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Cross-linking of Proteins by Maillard Processes—Characterization and Detection of a Lysine-Arginine Cross-link Derived from D-Glucose

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Abstract—Covalently cross-linked proteins are among the major modifications caused by the advanced Maillard reaction. So far, the chemical nature of these aggregates is largely unknown. Investigations are reported on how the cross-linking unit 2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-4-yl} hexanoate (**7**), designated as glucosepan, can be identified and quantified from D-glucose-bovine serum albumin (BSA) incubations. Independent synthesis and unequivocal structural characterization are given for glucosepan **7**. A protocol was established for its determination by LC–MS with electrospray ionization (ESI). BSA and D-glucose were incubated at 37 °C, pH 7.4 for eight weeks and the time-dependent formation of **7** was observed. Since glucosepan **7** is unstable under acid proteolytic conditions, BSA was cleaved enzymatically. The maximum value obtained from a solution containing 50 g/L BSA and 100 mM D-glucose after eight weeks incubation time corresponds to an arginine derivatization rate of 1.38 ± 0.07 mmol **7**/mol Arg (equivalent to 31.7 ± 1.6 mmol **7**/mol BSA). From these results, it seems justified to expect **7** to play an important role in the cross-linking of proteins in vivo as well as in foodstuffs. The structural similarity of glucosepan **7** and pentosidine **1** made it obvious to also look for an eventual parallelism in the respective formation pathways.

Introduction

The reaction between reducing carbohydrates and amines or proteins is known as the Maillard reaction or 'nonenzymatic browning'. This process initially leads to a rather unstable Schiff base which may rearrange to the more stable Amadori product. The Amadori compounds are slowly degraded, in complex reaction pathways via dicarbonyl intermediates, to a large number of compounds[1] summarized as 'advanced glycosylation end products' (AGEs); this overall reaction sequence has been shown to proceed in vitro and in vivo.[2] There is substantial evidence that such processes contribute to pathophysiological changes associated with diabetes and artheriosclerosis. Moreover, Maillard-type reactions are thought to be involved in the general aging process.[1–3] One major consequence of the advanced Maillard reaction is the formation of covalently cross-linked proteins which, in long-lived tissues, accumulate with age. Although not much is known about the chemical nature of the cross-linking units, several investigations indicate sugar-derived dicarbonyl compounds such as 3-deoxyosones, methylglyoxal, or glyoxal to be involved.[4] On the basis of various model reactions, different mechanisms for cross-linking of amino acid side chains in proteins have been discussed.[5]

2-Ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)-amino]-3*H*-imidazo[4,5-*b*]pyridin-4-ium-4-yl}hexanoate (**1**, pentosidine), a fluorescent compound linking lysine and arginine by a C_5 moiety, was found in a variety of human tissues, and its level in collagen is linearly correlated with human age[6a] (see Fig. 1). Since this compound, as well as fluorophore LM-1[6b] and imidazolysine,[6c] have been detected only in very low amounts, one would expect other structures to be involved in the extensive protein cross-linking in certain mammalian tissues. For a better understanding of the impact which the Maillard reaction has on aging and diabetes, and for developing effective therapeutic methods to prevent AGE accumulation in tissues, it is an absolute prerequisite to definitively establish the chemical nature of the major protein cross-links derived from this reaction.

Recently, we have shown that 2-acetylamino-5-[(5-butylimino-4-methyl-4,5-dihydro-1*H*-2-imidazolyl) amino] pentanoic acid (**2**) and 2-acetylamino-5-[(4-butyl-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-2-yl)amino] pentanoic acid (**3**) are formed from reactions of methylglyoxal or D-glucose with butylamine and α-*N*-acetyl-L-arginine.[7] These cross-link models,

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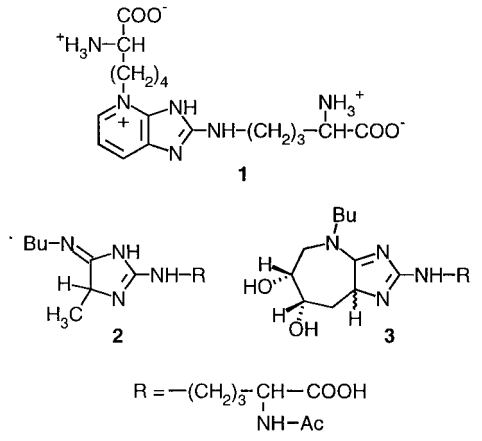


Figure 1. Structural formulae of 2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-3*H*-imidazo[4,5-*b*]pyridin-4-ium-4-yl}hexanoate (**1**, pentosidine), 2-acetylamino-5-[(5-butylimino-4-methyl-4,5-dihydro-1*H*-2-imidazolyl)amino]pentanoic acid (**2**), and 2-acetylamino-5-[(4-butyl-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-2-yl)amino]-pentanoic acid (**3**).

representing 1:1:1 adducts of the respective reaction partners, were isolated in fairly good yield. We now report on the synthesis of the quasihomologous 2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-4-yl}hexanoate (**7**, see Fig. 2) and its detection from D-glucose-bovine serum albumin (BSA) incubations. Due to the structural similarity of **7** and pentosidine **1**, we have also tried to shed some light on the still unknown formation pathway of these heterocyclic cores.

