Use of ^{13}C Labeling and NMR Spectroscopy for the Investigation of Degradation Pathways of Amadori Compounds

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The degradation pathways of Amadori compounds *in vivo* have not been fully understood because of the lack of suitable techniques although the compounds are considered to be key intermediates in glycation, which contributes to the development of pathologies associated with various chronic and age-related diseases. A new approach using ^{13}C labeling and NMR spectroscopy has thus been described to obtain more insight into the degradation pathways. A ^{13}C -labeled model Amadori compound, $[1-^{13}\text{C}]\text{N}^{\epsilon}$ -(1-deoxy-D-fructos-1-yl)hippuryl-lysine, was synthesized to investigate the degradation pathways of the sugar moiety. The labeled compound was then incubated under aerobic and physiologic conditions, followed by analysis using ^{13}C -NMR spectroscopy to obtain the degradation profile. Consequently, after 28-d incubation at least nine ^{13}C signals due to ^{13}C -labeled products were observed with those due to unlabeled hippuryl-lysine. These labeled products included not only carboxymethylated hippuryl-lysine as the major product and α - and β -glucose but also acetate and formate. These experiments demonstrate the potential of using a ^{13}C label and NMR spectroscopy in that the technique provides the comprehensive profiling of the degradation products containing the labeled position in spite of their chemical structures.

Key words Amadori compound; degradation; glycation; ^{13}C labeling; NMR

Glycation is a common posttranslational modification of proteins in the body, resulting from the reaction of glucose with amino groups on proteins.^{1,2)} The first stable adducts formed during glycation are Amadori compounds.³⁾ The adducts undergo dehydration and oxidative fragmentations to yield heterogeneous compounds collectively referred to as advanced glycation end-products (AGEs).^{2,4,5)} Glycation is thought to contribute to the development of pathologies associated with diabetes, atherosclerosis, chronic renal failure, and neurodegenerative diseases because the formation of chemically stable AGEs can alter protein structure and function. Amadori compounds are considered to be key intermediates in glycation and precursors of various reactive intermediates such as dicarbonyl and lower molecular weight sugars, which are more reactive with protein than glucose.³⁾ However, much of the evidence on the degradation of Amadori compounds and formation of intermediates is based on studies conducted at high temperature, high and low pH, and in nonaqueous systems.^{6,7)} In addition, there is insufficient information on the kinetics and products of the decomposition of sugar moieties of Amadori compounds because of the lack of suitable analytical techniques.

The usefulness of the stable isotope tracer technique using ^{13}C labeling of substrates and NMR spectroscopy has become accepted in metabolic investigations including degradation pathways of metabolites.^{8–11)} Owing to the high specificity of detection, the application of the tracer technique enables analysis of biofluids and reaction mixtures without resorting to extraction and chromatographic separations. Therefore the decomposition and loss of compounds under sample purification and separation procedures can be minimized. In addition, all products arising from a labeled substrate can be detected if they contain the labeled carbon and the detection sensitivity of NMR is sufficient. This technique is thus expected to provide information that is inaccessible by other methods and considered to be suitable for the

investigation of complicated degradation pathways of Amadori compounds. Thus, in this study, a ^{13}C -labeled model Amadori compound, $[1-^{13}\text{C}]\text{N}^{\epsilon}$ -(1-deoxy-D-fructos-1-yl)hippuryl-lysine, was synthesized and degradation mixtures of the labeled compound under aerobic conditions analyzed using ^{13}C -NMR spectroscopy to investigate the potential of the ^{13}C -labeling and NMR approach.

MATERIALS AND METHODS

General D- $[1-^{13}\text{C}]\text{Glucose}$ (99.49 atom% ^{13}C) and hippuryl-lysine (HL) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). N^{ϵ} -Carboxymethylhippuryl-lysine (CM-HL) was synthesized according to the method reported in the literature.¹²⁾ Other reagents were purchased from Wako (Osaka, Japan). TLC was performed on Silica Gel 60 F₂₅₄ plate (Merck, Darmstadt, Germany) using detection with UV absorption and color-producing reagents for amino acids (ethanolic 0.2% ninhydrin) and sugars [a mixture of phenol (5 g), concentrated sulphuric acid (5 ml) and ethanol (95 ml)]. The pH of the incubation mixture was measured with a pH meter F-8E with an electrode 6029-10T (Horiba, Kyoto, Japan). Mass spectra were measured on a ThermoQuest (San Jose, CA, U.S.A.) TSQ7000 spectrometer. ^1H -, ^{13}C -NMR, and two-dimensional NMR (COSY) spectra were measured using a 5-mm o.d. NMR tube on a Bruker DPX400 or DRX500 spectrometer operating at 400- or 500-MHz ^1H observation and 100- or 125-MHz ^{13}C observation frequency at probe temperature of 300K. The chemical shifts were referenced to that of sodium 3-trimethylsilyl[2,2,3,3- $^2\text{H}_4$]propionate (TSP) ($\delta^1\text{H}$ and $\delta^{13}\text{C}$ 0).

