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RESEARCH ARTICLE

Substituted benzenediol Schiff bases as promising new anti-glycation agents

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Abstract

A feature of diabetes is that the rate of protein glycation and the formation of advanced glycation endproducts (AGEs) increases spontaneously due to the abnormally elevated levels of sugar in the blood. The glycation of proteins is associated with a large number of late diabetic complications (retinopathy, neuropathy, atherosclerosis, end stage renal diseases, rheumatoid arthritis and neurodegenerative diseases). The increase in diabetic complications is a major cause of morbidity and mortality, which has increased significantly in the last two decades. Therefore, there is a considerable recent interest in the identification of lead molecules, which can inhibit the glycation process or slow it down considerably. A new class of anti-glycation agents has been identified, based on the spectrofluorimetric analysis of fluorescent advanced glycation endproducts (AGEs), benzenediol Schiff bases, and their structure-activity relationships have been studied. Some of these compounds have shown a promising anti-glycation potential *in vitro*.

Keywords: AGEs, anti-glycation agents, benzenediol Schiff bases, structure-activity relationships

Introduction

The Maillard's reaction, also known as non-enzymatic glycation or browning, is a series of non-enzymatic conversions [1]. This is a spontaneous reaction that induces changes in protein structure and function and plays an important role in the pathogenesis of late diabetic complications and aging. In this process, reducing sugars such as glucose bind to the amino acid residues of proteins, forming reversible Schiff bases which are converted to relatively stable Amadori compounds by rearrangement. The process prior to this conversion is known as the early stage glycation. Amadori compounds undergo a complicated dehydration and rearrangement, which result in the formation of dicarbonyl compounds, such as 3-deoxyglucosone (3DG), which promotes the advanced stage of glycation [2]. Advanced glycation endproducts (AGEs) form a complex and heterogeneous group of compounds that have been implicated in diabetes related complications, such as retinopathy, neuropathy, atherosclerosis, end stage renal diseases, rheumatoid arthritis and neurodegenerative disorders [3].

The formation of AGEs is a relatively slow process under physiological conditions and the accumulation of AGEs is more prominent in long-lived proteins, such as lens crystallins and tissue collagens. The amount of AGEs increases as a function of time and glucose concentration, but their concentration substantially increases with age [4]. As a consequence of the formation of AGEs, biologically active proteins and enzymes are deactivated through the formation of inter- and intra-molecular crosslinks leading to major functional impairment. In addition to their natural formation, other causes of the formation of AGEs in the human body are smoking and consumption of an AGEs-enriched diet [5]. There is an increased level of AGEs in serum and tissues due to a reduced removal of AGEs by the kidney, which results

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in end-stage renal failure in diabetic patients. A highly significant correlation has been shown between the quantity of AGEs deposits and the severity of diabetic complications [6].

Important AGEs include pentosidine, crossline, pyrraline and carboxy methyl lysine (CML). However, most of the AGEs have not been identified since most of them form inter- or intra-molecular cross-links and undergo complicated changes, such as polymerisation and insolubilisation. They begin to emit fluorescence and finally become brown pigments. The concentration of AGEs can be measured using the intensity of fluorescence which is one of their main characteristics [2].

Various attempts have been made to pharmacologically influence the process of glycation by preventing or slowing down the formation of AGEs [7]. Experiments have been conducted on diabetic dogs to investigate the effects of aminoguanidine and aspirin on the progression of retinopathy. These compounds can alter blood flow and vessel permeability and inhibit non-enzymatic glycation and oxidative stress. Aminoguanidine also has a beneficial effect on nerve conduction velocity [8]. In another study on rats, aminoguanidine inhibited the age-related increase in tail tendon break time (TBT), a parameter of crosslinking, with no effect on the skin [9].

Previously Schiff bases, derived from the aminoguanidine like aminoguanidine-pyridoxal adduct and the aminoguanidine-pyridoxal phosphate adduct, have been evaluated for anti-glycation activity *in vitro*. These compounds exhibited significant anti-glycation activities [10]. The Schiff bases of isatin also showed a promising anti-glycation potential *in vitro* [11]. As the strategy of influencing the development and prevention of diabetic complications in the near future is most likely to involve a potentially promising anti-glycation therapy, we have been evaluating diverse classes of natural and synthetic compounds for their anti-glycation potential using both *in vitro* and *in vivo* models [7].

