Tritiated deltorphin analogues with high specific radioactivity and high affinity and selectivity for delta opioid receptors

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Summary

New conformationally constrained deltorphin I and II analogues were designed and synthesized, using a more lipophilic amino acid (Ile) instead of Val at positions 5 and 6, and 2-aminotetralin-2-carboxylic acid (Atc) at position 3. Two analogues (Tyr-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2 and Tyr-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2) with high potency and selectivity for δ opioid receptors were chosen for tritiation, with 3,5-I_2-Tyr^1-deltorphin analogues as precursors. Catalytic dehalotritiation of these precursors resulted in tritiated peptides with high specific radioactivity (1.28 TBq/mmol (34.5 Ci/mmol) and 1.33 TBq/mmol (36.0 Ci/mmol), respectively).

Key words: deltorphins, dehalogenation, iodination, opioid receptors

Introduction .

The heterogeneity of opioid receptors is well established and it is accepted that there are at least three types of opioid receptors (μ, κ and δ). The development of selective opioid ligands has

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contributed enormously to the pharmacological characterization of opioid receptors, and the receptor types have recently been cloned.

To date, no opioid ligands have been found to exhibit absolute specificity for any particular receptor type, so the emphasis is to find ligands that have as high a specificity as possible for them. Detailed studies of these receptor types require radiolabelled ligands with high receptor binding affinity, high selectivity and specific activity in the radioreceptor assay.

The efforts of several laboratories over several years have led to the development of many new δ opioid receptor radioligands, including 4'-halogenated-Phe^4-DPDPE (D-Pen^2,D-Pen^5-enkephalin (1)), deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2, (2)), deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2, (3)) and its analogues (4), naltrindole (5) and its analogues (6), TIPP (7) and TIPP[Ψ] (8) labelled with ^3H and ^{125}I.

The currently most potent and most selective δ agonists are the deltorphins. In recent years numerous analogues have been synthesized for structure-activity relationship studies. One such analogue is Ile^{5,6}-deltorphin II (9). Substitution of the Val^{5,6} residues in deltorphin II with the more hydrophobic Ile^{5,6} increases the affinity and selectivity for δ receptors in rat brain membranes. We have synthesized this peptide in tritiated form (10) and applied it as a very useful and stable radioligand in the binding assay.

We earlier reported new analogues of Ile^{5,6}-deltorphin I and II with 2-aminotetralin-2-carboxylic acid (Atc) at position 3 (11). The new analogues are more active and more selective δ opioid ligands than the parent peptides. In the present paper we describe the synthesis and radiolabelling of these two new deltorphin analogues with highest specificity for δ receptors.

Results and discussion

Peptides were synthesized by usual solid-phase techniques with Boc strategy, using dicyclohexyl-carbodiimide (DCC)/hydroxybenzotriazole (HOBt) as coupling agents. Atc was prepared by a modified Strecker synthesis (12, 13). Boc-Atc was obtained in racemic form and incorporated as such into the peptides. The diastereomeric peptides were isolated separately by semipreparative HPLC. The configuration of Atc in the peptides was determined from the amino acid mixture

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obtained from the peptides after hydrolysis by chiral TLC (details in the Experimental). (*R*)- and (*S*)-Atc were prepared by enzymatic digestion, using N-trifluoroacetyl-Atc and carboxypeptidase A. The absolute configuration of (*R*)-Atc was determined by X-ray crystallography. Other supporting evidence for the identification of (*R*)-Atc and (*S*)-Atc was obtained by chiral TLC (14). In general, the L-amino acids have higher R_f values than the D-amino acids on chiral TLC plates with acetonitrile-methanol-water (4:1:1) as eluent. In this case (*S*)-Atc has a higher R_f than (*R*)-Atc (11).

The results of binding studies (15) demonstarated that the two most potent analogues, Tyr-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2 and Tyr-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2, were prepared in tritiated form. Diiodotyrosine (Dit) containing peptides were used as precursors; these were obtained by iodinating the peptides in the presence of 4-5 equivalents of NaI by the chloramine-T method (16). Under these circumstances, only Dit-containing peptides were obtained.

The radioactive ligands were stored in 37 MBq/mL (1 mCi/mL) concentration under liquid N_2. The purities were checked by TLC and HPLC from time to time, and the compounds were found to remain stable for more than 6 months (>95%).

