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Structure–Activity Study on the Spatial Arrangement of the Third Aromatic Ring of Endomorphins 1 and 2 Using an Atypical Constrained C Terminus

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The discovery of endomorphins (EMs) has opened the possibility of searching for new analgesics. However, the design of peptide analgesics has proven to be very difficult as a result of their conformational flexibility and a lack of clarity in structure–activity relationships (SAR). In EMs, the amino acid side chains exhibit considerable conformational flexibility, especially in the third aromatic ring, which is free to adopt a bioactive conformation. To resolve these problems, a series of C terminus EM analogues, [Xaa^4-R]EMs, modified through the substitution of Phe^4 with nonaromatic residues and termination with benzyl groups, were designed to generate conformational constrains of the third aromatic ring by amide bond and torsion angles (ϕ_4 and ψ_4) of Xaa^4. Introduction of (S)-α-methyl or (S)/(R)-α-carboxamide on the methylene unit of the benzyl group was designed to produce an atypical topographical constraint (ϕ_5) of the third aromatic ring rotation. Interestingly, some EM derivatives, with elimination of the C-terminal carboxamide group and significant changes in the address sequence (Phe^4-NH$_2$), still exhibited higher μ-opioid receptor (MOR) affinity than unmodified EMs. In contrast, some analogues with incorrectly constrained C termini displayed very low affinity and pharmacological activities. Thus, our results indicate that these EM analogues, with atypical constrained C termini, provide model compounds with potent MOR agonism. They also give evidence that the proper spatial orientation and conformational restriction of the third aromatic ring are crucial for the interaction of EMs with MOR.

Introduction

Opioid peptides have been studied extensively since their discovery, and many efforts have been dedicated to the determination of their intrinsic nature. However, the conformational flexibility of opioid peptides has hampered numerous attempts at determining the relationship between their structure and activity. Insight into the conformational requirements of peptide binding has been obtained through the synthesis of analogues[1,2] or peptide mimics[3–5] with a more rigid backbone scaffold, or by studies in media that promote structural stability such as viscous solvents,[6,7] lipids,[8] lyotropic liquid crystals,[9] aqueous/organic mixtures,[10] and membrane-mimicking environments.[11] An approach to determine these conformations by conformational constraint of the peptide backbone template (ϕ and ψ angles) and topographical constraint (χ^1, χ^2, etc.) of the side chains was described by Hruby et al.[12–14] It has been demonstrated that small changes in structure or in a single torsional angle are sufficient to dramatically modify complex behaviors.[15–17] This has been critical for peptide ligand design. Conformationally and topographically constrained peptide and peptide mimics[18,19] can provide critical insight into the preferred "biologically active" conformation[14] and have unique biophysical and biological properties.

The endogenous peptide ligands for MOR, endomorphin-1 (Tyr^1-Pro^2-Trp^3-Phe^4-NH$_2$; EM1), and endomorphin-2 (Tyr^1-Pro^2-Phe^3-Phe^4-NH$_2$; EM2), were initially discovered by Zadina et al.[20] These EMs exhibited the highest affinity for the MOR and extraordinarily high selectivity relative to the δ- and κ-opioid receptor systems of all known opioid substances.[20] Furthermore, these tetrapeptides have a strong antinociceptive effect on acute pain, similar to that of morphine. They are also more effective than the majority of the opioid peptides against neuropathic pain even at low doses, opening the possibility of

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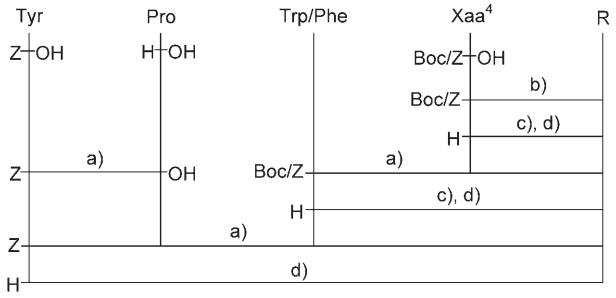
using the two peptides as drugs.[21] Therefore, it is meaningful to design and synthesize EM analogues to search for new analgesics and to study the structure–activity relationship (SAR) of MOR ligands. According to the "message-address" concept,[22] it is possible to consider that Tyr-Pro-Trp/Phe and Phe-NH_2 correspond to the message and address fragments, respectively.[23] The C-terminal address fragment Phe^4-NH_2 plays an important role in ligand recognition.[23–26] The replacement of Phe^4 by β-Phe,[27] L/D-Dmp,[28] aromatic, heteroaromatic, or aliphatic groups[29] considerably decreased affinity and selectivity toward the receptor; however, incorporation of (2*S*,3*S*)-β-MePhe^4 resulted in analogues with μ-opioid affinities fourfold higher than the parent peptides.[17]

To get insight into the important role of the third aromatic ring arrangement, we report herein the modification of the C termini of EM analogues ([Xaa^4-R]EMs) by substitution of the Phe^4 group with nonaromatic residues and by termination with benzyl groups. Such analogues are thus designed to generate conformational constrains of the third aromatic ring by amide bond and torsion angles (ϕ_4 and ψ_4) of Xaa^4. Introduction of (*S*)-α-methyl or (*S*)/(*R*)-α-carboxamide on the methylene unit of the benzyl group was designed to produce atypical topographical constraint (ϕ_5) of the third aromatic ring rotation (Figure 1). Investigations of these structures and bioactivities are described below.



Scheme 1. Synthesis of [Xaa^4-R]EMs: a) HOSu, DCC, THF/NaOH (4 N); b) DMAP, DCC, CH_2Cl_2/DMF; c) HCl/EtOAc; d) Pd/C, MeOH. Xaa^4 = Gly, Ala, β-Ala, Sar, D-Pro, D-Val, or D-Ala, R = benzylamine or (*S*)-α-methylbenzylamine.

mediates were characterized by TLC, ^{1}H NMR, and ESI-TOF MS. Deprotection of Boc was performed using HCl in EtOAc, and for deprotection of the Z group, catalytic hydrogenation over Pd/C was employed. All final products were easily purified by silica gel column chromatography, after which the purified peptides were crystallized from a solution of MeOH/EtOAc/PE and characterized by RP HPLC, TLC, ESI-TOF MS, $[\alpha]_D$, mp, elemental analysis and NMR. Purities were determined to be 95–99% by analytical RP HPLC. Data are reported in Table 1 or in the Supporting Information.

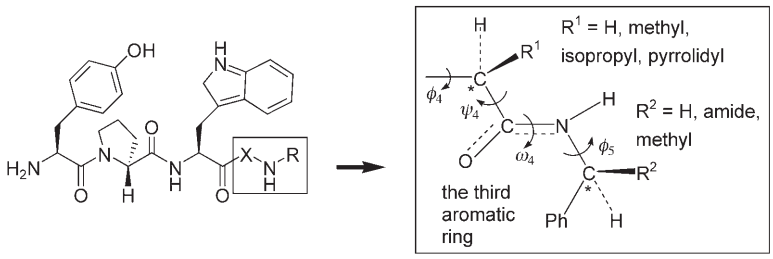


Figure 1. Design of EM1 analogues (similar C-terminal modifications were also applied to EM2 analogues **16**–**27**).

