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ORIGINAL PAPER

Chalcones and their pyrazine analogs: synthesis, inhibition of aldose reductase, antioxidant activity, and molecular docking study

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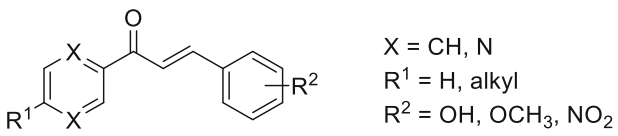
Abstract

Chalcones and their pyrazine analogs synthesized by Claisen–Schmidt condensation were tested for inhibition of aldose reductase, which is the key enzyme in the development of secondary diabetic complications. The most active compounds exerted IC_{50} values within the micromolar scale, and their interactions with the enzyme were described in a molecular docking study. Antioxidant activity of several representative compounds was explored in DPPH (2,2-diphenyl-1-picryl-hydrazyl) assay, revealing significant scavenging for 4-hydroxy-substituted derivatives endowed with electron-donating methoxy substituent in position 3 of the ring B. To conclude, the novel chalcones hydroxylated and methoxylated in the B-ring and their pyrazine analogs exhibited significant aldose reductase inhibition activity, albeit lower in comparison with the reference epalrestat. Medium antioxidant activity (not exceeding the antioxidant efficacy of the standard Trolox) was shown by the representative compounds tested.

Graphical abstract



Keywords Aldol reactions · Enones · Molecular modeling · Structure–activity relationships

Introduction

Chalcones (Fig. 1), chemical derivatives of 1,3-diphenyl-prop-2-en-1-ones, can be obtained by isolation from plants through isolation or by means of laboratory synthesis [1, 2]. The natural chalcones belong to the group of flavonoids and they are biosynthetically related to them. Therefore, a range of bio-activities is associated with various chalcone derivatives and their analogs [3, 4]. Despite being natural products, chalcones are easily synthesized, thus enabling a multiplicity of substitutions [5].

Aldose reductase (EC 1.1.1.21 [6], AKR1B1 [7], ALR2) acts as the first enzyme of the polyol metabolic pathway catalyzing the reduction of glucose to sorbitol with NADPH as a coenzyme [8]. Osmolyte sorbitol accumulates intracellularly, which may result in disruption of the

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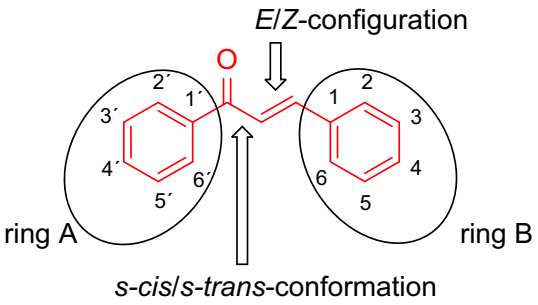


Fig. 1 General pattern of chalcone derivatives with rings and their numbering indicated; the depicted isomer represents (*E*)-(*s-cis*)-chalcone

osmotic homeostasis in tissues exposed to hyperglycemia. The polyol pathway thus represents one of the key molecular mechanisms contributing to the glucose toxicity responsible for the development of chronic diabetic complications such as diabetic neuropathy, nephropathy, retinopathy and cataracts. Carboxylic acid derivatives (e.g., drug epalrestat used for the prophylaxis of diabetic nephropathy and retinopathy, Fig. 2), spirohydantoins and related cyclic amides and phenolic derivatives (related to benzopyran-4-one and chalcones) belong to the chemical pattern exhibiting the inhibition of ALR2 [9]. Synthetic chalcones inhibiting ALR2 were the subject of a patent application [10]; several previous studies have been reviewed in Refs. [11, 12].

An antioxidant effect is advantageous for ALR2 inhibitors, as increased activity of ALR2 in the polyol pathway leads to oxidative stress [13]. Some natural chalcones (Fig. 3) perform this type of activity, e.g., isoliquritigenin, licochalcone B and D (all from *Glycyrrhiza* spp., Fabaceae), broussochalcone A (*Broussonetia papyrifera* L., Moraceae), xanthohumol (*Humulus lupulus* L., Cannabaceae) [8] and other natural prenylated chalcones [14]. At least one hydroxy substitution and α,β-unsaturated bond seem to be essential for phenoxy radical formation and its stabilization [8]. Concurrently, isoliquiritigenin is an ALR2 inhibitor [15].

