Conformationally Constrained Deltorphin Analogs with 2-Aminotetralin-2-Carboxylic Acid in Position 3

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Two approaches to the design of very active and highly selective δ opioid peptides were used to obtain new deltorphin analogs with altered hydrophobic and stereoelectronic properties. Deltorphin I and II analogs were synthesized involving the substitution of Ile instead of Val at positions 5 and 6 in the address domain and 2-aminotetralin-2-carboxylic acid (Atc) instead of Phe in the message domain. The peptides were agonists in the subnanomolar range in the MVD assay and in the micromolar or higher range in the GPI assay, showing a very high selectivity for δ receptors. A very similar trend could be observed in radioreceptor binding assays in which selective tritiated opioid ligands were used. (*R*)- and (*S*)-Atc-deltorphins exhibited similar K_i values in the binding assay, with almost complete loss of the stereospecificity of the binding. Conformational studies provided evidence for little disturbance of the backbone conformational equilibrium when Phe^3 is replaced by (*S*)- or (*R*)-Atc. The use of the Atc constraint gives additional evidence that, during its interaction with the δ receptor, the side chain of residue 3 adopts the trans conformation at χ_1.

Introduction

The incorporation of conformational constraints in analogs of biologically active peptides is a well-known approach for enhancing receptor selectivity and modulating efficacy. This approach has been especially successful in the opioid peptide area, where the natural small opioid peptides are known to be structurally flexible molecules whose conformations are strongly dependent on the environment. This is the main reason why these peptides lack significant selectivity toward one or the other opioid receptor type or subtype (μ, δ, and κ and their subtypes). In efforts to obtain better conformational integrity, various conformationally restricted opioid peptide analogs have been developed (for reviews, see Hruby and Gehrig, Schiller et al.[2,3]) for agonists and antagonists as well.

Currently, the most potent and most selective δ agonists are the deltorphins. H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 (deltorphin), 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.[4,5] In general deltorphin I and its analogs are more active in the δ opioid binding assay than deltorphin and deltorphin II analogs, while deltorphin II and its analogs are more δ selective compared to deltorphin and deltorphin I molecules because of their lower affinity for μ receptors.

Modifications in the side chains of the individual amino acids in deltorphins demonstrated that changes in the electronic[6] and hydrophobic structure significantly influenced the receptor binding properties. Alterations in the hydrophobicity of the positions 5 and 6 revealed a pivotal hydrophobic core in the deltorphins. Sasaki et al.[7] synthesized Ile^{5,6}-deltorphin II, replacing Val at positions 5 and 6 by Ile and obtained more lipophilic peptide, with eight times higher affinity and five times more δ selectivity. Ile^{5,6}-deltorphin II in tritiated form gave us[8] a useful tool to investigate the binding properties of different opioid compounds. This compound was very stable against protolytic enzymes[8,9] and during storage. The specific/nonspecific binding ratio was also excellent in the binding assay.

Recently, conformational restriction of the phenylalanine residue in deltorphins has resulted in drastic effects on receptor affinity and selectivity and on signal transduction. Tetrahydroisoquinoline-3-carboxylic acid (Tic),[10−12] different β-methylphenylalanines,[13,14] 2-aminotetralin-2-carboxylic acid (Atc), 2-aminoindan-2-carboxylic acid (Aic),[10,11] and 4-aminotetrahydro-2-benzazepin-3-one[12] were used instead of Phe at position 3 to investigate the effect of the side-chain conformation on the binding properties of deltorphins. Especially the Atc and Aic analogs displayed extraordinary δ receptor affinity and selectivity. Identification of the configuration of Atc in the diastereomeric peptides was not attempted, however.

