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***N*-(3-aminoalkyl)proline derivatives with potent antiglycation activity**

Harsha Chilukuri,^{a,b} Yogesh M. Kolekar,^a Govind S. Bhosle,^b Rashmi K. Godbole,^a Rubina S. Kazi,^a Mahesh J. Kulkarni^{a,*} and Moneesha Fernandes^{b,*}

The importance of amino acids in the therapy of conditions such as renal failure, neurological disorders and congenital defects has been documented. Some amino acids such as lysine and glycine have also been reported to have antiglycating activity. Herein we report the synthesis of a new series of *N*-(3-aminoalkyl)proline derivatives which are non-natural in nature. The compounds were unambiguously characterized by NMR, mass and IR spectroscopy. Their *in vitro* antiglycation activity was studied by circular dichroism and fluorescence spectrometry. The mechanism of action was also studied and found to take place by inhibition of Amadori product formation. The inhibition of AGE formation was further confirmed by Western blot and LC-MS/MS analyses and the IC₅₀ values of the potent compounds, determined. Compounds containing hydroxyl substituents at C4 were found to have superior antiglycation properties than those containing azide substituents at the same position. The compounds were additionally found to possess good anti-oxidant properties, which could lead to further reduction in AGE formation. Moreover, the title compounds were found to have low cytotoxicity in mammalian cells, another important attribute. Thus, the title compounds represent a novel promising class of antiglycating agents.

Introduction

Advanced Glycation End products (AGEs) are formed due to non-enzymatic reactions between protein and reducing sugars. They are involved in pathogenesis of diabetes and its complications. Thus, reducing AGE levels¹ and inhibition of protein glycation is crucial in the prevention of the above complications. Some molecules such as aminoguanidine that reduce AGE levels, but have toxic side-effects have not been approved by the FDA, while other FDA-approved drugs have been shown to display anti-glycation activity¹⁻⁵ in addition to their originally intended activities. These offer the possibility of repositioning these drugs for the treatment of diabetes and its complications. Reports from this laboratory have also been made for the use of *N*-(aminoalkyl)proline- and 4-hydroxy-*N*-(aminoalkyl)proline-derived compounds towards the synthesis of peptide nucleic acids⁶ and cell-penetrating oligomers,⁷ where these compounds were used as analogues of amino acids such as lysine and arginine.^{7b} Since amino acids such as glycine and lysine have been reported to exert antiglycating activity,⁸ we surmised that the use of non-natural analogues such as the title compounds, would have advantages in terms of stability *in vivo*.

In this study, we report the synthesis and anti-glycation activity of selected *N*-(3-aminoalkyl)proline derivatives and their mode of action by various physicochemical assays such as circular dichroism (CD), fluorescence spectroscopy, MALDI-TOF and LC-MS/MS assays. We also show that these compounds

^a Proteomics Facility, Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Pune- 411 008, India. E-mail: mj.kulkarni@ncl.res.in; Tel: +912025902541.

^b Organic Chemistry Division, CSIR-National Chemical Laboratory, Pune 411008, India. E-mail: m.dcosta@ncl.res.in; Tel: +912025902084.

† Footnotes relating to the title and/or authors should appear here.

Electronic Supplementary Information (ESI) available: [NMR, mass spectra of 1-9, mass spectra of glucose adducts with 4, 6, 8 & 9, Orbitrap analysis, database search & PTM analysis, Ponceau staining of anti-AGE & anti-CML blots, AGE fluorescence spectra of glycated BSA treated with 1, 3, 5 & 7]. See DOI: 10.1039/x0xx00000x

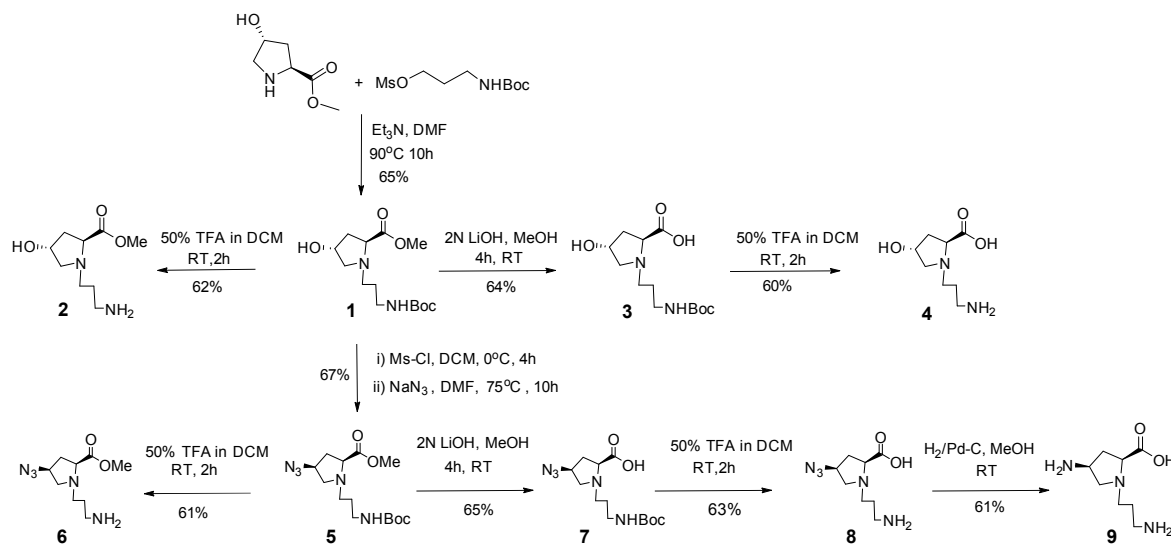
are capable of exerting good anti-oxidant properties and display low cytotoxicity, thus enhancing their value as potential anti-glycation agents.