Synthesis of $[1-^{13}\text{C}]\text{N}^{\epsilon}$ -(1-Deoxy-D-fructos-1-yl)hippuryl-lysine (^{13}C]DHL, See Chart 1) A mixture of D- $[1-^{13}\text{C}]\text{glucose}$ (407 mg, 2.25 mmol), hippuryl-lysine (230 mg, 0.75 mmol), and dry methanol (30 ml) was boiled under reflux for 22 h. When the reaction mixture was analyzed by

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TLC developed with *n*-propanol–acetic acid–H₂O (15:5:1, v/v), hippuryl-lysine (*R*_f 0.26) completely disappeared and a single spot (*R*_f 0.17) appeared. Color development for sugars virtually showed only two spots due to the product and the excess glucose. The methanol was then evaporated to dryness under reduced pressure to give a solid residue (652 mg). A solution of the solid residue (150–200 mg) in methanol (*ca.* 0.5 ml) was subjected to TLC (2 mm thick, 20×20 cm) developed with acetone–H₂O (9:2, v/v), where the product (*R*_f 0.1–0.2) was clearly separated from glucose (*R*_f *ca.* 0.8). The UV visible zone was scraped off and [¹³C]DHL was eluted with methanol. The eluates were evaporated to dryness under reduced pressure to give crude [¹³C]DHL (30–40 mg). The residue dissolved in H₂O (*ca.* 0.5–0.8 ml) was applied to Sep-Pak C₁₈ equilibrated with acetonitrile (6 ml) and H₂O (3 ml) and then eluted with H₂O (4 ml and then 20 ml) to obtain two fractions. The latter fraction was lyophilized to give white powdery [¹³C]DHL (12–16 mg, total yield 50 mg (14% based on hippuryl-lysine)); mp 129–130 °C (dec.); ¹H-NMR (deuterium oxide) δ¹H 4.25 (1H, dd, $J_{\text{Lys(H2),Lys(H3A)}}=8.4$ Hz, $J_{\text{Lys(H2),Lys(H3B)}}=4.8$ Hz, Lys(H2)), 1.64–1.77 (3H, multiplet, unresolved, Lys(H3A, H5)), 1.87 (1H, multiplet, unresolved, Lys(H3B)), 1.39 (2H, quintet, $J_{\text{Lys(H3A),Lys(H4)}}=J_{\text{Lys(H3B),Lys(H4)}}=J_{\text{Lys(H4),Lys(H5)}}=7.7$ Hz, Lys(H4)), 3.01 (2H, broad t, $J_{\text{Lys(H5),Lys(H6)}}=7.7$ Hz, $J_{\text{Lys(H6),Fru(C1)}}=1.7$ Hz, Lys(H6)), 4.12 (1H, d, $J_{\text{Gly(H2A),Gly(H2B)}}=16.7$ Hz, Gly(H2A)), 4.16 (1H, d, $J_{\text{Gly(H2A),Gly(H2B)}}=16.7$ Hz, Gly(H2B)), 7.85 (2H, d, $J_{\text{Benzoyl(o-H),Benzoyl(m-H)}}=7.5$ Hz, benzoyl(*o*-H)), 7.57 (2H, t, $J_{\text{Benzoyl(o-H),Benzoyl(m-H)}}=J_{\text{Benzoyl(m-H),Benzoyl(p-H)}}=7.5$ Hz, benzoyl(*m*-H)), 7.66 (1H, t, $J_{\text{Benzoyl(m-H),Benzoyl(p-H)}}=7.5$ Hz, benzoyl(*p*-H)), 3.21 (2H, $J_{\text{Fru(H1),Fru(C1)}}=143.3$ Hz, $J_{\text{Fru(H1A),Fru(H1B)}}=9.8$ Hz, Fru(H1A, H1B)), 3.73 (1H, dd, $J_{\text{Fru(H3),Fru(H4)}}=9.8$ Hz, $J_{\text{Fru(H3),Fru(C1)}}=1.7$ Hz, Fru(H3)), 3.88 (1H, dd, $J_{\text{Fru(H3),Fru(H4)}}=9.8$ Hz, $J_{\text{Fru(H4),Fru(H5)}}=3.3$ Hz, Fru(H4)), 4.00 (2H, unresolved, Fru(H5, H6B)), 3.74 (1H, dd, $J_{\text{Fru(H6A),Fru(H6B)}}=13.0$ Hz, $J_{\text{Fru(H5),Fru(H6A)}}=2.0$ Hz, Fru(H6A)); ¹³C{¹H}-NMR (phosphate buffer at pH 7.4): intense signal at δ¹³C 55.9 (Fru(C1)); mass (FAB⁺): *m/z* 471 ([M+H]⁺), 493 ([M+Na]⁺). *Anal.* Calcd for C₂₀H₃₁N₃O₉·5/2H₂O: C 48.93, H 7.04, N 8.15. Found: C 49.04, H 6.95, N 8.01. The chemical structure of [¹³C]DHL was confirmed by comparison of ¹H-NMR and COSY spectra with those of unlabeled DHL.

