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INHIBITORY ACTIVITY AND MECHANISM OF INHIBITION OF THE *N*-[[(4-BENZOYLAMINO)PHENYL]SULFONYL]AMINO ACID ALDOSE REDUCTASE INHIBITORS

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Abstract—A series of substituted *N*-[[(4-benzoylamino)phenyl]sulfonyl]amino acids (BAPS-amino acids) were synthesized by established methods, and the stereochemistry of the products was confirmed by HPLC analysis after chiral derivatization. When tested against aldose reductase (alditol:$NADP^+$ oxidoreductase; EC 1.1.1.21; ALR2) isolated from rat lens, all of the BAPS-amino acids were determined to be significantly more inhibitory than the corresponding *N*-(phenylsulfonyl)amino acids. Structure–inhibition and enzyme kinetic analyses suggest that the BAPS-amino acids inhibit ALR2 by a mechanism similar to the *N*-(phenylsulfonyl)amino acids. However, multiple inhibition analyses indicate that the increased inhibitory activity of the BAPS-amino acids is a result of interaction with multiple sites present on ALR2. Enzyme specificity studies with several of the BAPS-amino acids demonstrated that these compounds do not produce significant inhibition of other nucleotide-requiring enzymes including aldehyde reductase (alcohol: $NADP^+$ oxidoreductase; EC 1.1.1.2; ALR1).

The role of the enzyme aldose reductase (alditol: $NADP^+$ oxidoreductase; EC 1.1.1.21; ALR2) in the pathogenesis of chronic diabetic complications such as retinopathy, neuropathy and nephropathy has been detailed in numerous publications over the past decade [1–3]. This relationship has stimulated a significant effort to identify inhibitors of aldose reductase and determine the ability of these compounds to prevent or delay diabetic pathologies in test animals and human diabetics [4–6]. In earlier publications, we reported the synthesis and aldose reductase inhibitory activity of a variety of *N*-(phenylsulfonyl)amino acids. In these studies it was observed that *N*-phenylglycine derivatives such as **2** (Chart 1) were somewhat more inhibitory than the corresponding glycine analogues (**1**) [7, 8]. It was also noted that in the 2-phenylglycine series (**3**) the *S*-enantiomers produce substantially greater inhibition than the corresponding *R*-isomers, and slightly more inhibition than the corresponding glycines (**1**) [7]. Finally, in the *N*-(phenylsulfonyl)glycine series (**1**) optimal inhibitory activity was observed in those derivatives with a 4-benzoylamino substituent [9]. These observations prompted the present study of a number of substituted *N*-[[(4-benzoylamino)-phenyl]sulfonyl]amino acids (BAPS-amino acids) (**4–6**) (Chart 1) to investigate the combined effect of 4-benzoylamino and *N*- or 2-phenyl substitution on inhibitory activity as well as the mechanism of inhibition.

MATERIALS AND METHODS

Materials. The chemicals and reagents employed for the syntheses of the substituted BAPS-amino acids were obtained from the Aldrich Chemical Co. (Milwaukee, WI) except 4-nitrobenzenesulfonyl chloride and *N*-phenylglycine which were purchased from Eastman Kodak (Rochester, NY). All synthetic reagents were used as received. The cofactor NADPH Type I and D,L-glyceraldehyde were purchased from Sigma (St. Louis, MO). All cofactor, substrate and inhibitor stock solutions were prepared immediately prior to use.

Inhibitors. All of the substituted BAPS-amino acids were synthesized using the same general synthetic procedure. Reaction of commercially available 4-nitrobenzenesulfonyl chloride with glycine in aqueous NaOH yielded the intermediate *N*-[[(4-nitro)phenyl]sulfonyl]glycine which was reduced to the corresponding amine by catalytic hydrogenation (H_2/Pd-C) on a Parr Hydrogenator. Treatment of this amine with commercially available substituted benzoyl chlorides in solvent mixtures of tetrahydrofuran (THF), chloroform or dichloromethane and aqueous sodium bicarbonate or 20% aqueous potassium carbonate afforded the product BAPS-glycines (**4**). The BAPS-*N*-phenylglycines (**5**) were prepared by reaction of 4-nitrobenzenesulfonyl chloride with *N*-phenylglycine, followed by reduction and acylation as described above. The enantiomeric BAPS-2-phenylglycines (**6**) were synthesized by reaction of 4-nitrobenzenesulfonyl chloride with commercially available (*R*)- or (*S*)-2-phenylglycine in a solvent mixture of THF and aqueous sodium hydroxide, followed by reduction and acylation. All products were purified by recrystallization from mixtures of aqueous ethanol, and their structures were established by infrared and proton NMR spectroscopy. The purity of each product was determined by C, H and N elemental analysis (Atlantic Microlabs, Atlanta, GA) which demonstrated that all products have C, H, and N percentages within 0.4 of theoretical percentages.

