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Novel Carbohydrate-Induced Modification of Peptides: Crystal Structure and NMR Analysis of Ester-Bridged Bicyclic Galactose-Enkephalin Adduct Containing Imidazolidinone Moiety

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Abstract: Comparative studies based on x-ray crystallography and NMR spectroscopy were used for structural characterization of the novel minor, imidazolidinone moiety containing, product **2b** of the Maillard reaction obtained *in vitro* by using the galactose-modified endogenous opioid pentapeptide leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) **1**. The x-ray analysis uniquely defined the molecular structure as cyclo-{*N*-{[2-[-4)-D-*galacto*-pentitol-1-yl]-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl-(1 → O]acetyl}glycyl-L-phenylalanyl-L-leucyl-} (**3**), having an 18-membered ring with an ester bond between the secondary (C4′) hydroxyl group of a D-*galacto*-pentitolyl residue and the *C*-terminal carboxy group of leucine-enkephalin. The absolute configuration of the new chiral centre at the imidazolidinone moiety was established as C2(*S*), indicating a *cis* arrangement of C2 and C4 substituents at the 5-membered heterocyclic ring. The NMR analysis of compound **2b** carried out in CH_3CN-d_3 and DMSO-d_6, indicated the existence of two isomers in solution, differing only in the position of the ester group in the molecule. NMR data for the minor isomer (13%–16%) are in agreement with structure **3**. The migratory tendency of the peptidyl group from the primary (**2b**) to the secondary hydroxyl group (**3**) of a D-*galacto*-pentitolyl residue in methanol/water solution was confirmed by RP HPLC analysis.

Keywords: x-ray structure analysis; NMR spectroscopy; conformational analysis; leucine-enkephalin; imidazolidinone; carbohydrate; galactose; glycation

INTRODUCTION

Nonenzymatic reactions of carbohydrates with biological amines (proteins, amino acids, nucleic acids, amino phospholipids) have attracted considerable attention. These reactions have been implicated in a number of pathologies, most clearly in diabetes mellitus, but also in normal processes of ageing and neurodegenerative amyloid diseases such as Alzheimer's and Parkinson's disease [1–4]. The glycation reaction products, complex and heterogeneous group of compounds, are the major cause of tissue dysfunctions in the elderly and in diabetes due to the cross-linking of proteins which alters the normal cell–matrix interactions [5]. Protein glycation (the Maillard reaction) is initiated by a condensation reaction between available amino groups on proteins with either an aldehydo or a keto group of a reducing sugar or an oligosaccharide. It is generally accepted that either Amadori or Heyns intermediate rearrangement products may