In the present paper we describe new deltorphin analogs involving modifications in the side chains at positions 3, 5, and 6 causing changes in the hydrophobic and stereoelectronic properties. The new analogs were obtained by substitution of Atc at position 3, and Ile at positions 5 and 6 in deltorphin I and II. We describe

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Table 1. Opioid Receptor Binding Affinities of Deltorphin Analogs[a]

peptides	$K_{i\delta}$ (nM) [^{3}H]NTI	$K_{i\delta}$ (nM) [^{3}H]DIDII	$K_{i\delta}$ (nM) [^{3}H]TIPPΨ	$K_{i\mu}$ (nM) [^{3}H]DAGO	$K_{i\kappa}$ (nM) [^{3}H]U-69593	$K_{i\mu}/K_{i\delta}$
(1) Tyr-D-Ala-Phe-Asp-Ile-Ile-Gly-NH_2	0.047 ± 0.011	0.056 ± 0.010	0.018 ± 0.002	3224 ± 1037	>10000	57571
(2) Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2	5.750 ± 0.354	0.385 ± 0.078	0.535 ± 0.100	3188 ± 1039	>10000	8281
(3) Tyr-D-Ala-(*R*)-Atc-Asp-Ile-Ile-Gly-NH_2	3.950 ± 0.778	0.435 ± 0.021	1.650 ± 0.212	4526 ± 483	>10000	10405
(4) Tyr-D-Ala-(*S*)-Atc-Asp-Ile-Ile-Gly-NH_2	1.050 ± 0.071	0.030 ± 0.008	0.153 ± 0.030	660 ± 47	>10000	22000
(5) Tyr-D-Ala-(*R*)-Atc-Glu-Ile-Ile-Gly-NH_2	0.515 ± 0.106	0.035 ± 0.007	0.380 ± 0.028	731 ± 16	>10000	20886
(6) Tyr-D-Ala-(*S*)-Atc-Glu-Ile-Ile-Gly-NH_2	0.745 ± 0.007	0.310 ± 0.050	0.028 ± 0.011	1799 ± 723	>10000	5803

[a] $K_{i\mu}/K_{i\delta}$ ratios: $K_{i\delta}$ of [^{3}H]DIDII was used to calculate the selectivity ratio. Values in the table represent means of three to five measurements, each containing two parallels. SD is included.

the determination of the configuration of Atc in the peptides prepared from racemic Boc-Atc.

Results and Discussion

Synthesis. Peptides were synthesized by usual solid-phase techniques with Boc strategy, using dicyclohexylcarbodiimide (DCC)/hydroxybenzotriazole (HOBt) as coupling agents. Atc was prepared by a modified Strecker synthesis.[15,16] Boc-Atc was obtained in racemic form and incorporated as such into the peptides. The diastereomeric peptides were isolated separately by semipreparative HPLC. The configuration of Atc in the peptides was determined from the amino acid mixture obtained from the peptides after hydrolysis by chiral TLC and by HPLC of the amino acid mixture derivatized by GITC (details in the Experimental Section). (*R*)- and (*S*)-Atc were prepared by enzymatic digestion using *N*-trifluoroacetyl or methyl ester derivatives of Atc and carboxypeptidase A or α-chymotrypsin, respectively. The absolute configuration of the Atc enantiomer which was obtained from enzymatic digestion with carboxypeptidase A was determined by X-ray crystallography. This Atc enantiomer was treated with 48% HBr in water and crystallized from water and its absolute configuration was shown to be *R* by the Flack *x* value of 0.10(7). The substituted ring of (*R*)-Atc is in a screw-boat conformation with the ammonium group in a pseudoequatorial and the carboxyl group in a pseudoaxial position (Figure 1, Supporting Information). The packing of the molecules in the crystal structure presents a noticeable separation of the nonpolar and polar constituents into layers, of which the hydrophilic layer is stabilized by six different hydrogen bonds, i.e. all of the hydrogen bond donor possibilities are fully utilized (Figure 2, Supporting Information). The bromide ions sit in the center of a distorted tetrahedron, accepting hydrogen bonds from the carboxylic and ammonium moieties and from two separate water molecules. The water donates two hydrogen bonds to two separate bromide ions and accepts two from two separate ammonium ions from its crystallographic environment. The dihedral angles of the saturated ring are reported in Table 6 (Supporting Information). The angles corresponding to χ_1 and χ_2 of a conformationally constrained phenylalanine are 153.2° and −46.1°, respectively, in this crystal structure.

