Full Paper

Synthesis and Aldose Reductase Inhibitory Activities of Novel *O*-Substituted Hydroxyphenylacetic Acid Derivatives

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In continuation of our work aimed towards the preparation of novel aldose reductase inhibitors, several *O*-substituted hydroxyphenylacetic acid derivatives were investigated. The highest inhibitory activity was found for compounds **7b** and **7c** bearing a cyclohexylmethyl substituent. This result demonstrates that within these series, this moiety is a useful surrogate for the 4-bromo-2-fluorobenzyl residue which can be often found in potent aldose reductase inhibitors.

Keywords: Aldose reductase / Enzyme inhibitors / Inhibitor / Substituted phenylacetic acids

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Introduction

The number of people with diabetes mellitus is increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity [1]. Nevertheless, while prolonging life, antidiabetic therapy does not prevent the occurrence of disabling complications such as neuropathy, nephropathy, retinopathy, and cataracts. These problems arise from accumulation of sorbitol which is formed in the polyol pathway catalyzed by aldose reductase (EC 1.1.1.21, ALR 2). The latter is activated due to saturation of hexokinase, the key enzyme of the glycolytic pathway [2, 3]. These findings have led to the development of numerous aldose reductase inhibitors (ARIs) which offer the possibility to prevent or delay the occurrence of long-term complications. However, to date none is currently marketed for worldwide use due to lack of high efficacy and selectivity or due to toxicity [4].

Using previously defined pharmacophore requirements [5 – 9], we have recently designed compounds of type **I** (Fig. 1) as ARIs [10 – 12]. Modifications included the spacers X and Y, the substituent R, and the lipophilic as well as the hydrophilic (*i.e.* anionic) moieties. The latter two represent the suggested pharmacophores.

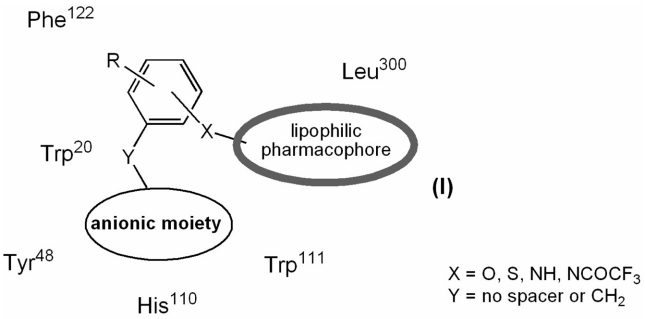

Figure 1. Model of ARIs of type **I** (characterized by hydrophilic and lipophilic pharmacophores) in the active site of the enzyme (the most important amino acids are Trp^{20}, Tyr^{48}, His^{110}, Trp^{111}, Phe^{122}, Leu^{300}).

So far, out of these series, the 3- and 4-(4-bromo-2-fluorobenzyloxy)phenyl acetic acids **II** (IC_{50} = 20.9 μM) and **III** (IC_{50} = 40.2 μM) emerged as the most potent aldose reductase inhibitors [12]. In order to get further insight into structure-activity relationships, we now became interested in derivatives bearing different lipophilic moieties.

Results and discussion

The compounds **2** were prepared starting from **1a – c** by benzylation in the presence of potassium carbonate in dry *N*,*N*-dimethylformamide. Whereas the use of phenylethyl bromide led to the desired compounds **3a – c**, employment of cyclohexylmethylbromide resulted in a

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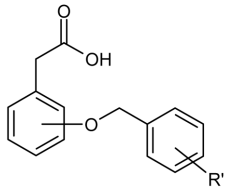
Table 1. Aldose reductase inhibition data of compounds **5**, **II**, and **III**.



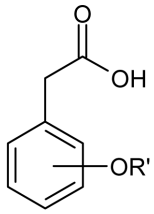
Compound	Position	R′	Inhibition at 100 μM	IC_{50} (μM)*
5aa	2	H	17%	
5ab	2	2-F	42%	
5ac	2	3-F	17%	
5ad	2	4-F	15%	
5ae	2	2-CF_3	24%	
5af	2	3-CF_3		58.6 (46.9–73.3)
5ag	2	4-CF_3	32%	
5ah	2	4-Cl	48%	
5ai	2	4-OCH_3	28%	
5ba	3	H		60.5 (58.8–62.3)
5bb	3	2-F		50.0 (35.6–70.4)
5bc	3	3-F		81.6 (60.3–110.3)
5bd	3	4-F		86.1 (85.3–86.9)
5be	3	2-CF_3		43.9 (34.2–56.4)
5bf	3	3-CF_3		38.6 (29.7–50.1)
5bg	3	4-CF_3		64.4 (52.2–79.4)
5bh	3	4-Cl		53.9 (40.0–72.2)
5bi	3	4-OCH_3		50.3 (48.2–52.7)
II**	3	4-Br-2F		20.9 (18.0–24.3)
5ca	4	H		78.8 (66.4–93.6)
5cb	4	2-F		73.9 (73.3–74.6)
5cc	4	3-F		92.4 (83.8–101.9)
5cd	4	4-F		100.2 (99.3–101.1)
5ce	4	2-CF_3		55.1 (50.0–60.8)
5cf	4	3-CF_3		50.7 (48.1–53.5)
5cg	4	4-CF_3		63.6 (56.9–71.1)
5ch	4	4-Cl		69.2 (49.7–96.5)
5ci	4	4-OCH_3		93.8 (77.3–113.7)
III**	4	4-Br-2F		40.2 (38.3–42.1)
Sorbinil				1.1 (1.0–1.2)

* IC_{50} values (95% CL).
** Data described in [12].

failure. However, **4a–c** became accessible by reaction of **1a–c** with the corresponding iodo derivative. Finally, the esters were converted to the desired free carboxylic acids **5–7** by alkaline hydrolysis. Inhibitory activities of the substituted phenylacetic acids **5aa–ci**, **6a–c**, and **7a–c** were evaluated in a spectrometric assay with D,L-glyceraldehyde as the substrate and NADPH as the cofactor.

