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Reversible and Covalent Binding of 5-(Hydroxymethyl)-2-furaldehyde (HMF) with Lysine and Selected Amino Acids

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ABSTRACT: The chemical reactivity of 5-(hydroxymethyl)-2-furaldehyde (HMF) with lysine, glycine, and proline was studied using isotope labeling technique. To confirm the formation of HMF adducts in glucose amino acid model systems, a useful strategy was developed in which products simultaneously possessing six glucose (HMF moiety) and any number of amino acid carbon atoms in addition to nitrogen were targeted using specifically labeled precursors such as [$^{15}N_{\alpha}$]lysine·2HCl, [$^{15}N_{\varepsilon}$]lysine·2HCl, [U-$^{13}C_6$]lysine·2HCl, [$^{13}C_6$]lysine·2HCl, and [U-$^{13}C_6$]glucose in the case of lysine model system. In addition, model systems containing HMF and amino acids were also studied to confirm specific adduct formation. Complete labeling studies along with structural analysis using appropriate synthetic precursors such as HMF Schiff base adducts of piperidine and glycine have indicated that HMF generated in the glucose/amino acid model systems initially forms a Schiff base adduct that can undergo decarboxylation through an oxazolidin-5-one intermediate and form two isomeric decarboxylated Schiff bases. Unlike the Schiff bases resulting from primary amines or amino acids such as glycine or lysine, those resulting from secondary amino acids such as proline or secondary amines such as piperidine can further undergo vinylogous Amadori rearrangement, forming N-substituted 5-(aminomethyl)furan-2-carbaldehyde derivatives.

KEYWORDS: lysine, proline, glycine, pent-4-en-1-amine, piperidine, HMF, vinylogous Amadori rearrangement and Schiff base adducts of HMF with amino acids

INTRODUCTION

The chemical reactivity of one of the most abundant Maillard reaction products, 5-(hydroxymethyl)-2-furaldehyde (HMF), is also the least studied. Although its origin and formation mechanism are well understood, its fate remains to be investigated, especially due to its abundant formation in many foods including honey,[1−3] bread,[4] infant formulas,[5] and citrus juices, freeze-dried pears, grape juice, tomato products, and syrup,[6] to name a few. Fructose and sucrose are considered to be the most efficient precursors in food that can be transformed into HMF through the formation of reactive fructofuranosyl cation.[7] According to Bachmann et al.,[8] the average content of HMF in various foods exceeds 1 g/kg levels with estimated daily consumption of 150 mg/person or 2.5 mg/kg body weight.[9] Its prevalence in thermally processed foods has made it a useful indicator of the severity of heat treatment or storage time.[10−12] Due to its demonstrated ability to break DNA strands[13] and its weakly positive genotoxicity response,[14] HMF has been the subject of intense study. Although some of its toxic metabolites have been identified, such as 5-hydroxymethyl-2-furoic acid and 5-sulfoxymethylfurfural, its reactivity with amino groups of proteins or peptides has only been investigated under physiological conditions. Janzowski et al.[15] demonstrated its reactivity with glutathione in different cell cultures. Abdulmalik et al.[16] showed reversible binding with human hemoglobin in transgenic mouse, and only recently has direct evidence for its ability to bind to N-terminal valine groups of human hemoglobin through Schiff base adduct formation been demonstrated.[17] This study has shown that in some human blood samples background levels of 10−35 pmol/g of globin of HMF−Schiff base adducts have been detected. On the other hand, using [U-^{14}C]-HMF-treated rats and mice, Godfrey et al.[18] concluded that HMF may also bind covalently with tissue proteins because extensive washing of tissue homogenates did not remove the radioactivity. Although reversible binding can be explained through Schiff base formation, nonreversible covalent binding of HMF has not been rationalized. Understanding the reactions of HMF with amino acids may provide further insight into its chemical reactivity in addition to its known ability to polymerize.

MATERIALS AND METHODS

Materials. DL-Lysine, glycine, 5-(hydroxymethyl)furfural, methylamine hydrochloride, sodium glycinate, and L-proline were purchased from Aldrich Chemical Co. (Milwaukee, WI). L-[$^{15}N_{\alpha}$]Lysine·2HCl, L-[$^{15}N_{\varepsilon}$]lysine·2HCl, L-[U-$^{13}C_6$]lysine·2HCl, L-[$^{13}C_6$]lysine·2HCl, D-[U-$^{13}C_6$]glucose, D-[$^{13}C_1$]glucose, D-[$^{13}C_2$]glucose, D-[$^{13}C_3$]glucose, D-[$^{13}C_4$]glucose, D-[$^{13}C_5$]glucose, D-[$^{13}C_6$]glucose, [$^{13}C_1$]glycine, [$^{13}C_2$]glycine, [^{15}N]glycine, [$^{13}C_1$]proline, and [U-$^{13}C_6$,^{15}N] proline were all >98% enriched and purchased from CIL (Andover, MA), DL-Lysine·2HCl (99%, Fluka, Buchs, Switzerland), D-glucose (99%, BDH, Toronto, Canada), and piperidine (99%, BDH, Poole, U.K.) were purchased as indicated. Melting points were determined on OptiMelt automated melting point system (Sunnyvale, Ca). The ^{13}C and ^{1}H NMR spectra were acquired in CD_3OD on a Varian VNMRS 500 MHz spectrometer. Infrared spectra were recorded on a Bruker Alpha-P spectrometer (Bruker Optic GmbH, Ettlingen, Germany) equipped with a deuterated triglycine sulfate (DTGS) detector, a temperature-

