Selective Irreversible Inhibitors of Aldose Reductase

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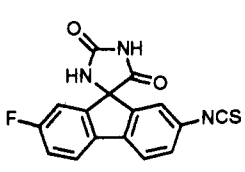
A series of 5-substituted-1,3-dioxo-1H-benz[*de*]isoquinoline-2(3*H*)-acetic acid analogues have been examined as irreversible inhibitors of aldose reductase. The 5-α-bromoacetamide and 5-α-iodoacetamide analogues **5** and **6** gave irreversible inhibition of aldose reductase while the 5-α-chloroacetamide analogue **3** did not show this type of inhibition. Protection studies indicate that irreversible inhibitions are occurring at the inhibitor binding site. Comparative irreversible inhibition studies with rat lens aldose reductase (RLAR) and rat kidney aldehyde reductase (RKALR) indicate that 5-α-haloacetamide analogues **5** and **6** are much more effective inhibitors of RLAR.

Introduction

Despite increasingly sophisticated treatments for diabetes mellitus, diabetics often suffer the eventual development of tissue-damaging complications which are largely responsible for the morbidity and mortality of diabetes.[1] The average lifespan of diabetics is only two-thirds that of nondiabetics.[2] Complications associated with diabetes are wide-ranging and include retinopathy, neuropathy, cataract, keratopathy, nephropathy, and angiopathy.[3-5] Although the mechanism(s) through which hyperglycemia causes tissue damage are unknown, mounting experimental evidence indicates that increased aldose reductase [EC 1.1.1.21] activity initiates biochemical changes which result in the onset of some secondary diabetic complications.[6] Aldose reductase is an enzyme in the polyol pathway which utilizes NADPH to catalyze the reduction of glucose to the sugar alcohol sorbitol (Figure 1). Experimental evidence indicates that inhibition of aldose reductase represents a possible approach to the treatment of some of the secondary complications of diabetes.[7]

Attempts at producing clinically useful aldose reductase inhibitors have led to the identification of large numbers of structurally diverse compounds with aldose reductase inhibitory activity.[7,8] Some of these compounds are illustrated in Chart I. Invariably, aldose reductase inhibitors have been shown to act as either noncompetitive or uncompetitive inhibitors of aldose reductase, suggesting the existence of three distinct binding sites on the enzyme: the substrate binding site, the nucleotide cofactor fold, and the inhibitor binding site.[8] Competition studies using aldose reductase inhibitors of different structural types suggest that these inhibitors bind to a common site.[9] Using the protein modifying reagents phenylsulfonyl fluoride and 2-bromo-4′-nitroacetophenone, the existence of a nucleophilic residue, possibly tyrosine, in or near the inhibitor binding site has been demonstrated.[10]

To exploit the possible existence of a nucleophilic residue within the inhibitor binding site, a series of affinity labels **1–6**, based on the reversible inhibitors alconil, alrestatin, and sorbinil, have been synthesized and examined



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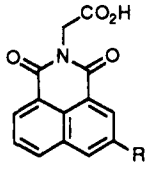

2: R = NCS
3: R = $COCH_2Cl$
5: R = $COCH_2Br$
6: R = $COCH_2I$
10: R = $COCH_3$

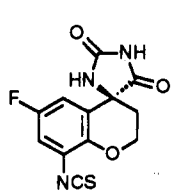

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Chart I

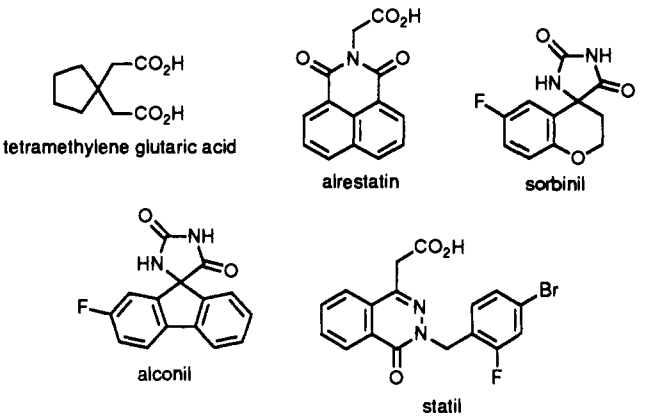

Table I. Inhibition of Rat Lens Aldose Reductase (RLAR) and Rat Kidney Aldehyde Reductase (RKALR)

