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Differential stereochemical requirements of μ vs. δ opioid receptors for ligand binding and signal transduction: Development of a class of potent and highly δ-selective peptide antagonists

(opioid peptides/conformational restriction/opioid receptor selectivity/intrinsic activity)

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ABSTRACT **Opioid peptide analogs consisting entirely of aromatic amino acid residues and containing conformationally restricted phenylalanine derivatives in position 2 of the peptide sequence were synthesized and pharmacologically characterized *in vitro*. Both diastereoisomers of H-Tyr-(D or L)-NMePhe-Phe-Phe-NH_2 (NMePhe is N^{α}-methylphenylalanine) were μ-receptor-selective, were full agonists in the μ-receptor-representative guinea pig ileum assay, and were partial agonists in the mouse vas deferens assay, with the L-NMePhe^2 analog displaying somewhat higher intrinsic activity than the D-NMePhe^2 analog. Further conformational restriction at position 2 in the sequence, as achieved through substitution of D- or L-tetrahydro-3-isoquinoline carboxylic acid (Tic), produced a configuration-dependent differential effect on receptor selectivity and intrinsic activity, leading to a potent μ-selective μ agonist (the D-Tic^2 analog) with increased intrinsic activity in the mouse vas deferens assay and to a potent δ-selective δ antagonist (the L-Tic^2 analog). These results demonstrate that imposition of conformational constraints in a peptide not only may alter receptor selectivity but also may decrease, totally abolish, or even enhance intrinsic activity. The tetrapeptide H-Tyr-Tic-Phe-Phe-NH_2 was a moderately potent full agonist in the guinea pig ileum assay and, thus, represents a compound with mixed μ-agonist/δ-antagonist properties. The corresponding peptide with a free C-terminal carboxyl group H-Tyr-Tic-Phe-Phe-OH showed high δ-receptor affinity (K_i^{δ} = 1.2 nM), unprecedented δ selectivity (K_i^{μ}/K_i^{δ} = 1410), high potency as δ antagonist (K_e = 3–8 nM against various δ agonists in the mouse vas deferens assay) and, unlike other δ antagonists, had no μ-antagonist properties. The tripeptides H-Tyr-Tic-Phe-OH and H-Tyr-Tic-Phe-NH_2 were also δ antagonists.**

Whereas the existence of at least three major opioid receptor classes (μ, δ, and κ) is now well-established, the development of potent opioid agonists and antagonists with high specificity for each receptor type and of ligands with receptor-specific agonist/antagonist properties continues to be an important goal in opioid pharmacology. The fact that μ and δ opioid receptors differ from one another in their conformational requirements for peptide ligands was first established through comparison of the receptor binding profiles of a cyclic enkephalin analog and its linear correlate (1). This observation led to the realization that conformational restriction of peptides either locally through incorporation of backbone or side-chain conformational constraints at a specific amino acid residue or more globally through peptide cyclizations may often result in improved receptor selectivity. The use of this strategy resulted in a number of conformationally restricted opioid peptide analogs with agonist properties that showed high preference for either μ or δ receptors (for a review, see ref. 2). It has often been speculated but never demonstrated unambiguously that conformational restriction of peptides in some cases might also reduce or even totally abolish their intrinsic activity ("efficacy") and, thus, may produce partial agonists or antagonists. No examples of opioid peptide analogs with significant antagonist properties as a consequence of conformational restriction have been reported to date. The only opioid-peptide-derived antagonists with reasonable potency described so far were obtained through diallylation of the N-terminal amino group. An enkephalin analog of this type, *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174,864; Aib = aminoisobutyric acid) (3), has been useful as a δ-selective antagonist.