Results and discussion

A series of Schiff bases **2**-**7**, were prepared from substituted benzenediol, and then subjected to a mechanism-based *in vitro* anti-glycation assay. A spectrofluorimetric analysis at 370 nm excitation and 440 nm emission was used to evaluate the anti-glycation potential. An aliquot with a 3 mM concentration for each compound (DMSO) was used to perform the initial screening, as shown in Figure 1. All the samples showed a significant inhibitory potential at the given concentration, when compared with reference inhibitor (rutin). Rutin, a common flavonoid found in fruits and vegetables, has been reported to modulate the formation of AGEs. Previous studies conducted on diabetic rats, demonstrated that rutin protected against the formation of skin-collagen fluorescence and also inhibited early glycation product formation [12]. Rutin also exhibited a significant inhibitory effect on glycation of haemoglobin and it was found to be more effective than aminoguanidine [13]. The percentage inhibition was calculated as follows (Equation 1):

$$\text{Percent inhibition} = 100 - \text{OD(sample)} / \text{OD(blank)} \times 100 \quad (1)$$

The half maximal inhibitory concentration (IC_{50}) values were measured using different concentrations of the potential inhibitors. The EZ-fit software (Perrella Scientific, Amherst, USA) was used to calculate the IC_{50} values (μM). The cytotoxicity potential of these compounds was determined using the MTT assay on mouse fibroblast 3T3 cells [14]. Cycloheximide was used as a standard in the cytotoxicity assays with IC_{50} values of 0.3 ± 0.089 μM.

Structure-activity relationship studies indicated that compound **1**, a simple brominated benzylic amine, has a moderate inhibitory activity with an IC_{50} value of 440.01 ± 12.71 μM. However, its corresponding Schiff base **2**, exhibited a markedly enhanced anti-glycation activity. This increase in activity may be attributed to the presence of the Schiff base moiety, which resulted in a 10-fold increase in activity ($IC_{50} = 43.39 \pm 2.37$ μM).

Schiff bases with different substituents at the benzene ring have considerably different inhibitory potentials. Chlorination at the *ortho*, *meta* and *para* positions of the benzene ring of the imino benzenediol moiety resulted in a potent inhibitory potential, for example compounds **3**, **4** and **5** with IC_{50} values of <16, 54. 82 ± 1.66 and 272.10 ± 11.71 μM, respectively. Chlorination at the *ortho* position was inferred to be more favourable for inhibition, as compared to the *meta* and *para* positions. Compound **6** with a dichlorinated benzene ring at the *ortho* and *para* positions is a more potent inhibitor with an IC_{50} <16 μM, than compounds **4** and **5** with *para* and *meta* chlorinated benzene rings. Compound **7** with a trifluoromethyl at the *meta* position of the benzene ring exhibited a significant anti-glycation activity with an IC_{50} of 71.672 ± 4.16 μM. the lowest activity among the series was shown by the *meta* substituted Schiff base. The cytotoxicity studies showed that compounds **1**, **3**, **5** and **6** were moderately toxic against the mouse fibroblast 3T3 cells, while the others were relatively non-toxic.

The mechanism of the formation of advanced glycation end products involves the formation of Schiff base, followed by Amadori rearrangement, which ultimately results in the emergence of AGEs. In order to determine the mechanism of action of the Schiff bases and to identify the active moiety responsible for the inhibition, various halogenated anilines were tested for their anti-glycation activity along with their corresponding Schiff bases.

Bovine Serum Albumin (BSA) was incubated with glucose in the presence of various halogenated anilines like 4-bromoaniline, 4-chloroaniline, 3-chloroaniline and hydroxy benzaldehyde and their corresponding Schiff bases. The control sample contained only BSA and glucose. The effect of halogenated anilines and

their corresponding Schiff bases on the formation of advanced glycation end products was assessed by fluorescence intensity. In Figure 3, column 1 is the control (glycated), while the other columns are the halogenated anilines, hydroxy benzaldehyde and Schiff bases, respectively.

