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Improved Synthesis of Lysine- and Arginine-Derived Amadori and Heyns Products and in Vitro Measurement of their Angiotensin I-Converting Enzyme Inhibitory Activity

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Supporting Information

ABSTRACT: The L-lysine- and L-arginine-derived Amadori and Heyns products consisting of *N*-(1-deoxy-D-fructos-1-yl)amino acid and *N*-(2-deoxy-D-glucos-2-yl)amino acid were prepared by reaction of D-fructose and D-glucose with L-lysine hydrochloride and L-arginine hydrochloride using commercial zinc powder as deprotonating reagent and also as catalyst precursor in a simple synthetic route in high yield. These compounds were screened for angiotensin I-converting enzyme (ACE) inhibitory activity using a high-throughput colorimetric assay (utilizing porcine kidney ACE). The IC_{50} values fall in the range of 1030−1175 μM, with N^{α}-(1-deoxy-D-fructos-1-yl)arginine showing the best IC_{50} value (1030 ± 38 μM). This study demonstrates an improved synthetic method for simple Amadori and Heyns products and their moderate ACE inhibitor activity.

KEYWORDS: *Amadori products, Heyns products, zinc, ACE inhibition*

INTRODUCTION

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) is a zinc-containing carboxy peptidase distributed in many tissues including endothelial and epithelial cells as somatic ACE (sACE) and also in germinal cells of testis as germinal ACE (gACE). ACE plays a major role in the regulation of blood pressure by catalyzing formation of potent vasoconstrictor angiotensin II by cleavage of C-terminal dipeptide His-Leu and inactivation of vasodilator bradykinin by cleavage of terminal dipeptide Phe-Arg.[1] As a result of this dual role, inhibition of ACE is a key therapeutic target for hypertension. In addition, ACE is also a major drug target for congestive heart failure, myocardial infarction, renal failure, and diabetic nephropathy.[2,3] Captopril (D-3-mercapto-2-methylpropionyl]-L-proline), based on the Brazilian snake venom, is the first pharmaceutical developed for treatment of mild to moderate hypertension.[4,5] Subsequently, many synthetic inhibitors have been developed including lisinopril, enalapril, zofenoprilat, and fosinoprilat.[6,7] The main drawback of the synthetic inhibitors is adverse side effects. Currently, there is great focus on developing natural and safe inhibitors from animal sources consisting of fermented milk, casein, various fish varieties including tuna, sardine, shark, and yellowfin sole, and plant sources consisting of rape seed, soy bean protein, and corn gluten.[8−16]

Amadori and Heyns compounds are products formed due to the rearrangement reaction of amino acid with aldoses and ketoses, respectively.[17,18] Amadori and Heyns reactions are part of the general Maillard reaction which plays a pivotal role in protein glycation.[19] In addition, it affects the flavor and nutritional quality of food.[20] The Maillard reaction products (MRPs) have been shown to form metal complexes.[5,21] In a study involving humans undergoing heat-sterilized parenteral nutrition, the observed extreme urinary loss of zinc, copper, and iron ions was attributed to chelation of MRPs resulting from the glucose and amino acids.[22] In another study, it was shown that rats fed with fructosyl lysine resulted in the lowering of kidney zinc.[23] Maillard-derived polymeric melanoidins have been shown to inhibit ACE.[24] On considering these previous observations, we hypothesized that lysine- and arginine-derived simple Amadori and Heyns compounds due to their ability to chelate zinc may result in inhibition of ACE. In addition, our aim was also to develop an easy and efficient synthetic method for lysine- and arginine-derived simple Amadori and Heyns compounds. We present herein an improved method of synthesis for Amadori compounds consisting of *N*-(1-deoxy-D-fructos-1-yl)amino acid (amino acid = L-arginine and L-lysine) and Heyns compounds consisting of *N*-(2-deoxy-D-glucos-2-yl)amino acid (amino acid = L-arginine and L-lysine). In addition, we report in vitro measurement of angiotensin I-converting enzyme inhibitory activity of these compounds.