Results

D-Glucose, α-*N*-t-BOC-L-lysine, and α-*N*-t-BOC-L-arginine were reacted in aqueous phosphate buffer for 17 h at 70 °C. The resulting diastereoisomers of 2-[(*tert*-butoxycarbonyl)amino]-6-[2-({4-[(*tert*-butoxycarbonyl)-amino]-4-carboxybutyl}amino)-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-4-yl]hexanoic acid (**6**) were identified by HPLC with diode array detection (DAD), and by comparing their UV spectra (λ_{max} 251 and 253 nm, respectively) with those of **3**.[7] A hypothetical pathway for the formation of **6** is outlined in Fig. 2; it will be discussed below.

HPLC monitoring of preliminary experiments had shown the yield of **6** to be increased slightly with extended incubation time; rather, the abundance of by-products, formed with longer reaction times, severely hampers work-up and purification of the diastereoisomers. Incubation was therefore limited to 17 h. The assignment of the twin HPLC peaks (see Fig. 6) to **6a,b** was validated by LC-MS with electrospray ionization (ESI) which shows coincidence of the diode array trace with that for the quasimolecular ion ($[M+H]^+$) of the diastereoisomers at *m/z* 629.4. Both compounds were isolated by preparative HPLC. LC-MS analysis of the fractions collected for the two diastereoisomers proved **6a** to be chromatographically pure whereas the **6b** fraction still contained two minor compounds with $[M+H]^+$ peaks at *m/z* 647.4 and 613.4, respectively. The protective groups were cleaved from both diastereoisomers with aqueous 3N HCl at room temperature, and the products again purified by preparative HPLC. LC–MS measurements gave quasimolecular ions at *m/z* 429.2 for the two compounds confirming that both *t*-BOC groups in **6a,b** are eliminated. Fortunately, a pure product was obtained from **6b**; the two minor constituents which now show $[M+H]^+$ signals at *m/z* 447.2 and 413.2 could be separated chromatographically. Accurate mass determination of the isolated compounds gave $[M+H]^+$ at *m/z* 429.2466, corresponding to an elemental composition of $C_{18}H_{33}N_6O_6$. The NMR data, compiled in Table 1, unequivocally prove the formation of 2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)-

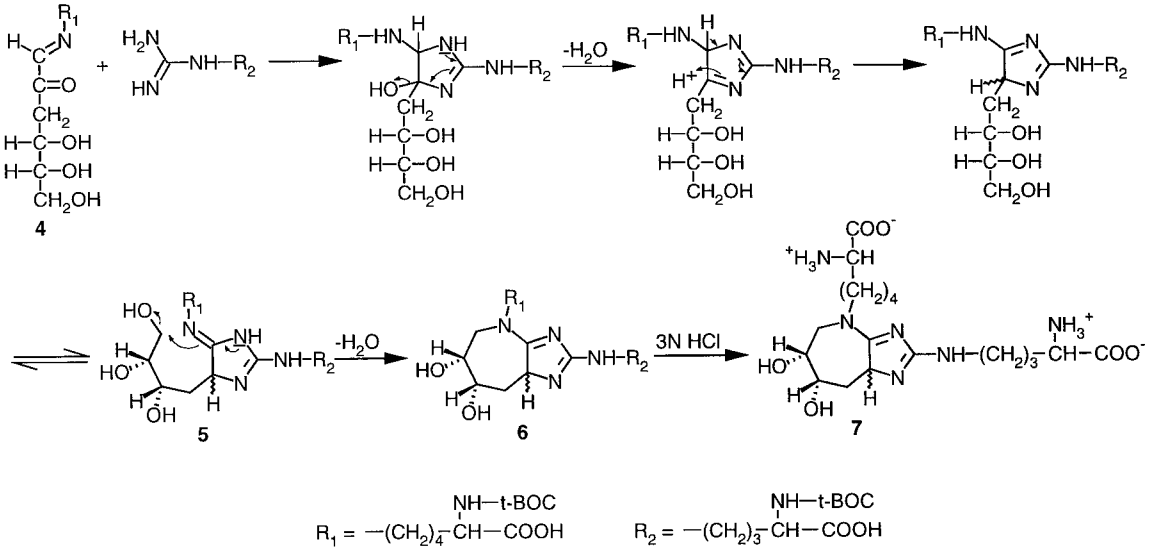
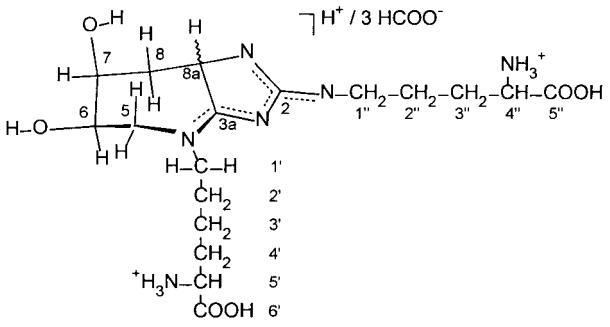


Figure 2. Hypothetic reaction pathway for the formation of 2-[(*tert*-butoxycarbonyl)amino]-6-[2-({4-[(*tert*-butoxycarbonyl)amino]-4-carboxybutyl}amino)-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-4-yl]hexanoic acid (**6**), and its cleavage to 2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-4-yl} hexanoate (**7**, glucosepan).

