Crosslines A and B as Candidates for the Fluorophores in Age- and Diabetes-related Cross-linked Proteins, and their Diacetates produced by Maillard Reaction of α-*N*-Acetyl-L-lysine with D-Glucose

Ko Nakamura, Taisuke Hasegawa, Yasutsugu Fukunaga and Kazuharu Ienaga*

Department of Natural Products Chemistry, Institute of Bio-Active Science, Nippon Zoki Pharmaceutical Co. Ltd., Yashiro, Kato-gun, Hyogo 673-14, Japan

A pair of novel fluorophores (*N*-diacetates of crosslines A and B), (3*R*,4*S*)-3,4-dihydroxy-5-[(1*S* or 1*R*,2*S*,3*R*)-1,2,3,4-tetrahydroxybutyl]-1,7-bis[6-(*N*-acetyl-L-norleucyl)]-1,2,3,4-tetrahydro-1,7-naphthyridinium chloride and its epimer, were isolated from the Maillard reaction mixture of α-*N*-acetyl-L-lysine and D-glucose, and their properties are similar to those of fluorophores in age- and diabetes-related cross-linked proteins, and a pair of homologues derived from n-pentylamine.

Free amino groups, such as ε-groups of lysine residues, in protein(s) can react with reducing sugar(s) to form fluorogenic cross-linkage(s) by the Maillard reaction.[1,2] Recently, this chemical reaction has attracted biologists' and medical doctors' attention, because the resulting protein cross-linkages have been recognized[3–5] to be important in connection with ageing and diseases such as diabetic complications; fluorogenic parts of such cross-linked proteins are named AGE (advanced glycation end products). Although, several Maillard reaction products, FFI,[6] pyrrolecarbaldehyde,[7] pentosidine[8] *etc.*, have been isolated, none of them properly satisfied the features of AGE to date. In the present paper, we describe two fluorophores, named crosslines A and B, which are hypothetical candidates for those in age- and diabetes-related cross-linked proteins.

Since it is most probable that two lysine residues in protein(s) would be linked by sugar(s), an equimolar Maillard reaction of α-*N*-acetyl-L-lysine and D-glucose in 0.25 mol dm^{-3} phosphate buffer (pH 7.4) was first investigated. After 6 weeks incubation at 37 °C, a pair of major fluorophores, **3** and **4**, was obtained as chlorides.† We then isolated similarly a pair of fluorogenic products **1** and **2**, akin to **3** and **4**,

† After treatment of the reaction mixture with a sulfonic acid type ion exchange column (H^+ form), the concentrated ammoniacal eluent was purified by reverse phase preparative column chromatography and reverse phase HPLC. Yields: **3**, 0.06%; **4**, 0.04%.

Table 1 NMR data for the fluorophores **2** and **3**[a]

	Fluorophore **2**		Fluorophore **3**	
Position[b]	^{13}C	^{1}H	^{13}C	^{1}H
2	50.0 t	3.37 ddd (1, 1, 13) 3.69 dd (2, 13)	49.9 t	3.35 ddd (1, 1, 13) 3.69 dd (2, 13)
3	66.1 d	4.02 ddd (1, 2, 3)	65.7 d	4.03 ddd (1, 2, 3)
4	63.4 d	4.73 dd (1, 3)	63.7 d	4.77 dd (1, 3)
4a	133.2 s		133.3 s	
5	145.5 s		145.3 s	
6	131.4 d	8.14 br. s	131.1 d	8.09 br. s
8	124.8 d	8.09 br. s	124.9 d	8.08 br. s
8a	144.6 s		144.5 s	
9	67.8 d	5.53 d (1)	67.3 d	5.64 d (1)
10	74.9 d	3.84 dd (1, 9)	75.2 d	3.51 dd (1, 9)
11	73.6 d	3.85 m	72.7 d	3.84 m
12	65.1 t	3.66 dd (5, 11) 3.83 dd (3, 11)	64.9 t	3.70 dd (6, 12) 3.84 dd (3, 12)
1′	52.5 t	3.42 m 3.58 m	52.1 t	3.40 m 3.56 m
2′	26.7 t	1.66 m 2H	25.9 t	1.70 m 2H
3′	29.4 t	1.3–1.45 m 2H	23.6 t[c]	1.50 m 2H
4′	23.3 t	1.3–1.45 m 2H	32.2 t[d]	1.7–2.0 m 2H
5′	14.4 q	0.94 t (7) 3H	53.1 d	4.40 dd (5, 9)
6′	—	—	175.1 s[e]	
Ac-Me	—	—	22.5 q	1.96 s 3H
Ac-CO	—	—	173.3 s[f]	
1″	63.2 t	4.49 t (7) 2H	62.9 t	4.51 t (7) 2H
2″	32.4 t	2.00 m 2H	31.9 t	2.05 m 2H
3″	30.1 t	1.3–1.45 m 2H	23.9 t[c]	1.50 m 2H
4″	23.8 t	1.3–1.45 m 2H	32.6 t[d]	1.7–2.0 m 2H
5″	14.6 q	0.95 t (7) 3H	53.1 d	4.42 dd (5, 9)
6″	—	—	175.4 s[e]	
Ac-Me	—	—	22.5 q	1.99 s 3H
Ac-CO	—	—	173.4 s[f]	

