Journal of Peptide Science
J. Peptide Sci. **10**: 578–587 (2004)
Published online 2 April 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.566

JOURNAL OF PEPTIDE SCIENCE

[D-Ala[2]]-Deltorphin I Peptoid and Retropeptoid Analogues: Synthesis, Biological Activity and Conformational Investigations

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Received 24 November 2003
Revised 19 December 2003
Accepted 5 January 2004

Abstract: The synthesis is described of a [D-Ala[2]]-deltorphin I peptoid analogue in which all amino acid residues have been substituted by the corresponding *N*-alkylglycine residues. The [D-Ala[2]]-deltorphin I retropeptoid was also prepared as well as [Ala[1],D-Ala[2]]-deltorphin 1 and the corresponding peptoid. Structural investigations by FT-IR and fluorescence measurements were carried out on the synthetic analogues and on some [D-Ala[2]]-deltorphin 1 peptide–peptoid hybrids previously prepared. According to the fluorescence measurements the distance between the aromatic residues in the deltorphin I peptoid and retropeptoid is similar to that suggested for the δ- and μ-opioids, respectively. Measurements of CD in the presence of β-cyclodextrin, and some preliminary pharmacological experiments were also performed. No dichroic bands are present in the spectrum of the [Ntyr[1],D-Ala[2]]-deltorphin I, but an increasing dichroic effect appears in the spectra of both the deltorphin I peptoid and retropeptoid. Activity tests on isolated organ preparations showed that the modifications made produced a dramatic decrease in the agonistic activity of the synthetic derivatives. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

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

INTRODUCTION

Deltorphins are heptapeptides isolated from frog skin, and are highly selective ligands at δ-opioid receptors [1–3]. They possess an interesting therapeutic potential [4–9] but, as for other bioactive peptides, their use meets with some severe limitations and intensive efforts have been made to

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 Nomeclature (1984) *Eur. J. Biochem.* **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*-ethylcarbodiimide; ESI-MS, electrospray ionization-mass spectrometry; NMP, *N*-methylpyrrolidone; Nala, *N*-methylglycine (i.e. sarcosine); Nasp, *N*-carboxymethyl-glycine; Nphe, *N*-benzylglycine; Ntyr, *N*-(4-hydroxy-benzyl)glycine; Ntyr(tBu), *N*-(4-*tert*-butyloxybenzyl)glycine; Nval, *N*-isopropylglycine; Rink amide MBHA resin, [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-4-methyl-4-benzydrylamine polystyrene]; TDM, 4,4′-tetramethyldiphenylmethane; TES, tri-ethyl silane.

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Contract/grant sponsor: MUIR.
Contract/grant sponsor: National Research Council of Italy.

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develop peptidomimetics displaying pharmacological properties more favourable than the prototypes with regard to specificity of action, resistance towards enzymatic degradation, pharmacokinetics and bioavailability. There is 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).

An interesting idea was the proposal to shift the amino acid side chains from the α-carbon to the α-nitrogen, yielding *N*-substituted glycine derivatives which can be used for the synthesis of oligomeric peptidomimetics, called peptoids or peptide–peptoid hybrids [10,11]. This communication will focus on the solid phase synthesis of the [D-Ala^2]-deltorphin I peptoid (**II**) and retropeptoid (**IV**) analogues, shown in Table 1. [D-Ala^2]-Deltorphin I, [Ala^1,D-Ala^2]-deltorphin I (**I**) and the peptoid **III** were also prepared for comparison. The synthesis and some preliminary pharmacological experiments on the [D-Ala^2]-deltorphin I analogues in which various amino acid residues (Phe, Tyr or Val) were substituted by the corresponding *N*-alkyl-glycine residues (compounds **V–IX**) have been previously described [12].

In an attempt to correlate the biological activity of the synthetic peptoids and peptide–peptoid hybrids with their conformational features, structural investigations were carried out by IR and fluorescence measurements. The ability of β-cyclodextrin to induce chirality into the achiral Ntyr residue of the peptoid and retropeptoid analogues has been investigated by CD measurements.

MATERIALS AND METHODS

All chemicals were commercial products of the best grade available. NMM was supplied by Fluka and acetonitrile (HPLC grade) by Carlo Erba. Rink amide MBHA resin was obtained from Novabiochem and HATU from PerSeptive Biosystems. All other chemicals for the solid phase synthesis were supplied by Advanced ChemTech. Ascending thin-layer chromatography was routinely performed on TLC plates silica gel 60, UV_{254}, Machery-Nagel, using the following solvent systems: E1bis: ethyl acetate/butan-1-ol/acetic acid/water (5 : 3 : 1 : 1 by vol.); E6: chloroform/methanol/acetic acid (90 : 8 : 2 v/v). Amino acid derivatives and peptides were visualized by one or more of the following procedures: ninhydrin, TDM reagent [13] and UV light.