Table 1. ^{1}H and ^{13}C NMR spectroscopic data of glucosepan **7a,b** (in D_2O): δ [ppm], chemical shift for the indicated hydrogen/carbon; *J* [Hz], coupling constant between the indicated protons; hydrogen/carbon assignment is validated by ^{1}H,^{1}H-COSY, ^{1}H, ^{13}C-COSY and ^{13}C-DEPT measurements



7a **7b**

^{1}H NMR	δ [ppm]	
H-5_A	3.55	4.09
H-5_B	3.55	3.52
H-6	3.51	3.93
H-7	3.75	4.06
H-8_A	2.41	2.11
H-8_B	1.68	2.01
H-8_a	4.95	5.18
H-$1'_A$	3.55–3.70	3.82
H-$1'_B$	3.55–3.70	3.39
H_2-2′	1.75	1.70
H_2-3′	1.43	1.41
$H_{A/B}$-4′	1.90	1.89
H-5′	3.74[a]	3.72[b]
H_2-1″	3.35	3.32
H_2-2″	1.72	1.70
$H_{A/B}$-3″	1.92	1.90
H-4″	3.78[a]	3.76[b]
$HCOO^-$	8.45	8.43
	J[Hz]	
$^2J_{5A,5B}$	—	(−)15.6
$^2J_{8A,8B}$	(−)13.2	(−)14.6
$^2J_{1'A,1'B}$	[c]	(−)14
$^3J_{5A,6}$	[c]	< 1
$^3J_{5B,6}$	[c]	5.5
$^3J_{6,7}$	[c]	4–5
$^3J_{7,8A}$	4.4	3.5–4
$^3J_{7,8B}$	[c]	2.3
$^3J_{8A,8a}$	2.9	3.0
$^3J_{8B,8a}$	12.2	12.3
$^3J_{1'A,2'}$	[c]	6–7
$^3J_{1'B,2'}$	[c]	6–7
$^3J_{4',5'}$	6.1	6.1
$^3J_{1'',2''}$	6.9	6.9
$^3J_{3'',4''}$	6.1	6.1
^{13}C NMR	δ [ppm]	
C-2	166.9	167.1
C-3a	181.7	182.4
C-5	53.4	50.3
C-6	72.7	68.5
C-7	73.9	69.1
C-8	35.5	31.0
C-8a	59.3	57.9
C-1′	52.3	53.4
C-2′	26.5	26.1
C-3′	21.9	21.9
C-4′	30.5	30.5
C-5′	55.1[d]	55.1[f]
C-6′	175.1[e]	175.1[g]
C-1″	42.1	42.1
C-2″	24.3	24.3
C-3″	28.0	28.0
C-4″	54.8[d]	54.7[f]
C-5″	174.8[e]	174.8[g]
$HCOO^-$	171.2	171.3

[a,b,d,e,f,g]Assignment may have to be reversed.
[c]No coupling constant can be determined, due to overlapping multiplets.

amino]-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-4-yl}hexanoate (**7**) existing as a pair of diastereoisomers **7a** and **7b**.

The NMR data sets for the heterocyclic core of **7a** and **7b** are close to identical with those of the diastereoisomers **3**.[7] Since the bicyclic structure of **3** has been definitely proven by various NMR techniques, the close homology made it unnecessary to repeat all these experiments with **7**, and to once again present the rationale by which we have arrived at these structures. If chromatographically pure **7a** and **7b**, respectively, are kept in aqueous solution at room temperature for several days they show partial interconversion as already found for the diastereoisomers **3**.[7] This must be taken into consideration if **7a** or **7b** are to be stored as quantification standards. Since the structure of the cross-link **7**, for which we have coined the designation glucosepan, is definitely established, an LC–ESI-MS method for its quantification was developed. Due to its high polarity, **7** is very poorly retained on reversed phase (RP) material when common eluents are used. The retention behavior is clearly improved if *n*-heptafluorobutyric acid (HFBA) is added to the eluent for providing the counter ion. HFBA is widely used in HPLC analysis of peptides and proteins and has also been successfully employed for the chromatography of pentosidine **1**.[6a] Additionally, HFBA is volatile in high vacuum and therefore poses no problem to the MS system.

Figure 3 shows structure **7** incorporated in a protein. To prove that the cross-linking unit **8** is actually formed, either intra- or intermolecularly, BSA and D-glucose were incubated at two different concentrations (series A and B; 37 °C, pH 7.4) for several weeks.

In two to three week intervals, an aliquot was dialyzed against water using a membrane with 30 kDa molecular weight cut-off (MWCO). As experiments with authentic synthetic material have shown, glucosepan **7** is unstable under the conditions for conventional acidic hydrolysis of proteins (6N HCl, 110 °C, 24 h). Therefore, the lyophilized BSA was cleaved enzymatically as described by Klostermeyer and Henle[8] for casein. Our own amino acid analyses have shown that this procedure is also suitable for BSA, resulting in almost quantitative hydrolysis. The hydrolyzates were diluted to avoid disturbance of the chromatography by the ion-pair activity of the tris(hydroxymethyl)aminomethane (TRIS)–HCl-buffer, being employed for enzymatic cleavage. The resulting solutions were applied to the LC–MS system operating in single ion monitoring (SIM) mode at *m*/*z*

