Carbohydrate Research, 59 (1977) 363–377

THE REACTION OF SODIUM D-GLUCURONATE WITH AN L-LYSINE-CONTAINING PEPTIDE

YASUKO TAKEDA,
Research Laboratories, Chugai Pharmaceutical Co., Ltd., Toshima-ku, Tokyo 171 (Japan)
YOSHIMASA KYOGOKU,
Institute for Protein Research, Osaka University, Suita, Osaka 565 (Japan)
AND MORIZO ISHIDATE
Tokyo Biochemical Research Institute, Toshima-ku, Tokyo 171 (Japan)
(Received July 7th, 1976; accepted for publication in revised form, February 14th, 1977)

ABSTRACT

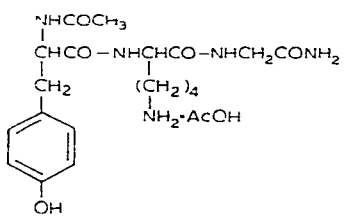
The reaction of sodium D-glucuronate with a synthetic peptide, AcTyrLysGlyNH_2 acetate, under physiological conditions, gave as major product the sodium salt of AcTyr-*N*-(D-*arabino*-5-carboxy-2,3,4,5-tetrahydroxy-1-pentenyl)-*N*-(D-*arabino*-5-carboxy-3,4,5-trihydroxy-2-oxopentylidene)LysGlyNH_2 (**2**). The structure was elucidated on the basis of p.m.r., ^{13}C-n.m.r., i.r., and u.v. spectra, and pH titration. Compound **2** is the product of oxidation of the sodium salt of AcTyr-*N*,*N*-bis(D-*arabino*-5-carboxy-2,3,4,5-tetrahydroxy-1-pentenyl)LysGlyNH_2, the bis-enol form of the di-D-fructuronic acid peptide obtained through the Amadori rearrangement. A new type of condensation that gives a product having a conjugated enol–keto–immonium group might take place when D-glucuronic acid reacts with peptides or proteins containing a lysine residue.

INTRODUCTION

D-Glucuronic acid may inactivate some viruses[1–3] and greatly reduce the toxicity of bacterial exotoxins[4–8], while leaving the antigenicity virtually unimpaired. These phenomena may be explained by assuming that the biologically active amino groups of toxins or virus proteins are modified chemically by D-glucuronic acid. A study of the reaction of D-glucuronic acid with ovalbumin[9,10] and with a purified protein derivative (PPD) of tuberculin[11–13] has shown that the acid reacts with the ω-amino groups of the basic amino acid residues. As a model reaction, AcTyrLysGlyNH_2 acetate (**1**), a synthetic peptide having an ω-amino group in the L-lysyl residue, was treated with sodium D-glucuronate, and the reaction product (**2**) was characterized.

RESULTS AND DISCUSSION

Compound **1** was incubated at 37° for 110 h with five times its weight of sodium D-glucuronate, the final sugar concentration being ~20%. The dark-yellow reaction mixture was fractionated by column chromatography on a CM-Sephadex C-25 column. The fractions that were eluted with distilled water were inadequately separated into three peaks, which gave u.v. absorption maxima at ~285 nm. They gave a positive reaction with Pauly's reagent but negatives one with ninhydrin and with sugar reagents on t.l.c. As the sugar moiety of the reaction products of sodium D-glucuronate with ovalbumin[9] and with PPD tuberculin[11] gave no specific color reactions, **1** was treated with sodium D-[6-^{14}C]glucuronate. After separation of the reaction mixture, the largest fraction showed ^{14}C radioactivity. Rechromatography of this fraction gave ^{14}C-labeled **2** as a homogeneous compound, as shown by t.l.c. (coincidence of a single, Pauly-positive spot with the radioactive spot). Compound **2** could not be crystallized owing to its highly hygroscopic character; the proportion of lysine was only 6% of that of tyrosine or glycine by amino acid analysis, indicating a modification of the lysyl residue. The molar ratio of sugar to peptide was ~2:1, in agreement with a mol. wt. estimated at ~790, on the basis of the tyrosine content, determined by colorimetry. The molecular formula could not be derived from the elemental analysis because **2** was fairly hygroscopic. The molar ratio of C to N was 31:5 by elemental analysis. As **1** contains 19 C and 5 N atoms in its free form, 12 C atoms should be ascribed to the sugar moiety, indicating the presence of two hexose residues. The presence of two carboxyl groups suggests two sugar residues where the uronic acid skeleton is retained. One equiv. of Na ion was found by flame analysis after neutralization of the two carboxyl groups, which indicated the presence of one equiv. of a positive charge.



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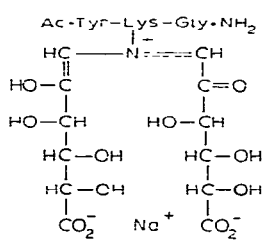

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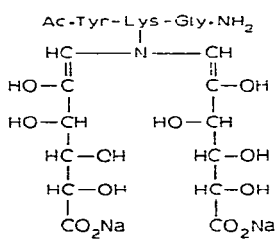

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The pKa value of the hydroxyl group of the tyrosyl residue and that of the two carboxyl groups of the sugar moiety in **2** were estimated to be 9.9 and 3.2, respectively (Fig. 1). The titration curve of **1** shows the presence of a dissociable proton in addition to those attributable to the hydroxyl group of the tyrosyl residue and to the carboxyl group of the acetate residue. It was attributed to an ε-amino group, the pKa value of which can hardly be differentiated from that of the hydroxyl group.

