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Design, Synthesis, Pharmacological Evaluation, and Structure−Activity Study of Novel Endomorphin Analogues with Multiple Structural Modifications

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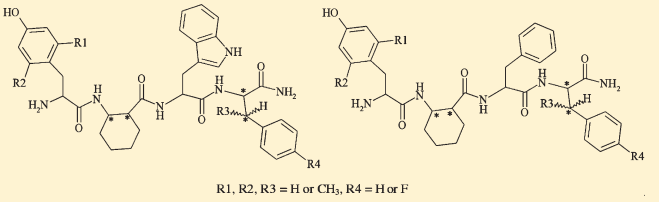
Supporting Information

ABSTRACT: This study reports on new proteolytically stable, pharmacologically active endomorphin analogues, incorporating Dmt[1], Achc[2], *p*FPhe[4], or *β*MePhe[4] unnatural amino acids. Consistent with earlier results, it was found that the analogues carrying Dmt[1] and Achc[2] residues displayed the highest *μ*-opioid receptor affinities, depending upon the configuration of the incorporated Achc[2]. Combination of such derivatives with *p*FPhe[4] or *β*MePhe[4] yielded further compounds with variable binding potencies. Combined application of Dmt[1], *cis*-(1*S*,2*R*)Achc[2], and *p*FPhe[4] (compound **16**) resulted in the most potent analogue. Ligand stimulated [^{35}S]GTP*γ*S binding assays indicated that the analogues retained their agonist activities and opioid receptor specificities. NMR and molecular modeling studies of the analogues containing *β*MePhe[4] or *p*FPhe[4] confirmed the predominance of bent structures, however, it is apparent that bent structures are energetically more favored than random/extended structures for all studied compounds.



INTRODUCTION

Morphine and related compounds that are clinically valuable for the relief of pain, act primarily at the *μ*-opioid receptor (MOR), a member of the G-protein-coupled receptor (GPCR) superfamily.[1] A major goal in opioid peptide research is the development of novel analgesics that could substitute for morphine without its well-known side-effects of dependence, tolerance, respiratory depression, and reward-seeking behavior.[2] The study of naturally occurring peptides provides a rational and powerful approach in the design of peptide medications. Endomorphin-1 (EM-1, YPWF-NH_2) and endomorphin-2 (EM-2, YPFF-NH_2) are high-affinity, *μ*-selective endogenous opioids which inhibit pain without some of the undesired side effects of plant opiates.[3] The precursor protein and encoding gene of the EMs has not yet been identified, although a putative biosynthetic pathway has been proposed and is still under investigation.[4] The exogenous application of EMs encounters serious limitations, including a short duration of action, a lack of activity after oral administration, a relative inability to cross the blood−brain barrier (BBB) into the central nervous system (CNS), and poor metabolic stability.[5−7] Aminopeptidases play a key role in the biodegradation of the EMs, during which the main cleavage occurs at the Pro[2]−Trp[3] and Pro[2]−Phe[3] peptide bonds. Even though the EMs have been shown to have the longest half-lives among all endogenous opioid ligands,[5,8] for their consideration as valuable therapeutic drugs it is essential to enhance their ability to enter the CNS and their resistance to enzymatic degradation.[9] Such objectives may possibly be achieved through systematic modification of the peptide sequence.[10−12]

According to the message-address concept of neuropeptides, the N-terminal free amine, the phenolic group of Tyr[1], and the aromatic side chain at position 3 of the sequence are essential for the binding of EMs to the MOR, while the C-terminal aromatic side chain and amine function have been found to be responsible for MOR vs DOR selectivity.[10] The replacement of Tyr[1] by Dmt results in marked increases in receptor-binding affinity and bioactivity in numerous opioid peptide agonists and antagonists.[13] Proline at position 2 of the EMs is considered to be a spacer residue, connecting two pharmacophoric aromatic residues, Tyr[1] and Trp[3]/Phe[3].[14] The L configuration of Pro is regarded as critical for *μ*-opioid activity and selectivity.[14,15] Replacement of Pro[2] by alicyclic *β*-amino acids,[16,17] 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (TIC),[18] pseudoprolines (ΨPro),[19] or piperidine-2-, -3-, and -4-carboxylic acids [(*S*)-Pip, (*R*)-Nip and Inp, respectively][20] resulted in increased affinity for the MOR and enhanced proteolytic stability.

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β-Methylated amino acids have been used to modify the conformational mobility of the side chains by biasing the population of the χ^1 torsional angle rotamers.[21] Phe^4 in EM-1 has been suggested to be flexible and independent of the preceding three amino acids in terms of the orientation required by the receptor binding site.[14] However, the results obtained for EM analogues in which Phe^3 and Phe^4 were replaced by βMePhe showed not only that the N-terminal tripeptide portion of the EMs contains key factors for the binding to the MOR but also that the appropriate orientation of the C-terminal aromatic chain is important.[22] The insertion of *p*FPhe in place of the Phe^4 in enkephalin resulted in increased potency in functional assays,[23] and similar results were observed in the EMs.[24]

The solution structures of the EMs and their analogues have been investigated in detail in relation to their bioactivity, and the results have been summarized in several review articles.[12,25] The backbone and side chain conformations of the MOR ligands contribute concomitantly to the orientation of the pharmacophore groups. Since no experimentally determined structure of a MOR−ligand complex has yet been published, no direct evidence of a bioactive backbone structure of μ-opioid peptides is available. While the first NMR spectroscopic investigations of EM-1 indicated an extended backbone structure,[26] several recent findings supported the bent backbone structure of the bound MOR ligands.[16,27,28] The EMs exist in *cis* and *trans* conformations with respect to the Tyr^1−Pro^2 peptide bond. Published observations on EM-1 and EM-2 revealed that they are predominantly in the *trans* configuration, with *cis*/*trans* ratios of 1:3 and 1:2, respectively.[26,29] This balance of configurations has been shown to be altered by the presence of Dmt and ΨPro in positions 1 and 2, respectively.[13,19] The synthesis and biological evaluation of stereoisomeric analogues of EM-2 demonstrated that different stereoisomers adopt different backbone structures, which results in marked variations in bioactivity.[15]

A structural model of MOR activity was first proposed on the basis of ^{1}H NMR studies of morphiceptin and its stereoisomeric analogues, where the bioactive structure is laid out by well-defined distances between the pharmacophore groups mentioned above.[30] Another topographical model for MOR-selective ligands was proposed following the structural analysis of cyclic somatostatin analogues. In this model, the optimal spatial arrangement of pharmacophores is provided by the bent backbone structure, the *gauche*+ conformation of the first, the *gauche*− conformation of the second, and the increased flexibility of the third aromatic side chain.[31] Previous studies by our group suggested a slightly bent backbone structure for receptor-bound ligands,[32] and more recently, four structural parameters of μ-opioid activity were confirmed.[33]

In the present paper, we report on the synthesis and structure−activity study of new analogues obtained as a result of the systematic replacement of natural amino acids by Dmt, *cis*-(1*S*,2*R*)Achc/*cis*-(1*R*,2*S*)Achc, (2*R*,3*R*)βMePhe/(2*S*,3*S*)βMePhe, and *p*FPhe in EMs (Figure 1). Such modifications were made in order to increase the proteolytic stability while retaining or enhancing the biological activity. The biological properties of these peptide analogues were evaluated in radioligand receptor binding experiments in rat brain membrane homogenates, followed by the determination of agonist/antagonist potencies by means of ligand-stimulated [^{35}S]GTPγS functional assays. The most promising compounds were studied by using NMR spectroscopy and molecular modeling in order to gain further insight into the structural determinants of MOR activity.