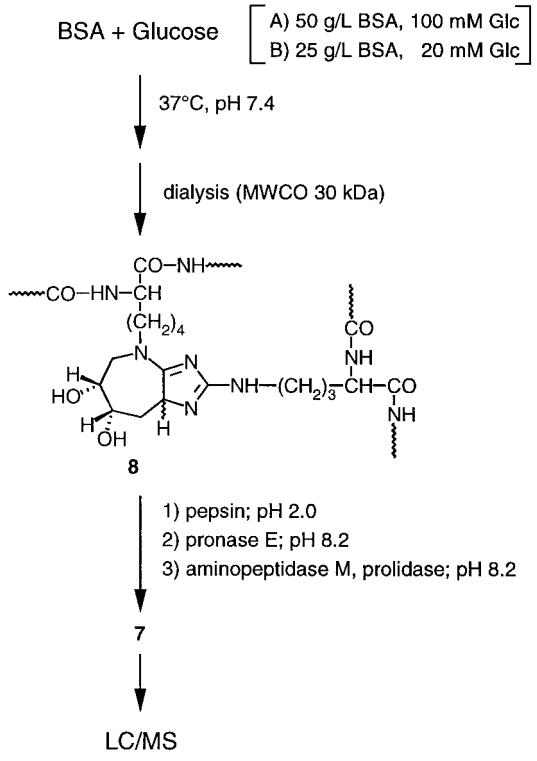


Figure 3. Schematic workup procedure for D-glucose-BSA incubations.

429.2. Typical chromatograms for the detection of **7** are given in Figure 4.

The lower trace shows the result for a hydrolyzate of BSA incubated without D-glucose (blank). The upper trace, from a work-up of incubation series A (see Fig. 3) after eight weeks reaction time, clearly displays the signals for the diastereoisomers **7a** and **7b**. Unfortunately, the **7b** peak cannot be separated from an overlaying signal and thus is unsuitable for proper quantification. The diastereoisomeric ratio **7a:7b** has been established as constant (2:1); thus, the **7a** peak only is quantified, and the concentration obtained multiplied by 1.5. The LC–MS system (SIM-mode, *m/z* 429.2) was calibrated in the range of 0.012–0.92 mg **7a**/L. The linear calibration graph can be described by the equation area = $(-282 \pm 85) + (28202 \pm 220)$ L/mg×c(**7a**), the values in brackets representing means ± confidence intervals ($p = 95\%$). The standard error (s_R) was determined as 62. Limit of detection (LOD) 0.014 mg/L and limit of quantitation (LOQ) 0.029 mg/L were calculated according to recommendations of the Deutsche Forschungsgemeinschaft (DFG).[9] The amounts of **7a**, determined for the incubation series A and B, can be correlated with the derivatization rate of arginine side chains in the BSA molecule as shown in Figure 5.

The concentrations found for incubation series B are below the calculated LOQ. Nevertheless, Figure 5 includes two of these values, towards the end of the reaction period, to give a rough impression of the conversion rate to structure **8**. Since the BSA molecule contains **23** arginine units, the highest determined value in series A of 1.38 ± 0.07 mmol **7**/mol Arg means that statistically every thirtieth BSA molecule contains a cross-linking unit **8**.

Discussion

Cross-linking and insolubilization are among the major biochemical changes which proteins suffer during aging. Enhancement of this process in extracellular matrix is implicated in a number of age- and diabetes-associated complications (e.g. artheriosclerosis and cataract formation). Thus, in recent years, much effort has focused on the chemical characterization of cross-linking structures derived from the Maillard reaction. The cross-links, described so far in the literature, either have pentoses and dehydroascorbic acid as the main precursors (e.g. pentosidine **1**,[6a] fluorophore LM-1[6b]), or stem from reactions with sugar-derived α-dicarbonyl compounds such as methylglyoxal, glyoxal, or 3-deoxyglucosone (e.g. imidazolysine derivatives MOLD[6c] and GOLD,[5f] C-2-imine cross-links,[5e] pyrraline ether[5j]). The concentration of these precursors in plasma and most tissues is very low (μM range or below[10]). Despite their high reactivity, it is questionable whether they actually can be responsible for the formation of the major cross-linking structures.

The main purpose of this study was to clarify whether and to which extent the cross-linking product **8** (Fig. 3) is formed in D-glucose-BSA incubations under physiological conditions. This type of cross-link so far has been studied only in model reactions of butylamine and

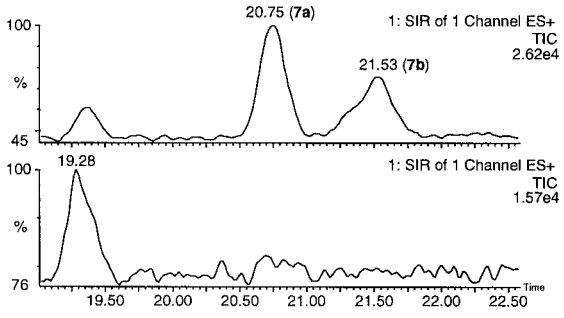


Figure 4. LC-ESI-MS chromatograms (SIM mode, *m/z* 429.2) for the detection of glucosepan **7** (upper trace); BSA blank run (lower trace).

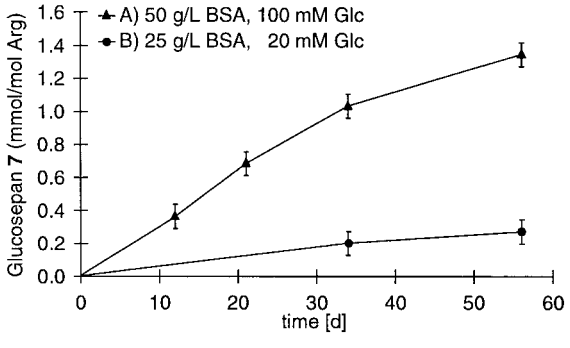


Figure 5. Time course for the formation of cross-link **8** in the D-glucose-BSA incubations.