Results

Synthesis of EM analogues with atypical C termini

EMs and their analogues were synthesized by solution-phase methods and are detailed in Figure 1. The peptide bond between Xaa^4 and benzylamine or (*S*)-α-methylbenzylamine was formed by a DMAP/DCC coupling method. The N-terminal dipeptide (Z-Tyr-Pro-OH or Z-Tyr-D-Ala-OH) and C-terminal dipeptides or tripeptides were prepared by HOSu ester (the protecting groups of H-Pro-OH and H-D-Ala-OH were not required in the progress of synthesis).[30] After synthesis of fragments, peptide coupling between the N- and C-terminal fragments was performed by an active ester method (Scheme l), and DCC/HOSu were used as coupling agents. Protected peptide inter-

Opioid receptor affinity and selectivity

The affinity and selectivity of EM analogues were evaluated by radioligand binding assays using rat brain membranes. In the binding assays [H^3]DAMGO and [H^3]DPDPE were used as μ- and δ-opioid receptor radioligands, respectively. EM1 and EM2 were also characterized for comparison, and the affinity values are in agreement with those published previously ($K_i(\mu)$ = 4.55 and 8.23 nM, respectively).[17,25]

The opioid receptor binding properties of the new EM analogues are summarized in Table 2. The EM1 analogue in which Phe^4 was replaced by Gly and terminated with benzyl (compound **2**) exhibited about 12-fold lower μ affinity than EM1. However, a similarly modified analogue, [Gly^4-NH-Bn]EM2 (**16**), exhibited only about threefold lower μ affinity than EM2. Moreover, both **2** and **16** displayed high μ selectivity ($K_i(\delta)/K_i(\mu)$ = 154 and 328, respectively). N-Methylation of Gly^4 of analogues **2** and **16** (to give compounds **5** and **19**) resulted in about four- and twofold higher μ affinity than **2** and **16**. Despite the enhanced conformational freedom given by the extra carbon atom, [β-Ala^4-NH-Bn]EM1 (**4**) and [β-Ala^4-NH-Bn]EM2 (**18**) displayed only marginal differences in μ affinity and selectivity compared with analogues **2** and **16**. However, a drastic loss of

Table 1. Analytical data for EMs and EM analogues with modified (Xaa^4-R) C termini.

Peptide	Sequence	TLC[a] (I)	TLC[a] (II)	RP HPLC[b]	$[\alpha]_D$ [°][c]	TOF MS [M+1] calcd	TOF MS [M+1] found	mp [°C]
1	Tyr-Pro-Trp-Phe-NH_2	0.23	0.57	19.19	−27	611	611.3	144–146
2	Tyr-Pro-Trp-Gly-Bn	0.25	0.59	19.69	−11	611	611.3	129–131
3	Tyr-Pro-Trp-Ala-Bn	0.27	0.69	20.41	−20	625	625.3	141–143
4	Tyr-Pro-Trp-β-Ala-Bn	0.24	0.63	19.98	−28	625	625.3	118–120
5	Tyr-Pro-Trp-Sar-Bn	0.27	0.67	20.13	−2	625	625.3	124–125
6	Tyr-Pro-Trp-D-Pro-Bn	0.22	0.46	20.36	+2	651	651.3	149–151
7	Tyr-Pro-Trp-D-Val-Bn	0.32	0.62	21.93	−21	653	653.3	145–146
8	Tyr-Pro-Trp-D-Ala-Bn	0.29	0.57	20.13	−19	625	625.3	121–124
9	Tyr-Pro-Trp-D-Ala-(*S*)-α-Me-Bn	0.26	0.57	20.13	−49	639	639.3	133–135
10	Tyr-Pro-Trp-D-Ala-Phe-NH_2	0.20	0.56	19.23	−9	682	682.3	138–140
11	Tyr-Pro-Trp-D-Val-D-Phg-NH_2	0.23	0.56	20.24	−25	696	696.3	164–166
12	Tyr-Pro-Trp-D-Val-Phg-NH_2	0.23	0.56	20.12	−63[d]	696	696.3	198–201
13	Tyr-Pro-Trp-Gly-Ph	0.31	0.62	20.17	−16	597	597.3	130–133
14	Tyr-D-Ala-Trp-D-Val-Bn	0.57	0.79	22.28	+5[e]	627	627.3	194–195
15	Tyr-Pro-Phe-Phe-NH_2	0.29	0.58	18.53	−23	572	572.3	130–131
16	Tyr-Pro-Phe-Gly-Bn	0.30	0.60	19.26	−21	572	572.3	114–116
17	Tyr-Pro-Phe-Ala-Bn	0.29	0.70	20.03	−7	586	586.3	149–151
18	Tyr-Pro-Phe-β-Ala-Bn	0.26	0.72	19.53	−56	586	586.3	207–208
19	Tyr-Pro-Phe-Sar-Bn	0.29	0.69	19.97	−9	586	586.3	110–112
20	Tyr-Pro-Phe-D-Pro-Bn	0.24	0.55	19.95	+14	612	612.3	142–144
21	Tyr-Pro-Phe-D-Val-Bn	0.38	0.63	20.84	−12	614	614.3	116–117
22	Tyr-Pro-Phe-D-Ala-Bn	0.30	0.61	19.54	−14	586	586.3	102–104
23	Tyr-Pro-Phe-D-Ala-(*S*)-α-Me-Bn	0.32	0.62	19.45	−47	600	600.3	123–125
24	Tyr-Pro-Phe-D-Ala-Phe-NH_2	0.23	0.58	18.82	−13	643	643.3	131–133
25	Tyr-Pro-Phe-D-Val-D-Phg-NH_2	0.24	0.60	19.65	−35	657	657.3	192–193
26	Tyr-Pro-Phe-Gly-Ph	0.34	0.66	19.85	−18	558	558.3	125–127
27	Tyr-D-Ala-Phe-D-Val-Bn	0.59	0.80	22.27	+17[f]	588	588.3	193–195

[a] Retention factors on silica gel 60 F_{254} precoated glass plates; solvent system: (I) EtOAc/MeOH/aq. NH_3 (25:5:1), (II) $(CH_3)_2O$/glacial HOAc/H_2O (8:1:1). [b] t_R with Delta–Park C_{18} column (3.9 mm × 150 mm), A/B = 90:10 to A/B = 10:90 for 30 min, A/B = 10:90 to A/B = 90:10 for 5 min. [c] $c = 0.3$, MeOH. [d] $c = 0.1$, MeOH. [e] $c = 0.1$, DMF. [f] $c = 0.3$, DMF.