As shown in Figure 3, when 4-bromoaniline, 4-chloroaniline, 3-chloroaniline and hydroxy benzaldehyde were evaluated separately at 1 mM, they did not show any inhibition of protein glycation, as compared to the control. On the other hand, their corresponding Schiff bases, such as 4-bromoaniline, 4-chloroaniline and 3-chloroaniline Schiff bases showed a significant inhibitory potential at 1 mM. It was thus concluded that the Schiff bases actually interact with the post Amadori step to inhibit the formation of AGEs.

In order to further prove this, halogenated aniline hydrochloride salts were prepared from their corresponding anilines and were evaluated for their ability to inhibit protein glycation, as shown in Figures 4 and 5. This again showed that the halogenated anilines, as well as their salts, did not show any inhibiton of the protein glycation, as compared to the glycated control.

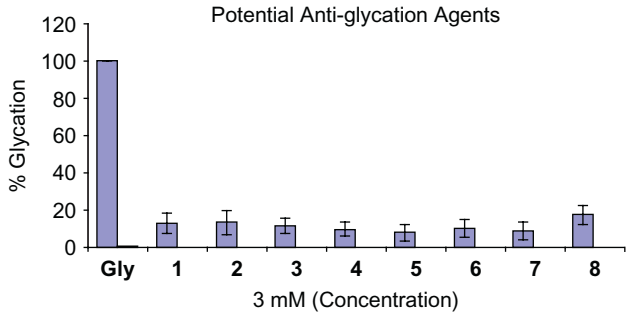


Figure 1. Anti-glycation activity of compounds **1**–**7**, rutin (**8**) and Gly (control).

Materials and methods

The chemicals used were purchased from different sources such as bovine serum albumin (BSA) from Research Organics (Cleveland, OH, USA), anhydrous D-glucose from Fisher Scientific (Leicestershire, UK), sodium azide and trichloro acetic acid (TCA) from

Table 1. The anti-glycation activity and cytotoxicity of compounds **1**–**8**.

Compound name		Anti-glycation IC_{50} (μM)	Cytotoxicity IC_{50} (μM)
3-[(4-Bromoanilino)methyl]1, 2-benzenediol	(**1**)	440.01 ± 12.71	17.41 ± 0.77
3-[(4-Bromophenylimino) methyl]benzene-1,2-diol	(**2**)	43.39 ± 2.37	> 100
3-[(2-Chlorophenylimino) methyl]benzene-1,2-diol	(**3**)	< 16	16.27 ± 0.28
3-[(4-Chlorophenylimino) methyl]benzene-1,2-diol	(**4**)	54.82 ± 1.66	22.04 ± 0.08
3-[(3-Chlorophenylimino) methyl]benzene-1,2-diol	(**5**)	272.10 ± 11.71	10.69 ± 0.42
3-[{(2,4-Dichlorophenyl)imino} methyl]1,2- benzenediol	(**6**)	< 16	11.91 ± 0.93
3-[(3-Triflouromethylphenylimino) methyl] benzene-1,2-diol	(**7**)	71.672 ± 4.16	26.83 ± 0.99
Rutin (Standard)	(**8**)	98.01 ± 2.03	> 100
Cycloheximide (cytotoxicity standard)		–	0.3 ± 0.089

