

## Research Article

# Structural Characterization of Amadori Rearrangement Product of Glucosylated N<sup>α</sup>-Acetyl-Lysine by Nuclear Magnetic Resonance Spectroscopy

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Maillard reaction is a nonenzymatic reaction between reducing sugars and free amino acid moieties, which is known as one of the most important modifications in food science. It is essential to characterize the structure of Amadori rearrangement products (ARPs) formed in the early stage of Maillard reaction. In the present study, the N<sup>α</sup>-acetyl-lysine-glucose model had been successfully set up to produce ARP, N<sup>α</sup>-acetyl-lysine-glucose. After HPLC purification, ARP had been identified by ESI-MS with intense [M+H]<sup>+</sup> ion at 351 *m/z* and the purity of ARP was confirmed to be over 90% by the relative intensity of [M+H]<sup>+</sup> ion. Further structural characterization of the ARP was accomplished by using nuclear magnetic resonance (NMR) spectroscopy, including 1D <sup>1</sup>H NMR and <sup>13</sup>C NMR, the distortionless enhancement by polarization transfer (DEPT-135) and 2D <sup>1</sup>H-<sup>1</sup>H and <sup>13</sup>C-<sup>1</sup>H correlation spectroscopy (COSY) and 2D nuclear overhauser enhancement spectroscopy (NOESY). The complexity of 1D <sup>1</sup>H NMR and <sup>13</sup>C NMR was observed due to the presence of isomers in glucose moiety of ARP. However, DEPT-135 and 2D NMR techniques provided more structural information to assign the <sup>1</sup>H and <sup>13</sup>C resonances of ARP. 2D NOESY had successfully confirmed the glycosylated site between 10-N in N<sup>α</sup>-acetyl-lysine and 7'-C in glucose.

## 1. Introduction

Maillard reaction, also called nonenzymatic reaction, occurs between reducing sugars and amino acids, peptides, or proteins. The reaction had been studied by many researchers after it was first observed by Maillard [1–4]. As one of the most important reactions in food science, Maillard reaction produces color and flavor compounds during food process. There are different mechanisms of Maillard reaction to produce various final products via the formation of complex intermediates [3, 5, 6]. To simplify the process of Maillard reaction, the mechanisms are described as early stage, intermediate stage, and final stage [7, 8]. For example, the reversible condensation between the aldehyde group of glucose and the amino group of protein produces a Schiff base; then an essentially irreversible rearrangement changes the Schiff base to colorless Amadori rearrangement products (ARPs)

[8, 9] (Figure 1). This ARP intermediate undergoes cycles of condensations with additional amines, dehydrations, and oxidative fragmentations to yield final heterogeneous chemical compounds as advanced glycation end-products (AGEs) [10, 11]. Nowadays, it is still a great challenge to control the reaction in food quality, nutrition assessment, and medicinal aspects due to its unclear mechanisms. Modern analytical techniques have been applied in structural characterization of ARPs, such as circular dichroism, infrared spectroscopy, fluorescence spectroscopy, mass spectrometry, and nuclear magnetic resonance (NMR) spectroscopy [12]. It has been found that the presence of tautomers in the ketose moiety of ARPs limits the structure determination by 1D <sup>1</sup>H and <sup>13</sup>C NMR due to the complexity of the spectra [13, 14]. However, 2D NMR techniques can provide more information for the structural characterization of ARPs.