Atc can be regarded as a constrained analog of both Phe and Hfe (with a reversed configurational relationship).[17] It is interesting to note that the resolution of racemic Atc by enzymatic methods resulted in Atc with different configurations, depending on the enzyme used. With carboxypeptidase A and *N*-trifluroacetyl-Atc as substrate, Atc with the *R* configuration could be obtained, while Atc with the *S* configuration was formed from racemic Atc-methyl ester when α-chymotrypsin was used.

Other supporting evidence for the identification of (*R*)-Atc and (*S*)-Atc was provided by chiral TLC. In general, L-amino acids have higher R_f values than those of D-amino acids on chiral TLC plates with an eluent mixture of acetonitrile−methanol−water, 4:1:1.[18] In this case, (*S*)-Atc has a higher R_f than that of (*R*)-Atc.

Biological Data. The synthetic deltorphin I and II 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).

[^{3}H]Ile^{5,6}-deltorphin II,[8] [^{3}H]TIPP[Ψ],[19] and [^{3}H]naltrindole[20,21] were used as δ radioligands, and [^{3}H]DAGO and [^{3}H]U-69,593 as μ and κ radioligands, respectively. The opioid receptor binding affinities of the deltorphin analogs are summarized in Table 1. All six peptides exhibited very high affinity for δ receptors (K_i values were in the subnanomolar range). We used tritiated agonist ([^{3}H]DIDII) and tritiated antagonist ([^{3}H]TIPP[Ψ] and [^{3}H]naltrindole) in the binding assays. These three relatively new tritiated ligands are very 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. We used K_i values from [^{3}H]DIDII binding study to calculate the selectivity ($K_{i\mu}/K_{i\delta}$). As can be seen from Table 1, the most δ-selective compound was the Ile^{5,6}-deltorphin I (compound **1**), but the selectivities of compounds **4** and **5** were also more than 20 000. In the Atc analogs of deltorphin I, the (*S*)-Atc analog **4** is the more potent and selective, whereas in the Atc analogs of deltorphin II, the (*R*)-Atc analog **5** is better in potency and in selectivity. μ Receptor binding affinities were in the interval 660−4526 nM, while all K_i values obtained for the κ receptors were higher than 10 000 nM. Deltorphin I with D-Phe at position 3 was 20−50 times less potent than the parent peptide,[10] while the Atc analogs had more comparable K_i values in the case of (*R*)- or (*S*)-Atc-deltorphin I and II analogs.

In the *in vitro* bioassay (Table 2), all six peptides displayed properties similar to those in the binding assay. In the MVD assay (where the δ opioid receptors are predominant), the analogs were agonists in the subnanomolar range, while in the GPI assay (which constains μ and κ opioid 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 peptide.[4] The ratio IC_{50}(GPI)/IC_{50}(MVD) for the deltorphin II analogs was >100 000.

Table 2. *In Vitro* Bioactivities of Synthetic Deltorphin Analogs

peptides	IC_{50} (nM) MVD	IC_{50} (nM) GPI	GPI/MVD ratios
Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2[b]	0.32	≥3000	≥9375
Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2[b]	0.21	≥1500	≥7143
(**1**) Tyr-D-Ala-Phe-Asp-Ile-Ile-Gly-NH_2	0.082 ± 0.010	4210 ± 470	51342
(**2**) Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2	0.500 ± 0.080	67250 ± 27600	185841
(**3**) Tyr-D-Ala-(*R*)-Atc-Asp-Ile-Ile-Gly-NH_2	0.070 ± 0.008	6618 ± 1750	91917
(**4**) Tyr-D-Ala-(S)-Atc-Asp-Ile-Ile-Gly-NH_2	0.038 ± 0.004	2162 ± 754	56895
(**5**) Tyr-D-Ala-(R)-Atc-Glu-Ile-Ile-Gly-NH_2	0.090 ± 0.030	19570 ± 3180	217444
(**6**) Tyr-D-Ala-(S)-Atc-Glu-Ile-Ile-Gly-NH_2	0. 270 ± 0.010	31270 ± 5360	115815

[a] Values in the table are mean ± SD. [b] Reference 4.