METHODS AND MATERIALS

L-Lysine hydrochloride, L-arginine hydrochloride, hippuryl-histidyl-leucine (HHL), hippuric acid (HA), sodium tetraborate, and Triton X-100 were procured from Sigma Aldrich. D-Fructose, D-glucose, and glacial acetic acid were purchased from Merck India. Zinc powder, pyridine, and benzene sulfonyl chloride (BSC) were from Spectrochem Pvt Ltd. Mass spectra were recorded in the Q-TOF ULTIMA instrument of Waters Corp. in the ESI positive mode. NMR spectra were recorded on a Bruker Avance 400 instrument (Reinstetten, Germany) with 400 MHz for ^{1}H and 100 MHz for $^{13}C\{H\}$ experiments. Proton chemical shifts are given relative to internal HOD signal (4.79 ppm) for D_2O solutions. Carbon chemical shifts are given relative to the signal of the external standard

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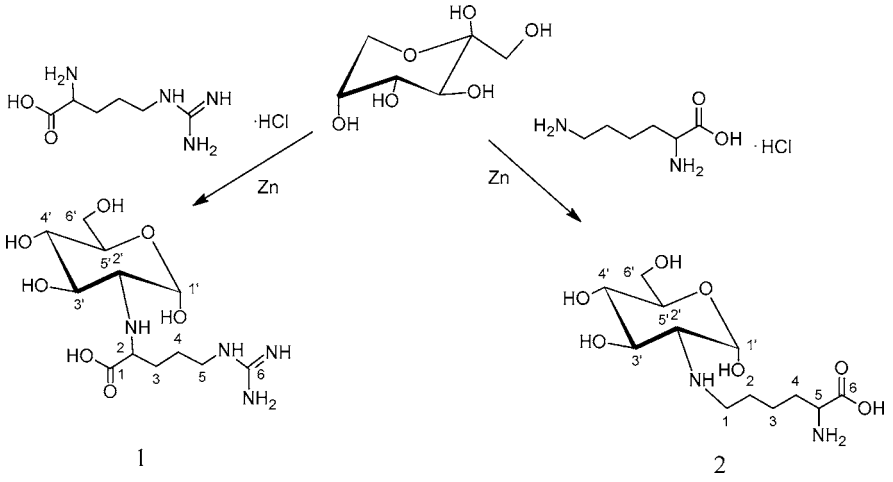

Figure 1. Synthesis of Heyns compounds N^{α}-(2-deoxy-D-glucos-2-yl)arginine (**1**) and N^{ε}-(2-deoxy-D-glucos-2-yl)lysine (**2**) from D-fructose with L-arginine hydrochloride and L-lysine hydrochloride. Reaction conditions: C_6H_5N/CH_3COOH, room temperature, 2 days.

tetramethylsilane for D_2O solutions. Assignments of ^1H and ^{13}C signals were based on ^1H, ^{13}C, HSQC (heteronuclear single-quantum coherence), HMBC (heteronuclear multiple bond correlation), and DEPT (distortionless enhancement by polarization transfer) experiments.

