Side Chain Methyl Substitution in the δ-Opioid Receptor Antagonist TIPP Has an Important Effect on the Activity Profile

Dirk Tourwé,*,† Els Mannekens,† Trang Nguyen Thi Diem,† Patricia Verheyden,† Hendrika Jaspers,† Géza Tóth,‡ Antal Péter,‖ István Kertész,‡ Gabriella Török,‖ Nga N. Chung,§ and Peter W. Schiller§

Eenheid Organische Chemie, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium, Isotope Laboratory, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary, Department of Inorganic and Analytical Chemistry, Attila József University, P.O. Box 440, H-6701 Szeged, Hungary, Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, Canada H2W1R7

Received March 9, 1998

The δ-opioid antagonist H-Tyr-Tic-Phe-Phe-OH (TIPP-OH) or its C-terminal amide analogue was systematically modified topologically by substitution of each amino acid residue by all stereoisomers of the corresponding β-methyl amino acid. The potency and selectivity (δ- vs μ- and κ-opioid receptor) were evaluated by radioreceptor binding assays. Agonist or antagonist potency were assayed in the mouse vas deferens and in the guinea pig ileum. In the TIPP analogues containing L-β-methyl amino acids the influence on δ-receptor affinity and on δ-antagonist potency is limited, the [(2*S*,3*R*)-β-MePhe^3]TIPP-OH analogue being among the most potent δ-antagonists reported. In the D-β-methyl amino acid series, the [D-β-MeTic^2] analogues are δ-selective antagonists whereas [D-Tic^2]TIPP-NH_2 is a δ-agonist. NMR studies did not indicate any influence of the β-methyl substituent on the conformation of the Tic residue. The [(2*R*,3*S*)-β-MePhe^3]TIPP-NH_2 is a potent δ-agonist, its C-terminal carboxylic acid analogue being more δ-selective but displaying partial agonism in both the δ- and μ-bioassay. These results constitute further examples of a profound influence of β-methyl substitution on the potency, selectivity, and signal transduction properties of a peptide.

Introduction[1]

The tetrapeptide H-Tyr-Tic-Phe-Phe-OH (TIPP-OH) **1** represents the prototype of a new class of potent and selective δ-opioid antagonists.[2] The δ-antagonist properties are a consequence of the conformational constraints imposed by the Tic residue. The aromatic residues at position 3 and 4 are not essential for high δ-antagonism since they can be replaced by lipophilic residues[3,4] or even omitted.[5] Several theoretical models of the receptor-bound conformation of TIP(P) peptides have been proposed and compared to the naltrexone-derived δ-antagonist naltrindole (NTI).[6] An important structural parameter in these models is the intramolecular distance between the Tyr^1 and Tic^2 aromatic rings which should be close to the distance between the corresponding aromatic rings in naltrindole. In the most plausible model, a close hydrophobic interaction (collapse) between the Tyr^1 and Tic^2 aromatic rings is prevented by intercalation of the Phe^3 aromatic ring in the tripeptide and of the Tic^2-Phe^3 peptide bond in the tetrapeptide. The Phe^3 and Phe^4 aromatic rings are then quite exposed and could engage in receptor interactions that might explain the relatively higher antagonist potencies of the tetrapeptides as compared to the tripeptides. It is clear that in such small peptides the amino acid side chains exhibit considerable conformational flexibility. Therefore, the establishment of an exact three-dimensional arrangement of the structural moieties constituting the δ-antagonist pharmacophore of TIP(P) remains a challenging problem. The use of β-methylated amino acids to constrain the conformational mobility of the side chain by biasing the population of the χ_1 torsion angle rotamers has been pioneered by V. J. Hruby.[7−10]

The effects of methylation of the β-carbon of a side chain on the biological properties of a peptide depends on the chiralities of the stereoisomers. Important effects on the receptor affinity, selectivity, and on the agonist/antagonist character have been observed in opioid peptides,[9−14] analogues of somatostatin,[15,16] α-MSH,[10,17,18] CCK,[19,20] oxytocin,[21] substance P[22] and angiotensin II,[23] and glucagon.[24]

We now report a systematic study of the effect of β-methyl substitution at each of the four amino acids of the δ-antagonist TIPP-OH and of the mixed μ-agonist/δ-antagonist TIPP-NH_2 on the opioid activity profile. In the latter case it was hoped that the β-methyl substitution might result in more balanced μ- and δ-affinities, thereby leading to new analgesics with diminished propensity to produce tolerance and dependence.[25]

Results and Discussion

Synthesis. Since this study required all stereoisomers of the β-methylated amino acids, a rapid synthetic route resulting in racemates was chosen, rather than a stereoselective synthesis for each isomer.[26]

Racemic *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-MePhe and *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-MePhe isomers were prepared as described in the literature.[9] The β-MeTic isomers were prepared by a Pictet−Spengler reaction,[21,27] starting from pure *erythro*- or *threo*-β-MePhe. For the preparation of the β-MeTyr stereoisomers, an

† Vrije Universiteit Brussel.
‡ Hungarian Academy of Sciences.
‖ Attila Jozsef University.
§ Clinical Research Institute of Montreal.

10.1021/jm981011u CCC: $15.00
Published on Web 12/01/1998

Scheme 1. Synthesis of Racemic *erythro* and *threo* Isomers of β-methyltyrosine

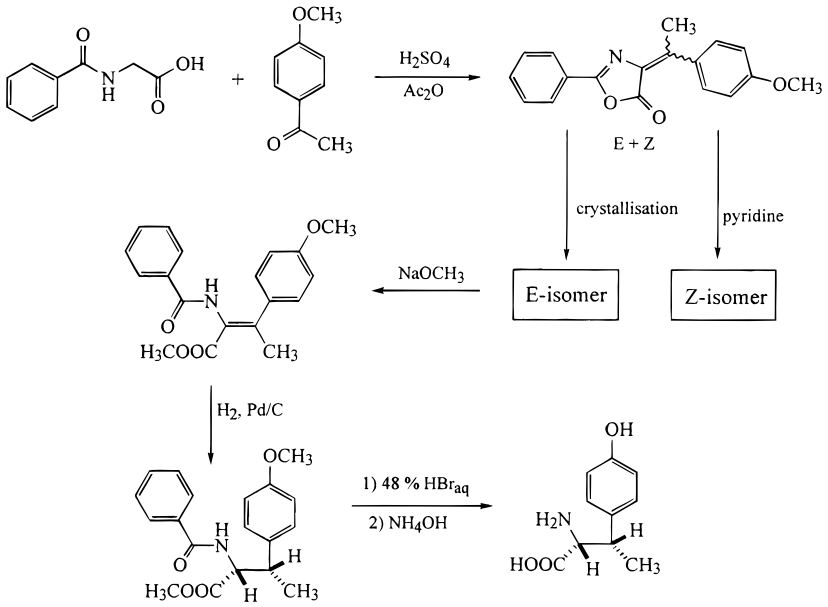

Table 1. Influence of the Configuration of Aromatic β-Methyl Amino Acids on the Population of the Side Chains

	(2*S*,3*S*)	(2*S*,3*R*)	(2*R*,3*R*)	(2*R*,3*S*)
gauche(−)	++	+	+	−
trans	−	+	−	+
gauche(+)	+	−	++	++

[a] Legend: −, strongly disfavored; +, allowed; ++, favored.

Erlenmeyer condensation between 4-methoxyacetophenone and hippuric acid was performed using a modification of the previously described protocol (Scheme 1).[28−30] From the resulting 70/30 mixture of *E*- and *Z*-oxazolone, the *E* isomer was easily isolated by crystallization.[31] The *E*-oxazolone can be isomerized to the *Z* isomer in pyridine.[28] Further catalytic hydrogenation and subsequent deprotection steps afforded the *erythro*-β-MeTyr and *threo*-β-MeTyr racemates.

After Boc protection all the β-methyl amino acids were incorporated into the TIPP analogues as racemic pairs. Peptide synthesis was performed by the solid-phase method using either a Merrifield resin or a 4-methylbenzhydrylamine resin. After cleavage from the resin by treatment with liquid HF, the resulting epimeric peptides were separated by reversed phase HPLC (Table 2). A very good separation of the L-(2*S*) from the D-(2*R*)-β-Me amino acid containing peptides was obtained. However, a systematic study had shown that the separation of epimers at the β-carbon (e.g. L-*erythro*-(2*S*, 3*S*) from L-*threo*-(2*S*, 3*R*)) can be problematic.[32] Therefore, in this synthetic strategy, special attention should be paid to the diastereomeric purity of the starting β-Me amino acids. The absolute configuration at the α-carbon of the β-Me amino acid in each peptide was determined after acid hydrolysis and derivatization of the resulting amino acids with GITC or FDAA.[32,33]

Biological Data. Affinities of the TIPP analogues for μ-opioid receptors were determined in a binding assay using rat forebrain membranes with [^{3}H] sufentanil as a μ-radioligand. Affinities for the δ-opioid receptor were measured by displacement of [^{3}H]DPDPE from NxG108CC15 cell membrane binding sites and

