBPC
D-glucose	9.90 ± 1.00	0.42 ± 0.06	0.56 ± 0.04	0.48 ± 0.04
3-pyridine-carbox-aldehyde	24.66 ± 0.39	1.32 ± 0.05	37.49 ± 0.87	35.43 ± 2.24
DL-glycer-aldehyde	26.59 ± 1.51	0.84 ± 0.16	3.50 ± 0.01	3.65 ± 0.24

[a] Rat lens aldose reductase (7.5 ng) and pig kidney aldehyde reductase (187 ng) enzyme activities were determined in the presence and absence of 1 μM EBPC with 0.5 M D-glucose, 5 mM DL-glyceraldehyde, and 10 mM 3-pyridinecarboxaldehyde. Data are expressed as the mean of triplicate determinations ± SEM.

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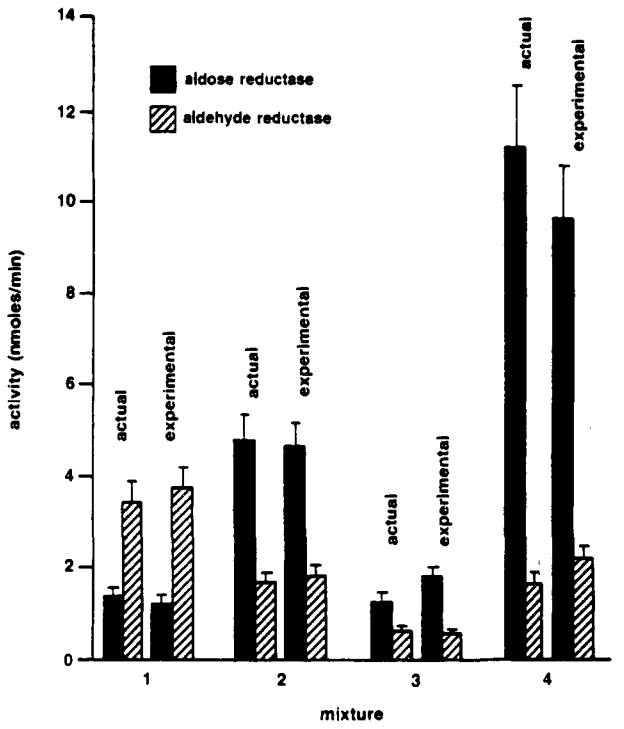
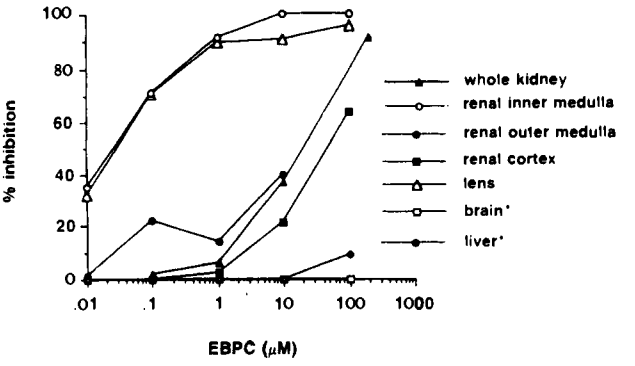


Figure 4. Quantitation of aldose and aldehyde reductase activities in simulated tissue extracts with EBPC as a selective inhibitor. Mixtures were prepared by adding known amounts of purified rat lens aldose (28%–87%) and rat kidney aldehyde (72%–13%) reductase activities. Each mixture was then assayed in the presence and absence of 1 μM EBPC with 3-pyridinecarboxaldehyde as the substrate. Activity in the presence of inhibitor was defined as experimentally determined aldehyde reductase activity. The mathematical difference between activities measured in the absence and presence of inhibitor was defined as experimentally determined aldose reductase activity. Data are expressed as the mean of three determinations ± SEM.

aldehyde reductase activity. The mathematical difference between NADPH oxidation in the presence and absence of EBPC accounted for the activity of aldose reductase. The choice of substrate used in these determinations would be expected to affect only absolute enzyme activities (due to catalytic efficiency) and not the ratio of enzyme activities.

