N-(aroyl)-N-(arylmethyloxy)-α-alanines: selective inhibitors of aldose reductase

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Abstract

Aldose reductase (ALR2), a NADPH-dependent reductase, is the first and rate-limiting enzyme of the polyol pathway of glucose metabolism and is implicated in the pathogenesis of secondary diabetic complications. In the last decades, this enzyme has been targeted for inhibition but despite the numerous efforts made to identify potent and safe ALR2 inhibitors, many clinical candidates have been a failure. For this reason the research of new ALR2 inhibitors highly effective, selective and with suitable pharmacokinetic properties is still of great interest. In this paper some new N-(aroyl)-N-(arylmethyloxy)alanines have been synthesized and tested for their ability to inhibit ALR2. Some of the synthesized compounds exhibit IC_{50} in the low micromolar range and all have proved to be highly selective towards ALR2. The N-(aroyl)-N-(arylmethyloxy)-α-alanines are a promising starting point for the development of new ALR2 selective drugs with the aim of delaying the onset of diabetic complications.

Keywords: Diabetes mellitus, aldose reductase (ALR2), aldose reductase selective inhibitors, α-alanines.

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1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by elevated levels of glucose in the blood. Type 1 diabetes is characterized by insufficient insulin production and needs a daily administration of insulin. Actually, no prevention is known for Type 1 diabetes. Type 2 diabetes normally occurs in adults, only recently seen also in children, and results from an ineffective use by the body of the insulin produced. Healthy lifestyle and body weight, and regular physical activity are recommended to prevent or delay the onset of Type 2 diabetes. The World Health Organization (WHO) estimates that 422 million people worldwide suffer from diabetes. In 2012 1.5 million deaths were directly caused by this pathology and it is believed that it will be the 7th leading cause of death in 2030[1].

Diabetes can be successfully controlled by the administration of insulin and/or effective oral antidiabetic drugs; however it remains a significant cause of morbidity and mortality because of the gradual development of debilitating complications such as cardiovascular disease, neuropathy, nephropathy, retinopathy, cataracts, which negatively affect the quality of life and life expectancy of diabetic patients. These complications arise from chronic hyperglycemia, which is the initiating cause of diabetic tissue damage. In fact, diabetes selectively damages cells that does not efficiently reduce the glucose transport inside the cell when these are exposed to hyperglycemia, such as endothelial cells and mesangial cells, leading to high glucose levels inside the cell[2-4].

Among the hypotheses that have been proposed the most accredited theory supported by experimental and clinical evidence suggests that the pathogenic mechanisms leading to diabetes complications is connected to glucose metabolism *via* the activation of the polyol pathway[5]. In normal physiological conditions, glucose is phosphorylated by hexokinase while in hyperglycemic conditions the excess of glucose is metabolized through the polyol pathway. Aldose reductase (alditol: NADP+ oxidoreductase, EC1.1.1.2, ALR2), is a monomeric ubiquitous cytoplasmatic protein member of the aldo-keto reductase super family (AKR) which consists of several enzymes that perform oxidoreduction reactions on a wide variety of substrates[6,7]. ALR2 is a widely

expressed enzyme. It has been identified in various tissues like the brain, kidney, liver, lens and skeletal muscle tissue. ALR2 is the first and rate-limiting enzyme in the polyol pathway of glucose metabolism[8,9] and normally catalyzes the reduction of toxic aldehydes in cells to inactive alcohols; but when the concentration of glucose in the cell becomes too high it also catalyzes the NADPH-dependent reduction of glucose to sorbitol which is later oxidized to fructose by sorbitol dehydrogenase (SDH), with NAD^+ as the cofactor[10] (Figure 1). In pathological conditions the accumulation of the high concentration of sorbitol inside the cell causes osmotic stress and it has been postulated that it contributes to the development of diabetic complications.

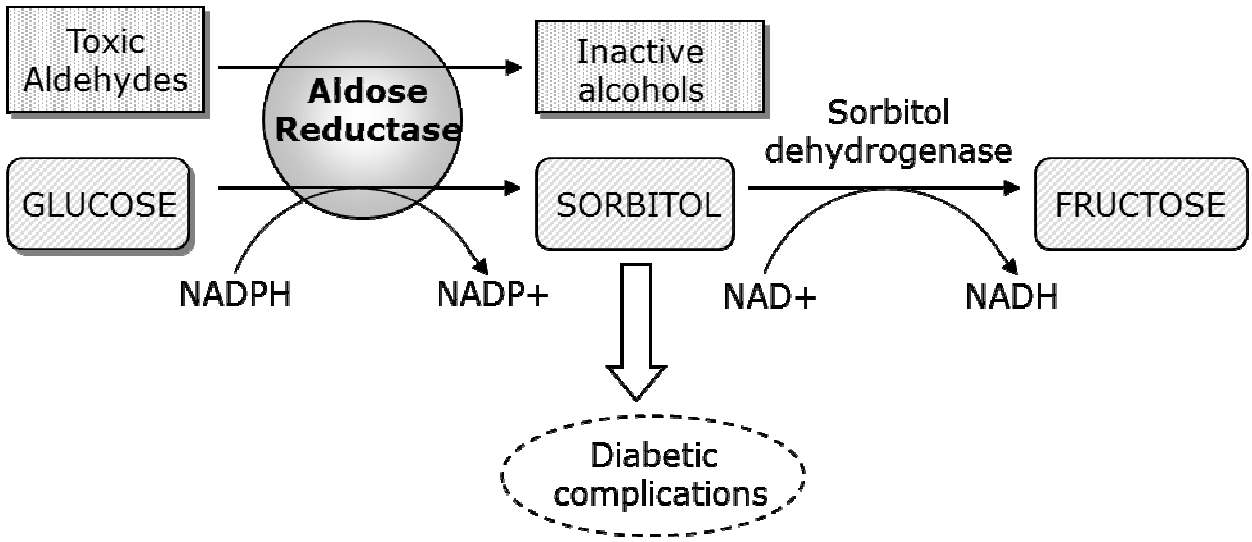


Figure 1. Aldose reductase and the polyol pathway of glucose metabolism.

In hyperglycemic conditions an increase in the activity of ALR2 causes oxidative stress due to imbalance in the ratios of NADPH/NADP+ and NADH/NAD+ that may mediate the pathogenesis of several diabetic complications like caractogenesis, retinopathy, nephropathy, neuropathy and microangiopathy (8,11,12). It has been demonstrated that the polyol pathway has a central role in ischemic injury in fact, the increase of ALR2 activity is detrimental under ischemic conditions and impedes functional and metabolic recovery during reperfusion[12]. Recently, its implication has been established in inflammatory and neoplastic processes[13-15].

In this context, the inhibition of ALR2 is a promising therapeutic strategy useful in preventing or delaying the onset of diabetes complications and controling their evolution[16]. During the last decades extensive efforts have been directed towards the development of aldose reductase inhibitors (ARIs) as therapeutic tools for the prevention and/or arrest of the progression of long-term diabetic complications[17,18]. Many compounds belonging to structurally different classes have been identified as potent *in vitro* ALR2 inhibitors. Generally, these inhibitors contain a cyclic amide group, such as in sorbinil, fidarestat, minalrestat, ranirestat, or acetic acid moiety, like in tolrestat, alrestatin, epalrestat, zopolrestat, but also phenolic groups as in quercetin (Figure 2).