As can be seen from Table 2, peptides containing Atc with *R* or *S* configuration were almost equipotent in the MVD assay (only a 2−3-fold difference). 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[17] and (*R*)- and (*S*)-Atc[3] analogs of deltorphin I.[10,11] The bioselectivity of Ile[5,6]-deltorphin I with (*R*)-Atc at position 3 (compound **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 (compounds **1** and **2**), [(*S*)-Atc][3] and [(*R*)-Atc][3] substitutions, respectively, resulted in the increased affinity in the MVD assay. The most selective was (*R*)-Atc[3], Ile[5,6]-deltorphin II (compound **5**), where the ratio GPI/MVD was higher than 200 000.

The sequence of potency and selectivity of the analogs in the *in vitro* bioassay differs from the one in the binding assay, which was already observed for other opioids.[10,17] A possible difference between peripheral and central opioid receptors has been proposed as an explanation.[22]

These results confirmed that an increase in the lipophilicity of deltorphins in the address part at positions 5 and 6 results in more active and more δ selective analogs. Very recently, Sasaki and Chiba reported new deltorphin (Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2) analogs with N^{α}-alkylglycine at positions 5 and 6 wich also exhibit higher affinity and δ selectivity as compared to the parent peptide.[23]

Conformational Studies. Besides the lipophilicity, our results and those of several other studies[10,11,24] proved that properly oriented side chains are needed for high affinity and selectivity.

From molecular modeling studies, Salvadori et al.[11] concluded that in deltorphin I the preferred side chain conformation of Phe[3] is trans ($\chi_1 = 138.3°$), whereas in the [(*S*)-Atc[3]] analog it is gauche (−) ($\chi_1 = -56.8°$). Moreover, the low energy backbone conformation of the [(*S*)-Atc[3]] analog is calculated to be distinctly different from that of deltorphin I. We also investigated the effect of the side chain conformation of the aromatic amino acids in deltorphins and dermorphins.[12,25] 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 (−).[13] With 4-aminotetrahydro-2-benzazepin-3-one as a conformational constraint for Phe[3]-Gly[4] in the deltorphin sequence, and also in the dermorphin sequence, high δ affinity was observed for both analogs.[12,25] This finding indicates that the preferred gauche (−) conformation for Phe[3] in deltorphin cannot be the bioactive one. The benzazepine constraint excludes the gauche (−), but still allows two conformations at χ_1: trans and gauche (+).[12] Although it was shown that trans was preferred in solution, a receptor-induced change to gauche (+) cannot be excluded. Earlier Tic[3]-deltorphin II was synthesized by us[12] and Tic[3]-deltorphin I by Shiller and Salvadori and their co-workers.[10,11] The Tic residue can only adopt gauche (−) and gauche (+) conformations in these peptides. The binding and bioactivity were significantly decreased by this substitution. This finding could be an indirect evidence that the preferred bioactive conformation of Phe[3] in deltorphin is trans.

Replacement of Phe[3] with Atc in the deltorphins can produce conformational constraints both in the side chains and in the backbone. The Atc residue is essentially able to adopt two side chain conformations,[17] which are characterized by the torsion angles $\chi_1 = 180°$ (t); $\chi_2 = +25°$ and $\chi_1 = -60°$ (g(−)); $\chi_2 = -25°$ for (*S*)-Atc and $\chi_1 = 180°$ (t); $\chi_2 = -25°$ and $\chi_1 = +60°$ (g(+)); $\chi_2 = +25°$ for (*R*)-Atc. The (*R*)-amino acid adopts a conformation in the crystal which is close to trans: $\chi_1 = 153.2°$ and $\chi_2 = -46.1°$ (Table 6, Supporting Information). This does not, however, exclude a conformational change when this residue is incorporated into a peptide or during receptor interaction. However, since the benzazepin[3]-deltorphin analog has a high potency ($IC_{50} = 5.0$ nM, despite the absence of the important Glu[4] side chain),[12] and since also the (*S*)-Atc[3]-deltorphins are very potent, and considering that the only common side chain conformation which is possible in both analogs is trans, this argues in favor of trans as the δ receptor-bound conformation. Our results, therefore, do not support the model proposed by Mosberg[26] for the cyclic peptide JOM-13, in which Phe[3] has a gauche (−) conformation. It is, however, consistent with the model proposed by Nikiforovich[27,28] for JOM-13 and other δ agonists.

