Amino Acids (2007) 32: 285–289
DOI 10.1007/s00726-006-0338-6
Printed in The Netherlands

Amino Acids

Convenient synthesis of GOLD and MOLD and identification of their oxidation products in vitro and in vivo

M. D. Linetsky and **E. V. Shipova**

Mason Eye Institute, University of Missouri-Columbia, Columbia, Missouri, U.S.A.

Received January 16, 2006
Accepted March 30, 2006
Published online May 26, 2006;

Summary. Two Lys–Lys crosslinks, 1,3-bis-(5-amino-5-carboxypentyl)-1H-imidazolium (GOLD) and 1,3-bis(5-amino-5-carboxypentyl)-4-methyl-1H-imidazolium (MOLD) salts, have been synthesized by the reaction of imidazole or 4(5)-methyl imidazole with 5-(4-bromobutyl)-hydantoin followed by the hydrolysis of 1,3-substituted imidazolium derivatives by 6.0 N HCL at 110 °C. Treatment of GOLD and MOLD with hydrogen peroxide in acetic acid leads to MOLD oxidation only. The oxidation product of MOLD was detected in cataractous lens proteins.

Keywords: Advanced Glycation End Products (AGEs) – GOLD – Lysine crosslinks – Maillard reaction – MOLD

Abbreviations: GOLD, 1,3-bis-(5-amino-5-carboxypentyl)-1H-imidazolium salt; MOLD, 1,3-bis(5-amino-5-carboxypentyl)-4-methyl-1H-imidazolium salt; GO, glyoxal; MGO, methylglyoxal

Introduction

The acid-stable Lys–Lys crosslinks, 1,3-bis-(5-amino-5-carboxypentyl)-1H-imidazolium (GOLD; **6**) (Chellan and Nagaraj, 1999; Odani et al., 1998; Wells-Knecht et al., 1995) and 1,3-bis(5-amino-5-carboxypentyl)-4-methyl-1H-imidazolium (MOLD; **7**) (Biemel et al., 2002; Brinkmann et al., 1995; Nagaraj et al., 1996) salts, are advanced glycation end products (AGEs). GOLD and MOLD increase significantly in tissue proteins such as lens crystallins in cataract (Chellan and Nagaraj, 1999) and collagen in uremia and diabetes (Degenhardt et al., 1998). Imidazole-type AGEs are formed by reaction of glyoxal (GO) (Phillips and Thornalley, 1993) or methylglyoxal (MGO) (Haik et al., 1994) with two protein Lys residues via the formation of diimine intermediates. Subsequent reaction with a second molecule of GO or MGO, followed by dehydration and elimination reactions, yields the five-membered imidazolium ring crosslinks between two Lys residues.