Results and Discussion

Synthesis of title compounds

The title compounds were synthesized using a simple strategy outlined in Scheme 1. Accordingly, 4(5)-hydroxy-2(R)-proline

Scheme 1. Synthesis of the title compounds



The removal of the Boc protecting group in 3 resulted in compound 4, which was obtained in 60% yield. Mesylation of the 4-OH group in compound 1 yielded the 4-*O*-mesyl derivative, which was further converted to its azide counterpart 5 in 67% yield over two steps after purification by column chromatography. Compound 6 was obtained by acidolytic removal of the Boc protecting group in 5, and purified by column chromatography. Compound 5, upon saponification with lithium hydroxide yielded 7 in 65% yield after column purification. Compound 7 was further subjected to removal of the Boc protecting group by treating it with TFA in CH₂Cl₂ to yield compound 8 in 63%. Reduction of the azide function in 8 by hydrogenation in the presence of Pd-C further gave compound 9 in 61% yield. All the compounds were unambiguously characterized by appropriate spectroscopic techniques.

Glycation inhibition studies

Circular Dichroism (CD)

CD is a powerful tool for investigating the structure and conformational changes of proteins, such as those occurring upon glycation,⁹ where an increase in the beta sheet conformation is observed. A decrease in the beta sheet conformation is therefore, indicative of antiglycation ability. Analysis of the CD spectra of BSA upon glycation in the presence of the title compounds (Fig. 1) revealed a decrease in

methylester was *N*-alkylated by treating it with 3-((tert-butoxycarbonyl)amino)propyl methanesulfonate in CH₂Cl₂ in the presence of triethylamine to yield compound 1 which was subjected to acidolytic removal of the Boc protecting group to afford compound 2 in 62% yield. Compound 3 was obtained from 1 upon saponification with lithium hydroxide and subsequent purification by column chromatography on neutral alumina.

the beta sheet percentage in comparison to glycated BSA, when no compound was present. In particular, the beta sheet conformation in the presence of compounds 2, 4, 6, 8 and 9 was 3%, 1.7%, 2.5%, 2% and 5.2% respectively, while that in glycated BSA was 18.7% and 5.7% in the presence of known anti-glycating agent, aminoguanidine (Amg). Thus, the title compounds are able to protect the conformation of BSA and inhibit beta sheet formation significantly, even in comparison to aminoguanidine.

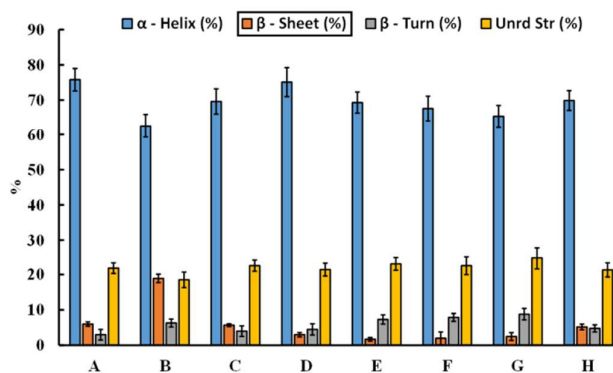


Fig. 1. CDPro analysis of (A) native BSA, (B) glycated BSA and (C-H) glycated BSA in the presence of 20mM aminoguanidine and compounds 2, 4, 8, 6 and 9 respectively. (Unrd= unordered.)

In vitro glycation inhibition by fluorescence spectroscopy

The degree of glycation can be measured by measuring the fluorescence intensity at 440nm, using an excitation wavelength of 370nm,¹⁰ since most AGEs have a characteristic fluorescence with an excitation maximum approximately at 370nm, and emission around 440nm. A decrease in the fluorescence emission intensity is thus, indicative of inhibition of AGE formation. BSA was incubated with glucose in the presence and absence of title compounds and the fluorescence emission of the compounds was monitored after excitation at 370nm (Fig. 2). The formation of AGEs was monitored after 14 days by measuring the fluorescence emission intensity at 440nm. The fluorescence-based assay for AGEs was also used to determine the IC₅₀ values of representative title compounds **2** and **6**. Accordingly, these were found to be 19.2mM and 29.5mM respectively. In comparison, the IC₅₀ value of aminoguanidine, considering the fluorescence excitation/emission as 370nm/440nm is reported to be 10mM.¹¹ However, this fluorescence-based method may be less specific¹² because some fluorescent AGEs differ in their excitation-emission wavelengths.

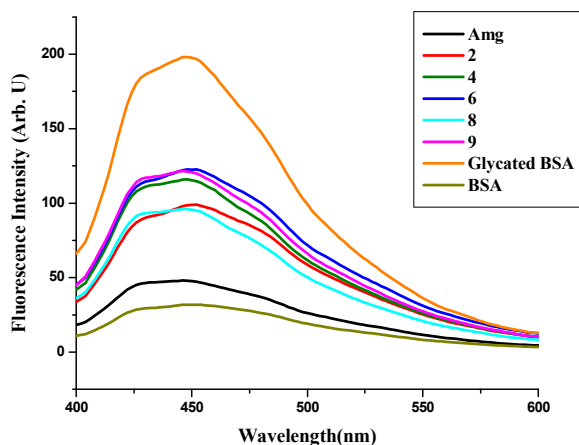


Fig. 2. AGE fluorescence spectra of BSA, glycated BSA, and glycated BSA treated with 20mM title compounds and aminoguanidine.

Western Blot Assay

Western blot analyses of the glycation products of BSA in the presence and absence of the title compounds were carried out using both anti-AGE as well as anti-CML (anti-Carboxymethyl lysine) antibodies, since CML is known to be the most abundant non-fluorescent AGE. In both the cases, compounds **2** and **4** were found to exhibit potent anti-glycation activity, which was even superior to aminoguanidine (Fig. 3). Ponceau staining of the gels was also carried out (ESI, Fig. S16) to illustrate the protein (BSA) content in the samples.



Fig. 3. Western blot analysis: Anti-CML Blot: Lane (1-9) compound **9**, **6**, **8**, **4**, **2**, aminoguanidine, glycated BSA, BSA, ladder; Anti-AGE Blot: Lane (1-8) compound **9**, **6**, **8**, **4**, **2**, aminoguanidine, glycated BSA, BSA.

From the above studies, compounds **2** and **4** emerged as the most promising title compounds with potent anti-glycation activity, which was found to be superior to known anti-glycating agent, aminoguanidine.

