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Synthesis, Conformation and Biological Activity of Dermorphin and Deltorphin I Analogues Containing *N*-Alkylglycine in Place of Residues in Position 1, 3, 5 and 6

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Abstract: Syntheses are described of new dermorphin and [D-Ala^2]deltorphin I analogues in which the phenylalanine, the tyrosine or the valine residues have been substituted by the corresponding *N*-alkylglycine residues. Structural investigations by CD measurements in different solvents and preliminary pharmacological experiments were carried out on the resulting peptide–peptoid hybrids. The contribution from aromatic side chain residues is prominent in the CD spectra of dermorphin analogues and the assignment of a prevailing secondary structure could be questionable. In the CD spectra of deltorphin analogues the aromatic contribution is lower and the dichroic curves indicate the predominance of random conformer populations. The disappearance of the aromatic contribution in the [Ntyr^1,D-Ala^2]-deltorphin spectrum could be explained in terms of high conformational freedom of the *N*-terminal residue. The kinetics of degradation of the synthetic peptoids digestion by rat and human plasma enzymes were compared with that of [Leu^5]-enkephalin. The binding to opioid receptors was tested on crude membrane preparations from CHO cells stably transfected with the μ- and δ-opioid receptors. The biological potency of peptoids was compared with that of dermorphin in GPI preparations and with that of deltorphin I in MVD preparations. All the substitutions produced a dramatic decrease in the affinity of the peptide–peptoid hybrids for both the μ- and δ-opioid receptors. Nval^5 and/or Nval^6 containing hybrids behaved as μ-opioid receptor agonists and elicit a dose-dependent analgesia (tail-flick test) when injected i.c.v. in rats.

Keywords: biological activity; conformation; deltorphin; dermorphin; opioid peptides; peptide synthesis; peptoids

Abbreviations: With the exception of D-Ala the amino acid residues are of the L-configuration. Standard abbreviations for amino acid derivatives and peptides are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984; **138**: 9–37). Abbreviations listed in the guide published in *J. Peptide Sci.* 2003; **9**: 1–8 are used without explanation. Other abbreviations: EDC, N-(3-dimethylamino-isopropyl)-N-ethyl-carbodiimide; NMP, N-methylpyrrolidone; Nphe, N-benzylglycine; Ntyr, N-(4-hydroxy-benzyl)glycine; Ntyr(tBu), N-(4-*tert*-butyloxy-benzyl)glycine; Nval, N-isopropylglycine; Rink amide MBHA resin, [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-4-methyl-4-benzydrylamine polystyrene].

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INTRODUCTION

Dermorphin and deltorphin are opioid heptapeptides isolated from frog skin and are highly selective ligands at μ- and δ-opioid receptors, respectively [1–3]. As an antinociceptive agent, dermorphin is 40 000-fold less potent after subcutaneous (s.c.) administration than after intracerebroventricular (i.c.v.) administration [4]. When injected into the brain of mice or rats the highly selective δ-opioid ligands deltorphins I and II produce analgesia [5,6], locomotor stimulation [7] and motivational rewarding [8] without the development of physical dependence [9] or respiratory depression [10].

However, the exploration of the therapeutic potential of these opioid agents has been hampered by the lack of derivatives active on peripheral injection.

There is a considerable interest in the development of peptides retaining high activity and receptor selectivity after peripheral administration and several strategies have been used to enhance peptide delivery to the central nervous system (CNS). In our earlier papers [11–13] the synthesis of some dermorphin and deltorphin glycosylated analogues was reported with their opioid receptor affinity and selectivity as well as their analgesic potency after subcutaneous injection in mice. An interesting idea for modifying the structure of a biologically active peptide was the proposal to shift the side chain of a certain amino acid residue, from the α-carbon to the α-nitrogen atom of the peptide bond. The resulting *N*-substituted glycine derivatives are achiral and can be used for the synthesis of peptide derivatives, called peptoids or peptide–peptoid hybrids, possessing proteolytic stability and high flexibility [14,15].

In the present paper the synthesis and some preliminary pharmacological experiments of new analogues are described in which various amino acid residues (phenylalanine, tyrosine and valine) in the dermorphin and [D-Ala^2]-deltorphin sequences have been substituted by the corresponding *N*-alkyl-glycine residues (Table 1). In an attempt to correlate the biological activity of the resulting peptide–peptoid hybrids with their conformational features, structural investigations were carried out by CD measurements in different solvents.