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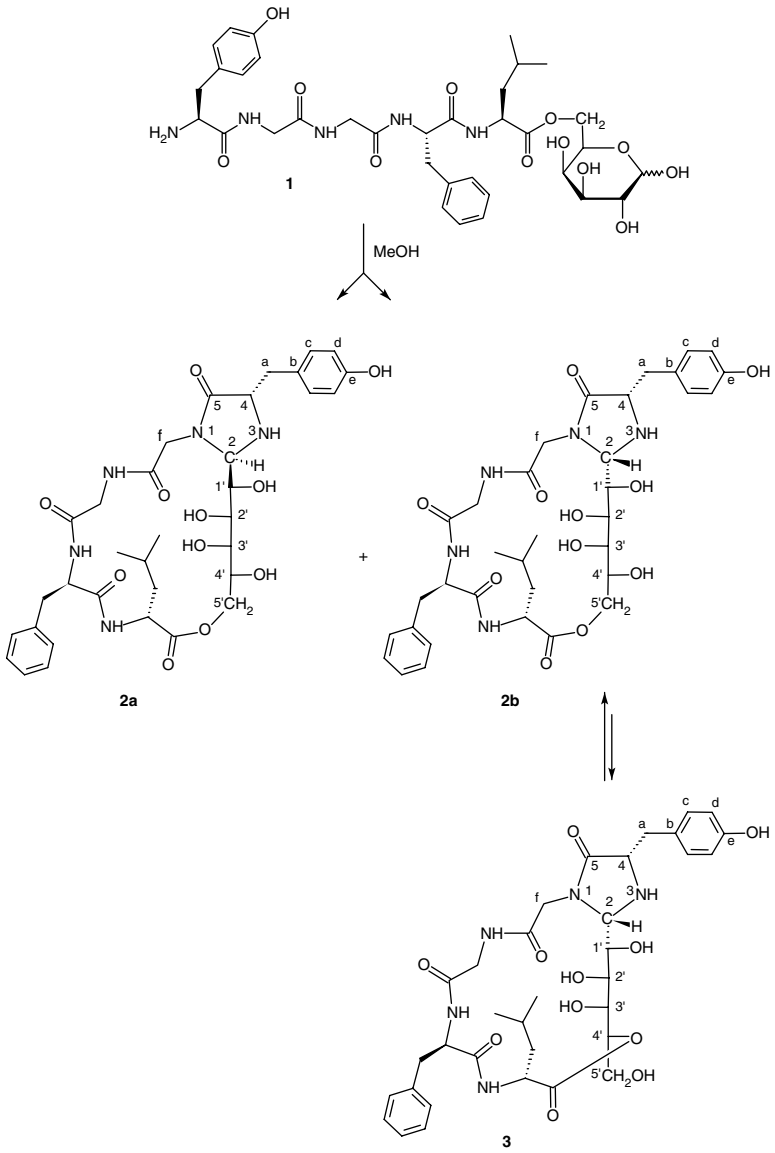
be formed *via* Schiff bases from the initial condensation reaction depending on whether the reducing sugar is an aldose or ketose [6]. A complex series of reactions follows [7] leading to the formation of highly reactive compounds having a pathogenic role, particularly in relation to the diabetic microvascular complications of retinopathy, nephropathy, neuropathy with accelerated vasculopathy observed in diabetes [5,8]. The chemistry of these later-stage glycation processes is still incompletely understood and it is not clearly known what chemical constituents of glycated proteins are involved in the generation of reactive oxygen and nitrogen species.

In an effort to better understand the role of the early glycation products in biologically relevant processes, in our recent studies carbohydrate-peptide esters were used in which the sugar moiety is linked to the *C*-terminal carboxy group of the endogenous opioid pentapeptide leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) [9] as model compounds for the study of the Maillard reaction *in vitro* [10–13]. It was found for the first time that, depending on the reaction conditions, in addition to an Amadori rearrangement, an alternative pathway for the carbohydrate-induced modification of peptides is possible, yielding hexose-related imidazolidinones [10,11]. Intramolecular cyclization



Scheme 1

of D-*galacto*-related 6-*O*-(leucine-enkephalyl)-ester **1** gave imidazolidinone derivative **2** as a mixture of isomers in a 1.3 : 1 diastereoisomeric ratio, supposed to have the *trans* and *cis* relative geometry of the substituents at the imidazolidinone ring moiety (Scheme 1) [11].

Our interest in this work has been focused on the comparative structural analysis based on x-ray crystallographic studies and NMR spectroscopic data carried out on the minor diastereoisomer of D-*galacto*-related bicyclic imidazolidinone, in order to determine absolute configuration at the new chiral centre (C2) and to gain definite confirmation about the relative arrangement of the substituents at the imidazolidinone ring moiety.

MATERIALS AND METHODS

Compounds

Bicyclic D-*galacto*-pentitolyl-imidazolidinone compounds **2** (Scheme 1) were prepared according to the reported procedure [11]. In brief, incubation of 6-*O*-(L-tyrosylglycylglycyl-L-phenylalanyl-L-leucyl)-D-galactopyranose **1** [14] in dry MeOH for 2 days at 60 °C afforded a mixture of imidazolidinones **2** which were purified and separated by semipreparative RP HPLC using 47% MeOH/0.1% TFA as the eluent. Major isomer: yield 13%, retention time: 27.1 min; minor isomer: yield 10%, retention time: 27.5 min.

