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δ Opioid Receptor Selectivity Induced by Conformational Constraints in Linear Enkephalin-Related Peptides: ^{1}H 400-MHz NMR Study and Theoretical Calculations†

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ABSTRACT: Introduction into the structure of the linear hexapeptide DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) or DTLET (Tyr-D-Thr-Gly-Phe-Leu-Thr) of *tert*-butyl groups as constraints different from cyclization leads to a large increase in the selectivity for δ opioid binding site in the case of DSTBULET [Tyr-D-Ser-(OtBu)-Gly-Phe-Leu-Thr] ($K_{i\delta}$ = 6.14 nM; $K_{i\mu}$ = 374 nM) and BUBU [Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu)] ($K_{i\delta}$ = 4.68 nM; $K_{i\mu}$ = 475 nM) or a loss of affinity for DTTBULET [Tyr-D-Thr(OtBu)-Gly-Phe-Leu-Thr] ($K_{i\delta}$ = 866 nM; $K_{i\mu}$ = 4500 nM). This puzzling behavior is studied here by 400-MHz ^{1}H NMR spectroscopy in DMSO-d_6 solution and by theoretical calculations. When DSLET and DTLET are compared, the reduction in energetically accessible ϕ and ψ angles induced by the *tert*-butyl group in the D-Ser^2 residue decreases the degree of freedom in the N-terminal part of the peptides. For DSTBULET and BUBU, the rigidification of the backbone evidenced by the appearance of the large NOE's of Phe^4 NH–Gly^3 α and Gly^3 NH–α and by the loss of the C_7 folding around the D-Ser^2 residue found in DSLET could explain the drastic loss of affinity for μ opioid receptors. In DTTBULET, a large change in the spatial orientation around the D-Thr^2 (OtBu) residue forces the aromatic rings far from each other. Conformational analysis of these peptides by Metropolis calculations is in agreement with NMR analysis suggesting that the preferential g^- orientation of the Phe^4 side chain, yielding a short distance (≤10 Å) between the two aromatic rings, plays a crucial role in δ receptor selectivity. The most stable conformation of BUBU deduced from NMR is very similar to the conformation of δ-selective *cyclo*-[D-Pen^2,L-Pen^5]enkephalin and *cyclo*-[D-Pen^2,D-Pen^5]enkephalin, two severely constrained cyclic peptides. Moreover, the Tyr^1 and Phe^4 aromatic rings of BUBU can be easily superimposed on the corresponding rings in the rigid and selective δ-antagonist naltrindole.

Characterization of the biological and pharmacological responses associated with μ or δ opioid receptor stimulation requires the design of highly specific ligands for these binding sites (Hansen & Morgan, 1984; Roques, 1988).

The conformation of the enkephalins has been extensively studied by NMR spectroscopy [for reviews, see Schiller et al. (1984) and Khaled et al. (1985)], and these studies have shown that in solution the endogenous pentapeptides behave as highly flexible molecules able to assume various conformations of comparable low energy, allowing conformational adaptation to both μ and δ receptors. Sterically restricted linear or cyclic peptides, such as morphiceptin and Tyr-*c*-(N-γ-D-A_2Bu-Gly-Phe-Leu), respectively, which behave as μ-agonists (Chang et al., 1981; Schiller & DiMaio, 1982), represent convenient models to probe the bioactive conformation at the μ opioid receptor. Conformational studies by energy calculations have shown that these compounds present a few structures stabilized mainly by intramolecular H-bonds involved in β-turns eventually reinforced by a C_7 turn between the carbonyl of Tyr^1 and the NH of Gly^3 (Loew et al., 1986; Maigret et al., 1986). The occurrence of folded structures in μ-selective peptides is in agreement with the proposed bioactive conformation at the μ opioid receptor (Roques et al., 1976; Clarke et al., 1978; Loew et al., 1978; DiMaio & Schiller, 1980; Fournié-Zaluski et al., 1981; Castiglione et al., 1987; Schiller et al., 1987; Keys et al., 1988).

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Until recently, two series of δ-agonists were available, one belonging to linear hexapeptides such as DSLET [Tyr-D-Ser-Gly-Phe-Leu-Thr (Gacel et al., 1981)] and DTLET [Tyr-D-Thr-Gly-Phe-Leu-Thr (Zajac et al., 1983)] and the other one to the cyclic pentapeptides DPLPE [Tyr-*c*-(D-Pen-Gly-Phe-Pen) (Pen = β,β-dimethylcysteine)] and DPDPE [Tyr-*c*-(D-Pen-Gly-Phe-D-Pen) (Mosberg et al., 1983)], which exhibit a lesser affinity but a better δ-selectivity than the former (Cotton et al., 1985; Delay-Goyet et al., 1988). Recent studies by ^{1}H NMR spectroscopy and theoretical calculations have shown that, despite large differences in their intrinsic flexibility, DTLET and DPLPE presented large conformational similarities (Belleney et al., 1987; Keys et al., 1988). Thus, a close correspondence between the penicillamino groups of the cyclic peptides and the threonyl and leucyl side chains of DTLET was observed by both methods. Moreover, in both compounds, the folding tendency of the peptide backbone induces similar distances (about 10 Å) and orientations of the aromatic side chains of Tyr and Phe, under their preferential g^- orientations.

The higher δ-selectivity of DPLPE related to DTLET was tentatively explained by a stronger unfavorable steric hindrance to fit the μ-site in the cyclic peptide (Belleney et al., 1987). Indeed, owing to the folding tendency exhibited by their N-terminal part and the degree of freedom of their second residue, DSLET and DTLET are able to fit the conformational space occupied by a rigid and compact structure such as morphine (Maigret et al., 1981).

Therefore, introduction of various constraints different from cyclization in the structure of the linear hexapeptides DSLET and DTLET was proposed as an alternative means for obtaining highly potent and selective δ-ligands. Interestingly, etherification of the side chains of Ser and Thr residues by *tert*-butyl groups in position 2 and/or position 6 led to opposite effects. In the case of DSLET ($K_{i\delta}$ = 4.8 nM; $K_{i\mu}$ = 31 nM), this leads to an increase in δ-selectivity without significant loss of affinity in DSTBULET [Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr] ($K_{i\delta}$ = 6.14 nM; $K_{i\mu}$ = 374 nM) and especially in BUBU [Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu)] ($K_{i\delta}$ = 4.68 nM; $K_{i\mu}$ = 475 nM) (Gacel et al., 1988b). According to these binding properties and to their enhanced resistance toward peptidases, DSTBULET and BUBU behave as the most suitable δ-agonists for binding, autoradiographic, and pharmacological studies (Delay-Goyet et al., 1988).