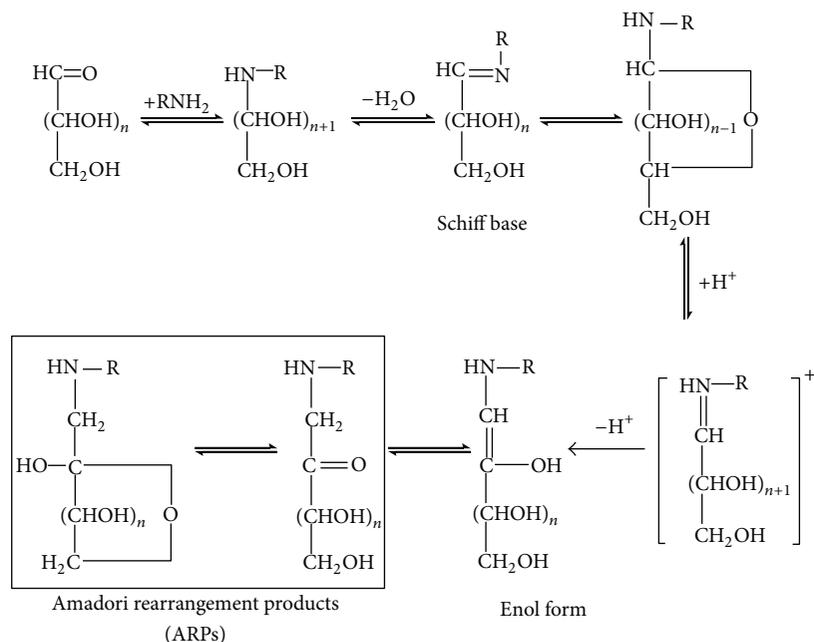


FIGURE 1: Mechanism of Amadori rearrangement in the Maillard reaction.

In the present study, the  $N^\alpha$ -acetyl-lysine-glucose model had been set up for a detailed illustration of structural information of ARP ( $N^\alpha$ -acetyl-lysine-glucose). 1D  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR, the distortionless enhancement by polarization transfer (DEPT-135) spectrum, and 2D  $^1\text{H}$ - $^1\text{H}$  and  $^{13}\text{C}$ - $^1\text{H}$  correlation spectroscopy (COSY) were used to assign correlations between the signals in the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra. To confirm the connection between the amino acid and glucose, 2D nuclear overhauser enhancement spectroscopy (NOESY) experiments were performed.

## 2. Experimental

*D*-glucose and  $N^\alpha$ -acetyl-lysine were purchased from Sigma (St. Louis, MO, USA) with the highest purity. Water was purified by Milli Q distilled water (Millipore, Bedford, MA, USA). All other reagents and solvents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

To set the reaction model, 0.1 M of  $N^\alpha$ -acetyl-lysine was dissolved in 1 M *D*-glucose solution with the ratio of 1:10 by molecular weight. Mixture solution was freeze-dried by SC250DDA Speedvac Plus (Thermo Electron Corporation, Waltham, MA) and heated in sealed vials for 1 hour at  $90^\circ\text{C}$ . For structural analysis of Amadori rearrangement product, glucosylated  $N^\alpha$ -acetyl-lysine, the reaction mixture was purified using high-performance liquid chromatography (HPLC) with a  $250 \times 5.0$  mm i.d. C18 reverse-phase semipreparative column and ARP-containing fractions were collected and lyophilized. Before NMR analysis, purified glucosylated  $N^\alpha$ -acetyl-lysine was identified by electrospray ionization mass spectrometry (ESI-MS) with intense  $[\text{M}+\text{H}]^+$  ion in the spectrum from  $m/z$  100 to 500.

