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Note

Identification of per-O-(trimethylsilyl) derivatives of aldoses generated from thermal decomposition of N-(1-deoxy-D-fructopyranos-1-yl)proline: reversibility of the Amadori rearrangement

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One manifestation of the widely occurring carbonyl–amine reaction in nature, is the non-enzymatic interaction of reducing sugars such as D-glucose with amino acids and proteins. The whole network of reactions stemming from this initial interaction is known as the Maillard reaction [1]. The initial phase of the reaction proceeds by the formation of a glycosylamine, which under acidic conditions can rearrange into the more stable 1-amino-1-deoxy-2-ketose form, a process known as the Amadori rearrangement [2]. The importance of this reaction stems from the fact that it can be initiated under physiological conditions. Long-lived body proteins and enzymes can be modified by glucose or other reducing sugars through the formation of Amadori compounds, a process known as glycation that subsequently can lead to the impairment of physiological functions, especially in diabetic patients and during aging [3]. Alternatively, at high temperature — conditions of cooking such as roasting or baking — Amadori products can be formed within hours and degrade or react with other food components to produce the characteristic aroma of baked and roasted food, in addition to the characteristic brown color [2].

As a succession of equilibrium reactions, the Amadori rearrangement is theoretically expected to be a reversible process [4]. The tendency of Amadori products (formed from

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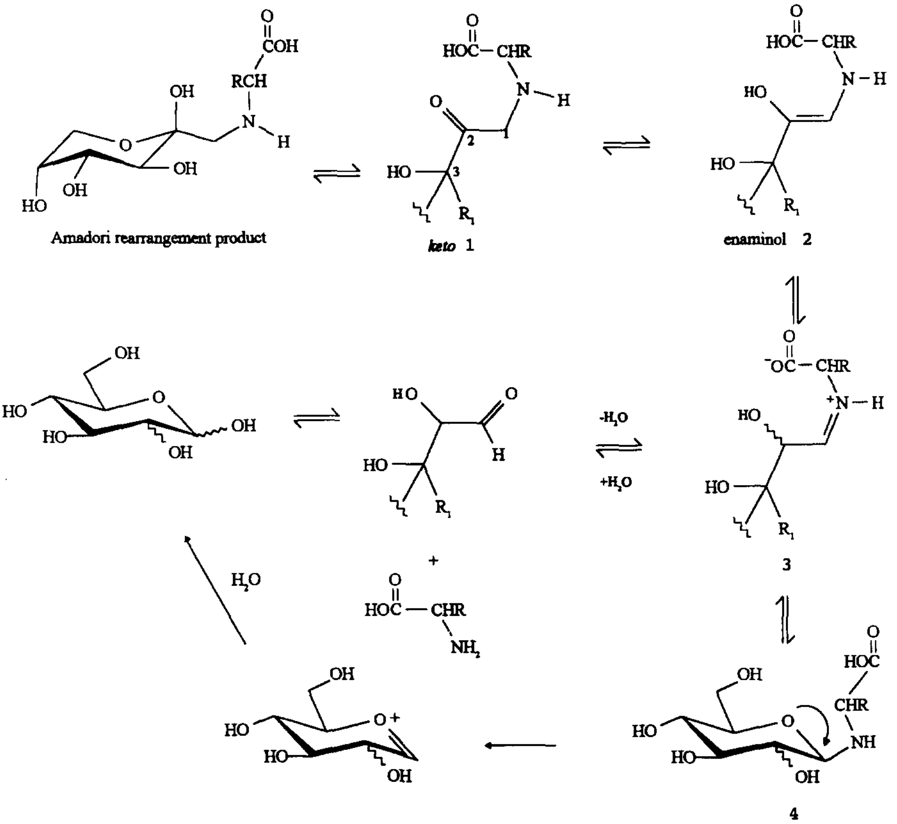
5′-ribosylphosphate) to undergo a non-enzymatic reversal into enolamines, and subsequently into the free sugar and amino acid, has been recently demonstrated [5] from the data obtained on the kinetics of *N*-(5′-*O*-phosphono-β-D-ribosyl)anthranilate ketol isomerase (EC 5.3.1.24). However, the reversibility of Amadori rearrangement is still a contentious issue [6], even though evidence has been presented in support of reversibility of both protein-bound and free Amadori products, under physiological conditions using glyceraldehyde [7] and *N*-α-formyl-*N*-ε-fructosyllysine [8]. When 2-oxo-3-(hydroxypropyl)-hemoglobin, produced by reacting hemoglobin with glyceraldehyde, was incubated in a phosphate–saline buffer at pH 7.4, 20% of the glyceraldehyde was released in 1 day at 37 °C [7]. However, *N*-(5′-phosphoribosyl)anthranilate spontaneously and irreversibly hydrolyzed to anthranilate with a half-life of 3.5 min at pH 7.6 [5]. According to Baynes et al. [4], if glyceraldehyde is replaced with glucose, the extra stability of Amadori rearrangement product gained by the formation of cyclic hemiketals, would probably decrease the rate of the reverse reaction and at the same time produce D-glucose and D-mannose, both being C-2 epimers. Indirect evidence for the formation of glucose and mannose was obtained when *N*-α-formyl-*N*-ε-fructosyllysine (15 mM) was incubated in a phosphate buffer (0.2 M, pH 7.4) in the dark at 37 °C under nitrogen for 15 days [8]. GLC–MS analysis of the reaction mixture after reduction with $NaBH_4$ revealed the formation of mannitol and glucitol. The arguments put forward against such evidence is based on the possibility of formation of "new" glucose and/or mannose by aldol condensation of two smaller sugar units such as hydroxyacetaldehyde and D-erythrose. The reversibility of Amadori rearrangement can have significant implications if the factors promoting the reverse reaction are identified and eventually controlled under physiological conditions. The reverse reaction might limit protein glycation and prevent further tissue damage. On the other hand, the presumed unavailability of ingested Amadori products as a nutritional source of essential amino acids could be viewed to be a less severe problem.

