β-Methyl Substitution of Cyclohexylalanine in Dmt-Tic-Cha-Phe Peptides Results in Highly Potent δ Opioid Antagonists

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The opioid peptide TIPP (H-Tyr-Tic-Phe-Phe-OH, Tic:1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) was substituted with Dmt (2′,6′-dimethyltyrosine) and a new unnatural amino acid, β-MeCha (β-methylcyclohexylalanine). This double substitution led to a new series of opioid peptides displaying subnanomolar δ antagonist activity and μ agonist or antagonist properties depending on the configuration of the β-MeCha residue. The most promising analog, H-Dmt-Tic-(2*S*,3*S*)-β-MeCha-Phe-OH was a very selective δ antagonist both in the mouse vas deferens (MVD) assay ($K_e = 0.241 \pm 0.05$ nM) and in radioligand binding assay ($K_i^{\delta} = 0.48 \pm 0.05$ nM, $K_i^{\mu}/K_i^{\delta} = 2800$). The epimeric peptide H-Dmt-Tic-(2*S*,3*R*)-β-MeCha-Phe-OH and the corresponding peptide amide turned out to be mixed partial μ agonist/δ antagonists in the guinea pig ileum and MVD assays. Our results constitute further examples of the influence of Dmt and β-methyl substitution as well as C-terminal amidation on the potency, selectivity, and signal transduction properties of TIPP related peptides. Some of these compounds represent valuable pharmacological tools for opioid research.

Introduction

The tetrapeptide H-Tyr-Tic-Phe-Phe-OH (TIPP)[1] represents the prototype of a class of potent and selective δ opioid antagonists that contain a 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) in the position 2 of the peptide sequence.[2]

In an effort to further improve the δ opioid antagonist potency and δ receptor selectivity of TIPP, numerous analogs have been prepared and pharmacologically characterized *in vitro*. First of all, the parent peptide, TIPP, was shown to undergo slow spontaneous Tyr-Tic diketopiperazine formation with cleavage of the Tic-Phe peptide bond. To prevent this degradation, a pseudo peptide containing a reduced peptide bond between the Tic and Phe residues (Tyr-Tic-ψ[CH_2-NH]-Phe-Phe-OH, TIPPψ) was synthesized. This peptide analog showed considerable higher stability against chemical and enzymatic degradation[3] and proved to be a more potent and more selective δ opioid antagonist compared to the parent peptide. Replacement of Tyr^1 with 2′,6′-dimethyltyrosine (Dmt) in TIPP resulted in a significant enhancement of δ antagonist potency, but it was accompanied by a slight decrease in δ opioid selectivity.[4] This observation confirms that the replacement of Tyr with Dmt in general results in an increase both in μ- and in δ-receptor affinity in a wide variety of opioid peptides.[5−7] Numerous substitutions with natural and unnatural amino acids were performed in position 3, and some Phe^3-substituted TIPP analogs exhibited very potent δ opioid antagonist properties.[8] Most interestingly, saturation of the Phe^3 aromatic ring in TIPP led to H-Tyr-Tic-Cha-Phe-OH [TICP] with substantially increased δ antagonist potency.[8] TIPP and its C-terminal amide analog were systematically modified topologically by substitution of each amino acid residue with all stereoisomers of the corresponding β-methyl amino acid. The TIPP analogs containing (2*S*,3*R*)-β-MeTic in position 2 or (2*S*,3*R*)-β-MePhe in position 3 displayed extraordinarily high δ antagonist potency and δ selectivity, while substitution of (2*S*,3*S*)-β-MePhe for Phe^3 in TIPP resulted in a peptide with mixed μ agonist/δ antagonist properties. These results suggest a profound influence of β-methyl substitution on the potency, selectivity, and signal transduction properties of TIPP peptides.[9]

The first known compound with a mixed μ agonist/δ antagonist profile was TIPP-NH_2.[2] Substitution of Dmt for the Tyr^1 in the TIPP-NH_2 resulted in the compound H-Dmt-Tic-Phe-Phe-NH_2 (DIPP-NH_2) with increased μ agonist potency in the guinea pig ileum (GPI[a]) assay and unchanged high δ antagonist activity in the mouse vas deferens (MVD) assay.[10] Reduction of the peptide bond between Tic^2 and Phe^3 of DIPP-NH_2 led to the compound DIPPψ-NH_2, which displayed further enhanced μ agonist potency and still high δ antagonist activity. In the rat tail-flick test, DIPPψ-NH_2 produced a potent analgesic effect, about three times higher than morphine. This peptide produced less-acute tolerance upon continuous infusion, and no physical dependence was observed after chronic administration.[10]