Heretofore, methodologies for distinguishing between aldose and aldehyde reductase have relied on unique immunological properties or substrate specificities.[23] Assays have been designed to preferentially measure one enzyme activity in the presence of the other, but these measurements have not been shown to be quantitative. While immunoquantitation has been used, it is not only labor intensive but capricious. Polyclonal antibodies raised against aldehyde reductase from one tissue have not always recognized aldehyde reductases from other tissues, and immunological cross-reactivity of the antibody has not always been evident among different species.[12b,22a,e] Recent work[22a,d,i] has demonstrated the utility of monospecific antiserum for the detection and quantitation of aldose and/or aldehyde reductase in rat, bovine, and human tissues and provided evidence of interspecies cross-reactivity. Since the readily prepared EBPC could be used to quantitate aldose reductase in crude tissue extracts, it offers a more practical alternative to immunological methodology.

Results with clinically important compounds are presented in Table III. Epalrestat and ponalrestat, the most selective of this group, showed 115- and 71.4-fold selectivity, respectively, for aldose reductase. Even this range



*Data for aldehyde reductase

	IC_{50} (M)
whole kidney	1.4×10^{-5}
renal cortex	4.8×10^{-4}
renal inner medulla	2.8×10^{-5}
renal outer medulla	2.1×10^{-4}
lens	4.7×10^{-5}
liver*	9% at 10^{-4}
brain*	0% at 10^{-4}

Figure 5. Intrinsic activity of EBPC against aldose/aldehyde reductase from various tissues. Enriched preparations (5–13-fold) of aldose/aldehyde reductases were prepared from each of the indicated tissues (see Experimental Section). Enzyme activities in the presence of the indicated concentrations of EBPC were determined with 3-pyridinecarboxaldehyde as the substrate. Data are presented as the mean of triplicate determinations; the standard deviation is no greater than ±4%. IC_{50} values were calculated by a log linear regression analysis.

Table III. Selectivity of N-Substituted Ethyl 3-Hydroxy-2(5*H*)-oxopyrrole-4-carboxylates, Related Compounds, and Known Inhibitors for Aldose Reductase[a]

compd	IC_{50}, M: aldose reductase (rat lens)	IC_{50}, M: aldehyde reductase (pig kidney)	selectivity: IC_{50} aldehyde/ IC_{50} aldose
EBPC (1)	4.7×10^{-8}	2% at 2×10^{-4}	>4000
32	1.1×10^{-7}	9% at 2×10^{-4}	>1800
12	9.1×10^{-7}	11% at 2×10^{-4}	>200
epalrestat[b,g]	1.3×10^{-8}	1.5×10^{-6}	115
ponalrestat[c,g]	2.8×10^{-8}	2.0×10^{-6}	71.4
tolrestat[d,g]	2.9×10^{-8}	5.4×10^{-7}	18.6
sorbinil[e]	2.8×10^{-7}	1.9×10^{-6}	6.8
alconil[f,g]	3.0×10^{-7}	2.7×10^{-8}	0.1

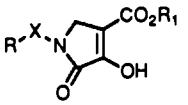
[a] Rat lens aldose reductase and pig kidney aldehyde reductase were incubated in the presence of the indicated inhibitors, and enzyme activity was measured with use of 3-pyridinecarboxaldehyde as the substrate. IC_{50} values were calculated by a log linear regression analysis. [b] (*Z*)-3-(Carboxymethyl)-5-[2(*E*)-(*o*-methylphenyl)propenylidene]rhodanine. [c] 3-(4-Bromo-2-fluorobenzyl)-4-oxo-3,4-dihydrophthalazin-1-yl)acetic acid. [d] *N*-[[5-(Trifluoromethyl)-6-methoxy-1-naphthalenyl]thioxomethyl]-*N*-methylglycine. [e] *d*-6-Fluorospiro(chroman-4,4'-imidazolidine)-2',5'-dione. [f] 2-Fluorospiro(9*H*-fluorene-9,4'-imidazolidine)-2',5'-dione. [g] For structures, see ref 10.

of selectivity falls far short of the more than 4000-fold selectivity observed with EBPC. It is also significant to note that our data for ponalrestat is in remarkable agreement with literature finding[16] in spite of differences in methodology.

Preliminary experiments with **12** and **32** suggest that selective aldose reductase inhibition activity may be characteristic of the family of compounds described here (Table IV).