It was previously demonstrated that the NMR parameters (chemical shift, nuclear Overhauser effects (NOE's), 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 in the N-terminal.[12,25] We have determined these NMR parameters for Ile[5,6]-deltorphin II and for the (*S*)- and (*R*)-Atc analogs in dimethyl sulfoxide (DMSO) solution. The chemical shifts (Table 7, Supporting Information) were assigned by using two-dimensional homonuclear Hartmann−Hahn and rotating frame NOE's spectroscopy. A complete sequential analysis was possible on the basis of the $C^{\alpha}H$ (residue *i*)−NH (residue *i* + 1) NOE's. The temperature dependence of the NH chemical shifts, together with the NH−$C^{\alpha}H$ vicinal coupling constants, are reported in Table 8 (Supporting Information). The conformationally indica-

tive NMR parameters for the D-Ala[2] methyl protons are listed in Table 9 (Supporting Information). The chemical shifts reveal only very 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.[11] 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 NOE's for the Atc residue (Table 9, Supporting Information), this argues in favor of 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 freely rotating phenyl ring in the [Phe[3]] analog, cannot be excluded. Because of the absence of an α proton, this conformation cannot be confirmed by coupling constants with the β protons.

Conclusion

The present paper describes the simultaneous application of two approaches to the design of highly selective opioid peptides. In the address domain of deltorphins with the substitution of Ile instead of Val at positions 5 and 6, and in the message domain with the substitution of Atc instead of Phe at position 3, we synthesized very active and highly selective δ opioid ligands, which are the most active and most selective deltorphin analogs reported to date. Conformational studies provide evidence for little disturbance of the backbone conformational equilibrium when Phe[3] is replaced by (*S*)- or (*R*)-Atc. These conformational studies in solution cannot rule out that a higher energy conformer may be the one interacting with the receptor.[10] However, the use of the Atc constraint, in conjunction with Tic and the 4-amino-2-benzazepin-3-one constraint, allows the conclusion that, during its interaction with the δ opioid receptor, the side chain of residue 3 adopts the trans conformation at χ_1.

Experimental Section

General Methods. Protected and unprotected amino acids (except Atc), coupling reagents, 4-methylbenzhydrylamine resin, carboxypeptidase A, α-chymotrypsin, and 7,8-benzo-1,3-diazaspiro[4.5]decane-2,4-dione were purchased from Sigma-Aldrich Kft, Budapest, Hungary; Bachem Feinchemikalien AG, Bubendorf, Switzerland. Precoated plates (silica gel F_{254} (Merck, Darmstadt, Germany) were used for TLC, while for the optical purity control of Atc isomers, chiralplate (Macherey-Nagel, Düren, Germany) was used. The following solvent systems were used: (I) butanol−acetic acid−water (4:1:1); (II) butanol−acetic acid−pyridine−water (15:3:10:12); (III) acetonitrile−methanol−water (4:1:1). Ninhydrin, UV light, and I_2 were applied to detect the peptides. Reversed-phase HPLC was performed on a Merck-Hitachi liquid chromatograph with a Vydac 218TP54 C_{18} column for analytical purposes or a Vydac 218TP1010 C_{18} column for semipreparative separations.

Quantitative amino acid analyses were performed on an HP 1090 Amino Quant amino acid analyzer (Hewlett-Packard, Waldbronn, Germany). A Hypersyl ODS C_{18} column (200 × 2 mm i.d., 5 μm particle size, Shandon Scientific) was used. Molecular weights of peptides were determined by FAB mass spectrometry on a double-focusing MS 902S spectrometer (A.E.I. Scientific Division, Manchester, U.K.).