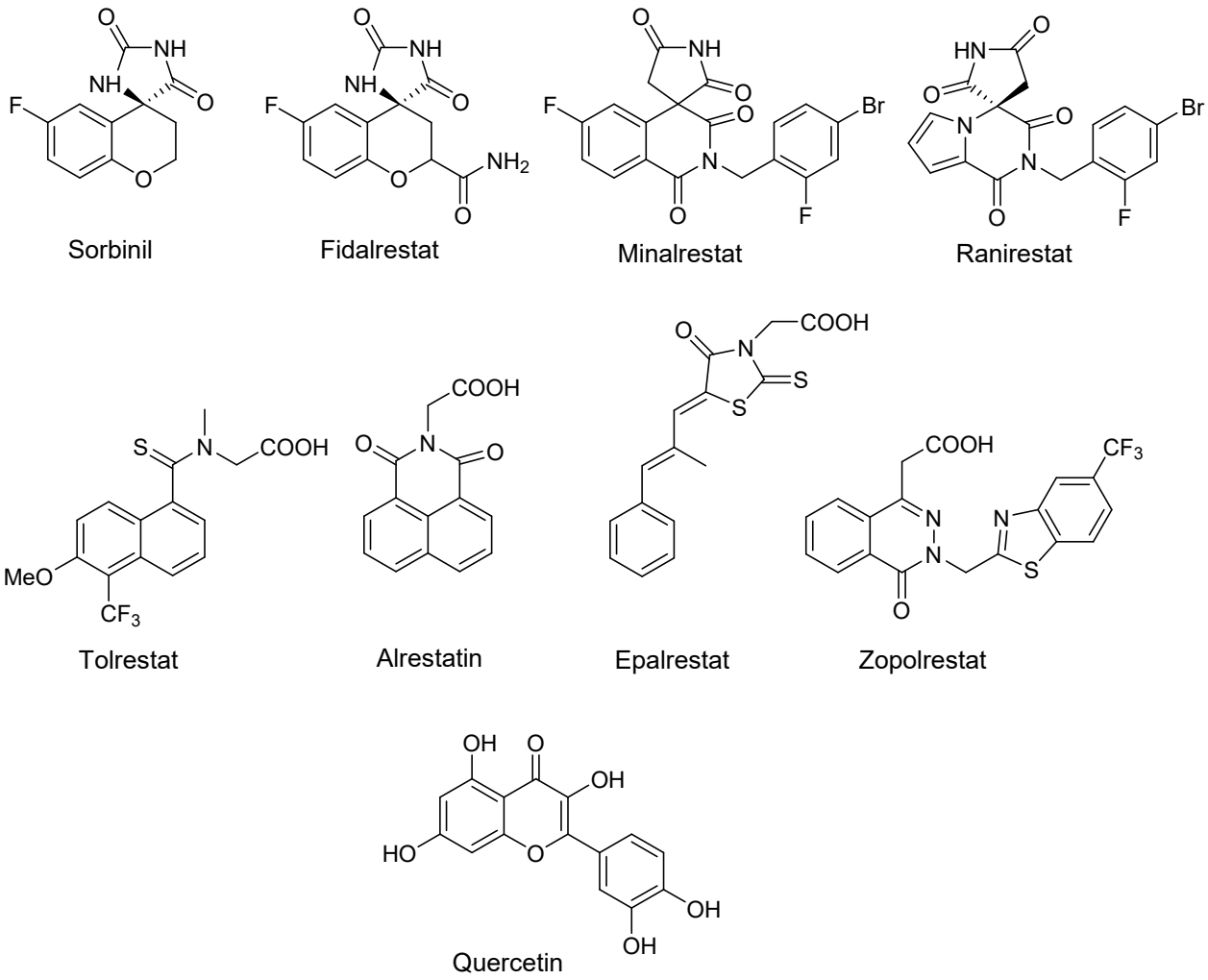


Figure 2. Chemical structure of known ALR2 inhibitors.

Although several ARIs have reached various phases of clinical experimentation however most of them have been withdrawn either due to an insufficient bioavailability[13], their poor efficacy or adverse side effects. Currently Epalrestat is in clinical use for the treatment of diabetic neuropathy. The side effects are due to a lack of selectivity towards the aldehyde reductase (ALR1, EC 1.1.1.2), which is a cytosolic enzyme of the AKR superfamily and is closely related to ALR2, showing high structural homology (65% identity in their amino acid sequences), substrate specificities and kinetic mechanisms[19]. ALR1 plays a detoxification role in the efficient removal of toxic aldehydes such as hydroxynonenal (HNE), methylglyoxal and 3-deoxyglucosone arising from pathological conditions connected with oxidative stress, like diabetes[20,21].

ALR2 is a monomeric (α/β)8-barrel cytoplasmatic protein of 36kDa composed of 316 amino acid residues. The active site is located in the barrel core at the bottom of a large and deep hydrophobic pocket at the C-terminal of the barrel[22]. The pyridine cofactor $NADP^+$ is bound at the bottom of the cleft through a flexible loop named the "safety-belt" loop[23]. Through a comparative analysis of the ALR2-inhibitor complex crystal structures it has been suggested that the ALR binding site splits into two parts with different flexibility properties. The first portion, mainly formed by the residues of the catalytic site (Trp20, Tyr48, Val47, His110, Trp49 and Trp111) and the flanking cofactor $NADP^+$, is named the "anion binding pocket". The second portion of the binding site is flexible and affects the segments Leu300 and Trp111 that are determinant for the appearance of a "specificity pocket", also named the "induced cavity region", which posseses hydrophobic features and shows a high degree of flexibility [24]. This pocket can adopt different conformations depending on the size and nature of the bound ligand[25,26]. These features constitute important pharmacophoric requirements for the design of selective inhibitors binding to ALR2. In particular, among the members of the AKR superfamily the hydrophobic amino acid residues present in the specific pocket of the ALR2 active site are less conserved than the hydrophilic ones providing the structural basis for selectivity.

The flexibility of the binding site of ALR2 permits accommodating a wide variety of small molecules with different shape and size. Although ALR2 inhibitors are structurally different they have two common features: one or more aromatic moieties able to open the "specificity pocket" which posseses a high degree of induced-fit adaptations (useful to arrange structurally different ligands), and an acidic moiety which can interact with the "anionic binding pocket" by establishing ionic and/or hydrogen bond interactions with Tyr48 and His110[26].

In previous papers from our laboratory we have studied several series of glycine (R = H, n=0) and β-alanine (R = H, n=1) derivatives (Figure 3) as ARIs[27-29]. In these studies, we have extensively examined the structure-activity relationships (SARs) within this class and explored the effects on inhibitory activity of aldose reductase by increasing both the distance between the aromatic portion and the carboxylic group and the nitrogen core, and by inserting a methylene spacer between them. Moreover, we have explored the effect obtained by shifting the phenyl ring away from the carbonyl group by inserting various spacers of different lengths and degrees of rigidity. The biological results indicate that the lengthened derivatives are in general less active with respect to the shorter ones. Thus, these distances between the pharmacophoric groups appeared to be a crucial element in determining the best spatial relationship between them within this structural class of compounds.

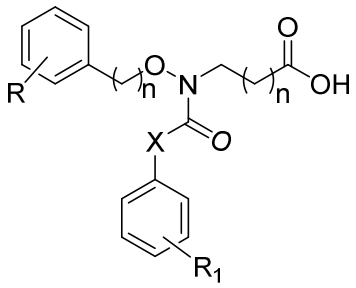


Figure 3. General formula of glycine and β-alanine derivatives.

In the present study we investigated three series of compounds starting from the structure of the most active compounds reported previously[27-29] (R =H,: Cl and R_1=OMe, n=0, IC_{50}= 3.5 μM and 6.3 μM, respectively **A** and **B** of Figure 4).

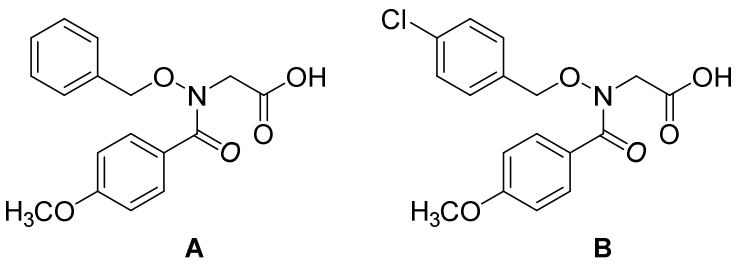


Figure 4. Our lead compounds.

For the first series we decided to verify the effects induced, on the ALR2 inhibitory properties, of an additional steric hindrance on the carbon in α position with respect to the carboxylic group, by introducing a methyl group (R_2 = Me). Therefore, we synthesized a series of new α-alanine derivatives of Type **1** (R_1= H) and **2** (R_1 = OMe) (Figure 5) in order to explore the structure activity relationship and obtain useful information for further structure-based optimization within this class of compounds. Similar to other well known carboxylate Type ALR2 inhibitors these compounds possess a carboxylic head group that should occupy the anion binding pocket, attached through a spacer to two aromatic moieties that may contribute to an efficient fit in the lipophilic region of the ALR2 binding site including the specificity pocket. The electron-withdrawn (Cl) substituent was introduced around the benzylic ring of all the synthesized compounds.

To investigate if the novel α-alanine compounds displayed a parallel SARs to those previously studied[27-29], for the second series we synthesized the compounds of Type **3** (R_2 = Me, n= 0). Then for the third, compounds of Type **4** (R_2 = Me, n= 1), superior homologous of Type **3** in which a benzylamidic group instead of a benzoylic group, was present on the nitrogen (Figure 5).

