Glycylglycine-Derived 1,3-Disubstituted Imidazole in Nonenzymatic Browning Reactions

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5-[3-(3-Aza-4-carboxy-2-oxo-1-butyl)-1-imidazolium]-3-aza-4-oxopentanoate was identified in a model reaction mixture containing glycylglycine, glyoxal, and formaldehyde. The structure of this compound was elucidated from the measured mass, ^1H NMR, and ^{13}C NMR spectral data. The stability of the compound as well as the dependence of its formation on pH value of the reaction mixture was studied. This 1,3-symmetrically substituted imidazole represents a new imidazole derivative not previously described either as a product of nonenzymatic browning reactions or as a product of any other reaction.

1,3-Disubstituted imidazoles, in the formation of which the whole molecules of amino acids serve as a source of nitrogen, were found quite recently. The simplest representative of these imidazole derivatives, 3-(carboxymethyl)-1-imidazoliumethanoate [1,3-bis(carboxymethyl)-imidazole], was first isolated as a product of nonenzymatic browning reactions of glycine with glyoxal and formaldehyde (Kratochvíl et al., 1988; Velíšek et al., 1989). Later on, some other 1,3-disubstituted imidazoles, derived from α-dicarbonyls and monocarbonyls other than glyoxal and formaldehyde as well as from amino acids other than glycine, were identified (Velíšek et al., 1989; Davídek, 1990). For example, the reaction of methylglyoxal with glycine and formaldehyde leads to the formation of 4-methyl-3-(carboxymethyl)-1-imidazoliumethanoate; similarly the reaction of methylglyoxal with glycine and acetaldehyde leads to the formation of 2,4-dimethyl-3-(carboxymethyl)-1-imidazoliumethanoate, etc.

1,3-Disubstituted imidazoles arise not only in simple reaction systems containing an α-dicarbonyl compound, amino acid, and aliphatic aldehyde but also during the reactions of amino acids with sugars (Davídek et al., 1990).

1,3-Disubstituted imidazoles could theoretically arise also from dipeptides. It is known that the reaction of dipeptides (as well as tripeptides or tetrapeptides) with α-dicarbonyl compounds leads to the formation of 2-pyrazinone derivatives (Chuyen et al., 1972, 1973a,b).

This study is a part of research on the Maillard reaction of proteins with carbonyls arising by the breakdown of sugars. Its purpose was to prove whether dipeptides (similarly to amino acids) can react with α-dicarbonyl compounds and aliphatic aldehydes under the formation of 1,3-disubstituted imidazoles.

MATERIALS AND METHODS

Chemicals. All chemicals used in this study were of analytical grade. Glycine and formaldehyde (30% aqueous solution) were purchased from Lachema (Brno, Czechoslovakia). Glyoxal (trimeric hydrate) was obtained from Sigma Chemical Co. (St. Louis, MO) and glycylglycine from Reanal (Budapest, Hungary). 3-(Carboxymethyl)-1-imidazoliumethanoate was prepared according to an already described procedure (Velíšek et al., 1989). 2-Oxo-1-(carboxymethyl)pyrazine was prepared according the method of Chuyen et al. (1973a,b).

High-Performance Liquid Chromatography. HPLC measurements were performed on a Spectra Physics SP 8810 instrument equipped with a variable-wavelength detector [Hewlett-Packard HP 1050 (set at 220 nm)]. A 150 × 3.3 mm glass column with Separon TM SGX C_{18} (5 μm) (Tessek Ltd., Czechoslovakia) and acetic acid (c = 0.05 mol L^{-1}) as a mobile phase were used. Flow rate was 0.5 mL min^{-1}, and column temperature was 40 °C.

UV spectra of 1,3-disubstituted imidazoles were measured on a Hewlett-Packard HP 1090 instrument, equipped with a diode array detector, which operated under the above conditions.

Mass Spectrometry. For the mass spectrometric measurements, a Shimadzu QP 1000 quadrupole mass spectrometer was used under the following conditions: The sample was directly introduced into the ion source. The temperrature of the probe was programmed from 25 to 300 °C at 20 °C min^{-1}. The ion source temperature was 305 °C. The electron energy 70 eV.

NMR Spectrometry. The ^1H NMR and ^{13}C NMR spectra were measured on a Bruker AM 400 apparatus (400.13 MHz for ^1H and 100.62 MHz for ^{13}C) in D_2O. Chemical shifts were referred to 2,2-dimethyl-2-silapentane-5-sulfonic acid (Na^+ salt) and transformed to the TMS scale.