Probable mechanism of glycation inhibition

Circular dichroism, AGE fluorescence and Western blotting suggested that at least some of the title compounds are capable of inhibiting AGE formation in proteins, using BSA as a representative protein. In order to probe the mechanism by which this occurs, a series of studies were undertaken, including MALDI-TOF-MS based insulin glycation assay, fructosamine assay, LC-MS/MS analysis of glycated peptide fragments formed after trypsin treatment and mass spectrometry of glucose adduct formation by the title compounds.

MALDI-TOF-MS based insulin glycation assay. Following a previously reported MALDI-TOF assay,^{2,5} insulin (m/z 5808) was glycated in presence of glucose to form Amadori modifications (m/z 5970). The intensity of glycated insulin was monitored in the presence or absence of inhibitors. Fig. 4 shows that all the title compounds were able to inhibit Amadori modification of insulin which is indicated by the decreased intensity of the glycated insulin peak, with compound **9** showing the highest inhibition.

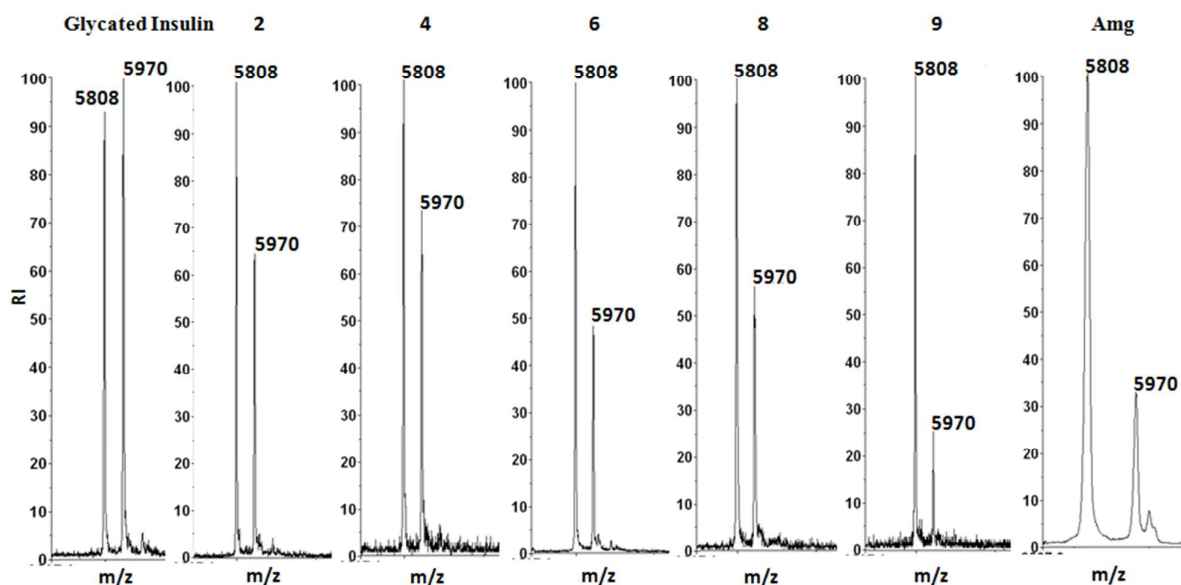


Fig. 4. MALDI-TOF assay for glycation inhibition for glycated insulin, insulin in presence of compounds **2**, **4**, **6**, **8**, **9** and aminoguanidine.⁵

The ability of the title compounds to inhibit Amadori product formation was further studied by high resolution mass spectrometry (Q-Exactive, Orbitrap mass spectrometer). BSA was glycated in the absence or presence of the title compounds and the formation of Amadori product-modified peptides after digestion with trypsin was monitored by LC-MS/MS. Glycated BSA was found to have the highest number of Amadori-modified peptides (34), while all the title compounds had lower number of the same- 29, 32, 28, 31 and 27 for compounds **2**, **4**, **6**, **8** and **9** respectively (Fig. 5), while with aminoguanidine, the number was 24. The detailed analysis is delineated in the ESI, S19-S25.

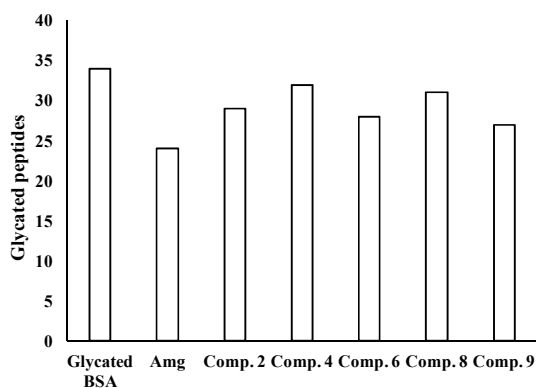


Fig. 5. LC-MS/MS analysis depicting the number of AGE-modified peptides in glycated BSA and glycated BSA after treatment with aminoguanidine or title compounds.

Fructosamine Assay. In the early stages of glycation, unstable Schiff bases are formed and turned into Amadori products such as fructosamine,¹³ which is clinically used as an indicator for short-term control of blood sugar in diabetic patients. Reduction of fructosamine, therefore, is a therapeutic strategy to delay incident vascular complications.¹⁴ The fructosamine

assay is a simple colorimetric test that measures glycated serum protein concentrations. Colour change is based on the reduction of nitroblue tetrazolium (NBT) to monoformazan (MF) by Amadori rearrangement products.¹⁵ The fructosamine levels in presence of the title compounds were found to be significantly lower than that in glycated BSA (Fig. 6). Specifically, the fructosamine levels were 539, 551, 476, 592 and 526 $\mu\text{mol L}^{-1}$ in the presence of compounds **2**, **4**, **6**, **8** and **9** respectively, which were even lower than that in the presence of aminoguanidine (735 $\mu\text{mol L}^{-1}$), while the fructosamine level in glycated BSA was 976 $\mu\text{mol L}^{-1}$. This data is thus, in accordance with that obtained from the MALDI-TOF assay, which suggested that the title compounds inhibited Amadori product formation.