***N*$^{\alpha}$-(2-Deoxy-D-glucos-2-yl)arginine (1).** A mixture of pyridine (20 mL), glacial acetic acid (20 mL), L-arginine hydrochloride (1.69 g, 0.008 mol), and zinc powder (0.53 g, 0.008 mol) was stirred for 40 min followed by addition of D-fructose (1.72 g, 0.0095 mol). The reaction mixture was stirred for 2 days at room temperature, and then it was filtered. Acetone was added (125 mL) to the filtrate to get fluffy solid, which settles to the bottom. Solvent was removed under nitrogen pressure using cannula; subsequently, the solid was washed several times with acetone (250 mL) and followed by diethyl ether (100 mL) to remove completely pyridine. Further, solvents were removed under vacuum, and product was redissolved in methanol (25 mL) and filtered. The filtrate was concentrated, and solvent was removed completely to get an off-white solid. Yield: 0.0079 mol, 83%. ESI-MS positive mode $(M)^+$ m/z: 336.42. Exact mass $(M)^+$: 336.1645. ^1H NMR of α-pyranose form (δ): 1.65 (m, 2H, $H_2C(4)$), 1.88 (m, 2H, $H_2C(3)$), 3.21 (t, 2H, J = 6.9 Hz, $H_2C(5)$), 3.62 (m, 1H, HC(2)), 3.52 (dd, 1H, HC(2′)), 3.73 (dd, 1H, HC(5′)), 3.76 (dd, 1H, HC(4′)), 3.85 (dd, 2H, HC(6′)), 3.99 (dd, 1H, HC(3′)), 5.34 (d, J = 3.1 Hz, 1H, HC(1′)). ^{13}C NMR of α-pyranose form (δ): 183.71 (C1), 66.10 (C2), 30.21 (C3), 26.53 (C4), 43.15 (C5), 159.44 (C6), 92.75 (C1′), 66.57 (C2′), 66.02 (C3′), 70.23 (C4′), 56.15 (C5′), 71.86 (C6′). ^1H NMR of β-pyranose form (δ): 5.01 (d,1H). ^{13}C NMR of β-pyranose form (δ): 95.12 (C1′).

***N*$^{\varepsilon}$-(2-Deoxy-D-glucos-2-yl)lysine (2).** This compound was prepared utilizing L-lysine hydrochloride (1.46 g, 0.008 mol), zinc powder (0.53 g, 0.008 mol), and D-fructose (1.72 g, 0.0095 mol) following the similar procedure presented for compound **1**. Yield: 0.008 mol, 84%. ESI-MS positive mode $(M + H)^+$ m/z: 309.42. Exact mass $(M + H)^+$: 309.1662. ^1H NMR of α-pyranose form (δ): 1.46 (m, 2H, HC(3)), 1.69 (m, 2H, HC(2)), 1.87 (m, 2H, HC(4)), 2.99 (m, 2H, $H_2C(1)$), 3.17 (dd, 1H, HC(2′)), 3.57 (dd, 1H, HC(4′)), 3.72 (t, 1H, J = 7 Hz, HC(5)), 3.78 (dd, 1H, HC(5′)), 3.86 (dd, 2H, HC(6′)), 3.98 (dd, 1H, HC(3′)), 5.52 (d, 1H, J = 3 Hz, HC(1′)). ^{13}C NMR of α-pyranose form (δ): 38.65 (C1), 25.88 (C2), 20.93 (C3), 29.41 (C4), 54.02 (C5), 180.48 (C6), 87.38 (C1′), 62.86 (C2′), 62.84 (C3′), 63.42 (C4′), 67.06 (C5′) 69.18 (C6′). ^1H NMR of β-pyranose form (δ): 5.02 (d, 1H). ^{13}C NMR of β-pyranose form (δ): 91.76 (C1′).

***N*$^{\alpha}$-(1-Deoxy-D-fructos-1-yl)arginine (3).** Synthesis of this compound was performed similar to compound **1** utilizing L-arginine hydrochloride (1.69 g, 0.008 mol), zinc powder (0.53 g, 0.008 mol), and D-glucose (1.72 g, 0.0095 mol). Yield: 0.008 mol, 84%. ESI-MS positive mode $(M)^+$ m/z: 336.39. Exact mass $(M)^+$: 336.1645. ^1H NMR of α-pyranose form(δ): 1.56 (m, 2H, $H_2C(4)$), 1.56 (m, 2H, $H_2C(3)$), 3.15 (t, 2H, J = 6.4 Hz, $H_2C(5)$), 3.09 (t, 1H, J = 5.9 Hz, HC(2)), 3.70 (d, 1H, J = 10.1 Hz, HC(2′)), 3.62 (dd, 1H, J = 1.8 Hz, J = 12.8 Hz, HC(5′)), 3.96 (d, 1H, J = 12.8 Hz, HC(5′)), 3.92 (d, 1H, J = 1.8 Hz HC(4′)), 2.72 (bs, 2H, HC(6′)), 3.81 (dd, 1H, J = 3.5 Hz, J = 9.8 Hz, HC(3′)). ^{13}C NMR of α-pyranose form (δ): 184.78 (C1), 66.52 (C2), 32.48 (C3), 27.36 (C4), 43.61 (C5), 159.42 (C6), 100.44 (C1′), 71.94 (C2′), 72.42 (C3′), 71.85 (C4′), 65.96 (C5′), 55.51 (C6′).