The conformational preferences of the μ-selective dermorphin and the δ-selective [D-Ala^2]-deltorphin I and II have been widely investigated, by several authors, by two-dimensional ^{1}H-NMR spectroscopy and molecular dynamics calculations in different solvents. These compounds exist in solution as a mixture of flexible conformers showing some conformational preferences. The presence, in DMSO, of an extended structure in the dermorphin central sequence Phe^3-Gly^4-Tyr^5 and the predominance of type I β-turn around the Pro^6-Ser^7 peptide bond was suggested by Toma *et al.* [16]. Tancredi *et al.* [17] showed that both dermorphin and deltorphin, adopt similar β-turns in the *N*-terminal sequence, but differentiate owing to the conformation and polarity of the *C*-terminal part. Investigations in aqueous solution [18,19] and in membrane mimetic solvents [20] provided evidence of the occurrence of a *cis*/*trans* isomerization (rotamers ratio about 1 : 5) around the Tyr^5-Pro^6 amide bond of dermorphin. In aqueous solution [18] many dermorphin conformers are characterized by a structure rounded at the *N*-terminal Tyr-D-Ala-Phe-Gly-Tyr and the *C*-terminal Gly-Tyr-Pro-Ser-NH_2 moieties which are almost at right angles to each other. In particular, the β-turn like folded conformation of the *C*-terminal Gly-Tyr-Pro-Ser-NH_2 dermorphin sequence is primarily due to the *trans* orientation of the Tyr-Pro amide bond and is stabilized by the Ser^{7}NH. . .Tyr^{5}C=O hydrogen bond. In the same solvent [D-Ala^2]-deltorphin II conformers [18] are characterized by a 'hook'-shaped backbone structure in which the nearly extended conformation of the Val-Val-Gly-NH_2 sequence is

Table 1 Amino Acid Sequence of Dermorphin and [D-Ala^2]-deltorphin I Peptide–peptoid Analogues

I	H-Ntyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	[Ntyr^1]-dermorphin
II	H-Tyr-D-Ala-Nphe-Gly-Tyr-Pro-Ser-NH_2	[Nphe^3]-dermorphin
III	H-Ntyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	[Ntyr^1,D-Ala^2]-deltorphin I
IV	H-Tyr-D-Ala-Nphe-Asp-Val-Val-Gly-NH_2	[D-Ala^2,Nphe^3]-deltorphin I
V	H-Tyr-D-Ala-Phe-Asp-Nval-Val-Gly-NH_2	[D-Ala^2,Nval^5]-deltorphin I
VI	H-Tyr-D-Ala-Phe-Asp-Val-Nval-Gly-NH_2	[D-Ala^2,Nval^6]-deltorphin I
VII	H-Tyr-D-Ala-Phe-Asp-Nval-Nval-Gly-NH_2	[D-Ala^2,Nval^5,Nval^6]-deltorphin I

located under the folded conformation of the *N*-terminal Tyr-D-Ala-Phe-Glu sequence.

In the membrane-mimetic phospholipid micelles [20] a turn conformation bent at the Gly^4 residue could be described as the representative averaged structure of the dermorphin, where both the *N*-terminal and the *C*-terminal tripeptide sequences take an extended form. Intramolecular hydrogen bonds (D-Ala^2NH...Ser^7C=O and *C*-terminal NH_2...Gly^4C=O) stabilize the folded conformation. On the other hand, most of the [D-Ala^2]deltorphin I conformers could be characterized as a S-shaped backbone conformation. The *N*-terminal Tyr-D-Ala-Phe-Asp and *C*-terminal Val-Val-Gly-NH_2 sequences take turn structures, respectively, and they are almost at right angles on the border of the Asp-Val sequence; the $Asp^4C{=}O^{\delta}$ is simultaneously hydrogen bonded to NH groups of Tyr^1, D-Ala^2 and *C*-terminal NH_2.

MATERIALS AND METHODS

TFA, EDC and NMM were Fluka products, HATU was obtained from Perseptive Biosystem and Rink Amide MBHA resin was obtained from Novabiochem. All other chemicals for the solid phase synthesis were supplied by Applied Biosystems. Nphe-OH, Nval-OH and Ntyr(tBu)-OH were prepared according to the literature [21]. All other chemicals were of the best grade available.

Melting points were taken on a Buchi model 150 melting point apparatus in open capillaries and are not corrected. Optical rotations were determined with a Perkin Elmer model 241 polarimeter. Amino acid analyses were made with a Carlo Erba model 3A 29 amino acid analyser equipped with a Perkin Elmer Sigma 10 chromatography data station following hydrolysis for 22 h (46 h for Val) at 110 °C, in sealed, evacuated vials in constant boiling hydrochloric acid. Analytical HPLC separations were performed on an Aquapore RP 300 column (220 × 4.6 mm, 7 μm, Brownlee Laboratories, flow rate 1.5 ml/min), using a Perkin Elmer series 3B liquid chromatograph equipped with a LC-90 UV detector and LCI-100 integrator. Eluant A (0.1% TFA in 90% aqueous CH_3CN) and B (aqueous 0.1% TFA) were used for preparing binary gradients (elution conditions: isocratic 10% A for 2 min, linear gradient 10%–90% A in 30 min). In the case of the metabolic stability assays the conditions used were the following: column Spherisorb 5-OD52, 250 × 4.6 mm; eluant A (0.1% TFA in 60% aqueous acetonitrile), eluant B (0.1% TFA in 5% aqueous acetonitrile), flow rate 1.0 ml/min, elution conditions: isocratic 30% A for 5 min, linear gradient 30%–50% A in 15 min. Semipreparative HPLC separations (Aquapore RP-300 column, 250 × 10 mm, 20 μm, Brownlee Laboratories; flow rate 15 ml/min) was performed on a Shimadzu series LC-6A chromatograph equipped with two independent pump units model LC-8A, a SPD-6A detector and a C-R6A integrator (eluants and elution conditions as those used for the analytical separations). Molecular weight determinations were made by MALDI-TOF MS carried out on a Maldi I Kratos Shimadzu instrument operating in linear mode at a nominal accelerating potential of +10 KeV (matrix: α-cyano-4-hydroxycinnamic acid). Solvents were dried and freshly distilled and evaporations were carried out under reduced pressure at 40°–45 °C, using a rotary evaporator. Yields are based on the weight of vacuum-dried products. Sodium sulphate was used for drying purposes.