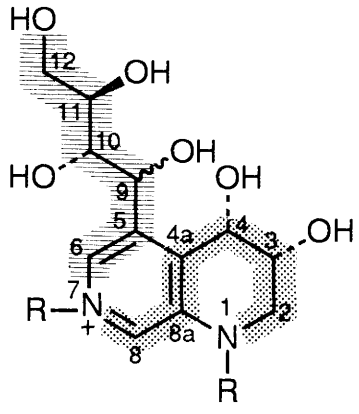
[a] ^{1}H (400 MHz in CD_3OD) and ^{13}C NMR (100 MHz in CD_3OD NMR chemical shifts are δ values from internal $SiMe_4$. ^{13}C assignments are based on C–H COSY and COLOC experiments. Figures in parentheses are coupling constants in Hz. Data for **1** are similar to those for **2** except for C9 [δ 67.3, d; 9-H δ 5.64, d (1)] and C10 (δ 75.2, d; 10-H δ 3.50, dd (1, 9)]. Data for **4** are similar to those for **3** except for C9 [δ 67.8, d; 9-H δ 5.53, d (1)] and C10 [δ 74.9, d; 10-H 3.82, dd (1, 9)]. [b] Primed atoms are those in the side chain at N1 and double primed atoms those in the side chain at N7. [c–f] Assignments with the same letter may be interchanged.

respectively, as chlorides‡ from a simpler Maillard reaction mixture of n-pentylamine and D-glucose in 0.25 mol dm^{-3} phosphate buffer (pH 7.4) for 3 weeks at 37 °C.

For **3** and **4** and for **1** and **2**, the same cationic empirical formulae $C_{28}H_{45}N_4O_{12}$ and $C_{22}H_{39}N_2O_6$, respectively, were assigned, because they showed common peaks at *m/z* 629 and 427, respectively, in positive SIMS; ^{13}C NMR spectra showed 28 and 22 carbons, respectively. Therefore, the components of these two pairs of fluorophores were thought to be obtained from two molecules of amine and two molecules of glucose, with elimination of six molecules of water. Our structural analyses started with the simpler products **1** and **2**.

Examination of the well dispersed 400 MHz ^{1}H NMR spectra of **2** (in D_2O at 40 °C or CD_3OD at room temperatures) involving double resonance and COSY experiments, allowed the assignments C2(CH_2)–C3(CH)–C4(CH) and C9(CH)–C10(CH)–C11(CH)–C12(CH_2). The proton attached to C4 was further coupled to the proton at C9 with a small coupling constant (<1 Hz). The downfield chemical shift of these protons indicated that above carbon atoms were attached to heteroatoms.

‡ Extraction of the acidified reaction mixture with n-butanol, followed by evaporation *in vacuo*, gave a yellowish oil, which was purified by Amberlite XAD-2 column and reverse phase preparative column chromatography, and reverse phase HPLC. Yields: **1**, 0.98%; **2** 0.76%.



	R	C9
Crossline	A; $-[CH_2]_4CH(NH_2)CO_2H$	(*R*)
	B; $-[CH_2]_4CH(NH_2)CO_2H$	(*S*)
	R	C9
	1; $-[CH_2]_4Me$	(*R*) or (*S*)
	2; $-[CH_2]_4Me$	Epimer of **1**
	3; $-[CH_2]_4CH(NHAc)CO_2H$	(*R*) or (*S*)
	4; $-[CH_2]_4CH(NHAc)CO_2H$	Epimer of **3**

Structures of crosslines A and B and their derivatives

The correlations observed in C–H COSY and the long range C–H correlation experiments (COLOC)[9] could be used to link the fragments and aromatic part of this molecule; the presence of the fragment C2–C3—C4—C4a(–C8a)–C5–C9 was first demonstrated and it was then extended as C2–C3–C4–C4a(—C8a–C8)–C5(–C6)–C9–C10–C11–C12. Because the coupling constant between 6-H and 8-H was very small (<1 Hz), C6 and C8 were not thought to be adjacent each other. This problem was solved by assuming that the aromatic part of **2** was pyridinium, and 6-H and 8-H were attached to adjacent sides of the nitrogen atom.

The remaining part of **2** needed to account for one degree of unsaturation and the positions of the heteroatoms. Acetylation of **2** by acetic anhydride–pyridine provided the hexaacetate **5**.§ ^{1}H NMR spectra of **5** showed downfield shifts for 3-H, 4-H, 9-H, 10-H, 11-H and 12-H, whereas the 2-H signal was not changed significantly. This indicates the existence of C8a–N(R)–C2, with hydroxy groups attached at C3, C4, C9, C10, C11 and C12. Each hydroxy proton was observed in the ^{1}H NMR spectrum (CD_3SOCD_3), and the couplings to the adjacent protons were confirmed (data not shown). The stereochemistry at C4 and C10 was (*S*) and that at C3 and C11 (*R*), because C4 and C10 corresponded to C4 of the original D-glucose, and C3 and C11 to C5.

