Synthesis and *cis–trans* isomerism in novel Leu-enkephalin-related peptidomimetics containing N-glycated glycine residues

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The influence of a new peptoid residue on amide-bond stereochemistry has been explored *via* the synthesis and NMR analysis of novel peptidomimetics related to the endogenous opioid pentapeptide Leu-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH). The compounds studied include protected and unprotected enkephalin analogues N-alkylated at either the second (Gly^2) or both the second and the third (Gly^2,Gly^3) amino acid residues with a 6-deoxy-D-galactose moiety. The syntheses of the mono- (7) and the bis-glycated pentapeptide (11) were performed in a stepwise manner in solution by employing N-glycated glycine as the building block. The relative populations of the *cis* and *trans* isomers in the compounds studied were estimated by NMR spectroscopy. In the fully protected N-glycated dipeptide 3 the most abundant isomer (64%) was shown to contain a *cis* Tyr^1-(X)Gly^2 amide bond. NMR analysis of mono-N-glycated pentapeptide 6 provided evidence that elongation of the peptide chain disfavours the *cis* and augments the *trans* isomer population (*cis* : *trans* 35 : 65). For the unprotected monoglycated peptides 5 and 7 both the *α*- and *β*-pyranose forms of the galactose moieties were detected, the β-pyranose tautomer being the most abundant (~70%). Removal of the protecting groups decreased the proportion of *cis*-rotamers relative to the corresponding protected peptides 3 and 6. The NMR spectra of enkephalin-related peptides 8–11, which contain two N-glycated glycine residues, were extremely complex; both proximal and distal isomerization effects were observed. For the bis-glycated tripeptide 8 significant amounts of both the *cis* and *trans* rotamers were observed for the Tyr^1-(X)Gly^2 and (X)Gly^2-(X)Gly^3 peptide bonds. In the fully protected pentapeptide 10 the configurational equilibrium was markedly shifted in favour of the *trans* isomers; only 15% of the *cis* isomer was observed for the Tyr^1-(X)Gly^2 bond, whereas the (X)Gly^2-(X)Gly^3 bond was completely in the *trans* configuration.

Introduction

The opioid receptors (μ,δ,κ) and their subtypes are involved in the control of various aspects of the perception of pain, pleasure, and mood as well as regulation of immune function (for review see Ref. 1), and the development of selective opioid receptor ligands offers the potential for improving clinical treatments involving these systems. In the search for potent opioid ligands the two endogenous opioid peptides Leu- and Met-enkephalin (H–Tyr–Gly–Gly–Phe–Leu/Met–OH) make an ideal template and many selective and conformationally restricted analogues of these peptides have been prepared.[2]

In the course of such studies we have shown that introduction of a carbohydrate moiety at the fifth position of enkephalins significantly influences the opioid activity profile of the parent peptide. Both the type of linkage used and the kind of sugar moiety introduced were of major consequence with regard to bioactivity[3] as well as reactivity[4] of these compounds. Recently, the modification of opioid peptides by monosaccharide molecules has gained interest in other groups as well.[5–7]

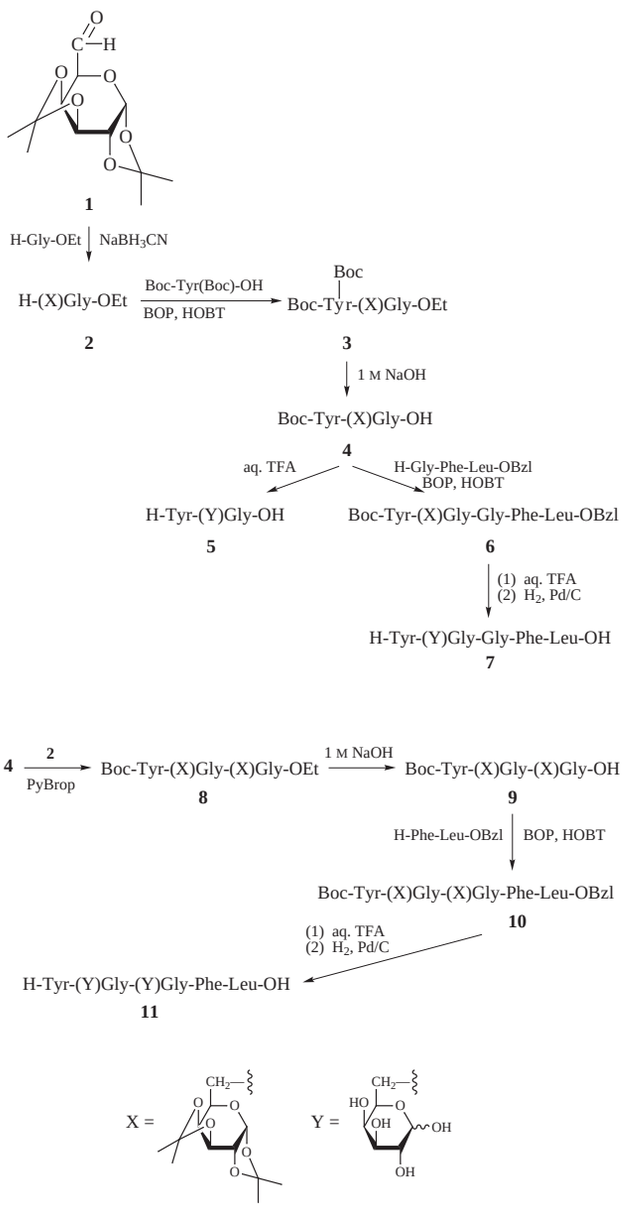
In this study we have designed and synthesized novel sugar-based peptidomimetics related to the physiologically important endogenous opioid peptide Leu-enkephalin (H–Tyr–Gly–Gly–Phe–Leu–OH). We replaced Gly^2, or both the Gly^2 and Gly^3 residues, in Leu-enkephalin by an N-alkylated glycine bearing a 6-deoxy-D-galactose moiety and studied the consequences of these changes by NMR spectroscopy. We reasoned that N-glycation of the amide bond could offer several advantages compared with simple N-methylation of the peptide backbone. First, the carbohydrate moiety will allow attachment of these neoglycopeptides to other relevant molecules, or could be modified systematically to achieve the optimum biological response. Secondly, based on our recent studies on Amadori compounds related to small biologically active peptides,[8,9] it could be expected that N-glycation will influence the backbone conformer distribution. Finally, the present study provides the first model of the compounds which might result from the reaction between the amino groups of proteins (peptides) and C–6 oxidized galactose residues obtained in biological systems by treatment with galactose oxidase.[10,11]

