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Demonstration of the presence of aminoreductone formed during the Maillard reaction in milk

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ABSTRACT

The Maillard reaction is a common chemical reaction that occurs in food and it generates multiple reaction products. Aminoreductone (AR) is one of the early-stage Maillard reaction products. At present the formation of AR has only been demonstrated in a model system consisting of a monosaccharide or disaccharide and an amino group-containing compound. There is no direct evidence to show the presence of AR in food. In this study, we demonstrated the formation and presence of AR in milk using a combination of 2,4-dinitrophenylhydrazine (DNP) and Cu²⁺. A DNP derivative of AR oxidised by Cu²⁺ was isolated and its detailed structure was identified by NMR analysis. We thus directly demonstrated the formation and presence of AR in milk.

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1. Introduction

The Maillard reaction (nonenzymatic glycation) is a chemical reaction between amino and carbonyl groups; it is an extremely complex reaction that usually takes place during food processing or storage. The products formed during the Maillard reaction are largely responsible for the colour, taste, flavour, and nutritional value of food (van Boekel, 2006; Somoza, 2005). Therefore, it is considered as one of the most important reactions influencing food quality and acceptance. Milk is usually subjected to heat treatment to ensure microbiological safety before retail and consumption, and the Maillard reaction occurs during this process (van Boekel, 1998). Lactose reacts primarily with the free amino groups of the milk proteins to proceed to the early, intermediate, and advanced stages of the Maillard reaction, and forms enormous amounts of Maillard reaction products. The reactions of lactose have therefore frequently been investigated and many researchers have demonstrated chemical changes and formation of various Maillard reaction products in milk during the heating process (van Boekel, 1998). In the general Maillard reaction, the Amadori product progresses down the 3-deoxyosone or 1-deoxyosone route, depending on reaction pH. In the case of Maillard reaction of disaccharides, there is a third degradation route of the Amadori product. It is the 4-deoxyosone route. Aminoreductone (AR) is generated in the 4-deoxyosone route (van Boekel, 1998). AR derived from the

Maillard reaction of lactose was first reported by Pischetsrieder, Schoetter, and Severin (1998). It is an early-stage Maillard reaction product obtained by heating a model solution of lactose and N^{α} -acetyllysine.

An XTT (3'-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro) benzenesulfonic acid) assay has been proposed as a useful method for evaluating the heat treatment of milk (Ukeda et al., 1996). Compared with previously reported methods, such as the quantification of lactulose, hydroxymethylfurfural, and furosine, the XTT assay is both rapid and convenient, and it has been found to be applicable for estimating not only the extent of thermal treatment but also the storage conditions. In a series of studies using a model system of lactose and butylamine, it was shown that AR was the XTT-reducing substance (Shimamura, Ukeda, & Sawamura, 2000, 2004). As described above, AR is an early-stage Maillard reaction product. The XTT assay can differentiate the severity of heat treatment in milk production by assessing the progress of the Maillard reaction, which is influenced by the temperature and duration of the heating process. We have attempted to elucidate the formation of AR in the Maillard reaction of lactose from the perspective of the relationship between formation of AR and galactose (Trang, Shimamura, Kashiwagi, Ukeda, & Katsuno, 2011).

The functionalities of aminoreductone have attracted interest, and so far, the protective effect against riboflavin photolysis (Trang, Kurogi, Katsuno, Shimamura, & Ukeda, 2008) and anti-*Helicobacter pylori* (*H. pylori*) activity (Trang et al., 2009) were revealed using the model system of lactose and butylamine. Antioxidative activity of AR from lactose and N^{α} -acetyllysine has also been