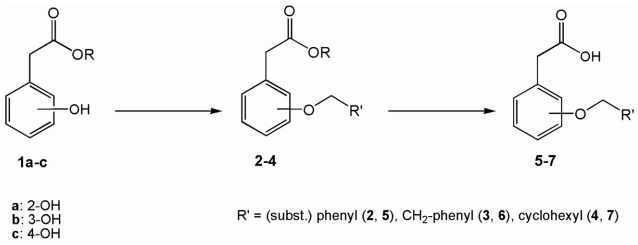
According to the *in vitro* results obtained (Tables 1 and 2), the substituted phenylacetic acids show satisfactory but less inhibitory activity than the reference sorbinil. Hence, the following conclusions can be drawn: The presence of an acidic proton appears to be an essential structural requisite since compounds devoid of any acidic proton **2–4** do not show any or only poor inhibitory activity. This, however, is in accordance with the known importance of a negative charge for the interaction of aldose reductase inhibitors with the active site of the enzyme [5–9]. Concerning the position of the lipophilic pattern on the benzene core, derivatives of the 3-hydroxy- and 4-hydroxyphenylacetic acids are favourable to inhibition of the enzyme compared to the corresponding 2-substituted congeners. Additionally, in most cases the former exhibit higher activity than the 4-isomers. Although no general structure-activity relationships can be given, introduction of substituents on the aryl unit of the benzyl residue exhibits an effect on the inhibitory activity. However, it can be seen that 3- and 4-fluoro are detrimental whereas 2- and 3-trifluoromethyl substituents are ben-

Table 2. Aldose reductase inhibition data of compounds **6** and **7**.



Compound	Position	R′	Inhibition at 100 μM	IC_{50} (μM)*
6a	2	2-phenylethyl	27%	
6b	3	2-phenylethyl		62.0 (56.5 – 68.1)
6c	4	2-phenylethyl		81.5 (66.1 – 100.6)
7a	2	cyclohexylmethyl	43%	
7b	3	cyclohexylmethyl		32.1 (23.1 – 44.5)
7c	4	cyclohexylmethyl		45.0 (44.1 – 45.9)
Sorbinil				1.1 (1.0 – 1.2)

* IC_{50} values (95% CL).



Scheme 1. Synthesis route for presented compounds **2**–**7**.

eficial. Formal introduction of a methylene group between X and the lipophilic pharmacophore (*i.e.* phenylethyl, **6a**–**c**) does not cause a significant change on the *in vitro* activity. Surprisingly, the most intriguing result was found in the case of cyclohexylmethyl substituted derivatives **7a**–**c** corresponding to a formal reduction of the benzyl residue brought a 1.5-to 2-fold increase in inhibitory activity [IC_{50} values: 32.1 μM (**7b**) *versus* 60.5 μM (**5ba**) and 45.0 μM (**7c**) *versus* 78.8 μM (**5ca**)].

Conclusions

In brief, we have discovered that the inhibitory activity of compounds **7b** and **7c** is in the same range as those of compounds **II** and **III** bearing a 4-bromo-2-fluorobenzyl moiety. Thus, the cyclohexylmethyl side chain seems to be a useful surrogate for the 4-bromo-2-fluorobenzyl residue which can be often found in potent aldose reductase inhibitors (*e.g.* zenarestat, ponalrestat, minalrestat, and AS-3201 [4]). To our knowledge, no comparable findings for aldose reductase inhibitors have been published before. Based on these results, further modifications on the nature of the lipophilic pharmacophore are in progress.

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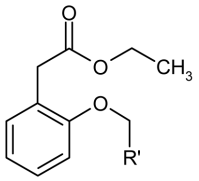
Experimental

Chemistry

Melting points were determined with a Kofler hot-stage microscope (C. Reichert, Vienna, Austria) and are uncorrected. Infrared spectra (KBr pellets) were recorded on a Mattson Galaxy Series FTIR 3000 spectrophotometer (Mattson Instruments, Inc., Madison, WI, USA). Mass spectra were obtained on a Finnigan MAT SSQ 7000 spectrometer (EI, 70 eV; Thermo Electron Corporation, Bremen, Germany). The ^{1}H-NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 solution in 5 mm tubes at 30°C on a Varian Gemini 200 spectrometer (199.98 MHz; Varian Inc., Palo Alto, CA, USA) with the deuterium signal of the solvent as the lock and TMS as internal standard. Chemical shifts are expressed in parts per million. Reactions were monitored by TLC using Polygram® SIL G/UV_{254} (Macherey-Nagel) plastic-backed plates (0.25 mm layer thickness). The yields given are not optimized. Light petroleum refers to the fraction of b.p. 40 – 60°C. Elemental analyses were performed by Mag. J. Theiner, "Mikroanalytisches Laboratorium", Faculty of Chemistry, University of Vienna, Austria, and the data for C and H are within ± 0.4% of the calculated values.

Compounds not listed were commercial samples of good grade. Cyclohexylmethyl iodide was prepared starting from the corresponding hydroxy compound according to [13] and [14]. The following compounds are already known but not tested as

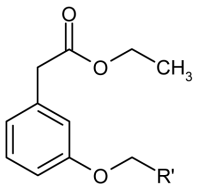
Table 3a. Physicochemical data of compounds of type **2a**.