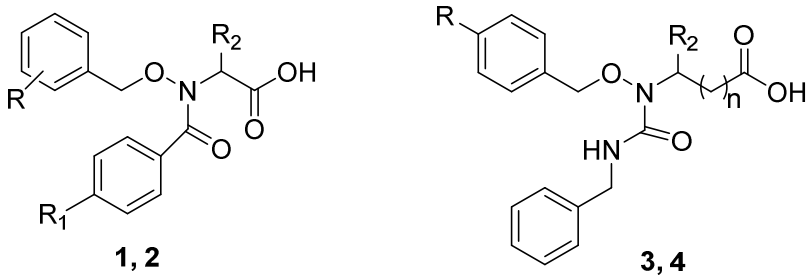


Figure 5. General formulas of synthesized compounds.

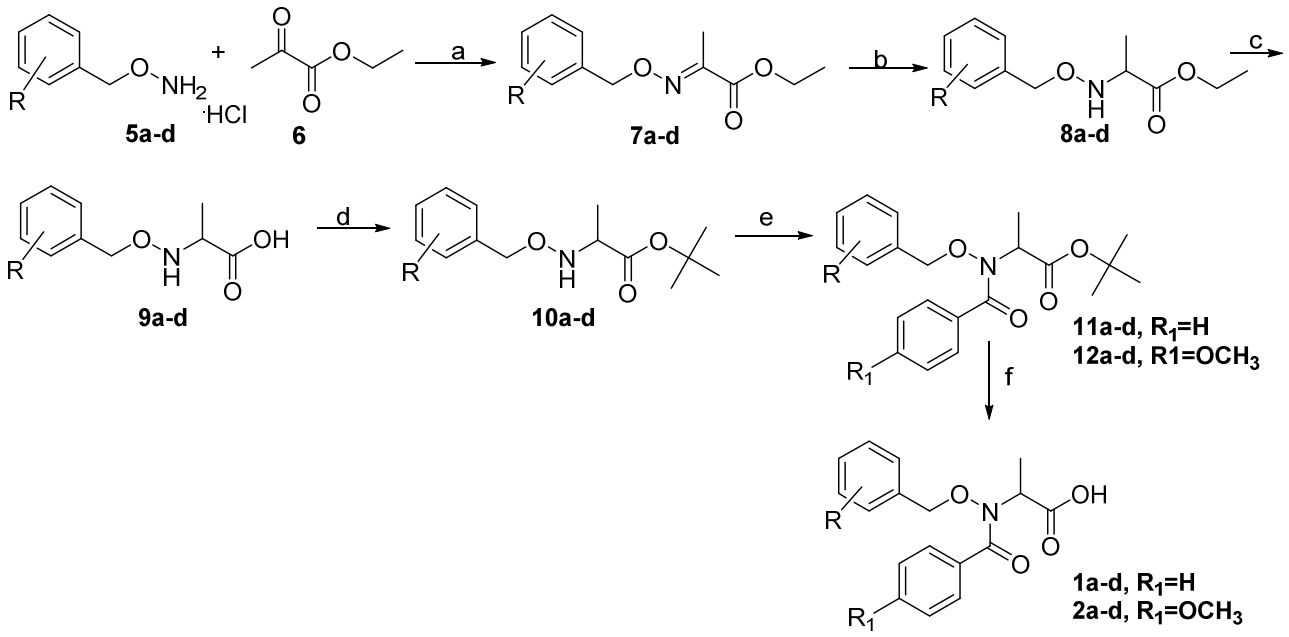
All the synthesized compounds were tested for their ALR2 inhibitory activity and selectivity.

2. Material and methods

2.1 Chemistry

The 2-(N-(arylmethyloxy)benzamido)propanoic acid (**1a-d**, **2a-d)** were prepared as described in Scheme 1. For the preparation of the intermediate ethyl 2-(aryloxyamino)propanoates **8a-d** we followed the one previously described[30] slightly modifying the synthetic route consisting of the reaction of the opportune *O*-(arylmethyl)hydroxylamines hydrochlorides **5a-d** with ethyl 2-oxo-propanoate (**6**) and consequent reduction of the resulting oximinoacid **7a-d** with borane-trimethylamine complex to obtain the ethyl 2-(aryloxyamino)propanoates **8a-d.** The alkaline hydrolysis of **8a-d** yielded the corresponding aminoacids **9a-d** which in turn were transformed in the *tert*-butyl esters **10a-d** by reaction with *tert*-butyl acetate in the presence of perchloric acid. The acylation of **10a-d** in heterogenic phase with the opportune aroylchlorides gave the amides **11a-d** and **12a-d.** The clevage of the *tert*-butyl esters with trifluoroacetic acid yielded the 2-(N-(arylmethyloxy)benzamido)propanoic acid **1a-d** and **2a-d.**

Scheme 1. Synthesis of 2-(N-(arylmethyloxy)benzamido)propanoic acid derivatives **1a-d** and **2a-d**.

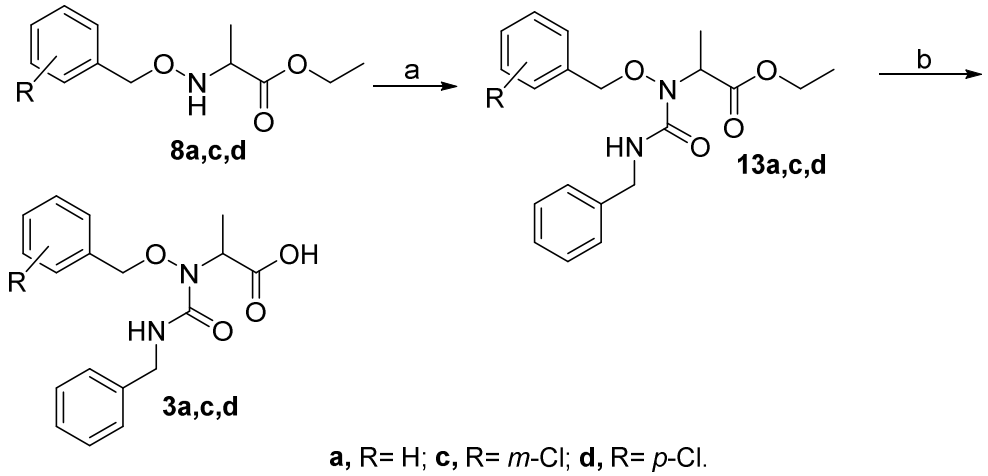


a, R= H, **b,** R= *o*-Cl; **c,** R= *m*-Cl; **d,** R= *p*-Cl.

Reagents and conditions: a) MeOH/H_2O, t.a., 1h; b) $(Me)_3N{:}BH_3$/ EtOH.HCl; c) NaOH, THF/H_2O, 0°C; d) AcO*t*-Bu, $HClO_4$; e) ArCOCl, $NaHCO_3$, AcOEt; f) CF_3COOH, Anisolo, 0°C.

The synthesis of 2-(3-benzyl-1-(arylmethyloxy)ureido)propanoic acid derivatives **3a,c,d** is outlined in Scheme 2. The reaction of the previously described ethyl 2-(arylmethyloxyamino)propanoate **8a,c,d** with (isocyanatomethyl)benzene in the presence of a catalytic amount of 4-N,N-dimethylamino pyridine yielded the ureido derivatives **13a,c,d** which were saponified with potassium hydroxide to obtain the ureido derivatives **3a,c,d.**

Scheme 2. Synthesis of 2-(3-benzyl-1-(arylmethyloxy)ureido)propanoic acid derivatives **3a,c,d**.

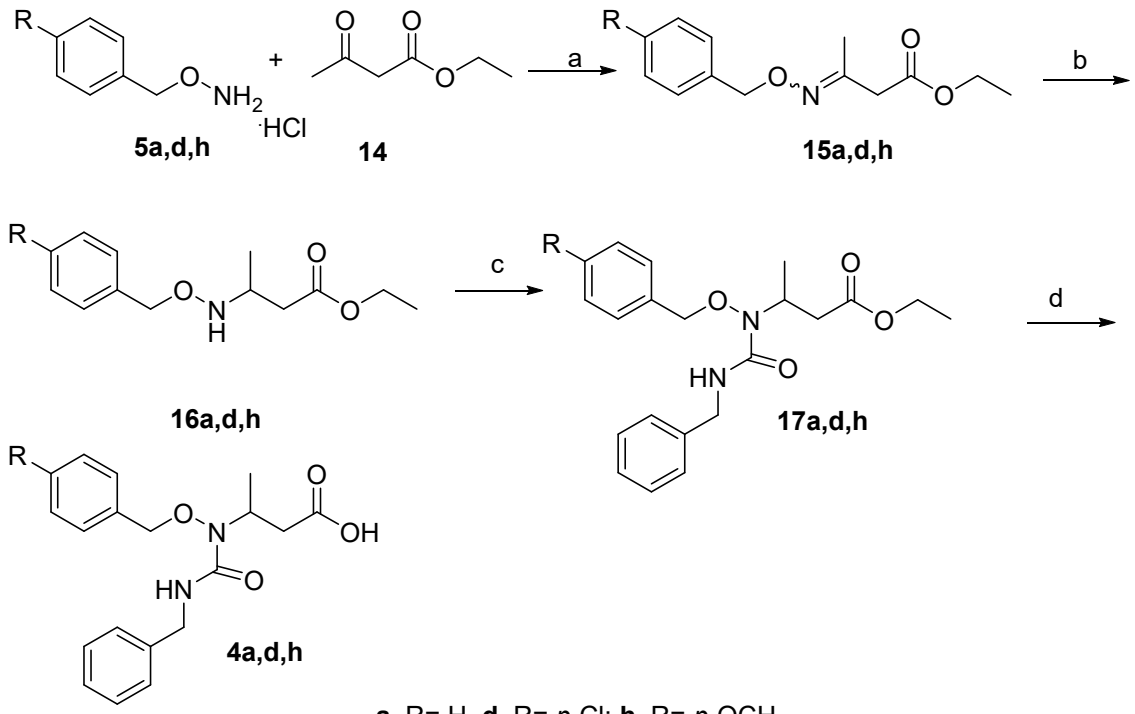


a, R= H; **c,** R= *m*-Cl; **d,** R= *p*-Cl.