After HPLC purification, freeze-dried glucosylated  $N^\alpha$ -acetyl-lysine was dissolved in dimethyl sulfoxide- $d_6$  (DMSO- $d_6$ ,  $500 \mu\text{L}$ ) and transferred to a 5 mm NMR tube for NMR analysis. All NMR experiments were performed on a Bruker DRX 500 spectrometer (Bruker BioSpin, Germany) equipped with a 5 mm TBO probe and operated at  $25^\circ\text{C}$  (298 K) with a proton frequency of 500.13 MHz. The chemical shifts ( $\delta$  values), given in parts per million (ppm), were referenced to the signals of the residual protons ( $\delta$  2.50 ppm) and carbon atom ( $\delta$  39.5 ppm) in DMSO- $d_6$ . All 1D ( $^1\text{H}$ ,  $^{13}\text{C}$ , and DEPT-135) and 2D ( $^1\text{H}$ - $^1\text{H}$  COSY,  $^{13}\text{C}$ - $^1\text{H}$  COSY, and NOESY) NMR measurements were performed using standard Bruker pulse sequences. Sweep widths of 5000 and 25000 Hz were used in  $^1\text{H}$  and  $^{13}\text{C}$  NMR, respectively. 2D  $^1\text{H}$ - $^1\text{H}$  COSY and  $^{13}\text{C}$ - $^1\text{H}$  COSY spectra were collected in quadrature with 1024 points in  $t_2$  and 256 points in  $t_1$ , and the sweep widths were 5000 and 15000 Hz of  $^1\text{H}$  and  $^{13}\text{C}$  dimensions, respectively. 2D NOESY was recorded with mixing time of 400 ms and 256  $t_1$  increments containing 16 transients of 2048 complex data points. 2D NMR data were applied with a  $90^\circ$  phase-shifted, squared sine-bell window function in both dimensions prior to Fourier transformation.

## 3. Results and Discussion

$[\text{M}+\text{H}]^+$  ion at 351.13  $m/z$  had been found in ESI-MS spectrum, and the purity of ARP had been determined as over 90% by the relative intensity of the  $[\text{M}+\text{H}]^+$  ion.

Structural characterization of purified ARP, glucosylated  $N^\alpha$ -acetyl-lysine (Figure 2), was carried out by performing a series of 1D and 2D NMR experiments, and the resonance assignments of  $^1\text{H}$  and  $^{13}\text{C}$  had been accomplished.

The signals with the chemical shifts from 1.0 to 4.0 ppm in  $^1\text{H}$  NMR spectrum had been grouped and assigned to

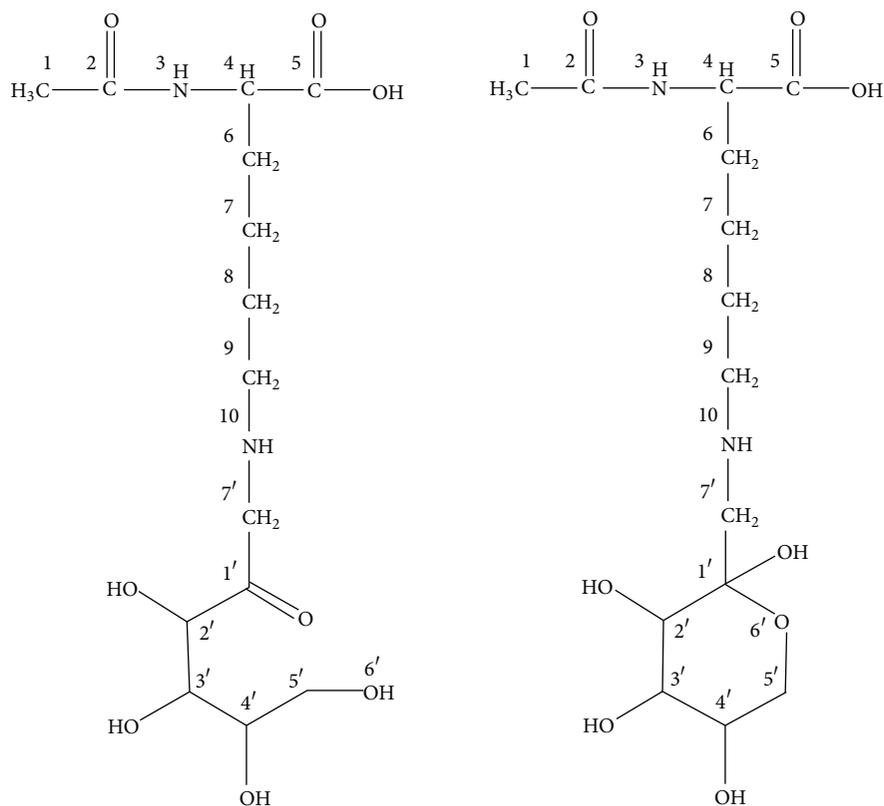


FIGURE 2: Two isomer structures of glucosylated  $N^{\alpha}$ -acetyl-lysine.