***N*$^{\varepsilon}$-(1-Deoxy-D-fructos-1-yl)lysine (4).** Synthesis of this compound was accomplished similar to compound **1** utilizing L-lysine hydrochloride (1.46 g, 0.008 mol), zinc powder (0.53 g, 0.008 mol), and D-glucose (1.72 g, 0.0095 mol). Yield: 0.0078 mol, 82%. ESI-MS positive mode $(M)^+$ m/z: 308.29. Exact mass $(M)^+$: 308.1584. ^1H NMR of α-pyranose form (δ): 1.46 (m, 2H, $H_2C(3)$), 1.73 (m, 2H, $H_2C(4)$), 1.90 (m, 2H, HC(2)), 3.02 (t, 2H, J = 7.9 Hz, $H_2C(1)$), 4.00 (s, 1H, HC(5)), 3.74 (d, 1H, J = 10 Hz, HC(2′)), 3.75 (d, 1H, J = 12 Hz, HC(5′)), 3.96 (d, 1H, J = 12 Hz, HC(5′)), 3.72 (m, 1H, HC(4′)), 3.28 (s, 2H, HC(6′)), 3.87 (d, 1H, J = 10 Hz, HC(3′)). ^{13}C NMR of α-pyranose form (δ): 41.83 (C1), 31.81 (C2), 24.32 (C3), 27.58 (C4), 71.65 (C5), 183.76 (C6), 98.15 (C1′), 72.48 (C2′), 72.03 (C3′), 72.38 (C4′), 66.66 (C5′), 55.53 (C6′).

Preparation of Porcine Lung ACE. ACE was extracted from the porcine kidney acetone powder in the laboratory as described earlier.[12]

In Vitro Colorimetric Assay of ACE Inhibitor Activity. ACE activity was assayed by monitoring the release of HA from hydrolysis of the substrate HHL. Porcine kidney ACE was preincubated at 37 ± 2 °C for 10 min with Amadori or Heyns compound, and the residual ACE activity was determined as follows.

The assay mixture contained 0.125 mL of 0.05 M sodium borate buffer pH 8.2 containing 0.3 M NaCl, 0.05 mL of 5 mM HHL, and 0.025 mL of ACE enzyme or ACE enzyme extract (the enzyme extract incubated at 37 ± 2 °C with Amadori or Heyns compound for 10 min). Reaction was arrested after incubation at 37 °C for 30 min by addition of 0.2 mL of 1 M HCl. After stopping the reaction, 0.4 mL of pyridine was added followed by 0.2 mL of BSC, and the solution was mixed by inversion for 1 min and cooled on ice. The yellow color developed was measured at 410 nm. One unit of ACE activity is defined as the amount of enzyme that releases 1 μmol of HA per min at 37 °C and pH 8.2. The IC_{50} value is defined as the concentration of the inhibitor required to decrease the ACE activity by 50%. The percent inhibition curves (residual activity) were plotted using a minimum of seven determinations for each inhibitor concentration, and IC_{50} values were computed from the semilogarithmic plots. Linear regression analysis was performed using Microsoft Excel. IC_{50} values for Amadori and Heyns compounds were determined in triplicate. IC_{50} values are expressed as mean ± 2SD.