Conversely, in the case of DTLET ($K_{i\delta}$ = 1.35 nM; $K_{i\mu}$ = 23.3 nM), introduction of the *tert*-butyl group in position 6 increases slightly the δ-selectivity while, when introduced in position 2 in DTTBULET [Tyr-D-Thr(OtBu)-Gly-Phe-Leu-Thr] ($K_{i\delta}$ = 866 nM; $K_{i\mu}$ = 4500 nM), this substituent induces a drastic loss of activity (Gacel et al., 1988b). In order to tentatively correlate these biological results with structural parameters, a conformational study by ^{1}H NMR spectroscopy in DMSO-d_6 and theoretical calculations were performed on these various linear peptides.

Experimental Procedures

Materials

The peptides were synthesized in the laboratory as described elsewhere (Gacel et al., 1988b), and their purity was checked by high-performance liquid chromatography (HPLC) (Waters apparatus) on a C_{18} μBondapak column with TEAP/CH_3CN systems as eluents. The retention times of the various peptides, at a flow rate 1.5 mL/min, were as follows: DSLET (TEAP/CH_3CN = 75/25), 14.4 min; DSTBULET (TEAP/CH_3CN = 75/25), 10.8 min; BUBU (TEAP/CH_3CN = 68/32), 9.4 min; DTTBULET (TEAP/CH_3CN = 75/25), 12.1 min.

Methods

NMR Studies. The NMR samples were prepared by dissolving the peptides in H_2O. The solutions were adjusted to pH 5.5 by HCl or NaOH and lyophilized. The dried peptides were redissolved in $(CD_3)_2SO$ at a concentration of 5×10^{-3} M. Fourier transform ^{1}H NMR spectra were run at 400 MHz on a Bruker AM 400 spectrometer equipped with a Bruker Aspect 3000 computer and a Bruker temperature controller (±1 °C); 16K data points were used for 1D spectra acquisition, and Fourier transformation was performed after zero filling to 32K data points with a final digital resolution of 0.2 Hz/point. To improve the spectra resolution, Gaussian multiplications were applied to the free induction decays. Chemical shifts were reported in ppm (±0.01 ppm) from hexamethyldisiloxane as internal reference, and the coupling constants were determined at ±0.2 Hz. The techniques used in these studies include two-dimensional proton-correlated spectroscopy (DQFCOSY) (Piantini et al., 1982) and Overhauser enhancement spectroscopy in rotation frame (ROESY) (Bax & Davis, 1985). In the case of ROESY the carrier frequency was positioned at 5 ppm, and a 4-kHz spin-lock field was used during the 200-ms mixing period. ROESY experiments were performed with the time-proportional phase increment (TPPI) method (Marion & Wüthrich, 1983), and the data were displayed in the phase-sensitive mode.

Conformational Calculations. The method used in previous conformational studies of linear peptides (Fourniê-Zaluski et al., 1986) was applied. A first sample of conformations is obtained according to the Metropolis method (Prêmilat & Maigret, 1977).

The force field of Scheraga and co-workers for peptides has been used to calculate the conformational energy (Momamy et al., 1979). A screening factor of 0.5 has been introduced on each ionic group to decrease their atomic charges in order to mimic crudely the solvent effect and to avoid dominance of folded structures.

The dihedral angles (ϕ, ψ) are used to generate conformations selected at random in the energetically allowed area of the well-known Ramachandran plots for the consecutive residues in the chain.

Several selected interatomic distances were calculated and stored during Metropolis calculations. Each conformer is thus represented by a point in the hyperspace defined by the set of these interatomic distances. This cloud of conformations was next separated into conformers of similar aspect according to clustering procedures. The most representative conformers in each of the different conformational classes thus obtained were finally refined with minimization procedures.

In the Metropolis sampling, 100 000 conformers were generated by 20 attempts of 5000 improvement steps each [see Fourniê-Zaluski et al. (1986) for details]. Only about 600 different conformations were found in the sample of each molecule of that series so that $100\,000 = \sum\omega_i$ ($1 \le i \le 600$), where ω_i represents the weight of each different conformation.

Among all the possible interatomic distances which should be used in further analysis of the conformational behavior of the molecules, three were found to have a strong noncorrelated discriminant effect. These distances are the N-terminal to C-terminal atomic distances, the aromatic rings' center to center distance, and the O^{γ_i}–O^{γ_i} distance between the oxygen atoms of the seryl and threonyl side chains. The resulting matrix (~600 conformers described by the three above distances) was next used in cluster analysis to separate the

Table I: Chemical Shifts,[a] Temperature Dependencies,[b] and $^3J_{NH-H\alpha}$ Coupling Constants[c] for DSLET (**1**), DSTBULET (**2**), BUBU (**3**), DTLET (**4**), and DTTBULET (**5**) in DMSO-d_6 at 297 K (Bis-Ionic Forms)

		δ_α	δ_β	δ_{NH}	δ_{other}[d]	$^3J_{NH-H\alpha}$
Tyr[1]	**1**	3.76	2.86/2.63		Ar, 6.98/6.61; OH, 9.20	
	2	3.74	2.83/2.64		Ar, 6.98/6.60; OH, 9.19	
	3	3.55	2.80/2.55		Ar, 6.97/6.59; OH, 9.17	
	4	3.70	2.85/2.55		Ar, 6.97/6.60; OH, 9.16	
	5	3.71	2.83/2.60		Ar, 6.98/6.61; OH, 9.18	
D-Ser[2]	**1**	4.14	3.53/3.43	8.53 (−10)	OH, 4.9	7
	2	4.22	3.39[e]	8.45 (−7.3)	tBu, 1.04	6.5
	3	4.22	3.38[e]	8.38 (−10)	tBu, 1.04	7
D-Thr[2]	**4**	4.00	3.94	8.26 (−5.0)	OH, 4.9; Me, 0.86	5.5
	5	4.11	3.73	8.20 (−7.5)	tBu, 1.05; Me, 0.76	8.0
Gly[3]	**1**	3.73/3.56		8.22 (−3.6)		5/6.5
	2	3.77/3.55		8.15 (−5.0)		4/7
	3	3.73/3.57		8.20 (−7.0)		4/7
	4	3.71/3.60		8.08 (−3.4)		5.5/6.5
	5	3.68/3.61		7.81 (−5.0)		5/5.5
Phe[4]	**1**	4.38	3.00/2.76	8.00 (−5.0)	Ar, 7.19	8
	2	4.39	3.00/2.76	8.06 (−5.0)	Ar, 7.20	8.5
	3	4.41	3.00/2.80	7.94 (−5.0)	Ar, 7.20	8
	4	4.41	3.03/2.76	8.04 (−4.2)	Ar, 7.19	8.5
	5	4.44	3.00/2.72	7.98 (−5.0)	Ar, 7.19	8.5
Leu[5]	**1**	4.11	1.48/1.44	8.31 (−7.0)	γ_H, 1.47; Me, 0.82/0.75	8
	2	4.10	1.48/1.44	8.36 (−6.0)	γ_H, 1.47; Me, 0.81/0.75	8
	3	4.20	1.51/1.49	8.26 (−7.0)	γ_H, 1.50; Me, 0.84/0.77	8.5
	4	4.14	~1.48	8.30 (−5.5)	γ_H, 1.48; Me, 0.82/0.75	8.5
	5	4.18	1.48/1.44	8.28 (−8.0)	γ_H, 1.54; Me, 0.83/0.77	8
Thr[6]	**1**	3.87	3.92	7.23 (−0.8)	OH, 4.9; Me, 0.88	7
	2	3.83	3.89	7.20 (−1.0)	Me, 0.87	6
	3	3.98	3.98	7.05 (−1.8)	tBu, 1.01; Me, 0.96	8.5
	4	3.86	3.92	7.24 (−0.6)	OH, 4.9; Me, 0.88	7
	5	3.90	3.94	7.32 (−3.0)	Me, 0.90	7.5