Analytical HPLC separations were performed on a Vydac C_{18} 218 TP-104 column (250 × 4.6 mm, 10 μm, flow rate 1.5 ml/min) using a Perkin Elmer series 410 liquid chromatograph equipped with a LC-90 UV detector and LCI-100 integrator. Eluants A (0.1% TFA in 90% aqueous acetonitrile) and B (0.1% aqueous TFA) were used for preparing binary gradients (elution conditions: isocratic 10% A for 3 min, linear gradient 10%–90% A in 30 min). Semipreparative HPLC separations (Vydac C_{18} 218 TP-1022 column, 250 × 22 mm, 10 μm, flow rate 15 ml/min) were performed on a Shimadzu series LC-8A chromatograph equipped with a SPD-6A detector and a C-R6A integrator (eluants as those used for the analytical separations; elution conditions: isocratic 15% A for 5 min, linear gradient 10%–60% A in 30 min). Solvents were dried and freshly distilled and evaporations were carried out under reduced pressure at 40°–45 °C, using a rotary evaporator. Sodium sulphate was used for drying purposes. Yields are based on the weight of vacuum-dried products.

Proton Nuclear Magnetic Resonance

Proton NMR spectra at 200 and 250 MHz were recorded at 298 K, unless stated otherwise, on

Table 1 [D-Ala^2]-deltorphin I Peptide, Peptoid and Retropeptoid Analogues

I	H-Ala-(D)Ala-Phe-Asp-Val-Val-Gly-NH_2	[Ala^1,D-Ala^2]-deltorphin I
II	H-Ntyr-Nala-Nphe-Nasp-Nval-Nval-Gly-NH_2	Deltorphin I peptoid
III	H-Nala-Nala-Nphe-Nasp-Nval-Nval-Gly-NH_2	[Nala^1]-deltorphin I peptoid
IV	H-Gly-Nval-Nval-Nasp-Nphe-Nala-Ntyr-NH_2	Deltorphin I retropeptoid
V	H-Ntyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	[Ntyr^1,D-Ala^2]-deltorphin I
VI	H-Tyr-D-Ala-Nphe-Asp-Val-Val-Gly-NH_2	[D-Ala^2,Nphe^3]-deltorphin I
VII	H-Tyr-D-Ala-Phe-Asp-Nval-Val-Gly-NH_2	[D-Ala^2,Nval^5]-deltorphin I
VIII	H-Tyr-D-Ala-Phe-Asp-Val-Nval-Gly-NH_2	[D-Ala^2,Nval^6]-deltorphin I
IX	H-Tyr-D-Ala-Phe-Asp-Nval-Nval-Gly-NH_2	[D-Ala^2,Nval^5,Nval^6]-deltorphin I

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Brucker spectrometers (WP 200 SY and AC-250-F, respectively). Sample concentrations were in the range 8–10 mg/ml in $CDCl_3$ or DMSO-d_6 (99.996%). Chemical shifts (δ) are expressed relative to the residual signals at 7.26 ppm in $CDCl_3$ and 2.49 ppm in DMSO-d_6. Proton assignments were determined by selective homo-spin decoupling.

Mass Spectra

Electrospray ionization mass spectrometry (ESI-MS) was performed on a PerSeptive Biosystem Mariner instrument. Ionization potential 4200 V, acceleration potential 100 V.

Infrared Absorption

Solid state IR absorption spectra (KBr disk technique) were recorded at room temperature using a nitrogen flushed Perkin Elmer Model 1720X FT-IR spectrophotometer. Elaboration of the spectra by baseline subtraction and second derivative formation was achieved using the Spectra Calc program (Galactic, Salem, USA).

Ultraviolet Absorption

UV absorption spectra were recorded over 250–350 nm at room temperature on a Perkin Elmer Lambda 5 spectrophotometer. A Hellma Suprasil quartz cell (10 mm path length) was used. The peptide concentration ($\sim 3-4 \times 10^{-4}$ M in Tris buffer 10^{-2} M, pH 7.4) was determined by the molar extinction coefficients of the aromatic residues (tyrosine: λ_{max} 274.6 nm, ε 1420; phenylalanine: λ_{max} 257.4 nm, ε 197, in aqueous neutral solution) [14].

Circular Dichroism

Circular dichroism spectra were obtained in water and in aqueous 10 mM β-cyclodextrin solution, at 298 K, over 250–305 nm, with a Jasco 715 spectropolarimeter connected with a PC IBM PS/2 Model 40 SIC for the spectra elaboration (J700 Windows program). A Hellma Suprasil quartz cell of 0.5 cm path length was used and six scans were accumulated for all spectra. The sample concentration was about 5 mg/ml. The spectra reported are original computer-drawn CD curves: $[\Theta]_R$ represents the molar ellipticity (deg cm^2 $dmol^{-1}$).

