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Synthesis and conformational analysis of a series of galactosyl enkephalin analogues showing high analgesic activity

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Two galactosyl derivatives of [DMet^2,Pro^5] enkephalinamide (compound 1), namely [DMet^2,Pro^5] enkephalin [$N^{1,5}$-β-D-galactopyranosyl] amide (compound 2) and $O^{1,5}$-(β-D-galactopyranosyl) [DMet^2,Hyp^5] enkephalinamide (compound 3) have been synthesized. Such glycosylpeptides have been shown to be extremely potent analgesic agonists. The conformational analysis of these three compounds in DMSO-d_6 solution has been carried out using two-dimensional NMR methods. Both the parent compound (1) and the βN-galactosyl derivative (2) show similar NMR parameters which are consistent with fairly rigid β-strands at both the N-terminus and C-terminus, connected by a glycine residue that displays a mixture between multiple conformational states. Thus, although the βN-galactosyl derivative (2) has been shown to be significantly more potent than the parent compound (1) in the tail immersion and paw pressure tests of analgesia, no correlation can be established between the conformation of (1) and (2) in DMSO and the difference in analgesic activity. In contrast, important conformational differences with respect to (1) and (2) have been detected in the βO-galactosyl derivative (3). In this case, only one of the likely conformations for (1) and (2) are consistent with the experimental data. These data show that the position of the galactose residue in compound (3) causes Gly^3 to loose flexibility leading to a more rigid folded conformation. Such a change in conformation could be related to the difference in analgesic activity between (2) and (3).

Key words: glycosyl peptides/enkephalin analogues/analgesic peptides/conformational analysis/two-dimensional NMR

Introduction

There is increasing evidence that the carbohydrate moieties of glycopeptides and glycoproteins play an important role in their biological activity and selectivity. It has become widely accepted that exposed sugar residues of glycoproteins or glycolipids serve as determinants for receptor recognition. In this sense, carbohydrate residues linked to proteins have been reported to be involved in such crucial processes as cell growth and cell differentiation (Olden *et al.*, 1985) and infection (Ofek *et al.*, 1978). It has also been found that different mammalian receptors exhibit a considerable and specific affinity for either D-glucose or D-galactose containing glycoproteins (Ashwell and Harford, 1982; Lunney and Ashwell, 1976). Considering that this selection mechanism might hold for many analogous glycoproteins and for mammals in general (Ashwell and Morell, 1974), the incorporation of sugar moieties could give an improvement of the selectivity of pharmaceuticals towards different receptors.

In earlier papers we reported a considerable increase in analgesic activity after incorporation of D-glucose and D-galactose residues into enkephalins (Torres *et al.*, 1988; García-Antón *et al.*, 1987). These results encouraged us to further investigate the influence of the sugar moieties in the activity and selectivity of such glycopeptides. Thus, we have incorporated the same sugar moieties into different positions in the parent peptide.

In the present work we describe the synthesis of a series of potent analgesic galactosyl enkephalins. For comparative purposes we selected the same parent compound that we used before (Torres *et al.*, 1988; García-Antón *et al.*, 1987), [DMet^2,Pro^5] enkephalinamide (Bajusz *et al.*, 1977), (compound **1**). This enkephalin analogue is a potent μ-selective agonist (Shaw and Turnbull, 1978) that shows a selectivity ratio $\mu/\delta = 30$. The galactosyl moieties have been incorporated into the fifth amino acid both via a βN-glycosyl linkage between the C-terminal proline and β-D-galactopyranosylamine to give [DMet^2,Pro^5] enkephalin [$N^{1,5}$-β-D-galactopyranosyl] amide (compound **2**) and via βO-glycosyl bond formation between the hydroxyl group of hydroxyproline and β-D-galactopyranose to give $O^{1,5}$-(β-D-galactopyranosyl) [DMet^2,Hyp^5] enkephalinamide (compound **3**). The schematic structures of both the *N*- and *O*-glycosyl enkephalin derivatives, as well as the parent compound, are shown in Figure 1.

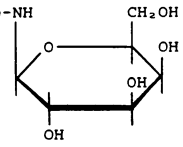
The *in vivo* analgesic activity of these three compounds has been studied. Even though this material is due to be published elsewhere, we wish to summarize here some significant data related to the NMR data. The *O*-galactosyl derivative (**3**) is the most active of these analogues and one of the most potent *in vivo* opioid peptide agonists synthesized up to now. This galactosyl analogue has proved to be >50 000-fold more potent than morphine and 1000-fold more potent than the parent compound (**1**) in rats, in both the tail immersion and paw pressure tests of analgesia after intraventricular administration (10 animals) (Rodríguez *et al.*, 1989). Compound (**2**) is also a potent analogue but ~10 times less potent than compound (**3**) in the same test of analgesia (Rodríguez *et al.*, 1989). These significant results have led us to question whether any conformational changes could be related to such an important increase in analgesic activity induced by the sugar moieties of such glycosyl enkephalins.

The conformational properties of both naturally occurring enkephalins and their synthetic analogues have been widely studied by many authors using spectroscopic methods, X-ray

Tyr-D-Met-Gly-Phe-Pro-NH_2

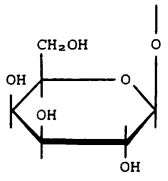
[$DMet^2$,Pro^5] enkephalin amide (1)

Tyr-D-Met-Gly-Phe-Pro-NH



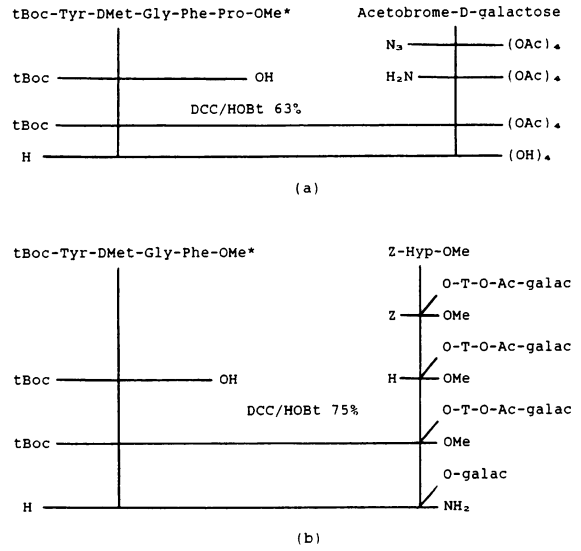
[$DMet^2$,Pro^5] enkephalin [$\underline{N}^{1\cdot5}$-ß-D-galactopyranosyl] amide (2)