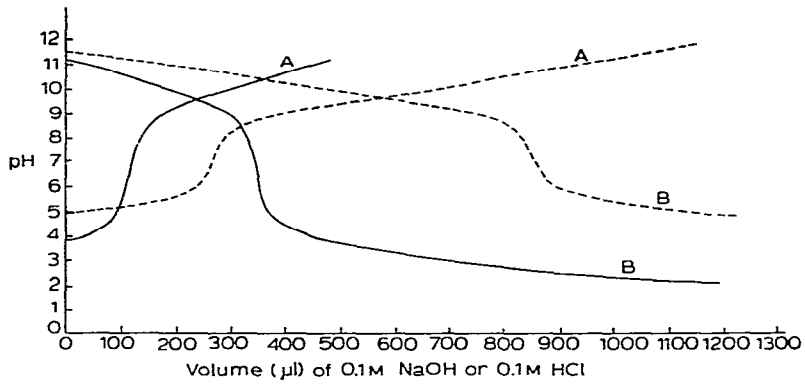

Fig. 1. Titration curves of **1** (3.57×10^{-5} mol, dotted line), and **2** (2.40×10^{-5} mol, full line): A, titration curve with 0.1M NaOH; B, back-titration curve with 0.1M HCl.

The titration curve of **2**, shows, however, only the dissociable protons of the carboxyl and the hydroxyl groups, suggesting that no hydrogen atom is attached to the amino nitrogen atom.

The u.v. spectrum of **1** closely resembles that of L-tyrosine, having an absorption maximum at 275 nm and a molar absorptivity of 1200 in the pH range from acidic to neutral. On the other hand, the spectrum of **2** exhibits the absorption maximum at 284 nm and a molar absorptivity of 6500. At alkaline pH, the absorption maxima of both spectra shift to longer wavelengths, and the intensity of the spectra increases (Fig. 2). It is related to the dissociation of the hydroxyl group of

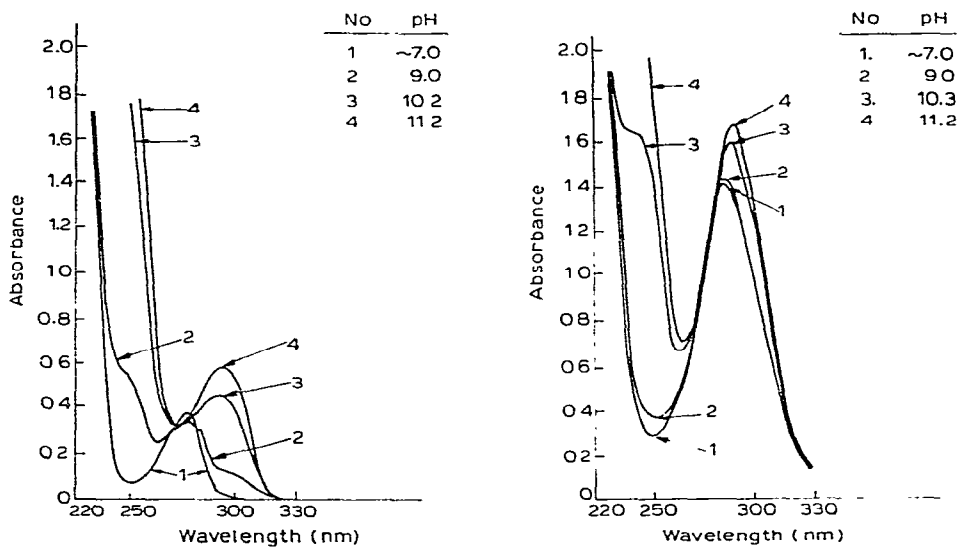

Fig. 2. U.v. spectra of **1** (2.7×10^{-4}M, left), and **2** (2.1×10^{-4}M, right) at various pHs.

the tyrosyl residue. The u.v. difference-spectra (Fig. 3) show the absorption maximum at 289 nm and a molar absorptivity of 6200, which is indicative of the presence of a conjugated double-bond in the sugar moiety. The position and intensity of the difference spectra did not vary with change of pH throughout the range 1.2–12.7. This observation is in accord with the result of the pH titration, indicating that no dissociable proton is attached to the amino nitrogen atom, and consequently that the amino nitrogen atom is quaternary. The appearance of an absorption band in the 310–330 nm region at a pH value higher than 13 may be attributed to a conjugated double-bond of a degradation product derived from **2**. It is known that D-glucurono-

