Identification and Quantification of Major Maillard Cross-links in Human Serum Albumin and Lens Protein

EVIDENCE FOR GLUCOSEPANE AS THE DOMINANT COMPOUND*

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Glycation reactions leading to protein modifications (advanced glycation end products) contribute to various pathologies associated with the general aging process and long term complications of diabetes. However, only few relevant compounds have so far been detected in vivo. We now report on the first unequivocal identification of the lysine-arginine cross-links glucoselopane 5, DOGDC 6, MODIC 7, and GODIC 8 in human material. For their accurate quantification by coupled liquid chromatography-electrospray ionization mass spectrometry, 13C-labeled reference compounds were synthesized independently. Compounds 5–8 are formed via the α-dicarbonyl compounds N⁴-(2,3-dihydroxy-5,6-dioxohexyl)-L-lysinate (1a,b), 3-deoxyglucosone (2), methylglyoxal (3), and glyoxal (4), respectively. The protein-bound dideoxyosone 1a,b seems to be of prime significance for cross-linking because it presumably is not detoxified by mammalian enzymes as readily as 2–4. Hence, the follow-up product glucoselopane 5 was found to be the dominant compound. Up to 42.3 pmol of 5/mg of protein was identified in human serum albumin of diabetics; the level of 5 correlates markedly with the glycation state of hemoglobin HbA1c. In the water-insoluble fraction of lens proteins from normoglycemics, concentration of 5 ranges between 132.3 and 241.7 pmol/mg. The advanced glycation end product GODIC 8 is elevated significantly in brunescent lenses, indicating enhanced oxidative stress in this material. Compounds 5–8 thus appear predestined as markers for pathophysiological processes.

The Maillard reaction, also designated as “nonenzymatic browning” or “glycation,” usually is initiated by the reaction of reducing carbohydrates with lysine side chains and N-terminal amino groups of proteins, yielding Amadori compounds (amino ketoses) as primary products. The plethora of compounds, formed in later stages of this process, is summarized under the term “advanced glycation end products” (AGEs) † 1, 2. α-Dicarbonyl compounds such as 3-deoxyglucosone (3-DG, 2; Fig. 1), methylglyoxal (MG, 3), and glyoxal (GO, 4) are established key intermediates in these complex reaction cascades, which proceed both in vitro and in vivo. Whereas 3-DG 2 is formed almost exclusively via the Amadori product, MG 3 derives in vivo mainly from enzymatic or nonenzymatic degradation of triose phosphate intermediates (3, 4) as well as from the metabolism of acetone (5) and threonine (6). Generation of GO 4 requires an oxidation process and proceeds predominantly under physiological conditions via oxidative degradation of Amadori products (7) or lipid peroxidation (8).

A major consequence of the advanced Maillard reaction is the formation of covalently cross-linked proteins; the involvement of α-dicarbonyl compounds in this route may be considered as firmly established. Especially in long lived tissue proteins, the cross-links are supposed to be the most significant AGEs because they are likely responsible, e.g. for the decreased flexibility of collagen (9) and the high level of urea-insoluble proteins in human cataracts (10). They may thus contribute to pathophysiology associated with aging and long term complications of diabetes and atherosclerosis. Although some cross-links have been isolated from tissues (11, 12), the majority has been obtained from in vitro incubations and later demonstrated to be present in vivo. Among these are crossline A,B (13), the imidazolium compounds MOLD 9 and GOLD 10 (Fig. 2) (14–18), as well as GOLA (19). Compounds 9 and 10 are formed by the reaction of two lysine moieties with two molecules each of MG 3 and GO 4, respectively, and they represent the quantitatively most important in vivo cross-links according to the literature data. Studies of Eble et al. (20) indicate, however, that the major cross-links are labile under conventional acid and alkaline hydrolysis conditions; the significance of the acid-