Degradation of [¹³C]DHL A solution of [¹³C]DHL (8.6 mM) or [1-¹³C]glucose (8.6 mM) was prepared in sodium phosphate buffer (0.1 M, pH 7.4, 1.9 ml). The solution was sterilized by 0.22-μm filtration into a sterile polypropylene tube (Asahi Technoglass, Funabashi, Japan). The mixture was incubated under a sterile air atmosphere in the dark at 37 °C for 4 weeks, and aliquots (0.5 ml) were withdrawn and subjected to NMR analysis after 2 and 4 weeks of incubation. The pH values of the reaction mixtures of [¹³C]DHL after incubation for 2 and 4 weeks were 7.21 and 7.07, respectively. In separate experiments, a solution of [¹³C]DHL (13 mM) in sodium phosphate buffer (0.1 M, pH 7.4) was incubated in an NMR tube at 65 °C for 25 h under aerobic conditions and then analyzed using ¹³C-NMR spectroscopy.

NMR Measurements of Degradation Mixtures Unless otherwise stated, NMR measurements were performed on a Bruker DRX500 spectrometer using a 5-mm NMR tube with a coaxial capillary tube (1.7 mm o.d., 1.2 mm i.d., Shigemi,

Tokyo, Japan) containing TSP dissolved in deuterium oxide (13 mg/ml). NMR spectra were measured at a probe temperature of 300 K without sample spinning. ¹³C-NMR spectra were obtained under the conditions of the usual ¹H decoupling (¹³C{¹H}) and distortionless enhancement by polarization transfer (DEPT) with ¹H decoupling. Parameters for ¹³C{¹H}-NMR were spectral width, 30581 Hz; time domain points, 65536; 45° pulse; acquisition time, 1.07 s; pulse delay, 2 s; and accumulation, *ca.* 17000 (*ca.* 15 h). The flip angle of the θ_y pulse was set at 90° and 135° in the DEPT experiments. ¹H-NMR spectra were obtained using the standard presaturation pulse sequence for water suppression,¹³⁾ where 40 free-induction decays were collected into 32768 data points with a spectral width of 7507 Hz and an acquisition time of 2.18 s, with a further 1-s delay to ensure full *T*₁ relaxation. Spectra were Fourier transformed after application of a line-broadening function of 0.3 Hz for ¹H-NMR and 1.0 Hz for ¹³C-NMR.

RESULTS AND DISCUSSION

Smith and Thornalley¹⁴⁾ described the pathways and mechanisms of degradation of a model Amadori compound, DHL, at pH 7.4 and 37 °C under aerobic conditions. They analyzed the degradation products by reverse-phase HPLC where the benzene moiety was used as a chromophore for UV detection of effluents. The study revealed a decrease of 55% in the initial concentration of DHL after 29 d of incubation and concomitant formation of CM-HL and HL, corresponding to 21% and 10% of the initial DHL concentration, respectively. However, 24% of the initial concentration of DHL was not accounted for by CM-HL and HL measurements. In addition, the HPLC method with UV detection provided only limited information on the decomposition pathways of the sugar moiety. Zyzak *et al.*¹⁵⁾ investigated the degradation fate of another model Amadori compound, *N*^α-formyl-*N*^ε-(1-deoxy-D-fructos-1-yl)lysine, at pH 7.4 and 37 °C under aerobic conditions. They described the same main degradation pathways as described by Smith and Thornalley, namely, formation of carboxymethyl-lysine (CML) by oxidative degradation and liberation of lysine by reversal of the Amadori rearrangement.³⁾ They also investigated the fate of the fructose moiety, focusing on formation of tetrose and larger sugars including deoxyglucosone, and observed that the main products included glucose and mannose formed by reversal of the Amadori rearrangement. However, smaller sugars and some products other than these sugars escape detection by the selected ion-monitoring gas chromatography-mass spectrometry used by them.

