Structure−Activity Study on the Phe Side Chain Arrangement of Endomorphins Using Conformationally Constrained Analogues

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Endomorphins-1 and -2 were substituted with all the β-MePhe stereoisomers in their Phe residues to generate a conformationally constrained peptide set. This series of molecules was subjected to biological assays, and for β-MePhe^4-endomorphins-2, a conformational analysis was performed. Incorporation of (2*S*,3*S*)-β-MePhe^4 resulted in the most potent analogues of both endomorphins with enhanced enzymatic stability. Their μ opioid affinities were 4-times higher than the parent peptides, they stimulated [^{35}S]GTPγS binding, and they were found to be full agonists. NMR experiments revealed that C-terminal (2*S*,3*S*)-β-MePhe in endomorphin-2 strongly favored the gauche (−) spatial orientation which implies the presence of the $\chi^1 = -60°$ rotamer of Phe^4 in the binding conformer of endomorphins. Our results emphasize that the appropriate orientation of the C-terminal aromatic side chain of endomorphins is substantial for binding to the μ opioid receptor.

Introduction

Opioid systems play important roles in a number of physiological processes which are mediated by endogenous opioid peptides through the activation of specific membrane-bound opioid receptors. Endomorphin-1 (H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2) are the likely endogenous ligands of the μ opioid receptors;[1] however, their precursor protein(s) and gene(s) encoding is (are) unidentified. These two neuropeptides have been extensively investigated since their first description in order to explore their central and peripheral actions.[1−6] Immunoreactivity for these peptides has been shown to occur in brain regions where μ opioid receptors are located.[1] Endomorphins mediate analgesia that can be blocked by naloxone and other μ antagonists.[7,8] They showed high affinity and selectivity for μ opioid receptors of rat and mouse brain homogenates and for recombinant μ opioid receptors in direct and indirect radioreceptor binding assays,[4,7,9] but in membranes of mice lacking the μ receptor gene, no binding was observed.[10] Both tetrapeptides inhibit adenylyl cyclase activity, stimulate [^{35}S]GTPγS binding to G-proteins in CHOμ cells and brains of wild-type mice,[10] and decrease the electrical evoked muscle contractions in GPI and MVD preparations.[11]

Since the μ opioid receptors mediate the most prominent pharmacological effects of morphine, endomorphins are important model peptides in the search toward new analgesics. Several attempts have been made to determine the bioactive conformation of endomorphins using NMR spectroscopy and molecular modeling, and the solution conformations of endomorphins were reported by numerous laboratories. In short peptides, the Xaa-Pro peptide bond exists as a mixture of the cis and trans isomers,[12] and an extended conformation of trans endomorphin-1 was proposed to be a bioactive form.[13] When the environment of endomorphin-1 was modified from water to amphipathic micelles in NMR experiments, the orientation of the aromatic side chains changed from an interaction of the Tyr^1 and Phe^4 residues to a close contact between the Trp^3 indole and the phenolic groups and thus forced the Phe^4 side chain into the opposite direction. This conformational switch was accompanied by a stabilization of the cis Pro^2 isomer.[14] The Phe^4 residue was found to be less structurally defined in endomorphin-1 because of the lack of NOE contacts between the protons of the aromatic ring and the other residues. The Tyr^1 and Trp^3 side chains, however, were well characterized with trans and gauche (−) rotamers, respectively.[15] The spatial orientations of Tyr^1, Pro^2, and Phe^3 in the most extended conformers of trans endomorphin-2 could approach to those of trans endomorphin-1, and the respective aromatic rings occupy the same spatial regions.[16] The marginally decreased binding affinity of N-MePhe^3-endomorphin-2 supports the extended backbone structure model for endomorphin-2, because peptides containing N-Me amide bonds in the backbone tend to adopt an extended conformation.[17] Furthermore, the C-terminal amide function of endomorphin-2 was found to be essential to decrease the flexibility of the tetrapeptide,[16] to regulate the binding and agonist/antagonist properties,[17] and also to increase the enzymatic stability.[18]

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Table 1. Analytical Data of β-MePhe-Containing Endomorphin Analogues

		ESI-MS $[M + H]^+$		RP-HPLC[a] k'		TLC[b] R_f		
no.	peptide	calcd	found	(A)	(B)	(III)	(IV)	(V)
1	H-Tyr-Pro-Trp-Phe-NH_2	611	611	3.33	2.31	0.45	0.56	0.34
2	H-Tyr-Pro-Trp-(2*S*,3*S*)-β-MePhe-NH_2	625	625	3.80	2.84	0.43	0.57	0.35
3	H-Tyr-Pro-Trp-(2*R*,3*R*)-β-MePhe-NH_2	625	625	4.37	3.13	0.36	0.53	0.31
4	H-Tyr-Pro-Trp-(2*S*,3*R*)-β-MePhe-NH_2	625	625	3.69	2.71	0.44	0.57	0.36
5	H-Tyr-Pro-Trp-(2*R*,3*S*)-β-MePhe-NH_2	625	625	4.41	3.06	0.37	0.55	0.34
6	H-Tyr-Pro-Phe-Phe-NH_2	572	572	2.83	2.03	0.43	0.55	0.37
7	H-Tyr-Pro-(2*S*,3*S*)-β-MePhe-Phe-NH_2	586	586	3.67	2.50	0.43	0.55	0.42
8	H-Tyr-Pro-(2*R*,3*R*)-β-MePhe-Phe-NH_2	586	586	5.55	3.23	0.37	0.56	0.36
9	H-Tyr-Pro-(2*S*,3*R*)-β-MePhe-Phe-NH_2	586	586	3.68	2.46	0.47	0.53	0.37
10	H-Tyr-Pro-(2*R*,3*S*)-β-MePhe-Phe-NH_2	586	586	5.56	3.34	0.39	0.56	0.38
11	H-Tyr-Pro-Phe-(2*S*,3*S*)-β-MePhe-NH_2	586	586	3.28	2.82	0.42	0.56	0.38
12	H-Tyr-Pro-Phe-(2*R*,3*R*)-β-MePhe-NH_2	586	586	4.39	4.29	0.39	0.53	0.36
13	H-Tyr-Pro-Phe-(2*S*,3*R*)-β-MePhe-NH_2	586	586	3.23	2.77	0.44	0.57	0.40
14	H-Tyr-Pro-Phe-(2*R*,3*S*)-β-MePhe-NH_2	586	586	4.47	4.30	0.41	0.55	0.38

