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Brief Article

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Opioid Activity Profiles of Oversimplified Peptides Lacking in the Protonable N-Terminus

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Supporting Information

ABSTRACT: Recently, we described cyclopeptide opioid agonists containing the D-Trp-Phe sequence. To expand the scope of this atypical pharmacophore, we tested the activity profiles of the linear peptides Ac-Xaa-Phe-Yaa (Xaa = L/D-Trp, D-His/Lys/Arg; Yaa = H, $GlyNH_2$). Ac-D-Trp-$PheNH_2$ appeared to be the minimal binding sequence, while Ac-D-Trp-Phe-$GlyNH_2$ emerged as the first noncationizable short peptide (partial) agonist with high μ-opioid receptor affinity and selectivity. Conformational analysis suggested that **5** adopts in solution a β-turn conformation.

INTRODUCTION

It is generally accepted that the fundamental binding interaction between the opioid receptors and their agonists is represented by the ionic bridge between the conserved Asp(3.32) of the receptor and a positively charged N-terminal amine of the ligand.[1] Indeed, the removal or derivatization of this group normally gave inactive ligands or antagonists.[2−7] Neverthelsess, there is recent evidence that some compounds deprived of a protonable amine exhibit full or partial opioid agonism: the diterpene salvinorin A[8] and its many derivatives;[9,10] a bicyclic enkephalin mimetic;[11] the cyclic analogue of endomorphin-1 (EM1)[12] *c*[Tyr-D-Pro-D-Trp-Phe-Gly] (**1**)[13] and the correlated *c*[Tyr-Gly-D-Trp-Phe-Gly] and *c*[D-Asp-1-amide-β-Ala-D-Trp-Phe] (**2**);[14] a cyclic enkephalin analogue containing 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (Dhp) in the place of Tyr;[15] a "carba" analogue of fentanyl, in which the classic piperidine nitrogen was replaced by a carbon but equipped with a ionizable guanidino group.[16]

Compounds **1** and **2** represent the first examples of peptides described in the literature whose μ-opioid receptor (MOR) agonist activities reside in the aromatic side chains. Molecular docking analysis revealed a inverse type II β-turn conformation centered on D-Trp-Phe, relevant contacts with Trp318(7.35), Trp293(6.48), and a fundamental binding interaction between the indolic NH of D-Trp and the carboxylate of Asp147(3.32), as sketched for **2** in Figure 1.

In the past few years, very similar sequences have been reproposed in the derivatives of the naturally occurring cyclotetrapeptide *c*[D-Pro-Phe-Trp-Phe] (**3**, CJ-15,208),[17] a opioid ligand that preferentially binds to κ-opioid receptor (KOR). The introduction of a D-configured Trp gave *c*[D-Pro-Phe-D-Trp-Phe] and *c*[D-Pro-Phe-D-Trp-Ala], more potent than the natural product.[18] These compounds did not exhibit any agonist activity in vitro; however, unexpected agonist activity in vivo was observed for **3** and some analogues in the warm water tail withdrawal antinociceptive assay, mediated predominantly by MOR.[19]

These results support the hypothesis that the D-Trp-Phe sequence constitutes an unusual kind of MOR pharmacophoric motif. Apparently, the stereochemistry and relative disposition of Trp and Phe, the macrocycle size, and secondary structure strongly impact affinity and selectivity and agonism vs antagonism.

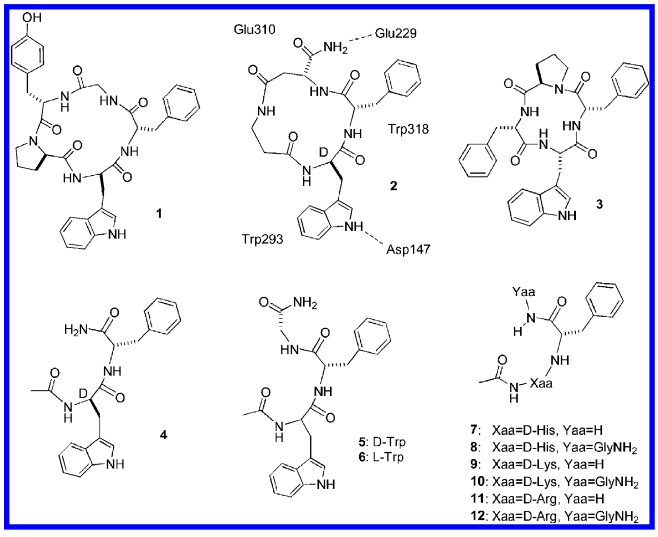

Figure 1. Structures of the cyclic EM1 analogues **1** and **2**, of **3**, and of the Ac di- and tripeptides **4**−**12**.

