DESIGN, SYNTHESIS AND TRITIUM LABELING OF CONFORMATIONALLY CONSTRAINED DELTA OPIOID PEPTIDES

PhD Thesis

Zsuzsanna Darula

Institute of Biochemistry

Biological Research Center

Hungarian Academy of Sciences

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LIST OF ABBREVIATIONS

Aba	4-aminotetrahydro-2-benzazepin-3-one
AcOH	acetic acid
ACN	acetonitrile
Aic	2-aminoindane-2-carboxylic acid
Atc	2-aminotetralin-2-carboxylic acid
Boc	*tert*-butyloxycarbonyl
BOP	benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluoro-phosphate
DAMGO	Tyr-D-Ala-Gly-NMe-Phe-Gly-ol
DCC	N,N'-dicyclohexylcarbodiimide
DCM	dichloromethane
deltorphins:	
	deltorphin (dermenkephalin) H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2
	deltorphin I H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2
	deltorphin II H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2,
DIEA	diisopropylethylamine
Dit	3',5'-diiodotyrosine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DPDPE	H-Tyr-D-Pen-Gly-Phe-D-Pen-OH
ee	enantiomeric excess
enkephalins:	
	Leu-enkephalin H-Tyr-Phe-Gly-Gly-Leu-OH
	Met-enkephalin H-Tyr-Phe-Gly-Gly-Met-OH
ESI	electrospray ionization
EtOAc	ethyl acetate
FAB	fast atom bombardment (ionization)
Fmoc	fluorenylmethoxycarbonyl
GC	gas chromatography
GITC	2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate
GPI	guinea pig ileum
GTPγS	guanosine 5'-O-(γ-thio)triphosphate
Hat	2-aminotetralin-2-carboxylic acid

Hba	8-hydroxy-4(*S*)-amino-1,3,4,5-tetrahydro-3-oxo-2H-2-benzazepine-2-acetic acid
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate
Hfe	homo-phenylalanine
Htc	7-hydroxytetrahydroisoquinoline-3-carboxylic acid
HOBt	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
IC_{50}	50% inhibitory concentration
JOM-13	Tyr-c[D-Cys-Phe-D-Pen]OH
k'	capacity factor
K_i	equilibrium inhibition constant
Marfey's reagent	1-fluoro-2,4-dinitrophenyl-5-L-alanine amide
MeOH	methanol
mp	melting point
MS	mass spectrometry
MVD	mouse vas deferens
NMR	nuclear magnetic resonance (spectroscopy)
NOE	nuclear Overhauser effect
norBNI	norbinaltorphimine
Pen	penicillamine
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluro-phosphate
PyBroP	bromo-tris-pyrrolidino-phosphonium hexaflurophosphate
RP	reversed-phase
RT	room temperature
TBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoroborate
TEA	triethyl amine
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulfonic acid
THF	tetrahydrofuran
Tic	tetrahydroisoquinoline-3-carboxylic acid
TIPPΨ	Tyr-TicΨ[CH_2NH]Phe-Phe-OH
TLC	thin-layer chromatography

1. INTRODUCTION

1.1 The opioid receptor and opioid peptides

1.1.1 The opioid receptor

The history of opioid compounds started some 5000 years ago with the medical use of opium. Besides its analgesic effect, opium has several side effects among others mood changes which are the basis of its non-medical use and of its abuse. Morphine, the active compound of opium was isolated from the poppy plant in 1805 by Friedrich Sertürner. Unfortunately, it proved to be as addictive as opium causing also respiratory depression (Table 1) which is the main limit of clinical use of morphine and related compounds. All these discoveries prompted biologists to find the mechanism of action morphine has in the human body and chemists to develop more efficacious and safe drugs.

As soon as ligands of high specific radioactivity were readily available, the presence of stereospecific opioid binding sites in mammalian brain were demonstrated [1,2]. Opioid receptors are membrane proteins which belong to the seven transmembrane domain containing neuroreceptor family that is linked to regulatory guanine nucleotide binding protein [3]. The heterogeneity of these receptors was also proven. On the basis of behavioral and neurophysiological observations on the chronic spinal dog, three different opioid receptors were distinguished [4] and named after the drugs used in the tests, namely the mu (μ, from morphine), the kappa (κ, from ketocyclazocine) and sigma (σ, from SKF 10047 or N-allylnormetazocine). Later the σ receptor proved to be non-opioid, and another type of opioid receptors, the delta (δ) receptor was described by Lord et al. [5] on the basis of results of bioassays carried out on peripheral tissue preparations.

Indeed, opioid receptors are present not only in the central nervous system but also on the periphery, which rendered probable the use of isolated organs in functional assays. Thus, several experiments on different tissue preparations, isolated from the guinea pig ileum or vas deferens of mouse, rat, rabbit or hamster were reported. These isolated organs have been successfully applied for more than 30 years to characterize the agonistic/antagonistic properties of newly synthesized opioid ligands.

Molecular biology approaches were also applied to demonstrate the existence of opioid receptors. In the early 90s, cloning of the opioid receptor cDNAs was reported by different groups [6-8]. The binding and functional properties of the cloned receptors are identical with those described by pharmacological experiments for the μ, δ and κ receptors. Recently, mice

lacking the μ opioid receptor have been generated by two independent laboratories [9,10]. Experiments performed on these animals proved that all the beneficial and non-desired effects of morphine are mediated *in vivo* exclusively by the μ receptors. Therefore, drugs targeting this receptor are not likely to provide the ideal analgesic drug. Thus, ligands acting on the δ and κ receptors are of great interest. However, ligands acting on κ receptors cause dysphoria (Table 1) which hampers their medical use.

Table 1. Main pharmacological effects mediated by opioid receptors

μ	δ	κ
analgesia (supraspinal)	analgesia (spinal and supraspinal)	analgesia (spinal)
respiratory depression	respiratory depression	
sedation	sedation	sedation
euphoria		dysphoria
obstipation	obstipation	diuresis
bradycardia		
hypothermia	hyperthermia	

There is a functional interaction between μ and δ receptors [11]. The μ "knockout" mice provided means to investigate if analgesia mediated by δ receptors is maintained in the absence of μ receptors. However, these investigations did not lead to an unambiguous conclusion. Spinal and supraspinal analgesia, evoked by DPDPE [12-14] and deltorphin II [12] was reported to be unchanged [13], slightly lower [12] or drastically reduced [14] in the μ receptor deficient mice. Biochemical pathways activated by δ agonists, namely the G-protein activation and inhibition of adenylate cyclase, were retained in the μ knockout mice. These data suggest, that antinociception mediated by δ receptors is at least in part independent from the μ receptors. This fortifies that highly potent δ agonists could gain clinical importance.

In the early 90s, the heterogeneity of the δ receptor was suggested on the basis of pharmacological investigations. These studies showed that the effect of different δ agonists are blocked unevenly by different, highly selective δ antagonist ligands [15-18]. These studies also proved the lack of two-way cross-tolerance between subtype-selective δ agonists [19]. Two subtypes, the so-called δ_1 and δ_2 receptors were distinguished. 7-benzylidenenaltrexon [20] is a selective antagonist of the δ_1 subtype. DPDPE acts on this site too, but also shows some affintiy towards the δ_2 subtype. Nonequlibrium antagonists of the δ_2 receptor are naltrindole-5'-isothiocyanate [21] and naltribene [15], while deltorphin II is a selective agonist of the δ_2

receptor. However, to date these pharmacologically defined subtypes have not yet been identified at a molecular level.

1.1.2 Opioid peptides

Enkephalins, the first discovered endogenous opioid ligands were described in the mid 70s [22,23]. These peptides act on δ receptors. Later, another peptide family, the dynorphins, the endogenous κ ligands were isolated [24]. Meanwhile it became evident that endogenous opioids are formed by posttranslational cleavage of larger precursors. In mammals, three such precursors are known: the proenkephalin A [25], which gives rise to enkephalins and related peptides; prodynorphine [26], from which dynorphins and neoendorphins are formed; and proopiomelanocortin [27] from which, amongst other hormonally active peptides, β endorphin is formed (Table 2). Although it is not very potent neither selective, β endorphin was regarded as the possible endogenous ligand of the μ receptor before the discovery of endomorphins [28] (Table 2). Discovery of the endomorphin family, the 'real' endogenous ligands of μ receptors, was undoubtedly the big sensation of the late 90s opioid research. The precursor molecule of these peptides is still unknown.

New δ peptide agonists were developed in order to improve the affinity and rather poor selectivity of the natural enkephalins. DADLE [29], DSLET [30], DTLET [31], BUBU [32] and BUBUC [33] (Table 3) are potent analogs, however, their δ selectivity remained low. Therefore, conformational restriction [34-37] of the enkephalin molecule was carried out by cyclization [35,36] resulting in a set of peptides including DPDPE [38] (Table 3), which became widely used in radioligand binding assays. Compared with the previously reported δ receptor selective analogs, the bis-Pen-containing analogs provide an order of magnitude increase in δ receptor selectivity. However, a cyclic peptide can still have considerable conformational freedom which is reducible by the insertion of local constraints, by replacing the naturally occurring amino acids to conformationally constrained ones in the peptide sequence. Indeed, conformationally constrained DPDPE analogs were synthesized in order to enhance the selectivity of the parent peptide [39-41].

Amongst many other biologically active compounds, frog skin contains opioid peptides, too. These peptides are dermorphin [42] and deltorphins [43,44] (Table 4). Dermorphin is a highly potent and selective μ ligand, while deltorphins, especially deltorphin I and II, are selective δ ligands with subnanomolar K_i values in the binding assay. Deltorphin I and II are the most δ selective natural opioids with selectivity values of 21000 and 3450, respectively.

Table 2. Endogenous opioid peptides

	Sequence	Precursor molecule
Met-enkephalin	H-Tyr-Gly-Gly-Phe-Met-OH	proenkephalin A
Leu-enkephalin	H-Tyr-Gly-Gly-Phe-Leu-OH	
Met-enkephalin-Arg^6-Phe^7	H-Tyr-Gly-Gly-Phe-Met-Arg-Gly-OH	
Met-enkephalin-Arg^6-Gly^7-Leu^8	H-Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu-OH	
dynorphin A	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gly-OH	prodynorphine
dynorphin B	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Glu-Glu-Leu-Phe-Asp-Val-OH	
α neoendorphin	H-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-OH	
β neoendorphin	H-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-OH	
β endorphin	H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH	proopiomelanocortin
endomorphin 1	H-Tyr-Pro-Trp-Phe-NH_2	unknown
endomorphin 2	H-Tyr-Pro-Phe-Phe-NH_2	

Table 3. Sequences of synthetic opioid peptide ligands mentioned in the text

DADLE	H-Tyr-D-Ala-Gly-Phe-D-Leu-OH
DSLET	H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH
DTLET	H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH
BUBU	H-Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu)-OH
BUBUC	H-Tyr-D-Cys(StBu)-Gly-Phe-Leu-Thr(OtBu)-OH
DPDPE	H-c-Tyr-D-Pen-Gly-Phe-D-Pen-OH

Table 4. Opioid peptide ligands isolated from tropical poison arrow frog skin

dermorphin	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2
deltorphin (also called dermenkephalin)	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2
deltorphin I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2
deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2

The tyrosine moiety is one of the key pharmacophores of opioid peptides. Replacement of L- with D-Tyr^1 in deltorphin resulted in a 1200-fold decreased δ affinity [45]. Replacement of Tyr^1 in deltorphin I to Phe^1 produced a 32-fold decrease in δ receptor affinity but only a 7-fold drop in antinociceptive potency [46], which indicates that the phenolic hydroxyl group of Tyr contributes to the effective interaction with the opioid receptor but is not an absolute requirement for opioid activity. Effect of conformational restriction of the first residue was not much investigated. Qian et al. synthesized (2*S*,3*S*)-2',6'-dimethyl-β-methyltyrosine substituted deltorphin I and found that the new analog had two to fivefold decrease of potency while retaining the high selectivity of the parent ligand [47]. Guerrini et al. synthesized 2',6'-dimethyl-L-tyrosine substituted deltorphin II and the new ligand exhibited high dual affinity and bioactivity toward δ and μ opioid receptors [48]. Enhanced interaction with the μ receptor was interpreted by the increased hydrophobicity of the first residue and/or by the possible changes in the peptide topography. It seems that the degree of methylation of the Tyr^1 affects the conformation of the peptide and its ability to interact differentially with δ and μ opioid receptors.

Conformational restriction of the Phe^3 residue in deltorphins has resulted in drastic effects on the ligands' affinity, selectivity, and ability to generate intracellular responses. Tetrahydroisoquinoline-3-carboxylic acid (Tic) [49-51], different β-methyl-phenylalanines [52,53], 2-aminotetralin-2-carboxylic acid (Atc) [49,50], 2-aminoindane-2-carboxylic acid (Aic) [49,50] and 4-aminotetrahydro-2-benzazepin-3-one (Aba) [51] were used to investigate the effect of the Phe^3 side-chain conformation on the binding properties of deltorphins. Especially the Atc and Aic analogs displayed extraordinary δ receptor affinity and selectivity.

Replacement of the Val residues by Ile in positions 5 and 6 [54] resulted in a more lipophilic compound with 8 times higher affinity and 5 times better δ selectivity than that of deltorphin II.

1.2 Synthesis of α,α-disubstituted amino acids

There is a growing interest in the synthesis, pharmacology and conformational properties of non-proteinogenic amino acids. In the opioid peptide field, lots of conformationally constrained peptide analogs were synthesized by the incorporation of unnatural amino acids. Since these amino acids are generally not commercially available or they are expensive, it is reasonable to look for an appropriate synthesis route. Racemic and enantiomerically pure α,α-disubstituted amino acids were prepared by a number of different routes:

- by the Strecker synthesis
- by hydrolysis of hydantoins obtained from the Bücherer reaction
- by the Schmidt rearrangement on disubstituted mono-esters of malonic acid [55]
- by diastereoselective enolate alkylation of oxazolidin-4-ones [56,57]
- by metallation and subsequent alkylation of Schöllkopf's bis-lactim ethers providing after hydrolysis under mild acidic conditions, a wide range of α,α-disubstituted amino acids [58]
- by alkylation of substituted isonitriles [59]
- by alkylation of imines followed by cleavage of the auxiliary [60].