The goal of this work was the synthesis and study of a group of chalcone derivatives and their pyrazine analogs (Scheme 1) as potential inhibitors of aldose reductase. These compounds have never been tested on this type of activity. Inhibitory activity on ALR2 was expected, especially with chalcones containing an acidic functional group in the ring (phenolic hydroxyl). Additionally, one representative with an electron-donating group (methoxyl) and one derivative with an electron-withdrawing group (nitro group) were included in the study. A pyrazine ring in place of a benzene ring A of chalcones represents an interesting alternative in the search for new aldose reductase inhibitors. Pyrazine is a stable and sufficiently aromatic analog ring to benzene in comparison with other diazines (pyrimidine and pyridazine) [16]. It is very slightly basic (pK_a 0.65, − 6.6) [17] and might help to improve the water-solubility of chalcones. A comparison of two series with identical substitution could also provide information about the importance of the ring analogy. The antioxidant effect of the synthesized compounds was also explored as a beneficial side effect of potential ALR2 inhibitors [13].

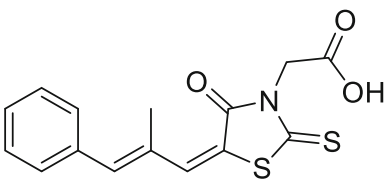


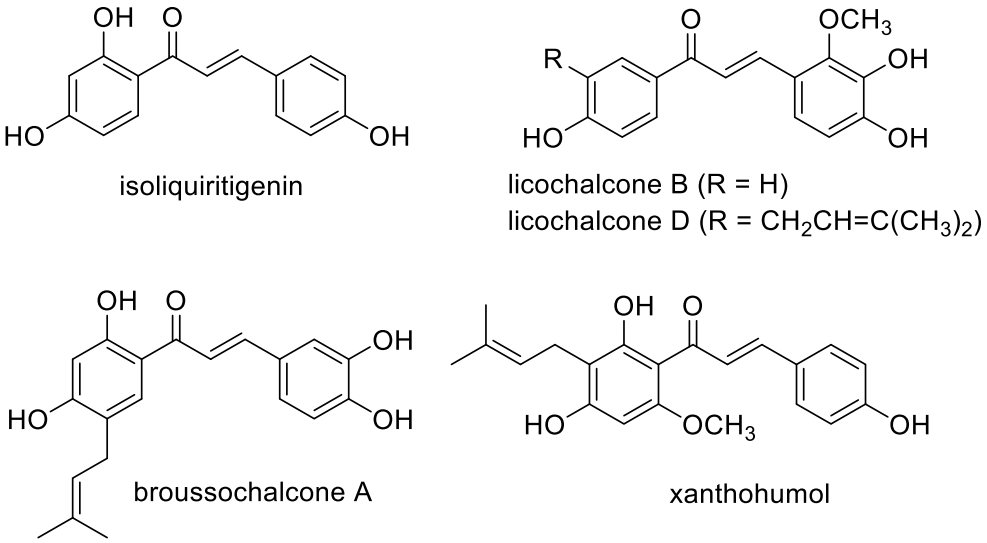
Fig. 2 Structure of epalrestat, drug used for the prophylaxis of diabetic nephropathy and retinopathy

Results and discussion

Chemistry

Chalcones **1**–**14** were synthesized by Claisen–Schmidt condensation combining aryl/pyrazine-2-yl methyl ketone and variously substituted aldehyde under different conditions (Scheme 1, for substitution see Table 1). Method A—methanol with a sodium hydroxide pellet [18–20] was used for synthesis of 1,3-diphenylprop-2-en-1-ones **2**, **3**, **5**, and **6**. The procedure differed from the published ones in the working-up (acidification necessary) and purifying process (column chromatography). Method B—pyridine with diethylamine was applied for synthesis of substituted 3-phenyl-1-pyrazin-2-ylprop-2-en-1-ones **7**–**14**, as was previously shown [21–24], and for the synthesis of (*E*)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one (**1**) as well. We faced some difficulties throughout the synthesis of 3-(4-hydroxy-3-methoxyphenyl)-1-phenylprop-2-en-1-one (**4**). Neither method A nor method B resulted in the desired product, despite method A being modified in the amount of solvent or sodium hydroxide and working-up (column chromatography). Using potassium hydroxide as a basic catalyst [25] helped obtain the compound **4**. All the methods used a basic catalysis. The best yields were obtained with method A (31–67%) in comparison with methods C (40%) and B (10–44%). Method B is more suitable for synthesis of 3-phenyl-1-pyrazinylprop-2-en-1-ones, with the lower yields being caused by the chromatographic cleaning process. In all cases, *E*-isomers were obtained. This was confirmed by the value of interaction constants *J* in ^{1}H NMR spectra.