To determine the minimal bioactive sequence of **1** and **2** and to expand the scope of the novel opioid pharmacophore, we designed (Figure 1) and tested the linear analogues Ac-D-Trp-$PheNH_2$ (**4**), Ac-D-Trp-Phe-$GlyNH_2$ (**5**), the tripeptide **6** containing L-Trp, and the di- or tripeptides **7**−**12** containing amino acids with a protonable side chain, D-His, D-Lys, and D-Arg.

RESULTS AND DISCUSSION

The peptides were prepared in solution under MW irradiation from *N*-Ac amino acids and H-$PheNH_2$ or H-Phe-$GlyNH_2$,

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Table 1. In Vitro Activity Profiles of the Ac Di- or Tripeptides 4−12 and of the Reference Compounds 2, DAMGO, DPDPE, U50,488 toward the Cloned Human Opioid Receptors Expressed on HEK-293 Cells[a]

compd	sequence	purity [%][b]	ESI-MS (m/z) [M + 1] vs calcd	K_i^{μ} (nM)	K_i^{δ} (nM)	K_i^{κ} (nM)	n_H
DAMGO	H-Tyr-D-Ala-Gly-*N*MePhe-glyol			1.5 ± 0.1			
DPDPE	H-Tyr-*c*[D-Pen-Gly-Phe-D-Pen]OH				3.30 ± 0.05		
U50,488						2.90 ± 0.04	
2	*c*[D-Asp-1-amide-β-Ala-D-Trp-Phe][c]			5.9 ± 0.1	>10^5	>10^5	
4	Ac-D-Trp-PheNH$_2$	96	393.0/393.2	15.5 ± 0.5	24 ± 9[d]	>10^5	0.8 ± 0.2
5	Ac-D-Trp-Phe-GlyNH$_2$	97	450.1/450.2	5.6 ± 0.2	>10^5	>10^5	0.9 ± 0.1
6	Ac-Trp-Phe-GlyNH$_2$	98	450.2/450.2	(7.4 ± 0.8) × 10^4	12.1 ± 0.4[d]	(3.3 ± 0.8) × 10^4	
7	Ac-D-His-PheNH$_2$[e]	96	344.1/344.2	>10^5	>10^5	>10^5	
8	Ac-D-His-Phe-GlyNH$_2$[e]	97	401.0/401.2	>10^5	>10^5	>10^5	
9	Ac-D-Lys-PheNH$_2$[e]	97	335.3/335.2	>10^5	>10^5	>10^5	
10	Ac-D-Lys-Phe-GlyNH$_2$[e]	96	392.2/392.2	>10^5	>10^5	>10^5	
11	Ac-D-Arg-PheNH$_2$[e]	96	363.2/363.2	>10^5	>10^5	>10^5	
12	Ac-D-Arg-Phe-GlyNH$_2$[e]	95	420.0/420.2	>10^5	>10^5	>10^5	

[a]Mean of four to six determinations ± SE. [b]Determined by analytical RP-HPLC; see General Methods. [c]Reference 14. [d][^{3}H]Diprenorphine displacement of <50%. [e]TFA.

using hydroxybenzotriazole/*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate/diisopropylethylamine (HOBt/HBTU/DIPEA) as coupling agents. H-Phe-GlyNH$_2$ was obtained by coupling Boc-PheOH and H-GlyNH$_2$; Boc cleavage was performed with TFA. The peptides were obtained pure (95−98%) after semipreparative reversed-phase (RP) HPLC. The presence of traces of free amino groups at the N-terminus was excluded on the basis of RP-HPLC/ESI-MS and spectroscopic analyses.

The affinities toward the cloned human MOR, δ-opioid receptor (DOR), and KOR, stably expressed on HEK-293 cells, were determined by displacement binding assays. [^{3}H]-DAMGO, [^{3}H]diprenorphine, and [^{3}H]U69,593 were employed as specific radioligands for MOR, DOR, and KOR, respectively. DAMGO, DPDPE, and U50,488 were chosen as reference compounds for MOR, DOR, and KOR. The tested peptides and the reference compounds were all used in the 10^{-12}−10^{-4} M range; the calculated K_i values are reported in Table 1.