In the Strecker synthesis, a given aldehyde can be converted into an α-amino acid containing one carbon atom more than the parent compound by reaction with ammonia then with hydrocyanic acid to give first the amino nitrile followed by hydrolysis to the amino acid. Modifications of this procedure have been introduced in order to increase the generally low yields and to overcome the technical difficulties imposed by handling the highly toxic hydrogen cyanide gas. One of these useful methods is the Bücherer reaction [61], where first a hydantoin is formed from the appropriate ketone by reaction with ammonium carbonate and an alkali cyanide, then the hydantoin is converted into the appropriate amino acid either by alkali or acidic hydrolysis. Hydrolysis of dialkyl hydantoins in 60% sulfuric acid gives reasonable yield of the amino acid, however, the hydrolysis of unsaturated alkyl- and aryl-substituted hydantoins is less successful in acid due to their lower solubility and significant decomposition. In order to overcome these difficulties, alkali hydrolysis by barium hydroxide was successfully applied for the hydrolysis of these hydantoins [62]. In comparison with the Strecker synthesis, the hydantoin method is preferable as it proceeds with less decomposition. In case of uncomplete reaction the starting material is easy to recover, and hydantoins, unlike amino nitriles, are stable and easy to purify.

Since the starting material for these reactions is an aldehyde or a ketone, these procedures obviously result in racemic products and a suitable resolution procedure is required before

peptide synthesis or, in case of incorporation in racemic form, the resulting diastereomeric peptides can be separated by HPLC during purification. The drawback of the hydantoin method as well as of the Strecker synthesis is that they require drastic hydrolysis conditions which are not applicable if sensitive functionalities are present in the target compound.

1.3 Separation of enantiomeric amino acids

Synthesis of amino acids often leads to a racemic mixture. Enantiomers have identical chemical and physical properties in symmetrical environment, but in a chiral environment such as the human body they act as two different molecules with quite different biological activities. Thus, separation of the enantiomers and determination of the enantiomeric purity are of high importance.

Separation procedures can be divided in two classes depending on the scale in which they are applicable, namely preparative and analytical scale.

1.3.1 Resolution of amino acids on preparative scale

Preferential crystallization by providing seed crystals of one antipode in a supersaturated solution of a racemate was employed in the resolution of a few amino acids. However, this method is time consuming, tedious and often fails to accomplish total separation of the enantiomers.

Diastereomeric salts can be formed by the use of enantiomerically pure chiral ion-pair forming reagents. Due to different solubility of the resulting diastereomeric salts, they can be separated via differential crystallization from an appropriate solvent. However, successful one-step resolution of a racemate presumes that the resolving agent forms a relatively tightly bound salt with at least one element of the racemate and that this salt is cleanly and quantitatively precipitable from that of the other isomer. Unfortunately, this is rarely the case. Complete purification of each salt is therefore tedious and time consuming, requiring multiple crystallization and subsequent control of the enantiomeric purity. Despite these difficulties, this method has been successfully applied to the resolution of most α-amino acids. N-benzoyl or N-formyl amino acids have been resolved as their brucine or strychnine salts, followed by acid hydrolysis to afford the enantiomerically pure free amino acids. Some amino acid esters or amides have been resolved by optically active acids such as camphoric acid and its derivatives.

The advantage of using biological procedures to resolve amino acid racemates is that their action towards amino acids of unknown optical structure can be predicted. However, in some

cases this specificity is not absolute. The method also permits a more general approach and a more uniform resolution procedure.

Although resolution can be performed by using a whole animal followed by the isolation of the intact enantiomer from the urine, or by action of microorganisms for asymmetric oxidation or decarboxylation, these procedures are not applicable when both antipodes are needed. In this case, asymmetric synthesis by a protease or asymmetric hydrolysis by amidases, esterases or acylases can be methods of choice.

Several N-protected amino acids have been resolved by proteases using bases as aniline [63] or p-toluidine [64] with papain as enzyme. However, this method is not recommended as a general procedure, as incomplete precipitation of the amide product leads to an equlibrium in the solution thus only partial resolution can be achieved.

Asymmetric enzymatic hydrolysis takes place in solution and analytic methods are avalaible to follow the course of the reaction. The method applies L-directed enzymes such as pancreatic chymotrypsine and carboxypeptidase, or renal acylases and amidases. Extracts of pancreas have been successfully applied to resolve esters of some amino acids. However, the application of this method is limited as under the reaction conditions required the spontaneous hydrolysis of the ester [65] as well as polymerization to form higher peptide esters may occur [66].

The most extensively used enzymes for the resolution of α-amino acids are acylases and amidases. L Antipodes of N-acetyl, chloroacetyl or trifluoroacetyl amino acids as substrates, except Pro, are readily hydrolyzed by acylases or carboxypeptidases.

1.3.2 Resolution of amino acids on analytical scale

Among the analytical methods applied for separation of enantiomers of amino acids, chromatography, including GC, HPLC and TLC, is the most widely used. Polarimetry is also popular, however, this method cannot be regarded as reliable as the measurement of specific rotation and the determination of *ee* value is influenced by the presence of impurities, change in concentration, solvent used and temperature. Moreover, the knowledge of the specific rotation value of the pure enantiomer is required which is generally not available in the case of unnatural amino acids.

Chromatographic methods can be classified to direct and indirect methods. In direct methods the enantiomers are separated either on achiral stationary phases by a chiral additive in the mobile phase or on chiral stationary phases. Indirect methods involve the conversion of the enantiomeric mixture to diastereomers by reaction with an enantiomerically pure chiral reagent, and the products are subsequently separated on achiral chromatography phases. In

principle, these considerations apply to all stationary phases but nowadays HPLC is the most widely used method. The GC analysis of amino acids, as they are not volatile, needs previous derivatization [67,68].

For direct separation, appropriate chiral selectors can be chiral ligands with metal ions [69], proteins [70], cyclodextrins [71], crown-ethers [72] and chiral counter ions [73]. These compounds, bound to a solid support can also serve as a chiral stationary phase. Indeed, chiral crown-ether phases [72], cyclodextrin phases [74], π acid and π base phases [75,76], ligand-exchange phases and macrocyclic antibiotic phases [77] were applied to analyse amino acids and their derivatives.

Chiral derivatizing reagents, applied for indirect enantiomeric resolution, have to fulfill a lot of requirements. The reagent has to be enantiomerically pure and should provide quantitative reaction without racemization or metabolism. It is advantageous to have both enantiomeric forms if the task is to determine the amount of enantiomeric impurity. However, when choosing a reagent, the available functional groups of the analyte primary define the type of the reagent possible. Chiral derivatization of amino acids includes the formation of diastereomeric amides, ureas, thioureas, isoindoles and dipeptides. The most widely used derivatizing agents for amino acids are Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) [78] and the GITC reagent (2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate) [79,80]. Lower limits of detection can be achieved by the use of fluorescent derivatizing agents such as ortho-phthalaldehyde together with chiral thiols [81].

As a comparison, direct separation rules out racemization, and accurate determination is possible even though the chiral selector may be enantiomerically impure. However, chiral phases are rather expensive. The use of chiral mobile phases is a flexible and versatile technique, while indirect methods provide lower detection limits.

Chiral TLC also provides a direct method for the resolution and analytical control of enantiomeric purity. This method is sensitive, easy to carry out and relatively inexpensive. Ligand exchange (Chiralplate), ion exchange (optically pure chiral selector) and molecular inclusion complexation (cellulose [82], cyclodextrin [83]) constitute the basis of these TLC separations. Ligand exchange chiral TLC, inroduced by Gunther et al. [84] on Chiralplate®, provides a direct and simple approach for separation of amino acid antipodes. The plate consist of RP-18 silica support soaked with the solution of Cu(II)-acetate and of the chiral selector, (2*S*,4*R*,2'*RS*)-4-hydroxy-1-(2'-hydroxydodecyl)-proline. Quantification can be carried out by densitometry, and respective antipodes can be evaluated at trace levels (the limit of the determination was reported to be >0.1%) [85].

The advantage of TLC over HPLC is that more organic solvents can be used because they evaporate off the thin-layer plate and therefore do not disturb the final detection. Furthermore, neither pressure resistance of the stationary phase nor re-use of the chromatographic bed is required. Under proper conditions, several analysis can be carried out in a single TLC plate while with GC or HPLC techniques all the analyses have to be performed sequentially. TLC also allows an easy control of synthetic processes.

1.4 Solid-phase peptide synthesis [86,87]

Solid-phase peptide synthesis is based on the sequential addition of α-amino or side-chain protected amino acids to an insoluble polymeric support. The growing peptide is attached to this support through its C-terminus via a linker. For N-α protection, the acid labile *tert*-butoxycarbonyl (Boc) or the base labile 9-fluorenylmethoxycarbonyl (Fmoc) groups are used. After the removal of the protecting group, the next protected amino acid is coupled using either a coupling reagent or a preactivated protected amino acid derivative.

The peptide can be cleaved to yield a peptide acid or amide depending on the linker to the polymer. Side-chain protecting groups are chosen so as to be cleaved simultaneously with detachment of the peptide from the resin. In principle, Boc-chemistry applies benzyl- and cyclohexyl-type protecting groups, while in Fmoc-chemistry mainly tert-butyl- and trityl-type protecting groups are used. Removing of the N-protecting group takes place by TFA in the case of the Boc group and by piperidine in the case of the Fmoc group. Final cleavage of the peptidyl-resin applies HF or trifluoromethanesulfonic acid (TFMSA) for Boc-chemistry and TFA for Fmoc-chemistry. Primary solvents for the coupling, deprotection and washing are dichloromethane and dimethylformamide for Boc- and Fmoc-chemistry, respectively.

For monitoring the presence or absence of free amino groups, the most widely used qualitative test is the Kaiser test [88]. The test reaction is quick and easy to carry out. However some deprotected amino acids (Ser, Asn, Asp and Pro) do not show the typical dark blue color. Moreover, in case of peptide aggregation false negative test results can be obtained. For monitoring the coupling or deprotection of Pro residues, the chloranil test [89] is recommended.

As a comparison, Fmoc-chemistry provides milder reaction conditions because the repetitive deprotection steps do not require TFA which can lead to alteration of the peptide bonds and acid catalysed side-reactions. Use of Fmoc-chemistry is highly recommended when

difficult sequences are expected. However, Fmoc-chemistry is not as economical as Boc-chemistry.

1.4.1 Coupling methods

Efficient peptide bond formation requires the activation of the α carboxyl group. There are basically four main types of coupling techniques and these are the carbodiimide, symmetrical anhydride, active ester and *in situ* activation methods.

Carbodiimides such as dicyclohexylcarbodiimide (DCC) [90] or diisopropylcarbodiimide [91] are often used as *in situ* activating reagents. However, the coupling reaction requires long time (up to 12 h) and dehydration of Asn and Gln residues can also take place. Addition of HOBt to the reaction mixture prevents both problems.

Symmetrical anhydrides are formed *in situ* when using two equivalents of the N-protected amino acid and one equivalent of DCC. These derivatives are highly reactive, but the method is quite wasteful and the anhydrides have to be prepared freshly prior to use.

The most important active esters are the HOBt and pentafluorophenyl esters. HOBt esters [92], which normally react extremely fast, are formed *in situ* when using HOBt, BOP, PyBOP, TBTU and HBTU and are not isolated. Pentafluorophenyl esters [93] react somewhat slower and they are also quite expensive.

Recently, *in situ* activating reagents have become very popular because of their easy use, fast reactions and lack of undesired side reactions. They are mainly phosphonium and uronium salts and react in the presence of a tertiary base. The most commonly used ones are BOP [94,95], PyBOP [96], HBTU [97] and TBTU [98], while the difficult and time consuming coupling of α-methyl amino acids can be carried out easily in the presence of PyBroP [99].

1.4.2 Cleavage

In case of Boc-chemistry, the generally used cleaving agent is anhydrous HF. Standard HF cleavage takes place at 0-5 °C for 30-60 min. Side reactions can be avoided by the use of appropriate scavengers. The most widely used one is anisole, which, in combination with dimethylsulfide and p-thiocresol, can prevent the alkylation of Met and Cys residues. The drawback of this method is that HF is highly toxic and reactive, therefore special cleavage apparatus is required. As an alternative to HF other strong acids, such as TFMSA, or for amide C-terminus peptides, 30% HBr/AcOH [100], can be applied in normal laboratory glassware.

In case of Fmoc-chemistry, cleavage of the peptide-resin bond and simultaneous deprotection is carried out by TFA. During cleavage, reactive cations are formed from the side-chain protecting groups and from the handles of the resin. Hence appropriate scavengers have to be applied to avoid modification of Trp, Met, Tyr and Cys residues. As an alternative to TFA cleavage, stronger acids, such as HBF_4 [101] or trimethylsilylbromide can be used to shorten cleavage time and to remove less acid sensitive protecting groups.

1.4.3 Post-cleavage work-up

In most cases, post-cleavage work-up consists of precipitation of the crude peptide from the cleavage mixture with ether. Then the peptide is washed with ether to remove scavengers and is lyophilized from AcOH or from buffer solutions.

In order to achieve high peptide purity, purification by high-performance liquid chromatography is necessary. Normally, reversed phase chromatography is used for this purpose on C_{18}, C_8, or C_4 derivatized silica depending on the hydrophobicity and the size of the peptide. Water/acetonitrile, water/methanol (MeOH) or water/isopropanol are used as eluents with acidic ion-pairing agents such as TFA, phosphonic acid, ammonium acetate or triethylammonium phosphate. The latter usually gives better resolution than TFA but the buffer has to be removed by desalination after chromatography. UV absorption at 210-220 nm or, in case of aromatic amino acid residues, 240-280 nm is used for monitoring the purification. If the peptide carries similarly charged side-chain groups, ion exchange chromatography can also be used for purification. Purified peptides can be stored as lyophilizates at -20 °C.

1.5 Tritium labeling of peptides

Investigation of neuropeptide receptors requires biologically active ligands containing radioactive, fluorescent, chemiluminescent and/or (photo)affinity labels. For peptides, radioisotopes are the most frequently used markers. Tritium is a soft β-emitting radionuclide with a half-life of 12.47 years. One tritium atom per molecule represents a specific radioactivity of 28.6 Ci/mmol (1080 GBq/mmol). Thus, tritium incorporation is appropriate for studies where only nanomolar concentration of peptides are needed.

In the opioid field, tritiated peptide ligands proved to be essential to perform binding experiments, autoradiographic localization and distribution studies of the receptors and to confirm that the cloned receptors are identical with the pharmacologically characterized ones. All these studies require selective opioid ligands, therefore radioactive labeling and characterization of new, potent and selective ligands are of great importance. As for δ opioid

peptides, tritium labeling and characterization of DPDPE [102] and deltorphin II and its analogs [103,104], just as that of highly potent antagonists, TIPPψ [105] and Dmt-Tic [3] was reported.

There are four major approaches to prepare tritiated peptides, i. e. isotope exchange reactions [106], chemical or enzymatic synthesis of peptides from labeled amino acids, derivatization of the neuropeptides by [^{3}H]methyliodide [107] or reductive methylation using tritiated metal hydrides [108] and synthesis of [^{3}H]neuropeptides using precursor peptides (Figure 1).