Fluorescence

Fluorescence measurements were performed at 25 °C in Tris 10^{-2} M buffer pH 7.4, on a Perkin Elmer LS50B spectrofluorometer using Hellma Suprasil quartz cells (1 cm path length). Eight scans were accumulated for all spectra and final fluorescence intensities were obtained after subtraction of the blank contribution. Sample solutions were deoxygenated by flushed nitrogen. An excitation and emission slit of 5 nm bandpass was used. The quantum yield of Nphe fluorescence in the model [$Nala^1$]-deltorfin peptoid **III** was determined by comparison with the emission spectrum of L-tyrosine in aqueous solution, (quantum yield, 0.14) [15]. The peptide concentration was measured by UV absorbance. Excitation wavelengths for L-tyrosine and Nphe were 275 and 257 nm, respectively. Relative fluorescence intensities of solutions having the same absorbance ($A < 0.05$) at the excitation wavelengths were determined by integration of the spectral areas and the quantum yield of the peptoid fluorophore was calculated according to Equation (1)

$$\Phi^o{}_D = \frac{Area_{Nphe}}{Area_{L-Tyr}} \times 0.14 \quad (1)$$

Energy Transfer Experiments

Förster excitation energy transfer was used to determine the average intramolecular distances between Nphe (donor) and Ntyr or Tyr (acceptors), in **II**, **IV** and **VI**.

The quantum-mechanical theory advanced by Förster [16] describes the transfer mechanism as weak resonance coupling. The efficiency, E, of the transfer is:

$$E = 1 - F/F_0 = R_0{}^6/(R^6 + R_0{}^6) \quad (2)$$

where F and F_0 are the fluorescence intensities of the donor in the presence and the absence of the acceptor, respectively. R is the distance between the centres of the two chromophores and R_0 (Förster critical distance) is the distance between the donor and the acceptor when the transfer efficiency is 50%.

The emission spectra of Nphe in the deltorphin analogues **II**, **III**, **IV** and **VI** were recorded between 267 and 278 nm with excitation at 257 nm, using 2.8×10^{-5} M solutions (Tris 10^{-2} M buffer, pH 7.4). The areas of the emission spectra, F and F_0, have been calculated using the FL WinLab program

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provided by Perkin Elmer. The characteristic distance R_0, was determined by Equation (3)

$$R_0 = [(8.97 \times 10^{-25})(k^2/n^4)\Phi^o{}_D J_{AD}]^{1/6} \text{ (cm)} \quad (3)$$

where k^2 is the dipole–dipole orientation factor and can assume values in the range 0 to 4; in a situation of dynamic random orientation the value 0.66 can be applied. In Equation (3) n is the refractive index of the solvent medium between the donor and the acceptor, for aqueous solvents a value of 1.38 is applied [17]; $\Phi^o{}_D$ is the donor fluorescence quantum yield in the absence of the acceptor and J_{AD} is the overlap integral of the donor emission and of the acceptor absorption spectra, determined by Equation (4)

$$J_{AD} = \frac{\Sigma F_D(\lambda)\varepsilon_A(\lambda)\lambda^4}{\Sigma F_D(\lambda)} \quad (4)$$

where $F_D(\lambda)$ are the spectral data of the donor emission and $\varepsilon_A(\lambda)$ are the molar absorption coefficents of the acceptor.

Förster parameters and average intramolecular distances of peptoid **II**, retropeptoid **IV** and peptide–peptoid hybrid **VI** are shown in Table 2.

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 [18]. Agonists were evaluated for their ability to inhibit the electrically evoked twitch. The biological potency of the 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) (Table 3).

Table 2 Förster Parameters and Average Intramolecular Distances

Peptoid	Donor-acceptor pair	E (Transfer efficiency)	J_{AD} ($\times 10^{-16}$ cm^4) Spectral overlap integral	R_0 (Å) Förster critical distance	R (Å)
II	$Ntyr^1$-$Nphe^3$	0.434	3.4	8.76	9.16
IV	$Nphe^5$-$Ntyr^7$	0.161	4.34	9.12	12.01
VI	Tyr^1-$Nphe^3$	0.221	4.04	9.02	11.12

Peptoid concentration 2.8×10^{-5} M in Tris 10^{-2} M, pH 7.4. Quantum yield of fluoresence of Nphe (8.23×10^{-3}) was determined from the fluorescence spectrum of the model peptoid III by comparison with the quantum yield (0.14) of L-tyrosine in aqueous solution [15].

Table 3 Biological Activity of the Indicated Analogues on Mouse Vas Deferens and Guinea-pig Ileum Preparations

		IC_{50} (nM) MVD	IC_{50} (nM) GPI
Dermorphin	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	16 ± 3	1.2 ± 0.2
[D-Ala^2]Deltorphin I	H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	0.3 ± 0.01	65 ± 10
II	H-Ntyr-Nala-Nphe-Nasp-Nval-Nval-Gly-NH_2	2500 ± 300	—
IV	H-Gly-Nval-Nval-Nasp-Nphe-Nala-Ntyr-NH_2	$16\,600 \pm 1500$	$\gg 65\,000$

IC_{50} agonist concentration that produced 50% inhibition of the electrically evoked twitch; the values are the mean of not less than five tissue samples ± SEM.