Tyr-D-Met-Gly-Phe-Hyp-CO-NH_2



$\underline{O}^{1\cdot5}$-(ß-D-galactopyranosyl)[D-Met^2,Hyp^5] enkephalin amide (3)

Fig. 1. Schematic structures of the parent compound (**1**) and the galactosyl derivatives (**2**) and (**3**).



* Prepared following the stepwise liquid phase procedure by using mixed anhydrides (Isobutylchloroformate) as activated N-protected amino acids (Torres et al, 1988).

Fig. 2. Synthesis of compounds (**2**) and (**3**). Glycosylation strategies.

crystallography and energy calculations (for reviews see Rapaka *et al.*, 1986; Schiller, 1984). Theoretical studies on low energy conformations of enkephalins indicate that both β-turns and extended forms are possible stable conformations in equilibrium (Loew and Burt, 1978; De Coen *et al.*, 1977; Wijne, 1980; Manavalan and Momany, 1981; Paine and Scheraga, 1985, 1986; Purisima and Scheraga, 1987). Experimental methods have also led to many different conclusions. Thus, several authors have detected folded conformations of enkephalins in DMSO or water solutions, mainly by NMR measurements (Garbay-Jaureguiberry *et al.*, 1976, 1980; Jones *et al.*, 1976; Momany, 1977), whereas some other authors find no proof for the existence of stable folded conformations in solution for such small peptides, neither from NMR data (Motta *et al.*, 1987; Higashijima *et al.*, 1979) nor from UV and circular dicroism data (Spirtes *et al.*, 1978). Nevertheless, Highashijima *et al.* (1979) pointed out the importance of ionization in the resulting conformation. They suggested that folded dipolar forms could be stabilized by head to tail interactions between the two charged ends of the peptide.

Most of these conformational analyses of enkephalins were based on 1D NMR measurements. For many years, the presence of folded conformations in solution has been mainly supported by both low temperature coefficients of N*H* chemical shifts and coupling constants. Modern NMR techniques allow the measurement of other spectroscopical parameters, in particular nuclear Overhauser effects (NOEs), which lead to more unequivocal conclusions. Such techniques have been successfully used in the determination of protein structures (Wüthrich, 1986). However, two problems hamper the application of NOE techniques to peptides of the size of enkephalins. Firstly their unfavourable $\omega_0\tau_0$ values make it difficult to detect NOEs. This problem can be avoided either by changing the magnetic field strength or by increasing the solvent viscosity at low temperatures (Motta *et al.*, 1988). A different approach is to measure the NOEs in the rotating frame, for which no $\omega_0\tau_0$ condition yields zero NOEs (Bothner-By *et al.*, 1984). Secondly, the flexibility of small linear peptides causes them to show a wide number of conformations in solution and averages the measured conformation-dependent parameters, NOEs in particular (Jardetzky, 1980; Kessler *et al.*, 1988). In the present work we describe the conformational study of the new series of galactosyl enkephalins in DMSO-d_6 solutions using NOESY and ROESY experiments.

Results

Synthesis

Compounds (**2**) and (**3**) have been synthesized following different strategies. The best reaction pathways among those tested are shown in Figure 2. The preparation of (**2**) includes the β*N*-glycosylation of the pentapeptide tBoc-Tyr−DMet−Gly−Phe−Pro-OH (63% yield) (Figure 2a) whereas compound (**3**) was obtained by previous β*O*-glycosylation of protected hydroxyproline and subsequent incorporation of this galactosyl amino acid, after saponification, to the rest of the peptide fragment (Figure 2b). In this case two hydroxyl protecting groups on the sugar moiety have been used. The best chemical yields were obtained by using acetylated sugars instead of the bulkier benzyl derivatives. Thus in the coupling between O^1-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) hydroxyproline methyl ester and tBoc-Tyr−DMet−Gly−Phe-OH 75% yield was achieved compared to 29% using O^1-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl) hydroxyproline methyl ester.

Conformational analysis

The conformation of (**1**), (**2**) and (**3**) in DMSO-d_6 was studied by ^{1}H-NMR at 270 MHz. All the signals were

Table I. The 270 MHz ^{1}H-chemical shifts of (**1**), (**2**) and (**3**) in DMSO-d_6

	Compound		
	(1)	(2)	(3)
Tyr NH_3^+	–[a]	–[a]	8.10
α	3.92	3.98	3.95
β_1	2.82	2.82	2.82
β_2	2.82	2.82	2.91
ortho H	6.96	6.96	6.96
meta H	6.62	6.65	6.65
DMet NH	8.57	8.62	8.58
α	4.25	4.26	4.33
β_1	1.70	1.72	1.76
β_2	1.55	1.58	1.61
$\gamma_{1,2}$	2.15	2.18	2.18
S-CH_3	1.95	1.98	1.96
Gly NH	8.13	8.20	8.18
α_1	3.65	3.68	3.70
α_2	3.50	3.55	3.52
Phe NH	8.19	8.12	8.15
α	4.65	4.67	4.66
β_1	3.00	2.98	3.04
β_2	2.68	2.73	2.71
aromatic H	7.15–7.27	7.15–7.27	7.15–7.27
Pro/Hyp α	4.18	4.31	4.27
β_1	1.92	2.00	2.17
β_2	1.74	1.94	1.95
γ_1	1.78	1.80	4.40
γ_2	1.78	1.80	
δ_1	3.56	3.62	3.82
δ_2	3.56	3.44	3.69
galac NH		8.25	
H1		4.65	4.17
H2		3.40	3.20
H3		3.30	3.25–3.30
H4		3.36	3.25–3.30
H5		3.30	3.25–3.30
H6		3.67	3.59

[a]At the current stage of the research it was not possible to assign neither hydroxyl chemical shifts nor Tyr NH_3^+ chemical shifts except for (**3**) in the latter case.

assigned by phase sensitive 2QF-COSY (Marion and Wüthrich, 1983) and Relayed-COSY (Weber and Müller, 1987) experiments. All assignments are listed in Table I.