In this paper we report that the tetrapeptide amide H-Tyr-D-Phe-Phe-Phe-NH_2 (**1a**) is a potent μ-selective opioid agonist. This compound consists entirely of aromatic amino acids that can be conformationally restricted in a number of interesting ways. We show that substitution of the D and L isomers of the conformationally restricted phenylalanine analogs N^{α}-methylphenylalanine (NMePhe) and tetrahydro-3-isoquinoline carboxylic acid (Tic) (Fig. 1) for D-Phe^2 in peptide **1a** produced astonishing changes in receptor affinities and intrinsic activities. Most importantly, these structure–activity studies defined a class of potent and selective δ antagonists, characterized by the N-terminal sequence H-Tyr-Tic-Phe-.

MATERIALS AND METHODS

Peptide Synthesis. Peptide analogs **1–7** were synthesized by the usual solid-phase technique with N^{α}-*t*-butyloxycarbonyl-protected amino acids and with benzotriazol-1-yl-oxytris-(dimethylamino)phosphonium hexafluorophosphate as coupling agent as described elsewhere (4, 5). Crude peptides were purified by gel filtration and by reversed-phase chromatography as described (5). Homogeneity of the peptides was established by TLC in two systems and by analytical HPLC, and their molecular weights were determined by fast atom bombardment mass spectrometry. Analytical data are presented in Table 1.

Binding Assays and Bioassays. Opioid-receptor binding studies were performed as described in detail elsewhere (5). Binding affinities for μ and δ receptors were determined by displacing, respectively, tritiated H-Tyr-D-Ala-Gly-NMePhe-

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Abbreviations: GPI, guinea pig ileum; MVD, mouse vas deferens; NMePhe, N^{α}-methylphenylalanine; Tic, tetrahydro-3-isoquinoline carboxylic acid; TIP, H-Tyr-Tic-Phe-OH; TIPP, H-Tyr-Tic-Phe-Phe-OH; Aib, aminoisobutyric acid; DAGO, H-Tyr-D-Ala-Gly-NMePhe-Gly-ol; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; DPDPE, [D-Pen^2,D-Pen^5]enkephalin, where Pen is penicillamine.

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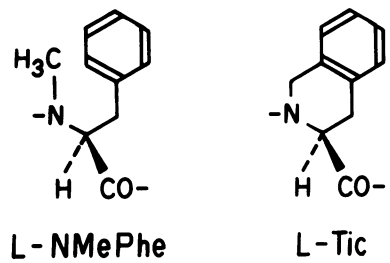

FIG. 1. Structural formulas of L-NMePhe and L-Tic.

Gly-ol ([^{3}H]DAGO; New England Nuclear) and tritiated H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH ([^{3}H]DSLET; Amersham) from rat brain membrane preparations, and κ opioid receptor affinities were measured by displacement of tritiated 5α,7α,8β-(−)-*N*-methyl-*N*-[7-(pyrrolidinyl)-1-oxaspiro-(4,5)dec-8-yl]benzeneacetamide ([^{3}H]U-69,593; New England Nuclear) from guinea pig brain membranes. Incubations were performed for 2 h at 0°C with [^{3}H]DAGO, [^{3}H]DSLET, and [^{3}H]U-69,593 at respective concentrations of 0.72, 0.78, and 0.80 nM. IC_{50} values were determined from logarithmic dose-displacement curves, and K_i values were calculated from the obtained IC_{50} values by the equation of Cheng and Prusoff (6), using values of 1.3, 2.6, and 2.9 nM for the dissociation constants of [^{3}H]DAGO, [^{3}H]DSLET, and [^{3}H]U-69,593, respectively.

The guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays were carried out as reported in detail elsewhere (1). K_e values for naloxone or for the Tic^2-peptide δ antagonists were determined from the ratio of IC_{50} values obtained in the presence and absence of a fixed antagonist concentration (7). The GPI assay is usually considered to be representative of μ-receptor interactions, even though the ileum also contains κ receptors. κ-receptor interactions in the GPI assay are indicated by relatively high K_e values for naloxone as antagonist (20–30 nM), in contrast to the low K_e values (1–3 nM) observed with μ-receptor ligands (8). The MVD assay is generally used for measuring δ-receptor-mediated agonist or antagonist activities. However, aside from δ receptors, μ and κ receptors also exist in the vas tissue. δ and κ agonists show relatively high K_e values (10–30 nM) for naloxone as antagonist in the MVD assay, whereas μ agonists show (9) lower K_e values (1–3 nM) again for naloxone as antagonist.