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Synthesis of the Fmoc-*N*-alkylglycines

Fmoc-Nala-OH, Fmoc-Nphe-OH, Fmoc-Nval-OH and Fmoc-Ntyr(Bu^t)-OH were prepared from the appropriate amine and an alkyl (ethyl, benzyl or *tert*-butyl) bromoacetate as previously described [20]. An attempt to prepare Fmoc-Nasp(OBu^t)-OH starting from ethyl glycinate and *tert*-butyl bromoacetate failed (Procedure A) and the desired compound was obtained by reacting *tert*-butyl glycinate with benzyl bromoacetate followed by catalytic hydrogenation and reaction with Fmoc-OSu (Procedure B). The synthesis of Fmoc-Nasp(OBu^t)-OH by reductive amination of glycine tert-butyl ester with glyoxylic acid has also been described [10] but no analytical data were reported.

Fmoc-Nasp(OBu^t)-OH

Procedure A. *tert*-Butyl bromoacetate (4 ml, 24.4 mmol) was added dropwise to a stirred solution of H-Gly-OEt hydrochloride (8.52 g, 61 mmol) and TEA (8.5 ml, 61 mmol) in anhydrous THF (40 ml). The reaction mixture was kept overnight at room temperature, the precipitate was removed by filtration and the filtrate was evaporated to dryness. The oily residue was dissolved in ethyl acetate (70 ml), throughly washed with saturated aqueous NaCl (10 × 20 ml) and dried. Evaporation of the solvent gave an oil (5.6 g) which was dissolved in ethyl acetate (4 ml) and *n*-hexane (4 ml) was added. Precipitation occurred on standing overnight. The precipitate was filtered off and the filtrate evaporated to dryness: yield 5.05 g of H-Nasp(OBu^t)-OEt (95%, oil). ^{1}H-NMR, 200 MHz, $CDCl_3$: 1.26 (t, 3H, O-CH_2-$\underline{CH_3}$), 1.44 (s, 9H, C($\underline{CH_3}$)$_3$), 3.36 (s, 2H, $\underline{CH_2}$-$COOBu^t$), 3.45 (s, 2H, $\underline{CH_2}$-COOEt), 4.20 (q, 2H, O-$\underline{CH_2}$-CH_3). The oil (5.04 g, 23.24 mmol) was dissolved in a mixture of dioxane (107 ml) and methanol (43 ml) and 4N NaOH (5.8 ml, 23.24 mmol) was added. After 4 h the solvent was removed, the solid residue was dissolved in water (170 ml) and the pH of the solution was adjusted to ~9.0 by adding a few drops of concentrated HCl. A solution of Fmoc-OSu (7.84 g, 23.24 mmol) in acetonitrile (170 ml) was added under stirring and the reaction was monitored by TLC in E6. The pH was kept in the range 8.0–8.5 by adding TEA. After 6 h at 25 °C the organic solvent was removed and the aqueous phase was washed with ethyl acetate (2 × 60 ml), acidified to pH 2–3 by adding citric acid and re-extracted with ethyl acetate (3 × 50 ml). The combined organic layers were washed with saturated aqueous NaCl (3 × 40 ml), dried and taken to dryness. The oily residue was dissolved in the minimum amount of ethyl acetate and precipitated with *n*-hexane: yield 2.57 g (solid). ^{1}H-NMR, 250 MHz, DMSO-d_6: 4.0 (s, 2H, $\underline{CH_2}$-COOH), 4.1 (s, 2H, $\underline{CH_2}$-COOH), 4.25 (s broad, 3H, $\underline{CH}$-Fmoc, $\underline{CH_2}$-Fmoc), 7.40 (m, 4H, ArH-Fmoc), 8.15 (d, 2H, ArH-Fmoc), 8.40 (d, 2H, ArH-Fmoc). No signals related to the *tert*-butyl group appeared in the NMR spectrum. $[M + H]^+$ 356.12; calculated for Fmoc-Nasp-OH 355.35. According to the analytical data both carboxyl groups are free in the isolated compound.

Procedure B. DIEA (21.4 ml, 125 mmol) was added dropwise to a stirred suspension of H-Gly-OBu^t hydrochloride (8.35 g, 50 mmol) in THF (30 ml). The DIEA hydrochloride was removed by filtration and a solution of benzyl bromoacetate (7.85 ml, 50 mmol) in THF (30 ml) was added dropwise to the filtrate. The mixture was refluxed by monitoring the reaction by TLC in E1bis. After 18 h, the solution was cooled to 20 °C, the solvent was removed *in vacuo* and the residue was taken up with ethyl acetate (150 ml), filtered to eliminate the DIEA hydrobromide, washed with 0.05 M aqueous $KHSO_4$ (3 × 50 ml), saturated aqueous NaCl (2 × 50 ml), dried and evaporated to dryness: yield 9.11 g of H-Nasp(OBu^t)-OBzl (64.5%, oil); single spot by TLC in E1bis; ^{1}H-NMR, 200 MHz, $CDCl_3$: 1.42 (s, 9H, C($\underline{CH_3}$)$_3$), 3.43 (s, 2H, $\underline{CH_2}$-$COOBu^t$), 3.58 (s, 2H, $\underline{CH_2}$-COOBzl), 5.20 (s, 2H, $\underline{CH_2}$-Ph), 7.35 (s, 5H, Ph). $[M + H]^+$ 280.15; calculated for H-Nasp(OBu^t)-OBzl 279.33.