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Table 1. Compositions of Model Systems Analyzed by Py-GC/MS[a]

target ion	model system studied
m/z 191	glucose + [^{15}Nα]lysine·2HCl[b] (1:1)
m/z 353	glucose + lysine·2HCl[b] (1:1)
	glucose[U-$^{13}C_6$] + lysine·2HCl[b] (1:1)
m/z 193	glucose or sucrose + [$^{15}N_\alpha$]lysine·2HCl + lysine
m/z 191	glucose or sucrose + [$^{15}N_\varepsilon$]lysine·2HCl + lysine
	glucose or sucrose + lysine + [^{13}C-6]lysine·2HCl
	glucose or sucrose + lysine + [U-$^{13}C_6$]lysine·2HCl
	[U-$^{13}C_6$]glucose + lysine + lysine·2HCl
	HMF + piperidine
	[5-(dipiperidin-1-ylmethyl)furan-2-yl]methanol (**3**)
m/z 139	HMF + [$^{13}C_1$]glycine
	HMF + [$^{13}C_2$]glycine
	HMF + [^{15}N]glycine
	HMF + glycine
	[U-$^{13}C_6$]glucose + methylamine-HCl + glycine
	[U-$^{13}C_6$]glucose + methylamine-HCl
	glucose + methylamine·HCl + glycine
	glucose + methylamine·HCl
	HMF + methylamine·HCl
	sodium ({[5-(hydroxymethyl)furan-2-yl]methylidene}-amino)acetate (**7**)
m/z 161	HMF + [U-$^{13}C_5$,^{15}N]proline
m/z 179	HMF + [$^{13}C_1$]proline
	HMF + proline
	HMF + pyrrolidine

[a] Unless otherwise specified. [b] Analyzed by TOF-MS (see Materials and Methods).

controlled single-bounce diamond attenuated total reflectance (ATR) crystal, and a pressure application device for solid samples.

Sample Preparation. The dihydrochloride salts of the commercially available isotopically labeled lysines were unreactive when pyrolzed as such; however, mixing the salts with an equimolar amounts of unlabeled free lysine resulted in increased reactivity when pyrolyzed. Consequently, equimolar amounts of unlabeled DL-lysine and specifically labeled DL-lysine·2HCl were mixed and homogenized before mixing with an equimolar amount of D-glucose (see Table 1).

Preparation of ESI-TOF MS Samples. Glucose (10 mg) and lysine·2HCl (14 mg) were dissolved in distilled water (0.8 mL) and heated in an open vial (3 mL) at 110 °C for 45 min or until dryness, generating a brown powder. The experiments were repeated with [U-$^{13}C_6$]glucose and [$^{15}N_\alpha$]lysine. The pyrolysis of this powder generated a profile similar but not identical to that of lysine glucose samples pyrolyzed without prior heating as described above.

Pyrolysis-GC/MS. Analyses were conducted using a Varian CP-3800 GC coupled with a Saturn 2000 ion trap mass spectrometer (Varian, Walnut Creek, CA). The pyrolysis unit included a CDS Pyroprobe 2000 and a CDS 1500 valved interface (CDS Analytical, Oxford, PA) installed onto the GC injection port. About 2.5 mg of a sample mixture (see Table 1) was packed inside a quartz tube (0.3 mm thickness), plugged with quartz wool, inserted inside the coil probe, and pyrolyzed for 20 s at a temperature of 250 °C. The sample separation was carried out on a DB-5MS (5% diphenyl, 95% dimethyl polysiloxane) capillary column with dimensions of 50 m length by 0.2 mm internal diameter and 0.33 μm film thickness (J&W Scientific, ON, Canada), using helium as the carrier gas. The GC column flow rate was regulated by an electronic flow controller (EFC) and set at a pressure pulse of 70 psi for the first 4 min and later maintained with a constant flow of 1.5 mL/min for the remainder of the run. The GC oven temperature was set at −5 °C for 5 min using CO_2 as the cryogenic cooling source. The temperature was increased to 50 °C at a rate of 50 °C/min and then to 270 °C at a rate of 8 °C/min, and kept at 270 °C for 5 min. The samples were detected by using an ion trap mass spectrometer with a scan range of *m/z* 20−650. The MS transfer line temperature was set at 250 °C, the manifold temperature was set at 50 °C, and the ion trap temperature was set at 175 °C. The ionization voltage of 70 eV was used, and EMV was set at 1700 V. Compound identification was performed using AMDIS (ver 2.65) and NIST Standard Reference Databases (data version 05 and software ver 2.0d) to compare the target compounds with the existing mass spectral libraries or by injecting commercially available standards. The reported percent label incorporation values (corrected for natural abundance and for percent enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

ESI-TOF MS Analysis. Samples were diluted in 1 mL of water and then again 1/100 with 50% methanol and 0.1% formic acid. Each sample (5 μL injections) was directly analyzed by liquid chromatography−mass spectrometry (LC-MS), on a 1200 series Agilent rapid resolution LC system coupled to an Agilent 6210 time-of-flight (ESI-TOF) instrument. The mobile phase consisted of 50% methanol and 0.1% formic acid at a flow rate of 0.3 mL/min. Data were acquired in positive electrospray mode with an acquisition mass range of *m/z* 100−1000 and internal calibration using *m/z* 121.050873 and 922.009798 (Agilent ESI tuning mix) for accurate mass measurements with a dual sprayer ESI source and constant infusion of calibrant ions. Source conditions were as follows: gas temperature, 350 °C; ESI voltage, 4000 V; dry gas flow (nitrogen), 12 L/min; nebulizer gas pressure, 35 psig; fragmentor and skimmer voltages, 100 and 60 V, respectively. In the MS/MS mode the CID spectra of the selected ions were similarly acquired in the positive ion mode with a collision energy of 30 V.