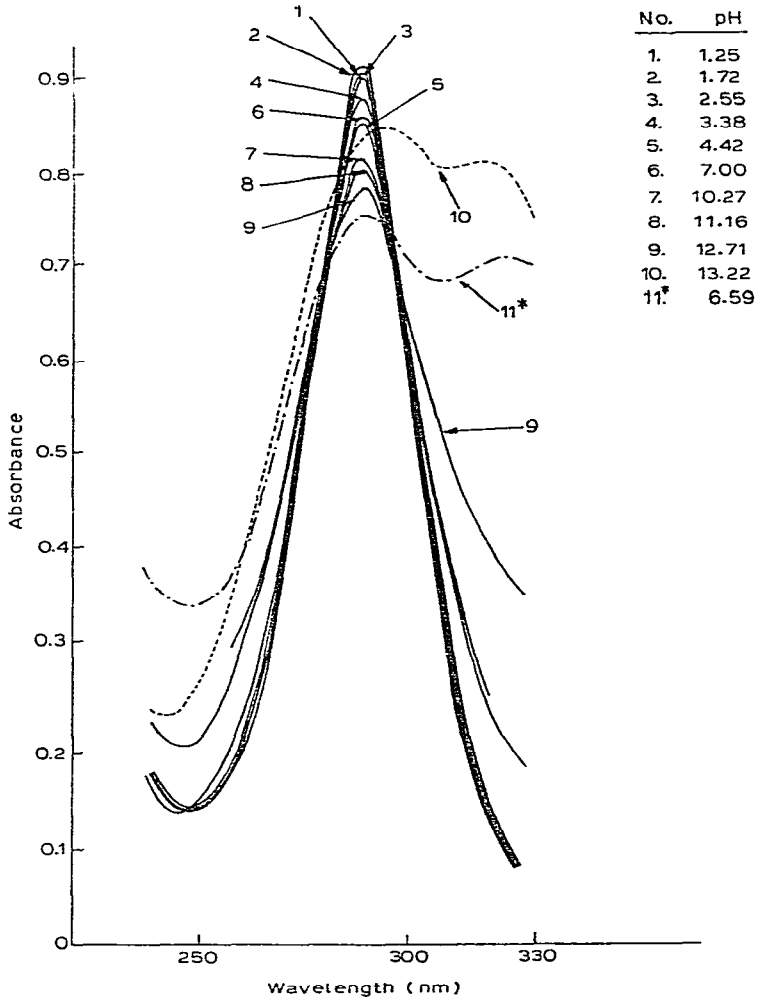

Fig. 3. The u.v. difference spectra of **2** *vs.* **1** at different pH values: concentrations of **1** and **2** were both 1.42×10^{-4}M at the starting pH of 4.42. The pH was varied first from 4.42 to 1.25 by addition of HCl and then to 13.22 with NaOH; 11*, difference spectrum recorded after readjustment of the pH from 13.22 to 6.59.

lactone is converted into 4,5-dideoxy-*aldehydo*-D-*glycero*-hex-4-enos-3-ulosuronic acid in an aqueous sodium hydroxide solution[14]. The absorption band did not disappear during the adjustment of pH from 13 to <7, indicating that the degradation product is no more converted into **2**.

The interpretation of the p.m.r. spectrum of **2** in D_2O, in the pD range 3.0–10.1, adds further support to the quaternary nature of the amino nitrogen atom, which is a counter-cation against one of the two carboxyl groups of the sugar moiety. Except for a doublet centered at δ 6.85 due to the aromatic ring protons at C-3 and C-5, which shows an upfield shift with increasing pD in relation to the dissociation of the hydroxyl group of the tyrosyl residue, all other signals were virtually invariant (Fig. 4).

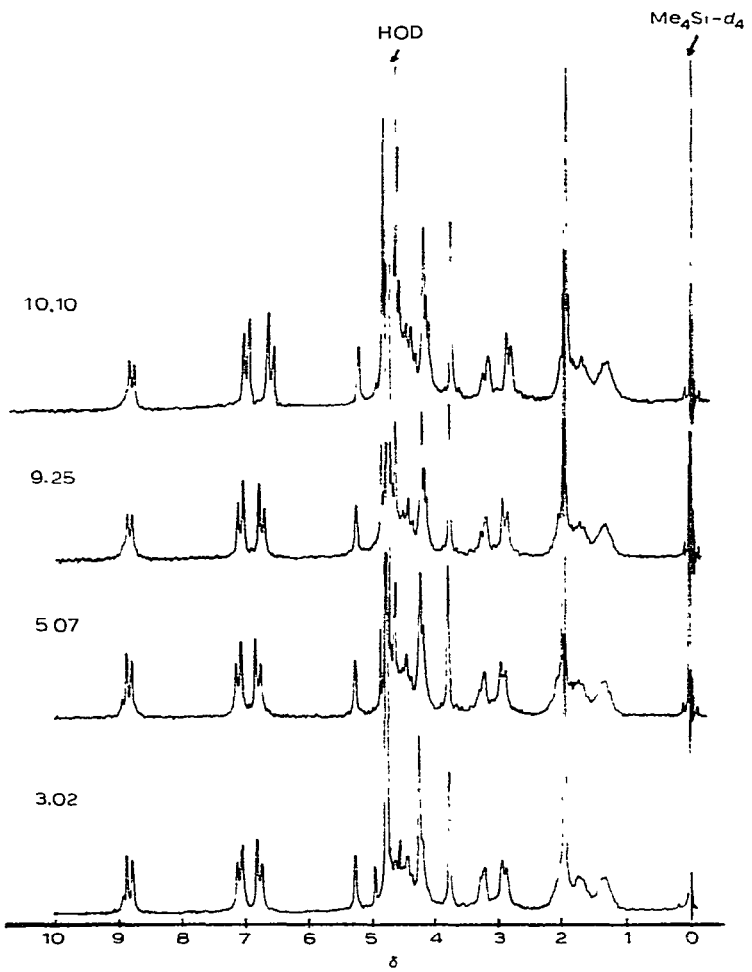

Fig. 4. 100-MHz p.m.r. spectra of **2** in D_2O at various pD values.