^{1}H-NMR spectra were measured on a Brucker AM 400 spectrometer (Brucker, Zug, Switzerland).

Radioligands except DAGO (Amersham) were prepared in our laboratory as described earlier for naltrindole,[21] Ile[5,6]-deltorphin II,[8] and TIPP[ψ].[19]

2-Aminotetralin-2-Carboxylic Acid (Atc, 1). (*R,S*)-Atc was synthesized as described by Schiller et al.[17] Briefly, a hot mixture of aqueous sodium hydroxide (50 mL, 40%) and propylene glycol (100 mL) was added to 7,8-benzo-1,3-diazaspiro-[4.5]decane-2,4-dione (21.6 g, 0.1 mol), and the mixture was stirred and refluxed for 20 h. The solution was diluted with water (100 mL). The solution was acidified with aqueous HCl (5 N) to pH 5.5. The desired amino acid slowly precipitated as a white powder: 16.8 g (88%); mp 309−310 °C; MS *m/e* 191 (M^+). The purity of the crude product was 98%, and this was used for further transformations.

N-α-Boc-(*R,S*)-2-aminotetralin-2-carboxylic acid (2) was prepared by literature procedure:[29,30] mp 182 °C; ^{1}H-NMR (ppm) ($CDCl_3$) δ 1.43 (s, 9H), 2.13 (m, 2H), 2.85 (s, 3H), 3.38 (d, 1H), 7.13 (m, 4H), 7.25 (s, 1H).

(*R,S*)-*N*-(Trifluoroacetyl)-2-aminotetralin-2-carboxylic Acid (3). To a solution of **1** (2.87 g, 15 mmol) in TFA (20 mL) was added dropwise trifluoroacetic anhydride (5.21 mL), and the mixture was vigorously stirred at 0 °C for 2 h and then for 2 days at room temperature. After the solvent and excess anhydride had been removed under reduced pressure, the residue was diluted with water (30 mL), extracted with ethyl acetate (two times), brine (two times), and water (two times), dried over $MgSO_4$, and concentrated. The resulting yellow oil was crystallized from EtOAc and petroleum ether: yield 2.80 g (65%); mp 186 °C; TLC (chloroform−ethanol−acetic acid, 90:10:1) R_f = 0.48; MS *m/e* 287 (M^+); ^{1}H-NMR (DMSO) δ 2.05 (m, 1H), 2.40 (m, 1H), 2.73 (m, 2H), 3.12 (d, 1H), 3.25 (d, 1H), 7.1 (m, 4H), 9.5 (s, 1H), 13 (s, 1H).

Enzymatic Separation of 1 by Carboxypeptidase A. 3 (1.5 g) was suspended in 150 mL of water. The pH was adjusted to 7.5−8 with NaOH, 200 μL of carboxypeptidase A (∼2−3 mg) was added, and the mixture was stirred for 1 day. From time to time the pH was adjusted to 7.5−8. After completition of the reaction, charcoal was added to the solution to remove the enzyme. After filtration, the pH of the solution was adjusted to 3 with 1 N HCl. Unreacted **3** was extracted with ethyl acetate. 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, 93%; $[\alpha]^{25}_D$ = −6.77° (*c* = 1.0, 0.3 M HCl/water). The ethyl acetate solution after drying over $MgSO_4$ was evaporated, and the oil was crystallized from ethyl acetate/petroleum ether: yield 734 mg (98%); $[\alpha]^{25}_D$ = +4.1° (*c* = 1.0, ethanol). The resulting *N*-TFA-(*S*)-Atc was hydrolyzed by refluxing with 6 N HCl (40 mL) for 6 h. The solution was evaporated. The residue was dissolved in 20 mL of water. The pH was adjusted to 6 with 1 N NH_4OH. The crystals were filtered off: yield 210 mg (43%); $[\alpha]^{25}_D$ = +6.3° (*c* = 1.0, 0.3 M HCl/water). (*R*)-Atc·HBr was prepared by treating (*R*)-Atc with 48% HBr/water and crystallized from water: yield 170 mg (25%); mp 268-270 °C; $[\alpha]^{25}_D$ = −7.16° (*c* = 1.0, water); chiral TLC, eluent acetonitrile−methanol−water (6:1:1), R_f (Atc from aqueous solution) 0.57; R_f (Atc from organic solution) 0.63.