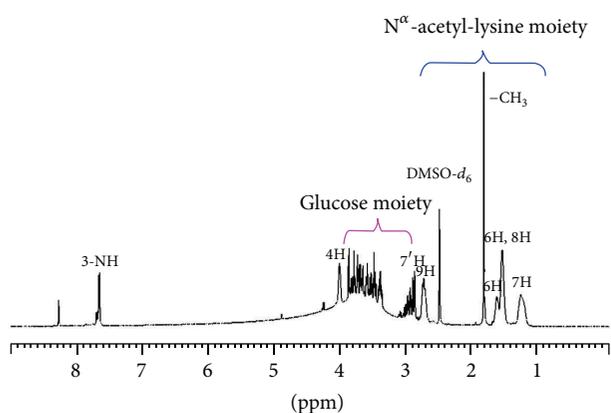


FIGURE 3: 1D  $^1\text{H}$  NMR spectrum of glucosylated  $N^{\alpha}$ -acetyl-lysine. The assignments of the protons are labeled according to the numerals in Figure 2.

aliphatic protons in glucose moiety ( $\delta$  2.80 to 3.90 ppm) and  $N^{\alpha}$ -acetyl-lysine moiety ( $\delta$  1.0 to 2.80 ppm) of glucosylated  $N^{\alpha}$ -acetyl-lysine, respectively (Figure 3). The complexity of  $^1\text{H}$  NMR spectrum in glucose moiety suggested that different conformations existed in the glucose moiety. Signals in  $^{13}\text{C}$  NMR spectrum also presented as groups of two moieties: glucose moiety ( $\delta$  50 to 100 ppm) and  $N^{\alpha}$ -acetyl-lysine moiety ( $\delta$  20 to 60 ppm) (Figure 4(a)). The finding of multiple chemical shifts of  $^{13}\text{C}$  signals in glucose moiety as  $^1\text{H}$  signals

had confirmed the presence of isomers with open-chain and closed-chain structures in the moiety (Figure 2) [15]. In  $^{13}\text{C}$  NMR spectrum, two carbonyl signals had been observed at 169 ppm and 175 ppm that were assigned to 5-C and 2-C in ARP structures.

The DEPT-135 spectrum was a spectral editing sequence, which could verify  $-\text{CH}$ ,  $-\text{CH}_2$ , and  $-\text{CH}_3$  carbons by the phase of signals, and signals from quaternary carbons and other carbons with no attached protons were always absent. In DEPT-135 spectrum of ARP, three  $-\text{CH}$  and two  $-\text{CH}_2$  in glucose moiety and one  $-\text{CH}$ , four  $-\text{CH}_2$ , and one  $-\text{CH}_3$  in  $N^{\alpha}$ -acetyl-lysine moiety had been found (Figure 4(b)). The signal at  $\sim 83$  ppm was verified as  $-\text{CH}$  group and assigned to 2'-C in the open-chain structure while the signal at 102 ppm was assigned to 1'-C in the closed-chain structure. The  $^{13}\text{C}$  signal at 22 ppm was also identified as the overlapping signal of two carbons  $-\text{CH}_3$  and  $-\text{CH}$  that were assigned to 1-C and 7-C, respectively.