The $^3J_{HN\alpha}$ coupling constants were measured from 1D spectra and are given in Table II. $^3J_{\alpha\beta}$ coupling constants for Phe^4 were also measured from 1D spectra (see Table V).

Amide chemical shifts, their temperature coefficients and the $^3J_{HN\alpha}$ coupling constants are listed in Table II. The values are independent of concentration within experimental error.

NOEs were measured using NOESY (Kumar *et al.*, 1980) and ROESY (Bothner-By *et al.*, 1984) experiments. For qualitative purposes ROESY was found to be better than NOESY since more NOEs were detected. Some different values of mixing times (τ_m) were tested (50–200 ms). A time of 100 ms was found to be optimal for (**2**), whereas for (**1**) and (**3**) a τ_m of 200 ms was used. ROESY data can

Table II. Chemical shifts, temperature coefficients and coupling constants of the NH protons

		Residue			
		D-Met^2	Gly^3	Phe^4	galac^6
δ (p.p.m.)	(1)	8.57	8.13	8.19	
	(2)	8.62	8.20	8.12	8.25
	(3)	8.52	8.18	8.15	
$\Delta\delta/\Delta T$	(1)	−3.0	−3.0	−4.5	
(p.p.b./K)	(2)	−2.5	−3.0	−4.5	−4.5
	(3)	−5.5	−8.5	−4.5	
$^3J_{HN\alpha}$	(1)	7.9	5.8[a]	8.4	
(Hz)	(2)	7.8	6.1[a]	8.4	9.1
	(3)	7.9	5.7[a]	8.4	

[a]Both $^3J_{HN\alpha}$ measured were equal.

Table III. Intrarresidual NOEs observed by ROESY in DMSO-d_6

	Correlation[a]	Compound		
		(1)	(2)	(3)
Tyr NH_3^+–αH		–	–	++
Tyr NH_3^+–βH		–	–	+
Tyr *ortho*H–αH		++	++	++
Tyr *ortho*H–βH		+	+	+
Tyr *meta*H–βH		–	+	+
Tyr αH–βH		+	+	+
D-Met NH–αH		+	+	+
D-Met NH–β_1H				+
D-Met NH–β_2H		+	++	++
D-Met NH–γH		++	++	++
D-Met αH–β_1H		++	++	++
D-Met αH–β_2H		++	++	++
D-Met αH–γH		++	++	+++
Gly NH–α_1H	D_1		+++	–
Gly NH–α_2H	D_2		+++	+++
Phe NH–αH		+	+	+
Phe NH–β_1H			++	
Phe NH–β_2H		+	++	++
Phe αH–β_1H		++	++	++
Phe αH–β_2H		+	++	+
Pro αH–βH		++	++	++
Pro γH–δH		++	++	
Hyp γH–βH				++
galac NH–H1			+	
galac NH–H2			++	

[a]Letter correlates with ROESY cross-peaks in Figures 4 and 5 and with short distances in Figure 6.

be found in Tables III (intrarresidual NOEs) and IV (interresidual NOEs).

Our proposal for the backbone conformation of (**1**), (**2**) and (**3**) is based on (i) measurements of the $^3J_{HN\alpha}$ coupling constants, (ii) the occurrence of NOEs as measured by ROESY and NOESY experiments and (iii) the solvent exposure as revealed by the temperature dependence of the NH chemical shifts.

Experimental data seem to be similar for both the N-terminus and C-terminus of the three compounds in contrast with the obtained parameters for Gly^3.

DMet^2 and Phe^4 show $^3J_{HN\alpha}$ values of ~8 Hz, which correspond to ϕ_i torsion angles around −90° or −150°

(Phe[4]) and +90° or +150° (DMet[2]) when the equation:

$$^3J_{HN\alpha} = 6.4\cos^2\Theta - 1.4\cos\Theta + 1.9,$$

with $\Theta = |\phi\text{-}60°|$ (L-AA) or $|\phi+60°|$ (D-AA), is used (Pardi *et al.*, 1984). Since in proteins (L-amino acids) ϕ angles of all residues except glycine have been reported to be in the range −30° to −180° (Richardson, 1981), both of the angles obtained for each amino acid are possible. Nevertheless, the detection of the similarly intense NH−αH, NH−β_1H and NH−β_2H ROESY cross-peaks both in DMet[2] and Phe[4] leads to ϕ_i angles around +150° and −150°, respectively, since ϕ_2 = +90° and ϕ_4 = −90° are not consistent with NH−αH NOEs (Sherman *et al.*, 1987). In addition, the strong TyrαH−DMetNH, DMetαH−GlyNH and either PheαH−Proδ_1H and PheαH−Proδ_2H or PheαH−Hypδ_1H and PheαH−Hypδ_2H interresidual ROESY cross-peaks indicate that $\psi_1 \approx \psi_4 \approx +120°$ and $\psi_2 \approx -120°$ in the three compounds and ProαH−galacNH NOE leads also to $\psi_5 \approx +120°$ in (**2**) (Billeter *et al.*, 1982). The ϕ_i and ψ_i torsion angles obtained are consistent with the existence of two fairly rigid β-structures involving on one hand Tyr[1] and DMet[2] and on the other hand Phe[4] and Pro[5], extended to the galactose moiety in (**2**).