In the present article, we describe further structural modifications of the TIPP peptide, which resulted not only in highly potent δ opioid antagonists with very high δ selectivity, but

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[a] Abbreviations: Boc, *tert*-butyloxycarbonyl; DAMGO, H-Tyr-D-Ala-Gly-NMe-Phe-Gly-ol; DPDPE, H-Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH; FDAA, N^{α}-(2,4-dinitro-5-fluorophenyl)-L-alaninamide; GITC, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate; GPI, guinea pig ileum; MVD, mouse vas deferens; RP-HPLC, reversed-phase high-performance liquid chromatography; TAPP, H-Tyr-D-Ala-Phe-Phe-NH_2; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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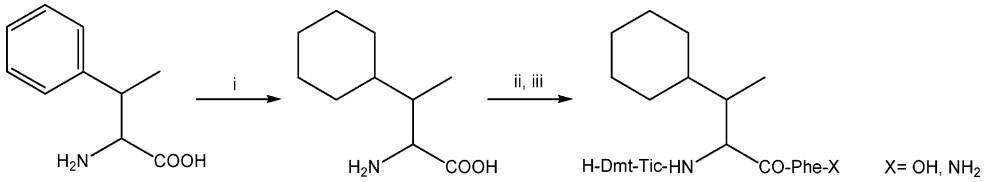
Scheme 1. Synthesis of Dmt-Tic-β-MeCha-Phe Peptides[a]



[a] Reagents and conditions: (i) 50 psi H_2, 50 °C, PtO_2, AcOH−H_2O (2:1); (ii) $(Boc)_2O$, pH 9; (iii) Boc solid-phase peptide synthesis on Phe-Merrifield or Phe-MBHA resin

Table 1. Analytical Properties of TIPP and TIPP-NH_2 Analogs

		TLC[a]				
no.	peptides	R_f(A)	R_f(B)	R_f(C)	HPLC[b] k′	ESI-MS $[M + H]^+$
1	H-Dmt-Tic-(2*S*,3*S*)-β-MeCha-Phe-OH	0.65	0.56	0.48	8.94	683
2	H-Dmt-Tic-(2*R*,3*R*)-β-MeCha-Phe-OH	0.65	0.55	0.53	11.17	683
3	H-Dmt-Tic-(2*S*,3*R*)-β-MeCha-Phe-OH	0.66	0.60	0.52	9.44	683
4	H-Dmt-Tic-(2*R*,3*S*)-β-MeCha-Phe-OH	0.64	0.50	0.46	10.94	683
5	H-Dmt-Tic-(2*S*,3*S*)-β-MeCha-Phe-NH_2	0.64	0.58	0.64	8.31	682
6	H-Dmt-Tic-(2*R*,3*R*)-β-MeCha-Phe-NH_2	0.60	0.56	0.49	10.23	682
7	H-Dmt-Tic-(2*S*,3*R*)-β-MeCha-Phe-NH_2	0.63	0.62	0.63	8.76	682
8	H-Dmt-Tic-(2*R*,3*S*)-β-MeCha-Phe-NH_2	0.60	0.60	0.45	9.82	682

[a] Retention factor on silica gel 60 F_{254} precoated glass plates. Solvent systems: (A) butanol/acetic acid/water (4:1:1), (B) acetonitrile/methanol/water (4:1:1), (C) ethyl acetate/pyridine/acetic acid/water (60:20:6:11). [b] Capacity factors for Vydac 218TP54 (25 × 0.46 cm, $d_p = 5$ μm) column with gradient of 0.75%/min acetonitrile (0.08% (v/v) TFA) in water (0.1% TFA) over 40 min starting from 25% acetonitrile. Flow rate: 1 mL/min, $t_0 = 3.1$ min, $\lambda = 216$ nm.

also led to peptides with mixed partial μ agonist/δ antagonist properties. For this purpose, we substituted β-methylcyclohexylalanine (β-MeCha) for Phe in position 3, and the effects of the different configurations of the isomers of this amino acid on the biological properties of TIPP and TIPP-NH_2 were explored.

