TIPP[ψ]: A Highly Potent and Stable Pseudopeptide δ Opioid Receptor Antagonist with Extraordinary δ Selectivity

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Pseudopeptide analogues of the δ opioid antagonists H-Tyr-Tic-Phe-Phe-OH (TIPP) and H-Tyr-Tic-Phe-OH (TIP) containing a reduced peptide bond between the Tic[2] and Phe[3] residues were synthesized. The two compounds, H-Tyr-Ticψ[CH_2NH]Phe-Phe-OH (TIPP[ψ]) and H-Tyr-Ticψ-[CH_2NH]Phe-OH (TIP[ψ]), were tested in μ-, δ-, and κ-receptor-selective binding assays and in the guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays. In comparison with their respective parent peptides, both pseudopeptide analogues showed increased δ antagonist potency in the MVD assay, higher δ receptor affinity and further improved δ receptor selectivity. The more potent compound, TIPP[ψ], displayed subnanomolar δ receptor affinity and in direct comparisons with other selective δ ligands was shown to have unprecedented δ specificity (K_i^{μ}/K_i^{δ}= 10 500). Furthermore, this compound turned out to be highly stable against enzymatic degradation and, unlike other δ antagonists, showed no μ or κ antagonist properties. TIPP[ψ] is likely to find wide use as a pharmacological tool in opioid research.

Receptor-selective opioid antagonists are of interest both as pharmacological tools and as potential therapeutic agents. In the past few years substantial progress has been made in the development of both peptide- and non-peptide antagonists with high potency and markedly improved selectivity for each of the three opioid receptor types (μ, δ, κ). Opioid antagonists with highest selectivity for μ receptors are somatostatin-derived cyclic peptide analogues (TCTP, TCTOP, and TCTAP)[2] and the most selective κ antagonist is the bivalent ligand norbinaltorphimine (norBNI).[3] Among various reported δ antagonists, the enkephalin analogue *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864) shows considerable δ selectivity but only moderate potency,[4,5] whereas the δ-selective non-peptide antagonist naltrindole (NTI) is highly potent.[6] A benzofuran analog of naltrindole, NTB, showed further improved δ selectivity but somewhat lower δ antagonist potency.[7] However, both NTI and NTB also turned out to be antagonists against μ and κ agonists in the guinea pig ileum (GPI) assay with potencies (K_e = 29–48 nM) about 100–300 times lower than those observed against δ agonists in the mouse vas deferens (MVD) assay (K_e = 0.13 and 0.27 nM, respectively).[7]

Recently, we reported the discovery of a new class of opioid peptide-derived δ antagonists that contain a 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) residue in the 2-position of the peptide sequence.[8] The two prototype antagonists were the tetrapeptide H-Tyr-Tic-Phe-Phe-OH (TIPP) and the tripeptide H-Tyr-Tic-Phe-OH (TIP). TIPP showed high antagonist potency against various δ agonists in the MVD assay (K_e = 3–5 nM), high δ affinity (K_i^{δ} = 1.22 nM), and extraordinary δ selectivity (K_i^{μ}/K_i^{δ} = 1410). Furthermore, TIPP displayed no μ or κ antagonist properties in the GPI assay at concentrations as high as 10 μM. The compound was recently prepared in tritiated form and [^3H]TIPP was shown to be an excellent new radioligand for the study of δ opioid receptor interactions.[9] In comparison with TIPP, TIP was a somewhat less potent and less selective δ antagonist.

Both TIPP and TIP were stable in the aqueous buffer solution (pH 7.7) used for biological testing for periods up to 6 months. However, these peptides were shown to undergo slow, spontaneous Tyr-Tic diketopiperazine formation with concomitant cleavage of the Tic-Phe peptide bond in DMSO and MeOH.[10] This observation prompted the design of corresponding peptides containing a reduced peptide bond between the Tic[2] and Phe[3] residues, since this structural modification altogether eliminates the possibility of Tyr-Tic diketopiperazine formation. Here we describe the syntheses, opioid activity profiles, and stability of the two pseudopeptide analogues H-Tyr-Ticψ-[CH_2NH]Phe-Phe-OH (TIPP[ψ]) and H-Tyr-Ticψ[CH_2-NH]Phe-OH (TIP[ψ]).