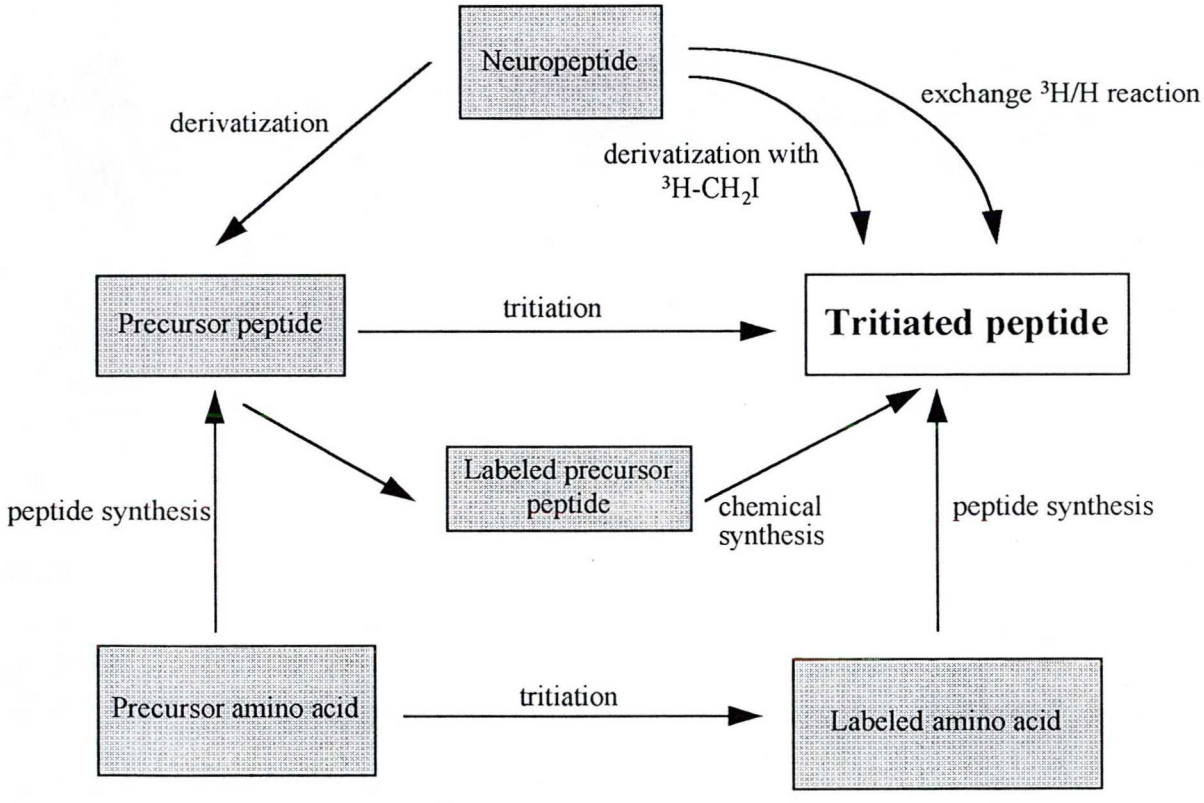


Figure 1. Possible reaction routes to obtain tritiated peptides

Tritium labeling can be performed on precursor peptides gained by direct synthesis or by postsynthetic modifications [109,110]. Due to its simplicity, the specifity of the label and the unnecessity of time consuming peptide synthesis, tritiation of modified precursor peptides is a very popular method. The most important postsynthetic modification is the iodination of peptides.

Tyr and His residues can be iodinated by ICl or I_2. For this purpose, I_2 can be used directly in a MeOH solution or can be generated from HI and HIO_3 under highly acidic conditions;

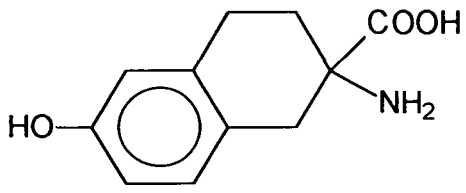
from I^- by oxidation with chloramin-T or iodogen; by electrolysis; by enzymatic reaction using peroxidases etc. Depending upon the iodine/substrate ratio, temperature and reaction time, mono- and diiodinated analogs can be prepared. The crude reaction mixture usually contains mono- and diiodinated as well as noniodinated peptides. The diiodinated analog, favorable for tritiation, can be separated by HPLC purification. Tritium labeling of these precursor peptides results in labels in the aromatic rings in positions 3' and 5' in the Tyr or in positions 2' and 4' in the His residue. Direct halogenation of peptides containing Trp, Met or Cys may be associated with irreversible damage of these sensitive residues. To avoid such difficulties fragment condensation is a plausible approach [111].

Tritiated neuropeptides can also be prepared by using precursor peptides obtained by peptide synthesis. Amino acid derivatives containing a halogen atom, a double or a triple bond can be appropriate starting materials for peptide synthesis. Boc- and Fmoc-chemistry can equally be used but in the case of double bonds Fmoc-chemistry is preferred. The most popular amino acids are 3',5'-diiodo-tyrosine (Dit) or 3',5'-dibromo-tyrosine. 2',6'-Dibromo-tyrosine can be used to place the labels in more stable positions [112]. The use of p-iodo-phenylalanine or other p-halogenated Phe residues for precursor synthesis results in lower specific activity in the tritiated peptide, but the label itself will be more stable [103]. There are other frequently used precursor amino acids, e. g. 2',4'-diiodo-histidine [113], 5'-bromo-Trp [114], dehydro-proline [115], dehydro-leucine [116], dehydro-isoleucine and propargyl- [117] or allyl-glycine.

Pd/ $BaSO_4$ is the most widely applied catalyst for tritiation of halogen-containing peptides [104,105,118], but Pd/C and carrier-free Pd catalyst were also applied for labeling of opioid peptides [103,119,120].

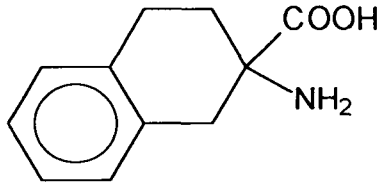
2. AIMS AND SCOPES

The aim of the presented work was the synthesis of new, highly potent and selective δ opioid peptide ligands. The naturally occurring, highly potent and selective deltorphins were promising templates for this purpose. Modification of the pharmacological key elements of deltorphins, namely the Tyr^1 and the Phe^3 moieties were expected to influence the binding characteristics of these ligands. In order to reduce the conformational freedom of the side-chains of these residues, the tetraline-ring-containing amino acids, Hat (6-hydroxy-2-aminotetralin-2-carboxylic acid) and Atc (2-aminotetralin-2-carboxylic acid) were incorporated into $Ile^{5,6}$ deltorphin I and II.



6-hydroxy-2-aminotetralin-2-carboxylic acid

(Hat)



2-aminotetralin-2-carboxylic acid

(Atc)

For further biological characterization and potential application in the radioligand binding assay and/or autoradiographic study of the opioid receptors, two new, highly potent and selective ligands were synthesized in radiolabeled form from their corresponding halogen-containing precursor peptides to yield tritiated ligands of high specific radioactivity.

Based on the results of biological characterization and NMR experiments, both carried out by collaborating groups, structure-activity relationships of the new compounds are discussed.

3. MATERIALS AND METHODS

TLC analyses were performed on precoated plates (silica gel F_{254}) using the following solvent systems: (1) acetonitrile-methanol-water (4:1:1)

(2) chloroform-methanol-acetic acid (90:10:1)

(3) ethyl acetate

(4) n-butanol-acetic acid-water (2:1:1)

(5) n-butanol-acetic acid-water (4:1:1)

(6) acetonitrile-methanol-water (6:1:1)

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

UV light, I_2 and ninhydrin were applied to detect the compounds.

Chiral TLC was performed on Chiralplate (Macherey-Nagel, Düren, Germany) **[IV]**.

RP-HPLC analyses were carried out on a Merck-Hitachi chromatograph applying a Vydac 218TP54 column for analytical and a Vydac 218TP1010 column for preparative purposes. UV detection at 215 nm was applied for detection. Final products were obtained as lyophilizates.

Analytical characterizations were performed using a linear gradient of ACN from 0 to 80 % over 30 min.

Atc-containing diastereomeric peptides were separated and purified to homogeneity with a linear gradient of water-0.1% TFA (eluent A) and acetonitrile (ACN) -0.1% TFA (eluent B); starting from 25 to 30% ACN over 25 min.

Hat-containing diastereomeric peptides did not separate in solvent systems containing ACN, MeOH or THF. *(S)*-Hat- and *(R)*-Hat-containing crude peptides were purified using the following gradient program: 0 min, 30% MeOH; 5 min, 50% MeOH; 25 min, 55% MeOH; t_R:12.4 min. (For analytical data see Table 5.)

Iodinated peptides were purified using a linear gradient from 28% to 35% ACN in 0.1% TFA in 35 min.

Synthesis of amino acids and their derivatives

(R,S)-2-Aminotetralin-2-carboxylic acid (Atc)

A hot mixture of 70 g of aqueous barium hydroxide (222 mmol of $Ba(OH)_2$, 2.4 equiv.) and 20.0 g (93 mmol) of 7,8-benzo-1,3-diazaspiro[4.5]decane-2,4-dione in 300 mL of water was stirred and refluxed for 24 h. After dilution with water (50 mL), the solution was acidified with

aqueous H_2SO_4 (1 M) to pH 1 and the mixture was stirred at 100 °C in a waterbath for 1 h. The precipitated $BaSO_4$ was filtered and washed with water (2×50 mL), then the combined mother liquor was evaporated to 200 mL and the pH was set to 6 with 17% NH_3. The desired amino acid precipitated as a white powder. 15.5 g (88%); mp: 309-310 °C. ^{1}H-NMR (D_2O-TFA); 7.18 (s, 4 arom. H); 3.42 , 3.02 (2d, J=17.0 Hz, 2 aliph. H); 3.03-2.73 (m, 2 aliph. H); 2.42-2.30 (m, 1 aliph. H); 2.18-2.08 (m, 1 aliph. H). MS (ESI): 192 (MH^+); TLC R_f(1): 0.43; R_f(5): 0.48; HPLC t_R:11.58 min.

N-tert-Butyloxycarbonyl-(R,S)-2-aminotetralin-2-carboxylic acid (Boc-Atc)

To the solution of 1.91 g (10 mmol) of Atc in 210 ml of dioxane-water 2:1 (pH adjusted to 9 with 2N NaOH), 2.40 g (11 mmol, 1.1 equiv.) of Boc_2O was added and the mixture was stirred in an icebath for 1.5 h. Then an additional portion of 0.66 g (3 mmol, 0.3 equiv.) of Boc_2O was added and stirring was continued at RT. The pH of the solution was kept at 9. After stirring overnight, the dioxane was removed under reduced pressure and the mixture was extracted with ether (2×50 mL) then with EtOAc (3×50 mL). The combined EtOAc layer was extracted with brine (1×80 mL) then was dried over $MgSO_4$ and evaporated. The resulted oil was crystallized from EtOAc/petroleum ether to give 1.89 g (6.5 mmol) of Boc-Atc in 65 % yield.

mp: 182 °C; MS (ESI) 290 ($M-H^-$); ^{1}H-NMR (DMSO-d_6): 7.40-7.02 (m, 4 arom. H); 3.17; 3.02 (2 d, J=16.32 Hz, 2 aliph. H); 2.63-2.88 (m, 2 aliph. H); 2.20-2.35 (m, 1 aliph. H); 1.87-2.08 (m, 1 aliph. H); 1.37 (s, 9 tBu-H). TLC R_f(1): 0.74; R_f(5): 0.75; HPLC t_R: 22.19 min.

N-Trifluoroacetyl-(R,S)-2-aminotetralin-2-carboxylic acid (N-TFA-Atc)

To a solution of Atc (2.87 g, 15 mmol) in TFA (20 mL), trifluoroacetic anhydride (5.21 mL) was added dropwise and the mixture was vigorously stirred at 0 °C for 2 h and then for 2 days at RT. After the solvent and excess anhydride had been removed under reduced pressure, the residue was diluted with water (30 mL), extracted with ethyl acetate (3×30 mL), brine (2×30 mL) and water (2×30 mL), dried over $MgSO_4$ and concentrated. The resulting yellow oil was crystallized from EtOAc / petroleum ether; yield 2.80 g (65%), mp: 186 °C. ^{1}H-NMR (DMSO-d_6): 13.0 (s, 1 COOH); 9.5 (s, 1 NH); 7.1 (m, 4 arom. H); 3.12; 3.25 (2 d, J=10.7 Hz, 2 aliph. H); 2.73 (m, 2 aliph. H); 2.40 (m, 1 aliph. H); 2.05 (m, 1 aliph. H). TLC R_f(2): 0.48. MS ESI: 288 (MH^+).

Enzymatic separation of N-Trifluoroacetyl-(R,S)-2-aminotetralin-2-carboxylic acid

1.5 g (5.23 mmol) of N-TFA-Atc was suspended in 150 mL of water. The pH was adjusted to ~7.5 with NaOH, 200 μL of carboxypeptidase A (23 mg protein/ml suspension, 56 unit/mg protein) was added and the mixture was stirred at 37 °C for 2 h. From time to time the pH was adjusted to 7.5-8. After the completion of the reaction, which was followed by chiral TLC and HPLC, charcoal was added in order to remove the enzyme. After filtration, the pH of the solution was adjusted to 3 with 1 M HCl. Unreacted N-TFA-Atc was extracted with EtOAc. The aqueous solution was evaporated to ~50 mL and the pH was adjusted to 5-6. The precipitated crystals were filtered off and dried. Yield: 478 mg; 47%, $[\alpha]^{25}_D$ = -6.77° (c = 1.0, 0.3 M HCl/water).

After drying over $MgSO_4$, the organic phase was evaporated and the resulting oil was crystallized from ethyl acetate /petroleum ether. Yield: 734 mg (49%), $[\alpha]_D^{25}$ = +4.1° (c = 1.0, ethanol). The resulting N-TFA-*(S)*-Atc was hydrolyzed by refluxing with 6 M HCl (40 mL) for 6 hours. The solution was evaporated, the residue was dissolved in water (20 mL) and the pH was adjusted to 6 with 1 N NH_4OH. The crystals were filtered to give 210 mg (43%) of *(S)*-Atc. $[\alpha]^{25}_D$ = +6.3° (c = 1.0, 0.3 M HCl/water).

Chiral TLC: (*R*)-Atc (Atc from aqueous solution) R_f(6): 0.57 (identical with the lower spot of racemic Atc); (*S*)-Atc (Atc from organic solution) R_f(6): 0.63 (identical with the upper spot of racemic Atc).

(R)-Atc.HBr was prepared by treating *(R)*-Atc with 48% HBr/water, and crystallized from water to give the desired salt in 25% of yield. mp: 268-270 °C, $[\alpha]_D^{25}$=-7.16° (c=1.0, water).