Reagents and conditions: a) $C_6H_5CH_2NCO$, DMAP, CH_2Cl_2, t.a.; b) THF/H_2O, NaOH 1N, 0 °C.

The synthesis of 3-(3-benzyl-1-(arylmethyloxy)ureido)butanoic acid derivatives **4a,d,h** is described in Scheme 3. The reaction of the opportune *O*-(arylmethyl)hydroxylamines hydrochlorides **5a,d,h** in MeOH/H_2O mixture with ethyl 3-oxo-butanoate (**14**) afforded a mixture of the corresponding 3-(arylmethyloxyimino)butanoate ethyl esters **15a,d,h***(E/Z)* in a ratio of 1:1**.** Reduction of the isomeric mixture **15a,d,h** with the borane-trimethylamine complex in alcoholic solution in the presence of HCl, yielded the corresponding ethyl 3-(aryloxyamino)butanoate **16a,d,h.** The reaction of **16a,d,h** with (isocyanatomethyl)benzene in the presence of a catalytic amount of 4-N,N-dimethylamino pyridine gave the ureido derivatives **17a,d,h** which were treated with potassium hydroxide to obtain the acids **4a,d,h.**

Scheme 3. Synthesis of 3-(3-benzyl-1-(arylmethyloxy)ureido)butanoic acid derivatives **4a,d,h**.



a, R= H, **d,** R= *p*-Cl; **h,** R= *p*-OCH_3.

Reagents and conditions: a) AcONa, abs.EtOH, t.a.; b) $(Me)_3N{:}BH_3$/ EtOH.HCl; c) $C_6H_5CH_2NCO$, DMAP, CH_2Cl_2, t.a.; b) THF/H_2O, NaOH 1N, 0°C.

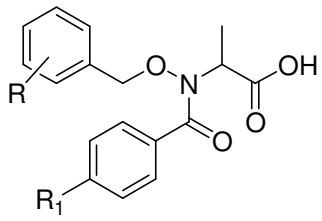
2.2 Biology

The newly synthesized 2-(N-(arylmethyloxy)benzamido)propanoic acid derivatives **1a-d**, **2a-d**, and the intermediate aminoacids **7a-d**, were tested *in vitro* as inhibitors of aldose reductase ALR2, isolated and purified from rat lenses. In order to evaluate the selectivity towards ALR2, all the compounds were also tested for their ability to inhibit ALR1. The results (Table 1) are expressed as IC_{50} values (μM) and were determined by linear regression analysis of the log of the concentration response curve. Their effectiveness was evaluated using as reference drugs Sorbinil and Tolrestat, which are ALR2 selective drugs.

3. Results and discussion

All the synthesized compounds were tested for their activity and against ARL2 and selectivity towards ARL1. Results obtained are shown in Table 1.

Table 1. ARL2 and ALR1 inhibitory activity (μM) of compounds **1a-d** and **2a-d.**



compd	R	R_1	ALR2 (IC_{50}, μM)[a]	ALR1(IC_{50}, μM)[a]
1a	H	H	46.7 (44.5-48.9)	>100
1b	*o*-Cl	H	4.88 (4.64-5.11)	>100
1c	*m*-Cl	H	12.5 (11.8±13.1)	>100
1d	*p*-Cl	H	2.69 (2.56±2.82)	>100
2a	H	H	>100	>100
2b	*o*-Cl	OMe	2.38 (2.31±2.47)	>100
2c	*m*-Cl	OMe	>100	>100
2d	*p*-Cl	OMe	10.1 (9.60±10.6)	>100
Sorbinil			2.0 (1.7-2.10)	0.029
Tolrestat			0.05 (0.03-0.06)	>100

[a]IC_{50} (95% CL) values represent the concentration required to produce 50% of enzyme inhibition.

Compounds **1a-d**, **2a-d**, and **7a-d** were assayed as racemic mixtures, to identify the best ALR2 inhibitor that will be further studied for the functional properties of each enantiomeric form. Within these series, the electron-withdrawn (Cl) substituent is introduced around the benzylic ring of all the synthesized compounds.

Most of the tested compounds of Type **1** and **2** proved to inhibit ALR2, exhibiting potency levels in the micromolar range. By observing the data of Table I we can evidence the following structure−activity relationship (SAR):

- the insertion of electron-withdrawing chlorine atom as R substituent gave rise to a general increase in the inhibitory potency with respect to unsubstituted parent compound **1a**;
- compounds **1b** and **1d**, bearing the *orto*- and *para*-chlorine atom as R moiety and a hydrogen as R_1 substituent showed appreciable inhibitory activity with IC_{50} value of 4.88 and 2.69 μM, respectively;
- the trend of the inhibitory potency in compounds bearing a methoxy group as R_1 substituent shows a different trend in the potency: the unsubstituted compound **2a** is completely inactive as well as compound **2c** *m*-chloro substituted;
- the *o*-chloro substituted compound **2b** possesses a higher potency with respect to the parent compound **1b** (R_1=H) showing an IC_{50} value of 2.38μM *vs* 4.88μM and is the most effective ALR2 inhibitors of the series. On the contrary among the *p*-chloro substituted compounds **1d** (R_1=H) and **2d** ($\mathbf{R}_1$=OCH_3) is the compound **1d** that shows a 4-fold enhancement in potency;
- 2-(3-benzyl-1-(arylmethyloxy)ureido)propanoic acid derivatives **3a,c,d** and 3-(3-benzyl-1-(arylmethyloxy)ureido)butanoic acid derivatives **4a,d,h** showed no ability to inhibit the enzyme ALR2.

In order to assess the importance of the amide function were also tested acids **9a-d** devoid of this group and intermediate for the synthesis of Type **2** compounds. Compounds **9a-d** were found to be completely inactive.

Unlike sorbinil (IC_{50}ALR1 > 10 μM *versus* ARL1 = 0.029 μM, respectively), none of the synthesized compounds **1-4** tested at 100 μM showed to have an appreciable activity against ARL1, proving to be completely selective towards the ALR2 enzyme.

The inhibition profile of the most active compounds, **1d** and **2b**, is quite similar to that of sorbinil on ALR2 isoform whereas they prove to possess a better selectivity ALR2/ALR1 than sorbinil being completely selective towards the ALR2 enzyme. ALR1, in fact, is thought to be the main responsible of side effects and for this reason a clinically effective and safe ARI should be able to inhibit ALR2 selectively.

4. Conclusions

ALR2 is involved in the pathogenesis of several diabetic complications. On the basis of the above considerations, we investigated a new series of N-(aroyl)-N-(arylmethyloxy)alanines (**1-4**) obtained by an original synthesis. These compounds were found highly selective for ALR2 enzyme because they have no inhibitory activity against ALR1. The selectivity for ALR2 is considered as a great advantage for aldose reductase inhibitors because of reduction of the risk of side effects. The results of this work have allowed us to consider **1d** and **2b** a good starting point for further optimization to obtain new drugs useful in preventing or delaying the onset of diabetes complications.