N°	R′ Formula (MW)	Yield Purification Properties	IR (cm^{-1}) MS	^{1}H-NMR ($CDCl_3$)
2aa	C_6H_5 $C_{17}H_{18}O_3$ (270.33)	71% cc with CH_2Cl_2 followed by recryst. from EtOH colorless crystals mp 32–33°C	1733 270	7.44–7.20 (m, 7H, phenyl-H), 6.97–6.90 (m, 2H, phenyl-H), 5.08 (s, 2H, OCH_2), 4.10 (q, *J* = 7.1 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.67 (s, 2H, CH_2), 1.19 (t, *J* = 7.1 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2ab	2-F(C_6H_4) $C_{17}H_{17}FO_3$ (288.32)	86% recryst. from EtOH colorless crystals mp 56–57°C	1731 288	7.56–7.48 (m, 1H, phenyl-H), 7.36–6.92 (m, 7H, phenyl-H), 5.15 (s, 2H, OCH_2), 4.11 (q, *J* = 7.1 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.67 (s, 2H, CH_2), 1.19 (t, *J* = 7.1 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2ac	3-F(C_6H_4) $C_{17}H_{17}FO_3$ (288.32)	88% recryst. from EtOH colorless crystals mp 38–40°C	1733 288	7.39–7.13 (m, 5H, phenyl-H), 7.04–6.87 (m, 3H, phenyl-H), 5.08 (s, 2H, OCH_2), 4.13 (q, *J* = 7.2 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.68 (s, 2H, CH_2), 1.21 (t, *J* = 7.2 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2ad	4-F(C_6H_4) $C_{17}H_{17}FO_3$ (288.32)	43% cc with CH_2Cl_2 colorless oil	1735 288	7.42–7.35 (m, 2H, phenyl-H), 7.29–7.20 (m, 2H, phenyl-H), 7.11–6.89 (m, 4H, phenyl-H), 5.04 (s, 2H, OCH_2), 4.10 (q, *J* = 7.1 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.65 (s, 2H, CH_2), 1.19 (t, *J* = 7.1 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2ae	2-CF_3(C_6H_4) $C_{18}H_{17}F_3O_3$ (338.33)	59% recryst. from EtOH colorless crystals mp 38–41°C	1722 338	7.79–7.67 (m, 2H, phenyl-H), 7.60–7.53 (m, 1H, phenyl-H), 7.44–7.37 (m, 1H, phenyl-H), 7.28–7.19 (m, 2H, phenyl-H), 7.00–6.84 (m, 2H, phenyl-H), 5.30 (s, 2H, OCH_2), 4.13 (q, *J* = 7.2 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.71 (s, 2H, CH_2), 1.21 (t, *J* = 7.2 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2af	3-CF_3(C_6H_4) $C_{18}H_{17}F_3O_3$ (338.33)	39% recryst. from EtOH colorless crystals mp 54–56°C	1729 338	7.70–7.46 (m, 4H, phenyl-H), 7.31–7.22 (m, 2H, phenyl-H), 7.00–6.89 (m, 2H, phenyl-H), 5.13 (s, 2H, OCH_2), 4.11 (q, *J* = 7.1 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.68 (s, 2H, CH_2), 1.19 (t, *J* = 7.1 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2ag	4-CF_3(C_6H_4) $C_{18}H_{17}F_3O_3$ (338.33)	72% recryst. from EtOH colorless crystals mp 93–94°C	1725 338	7.64 (′d′, *J* = 8.4 Hz, 2H, phenyl-H), 7.54 (d, *J* = 8.4 Hz, 2H, phenyl-H), 7.29–7.21 (m, 2H, phenyl-H), 7.00–6.87 (m, 2H, phenyl-H), 5.14 (s, 2H, OCH_2), 4.11 (q, *J* = 7.1 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.68 (s, 2H, CH_2), 1.20 (t, *J* = 7.1 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2ah	4-Cl(C_6H_4) $C_{17}H_{17}ClO_3$ (304.78)	56% recryst. from EtOH colorless crystals mp 44–51°C	1725 304/306	7.35 (s, 4H, phenyl-H), 7.28–7.20 (m, 2H, phenyl-H), 6.98–6.87 (m, 2H, phenyl-H), 5.04 (s, 2H, OCH_2), 4.10 (q, *J* = 7.1 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.66 (s, 2H, CH_2), 1.20 (t, *J* = 7.1 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2ai	4-OCH_3(C_6H_4) $C_{18}H_{20}O_4$ (300.36)	59% cc with CH_2Cl_2 colorless oil	1728 300	7.36–7.19 (m, 4H, phenyl-H), 6.96–6.87 (m, 4H, phenyl-H), 5.01 (s, 2H, OCH_2), 4.10 (q, *J* = 7.1 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.82 (s, 3H, OCH_3), 3.65 (s, 2H, CH_2), 1.19 (t, *J* = 7.1 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)

cc = column chromatography

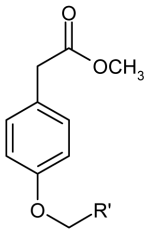
Table 3b. Physicochemical data of compounds of type **2b**.



N°	R′ Formula (MW)	Yield Purification Properties	IR (cm^{-1}) MS	^{1}H-NMR ($CDCl_3$)
2bb	2-F(C_6H_4) $C_{17}H_{17}FO_3$ (288.32)	75% cc with CH_2Cl_2 colorless oil	1734 288	7.56 – 7.47 (m, 1H, phenyl-H), 7.37 – 7.04 (m, 4H, phenyl-H), 6.93 – 6.88 (m, 3H, phenyl-H), 5.13 (s, 2H, OCH_2), 4.15 (q, *J* = 7.2 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.59 (s, 2H, CH_2), 1.25 (t, *J* = 7.2 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2bc	3-F(C_6H_4) $C_{17}H_{17}FO_3$ (288.32)	86% cc with CH_2Cl_2 colorless oil	1733 288	7.40 – 7.13 (m, 4H, phenyl-H), 7.05 – 6.84 (m, 4H, phenyl-H), 5.05 (s, 2H, OCH_2), 4.15 (q, *J* = 7.2 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.58 (s, 2H, CH_2), 1.25 (t, *J* = 7.2 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2bd	4-F(C_6H_4) $C_{17}H_{17}FO_3$ (288.32)	61% cc with CH_2Cl_2 colorless oil	1733 288	7.44 – 7.37 (m, 2H, phenyl-H), 7.28 – 7.20 (m, 1H, phenyl-H), 7.12 – 7.01 (m, 2H, phenyl-H), 6.91 – 6.84 (m, 3H, phenyl-H), 5.01 (s, 2H, OCH_2), 4.15 (q, *J* = 7.2 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.58 (s, 2H, CH_2), 1.25 (t, *J* = 7.2 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2be	2-CF_3(C_6H_4) $C_{18}H_{17}F_3O_3$ (338.33)	80% cc with CH_2Cl_2 colorless oil	1735 338	7.77 – 7.68 (m, 2H, phenyl-H), 7.61 – 7.53 (m, 1H, phenyl-H), 7.45 – 7.38 (m, 1H, phenyl-H), 7.29 – 7.21 (m, 1H, phenyl-H), 6.92 – 6.84 (m, 3H, phenyl-H), 5.27 (s, 2H, OCH_2), 4.15 (q, *J* = 7.2 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.59 (s, 2H, CH_2), 1.25 (t, *J* = 7.2 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2bf	3-CF_3(C_6H_4) $C_{18}H_{17}F_3O_3$ (338.33)	83% cc with CH_2Cl_2 colorless oil	1735 338	7.71 – 7.47 (m, 4H, phenyl-H), 7.30 – 7.22 (m, 1H, phenyl-H), 6.93 – 6.86 (m, 3H, phenyl-H), 5.11 (s, 2H, OCH_2), 4.15 (q, *J* = 7.2 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.60 (s, 2H, CH_2), 1.25 (t, *J* = 7.2 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2bg	4-CF_3(C_6H_4) $C_{18}H_{17}F_3O_3$ (338.33)	87% cc with CH_2Cl_2 colorless oil	1735 338	7.65 (d, *J* = 8.4 Hz, 2H, phenyl-H), 7.55 (d, *J* = 8.4 Hz, 2H, phenyl-H), 7.29 – 7.21 (m, 1H, phenyl-H), 6.92 – 6.84 (m, 3H, phenyl-H), 5.12 (s, 2H, OCH_2), 4.15 (q, *J* = 7.1 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.59 (s, 2H, CH_2), 1.25 (t, *J* = 7.1 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)
2bh	4-Cl(C_6H_4) $C_{17}H_{17}ClO_3$ (304.78)	60% cc with CH_2Cl_2 colorless oil	1735 304/306	7.35 (s, 4H, phenyl-H), 7.28 – 7.19 (m, 1H, phenyl-H), 6.92 – 6.82 (m, 3H, phenyl-H), 5.02 (s, 2H, OCH_2), 4.15 (q, *J* = 7.1 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.58 (s, 2H, CH_2), 1.25 (t, *J* = 7.1 Hz, 3H, OCH_2**C**$\mathbf{H}_3$**)**
2bi	4-OCH_3(C_6H_4) $C_{18}H_{20}O_4$ (300.36)	66% cc with CH_2Cl_2 colorless oil	1737 300	7.39 – 7.20 (m, 3H, phenyl-H), 6.95 – 6.85 (m, 5H, phenyl-H), 4.98 (s, 2H, OCH_2), 4.15 (q, *J* = 7.1 Hz, 2H, O**C**$\mathbf{H}_2$$CH_3$), 3.82 (s, 3H, OCH_3), 3.58 (s, 2H, CH_2), 1.25 (t, *J* = 7.1 Hz, 3H, OCH_2**C**$\mathbf{H}_3$)