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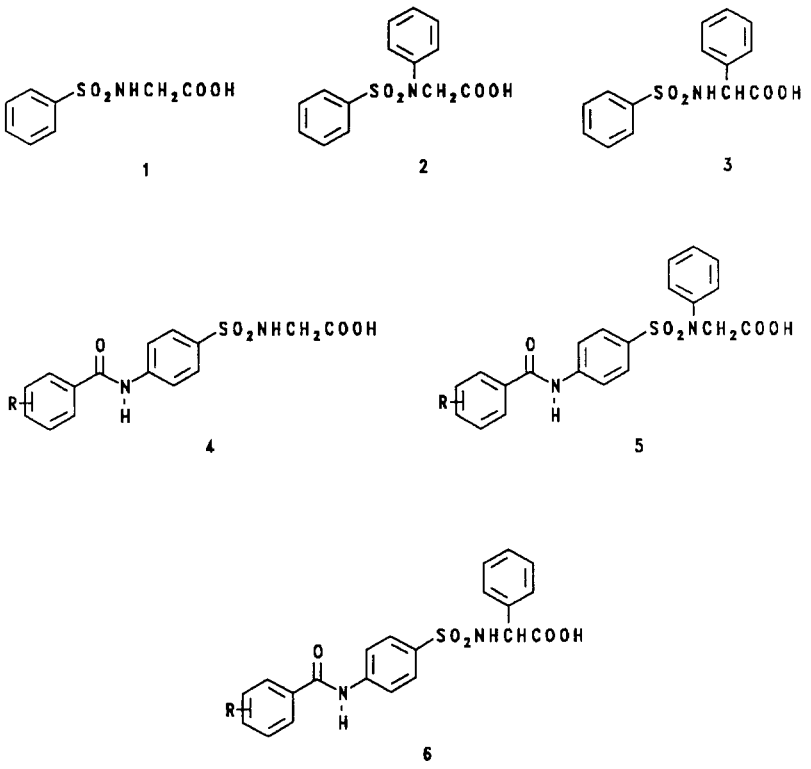


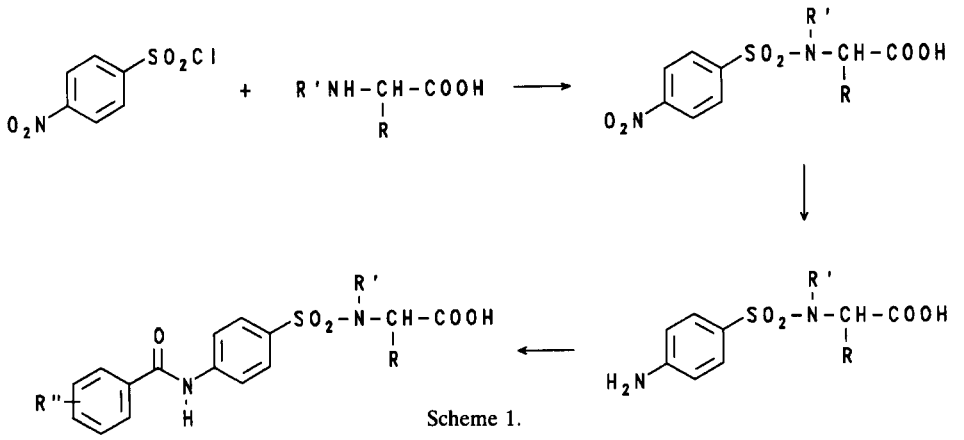
Chart 1.

Enantiomeric analysis. The enantiomeric purity of the intermediate amines and BAPS-2-phenylglycines (**6**) was established using the chiral derivatization/HPLC analysis method of Clark and Barksdale [10]. The derivatization process involved conversion of a sample of the intermediate (*R*)- and (*S*)-*N*[[(4-amino)phenyl]sulfonyl]-2-phenylglycines and BAPS-2-phenylglycine products (**6**) to the corresponding acid chlorides by treatment with thionyl chloride in benzene. Reaction of these acid chlorides with (*R*)-α-methylbenzylamine then afforded amide derivatives which were analyzed by HPLC. A modular isocratic system including a Waters Associates 6000A pump (Milford, MA), U6K injector and a 440 absorbance detector was used for these analyses. A normal phase analytical column (150 × 4.6 mm i.d.) packed with Hypersil 5 Silica (Phenomenex, Ranchos Palos Verdes, CA) was used as the stationary phase of for the separations. The mobile phase consisted of mixtures of HPLC grade chloroform and methanol, chloroform and ethanol, and chloroform and acetonitrile at flow rates of 1.5 mL/min. The UV detector was operated at 254 nm and 1.0 AUFS. The separations were carried out at ambient temperature, and the column void volume was determined using toluene.