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† To whom correspondence should be addressed. Tel.: 49-711-459-4095; Fax: 49-711-459-4096; E-mail: lederem@uni-hohenheim.de.
‡ The abbreviations used are: AGE(s), advanced glycation end products(a); AGOE(s), advanced glycoxidation end product(s); BSA, bovine serum albumin; 3-DG, 3-deoxyglucosone; DOGDC 6, N⁴-[2-([(4S)-4-ammonio-5-oxido-5-oxopentyl][amino]-5-[(25S,2R)-2,3,4-trihydroxybut- yl]-3,5-dihydro-4H-imidazol-4-ylidene]-L-lysinate; DQFCOSY, double quantum-filtered correlation spectroscopy; glucoselopane 5, 6-[(2-[(4S)-4-ammonio-5-oxido-5-oxopentyl][amino]-6,7-dihydroxy-6,7,8a-tetrahydroimidazo[4,5-b]azepin-4(5H)-yl]-L-lysinate; GO, glyoxal; GODIC 8, N⁴-[2-([(4S)-4-ammonio-5-oxido-5-oxopentyl][amino]-3,5-dihydro-4H-imidazol-4-ylidene]-L-lysinate; GOLA, N⁴-[2-[(4S)-5-ammonio-6-oxido-6-oxohexyl][amino]-2-oxoethyl]-L-lysinate; GODIC 8, 6-[(1-[(4S)-5-ammonio-6-oxido-6-oxohexyl][imidazolium-3-yl]-L-lysinate; HbA1c value, percentage of adult hemoglobin, fructosylated at β-Val-1, relative to native hb (HbA0); HMBC, heteronuclear multiple bond correlation; HPLC, high performance liquid chromatography; HSA, human serum albumin; HSCQ, heteronuclear single quantum coherence; ISTD, internal standard; LC-(ESI)MS, coupled liquid chromatography-electrospray ionization mass spectrometry; MG, methylglyoxal; MODIC 7, N⁴-[(2-[(4S)-4-ammonio-5-oxido-5-oxopentyl][amino]-5-methyl-3,5-dihydro-4H-imida zol-4-ylidene]-L-lysinate; MOLD 9, 6-[(1-[(4S)-5-ammonio-6-oxido-6-oxohexyl][4-methylimidazolium-3-yl]-L-lysinate; MS/MS, tandem mass spectrometry; PBS, phosphate-buffered saline; SIM, selected ion monitoring; t-Boc, tert-butoxycarbonyl.

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Maillard Cross-links in Vivo

**FIG. 1.** Structural formulas of important α-dicarbonyl intermediates. The dideoxyose N^9-(2,3-dihydroxy-5,6-dioxoethyl)-l-lysinate (1a,b) generally is incorporated into a protein and thus differs strikingly from 3-DG (2), MG (3), and glyoxal (4).

![Structural formulas of important α-dicarbonyl intermediates](image1.png)

**FIG. 2.** Structural formulas of major protein cross-links. The stable isotope-labeled compounds glucosepane 5a-d-13C_6, DOGDIC 6a,b-13C_6, MODIC 7-13C_3, and GODIC 8-13C_2 were synthesized independently to allow for an accurate quantification of these cross-links by LC-(ESI)MS. Reference material for MOLD 9 and GOLD 10 has also been prepared.

![Structural formulas of major protein cross-links](image2.png)

stable compounds 9 and 10 might thus have been overestimated. This hypothesis is supported by model experiments that clearly show formation of 9 and 10 to be a secondary process at low α-dicarbonyl levels (19, 21); the physiological concentration, e.g., in plasma of diabetics, is in the lower μM region for both MG 3 and GO 4 (22).

We have shown previously that the lysine-arginine cross-links DOGDIC 6a,b, MODIC 7, and GODIC 8 are obtained in high yield from model reactions of proteins with 3-DG 2, MG 3, and GO 4, respectively (21, 23). Furthermore, we could recently prove formation of the bicyclic cross-link glucosepane 5a-d to proceed via the so far unknown N^9-(2,3-dihydroxy-5,6-dioxo-

hexyl)-l-lysinate (1a,b) (24). In this dideoxyose, the lysine N^9 is bonded directly to C-1 of the original carbohydrate backbone; generation of 1a,b requires carbonyl shifts along the entire carbohydrate backbone.

We now report on the identification and quantification of the cross-links 5–10 in human serum albumin (HSA) from healthy controls and diabetics as well as in the water-insoluble fraction of lens proteins from normoglycemic subjects. To guarantee accurate quantification by LC-(ESI)MS, 13C-labeled standards for 5–8 were synthesized independently and their structures established unequivocally. Additionally, compounds 5–8 were tested for their stability under quasiphysiological conditions. A rationale is given for the finding that glucosepane 5a-d represents the dominant in vivo cross-link.

**EXPERIMENTAL PROCEDURES**

**Materials**

Milli-Q water (purified to 18 megohms-cm; Millipore, Eschborn, Germany) was used in the preparation of all solutions. HPLC grade methanol was employed for LC and LC-MS. For preparative HPLC, solvents were degassed by flushing with helium. Propionic acid, 2-propanol, N^9-t-Boc-l-lysine and N^9-t-Boc-l-arginine, formaldehyde solution (−37%), glyoxylic solution (−40%), and methylyglyoxal solution (−40%) were purchased from Fluka (Neu-Ulm, Germany). n-Heptafaurobutyric acid was from Aldrich (Steinheim, Germany). Phosphate-buffered saline (PBS), peptidase, and aminopeptidase M were from Sigma (Steinheim). N-glucose and Pronase E were from Merck (Darmstadt, Germany), and D-glucose-13C_6 was from Dr. Glaser AG (Basel, Switzerland). For a PBS mixture giving solutions with pH 7.4, KH_2PO_4 (2.68 g, 20 mmol) and Na_2HPO_4·2H_2O (14.30 g, 80 mmol) were mixed vigorously.