DAMGO,[13,20] DPDPE,[21] and U50,488[22] all showed K_i in the nanomolar range, in agreement with the literature. As for the tested peptides, Ac-D-Trp-PheNH$_2$ (**4**) and Ac-D-Trp-Phe-GlyNH$_2$ (**5**) displayed an easily measurable, concentration-dependent displacement of [^{3}H]DAMGO from MOR, with K_i^{μ} values of 15.5 × 10^{-9} and 5.6 × 10^{-9} M, respectively (Table 1), comparable to that of cyclic **2**.[14] The Hill coefficients (n_H) are not significantly different from unity. Interestingly, **5** did not show any relevant affinity toward DOR or KOR, as it does not displace [^{3}H]diprenorphine and [^{3}H]U69,593. On the other hand, **4** was less selective, showing a modest ability to displace [^{3}H]diprenorphine from DOR (Table 1). The tripeptide Ac-Trp-Phe-GlyNH$_2$ (**6**) displayed very poor affinity toward MOR and KOR and displaced [^{3}H]diprenorphine from DOR only slightly (Table 1). Finally, compounds **7**−**12** did not show any significant displacement of the radioligands employed in this study (Table 1).

To our knowledge, peptides **4** and **5**, derived from the cyclopeptides **1** and **2** and designed on the basis of molecular docking insights of the bioactive conformation, represent the first noncationizable linear peptides with nanomolar affinity for MOR. The dipeptides *c*[Dmt-Tic] (Dmt, 2′,6′-dimethyl-Tyr) and Ac-Dmt-Tic-NH$_2$, previously described by Balboni et al.,[23] exhibited low to moderate δ antagonist activity. The recently reported tetrapeptides Ac-D-Pro-Phe-D-Trp-PheNH$_2$ and Ac-Phe-D-Trp-Phe-D-ProNH$_2$, derived from **3** by systematic single amide bond cleavage, displayed a modest 10^{-7} M potency at the MOR.[18]

Given that **4** and **5** were the only peptides with a significant affinity for MOR, their biological activities were measured by evaluating their effects on forskolin-stimulated cAMP accumulation in whole HEK-293 cells stably expressing MOR (Table 2). Morphine was assayed under the same experimental conditions to compare the activity of the tested peptides to this prototypical opioid analgesic drug. Furthermore, we report the activity of cyclic **2**, which we described in a previous study.[14]

Table 2. Inhibitory Effects of Morphine, 2, 4, 5 on Forskolin-Induced cAMP Formation in HEK-293 Stably Expressing MOR[a]

compd	IC_{50} (nM)[b]	E_{max} (% vehicle)[c]
morphine	4.3 ± 0.4	77 ± 4
2[d]	37 ± 5	58 ± 5
4	>10^5	
5	200 ± 30	52 ± 3**

[a]Mean ± SE of five to six independent experiments performed in triplicate; data were analyzed by one-way ANOVA followed by Tukey's test. [b]IC_{50} is the half-maximal inhibitory concentration. [c]E_{max} is the maximal obtainable effect. [d]Reference 14. **, P < 0.01 vs morphine and **2**.

As expected, morphine significantly inhibited forskolin-induced cAMP accumulation with IC_{50} = 4.3 nM and E_{max} = 77%. The cyclic peptide **2** had IC_{50} = 37 nM and E_{max} = 58% (Table 2). For peptide **5**, it also significantly inhibited forskolin-induced cAMP accumulation; however, it was less potent than morphine or the cyclic **2**, with IC_{50} = 200 nM and a E_{max} = 52% (Table 2). This latter finding suggests that **5** behaves as a partial agonist. The dipeptide **4** did not produce any significant effect, thus appearing to lack agonist activity albeit it is capable of binding to the MOR (Table 1).

The "partial" agonist activity of **5** makes sense, as EM1 and EM2 have been reported to behave as partial agonists at MOR,[24,25] their full agonism being shown only in a few cases.[26]

The tripeptide **5** is probably less effective in activating the MOR compared to morphine as a consequence of its minimal and more flexible structure, whereas the dipeptide **4**, deprived of the glycine, does not bind the MOR in the same way as **5** and lacks of MOR agonist activity on forskolin-induced cAMP accumulation.