Although the reversibility of Amadori rearrangement is documented under physiological conditions, no conclusive evidence yet exists on its occurrence at high temperatures. Due to extensive thermal decompositions, it is expected that the reversibility of Amadori rearrangement becomes less important as the temperature is increased. However, providing evidence for reversibility can further support the phenomenon of reversibility under physiological conditions and provide additional insight into the kinetics and mechanism of decomposition of Amadori products.

To identify the carbohydrates produced during the thermal decomposition of Amadori rearrangement products, including the D-glucose and D-mannose that are expected to be formed as the result of reversibility of Amadori rearrangement (Scheme 1), *N*-(1-deoxy-D-fructopyranos-1-yl)proline (proline Amadori product) was thermally decomposed at 130 °C for 20 min and immediately reacted with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide to convert non-volatile carbohydrate degradation products into volatile trimethylsilyl ether derivatives that could be detected and identified by GLC–MS, since the mass spectra of per-*O*-(trimethylsilyl)ated sugar derivatives have been investigated in great detail [9]. The GLC-MS analysis of the decomposition mixture (Fig. 1) revealed the presence of per-*O*-(trimethylsilyl)ated derivatives of D-glucose, *aldehydo*-D-glucose, D-mannose, D-arabinose, D-arabinonic acid γ-lactone, 2-deoxy-pentonic acid, D-erythrose



Scheme 1. Proposed mechanism of reverse Amadori rearrangement.