cc = column chromatography

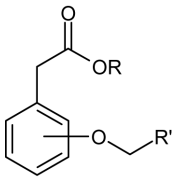
Table 3c. Physicochemical data of compounds of type **2c**.



N°	R′ Formula (MW)	Yield Purification Properties	IR (cm^{-1}) MS	^{1}H-NMR ($CDCl_3$)
2cb	2-F(C_6H_4) $C_{16}H_{15}FO_3$ (274.29)	73% recryst. from dipe/lp colorless crystals mp 29–32°C	1728 274	7.54–7.46 (m, 1H, phenyl-H), 7.37–7.03 (m, 5H, phenyl-H), 6.98–6.91 (m, 2H, phenyl-H), 5.12 (s, 2H, OCH_2), 3.69 (s, 3H, OCH_3), 3.57 (s, 2H, CH_2)
2cc	3-F(C_6H_4) $C_{16}H_{15}FO_3$ (274.29)	97% cc with CH_2Cl_2 colorless oil	1737 274	7.39–7.12 (m, 5H, phenyl-H), 7.05–6.89 (m, 3H, phenyl-H), 5.04 (s, 2H, OCH_2), 3.68 (s, 3H, OCH_3), 3.56 (s, 2H, CH_2)
2cd	4-F(C_6H_4) $C_{16}H_{15}FO_3$ (274.29)	76% recryst. from EtOH colorless crystals mp 42–47°C	1734 274	7.43–7.36 (m, 2H, phenyl-H), 7.23–7.16 (m, 2H, phenyl-H), 7.11–7.02 (m, 2H, phenyl-H), 6.95–6.88 (m, 2H, phenyl-H), 5.00 (s, 2H, OCH_2), 3.69 (s, 3H, OCH_3), 3.57 (s, 2H, CH_2)
2ce	2-CF_3(C_6H_4) $C_{17}H_{15}F_3O_3$ (324.30)	83% cc with CH_2Cl_2 colorless oil	1740 324	7.76–7.67 (m, 2H, phenyl-H), 7.59–7.52 (m, 1H, phenyl-H), 7.45–7.37 (m, 1H, phenyl-H), 7.24–7.17 (m, 2H, phenyl-H), 6.96–6.88 (m, 2H, phenyl-H), 5.26 (s, 2H, OCH_2), 3.69 (s, 3H, OCH_3), 3.57 (s, 2H, CH_2)
2cf	3-CF_3(C_6H_4) $C_{17}H_{15}F_3O_3$ (324.30)	73% cc with CH_2Cl_2 colorless oil	1737 324	7.70–7.46 (m, 4H, phenyl-H), 7.24–7.18 (m, 2H, phenyl-H), 6.97–6.89 (m, 2H, phenyl-H), 5.10 (s, 2H, OCH_2), 3.69 (s, 3H, OCH_3) 3.58 (s, 2H, CH_2)
2cg	4-CF_3(C_6H_4) $C_{17}H_{15}F_3O_3$ (324.30)	84% recryst. from EtOH colorless crystals mp 75–82°C	1728 324	7.64 (d, J = 8.1 Hz, 2H, phenyl-H), 7.54 (d, J = 8.1 Hz, 2H, phenyl-H), 7.24–7.17 (m, 2H, phenyl-H), 6.95–6.88 (m, 2H, phenyl-H), 5.11 (s, 2H, OCH_2), 3.69 (s, 3H, OCH_3), 3.57 (s, 2H, CH_2)
2ch	4-Cl(C_6H_4) $C_{16}H_{15}ClO_3$ (290.75)	74% recryst. from EtOH colorless crystals mp 53–58°C	1734 290/292	7.35 (s, 4H, phenyl-H), 7.23–7.16 (m, 2H, phenyl-H), 6.94–6.87 (m, 2H, phenyl-H), 5.01 (s, 2H, OCH_2), 3.68 (s, 3H, OCH_3), 3.56 (s, 2H, CH_2)
2ci	4-OCH_3(C_6H_4) $C_{17}H_{18}O_4$ (286.33)	55% recryst. from EtOH colorless crystals mp 54–60°C	1740 286	7.35 (′d′, J = 8.8 Hz, 2H, phenyl-H), 7.19 (′d′ J = 8.8 Hz, 2H, phenyl-H), 6.94–6.89 (m, 4H, phenyl-H), 4.97 (s, 2H, OCH_2), 3.81 (s, 3H, OCH_3), 3.68 (s, 3H, $COOCH_3$), 3.56 (s, 2H, CH_2)

cc = column chromatography; dipe = diisopropyl ether; lp = light petroleum.