There has been no study on degradation pathways of Amadori compounds using ¹³C labeling and NMR spectroscopy although reaction mixtures of ¹³C-labeled glucose and amino acids have been directly analyzed by gas chromatography/mass spectrometry,¹⁶⁾ and chemical structures of ¹³C-labeled Amadori compounds^{17,18)} and their reactivity with pyridoxal¹⁹⁾ investigated using ¹³C-NMR spectroscopy. In this study, ¹³C-labeled glucose was used as the labeling substrate for the synthesis of labeled Amadori compound to investigate the degradation fate of the sugar moiety, and hippuryl-lysine was selected as the peptide moiety as in the literature mentioned above¹⁴⁾ because the synthetic reactions

and the purity of products can be examined using the UV absorption due to the benzoyl group. Although various types of ^{13}C -labeled glucose are commercially available, and different information is obtained depending upon the labeled position, $[1-^{13}\text{C}]\text{glucose}$ was used here because this position is contained in most degradation products reported to date.^{6,7,14,15)}

$[^{13}\text{C}]\text{DHL}$ (see Chart 1) was synthesized by a direct condensation of glucose and HL, as in the synthesis of unlabeled DHL,²⁰⁾ purified by TLC followed by solid-phase extraction, and characterized by ^1H -NMR spectroscopy and COSY. The ^1H -NMR spectrum in deuterium oxide was similar to the reported spectrum of unlabeled DHL in phosphate buffer (pH 7.4),²⁰⁾ except the H1 resonance of the fructose moiety (Fru) was split by ^{13}C incorporation into the C1 position, as shown in Fig. 1. $[^{13}\text{C}]\text{DHL}$ showed a single intense C1 (Fru) resonance at $\delta^{13}\text{C}$ 55.9 in the ^1H -decoupled ^{13}C -NMR spectra, as shown in Fig. 2A. The isotopic purity of $[^{13}\text{C}]\text{DHL}$ was more than 99% based on the integral intensities of the H1 signals ($\delta^1\text{H}$ 3.21) due to the labeled and unlabeled DHL shown in

Chart 1. Chemical Structures of $[1-^{13}\text{C}]\text{N}^{\epsilon}$ -(1-Deoxy-D-fructos-1-yl)hippuryl-lysine ($[^{13}\text{C}]\text{DHL}$) and Its Degradation Products $[^{13}\text{C}]\text{N}^{\epsilon}$ -Carboxymethylhippuryl-lysine ($[^{13}\text{C}]\text{CM-HL}$) and Hippuryl-lysine (HL)

The labeled position is indicated by an asterisk. The fructose moiety of $[^{13}\text{C}]\text{DHL}$ predominantly exists as the β -pyranose form.²¹⁾

Fig. 1.

When NMR spectroscopy of the labeled compound was measured in deuterium oxide, the ^{13}C signal at δ 55.9 [Fru(C1)] gradually decreased with the concurrent appearance of a broad triplet resonance shifted upfield by 0.3 ppm. This observation means that the protons on the C1 position of the sugar moiety of the Amadori compound were partially lost due to deuterium exchange through enolization, which was also mentioned in the literature.^{16,21)} Thus the NMR spectroscopic monitoring of the degradation must be performed without mixing the incubation mixture with deuterium oxide for field-frequency lock because this phenomenon makes it difficult to interpret the spectra quantitatively. Thus, in this paper, deuterium oxide was placed in a capillary tube inserted into an NMR tube containing the incubation mixture.