Solid Phase Synthesis

The assembly of peptide–peptoid hybrids **I–VII** (Table 1) on the Applied Biosystems Model 431 A Peptide Synthesizer was performed on a 0.25 mmol scale by the FastMoc methodology (HBTU/HOBt/DIEA) starting with Fmoc-Ser(tBu)-Rink Amide MBHA resin or Fmoc-Gly-Rink-Amide MBHA resin. A single acylation protocol was used through all syntheses for the acylation of L-amino acids and D-alanine. For the acylation of *N*-alkylglycines, activation of the carboxyl component was achieved by adding HATU, as a solid, into the cartridge containing the Fmoc-amino acid. The mixture was dissolved with DMF without adding HOBt and the time of coupling was doubled (50 min). To prevent the possible intrachain aminolysis at the dipeptide stage [22] and the consequent peptide chain loss in form of diketopiperazine, the dipeptide Fmoc-Tyr(tBu)-Pro-OH [13] containing the third and the second residues was used in the first acylation step during the synthesis of the dermorphin peptoid analogues. Coupling yields were determined by ninhydrin analysis [23] of a small aliquot of peptide resin removed automatically after each coupling step. The final peptide resin was N^{α}-deprotected with 20% piperidine in NMP, thoroughly washed and dried. Cleavage from the resin and removal of the side chain protecting groups were simultaneously achieved on portions

Table 2 Characterization of Dermorphin and [D-Ala^2]-deltorphin I Peptide–peptoid Analogues

Peptide	Yield%	HPLC (R_t) min	$[\alpha]_D^{20}$ c 1.0, H_2O	Molecular weight		Amino acid ratios[a]										
				Calcd.	Found[b]	Asp	Ser	Pro	Gly	Ala	Val	Tyr	Phe	Nphe	Nval	Ntyr
I	68	15.41	−3.4°	802.9	802.5	—	−0.97	0.98	1.11	0.97	—	0.99	0.97	—	—	n.d.
II	89	15.33	+8.5°	802.9	802.3	—	0.99	1.01	1.01	0.97	—	2.00	—	1.01	—	—
III	64	16.72	−16.5°	768.9	769.2	0.96	—	—	1.04	0.94	1.87	—	0.96	—	—	n.d.
IV	65	16.84	−2.5°	768.9	768.4	1.04	—	—	1.06	1.00	1.89	0.95	—	1.03	—	—
V	58	16.97	−7.6°	768.9	768.1	1.00	—	—	1.04	1.00	1.00	0.95	0.98	—	n.d.	—
VI	30	17.26	−7.4°	768.9	769.0	1.00	—	—	0.98	1.02	0.99	0.97	1.02	—	n.d.	—
VII	65	16.73	+5.7°	768.9	768.5	1.02	—	—	1.05	0.99	—	0.97	0.97	—	n.d.	—

[a] The values denotes the number of residues per molecule, n.d., not determined.
[b] MALDI-TOF MS, matrix: α-cyano-4-hydroxycinnamic acid; the experimental value refers to [M + H].

of the peptide resin by treatment with aqueous 95% TFA (about 6 ml/300 mg peptide resin, 3.5 h at room temperature). The acid solution was evaporated *in vacuo* and the residue was dissolved in a small volume of TFA and precipitated with excess *tert*-butyl-methyl ether. Precipitation from TFA was repeated and the product was collected and dried in the presence of P_2O_5 and KOH pellets. When necessary peptide–peptoid analogues were further purified by semipreparative HPLC. All products were characterized by reverse phase analytical HPLC, optical rotation, amino acid composition and molecular weight determination.