Table 2. Analytical Data of TIPP Analogues

		HPLC[a]		TLC[b]		
no.	peptide	R_t(min)	k'	R_{f_A}	R_{f_B}	R_{f_C}
1	TIPP-OH	14.7	3.9	0.65	0.82	0.53
2	TIPP-NH_2	12.7	3.23	0.71	0.88	0.62
3	(*SS*)-βMeTyr^1-TIPP-NH_2	15.6	4.20	0.83	0.88	0.68
4	(*SR*)-βMeTyr^1-TIPP-NH_2	12.7	3.23	0.83	0.88	0.72
12	(*RR*)-βMeTyr^1-TIPP-NH_2	15.9	4.30	0.71	0.89	0.78
13	(*RS*)-βMeTyr^1-TIPP-NH_2	10.7	2.56	0.72	0.89	0.72
5	(*SS*)-βMeTic^2-TIPP-OH	20.5	5.83	0.76	0.82	0.47
6	(*SR*)-βMeTic^2-TIPP-OH	21.1	6.03	0.75	0.82	0.41
15	(*RR*)-βMeTic^2-TIPP-OH	21.3	6.10	0.84	0.83	0.53
16	(*RS*)-βMeTic^2-TIPP-OH	24.7	7.23	0.84	0.82	0.49
7	(*SS*)-βMePhe^3-TIPP-NH_2	14.5	3.83	0.78	0.81	0.74
8	(*SR*)-βMePhe^3-TIPP-NH_2	12.7	3.23	0.78	0.81	0.71
19	(*RR*)-βMePhe^3-TIPP-NH_2	53.0	16.66	0.78	0.75	0.62
20	(*RS*)-βMePhe^3-TIPP-NH_2	47.3	14.76	0.75	0.79	0.61
9	(*SR*)-βMePhe^3-TIPP-OH	13.28[c]	3.15	0.77	0.82	0.69
21	(*RS*)-βMePhe^3-TIPP-OH	37.67[c]	10.77	0.77	0.88	0.55
10	(*SS*)-βMePhe^4-TIPP-NH_2	16.3	4.43	0.77	0.79	0.70
11	(*SR*)-βMePhe^4-TIPP-NH_2	28.5	8.5	0.76	0.76	0.67
22	(*RR*)-βMePhe^4-TIPP-NH_2	29.1	8.7	0.76	0.75	0.67
23	(*RS*)-βMePhe^4-TIPP-NH_2	12.0	3.0	0.75	0.82	0.76

[a] Retention time and capacity factor for Vydac 218 TP54 reversed phase column (25 × 0.46 cm) with 0.1% trifluoroacetic acid/acetonitrile (73:27 (v/v)). Flow rate 1 mL/min; t_o = 3 min; detection at λ = 210 nm. [b] Kieselgel GF254 (Merck plates). Solvent systems: (A) 1-butanol/acetic acid/water 4:1:1; (B) acetonitrile/methanol/water 4:1:1; (c) ethyl acetate/pyridine/acetic acid/water 12/4/1.2/2.2. [c] Composition of eluent: 0.1% aqueous TFA/acetonitrile (70/30, v/v, isocratic).

those for the κ-opioid receptor using guinea pig cerebellum membranes and [^{3}H]U69593 (Tables 3 and 4).

The L-β-Me amino acid containing TIPP analogues showed only relatively minor changes in δ-receptor affinity as compared to the parent peptides TIPP-OH or TIPP-NH_2. The effect of changing the configuration at the β-carbon on δ-receptor binding was generally found to be quite small, except in the case of the (2*S*,3*S*)-β-MeTic analogue **5** which showed 14-fold reduced δ-receptor affinity. On the other hand the μ-receptor affinity was affected to a somewhat larger extent by the configurational change at the β-carbon, particularly at the 4-position residue. This is indicated by the fact that the (2*S*,3*S*)-β-MePhe^4 analogue **10** showed a receptor

Table 3. Receptor Binding Affinities and Activities in MVD and GPI Bioassays of TIPP Analogues Containing L-β-Me Amino Acids

		receptor binding (K_i, nM)			bioassay	
no.		μ^a $N = 3-5$	δ^b $N = 3-6$	κ^c $N = 2$	GPI (IC_{50}) agonist [nM]	MVD (K_e DPDPE) antagonist [nM]
1	H-Tyr-Tic-Phe-Phe-OH	>10000	0.48 ± 0.07	>10000	>10000	4.80 ± 0.20
2	H-Tyr-Tic-Phe-Phe-NH_2	178 ± 26	0.83 ± 0.11	838 ± 116	1700 ± 220	18.0 ± 2.2
3	H-(2*S*,3*S*)-β-MeTyr-Tic-Phe-Phe-NH_2	352 ± 100	2.88 ± 0.25	>10000	>10000	102 ± 6
4	H-(2*S*,3*R*)-β-MeTyr-Tic-Phe-Phe-NH_2	284.0 ± 63.5	1.21 ± 0.06	>10000	1210 ± 260	partial agonist (max 50%) IC_{25} = 1030 ± 120
5	H-Tyr-(2*S*,3*S*)β-MeTic-Phe-Phe-OH	>10000	6.81 ± 0.31	>10000	10000	2.09 ± 0.10
6	H-Tyr-(2*S*,3*R*)-β-MeTic-Phe-Phe-OH	>10000	0.53 ± 0.08	>10000	>10000	1.61 ± 0.29
7	H-Tyr-Tic-(2*S*,3*S*)-β-MePhe-Phe-NH_2	973 ± 309	2.91 ± 0.40	3396 ± 655	5520 ± 640	18.2 ± 3.4
8	H-Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-NH_2	149 ± 31	0.66 ± 0.04	4324 ± 1978	5080 ± 700	7.24 ± 0.11
9	H-Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH	>10000	0.38 ± 0.01	>10000	>10000	0.192 ± 0.025
10	H-Tyr-Tic-Phe-(2*S*,3*S*)-β-MePhe-NH_2	47.0 ± 12.4	1.60 ± 0.18	1282 ± 87	636 ± 62	5.36 ± 1.05
11	H-Tyr-Tic-Phe-(2*S*,3*R*)-β-MePhe-NH_2	2021 ± 202	1.68 ± 0.15	253 ± 10	2740 ± 360	5.37 ± 0.84

[a] μ: [^{3}H]sufentanil (rat forebrain). [b] δ: [^{3}H]DPDPE (NxG 108 CC 15 cells). [c] κ: [^{3}H]U69596 (guinea pig cerebellum).

Table 4. Receptor Binding Affinities and Activities in MVD and GPI and Bioassays of TIPP Analogues Containing D-β-Me Amino Acids

		receptor binding (K_i, nM)			bioassay	
no.		μ^a $N = 5$	δ^b $N = 5$	κ^c $N = 2$	GPI (IC_{50}) agonist [nM]	MVD (K_e DPDPE) antagonist [nM]
12	H-(2*R*,3*R*)-β-MeTyr-Tic-Phe-Phe-NH_2	1596 ± 412	28 ± 2	1180 ± 63.5	>10000	1020 ± 150
13	H-(2*R*,3*S*)-β-MeTyr-Tic-Phe-Phe-NH_2	>10000	113 ± 14	2509 ± 300	>10000	317 ± 62
14	H-Tyr-D-Tic-Phe-Phe-NH_2[2]	7.30[d] ± 0.52	519[e] ± 46		37.1 ± 2.6	agonist: IC_{50} = 454 ± 72
15	H-Tyr-(2*R*,3*R*)-β-MeTic-Phe-Phe-OH	>10000	543 ± 60	>10000	>10000	1560 ± 120
16	H-Tyr-(2*R*,3*S*)-β-MeTic-Phe-Phe-OH	>10000	474 ± 81	>10000	>10000	401 ± 96
17	H-Tyr-Tic-D-Phe-Phe-NH_2[60]	658[d]	7.83[e]		>10000	125 ± 6
18	H-Tyr-Tic-D-Phe-Phe-OH[60]	4000[d]	6.76[e]		>10000	partial agonist (max 45%)
19	H-Tyr-Tic-(2*R*,3*R*)-β-MePhe-Phe-NH_2	>10000	74 ± 5.1	442 ± 52	>10000	54.4 ± 5.8
20	H-Tyr-Tic-(2*R*,3*S*)β-MePhe-Phe-NH_2	84 ± 21	0.50 ± 0.07	1190 ± 452	127 ± 19	agonist: IC_{50} = 1.78 ± 0.25
21	H-Tyr-Tic-(2*R*,3*S*)β-MePhe-Phe-OH	810 ± 185	0.76 ± 0.10	>10000	partial agonist (max 70.%) IC_{35} = 519 ± 62	partial agonist max 60%) IC_{30} = 0.623 ± 0.12
22	H-Tyr-Tic-Phe-(2*R*,3*R*)-β-MePhe-NH_2	498 ± 150	4.1 ± 0.3	766 ± 18	partial agonist (max 70%) IC_{35} = 1710 ± 270	20.5 ± 0.7
23	H-Tyr-Tic-Phe-(2*R*,3*S*)-β-MePhe-NH_2	>10000	11.00 ± 1.01	2844 ± 519	4510 ± 750	27.7 ± 5.5

[a] μ: [^{3}H]sufentanil. [b] δ: [^{3}H]DPDPE (NXG 108CC15 cells). [c] κ: [^{3}H]U69593 (guinea pig cerebellum). [d] μ: [3H]DAGO (rat brain, see ref 2). [e] δ: [^{3}H]DSLET (rat brain, see ref 2).

binding profile similar to that of the parent peptide **2**, whereas the epimeric (2*S*,3*R*)-β-MePhe[4] analogue **11** displayed 11-fold reduced μ-receptor affinity and, consequently, increased δ-selectivity.

In the D-β-Me amino acid containing TIPP analogues, the effects of the β-methyl substitution are much more dramatic. Substitution at Tyr[1] results in δ-selective compounds, the (2*R*,3*R*) epimer **12** being more potent than the (2*R*,3*S*) analogue **13**. In contrast to [L-Tic[2]]-TIPP-NH_2 **2** which is δ-selective, the [D-Tic[2]] analogue **14** is μ-selective.[2] The β-methyl substitutions of the D-Tic residues **15** and **16** did not appreciably affect the moderate δ-affinity observed with the parent peptide, but abolished the μ-affinity. [D-Phe[3]]TIPP-NH_2 **17** and [D-Phe[3]]TIPP-OH **18** are both approximately 10 times less potent at the δ-receptor than **1** or **2**. Whereas the (2*R*,3*R*)-β-Me-Phe[3] analogue **19** showed 10 times lower δ-affinity than its corresponding parent peptide and no μ-affinity, the epimeric (2*R*,3*S*) analogue **20** displayed subnanomolar δ-affinity. As expected, replacement of the C-terminal carboxamide function in **20** with a carboxylic acid group resulted in a compound **21** with further improved δ-receptor selectivity. The β-methyl substitutions performed at D-Phe[4] resulted in the still quite potent and very δ-selective analogues **22** and **23**.

The binding to κ-opioid receptors remains low in all cases.