[a] Capacity factors for Vydac 218TP54 column (25 × 0.46 cm, $d_p = 5$ μm) with gradient (A) of 1%/min acetonitrile (0.08% (v/v) TFA) in water (0.1% (v/v) TFA) within 20 min starting from 20% acetonitrile, and gradient (B) of 2%/min methanol in 20 mM phosphate buffer pH = 2.1 within 20 min starting from 20% methanol. The flow rate was 1 mL/min; $t_0 = 2.6$ min; detection at $\lambda = 216$ nm. [b] Retention factors on silica gel 60 F_{254} precoated glass plates. Solvent systems: (III) acetonitrile−methanol−water (4:1:1), (IV) *n*-butanol−acetic acid−water (4:1:1), (V) ethyl acetate−pyridine−acetic acid−water (60:20:6:11).

The bioactive conformation and structural determinants of the endomorphins have also been probed by testing different types of analogues. On the basis of a similarity between the three-dimensional structure and the receptor selectivity profile of endomorphins and a β-turn mimetic with 4,7-dioxo-hexahydro-pyrazino[1,2-*a*]pyrimidine scaffold, a type III 4→1 β-turn was suggested as the biologically active conformation.[19] Endomorphin-2 derivatives containing the cis inducer pseudoprolines provided evidence that the receptor-bound conformation of the Tyr^1-Pro^2 peptide bond in endomorphin-2 was cis.[20] Similarly to morphiceptin,[21] PLO17,[22] and Tyr-W-MIF-1,[23,24] the Pro^2 residue of endomorphins was substantiated to direct the Tyr^1 and Trp^3/Phe^3 side chains into the required orientation. The L-configuration of Pro was considered vital for μ opioid activity and selectivity.[15,25] Introduction of β-(*R*)-Pro or L-homo-Pro instead of Pro^2 residue resulted in endomorphin-1 derivatives with higher or similar affinity compared to the parent peptide,[26,27] further emphasizing the stereochemical spacer role of the Pro^2 residue in endomorphins. It is well established that the amino and the phenolic functional groups of Tyr^1 together with the aromatic side chains of Trp^3 or Phe^3 and Phe^4 are essential for μ opioid receptor recognition.[1,28] Consequently, the side chain structure and conformation of these pharmacophore aromatic residues play an important role in generating favorable interactions between opioid peptides and their receptors. For instance the substitution of Gly^4 in Tyr-W-MIF-1 with Phe increased the affinity nearly 200-times and led to endomorphin-1 possessing subnanomolar μ opioid receptor affinity.[1] The C-terminal L-Phe in endomorphin-2 appears to generate the optimum μ receptor binding activity; however, D-Phe^4 and des-Phe^4 derivatives provided only a little lower affinity.[17,25,29] The negative impact of the N-terminal D-Tyr on μ affinity was found to be minimal. However, Phe^3 residue was quite essential, because its enantiomer reduced the μ affinity.[25] A similar tendency was observed in the case of endomorphin-1, where the D-Trp^3 derivative possessed lower μ affinity than the parent peptide, while the inversion of the configuration of the N- and C-terminal aromatic amino acids caused a moderate alteration.[15] The substitution of Tyr^1, Trp^3, or Phe^4 residues with the isomeric β-amino acids resulted in analogues with decreased μ affinity,[26] and similarly, replacing these residues with the corresponding homo-amino acids also caused a significant loss of affinity.[27]

In small peptides such as endomorphins, the amino acid side chains exhibit considerable conformational flexibility; therefore, the establishment of the three-dimensional arrangement of the structural moieties constituting the μ agonist pharmacophores is not yet resolved. The use of β-methylated amino acids to constrain the conformational mobility of the side chain by biasing the population of the χ^1 torsional angle rotamers has been introduced by V. J. Hruby.[30−32] In the present paper we report a systematic study of the effect of β-methylation at Phe^4 residue of endomorphin-1, and at Phe^3 and Phe^4 residues of endomorphin-2 on biological activity. The new analogues were obtained by substitution of the Phe residues with each of the four conformationally constrained β-MePhe isomers. Investigations of their structures and bioactivities are described here.

Results

Synthesis. Since this study required all stereoisomers of β-MePhe, a synthetic route[31] resulting in racemates was chosen. Peptide synthesis was performed by the solid-phase method using 4-methylbenzhydrylamine resin. RP-HPLC analyses of the crude peptides indicated that the ratio of the diastereomeric peptides was nearly 1:1. Optically pure peptides were obtained by semipreparative RP-HPLC (Table 1). The absolute configuration of β-MePhe in the peptides was determined by chiral TLC analyses of the acidic hydrolysates of peptides. The (2*S*)-β-MePhe isomers had higher R_f values than the corresponding (2*R*)-isomers in an eluent mixture of acetonitrile−methanol−water (4:1:1).[33] Comparison of the TLC data with the RP-HPLC chromatograms clearly revealed that the compound eluting first from the reversed-phase column contained (2*S*)-β-MePhe isomer.