Table 3d. Physicochemical data of compounds of type **3** and **4**.



N°	R	Position	R′ Formula (MW)	Yield Purification Properties	IR (cm^{-1}) MS	^{1}H-NMR ($CDCl_3$)
3a	C_2H_5	2	$CH_2C_6H_5$ $C_{18}H_{20}O_3$ (284.36)	33% cc with CH_2Cl_2 + lp (1/1) colorless oil	1734 284	7.32 – 7.14 (m, 7H, phenyl-H), 6.94 – 6.82 (m, 2H, phenyl-H), 4.17 (t, *J* = 6.8 Hz, 2H, O**CH**$_2$$CH_2$), 4.14 (q, *J* = 7.1 Hz, 2H, O**CH**$_2$$CH_3$), 3.59 (s, 2H, CH_2), 3.08 (t, *J* = 6.8 Hz, 2H, OCH_2**CH**$_2$), 1.25 (t, *J* = 7.1 Hz, 3H, OCH_2**CH**$_3$)
3b	C_2H_5	3	$CH_2C_6H_5$ $C_{18}H_{20}O_3$ (284.36)	11% cc with CH_2Cl_2 colorless oil	1734 284	7.36 – 7.17 (m, 6H, phenyl-H), 6.87 – 6.77 (m, 3H, phenyl-H), 4.17 (t, *J* = 7.1 Hz, 2H, O**CH**$_2$$CH_2$), 4.14 (q, *J* = 7.2 Hz, 2H, O**CH**$_2$$CH_3$), 3.56 (s, 2H, CH_2), 3.09 (t, *J* = 7.1 Hz, 2H, OCH_2**CH**$_2$), 1.24 (t, *J* = 7.2 Hz, 3H, OCH_2**CH**$_3$)
3c	CH_3	4	$CH_2C_6H_5$ $C_{17}H_{18}O_3$ (270.33)	41% cc with CH_2Cl_2 colorless oil	1736 270	7.35 – 7.14 (m, 7H, phenyl-H), 6.88 – 6.82 (m, 2H, phenyl-H), 4.16 (t, *J* = 7.2 Hz, 2H, O**CH**$_2$$CH_2$), 3.67 (s, 3H, OCH_3), 3.55 (s, 2H, CH_2), 3.09 (t, *J* = 7.2 Hz, 2H, OCH_2**CH**$_2$)
4a	C_2H_5	2	$C_6H_{11}C_{17}H_{24}O_3$ (276.38)	43% cc with CH_2Cl_2 + lp (1/1) colorless oil	1733 276	7.26 – 7.16 (m, 2H, phenyl-H), 6.90 (dd, *J* = 0.9 Hz, *J* = 7.6 Hz, 1H, phenyl H), 6.83 (d, *J* = 7.6 Hz, 1H, phenyl H), 4.14 (q, *J* = 7.0 Hz, 2H, O**CH**$_2$$CH_3$), 3.75 (d, *J* = 5.8 Hz, 2H, O**CH**$_2$$C_6H_{11}$), 3.61 (s, 2H, CH_2), 1.87 – 1.69 (m, 6H, alkyl-H), 1.39 – 0.98 (m, 5H, alkyl-H), 1.25 (t, *J* = 7.0 Hz, 3H, OCH_2**CH**$_3$)
4b	C_2H_5	3	$C_6H_{11}C_{17}H_{24}O_3$ (276.38)	24% cc with CH_2Cl_2 + lp (1/1) colorless oil	1734 276	7.25 – 7.17 (m, 1H, phenyl-H), 6.86 – 6.77 (m, 3H, phenyl-H), 4.15 (q, *J* = 7.2 Hz, 2H, O**CH**$_2$$CH_3$), 3.74 (d, *J* = 6.2 Hz, 2H, **OCH**$_2$$C_6H_{11}$), 3.57 (s, 2H, CH_2), 1.90 – 1.68 (m, 6H, alkyl-H), 1.40 – 0.96 (m, 5H, alkyl-H), 1.26 (t, *J* = 7.2 Hz, 3H, OCH_2**CH**$_3$)
4c	CH_3	4	$C_6H_{11}C_{16}H_{22}O_3$ (262.35)	22% cc with CH_2Cl_2 + lp (1/1) colorless oil	1728 262	7.17 (d, *J* = 8.4 Hz, 2H, phenyl-H), 6.87 – 6.80 (m, 2H, phenyl-H), 3.73 (d, *J* = 5.8 Hz, 2H, OCH_2-C_6H_{11}), 3.68 (s, 3H, OCH_3), 3.55 (s, 2H, CH_2), 1.89 – 1.72 (m, 6H, alkyl-H), 1.39 – 0.95 (m, 5H, alkyl-H)

cc = column chromatography; lp = light petroleum

aldose reductase inhibitors: ethyl 3-benzyloxyphenyl acetate **2ba** [15], methyl 4-benzyloxyphenyl acetate **2ca** [16], the benzyloxyphenyl acetic acids **5aa**, **5ba**, **5ca** [17] and (4-cyclohexylmethyloxy)phenyl acetic acid **7c** [18]. Moreover, **5ah** [CAS: 52804-00-9], **5ce** [CAS: 574005-40-6], **5cf** [CAS: 574005-41-7], **5ci** [CAS: 76968-91-7], and **6a** [19] were published as intermediates; however, no spectroscopic as well as analytical data are given.

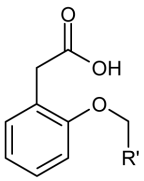
*General procedure for the O-substitution to prepare compounds of type **2 – 4***

Powdered potassium carbonate (4 equiv., 11.1mmol) was added to a solution of the appropriate compound **1** (2 equiv., 5.55 mmol) in 10 mL of dry *N,N*-dimethylformamide under atmosphere of nitrogen. After stirring for 30 min at room temperature, one equivalent of the appropriate aralkyl halide or cyclohexylmethyl iodide, respectively, was added and stirring was continued until TLC indicated no further conversion. Then, the mixture was poured into cold water, the mixture was acidified with 2N HCl and the product was extracted exhaustively with diethyl ether or dichloromethane, respectively. The organic layer was washed successively with 2N NaOH, water and brine, dried over anhydrous sodium sulphate, and evaporated to dryness. The residue thus obtained was purified as described in Tables 3a – d.