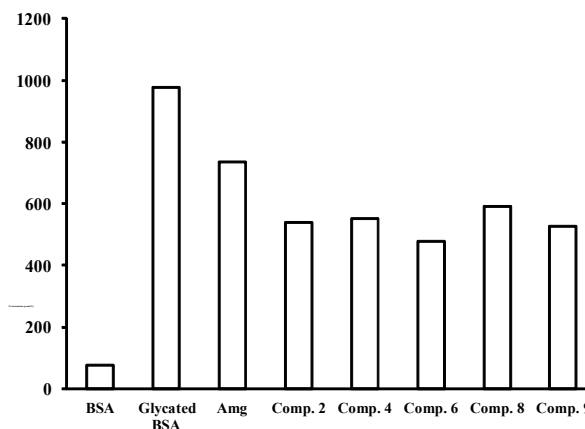


Fig. 6. Fructosamine levels of BSA, glycated BSA and glycated BSA in presence of aminoguanidine or title compounds.

Adduct formation of title compounds with glucose. The title compounds were incubated in the presence of glucose and the

reaction mixture was subjected to LC-MS analysis. All the compounds were found to form adducts with glucose. These are probably Schiff base type of adducts formed by the reaction of the free amino group in the title compounds with the aldehyde group of the sugar (Fig. 7, for a representative title compound, **2**. The adduct formation with other title compounds is depicted in the ESI, page S17 and S18). The formation of adduct with glucose could thus, be one of the ways by which the title compounds inhibit glycation.

Cytotoxicity assay

The cytotoxicity of the title compounds was evaluated in L6 rat muscle cells by measuring the cell viability using the standard MTT assay. It is a colourimetric assay that is based on the reduction of the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan derivative, that is achieved by enzymes present in viable cells. A decreased colour intensity is therefore indicative of low cell viability as a result of increased cytotoxicity. As seen in Fig. 8, all the title compounds were non-toxic at almost all concentrations tested, except at 40mM concentration, where a slight decrease in cell viability was observed, this being more pronounced in compounds **6** and **8**. However, this concentration is double that used for the glycation inhibition studies described herein.

Anti-oxidant properties of title compounds

AGEs, along with advanced lipoxidation products, have also been implicated in the generation of free radicals and reactive oxygen species (ROS), that are known to be involved in a variety of cellular processes ranging from apoptosis and necrosis to carcinogenesis.¹⁶ It therefore follows that any candidate, that is capable of inhibiting AGE formation and additionally possess anti-oxidant properties, would have increased therapeutic value. Keeping this in mind, the title compounds were evaluated for their ability to reduce the concentration of intracellular ROS, using ascorbic acid as a control anti-oxidant, in a fluorescence-based assay. The anti-oxidant activity of a compound is evidenced by a decrease in the fluorescence intensity. Fig. 9 shows a comparative study of the title compounds for anti-oxidant activity, wherein, it was found that compounds **2** and **4** exhibited higher anti-oxidant activity than other compounds, including ascorbic acid. More specifically, the percent fluorescence values were 57, 56, 83, 62 and 70 for compounds **2**, **4**, **6**, **8** and ascorbic acid respectively. Compound **9** did not show anti-oxidant activity as observed for other compounds.

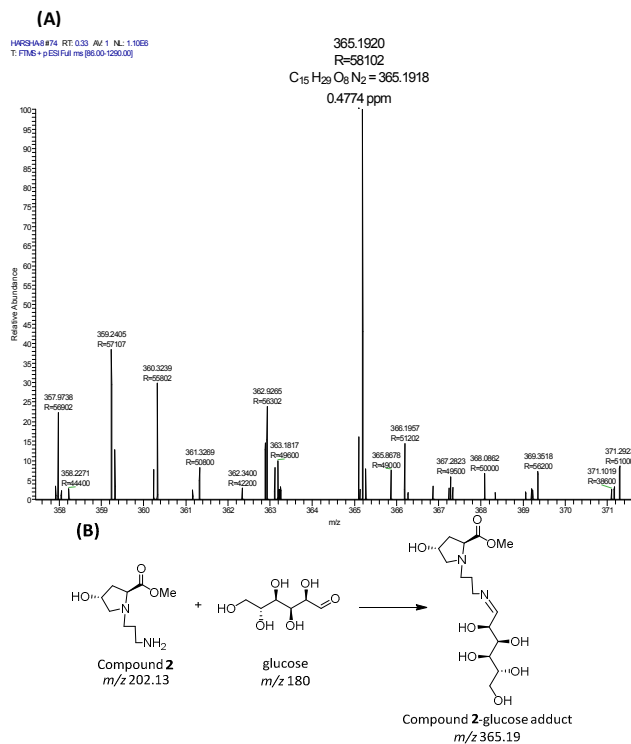


Fig. 7. (A) LC-MS spectrum of compound 2-glucose adduct and (B) Probable mechanism of compound 2-glucose adduct formation.

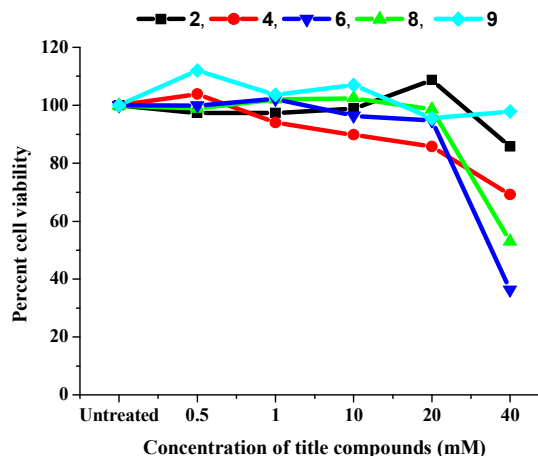


Fig. 8. MTT assay depicting viability of L6 rat muscle cells in presence of title compounds.

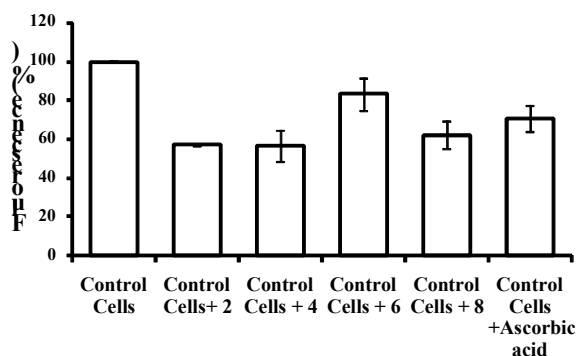


Fig. 9. Anti-oxidant properties of title compounds in comparison to ascorbic acid.