Biological Data. The potency and selectivity of the new β-MePhe containing endomorphin analogues were

Table 2. Opioid Receptor Binding Affinities of Endomorphin Analogues[a]

peptide	$K_{i\mu}$ (nM)[b]	$K_{i\mu}$ (nM)[c]	$K_{i\delta}$ (nM)[d]	$K_{i\kappa}$ (nM)[e]	$K_{i\delta}/K_{i\mu}$[c]
1	1.62 ± 0.11	4.21 ± 0.31	6390 ± 539	52.7 ± 12.7	1518
2	0.47 ± 0.08	0.80 ± 0.09	567 ± 22	33.7 ± 7.5	709
3	43.6 ± 11.3	45.3 ± 7.7	1460 ± 117	467 ± 52	32
4	23.4 ± 9.7	26.3 ± 5.8	4630 ± 369	65.4 ± 9.7	176
5	47.0 ± 15.3	107 ± 32	745 ± 43	316 ± 42	7
6	4.00 ± 1.22	9.53 ± 2.19	2650 ± 175	10.6 ± 2.5	278
7	73.1 ± 7.1	45.3 ± 4.1	179 ± 15	83.0 ± 15.3	4
8	6980 ± 407	7090 ± 131	6760 ± 865	4470 ± 340	–
9	35.0 ± 11.4	106 ± 9	>10000	55.7 ± 3.5	–
10	>10000	4910 ± 328	>10000	990 ± 93.0	–
11	0.97 ± 0.30	1.67 ± 0.31	6360 ± 238	11.9 ± 2.4	3808
12	127 ± 25	250 ± 35	5180 ± 438	181 ± 35	21
13	23.4 ± 5.5	69.5 ± 6.6	4900 ± 399	28.1 ± 5.2	71
14	47.7 ± 6.8	104 ± 15	>10000	94.3 ± 12.5	–

[a] Values are arithmetic means of 3−5 measurements, each containing two parallels ± SE. The following radioligands were used. [b] [^{3}H]Endomorphin-2. [c] [^{3}H]DAMGO. [d] [^{3}H]Ile^{5,6}-deltorphin II. [e] [^{3}H]Dynorphin A in the presence of 10 nM DAMGO and 100 nM DADLE to quench the μ and δ binding.

evaluated by radioligand binding assays using rat brain membranes, and those of the β-MePhe^4-endomorphin-2 isomers by bioassays using MVD and GPI preparations. In the binding assays [^{3}H]endomorphin-2 prepared in our laboratory (G.T.)[34,35] and [^{3}H]DAMGO were used as μ radioligands, and [^{3}H]Ile^{5,6}-deltorphin II and [^{3}H]-dynorphin A as δ and κ radioligands, respectively. Although [^{3}H]dynorphin A is reported to be a selective κ opioid receptor ligand, it also binds to μ receptors with considerable affinity.[36] Therefore, the $K_{i\kappa}$ values are relatively low. Endomorphins-1 and -2 were also characterized for comparison. Since no appreciable degradation of endomorphins-1 and -2 was observed in the presence of rat brain membrane preparation under the binding conditions,[34,35] the nonproteinogenic amino acid containing derivatives were supposed to remain intact under the same conditions.

The binding properties of the new analogues are summarized in Table 2. The endomorphin-1 analogues substituted with L-β-MePhe^4 isomers (2*S*,3*S*) or (2*S*,3*R*) exhibited higher μ affinities than their D-β-MePhe^4 containing epimers. Between the diastereomers with (2*S*) chirality, analogue **2** exhibited 50- and 33-fold higher μ affinity measured with [^{3}H]endomorphin-2 and [^{3}H]DAMGO, respectively, than its epimer **4**. Moreover, compound **2** displayed subnanomolar μ affinity ($K_{i\mu}$ = 0.47 nM) and was found to be more potent than the parent endomorphin-1. In contrast, the β-methyl substitution performed at D-Phe^4 resulted in analogues **3** and **5** with decreased μ affinity and increased δ affinity, consequently decreased μ selectivity. The selectivity of the most potent analogue **2** also decreased slightly as it exhibited an 11-fold increased δ and 1.5-fold increased κ receptor affinity compared to the parent peptide. In the case of endomorphin-2, two isomeric series were prepared by replacing both Phe residues with β-MePhe isomers. Substitution of the Phe^3 residue resulted in peptides **7**−**10** with reduced potencies compared to the parent endomorphin-2. Peptides **7** and **9** in which the β-MePhe^3 residue had (2*S*) stereochemistry displayed higher μ affinity than their epimers **10** and **8**, respectively. It is noteworthy that compound **7** displayed the highest δ affinity ($K_{i\delta}$ = 179 nM), 15-fold higher than that of the parent endomorphin-2, among all synthesized peptides. The effects of β-methyl substitution in the C-terminal residue of endomorphin-2 was similar to that observed in the case of endomorphin-1. The

Table 3. In Vitro Biochemical Data for Estimating Agonist/Antagonist Nature of the Endomorphin Analogues

peptide	[^{35}S]GTPγS binding stimulation			sodium index[b]
	maximum (%)[a]	ED_{50} (nM)	log ED_{50} ± SE	
1	237 ± 7	289	−6.87 ± 0.15	30.5
2	163 ± 2	114	−6.94 ± 0.06	33.3
3	114 ± 2	1486	−5.82 ± 0.21	68.0
4	126 ± 1	209	−6.68 ± 0.11	5.6
5	124 ± 2	784	−6.10 ± 0.16	2.1
6	213 ± 4	474	−6.32 ± 0.11	30.4
7	160 ± 2	1612	−5.79 ± 0.06	1.9
8	105 ± 1	7.7	−8.11 ± 0.24	0.1
9	146 ± 3	1799	−5.75 ± 0.10	10.4
10	103 ± 1	53	−7.77 ± 0.30	3.5
11	148 ± 2	139	−6.86 ± 0.07	20.0
12	108 ± 1	104	−6.98 ± 0.37	2.1
13	132 ± 2	455	−6.34 ± 0.11	84
14	115 ± 2	572	−6.24 ± 0.25	3.7

[a] Maximal stimulation % mean values ± SE. [b] Sodium index values were calculated by dividing the IC_{50} values obtained in [^{3}H]naloxone displacement experiments in the presence and in the absence of NaCl.