Enzyme isolation and assay. ALR2 was isolated from rat lenses (Charles River Professional Services, Wilmington, MA) as described previously [11]. Enzyme activity was measured spectrophotometrically at 30° by determining the decrease in absorbance at 340 nm in a Shimadzu UV-160 spectrophotometer equipped with a thermo-controlled, multicell positioner. The control reaction mixtures contained 0.104 mM NADPH in 0.1 M phosphate buffer (pH 6.2), 0.2 mL of enzyme solution, and 0.2 mL of substrate in a total volume of 2.0 mL. A blank consisting of all reagents except substrate was used to correct for any oxidation of NADPH not associated with reduction of substrate. Substrates used for the reactions included 10 mM glyceraldehyde, 0.01 mM 4-nitrobenzaldehyde, 100 mM glucose, 50 mM galactose or 50 mM xylose. All enzymatic reactions were initiated by the addition of substrate solution and were monitored for 3 min following a 45-sec incubation period. Enzyme activity was adjusted by dilution with distilled water such that 0.2 mL of enzyme solution gave an average reaction rate for the control reactions of 0.0120 ± 0.002 absorbance units/min.

Effects of inhibitors. The inhibitory activities of the BAPS-amino acids **4**, **5** and **6** were determined by including 0.2 mL of an aqueous solution of each compound at the desired concentration in the reaction mixtures. For IC_{50} determinations, each inhibitor was tested at no fewer than four different concentrations with a minimum of two determinations at each concentration. The percent inhibition for each inhibitor was calculated at all concentrations by comparing the rate of reactions containing inhibitor to control reactions with no inhibitor. Inhibitor IC_{50} values were then obtained by least squares analyses of the linear portion of the log concentration–response curves using the LINEFIT program of Barlow [12].

Kinetic studies. Kinetic analyses with **1**, **4d** and **5a**



Scheme 1.

were conducted using a minimum of four concentrations of each inhibitor (see Figs. 2–4). For substrate kinetics, the concentrations of D,L-glyceraldehyde ranged from 5.0 to 0.078 mM, and the concentration of cofactor was held constant at 0.104 mM. For cofactor kinetics, the concentrations of NADPH were varied from 3.25 to 105 μM and the substrate concentration held constant at 10 mM. The nature of inhibition produced by each concentration of inhibitor was determined by analysis of double-reciprocal plots of enzyme velocity versus D,L-glyceraldehyde or NADPH concentration as generated by least squares fit of the data using the program of Barlow [12].

Multiple inhibition analyses. Multiple inhibition analyses were performed as outlined by Semenza and von Balthazar [13]. The rates of inhibited reactions (v_i) were measured with varying concentrations of one inhibitor (I_1) in the presence of different, fixed concentrations of a second inhibitor (I_2) at constant substrate and cofactor concentrations. Control reaction rates were also measured in which no inhibitor was present to generate an uninhibited velocity (v_0). These data were then used to construct Yonetani–Theorell (v_0/v_i versus [I]) plots [14, 15]. The superimposed Yagi–Ozawa plots (v_0/v_i versus $[I_1]$ at $I_2 = n[I_1]$) in Figs. 4–6 were constructed from data in which the fixed concentrations of one inhibitor were varied by the same factor as the increments of the other inhibitors [16]. Those concentrations of inhibitor which did not give distinguishably separate lines due to a lack of significant differences in measured rates are not shown. All linear plots of data were generated by least squares fit.

Enzyme specificity studies. The inhibitory potential of the BAPS-amino acids versus other adenine nucleotide-requiring reductases and dehydrogenases were determined using published procedures. Aldehyde reductase (alcohol:NADP$^+$ oxidoreductase; EC 1.1.1.2, ALR1) was isolated from rat kidney and assayed as described by Sato *et al.* [17] and glutathione reductase (oxidized glutathione:NADPH oxidoreductase; EC 1.6.4.2; GR) was obtained from Sigma (Type III, Bakers yeast) and assayed by the method of Carlberg and Mannervik [18]. Succinic semialdehyde reductase (succinate semialdehyde:NAD$^+$ oxidoreductase; EC 1.2.1.16; SSDH) was purchased from Sigma (GABASE from *Pseudomonas fluorescens*) and assayed by the method of Anlezark and coworkers [19]. Lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase; EC 1.1.1.27; LDH) was purchased from Sigma (Type I, rabbit muscle) and assayed by the method of Eichner [20].