Fig. 3 Natural chalcones with antioxidant activity



Scheme 1

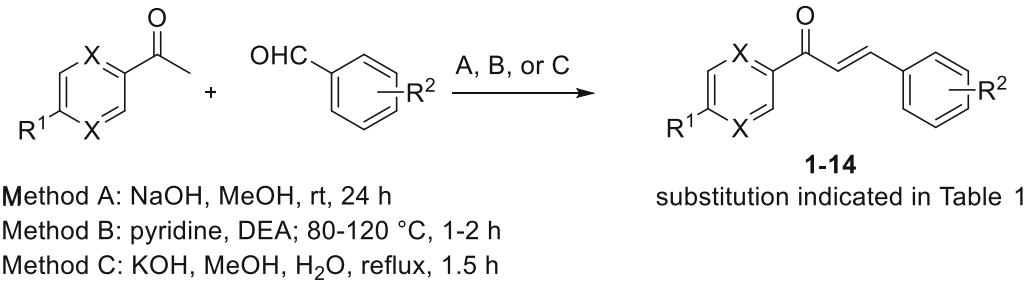


Table 1 Inhibition of ALR2 isolated from rat eye lenses by the synthesized compounds in comparison with the reference epalrestat

Compound	X	Ring A substitution R^1	Ring B substitution R^2	Inhibition of ALR2 $IC_{50}/\mu M$
1	CH	–	2-OH	> 100
2	CH	–	3-OH	> 100
3	CH	–	4-OH	$25 < IC_{50} < 50$
4	CH	–	4-OH-3-OCH_3	> 100
5	CH	–	4-OCH_3	> 50
6	CH	–	4-NO_2	> 50
7	N	–	2-OH	> 100
8	N	–	3-OH	$25 < IC_{50} < 50$
9	N	–	4-OH	$25 < IC_{50} < 50$
10	N	$CH_2CH_2CH_3$	4-OH	$25 < IC_{50} < 50$
11	N	$CH(CH_3)_2$	4-OH	18.59 ± 1.81
12	N	–	4-OH-3-OCH_3	> 100
13	N	–	4-OCH_3	> 100
14	N	–	4-NO_2	> 50
Epalrestat	–	–	–	0.23 ± 0.02

Enzyme inhibition

All synthesized compounds were tested for inhibition of aldose reductase isolated from rat eye lenses. The inhibitory concentrations corresponding to 50% inhibition (IC_{50}) in comparison with the reference epalrestat are provided in Table 1. From the results, it is apparent that in the series of chalcone derivatives **1**–**6** a significant inhibition was recorded only for derivative **3**, with 4-hydroxy substituent at the B-ring. In the group of the pyrazine analogs, significant inhibition activity was recorded for derivatives hydroxylated in the 3- or 4-position of the B-ring; for 4-hydroxy-substituted derivatives, the highest inhibition was recorded for compound **11** with an *iso*-propyl substituent in position 5 of the A-ring. Based on IC_{50} values, a one to two orders lower inhibition efficacy was recorded for the tested compounds in comparison to previously published chalcones hydroxylated in the 4- and 2-positions in ring A [26–28].

Antioxidant activity

Four representative compounds of the tested group were inspected for antioxidant action in the DPPH (2,2-diphenyl-1-picrylhydrazyl) test. As a weak hydrogen atom abstractor, DPPH is considered a good kinetic model for peroxyl $ROO^{\cdot}$ radicals [29]. DPPH assay is routinely used as a primary screening test for antiradical efficacy. The time-dependent decrease of the characteristic absorbance of the ethanolic solution of DPPH at 518 nm in the presence of the compounds tested and in comparison with the reference Trolox is shown in Fig. 4. The initial fast absorbance decrease, corresponding to the transfer of the most labile H atoms, was followed by a much slower absorbance decline. This represented the residual antiradical activity of the antioxidant degradation products. The initial velocity of DPPH decolorization was used as a marker of the antiradical activity and is shown in Table 2. As shown in Fig. 4 and Table 2, significant antioxidant activity was recorded for 4-hydroxy-substituted derivatives **4** and **12** endowed with electron-donating methoxy substituent in position 3. Nonetheless, the efficacy was lower than that of standard Trolox.