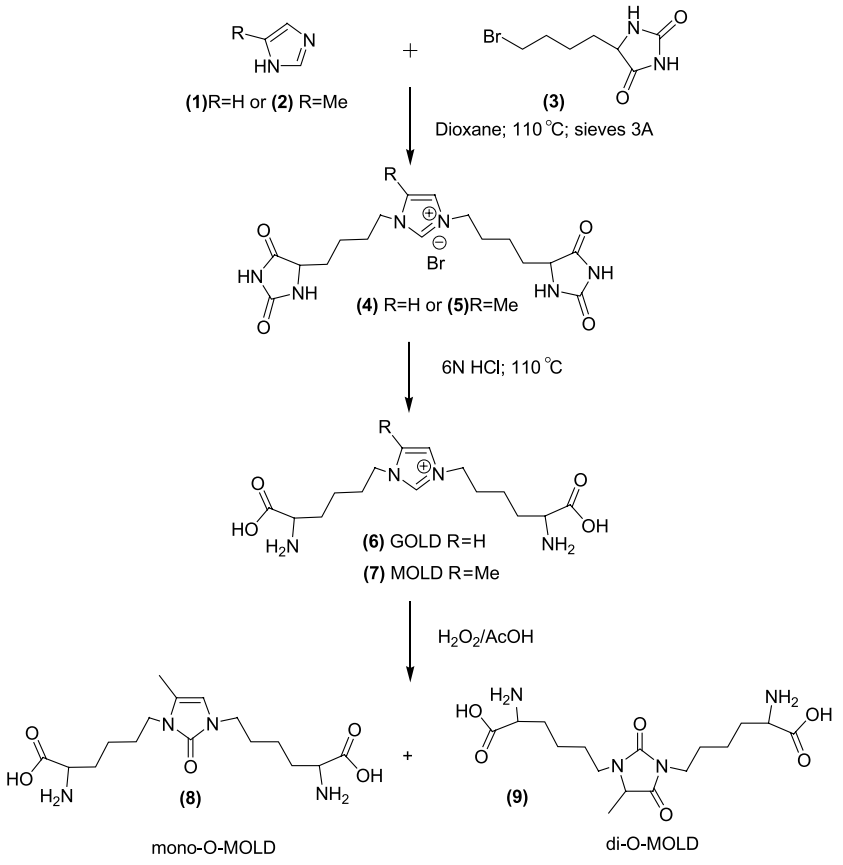
The first step in the chemical syntheses of these crosslinks typically used incubation of N^{α}-hippuryllysine or N^{α}-t-Boc-Lys with GO or MGO in phosphate buffers (Brinkmann et al., 1995; Chellan and Nagaraj, 1999; Nagaraj et al., 1996; Odani et al., 1998; Wells-Knecht et al., 1995). In the second step, the N^{α}-benzoylglycine- or N^{α}-t-Boc- blocking groups were removed by acid hydrolysis and the MOLD and GOLD purified by HPLC, thus producing a relatively low yield of products. Because of these limitations, we developed a high-yield, two-step method for synthesis of GOLD and MOLD (Scheme 1). Given the availability of significant amounts of GOLD (**6**) and MOLD (**7**), we were able to study their oxidation at the imidazolium ring (**8**, **9**).

GOLD and MOLD are di-N, N-substituted imidazole and 4(5)-methylimidazole derivatives (see Scheme 1), respectively. Synthesis of di-substituted imidazoles by alkylhalides has been shown to proceed smoothly, with relatively high yields and only a few side-products in organic solvents (Harlow et al., 1996). In this study we synthesized GOLD and MOLD by a two-step procedure that employs alkylation of commercially available imidazole (**1**) and 4(5)-methylimidazole (**2**), respectively, using 5-(4-bromobutyl) hydantoin (**3**) as the alkylating agent. This reactant has been successfully used for the preparation of anabilysine crosslink (Hardy et al., 1979).

Materials and methods

*Synthesis of di-N, N-hydantoin derivatives of GOLD (**4**) and MOLD (**5**)*

Solutions of (**1**) or (**2**) (5.0 mmol in 5.0 ml of anhydrous dioxane) and (**3**) (10.5 mmol; 2.47 g; in 15.0 ml of anhydrous dioxane) were mixed in a 50-ml glass screw-top vial, containing 10.0 g of 3.0 Å molecular sieves (Aldrich, Milwaukee, WI), blanketed with dry argon, capped and heated at 110 °C for 24 h. The reaction mixture was then cooled, and the dioxane was decanted. The sieves were washed with 3×25.0 ml of anhydrous dioxane and then with 4×25.0 ml of water. Aqueous fractions were