Structure–activity relationships (SAR) around EBPC were pursued in order to explore whether potency of EBPC could be improved by chemical modifications. In vitro screening (see Experimental Section) was done with use of human placental aldose reductase. SAR included

Table IV. Physicochemical and in Vitro Aldose Reductase Inhibition Data for 1-Substituted Ethyl 3-Hydroxy-2(5*H*)-oxopyrrole-4-carboxylates



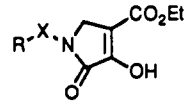
compd	R	X	R_1	formula	mp, °C	human placental aldose reductase: IC_{50},[a] M
1 (EBPC)	Ph	CH_2	Et	$C_{14}H_{15}NO_4$	135–136[b]	2.6×10^{-7}
21	Ph	CH_2	Me	$C_{13}H_{13}NO_4$	183–184[b]	2.6×10^{-6}
22	Ph	CH_2	*n*-Pr	$C_{15}H_{17}NO_4$	109	1.9×10^{-6}
23	Ph	CH_2	*t*-Bu	$C_{16}H_{19}NO_4$	178–179	27% at 10^{-5}[c]
24[d]	2-ClC_6H_4	CH_2	Et	$C_{14}H_{13}ClNO_4Na$		2.3×10^{-6}
25	4-ClC_6H_4	CH_2	Et	$C_{14}H_{14}ClNO_4$	160–161	3.1×10^{-7}
26	3,4-$Cl_2C_6H_3$	CH_2	Et	$C_{14}H_{13}Cl_2NO_4$		2.4×10^{-7}
27	4-BrC_6H_4	CH_2	Et	$C_{14}H_{14}BrNO_4$	165–167	5.0×10^{-7}
28	4-$OMeC_6H_4$	CH_2	Et	$C_{15}H_{17}NO_5$	130–133	4.1×10^{-7}
29	2-thienyl	CH_2	Et	$C_{12}H_{13}NO_4S$	98–100	3.8×10^{-7}
30	2-thienyl	CH_2	*t*-Bu	$C_{14}H_{17}NO_4S$	157–159	9% at 10^{-5}[c]
31	2-pyridyl	CH_2	Et	$C_{13}H_{14}N_2O_4$	130–132	4.4×10^{-7}
32	4-pyridyl	CH_2	Et	$C_{13}H_{14}N_2O_4$	173–175	2.0×10^{-7}
33[d]	2-furyl	CH_2	Et	$C_{12}H_{12}NO_5Na$		3.3×10^{-6}
34	1-naphthyl	CH_2	Et	$C_{18}H_{17}NO_4$	159–161	2.3×10^{-6}
35	9-fluorenyl	–	Et	$C_{20}H_{17}NO_4$	177	3.0×10^{-7}
36	Ph	$CH(CH_3)$[S-(–)]	Et	$C_{15}H_{17}NO_4$	111–112	1.2×10^{-6}
37	Ph	$CH(CH_3)$[*R*-(+)]	Et	$C_{15}H_{17}NO_4$	110–111	8.4×10^{-7}
38[d]	Ph	CH_2CH_2	Et	$C_{15}H_{16}NO_4Na$		2.5×10^{-6}
39	Ph	–	Et	$C_{13}H_{13}NO_4$	153[b]	28% at 10^{-5}[c]
40	1-naphthyl	–	Et	$C_{17}H_{15}NO_4$	174	36% at 10^{-5}[c]
41	cyclohexyl	CH_2	Et	$C_{14}H_{21}NO_4$	161–163	64% at 10^{-5}[c]
42	cyclohexyl	–	Et	$C_{13}H_{19}NO_4$	188[b]	22% at 10^{-5}[c]

[a] IC_{50}s were calculated by using a log linear regression analysis. Sorbinil was used as a positive control, and its inhibition values (including range) are as follows: 10^{-5} M, 87 ± 9%; 10^{-6} M, 70 ± 10%, 10^{-7} M, 36 ± 12%; $n = 120$. Its average IC_{50}, based on 120 determinations, is 3.47×10^{-7} M with SEM = 0.25×10^{-7} M. [b] Identical with literature (ref 1). [c] Inhibition determined at only one concentration. [d] Isolated as sodium salt.

