Deltorphin II Analogues with 6-Hydroxy-2-aminotetralin-2-carboxylic Acid in Position 1

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Two approaches to the design of very active and highly selective δ opioid peptides were used to obtain new deltorphin analogues with altered hydrophobic and stereoelectronic properties. Deltorphin II analogues were synthesized with the substitution of Ile instead of Val at positions 5 and 6 in the address domain and 6-hydroxy-2-aminotetralin-2-carboxylic acid (Hat) instead of Tyr[1] in the message domain. In the radioreceptor-binding studies, in which type-specific tritiated opioid ligands were used, *(R)*- and *(S)*-Hat-deltorphins exhibited similar K_i values, revealing high δ selectivity. The peptides displayed agonist properties in the in vitro bioassay, with IC_{50} values in the subnanomolar range in the mouse vas deferens assay and in the micromolar or higher range in the guinea pig ileum assay, again demonstrating a high selectivity toward δ receptors. The agonist property of the new ligands was confirmed by means of [^{35}S]-GTPγS-binding experiments in membranes of the rat frontal cortex.

Introduction[1]

The incorporation of conformational constraints into biologically active peptides is a well-known approach for enhancing their receptor selectivity and modulating their efficacy. This approach has been especially successful in the opioid peptide area, where the conformation of the small, structurally flexible natural ligands is strongly dependent on the environment. This is the main reason these peptides lack significant selectivity toward one or other opioid receptor types (μ, δ, κ) or subtypes. To obtain better conformational integrity, various conformationally restricted opioid peptide analogues, agonists, and antagonists have been developed.[2−4]

The most potent and most selective δ agonists belong in the deltorphin family. H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 (dermenkephalin), H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (deltorphin I), and H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 (deltorphin II) were isolated from frog skin.[5,6] As discussed below, structure−activity relationship studies revealed the roles of the individual amino acid residues in the receptor-binding abilities of the ligands.

Conformational restriction of the Phe[3] residue in deltorphins has resulted in drastic effects on the affinity, selectivity, and ability of the ligands to generate intracellular responses. 1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid (Tic),[7−9] different β-methylphenylalanines,[10,11] 2-aminotetralin-2-carboxylic acid (Atc),[7,8,12] 2-aminoindan-2-carboxylic acid (Aic),[7,8] and 4-aminotetrahydro-2-benzazepin-3-one[9] were used to investigate the effects of the Phe[3] side-chain conformation on the binding properties of deltorphins. Especially the Atc and Aic analogues displayed extraordinary δ receptor affinities and selectivities.

Systematic replacement of residue 4 in deltorphins I and II revealed the great importance of this residue. Although Asn-, Gln-,[13,14] Ser-, and Cys[15]-substituted deltorphin I or II analogues retained the high δ affinity of the parent compounds, the selectivities of these ligands were significantly lower. The location of the charged group relative to the hydrophobic residues in the address domain of the peptide is also critical for δ affinity and selectivity.[13] The role of this charged residue is to prevent μ site recognition and/or binding[16] or to stabilize the appropriate conformation of the ligands for δ receptors.[17,18]

Replacement of the Val residues in positions 5 and 6 by Ile in deltorphin II[19] resulted in a more lipophilic compound, with 8 times higher affinity and 5 times better δ selectivity than that of the parent compound.

The Tyr moiety is one of the key pharmacophores of opioid peptides. Replacement of L- with D-Tyr[1] in dermenkephalin resulted in a 1200-fold lower δ affinity.[20] Replacement of Tyr[1] in deltorphin I with Phe[1] produced a 32-fold decrease in δ receptor affinity, but only a 7-fold drop in antinociceptive potency,[21] which indicates that the phenolic hydroxy group of Tyr contributes to the effective interaction with the opioid receptor, but is not an absolute requirement for opioid activity. The effect of conformational restriction of the first residue has not been investigated as widely as that of the Phe[3] residue. Qian et al.[22] synthesized (2*S*,3*S*)-2′,6′-dimethyl-β-methyltyrosine-substituted deltorphin I and found that the

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Table 1. ^{1}H Chemical Shifts (δ in ppm), Coupling Constants (J in Hz), and Temperature Coefficients of NH Protons (−ppb/K) for *(S)*- and *(R)*-Hat[1],Ile[5,6]-deltorphin II (T = 300 K, DMSO-d_6)