RP HPLC analysis of the 2b → 3 isomerization. Compound **2b** (1.0 mg, 1.43 μmol) was dissolved in the mixture methanol/water (5 : 4 v/v) (0.9 ml) and kept at room temperature for 2 months (Figure 1). The appearance of compound **3** was monitored on the aliquots removed periodically from the incubation mixture and directly analysed by RP HPLC by using 53% MeOH/0.1% TFA as the eluent.

X-ray Analysis

Compound **3** crystallized from the solution of compound **2b** in methanol/water (5 : 4 v/v) mixture by slow evaporation at room temperature for 2 months. The crystal data and summary of experimental details are listed in Table 1. The x-ray intensity data were collected with a Nonius CAD4 diffractometer using graphite monochromated Cu$K\alpha$ radiation at 100 K. Data reduction was performed by HELENA [15] and the absorption correction using Ψ-scan data. The structure was solved by SIR97

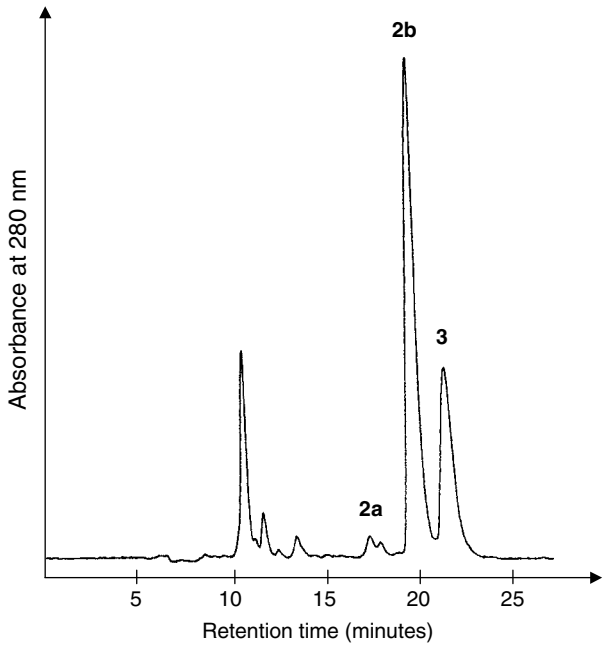


Figure 1 RP HPLC chromatogram of compound **2b** in methanol–water (5 : 4) mixture after 2 months at room temperature.

[16] and refined on F^2 by SHELXL97 [17]. The H-atom coordinates were calculated geometrically and refined using the SHELX97 [17] riding model. Details of the refinement are given in Table 1. During the structure determination, the chirality of amino acid moieties was used as an internal standard to define the absolute configuration of the atoms involved in the chemical reactions. The absolute configuration is C1(*S*), C2(*S*), C3(*R*), C4(*R*), C5(*R*), C7(*S*), C10(*S*), C11(*S*). The absolute structure parameter of 0.5 (3) obtained during refinement was not informative in spite of the good-quality low temperature data set (Table 1). The molecular geometry was calculated by program PLATON [18] and the molecular structure presented by ORTEP [19].

Crystallographic data for the structure reported has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-195737. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44/1223336033; e-mail: deposit@ccdc.cam.ac.uk).

NMR Spectroscopy

All one- and two-dimensional NMR spectra were recorded on a Bruker Avance DRX500 spectrometer equipped with a 5 mm diameter inverse detection probe and z-gradient accessory working at

Table 1 Crystallographic Data, Structure Solution and Refinement of **3**

Compound	**3**
Formula	$C_{34}H_{45}N_5O_{11}$
M_r	699.75
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
a/Å	35.235 (4)
b/Å	9.7639 (7)
c/Å	9.8667 (10)
V/Å^3	3394.5 (6)
Z	4
D_x/Mg m^{-3}	1.369
Temperature/K	100(3)
Wavelength/Å	1.54180
Absorption coefficient/mm^{-1}	0.860
F(000)	1488
Absorption correction	Ψ-scan
Max and min transmission	0.9833, 0.9111
Crystal size/mm	0.3 × 0.17 × 0.05
Total data	3835
Observed data [I > 2σ(I)]	2410
Θ_{max}, completeness of data	74, 97.5%
Range of h,k,l	−44, 0; −12, 0; 0, 12
Absolute structure parameter	0.5(3)
R_1 [$F_o > 4\sigma(F_o)$]	0.0395
R_1, wR_2 (F^2) (all data)	0.1418, 0.101
S	1.019
No. of parameters	489
$\Delta\rho_{max}$, $\Delta\rho_{min}$/eÅ^{-3}	0.305, −0.313
Weighting scheme	w = 1/[$\sigma^2(F_o^2)$ + (0.0468 P)^2] $P = (F_o^2 + 2F_c^2)/3$.