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reported (Pischetsrieder, Rinaldi, Gross, & Severin, 1998), and AR from lactose and butylamine has been shown to be an active component inducing nuclear NF- κ B translocation in macrophages (Wühr, Deckert, & Pischetsrieder, 2010). Thus, AR, which was formed during the Maillard reaction of lactose, can clearly affect the quality and function of foods such as milk and dairy products. At present the formation of AR in the Maillard reaction mixture has only been shown in model systems such as lactose and butylamine or N^{α} -acetyllysine (Pischetsrieder, Schoetter et al., 1998; Shimamura et al., 2000), and the generation of AR in milk has only been presumed (Pischetsrieder, Schoetter et al., 1998). Presence of AR in milk has not yet been demonstrated because AR is very labile and difficult to isolate. In this study, we demonstrated the presence of AR in milk using a 2,4-dinitrophenylhydrazine (DNP) derivatisation method.

2. Materials and methods

2.1. Reagents and milk sample

Lactose monohydrate was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). *n*-Butylamine and DNP were obtained from Wako Pure Chemical Industries (Osaka, Japan), and XTT and α -lactalbumin from Sigma–Aldrich Co. (St. Louis, MO). Chloroform-*d* (CDCl₃) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). All other reagents were of the highest grade commercially available. Milli-Q water was used throughout all experiments. Long-life (LL) milk (Nippon Milk Community Co., Ltd., Saitama, Japan; 140 °C, 3 s) which had an expiration date after 60 days was purchased from the local supermarket.

2.2. Heating condition

A model solution consisting of a lactose and amino group compound was heated as described in our previous studies (Shimamura et al., 2000, 2004). Lactose monohydrate (262 mM) and butylamine (1.16 M) were dissolved in 1.28 M phosphate buffer (pH 7.0), and then the final pH was adjusted to 7.0 with phosphoric acid. In the case of the milk protein, lactose (4.6%) and α -lactalbumin (2.3%) were dissolved in 20 mM phosphate buffer (pH 6.7). A 1.5-ml sample tube was filled with 1.2 ml of the model solution and heated at 100 or 130 °C for the indicated duration using a dry heater (Dry Thermo Unit DTU-1C, Taitec Co., Saitama, Japan). Immediately after heating, the sample was cooled on ice to stop the reaction. The milk was heated in a test tube in order to scale up.

2.3. Preparation of crude AR

Crude AR was prepared from the heated model solution of lactose and butylamine by the method described in our previous studies (Shimamura et al., 2000, 2004). Briefly, the heated model solution was extracted three times with a double volume of ethyl acetate. The ethyl acetate layer was collected and the solvent in this fraction was evaporated on a water bath at 70 °C under nitrogen atmosphere. The residue (crude AR) was used in subsequent experiments.

2.4. XTT assay

The XTT assay was performed in a 96-well microtiter plate according to the method described in our previous studies (Shimamura et al., 2000, 2004). A 60 μ l of 0.5 mM XTT prepared with 0.2 M potassium phosphate buffer (pH 7.0) saturated with menadione was added into each well. Afterwards, the sample (40 μ l) was added to the well. After mixing on a microplate shaker

at 500 rpm for 15 s, the difference in the absorbance between 492 and 600 nm was read on a microplate reader MPR A4i (Tosoh, Tokyo, Japan) as the absorbance at 0 min. After 20 min at room temperature, the absorbance difference was read again. An increase in the absorbance was recorded as the ability of the sample to reduce XTT (XTT reducibility).

2.5. DNP derivatisation of AR

The sample (200 μ l) was mixed in a test tube with 200 μ l of 5 mg/ml CuSO₄ and 800 μ l of 7 mM DNP in 2 M HCl and incubated for 1 h at room temperature. One millilitre of chloroform was added to this solution and the mixture was vigorously stirred. The chloroform layer containing the DNP derivative of oxidised AR (OAR-DNP) was then collected and subjected to HPLC analysis and purification.

