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Cross-linking of Proteins by Maillard Processes—Model Reactions of D-Glucose or Methylglyoxal with Butylamine and Guanidine Derivatives

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Abstract—Advanced Maillard reaction in proteins leads to formation of covalently cross-linked aggregates the chemical nature of which is largely unknown. From model reactions of methylglyoxal and butylamine with creatine or α-*N*-acetyl-L-arginine, one main product each was isolated. These two compounds were identified, on the basis of unequivocal spectroscopic evidence, as 2-[(5-butylimino-4-methyl-4,5-dihydro-1*H*-2-imidazolyl)(methyl)amino]acetic acid and 2-acetylamino-5-[(5-butylimino-4-methyl-4,5-dihydro-1*H*-2-imidazolyl)amino]pentanoic acid, respectively. Using D-glucose instead of methylglyoxal, two main products each were obtained from reaction with the respective guanidine derivative. The spectroscopic data definitively establish the formation of the diastereoisomeric 2-[(4-butyl-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-2-yl)(methyl)amino]acetic acid from the reaction with creatine, and of the diastereoisomeric 2-acetylamino-5-[(4-butyl-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-2-yl)amino]pentanoic acid from the reaction with α-*N*-acetyl-L-arginine. All products were isolated in fairly good yield and represent 1:1:1 adducts of the respective reaction partners. Formation of these compounds thus constitutes an efficient reaction pathway for linking primary amines to guanidine derivatives. It seems justified, therefore, to expect cross-linking of proteins by action of reducing carbohydrates to proceed analogously.

Introduction

Nonenzymatic reaction of glucose and other reducing carbohydrates with lysine side chains and N-terminal amino groups of proteins initially leads to a rather unstable Schiff base which may rearrange to the more stable Amadori product. This early stage of the overall reaction sequence is called nonenzymatic glycation. 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). The complex reaction between reducing sugars and proteins, also known as Maillard reaction, 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 artherosclerosis. Moreover, Maillard-type reactions are thought to be involved in the ageing process.[1–3] Advanced glycation has also been implicated in the pathology of Alzheimer's disease.[4]

One of the major consequences 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.[5] On the basis of various model reactions, different mechanisms for cross-linking of amino acid side chains in proteins have been discussed.[6]

2-Ammonio-7-[2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-3*H*-imidazo[4,5-*b*]pyridin-4-ium-4-yl]heptanoate (**1**, pentosidine), a fluorescent compound linking lysine

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and arginine by a C_5 moiety, was found in a variety of human tissues and its levels in collagen correlate linearly with human age[7a] (see Figure 1). Since this compound, as well as fluorophore LM-1 and imidazolysine,[7b–d] have been detected only in very low amounts, one would expect other structures beside these to be responsible for the extensive protein cross-linking in certain mammalian tissues. Therefore, to better understand the impact of the Maillard reaction in ageing and diabetes and to develop effective therapeutic methods to prevent AGE accumulation in tissues, it is necessary to elucidate the chemical nature of the major protein cross-links derived from this reaction.

Recently, it has been shown that 3-deoxyhexosulose, methylglyoxal, and 3-deoxypentosulose react with guanidine derivatives to give aminoimidazolinones of the general structure **2**[8] (see Figure 1). We now report on new cross-linking products obtained in good yield by reaction of D-glucose or methylglyoxal with butylamine and guanidine derivatives. In this context, butylamine is considered a model for the lysine side chains or terminal amino groups of proteins.

Results and Discussion

Methylglyoxal, butylamine, and creatine (**5**) or α-*N*-acetyl-L-arginine (**6**) were reacted in aqueous phosphate buffer for 24 h at 40 °C; the pH was adjusted to 7.4 at the outset of the reaction. HPLC analysis showed a main peak with a characteristic UV maximum at 246 nm for the reaction with **5**. For the α-*N*-acetyl-L-arginine incubation, the respective product (λ_{max} 243 nm) is less predominant. Both compounds were isolated by preparative HPLC. Preliminary experiments had shown that the yield of these products increased with extended incubation time. The incubation was nevertheless limited to 24 h since the abundance of by-products formed with longer reaction times severely hampered work-up and purification of the desired compounds. FABHRMS gave the accurate mass of the quasimolecular ion ($[M+H]^+$) as *m/z* 241.1664 for the creatine product, and as *m/z* 326.2193 for the α-*N*-acetyl-L-arginine derivative, corresponding to elemental compositions of $C_{11}H_{21}N_4O_2$ and $C_{15}H_{28}N_5O_3$, respectively. Thus, in both reactions a 1:1:1 adduct of methylglyoxal, butylamine, and the guanidine derivative has been formed, with elimination of 1 mol of water. The unequivocal structural assignment rests on various NMR techniques, and will be discussed in detail below. From these data sets, the two structures 2-[(5-butylimino-4-methyl-4,5-dihydro-1*H*-2-imidazolyl)(methyl)amino]acetic acid (**7**) from the reaction with **5**, and 2-acetylamino-5-[(5-butylimino-4-methyl-4,5-dihydro-1*H*-2-imidazolyl)amino]-pentanoic acid (**8**) from the reaction with **6** may be considered as firmly established.