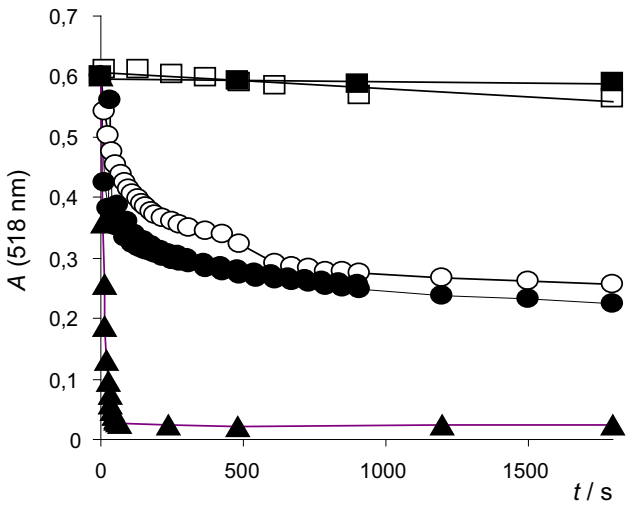


Fig. 4 Continual absorbance decrease of ethanolic solution of DPPH radical (50 μM) in the presence of 100 μM concentration of the compounds tested (filled square) **3**; (filled circle) **4**; (open square) **9**; (open circle) **12**; (filled triangle) Trolox

Table 2 Antiradical activity measured by the initial velocity of DPPH absorbance decrease at 518 nm

Compound	$(-\Delta A/s)$
3	< 0.001
4	0.0032 ± 0.0002
9	< 0.001
12	0.0023 ± 0.0004
Trolox	0.0157 ± 0.0006

The ethanolic solution of the DPPH radical (50 μM) was incubated in the presence of the 100 μM compound tested. Absorbance decrease at 518 nm during the first 70-s interval (**3**, **4** and **9**, **12**) or 30-s interval (Trolox) was determined. Results are mean values ± SD of the three measurements.

Molecular modeling

To explain the experimental ALR2 inhibition data obtained, molecular modeling in silico was performed. Conformation analysis revealed that, owing to stronger conjugation in pyrazine-based chalcones, their molecules are strictly planar. In the chalcone group, rotation of the benzene ring is looser, resulting in slight distortion of the ring. This conclusion is in accordance with our previous results concerning conformation analysis of 3-(2-hydroxyphenyl)-1-pyrazin-2-ylprop-2-en-1-ones [30]. Other authors concerned with conformational analysis calculations [31–33] or crystallographic studies [34–40] on substituted 1,3-diphenylprop-2-en-1-ones or their analogs [41–43] revealed that the dihedral angle between the aromatic rings amounts to 0°–20°. Another stereochemical characteristic of chalcones represents the conformation of a single bond connecting carbonyl functional group and double bond in the linkage chain [31] (indicated in Fig. 1). In solid phase, the chalcones usually exert in (*E*)-*s*-*cis* form, whereas in solution, a minor part of the compound can occur as (*E*)-*s*-*trans*-conformer [33]. Based on the IR spectra, we have concluded that the structures characterized here were in *s*-*cis* conformation due to the missing band around 1636 cm^{-1}. Compounds **7**, **9**, and **12** described earlier have shown the presence of both conformers [30].

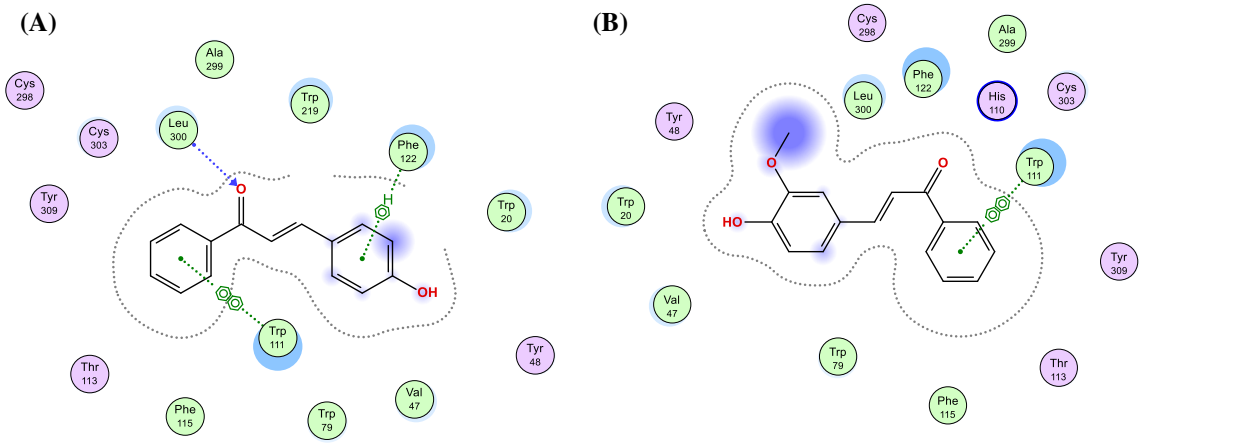


Fig. 5 **a** Key interactions of compound **3** with the active binding site of aldose reductase: O interacts with N of LEU300 as H-acceptor at a distance of 2.75 Å and energy − 11.7 kJ/mol, ring B interacts with PHE122 via π–H interaction at a distance of 4.02 Å and energy − 2.9 kJ/mol, and ring A interacts with 6-ring of TRP111 via π–π interaction at a distance of 3.49 Å. **b** Key interactions of compound **4** with the active binding site of aldose reductase: ring A interacts with 6-ring of TRP111 via π–π interaction at a distance of 3.45 Å

