Eur. J. Med. Chem. 36 (2001) 697–703

PII: S0223-5234(01)01272-7/FLA

Original article

7-Hydroxy-2-substituted-4-*H*-1-benzopyran-4-one derivatives as aldose reductase inhibitors: a SAR study

Luca Costantino[a,*], Antonella Del Corso[b], Giulio Rastelli[a], J. Mark Petrash[c], Umberto Mura[b]

[a]Dipartimento di Scienze Farmaceutiche, Modena e Reggio Emilia University, Via Campi 183, I-41100 Modena, Italy
[b]Dipartimento di Fisiologia e Biochimica, Pisa University, Via S. Maria 55, I-56100 Pisa, Italy
[c]Department of Ophthalmology and Visual Sciences, Washington University, School of Medicine, St. Louis, MO 63110, USA

Received 16 May 2001; accepted 23 July 2001

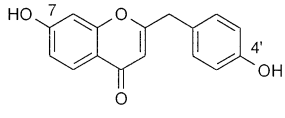
Abstract – On the basis of the results of molecular modelling studies performed on the aldose reductase (ALR2) inhibitor 7-hydroxy-2-(4′-hydroxybenzyl)-4*H*-1-benzopyran-4-one (compound **A**) bound at the active site of the enzyme, we synthesised and tested on bovine and human ALR2 several derivatives modified at position 2 of the benzopyran moiety, in order to confirm the hypothesised binding mode of this compound. The substitution of the methylene bridge with the isosteric sulphur substituent gives an active derivative, while substitution with a polar NH causes a decrease in inhibitory activity; this is in accordance to the previously reported structure in which the methylene linker was found to be adjacent to a hydrophobic aminoacid (Leu300). Among the substituents at 4′ position examined, the most favourable for inhibitory activity are those able to act as hydrogen bond donors, supporting the hypothesis of the importance of the interaction with Thr113 for the inhibition of the enzyme. © 2001 Éditions scientifiques et médicales Elsevier SAS

aldose reductase inhibitors / benzopyran-4-one derivatives / diabetic complications

1. Introduction

Aldose reductase (ALR2) is the first enzyme of the polyol pathway; it converts glucose to sorbitol. Sorbitol is then converted to fructose by the second enzyme of this pathway, sorbitol dehydrogenase. Glucose, metabolised through this pathway, has been linked to long-term diabetic complications such as cataract, retinopathy, nephropathy and neuropathy. Thus aldose reductase inhibitors (ARIs) have been proposed as therapeutic agents able to prevent the development of diabetic complications [1].

In a recent publication, we reported the discovery of a new and more potent ARI, a compound of formula **A** (IC50: 2.50 μM) [2], starting from the activity of the flavonoid Quercetin.



A

The 7-hydroxyl substituent was identified as the most important structural requirement for ALR2 inhibition, because in its dissociated form it hydrogen bonds to Tyr48 and His110. The 2-benzyl substituent was found, from molecular docking experiments, to fit optimally an additional hydrophobic pocket of the enzyme, lined by Trp111 and Leu300, with the 4′-hydroxy group hydrogen bonding to Thr113 [2, 3].

Given the interest in this class of compounds, owing to their lower acidity with respect to carboxylic acids (a well known class of ARIs), which potentially make for better pharmacokinetic properties, we decided to expand structure–activity relationships by modification of compound **A** in two different ways: first, in order to investigate the importance of the hydrogen bond between the 4′-hydroxyl and Thr113, we introduced different functional groups at 4′ position; then the methylene connecting the phenyl to the benzopyran-4-one nucleus was changed to sulphur and amine analogues.

* Correspondence and reprints
E-mail address: costantino.luca@unimo.it (L. Costantino).

Moreover, while IC50s were determined only on partially purified bovine ALR2 in our previous study and the modelling experiments were performed on human ALR2 [2], here inhibition constants (Ki) are determined also for both highly-purified bovine and human enzyme, so as to investigate possible differences between the inhibition of the two enzymes.