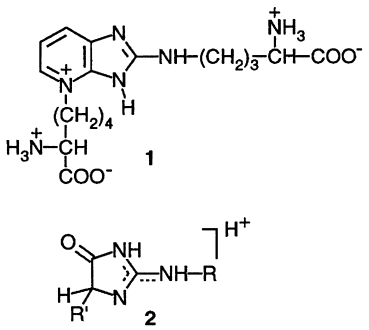
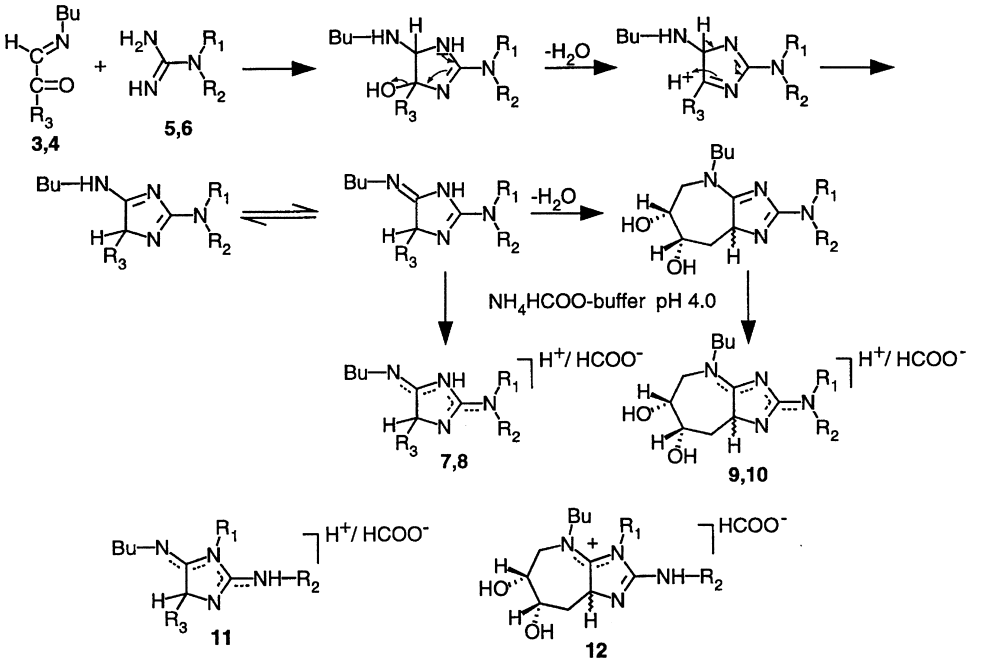


Figure 1. Structural formula of 2-ammonio-7-[2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-3*H*-imidazo[4,5-*b*]pyridin-4-ium-4-yl]heptanoate (**1**, pentosidine), and generalized structure of the aminoimidazolinone derivatives **2**.

The postulated reaction pathway, outlined in Figure 2, is analogous to that proposed for the formation of the aminoimidazolinones **2** (see Figure 1).[8b] In the case of **2**, the α-dicarbonyl compound reacts with guanidine derivatives to form dihydroxyimidazolines which rearrange to imidazolinones. Formally, this corresponds to an intramolecular redox reaction: The aldehyde group of the α-dicarbonyl structure is transformed to the oxidation level of the corresponding carboxylic acid, and the keto function is reduced to an amine. Since the aminoimidazoline imines (**7** and **8**) incorporate an amidine partial structure instead of the amide in **2**, one might expect the aldimine (e.g. **3** and **4**, see Figure 2) of an α-dicarbonyl compound as precursor for compounds **7** and **8**. Such an aldimine can be derived directly from an Amadori product by water elimination from its enaminol tautomer, or can arise upon Schiff base formation from the α-dicarbonyl compound.

From the reaction of D-glucose and butylamine with **5** or **6** at pH 7.4 and 70 °C, two main products each were isolated by preparative HPLC. The same compounds were obtained from reaction at 40 °C, or when 1-deoxy-1-butylamino-D-fructose and 3-deoxyglucosone, respectively, were used as starting material instead of D-glucose. Incubation was stopped once more after 24 h for the same reasons as for the reaction with methylglyoxal. The UV maximum at 256 nm for the two compounds from the creatine incubation, and at 253 nm for the α-*N*-acetyl-L-arginine derivatives, shows a bathochromic shift of 10 nm relative to the products derived from the reactions with methylglyoxal. This shift cannot be due only to the different alkyl substituents at C-4 of the imidazoline ring (–CH_3 for **7** and **8**, –CH_2–CHOH–CHOH–CH_2OH for the D-glucose products); rather, the heterocylic core of these structures must be different from that in compounds **7** and **8**. FABHRMS analysis