Scheme 1. Synthesis of GOLD and MOLD by the reaction of 5-bromobutyl hydantoin with imidazoles and the formation of MOLD mono- and di-oxidation products by the reaction of MOLD with hydrogen peroxide

combined, filtered through a filter paper and lyophilized, yielding 1.1 g of (**4**) and 1.2 g of (**5**) (58% and 61% yields, respectively; 95% pure based on their MS spectra). For precise mass determination by the high resolution mass spectroscopy, compounds (**4**) and (**5**) were re-purified on a Prodigy ODS (3) preparative column (5 µm particle diameter, 21.2 × 250 mm) using the following gradient: solvent A: 0.1% (v/v) formic acid in water; solvent B: 0.1% (v/v) formic acid in acetonitrile; 2–30% acetonitrile in 45 min at a flow rate of 7.0 ml/min. Reaction products were monitored at λ = 230 nm. Compound (**4**): R_t = 20.2 min; M^+ = 377.1932 Th; molecular formula: $C_{17}H_{25}N_6O_4^+$; ***¹H-NMR*** (250 MHz, DMSO-d_6): 1.28–1.33 (m, 12H, C*H*$_2$-2′,2″,3′,3″,4′,4″); 4.00 (t, J = 6.25 Hz, 2H, C*H*-Hyd); 4.16 (t, $J_{1',2'} = J_{1'',2''}$ = 7.0 Hz, 4H, C*H*$_2$-1″,1″); 7.79 (s, 2H, Im-4,5); 7.99 (s, 2H, N*H*-Hyd); 9.24 (s, 1H, Im-2); 10.65 (s, 2H, N*H*-Hyd). ***¹³C-NMR*** (250 MHz, DMSO-d_6): 20.82 (CH$_2$-3′,3″); 28.87 (CH$_2$-2′,2″); 30.42 (CH$_2$-4′,4″); 48.50 (CH$_2$-1′,1″); 57.23 (CH-Hyd); 122.43 (Im-4,5); 136.00 (Im-2); 157.44 (NC(O)C); 175.99 (NC(O)N). Compound (**5**): R_t = 20.6 min; M^+ = 391.2081 Th; molecular formula: $C_{18}H_{27}N_6O_4^+$; ***¹H-NMR*** (250 MHz, DMSO-d_6): 1.28–1.35 (m, 12H, C*H*$_2$-2′, 2″, 3′, 3″, 4′, 4″); 2.28 (s, 3H, C*H*$_3$-Im); 3.99 (m, 2H, C*H*-Hyd); 4.00–4.13 (m, 4H, C*H*$_2$-1″,1″); 7.54 (s, 1H, Im-5); 7.94 (s, 2H, N*H*-Hyd); 9.13 (s, 1H, Im-2); 10.59 (s, 2H, N*H*-Hyd). ***¹³C-NMR*** (250 MHz, DMSO-d_6): 8.61 (CH$_3$); 20.86 (CH$_2$-3″); 21.02 (CH$_2$-3′); 28.38 (CH$_2$-2″); 28.81 (CH$_2$-2′); 30.43 (CH$_2$-4″); 30.52 (CH$_2$-4′); 45.88 (CH$_2$-1″); 48.35 (CH$_2$-1′); 57.24 (CH-Hyd); 119.23 (Im-5); 130.83 (Im-4); 135.48 (Im-2); 157.40 (NC(O)C); 175.97(NC(O)N).

Acid hydrolysis of dihydantoin derivatives of GOLD (4) and MOLD (5)