2. Chemistry

7-Hydroxy-2-(4′-hydroxyphenylamino)-4*H*-1-benzopyran-4-one (**1**) and 7-hydroxy-2-(4′-hydroxyphenylthio)-4*H*-1-benzopyran-4-one (**2**) were obtained following general methods [4, 5] starting from the intermediate 2-methylthio-7-[2-(tetrahydropyranyl)oxy]-4*H*-1-benzopyran-4-one (**1c**) (*figure 1*). Treatment of this intermediate with 4-aminophenol at 160 °C in the presence of a catalytic amount of *p*-toluenesulfonic acid yielded **1**, while compound **2** was obtained by treatment of the corresponding sulfoxide **2a** with 4-hydroxythiophenol, followed by deprotection of the 7-hydroxyl.

7-Hydroxy-2-(4′-chlorobenzyl)-4*H*-1-benzopyran-4-one (**3**) was synthesised by the reaction between 2-hydroxy-4-[2-(tetrahydropyranyl)oxy]acetophenone (**1a**) and methyl 4-chlorophenylacetate in the presence of NaH–pyridine (*figure 2*). Treatment of the intermediate 1,3-diketone with hydrochloric acid–acetic acid enabled the cyclisation and removal of the protecting group simultaneously, to yield **3**.

7-Hydroxy-2-(4′-nitrobenzyl)-4*H*-1-benzopyran-4-one (**4**) was synthesised starting from 2-hydroxy-4-methoxy(methoxycarbonyl)acetophenone (**4a**) by reaction with 4-nitrophenyl acetylchloride in the presence of $Mg(OEt)_2$ (*figure 3*) following a general

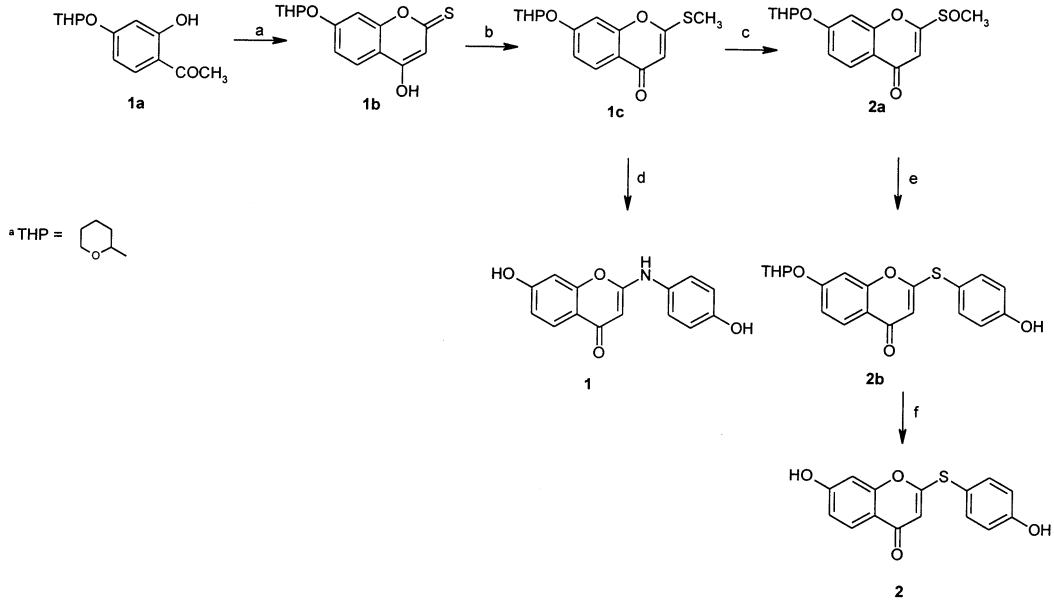


Figure 1. (a): CS_2, ^{t}BuOK; (b): CH_3I; (c): mCIPBA; (d): 4-aminophenol, Δ; (e): 4-hydroxythiophenol, K_2CO_3; (f): *p*-toluenesulfonic acid.