	R_1	R_2	R_3
3	—	—	$-CH_3$
4	—	—	$-CH_2-CH(OH)-CH(OH)-CH_2(OH)$
5	$-CH_2-COOH$	$-CH_3$	—
6	$-(CH_2)_3-CH(NH-C(=O)-CH_3)-COOH$	H	—
7	$-CH_2-COOH$	$-CH_3$	$-CH_3$
8,11	$-(CH_2)_3-CH(NH-C(=O)-CH_3)-COOH$	H	$-CH_3$
9	$-CH_2-COOH$	$-CH_3$	—
10,12	$-(CH_2)_3-CH(NH-C(=O)-CH_3)-COOH$	H	—

Figure 2. Hypothetic reaction pathway for the formation of 2-[(5-butylimino-4-methyl-4,5-dihydro-1*H*-2-imidazolyl)(methyl)amino] acetic acid (**7**), 2-acetylamino-5-[(5-butylimino-4-methyl-4,5-dihydro-1*H*-2-imidazolyl)amino]pentanoic acid (**8**), 2-[(4-butyl-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-2-yl)(methyl)amino]acetic acid (**9**), 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 (**10**).

gave $[M+H]^+$ at *m/z* 313.1874 for the creatine products, and at *m/z* 398.2402 for the α-*N*-acetyl-L-arginine derivatives, corresponding to empirical formulae of $C_{14}H_{25}N_4O_4$ and $C_{18}H_{32}N_5O_5$, respectively. Once again, 1:1:1 adducts of the three reaction partners were formed. However, 3 or 2 mol of water were eliminated using D-glucose or 1-deoxy-1-butylamino-D-fructose and 3-deoxyglucosone, respectively, as starting material (i.e., an additional mole of water has been lost compared to the reaction with methylglyoxal). The low

resolution FABMS spectra show adduct ion signals for $[M+Na]^+$ and, in the case of the α-*N*-acetyl-L-arginine derivatives, also for $[M+H+M]^+$ and $[M+Na+M]^+$ (see Experimental). Therefore, assignment of the signals at *m*/*z* 313 and *m*/*z* 398 to the respective quasimolecular ion $[M+H]^+$ is unequivocal. The loss of an additional H_2O thus must have occured in the course of the reaction, and cannot be the result of a fragmentation process in the mass spectrometer. The NMR data unequivocally prove the formation of 2-[(4-butyl-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-2-yl)(methyl)amino]acetic acid (**9**) from the reaction with **5**, and of 2-acetylamino-5-[(4-butyl-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepin-2-yl)amino] pentanoic acid (**10**) from the reaction with **6** (see Figure 2); both products exist as a pair of diastereoisomers. Since these bicyclic compounds, incorporating a 7-membered ring, were totally unexpected, the rationale by which these structures were derived will be presented in detail below, and their spectroscopic data discussed exhaustively. No plausible reaction mechanism can be presented yet for how the terminal OH-function of the polyhydroxyalkyl side chain is substituted by the nitrogen of the butylamino group.

The yields of **7** and **8** and **9** and **10** in the model incubations are relatively high (0.9, 0.7% and 6.4, 6.1%, respectively), compared to other cross-link reactions described in the literature, and formation of these compounds thus may be considered a major reaction pathway for linking primary amines to guanidine derivatives. On the basis of these results, it seems justified to formulate cross-linking of proteins by the action of reducing carbohydrates in an analogous manner. Investigations are in progress to substitute the butylamine model by α-*N*-acetyl-L-lysine, and to obtain the respective cross-linking structures expected from the interaction of D-glucose or methylglyoxal with proteins. A method for their detection in biological systems shall also be developed, and the reaction kinetics studied. These results will then show whether the products **8** and **10**, obtained after 24 h from the model incubations, are in fact of biological and physiological significance for the cross-linking of proteins with respect to the long-term effects of AGEs. Furthermore, HPLC analysis of a preliminary test with xylose, α-*N*-acetyl-L-lysine, and α-*N*-acetyl-L-arginine has shown, besides the peak for the pentosidine analogue, two signals with UV characteristics similar to **10**, though in much lower yield. Further investigations will have to prove whether these compounds likewise are bicyclic, incorporating a six-membered instead of the seven-membered ring in the hexose products. Perhaps, these structures represent precursors for the, until now, most famous cross-linking product pentosidine (**1**), and may help to understand its formation pathway which is still unknown.

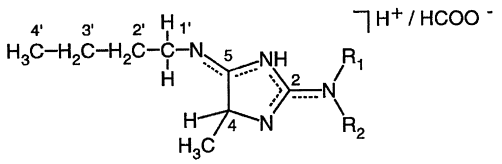
Structural Assignments

Since compounds **7** and **8** and **9** and **10** represent two new types of cross-links between primary amines and guanidine derivatives, special effort was invested in unequivocally establishing their structures. The ^1H and ^{13}C NMR chemical shifts (δ) and coupling constants (*J*) of **7** and **8** are compiled in Table 1.

