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Differences in the conformational behaviour of the potent and selective, Tyr-D.Thr-Gly-Phe-Leu-Thr(OtBu) and of the inactive Tyr-D.Thr(OtBu)-Gly-Phe-Leu-Thr δ-opioid ligands evidenced by ^{1}H NMR.

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Abstract : Introduction of a bulky tertiobutyl group on the hydroxyl group of only one threonyl residue in DTLET, Tyr-D.Thr-Gly-Phe-Leu-Thr leads to opposite results : increase in the selectivity for δ opioid binding site for DTLETBU, Tyr-D.Thr-Gly-Phe-Leu-Thr(OtBu) or complete loss of potency for DTBULET, Tyr-D.Thr(OtBu)-Gly-Phe-Leu-Thr. The 400 MHz spectra of the three peptides in $(CD_3)_2SO$ solution showed the occurrence of large structural analogies in DTLET and DTLETBU characterized by a C-terminal folded conformation (β-turn around the Phe^4-Leu^5 residues). In DTBULET all the temperature coefficients are in the range of more or less solvent exposed amide protons. This conformational change is likely induced by a steric hindrance between the bulky O-tert-butyl group of Thr^2 and the vicinal residues. This assumption is supported by a strong NOE between the CH_3 of the Thr^2(OtBu) side chain and the Gly^3NH. These findings were confirmed by conformational calculations using the Metropolis procedure. The biological results show that the Thr^2(OtBu) prevents the conformational adaptation of the peptide to the opioid receptors. This underlines the usefulness of conformational analysis of peptide as pre-requisite to a rational design of selective ligand.

[Met]-enkephalin and [Leu]-enkephalin, Tyr-Gly-Gly-Phe-Met(Leu) are endogenous flexible peptides which interact preferentially with δ-opioid receptor but also with the mu binding site (1). As previously discussed investigations on the physiological relevance of these two receptor

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types require molecules which display a binding affinity at least 100 times higher for one class of receptor. Along this way linear hexapeptides as DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr and DTLET, Tyr-D-Thr-Gly-Phe-Leu-Thr where proposed as relatively δ selective ligands and used to characterize specific responses induced by δ-receptor stimulations (reviews in 2 and 3). These peptides were designed by taking into account the conformational flexibility of the native enkephalins (4) and the initial assumption (5) of their binding to μ or δ opioid receptors through the zipper mechanism (6,7). Recently, the cyclic enkephalin DPLPE Tyr-D-Pen-Gly-Phe-Pen characterized by the presence of a disulfide linking two highly constrained penicillamine residues has been shown to display a higher selectivity than DTLET (8). However this enhanced δ-specificity is associated with a decreased δ-affinity (9). Using ^{1}H NMR spectroscopy similar conformations were found in solution for the constrained peptide DPLPE and the flexible hexapeptide DTLET (10). Therefore it was hypothesized that the enhanced δ-selectivity of DPLPE as compared to DTLET was not due to large differences in the solvated forms of both peptides but may be related to a very large conformational expense of energy needed for DPLPE to interact with the μ opioid receptor, a feature not encountered with DTLET. Moreover the weaker affinity of DPLPE was attributed to a lower intrinsic flexibility leading to a higher energetic penalty during the binding process to δ-sites (10). From these results we have hypothesized that an increase in the size of the residue in position 2 and/or 6 of DTLET or DSLET could reinforce the structural analogy with the Pen containing peptides leading therefore to a decrease in μ receptor affinity without modification of δ-receptor recognition.

Accordingly a threonine residue etherified by the bulky tertiobutyl (OtBu) group was introduced in position 2 or 6 of DTLET. The obtained peptide Tyr-D.Thr-Gly-Phe-Leu-Thr(OtBu) has shown as expected a large decrease in μ affinity without great modification in the recognition of δ-sites. Contrastingly a dramatic loss of affinity for both μ and δ receptors was observed with the Tyr-D.Thr(OtBu)-Gly-Phe-Leu-Thr, DTBULET. This striking result was investigated through a conformational analysis of both peptides by ^{1}H NMR spectrocopy, the results of which being presented in this paper.

