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Oxidative Damage of Protein Induced by the Amadori Compound–Copper Ion System

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Generation of oxygen radicals from the browning solution of glucose and amino acids in the presence of copper ion and their oxidative action on protein were investigated in detail. Reaction mixtures of the browning solution, bovine serum albumin (BSA) and copper(II), incubated for 24 h at 40 °C under aerated conditions markedly accelerated the oxidative depolymerization of BSA and the oxidative degradation of its histidine residue. Among the many amino acids used in these browning solutions, β-alanine, 4-aminobutyric acid, and 6-aminocaproic acid containing solutions exhibited very high activities for oxidative degradation of *N*-benzoyl-L-histidine (Bz-His). The active product in the browning solution of glucose and β-alanine was estimated to be an Amadori compound (AC), 1-deoxy-1-β-alanino-D-fructose.

Amino–carbonyl reaction, e.g., the Maillard reaction, between reducing sugars and amino acids or proteins produces the brown color and specific flavor in food processing that contribute to the development of good qualities in food. On the other hand, the formation of Amadori compounds from glycated proteins in the human body is thought to promote several diseases associated with diabetes and aging (Pongor et al., 1984; Brownlee et al., 1986; Cerami et al., 1987). In the amino–carbonyl reaction, many dicarbonyl compounds and keto amines are produced as intermediates of the browning reaction (Feather, 1985), and then these intermediates must be reversibly transformed to enediols and enaminols, respectively, in the reaction processes. Those compounds may easily form complexes with metal ions. The formation of such complexes under aerated conditions may cause one-electron transfer from enediols and enaminols to an oxygen molecule through metal ions to give a superoxide anion radical (O_2^-) (Fridovich, 1979). In general, a solution containing ascorbic acid and metal ion is well-known as a generating system for active oxygen, which means a series of oxygen radicals formed by a one-electron reduction of oxygen. The interaction of the ascorbic acid–metal ion system and some biological substrates spontaneously produced the oxidation and oxidative changes in their molecules, that is, protein (Marx and Chevion, 1986; Garland et al., 1986; Kano et al., 1987; Uchida and Kawakishi, 1988b), enzymes (Shinar et al., 1983; Levine, 1983; Nakanishi et al., 1985), polysaccharides (Matsumura and Pigman, 1965; Wong et al., 1981; Uchida and Kawakishi, 1986b, 1987), and DNA (Shinohara et al., 1983; Aronovitch et al., 1987).

The Amadori compound formed in glycated protein, N^{ϵ}-fructosyllysine, is oxidatively degraded in phosphate buffer under aerated conditions to give N^{ϵ}-(carboxymethyl)lysine residue (Ahmed et al., 1986). Kashimura et al. (1986) reported the cleavage of DNA strand and virus inactivation by the action of some Amadori compounds with copper ion.

With this background, it is considered that reaction mixtures of glucose and amino acids may generate some oxygen radicals by addition of metal ion and these radical species will oxidatively attack some biological and food constituents, if they exist together with the radical-generating system. By using the ascorbic acid–copper ion system as an oxygen radical generator, we have investigated the oxidative depolymerization of polysaccharides (Uchida and Kawakishi, 1986b, 1987) and protein (Uchida and Kawakishi, 1988b) and the oxidative degradation of oligosaccharides (Uchida and Kawakishi, 1988a) and histidine residue in protein (Uchida and Kawakishi, 1986a).

In this paper, we report the generation of active oxygen from the browning reaction mixture–copper ion system and its oxidative damage to protein and also prove the inducer of this oxidation to be mainly Amadori compounds.