6-Methoxy-β-tetralone

To the solution of 38.2 g (0.28 mmol, 2 equiv.) of $AlCl_3$ in 560 ml of DCM cooled in dry ice-acetone bath, 26.1 g (0.14 mol) of p-methoxy-phenylacetyl chloride in 140 ml of DCM was added dropwise over 30 min, then ethylene was bubbled vigorously into the flask for ~10 min. Cooling was finished and the mixture was stirred at RT for 4 h. The reaction mixture was cooled in an ice bath while 170 ml of water was added dropwise and the mixture was stirred until the solid material was dissolved. The yellow organic layer was separated, washed with 5% HCl (2×100 ml), 7% $NaHCO_3$ (2×100 ml), water (1×100 ml), was dried and evaporated. The crude oil was distilled under reduced pressure (bp: 108-115 °C (0.2 Hgmm)) to give 17.2 g (mmol, 69 %) of 6-methoxy-β-tetralone.

6-Methoxy-tetralin-spirohydantoin

49.2 g (0.28 mol) of 6-methoxy-β-tetralone, 26.8 g (0.55 mol, 2 equiv.) of NaCN and 135 g (1.4 mol, 5 equiv.) of $(NH_4)_2CO_3$ were suspended in EtOH-water (1:1) (400 mL) and the mixture was stirred under N_2 at 70 °C for 23 hours. The resulting mixture was filtered, washed with water (2×50 ml) and chloroform (2×50 ml) and dried to give 56.6 g (0.23 mol) of the desired product in 82% yield.

mp: 238-239 °C; MS ESI+: 247 (MH^+), HPLC t_R:16.87 min; TLC (2): R_f: 0.48 (3): R_f:0.47; ^{1}H-NMR (DMSO-d_6); 10.61 (s, 1 NH); 8.21 (s, 1 NH); 6.98, 6.70 (2d, J=7.9 Hz, 2 arom. H); 6.69 (s, 1 arom. H); 3.70 (s, 3 OCH_3); 3.02, 2.68 (2d, J=16.6 Hz, 2 aliph. H); 2.84-2.90 (m, 2 aliph. H); 1.86-1.98 (m, 1 aliph. H); 1.75-1.82 (m, 1 aliph. H).

Synthesis of 6-hydroxy-(R,S)-2-aminotetralin-2-carboxylic acid (Hat)

8.79 g (35.7 mmol) of 6-methoxy-tetralin-spirohydantoin was refluxed in aqueous 47% HBr (120 mL) for 48 h. After evaporation, the residue was dissolved in 200 mL of hot water and the pH was adjusted to 5-6 with 25% NH_3. The resulting brown solid was crystallized from ethanol to give 4.96 g of white powder in 67 % yield.

mp 280-282 °C. ^{1}H-NMR (DMSO-d_6); 7.02, 6.70 (2d, J=8.2 Hz, 2 arom. H); 6.67 (s, 1 arom. H); 3.33 , 2.96 (2d, J=16.8 Hz, 2 aliph. H); 2.96-2.72 (m, 2 aliph. H); 2.28-2.40 (m, 1 aliph. H); 1.99-2.15 (m, 1 aliph. H). MS (ESI): 208 (MH^+) TLC(1) R_f: 0.44. HPLC t_R: 10.6 min.

N-tert-Butyloxycarbonyl-6-hydroxy-(R,S)-2-aminotetralin-2-carboxylic acid (Boc-Hat)

Boc-Hat was prepared from 11.73 g (57 mmol) of Hat in dioxane-water 2:1 at pH=10 with 1.1 equivalent of di-tert-butyl-dicarbonate (Boc_2O). After 3 h of stirring, an additional portion of Boc_2O (0.3 equivalent) was added and stirring was continued for 4 days at RT. After extraction with EtOAc, the resulting brownish oil was dissolved in EtOAc and was precipitated by petroleum ether.

10.08 g (58 %). mp 141 °C. ^{1}H-NMR (CD_3OD); 6.87, 6.54 (2d, J=8.0 Hz, 2 arom. H); 6.53 (s, 1 arom. H); 3.30-3.35 (m, 2 aliph. H); 3.14, 2.92 (2d, J=16.1 Hz, 2 aliph. H) 2.68-2.81 (m, 2 aliph. H); 1.42 (s, 9 tBu-H). MS (ESI): 307 (MH^+). TLC R_f(1): 0.74; R_f(2): 0.32. HPLC t_R: 18.8 min.

6-Methoxy-(R,S)-2-aminotetralin-2-carboxylic acid (6-OCH_3-Atc)

A mixture of 5 g (20.3 mmol) of 6-methoxy-tetralin-spirohydantoin and 35 g (131 mmol, 5.5 equivalent) of $Ba(OH)_2.8H_2O$ in 150 mL of hot water was refluxed in a pyrolysis tube for 47 h.

The reaction mixture was then poured into a flask, 200 mL of extra water was added and the unsoluble white residue was filtered. The pH of the solution was set to 2 with 1 M H_2SO_4 in a waterbath and stirred for an hour, the precipitated $BaSO_4$ was filtered, washed with hot water, then the pH of the combined mother liquor was adjusted to 6 with 25% NH_3. The mixture was evaporated to half of its original volume and the resulting white precipitate was crystallized from EtOH-water (1:1) to give 3.81 g of white crystals in 85% yield. mp 231-232 °C. ^{1}H-NMR (DMSO-d_6/TFA); 7.05 (d, J=8.4 Hz, 1 arom. H); 6.76, 6.74 (2d, J_1=8.4 Hz, J_2=2.2 Hz; 1 arom. H); 6.72 (m, 1 arom. H); 3.72 (s, 3 OCH_3 H); 3.25 , 2.90 (2d, J=16.7 Hz, 2 aliph. H); 2.84 (t, J=6.4 Hz, 2 aliph. H); 2.16-2.22 (m, 1 aliph. H); 2.01-2.06 (m, 1 aliph. H). MS (ESI): 222 (MH^+). TLC R_f(4): 0.60. HPLC t_R: 13.2 min.

N-Trifluoroacetyl-6-methoxy-(R,S)-2-aminotetralin-2-carboxylic acid (N-TFA-6-OCH$_3$-Atc)

1.75 g (7.91 mmol) of 6-OCH_3-Atc and 7.5 mL (54.0 mmol, 6.8 equivalent) of trifluoroacetic anhydride in 28 mL of TFA were stirred in an icebath for 2 h followed by stirring at RT for 21 h. The brown solution was evaporated and water was added to the resulted brown oil. The oil solidified and the resulting white precipitate was crystallised from EtOH-water 1:1 to give 1.67 g of white crystals in 68% yield.

mp 145-147 °C. ^{1}H-NMR (CD_3OD); 6.98 (d, J=8.4 Hz, 1 arom. H); 6.72, 6.70 (2d, J_1=8.4 Hz, J_2=2.6 Hz, 1 arom. H); 6.67 (d, J=2.5 Hz, 1 arom. H); 3.75 (s, 3 OCH_3 H); 3.25, 3.10 (2d, J=16.5 Hz, 2 aliph. H); 2.76-2.85 (m, 2 aliph. H); 2.52-2.56 (m, 1 aliph. H) 2.15-2.21 (m, 1 aliph. H). MS (ESI): 318 (MH^+). TLC R_f(4): 0.71. HPLC t_R: 21.4 min.

Resolution of N-trifluoroacetyl-6-methoxy-(R,S)-2-aminotetralin-2-carboxylic acid

7.32 g (23.1 mmol) of N-TFA-6-OCH_3-Atc was dissolved in 500 mL of water, the pH was set to 7-8 with 2 N NaOH. 500 µl of carboxypeptidase A (22 mg protein/mL, 70 U/mg protein) was added and the reaction mixture was stirred for 4 days at 37 °C. The mixture was acidified to pH=2 with cc. HCl, the solution was boiled for 2 min, charcoal was added and the mixture was boiled again, then the charcoal was filtered and the solution was extracted with 4×100 mL of EtOAc, then the combined organic fractions were extracted with 3×80 mL of water. Both solutions were evaporated to dryness and 50 mL of 48% HBr was added to each and refluxed for 24 h under stirring. Then the solutions were evaporated, the residues were dissolved in 30

mL of hot water, the pH was adjusted to 6-7 with 25% NH_3, the resulting precipitate was filtered and crystallized from MeOH-water (1:1).

(R)-Hat (Hat from the aqueous phase): m: 1.26 g (26%). mp >280 °C. ^{1}H-NMR (DMSO-d_6); 6.84 (d, J=8.2 Hz, 1 arom. H); 6.54 (2d, J_1=8.2 Hz, J_2=2.3 Hz, 1 arom H); 6.49 (d, J=2.0 Hz, 1 arom. H); 3.18 , 2.61 (2d, J=16.9 Hz, 2 aliph. H); 2.64-2.73 (m, 2 aliph. H); 2.02-2.08 (m, 1 aliph. H); 1.77-1.80 (m, 1 aliph. H). MS (ESI): 208 (MH^+). TLC R_f(1): 0.37; R_f(5): 0.50. Chiral TLC R_f(1): 0.48 (identical with the lower spot of racemic Hat). $[\alpha]_D^{20}$: -4.1 ° × mL/g × dm (c=6.67 g / 100 mL 1M HCl (0.32 M)). HPLC t_R: 10.7 min.

(S)-Hat (Hat from the organic phase): m: 1.59 g (33%). mp >280 °C. ^{1}H-NMR (DMSO-d_6) in close agreement with those of *(R)*-Hat: 6.84 (d, J=8.2 Hz, 1 arom. H); 6.55 (2d, J_1=8.1 Hz, J_2=2.2 Hz, 1 arom. H); 6.49 (s, 1 arom. H); 3.18 , 2.62 (2d, J=16.9 Hz, 2 aliph. H); 2.66-2.72 (m, 2 aliph. H); 2.02-2.08 (m, 1 aliph. H); 1.78-1.81 (m, 1 aliph. H). MS (ESI): 208 (MH^+). TLC R_f(1): 0.37; R_f(5): 0.50. Chiral TLC R_f(1):0.56 (identical with the upper spot of racemic Hat). $[\alpha]_D^{20}$: +3.7 ° × mL/g × dm (c=6.67 g / 100 mL 1M HCl (0.32 M)). HPLC t_R: 10.7 min.

Boc-(S)-Hat and ***Boc-(R)-Hat*** were prepared the same way as described above for Boc-Hat to yield the desired products as yellow oils in 40% yield. MS, TLC and HPLC parameters of the products were very similar or identical with those of racemic Boc-Hat.

Solid-phase peptide synthesis was performed by the manual solid-phase technique using 4-methylbenzhydrylamine resin (0.8 mmol/g of titratable amine).

Coupling was performed by shaking the resin with two equivalents of Boc-amino acid, HOBt and DCC for 1-3 h, then the resin was washed with DCM, EtOH and DCM again (3 times each). Coupling was monitored with the ninhydrin test.

Boc-deprotection was carried out by treating the peptidyl-resin with 50% TFA/DCM containing 2 % of anisole (5+20 min), then the resin was washed with DCM, (3 times), neutralized with 10% DIEA/DCM (2 times) and washed with DCM again (3 times).

Atc and Hat were incorporated as racemic mixtures to the peptide sequences. Side-chain protections were benzyl for Asp and Glu, and dichlorobenzyl for Tyr.

Simultaneous side-chain deprotection and cleavage from the resin were accomplished by treatment with HF and anisole as scavenger (1 mL of anisole and ~10 mL of HF /g peptide-resin) at 0 °C for 1 h. After evaporation of the HF, the peptide resin was washed with diethylether and the peptide was extracted with glacial acetic acid and lyophilized.

Purified peptides were characterized by HPLC, TLC and MS analyses (Table 5).

Table 5. Physicochemical data of deltorphin analogs

Peptide	HPLC k'	TLC R_f			MS Mol. Wt.
		(1)	(5)	(7)	
Tyr-D-Ala-Phe-Asp-Ile-Ile-Gly-NH_2	2.49[a]	0.53	0.56	0.67	796.6[d]
Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2	2.37[a]	0.51	0.61	0.65	810.6[d]
Tyr-D-Ala-(*R*)-Atc-Asp-Ile-Ile-Gly-NH_2	5.90[a]	0.43	0.53	0.62	822.6[d]
Tyr-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2	6.90[a]	0.48	0.53	0.64	822.6[d]
Tyr-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2	4.82[a]	0.46	0.54	0.63	837[d]
Tyr-D-Ala-(*S*)-Atc-Glu-Ile-Ile-Gly-NH_2	5.75[a]	0.49	0.57	0.64	837[d]
Dit-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2	4.61[b]	0.54	0.62	0.63	1075[e]
Dit-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2	3.94[b]	0.58	0.61	0.59	1089[e]
(S)-Hat-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2	4.73[c]	0.58	0.60		837[e]
(R)-Hat-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2	4.75[c]	0.59	0.59		837[e]

HPLC k' on a Vydac 218TP1010 column;
[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. Solvent system was 0.1% TFA in water, 0.1% TFA in ACN. Solvent front breakthrough at 3.0 min.
[c] *HPLC k' on a Vydac 218TP54 column; gradient of 0-80% organic component in 30 min; flow rate 1 mL/min. Solvent system was 0.1% TFA in water, 0.1% TFA in ACN. Solvent front breakthrough at 3.0 min.*
[d] *FAB ionization*
[e] *ES ionization*

Determination of the configuration of Atc in the peptides

After RP-HPLC purification, two diastereomeric peptides were obtained. 1 mg of each peptide was hydrolyzed separately by refluxing with 1 ml of 6 N HCl under nitrogen in a glass ampulle for 25 h. The solvent was removed by evaporation, Atc was separated from the amino acid mixture by HPLC (Vydac column 218TP1010 C_{18}, 254 nm, linear gradient from 10 to 15% of ACN in 0.1% TFA solution within 30 min (k' of Atc: 2.73)). Half of the amount of Atc was spotted onto a chiral TLC plate to determine the R_f value in ACN-MeOH-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). The other part of the Atc from the HPLC separation was used for GITC derivatization. Upon comparison with *(R)*- and *(S)*-Atc standards, the k' values of the Atc derivatives (Vydac 218TP54 column, eluent: 0.1% TFA in water-MeOH (55:45), k' of *(S)* isomer: 2.41; k' of *(R)* isomer: 2.97) indicated that the first eluting peptide contains *(R)*-Atc. This is true for deltorphin I and deltorphin II analogs, too.

Iodination of Tyr-D-Ala-(R)-Atc-Glu-Ile-Ile-Gly-NH_2 and Tyr-D-Ala-(S)-Atc-Asp-Ile-Ile-Gly-NH_2

3.5-4.0 μmol of peptide was dissolved in 1 mL of Na_2HPO_4 buffer (0.25 M, pH 7.4) and 2 equivalents of NaI (10 mg/ml aqueous solution) and 2-2.5 equivalents of chloramine-T (2.5 mg/ml aqueous solution) were added. After stirring for 10 s at RT, products were separated by HPLC to give the desired diiodinated peptides in 50-60 % yield. Purified Dit-containing precursor peptides were characterized by HPLC, TLC and MS analyses (Table 5).