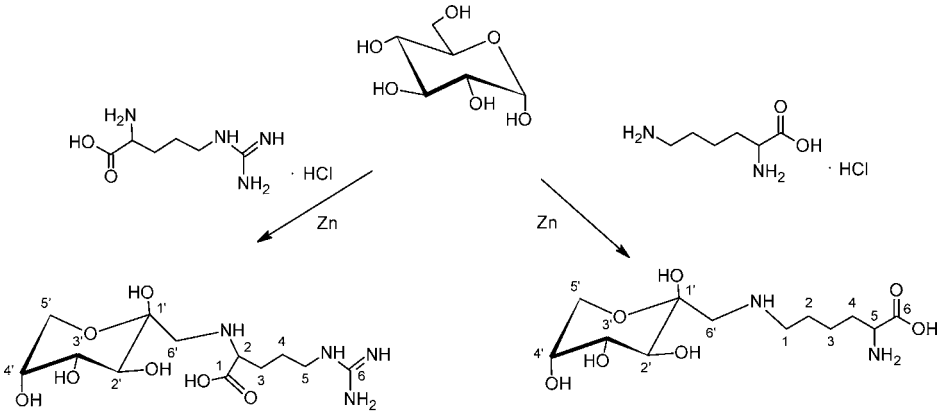

Figure 2. Synthesis of Amadori compounds N^{α}-(1-deoxy-D-fructos-1-yl)arginine (**3**) and N^{ε}-(1-deoxy-D-frutos-1-yl)lysine (**4**) from D-glucose with L-arginine hydrochloride and L-lysine hydrochloride. Reaction conditions: C_6H_5N/CH_3COOH, room temperature, 2 days.

RESULTS

A majority of the synthetic efforts in accomplishing Amadori and Heyns compounds utilize methanol or dimethyl sulfoxide as solvent with vigorous reaction conditions. In addition, tedious column separations are employed to achieve spectroscopically pure compounds. Kiel et al. reported a simple method for fructosyl valine utilizing pyridine and glacial acetic acid as solvents under mild conditions.[25] Even this method delivers low yield and requires 4 days of stirring time. We modified this method by utilizing an inexpensive reagent commercial zinc powder.

Synthesis of N^{α}-(2-Deoxy-D-glucos-2-yl)arginine (1) and N^{ε}-(2-Deoxy-D-glucos-2-yl)lysine (2). Reaction of D-fructose with L-arginine hydrochloride or L-lysine hydrochloride in the solvent combination of pyridine and glacial acetic acid for 4 days resulted in unidentified products. Then, Heyns compounds N^{α}-(2-deoxy-D-glucos-2-yl)arginine (**1**) and N^{ε}-(2-deoxy-D-glucos-2-yl)lysine (**2**) (Figure 1) were achieved by reaction of L-arginine hydrochloride and L-lysine hydrochloride with D-fructose and utilizing commercial zinc powder in pyridine and glacial acetic acid for 2 days at room temperature, respectively. Work up of the reaction involved simple steps, and it produced spectroscopically pure compounds with 80−85% yield.

Compounds **1** and **2** were characterized by ESI mass and NMR spectroscopy. Previous NMR spectroscopic investigation of different Heyns rearrangement products has shown that these compounds exist mainly as an equilibrium of the α-pyranose and β-pyranose forms in the $^4C_1$ conformation.[26] The other possible forms consisting of the open form and the furanose form were below the detection limit as indicated by ^{13}C NMR. We observed from our studies that glucosyl arginine is present in the α-pyranose form to an extent of 95% and β-pyranose form in about 5%. Similar to glucosyl arginine, glucosyl lysine occurs in the α-pyranose form to an extent of 95% and β-pyranose form in less than 5%. Glucosyl lysine was found to be more hygroscopic compared to glucosyl arginine. These compounds upon exposure to air become syrupy liquid; however, there is no indication of decomposition as revealed by ^1H NMR spectra.