The significant receptor affinity of **4** indicates that the dipeptide D-Trp-Phe represents the minimal bioactive sequence for receptor binding but not for agonism. The opposite configuration of Trp in **5** and **6** seems to be responsible for tuning selectivity from exclusively binding MOR to some affinity for DOR. A comparison between the different results of **4**−**6** and the other peptides highlighted the uniqueness of the indole of D-Trp for efficient binding to MOR in this class of atypical opioid peptides. Despite the presence of amino acids with protonable side chains, **7**−**12** had no opioid activity.

The different profiles of the structurally correlated **4** and **5** prompted us to investigate the preferred conformations in solution. Conformational analyses were performed by NMR spectroscopy and molecular dynamics (MD) simulations. Because of the negligible solubility in water, NMR spectra were recorded in the biomimetic medium 8:2 DMSO-d_6/H_2O.[27] The higher ability of DMSO to mimic the environment near the receptor with respect to pure water has been recently commented.[28]

The ^{1}H NMR of **4** and **5** revealed a single set of sharp resonances suggestive of conformational homogeneity or a fast equilibrium between conformers; gCOSY allowed the unambiguous assignment of all the resonances.

VT-^{1}H NMR experiments were used to deduce the presence of H-bonds (Table 3). For **4**, the $\Delta\delta/\Delta T$ (ppb/K) of the PheCONH$_2$ proton at 7.1 ppm was −1.1, comparatively lower than that of the other amide protons, indicating some folded population stabilized by a H-bond ($|\Delta\delta/\Delta t| \lesssim 2$).[29] For **5**, the very low $\Delta\delta/\Delta T$ of GlyNH of 0.3 is compatible with structures in which the amide proton is involved in a strong H-bond, supporting the speculation that **5** could be conformationally more homogeneous and well-defined.

Table 3. $\Delta\delta/\Delta t$ [ppb/K]^a of Amide Protons for 4 and 5

compd	D-TrpNH	PheNH	YaaNH	$CONH_2$
4	−6.6	−10.1	*b*	−1.1/−8.6
5	−8.0	−7.6	0.3^c	−2.4/−7.6

^{a}VT-NMR analysis in 8:2 DMSO-d_6/H_2O at 400 MHz over the range 298−348 K. ^{b}Yaa = H. ^{c}Yaa = $GlyNH_2$.

Molecular backbone conformations were investigated by 2D ROESY; the intensities of the cross-peak were ranked to infer plausible interproton distances (Tables S1 and S2). Structures consistent with the spectroscopic analyses were obtained by MD simulations[30] in a box of explicit water molecules,[31] starting with a set of random geometries. The structures were subjected to high-temperature MD using the distances derived from ROESY as constraints with a scaled force field, followed by a simulation with full restraints. The system was gradually cooled, and the structures were minimized with the AMBER[32] force field. The results were clustered by the rmsd analysis of the backbone atoms.

Computations essentially gave one major cluster for each compound. The representative geometries of **4** and **5** with the lowest internal energy were selected and analyzed (Figure 2). These structures are roughly compatible with an inverse type II β-turn centered on D-Trp-Phe. Generally, the formation of inverse turns is favored by a D-residue in the position $i + 1$.[29] Explicit H-bonds predicted by VT-NMR are not present probably because of a fast equilibrium between different geometries.

Figure 2. (Left and middle) Representative low-energy structures of **4** and **5**, consistent with ROESY analysis, calculated by restrained MD, and representative low-energy structure **5A** (right) showing a well-defined inverse β-turn, determined by unrestrained MD simulations.

To investigate the dynamic behavior, the structures were analyzed by unrestrained MD in a box of water molecules. Besides the different random conformations, the analyses of the trajectories of **5**, but not **4**, revealed the occurrence of well-defined β-turn secondary structures stabilized by a clear H-bond between D-TrpCO and GlyNH (Figure 2, **5A**).

The predisposition to adopt the β-turn might be correlated to the high affinity and selectivity of **5** and to the agonist behavior. Nevertheless, the presence of the third residue $GlyNH_2$ can also strongly influence the binding mode by interacting with an individual residue within the address locus of the MOR, such as Glu229(5.35), in a similar way as proposed for **2** (Figure 1).[14]

CONCLUSIONS

In this work we discussed a minilibrary of minimalist peptides having the N-terminus acetylated. The peptides Ac-D-Trp-$PheNH_2$ (**4**) and Ac-D-Trp-Phe-$GlyNH_2$ (**5**) turned out as opioid ligands with nanomolar affinity. While **4** did not show any agonist activity on MOR, **5** was a selective, partial MOR agonist. The comparison with the structurally correlated **6**−**12** stressed the role of D-Trp and pointed out that the presence of a basic amino group is not a sine qua non[1,13,15] for a proper interaction and activation of MOR. The peptides **4** and **5** might represent interesting candidates for the development of a new class of liphophilic MOR-active peptidomimetics.[33] Conformational analysis showed that **4** and **5** adopt in solution similar conformations; nevertheless, **5** tends to fold into a well-defined β-turn, which might contribute to higher affinity and selectivity.