[5-(Dipiperidin-1-ylmethyl)furan-2-yl]methanol (3). HMF (124 mg) was dissolved in two times excess piperidine and mixed thoroughly and left at room temperature without solvent for 24 h. Acetonitrile (0.2 mL) was then added to the mixture, and the resulting powder was filtered and washed with excess acetonitrile. The resulting solid had a chemical purity of 98% based on ^{1}H NMR: mp 80.6−81.6 °C; ^{1}H NMR δ 1.44−1.57 (m, 12H, H-3′ to 5′ and H-3″ to H-5″), 2.57 (t, 2H, H-2′), 2.65 (t, 2H, H-2″), 2.74 (t, 4H, H-6′, 6″) 3.30 (s, 1H, H-1), 4.49 (s, 2H, H-6), 6.28 (s, 2H, H-3,4); ^{13}C NMR δ 154.4 (C-5), 151.2 (C-2), 109.0 (C-4), 107.4 (C-3), 92.6 (C-1), 56.0 (C-6), 48.5 (C-6′,6″), 46.2 (C-2′,2″), 25.9 (C-3′,3″), 25.4 (C-5′,5″), 24.3 (C-4″), 24.2 (C-4′); FTIR (solid) 3226 cm^{-1} (C—OH stretch), 3122 cm^{-1} (=C—H stretch), 2927 cm^{-1} (CH_2—CH_2 stretch), 2855 cm^{-1} (CH_2—CH_2 stretch), 1011 cm^{-1} (C—O—C stretch); MS *m/z* (% abundance) 39 (8.6), 42 (11.5), 53 (15.2), 81 (19.8), 84 (11.0), 109 (68.8), 136 (6.4), 164 (24.3), 192 (100), 193 (45.7), 194 (12.7).

Sodium ({[5-(Hydroxymethyl)furan-2-yl]methylidene}-amino)acetate (7). HMF (124 mg) and sodium glycinate (97 mg) were intimately mixed at room temperature without a solvent until a homogeneous mixture was produced as a light brown oil (15 min). ^{1}H NMR analysis indicated the presence of two sets of peaks; one set was specific to HMF (25%), and the second set was consistent with the title compound (75%). ^{1}H NMR δ 4.2 (s, 2H, H-2′), 4.6 (s, 2H, H-6), 6.4 (d,

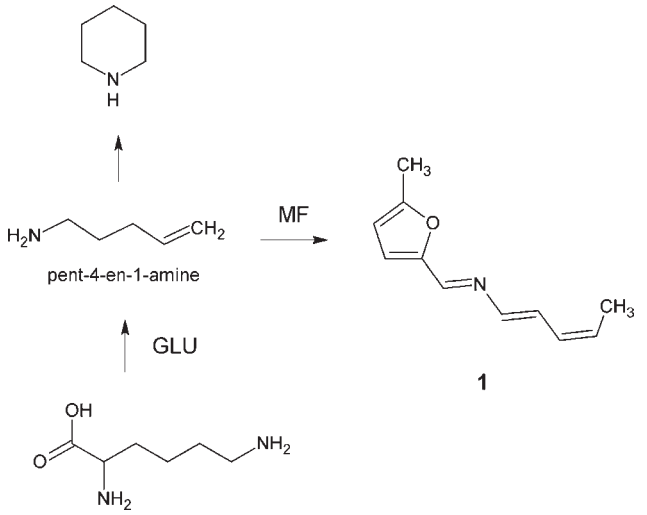

Figure 1. Formation of pent-4-ene-1-amine and its Schiff base adduct with 5-methylfurfural (MF) based on ref 19. GLU, glucose.

1H, H-3), 6.9 (d, 1H, H-4), 8.0 (s, 1H, H-1); ^{13}C NMR δ 176.0 (C-1′), 157.8 (C-5), 152.5 (C-1), 150.8 (C-2), 116.2 (C-4), 109.0 (C-3), 63.7 (C-2′), 56.2 (C-6); FTIR (solid) 3243 cm^{-1} (C—OH stretch), 3122 cm^{-1} (=C—H stretch), 1646 cm^{-1} (C=N stretch), 1582 cm^{-1} (COO− stretch), 1017 cm^{-1} (C—O—C stretch); MS *m/z* (% abundance) 39 (30.4), 41 (24.7), 42 (59.2), 51 (19.7), 69 (27.0), 81 (17.3), 97 (23.9), 110 (35.7), 122 (20.5), 138 (57.1), 139 (100), 140 (17.8).

Table 2. Number of Labeled Atoms Incorporated in the Major Mass Spectral Fragments of Compound 2[a] Shown in Figure 2

	m/z 193	*m/z* 192	*m/z* 164	*m/z* 109
[^{15}N$_\alpha$]lysine	0	0	0	0
[^{15}N$_\varepsilon$]lysine	1	1	1	0
[^{13}U$_6$]lysine	5	5	5	0
[^{13}C$_6$]lysine	1	1	1	0

[a] Generated through pyrolysis of glucose/lysine or sucrose/lysine model systems.