5. Experimental

5.1 Material and methods

Analytical grade reagents and solvent were purchased from Sigma-Aldrich (St. Louis, MO), and were used as supplied. Solvents were dried according to standard methods. Melting points were determined on a Köfler hot-stage apparatus and are uncorrected. IR spectra for comparison between compounds were recorded on a Unicam Mattson 1000 FT-IR spectrometer (Cambridge, UK), as Nujol mulls in the case of solid substances, or as liquid film in the case of liquids. 1H NMR spectra were recorded with a Varian CFT20 spectrometer (Mountain View, CA) operating at 80 MHz at 25°C in a ca. 5% solution of $CDCl_3$ and Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) as internal standard. ^{1}H-NMR spectra for compounds

3a,c,d and **4a,d,h** were determined with a Bruker Ultrashield^{TM} 400 MHz (Fällander, Switzerland). Coupling constants *J* are reported in Hertz. The following abbreviations are used: singlet (s), doublet (d), triplet (t), broad (br) and multiplet (m). Reactions were monitored by thin layer chromatography (TLC) on silica gel plates containing a fluorescent indicator (Merck Silica Gel 60 F254) and spots were detected under UV light (254 nm). Preparative TLC were performed with silica gel plates (2mm, Merck Silica Gel 60 F254). Chromatographic separations were performed on silica gel columns by flash column chromatography (Kieselgel 40, 0.040−0.063 mm; Merck). GC analyzes were performed on a Perkin-Elmer model 8410 apparatus with a flame ionization detector, using a 1.5 m x 2.5mm column packed with methylsilicone OV-1 3% on Supelcoport 80-100 mesh. Na_2SO_4 was always used as the drying agent. Evaporation was carried out "in vacuo" (rotating evaporator). Elemental analyzes were performed by our analytical laboratory and agreed with the theoretical values to within ± 0.4%.

5.2 Synthetic procedure

5.2.1 General procedure for the synthesis of 2-(arylmethyloxyamino)propionic acids (9a-d)

These compounds were prepared slightly modifying the synthetic route previously described[30]. Treatment of aqueous 1:1 solution of the appropriate O-(arylmethyl)hydroxylamine hydrochloride **5a-d** and ethyl 2-oxo-2-propanoate (**6**) in MeOH (20 ml) at room temperature for 1 h afforded the (*E*)-ethyl 2-(benzyloxyimino)propanoate (**7a-d**). Reduction of **7a-d** with borane-methylamine complex in EtOH in the presence of 10% aqueous HCl, yielded after the usual work-up the amino esters **8a-d** which afforded the corresponding acids **9a-d** upon alkaline hydrolysis.

5.1.2 General procedure for the synthesis of tert-Butyl 2-(arylmethyloxyamino)propanoates (10a-d)

To a solution of the opportune amino acids **9a-d** (1.29 mmol) in *tert*-butylacetate (15.40 ml) was added $HClO_4$ (0.128 ml). The reaction mixture was stirred at room temperature for 1 day, then

washed with a saturated solution of $NaHCO_3$. Organic layers were dried over Na_2SO_4, and concentrated *in vacuo* to give **10a-d** practically pure (GC)**.**

10a (98% yield); purity 98,7% (GC); IR ν 3269 (NH), 1742 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.2 (d, 3H, *J* = 7.0 Hz), 1.50 (s, 9H). 3.61 (m, 1H), 4.82 (s, 2H), 5.45 (brs, 1H), 7.47 (s, 5H); Anal. Calcd for $C_{14}H_{21}NO_3$ (251.15) C, 66.91; H, 8.42; N, 5.57; Found: C, 66.56; H, 8.56; N, 5.46. **10b** (70% yield); purity 99% (GC); IR ν 3269 (NH), 1742 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.2 (d, 3H, *J* = 7.0 Hz), 1.50 (s, 9H). 3.70 (m, 1H), 4.97 (s, 2H), 5.50 (brs, 1H), 7.53 (m, 4H); Anal. Calcd for $C_{14}H_{20}ClNO_3$ (285.77) C, 58.84; H, 7.05; N, 4.90; Found: C, 58.75; H, 6.97; N, 5.06. **10c** (71% yield); purity 99,0% (GC); IR ν 3269 (NH), 1742 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.18 (d, 3H, *J* = 7.0 Hz), 1.47 (s, 9H). 3.64 (m, 1H), 4.73 (s, 2H), 5.53 (brs, 1H), 7.45 (m, 4H); Anal. Calcd for $C_{14}H_{20}ClNO_3$ (285.77) C, 58.84; H, 7.05; N, 4.90; Found: C, 58.95; H, 7.18; N, 5.01. **10d** (71% yield); purity 99,7% (GC); IR ν 3269 (NH), 1742 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.16 (d, 3H, *J* = 7.0 Hz), 1.47 (s, 9H). 3.58 (m, 1H), 4.77 (s, 2H), 5.24 (brs, 1H), 7.37 (s, 4H); Anal. Calcd for $C_{14}H_{20}ClNO_3$ (285.77) C, 58.84; H, 7.05; N, 4.90; Found: C, 58.79; H, 7.11; N, 4.82.

5.2.3 General procedure for the synthesis of tert-Butyl 2-(N-(arylmethyloxy)benzamido)propanoate (11a-d) and tert-Butyl 2-(N-(arylmethyloxy)-4-methoxybenzamido)propanoate (12a-d)

To a stirred solution of the appropriate *tert*-butyl 2-(aryloxyamino)propanoates (**10a-d**) (3.82 mmol) in EtOAc (30 ml) was added a saturated solution of $NaHCO_3$ (30 ml). The resulting mixture was treated dropwise with a solution of benzoyl chloride (3.82 mmol) or with a solution of 4-methoxybenzoyl chloride (3.82 mmol) in EtOAc (30 ml) for **11a-d** or **12a-d,** respectively. The mixture was stirred at room temperature for 16h. The organic phase was then separated, washed (H_2O, aqueous 5% HCl, saturated solution of $NaHCO_3$, H_2O), dried over Na_2SO_4, and evaporated to give the amides **11a-d** as oils practically pure (GC), while the pure amides **12a-d** were obtained by

flash chromatography of the crude oils on silica gel column, eluting with a EtOAc /hexane 4:1 mixture.

11a (78% yield); purity 97,2% (GC); IR ν 1742 (C=O), 1665 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.55 (m, 12H), 4.83 (s 2H), 4.90 (m, 1H), 7.45 (m, 10H); Anal. Calcd for $C_{21}H_{25}NO_4$ (355.43) C, 70.96; H, 7.09; N, 3.94; Found: C, 70.78; H, 7.16; N, 3.86. **11b** (60% yield); purity 94,3% (GC); IR ν 1742 (C=O), 1665 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.60 (m, 12H), 4.96 (s 2H), 4.90 (m, 1H), 7.59 (m, 9H); Anal. Calcd for $C_{21}H_{24}ClNO_4$ (389.87) C, 64.69; H, 6.20; N, 3.59; Found: C, 64.47; H, 6.12; N, 3.68. **11c** (63% yield); purity 95,5% (GC); IR ν 1742 (C=O), 1665 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.56 (m, 12H), 4.80 (s 2H), 4.86 (m, 1H), 7.43 (m, 9H); Anal. Calcd for $C_{21}H_{24}ClNO_4$ (389.87) C, 64.69; H, 6.20; N, 3.59; Found: C, 64.51; H, 6.18; N, 3.47. **11d** (83% yield); purity 96,4% (GC); IR ν 1742 (C=O), 1665 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.60 (m, 12H), 4.80 (s 2H), 4.88 (m, 1H), 7.70 (m, 9H); Anal. Calcd for $C_{21}H_{24}ClNO_4$ (389.87) C, 64.69; H, 6.20; N, 3.59; Found: C, 64.77; H, 6.16; N, 3.65.

12a (40% yield); IR ν 1742 (C=O), 1665 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.56 (m, 12H), 3.85 (s 3H), 4.78 (s, 2H), 4.87 (m, 1H), 7.10 (m, 6H), 7.70 (m, 2H); Anal. Calcd for $C_{22}H_{27}NO_5$ (385.45) C, 68.55; H, 7.06; N, 3.63; Found: C, 68.71; H, 7.13; N, 3.55. **12b** (38% yield); IR ν 1742 (C=O), 1665 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.44 (m, 12H), 3.86 (s, 3H), 4.75 (s, 2H), 4.86 (m,1H), 6.96 (m, 6H), 7.70 (m, 2H); Anal. Calcd for $C_{22}H_{26}ClNO_5$ (419.90); C, 62.93; H, 6.24; N, 3.34;Found: C, 62.88; H, 6.17; N, 3.42. **12c** (25% yield); IR ν 1742 (C=O), 1657 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.43 (m, 12H), 3.85 (s, 3H), 4.75 (s, 2H), 4.86 (m, 1H), 7.02 (m, 6H), 7.69 (m, 2H); Anal. Calcd for $C_{22}H_{26}ClNO_5$ (419.90); C, 62.93; H, 6.24; N, 3.34;Found: C, 63.05; H, 6.16; N, 3.26. **12d** (32% yield); IR ν 1742 (C=O), 1649 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.42 (m, 12H), 3.85 (s, 3H), 4.75 (s, 2H), 4.86 (m, 1H), 7.02 (m, 6H), 7.69 (m, 2H); Anal. Calcd for $C_{22}H_{26}ClNO_5$ (419.90); C, 62.93; H, 6.24; N, 3.34;Found: C, 62.85; H, 6.02; N, 3.45.