EXPERIMENTAL SECTION

General Methods. Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. The MW-assisted synthesis was performed using a MicroSYNTH microwave labstation. Flash chromatography was performed on silica gel (230−400 mesh), using mixtures of distilled solvents. Purities were determined to be ≥95% by analytical RP-HPLC and combustion analysis. Analytical RP-HPLC was performed on an ODS column (4.6 μm particle size, 100 Å pore diameter, 250 μm, DAD 210 nm, from a 9:1 H_2O/CH_3CN to a 2:8 H_2O/CH_3CN in 20 min) at a flow rate of 1.0 mL/min, followed by 10 min at the same composition. Elemental analyses were performer using a Thermo Flash 2000 CHNS/O analyzer. Semipreparative RP-HPLC was performed on a C18 column (7 μm particle size, 21.2 mm × 150 mm, from 8:2 H_2O/CH_3CN to 100% CH_3CN in 10 min) at a flow rate of 12 mL/

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min. RP-HPLC of **7**−**12** were performed as reported above, with the addition of 0.1% TFA in the mobile phase. Mass analysis was done by ESI. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, in 5 mm tubes, at rt. Chemical shifts are reported as δ values relative to the solvent peak. 2D spectra were recorded in the phase sensitive mode and processed using a 90°-shifted, squared sine-bell apodization.

Synthesis of 5. HOBt (1.2 mmol) and HBTU (1.2 mmol) were added to a stirred solution of Boc-PheOH (1.0 mmol) in 4:1 DCM/DMF (5 mL) at rt under an inert atmosphere. After 5 min, H-GlyNH$_2$-HCl (1.2 mmol) and DIPEA (2.4 mmol) were added at rt, and the mixture was stirred under MW irradiation at 150 W. After 10 min the mixture was diluted with DCM, and the solution was washed with 0.5 M HCl (5 mL) and saturated $NaHCO_3$ (5 mL). The organic layer was dried over Na_2SO_4, and solvent was removed at reduced pressure. Boc deprotection was performed by treatment with 1:3 TFA/DCM (4 mL). After 30 min the mixture was concentrated at reduced pressure, and the procedure was repeated. The resulting H-Phe-GlyNH$_2$-TFA was coupled with Ac-D-TrpOH under the same conditions described above. The procedure was also utilized to obtain the other peptides. Purification by semipreparative RP-HPLC (general methods) gave **4**−**12** as waxy solids (70−85% yield, 95−98% pure by analytical RP-HPLC).

Receptor Binding to Cloned Human OR. Displacement binding assays have been performed in HEK-293 cells stably expressing human MOR, human DOR, or human KOR obtained as previously reported.[14] Cell surface human MOR receptors were measured on intact cells using [^{3}H]DAMGO (0.1−5.0 nm) as radioligand and naloxone (30 μM) to determine nonspecific binding. For displacement binding assays, HEK-293 cells expressing human MOR were incubated at rt for 2 h with [^{3}H]DAMGO (2.5 nM) in the presence or absence of compounds at various concentrations (10^{-12}−10^{-4} M); nonspecific binding was determined in the presence of naloxone (30 μM). After incubation with the ligands, cells were washed in PBS (pH 7.4) and lysed with 0.1 N NaOH. Lysed samples were buffered with an equal amount of 0.1 N HCl and left in scintillation fluid for 8 h before counting. Cell membranes from DOR and KOR expressing HEK-293 cells were prepared as previously reported.[14] Receptor binding assays were carried out by using [^{3}H]diprenorphine to label DOR and [^{3}H]U69,593 to label KOR and by incubating the membrane preparations at 25 °C for 90 min in buffer containing 100 mM Tris-HCl and 0.3% BSA. For saturation binding assays, the concentrations of [^{3}H]diprenorphine and [^{3}H]U69,593 ranged from 40 pM to 3 nM and from 20 pM to 5 nM, respectively. For competition binding assays, the concentration of [^{3}H]diprenorphine or [^{3}H]U69,593 was 1 and 2 nM, respectively. Nonspecific binding was determined in the presence of 10 μM DPDPE (DOR) or 10 μM U50,488 (KOR) and corresponded to 8−12% and 12−15% of total [^{3}H]diprenorphine and [^{3}H]U69,593 binding, respectively. Triplicate determinations were made for each experiment. Reactions were terminated by filtration through Whatman GF/C filters presoaked with 0.3% polyethylenimine, which were washed three times with 5 mL of ice-cold buffer containing 50 mM Tris-HCl, pH 7.4. The radioactivity trapped was determined by liquid scintillation spectrometry. Data from at least three independent experiments were fitted by nonlinear regression analysis using GraphPad Prism. K_i values were calculated from the IC_{50} using the Cheng−Prusoff equation.[34] IC_{50} values represent mean values from no less than four experiments. IC_{50} values, relative potency estimates, and their associated standard errors were determined by fitting the data to the Hill equation by a computerized nonlinear least-squares method.