β-MePhe derivatives with (2*S*) chirality (**11** and **13**) displayed higher affinity for μ opioid receptors than those with (2*R*) chirality (**12** and **14**). Among all synthesized peptides, **11** was the most selective compound with high μ affinity ($K_{i\mu}$ = 0.97 nM ([^{3}H]endomorphin-2) and 1.67 nM ([^{3}H]DAMGO)), and it exhibited even a 10-fold higher μ vs δ and a 5-fold higher μ vs κ selectivity as compared to the parent endomorphin-2.

Agonist−antagonist properties of the new peptides were measured and suggested by functional biochemical means, i.e., determining in vitro effects of sodium ions on equilibrium [^{3}H]naloxone binding, and measuring the binding of the nonhydrolyzable GTP analogue GTPγS to G-proteins (Table 3). Endomorphins-1 and -2 (**1**, **6**) and peptides **2**, **3**, **9**, **11**, **13** are likely the best agonists among all the compounds tested. These ligands showed sodium index values above 10 in the binding assays and gave a 14−63% maximal stimulation of [^{35}S]GTPγS binding over the basal level. The (2*S*,3*S*)-β-MePhe^4 containing derivatives **2** and **11** displayed not only high [^{35}S]GTPγS binding stimulation (63% and 48% over the basal level, respectively) but had better ED_{50} values than their parent peptides. The sodium index values for endomorphins and their derivatives appear to carry different information value as compared to those obtained for non-peptide opioids or Tyr-Gly-Gly-Phe-based

Table 4. Opioid Action of β-MePhe⁴-Endomorphin-2 Isomers in Isolated Tissues[a]

peptide	IC_{50} (nM) GPI	IC_{50} (nM) MVD	GPI/MVD potency ratio
1	10.1 ± 1.2	36.3 ± 5.2	0.27
6	9.22 ± 0.96	24.1 ± 4.6	0.38
11	3.46 ± 0.55	9.15 ± 2.90	0.37
12	816 ± 84	>10000	<0.08
13	89.9 ± 16.1	235 ± 47	0.38
14	589 ± 88	>10000	<0.05

[a] Values are arithmetic means ± SE obtained in 4−14 independent experiments.

peptides. Low ratios are likely to indicate partial agonism but even high ratios may not be inconsistent with a possible partial agonism. Compounds **4**, **5**, **7**, **8**, **10**, **12**, **14** displayed sodium index values in the range of 0.1−5.6. All these peptides stimulated receptor-mediated G-protein activation in the [^{35}S]GTPγS binding assays, although **8**, **10**, and **12** turned out to be very weak in terms of maximal stimulation. Since **8** and **10** were almost inactive in the receptor binding assays, their apparent antagonist properties predicted from the sodium-shift data have no pharmacological relevance. ED_{50} values in the [^{35}S]GTPγS binding assays were higher comparing with K_i values observed in the receptor binding studies because 100 mM NaCl was present in the functional assay. Such concentration of sodium salt substantially decreased the ligand binding, resulting in a rightward shift on [^{35}S]GTPγS dose−response curves.

Since β-MePhe⁴-endomorphin-1 and β-MePhe⁴-endomorphin-2 derivatives displayed similar alteration in binding assays, and β-MePhe³-endomorphin-2 derivatives lost the μ opioid receptor affinity, only compounds **11**−**14** were subjected to GPI and MVD bioassays. The GPI preparation contains predominantly μ opioid receptors, but also κ receptors, while in the MVD preparation δ receptors are predominant, but contains μ and κ receptors too.[37] The antagonism between the endomorphin derivatives and the antagonist naltrexone was determined in both preparations. The K_e values of naltrexone fell into the range of 0.20−0.99 nM in GPI assay and 0.21−0.35 nM in MVD assay. These K_e values indicate that the inhibitory effect of endomorphin derivatives **11**−**14** is exerted mainly or exclusively on μ opioid receptors in both preparations. The potencies to inhibit an electrically evoked neurotransmitter release and the resulting muscle contractions in the GPI and MVD preparation are summarized in Table 4. Results for the parent endomorphins are included for comparison. Analogues **11**−**14** of endomorphin-2 exhibited similar structure−activity relationship in GPI and MVD bioactivity assays than in radioligand binding assays, i.e. compound **11** with (2*S*,3*S*) stereochemistry was the most potent. None of the β-MePhe⁴-endomorphin-2 derivatives had overt partial agonist properties in the isolated organs. However, since the partial agonism of endomorphins and their synthetic analogues can be revealed in these preparations only by partial μ opioid receptor pool inactivation,[38] it is possible that some of these novel analogues may possess partial agonist properties.

Beside the binding and bioactivity data, the enzymatic resistance of a peptide ligand is also a very important property to investigate. Therefore, the in vitro stabilities of the most potent compounds **2** and **11** were examined in a rat brain homogenate as described elsewhere.[35] The kinetics of the metabolism of these endomorphin derivatives was characterized by analyzing the digestion mixtures with RP-HPLC. The velocity constants and half-lives were then calculated on the basis of pseudo-first-order kinetics (Table 5). In contrast with the enkephalins, the endomorphins-1 and -2 were degraded relatively slowly in the rat brain homogenate,[35] but **2** and **11** displayed even higher enzymatic stability than their parent peptides. The half-life of **2** was found to be 10.7 min, and that of **11** was 16.1 min at 5.4 mg/mL protein content in the homogenate.

Table 5. Half-Lives of the Most Potent Endomorphin Derivatives in Rat Brain Homogenate[a]

peptide	$100 \times k$ (min^{-1})[b]	$t_{1/2}$ (min)[c]
1	14.1 ± 0.5[35]	4.9 ± 0.2[35]
2	6.4 ± 0.2	10.7 ± 0.3
6	18.4 ± 0.9[35]	3.8 ± 0.2[35]
11	4.4 ± 0.6	16.1 ± 2.1

[a] Values are arithmetic means of 3−5 measurements ± SE. The protein content of the homogenate was 5.4 mg/mL. [b] Velocity constants. [c] Half-lives were calculated on the basis of pseudo-first-order kinetics of the disappearance of the peptides.