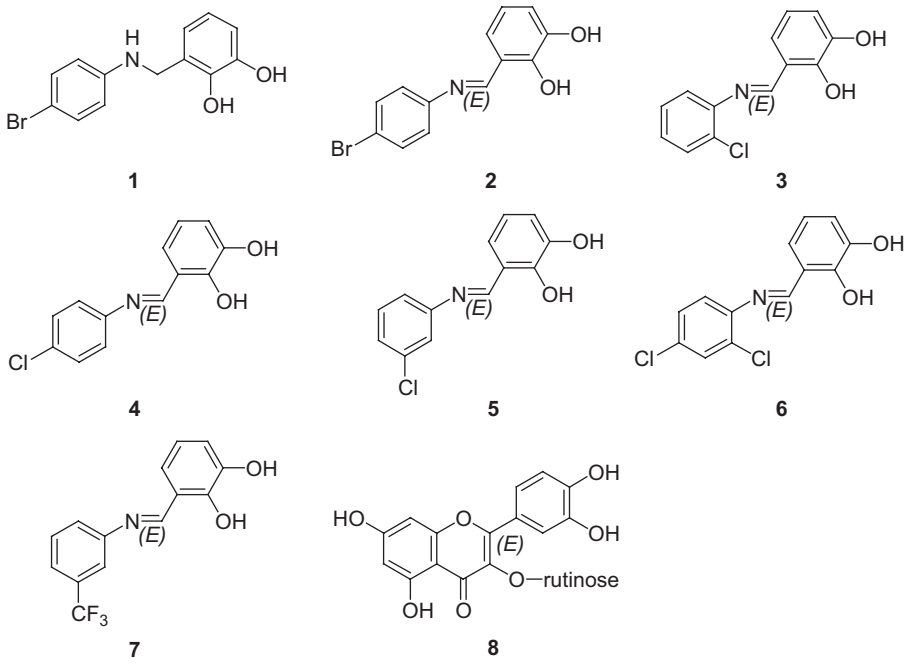


Figure 2. Compound **1** is brominated benzylic amine, while **2**–**7** are Schiff bases and rutin (**8**) was used as standard.

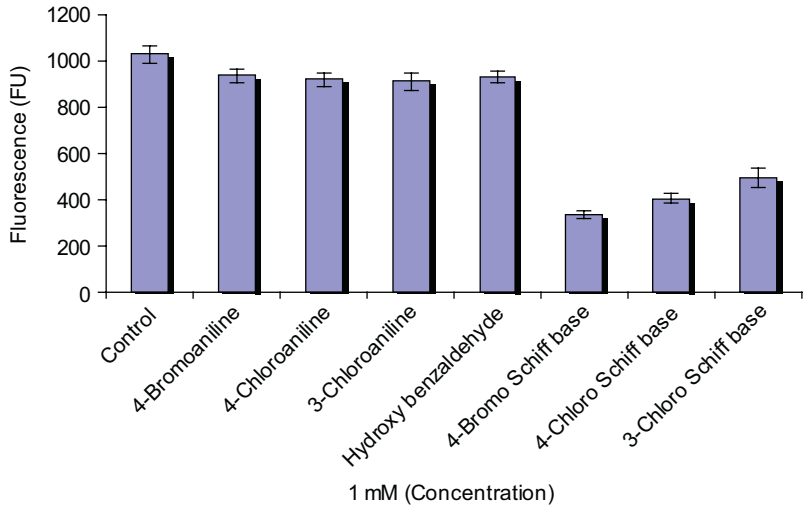
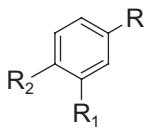


Figure 3. Anti-glycation activity of halogenated anilines and their corresponding Schiff bases as compared to control.



R = NH_2, R_2 = Br (4-Bromoaniline)

R = $\overset{+}{N}H_3\overset{-}{Cl}$, R_2 = Br (4-Bromoaniline hydrochloride)

R = NH_2, R_2 = Cl, (4-Chloroaniline)

R = $\overset{+}{N}H_3\overset{-}{Cl}$, R_2 = Cl, (4-Chloroaniline hydrochloride)

R = NH_2, R_1 = Cl (3-Chloroaniline)

R = $\overset{+}{N}H_3\overset{-}{Cl}$, R_1 = Cl (3-Chloroaniline hydrochloride)

Figure 4. Halogenated anilines and their hydrochloride salts.