**Synthesis of Glucosepane 5a-d-13C_6, DOGDIC 6a,b-13C_6, MODIC 7-13C_3, and GODIC 8-13C_2**

N^9-t-Boc-l-lysine (1.52 g, 6.2 mmol), N^9-t-Boc-l-arginine (1.15 g, 4.2 mmol), and N-glucose-13C_6 (363 mg, 1.95 mmol) were dissolved in 10 ml of water, and the pH was adjusted to 7.4 by adding solid NaHCO_3 (synthesis of 5a-d-13C_6 or phosphate buffer (1.7 g, 10 mmol); synthesis of 5a-d-13C_6) or phosphate buffer (1.7 g, 10 mmol). The mixtures were kept at 50 °C for 10 days and purified by preparative HPLC (column A, gradient A). Fractions with t_R 14.1 min (fraction I), 14.6 min (fraction II), 15.1 min (fraction III), and 15.6 min (fraction IV) were isolated and lyophilized.

**Fractions—**Each fraction was dissolved in 1.5 ml of aqueous 3 N HCl—D_2O (25 °C).

![Structural formulas of major protein cross-links](image2.png)

![Structural formulas of glucosepane](image3.png)

![Structural formulas of important α-dicarbonyl intermediates](image1.png)
The mixture was kept at 50 °C (column A, gradient A). Fractions with the mixture was subjected to preparative HPLC (column A, gradient B). The NMR data of the unlabeled compounds 6–8 are given in Refs. 21 and 23.

\( ^{13}C \) NMR data for the labeled positions of DOGDIC 6a,b, 12C<sub>3</sub> MODIC 7, 13C<sub>4</sub> and GODIC 8-13C<sub>3</sub> (D<sub>2</sub>O, 25 °C)

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<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
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<th>7-13C&lt;sub&gt;3&lt;/sub&gt;</th>
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\( ^{1}{H} \) NMR and \( ^{13}C \) NMR data of MOLD 9 and GOLD 10 (D<sub>2</sub>O, 25 °C)

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<tr>
<td>J(Hz)</td>
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\( ^{13}C \) NMR data of MOLD 9 and GOLD 10 (D<sub>2</sub>O, 25 °C)

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**Type 1 Diabetics and Control Subjects**

Permission to use blood from type 1 diabetic patients was obtained from the local ethics committee, and each patient was informed about the aims of the study. The patients were selected on the following criteria: age, ≤ 40 years; duration of diabetes, ≤ 2 years; normal triglyceride (75–150 mg/dl) and cholesterol (120–200 mg/dl) levels; no blood transfusion within the previous 3 months. The HbA<sub>1C</sub> values were supplied by the hospital laboratory; they were determined by ion exchange chromatography according to a standard protocol and range between 4.3 and 6.1% for normoglycemics. Controls were healthy, non-diabetic volunteers (age ≤ 40 years) from the Institute of Food Chemistry of the University of Hohenheim and from the Burgerhospital.

**Water-insoluble Proteins from Human Lenses**

These were generously supplied by R. H. Nagaraj (Department of Ophthalmology, Case Western Reserve University, Cleveland, OH). The samples were obtained from normoglycemic subjects (age 59–87 years) and comprised material with the following classifications: normal, type I + III cataract, and brunescent cataract.

**Isolation of HSA**

Fresh blood (9 ml) from type 1 diabetics and non-diabetic individuals was collected into sterile tubes containing EDTA and centrifuged within 2 h (2,500 x g, 4 °C, 30 min). Trichloroacetic acid (2 ml, 20%) was added to the plasma (2 ml), and the suspension was mixed vigorously and kept for 15 min. Precipitated proteins were pelleted by centrifugation (2,500 x g, 4 °C, 15 min). The pellet was taken up in phosphate buffer (3 ml, 0.1 M, pH 7.4) and centrifuged again. The proteins were suspended in the phosphate buffer (3 ml), transferred to an Amicon
Enzymatic Protein Hydrolysis and LC Clean-up of the Cross-links

Aliquots of plasma proteins (3 mg) and water-insoluble lens proteins (1.5 mg), respectively, were hydrolyzed enzymatically according to the procedure of Glomb and Pfahler (19), protocol C. A stock solution (10 μl) containing 4.5 mg/liter 5a-d,13C6, 2.4 mg/liter 6a,b,13C6, 2.4 mg/liter 7,13C5, and 2.4 mg/liter 8,13C5 was added at the outset of the digestion procedure. The hydrolysates were ultrafiltered by centrifugation (Millipore Ultrafree MC, molecular mass cutoff, 5 kDa) and the filtrates (1 ml) subjected to LC-(ESI)MS analysis (gradient B, SIM mode). The remaining solutions were diluted to 1.5 ml and subjected to preparative HPLC (column B, gradient C). The eluate within 3.5–10 min was collected, lyophilized, and the residue was dissolved in 150 μl of water and subjected to LC-(ESI)MS analysis (gradient B, SIM mode).