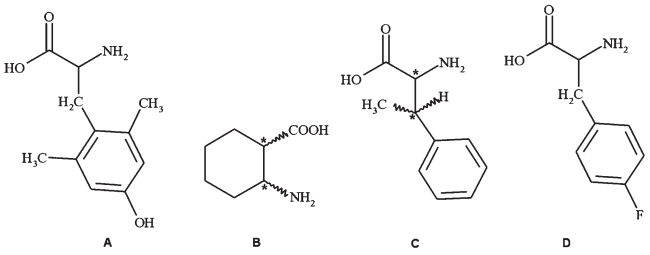

Figure 1. Structures of unnatural amino acids incorporated into endomorphins: (A) 2′,6′-dimethyltyrosine, (B) *cis*-(1*S*,2*R*)/(1*R*,2*S*)-2-aminocyclohexanecarboxylic acid, (C) (2*S*,3*S*)/2*R*,3*R*)β-methyl phenylalanine, (D) *para*-fluoro-phenylalanine.

RESULTS

Synthesis. Solid-phase peptide synthesis was performed on 4-methylbenzhydrylamine (MBHA) resin. Racemic *erythro*-(2*S*,3*S*)/(2*R*,3*R*)βMePhe was used to obtain the respective peptide diastereomers. Pure *cis*-(1*S*,2*R*)Achc was applied for the synthesis of peptides containing βMePhe and racemic *cis*-Achc for *p*FPhe^4-containing EM derivatives. Pure peptides were obtained by semipreparative RP-HPLC. The configurations of βMePhe residues in the peptide analogues were determined by chiral TLC analysis of the acidic hydrolysates of the peptides. The (2*S*,3*S*)βMePhe isomers had higher R_f values than those of the corresponding (2*R*,3*R*)βMePhe isomers in an eluent mixture of acetonitrile−methanol−water (4:1:1). These R_f values were compared with those of standard amino acids.[34] The configurations of (1*S*,2*R* or 1*R*,2*S*)Achc were determined by analytical RP-HPLC analysis of GITC-derivatized peptide hydrolysates. The retention times were compared with those of standard β-amino acid derivatives.[35] The RP-HPLC analysis of the crude peptides indicated that the ratio of the diastereomeric peptides was nearly 1:1. Molecular masses of all analogues were established by high-resolution mass spectrometry (HRMS) measurements. (The detailed analytical properties of the synthetic analogues are provided in Table S1 in the Supporting Information.)

Biological Evaluation. The potency, selectivity, and efficacy of the new EM analogues were evaluated via radioligand binding assays and functional [^{35}S]GTPγS binding assays[36] using a rat brain membrane homogenate. The MOR prototype enkephalin derivative [^{3}H]DAMGO and DOR-specific [^{3}H]Ile^{5,6}-deltorphin II peptide ligands were used as radioligands in the receptor binding assays. All the new compounds competed in a concentration-dependent manner with the radiolabeled MOR and DOR ligands for the receptor binding sites. The inhibitory constants (K_i) and selectivities (K_i^{δ}/K_i^{μ}) of the 14 new analogues and the parent ligands are listed in Table 1. EM-1 and EM-2 were also evaluated for comparison. The receptor binding values obtained for the parent ligands agreed well with literature data.[3,16,37] On the basis of the competition experiments, four EM analogues were selected for enzymatic degradation studies. These analogues demonstrated prolonged half-lives (>20 h) relative to the EMs ($t_{1/2}$ = 5−7 min), proving the enzymatic resistance of the new analogues (Table 4). All such analogues can be considered to be as resistant as tested ligands because of the similarities in their chemical structures. No appreciable degradation of EM-1 or EM-2 was previously observed in a rat brain membrane homogenate under the given binding conditions,[7] and therefore no protease inhibitors were added to the incubation mixtures.

Table 1. Opioid Receptor Binding Affinities and Selectivities of EM Analogues, Measured in Rat Brain Membrane Preparation

		inhibitory constants		selectivity
no.	peptide	K_i^{μ} (nM)[a]	K_i^{δ} (nM)[b]	K_i^{δ}/K_i^{μ}
1	Tyr-Pro-Trp-Phe-NH_2	1.6 ± 0.3	4169 ± 881	2605
2	Tyr-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	4.2 ± 0.9	1444 ± 182	343
3	Tyr-(1*S*,2*R*)Achc-Trp-(2*R*,3*R*)βMePhe-NH_2	34.6 ± 3.5	3364 ± 1093	97
4	Dmt-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	0.93 ± 0.07	123 ± 17	132
5	Dmt-(1*S*,2*R*)Achc-Trp-(2*R*,3*R*)βMePhe-NH_2	4.11 ± 0.7	287 ± 55	69
6	Tyr-(1*S*,2*R*)Achc-Trp-*p*FPhe-NH_2	3.2 ± 0.5	571 ± 92	178
7	Tyr-(1*R*,2*S*)Achc-Trp-*p*FPhe-NH_2	143.1 ± 5.9	7823 ± 1039	54
8	Dmt-(1*S*,2*R*)Achc-Trp-*p*FPhe-NH_2	11.1 ± 2.0	2636 ± 670	237
9	Tyr-Pro-Phe-Phe-NH_2	1.35 ± 0.2	8771 ± 1316	6497
10	Tyr-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	0.82 ± 0.2	661 ± 43	816
11	Tyr-(1*S*,2*R*)Achc-Phe-(2*R*,3*R*)βMePhe-NH_2	33.9 ± 6.0	1268 ± 33	37
12	Dmt-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	0.47 ± 0.06	142 ± 8	302
13	Dmt-(1*S*,2*R*)Achc-Phe-(2*R*,3*R*)βMePhe-NH_2	9.7 ± 1.3	198 ± 48	20
14	Tyr-(1*S*,2*R*)Achc-Phe-*p*FPhe-NH_2	1.5 ± 0.3	366 ± 61	244
15	Tyr-(1*R*,2*S*)Achc-Phe-*p*FPhe-NH_2	2.8 ± 0.5	689 ± 98	246
16	Dmt-(1*S*,2*R*)Achc-Phe-*p*FPhe-NH_2	0.13 ± 0.02	96 ± 9	738

[a] [^{3}H]DAMGO (K_d = 0.5 nM) was used as radioligand for the μ-opioid receptor. [b] [^{3}H]Ile^{5,6}deltorphin-2 (K_d = 2.0 nM) was used as radioligand for the δ-opioid receptor. K_i values were calculated according to the Cheng−Prusoff equation: $K_i = EC_{50}/(1 + [\text{ligand}]/K_d)$, where the shown K_d values were taken from literature data and calculated from direct isotope saturation plots.[16] Data are expressed as means ± SEM, $n \geq 3$.

Analysis of the binding results revealed that the coapplication of (1*S*,2*R*)Achc[2] and (2*S*,3*S*)βMePhe[4] in both EMs resulted in analogues (compounds **2** and **10**) with potencies comparable to those of the native peptides. These binding potencies were further enhanced by the combined substitution of Dmt[1], (1*S*,2*R*)Achc[2], and (2*S*,3*S*)βMePhe[4] in compounds **4** and **10**. It was also confirmed that the individual or combined use of Dmt[1] increased the affinity for MOR but at the same time decreased the selectivity of the ligands. Analogues containing the corresponding (2*R*,3*R*)βMePhe[4] residue exhibited lower affinities than those containing (2*S*,3*S*)βMePhe[4], but the replacement of Tyr[1] with Dmt enhanced the binding potency (compounds **5** and **13**). No significant changes were observed in the binding affinities of these ligands. Co-substitution with the halogenated *p*FPhe[4] and Achc[2] resulted in ligands with different potencies depending on the chirality of the alicyclic β-amino acids (compounds **6**−**8** and **14**−**16**). In comparison with compounds **5** and **13**, it is interesting to note that compound **16** showed higher potency than its corresponding EM-1 analogue, and it may therefore be assumed that only minor differences in structures (Trp/Phe) may be responsible for the observed changes in ligand binding. Furthermore, *p*FPhe was found to compensate the detrimental effects of (1*R*,2*S*)Achc[2] incorporation as demonstrated for compound **15** and other EM derivatives.[16] Among the *p*FPhe[4]-containing analogues, compound **16** displayed the highest MOR affinity with relatively high selectivity. The rank order of potency of the compounds measured against [^{3}H]DAMGO was as follows: **16** > **12** > **10** > **4** > **9** > **14** > **1** > **15** > **6** > **5** > **2** > **13** > **8** > **11** > **3** > **7**. Each ligand showed moderate to low binding affinities for the DORs, which indicates that these types of modifications primarily provide MOR-selective ligands.