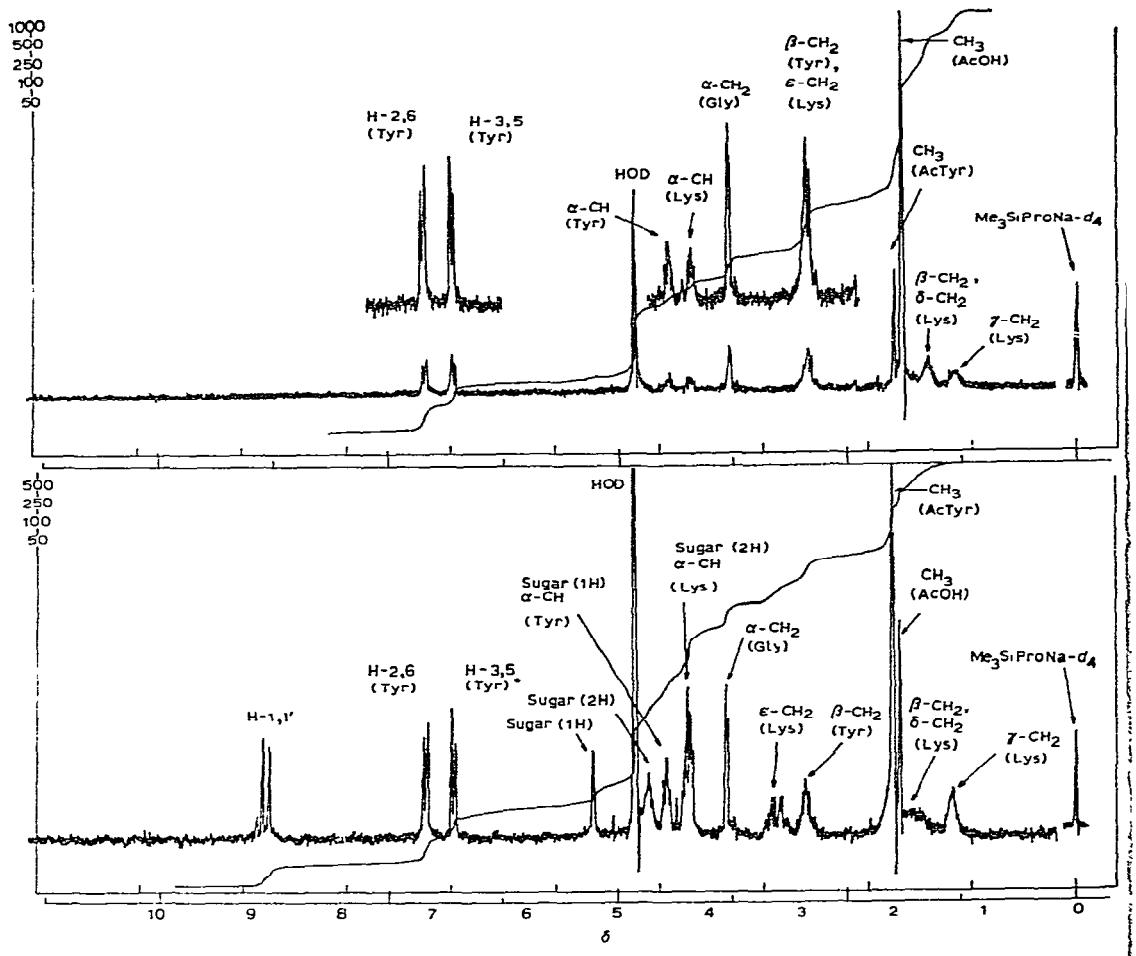

Fig. 5. 220-MHz p.m.r. spectra of **1** (upper) and **2** (lower) in D_2O.

In the 220-MHz p.m.r. spectra of **1** and **2** in D_2O (Fig. 5), the spectral assignments for **1** and the peptide portion of **2** are based on the assignments given for lysine-vasopressin[15–17], which has an amino acid sequence in part similar to that of **1**, and for α-amino acids[18] at pD 5.5–6.0. A comparison of the resonances of the two spectra for consistency of resonance positions of the peptide moiety reveals a downfield shift of the resonances due to the ε-methylene protons of **2**, the ε-methylene protons of **1** and **2** resonating at δ 2.93 and 3.28, respectively. The relatively large downfield shift indicates that a condensation reaction had occurred with the ε-amino groups. Upon integration of the peak areas, 8 protons were attributed to the sugar moiety, with some ambiguity due to the overlap of peaks caused by the resonances of α-methine protons of the tyrosyl and lysyl residues. The loss of two protons suggests the presence of two double bonds formed through intramolecular dehydra-

tion. Two of the signals of the sugar protons, at δ 8.83 and 8.90, are of interest. As they are not coupled, no vicinal proton is present and the resonance positions indicate that the electrons withdraw from these protons, which may be adjacent to the olefinic or carbonyl carbon atoms and are assigned to H-1 and H-1′ (see ^{13}C-n.m.r. spectra discussed later). The peak areas of several resonances in the range 4–5 p.p.m. correspond to six protons. These six resonances and the H-1 and H-1′ resonances suggest a nonequivalence of the chemical environment in the two sugar moieties with respect to the central nitrogen atom. In Me_2SO-d_6 solution, the chemical shift difference between the two sugar moieties became smaller: the two singlets collapsed to one single signal at δ 8.87, and the signals around 5 p.p.m. appeared in a narrower frequency range, indicating that the asymmetry is solvent dependent. The influence of the temperature on the p.m.r. spectra was studied for a change of the temperature from 5° to 80° for D_2O solutions, and from 20° to 100° for Me_2SO-d_6 solutions; no appreciable change was observed for either medium.

The 25.1-MHz ^{13}C-n.m.r. spectra of **1** and **2** in D_2O solution and the chemical shifts relative to Me_4Si are given in Fig. 6 and Table I. The spectral assignments of **1** and of the peptide portion of **2** were also made by comparison with those for lysine-