μ affinity and selectivity were found when a chiral residue (L-Ala) was introduced into the Xaa^4 position (peptides **3** and **17**). Derivatives **3** and **17**, in which Xaa^4 residue has *S* stereochemistry, exhibited distinctly low μ affinity, 23- and 15-fold lower than that of their respective parent compounds. Interestingly, stereochemical inversion at Ala^4 of analogues **3** and **17** markedly increased μ affinity. [D-Ala^4-NH-Bn]EM1 (**8**) and [D-Ala^4-NH-Bn]EM2 (**22**) exhibited 22- and 14-fold higher μ affinity ($K_i(\mu) =$ 4.56 and 8.67 nM, respectively) relative to their diastereomers. Furthermore, D-Val substitution at D-Ala^4 of **8** and **22** further increased μ affinity and δ affinity. The μ affinity of [D-Val^4-NH-Bn]EM1 (**7**), which is the most potent EM1 analogue, increased about twofold ($K_i(\mu) = 2.32$ nM), and δ affinity increased 1.6-fold over its parent, consequently giving a slight increase in μ selectivity ($K_i(\delta)/K_i(\mu) = 1417$). Although both μ and δ affinity of the most potent EM2 derivative **21** increased in binding assays, the selectivity decreased about threefold ($K_i(\delta)/K_i(\mu) =$ 676) relative to that of EM2. On the other hand, probably due to the presence of *trans* and *cis* conformations in the C terminus generated by introduction of D-Pro at the Xaa^4 position, [D-Pro^4-NH-Bn]EM1 (**6**) and [D-Pro^4-NH-Bn]EM2 (**20**) exhibited about 13- and fourfold less potent than their parent compounds, respectively. Notably, [D-Ala^2, D-Val^4-NH-Bn]EM2 (**27**) displayed the highest δ affinity ($K_i(\delta) = 71.33$ nM), 206-fold higher than that of the parent, among all the peptides synthesized. Simultaneous substitution of D-Ala for Pro^2 and conversion of Phe^4-NH_2 to D-Val^4-NH-Bn did not markedly alter the μ affinity of derivative **14**, which still displays high affinity, similar to that of EM1.

To generate topographical constraints of the third aromatic pharmacophore, (*S*)-methyl (in **9** and **23**) or (*S*)/(*R*)-carboxamide (in **11**, **12**, and **25**) groups were introduced on the methylene unit of the benzyl group. All the derivatives modified with the above-mentioned methods exhibited lower μ affinity than their parents. Methylation *S*-configured derivatives **9** and **23** displayed slightly lower μ affinity than their parents; however, both (*S*)-**12**, and (*R*)-**11** and (*R*)-**25** (carboxamide) introductions significantly decreased μ affinity and selectivity. Introduction of an extra carbon atom between the phenyl and carboxamide groups, given by replacement of Phg^5-NH_2 with Phe^5-NH_2, produced [D-Ala^4-Phe^5-NH_2]EM1 (**10**), which exhibited about fivefold higher μ affinity than derivatives **11** and **12**. Elimination of the methylene unit of the benzyl group, in peptides **13** and **26**, resulted in a significant decrease in μ affinity ($K_i(\mu) = 216.4$ and 256.95 nM, respectively).

Pharmacological activity in vitro

Pharmacological activities were evaluated in vitro using isolated guinea pig ileum (GPI) for the μ-opioid receptor and mouse vas deferens (MVD) for the δ-opioid receptor. EM1 and EM2 were also subjected to GPI and MVD bioassays for comparison. The potencies of new EM analogues to inhibit an electrically evoked neurotransmitter release and the resulting muscle contractions in GPI and MVD preparations are summarized in Table 2. All derivatives exhibited agonism below 1 μM in GPI assays except peptides **3**, **11**, **12**, and **25**. In particular, [D-Val^4-NH-Bn]EM1 (**7**) and [D-Val^4-NH-Bn]EM2 (**21**) displayed potent agonism toward MOR ($IC_{50} < 10$ nM), about four- and threefold higher than that of their parents, respectively. In the GPI assay, [D-Ala^4-NH-Bn]EM1 (**8**) displayed higher potency than its parent, although it exhibited similar affinity to EM1 in radioligand binding assays. However, the potency of analogues **3**, **11**, **12**, and **25** was observed to nearly disappear in the GPI assays, although they exhibited fairly potent affinity toward MOR in radioligand binding assays. Furthermore, despite similar or de-

Table 2. Opioid receptor binding affinities[a] and in vitro pharmacological activity of EMs and EM analogues.

Compd	Peptide	$K_i(\mu)$ [nM][b]	$K_i(\delta)$ [nM][c]	$K_i(\delta)/K_i(\mu)$	IC_{50} [nM][d] GPI	IC_{50} [nM][d] MVD	GPI/MVD[e]
1	EM1	4.55 ± 0.16	5093 ± 660	1121	11.9 ± 0.68	42.8 ± 9.1	0.26
2	[Gly^4-NH-Bn]EM1	56.2 ± 7.0	8690 ± 767	154	1209 ± 175	68.3 ± 25.6	17.7
3	[Ala^4-NH-Bn]EM1	102 ± 6.6	6263 ± 634	61	> 10000	3764.76 ± 271	–
4	[β-Ala^4-NH-Bn]EM1	35.6 ± 2.6	4735 ± 360	133	248 ± 52	> 10000	–
5	[Sar^4-NH-Bn]EM1	14.2 ± 2.1	4684 ± 21	343	374 ± 78	195.78 ± 7.86	1.91
6	[D-Pro^4-NH-Bn]EM1	57.5 ± 5.1	722 ± 50.2	13	8424 ± 1317	> 10000	–
7	[D-Val^4-NH-Bn]EM1	2.32 ± 0.15	3287 ± 456	1417	3.06 ± 0.15	4.36 ± 0.56	0.70
8	[D-Ala^4-NH-Bn]EM1	4.56 ± 0.84	906 ± 112	199	5.2 ± 1.03	34.5 ± 6.6	0.15
9	[D-Ala^4-NH-α-Me-Bn]EM1	17.8 ± 1.5	765 ± 74	43	379 ± 80	167 ± 27	2.27
10	[D-Ala^4-Phe^5-NH_2]EM1	11.6 ± 1.2	1959 ± 141	168	271 ± 63	163 ± 17	1.66
11	[D-Val^4-D-Phg^5-NH_2]EM1	57.8 ± 6.9	16230 ± 1527	281	> 10000	> 10000	–
12	[D-Val^4-Phg^5-NH_2]EM1	55.5 ± 7.3	3478 ± 114	63	> 10000	> 10000	–
13	[Gly^4-NH-Ph]EM1	216 ± 31	13706 ± 1939	63	5006 ± 631	> 10000	–
14	[D-Ala^2, D-Val^4-NH-Bn]EM1	4.99 ± 0.9	2405 ± 327	482	–	–	–
15	EM2	8.23 ± 0.48	14670 ± 1868	1782	9.67 ± 0.84	25.8 ± 5.2	0.37
16	[Gly^4-NH-Bn]EM2	22.1 ± 2.2	5313 ± 213	328	99.8 ± 10.4	98.3 ± 12.6	1.02
17	[Ala^4-NH-Bn]EM2	122 ± 17	9757 ± 992	79	1055 ± 245	1513 ± 234	0.696
18	[β-Ala^4-NH-Bn]EM2	55.1 ± 5.6	10630 ± 563	193	377 ± 79	> 10000	–
19	[Sar^4-NH-Bn]EM2	13.2 ± 0.2	6316 ± 1438	479	191 ± 32	96.7 ± 22.1	1.97
20	[D-Pro^4-NH-Bn]EM2	30.6 ± 4.7	1302 ± 153	43	78.6 ± 10.2	111 ± 15	0.7153
21	[D-Val^4-NH-Bn]EM2	4.97 ± 1.24	3358 ± 414	676	3.09 ± 0.65	5.43 ± 0.61	0.56
22	[D-Ala^4-NH-Bn]EM2	8.67 ± 1.27	2860 ± 187	330	34.6 ± 6.2	42.5 ± 5.5	0.81
23	[D-Ala^4-NH-α-Me-Bn]EM2	17.1 ± 1.8	506 ± 72	30	224 ± 47	105 ± 11	4.72
24	[D-Ala^4-Phe^5-NH_2]EM2	14.5 ± 1.5	3681 ± 181	255	79.2 ± 11.4	143 ± 30	0.55
25	[D-Val^4-D-Phg^5-NH_2]EM2	68.5 ± 9.4	3629 ± 78	53	> 10000	> 10000	–
26	[Gly^4-NH-Ph]EM2	257 ± 26	22850 ± 3379	89	4339 ± 631	3437 ± 78	1.26
27	[D-Ala^2, D-Val^4-NH-Bn]EM2	28.9 ± 2.7	71.3 ± 9.8	2.46	–	–	–