Conformational Studies. All calculations were performed using the molecular modeling software SYBYL (Tripos Associates, St. Louis) on a VAXstation 3510, by following procedures described elsewhere (5, 10). Both cis and trans peptide bonds were allowed in the molecular mechanics calculations (energy minimization studies). Molecular dynamics simulations were carried out for 300 ps at 300 K, by using a dielectric constant of 78 to simulate an aqueous environment. Starting conformations were low-energy conformers that had been obtained in the molecular mechanics studies.

RESULTS AND DISCUSSION

In the opioid-receptor binding assays (Table 2), the tetrapeptide amide H-Tyr-D-Phe-Phe-Phe-NH_2 (**1a**) showed high μ-receptor affinity and considerable preference for μ receptors over δ receptors. In agreement with the receptor binding data, this analog was found to be a potent full agonist in the GPI assay and to have relatively weak but full agonist activity in the MVD assay (Table 3). In the latter assay, the agonist effect was reversed by low concentrations of naloxone (K_e = 3.02 ± 0.46 nM) but not by the δ antagonist H-Tyr-Tic-Phe-Phe-OH (TIPP; see below) at concentrations up to 100 nM, suggesting that it was mediated by μ receptors that are also present in the MVD. The observation that the diastereoisomeric peptide H-Tyr-Phe-Phe-Phe-NH_2 (**1b**) had weak potency is in agreement with the well-known fact that in general substitution of an L-amino acid in position 2 of opioid peptides is detrimental to activity.

N-methylation of Phe^2 had a divergent effect on the opioid activity profiles of these two diastereoisomeric tetrapeptide amides. In comparison with the D-Phe^2 parent peptide (**1a**), the D-NMePhe^2 analog (**2a**) showed a 12-fold decrease in μ affinity and slightly reduced affinity for δ receptors. Most interestingly, N-methylation of the L-Phe^2 analog resulted in a compound (**2b**) with greatly increased μ-receptor affinity and slightly enhanced δ-receptor affinity. Analog **2b** displayed quite high preference for μ receptors over δ receptors (K_i^δ/K_i^μ = 97.3), whereas its diastereoisomer (**2a**) was only moderately μ-selective. In the μ-receptor-representative GPI assay, **2a** and **2b** were both full agonists with relative potencies that were in agreement with the μ-receptor affinity constants determined in the receptor binding assays. Interestingly, both diastereoisomers were partial agonists in the MVD assay (Fig. 2). Depending on the individual vas preparation, maximal inhibitions of the electrically induced contractions obtained with compounds **2a** and **2b** were 10–20% and 30–50%, respectively, indicating that the L-NMePhe^2 analog had somewhat higher intrinsic activity than the D-NMePhe^2 analog. For analog **2b**, a K_e value of 1.83 ± 0.26 nM was determined for naloxone as antagonist and naloxone reversibility could also be demonstrated for its diastereoisomer, even though the low intrinsic activity of **2a** precluded the accurate determination of a K_e value. The effects of both **2a** and **2b** were not antagonized by the potent δ antagonist TIPP at 100 nM. These results indicated that the partial agonist

Table 1. Analytical data on synthetic peptides

Peptide		TLC, R_f		HPLC	FAB-MS
No.	Sequence	BAW	BPAW	K' value	(MH^+), m/e
1a	H-Tyr-D-Phe-Phe-Phe-NH_2	0.74	0.77	2.50	622
1b	H-Tyr-Phe-Phe-Phe-NH_2	0.87	0.79	1.33	622
2a	H-Tyr-D-NMePhe-Phe-Phe-NH_2	0.74	0.80	3.33	636
2b	H-Tyr-NMePhe-Phe-Phe-NH_2	0.75	0.82	2.00	636
3a	H-Tyr-D-Tic-Phe-Phe-NH_2	0.72	0.78	1.83	634
3b	H-Tyr-Tic-Phe-Phe-NH_2	0.75	0.81	1.33	634
4	H-Tyr-Tic-Phe-Phe-OH	0.77	0.79	2.08	635
5a	H-Tyr-D-Tic-Phe-NH_2	0.68	0.78	0.83	487
5b	H-Tyr-Tic-Phe-NH_2	0.67	0.80	0.58	487
6	H-Tyr-Tic-Phe-OH	0.69	0.77	0.91	488