Results and discussion

Synthesis

Isopropylidene-protected D-*galacto*-hexodialdopyranose **1** was obtained from 1,2 : 3,4-di-*O*-isopropylidene-α-D-galactose by oxidation with the chromium trioxide–dipyridine complex.[12] Reductive amination of aldehyde **1** by glycine ethyl ester in the presence of sodium cyanoborohydride gave compound **2** (51% yield), the key building block in the synthesis of enkephalin derivatives **7** and **11**. N-Acylation of compound **2** with Boc–Tyr(Boc)–OH by using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) as the coupling reagent gave the fully protected N-glycated dipeptide Boc–Tyr(Boc)–(X)Gly–OEt (X = 6-deoxy-1,2 : 3,4-di-*O*-isopropylidene-α-D-galactopyranos-6-yl) **3** in 77% yield. The initial target, monoglycated pentapeptide **7**, was synthesized in four steps from dipeptide **3** (Scheme 1). Deprotection of the

ethyl ester in compound **3**, accomplished with aq. sodium hydroxide in methanol, was accompanied by the loss of Boc protection at the tyrosine phenol moiety, giving Boc–Tyr–(X)Gly–OH **4** in 82% yield. The fully protected pentapeptide Boc–Tyr–(X)Gly–Gly–Phe–Leu–OBzl **6** was assembled by coupling of tripeptide H–Gly–Phe–Leu–OBzl with dipeptide **4** in solution with BOP as the coupling reagent (83% yield). The Boc group, and the isopropylidene groups at the galactose moiety in pentapeptide **6**, were removed by short treatment with TFA containing water. The obtained intermediate, H–Tyr–(Y)Gly–Gly–Phe–Leu–OBzl (Y = 6-deoxy-D-galactos-6-yl), was subjected to hydrogenolysis in the presence of Pd/C to give, after reversed-phase high-performance liquid chromatography (RP HPLC) purification, H–Tyr–(Y)Gly–Gly–Phe–Leu–OH **7** in 76% yield.



Scheme 1

The synthesis of Leu-enkephalin analogue **11**, in which two carbohydrate moieties are attached to the peptide backbone, is shown in Scheme 1. The key precursor tripeptide Boc–Tyr–(X)Gly–(X)Gly–OEt **8** was prepared by reaction of N-glycated dipeptide **4** with the glycine ester derivative **2**. In contrast to the synthesis of dipeptide **3**, BOP coupling led to only minor formation of tripeptide **8** (8%) while the major products were polymers. In interpreting these results we assumed that, instead of N-acylation, O-acylation of the phenolic part of the tyrosine residue had occurred, the phenolate anion, formed under the reaction conditions, being a better nucleophile than amino component **2**. This assumption was based on fast-atom bombardment mass spectrometry (FABMS) analysis and on the observation that the polymerization products did not show a UV absorption at 275 nm, which is characteristic for a tyrosine residue with a free OH group. In contrast to BOP, condensation of dipeptide **4** with ester **2** in the presence of PyBroP, highly efficient in the coupling of N-methylated amino acids,[13] provided key intermediate **8** in 94% yield. Subsequent hydrolysis of tripeptide **8** followed by BOP-mediated coupling with H–Phe–Leu–OBzl gave pentapeptide Boc–Tyr–(X)Gly–(X)Gly–Phe–Leu–OBzl **10** (53% yield). All the protecting groups in compound **10** were removed in two steps as described for compound **7**, to afford N,N′-bisglycated Leu-enkephalin derivative H–Tyr–(Y)Gly–(Y)Gly–Phe–Leu–OH **11** in 61% yield.

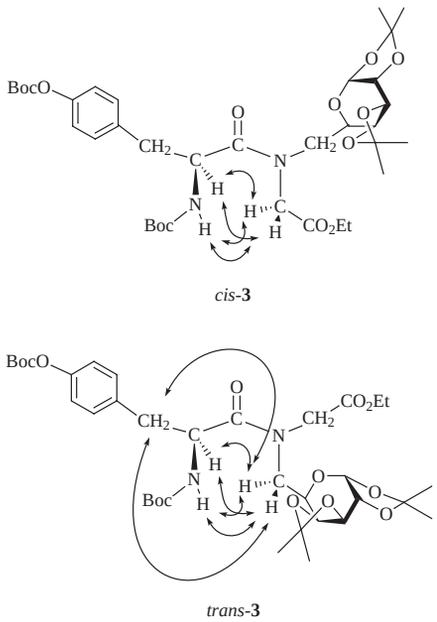


Fig. 1 NOEs observed for the *cis*- and *trans*-isomer of the N-glycated dipeptide **3**

Consequences of N-glycation for *cis–trans* isomerization of the amide bond – NMR studies

For glycated dipeptide **3**, two sets of resonances, arising from the *cis–trans* isomerization around the Tyr[1]–(X)Gly[2] amide bond, were clearly observed in the ^{1}H and ^{13}C NMR spectra obtained in $CDCl_3$. As explained in Fig. 1, characteristic NOEs [from a rotating-frame nuclear Overhauser effect spectroscopy (ROESY) experiment] were observed for the two conformations and used for the complete assignment of the individual isomer (Table 1). The *cis*:*trans* ratio for compound **3**, calculated from the integrated intensities and/or peak heights of the appropriate resonances, was 64:36. The *cis*/*trans* equilibria of the other N-glycated peptides were corroborated by similar characteristic NOEs as observed for compound **3**.

To determine the influence of the unprotected galactose moiety on the *cis–trans* isomerization about the Tyr[1]–Gly[2] bond, fully deprotected H–Tyr–(Y)Gly–OH **5** was prepared. Four sets of resonances attributable to the *cis* and *trans* isomers of compound **5**, bearing on the glycine residue a 6-deoxy-D-galactos-6-yl moiety in α- (**5α**) or β-pyranose (**5β**) form, were observed in D_2O solution (Table 1). Integration of signal intensities in the ^{13}C NMR spectra yielded the following equilibrium composition of dipeptide **5** at pH 5.80 and 25 °C: *cis*-**5α** 12%, *trans*-**5α** 16%, *cis*-**5β** 34% and *trans*-**5β** 38%. The observed predominance of the β-pyranose form in water (72%) is in accordance with the view that the pyranose chair conformation with

Table 1 NMR Chemical-shift data (δ, ppm) of glycated dipeptides **3** and **5**[a]

	3				**5β**				**5α**			
	cis		*trans*		*cis*		*trans*		*cis*		*trans*	
Residue	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Gal-1	5.41	96.21	5.46	96.24	4.49	99.25	4.32	99.17	5.19	95.08	5.20	95.20
Gal-2	4.23	70.29	4.26	70.29	3.45	74.63	3.40	74.58	3.74	71.07	3.74	71.07
Gal-3	4.52	70.61	4.57	70.74	3.62	74.97	3.46	75.46	3.66	71.07	3.80	71.90
Gal-4	3.80	71.11	4.08	71.23	3.66	72.29	3.58	71.95	3.71	73.11	3.56	72.21
Gal-5	3.98	65.79	3.82	66.12	3.57	75.53	3.14	75.26	3.67	70.69	4.01	70.21
Gal-6/6′	3.41/3.55	48.62	3.50	48.62	3.15/3.46	51.45	3.19/3.42	52.91	3.25/3.53	51.33	3.25/3.44	52.80
Tyr^1-NH	5.17		5.21		n.o.		n.o.		n.o.		n.o.	
Tyr^1-α	4.63	51.23	4.94	51.33	4.38	54.64	4.72	54.45	4.42	54.56	4.72	54.56
Tyr^1-β,β′	2.90/3.00	38.89	2.90/3.09	38.78	3.05	38.66	3.10	38.75	3.10	38.66	3.10	38.75
Tyr^1-γ		135.24		135.24		128.23		128.54		128.23		128.36
Tyr^1-δ	7.16	130.32	7.23	130.55	7.18	133.91	7.19	134.05	7.18	133.91	7.19	134.05
Tyr^1-ε	7.07	121.20	7.07	121.01	6.89	118.47	6.89	118.47	6.89	118.47	6.89	118.47
Tyr^1-ζ		154.90		154.77		158.30		158.30		158.30		158.30
Gly^2-α,α′	4.01/4.21	51.02	3.93/4.39	48.16	3.62/3.70	55.42	3.78/4.10	54.33	3.62/3.70	55.42	3.96/n.o.	54.33