2.6. HPLC conditions

HPLC analysis of OAR-DNP was performed using either of the two systems: Hitachi L-7100 pump, UV–vis detector L-4000 UV detector, and D-2500 chromato-integrator (Tokyo, Japan) or Shimadzu LC-10A pump, SPD-M10A diode array detector, SLC-10A system controller, and CLASS VP-5 software for data analysis (Kyoto, Japan). The HPLC conditions were as follows: column, Wakosil 5SIL (4.6 mm \times 250 mm, Wako Pure Chemical Industries); wavelength, 525 nm; mobile phase, hexane/chloroform/methanol (6.5:2.5:1); flow rate, 0.75 ml/min; injection volume, 15 µl.

2.7. Purification of OAR-DNP

Crude OAR-DNP was dissolved in 10 ml of chloroform, and applied to a silica gel column (10 mm \times 550 mm) packed with 10 g of Wakogel C300 gel (Wako Pure Chemical Industries), and eluted with 300 ml of hexane/chloroform/methanol (6.5:2.5:1). The OAR-DNP fraction, which could be differentiated by its orange colour, was collected and subsequently purified by normal-phase HPLC and reversed-phase HPLC to isolate OAR-DNP. The HPLC conditions were as follows: normal-phase HPLC; column, Cosmosil 5SL-II (10 mm \times 250 mm, Nacalai Tesque, Inc.); wavelength, 525 nm; mobile phase, hexane/chloroform/methanol (6.5:2.5:1); flow rate, 3.0 ml/min; injection volume, 100 µl: reversed-phase HPLC; column, Cosmosil 5C₁₈-MS-II (10 mm \times 250 mm); wavelength, 525 nm; mobile phase, 60% methanol; flow rate, 2.0 ml/min; injection volume, 100 µl.

2.8. Instrument analyses

¹H NMR and ¹³C NMR spectra, including two-dimensional correlation spectra, were measured with a JEOL JNM-AL 400 (400 MHz) spectrophotometer (Tokyo, Japan). TMS was used as an internal standard. Letters s, d, t, q, and m represent singlet, doublet triplet, quartet, and multiplet, respectively, and coupling constants are given in Hz. The IR spectrum was recorded on IR Prestige-21 (Shimadzu Co.) by the liquid film method.

OAR-DNP derived from lactose-butylamine model system, ((S)-5,6-dihydroxy-2,3-bis(2,4-dinitrophenylhydrazono)hexanoic acid). ¹H NMR (CDCl₃) δ : 9.18 (d, *J* = 2.4, 1H, H-3 in DNP), 8.92 (d, *J* = 2.4, 1H, H-3' in DNP'), 8.61 (dd, *J* = 9.8 and 2.4, 1H, H-6' in DNP'), 8.51 (dd, *J* = 9.8 and 2.4, 1H, H-6 in DNP), 8.04 (d, *J* = 9.8, 1H, H-5' in DNP'), 7.91 (d, *J* = 9.8, 1H, H-5 in DNP), 4.27 (m, 1H, H-5 in OAR), 3.84 (dd, *J* = 11.2 and 3.6, 1H, H-6 in OAR), 3.68 (dd, *J* = 11.2 and 6.0, 1H, H-6 in OAR), 2.96 (m, 2H, H-4 in OAR). ¹³C NMR (CDCl₃) δ : 175.9 (s, C-1 in OAR), 147.8 (s, C-1' in DNP'), 144.7 (s, C-3 in OAR), 134.9 (s, C-2 in OAR), 133.7 (s, C-2' in DNP'), 133.1 (s, C-2 in DNP), 130.2 (d, C-6 in DNP), 128.1 (d, C-6' in DNP'), 125.8 (d, C-5' in DNP'), 122.8 (d, C-3 in DNP), 121.7 (d, C-3' in DNP'), 117.2 (d, C-5 in DNP), 69.4 (d, C-5 in OAR), 66.0 (t, C-6 in OAR), 29.5 (t, C-4 in OAR). IR ν_{MAX} (liquid film) cm⁻¹: 3400 (OH).