Compounds (**4**) (10 mg, 26.5 µmol) or (**5**) (10 mg, 25.6 µmol) were dissolved in 4.0 ml of 6.0 N HCl and placed in a 10-ml glass screw-top vial, blanketed with dry argon, capped and heated at 110 °C for 48 h. The reaction mixture was then cooled, frozen and lyophilized. The contents were redissolved in 0.1% (v/v) formic acid and purified to homogeneity on a Prodigy ODS (3) semi-preparative column (5 µm particle diameter, 10.0 × 250 mm) using the solvents and HPLC gradient described above. The flow rate was 2.5 ml/min. GOLD (**6**, 7.8 mg) and MOLD (**7**, 7.2 mg) were isolated, with 76% and 65% yields, respectively. Compound (**6**): R_t = 26.9 min; high resolution FT-MS (caffeine as an internal standard): M^+ = 327.2027 Th; molecular formula: $C_{15}H_{27}N_4O_4^+$; ***¹H-NMR*** (250 MHz, CD_3OD): 1.53–1.58 (m, 4H, C*H*$_2$-3′,3″); 1.93–2.05 (m, 8H, C*H*$_2$-2′,2″,4′,4″); 4.00 (t, $J_{4',5'} = J_{4'',5''}$ = 6.3 Hz, 2H, C*H*-5′,5″); 4.28(t, $J_{1',2'} = J_{1'',2''}$ = 7.0 Hz, 4H, C*H*$_2$-1″,1″); 7.68 (d, $J_{4,5}$ = 1.47 Hz, 2H, Im-4,5); 9.18 (s, 1H, Im-2). ***¹³C-NMR*** (250 MHz, CD_3OD): 22.97 (CH$_2$-3′,3″); 30.51 (CH$_2$-2′,2″); 30.87 (CH$_2$-4′,4″); 50.42 (CH$_2$-1′,1″); 53.60 (CH-5′,5″); 123.85 (Im-4,5); 137.43 (Im-2); 171.60 (COOH). Compound (**7**): R_t = 30.9 min; high resolution FT-MS: M^+ = 341.2181 Th; molecular formula: $C_{16}H_{29}N_4O_4^+$; ***¹H-NMR*** (250 MHz, CD_3OD): 1.21 (m, 1H); 1.45–1.51 (m, 3H); 1.91–1.99 (m, 8H, C*H*$_2$-2′,2″,4′,4″); 2.37 (s, 3H, C*H*$_3$-Im); 3.71 (m, 2H, C*H*-5′,5″); 4.21–4.25 (m, 4H, C*H*$_2$-1″,1″); 7.42 (s, 1H, Im-5); 8.98 (d, J = 9 Hz,1H, Im-2). ***¹³C-NMR*** (250 MHz, CD_3OD): 7.58 (CH$_3$); 20.67 (CH$_2$-3″); 21.22 (CH$_2$-3′); 28.15 (CH$_2$-2″); 28.68 (CH$_2$-2′); 29.37 (CH$_2$-4″); 29.73 (CH$_2$-4′); 46.11 (CH$_2$-1″); 48.55 (CH$_2$-1′); 53.82 (CH-5′,5″); 119.29 (Im-5); 130.83 (Im-4); 135.71 (Im-2); 175.40 (COOH); 175.97(COOH).

Results and discussion

Imidazole (**1**) or 4(5)-methylimidazole (**2**), when reacted with 5-(4-bromobutyl) hydantoin (**3**) in anhydrous dioxane

at 110 °C for 24 h, produced 1,3-substituted dihydantoin imidazolium salts (**1**) and (**2**), respectively (see Scheme 1). The presence of zeolitic molecular 3 Å sieves (Sigma-Aldrich, Milwaukee, WI) was important in these reaction mixtures because the sieves selectively absorbed sparingly soluble in dioxane di-substituted dihydantoin