Circular Dichroism

CD measurements were performed, in the solvents indicated, at 298 K, over 250–185 nm, using a Jasco 715 spectropolarimeter connected with a PC IBM PS/2 Model 40 SIC for the spectra elaboration (J700 Windows program). A Suprasil quartz cell of 0.5 mm path length was used, and six scans were accumulated for all spectra. The sample concentration was about 2×10^{-4} M. Solutions of the desired concentration were prepared by dissolving samples of the different peptides in the minimum amount of water; the peptide concentration was determined by quantitative amino acid analysis. Aliquots of the mother solutions were diluted with the appropriate volumes of 5 mM Tris.HCl buffer (pH 7.0), aqueous SDS or TFE. Final aqueous solutions were 30 mM SDS or 97% TFE (v/v). The spectra reported are original computer-drawn CD curves, $[\Theta]_R$ represents the mean residue molar ellipticity (deg cm^2 dmol^{-1}).

Pharmacological Tests

Receptor binding assays. The binding of peptide–peptoid hybrids to opioid receptors was tested on crude membrane preparations from CHO cells stably transfected with the μ- and δ-opioid receptors. The μ-binding sites were selectively labelled with [^{3}H][D-Ala^2, MePhe^4, Glyol^5]-enkephalin ([^{3}H]DAMGO, 0.5 nM; 55.3 Ci/mmol) and the δ-sites with [^{3}H]-deltorphin II (0.3 nM; 53 Ci/mmol). Compounds were assayed on 60 µg cell membrane protein, in a final volume of 2 ml Tris-HCl buffer (50 mM, pH 7.4), at 35 °C for 90 min. All experiments were performed in triplicate and total binding, nonspecific binding (10 µM naloxone) and 12 inhibitor concentrations were determined. The inhibition constant (K_i) of the opioids was calculated from competitive binding curves with the computer program Prism. Data are presented as the arithmetic mean ± SEM of four independent measurements.

Activity on isolated organ preparations. Preparations of the myenteric plexus-longitudinal muscle obtained from male guinea-pig ileum (GPI, rich in μ-opioid receptors) and preparations of mouse vas deferens (MVD, rich in δ-opioid receptors) were used for field stimulation with bipolar rectangular pulses of supramaximal voltage [24]. Agonists were evaluated for their ability to inhibit the electrically evoked twitch. The biological potency of peptoid analogues was compared with that of the μ-opioid receptor agonist dermorphin in GPI preparations and with that of the δ-opioid receptor agonist deltorphin I in MVD preparations. The results are expressed as the IC_{50} values obtained from concentration-response curves (Prism). IC_{50} values represent the mean of not less than six tissue samples ± SEM.

Antinociception studies. Compounds were injected into the lateral brain ventricle (i.c.v.) of rats through a guide cannula, using a Hamilton microlitre syringe, in a volume of 5 µl (10% DMSO). Each animal received one injection only. Every dose of each compound was evaluated in groups of eight animals. Antinociception was measured by the tail-flick test [25] and expressed as percentage maximum possible effect: %MPE = 100 × (test latency − control latency)/(15 s − control latency). The AD_{50} values of each peptide was the dose producing 50% of the MPE.

Metabolic Stability Assays

The kinetics of degradation of the synthetic peptoid analogues, in the presence of rat or human plasma, were compared with that of [Leu^5]-enkephalin, used as the standard reference. Dermorphin and [D-Ala^2]deltorphin I were also tested. The stability assays were carried out in 10 mM Tris-HCl buffer, pH 7.5, and the compounds (final concentration 4×10^{-4} M) were incubated with plasma (300 µl) in a total volume of 750 µl. Incubation was performed, at 37 °C, for increasing periods of time (up to 360 min), terminated by adding ethanol (1.0 ml), and the mixture was cooled to −21 °C. After centrifugation (5 min at 5000 rpm), an aliquot (10 µl) of the clear supernatant was analysed by analytical HPLC. The degradation half-time ($T_{1/2}$) was obtained by a least-square linear regression analysis of a plot of the

logarithm of peptoid concentration versus time, using at least five points.

RESULTS AND DISCUSSION

Metabolic Stability Assays

Measurements carried out in the presence of rat plasma showed that [Leu^5]-enkephalin was rapidly degraded ($T_{1/2} = 2.1$ min) but compounds **I–VII** were very resistant to digestion by the rat plasma enzymes ($T_{1/2} > 360$ min). In agreement with previous observations [26,27] dermorphin and [D-Ala^2]-deltorphin I were slowly metabolized ($T_{1/2} = 190.6$ and 131.6 min, respectively). In the presence of human plasma the degradation rate of compounds **I–VII** was essentially identical ($T_{1/2} > 360$ min) and, as previously reported [26], the rate of the [Leu^5]-enkephalin cleavage was slower ($T_{1/2} = 4.5$ min) than that observed in the presence of rat plasma.

Conformational Studies

The CD spectra of [Nphe^3]-dermorphin in H_2O, TFE and SDS are shown in Figure 1. The far UV CD spectrum in water is characterized by a negative minimum below 190 nm, two positive bands at 226–227 nm and 200–201 nm respectively, and a positive minimum at 215 nm. In TFE the minimum at 215 nm is negative and the maximum at lower wavelength is shifted at 198 nm. The spectra are similar to those already reported for dermorphin [28] in the same solvents.