and glyceric acid among other unidentified products. In order to verify the presence of the unreacted Amadori compound and other degradation products, synthetic Amadori products of proline with glyceraldehyde and with glucose were silylated and analyzed by GLC–MS. Scheme 2 shows the initial electron-impact fragmentations of the per-*O*-(trimethylsilyl)ated glucose Amadori compound. As with other trimethylsilyl derivatives of Amadori products reported in ref. [10], the main fragmentations lead to the formation of oxonium and imminium ions. The main chromatographic peak in the heated mixture of the proline Amadori compound (Fig. 1) was identified as the unreacted Amadori compound detected as the trimethylsilyl ester of *N*-[1-deoxy-2,3,4,5-tetrakis-*O*-(trimethylsilyl)-D-fructopyranos-1-yl]proline by comparison with the spectrum obtained by silylation of the authentic Amadori compound. However, the glyceraldehyde Amadori product of proline was not detected in the mixture. Pyrolysis-GLC–MS analysis [11] of this product indicated that it can quickly decompose and produce mainly 1-pyrrolidino-2-propanone.

The origin of aldoses detected as per-O-(trimethylsilyl)ated derivatives in the thermal decomposition mixture of the proline Amadori product.—Scheme 1 depicts the proposed mechanism of the reverse Amadori rearrangement. The open chain *keto* form **1** of the

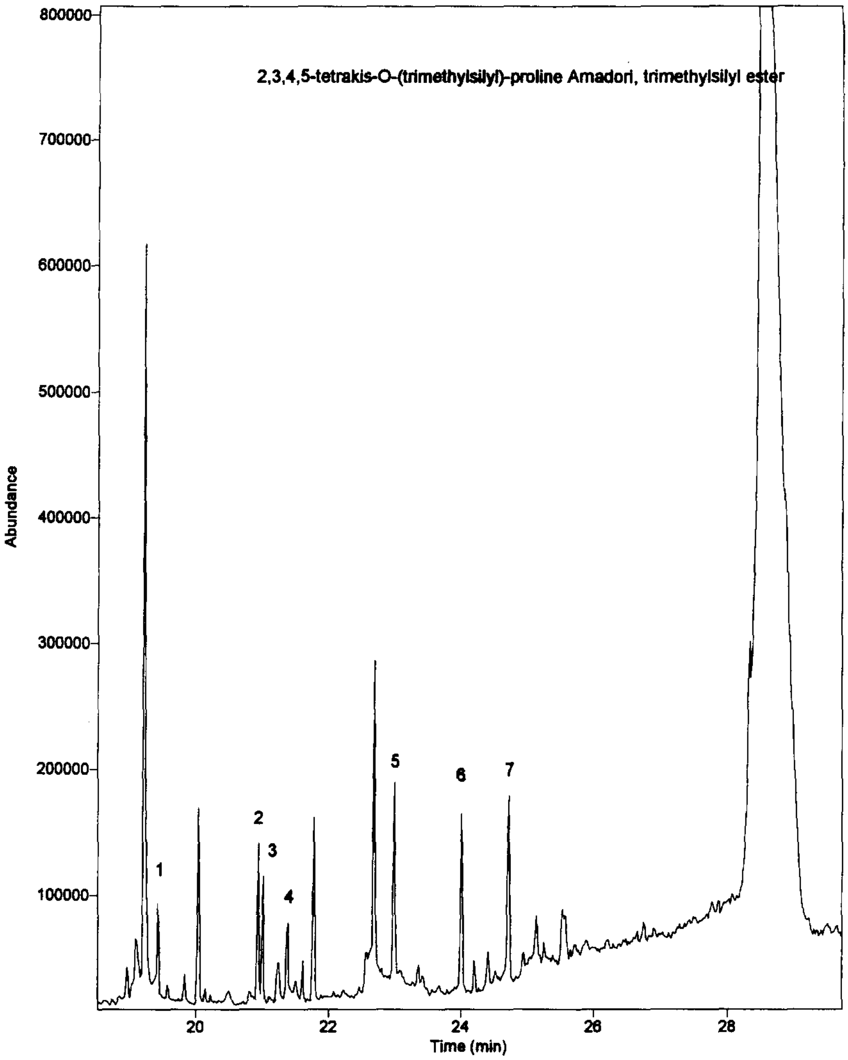
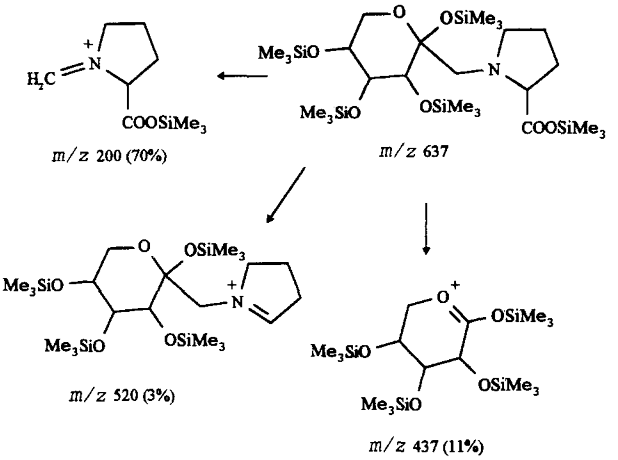