In the binding assay, both ligands proved very potent and highly selective for δ opioid receptors in rat brain membrane; the nonspecific binding was less than 20% in both cases, so there is a good chance to use them in the binding assays as δ ligands (15).

Experimental

Materials

Protected and unprotected amino acids, except for Atc, coupling reagents and 4-methyl-benzhydrylamine resin were purchased from Aldrich Chemical Co. and Bachem Feinchemicalien. Carboxypeptidase A was a Sigma product. PdO/$BaSO_4$ catalyst (10% Pd) was from Merck. Precoated plates (silica gel F254 (Merck, Darmstadt, Germany)) were used for TLC while Chiralplate (Macherey-Nagel, Düren, Germany) was applied for determination of the optical purity of Atc isomers. The following solvent systems were used: (I) butanol-acetic acid-water (BAW) (4:1:1); (II) butanol-acetic acid-pyridine-water (15:3:10:12) and (III) acetonitrile-methanol-water

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(4:1:1). Ninhydrin, UV light and I_2 were employed to detect the peptides. Reversed-phase HPLC (RP-HPLC) was performed on a Merck-Hitachi liquid chromatograph, utilizing a Vydac 218TP54 C_{18} column for analytical purposes or a Vydac 218TP1010 C_{18} column for semipreparative separations.

Quantitative amino acid analyses were performed on a HP 1090 Amino Quant amino acid analyser (Hewlett-Packard, Waldbronn, Germany), on a Hypersyl ODS C_{18} column (200x2 mm I.D., 5 μm particle size, Shandon Scientific). Molar masses of peptides were determined by mass spectrometry. ^{1}H-NMR spectra were measured on a Brucker AM 400 spectrometer (Brucker, Zug, Switzerland).

$^3H_2$ gas was purchased from Technabexport, Russia, and contained at least 98% $^3H_2$. Radiochemical purity was checked with a Berthold Radiochromatogram Tracemaster. The amount of tritiated material was measured by UV detection on a Shimadzu-160 spectrophotometer. Radioactivity was counted in a toluene-Triton X-100 scintillation cocktail with a Searle-Delta-300 liquid scintillation counter.

Methods

2-Aminotetralin-2-carboxylic acid (Atc) (1)

A warm mixture of water (50 mL) and ethylene glycol (50 mL) was added to 7,8-benzo-1,3-diazaspiro[4.5]decane-2,4-dione (5.4 g, 25 mmol) and 20 g of $Ba(OH)_2.8H_2O$, and the mixture was stirred and refluxed for 27 h. The solution was diluted with water (100 mL) and carefully acidified in a waterbath with 4 N H_2SO_4 to pH 2. The mixture was stirred for 1 h, filtered and washed with water. The pH of the filtrate was set to 5 with 25% NH_3. The desired amino acid slowly precipitated as a white powder (4.63 g, 24 mmol).

Yield: 97%, mp: 309-310 °C.

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N-Trifluoroacetyl-2-aminotetralin-2-carboxylic acid (2)

To a solution of racemic 1 (2.87 g, 15 mmol) in trifluoroacetic acid (TFA) (20 mL), trifluoroacetic anhydride (5.21 mL, 37 mmol) was added dropwise and the mixture was vigorously stirred for 2 h at 0 °C, and then for 2 days at room temperature. After the solvent and excess anhydride had been removed under reduced pressure, the residue was diluted with water (30 mL), extracted in turn with ethylacetate (EtOAc) (2x30 mL), brine (2x30 mL) and water (2x30 mL), dried over $MgSO_4$ and concentrated. The resulting yellow oil was crystallized from EtOAc and petroleum ether (bp: 30-40 °C): Yield:1.94 g (45%).

TLC: $CHCl_3$:EtOH:AcOH 90:10:1 R_f=0.48

^{1}H-NMR (DMSO): δ:2.05 (m, 1H); δ:2.40 (m, 1H); δ:2.73 (m, 2H); δ:3.12 (d, 1H); δ:3.25 (d, 1H); δ:7.1 (m, 4H); δ:9.5 (s, 1H); δ:13 (s, 1H).