H-Nasp(OBu^t)-OBzl (9 g, 32.2 mmol) was dissolved in methanol (100 ml) and hydrogenated in the presence of 10% Pd/C. The reaction was monitored by TLC in E1bis and after 1 h the catalyst was removed by filtration and the filtrate was evaporated to dryness: yield 5.92 g (97%); single spot by TLC in E1bis. ^{1}H-NMR, 200 MHz, $CDCl_3$: 1.4 (s, 9H, C($\underline{CH_3}$)$_3$), 3.25 (s, 2H, $\underline{CH_2}$-$COOBu^t$), 3.39 (s, 2H, $\underline{CH_2}$-COOH).

Fmoc-OSu (11.8 g, 35 mmol) was dissolved in acetonitrile (100 ml) and added dropwise to a stirred solution of H-Nasp(OBu^t)-OH (5.9 g, 31.2 mmol) in 50% aqueous acetonitrile (100 ml). The pH of the mixture was adjusted at 8.5–9.0 with DIEA and the reaction was monitored by TLC in E1bis. After 90 min the organic solvent was removed *in vacuo*, water (100 ml) was added and the resulting solution was extracted with ethyl ether. The title

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compound separated from the aqueous phase as an oil which was collected, washed with ether (3 × 50 ml), dissolved in ethyl acetate (150 ml), washed with saturated aqueous NaCl (2 × 50 ml) and dried.

Evaporation of the solvent gave a solid: yield 9.67 g (75%); single spot by TLC in E1bis. ^{1}H-NMR, 200 MHz, $CDCl_3$: 1.32 (s, 9H, C($\underline{CH_3}$)$_3$), 3.85 (s, 2H, $\underline{CH_2}$-COOBu^t), 3.96 (s, 2H, $\underline{CH_2}$-COOH), 4.06 (m, 1H, $\underline{CH}$-Fmoc), 4.19 (d, 2H, $\underline{CH_2}$-Fmoc), 7.09–7.65 (m, 8H, $\underline{ArH}$-Fmoc).

Solid Phase Synthesis

Tha assembly of peptide **I**, peptoids **II** and **III** and retropeptoid **IV** on the Advanced ChemTech 348Ω Peptide Synthesizer, was performed on a 0.06 mmol scale, starting with Rink amide MBHA resin (0.085 g, substitution 0.73 mmol/g). The synthesis of **I** was achieved by the FastMoc methodology (HBTU/HOBt/DIEA, NMP as the solvent, single acylation protocol, coupling time 45 min). For the synthesis of **II**, **III** and **IV**, activation of the carboxyl component was achieved by adding HATU, and the coupling time was doubled. Owing to their low solubility in NMP, freshly prepared solutions of Fmoc-Nasp(OtBu)-OH and Fmoc-Nphe-OH were added to the reaction vessel. The final peptide, or peptoid, resin was N^α-deprotected with 20% piperidine in NMP, thoroughly washed with NMP and dried. Cleavage from the resin and removal of the side-chain protecting groups were simultaneously achieved by treatment with a mixture of TFA : H_2O : TES (95/2.5/2.5 by vol) (4 ml/100 mg of peptide resin, 3.5 h at room temperature). The acid solution was concentrated *in vacuo* and the peptide analogue was precipitated with excess *tert*-butyl-methyl ether, collected by centrifugation, dissolved in a small volume of TFA and reprecipitated twice with *tert*-butyl-methyl ether and finally dried. Peptoid analogues were further purified by semipreparative HPLC. All products were characterized by reverse phase analytical HPLC and molecular weight determination. The synthesis and the characterization of the peptide–peptoid hybrids **V–IX** have been already reported [12].

H-Ala-(D)Ala-Phe-Asp-Val-Val-Gly-NH$_2$, I (Ala^1, D-Ala^2)-deltorphin I. The synthesis started with Rink amide MBHA resin and the peptide was cleaved from the resin and worked up as described previously: yield 29.4 mg (73%), homogeneous by analytical HPLC. $[M+H]^+$ 677.4; calculated for **I** 676.4.

H-Ntyr-Nala-Nphe-Nasp-Nval-Nval-Gly-NH$_2$, II Deltorphin I peptoid. The peptoid was assembled in seven consecutive Fmoc-cleavage-coupling cycles, as previously described. The peptoid resin (126 mg) gave the crude peptoid (38.2 mg) which was purified by semipreparative HPLC. Final yield 11.5 mg (25%), single peak by analytical HPLC. $[M+H]^+$ 769.4; calculated for **II** 768.4.