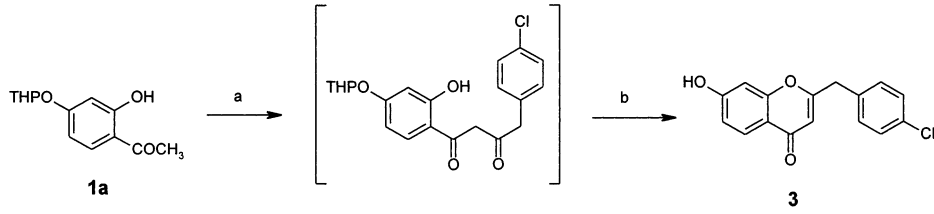


Figure 2. (a): $ClC_6H_4CH_2COOCH_3$, NaH; (b): CH_3COOH/HCl.

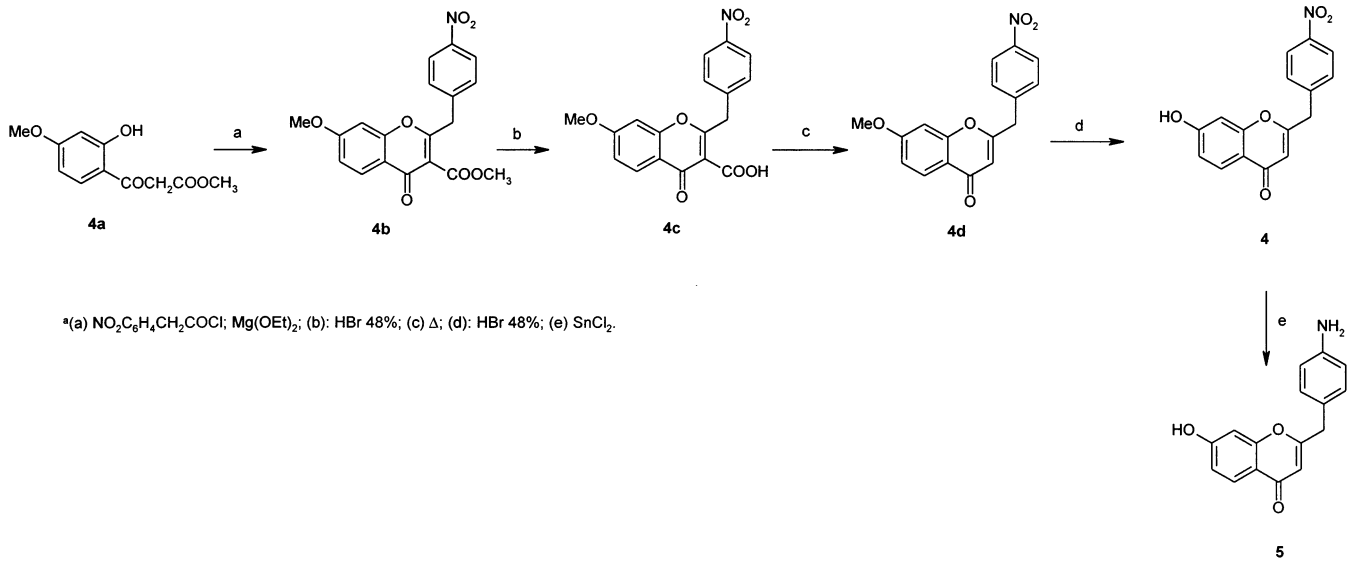
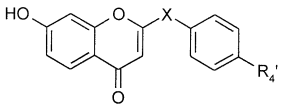


Figure 3. (a): $NO_2C_6H_4CH_2COCl$; $Mg(OEt)_2$; (b): HBr 48%; (c): Δ; (d): HBr 48%; (e) $SnCl_2$.

Table I. Inhibitory activity toward ALR2.



