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Note

Preparation and characterisation of N_ε-(1-deoxy-D-fructos-1-yl)hippuryl-lysine

Patricia R. Smith and Paul J. Thornalley*
Department of Chemistry and Biological Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SQ (Great Britain)

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Monosaccharides react non-enzymically with the N-terminal amino group and lysyl side-chain amino groups of proteins, under physiological conditions, to produce glycated proteins[1]. Examples of proteins known to undergo this reaction are haemoglobin, serum albumin, immunoglobulins, collagen, and lens crystallins[2–6]. The acyclic aldehyde or ketone form of the monosaccharide reacts with the amino group to form a Schiff's base or imine that undergoes an Amadori rearrangement to form a ketoamine[7]. With glucose, the ketoamine is an N_ε-(1-deoxy-D-fructos-1-yl)-amino acid residue, a fructosamine[8].

The concentration of fructosamines in samples of blood plasma from diabetic patients is used as a measure of dietary or pharmacological control of blood-glucose concentration[9]. This concentration is assayed routinely by a simple, colorimetric assay based on the reducing activity of glycated serum proteins in alkaline solution. At pH 10.35, the fructosamine reduces Nitro Blue Tetrazolium (NBT) to formazan; the corresponding rate of change in absorbance at 520 nm is directly proportional to the concentration of fructosamine[10].

Fructosamines are also oxidised slowly under physiological conditions[11]. Oxidative reactions and rearrangements lead to the irreversible formation of advanced glycation end-products (AGEs). AGEs alter the physicochemical and biochemical characteristics of proteins, usually associated with a deterioration in the biological function of the proteins[12]. Several AGEs have been identified and include 3-deoxyglucosone[13], 5-hydroxymethyl-1-alkylpyrrole-2-carbaldehyde[14], N_ε-carboxymethyl-lysine[15], N_ε-(3-lactato)lysine[16], and 2-furoyl-4(5)-(2-furanyl)-1*H*-imidazole (FFI)[17]; although the formation of FFI is now thought to be artifactual[12,19,20]. The mechanism by which AGEs are formed has not yet been fully explained[1].

The synthesis and characteristics of fructosamines derived from amino acids and peptides are currently under investigation as prospective standards in the fructosamine test[21] and to study the mechanism of fructosamine degradation[13–20]. The fructosamine

* To whom correspondence should be addressed.

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derivative of hippuryl-lysine, N_ε-(1-deoxy-D-fructos-1-yl)hippuryl-lysine (DHL), may be a suitable model fructosamine. The benzoyl group protects the N-terminal amino group and provides a chromophore such that DHL and peptide-based degradation products may be resolved rapidly and detected by h.p.l.c. with u.v. detection. The precursor, hippuryl-lysine is relatively inexpensive and available commercially.

We now describe the preparation, characterisation, and the alkaline reducing activity of DHL.

DHL (**1**) was prepared by reacting hippuryl-lysine with a 12-fold molar excess of D-glucose in refluxing methanol for 4 h, purified (see Experimental) by solid-phase extraction followed by phenylboronate affinity chromatography, and characterised by ^{1}H- and ^{13}C-n.m.r. spectroscopy (Tables I and II). Assignments were made by comparison with data for hippuryl-lysine and for amino-acid fructosamine derivatives[22]. The major conformer in solution was the β-pyranose form ($J_{3,4}$ 9.9 Hz indicated H-3,4 to be *trans*-diaxial and the resonances for C-3,4,5 at 71.0–71.6 p.p.m. indicated the β-pyranose form). These data were obtained using a freshly prepared solution of DHL in deuterated phosphate buffer. On storage, the H-1 resonance of the fructosamine group

TABLE I

^{1}H-N.m.r. data[a] for hippuryl-lysine and N_ε-(1-deoxy-D-fructos-1-yl)hippuryl-lysine (DHL)

Assignment	*Hippuryl-lysine*		*DHL*	
	δ (p.p.m.)	*J (Hz)*	*δ (p.p.m.)*	*J (Hz)*
Lysine				
H-2	4.10	$J_{2,3A}$ 8.3, $J_{2,3B}$ 4.8	4.05	$J_{2,3A}$ 8.7, $J_{2,3B}$ 4.9
H-3A	1.62	unresolved	1.57	unresolved
H-3B	1.72	unresolved	1.68	unresolved
H-4 (2 H)	1.21	$J_{3A,4} = J_{3B,4} = J_{4,5} = 7.5$	1.21	$J_{3A,4} = J_{3B,4} = J_{4,5} \approx 7$
H-5 (2 H)	1.54	$J_{4,5} = J_{5,6} = 7.5$	1.53	unresolved
H-6 (2 H)	2.89	$J_{5,6}$ 7.5	2.89	$J_{5,6} \sim 8$
Glycine				
H-2A	3.95	$J_{2A,2B}$ −16.7	3.90	$J_{2A,2B}$ −16.7
H-2B	4.03	$J_{2A,2B}$ −16.7	3.97	$J_{2A,2B}$ −16.7
Benzoyl				
o-H (2 H)	7.70	$J_{o,m}$ 7.1	7.64	$J_{o,m}$ 7.1
m-H (2 H)	7.41	$J_{m,p} = J_{m,o} = 7.1$	7.36	$J_{m,p} = J_{m,o} = 7.1$
p-H	7.50	$J_{m,p}$ 7.1	7.45	$J_{m,p}$ 7.1
Fructose				
H-1 (2 H)			3.08	
H-3			3.53	$J_{3,4}$ 9.9
H-4			3.69	$J_{3,4}$ 9.9, $J_{4,5}$ 3.4
H-5			3.82	$J_{4,5}$ 3.4, $J_{6,5}$ 2.0
H-6A			3.56	$J_{6A,6B}$ 13.1, $J_{5,6A}$ 2.0
H-6A			3.81	$J_{6A,6B}$ 13.1, $J_{5,6B}$ unresolved