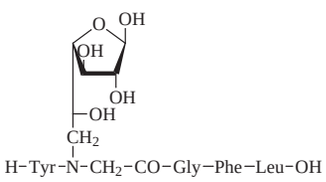
[a] In $CDCl_3$ for compound **3** and in D_2O for compounds **5** (*cf.* conditions in Materials and methods in the Experimental section). n.o. = not observed.

equatorially oriented hydroxy groups is well accommodated into the tridymite structure of water[14] and agrees with published data for the composition of 6-deoxy-D-galactose in water.[15] In compound **5**, however, no signals were observed which could be attributed to furanose forms of the sugar moiety, even though published information concerning the tautomeric equilibrium of free 6-deoxy-D-galactose stated that these forms were present in an amount of 3% in aqueous solution.[15]

By comparison of the influence exerted by introduction of an N-methylated glycine residue into a protected dipeptide model[16] with those induced by N-glycation in compounds **3** and **5**, it appears that N-methylation, resulting in nearly 25% as *cis* conformers, has less effect on amide-bond isomerization. Although it could be expected that the rotational barrier should be lowered as the N-alkyl substituent becomes bulkier,[17] the significant predominance of the *cis* form in compound **3** implies that conformations favourable for CH-π interactions are adopted.[18] This was borne out from the NOEs between Tyr^1H^{ε}, as well as the Tyr^1H^{β}, and H–4 (Gal) observed only in *cis*-**3** and may explain the strong upfield shift of H–4 (Gal) (0.28 ppm) in the *cis* conformer by its proximal location to the aromatic ring of the Tyr residue. The unprotected N- and C-termini, as well as the decrease in the size of the N-alkyl substituent in **5**, resulted in a different charge distribution and the *trans* conformer become slightly more stable (54%).

NMR analysis of the fully protected N-glycated pentapeptide Boc–Tyr–(X)Gly–Gly–Phe–Leu–OBzl **6** in $CDCl_3$ (Table 2) provided evidence that elongation of the peptide chain resulted in a decreased amount of the *cis* isomer (*cis*:*trans* 35:65) in relation to that in protected dipeptide **3**. The 1D NMR spectrum of compound **6** revealed resonances which indicate the presence of multiple conformers in $CDCl_3$ solution. Thus, the 5.1–5.2 ppm region contains six signals corresponding to the CH_2 group of the benzyl ester in the proportions 1:5:6:10:2:1. In addition, the galactose anomeric region (δ 5.3–5.5) contained, together with two major H–1 resonances, three minor doublets (less than 10% as estimated by integrals) assigned to Gal H–1 in minor conformations (data not shown in Table 2).

In the case of the target Leu-enkephalin-related peptide H–Tyr–(Y)Gly–Gly–Phe–Leu–OH **7**, NMR studies performed in H_2O/D_2O (9:1) indicated a pronounced preference for *trans*-**7** (~90%) at pH 5.65 and 5 °C. The 6-deoxy-D-galactose unit resided both in the α- (**7α**) and β-pyranose (**7β**) form. The ^{13}C NMR spectrum also revealed the presence of a third tautomer in the anomeric region (Gal C–1 δ_C 103.91) which was assigned[15] to the β-furanose form **7f** of the sugar moiety.



7f

According to the NMR analysis the following equilibrium composition was found for pentapeptide **7**: *cis*-**7α** 5%, *trans*-**7α** 20%, *cis*-**7β** 5%, *trans*-**7β** 67% and **7f** 3%. A summary of the chemical shifts determined for the *cis* and *trans* isomers of **7α** and **7β** is presented in Table 2.

The NMR spectra of the Leu-enkephalin-related peptides containing two N-glycated glycine residues were extremely complex, the signals of various conformers appearing within a narrow range, thus rendering the assignments ambiguous. Both proximal and distal isomerization effects were observed by NMR spectroscopy. In this regard, we took an initial structural approach in which we limited our analysis to some characteristic features observed in the NMR spectra of tripeptide **8**, and pentapeptides **10** and **11**.

As shown in Fig. 2A the Tyr^1NH resonance in bis-glycated tripeptide Boc–Tyr–(X)Gly–(X)Gly–OEt **8** in $CDCl_3$ solution is divided into four doublets. In analogy with assignment of the monoglycated peptides **3** and **6** the two downfield doublets were assigned to Tyr^1NHs in the *trans*-rotamer and the upfield-shifted pair of doublets were assigned to the *cis*-rotamers of compound **8**. In the galactose anomeric region (δ 5.35–5.60) (Fig. 2A), seven doublets (out of eight) corresponding to H–1 of the protected galactose residues attached to Gly^2 and Gly^3 of tripeptide **8** were observed, thus reflecting each sugar moiety in four *cis*/*trans* isomers of compound **8**. The *cis*:*trans* ratios, determined from the integrated intensities of eight well separated signals for the Gal^2 and Gal^3 C–5 atoms in the region δ_C 65–67 of the ^{13}C NMR spectrum of tripeptide **8**, were for the Tyr^1–(X)Gly^2 bond (66:34) and for the (X)Gly^2–(X)Gly^3 amide bond (46:54).

