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Note

Formation of α-amino-acid amides and α-hydroxy-acid amides by degradation of sugars with primary amines

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Abstract

Reactions of glucose and ribose with an excess of propylamine in phosphate buffered nearly neutral solution lead to the formation of *N*-propylalanine propylamide, *N*-propylglycine propylamide, lactic acid propylamide, and glycolic acid propylamide. The production of these amides is favoured by alkaline conditions. These model reactions represent a new mechanism for cross-linking of proteins, through lysine side chains by sugars. © Elsevier Science Ltd.

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Reactions of reducing sugars with proteins, amino acids or simple amines (Maillard reaction) are complex processes that are only partially understood. During past decades, nonenzymatic glycosylation reactions have been intensively studied in connection with problems of food chemistry, whereas the importance of Maillard chemistry in living organisms has been recognized only recently. Sugar–protein interactions are thought to be responsible for deleterious effects associated with diabetes. Furthermore, a number of functional consequences of nonenzymatic glycosylation of proteins have been described. These processes have been posited to be involved in arteriosclerosis or more generally in biological aging [1].

At present there is an intense search for so called 'advanced glycosylation end-products' (AGEs) which result from the interaction of glucose with proteins in the human body. Carboxymethyllysine (CML) appears to meet these criteria and has been shown to be a valuable indicator of protein glycosylation in medical biochemistry [2]. Recently, we have shown that glucose reacts with primary amines like propylamine or α-*N*-acetyllysine to give *N*-substituted alanine derivatives in comparable quantity [3].

It is well established that sugar–protein interactions can lead to the cross-linking of proteins. This process is thought to be of considerable biological importance, but the underlying mechanism has been explained only to a limited extent [4]. Only a few cross-linking reactions of proteins by sugars have been established so far. Sell and Monnier were able to isolate a fluorescent Maillard reaction product called pentosidine [5]. Comparatively high levels of pentosidine are associated with diabetics and uraemia. Furthermore, pentosidine concentration in tissues may

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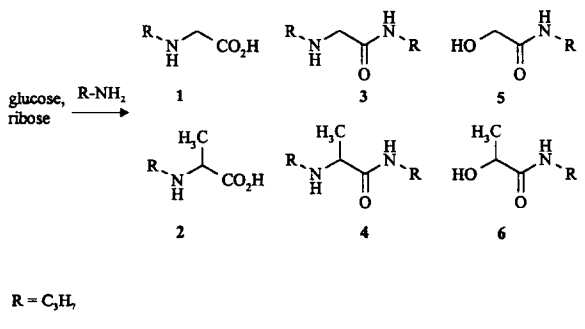
reflect the aging process, but the absolute concentration of this heterocycle is very small.

Aldohexoses and aldopentoses react with primary amines to give formylpyrroles [6], and analogous products are formed with lysine side-chains of proteins [7]. These pyrroles are thought to be involved in cross-linking reactions based on studies of in model systems. Hydroxy groups in the α-position of pyrrole side chains are easily substituted by nucleophiles, such as cysteine residues, under physiological conditions [8]. On the other hand aldimines are formed by interaction with lysine-side chains of proteins. Reactions of this type may participate in the cross-linking of proteins [9].

Here we report the formation of α-amino-acid amides through the reaction of reducing sugars with primary amines. This interaction represents a possible new mode of cross-linking of primary amines and proteins by sugars.

1. Results and discussion

When D-ribose and propylamine are heated in a molar ratio of 1:2 or 1:5 in a nearly neutral phosphate buffered (pH 7.4) aqueous solution at 70°C for about 5 h, the mixture turns dark brown with the formation of several hydrophilic and lipophilic products. The compounds, which were extractable by ethyl acetate, were acetylated and analysed by GC/MS. The major products, *N*-propylglycine propylamide (**3**), *N*-propylalanine propylamide (**4**), glycolic acid propylamide (**5**), and lactic acid propylamide (**6**), were detected as their corresponding acetyl derivatives (Scheme 1, Fig. 1). Reference compounds were obtained through the reaction of the ethyl esters of bromoacetic acid, *N*-propylalanine, lactic acid, and the butyl ester of glycolic acid with propylamine. Degradation of glucose in the presence of propylamine in phosphate buffered solution gave the same products, but glucose reacted slowly and afforded smaller quantities compared to ribose.