modifications with respect to the ester group, spacer between the benzene and the pyrrolidinedione rings and substituents on the benzene ring, and replacements for the benzene ring. A compilation of compounds prepared and tested is shown in Table IV. The ethyl ester group was found to be optimum. While the methyl and *n*-propyl esters were less active, the *tert*-butyl esters were devoid of activity, suggesting a limit for steric toleration. That the ester grouping may not be essential for activity is indicated by the attractive inhibitory activity observed ($IC_{50} = 1.2 \times 10^{-6}$ M) with the cyano compound **13**. Activity of **1** was maintained with both electron-releasing and -withdrawing substituents at the 4-position of the benzene ring (cf. **25** and **28**). Ortho substutition led to diminished activity (**24** and **34**). Among the spacers, methylene was the best. Analogues with no spacer were inactive (**39** and **40**). Branching at the methylene spacer (**36** and **37**) resulted in less active compounds. However, the 9-fluorenyl analogue **35**, which can be considered as a tied-back analogue of either **36** or **37**, was nearly equipotent to **1**. The benzene ring could be replaced by either pyridine (**29**) or thiophene (**31**) without loss of activity; furan (**33**) in place of benzene led to decreased activity. Replacement by cyclohexyl (**41**) resulted in further diminution of activity.

The high potency of EBPC and some of its congeners against human placental aldose reductase prompted us to explore whether a pyrrolidinedione ring could function as a bioisostere of the spirohydantoin ring present in sorbinil. The spiro compound **12**, designed to pursue this possibility, was found to be quite active ($IC_{50} = 1.5 \times 10^{-6}$ M) but was less potent than EBPC. This observation is in contrast to an earlier unsuccessful effort to seek alternative pharmacophores to the spirohydantoin moiety.[24] No attempt was made to resolve compound **12** to examine the relationship between stereochemistry and biological activity.

Table V. In Vivo Activity of Selected Hydroxypyrrolecarboxylates[a]



compd	R	X	% inhibn of sorbitol accumulation: sciatic nerve	% inhibn of sorbitol accumulation: lens
1	phenyl	CH_2	51	42
30	2-thienyl	CH_2	37	36
32	4-pyridyl	CH_2	28	37

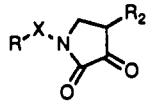
[a] Diabetes was induced in rats by administration of streptozotocin at 85 mg/kg, intravensouly, at 0 h. Compounds were administered orally (100 mg/kg) at 4, 7, and 24 h and rats sacrificed at 27 h. Inhibition was calculated on the basis of comparison to untreated diabetic rats ($n = 6$ for both treated and untreated groups) and significance was calculated by using the Student's *t* test ($p < 0.05$). All values were significant ($p < 0.05$) relative to untreated diabetic group. In this model, sorbinil's ED_{50} for inhibition of sorbitol accumulation in sciatic nerve was 0.25 mg/kg (see ref 19).

EBPC was tested in vivo, at 100 mg/kg, in an acute rat model of diabetic complications and was found to inhibit sorbitol accumulation, both in nerve and lens, but only to a moderate degree. Although several analogues of EBPC showed high in vitro activity, only compounds **30** and **32** were active in vivo. However, they were less potent than **1** (Table V).

EBPC and its congeners are highly acidic. For example, the pK_a of EBPC (6.4, 1:1 dioxane–H_2O) is nearly the same as that of phenylacetic acid (6.20) and significantly lower than that of sorbinil (9.7) in the same solvent system. Since such compounds, which predominantly exist in their

(24) Cox, M. T.; Godfrey, A. A.; Ridsdale, C. M. *J. Chem. Soc., Perkin Trans.* 1988, 3217.

Table VI. Physicochemical Data for Selected Pyrrolidinediones



compd	R	X	R_2	formula	mp, °C	pK_a
43	Ph	CH_2	H	$C_{11}H_{11}NO_2$	98–99[a]	could not be measured[b]
44	Ph	CH_2	CH_3	$C_{12}H_{13}NO_2$	145–146	10.6 (H_2O/dioxane, 1/1)
45	Ph	CH_2	SMe	$C_{12}H_{13}NO_2S$	168	7.3 (H_2O)
46	Ph	CH_2	SO_2Me	$C_{12}H_{13}NO_4S$	193–195	5.1 (H_2O/dioxane, 1/1)
47	Ph	CH_2	Ph	$C_{17}H_{15}NO_2$	247 dec	not measured