The  $^1\text{H}$  signal at 7.7 ppm suggested an amide proton (3-NH) of lysine moiety, and this proton was strongly correlated with the proton at 4.0 ppm according to the 2D  $^1\text{H}$ - $^1\text{H}$  COSY spectrum in Figure 5. Meanwhile, the correlated carbon of the proton at 4.0 ppm was the signal at 51 ppm in  $^{13}\text{C}$ - $^1\text{H}$  COSY spectrum in Figure 6, which was verified as a  $-\text{CH}$  group in DEPT-135 spectrum. Based on this information, the  $^1\text{H}$  signal at 4.0 ppm was assigned to  $\alpha$ -H (4-H) of lysine moiety. The  $^1\text{H}$ - $^1\text{H}$  COSY spectrum showed the other correlations between the protons at 4.0 ppm and 1.5 ppm,

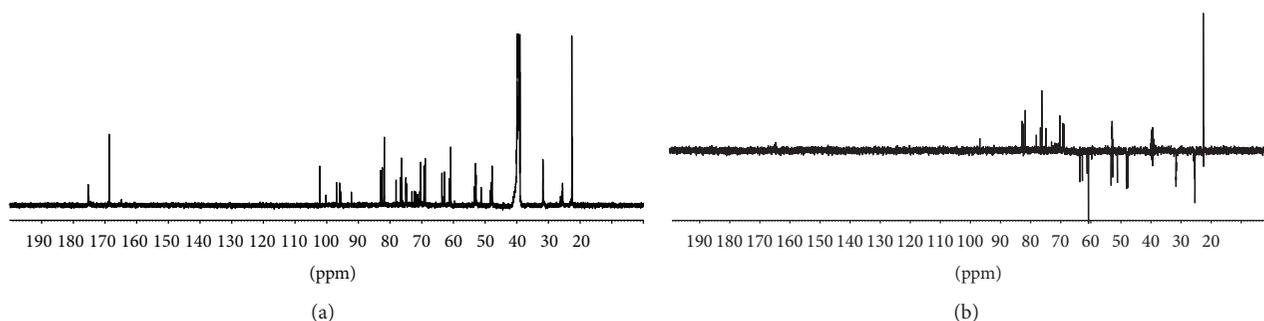


FIGURE 4: 1D  $^{13}\text{C}$  NMR spectrum (a) and DEPT-135 spectrum (b) of glucosylated  $\text{N}^\alpha$ -acetyl-lysine.

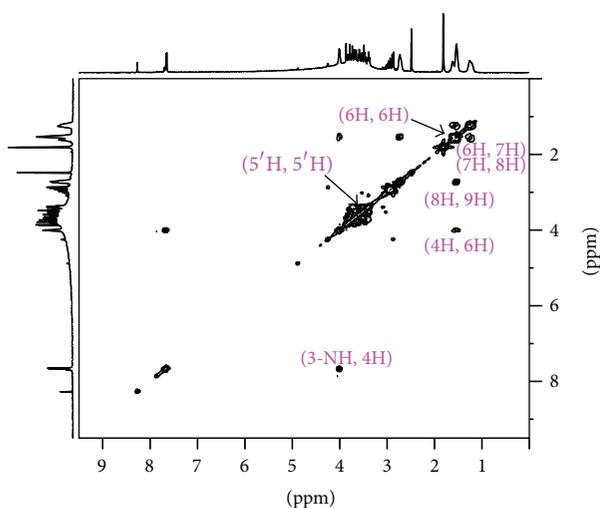


FIGURE 5: 2D  $^1\text{H}$ - $^1\text{H}$  COSY spectrum of glucosylated  $\text{N}^\alpha$ -acetyl-lysine. The assignments of the cross-peaks are labeled according to the numerals in Figure 2.