	NH		H^{α}		H^{β}		$H^{\gamma\,a}$		$H^{\delta\,a}$		H^{Ar}	
residue	*(S)*	*(R)*	*(S)*	*(R)*	*(S)*	*(R)*	*(S)*	*(R)*	*(S)*	*(R)*	*(S)*	*(R)*
((S)/(R))-Hat[1]					3.29 β 2.75 β' $J_{\beta\beta'} = 17.0$	3.29 β 2.77 β' $J_{\beta\beta'} = 17.1$	2.78 2.71	2.74	2.25 1.88	2.31 1.94	6.91 6.59 6.54	6.92 6.61 6.54
D-Ala[2]	8.09 $J_{NH\alpha} = 8.2$ (5.3)	8.07 $J_{NH\alpha} = 7.5$ (4.2)	4.35 $J_{\alpha\beta} = 7.2$	4.34 $J_{\alpha\beta} = 7.0$	0.95	0.95						
Phe[3]	8.17 $J_{NH\alpha} = 8.7$ (5.1)	8.18 $J_{NH\alpha} = 8.7$ (4.4)	4.60 $J_{\alpha\beta} = 3.6$ $J_{\alpha\beta'} = 10.4$	4.61 $J_{\alpha\beta} = 3.8$ $J_{\alpha\beta'} = 9.9$	3.02 β *(S)* 2.69 β' *(R)* $J_{\beta\beta'} = 13.7$	3.05 β *(S)* 2.72 β' *(R)* $J_{\beta\beta'} = 13.8$					7.1−7.3	7.1−7.3
Glu[4]	8.29 $J_{NH\alpha} = 7.9$ (5.7)	8.26 $J_{NH\alpha} = 7.8$ (5.7)	4.32 $J_{\alpha\beta} = 6.2$ $J_{\alpha\beta'} = 7.8$	4.34 $J_{\alpha\beta} = 7.0$	1.87 β 1.77 β'	1.90 β 1.79 β'	2.25	2.26				
Ile[5]	7.90 $J_{NH\alpha} = 8.7$ (4.3)	7.88 $J_{NH\alpha} = 8.9$ (4.2)	4.23 $J_{\alpha\beta} = 8.1$	4.24 $J_{\alpha\beta} = 7.5$	1.72	1.74	1.44 γ 1.06 γ' 0.81 CH_3	1.45 γ 1.09 γ' 0.82 CH_3	0.81	0.82		
Ile[6]	7.98 $J_{NH\alpha} = 8.2$ (6.2)	7.93 $J_{NH\alpha} = 8.3$ (6.0)	4.12 $J_{\alpha\beta} = 7.8$	4.13 $J_{\alpha\beta} = 7.6$	1.73	1.74	1.44 γ 1.09 γ' 0.81 CH_3	1.45 γ 1.10 γ' 0.82 CH_3	0.81	0.82		
Gly[7]	8.09 $J_{NH\alpha} = 6.1$ $J_{NH\alpha'} = 5.5$ (6.0)	8.05 $J_{NH\alpha} = 6.2$ $J_{NH\alpha'} = 5.5$ (5.6)	3.67 α 3.57 α' $J_{\alpha\alpha'} = 16.7$	3.68 α 3.57 α' $J_{\alpha\alpha'} = 16.8$								

[a] For Hat, C^{γ} corresponds to C(4) and C^{δ} to C(3); see also Figure 1. Temperature coefficients of NH protons (−ppb/K) are given in parentheses.

new analogue was 2 and 5 times less potent in the binding assay and in the MVD bioassay, respectively, 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 the *gauche(−)*. Guerrini et al.[23] synthesized 2′,6′-dimethyl-L-tyrosine-substituted deltorphin II, and the new ligand was found to exhibit high dual affinity and bioactivity toward δ and μ opioid receptors. Enhanced interaction with the μ receptor was interpreted in terms of the increased hydrophobicity of the first residue and/or 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.

Hat, a conformationally restricted bicyclic Tyr analogue, in which rotation about χ^1 and χ^2 is restricted, was incorporated into enkephalin[24,25] and DPDPE analogues.[26,27] These ligands displayed high affinity toward δ receptors tested either as a mixture[24,25] or after separation of the diastereomers on HPLC.[26,27] This indicates that both *(R)* and *(S)* stereochemistries allow superimposition of the key elements of the δ pharmacophore within the Tyr residue, i.e., the α-amino group and the phenolic aromatic and hydroxy elements. These findings made this amino acid a promising candidate to incorporate into deltorphin II to obtain more information about its bioactive conformation by comparing the accessible conformations and biological activities of the new ligands.

In the present paper, we describe the synthesis and conformational evaluation of two new conformationally constrained Ile[5,6]-deltorphin II analogues in which the Tyr[1] is replaced by *(R)*- or *(S)*-Hat[1]. The new peptides were tested in different in vitro assays to investigate their receptor affinity, specificity, and agonist potency. Finally, on the basis of the NMR data, a proposal is put forward as concerns the preferred solution conformation.

Synthesis

Hat was synthesized according to well-known methods described in the literature.[28] Racemic Hat was built into Ile[5,6]-deltorphin II by standard solid-phase peptide chemistry. However, RP-HPLC separation of the resulting diastereomeric peptides was not successful, although acetonitrile, methanol, and tetrahydrofuran were tried as organic modifiers in TFA/water at different flow rates in order to separate the peptides. Thus, the amino acid had to be synthesized in enantiomerically pure form first. Mosberg et al.[27] applied α-chymotrypsin to resolve the methyl ester of Hat. However, this approach resulted only in enantiomerically enriched amino acid. Similar results were obtained in the resolution of Atc with α-chymotrypsin by Tóth et al.[12] In contrast, resolution of *N*-trifluoroacetyl-Atc with carboxypeptidase A resulted in high enantiomeric purity. For this reason, carboxypeptidase A was applied to digest *N*-trifluoroacetyl-6-methoxy-Atc. According to chiral TLC and GITC derivatization,[29] the products of the resolution have high optical purity (ee ≥ 95%). The absolute configurations of the enantiomers were not determined but can be assigned unambiguously by comparison of the $[\alpha]_D$ values with literature data where crystallographic assignment was carried out.[30] It is interesting to note that, while α-chymotrypsin follows the "normal" stereochemistry in the resolution of Atc[12] and Hat[27], carboxypeptidase A digests the *(R)* enantiomer of these amino acids.