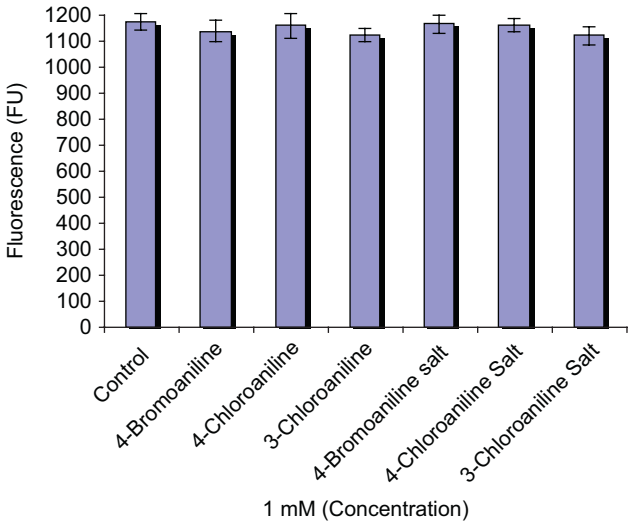


Figure 5. Anti-glycation activity of the halogenated anilines and their corresponding salts as compared to the control.

Scharlau (Barcelona, Spain). Phosphate buffer (pH 7.4), phosphate buffer saline (pH 10) and rutin were purchased from Carl Roth (Karlsruhe, Germany). Reagents for cytotoxicity assay were purchased as mouse fibroblast (3T3) from European American Culture Collection (EACC, Porton Down), Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from GIBCO-BRL (San Francisco, USA), 3-[4,5-dimethylthiazole- 2-yl]-2-,5-diphenyltetrazolium bromide (MTT) from Amresco (Cochran Solon, OH, USA), Penicillin and streptomycin from Sigma-Aldrich (St. Louis, MO, USA). The compounds were synthesized in our laboratory. All physical and spectroscopic data are given in the experimental part and found satisfactory.

Anti-glycation assay (in vitro)

BSA 10 mg/mL was dissolved in 67 mM phosphate buffer at pH 7.4. Anhydrous glucose was used as a 50 mg/mL solution in phosphate buffer at pH 7.4. A 3 mM sodium azide solution was added in sufficient quantity to inhibit any bacterial growth in the buffer. A 3 mM concentration of the unknown inhibitors were dissolved in DMSO (USA Scientific, Ocala, FL, USA), along with the standard inhibitor then tested. A 96-well plate containing 60 µL of the test sample per well was used (in triplicate). A blank sample containing only the BSA dissolved in phosphate buffer and a positive control sample with BSA and glucose, were prepared and incubated for a week at 37°C. After incubation in the 96-well plate for a week, samples were removed and cooled to room temperature. Then an aliquot of 6 µL of 100% TCA (trichloroacetic acid) was added to each well. The supernatant containing the unbound glucose, inhibitor and test sample were removed after centrifugation at 14 000 rpm for 4 minutes, and pellets were obtained at the bottom of the wells. The supernatants were removed from each well and 60 µL of PBS (pH 10) was added to dissolve the pellets for screening. The comparison of fluorescence intensity at 370 nm excitation and emission at 440 nm was obtained by using a spectrofluorimeter (RF-1500, Shimadzu, Kyoto, Japan) [15-18]. Rutin, a standard inhibitor, showed an 82.5%

inhibition of glycation at a 3 mM concentration with an IC_{50} of 98.01 ± 2.03 μM.

Cytotoxicity bioassay (In vitro)

The cytotoxicity of these compounds was evaluated in 96-well flat-bottom microplates using the standard 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. In this assay, 3T3 cells (mouse fibroblasts) were cultured in Dulbecco's modified Eagle's medium, supplemented with 5% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin by using a 25 cm^2 flask in a 5% CO_2 incubator at 37°C. The exponentially growing cells were harvested, counted with a haemocytometer (Precicolor, Giessen, Germany) and diluted with a particular medium (GIBCO, San Francisco, USA). The cell cultures with a concentration of 3×10^4 cells/mL were prepared and plated (100 μL/well) onto 96-well plates. After an overnight incubation, the medium was removed and 200 μL of fresh medium was added with different concentrations of each compound (1–100 μM). After 72 h, 50 μL of MTT (2 mg/mL) was added to each well and the incubation was continued for 4 h. Subsequently, 100 μL of DMSO was added to each well. The extent of the MTT reduction to formazan within the cells was calculated by measuring the absorbance at 540 nm by using a microplate ELISA reader (Molecular Devices, CA, USA). The cytotoxicity of these compounds was recorded as the concentration causing 50% growth inhibition. Cycloheximide was used as a standard (IC_{50} = 0.3 ± 0.089 μM).