The NMR spectra of compound **7** show the presence of two isomers **7a** and **7b** in a 2:1 ratio which cannot be separated by HPLC. One NMR signal set can be assigned to each isomer on the basis of the relative intensities. Significant differences in the chemical shifts of **7a** and **7b** are observed especially for C-1″, C-2″, and C-1‴, as well as for the respective protons H-1″$_{A/B}$ and H_3-1‴. These can only be rationalized in terms of a change in the orientation of the methyl and carboxymethyl substituent relative to the imidazoline ring. Thus, the two isomers were assigned as the respective *Z*- and *E*-form, **7a** and **7b**. The strongly hindered rotation around the exocyclic C–N bond clearly demonstrates the substantial double-bond character. This fact supports the formulated delocalization of the positive charge in the heterocyclic core. When α-*N*-acetyl-L-arginine (**6**) is used instead of creatine (**5**), at least two positional isomers are plausible: The N^{δ} of the arginine moiety may be incorporated in an exocyclic position as in compound **8**, or in the imidazoline ring as in **11** (see Figure 2). Schwarzenbolz et al.[9] have identified 5-(2-amino-4- oxo-4,5-dihydro-1*H*-imidazol-3-ium-3-yl)-2-ammonio pentanoate from the reaction of glyoxal with proteins, a product with N^{δ} of the L-arginine side chain being endocyclic like in **11**. The differentiation between the potential structures **8** and **11** rests on ^1H,^{13}C long range correlation spectra (COLOC).[10a] The results of these spectra are summarized in Figure 3; the arrows indicating significant carbon–proton long-range coupling across two or three bonds (^2J or ^3J).

The triplet for H_2-1″ only shows a single cross-peak with C-2 (for the numbering see Table 1) and N^{δ} of the L-arginine side chain thus has remained exocyclic in the formation of the imidazoline ring. For structure **11**, in contrast, correlation of H_2-1″ with both quasi-carbonyl C-atoms C-2 and C-5 would be required. Hence, **8** is definitively established as the structure of the major product from this reaction, incorporating all three constituent moieties, methylglyoxal, butylamine, and **6**. The NMR spectra, however, show an additional set of signals from a minor constituent with one fourth the relative intensity compared to **8**; the corresponding compound cannot be separated chromatographically. Unfortunately, the weak signals for this product prevent detection of ^1H,^{13}C long-range cross-peaks. No definite decision is possible, therefore, between structure **11** and

Table 1. ^{1}H and ^{13}C NMR spectroscopic data of compounds **7a,b** and **8** (in D_2O)



7a **7b** **8**

^{1}H NMR	δ[ppm]		
R_1	1" 2" $-CH_2-COOH$	1''' $-CH_3$	5" COOH, HN–CH 4", O=C CH2 3", H3C CH2 2", CH2 1", H
R_2	1''' $-CH_3$	1" 2" $-CH_2-COOH$	
H-4	4.72	4.69	4.72
H_3C-4	1.46	1.43	1.46
H-1′$_A$	3.46	3.51	3.42
H-1′$_B$	3.45	3.49	3.42
H_2-2	1.59	1.63	1.57–1.75
H_2-3′	1.34	1.38	1.38
H_3-4′	0.90	0.92	0.91
H-1″$_A$	4.20	3.96	3.31
H-1″$_B$	4.18	3.94	3.31
H_3-1‴	3.09	3.24	—
H-2″	—	—	1.57–1.75
H-3″$_A$	—	—	1.85
H-3″$_B$	—	—	1.72
H-4″	—	—	4.18
H_3C-CO-NH-4″	—	—	2.03
$HCOO^-$	8.45	8.45	8.45
	J[Hz]		
$^{2}J_{1'A,1'B}$	(−)13.3	(−)13.4	—
$^{2}J_{1''A,1''B}$	(−)17.2	(−)18.0	—
$^{3}J_{4,H_3C\text{-}4}$	6.9	6.9	6.9
$^{3}J_{1'A,2'}$	7.0	7.0	7.0
$^{3}J_{1'B,2'}$	7.0	7.0	7.0
$^{3}J_{2',3'}$	7.3	7.2	7.2
$^{3}J_{3',4'}$	7.4	7.4	7.4
$^{3}J_{1'',2''}$	—	—	6.7
$^{3}J_{3''A,4''}$	—	—	4.7
$^{3}J_{3''B,4''}$	—	—	6.4
^{13}C NMR	δ[ppm]		
C-2	167.9	167.7	170.0
C-4	58.8	58.7	58.5
H_3C-4	18.1	18.0	18.1
C-5	181.2	181.4	181.1
C-1′	43.1	43.3	43.4
C-2′	30.2	30.1	30.3
C-3′	19.5	19.6	19.7
C-4′	13.2	13.2	13.3
C-1″	54.9	54.0	42.3
C-2″	175.8	174.2	24.9
C-1‴	36.8	38.1	—
C-3″	—	—	29.1
C-4″	—	—	55.1
C-5″	—	—	179.2
H_3C-CO-NH-4″	—	—	22.3
H_3C-CO-NH-4″	—	—	174.0
$HCOO^-$	171.2	171.2	171.2