NMR Experiments

One-dimensional (1D) proton and band-selective TOCSY spectra and also two-dimensional (2D) TOCSY[31,32] and ROESY[33,34] spectra of the *(S)*- and *(R)*-Hat[1] analogues of Ile[5,6]-deltorphin II were recorded in DMSO-d_6 at 500 MHz. A complete sequential assignment[35] of the proton resonances could be achieved in a straightforward manner by the combined use of TOCSY and ROESY correlation maps. All ^{1}H chemical shifts and

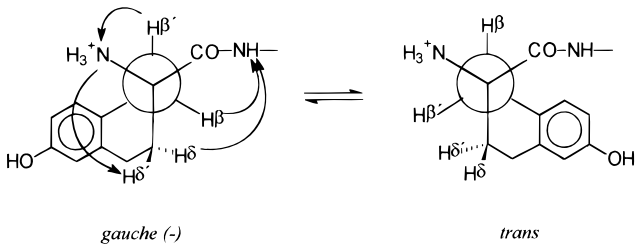
Table 2. NOEs Observed in ROESY Spectra of *(S)* and *(R)* Stereoisomers of Hat[1],Ile[5,6]-deltorphin II (T = 300 K, DMSO-d_6)

from		to		NOE intensities[b]	
residue	proton[a]	residue	proton[a]	*(S)*	*(R)*
Hat[1]	βH	Ala[2]	NH	s[d]	s[d]
Hat[1]	δH	Ala[2]	NH	s[d]	s[d]
Hat[1]	βH	Hat[1]	δH	w[d]	w[d]
Hat[1]	βH′	Hat[1]	NH_2	m[d]	w[d]
Hat[1]	δH′	Hat[1]	NH_2	w[d]	w[d]
Ala[2]	αH	Phe[3]	NH	s	s
Ala[2]	βH	Phe[3]	NH	w	w
Ala[2]	NH	Phe[3]	NH	w	w
Phe[3]	αH	Glu[4]	NH	s	s
Phe[3]	βH	Glu[4]	NH	w−m	w−m
Phe[3]	βH′	Glu[4]	NH	w	w
Phe[3]	NH	Glu[4]	NH	w	vw
Phe[3]	βH′	Phe[3]	NH	s[c]	s[c]
Phe[3]	βH′	Phe[3]	αH	w[c]	w[c]
Phe[3]	βH	Phe[3]	αH	s[c]	s[c]
Phe[3]	Ar-H	Phe[3]	NH	w[c]	w[c]
Phe[3]	Ar-H	Ala[2]	βH	w[c]	w[c]
Phe[3]	Ar-H	Ala[2]	NH	vw[c]	vw[c]
Glu[4]	αH	Ile[5]	NH	s	s
Glu[4]	NH	Ile[5]	NH	w	w
Ile[5]	αH	Ile[6]	NH	s	s
Ile[5]	NH	Ile[6]	NH	w	
Ile[6]	αH	Gly[7]	NH	s	s
Ile[6]	NH	Gly[7]	NH	w	w

[a] High-field protons of nonequivalent CH_2's are denoted as H′. [b] NOE cross-peak intensities are qualitatively classified as strong (s), medium (m), or weak (w), corresponding to distance limits of 1.8−2.5, 1.8−3.0, and 1.8−3.5 Å, respectively. [c] For the Phe[3] residue, these NOE correlations, together with the $^3J_{H\alpha H\beta}$ coupling constants, indicate the dominance of the *gauche(−)* conformation: *gauche(−)* 66%, *gauche(+)* 33%, *trans* 1% for the (*S*) analogue; *gauche(−)* 62%, *gauche(+)* 36%, *trans* 2% for the (*R*) analogue.[d] For the Hat[1] residue, these NOE correlations corroborate the presence of the *gauche(−)* and *gauche(+)* conformations for the (*S*) and (*R*) analogues, respectively.

coupling constants, together with the temperature coefficients of NH protons, are collected in Table 1. The ^{1}H NMR spectra remained the same after 10-fold dilution which excludes the occurrence of peptide self-aggregation. For the *(R)* analogue, where severe signal overlap between the NH and NH_2 resonances hampered the conventional temperature shift measurement (simply acquiring 1D spectra at several temperatures), a series of 1D TOCSY spectra with band-selective excitation[36] of the aliphatic proton resonances were recorded. The TOCSY transfer from aliphatic to NH protons resulted in clean, NH_2-free proton spectra and consequently allowed an accurate evaluation of the temperature dependence of the NH resonances. The temperature coefficients were found to vary between −4.2 and −6.2 ppb/K, which indicates that none of the amide protons are involved in stable hydrogen bonding. ROE peak intensities measured for 120 ms of mixing are reported in Table 2.

The observed NOE effects, and in particular the intense $C_\alpha H_i$−NH_{i+1} and weak NH_i−NH_{i+1} NOEs detected simultaneously for residues 2−7, indicate a time-averaged backbone conformation in solution. The consecutive NH_i−NH_{i+1} NOEs, and also the medium-range NOE observed between Phe[3]-H_β and Ile[5]-NH, strongly suggest that some kind of folded conformers may exist in conformational equilibrium with the extended ones. The assumption of fast exchange between partially folded and extended conformations is supported by the measured $J_{NH\alpha}$ couplings, ranging from 7.9 to 8.3 Hz.



gauche (-) *trans*

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

The large similarities of the ^{1}H NMR parameters and the ROE patterns observed for the two analogues indicate their very similar conformational characteristics. The side-chain conformations of the Hat[1] and Phe[3] residues, which have a crucial impact on the receptor-binding affinity and/or selectivity, were deduced from the observed NOE effects and also, in the case of Phe[3], from the measured homonuclear $J_{\alpha\beta}$ vicinal coupling constants. For Phe[3], the *gauche(−)* conformation was found to be the most populated in both analogues (66% and 62% for *(S)* and *(R)* analogues, respectively). However, the *gauche(+)* conformer also made a significant contribution to the conformational equilibrium (33% and 36% for *(S)* and *(R)* analogues, respectively). Analysis of the NOE patterns involving Hat[1] and D-Ala[2] resonances (see correlations denoted by footnote d in Table 2) allowed an unambiguous assignment of the preferred rotamer for Hat[1], namely *gauche(−)* for the *(S)* and *gauche(+)* for the *(R)* analogue. These NOE patterns are indicated on the *gauche(−)* conformer in Figure 1. These patterns cannot be fitted to the *trans* conformer without creating major discrepancies.