Compound	X	R'_4	IC50 (bovine enzyme) [a]	Ki (bovine enzyme) [b]	Ki (human enzyme) [b]
1	NH	OH	9.08 (7.21–11.43)	2.52 ± 0.17	0.76 ± 0.17
2	S	OH	1.17 (0.98–1.39)	0.29 ± 0.01	0.30 ± 0.02
3	CH_2	Cl	12.89 (11.23–14.80)	N.T. [c]	N.T. [c]
4	CH_2	NO_2	18.70 (15.92–21.97)	N.T. [c]	N.T. [c]
5	CH_2	NH_2	2.74 (2.23–3.37)	0.53 ± 0.05	0.43 ± 0.03

[a] Partially purified enzyme; IC50 values (μM) (95% C.L.).
[b] Highly purified enzyme; Ki values (μM) (± S.D.).
[c] N.T.: not tested.

procedure [6]. The methyl 7-methoxy-2-(4′-nitrobenzyl)-4*H*-1-benzopyran-4-one-3-carboxylate (**4b**) thus obtained was saponified using HBr 48%, then decarboxylated by heating in quinoline. Subsequent treatment with HBr 48% yielded the desired compound **4**. Reduction of **4** with $SnCl_2$ afforded compound **5**.

3. Results

The substitution of the methylene bridge in the lead compound **A** (IC50: 2.50 μM towards partially purified bovine lens ALR2 [2]) with the isosteric sulphur substituent (compound **2**) gives a compound with similar activity (IC50: 1.17 μM, *table I*) while substitution with a polar NH (compound **1**) causes a decrease in the inhibitory activity of about one order of magnitude (IC50: 9.08 μM, *table I*). The same trend is obtained from the Ki values (uncompetitive inhibition) determined on highly purified bovine and human ALR2 (*table I*). These conclusions are consistent with the previously reported structure of the complex between compound **A** and ALR2 [2], in which the methylene linker was found to be adjacent to a hydrophobic aminoacid (Leu300).

The effect of the substituents at 4′ position able to act as hydrogen bond donors to Thr113 (OH, compound **A**, NH_2, compound **5**) was compared with that of substituents which cannot act as donors like Cl and NO_2 (compounds **3** and **4**). While the derivative with the NH_2 moiety at 4′ (compound **5**) retains activity toward ALR2 in all inhibitory assays here performed (*table I*), the derivatives with Cl and NO_2 at 4′ (compounds **3** and **4**) are considerably less active (*table I*). Similar conclusions were obtained for 4′-OCH_3 (IC50: 7.01 μM, bovine enzyme [2]), and for 4′-CH_3 or 4′-CF_3, which are one order of magnitude less active than **A** on both bovine and human ALR2 [unpublished results]. Taken together, these results support the hypothesis that a hydrogen bond donor substituent interacting with Thr113 is important for activity.

In conclusion, the results obtained from this limited number of derivatives seem to confirm the hypothesised binding mode of this class of compounds, given the effects of substituents on the hydrophobic linker region and on the hydrogen bond donor region of Thr113. These conclusions hold for both bovine (partially or highly purified) enzyme and for human enzyme (*table I*). This last finding is consistent with the high sequence identity between the bovine and human enzymes [7, 8] (86% identity), with particular emphasis on the binding site, where the aminoacidic residues in contact with the inhibitors are completely conserved between the two related enzymes [2]. However, it should be noted that compound **1** is more active in the presence of human than bovine ALR2 (*table I*), and other studies are needed in order to explain this difference.

These data will be useful in the future development of new inhibitors based on the benzopyran-4-one scaffold.

4. Experimental protocols

4.1. Chemistry

Melting points were determined on a Buchi 510 melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on a Bruker AC200 spectrometer (Centro Interdipartimentale Grandi Strumenti, Modena University) in DMSO-d_6 solution. Chemical shifts are reported in ppm from tetramethylsilane as internal standard. Unless otherwise stated, spectra were recorded in DMSO-d_6 solution. *J* values are given in Hz. Microanalyses were carried out in the Microanalysis Laboratory of the Dipartimento di Scienze Farmaceutiche, Modena University. Analyses indicated by the symbol of the elements were within ±0.4% of the theoretical values. TLCs were performed on precoated silica gel F254 plates (Merck). Silica gel (Merck, 70-230 Mesh) was used for column chromatography.