[a] Literature (ref 1), mp 99–100 °C. [b] Compound unstable to pH > 7; pK_a of 11.3 (H_2O/dioxane) corresponded to a dimeric structure (neutral equivalent, 296), presumably arising from intermolecular Michael reaction and dehydration.

ionized form at physiological pH, are expected to penetrate nerve and lens tissues very poorly,[19,25] it was desirable to examine modifications with higher pK_a. Replacement of acidity enhancing ester group by methyl, methylthio, or methylsulfonyl resulted in compounds with pK_a's ranging from 10.6 to 5.1 (Table VI). However, these compounds, including the more acidic methylsulfonyl compound, were very poor inhibitors in vitro (no significant inhibition even at 10^{-4} M). The nonacidic compound 14 was also devoid of activity (7% inhibition at 10^{-5} M).

In conclusion, EBPC is a potent aldose reductase inhibitor in vitro with unprecedented selectivity and is potentially a convenient tool for measuring relative contributions of aldose and aldehyde reductase to sorbitol accumulation in diabetic target tissues, ex vivo. Consistent with published results, we found that aldose reductase was predominant in rat lens and sciatic nerve and that aldehyde reductase was predominant in rat kidney cortex. We believe that high acidity and polarity associated with the heterocyclic pharmacophore were principal causes for lack of expected translation of potency from in vitro to in vivo among our hydroxypyrrole esters.

Experimental Section

Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. Structures of new compounds were confirmed by mass and NMR spectroscopy. NMR spectra were recorded on either Brucker (AM 300) or Varian (XL250) instruments. High-resolution mass spectra were run on a Kratos (MS 30) mass spectrometer. All new target compounds in Tables IV and VI gave satisfactory elemental analysis. Structures of intermediate aminopropionates were checked by spectral methods. X-ray data are available as supplementary material.

Ethyl 3-[(2-Thienylmethyl)amino]propionate (4, R = 2-Thienyl, X = CH_2, R_2 = H). To an ice-cold solution of 2-thiopheneylmethylamine (16.1 g, 143 mmol) in EtOH (30 mL) was gradually added ethyl acrylate (15.5 mL, 143 mmol). After this was stirred overnight, excess EtOH was removed and the resulting syrupy liquid was distilled to obtain the product (56%): bp 118–126 °C (3 mm).

Ethyl 1-(2-Thienylmethyl)-3-hydroxy-2(5*H*)-oxopyrrole-4-carboxylate (29). NaOEt, prepared by dissolving Na (2.2 g, 95.6 mmol) in EtOH (20 mL), was added to a solution of ethyl 3-[(2-thienylmethyl)amino]propionate (17.0 g, 79.7 mmol) in diethyl oxalate (11.6 g, 79.7 mmol). It was then heated on a steam bath for 1 h. The heavy precipitate obtained upon cooling in an ice bath was filtered. The precipitate was dissolved in H_2O and was acidified to pH 3 with dilute HCl to obtain a white solid. This was collected and air-dried (33%): mp 98–100 °C.

Methyl 3-(Benzylamino)-2-(methylthio)propionate (4, R = Ph, X = CH_2, R_2 = SMe). A mixture of benzylamine (5–36 g, 50 mmol), methyl 1-(methylthio)acrylate (6.6 g, 50 mmol), and EtOH (25 mL) was stirred at room temperature for 4 h. It was then distilled to obtain the product (79%): bp 117–124 °C (17 mm); ^{1}H NMR ($CDCl_3$) δ 2.06 (s, 3 H), 2.9 (m, 2 H), 3.4 (m, 1 H), 3.7 (s, 3 H), 3.8 (s, 2 H), 7.2 (s, 5 H).

1-Benzyl-3-(methylthio)-4,5-dioxopyrrolidine (45). NaOEt, prepared by dissolving Na (0.80 g, 35 mmol) in EtOH (10 mL), was added to a solution of methyl 3-(benzylamino)-2-(methylthio)propionate (5.98 g, 25 mmol) in diethyl oxalate (3.66 g, 25 mmol). The reaction was stirred overnight at room temperature. Excess EtOH was evaporated, the residue was diluted with H_2O (30 mL), the pH was adjusted to about 7.0 with dilute HCl, and the mixture was then extracted with EtOAc (2 × 20 mL). The EtOAc extract was evaporated and the solid residue was crystallized from toluene (32%): mp 168 °C; ^{1}H NMR ($CDCl_3$) δ 2.3 (s, 2 H), 3.8 (s, 2 H), 4.55 (s, 2 H), 7.2–7.5 (m, 5 H), 9.7 (s, 1 H).