[a] Values represent the average of 3–5 measurements, each containing two parallels ± SE. [b] Ligand used: [H^3]DAMGO. [c] Ligand used: [H^3]DPDPE. [d] Values represent the average of 10–15 measurements. [e] Potency ratio.

creased μ affinity in binding assays, derivates **5**, **9**, **10**, **19**, **22**, and **23** displayed about 4–31-fold lower potencies than their parents. In the MVD bioassays, all analogues were relatively weak δ-opioid receptor agonists ($IC_{50} > 10$ nM), except **7** and **21**, which exhibited inhibition in the nanomolar range. It is possible, that for the most part, this observation with **7** and **21** is elicited by a MOR that coexists in the MVD tissues.[31] Moreover, all the inhibition effects were blocked by naloxone (data not shown).

Solution conformation analysis

NMR studies of EM analogues were conducted in [D_6]DMSO at 298 K. The relative peak intensities from the 1D spectrum indicated the populations of the *cis*–*trans* isomers. Each 2D spectrum was acquired with 1024×1024 data matrix complex points in *t*1 and *t*2. Analyzed by the FELIX 2000 program, ROE cross-peaks were determined from ROESY spectra using the correlation between signal strength and interatomic distance. Random structures were minimized in vacuo and generated average structures by ROE distance data using the DG II standard program as described by Moroder and co-workers.[11] The torsion angles of these atypical structures were measured and compared with those of EM1, the values of which are in accord with the data reported by Podlogar et al.[32] (Table 3).

The comparisons of molecular model and torsion angles of [D-Ala^4-NH-Bn]EM1 (**8**) and its diastereomer [Ala^4-NH-Bn]EM1 (**3**) generated by DG II calculation are shown in Figure 2 and Table 3. Notably, inversion of the Xaa^4 chiral center (from Ala to

Table 3. Torsion angles of DG-calculated structures of EM1 and EM analogues used in the structure comparison.[a]

Peptide	Tyr^1		Pro^2		Trp^3/Phe^3			Phe^4/Xaa^4			Bn
	ψ_1	$\chi_1{}^1$	ϕ_2	ψ_2	ϕ_3	ψ_3	$\chi_3{}^1$	ϕ_4	ψ_4	$\chi_4{}^1$	ϕ_5
trans-EM1 (**1**)	154	−73	−49	154	−96	154	−92	−114	132	−94	–
cis-EM1 (**1**)	177	−50	−63	−48	−123	−100	42	−131	159	−120	–
trans-[Ala^4-NH-Bn]EM1 (**3**)	−170	−120	−80	108	71	84	174	−41	−176	–	135
cis-[Ala^4-NH-Bn]EM1 (**3**)	−145	−134	−78	137	−20	115	149	−112	−88	–	−155
trans-[D-Ala^4-NH-Bn]EM1 (**8**)	99	−96	−92	−146	−75	46	64	1	100	–	132
cis-[D-Ala^4-NH-Bn]EM1 (**8**)	−178	−112	−118	−133	−6	145	162	154	57	–	−129
trans-[D-Val^4-NH-Bn]EM1 (**7**)	165	−127	−80	−160	51	60	−92	134	139	−61	−62
cis-[D-Val^4-NH-Bn]EM1 (**7**)	127	172	−72	14	−58	104	−155	88	169	−31	44
trans-[D-Val^4-NH-Bn]EM2 (**21**)	−176	−175	−94	−157	−119	51	−160	53	−174	5	−60
cis-[D-Val^4-NH-Bn]EM2 (**21**)	140	170	−84	−154	−75	138	47	64	−144	144	51

[a] All angles reported in degrees.

D-Ala) resulted in different spatial orientations of Tyr[1], Trp[3], and Bn in *trans* and *cis* isomers. In the *trans* isomer of [Ala[4]-NH-Bn]EM1 (**3**), the introduction of *S* stereochemistry at the position of Xaa[4] produced different torsion angles ($\phi_4 = -41^\circ$, $\psi_4 = -176^\circ$) in the C terminus relative to those of its diastereomer [D-Ala[4]-NH-Bn]EM1 (**8**) ($\phi_4 = 1^\circ$, $\psi_4 = 100^\circ$). The diverse conformation constraint of the backbone template (ϕ_4 and ψ_4 angles) led to a different spatial orientation of the third aromatic ring between the *trans* isomers of these diastereomers. Possibly due to the steric hindrance induced by incorrect spatial orientation of the third aromatic ring and interactions of the phenol ring (Tyr[1]) and benzyl group, the phenol ring was forced to point in nearly the opposite direction in the *trans* isomer of [Ala[4]-NH-Bn]EM1 (**3**; $\psi_1 = -170^\circ$; Figure 2A) relative to [D-Ala[4]-NH-Bn]EM1 (**8**; $\psi_1 = 99^\circ$; Figure 2B). Consequently, the spatial orientation of the side chain of Trp[3] was also altered (ϕ_3 (**3**) = 71°, ϕ_3(**8**) = −75°; χ_3^1(**3**) = −174°, χ_3^1(**8**) = 64°). Furthermore, although the orientation of Tyr[1] displayed no difference for the *cis* isomer between [Ala[4]-NH-Bn]EM1 (**3**; Figure 2D) and its diastereomer [D-Ala[4]-NH-Bn]EM1 (**8**; Figure 2E), the torsion angles and spatial arrangements of Trp[3] and Xaa[4]-NH-Bn changed significantly (Table 3). With the presence of a proline residue in the second position of the peptide sequence, *cis–trans* isomerization readily occurred at the Xaa[1]-Pro[2] peptide bond. According to the analysis, the atypical C-terminal modification did not significantly alter the *trans*/*cis* conformer ratio of EM analogues in DMSO relative to their parent compounds (data are shown in the Supporting Information).