[a] δ given in ppm (±0.01 ppm) from HMDS used as internal reference. [b] The NH temperature dependencies in ppm/°C × 10^3 are given in parentheses. [c] The coupling constants are given in Hz (±0.25 Hz). [d] Ar and Me correspond to aromatic and methyl protons, respectively. [e] The side-chain protons for Ser[2] are degenerate.

conformational space into families presenting similarities in the set of selected distances. The clustering algorithm used here was the MICKA algorithm (Marchionini et al., 1983).

The calculations were performed on a CRAY 1 computer and the structures visualized on a PS 390 using the software SYBYL/MENDYL from Tripos Associates.

RESULTS

^{1}H NMR Studies

The conformational parameters of the various hexapeptides (DSLET, DSTBULET, BUBU, DTLET, and DTTBULET), under their bis-ionic forms, were determined by ^{1}H NMR spectroscopy in $(CD_3)_2SO$ at 297 K. Sequential connectivities between successive residues provide individual assignments of spin system. This is achieved through a combined use of COSY and ROESY spectra of the molecules recorded under identical conditions. Figure 1 shows the COSY–ROESY connectivity diagram of BUBU. The ROESY spectrum indicates through-space NOE connectivities, and the COSY spectrum indicates through-bond ^3J connectivities.

Conformational Parameters of the Peptide Backbones. The proton chemical shifts and NH temperature dependencies are reported in Table I and the NOE effects in Figure 2 for the five linear hexapeptides.

The comparison of the proton chemical shifts indicates large similarities between DSLET and DSTBULET. Nevertheless, the introduction of the *tert*-butyl group in D-Ser[2] induces small modifications in the NMR parameters of this residue, i.e., a deshielding of 0.08 ppm on the α proton, a small shielding associated with a degeneration of the β protons, and an upfield shift of the amide NH (−0.08 ppm). The modification of the

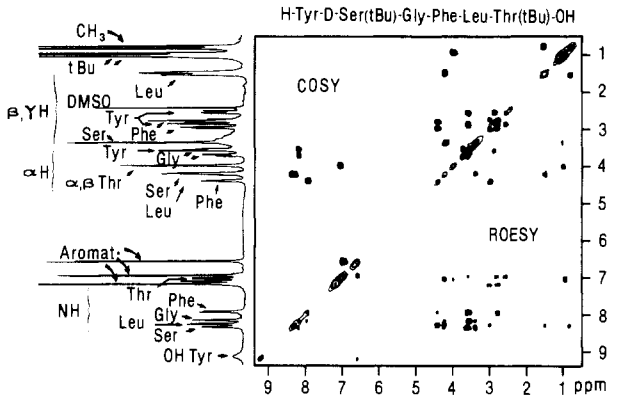

FIGURE 1: ^{1}H 400-MHz 2D COSY and ROESY spectra of BUBU (**3**) in DMSO-d_6 solution at 297 K.

D-Ser[2] residue is also responsible for an increase in the temperature dependency of the Gly[3] NH. Large values for this parameter (>−4 × 10^{-3} ppm/°C) are indicative of exposure to and exchange with solvent protons while small values (<2 × 10^{-3} ppm/°C) suggest inaccessibility to the solvent or participation in intramolecular hydrogen bonding (Wüthrich, 1976). For all the other residues, the differences in the chemical shifts are lower than 0.05 ppm, and the NH temperature dependencies are almost identical in DSLET and DSTBULET. Thus, in both peptides, the Thr[6] NH is buried from the solvent with slopes $\Delta\delta/\Delta T = -0.8 \times 10^{-3}$ ppm/°C in DSLET and -1.0×10^{-3} ppm/°C in DSTBULET.

As compared to the case of DSTBULET, the introduction of the second *tert*-butyl group on Thr[6] in BUBU leads to a deshielding of 0.15 and 0.09 ppm for the α and β protons of