Solvent systems for TLC are BAW [1-butanol/acetic acid/water, 4:1:5 (vol/vol) (upper phase)] and BPAW [1-butanol/pyridine/acetic acid/water, 15:10:3:12 (vol/vol)]. For HPLC, the capacity factor is presented for the following system: Vydac C_{18} RP column (25 cm × 4.6 mm) with 0.1% trifluoroacetic acid/MeOH, 45:55 (vol/vol), at a flow rate of 1.2 ml/min. Peptides were monitored at 280 nm. FAB-MS, fast atom bombardment mass spectrometry.

Table 2. Opioid-receptor binding assays of peptide analogs

Peptide		[^{3}H]DAGO		[^{3}H]DSLET		Selectivity ratio	
No.	Sequence	K_i^μ, nM	Relative potency	K_i^δ, nM	Relative potency	K_i^δ/K_i^μ	K_i^μ/K_i^δ
1a	H-Tyr-D-Phe-Phe-Phe-NH_2	3.63 ± 0.59	2.60 ± 0.42	137 ± 9	0.0185 ± 0.0013	37.7	
1b	H-Tyr-Phe-Phe-Phe-NH_2	1600 ± 170	0.00589 ± 0.00064	2510 ± 610	0.00101 ± 0.00025	1.57	
2a	H-Tyr-D-NMePhe-Phe-Phe-NH_2	44.5 ± 8.4	0.212 ± 0.040	209 ± 45	0.0121 ± 0.0026	4.70	
2b	H-Tyr-NMePhe-Phe-Phe-NH_2	11.3 ± 0.5	0.835 ± 0.032	1100 ± 70	0.00230 ± 0.00015	97.3	
3a	H-Tyr-D-Tic-Phe-Phe-NH_2	7.30 ± 0.52	1.29 ± 0.09	519 ± 46	0.00488 ± 0.00043	71.1	
3b	H-Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2)	78.8 ± 7.1	0.120 ± 0.011	3.00 ± 0.15	0.843 ± 0.043		26.3
4	H-Tyr-Tic-Phe-Phe-OH (TIPP)	1720 ± 50	0.00546 ± 0.00017	1.22 ± 0.07	2.08 ± 0.12		1410
5a	H-Tyr-D-Tic-Phe-NH_2	121 ± 15	0.0777 ± 0.0096	396 ± 11	0.00639 ± 0.00018	3.27	
5b	H-Tyr-Tic-Phe-NH_2 (TIP-NH_2)	624 ± 79	0.0151 ± 0.0019	12.0 ± 1.3	0.212 ± 0.023		52.0
6	H-Tyr-Tic-Phe-OH (TIP)	1280 ± 140	0.00734 ± 0.00083	9.07 ± 1.02	0.280 ± 0.032		141
	Naltrindole	12.2 ± 1.9	0.771 ± 0.121	0.687 ± 0.100	3.69 ± 0.54		17.8
	DPDPE	943 ± 181	0.0100 ± 0.0019	16.4 ± 1.8	0.154 ± 0.017		57.5
	[D-Ala^2]Deltorphin II	3930 ± 480	0.00240 ± 0.00029	6.43 ± 0.73	0.393 ± 0.045		611
	[Leu^5]Enkephalin	9.43 ± 2.07	1	2.53 ± 0.35	1		3.73