D-glucose with α-*N*-acetyl-L-arginine or creatine.[7] The respective products were obtained in fairly good yields and it seemed justified, therefore, to expect cross-linking of proteins to proceed analogously. The results of the present investigation clearly show that **8** is formed in substantial amount from the reaction of D-glucose with BSA at 37 °C and pH 7.4 (see Fig. 5). In contrast to other published in vitro cross-link studies, the physiologically most important monosaccharide D-glucose was for the first time successfully employed in native form to observe the formation of a cross-linking structure. A hypothetical reaction pathway for the generation of compound **6**, which should be identical to that of **8**, is outlined in Figure 2. The aldimine **4** can be derived from an Amadori product by water elimination from its enaminol tautomer. Reaction of **4** with the guanidine function of an arginine side chain, followed by H_2O elimination and prototropic rearrangement, leads to compound **5**. This reaction sequence is similar to that proposed for the formation of aminoimidazolinone derivatives.[11] The model incubation of butylamine, methylglyoxal, and α-*N*-acetyl-L-arginine yields **2** (see Fig. 1) as the main product,[7] with a heterocyclic core identical to **5**. The results of an LC–MS analysis of the reaction mixture from the synthesis of **6**, shown in Figure 6, indicate the formation of **5** ($[M+H]^+$, *m*/*z* 647.4) as a minor product; its UV spectrum is similar to that of **2**. By employing 6-*O*-protected hexoses, we now try to obtain **5** in higher yield and to clarify whether it is in fact a precursor of **6** as formulated in Figure 2.

Protein-bound Amadori products (fructosylated proteins) have been detected in human blood and several tissues in mM range,[1a] with the level correlating in most cases with age and D-glucose concentration. The proposed reaction pathway explains the formation of the cross-linking structure **8** from the Amadori compound without free α-dicarbonyl intermediates. This straightforward reaction sequence might be responsible for the efficient linking of lysine and arginine side chains in the protein by compound **8**. Further investigations will have to prove the significance of **8** for the cross-linking of proteins in vivo and also in foodstuffs.

The structural similarity between glucosepan **7** and pentosidine **1** suggests parallel formation pathways for **7** and **1**. D-Xylose, α-*N*-*t*-BOC-L-arginine, and α-*N*-*t*-BOC-L-lysine were reacted under the same conditions as described for the D-glucose mixture. The results of the LC–MS analysis are given in Figure 7.

The signal for the pentosidine derivative **12** is assigned on the basis of the quasimolecular ion at *m*/*z* 579.4; the assignment is validated by HPLC with fluorescence detection. The ion trace at *m*/*z* 599.4 shows a double peak. This mass corresponds to the $[M+H]^+$ for the postulated precursor **11**, and the peak pattern is almost identical to that for compound **6** (Fig. 6). It seems fully justified, therefore, to formulate **11** as an analogue of **6**, incorporating a 6- in lieu of a 7-membered ring. At the moment, we are trying to isolate **11**, to prove its structure, and to test whether **11** yields the pentosidine derivative **12** by elimination of one mole of water and subsequent oxidation as postulated in the reaction scheme in Figure 8.

A peak, to which structure **10** ($[M+H]^+$, *m*/*z* 617.4) as possible precursor of **11** could be assigned, has also been found. It is noteworthy that the signals for compounds **10–12** have similar and low intensities (see ion intensities and total ion chromatograms (TICs) in Fig. 7). Rather, the TICs are absolutely dominated by the signals for the