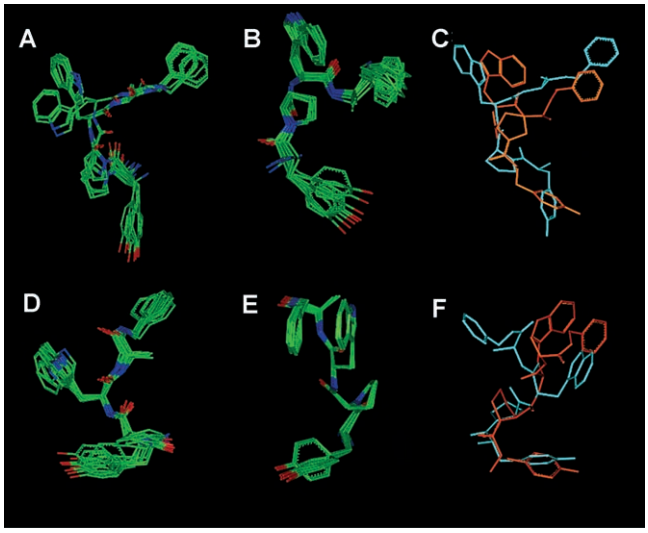


Figure 2. *Trans* and *cis* conformations of [Ala[4]-NH-Bn]EM1 (**3**) and its diastereomer [D-Ala[4]-NH-Bn]EM1 (**8**): A) *trans* conformations of analogue **3**; B) *trans* conformations of analogue **8**; C) superimposition of *trans* conformations of analogue **3** (in blue) and **8** (in red); D) *cis* conformations of analogue **3**; E) *cis* conformations of analogue **8**; F) superimposition of *cis* conformations of analogue **3** (in blue) and **8** (in red). All the *trans* and *cis* conformations were generated by DG calculation with NOE data in DMSO. Inversion of the Xaa[4] chiral center (from Ala to D-Ala) resulted in significantly different spatial orientations of Tyr[1], Trp[3], and Bn between the *trans* and *cis* isomers of analogues **3** and **8**.

Discussion

C-terminal residues play an important role in the biological activity of MOR-specific ligands.[33] In fact, the C-terminal residue is essential for high-affinity binding of EMs to the MOR.[23–26] Many results have suggested that the bioactivity of this sequence (Tyr-Pro-Trp/Phe-X-NH_2) is determined by the nature of the fourth residue,[24] and the third aromatic pharmacophore is crucial for bioactivity.[20,24] For example, in Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2), the affinity increases about 200-fold[20] if Gly[4] is substituted by the hydrophobic residue Phe, resulting in an observation that actually led to the discovery of EMs. Herein, analogues **2** (Tyr-Pro-Trp-Gly-NH-Bn) exhibited only 12-fold lower affinity than EM1, possibly due to conformational freedom of the third aromatic ring induced by the methylene group of Gly[4]. However, they showed about 20-fold higher potency than Tyr-W-MIF-1, a result that further emphasizes the importance of the third aromatic pharmacophore in the binding of EM1 to the MOR. The slightly increased binding affinity of [Sar[4]-NH-Bn]EM1 (**5**) and [Sar[4]-NH-Bn]EM2 (**19**) compared with analogues **2** and **16** supports the extend backbone structure model for EMs, because peptides containing *N*-methyl amide bonds in the backbone tend to adopt an extended conformation.[25] Although more flexible than analogues **2** and **16** owing to the extra methylene group present, [β-Ala[4]-NH-Bn]EM1 (**4**) and [β-Ala[4]-NH-Bn]EM2 (**18**) displayed only marginal differences from analogues **2** and **16**, evidence that the MOR might have the capacity of conformational adjustment to accommodate different ligands.[34] Simultaneous substitution of D-Ala for Pro[2] and conversion of Phe[4]-NH_2 to D-Val[4]-NH-Bn did not markedly alter the μ affinity of derivative **14**, which still displays high affinity, similar to that of EM1. However, these same alterations slightly decreased the μ affinity of derivative **27** and significantly increased its δ affinity relative to EM2. It further demonstrated that Tyr-D-Ala-Phe as a message sequence strongly favors ligand interaction with the δ-opioid receptor.

Notably, [D-Ala[4]-NH-Bn]EM1 (**8**) displayed μ affinity and bioactivity significantly different from those of its diastereomer [Ala[4]-NH-Bn]EM1 (**3**). Analogue **8** exhibited 22-fold higher μ affinity in binding assays and was over 200-fold more potent in GPI assays than its diastereomer peptide **3**. These findings demonstrate that the chirality of Xaa[4] is crucial for MOR recognition in these C-terminally modified structures. In addition, in SAR of EMs, a subtle requirement is the proper spatial arrangement of side chains,[14] especially the Tyr[1] moieties.[34] Herein, conformational analysis of these diastereomers (**3** and **8**) demonstrates that differences in the stereochemistry of Xaa[4] generates different positioning of the side chains of Tyr[1], Trp[3], and the benzyl group through the constraints of amide bond and torsion angles (ϕ_4 and ψ_4), not only in the *cis* isomer but in the *trans* isomer as well. It indicates that *R* stereochemistry of Xaa[4] produces favorable conformational constraints on the third aromatic pharmacophore and gives proper positioning of other side chains; this is not the case with *S* stereochemistry.

Recently, more endeavors have been made to design topographically constrained bioactive peptides in which side chain groups of key amino acid residues have a particular χ angle

(χ^1, χ^2, etc.).[12,17] Tömböly et al. emphasized that the presence of the $\chi^1 = -60^\circ$ rotamer of Phe^4 in EMs produces the appropriate orientation of the C-terminal aromatic side chain, and these derivatives exhibit fourfold higher affinity than their parent compounds.[17] Herein, the torsion angle ϕ_5 (Figure 1) was designed to generate atypical topographical constraints of the third aromatic ring (benzyl group), which displayed the same function of the χ^1 angle of Phe^4 in parent molecules. However, all the topographically constrained modifications given by the introduction of (*S*)-α-methyl or (*S*)/(*R*)-α-carboxamide on the methylene unit of the benzyl group decreased the affinity and bioactivity of the resulting analogues. In particular, derivatives **13** and **26**, which lack the methylene unit of the benzyl group and in which the third aromatic rings are further constrained by an amide bond, displayed extraordinarily low affinity and bioactivity. This indicates that all the modifications mentioned above generated unfavorable topographical constraints to the third aromatic ring and further demonstrates the important role of the proper orientations of the third aromatic pharmacophore. Interestingly, [D-Val^4-NH-Bn]EM1 (**7**) and [D-Val^4-NH-Bn]EM2 (**21**), which have no extra modifications on the methylene unit of the benzyl group to generate topographical constraint, still exhibit the highest affinity and bioactivity of all analogues, both in vivo and in vitro. This is possibly due to the isopropyl group, the torsion angles (ϕ_4 and ψ_4) of D-Val^4, and the amide bond producing the favorable constraints of the third aromatic ring. It is notable that peptides **7** and **21** exhibited the same ϕ_5 torsion angle ($\phi_5 = -60^\circ$ and -62°, respectively) in the *trans* isomers, which might strongly favor the third aromatic ring interactions with MOR.