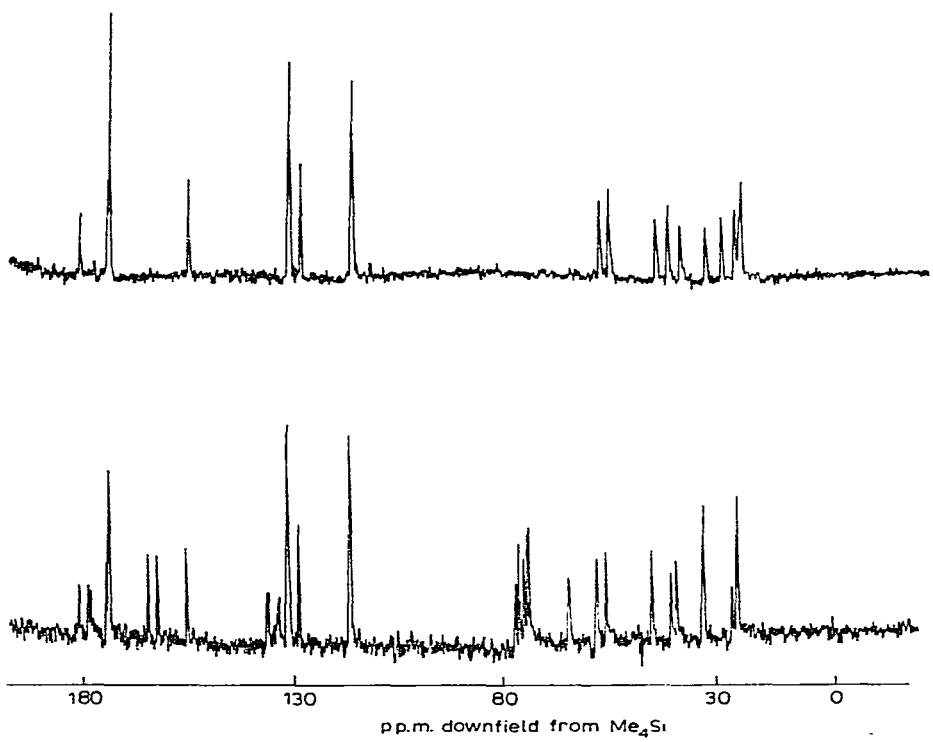

Fig. 6. 25.1-MHz ^{13}C-n.m.r. spectra of **1** (upper) and **2** (lower) in D_2O: pulse interval, 2.0 sec.; the spectra were accumulated 800 and 1200 times, respectively, for **1** and **2**.

TABLE I

^{13}C CHEMICAL SHIFTS (p.p.m.) AND TENTATIVE ASSIGNMENTS FOR THE CARBON ATOMS OF **1** AND **2**

Carbon assignments	*Chemical shifts*[a]			*Shift differences*[b]		*Partially decoupled spectra*[c]
	1	**2**	**L**[d]	**1−L**	**1−2**	
Peptide moiety						
Lysine						
C-α	54.8	54.6	54.7	0.1	0.2	d
C-β	31.5	31.3	31.1	0.4	0.2	t
C-γ	24.5	31.3	23.0	0.5	−6.8	t
C-δ	27.7	38.7	27.3	0.4	−11.0	t
C-ε	40.6	63.8	41.5	0.9	−23.2	t
Glycine						
C-α	43.5	43.4	43.1	0.4	0.1	t
Tyrosine						
C-α	57.0	56.9	56.4	0.6	0.1	d
C-β	37.6	37.4	36.9	0.7	0.2	t
C_{arom}-1	129.1	129.1	128.6	0.5	0.0	s
C_{arom}-2,6	132.0	131.9	131.3	0.7	0.1	d
C_{arom}-3,5	116.9	116.8	116.5	0.4	0.1	d
C_{arom}-4	156.0	155.9	155.4	0.6	0.1	s
Carbonyl carbons						
amides	175.0	174.7	174.9	0.1	0.3	s
N-Acetyl carbons						
methyl	23.4	23.0			0.4	q
carbonyl	175.3	175.3			0.0	s
Acetate carbons						
methyl	24.4		24.1	0.3		q
carboxyl	182.0		181.6	0.4		s

Sugar moiety of **2**							
Chemical shifts[a]	179.5	179.0	165.1	162.8	137.7	134.1	76.5–73.0
Partially decoupled spectrum[c]	s	s	s	s	d	d	d
Number of carbons	1	1	1	1	1	1	6
Carbon assignments	C-6 or C-6′		C-2 or C-2′		C-1 or C-1′		C-3,3′–C-5,5′

[a]From the signal of Me_4Si. [b]Chemical shift difference: a positive value denotes upfield shift and a negative value, downfield shift. [c]Key: s, singlet; d, doublet; t, triplet; q, quartet. [d]**L**, lysine-vasopressin; the chemical shift data were converted by use of the value $\delta_C^{CS_2} = 192.8$ p.p.m.

vasopressin[19] and for α-amino acids[20], and partly with the partially proton-decoupled spectra. The signal due to the ε-methylene carbon atom of **2** was observed in a field lower than that of **1** by 23.2 p.p.m. This large downfield shift not only reflects the site of condensation, but also indicates that the ε-amino nitrogen atom may be in the tertiary or quaternary form[21]. The 12 resonances attributed to the sugar moiety suggest a 2:1 binding ratio of sugar to peptide moieties. Two signals at δ 136.7 and

134.1 that are doublets in the partially decoupled spectrum may be ascribed to an olefinic carbon atom linked to one proton. These signals are correlated with the two singlets at δ 8.83 and 8.90 in the p.m.r. spectrum of **2**, since no other proton appears in the lower spectral region of unsaturated carbon atoms and gives doublets in the partially decoupled spectrum. The two protons on the carbon atoms do not show the presence of geminal and vicinal protons, thus these carbon atoms should be adjacent to quaternary carbon atoms. The existence of such a quaternary carbon atoms is confirmed by the appearance of two resonances at δ 162.8 and 165.1, which are observed separately from the two resonances at δ 179.0 and 179.5, assignable to the carboxyl C-6 and C-6′ atoms. The chemical shifts for two resonances (C-6 and C-6′) indicate that these carbon atoms are not conjugated with olefinic carbon atoms, as conjugation tends to shield carbonyl carbon atoms[22–24]. This observation excludes the possibility that C-5 and C-5′ are olefinic carbon atoms, and consequently the olefinic carbon atoms are C-1 and C-1′. These assignments are further supported by the observation that these signals exhibit smaller and broader peaks than other signals, and thus the corresponding ^{13}C nuclei possess shorter relaxation times and should be adjacent to the amino nitrogen atom. Consequently, the two quaternary carbon atoms are C-2 and C-2′; they are probably the carbonyl carbon atoms of highly conjugated ketones or enolic carbon atoms, as estimated from the chemical shifts. The nonequivalence of the two sugar moieties is also observed in the ^{13}C-