H-Nala-Nala-Nphe-Nasp-Nval-Nval-Gly-NH$_2$, III (Nala^1)-deltorphin I peptoid. The synthesis was carried out as described for **II** yielding 122 mg of peptoid resin. An aliquot of the peptoid resin (50 mg) was cleaved and the crude product (13.1 mg) was purified by semipreparative HPLC. Final yield 5.5 mg (33%), single peak by analytical HPLC. $[M+H]^+$ 677.4; calculated for **III** 676.4.

H-Gly-Nval-Nval-Nasp-Nphe-Nala-Ntyr-NH$_2$, IV Deltorphin I retropeptoid. The synthesis was carried out as described for **III.** Yield 135 mg of peptoid resin. An aliquot (50 mg) was cleaved and the crude peptoid (16 mg) was purified by semipreparative HPLC. Final yield 9.3 mg (55%), single peak by analytical HPLC. $[M+H]^+$ 769.4; calculated for **IV** 768.4.

RESULTS AND DISCUSSION

Infrared Absorption Analysis

The poor solubility of the peptide, peptoid and retropeptoid analogues listed in Table 1 prevented FT-IR absorption measurements in solution and their conformational preferences were examined in the solid state only (KBr pellets). The IR absorption spectra of **II**, **IV**, **V**, **VI**, **VII**, **IX**, in the 3500–3200 cm^{-1} region (amide A), are shown in Figure 1. The spectrum of [D-Ala^2]-deltorphin I, **C**, is also shown for comparison. The main amide I and amide II bands (1800–1500 cm^{-1}) are reported in Table 4. In the amide A region, [D-Ala^2]-deltorphin I shows a strong IR band at 3280 cm^{-1}, associated with strongly H-bonded NH groups. The band is not significantly affected by the Tyr/Ntyr and Phe/Nphe substitution (**V** and **VI**, respectively) but the substitution of one (**VII**) or both (**IX**) valine residues dramatically decreases the extent of H-bonding and there are no significant bands near 3280 cm^{-1}. In **II** and **IV**, the NH residues are almost

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Table 4 IR Frequencies (cm^{-1}) in the Solid State (KBr pellets) of [D-Ala[2]]-deltorphin I, Deltorphin I peptoid, Retropeptoid and Peptide-peptoid Hybrids

Peptide		Amide I				Amide II	
[D-Ala[2]]-deltorphin I	**C**	1689 m/w		1665 m/w	1639 m	1547 b	1535 b
[Ala[1],D-Ala[2]]-deltorphin I	**I**	1687 sh		1665 sh	1647 s	1553 sh	1539 m/w
[Ntyr[1],D-Ala[2]]-deltorphin I	**V**	1689 m/w		1667 m/w	1645 s	1555 b	1539 b
[D-Ala[2],Nphe[3]]-deltorphin I	**VI**	1683 b		Broad	1645 m	broad	1537 b
[D-Ala[2],Nval[5]]-deltorphin I	**VII**	1685 sh		1663 m		1555 sh	1535 b
[D-Ala[2],Nval[5,6]]-deltorphin I	**IX**	1685 sh		1667 m/w		1555 b	1539 m/w
Deltorphin I peptoid	**II**		1673 s		1645 sh	1555 sh	1543 w
Deltorphin I retropeptoid	**IV**		1671 m		1645 sh	1545 sh	1537 w
[Nala[1]]-deltorphin I peptoid	**III**		1675 s		1651 sh	1545 sh	1539 w

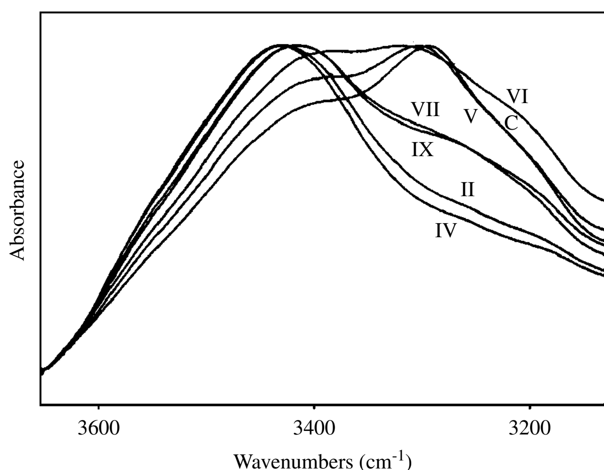

Figure 1 IR Spectra (KBr pellets) of the indicated compounds in the amide A zone.

lacking and no appreciable amounts of H-bonding are observed.