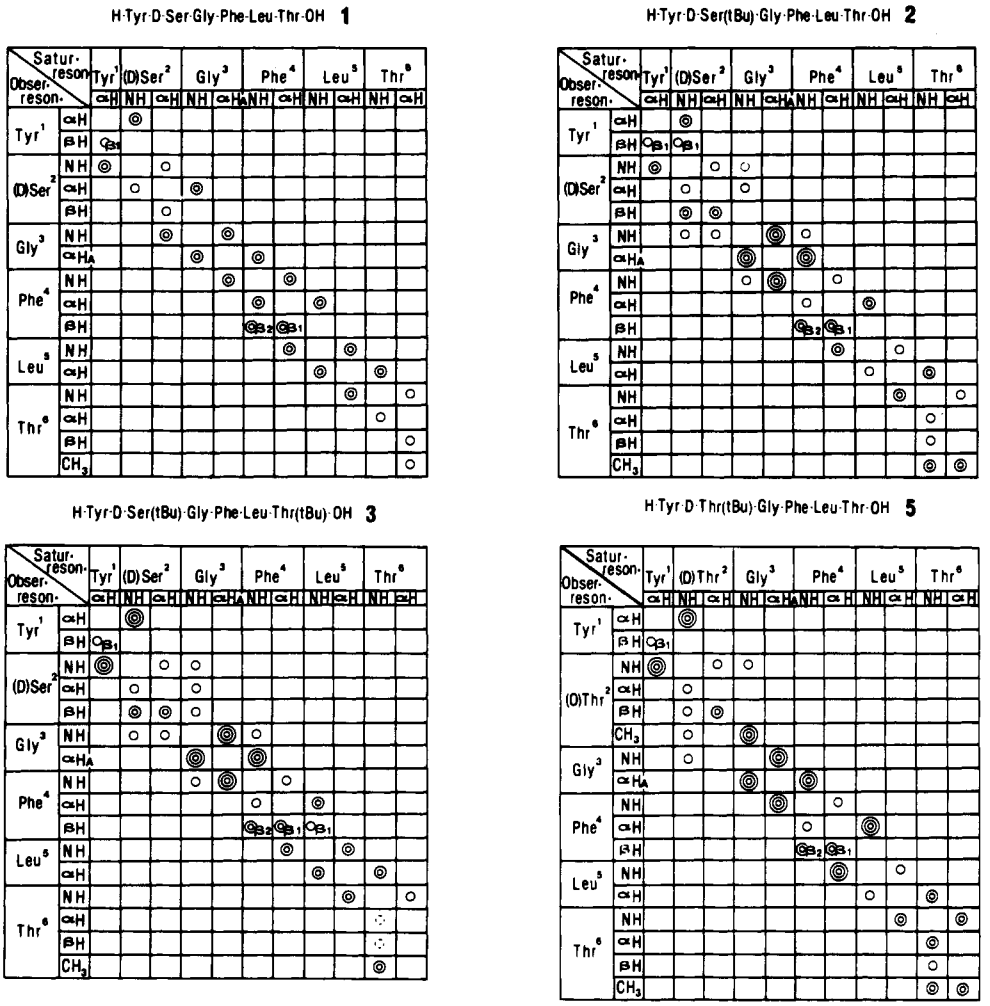

FIGURE 2: Nuclear Overhauser enhancements measured by ROESY experiments in DSLET (**1**), DSTBULET (**2**), BUBU (**3**), and DTTBULET (**5**). β_1 and β_2 refer to the lower and higher field β protons, respectively. The signals are calibrated by referring to the NOE between the ortho and meta aromatic protons of tyrosine. NOE: (circle) <5%; (two concentric circles) <15%; (three concentric circles) >16%.

this residue, associated with an upfield shift of the amide group (−0.15 ppm). However, the temperature coefficient of Thr^6 NH (-1.8×10^{-3} ppm/°C) remains in the range of the solvent-buried amide proton. Two opposite effects are measured on the vicinal Leu^5 protons, i.e., a deshielding (0.14 ppm) of the α proton an a shielding (−0.10 ppm) of the amide group. Interestingly, the N-terminal tyrosine residue is also influenced by the Thr^6(OtBu) residue as shown by the large upfield shift of the α proton (−0.19 ppm) and the higher field for both β protons.

The observation of ROESY cross-peaks which reflect the spatial disposition of various hydrogen atoms can provide insight into the three-dimensional structure of the molecules in solution. Figure 2 shows the NOE effects between the various protons for DSLET, DSTBULET, BUBU, and DTTBULET. The ortho and meta protons of Tyr^1 are fixed at a short distance (~2.5 Å), yielding strong NOE enhancements which were found similar in all the studied peptides. This spin system was therefore used to calibrate the magnitude of NOE's in the different compounds. For DSTBULET and BUBU the appearance of large NOE's (>20%) occurring between Gly^3 α–NH and between Gly^3 α–Phe^4 NH and a small but significant NOE between D-Ser^2 NH–Gly^3 NH indicating the occurence of a turn reflects a more constrained backbone conformation as compared to that of DSLET.

The comparison of the proton chemical shifts of DSLET and DTLET shows only minor differences for the common residues. An upfield shift for the α and β protons of Tyr^1 and a smaller inequivalence of the Gly^3 CH_2 protons was observed in DTLET as compared to those in DSLET. The amide proton temperature dependency reinforces these analogies, since in both peptides the smallest coefficients occur for the C-terminal residue ($\Delta\delta/\Delta T = -0.8 \times 10^{-3}$ ppm/°C in DSLET and −0.6 $\times 10^{-3}$ ppm/°C in DTLET) and for the Gly^3 NH ($\Delta\delta/\Delta T = -3.6 \times 10^{-3}$ ppm/°C in DSLET and -3.4×10^{-3} ppm/°C in DTLET).

Introduction of a bulky *tert*-butyl group on the hydroxyl of the threonyl-2 residue in DTLET induces significant differences at the level of the D-Thr^2 and Gly^3 residues. Indeed, in DTTBULET the α and β protons of the substituted threonine are downfield and upfield shifted, respectively (αH, 0.11 ppm; βH, −0.21 ppm), and the Gly^3 NH is shielded by 0.27 ppm. In addition, the difference in the chemical shift of Gly^3 CH_2 protons is lowered with respect to all other peptides. The variation in amide proton temperature dependency shows that the smallest coefficient occurs again for the C-terminal Thr^6 residue ($\Delta\delta/\Delta T = -3 \times 10^{-3}$ ppm/°C). However, this temperature slope is clearly larger than the corresponding value in DTLET (-0.6×10^{-3} ppm/°C), indicating that the Thr^6 NH in DTTBULET is only slightly buried from the solvent. Likewise, by comparison to the Gly^3 NH in DTLET, the temperature dependency of the glycine exchangeable proton in DTTBULET ($\Delta\delta/\Delta T = -5 \times 10^{-3}$ ppm/°C) corresponds to a well-exposed NH group. Compared to DTLET (Belleney et al., 1987), it is interesting to note an

Table II: $^3J_{\alpha-\beta}$ Coupling Constants[a] and Derived Rotamer Populations for DSLET (**1**), DSTBULET (**2**), BUBU (**3**), DTLET (**4**), and DTTBULET (**5**) in DMSO-d_6 at 297 K (Bis-Ionic Forms)[b]

	Tyr[1]				D-Ser[2], D-Thr[2]				Phe[4]				Thr[6]			
	^3J	g^-	t	g^+	^3J	g^-	t	g^+	^3J	g^-	t	g^+	^3J	g^-	t	g^+
1	5.5/8	49	26	25	5/6	30	22	47	4.5/10	67	18	15	4	13	87	
2	5/8	49	22	29	6[c]				4/10	67	13	20	3.5	8	92	
3	4.5/8.5	53	17	30	5.5[c]				3.5/9	58	8	34	*d*			
4	5.5/8.5	53	26	21	3.3		94	6	4.5/10	67	17	16	3.5	8	92	
5	6.2/8	49	33	18	3.5		92	8	4/10	67	13	20	3.3	6	94	

[a] The coupling constants are given in Hz (±0.25 Hz). [b] Percentage of each conformer was determined following Pachler (1964). [c] Degenerate system. [d] Overlapping system.

increase in the intensity of NOE effects for Leu[5] α–NH, Gly[3] α–NH, and Tyr[1] α–Thr[2] NH. Moreover, in opposition to DTLET, a strong NOE (~25%) is observed in DTTBULET between the CH_3 of the threonyl-2 residue and the Gly[3] NH.