Fig. 1. GLC–MS chromatogram (fused silica DB-5 column, 30 m×0.25 mm i.d.; 0.25 m film thickness; Supelco, Inc.) of the *N*,*O*-bis(trimethylsilyl)trifluoroacetamide-treated sample of the proline Amadori compound heated at 130 °C for 20 min. (1) D-Erythrose, (2) D-arabinonic acid γ-lactone, (3) 2-deoxy-pentonic acid, (4) D-arabinose, (5) *aldehydo*-D-glucose, (6) D-mannose, (7) D-glucose. (Trimethylsilyl 2,3-di-*O*-trimethylsilylpropanoate appears at a retention time of 16.4 min, not shown in here.)

Amadori product undergoes a 1,2-enolization to produce an enaminol **2** in equilibrium with the *keto* form. The enaminol epimerizes the sugar at C-2 by isomerization into the α-hydroxy imminium ion **3** that can exist in equilibrium with glycosylamine **4**. Hydrolysis of either **3** or **4**, in theory, can generate the two epimeric aldohexoses, D-glucose and D-mannose, in addition to free proline that has been detected as the cyclic dimer 3,9-diazatricyclo[7.3.0.0*.3*.7*]dodecane-2,8-dione [12]. However, hydrolytic cleav-



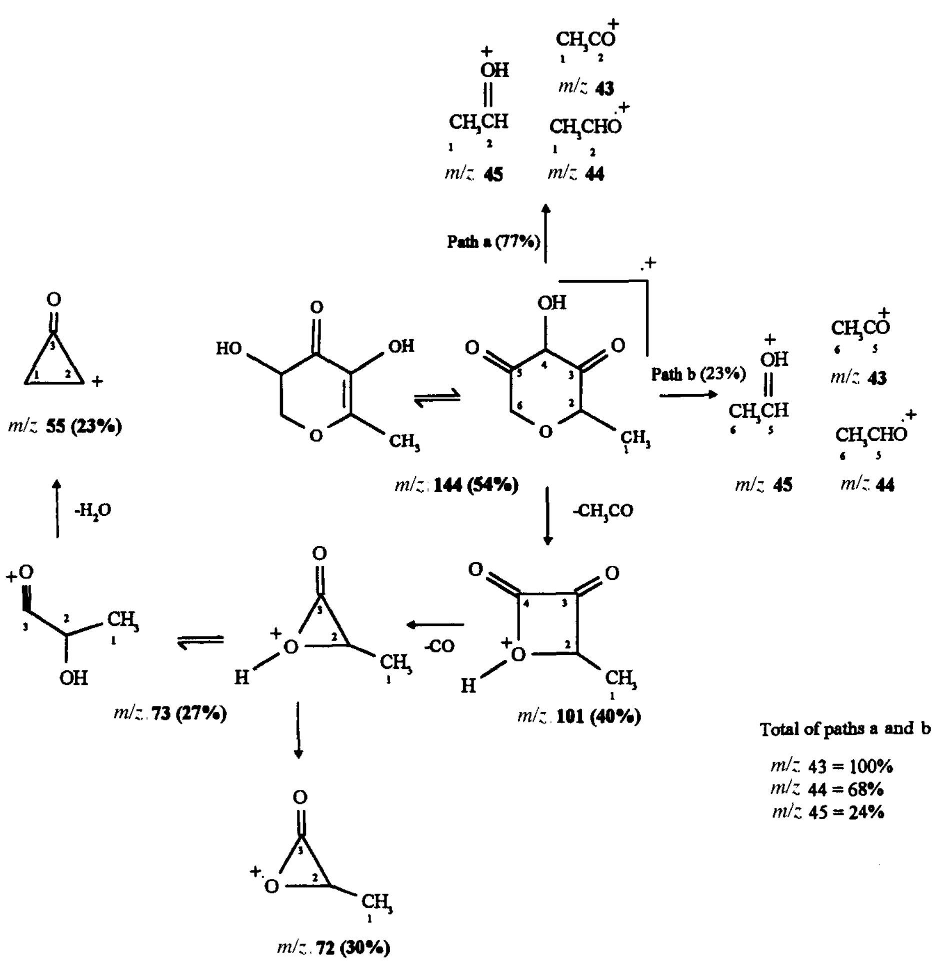
Scheme 2. The main electron-impact fragmenations of 1-deoxy-1-prolino-2,3,4,5-tetrakis-*O*-(trimethylsilyl)-D-fructose, trimethylsilyl ester.

age of the imminium ion **3** dictates the initial release of the epimeric aldoses from the Amadori product to be in their respective open-chain *aldehydo* forms. When free glucose was silylated at 80 °C, the amount of *aldehydo* form was less than 1% [13]. Although silylation of the decomposition mixture of the Amadori product was performed at 70 °C, still the trapped *aldehydo* form was present in amounts comparable to the cyclic form (see peaks 5 and 7 in Fig. 1). This finding could be considered as further evidence for the proposed mechanism of reversibility of the Amadori rearrangement.