RESULTS AND DISCUSSION

All of the substituted BAPS-amino acids were prepared using a common synthetic method (Scheme 1). Reaction of 4-nitrobenzenesulfonyl chloride with glycine, *N*-phenylglycine, (*S*)-2-phenylglycine or (*R*)-2-phenylglycine in aqueous base afforded the intermediate *N*-[[(4-nitro)phenyl]sulfonyl]amino acids. These intermediates were reduced catalytically to yield the corresponding amino intermediates which were acylated with commercially available benzoyl chlorides to give the desired products **4**–**6**. The enantiomeric purity of the intermediate *N*-[[(4-amino)phenyl]sulfonyl]-2-phenylglycines and BAPS-2-phenylglycine products (**6**) was determined using a chiral derivatization/HPLC method reported by Clark and Barksdale [10]. The derivatization process involved conversion of each BAPS-2-phenylglycine analogue to the corresponding acid chloride, followed by treatment with (*R*)-α-methylbenzylamine to yield diastereomeric amides. With this method, 2-phenylglycines which racemized upon formation would yield a pair of diastereomeric amides (*S*,*R* and *R*,*R*-amides) when derivatized, and these diastereomers could be separated by HPLC using an achiral stationary phase. If racemization did not occur, then each BAPS-2-phenylglycine product (**6**) would yield only a single diastereomeric amide upon derivatization—the *S*,*R*-amide from the (*S*)BAPS-2-phenylglycine products and the *R*,*R*-amide from the (*R*)-BAPS-2-phenylglycine products.

The diastereomeric amide derivatives were analyzed via normal phase HPLC using a silica stationary phase and mobile phases consisting of chloroform and various polar modifiers. The analysis of the diastereomeric amides of the amino intermediates used for the synthesis of **6a**–**6j** (see Table 1) is shown in Fig. 1. Using a mobile phase of 0.5% methanol in

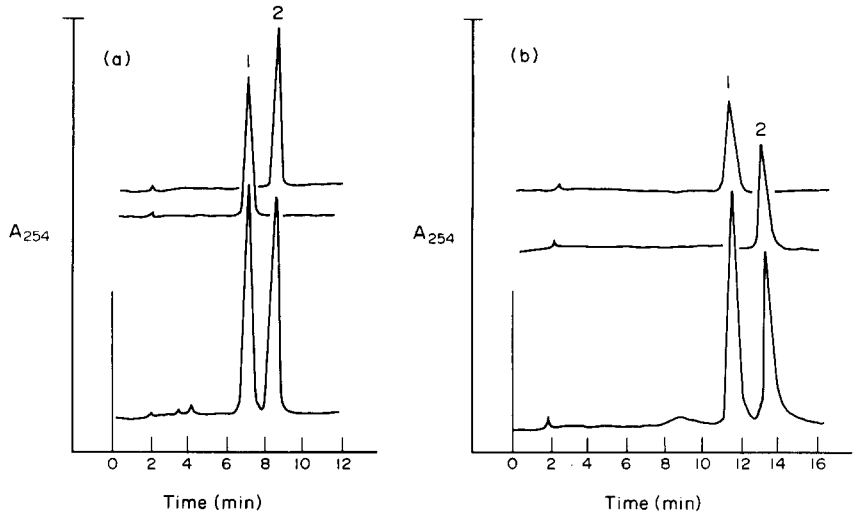


Fig. 1. (a) Normal phase liquid chromatographic separation of the intermediate *N*-[[(4-amino)phenyl]sulfonyl]-2-phenylglycine diastereomeric amides. Peak 1 = *S*,*R*-amide and 2 = *R*,*R*-amide. A solvent system of 0.5% methanol in chloroform was used. (b) Normal phase liquid chromatographic separation of the 4-fluoro BAPS-2-phenylglycine diastereomeric amides. Peak 1 = *S*,*R*-amide and 2 = *R*,*R*-amide. A solvent system of 0.5% methanol in chloroform was used.

chloroform, each amine enantiomer yielded only a single diastereomeric amide (Fig. 1). Furthermore, the retention time for the amide obtained from the (*S*)-amino intermediate differs significantly from that of the amide obtained from the (*R*)-amine (Fig. 1a), demonstrating that racemization did not occur in the reactions to form the enantiomeric amine intermediates.

The HPLC analysis of the diastereomeric amides of the BAPS-2-phenylglycine products **6a–6j** yielded similar results. For example, as illustrated in Fig. 1b, single diastereomers of different retention were obtained from (*S*)-fluoro enantiomer (**6g**) and the corresponding (*R*)-enantiomer (**6h**). These retention data demonstrate that racemization did not occur in the acylation reactions to form **6a–6j** from the intermediate enantiomeric amines.