The far UV region is of most concern in the application of CD to the conformational analysis of peptides and proteins and is apparently dominated by peptide contribution. However, contribution from aromatic side chains to the CD spectrum of peptides and proteins have been acknowledged in both experimental and theoretical studies [29,30] being more prominent in systems where several aromatic groups are in close proximity [31]. Tyrosyl residues have been reported to contribute to a CD spectrum with four additional transitions: the L*b* band at 275 nm, the L*a* band at 227 nm and the B*a*, B*b* bands at 190 nm. Therefore the maximum at 226–227 nm in the dermorphin CD spectrum [28] could be attributed essentially to the tyrosine residues in position 1 and 5. The assignment of the band at 200–201 nm is rather questionable, owing to the presence of contributions arising from the peptide chromophores and from the B-transitions of both Tyr and Phe chromophores. On the other hand, according to Woody [32], the negative band at 215 nm, the strong positive one at about 201 nm and the negative band below 190 nm could agree with the CD spectrum of peptides folded in β-turns. Moreover dermorphin contains D-Ala and the Pro-Ser sequence which are β-turn inducers [33] and β-turn like structures have been determined by NMR studies [16]. The CD spectrum in the far UV region could also reflect the contribution of folded structures. The general similarity of the CD spectra of dermorphin and [Nphe^3]-dermorphin in H_2O and in TFE, suggests that the afforded substitution did not significantly affect the peptide conformation. The [Nphe^3]-dermorphin CD spectrum in SDS solution could indicate a conformation more ordered and more rigid than that found in water and in TFE. The spectrum shows an intense negative band with a peak at 215–220 nm, a shoulder at 200 nm and a positive band at 190 nm suggesting that SDS contributes to stabilize preferentially a β-sheet structure. Possibly, the hydrophobic interaction between the peptide aromatic residues and the SDS long chain acyl groups promotes the extension of the peptide backbone and the stabilization of β-sheet structure [34]. This feature could agree with the average structure of dermorphin described by NMR measurements [20] in phospholipid micelles.

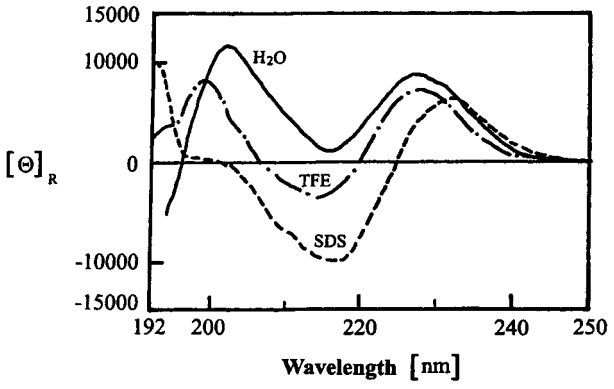

Figure 1 CD spectra of [Nphe^3]-dermorphin in the indicated solvents.

The C$\alpha \rightarrow$ Nα shift of the Tyr^1 side chain (compound II) causes a general decrease in the band's intensity of the dermorphin CD spectra (Figure 2). This feature could arise from an increased conformational freedom of the *N*-terminal achiral residue preventing the interaction between the aromatic side chains of Phe^3 and Ntyr^1. In water and in SDS solution the bands are positive in the overall spectra and do not indicate a prevailing secondary

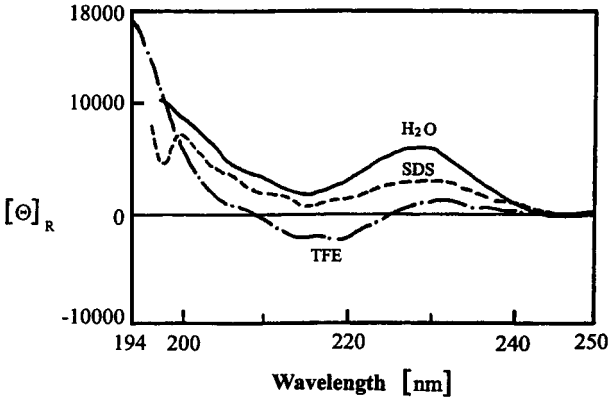

Figure 2 CD spectra of [Ntyr^1]-dermorphin in the indicated solvents.

structure. In TFE the CD spectrum shows a strong positive band at about 190 nm and a negative weak absorption at 210–220 nm suggesting the presence of some amount of β-turns.