*4.1.1. 4-Hydroxy-7-[2-tetrahydropyranyl)oxy]-2-thio-2H-1-benzopyran (**1b**)*

A solution of 2-hydroxy-4-[2-(tetrahydropyranyl)-oxy]acetophenone (**1a**) [9] (6.62 g, 28.1 mmol) and CS_2 (1.69 mL, 28.1 mmol) in anh. THF (20 mL) was slowly added to a stirred suspension of potassium *t*-butoxide (9.40 g, 83.8 mmol) in anh. THF (20 mL) at 15 °C. The red mixture was stirred overnight at r.t. under N_2 atmosphere and then poured into water, extracted with CH_2Cl_2 (2×20 mL), acidified to pH 5 and extracted with EtOAc (2×25 mL). The solvent was dried (Na_2SO_4) and removed under reduced pressure. Yield 4.00 g (51%), m.p. 121–123 °C (EtOAc), ^{1}H-NMR: 7.82 (1H, d, J = 8.78), 7.19 (1H, d, J = 2.81), 7.10 (1H, dd, J = 8.78, J = 2.81), 6.59 (1H, s), 5.70 (1H, broad s), 3.70 (2H, m), 1.75 (6H, m).

*4.1.2. 2-Methylthio-7-[2(tetrahydropyranyl)oxy]-4H-1-benzopyran-4-one (**1c**)*

A stirred mixture of **1b** (4.00 g, 14.4 mmol), K_2CO_3 (2.24 g, 16.2 mmol) in anh. acetone (50 mL) and iodomethane (4.00 mL, 64.3 mmol) was refluxed for 30 min. The mixture was then filtered, water was added to the filtrate and the mixture was extracted with $CHCl_3$ (3×25 mL); the solvent was dried (Na_2SO_4) and removed under reduced pressure and the residue purified by column chromatography (CH_2Cl_2:CH_3OH 97.5:2.5). Yield 2.20 g (52%) (viscous oil); ^{1}H-NMR ($CDCl_3$): 8.07 (1H, m), 7.05 (2H, m), 6.16 (1H, s), 5.55 (1H, m), 3.80 (2H, m), 2.54 (3H, s), 1.80 (6H, m).

*4.1.3. 7-Hydroxy-2-(4′hydroxyphenylamino)-4H-1-benzopyran-4-one (**1**)*

A mixture of **1c** (0.070 g, 0.24 mmol), 4-aminophenol (0.50 g, 4.58 mmol), *p*-toluenesulfonic acid monohydrate (0.010 g, 0.05 mmol) and ethylene glycol (1 mL) was heated at 160 °C for 20 min. The mixture was then cooled, water was added and the precipitate was filtered, washed with water and purified by means of column chromatography (cyclohexane:EtOAc 87.5:12.5, then acetone 100%). Yield 0.010 g (16%), m.p. 254 °C (dec.);

^{1}H-NMR: 10.46 (1H, s), 9.51 (2H, s), 7.75 (1H, d, $J = 8.54$), 7.10 (2H, m), 6.75 (4H, m), 5.14 (1H, s). Analysis: $C_{15}H_{11}NO_4$: C, H, N.

4.1.4. 2-Methylsulphinyl-7-[2-(tetrahydropyranyl)oxy]-4H-1-benzopyran-4-one (**2a**)

A solution of *m*-chloroperbenzoic acid (1.11 g, 85% pure, 5.47 mmol) in $CHCl_3$ (10 mL) was slowly added to a solution of **1c** (1.60 g, 5.5 mmol) in $CHCl_3$ (10 mL) maintained at −10 °C. After 2 h, the suspension was filtered and the filtrate was washed with 5% $NaHCO_3$ solution, dried (Na_2SO_4) and the solvent evaporated under reduced pressure. The residue was then purified by means of column chromatography (CH_2Cl_2:CH_3OH 97.5:2.5). Yield 0.90 g (53%) (oil), ^{1}H-NMR ($CDCl_3$): 8.05 (1H, m), 7.00 (2H, m), 6.78 (1H, s), 5.50 (1H, broad s), 3.70 (2H, m), 2.92 (3H, s), 1.70 (6H, m).