General procedure for the synthesis of compound 1 and Schiff bases 2–7

The Schiff bases **2**–**7** were prepared by refluxing 6 mmol of 2,3-dihydroxybenzaldehyde with 6 mmol of substituted anilines in 100 mL of ethanol for 3 hours. The solution was cooled and left overnight. The solid product obtained was filtered and recrystallised from methanol. Compound **1** was synthesised by reducing compound **2** with sodium borohydride in ethanol using a reported procedure [21].

3-[(4-Bromoanilino)methyl]1,2-benzenediol (1)

Yield 78%, mp 171 °C, analysis for $C_{13}H_{12}BrNO_2$ (293): calcd. C, 53.08; H, 4.11; N, 4.76. found: C, 53.05; H, 4.08; N, 4.8. EI MS: *m/z* (%) at 70 eV $[C_{13}H_{12}BrNO_2]^+$ 293 (100), $[C_{13}H_{11}BrNO]^+$ 276 (18.2), $[C_7H_7BrN]^+$ 184 (10.9), $[C_6H_4Br]^+$ 155 (33.51), $[C_5H_5]^+$ 65 (22.35), $[C_4H_3]^+$ 51 (18.66). *¹H*-NMR: Azomethine hydrogen δ 8.62, Aromatic protons δ 7.62-6.85.

3-[(4-Bromophenylimino)methyl]benzene-1,2-diol (2)

Yield 88%, mp 149 °C, analysis for $C_{13}H_{10}BrNO_2$ (291): calcd. C, 53.45; H, 3.45; N, 4.79. found: C, 53.47; H, 3.43; N, 4.82. EI MS: *m/z* (%) at 70 eV $[C_{13}H_{10}BrNO_2]^+$ 291 (100), $[C_{13}H_9BrNO]^+$ 274 (10.54), $[C_{13}H_{10}NO_2]^+$ 212 (12.03), $[C_7H_5BrN]^+$ 182 (17.4), $[C_6H_4Br]^+$ 155 (69.28), $[C_7H_6NO_2]^+$ 136 (52.03), $[C_6H_6O_2]^+$ 110 (13.87), $[C_6H_5O]^+$ 93 (7.24), $[C_6H_5]^+$ 77 (44.25), $[C_5H_5]^+$ 65 (26.88), $[C_4H_3]^+$ 51 (26.56).

3-[(2-Chlorophenylimino) methyl] benzene-1,2-diol (3)

Yield 80%, mp 127 °C, Analysis for $C_{13}H_{10}ClNO_2$ (247): calcd. C, 63.04; H, 4.07; N, 5.66. Found: C, 63.01; H, 4.08; N, 5.68. EI MS: *m/z* (%) at 70 eV. $[C_{13}H_{10}ClNO_2]^+$ 247 (56.27), $[C_{13}H_9ClNO]^+$ 230 (2.15), $[C_{13}H_{10}NO_2]^+$ 212 (100), $[C_7H_5ClN]^+$ 138 (9.09), $[C_7H_6NO_2]^+$ 136 (16.81), $[C_6H_4Cl]^+$ 111 (34.62), $[C_6H_6O_2]^+$ 110 (5.44), $[C_6H_5O]^+$ 93 (4.59), $[C_6H_5]^+$ 77 (24.53), $[C_5H_5]^+$ 65 (13.90), $[C_4H_3]^+$ 51 (17.28).

3-[(4-Chlorophenylimino)methyl]benzene-1,2-diol (4)

Yield 79%, mp 148 °C, analysis for $C_{13}H_{10}ClNO_2$ (247): calcd.: C, 63.04; H, 4.07; N, 5.66. found: C, 62.99; H, 4.1; N, 5.62. EI MS: *m/z* (%) at 70 eV $[C_{13}H_{10}ClNO_2]^+$ 247 (100), $[C_{13}H_9ClNO]^+$ 230 (21.34), $[C_{13}H_{10}NO_2]^+$ 212 (11.4), $[C_7H_5ClN]^+$ 138 (15.66), $[C_7H_6NO_2]^+$ 136 (27.33), $[C_6H_4Cl]^+$ 111 (56.11), $[C_6H_6O_2]^+$ 110 (7.27), $[C_6H_5O]^+$ 93 (5.1), $[C_6H_5]^+$ 77 (27.84), $[C_5H_5]^+$ 65 (11.41), $[C_4H_3]^+$ 51 (33.57).