Chemistry. Both peptides were synthesized by the solid-phase method using *tert*-butyloxycarbonyl (Boc) protected amino acids and 1,3-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) as coupling agents. Introduction of the reduced peptide bond between the Tic[2] and Phe[3] residues required a reductive alkylation reaction[11] between 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde and the amino group of the resin-bound phenylalanine or H-Phe-Phe dipeptide. 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde was synthesized via preparation of 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-(*N*-methoxy-*N*-methylamide) by using a published procedure.[12] Peptides were cleaved from the resin by HF/anisole treatment in the usual manner. Crude products were purified by reversed-phase chromatography.

Receptor Binding Assays, in Vitro Bioassays and Enzymatic Degradation Studies. Binding affinities for μ and δ opioid receptors were determined by displacing, respectively, [^3H]DAMGO and [^3H]DSLET from rat brain membrane binding sites, and κ opioid receptor affinities were measured by displacement of [^3H]U69,593 from guinea pig brain membrane binding sites. For the determination of their in vitro opioid activities, analogues were tested in bioassays based on inhibition of electrically evoked contractions of the GPI and of the MVD. The GPI assay is usually considered as being representative for μ receptor interactions, even though the ileum does

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Table I. K_e Values Determined for δ Antagonists against Various δ Agonists in the MVD Assay

antagonist	K_e,[a] nM		
	[Leu[5]]enkephalin	[D-Ala[2]]deltorphin I	DPDPE
H-Tyr-Ticψ[CH_2NH]Phe-Phe-OH (TIPP[ψ])	2.11 ± 0.58	2.58 ± 0.29	2.89 ± 0.14
H-Tyr-Tic-Phe-Phe-OH [TIPP]	5.86 ± 0.33	2.96 ± 0.02	4.80 ± 0.20
H-Tyr-Ticψ[CH_2NH]Phe-OH (TIP[ψ])	9.12 ± 1.57	8.23 ± 0.12	9.06 ± 0.72
H-Tyr-Tic-Phe-OH (TIP)	11.7 ± 0.9	12.6 ± 1.9	16.1 ± 1.7
naltrindole	0.850 ± 0.221	0.632 ± 0.161	0.636 ± 0.103

[a] Mean of three or four determinations ± SEM.

Table II. Binding Affinities of δ-Selective Compounds at μ and δ Receptors in Rat Brain Homogenates

compd	[3H]DAMGO		[3H]DSLET		
	K_i^{μ},[a] nM	rel potency[b]	K_i^{δ},[a] nM	rel potency[b]	K_i^{μ}/K_i^{δ}
TIPP[ψ]	3228 ± 439	0.00292 ± 0.00040	0.308 ± 0.060	8.23 ± 1.60	10500
TIPP	1720 ± 50	0.00546 ± 0.00017	1.22 ± 0.07	2.08 ± 0.12	1410
TIP[ψ]	10800 ± 1300	0.000868 ± 0.000103	1.94 ± 0.14	1.30 ± 0.09	5570
TIP	1280 ± 140	0.00734 ± 0.00083	9.07 ± 1.02	0.280 ± 0.032	141
naltrindole	3.86 ± 0.74	2.44 ± 0.47	0.182 ± 0.024	13.9 ± 1.8	21.2
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 ± 0.53	1	3.73

[a] Mean of three to seven determinations ± SEM. [b] Potency relative to that of [Leu[5]]enkephalin.

also contain κ receptors. In the MVD assay opioid effects are primarily mediated by δ receptors, but μ and κ receptors also exist in this tissue. The stability of the TIPP-related antagonists against degradation by brain peptidases was examined by incubating them with rat brain membrane suspensions at 37 °C for various periods of time. At the end of the incubations enzymatic activity was destroyed and the extent of enzymatic degradation was determined in the [3H]DSLET receptor binding assay.