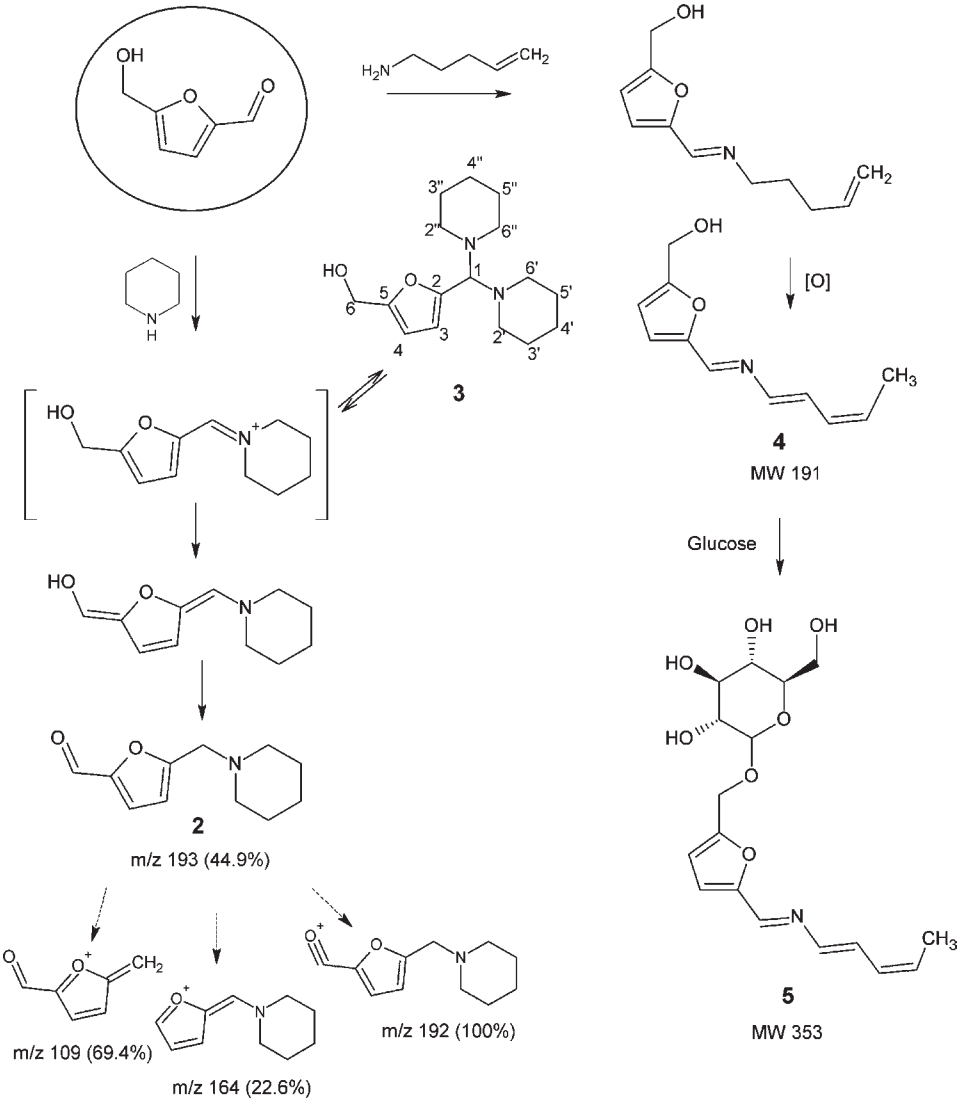

Figure 2. Schiff base formation of 5-(hydroxymethyl)-2-furaldehyde (HMF) with pent-4-ene-1-amine and its vinylogous Amadori rearrangement with piperidine. For compound **2** (*m/z* 193) important EI mass spectral fragments are also shown. [O], oxidation; MW, molecular weight.

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RESULTS AND DISCUSSION

The chemical reactivity of HMF with amino acids has not been investigated in detail, although Schiff base formation could logically be assumed to be a viable end product due to the stability of the resulting conjugated system. 5-Methylfurfural (MF), for example, has been shown to form a Schiff base adduct (**1**) with pent-4-en-1-amine, the counterpart of acrylamide from lysine (see Figure 1) in glucose/lysine model systems.[19] Similar adducts could be expected to be formed with HMF; however, the latter is also considered to be α,β-unsaturated aldehyde derivative that can undergo Michael addition with sulfur nucleophiles or vinylogous Amadori rearrangement with nitrogen nucleophiles.[20,21] However, the fact that an α,β-unsaturated moiety constitutes a part of the HMF aromatic ring system drastically reduces its reactivity, requiring a driving force to overcome the activation energy needed to disrupt the conjugated system. To explore the chemistry of HMF reactions with various amino acids and particularly with lysine, appropriate model systems were investigated through an isotope labeling technique (see Table 1).

Table 3. Number of Labeled Atoms Incorporated in the Major EI and ESI Fragments of Compound 4 Shown in Figure 2

	EI[a]					
	m/z 191	*m/z* 176	*m/z* 163	*m/z* 135	*m/z* 107	*m/z* 93
[$^{15}N_{\alpha}$]lysine	0	0	0	0	0	0
[$^{15}N_{\varepsilon}$]lysine	1	1	1	1	0	1
[$^{13}U_6$]lysine	5	4	5	5	5	2
[$^{13}C_6$]lysine	1	0	1	1	0	0
[$^{13}U_6$]glucose	6	6	4	3	2	3

	ESI[b]			
[M + H] ions	*m/z* 192	*m/z* 175	*m/z* 146	*m/z* 132
[$^{15}N_{\alpha}$]lysine	0	0	0	0
[$^{13}U_6$]glucose	6	6	5	4

[a] From pyrolysis experiments. [b] From aqueous model system using TOF MS/MS, $[M + H]^+$ = 192.1043 and molecular formula of $C_{11}H_{13}NO_2$.

Table 4. TOF MS/MS[a] ESI Fragment Ions [M + H] of Compound 5 Shown in Figure 2

	m/z 354	*m/z* 294	*m/z* 264	*m/z* 192	*m/z* 174
[$^{15}N_{\alpha}$]lysine	0	0	0	0	0
[$^{13}U_6$]glucose	12	10	9	6	6

[a] From aqueous model system, $[M + H]^+$ = 354.1547 and molecular formula of $C_{17}H_{24}NO_7$.