Preparative HPLC

The preparative HPLC system consisted of a Konrak (Sinheim, Germany) KD200/100SS gradient pump system combined with a Knauer (Berlin, Germany) A0293 variable wavelength detector and a (A) Konrak HPLC column (guard column 50 × 20 mm, column 250 × 20 mm, Nucleosil C18, 100 Å, 7 μm), (B) YMC HPLC column (YMC Europe; Schermbeck, Germany; 250 × 20 mm, YMC-Pack ODS A, 120 Å, 10 μm); injection volume, 1.5 ml; flow rate, 18 ml/min. The following gradients were applied: (A) ammonium formate buffer (10 mM, pH 4.0) and 30% MeOH at 0 min, 70% at 15 min, 100% at 16–21 min, and 30% at 24–30 min. (B) 0% MeOH at 0 min, 6% at 6–10 min, and 0% at 11–16 min. (C) 0% MeOH at 0 min, 5% at 6–10, 95% at 13–15 min, and 0% at 18–23 min. Detection was at 244 nm (5–8) or 230 nm (9, 10).

LC-(ESI)MS Analyses

These were run on an HP1100 (Hewlett Packard, Waldbronn, Germany) HPLC system coupled to a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer equipped with an electrospray (ESI) interface. The HPLC system comprised an HP1100 autosampler, HP1100 gradient pump, HP1100 thermostor, and HP1100 diode array detector (DAD) module. The column was a YMC- Pack Pro C18, 120 A, 5 μm (guard column 10 × 4.6 mm, column 150 × 4.6 mm); column temperature 25 °C; flow rate 1.0 ml/min; injection volume 20 μl. The gradients were: (A) 10 mM ammonium formate buffer (pH 4.0) and 5% MeOH at 0 min, 95% at 30–40 min, and 5% at 45–50 min. (B) 10 mM n-heptafluorobutyric acid and 5% MeOH at 0 min; 50% at 25 min, 95% at 30–35 min, and 5% at 40–47 min. For acquisition in the SIM mode, postcolumn addition of propionic acid/2-propanol (3:1, v/v) in a 1:3 ratio with the HPLC mobile phase was performed with a Knauer WellChrom Maxi-Star K-1000 HPLC pump. The eluent was finally split 1:20 before being introduced into the ion source. MS parameters: ESI1, source temperature, 120 °C; capillary, 3.5 kV; HV lens, 0.5 kV; cone, 55 V. The MS system was operated either in full scan (m/z 200–1,000) or in SIM mode (cone, 65 V; span, 0.5 Da; dwell time, 0.2 s; monitoring masses at m/z 453.3, 447.3, 429.3, 360.25, 357.25, 345.25, 343.25, or 341.25, and 327.25).

NMR Spectroscopy

1H and 13C spectra were recorded at 25 °C, in D2O, on a Bruker (Karlsruhe, Germany) ARX 500 spectrometer (500 and 126 MHz nominal frequency, respectively) and/or a Varian (Darmstadt, Germany) Unity-plus 300 instrument (300 and 75 MHz). The Unity-plus 300 was also used for the acquisition of 2H,1H DQF COSY, HSQC, and HMBC (Varian standard library) spectra.

Lyoophilization

A Leybold-Heraeus (Koln, Germany) Lyovac GT 2 and a Labconco Centrivap Concentrator (Kansas City, MO) were applied.

Statistics

The significance was determined according to the U test by Wilcoxon, Mann, and Whitney; p < 0.05 was considered to represent statistically significant differences.

RESULTS

Synthesis of 13C-Labeled Reference Material and Determination of the Cross-links by LC-(ESI)MS—as reported previously, the cross-links glucosepane 5a-d, DOGDIC 6a,b, MODIC 7, and GODIC 8 (Fig. 2) are unstable under the conventional conditions for acid protein hydrolysis (110 °C, 24 h); this also holds for the pentose-derived structures pentosanone and DOP-DIC (21, 23). Determination of such compounds hence requires enzymatic digestion of the protein and follows a protocol developed by Glomb and Pfahler (19), proven to yield nearly quantitative hydrolysis even for highly cross-linked proteins. In vivo, hexoses clearly dominate over pentoses, which are either originally present in the living organism or formed from hexoses by the so-called autoxidative glycosylation (25). Pentose follow-up products thus are expected to be quantitatively less significant; the prominent cross-link pentosidine, for instance, is a well established AGE marker but known to be a minor product (18). Our investigation therefore was focused on the hexose-derived structures 5a-d and 6a,b as well as the products 7,8, resulting from the reaction with major short chain α-diarylcarboxyl compounds. To allow for an accurate quantification, reference compounds of 5–8 (see Fig. 2) labeled with stable isotopes were synthesized independently from d-glucose-13C6 and their structures unequivocally established; relevant 13C NMR data are compiled in Tables I and II. The 13C-labeled material was used as an internal standard (ISTD) and added prior to the enzymatic digestion procedure to compensate for any losses which might occur during work-up.