Experimental

Synthetic procedures

Methyl (2S,4R)-1-(3-((tert-butoxycarbonyl)amino)propyl)-4-hydroxypyrrolidine-2-carboxylate (1). Methyl (2S,4R)-4-hydroxypyrrolidine-2-carboxylate (1.00g, 6.8mmol) was dissolved in dry DMF; to it dry Et_3N (3.6mL, 20.0mmol) was added and stirred for 5 min at RT. Then to the above solution, 3-((tert-butoxycarbonyl)amino)propyl methanesulfonate (2.09g, 8.0mmol) was added drop-wise with continuous stirring. The reaction mixture was heated at 90°C for 10h. The reaction was monitored by TLC and after completion of the reaction, DMF was removed under vacuum and the compound was extracted from water with ethyl acetate. The organic layer was concentrated to get the crude compound, which was purified by column chromatography to get yellow coloured gum (1.3g, 65%). $[\alpha]_D = -48.31$ ($c = 0.135$ in MeOH); $^1\text{H NMR}$ (200 MHz, CDCl_3) δ : 1.44 (s, 9H), 1.59-1.69 (m, 2H), 2.08-2.17 (m, 2H), 2.38-2.56 (m, 2H), 2.69-2.83 (m, 1H), 3.08 (br s, 1H), 3.19 (br m, 2H), 3.36-3.34 (dd, $J = 5.4, 10.1\text{Hz}$, 1H), 3.50-3.58 (t, $J = 7.6\text{Hz}$, 1H), 3.72 (s, 3H), 4.43-4.46 (m, 1H), 5.38 (br s, 1H) ppm. $^{13}\text{C NMR}$ (50 MHz, CDCl_3) δ : 28.0, 28.4, 39.3, 51.8, 60.9, 64.4, 70.0, 78.9, 156.3, 174.4 ppm; $^{13}\text{C DEPT}$ (50 MHz, CDCl_3) δ : 28.0 (CH_2), 28.4, 38.5 (CH_2), 39.3 (CH_2), 51.8 (CH_2), 51.8, 60.9 (CH_2), 64.4, 70.0 ppm. HRMS: Calculated Mass for $\text{C}_{14}\text{H}_{26}\text{N}_2\text{O}_5$: 302.1842, Observed Mass (M+H): 303.1915, (M+Na): 325.1713.

Methyl (2S,4R)-1-(3-aminopropyl)-4-hydroxypyrrolidine-2-carboxylate (2). To compound **1** (0.10g, 0.33 mmol), a 50% solution of TFA in DCM (5mL) was added with vigorous stirring at RT. After completion of the reaction, solvents were removed under vacuum and crude compound was purified by column chromatography. The resultant TFA salt of the compound was obtained in 62% yield (0.065g). $[\alpha]_D = -24.47$ ($c = 0.11$ in MeOH). IR (neat) ν : 3352, 2946, 2833, 1749, 1683 cm^{-1} . $^1\text{H NMR}$ (200 MHz, D_2O) δ : 2.09 - 2.21 (m, 2H), 2.33-2.41 (m, 1H), 2.49 - 2.60 (m, 1H), 3.05-3.13 (m, 2H), 3.28-3.41 (m, 2H), 3.54-3.60 (m, 1H), 3.86 (s, 3H), 3.91-3.99 (m, 1H), 4.66 (br m, 2H) ppm. $^{13}\text{C NMR}$ (50 MHz, D_2O) δ : 24.6, 38.0, 38.8, 52.7, 53.2, 60.2, 64.8, 69.2, 175.5 ppm. $^{13}\text{C DEPT}$ (50 MHz, D_2O) δ : 34.6 (CH_2), 38.0 (CH_2), 38.8 (CH_2), 52.7, 53.2 (CH_2), 60.2 (CH_2)

64.8, 69.2 ppm. HRMS: Calculated Mass for $\text{C}_9\text{H}_{18}\text{N}_2\text{O}_3$: 202.1317, Observed Mass (M+H): 203.1392.

(2S,4R)-1-(3-((tert-butoxycarbonyl)amino)propyl)-4-hydroxypyrrolidine-2-carboxylic acid (3). Compound **1** (0.2g, 0.66 mmol) was dissolved in methanol and to it, 2N LiOH (3mL) was added with vigorous stirring. The reaction was monitored by TLC and after completion of reaction, the reaction mixture was neutralised using Dowex H^+ resin; the resin was subsequently filtered off. The filtrate was concentrated under vacuum and the compound was purified by column chromatography (0.12g, 64%). $[\alpha]_D = -35.06$, ($c = 0.09$ in MeOH). $^1\text{H NMR}$ (200 MHz, D_2O) δ : 1.39 (s, 9H), 1.82-1.86 (m, 2H), 2.11-2.51 (m, 2H), 3.16 (br m, 2H), 3.23 (br s, 1H), 3.31 (br. m, 2H), 3.85-3.93 (dd, $J = 4.2, 13.0\text{Hz}$, 1H), 4.14-4.23 (m, 1H), 4.59 (br s, 1H) ppm. $^{13}\text{C NMR}$ (50 MHz, D_2O) δ : 28.3, 30.1, 40.5, 58.0, 64.2, 71.1, 71.8, 83.6, 160.7, 175.6 ppm. $^{13}\text{C DEPT}$ (50 MHz, D_2O) δ : 28.3 (CH_2), 30.1, 39.3 (CH_2), 40.5 (CH_2), 58.0 (CH_2), 64.2 (CH_2) 71.1, 71.8 ppm. HRMS: Calculated Mass for $\text{C}_{13}\text{H}_{24}\text{N}_2\text{O}_5$: 288.1685, Observed Mass (M+H): 289.1757.