Results and Discussion

Chemistry. The racemic *erythro*-(2*S*,3*S* and 2*R*,3*R*) and *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-MeCha diastereoisomers were obtained from the corresponding *erythro*- or *threo*-β-MePhe[9,11,12] by hydrogenation at 60 psi H_2 and 50 °C using a PtO_2 catalyst in aqueous acetic acid solution, as was previously reported for the preparation of cyclohexylalanine from phenylalanine.[13−17] Boc-protection of *erythro*- or *threo*-β-MeCha and peptide synthesis on a Merrifield or MBHA resin were carried out by known, published methods (Scheme 1).[9] The relative configurations were confirmed indirectly by NMR analysis of the Boc-β-MeCha diastereomers. Similarly to the β-MePhe and β-MeNal isomers, the coupling constant between C_α-H and C_β-H of the Boc-*erythro*-β-MeCha was larger ($J = 8.5$ Hz) than in the Boc-*threo*-β-MeCha ($J = 5.0$ Hz).[9,11,12,18] Furthermore, the relative NOE intensity between the NH and β-methyl protons of the Boc-*threo*-β-MeCha was found to be significantly higher than that of the *erythro* isomers, as published for the β-MePhe isomers.[19] The configuration of the α-carbon was determined directly by enzymatic digestion of the β-MeCha isomers with L-amino acid oxidase,[20] followed by RP-HPLC separation of the chiral derivatized β-MeCha isomers or by chiral TLC separation of the amino acid isomers. The GITC derivatization[21] was more useful, as it made it possible to separate all four β-MeCha isomers and, thus, the 2*R* and 2*S* diastereomers were unambiguously identified (see Supporting Information). In contrast, the resolution of the FDAA derivatives of the 2*S* and 2*R* diastereomers was very low. Racemic *erythro*- and *threo*-β-MeCha was digested with L-amino acid oxidase in 250 mg scale as well to obtain (2*R*,3*R*)- and (2*R*,3*S*)-β-MeCha as standards.

The solid-phase peptide synthesis resulted in diastereomeric peptides that were separated by preparative RP-HPLC. Analytical properties of the new TIPP and TIPP-NH_2 analogs are summarized in Table 1. The configuration of the β-MeCha residue in the new TIPP analogs was determined by chiral TLC analysis of the peptide hydrolysates and by analytical HPLC analyses of the GITC-derivatized hydrolysates.[20,21]

Table 2. Equilibrium Competition Binding Data for [β-MeCha[3]]TIPP Tetrapeptides in Rat Brain Membranes[a]

peptides	K_i^μ (nM)	K_i^δ (nM)	K_i^μ/K_i^δ
H-Tyr-Tic-Phe-Phe-OH[b]	1720 ± 50	1.22 ± 0.07	1410
H-Tyr-Tic-Cha-Phe-OH[b]	3600	0.61	5890
1	1400 ± 149	0.48 ± 0.05	2800
2	580 ± 80	2.0 ± 0.6	290
3	280 ± 5	2.0 ± 0.3	140
4	55 ± 1	1.5 ± 0.1	37
5	330 ± 106	4.0 ± 1.4	82
6	290 ± 99	13 ± 2.6	22
7	170 ± 11	4.1 ± 0.7	41
8	200 ± 37	7.0 ± 1.3	29

[a] Receptor binding data are presented as the means ± SEM of two or three independent assays. [^{3}H]DAMGO as μ ligand and [^{3}H]Ile^{5,6}-deltorphin-2 as δ ligand were used. [b] See ref 5.

Biological Evaluation. The opioid receptor binding assays[22] were performed by displacement of [^{3}H]DAMGO and [^{3}H]Ile^{5,6}-deltorphin-2[23] as μ and δ opioid receptor selective radioligands by the novel TIPP analogs in rat brain membrane preparations. The opioid receptor binding affinities (K_i values) of the eight novel peptides (four peptide acids and four C-terminal amidated analogs) are presented in Table 2. Compound **1**, [Dmt,[1] (2*S*,3*S*)-β-MeCha[3]]TIPP, exhibited 2.5-fold higher δ opioid receptor affinity ($K_i^\delta = 0.48 \pm 0.05$ nM) and 2-fold higher selectivity for the δ opioid receptor ($K_i^\mu/K_i^\delta = 2800$) than the parent TIPP. The other analogs had lower δ binding affinity than TIPP and were less δ opioid receptor selective. Comparing the peptide acid and amide analogs of TIPP, it turned out that the TIPP amides in general had higher affinities for the μ opioid receptor, with the exception of compound **8**, and slightly decreased δ affinities.