3-[(3-Chlorophenylimino)methyl]benzene-1,2-diol (5)

Yield 81%, mp 138 °C, Analysis for $C_{13}H_{10}ClNO_2$ (247): calcd.: C, 63.04; H, 4.07; N, 5.66. found: C, 63.01; H, 4.1; N, 5.7. EI MS: *m/z* (%) at 70 eV $[C_{13}H_{10}ClNO_2]^+$ 247 (100), $[C_{13}H_9ClNO]^+$ 230 (18.76), $[C_7H_5ClN]^+$ 138 (10.74), $[C_{13}H_{10}NO_2]^+$ 212 (7.40), $[C_7H_6NO_2]^+$ 136 (39.95), $[C_6H_4Cl]^+$ 111 (48.49), $[C_6H_6O_2]^+$ 110 (9.31), $[C_6H_5O]^+$ 93 (4.37), $[C_6H_5]^+$ 77 (22.9), $[C_5H_5]^+$ 65 (17.19), $[C_4H_3]^+$ 51 (27.34).

3-[(2, 4-Dichlorophenylimino)methyl]-1,2-benzenediol (6)

Yield 86%, mp 160 °C, analysis for $C_{13}H_9Cl_2NO_2$ (281) calcd.: C, 55.34; H, 3.22; N, 4.96. found: C, 55.37; H, 3.25; N, 5. EI MS: *m/z* (%) at 70 eV $[C_{13}H_9Cl_2NO_2]^+$ 281 (33.3), $[C_{13}H_8Cl_2N]^+$ 248 (30.57), $[C_{13}H_9ClNO_2]^+$ 246 (100), $[C_7H_4Cl_2N]^+$ 172 (7.33), $[C_6H_3Cl_2]^+$ 145 (25.54), $[C_7H_7NO_2]^+$ 136 (23.09), $[C_6H_5O_2]^+$ 109 (33.53), $[C_5H_5]^+$ 65 (41.27). *¹H*- NMR: hydroxy hydrogen δ 13.48, azomethine hydrogen δ 8.63.

3-[(3-Triflouromethylphenylimino)methyl]benzene-1, 2-diol (7)

Yield 82%, mp 116 °C, analysis for $C_{14}H_{10}F_3NO_2$ (281): calcd.: C, 59.79; H, 3.58; N, 4.98. found: C, 59.72; H, 3.59; N, 5.01. EI MS: *m/z* (%) at 70 eV $[C_{14}H_{10}F_3NO_2]^+$ 281 (100), $[C_{14}H_9F_3NO]^+$ 264 (11.81), $[C_{13}H_{10}NO_2]^+$ 212 (5.48), $[C_8H_5F_3N]^+$ 172 (7.23), $[C_7H_4F_3]^+$ 145 (40.04), $[C_7H_6NO_2]^+$ 136 (37.56), $[C_6H_6O_2]^+$ 110 (6.04), $[C_6H_5O]^+$ 93 (2.79), $[C_6H_5]^+$ 77 (5.15), $[C_5H_5]^+$ 65 (7.51), $[C_4H_3]^+$ 51 (10.44).

Conclusion

In the future, anti-glycation therapy for the prevention of diabetic complications with a high efficacy and low

toxicity is a possibility. To inhibit protein glycation, a class of substituted benzenediol Schiff bases were evaluated which exhibited excellent *in vitro* anti-glycation activity. We concluded from the mechanism-based studies that although Schiff bases were hydrolysed to their corresponding amines, albeit in a very minute percentage, these Schiff bases interacted with the post Amadori step to inhibit the formation of advanced glycation end products (AGEs).

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Declaration of interest

The author declare no conflict of interest.

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