5.2.4 General procedure for the synthesis of 2-(N-(arylmethyloxy)benzamido)propanoic acids (1a-d) and 2-(N-(arylmethyloxy)-4-methoxybenzamido)propanoic acids (2a-d).

To a stirred and cooled (0°C) solution of the appropriate ester **1a-d** or **2a-d** (2.20 mmol) in anisole (5.0 ml) and CH_2Cl_2 (2 ml) was added dropwise CF_3COOH (25 ml). After stirring at 0°C for 3 h the solution was evaporated at reduced pressure, diluted with EtOAc and extracted with a saturated solution of $NaHCO_3$ and brine. Aqueous basic phases were then acidified with 1 N HCl until pH 3 and the product was extracted with EtOAc. Organic layers were collected, dried over Na_2SO_4, and concentrated *in vacuo* to give **1a-d** and **2a-d** as white solids and crystallized by EtOAc/hexane. **1a** (38% yield); IR ν 1749 (C=O), 1649 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1,63 (d, 3H, *J* = 7.0 Hz), 4,76 (s, 2H), 5.11 (q, 1H, *J* = 7.0 Hz), 7.48 (m, 10H), 10.10 (brs, 1H); Anal. Calcd for $C_{17}H_{17}NO_4$ (299,32) C, 68.21; H, 5.72; N, 4.68; Found: C, 68.32; H, 5.63; N, 4.76. **1b** (45% yield); IR ν 1749 (C=O), 1649 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1,68 (d, 3H, *J* = 7.0 Hz), 4,96 (s, 2H), 5.11 (q, 1H, *J* = 7.0 Hz), 7.86 (m, 9H), 10.16 (brs, 1H); Anal. Calcd for $C_{17}H_{16}Cl\,NO_4$ (333,77) C, 61.18; H, 4.83; N, 4.20; Found: C, 61.07; H, 5.04; N, 4.38. **1c** (67% yield); IR ν 1757 (C=O), 1634 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1,66 (d, 3H, *J* = 7.0 Hz), 4,77 (s, 2H), 5.03 (q, 1H, *J* = 7.0 Hz), 7.63 (m, 9H), 9.11 (brs, 1H); Anal. Calcd for $C_{17}H_{16}Cl\,NO_4$ (333,77) C, 61.18; H, 4.83; N, 4.20; Found: C, 61.28; H, 4.71; N, 4.32. **1d** (71% yield); IR ν 1757 (C=O), 1634 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1,63 (d, 3H, *J* = 7.0 Hz), 3.83 (s, 3H), 4,87 (m, 2H), 4,91 (m, 1H), 6.84 (d 2H, *J* = 8.8 Hz), 7.19 (m, 4H), 7.68 (d, 2H, *J* = 8.8 Hz), 9.80 (brs, 1H); Anal. Calcd for $C_{17}H_{16}Cl\,NO_4$ (333,77) C, 61.18; H, 4.83; N, 4.20; Found: C, 61.32; H, 4.71; N, 4.09.

2a (40% yield); IR ν 1749 (C=O), 1650 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1,65 (d, 3H, *J* = 7.2 Hz), 3.83 (s, 3H), 4,87 (m, 2H), 4.91 (m, 1H), 6.84 (d 2H, *J* = 8.8 Hz), 7.19 (m, 4H), 7.68 (d, 2H, *J* = 8.8 Hz). Anal. Calcd for $C_{18}H_{19}NO_5$ (329.35) C, 65.64; H, 5.82; N, 4.25; Found: C, 65.58; H, 5.87; N, 4.45. **2b** (37% yield); IR ν 1749 (C=O), 1649 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1,64 (d, 3H, *J* = 7.2 Hz), 3.83 (s, 3H), 4,87 (m, 2H), 4.90 (m, 1H), 6.83 (d, 2H, *J* = 8.8 Hz), 7.16 (m, 4H), 7.70 (d, 2H, *J* = 8.8 Hz). Anal. Calcd for $C_{18}H_{18}ClNO_5$ (363,79) C, 59.43; H, 4.99; N, 3.85; Found:

C, 59.57; H, 4.87; N, 3.67. **2c** (55% yield); IR ν 1749 (C=O), 1649 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.64 (d, 3H, *J* = 7.2 Hz), 3.85 (s, 3H), 4,68 (m, 2H), 4.93 (m, 1H), 6.86 (d, 2H, *J* = 8.8 Hz), 7.09 (m, 4H), 7.67 (d, 2H, *J* = 8.8 Hz); Anal. Calcd for $C_{18}H_{18}ClNO_5$ (363,79) C, 59.43; H, 4.99; N, 3.85; Found: C, 59.24; H, 4.83; N, 3.78. **2d** (43% yield); IR ν 1749 (C=O), 1649 (N-C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1,63 (d, 3H, *J* = 7.2 Hz), 3.85 (s, 3H), 4,68 (m, 2H), 4.94 (m, 1H), 6.85 (d, 2H, *J* = 8.8 Hz), 7.10 (m, 4H), 7.67 (d, 2H, *J* = 8.8 Hz)Anal. Calcd for $C_{18}H_{18}ClNO_5$ (363,79) C, 59.43; H, 4.99; N, 3.85; Found: C, 59.57; H, 4.81; N, 3.66.

5.2.5 General procedure for the synthesis of ethyl 2-(3-benzyl-1-(arylmethyloxy)ureido)propanoate (13a,c,d).

To a stirred solution of the appropriate amino esters **8a,c,d** (1.16 mmol) in anhydrous CH_2Cl_2 (31 ml), Et_3N (1.16 mmol) and a catalytic amount of DMAP (5 mg) was added dropwise with a solution of phenylisocyanate (2.0 mmol) in anhydrous CH_2Cl_2 (2 ml). The reaction mixture was stirred at room temperature for 12h. The organic phase was evaporated and the residue was purified by column chromatography, eluting with a 3:2 hexane/ EtOAc mixture to yield pure derivatives **13a,c,d.**

13a (70% yield); IR ν 1742 (C=O), 1688 (N-C=ON) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.22 (t, 3H, *J* = 7.2 Hz), 1.54 (d, 3H, *J* = 7.2 Hz), 4.15 (q, 2H, *J* = 7.2 Hz), 4.30 (d, 2H, *J* = 5.6 Hz), 4.84 (m, 3H), 7.23 (m, 10H); Anal. Calcd for $C_{20}H_{24}N_2O_4$ (356.42); C, 67.40; H, 6.79; N, 7.86; Found: C, 67.58; H, 6.62; N, 7.74. **13c** (81% yield); IR ν 1742 (C=O), 1688 (N-C=ON) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.23 (t, 3H, *J* = 7.2 Hz), 1.53 (d, 3H, *J* = 7.2 Hz), 4.16 (q, 2H, *J* = 7.2 Hz), 4.33 (d, 2H, *J* = 5.6 Hz), 4.80 (m, 3H), 7.22 (m, 9H); Anal. Calcd for $C_{20}H_{23}ClN_2O_4$ (390.86); C, 61.46; H, 5.93; N, 7.17; Found: C, 61.62; H, 6.02; N, 7.31. **13d** (45% yield); IR ν 1742 (C=O), 1688 (N-C=ON) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.23 (t, 3H, *J* = 7.2 Hz), 1.53 (d, 3H, *J* = 7.2 Hz), 4.16 (q, 2H, *J* = 7.2 Hz), 4.31 (d, 2H, *J* = 5.6 Hz), 4.80 (m, 3H), 7.20 (m, 9H); Anal. Calcd for $C_{20}H_{23}ClN_2O_4$ (390.86); C, 61.46; H, 5.93; N, 7.17; Found: C, 61.38; H, 5.86; N, 7.08.