NMR analysis of the spectra of the fully protected pentapeptide Boc–Tyr–(X)Gly–(X)Gly–Phe–Leu–OBzl **10** in $CDCl_3$ revealed that elongation of the peptide chain changed the population of the amide bond isomers in favour of the *trans* isomers. Comparison of the anomeric proton region in compound **10** (Fig. 2B) with the same region in tripeptide **8** documents, with only two major H–1 Gal^2 and Gal^3 doublets observed, the predominance of only one amide bond rotamer in pentapeptide **10**. The ^{13}C NMR spectrum of pentapeptide **10** revealed only

Table 2 NMR Chemical-shift data (δ, ppm) of glycated pentapeptides **6** and **7**[a]

	6				**7β**				**7α**			
	cis		*trans*		*cis*		*trans*		*cis*		*trans*	
Residue	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Gal-1	5.39	96.19	5.43	96.20	4.30	98.86	4.26	99.13	5.08	95.05	5.11	95.05
Gal-2	4.21	70.60	4.29	70.33	3.35	74.66	3.35	74.53	3.67	71.01	3.67	71.01
Gal-3	4.54	70.34	4.57	70.34	3.54	75.00	3.43	75.37	*b*	*b*	3.59	71.76
Gal-4	4.10	71.33	4.06	70.99	3.70	72.23	3.60	71.90	*b*	71.82	3.70	72.57
Gal-5	4.15	65.52	3.80	66.18	3.59	76.13	3.27	74.53	*b*	70.18	3.77	69.93
Gal-6/6′	2.91/3.87	50.06	3.14/3.68	49.39	3.01/3.54	51.82	3.01/3.44	52.82	*b*	51.45	3.03	52.77
Tyr^1-NH	5.02		5.62		n.o.		n.o.		n.o.		n.o.	
Tyr^1-α	4.51	51.94	4.87	51.95	4.39	54.52	4.67	54.44	4.39	54.59	4.67	54.59
Tyr^1-β,β′	2.79/3.00	38.89	2.83/3.00	38.67	2.96/3.02	38.74	3.03	38.80	2.96/3.02	38.74	3.03	38.80
Tyr^1-γ		135.32		135.48		127.76		127.99		127.76		127.85
Tyr^1-δ	7.00	130.49	7.00	130.45	7.06	133.65	7.10	133.71	7.06	133.44	7.10	133.65
Tyr^1-ε	6.69	115.81	6.69	115.68	6.80	118.28	6.80	118.65	6.80	118.59	6.80	118.68
Tyr^1-ζ		155.71		155.45		158.10		158.10		158.10		158.10
Gly^2-α,α′	3.85/4.08	53.75	3.78/4.03	52.18	3.83/3.90	53.91	3.92/4.09	53.52	3.75	53.75	4.01	53.08
Gly^3-NH	6.25		6.28		8.29		8.20		8.29		8.13	
Gly^3-α,α′	3.45/3.85	43.52	3.70/3.85	43.17	3.61/3.79	44.90	3.47/3.77	45.00	3.61/3.79	44.90	3.44/3.77	45.10
Phe^4-NH	6.39		7.05		8.36		8.06		8.36		8.02	
Phe^4-α	4.52	54.83	4.65	54.64	4.59	*b*	4.60	57.56	4.59	*b*	4.60	*b*
Phe^4-β,β′	3.02	37.47	3.09/3.19	37.78	2.85/3.13	39.66	2.91/3.13	39.76	2.85/3.13	39.66	2.91/3.13	39.76
Phe^4-γ		136.80		136.80		139.10		139.28		139.25		139.25
Phe^4-δ	7.15	129.21	7.20	129.21	7.17–7.30	131.86	7.17–7.30	131.95	7.17–7.30	131.86	7.17–7.30	131.88
Phe^4-ε	7.20–7.30	128.63	7.20–7.30	128.57	7.17–7.30	131.45	7.17–7.30	131.39	7.17–7.30	131.45	7.17–7.30	131.39
Phe^4-ζ	7.20–7.30	128.22	7.20–7.30	128.17	7.17–7.30	129.86	7.17–7.30	129.76	7.17–7.30	129.86	7.17–7.30	129.76
Leu^5-NH	6.32		6.54		7.90		8.04		7.90		8.05	
Leu^5-α	4.60	51.26	4.60	51.11	4.12	56.77	4.10	56.87	4.12	56.77	4.10	56.77
Leu^5-β	1.58	41.20	1.56	40.97	1.50	*b*	1.50	43.49	1.50	*b*	1.50	*b*
Leu^5-γ	1.58	25.12	1.58	25.12	1.52	27.20	1.52	27.20	1.52	27.20	1.52	27.20
Leu^5-δ	0.88	21.92	0.88	21.90	0.78	23.68	0.78	23.68	0.78	23.71	0.78	23.71
Leu^5-δ′	0.90	22.74	0.90	22.76	0.82	25.57	0.82	25.08	0.82	25.09	0.82	25.09

[a] In $CDCl_3$ for compound **6** and in water–D_2O (9 : 1) for compounds **7** (*cf.* conditions in Materials and methods in the Experimental section). [b] Exact chemical shift not determined due to peak overlap of various conformers in this region; n.o. = not observed.

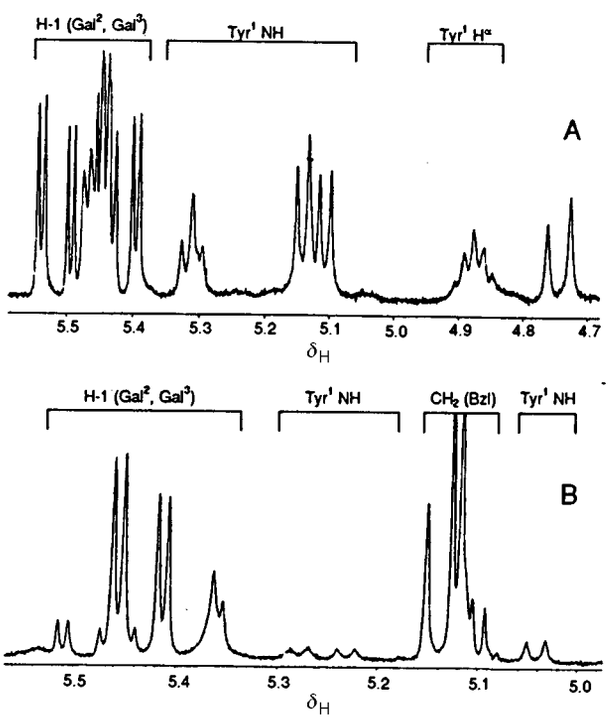


Fig. 2 Expanded anomeric (H–1) and Tyr^1NH region of 1D spectra of protected bis-glycated tripeptide **8** (A) and pentapeptide **10** (B) in $CDCl_3$

two signals for Gal^2 C–5, reflecting *cis–trans* isomerization of the proximal Tyr^1–(X)Gly^2 bond (ratio 15 : 85), while for Gal^3 C–5 only one signal was observed, indicating no isomerization around the (X)Gly^2–(X)Gly^3 bond. As illustrated in Fig. 2B, the line pattern of the CH_2 resonance of the benzyl ester group, and several minor resonances observed for H–1 in the Gal^2, Gal^3 anomeric region, as well as for the Tyr^1NHs, suggest the presence of multiple minor conformers of compound **10** in addition to the major *trans*,*trans* amide-bond conformer.