In vitro agonist and antagonist activities of the novel TIPP compounds were determined in the GPI and MVD assays. GPI preparations contain mainly μ opioid receptors, whereas δ opioid

Table 3. *In Vitro* Opioid Activity (GPI and MVD Assays) of [β-MeCha^3]TIPP Analogs

peptides	K_e (nM)		
	GPI	GPI[a]	MVD[b]
H-Tyr-Tic-Phe-Phe-OH[c]	>10 000		4.8 ± 0.2
H-Tyr-Tic-Phe-Phe-NH$_2$[c]	1700 ± 220		18.0 ± 2.2
H-Dmt-Tic-Phe-Phe-OH[c]			0.20
H-Tyr-Tic-Cha-Phe-OH[c]			0.44
H-Dmt-Tic-Phe-Phe-NH$_2$[c]	18.0 ± 1.8		0.21 ± 0.04
1		970 ± 31	0.24 ± 0.05
2		290 ± 53	0.55 ± 0.02
3	150 ± 28 (IC_{30})		0.81 ± 0.81
4		19.0 ± 2.6	0.11 ± 0.03
5		340 ± 50	0.77 ± 0.09
6		670 ± 50	1.70 ± 0.33
7	110 ± 17 (IC_{40})		0.68 ± 0.04
8		120 ± 12	1.94 ± 0.02

[a] Determined against H-Tyr-D-Ala-Phe-Phe-NH$_2$ (TAPP). [b] Determined against DPDPE. [c] See ref 5 and references therein.

receptors are predominantly present in MVD tissues. δ Opioid antagonist potencies were determined against the δ agonist DPDPE, while μ opioid antagonist potencies were determined against the μ agonist Tyr-D-Ala-Phe-Phe-NH$_2$ (TAPP). The results are presented in Table 3. All compounds were found to be δ opioid antagonists with high potencies in the MVD assay. Compound **1** had the highest δ opioid selectivity (K_e^{μ}/K_e^{δ} = 4042) in these functional assays. In the GPI assays, compounds **3** and **7** behave as partial μ opioid agonists, whereas compounds **1**, **2**, **5**, **6**, and **8** displayed moderate μ antagonist properties. In this assay, compound **4** had quite high μ antagonist potency (K_e = 19.0 ± 2.6 nM).

Discussion

Earlier structural modifications of TIPP peptides resulted in analogs with interesting pharmacological profiles. For example, Dmt-Tic-Phe-Phe-NH$_2$ showed high affinity for δ opioid receptors and μ opioid receptors with moderate δ receptor selectivity in radioligand binding assays. This compound behaved as μ agonist and δ antagonist in the *in vitro* GPI and MVD assays.[10,24] Saturation of the Phe^3 aromatic ring in TIPP led to a compound (Tyr-Tic-Cha-Phe-OH, TICP) with substantially increased δ antagonist potency and higher δ selectivity than the parent peptide.[8] Methylation of the β-carbon in the Phe^3 in TIPP resulted in different activity profiles depending on the configuration of the β-MePhe in the TIPP and TIPP-NH$_2$.[9] It demonstrated that β-methyl substitution of the Phe^3 residue in TIPP can have important effects on receptor selectivity and on agonist/antagonist properties. On the basis of these earlier results, we decided to prepare new TIPP and TIPP-NH$_2$ analogs containing L-Dmt,[1] and the four different β-MeCha^3 isomers. Consequently, we have prepared *erythro*- and *threo*-β-MeCha by saturation of the aromatic ring of β-MePhe using catalytic hydrogenation in the presence of PtO_2. The racemic Boc-*erythro*- and Boc-*threo*-β-MeCha isomers were introduced into the TIPP or TIPP-NH$_2$ sequences by solid-phase peptide synthesis. The resulting diasteromeric peptides were separated, and the configuration of the β-MeCha residue was assigned by chiral HPLC or TLC methods.