On the basis of the heterologue displacement binding results, the most potent analogues were selected for [^{35}S]GTPγS functional assays. The results of the ligand-stimulated [^{35}S]-GTPγS functional assays are reported in Table 2. From the pool of ligands, compounds **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16** and the parent peptides were subjected to overall functional characterization and comparison. To confirm opioid receptor functionality, the newly synthesized ligands were also assayed in the presence of the opioid-selective antagonist naloxone. Potency (EC_{50}) and efficacy (E_{max}) values were compared with those of the full μ-receptor agonist DAMGO. Dose-dependent increases were observed for selected compounds in [^{35}S]GTPγS binding.

Table 2. Summary of [^{35}S]GTPγS Functional Assays with Selected EM Analogues in a Rat Brain Membrane Preparation[a]

no.	peptide	EC_{50} (nM)	E_{max} (%)
0	DAMGO	214 ± 39	178 ± 5
1	Tyr-Pro-Trp-Phe-NH_2	166 ± 27	149 ± 4
2	Tyr-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	264 ± 8	162 ± 2
4	Dmt-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	22.1 ± 3	162 ± 9
6	Tyr-(1*S*,2*R*)Achc-Trp-*p*FPhe-NH_2	380 ± 38	174 ± 9
8	Dmt-(1*S*,2*R*)Achc-Trp-*p*FPhe-NH_2	8.1 ± 3	163 ± 13
9	Tyr-Pro-Phe-Phe-NH_2	212 ± 6	163 ± 6
10	Tyr-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	273 ± 31	173 ± 6
12	Dmt-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	17 ± 5	159 ± 4
14	Tyr-(1*S*,2*R*)Achc-Phe-*p*FPhe-NH_2	92 ± 8	168 ± 6
16	Dmt-(1*S*,2*R*)Achc-Phe-*p*FPhe-NH_2	51 ± 8	176 ± 7

[a] Sigmoid dose−response curves of the listed peptides were determined as described in the Methods section. EC_{50} and E_{max} values were calculated by using the sigmoid dose−response fitting option of the GraphPad Prism software. Data are expressed as the % stimulation of the basal activities, i.e. the binding in the absence of peptides, which was defined as 100%. Data are means ± SEM, $n \geq 3$, each performed in triplicate.

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Table 3. Summary of [^{35}S]GTPγS Functional Assays with Selected EM Analogues in the Presence of Naloxone, Measured in a Rat Brain Membrane Preparation[a]

no.	peptide	E_{max} (%)
0	DAMGO	119 ± 2
1	Tyr-Pro-Trp-Phe-NH_2	124 ± 10
2	Tyr-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	130 ± 7
4	Dmt-(1*S*,2*R*)Achc-Trp-(2*S*,3*S*)βMePhe-NH_2	145 ± 8
6	Tyr-(1*S*,2*R*)Achc-Trp-*p*FPhe-NH_2	125 ± 3
8	Dmt-(1*S*,2*R*)Achc-Trp-*p*FPhe-NH_2	127 ± 6
9	Tyr-Pro-Phe-Phe-NH_2	123 ± 5
10	Tyr-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	120 ± 7
12	Dmt-(1*S*,2*R*)Achc-Phe-(2*S*,3*S*)βMePhe-NH_2	146 ± 5
14	Tyr-(1*S*,2*R*)Achc-Phe-*p*FPhe-NH_2	114 ± 4
16	Dmt-(1*S*,2*R*)Achc-Phe-*p*FPhe-NH_2	145 ± 4

[a] Sigmoid dose−response curves of the listed peptides were determined as described in the Methods section. EC_{50} and E_{max} values were calculated by using the sigmoid dose−response fitting option of the GraphPad Prism software. The concentration of naloxone used to block ligand binding and subsequent G-protein activation was set at 10^{-5} M. Data are expressed as the % stimulation of the basal activities in the absence of peptides, which was defined as 100%. Data are means ± SEM, $n \geq 3$, each performed in triplicate.

Table 4. Half-Lives of the EMs and Their Potent Analogues in a Crude Rat Brain Membrane Homogenate[a]

peptide	half-life
1	6.3 ± 0.1 min
2	>20 h
6	>20 h
9	5.7 ± 0.1 min
12	>20 h
16	>20 h

[a] Data are means of at least three individual experiments ± SEM. The protein content of the brain homogenate was 5.0 mg/mL. Half-lives were calculated on the basis of pseudo-first-order kinetics of the disappearance of the peptides.

The parent ligands exhibited comparable potencies but lower efficacies (E_{max} = 166% for EM-1 and E_{max} = 163% for EM-2) were observed as compared with DAMGO, confirming that the EMs are partial agonists, as reported earlier.[38,39] Compounds **6**, **10**, and **16** displayed the highest efficacies (E_{max} = 174%, 173%, and 176%, respectively), acting as full agonists. Additionally, compound **16** exhibited a 4 times higher potency (EC_{50} = 51 nM) than that of EM-2 (EC_{50} = 212 nM). Compounds **2**, **4**, **8**, **12**, and **14** were slightly less efficacious than DAMGO, which indicates partial agonist or full agonist properties. Interestingly, compound **12** displayed the highest potency (EC_{50} = 17 nM) among all the analogues. The rank order of efficacy (E_{max}) measured by [^{35}S]GTPγS functional assay was **0** > **16** > **6** > **10** > **14** > **8** = **9** > **2** = **4** > **12** > **1**. The analysis of the [^{35}S]GTPγS binding results in the presence of naloxone revealed that the ligands predominantly activate G-proteins through MOR. However, compounds **4**, **12**, and **16** showed only minor decreases in efficacy in the presence of naloxone (Table 3). These findings suggest that these ligands may also bind to other nonopioid receptors coupled with G-proteins and similarly activate primary downstream effectors.

Structural Study. Because of their exceptional biological properties, compounds **10**, **12**, **14**, and **16** were selected for structural studies. The NMR studies were carried out in deuterated dimethylsulfoxide (DMSO-d_6), a solvent widely used in NMR studies of peptides, including enkephalins, because of its fairly good physical approximation to transport fluid environments: its relative permittivity and viscosity are in the ranges of those of intersynaptic fluids. Furthermore, as a good hydrogen bond acceptor, DMSO induces inter- rather than intramolecular interactions, which may reveal intrinsic conformational preferences and may mimic the physical circumstances of receptor−ligand interactions.[40] ^{1}H NMR parameters including the chemical shifts (δ) of amide and aliphatic protons, and the intraresidue proton−proton geminal (^2J) and vicinal (^3J) coupling constants are reported in Table 5, together with the NH temperature coefficients. The ^{13}C NMR chemical shifts of all protonated carbons and the $^3J_{C\gamma H\alpha}$ coupling constants for the β-Me derivatives (**10** and **12**) are listed in Table 6.