500.13 MHz for ^{1}H. In ^{1}H NMR experiments the spectral width was 6000 Hz, the number of data points 65 K and the number of scans 32. TMS was used as the internal standard. The sample concentration was 12 mg ml^{-1} in CH_3CN-d_3 and DMSO-d_6 solutions. The digital resolution was 0.1 Hz per point.

Two-dimensional DQFCOSY and ROESY spectra were recorded under the following conditions. The spectral width was 6000 Hz in both dimensions, 2 K data points were applied in time domain and 512 increments were collected for each data set with linear prediction and zero filling to 2 K. 8–16 scans were applied for each increment. A relaxation delay was 1.5 s. States-TPPI ROESY spectra were obtained with a mixing time of 250 ms and processed with sine squared function shifted by $\pi/2$ in both domains, while DQFCOSY spectra were processed with unshifted sine function. The digital resolution was 2.7 and 10.7 Hz per point in f2 and f1 domains, respectively.

HSQC and HMBC spectra were recorded with a relaxation delay of 1.5 s and 32 scans per increment. The spectral width was 31 000 Hz in acquisition domain f2 and 6000 Hz in time domain f1. Data were collected into a 2048 × 256 acquisition matrix and processed using a 2K × 1K transformed matrix with zero filling in f1 domain. Sine multiplication was performed prior to Fourier transformations. In HMBC spectra the delay for long-range couplings was set to 60 ms.

RESULTS AND DISCUSSION

Synthesis

The synthetic route to bicyclic imidazolidinone compounds **2** involved intramolecular cyclization of 6-*O*-(Tyr-Gly-Gly-Phe-Leu-)-D-galactopyranose (**1**), in methanol as the solvent, resulting in the formation of imidazolidinone diastereoisomers **2**. It was assumed that, for steric reasons, the major reaction product has *trans* (**2a**) and minor *cis* (**2b**) relative geometry of the substituents at the imidazolidinone ring moiety (Scheme 1) [11]. Compounds **2a** and **2b** were separated by RP HPLC and a suitable crystal of compound **3** for x-ray analysis was obtained from the methanol–water solution of product **2b**.

Structural Characterization of 3 by x-ray Structure Analysis

For the labelling of the atoms in the crystal structure see Figure 2. X-ray analysis uniquely defined the molecular structure of **3** (Figure 2) revealing the intramolecular esterification *via* a secondary hydroxyl group involving the C5 position of D-galacto-pentitolyl residue and the *C*-terminus of the Leu moiety resulting in an 18-membered ring; its conformation is described by a set of torsional angles listed in Table 2. The absolute configuration of **3** is defined according to the internal standard [using the (*S*)-configuration of the amino acid residues] being: C1(*S*), C2(*S*), C3(*R*), C4(*R*), C5(*R*), C7(*S*), C10(*S*), C11(*S*) (C1 → C5 belong to a *galacto*-moiety and C7 → C11 to amino acid residues). The 18-membered ring conformation can be simply described as: t^-, t^+, g^-, t^+, t^-, g^-, g^-, t^-, g^-, g^-, t^+, t,$^-$ g^+, t^+, t^+, g^-, g^+, g^+ (Table 2, Figure 3). Significant

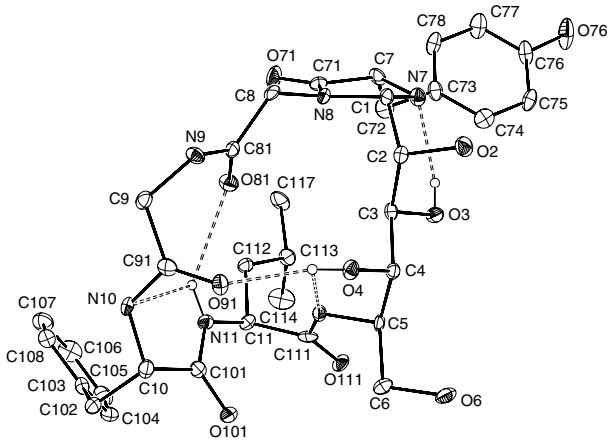


Figure 2 ORTEP drawing of **3** with thermal ellipsoid scaled at the 30% probability. The intramolecular hydrogen bonds are shown.