EXPERIMENTAL

DTLET, DTBULET and DTLETBU were synthesized in our laboratory as described (11). The NMR samples were prepared by dissolving the peptides in H_2O. The solutions were adjusted to pH 5.5 and lyophilized. The dried peptides were redissolved in $(CD_3)_2SO$ at a concentration of 5×10^{-3}M. Spectra were run in the Fourier transform mode at 400 MHz on a Bruker AM 400 equipped with Aspect 3000 computer and a Bruker temperature controller (± 1°C). Chemical shifts are given in ppm (± 0.01) using hexamethyldisiloxane (HMDS) as an internal reference. The coupling constants were determined at ± 0.2 Hz. For Leu^5 fragment, $H_{\alpha-\beta}$ protons exhibits an ABX spin system in which A and

B are the β protons and X is the α proton. $^{3}J_{\alpha-\beta}$ coupling constants were obtained from spectral simulation using the PANIC program (Bruker). Resolution enhancement was achieved using a gaussian multiplication of the FID. To record the COSY spectra, the basic two pulses Jeener sequence was used in its improved version known as double quantum filter (DQF) COSY (12) $[t_0\text{-}90(X)\text{-}T_1\text{-}90(X)90\ (\psi)\text{-acquisition}\ (\psi)]$.The ROESY pulse sequence $[90^{\circ}_{\phi} - t_1 - [\text{spin lock}]_{\phi + \frac{\pi}{2}} - \text{acquisition}\ (t_2)]$ was used (13). The carrier frequency was positioned at 5 ppm and a 4 kHz spin-lock field used during the 200 ms mixing period. ROESY experiments were performed using the time-proportional phase increment (TPPI) method (14) and the data displayed in the phase-sensitive mode.

The theoretical conformational analysis was performed in three steps (18) : i) sampling of the conformational space of each compound using the Metropolis procedure ; ii) grouping of the conformers of each individual sample into families showing the conformational possibilities of each compound ; ii) refinement of the most representative conformations by energy minimisation and their comparison with the stable conformations obtained for cyclic δ-selective peptides.

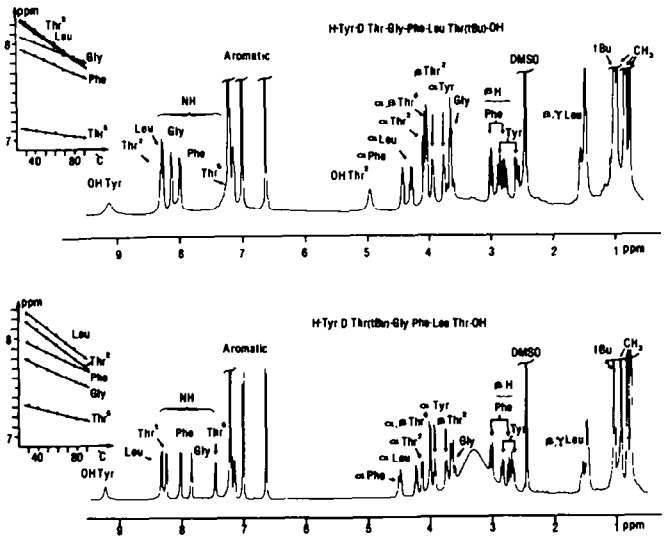

Figure 1.

^{1}H NMR spectra and assignments of DTLETBU (above) and DTBULET (below) in DMSO-d_6 at 400 MHz.

Insert : temperature dependence of peptide NH chemical shifts.

RESULTS

The conformational characteristics of DTBULET and DTLETBU were determined by ^{1}H NMR study in $(CD_3)_2SO$ solution (Fig. 1). For both peptides proton assignment was done by 2D correlation spectrocopy and the discrimination between Thr^{2}and Thr^6 residues in each peptide was performed by sequential NOE experiments as described (10). The NMR parameters of DTBULET and DTLETBU are reported in Table 1 and compared with the already reported data (10) of their parent compound DTLET.

Table 1. Chemical shifts (a), NH temperature dependency (b) for DTLET (1), DTBULET (2) and DTLETBU (3) in DMSO-D_6 (bisionic forms).