The ^{1}H NMR spectrum of each peptide invloved only one set of signals, indicating the presence of one predominant isomer, and the ROESY spectra yielded no experimental evidence of a *cis*-peptide bond in any of the studied analogues. The 1*S*,2*R* configuration of Achc[2] in peptides **10**, **12**, **14**, and **16** was confirmed by the strong ROESY crosspeaks observed between H_α and H_β for each analogue. Interestingly, in the cases of **12** and **16**, ROESY crosspeaks were found between protons of the opposite termini, indicating a kind of bent backbone structure. However, such striking evidence was not seen for **10** and **14**. A complete list of the ROE assignments is given in the Supporting Information. The populations of the three staggered rotamers around the $C_\alpha-C_\beta$ bond were calculated for the aromatic side chain groups (Tyr, Dmt, *p*FPhe, and βMePhe). The *gauche*− and *trans* χ^1 rotamers could be assigned via the stereospecific assignments of the H_βs obtained from the 2D ROESY experiments. The calculated rotamer populations are given in Table 7. An exclusive predominance of the *trans* conformation was found for the Dmt[1] side chains, while for peptides **10** and **14** the three staggered conformers of the Tyr[1] side chain were almost equally populated. The Phe[3] side chain was found to prefer the *gauche*− conformational state in all peptides. For the *p*FPhe[4] and βMePhe[4] side-chains the *gauche*− and *gauche*+ conformers proved to be favored over the *trans* rotamer.

The three-dimensional structures of **10**, **12**, **14**, and **16** were built up by using the NMR distance restraints obtained from the ROESY spectra and were included throughout the structure refinement process. To avoid biasing the structures, only unambiguous, manually assigned ROESY crosspeaks were used in the calculations. A 1000-membered conformational ensemble was generated for the peptides by using distance geometry, and the unique geometries were then optimized by means of the AM1 method and analyzed after removal of the duplicate structures and structures with relative potential energies higher than 15 kcal/mol. As a result of this structure refinement process, 30, 25, 63, and 44 unique, low-energy structures were obtained for peptides **10**, **12**, **14**, and **16**, respectively. It was shown that the secondary structures of short peptides containing a β-amino acid differed from those of all α-amino acid-containing peptides. The backbone conformation could not be described by the classical secondary structural definitions alone; accordingly, a previously established noncanonical classification scheme was applied here.[16] In general, the backbone structures of the final low-energy conformers were found to be bent and the occurrence of random/extended structures was evanescent for all compounds.

Table 5. ^{1}H NMR Chemical Shifts (ppm), Coupling Constants (J in Hz), and NH Temperature Coefficients in Brackets (−ppb) for Compounds 10, 12, 14, and 16

peptide	Tyr^1 (NH_2) or Dmt^1 (NH_2)	Achc^2	Phe^3	(2*S*,3*S*)-βMePhe^4	*p*FPhe^4
			NH		
10		7.96	7.91	7.67	N/A
		$J_{NH\alpha}$ = 8.5 (6.3)	$J_{NH\alpha}$ = 8.2 (4.0)	$J_{NH\alpha}$ = 9.1 (5.6)	
12	8.33 (2.4)	7.46	7.76	7.69	N/A
		$J_{NH\alpha}$ = 8.7 (6.0)	$J_{NH\alpha}$ = 8.3 (4.1)	$J_{NH\alpha}$ = 9.1 (7.4)	
14	8.01 (1.5)	7.96	7.85	N/A	7.93
		$J_{NH\alpha}$ = 8.5 (6.0)	$J_{NH\alpha}$ = 8.3 (5.4)		$J_{NH\alpha}$ = 8.2 (7.0)
16	8.25 (2.1)	7.47	7.73	N/A	8.01
		$J_{NH\alpha}$ = 8.7 (6.1)	$J_{NH\alpha}$ = 8.3 (4.2)		$J_{NH\alpha}$ = 8.1 (7.0)
			H^{α}		
10	3.98	3.89[a]	4.33	4.53	N/A
	$J_{\alpha\beta}$ = 7.0, $J_{\alpha\beta'}$ = 7.2		$J_{\alpha\beta}$ = 4.2, $J_{\alpha\beta'}$ = 10.2	$J_{\alpha\beta}$ = 9.0	
12	3.91	3.85[a]	4.34	4.53	N/A
	$J_{\alpha\beta}$ = 12.0, $J_{\alpha\beta'}$ = 5.0		$J_{\alpha\beta}$ = 4.4, $J_{\alpha\beta'}$ = 10.4	$J_{\alpha\beta}$ = 8.4	
14	8.01 (1.5)	7.96	7.85	N/A	7.93
		$J_{NH\alpha}$ = 8.5 (6.0)	$J_{NH\alpha}$ = 8.3 (5.4)		$J_{NH\alpha}$ = 8.2 (7.0)
16	8.25 (2.1)	7.47	7.73	N/A	8.01
		$J_{NH\alpha}$ = 8.7 (6.1)	$J_{NH\alpha}$ = 8.3 (4.2)		$J_{NH\alpha}$ = 8.1 (7.0)
			H^{β}		
10	2.90$^{\beta}$, 2.84$^{\beta'}$	2.51[a]	2.78$^{\beta}$, 2.52$^{\beta'}$	3.14	N/A
	$J_{\beta\beta'}$ = 14.0		$J_{\beta\beta'}$ = 14.6	$J_{\beta\beta}$ = 7.2	
12	3.02$^{\beta}$ 2.87$^{\beta'}$	2.43[a]	2.81$^{\beta}$ 2.49$^{\beta'}$	3.20	N/A
	$J_{\beta\beta'}$ = 13.6		$J_{\beta\beta'}$ = 14.3	$J_{\beta\beta}$ = 7.1	
14	3.97	3.95[a]	4.47	N/A	4.44
	$J_{\alpha\beta}$ = 6.6, $J_{\alpha\beta'}$ = 7.3		$J_{\alpha\beta}$ = 4.1, $J_{\alpha\beta'}$ = 10.5		$J_{\alpha\beta}$ = 5.5, $J_{\alpha\beta'}$ = 8.5
16	3.89	3.89[a]	4.45	N/A	4.44
	$J_{\alpha\beta}$ = 12.6, $J_{\alpha\beta'}$ = 4.9		$J_{\alpha\beta}$ = 3.8, $J_{\alpha\beta'}$ = 10.7		$J_{\alpha\beta}$ = 4.9, $J_{\alpha\beta'}$ = 8.3
			$H^{\gamma\text{-}\varepsilon}$		
10		1.1−1.7			
12		1.0−1.5			
14		1.1−1.7			
16		1.0−1.5			
			H^{Me}		
10				1.22	N/A
12	2.17			1.20	N/A
14				N/A	
16	2.16			N/A	
			H^{Ar}		
10	7.04, 6.70		7.1−7.3	7.1−7.3	N/A
12	6.40		7.1−7.3	7.1−7.3	N/A
14	7.03, 6.69		7.1−7.3	N/A	7.27, 7.09
16	6.39		7.1−7.3	N/A	7.28, 7.09

[a] Note that in Achc H^{α} (C^{α}) is attached to NH, while H^{β} (C^{β}) is next to CO. Other Achc-ring Hs are labeled as γ−ζ.