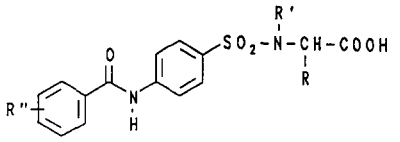
All of the substituted BAPS-amino acids were tested for their ability to inhibit ALR2 isolated from rat lens using the published method [11]. Each compound was tested at four or more concentrations, and log concentration–response curves constructed from the data. Inhibitor IC_{50} values were then calculated from the linear portions of the log concentration-response curves using the least squares program of Barlow [12]. Examination of the inhibitory data obtained for the substituted BAPS-glycines (**4**) reveals that all of these compounds were substantially more inhibitory than *N*-(phenylsulfonyl)glycine (**1**) (Table 1). These data demonstrate that addition of an aroylamino moiety at the 4-position of the *N*-(phenylsulfonyl)glycine structure (**1**) resulted in significantly enhanced ALR2 affinity. These data also reveal that the incorporation of substituents of varying electronic character, as in **4a–4g**, did not alter dramatically the efficacy of interaction with ALR2. This is demonstrated by the relative narrow range of IC_{50} values observed for the members of series **4**; the IC_{50} values range from 0.41 μM for **4a** to 5.6 μM for **4c**.

Examination of the inhibitory data for the BAPS-*N*-phenylglycine derivatives (**5**) reveals that these compounds were significantly more inhibitory than the parent compound *N*-(phenylsulfonyl)-*N*-phenylglycine (**2**) or the glycine derivative (**1**) [8]. Therefore, substitution of an aroylamino moiety at the 4-position of **2** also resulted in substantially higher ALR2 affinity. Comparison of derivatives **5a–5e** again shows that addition of substituents of varying electronic nature on the 4-aroylamino moiety did not influence significantly inhibitor binding (Table 1).

Evaluation of the substituted BAPS-2-phenylglycine enantiomers (**6**) reveals that these analogues displayed enantioselectivity comparable to the *N*-(phenylsulfonyl)-2-phenylglycines (**3**) reported earlier [8]. In this series, the *S*-enantiomers range from 30 (**6i**) to 230 times (**6a** and **6c**) more active than the corresponding *R*-stereoisomers. Also, while additional substituents on the 4-benzoylamino moiety again did not alter dramatically inhibitory activity of the more active *S*-enantiomers, these substituents did appear to contribute to differences in enantiomeric potency ratios. For example, the difference in inhibitory activities for *S*- and *R*-stereoisomers possessing electron donating moieties (**6c–6f**) was substantially greater than for those enantiomers with electron withdrawing groups (**6g–6j**). Also, the *S*-enantiomers of series **6** were significantly more inhibitory than the corresponding *S*-isomers of the *N*-(phenylsulfonyl)-2-phenylglycine series (**3**), again indicating that addition of a 4-benzoylamino substitutent results in considerably higher ALR2 affinity.

Comparison of the inhibitory data obtained for the BAPS-*N*-phenyl (**5**) and BAPS-2-phenyl derivatives (**6**) (Table 1) to that of the parent BAPS-glycine

Table 1. Aldose reductase inhibitory activity of the *N*-[[(4-aroylamino)phenyl]sulfonyl]amino acids*†



Compound No.	R	R′	R″	Config.‡	IC_{50}, μM (95% CI)§
4a	H	H	H	—	0.41 (0.08–8.0)
4b	H	H	4-OCH_3	—	4.7 (0.6–35)
4c	H	H	4-F	—	5.6 (1.1–32)
4d	H	H	4-NO_2	—	1.4 (0.7–3.0)
4e	H	H	3-NO_2	—	1.7 (0.5–6.0)
4f	H	H	2-NO_2	—	1.5 (0.3–7.0)
4g	H	H	4-CHO	—	0.7 (0.09–6.0)
5a	H	C_6H_5	H	—	0.38 (0.1–1.4)
5b	H	C_6H_5	4-CH_3	—	0.80 (0.2–3.5)
5c	H	C_6H_5	4-OCH_3	—	1.5 (0.3–8.0)
5d	H	C_6H_5	4-F	—	2.6 (0.9–7.0)
5e	H	C_6H_5	4-NO_2	—	1.7 (0.5–5.5)
6a	C_6H_5	H	H	*S*	0.60 (0.2–2.0)
6b	C_6H_5	H	H	*R*	140 (50–380)
6c	C_6H_5	H	R-CH_3	*S*	0.11 (0.05–0.2)
6d	C_6H_5	H	4-CH_3	*R*	26 (15–45)
6e	C_6H_5	H	4-OCH_3	*S*	0.16 (0.02–0.8)
6f	C_6H_5	H	4-OCH_3	*R*	22 (7–75)
6g	C_6H_5	H	4-F	*S*	0.54 (0.07–3.0)
6h	C_6H_5	H	4-F	*R*	23 (15–34)
6i	C_6H_5	H	4-NO_2	*S*	0.28 (0.15–0.5)
6j	C_6H_5	H	4-NO_2	*R*	9.3 (1.4–68)

* The reported IC_{50} values were obtained using rat lens ALR2 and DL-glyceraldehyde as the substrate.