Compound **1** was similar to 2 except for the chemical shifts of 9-H and 10-H. This strongly suggested that **1** was the epimer of **2** at C9 and that the epimerization resulted from formation of the pyridinium ring. The stereochemistry at C9 has yet to be clarified; **2** was thus (3*R*,4*S*)-3,4-dihydroxy-5-[(1*S* or 1*R*,2*S*,3*R*)-1,2,3,4-tetrahydroxybutyl]-1,7-dipentyl-1,2,3,4-tetrahydro-1,7-naphthyridinium chloride and **1** was its epimer.

Similarly the ^{1}H and ^{13}C NMR spectra of the pair of compounds **3** and **4** indicated structures corresponding to **1** and **2**, respectively: the structure of **4** was assigned as (3*R*,4*S*)-3,4-dihydroxy-5-[(1*S* or 1*R*,2*S*,3*R*)-1,2,3,4-tetrahydroxybutyl]-1,7-bis[6-(*N*-acetyl-L-norleucyl)]-1,2,3,4-tetrahydro-1,7-naphthyridinium chloride and **3** was its epimer.

§ Hexaacetate chloride **5**; ^{1}H NMR (400 Mhz, CD_3OD): δ 8.39 (1H, br s), 8.17 (1H, br s), 6.16 (1H, br d, *J* 3 Hz), 5.95 (1H, d, *J* 2 Hz), 5.37 (1H, ddd, *J* 2, 4, 10 Hz), 5.29 (1H, ddd, *J* 1, 2, 4 Hz), 5.26 (1H, dd, *J* 2, 10 Hz), 4.54 (1H, m), 4.51 (1H, m), 4.32 (1H, dd, *J* 4, 13 Hz), 4.20 (1H, dd, *J* 2, 13 Hz), 3.70 (1H, dd, *J* 2, 13 Hz), 3.66 (1H, dd, *J* 1, 13 Hz), 3.55 (2H, t, *J* 7 Hz), 2.25 (3H, s), 2.14 (3H, s), 2.12 (3H, s), 2.10 (3H, s), 1.98 (3H, s), 1.96 (3H, s), 1.95 (2H, m), 1.65 (2H, m), 1.3–1.4 (8H, m) and 0.94 (3H × 2, t, *J* 7 Hz); positive SIMS: *m/z* 679.

In the structural formula, the two carbon chains derived from the two molecules of D-glucose are shaded; carbon atoms from the same sugar are not branched but linearly extended. Crossline A retains all the stereogenic centres of aliphatic and alicyclic carbon atoms derived from the D-glucoses units; it has C9(*R*). Crossline B has C9(*S*).

The four fluorophores **1**–**4** gave the nearly same fluorescent spectra: λ_{max} (exc.) 379 nm, λ_{max} (em.) 463 nm. These fluorescent characteristics are not identical with those of the known natural AGE, but they are similar. Since antisera against the haptenic fluorophores (1 : 1 mixture of **3** and **4**) recognize the AGE produced not only *in vitro* but also *in vivo*, crosslines A and B can be hypothetical candidates of fluorophores, AGE, in age- and diabetes-related cross-linked proteins. Immunohistochemical staining of renal glomerular basement membranes for STZ (storeptozotocin) induced diabetic rat with our antisera increased in comparison with that for a normal rat. Details will be published in a biological journal.

Further studies on the determination of the C9 chirality for **3** and **4**, and isolation of other minor fluorophores from the same Maillard reaction mixture are now in progress.

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References

1 L. C. Maillard, *C.R. Acad. Sci (Paris)*, 1912, **154**, 66.
2 H. F. Bunn, D. N. Hansy, H. H. Gabbay and P. M. Gallop, *Biochem. Biophys. Res. Commun.*, 1975, **67**, 103.
3 M. Brownlee, H. Vlassara and A. Cerami, *Ann. Int. Med.*, 1984, **101**, 527.
4 V. M. Monnier, R. R. Cohen and A. Cerami, *Proc. Natl. Acad. Sci. USA*, 1983, **81**, 583.
5 V. M. Monnier and A. Cerami, *Science*, 1981, **211**, 491.
6 S. Pengor, P. C. Ulrich, F. A. Bencsath and A. Cerami, *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 2684.
7 F. Hayase, R. H. Nagarej, S. Miyata, F. G. Njoroge and V. M. Monnier, *J. Biol. Chem.*, 1989, **263**, 3758.
8 D. R. Sell and V. M. Monnier, *J. Biol. Chem.*, 1989, **264**, 21597.
9 H. Kessler, C. Griesinger, J. Zarbrock and H. R. Loosli, *J. Magn. Reson.*, 1984, **57**, 331.