The CD spectra of [D-Ala^2,Nphe^3]-deltorphin I in H_2O, TFE and SDS are shown in Figure 3. The spectrum in H_2O exhibits a negative band near 190 nm, characteristic of a random coil conformation, and a positive band at the aromatic chromophore absorption wavelength (227 nm). In SDS solution the CD spectrum displays a positive band at 190 nm, a large negative band, with a minimum at 198 nm and a shoulder at about 215 nm, and a positive aromatic band at 230 nm. The spectrum indicates the predominance of a random conformer population, however, the widening of the band and the shoulder could suggest the presence of more or less ordered conformers. The CD spectrum in TFE is red shifted, the amplitude of both the positive and

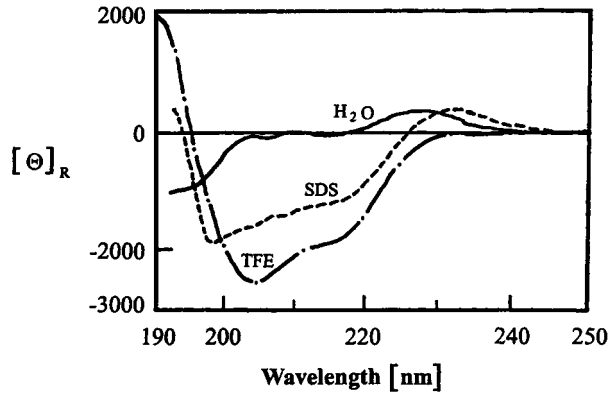

Figure 3 CD spectra of [D-Ala^2,Nphe^3]-deltorphin I in the indicated solvents.

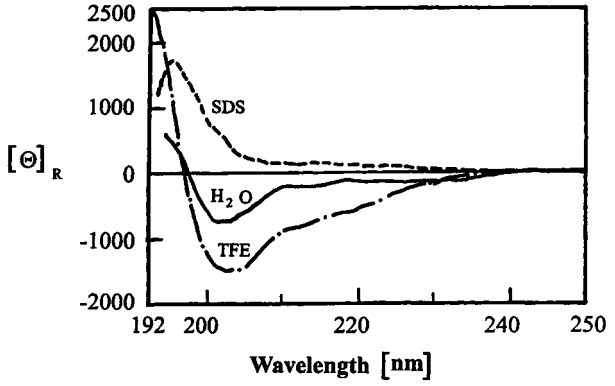

Figure 4 CD spectra of [Ntyr^1,D-Ala^2]-deltorphin I in the indicated solvents.

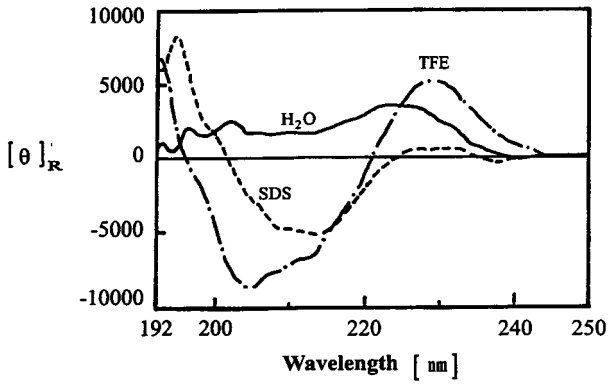

Figure 5 CD spectra of [D-Ala^2,Nval^5]-deltorphin I in the indicated solvents.

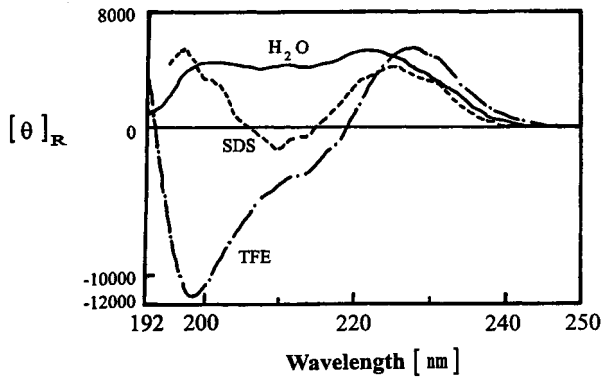

Figure 6 CD spectra of [D-Ala^2,Nval^5,Nval^6]-deltorphin I in the indicated solvents.

negative bands is increased and the aromatic contribution at 230 nm is missing. The peptide clearly adopts a significant amount of ordered secondary structure and the spectrum can be considered as

C-like class, indicative of the presence of type I β turns [32]. In the membrane mimetic solvents the [D-Ala^2,Nphe^3]-deltorphin I structure could be similar to the folded structure determined by NMR [17,20] for deltorphin I in phospholipid micelles.

The side chain shift from Cα to Nα in [Ntyr^1,D-Ala^2]-deltorphin I significantly modify the CD spectra, as shown in Figure 4. In any solvent the most striking feature is the complete disappearance of the aromatic contribution at about 230 nm which could be explained in terms of high conformational freedom of the *N*-terminal residue. The CD curve in SDS shows only a positive band at 195 nm. The shape of the spectrum, but not its amplitude, is similar to a class C′ spectrum which has been observed in L-Aaa-D-Aaa- models [35] adopting mainly a type II β-turn. On the contrary the spectrum in TFE is characterized by a positive band at 190 nm and a negative one at 205 nm with a shoulder at $\approx$ 215 nm. The curve can still be considered as a C-like CD spectrum [36] suggesting the predominance of a random conformer population with the presence of type I β-turn structures. The spectrum in H_2O is similar to that in TFE but the decreased band intensity indicates an increase of conformational mobility.