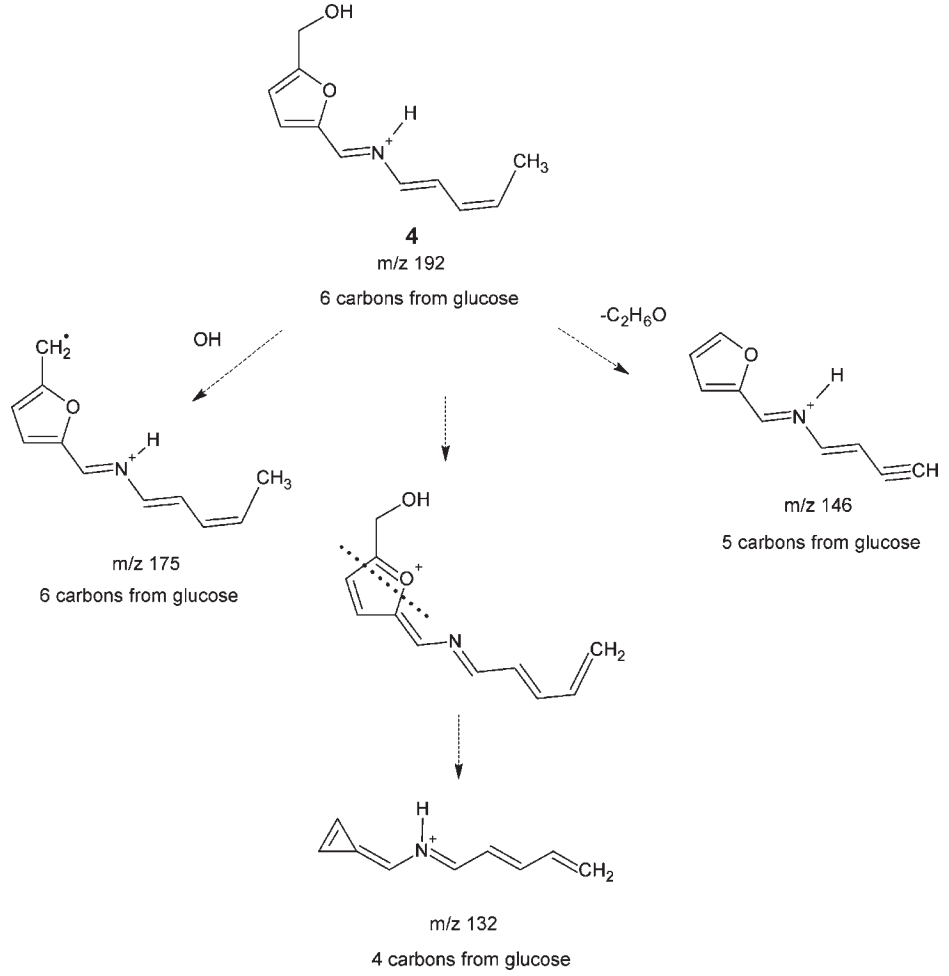

Figure 3. TOF-MS/MS fragmentations of structure **4** (see also Table 3).

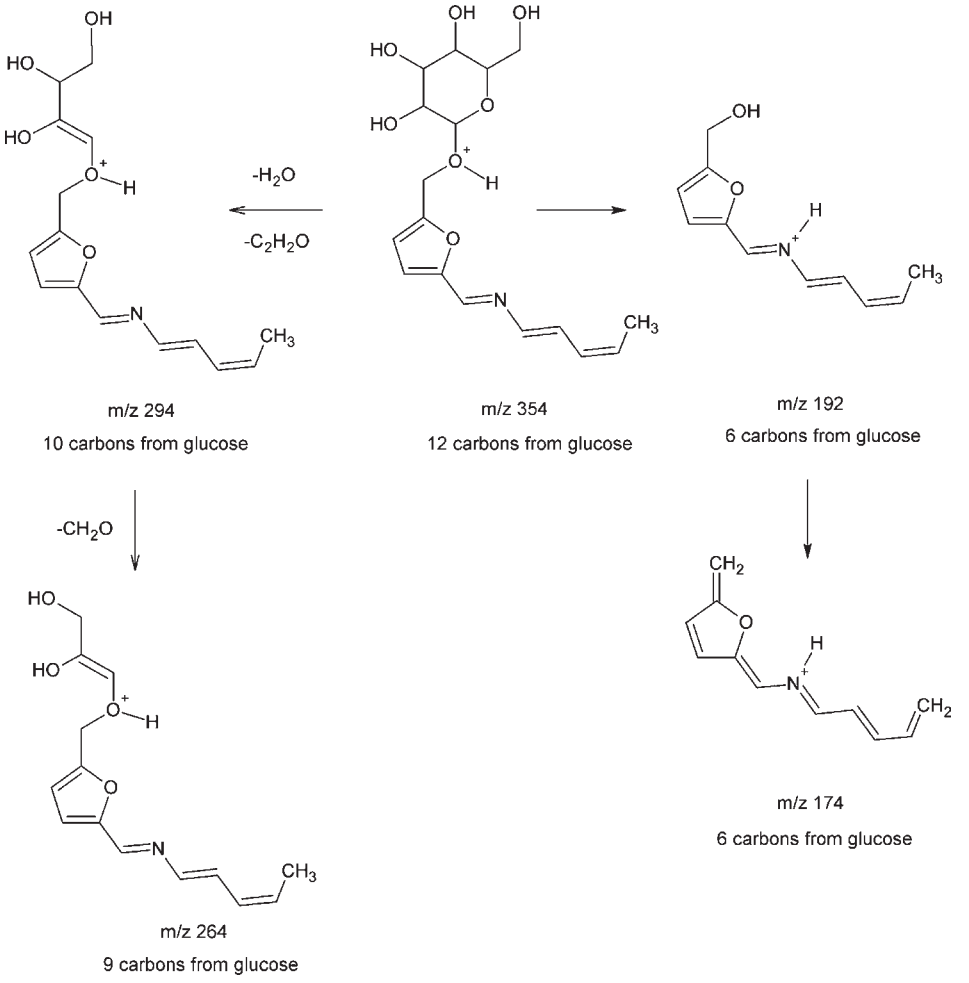

Figure 4. TOF-MS/MS fragmentations of structure **5** (see also Table 4).