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.

that of the other diastereoisomer of compound **8** for the minor constituent. However, a 1:4 diastereoisomer ratio is rather unlikely since the chiral carbon in **6** is far away from the stereogenic center formed at C-4 in the course of the reaction, and thus should not severely influence the respective stereochemistry.

The ^{1}H and ^{13}C NMR data of the proposed bicyclic structures **9** and **10** are listed in Table 2. The FABHRMS spectra prove additional loss of 1 mol of water for these compounds, compared to products **7** and **8** (see above). The most striking difference between **7** and **8**, and **9** and **10** are the ^{13}C chemical shifts for C-1′: 43.1–43.4 and 52.1–53.8 ppm, respectively. Such a pronounced low field shift can be rationalized only if the secondary nitrogen of the butylamino group in compounds **7** and **8** is converted to a tertiary one in **9** and **10**. Furthermore, the carbon resonance for the terminal CH_2OH group of a polyhydroxyalkyl side chain is expected in the 63–68 ppm range, where the spectra of **9** and **10** show no signals at all. A heterocyclic core with a polyhydroxyalkyl side chain instead of the methyl group in compounds **7** and **8** thus is definitively ruled out. Rather, ^{13}C DEPT (distorsionless enhancement by polarization transfer) spectra each show a methylene carbon in the range of 48.8–53.4 ppm; these resonances can arise only from a second *N*-substituted CH_2 function. The terminal CH_2OH group of the sugar backbone obviously has suffered nucleophilic substitution by the butylamino group, with closure of a second ring. This structural hypothesis is strengthened by the remarkable shift difference of the diastereotopic protons at C-5 and C-8 (up to 0.7 ppm) as well as by their respective ^{3}J coupling constants with the neighbouring methine hydrogens; these values exclude free rotation expected for the polyhydroxyalkyl side chain. The $^{1}H,^{13}C$ COLOC spectrum of **10b** displays a clear correlation between 5-H_B and C-3a (see the respective arrow in Figure 3) and thus definitively proves the bicyclic structure, incorporating a 7-membered ring. The N^{δ} of the

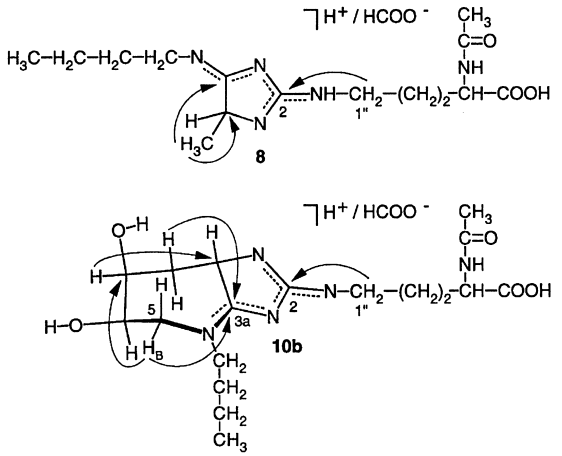


Figure 3. Identified structures of **8** and **10b**, with arrows indicating the characteristic carbon–proton long-range coupling detected in the COLOC spectra.

α-*N*-acetyl-L-arginine side chain again is established as exocyclic by the single crosspeak correlating H_2-1″ with C-2. This excludes the alternative structure **12** (see Figure 2) for this product.

As mentioned previously, two compounds each were isolated from the reactions of D-glucose, butylamine, and **5** or **6**. In the case of **10**, these are characterized as a pair of diastereoisomers, **10a** (6*R*,7*S*,8a*R*) and **10b** (6*R*,7*S*,8a*S*). The assignment of the respective signal sets to the structures shown in the lead of Table 2 is based predominantly on the coupling constants within the proton spin system H-$5_{A/B}$ to H-8a, and the ^{13}C resonance sets C-3a to C-8a. If chromatographically pure products are kept at room temperature in D_2O for a week, HPLC analysis shows 8% of **10a** to be converted to **10b**, and 21% of **10b**, respectively, to **10a**. This interconversion proceeds even faster in dimethylsulfoxide solution; the thermodynamic equilibrium **10a**:**10b** is established as 30:70 within 5 h, starting either from neat **10a** or **10b**. Furthermore, ^{1}H NMR spectra of the D_2O solutions of **10a** or **10b** show no H/D exchange for H-8a in the course of this process. Inversion of the configuration at this stereogenic center, therefore, very likely proceeds via a [1,5]-H shift, without contribution of the protic solvent. Such a [1,5]-H shift is a suprafacial, symmetry-permitted process with a six-electron transition state, and can be formulated between positions 8a and 1 of the imidazole ring in the bicyclic structures **9** and **10**.