The ^{1}H NMR spectrum of the unprotected bis-glycated pentapeptide H–Tyr–(Y)Gly–(Y)Gly–Phe-Leu-OH **11**, in water–D_2O (9 : 1) at pH 5.6 and 5 °C, indicates the presence of multiple conformers due to the *cis–trans* isomerism of the N-alkylated peptide bonds and tautomerism of the two galactose moieties. In the amide region (Fig. 3A), strongly downfield-shifted resonances (δ 8.4–8.6) were assigned to Phe^4NH in *cis* conformers of **11α** and **11β** based on the combined use of chemical-shift-correlation spectroscopy (COSY), rotating-frame nuclear Overhauser effect spectroscopy (ROESY) and phase-sensitive two-dimensional total correlation spectroscopy (TOCSY) experiments and on comparison with monoglycated pentapeptide **7**. The same proton resonance in the *trans* conformers of compounds **11** appeared in the δ 8.1–8.2 region. This large chemical-shift difference suggests a different contribution of the phenylalanine residue to the structure stabilization of individual conformers. Concerning the Leu^5NH proton resonances, two groups of peaks were observed in the region δ 7.9–8.0 and 8.0–8.1 and were ascribed to *trans* and *cis* conformers, respectively (Fig. 3A). The main conformers of compound **11** in solution show a *trans* arrangement of the peptide bonds as judged by the integration of the amide-bond region (the *cis*/*trans* ratio being ~1 : 4). The assignment of the Phe^4NH resonances was further confirmed by the chemical shifts of the Phe^4β-Hs, which for the main *trans* conformers showed a typical narrower splitting pattern than did the same protons of the *cis* conformers (Fig. 3B). The H–1 resonances of the Gal^2 and Gal^3 residues in the α-pyranose form **11α** appeared in the δ 5.0–5.3 region as six ill resolved doublets (the major

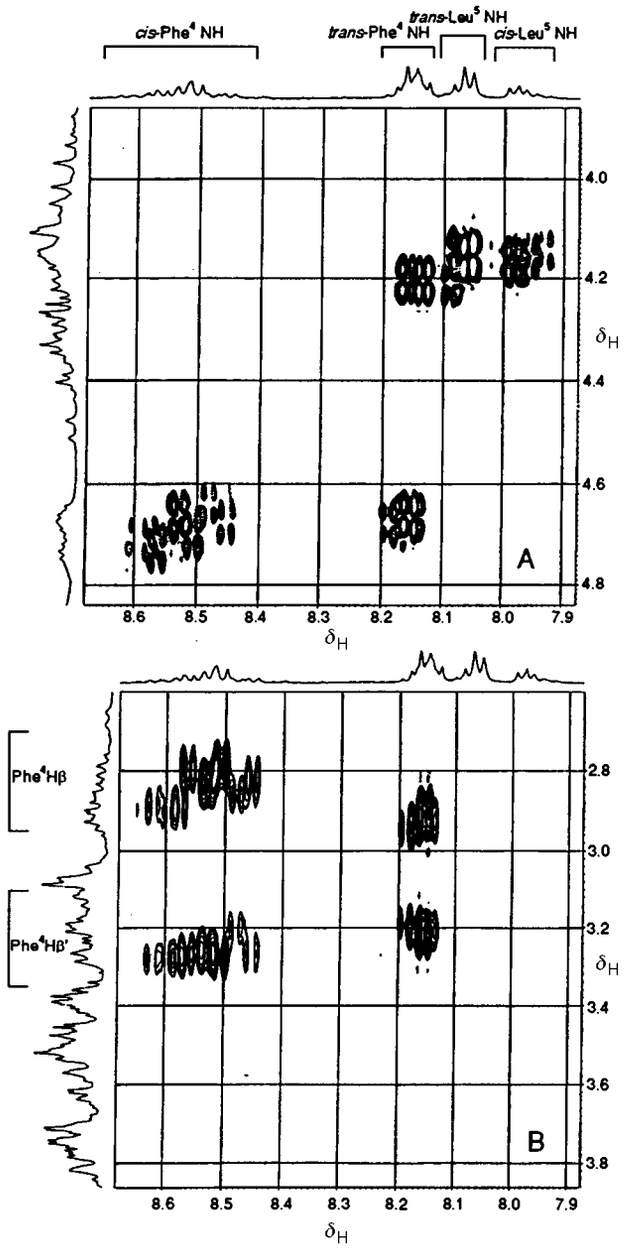


Fig. 3 Amide and α-CH region of the double-quantum-filtered COSY (A) and amide and $Phe^4H^{\beta,\beta'}$ region of the TOCSY (B) spectra of the unprotected bis-glycated pentapeptide **11** in water–D_2O (9 : 1)

conformer at δ 5.17), whereas at least seven H–1 resonances for the galactose moieties in their β-pyranose forms **11β** were detected in the δ 4.2–4.4 region, overlapping with other proton resonances. This implies isomerization around both N-glycated amide bonds in compounds **11** and the presence of different conformers of each anomeric entity in aqueous solution.

In analysing the consequences of the N-glycation on the *cis*↔*trans* equilibria about peptide bonds in the compounds studied it appears that isomerization takes place most easily at the N-terminal Tyr^1–Gly^2 bond, with the *cis*-conformer predominant in the short, fully protected peptides **3** and **8**. Concerning the stabilization of the *trans*-configuration in the mono- and bis-glycated pentapeptides (**6**, **7**, **10**, **11**) it is reasonable to assume that elongation of the peptide chain induced different interactions between the sugar and aromatic moieties present, resulting in the decreased amount of the *cis*-conformers. Evidence has recently been provided by CD and FTIR spectroscopy[9] that attachment of a sugar to the N-terminus of Leu-enkephalin, and related peptides, influences not only the distribution of backbone conformers but also the side-chain conformer distribution of Tyr. However, the observed effects cannot at present be defined without more detailed conformational analysis.

Conclusions

Two glycated enkephalin peptidomimetics have been prepared using solution synthesis from *N*-(6-deoxy-1,2 : 3,4-di-*O*-isopropylidene-α-D-galactos-6-yl)glycine ethyl ester **2**. It is expected that compound **2**, and similar glycated amino acid building blocks, will also be able to be used in solid-phase synthesis.

Using NMR spectroscopy it was found that incorporation of either one or two N-glycated glycine residues into the peptide backbone results in variable *cis* : *trans* ratios of the glycated amide bond. The conformational preference is essentially driven by the nature of the preceding amino acid residue, by the length of the peptide chain, and by the anomeric configuration of the 6-deoxy-D-galactose moiety. The structural details highlighted in this study thus provide a basis for rational drug design in the field of novel peptidomimetics. The hydroxy groups present on the carbohydrate moiety allow convenient derivatization with a multitude of hydrophobic and hydrophilic groups which can change the physical and chemical properties without changing the peptide backbone structure. Most notably, the incorporated carbohydrate element offers applications in molecular recognition studies and may serve as point of attachment (through the unsubstituted anomeric centre) for amino groups of proteins and other biologically active amines.