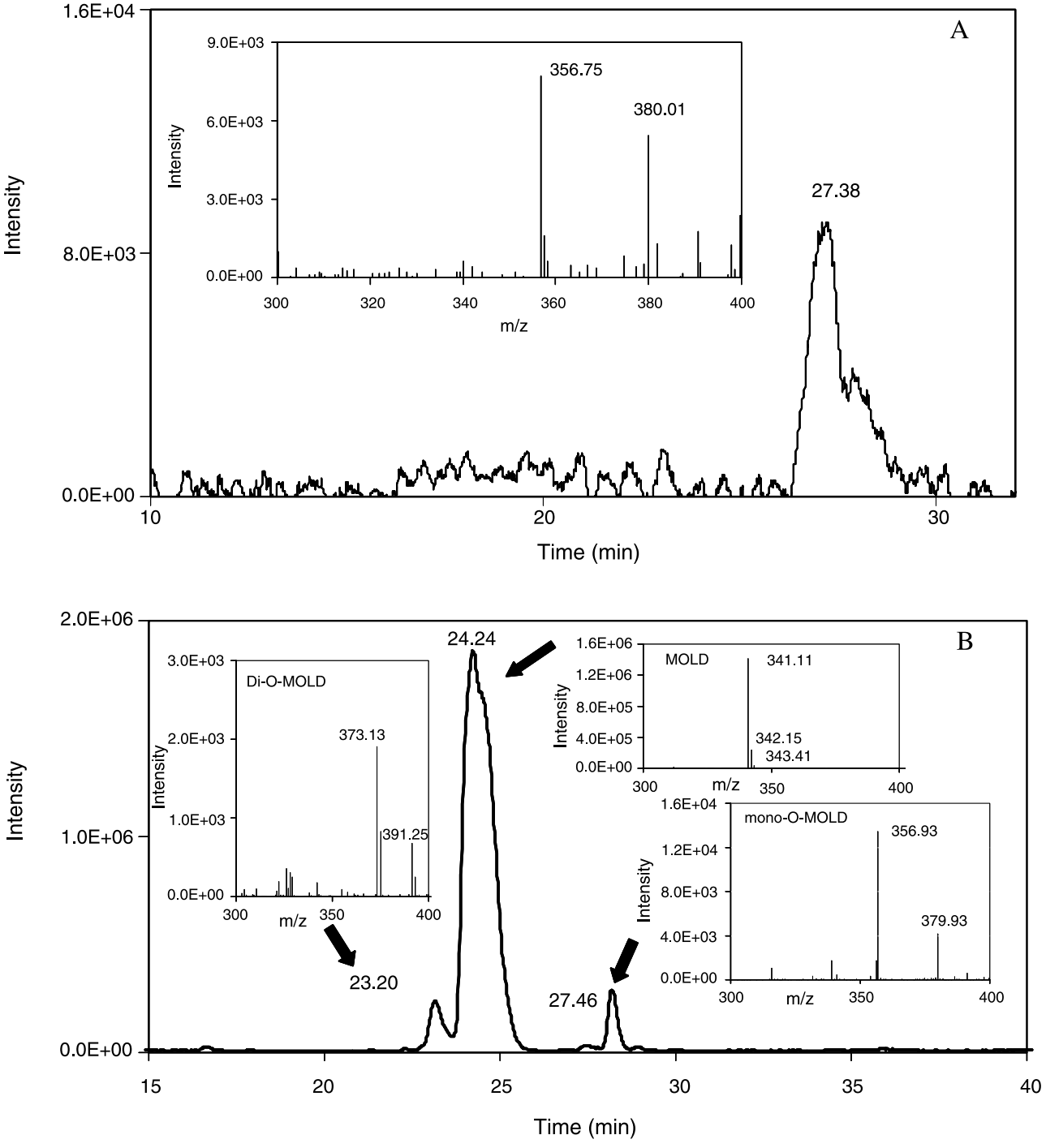


Fig. 1. LC-MS analysis of the brunescent cataract lens protein digest (Cheng et al., 2004), with selective ion monitoring for (**8**) at m/z = 355–358 Th (**A**), and LC-MS analysis with the selective ion monitoring at m/z = 300–400 Th of the reaction mixture of MOLD (**7**; 8.0 mg; 23.5 μmol) and H_2O_2 (0.45 ml of 35% aqueous solution) in glacial acetic acid (1.0 ml) incubated for two hours at ambient temperature (**B**). In **A**, 30.0 mg of a protein digest from brunescent cataracts was applied to a G-10 column (1 × 50.0 cm) operating at flow rate 7.0 ml/h, using 0.1% acetic acid as the eluent with the monitoring at λ = 254 nm. Peak #3 from this separation was used for the LC-MS analysis. The insets in **B** show the partial mass spectra of MOLD and its oxo-derivatives