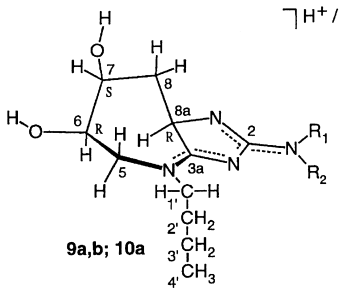
Compound **9** likewise is isolated as a pair of diastereoisomers which also can be separated chromatographically and show the same interconversion as **10a**,**b**. Each of these diastereoisomers exists, however, as a mixture of *E*/*Z* isomers **9a**/**9b** and **9c**/**9d**, respectively, in a 2:1 ratio. Due to overlapping signals of the complex multiplets in the ^{1}H spectra caused by this *E*/*Z* isomerism, a number of coupling constants, marked with index a in Table 2, could not be determined.

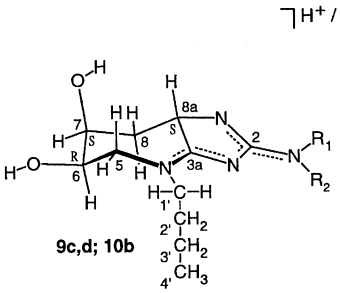
Experimental

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[10b], and $d_1 = 2$ s, $d_2 = 3.45$ ms, and $d_3 = 2.30$ ms for ^{1}H,^{13}C.[10c] The COLOC data were recorded with $d_1 = 2$ s and a 15 ms (mixing time) delay for evolution of long range couplings.[10a] Liquid secondary-ion high resolution mass spectra (SIMS-HRMS, analogous to FABHRMS) were obtained on a Finnigan (Bremen, Germany) MAT 95 instrument. Analytical HPLC was run with a Merck (Darmstadt, Germany) L-7100 gradient pump and a Merck L-7450 photodiode array detector (DAD) including Merck-Hitachi Model D-7000 chromatography data station software. Separations were performed on a Macherey-Nagel (Oensingen, Switzerland) HPLC column (guard column 8×3 mm, column 125×3 mm; Nucleosil 100 RP 18, 5 μm); flow rate, 0.5 mL/min. Solvent A was ammonium formate buffer (5 mM, pH 4.0; adjusted with formic acid): MeOH = 95:5, solvent B was MeOH; gradient: % B [*t*(min)] 0 [0]-100 [25]-100 [30]. For analyte detection and recording of the UV spectra the DAD was operated from 220 to 400 nm. The preparative HPLC systems consisted of: (i) A Merck L-6250 pump combined with a Merck L-4000 UV detector and a Macherey-Nagel HPLC column (Nucleosil 100 RP 18, 7 μm, 250×21 mm); flow rate, 10 mL/min; ammonium formate buffer (5 mM, pH 4.0): MeOH = 75:25; injection volume, 2 mL. (ii) 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 gradient: % MeOH [*t*(min)], 0 [0]-45 [30]; injection volume, 1.5 mL; detection, 253 nm. All reaction mixtures were adjusted to pH 7.4 with phosphoric acid. After filtration (membrane filter, 0.45 μm), the incubations were subjected to preparative

Table 2. ^{1}H and ^{13}C NMR spectroscopic data of compounds **9a–d** and **10a,b** (in D_2O)