Conformational Analysis. NMR investigations of β-MePhe⁴-substitited endomorphin-2 isomers were performed using standard one- and two-dimensional homo- and heteronuclear techniques[39] in DMSO-d_6 at 300 K. The one-dimensional ^{1}H spectra of **11**−**14** showing two sets of signals indicated the presence of a conformational exchange slow on the NMR time scale (e.g. Table 6 for **11**). The relative integrated intensities of proton signals corresponding to the different isomers indicated that β-MePhe⁴-endomorphins-2 reside in the cis and trans isomers in a population ratio of 1:2, respectively, with respect to the Tyr¹-Pro² peptide bond. The NMR assignments of the cis−trans isomers were based on the characteristic sequential NOEs observed between the Tyr¹ and Pro² residues, and were supported by the characteristic ^{13}C chemical shift differences of Pro²-$C_{\beta,\gamma}$ carbons. A significant difference of ca. 0.8 ppm was observed between the proton chemical shifts of Pro²-H_α in the cis and trans isomers. The large upfield shift of the Pro²-H_α signals of the cis isomers can only be explained by the ring current effect of the aromatic rings of the two neighboring residues.[40−42] The cis Tyr¹-Pro² peptide bond is stabilized by the clustering of the side chains of Tyr¹ and Phe³. This folded state can only be formed with the preferred χ^1 rotamers of Tyr¹ and Phe³, namely trans and gauche (−), respectively. A similar cis/trans isomeric ratio with the predominance of the trans isomers was reported for endomorphins-1 and -2.[13−16] Only a few nonsequential ROESY cross-peaks were observed for the investigated peptides, which is an indication of the existence of extended conformations in DMSO solution. However, the sequential NH_i to NH_{i+1} NOEs observed between Phe³ and β-MePhe⁴, and the small temperature coefficient of Phe³-NHs (between −4.5 and −2.6 ppb/K), suggest that some folded conformers may still also exist in conformational equilibrium with the extended ones. The side chain conformations of the Tyr¹, Phe³, and β-MePhe⁴ residues were deduced from the three-bond homo- and heteronuclear coupling constants[43−45] and were corroborated by the

Table 6. ^{1}H NMR Chemical Shifts[a] (ppm), Coupling Constants (J in Hz), and Temperature Coefficients (ppb/K) in Brackets for **11**

residue	Tyr-Pro[b]	NH	H^{α}	H^{β}	$H^{\gamma,\gamma'}$	$H^{\delta,\delta'}$
Tyr^1	trans	8.01	4.18, $J_{\alpha\beta} = 6.0$, $J_{\alpha\beta'} = 7.2$	2.93 (β), 2.79 (β'), $J_{\beta\beta'} = 14.3$		
	cis	8.01	3.27, $J_{\alpha\beta} = 7.2$, $J_{\alpha\beta'} = 8.3$	2.83 (β), 2.76 (β') $J_{\beta\beta'} = 13.7$		
Pro^2	trans	NA	4.37	1.94, 1.63	1.74	3.55, 3.06
	cis		3.58	1.59	1.46	3.37, 3.24
Phe^3	trans	7.89, $J_{NH\alpha} = 7.7$, (−2.8)	4.41, $J_{\alpha\beta} = 4.9$, $J_{\alpha\beta'} = 7.7$	2.84 (β), 2.72 (β'), $J_{\beta\beta'} = 13.9$		
	cis	8.18, $J_{NH\alpha} = 8.6$, (−3.3)	4.42, $J_{\alpha\beta} = 4.9$, $J_{\alpha\beta'} = 9.8$	2.84 (β), 2.65 (β'), $J_{\beta\beta'} = 13.9$		
β-MePhe^4	trans	7.66, $J_{NH\alpha} = 8.6$, (−7.0)	4.49, $J_{\alpha\beta} = 9.0$	3.13, $J_{\beta\gamma} = 7.2$	1.20	
	cis	7.78, $J_{NH\alpha} = 8.9$, (−7.1)	4.52, $J_{\alpha\beta} = 9.2$	3.09, $J_{\beta\gamma} = 7.2$	1.19	

[a] Proton chemical shifts are referenced to the residual solvent signal of DMSO-d_6 at 2.49 ppm. ^{1}H NMR signals of aromatic protons for Tyr^1 (6.69/7.13) in *trans*-**11** and Tyr^1 (6.69/6.89) in *cis*-**11**, for Phe^3 and (2*S*,3*S*)-β-MePhe^4 (7.1−7.3). [b] Tyr-Pro conformer.

Table 7. Rotamer Populations (P)[a] of Tyr^1, Phe^3, and β-MePhe^4 Side Chains in β-MePhe^4-Endomorphin-2 Isomers

		Tyr^1			Phe^3			β-MePhe^4		
peptide	Tyr-Pro[b]	P($g-$)	P(t)	P($g+$)	P($g-$)	P(t)	P($g+$)	P($g-$)	P(t)	P($g+$)
11	trans	35	24	41	40	13	47	53	8	39
	cis	35	46	19	60	13	27	55	8	37
12	trans	35	23	42	49	13	38	35	0	65
	cis	38	49	13	74	7	19	25	10	65
13	trans	38	27	35	48	15	37	30	45	25
	cis	29	44	27	62	13	25	39	44	17
14	trans	38	24	38	49	16	35	42	28	30
	cis	24	43	33	68	13	19	38	26	36

[a] Rotamer populations of β-MePhe^4 residues were derived from the measured $J_{H\alpha H\beta}$ and $J_{H\alpha C\gamma}$ coupling constants,[43−45] and that of Tyr^1 and Phe^3 were calculated from the $J_{H\alpha H\beta}$ coupling constants. The stereospecific assignment of β-protons were deduced from the ROE patterns. Data given in percentage. [b] Tyr-Pro conformer.