imidazolium derivatives (**4**, **5**) from the reaction mixtures. The structures of these compounds were confirmed by high-resolution FT-MS. Both compounds showed characteristic hydantoin resonances at 57.2 ppm (CH-Hyd); 157.4 ppm (N*C*(O)C) and 176 ppm (NC(O)N) in their ^{13}C-NMR spectra, consistent with previously reported data for amino acid hydantoins (Huang and Ough, 1991). In addition, the hydantoin derivative (**5**) showed a resonance at 8.6 ppm (CH_3-4), indicating the presence of a methyl group attached to the C_4 carbon of the imidazolium ring (Biemel et al., 2002; Brinkmann et al., 1995; Nagaraj et al., 1996). Both molecules have been shown to possess characteristic resonances for imidazolium ring at 119.2 ppm (C_5), 130.8 (C_4) and 135.5 ppm (C_2) in their ^{13}C spectra (Biemel et al., 2002).

Hydrolysis of (**4**) and (**5**) in 6.0 N HCl for 48 h at 110 °C produced diastereomeric GOLD (**6**) and MOLD (**7**) (see Scheme 1) in the yields of 44% and 40%, respectively, after two-step procedure and preparative HPLC purification. Their identities were confirmed by high-resolution FT-MS analysis, ^{1}H NMR and ^{13}C NMR, and the results correlate well with previously published data for GOLD and MOLD (Biemel et al., 2002; Brinkmann et al., 1995; Wells-Knecht et al., 1995). Thus, unlike the previously published methods for the synthesis of GOLD and MOLD (Brinkmann et al., 1995; Chellan and Nagaraj, 1999; Nagaraj et al., 1996; Odani et al., 1998; Wells-Knecht et al., 1995), an alternative procedure presented here, which is based on alkylation of imidazoles by commercially available 5-(4-bromobutyl) hydantoin, shortens time for the preparation of these crosslinks to 2–3 days, produces reaction mixtures that are easy to purify by HPLC, and affords pure crosslinks in 40–50% overall yields.

Following our study that showed that the enzymatic digests (Cheng et al., 2004) from brunescent cataract lens proteins contained peak with positively charged ion at $m/z = 357$ Th in the peak with the $R_t = 27.5$ min) (Fig. 1A), we hypothesized, by analogy with 2-oxohistidine (Roberts et al., 2001), that this substance in LC-MS profiles represents an oxidized form of MOLD. To verify this hypothesis, we attempted to oxidize unprotected GOLD and MOLD with hydrogen peroxide in acetic acid at ambient temperature for 2 h. GOLD was resistant to oxidation by H_2O_2 (20-fold excess), while MOLD yielded two additional products (**8**, **9**). LC-MS analysis of the MOLD-H_2O_2–AcOH mixture, using specific ion monitoring in the range of 300–400 Th (Fig. 1B), showed that the peak with $R_t = 23.2$ min contained a positively charged ion with $m/z = 373.13$ Th ($[M + H]^+$; 340 Th + 32 Th + H). The presence of this positively charged ion could be tentatively considered to signify the addition of 32 Da to the MOLD molecule (**9**) to positions 2 and 4 in the imidazolium ring. The peak with $R_t = 27.5$ min eluted exactly as the compound found in the digests from brunescent cataractous lenses (see Fig. 1). Similar to digests from brunescent cataractous lenses, it contained a positively charged ion with $m/z = 356.93$ Th ($[M + H]^+$; 340 Th +16 Th + H). The data suggest that a compound with such an m/z ratio could be tentatively identified as the monooxo (M + 16) derivative of MOLD (**8**) at the 2 or 4 position of the imidazolium ring. Work is in progress to establish the precise structure of the compound with $m/z =$ 357 Th and to measure levels of this compound in the normal and cataractous lens.

Acknowledgements

This study was supported by NIH grant EYE13244 and by an unrestricted departmental grant from Research to Prevent Blindness, Inc. We thank Dr. N. D. Leigh, manager of the Mass Spectrometry facility at the University of Missouri-Columbia, for assistance with MS analysis and helpful discussions. We would like to thank Ms S. S. Morey for a skillful editorial assistance.