ROESY experiments confirmed stable bend formation only for compounds **12** and **16**, although it is apparent that bent structures are energetically favored for all the studied compounds. Besides the very few random/extended structures, the following 10 structural elements were identified: a C_9-turn around Achc^2−Phe^3 (Figure 2A), a C_{11}-turn around Achc^2−Phe^3 (Figure 2B), a C_8-turn around Achc^2 (Figure 2C), a γ-turn around Phe^3 (Figure 2D,E), a C-terminal β-turn around Phe^3−βMePhe^4/*p*FPhe^4 (Figure 2F), a C_{12} loop formed by an H-bond between the N-terminal free amine, and the C═O group of Phe^3 (Figure 2G), a C_{14} loop formed by an H-bond between the C-terminal NH_2 group and the C═O group of Tyr^1/Dmt^1 (Figure 2H), and a bent structure stabilized by an H-bond between the N-terminal free amine and the C-terminal C═O group (Figure 2I). These structural components led to the identification of 7, 6, 8, and 9 possible conformational states

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Table 6. ^{13}C-NMR Chemical Shifts (ppm) for Peptides 10, 12, 14, and 16[a]

	C_α				C_β				C_γ + others			
	10	**12**	**14**	**16**	**10**	**12**	**14**	**16**	**10**	**12**	**14**	**16**
Tyr[1] or Dmt[1]	54.1	51.9	54.0	52.0	37.0	31.4	36.9	31.0				
Achc[2]	48.2	48.3	48.0	48.1	44.0	43.9	44.1	44.1	28.7; 6.6	28.5; 6.6	29.0; 6.2	28.6; 26.2
									23.1; 22.2	23.0; 22.2	22.6	22.6
Phe[3]	54.8	54.5	54.2	54.3	37.5	37.5	37.8	37.8				
βMePhe[4]or *p*FPhe[4]	57.8	57.8	54.2	54.3	41.7	41.7	37.2	37.2	19.3	19.3		

[a] **10**: ^{13}C for aromatic ring of Tyr (130.8/115.6), and Phe (126-130). $^3J_{C\gamma H\alpha}$= 1.2 ± 0.3 Hz in (2*S*,3*S*)βMePhe[4]. **12**: ^{13}C for aromatic ring and methyl of Dmt (115.3; 20.4), and Phe (126-130). $^3J_{C\gamma H\alpha}$= 1.4 ± 0.3 Hz in (2*R*,3*R*)βMePhe[4]. **14**: ^{13}C for aromatic ring of Tyr (130.8/115.7), *p*FPhe (131.5/115.3), and Phe (126-130). **16**: ^{13}C for aromatic ring and methyl of Dmt (115.3; 20.3), pFPhe (131.5/115.3), and Phe (126-130).

Table 7. Rotamer Populations[a] of Aromatic Side Chains in 10, 12, 14, and 16

	rotamer populations, %								
	Tyr[1] / Dmt[1]			Phe[3]			*p*F-Phe[4] /βMePhe		
peptides	P(g−)	P(t)	P(g+)	P(g−)	P(t)	P(g+)	P(g−)	P(t)	P(g+)
10	35	33	32	64	6	30	53	0	47
12	14	82	4	66	8	26	47	0	53
14	36	30	34	67	5	28	48[b]	19[b]	33
16	13	87	0	69	3	28	46[b]	13[b]	41

[a] Rotamer populations of aromatic side chains were calculated from the $J_{H\alpha H\beta}$ coupling constants. Rotamer populations of the βMePhe residue were assessed from the $J_{H\alpha H\beta}$ and $J_{H\alpha C\gamma}$ coupling constants in **10** and **12**. The stereospecific assignments of β-protons were deduced from the ROE patterns. [b] Interchangeable populations due to the ambiguous stereospecific assignment of β-protons.

for compounds **10**, **12**, **14**, and **16**, respectively, as presented in Table 8. The lowest-energy structure was found to be the C_{12} loop formed by an H-bond between the N-terminal free amine and the C=O group of Phe[3] for all the studied compounds. The χ^1 torsional angle of the Phe[3] side chain in the final low-energy structures was found to adopt a predominantly *gauche*− conformation, while the terminal aromatic side chains did not show any significant preference for any of the three rotameric states.

DISCUSSION

The introduction of Dmt, alicyclic β-amino acids, βMePhe, and *p*FPhe into different positions in the EMs resulted in proteolytically stable compounds with exceptionally high MOR affinity in some cases. Similar potencies were measured and no *cis/trans* isomerization of the amide bond was observed between the Dmt[1]/Tyr[1] and Achc[2] residues, in accordance with previous reports on *cis*-Achc[2]-containing EM analogues.[16] Compounds with *cis*-(1*S*,2*R*)Achc[2] residues were found to be more potent than those of the *cis*-(1*R*,2*S*)Achc[2] EMs, suggesting that the configuration of the Achc[2] residue plays a crucial role in orienting the pharmacophores in space. This is in agreement with earlier reports, where the configuration of the spacer residue was found to be decisive.[14−16] EM analogues containing *p*FPhe[4] retained the activity of the parent compound in receptor binding assays, confirming previous observations for derivatives incorporating *para*-halogenated amino acids.[24] Introduction of (2*S*,3*S*)βMePhe[4] resulted in an increased affinity and potency as compared with the parent ligands, while (2*R*,3*R*)βMePhe[4] substitution decreased the affinity and selectivity toward the MOR. These results confirm that the modulation of side chain rotations is a very important feature determining the biological activity of a peptide.[21,22] Combined application of Dmt[1], *cis*-(1*S*,2*R*)Achc[2] and *p*FPhe[4] resulted in the most potent analogue, with a 1 order of magnitude higher receptor affinity than that of EM-2. Furthermore, this compound proved to be the most active in the [^{35}S]GTPγS functional binding assays.

Most of the EM derivatives exhibited higher efficacies than those of the parent compounds. The [^{35}S]GTPγS binding assay results indicated that all the examined EM analogues retained agonist or partial agonist properties. The observed efficacy for compound **16** (E_{max} = 176%) was comparable with that of the prototypical MOR agonist DAMGO (E_{max} = 178%). The results of [^{35}S]GTPγS binding experiments in the presence of the general antagonist naloxone showed that the ligands bind to the opioid receptors and activate G-proteins and presumably downstream signaling. It is interesting that compounds **4**, **12**, and **16** stimulated G-protein binding even in the presence of 10^{-5} M naloxone. We therefore hypothesize that these ligands may bind to other G-protein-coupled receptors or a nonspecific opioid binding site.

In our earlier study, EM analogues in which Pro[2] was replaced by (1*S*,2*R*)-Acpc[2] were shown to adopt a bent structure more readily than (1*R*,2*S*)-Acpc-containing analogues. Moreover, the (1*S*,2*R*)-Acpc[2] analogues displayed significantly higher affinities for the MOR, suggesting that a higher tendency to form a bend or a turn may be important for receptor binding.[16] In the present study, (1*S*,2*R*)-Achc[2]-substituted analogues were found to act similarly. In addition to their exceptionally high MOR-binding affinity, a bent backbone structure was indicated in solution by NMR spectroscopy-based molecular modeling studies. This is in accordance with previous results, when potent opioid peptides were obtained via turn structure-inducing chemical constraints.[27,28] Various classical and noncanonical turn structures were identified as possible conformations of the analogues studied by the analysis of specific intramolecular H-bonds. Unique structural elements were identified as a consequence of the elongation of the peptide backbone by incorporation of a β-amino acid. As biological data suggest, this elongation does not significantly affect the folding of these peptides or the orientation of the pharmacophore elements, confirming the role of Pro[2] as a stereochemical spacer. Although the relative population of the different conformational families can not be given with our modeling data, the high occurrence of bent structures among the low-energy structures suggests