Enzymatic Separation of 1 with α-Chymotrypsin. (*R,S*)-Atc-OCH_3 (600 mg) was prepared by a known method,[15] using methanol and $SOCl_2$ dissolved in 10 mL of methanol and 40 mL of water. The pH was adjusted to 7.9 with NaOH, 6 mg of enzyme (α-chymotrypsin) was added, and the mixture was stirred for 1 day. The unreacted ester was extracted with ethyl acetate. The aqueous layer was treated with charcoal, and after evaporation to 10 mL, the pH was adjusted to 3. The crystals were filtered off: yield 108 mg (38.6%). The ethyl acetate solution was dried on $MgSO_4$ and evaporated. The oil was crystallized as the HCl salt from ether−HCl. The crystals were filtered off and dried: yield 135 mg (45%). According to chiral TLC, the Atc formed from aqueous solution was an *S*/*R* mixture, but the *S* isomer predominanted: ∼3:1.

Solid-Phase Synthesis and Purification of the Diastereometric Peptides. Peptide synthesis was performed by the manual solid-phase technique using 4-methylbenzhydrylamine resin (0.8 mmol/g of titratable amine). Boc-protected

amino acids and dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) as coupling agents were used. Side chain protections were benzyl for Asp and Glu and dichlorocarbobenzyloxy for Tyr. 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,[31] (9) Boc deprotection with 50% TFA in DCM (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 to homogenity by semipreparative reversed-phase high-performance chromatography (RP-HPLC) on a Vydac 218TP1010 C_{18} column with a linear gradient of 0.1% TFA of water (eluent A) and acetonitrile−0.1% TFA (eluent B); starting 25% acetonitrile to 30% acetonitrile for 25 min. After the column was washed with 80% acetonitrile for 10 min, it was equilibrated with 25% acetonitrile to be ready for the next run. The final products were obtained as lyophilizates. Peptide purity was assessed by analytical RP-HPLC and TLC in three different solvent systems. Fast atom bombardment mass spectrometry confirmed the appropriate molecular weight (Table 10, Supporting Information). Amino acid analyses gave the expected amino acid patterns.

Determination of the Configuration of Atc in the Peptides. After RP-HPLC purification, two diastereomeric peptides were obtained. One milligram of each peptide was hydrolyzed separately in 6 N HCl under argon pressure in Teflon bombs in a microwave oven.[32] The solvent was removed by flushing with argon. The dried samples were used for separation of the amino acid mixture, and the Atc was collected (Vydac column 218TP54 C_{18}, 254 nm, linear gradient from 10 to 15% of acetonitrile 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 acetonitrile−methanol−water, 5:1:1. These R_f values were compared with those of standard (*R*)- and (*S*)-Atc (R_f of (*R*)-Atc 0.37; R_f of (*S*)-Atc 0.43). The other part of the Atc from the HPLC separation was used for GITC derivatization.[33,34] When this derivatization was performed with (*R*)- and (*S*)-Atc standards, the k' values of the Atc derivatives (Vydac 218TP54 column, eluent 0.1% TFA in water−methanol (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.

NMR Study of Ile^{5,6}-Deltorphin II and Its [Atc^3] Analogs. Samples were prepared by dissolving 2 mg of peptide in 0.5 mL of 0.1% TFA in water (pH = 2). This solution was lyophilized, and the remaining powder was dried over P_2O_5 *in vacuo* and then dissolved in 0.5 mL of DMSO-d_6 (>99.96% Aldrich). 1D spectra were measured at 303, 313, 323, and 333 K. 2D HOHAHA, ROESY, and E. COSY spectra were measured at 313 K.