The affinity for the μ or δ opioid receptor was determined in rat brain membranes. As expected, all compounds show high affinity for the δ receptor and are less potent at the μ receptor. The δ selectivity was higher for the C-terminal carboxylic acid analogs than for the C-terminal amides; Dmt-Tic-(2*S*,3*S*)-β-MeCha-Phe-OH (**1**) displaying the highest δ selectivity.

In the functional assays, compounds **3** and **7** were mixed partial μ agonist/δ antagonists. The other compounds **1**, **2**, **4**, **5**, **6**, and **8** behaved as μ and δ antagonists. The configuration of the β-MeCha residue has a profound influence on the μ affinity and μ agonist properties, similar to our earlier results obtained with [β-MePhe^3]TIPP analogs.[9,25] On the other hand, all compounds displayed high δ antagonist activity, indicating that the configuration or the conformation of the β-MeCha^3 in the TIPP analogs does not influence the antagonist properties. This further confirms that the aromatic ring in position 3 in TIPP or TIPP-NH$_2$ is not required for δ antagonist properties.

We also tested the G-protein activation of these analogs in [^{35}S]GTPγS binding assays using rat brain membranes or Chinese hamster ovary (CHO) cells stably expressing μ or δ opioid receptor.[26] In rat brain membranes, none of the compounds activated [^{35}S]GTPγS binding. Similarly, in δ_hCHO cell membranes, no increase in [^{35}S]GTPγS binding was found in the presence of these new analogs. In contrast, in μ_hCHO cell membranes, all amidated analogs displayed weak-to-moderate partial agonist properties. These results are in agreement with the data obtained in the GPI and MVD assays presented here.

In conclusion, we prepared new β-MeCha^3-containing TIPP analogs that show very potent δ antagonist properties. The configuration of the β-methyl-substituted carbon has a large influence on the affinity and activity profile of the peptide analogs. H-Dmt-Tic-(2*S*,3*S*)-β-MeCha-Phe-OH (**1**) is a δ opioid antagonist with increased potency and higher δ selectivity as compared to the parent TIPP. Compounds **3** and **7** are mixed partial μ agonist/δ antagonists.

Experimental Section

General Methods. TLC was performed on 2.5 × 10 cm plates precoated with silica gel 60 F_{254} from Merck (Darmstadt, Germany) or on chiral TLC plates (Macherey Nagel, Dürer, Germany), using the following solvent systems: (A) 1-butanol/acetic acid/water (4:1:1), (B) acetonitrile/methanol/water (4:1:1), and (C) ethyl acetate/pyridine/acetic acid/water (60:20:6:11). TLC spots were visualized under UV light or with ninhydrin reagent. Melting points were measured with a Büchi 510 apparatus. Enhanced NMR spectra were obtained on a Bruker AC 250-P 250 MHz and on a Bruker Avance DRX 400 MHz spectrometer. The assignments were based on ^{1}H, ^{13}C, DEPT, HMQC, and HMBC NMR experiments.

RP-HPLC was performed on a Merck-Hitachi instrument equipped with a Vydac 218TP54 column for analytical purposes or with a Vydac 218TP1010 column for semipreparative separations. UV detection was carried out at 216 nm or 275 nm. Mass spectra were recorded on a VG Quattro II spectrometer (ES ionization; cone voltage, 70 V; source temperature, 80 °C).

Specific optical rotation was determined on an Optical Activity AA-5 automatic polarimeter at 589.4 nm.

Protected amino acids (except β-MeCha) and Merrifield and 4-methylbenzhydrylamine resins were purchased from Sigma Aldrich Kft. (Budapest, Hungary) or from BACHEM Feinchemicalen AG (Bubendorf, Switzerland). Boc-L-Dmt was obtained from RSP Amino Acids LLC (Sherley, MA). PtO_2 catalyst was purchased from Acros Chimica N.V. (Geel, Belgium).

erythro-β-MePhe and *threo*-β-MePhe were prepared in racemic form in our laboratory by fractional crystallization[12] using the method of Kataoka et al.[11]

Radioligands were prepared in our laboratory using the precursor peptides [Dit^1]DAMGO, [Dit^1]Ile^{5,6}-deltorphin-2 and tritium gas in the presence of PdO/$BaSO_4$ catalyst of Merck (Darmstadt, Germany).[27]

