Characterization of an imidazolium compound formed by reaction of methylglyoxal and N^{α}-hippuryllysine

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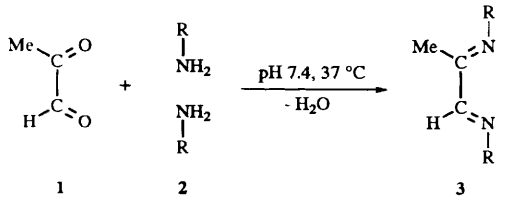
The 1,3-di-N^{α}-hippuryllysino-4-methylimidazolium salt **6** has been identified as a major product of reaction of the α-dicarbonyl compound, methylglyoxal **1**, with the lysine derivative, N^{α}-hippuryllysine (N^{α}-benzoylglycyllysine) **2**, in phosphate buffer at neutral pH, suggesting a mechanism for crosslinking of protein by **1** and related dicarbonyl compounds during the Maillard reaction.

α-Dicarbonyls such as glyoxal, methylglyoxal **1** and 3-deoxyglucosone are intermediates in the browning and crosslinking of proteins by reducing sugars during the Maillard reaction.[1,2] Compound **1** may be formed *in vivo* either enzymatically (from dihydroxyacetone phosphate by methylglyoxal synthase and other pathways) or non-enzymatically (by elimination of phosphate from glyceraldehyde phosphate or dihydroxyacetone phosphate).[3,4] The concentration of **1** undergoes a 2–6-fold



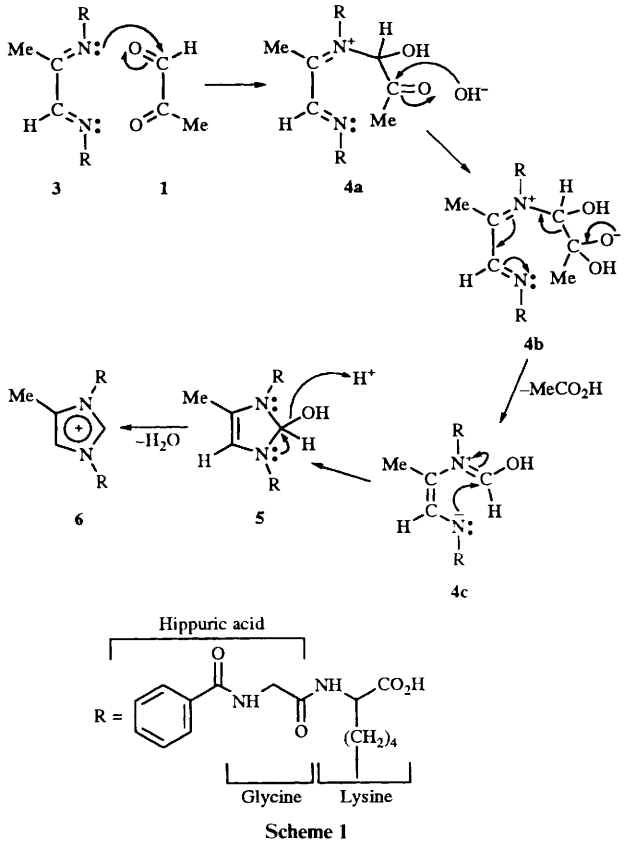
1 **2** **3**

increase in blood of diabetic patients where it is thought to be involved in the increased chemical modification of tissue proteins in diabetes, contributing to the development of diabetic complications.[5,6] Since no specific products of reaction of **1** with amino groups in proteins have been identified, we have studied the reaction of **1** with the lysine derivative, N^{α}-hippuryllysine (N^{α}-benzoylglycyllysine) **2**, as a model for reaction of **1** with the ε-amino group of lysine residues in proteins

Compounds **1** (200 mmol dm^{-3}) and **2** (100 mmol dm^{-3}) were allowed to react in 0.2 mmol dm^{-3} phosphate buffer, pH 7.4, at 37 °C. Reversed-phase HPLC analysis showed that one major product was formed, reaching a maximum at 24 h and remaining stable for at least 3 days. The yield at 24 h was 15%, based on total absorbance at 228 nm. Reaction of **1** (200 mmol dm^{-3}) with **2** (100 mmol dm^{-3}) in water (unbuffered) yielded 27% of the product at 24 h and 32% at 3 days. For preparative purposes, since **1** was rapidly consumed during the reaction ($t_{\frac{1}{2}}$ 30 min), a reaction mixture containing **1** (200 mmol dm^{-3}) and **2** (100 mmol dm^{-3}) in water was supplemented 4 times by addition of **1** (100 mmol dm^{-3}) at hourly intervals, yielding a 71% conversion of **2** into **6**.