Displacement of [^{3}H]DAGO (μ selective) and [^{3}H]DSLET (δ selective) from rat brain membrane preparations is shown. The K_i values are the mean ± SEM of three determinations. The potency is relative to that of [Leu^5]enkephalin.

effects of **2a** and **2b** were again produced through interaction with μ receptors in the vas preparation. Since the full agonist effect observed with parent peptide **1a** in the MVD assay was also mediated by μ receptors, it appears that the conformational constraints imposed in **2a** through methylation of the D-Phe^2 residue resulted in a reduced intrinsic activity at the μ receptor. This decrease in the intrinsic activity is not observed in the GPI assay because of the very large reserve of μ receptors known to exist in this preparation (11).

Further conformational restriction in the residue at position 2 through ring closure between the 2′ position of the aromatic ring and the N^α-methyl group in analogs **2a** and **2b** was achieved by synthesis of the corresponding D- and L-Tic^2-tetrapeptide analogs (compounds **3a** and **3b**). This additional conformational constraint produced an increase in μ affinity and a decrease in δ affinity for the D-Tic^2-analog. On the other hand, the L-Tic^2 analog showed reduced μ affinity and, very surprisingly, greatly enhanced δ affinity. Thus, H-Tyr-D-Tic-Phe-Phe-NH_2 (**3a**) turned out to be μ-selective, whereas its diastereoisomer, H-Tyr-Tic-Phe-Phe-NH_2 (**3b**), showed considerable δ-selectivity (K_i^μ/K_i^δ = 26.3). The opposite receptor selectivity profiles of analogs **3a** and **3b**, which contain stereoisomers of a rigid amino acid at position 2, demonstrate that μ and δ opioid receptors have different stereochemical architectures.

Analogs **3a** and **3b** were both full agonists in the GPI assay with potencies that were in agreement with their relative μ-receptor affinities determined in the binding assays. In the MVD assay, the D-Tic^2 analog was nearly a full agonist, able to inhibit the electrically evoked contractions to a maximal extent of ≈70% (Fig. 2) with a potency ≈20 times lower than that of [Leu^5]enkephalin. Again, this agonist effect of **3a** was reversed by naloxone at low concentration (K_e = 1.33 ± 0.25 nM) but not by the δ antagonist TIPP (100 nM), indicating that μ receptors rather than δ receptors in the MVD preparation were implicated. Since compound **3a** had higher intrinsic activity than **2a** in the MVD assay, it appears that the further conformational restriction achieved through ring closure at the residue in position 2 (D-Tic^2 analog) resulted in enhanced intrinsic activity at the μ receptor. Thus, H-Tyr-D-Tic-Phe-

Table 3. GPI and MVD assays of opioid peptide analogs

Peptide		GPI		MVD		MVD/GPI
No.	Sequence	IC_{50}, nM	Relative potency	IC_{50}, nM	Relative potency	IC_{50} ratio
1a	H-Tyr-D-Phe-Phe-Phe-NH_2	247 ± 87	0.997 ± 0.353	2,680 ± 680	0.00425 ± 0.00108	10.9
1b	H-Tyr-Phe-Phe-Phe-NH_2	25,300 ± 4000	0.00973 ± 0.00155	112,000 ± 27,000	0.000102 ± 0.000025	4.43
2a	H-Tyr-D-NMePhe-Phe-Phe-NH_2*	774 ± 100	0.318 ± 0.041	—	—	—
2b	H-Tyr-NMePhe-Phe-Phe-NH_2	56.0 ± 4.3	4.39 ± 0.34	442 ± 38	0.0258 ± 0.0022	7.89
3a	H-Tyr-D-Tic-Phe-Phe-NH_2	37.1 ± 2.6	6.63 ± 0.47	454 ± 72	0.0251 ± 0.0040	12.2
3b	H-Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2)	1,700 ± 220	0.145 ± 0.019	>10,000 (antagonist)		—
4	H-Tyr-Tic-Phe-Phe-OH (TIPP)	>10,000	<0.0246	>10,000 (antagonist)		—
5a	H-Tyr-D-Tic-Phe-NH_2	2,030 ± 120	0.121 ± 0.007	28,900 ± 4,800	0.000394 ± 0.000066	14.2
5b	H-Tyr-Tic-Phe-NH_2 (TIP-NH_2)	16,500 ± 3400	0.0149 ± 0.0031	>10,000 (antagonist)		—
6	H-Tyr-Tic-Phe-OH (TIP)	>10,000	<0.0246	>10,000 (antagonist)		—
	[Leu^5]Enkephalin	246 ± 39	1	11.4 ± 1.1	1	0.0463