***erythro*-β-Methylcyclohexylalanine.** An amount equal to 1.9 g of *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-MePhe hydrochloride (8.8 mmol) was dissolved in 100 mL of acetic acid/water (2:1). PtO_2 (250 mg) was added to the solution, and it was shaken under 50 psi of hydrogen at 50 °C in a Parr-hydrogenator for 20 h. The reaction mixture was cooled to room temperature, the catalyst was filtered over a layer of Celite, and the filtrate was evaporated in vacuum. The remaining solid was dissolved in hot water and kept in a refrigerator overnight. The precipitate was filtered, and a yield of 1.77 g (81%) was obtained; mp 258 °C; R_f(A) 0.48; R_f(B) 0.56 and 0.36 on chiral plate; ESI-MS 186 $[M + H]^+$; ^{1}H NMR (DCl/DMSO-d_6) δ 8.60 (m, NH_3^+), 3.82 (d, 1H, J = 4.2 Hz, α-H), 1.79−0.78 (m, 12H, β-H, chx), 0.85 (d, 3H, J = 7.0 Hz, β-CH_3); ^{13}C NMR (DCl/DMSO-d_6) δ 170.2 (COOH), 54.2 (C-α), 39.7 (C-β), 38.4 (chx-CH), [31.0, 29.2, 26.2, 26.1, 25.9] (chx-CH_2), 12.0 (β-CH_3); Anal. ($C_{10}H_{19}NO_2$) C, H, N.

***threo*-β-Methylcyclohexylalanine.** An amount equal to 0.85 g of *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-MePhe was dissolved in 40 mL of acetic acid/water (2:1) and, in the presence of 100 mg of PtO_2 catalyst, was hydrogenated under 55 psi of hydrogen at 50 °C for 23 h, followed by the above work up. Yield was 0.79 g (88%); mp 260.1 °C; R_f(A) 0.48; R_f(B) 0.54 and 0.45 on chiral plate; ESI-MS 186 $[M + H]^+$; ^{1}H NMR (DCl/DMSO-d_6) δ 8.42 (m, NH_3^+), 3.82 (d, 1H, J = 4.0 Hz, α-H), 1.79 (1H, m, β-H), 1.63−0.77 (m, 10H, chx), 1.32 (m, 1H, chx-CH), 0.82 (d, 3H, J = 7.0 Hz, β-CH_3); ^{13}C NMR (DCl/DMSO-d_6) δ 171.2 (COOH), 54.5 (C-α), 39.3 (C-β), 37.6 (chx-CH), [31.1, 29.3, 26.1(2C), 25.9] (chx-CH_2), 12.0 (β-CH_3); Anal. ($C_{10}H_{19}NO_2$) C, H, N.

Identification of Enantiomers of β-Methylcyclohexylalanine. For the preparation of the (2*R*)-β-MeCha isomers, 75 mg (16.5 units) of L-amino acid oxidase (Sigma-Aldrich Kft, Budapest) was added to the solution of 250 mg of *erythro*- or *threo*-β-MeCha in 200 mL of water at pH 7.3. The solution was stirred at room temperature overnight. After gel filtration on Sephadex G-10, the (2*R*,3*R*)- or (2*R*,3*S*)-β-MeCha was obtained. On analytical scale, 250 μL of a 10 mM solution of racemic *erythro*- or *threo*-β-MeCha in 100 mM Tris buffer pH 7.2 was digested with 0.05 units of L-amino acid oxidase at room temperature overnight.[20] The chiral TLC spots and the HPLC peaks of the GITC derivatives were identified with these (2*R*)-diastereomers (see Supporting Information).

Optically pure (2*S*,3*S*)- and (2*R*,3*R*)-β-MeCha were prepared from resolved benzyloxycarbonyl *erythro*-β-MePhe. The optical resolution of the *erythro*-β-MePhe was attained by fractional crystallization of the quinine salt of the benzyloxycarbonyl derivative.[11] Hydrogenation of the resolved *erythro*-β-MePhe resulted in the removal of the benzyloxycarbonyl group and the saturation of the aromatic ring (see Supporting Information, p. S2).