OAR-DNP derived from LL milk, ((S)-5,6-dihydroxy-2,3-bis(2,4-dinitrophenylhydrazono)hexanoic acid). ¹H NMR (CDCl₃) δ : 9.19 (d, *J* = 2.4, 1H, H-3 in DNP), 8.93 (d, *J* = 2.8, 1H, H-3' in DNP'), 8.61 (dd, *J* = 9.2 and 2.4, 1H, H-6' in DNP'), 8.51 (dd, *J* = 9.2 and 2.4, 1H, H-6 in DNP), 8.04 (d, *J* = 8.8, 1H, H-5' in DNP'), 7.91 (d, *J* = 9.6, 1H, H-5 in DNP), 4.22 (m, 1H, H-5 in OAR), 3.84 (dd, *J* = 11.2 and 3.6, 1H, H-6 in OAR), 3.69 (dd, *J* = 11.2 and 5.2, 1H, H-6 in OAR), 2.96 (m, 2H, H-4 in OAR). ¹³C NMR (CDCl₃) δ : 175.9 (s, C-1 in OAR), 130.2 (d, C-6 in DNP), 128.1 (d, C-6' in DNP'), 125.8 (d, C-5' in DNP'), 123.2 (d, C-3 in DNP), 121.7 (d, C-3' in DNP'), 117.1 (d, C-5 in DNP), 69.3 (d, C-5 in OAR), 66.0 (t, C-6 in OAR), 29.5 (t, C-4 in OAR).

3. Results and discussion

3.1. Postulated mechanism of DNP derivatisation

In a series of studies (Shimamura et al., 2000, 2004; Trang et al., 2011), the formation of AR during the Maillard reaction of lactose and the involvement of AR in the reduction of XTT had been elucidated using a model system consisting of a lactose and amino compound. However, direct demonstration of the presence of AR in milk had not been accomplished previously because of the difficulty in isolation of an intact AR from milk protein. AR is labile and hence not suitable for enzyme hydrolysis and multiple extraction steps (Wühr et al., 2010). In order to achieve the practical application of the XTT assay in food industries, it is essential to demonstrate the presence of AR in milk. Hence, in this study, we attempted to isolate AR from milk protein using a DNP reagent, a common labelling reagent for the carbonyl group (Levine et al., 1990). Shinohara, Fukumoto, Tseng, Inoue, and Omura (1974) successfully performed DNP derivatisation of AR generated from triose reductone and amino acid (TR-AA); wherein, TR-AA was first treated with \mbox{Cu}^{2+} to produce mesoxal-dialdehyde with 3 carbonyl groups followed by addition of DNP, which formed a DNP-hydrazone derivative that reacted with two of the carbonyl groups. In this study, we applied the above-mentioned protocol to the AR generated using the milk and the lactose-butylamine model solution. The postulated mechanism of DNP derivatisation of AR in milk is shown in Fig. 1. OAR-DNP was subjected to HPLC, NMR, and IR.

3.2. DNP derivatisation of AR derived from the model system

The DNP derivatisation was applied to AR generated in the lactose-butylamine model solution heated at 100 $^\circ C$ for 15 min and

the DNP derivative obtained was analysed by HPLC. For comparison, DNP derivatisation was performed in the absence (Fig. 2A) and presence (Fig. 2B) of Cu^{2+} . In Fig. 2B, the new peaks (peak 2, 3, and 6) were recognised in comparison with Fig. 2B. These peaks were thought to be generated by the addition of Cu^{2+} . Among them, it is evident that the peak 6 at 21 min was markedly increased by the addition of Cu^{2+} . In the spectrum of peak 6, absorption maxima occurred at around 450 and 525 nm, which are characteristic of the DNP derivative, were detected (Jones, Holmes, & Seligman, 1956). In addition, the crude AR prepared from the lactose-butylamine model solution using ethyl acetate was also treated with DNP and subjected to HPLC analysis. Peak 6 at 21 min was also detected in the DNP derivative of the crude AR chromatogram. Given these results, there is a strong possibility that peak 6 corresponds to OAR-DNP.