Table 2 Selected Torsional Angles (°) for **3** Defining 18-membered Ring Conformation

C1–C2–C3–C4	−168.4(3)
C2–C3–C4–C5	176.4(3)
C3–C4–C5–O5	−63.4(4)
C4–C5–O5–C111	156.1(3)
C5–O5–C111–C11	−178.4(3)
O5–C111–C11–N11	−36.7(4)
C111–C11–N11–C101	−54.6(5)
C11–N11–C101–C10	−174.4(3)
N11–C101–C10–N10	−23.5(5)
C101–C10–N10–C91	−63.8(4)
C10–N10–C91–C9	176.6(3)
N10–C91–C9–N9	−156.3(3)
C91–C9–N9–C81	66.3(4)
C9–N9–C81–C8	165.6(3)
N9–C81–C8–N8	158.5(3)
C81–C8–N8–C1	−111.9(4)
C8–N8–C1–C2	63.9(4)
N8–C1–C2–C3	61.8(4)

distortion from *gauche* conformation is introduced by: intramolecular 3-centred hydrogen bonds O4-H4···O5 and O4-H4···O91; N11-H11···O81 and N11-H11···N10, and also by embedding an imidazolidinone ring into the 18-membered ring. The values of involved torsional angles of the atom sequences are: O5–C111–C11–N11 [−36.7(4)°], N11–C101–C10–N10 [−23.5(5)°] and C81–C8–N8–C1 [−111.9(4)°]. The imidazolidinone ring adopts an envelope conformation with pseudorotation parameters $P = 347.4(7)^\circ$ and $\tau = 20.2(3)^\circ$ [20] with the average endocyclic torsion angle value of 12.8° and N7 being the out-of-plane atom [0.119(4)Å] that participates in the intramolecular hydrogen bond O3-H3···N7 (Table 3, Figure 2). The *cis*-orientation of the substituents of the imidazolidinone ring are determined by the values of torsional angles: C71–N8–C1–C2, −132.4(3)°; C1–N7–C7–C72, −142.4(3)°. In the peptide backbone of the cyclic galactose-peptide conjugate the intramolecular hydrogen bonds are related by a β-turn of the type III [21] involving Gly (i), Phe (i + 1) and Leu (i + 2) according to the definition of the torsional angles: $\Phi_{i+1} = -60^\circ$, $\Psi_{i+1} = -30^\circ$; $\Phi_{i+2} = -60$, $\Psi_{i+2} = -30^\circ$; in the present structure these values are −63.8°, −23.5°, −54.6° and −36.7°, respectively.

Theoretically, linear peptides can adopt many different backbone conformations, whereas in the cyclic ones the conformation can be restricted. In the crystalline state of enkephalins three types have been observed: an extended, a single β-fold and a double β-fold [22]. Generally, for enkephalins and related compounds a single-fold conformation is the most common one with a predominance of the type I′ β-fold often stabilized by two intramolecular hydrogen bonds N(i+3)–H···O(i) and N(i)–H···O(i + 3) [22,23]. A double β-fold conformation has been observed for Leu-enkephalin itself [24] and for two analogues [23,25]. However, in the cyclic peptides the conformational flexibily is reduced. In **3** Gly, Phe, and the termini of Leu and *galacto*-pentitol moieties are involved in intramolecular hydrogen bonds that reduce the conformational flexibility [26] (Figures 2, 3 and Table 3). Two intramolecular contacts (Table 3) involving the proton donor groups