The *gauche(−)* conformation of the *(S)*-Hat-deltorphin analogue 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 unusually high field position of 0.62 ppm, which is ascribed to shielding by the Tyr[1] aromatic ring in the *trans* orientation. However, it is close to the value of 0.91 ppm observed for the Htc[1]-deltorphin II, which also has a preferred *gauche(−)* conformation of the Htc residue.[9]

Biological Data

The new analogues were tested with regard to their binding properties to rat brain opioid receptors, their ability to activate the GTP-binding proteins coupled to the opioid receptors, and their in vitro bioactivities to inhibit the electrically induced contractions of MVD and GPI preparations.

The results of the radioligand-binding assays are shown in Table 3. Both new ligands exhibited high affinities toward δ opioid receptors, with K_i values in the subnanomolar range. The *(S)* analogue was more potent against high-affinity and specificity δ ligands, [^{3}H]pClPhe[4]-DPDPE and [^{3}H]Ile[5,6]-deltorphin II, but also displayed a several times higher μ affinity too. Thus, despite the higher K_i values observed, the *(R)* analogue proved to be the more selective (μ/δ selectivities of 2176 and >10000 for the *(S)* and the *(R)* analogue, respectively). Moreover, the *(R)* analogue demonstrated a higher selectivity as compared to the parent peptide

Table 3. K_i (nM) Values[a] of Deltorphin II Analogues in the Radioligand-Binding Assay

	[^{3}H]pClPhe^4-DPDPE		[^{3}H]Ile^{5,6}-deltorphin II		[^{3}H]DAMGO		[^{3}H]norBNI
	K_i (nM)	Hill slope	K_i (nM)	Hill slope	K_i (nM)	Hill slope	K_i (nM)
Ile^{5,6}-deltorphin II[b]			0.39		3188		
5a	0.16 ± 0.02	−0.34 ± 0.03	0.34 ± 0.02	−0.69 ± 0.09	740 ± 72.8	−1.01 ± 0.04	>10000
5b	0.94 ± 0.10	−0.42 ± 0.08	1.00 ± 0.18	−0.50 ± 0.08	>10000	−1.094 ± 0.22	>10000

[a] Values listed are arithmetic means ± SEM of 3−5 measurements carried out in duplicate. [b] Cited from ref 12.

Table 4. In Vitro Bioactivities of Deltorphin II Analogues

	isolated organ IC_{50} (nM)[a]		MVD/GPI potency ratio
ligand	MVD	GPI	IC_{50}(GPI)/IC_{50}(MVD)
deltorphin II	0.39 ± 0.05	>1000[b]	>2564
5a	2.06 ± 0.47	5129 ± 816	2490
5b	6.57 ± 0.87	>10000[c]	>1522

[a] 50% inhibitory concentrations; arithmetic means ± SEM 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.

Ile^{5,6}-deltorphin II, too. None of the ligands revealed a detectable affinity toward κ receptors, as measured with [^{3}H]norBNI.

[^{3}H]pClPhe^4-DPDPE and [^{3}H]Ile^{5,6}-deltorphin II are presumed to label δ_1 and δ_2 opioid receptors, respectively. The new deltorphin analogues seem to label both sites equipotently. Furthermore, the values of the Hill slope (Table 3) are far from unity in experiments involving the use of δ radioligands, which suggests the labeling of a heterogeneous receptor population, too.

Similar results were obtained in the in vitro bioassays (Table 4), where the *(S)* analogue was about 4 times more potent than the *(R)* analogue in the MVD test. The maximum effects in MVD were identical for the two analogues. However, the slope function calculated from the logarithmic regressions of dose−response curves was significantly higher for the *(S)* analogue (36.7 (31.8−42.2) versus 20.7 (19.5−27.0)). In accordance with the results obtained in the radioligand-binding assays, the *(S)* analogue acted more potently in the GPI preparations, where predominantly μ receptors are present. Thus, both in bioassays and in radioligand-binding experiments, the *(S)* analogue is the more potent while the *(R)* analogue is the more selective.

The effects of agonists on the activation of opioid receptors and their potency and efficacy in the activation can be determined in functional assays. Similarly to other G protein-coupled receptors, the δ opioid receptor achieves its cellular activity by first activating G proteins. Thus, receptor-activated [^{35}S]GTPγS binding serves as a valuable marker of the ability of an agonist to activate a receptor quantitatively. The G protein-activating properties of the new ligands were tested in [^{35}S]GTPγS-binding experiments in membranes of the rat frontal cortex, a brain area known to be rich in δ opioid-binding sites. This assay reflects the GDP−GTP exchange reaction evoked by receptor agonists on the α subunit of the G protein, by measurement of the binding of the nonhydrolyzable GTP analogue.[37] [^{35}S]GTPγS-binding experiments (Table 5) confirmed that the new peptides have agonist properties since both of them stimulated [^{35}S]GTPγS binding (i.e. activated G proteins) significantly (about 1.7 times the control value). However, different activation pattern by the two analogues may suggest a partial agonist property for the *(R)* analogue. In the MVD bioassay, the differences in the slope function may support this notion. The *(S)* analogue showed very similar effect to the parent compound Ile^{5,6}-deltorphin II. In summary, the two new analogues and the parent peptide all have about the same intrinsic efficacy.