IC_{50} values are the mean ± SEM of 3–10 determinations. The potency is relative to that of [Leu^5]enkephalin.
*Low efficacy (10–20%) precludes accurate determination of the IC_{50} value in the MVD assay.

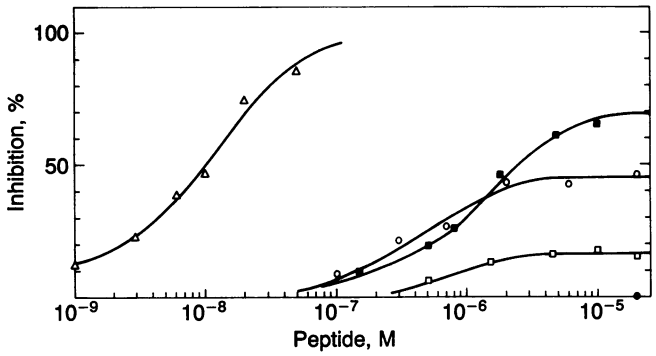

FIG. 2. Inhibition of electrically evoked contractions in the MVD by [Leu^5]enkephalin (△), H-Tyr-D-NMePhe-Phe-Phe-NH$_2$ (□), H-Tyr-NMePhe-Phe-Phe-NH$_2$ (○), H-Tyr-D-Tic-Phe-Phe-NH$_2$ (■), and H-Tyr-Tic-Phe-Phe-NH$_2$ (antagonist) (●). Logarithmic dose–response curves were obtained with a single representative vas preparation.

Phe-NH$_2$ is a potent and very selective μ agonist. Most surprisingly, the L-Tic^2 analog showed no agonist potency in the MVD assay at concentrations as high as 10 μM, despite its demonstrated high δ-receptor affinity and it turned out to be a potent selective δ antagonist with K_e values from 14 to 18 nM against various selective δ agonists, including [D-Ala^2]deltorphin I (12) and [D-Pen^2,D-Pen^5]enkephalin (DPDPE, where Pen is penicillamine) (13) (Table 4). The observation that configurational inversion at a single conformationally restricted amino acid residue can turn an agonist with selectivity for one receptor type into an antagonist with preference for another receptor class is unique and, to the best of our knowledge, has not been made previously in the peptide field.

The fact that the additional conformational restriction achieved through ring closure between the 2′ position of the aromatic ring and the N^α-methyl group in the D- and L-tetrapeptide analogs produced a significant intrinsic activity increase at the μ receptor in the case of the D-Tic^2 analog and pure δ antagonism in the case of the L-Tic^2 analog is most intriguing. It should be realized that the Tic^2 analogs are structurally distinguished from the NMePhe^2 analogs merely by the closing of a carbon–carbon bond (Fig. 1) and that the observed effects of this ring closure on the biological activity profile are due to the conformational restriction *per se* and not to any other structural differences. It has often been speculated that structural rigidification may interfere with signal transduction, resulting in reduced intrinsic activity or antagonism. The present data suggest that this assumption may not be generally true and that conformational restriction of a receptor ligand may in some cases decrease or totally abolish intrinsic activity and in other cases may produce an increase in intrinsic activity.