ROE patterns observed between the backbone and side chain protons (Table 7). Tyr^1 prefers the trans conformation (up to 45−50% of the population) in the cis peptides, but in the trans isomers all three staggered conformers are almost equally populated. The trans rotamer of Tyr^1 and the gauche (−) rotamer of Phe^3 side chains allow favorable interactions between the aromatic rings and Pro^2 residue in the cis isomers.[40−42] In the most potent compound **11**, the β-MePhe^4 side chain prefers gauche (−) (53−55%) and gauche (+) conformations (37−39%), while the gauche (+) conformation is favored (65%) in the rotamer equilibrium of **12**. In the *threo*-β-MePhe^4-endomorphin-2 isomers **13** and **14**, all three rotamers of β-MePhe^4 are populated without any significant preference for one of the rotamers.

Discussion

The introduction of a methyl group at the β position of Phe alters the population of χ^1 rotamers because the torsional angle χ^1 of the substituted amino acid is affected by van der Waals interactions. These interactions depend on the stereochemistry and so the effect of this β-carbon methylation on the biological properties of endomorphins depends on the chirality of the α- and β-carbons of β-MePhe. In all cases, endomorphins with L-β-MePhe (2*S*,3*S* or 2*S*,3*R*) had higher μ opioid receptor affinities compared to the D-β-MePhe (2*R*,3*R* or 2*R*,3*S*) containing diastereomeric peptides. The effect of β-methylation in endomorphin-2 further depended on which Phe residue was modified. All β-MePhe^3 derivatives of endomorphin-2 exhibited lower μ affinity than β-MePhe^4 derivatives. The differences between the μ affinities of the (2*S*)- and (2*R*)-β-MePhe^3-endomorphins-2 were higher than those between the μ affinities of the (2*S*)- and (2*R*)-β-MePhe^4 analogues. It is in agreement with the importance of L-Phe^3 residue of endomorphin-2 which was previously reported to be quite essential for μ opioid receptor affinity, and with that endomorphin-2 can tolerate the inversion of the C-terminal Phe.[25] A similar tendency was observed in the case of endomorphin-1, where D-Trp^3-endomorphin-1 possessed lower μ opioid receptor affinity than the parent peptide, while the C-terminal amino acid inversion caused a moderate alteration.[15] The 15-fold increased δ affinity of **7** can be the consequence of the orientation of the second aromatic pharmacophore relative to the Tyr^1 side chain which dictates the μ or δ receptor selectivity. Incorporation of (2*S*,3*S*)-β-MePhe residue at the C-terminus resulted in the most potent ligands **2** and **11** with 4−5-fold higher activities compared to the parent peptides. **11** was not only one of the most potent but was the most selective compound for the μ opioid receptors. Further, these two peptides were found to be 2−3-fold more active in [^{35}S]GTPγS binding experiments than the parent peptides. Both [^{35}S]GTPγS binding stimulation data and sodium index values support the full agonist properties of **2** and **11**. The enzymatic stability of **2** and **11** also increased 2- and 4-fold. The alteration of the in vitro bioactivity of the β-MePhe^4-endomorphin-2 series in GPI and MVD assays was similar to those in the binding assays, i.e. **11** was found to be the most potent peptide, and to evolve its effect on μ opioid receptors. According to our NMR experiments, the incorporation of each stereoisomer of β-MePhe into the C-terminus of endomorphin-2 resulted in the same backbone conformation but produced different side-chain conformations in the peptides. The gauche (−) side chain conformation is preferred in **11**, which can ensure a favorable arrangement for μ opioid receptor binding. The same side chain rotamer of (2*S*,3*S*)-β-MePhe residue was found to be preferred when it was incorporated in the δ agonists DPDPE[31] and JOM-13.[46,47] A *trans*-Tyr^1-Pro^2 peptide bond was previously published to predominate over the cis rotamer in endomorphin-1.[13] Peptide **11** displayed a similar distribution preferring the trans isomeric form. However, the cis/trans equilibrium in solution does not

permit definitive conclusions concerning the receptor-bound conformation of these peptides.

Conclusion

A conformationally restricted Phe derivative was incorporated instead of Phe residue into the endomorphins to examine the conformational requirements of this amino acid side chain for bioactivity and with the aim of obtaining more selective and potent μ opioid ligands. The remarkable differences observed in the biochemical properties of the β-MePhe-substituted endomorphins with different stereochemistry suggest the importance of the proper orientation of the Phe^4 aromatic side chain for the μ opioid activity. Paterlini et al. supposed that Phe^4 of endomorphin-1 is free to adopt a bioactive conformation at the receptor site and that activation can occur independently of the correct orientation and stereochemistry of this residue.[15] Furthermore, it was reported that Tyr-Pro-Phe-OH binds to the μ opioid receptor with a K_i value of 46.3 nM[25] or with an IC_{50} value of 160.5 nM.[17] Our presented findings suggest that not only the N-terminal tripeptide portion of endomorphins contains key factors for binding to the μ opioid receptor, but the appropriate orientation of the C-terminal aromatic side chain is also substantial. Peptides **2** and **11** containing (2*S*,3*S*)-β-MePhe^4 exhibited μ opioid receptor affinities 4-fold higher than the parent endomorphins, they were 2−3 times more potent in the stimulation of [^{35}S]GTPγS binding, and their enzymatic stability was increased. These two derivatives were previously studied in vivo on animal models of different types of pain, and they exhibited higher analgesic potencies than the endogenous endomorphins.[48] Since the C-terminal side chain of isomer **11** strongly favors the gauche (−) spatial orientation, our data imply the presence of the $\chi^1 = -60°$ rotamer of Phe^4 in the binding conformer of endomorphins. Our results are to be useful in the development of new native peptide-based analgesics, when the transformation of the peptide structure to a nonpeptidic molecule retaining the three-dimensional array of pharmacophores is required.