EM1 and EM2 are quite distinct from conventional endogenous opioid receptor ligands in their N-terminal sequence (Tyr-Pro versus Tyr-Gly) and C-terminal amide. Both opioid tetrapeptides contain Pro in position 2 and aromatic amino acids in positions 1, 3, and 4. Aromatic–aromatic noncovalent interactions between different aromatic amino acids (Tyr, Trp, and Phe) may be important in the determination and stabilization of the structures of EMs,[35] and may generate a favorable MOR binding pocket.[32] The third aromatic ring of EMs is free to adopt a "bioactive" conformation compared with other side chains.[36] As observed in the present study, possibly owing to the steric hindrance induced by incorrect spatial orientation of the third aromatic ring and interactions of the phenol ring (of Tyr^1) and the benzyl group, the phenol ring was forced to point in nearly the opposite direction in the *trans* isomer of [Ala^4-NH-Bn]EM1 (**3**) compared with [D-Ala^4-NH-Bn]EM1 (**8**). This could explain the drastic loss of activity observed with [Ala^4-NH-Bn]EM1 (**3**). This indicates that the correct arrangement of the third aromatic ring is crucial for the stabilization of the positions of other aromatic side chains and in facilitating the formation of a proper hydrophobic binding pocket by all the aromatic rings. The incorrect spatial arrangement of the third aromatic ring might disrupt the proper conformations of other side chains and lead to loss of activity. The poor affinity and bioactivity of [D-Val^4-D-Phg^5-NH$_2$]EM1 (**11**), [D-Val^4-Phg^5-NH$_2$]EM1 (**12**), [D-Val^4-D-Phg^5-NH$_2$]EM2 (**25**), [Gly^4-NH-Ph]EM1 (**13**), and [Gly^4-NH-Ph]EM2 (**26**) may be induced by improper restrictions of the third aromatic ring. Such incorrect constraints of the third aromatic ring might produce significant effects on the orientations of other aromatic side chains. However, the presence of an extra methylene group results in a significant increase in the affinity and bioactivity of [D-Ala^4-D-Phe^5-NH$_2$]EM1 (**10**) and [D-Ala^4-D-Phe^5-NH$_2$]EM2 (**24**) relative to the respective Phg-containing peptides **11** and **25**. This indicates that the introduction of an extra carbon atom in the side chain weakens the negative effects of the improper orientation of the third aromatic ring on the interactions of all the side chains. It further demonstrates that the distance between the C-terminal aromatic ring and peptide backbone has a strong effect on MOR binding.[25]

EM1 and EM2 only differ in one amino acid. The side chain of Trp^3 of EM1 is an indole ring, which is a relatively bulky group compared with Phe^3 of EM2. The difference in steric properties between the indole and phenyl rings leads to different solution conformations[34] and distinctive bioactivity.[37,38] Thus, it is not surprising that [Gly^4-NH-Bn]EM1 (**2**), [D-Pro^4-NH-Bn]EM1 (**6**), [Gly^4-NH-Bn]EM1 (**16**), and [D-Pro^4-NH-Bn]EM1 (**20**) exhibit similar μ receptor affinities, about 3–10-fold lower than their respective parent compounds. However, a drastic loss of functional bioactivity was observed in GPI assays of EM1 analogues (**2** and **16**), but not EM2 analogues (**6** and **20**), despite having the same C-terminal modification. These results indicate that the difference in steric bulk between the side chains of Phe^3 and Trp^3 produce different effects on the ligand interactions with the receptors, and thus variations in bioactivity.

Although the C-terminal amide function of EMs was found to be essential to decrease conformational flexibility of the tetrapeptides[23,24] to regulate binding and agonist/antagonist properties[25] and also to increase enzymatic stability,[39] the results presented herein show that [D-Val^4-NH-Bn]EM1 (**7**) and [D-Val^4-NH-Bn]EM2 (**21**), in which the C-terminal amide is eliminated, still exhibit higher affinity and more potent bioactivity both in vivo and in vitro than their respective parent compounds. It was shown that Xaa^4-NH-Bn can act at the opioid receptor as a Phe^4-NH$_2$ mimic.

Experimental Section

Mass spectra were measured with a Mariner 5074 ESI-TOF analyzer (Applied Biosystems, USA). Melting points were determined on a micro-melting point apparatus (AII-E, China). TLC was performed on precoated plates of silica gel G F_{254}. Optical rotations were determined with Polarimeter 341 (PerkinElmer). Analytical RP HPLC was carried out with a Waters Delta 600 instrument equipped with a Waters Deltapak C_{18} column (3.9 mm × 150 mm); absorbance was monitored at $\lambda = 220$ nm. The solvents for analytical HPLC were as follows: A, 0.05% TFA in water; B, 0.05% TFA in CH_3CN. The column was eluted at a flow rate of 0.6 mL min^{-1} with a linear gradient of A/B = 90:10 to A/B = 10:90 for 30 min, and a gradient of A/B = 10:90 to A/B = 90:10 for 5 min. The retention time is reported as t_R (min). Elemental analyses were determined on a Vario EL instrument (Elementar, Germany). 1D and 2D ^{1}H NMR were measured on a Varian INOVA 500 spectrometer. Chemical shift values are expressed as δ (ppm) with tetramethylsilane as standard. Peptide samples were dissolved in [D_6]DMSO (99.9% isotopic purity;

Cambridge Isotope Laboratories, Andover, MA, USA) at a concentration of 20 mg mL^{-1}. 2D COSY, TOCSY, and ROESY spectra were acquired using standard pulse programs available in the Varian software library. Mixing times of 60 and 600 ms were used for TOCSY and ROESY spectra, respectively.

General procedure for the synthesis of Z-Xaa-NH-Bn (Xaa = Gly, Ala, Sar, D-Pro): Benzylamine (4.5 mmol) was added to distilled CH_2Cl_2/DMF (10:1 *v/v*) containing Z-Xaa-OH (3 mmol) and DCC (3.6 mmol) at 0 °C. After 30 min, DMAP (1.5 mmol) was added to the mixture. The mixture was stirred at room temperature overnight. DCU was then removed by filtration. After removal of the solvent, the residue was extracted with EtOAc. The extract was washed with citric acid (5%), saturated $NaHCO_3$, and saturated NaCl, dried over Na_2SO_4, and evaporated at reduced pressure. Petroleum ether (PE) was added to the residue to form crystals (EtOAc/PE 2:25) which were collected by filtration. Yield, mp, R_f, ESI-TOF MS and ^{1}H NMR data are summarized in the Supporting Information.