Results and Discussion

In the MVD assay the pseudotetrapeptide analogue H-Tyr-Ticψ[CH_2NH]Phe-Phe-OH (TIPP[ψ]) was a potent δ antagonist against the δ agonists [Leu[5]]enkephalin, [D-Pen[2],D-Pen[5]]enkephalin (DPDPE)[13] and [D-Ala[2]]-deltorphin I,[14] with K_e values ranging from 2.2 to 2.9 nM (Table I). TIPP[ψ] was somewhat more potent (1.2–2.6-fold) as δ antagonist than the parent peptide TIPP and 2.6–4.5 times less potent than the non-peptide δ antagonist naltrindole. TIPP[ψ] had no agonist effect at concentrations up to 10 μM both in the MVD assay and in the GPI assay, and again showed no antagonist activity against the μ agonist DALDA[15] and the κ agonist U69,593[16] in the GPI assay at concentrations as high as 10 μM. These results indicate that, like its parent peptide TIPP, TIPP[ψ] is a pure δ antagonist, in contrast to naltrindole and NTB which showed considerable μ and κ antagonist potency in the GPI assay.[7] The pseudopeptide H-Tyr-Ticψ[CH_2NH]Phe-OH (TIP[ψ]) also was a δ antagonist in the MVD assay with a potency (K_e = 8.2–9.1 nM) slightly higher than that of the tripeptide parent peptide TIP and was also found to be a pure δ antagonist, showing no agonist activity in the GPI and MVD assay at doses up to 10 μM and no antagonist effect against μ and κ agonists in the GPI assay at concentrations as high as 10 μM.

In the rat brain membrane opioid receptor binding assays, TIPP[ψ] showed 4 times higher affinity for δ receptors and about half the affinity for μ receptors in comparison with the TIPP parent peptide (Table II). Consequently, TIPP[ψ] turned out to be about 7.5 times more δ-selective than TIPP with a K_i^{μ}/K_i^{δ} ratio of 10 500. The δ receptor affinity of the pseudotetrapeptide is in the subnanomolar range (K_i^{δ} = 0.308 nM) and nearly as high as that of naltrindole (K_i^{δ} = 0.182 nM). In a direct comparison (Table II), TIPP[ψ] was at least 1 order of magnitude more selective than the δ-selective agonists DPDPE and [D-Ala[2]]deltorphin II.[14] It would also appear that TIPP[ψ] is about 30 times more δ-selective than [Phe-(p-Cl)[4]]DPDPE, which has been reported to have about 5 times higher δ selectivity than the DPDPE parent peptide.[17] Direct comparison revealed that TIPP[ψ] has about 500 times higher δ selectivity than naltrindole (Table II) and it appears to be about 40 times more δ-selective than NTB, which has been found to be about 12 times more selective than NTI.[7] Furthermore, this compound is also about 2 orders of magnitude more δ-selective and 20 times more potent than the enkephalin-derived δ antagonist *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864).[4,5] On the basis of these results and comparisons, TIPP[ψ] appears to have unprecedented δ selectivity. The pseudotripeptide TIP[ψ] also showed an about 4-fold δ affinity enhancement relative to its parent peptide (TIP) and an even more pronounced increase (40-fold) in δ selectivity. As it had been the case with TIPP and TIP,[8] the pseudopeptides TIPP[ψ] and TIP[ψ] showed K_i^{κ} values > 1 μM in the binding assay based on displacement of [3H]U69,593 from guinea pig brain membranes and, thus, did not have any significant affinity for κ opioid receptors.