The in vitro opioid activities of the TIPP analogues were tested in bioassays based on inhibition of electrically evoked contractions of the GPI and of the MVD (Tables 3 and 4). The GPI preparation contains mainly μ-receptors, whereas in the MVD assay the opioid effects are primarily mediated by δ-receptors.[34] δ-Antagonist potencies were determined against the agonist [D−Pen[2], D-Pen[5]]enkephalin (DPDPE).[35] In the L-β-Me amino acid series (Table 3), all analogues retained the δ-antagonist properties of **1** and **2**, with the exception of the (2*S*,3*R*)-β-MeTyr[1] analogue **4**, which was a partial δ-agonist. In general, the δ-antagonist potencies correlated quite well with the δ-receptor affinities determined in the binding assay. The (2*S*,3*R*)-β-MePhe[3] analogue **9** has a very high δ-antagonist potency with no μ-agonist or μ-antagonist effect at concentrations up to 10 μM. In comparison with **2**, compounds **10** and **11** are mixed μ-agonist/δ-antagonists with 3-fold increased δ-antagonist potency and with 3-fold enhanced μ-agonist potency in the case of **10** and 1.6-fold decreased μ-agonist potency in the case of **11**. Compounds **10** and **11** are less potent and less balanced than other mixed μ-agonist/δ-antagonists derived from TIPP-NH_2.[25] In the D-β-Me amino acid containing analogue series (Table 4), the β-MeTyr[1] analogues **12** and **13** were found to be weakly potent δ-antagonists, in agreement with their relatively low δ-receptor affinities. The D-β-MeTic[2] isomers **15** and **16** both turned out to be weak δ-antagonists, in contrast to compound **14** which is a δ-agonist. Very interestingly, the (2*R*,3*R*)-β-MePhe[3] analogue **19** is a moderately potent but quite selective δ-antagonist.

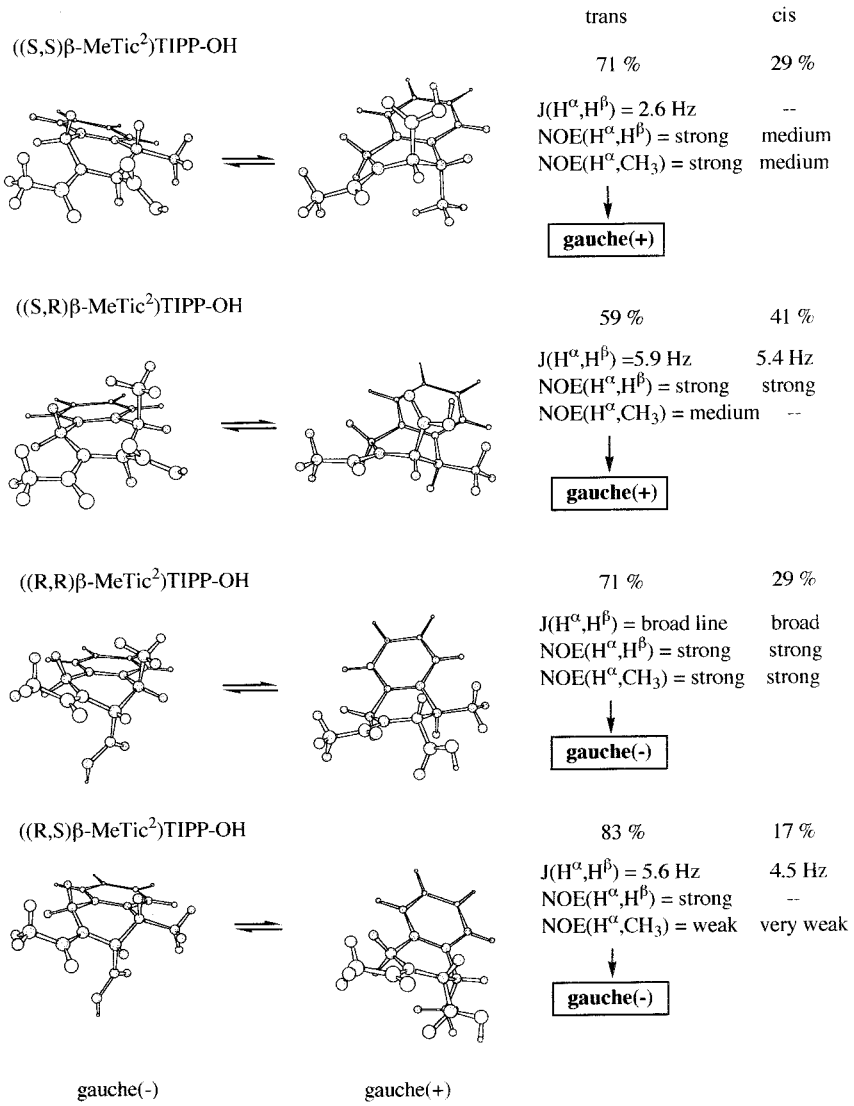

Figure 1. NMR parameters of β-MeTic protons relevant for the conformation of the β-MeTic residue in TIPP-analogues.

On the other hand, the (2*R*,3*S*) epimer **20** is a very potent full δ-agonist and a moderately potent μ-agonist. The corresponding C-terminal carboxylic acid analogue **21** is a partial agonist at both receptors. The D-β-MePhe[4] analogues **22** and **23** maintain moderate δ-antagonist potency. Whereas **23** is a very weak but full μ-agonist, the epimeric **22** is a partial μ-agonist.

Conformational Studies. Previous conformational studies on L-Tic containing peptides have indicated that the conformation of the Tic heterocyclic ring corresponds to a *gauche(+)* conformation of the side chain (*gauche(−)* for D-Tic) when the Tic residue is at an internal position in the peptide sequence.[7,36,37] The introduction of a β-methyl substituent in the (2*S*,3*R*) or (2*R*,3*S*) isomer is expected to stabilize this conformation because of its pseudoequatorial position (Figure 1). In contrast, in the (2*S*,3*S*) or (2*R*,3*R*) isomers the pseudoaxial position of the methyl in the *gauche(+)* or, respectively, *gauche(−)* configuration may shift the equilibrium toward the conformation having the two substituents in an equatorial position. Therefore, NMR studies in DMSO solution were performed on the four β-MeTic containing TIPP analogues **5, 6** and **15, 16**. As in TIPP, these four isomers all display a slow *cis*/*trans* isomerization around the Tyr-Tic peptide bond. In all cases this peptide bond in the major conformer was assigned the *trans* configuration on the basis of the characteristic Tyr C$^{\alpha}$H nuclear Overhauser effect (NOE) with Tic C^{1}H$_2$ in the *trans* or with Tic C^{3}H in the *cis* conformation.[38] The chemical shifts were assigned using two-dimensional homonuclear Hartmann−Hahn and rotating frame NOE spectroscopy. A distinction between the Phe[3] and Phe[4] signals was made on the basis of C$^{\alpha}$H−NH NOEs. The NMR parameters which are relevant for β-MeTic are presented in Figure 1. It is clear that the β-methyl substituent does not change the side chain conformation to an appreciable extent: both the vicinal coupling constants and the NOEs indicate a predominant *gauche(+)* orientation in both (2*S*)-β-MeTic[2] analogues **5** and **6** and a *gauche(−)* orientation in both (2*R*)-β-MeTic[2] analogues **15** and **16**, as observed previously for L-Tic and D-Tic containing peptides.[7,36,37]

Discussion

The intramolecular distance between the aromatic rings of Tyr[1] and Tic[2] is a crucial factor determining the δ-antagonism in TI(PP). The Phe[3] or Phe[4] residues may prevent the hydrophobic collapse of the Tyr-Tic rings and/or can engage in additional receptor interactions. The introduction of a β-methyl group on each of

the residues in the tetrapeptides may therefore have profound effects on receptor affinity, on selectivity, and on the agonist/antagonist character by biasing the side chain topology to a particular *gauche* conformation. NMR studies of CCK-8 analogues have indicated that the introduction of a β-methyl substituent in the Phe side chain virtually eliminates the *trans* χ_1-rotamer in the *erythro* isomers (2*S*,3*S* and 2*R*,3*R*) and strongly reduces the population of the *gauche(+)* rotamer in the (2*S*,3*R*) *threo* isomer and of the *gauche(−)* rotamer in the (2*R*,3*S*) *threo* isomer. Both other rotamers are populated to an appreciable extent (Table 1).[39,40] These results were confirmed by theoretical calculations, indicating that there is a small (less than 2 kcal/mol) energy preference to the χ_1-rotamers which places both C^β-substituents in a *gauche* conformation to H^α and a slight disfavor for the χ_1-rotamer that places both C^β-substituents *gauche* to the CO group.[10] Overall, substitution of L-β-methyl amino acids in TIPP had a minimal effect on δ-receptor affinity and on δ-antagonist potency. The overlap studies of TIP(P) peptides with naltrindole indicate a trans χ_1 for Tyr^1 in all models.[6] Clearly, the small differences in opioid activities seen between the two β-$MeTyr^1$ isomers **3** and **4** do not allow a conclusion with regard to the required side chain conformer. The common allowed rotamer for both the (2*S*,3*R*) and (2*S*,3*S*) isomers is *gauche(−)*, which is not consistent with the theoretical models and with the observed solution conformation for Tyr-Tic dipeptide analogues [41] and with the solid-state conformation of **1**.[42] The difference in δ-affinity is much higher in the highly constrained dipeptide β-methyl-2′,6′-dimethyltyrosine-Tic, for which a trans χ_1 is proposed.[43] For the diketopiperazine c[Tyr-Tic] a *gauche(−)* orientation for Tyr is observed in the NMR spectra and in the crystal.[44] The cyclic compound displays, however, much weaker δ-affinity and antagonism than the linear ones. The (2*S*,3*R*)-β-$MeTyr^1$ analogue **4** becomes a partial δ-agonist (max = 50%). A similar effect of β-methylation on the transduction properties of somatostatin-derived opioids has been observed and was interpreted as due to differences in geometrical arrangement of the pharmacophores.[16]

For the Tic^2 residue, β-methylation does not change the preferred *gauche(+)* orientation. The (2*S*,3*S*)-β-$MeTic^2$ analogue **5**, having the β-methyl group in the pseudoaxial orientation has, approximately 13 times less δ-affinity than the (2*S*,3*R*)-β-$MeTic^2$ analogue **6**, having the methyl group in the pseudoequatiorial position, but both have very similar δ-antagonist potency.