Enzymatic separation of 2 with carboxypeptidase A

1.5 g of 2 was suspended in 150 mL of water. The pH was adjusted to 7.5-8 with NaOH, 200 μL of carboxypeptidase A (~2-3 mg) was added and the mixture was stirred for 1 day. From time to time the pH was adjusted to 7.5-8. After completion of the reaction, charcoal was added to the solution to remove the enzyme. After filtration, the pH of the solution was adjusted to 3 with 1 N HCl. The unreacted 2 was extracted with EtOAc. The aqueous solution was evaporated to ~50 mL and the pH was adjusted to 5-6. The precipitated crystals of 1 were filtered off and dried. Yield: 478 mg (93%). The EtOAc solution was dried over $MgSO_4$ and evaporated, and the oil was hydrolysed by refluxing in 6 N HCl (40 mL) for 6 h. The solution was evaporated to dryness and the residue was dissolved in 20 mL of water. The pH was adjusted to 6 with 1 N NH_4OH. The crystals were filtered off. Yield: 210 mg (41%).

Chiral TLC: eluent: acetonitrile-methanol-water (6:1:1).

R_f (1 from aqueous solution): 0.57;

R_f (1 from organic solution): 0.63.

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The absolute configuration was determined by X-ray crystallography on **1**.HBr (in aqueous solution) and was found to be *R*; optical rotation: -7.16° in water.

N-α-Boc-(R,S)-2-aminotetralin-2-carboxylic acid (3) was prepared by a literature procedure (17). Mp: 182 °C.

^{1}H-NMR (ppm) ($CDCl_3$): δ:1.43 (s, 9H); δ:2.13 (m, 2H); δ:2.85 (s, 3H); δ:3.38 (d, 1H); δ:7.13 (m, 4H); δ:7.25 (s, 1H).

Solid-phase peptide synthesis and purification of diastereomeric peptides

Peptide synthesis was performed by the manual solid-phase technique, with 4-methylbenzhydryl-amine resin (0.8 mmol/g of titratable amine). Boc-protected amino acids, with DCC and HOBt as coupling agents were used. Side chain protection was benzyl for Asp and Glu, and dichlorocarbobenzyloxy for Tyr. The deprotection solution was 50% TFA and 2% anisole in dichloromethane (DCM). Simultaneous deprotection and cleavage from the resin were accomplished by treatment with 90% HF and 10% anisole (9 mL HF and 1 mL anisole/g peptide resin) at 0 °C for 1 h. After evaporation of the HF, the peptide resin was washed with diethyl ether and the peptide was extracted with glacial acetic acid and lyophilized. Crude peptides were purified to homogeneity by RP-HPLC on a Vydac 218TP1010 C_{18} column with a linear gradient of 0.1% TFA (eluent A) and acetonitrile (AcCN) (0.1% TFA) (eluent B), from the starting 25% AcCN to 30% AcCN in 25 min. The final products were obtained as lyophilizates. Peptide purity was assesed by RP-HPLC and TLC in three different solvent systems. Mass spectroscopy confirmed the appropriate molar mass. Amino acid analyses gave the expected amino acid patterns.

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Table 1.

Physicochemical data on deltorphin analogues

Peptide	HPLC	TLC R_f[c]			MS
	k'	A	B	C	Mol. wt.
Tyr-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2	6.90[a]	0.533	0.480	0.644	822.6
Tyr-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2	4.82[a]	0.541	0.458	0.629	837
Dit-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2	4.61[b]	0.621	0.536	0.629	1075
Dit-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2	3.94[b]	0.614	0.580	0.593	1089

HPLC k' on a Vydac 218TP1010 C_{18} column (1.0 x 25 cm);

[a] gradient of 25-30% organic component in 25 min;

[b] gradient of 30-40% organic component in 20 min;

flow rate 4 mL/min. The solvent system was 0.1% TFA in water, 0.1% TFA in AcCN. Solvent breakthrough at 3.0 min.

[c] R_f values for thin-layer chromatograms in the following solvent systems:

A: n-butanol:acetic acid:water (4:1:1);

B: acetonitrile:methanol:water (4:1:1);

C: n-butanol:acetic acid:pyridine:water (38:6:24:20).