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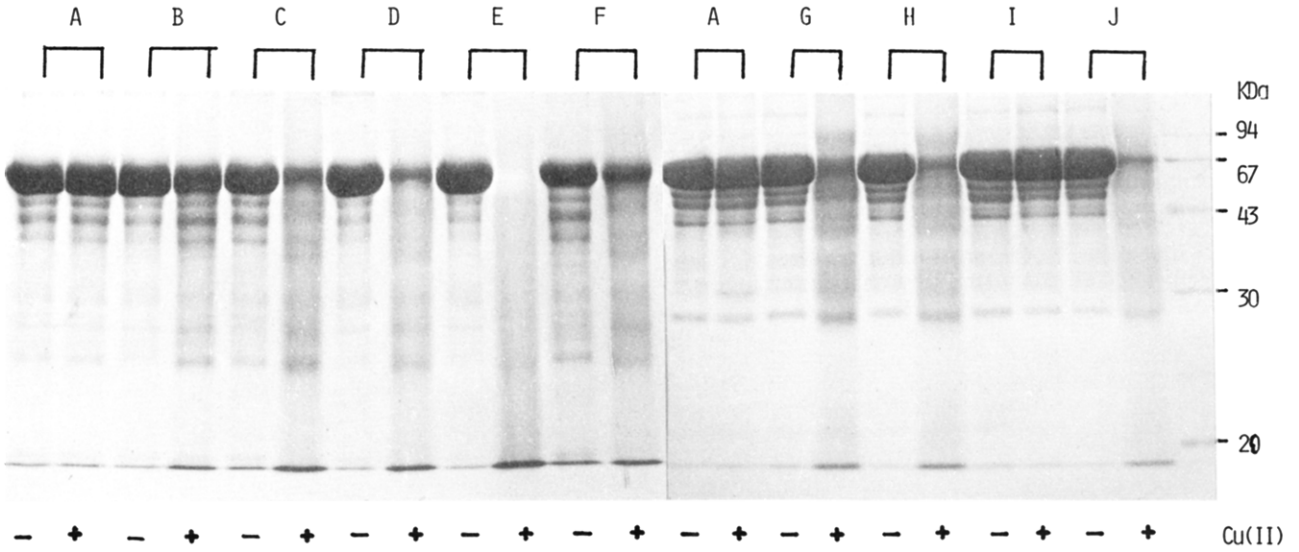

Figure 1. SDS-PAGE of BSA treated with several browning solution–copper(II) systems. The reaction mixtures were incubated at 40 °C for 24 h. Browning solutions: A, none (untreated BSA); B, glucose; C, glucose and glycine; D, glucose and α-alanine; E, glucose and β-alanine; F, glucose and DL-α-alanine; G, glucose and arginine; H, glucose and arginine hydrochloride salt; I, glucose and histidine hydrochloride salt; J, glucose and lysine hydrochloride salt.

EXPERIMENTAL SECTION

Reagents. Fructose–β-alanine (1-deoxy-1-β-alanino-D-fructose) and fructose–*p*-toluidine (1-deoxy-1-*p*-toluidino-D-fructose) were prepared by the procedures of Anet (1957) and Weygand (1940), respectively. All other reagents were of the best grades commercially available.

Preparation Conditions of the Browning Mixtures of Glucose–Amino Acids. Equimolar amounts of D-glucose and amino acid, 0.5 M, were dissolved in 50 mL of water, the solutions were heated in boiling water for 2 h under reflux, and the reaction mixture was cooled to an ice bath.

Reaction Conditions. All reactions were carried out in 10-mL total volume with BSA (0.4 mg/mL) and each browning mixture (50 μL) or Amadori compound (5 mM) in the absence or presence of copper(II) (50 μM) in phosphate buffer (50 mM, pH 7.2) at 40 °C. With use of *N*-benzoylhistidine (Bz-His), instead of BSA, the composition of the reaction mixture was 100 μL of browning mixture or Amadori compound (1 mM) and Bz-His (0.2 mM) in the absence or presence of copper(II) (100 μM). The reactions were started by addition of browning mixture or Amadori compound and were stopped by the addition of EDTA.

Determination of Bz-His and Fructose–β-Alanine. Bz-His was determined by the HPLC method (Uchida and Kawakishi, 1986). Amadori compound, fructose–β-alanine, was determined by HPLC on a Develosil NH_2-5 column (4.6 × 150 mm) and eluted with acetonitrile–water (70/30, v/v) at a flow rate of 2 mL/min. The elution was monitored at 210 nm.

Fractionation of the Browning Mixture of Glucose and β-Alanine. The browning mixture was dialyzed against water, and the dialysate was fractionated with a Dowex 50X4 (H form, 1.6 × 62 mm) column into two parts, the unadsorbed and adsorbed parts, and eluted with water and 1 M ammonia, respectively.