*General procedure for the synthesis of phenylacetic acids of type **5 – 7***

A solution of the appropriate ester **2 – 4** in 10 mL of ethanol was treated with 2N NaOH (1.4 equivalents) and stirred overnight at room temperature. The solvent was then evaporated, the residue treated with a small amount of water and the pH adjusted to 5 with 2N HCl. The mixture thus obtained was extracted with ethyl acetate, the organic layer was washed successively with water and brine, dried over anhydrous sodium sulphate, and

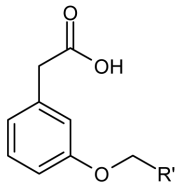
Table 4a. Physicochemical data of compounds of type **5a**.



N°	R′ Formula (MW)	Yield Purification Properties	IR (cm^{-1}) MS	^{1}H-NMR ($CDCl_3$)
5ab	2-F(C_6H_4) $C_{15}H_{13}FO_3$ (260.27)	60% recryst. from dipe colorless crystals mp 107 – 113°C	1706 260	7.51 – 7.43 (m, 1H, phenyl-H), 7.33 – 6.93 (m, 7H, phenyl-H), 5.15 (s, 2H, OCH_2), 3.71 (s, 2H, CH_2)
5ac	3-F(C_6H_4) $C_{15}H_{13}FO_3$ (260.27)	55% recryst. from dipe colorless crystals mp 85 – 90°C	1713 260	7.36 – 6.88 (m, 8H, phenyl-H), 5.08 (s, 2H, OCH_2), 3.73 (s, 2H, CH_2)
5ad	4-F(C_6H_4) $C_{15}H_{13}FO_3$ (260.27)	54% recryst. from dipe/lp colorless crystals mp 76 – 84°C	1710 260	7.38 – 7.20 (m, 4H, phenyl-H), 7.06 – 6.89 (m, 4H, phenyl-H), 5.03 (s, 2H, OCH_2), 3.70 (s, 2H, CH_2)
5ae	2-CF_3(C_6H_4) $C_{16}H_{13}F_3O_3$ (310.27)	63% recryst. from dipe/lp colorless crystals mp 100 – 107°C	1718 310	7.72 – 7.64 (m, 2H, phenyl-H), 7.53 – 7.21 (m, 4H, phenyl-H), 7.00 – 6.83 (m, 2H, phenyl-H), 5.26 (s, 2H, OCH_2), 3.74 (s, 2H, CH_2)
5af	3-CF_3(C_6H_4) $C_{16}H_{13}F_3O_3$ (310.27)	34% recryst. from dipe colorless crystals mp 96 – 102°C	1718 310	7.66 – 7.21 (m, 6H, phenyl-H), 7.00 – 6.88 (m, 2H, phenyl-H), 5.11 (s, 2H, OCH_2), 3.72 (s, 2H, CH_2)
5ag	4-CF_3(C_6H_4) $C_{16}H_{13}F_3O_3$ (310.27)	66% recryst. from dipe colorless crystals mp 109 – 115°C	1710 310	7.59 (d, *J* = 8.2 Hz, 2H, phenyl-H), 7.48 (d, *J* = 8.2 Hz, 2H, phenyl-H), 7.31 – 7.22 (m, 2H, phenyl-H), 7.01 – 6.86 (m, 2H, phenyl-H), 5.11 (s, 2H, OCH_2), 3.72 (s, 2H, CH_2)
5ah	4-Cl(C_6H_4) $C_{15}H_{13}ClO_3$ (276.72)	86% recryst. from dipe/lp colorless crystals mp 90 – 100°C	1714 276/278	7.30 (s, 4H, phenyl-H), 7.26 – 7.20 (m, 2H, phenyl-H), 6.99 – 6.87 (m, 2H, phenyl-H), 5.03 (s, 2H, OCH_2), 3.70 (s, 2H, CH_2)
5ai	4-OCH_3(C_6H_4) $C_{16}H_{16}O_4$ (272.30)	73% recryst. from dipe colorless crystals mp 128 – 132°C	1710 272	7.33 – 7.20 (m, 4H, phenyl-H), 6.97 – 6.85 (m, 4H, phenyl-H), 5.00 (s, 2H, OCH_2), 3.78 (s, 3H, OCH_3), 3.69 (s, 2H, CH_2)

dipe = diisopropyl ether; lp = light petroleum

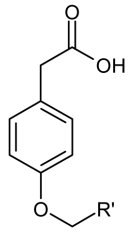
Table 4b. Physicochemical data of compounds of type **5b**.