In the enzymatic degradation studies 1 × 10^{-4} M solutions of [Leu[5]]enkephalin, TIPP, TIPP[ψ], and TIP were incubated with rat brain membrane preparations for periods varying from 10 min to 24 h (Figure 1). As expected, enzymolysis of [Leu[5]]enkephalin was rapid and essentially complete after 1 h. In the case of the tetrapeptide TIPP, enzymatic degradation occurred slowly to an extent of about 50% after 24 h of incubation and, therefore, this compound still represents a useful tool for most pharmacological studies. Experiments aimed at identifying the cleavage site in TIPP have been initiated. In contrast to TIPP, the pseudotetrapeptide antagonist TIPP[ψ] was completely stable for periods of up to 24 h of incubation. HPLC analyses performed with aliquots of the TIPP[ψ] supernatants confirmed the complete absence of enzymatic degradation (data not shown). Interestingly, the tripeptide antagonist TIP was also found

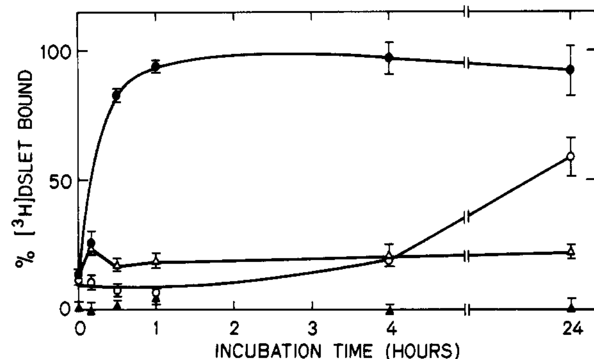

Figure 1. Enzymatic degradation study of [Leu[5]]enkephalin (●), TIPP (○), TIPP[ψ] (▲), and TIP (△) by incubation of 10^{-4} M peptide solutions with rat brain membranes at 37 °C. The remaining δ receptor binding capacity after various times of incubation was determined with the rat brain membrane binding assay ([^{3}H]DSLET displacement).

Table III. Parameters of Lowest Energy Conformers of TIP[ψ] and TIP[a]

	TIP[ψ] conformers		TIP conformers	
	1–1313	1–1205	1–996	1–1070
	Torsional Angles, deg			
ψ_1	92.8	140.3	143.2	70.6
ω_1	−179.3	−176.3	−177.8	−176.7
ϕ_2	−99.2	−82.3	−76.3	−78.4
ψ_2	59.3	81.2	83.1	77.7
ω_2	−171.8	−173.3	178.0	178.9
ϕ_3	−149.5	−63.9	−67.0	−150.2
χ_{11}	176.2	−177.6	174.6	−175.6
χ_{12}	69.5	91.0	98.7	70.8
χ_{21}	57.4	58.3	53.6	55.1
χ_{22}	−38.7	−48.4	−46.6	−46.9
χ_{31}	−56.2	−63.9	−61.4	−57.0
χ_{32}	109.4	101.7	104.5	98.7
	Energy (kcal/mol)			
	1.519	2.247	−0.024	1.028

[a] The designation of the lowest energy conformers is based on the numbering of the conformations used in the conformational search procedure.

to be completely resistant to enzymolysis under the conditions of the performed experiments.

These results indicate that TIPP[ψ] is stable against enzymatic degradation and, therefore, can be expected to be useful for *in vivo* studies. Naltrindole has recently been shown to prevent the development of morphine tolerance and dependence in mice.[18] In other studies it has been demonstrated that naltrindole has an immunosuppressive effect on T cell immunity in the mixed lymphocyte reaction and in rat allograft survivals.[19] It will be of considerable interest to examine whether TIPP[ψ] is able to produce similar effects.

A theoretical conformational analysis of TIP[ψ] was carried out in order to examine its conformational behavior in comparison with the parent peptide TIP. A molecular mechanics study of TIP[ψ] resulted in compact lowest energy conformations similar to those obtained for TIP (Table III, Figure 2). However, the lowest-energy conformer of TIP[ψ] was found to correspond to the second lowest energy conformer of TIP and the second lowest energy conformer of TIP[ψ] corresponded to the lowest energy conformer of TIP. The difference in energy between the two lowest energy conformers is about 0.7 kcal/mol in the case of TIP[ψ] and about 1.0 kcal/mol in the case of TIP. For TIP five structures with energies less than 2 kcal/mol higher than that of the lowest energy