SDS–Polyacrylamide Gel Electrophoresis (SDS-PAGE). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 10% acrylamide gel was performed according to Laemmli (1970). Gel sheets were stained with a solution of 0.25% Coomasie Brilliant Blue R-250 in water–2-propanol–acetic acid (5:5:1) and destained with 7% acetic acid containing 5% methanol. Decrease of the BSA band at the position of near 67 kDa on an SDS-PAGE gel sheet was quantitively determined by scanning densitometry.

Amino Acid Analyses. The reaction solutions with BSA and browning mixture or Amadori compound were treated with 6% TCA solutions, and the precipitated protein was hydrolyzed in a sealed tube with 6 N HCl at 120 °C for 24 h. The hydrolysates were concentrated to dryness, dissolved in dilute HCl solution (pH 2.2), and then submitted to an amino acid analyzer (JEOL, Model JLC-6AH).

Table I. Changes in Amino Acid Composition of BSA Treated with the Glucose–β-Alanine–Copper(II) System[a]

	incubation time, h				
	0	2	4	8	24
Asp	10.1	10.5	10.3	11.3	10.8
Thr	5.9	6.1	5.7	6.3	5.7
Ser	3.9	4.1	4.6	4.1	4.7
Glu	13.9	14.0	14.0	14.9	14.4
Pro	7.3	7.4	8.4	8.1	8.3
Gly	3.4	3.5	3.3	3.7	3.5
Ala	8.0	8.1	8.0	8.3	8.0
Cys	2.8	2.7	2.6	2.4	2.4
Val	6.6	6.9	6.6	7.2	7.0
Met	0.6	0.6	0.7	0.5	0.5
Ile	2.5	2.6	2.7	2.3	2.4
Leu	10.2	10.0	10.8	9.7	10.5
Tyr	3.5	3.4	3.5	3.3	3.4
Phe	4.9	5.2	5.5	5.4	5.5
His	3.0	2.2	1.7	1.0	0.5
Lys	9.1	8.6	7.4	7.4	8.2
Arg	4.3	4.1	4.2	4.1	4.2

[a] Amino acid compositions are expressed in molar ratios.

RESULTS

Oxidative Damage of BSA with Several Browning Reaction Mixture–Copper(II) Systems. The SDS-PAGE of BSA treated with the browning solution of glucose–amino acid–copper(II) is shown in Figure 1, which reveals the disappearance of BSA bands in the presence of copper ion in all glucose–amino acid systems besides glucose–histidine. However, any changes in BSA were not observed in the absence of copper ion. BSA at 67 kDa was oxidatively depolymerized to lower molecular weight polypeptides (less than 14.4 kDa), and moreover, the polymer bands above 67 kDa were not detected. Among the amino acids used in this experiment, β-alanine-, lysine-, and ariginine-containing browning solutions caused the most remarkable changes in the BSA molecule, and the histidine-containing solutions did not cause any change in protein. BSA treated with the glucose and β-alanine–copper(II) system was hydrolyzed with 6 N HCl in a sealed tube over time, and amino acid analyses were performed. The data (Table I) showed a marked decrease in histidine residue, e.g., about 83% decrease over 24 h, but other amino acids did not appreciably

Table II. Oxidative Degradation of Benzoylhistidine Treated with Browning Solutions in the Absence and Presence of Copper(II)

browning system	residual benzoylhistidine, %	
	0 mM copper(II)	0.1 mM copper(II)
Glc	100	100
Glc + Gly	100	75.0
Glc + Ala	100	74.0
Glc + β-Ala	100	8.6
Glc + DL-α-Ala	100	79.1
Glc + Pro	100	92.7
Glc + Ser	100	78.0
Glc + Glu	100	37.1
Glc + Asp	100	71.4
Glc + Arg	100	74.0
Glc + His	100	100
Glc + Lys	100	58.5
Glc + GABA[a]	95.5	1.7
Glc + EACA[b]	94.9	2.3

[a] 4-Aminobutyric acid. [b] 6-Aminocaproic acid.

change, except for a small decrease in lysine. From these results, it was proven that these browning solution–copper ion systems caused both oxidative depolymerization of protein and selective degradation of its histidine residue.