Reaction of Glucose or Sucrose with Lysine. Sugar/lysine model systems were prepared by two methods. In one approach glucose/lysine mixtures were dissolved in distilled water and heated in an oven at 110 °C using an open vial for 45 min or until dryness, generating a free-flowing brown powder. The powder was analyzed through high-resolution TOF-MS using mainly [U-$^{13}C_6$]glucose labeled precursors. In the second approach glucose/lysine and sucrose/lysine model systems were pyrolyzed at 250 °C for 20 s using variously labeled lysine precursors (see Table 1). Sucrose/lysine model systems were used to enhance the formation of HMF generated products. Furthermore, to confirm the formation of HMF adducts a useful strategy based on an isotope labeling technique was developed in which products simultaneously possessing six glucose (HMF moiety) and any number of lysine carbon atoms in addition to nitrogen were targeted using specifically labeled precursors such as [$^{15}N_\alpha$]lysine·2HCl, [$^{15}N_\varepsilon$]lysine·2HCl, [U-$^{13}C_6$]lysine·2HCl, [$^{13}C_6$]lysine·2HCl, and [U-$^{13}C_6$]glucose. In addition, model systems containing HMF and lysine or HMF and piperidine were also studied to confirm specific adduct formation. Complete labeling studies along with structural analysis using synthetic and other available precursors have shown the presence of two such peaks at retention times of 26.9 min (m/z 193) and 30.9 min (m/z 191) that satisfied the above criteria. The peak at a retention time of 26.9 min was detected only in the pyrolyzed sucrose/lysine samples; however, the peak at a retention time of 30.9 min was detected both in pyrolyzed glucose/lysine and in the aqueous heated samples that was analyzed by TOF-MS/MS. Furthermore, the aqueous heated sample also generated a related compound possessing 12 carbon atoms from glucose ($[M + H]^+$ = 354.1547; molecular formula $C_{17}H_{24}NO_7$).

Identification of Compound 2 Eluting at 26.9 min. This compound was detected only in sucrose/lysine and piperidine/HMF pyrolyzed samples. In fact, the major peak in the model system of HMF/piperidine eluted at a retention time of 26.9 min. Preliminary analysis of the isotope labeling pattern generated from the sucrose/lysine model systems have indicated the incorporation of five lysine carbon atoms (including C_6) and one N_ε atom. This pattern of lysine atom distribution is indicative of either a piperidine or a pent-4-en-1-amine moiety.[19] In a previous study[19] lysine was found to release pent-4-en-1-amine through interaction with glucose, and isotope labeling studies confirmed its conversion into piperidine (see Figure 1). The precursors of compound **2** were confirmed when the reaction of HMF with piperidine produced the major peak at the same retention time and with an identical mass spectrum. Piperidine being a secondary amine can initially form an iminium ion when reacted with HMF (see Figure 2). This ion can be either stabilized through its reaction with a second mole of piperidine to form [5-(dipiperidin-1-ylmethyl)furan-2-yl]methanol

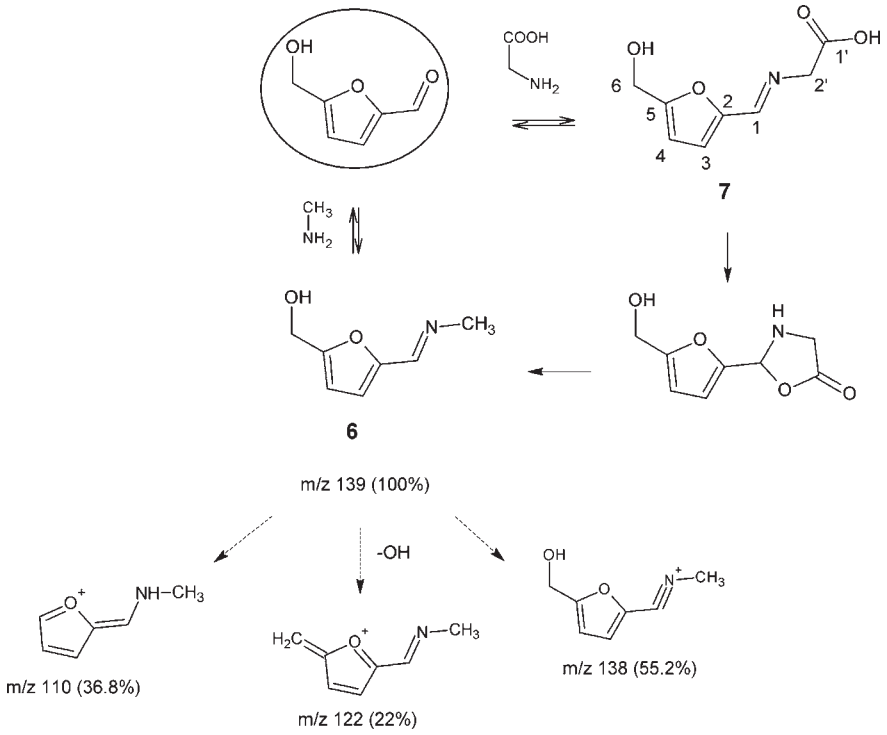

Figure 5. Reaction of 5-(hydroxymethyl)-2-furaldehyde (HMF) with glycine (see also Table 5). For compound **6** (m/z 139) important EI mass spectral fragments are also shown.