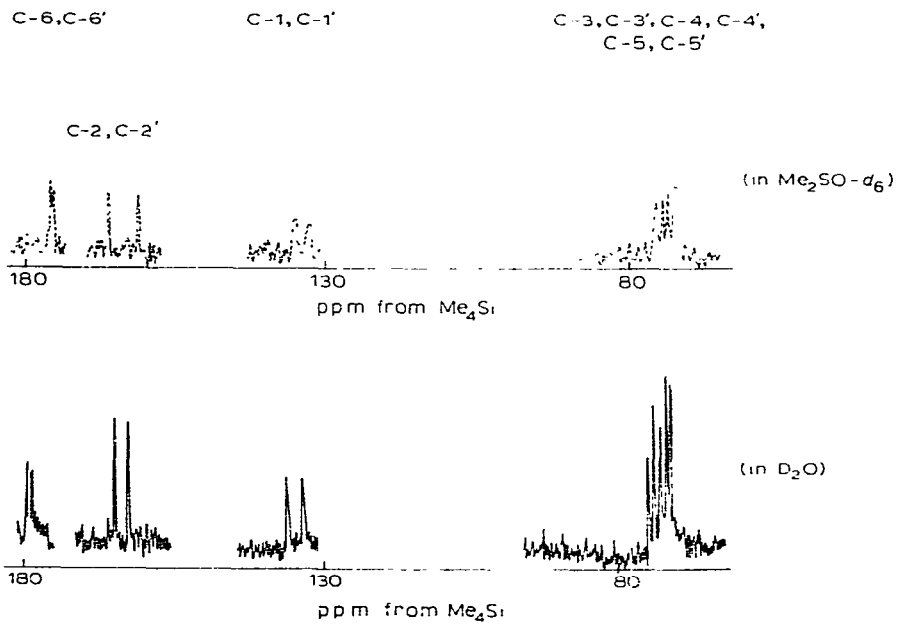

Fig. 7. ^{13}C-Chemical shift differences between the corresponding resonances of the two sugar moieties of **2** in D_2O (full line) and in Me_2SO-d_6 (dotted line).

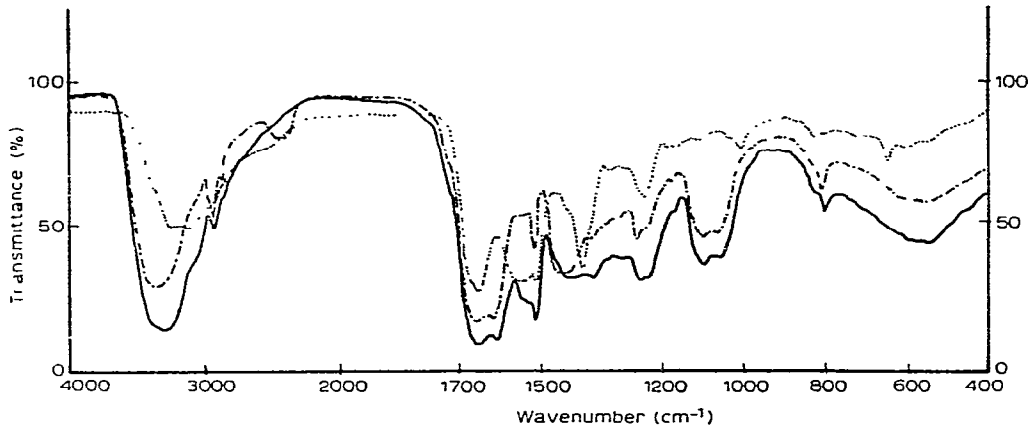

Fig. 8. I.r. spectra for KBr discs of **1** (·····), **2** (———), and largely-deuterated **2** (—.—.—).

n.m.r. spectrum. The extent of nonequivalency is decreased in Me_2SO-d_6, with the exception of C-2 and C-2′ (Fig. 7). This observation suggests that the nonequivalence due to the peptide moiety is reduced in Me_2SO-d_6 and that, in Me_2SO-d_6 solution, one half of the sugar moiety has a greater tendency to take the enol form, and the other half the keto form than they do in D_2O solution.

The i.r. spectra of **1** and **2** (see Fig. 8) show differences in the regions of 3500–3300, 1620–1600, and 1120–1070 cm^{-1}. Since the peaks in these regions may be assigned to the O–H stretching-vibrations, to the carboxylate ion stretching-vibrations, and to the C–O stretching-vibrations, respectively, some structural change occurs in the sugar moiety. The absorption bands at 815 and 800 cm^{-1}, which are different from the peaks of the tyrosyl residue, are assigned to the out-of-plane, bending vibrations of the trisubstituted olefinic group in the sugar moiety.

Among the products of the reaction of reducing sugars with amines or amino acids to yield humin substances, *N*-glycosylamines and the Amadori rearrangement products[25–27] are composed of an amino component and two sugars. Thus, it is of interest to compare the structure of **2** with those of (*N*-glycosylamine)uronic acids and their Amadori rearrangement products. Proton resonances due to anomeric H-1 of sodium (*N*-benzyl-β-D-glucopyranosylamine)uronate and sodium [*N*,*N*-(2-isonicotinyl)-β-D-glucopyranosylhydrazine]uronate in D_2O solutions were observed[28] as a doublet at δ 4.0 and 4.2. The chemical shift of the ^{13}C signal for C-1 of sodium (*N*-*p*-tolyl-β-D-glucopyranosylamine)uronate has been reported[29] to be δ 86.0. On the other hand, the p.m.r. and the ^{13}C-n.m.r. spectra of **2** exhibit no signal in the regions where the proton and ^{13}C resonances for H-1 and C-1 of (*N*-glucosylamine)-uronic acids appear. Thus, this structure is ruled out for **2**. Methylene protons (H-1 and H-1′) of (*N*-*p*-tolyl-β-D-fructofuranosylamine)uronamide give signals of AB-type pattern at δ 3.09 and 3.17 in the p.m.r. spectrum of the Me_2SO-d_6 solution[28]. The ^{13}C chemical shift for the C-1 methylene carbon atom of sodium (*N*-*p*-tolyl-β-D-