Oxidative Degradation of *N*-Benzoylhistidine. To make clear the oxidative degradation of histidine residue in BSA, the action of the browning solution of glucose and amino acid–copper(II) on Bz-His, which was a suitable substrate for the reaction of active oxygens from the ascorbic acid–copper(II) system (Uchida and Kawakishi, 1986, 1988), was examined here. The browning solutions with glucose were prepared from ten kinds of α-amino acids and three kinds of other amino acids, i.e., β-alanine, 4-aminobutyric acid (GABA), and 6-aminocaproic acid (EACA). As shown in Table II, most of browning mixtures, besides proline- and histidine-containing solutions, induced oxidative degradation of Bz-His in the presence of copper ion. Especially, the oxidative degradation was most remarkable in the case of β-alanine, GABA, and EACA.

Effects on the Degradation of Benzoylhistidine with the Glucose–β-Alanine–Copper(II) System and Its Fractionated Components. From the above results, it was evident that the β-alanine-containing browning mixture exhibited strong activity for oxidative damage of BSA and Bz-His. Then, to characterize the active component in the browning mixture, the browning solution from glucose and β-alanine was fractionated. The solution was dialyzed, and the dialyzable materials were fractionated by a Dowex 50X4 column into a nonadsorbed part and an adsorbed part and eluted with 0.6 N ammonia. These fractions were assayed by addition of copper(II) for their degradation activities on Bz-His (Figure 2A). Among them, the fraction eluting with ammonia, that is, the part adsorbed on Dowex 50X4 resin, exhibited strong activity as well as the browning solution, and this fraction contained mainly unreacted β-alanine and Amadori compound. This activity was then compared with that of a typical Amadori compound, 1-deoxy-1-β-alanino-D-fructose, synthesized from D-glucose and β-alanine. As shown in Figure 2B, the activity for the oxidative degradation of Bz-His with the glucose–β-alanine–copper(II) system seemed to be almost identical with that of the Amadori compound. The Amadori compound was produced as an intermediate in the initial stage of amino–carbonyl reaction through a Schiff-type base, and a relationship between the formation of the Amadori compound and the oxidative degradation of Bz-His in the presence of copper(II) was investigated with glucose and β-alanine. The results are shown in Figure 3. A clear relation was shown between both these phenomena. In particular, the browning solution heated more than 3 h decomposed Bz-His nearly completely. For the browning solution heated 6 h, the yield of Amadori compound reached about 35% of the amount of glucose consumed. Moreover, the formation of fructose–β-alanine in the heating solution was proportional to the development of browning. From these data, it is concluded that the principal product relating to the oxidative damage of protein and its histidine residue with copper ion may be Amadori compound in the browning solution. Histidine was degraded to some oxidative products not identified in this experiment; however, the oxidative products of Bz-His by the ascorbate–copper(II) system will be published in another place. Related to those, Yong and Karel (1979) reported the oxidative degradation of its imidazole ring by the associated lipid peroxidation.

Since these reactions might arise from some oxygen radicals formed through the interaction between the Ama-

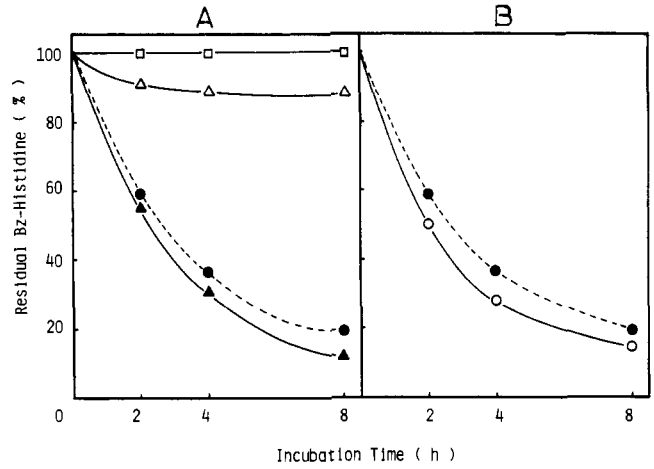

Figure 2. Oxidative degradation of benzoylhistidine induced by fractionated browning solutions of glucose and β-alanine in the presence of copper(II): (●) browning solution of glucose and β-alanine; (□) nondialyzable fraction of browning solution; (△) nonadsorbed part on Dowex 50X4 column of dialyzable fraction in browning solution; (▲) adsorbed part (eluted with 0.6 N ammonia) on Dowex 50X4 column of dialyzable fraction in browning solution; (○) 1-deoxy-1-β-alanino-D-fructose (fructose–β-alanine).