Side-Chain Orientations. The $^3J_{\alpha-\beta}$ coupling constants allow determination of the preferential orientation of the side chains in the studied compounds (Table II). The population of the three staggered conformations was calculated with Pachler's parameters (Pachler, 1964). The proportions of the three staggered conformers for Phe and Tyr residues were calculated by assuming that the relative positions of *β-pro-R* and *β-pro-S* protons in the spectra corresponded to those determined in small Phe- and Tyr-containing peptides and in Met-enkephalin. In these compounds the lower field and higher field components of β proton signals were unambiguously assigned to the *pro-S* and *pro-R* protons, respectively, by use of selectively deuterated residues (Kobayashi et al., 1979, 1980) or simultaneous use of ^1H, ^{13}C, and ^{15}N NMR (Garbay et al., 1982). In all the peptides, there is a large predominance of the g^- conformer (about 50%) for the tyrosine residue. The phenylalanine side chain is also preferentially oriented in the g^- position in all peptides, a result confirmed by the consistently observed intraresidue Phe α–β_1 and Phe NH–β_2 NOE's.

The population of the three conformers of D-Ser[2] can be determined only for DSLET in which a slight predominance of g^+ population is observed. For DSTBULET and BUBU, the degeneracy of the β proton shifts precludes this determination. For the threonine moiety, the presence of the unique coupling constant $^3J_{\alpha-\beta}$, when this two-spin system is not degenerate, allows only the determination of the g^- population for L-threonine and the g^+ population for D-threonine, which are in all cases very low. However the observation in DSLET, DSTBULET, and DTTBULET of large NOE effects for Thr[6] α–CH_3 and Thr[6] NH–CH_3 may be related to the predominance of the g^+ population. Conversely, in BUBU the absence of NOE Thr[6] α–CH_3 probably reflects a preferential t orientation.

In DTLET the NOE's observed in the two threonine residues between the CH_3 group and the α proton seem to indicate a preferential g^- conformer for D-Thr[2] and a g^+ conformer for L-Thr[6] side chains. In contrast to DTLET, no NOE was observed between Hα and CH_3 in the D-threonyl-2 residue of DTTBULET, indicating a predominance of t population for this side chain.

Conformational Calculations

A preliminary statistical analysis of the Metropolis samples obtained for each molecule shows that all the studied peptides have roughly the same conformational behavior. Due to ionization of the N- and C-terminal amino acids considered in the calculations, these molecules appear as folded compact structures stabilized by electrostatic interactions between the N- and C-terminal residues (Table III). Nevertheless, conformational differences due to the various sequences seem to occur. Indeed, the histograms related to interatomic distance distributions show the occurrence of extended and compact conformers with a decreasing order of probability from DSLET to DSTBULET for the former. Similarly, the distribution obtained for the distance between the Tyr and Phe aromatic rings shows that, in addition to conformers in which both side chains are far away (12–13 Å), depending on the sequence other possibilities exist for finding short distances (<10 Å) between rings. The same pattern is found for the distribution of distances between the oxygen atoms of alcohol side chains of the second and last residues (Table III).

A clustering analysis performed on all the samples gives more precise information concerning the relative conformational possibilities given to the different peptides. The same partition of the conformational space can be roughly retained for all compounds. The differences arise from the relative weights of conformers. Thus, three fundamental classes characterized by the conformational characteristics of residues 2 and 3 on one side and of residues 4 and 5 on the other side were obtained (Table IV). These classes can be associated with the existence of particular H-bonded conformations, mainly β turns or C_7 types. Differences in the conformational

Table III: Mean Distances (Å) Observed from the Sampling Procedure

compd	Tyr[1]···Phe[4] (aromatic side chains)	O···O[a]	N^+···CO^-
DSLET (**1**)	11.8	9.8	4.6
DSTBULET (**2**)	12.1	10.7	4.6
BUBU (**3**)	12.4	9.5	5.6
DTLET (**4**)	13.0	10.6	5.3
DTTBULET (**5**)	11.2	9.4	6.6

[a] Distances between alcohol oxygens of the side chains of the second and last residue.

Table IV: Residue Dihedral Angles (deg) of Some of the Most Representative Computed Conformations of the Studied Hexapeptides

		ϕ	ψ		χ^1	χ^2
conformation A	Tyr[1]		−174		−138	−118
	D-X[2]	70	−100	C_7	176	
	Gly[3]	−55	−33			
	Phe[4]	−146	103		−150	59
	Leu[5]	−62	84	C_7	−63	−146
	Thr[6]	−153	158		53	
conformation B	Tyr[1]		154		−178	−107
	D-X[2]	73	−90	C_7	178	
	Gly[3]	−82	−87			
	Phe[4]	−73	132	β_{II}	163	−93
	Leu[5]	41	59		−173	110
	Thr[6]	−47	140		−49	
conformation C	Tyr[1]		155		177	−106
	D-X[2]	68	22		−170	
	Gly[3]	−95	−45			
	Phe[4]	−75	134		−55	109
	Leu[5]	−83	64	C_7	−51	−162
	Thr[6]	−145	171		74	

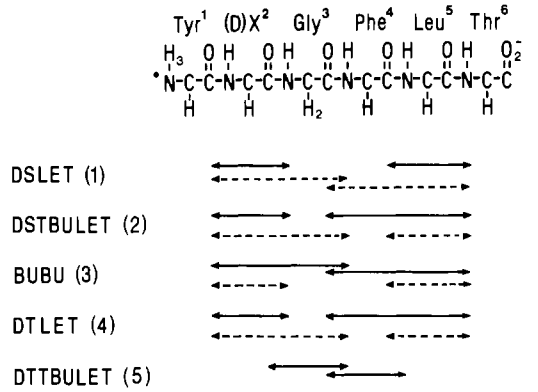

FIGURE 3: Schematic representation of the various turns (solid and broken arrows) observed in the two most stable conformations of DSLET (**1**), DSTBULET (**2**), BUBU (**3**), DTLET (**4**), and DTTBULET (**5**) obtained by Metropolis calculations (conformations indicated by solid arrows are more stabilized than those indicated by broken arrows).

behavior of the studied peptides appear clearly from this analysis. The amount of conformer presenting the type A organization between residues 2 and 3 (C_7 turn) decreases from 43% of the whole sample in DSLET to 17% in DSTBULET. Conformers presenting type B organization between residues 4 and 5 (β_{II} turn) are more important for BUBU compared to other sequences. A schematic representation of the H bonded forms is shown in Figure 3.