6-Fluoro-3,4-dihydro-2*H*-1-benzopyran-4-carboxylic Acid Phenylmethyl Ester (7). A solution of **9** (50.0 g, 0.25 mol) in saturated aqueous $NaHCO_3$ (200 mL) was added to a solution of benzyl bromide (42.8 g, 0.25 mol) in Ch_2Cl_2 (200 mL) containing benzyltributylammonium chloride (78.2 g, 0.25 mol) and stirred overnight at room temperature. The reaction mixture was washed with H_2O (3 × 30 mL), and the CH_2Cl_2 layer was collected and evaporated to dryness. The residue was purified by flash chromatography on silica gel using CH_2Cl_2 to obtain **7** as an oil (74%): ^{1}H NMR ($CDCl_3$) δ 2.1 (m, 2 H), 3.6 (t, 1 H), 4.0 (m, 2 H), 5.0 (s, 2 H), 6.8 (m, 3 H), 7.1 (s, 5 H).

6-Fluoro-3,4-dihydro-4-[(phenylmethoxy)carbonyl]-2*H*-1-benzopyran-4-acetic Acid Ethyl Ester (8). To a suspension of sodium hydride (1.05 g, 22 mmol) in DMF (15 mL) was added a solution of **7** (5.72 g, 20 mmol) in DMF (5 mL). After 15 min ethyl bromoacetate (3.34 g, 22 mmol) was slowly added. The reaction was complete immediately after the addition. The reaction mixture was poured onto cold H_2O (50 mL): sufficient HCl was added to adjust the pH to about 4.0 and then extracted with EtOAc (2 × 25 mL). The extract was washed with H_2O, collected, dried, and then evaporated to a pale yellow oil (80%): ^{1}H NMR ($CDCl_3$) δ 1.2 (t, J = 8 Hz, 3 H), 2.2 (m, 2 H), 2.8–3.2 (m, 2 H), 4.1 (m, 4 H), 5.1 (s, 2 H), 6.9–7.2 (m, 8 H).

4-(Ethoxycarbonyl)-3,4-dihydro-2*H*-1-benzopyran-4-acetic Acid (9). To a solution of **8** (5.0 g, 13.4 mmol) in MeOH (40 mL) was added Pd–C catalyst (0.5 g) and the mixture hydrogenated in a Parr apparatus at 50 psi for 1.5 h. The mixture was filtered and the filtrate was evaporated to obtain the desired compound (80%): ^{1}H NMR ($CDCl_3$) δ 1.2 (t, J = 8 Hz, 3 H), 2.2 (m, 2 H), 2.6–2.9 (m, 2 H), 3.9–4.3 (m, 4 H), 5.1 (s, 2 H), 6.8–7.2 (m, 8 H).

6-Fluoro-3,4-dihydro-4-[[[(4-methoxyphenyl)methoxy]carbonyl]amino]-2*H*-1-benzopyran-4-acetic Acid Ethyl Ester (10). To a solution of **9** (2.82 g, 10 mmol) in benzene (75 mL) and Et_3N (1.7 mL) was added diphenyl phosphorazidate (3.30 g, 12 mmol). After the reaction mixture was stirred overnight, *p*-methoxybenzyl alcohol (1.66 g, 12.0 mmol) was added and the mixture refluxed for 4 h. The reaction mixture was cooled and washed successively with Na_2CO_3 solution (10%, 15 mL), dilute HCl (1 N, 5 mL), and H_2O (20 mL). The organic portion was collected, dried, and then chromatographed to obtain the product (82%): ^{1}H NMR ($CDCl_3$) δ 1.2 (t, J = 8 Hz, 3 H), 2.2 (m, 2 H), 2.6–3.2 (m, 2 H), 3.1 (s, 3 H), 4.1–4.3 (m, 4 H), 4.9 (s, 2 H), 5.9 (s, H), 6.6–7.3 (m, 7 H).