		δ_α [a]	δ_β [a]	δ_{NH} [a(b)]	$^3J_{NH-\alpha}$ [c]	δ other [a]
Tyr^1	(1)	3.70	2.85;2.55	-	-	Ar.=6.97;6.60 OH=9.16
	(2)	3.71	2.83;2.60	-	-	Ar.=6.98;6.61 OH=9.18
	(3)	3.65	2.85;2.52	-	-	Ar.=6.97;6.59 OH=9.10
(D)Thr^2	(1)	4.00	3.94	8.26(-5.2)	5.5	Me=0.86 OH=4.91
	(2)	4.11	3.73	8.20(-7.5)	8.0	Me=0.76 Bu=1.05
	(3)	4.00	3.93	8.25(-8.0)	7.5	Me=0.87 OH=4.94
Gly^3	(1)	3.71;3.60		8.08(-3.4)	5.5;6.5	
	(2)	3.68;3.61		7.81(-5.0)	5.0;5.5	
	(3)	3.67;3.57		8.07(-4.0)	4.0;7.0	
Phe^4	(1)	4.41	3.03;2.76	8.04(-4.2)	8.5	Ar.=7.19
	(2)	4.44	3.00;2.72	7.98(-5.0)	8.5	Ar.=7.19
	(3)	4.42	3.00;2.78	7.94(-5.0)	8.5	Ar.=7.19
Leu^5	(1)	4.14	-1.48	8.30(-5.5)	8.5	YH=1.48 Me=0.82;0.75
	(2)	4.18	1.48;1.44	8.28(-8.0)	8.0	YH=1.54 Me=0.83;0.77
	(3)	4.26	1.51;1.47	8.22(-7.0)	8.5	YH=1.55 Me=0.83;0.77
Thr^6	(1)	3.86	3.92	7.24(-0.6)	7.0	Me=0.88 OH=4.91
	(2)	3.90	3.94	7.32(-3.0)	7.5	Me=0.90
	(3)	4.05	4.04	7.13(-2.0)	9.0	Me=0.98 Bu=1.01

(a) δ given in ppm ($\pm$0.01) from HMDS used as internal reference ; (b) the NH temperature dependencies in ppm/°C x 10^{-3} are given in brackets ; (c) the coupling constants are given in Hz ($\pm$0.25 Hz) ; (d) Ar. and Me correspond to aromatic and methyl protons respectively.

In the whole the proton chemical shifts of the corresponding residues in the three peptides were closely related. Nevertheless significant differences occurred at the level of the D.Thr^2(OtBu) and Gly^3 residues in DTBULET 2. Thus the α and β protons of the substituted threonine were downfield and upfield shifted respectively while the Gly^{3}NH was shielded by 0.27 ppm. All three peptides were characterized by a large inequivalence of the Gly^3 CH_2 protons but the chemical shift difference is weaker in DTBULET. The variations in amide proton temperature dependencies were parallel in the three compounds with the smallest coefficient occurring for the C-terminal Thr^6 residue : DTLET ($\Delta\delta/\Delta T$ = -0.6×10^{-3}ppm/°C) (10) ; DTLETBU ($\Delta\delta/\Delta T$ = $-2.\times10^{-3}$ppm/°C) ; DTBULET ($\Delta\delta/\Delta T$ = -3×10^{-3}ppm/°C). However while the Gly^{3}NH may be considered as a buried proton in DTLET (10), and to a lesser extent in DTLETBU, the temperature dependency of the glycine amide proton in DTBULET ($\Delta\delta/\Delta T$ = -5×10^{-3}ppm) corresponds to a more solvent-exposed NH group. For the other amide groups, the temperature slopes were rather large especially in DTBULET. Precise informations about the conformational characteristics of peptides can be obtained through the study of both the $^3J_{\alpha-NH}$ coupling constants which are related to the ϕ dihedral angles and interproton NOE's which reflected the distance between them.

Table 2 . $^3J_{\alpha-\beta}$ coupling constants for DTLET (1), DTBULET (2) and DTLETBU (3) in DMSO-d_6 (bisionic forms).

	Tyr^1			(D)Thr^2			Phe^4			Leu^5			Thr^6		
	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
$^3J_{\alpha-\beta}$	5.5 8.5	6.2 8.0	5.5 8.5	3.3	3.5	3.0	4.5 10	4.0 10	3.5 10.5	...	6.0 8.5	5.5 8.5	3.5	3.3	2.5
χ^1=-60°	53%	49%	54%	}94%	}92%	}96%	67%	67%	72%	...	54%	54%	8%	6%	2%
χ^1=+180°	26%	33%	26%				17%	13%	8%	...	31%	26%	}92%	}94%	}98%
χ^1=+60°	21%	18%	20%	6%	8%	4%	16%	20%	20%	...	15%	20%			

(a) the coupling constants are given in Hz (± 0.25 Hz) ; (b) percentage of each conformer was determined following Pachler (17).

Conformational characteristic of the aminoacids side chains of DTBULET and DTLETBU.