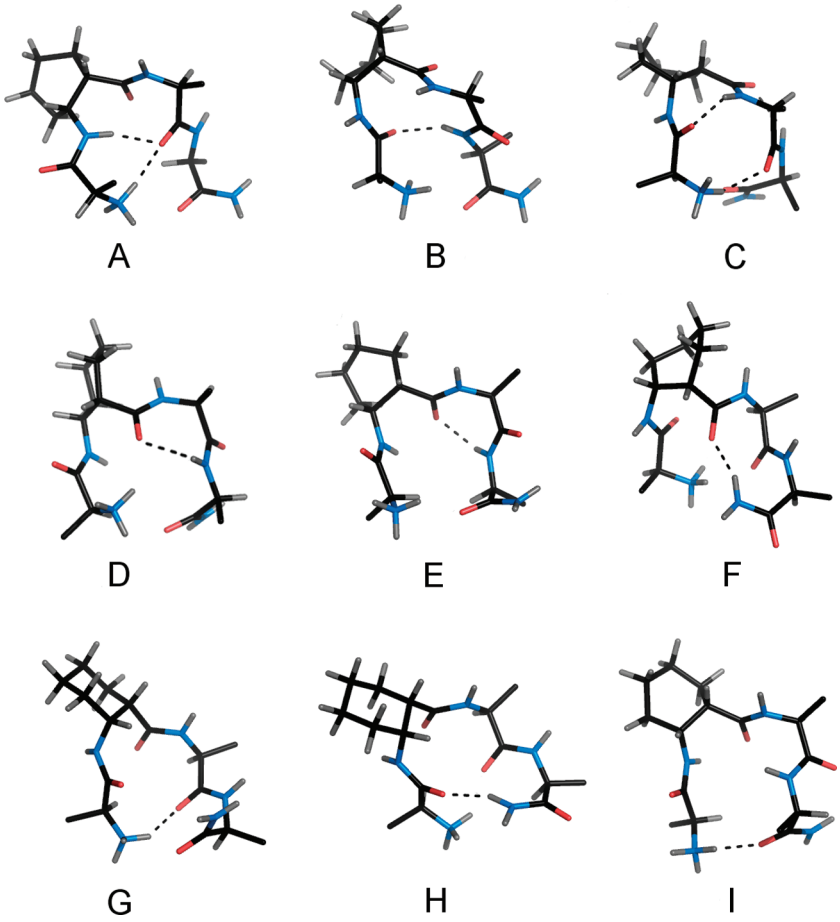

Figure 2. Folded structures identified for peptides **10**, **12**, **14**, and **16**. Aromatic side chains are omitted for clarity. (A) C_9-turn, (B) C_{11}-turn, (C) "classic" C_8-turn, (D) classic γ-turn, (E) inverse γ-turn, (F) C-terminal β-turn, (G) N-terminal C_{12}-loop, (H) C-terminal C_{14}-loop, (I) N to C-terminal loop.

Table 8. Conformations of Compounds 10, 12, 14, and 16 Identified via NMR Spectroscopy-Based Molecular Modeling

	compound			
conformation	**10**	**12**	**14**	**16**
random/extended	−	+	+	−
C_9-turn	+	+	+	+
C_{11}-turn	+	−	+	+
"classic" C_8-turn	+	−	+	+
classic γ-turn around Phe^3	+	+	−	+
inverse γ-turn around Phe^3	+	+	+	+
C-terminal β-turn	−	−	−	+
N-terminal C_{12}-loop	+	+	+	+
C-terminal C_{14}-loop	−	−	+	+
N- to C-terminal loop	+	+	+	+

that such a backbone conformation facilitates an advantageous orientation of the pharmacophores in terms of MOR binding. Most of the special, noncanonical secondary structural elements were found to be stabilized by intramolecular H-bonds. Aromatic−aromatic and proline−aromatic interactions may also play a significant role in the stabilization of such structures. Those interactions were not analyzed in this study, but there have been a number of reports signifying their importance in the stabilization of local structures in opioid and other peptides.[41,42] Dmt, *p*FPhe, and βMePhe incorporated into the sequence of the EMs modulated the distribution of χ^1 rotamers.[13,21,22] Here, the conformational preference of χ^1 rotamers (*gauche*−, *trans*, and *gauche*+) in the Dmt^1-substituted analogues, calculated from the measured $^3J_{H\alpha\text{-}H\beta}$ and $^3J_{H\alpha C\gamma}$ coupling constants, was found to be in perfect agreement with a recently proposed possible bioactive structure model of μ-opioid peptides. In this proposed bioactive structure, the appropriate orientation of the pharmacophore groups is provided by the *trans* χ^1 rotamer of Tyr^1/Dmt^1, the *gauche*− conformation of Phe^3 and the bent backbone structure.[33] An even distribution of χ^1 rotamers was found in the case of Tyr^1 analogues, but it did not diminish the MOR binding. However, a slight decrease in binding affinity was observed relative to the corresponding Dmt^1-substituted analogues. These findings suggest that an intrinsic propensity of the N-terminal aromatic side chain to adopt a *trans* conformation is advantageous but not a strict requirement for MOR binding. As long as this conformation is accessible upon receptor−ligand interaction, no significant loss of binding affinity should be expected. The preference for the *gauche*− conformation of the χ^1 torsional angle of Phe^3 was established for all four peptides studied in the present work, reinforcing previously proposed hypotheses relating to the role of this structural parameter.[31,33]

CONCLUSIONS

A small pool of EM analogues were synthesized by incorporating the unnatural amino acids Dmt[1], Achc[2], βMePhe[4], and *p*FPhe[4] in order to examine the pharmacological properties and bioactive structures of the EMs. The multiple structural modifications resulted in proteolytically stable and pharmacologically active analogues, presumably because of retention of the structural properties required for MOR binding. In general, the analogues containing Dmt[1] and (2*S*,3*S*)βMePhe[4] exhibited higher MOR affinities than those of the parent EMs. Of all the analogues, compound 16 displayed the highest affinity, with high MOR selectivity, and also the highest efficacy (E_{max} = 176%), which was comparable with that of DAMGO. The importance of the configurations of the alicyclic β-amino acids and βMePhe was confirmed by our data. Various possible conformations are proposed for compounds **10**, **12**, **14**, and **16** on the basis of NMR spectroscopic and the corresponding molecular modeling studies. Conformational studies on isolated molecules in the absence of their receptor do not necessarily provide unambiguous information on the bioactive structure. Nevertheless, intrinsic structural properties detected for isolated molecules are often found to correlate well with biological data and may reveal important structural properties regarding receptor binding. In the study presented here, the solution structures of the selected high-affinity MOR ligands were found to be in agreement with previous proposals of MOR ligand structure−activity relationships, in which the *trans* χ^1 rotamer of Tyr[1]/Dmt[1], the *gauche*− conformer of Phe[3], and the bent backbone structure are assumed to be responsible for the high MOR activity.[33]

EXPERIMENTAL SECTION

Materials and Methods. The Boc-protected amino acids (with the exception of βMePhe, it was prepared in our laboratory[22]) and 4-methylbenzhydralamine (MBHA) resin were purchased from Sigma-Aldrich Kft. (Budapest, Hungary) or from Bachem Feinchemikalen AG (Bubendorf, Switzerland). Coupling reagents were purchased from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland). Pure *cis*-(1*S*,2*R*)Achc was purchased from Peptides NeoMPS, USA, in optically pure form. Racemic *cis*-(1*S*,2*R*) Achc was generously provided by Prof. Ferenc Fülöp (University of Szeged, Hungary). TLC was performed on silica gel 60 F_{254} from Merck (Darmstadt, Germany) or on chiral TLC plates (Macherey-Nagel, Dürer, Germany) with the following solvent systems: (A) acetonitrile/methanol/water (4:1:1), (B) 1-butanol/acetic acid/water (4:1:1). Spots were visualized with ninhydrin reagent. Analyses and separations of all compounds were carried out with a complete HPLC system consisting of an L-7100 pump (Merck, KGaA, Darmstadt, Germany), a SIL-6B auto sampler (Shimadzu Co., Kyoto, Japan), a Shimadzu SCL-6B system controller, and a Merck l-7400 UV−vis detector, operating at 216 nm with a Hitachi D-7000 HPLC system manager. Samples were analyzed on an Altima analytical C_{18} column (250 mm × 4.6 mm, 5 μm) at a flow rate of 1 mL/min. Compounds were separated on a Vydac 218TP1010 (250 mm × 10 mm, 12 μm) with a flow rate of 4 mL/min. The mobile phase consisted of 0.1% (v/v) TFA in water and 0.08% (v/v) TFA in acetonitrile (Merck). Mass spectra were recorded on a VG Quattro II triple quadruple spectrometer (Micromass, Manchester, UK) with electron spray ionization.