Another way to analyze these data is to cluster the conformational space of each compound into similar families. The most highly populated classes obtained for each residue in each compound are shown on the ϕ, ψ maps of Figure 4. Very interestingly, for the Gly³ residue, the corresponding, ϕ, ψ map shows differences between the active and inactive peptides. Indeed, the highest peaks are centered around $\phi = -80°$, $\psi = -60°$ for the biologically active δ-selective peptides DSLET, DSTBULET, BUBU, and DTLET, while these maxima are found in two quadrants, $\phi = -130°$, $\psi = 40°$ and $\phi = 100°$, $\psi = -30°$, for the inactive DTTBULET. Concerning the D-X² residue, the most stabilized conformers are located in two quadrants with positive and negative ψ values for DSLET and DTLET. In the higher δ-selective compounds DSTBULET and BUBU, if the limits for ϕ values are in the same area as those occurring in DSLET, the ψ range is restricted to negative values. For all the other residues, ϕ, ψ maps show only minor differences.

Finally, the conformations selected as best representing each conformational class have been refined with usual energy minimization procedures. Residue Leu⁵ appears from these calculations as very flexible because all the energy minima corresponding to different conformations of this amino acid are very close to each other with an energy range of 3 kcal/mol above the deepest minimum of each set of calculations. The influence of sequence modifications appears in the relative energy values of these Leu⁵ conformers, the best one being a β_{II} type for DTLET and C_7 type for DSLET, DSTBULET, and BUBU. Some of the most representative minima are illustrated in Table IV.

Discussion

In this study the ^{1}H NMR spectra of the studied compounds have been recorded in DMSO-d_6 solution. The choice of DMSO as solvent was imposed by the low water solubility of DSTBULET and BUBU under their zwitterionic forms, which correspond to the ionic species present in physiological conditions. Moreover, due to its higher viscosity than H_2O, the use of DMSO allowed us to carry out NOE experiments of great utility in evaluating conformationally dependent proton–proton distances in these small peptides. In this line, it

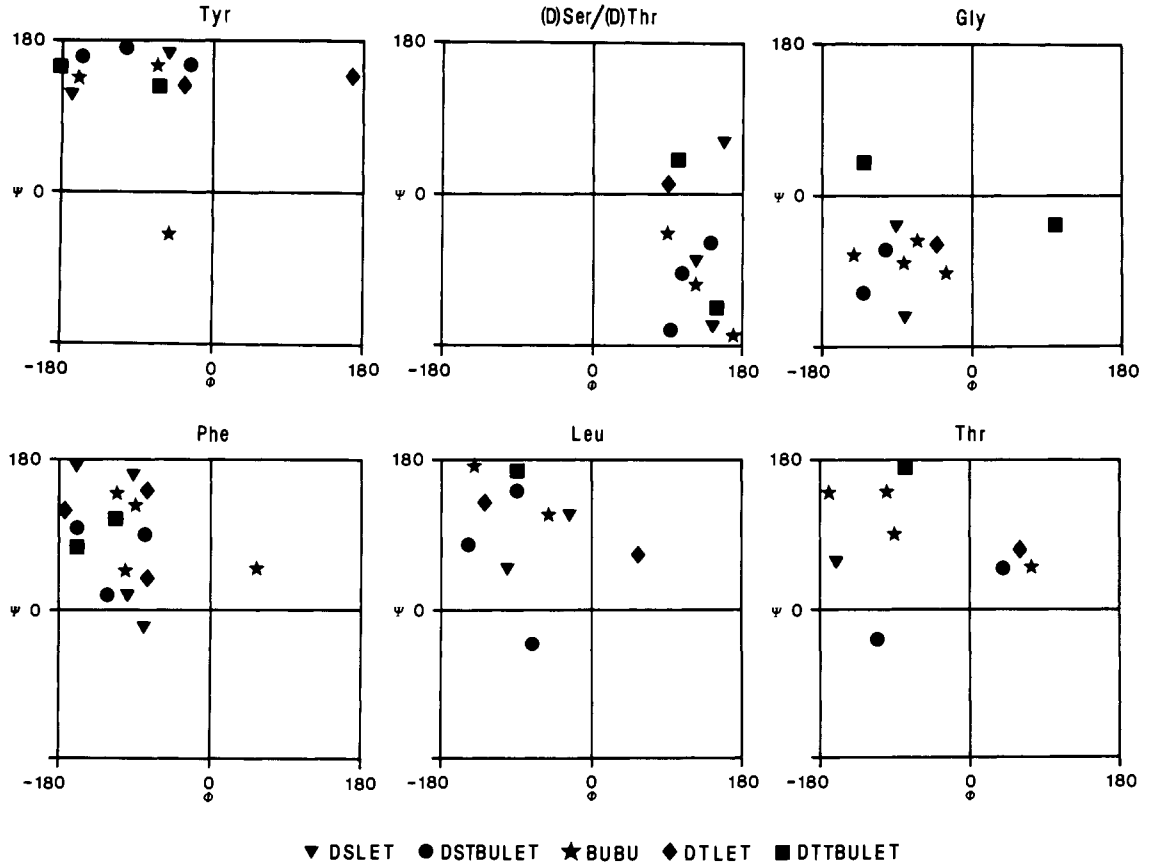

FIGURE 4: ϕ, ψ maps for all the residues in DSLET (**1**), DSTBULET (**2**), BUBU (**3**), DTLET (**4**), and DTTBULET (**5**).

can be observed that the viscosity and hydrophobicity of DMSO are probably closer to the situation encountered by peptides in the opioid receptor environment (Hashin, 1978). On the other hand, this study is focused on comparing the conformational behavior of a homogeneous series of peptides to their biological properties, thus minimizing the proper role of the solvent. Finally, the structures of the peptides which exhibit highly energetically favored conformations such as CCK_8 (Fourniē-Zaluski et al., 1986) or which are endowed with severe conformational constraints such as [Tyr-*c*-(D-A_2bu-Gly-Phe-Leu)] (Mammy et al., 1985) were found very similar in both DMSO-d_6 and H_2O/D_2O solutions (see below).