The preferential orientations of the aminoacids side chains were estimated from the measured $^3J_{\alpha-\beta}$ (Table 2) and NOE experiments in both peptides.

The proportions of the three staggered conformers were calculated according to Pachler, assuming that for Phe and Tyr, the lower field and high field resonances of the CH_2 group correspond to the Pro-S and Pro-R protons respectively (15,16). As shown in table 2, the same type

of conformational equilibrium occurred in both peptides with a large preference in the population of g^- conformers for the Tyr and Phe side chains. This preferential orientation already found in DTLET (10) was confirmed by the large NOE-related increase in the intensity of the β_2 and β_1 protons of Phe through irradiation of its amide and α protons respectively. In these peptides the determination of a single $^3J_{\alpha-\beta}$ coupling constants for the two threonine side chains allowed the determination of only the population of trans conformer which was found inferior to 10%. Nevertheless a very large difference occurred in the spatial orientation of the side chain of the D-Thr^2 residue in the three enkephalin analogs. Indeed a strong NOE (~25%) was observed between the CH_3 of the Thr^2(OtBu) residue and the NH of Gly^3 only in DTBULET. Moreover in this peptide the $^3J_{NH-\alpha}$ of the Thr^2(OtBu) residue was found higher (~ 8Hz) as in DTLET (~5.5 Hz) suggesting a quasi-trans orientation (~ 180°) of these two protons. This backbone conformation leads to a close proximity between the NH of Thr^2(tBu) and the Hα of Tyr^1 as shown by the significant NOE (~ 10%) occurring between these protons.

DISCUSSION

The NMR parameters obtained in this study showed large structural analogies in DTLET (10) and DTLETBU but significant difference between this latter and DTBULET. In DTLET the very low temperature coefficient of Thr^6NH (-0.6×10^{-3} ppm/°C) was interpreted by the occurrence of a C-terminal folded conformation i.e. a β turn around the Phe^4-Leu^5 residues. This assumption was supported by the large NOE observed between Phe-H_α Leu-NH, Leu-NH Thr-NH and Leu-H_α Thr-NH. Besides a folding tendency of the N-terminal part of DTLET was supported by the relatively low Gly^3NH coefficient.

In the whole the NMR parameters of DTLETBU are not very different from those of DTLET. The most significant changes are the larger temperature depencies of the Gly^3 and Thr^6 amide protons. Nevertheless according to its relatively low coefficient (-2×10^{-3} ppm/°C) the Thr^6NH can be considered either as an hydrogen-bonded or at least buried proton.

This is not the case for DTBULET in which all the temperature coefficients, including that of Thr^6 are in the range of more or less solvent-exposed amide protons. Interestingly introduction of the bulky tert-butyl group in D.Thr^2 or Thr^6 leads in both cases to an increase in the $^3J_{NH-\alpha}$ of the D.Thr^2 residue as compared to that found in DTLET. Furthermore the NOE occurring between the amide groups of Leu^5 and Thr^6 in DTLET were not observed in the substituted analogs. Finally the most important change was found at the level of the Thr^2(OtBu) residue in DTBULET where a strong NOE was obtained between the CH_3 group of the threonyl side chain and the NH of the adjacent Gly residue. This indicates a large change in the spatial orientation of the lateral chain with an assumed large population of trans conformer around the Cα-Cβ bond. This results very likely from a steric hindrance between the bulky tert-butyl group and the peptide backbone. Such a

conformational modification was not observed for the Thr^6(tBu) moiety because this modified aminoacid is located in the C-terminal position.

It can be noticed that the conformer populations of Tyr^1 and Phe^4 were not significantly modified by the different spatial orientations of the Thr^2 side chain in the three peptides. All these results seem to indicate that introduction of the bulkyl tert-butyl groups decreased the folding tendency observed in DTLET with a disappearence of the C-terminal β-turn which could be replaced in DTLETBU by a γ turn centered on Leu^5. The occcurrence of this type of preferential conformation is in agreement with the relatively low temperature coefficient of the Thr^6(OtBu) amide proton and with the lack of NOE between Hα-Phe and NH-Leu. The existence of large differences in the averaged conformations of DTLET, DTLETBU and DTBULET is supported by energy calculations (fig. 2). Even at the level of the Metropolis sampling, significant different behaviours have been found for the active peptide D.Thr^2-Thr^6 and the inactive analogue D.Thr^2(OtBu)Thr^6. These differences mainly occurs at the level of distances between Tyr^1 and Phe^4 aromatic rings and between the oxygen atom of the side-chain of residues 2 and 6 respectively. The clustering analysis performed on these data shows that the conformational characteristics which are roughly conserved for DTLET and DTLETBU are strongly modified in DTBULET. Energy refinement of the three peptides shows that for the active compounds, the residue in position 2 is mainly found in the C_7 (ϕ = -60° ; ψ = -90°) conformation, the residue 3 near the right-handed α helix (ϕ = -60°, ψ = -60°), so that a β-turn of type II' can also be imagined. One of these conformational tendencies as found with DTBULET. From the previous comparative conformational studies of DTLET and DPLPE we hypothesized that an increase in the size of the residue in position 2 or 6 could