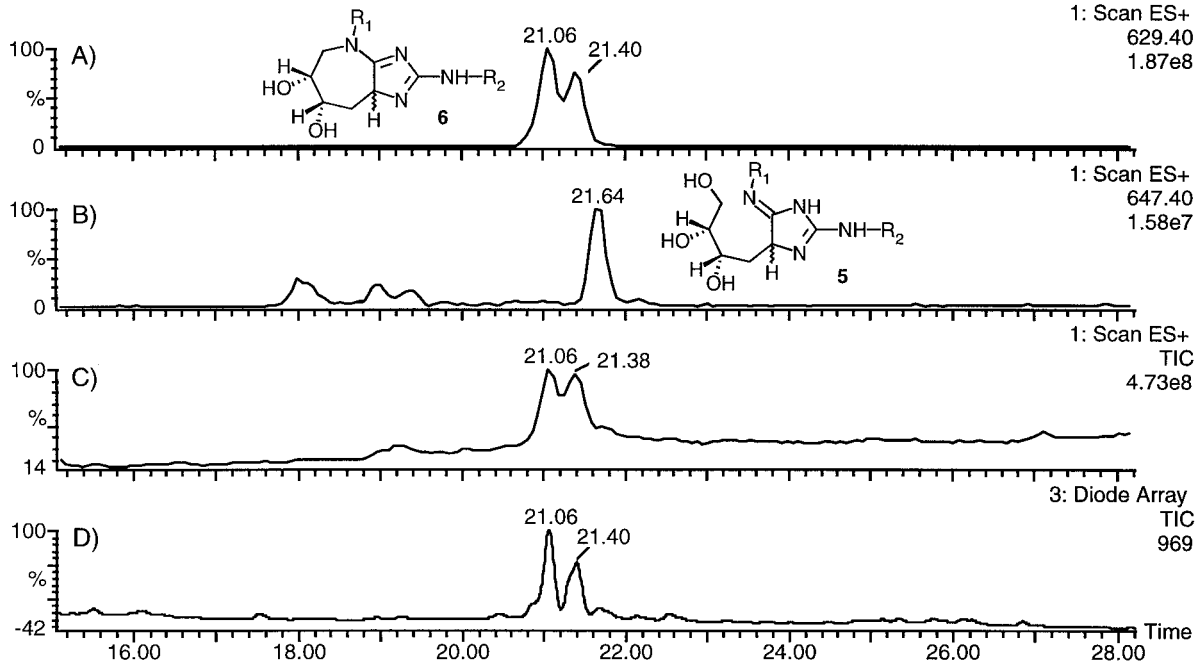


Figure 6. Chromatograms for a reaction mixture of D-glucose, α-*N*-*t*-BOC-L-lysine, and α-*N*-*t*-BOC-L-arginine: (A and B) $[M+H]^+$ ion traces of the glucosepan derivative **6** (*m*/*z* 629.4) and its postulated precursor **5** (*m*/*z* 647.4); (C) MS-TIC recorded for the mass range *m*/*z* 200–1000; (D) 'TIC' of the DAD, operating from 220–500 nm.

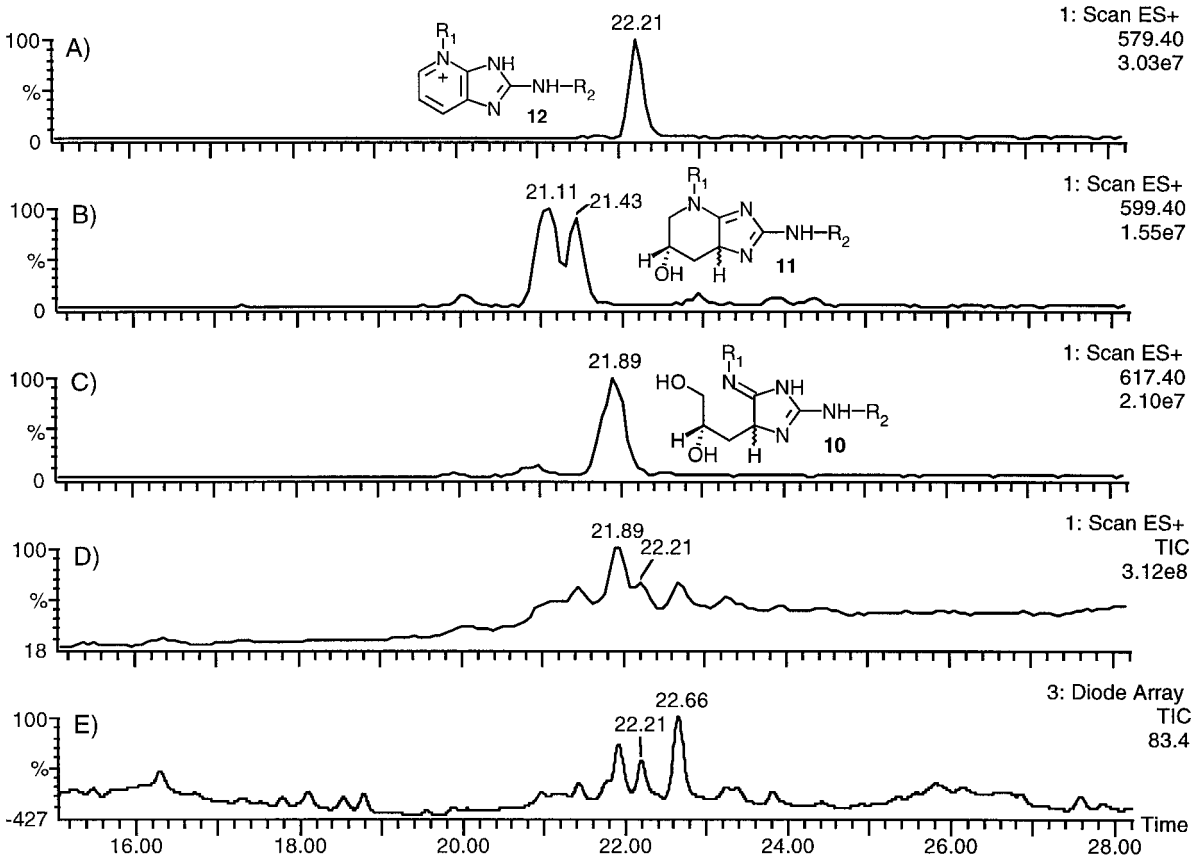


Figure 7. Chromatograms for a reaction mixture of D-xylose, α-*N*-*t*-BOC-L-lysine, and α-*N*-*t*-BOC-L-arginine: (A–C) $[M+H]^+$ ion traces of the pentosidine derivative **12** (*m*/*z* 579.4) and its postulated precursors **11** (*m*/*z* 599.4) and **10** (*m*/*z* 617.4); (C) MS-TIC recorded for the mass range *m*/*z* 200–1000; (D) 'TIC' of the DAD, operating from 220–500 nm.

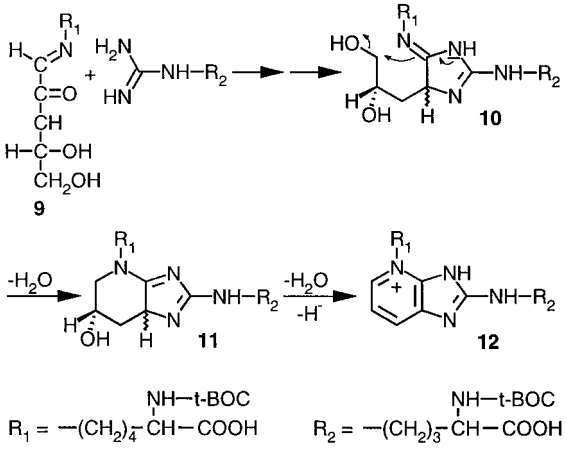


Figure 8. Postulated pathway for the formation of the pentosidine derivative **12**.

glucosepan derivative **6** (see Fig. 6); this clearly emphasizes the leading role of glucosepan **7** in protein cross-linking.