The ^{1}H NMR data obtained for DSLET and DTLET support a close correspondence between the conformational behavior of both peptides. Indeed, the very low temperature coefficient of Thr^6 NH in both compounds suggests that this amide proton adopts a solvent-buried orientation probably associated with a folding tendency of the C-terminal part of these peptides. For DTLET, the presence of the NOE's Phe α–Leu NH, Leu NH–Thr NH, and Leu α–Thr NH has been previously interpreted (Belleney et al., 1987) by the occurrence of a β turn of type II, around Phe^4-Leu^5 residues. For DSLET the observation of weaker sequential NOE's (6%–15%) seems to indicate a larger flexibility of the backbone, very likely related to an equilibrium between various folded conformations. Nevertheless, in spite of the absence of a clear dipolar effect between the amide protons Leu^5 NH and Thr^6 NH, the relatively high values of the NOE's of Phe^4 α–Leu^5 NH and Leu^5 α–Thr^6 NH in both peptides are in favor of a preferential conformation with a β turn of type II, although the possibility of a C_7 equatorial conformer may be also considered.

In the N-terminal part of both peptides, the intermediate slope of the temperature dependency encountered for Gly NH led to the proposal of a folding of the backbone around the D-residue in position 2. In DSLET, as in DTLET, the occurrence of a relatively large NOE, D-X^2 α–Gly^3 NH, and of a low intraresidue dipolar effect, D-X^2 α–NH, may reflect the existence of a C_7 turn ($\phi = 80°$, $\psi = -80°$). The different folded forms proposed for the C- and N-terminal parts from NMR studies are in good agreement with Metropolis calculations. Indeed, for both peptides the folding of the backbone around the D-X^2 residue suggests the existence of a C_7 turn, and for the C terminal part the two approaches converge to the same conformational pattern, i.e., a β turn for DTLET and a C_7 turn for DSLET.

Furthermore, as previously outlined for DTLET (Belleney et al., 1987), the Gly^3 residue in DSLET seems to play the role of a hinge between the two folded parts of the backbone illustrated by the large inequivalence of the Gly^3 methylene protons. The significant intra- and interresidue NOE's Gly^3 NH–D-X^2 α, Gly^3 NH–α, and Phe^4 NH–Gly^3 α, in conjunction with J coupling constants, allow us to propose the following dihedral angles: ψ (D-X^2) $\sim -80°$ and ψ (Gly^3) = $\pm 60°$ to $\pm 120°$. On the other hand, the orientation of the N-terminal tyrosine related to the backbone seems to be identical with that proposed to DTLET: the intraresidue Tyr β(*pro-S*)–α and the interresidue Tyr^1 α–D-Ser^2 NH NOE's are in favor of a ψ angle of about $-150°$ for this amino acid. We note that these NOE effects were observed for bis-ionic forms of peptides while a lack of such effect was reported for cationic forms (Dhingra & Saran, 1987). For both peptides, the $^3J_{\alpha-\beta}$ coupling constants support a preferential g^- orientation for the aromatic side chains. The large preference ($\sim$67%) of the g^- conformer of Phe minimizes the spatial interactions with Leu^5 and Thr^6 side chains. In this situation, a distance around 10 Å is observed between the aromatic rings. Finally, in DSLET as in DTLET, the backbone folding induces a proximity between the side chains of the hydrophilic amino acids in positions 2 and 6.

The introduction of a *tert*-butyl group on D-Ser^2 in DSTBULET reduces the energetically accessible ϕ, ψ angles of this amino acid. A rotation of the N-terminus around the ϕ and ψ angles of the D-Ser^2(OtBu) residue in the ranges 60° to 90° and $-80°$ to $+20°$, respectively, may be proposed according to the increased temperature coefficient of the glycine amide proton, the appearance of a small but significant inter-NH NOE D-Ser^2(OtBu)–Gly^3, and the decreased NOE Gly^3 NH–D-Ser^2(OtBu) α. Moreover, the rigidification of the backbone around the Gly residue evidenced by the occurrence of the very large NOE's Phe^4 NH–Gly^3 α and Gly^3 NH–α is in agreement with energy calculations showing a statistical decrease of the C_7 turn around the χ^2 residue from 43% in DSLET to 17% in DSTBULET.

This movement around the D-Ser^2 residue leads to a short distance between the two aromatic rings. The C-terminal folded part of DSTBULET was not significantly perturbed by the introduction of the *tert*-butyl group, although the large NOE Thr^6 NH–Leu α and the small intraresidue NOE Leu NH–α are in full accordance with a preferential C_7 equatorial folding around the leucine residue.

BUBU does not present very large conformational modifications relative to DSTBULET. In spite of a small increase in the solvent accessibility of Thr^6 NH, a folded C-terminal backbone is also observed around the Leu residue. Nevertheless, in order to minimize the steric hindrance between the bulky side chains D-Ser^2(OtBu) and Thr^6(OtBu), the threonyl side chain occurs preferentially in the t conformation. The ϕ, ψ maps for the D-Ser^2 residue in the various peptides (Figure 4) illustrate the loss of flexibility induced by the bulky *tert*-butyl group. Thus, in DSLET the ψ values are both positive and negative, while in DSTBULET and BUBU they are only found in the negative part of the map.

Introduction of the *tert*-butyl group in DTTBULET leads to an increase in the ^3J NH–α of the D-Thr^2(OtBu) residue as compared to that found in DTLET. The most important change is observed at the level of the Thr^2(OtBu) residue, with large NOE Thr^2(OtBu) CH_3–Gly^3 NH, small NOE Thr^2(OtBu) NH–CH_3, and no NOE Thr^2(OtBu) α–CH_3 suggesting a different spatial orientation of the side chain relative to DTLET with a larger population of t conformer around the Cα–Cβ bond of this sterically hindered residue. The strong NOE between the CH_3 group of the threonyl side chain and the NH of the adjacent Gly residue implies a close proximity between these groups. Accordingly, it is possible to propose a ψ value in the range of 60°–120° for the Thr^2(OtBu) residue in DTTBULET. As compared to Ser^2(OtBu) in DSTBULET and Thr^6(OtBu) in BUBU, the large shielding of the β proton and the methyl group observed in Thr^2(OtBu) are probably related to a steric hindrance between the bulky *tert*-butyl group and the peptide backbone in DTTBULET. This is supported by the 0.27-ppm shielding of the vicinal NH Gly^3 proton and the theoretical dihedral angles ϕ and ψ, which are very different for Gly^3 in DTLET and DTTBULET (Figure 4). In addition, the NOE occurring between the amide groups of Leu^5 and Thr^6 in DTLET was not observed in the substituted analogue.