Although the detection of D-glucose, *aldehydo*-D-glucose, and D-mannose in the decomposition mixture of the proline Amadori product could be considered as sufficient evidence for the reversibility of the rearrangement, it could be argued [6], however, that they might arise by aldol-type condensations between smaller sugar fragments generated from the decomposition of Amadori products. This is a theoretically valid argument considering the fact that many sugar fragments such as pyruvaldehyde, glyceraldehyde, erythrose, 1-hydroxy-2-propanone, glycoaldehyde, etc. have been detected in different model systems consisting of glucose and amino acids [2]. The logical candidates for this condensation reaction to form glucose and/or mannose are glycoaldehyde (hydroxyacetaldehyde) and D-erythrose, since the stereochemistry of the last three carbon atoms in D-glucose is already set as part of the D-*erythro* configuration. Such a condensation should generate a mixture of aldohexoses: D-allose, D-altrose, D-glucose, and D-mannose. However, considering the low concentrations of such sugar fragments relative to the amino acid (which does not degrade as extensively as the sugar) and the relative ease of carbonyl–amine reactions compared to reactions that lead to C–C bond formation, the likelihood of a reaction with an amino compound is much higher in such mixtures than a reaction among carbohydrate degradation products to form a hexose. Even if such encounters are possible among carbohydrate fragments, the chances of an aldohexose formation in general, and D-glucose and D-mannose in particular, are even slimmer.