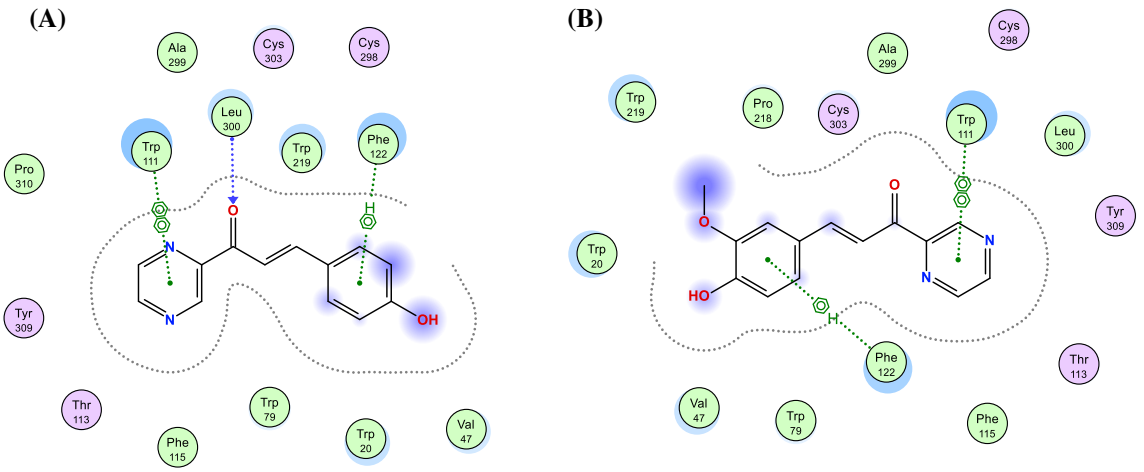


Fig. 6 **a** Key interactions of compound **9** with the active binding site of aldose reductase: O interacts with N of LEU300 as an H-acceptor at a distance of 3.04 Å and energy − 3.8 kJ/mol, ring B interacts with PHE122 via π–H interaction at a distance of 3.85 Å and energy − 2.9 kJ/mol, and ring A interacts with 6-ring of TRP111 via π–π interaction at a distance of 3.35 Å. **b** Key interactions of compound **12** with the active binding site of aldose reductase: ring B interacts with PHE122 via π–H interaction at a distance of 4.08 Å, and ring A interacts with ring A of TRP111 via π–π interaction at a distance of 3.41 Å

Molecular docking of the compounds tested into a human aldose reductase (pdb code: 1z3n) binding site revealed a common feature in the binding of the compounds. All derivatives were found in the cavity of the active site anchored by the π–π interaction of a pyrazine or benzene ring with TRP111. Lacking negatively charged group, the compounds were not visibly attracted to the positively charged nicotinamide. Derivatives **3** and **9** with a benzene ring substituted only by a hydroxyl group were able to approach LEU300 by carbonyl oxygen to create a hydrogen bond (Figs. 5, 6).

Methoxy-substituted derivatives **4** and **12** were not able to adopt a proper position for the formation of the hydrogen bond. However, **12** was bound via π–H interaction with PHE122, as was **9** (Fig. 6).

Considering the binding mode of our compounds, we detected an interaction with TRP111 which is able to interact with the hydrophilic (pyrazine in **9** and **12**) as well with the hydrophobic part (benzene in **3** and **4**) of chalcone derivatives. TRP111 and LEU300 are shared by the catalytic anionic pocket and by the hydrophobic pocket [44, 45]. Although some authors claim that LEU300 tends to bind to lipophilic scaffold of inhibitors [46], in the case of our compounds it interacted slightly with carbonyl oxygen via hydrogen bonding. Generally, amino acids TYR48, HIS110 [9, 46, 47] and TRP111 [46] are