Table 5. Number of Labeled Atoms Incorporated in the Major EI Fragments of Compound 6[a] Shown in Figure 5

	m/z 139	m/z 138	m/z 122	m/z 110
[$^{13}C_1$]glycine	0	0	0	0
[$^{13}C_2$]glycine	1	1	1	1
[^{15}N]glycine	1	1	1	1
[$^{13}U_6$]glucose	6	6	6	5

[a] Generated through pyrolysis of HMF/glycine and glucose/methylamine·HCl model systems.

(**3**) or undergo vinylogous Amadori rearrangement to form a covalent adduct (**2**). Although the dipiperidine adduct **3** (equivalent to the Schiff base) was not detected, when a synthetic sample was pyrolyzed, it generated an identical compound eluting at the same retention time and with a mass spectrum identical to that of **2**. Formation of compound **2** indicates secondary amines such as piperidine that can form imminum ions are also able to provide the necessary driving force for its isomerization into the corresponding Amadori product to neutralize the positive charge. The main mass spectral fragments of **2** are also shown in Figure 2, and the corresponding label incorporation patterns are listed in Table 2. The fragmentation pattern observed is consistent with the presence of an aldehyde functionality, and the label incorporations in the fragment ions are also consistent with the proposed structure.

On the other hand, the primary amine pent-4-en-1-amine is also expected to be formed in glucose/lysine model systems[19] and subsequently generate a stable Schiff base adduct with HMF (expected MW 191 amu), similar to 5-methylfurfural (**1**). A peak eluting at 30.92 min (nominal molecular weight m/z 191) and incorporating six glucose carbon atoms, five lysine carbon atoms (including C_6), and one N_ε atom was detected in glucose/lysine model systems and also in the aqueous heated glucose/lysine sample analyzed by TOF MS/MS (see Table 3).

Tentative Identification of Compound 4 Eluting at 30.9 min. As mentioned above, this compound was detected in both pyrolyzed and aqueous heated samples of glucose and lysine. Analysis of the label incorporation pattern from the TOF-MS analysis indicated the incorporation of six carbon atoms from glucose and showed a consistent molecular formula ($C_{11}H_{13}NO_2$) and MS/MS data (Table 3). Similarly, analysis of the label incorporation pattern from pyrolysis experiments indicated the incorporation of six carbon atoms from glucose, five lysine carbon atoms (including C_6), and one N_ε atom. Because this pattern of label incorporation is indicative of either a piperidine or pent-4-en-1-amine moiety[19] and because the peak was not generated in the piperidine/HMF model, the evidence therefore points to a structure equivalent to the Schiff base formed between 5-methylfurfural (MF) and pent-4-en-1-amine shown in Figure 1 and detected in the same model system of glucose and lysine.[19] The TOF MS/MS fragmentations shown in Figure 3 provide further evidence to the proposed structure **4**. Tentative identification of the glycosylated derivative (**5**) of structure **4** in the same aqueous heated sample provides further evidence for its formation. Isotope labeling experiments using TOF-MS analysis indicated the presence of 12 carbon atoms from glucose (see Table 4) with a molecular weight of $[M + H]^+ = 354.1547$ and a calculated molecular formula of $C_{17}H_{24}NO_7$ consistent with the proposed structure **5**. The remaining five carbons and the one nitrogen atom should therefore originate from the lysine component. In fact, a fragment ion (m/z 192) indicative of structure **4** incorporating six carbon atoms from glucose (see Figure 4) was the major daughter ion in the MS/MS spectrum of **5**