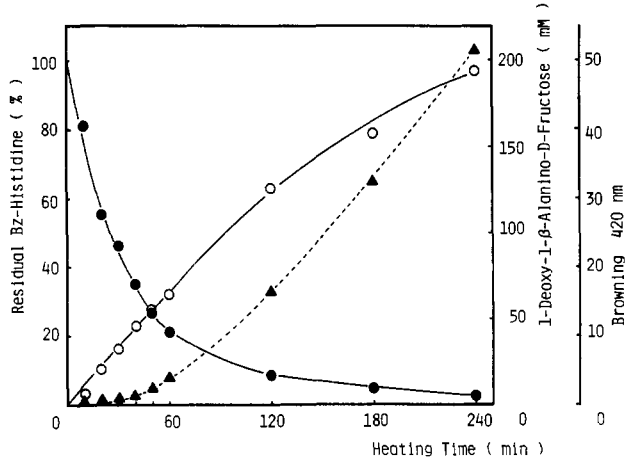

Figure 3. Relationship between formation of Amadori compound and oxidative degradation of benzoylhistidine in the glucose–β-alanine–copper(II) system: (○) Amadori product; (●) benzoylhistidine; (▲) browning.

Table III. Effects of Several Radical Scavengers on Oxidative Degradation of BSA with the Fructose–β-Alanine–Copper(II) System

scavenger	inhibition, %
none	0
EDTA[a] (500 μM)	100
DTPA[b] (500 μM)	98.7
GGH[c] (500 μM)	56.4
mannitol (10 mM)	0
urea (10 mM)	2.3
SOD[d] (500 units/mL)	10.1
catalase (500 units/mL)	90.3

[a] Ethylenediaminetetraacetic acid. [b] Diethylenetriaminepentaacetic acid. [c] Glycylglycyl-L-histidine. [d] Superoxide dismutase.

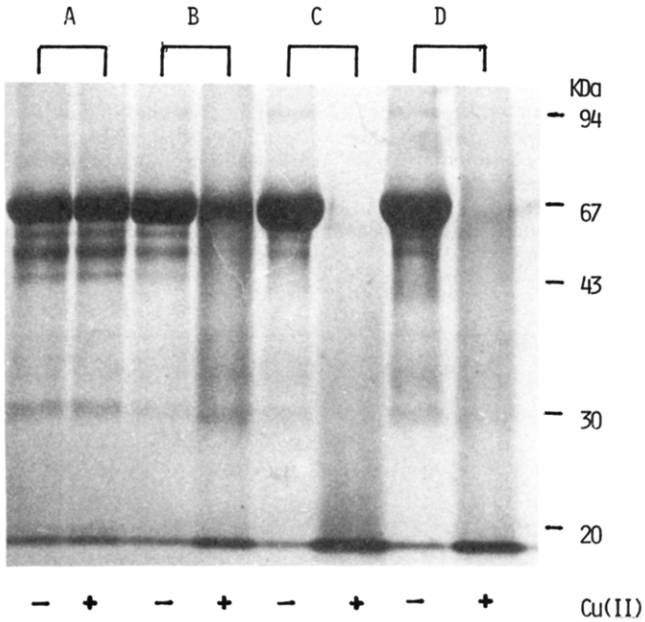

Figure 4. SDS-PAGE of BSA treated with fructose and Amadori compounds–copper(II) systems: A, untreated BSA; B, fructose; C, fructose–β-alanine; D, fructose–p-toluidine. Fructose was used in 5 mM concentration, and other conditions were described in the Experimental Section.

dori compound–copper(II)–histidine complex and oxygen, the inhibitory effects of radical scavengers and enzymes relating to the decomposition of these active oxygens on the oxidative depolymerization of BSA with these systems were studied. As an Amadori compound, fructose–β-alanine was used and the inhibitory effects of several additives were determined by densitometric measurement of the decreases in 67-kDa band (BSA) on the SDS-PAGE gel sheet. Table III showed that metal-chelating agents, EDTA and DTPA, and catalase completely depressed the oxidative degradation of BSA by the above system. However, these oxidative reactions were little affected by the addition of mannitol, a hydroxyl radical scavenger, and slightly by SOD.