4-Amino-6-fluoro-3,4-dihydro-2*H*-1-benzopyran-4-acetic Acid Ethyl Ester (11). A mixture of **10** (3.3 g, 7.49 mmol), MeOH (20 mL), and Pd–C catalyst (0.5 g) was hydrogenated in a Paar apparatus at 20 psi for 1 h. The catalyst was filtered off and the

(25) Wrobel, J.; Millen, J.; Sredy, J.; Dietrich, A.; Kelly, J. M.; Gorham, B. J.; Sestanj, K. *J. Med. Chem.* **1989**, *32*, 2493.

filtrate was evaporated. The residue was extracted with EtOAc and the EtOAc layer was extracted with dilute HCl. The acidic portion was collected, basified to pH 7.0 with 10% KOH, and extracted with Et_2O. The Et_2O layer was collected, dried, and evaporated to obtain the product as an oil (14%): ^1H NMR ($CDCl_3$) δ 1.2 (t, J = 8 Hz, 3 H), 2.2 (m, 2 H), 3.2 (q, J = 8 Hz, 2 H), 4.1–4.4 (m, 4 H), 6.6–7.2 (m, 3 H).

6-Fluoro-2,3-dihydro-4',5'-dioxospiro[4*H*-1-benzopyran-4,2'-pyrrolidine]-3'-carboxylic Acid Ethyl Ester (12). To a solution of NaOEt prepared from Na (11 mg, 0.5 mmol) and EtOH (5 mL) was added a solution of 11 (75 mg, 0.3 mmol) and diethyl oxalate (58 mg, 0.4 mmol) in EtOH (2 mL). The reaction mixture was stirred overnight, and to the precipitated white solid was added sufficient 10% HCl to adjust the pH to about 2.0. The resulting solid was collected and air-dried (84%): mp 211–214 *C*; ^1H NMR ($CDCl_3$) δ 1.05 (t, J = 8 Hz, 3 H), 2.8 (m, 2 H), 4.4 (q, J = 8 Hz, 2 H), 4.55 (m, 2 H), 7.1 (m, 1 H), 7.4 (m, 2 H).

Single-Crystal X-ray Analysis of 1. A representative crystal of 1 ($C_{14}H_{15}NO_4$, MW = 261.3, D_c, X-ray = 1.32 g cm^{-3}) with appropriate dimensions 0.06 × 0.14 × 0.28 mm was surveyed and a 1-Å data set (maximum $\sin\theta/\lambda$ = 0.5) was collected on a Nicolet R3M/μ diffractometer. Atomic scattering factors were taken from ref 26. All crystallographic calculations were facilitated by the SHELXTL system.[27] All diffractometer data were collected at room temperature. Crystal parameters were as follows: cell dimensions, a = 8.475 (5) Å, b = 13.741 (4) Å, c = 11.96 (1) Å, α = 90.00°, β = 109.46 (5)°, γ = 90.00°; space group $P2_1/a$; molecules/unit cell, 4 and linear absorption factor = 7.70 cm^{-1}. Refinement parameters were as follows: number of reflections, 1342 nonzero reflections ($I > 3.0\sigma$), 1033; R index, 0.065; GOF, 1.08; scale factor, 1.883 (7); secondary extinction factor, none. A trial structure was obtained by direct methods. This trial structure refined routinely. Hydrogen positions were calculated wherever possible. The methyl hydrogens and the hydrogen on oxygen were located by different Fourier techniques. The hydrogen parameters were added to the structure factor calculations but were not refined. The shifts calculated in the final cycle at least squares refinement were all less than 0.1 of their corresponding standard deviations. The final R index was 0.065. The weighted R index was 0.0834. A final difference Fourier revealed no missing or misplaced electron density.

The refined structure was plotted by using the SHELXTL plotting package (Figure 1). Coordinates, anisotropic temperature factors, distances, angles, and packing diagrams are available as supplementary material. Packing diagrams revealed that the hydrogen on the hydroxyl group was hydrogen bonded to the carbonyl group of a symmetrically related molecule. The hydroxyl group of the symmetry related molecule was in turn hydrogen bonded to the carbonyl group of the first molecule.