For Phe^3, the (2*S*,3*R*)-β-MePhe isomer **8** is more potent than its (2*S*,3*S*) analogue **7**. As expected, the receptor selectivity of **8** can be increased by changing the C-terminal amide to the carboxylic acid **9**. The high antagonist potency of **9** suggests that models having the side chain of Phe^3 in a *gauche(+)* conformation[6] are to be considered unlikely candidates of the bioactive conformation. The higher potency of the (2*S*,3*R*) epimer **8** compared to the (2*S*,3*S*) epimer **7** may indicate that a *trans* χ_1 is preferred. In the lowest energy conformation of H-Tyr-Ticψ(CH_2NH)Phe-Phe-OH, the Phe^3 takes the *trans* orientation, which makes it more exposed and accessible for interaction with the δ-receptor.[6]

Compound **9** ranks among the most potent δ-antagonists reported to date with a K_e-value (0.192 nM) comparable to that of the highly δ-selective pseudopeptide H-Tyr-Ticψ(CH_2NH)Cha-Phe-OH (K_e = 0.219 nM)[4] and to that of H-Dmt-Tic-Phe-Phe-OH (K_e = 0.152 nM).[45] In Tyr-Tic peptides, the substitution by 2′,6′-dimethyltyrosine increases antagonist potency and selectivity by several orders of magnitude.[46] The K_e for H-Dmt-Tic-OH of 5.7 nM and for the *N*,*N*-$(Me)_2$-Dmt-Tic-OH (K_e = 0.25 nM)[47] are also comparable to that of **9**, as well as their receptor selectivity. In the β-$MePhe^4$ analogues **10** and **11** the configuration at the β-carbon has no effect on δ-affinity and potency. This can be consistent with the fact that both isomers can adopt a common *gauche(−)* orientation. Since μ-affinity is affected to a larger extent, the (2*S*,3*R*)-β-$MePhe^4$ isomer **11** becomes more δ-selective.

In the D amino acid containing analogue series, some remarkable effects of the β-methyl substitution on opioid activity profile are observed. The low potency of both (2*R*,3*R*)- and (2*R*,3*S*)-β-$MeTyr^1$ analogues **12** and **13** is not surprising, since a D-Tyr^1 residue is not tolerated in opioid peptides. According to our NMR observations, the conversion of the μ-selective agonist H-Tyr-D-Tic-Phe-Phe-NH_2 into the weak δ-selective antagonists **15** and **16** cannot be explained by a change in the side chain (or ring) conformation of the Tic residue. The added methyl group therefore either causes an overall conformational change of the backbone or has an effect on the transduction mechanism. The former is rather unlikely in view of its orientation outside the cleft formed by the Tyr^1/Tic^2/Phe^3 aromatic rings, whereas precedent for the latter has been reported.[16]

A similar but stereospecific effect of the β-methyl substitution is observed in the (2*R*)-Phe^3 analogues: whereas the introduction of a β-methyl with (3*R*) configuration in [D-Phe^3]TIPP-NH_2 **17** to give analogue **19** does not change the antagonist character, the epimer with (2*R*,3*S*) configuration **20** is a very potent δ-agonist and a moderately potent μ-agonist. It is therefore tempting to speculate that the *trans* χ_1-rotamer, which is highly disfavored in the (2*R*,3*R*) isomer but allowed in the (2*R*,3*S*) isomer, is causing the effect. Very subtle effects, even remote from the essential Tyr-Tic pharmacophore can influence the antagonism: the change from amide **17** to acid **18** results in partial δ-agonism, which is maintained on (3*S*)-β-methyl substitution. In contrast the D-β-$MePhe^4$ analogues, **22** and **23** remain moderate δ-antagonists, but in **22** partial μ-agonism is observed. This observation is similar to the one observed in the L-β-$MePhe^4$ isomers **10** and **11**.

Conclusions

The introduction of a β-methyl substituent on each residue in TIPP can have important effects on receptor selectivity and on agonist/antagonist properties. Although it has been demonstrated that the dipeptides Tyr-Tic and especially Dmt-Tic have the necessary requirements for δ-antagonism, the present results demonstrate that substitutions at Phe^3 and Phe^4 can strongly influence the biological profile. Despite the fact that a single β-methyl substituent is reported not to dramatically constrain the conformational mobility of aromatic amino acids in χ-space,[10] the usefullness of

these topographically constrained amino acids for creating differences in biological activity of peptides is demonstrated. As equally stated,[10] the results of such β-methyl substitutions are not easily interpreted in terms of topographical requirements of the receptor to the side chain conformations. More bulky substituents, such as β-isopropyl, or combined substitutions, such as β-methyl-2′,6′-dimethyl, may provide additional information due to the higher energy differences between the rotamers.[10]

Experimental Section

General Methods. TLC was performed on 2.5 × 10 cm plates precoated with Merck silica gel $60F_{254}$ using the following solvent systems: (A) 1-butanol/acetic acid/water (4:1:1), (B) acetonitrile/methanol/water (4:1:1), (C) ethyl acetate/pyridine/acetic acid/water (12/4/1.2/2.2), (D) chloroform/methanol/acetic acid (90:8:2), and (E) hexane/ethyl acetate (1:1). TLC spots were detected under UV light and using iodine vapor.

Melting points were measured with a Büchi 510 apparatus. NMR spectra were obtained in either $CDCl_3$, D_2O, or D_2O/TFA on a Bruker AC 250-P 250 MHz spectrometer.

Mass spectra were recorded on a AEI/902*S* spectrometer with fast atom bombardment (FAB) ionization using xenon gas. Reversed phase HPLC (RP-HPLC) was performed on a Vydac 218TP54 analytical column (25 × 0.46 cm) or on a Vydac 218TP510 semipreparative column (25 × 1.0 cm) using UV detection at $\lambda = 210$ nm.

Synthesis of N^{α}-*tert*-Butyloxycarbonyl-*erythro*-β-methylphenylalanine and N^{α}-*tert*-Butyloxycarbonyl-*threo*-β-methylphenylalanine. The pure *erythro*-(2*S*,3*S* and 2*R*,3*R*) racemate and the pure *threo*-(2*S*,3*R* and 2*R*,3*S*) racemate were obtained by fractional crystallization [9] of the isomeric mixture prepared by the method of Kataoka.[48] A solution of 5 g (23 mmol) of *erythro*-β-methylphenylalanine hydrochoride salt or 5 g (23 mmol) of *threo*-(*S*,*R* and *R*,*S*)-β-methylphenylalanine hydrochloride salt in 60 mL of dioxane/water (2:1) is cooled in an ice bath. To this solution are added 30 mL of 1 N sodium hydroxide and 5.22 g (24 mmol) of di-*tert*-butyldicarbonate. The solution is stirred at room temperature for 25 h, whereby the pH is continuously adjusted to 7.5 with 1 N sodium hydroxide. After evaporation of the dioxane, the residue is diluted with water and the pH is adjusted to 3 with a saturated $KHSO_4$ solution under cooling in an ice bath. The aqueous phase is extracted with 3 × 100 mL portions of ethyl acetate. The combined organic phases are dried over $MgSO_4$ and evaporated. The residue is then dissolved in a minimum amount of ethyl acetate, and petroleum ether is then added slowly to give white crystalline products. N^{α}-Boc-*erythro*-β-methylphenylalanine: yield 6.42 g (75%); mp 109−112 °C (lit.[9] mp 108 °C), R_f 0.82 (B). N^{α}-Boc-*threo*-β-methylphenylalanine: yield 4.28 g (50%); mp 96−97 °C (lit.[9] mp 96 °C); R_f 0.82 (B).

Synthesis of *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-Methyltyrosine and *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-Methyltyrosine. 2-Phenyl-4-[α-*E*/*Z*-*p*-methoxyphenylethylidene]-5(4*H*)-oxazolone. A mixture of 15 g (0.08 mol) of hippuric acid and 23 mL (0.24 mol) of acetic anhydride is cooled to approximately 10 °C. Dropwise, 9 mL of sulfuric acid is added under stirring. To the yellow solution is added 12.5 g (0.09 mol) of solid *p*-methoxyacetophenone, and the reaction mixture is left stirring until room temperature is reached. The solution is then heated at 60 °C for 15 min and left stirring overnight at room temperature.

The resulting dark brown oil is poured into 150 mL of ice cold water, whereby the product precipitates. The water is decanted, and the residue is washed 3 times with ice cold water. Then, 100 mL of hot water is added under stirring. The residue is filtered, dried over P_2O_5, and dissolved in 350 mL of hot ethanol. After cooling, the yellow crystals are filtered to yield 20.37 g (83%) of product (*E*:*Z* = 70:30 as determined from the methyl signal in ^{1}H NMR).

Isolation of 2-Phenyl-4-[α-*E*-*p*-methoxyphenylethylidene]-5(4*H*)-oxazolone. The *E*/*Z* mixture of oxazolone (*E*:*Z* = 70:30) is crystallized from ethanol once again and gives the oxazolone (*E*:*Z* = 98/2) with a yield of 68%: NMR ($CDCl_3$) δ 8.08 (2H, aromatic, d, $J = 8.4$ Hz), 7.50 (5H, aromatic), 6.93 (2H, aromatic, d, $J = 8.4$ Hz), 3.85 (3H, s, OCH_3), 2.64 (3H, s, CH_3); mp 123−127 °C; R_f 0.77 (E); MS (*m*/*z*, RI) 294 ($M^+ + 1$).

2-Phenyl-4-[α-*Z*-*p*-methoxyphenylethylidene]-5(4*H*)-oxazolone. A solution of 0.3 g (1.02 mmol) of the oxazolone (*E*:*Z* = 70:30) in 15 mL of pyridine is stirred for 10 min at room temperature. The solution is poured into a beaker which contains 20 mL of 0.6 M hydrochloric acid and 30 g of ice water. The oxazolone precipitates, and the solid is filtered as quickly as possible over a layer of dicalite.