Scheme 1. Formation of amino-acid amides and hydroxy-acid amides from glucose or ribose.

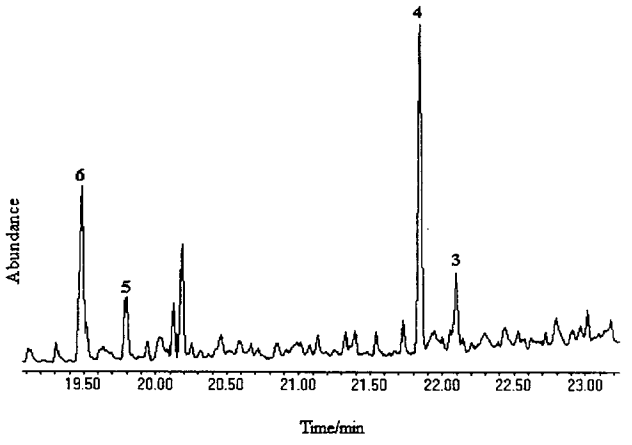
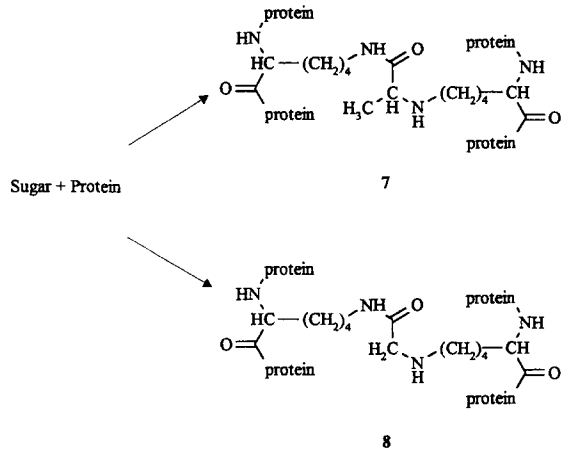


Fig. 1. Gas-chromatogram of a Maillard reaction mixture at pH 7.4 with ribose after extraction with ethyl acetate and acetylation (compounds **3** and **5** were not extracted completely).

The products isolated from the pentose or hexose/amine reaction mixtures and the reference compounds showed identical mass-spectra after acetylation (see Section 3, Experimental). In addition, the reaction mixtures were spiked with the synthesized reference compounds and derivatized. Thus the structures were unambiguously established. Detection by GC after acetylation did not interfere with other products of the Maillard reaction.

The amounts of acid amides formed is dependent upon the molar ratio of the reactants. A high amine/sugar molar ratio leads to a comparatively high yield of amino-acid amides. Furthermore, the yield of the amino-acid amides is highly dependent upon reaction conditions such as pH, temperature, concentration and solvent. Alkaline conditions favour the C,C-cleavage of the carbon chain. In aprotic solvents such as acetonitrile containing a small amount of water, the yields are higher than they are in dilute aqueous solution. For instance, heating of a 0.04 M aqueous solution of a glucose-propylamine Amadori compound mixture with a 5-fold molar excess of propylamine at pH 7.4 for 5 h produced approximately 0.5% *N*-propylalanine propylamide (**4**) and 2.2% *N*-propylglycine propylamide (**3**). When the same mixture was heated at pH 9, the yield of **4** increased to 1.5% and the yield of **3** increased to 2.7%. Under acidic conditions the amides could not be detected. Heating in acetonitrile containing 10% water at about pH 8.5 (measured after dilution with water) resulted in yields of 3.1% of **3** and 3.2% of **4**.



Scheme 2. Putative cross-links between sugars and proteins.