(2S,4R)-1-(3-Aminopropyl)-4-hydroxypyrrolidine-2-carboxylic acid (4). Compound **3** (0.30g, 1.04mmol) was dissolved in a 50% solution of TFA in DCM (5mL) with vigorous stirring at RT. The reaction was monitored by TLC and after the completion of reaction, solvents were removed under vacuum. Yield 0.19g (60%). $[\alpha]_D = -12.18$ ($c = 0.11$ in MeOH). IR (neat) ν : 3375, 2948, 2834, 1684 cm^{-1} . $^1\text{H NMR}$ (400 MHz, D_2O) δ : 2.12 - 2.13 (m, 2H), 2.25 - 2.32 (m, 1H), 2.52 - 2.56 (m, 1H), 3.06-3.10 (m, 2H), 3.27-3.38 (m, 2H), 3.52-3.54 (m, 1H), 3.94-3.97 (m, 1H), 4.51-4.55 (dd, $J = 7.1, 11.3\text{Hz}$, 1H), 4.64 (br s, 1H) ppm. $^{13}\text{C NMR}$ (50 MHz, D_2O) δ : 23.4, 36.3, 38.0, 48.8, 54.5, 61.8, 69.3, 172.9 ppm. $^{13}\text{C DEPT}$ (50 MHz, D_2O) δ : 23.4 (CH_2), 36.3 (CH_2), 38.0 (CH_2), 54.5 (CH_2), 61.8 (CH_2), 68.6, 69.3 ppm. HRMS: Calculated Mass for $\text{C}_8\text{H}_{16}\text{N}_2\text{O}_3$: 188.1161, Observed Mass (M+H): 189.1235.

Methyl (2S,4S)-4-azido-1-(3-((tert-butoxycarbonyl)amino)propyl)pyrrolidine-2-carboxylate (5). To a solution of **1** (0.7g, 2.30mmol) in dry DCM, dry Et_3N (0.9mL, 6.90mmol) was added and stirred for 5min at RT. Ms-Cl (0.3mL, 0.003mol) was added dropwise at 0°C, with continuous stirring. After completion of the reaction, DCM was evaporated under vacuum and the crude mesylated compound was used for further reaction without further purification. The mesylated compound was dissolved in dry DMF, to it NaN_3 was added with continuous stirring (1.5g, 23.1mmol). The reaction was heated for 10h at 75°C. After completion of the reaction, DMF was removed under vacuum and the compound was purified by column chromatography (0.58g, 67%). $[\alpha]_D = -0.0686$ ($c = 0.1$ in CHCl_3). $^1\text{H NMR}$ (200 MHz, CDCl_3) δ : 1.44 (s, 9H), 1.65-1.68 (m, 2H), 2.02-2.15 (br m, 1H), 2.34 - 2.60 (m, 3H), 2.78 - 2.88 (m, 1H), 3.15 - 3.25 (br m, 4H), 3.77 (s, 3H), 3.96 (br s, 1H), 5.47 (br s, 1H) ppm. $^{13}\text{C NMR}$ (50 MHz, CDCl_3) δ : 27.8, 28.4, 35.4, 38.4, 51.7, 52.1, 58.0, 58.7, 64.8, 77.2, 78.6, 156.2, 173.3 ppm. $^{13}\text{C DEPT}$ (50 MHz, CDCl_3) δ : 27.7 (CH_2), 28.4, 35.4 (CH_2), 38.4 (CH_2), 51.7 (CH_2), 52.1, 58.0 (CH_2), 58.7, 64.8 ppm. HRMS:

Calculated Mass for $C_{14}H_{25}N_5O_4$: 327.1907, Observed Mass (M+H): 328.1979, (M+Na): 350.1797.

Methyl (2S,4S)-1-(3-aminopropyl)-4-azidopyrrolidine-2-carboxylate (6). To compound **5** (0.3g, 0.91mmol), a 50% solution of TFA in DCM (5mL) was added with vigorous stirring at RT. The reaction was monitored by TLC and after the completion of reaction, solvents were removed under vacuum and the product was purified by column chromatography (0.19g, 61%). $[\alpha]_D = -8.83$ ($c = 0.105$ in MeOH). IR (neat) ν : 3376, 2188, 1745, 1678 cm^{-1} . 1H NMR (200 MHz, D_2O) δ : 0.90-1.01 (m, 1H), 1.82-1.94 (m, 2H), 2.23-2.30 (m, 1H), 2.58-2.60 (m, 1H), 2.80-2.87 (m, 2H), 3.17-3.27 (m, 2H), 3.64 (s, 3H), 4.40-4.45 (m, 2H) ppm. ^{13}C NMR (50 MHz, D_2O) δ : 23.1, 33.7, 36.2, 52.9, 54.2, 58.6, 59.9, 65.7, 169.2 ppm. ^{13}C DEPT (50 MHz, D_2O) δ : 23.0 (CH_2), 33.6 (CH_2), 52.8 (CH_2), 54.1, 58.5, 59.8 (CH_2), 65.7 ppm. HRMS (ESI): Calculated Mass for $C_9H_{17}N_5O_2$: 227.1382, Observed Mass (M+H): 228.1457.

(2S,4S)-4-Azido-1-(3-((tert-butoxycarbonyl)amino)propyl)pyrrolidine-2-carboxylic acid (7). Compound **5** (0.2g, 0.61 mmol) was dissolved in methanol and to it 2N LiOH (3mL) was added with vigorous stirring. After completion of the reaction, the reaction mixture was neutralised using Dowex H^+ resin; the resin was subsequently filtered off. The filtrate was concentrated under vacuum and the compound was purified by column chromatography (0.125g, 65%). $[\alpha]_D = -4.54$ ($c = 0.11$ in MeOH). 1H NMR (200 MHz, D_2O) δ : 1.42 (s, 9H), 1.87-1.89 (br m, 2H), 2.34-2.41 (m, 1H), 2.68 - 2.79 (m, 1H), 3.06 - 3.39 (br m, 6H), 3.76-3.82 (m, 1H), 4.08-4.12 (m, 1H), 4.57 (br s, 1H) ppm. ^{13}C NMR (50 MHz, D_2O) δ : 25.7, 27.6, 34.3, 53.5, 59.0, 59.6, 67.2, 81.1, 158.2, 173.0 ppm. ^{13}C DEPT (50 MHz, D_2O) δ : 25.7 (CH_2), 27.6, 34.3 (CH_2), 36.8 (CH_2), 53.5 (CH_2), 59.0, 59.6 (CH_2), 67.2 ppm. HRMS (ESI): Calculated Mass for $C_{13}H_{23}N_5O_4$: 313.1750, Observed Mass (M+H): 314.1817, (M+Na): 336.1635.