Tritium labeling of Dit-D-Ala-(R)-Atc-Glu-Ile-Ile-Gly-NH_2 and Dit-D-Ala-(S)-Atc-Asp-Ile-Ile-Gly-NH_2

1.5 μmol of the iodinated precursor peptide was dissolved in 1 mL of 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. 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 at RT for 80-90 min. Tritiation was controlled by following the tritium pressure with a manometer. After the reaction had been completed, the reaction mixture was frozen with liquid N_2 and unreacted tritium was absorbed on pyrophoric uranium. The catalyst was filtered off through a Whatman GF/C filter and washed three times with EtOH:H_2O (1:1). The mother liquor was evaporated and the labile tritium was removed by repeated evaporation from EtOH:H_2O (1:1). The total radioactivity of the labeled peptide was measured by liquid scintillation counting, and was found to be 2.14 GBq (57.7 mCi) and 1.23 GBq (33.2 mCi) for tritiated Dit-D-Ala-(R)-Atc-Glu-Ile-Ile-Gly-NH_2 and Dit-D-Ala-(S)-Atc-Asp-Ile-Ile-Gly-NH_2, respectively.

Crude tritiated peptides were purified by TLC in solvent (5). Purity was checked by analytical HPLC and by TLC. Specific radioactivity was determined by comparing the UV absorption of the labeled peptides to that of unlabeled standards of known weight, and proved to be 1.33 GBq/mmol (36.0 Ci/mmol) and 1.28 TBq/mmol (34.5 Ci/mmol) for [^{3}H]-Tyr-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2 and [^{3}H]-Tyr-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2, respectively. Purified peptides were stored in 37 MBq/mL (1 mCi/mL) concentration under liquid N_2.

4. RESULTS AND DISCUSSION

4.1 Synthesis

Syntheses of 6-hydroxy-2-aminotetralin-2-carboxylic acid (Hat) and 2-aminotetralin-2-carboxylic acid (Atc) were accomplished by well-known literature methods.

Atc was synthesized from the commercially available hydantoin derivative in one step [**I**]. In principle, hydantoins can be cleaved both by acid and alkali [62]. However, according to HPLC analysis, refluxing with concentrated HCl for one week did not turn the hydantoin quantitatively to the desired amino acid. Therefore, aqueous $Ba(OH)_2$, which converts the hydantoins into the corresponding amino acids smoothly under reflux in 16 h [62,121], was used for hydrolysis.

Hat [**II**] was synthesized in three steps starting from p-methoxy-phenylacetyl chloride (Figure 2) to give 6-methoxy-β-tetralone. This compound is not stable and readily decomposes during storage at -20 °C, therefore it was converted either into the hydantoin derivative in a short time or was stored as its bisulphite derivative which is stable at RT for several months and can be converted into the hydantoin under the same reaction conditions as the ketone. Hydrolysis of the hydantoin was performed by 47% aqeous HBr which cleaves the hydantoin and the methoxy group simultaneously.

Boc-protection of the racemic amino acids was performed by well-known literature procedure [122]. However, standard protocol, which applies 1.1 equivalent of the Boc_2O for overnight reaction, did not prove to be sufficient and an additional portion of the reagent and long reaction time (3-5 days) was required to yield Boc-Hat in approximately 60% yield. Beside N-Boc-Hat, some (~ 2%) bis-Boc-Hat was also formed which precipitated together with N-Boc-Hat. This mixture was used for peptide synthesis without further purification.

Deltorphin analogs were synthesized manually by the solid-phase peptide synthesis method using Boc-chemistry [**I,II**]. As an amide terminus already provides some resistance towards enzymatic degradation, and the native deltorphins also have amidated C termini, new analogs were designed to have amide termini, too. Therefore the p-methyl-benzhydrylamine resin was chosen as support for the synthesis. HOBt and DCC were utilized as coupling agents, and 50% TFA in DCM, which contained 1% of anisole as scavenger, was used for deprotection of Boc-peptidyl-resin.

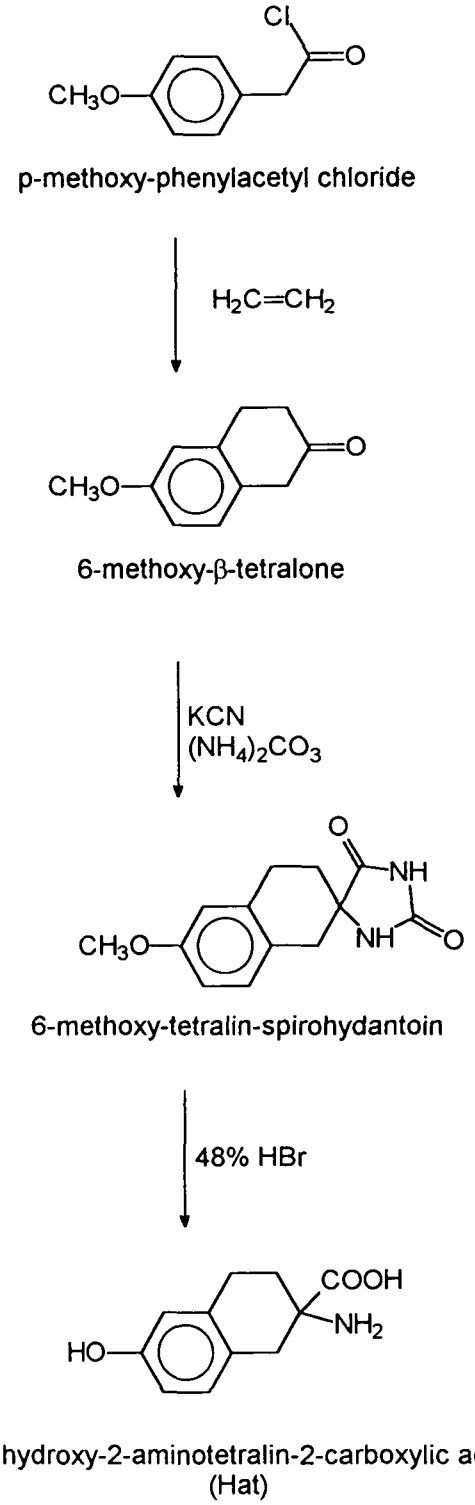


Figure 2. Synthesis of 6-hydroxy-2-aminotetralin-2-carboxylic acid (Hat)

In the course of peptide synthesis, some difficulties arose. Coupling of Boc-Atc to the Asp/Glu-Ile-Ile-Gly-resin yielded a sterically hindered protected peptidyl-resin. As a result, deprotection was not accomplished by the usual 5+20 min deprotection time. In order to complete deprotection, an additional step with 2% anisole in TFA was applied. After this, the ninhydrine test showed the desired blue color indicating the presence of a free amino group. However, in spite of a negative ninhydrine test after coupling of Boc-D-Ala, HPLC analysis of the crude peptide mixture obtained after HF cleavage revealed the presence of three big peaks instead of the expected two for the desired diastereomeric peptides. Mass spectrometry proved, that the first eluting (the least hydrophobic) product has a molar mass which is equivalent with that of a peptide which does not contain D-Ala (Tyr-Atc-Glu-Ile-Ile-Gly-NH_2). A question arises: why were there only three peaks? The des-D-Ala-peptide should be also diastereomeric. There are two possible explanations. First, HPLC conditions applied for the separation of *(R)*- and *(S)*-Atc-containing deltorphin analogs were not sufficient to resolve those diastereomers which did not contain the D-Ala moiety. Second, the peptide which did not contain the D-Ala residue resulted as a failure of coupling Boc-D-Ala only to one Atc enantiomer. In other words, peptide bond formation has different reaction velocity for coupling Boc-D-Ala to the diastereomers of Atc-peptidyl-resins.

The first assumption is not likely. Although HPLC conditions used for the separation of the diastereomeric peptides with the desired sequence provide a k' value to the des-D-Ala peptide of only 3.5, the k' values of the desired peptides were 4.8 and 5.8 thus providing a Δk'of 0.9. According to this, one may expect that the des-D-Ala peptides could also have been resolved at least in part.

There is some supporting evidence for the second presumption. The sum of the area of the first two eluting peaks, one of which is the des-D-Ala peptide and the other is one of the diastereomers of Atc^3,$Ile^{5,6}$-deltorphin II, is roughly the same as that of the third peak, which stands for the other diastereomer of Atc^3,$Ile^{5,6}$-deltorphin II. However, as the focus of this project was to develop highly potent deltorphin analogs, and a peptide missing the D-Ala residue was suspected to lack biological activity, configuration of Atc in the des-D-Ala peptide was not investigated. The conclusion of this synthetic difficulty was that longer deprotection and coupling times (2-3 h instead of 1 h which is generally sufficient) and larger excess of Boc-D-Ala (such as 4 equivalent instead of the two-fold excess which was routinely applied) are needed in order to achieve complete coupling. During further peptide synthesis, these

modifications did not prevent completely the formation of des-D-Ala peptides but reduced its amount significantly.

Coupling to Hat, being the N-terminal amino acid, could not cause similar problems. However, coupling of Boc-Hat to the D-Ala-peptidyl resin proved to be difficult but the problem could be overcome by long coupling times (ranging from 5 h to overnight coupling). For monitoring of this coupling, the Kaiser test did not prove to be reliable, therefore use of the chloranil test [89] is advisable.

Peptides were cleaved from the polymeric support by liquid HF (appr. 10 ml of HF/g of peptide-resin) with anisole as scavenger (1 ml of anisole/g of peptide resin). After cleavage, the scavenger was removed by washing the reaction mixture with diethyl ether, and peptides were extracted from the peptide-resin mixture by glacial acetic acid, and were lyophilyzed. Crude peptides were obtained generally in appr. 60% yield (referring to the amount of NH_2 groups on the resin) and were purified by reversed phase HPLC on a semipreparative C-18 column. In the case of the Atc-containing peptides, a biner eluent made up of ACN as organic modifier and of water, both containig 0.1% of TFA as ion-pairing agent, afforded good separation for the diastereomeric peptides. However, Hat-containing diastereomers did not separate well enough for semipreparative purification in any biner eluents of water and organic modifiers such as ACN, MeOH or THF even under isocratic conditions with k' value >10. A similar purification problem was observed for the Hat^1-DPDPE analog [41], but in that case separation of diastereomers could have been performed in methanolic eluent at the expense of long separation times (k' >8). In general, incorporation of a racemic amino acid to the N-terminal of a peptide may not generate such changes in the conformation or in the overall polarity of the molecule which would allow a quick HPLC separation on semipreparative scale. Identical HPLC and TLC data for the Hat-containing diastereomers indicated that conformational and biological properties of these peptides may also be very similar.

Owing to the failure of the separation of the Hat-containing diastereomers, synthesis of Hat in enantiomerically pure form had to be carried out on preparative scale [**II**]. Determination of the configuration of Atc in the new analogs also required optically pure standards [**I**]. Enzymatic resolution of the amino acids seemed to be an appropriate solution. α-Chymotrypsine and carboxypeptidase A both can be applied for the resolution of aromatic amino acids. Since carboxypeptidase A was successfully applied for the resolution of β-methyl-Phe [123], this enzyme was chosen. A pseudopeptide bond was formed by trifluoroacetylation

of the NH_2 group. In order to avoid possible side-reactions, trifluoroacetylation was performed on 6-methoxy-Atc instead of Hat. This compound was synthesized by alkali hydrolysis of the corresponding hydantoin.

Enzymatic digestion of N-TFA-Atc and N-TFA-6-methoxy-Atc (Figure 3) resulted in good resolution with interesting results. According to TLC and RP-HPLC, carboxypeptidase A can digest both enantiomers of N-TFA-Atc in 24 h at RT. However, the reaction can be stopped halfway to yield an enantiomerically pure product, according to chiral TLC and chiral derivatization. The amino acid digested by carboxypeptidase A was the enantiomer giving the lower spot of racemic Atc on chiral TLC **[IV]** and the second peak on HPLC after derivatization of racemic Atc with GITC. Polarimetry showed that this is the levorotary compound. Among the enantiomers of Phe, L-Phe (in terms of absolute configuration, *(S)*-Phe) is the levorotary enantiomer. However, Atc can be regarded as a ring-closed analog of both Phe and homo-Phe (Hfe), which results in opposite relative configuration for the same compound. Moreover, specific rotatory values alone do not give a proof for absolute configuration. Therefore a conclusive proof was required to assign the absolute configuration of the product of the enzymatic digestion, which was provided by X-ray diffraction. This experiment needs the presence of a "heavy" atom in the molecule, thus the hydrobromic salt of the digested Atc isomer was prepared and crystallized from EtOH-water (1:1). Interestingly, X-ray diffraction verified that the absolute configuration of this Atc enantiomer is *(R)*. A possible explanation for the "strange" behaviour of carboxypeptidase A can be that the enzyme recognizes its substrate as a Hfe analog, because this provides a longer and probably more hydrophobic side-chain for the hydrophobic pocket, and the C1 carbon of the tetralin ring stands for the α-hydrogen of natural amino acids. This opposite behaviour does not hold for α-chymotrypsine, which was reported to digest the dextrorotary enantiomer of Atc **[I]** and Hat [124].

Resolution of the Hat derivative was performed under the same conditions as that of the Atc derivative **[II]**. Surprisingly, digestion of only one enantiomer was observed over a reaction time of 2 weeks at 37 °C. The product of the digestion was separated from the unreacted starting material and both were hydrolyzed (separately) by 47 % HBr to give optically pure Hat enantiomers.