In all solvents the CD spectra of [D-Ala^2,Nval^5]-deltorphin I, (Figure 5), [D-Ala^2,Nval^6]-deltorphin I (not shown) and [D-Ala^2,Nval^5,Nval^6]-deltorphin I (Figure 6) show the positive band of the aromatic residue at 225–235 nm, although with different ellipticity values. In the [D-Ala^2,Nval^5]-deltorphin I spectrum the aromatic band is more intense in TFE rather in the SDS solution. Apparently the modification in position 5 and 3 affects in the opposite way the dichroic environment of Tyr^1 in the different solvent systems. In TFE the remaining portion of the spectrum of **IV** and **V** is similar. However, the lower ellipticity value of the band at 205 nm with the shoulder at 215 nm for [D-Ala^2,Nval^5]-deltorphin I reflects a different equilibrium mixture of conformers. In SDS solution the negative band of the Nval^5 derivative is red shifted, in comparison with that of **IV**, suggesting, also in this case, a different mixture of conformers.

The CD profiles of [D-Ala^2,Nval^5]- and [D-Ala^2,Nval^5,Nval^6]-deltorphin I in water are positive in the overall spectra. They show only the aromatic transitions and do not indicate a prevailing secondary structure. A similar situation is shown in SDS by the analogue **VII**. In the spectrum of [D-Ala^2,Nval^5,Nval^6]-deltorphin I in TFE, the intense negative band is shifted at 198 nm, suggesting the presence of unordered structures.

In conclusion the contribution from aromatic side chain residues is prominent in the CD spectra of dermorphin analogues and the assignment of a prevailing secondary structure could be questionable. In the CD spectra of deltorphin analogues the aromatic contribution is lower and in all solvents the dichroic curves indicate the predominance of random conformer populations. A striking feature is the disappearance in the [Ntyr^1,D-Ala^2]-deltorphin I, of the aromatic contribution which could be explained

Table 3 Opioid Receptor Affinity and Selectivity of Peptide–peptoid Analogues Compared with those of Dermorphin and Deltorphin I

Peptide	K_i (μM)		δ/μ selectivity
	CHO/μ	CHO/δ	
Dermorphin	0.002	1.73	1017
[Ntyr^1]-dermorphin	55 ± 8	>100	—
[Nphe^3]-dermorphin	>100	>100	—
Deltorphin I	1.63	0.003	1.8×10^{-3}
[Ntyr^1,D-Ala^2]-deltorphin I	>100	32 ± 4	—
[D-Ala^2,Nphe^3]-deltorphin I	>100	>100	—
[D-Ala^2,Nval^5]-deltorphin I	25 ± 4	3.3 ± 0.4	1.3×10^{-1}
[D-Ala^2,Nval^6]-deltorphin I	28 ± 7	2.5 ± 0.3	8.9×10^{-2}
[D-Ala^2,Nval^5,Nval^6]-deltorphin I	36 ± 5	52 ± 8	1.4

K_i, inhibition constant; the values are the mean of four experiments $\pm$ SEM. μ Opioid receptors were labelled with [^{3}H]DAMGO (0.5 nM; 55.3 Ci/mmol), δ opioid receptors were labelled with [^{3}H]deltorphin II (0.3 nM; 53 Ci/mmol).

Table 4 Biological Activity of Peptide–peptoid Analogues on Guinea-pig Ileum (GPI) and Mouse Vas Deferens (MVD) Preparations

Peptide	$IC_{50}(\mu M)$		MVD/GPI
	GPI	MVD	
Dermorphin	0.002	0.016	8
[Ntyr^1]-dermorphin	55	>100	—
[Nphe^3]-dermorphin	55	>100	—
Deltorphin I	1.25	0.0002	16×10^{-5}
[Ntyr^1,D-Ala^2]-deltorphin I	>100	59	—
[D-Ala^2,Nphe^3]-deltorphin I	>100	>100	—
[D-Ala^2,Nval^5]-deltorphin I	24 ± 4	4 ± 0.5	1.7×10^{-1}
[D-Ala^2,Nval^6]-deltorphin I	17 ± 3	3 ± 0.3	1.7×10^{-1}
[D-Ala^2,Nval^5,Nval^6]-deltorphin I	26 ± 5	68 ± 8	2.6

IC_{50}, agonist concentration that produced 50% inhibition of the electrically evoked twitch; the values are the mean of six experiments ± SEM.

in terms of high conformational freedom of the *N*-terminal residue.