[a] Solvent: 100mM NaD_2PO_4/Na_2DPO_4 (pD 7.4) at 25°.

TABLE II

^{13}C-N.m.r. data[a] for hippuryl-lysine and N_ε-(1-deoxy-D-fructos-1-yl)hippuryl-lysine (DHL)

Assignment	*Hippuryl-lysine* δ *(p.p.m.)*	*DHL* δ *(p.p.m.)*
Lysine		
C-1	178.1	180.7
C-2	38.6	33.0
C-3	21.3	24.1
C-4	25.6	25.1
C-5	30.4	26.7
C-6	54.1	56.7
Glycine		
C-1	170.6	173.1
C-2	42.5	45.2
Benzoyl		
C-1	170.2	172.9
o-C	128.2	129.2
m-C	126.6	130.9
p-C	131.9	134.5
Fructose		
C-1	–	54.9
C-2	–	97.4
C-3	–	71.6
C-4	–	71.3
C-5	–	71.0
C-6	–	65.9

[a] Solvent: 100mM NaD_2PO_4/Na_2DPO_4 (pD 7.4) at 25°.

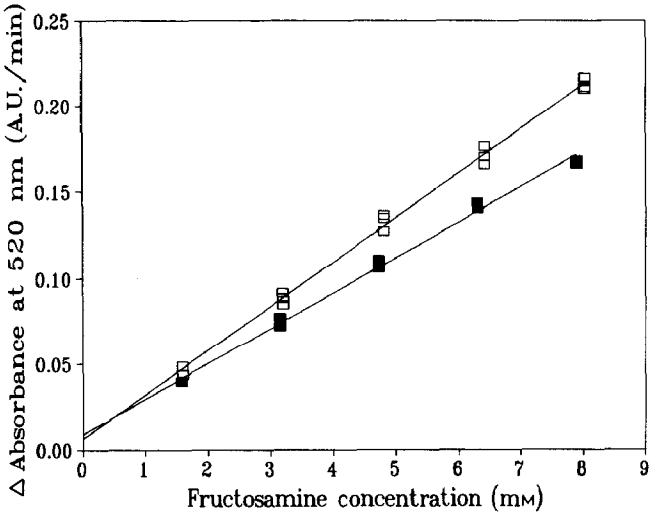
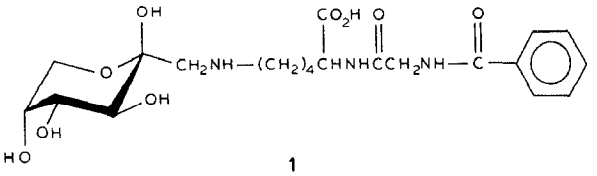


Fig. 1. Alkaline reducing activities of N_ε-(1-deoxy-D-fructos-1-yl)hippuryl-lysine (**1**, DHL, ■) and 1-deoxy-1-*N*-morpholino-D-fructose (DMF, □) (see Experimental). Least-squares linear regression: DHL, $y = (0.0206 \pm 0.0004)x + 0.0088$, $r^2 = 0.995$, $n = 16$; DMF, $y = (0.0257 \pm 0.0004)x + 0.0064$, $r^2 = 0.996$, $n = 16$.

was lost due to deuterium exchange through enolisation. It is also probable that minor proportions of other forms (α-pyranose, α- and β-furanose, and keto) exist in solution, but this aspect was not investigated further.



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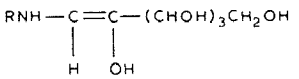

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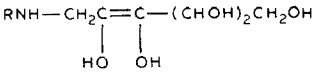

3

Reverse-phase h.p.l.c. provided a rapid assessment of the purity of DHL, particularly from hippuryl-lysine and other hippuryl-lysine-based degradation products[23].

The alkaline reducing activity of DHL and the synthetic standard used in the fructosamine test, 1-deoxy-1-*N*-morpholino-D-fructose (DMF), were evaluated by studying the rate of reduction of Nitro Blue Tetrazolium (NBT) at pH 10.35 by following the increase in absorbance at 520 nm. DHL reduced NBT at a slightly lower rate than DMF (Fig. 1). Although similar low-molecular-weight fructosamines have been synthesised[15,22], no comparable studies of alkaline reducing activity were reported.