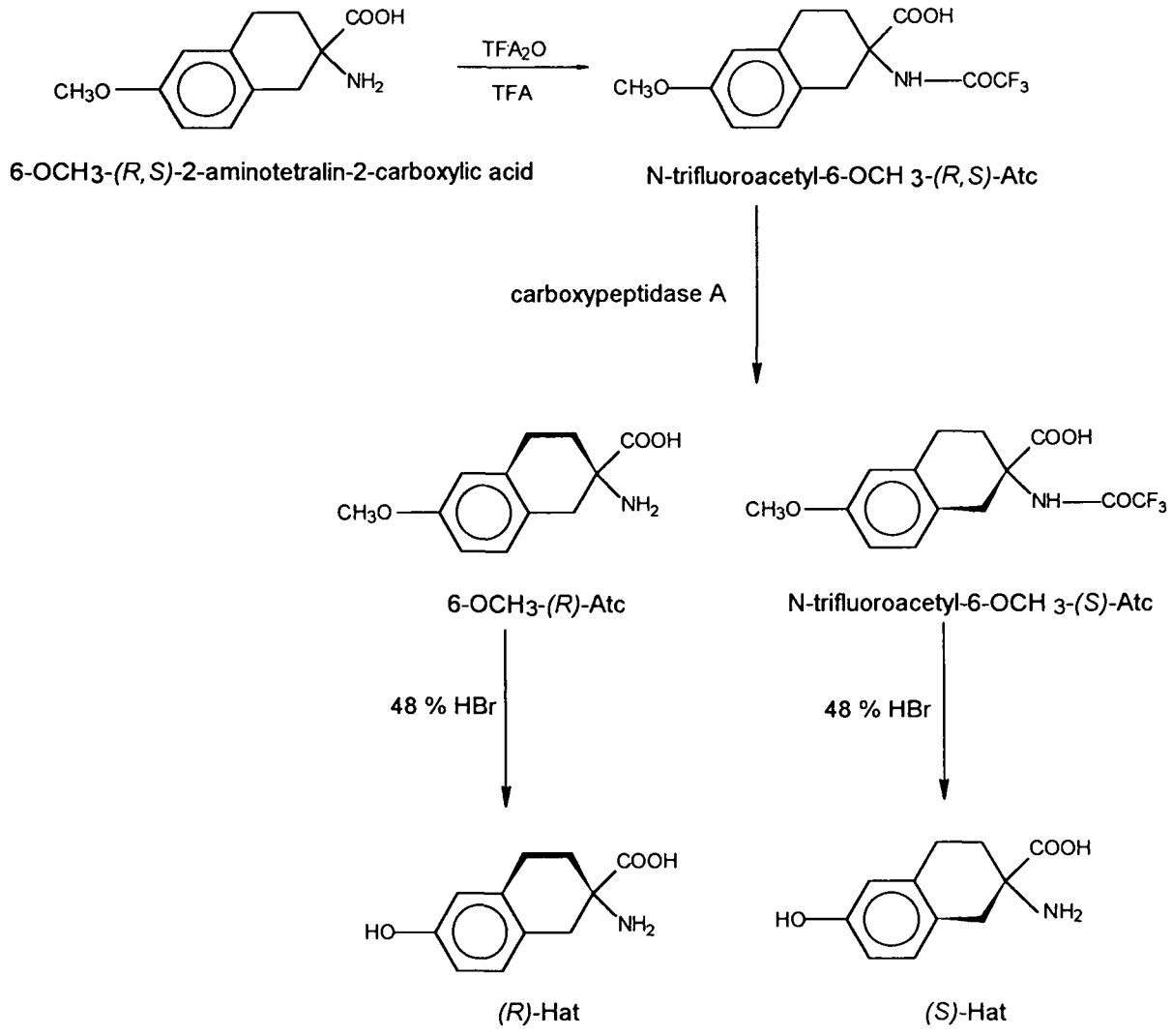


Figure 3. Enzymatic resolution of N-trifluoroacetyl-6-methoxy-(*R,S*)-2-aminotetralin-2-carboxylic acid

This was proved by chiral TLC [**IV**] and GITC derivatization. The derivative which was digested by carboxypeptidase A, after hydrolysis, proved to be identical with the Hat enantiomer giving the lower spot on chiral TLC and the second peak on HPLC after GITC derivatization. Polarimetry showed that this is the levorotary enantiomer of Hat, thus there was an indirect proof that carboxypeptidase A cleaves the TFA group from the amino acid derivative of *(R)* configuration again. In this case X-ray diffraction was not carried out as high quality hydrobromic acid crystals of the digest could not be obtained. However, stereochemistry could be assigned unambiguously by comparison of the $[\alpha]_D$ values with

literature data where crystallographic assignment was carried out [125]. The enantiomerically pure Hat isomers were converted into their Boc-protected derivatives and were incorporated to $Ile^{5,6}$-deltorphin II the same way as was described above. Crude peptides were purified by RP-HPLC in MeOH-water biner eluent.

For the Atc-containing diastereomers, configuration of Atc in the peptides had to be assigned [**I**]. 1-1 mg sample of the collected HPLC peaks of the diastereomeric peptides were hydrolyzed under reflux with 6 M HCl for 25 h, and Atc was separated from the resulting amino acid mixture by HPLC. Then the R_f value of the separated Atc was compared with that of racemic Atc. The first eluting peak contained Atc with *(R)* configuration both for deltorphin I and II analogs.

The commercially available chiral TLC plate of Macherey-Nagel proved to be an extremely useful tool during this project for several purposes: for the assignment of Atc in the diastereomeric peptides, for following the course of enzymatic resolution and for monitoring the optical purity of enzymatically resolved Atc and Hat [**IV**]. However, due to the lack of a densitometer, quantification of the results could not be performed by this method. Fortunately, HPLC analysis after chiral derivatization of a putative mixture of enantiomers can serve as a simple method for this purpose. There are two widely used derivatizing reagents for analysis of amine compounds: Marfey's reagent (1,5-difluoro-2,4-dinitrobenzene) and the GITC (2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate) reagents. Both of them were succesfully applied to produce diastereomeric compounds of Atc and Hat which are easy to separate by HPLC [126]. Derivatization with Marfey's reagent takes place at 40 °C in 1-1.5 h producing a yellow product of the amino acid(s) arylated on the amine functionality, which can be detected at 340 nm. However, both the reagent and the products are sensitive to light. For this reason GITC derivatization was chosen for routine monitoring of optical purity of Atc and Hat, which proceeds smoothly at RT in 1 h and yields the thiourea derivative of the amino acid which can be detected at 250 nm and is stable for at least 1 week.

According to GITC derivatization, enzymatically resolved Atc enantiomers did not contain enantiomeric impurity, thus, enantiomeric excess value is practically 100. Hat isomers contained 2-3% of enantiomeric impurity, thus *ee* was found to be ~95. Thus, carboxypeptidase A was an appropriate choice for resolution of Atc and Hat, as resolution of Atc [**I**] and Hat [124] by α-chymotrypsin yields only enantiomerically enriched amino acids.

Biological characterization revealed the high affinity and selectivity of the new deltorphin analogs (see below). Two potent and selective new analogs, *(R)*-Atc^3,Ile^{5,6}-deltorphin I and *(S)*-Atc^3,Ile^{5,6}-deltorphin II were chosen for radioactive labeling [**III**] for further biological characterization [**V**]. The most widely used radioactive labels for peptides are C-14, H-3, S-35 and I-125. Incorporation of I-125 provides high specific radioactivity but evidently changes the physicochemical properties of the molecule, which in turn results in different biological characteristics. Therefore, radioiodinated peptides cannot be applied as labeled equivalents of the nonlabeled parent peptides. Monoiodinated peptides sometimes retain most of the biological activity of the parent compounds, but diiodination of the Tyr residue in most cases destroys potency. This phenomenon is not striking if we consider the modified size, hydrophobicity and acidity of the Tyr side-chain which may influence the characteristics of the whole molecule. The Tyr moiety of opioid ligands is a key pharmacophore, therefore it is highly recommended to keep this moiety intact. Thus, tritium labeling, which does not change significantly the properties of the parent peptide and can easily be carried out on the activated aromatic ring of Tyr, can be the matter of choice. Incorporation of one tritium atom into the molecule results in a specific activity of 28.6 Ci/mmol, which is satisfactory not only for radioligand binding assays but also to perform autoradiography [127,128]. Last, but not least, tritium is a soft β emitting nuclide with 37 keV average electron energy which makes the use of this isotope ideal in terms of safety.

Tritium labeling can be carried out in several ways [129]. Tritiation of halogen- or double bond-containing precursor peptides is easily applicable if a suitable vacuum manifold and tritium gas are available. Halogen-containing precursor peptides are readily available either by the synthesis of peptides involving the incorporation of halogenated amino acids such as diiodotyrosine (Dit), or by iodination of the aromatic side-chain of the parent peptide. Precursor peptides for tritium labeling of *(R)*-Atc^3,Ile^{5,6}-deltorphin I and *(S)*-Atc^3,Ile^{5,6}-deltorphin II were obtained by the latter approach.

HPLC purified *(R)*-Atc^3,Ile^{5,6}-deltorphin I and *(S)*-Atc^3,Ile^{5,6}-deltorphin II were iodinated by the chloramine T method [130]. Chloramine T oxidizes iodide ions and subsequently polarizes the resulting iodine the positively polarized part of which attacks the aromatic ring of Tyr activated by the phenolic hydroxyl group. As a result of electrophylic substitution monoiodo- and diiodotyrosine-containing peptides are formed depending on the ratio of the peptide and iodine. The reaction can be terminated by sodium thiosulphate, but in order to avoid

purification problems, rapid cooling was applied instead. The reaction mixture was purified by HPLC and it was found that 4 equivalents of NaI and chloramine T generates only Dit-containing peptides. These Dit-containing precursor peptides were dehalogenated by tritium gas in a tritiating apparatus manufactured in our institute. The reaction was carried out in DMF solution with triethylamine to neutralize the HI formed. 10% Pd (oxidized form) on $BaSO_4$ was applied as catalyst.

Tritium labeled peptides were purified on reaction-zone type preparative TLC plates. It was found earlier by practice that appr. 30 mCi of radioactivity can be conveniently purified in one step thus this was applied for the new tritiated peptides too. Radioactivity was detected by radiography of a photography emulsion, then the silica gel which contained adsorbed radioactive material was extracted by MeOH. Purity control of the resulted compounds was performed by TLC and HPLC with detection of radioactivity by a TLC plate scanner and liquid scintillation detector, respectively. It was found that the tritiated compounds were pure and identical with the nonlabeled parent compounds. Specific radioactivity was determined by compairing the UV spectra of samples of known radioactivity with that of nonlabeled peptides of known weight, and it was found to be 1.28 TBq/mmol (34.5 Ci/mmol) for [^{3}H]-*(S)*-Atc^3,Ile^{5,6}-deltorphin I and 1.33 GBq/mmol (36.0 Ci/mmol) for [^{3}H]-*(R)*-Atc^3,Ile^{5,6}-deltorphin II. These specific radioactivity values are high enough to perform biological tests properly and are 60.5% and 63.2% of the theoretical maximum (57 Ci/mmol for 2 tritium atoms per molecule). The difference could be the result of hydrogen impurity of the tritium gas or exchange reactions with labile protons of the reagents, target compound, solvent etc. Tritiated compounds were stored as 37 MBq/ml ethanolic solutions under liquid nitrogen and were found to be stable for more than 6 months.

4.2 Conformational considerations

In order to find molecular determinants for high δ affinity and selectivity, structure of the native deltorphins were investigated by 2D NMR studies [131] in combination with molecular modeling [132-135].

Conformation of deltorphin I was examined in a DMSO/water cryoprotective mixture [133] and it was found that there were at least two conformations in equilibrium, a folded and an extended one. The folded conformer was characterized by a relative arrangement of the two aromatic rings similar to those of many rigid opiates and by a hydrophobic surface on the C-

terminal part. In this conformer, the Tyr^1 and the Phe^3 side-chains adopt the *trans* and the *gauche(–)* position, respectively (Figure 4). Similar observations were reported for deltorphin I in a DMSO solution [131].

In order to mimic the biological environment during receptor-ligand interaction, conformations of dermorphin and deltorphin I were investigated also in micelles of perdeuterated dodecylphosphocholine [135]. The presence of equilibrium mixture of multiple conformations was observed. Deltorphin I conformers generated by distance geometry calculations in accordance with the observed NOE data showed the common feature that the N-terminal Tyr-D-Ala-Phe-Asp and the C-terminal Val-Val-Gly-NH_2 sequences took turn structures resulting in a twisted S-shaped backbone conformation. In five low energy conformers, Tyr^1 side-chains were exclusively in *trans* position, while the Phe^3 side-chain could adopt either of the *gauche* conformations.

The same authors reported the conformational analysis of deltorphin II in the same biomimetic environment [134] and in water [136]. In the phospholipid micelles, the presence of the two β turns of the message and address domain was observed again. Five low energy conformers were described in which Tyr^1 side-chain could assume either of the *gauche* conformations, while the Phe^3 side-chain took on either the *trans* or the *gauche(–)* position. High selectivity of deltorphin I and II was ascribed to the presence of the C-terminal β turn. However, in aqueous solution (where low energy conformers for the aromatic side-chains were *trans* for Tyr^1 and *gauche(+)* for Phe^3), the presence of the C-terminal β turn was not observed [136].

All these data suggest, that side-chains of these residues are highly flexible in solution.

Tyr and Phe both can adopt three different low energy side-chain conformations, and these are *gauche(–)*, *trans* and *gauche(+)* characterized by the torsion angle χ^1 of -60°, 180° and 60°, respectively. The number of the available side-chain conformations can be reducted by conformational constriction. In Atc and Hat, there are two available side-chain conformations, namely *gauche(–)* and *trans* for *(S)*-Hat and *(S)*-Atc, and *trans* and *gauche(+)* for *(R)*-Hat and *(R)*-Atc (Figure 4).

Bioactive conformations can be assigned in terms of bioactivity and conformational data obtained by NMR experiments or molecular modeling.

From molecular modeling studies, Salvadori et al. [137] concluded that in deltorphin I the preferred side-chain conformation of Phe^3 is *trans* (χ_1 = 138.3°), whereas in the *(S)*-Atc^3

analog it is *gauche(–)* (χ_1 = -56.8°). Moreover, the low energy backbone conformation of the *(S)*-Atc[3] analog was calculated to be distinctly different from that of deltorphin I. NMR studies comparing deltorphin I and the (*S,S*)-βMePhe[3] and (*S,R*)-βMePhe[3] analogs indicated that the preferred side-chain conformation of Phe[3] in solution is *gauche(–)* [52]. For Hat[1] analogs of deltorphin II it was also found that the *gauche(–)* conformer also had a significant contribution to the conformational equilibrium [**II**].