	IC_{50} (μM)		
compd	RLAR (mean ± SD)	RKALR (mean ± SD)	RKALR/RLAR
10	0.64 ± 0.02	6.4 ± 1.6	10
3	0.55 ± 0.04	10.4 ± 1.4	19
5	0.60 ± 0.05	8.0 ± 1.1	13
6	0.40 ± 0.02	6.5 ± 1.6	16

for aldose reductase inhibitory activity.[11-13] The IC_{50} values for the parent 5-acetamide and 5-haloacetamides

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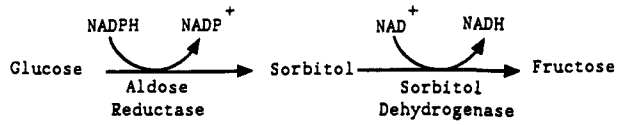


Figure 1.

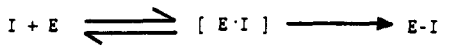


Figure 2.

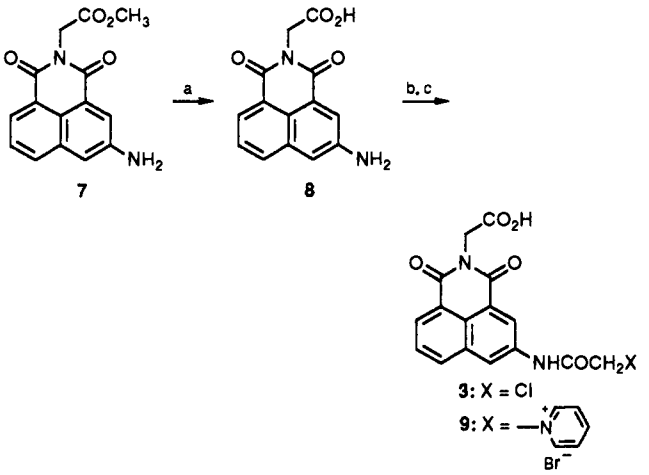
are given in Table I. As shown, all of the compounds have very similar IC_{50} values, indicating that the increased steric bulk of the halogens had little effect on the interactions of the inhibitor with the inhibitor binding site.

As illustrated in Figure 2, affinity labeling is a two-step process.[14] In the first step, an equilibrium between free enzyme, inhibitor, and the enzyme–inhibitor complex is rapidly established while the second step leads to alkylation and irreversible inhibition of the enzyme. This second step is dependent upon both the chemical reactivity of the electrophile and the spatial relationship established, after binding, between the electrophile and the nucleophile of the enzyme.[14] Therefore, the extent of enzyme alkylation is dependent upon three criteria: the affinity of the label for the enzyme, the chemical reactivity of the electrophile of the label, and the spatial relationship established, after binding, between the electrophile of the affinity label and the nucleophile of the enzyme.

The similar IC_{50} values of the isothiocyanato (0.3 μM)[12] and the chloroacetamido (0.5 μM) analogues of alrestatin (2 and 3, respectively) suggest that these analogues have similar apparent affinities for aldose reductase and that the structural differences between the isothiocyanate and chloroacetamide groups have little or no effect on the apparent affinities. The poor irreversible inhibitor activity of 3, relative to 2, may be due to either a lack of sufficient chemical reactivity of the chloroacetamide of 3, relative to the isothiocyanate of 2, or a difference in the spatial relationships between these two electrophiles relative to the nucleophile on the enzyme. To gain further insight into this observed difference in irreversible binding, the chemically more reactive but structurally similar bromo- and iodoacetamide analogues of 3, namely 5 and 6, respectively, have been synthesized and tested for their ability to irreversibly inhibit rat lens aldose reductase.