Experimental

Materials and methods

Flash column chromatography (FCC) was performed with the solvents indicated, on Silica Gel 60, 200–400 mesh (Merck). Reactions were monitored by TLC on Silica Gel 60 plates (Merck) using detection with ninhydrin, the chlorine–iodine reagent, or heating with H_2SO_4. Mps were obtained on a Büchi-Tottoli apparatus and are uncorrected. Optical rotations were measured at room temperature (rt) using an Optical Activity LTD automatic AA-10 Polarimeter and $[a]_D$-values are given in units of 10 cm^{-1} deg cm^2 g^{-1}. Positive FABMS were recorded on a JEOL SX 102 mass spectrometer. Ions were produced by a beam of xenon atoms (6 keV) from a matrix of glycerol or thioglycerol. Elemental analyses were carried out at the Microanalytical Laboratory, Ruđer Bošković Institute. HPLC was performed on a Varian 9010 HPLC system with Kromasil C–8, 5 mm, and Kromasil silica, 5 mm, analytical columns (4.6 × 250 mm) and with a Eurospher 100 C–18, 5 mm, semipreparative column (8 × 250 mm), detection at 280 nm. ^1H and ^{13}C NMR spectra were recorded at 500 and 126 MHz, respectively, on a Bruker ARX-500 spectrometer. Spectra were obtained for solutions in $CDCl_3$ [residual $CHCl_3$ (δ_H 7.25) or $CDCl_3$ (δ_C 77.0) as internal standard] at 25 °C, in D_2O [residual HOD (δ_H 4.98) as internal standard or sodium 3-(trimethylsilyl)propanesulfonate (δ_C 00.0 for $SiMe_4$) as external standard] at pH 5.80 and 25 °C or in a 9 : 1 mixture of water and D_2O [H_2O (δ_H 4.98) as internal standard or sodium 3-(trimethylsilyl)propanesulfonate (δ_C 00.0 for $SiMe_4$ as external standard] at pH 5.6 and 5 °C. First-order ^1H chemical shifts and coupling constants (*J*/Hz) were obtained from one-dimensional spectra and resonances were assigned from COSY, TOCSY and ROESY experiments. ^{13}C resonances were assigned from ^1H–^{13}C heteronuclear shift-correlation spectroscopy HETCOR, two-dimensional heteronuclear multiple quantum-filtered coherence spectroscopy (HMQC) and long-range HETCOR two-dimensional heteronuclear multiple-bond correlation spectroscopy (HMBC) experiments. Petroleum spirit refers to the fraction with distillation range 55–65°C.

N-(6-Deoxy-1,2 : 3,4-di-*O*-isopropylidene-α-D-galactos-6-yl)-glycine ethyl ester 2

To a solution of 1,2 : 3,4-di-*O*-isopropylidene-α-D-*galacto*-

hexodialdo-1,5-pyranose **1**[12] (258 mg, 1 mmol) in MeCN (10 cm^3) were added HOAc (57 cm^3, 1 mmol) and H–Gly–OEt (103 mg, 1 mmol). The mixture was stirred for 1 h and then $NaBH_3CN$ (63 mg, 1 mmol) was added and the stirring was continued overnight. The unchanged hydride was destroyed by addition of Silica Gel 60, 200–400 mesh (1 g), the solvent was evaporated off and the purification was achieved by FCC (EtOAc–toluene, 10 : 1) to yield 175 mg (51%) of *compound* **2** as a yellowish oil; $[\alpha]_D$ −45 (*c* 1.0, MeOH); $\delta_H(CDCl_3)$ 1.27 (t, 3 H, CH_2CH_3), 1.32, 1.33, 1.45 and 1.54 (4 s, 3 H each, isopropylidene CH_3), 2.82 (dd, 1 H, *J* 12.3, *J* 4.9 GalH-6), 2.90 (dd, 1 H, *J* 12.3 and 7.9, GalH-6′), 3.44 (q, 2 H, *J* 17.3, $GlyH^{\alpha,\alpha'}$), 3.88 (m, 1 H, GalH-5), 4.18 (q, 2 H, CH_2CH_3), 4.21 (dd, 1 H, *J* 4.9 and 1.9, GalH-4), 4.31 (dd, 1 H, *J* 5.1 and 2.4, GalH-2), 4.59 (dd, 1 H, *J* 7.9 and 1.9, GalH-3) and 5.54 (d, 1 H, *J* 5.1, GalH-1) (Found: C, 55.7; H, 8.2; N, 3.9. $C_{16}H_{27}NO_7$ requires C, 55.6; H, 7.9; N, 4.1%).

N,*O*-Bis(*tert*-butoxycarbonyl)-L-tyrosyl-*N*-(6-deoxy-1,2 : 3,4-di-*O*-isopropylidene-α-D-galactos-6-yl)glycine ethyl ester 3

Boc–Tyr(Boc)–OH (133 mg, 0.35 mmol) was dissolved in dry CH_2Cl_2 (15 cm^3) and to this solution **2** (120 mg, 0.35 mmol), BOP (174 mg, 0.39 mmol), HOBT (40 mg, 0.35 mmol), *N*-methylmorpholine (NMM) (0.043 cm^3, 0.39 mmol), and molecular sieves (4 Å, 200 mg) were added. The reaction mixture was then stirred for 36 h at rt and concentrated under reduced pressure. The residue was purified by column chromatography (EtOAc–toluene, 1 : 3) to give, after crystallization from propan-2-ol–water, 190 mg (77%) of pure *dipeptide* **3**, mp 68–70 °C; $[\alpha]_D$ −33 (*c* 1.0, MeOH). For NMR data see Table 1; *m*/*z* (FABMS, +) 709 $(M + H)^+$ (Found: C, 59.4; H, 7.6; N, 4.0. $C_{35}H_{52}N_2O_{13}$ requires C, 59.3; H, 7.4; N, 3.9%).

N-(*tert*-Butoxycarbonyl)-L-tyrosyl-*N*-(6-deoxy-1,2 : 3,4-di-*O*-isopropylidene-α-D-galactos-6-yl)glycine 4

Dipeptide **3** (71 mg, 0.1 mmol) was dissolved in MeOH (10 cm^3) and the solution was cooled to 0 °C. To this solution was added 1 M NaOH (2 cm^3). The reaction mixture was stirred for 0.5 h at 0 °C and for 3 h at rt. The MeOH was evaporated off and, after cooling, the pH of the mixture was adjusted to 3 with 2 M HCl and extracted twice with EtOAc. The combined extracts were dried (Na_2SO_4), and purified, after removal of solvent, by column chromatography (EtOAc–HOAc–toluene, 4 : 1 : 8) to yield 56 mg (82%) of *compound* **4**, mp 140–144 °C; $[\alpha]_D$ −39 (*c* 1.0, MeOH) (*cis* : *trans* = 3 : 1); *cis*-**4**: $\delta_H(CDCl_3)$ 2.81 (dd, *J* 13.3 and 5.5, Tyr^1H^{β}), 2.97 (m, $Tyr^1H^{\beta'}$), 3.19 (m, GalH-6), 3.80 (m, GalH-5), 4.03 (m, Gly^2H^{α}), 4.03 (m, GalH-4), 4.04 (m, GalH-6′), 4.23 (dd, *J* 4.8 and 2.3, GalH-2), 4.31 (m, $Gly^2H^{\alpha'}$), 4.54 (dd, *J* 7.9 and 2.3, GalH-3), 4.62 (m, Tyr^1H^{α}), 5.40 (d, *J* 7.1, Tyr^1NH), 5.41 (d, *J* 4.8, GalH-1), 6.69 (d, *J* 8.3, Tyr^1H^{ε}) and 6.98 (d, *J* 8.3, Tyr^1H^{δ}); *trans*-**4**: $\delta_H(CDCl_3)$ 2.91 (dd, *J* 13.6 and 4.9, Tyr^1H^{β}), 2.97 (m, $Tyr^1H^{\beta'}$), 3.44 (m, GalH-6), 3.57 (dd, *J* 15.2 and 7.1, GalH-6′), 3.89 (m, GalH-5), 3.95 (d, *J* 17.3, Gly^2H^{α}), 4.10 (m, GalH-4), 4.28 (m, GalH-2), 4.41 (d, *J* 17.3, $Gly^2H^{\alpha'}$), 4.58 (dd, *J* 7.9, GalH-3), 4.91 (m, Tyr^1H^{α}), 5.34 (d, *J* 8.1, Tyr^1NH), 5.49 (d, *J* 4.8, GalH-1), 6.69 (d, *J* 8.3, Tyr^1H^{ε}) and 7.04 (d, *J* 8.3, Tyr^1H^{δ}); *m*/*z* (FABMS, +) 581 $(M + H)^+$ (Found: C, 58.2; H, 7.0; N, 4.7. $C_{28}H_{40}N_2O_{11}$ requires C, 57.9; H, 6.9; N, 4.8%).