2D HOHAHA spectra were recorded with an MLEV 17 spin−lock pulse of 60 ms, preceded and followed by 2.5 ms trim pulses. For NOE spectroscopy (ROESY spectra), a mixing time of 200 ms was used. The 2D ROESY and HOHAHA spectra were measured as 256 free induction decays of 2K data points. Processing was done by multiplication with a $\pi/3$ shifted sine-bell window in both dimensions, zero filling to 2K in ω_1, Fourier transformation, phase correction, and baseline correction in both dimensions.

Radioligand Binding Assays. Membranes were prepared from Wistar rat brain (minus cerebellum) according to the method of Simon et al.[35] The binding experiments were performed in 50 mM TRIS-HCl buffer, pH 7.4, at a final volume of 1.0 mL containing 300−500 μg of protein (protein concentration was determined by the method of Bradford[36]). In competition experiments, the following conditions were used for incubations: [^{3}H]DAGO (35 °C, 45 min); [^{3}H]naltrindole (25 °C, 60 min); [^{3}H]Ile^{5,6}-deltorphin II (35 °C, 45 min); [^{3}H]-TIPP[ψ] (25 °C, 30 min). Incubations were started by the addition of membrane suspension, continued under gentle vortexing and shaking in a thermal water bath, and terminated by rapid vacuum filtration through Whatman GF/C filters using a Brandell cell harvester. After two washings with 10 mL portions of ice-cold buffer, the filters were dried for 3 h at 37 °C and the radioactivity was measured in a toluene-based scintillation cocktail, using a Beckman LS 5000TD scintillation spectrometer. The extent of nonspecific binding was determined in the presence of 10 μM naloxone. Experimental data were calculated by using the LIGAND program of Munson and Rodbard.[37]

GPI and MVD *in Vitro* Bioassays. The bioassays of all the peptides were based on electrically induced smooth muscle contractions of mouse vas deferens (MVD) and guinea pig ileum (GPI) myenteric plexus−longitudinal muscle strips.[38]

The tissues were suspended between platinum axial wire electrodes in warm (37 °C), oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (34 °C and Mg-free for MVD) in an 18 mL bath and stretched to an optimal tension of 1 g for GPI and 0.5 g for MVD. The tissues were then allowed to equilibrate for 15 min and stimulated transmurally with square-wave impulses (0.1 Hz, 1 ms duration for GPI and 2 ms for MVD) at supramaximal (40 V) voltage. The GPI preparation was equilibrated for 60 min before the application of the peptides; the contraction then was stable. The opioid agonist activity of the drugs was determined by constructing dose−response curves with the single dose method. The concentration causing a 50% reducting the contractions was also established: thus, the IC_{50} values were estimated. The IC_{50} estimates and their associated standard errors were determined by fitting the mean data to the Hill equation, using a computerized least squares method.[39]

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Supporting Information Available: Crystal structure determination, crystal data and structure refinement, atomic coordinates and equivalent isotropic displacement parameters, bond length, bond angles, torsion angles, anisotropic displacement parameters, and hydrogen coordinates for (*R*)-Atc; two figures for the crystal structure of (*R*)-Atc·HBr and the packing of the (*R*)-Atc·HBr molecules in the crystal structure, as well as chemical shifts, temperature dependence of NH chemical shifst and NH-C$^\alpha$H coupling constants, D-Ala^2 methyl parameters and physicochemical data for deltorphin analogs (12 pages). Ordering information is given on any current masthead page.

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC−IUB Commission on Nomenclature (*J. Biol. Chem.* **1972,** *247*, 977−983). All optically active amino acids are of the L variety unless otherwise noted. Other abbreviations used are Hfe, homophenylalanine; Atc, 2-aminotetralin-2-carboxylic acid; TFA, trifluroacetic acid; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide, DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; GITC, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylisothiocyanate; DAGO, Tyr-D-Ala-Gly-NMe-Phe-Gly-ol; TIPP[Ψ], Tyr-TicΨ[CH_2NH]Phe-Phe-OH; U69593, (5α,7α,8α)-(−)-*N*-methyl-[7-(1-pyrrolidinyl)-1-oxaspiro-[4.5]dec-8-yl]benzeneacetamide; NTI, naltrindole; DIDII, Ile^{5,6}-deltorphin II.

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