Following the Karplus equation given previously (Pardi *et al.*, 1984) and the Bystrov curves for glycyl residues (Bystrov, 1976) the measured $^3J_{HN\alpha1\alpha2}$ vicinal coupling constants of Gly[3], around 6 Hz, lead to $\phi_3 \approx +30°$ or −30° for the three compounds. These torsion angles are consistent with the detection of NH−α_1H but not NH−α_2H NOEs, and NH−α_2H but not NH−α_1H, respectively. The observation of both NOEs in (**2**) (ROESY cross-peaks D_1, D_2 in Table III and Figure 4) is then incompatible with the $^3J_{HN\alpha}$ data and indicates conformational heterogeneity. In contrast, only the NH−α_2H

Table IV. Interresidual NOEs observed by ROESY in DMSO-d_6 solutions

	Correlation[a]	Compound			
		(1)	(2)	(3)	
				303 K	333 K
α_iH−NH$_{i+1}$					
Tyr αH−D-Met NH	A	+++	+++	+++	−
D-Met αH−Gly NH	B	+++	+++	+++	+
Gly α_1H−Phe NH	C_1	+++	+++	−	−
Gly α_2H−Phe NH	C_2	+++	+++	+++	+
Pro αH−galac NH			++		
β_iH−NH$_{i+1}$					
Tyr βH−D-Met NH		+	++	+	−
α_iH−δ_{i+1}H					
Phe αH−Pro δ_1H		+++	+++		
Phe αH−Pro δ_2H		+++	+++		
Phe αH−Hyp δ_1H	E_1			+++	++
Phe αH−Hyp δ_2H	E_2			+++	++
Others					
Hyp γH−galac H1	F			+++	++

[a]Letters correlate with ROESY cross-peaks in Figures 3−5 and with short distances in Figure 6.

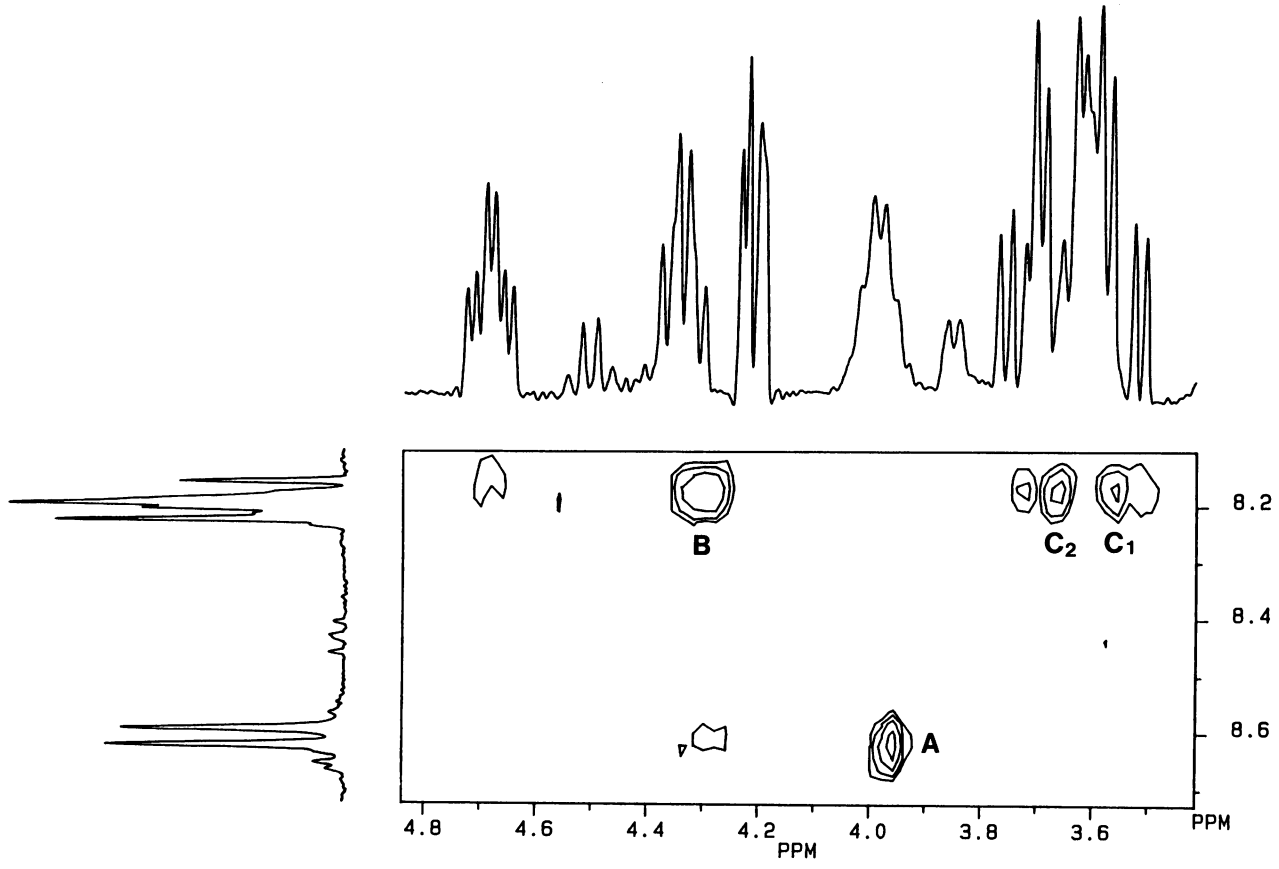


Fig. 3. ROESY spectrum of compound (**1**) in DMSO-d_6. NH−CαH extensions where (**A**) Tyr αH−DMet NH; (**B**) DMet αH−Gly NH; (**C_1**) Gly α_1H−Phe NH; (**C_2**) Gly α_2H−Phe NH. 10 mM, 303 K, mixing time τ_m = 200 ms. Negative levels are shown.