The residue is dissolved in 300 mL of hot ethanol and the dicalite is filtered off. By adding water to the solution, the oxazolone (*E*:*Z* = 4:96) crystallizes with a yield of 73% (0.22 g): NMR ($CDCl_3$) δ 8.07 (2H, aromatic, d, $J = 8.2$ Hz), 7.50 (5H, aromatic), 6.98 (2H, aromatic, d, $J = 8.2$ Hz), 3.877 (3H, s, OCH_3), 2.77 (3H, s, CH_3); mp 95−98 °C; R_f 0.77 (E); MS (*m*/*z*, RI) 294 ($M^+ + 1$).

***E*-2-benzoylamino-3-*p*-methoxyphenyl-2-butenoic Acid Methyl Ester.** To a solution of 0.125 g (5.43 mmol) of sodium in 50 mL of anhydrous methanol is added 5 g (17.06 mmol) of oxazolone (*E*:*Z* = 98/2) in portions. This mixture is stirred for 2 h at room temperature. The precipitated solid is filtered and washed with methanol. After neutralization of the filtrate with concentrated hydrochloric acid, additional material precipitates and is collected by filtration. The remaining filtrate is then evaporated in vacuo. The combined solids are crystallized from ethanol/water and a yield of 4.77 g (86%) of the *E*-isomer is obtained: NMR ($CDCl_3$) δ 7.86 (2H, aromatic, d, $J = 8.3$ Hz), 7.35 (4H, aromatic), 7.16 (2H, aromatic, d, $J = 8.7$ Hz), 6.86 (2H, aromatic, d, $J = 8.7$ Hz), 3.87 (3H, s, $COOCH_3$), 3.50 (3H, s, OCH_3), 2.16 (3H, s, CH_3); mp 147−148 °C; R_f 0.33 (E); MS (*m*/*z*, RI) 326 ($M^+ + 1$, 9), 185 (30), 105 (70).

***Z*-2-Benzoylamino-3-*p*-methyloxyphenyl-2-butenoic Acid Methyl Ester.** The synthesis was started from *Z*-oxalozone, and the procedure was the same for the *E*-oxazolone: NMR ($CDCl_3$) δ 7.86 (2H, aromatic), 7.35 (3H, aromatic), 7.16 (2H, aromatic, d, $J = 8.7$ Hz), 6.86 (2H, aromatic, d, $J = 87$ Hz), 3.8 (3H, s, $COOCH_3$), 3.5 (3H, s, OCH_3), 2.29 (3H, s, CH_3); mp 138−140 °C; R_f 0.33 (E); MS identical that of the *E* isomer.

***erythro*-*N*-Benzoyl-β-methyl-*p*-methoxy-phenylalanine Methyl Ester.** A mixture of 4.73 g (14.55 mmol) of the *E* isomer of the foregoing butenoic acid methyl ester in 190 mL of methanol and 0.4 g of Pd/C catalyst (10% type 90, Johnson Matthey, powder) is hydrogenated for 4 days at room temperature at a pressure of 50 psi in a Parr-hydrogenator. Then the catalyst is filtered over a layer of Celite, and the filtrate is evaporated in vacuo. The yellow oil is dissolved in hot ethanol and crystals appear on addition of water to the solution: yield 3.77 g (76%); NMR ($CDCl_3$) δ 7.67 (2H, aromatic, d, $J = 8.7$ Hz), 7.45 (3H, aromatic), 7.12 (2H, aromatic, d, $J = 8.6$ Hz), 6.86 (2H, aromatic, d, $J = 8.6$ Hz), 6.28 (1H, NH, d, $J = 8.1$ Hz), 5.00 (1H, Hα, dd, $J = 8.1$ and 5.2 Hz), 3.78 (3H, s, OCH_3), 3.72 (3H, s, $COOCH_3$), 3.46 (1H, m, Hβ), 1.38 (3H, CH_3, d, $J = 7.2$ Hz); mp 80−83 °C; R_f 0.42 (E); MS (*m*/*z*, RI) 328($M^+ + 1$, 15), 268 (13), 135 (19), 105 (100).

***threo*-*N*-Benzoyl-β-methyl-*p*-methoxy-phenylalanine Methyl Ester.** A mixture of the foregoing butenoic acid methyl esters (*Z*:*E* = 80:20) was submitted to the hydrogenation as for the *E* isomer: NMR ($CDCl_3$) (the spectrum contains additional signals of the *erythro* isomer as described above, for about 20%) δ 7.67 (2H, aromatic, d, $J = 8.7$ Hz), 7.45 (3H, aromatic), 7.12 (2H, aromatic, d, $J = 8.6$ Hz), 6.86 (2H, aromatic, d, $J = 8.6$ Hz), 6.54 (1H, NH, d, $J = 8.2$ Hz), 4.96 (1H, Hα, d, $J = 5.2$ Hz), 3.78 (3H, s, OCH_3), 3.72 (3H, s, $COOCH_3$), 3.46 (1H, m, Hβ), 1.43 (3H, CH_3, d, $J = 7.3$ Hz); mp 125−128 °C; R_f 0.42 (E); MS identical to that of the *erythro* isomer.

***erythro*-β-Methyltyrosine.** To a solution of 5.2 mL of 48% HBr (aqueous) and 10.4 mL of acetic acid is added 1 g (3 mmol) of *erythro*-*N*-benzoyl-β-methyl-*p*-methoxy-phenylalanine meth-

yl ester. The solution is refluxed for 24 h and evaporated in vacuo. The resulting oil is dissolved in water and evaporated again. To the residue is added 5 mL of water, and the flask is placed in a refrigerator. Purple-colored crystals of benzoic acid are filtered, and the remaining solution is extracted with ethyl acetate. The aqueous phase is adjusted to pH 6 with ammonium hydroxide, and *erythro*-β-methyltyrosine precipitates. The precipitate is filtered, and a yield of 0.361 g (58%) is obtained after recrystallization from hot water: NMR (D_2O/TFA) δ 13.6 (1H, s, COOH), 6.75 (2H, aromatic, d, J = 8.6 Hz), 6.43 (2H, aromatic, d, J = 8.6 Hz), 3.65 (1H, Hα, d, J = 7.2 Hz), 3.07 (1H, Hβ, m), 0.94 (3H, CH_3, d, J = 7.2 Hz); mp 228−231 °C; R_f 0.47 (A); MS (*m*/*z*, RI) 196 ($M^+ + 1$, 50), 110 (50).

***threo*-β-Methyltyrosine.** *Threo*-*N*-Benzoyl-β-methyl-*p*-methoxy-phenylalanine methyl ester was hydrolyzed in 48% HBr (aqueous) and acetic acid as above. Any *erythro* isomer was removed by chromatography on a Lobar (Merck) LiChroprep RP8 (40-63) column, using 1% aqueous methanol as eluent: NMR (D_2O/TFA) δ 13.6 (1H, s, COOH), 6.81 (2H, aromatic, d, J = 8.6 Hz), 6.43 (2H, aromatic, d, J = 8.6 Hz), 4.11 (1H, Hα, d, J = 7.2 Hz), 3.37 (1H, Hβ, m), 1.31 (3H, CH_3, d, J = 7.0 Hz); mp 228−231 °C; *Rf* 0.47 (A); MS identical to that of the *erythro* isomer.

N^{α}-*tert*-Butyloxycarbonyl-*erythro*-β-methyltyrosine and N^{α}-*tert*-Butyloxycarbonyl-*threo*-β-methyltyrosine. A solution of 1.41 g (5.13 mmol) of *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methyltyrosine or 1.41 g (5.13 mmol) of *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methyltyrosine in 30 mL of dioxane/water (2:1) is cooled in an ice bath. To this solution is added a 4 N sodium hydroxide solution under stirring until a pH of 10 is reached and then 1.3 mL (1.1 equiv) of Boc_2O is added. For 2 h this solution is stirred at 0 °C while the pH is continuously adjusted to 10. Then another 0.4 mL of Boc_2O is added, and the mixture is stirred overnight at room temperature.

After evaporation of the dioxane, the residue is diluted with a few milliliters of water and the pH is adjusted to 2−3 with a saturated $KHSO_4$ solution under ice cooling. The aqueous layer is then extracted 3 times with 50 mL of ethyl acetate. The combined organic phases are washed with water, a saturated NaCl solution, and again with water. After drying over $MgSO_4$ and evaporation in vacuo, the oil is diluted in a minimum amount of ethyl acetate and slowly added to 130 mL of ice-cooled petroleum ether with vigorous stirring. A white precipitate is formed, filtered, and immediately dried in vacuo over P_2O_5: yield 1.40 g (92.3%) of N^{α}-Boc-*erythro*-β-methyltyrosine or 1.06 g (70%) of N^{α}-Boc-*threo*-β-methyltyrosine.

N^{α}-Boc-*erythro*-β-methyl-Tyr: NMR (DMSO-d_6) δ 12.55 (1H, s, COOH), 9.17 (1H, s, OH), 7.06 (2H, aromatic, d, J = 8.3 Hz), 6.64 (2H, aromatic, d, J = 8.3 Hz), 6.55 (1H, NH, d, J = 8.6 Hz), 3.98 (1H, Hα, dd, $J_1 \sim J_2$ = 8.6 Hz), 2.94 (1H, Hβ, m), 1.33 (9H, s, *t*-Bu), 1.13 (3H, CH_3, d, J = 7.0 Hz); mp 79−82 °C; R_f 0.80 (A); MS (*m*/*z*, RI) 296 ($M^+ + 1$, 40), 240 (60),-196 (55).

N^{α}-Boc-*threo*-β-methyl-Tyr: NMR (DMSO-d_6) δ 12.35 (1H, s, COOH), 9.16 (1H, s, OH), 7.26 (2H, aromatic, d, J = 8.4 Hz), 7.16 (1H, NH, d, J = 8.7 Hz), 7.00 (2H, aromatic, d, J = 8.4 Hz), 4.08 (1H, Hα, dd, $J_1 \sim J_2$ = 8.7 Hz), 3.22 (1H, Hβ, m), 1.33 (9H, s, *t*-Bu), 1.15 (3H, CH3, d, J = 7.1 Hz); mp 87−90 °C; R_f 0.80 (A); MS (*m*/*z*, RI) 296 ($M^+ + 1$, 40), 240 (100), 194 (70), 185 (90).