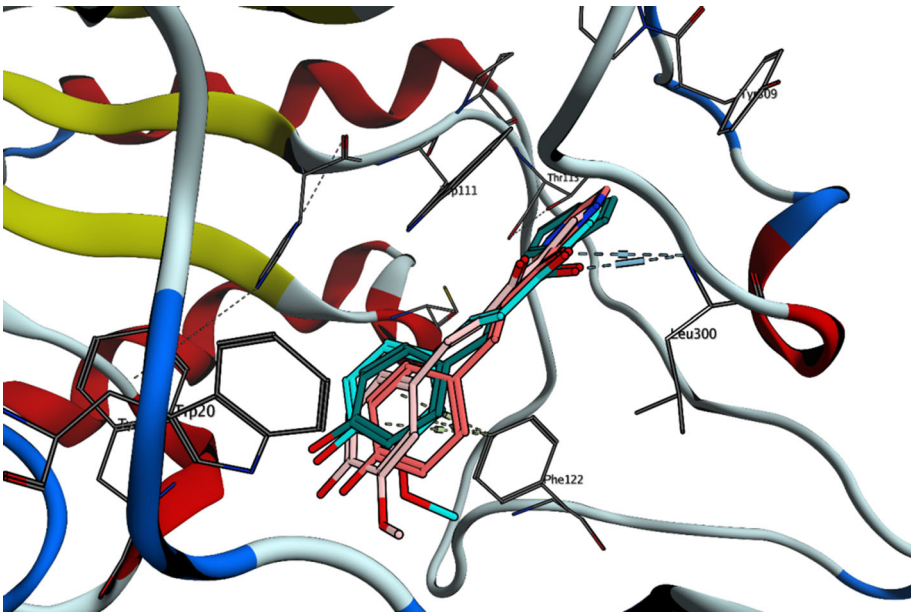


Fig. 7 Positions of **3** (cyan), **4** (dark green), **9** (light pink), and **12** (dark pink) anchored by the π–π interaction with TRP111

highlighted for interaction with inhibitory ligands in the active binding site. However, PHE122 was included in the amino acids of the specific ALR2 pocket [48]. PHE122 was reported to interact with the more lipophilic ring of chalcone derivatives [49]; in the case of chalcones synthesized in frame of this work, PHE122 always interacted with the ring B. Superposition of all four compounds in the binding site of the enzyme is shown in Fig. 7.

Conclusion

To conclude, the synthesized chalcones hydroxylated and methoxylated in ring B and their pyrazine (ring A) analogs exhibited significant aldose reductase inhibition activity, albeit lower in comparison with the reference drug epalrestat. Generally, the pyrazine analogs of chalcone showed higher inhibition of aldose reductase than chalcones (diphenylpropenones) and presence of 4-hydroxy group in the ring B seems to be essential for the inhibitory activity. The molecular modeling study confirmed interaction with the same amino acid residues involved in the binding mode of other inhibiting compounds described in literature. Medium antioxidant activity was demonstrated for both derivatives with 4-hydroxy-3-methoxy substitution, but it did not exceed the antioxidant efficacy of the standard Trolox.

Experimental

Acetophenone, vanillin, 2-, 3-, and 4-hydroxybenzaldehyde, 4-methoxybenzaldehyde, and 4-nitrobenzaldehyde were used as starting materials and were purchased from Merck (Prague, Czech Republic). 1-(Pyrazin-2-yl)ethan-1-one was available in our laboratory prepared previously according to Refs. [50, 51]. Silica gel 0.040–0.063 nm (Merck, Prague, Czech Republic) was used for column chromatography. The purity of the products was checked by TLC on aluminium sheets, silica gel 60 F254 (Merck, Prague, Czech Republic). Analytical samples were dried over anhydrous phosphorus pentoxide under reduced pressure at room temperature. Melting points were determined either on a Boëtius apparatus or Stuart SMP20 and were corrected. Elemental analyses (C, H, N, S) were conducted using an EA 1110 CHNS instrument (CE Instruments, Milano, Italy) or Vario Micro Cube Elemental Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Their results were found to be in good agreement (± 0.3%) with the calculated values. Infrared spectra were recorded either in KBr pellets with a Nicolet Impact 400 IR spectrophotometer (Thermo Scientific, Waltham, MA, USA) or on germanium crystal using ATR method with a Nicolet Impact 6700 IR spectrophotometer (Thermo Scientific). ^{1}H and ^{13}C NMR spectra were corroborated at ambient temperature with a Varian Mercury-Vx BB 300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C or with a VNMR S500 spectrometer operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR (both from Varian Corp., Palo Alto, CA, USA). Chemical shifts are indirectly referenced to tetramethylsilane (TMS) via the solvent signal (2.49 ppm for ^{1}H, 39.7 ppm for ^{13}C in DMSO-d_6 and 7.26 ppm for ^{1}H, 77.0 ppm for ^{13}C in $CDCl_3$).