NOE was detected in (**3**) (ROESY crosspeak D_2 in Table III and Figure 5) which accounts for the existence of only one of those ϕ_3 possibilities. Which ϕ_3 corresponds to this observation is not known without stereospecific assignment of α_1H and α_2H. As far as the ψ_3 torsion angle is concerned, the strong Glyα_2H−PheNH (ROESY cross-peak C_2 in Table IV and Figure 5) and the absence of the Glyα_1H−PheNH sequential ROESY cross peak in (**3**) agree with $\psi_3 \approx +120°$ or $-120°$. Again the lack of stereospecific assignment of α_1H and α_2H prevent a choice between ψ_3 values. Nevertheless, since GlyNH−αH and GlyαH−PheNH NOEs detected involve the same GlyαH, the only possible combinations for (ϕ_3,ψ_3) are $(+30°,+120°)$ and $(-30°,-120°)$. On the other hand, the presence of two equally intense $\alpha H_i - NH_{i+1}$ for (**1**) (ROESY cross-peaks C_1, C_2 in Table IV and Figure 3) and (**2**) (ROESY cross peaks C_1, C_2 in Table IV and Figure 4) corresponds to $\psi_3 = 0°$ which is energetically unfavourable. Considering the conformational heterogeneity at Gly^3, the detection of NOEs involving both GlyαH is likely to be due to the co-existence of conformations with different ψ_3. A possible combination would involve both conformations under consideration for (**3**).

Further restrictions can be introduced considering the conformation of the Phe^4 side-chain (Table V). The data concerning compound (**3**) are consistent with a large population of the gauche (−) rotamer. In such a case, the conformation corresponding to $\phi_3 \approx +30°$ and $\psi_3 \approx +120°$ is clearly unfavourable and should be discarded. On the other hand, the data corresponding to (**1**) and (**2**) are more ambiguous. In that case, although $\psi_3 \approx +120°$ should be also unfavourable, the consideration of all the significant

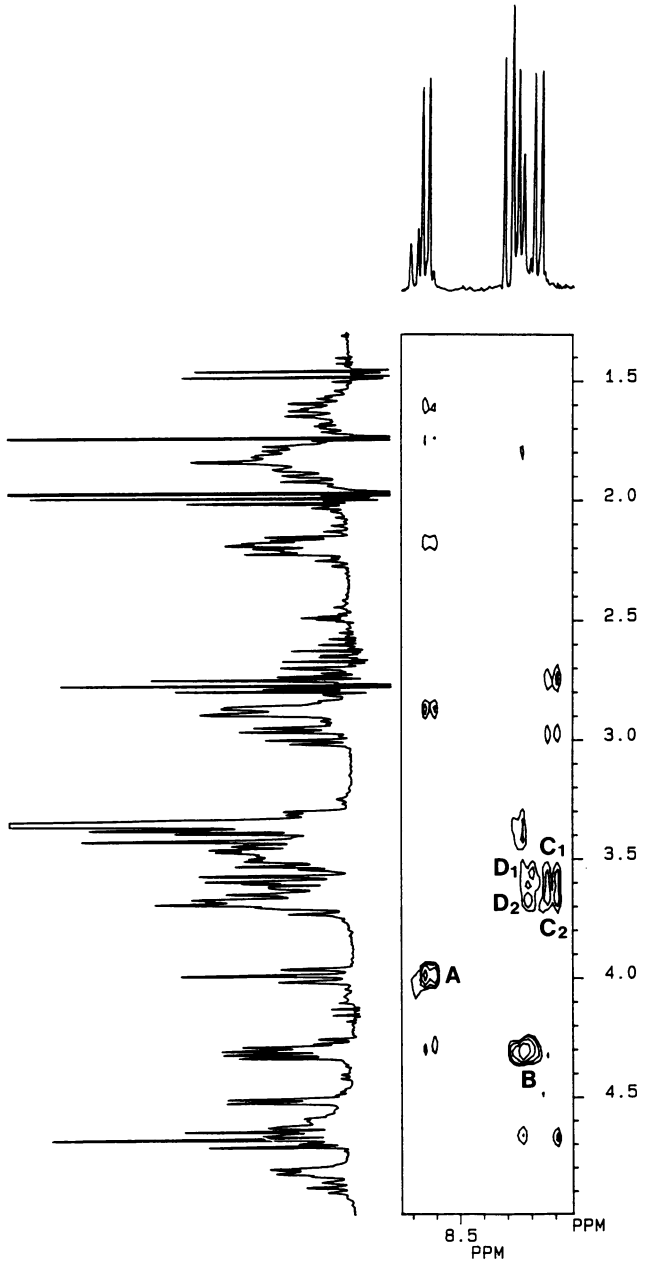


Fig. 4. ROESY spectrum of compound (**2**) in DMSO-d_6. NH−CαH extensions where (**A**) Tyr αH−DMet NH; (**B**) DMet αH−Gly NH; (**C_1**) Gly α_1H−Phe NH; (**C_2**) Gly α_2H−Phe NH; (**D_1**) Gly NH−α_1H; (**D_2**) Gly NH−α_2H. 10 mM, 303 K, mixing time τ_m = 100 ms. Negative levels are shown.