[^{35}S]GTPγS (>1000 Ci/mmol) was obtained from the Institute of Isotopes Co., Ltd., Budapest, Hungary. [^{3}H]DAMGO (1.6 TBq/mmol, 43 Ci/mmol) and [^{3}H]Ile^{5,6}deltorophin II (1.5 TBq/mmol, 39 Ci/mmol) were prepared in our laboratory from the appropriate halogenated peptide derivatives.[43,44] Incubation mixtures were filtered by using a M24R Brandel cell harvester (Gaithersburg, MD). Filter-bound radioactivities were detected and measured in a TRI-CARB 2100TR liquid scintillation analyzer (Packard) with a biodegradable Optiphase Supermix cocktail developed by Perkin-Elmer, USA.

Solid-Phase Peptide Synthesis and Peptide Purification. The peptide analogues were synthesized by a manual solid-phase technique by using N-α-*t*-Boc-protected amino acids and MBHA resin to obtain C-terminal amides. Boc-βMePhe and in most cases Boc-*cis*-Achc were incorporated into the peptides in racemic form. Boc-*cis*-(1*S*,2*R*)-Achc was applied in optically pure form only in the βMePhe-containing analogues. *N*-Hydroxybenzotrioazole (HOBt, Novabiochem) and *N*,*N*-dicyclohexylcarbodiimide (DCC, Merck) were used as coupling reagents for peptide chain elongation, and coupling was monitored with the ninhydrin test. The protective groups were removed with a solution containing 50% TFA, 48% DCM and 2% anisole, followed by neutralization with 10% diisopropyl-ethylamine (DIEA) in DCM. The peptides were removed from the solid support with anhydrous HF (10 mL/g resin) in the presence of anisole (1 mL/g resin) and dimethylsulfide (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. Peptides were extracted with 30% (v/v) aqueous acetic acid. Crude peptides were obtained in solid form after lyophilization of the diluted extract (yields 70−80%). The diastereomers were separated by using semipreparative RP-HPLC and a Vydac 218TP1010 C_{18} column. Erythro (2*S*,3*S* and 2*R*,3*R*)βMePhe-containing peptides were purified by silica gel column chromatography because of the same RP-HPLC retention time of the diastereomers. The eluent used for compounds **2**−**5**, **11**, and **12** was 80:20 (v/v) DCM/MeOH and that for compounds **13** and **14** was 70:30 (v/v) EtOAC/AcOH. Then peptides were subjected to RP-HPLC purification. Purity of peptides was determined by analytical RP-HPLC. The molecular weights of the peptides were confirmed by ESI-MS and HRMS. HRMS spectra were collected on a Waters QTOF Premier mass spectrometer (Waters Corporation, Milford, MA), using direct infusion to the nanoelectronspray source. Samples were dissolved in 0.1% (v/v) formic acid/20% (v/v) acetonitrile/water solvent to a final concentration of ∼0.01 mg/mL. The mass spectra were collected in the 50−990 Da range; the spectrometer was calibrated by using the MS/MS fragments of a 100 fmol [Glu[1]]-fibrinopeptide B/μL solution. The identities of all peptides were confirmed and their purities were found to be >98%.

Determination of the Configuration of 2-Aminocyclohexanecarboxylic Acid (Achc) in Peptides. The configurations of the incorporated Achc were determined after GITC derivatization of the acidic hydrolysates of the peptides (for acidic hydrolysis: 1 mg of peptide, 1 mL of 6 M HCl, 24 h, 110 °C) followed by analytical HPLC analysis. The retention times of the derivatized alicyclic β-amino acid were compared with those of the derivatized β-amino acid standards.[35]

Determination of the Configuration of βMePhe in Peptides. One milligram of each diastereomeric peptide was hydrolyzed separately in 1 mL of 6 M HCl solution under N_2 pressure at 110 °C for 24 h. The solvent was then removed by evaporation, and the mixture of amino acids was analyzed by chiral TLC in acetonitrile/methanol/water (4:1:1). The R_f values were compared with those of standard optically pure βMePhe isomers.[34]

Rat Brain Membrane Preparation. The preparation of the rat brain membrane homogenate has been described in detail elsewhere.[16,45] Briefly, rats (male, Wistar, 250−300 g body weight) kept on a standard diet and water were sacrificed, and the whole brains minus the cerebellum were dissected and then homogenized. The homogenate was centrifuged at 20000*g* for 25 min, and the supernatant was discarded. The resulting pellet was resuspended in ice cold Tris-HCl buffer (50 mM, pH 7.4), followed by incubation at 37 °C for 30 min to remove endogenous ligands. The centrifugation was then repeated. The final pellets were taken up in 50 mM Tris-HCl (pH 7.4) buffer containing 0.32 M sucrose and stored at −80 °C.

Sucrose was removed before the experiments. The pellet was resuspended in an appropriate volume of the reaction buffer and was used for

binding experiments. The protein content of the membrane preparation was determined by the method of Bradford, with bovine serum albumin as standard.[46]

Radioligand Binding Assay. A more comprehensive description of the competition binding experiments is to be found elsewhere.[47] Briefly, heterologue competition binding experiments were performed by incubating rat brain membranes (0.2−0.5 mg protein/tube) with 1 nM [^{3}H]DAMGO or 2 nM [^{3}H]Ile^{5,6}-deltorphin II and 10^{-10}−10^{-5} M unlabeled ligands for various times. The total bindings were corrected for the nonspecific bindings measured with 10 μM naloxone in order to obtain the specific binding values. The filter-bound radioactivities were measured in an Optiphase Supermix scintillation cocktail by using a TRI-CARB 2100TR liquid scintillation counter (Canberra-Packard, Perkin-Elmer Life Sciences, Boston, MA), and were analyzed with the GraphPad Prism software (version 4.0, San Diego, CA).

Ligand-Stimulated [^{35}S]GTPγS Functional Binding Assay. The experimental procedure has been detailed elsewhere.[45] Briefly, rat brain membranes (10−15 μg protein/tube) were incubated with 0.05 nM [^{35}S]GTPγS and 10^{-10}−10^{-5} M unlabeled ligands in the presence of 30 μM GDP in Tris-EGTA buffer (50 mM Tris-HCl, 100 mM NaCl, 3 mM $MgCl_2$ and 1 mM EGTA, pH 7.4) at 30 °C for 60 min in the absence or presence of naloxone (10^{-5} M). Basal binding was determined in the absence of ligands and set at 100%. Nonspecific binding was measured with 10 μM unlabeled GTPγS. The radioactivity was counted in an Optiphase Supermix cocktail with a TRI-CARB 2100TR liquid scintillation counter (Canberra-Packard, Perkin-Elmer Life Sciences).