Because only minor amounts of the 13C standards are available, no stock solutions with a defined content could be prepared directly. The ISTD concentrations of 5a-d,13C6, 6a,b,13C6, 7,13C5, and 8,13C5 therefore were determined by adding known amounts of the unlabeled compounds 5–8 to constant aliquots of the respective stock solution. This procedure is justified because of the virtually identical (ESI)MS response for labeled and unlabeled cross-links. This way, the LC-(ESI)MS system was calibrated in the range of 1.4–364.4 μg of 5/liter (ISTD 20.9 μg of 5,13C5/liter), 2–274.1 μg of 6/liter (ISTD 17.0 μg of 6,13C5/liter), 2.1–540.6 μg of 7/liter (ISTD 23.7 μg of 7,13C5/liter), and 2.1–538.6 μg of 8/liter (ISTD 16.7 μg of 8,13C5/liter), respectively. The linear calibration graphs are described by the equations area = (−53 ± 294) + (1,041 ± 4) liter/μg × c(5), area = (267 ± 581) + (928 ± 10) liter/μg × c(6), area = (571 ± 543) + (1,127 ± 9) liter/μg × c(7), and area = (−4,532 ± 2,792) + (1,477 ± 45) liter/μg × c(8), the values in parentheses representing means ± confidence intervals (p = 95%). The standard error was determined as 179, 455, 425, and 1,521, respectively. Limits of detection 1.2, 2.8, 2.2, or 25 μg/liter and limits of quantitation 1.8, 4.2, 3.7, or 12.6 μg/liter for 5, 6, 7, or 8 were calculated according to the recommendations of the Deutsche Forschungsgemeinschaft (26). With this ISTD method, cross-links 5–8 were identified and quantified in HSA and water-insoluble lens proteins from diabetic and healthy controls by LC-(ESI)MS in the SIM mode. All samples were screened also for MOLD 9 and GOLD 10. The respective reference compounds were likewise synthesized independently and their structures established unequivocally; the fully assigned 1H and 13C NMR data for 9 and 10 are listed in Table III. LC-(ESI)MS analyses were performed directly from the enzymatic hydrolysates as well as after preparative HPLC clean-up on a C18 column. This step affords both an enrichment of the monitored compounds and less interference with coeluting material.

Cross-links in HSA—HSA was obtained from 11 healthy, nonobese individuals and 18 diabetics (Hba1c, 5.4–16.8%; median, 7.7%); for two controls, the Hba1c values were determined as 5.2 and 5.3%. Typical LC-(ESI)MS chromatograms for the work-up of HSA from a diabetic are given in Fig. 3. The ion traces for 5–8 and the corresponding 13C reference compounds show clearly that all of these cross-links except for GODIC 8...
can be detected unequivocally. Chromatogram D in fact displays a small signal with the retention time of 8-$^{13}$C$_2$ (trace C); however, the peak shape is different, i.e. the typical fronting cannot be observed. Because the peak area is below that of the calculated limit of detection, GODIC 8 is classified as not detected. In the case of MOLD 9 and GOLD 10, no signals were observed at the established retention times in the respective quasimolecular ion traces. The corresponding chromatograms appear virtually identical to those shown in Fig. 6, traces B and D (see below), and therefore are not included in Fig. 3. This result was very surprising because inconsistent but relatively high amounts of 9 and 10 in plasma proteins are reported in the literature (14, 16, 17). The effectiveness of the enzymatic digestion procedure, employed in our experiments, was verified by the determination of GOLD 10 (19) and confirmed to be analogous for MOLD 9. Nevertheless, we also worked up the incubations, following the protocols in the respective references. In our hands, neither 9 or 10 could be detected with these methods. When independently synthesized reference material was added to some samples in a concentration comparable with that given in the literature, the signals for 9 and 10 were clearly evident. It is thus proven that these signals are not suppressed by ESI quenching effects that might result from coeluting material.