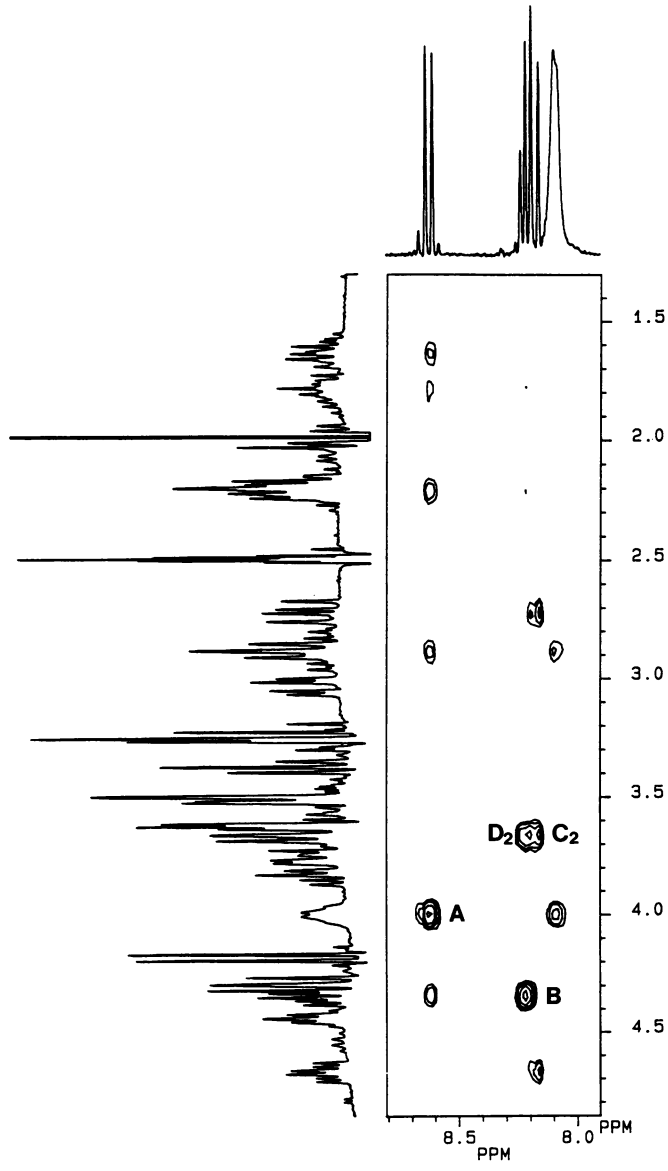


Fig. 5. ROESY spectrum of compound (**3**) in DMSO-d_6. NH−CαH extensions where (**A**) Tyr αH−DMet NH; (**B**) DMet αH−Gly NH; (**C_2**) Gly α_2H−Phe NH; (**D_2**) Gly NH−α_2H. 20 mM, 303 K, mixing time τ_m = 200 ms. Negative levels are shown.

Table V. Data determining Phe[4] side-chain conformation

	$^3J_{\alpha\beta1}$ (Hz)	$^3J_{\alpha\beta2}$ (Hz)	$NOE_{\alpha H-\beta 1H}$	$NOE_{\alpha H-\beta 2H}$	$NOE_{NH-\beta 1H}$	$NOE_{NH-\beta 2H}$
(1)	4.3	9.1	++	+	−	+
(2)	−	8.8	++	++	++	++
(3)	4.0	8.8	++	+	−	++

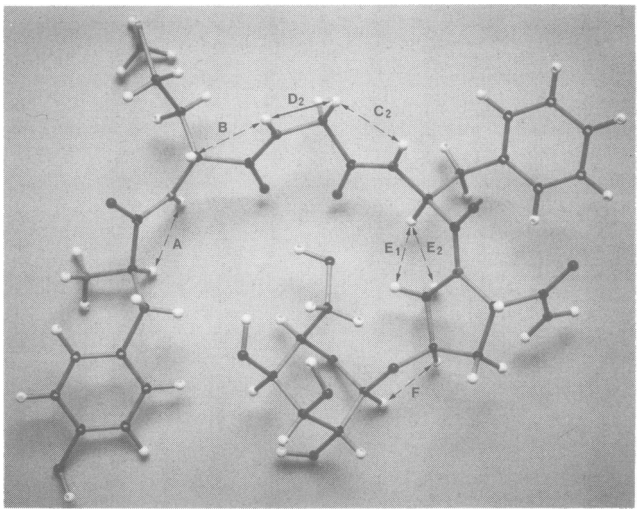


Fig. 6. Proposed conformation for compound (**3**). Solid arrows, short distances corresponding to intra-residual ROESY cross-peaks; broken arrows, short distances corresponding to inter-residual ROESY cross-peaks. Letters correlate with ROESY cross-peaks in Tables III and IV and Figure 5.

NMR data obtained in the present work does not allow us to discard any of the possible torsion angles.

In summary, the three compounds tested present two β strands connected to each other by a fairly flexible glycyl residue at position 3. For compounds (**1**) and (**2**), experimental data are inconsistent with a single conformation of Gly[3]. In contrast, compound (**3**) seems to preferentially exist in a more rigid folded conformation in which $\phi_3 \approx -30°$ and $\psi_3 \approx -120°$ (Figure 6).

The temperature coefficients summarized in Table II show no evidence for hydrogen bonds involving the amide protons.

Furthermore, other experimental evidence has led us to suggest an explanation for the preferential existence of the proposed folded conformation in the βO-galactosyl analogue (**3**) and not in the βN-galactosyl analogue (**2**). Three strong NOEs detected even at 333 K, namely the previously mentioned PheαH−Hypδ_1H, PheαH−Hypδ_2H and HypγH−galacH1 (ROESY cross peaks E_1, E_2 and F, respectively, in Table IV and Figure 5) prove that the C-terminus of (**3**) is an especially rigid part of the molecule compared with the N-terminus, since the rest of the sequential NOEs were weaker at high temperatures. In the proposed conformation for compound (**3**) (Figure 6) the hydroxyl groups in positions 4 and 6 of the galactosyl residue are faced to the carbonyl groups of DMet[2] and Gly[3], respectively, so we suggest that such a conformation could be stabilized by hydrogen interactions between hydroxyl groups of galactose and carbonyl groups of the peptide backbone. Unfortunately, such a hypothesis cannot be supported by NOE data since all the non-exchangeable hydrogens of the galactosyl residue are too far from any hydrogen of the peptide moiety to show an observable NOE.

Discussion

The experimental NMR data presented are consistent with two β-strands (N-terminus and C-terminus) connected to each other by Gly[3] for the three compounds tested. Both the parent compound [DMet[2],Pro[5]] enkephalin amide (1), and the $N^{1,5}$ galactosyl analogue [DMet[2],Pro[5]] enkephalin [$N^{1,5}$-β-D-galactopyranosyl] amide (**2**) have been found to exhibit a similar conformational equilibrium with great flexibility at position 3. In contrast, $O^{1,5}$-(β-D-galactopyranosyl) [DMet[2],Hyp[5]] enkephalin amide (**3**) shows a preferential folded structure.