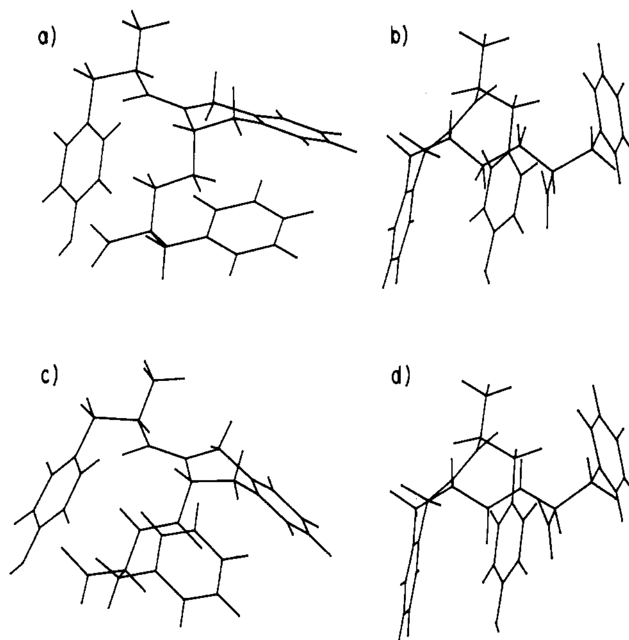

Figure 2. Low-energy conformations of TIP[ψ] and TIP: (a) TIP[ψ] (lowest), (b) TIP[ψ] (second lowest), (c) TIP (second lowest), (d) TIP (lowest).

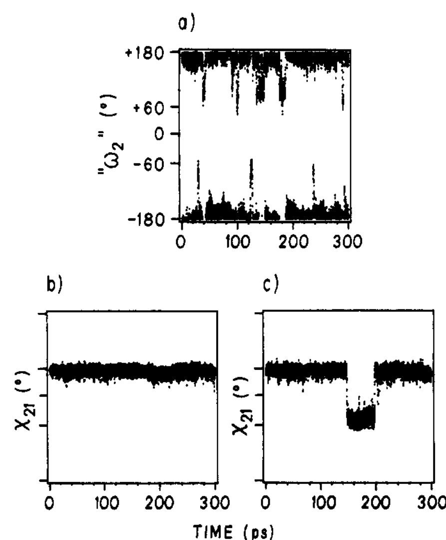

Figure 3. Monitoring of dihedral angles in a molecular dynamics simulation of TIP[ψ] and TIP: (a) "ω_2" (TIP[ψ]), (b) χ_{21} (TIP[ψ]), (c) χ_{21} (TIP).

conformer were found, whereas in the case of TIP[ψ] the number of conformers within 2 kcal/mol of its lowest energy structure was six. For both TIP and TIP[ψ] these various low-energy conformers were all characterized by similar ϕ_2, χ_{11}, χ_{21}, and χ_{31} angles but showed considerable variation in the ψ_1, ψ_2, and ϕ_3 angles. These results demonstrate the considerable structural flexibility of these tripeptides and clearly do not permit an unambiguous conclusion with regard to their bioactive conformation.

Molecular dynamics simulations carried out at 300 K for 300 ps revealed that the reduced peptide bond in TIP[ψ] assumed mostly the trans configuration (Figure 3), as was the case for the Tic[2]-Phe[3] peptide bond in the parent peptide, which showed no trans–cis peptide bond transitions in the course of a 300-ps run carried out under the same conditions (data not shown). However, the "ω_2" angle in TIP[ψ] occasionally did assume values of +60° or −60° for short time intervals, reflecting the increased structural

flexibility around the reduced peptide bond in the pseudopeptide. Furthermore, the Tic[2] residue in TIP[ψ] assumed exclusively the g^+ configuration for the entire duration of the simulation. In the case of TIP the conformation of the Tic[2] residue was g^+ as well most of the time, but the g^- conformation was also observed during a relatively short time interval (~50 ps). The fact that TIP[ψ] and TIP show similarities in their overall conformational behavior may explain their qualitatively similar opioid activity profile. However the increased structural flexibility around the reduced peptide bond in the pseudopeptide may be the reason for the observed quantitative differences in receptor affinities and in receptor selectivity. TIP analogues containing additional conformational constraints need to be developed for the elaboration of a pharmacophore model.