***erythro*- and *threo*-β-Me-Tic.** Pure *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-MePhe·HCl (3.15 g) was suspended in 20 mL of concentrated HCl, 5 mL of a formaldehyde solution (37%) was added, and the mixture was refluxed for 30 min at 110 °C. Then 5 mL of concentrated HCl and 2.5 mL of formaldehyde solution were added to the reaction mixture, which was further refluxed for an additional hour. The liquid was evaporated and the remaining solid was crystallized from water. *erythro*-β-Me-Tic·HCl: yield 2.4 g (72.2%); NMR (D_2O) δ 7.57−7.5 (4H, aromatic, m), 4.53 (2H, H_1, d, J = 18 Hz), 3.89 (1H, H_3, m), 3.54 (1H, H_4, m), 1.59 (CH_3, d, J = 7.5 Hz); mp 278−281 °C; R_f 0.47 (A); MS (*m*/*z*) 192 ($M^+ + 1$, 100). *Threo*-β-Me-Tic was prepared in the same way starting with 2 g of *threo*/*erythro* (8:2) β-Me-Phe: yield 1.60 (66%) g of *threo*-β-Me-Tic·HCl (10% *erythro*); NMR (D_2O) δ 7.52−7.35 (4H, aromatic, m), 4.58 (2H, H_1, d, J = 18 Hz), 4.47 (1H, H_3, m), 3.74 (1H, H_4, m), 1.39 (CH_3, d, J = 7.5 Hz); mp 253−257 °C; R_f 0.45 (A); MS (*m*/*z*) 192 ($M^+ + 1$, 100).

N^{α}-Boc-*erythro*- and *threo*-β-Me-Tic. The procedure described above for the preparation Boc-*erythro*- or *threo*-β-Me-Phe was also used for the synthesis of *erythro*- or *threo*-β-Me-Tic, starting with 1 g of amino acids. N^{α}-Boc-*erythro*-β-Me-Tic: yield 1.00 g (66.3%); NMR ($CDCl_3$) (the spectrum shows signals of the *cis*−*trans* isomers around the *N*−Boc bond in a ration of 43/57) (7.2−7.0 (4H, aromatic, m), 4.9−4.45 (4H, H_1, H_3, m), 3.45 (0.57 H, H_4, m), 3.25 (0.43 H, H_4, m), 1.57 and 1.43 (9H, *t*-Bu, s), 1.27 (3H, CH_3, d, J = 9 Hz); mp 80−82 °C; R_f 0.48 (D); MS (*m*/*z*) 292 ($M^+ + 1$, 86), 236 (100) and 192 (98). Yield 0.84 g (55.7%) N^{α}-Boc-*threo*-β-Me-Tic: NMR ($CDCl_3$) δ 7.25−7.05 (4H, aromatic, m), 4.85−4.40 (3H, H_1,H_3, m), 3.30 (1H, H_4, quintuplet, J = 6.8 Hz), 1.47−1.27 (12H, *t*-Bu, CH_3); mp 149 °C; *Rf* 0.48 (D); MS identical to the one of the *erythro* isomer.

General Methods for the Synthesis of TIPP Analogues. All the peptide analogues of TIPP were synthesized by the solid-phase method using a semiautomatic Labortec SP640 synthesizer. Chloromethylated (1.7 mmol/g resin) polystyrene resin 1% cross-linked with divinylbenzene (Fluka) was used for the C-terminal carboxylic acid analogues; 4-methylbenzhydrylamine (0.53 mmol/g resin) polystyrene resin (Neosystem, Strasbourg, France) was used for the C-terminal amides. N^{α}-Boc-protected amino acids were obtained from Neosystem. The N^{α}-*tert*-butyloxycarbonyl-phenylalanine was attached to the Merrifield resin by the Gisin method.[49] Removal of the Boc protecting group was performed by washing with a TFA/DCM/anisole (33:65:2) solution during 5 and 20 min, followed by DCM washes (4 × 0.5 min), a neutralization with 20% DIEA (3 × 1 min), and DCM washes (4 × 0.5 min). Amino acids were coupled in 1.5 (β-Me amino acids) to 2-fold (Phe, Tic, Tyr) excess using either diisopropylcarbodiimide/HOBt or BOP/HOBt/DIEA. Completion of the coupling reactions was monitored by the ninhydrin [50] or chloranil test.[51] Since important amounts of Tyr^1-deletion peptides were detected after purification, Boc-Tyr-OH or Boc-Tyr(2,6-Cl_2Bzl)-OH were used in either single or double couplings, without, however, any significant improvement. The peptides were cleaved from the resin with anhydrous HF (5 mL/g resin) with anisole added as scavenger (1 mL/g resin) for 1 h at 0 °C. The peptide was extracted from the resin by washing with anhydrous ethyl ether and then stirring of the resin in 60 mL of a 50% aqueous solution of acetic acid for 0.5 h.

The filtrate was lyophilized. Purity was determined by analytical RP-HPLC. Purification and separation of the diastereomeric peptides was performed by preparative RP-HPLC (Gilson 712 HPLC system) using an acetonitrile/water system with 0.1% TFA as eluent. The $(M + 1)^+$ molecular ions and fragmentation patterns were obtained by FAB-MS and were in agreement with the calculated molecular weights for each peptide (*m*/*z* = 648 for peptide amides, 649 for peptide acids). Yields varied from 2 to 12% for each isomer, and in almost every peptide synthesis a similar amount of tyrosine-deletion peptide isomers was isolated.

Identification of Enantiomers of β-Methyl Amino Acids in Peptides.[32,33] Identification of enantiomers of β-methyl amino acids in peptides involved three steps: acid hydrolysis of peptides, derivatization of the resulting amino acids with chiral derivatizing reagent GITC or FDAA, and separation and identification of derivatized amino acids by HPLC. The separation of derivatized amino acids was carried out on a Vydac 218TP54 C_{18} column. The peaks of derivatized β-methyl amino acids were identified by application of standards of (2*R*,3*S*)- and (2R,3*R*)-β-methyl amino acids, obtained by L-AA oxidase digestion of the racemic *erythro* or *threo* isomers.

Radioligand Binding Methods. μ-Opioid Receptor Assay.[52] Wistar rats (150−200 g) are sacrificed, the brain is removed and forebrain is dissected. The forebrain is homogenized in 10 volumes (v/w) of homogenization buffer (0.25 M sucrose, 85.5 g/L) using a Dual homogenizer. The homogenate is centrifuged at 0−4 °C for 10 min at 350g (2000 rpm), the supernatant is carefully removed and the pellet is homogenized again in 5 volumes (v/w) of buffer and centrifuged at 830*g* (3000 rpm) for 10 min. The combined supernatants are diluted to the double volume with incubation buffer (50 mM Tris-HCl, pH 7.4, 6 g/L, containing 5 mM $MgCl_2$, 5 mM EGTA, 0.1 mM toluenesulfonyl fluoride, 0.1 mM bacitracin, and 0.1% bovine albumin) and centrifuged at 23500*g* (16000 rpm) for 20 min. The pellet is rehomogenized in fresh incubation buffer, incubated at 37 °C for 30 min, and then centrifuged, and the resulting pellet is suspended in 200 volumes of (v/w) incubation buffer using a Dual homogenizer. Radioligand binding inhibition assay samples consisted of 0.4 mL of rat forebrain membrane suspension, 0.05 mL of [^{3}H]sufentanil (0.5 mM in 10% DMSO, specific activity 15 Ci/mmol, Janssen Research Foundation, Belgium), and 0.05 mL of solvent (10% DMSO), peptide analogue or blank. The blank consisted of dextromoramide (1.1×10^{-3} M in DMSO stock solution, diluted 1/50 into 10% DMSO). Peptide analogues were assayed over a concentration range of 0.3 mM to 10 μM. Incubations were performed at 25 °C for 60 min, after which 5 mL cold Tris-HCl buffer was added and the samples were filtered through GF/B glass fiber filters, presoaked in 0.1% poly(ethyleneimine) for 1 h. The filters were washed two times with 5 mL of Tris-HCl buffer and transferred to scintillation vials. The radioactivity was measured using a Tri Carb 1500 liquid scintillation analyzer after adding 2 mL of Ultima Gold (Packard) scintillation fluid, shaking for 5 min, and allowing the samples to equilibrate over 12 h.

Inhibition curves were plotted and were analyzed by least-squares regression.

δ-Opioid Receptor Assay.[53] NXG108CC15 cells were cultured in DMEM medium, supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, antibiotics (100 μU/mL penicillin and 0.1 mg/mL streptomycin), 0.1 mM hypoxantine, 0.001 mM aminopteridine, and 0.016 mM thymidine. After at least two passages, cells are scraped off carefully and centrifuged at 200*g* (1500 rpm) in a Sorvall RC-5B s-centrifuge. The cells are washed once with 0.9% saline, and the cell pellet is suspended in 0.9% saline in a concentration of 10^7 cells/mL, which is divided into 2 mL aliquots and stored at −80 °C for several weeks. For the receptor binding experiments, a vial is thawed and homogenized in incubation buffer (see μ-receptor assay). Radioligand binding inhibition assay samples consisted of 0.4 mL of cell suspension, containing 5×10^5 cells, 0.05 mL of [^{3}H]DPDPE (2 nM, specific activity 30−32 Ci/mmol, New England Nuclear, Boston, MA), and 0.05 mL of solvent (10% DMSO), peptide analogue or blank. The blank consisted of naltrindole (1 μM). Assay conditions and analysis were as described for the μ-receptor assay.