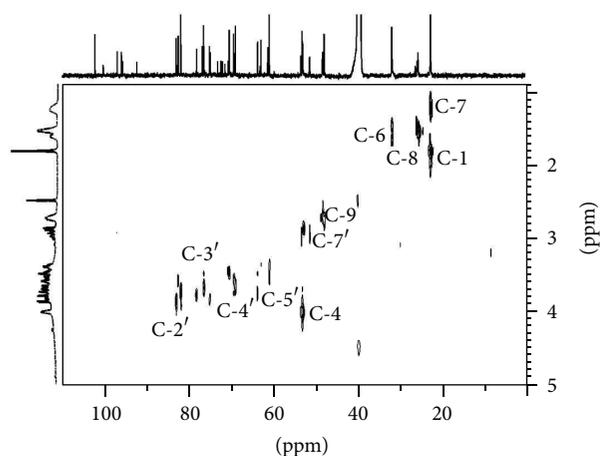


FIGURE 6: 2D  $^{13}\text{C}$ - $^1\text{H}$  COSY spectrum of glucosylated  $\text{N}^\alpha$ -acetyl-lysine. The assignments of the cross-peaks are labeled according to the numerals in Figure 2.

1.5 ppm and 1.3 ppm, and 1.5 ppm and 2.7 ppm, indicating the connection of lysine aliphatic chain from 4-H to 9-H in  $^1\text{H}$ - $^1\text{H}$  COSY spectrum (Figure 5). The  $^1\text{H}$  signal at 2.7 ppm was assigned to 9-H as the connection to 10-NH in lysine chain. According to the integral of the signals, the overlapping of 6-H and 8-H at 1.5 ppm had been found. The correlations between the protons at 1.6 ppm and 1.5 ppm and 1.5 ppm and 2.7 ppm suggested that the  $^1\text{H}$  signals at 1.5 ppm and 1.6 ppm were assigned to the two protons at 6-H, and  $^1\text{H}$  signals at 1.5 ppm were also assigned to 8-H. Consequently, signal at 1.3 ppm was assigned to 7-H and the assignment of 9-H was also confirmed. The carbon resonances in lysine aliphatic chain were further assigned by the correlations in  $^{13}\text{C}$ - $^1\text{H}$  COSY spectrum with their corresponding proton assignments (Table 1). The  $^{13}\text{C}$ - $^1\text{H}$  COSY spectrum suggested that the protons at position 6 had split at 1.6 ppm and 1.5 ppm, which was also presented by the strong correlation in  $^1\text{H}$ - $^1\text{H}$  COSY spectrum. The  $^{13}\text{C}$  assignments in  $\text{N}^\alpha$ -acetyl-lysine moiety were also confirmed by 1D  $^{13}\text{C}$  NMR and DEPT-135 spectra.

The multiple correlations in the glucose moiety of  $^1\text{H}$ - $^1\text{H}$  COSY spectrum also suggested the presence of isomers in the moiety. The strong correlations in  $^1\text{H}$ - $^1\text{H}$  COSY spectrum between 3.4 ppm and 3.5 ppm and 3.5 ppm and 3.8 ppm had been observed; the two sets of  $^1\text{H}$  signals were correlated to the  $-\text{CH}_2$  at 61 ppm and 64 ppm in the  $^{13}\text{C}$ - $^1\text{H}$  COSY spectrum, respectively. Consequently,  $^1\text{H}$  signals at 3.4 ppm and 3.5 ppm were assigned to  $5'$ -H in the open-chain structure, while  $^1\text{H}$  signals at 3.5 ppm and 3.8 ppm were assigned to  $5'$ -H of glucose moiety in the closed-chain structure. The  $-\text{CH}_2$  at  $\sim 50$  ppm in glucose moiety was assigned to  $7'$ -C, and the correlated proton signals had been found at  $\sim 2.9$  ppm in  $^{13}\text{C}$ - $^1\text{H}$  COSY spectrum. The assignment of  $7'$ -H was essential to the confirmation of the ARP that contained the chemical bonding between 10-N and  $7'$ -C. The  $-\text{CH}$  signals at 78 ppm and 70 ppm were assigned to  $3'$ -C and  $4'$ -C in the open-chain structure, and the  $-\text{CH}$  signals at 82 ppm, 76 ppm, and 69 ppm were assigned to  $2'$ -C,  $3'$ -C, and  $4'$ -C in the closed-chain structure, respectively. The signals of aliphatic protons in glucose moiety were consequently assigned according to the correlations in  $^{13}\text{C}$ - $^1\text{H}$  COSY spectrum (Table 1).