† The IC_{50} values for inhibitors **1** and **2** in the rat lens ALR2 assay with D,L-glyceraldehyde as the substrate were 29 and 25 μM respectively. The IC_{50} value for the *s*-enantiomer of **3** was 11 μM, whereas the *R*-enantiomer had an IC_{50} of 460 μM in the same assay.

‡ *S* represents the *S*-enantiomer and *R* represents the *R*-enantiomer.

§ CI represents the 95% confidence interval.

derivatives (**4**) shows that *N*- and 2-phenyl substitution resulted in a modest alteration of inhibitory activity across these structurally related series. The BAPS-glycine derivatives (**4**) appeared to be slightly less inhibitory than the corresponding BAPS-*N*-phenyl derivatives (**5**). For example, the methoxy glycine derivative **4b** has an IC_{50} of 4.7 μM, whereas the corresponding methoxy *N*-phenylglycine derivative **5c** has an IC_{50} of 1.5 μM. Also, the BAPS-*N*-phenylglycine analogues (**5**) were slightly less inhibitory than the corresponding (*S*)-BAPS-2-phenylglycines (**6**). For example, the (*S*)-methoxy 2-phenylglycine (**6e**) (IC_{50} = 0.16 μM) was six times more inhibitory than the methoxy *N*-phenylglycine (**5c**) (IC_{50} = 1.5 μM). The relative structure-inhibition trend of (*S*)-BAPS-phenylglycine > BAPS-*N*-phenylglycine > BAPS-glycine > (*R*)-BAPS-2-phenylglycine observed for **4–6** was the same relative pattern of activity reported earlier for the *N*-(phenylsulfonyl)amino acids (**1–3**) [7, 8].

Lineweaver–Burk analysis of several of the substituted BAPS-amino acid derivatives of series **4**, **5** and **6** demonstrated that the kinetics of inhibition of these compounds are similar. As illustrated in Figs. 2 and 3 with the *N*-phenyl derivative **5a**, these compounds produced uncompetitive inhibition with respect to both the substrate glyceraldehyde and the cofactor NADPH at low inhibitor concentrations. At higher inhibitor concentrations, however, the nature of inhibition relative to both substrate and cofactor was mixed-type. These kinetic results are similar to those observed earlier for the *N*-(phenylsulfonyl)glycine (**1**), *N*-phenylglycine (**2**) and 2-phenylglycine (**3**) derivatives. The kinetic data, along with the structure–inhibition data detailed above, indicate that, while 4-benzoylamino substitution resulted in significantly enhanced ALR2 affinity (as much as 400-fold), it did not appear to change the kinetic mechanism of inhibition. Furthermore the structure–inhibition data and kinetic similarities suggest that all series **1–6** share, at least partially, a common site of interaction on ALR2.

The similarity in binding between compounds of these series was substantiated by multiple inhibition analyses. For example, inhibition by mixtures of **4d** and **1** yielded parallel Yonetani–Theorell plots (v_0/v_i vs [I]) and a linear Yagi–Ozawa plot (Fig. 4), indicating that these two inhibitors compete for a

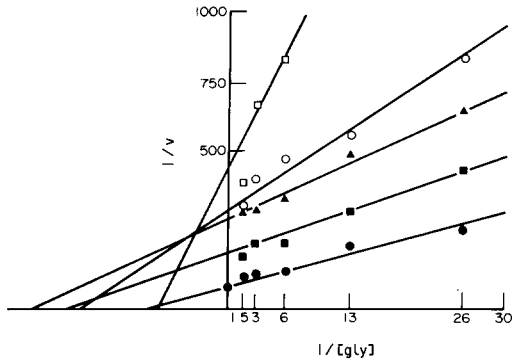


Fig. 2. Lineweaver–Burk double-reciprocal plots of initial enzyme velocity versus concentration of the substrate DL-glyceraldehyde in the presence of BAPS-*N*-phenylglycine (**5a**) at 0 μM (●), 1.0 μM (■), 5.0 μM (▲), 10.0 μM (○) and 50.0 μM (□).