Conclusions

Reduction of the Tic[2]-Phe[3] peptide bond in the δ antagonist TIPP to prevent spontaneous peptide bond cleavage resulted in a compound, H-Tyr-Ticψ[CH_2NH]-Phe-Phe-OH (TIPP[ψ]), which also was highly stable against enzymatic degradation over extended periods of time. Furthermore, TIPP[ψ] showed improved δ antagonist potency, subnanomolar δ receptor affinity, unprecedented δ selectivity, and, unlike other δ antagonists, no μ or κ antagonist properties. TIPP[ψ] is likely to find wide use as a pharmacological tool in opioid research and may also have potential as a therapeutic agent.

Experimental Section

General Methods. Precoated plates (silica gel 60 F_{254}, 250 μm, Merck, Darmstadt, FRG) were used for ascending TLC in the following solvent systems (all v/v): (I) EtOAc/hexane (1:1); (II) EtOAc/hexane (2:1); (III) *n*-BuOH/AcOH/H_2O (4:1:5, organic phase) and (IV) *n*-BuOH/pyridine/AcOH/H_2O (15:10:3:12). Reversed-phase HPLC was performed on a Varian VISTA 5500 liquid chromatograph, utilizing a Vydac 218-TP column (4.6 × 250 mm). Proton nuclear magnetic resonance spectra were recorded at 25 °C on a Varian VXR-400S spectrometer using tetramethylsilane as an internal standard. Molecular weights of compounds were determined by FAB mass spectrometry on an MS-50 HMTCTA mass spectrometer interfaced to a DS-90 data system (Drs. M. Evans and M. Bertrand, Department of Chemistry, University of Montreal).

Synthesis of 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde. The aldehyde was synthesized via preparation of 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-(*N*-methoxy-*N*-methylamide) by using a published procedure.[12] (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (3.48 g, 10 mmol) was added to a stirred solution of Boc-Tic-OH (2.8 g, 10 mmol) and triethylamine (1.33 mL, 10 mmol) in CH_2Cl_2. After 5 min, *N,N*-dimethylhydroxylamine hydrochloride (1.2 g, 12 mmol) and triethylamine (1.68 mL, 12 mmol) were added to the solution. The reaction was carried out for 17 h. Subsequently, the reaction mixture was diluted with CH_2Cl_2 and washed with 3N H Cl, a saturated aqueous solution of $NaHCO_3$ and a saturated aqueous solution of NaCl. The organic solution was dried over $MgSO_4$ prior to evaporation of the solvent. The crude product was purified by silica gel column chromatography (EtOAc/hexane, 1:2) to afford 2.1 g (65%) 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-(*N*-methoxy-*N*-methylamide) as an oil: TLC R_f 0.57 (I), R_f 0.30 (II); ^1H NMR ($CDCl_3$) δ 1.45 (s, 9H, $C(CH_3)_3$), 3.00 (m, 2H, H-4), 3.18 (s, 3H, NCH_3), 3.77 (s, 3H, OCH_3), 4.42–4.86 (m, 3H, 2 H-1 + H-3), 7.07–7.22 (m, 4H, ar); FAB-MS *m/e* 321 (M^+).