It was most interesting to note that the δ antagonist H-Tyr-Tic-Phe-Phe-NH$_2$ (TIPP-NH$_2$) was a full agonist in the GPI assay with ≈15% the potency of [Leu^5]enkephalin. The effect of this compound on the ileum was very sensitive to naloxone inhibition (K_e = 1.74 ± 0.45 nM), indicating that it was mediated by μ opioid receptors. Therefore, it appears that TIPP-NH$_2$ represents an opioid compound with mixed μ-agonist/δ-antagonist properties. Recently, it has been shown that pretreatment of mice with the nonpeptide δ antagonist naltrindole (14) prevented the development of morphine tolerance and dependence (15). This important observation led to the suggestion that compounds with mixed μ-agonist/δ-antagonist properties might have potential as analgesics that do not produce tolerance and physical dependence.

Replacement of the C-terminal carboxamide function in TIPP-NH$_2$ with a free carboxylate group resulted in a compound, TIPP (**4**), with further improved δ-receptor affinity (K_i^δ = 1.22 ± 0.07 nM) and drastically diminished affinity for μ receptors. In fact, TIPP displayed extraordinary δ-selectivity (K_i^μ/K_i^δ = 1410) and in a direct comparison (Table 2) turned out to be more selective than DPDPE, [D-Ala^2]-deltorphin II, and naltrindole, which represent the most selective δ-receptor ligands reported until now. In the MVD assay, TIPP was a potent antagonist (K_e = 3–6 nM) against various δ agonists (Table 4) and had no agonist effects at concentrations as high as 10 μM. In agreement with the receptor binding data, TIPP was a very weak agonist in the GPI assay (IC_{50} > 10 μM) and, most importantly, displayed no antagonist properties at concentrations up to 10 μM in this μ-receptor-representative bioassay system.

In comparison with the nonpeptide antagonist naltrindole (14), TIPP showed about half the affinity for δ receptors in the binding assay (Table 2) and 5–7 times higher K_e values against δ agonists in the MVD assay (Table 4). Thus, TIPP was a slightly less potent δ antagonist than naltrindole but, on the other hand, was 80 times more δ-selective than the nonpeptide δ antagonist (Table 2). Naltrindole has μ-receptor affinity in the nanomolar range and is a μ antagonist with a reported K_e value of 29 nM against morphine in the GPI assay (16). TIPP is also at least 10 times more potent and 10 times more δ-selective than the δ antagonist *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174,864) (3, 17). In summary, because of its high potency, extraordinary δ selectivity, and complete lack of μ-antagonist properties, TIPP should turn out to be an attractive δ antagonist for use in opioid pharmacology.

Deletion of Phe^4 in compounds **3a**, **3b**, and **4** resulted in tripeptide analogs that, in comparison with the corresponding parent tetrapeptides, had qualitatively similar activity profiles but were somewhat less potent and less selective. Thus, H-Tyr-D-Tic-Phe-NH$_2$ (**5a**) was a μ-selective agonist, whereas H-Tyr-Tic-Phe-NH$_2$ (TIP-NH$_2$) (**5b**) and H-Tyr-Tic-Phe-OH (TIP) (**6**) were δ-selective antagonists.

All peptide analogs described in this paper showed K_i^κ values >1 μM in the binding assay based on displacement of [^{3}H]U-69,593 from guinea pig brain membranes and, thus, did not have significant affinity for κ receptors. Enzymatic degradation could be excluded as a factor affecting the opioid-receptor affinity profiles of the peptides, since incubations in the receptor binding experiments were performed at 0°C (18). Furthermore, the structural characteristics of most of the Tic^2, NMePhe^2, and D-Phe^2 analogs examined

Table 4. K_e values determined for δ antagonists against various δ agonists in the MVD assay

	K_e, nM		
Antagonist	[Leu^5]Enkephalin	[D-Ala^2]Deltorphin I	DPDPE
TIPP	5.86 ± 0.33	2.96 ± 0.02	4.80 ± 0.20
TIPP-NH$_2$	15.7 ± 2.4	14.4 ± 2.2	18.0 ± 2.2
TIP	11.7 ± 0.9	12.6 ± 1.9	16.1 ± 1.7
TIP-NH$_2$	43.9 ± 8.9	58.9 ± 7.7	96.8 ± 14.1
Naltrindole	0.850 ± 0.221	0.632 ± 0.161	0.636 ± 0.103

Data are the mean ± SEM of 4–10 determinations.