Oxidative Damage of BSA with the Amadori Compound–Copper(II) System. Two Amadori compounds, fructose–β-alanine and fructose–p-toluidine, synthesized from glucose and β-alanine and from glucose and p-toluidine, respectively, were used. The SDS-PAGE of the reacted BSA with two Amadori compounds and fructose–copper(II) systems are shown in Figure 4. Similar to Figure 1, BSA was notably depolymerized with the oxidative action of Amadori compound–copper(II) systems. These results indicated that the oxidative action of the browning reaction mixture–copper(II) systems could be replaced with that of the Amadori compound–copper(II) system.

DISCUSSION

The most important intermediates in amino–carbonyl reactions are Amadori compounds (AC), which are spontaneously converted to melanoidin and many other carbonyl compounds. Among them, AC and α-dicarbonyl compounds exist in partially isomerized forms, enaminols and enediols, respectively, which easily form complexes with metal ions. The formation of these complexes under aerated conditions naturally causes a one-electron transfer to the oxygen molecule from enaminol and enediol to give a superoxide anion radical (O_2^-), similar to the ascorbate–copper(II) system (Levine, 1983; Uchida and Kawakishi, 1986–1988). Active oxygen radicals originating in O_2 formed from the ascorbate–copper(II) system cause oxidative damage to other biological constituents. On the reaction species in this system, there were large differences between polysaccharide and protein as targets; that is, the oxidative depolymerization of the former arose from hydroxyl radical (Uchida and Kawakishi, 1986b, 1987, 1988a), but that of the latter did not (Uchida and Kawakishi, 1988b). However, Hunt et al. (1988) reported the hydroxyl radical production from the protein exposed to glucose in the presence of copper ion.

To determine the radical species participating in this oxidation reaction using the AC–copper(II) system, several inhibitors were applied to the Amadori compound–copper(II) system with Bz-His as a target material. Metal-chelating agents, EDTA, DTPA, and glycylglycylhistidine, and catalase completely inhibited this oxidation reaction. This means that copper ion strongly participates in this reaction and the formation of hydrogen peroxide is required for the progress of this oxidation reaction. Copper(II) may be a key substance for the complex formation between AC and BSA, and via copper ion, a one-electron transfer to a protein or oxygen molecule from AC will easily occur. The former case of electron transfer may be a one-electron reduction of a specific amino acid residue, histidine, in BSA to give the imidazole radical, which will be oxidatively degraded by the addition of oxygen. The latter case must be followed by the formation of O_2^-, and then this oxidant or its relating radicals will attack the specific site in protein molecule near the place of complex formation. In this case, the oxidation must be inhibited with the addition of SOD and some radical scavengers. However, the reaction was not depressed with these additives as shown in Table III. Two oxidation processes of BSA by the action of the AC–copper(II) system, the depolymerization of a protein molecule and the oxidative degradation of its histidine residue, were thought to be promoted through the above radical reaction processes; however, the radical species in this reaction and the radical reaction mechanism are still unknown.

Moreover when AC in the above oxidation reaction acts as a strong electron donnor, AC itself must be oxidized automatically. Ahmed et al. (1986) reported the oxidative degradation of N^{ϵ}-fructosyllysine in phosphate buffer at 37 °C to N^{ϵ}-(carboxymethyl)lysine and erythronic acid under aerated conditions. We have also studied the oxidative products of AC and identified β-alanine from fructose–β-alanine (data not shown), but a sugar moiety as an oxidative product has not yet been detected.

Registry No. L-His, 71-00-1; Bz-His, 5354-94-9; L-Lys, 56-87-1; Gly, 56-40-6; Ala, 56-41-7; β-Ala, 107-95-9; DL-α-Ala, 302-72-7; L-Pro, 147-85-3; L-Ser, 56-45-1; L-Glu, 56-86-0; L-Asp, 56-84-8; L-Arg, 74-79-3; GABA, 56-12-2; EACA, 60-32-2; O_2, 7782-44-7; copper, 7440-50-8; D-glucose, 50-99-7; 1-deoxy-1-β-alanino-

D-fructose, 37721-43-0; 1-deoxy-1-*p*-toluidino-D-fructose, 5469-72-7.

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