	9a	**9b**	**9c**	**9d**	**10a**	**10b**
^{1}H NMR			δ[ppm]			
R_1	1" 2" $-CH_2-COOH$	1''' $-CH_3$	1" 2" $-CH_2-COOH$	1''' $-CH_3$	1" 2" 3" 4" 5" $-CH_2-CH_2-CH_2-CH(NH-C(=O)-CH_3)-COOH$	
R_2	1''' $-CH_3$	1" 2" $-CH_2-COOH$	1''' $-CH_3$	1" 2" $-CH_2-COOH$	H	
H-5_A	3.48	3.48	4.06	4.06	3.52	4.09
H-5_B	3.48	3.48	3.52	3.52	3.52	3.54
H-6	3.48	3.48	3.93	3.93	3.52	3.95
H-7	3.75	3.74	4.10	4.10	3.78	4.08
H-8_A	2.41	2.37	2.13	2.08	2.40	2.13
H-8_B	1.67	1.67	2.02	2.02	1.65	2.02
H-8a	4.94	4.91	5.18	5.15	4.95	5.18
H-$1'_A$	3.73	3.76	3.92	3.92	3.70	3.82
H-$1'_B$	3.59	3.59	3.33	3.33	3.58	3.36
H_2-2′	1.67	1.67	1.62	1.62	1.67	1.65
H_2-3′	1.35	1.35	1.33	1.33	1.35	1.33
H_3-4′	0.92	0.93	0.92	0.92	0.94	0.92
H_2-1″	4.15	3.92	4.15	3.92	3.32	3.31
H_3-1‴	3.09	3.24	3.08	3.24	—	—
H_2-2″	—	—	—	—	1.67	1.69
H-$3''_A$	—	—	—	—	1.86	1.88
H-$3''_B$	—	—	—	—	1.74	1.74
H-4″	—	—	—	—	4.20	4.20
H_3C-CO-4″	—	—	—	—	2.04	2.04
$HCOO^-$	8.45	8.45	8.44	8.44	8.44	8.43
			J[Hz]			
$^{2}J_{5A,5B}$	—	—	a	a	—	(−)15.5
$^{2}J_{8A,8B}$	(−)13.8	a	(−)14.7	a	(−)13.4	(−)14.5
$^{2}J_{1'A,1'B}$	a	a	a	a	(−)13.7	(−)13.7
$^{3}J_{5A,6}$	a	a	a	a	a	< 1
$^{3}J_{5B,6}$	a	a	a	a	a	5.8
$^{3}J_{6,7}$	a	a	a	a	8.4[b]	4–5
$^{3}J_{7,8A}$	4.4	a	4.0	a	4.5	3–4
$^{3}J_{7,8B}$	a	a	a	a	11.7[b]	2.2
$^{3}J_{8A,8a}$	3.0	a	3.1	a	2.8	3.1
$^{3}J_{8B,8a}$	12.0	a	12.1	a	12.1	12.1
$^{3}J_{1'A,2'}$	a	a	a	a	6.6	6.6
$^{3}J_{1'B,2'}$	a	a	a	a	8.5	8.5

(continued)

Table 2—*contd*

				J[Hz]		
$^3J_{2',3'}$	[a]	[a]	[a]	[a]	7.5	7.5
$^3J_{3',4'}$	7.4	7.4	7.3	7.3	7.4	7.4
$^3J_{1',2'}$	—	—	—	—	6.7	6.8
$^3J_{3''A,4''}$	—	—	—	—	5.0	5.0
$^3J_{3''B,4''}$	—	—	—	—	8.0	8.0
^{13}C NMR				δ[ppm]		
C-2	167.6	167.4	167.6	167.4	166.8	167.0
C-3a	181.4	181.6	182.3	182.5	181.4	182.0
C-5	52.9	53.0	49.8	49.9	53.4	50.3
C-6	72.6	72.6	68.6	68.6	72.7	68.6
C-7	73.9	73.9	69.1	69.2	73.9	69.1
C-8	35.6	35.7	31.1	31.2	35.6	31.1
C-8a	59.5	59.4	58.1	58.0	59.2	57.8
C-1′	52.1	52.3	53.3	53.5	52.7	53.8
C-2′	28.7	28.7	28.4	28.5	29.0	28.6
C-3′	19.4	19.4	19.5	19.5	19.7	19.7
C-4′	13.3	13.3	13.3	13.3	13.5	13.5
C-1″	55.0	53.9	55.0	53.9	42.3	42.3
C-2″	175.8	174.2	175.9	174.3	24.9	24.9
C-1‴	36.8	38.2	36.7	38.2	—	—
C-3″	—	—	—	—	29.2	29.1
C-4″	—	—	—	—	55.0	55.0
C-5″	—	—	—	—	179.0	178.9
H_3C-CO-NH-4″	—	—	—	—	22.4	22.3
H_3C-**C**O-NH-4″	—	—	—	—	174.0	174.1
$HCOO^-$	171.3	171.3	171.3	171.3	170.7	170.6

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.
[a]No coupling constant can be determined, due to overlapping multiplets.
[b]Assignment of coupling constants may have to be reversed.

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. The water used for HPLC was distilled and filtered through a nylon membrane (0.45 μm). HPLC grade methanol was used without further purification. All 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*-Acetyl-L-arginine was purchased from Aldrich (Steinheim, Germany), methylglyoxal (solution in water, ≈40%), *n*-butylamine, and creatine from Fluka (Neu-Ulm, Germany), D-(+)-glucose anhydrous from Merck.

Model reactions. Methylglyoxal (70 μL, 0.4 mmol), *n*-butylamine (43 mg, 0.6 mmol), phosphate buffer pH 7.4 (170 mg, 1.0 mmol), and creatine (**5**) (52 mg, 0.4 mmol) or α-*N*-acetyl-L-arginine (**6**) (86 mg, 0.4 mmol) were dissolved in water (1.0 mL). After incubation (40 °C, 24 h), aliquots of 20 μL of the membrane filtrate were injected into the analytical HPLC system. A main peak was detected for reactions with **5** (t_R 7.7 min, UV maximum (λ_{max}) 246 nm) and a less predominant peak for reactions with **6** (t_R 7.5 min, λ_{max} 243 nm). Employing D-glucose (36 mg, 0.2 mmol) instead of methylglyoxal, two main peaks (t_R 6.6/7.3 min, λ_{max} 256 nm) were found for the reaction with **5** (70°C, 24 h). For the respective α-*N*-acetyl-L-arginine incubation (70°C, 24 h), signals with t_R 6.8/7.4 min and λ_{max} 253 nm were detected.