4.1.5. 2-(4-Hydroxyphenylthio)-7-[2-tetrahydropyranyl)-oxy]-4H-1-benzopyran-4-one (**2b**)

4-Hydroxythiophenol (0.13 g, 1.03 mmol) in anh. acetone (2 mL) and K_2CO_3 (0.14 g, 1.02 mmol) were added at r.t. to a stirred solution of **2a** (0.20 g, 0.65 mmol) in anh. acetone (10 mL). After 8 h, HCl 1N was added until pH 5. The precipitate was filtered and washed with water. Yield 0.15 g (63%), m.p. 182–183 °C; ^{1}H-NMR: 10.31 (1H, s), 7.86 (1H, m), 7.55 (2H, m), 7.15 (2H, m), 6.95 (2H, m), 5.70 (1H, broad s), 5.56 (1H, s), 3.77 (2H, m), 1.80 (6H, m).

4.1.6. 7-Hydroxy-2-(4-hydroxyphenylthio)-4H-1-benzopyran-4-one (**2**)

p-Toluenesulfonic acid monohydrate (0.010 g, 0.053 mmol) was added to a suspension of **2b** (0.15 g, 0.41 mmol) in CH_3OH (20 mL), and refluxed for 15 min. Water was then added to the solution thus obtained and the precipitate was filtered and washed with water. Yield 0.10 g (86%), m.p. 278–280 °C (acetone), ^{1}H-NMR: 10.80 (1H, s), 10.26 (1H, s), 7.80 (1H, d, $J = 8.69$), 7.52 (2H, m), 6.97 (2H, m), 6.88 (1H, dd, $J = 8.69$, $J = 2.43$), 6.75 (1H, d, $J = 2.43$), 5.51 (1H, s). Analysis $C_{15}H_{10}O_4S$: C, H.

4.1.7. 7-Hydroxy-2-(4′-chlorobenzyl)-4H-1-benzopyran-4-one (**3**)

A solution of 2-hydroxy-4-[2-(tetrahydropyranyl)-oxy]acetophenone (**1a**) [9] (1.0 g, 4.2 mmol) and methyl 4-chlorophenyl acetate (0.86 g, 4.6 mmol) in anh. pyridine (8 mL) was added dropwise to a well-stirred suspension of NaH (60% dispersion in mineral oil) (0.50 g, 12.6 mmol) in anh. pyridine (8 mL). When the reaction subsided, the mixture was heated at 90 °C for 15 min. After cooling, the mixture was decomposed in 2N HCl and extracted with CH_2Cl_2 (3×25 mL). The combined organic layers were washed with 1N HCl (2×30 mL) and water (30 mL) and dried (Na_2SO_4); then the solvent was removed under reduced pressure. The residue was then purified by means of column chromatography (cyclohexane:EtOAc 75:25). Yield 0.76 g (63%), m.p. 216–217 °C, ^{1}H-NMR: 10.60 (1H, s), 7.83 (1H, d, $J = 8.21$), 7.40 (4H, m), 6.90 (1H, dd, $J = 8.21$, $J = 2.30$), 6.80 (1H, d, $J = 2.30$), 6.08 (1H, s), 4.00 (2H, s). Analysis: $C_{16}H_{11}ClO_3$: C, H.

4.1.8. Methyl 7-methoxy-2-(4′-nitrobenzyl)-4H-1-benzopyran-4-one 3-carboxylate (**4b**)

A solution of 2-hydroxy-4-methoxy-(methoxycarbonyl)acetophenone (**4a**) [6] (6.00 g, 26.8 mmol) in anh. EtOH (30 mL) was added to a suspension of $Mg(OEt)_2$ (3.07 g, 26.8 mmol) in anh. EtOH (100 mL). A white precipitate formed. EtOH was then removed under reduced pressure, and anh. benzene was added (70 mL), followed by 4-nitrophenylacetyl chloride (5.35 g, 26.9 mmol) dissolved in benzene (30 mL). The mixture was refluxed for 3 h, then it was cooled and poured in 10% acetic acid in water (300 mL) at 0 °C. The suspension was extracted with EtOAc (3×100 mL), the organic layer was dried (Na_2SO_4) and the solvent removed under reduced pressure. Finally, the resultant residue was purified by column chromatography (cyclohexane:EtOAc 50:50). Yield 6.00 g, (61%), m.p. 146–147 °C, ^{1}H-NMR ($CDCl_3$): 8.26 (2H, m), 8.12 (1H, d, $J = 8.92$), 7.60 (2H, m), 7.00 (1H, dd, $J = 8.92$, $J = 2.38$), 6.77 (1H, d, $J = 2.38$), 4.20 (2H, s), 3.99 (3H, s), 3.92 (3H, s).