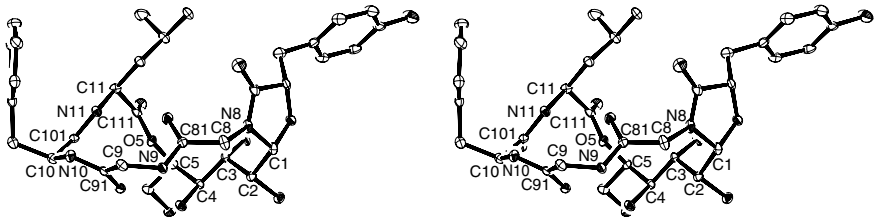


Figure 3 A stereo view illustrating the conformation of the 18-membered ring.

Table 3 Hydrogen Bonds and Intramolecular Contacts for **3**

Donor–H···Acceptor	D–H (Å)	H···A (Å)	D···A (Å)	D–H···A (°)	Symmetry operation on A
O2–H2···O91	0.91(5)	1.93(5)	2.823(4)	168(5)	$\frac{1}{2}-x, -y, \frac{1}{2}+z$
O3–H3···N7	0.81(5)	2.02(5)	2.733(5)	147(5)	x, y, z
O4–H4···O5	0.87(4)	2.46(4)	2.802(4)	104(3)	x, y, z
O4–H4···O91	0.87(4)	1.99(4)	2.788(4)	151(4)	x, y, z
O6–H6···O101	0.75(5)	1.94(5)	2.662(4)	165(4)	$\frac{1}{2}-x, 1-y, \frac{1}{2}+z$
N9–H9···O6	0.99(4)	1.93(4)	2.878(4)	158(3)	$\frac{1}{2}-x, -y, -\frac{1}{2}+z$
N10–H10···O3	0.89(4)	2.18(4)	3.044(4)	165(3)	$x, y, -1+z$
N11–H11···O81	0.95(5)	2.52(5)	3.321(5)	141(4)	x, y, z
N11–H11···N10	0.95(5)	2.34(5)	2.735(4)	105(3)	x, y, z
O76–H76···O71	0.85(5)	1.85(5)	2.693(4)	171(6)	$x, y, 1+z$

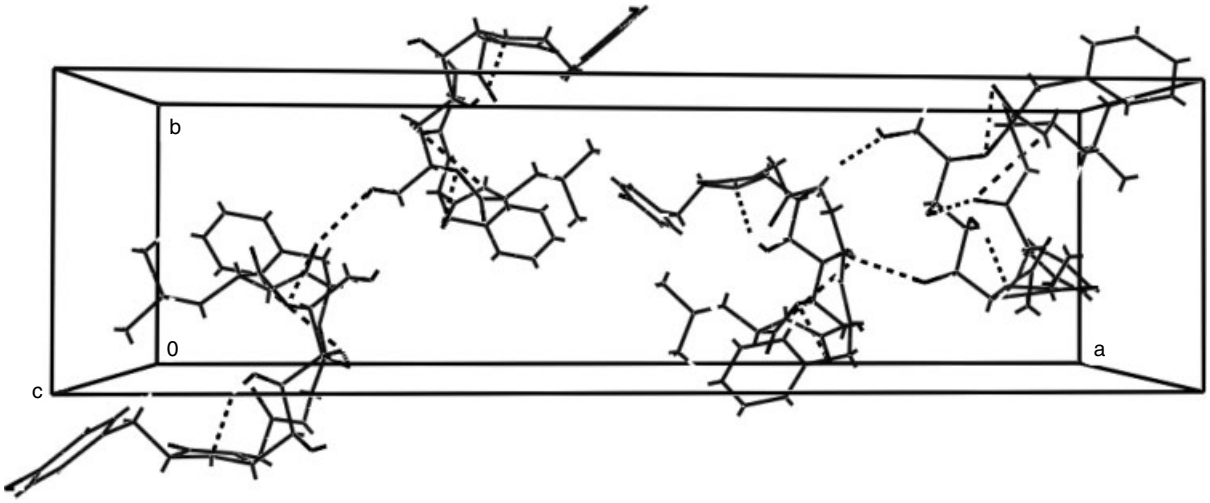


Figure 4 The crystal packing of **3** with the two-dimensional hydrogen bond network.