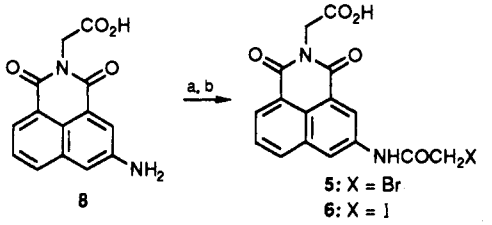
Recent studies indicate that many aldose reductase inhibitors are nonspecific and that they also inhibit the closely related enzyme aldehyde reductase (EC 1.1.1.20).[7,15,16] The primary sequence of aldose and aldehyde reductase are quite similar.[17,18] Because of the sequence homology and the nonspecificity of inhibition, it has been suggested that the inhibitor binding site of both enzymes are structurally similar.[18] Therefore, compounds 3, 5, and 6 were assayed for their ability to reversibly and irreversibly inhibit aldehyde reductase to determine structural similarities/differences between the inhibitor binding sites of both enzymes.

Scheme I[a]



[a] (a) KOH, MeOH/CH_3CN; (b) $O(COCH_2X)_2$ DMAP, Pyr, 0 °C; (c) dilute HX (X = Cl or Br).

Scheme II[a]



[a] (a) $O(COCH_2X)_2$ (X = Br or I), DMAP, THF, 0 °C; (b) H_2O.

Chemistry

The synthesis of chloroacetamide 3 is shown in Scheme I. The synthesis of 3 from methyl ester 7 has been previously described in the literature.[11,13] However, the previously reported procedure required the cautious hydrolysis of the methyl ester of 3. Although this procedure proved useful for the synthesis of 3, an alternate procedure was sought which would avoid exposing the more reactive bromo- and iodoacetamide functional groups to such conditions. Therefore, the methyl ester was hydrolyzed before acetamide formation. Reaction of amino acid 8 with chloroacetic anhydride and 4-(dimethylamino)pyridine in pyridine at 0 °C for 1 h gave, after workup, the desired product, 3. When these reaction conditions were extended to the synthesis of bromoacetamide 5, however, only the pyridinium bromide acetamide 9 could be isolated. Shortening of the reaction time to 15 min and lowering the

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Table II. Irreversible Inhibition of Rat Lens Aldose Reductase (RLAR) and Rat Kidney Aldehyde Reductase (RKALR) by 5-Substituted Alrestatin Analogues

compd	% inhibn at 10^{-4} M	
	RLAR	RKALR
2	56	0
3	0	0
5	46	0
6	89	11

Table III. Protection by 10 of the Irreversible Inhibition of Rat Lens Aldose Reductase (RLAR) by 6

concn of 6 (mM)	% protection of 10		
	0.01 mM	0.1 mM	1 mM
0.1	17	19	39
0.01	11	43	100

reaction temperature to −20 °C produced a 3:2 mixture of the desired bromoacetamide **5** versus the pyridinium acetamide **9**. Thus, although pyridine proved to be a poor choice of solvent for the synthesis of **5**, the greater chemical reactivity of the bromoacetamide relative to the chloroacetamide is clearly evident. THF proved to be a useful solvent for the synthesis of **5** and **6** as shown in Scheme II.