L-Tyrosyl-*N*-(6-deoxy-α,β-D-galactos-6-yl)glycine 5

N-Protected dipeptide **4** (58 mg, 0.1 mmol) was treated with TFA–water (9 : 1; 2 cm^3) in the presence of anisole (0.4 cm^3) for 0.5 h. After addition of diisopropyl ether (80 cm^3) at 0 °C, the precipitate was collected by centrifugation and triturated several times with diisopropyl ether. The obtained residue was purified by semipreparative RP HPLC by using 54% MeOH–0.1% TFA. The product was desalted on a short (5 × 1 cm) Dowex 1 × 2 200 (Ac) column and crystallized from ethanol–diisopropyl ether to give 25 mg (62%) of pure *dipeptide* **5**, mp 225 °C (decomp.); $[\alpha]_D$ +75 (*c* 1.0, water). For NMR data see Table 1; *m*/*z* (FABMS, +) 401 $(M + H)^+$ (Found: C, 50.7; H, 6.2; N, 6.9. $C_{17}H_{24}N_2O_9$ requires C, 51.0; H, 6.0; N, 7.0%).

N-(*tert*-Butoxycarbonyl)-L-tyrosyl-*N*-(6-deoxy-1,2 : 3,4-di-*O*-isopropylidene-α-D-glactos-6-yl)glycylglycyl-L-phenylalanyl-L-leucine benzyl ester 6

The TFA salt of H–Gly–Phe–Leu–OBzl (54 mg, 0.1 mmol) was dissolved in dry CH_2Cl_2 (30 cm^3) and neutralized by addition of NMM (0.011 cm^3, 0.1 mmol). To this solution were added dipeptide **4** (58 mg, 0.1 mmol), BOP (49 mg, 0.1 mmol), HOBT (11 mg, 0.1 mmol), NMM (0.012 cm^3, 0.11 mmol), and molecular sieves (4 Å, 100 mg). The reaction mixture was stirred overnight at rt and the solvent was evaporated off. The residue was purified by column chromatography (EtOAc–toluene, 3 : 1) to give *compound* **6**, which was crystallized from EtOAc–petroleum spirit to give a crop of 90 mg (83%), mp 110–115 °C; $[\alpha]_D$ −36 (*c* 1.0, MeOH). For NMR data see Table 2; *m*/*z* (FABMS, +) 988 $(M + H)^+$ (Found: C, 63.1; H, 7.0; N, 7.3. $C_{52}H_{69}N_5O_{14}$ requires C, 63.2; H, 7.0; N, 7.1%).

L-Tyrosyl-*N*-(6-deoxy-α,β-D-galactos-6-yl)glycylglycyl-L-phenylalanyl-L-leucine 7

Protected pentapeptide **6** (99 mg, 0.1 mmol) was treated with TFA–water (9 : 1; 2 cm^3) in the presence of anisole (0.4 cm^3) for 0.5 h. After addition of diisopropyl ether (80 cm^3) at 0 °C, the precipitate was collected by centrifugation and triturated several times with diisopropyl ether. The obtained H–Tyr–(Y)Gly–Gly–Phe–Leu–OBzl (81 mg, 0.1 mmol) was hydrogenated for 3 h in a mixture of MeOH (25 cm^3) and HOAc (2 cm^3) in the presence of 10% Pd/C (100 mg). After filtration to remove the catalyst, the filtrate was evaporated *in vacuo* and the residue was purified by semipreparative RP HPLC by using 54% MeOH–0.1% TFA. The product was desalted on a short (5 × 1 cm) Dowex 1 × 2 200 (Ac) column and crystallized from ethanol–diisopropyl ether to give 55 mg (76%) of pure *pentapeptide* **7**, mp 142 °C; $[\alpha]_D$ +26 (*c* 1.0, MeOH). For NMR data see Table 2; *m*/*z* (FABMS, +) 718 $(M + H)^+$ (Found: C, 54.1; H, 7.0; N, 9.4. $C_{34}H_{47}N_5O_{12}\cdot 2H_2O$ requires C, 54.2; H, 6.8; N, 9.3%).

N-(*tert*-Butoxycarbonyl)-L-tyrosyl-*N*-(6-deoxy-1,2 : 3,4-di-*O*-isopropylidene-α-D-galactos-6-yl)glycyl-*N*-(6-deoxy-1,2 : 3,4-di-*O*-isopropylidene-α-D-galactos-6-yl)glycine ethyl ester 8

To a chilled (0 °C) solution of N-glycated dipeptide **4** (58 mg, 0.1 mmol) and glycine derivative **2** (35 mg, 0.1 mmol) in dry CH_2Cl_2 (25 cm^3) were added bromotripyrrolidinophosphonium hexafluorophosphate (PyBroP) (47 mg, 0.11 mmol) and NMM (0.022 cm^3, 0.2 mmol). The reaction mixture was stirred for 5 min at 0 °C and then at rt overnight. The solvent was evaporated off and the residue was purified by flash chromatography (EtOAc–toluene, 3 : 2) to give *compound* **8**, which was crystallized from acetone–petroleum spirit (85 mg, 94%), mp 104–105 °C; $[\alpha]_D$ −28 (*c* 1.0, MeOH) (mixture of four *cis*/*trans* isomers): $\delta_H(CDCl_3)$ 1.20–1.50 (isopropylidene, CH_2CH_3), 2.75/2.98 and 2.98/3.02 ($Tyr^1H^{\beta,\beta'}$), 3.00/3.95, 3.13/3.85, 3.13/3.95 and 3.45/3.67 (GalH-6,6′), 3.89, 4.08 and 4.13 (GalH-5), 4.05–4.15 (GalH-4), 4.10–4.23 (CH_3CH_2), 4.20–4.30 (GalH-2), 4.28/4.08, 4.31/4.20, 3.86/4.49 and 4.58/4.73 ($GlyH^{\alpha,\alpha'}$), 4.50–4.63 (GalH-3), 4.55, 4.89 (Tyr^1H^{α}), 5.11 (d, *J* 8.6, Tyr^1NH), 5.14 (d, *J* 8.7, Tyr^1NH), 5.30 (d, *J* 7.6, Tyr^1NH), 5.33 (d, *J* 8.0, Tyr^1NH), 5.35–5.60 (GalH-1), 6.62–6.68 (Tyr^1H^{ε}) and 7.01–7.08 (Tyr^1H^{δ}); *m*/*z* (FABMS, +) 908 $(M + H)^+$ (Found: C, 58.5; H, 7.1; N, 4.9. $C_{44}H_{65}N_3O_{17}$ requires C, 58.2; H, 7.2; N, 4.6%).