2-[(5-Butylimino-4-methyl-4,5-dihydro-1*H*-2-imidazolyl) (methyl)amino]acetic acid (7). Methylglyoxal (1.4 mL, 7.78 mmol), *n*-butylamine (4.3 g, 58.9 mmol), **5** (2.0 g, 15.3 mmol), and phosphate buffer pH 7.4 (3.0 g, 17.7 mmol) were dissolved in water (19.0 mL). After incubation (40°C, 24 h), the reaction mixture was purified by preparative HPLC (I) (monitoring wavelength 246 nm). Fractions with t_R 21.1 min were combined, lyophilized, taken up in eluent (10 mL), and once again subjected to preparative HPLC (I) and subsequent lyophilization procedure. After all, **7** was obtained as the respective formate (20 mg; 0.9%); ^{1}H and ^{13}C NMR

(D_2O): see Table 1; FABHRMS (glycerol): m/z 241.1664 $[M+H]^+$ (241.1665, calculated for $C_{11}H_{21}N_4O_2$).

2-Acetylamino-5-[(5-butylimino-4-methyl-4,5-dihydro-1*H*- 2-imidazolyl)amino]pentanoic acid (8). Methylglyoxal (2.8 mL, 15.6 mmol), *n*-butylamine (2.1 g, 28.8 mmol), **6** (4.2 g, 19.4 mmol), and phophate buffer pH 7.4 (6.9 g, 40.8 mmol) were dissolved in water (19.0 mL) and kept at 40°C for 24 h. The reaction mixture was purified as described for **7**. Fractions with t_R 20.3 min (detection at 243 nm) yielded the formate of **8** (39.1 mg, 0.7%); ^1H and ^{13}C NMR (D_2O): see Table 1; FABHRMS (glycerol): m/z 326.2193 $[M+H]^+$ (326.2192, calcd for $C_{15}H_{28}N_5O_3$).

2-[(4-Butyl-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo [4,5-*b*]azepin-2-yl)(methyl)amino]acetic acid (9). D-Glucose (0.7 g, 3.9 mmol), *n*-butylamine (0.9 g, 12.3 mmol), **5** (1.0 g, 7.6 mmol), and phosphate buffer pH 7.4 (3.2 g, 18.9 mmol) were dissolved in water (20.0 mL) and kept at 70 °C for 24 h. The reaction mixture was purified as described for **7**. Fractions with t_R 14.7 min and t_R 19.4 min (detection at 256 nm) were collected, yielding **9a,b** (32 mg, 2.3%) and **9c,d** (57.1 mg, 4.1%) as the respective formates; ^1H and ^{13}C NMR (D_2O): see Table 2; FABMS (glycerol): m/z 313 ($[M+H]^+$, 100), 335 ($[M+Na]^+$, 5); FABHRMS (glycerol): m/z 313.1874 $[M+H]^+$ (313.1876, calcd for $C_{14}H_{25}N_4O_4$).

2-Acetylamino-5-[(4-butyl-6,7-dihydroxy-4,5,6,7,8,8a- hexahydroimidazo[4,5-*b*]azepin-2-yl)amino]pentanoic acid (10). D-Glucose (0.7 g, 3.9 mmol), *n*-butylamine (0.9 g, 12.3 mmol), **6** (1.8 g, 8.3 mmol), and phosphate buffer pH 7.4 (3.2 g, 18.9 mmol) were dissolved in water (20 mL) and kept at 70 °C for 24 h. The reaction mixture was first subjected to preparative HPLC (I). Fractions with t_R 12.8 min and t_R 18.1 min (monitoring wavelength 253 nm) were combined and lyophilized. For further purification, the residue was dissolved in ammonium formate buffer (10 mM, pH 4.0, 9 mL) and subjected to preparative HPLC (II). Fractions with t_R 22.6 min and t_R 25.3 min gave **10a** (60 mg, 3.5%) and **10b** (44 mg, 2.6%) as the respective formates; ^1H and ^{13}C NMR (D_2O): see Table 2; FABMS (glycerol): m/z 398 ($[M+H]^+$, 100), 420 ($[M+Na]^+$, 16), 795 ($[M+H+M]^+$, 3), 817 ($[M+Na+M]^+$, 2); FABHRMS (glycerol): m/z 398.2402 $[M+H]^+$ (398.2403, calcd for $C_{18}H_{32}N_5O_5$).

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