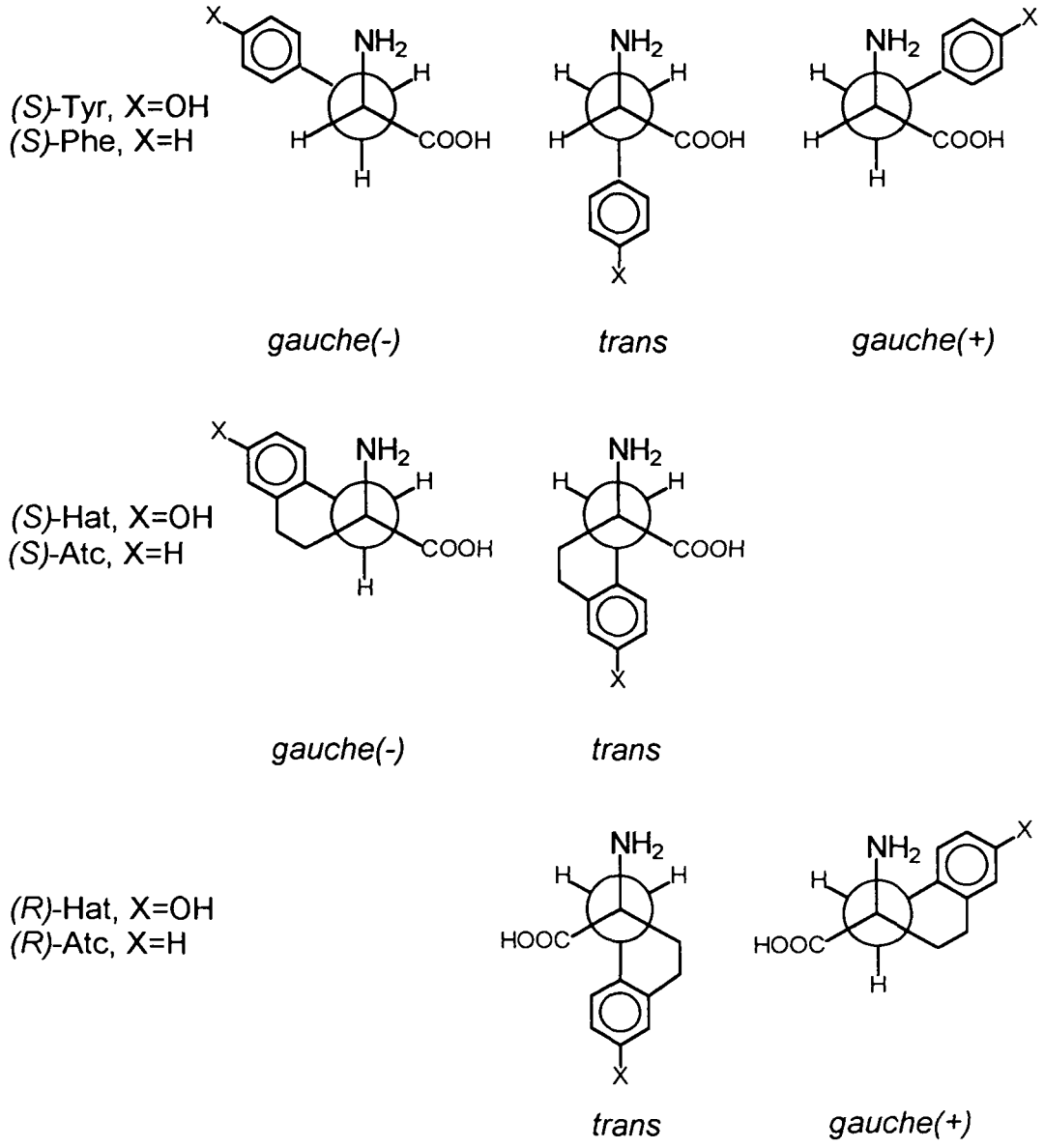


Figure 4. Possible side-chain conformations of enantiomers of Tyr, Phe, Hat and Atc

However, these findings are not consistent with the biological activities of other opioid ligands. Incorporation of 4-aminotetrahydro-2-benzazepine-3-one (Aba) as a conformational constraint for Phe^3-Glu^4 into the deltorphin II and also in the dermorphin sequence, resulted in high δ affinity for both analogs [51,138]. This finding indicates that the preferred *gauche(–)* conformation for Phe^3 in deltorphin cannot be the bioactive one, as the benzazepine constraint excludes the *gauche(–)* conformation, but it still allows two conformations at χ_1: *trans* and *gauche(+)*. Although it was shown that *trans* position was preferred by the Aba side-chain in solution, a receptor-induced change to *gauche(+)* cannot be excluded.

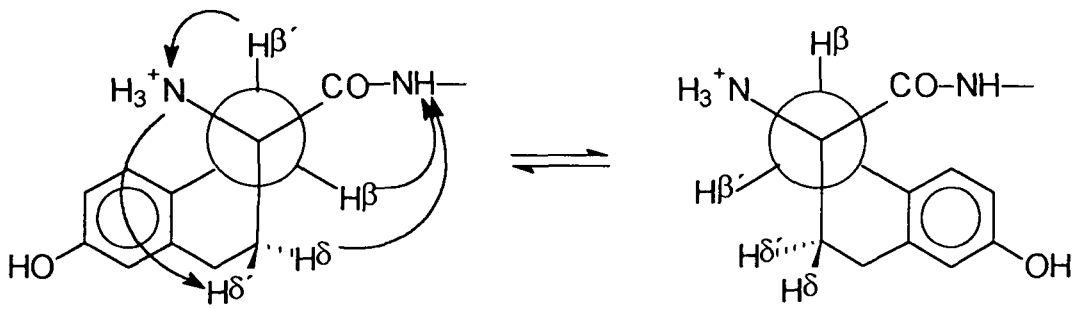
Atc^3-deltorphin I [49,50] and Atc^3,$Ile^{5,6}$-deltorphin II analogs [**I**] both have high affinity and selectivity towards δ receptors independently on the absolute configuration of the Atc^3 residue. Tic^3-deltorphin I [49,50] and Tic^3-deltorphin II [51] were also synthesized. The Tic residue can only adopt the *gauche(–)* and *gauche(+)* conformations. Binding ability and bioactivity were significantly decreased by this substitution. As neither *gauche(–)* nor *gauche(+)* is the bioactive conformation, it comes as an indirect evidence that the preferred bioactive conformation of Phe^3 in deltorphins is *trans*. Since the Aba^3-deltorphin II analog has a high potency (IC_{50} = 5.0 nM, despite the absence of the important Glu^4 side-chain) [51] and the *(S)*-Atc^3-deltorphins are also very potent, these argue in favor of *trans* as the δ receptor-bound conformation, too.

This conclusion does not support the model proposed by Mosberg [39] for the cyclic peptide JOM-13, in which Phe^3 has a *gauche(–)* conformation, but it is consistent with the model proposed by Nikiforovich [139,140] for JOM-13 and other δ agonists. The latter author applies the concept of "topology" introduced by Hruby [35]. This approach presumes, that the function of the peptide backbone is merely to fix the pharmacological key elements of the ligand in the correct spatial arrangement. Based on this concept, Nikiforovich et al. [139] developed a model for the δ receptor-bound conformer(s) of δ ligands which suggests that the Phe side-chain adopts the *gauche(–)* conformation in peptides containing Phe^4 (enkephalin and its analogs) and the *trans* rotamer for peptides containing Phe^3 (deltorphin and analogs).

It was previously demonstrated that the NMR parameters (chemical shift, nuclear Overhauser effects (NOEs) and temperature dependence) of the D-Ala^2 methyl group in deltorphins are very good probes for detecting changes in side-chain or backbone conformations at the N-terminus [51,138]. These NMR parameters have been determined for $Ile^{5,6}$-deltorphin II and for the Atc- and Hat-containing analogs in DMSO solution [**I,II**]. The

chemical shifts reveal only minor differences between the *(S)*- and *(R)*-Atc[3] analogs. Moreover, chemical shift differencies with the values for the parent Ile[5,6]-deltorphin II are limited to the D-Ala[2] methyl signal. These similarities do not support significantly different conformational behavior as predicted by Salvadori [137]. The observed low-field shift of the D-Ala-methyl signal and the small temperature dependence indicate that the shielding effect due to the aromatic side-chain at position 3 is absent in both Atc[3] analogs. Together with the absence of NOEs for the Atc residue this favors a common *trans* side-chain orientation for both Atc residues although an influence of the fixation of the χ_2 in Atc compared to a free rotating phenyl ring in the Phe[3] analog cannot be excluded.

Similar discrepancies came up by comparing the conformation determined from NMR data with bioactivity of deltorphin analogs restricted in the Tyr[1] residue. Qian et al. [47] synthesized (2*S*,3*S*)-2',6'-dimethyl-β-methyltyrosine substituted deltorphin I and found that the new analog showed two to fivefold decrease of affinity, while retaining the high selectivity of the parent ligand. Results of NMR studies suggested that the most populated side-chain conformation of the first residue is *gauche(–)*. For the Hat[1] analogs of deltorphin II described in this project, the analysis of the NOE patterns involving Hat[1] and D-Ala[2] resonances allowed an unambiguous assignment of the preferred rotamer for Hat[1], namely *gauche(–)* for the *(S)*- and *gauche(+)* for the *(R)*-analog [**II**]. These NOE patterns are indicated on the *gauche(–)* conformer in Figure 5.



gauche(–) *trans*

Figure 5. NOE patterns used to assign the *gauche(–)* conformation in the *(S)*-Hat[1]-deltorphin analog

The *gauche(–)* conformation of the *(S)*-Hat[1]-deltorphin analog is in agreement with the absence of an NOE effect between the Hat[1] aromatic protons and the D-Ala[2] methyl protons, and with the chemical shift of 0.95 ppm of these methyl protons. In deltorphin II, the

corresponding signal is observed at the unusual high field position of 0.62 ppm, which is ascribed to a shielding by the Tyr^1 aromatic ring in the *trans* orientation. It is however close to the value of 0.91 ppm observed in the hydroxy-Tic $(Htc)^1$-deltorphin II, which prefers the *gauche(–)* conformation in the Htc residue [51].

Nevertheless, just as in the case of Phe^3 substituted deltorphins, NMR data are not conclusive for the bioactive confomation. Htc^1 substitution in dermorphin and deltorphin II [51] destroyed the bioactivity of the parent peptides. This suggests, that *gauche(–)* and *gauche(+)*, the two available side-chain conformations for the Htc residue, cannot be the bioactive ones. The high δ affinity of the Hba^1-deltorphin II analogue [138] which cannot adopt the *gauche(–)* conformation, also does not support the idea that the bioactive side-chain conformation of the first residue in the deltorphins is the *gauche(–)* but that it is the *trans*. All these data strongly suggests that the Hat^1-deltorphin analogs switch from their preferred solution conformation to the *trans* conformation during receptor interaction.

The above described discrepancies point out the inevitable deficiency of drawing conclusions merely on the basis of NMR data for the bioactive conformation of a receptor ligand. One has to keep in mind that the bioactive structure is not necessarily of the lowest energy and that this structure may not exist in solution but occurs only when the ligand is bound to the receptor site. A more accurate approach would include the direct analysis of the ligand-receptor complex. This study would require the preparation of NMR-active isotopically labeled peptides or the deuteration of the target receptor in order to distinguish the signals of the two species. Although this approach faces enormous synthetic and spectroscopic difficulties, there are a few examples when this type of study was applied for other biological systems, but it was not yet performed on opioids.

4.3 Biological data

The new deltorphin analogs were examined with regard to their binding properties to rat brain opioid receptors and *in vitro* bioactivities via their abilities to inhibit an electrically induced contraction of the mouse vas deferens (MVD) and guinea pig ileum (GPI).

For characterization of Atc^3-containing analogs [**I**], $[^3H]Ile^{5,6}$-deltorphin II [104], [^{3}H]TIPPΨ [105] and [^{3}H]naltrindole [141,142] were used as δ radioligands, and [^{3}H]DAMGO and [^{3}H]U-69,593 as μ and κ radioligands, respectively. $Ile^{5,6}$-deltorphin I and II

were also characterized for comparison. Binding affinities of these ligands are summarized in Table 6.

All six peptides exhibited very high affinity for the δ receptors (K_i values were in the subnanomolar range) by replacing tritiated agonist ([^{3}H]Ile^{5,6}-deltorphin II) or antagonist ([^{3}H]TIPP[Ψ] and [^{3}H]naltrindole) in the binding assays. These three relatively new tritiated ligands, prepared in our laboratory, are highly selective for δ opioid receptors. In the competition studies the peptides were less active against [^{3}H]naltrindole, compared to data against the two peptide ligands.

δ Selectivity ($K_{i\mu}/K_{i\delta}$) was calculated from K_i values measured with [^{3}H]Ile^{5,6}-deltorphin II and [^{3}H]DAMGO (Table 6). The most selective compound was Ile^{5,6}-deltorphin I, but the selectivity of *(S)*-Atc^3,Ile^{5,6}-deltorphin I and *(R)*-Atc^3,Ile^{5,6}-deltorphin II were also more than 20000. In the Atc^3 analogs of deltorphin I, the *(S)* analog is the more potent and selective, whereas in the Atc^3 analogs of deltorphin II, the *(R)* analog has better potency and selectivity. μ Receptor binding affinities were in the interval of 660-4526 nM, while all K_i values obtained for the κ receptors were higher than 10000 nM. Deltorphin I with D-Phe at position 3 was 20-50 times less potent than the parent peptide [49], while the Atc^3 analogs had more comparable K_i values for the *(R)* and *(S)* analogs.

In the *in vitro* bioassay (Table 7), all six peptides displayed properties similar to those in the binding assay [I]. In the MVD assay (where δ receptors are predominant), the new analogs were agonists in the subnanomolar range, while in the GPI assay (which contains predominantly μ receptors) the IC_{50} values were in the micromolar or higher range. These results indicate, that the δ opioid receptor selectivity of these peptides is higher than that of the parent peptides [44]. With ratios of IC_{50}(GPI)/IC_{50}(MVD) higher than 100000, *(R)*- and *(S)*-Atc^3,Ile^{5,6}-deltorphin II analogs are the most δ selective peptides reported to date.

Peptides containing Atc with *(R)* or *(S)* configuration were almost equipotent in the MVD assay (only a 2 to 3 fold difference) (Table 7). An almost complete loss of stereospecificity was earlier observed for the *(R)*- and *(S)*-Atc^3 analogs of Tyr-D-Orn-Phe-Glu-NH_2, a slightly μ selective peptide [143] and *(R)*- and *(S)*-Atc^3 analogs of deltorphin I [49,50]. The bioselectivity of Ile^{5,6}-deltorphin I with *(R)*-Atc at position 3 was about 2 times higher than that of Ile^{5,6}-deltorphin I. In the cases of Ile^{5,6}-deltorphin I and Ile^{5,6}-deltorphin II, *(S)*-Atc^3 and *(R)*-Atc^3 substitutions, respectively, resulted in an the increased affinity in the MVD assay.

Table 6. Opioid receptor binding affinities of Atc^3-containing deltorphin analogs

Peptides	$K_{i\delta}$ (nM)			$K_{i\mu}$ (nM)	$K_{i\kappa}$ (nM)	$K_{i\mu}/K_{i\delta}$
	[^{3}H]NTI	[^{3}H]$Ile^{5,6}$-deltorphin II	[^{3}H]TIPPΨ	[^{3}H]DAMGO	[^{3}H]U-69593	
$Ile^{5,6}$-deltorphin I	0.047±0.011	0.056±0.010	0.018±0.002	3224±1037	>10000	57571
$Ile^{5,6}$-deltorphin II	5.750±0.354	0.385±0.078	0.535±0.100	3188±1039	>10000	8281
(R)-Atc^3,$Ile^{5,6}$-deltorphin I	3.950±0.778	0.435±0.021	1.650±0.212	4526±483	>10000	10405
(S)-Atc^3,$Ile^{5,6}$-deltorphin I	1.050±0.071	0.030±0.008	0.153±0.030	660±47	>10000	22000
(R)-Atc^3,$Ile^{5,6}$-deltorphin II	0.515±0.106	0.035±0.007	0.380±0.028	731±16	>10000	20886
(S)-Atc^3,$Ile^{5,6}$-deltorphin II	0.745±0.007	0.310±0.050	0.028±0.011	1799±723	>10000	5803

$K_{i\mu}/K_{i\delta}$ ratios: $K_{i\delta}$ of [^{3}H]$Ile^{5,6}$-deltorphin II was used to calculate the selectivity ratio.
Values in the table represent means of 3-5 measurements±SD, each containing two parallels.