Isolation of 5-[3-(3-Aza-4-carboxy-2-oxo-1-butyl)-1-imidazolium]-3-aza-4-oxopentanoate (GlyGly-im-GlyGly). An aqueous solution (50 mL) containing 0.1 mol of glycylglycine, 0.05 mol of glyoxal, and 0.05 mol of formaldehyde was heated for 8 h at 80 °C. After cooling to room temperature, the reaction mixture was evaporated under reduced pressure to 25 mL and stored for 48 h at 5 °C. The resulting crystals were filtered off, recrystallized from water, dried in a vacuum desiccator over silica gel, and analyzed by mass spectrometry and NMR spectrometry.

Stability Study. The solutions containing 0.2 g of GlyGly-im-GlyGly dissolved in 100 mL of Britton-Robinson buffer of pH 1, 4, 8, and 12 were heated at 80 °C. At given intervals, aliquots of these solutions were taken, diluted 100 times with acetic acid (c = 0.05 mol L^{-1}), and analyzed by HPLC.

Influence of pH Value on the Formation of GlyGly-im-GlyGly. Twelve solutions containing 0.005 mol of glycylglycine, 0.0025 mol of glyoxal, and 0.0025 mol of formaldehyde in 25 mL of Britton–Robinson buffer of pH 1–12 were heated for 8 h at 80 °C. After cooling to room temperature, the solutions were 100 times diluted with acetic acid (c = 0.05 mol L^{-1}) and analyzed by HPLC.

Formation of GlyGly-im-GlyGly and 2-Pyrazinone at pH 4. The solution containing 0.005 mol of glycylglycine, 0.0025 mol of glyoxal, and 0.0025 mol of formaldehyde in 25 mL of Britton–Robinson buffer of ph 4 and the solution containing 0.0025 mol of glycylglycine, 0.0025 mol of glycine, 0.0025 mol of glyoxal, and 0.0025 mol of formaldehyde in 25 mL of Britton–Robinson buffer of pH 4 were heated at 80 °C. At given intervals, aliquots of these solutions were taken, diluted 100 times with acetic acid (c = 0.05 mol L^{-1}), and analyzed by HPLC.

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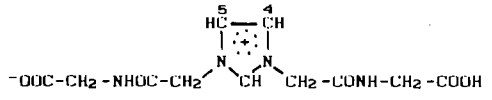
Table I. Mass Spectrum of 5-[3-(3-Aza-4-carboxy-2-oxo-1-butyl)-1-imidazolium]-3-aza-4-oxopentanoate

ions[a]	m/z	%
$(M - R)^+$	183	16
$(M - R - COOH)^+$	138	7
$(C_5H_6N_2O_2)^+$	126	13
$(M - R - NHCH_2COOH)^+$	109	2
$(C_4H_5N_2)^+$	81	100
$(C_2H_4O_2N)^+$	74	9
$(C_3H_4N_2)^+$	68	7
$(COO)^+$ or $(C_2H_6N)^+$	44	46

[a] R = $CH_2CONHCH_2COO^-$.

Table II. ^1H and ^{13}C NMR Spectra of 5-[3-(3-Aza-4-carboxy-2-oxo-1-butyl)-1-imidazolium]-3-aza-4-oxopentanoate in D_2O



position	chemical shift, ppm ^1H NMR	chemical shift, ppm ^{13}C NMR	position	chemical shift, ppm ^1H NMR	chemical shift, ppm ^{13}C NMR
2	9.33	139.50	b		168.28
4, 5	7.97	125.16	c	4.36	44.10
a	5.54	52.65	d		175.67

Table III. Stability of GlyGly-im-GlyGly in Aqueous Solutions at 80 °C

time, h	retention, %, in pH 1	4	8	12
0	100.0	100.0	100.0	100.0
12	50.3	102.1	97.2	0.1
24	29.1	101.1	92.3	0
36	14.4	99.3	91.2	0
48	4.6	101.4	92.4	0
60	3.8	98.6	89.3	0
72	1.7	101.2	87.3	0

RESULTS AND DISCUSSION

The crystalline product isolated from the glycylglycine–glyoxal–formaldehyde model system was analyzed by mass spectrometry and NMR spectrometry.

The mass spectrum of the compound had the base peak $(C_4H_5N_2)^+$ at m/z 81. Other important peaks are $(M - CH_2CONHCH_2COO)^+$ at m/z 183, $(M - CH_2CONHCH_2COO - COOH)^+$ at m/z 138, $(C_5H_6N_2O_4)^+$ at m/z 126, and a peak at m/z 44, which is probably the mixture of $(COO)^+$ and $(C_2H_6N)^+$ ions (Table I).