Biological Evaluation

Evaluation of haloacetamides **3**, **5**, and **6** and nonhalogenated acetamide **10** for their ability to inhibit purified rat lens aldose reductase as compared to rat kidney aldehyde reductase in vitro revealed that aldose reductase was inhibited to a greater extent than aldehyde reductase. No significant difference in inhibition, expressed as IC_{50} values for reversible inhibition, could be observed between the haloacetamide analogues **3**, **5**, and **6** and the parent **10** with either enzyme (Table I); however, inhibition of aldose reductase was ca. 10–19-fold greater than that of aldehyde reductase. Although similar IC_{50} values were observed, analogues **3**, **5**, and **6** demonstrate differences with respect to irreversible inhibition (Table II). Compared to the isothiocyanate analogue **2**, where 0.1 mM resulted in 56% irreversible inhibition, a progressive increase in irreversible inhibition was observed with 0.1 mM of these inhibitors, with no inhibition observed with the chloro analogue **3**, 46% with bromo analogue **5**, and 89% with iodo analogue **6** (Table II). Similar studies with aldehyde reductase resulted in no inhibition with either isothiocyanate analogue **2** or the chloro (**3**) or bromo (**4**) analogue and only slight (11%) inhibition of aldehyde reductase with iodo analogue **6**.

These studies indicate that the extent of irreversible inhibition is directly linked to the chemical reactivity of the haloacetamide group and its ability to undergo nucleophilic attack. Time course studies revealed that irreversible inhibition as a result of an apparent S_N2 attack occurred rapidly (Figure 3). Since the aldose reductase protein contains a number of reactive residues, protection studies were conducted to investigate whether irreversible inhibition resulting from the chemical "reactivity" of the haloacetamide analogue was specific for the inhibitor site. These studies, summarized in Table III, demonstrate that irreversible inhibition resulted from a specific interaction with a nucleophile at the aldose reductase inhibitor binding site. It is noteworthy that the enzyme may be completely protected from irreversible inhibition through the use of a 100-fold excess of reversible inhibitor **10**. These studies confirm the premise that aldose reductase contains a reactive nucleophile in the inhibitor binding site, and demonstrates the utility of iodoacetamide analogue **6** as a selective affinity label for the enzyme aldose reductase relative to aldehyde reductase.

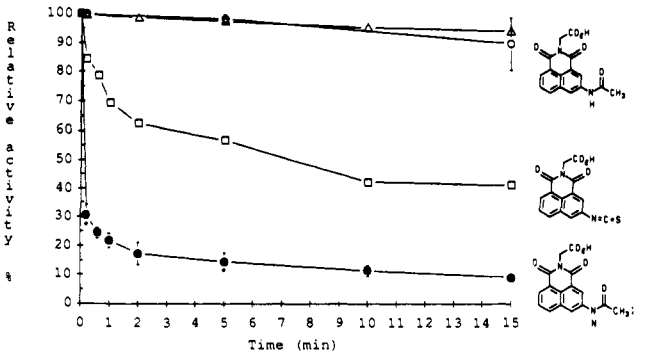

Figure 3. Irreversible inhibition of rat lens AR: control (○), acetamide (△), isothiocyanate (□), iodoacetamide (●).

Experimental Section

Melting points are uncorrected and were determined with a Thomas-Hoover melting point apparatus. IR spectra were obtained with a Beckman 4230 infrared spectrophotometer. NMR spectra were obtained with a IBM NR/250 FTNMR (250 MHz). Mass spectra were obtained with either a Kratos MS25RFA or a Kratos MS-30 mass spectrometer. Anhydrous THF was produced by distillation over sodium using benzophenone as an indicator for dryness. Dry pyridine was produced by distillation over solid KOH and was stored over type 4A molecular sieves. Any reference to THF or pyridine refers to the solvents dried according to the procedures given above. Chemical analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN). All analytical results for the indicated elements were within ±0.4% of the theoretical values.