In plasma proteins from normoglycemic subjects, 13.1–19.8 pmol/mg of protein (median, 17.1 pmol/mg; n = 11) of the bicyclic structure glucosepane 5 and 3.7–4.5 pmol/mg of protein (median, 4.1 pmol/mg; n = 11) of MODIC 7 were detected. In diabetics, the levels of 5 are significantly higher: 13.4–42.3 pmol/mg of protein (median, 29.2 pmol/mg; n = 18; p < 0.001 (27)). With 3.7–5.5 pmol/mg of protein (median, 3.9 pmol/mg; n = 18), the values for 7, in contrast, are in the same range as for the nondiabetic group. A significant correlation becomes apparent between the HbA$_{1c}$ values and the glucosepane 5 levels (r = 0.844, p < 0.001 (28)), with the relationship described by the linear regression straight line given in Fig. 4; as mentioned above, the MODIC 7 levels are almost independent from HbA$_{1c}$. The concentrations determined for DOGDIC 6 in plasma proteins were in the range of the limit of quantitation: 1.0–2.1 pmol/mg of protein for normoglycemics (median, 1.9 pmol/mg; n = 11) and 1.6–4.0 pmol/mg of protein for diabetics. This represents a significant increase (median, 2.3 pmol/mg; n = 15; p < 0.001 (27)).

Cross-links in Human Lens Proteins—Water-insoluble lens proteins were obtained from normoglycemic (59–87 years; n = 14), including patients with cataract types I and III as well as brunescent cataract. Compounds 5–8 were clearly identified by LC-(ESI)MS analysis, as outlined for a brunescent cataractous lens in Figs. 5 and 6. Contrary to the HSA samples, the presence of GODIC 8 is now definitely established. From a comparison of trace H in Fig. 3 and trace D in Fig. 5, the ratio of the diastereoisomer pairs a,b and c,d for glucosepane 5 (24) is shown to change from 2:1 to almost 1:1. The ratio thus seems to depend on the protein that is cross-linked by 5. The ion chromatograms B and D in Fig. 6 for MOLD 9 and GOLD 10, respectively, display no signal at the retention time of the

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**Fig. 3.** LC-(ESI)MS analysis of enzymatically hydrolyzed HSA from a diabetic. Total ion current (trace I) and quasimolecular ion traces (A–H) for the cross-links 5–8 and their respective $^{13}$C-isotopomers are given. Except for GODIC 8 (trace D), all compounds could be identified unequivocally. The enzymatic digest was purified by preparative HPLC prior to LC-(ESI)MS analysis.

**Fig. 4.** HbA$_{1c}$ values of 11 controls and 18 diabetics plotted versus the glucosepane 5 and MODIC 7 levels in HSA. Whereas the amount of 5 shows a positive correlation with the HbA$_{1c}$ value, the MODIC 7 contents are virtually identical for both subject groups, suggesting the MG 3 concentration to be independent from HbA$_{1c}$.
reference compounds (traces A and C). As in the HSA specimen, no 9 and 10 are detected in the brunescent lens material; this result has also been validated by employing different digestion procedures and standard addition.

No significant cataract-related increase was observed for the cross-links glucosepane 5 and MODIC 7 (Fig. 7); the amounts range from 132.3 to 241.7 pmol/mg of protein (median, 185.1 pmol/mg; n = 4) for 5 and 40.7 to 97.2 pmol/mg of protein (median, 65.5 pmol/mg; n = 4) for 7. A marginal increase was found for DOGDIC 6 in proteins from brunescent lenses: 5.6–8.0 pmol/mg of protein (median, 6.4 pmol/mg; n = 5; p < 0.001 (27)) compared with 1.3–6.0 pmol/mg of protein (median, 3.0 pmol/mg; n = 9) in the normal as well as cataractous lenses. In contrast, the levels for GODIC 8 (Fig. 8) with 2.4–43.5 pmol/mg of protein (median, 21.1 pmol/mg; n = 5; p < 0.001 (27)) were up to 40-fold in brunescent lenses compared with normal and nonbrunescent cataractous lenses. In the latter, the GODIC contents are close to the limit of detection and hence can only be estimated to range between 1.2 and 2.3 pmol/mg of protein (median, 1.8 pmol/mg; n = 9).