^1H NMR and ^{13}C NMR spectra of this compound are listed in Table II.

The NMR and MS data revealed that the analyzed compound was 5-[3-(3-aza-4-carboxy-2-oxo-1-butyl)-1-imidazolium]-3-aza-4-oxopentanoate (GlyGly-im-GlyGly).

The mechanism of the formation of this imidazole derivative seems to be similar to that of 3-(carboxymethyl)-1-imidazoliumethanoate [1,3-bis(carboxymethyl)imidazole, Gly-im-Gly] (Velíšek et al., 1989). This mechanism includes the interaction of two molecules of glycylglycine with glyoxal and formaldehyde (Figure 1).

GlyGly-im-GlyGly has two dipeptide bonds in its molecule. As the hydrolysis of these bonds can be expected, the stability of GlyGly-im-GlyGly in water solutions of pH 1, 4, 8, and 12 at 80 °C was studied. The results are summarized in Table III. The data demonstrate that GlyGly-im-GlyGly is stable at pH 4. Under alkaline or acidic conditions, it decomposes to 3-(carboxymethyl)-1-imidazoliumethanoate (Gly-im-Gly).

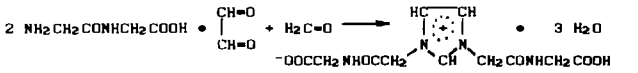


Figure 1. GlyGly-im-GlyGly formation.

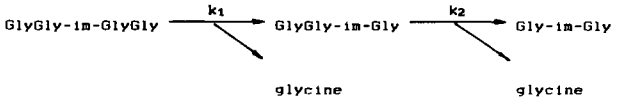


Figure 2. GlyGly-im-GlyGly hydrolysis.

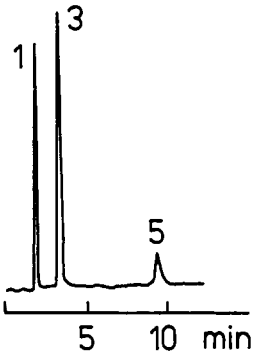


Figure 3. HPLC separation of GlyGly-im-GlyGly solution of pH 12. Peak identification: (1) Gly-im-Gly; (3) GlyGly-im-Gly; (5) GlyGly-im-GlyGly.

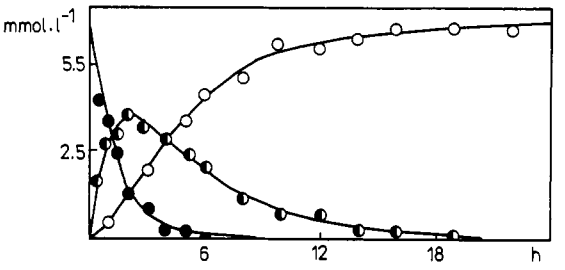


Figure 4. GlyGly-im-GlyGly hydrolysis at pH 12. (●) GlyGly-im-GlyGly; (◐) GlyGly-im-Gly; (○) Gly-im-Gly.

We found that GlyGly-im-GlyGly is degraded by two subsequent reactions of the first order, as illustrated in Figure 2.

HPLC separation of GlyGly-im-GlyGly solution of pH 12, which was heated for 3.5 h at 80 °C, is shown in Figure 3. This solution contained three compounds. Two of them, compounds 1 and 5, were Gly-im-Gly and GlyGly-im-GlyGly, respectively. Compound 3 was the intermediate of GlyGly-im-GlyGly hydrolysis to Gly-im-Gly, as was obvious from the dependence of the concentration of compound 3 on the time of hydrolysis (Figure 4). This intermediate is 3-(3-aza-4-carboxy-2-oxo-1-butyl)-1-imidazoliumethanoate (GlyGly-im-Gly), which arises from GlyGly-im-GlyGly by cleavage of one peptidic bond. The chromatographic behavior of compound 3 (elution between Gly-im-Gly and GlyGly-im-GlyGly) and its UV spectrum (similar to those of Gly-im-Gly and GlyGly-im-GlyGly) correspond well to the proposed structure.