Pharmacological Screenings

All the substitutions produced a dramatic decrease in the affinity of the resulting peptide–peptoid hybrids for the μ- and δ-opioid receptors (Table 3). The substitution of Tyr^1 or Phe^3, (**I**, **II**, **III** and **IV**) practically cancelled any agonistic activity of both dermorphin and [D-Ala^2]-deltorphin I when evaluated on both GPI and MVD preparations. The substitution of Val^5 and/or Val^6 residues in the [D-Ala^2]-deltorphin I sequence (**V**, **VI** and **VII**) highly decreased the δ agonistic activity on MVD, but slightly reduced the μ agonistic activity on GPI, yielding compounds endowed with similar potency on GPI and MVD preparations (Table 4). When injected i.c.v. in rats, **V**, **VI** and **VII** elicited a dose-dependent analgesia (Figure 7). The rank order of analgesic potency was: **VII** (AD_{50} = 27.9 (23.7–32.9) μg/rat) > **V** (AD_{50} = 48.2 (35–66.4) μg/rat) = **VI** (AD_{50} = 52.6 (35.3–78.3) μg/rat). The analgesic effect of the three peptoids tested peaked within 30–45 min, and lasted more than 2 h (Figure 8), showing a time course highly different from that of deltorphin I. Preadministration (15 min) of 0.1 mg/kg of naloxone significantly reduced or completely abolished the peptoid induced antinociception (data not shown). These results confirm that Nval^5 and/or Nval^6 substitutions produced compounds that behaved as μ-opioid receptor agonists.

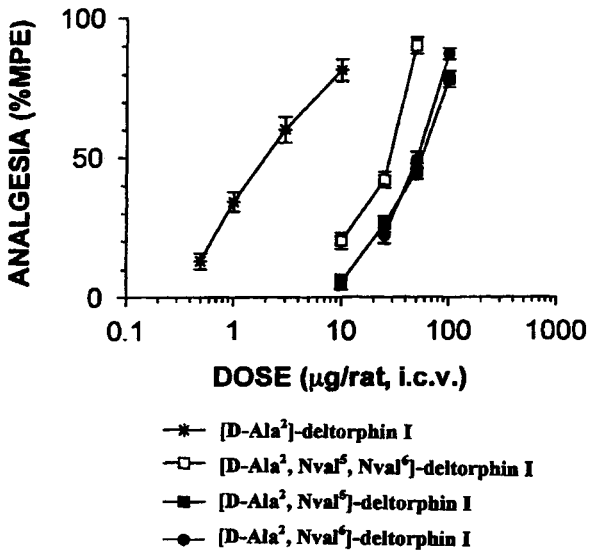

Figure 7 Dose-response curves of the antinociception produced by i.c.v. injection of [D-Ala^2]-deltorphin I (AD_{50} = 2.0 (1.7–2.4) μg/rat), [D-Ala^2,Nval^5,Nval^6]-deltorphin I (AD_{50} = 27.9 (23.7–32.9) μg/rat), [D-Ala^2,Nval^5]-deltorphin I (AD_{50} = 48.2 (35–66.4) μg/rat), [D-Ala^2,Nval^6]-deltorphin I (AD_{50} = 52.6 (35.3–78.3) μg/rat), evaluated by the tail-flick test in rats. Curves were analysed by a linear regression computer program [37]. Each point represents the mean antinociceptive effect of 8 rats ± SEM.

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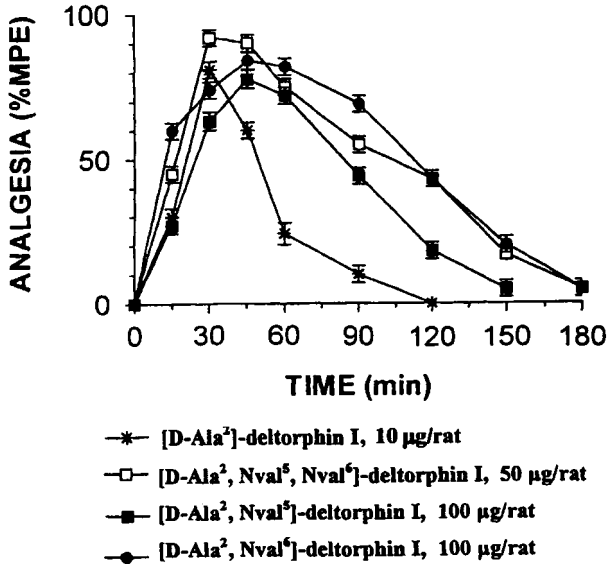

Figure 8 Time course of the antinociception produced by i.c.v. administration of equianalgesic doses of [D-Ala^2]-deltorphin I, [D-Ala^2,Nval^5]-deltorphin I, [D-Ala^2, Nval^6]-deltorphin I and [D-Ala^2,Nval^5,Nval^6]-deltorphin I, evaluated by the tail-flick test. Each point represents the mean antinociceptive effect of 8 rats ± SEM.

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