4.1.9. 7-Methoxy-2-(4′-nitrobenzyl)-4H-1-benzopyran-4-one-3-carboxylic acid (**4c**)

A suspension of **4b** (0.50 g, 1.4 mmol) in HBr 48% (15 mL) was heated at 110–120 °C for 30 min. After cooling, the precipitate was filtered and washed with water. Yield 0.40 g (83%), m.p. 185–186 °C, ^{1}H-NMR: 8.22 (2H, m), 8.05 (1H, m), 7.69 (2H, m), 7.11 (2H, m), 4.51 (2H, s), 3.90 (3H, s).

4.1.10. 7-Methoxy-2-(4′-nitrobenzyl)-4H-1-benzopyran-4-one (**4d**)

A solution of **4c** (0.40 g, 1.12 mmol) in quinoline (65 mL) was heated at 180 °C for 10 min, then cooled and poured in water (100 mL) containing conc. HCl (12

mL). The suspension was extracted with EtOAc (3×40 mL), the organic phase was dried (Na_2SO_4) and the solvent removed under reduced pressure. Yield 0.25 g (71%), m.p. 153–155 °C (EtoAc), ^{1}H-NMR ($CDCl_3$): 8.25 (2H, m), 8.10 (1H, d, $J = 8.90$), 7.51 (2H, m), 6.95 (1H, dd, $J = 2.91$, $J = 8.90$), 6.80 (1H, d, $J = 2.91$), 6.16 (1H, s), 4.04 (2H, s), 3.91 (3H, s).

*4.1.11. 7-Hydroxy-2-(4′-nitrobenzyl)-4H-1-benzopyran-4-one (**4**)*

A solution of **4d** (0.5 g, 1.6 mmol) in HBr 48% (15 mL) was heated at 140 °C for 5 h. After cooling, the precipitate was collected and washed with water. Yield 0.22 g (46%), m.p. 210 °C (dec.) (acetone–petroleum ether); IR (cm^{-1}) (Nujol): 1639, 1555, 1299; ^{1}H-NMR: 10.76 (1H, s), 8.25 (2H, m), 7.85 (1H, d, $J = 8.90$), 7.66 (2H, m), 6.90 (1H, dd, $J = 2.33$, $J = 8.90$), 6.75 (1H, d, $J = 2.33$), 6.18 (1H, s), 4.18 (2H, s). Analysis: $C_{16}H_{11}NO_5$: C, H, N.

*4.1.12. 7-Hydroxy-2-(4′-aminobenzyl)-4H-1-benzopyran-4-one (**5**)*

$SnCl_2 \cdot 2H_2O$ (0.76 g, 3.4 mmol) was added to a solution of **4** (0.20 g, 0.7 mmol) in EtOH. The resulting suspension was stirred and refluxed for 20 min, then the solvent was removed under reduced pressure. The residue was diluted with water (7 mL), filtrated and the filtrate was brought to pH 6 with NaOH 0.1N and extracted with EtOAc (3×30 mL); the organic phase was dried (Na_2SO_4) and the solvent removed under reduced pressure. Yield 0.1 g (56%), m.p. 215–218 °C; IR (cm^{-1}) (Nujol): 3465, 3358, 1643; ^{1}H-NMR: 10.70 (1H, s), 7.86 (1H, d, $J = 8.60$), 7.04 (2H, m), 6.88 (1H, dd, $J = 8.60$, $J = 2.20$), 6.80 (1H, d, $J = 2.20$), 6.55 (2H, m), 6.00 (1H, s), 5.00 (2H, broad s), 3.75 (2H, s). Analysis: $C_{16}H_{13}NO_3$: C, H, N.