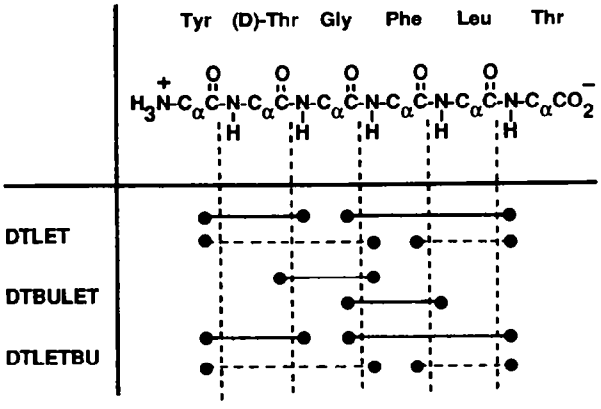

Figure 2.

Schematic representation of the various turns observed in the most stable conformations of DTLET, DTBULET and DTLETBu obtained by Metropolis calculations.

improve the recognition of the opioid μ-receptor without important change in δ-receptor binding. This is indeed the case for DTLETBU but not for DTBULET in which the tert.butyl group was introduced in the Thr^2 residue (table 3). The dramatic decrease in binding affinity of this peptide for both μ and δ sites shows that the steric hindrance induced by the Thr^2(OtBu) moiety hinders the conformational adaptation of the peptide to both types of opioid receptors (table 3). It is therefore necessary to increase the degree of freedom of the aminoacid in position 2 for instance by introduction of tert-butyl groups in less constrained aminoacids as serine or allo-threonine. This work is now in progress in our laboratories.

Table 3. Selectivities of enkephalin derivatives on MU or DELTA opioid receptors of rat brain.

Compounds	δ site * K_I, nM	μ site ** K_I, nM	$K_I(\delta)/K_I(\mu)$
DTLET	1.35±0.15	25.3±2.5	0.053
DTBULET	866±120	4500±920	0.192
DTLETBU	2.57±2.70	66.3±5.6	0.039

* labelled with [^{3}H]DTLET (1 nM) (35°C/40 mn) ; ** labelled with [^{3}H]DAGO (1 nM) (35°C/40 mn).

CONCLUSION

It is obvious that neither the solvated conformations nor the computed forms correspond exactly to the biologically active structures at the receptor sites. However comparison of data from crystallographic studies of enzyme-inhibitor as pepstatin-rhizopus chineusis (19) or β-phenylpropionyl-L-phenylalanine, βPPP-thermolysin respectively (20) and from NMR studies in solution of pepstatin (21) and βPPP (20) have shown that no drastic changes occurred at the level of the peptide backbone between the solvated and the bound forms. Moreover a good relationship appears between the immunogenicity of peptides which exhibit a high tendency to form turns both in proteins and in solution (22,23). Likewise, purification of receptors by means of antiidiotypes is based on the structural analogies between the solvated form of a potent and selective ligand recognized by the primary antibody and the peptidic epitope of the antiidiotype able to fit the receptor binding site (24-26). Concerning the receptor recongition process the faster association rate of a flexible peptide through the zipper mechanism is theoretically associated with a faster dissociation rate (6). Stabilisation of flexible parts of the peptide through specific and stable

interactions in the receptor binding site results in an enthalpic gain but also in an entropic destabilization. In fact the minimum free energy corresponding to the binding constant of a given flexible ligand to a macromolecule seems to result from the sum of enthalpic gain and transfer of flexibility from the binding site to another part of the macromolecule (27-29). Such a process minimizes the entropic destabilization decreasing therefore the dissociation rate. According to this receptor recognition process the knowledge of solvated conformation of a peptide is an essential pre-requisite for a rational design of selective ligands.

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