Synthesis of N^{α}-(1-Deoxy-D-fructos-1-yl)arginine (3) and N^{ε}-(1-Deoxy-D-fructos-1-yl)lysine (4). Amadori compounds N^{α}-(1-deoxy-D-fructos-1-yl)arginine (**3**) and N^{ε}-(1-deoxy-D-fructos-1-yl)lysine (**4**) (Figure 2) were achieved by reaction D-glucose with L-arginine hydrochloride and L-lysine hydrochloride utilizing commercial zinc powder in pyridine and glacial acetic acid for 2 days at room temperature, respectively. The yield obtained was 80−85%.

Compounds **3** and **4** were characterized by ESI mass and NMR spectroscopy. NMR spectra of compounds **3** and **4** matched with the reported data.[27,28]

ACE Inhibitor Activity. Compounds **1**−**4** were screened for ACE inhibitor activity utilizing a high-throughput colorimetric assay reported recently.[29] The ACE used was extracted from porcine kidney. The porcine kidney ACE was preincubated for 10 min with different concentrations of each of the Amadori and Heyns compounds. The residual ACE activity was assayed by monitoring the release of HA from the substrate of HHL. The extent of HA released is directly proportional to ACE activity. Among the compounds screened, fructosyl arginine showed the lowest IC_{50} value (1038 ± 36 μM). The IC_{50} values for the other three compounds were in the range 1135−1175 μM (Table 1). We anticipated significantly different IC_{50} values for Amadori compounds compared to Heyns compounds as they differ in their structure. Among the compounds, only fructosyl arginine showed a slightly different value, whereas fructosyl lysine showed a similar value compared to glucosyl lysine and glucosyl arginine. Even when we compare among the Heyns compounds, similar IC_{50} values were observed, although the amino acid attached to the glucosyl moiety was different.

Table 1. IC_{50} Values for Inhibition of ACE by Lysine- and Arginine-Derived Amadori and Heyns Compounds

compounds	IC_{50} (μM)
N^{α}-(2-deoxy-D-glucos-2-yl)arginine (**1**)	1173 ± 22
N^{ε}-(2-deoxy-D-glucos-2-yl)lysine (**2**)	1163 ± 36
N^{α}-(1-deoxy-D-fructos-1-yl)arginine (**3**)	1038 ± 36
N^{ε}-(1-deoxy-D-fructos-1-yl)lysine (**4**)	1175 ± 40

IC_{50} values obtained for Amadori and Heyns compounds from our work are comparable to glucose esters of amino acids.[30] L-Isoleusyl glucose was shown to have an IC_{50} value of 0.7 ± 0.067 mM, whereas L-phenylalanyl D-glucose showed 1.0 ± 0.0089 mM. Maillard reaction derived melanoidins have

shown 45.1−62.1% of ACE inhibitiory activity for a concentration of 2 mg/mL.

DISCUSSION

The lysine- and arginine-derived simple Amadori and Heyns compounds were prepared utilizing the commercial zinc powder as deprotonating agent for lysine hydrochloride and arginine hydrochloride. Deprotonation of amino acid hydrochloride by zinc results in amino acid and zinc chloride. Then, the zinc chloride acts as a catalyst for subsequent Amadori or Heyns rearrangement reactions. The zinc halides have been demonstrated as catalysts in the preparation of carbohydrate-based surfactants via Heyns/Amadori rearrangement.[31] Our developed method uniquely incorporates Zn as reactant (deprotonating agent) and also as a precursor for the catalyst zinc chloride. As a result of catalysis by zinc chloride, a significant yield of 80−85% was observed for the first time in the synthesis of simple lysine- and arginine-derived Amadori and Heyns compounds.