The amide I bands of [D-Ala[2]]-deltorphin I at 1639, 1665 and 1689 cm^{-1}, may be indicative of antiparallel β-sheet conformations [20,21] together with some folded conformations stabilized by $1 \leftarrow 4$ intramolecular H bonds. The presence of folded structures in deltorphin analogues has been suggested by NMR analysis and distance-geometry calculations [22]. In **V** and **VI**, the main amide I band shifts to 1645 cm^{-1}, indicating the existence of predominantly random coil conformations along with $1 \leftarrow 4$ intramolecular H bonded structures. The IR spectra of **VII** and **IX** show only an amide I peak at $\approx$1665 cm^{-1} with a shoulder at 1685 cm^{-1}. The introduction of *N*-substituted amino acids into the peptide sequences restricts their conformational flexibility and increases the occurrence of *cis* structures in the tertiary amide bonds, making more difficult the β-sheet formation [23]. A parallel trend is shown in the amide A region where the absorption at 3280 cm^{-1} of **VII** and **IX** is significantly decreased. However, $1 \leftarrow 4$ hydrogen bonds are still possible and the secondary structure of **VII** and **IX** is probably characterized by the presence of type II and type VI β-turns [23]. The IR spectra of **II** and **IV** show the amide I band at $\approx$1673 cm^{-1} arising from stretching vibrations of unbonded C=O. In peptoids the hydrogen bond formation is impossible and the steric requirements of the *N*-alkylglycine residues probably promote the formation of polyglycine II extended conformations. A systematic conformational analysis of sequences with up to five peptoid units, performed on the basis of quantum chemical calculations and molecular dynamic simulations [23], shows a strong tendency to accept periodically secondary structures as polyglycine II or α helix. Polyglycine II remains more stable than the α helix because the larger space requirements of the *N*-alkyl groups are better compensated by an increase of bond angles in the peptoid chain than by torsion angle changes.

Fluorescence Measurements

The insolubility of the model peptide **I** in Tris 10^{-2} M, pH 7.4, prevented the determination of the quantum yield of fluorescence of the donor residue Phe. Measurements were only performed with compounds containing the Nphe residue (**II**, **IV** and **VI**), using [Nala[1]]-deltorphin I peptoid **III** as a model. The benzene bands in the absorption spectrum of **III** in water, are located at 252, 257,4 (maximum) and 266 nm. The emission spectrum

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obtained by excitation at 257 nm is centred at 285 nm and partially superimposes the absorption band of the acceptor Ntyr in **II** and **IV** (λ max 272.5 nm and 275 nm, respectively) and Tyr in **VI** (λ max 275 nm). The overlap integral J that appears in Equation (4) is not zero. A fluorescent quantum yield $\Phi^o{}_D$ of 8.23×10^{-3} was obtained for the Nphe^3 residue in **III**. The Förster parameters and average intramolecular distances obtained from the fluorescent measurements are summarized in Table 2. The intramolecular donor–acceptor distance results in an average separation of about 9 Å between the aromatic side chains of Nphe^3 and Ntyr^1 in **II**. A larger intramolecular distance is obtained between Nphe^5 and Ntyr^7 in retropeptoid **IV** (~12 Å) and between Tyr^1 and Nphe^3 in **VI** (~11 Å). The mean distances between the aromatic residues could indicate that the conformation of the *C*-terminal tripeptide of **IV** (-Nphe -Nala-Ntyr-) is more extended in comparison with that of the *N*-terminal tripeptide of **II** (Ntyr-Nala-Nphe-). In the peptide–peptoid hybrid **VI** the average distance of the aromatic rings is intermediate between those measured in **II** and **IV**. Many conformational studies have been carried out to explain the selectivity of the opioid peptides and to identify the bioactive conformations that bind the δ- and μ-receptors, respectively. Careful conformational analyses on deltorphin analogues, carried out by NMR and distance-geometry techniques [22], indicate that the selectivity for the δ- and μ-opioid receptors could be ascribed to the spatial arrangements of the aromatic moieties. Conformations in which the distance between the Tyr^1 and Phe^3 aromatic rings is about 8 Å recognize the δ-opioid receptors, and μ-receptor recognition is attained by topologies in which the distance between the aromatic rings is larger than 12–13 Å. The distance values calculated for **IV** and **II** are thus similar to those considered the best for binding μ-receptors and δ-receptors, respectively [22].

Induced Circular Dichroism (ICD)