fructofuranosylamine)uronate has been reported[29] to be δ 49.5. In contrast, in these regions in the p.m.r. and ^{13}C-n.m.r. spectra of **2**, no signal was observed, and the partially decoupled ^{13}C-n.m.r. spectrum of **2** revealed the absence of a methylene carbon in the sugar moiety. This means that the structure of **2** cannot be that of an Amadori rearrangement product, except for the 1,2-enol form where no methylene carbon is involved. The possibility that **2** is the sodium salt of AcTyr{*N*,*N*-bis(D-*arabino*-5-carboxy-2,3,4,5-tetrahydroxy-1-pentenyl)}LysGlyNH_2 (**3**), namely a bis(1,2-enolic)amine of a di-D-fructuronic acid peptide is excluded, because the amino nitrogen atom of **3** is tertiary and, in both the p.m.r. and u.v.-difference spectra of **2**, no appreciable change was observed with change of pH. In addition, the two singlets at δ 8.84 and 8.90 in the p.m.r. spectrum of **2**, which are assignable to H-1 and H-1′, appear at a much lower field than expected for the olefinic protons of simple enol compounds. Thus, it is clear that the structure of **2** is different from that of any of the products reported for the Maillard reaction. The structure that satisfies all the experimental results was found neither in the products of the β-eliminative degradation[30] nor in the dehydration[31] products. The only conceivable structure for **2** is that of the sodium salt of AcTyr-(*N*-D-*arabino*-5-carboxy-2,3,4,5-tetrahydroxy-1-pentenyl)-*N*-D-*arabino*-5-carboxy-3,4,5-trihydroxy-2-oxopentylidene)LysGlyNH_2. This structure, which is one of the oxidized forms of **3**, is a limiting structure, and delocalization of the π-electron occurs, as shown by the Hückel molecular orbital calculation for the enol–keto–immonium structure (Fig. 9). A similar calculation for the bis(1,2-enolic)amine structure, which corresponds to the structure of **3**, shows a difference of delocalization between both structures. The ^{13}C resonances of C-2 and C-2′ of the sugar moieties are at unusually higher and lower fields, respectively, when compared with those of the corresponding resonances of similar compounds[29,32–34]. However, the delocalization in the conjugated system is remarkable as shown in the HMO calcula-

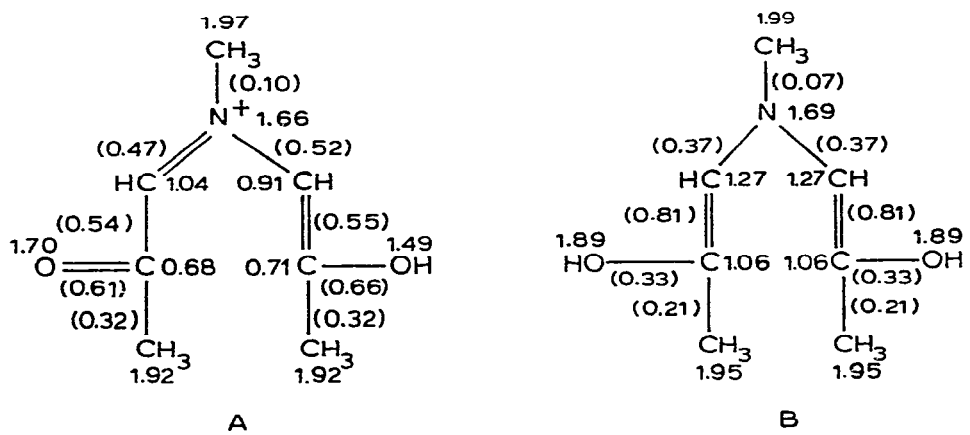

Fig. 9. Bond orders and electron densities of (A) enol-keto-immonium structure and (B) bis-enolic amine structure. Hückel molecular orbital calculations were made by the simple LCAO–MO method. Values with and without parentheses denote bond orders and electron densities, respectively.

tion (Fig. 9), and the phenomenon may also be attributed to the average of the resonance positions due to the rapid exchange between keto and enol forms.

The structure of **2** is, to our knowledge, the first example of two residues of a sugar bound to one amino group of a peptide through a conjugated enol–keto–immonium structure. Heyns and Schulz[35] reported that sodium D-glucuronate reacts with L-lysine in water–methanol medium to give two Amadori rearrangement products, namely: (ε-D-fructuronic acid)-L-lysine and (α,ε-di-fructuronic acid)-L-lysine. The reaction conditions used, however, were different from those under which bacterial exotoxins and viruses are inactivated by the sugar. We were unable to isolate a product from the reaction of sodium D-glucuronate with L-lysine or its derivative, α-*N*-AcLysNH$_2$ acetate. In contrast to unstable (*N*-glucosylamine)uronic acids of simple aliphatic amines[36] or to Amadori di-rearrangement products[25], **2** is a fairly stable compound which did not decompose in aqueous solution in the pH range of 1.2–12.7, nor did it change at temperatures up to 80° for 20 min or longer.

As the reaction conditions of sodium D-glucuronate with ovalbumin and with PPD tuberculin were similar to those under which **2** was obtained, and the stability of the reaction products and the lack of reactivity for sugar reagents were similar to those of **2**, a structure similar to that of **2** can be expected for the reaction products. Such a new type of condensation may also take place during the treatment of sodium D-glucuronate with peptides or proteins containing L-lysyl residues under the mild conditions used for inactivation of toxins or virus proteins.