Fructosyl lysine and fructosyl arginine are initial glycation products,[27] whereas glucosyl lysine and glucosyl arginine are initial fructation products.[32,33] Among these compounds, fructosyl lysine is precursor for the glucosepane, which is a major protein cross-linker of extracellular matrix.[34,35] Recent human studies have examined the bioavailability of injested early Maillard reaction products (expressed as fructosyl lysine). The studies revealed that about 95% of the ingested MRPs is not recovered in the excretion, indicating degradation in the intestine or plasma to unknown metabolites.[36,37] In addition, the small fraction of fructosyl lysine excreted in the urine is dependent on the content of the MRPs in the diet.[37] The low recovery in faeces was attributed to microbial degradation by *Escherichia coli*.[38] Koschinsky et al.[39] found that 10% of the oral load of MRPs (reported by the authors as reactive glycation products/glycotoxins) was intestinally absorbed and transported to the bloodstream. In addition, one-third of absorbed MRPs were excreted in the urine. Data on the absorption mechanism of MRPs is limited. Studies involving feeding experiments on fructosyl lysine[40] and in vitro studies[41] have indicated that fructosyl lysine is absorbed by passive diffusion after its release from the protein by digestive enzymes.

The in vivo concentration of free/protein-bound fructosyl lysine and also similar Amadori products is likely to be significant from the exogenous sources consisting of mainly diet and endogenous sources consisting of mainly glycation.[37] Free/protein-bound fructosyl lysine or fructosyl arginine may not bring a significant therapeutic effect for hypertension with IC_{50} values in the millimolar (mM) range (Table 1) compared to lisinopril or captopril. However, it is clear that these compounds from exogenous or endogenous sources may contribute to a small effect in total ACE inhibition happening in vivo. Inhibition studies of ACE by free fructosyl lysine or fructosyl arginine reported here serves as a model for inhibition by protein-bound fructosyl lysine and other MRPs. Precise in vivo experiments are required to understand further the role of early and advanced MRPs toward inhibition of ACE and subsequent effects against hypertension. Efforts are on toward this goal in our laboratory.

Possible Mechanism of ACE Inhibition. The mechanism of action for ACE-inhibitory activity of Maillard reaction derived melanoidins remains unknown as the structure of polymeric melanoidins is not elucidated until now. In our case, the structure of Amadori and Heyns compounds are well defined. As ACE is a Zn-dependent enzyme it could be plausible that the inhibitory activity is mainly due to metal-chelating properties of Amadori and Heyns compounds. Further, it has been well established that the MRPs form metal complexes.[5,21,42]

The crystal structure of t-ACE with lisinopril and captopril[43,44] revealed that ACE−inhibitor complex is predominantly α-helical with a deep inhibitor binding channel having catalytic Zn^{2+} (coordination provided by two histidine and glutamic acid and the fourth ligand by inhibitor to complete the tetraheadron). The thiol group of captopril interacts with catalytic Zn^{2+} ion (2.32 Å) forming a zinc−thiolate complex deep inside the channel at the active site of t-ACE. On the other hand, the carboxyalkyl carboxylate of lisinopril is well positioned to bind to the active-site zinc atom (2.14 Å) and provides one coordinating ligand. Although we do not have any evidence for binding of any particular group including guanidine NH_2 or carboxylate of fructosyl arginine, we expect weak interaction of the guanidine group. If the carboxylate group were to interact, probably a still lower IC_{50} value was expected. In the fructosyl lysine case, probably the lysine ε-NH_2 group is having an interaction with Zn^{2+}. Among all compounds, fructosyl arginine acts as a better donor for Zn^{2+} of the active site compared to fructosyl lysine, glucosyl arginine, and glucosyl lysine as indicated by their IC_{50} values. In addition to the weak ligand effect, steric hindrance of these compounds at the active site cannot be ruled out as an additional factor responsible for the observed moderate ACE inhibition.

In conclusion, we developed a simple, efficient catalytic synthetic method using inexpensive zinc in a dual role of deprotonating agent and catalyst precursor for lysine- and arginine-derived Amadori and Heyns compounds. In addition, we established moderate inhibitory activity of angiotensin-I converting enzyme of these compounds. This study may lead to screening of more MRPs available from food sources and glycation, for inhibitory activity of ACE, and for establishment of a beneficial effect against hypertension.

ASSOCIATED CONTENT

Supporting Information

IC_{50} determination for all compounds in triplicate for ACE inhibitory activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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