Experimental

General methods

^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) spectra were recorded on a Bruker (Karlsruhe, Germany) ARX 500 spectrometer in D_2O. Chemical shifts (δ) are given in ppm relative to external Me_4Si, coupling constants (*J*) in Hz. For the 2-D NMR experiments, Bruker standard software (X-WIN-NMR 2.0) was employed. The relevant spectrometer parameters were: 90° pulse widths 13 μs and 10 μs for ^{1}H and ^{13}C, respectively; digital FID resolution 34.3 Hz/point for ^{1}H and 7.2 Hz/point for ^{13}C. COSY Spectra were recorded with a relaxation delay $d_1 = 2$ s for ^{1}H,^{1}H[12a] and $d_1 = 2$ s, $d_2 = 3.45$ ms, and $d_3 = 2.30$ ms for ^{1}H,^{13}C.[12b] The analytical high performance liquid chromatography (HPLC) system comprised an HP1100 autosampler, HP1100 gradient pump, HP1100 diode array detector (DAD), and HP1046A fluorescence detector (FLD) module (Hewlett Packard; Waldbronn, Germany). For data acquisition and processing, an HP Chem Station (Rev. A. 04.02) software was used. Column (YMC Europe; Schermbeck, Germany): YMC-Pack Pro C 18, 5 μm, 150×4.6 mm; flow rate 1.0 mL/min; injection volume 20 μL; 10 mM phosphate buffer (pH 4.0)-methanol gradient: % MeOH(t[min]) 0(0)-95(30)-95(40)-0(43)-0(48); DAD detection wavelengths 220, 243, and 253 nm, spectral band width (SBW) 4 nm, reference 500 nm (SBW 100 nm); FLD λ_{ex} 335 nm, λ_{em} 385 nm. LC-MS was run on an HP1100 HPLC system (Modules as described above except the FLD), coupled to a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer equipped with an electrospray (ESI) interface. Chromatographic conditions: Column, flow rate, and injection

volume were as described above; gradients: A) 0.01 M ammonium formate buffer (pH 4.0)-MeOH, % MeOH (t [min]) 0(0)-95(30)-95(40)-0(43)-0(48); B) 20 mM *n*-heptafluorobutyric acid (HFBA)-MeCN, % MeCN(t[min]) 5(0)-30(30)-95(35)-95(40)-5(45)-5(50); post-column splitting ratio 1:20. MS parameters: ESI+, source temperature 120 °C, capillary 3.5 kV; HV lens 0.5 kV; cone 60 V. For LC analyses, the MS system was operated either in full scan mode (*m*/*z* 200-1000) or in single ion mode (SIM; *m*/*z* 429.2, span 0.5 Da, dwell time 1.0 s). For accurate mass determination,[13] data were collected in the multichannel acquisition (MCA) mode with 128 channels per *m*/*z* unit using 11 scans (5 s) with 0.1 s reset time. The resolution was 1150 (10% valley definition). The sample was dissolved for analysis in water/MeCN (1:1) containing poly(ethylene glycol) 400 (0.1 μg/μL) as reference material; the sample concentration was similar to that of PEG 400. The solution was introduced into the ESI source at a flow rate of 5 μL/min. With a *m*/*z* 360–570 scan range, 5 reference peaks could be used for calibration: *m*/*z* 371.2281, 415.2543, 459.2805, 503.3068, 564.3595. The preparative HPLC system consisted of a Kronlab (Sinsheim, Germany) KD200/100SS gradient pump system combined with a Knauer (Berlin, Germany) A0293 variable wavelength detector and a Kronlab HPLC column (guard column 50×20 mm, column 250×20 mm; Nucleosil 100 RP 18, 7 μm); flow rate 20 mL/min; ammonium formate buffer (10 mM, pH 4.0)-MeOH gradients: % MeOH (t [min]), A) 30(0)-70(15)-100(16)-100(21)-30(24)-30(30), B) 0(0)-5(5)-0(6)-0(11); injection volume 1.5 mL; detection wavelength 253 nm. An Amicon (Witten, Germany) stirred cell 8050 equipped with an Amicon PM 30 ultrafilter was employed for dialysis. Reaction mixtures from synthetic procedures were filtered (membrane filter, 0.45 μm) before preparative HPLC. MeOH was removed from the eluent in vacuo, and the remaining aqueous layer lyophilized with a Leyboldt–Heraeus (Köln, Germany) Lyovac GT 2.

Materials

Ultrapure water, obtained from a Milli-Q 185 Plus apparatus (Millipore; Eschborn, Germany), HPLC grade methanol, and HPLC grade acetonitrile were used for LC and LC–MS. For preparative HPLC, solvents were degassed by flushing with helium. For a phosphate buffer salt mixture giving solutions with pH 7.4, KH_2PO_4 (2.68 g; 20 mmol) and $Na_2HPO_4{\cdot}2H_2O$ (14.30 g; 80 mmol) were mixed vigorously. α-*N*-*t*-BOC-L-Arginine, α-*N*-*t*-BOC-L-lysine, and prolidase were purchased from Fluka (Neu-Ulm, Germany), *n*-heptafluorobutyric acid and poly(ethylene glycol) 400 from Aldrich (Steinheim, Germany), bovine serum albumin (fraction V), phosphate buffered saline, pH 7.4 (PBS), and pepsin from Sigma (Steinheim, Germany), pronase E from Merck (Darmstadt, Germany), aminopeptidase M from Boehringer Mannheim (Mannheim, Germany).