The C conformer (Table IV), which is consistent with most NMR results, was selected as the most representative structure of the biologically active δ-selective peptides DSTBULET and BUBU. Figure 5 shows a stereospecific representation of the

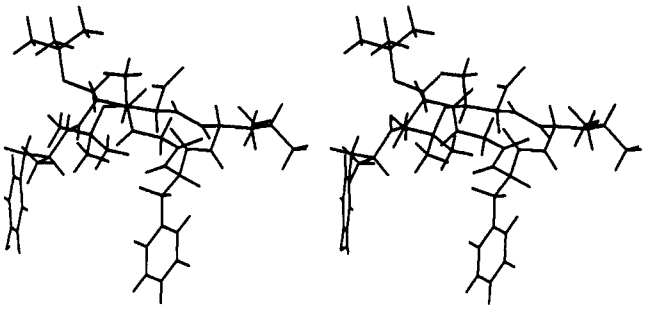

FIGURE 5: Stereo structure of BUBU under conformation C (Table IV).

three-dimensional structures of BUBU under conformation C. This structure is endowed with several interesting characteristics. Thus the amide protons of the successive amino acids D-Ser^2(OtBu), Gly^3, and Phe^4 are oriented toward the aromatic rings of Tyr^1 and Phe^4 and therefore somewhat buried from the solvent. As already noted for DTLET (Fournié-Zaluski et al., 1981; Belleney et al., 1987), the glycine residue plays a hinge role and directs the Tyr-Ser(OtBu) and the Phe-Leu-Thr moieties in different spatial orientations. This probably accounts, in biologically active peptides, for the large nonequivalence of the two Gly^3 α protons, which are located in different environments with respect to both vicinal carbonyl groups (Hruby et al., 1988). This conformational feature leads to a proximity (<10 Å) between the aromatic rings of Tyr^1 and Phe^4 ocurring preferentially under g^- conformations, while the hydrophobic side chains of D-Ser^2(OtBu), Leu^4, and Thr^6(OtBu) residues are located on the opposite side of the peptide.

Very interestingly, this conformation is close to the low-energy conformation of DPDPE proposed by Hruby et al. (1988) from conformational calculations and NMR studies performed in aqueous medium with this peptide in its positively charged form. This is clearly illustrated by the reported dihedral angles of DPDPE (Tyr^1, ψ = 164°; D-Pen^2, ϕ = 111°, ψ = 14°; Gly^3, ϕ = −98°, ψ = −18°; Phe^4, ϕ = −72°, which are very similar to those reported in Table IV for the C conformer of BUBU. A candidate conformer for high-affinity binding of both linear hexapeptides studied here and the cyclic penicillamino-containing peptides has been recently proposed (Keys et al., 1988). This conformer presents some differences with the structure reported in Figure 5, probably due to the more important role played by intramolecular H-bonds in the computed structure. Nevertheless, the most important characteristics for δ-selectivity (proximity between Tyr and Phe rings, g^- orientation of Phe, and opposite spatial localizations of the Tyr^1 and Phe^4 rings with respect to the various amino acid side chains) are conserved. The peculiar conformational behavior of DTTBULET, as given by the computed ϕ, ψ angle values of Gly^3, is very different from those obtained for active peptides and inconsistent with a distance of about 10 Å between the two aromatic rings; this accounts probably for the very weak affinity for δ receptors exhibited by this peptide (Delay-Goyet et al., 1988).

All the results of this study suggest that the drastic loss of μ receptor affinity relative to that of DSLET, exhibited by the recently designed highly δ-selective peptides DSTBULET and BUBU, is a direct consequence of the conformational restriction introduced into these peptides by the bulky *tert*-butyl group in the Ser^2 residue. A similar result was observed in cyclic enkephalins since a D-Pen^2 residue is more favorable for δ-selectivity than a D-Cys^2 one (Mosberg & Schiller, 1984). The inequivalence of Gly^3 CH_2 protons is considerably larger

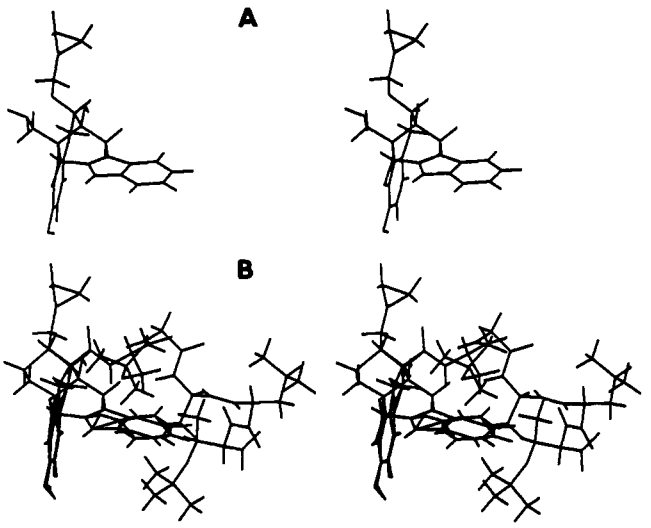

FIGURE 6: Stereoview of the δ antagonist naltrindole, NTI (A), and relative orientation of BUBU with NTI (B) with maximum overlap.

in DPDPE as in DSTBULET and BUBU, suggesting for this amino acid a stronger restricted degree of freedom around the ϕ and ψ angles in the cyclic peptide. According to the well-accepted zipper mechanism for peptide receptor recognition, this decreased flexibility could explain the significantly lower affinity for opioid δ receptor of DPDPE vs DSTBULET or BUBU (Delay-Goyet et al., 1987).

Furthermore, the δ-affinity displayed by DSTBULET and BUBU confirms the importance of the phenyl side chain previously proposed as a crucial component for δ receptor interaction (Roques et al., 1979; Fournié-Zaluski et al., 1981) and more precisely of its spatial proximity to the Tyr residue (Belleney et al., 1987). These proposals were recently reinforced by the synthesis of a new series of highly potent and selective nonpeptide δ opioid antagonists such as naltrindole (NTI) (Portoghese et al., 1988). These compounds are obtained by introduction of an additional aromatic ring to naltrexone. Due to the rather rigid structure of NTI, the two aromatic components of this antagonist can adopt a limited number of conformations. Interestingly enough, minor changes in the ϕ, ψ, and χ values of Phe^4 proposed for conformation C in DSTBULET and BUBU (Table IV) allow a good overlap of the corresponding aromatic rings in both structures (Figure 6).

These results suggest that conformational changes of the phenyl ring in the appropriate binding subsite could be one of the most important factors involved in agonist-induced δ receptor transduction process.

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Registry No. DSLET, 75644-90-5; DTLET, 85286-38-0; DSTBULET, 111035-56-4; BUBU, 114414-60-7; DTTBULET, 114442-55-6.

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