5.2.6 General procedure for the synthesis of 2-(3-benzyl-1-(arylmethyloxy)ureido)propanoic acid (3a,c,d).

A THF (3 ml) solution of **13a,c,d** (0.28 mmol) was treated with a solution of $NaHCO_3$ (0.83 mmol) in H_2O (1 ml). The reaction mixture was stirred at room temperature (24h) until the disappearance of the starting ester (TLC, hexane/EtOAc 1:1). The solution was concentrated and the aqueous residue was washed with Et_2O, acidified to pH 3 with 10% aq. HCl and extracted with $CHCl_3$. Evaporation of the filtered organic layers afforded a residue which was purified by trituration with *n*-hexane to give the desired acids **3a,c,d.**

3a (90% yield); IR ν 1796 (N-C=ON); 1734 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ: 1.26 (d, 3H, *J* = 7.0 Hz), 3.92 (q, 1H, *J* = 7.0 Hz), 4,63 (dd, 2H, *J* = 8.8 Hz), 4.98 (dd, 2H, *J* = 8.8 Hz), 7.34 (m, 10H). Anal. Calcd for $C_{18}H_{20}N_2O_4$ (328.37) C, 65.84; H, 6.14; N, 8.53; Found: C, 66.02; H, 6.28; N, 8.39. **3c** (85% yield); IR ν 1796 (N-C=ON); 1734 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ: 1.27 (d, 3H, *J* = 7.0 Hz), 3.92 (q, 1H, *J* = 7.0 Hz), 4,62 (dd, 2H, *J* = 8.8 Hz), 4.97 (dd, 2H, *J* = 8.8 Hz), 7.33 (m, 9H). Anal. Calcd for $C_{18}H_{19}ClN_2O_4$ (362.81) C, 59.59; H, 5.28; N, 7.72; Found: C, 59.72; H, 5.44; N, 7.60. **3d** (82% yield); IR ν 1796 (N-C=ON); 1734 cm^{-1} (C=O);); ^1H NMR ($CDCl_3$) δ: 1.25 (d, 3H, *J* = 7.0 Hz), 3.99 (q, 1H, *J* = 7.0 Hz), 4,60 (dd, 2H, *J* = 8.8 Hz), 4.95 (dd, 2H, *J* = 8.8 Hz), 7.32 (m, 9H). Anal. Calcd for $C_{18}H_{19}ClN_2O_4$ (362.81) C, 59.59; H, 5.28; N, 7.72; Found: C, 59.69; H, 5.48; N, 7.88.

5.2.7 General procedure for the synthesis of (E/Z)-ethyl 3-(arylmethyloxyimino)butanoate (15a,d,h).

A solution of the appropriate O-(arylmethyl)hydroxylamine hydrochloride **5a,d,h** (0.084 mol) and ethyl 3-oxobutanoate (0.084 mol) in anhydrous EtOH (65 ml) was treated dropwise, under stirring, with a solution of AcONa (0.126 mol) in anhydrous EtOH (27 ml). The resulting mixture was

stirred at room temperature for 24 h and then evaporated at reduced pressure. The residue was added to H_2O and extracted with Et_2O. The organic phase was separated, washed (5% aqueous HCl, 10% aqueous $NaHCO_3$ and H_2O) and evaporated to dryness to yield a 1:1 (GC) mixture of *E* and *Z* isomers (**15a,d,h**) which, without any further purification, was used for the following reaction.

(*E/Z*)**15a** (76% yield); IR ν 1742 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.23 (t, 3H, *J* = 7.2 Hz), 1.95, 2.03 (s, 3H), 3.36 e 3.43 (s, 2H), 4.13 (q, 2H, *J* = 7.2 Hz), 5.13 (s, 2H), 7.35 (m, 5H); Anal. Calcd for $C_{13}H_{17}NO_3$ (235.28); C, 66.36; H, 7.28; N, 5.95; Found: C, 65.28; H, 7.35; N, 5.71. (*E/Z*)**15d** (80% yield); IR ν 1726 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.23 (t, 3H, *J* = 7.2 Hz), 1.93, 2.03 (s, 3H), 3.33 e 3.43 (s, 2H), 4.16 (q, 2H, *J* = 7.2 Hz), 5.05 (s, 2H), 7.30 (m, 5H); Anal. Calcd for $C_{13}H_{16}ClNO_3$ (269,72); C, 57.89; H, 5.98; N, 5.19; Found: C, 57.78; H, 5.81; N, 5.36. (*E/Z*)**15h** (80% yield); IR ν 1726 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.23 (t, 3H, *J* = 7.2 Hz), 1.93, 1.96 (s, 3H), 3.20 e 3.30 (s, 2H), 3.80 (s, 3H), 4.85 (q, 2H, *J* = 7.2 Hz), 5.05 (s, 2H), 6.88 (d,2H, *J* = 8.0 Hz), 7.32 (d, 2H, *J* = 8.0 Hz); Anal. Calcd for $C_{14}H_{19}NO_4$ (265,13); C, 63.38; H, 7.22; N, 5.28; Found: C, 63.27; H, 7.36; N, 5.43.

5.2.8 General procedure for the synthesis of ethyl 3-(arylmethyloxyamino)butanoate (16a,d,h)

To a stirred and cooled (0°C) mixture of borane–trimethylamine complex (4.25 mmol) and the appropriate oximether (**15a,d,h**) (4.25 mmol) in absolute EtOH (10 ml) was added dropwise ethanolic hydrochloric acid (8 N, 34 ml). After stirring for 8h the solvent was evaporated and the residue was dissolved in CH_2Cl_2 and supplemented with solid $NaHCO_3$. After stirring for several hours, the suspension was filtered and the solvent was evaporated. The residue was purified by filtration through a silica gel column eluting with a hexane/EtOAc (4:1) mixture. Evaporation of the final fraction yielded pure **16a,d,h** as oils.

16a (90%): IR ν 1734(C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.11(d, 3H, *J* = 6.0 Hz), 1.23 (t, 3H, *J* = 7.0 Hz), 2.46 (t, 2H, *J* = 6.0 Hz), 3.46 (m, 1H), 4.13 (q, 2H, *J* = 7.0 Hz), 4.72 (s, 2H), 7.38 (m,5H);

Anal. Calcd for $C_{13}H_{19}NO_3$ (237,29); C, 65.80; H, 8.07; N, 5.90; Found: C, 65.72; H, 7.94; N, 5.86. **16d** (95%): IR ν 1734(C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.11(d, 3H, *J* = 7.0 Hz), 1.23 (t, 3H, *J* = 7.0 Hz), 2.45 (t, 2H, *J* = 6.0 Hz), 3.46 (q, 1H, *J* = 7.0 Hz), 4.13 (q, 2H, *J* = 7.0 Hz), 4.66 (s, 2H), 7.33 (m,4H); Anal. Calcd for $C_{13}H_{18}ClNO_3$ (271,74); C, 57.46; H, 6.68; N, 5.15; Found: C, 57.35; H, 6.81; N, 5.33. **16h** (97%): IR ν 1734(C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.08(d, 3H, *J* = 7.0 Hz), 1.18 (t, 3H, *J* = 7.0 Hz), 2.40 (t, 2H, *J* = 7.0 Hz), 3.46 (q, 1H, *J* = 7.0 Hz), 3.76 (s, 3H), 4.13 (q, 2H, *J* = 7.0 Hz), 4.56 (s, 2H), 6.87 (d, 2H, *J* = 8.0 Hz), 7.33 (d,2H, *J* = 8.0 Hz); Anal. Calcd for $C_{14}H_{21}NO_4$ (267,32); C, 62.90; H, 7.92; N, 5.24; Found: C, 62.79; H, 7.85; N, 5.36.

5.2.9 General procedure for the synthesis of ethyl 3-(3-benzyl-1-(arylmethyloxy)ureido)butanoate (17a,d,h)

To a stirred solution of the appropriate amino esters **16a,d,h** (6.32 mmol) in anhydrous CH_2Cl_2 (169 ml), Et_3N (6.32 mmol) and a catalytic amount of DMAP (5 mg) was added dropwise with a solution of benzylisocyanate (10.83 mmol) in anhydrous CH_2Cl_2 (20 ml). The reaction mixture was stirred at room temperature for 1 day and monitored by TLC (hexane/EtOAc 3:2). The organic phase was evaporated and the residue was purified by column chromatography, eluting with a 3:2 hexane/EtOAc mixture to yield pure derivatives **17a,d,h.**