The effect of heating time on the area of peak 6 at 21 min was examined using the lactose-butylamine model solution heated at 100 °C for 0–15 min (Fig. 3). An increase in the area of peak 6 was dependent on the heating time. It has been reported that AR is an XTT reducing-substance (Shimamura et al., 2000, 2004), hence, the correlation between XTT reducibility and the area of peak 6 was investigated, and a significant linear correlation was identified (r = 0.992, n = 4, p < 0.05). This result strongly suggests that peak 6 is derived from AR and corresponds to OAR-DNP.

3.3. DNP derivatisation of AR derived from a lactose-milk protein model solution

In the milk, milk proteins such as casein, α -lactalbumin, and β lactoglobulin are associated with both the Maillard reaction and generated AR bound to amino groups in the milk proteins. As DNP derivatisation was found possible for AR derived from a lactose-butylamine model system, we next tried to apply this method to AR derived from lactose and α -lactalbumin. After DNP derivatisation in the absence and presence of Cu²⁺, HPLC analyses were performed. In the HPLC chromatogram, the peak at 21 min was increased by the addition of Cu²⁺ as well as the above mentioned result of lactose-butylamine model system (data not shown). From this result, it was presumed that DNP derivatisation in the presence of Cu²⁺ was effective in liberating AR from milk protein and forming OAR-DNP.

3.4. DNP derivatisation of AR derived from LL milk

The DNP derivatisation using Cu²⁺ was applied to LL milk and the derivative obtained was analysed by HPLC. However, contrary to our expectations, the peak at 21 min, which was thought to be corresponding to OAR-DNP, was not observed in the chromatogram. This unfortunate consequence was believed to have occurred



Fig. 1. Postulated mechanism of DNP derivatisation of AR in milk.



Fig. 2. Chromatograms of the DNP derivative of a lactose-butylamine model solution heated at 100 °C for 15 min. DNP derivatisation was performed in the absence (A) and presence (B) of Cu^{2+} .



Fig. 3. Effect of heating time on the area of peak 6 at 21 min in the chromatogram of DNP derivative from lactose-butylamine model system heated at 100 $^\circ C$ for 0–15 min.

because of the very low AR content in LL milk. Thus, the concentration of the DNP derivative used for HPLC analysis was increased 100 times by scaling up the reaction for the derivatisation and the concentration of the DNP derivative. As a result, the peak at 21 min was then detected in the HPLC chromatogram, as can be seen in model system of lactose-butylamine and lactose- α -lactalbumin. There is thus a strong possibility that DNP derivatisation was available for the milk and AR was generated in the LL milk.

3.5. Structural determination and assignment of NMR signals of OAR-DNP derived from the lactose-butylamine model solution

Since the compound corresponding to peak 6 in Fig. 2B was expected to be OAR-DNP and the peak which showed the same retention time with peak 6 in Fig. 2B was detected in the LL milk and lactose- α -lactalbumin model system, its structural elucidation was performed. To purify the compound corresponding to peak 6 derived from the DNP derivative of a model system of lactose and butylamine, it was sequentially subjected to silica gel open column chromatography, preparative normal-phase HPLC, and preparative reversed-phase HPLC. The purified compound (4.2 mg) corresponding to peak 6 was then analysed by NMR.