EXPERIMENTAL

General methods. — Optical rotations were measured for aqueous solutions with a Yanaco OR-50 Automatic Polarimeter at the D line (Na). Amino acids were analyzed with a Yanagimoto LC-2 Amino Acid Analyzer; samples were hydrolyzed with a redistilled HCl solution at 110±1° for 24 h. Compound **1** and the peptide moiety of **2** were determined with the 1-nitroso-2-naphthol method[37], L-tyrosine being the standard. The ^{14}C radioactivity was measured with a Nuclear Chicago Mark I Liquid Scintillation Counter. The sugar moiety of **2** was determined by measurement of the ^{14}C radioactivity, sodium D-[6-^{14}C]glucuronate being the control. Carboxyl groups were determined by the procedure of Kasai and assoc.[38,39], sodium D-glucuronate being the control. The pH was determined with a Metrohm E 436 Potentiograph Herisau (Switzerland), the reagent being added with a microsyringe to control a volume as small as 1 μl. PKa values were estimated from titration curves in the usual way. The amount of Na ions was measured at 589.2 nm with a Seiko SAS 721 Atomic Absorption Spectrophotometer, after neutralization of the carboxyl groups by addition of NaOH.

U.v. spectra were recorded with a Hitachi 624 Digital Spectrophotometer, and i.r. spectra for KBr discs and hexachlorobutadiene mulls with a Hitachi EPI G3 instrument, the sample being previously treated several times with D_2O followed by lyophilization. N.m.r. spectra were recorded for solutions in D_2O and Me_2SO-d_6

with a JEOL PS-100 spectrometer and with a Varian HR-220 spectrometer, Me_4Si and $(CH_3)_3SiCD_2CD_2CO_2Na$ being the internal standards. ^{13}C-N.m.r. spectra were obtained for solutions of D_2O and Me_2SO-d_6 at 25.1-MHz with a JEOL PFT-100 pulse, Fourier-transform n.m.r. system locked on deuterium; chemical shifts were measured relative to the proton-decoupled ^{13}C resonance of Me_4Si by data reduction.

Thin-layer chromatography. — T.l.c. was performed on glass plates coated with Silica Gel G (E. Merck, Darmstadt, Germany) with 4:1:5 (A) and 5:2:3 (B) butanol-acetic acid–water. Amino groups, phenol groups, and D-glucuronic acid were detected by spraying with ninhydrin, Pauly's reagent, and naphthoresorcinol reagent, respectively. Radioactive spots were detected by autoradiography.

AcTyrLysGlyNH$_2$ acetate (**1**). — Compound **1** was provided by Dr. Okada. The homogeneity of **1** was verified by t.l.c., amino acid analysis, and elemental analysis. It could be crystallized through evaporation of water, after purification on a CM-Sephadex C-25 column, but, as it was extremely hygroscopic, the lyophilized powder was used: $[\alpha]_D^{20}$ +2.5° (*c* 4, water); t.l.c.: R_F 0.32 (A), 0.54 (B).

Anal. Calc. for $C_{19}H_{29}N_5O_5 \cdot 2AcOH \cdot 2H_2O$: C, 50.64; H, 7.16; N, 12.84. Found: C, 50.78; H, 6.99; N, 12.73.

Preparation and purification of **2**. — Peptide **1** (100 mg) was dissolved in distilled water (0.7 ml), and the pH of the solution was adjusted with NaOH to ~7. Sodium D-glucuronate (500 mg), 0.5M phosphate buffer solution (0.8 ml, pH 7.2), and a small amount of the antiseptic thimerosal were added. The solution was incubated for 110 h at 37°. An aliquot (0.35 ml) of the solution was diluted with 0.02M acetic acid (pH 3.3) to 40 ml, and applied to a CM-Sephdex C-25 column (2.5 × 32 cm), which had been previously washed with the acetic acid solution. The column was successively eluted with acetic acid (280 ml), distilled water (450 ml), and 0.1M ammonium acetate (320 ml). The fractions that exhibited almost identical u.v. absorption curves were combined, and analyzed by t.l.c. The fractions that were eluted with acetic acid contained unreacted sodium D-glucuronate and some degradation products. With sodium D-[6-^{14}C]glucuronate, the reaction product **2** was found in the largest of the

TABLE II

PARAMETERS OF COULOMB AND RESONANCE INTEGRALS

Substituent group X	a^a	b^b	l^c
=O	2.0	0.2	1.4
–OH	0.6	0.0	0.7
–N–	1.0	0.1	1.0
=N±	3.0	0.2	1.0
$-CH_3$	3.0	−0.1	1.0

[a]Coulomb integral of the substituent X: $\alpha_X = \alpha + a\beta$. [b]Coulomb integral of the carbon atom adjacent to X: $\alpha_{adj} = \alpha + b\beta$. [c]Resonance integral between the carbon atom and X: $\beta_{C-X} = l\beta$.

three peaks that had been eluted with distilled water. It was purified by rechromatography on a CM-Sephdex C-25 column (0.9×30 cm) to give a homogeneous compound (~20% yield), as verified by t.l.c. [R_F 0.16 (A) and 0.24 (B)]; $[\alpha]_D^{20}$ $-27°$ (*c* 4, water).

Calculations of Hückel molecular orbitals. — Molecular orbitals were calculated by the simple LCAO-MO method, neglecting overlap integrals. The parameters of coulomb and resonance integrals for substituent groups are described in Table II.

ACKNOWLEDGMENTS

The authors thank Profs. T. Akiba and T. Tanimura (University of Tokyo), and Dr. K. Morita (Chugai Pharmaceutical Co., Ltd., Tokyo) for valuable discussions; Dr. M. Okada (Chugai Pharmaceutical Co.) for synthesizing the model peptide; the Administration Committee of the 220-MHz NMR System at the Department of Hydrocarbon Chemistry of Kyoto University and Dr. H. Kobayashi (University of Tokyo) for the measurements of n.m.r. spectra; Dr. Y. Kasai for the determinations of carboxylic groups; and Dr. I. Matsuura for HMO calculations.

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