As mentioned before, the $N^{1,5}$-galactosyl derivative (**2**) is significantly more potent than the parent compound (**1**) in rats, in both the tail immersion and paw pressure tests of analgesia after intraventricular administration. This difference in the analgesic activity of (**1**) and (**2**) cannot be related to any conformational change in DMSO solution since all the significant NMR parameters measured are very similar and consistent with a similar conformational equilibrium. Nevertheless, the increase in the analgesic activity of the $O^{1,5}$-galactosyl analogue (**3**) with respect to the $N^{1,5}$-galactosyl analogue (**2**) could be related to the adoption of the preferential folded conformation proposed in this work.

In conclusion, the introduction of a galactosyl moiety into the fifth residue of [DMet[2],Pro[5]] enkephalinamide leads to an increase in analgesic activity of ~100-times which is independent of the conformation of the molecule in DMSO solutions. Nevertheless, folded conformations seem to play an additional important role. In fact, when the galactosyl moiety is incorporated into another position in the same residue which allows galactose−peptide backbone interactions, a preferential folded conformation is detected. Such a conformational change could be related to an extra increment in analgesic activity of ~10 times.

Materials and methods

Synthesis

Thin layer chromatography was performed on silica gel plates (0.25 mm) from Merck. Spots were detected by reaction with ninhydrin or chlorine followed by tolidine solution. The sugar was detected by Orcinol−HCl. Amino acid analysis was performed in a Beckman 119 C instrument or a Biotronik LC 5001. All the solvents used were of analytical grade. They were distilled and stored over molecular sieves when necessary.

Microanalyses were performed by the Microanalytical Laboratory, Department of Biological Organic Chemistry, CID, Barcelona. FAB mass spectra were determined with a MS9-V6 updated system equipped with a VG-11 250 data system by the Laboratory of Mass Spectrometry, CID, Barcelona. An ion tech atom gun and a standard FAB source were used. The samples, dissolved in glycerol, were bombarded with 8 keV xenon atoms and the ions produced accelerated through 8 kV.

Isolation and purification of the protected glycosyl derivatives of the amino acids and peptides was carried out by flash chromatography, using Silica gel (40−63 μm), 15 × 2 or 15 × 5 cm columns eluted with the appropriate solvent system at a flow rate of 5 cm/min.

The purity of the protected glycosyl amino acids and peptides was checked by reversed-phase HPLC [ODS, 5 μm column, H_2O 0.05% trifluoroacetic acid (TFA):CH_3CN gradient elution from 9 to 100% CH_3CN at a linear rate of 3.5% CH_3CN/min] with detection by absorbance rationing measurements at several wavelengths.

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl amine has been obtained from acetobrome-D-galactose according to the Bertho *et al.* (1932) procedure. The resulting product was characterized by ^{1}H-NMR. Melting point 137−139°C (literature value = 139°).

Two different amino acid N-protecting groups have been used, benzyloxycarbonyl (Z) and tert-butyloxycarbonyl (tBoc). Z groups were cleaved by hydrogenolysis in the presence of 10% palladium on charcoal (0.1 g/g) in

methanol. Removal of tBoc groups was achieved with TFA:CH_2Cl_2 (1:1) containing a few drops of 2-mercaptoethanol, 30 min at room temperature with a ratio of 10 ml acid−solvent mixture to 1 mmol of peptide or amino acid derivatives.

All optically active amino acids were of the L configuration with the exception of D-methionine, as indicated throughout the text.

^{1}H-NMR characterization of the final derivatives is not included in this experimental section.

βN-glycosylation. Synthesis of Tyrosyl, D-methionyl, glycyl, phenylalanyl, prolyl [N^1-β-D-galactopyranosyl] amide. Compound (**2**).

tBoc-Tyr−DMet−Gly−Phe−Pro-OH (0.750 g, 1.05 mmol) and 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosylamine (0.36 g, 1.05 mmol) were dissolved in 20 ml of tetrahydrofuran (THF) at −20°C. 1-hydroxybenzotriazol (HOBt) (0.15 g) and two drops of 2-mercaptoethanol were added to the solution. *N,N'*-dicyclohexylcarbodiimide (DCC) (0.21 g, 1.05 mmol) dissolved in 2 ml of THF was also added dropwise to the solution and the reaction mixture was kept at −20° for 1 h and then overnight at room temperature. The mixture was then filtered and the filtrate washed successively with 5% citric acid (3 × 20 ml), 5% sodium bicarbonate (3 × 20 ml) and water (3 × 20 ml). The organic layer was then dried over anhydrous Na_2SO_4 and the amorphous solid obtained after evaporation was purified by flash chromatography on silica gel using ethyl acetate:methanol (8:2) as eluent. Amino acid analysis: Tyr 0.94, D-Met 0.91, Gly 1.00, Phe 0.97, Pro 0.99. $[\alpha]^{20}_{295} = 7.58°$, $[\alpha]^{20}_{255} = -26.06°$, $[\alpha]^{20}_{240} = -12.12°$, $[\alpha]^{20}_{235} = -38.18°$ (c = 0.32, methanol). ^{13}C-NMR (20 MHz) ($CDCl_3$) 83.0 Cl galactose β anomer.