Table 5. Stimulation of [^{35}S]GTPγS Binding by Different Concentrations of Deltorphin II Analogues

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

[a] Stimulation is given as a percentage of the specific binding. Data were calculated from three independent experiments performed in triplicate and are presented here as means ± SEM. Nonspecific binding was 63%. Nonstimulated [^{35}S]GTPγS binding was 86.65 ± 13.42 fmol/mg of protein.

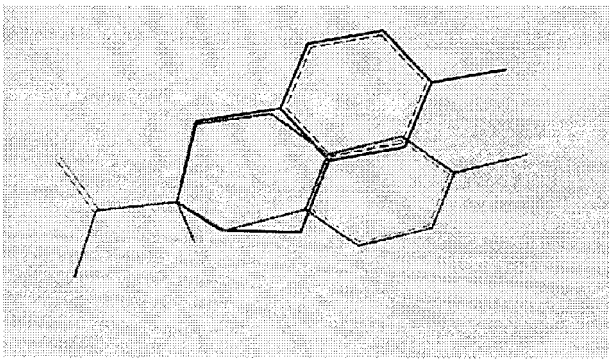


Figure 2. Superposition of the *gauche(−)* and *gauche(+)* conformations of *(S)*-Hat and *(R)*-Hat, respectively.

Conclusion

The two ligands display quite similar affinities and selectivities, indicating the similarity of the topography of the ligands during their binding to the opioid receptor. This also suggests that the bioactive side-chain conformation of the Hat^1 residue should be available for both the *(S)* and *(R)* analogues. Indeed, a superposition of the nitrogen, C$^\alpha$, and carbonyl groups of *(S)*- and *(R)*-Hat in the *gauche(−)* and *gauche(+)* conformations, respectively, indicates that topologically the two enantiomers differ by a translation of the aromatic rings relative to each other (Figure 2). However, the phenolic hydroxy groups are pointing in the same direction. This suggests that the δ receptor is able to interact equally well with both orientations.

However, the high δ affinity of the Hba^1-deltorphin II analogue,[38] which cannot adopt a *gauche(−)* conformation, strongly suggests that the Hat^1-deltorphin analogues switch from their preferred conformation in solution to a *trans* conformation during receptor interaction.

Experimental Section

General Methods. Protected and unprotected amino acids (except Hat), coupling reagents, 4-methylbenzhydrylamine resin, carboxypeptidase A, and 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate (GITC) were purchased from Sigma-Aldrich Kft, Budapest, Hungary, or from Bachem Feinchemikalien AG, Bubendorf, Switzerland. Precoated plates (silica gel F_{254} (Merck, Darmstadt, Germany)) were used for TLC with the following solvent systems: (1) acetonitrile−methanol−water (4:1:1), (2) chloroform−methanol−acetic acid (90:10:1), (3) *n*-butanol−acetic acid−water (2:1:1), (4) *n*-butanol−acetic acid−water (4:1:1). UV light, I_2, and ninhydrin were applied to detect the compounds. Chiral purity control of Hat isomers was performed on Chiralplate (Macherey-Nagel, Düren, Germany) and by GITC derivatization. Reversed-phase HPLC was performed on a Merck-Hitachi liquid chromatograph with a Vydac 218TP54 C_{18} column for analytical or a Vydac 218TP1010 C_{18} column for semipreparative separations. Analytical RP-HPLC gradients (0−80% of organic component over 30 min at a flow rate of 1 mL/min) were run with the solvent system 0.1% (v/v) TFA in water/0.1% (v/v) TFA in acetonitrile; peaks were monitored at 215 nm.

Molecular weights of peptides were determined by ESI mass spectrometry on a double focusing MS 902S spectrometer (A.E.I. Scientific Division, Manchester, U.K.). Routine ^{1}H NMR spectra were measured on a Bruker AC 250-P 250 MHz spectrometer. Radioligands, except for DAMGO (Amersham) and [pClPhe^4]-DPDPE (NEN), were prepared in our laboratory as described earlier for Ile^{5,6}-deltorphin II[39] and norBNI.[40]

Synthesis of 6-Hydroxy-2-aminotetralin-2-carboxylic Acid (Hat, 1).[28] A mixture of 6-methoxy-2-spiro[hydantointetralin] (8.79 g, 35.7 mmol), prepared from 6-methoxy-2-tetralone[41] and aqueous 48% HBr (120 mL), was refluxed for 48 h. The aqueous HBr was then evaporated off, the residue was dissolved in 200 mL of hot water and the pH was adjusted to 5−6 with 25% aqueous NH_3 solution. 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 ppm (2d, J = 8.2 Hz, 2 arom H); 6.67 ppm (s, 1 arom H); 3.33 ppm, 2.96 ppm (2d, J = 16.8 Hz); 2.96−2.72 ppm (m, 2 aliph H); 2.28−2.40 ppm (m, 1 aliph H); 1.99−2.15 ppm (m, 1 aliph H). MS (ESI): 208 (MH^+). TLC (1) R_f: 0.44. HPLC t_R: 10.6 min.