The most selective ligand was *(R)*-Atc[3],Ile[5,6]-deltorphin II, where the GPI/MVD ratio was higher than 200000.

Table 7. *In vitro* bioactivities of Atc[3]-containing deltorphin analogs

Peptides	IC_{50} (nM)		MVD/GPI potency ratio
	MVD	GPI	$IC_{50(GPI)}/IC_{50(MVD)}$
deltorphin I*	0.32	≥3000	≥9375
deltorphin II*	0.21	≥1500	≥7143
Ile[5,6]-deltorphin I	0.082±0.010	4210±470	51342
Ile[5,6]-deltorphin II	0.500±0.080	67250±27600	185841
(R)-Atc[3],Ile[5,6]-deltorphin I	0.070±0.008	6618±1750	91917
(S)-Atc[3],Ile[5,6]-deltorphin I	0.038±0.004	2162±754	56895
(R)-Atc[3],Ile[5,6]-deltorphin II	0.090±0.030	19570±3180	217444
(S)-Atc[3],Ile[5,6]-deltorphin II	0.270±0.010	31270±5360	115815

Values in the table are arithmetic means±SD.
**Reference [44]*

The sequence of potency and selectivity of the analogs in the *in vitro* bioassay differs from the one in the binding assay. This was already observed for other opioids [49,143]. A possible difference between peripheral and central opioid receptors has been proposed as an explanation [144].

These results confirmed that an increase in the lipophilicity of deltorphins in the so-called address part at positions 5 and 6 results in more active and more δ selective analogs. Recently, Sasaki and Chiba reported new deltorphin analogs with N^{α}-alkylglycine at positions 5 and 6 wich also exhibit higher affinity and δ selectivity than the parent peptide [145].

For the characterization of the Hat[1]-containing analogs in the radioligand binding assay, [^{3}H]pClPhe[4]-DPDPE and [^{3}H]Ile[5,6]-deltorphin II were used as δ radioligands, and [^{3}H]DAMGO and [^{3}H]norBNI as μ and κ ligands, respectively (Table 8) [**II**]. Both new ligands showed high affinities towards δ opioid receptors with K_i values in the subnanomolar range. The *(S)*-analog was more potent against high affinity and specificity δ ligands, [^{3}H]pClPhe[4]-DPDPE and [^{3}H]Ile[5,6]-deltorphin II, but showed several times higher μ affinity too. Thus, despite of the higher K_i values observed, the *(R)*-analog proved to be the more selective one (μ/δ selectivities are 1254 and >10000 for the *(S)*- and the *(R)*-analog, respectively). Moreover, the *(R)*-analog showed

higher selectivity compared to the parent peptide Ile^{5,6} deltorphin II too. None of the ligands showed detectable affinity towards κ receptors, measured by [^{3}H]norBNI.

Table 8. K_i (nM) values of deltorphin II analogs in the radioligand binding assay

radioligand	Ile^{5,6}-deltorphin II*	(*S*)-Hat^1,Ile^{5,6}-deltorphin II	(*R*)-Hat^1,Ile^{5,6}-deltorphin II
[^{3}H]pClPhe^4-DPDPE		0.16 ±0.02	0.94 ± 0.10
		(-0.34 ± 0.03)	(-0.42 ± 0.08)
[^{3}H]Ile^{5,6}-deltorphin II	0.39	0.34 ± 0.02	1.00 ± 0.38
		(-0.69 ± 0.09)	(-0.50 ± 0.08)
[^{3}H]DAMGO	3188	740± 72.8	> 10000
		(-1.01 ± 0.04)	(-1.094 ± 0.22)
[^{3}H]norBNI		> 10000	> 10000

values listed are arithmetic means ±S.E.M. of 3-5 measurements carried out in duplicates, Hill slopes are given in brackets
**Cited from [146].*

Similar results were obtained in the *in vitro* bioassays (Table 9), where the *(S)*-analog was about 4 times more potent in the MVD test than the *(R)*-analog. In accordance with the results obtained in the radioligand binding assays, the *(S)*-analog acted more potently in the GPI preparations, where predominantly μ receptors are present. Thus, in bioassays, as well as in radioligand binding experiments, the *(S)*-analog is more potent, while the *(R)*-analog is more selective.

Table 9. *In vitro* bioactivites of deltorphin II analogues.

ligand	IC_{50} (nM)[a]		MVD/GPI potency ratio
	MVD	GPI	$IC_{50(GPI)}/IC_{50\,(MVD)}$
deltorphin II	0.39±0.05	>1000[b]	>2564
(*S*)-Hat^1,Ile^{5,6}-deltorphin II	2.06±0.47	5129±816	2490
(*R*)-Hat^1,Ile^{5,6}-deltorphin II	6.57±0.87	>10000[c]	>1522

[a] *50% inhibitory concentrations, arithmetic means ±S. E. M. of 4-6 measurements are listed*
[b] *12.0±0.8% (mean±SEM, n=4) inhibition at 10^{-6} M*
[c] *11.0±0.9% (mean±SEM, n=4) inhibition at 10^{-5} M*

The G protein activating properties of the Hat-containing analogs were tested by [^{35}S]GTPγS binding experiments in membranes of rat frontal cortex [**II**], a brain area known to be rich in δ opioid binding sites. [^{35}S]GTPγS binding experiments confirmed that the new peptides have

agonist properties since both of them stimulated [^{35}S]GTPγS binding (i.e. activated G proteins) significantly (to about 1.7-fold over the control value). The two peptides had similar effects, being the *(S)*-analog slightly better. The stimulation values for both peptides were comparable to that obtained by testing the parent compound (Table 10).

Table 10. Stimulation of [^{35}S]GTPγS binding by different concentrations of *(S)*- and *(R)*-Hat^{1},Ile^{5,6}-deltorphin II.

	% stimulation of [^{35}S]GTPγS binding*		
	Ile^{5,6} deltorphin II	(*S*)-Hat^{1},Ile^{5,6}-deltorphin II	(*R*)-Hat^{1},Ile^{5,6}-deltorphin II
0.1 μM	152.2 ± 13.0	155.4 ± 16.3	126.1 ± 7.8
1 μM	179.6 ± 12.8	176.1 ± 19.6	136.9 ± 8.7
10 μ	173.9 ± 6.5	173.0 ± 26.0	160.9 ± 6.1

**Stimulation is given as a percent of the specific binding. Data were calculated from three independent experiments performed in triplicates and presented here as means ± S.E.M. Non-specific binding was 63%. Non-stimulated [^{35}S]GTPγS binding was 86.65 ± 13.42 fmol/mg protein.*

[^{3}H](*S*)-Atc^{3},Ile^{5,6}-deltorphin I and [^{3}H](*R*)-Atc^{3},Ile^{5,6}-deltorphin II were characterized in rat brain membrane preparations [**V**]. The K_d values of the tritiated ligands were in good agreement with the K_i values of the nonlabeled peptides obtained in the radioligand binding assay. The saturation isotherms were best fitted with the model for a single class of binding sites. DPDPE, which is thought to be a selective δ_1 agonist, and deltorphin II and DSLET, which are considered as δ_2 ligands showed one order of magnitude difference in inhibiting the binding of Atc^{3}-containing radioligands. This suggests that both new ligands recognize better the δ_2 binding site. However, deltorphin I is thought to be a δ_1 ligand. On this basis, [^{3}H](*S*)-Atc^{3},Ile^{5,6}-deltorphin I is also expected to be a δ_1 agonist.

As for the Hat^{1}-containing analogs, these ligands labeled equipotently binding sites occupied by [^{3}H]pClPhe^{4}-DPDPE and [^{3}H]Ile^{5,6}-deltorphin II. Furthermore, the Hill-coefficients (Table 8), being far from unity in experiments carried out by using δ radioligands, suggest labeling of a heterogenous receptor population, too. According to these results, Hat^{1}-containing peptides did not show subtype-specificity.

The existence of δ receptor subtypes does not necessarily mean that these receptors differ in their molecular structure. It is also possible that much of what has been interpreted as δ subtypes represents different ligand recognition dependent on the molecular environment, such as coupled and uncoupled state of a single receptor to another receptor molecule or a G protein.

5. SUMMARY

The aim of the study presented here was to develop new, highly potent and selective δ opioid peptide ligands. In order to investigate the bioactive side-chain conformations of Tyr^1 and Phe^3 residues, two pharmacological key-elements of deltorphins, the following, 6-hydroxy-2-aminotetralin-2-carboxylic acid (Hat) [**II**] and 2-aminotetralin-2-carboxylic acid (Atc) [**I**] substituted deltorphin analogs were synthesized:

(*S*)-Hat^1,$Ile^{5,6}$-deltorphin II ((*S*)-Hat-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2)
(*R*)-Hat^1,$Ile^{5,6}$-deltorphin II ((*R*)-Hat-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2)
(*S*)-Atc^3,$Ile^{5,6}$-deltorphin I (Tyr-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2)
(*R*)-Atc^3,$Ile^{5,6}$-deltorphin I (Tyr-D-Ala-(*R*)-Atc-Asp-Ile-Ile-Gly-NH_2)
(*S*)-Atc^3,$Ile^{5,6}$-deltorphin II (Tyr-D-Ala-(*S*)-Atc-Glu-Ile-Ile-Gly-NH_2)
(*R*)-Atc^3,$Ile^{5,6}$-deltorphin II (Tyr-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2)

Synthesis of the conformationally constrained aromatic amino acids was carried out from the corresponding acid chloride or ketone by literature methods. Peptides were synthesized by solid-phase peptide synthesis, the constrained building blocks were incorporated in racemic form. Purification and separation of the resulted diastereomeric peptides was performed by RP-HPLC.

However, no condition was found to separate diastereomeric Hat^1 analogs, thus the enzymatic resolution of Hat was carried out on large scale for peptide synthesis [**II**]. Enzymatic resolution of Atc to assign its configuration in the new deltorphin analogs was also carried out [**I**]. For this purpose, carboxypeptidase A was found to resolve its corresponding substrate with higher enantioselectivity than α-chymotrypsine. Furthermore, it was demonstrated that carboxypeptidase A digests the *(R)* enantiomer of these unnatural amino acids.

Biological characterization of the new ligands was performed by collaborating groups. Both new ligands exhibited high affinities towards δ opioid receptors in the radioligand binding assay with K_i values in the subnanomolar range. Two peptides, *(R)*-Hat^1- and *(R)*-Atc^3,$Ile^{5,6}$-deltorphin II, proved to be more selective than the parent compound $Ile^{5,6}$-deltorphin II. *In vitro* bioassays on MVD and GPI demonstrated that the new ligands, just as the parent compounds, are full agonists. The selectivity of the new compounds was showed to be one order of magnitude higher than that of deltorphins.

Interestingly, the configuration of the incorporated constrained amino acids has little influence on potency. On the other hand, D-Tyr^1 and D-Phe^3 substituted deltorphins show a two

to three order of magnitude lower δ affinity. These findings suggest that either the mode of binding to the δ receptor is different for the new analogs than that for the native peptides, or D-Tyr[1] and D-Phe[3] substitutions induce major disadvantageous changes in the ligands' conformation. For Hat[1] and also for Atc[3] substituted deltorphin analogs, the solution conformation of the corresponding *(R)* and *(S)* analogs were found to be very similar.

The high affinity of the new ligands also suggested that the bioactive side-chain conformations of residues 1 and 3 have to be available for both enantiomers of Hat and Atc. There are two pairs of side-chain conformations for Hat and Atc enantiomers, which are not equivalent, but differ only by a translation of the aromatic rings relative to each other (Figure 6). These are *gauche(–)* and *gauche(+)*for the *(S)* and *(R)* enantiomers and the two respective *trans* conformations (Figure 3). Htc[1] and Tic[3]-substituted deltorphin analogs, where *trans* orientation for the corresponding side-chains is ruled out, were reported to be inactive. On the other hand, *(S)*-Aba[3]- and *(S)*-Hba[1]-substituted analogs, which cannot adopt the *gauche(–)* conformation at χ^1, retain the high affinity of deltorphins. All these data strongly suggest, that during their interaction with the δ receptor, the side-chain conformations of residues 1 and 3 are *trans*.

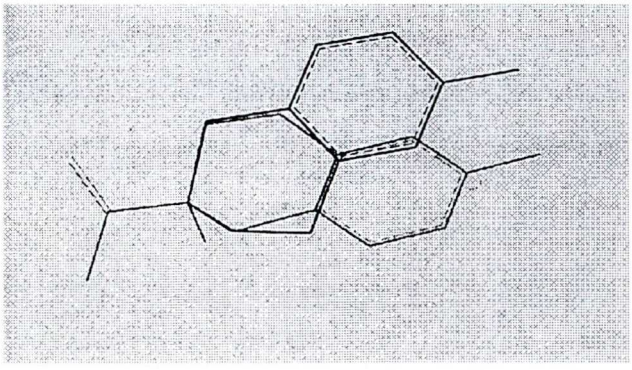


Figure 6. Superposition of the *gauche(–)* and *gauche(+)* conformations of *(S)*-Hat and *(R)*-Hat, respectively

Anatomical, pharmacological and functional investigation of receptors requires potent and preferably highly selective labeled ligands. In hope of being used in such studies, two highly potent and selective analogs,

(*S*)-Atc[3],Ile[5,6]-deltorphin I (Tyr-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2)
(*R*)-Atc[3],Ile[5,6]-deltorphin II (Tyr-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2)

were prepared in radiolabeled form from their corresponding halogen-containing precursors to yield tritiated ligands of high specific radioactivity (34.5 Ci/mmol for [^{3}H]-*(S)*-Atc[3],Ile[5,6]-deltorphin I and 36.0 Ci/mmol for [^{3}H]-*(R)*-Atc[3],Ile[5,6]-deltorphin II) [**III** These new ligands were characterized in rat brain membrane preparations [**V**] and were found to be the most δ selective radioligands reported to date. Therefore, they represent excellent tools for investigating the complexity of the opioid receptor system. Indeed, these new ligands have been already successfully applied for investigation of the putative δ subtypes in binding studies [147].

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