General procedure for the synthesis of Boc-Xaa-NH-Bn (Xaa = D-Ala, D-Val, β-Ala), Boc-D-Ala-NH-(*S*)-α-Me-Bn, and Boc-Gly-Ph: These compounds were prepared as for the synthesis of Z-Xaa-NH-Bn, starting from Boc-Xaa-OH (2 mmol), benzylamine, (*S*)-α-methylbenzylamine or phenylamine (3 mmol), and DCC (2.4 mmol) in CH_2Cl_2 (20 mL) at 0 °C. After 30 min, DMAP (1 mmol) was added to the mixture. The crude products were purified by silica gel column chromatography (eluent: PE/EtOAc 1:1), after which the purified peptide was crystallized from PE/EtOAc solution. Yield, R_f, and ESI-TOF MS data are summarized in the Supporting Information.

General procedure for the synthesis of Z-Trp/Phe-Xaa-NH-Bn (Xaa = Gly, Ala): A solution of Z-Phe or Z-Trp (3.36 mmol), HOBt (3.7 mmol), and DCC (4.0 mmol) in distilled CH_2Cl_2/DMF (15:1 *v/v*) was stirred at 0 °C for 30 min, and at room temperature for 8 h. After DCU was removed by filtration, the solution of Z-Phe/Z-Trp-OBt was retained for the next coupling step. Z-Xaa-NH-Bn (2.8 mmol) was dissolved in distilled MeOH containing Pd/C (50 mg) and H_2, and the reaction mixture was stirred at room temperature for 3 h. After the Pd/C was removed by filtration, the solvent was evaporated at reduced pressure, and the residue was dissolved in distilled CH_2Cl_2/DMF (5:1 *v/v*). The mixture of Z-Phe/Z-Trp-OBt and deprotected Z-Xaa-NH-Bn was then stirred at 0 °C for 30 min, and at room temperature overnight. After removal of the solvent, the residue was extracted with EtOAc. The extract was washed with 3% HCl, saturated $NaHCO_3$, and saturated NaCl, dried over Na_2SO_4, and evaporated at reduced pressure. PE was added to the residue to form crystals (EtOAc/PE 1:5) which were collected by filtration. Yield, mp, R_f, ESI-TOF MS and ^{1}H NMR data are summarized in the Supporting Information.

General procedure for the synthesis of Boc-Phe/Trp-Xaa-NH-Bn (Xaa = β-Ala, D-Ala, D-Val, Sar, D-Pro), Boc-Trp/Phe-(*S*)-α-Me-Bn, and Boc-Trp/Phe-Gly-Ph: A solution of Boc-Phe or Boc-Trp (0.72 mmol), HOSu (0.84 mmol), and DCC (0.84 mmol) in distilled THF (10 mL) was stirred at 0 °C for 30 min, and at room temperature for 6 h. After DCU was removed by filtration, the solution of Boc-Phe/Boc-Trp-OSu was retained for the next coupling step. Boc-Xaa-NH-Bn or Boc-D-Ala-NH-α-Me-Bn (0.60 mmol) was treated with 12.5 M HCl/EtOAc (1:4 *v/v*) with stirring for 2 h at room temperature. After the solution was brought to pH 9 with 4 mM NaOH, it was combined with the solution of Boc-Phe/Boc-Trp-OSu. The resulting mixture was stirred in THF/water at 0 °C for 30 min, and at room temperature overnight. After removal of the solvent, the residue was extracted with EtOAc. The extract was treated in a manner similar to that for Z-Xaa-NH-Bn, and crude products were purified by silica gel column chromatography (eluent: PE/EtOAc 1:3). Yield, R_f, and ESI-TOF MS data are summarized in the Supporting Information.

Synthesis of Z-D/L-Phg-NH$_2$: Z-D-Phg-OSu was prepared with a method similar to the preparation of Boc-Phe/Boc-Trp-OSu for the synthesis of Boc-Phe/Trp-Xaa-NH-Bn. It started from Z-D-Phg-OH (3 mmol), HOSu (3.6 mmol), and DCC (3.6 mmol) in THF (15 mL). The ammonia solution (1.5 mL, 18% $NH_3{\cdot}H_2O$) was added to the THF solution of Z-D-Phg-OSu. After the reaction mixture in THF/water was stirred at 0 °C for 30 min, and at room temperature overnight, the solvent was removed. The residue was dissolved in EtOH (10 mL), and distilled H_2O (150 mL) was added to the solution at 4 °C to obtain a precipitate that was collected by filtration and dried in vacuo. Yield: 0.84 g (98.69%); R_f = 0.45 (PE/EtOAc/ammonia solution 10:20:1); TOF MS: *m/z* calcd for $C_{16}H_{16}N_2O_3$ [M+H$^+$]: 285, [M+Na$^+$]: 307, found: 285.04, 307.01. Z-Phg-NH$_2$ was prepared by the same method as Z-D-Phg-NH$_2$. Yield: 0.73 g (85.68%); R_f = 0.45 (PE/EtOAc/ammonia water 10:20:1); TOF MS: *m/z* calcd for $C_{16}H_{16}N_2O_3$ [M+Na$^+$]: 307, found: 307.17.

General procedure for the synthesis of Z-D-Val-Xaa-NH$_2$ (Xaa = D-Phg, Phg), Boc-D-Ala-Phe-NH$_2$, and Z-Trp/Phe-NH$_2$: These compounds were synthesized according to the procedure described in the synthesis of Boc-Phe/Trp-Xaa-NH-Bn, starting from Z/Boc-Xaa-OH (2 mmol), HOSu (2.2 mmol), and DCC (2.4 mmol) in distilled THF (20 mL). Deprotection of the Z group was performed with Pd/C/H_2. Purification was in accord with the procedure for Z-D-Phg-NH$_2$. Yield, R_f, and ESI-TOF MS data are summarized in the Supporting Information.

General procedure for the synthesis of Boc-Trp/Phe-D-Val-Xaa-NH$_2$ (Xaa = D-Phg, Phg) and Boc-Trp/Phe-D-Ala-Phe-NH$_2$: These peptides were synthesized according to the procedure described in the synthesis of Boc-Phe/Trp-Xaa-NH-Bn. Purification was carried out according to the procedure for Z-D-Phg-NH$_2$. Yield, R_f, and ESI-TOF MS data are summarized in the Supporting Information.