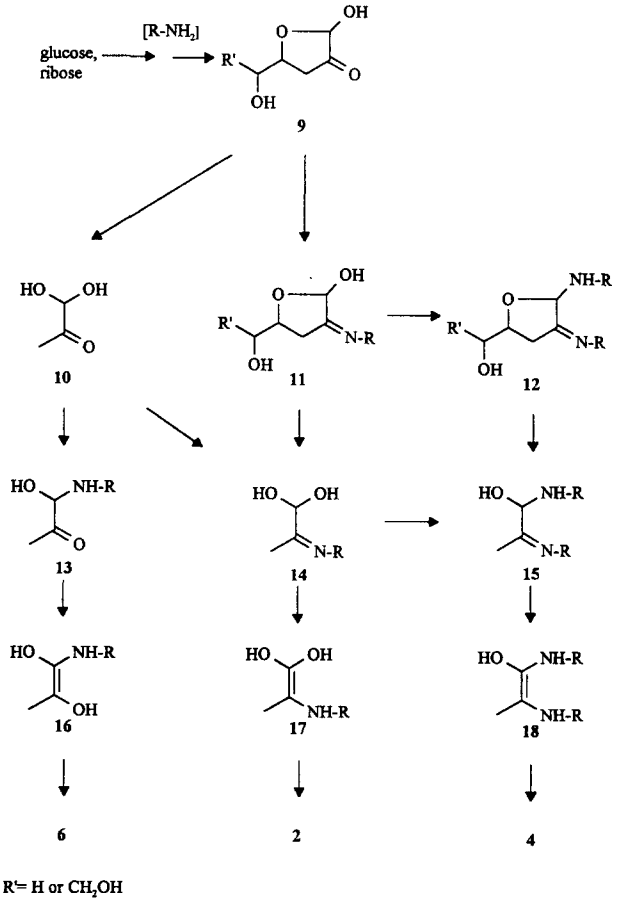
A higher concentrated mixture produced generally smaller quantities of the amides. The amounts of *N*-propylglycine propylamide (**3**) that formed depended on the presence of oxygen.

Heating of D-ribose or D-xylose under all conditions described above afforded **4** in a slightly higher amount, whereas the amount of **3** did not increase, compared to hexoses. The amides could also be detected after warming of the reaction mixture at 40°C for several days. Lactic acid propylamide (**6**) was produced in amounts similar to **4**, and glycolic acid propylamide (**5**) in amounts similar to **3**. In summary, the yields of the amides are not high, but they are in the same range as other important degradation products of the Amadori compounds.

The model reactions of a pentose or glucose with propylamine or other primary amines forming *N*-substituted alanine and glycine amides represent two possible different modes of cross-linking of proteins through lysine side chains. Cross-linking of proteins by pyruvaldehyde and glyoxal has already been studied in detail [10]. These dicarbonyl compounds are known to arise by the degradation of sugars under various conditions, but generally only in low amounts due to their high reactivity. The interaction of pyruvaldehyde with α-*N*-acetyllysine has been examined by NMR spectroscopy. When pyruvaldehyde and α-*N*-acetyllysine were allowed to react at pH 7.4 and 37 °C, the intensities of the methyl proton of the pyruvaldehyde hydrates decreased, and two new signals increased in intensity with increasing reaction time. The formation of a pyruvaldehyde/α-*N*-acetyllysine mono- and bis-adduct, as suggested by the authors, would explain these observations [11]. Furthermore, it has been proposed that a bis-imine may isomerize to give an alanine derivative, but products of definite structure have not been obtained so far. The isolation of *N*-substituted alanine amides described in this paper strongly supports the proposed mechanism of cross-linking of α-*N*-acetyllysine and proteins by an alanine moiety **7** (Scheme 2).

The formation of the alanine derivatives **2** and **4** from aldopentoses or aldohexoses can be explained as follows (Scheme 3). 3-Deoxypentosulose or 3-deoxyhexosuloses **9**, which are well known sugar degradation products, are proposed as intermediates. A retroaldol reaction is envisaged as an essential step during the formation of the alanine derivatives **2** and **4**. This C,C-cleavage may proceed before the generation of a Schiff base leading to pyruvaldehyde (**10**), but it is also plausible that ketoimine **11** could be formed followed by a retroaldol reaction. A simple tautomerisation of product **15**, obtained by addition of a second amine to the intermediate **14** or **13**, would lead directly to the alanine amide **4**.

The formation *N*-propylglycine propylamide (**3**), which depends on the presence of oxygen, can be



Scheme 3. Proposed reaction mechanisms.

explained by another reaction sequence. Glyoxal was identified as a sugar degradation product during autoxidation of glucose [12]. This dicarbonyl compound is involved in Maillard-type reactions leading to modifications of proteins. It has been proposed that the addition of two lysine residues to one molecule of glyoxal followed by tautomerisation might result in a protein cross-link by a glycine amide moiety **8** similar to the modification through pyruvaldehyde, but the appropriate *N*-substituted glycine amides have not been isolated [12]. As described in this paper, the model reactions of ribose or glucose with propylamine, which lead to the identification of the *N*-substituted glycine amide, substantiate this hypothesis.