References

Bambal R, Hanzlik RP (1994) Synthesis of Nε-(p-bromophenyl)-L-lysine and Nτ-(p-bromophenyl)-L-histidine as models for adducts of bromobenzene 3,4-oxide to protein. Observation of an unusual Pd-catalyzed Nτ to Nπ-Aryl substituent migration. J Organ Chem 59: 729–732

Biemel KM, Friedl DA, Lederer MO (2002) Identification and quantification of major Maillard cross-links in human serum albumin and lens protein. Evidence for glucosepane as the dominant compound. J Biol Chem 277: 24907–24915

Brinkmann E, Wells-Knecht KJ, Thorpe SR, Baynes JW (1995) Characterization of an imidazolium compound formed by reaction of methylglyoxal and N^{α}-hippuryllysine. J Chem Soc Perkin Trans 22: 2817–2818

Chellan P, Nagaraj RH (1999) Protein crosslinking by the Maillard reaction: dicarbonyl-derived imidazolium crosslinks in aging and diabetes. Arch Biochem Biophys 368: 98–104

Cheng R, Feng Q, Argirov OK, Ortwerth BJ (2004) Structure elucidation of a novel yellow chromophore from human lens protein. J Biol Chem 279: 45441–45449

Degenhardt TP, Thorpe SR, Baynes JW (1998) Chemical modification of proteins by methylglyoxal. Cell Mol Biol 44: 1139–1145

Haik GM Jr, Lo TW, Thornalley PJ (1994) Methylglyoxal concentration and glyoxalase activities in the human lens. Exp Eye Res 59: 497–500

Hardy PM, Hughes GJ, Rydon HN (1979) The nature of the crosslinking of proteins by glutaraldehyde. Pt 2. The formation of quaternary pyridinium compounds by the action of glutaraldehyde on proteins and the identification of a 3-(2-piperidyl) pyridinium derivative, anabilysine, as a crosslinking entity. J Chem Soc Perkin Trans 9: 2282–2288

Harlow KJ, Hill AF, Welton T (1996) Convenient and general synthesis of symmetrical N, N′-disubstituted imidazolium halides. Synthesis 6: 697–698

Huang Z, Ough CS (1991) Determination of amino acid hydantoins by HPLC with diode array detection. J Agr Food Chem 39: 2218–2222

Nagaraj RH, Shipanova IN, Faust FM (1996) Protein crosslinking by the Maillard reaction. Isolation, characterization, and in vivo detection of a lysine-lysine cross-link derived from methylglyoxal. J Biol Chem 271: 19338–19345

Odani H, Shinzato T, Usami J, Matsumoto Y, Brinkmann FE, Baynes JW, Maeda K (1998) Imidazolium crosslinks derived from reaction of lysine with glyoxal and methylglyoxal are increased in serum proteins of uremic patients: evidence for increased oxidative stress in uremia. FEBS Lett 427: 381–385

Phillips SA, Thornalley PJ (1993) The formation of methylglyoxal from triose phosphates. Investigation using a specific assay for methylglyoxal. Eur J Biochem 212: 101–105

Roberts JE, Finley EL, Patat SA, Schey KL (2001) Photooxidation of lens proteins with xanthurenic acid: a putative chromophore for cataractogenesis. Photochem Photobiol 74: 740–744

Wells-Knecht KJ, Brinkmann E, Baynes JW (1995) Characterization of an imidazolium salt formed from glyoxal and N^{α}-hippuryllysine: a model for Maillard reaction crosslinks in proteins. J Organ Chem 60: 6246–6247

Authors' address: Mikhail Linetsky, Ph.D., Mason Eye Institute, University of Missouri-Columbia, 404 Portland St., Columbia, MO 65201, U.S.A., Fax: +1-573-884-4868, E-mail: LinetskyM@health.missouri.edu