Gly-im-Gly is stable under the above conditions of hydrolysis (Davídek, 1990). The concentrations of GlyGly-im-GlyGly, GlyGly-im-Gly, and Gly-im-Gly are given by

$$c_A = c_A^0 e^{-k_1 t} \quad (1)$$

$$c_B = c_A^0 \frac{k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \quad (2)$$

$$c_C = c_A^0\left(1 - \frac{k_2}{k_2 - k_1}e^{-k_1 t} + \frac{k_1}{k_2 - k_1}e^{-k_2 t}\right) \quad (3)$$

Table IV. GlyGly-im-GlyGly and GlyGly-im-Gly Hydrolysis Rate Constants

pH	k_1, s^{-1}	k_2, s^{-1}	pH	k_1, s^{-1}	k_2, s^{-1}
1	1.5×10^{-5}	5.2×10^{-6}	8	4.8×10^{-7}	1.7×10^{-7}
4	0	0	12	1.8×10^{-4}	6.7×10^{-5}

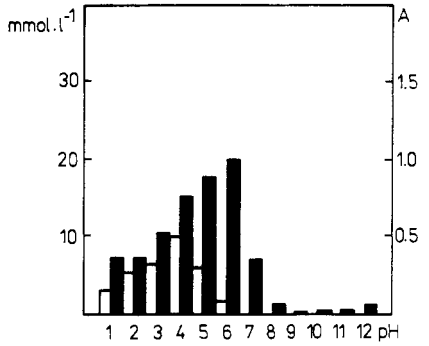


Figure 5. pH influence of GlyGly-im-GlyGly formation. A = absorbance at 420 nm; (□) GlyGly-im-GlyGly; (■) brown discoloration.

where c_A^0 is the concentration of GlyGly-im-GlyGly at time $\tau = 0$, c_A, c_B, and c_C are the concentrations of GlyGly-im-GlyGly, GlyGly-im-Gly, and Gly-im-Gly at time τ, respectively, and k_1 and k_2 are rate constants of the decomposition of GlyGly-im-GlyGly and GlyGly-im-Gly, respectively.

The rate constants k_1 and k_2, which were calculated by using eqs 1–3, are given in Table IV. The data show that the degradation of GlyGly-im-GlyGly is generally acid and base catalyzed.

The influence of pH value on the formation of GlyGly-im-GlyGly in the glycylglycine–glyoxal–formaldehyde reaction system was also studied. The obtained results are summarized in Figure 5. To illustrate the extent of the browning, the absorption of the reaction mixtures at 420 nm was measured, and it is given in the same figure. It is obvious that GlyGly-im-GlyGly arises mainly in weak acidic medium [similarly to 1,3-disubstituted imidazoles derived from amino acids (Davídek, 1990)]. The highest concentration of GlyGly-im-GlyGly was found in the reaction system at pH 4 (9.8 mmol L^{-1}, i.e., 9.8% of the theoretical amount).

The separation of the glycylglycine–glyoxal–formaldehyde reaction mixture of pH 4 by HPLC is shown in Figure 6. As can be seen, the above reaction mixture contained besides glycylglycine (compound 4) and GlyGly-im-GlyGly (compound 5) also one compound (compound 2) which was probably the main product of nonenzymatic browning reactions in this system.

The reaction of glycylglycine with glyoxal leads to the formation of 2-oxo-1-(carboxymethyl)pyrazine (2-pyrazinone) (Chuyen et al., 1973a,b). This compound, which has absorption at 322 nm, can be expected in the glycylglycine–glyoxal–formaldehyde model system, too. The UV spectrum of compound 2 was, therefore, measured. This spectrum showed a maximum at 322 nm. To confirm the structure of compound 2, 2-pyrazinone was synthesized and analyzed under the same conditions. The retention time and UV spectrum of the synthesized 2-pyrazinone were identical with those of compound 2.

The influence of pH value on the formation of 2-pyrazinone in the glycylglycine–glyoxal–formaldehyde model system is shown in Figure 7. It was found that the amount of 2-pyrazinone rises with rising pH value. The concentration of 2-pyrazinone in the analyzed model system of pH 9 or higher (about 81 mmol L^{-1}, corresponds to about

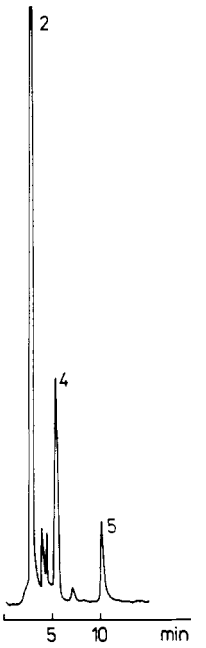


Figure 6. HPLC separation of the glycylglycine–glyoxal–formaldehyde reaction mixture of pH 4. Peak detection: (2) 2-pyrazinone; (4) glycylglycine; (5) GlyGly-im-GlyGly.