To a stirred solution of the *N*-methoxy-*N*-methylamide derivative (1.2 g, 4 mmol) in 30 mL of Et_2O 190 mg (5 mmol) lithium aluminum hydride were added. The reduction was carried out for 1 h at 0 °C and the reaction mixture was then hydrolyzed with a solution of $KHSO_4$ (954 mg, 7 mmol) in 20 mL of H_2O. Subsequently, the aqueous phase was separated and extracted with three 50-mL portions of ether. The four organic phases were combined, washed with 3 N HCl, a saturated aqueous solution of $NaHCO_3$, and a saturated aqueous solution of NaCl, and finally dried over $MgSO_4$. After solvent evaporation pure 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde (635 mg, 60%) was obtained as an oil: TLC R_f 0.84 (I), R_f 0.57 (II); ^1H NMR ($CDCl_3$) δ 1.48 (s, 9H, $C(CH_3)_3$), 3.03–3.27 (m, 2H, H-4), 4.42–4.81 (m, 3H, 2 H-1 + H-3), 7.04–7.21 (m, 4H, ar), 9.47 (d, 1H, CHO). The aldehyde decomposed rapidly and had to be used immediately for reductive alkylation of the resin-bound dipeptide or phenylalanine. Because of the rapid decomposition, a molecular weight determination by FAB-MS was not feasible.

Peptide Synthesis. Peptide synthesis was performed by the manual solid-phase technique using a Merrifield resin (1% cross-linked, 100–200 mesh, 0.61 mmol Boc-Phe/g of resin) obtained from Peninsula, Belmont, CA. Peptides were assembled using Boc-protected amino acids and 1,3-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as coupling agents. The side chain of Tyr was also Boc-protected. The following steps were performed in each cycle: (1) addition of Boc amino acid in CH_2Cl_2 (2.5 equiv); (2) addition of HOBt (2.5 equiv); (3) addition of DIC (2.5 equiv) and mixing for 2–3 h; (4) washing with CH_2Cl_2 (3 × 1 min); (5) washing with EtOH (1 min); (6) monitoring completion of the reaction with the ninhydrin test,[20] (7) Boc deprotection with 50% (v/v) TFA in CH_2Cl_2 (30 min); (8) washing with CH_2Cl_2 (5 × 1 min); (9) neutralization with 10% (v/v) DIEA in CH_2Cl_2 (2 × 5 min); and (10) washing with CH_2Cl_2 (5 × 1 min).

To introduce the reduced peptide bond between the Tic[2] and Phe[3] residues a reductive alkylation reaction[11] between 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde and the amino group of the resin-bound phenylalanine or H-Phe-Phe dipeptide was performed as follows. After two washes with DMF, 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde (2.5 equiv) in DMF containing 1% AcOH was added to the resin. Sodium cyanoborohydride (5.0 equiv) was then added portionwise over a period of 40 min and the reaction was allowed to continue for 3 h. The resin was then washed with DMF (2 × 1 min) and CH_2Cl_2 (3 × 1 min). Deprotection and coupling of Boc-Tyr(Boc)-OH were then performed according to the protocol described above. After final deprotection with 50% (v/v) TFA in CH_2Cl_2 (30 min), the resin was washed with CH_2Cl_2 (3 × 1 min) and EtOH (3 × 1 min) and was dried in a desiccator. Peptides were cleaved from the resin by treatment with HF for 60 min at 0 °C (20 mL of HF plus 1 mL of anisole/g resin). After evaporation of the HF, the resin was extracted three times with Et_2O and, subsequently, three times with glacial AcOH. The crude peptide was obtained in solid form through lyophylization of the acetic acid extract.

Crude peptides were purified by reversed-phase chromatography on an octadecasilyl silica column, with a linear gradient of 20–80% MeOH in 1% TFA. Each peptide was >98% pure as assessed by analytical RP-HPLC using a linear gradient of 25–75% acetonitrile in 0.1% TFA at a flow rate of 1.5 mL/min, and by TLC. Molecular weights were confirmed by FAB-MS.

TIPP[ψ]: HPLC K' 2.0; TLC R_f 0.48 (III), R_f 0.73 (IV); FAB-MS *m/e* 621 (M^+).

TIP[ψ]: HPLC K' 0.5; TLC R_f 0.25 (III), R_f 0.66 (IV); FAB-MS *m/e* 474 (M^+).