κ-Opioid Receptor Assay.[54] Guinea pigs (200−250 g) are sacrificed by decapitation, the brain is removed, and cerebellum is dissected. The cerebellum is homogenized in 10 volumes (v/w) Tris-HCl buffer (50 mM, pH 7.4, 6.06 g/L) using an Ultra Turrax homogenizer. The homogenate is centrifuged at 0−4 °C at 23500*g* (16000 rpm) in a Sorvall RC-5B centrifuge. The pellet is washed twice by rehomogenization and recentrifugation. Finally, the pellet is suspended in 100 v/w incubation buffer (see μ-receptor assay) using a Dual homogenizer. Radioligand binding inhibition assay samples consisted of 0.4 mL of guinea pig cerebellum membrane suspension, 0.05 mL of [^{3}H]U69593 (0.5 mM in 10% DMSO, specific activity 60−65 Ci/mmol, Amersham), and 0.05 mL of solvent (10% DMSO), peptide analogue or blank. The blank consisted of spiradoline (1 μM in 10% DMSO). Assay conditions and analysis were as described for the μ-receptor.

GPI and MVD Bioassays. In vitro opioid activities of the compounds were tested in the GPI [55] and MVD[56] bioassays as reported in detail elsewere.[57,58] A log dose response curve was determined with [Leu5]enkephalin as standard for each ileum and vas deferens preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure.[59] K_e values for the antagonists were determined from the ratio of IC_{50} values obtained in the presence and absence of a fixed antagonist concentration, as described in the literature.[34]

Acknowledgment. Supported by grants (to P.W.S.) from the Medical Research Council of Canada (MI-5655) and the National Institutes of Health (DA-04443) and by grants (to D.T., G.T., and A.P) of Copernicus (CIPA-CT94-0226), Flemish-Hungarian, Bilateral Intergovernmental S&T Cooperation B-3/96. Dr. J. E. Leysen and W. Gommeren (Janssen Research Foundation, Belgium) are thanked for the receptor binding assays.

References

(1) Abbreviations and definitions are those recommended by IUPAC-IUB Commission on Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977−983). All optically active amino acids are of L configuration unless otherwise noted. Other abbreviations used are Tic, tetrahydroisoquinoline-3-carboxylic acid; TFA, trifluoroacetic acid; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HOBt, 1-hydroxybenzotriazole; BOP, benzotriazole-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate; GITC, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylisothiocyanate; FDAA 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; DPDPE, Tyr-D−Pen-Gly-Phe-D-Pen-OH; DAGO, Tyr-D-Ala-Gly-NMe-Phe-Gly-ol; U69593, (5*a*,7*a*,8*a*)-(−)-*N*-methyl-[7-1-pyrrolidinyl)-1-oxa spiro-[4,5]dec-8-yl]benzeneacetamide; MVD, mouse vas deferens; GPI, guinea pig ileum, HPLC, high-performance liquid chromatography, TLC, thin layer chromatography.

(2) Schiller, P. W.; Nguyen, T. M.-D.; Weltrowska, G.; Wilkes, B. C.; Marsden, B. J.; Lemieux, C.; Chung, N. N. 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. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11871−11875.

(3) Mosberg, H. I.; Omnaas, J. R.; Sobczyk-Kojiro, K.; Dua, R.; Ho, J. C.; Ma, W.; Bush, P.; Mousigian, C.; Lomize, A. Pharmacophore elements of the TIPP class of delta opioid receptor antagonists. *Lett. Pept. Sci.* **1994**, *1*, 69−72.

(4) Schiller, P. W.; Weltrowska, G.; Nguyen, T. M.-D.; Lemieux, C.; Chung, N. N.; Zelent, B.; Wilkes, B. C.; Carpenter, K. A. Structure-agonist/antagonist activity relationships of TIPP analogs. In *Peptides: Chemistry, Structure and Biology*, Kaumaya, P. T. P., Hodges, R. S., Eds., Mayflower: Kingswinford, England, 1996; pp 609−611.

(5) Temussi, P. A.; Salvadori, S.; Amodeo, P.; Bianchi, C.; Guerrini, R.; Tomatis, R.; Lazarus, L. H.; Picone, D.; Tancredi, T. Selective opioid dipeptides. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 933−939.

(6) Wilkes, B. C.; Schiller, P. W. Comparative analysis of various proposed models of the receptor-bound conformation of H-Tyr-Tic-Phe-OH related δ-opioid antagonists. *Biopolym. (Pept. Sci.)* **1995**, *37*, 391−400.

(7) Hruby, V. J.; Al-Obeidi, F.; Kazmierski, W. Emerging approaches in the molecular design of receptor-selective peptide ligands: conformational, topographical and dynamic considerations. *Biochem. J.* **1990**, *268*, 249−262.

(8) Dharanipragada, R.; Nicolas, E.; Tóth, G.; Hruby, V. J. Asymmetric synthesis of unusual amino acids: synthesis of optically pure isomers of β-methylphenylalanine. *Tetrahedron Lett.* **1989**, *49*, 6841−6844.

(9) Hruby, V. J.; Tóth, G.; Gehrig, C. A.; Kao, L.-F.; Knapp, R.; Lui, G. K.; Yamamura, H. I.; Kramer, T. H.; Davis, P.; Burks, T. F. Topographically designed analogues of [D-Pen^2, D-Pen^5]enkephalin. *J. Med. Chem.* **1991**, *34*, 1823−1830.

(10) Hruby, V. J.; Li, G.; Haskell-Luevano, C.; Shenderovich, M. Design of peptides, proteins, and peptidomimetics in chi space. *Biopolym. (Pept. Sci.)* **1997**, *43*, 219−266.

(11) Tóth, G.; Péter, A.; Tourwé, D.; Jaspers, H.; Verheyden P. M. F.; Tóth, Z. New analogues of deltorphin C and enkephalin-Val^{5,6}-Gly^7-NH_2 with replacement of Phe by βMePhe. In *Peptides 1994*; Maia, H. L. S., Ed., ESCOM: Leiden, **1995**; pp 335−336.

(12) Misicka, A.; Cavagnero, S.; Horvath, R.; Davis, P.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. Synthesis and biological properties of β-MePhe^3 analogues of deltorphin I and dermenkephalin: influence of biased χ^1 of Phe^3 residues on peptide recognition for δ-opioid receptors. *J. Peptide Res.* **1997**, *50*, 48−54.

(13) Mosberg, H. I.; Omnaas, J. R.; Lomize, A.; Heyl, D. L.; Nordan, I.; Mousigian, C.; Davis, P.; Porreca, F. Development of a model for the δ opioid receptor pharmacophore. 2. Conformationally restricted Phe^3 replacements in the cyclic δ receptor selective tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13). *J. Med. Chem.* **1994**, *37*, 4384−4391.

(14) Shenderovich, M. D.; Köver, K. E.; Nikiforovich, G. V.; Jiao, D.; Hruby, V. J. Conformational analysis of β-methyl-para-nitrophenylalanine stereoisomers of cyclo[D-Pen^2, D-Pen^5]enkephalin by NMR spectroscopy and conformational energy calculations. *Biopolymers* **1996**, *38*, 141−156.

(15) Huang, Z.; He, Y.-B.; Raynor, K.; Tallent, M.; Reisine, T.; Goodman, M. Main chain and side chain chiral methylated somatostatin analogs: syntheses and conformational analyses. *J. Am. Chem. Soc.* **1992**, *114*, 9390−9401.

(16) Bonner, G. G.; Davis, P.; Stropova, D.; Ferguson, R.; Yamamura, H. I.; Porreca, F.; Hruby, V. J. Opioid peptides: simultaneous δ agonism and μ antagonism in somatostatin analogues. *Peptides* **1997**, *18*, 93−100.

(17) Haskell-Luevano, C.; Boteju, L. W.; Miwa, H.; Dickinson, C.; Gantz, I.; Yamada, T.; Hadley, M. E.; Hruby, V. J. Topographical modification of melanotropin peptide analogues with β-methyltryptophan isomers at position 9 leads to differential potencies and prolonged biological activities. *J. Med. Chem.* **1995**, *38*, 4720−4729.

(18) Haskell-Luevano, C.; Toth, K.; Boteju, L.; Job, C.; Castrucci, A. M. de L.; Hadley, M. E.; Hruby, V. J. β-Methylation of the Phe^7 and Trp^9 melanotropin side chain pharmacophores affects ligand−receptor interactions and prolonged biological activity. *J. Med. Chem.* **1997**, *40*, 2740−2749.

(19) Hruby, V. J.; Fang, S.; Tóth, G.; Matsunaga, T. O.; Collins, N.; Knapp, R.; Yamamura, H. I. Highly potent and selective cholecystokinin analogues for the CCK-B receptor. In *Peptides 1990;* Giralt, E., Andreu, D., Eds.; ESCOM: Leiden, 1995; pp 707−709.

(20) Boteju, W.; Nikiforovich, G. V.; Haskell-Luevano, C.; Fang, S.-N.; Zalewska, T.; Stropova, D.; Yamamura, H. I.; Hruby, V. J. The use of topographical constraints in receptor mapping: investigation of the topographic requirements of the tryptophan 30 residue for receptor binding of Asp-Tyr-D-Phe-Gly-Trp-(N-Me)Nle-Asp-Phe-NH_2 (SNF 9007), a cholecystokinin (26−33) analogue that binds to both CCK-B and δ-opioid receptors. *J. Med. Chem.* **1996**, *39*, 4120−4124.

(21) Lebl, M.; Tóth, G.; Slaninova, J.; Hruby, V. J. Conformationally biased analogs of oxytocin. *Int. J. Pept. Protein Res.* **1992**, *40*, 148−151.

(22) Birney, D. M.; Cole, D. C.; Crosson, C. E.; Kahl, B. F.; Neff, B. W.; Reid, T. W.; Ren, K.; Walkup, R. D. Use of β-methylphenylalanine (βMeF) residues to probe the nature of the interaction of Substance P with its receptor: effects of βMeF-containing Substance P analogs on rabbit iris smooth muscle contraction. *J. Med. Chem.* **1995**, *38*, 2478−2482.

(23) Samanen, J.; Narindray, D.; Cash, T.; Brandeis, E.; Adams, W.; Yellin, T.; Eggleston, D.; DeBrosse, C.; Regoli, D. Potent angiotensin II antagonists with non-β-branched amino acids in position 5. *J. Med. Chem.* **1989**, *32*, 466−472.