O4-H and N11-H can be considered as the three-centred hydrogen bonds; multifurcated hydrogen bonds are common in carbohydrates and amino acids [27]. The geometry observed is in agreement with those found in bifurcated systems [28]. The functionalities (NH, OH, C=O) that are not involved in intramolecular hydrogen bonds protrude out of the ring, ready for intermolecular hydrogen bonds forming a two-dimensional network in the (bc) plane (Table 3, Figure 4).

Structural Characterization of 2b and 3 by NMR Spectroscopy

NMR studies of compound **2b** were performed in CH_3CN-d_3 and DMSO-d_6 solutions. In general, spectral resolution in the carbohydrate region was better in the CH_3CN-d_3. Proton and carbon chemical shifts were determined by using ^1H, COSY and TOCSY experiments combined with HSQC and HMBC techniques. Atom enumeration is given in Scheme 1 and the chemical shifts are collected in Table 4. In both solvents, 1D and 2D spectra showed two sets of peaks indicating the presence of another compound in addition to the major one. According to the integrated proton peak intensities the percentage of the minor component was found to be 13%–16%.

The observed chemical shifts of the major component in both solvents point towards structure **2b**. Thus, the chemical shifts of C-2 atom found in the region of 73–74 ppm are typical for the five-membered ring in which the carbon atom is bonded to two nitrogen atoms, as in the imidazolidinone

Table 4 NMR Chemical Shift Data (δ, ppm) of Imidazolidinone Compounds **2b** and **3**[a]

Residue	Atom[b]	**2b**				**3**			
		CH_3CN-d_3		DMSO-d_6		CH_3CN-d_3		DMSO-d_6	
		δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Imidazolidinone ring	CH-2	4.72	74.6	4.80	73.5	4.89	75.3	4.91	76.0
	CH-4	4.03	58.7	3.94	59.4	4.05	57.9	4.03	58.0
p-Hydroxybenzyl	a/a′	2.91/3.18	35.1	2.72/3.15	36.0	2.58/3.32	36.3	2.55/3.25	—[c]
	b		126.7		—[c]		125.9		—[c]
	c	7.15	130.4	7.15	130.05	7.06	130.4	7.08	130.0
	d	6.75	115.0	6.73	115.19	6.80	115.3	6.77	115.2
	e		155.8		156.1		155.9		—[c]
N^1-CH_2	f/f′	3.94/4.20	45.8	4.01/4.18	45.6	3.66/4.27	43.4[d]	3.74/4.25	43.5
Sugar moiety	1′	4.15	64.0	4.18	64.5	4.14	70.4	3.98	63.3
	2′	3.94	70.0	3.97	65.2	3.79	68.9[e]	3.76	66.8
	3′	3.60	70.0	3.78	68.2	3.72	67.8[e]	—[c]	—[c]
	4′	3.97	66.5	3.52	68.9	5.08	72.2	4.99	72.0
	5′/5″	4.04/4.07	65.3	3.95/4.16	64.8	3.53/3.64	59.6	3.37/3.53	58.0
Gly	NH	7.40		8.37		7.38		8.74	
	α/α'	3.73/3.86	41.5	3.47/3.78	42.0	3.45/3.64	43.6[d]	3.52/3.72	43.2
Phe	NH	7.39		7.97		7.23		8.96	
	α	4.55	54.4	4.62	53.5	4.48	54.7	4.30	54.5
	β/β'	2.91/3.12	37.5	2.79/3.15	38.5	2.95/3.28	35.7	2.80/3.33	—[c]
	γ		136.7		138.0		137.0		—[c]
	δ	7.31	129.0	7.40	129.5	7.31	128.7	—[c]	128.9
	ε	7.31	128.1	7.29	127.9	7.31	128.2	—[c]	128.3
	ζ	7.25	126.5	7.22	126.3	7.25	—[c]	—[c]	126.6
Leu	NH	7.18		8.54		7.27		7.41	
	α	4.22	51.7	4.18	51.66	4.30	52.1	4.18	51.7
	β/β'	1.59/1.67	38.7	1.56/1.70	38.7	1.59/2.06	38.6	1.43/2.10	38.5
	γ	1.59	24.0	1.70	24.2	—[c]	24.3	—[c]	—[c]
	δ_1	0.88	20.3	0.89	21.1	0.88	19.7	0.86	21.0
	δ_2	0.92	21.8	0.95	22.8	0.92	22.2	0.89	23.0