Experimental Section

Abbreviations. Abbreviations and definitions are those recommended by IUPAC−IUB Commission of Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977−983). All optically active amino acids are of L configuration unless otherwise noted. The following additional abbreviations are used: Boc, *tert*-butyloxycarbonyl; DADLE, H-Tyr-D-Ala-Gly-Phe-D-Leu-OH; DAMGO, H-Tyr-D-Ala-Gly-NMe-Phe-Gly-ol; DMSO, dimethyl sulfoxide; GDP, guanosine-5′-diphosphate; GPI, guinea pig ileum; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; MVD, mouse vas deferens; NOE, nuclear Overhauser effect; ROESY, rotating-frame Overhauser effect spectroscopy; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

General Methods. Protected and unprotected amino acids (except β-MePhe) and 4-methylbenzhydrylamine resin were purchased from Sigma-Aldrich Kft. (Budapest, Hungary) or from Bachem Feinchemikalen AG (Bubendorf, Switzerland). Coupling agents were acquired from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland). Silica gel 60 F_{254} precoated glass plates of Merck (Darmstadt, Germany) were used for TLC with the following solvent systems: (I) acetonitrile−chloroform−acetic acid (8:1:1), (II) chloroform−methanol−acetic acid (90:8:2), (III) acetonitrile−methanol−water (4:1:1), (IV) *n*-butanol−acetic acid−water (4:1:1), (V) ethyl acetate−pyridine−acetic acid−water (60:20:6:11). UV light, I_2 vapor and ninhydrin were applied to visualize the TLC spots. Chiral separation of β-MePhe isomers was performed on Chiralplate of Macherey-Nagel (Düren, Germany). RP-HPLC was performed on a Merck-Hitachi liquid chromatographic system, equipped with a Vydac 218TP54 column for analytical purposes or with a Vydac 218TP1010 column for semipreparative separations. UV detection was used at $\lambda = 216$ nm. Mass spectra were recorded on a VG Quattro II triple quadrupole spectrometer (Micromass, Manchester, UK) with electrospray ionization (ESI).

Radioligands, except for [^{3}H]DAMGO (Amersham), were prepared in our laboratory as described earlier for [^{3}H]-endomorphin-2[34] and for [^{3}H]Ile^{5,6}-deltorphin II.[49]

All NMR parameters used in the present study have been obtained from 1D and 2D homo- and heteronuclear experiments performed with a 500 MHz Bruker Avance spectrometer (Brucker, Zug, Switzerland) equipped with a 5-mm inverse gradient probe. Peptide samples were dissolved in DMSO-d_6 at a concentration of 3−4 mg/500 μL. Proton and carbon assignments are established using a series of 2D experiments including TOCSY,[39,50,51] ROESY,[52,53] and HSQC[51,54] based on standard protocols.[39] Vicinal proton−proton and proton−carbon coupling constants were obtained with the use of gradient- and sensitivity enhanced TOCSY,[51] and X(ω_1) half-filtered TOCSY[55] or HECADE[56] experiments.

N$^\alpha$-*tert*-Butyloxycarbonyl-*erythro*-β-methylphenylalanine and N$^\alpha$-*tert*-Butyloxycarbonyl-*threo*-β-methylphenylalanine. The pure *erythro*-(2*S*,3*S* and 2*R*,3*R*)- and the pure *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-MePhe racemates were obtained by fractional crystallization[31] of the isomeric mixture prepared by the method of Kataoka.[57] 1.5 g of *erythro*-β-MePhe·HCl salt or 1.5 g of *threo*-β-MePhe·HCl salt was dissolved in 40 mL of dioxane−water (2:1), and the solution was cooled in an ice bath. Then the pH of the solution was adjusted to 9 with 4 M NaOH solution, and 1.85 g of di-*tert*-butyl dicarbonate was added. The solution was stirred at room temperature overnight, whereby the pH was continuously adjusted to 7.5 with NaOH solution. After evaporation of the dioxane, the residue was acidified with $KHSO_4$ solution and extracted with ethyl acetate. The extract was dried over $MgSO_4$ and evaporated. The resulting oil was crystallized from ethyl acetate/petroleum ether. N$^\alpha$-Boc-*erythro*-β-MePhe: yield 1.2 g (52%); mp 108−110 °C (lit. 108 °C[31]); TLC R_f(I) 0.75, R_f(II) 0.56; ^{1}H NMR (DMSO-d_6) δ 7.22 (m, 5H, aromatic), 4.14 (s, 1H, α-H), 3.17 (s, 1H, β-H), 1.33 (s, 9H, Boc-CH_3), 1.21 (s, 3H, β-CH_3). N$^\alpha$-Boc-*threo*-β-MePhe: yield 1.3 g (56%); mp 96−98 °C (lit. 96 °C[31]); R_f(I) 0.72, R_f(II) 0.57; ^{1}H NMR (DMSO-d_6) δ 7.23 (m, 5H, aromatic), 4.08 (s, 1H, α-H), 3.05 (s, 1H, β-H), 1.26 (s, 9H, Boc-CH_3), 1.19 (d, $J = 5.5$ Hz, 3H, β-CH_3).

Solid-Phase Synthesis and Purification of the Peptides. Peptide synthesis was performed by the manual solid-phase technique using 4-methylbenzhydrylamine resin (0.8 mmol/g of titratable amine). An excess (2 equiv) of Boc-protected amino acids, of dicyclohexylcarbodiimide, and of 1-hydroxybenzotriazole were used for coupling reactions, which were monitored by the ninhydrin test. The deprotection solution contained 50% (v/v) TFA and 0.5% (m/v) 1,4-dithiothreitol in dichloromethane. The removal of the peptides from the resin was accomplished with anhydrous HF (10 mL/g resin) in the presence of anisole (1 mL/g resin) and dimethyl sulfide (1 mL/g resin) at 0 °C for 60 min. After evaporation of the HF, the resin was washed with diethyl ether to remove the scavengers, and extracted subsequently with 30% (v/v) aqueous acetic acid. Crude peptides were obtained in solid form after lyophilization of the diluted extract (yields 70−75%). Purification was performed by semipreparative RP-HPLC on a Vydac 218TP1010 C_{18} column with a linear gradient of 0.1% (v/v) TFA in water and 0.08% (v/v) 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 (Table 1).