Biological. Aldose reductase from rat lens and aldehyde reductase from rat kidney were purified by a modification of a published procedure.[4] The enzymes were fractionated by a 40%–70% ammonium sulfate precipitation, and the digitonin wash was omitted. Purity was assessed by the presence of a single major staining polypeptide after analysis of an overloaded polyacrylamide gel electrophoresed under denaturing and reducing conditions. Aldehyde reductase purified to homogeneity from pig kidney was provided as a generous gift from Dr. T. Geoffrey Flynn (Queen's University, Ontario, Canada).

Tissues were homogenized (10% w/v) with a glass Teflon homogenizer, in 0.1 M potassium phosphate buffer, pH 7.2, containing 5 mM 2-mercaptoethanol and centrifuged at 40000g for 30 min at 4 °C. The resulting supernatant was chromatographed on DEAE-cellulose column employing a linear salt gradient (0–0.5 M NaCl). Peak fractions were pooled and aliquots stored frozen.

Aldose and aldehyde reductases (unless otherwise indicated) were assayed in 0.25 mL of 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM NADPH and 5 mM 3-pyridinecarboxaldehyde. Enzyme assays were initiated with substrate, and the rate of NADPH disappearance at 340 nm was monitored for 12 min at 25 °C.

In vitro screening using human placental aldose reductase was conducted as follows. Aldose reductase from human placentae was partially purified by a modification of a literature procedure.[4] Freshly obtained human placentae were homogenized in 3 volumes of 0.1 M potassium phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol and centrifuged for 20 min at 33000g at 4 °C. The supernatant was subjected to a 50%–75% ammonium sulfate fractionation, and the resulting pellets were pooled, resuspended in a minimum volume of buffer, and dialyzed overnight. The dialysate was chromatographed on a DEAE-cellulose column (2 cm × 25 cm) and aldose reductase was eluted with a linear salt gradient (0–1 M NaCl). Peak fractions containing aldose reductase activity were pooled and aliquots stored frozen. Enzyme activity was assayed by using an Abbot VP bichromatic clinical analyzer which measured the decrease in the rate of NADPH oxidation at 340 nm at 25 °C over 10 min in a reaction mixture of 0.25 mL of 50 mM potassium phosphate buffer (pH 7.1) containing 0.4 M ammonium sulfate, 0.067 mM NADPH, and 1.0 mM *dl*-glyceraldehyde. Sufficient enzyme was added to produce a rate of NADPH oxidation equal to 4 milliunits (unit equal to 1 μmol of NADPH oxidized at 25 °C/min). IC_{50}s were calculated by using a log linear regression analysis.

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Registry No. 1, 4450-98-0; 4, 131436-59-4; 6, 76301-93-4; 7, 131436-60-7; 8, 131436-61-8; 9, 131436-62-9; 10, 131436-63-0; 11, 131436-64-1; 12, 131436-65-2; 15, 131436-66-3; 16, 131436-67-4; 17, 131436-68-5; 18, 131436-69-6; 19, 131436-70-9; 20, 131436-71-0; 21, 96124-43-5; 22, 131436-72-1; 23, 26027-68-9; 24, 131436-73-2; 24·Na, 131436-93-6; 25, 131436-74-3; 26, 131436-75-4; 27, 131436-76-5; 28, 131436-77-6; 29, 131436-78-7; 30, 131436-79-8; 31, 131436-80-1; 32, 131436-81-2; 33, 131436-82-3; 33·Na, 131436-94-7; 34, 131436-83-4; 35, 131436-84-5; 36, 131436-85-6; 37, 131436-86-7; 38, 4661-35-2; 38·Na, 131436-95-8; 39, 57056-57-2; 40, 131436-87-8; 41, 131436-88-9; 42, 4563-90-0; 43, 58486-00-3; 44, 7151-49-7; 45, 131436-89-0; 46, 131436-90-3; 47, 131436-91-4; 2-thiopheneylmethylamine, 27757-85-3; ethyl acrylate, 140-88-5; diethyl oxalate, 95-92-1; benzylamine, 100-46-9; methyl 1-(methylthio)acrylate, 131436-92-5; ethylbromoacetate, 105-36-2; diphenyl phosphorazidate, 26386-88-9; *p*-methoxybenzyl alcohol, 105-13-5; aldose reductase, 9028-31-3.

Supplementary Material Available: Crystal packing diagram and Tables S1–S5 listing atomic coordinates, isotropic and anisotropic thermal parameters, bond lengths and angles, and H atom coordinates (6 pages). Ordering information is given on any current masthead page.

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