Synthesis of *tert*-Butyloxycarbonyl-Hat (Boc-Hat, 2). **2** was prepared from 11.73 g (57 mmol) of **1** by a standard literature procedure[42] in dioxane−water (2:1) at pH 10 with 1.1 equiv of di-*tert*-butyl dicarbonate (Boc_2O), but after stirring for 3 h, an additional portion of Boc_2O (0.3 equiv) was added and stirring was continued for 4 days at room temperature. After extraction, the resulting brownish oil was crystallized from EtOAc/petroleum ether. In accordance with the high excess of Boc_2O, about 2% of bis-Boc-Hat was also formed, which was revealed by RP-HPLC to crystallize together with Boc-Hat. This mixture of the protected amino acid was used for peptide synthesis. Yield: 10.08 g (58%). Mp: 141 °C. ^{1}H NMR (CD_3OD): 6.87, 6.54 ppm (2d, J = 8.0 Hz, 2 arom H); 6.53 ppm (s, 1 arom H); 3.30−3.35 ppm (m, aliph CH_2); 3.14, 2.92 ppm (2d, J = 16.1 Hz, aliph CH_2) 2.68−2.81 ppm (m, 2 aliph H); 1.42 ppm (s, 9 tBu-H). MS (ESI): 307 (MH^+). TLC (1) R_f: 0.74; (2) R_f: 0.32. HPLC t_R: 18.8 and 27.5 min for bis-Boc-Hat.

Synthesis of 6-Methoxy-2-aminotetralin-2-carboxylic Acid (6-OCH_3-Atc, 3). A mixture of 5 g (20.3 mmol) of 6-methoxy-2-spiro[hydantointetralin] and 35 g (111 mmol, 5.5 equiv) of $Ba(OH)_2 \cdot 8H_2O$ in 150 mL of hot water was refluxed in a pyrolysis tube for 47 h. The reaction mixture was then transferred to a flask, 2−300 mL of water was added, and the insoluble white residue was filtered off. The pH of the solution was adjusted to 2 with 1 M H_2SO_4 and stirred in a waterbath for 1 h, the precipitated $BaSO_4$ was filtered off and washed two times with hot water, and the pH of the combined filtrate was set to 6 with 25% aqueous NH_3 solution. The mixture was evaporated to half-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 (dd, 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 (d, J = 16.7 Hz, 1 aliph H); 2.90 (d, J = 16.7 Hz, 1 aliph H); 2.84 (t, J = 6.4 Hz, 2 aliph H); 2.19 (m, 1 aliph H); 2.04 (m, 1 aliph H). MS (ESI): 222 (MH^+). TLC (3) R_f: 0.60. HPLC t_R: 13.2 min.

Synthesis of *N*-Trifluoroacetyl-6-methoxy-2-aminotetralin-2-carboxylic Acid (*N*-TFA-6-OCH_3-Atc, 4). A mixture of 1.75 g (7.91 mmol) of **3** and 7.5 mL (54.0 mmol, 6.8 equiv) of trifluoroacetic anhydride in 28 mL of TFA was stirred in an icebath for 2 h, followed by stirring at room temperature for 21 h. The brown solution was evaporated, and water was added to the resulting brown oil. The oil solidified, and the white precipitate was crystallized 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 (dd, 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 (d, J = 16.5 Hz, 1 aliph H); 3.10 (d, J = 16.4 Hz, 1 aliph H); 2.81 (m, 2 aliph H); 2.54 (m, 1 aliph H); 2.18 (m, 1 aliph H). MS (ESI): 318 (MH^+). TLC (3) R_f: 0.71. HPLC t_R: 21.4 min.

Resolution of *N*-TFA-6-OCH_3-Atc. 7.32 g (23.1 mmol) of **4** was dissolved in 500 mL of water, the pH was adjusted to 7−8 with 2 M NaOH, 500 μL of carboxypeptidase A (Sigma C-0386; 22 mg of protein/mL, 70 U/mg of protein) was added and the reaction mixture was stirred for 4 days at 37 °C. The mixture was acidified to pH 2 with concentrated HCl, the solution was boiled, charcoal was added, the mixture was boiled again, the charcoal was filtered off, the solution was extracted with 4 × 100 mL of EtOAc, and the combined organic fraction was extracted with 3 × 80 mL of water. Both solutions were evaporated to dryness, 50 mL of 48% HBr was added to each residue, the solutions were refluxed for 24 h under stirring and then evaporated, the residues were dissolved in 30 mL of hot water, the pH was adjusted to 6−7 with 25% aqueous NH_3 solution, and the precipitate was filtered off and crystallized from MeOH−water (1:1).

***(R)*-Hat (Hat from the water layer):** Yield: 1.26 g (26%). Mp: >280 °C. ^{1}H NMR (DMSO-d_6): 6.84 ppm (d, J = 8.2 Hz, 1 arom H); 6.54 ppm (2d, J_1 = 8.2 Hz, J_2 = 2.3 Hz, 1 arom H); 6.49 ppm (d, J = 2.0 Hz, 1 arom H); 3.18 ppm, 2.61 ppm (2d, J = 16.9 Hz, aliph CH_2); 2.64−2.73 ppm (m, 2 aliph H); 2.02−2.08 ppm (m, 1 aliph H); 1.77−1.80 ppm (m, 1 aliph H). MS (ESI): 208 (MH^+). TLC (1) R_f: 0.37; (4) R_f: 0.50. Chiral TLC (1) R_f: 0.48 (identical with the lower spot of racemic Hat). $[\alpha]_D^{20}$: −4.1° × mL/g × dm (c = 6.67 g/100 mL of 1 M HCl (0.32 M)). HPLC t_R: 10.7 min.