Synthetic procedures

Syntheses and characteristics of pyrazine analogs of chalcone have already been described in our papers and

corresponded to the previously published data: (*E*)-3-(2-hydroxyphenyl)-1-(pyrazin-2-yl)prop-2-en-1-one (**7**), (*E*)-3-(4-hydroxyphenyl)-1-(pyrazin-2-yl)prop-2-en-1-one (**9**), (*E*)-3-(4-hydroxyphenyl)-1-(5-propylpyrazin-2-yl)prop-2-en-1-one (**10**), and (*E*)-3-(4-hydroxy-3-methoxyphenyl)-1-(pyrazin-2-yl)prop-2-en-1-one (**12**) in Ref. [21], (*E*)-3-(3-hydroxyphenyl)-1-(pyrazin-2-yl)prop-2-en-1-one (**8**) in Ref. [22], (*E*)-3-(4-hydroxyphenyl)-1-(5-isopropylpyrazin-2-yl)-2-en-1-one (**11**) in Ref. [24], and (*E*)-3-(4-nitrophenyl)-1-(pyrazin-2-yl)prop-2-en-1-one (**14**) in Ref. [23].

Method A

Acetophenone (0.01 mol) and appropriate substituted benzaldehyde (0.01 mol) were dissolved in 30 cm^3 methanol. Five NaOH pellets were added to this solution. The reaction mixture was stirred at r.t. for 24 h [18–20]. The reaction mixture was then diluted with 100 cm^3 of H_2O, acidified by 98% acetic acid to pH 6–7 and cooled in a refrigerator. The precipitated solid was filtered and recrystallized from anhydrous ethanol, repeatedly if needed. The following compounds of analytical purity were obtained (spectroscopic data for known compounds can be found in the Supplementary Material):

(*E*)-3-(3-Hydroxyphenyl)-1-phenylprop-2-en-1-one (2) Yield: 0.69 g (31%); $R_f = 0.56$ (hexane–ethyl acetate 6:4); m.p.: 162.0–166.0 °C (Ref. [52] 156–158 °C from ethanol).

(*E*)-3-(4-Hydroxyphenyl)-1-phenylprop-2-en-1-one (3) Yield: 1.00 g (45%); $R_f = 0.50$ (hexane–ethyl acetate 6:4); m.p.: 185.0–188.0 °C (Ref. [53] 187–188 °C).

(*E*)-3-(4-Methoxyphenyl)-1-phenylprop-2-en-1-one (5) Yield: 1.23 g (52%); $R_f = 0.45$ (hexane–ethyl acetate 8:2); m.p.: 72.0–75.0 °C (Ref. [54] 76 °C).

(*E*)-3-(4-Nitrophenyl)-1-phenylprop-2-en-1-one (6) Yield: 1.69 g (67%); $R_f = 0.48$ (hexane–ethyl acetate 8:2); m.p.: 166.0–168.0 °C (Ref. [55] 164.0–164.5 °C).

Method B

Acetophenone or 1-(pyrazin-2-yl)ethan-1-one (0.01 mol) and the corresponding substituted benzaldehyde (0.01 mol) were dissolved in 4.4 cm^3 pyridine. Diethylamine (0.01 mol) was added and the reaction mixture was stirred at 80–120 °C for 1 h. After cooling, the mixture was poured into 300 cm^3 ice water, acidified to pH 3 with acetic acid, and then refrigerated for 24 h. The mixture was extracted with diethyl ether and subjected to column chromatography. Hexane–ethyl acetate (ratio indicated at R_f values) was used as the eluent. The fractions containing the desired compounds were combined, evaporated, and recrystallized (repeatedly if needed) from absolute ethanol to obtain the following compounds as analytically pure crystals:

(*E*)-3-(2-Hydroxyphenyl)-1-phenylprop-2-en-1-one (1) Yield: 0.76 g (41%); $R_f = 0.45$ (hexane–ethyl acetate 7:3); m.p.: 152.0–155.0 °C (Ref. [56] 154–156 °C decomp.).

(*E*)-3-(4-Methoxyphenyl)-1-(pyrazin-2-yl)prop-2-en-1-one (13, $C_{14}H_{12}N_2O_2$) Yield: 0.24 g (10%); $R_f = 0.45$ (hexane–ethyl acetate 6:4); m.p.: 91.0–93.0 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 9.36$ (d, 1H, $J = 1.7$ Hz, H3′), 8.75 (d, 1H, $J = 2.5$ Hz, H6′), 8.68 (dd, 1H, $J = 2.5$ Hz, 1.7 Hz, H5′), 8.06 (d, 1H, $J = 15.9$ Hz, CH), 7.95 (d, 1H, $J = 15.9$ Hz, CH), 7.72–7.64 (m, AA′, BB′, 2H, H2, H6), 6.98–6.90 (m, AA′, BB′, 2H, H3, H5), 3.85 (s, 3H, OCH_3) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 188.4$, 162.0, 148.7, 147.2, 145.7, 144.8, 143.3, 130.8, 127.5, 117.6, 114.4, 55.4 ppm; IR (ATR-Ge): $\bar{\nu} = 3045$ (CH arom, alkene), 2958, 2921 (CH aliph), 1661 (C=O), 1591 (CH=CH), 1260 (C–O ether arom), 1167 (C=N pyrazine), 1057 (C=C pyrazine), 991 (C–H *trans*-alkene) cm^{-1}.