Determination of the configuration of 1 in the peptides

After RP-HPLC purification, two diastereomeric peptides were obtained. 1 mg of each peptide was hydrolysed separately in 6 N HCl under argon pressure in teflon bombs in a microwave oven (18). The solvent was removed by repeated evaporation from methanol-water 1:1. The dried samples were used for separation of the amino acid mixture and 1 was collected (Vydac 218TP1010 C_{18} column, linear gradient from 10 to 15% of AcCN in 0.1% TFA solution within 30 min; k' of 1: 2.73) and spotted onto a chiral TLC plate to determine the R_f value in acetonitrile-methanol-water (5:1:1). These R_f values were compared with those of standard (*R*)- and (*S*)-Atc (R_f of (*R*)-Atc: 0.37; R_f of (*S*)-Atc: 0.43).

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Dit-D-Ala-(R)-Atc-Glu-Ile-Ile-Gly-NH_2 (4)

4 mg of Tyr-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2 was dissolved in 1 mL of Na_2HPO_4 buffer (0.25 M, pH 7.4). 220 μL of 10 mg/mL NaI and (as an oxidizing agent) 1 mL of 2.5 mg/mL chloramine-T were added. After stirring for 10 sec, the resulting 4 was separated from the reaction mixture by HPLC using a linear gradient from 30% to 40% AcCN in 0.1% TFA in 20 min. Yield: 2.35 mg (47%).

$^3H_2$-Tyr-D-Ala-(R)-Atc-Glu-Ile-Ile-Gly-NH_2 (5)

1.8 mg (1.5 μmol) of 4 was dissolved in 1 mL of dimethylformamide (DMF) and 60 μL of 0.1 M triethylamine (TEA) in DMF, and 10 mg of $Pd/BaSO_4$ (10% Pd, oxidized form) was added as catalyst. After connection of the reaction vessel to the tritium manifold (19), the solution was frozen with liquid N_2 and evacuated. Tritium gas was introduced, liquid N_2 was removed, and the reaction mixture was stirred with a magnetic stirrer for 90 min. Tritiation was controlled by following the tritium pressure with a manometer. After the reaction was complete, the reaction mixture was frozen with liquid N_2, and unreacted tritium was absorbed on pyrophoric uranium. The liquid N_2 was removed and the reaction vessel was taken off the tritium manifold to work up the crude mixture. The catalyst was filtered through a Whatman GF/C filter and washed three times with $EtOH:H_2O$ (1:1). The solvent was evaporated and the labile tritium was removed by repeated evaporation from $EtOH:H_2O$ (1:1). The total radioactivity of the labelled peptide was measured by liquid scintillation counting, and proved to be 2.14 GBq (57.7 mCi).

The crude tritiated peptide was purified by TLC in BAW (4:1:1). The purity was checked by means of analytical HPLC. Quantitative analysis of the labelled peptide was performed via UV (210 nm) during elution (linear gradient rising from 23 to 28% AcCN in 25 min), with the unlabelled peptide as standard; the specific radioactivity was 1.33 GBq/mmol (36.0 Ci/mmol). The purified peptide was stored in 37 MBq/mL (1 mCi/mL) concentration under liquid N_2.

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Dit-D-Ala-(S)-Atc-Asp-Ile-Ile-Gly-NH_2 (6)

3.4 mg (3.63 μmol) of Tyr-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2 was dissolved in 1 mL of Na_2HPO_4 (0.25 M, pH 7.4) buffer, and 310 μL of 7 mg/mL NaI and 1 mL of 2.5 mg/mL chloramine–T solution were added. After rapid stirring, the resulting 6 was separated from the reaction mixture by HPLC, with a linear gradient rising from 30 to 40% AcCN in 0.1% TFA in 20 min. Yield: 2.4 mg (56%).

$^3H_2$-Tyr-D-Ala-(S)-Atc-Asp-Ile-Ile-Gly-NH_2 (7)

1.7 mg (1.4 μmol) of 6 was dissolved in 1 mL of DMF, and 100 μL of 10% TEA/DMF, and 10 mg of $Pd/BaSO_4$ (10% Pd, oxidized form) was added as catalyst. The tritiation procedure was the same as above. The reaction was complete in 80 min. The total radioactivity of the crude product was 1.23 GBq (33.2 mCi). The labelled peptide was purified by TLC in BAW (4:1:1). The purity was checked by analytical HPLC. The specific radioactivity was determined from a calibration curve made from unlabelled standards, and proved to be 1.28 TBq/mmol (34.5 Ci/mmol). The purified peptide was stored in 37 MBq/mL (1 mCi/mL) concentration under liquid N_2.

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