17a (61% yield); IR ν 1742 (C=O), 1680 (N-C=ON) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.24 (t, 3H, *J* = 7.0 Hz), 1.28 (d, 2H, *J* = 7.6 Hz), 2.54 (m, 1H), 2.72 (m, 1H), 4.13 (q, 2H, *J* = 7.0 Hz), 4.29 (d, 2H, *J* = 5.6 Hz), 4.63 (m, 1H), 4.79 (m, 2H), 7.26 (m, 10H); Anal. Calcd for $C_{21}H_{26}N_2O_4$ (370,44); C, 68.09; H, 7.07; N, 7.56; Found: C, 68.18; H, 7.12; N, 7.63. **17d** (40% yield); IR ν 1742 (C=O), 1680 (N-C=ON) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.23 (t, 3H, *J* = 7.0 Hz), 1.28 (d, 2H, *J* = 7.6 Hz), 2.53 (m, 1H), 2.72 (m, 1H), 4.13 (q, 2H, *J* = 7.0 Hz), 4.29 (d, 2H, *J* = 5.6 Hz), 4.63 (m, 1H), 4.76 (m, 2H), 7.24 (m, 9H); Anal. Calcd for $C_{21}H_{25}ClN_2O_4$ (404,89); C, 62.30; H, 6.22; N, 6.92; Found: C, 62.46; H, 6.32; N, 7.04. **17h** (72% yield); IR ν 1742 (C=O), 1680 (N-C=ON) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.26 (t, 3H, *J* = 7.1 Hz), 1.28 (d, 2H, *J* = 7.6 Hz), 2.52 (m, 1H), 2.74 (m, 1H), 3.78 (s,

3H), 4.14 (q, 2H, *J* = 7.1 Hz), 4.31 (d, 2H, *J* = 5.6 Hz), 4.63 (m, 1H), 4.73 (m, 2H), 6.81 (d, 2H, *J* = 8.7 Hz), 7.18 (m, 2H), 7.19 (d, 2H, *J* = 8.7 Hz), 7.28 (m, 3H); Anal. Calcd for $C_{21}H_{25}ClN_2O_4$ (404,89); C, 62.30; H, 6.22; N, 6.92; Found: C, 62.46; H, 6.32; N, 7.04.

5.2.10 General procedure for the synthesis of 3-(3-benzyl-1-(benzyloxy)ureido)butanoic acid (4a,d,h)

A stirred THF (6.5 ml)/H_2O (2.6ml) solution of **17a,d,h** (1.25 mmol) was cooled (0°C) and treated dropwise with NaOH 1N (1.29 ml). The reaction mixture was stirred at 0°C until the disappearance of the starting ester (TLC, hexane/EtOAc 1:1) (4h). The solution was concentrated and the aqueous residue washed with Et_2O, cooled and acidified to pH 3 with 10% aq. HCl, then extracted with $CHCl_3$. Evaporation of the filtered organic layers afforded a residue which was crystallized with $CHCl_3$/ *n*-hexane to give the desired acids **4a,d,h.**

4a (97% yield); IR ν 1711 (C=O), 1649 (N-C=ON) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.29 (d, 3H, *J* = 6.7 Hz), 2.56 (m, 1H), 2.81 (m, 1H), 4.29 (m, 2H), 4.56 (m, 1H), 4.80 (s, 2H), 6.03 (m, 1H), 7.26 (m, 10H); Anal. Calcd for $C_{19}H_{22}N_2O_4$ (342,39); C, 66.65; H, 6.48; N, 8.18; Found: C, 66.53; H, 6.33; N, 7.95. **4d** (65% yield); IR ν 1711 (C=O), 1649 (N-C=ON) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.30 (d, 3H, *J* = 6.7 Hz), 2.56 (m, 1H), 2.80 (m, 1H), 4.29 (m, 2H), 4.55 (m, 1H), 4.76 (s, 2H), 6.04 (m, 1H) 7.19 (m, 9H); Anal. Calcd for $C_{19}H_{21}ClN_2O_4$ (376,12); C, 60.56; H, 5.62; N, 7.43; Found: C, 60.51; H, 5.49; N, 7.58. **4h** (89% yield); IR ν 1711 (C=O), 1649 (N-C=ON) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.29 (d, 3H, *J* = 6.7 Hz), 2.57 (m, 1H), 2.81 (m, 1H), 3.77 (s, 3H), 4.30 (m, 2H), 4.56 (m, 1H), 4.74 (s, 2H), 6.06 (m, 1H), 6.81 (d, 2H, *J* = 8.4 Hz), 7.16 (m, 2H), 7.19 (d, 2H, *J* = 8.4 Hz), 7.29 (m, 3H); Anal. Calcd for $C_{20}H_{24}N_2O_5$ (372,41); 64.50; H, 6.50; N, 7.52; Found: C, 64.38; H, 6.37; N, 7.41.

5.3 Biology

Aldose reductase (ALR2) and aldehyde reductase (ALR1) were obtained from Sprague-Dawley albino rats, 120–140 g body weight, supplied by Harlan Nossan, Italy. To minimize cross-contamination between ALR2 and ALR1 in the enzyme preparation, rat lenses, in which ALR2 is the predominant enzyme, and kidneys, where ALR1 shows the highest concentration, were used for the isolation of ALR2 and ALR1, respectively. Pyridine coenzyme, D,L-glyceraldehyde, and sodium D-glucuronate were from Sigma-Aldrich. Sorbinil is a gift from Pfizer, Groton CT. Tolrestat was obtained from Lorestat Recordati, Italy. All other chemicals were of reagent grade.

5.3.1 Aldose reductase (ALR2)

A purified rat lens extract was prepared as described previously[31-33]. Briefly, lenses were quickly removed from rats following euthanasia and were homogenized (Glas-Potter) in 3 volumes of cold deionized water. The homogenate was centrifuged at 12 000 rpm at 0–48C for 30 min. Saturated ammonium sulphate solution was added to the supernatant fraction to form a 40% solution, which was stirred for 30 min at 0–48C and then centrifuged at 12 000 rpm for 15 min. Following this same procedure, the recovered supernatant was subsequently fractionated with saturated ammonium sulfate solution using first a 50% and then a 75% salt saturation. The precipitate recovered from the 75% saturated fraction, possessing ALR2 activity, was redissolved in 0.05 M NaCl and dialyzed overnight in 0.05 M NaCl. The dialyzed material was used for the enzymatic assay.

5.3.2 Aldehyde reductase (ALR1)

Rat kidney ALR1 was prepared as reported earlier[31-33]. Briefly, kidneys were quickly removed from normally killed rats and homogenized (Glas-Potter) in 3 volumes of 10 mM sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose, 2.0 mM EDTA dipotassium salt, and2.5 mM β-mercaptoethanol. The homogenate was centrifuged at 12 000 rpm at 0–48C for 30 min, and the supernatant was subjected to a 40–75% ammonium sulphate fractionation, following the same procedure previously described for ALR2. The precipitate obtained from the 75% ammonium

sulfate saturation, possessing ALR1 activity, was redissolved in 50 volumes of 10 mM sodium phosphate buffer, pH 7.2, containing 2.0 mM EDTA dipotassium salt and 2.0 mM b-mercaptoethanol, and was dialyzed overnight using the same buffer. The dialyzed material was used in the enzymatic assay.

5.3.3 Enzymatic assays.

The activity of the two test enzymes was determined spectrophotometrically by monitoring the change in absorbance at 340 nm, which is due to the oxidation of NADPH catalyzed by ALR2 and ALR1. The change in pyridine coenzyme concentration/min was determined using a Beckman DU-64 kinetics software program (Solf Pack TM Module).

ALR2 activity was assayed at 30 °C in a reaction mixture containing 0.25 ml of 10 mM D,L-glyceraldehyde, 0.25 ml of 0.104 mM NADPH, 0.25 ml of 0.1 M sodium phosphate buffer (pH 6.2), 0.1 ml of enzyme extract, and 0.15 mL of deionized water in a total volume of 1 ml. All the above reagents, except D,L-glyceraldehyde, were incubated at 30°C for 10 min; the substrate was then added to start the reaction, which was monitored for 5 min.

ALR1 activity was determined at 37°C in a reaction mixture containing 0.25 ml of 20 mM sodium D-glucuronate, 0.25 ml of 0.12 mM NADPH, 0.25 ml of dialyzed enzymatic solution, and 0.25 ml of 0.1 M sodium phosphate buffer (pH 7.2) in a total volume of 1 ml.

5.3.4 Enzymatic inhibition

The inhibitory activity of the newly synthesized compounds against ALR2 and ALR1 was assayed by adding 0.1 ml of the inhibitor solution to the reaction mixture described above. All the inhibitors were solubilized in water and the solubility was facilitated by adjustment to a favorable pH. After complete solution, the pH was readjusted to 7. To correct for the non-enzymatic oxidation of NADPH and for absorption by the compounds tested, a reference blank containing all the above

assay components except the substrate was prepared. The inhibitory effect of the new derivatives was routinely estimated at a concentration of 10^{-4} M. Those compounds found to be active were tested at additional concentrations between 10^{-5} and 10^{-8} M. The determination of the IC_{50} values was performed by linear regression analysis of the logdose response curve, which was generated using at least four concentrations of the inhibitor, causing an inhibition between 20% and 80%, with two replicates at each concentration. The 95% confidence limits (95% CL) were calculated from t values for n – 2, where n is the total number of determinations.

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