Determination of Inhibition of cAMP Accumulation. The agonist activity was determined by measuring the inhibition of forskolin-stimulated cAMP accumulation in whole HEK-293 cells stably expressing MOR. Cells were grown at 37 °C and 5% CO_2 in MEM, 2 mM Gln, and 1× nonessential amino acids supplemented with 10% FBS. Samples in a 75 cm^2 flask at 95−100% confluence were split into 24 wells and incubated overnight. When the confluence became 85−95%, the medium was removed and the cells were washed three times with PBS; thereafter, cells were incubated in serum-free medium containing 0.5 mM 3-isobutyl-1-methylxanthine (Sigma−Aldrich) and exposed for 15 min to 10 μM forskolin without and with each compound (0.01 nM to 100 μM) at 37 °C. Cells were then lysed in 0.1 N HCl, scraped off, and centrifuged (2000g, 5 min). Supernatants were assayed for cAMP concentration by using a cAMP EIA kit (Cayman Chemical Co., Ann Arbor, MI, U.S.) according to the manufacturer's instructions. Each well was determined individually. The triplicates were averaged, and IC_{50} values were determined. Activities were expressed as percent inhibition of forskolin-induced cAMP production.

Conformational Analysis. ROESY cross-peak intensities in 8:2 DMSO-d_6/H_2O were classified as very strong, strong, medium, and weak and associated with distances of 2.2, 2.6, 3.0, and 4.2 Å, respectively. Geminal and obvious correlations were discarded. For the absence of H$\alpha(i,i+1)$ cross peaks, the ω bonds were set at 180° (force constant: of 16 kcal mol^{-1} Å^{-2}). The restrained MD simulations were conducted using the AMBER force field in a 30 Å × 30 Å × 30 Å box of standard TIP3P models of equilibrated water. All water molecules with atoms that come closer than 2.3 Å to a solute atom were eliminated. A 50 ps simulation at 1200 K was used for generating 50 random structures that were subsequently subjected to a 20 ps restrained MD with a 50% scaled force field at the same temperature, followed by 20 ps with full restraints (distance force constant of 7 kcal mol^{-1} Å^{-2}), after which the system was cooled in 5 ps to 50 K. H-Bond interactions were not included nor were torsion angle restraints. The resulting structures were minimized with 3000 cycles of the steepest descent and 3000 cycles of the conjugated gradient (convergence of 0.01 kcal Å^{-1} mol^{-1}). The backbones of the structures were clustered by the rmsd analysis module of HyperChem. Unrestrained MD simulation in explicit water was performed for 10 ns at 298 K at constant temperature and pressure (Berendsen scheme,[35] bath relaxation constant of 0.2). For 1−4 scale factors, van der Waals and electrostatic interactions are scaled in AMBER to half their nominal values. The integration time step was set to 0.1 fs. Box equilibration was set to 10 ps.

■ ASSOCIATED CONTENT

Supporting Information

ROESY cross-peaks observed for **4** and **5**; analytical characterization of **4**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

MOR, μ-opioid receptor; DOR, δ-opioid receptor; KOR, κ-opioid receptor; Dmt, 2′,6′-dimethyltyrosine; VT, variable temperature; MW, microwave; DIPEA, diisopropylethylamine; HOBt, hydroxybenzotriazole; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; RP, reversed phase

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