Model reactions

D-Glucose (36 mg, 0.2 mmol) or D-xylose (150 mg, 0.2 mmol), α-*N*-*t*-BOC-L-lysine (152 mg, 0.62 mmol), α-*N*-*t*-BOC-L-arginine (114 mg, 0.42 mmol) and phosphate buffer pH 7.4 (170 mg, 1.0 mmol) were dissolved in water (1.0 mL). After incubation (70 °C, 17 h), an aliquot of 20 μL was diluted with water to 1 mL, and the membrane filtrate (0.45 μm) injected into the analytical HPLC or LC–MS system (gradient A).

2-Ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo [4,5-*b*] azepin-4-yl}hexanoate (7). D-Glucose (0.7 g, 3.9 mmol), α-*N*-*t*-BOC-L-lysine (3.04 g, 12.3 mmol), α-*N*-*t*-BOC-L-arginine (2.28 g, 8.3 mmol) and phosphate buffer pH 7.4 (3.2 g, 18.8 mmol) were dissolved in water (20 mL). The mixture was kept at 70 °C for 17 h and purified by preparative HPLC (gradient A). Fractions with t_R 13.7 min (I) and 14.2 min (II) were collected, and their purity checked by LC–MS. Fraction I proved to be chromatographically pure; fraction II contained two by-products with $[M+H]^+$ at *m*/*z* 647.4 and 613.4, respectively. After lyophilization, the diastereoisomers of 2-[(*tert*-butoxycarbonyl)amino]-6-[2-({4-[(*tert*-butoxycarbonyl)-amino]-4-carboxybutyl}amino)-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-4-yl]hexanoic acid **6a** (70.5 mg, 2.7%, neat compound) from fraction I and **6b** (60.5 mg, with by-products) from fraction II were obtained as the respective formates; UV(H_2O): λ_{max} [nm] 251 (**6a**), 253 (**6b**); LC–ESI-MS (gradient A): **6a**, t_R 21.2 min, *m*/*z* 667 (4.7, $[M+K]^+$), 651 (1.3, $[M+Na]^+$), 629 (100, $[M+H]^+$), 573 (5.9), 529 (7.5), 429 (1.1); **6b**, t_R 21.5 min, *m*/*z* 667 (3.4, $[M+K]^+$), 651 (0.4, $[M+Na]^+$), 629 (100, $[M+H]^+$), 573 (5.1), 529 (9.6), 429 (1.7).

6a (70.5 mg) and **6b** (60.5 mg) each were dissolved in aqueous 3N HCl (3 mL) and kept at room temperature for 30 min. The pH was adjusted to **7** by slowly adding solid $NaHCO_3$ (250–280 mg, 2.98–3.33 mmol), and the volume was filled up to 5 mL. Both mixtures were purified by preparative HPLC (gradient B). Fractions at t_R 4.4 min or t_R 6.6 min were collected, respectively, from the reactions of **6a** and **6b**, and yielded after lyophilization **7a**·3 HCOOH (18.4 mg, 31%) and **7b**·3 HCOOH (9.0 mg); ^1H and ^{13}C NMR (D_2O): see Table 1; UV(H_2O): λ_{max} [nm] (lg ε) **7a**, 251(4.25), **7b**, 253(4.26); LC–ESI-MS (gradient B): **7a**, t_R 20.8 min, *m*/*z* 467 (1.3, $[M+K]^+$), 451 (4.9, $[M+Na]^+$), 429 (100, $[M+H]^+$), 384 (0.8); **7b**, t_R 21.5 min, *m*/*z* 467 (2.1, $[M+K]^+$), 451 (1.9, $[M+Na]^+$), 429 (100, $[M+H]^+$), 384 (3); accurate mass (mean of 5 measurements ± standard deviation): *m*/*z* 429.2466 ± 0.0006 $[M+H]^+$ (429.2462, calcd for $C_{18}H_{33}N_6O_6$).

D-Glucose-bovine serum albumin (BSA) incubations. BSA (3.5 g, 52.8 μmol) and D-glucose (1.26 g, 7 mmol) were dissolved in phosphate buffer (70 mL, 0.1 M, pH 7.4) and passed through a 0.45 μm membrane filter (series A incubations). BSA (1.75 g, 26.4 μmol) and D-glucose (252 mg, 1.4 mmol) were dissolved in phosphate buffered saline (PBS, 70 mL, pH 7.4) and passed through a 0.45 μm membrane filter (series B incubations). Aliquots (5×10 mL) of each series were filtered (sterile filter, 0.2 μm) into tubes which were sealed tightly and kept at 37 °C with gentle shaking.

Work up of incubation mixtures and enzymatic cleavage of the protein

After 12, 21, 34, and 56 d, aliquots from both BSA incubation series (equivalent to 100 mg protein) were transferred to the Amicon stirred cell, diluted to 25 mL with water, and dialyzed with 0.8 L water by applying a pressure of 3 bar. After lyophilization, 7 mg of the obtained protein was cleaved enzymatically as described.[8] The hydrolyzates were filled up to 10 mL with water and applied to the LC–MS system, operating in the SIM mode.

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