The ability of all hexose sugars to invariably produce upon thermal decomposition

2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one — a universal indicator for the presence of hexoses [2] — has been taken advantage of, in conjunction with ^{13}C labeling experiments, to provide evidence that no "new" glucose or mannose was produced by condensation of smaller fragments, when glucose–amino acid mixtures were decomposed. 2,3-Dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one contains all the six carbon atoms of the starting hexose and as such can serve as a volatile indicator for the presence of non-volatile hexoses during GLC–MS analysis. In our laboratories, a pyrolysis-GLC–MS system has been successfully employed as an integrated reaction, separation, and identification unit [12]. The reactant(s) is (are) heated at the desired temperature for a desired period of time in the pyrolysis probe, and the products generated are swept by the carrier gas into the GLC column for separation, and eventual identification by MS. Most of the products generated by autocleaving a mixture of



Scheme 3. The main electron-impact mass spectral fragmentaion patterns of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one. Numbers indicate carbon atoms of D-glucose.

proline and glucose in water [14] were also found to be formed in the pyrolysis mixture of the same reactants and in the pure Amadori product, when analyzed by GLC–MS [12]. Application of pyrolysis-GLC–MS to analyze numerous Amadori products, glucose, and glucose–amino acid mixtures [11,12,15] has indicated that during pyrolysis (250 °C, 20 s) a similar thermal degradation of glucose and Amadori products occurs producing 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one as the main product. In addition, it has been demonstrated that an equimolar mixture of proline and glucose generates a strikingly similar pyrogram to that of the proline Amadori compound [12], indicating fast and efficient Amadori product formation during pyrolysis. 2,3-Dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one, therefore, can arise during pyrolysis of glucose–amino acid mixtures, or from any hexose, whether generated from condensation reactions or released intact from an Amadori product. However, the two processes of hexose formation can be distinguished using [^{13}C]glucoses. During pyrolysis of an

Table 1
Percent relative abundances of parent ion ($M = 144$) of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one generated in different model systems

Model system [a]	M 144	$M+1$ 145	$M+2$ 146
D-Glucose	49	3	0
D-Fructose	54	4	1
D-Glucose–glycine	58	4	1
Proline ARP	53	4	1
Glycine ARP	58	4	1
Histidine ARP	61	4	1
D-[1-^{13}C]Glucose	0	66	4
D-[1-^{13}C]Glucose–proline	0	64	4
D-[1-^{13}C]Glucose–glycine	0	57	3
D-[1-^{13}C]Glucose–phenylalanine	0	70	4
D-[1-^{13}C]Glucose–methionine	0	57	3
D-[2-^{13}C]Glucose–proline	0	78	5
D-[2-^{13}C]Glucose–methionine	0	56	3
D-[2-^{13}C]Glucose–glycine	0	68	4
D-[3-^{13}C]Glucose–proline	0	63	4
D-[3-^{13}C]Glucose–glycine	0	63	4
D-[4-^{13}C]Glucose–proline	0	66	4
D-[4-^{13}C]Glucose–glycine	0	66	4
D-[5-^{13}C]Glucose–proline	0	68	4
D-[5-^{13}C]Glucose–glycine	0	72	4
D-[5-^{13}C]Glucose–methionine	1	63	4
D-[6-^{13}C]Glucose–proline	0	82	5
D-[6-^{13}C]Glucose–glycine	0	71	4
[1-^{13}C]Glucose–[4-^{13}C]glucose–glycine	0	65	4
Expected [b]	0	71	4

[a] ARP = Amadori rearrangement product systems consisting of two components are equimolar.

[b] Based on 50% contribution from each intact sugar.