The cleavage of the protecting groups was carried out in two steps, TFA:CH_2Cl_2 (1:1) treatment for the elimination of the N-terminal tBoc group and NH_3:MeOH treatment for the cleavage of the acetyl groups. The resulting galactopyranosyl peptide was then purified by gel filtration on Sephadex G-25 and by semipreparative HPLC, C_{18}, solvent system H_2O−0.05% TFA:CH_3CN, isocratic mode elution 70:30, λ = 280 nm. Homogeneity was assessed by TLC and reversed-phase HPLC ODS 5 μm column, solvent system H_2O−0.05% TFA:CH_3CN 1 ml/min, gradient elution from 10 to 90% CH_3CN at a linear rate of 3.5% CH_3CN/min, 0.16 AUFS with detection by absorbance rationing measurements. Amino acid analysis: Tyr 0.95, D-Met 0.90, Gly 1.00, Phe 0.98, Pro 0.99. $[\alpha]^{20}_{295} = 6.07°$, $[\alpha]^{20}_{255} = 28.21°$, $[\alpha]^{20}_{240} = 68.21°$, $[\alpha]^{20}_{235} = -63.93°$ (c = 0.32, methanol). ^{1}H-NMR ROESY, different intensity of galac N-1 and galac N-2 cross-peaks consistent with β anomer. FAB mass spectrometry: m/z 775 (M + 1).

βO-glycosylation. Synthesis of Tyrosyl, D-methionyl, glycyl, phenylalanyl, [O^1-β-D-galactopyranosyl] hydroxyprolinamide. Compound (**3**).

Z-Hyp-OMe (0.700 g, 2.5 mmol) was dissolved in 40 ml of dry benzene and 40 ml of nitromethane and heated under anhydrous conditions until about 15 ml of solvent had distilled off. To this solution a double equivalent amount of both 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (2.056 g) and $Hg(CN)_2$ (1.286 g) were added in three portions: half of the given amounts at the beginning of the reaction and the rest in two equal portions after 2 and 3 h, respectively. The mixture was heated at 80°C for a total time of 4 h. The solution was then cooled and diluted with ether (80 ml) and filtered to eliminate the precipitated mercuric salts. The filtrate was then washed with water, dried over Na_2SO_4 and evaporated. The oily resulting mixture was submitted to flash chromatography on Silica gel (40−63 μm), 15 × 5 cm ID column eluted with hexane:ethyl acetate (3:7). ^{1}H-NMR (360 MHz, $CDCl_3$) d 4.35 $J_{1,2}$ = 7.5 Hz H-1 β anomer.

After hydrogenolysis of the Z group, the incorporation of the resulting O_1-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) hydroxyproline methyl ester to the tetrapeptide tBoc-Tyr−DMet−Gly−Phe-OH by the DCC/HOBt method and the purification of resulting protected galactosylated pentapeptide were carried out following the same procedure stated for compound (**2**).

The cleavage of the protecting groups and the C-terminal amide formation were also carried out in two steps, TFA:CH_2Cl_2 (1:1) treatment for elimination of N-terminal tBoc group and NH_3:MeOH (saturated at 0°C) treatment for the cleavage of acetyl groups and terminal amide formation from the methyl ester. The resulting galactopyranosyl peptide was then purified by gel filtration on Sephadex G-25 and semipreparative HPLC, C_{18}, solvent system H_2O−0.05% TFA:CH_3CN, isocratic mode elution 70:30, λ = 280 nm. Homogeneity was assessed by TLC and reversed-phase HPLC ODS 5 μm column, solvent system H_2O−0.05% TFA:CH_3CN 1 ml/min, gradient elution from 10 to 90% CH_3CN at a linear rate of 3.5% CH_3CN min, 0.16 AUFS with detection by absorbance rationing measurements. Amino acid analysis: Tyr 0.95, D-Met 0.90, Gly 1.00, Phe 1.05, Hyp 0.98. $[\alpha]^{20}_{295} = 4.37°$, $[\alpha]^{20}_{254} = 0°$, $[\alpha]^{20}_{240} = 19.37°$, $[\alpha]^{20}_{235} = 9.69°$ (c = 0.32, MeOH). FAB mass spectrometry m/z 791 (M + 1), 813 (M + 23). ^{1}H-NMR (270 MHz, DMSO-d_6) $^3J_{1,2}$ galac = 7.09 β anomer.

The parent compound (**1**) has been synthesized by coupling tBoc-Tyr−DMet−Gly−Phe-OH and H-Pro-OMe followed by deprotection and terminal amide formation.

Epimerization has been checked by gas chromatography on an enantioselective stationary phase (cyano ethyl-siloxane-L-valine-S-α-phenyl ethyl amide, Quirasil-valine). The extent of epimerization produced during each step in the synthesis of compounds (**1**−**3**) was not greater than 1%.

Conformational analysis

NMR spectra were recorded on a Bruker AM 270 spectrometer equipped with an Aspect 2000 computer running the DISN861 program. The obtained data were also processed with an Aspect 3000 computer running DISR87 or DISR88. For the conformational study the sample concentration was 10 or 20 mM in DMSO-d_6 (CEA 99.98%). Samples were degassed by several freeze−pump−thaw cycles. The types of 2D-NMR experiments carried out were phase-sensitive, double quantum filtered COSY (Marion and Wüthrich, 1983) and relayed COSY (Weber and Müller, 1987), NOESY (Kumar *et al.*, 1980) and ROESY (Bothner-By *et al.*, 1984).

All spectra were measured with a spectral width of 2703 Hz, covering the range 0.0−10.0 p.p.m.

The 1D spectra were measured with 8 K data. The interferograms were multiplied by a Gaussian function with LB = −3 and GB = 0.3, zero-filled to 16 K and Fourier transformed. The resulting spectra had a digital resolution of 0.3 Hz/point.

All the 2D spectra were measured in a phase-sensitive mode using TPPI (Marion and Wüthrich, 1983). In relayed COSY, the relay period amounted to 50 ms. Mixing times were 200−400 ms in NOESY and 50−200 ms in ROESY. In ROESY experiments the spin-lock field was 3.8 kHz, to minimize Hartman−Hahn effects (Bax and Davies, 1985).

All 2D spectra consisted of 256 or 512 interferograms of 1 K data. The interferograms were multiplied by a Gaussian function with LB = −3 and GB = 0.3 and Fourier transformed. The t_1-interferograms were multiplied by a sine bell function shifted by π/8, zero-filled to 1 K and Fourier transformed. The digital resolution in the resulting 512 × 512 W spectra was 5.3 Hz in both dimensions.

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