5-Amino-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2(3*H*)-acetic Acid (8). To a suspension of **7**[12] (1 g, 3.5 mmol) in 50 mL of a 1:2 mixture of MeOH/MeCN was added 10 mL of 30% aqueous KOH. The reaction was allowed to continue with stirring at room temperature for 2 h. During the reaction the starting material passed into solution. Upon completion of the reaction the organics were removed under reduced pressure, and the resulting basic solution was acidified to pH 5 with 10% HCl. The resulting suspension was allowed to stand for 5 min, and the solids were collected by filtration. The solids were dried in vacuo and recrystallized from MeOH to yield 0.85 g (90%) of **8** as a yellow solid: mp 290–291 °C (lit.[18] mp 292 °C).

5-(Chloroacetamido)-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2(3*H*)-acetic Acid (3). To a suspension of **8** (0.3 g, 1.1 mmol) in 10 mL of dry pyridine at 0 °C was added a mixture of chloroacetic anhydride (1.95 g, 5.5 mmol) and 5 mg of 4-(dimethylamino)pyridine (DMAP) in 2 mL of anhydrous THF. The reaction was allowed to continue for approximately 1 h with stirring at 0 °C. Upon completion of the reaction, 100 mL of water was added and the mixture allowed to stir at room temperature for an additional 20 min. CH_2Cl_2 (100 mL) was added and the aqueous layer was acidified with 10% HCl. The neutralized acid **3** fell out of solution, but was not soluble enough in CH_2Cl_2 or other organic solvents to allow for efficient extraction. Solid **3** formed at the organic/water interface. The organic layer was separated, dried ($MgSO_4$), filtered, and evaporated at reduced pressure to yield a solid. The solids remaining in the aqueous layer were collected by filtration and added to the solids collected from the organic phase. The combined solids were dried in vacuo and recrystallized from acetone to yield 0.255 g (67%) of **3** as a light yellow solid: mp 250–251 °C (lit.[11,13] mp 251 °C).

5-(Bromoacetamido)-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2(3*H*)-acetic Acid (5). To a suspension of **8** (0.3 g, 1.1 mmol) in 10 mL of anhydrous THF at 0 °C was added a mixture of bromoacetic anhydride (1.44 g, 5.5 mmol) and 5 mg of DMAP in a small amount of THF. The mixture was allowed to stir at 0 °C until the reaction was judged to be complete, approximately 1 h, after which time 20 mL of water was added. This mixture was allowed to stir at room temperature for an additional 20 min. The resulting mixture was extracted with a large volume of EtOAc

(3 × 200 mL). The EtOAc fractions were combined, dried ($MgSO_4$), filtered, and evaporated under reduced pressure to yield a light yellow solid. A small amount of **5** collected at the water/organic interface. These solids were collected by filtration and added to the solids isolated by extraction. The combined solids were recrystallized from acetone to yield 0.375 g (87%) of **5** as a light yellow solid: mp 259–260 °C dec; ^1H NMR (DMSO-d_6, 250 MHz) δ 13.1 (br s, 1 H, D_2O exchangeable), 11.03 (s, 1 H, D_2O exchangeable), 8.79 (d, J = 1.9 Hz, ArH), 8.6 (d, J = 1.9 Hz, 1 H, ArH), 8.44 (d, J = 7.7 Hz, 1 H, ArH), 7.84 (t, J = 7.7 Hz, ArH), 4.72 (s, 2 H, CH_2COOH), 4.15 (s, 2 H, CH_2Br); IR (KBr, cm^{-1}) 3600–2700 (COOH); MS (m/e) 391.983) M^+, ^{81}Br), 390.0005 (theoretical 389.985) M^+, ^{79}Br). Anal. ($C_{16}H_{11}BrN_2O_5$) C, H, N.