Stability of the Cross-links 5–8 under Quasiphysiological Conditions—A mixture of glucosepane 5, DOGDIC 6, MODIC 7, and GODIC 8, (20 mg/liter each) was incubated in PBS at 37 °C, pH 7.4, for several weeks. Aliquots were taken at regular intervals and analyzed by LC-(ESI)MS. Compounds 5, 6, and 7 proved to be fairly stable under the given conditions. In contrast, only about 40% of the initial amount of DOGDIC 6 was recovered after 3 weeks (see Fig. 9, curve B). It could be shown that 6 is in part transformed into the spiro structures \( N^\alpha[^65R*,^7R,^8S] - 2\{(4S) - 4\text{-amino-5-oxido-5-oxopentyl}[6\text{-oxa-1,3-diazaspiro}[4.4][2.1]dicyclohexylidene}-1\text{-lysine}\) (11) and \( N^\alpha[^{5R}*[^{5R}*[^{8R}*[^{8S}*] - 2\{(4S) - 4\text{-amino-5-oxido-5-oxopentyl}[6\text{-oxa-1,3-diazaspiro}[4.4][2.1]dicyclohexylidene}-1\text{-lysine}\) (12) (Fig. 10; data not shown). Compounds 11 and 12 are formed from 6 via an oxidation step and thus represent AGOEs; the reaction rate is pH-dependent and negligible at values < 5. For comparison, ultrafiltered BSA that had been modified with 5–8 by incubation with d-glucose was kept under conditions identical to those of the free compounds. Again, aliquots were taken at regular intervals, the protein digested enzymatically, and the cross-links quantified by LC-(ESI)MS. In this case, all investigated compounds proved to be stable during incubation. Protein-bound DOGDIC 6 thus has a different chemical reactivity as the free form. The amounts of 5–8 even increase slightly over time, as exemplified for 6 in Fig. 9, curve A. This can be rationalized by the well established fact that glycosylated proteins continue to cross-link in the absence of d-glucose via their protein-bound precursors (20).

### DISCUSSION

Cross-linking and insolubilization are among the most significant biochemical changes that proteins suffer during aging; these processes are especially prominent in long lived proteins such as those of the eye lens. Their enhancement in extracellular matrix is implicated in a number of age- and diabetes-associated complications, e.g. arteriosclerosis and cataract formation. In recent years, much effort has therefore been focused on the identification of the major cross-linking units derived from the Maillard reaction. In the present study, we have shown for the first time that the lysine-arginine cross-links glucosepane 5a-d, DOGDIC 6a-b, MODIC 7, and GODIC 8 can be detected unequivocally in human material; all compounds derive from \( \alpha \)-dicarbonyl precursors. Among these, the dideoxyosone 1 seems to hold an exceptional position because its follow-up product 5 turned out to be by far the quantita-
tively most important (see Figs. 4 and 7). This is very likely due to the fact that 1 is linked irreversibly to the protein by N\textsubscript{9280} of a lysine moiety, whereas 3-DG, MG, and GO exist in an equilibrium between free and loosely protein-associated form. Hence, 2–4 can be detoxified much easier by mammalian enzymes, widely distributed throughout the body to protect a large number of tissues against damage. Both the NADPH-dependent aldose reductase and aldehyde reductase were proven to convert 2-oxoaldehydes to monocarbonyl derivatives; 3-DG thus is transformed into 3-deoxy-D-fructose (29, 30). MG and GO are also disposed by these enzymes or the glyoxalase system; the enzyme kinetics of glyoxalase I indicate the activity of the enzyme \textit{in situ} to be proportional to the concentration of glutathione (31). Because the protein-bound dideoxyosone 1 is hardly accessed by such enzymes, it represents a persistent glycating agent. The high affinity of 1 toward the guanidine group of the arginine side chain is another argument for the high \textit{in vivo} derivatization quota of proteins by glucosepane 5.

It could be shown that formation of 5 in model incubations of D-glucose with N\textsubscript{9280}-t-Boc-L-lysine and N\textsubscript{9280}-t-Boc-L-arginine is suppressed only in the presence of a 2-fold molar excess of o-phenylenediamine relative to the N\textsubscript{9280}-t-Boc-L-arginine concentration (data not given). o-Phenylenediamine is one of the most effective \(\alpha\)-dicarbonyl trapping reagents, yielding quinoxaline derivatives (24); \textit{i.e.} the guanidine function is an excellent

\textbf{FIG. 7.} Levels of glucosepane 5, DOGDIC 6, and MODIC 7 in the water-insoluble fraction of lens proteins. The levels for these cross-links are more or less independent of the lens type. The values for 5 establish the prime quantitative significance of this compound.

\textbf{FIG. 8.} Levels of GODIC 8 in the water-insoluble fraction of lens proteins. Because GODIC 8 represents an AGOE, the highly elevated amounts found in most brunescent lenses suggest that such material suffers enhanced oxidative stress.

\textbf{FIG. 9.} Time course for the degradation of the cross-link DOGDIC 6 in PBS. Compounds 5, 7, and 8 prove fairly stable when stored at 37 °C in PBS; only about 40% of the initial amount of DOGDIC 6, in contrast, was recovered after 3 weeks (curve B). Surprisingly, 6 is much more stable when incorporated into a protein; curve A was obtained by analysis of enzymatically digested BSA, modified with 5–8 by incubation with \(\alpha\)-glucose, and kept under identical conditions as the free compounds.