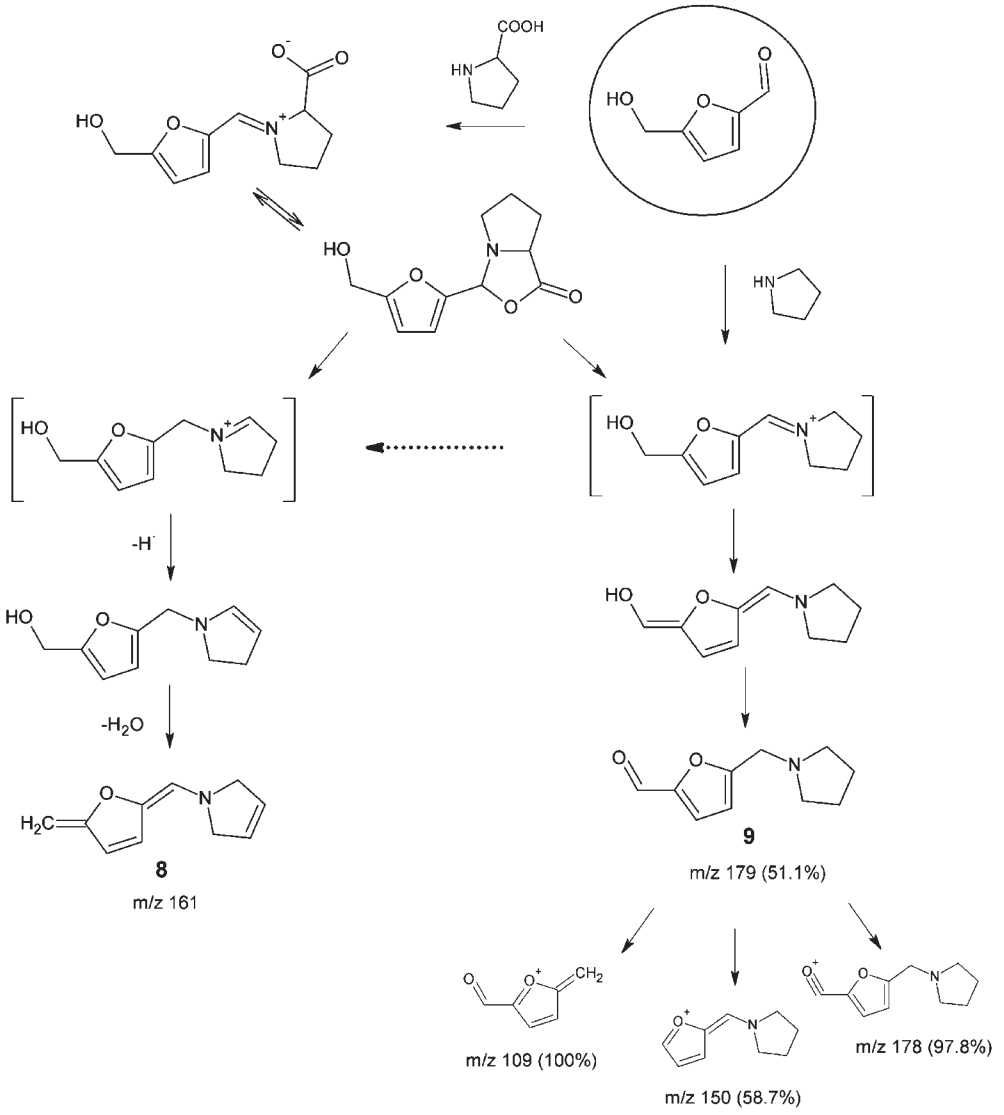

Figure 6. Reaction of 5-(hydroxymethyl)-2-furaldehyde (HMF) with proline (see also Table 6). For compound **9** (*m*/*z* 179) important EI mass spectral fragments are also shown.

Table 6. Number of Labeled Atoms Incorporated in the Major EI Fragments of Compounds 8 and 9 Shown in Figure 6

	Compound 9[a]			
	m/*z* 179	*m*/*z* 178	*m*/*z* 150	*m*/*z* 109
[U-$^{13}C_5$,^{15}N]proline	5	5	5	0
[$^{13}C_1$]-proline	0	0	0	0
	Compound 8[a]			
	m/*z* 161	*m*/*z* 146	*m*/*z* 132	*m*/*z* 118
[U-$^{13}C_5$,^{15}N]proline	5	5	5	5
[$^{13}C_1$]proline	0	0	0	0

[a] Generated through pyrolysis of proline/HMF model systems.

(see Table 4 and Figure 4). All of the proposed ESI fragments shown in Figure 4 were consistent with the proposed structure and the labeling data.

Reaction of HMF with Glycine and Proline. To confirm the assertion that secondary amines or secondary amino acids undergo vinylogous Amadori rearrangement and primary amino acids or primary amines form only Schiff bases with HMF, the reaction of HMF with glycine and proline was investigated. When glycine was pyrolyzed with HMF, a peak corresponding to the specification of a decarboxylated Schiff base adduct (**6**) and eluting at a retention time of 21.44 min was observed (see Figure 5). The peak incorporated one nitrogen atom and one C-2 atom of glycine and no C-1 atom from glycine (see Table 5). The same peak was also generated from a mixture of HMF/methylamine and glucose/methylamine. The latter model system allowed the confirmation of incorporation of six glucose carbon atoms into the structure of the peak eluting at 21.44 min when glucose was replaced with [U-$^{13}C_6$]glucose. Furthermore, when a synthetic HMF−glycine Schiff base adduct (**7**) was pyrolyzed, a peak eluting at the same retention time and having a mass spectrum identical to that of **6** was also generated, confirming unequivocally the structure **6** shown in Figure 5. Furthermore,

loss of an OH radical to form the ion at m/z 122 confirms the presence of an alcohol moiety in **6**.

On the other hand, the reaction of secondary amino acid proline with HMF generated two relevant adducts, one eluting at 24.5 min tentatively identified as structure **8** and the other eluting at 25.5 min tentatively identified as structure **9** (see Figure 6). Labeling studies using [U-$^{13}C_5$,^{15}N]proline and [$^{13}C_1$]proline indicated the incorporation of a pyrrolidine moiety in both structures (see Table 6), and a subsequent study on the reaction of pyrrolidine with HMF confirmed the generation of mainly the peak eluting at 25.5 min (structure **9**) and a trace amount of **8**. The Schiff base adducts of HMF with amino acids are prone to undergo decarboxylation through oxazolidin-5-one intermediate and formation of two isomeric imminium ions, one conjugated, which can undergo vinylogous Amadori rearrangement to generate **9**, and the other not conjugated, which can be stabilized through dehydration to form **8** as shown in Figure 6. The fact that the reaction of HMF with proline generates comparable amounts of **8** and **9** but only trace amounts of **8** when pyrrolidine was used further confirms the formation of an oxazolidin-5-one intermediate, the formation of which is possible only with amino acids. The formation of **8** in pyrrolidine models can be rationalized through a less desirable transamination reaction that requires basic conditions.[22] As expected, the major mass spectral fragments of **9** shown in Figure 6 are generated through fragmentation pathways identical to that of its piperidine analogue (structure **2**) shown in Figure 2.

Although some of the HMF formed in food is prone to polymerization,[23] as indicated above, some may also undergo amino acid specific reactions. The initially formed Schiff base adducts may partially undergo decarboxylation through oxazolidin-5-one intermediate[19] and form two isomeric decarboxylated Schiff bases. Unlike the Schiff bases resulting from primary amines or primary amino acids, those resulting from secondary amino acids such as proline or secondary amines such as piperidine can further undergo vinylogous Amadori rearrangement, forming N-substituted 5-(aminomethyl)furan-2-carbaldehyde derivatives. Lysine can exhibit both reversible and covalent adduct formation depending on the formation of either pent-4-en-1-amine or piperidine in the reaction mixture.

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