N°	R′ Formula (MW)	Yield Purification Properties	IR (cm^{-1}) MS	^{1}H-NMR ($CDCl_3$)
5bb	2-F(C_6H_4) $C_{15}H_{13}FO_3$ (260.27)	52% recryst. from dipe colorless crystals mp 99 – 104°C	1700 260	7.54 – 7.46 (m, 1H, phenyl-H), 7.37 – 7.03 (m, 4H, phenyl-H), 6.93 – 6.88 (m, 3H, phenyl-H), 5.13 (s, 2H, OCH_2), 3.63 (s, 2H, CH_2)
5bc	3-F(C_6H_4) $C_{15}H_{13}FO_3$ (260.27)	66% recryst. from dipe colorless crystals mp 90 – 96°C	1696 260	7.40 – 7.13 (m, 4H, phenyl-H), 7.05 – 6.86 (m, 4H, phenyl-H), 5.05 (s, 2H, OCH_2), 3.63 (s, 2H, CH_2)
5bd	4-F(C_6H_4) $C_{15}H_{13}FO_3$ (260.27)	68% recryst. from dipe colorless crystals mp 124 – 129°C	1700 260	7.43 – 7.36 (m, 2H, phenyl-H), 7.29 – 7.21 (m, 1H, phenyl-H), 7.10 – 7.02 (m, 2H, phenyl-H), 6.91 – 6.87 (m, 3H, phenyl-H), 5.01 (s, 2H, OCH_2), 3.62 (s, 2H, CH_2)
5be	2-CF_3(C_6H_4) $C_{16}H_{13}F_3O_3$ (310.27)	39% recryst. from dipe colorless crystals mp 93 – 99°C	1700 310	7.77 – 7.67 (m, 2H, phenyl-H), 7.60 – 7.52 (m, 1H, phenyl-H), 7.44 – 7.37 (m, 1H, phenyl-H), 7.30 – 7.22 (m, 1H, phenyl-H), 6.92 – 6.85 (m, 3H, phenyl-H), 5.26 (s, 2H, OCH_2), 3.63 (s, 2H, CH_2)
5bf	3-CF_3(C_6H_4) $C_{16}H_{13}F_3O_3$ (310.27)	54% recryst. from dipe colorless crystals mp 93 – 101°C	1700 310	7.70 (s, 1H, phenyl-H), 7.63 – 7.45 (m, 3H, phenyl-H), 7.31 – 7.22 (m, 1H, phenyl-H), 6.93 – 6.87 (m, 3H, phenyl-H), 5.10 (s, 2H, OCH_2), 3.63 (s, 2H, CH_2)
5bg	4-CF_3(C_6H_4) $C_{16}H_{13}F_3O_3$ (310.27)	42% recryst. from dipe colorless crystals mp 97 – 102°C	1700 310	7.64 (d, J = 8.8 Hz, 2H, phenyl-H), 7.54 (d, J = 8.8 Hz, 2H, phenyl-H), 7.30 – 7.22 (m, 1H, phenyl-H), 6.92 – 6.86 (m, 3H, phenyl-H), 5.11 (s, 2H, OCH_2), 3.63 (s, 2H, CH_2)
5bh	4-Cl(C_6H_4) $C_{15}H_{13}ClO_3$ (276.72)	65% recryst. from dipe colorless crystals mp 115 – 124°C	1696 276/278	7.35 (s, 4H, phenyl-H), 7.29 – 7.21 (m, 1H, phenyl-H), 6.91 – 6.84 (m, 3H, phenyl-H), 5.02 (s, 2H, OCH_2), 3.62 (s, 2H, CH_2)
5bi	4-OCH_3(C_6H_4) $C_{16}H_{16}O_4$ (272.30)	63% recryst. from dipe colorless crystals mp 125 – 132°C	1700 272	7.38 – 7.31 (m, 2H, phenyl-H), 7.28 – 7.20 (m, 1H, phenyl-H), 6.94 – 6.85 (m, 5H, phenyl-H), 4.97 (s, 2H, OCH_2), 3.81 (s, 3H, OCH_3), 3.62 (s, 2H, CH_2)

dipe = diisopropyl ether

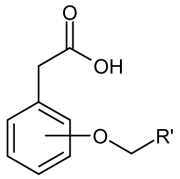
Table 4c. Physicochemical data of compounds of type **5c**.



N°	R′ Formula (MW)	Yield Purification Properties	IR (cm^{-1}) MS	^{1}H-NMR
5cb	2-F(C_6H_4) $C_{15}H_{13}FO_3$ (260.27)	50% recryst. from dipe colorless crystals mp 106 – 109°C	1700 260	($CDCl_3$) 7.53 – 7.45 (m, 1H, phenyl-H), 7.36 – 7.02 (m, 5H, phenyl-H), 6.95 (′d′, *J* = 8.8 Hz, 2H, phenyl-H), 5.12 (s, 2H, OCH_2), 3.59 (s, 2H, CH_2)
5cc	3-F(C_6H_4) $C_{15}H_{13}FO_3$ (260.27)	65% recryst. from dipe colorless crystals mp 98 – 103°C	1684 260	($CDCl_3$) 7.39 – 7.12 (m, 5H, phenyl-H), 7.05 – 6.88 (m, 3H, phenyl-H), 5.04 (s, 2H, OCH_2), 3.59 (s, 2H, CH_2)
5cd	4-F(C_6H_4) $C_{15}H_{13}FO_3 \times 0.2\ H_2O$ (263.87)	47% recryst. from dipe colorless crystals mp 128 – 130°C	1684 260	($CDCl_3$) 7.42 – 7.35 (m, 2H, phenyl-H), 7.24 – 7.16 (m, 2H, phenyl-H), 7.12 – 7.00 (m, 2H, phenyl-H), 6.95 – 6.88 (m, 2H, phenyl-H), 5.00 (s, 2H, OCH_2), 3.58 (s, 2H, CH_2)
5ce	2-CF_3(C_6H_4) $C_{16}H_{13}F_3O_3$ (310.27)	56% recryst. from dipe colorless crystals mp 94 – 99°C	1700 310	($CDCl_3$) 7.75 – 7.67 (m, 2H, phenyl-H), 7.59 – 7.52 (m, 1H, phenyl-H), 7.45 – 7.37 (m, 1H, phenyl-H), 7.21 (′d′, *J* = 8.4 Hz, 2H, phenyl-H), 6.96 – 6.89 (m, 2H, phenyl-H), 5.26 (s, 2H, OCH_2), 3.59 (s, 2H, CH_2)
5cf	3-CF_3(C_6H_4) $C_{16}H_{13}F_3O_3$ (310.27)	54% recryst. from dipe/lp colorless crystals mp 77 – 80°C	1700 310	($CDCl_3$) 7.69 (′s′, 1H, phenyl-H), 7.63 – 7.46 (m, 3H, phenyl-H), 7.27 – 7.18 (m, 2H, phenyl-H), 6.97 – 6.90 (m, 2H, phenyl-H), 5.09 (s, 2H, OCH_2), 3.59 (s, 2H, CH_2)
5cg	4-CF_3(C_6H_4) $C_{16}H_{13}F_3O_3$ (310.27)	46% recryst. from dipe colorless crystals mp 104 – 110°C	1706 310	($CDCl_3$) 7.66 – 7.52 (m, 4H, phenyl-H), 7.21 (d, *J* = 8.0 Hz, 2H, phenyl-H), 6.92 (d, *J* = 8.0 Hz, 2H, phenyl-H), 5.11 (s, 2H, OCH_2), 3.59 (s, 2H, CH_2)
5ch	4-Cl(C_6H_4) $C_{15}H_{13}ClO_3$ (276.72)	65% recryst. from dipe colorless crystals mp 144 – 157°C	1700 276/278	($CDCl_3$) 7.35 (s, 4H, phenyl-H), 7.19 (′d′, *J* = 8.4 Hz, 2H, phenyl-H), 6.91 (′d′, *J* = 8.4 Hz, 2H, phenyl-H), 5.01 (s, 2H, OCH_2), 3.58 (s, 2H, CH_2)
5ci	4-OCH_3(C_6H_4) $C_{16}H_{16}O_4$ (272.30)	77% recryst. from ea/THF colorless crystals mp 148 – 165°C	1710 272	(DMSO-d_6) 7.35 (d, *J* = 8.4 Hz, 2H, phenyl-H), 7.14 (d, *J* = 8.8 Hz, 2H, phenyl-H), 6.95 – 6.89 (m, 2H, phenyl-H), 6.91 (d, *J* = 8.4 Hz, 2H, phenyl-H), 4.98 (s, 2H, OCH_2), 3.74 (s, 3H, OCH_3), 3.46 (s, 2H, CH_2)

dipe = diisopropyl ether; ea = ethyl acetate; lp = light petroleum

Table 4d. Physicochemical data of compounds of type **6** and **7**.