$[^{13}\text{C}]\text{DHL}$ was incubated at pH 7.4 and 37°C under sterile and aerobic conditions. As can be seen from the ^{13}C -NMR spectra of the incubation mixture (Fig. 2), the concentration of DHL ($\delta^{13}\text{C}$ 55.9) decreased to 15% of the initial concentration after 28 d of incubation. There was, at least, a concomitant formation of nine labeled products at $\delta^{13}\text{C}$ 173.9 (CH), 98.9 (CH), 95.1 (CH), 84.9 (CH_2), 64.2 (CH_2), 53.5 (CH_2), 52.2 (CH_2), 26.2 (CH_3), and 23.0 (CH_3). The number

Fig. 1. Partial ^1H -NMR Spectrum of $[^{13}\text{C}]\text{DHL}$

Signals 1 and 2 are due to the H1 of fructose moiety of $[^{13}\text{C}]\text{DHL}$ and unlabeled DHL, respectively. The former signal is split ($J=143.3\text{ Hz}$) by ^1H - ^{13}C coupling with the labeled position [Fru(C1)].

Fig. 2. $^{13}\text{C}\{^1\text{H}\}$ -NMR Spectra of $[^{13}\text{C}]\text{DHL}$ in Phosphate Buffer at pH 7.4 before (A) and after Incubation at 37°C under Aerobic Conditions for 14 (B) and 28 d (C)

An aliquot was withdrawn and analyzed by $^{13}\text{C}\{^1\text{H}\}$ -NMR spectroscopy. Signals 1 and 2 are due to $[^{13}\text{C}]\text{DHL}$ and TSP, respectively. Signals 3—11 are ^{13}C -labeled products formed from $[^{13}\text{C}]\text{DHL}$. Assignments: 3, formate; 4 and 5, α - and β -anomer of glucose; 9, CM-HL; 10, acetate. Signals 6, 7, 8, and 11 remain to be identified.

of ^1H attached to each carbon observed in the spectra was determined in DEPT experiments. When ^{13}C]DHL was incubated at pH 7.4 and 65°C , the same signals, except the signal at $\delta^{13}\text{C}$ 84.9, were observed (Fig. 3) although the relative intensities of signals were considerably different from those in Fig. 2. These results demonstrate that various degradation products derived from ^{13}C]DHL are clearly detected by the ^{13}C -labeling and NMR approach.

The signal at $\delta^{13}\text{C}$ 52.2 (signal 9) was assigned to CM-HL formed by oxidative degradation of DHL by comparison of the chemical shift with that of the corresponding position ($\text{CH}_2\text{CO}_2\text{H}$) of authentic unlabeled CM-HL. Although the relative signal intensities in the spectra do not necessarily show the molar ratios of products in the solutions owing to differences in the nuclear Overhauser enhancement and T_1 relaxation time of each carbon,⁸⁾ CM-HL is considered to be the major product of all those with the C1 of the parent fructose moiety under physiologic conditions. On the other hand, at 65°C the signal at $\delta^{13}\text{C}$ 26.2 (identified as acetate below) was the most prominent and CM-HL was the next major product. Labeled glucose was observed as both the α - ($\delta^{13}\text{C}$ 95.1) and β -anomers ($\delta^{13}\text{C}$ 98.9). In addition, ^{13}C signals due to HL were appreciably detected irrespective of their *ca.* 1% natural abundances at $\delta^{13}\text{C}$ 181.6 (CO), 174.1 (CO), 173.8 (CO), 135.7 (phenyl, *ipso*), 135.4 (phenyl, *para*), 131.7 (phenyl), 130.1 (phenyl), 57.7 (Lys, C2), 46.2 (Gly, C2), 42.4, (Lys, C6) 34.0 (Lys, C5), 29.3 (Lys, C4), and 24.9 (Lys, C3). These observations at both temperatures may suggest that large amounts of HL were formed with glucose by reversal of Amadori rearrangement under physiologic conditions, as suggested in the literature.¹⁵⁾ Most of the glucose formed appeared to decompose to other products such as aliphatic acids^{6,7)} because the molar concentration of glucose was estimated to be much less than that of HL on the basis of their signal intensities. The observations that CM-HL and HL are the major products at pH 7.4 and 37°C under aerobic conditions are consistent with the previous report.¹⁴⁾

When the incubation mixture was reanalyzed using ^{13}C -NMR spectroscopy after being freeze-dried and reconstituted in deuterium oxide, not only the signal of DHL at $\delta^{13}\text{C}$ 55.9 but also the signals at $\delta^{13}\text{C}$ 173.9, 84.9, and 26.2 completely disappeared. Thus the latter three compounds were considered to have labile protons like DHL or be volatile. The signals at $\delta^{13}\text{C}$ 26.2 (CH_3) and 173.9 (CH) were identified as acetate and formate, respectively, by comparison of their chemical shifts with those of authentic compounds. These carboxylates have been reported to form from Amadori compounds at pH around 7 and 90 – 120°C ,^{6,7)} whereas under physiologic conditions (pH 7.4, 37°C) no data on their formation has been reported to date.