Determination of the Configuration of β-MePhe in Peptides. One milligram of each diastereomeric peptide was hydrolyzed separately in 1 mL of 6 M HCl solution under Ar pressure at 110 °C for 24 h. Then the solvent was removed by evaporation, and the mixture of amino acids was analyzed by chiral TLC in acetonitrile−methanol−water (4:1:1). These R_f values were compared with those of standard optically pure β-MePhe isomers.[33]

Radioligand Binding Assay. Membranes were prepared from Wistar rat brain (without cerebellum) according to the method of Simon et al.[58] The binding experiments were performed in 50 mM Tris-HCl buffer, pH 7.4, at a final volume of 1.0 mL containing 300−500 μg of protein (protein content was determined by the method of Bradford[59]). In competition experiments, the following conditions were used for incubations: [^{3}H]endomorphin-2 (25 °C, 45 min), [^{3}H]DAMGO (35 °C, 45 min), [^{3}H]Ile^{5,6}-deltorphin II (35 °C, 45 min), [^{3}H]-dynorphin A (25 °C, 45 min with 10 nM DAMGO and 100 nM DADLE to quench the μ and δ binding). Incubations were started by the addition of membrane suspension, continued under gentle vortexing and shaking in a thermal water bath, and terminated by rapid vacuum filtration through Whatman GF/C filters using a Brandel M24R cell harvester. The filters were washed twice with 10 mL of ice-cold buffer and then dried for 3 h at 37 °C, and the radioactivity was measured in Packard UltimaGOLD scintillation cocktail with a Packard TriCarb 2300TR scintillation spectrometer. To determine the sodium index values the displacement experiments with [^{3}H]-naloxone were repeated in the presence of 100 mM NaCl, and the resulting IC_{50} values were divided by the values obtained in the absence of NaCl. The extent of nonspecific binding was determined in the presence of 10 μM naloxone. All experiments were carried out in duplicate assays and repeated at least three times. The data were analyzed by nonlinear least-squares regression using the GraphPad Prism 2.01 software.

[^{35}S]GTPγS Binding Assays. Rat brain membrane fractions (≈ 10 μg of protein/sample) were incubated at 30 °C for 60 min in Tris-EGTA buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 μM NaCl; pH 7.4) containing [^{35}S]GTPγS (0.05 nM) and increasing concentrations (10^{-9}−10^{-5} M) of the compounds tested in the presence of excess GDP (100 μM) in a final volume of 1 mL as described by Traynor and Nahorsky[60] with slight modifications. Nonstimulated activity was measured in the absence of the endomorphin derivatives, and nonspecific binding was measured in the presence of 100 μM unlabeled GTPγS. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters with a Brandel M24R cell harvester. Filters were washed three times with 5 mL of ice-cold buffer, and the bound radioactivity was detected in Packard UltimaGOLD scintillation cocktail with a Packard TriCarb 2300TR counter. Data were calculated from three independent experiments performed in triplicates and analyzed using the GraphPad Prism 2.01 software. Stimulation is given as percent of the specific binding.

GPI and MVD Bioassays. In vitro opioid activities of the compounds were tested in the GPI[61] and MVD[62] bioassays as reported elsewhere.[38,63] In brief, tissues were suspended in thermostated (37 °C for GPI and 31 °C for MVD) Krebs solution (Mg-free media for MVD) under an initial tension of 0.8 g (GPI) or 0.1 g (MVD). Field electrical stimulation was used through platinum wire electrodes positioned at the top (ring formation) and bottom (straight wire) of organ bath. The parameters of stimulation were as follows: supramaximal (9 V/cm) rectangular pulses of 1 ms duration were delivered by 10 s (i.e. at 0.1 Hz frequency) in the case of GPI whereas pairs of pulses (100 ms pulse distance) with identical individual parameters were delivered also by 10 s in the case of MVD. GPI was equilibrated for 60 min, MVD for 30 min under stimulation. Inhibitory dose−response curves with the agonists were constructed in a noncumulative manner, and the IC_{50} values, characterizing agonist potencies, were obtained from a sigmoidal curve fitting with the software Origin ver.6.

Determination of the Half-Lives. The digestion of the peptides **2** and **11** was performed as recently reported.[35] Twenty microliters of a 1 mM peptide stock solution in 50 mM Tris-HCl buffer (pH = 7.4) was added to 180 μL of the rat brain homogenate (protein content: 5.4 mg/mL), and the mixture was incubated at 37 °C. Aliquots of 20 μL were withdrawn from the incubation mixtures and immediately acidified with 25 μL of 0.1 M aqueous HCl solution. Ten microliters of supernatant obtained after centrifugation (11340 g, 5 min, 25 °C) of the sample was analyzed by RP-HPLC. The rate constants of degradation (k) were obtained by least-squares linear regression analysis of logarithmic tetrapeptide peak areas ($\ln(A/A_0)$) vs time courses, using a minimum of five points. Degradation half-lives ($t_{1/2}$) were calculated from the rate constants as $\ln 2/k$.

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Supporting Information Available: Concentration−effect curves of **2**−**5** and **7**−**14** stimulated [^{35}S]GTPγS binding in rat brain membrane fraction; ^{1}H NMR chemical shifts, coupling constants, and temperature coefficients for **12**−**14**; ^{13}C NMR chemical shifts for **11**−**14**. This material is available free of charge via the Internet at http://pubs.acs.org.

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