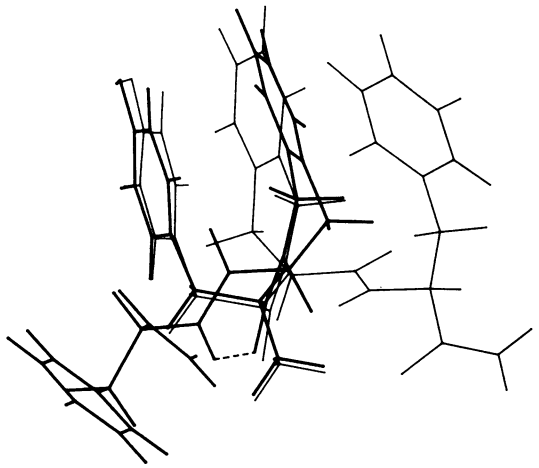

FIG. 3. Superimposition of lowest-energy conformers of H-Tyr-Tic-Phe-NH_2 (heavy lines) and H-Tyr-D-Tic-Phe-NH_2 (light lines). In both structures the Tyr^1–Tic^2 peptide bond is in the trans conformation. An inverse γ-turn stabilized by a Tyr^1-CO · · · HN-Phe^3 hydrogen bond is observed with the L-Tic^2 analog.

should make them quite enzyme-resistant even at higher temperatures.

The more pronounced conformational constraints existing in the Tic^2 analogs as compared to the $NMePhe^2$ analogs were analyzed in molecular mechanics studies and in molecular dynamics simulations. In the L-Tic^2-tetrapeptide analog (**3b**), the ϕ_2 angle was found to be limited to negative values from $-70°$ to $-100°$, whereas, for the corresponding L-$NMePhe^2$ analog (**2b**), low-energy conformers with ϕ_2 values around $+60°$ and $-140°$ were observed. With regard to the side-chain conformation of the Tic residue, the g^+ and g^- configurations are possible, whereas the t configuration is excluded. The theoretical conformational analyses revealed that the preferred conformation of the L-Tic^2 residue in **3b** was g^+. For the L-NMePhe residue, all three side-chain configurations (t, g^-, and g^+) are possible in principle, but mostly the t and g^- configurations were observed with the L-$NMePhe^2$ analog **2b**. As expected, the ϕ_2 angle in the D-Tic^2-tetrapeptide analog (**3a**) could only assume positive values (around $+90°$ and $+50°$) and, with the D-$NMePhe^2$ tetrapeptide (**2a**), the preferred value for ϕ_2 was $-50°$, even though positive values around $+140°$ also occurred. For the D-Tic^2 residue in **3a** both possible side-chain conformations (g^+ and g^-) were observed and low-energy conformers with the D-$NMePhe^2$ residue in each of the three possible side chain conformations (g^+, g^-, and t) were found for compound **2a**. Obviously, the distinct conformational preferences and constraints around the residue in position 2 are the reason for the drastic differences in receptor binding profile and intrinsic activity of the Tic^2 vs. the $NMePhe^2$ analogs. The lowest-energy conformers of the tripeptides H-Tyr-D-Tic-Phe-NH_2 (**5a**) and H-Tyr-Tic-Phe-NH_2 (**5b**) obtained in the molecular mechanics studies were compared by superimposing their Tyr and Tic residues (Fig. 3). Most strikingly, this spatial superimposition revealed that Phe^3 of these diastereoisomeric peptides is located on opposite sides of the plane defined by the Tic residue as a consequence of the conformational constraints existing at the Tic^2 residue. This finding may be relevant to the fact that configurational inversion from D to L at the Tic^2 residue turned a μ-selective agonist (**5a**) into a δ-selective antagonist (**5b**).

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