Product **6** was identified as the 1,3-di-N^{α}-hippuryllysino-4-methylimidazolium salt, based on spectroscopic evidence [high resolution fast atom bombardment (FAB)-MS, ^{1}H NMR, ^{13}C NMR, insensitive nuclei enhanced by polarization transfer (INEPT)-NMR, ^{1}H-correlation spectroscopy (COSY) and 2-dimensional ^{1}H ^{13}C NMR].

Scheme 1 shows a likely mechanism for the formation of **6**. The first step is the reaction of **1** and **2** to form the diimine intermediate **3**, which reacts further with **1** to form **4a**. Acetic



Scheme 1

acid is eliminated in an intramolecular Cannizzaro-type reaction *via* intermediates **4b** and **4c**, forming **5**, which undergoes dehydration to form the imidazolium end-product, **6**.

These experiments present an alternative to current methods for the synthesis of imidazolium salts,[7] using dicarbonyl sugars and amines, and suggest that imidizolium salts are involved in the crosslinking of proteins by dicarbonyl sugars during the Maillard reaction.

Experimental

Reaction conditions

Solutions of **1** (1 mol dm^{-3}; 0.8 cm^3), **2** (400 mmol dm^{-3}; 1 cm^3) and phosphate buffer (pH 7.4; 400 mmol dm^{-3}; 2 cm^3) were mixed in a 13 × 100 mm screw-top test tube. The pH was

adjusted to 7.4 with 1 mol dm $^{-3}$ NaOH and the volume diluted to 4 cm^3. For preparative reactions in water, the pH was not adjusted. Reaction mixtures were incubated at 37 °C and aliquots removed at various times and frozen at −20 °C prior to HPLC analysis.

Reversed-phase HPLC

To remove brown products, samples were applied to C-18 solid-phase extraction cartridges (1 cm^3), washed with aqueous 0.05% formic acid (3 cm^3), 0.05% acetic acid (pH 3.5), and then eluted with 50% acetonitrile in water and dried under nitrogen. Reversed-phase HPLC analyses were performed on a Zorbax C-18 analytical column (5 μm particle size, 4.6 mm × 25 cm), using a multi-step gradient; buffer A: 0.05% acetic acid, 0.05% formic acid, 0.1% triethylamine in water; buffer B, same components in 25% acetonitrile–water. Products were monitored at 228 nm using a Waters photodiode array detector (series 996).

Preparation of 6 for NMR analysis

A reaction mixture (2 cm^3) was applied to a 0.6 × 11 cm C-18 column. Products were eluted stepwise with mixtures of buffer and A and B without triethylamine. Fractions (1 cm^3) were collected and monitored for absorbance at 228 nm. Fractions containing **6** were pooled, dried by rotary evaporation and dissolved in D_2O for NMR spectroscopy.

Compound **2**: δ_H(500 MHz; D_2O) 1.21–1.89 (6 H, m), 2.8–3.1 (2 H, m), 3.98–4.14 (2 H, q), 4.16–4.24 (1 H, q) and 7.38–7.80 (5 H, m); δ_C(125 MHz; D_2O) 22.52 [γ-lysine(L)-CH_2], 26.81 (β-L-CH_2), 31.58 (δ-L-CH_2), 39.72 (ε-L-CH_2), 43.60 [α-glycine(G)-CH_2], 55.28 (α-L-CH), 127.68 (*meta*-CH), 129.32 (*ortho*-CH), 132.99 (*para*-CH) and 133.18 (*ipso*-C).

Compound **6**: high resolution FAB-MS (polyethyleneglycol as reference): M^+ = 663.3142 (−3.4 ppm deviation), molecular formula: $C_{34}H_{43}N_6O_8$; δ_H(500 MHz; D_2O) 1.23–1.38 (4 H, m), 1.65–1.90 (8 H, m), 2.19–2.23 (3 H, s), 3.96–4.15 (8 H, m), 4.27–4.33 (2 H, m), 7.09–7.11 (1 H, s), 7.48–7.86 (10 H, m) and 8.56–8.57 (1 H, s); δ_C(125 MHz; D_2O), 8.67 [imidazolium(I)-CH_3], 22.26/22.33 (γ-L-CH_2 a,b), 28.63/28.99 (β-L-CH_2, a,b), 30.94/31.03 (δ-L-CH_2, a,b), 43.55 (α-G-CH_2), 46.90 (ε-L-CH_2, a), 49.53 (ε-L-CH_2, b), 54.05 (α-L-CH), 11.59 (I-CH 2), 126.67 (*meta*-CH), 129.36 (*ortho*-CH), 132.24 (I-C 4), 132.99 (*para*-CH), 133.29 (*ipso*-C), 134.83 (I-CH 5), 171.24, 171.57 (G- and L-amide-C) and 177.55 (L-carboxyl-C).

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