\textbf{FIG. 10.} Oxidation of DOGDIC 6 yields the spiro compounds 11 and 12. Part of the degradation of 6 (see Fig. 9, curve B) can be rationalized by the transformation to 11 and 12. Trace amounts of these dehydrogenation products were detected also in brunescent lenses.
Maillard Cross-links in Vivo

![Diagram of reaction pathways](image)

Fig. 11. Formation of the cross-links glucosepane 5a-d and crossline 16a,b. a, reaction pathway as established for the generation of 5a-d. The dideoxyosone 1a,b very likely undergoes ring closure to the intramolecular aldimine 13a,b, which then reacts with an arginine side chain, b, hypothetical reaction pathway for 16a,b via the ketimine 15a,b and subsequent aldol condensation of this structure with an aminoketose.

The precursor 1 and 2 of glucosepane 5 and DOGDIC 6 are formed from d-glucose via the aminoketose, correlating with the levels for these cross-links in HSA with the HbA1c value was expected and is visualized for 5 in Fig. 4. Although the half-life of erythrocytes (120 days) is significantly longer than that of HSA (12-20 days (34)) and thus glycosylation might be different because of long term fluctuations in the blood glucose level, the correlation coefficient of 0.844 is quite good. Other than glucosepane 5, DOGDIC 6 is present only in minute amounts (for probable reasons see above); nevertheless it is the first 3-DG 2-derived cross-link detected in vivo. Skovsted et al. (35) have described another imidazolium cross-link, termed DOLD, which is structurally analogous to MOLD 9 and GOLD 10 and formed by reaction of 2 with protein, but this compound has not yet been identified in biological matrices. Surprisingly, the MODIC 7 levels in HSA showed no positive correlation with HbA1c, even though many reports in literature suggest an elevated plasma level for MG 3 in diabetes (36). Recent investigations of Beisswenger² have shown, however, that the MG 3 concentration in plasma ranges between 100 and 250 nM and may be independent from HbA1c (37). This finding is supported by our results, which prove the MODIC 7 levels of 3.7–5.5 pmol/mg HSA to be likewise almost constant for normoglycemics and diabetic individuals. Upon acid hydrolysis and HPLC determination with fluorescence detection of the α-phthalidialdehyde derivative of MOLD 9, Nagaraj et al. (14) found 261.3 ± 50.4 pmol of 9/mg of HSA for healthy subjects. In contrast, the estimates for 9 of Odani et al. (17) were significantly lower (34 ± 23 pmol of 9/mg of HSA) for normal controls. These authors applied enzymatic hydrolysis and quantified 9 and GOLD 10 (28 ± 21 pmol of 10/mg of HSA) by LC-(ESI)MS/MS with reference compounds labeled with stable isotopes. The apparent discrepancies may on one hand be traced back to the digestion procedure: acid hydrolysis proceeds under drastic conditions, and the formation of artifacts cannot be excluded. On the other hand, the LC-(ESI)MS/MS procedure provides a much greater specificity. The enzymatic digestion protocol, employed in this work, was proven in own experiments to liberate about two-thirds of MOLD 9 and GOLD 10 (19) from BSA, compared with acid hydrolysis. Although the limits of detection for 9 and 10 thus should definitely be < 10 pmol/mg of protein, we were not able to detect MOLD 9 and GOLD 10 in the HSA of our individuals. This result supports the hypothesis that the role of 9 and 10 in protein cross-linking so far has been overestimated.

Glucosepane 5 represents the dominant cross-link also in lens protein (132.3–241.7 pmol/mg of protein; see Fig. 7). MOLD 9, GOLD 10, vespertisine A, pentosidine, and the amide cross-link GOLA can be compared directly with our results because it has been determined upon identical work-up procedures. Once more we were not able to reproduce the findings for 9 and 10 with our methodology. As for DOGDIC 6 (1.3–8.0 pmol/mg of protein) and MODIC 7 (40.7–97.2 pmol/mg of protein), the values for 5 are more or less independent of the lens type. This result is not surprising because material only from normoglycemic subjects was investigated. In the case of GODIC 8, the situation is completely different: whereas the levels for 8 are close to the limit of quantitation in both normal and cataractous lenses (types I and III), most of the bruneosine ones show enormously elevated contents and additionally minute amounts of the spiro structures 11 and 12. Because GODIC 8 as well as 11 and 12 (Fig. 10), which stem from the dehydrogenation of DOGDIC 6, all are AGOE, these findings strongly

² P. J. Beisswenger, personal communication.
support the hypothesis that such lenses are exposed to an enhanced oxidative stress. The high levels for 4 seem to result from an increased GO 4 formation rather than from a deficiency in detoxification because the levels for MODIC 7, resulting from MG 3, are not elevated in brunescent lenses. As already discussed above, both of these α-dicarbonyl compounds are disposed via identical pathways. In this context, the Roche European Cataract Trial (REACT) has recently shown that the progression of cataracts is slowed down by a