(2S,4S)-1-(3-Aminopropyl)-4-azidopyrrolidine-2-carboxylic acid (8). To compound **7** (0.3g, 0.95mmol), a 50% solution of TFA in DCM (5mL) was added with vigorous stirring at RT. The reaction was monitored by TLC and after the completion of reaction, solvents were removed under vacuum. The crude compound was used without further purification (0.132g, 63%). $[\alpha]_D = -13.90$ ($c = 0.105$ in MeOH). IR (neat) ν : 3377, 2953, 2122, 1683 cm^{-1} . 1H NMR (200 MHz, D_2O) δ : 2.08-2.16 (m, 2H), 2.38-2.46 (m, 1H), 2.70-2.85 (m, 1H), 3.04-3.11 (m, 2H), 3.37-3.41 (m, 2H), 3.80-3.86 (m, 1H), 4.25-4.29 (dd, $J = 3.5, 10.8$ Hz, 1H), 4.56-4.59 (m, 1H) ppm. ^{13}C NMR (50 MHz, D_2O) δ : 23.3, 34.1, 36.4, 52.8, 58.8, 60.0, 66.4, 162.7 ppm. ^{13}C DEPT (50 MHz, D_2O) δ : 23.3 (CH_2), 34.1 (CH_2), 36.3 (CH_2), 52.8 (CH_2), 58.8, 60.0 (CH_2), 66.4 ppm. HRMS (ESI): Calculated Mass for $C_8H_{15}N_5O_2$: 213.1226, Observed Mass (M+H): 214.1299.

(2S,4S)-4-Amino-1-(3-aminopropyl)pyrrolidine-2-carboxylic acid (9). Compound **8** (0.2g, 0.61 mmol) was dissolved in methanol, to it Palladium on charcoal (0.02g) was added and the reaction was allowed to proceed under H_2 pressure (35-40psi). After completion of reaction, the solvent was removed

under vacuum and the crude compound (0.112g, 61%) was purified by HPLC. $[\alpha]_D = -13.9$ ($c = 0.1$ in MeOH). 1H NMR (400 MHz, D_2O) δ : 1.99-2.23 (m, 3H), 2.96-3.09 (m, 3H), 3.20-3.35 (m, 1H), 3.43-3.58 (m, 1H), 3.70-3.81 (m, 1H), 3.91-4.00 (m, 1H), 4.24-4.34 (m, 2H) ppm. ^{13}C NMR (100 MHz, D_2O) δ : 23.2, 32.7, 36.2, 47.2, 52.7, 55.8, 67.6, 163.0, 170.1 ppm. ^{13}C DEPT (100 MHz, D_2O) δ : 23.2 (CH_2), 32.7 (CH_2), 36.2 (CH_2), 47.2, 52.7 (CH_2), 55.8 (CH_2), 67.6 ppm. HRMS (ESI): Calculated Mass for $C_8H_{17}N_3O_2$: 187.1321, Observed Mass (M+H): 188.1392.

Materials and methods

All the chemicals were procured from Sigma-Aldrich unless otherwise mentioned. TLC analyses were carried out on precoated silica gel 60 F_{254} (Merck). Column chromatographic separations were performed using neutral alumina or silica gel (60-120 mesh or 200-400 mesh, Merck) and using the solvent systems EtOAc/petroleum ether or MeOH/DCM. 1H and ^{13}C NMR spectra were obtained using Bruker AC-200, AC-400 NMR spectrometers. The chemical shifts are reported in delta (δ) values and referred to internal standard TMS for 1H . High resolution mass spectra were recorded on a Thermo Fisher Scientific Q Exactive mass spectrometer. Specific rotations of samples were recorded on a Bellingham Stanley ADP220 Polarimeter, IR was recorded on Bruker Alpha spectrophotometer. Primary anti-AGE antibody, anti-CML antibody and secondary antibody conjugate were purchased from Merck Millipore (India).

Circular dichroism measurements. The CD spectra were measured as described earlier.⁵ The protein concentration was constant in all the samples (0.02 mg ml^{-1}). CD spectra were acquired on a JASCO J-815 spectropolarimeter at room temperature. The spectra were collected as accumulations of three scans and were corrected for respective blanks. Results are expressed as molar ellipticity, $[\theta]$ (deg cm^2 $dmol^{-1}$). The CD spectra of the protein samples were analysed to calculate the secondary structure content using CDPro software that has three algorithms: CONTINLL, CDSSTR and SELCON3.¹⁷

In vitro glycation of bovine serum albumin (BSA). The BSA glycation reaction was performed as described earlier by Kanska and Boratyński¹⁸ with minor modification described recently by Kolekar *et al.*⁵ with or without title compounds (20mM) and kept at 37°C for 10-15 days. *In vitro* glycation was monitored by fluorescence spectroscopy, excitation at 370nm and emission at 440nm by using a spectrofluorometer (Thermo, Varioskan Flash Multimode Reader).

IC₅₀ determination of representative title compounds. The percent inhibition of AGE formation was calculated using the formula: %Inhibition = $(1 - F_i/F_c) \times 100$, where F_i = fluorescence intensity of glycated BSA treated with inhibitor and F_c = fluorescence intensity of glycated BSA in the absence of any inhibitor. The apparent IC₅₀ was determined by plotting the percent glycation inhibition Vs inhibitor concentration.

Western blot analysis using anti-AGE and anti-CML antibodies. BSA, glycated BSA or glycated BSA with 20mM of aminoguanidine or 20mM of compounds 2, 4, 6, 8, and were incubated at 37°C for 10-15 days. 5µg of each protein sample was separated by 12% SDS-PAGE and transferred onto a PVDF membrane. The membranes were blocked with 5% skimmed milk powder dissolved in PBS. The proteins were probed by anti-AGE and anti-CML antibodies at the antibody dilution of 1:2000 each. Secondary anti-rabbit antibody conjugated with HRP was used at a dilution of 1:5000 for both the blots. Immunoreactive bands were visualized using Western Bright Quantum western blotting detection kit (Advansta) and documented by Syngene Imaging System.