***(S)*-Hat (Hat from the organic layer):** Yield: 1.59 g (33%). Mp: >280 °C. ^{1}H NMR (DMSO-d_6) in close agreement with that of *(R)*-Hat: 6.84 ppm (d, J = 8.2 Hz, 1 arom H); 6.55 ppm (2d, J_1 = 8.1 Hz, J_2 = 2.2 Hz, 1 arom H); 6.49 ppm (s, 1 arom H); 3.18 ppm, 2.62 ppm (2d, J = 16.9 Hz, aliph CH_2); 2.66−2.72 ppm (m, 2 aliph H); 2.02−2.08 ppm (m, 1 aliph H); 1.78−1.81 ppm (m, 1 aliph H). MS (ESI): 208 (MH^+). TLC (1) R_f: 0.37; (4) R_f: 0.50. Chiral TLC (1) R_f: 0.56 (identical with the upper spot of racemic Hat). $[\alpha]_D^{20}$: +3.7° × mL/g × dm (c = 6.67 g/100 mL of 1 M HCl (0.32 M)). HPLC t_R: 10.7 min.

Solid-Phase Synthesis and Purification of (*S*)-Hat-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2 (5a) and (*R*)-Hat-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2 (5b). Peptide synthesis was performed manually by the solid-phase technique, using 4-methylbenzhydrylamine resin (0.8 mmol/g of titratable amine). Boc-protected amino acids were used and dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) as coupling agents. Side-chain protection was benzyl for Glu. The deprotection solution was 50% TFA and 2% anisole in dichloromethane (DCM). The protocol for peptide synthesis in each cycle was as follows: (1) addition of Boc-amino acid in DCM (2 equiv), (2) addition of DCC (2 equiv), (3) addition of HOBt (2 equiv), (4) shaking for 1−3 h, (5) washing with DCM (3 × 2 min), (6) washing with EtOH (3 × 2 min), (7) washing with DCM (3 × 2 min), (8) monitoring completion of the reaction with the ninhydrin test,[43] (9) Boc deprotection with 50% TFA in DCM

Table 6. Physicochemical Data on Deltorphin II Analogues

	HPLC[a]	TLC		ESI MS
peptide	k'	$R_f(1)$	$R_f(4)$	MW
Ile^{5,6}-deltorphin II[b]		0.51	0.61	811
5a	4.73	0.58	0.60	837
5b	4.75	0.59	0.59	837

[a] Solvent front breakthrough at 3.0 min. [b] Cited from ref 12.

(2 min + 20 min), (10) washing with DCM (3 × 2 min), (11) neutralization with DIEA in DCM (2 × 2 min), and (12) washing with DCM (3 × 2 min). Simultaneous deprotection and cleavage from the resin were accomplished by treatment with 90% HF and 10% anisole (9 mL of HF and 1 mL of anisole/g peptide resin) at 0 °C for 1 h. After evaporation of the HF, the peptide resin was washed with diethyl ether and the peptide was extracted with glacial acetic acid and lyophilized. Crude peptides were purified by semipreparative RP-HPLC. Peptide purity was assessed by analytical RP-HPLC and TLC. ESI mass spectrometry confirmed the appropriate molecular weight (Table 6).

NMR Experiments.[35] These were carried out with a Bruker Avance DRX 500 (500.13 MHz for ^{1}H) spectrometer equipped with a 5-mm triple-resonance probe (^{1}H/^{13}C/^{15}N) and an actively shielded *z*-gradient coil. The samples contained 2.3 and 3.1 mg of *(S)* and *(R)* analogue, respectively, dissolved in 0.5 mL of DMSO-d_6. Proton chemical shifts are referred to the residual signal of the solvent, $\delta_{\mathrm{DMSO-d6}} = 2.49$ ppm. All ^{1}H NMR data, including the chemical shifts, coupling constants, and NH temperature coefficients reported in Table 1, were extracted from resolution-enhanced 1D spectra or, in the case of signal overlap, from 1D band-selective TOCSY spectra or the highly digitized 1D traces of gradient-enhanced TOCSY correlation maps.[31,32] Gradient-enhanced 2D TOCSY experiments were run by using the MLEV 17 sequence[32] for isotropic mixing with a duration of 62 ms. High-power spin−lock pulses of 2−3 ms with simultaneously switched gradients[31] were applied to generate pure absorption signals for coupling constant measurement. The 2D data matrix consisted of 4K × 320 complex data points. Zero-filling in F_1 and a squared cosine function in both F_1 and F_2 were applied prior to Fourier transformation. Eight transients were accumulated for each of the t_1 increments, with a relaxation delay of 1.8 s. A spin−lock field of 8300 Hz was used for the TOCSY transfer. A spectral width of 4740 Hz was set in both dimensions for the 2D homonuclear experiments. The 1D band-selective TOCSY experiments were performed by using the double-pulsed field gradient spin−echo sequence.[44] Band-selective excitation of aliphatic proton resonances was executed by means of a pulse-width-modulated DANTE-Z pulse,[36] using an I-BURP-1/DANTE train with 32 elementary pulses ($P = 20.6$-μs 90° pulse, $\tau = 50$-μs interpulse delay, resulting in a ±2 ppm excitation bandwidth).

The populations of side-chain rotamers of the Phe residues were quantitatively assessed from the homonuclear coupling constants, $J_{\alpha\beta}$,[45] using the Pachler equations[46,47] with Cung's parametrization proposed for aromatic amino acids.[48] The stereospecific assignment of the prochiral β-protons of Phe^3 indicated in Table 1 could be deduced from the concerted use of homonuclear vicinal couplings ($J_{\alpha\beta}$) and intraresidue ROE effects observed between NH and H_β and between H_α and H_βs, respectively.