2. Conclusion

Several investigations have shown that simple primary aliphatic amines react in the same way as α-*N*-acetyllysine or lysine side-chains of proteins with sugars. If these data are valid, the formation of *N*-propylglycine propylamide (**3**) and *N*-propylalanine propylamide (**4**) via the degradation of sugars under the influence of primary amines must be considered as a model for cross-linking of proteins.

3. Experimental

NMR and mass spectral analyses.—^{1}H and ^{13}C NMR spectra (internal standards; tetramethylsilane and [d_6]-acetone, respectively) were recorded with a JEOL 400 GSX spectrometer. Chemical shifts are reported in parts per million (ppm). Mass spectral analyses were obtained with a Varian MAT CH7 spectrometer (CI with CH_4, 70 eV).

Gas chromatography (GC) mass spectrometry (MS).—Solutions were prepared according to the described procedures and analysed by GC/MS using a 25 m × 0.25 μm i.d. fused silica capillary column coated with Permabond OV 1701-DF in a Hewlett–Packard gas chromatograph (Model 5890/2) equipped with a Hewlett–Packard MS computerised system (Model 5971A). The temperature was programmed as follows: $\theta_1 = 60$°C; Isotime 1 = 4 min; Ramp Rate 1 = 2°C/min. $\theta_2 = 80$°C; Isotime 2 = 0 min; Ramp Rate 2 = 20°C/min. $\theta_3 = 260$°C; Isotime 3 = 15 min. The mass spectrometer was operated in scan mode for compound identification, and mass spectra were recorded in the electron impact mode. The mass-range was chosen from 50 to 500 *m/z*.

Sample derivatization.—The compounds (0.5 mg each) were acetylated overnight with 0.2 mL acetic anhydride in 0.4 mL anhydrous pyridine, and reaction mixtures were poured into water and extracted with ether. The combined ether extracts were washed with aq $NaHCO_3$.

Sample preparation.—Sugar or Amadori compound (0.04 mmol) were heated with propylammonium acetate (0.2 mmol) in 1 mL 0.5 M phosphoric buffer (pH = 7.4) for 5 h at 70°C in a sealed tube. The solution was made alkaline with propylamine, extracted with EtOAc, and the EtOAc extract was dried under vacuum. The residue was treated as described above and analyzed by GC–MS.

N - *propylglycine propylamide* (**3**).—Bromoacetic acid ethyl ester (167 mg, 1 mmol) was stirred in propylamine (2 mL) for 48 h at rt. The reaction mixture was evaporated, and the residue dissolved in water and extracted with dichloromethane at pH 10, giving **3** (126 mg, 80%). ^{1}H NMR (CD_3OD): δ 0.90 (2 t, 6 H, *J* 7.4 Hz, 2 × CH_3), 1.49 (m, 4 H, 2 × CH_2–CH_3), 2.48 (t, 2 H, *J* 7.0 Hz, CH_2–NH–C-2), 3.13 (t, 2 H, *J* 7.0 Hz, CH_2–NH–C-1), 3.19 (s, 2 H, H-2); ^{13}C NMR (CD_3OD): δ 11.62 + 11.89 (2 × CH_3), 23.64 + 23.69 (2 × CH_2–CH_3), 41.95 (CH_2–NH–C-1), 52.3 (CH_2–NH–C-2), 52.5 (C-2), 173.62 (C-1); MS–CI: *m/z* = 159 (100%, M + 1$^+$), 72 (23); GC–MS-data after acetylation: t_{ret} = 22.1 min; *m/z* = 200 (2%, M$^+$), 157 (6), 142 (2), 129 (3), 115 (4), 114 (9), 101 (14), 100 (9), 87 (3), 84 (9), 73 (11), 72 (100), 58 (6), 56 (4). Anal. Calcd for $C_8H_{18}N_2O$: C, 60.72; H, 11.47; N, 17.70. Found: C, 61.37; H, 10.93; N, 17.59.