Determination of the Half-Lives. The half-lives of the EM analogues were determined as described earlier.[7] Briefly, 20 μL of 1 mM peptide stock solution in 50 mM Tris-HCl buffer (pH = 7.4) was digested with 180 μL of rat brain homogenate (protein concentration: 5.0 mg/mL) and the mixtures were incubated at 37 °C. Aliquots of 20 μL were withdrawn from the incubation mixtures, and the degradation reaction was halted by immediate acidification with 25 μL of 0.1 mM aqueous HCl solution. After centrifugation (11340g, 5 min, 25 °C), 10 μL of the supernatant obtained was analyzed by RP-HPLC. The degradation rate constants (k) were determined by least-squares linear regression analysis of logarithmic tetrapeptide peak area [$(\ln(A/A_o)$] vs time courses, with at least 5 time points. The rate constants obtained were used to establish the degradation half-lives ($t_{1/2}$) as ln 2/k.

NMR Spectroscopy. Approximately 10 mg of compound **10**, **12**, **14**, or **16** was dissolved in 0.6 mL of DMSO-d_6 (Cambridge Isotopes). All NMR spectra were acquired at 305 K by using an inverse multinuclear (bbi) single-axis gradient 5 mm probe. Proton and carbon chemical shifts were referenced directly to the internal DMSO peak at 2.49 and 39.5 ppm, respectively. TOCSY and ROESY spectra were recorded in phase-sensitive mode with mixing times of 60 and 150 ms, respectively. TOCSY experiments were carried out with 4096 data points in f_2 and 512 data points in f_1, and 16 scans were collected at each increment. The MLEV-17 mixing sequence was flanked by simultaneously switched spin-lock and gradient pulses to obtain the signals of the pure absorption phase for coupling constant measurements. ROESY experiments were performed with 2048 data points in f_2 and 512 data points in f_1, and 64 scans were collected at each increment. The ^{1}H chemical shifts were assigned by following the standard protocol of Wüthrich,[48] through 2D TOCSY and ROESY experiments.[49] The ^{13}C chemical shifts of protonated carbons were assessed on the basis of the gradient-enhanced HSQC experiment.[50] Proton−proton scalar coupling constants were measured from the 1D ^{1}H NMR and/or 2D TOCSY spectra. In the latter case, the corresponding rows extracted from the TOCSY spectra were inversely Fourier transformed and then zero-filled to 16 K real data points. A Gaussian function was applied prior to the Fourier transform, and the final digital resolution of the resulting 1D traces was ∼0.3 Hz. The coupling constants $^3J_{H\alpha\text{-}H\beta}$ were used to estimate the side-chain rotamer populations along the χ^1 angles in Tyr, Dmt and Phe via Pachler parametrization of the Karplus equation,[51,52] with parameters appropriate for aromatic residues ($^{ap}J_{H\alpha\text{-}H\beta}$ = 13.9 Hz and $^{sc}J_{H\alpha\text{-}H\beta}$ = 3.55 Hz). The stereospecific assignment of β-protons was deduced from the ROE patterns. For βMePhe residues, such rotamer populations were assessed from the $J_{H\alpha H\beta}$ and $J_{H\alpha C\gamma}$ coupling constants.[53] The relevant $J_{H\alpha C\gamma}$ coupling constants were obtained from gradient- and sensitivity-enhanced X(ω_1) half-filtered TOCSY[54] and/or HECADE[55] experiments, using a DIPSI-2 mixing time of 60−70 ms for efficient TOCSY transfer. Proton−carbon coupling constants were measured from the tilt of the E. COSY multiplets in the F2 acquisition dimension with an accuracy of 0.4 Hz. The volumes of ROESY crosspeaks were converted into distance bounds by using the intensities of the Phe^3-H_β,$H_{\beta'}$ peaks for calibration.

Computational Methods. The TINKER 4.2 program package[56] was used to perform distance geometry calculations, which included ROESY data-derived distance restraints with a ±10% tolerance interval. All peptide bonds were constrained to the *trans* configuration. For each peptide, 1000 structures were generated and the resultant structures were clustered to identify and eliminate duplicate structures from further analysis. Clustering was performed with the g_cluster utility of the GROMACS 4.0.5 program package[57] and the gromos[58] method with a 0.5 Å rmsd similarity cutoff. The positions of C_β, C_γ, and main chain atoms were compared via least-squares fitting. The C_β and C_γ atoms were included in the analysis in order to differentiate between the rotamers of the aromatic side chains. Only the middle structures of the resultant clusters were subjected to further analysis. These geometries were optimized by using the AM1 method implemented in the Gaussian 03 program package.[59] Optimized geometries with a relative potential energy of ≤15 kcal/mol were analyzed further. This relative potential energy cutoff was established on the basis of previous results for potential energy difference between different conformations of peptides of this size.[60] The pool of optimized, low-energy structures was clustered again, similarly as above, to identify and eliminate structures which converged to the same local energy minimum during geometry optimization. The middle structures of the resultant clusters, which were regarded as possible geometries of the studied peptides, were analyzed one by one, using the Pymol 0.99rc6 molecular visualization and analysis program. Structural classifications were carried out after the identification of intramolecular H-bonds with the following criteria: the cutoff distance between a hydrogen and an acceptor atom was set to 2.2 Å, and 60° was taken as the cutoff for the donor-hydrogen-acceptor angle.

Data Analysis. Receptor binding assay data are reported as means ± SEM of at least three independent measurements, each performed in duplicate. Inhibitory constants (K_i, nM) were calculated from the competition experiments by using nonlinear least-squares curve fitting and the Cheng−Prusoff equation[61] with GraphPad Prism software (version 4.0, San Diego, CA). The percentage stimulation of the specific [^{35}S]GTPγS binding over the basal activity is reported as the mean ± SEM. Each measurement was performed in triplicate and analyzed with the sigmoid dose−response curve-fitting option of the GraphPad Prism software (version 4.0, San Diego, CA) to obtain potency (ED_{50}) and efficacy (E_{max}) values.

■ ASSOCIATED CONTENT

Supporting Information. Chromatograms of the purified peptide diastereomers; RP-HPLC, TLC, and HRMS data on all diastereomers and EMs; ROE assignments for **10**, **12**, **14**, and **16**; number of possible geometries remaining after each step of the computational analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

Acpc, 2-aminocyclopentanecarboxylic acid; Achc, 2-aminocyclohexanecarboxylic acid; BBB, blood−brain barrier; Boc, *tert*-butyloxycarbonyl; CNS, central nervous system; DAMGO, H-Tyr-D-Ala-Gly-NMePhe-Gly-ol; Ile^{5,6}-deltorphin-2, H-Tyr-D-Ala-Phe-Glu-Ile-Ile-Gly-NH_2; DCC, *N*,*N*-dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DOR, δ-opioid receptor; DMSO, dimethylsulfoxide; Dmt, 2′,6′-dimethyltyrosine; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*,*N*-tetraacetic acid; ESI-MS, electron spray ionization mass spectrometry; EM-1, H-Tyr-Pro-Trp-Phe-NH_2; EM-2, H-Tyr-Pro-Phe-Phe-NH_2; GITC, 2,3,4,6-tetra-*O*-acetyl-β-glucopyranosyl isothiocyanate; GDP, guanosine-5′-diphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; GPCR, G-protein-coupled receptor; HRMS, high-resolution mass spectrometry; HOBt, 1-hydroxybenzotrioazole; MOR, μ-opioid receptor; MBHA, 4-methylbenzhydrylamine; βMePhe, β-methylphenylalanine; NMR, nuclear magnetic resonance spectroscopy; NOE, nuclear Overhauser effect; *p*FPhe, *para*-fluorophenylalanine; ΨPro, pseudoproline; ROESY, rotating-frame Overhauser effect spectroscopy; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TIC, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy

■ ADDITIONAL NOTE

The abbreviations and definitions in this manuscript are in line with those recommended by the IUPAC-IUB Commission of Biochemical Nomenclature.

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