The ¹H NMR and ¹³C NMR signals of peak 6 derived from lactose-butylamine model system are listed in Table 1 and Table 2, respectively. In ¹³C NMR spectrum, the fourteen peaks were detected in the region from 117.2 to 147.8 ppm. By DEPT spectrum, six signals at 117.2–130.2 ppm were revealed as methine carbons. Thus, these six signals at 117.2–130.2 ppm were assumed to be carbons in a benzene ring in DNP and DNP'. In the ¹H NMR spectrum, the signals corresponding to 6H were recognised at 7.91– 9.18 ppm and they showed two sets of a typical capping pattern of 1,2,4-substituted benzene ring. From these results, the introduction of two molecules of DNP was identified. Assignments of all carbons and protons in DNP and DNP' were established based on

HMOC and HMBC, as can be seen in Tables 1 and 2. In the ¹³C NMR spectrum, six signals were observed other than the signals assigned to DNP and DNP'. Among them, the signal at 175.9 ppm was assumed to be a carboxylic group. The signals at 134.9 ppm and 141.4 ppm were sp^2 carbons, that is, quaternary carbons confirmed by the DEPT spectrum. The signals at 69.4 ppm (CH) and 66.0 ppm (CH₂) were shifted downfield, indicating the binding to oxygen. The remaining signal at 29.5 ppm corresponded to CH₂. In addition, judging from the carping pattern with hydrogen and H-H COSY, the -CH2-CHOH-CH2OH structure in OAR-DNP (C-4, C-5, and C-6 in OAR) was revealed. The sp^2 quaternary carbons at 134.9 and 141.4 ppm were assigned to C=N (C-2 and C-3, respectively), based on the reaction pattern of DNP and AR. All signals in the ¹H NMR spectrum were then assigned, as shown in Table 1, based on the H-H COSY and HMQC. Although the signal at 175.9 ppm was assumed to be a carboxylic group of C-1 in OAR, further confirmation was required. To further clarify the presence of the carboxylic group in OAR-DNP, an IR analysis was performed. In the IR spectrum, the absorption corresponding to the carboxylic group was identified in the region of 2800–3400 cm⁻¹. This result strongly suggested the presence of a carboxylic group in OAR-DNP. In conclusion, all of the structure and formation of OAR-DNP were demonstrated, as proposed in Fig. 1. From these results, it was suggested that the DNP derivatisation of AR progressed in a predictable way, as shown in Fig. 1.

Table 1

 $^1\mathrm{H}$ NMR data for OAR-DNP from lactose-butylamine model system and LL milk in CDCl₃.^a

Position	δu (I Hz)					
robition						
	From lactose-butylamine	From LL milk				
OAR-1						
2						
3						
4	2.96 (m, 2H)	2.96 (m, 2H)				
5	4.27 (m, 1H)	4.22 (m, 1H)				
6	3.84 (dd, J = 11.2, 3.6, 1H)	3.84 (dd, J = 11.2, 3.6, 1H)				
	3.68 (dd, <i>J</i> = 11.2, 6.0, 1H)	3.69 (dd, <i>J</i> = 11.2, 5.2, 1H)				
DNP ^b -1						
2						
3	9.18 (d, <i>J</i> = 2.4, 1H)	9.19 (d, <i>J</i> = 2.4, 1H)				
4						
5	7.91 (d, <i>J</i> = 9.8, 1H)	7.91 (d, J = 9.6, 1H)				
6	8.51 (dd, <i>J</i> = 9.8, 2.4, 1H)	8.51 (dd, J = 9.2, 2.4, 1H)				
DNP ^b -1/						
2'						
3′	8.92 (d. <i>I</i> = 2.4, 1H)	8.93 (d. <i>I</i> = 2.8, 1H)				
4′						
5′	8.04 (d, <i>J</i> = 9.8, 1H)	8.04 (d, <i>J</i> = 8.8, 1H)				
6′	8.61 (dd, <i>J</i> = 9.8, 2.4, 1H)	8.61 (dd, J = 9.2, 2.4, 1H)				

^a Chemical shits are given in parts per million.

^b Assignments of suit of DNP and DNP' signal may be interchanged.