[a] At 25 °C; [b] Designations of particular atoms are given in Scheme 1; [c] Not assigned; [d,e] Assignation of signals can be interchangeable.

structures. Another characteristic feature is the disappearance of the Gly^2 amide proton caused by the imidazolidinone formation involving *N*-terminal tyrosine residue and the open-chain form of the sugar moiety in ester **1**, followed by the nucleophilic attack of the Gly^2 nitrogen and formation of a new N,N′-acetal centre [11]. The resonances in the carbohydrate region are in agreement with data for the sugar pentitol derivative with D-*galacto* structure. The lower field ^1H and ^{13}C chemical shifts observed for CH_2-5′, together with shielding of CH-4′ are in accordance with the 5′-*O*-ester position in compound **2b**.

NMR data for the minor component present in the solution of compound **2b** are in agreement with structure **3** (Scheme 1). Namely, the peptide carbon resonances of the respective amino acid residues are similar to those observed for **2b**. The chemical shifts of atoms at position 2 confirm the imidazolidinone part of compound **3**. However, a comparison of the CH-4′ chemical shifts in **3** with that of **2b** demonstrated a downfield shift by 1.1–1.5 ppm for proton and 3.1–5.7 ppm for carbon. On the other hand, an upfield shift by 0.4–0.6 ppm for proton and ~6 ppm for carbon have been observed for CH_2-5′ group. This provides an evidence that compounds **2b** and **3** are isomers differing only in the position of the ester group in the molecule.

Characteristic NOEs were observed only in DMSO-d_6 for 5′-*O*-ester **2b** and 4′-*O*-ester **3**. Specifically,

the strong sequential $d_{N,N}$ NOE along the Gly-Phe and the inter-residue dipolar contact $d_{N,N}$ $(i, i+2)$ between residues Gly and Leu were characteristic of **2b**, whereas sequential $d_{N,N}$ NOE between Phe and Leu was indicative for **3**. In addition, the following inter-residual cross-peaks LeuNH-PheβH and PheβH-H-4′ were observed only in **2b**. On the other hand, the weak H-4′-H-5′ and H-3′-H-4′ cross-peaks were observed in compound **3**, indicating different structures of positional isomers **2b** and **3** in solution.

To gain insight into the migratory tendency of the peptidyl group from the primary to the secondary hydroxyl group as well as to explain the results of the x-ray analysis, the relative amounts of the 5′-*O*-(**2b**) and 4′-*O*-ester (**3**) present in the solution under the conditions of the crystal formation from **2b** were examined. As evidenced by RP HPLC, the amount of compound **3** started to increase after dissolution of **2b** in methanol–water (5 : 4) mixture to reach 32% after 2 months at room temperature (Figure 1). Based on this result, it is conceivable to assume that under the above conditions compound **3** is less soluble than **2b** and crystallized from the solution.

CONCLUSIONS

The *cis*-arrangement of the substituents attached to C2 and C4 (corresponding to crystallographic numbering C1 and C7, respectively) of the 5-imidazolidinone ring moiety in minor diastereoisomeric product **2b**, obtained by intramolecular cyclization of D-*galacto*-related 6-*O*-(leucine-enkephalyl)-ester **1**, has been elucidated by comparative studies based on x-ray crystallography and NMR spectroscopy, suggesting *trans* orientation of C2 and C4 substituents in the major diastereoisomer **2a**. Additional studies of compound **2b** based on RP HPLC analysis and NMR spectroscopy revealed that under the crystallization conditions in methanol/water (5 : 4 v/v) solution, the peptidyl group has a tendency to migrate from the primary to the adjacent secondary hydroxyl group of the D-*galacto*-pentitolyl residue to give less soluble compound **3** that crystallized from the solution.

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