N - *propylalanine propylamide* (**4**).—*N*-propylalanine ethyl ester (55 mg, 0.34 mmol) (see [3]) (*N*-propylalanine: Anal. Calcd for $C_6H_{13}NO_2$: C, 54.96; H, 9.99; N, 10.68. Found: C, 54.90; H, 10.00; N, 10.67) was dissolved in propylamine (2 mL) and treated as described above to give **4** (40 mg, 67%). ^{1}H NMR (CD_3OD): δ 0.92 (2 t, 6 H, *J* 7.4 Hz, 2 × CH_3), 1.25 (d, 3 H, *J* 6.8 Hz, H-3), 1.51 (m, 4 H, 2 × CH_2–CH_3), 2.44 (t, 2 H, *J* 7.0 Hz, CH_2–NH–C-2), 3.15 (m, 3 H, CH_2–NH–C-1 + H-2); ^{13}C NMR (CD_3OD): δ 11.63 + 11.98 (2 × CH_3), 19.6 (C-3), 23.6 + 23.8 (2 × CH_2–CH_3), 41.9 (CH_2–NH–C-1), 50.9 (CH_2–NH–C-2), 58.79 (C-2), 177.7 (C-1); MS–CI: *m/z* = 173 (100%, M + 1$^+$), 115 (38), 86 (50); GC–MS-data after acetylation: t_{ret} = 21.8 min; *m/z* = 214 (1%, M$^+$), 129 (6), 128 (35), 115 (9), 87 (8), 86 (100), 84 (4), 72 (5), 70 (3), 58 (4), 57 (5), 56 (20), 55 (3), 54 (2).

Glycolic acid propylamide (**5**).—Butyl glycolate (132 mg, 1 mmol) was stirred in propylamine (3 mL) for 100 h at rt. The solution was evaporated in vacuum to give **5** (110 mg, 94%). ^{1}H NMR (D_2O): δ 0.88 (t, 3 H, J 7.2 Hz, CH_3–CH_2), 1.52 (sex, 2 H, J 7.2 Hz, CH_2–CH_3), 3.2 (t, 2 H, J 7.0 Hz, CH_2–NH), 4.0 (s, 2 H, H-2); ^{13}C NMR (D_2O): δ 10.78 (CH_3–CH_2), 22.22 (CH_2–CH_3), 40.94 (CH_2–NH), 61.17 (C-2), 174.91 (C-1); MS–CI: m/z = 118 (100%; M + 1$^+$); GC–MS-data after acetylation: t_{ret} = 19.8 min; m/z = 159 (41%, M$^+$), 144 (12), 130 (17), 118 (41), 101 (82), 86 (49), 74 (19), 73 (78), 58 (24). Anal. Calcd for $C_5H_{11}NO_2$: C, 51.26; H, 9.47; N, 11.96. Found: C, 51.81; H, 9.64; N, 11.25.

Lactic acid propylamide (**6**).—Ethyl L-lactate (118 mg, 1 mmol) was stirred in propylamine (2 mL) for 48 h at rt and the mixture was evaporated to give **6** (124 mg, 95%). ^{1}H NMR (D_2O): δ 0.86 (t, 3 H, J 7.2 Hz, CH_3–CH_2), 1.33 (d, 3 H, J 6.8 Hz, H-3), 1.49 (sex, 2 H, J 7.2 Hz, CH_2–CH_3), 3.15 (t, 2 H, J 7.0 Hz, CH_2–NH), 4.2 (q, 1 H, J 6.8 Hz, H-2); ^{13}C NMR (D_2O): δ 10.8 (CH_3–CH_2), 20.1 (C-3), 22.2 (CH_2–CH_3), 40.9 (CH_2–NH), 68.0 (C-2), 177.7 (C-1); MS–CI: m/z = 132 (100%, M + 1$^+$), 86 (17); GC–MS-data after acetylation: t_{ret} = 19.5 min; m/z = 173 (41%, M$^+$), 132 (26), 115 (74), 88 (100), 87 (94), 86 (92), 70 (56), 61 (41), 58 (24), 56 (30), 55 (28). Anal. Calcd for $C_6H_{13}NO_2$: C, 54.96; H, 9.99; N, 10.68. Found: C, 54.73; H, 9.91; N, 11.29.

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