N-(*tert*-Butoxycarbonyl)-L-tyrosyl-*N*-(6-deoxy-1,2 : 3,4-di-*O*-isopropylidene-α-D-galactos-6-yl)glycyl-*N*-(6-deoxy-1,2 : 3,4-di-*O*-isopropylidene-α-D-galactos-6-yl)glycine 9

Compound **9** was prepared from tripeptide ester **8** (91 mg, 0.1 mmol) in the same way as described for compound **4**. Column

chromatography (EtOAc–HOAc–toluene, 4:1:8) and crystallization from diethyl ether–petroleum spirit afforded 82 mg (93%) of *tripeptide* **9**, mp 158–161 °C; $[a]_D$ −20 (*c* 1.0, MeOH); *m*/*z* (FABMS, +) 880 $(M + H)^+$ (Found: C, 57.1; H, 7.1; N, 5.0. $C_{42}H_{61}N_3O_{17}$ requires C, 57.3; H, 7.0; N, 4.8%).

_N_-(*tert*-Butoxycarbonyl)-L-tyrosyl-_N_-(6-deoxy-1,2:3,4-di-_O_-isopropylidene-α-D-galactos-6-yl)glycyl-_N_-(6-deoxy-1,2:3,4-di-_O_-isopropylidene-α-D-galactos-6-yl)glycyl-L-phenylalanyl-L-leucine benzyl ester 10

To a solution of the TFA salt of H−Phe−Leu−OBzl (48 mg, 0.1 mmol) in dry CH_2Cl_2 (25 cm^3) neutralized with NMM (0.011 cm^3, 0.1 mmol) were added BOP (49 mg, 0.11 mmol), HOBT (11 mg, 0.1 mmol) and NMM (0.012 cm^3, 0.11 mmol). The reaction mixture was stirred overnight in the presence of molecular sieves (4 Å, 100 mg). The solvent was evaporated off and the residue was purified by column chromatography (EtOAc–toluene, 3:2) to yield, after crystallization (acetone–petroleum spirit), 65 mg (53%) of *pentapeptide* **10**, mp 90–98 °C; $[a]_D$ −49 (*c* 1.0, MeOH) (mixture of four *cis*/*trans* isomers): $\delta_H(CDCl_3)$ 0.82–0.90 ($Leu^5H^{\delta,\delta'}$), 1.24–1.48 (isopropylidene), 1.58–1.68 ($Leu^5H^{\beta,\beta',\gamma}$), 2.72/3.03, 2.87/3.08 and 3.01 ($Tyr^1H^{\beta,\beta'}$), 2.95 (dd, *J* 15.5 and 5.6, GalH-6), 3.20 (dd, *J* 14.0 and 5.8, Phe^4H^{β}), 3.27 (dd, *J* 14.2 and 10.1, $Phe^4H^{\beta,\beta'}$), 3.42/4.48, 3.69/4.44 and 4.50/4.72 ($GlyH^{\alpha,\alpha'}$), 3.50 (dd, *J* 15.9 and 9.1, GalH-6), 3.69 (GalH-5), 4.00–4.16 (GalH-4), 4.20–4.30 (GalH-2), 4.41 and 4.72 (Tyr^1H^{α}), 4.52–4.59 (GalH-3), 4.63 (Phe^4H^{α} + Leu^5H^{α}), 5.04 (d, *J* 9.3, Tyr^1NH), 5.09, 5.11, 5.12, 5.13 and 5.15 (Bzl CH_2), 5.23 (d, *J* 9.1, Tyr^1NH), 5.27 (d, *J* 9.2, Tyr^1NH), 5.36 (d, *J* 4.7, GalH-1), 5.41 (d, *J* 5.0, GalH-1), 5.45 (d, *J* 5.0, GalH-1), 5.51 (d, *J* 5.0, GalH-1), 6.17 (d, *J* 9.4, Tyr^1NH), 6.64–6.70 (Tyr^1H^{ϵ}), 6.72 (d, *J* 8.4, Phe^4NH), 7.01–7.08 (Tyr^1H^{δ} + Ph) and 7.56 (d, *J* 8.7 Hz, Leu^5NH); *m*/*z* (FABMS, +) 1230 $(M + H)^+$ (Found: C, 62.4; H, 7.2; N, 5.9. $C_{64}H_{87}N_5O_{19}$ requires C, 62.5; H, 7.1; N, 5.7%).

L-Tyrosyl-_N_-(6-deoxy-α,β-D-galactos-6-yl)glycyl-_N_-(6-deoxy-α,β-D-galactos-6-yl)glycyl-L-phenylalanyl-L-leucine 11

The bis-glycated pentapeptide **11** was prepared from the protected ester **10** (100 mg, 0.08 mmol) in two steps according to the procedure described for the synthesis of pentapeptides **7**. Purification by RP HPLC by using 47% MeOH–0.1% TFA, and crystallization from ethanol–diisopropyl ether afforded 43 mg (61%) of pure *compound* **11**, mp 150 °C; $[a]_D$ +28 (*c* 1.0, MeOH) (mixture of *cis*/*trans* isomers): δ_H(water–D_2O, 9:1) 0.80–0.90 ($Leu^5H^{\delta,\delta'}$), 1.50–1.65 ($Leu^5H^{\beta,\beta',\gamma}$), 2.60–3.30 ($Tyr^1H^{\beta,\beta'}$ + $Phe^4H^{\beta,\beta'}$), 3.31–3.60 (GalH-2), 4.17–4.46 (βGalH-1), 4.35 and 4.74 (Tyr^1H^{α}), 4.63–4.80 (Phe^4H^{α}), 5.05–5.30 (αGalH-1), 6.80–7.40 (Tyr^1ArH + Ph), 7.90–8.00 (*cis*-Leu^5NH), 8.00–8.10 (*trans*-Leu^5NH), 8.10–8.20 (*trans*-Phe^4NH) and 8.40–8.70 (*cis*-Phe^4NH); *m*/*z* (FABMS, +) 880 $(M + H)^+$ (Found: C, 52.6; H, 6.9; N, 7.8. $C_{40}H_{57}N_5O_{17}$ requires C, 52.4; H, 6.7; N, 7.6%).

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