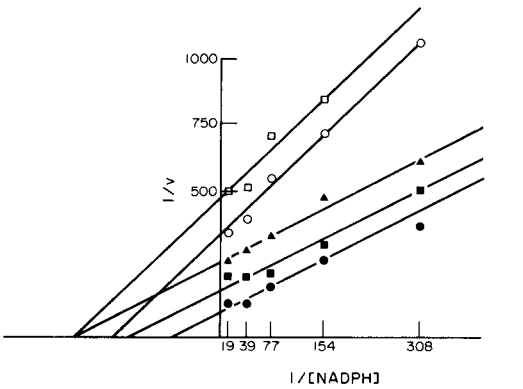


Fig. 3. Lineweaver–Burk double-reciprocal plots of initial enzyme velocity versus concentration of the cofactor NADPH in the presence of BAPS-*N*-phenylglycine (**5a**) at 0 μM (●), 1.0 μM (■), 5.0 μM (▲), 10.0 μM (○) and 50.0 μM (□).

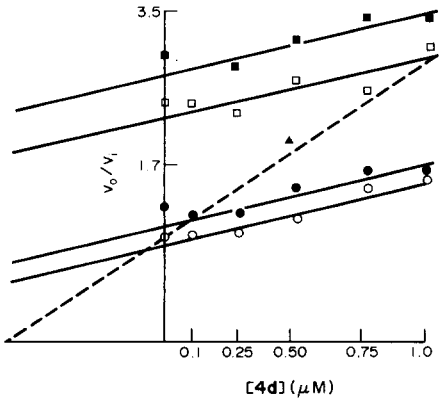


Fig. 4. Yonetani–Theorell plots of reciprocal relative inhibited velocity (v_0/v_i) versus concentration of **4d** in the presence of **1** at 0 μM (○), 5.0 μM (●), 25 μM (▲), 50 μM (□) and 75 μM (■). A Yagi–Ozawa plot is shown superimposed (-----).

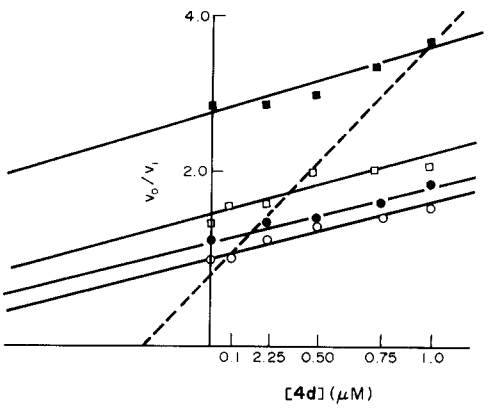


Fig. 5. Yonetani–Theorell plots of reciprocal relative inhibited velocity (v_0/v_i) versus concentration of **4d** in the presence of 4-nitroacetophenone at 0 μM (○), 250 μM (●), 500 μM (□) and 1.0 mM (■). A Yagi–Ozawa plot is shown superimposed (-----).

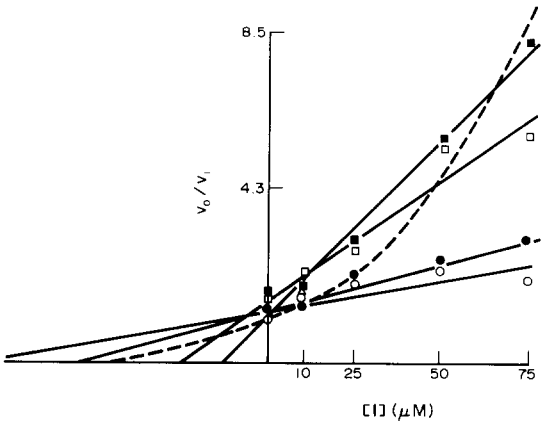


Fig. 6. Yonetani–Theorell plots of reciprocal relative inhibited velocity (v_0/v_i) versus concentration of **1** in the presence of 4-nitroacetophenone at 0 μM (○), 100 μM (●), 250 μM (△), 500 μM (□) and 750 μM (■). A Yagi–Ozawa plot is shown superimposed (-----).

common site on ALR2. Furthermore, inhibition by mixtures of **4d** and the substrate-competitive inhibitor 4-nitroacetophenone also gave parallel Yonetani–Theorell plots and a linear Yagi–Ozawa plot (Fig. 5), demonstrating that **4d** may bind at the substrate site of ALR2. *N*-(Phenylsulfonyl)glycine (**1**), however, did not appear to share a common site of interaction with 4-nitroacetophenone as evidenced by intersecting Yonetani–Theorell plots and a second order Yagi–Ozawa plot (Fig. 6) for inhibition by mixtures of **1** and 4-nitroacetophenone. The increased inhibition of ALR2 by the 4-benzoylamino derivatives **4–6** observed in these studies can therefore be explained by interaction at the substrate binding site in addition to a distinct inhibitor site present on the enzyme. Multiple site interaction with ALR2 is also consistent with the mixed-type Lineweaver–Burk kinetics observed for these compounds (Figs. 2 and 3) [14]. Mixed-type kinetics