The rates of reduction of NBT by DHL and DMF are similar in spite of the different amino substituents. The mechanism is thought to involve enolic intermediates which are the probable primary reductants; other secondary products, such as enoloxy and superoxide free radicals, and α,β-dicarbonyl compounds may also reduce NBT[24].

Two enols may be formed, namely, the 1,2-ene-aminol (**2**) and the 2,3-ene-diol (**3**), by a hydroxide-catalysed process. The formation of **3** may also be catalysed by an intramolecular deprotonation involving the fructosamine amino group. At pH 10.35, both processes may contribute significantly to the formation of reductants of NBT[25,26].

DMF has been rejected for use as a calibration standard in the clinical assay of fructosamines because it reduces NBT at a rate much lower than those of protein-based fructosamines (prepared by non-enzymic glycation of polylysine or serum albumin) under alkaline conditions. The use of DMF has led to a ten-fold overestimation of the concentration of serum fructosamine[27].

Since the NBT alkaline reducing activities (Fig. 1) of DHL and DMF are similar, DHL may also not be a suitable standard. However, because the rate of reduction of NBT in the clinical assay involves a biological matrix (diluted serum) and measurement of the increase in absorbance at 540 nm in the time interval 9–13 min after mixing the sample with NBT (to avoid interferences which rapidly reduce NBT in the first 9 min)[27], no direct comparison can be made with the simple chemical model system.

The evaluation of peptide-based fructosamines for use as calibration standards deserves further investigation, since they are relatively inexpensive and can be purified to homogeneity (protein-fructosamine standards contain typically only 20–30% of the available amine groups in the fructosamine form and may also contain some fructosamine degradation products)[27]. However, there appears to be an intrinsic difference in the reactivity of peptide-fructosamines and protein-fructosamines with NBT under alkaline conditions[28].

EXPERIMENTAL

General. — Hippuryl-lysine and hippurylglycylglycine were purchased from Sigma. T.l.c. was performed on Silica Gel 60 F_{254}, using 1-propanol–acetic acid–water (15:5:1) and detection with ethanolic 0.2% ninhydrin at 100° for 15 min.

Reverse-phase h.p.l.c. was performed with a C_{18} radial compression cartridge (8 mm × 10 cm; Waters-Millipore) by elution with 10mM sodium phosphate buffer (pH 7.0) with a non-linear, concave gradient of 5→10% of MeOH at 2 mL/min. The eluate was monitored by flow spectrophotometry at 230 nm. The internal standard was hippurylglycylglycine.

N_ε-(1-Deoxy-D-fructos-1-yl)hippuryl-lysine (DHL). — A mixture of hippuryl-lysine (46.1 mg, 0.15 mmol), D-glucose (329 mg, 1.83 mmol), and methanol (h.p.l.c. grade, 6.25 mL) was boiled under reflux for 4 h. The methanol was then evaporated, and a solution of the solid residue in 10mM sodium phosphate buffer (pH 7.4, 1.00 mL) was applied to a solid-phase extraction cartridge (C_{18}, 1 mL, SEP-PAK; Waters-Millipore) equilibrated with the same buffer and eluted with phosphate buffer (50 mL). The absorbance of the eluate was monitored at 275 nm. The alkaline reducing activity of each fraction of the eluate was assayed (see below). The appropriate 2-mL fractions (1–20) were combined and lyophilised. Subsequent to the development of the methods, the cartridge was eluted with 50 mL of phosphate buffer and the complete eluate was lyophilised.

A solution of the product in water (5 mL) was applied to a column (12.0 × 1.6 cm) of phenylboronate affinity matrix (Affi-Gel 601, Biorad), equilibrated with 10mM sodium phosphate buffer and eluted with water (100 mL), then with aqueous 5% acetic acid at 30 mL/h. The eluate was monitored at 254 nm, the alkaline reducing activity of each 2-mL fraction was assayed (see below), and the appropriate fractions (17–24) were combined to give DHL (**1**, 22.1 mg), m.p. 88–89° (dec.), $[\alpha]_D^{20}$ −24° (*c* 0.3–1.5, water); λ_{max} (10mM phosphate buffer) 229 (ε 6.92) and 275 nm (sh); t.l.c., R_F 0.18 (*cf.* 0.25 for hippuryl-lysine); h.p.l.c., *T* 21.4 min (*cf.* 19.0 min for hippuryl-lysine).

Anal. Calc. for $C_{21}H_{31}N_3O_5 \cdot 4H_2O$: C, 45.7; H, 6.8; N, 7.6. Found: C, 45.9; H, 6.9; N, 7.9.

Assay of alkaline reducing activity ("fructosamine test"). — 0.25mM Nitro Blue Tetrazolium (NBT) in 100mM sodium carbonate buffer (750 μL, pH 10.35) was diluted with water (180 μL) and the mixture was incubated at 37° for 3 min. An aliquot (60 μL) of the solution to be assayed was added to the incubation mixture and the absorbance

was monitored at 520 nm during 6 min. The rate of change in absorbance $\Delta A_{520}/\Delta t$ was linear from 2–6 min and this rate was taken as a measure of alkaline reducing activity. The results are given in Fig. 1.

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