4.2. Enzyme inhibition studies

Recombinant human aldose reductase was expressed as previously described [10]. Both bovine lens and human recombinant aldose reductase were purified to electrophoretic homogeneity following the same chromatographic steps as previously described for the bovine enzyme (ion exchange chromatography on DEAE-DE52, followed by affinity chromatography on Orange Matrex A and finally Sephadex G75) [11]. The pure enzymes were stored at 4 °C in 10 mM sodium phosphate buffer pH 7.0 in the presence of 2 mM DTT.

Protein concentration was evaluated by the method of Bradford [12]. The electrophoretic homogeneity of enzyme preparations was assessed by SDS-PAGE according to the method of Laemmli [13], and gels were stained according to the method of Wray et al. [14].

The assay for aldose reductase activity was performed at 37 °C using 4.7 mM D,L-glyceraldehyde as substrate, in 0.25 M sodium phosphate buffer pH 6.8 containing 0.11 mM NADPH, 0.38 M ammonium sulphate and 0.5 mM EDTA. One unit of enzyme activity is the amount of enzyme which catalyses the oxidation of 1 μmol of NADPH/min.

The sensitivity of aldose reductase to different compounds was tested in the above assay conditions in the presence of the inhibitor dissolved at appropriate concentration in DMSO. The concentration of DMSO in the assay mixture was kept constant at 1%. IC50 values on partially purified bovine lens aldose reductase were obtained using crude enzyme after the ion exchange chromatography on DEAE-DE52 purification step [2]. Each log dose–inhibition curve was generated using at least four concentrations of 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 [2]. For the determination of Ki, double reciprocal plots with D,L-glyceraldehyde as variable substrate at fixed concentrations of NADPH were obtained. For each inhibitor at least three different concentrations were used.

References

[1] Yabe-Nishimura C., Pharmacol. Rev. 50 (1998) 21–33.

[2] Costantino L., Rastelli G., Gamberini M.C., Vinson J.A., Bose P., Iannone A., Staffieri M., Antolini L., Del Corso A., Mura U., Albasini A., J. Med. Chem. 42 (1999) 1881–1893.

[3] Rastelli G., Antolini L., Benvenuti S., Costantino L., Bioorg. Med. Chem. 8 (2000) 1151–1158.

[4] Roma G., Di Braccio M., Grossi G., Marzano C., Simonato M., Bordin F., Il Farmaco 53 (1998) 494–503.

[5] Bantick J.R., Suschitzky J.L., J. Heterocyclic Chem. 18 (1981) 679–684.

[6] Cushman M., Nagarathnam D., Burg D.L., Gealen R.L., J. Med. Chem. 34 (1991) 798–806.

[7] Schade S.S., Sherrell L.E., Williams T.R., Kezdy F.J., Heinrikson R.L., Grimshaw C.E., Doughty C.C., J. Biol. Chem. 265 (1990) 3628–3635.

[8] Bohren K.M., Bullock B., Wermuth B., Gabbay K.H., J. Biol. Chem. 264 (1989) 9547–9551.

[9] Sogawa S., Nihro Y., Ueda H., Miki T., Matsumoto H., Satoh T., Biol. Pharm. Bull. 17 (1994) 251–256.

[10] Petrash J.M., Harter T.M., Devine C.S., Olins P.O., Bhatnagar A., Liu S., Srivastava S.K., J. Biol. Chem. 267 (1992) 24833–24840.

[11] Del Corso A., Barsacchi D., Giannessi M., Tozzi M.G., Camici M., Mura U., Arch. Biochem. Biophys. 283 (1990) 512–518.

[12] Bradford M.M., Anal. Biochem. 72 (1976) 248–254.

[13] Laemmli U.K., Nature 227 (1970) 680–685.

[14] Wray W., Boulikas T., Wray W.P., Hancock R., Anal. Biochem. 118 (1981) 197–203.