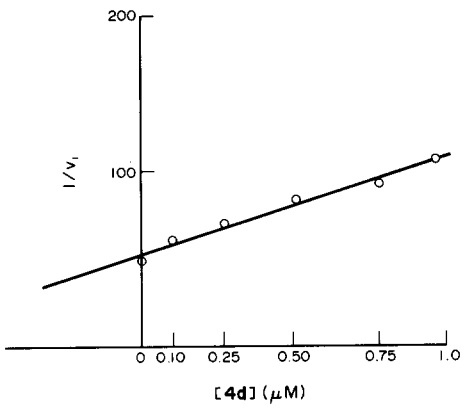


Fig. 7. Dixon plot of reciprocal inhibited velocity ($1/v_i$) versus concentration of **4d**.

Table 2. Inhibitory activity of **5a** as a function of substrate*

Substrate	Substrate concn (mM)	IC_{50} (μM)
DL-Glyceraldehyde	10	0.38
4-Nitrobenzaldehyde	0.01	0.46
D-Glucose	100	1.9
D-Galactase	50	1.9
D-Xylose	50	0.73

* The inhibitor **5a** was tested using rat lens ALR2 at six concentrations to generate the IC_{50} values: 10.0, 5.0, 2.50, 1.25, 0.625 and 0.313 μM.

result when inhibitors bind at two sites on an enzyme and interaction at one site excludes substrate binding while interaction at the second site may or may not alter binding at the first site. Such a two-site system is a unique mixed-type inhibition in that the reciprocal plots will not intersect at a common point when binding at the two sites is not mutually exclusive as with the BAPS-amino acids (Figs. 2 and 3). Also, while most mixed-type kinetic systems give curved Dixon plots ($1/v_i$ vs [I]), the two-site binding system yielded a linear Dixon plot as was obtained for the BAPS-amino acid derivative (**4d**) (Fig. 7).

Based on the results obtained from the kinetic and multiple inhibition studies, the inhibitory activity of 4-benzoylamino substituted compounds was also investigated as a function of varying substrate. For example, IC_{50} values were determined for the N-phenyl derivative (**5a**) in the presence of several aldose and aldehyde substrates. For these studies a constant concentration of enzyme and concentrations of substrate which provide maximal enzyme velocity were used. The data in Table 2 demonstrate that inhibitory potency varied as much as 4-fold depending on the nature of the substrate; IC_{50} values ranged from 1.9 μM for glucose to 0.38 and 0.46 μM for glyceraldehyde and 4-nitrobenzaldehyde respectively. These results are not surprising since the mechanism of inhibition for compounds of this type appears to involve at least partial interaction at the substrate site. The differences in inhibitory activities observed for **5a** with varying substrates do contrast significantly with results reported earlier by Poulsom [21] where it was observed that statil, a carboxylic acid inhibitor like **5a**, produces substantially greater inhibition versus glucose than 4-nitrobenzaldehyde.

The high level of inhibitory activity and unique kinetics of inhibition observed for compounds **4–6** prompted additional studies to determine the enzyme specificity of inhibitors of this structural class. Several compounds of series **4** and **6** were tested for their abilities to inhibit other NADPH, NADP and NADH-requiring enzymes including aldehyde reductase (ALR1), lactate dehydrogenase (LDH), succinic semialdehyde dehydrogenase (SSDH) and glutathione reductase (GR). In the ALR1 assay, none of the compounds tested were found to produce significant inhibition. For example, both the glycine and 2-phenyl derivatives, **4a** and **6a** had IC_{50} values substantially greater than 100 μM versus rat kidney ALR1. These findings are significant in comparison with other known inhibitors of ALR2 such as sorbinil, tolrestat, alconil, alrestatin and the flavonoids which have IC_{50} values in the micromolar or submicromolar range in the rat kidney and lens ALR1 assays [17, 22]. Also both **4a** and **6a** produced less than 10% inhibition of rabbit muscle LDH at concentrations of 100 μM. Finally, in the SSDH and GR assays, neither **4a** nor **6a** produced greater than 25% inhibition at concentrations of 100 μM. Since both **4a** and **6a** have IC_{50} values below 0.6 μM versus rat lens assay, they are significantly more inhibitory versus ALR2 than the other adenine nucleotide-requiring enzymes examined in this study.

In summary, addition of a 4-benzoylamino moiety as in the BAPS-amino acids **4**, **5** and **6** resulted in substantially increased ALR2 affinity. Kinetic and multiple inhibition analyses indicated that the enhanced inhibitory activities of these compounds are due to their abilities to interact with an additional site present on ALR2. Also, in spite of the relatively high ALR2 affinity observed for the BAPS-amino acids, these compounds did not produce significant inhibition of related enzymes such as aldehyde reductase.

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