Many different molecules may interact with the chiral hydrophobic cavity of β-cyclodextrin [24] allowing the formation of inclusion complexes that are considered good models for studying the protein–ligand interaction and the enzymatic catalysis. The complexes of β-cyclodextrin with phenolic compounds are particularly interesting. The H-bond formation between phenols and the hydroxyl groups of the β-cyclodextrin macrocycle mimics the interaction of aromatic amino acid residues within protein matrices. The parameters of complexation have been studied and several types of interaction have been proposed in terms of van der Waal interaction, H-bonding interaction, release of high-energy water and decrease in strain energy in the β-cyclodextrin cavity. The photophysical and photochemical properties of the guest molecule are modified to a different extent according to its molecular size and shape and the degree of interaction with the β-cyclodextrin cavity [24]. The circular dichroism induced in benzene derivatives provides a direct proof of the association with β-cyclodextrin and is very sensitive to their orientation and position in the host cavity [25,26]. Circular dichroism spectra of peptoid **II**, retropeptoid **IV**, [Nala^1]-peptoid **III** and [Ntyr^1,D-Ala^2]-deltorphin **V** in aqueous 10 mM β-cyclodextrin, are shown in Figure 2. The CD spectra of [D-Ala^2,Nphe^3]-deltorphin **VI** in water and in aqueous 10 mM β-cyclodextrin solution are reported for comparison (Figure 3). The position of the negative band of the Tyr^1 residue is not affected by the presence of β-cyclodextrin, but the optical activity is higher than in water. The dichroic band of the achiral Ntyr^1 residue (peptoid **II**) and Ntyr^7 residue (retropeptoid **IV**) is similar to that shown by the Tyr^1 residue of **VI**, but in **II** the intensity is much lower. The CD spectrum of the [Ntyr^1,D-Ala^2]-deltorphin **V,** does not exhibit any signal due to a possible inclusion complex and only shows the dichroic band of the Phe^3 residue. The [Nala^1]-deltorphin peptoid **III**, where the Ntyr^1 is missing and the residue in position 3 is achiral, does not show dichroic signals. The optical activity observed for the repropeptoid **IV,** more negative than that of peptoid **II**, indicates that the Ntyr^7 side chain is inserted more deeply into the host cavity than that of the Ntyr^1 and suggests a greater exposure of the aromatic residue in agreement with the more extended conformation determined by the fluorescent measurements. The Ntyr of **V** does not form an inclusion complex. Probably structural factors and/or the polarity of the next amino group play, to a different extent, a negative role in determining the complex formation of **II** and **V** with β-cyclodextrin.

Pharmacological Screening

We had already demonstrated that the substitution of some residues in the dermorphin and [D-Ala^2] deltorphin I sequences with the corresponding *N*-alkylglycine produced a dramatic decrease in the

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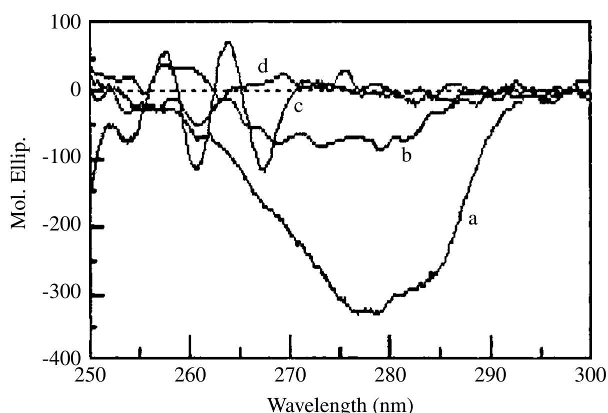

Figure 2 CD Spectra of **IV** (a), **II** (b), **V** (c) and **III** (d) in aqueous 10 mM β-cyclodextrin solution.

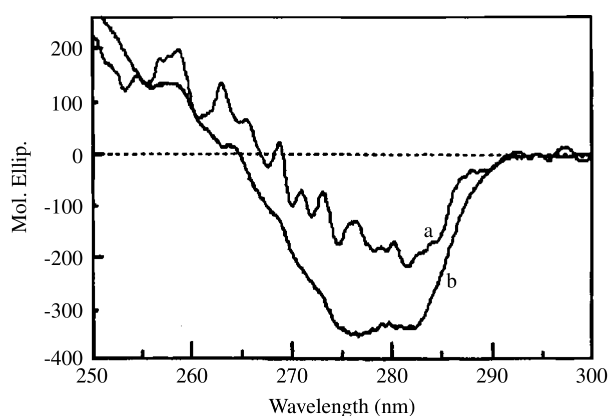

Figure 3 CD Spectra of **VI** in (a) water, (b) aqueous 10 mM β-cyclodextrin solution.

affinity of the resulting peptide-peptoid hybrids for the μ- and δ-opioid receptors [12]. The substitution of Tyr^1, or Phe^3, practically cancelled any agonistic activity of both peptides 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 greatly 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. Replacement of all amino acid residues with the corresponding *N*-alkylglycines produced a dramatic decrease (8000 to 50 000 fold) in the agonistic activity of both the deltorphin I peptoid and retropeptoid (**II** and **IV** respectively) in comparison with [D-Ala^2]-deltorphin I when evaluated on MVD preparations, and a complete loss of biological activity when tested on GPI preparations. The possible formation of a polyglycine II extended conformation promoted by the *N*-alkylglycine residues [23], and suggested by the IR spectra of **II** and **IV**, could contribute to the dramatic effect on the agonistic activity of both compounds.

Acknowledgements

We would like to thank Professor B. Pispisa (Roma Tor Vergata University) for his help in the interpretation of the fluorescence data. Financial support from MIUR (Cofin 2002) and the National Research Council of Italy is gratefully acknowledged.

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