(2*S*,3*S*)-β-MeCha: $[\alpha]^{25}_D = +25.3$ (c = 0.75; 25% (v/v) AcOH), R_f(B) 0.56 on chiral plate, t_R(GITC derivative) = 19.7 min. (2*R*,3*R*)-β-MeCha: $[\alpha]^{25}_D = -27.3$ (c = 0.75; 25% (v/v) AcOH), R_f(B) 0.36 on chiral plate, t_R(GITC derivative) = 16.7 min. (2*S*,3*R*)-β-MeCha: R_f(B) 0.54 on chiral plate, t_R(GITC derivative) = 18.8 min. (2*R*,3*S*)-β-MeCha: $[\alpha]^{25}_D = -7.0$ (c = 0.75; 25% (v/v) AcOH), R_f(B) 0.45 on chiral plate, t_R(GITC derivative) = 15.7 min.

This information was used for the identification of the configuration of the β-MeCha in the diastereomeric peptides (see below).

***N*$^\alpha$-Boc-*erythro*-β-methylcyclohexylalanine.** A solution of 2.74 g of *erythro*-β-MeCha·HOAc salt (11.2 mmol) in 50 mL of dioxane and 25 mL of water was cooled in an ice bath. The pH was adjusted to 9 with 2 N NaOH, and then 3.09 g of $(Boc)_2O$ was added to the solution with stirring for 3 h, followed by overnight stirring at room temperature. If the pH changed it was adjusted to 9. After evaporation of dioxane, the pH of water solution was adjusted to 2−3 with a saturated $KHSO_4$ solution under ice cooling. The aqueous layer was extracted three times with 50 mL of ethyl acetate. The combined organic layers were washed with water, brine, and again with water. The organic phase was dried over Na_2SO_4 and evaporated in vacuum. Because precipitation of the resulting oil from an ethyl acetate/petroleum ether mixture failed, the oil was stored in a refrigerator overnight, after which it solidified. Yield 3.0 g (94%); mp 86.7−88.7 °C; R_f(A) 0.9; ESI-MS 286 $[M + H]^+$; ^{1}H NMR (DMSO-d_6) δ 6.95 (d, 1H, J = 8.5 Hz, NH), 3.85 (t, 1H, J = 8.5 Hz, α-H), 1.67−0.80 (m, 11H, chx), 1.65 (br s, 1H, β-H), 1.38 (s, 9H, Boc-CH_3), 0.75 (d, 3H, J = 7.0 Hz, β-CH_3); ^{13}C NMR (DMSO-d_6) δ 174.2 (COOH), 155.8 (Boc-CO), 78.3 ($C(CH_3)_3$), 56.6 (C-α), 39.7 (C-β), 38.0 (chx-CH), 31.4 (chx-CH_2), 28.6 (C(CH_3)), [27.1, 26.8, 26.5] (chx-CH_2), 12.4 (β-CH_3); Anal. ($C_{15}H_{27}NO_4$) C, H, N.

***N*$^\alpha$-Boc-*threo*-β-methylcyclohexylalanine.** An amount equal to 2.0 g of *threo*-β-MeCha·HOAc was dissolved in 50 mL of dioxane and 25 mL of water. The pH was adjusted to 9 with 2 N NaOH, and 2.2 g of $(Boc)_2O$ was added to the solution. The reaction mixture was stirred for 3 h at 0 °C and then at room temperature overnight. The work up was as described above. Yield 2.45 g (99%); mp 132.2 °C; TLC R_f(A) 0.9; ESI-MS 286 $[M + H]^+$; ^{1}H NMR (DMSO-d_6) δ 6.77 (d, 1H, J = 9.2 Hz, NH), 4.15 (dd, 1H, J = 5.0 Hz, 9.2 Hz, α-H), 1.67−0.84 (m, 10H, chx), 1.70 (br s, 1H, β-H), 1.38 (s, 9H, Boc-CH_3), 1.10 (m, 1H, chx-C*H*), 0.79 (d, 3H, J = 7.0 Hz, β-CH_3); ^{13}C NMR (DMSO-d_6) δ 174.6 (COOH), 156.2 (Boc-CO), 78.3 ($C(CH_3)_3$), 55.8 (C-α), 39.7 (C-β), 39.3 (chx-CH), [30.9, 29.5] (chx-CH_2), 28.6 (C(CH_3)), 26.4 (chx-CH_2), 12.3 (β-CH_3); Anal. ($C_{15}H_{27}NO_4$) C, H, N.