(24) Azizeh, B. Y.; Shenderovich, M. D.; Triverdi, D.; Li, G.; Sturm, N. S. and Hruby, V. J. Topographical amino acid substitution in position 10 of glucagon leads to antagonists/partial agonists with greater binding differences. *J. Med. Chem.* **1996**, *39*, 2449−2455.

(25) Schiller, P. W., Weltrowska, G.; Schmidt, R.; Nguyen, T. M. D.; Berezowska, I.; Lemieux, C.; Chung, N. N.; Carpenter, K. A.; Wilkes, B. C. Four different types of opioid peptides with mixed μ agonist/δ antagonist properties. *Analgesia* **1995**, *1*, 703−706.

(26) Pasto, M.; Moyano, A.; Pericas, M. A.; Riera, A. A catalytic asymmetric synthesis of *N*-Boc-β-Methylphenylalanines. *J. Org. Chem.* **1997**, *62*, 8425−8431.

(27) Pictet, A.; Spengler, T. Über die Bildung von Isochinolinderivaten durch Einwirkung von Methylal auf Phenyl-äthylamin, Phenyl-alanin und Tyrosin. (About the formation of isoquinoline derivatives by reaction of methylol with phenylethylamine, phenylalanine and tyrosine.) *Chem. Ber.* **1911**, *44*, 2030−2046.

(28) Cativiela, C.; Meléndez, E. Geometric isomers of 2-phenyl-4-(α-arylethylidene)-5(4H)-oxazolones. *Synthesis* **1978**, 832−834.

(29) Cativiela, C.; Meléndez, E. Stereospecific synthesis of (*Z*/*E*)-isomers of 2-benzoylamino-3-phenyl-2-butenoic acids. *Synthesis* **1980**, 901−902.

(30) Cativiela, C.; Meléndez, E. Stereospecific synthesis of 2-amino-3-arylbutanoic acids from 2-phenyl-4-(1-arylethylidene)-5-oxo-4,5-dihydro-1,3-oxazoles. *Synthesis* **1981**, 805−807.

(31) Mannekens, E.; Tourwé, D.; Vanderstichele, S.; Nguyen Thi Diem, T.; Tóth, G.; Péter, A.; Chung, N. N.; Schiller, P. W. Synthesis of the diastereomers of β-Me-Tyr and β-Me-Phe and their effect on the biological properties of the delta opioid receptor antagonist TIPP. *Lett. Pept. Sci.* **1995**, *2*, 190−192.

(32) Péter A.; Tóth, G.; Török, G.; Tourwé, D. Separation of enantiomeric β-methyl amino acids and of β-methyl amino acid containing peptides. *J. Chromatogr. A*, **1996**, *728*, 455−465.

(33) Péter, A.; Tóth, G.; Cserpan, E.; Tourwé, D. Monitoring of optical isomers of β-methylphenylalanine in opioid peptides. *J. Chromatogr. A* **1994**, *660*, 283−291.

(34) Kosterlitz, H. W.; Watt, A. J. Kinetic parameters of narcotic agonist, with particular reference to N-allylnoroxymorphane (naloxone*). Br. J. Pharm. Chemother.* **1968**, *33*, 266−276.

(35) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. Bis-penicillamine enkephalins possess highly improved specificity towards δ opioid receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871−5874.

(36) Kazmierski, W. M.; Yamamura, H. I.; Hruby, V. J. Topographic design of peptide neurotransmitters and hormones on stable backbone templates: relation of conformation and dynamics to bioactivity. *J. Am. Chem. Soc.* **1991**, *113*, 2275−2283.

(37) Valle, G.; Kazmierski, W. M.; Crisma, M.; Bonora, G. M.; Toniolo, C.; Hruby, V. J. Constrained phenylalanine analogues. Preferred conformation of 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) residue. *Int. J. Pept. Protein Res.* **1992**, *40*, 222−232.

(38) Wüthrich, K. In *NMR of Proteins and Nucleic Acids*; J. Wiley: New York, **1986**; pp 123−125.

(39) Kövér, K. E.; Jiao, D.; Fang, S.; Hruby, V. J. Conformational analysis of four β-methylphenylalanine stereoisomers in a bioactive peptide by Z-filtered relay NMR spectroscopy. *Magn. Reson. Chem.* **1993**, *31*, 1072−1076.

(40) Köver, K. E.; Jiao, D.; Fang, S.; Hruby, V. J. Conformational properties of the unnatural amino acid β-methylphenylalanine in a linear octapeptide system; correlations of ^{13}C-NMR chemical shifts with the side chain stereochemistry of these amino acid residues. *J. Org. Chem.* **1994**, *59*, 991−998.

(41) Carpenter, K. A.; Wilkes, B. C.; Weltrowska, G.; Schiller, P. W. Role of hydrophobic substituents in the interaction of opioid Tyr-Tic dipeptide analogs with dodecylphosphocholine micelles. *Eur. J. Biochem.* **1996**, *241*, 756−764.

(42) Flippen-Anderson, J. L.; George, C.; Deschamps, J. R.; Reddy P. A.; Lewin, A. H.; Brine, G. A. X-ray structures of the δ opioid antagonist TIPP and a protected derivative of the δ opioid antagonist ICI 174,864. *Lett. Pept. Sci.* **1994**, *1*, 107−115.

(43) Liao, S.; Lin, J.; Shenderovich, M. D.; Han, Y.; Hasohata, K.; Davis, P.; Qiu, W.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. The stereochemical requirements of the novel δ-opioid selective dipeptide antagonist TMT-Tic. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 3049−3052.

(44) Ciajolo, M. R.; Balboni, G.; Picone, D.; Salvadori, S.; Tancredi, T.; Temussi, P. A.; Tuzi, A. Solution and solid-state structure of the diketopiperazine of tyrosyl-tetrahydroisoquinoline-3-carboxylic acid. *Int. J. Peptide Protein Res.* **1995**, *46*, 134−138.

(45) Schiller, P. W.; Nguyen, T. M.-D.; Weltrowska, G.; Wilkes, B. C.; Marsden, B. J.; Schmidt, R.; Lemieux, C.; Chung, N. N. TIPP Opioid peptides: development of extraordinarily potent and selective δ antagonists and observation of astonishing structure-intrinsic activity relationships. In *Peptides: Chemistry, Structure and Biology*, Hodges, R. S., Smith, J. A., Eds.; ESCOM: Leiden, **1994**; pp 483−486.

(46) Bryant, S. D.; Salvadori, S.; Cooper, P. S.; Lazarus, L. H. New δ-opioid antagonists as pharmacological probes. *Trends. Pharmacol. Sci.* **1998**, *19*, 42−46.

(47) Salvadori, S.; Balboni, G.; Guerrini, R.; Tomatis, R.; Bianchi, C.; Bryant, S. D.; Cooper, P. S.; Lazarus, L. H. Evolution of the Dmt-Tic pharmacophore: N-terminal methylated derivatives with extraordinary δ opioid antagonist activity. *J. Med. Chem.* **1997**, *40*, 3100−3108.

(48) Kataoka, Y.; Seto, Y.; Yamamoto, M.; Yamado, T.; Kuwata, S.; Watanabe, H. Studies of unusual amino acids and their peptides. VI. The synthesis and the optical resolution of β-methylphenylalanine and its dipeptide present in Bottromycin. *Bull. Chem. Soc. Jpn.* **1976**, *49*, 1081−1084.

(49) Gisin, B. F. The preparation of MerrifielD-resins through total esterification with cesium salts. *Helv. Chim. Acta* **1973**, *56* (143), 1476−1482.

(50) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595−598.

(51) Christensen, T. A chloranil test for monitoring coupling completeness in solid-phase peptide synthesis. In *Peptides: Structure and Biological Function;* Gross, E., Meienhofer, J., Eds.; Pierce Chemical Co.: Rockford, IL, 1979; pp 385−388.

(52) Leysen, J. E.; Gommeren, W.; Niemegeers, C. J. E. [^{3}H]-sufentanil, a superior ligand for μ-opiate receptors: binding properties and regional distribution in rat brain and spinal cord. *Eur. J. Pharmacol.* **1983**, *87*, 209−225.

(53) Akiyama, K.; Gee, K. W.; Mosberg, H. I.; Hruby, V. J.; Yamamura, H. I. Characterization of [^{3}H][2-D-penicillamine, 5-D-penicillamine]-enkephalin binding to delta opiate receptors in rat brain and neuroblastoma-glioma hybrid cell line (NG 108-15). *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2543−2547.

(54) Lahti, R. A.; Mickelson, M. M.; McCall, J. M.; Voightlander, P. F. [3H]U-69593: a highly selective ligand for the opioid-κ receptor. *Eur. J. Pharmacol.* **1985**, *109*, 281−284.
(55) Paton, W. D. M. The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br. J. Pharmacol.* **1957**, *12*, 119−127.
(56) Henderson, G.; Hughes, J.; Kosterlitz, H. W. A new example of a morphine sensitive neuroeffector junction. *Br. J. Pharmacol.* **1972**, *46*, 7164−7166.
(57) DiMaio, J.; Nguyen, T. M.-D.; Lemieux, C.; Schiller, P. W. Synthesis and pharmacological characterization in vitro of cyclic enkephalin analogues: effects of conformational constraints on opiate receptor selectivity. *J. Med. Chem.* **1982**, *25*, 1432−1438.
(58) Schiller, P. W.; Lipton, A.; Horrobin, D. F.; Bodanszky, M. Unsulfated C-terminal 7-peptide of cholecystokinin: a new ligand of the opiate receptor. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 1332−1338.
(59) Waterfield, A. A.; Leslie, F. M.; Lord, J. A. H.; Ling, N.; Kosterlitz, H. W. Opioid activities of fragments of β-endorphin and of its leucine^{65}-analogue. Comparison of binding properties of methionine- and leucine-enkephalin. *Eur. J. Pharmacol.* **1979**, *58*, 11−18.
(60) Schiller, P. W. Results to be published.

JM981011U