MALDI-TOF-based insulin glycation assay. The reaction mixture (100µl) comprising phosphate buffer (pH 7.4, 0.1M) containing title compounds (20mM), insulin (1.8 mg/ml, 25µl) and glucose (250mM, 25µl) was incubated at 37°C. The reaction was monitored till the relative intensity of glycated insulin reached 100% as seen by MALDI-TOF-TOF analysis (AB SCIEX TOF/TOF™ 5800) (5 days). The reaction mixture was mixed with sinapinic acid (ratio 1:5) and analysed by MALDI-TOF-TOF in linear mode using Anchor Chip 384 targets as described.¹

Fructosamine assay. The fructosamine level was measured by the NBT Labkit (Chemelx, S.A.) protocol. 300 µl of 0.75 mM NBT was added to a 96-well microplate containing 30 µl of 0.50µg BSA, glycated BSA, or glycated BSA with title compounds (20mM). Glycated BSA with aminoguanidine (20mM) was taken as a control. The reduction of NBT absorbance by fructosamine was measured at 520nm immediately after additions (considered as Abs1) and after incubation at 37°C for 15 min (considered as Abs2). The absorbance was monitored by using a UV spectrophotometer (UV 1800, Shimadzu).

Adduct formation with glucose. To elucidate the AGE inhibition mechanism of the title compounds, 2, 4, 6, 8, 9 (20mM each) were incubated with glucose (0.5M) in phosphate buffer pH 7.4 at 37°C for 6 days. The reaction was analysed by LC-MS on Q-Exactive Orbitrap to study and detect the glucose adduct formation with the title compounds.

MTT assay for cytotoxicity. L6 rat muscle cells were seeded at a cell density of 1×10^4 cells per well in a 96 well plate. After the cells adhered and attained their morphology, they were serum starved for 4h and treated with various concentrations of the title compounds (2, 4, 6, 8, and 9) in triplicate for 16h, while only serum starved cells served as control. After incubation, cells were given one wash with PBS and 100µL fresh serum free media was added. 6µL of 5mg/ml MTT (dissolved in PBS) was added to each well and incubated in dark at 37°C until violet formazan crystals were observed. Media from each well was discarded and crystals were dissolved in 100µL DMSO. Absorbance was measured at 555nm using Varioskan flash

multimode plate reader. This assay was performed using the Vybrant MTT Proliferation Assay Kit from Invitrogen.

Flow cytometry analysis of intracellular reactive oxygen species in *Saccharomyces cerevisiae* BY4743. Levels of intracellular ROS were measured in the presence of title compounds using ascorbic acid as control with 2', 7'-dichlorodihydrofluorescein diacetate (Molecular Probes). Cells were grown in SD medium with or without compounds for 3 days. Briefly, aliquots were taken after 3 days, cells were pelleted down and washed with PBS (137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 2mM KH₂PO₄). Cells were incubated with the dye (10µM) for 90min at 30°C. Cells were then washed twice in PBS and analyzed by flow cytometry analysis on BD Accuri C6 flow cytometer equipped with a 50mW argon laser emitting at 488nm. The green fluorescence was collected through a 488nm blocking filter. Data acquired from a minimum of 10,000 cells per sample at low flow rate were analyzed¹⁹ using BD accuri C6 software.

Orbitrap Analysis, database search and PTM analysis. 100µg of protein was reduced with 100mM dithiothreitol (DTT) at 60°C for 15 min, then alkylated with 200mM iodoacetamide in dark at room temperature for 30min. Proteins were digested by adding porcine trypsin in the ratio of 1:50 (final enzyme:proteins) at 37°C overnight. The digestion reaction was stopped by adding concentrated HCL and incubated for 10min at 37°C before vortexing and centrifugation and analysed on LC MS/MS (Q-Exactive Orbitrap, Thermo). The chromatographic separation was performed as described in our earlier report.⁵ The mass spectrometric data were processed using Proteome discoverer 1.4 (Version 1.4.0.288, (Thermo Fisher Scientific, Bremen, Germany). The data were searched against UniProt Bovine Serum Albumin (P02769) sequence database. Carbamidomethylation of cysteine (C) and oxidation at methionine (M) was considered as fixed and variable modification respectively. Additionally glycation modifications at lysine position were searched as dynamic variable modification included Amadori (+162.02 Da); Peptide and fragment mass tolerance were 10ppm, 0.5Da respectively with minimum of 2 missed cleavages and 1% false discovery rate (1%FDR). The identification of glycation modifications were selected based on the criteria described earlier.²⁰

Conclusions

The studies reported herein represent an important contribution towards the search for new molecules that not only inhibit glycation and AGE formation, but are also effective at controlling the concentration of intracellular reactive oxygen species. Moreover, the title compounds are easily accessible synthetically through simple chemical transformations, that should be amenable to scale-up. The superior antiglycation properties of the title compounds have been demonstrated by circular dichroism, fluorescence spectroscopy, Western blot assay and also mass spectrometry. Mass spectrometric analysis and the fructosamine assays

suggest that the title compounds form adducts with glucose, indicating that they probably act by inhibiting the formation of Amadori products. The low cytotoxicity of the title compounds is yet another favourable attribute. On the whole, the compounds-methyl (2*S,4R*)-1-(3-aminopropyl)-4-hydroxypyrrolidine-2-carboxylate (**2**) and (2*S,4R*)-1-(3-aminopropyl)-4-hydroxypyrrolidine-2-carboxylic acid (**4**) exhibited the best activity in the present study. These results suggest the contribution of the C4-hydroxyl group towards the antiglycation activity of the title compounds, in addition to the free amino function. The hydroxyl group offers possibilities of hydrogen-bonding and/or metal ion chelation, which could influence the activity of the respective title compound. Further, C4 being a chiral centre, the role of stereochemistry on the observed activity also cannot be ruled out. Studies towards dissecting and discerning these effects are currently underway in our laboratory and will be reported in due course.

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Graphical Abstract

***N*-(3-aminoalkyl)proline derivatives with potent antiglycation activity**

Harsha Chilukuri,^{a,b} Yogesh M. Kolekar,^a Govind S. Bhosle,^b Rashmi K. Godbole,^a Rubina S. Kazi,^a Mahesh J. Kulkarni^{a,*} and Moneesha Fernandes^{b,*}