5-(Iodoacetamido)-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2-(3*H*)-acetic Acid (6). A solution of iodoacetic anhydride (0.654 g, 1.85 mmol) and 5 mg of DMAP in a small amount of dry THF was added to a stirred suspension of **7** (0.1 g, 0.37 mmol) in dry THF at 0 °C. The mixture was allowed to stir at 0 °C until the reaction was judged to be complete, approximately 1 h, at which time 10 mL of water was added. This mixture was allowed to stir for an additional 15 min. The resulting mixture was extracted with a large volume of EtOAc (3 × 100 mL). The combine extracts were dried ($MgSO_4$), filtered, and evaporated under reduced pressure to provide a light yellow solid. The small amount of solid that collected at the organic/water interface was collected by filtration and combined with the solids obtained through extraction. The combined solids were recrystallized from acetone to yield 0.13 g (80%) of **6** as a light yellow solid: mp 222–223 °C dec; ^1H NMR (DMSO-d_6, 250 MHz) δ 13.1 (br s, 1 H, D_2O exchangeable), 10.97 (s, 1 H, D_2O exchangeable), 8.77 (d, J = 2.0 Hz, 1 H, ArH), 9.58 (d, J = 2.0 Hz, 1 H, ArH), 8.47–8.41 (m, 2 H, ArH), 8.38 (t, J = 7.8 Hz, 1 H, ArH), 4.72 (s, 2 H, CH_2 COOH), 3.91 (s, 2 H, CH_2I); IR (KBr cm^{-1}) 3650–2700 (COOH); MS (m/e) FAB 439 (M^+ + H). Anal. ($C_{16}H_{11}IN_2O_5$) C, H, N.

Purification of Enzymes. Rat lens aldose reductase (RLAR) and rat kidney aldehyde reductase (RKALR) were purified to apparent homogeneity through a series of steps which included gel filtration on Sephadex G-75, affinity chromatography on Amicon Matrex Gel Orange A, and chromatofocusing on Pharmacia Mono P as previously decribed.[16,20] Both enzymes appeared as single bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of Reductase Activity. Reductase activity was spectrophotometrically assayed on a Guilford Response spectrophotometer or a Shimazu UV2100U spectrophotometer by following the decrease in the absorption of NADPH at 340 nm over a 4-min period with DL-glyceraldehyde as substrate.[16] Each 1.0-mL cuvette contained equal units of enzyme, 0.10 M Na,K phosphate buffer (pH 6.2), 0.3 mM NADPH with/without 10 mM substrate, and inhibitor. One unit of enzyme activity was defined as that activity consuming or producing 1 μmol of NADPH per min at 22 °C. Appropriate controls were employed to negate potential changes in the absorption of nucleotide and/or affinity label or aldose reductase inhibitors at 340 nm in the absence of substrate.

Irreversible Inhibition Assay. Rat lens aldose reductase or rat kidney aldehyde reductase was passed through either a PD-10 or a NAP-5 desalting column (Pharmacia LKB, Piscataway, NJ) equilibrated with 0.1 M phosphate buffer (pH 7.4) to remove glycerol and 2-mercaptoethanol. Enzyme (ca. 0.3 unit/mL) was then added to test tubes containing only 0.1 M phosphate buffer (pH 7.4) (control) or the buffer and either the reversible aldose reductase inhibitor or its irreversible inhibitor analogue, and the reaction was allowed to proceed at room temperature for 15 min. Unreacted reagent was then removed from a 400-μL aliquot of the reaction mixture by passing it through the desalting column with 0.1 M phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol, and 50 μL of 1 mg/mL BSA solution was immediately added to the eluant to stabilize the reductase. The eluant was then kept on ice until the residual reductase activity was measured. All experiments were conducted at least in triplicate with the average values taken.

Protection Studies. Protection studies with irreversible inhibitors were essentially conducted as described above. In addition to the above reactions, test tubes were prepared containing 0.025, 0.25, and 2.5 mM of the reversible inhibitor 5-acetamido-1,3-dioxo-1*H*-benz[*de*]isoquinoline-2(3H)-acetic acid (**10**). After a 5-min incubation, the irreversible inhibitor analogue was added to each appropriate enzyme mixture, and the mixtures were incubated an additional 5 min prior to filtration.

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