Table 2								
13C NMR	data	for OAR-DNP	from	lactose-butylamine	model	system	and LL	milk in
CDCl ₃ . ^a				-		-		

Position	δ_{c}				
	From lactose-butylamine	From LL milk			
OAR-1	175.9 (s, C=O)	175.9 (s, C=O)			
2	134.9 (s, C)				
3	141.4 (s, C)				
4	29.5 (t, CH ₂)	29.5 (t, CH ₂)			
5	69.4 (d, CH)	69.3 (d, CH)			
6	66.0 (t, CH ₂)	66.0 (t, CH ₂)			
DNP ^b -1	144.7 (s, C)				
2	133.1 (s, C)				
3	122.8 (d, CH)	123.2 (d, CH)			
4	141.8 (s, C)				
5	117.2 (d, CH)	117.1 (d, CH)			
6	130.2 (d, CH)	130.2 (d, CH)			
DNP' ^b -1'	147.8 (s, C)				
2′	133.7 (s, C)				
3′	121.7 (d, CH)	121.7 (d, CH)			
4′	142.1 (s, C)				
5′	125.8 (d, CH)	125.8 (d, CH)			
6′	128.1 (d, CH)	128.1 (d, CH)			

^a Chemical shits are given in parts per million.

^b Assignments of suit of DNP and DNP' signal may be interchanged.

3.6. Demonstration of the formation of AR in milk

To demonstrate the formation of OAR-DNP derived from AR in the milk, NMR analysis was performed. In this case, the DNP derivatisation was conducted using LL milk reheated at 130 °C for 15 min in order to accelerate the formation of AR because the content of DNP derivatives from LL milk was very low. Reheating of milk could increase the content of the DNP derivatives by 40 times, judging from the peak area in the HPLC chromatogram. To purify the DNP derivative from LL milk, preparative normal-phase HPLC and preparative reversed-phase HPLC were sequentially performed. Finally, the purified substance (4.2 mg) was obtained and analysed by NMR. Thus, from 980 ml of LL milk, 4.2 mg of pure DNP derivative was obtained.

The NMR signals of the DNP derivative from LL milk in CDCl₃ are listed in Table 1 (¹H NMR) and Table 2 (¹³C NMR). Although there were some extraneous signals in the NMR spectra, the signals derived from the DNP derivative were clearly identified. The ¹H NMR signals and the H-H COSY spectrum of the DNP derivative from LL milk were nearly the same as those of OAR-DNP from the lactosebutylamine model system. In the ¹³C NMR spectrum, the signals of quaternary carbons other than the carboxylic group (C-1 in OAR) were not clear because of insufficient NMR sample purity. However, the other signals of the ¹³C NMR spectrum were almost the same as those of OAR-DNP from the lactose-butylamine model system shown in Fig. 1. Consequently, the recognised NMR signals shown in Tables 1 and 2 could be assigned to OAR-DNP (excluding the guaternary carbons). These results revealed that the OAR-DNP from milk was identical to that from the lactose-butylamine model system. Thus, this is the first report on direct demonstration of the presence of AR in milk.

At present the formation of AR in milk has been explained based on indirect demonstration only using model systems of the Maillard reaction. In the present study, the combination of DNP, which is a common carbonyl-labelling reagent, with Cu²⁺ was applied to AR formed in a lactose and amino compound model system and LL milk. By using this derivatising reaction, AR was liberated from milk protein and derivatised with DNP. Direct demonstration was achieved by structural elucidation of OAR-DNP, where the DNP derivative of AR was oxidised by Cu²⁺.

Since a direct demonstration of the presence of AR in milk has now been provided, the previously reported functionalities of AR such as its antioxidative activity (Pischetsrieder, Rinaldi et al., 1998), protective effect against photo-degradation of riboflavin (Trang et al., 2008), and its antimicrobial activity against *H. pylori* (Trang et al., 2009) now has a reality.

Detailed investigations of the relationship between heating conditions and the formation of AR in manufacturing of milk and dairy products may now be pursued. Further information on this would allow indexing of the heating process and also would help improve the functional qualities of various foods.

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