N°	Position	R′ Formula (MW)	Yield Purification Properties	IR (cm^{-1}) MS	^{1}H-NMR ($CDCl_3$)
6a	2	$CH_2C_6H_5$ $C_{16}H_{16}O_3$ (256.30)	19% recryst. from dipe/lp colorless crystals mp 69 – 75°C	1700 256	7.31 – 7.16 (m, 7H, phenyl-H), 6.95 – 6.84 (m, 2H, phenyl-H), 4.19 (t, *J* = 6.8 Hz, 2H, O**CH**$_2$CH$_2$), 3.63 (s, 2H, CH_2), 3.08 (t, *J* = 6.8 Hz, 2H, OCH_2**CH**$_2$)
6b	3	$CH_2C_6H_5$ $C_{16}H_{16}O_3$ (256.30)	12% recryst. from dipe/lp colorless crystals mp 96 – 104°C	1700 256	7.36 – 7.18 (m, 6H, phenyl-H), 6.87 – 6.78 (m, 3H, phenyl-H), 4.17 (t, *J* = 7.1 Hz, 2H, O**CH**$_2$CH$_2$), 3.60 (s, 2H, CH_2), 3.09 (t, *J* = 7.1 Hz, 2H, OCH_2**CH**$_2$)
6c	4	$CH_2C_6H_5$ $C_{16}H_{16}O_3$ (256.30)	15% recryst. from dipe/lp colorless crystals mp 85 – 91°C	1700 256	7.35 – 7.15 (m, 7H, phenyl-H), 6.85 (′d′, *J* = 8.8 Hz, 2H, phenyl-H), 4.16 (t, *J* = 7.2 Hz, 2H, O**CH**$_2$CH$_2$), 3.57 (s, 2H, CH_2), 3.09 (t, *J* = 7.2 Hz, 2H, OCH_2**CH**$_2$)
7a	2	C_6H_{11} $C_{15}H_{20}O_3 \times 0.3\ H_2O$ (253.73)	24% recryst. from lp colorless crystals mp 58 – 60°C	1700 248	7.28 – 7.16 (m, 2H, phenyl-H), 6.94 – 6.83 (m, 2H, phenyl-H), 3.77 (d, *J* = 5.8 Hz, 2H, OCH_2), 3.66 (s, 2H, CH_2), 1.86 – 1.71 (m, 6H, alkyl-H), 1.32 – 0.82 (m, 5H, alkyl-H)
7b	3	C_6H_{11} $C_{15}H_{20}O_3$ (248.32)	19% recryst. from dipe/lp colorless crystals mp 75 – 80°C	1700 248	7.23 – 7.18 (m, 1H, phenyl-H), 6.86 – 6.78 (m, 3H, phenyl-H), 3.74 (d, *J* = 6.2 Hz, 2H, OCH_2), 3.61 (s, 2H, CH_2), 1.89 – 1.73 (m, 6H, alkyl-H), 1.43 – 0.95 (m, 5H, alkyl-H)
7c	4	C_6H_{11} $C_{15}H_{20}O_3 \times 0.2\ H_2O$ (251.93)	28% recryst. from dipe colorless crystals mp 95 – 103°C	1700 248	7.17 (′d′, *J* = 8.8 Hz, 2H, phenyl-H), 6.84 (′d′, *J* = 8.8 Hz, 2H, phenyl-H), 3.73 (d, *J* = 5.8 Hz, 2H, OCH_2), 3.57 (s, 2H, CH_2), 1.88 – 1.72 (m, 6H, alkyl-H), 1.39-0.94 (m, 5H, alkyl-H)

dipe = diisopropyl ether; lp = light petroleum; compound **7c** has been already described in [18].

evaporated to dryness under reduced pressure. The residue thus obtained was purified as described in the Tables 4a – d.

Aldose reductase inhibitory assay

NADPH, D,L-glyceraldehyde, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. DEAE-cellulose (DE-52) was obtained from Whatman. Sorbinil was a gift from Prof. Dr. Luca Costantino, University of Modena (Italy) and was used as standard [IC_{50} = 1.1 (± 0.1) μM]. All other chemicals were commercial samples of good grade. Calf lenses for the isolation of ALR 2 were obtained locally from freshly slaughtered animals. The enzyme was purified by a chromatographic procedure as previously described [20]. Briefly, ALR 2 was released by carving the capsule and the frozen lenses were suspended in potassium phosphate buffer pH 7 containing 5 mM DTT and stirred in an ice-cold bath for 2 h. The suspension was centrifuged at 4000 rpm at 4°C for 30 min and the supernatant was subjected to ion exchange chromatography on DE-52. Enzyme activity was assayed spectrophotometrically on a Cecil Super Aurius CE 3041 spectrophotometer (Cecil Instruments, Cambridge, England) by measuring the decrease in absorption of NADPH at 340 nm which accompanies the oxidation of NADPH catalysed by ALR 2. The assay was performed at 37°C in a reaction mixture containing 0.25 M potassium phosphate buffer pH 6.8, 0.38 M ammonium sulfate, 0.11 mM NADPH and 4.7 mM D,L-glyceraldehyde as substrate in a final volume of 1.5 mL. All inhibitors were dissolved in DMSO. The final concentration of DMSO in the reaction mixture was 1%. To correct for the nonenzymatic oxidation of NADPH, the rate of NADPH oxidation in the presence of all the components except the substrate was subtracted from each experimental rate. Each dose-effect curve was generated using at least three concentrations of inhibitor causing an inhibition between 20 and 80%. Each concentration was tested in duplicate and IC_{50} values as well as the 95% confidence limits (95% CL) were obtained by using CalcuSyn software [21] for dose effect analysis.

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