ROESY spectra[33,34] were recorded for a mixing time of 120 ms with a CW spin−lock field of 3300 Hz. A relaxation delay of 1.8 s was allowed between successive transients. Sixty-four transients were recorded with 2K complex data points each for a total number of 512 experiments. For processing, the matrices were zero-filled and apodized by a squared cosine function in both dimensions. A polynomial baseline correction was also applied. Volume intensities of ROE peaks were measured and classified as strong (s), medium (m), or weak (w), corresponding to inter-proton distance ranges of 1.8−2.5, 1.8−3.0, and 1.8−3.5 Å, respectively.[49] All NOE correlations of conformational relevance are reported in Table 2.

Radioligand-Binding Assays. Membranes were prepared from Wistar rat brain (minus cerebellum) according to the method of Simon et al.[50] The binding experiments were performed in 50 mM Tris-HCl buffer, pH 7.4, in a final volume of 1.0 mL containing 300−500 μg of protein, different concentrations of *(S)*- and *(R)*-Hat^1,Ile^{5,6}-deltorphin II, and type-specific radioligands. Protein concentration was determined by the method of Bradford.[51] The following conditions were used for incubations: [^{3}H]DAMGO (0.5 nM, 35 °C, 45 min); [^{3}H]Ile^{5,6}-deltorphin II (0.5 nM, 35 °C, 45 min); [^{3}H]pClPhe^4-DPDPE (0.8 nM, 25 °C, 90 min); and [^{3}H]norBNI (0.1 nM, 25 °C, 60 min). Incubations were started by the addition of membrane suspension, continued under gentle shaking in a thermal water bath, and terminated by rapid vacuum filtration through Whatman GF/B or C filters using a Brandell cell harvester. After three washings with 6-mL portions of ice-cold buffer, the filters were dried and the radioactivity was measured in a toluene-based scintillation cocktail, using a Wallac 1409 scintillation spectrometer (Turku, Finland; $\eta = 0.55$). The extent of nonspecific binding was determined in the presence of 10 μM naloxone. Data were evaluated with the use of the software GraphPad Prism 2.01.

[^{35}S]GTPγS Binding. Agonist-stimulated [^{35}S]GTPγS binding was measured in crude membranes of the rat frontal cortex, a brain area known to be enriched in δ opioid receptors.[52] After dissection of the frontal cortex, the procedure of membrane preparation was the same as in the case of the radioligand-binding assays.[50] Experiments were carried out in 50 mM Tris-HCl buffer containing 1 mM EGTA, 3 mM $MgCl_2$, and 100 mM NaCl in a final volume of 1 mL. Assay tubes, containing 10 μg of protein, 30 μM GDP, 0.1/1/10 μM opioid ligands, and 0.05 nM [^{35}S]GTPγS, were incubated for 1 h at 30 °C. For positive control, Ile^{5,6}-deltorphin II was utilized. Nonstimulated activity was measured in the absence of tested compounds, while nonspecific binding was measured in the presence of 10 μM nonlabeled GTPγS. The incubation was terminated by filtering the samples through Whatman GF/F glass fiber filters in a Millipore filtration instrument. Filters were washed three times with 5 mL of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and then dried, and the radioactivity was measured in a Wallac 1409 scintillation counter (Turku, Finland), using a toluene-based scintillation cocktail.

GPI and MVD Bioassays. The bioassays of the peptides were based on the measurement of the inhibition of electrically induced smooth muscle contractions of MVD and GPI myenteric plexus-longitudinal muscle strips.[53] Longitudinal muscle strips taken from the ileum of male guinea pigs (GPI) weighing 400−600 g and the vas deferens of CFLP mice (MVD) weighing 35−40 g were prepared and used as described previously.[54] The experimental conditions for GPI were quite similar to the international routine. The technical parameters for the experiments with the MVD preparation were either a modified version[54] or the original description.[55] In brief, a single vas was used instead of a pair. The preparations were mounted in Mg^{2+}-free Krebs solution at 31 °C under an initial tension of 0.05−0.08 g. The field electrical stimulation parameters were as follows: rectangular pulses of 1-ms duration, 9 V/cm intensity. Twin pulses (100-ms pulse distance) were repeated at 10 s.

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Supporting Information Available: Analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) Abbreviations and definitions are those recommended by the IUPAC−IUB Commission on Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977−983). All optically active amino acids are of the L configuration unless otherwise noted. Other abbreviations: Tic, tetrahydroisoquinoline-3-carboxylic acid; Htc, 7-hydroxytetrahydroisoquinoline-3-carboxylic acid; Atc, 2-aminotetralin-2-carboxylic acid; Aic, 2-aminoindan-2-carboxylic acid; Hat, 6-hydroxy-2-aminotetralin-2-carboxylic acid; Hba, 8-hydroxy-4(*S*)-amino-1,3,4,5-tetrahydro-3-oxo-2*H*-2-benzazepine-2-acetic acid; 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; DPDPE, Tyr-D-Pen-Gly-Phe-D-Pen-OH; norBNI, norbinaltorphimine; DAMGO, Tyr-D-Ala-Gly-NMe-Phe-Gly-ol; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GTPγS, guanosine 5′-*O*-(γ-thio)triphosphate; MVD, mouse vas deferens; GPI, guinea pig ileum; TFA, trifluoroacetic acid; Boc_2O, di-*tert*-butyl dicarbonate; DCM, dichloromethane; DIEA, diisopropylethylamine; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; DMSO, dimethyl sulfoxide; GITC, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

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