equimolar mixture of a singly substituted [^{13}C]glucose and an amino acid, the free [^{13}C]glucose will decompose and produce the singly substituted indicator compound, 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one. Similarly, if the Amadori product formed in situ undergoes a reversal, it will also produce the same singly substituted 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one, since the carbon skeleton of the glucose remains intact during Amadori rearrangement. However, any hexose generated by the condensation of smaller fragments by the action of the amino acid, should produce unsubstituted, singly, and doubly substituted 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-ones and as a result exhibit scrambling of the labels. Scheme 3 depicts the mass spectral fragmentation patterns of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one. Table 1 summarizes the relative abundances of the major peaks of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one detected in different model systems consisting of both unsubstituted and singly substituted glucoses at different carbon atoms (1-^{13}C, 2-^{13}C, 3-^{13}C, 4-^{13}C, 5-^{13}C, and 6-^{13}C). The model systems were reacted and analyzed using the pyrolysis-GLC–MS system described above. The results of these studies indicate the absence of unlabeled and doubly labeled 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one formation in any of the model systems. In addition, the relative abundances of the peaks of the indicator compound remained unchanged in the presence of different amino acids reacting with similarly labeled glucoses. These observations strongly suggest the absence of hexose formation by condensation of smaller sugar fragments. To corroborate the above findings and to increase the statistical probability of doubly labeled and unlabeled hexose formation through condensation of sugar fragments such as $C_2 + C_4$, an equimolar mixture of D-[1-^{13}C]glucose and D-[4-^{13}C]glucose in the presence of glycine was analyzed by pyrolysis-GLC–MS. Again, no formation of unsubstituted and doubly substituted 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-ones was observed. When theoretically expected relative abundances of the peaks of the indicator compound were calculated, based on 50% contribution from each intact sugar or Amadori product formed thereof, they were found to be in close agreement with the experimentally obtained values as indicated in Table 1.

2. Experimental

All reagents and chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI). D-[1-^{13}C]glucose, D-[2-^{13}C]glucose, and D-[6-^{13}C]glucose were purchased from Aldrich Chemical Company (Milwaukee, WI). D-[3-^{13}C]glucose, D-[4-^{13}C]glucose and D-[5-^{13}C]glucose were purchased from ICON Services, Inc. (Summit, NJ). *N,O*-bis(trimethylsilyl)trifluoroacetamide was purchased from Sigma Chemical Company (St. Louis, MO). Syntheses of Amadori products of glucose and of glyceraldehyde were performed according to published procedures [11,16].

Pyrolysis-GLC–MS and GLC–MS analyses.—A Hewlett–Packard gas chromatography (GC) mass selective detector (MSD) (5890 GC/5971B MSD) interfaced to a CDS Pyroprobe 2000 unit was used for the pyrolysis-GLC–MS analysis. Solid samples (1–5 mg) were introduced inside the quartz tube (0.3 mm thickness) and pyrolyzed at 250 °C with a total heating time of 20 s. The GLC column flow rate was 0.8 mL/min for a split ratio of 92:1 and a septum purge of 3 mL/min. The pyroprobe interface was set at 250 °C and the pyroprobe was set at the desired temperature at a rate of 50 °C/ms. The capillary-direct MS interface temperature was 180 °C and the ion source temperature was 280 °C. The ionization voltage was 70 eV and the electron multiplier voltage was 1494 V. The mass range analyzed was 20–550 amu. The column was a fused silica DB-5 column (30 m × 0.25 μm i.d.; 0.25 μm film thickness; Supelco, Inc.). The column initial temperature was −5 °C for 3 min and was increased to 50 °C at a rate of 30 °C/min; immediately the temperature was further increased to 270 °C at a rate of 8 °C/min and kept at 270 °C for 5 min. Products were identified through the library search facility of MS ChemStation software.

Silylation of D-*glucose and* D-*glyceraldehyde Amadori compounds.*—To a solution of Amadori products (3 mg) in 50% acetonitrile-*N,N*-dimethylformamide (Me$_2$NCHO, 1 mL) was added *N,O*-bis(trimethylsilyl)trifluoroacetamide (1 mL). The mixture was heated at 70 °C for 10 min and injected into the GLC–MS for analysis.

Silylation of thermal degradation products of the proline Amadori compound.—The Amadori product of proline (3 mg) was decomposed in *N,N*-dimethylformamide (1 mL) at 130 °C for 20 min, and the resulting mixture was silylated as described above.

Acknowledgements

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