TABLE 1:  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR resonance assignments of the isomers of ARP.

Position	$^1\text{H}$ NMR (ppm)		$^{13}\text{C}$ NMR (ppm)	
	Open-chain	Closed-chain	Open-chain	Closed-chain
1	1.82	1.82	22	22
2	—	—	175	175
3	7.7	7.7	—	—
4	4.0	4.0	53	53
5	—	—	169	169
6	1.5, 1.6	1.5, 1.6	32	32
7	1.3	1.3	22	22
8	1.5	1.5	25	25
9	2.7	2.7	48	48
10	2.6	2.6	—	—
1'	—	—	175	102
2'	3.88	3.73	83	82
3'	3.8	3.7, 3.65	78	76
4'	3.5	3.6	70	69
5'	3.4, 3.5	3.5, 3.8	61	63
7'	2.88	2.93	53	51

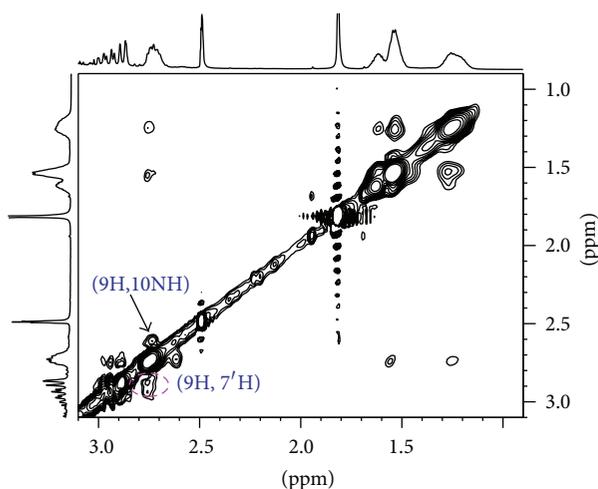


FIGURE 7: 2D NOESY spectrum of glucosylated  $\text{N}^\alpha$ -acetyl-lysine. The assignments of the cross-peaks are labeled according to the numerals in Figure 2.

2D NOESY experiments were carried out to confirm the crosslink between the glucose and  $\text{N}^\alpha$ -acetyl-lysine moieties (Figure 7). The correlations between 2.9 ppm and 2.7 ppm in NOESY spectrum suggested that the 7'-H had an NOE effect with proton at 9-H that could only be found when ARP formed in the reaction model. In NOESY spectrum, there was a correlation between 2.7 ppm and 2.6 ppm that suggested that  $^1\text{H}$  signal at 2.6 ppm had an NOE effect with 9-H, and the signal was then assigned as 10-NH. The  $^1\text{H}$  signal of 10-NH shifted from  $\sim 8$  ppm in free lysine to high magnetic field also suggested that the glucose moiety was chemically bonded to 10-NH.

In summary, structural characterization of ARP glycosylated  $\text{N}^\alpha$ -acetyl-lysine in Maillard reaction had been accomplished by serials of NMR experiments. The complexity of 1D  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR was observed due to the presence of isomers in glucose moiety of ARP with the open-chain structure and the closed-chain structure. However, DEPT-135 and 2D NMR techniques provided more structural information of ARP, and the assignment of  $^1\text{H}$  and  $^{13}\text{C}$  resonances had been accomplished. 2D NOESY had successfully confirmed the glycosylated site between 10-N in  $\text{N}^\alpha$ -acetyl-lysine and 7'-C in glucose.

## Conflict of Interests

There are no competing interests amongst the authors. The authors declare that there is no conflict of interests.

## Authors' Contribution

Hui Wang and Chuanjiang Li contributed to the paper as the first authors.

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