Bioassays, Receptor Binding Assays, and Enzymatic Degradation Studies. The GPI[21] and MVD[22] bioassays were carried out as reported in detail elsewhere.[23,24] A dose–response curve was determined with [Leu[5]]enkephalin as standard for each ileum and vas preparation and IC_{50} values of the compounds being tested were normalized according to a published procedure.[25] K_e values for the δ antagonists were determined from the ratio of IC_{50} values obtained with a particular δ agonist in the MVD assay in the presence and absence of a fixed antagonist concentration (5 nM).[26]

Opioid receptor binding studies were performed as described in detail elsewhere.[23] Binding affinities for μ and δ receptors were determined by displacing, respectively, [^3H]DAMGO (New England Nuclear) and [^3H]DSLET (Amersham) from rat brain membrane preparations, and κ opioid receptor affinities were measured by displacement of [^3H]U69,593 (New England Nuclear) from guinea pig brain membranes. Incubations were performed for 2 h at 0 °C with [^3H]DAMGO, [^3H]DSLET, and

[^{3}H]U69,593 at respective concentrations of 0.72, 0.78, and 0.80 nM. IC_{50} values were determined from log dose–displacement curves and K_i values were calculated from the obtained IC_{50} values by means of the equation of Cheng and Prusoff,[27] using values of 1.3, 2.6, and 2.9 nM for the dissociation constants of [^{3}H]-DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593, respectively.

The enzymatic degradation studies were performed as described elsewhere in detail.[28] Incubations of 1×10^{-4} M peptide solutions with rat brain membrane preparations were performed at 37 °C for 10 and 30 min and 1, 4, and 24 h. At the end of the incubations enzymatic activity was destroyed by dipping the sample tubes for 2 min into boiling water. After centrifugation aliquots of the supernatant were tested in the [^{3}H]DSLET binding assay as described above.

Theoretical Conformational Analyses. All calculations were carried out using the software package SYBYL (Tripos Associates, St. Louis, MO) on a Vaxstation 3500. Molecules were viewed on an Evans & Sutherland PS330 computer graphics display terminal and a Hewlett-Packard HP7475 plotter was used for the preparation of the figures. The standard SYBYL force field[29] was employed for the energy calculations, and a distance-dependent dielectric constant of 78 was chosen to simulate an aqueous environment. Basically, the molecular mechanics calculations were carried out as previously described.[30] Peptide structures containing the Tic residue in either the g^+ or the g^- side chain configuration were independently constructed and in the case of TIP[ψ] a positively charged (protonated) amino group in the reduced peptide bond was used. For each of the starting structures a grid search encompassing all rotatable bonds and using 30° increments was performed. Both trans and cis peptide bonds were allowed. Conformations within 5 kcal/mol of the lowest energy structure were grouped into families, with each family consisting of conformers showing similarity in all torsion angles (±30°). The lowest energy conformer in each family was then extensively minimized. A total of 119 and 157 conformations were generated and minimized for TIP and TIP[ψ], respectively, by using this procedure. Molecular dynamics simulations were carried out as described elsewhere in detail.[31] For each peptide the lowest energy conformer obtained in the molecular mechanics study was used as the starting conformation. The simulations were carried out for 300 ps at 300 K. Each dynamics trajectory was analyzed for torsion angles to generate Figure 3.

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References

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* **1984**, *219*, 345–373. The following other abbreviations were used: Boc, *tert*-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DALDA, H-Tyr-D-Arg-Phe-Lys-NH_2; DAMGO, H-Tyr-D-Ala-Gly-N^{α}MePhe-Gly-ol; DIC, 1,3-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DPDPE, H-Tyr-D-Pen-Gly-Phe-D-Pen-OH; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; FAB-MS, fast atom bombardment mass spectrometry; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; ICI 174864, *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH; MVD, mouse vas deferens; NTB, benzofuran analog of naltrindole; NTI, naltrindole; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIP, H-Tyr-Tic-Phe-OH; TIP[ψ], H-Tyr-Ticψ[CH_2NH]Phe-OH; TIPP, H-Tyr-Tic-Phe-Phe-OH; TIPP[ψ], H-Tyr-Ticψ[CH_2NH]Phe-Phe-OH; U69,593, ($5\alpha,7\alpha,8\beta$)-(–)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

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