Synthesis of Z-Tyr-Pro-OH: Z-Tyr-OSu was prepared by a method similar to the preparation of Boc-Phe/Boc-Trp-OSu for the synthesis of Boc-Phe/Trp-Xaa-NH-Bn, and started from Z-Tyr-OH (8 mmol), HOSu (8.8 mmol), and DCC (9.6 mmol) in THF (40 mL). H-Pro-OH (10.4 mmol) was dissolved in 4 nM NaOH/THF, and the pH value was adjusted to 9. Then, the reaction mixture containing Z-Tyr-OSu and H-Pro-ONa was stirred in THF/H_2O at 0 °C for 30 min, and at room temperature overnight. After removal of the solvent, the residue was extracted with EtOAc. The extract was washed with citric acid (5%) and saturated NaCl, dried over Na_2SO_4, and evaporated at reduced pressure. The residue was crystallized from PE/EtOAc/HOAc (60:10:1). Yield: 2.31 g (70.3%); R_f = 0.31 (PE/EtOAc/HOAc 10:30:1); $[\alpha]_D = -19°$ (c = 1.0, MeOH); mp: 102–104°; ESI-TOF MS: *m/z* calcd for $C_{22}H_{24}N_2O_6$ [M+H$^+$]: 413, found: 413.56.

General procedure for the synthesis of Tyr-Pro-Trp/Phe-Xaa-NH-Bn (Xaa = Gly, Ala, β-Ala, Sar, D-Pro, D-Val, or D-Ala), Tyr-Pro-Trp/Phe-D-Ala-R (R = (*S*)-α-Me-NH-Bn, Phe-NH$_2$), Tyr-Pro-Trp/Phe-Gly-NH-Ph, Tyr-Pro-Trp/Phe-D-Val-R (R = D-Phg-NH$_2$, Phg-NH$_2$), Tyr-D-Ala-Trp/Phe-D-Val-NH-Bn, and Tyr-Pro-Trp/Phe-Phe-NH$_2$: A solution of N-terminal fragments Z-Tyr-Pro-OH or Z-Tyr-D-Ala-OH (0.2 mmol), HOSu (0.22 mmol), and DCC (0.24 mmol) in distilled THF (5 mL) was stirred at 0 °C for 30 min, and at room temperature for 6 h. After removal of DCU by filtration, the solution of Z-Tyr-Pro-OSu/Z-Tyr-D-Ala-OSu was retained for the next coupling step. Deprotection of the Boc and Z groups of C-terminal fragments (0.2 mmol) was performed with HCl/EtOAc and Pd/C, respectively.

Then, the reaction mixture containing Z-Tyr-Pro/D-Ala-OSu and deprotected C-terminal fragments was stirred in THF/H_2O (1:1) at 0 °C for 30 min, and at room temperature overnight. After removal of the solvent, the residue was extracted with EtOAc. The extract was treated in a manner similar to that for Z-Xaa-NH-Bn, and the crude protected tetrapeptides or pentapeptides were purified by silica gel column chromatography (eluent: PE/EtOAc/ammonia solution 10:50:1) with a yield range of 60–90%.

The Z-group deprotection was performed by treatment with Pd/C in MeOH containing H_2 at room temperature. After 5 h the solvent was evaporated at reduced pressure. The residue was easily purified by silica gel column chromatography (eluent: EtOAc/MeOH/ammonia solution 50:10:1), after which the purified peptide was crystallized from a solution of PE/EtOAc/MeOH and characterized by RP HPLC, TLC, TOF MS, $[\alpha]_D$, mp, and NMR analysis. All the data are summarized in Table 1, and spectroscopic characterization of final products is supplied in the Supporting Information.

Radioligand binding assays: This study was approved by the Ethics Committee of Lanzhou Medical College. Membranes were prepared from Wistar rat brain (without cerebellum) according to published methods.[40,41] All binding experiments were performed in 50 mM Tris-HCl buffer (pH 7.4) at a final volume of 0.5 mL containing 300–500 $\mu g\,mL^{-1}$ protein[17] (protein concentration was determined by the method of Bradford[42]). For competition experiments, the following incubation conditions were used: [^{3}H]DAMGO, 25 °C, 1 h; and [^{3}H]DPDPE, 25 °C, 3 h.[43] Incubations were started by the addition of the membrane suspension under continuous shaking in a thermal water bath, and terminated by rapid vacuum filtration through GF/C filters using a cell harvester. The filters were washed twice with 6 mL ice-cold buffer and then dried for 1 h at 80 °C. The radioactivity was measured by a Wallac Microbeta 1450 Trilux scintillation counter after 12 h incubation in the scintillation cocktail. The extent of nonspecific binding was determined in the presence of 10 μM naloxone. All experiments were carried out in duplicate, and repeated at least three times. Affinity constants (K_i) were determined as described earlier.[44]

In vitro bioactivity assays: For the GPI assay, the myenteric plexus longitudinal muscle was obtained from guinea pig (200–300 g) ileum as described by Rang.[45] For the MVD assay, the vas deferens of male Kunming strain mice (30–35 g) were prepared as described by Hughes et al.[46] The GPI tissue and MVD tissues were mounted in a 10-mL bath containing aerated (95% O_2, 5% CO_2) Krebs–Henseleit solution at 37 and 35 °C, respectively. Both tissues were used for field stimulation with bipolar rectangular pulses of supramaximal voltage. Moreover, in both assays, three to four washings were done with intervals of 15 min between each dose. Dose–response curves were constructed, and IC_{50} values (concentration causing a 50% decrease in electrically induced twitches) were calculated graphically. The values are arithmetic means of 10–15 measurements.

Molecular modeling: All the molecular modeling calculations were performed on an Origin 2000 workstation running the Irix 6.5 operation system (Silicon Graphics Inc., Mountain View, CA, USA). The 2D NMR matrixes were created and analyzed using the Felix 2000 computer program (Biosym Technologies, Inc., San Diego, CA, USA). Molecular modeling was carried out by using the Discover 98 and NMR Refine modules in the Insight II 2000 package. The AMBER force field was used to calculate energy values.[47–48] Final structures were generated from the standard protocol of the DG II package in NMR Refine modules.

Abbreviations

Abbreviations used herein for amino acids, peptides, and their derivatives are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature: *Biochemistry* **1966**, *5*, 2485–2489; **1966**, *6*, 362–364; **1972**, *11*, 1726–1732. The notation for the customary L configuration of amino acid residues is omitted. The following additional abbreviations are used: AD_{50}, 50% antinociception dose; Boc, *tert*-butyloxycarbonyl; Bn, benzyl; COSY, correlation spectroscopy; DAMGO, Tyr-D-Ala-Gly-*N*-MePhe-glycinol; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; Dmp, 2′,6′-dimethylphenylalanine; Dmt, 2′,6′-dimethyl-L-tyrosine; DPDPE, Tyr-*c*(D-Pen-Gly-Phe-D-Pen); EM1, endomorphin-1; EM2, endomorphin-2; ESI-TOF MS, electrospray ionization time-of-flight mass spectrometry; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; IC_{50}, concentration required for 50% inhibition of electrically induced contraction in muscle derived from a dose–response curve; MVD, mouse vas deferens; NOE, nuclear Overhauser effect; RP HPLC, reversed-phase high performance liquid chromatography; NOESY, nuclear Overhauser effect spectroscopy; PE, petroleum ether; Phg, phenylglycine; Sar, sarcosine; ROESY, rotating frame Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

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Keywords: drug design · endomorphins · opioid receptors · spatial arrangements · synthesis

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