As shown in Fig. 4, the ^1H -NMR spectra of the incubation mixture showed a singlet signal due to unlabeled formate together with a doublet signal due to ^{13}C -labeled formate at virtually the same chemical shift ($\delta^1\text{H}$ 8.44). The latter signal was assigned by comparison of the frequencies with those of the ^{13}C satellite signal for the ^1H signal of unlabeled formate. These results showed that formate originates from not only the labeled C1 of the parent fructose moiety but also from other unlabeled carbons. Formate has been suggested to be formed from Amadori compounds through glucose at higher temperatures.^{6,7)} Thus $[1\text{-}^{13}\text{C}]\text{glucose}$ was incubated under

Fig. 3. $^{13}\text{C}\{^1\text{H}\}$ -NMR Spectrum of ^{13}C]DHL in Phosphate Buffer at pH 7.4 after Incubation at 65°C under Aerobic Conditions for 25 h

A Bruker DPX400 spectrometer was used. Parameters for $^{13}\text{C}\{^1\text{H}\}$ -NMR were spectral width, 26178 Hz; time domain points, 65536; 80° pulse; acquisition time, 1.25 s; pulse delay, 2 s; and accumulation, *ca.* 12600 (*ca.* 11 h). Key is identical to that in Fig. 2.

Fig. 4. Partial ^1H -NMR Spectrum of ^{13}C]DHL in Phosphate Buffer at pH 7.4 after Incubation at 37°C for 28 d under Aerobic Conditions

Signals 1 and 2 show the H1 of ^{13}C -labeled and unlabeled formate, respectively. The former signal is split by ^1H – ^{13}C coupling ($J=195$ Hz).

physiologic conditions to investigate the mechanism of formate formation from DHL. After 28 d of incubation, only a low percentage of the substrate was degraded to produce several minor ^{13}C signals, including formate in the ^{13}C -NMR spectra (data not shown). Minor ^1H signals due to ^{13}C -labeled and unlabeled formate were also observed with a similar intensity in the ^1H -NMR spectra, and both compounds were estimated to be about one-fourth and one-eighth of those observed in the incubation mixture of ^{13}C -DHL, respectively, on the basis of the signal intensities of TSP. These results mean that formate is, at least partially, formed *via* glucose liberated from DHL under physiologic conditions, and the formation is due to not only a cleavage between the labeled C1 and C2 of glucose⁷⁾ but also to additional pathways.

Under physiologic conditions, 3-deoxyglucosone²²⁾ and 2-glucosulose²³⁾ have been reported to form as well. If they are produced in the present experiments, they have methine carbons labeled with ^{13}C derived from the parent labeled compound. However, the remaining major ^{13}C signals observed at $\delta^{13}\text{C}$ 84.9 (CH_2), 64.2 (CH_2), 53.5 (CH_2), and 23.0 (CH_3) in Fig. 2 are due to methylene or a methyl group. Thus 3-deoxyglucosone and 2-glucosulose do not correspond to any of these four signals and their formation is not appreciable under physiologic conditions. Thus the four signals are

due to new products unreported to date.

In summary, clear degradation profiles of [^{13}C]DHL under physiologic conditions were obtained using the ^{13}C labeling and NMR approach, in which nine major products were observed including CM-HL, α - and β -glucose, and six new products, and two of the new products were identified to be acetate and formate although the others remain unknown. This preliminary study suggests that the combined use of ^{13}C labeling and NMR spectroscopy is a promising approach for the investigation of the degradation pathways of Amadori compounds in that it provides a clear and comprehensive profiling of the degradation products containing the labeled position in spite of their chemical structures, by a single measurement of complicate reaction mixtures. Further NMR spectroscopic examination of the reaction mixtures coupled with SPEC-NMR²⁴⁾ and LC-NMR²⁵⁾ and the use of DHL ^{13}C labeling at various positions would enable identification of the remaining products and give more insight into the degradation pathways of Amadori compounds.

Acknowledgments This research was supported by grants for private universities provided by the Ministry of Education, Culture, Sports, Science and Technology and the Promotion and Mutual Aid Corporation for Private Schools of Japan.

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