Method C

Solid KOH (16.8 g. 0.3 mol) was added to a mixture of 1.01 g acetophenone (8.4 mmol) and 1.28 g vanillin (8.4 mmol) in 16.8 cm^3 MeOH and 8.4 cm^3 H_2O. The resulting solution was refluxed for 1.5 h, cooled in an ice-water bath, then acidified with 33.6 cm^3 conc. HCl. The solution was diluted with 80 cm^3 water and stored in a refrigerator overnight. The reaction mixture was extracted into ethyl acetate and prepared for column chromatography. Recrystallization from anhydrous ethanol resulted in the following product:

(*E*)-3-(4-Hydroxy-3-methoxyphenyl)-1-phenylprop-2-en-1-one (4) Yield: 0.86 g (40%); $R_f = 0.13$ (hexane–ethyl acetate 8:2); m.p.: 92.6–94.6 °C (Ref. [57] 86–87 °C).

Biology

Male Wistar rats 8–9 weeks old, weighing 200–230 g, were used as organ donors. The animals came from the Breeding Facility of the Institute of Experimental Pharmacology Dobra Voda (Slovak Republic). The study was approved by the Ethics Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83-25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9, 2003).

Inhibition of aldose reductase

Aldose reductase preparation from rat lenses has been reported previously [58]. Aldose reductase activity was assayed spectrophotometrically by determining NADPH consumption at 340 nm and was expressed as a decrease in optical density (OD)/s/mg protein. The effect of the compounds on enzyme activities was determined by including the inhibitor in the reaction mixture at required concentrations dissolved in DMSO at 1% final concentration. At the same concentration, the inhibitor was included in the reference blank. The reference blank contained all the above reagents except the substrate, in order to correct for oxidation of NADPH not associated with reduction of the substrate. The enzyme reaction was initiated by addition of substrate and monitored for up to 4 min after an initial period of 1 min. Enzyme activities were adjusted by diluting the enzyme preparations with distilled water. Thus 0.05 cm^3 of the preparation gave an average reaction rate for the control sample of 0.020 ± 0.005 absorbance units/min. IC_{50} values (the concentration of the inhibitor required to produce 50% inhibition of the enzyme reaction) were determined both from the least-square analysis of the linear portion of the semi-logarithmic inhibition curves and non-linear regression analysis. Each curve was generated using at least four concentrations of inhibitor, resulting in an inhibition in the range of 25–75%.

Antioxidant assay

To investigate the antiradical activity of the compounds studied, the ethanolic solution of DPPH (50 μM) was incubated in the presence of the given compound tested (100 μM) at laboratory temperature. The absorbance decrease, recorded at $\lambda_{max} = 518$ nm, during the first 70 s interval was taken as a marker of the antiradical activity. During the 70 s interval used, an approximately linear decrease of DPPH absorbance was observed. This was considered to be a good assessment of the initial velocity of the radical reaction. The radical studies were performed at the laboratory temperature.

Molecular modeling

A Monte Carlo equilibrium conformer search (MMFF94) was used for obtaining optimal conformers of the compounds. The geometries were subsequently fully optimized by the DFT B3LYP 6-31G* method in the program SPARTAN'08 (Wavefunction Inc., USA [59]). Human recombinant enzyme AKR1B1 in complex with NADP+ and lidorestat (PDB: 1Z3 N [60]) was used for docking. The optimal conformers of the inhibitors were docked into the enzyme-cofactor complex by the program YASARA [61] by using YAMBER3 force field and utilizing the flexible ligands option. The first five clusters (if there) were then optimized in water. The simulation cell (overlapping the complex by 8 Å) was filled by water molecules (final density 0.997 g/cm^3) and ions Na^+ and Cl^- in the amount of 0.9% of the overall water environment. The ratio of ions was chosen to eliminate the total charge of the complex. Re-calculation of pK_a values of amino acids was performed and a pH of 6.2 was maintained. A standard optimization protocol was used, consisting of the steepest gradient optimization, molecular dynamics and simulated annealing. The final geometries were then analyzed according to the binding place of the inhibitor and the value of the binding energy.

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