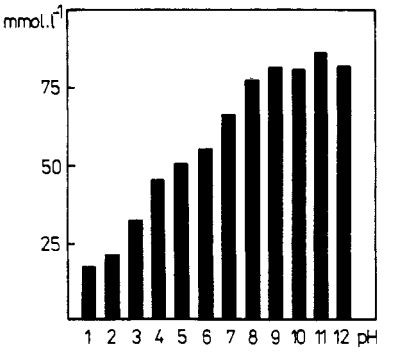


Figure 7. pH influence on 2-pyrazinone formation. (■) 2-Pyrazinone.

81% of the theoretical amount. This fact is probably one of the reasons the extent of the browning was relatively low in alkaline solutions (Figure 5). The concentration of 2-pyrazinone that was found in the reaction mixture of pH 4 (at this pH the highest concentration of GlyGly-im-GlyGly was found) was 44.1 mmol L^{-1} (i.e., 44% of the theoretical amount), whereas that of GlyGly-im-GlyGly was 9.8 mmol L^{-1} (i.e., 9.8% of the theoretical amount). It can be seen that 2-pyrazinone is the main product of nonenzymatic browning reactions in this model system.

As it was already stated, GlyGly-im-Gly arises as an intermediate during the hydrolysis of GlyGly-im-GlyGly. It is highly probable that this compound can arise also by the reaction of glycylglycine with glycine, glyoxal, and formaldehyde. The reaction mixture containing all of these compounds was, therefore, prepared and analyzed. The data shown in Figure 8 demonstrate that GlyGly-im-Gly is actually formed in this model system. Besides this compound, Gly-im-Gly, GlyGly-im-GlyGly, and 2-pyrazinone are formed, too. 2-Pyrazinone is the main product of nonenzymatic browning reactions in this reaction system. The concentrations of 2-pyrazinone, Gly-im-Gly, GlyGly-im-Gly, and GlyGly-im-GlyGly that were found after 8 h of reaction were 26.4, 9.9, 3.5, and 1.3 mmol L^{-1}, respectively.

It seems that in the presence of both amino acid and dipeptide 1,3-disubstituted imidazoles arise preferably

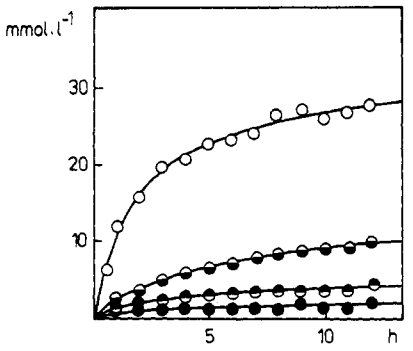


Figure 8. 1,3-Disubstituted imidazoles and 2-pyrazinone formation in the glycylglycine–glycine–glyoxal–formaldehyde reaction system of pH 4. (○) 2-Pyrazinone; (◒) Gly-im-Gly; (◓) GlyGly-im-Gly; (●) GlyGly-im-GlyGly.

from amino acid, but 1,3-disubstituted imidazoles derived from one molecule of amino acid and one molecule of dipeptide as well as those derived from two molecules of dipeptide are formed, too.

From all the obtained data, it is obvious that 1,3-disubstituted imidazoles can arise not only from amino acids but also from dipeptides. The amounts of 1,3-disubstituted imidazoles arising from dipeptides will probably be generally lower than the amounts of 1,3-disubstituted imidazoles arising from amino acids under the same conditions. This is caused mainly by cyclization of dipeptide in the presence of α-dicarbonyl compounds which leads to the formation of 2-pyrazinone derivatives.

It is probable that, similarly to free amino acids and dipeptides, N-terminal amino acids of proteins can also react. In that case, 1,3-disubstituted imidazoles would be bound to proteins. Of course, to confirm this hypothesis, further study would be necessary.

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Registry No. GlyGly-im-GlyGly, 134310-88-6; glycylglycine, 556-50-3; glyoxal, 107-22-2; formaldehyde, 50-00-0; 3-(carboxymethyl)-1-imidazoliumethanoate, 117255-11-5; 3-(3-aza-4-carboxy-2-oxo-1-butyl)-1-imidazoliumethanoate, 134310-89-7; 2-pyrazinone, 42352-55-6.