Solid-Phase Peptide Synthesis and Peptide Purification. The TIPP peptide analogs were prepared by manual solid-phase peptide synthesis using Boc chemistry on Merrifield resin for the C-terminal carboxylic acid analogs and 4-methylbenzhydrylamine resin for the C-terminal amides. Boc-Phe was attached to the Merrifield resin by the Gisin method.[28] Diisopropylcarbodiimide and 1-hydroxybenzotriazole were used as coupling reagents. The coupling steps were monitored by ninhydrin test[29] or chloranil test. Removal of the Boc protecting group was performed by washing with a TFA/DCM/anisole (50:48:2) solution for 2 and 20 min, followed by DCM washes (4), a neutralization with 20% DIEA in DCM (2 × 2 min), and DCM washing (3×) again. The peptides were cleaved from the resin with anhydrous HF (5 mL/g resin) in the presence of anisole as scavenger for 1 h at 0 °C. The peptide was separated from the resin by dissolving in 30% acetic acid solution. The solution was filtered and lyophilized. The purification of crude peptide was performed by semipreparative RP-HPLC using a Vydac 218TP1010 column with a linear gradient of 0.1% TFA in water and 0.08% TFA in acetonitrile. Each peptide was at least 98% pure as assessed by TLC and analytical RP-HPLC. The molecular weights of the peptides were confirmed by ESI-MS (see Table 1).

Identification of Enantiomers of β-Methylcyclohexylalanine in Peptides. The acidic hydrolysate (6 N HCl, 24 h, 110 °C) of 1 mg of peptide was separated by analytical HPLC to isolate β-MeCha. The configuration of the pure amino acid was determined by chiral TLC in acetonitrile/methanol/water (4:1:1). The R_f value was compared with that of standard optical pure β-MeCha isomers.[20] GITC derivatization of the amino acid mixture obtained after hydrolysis was also used as an alternative method.[21] The derivatized amino acid mixture was separated by analytical HPLC on a Vydac 218TP54 column, and the peak of the derivatized β-MeCha was compared with those of the prepared standard β-MeCha isomers (see Supporting Information, p. S5).

Radioligand Binding Assays. Rat brain membranes were prepared from Wistar rat brain according to Simon et al.[22] The binding experiments were performed in 50 mM Tris·HCl buffer, pH 7.4, at a final volume of 1 mL containing 200−300 μg protein. In competition experiments the following conditions were used for incubations: [^{3}H]DAMGO (35 °C, 45 min), [^{3}H]Ile^{5,6}-deltorphin-2 (35 °C, 45 min).[23] Incubations were started by the addition of the membrane suspension and terminated by rapid filtration through Whatman GF/C glass fiber filters using Brandel M24R Cell Harvester. The filters were washed three times with ice cold Tris·HCl buffer and dried for 3 h at 37 °C, and the radioactivity was measured in UltimaGOLD scintillation cocktail using a Packard TriCarb 2300 TR counter. Affinities of competing ligands were determined by co-incubation with 10^{-12}−10^{-5} M freshly prepared

solution of the unlabeled peptides with 0.5−1 nM tritiated ligand. Nonspecific binding was defined as a radioactivity bound in the presence of 10 μM naloxone. All assays were performed in duplicate and repeated several times. Experimental data were analyzed by GraphPad Prism 2.01 software.[30]

GPI and MVD *In Vitro* Bioassays. The bioassays of the peptides were based on electrically induced smooth muscle contractions of MVD and GPI myenteric plexus-longitudinal muscle strips, as reported in detail elsewere.[31,32] The dose−response curve was determined with Leu-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.[33] K_e values for the δ antagonist were determined from the ratio of IC_{50} values obtained with δ agonist, DPDPE in MVD assay in the presence and absence of a fixed antagonist concentration (5 nM). K_e values of compounds for μ antagonist properties were determined in GPI assay against the μ agonist TAPP.[34]

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Supporting Information Available: ^{1}H NMR spectra of Boc-*erythro*- and *threo*-β-MeCha, 1D NOESY slices taken at the chemical shifts of H_N for Boc-*erythro* and *threo*-β-MeCha, GITC derivatization, RP-HPLC analysis of β-MeCha, and elemental analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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