A Topochemical Approach To Explain Morphiceptin Bioactivity

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A topochemical model to explain the bioactivity of morphiceptin (Tyr[1]-Pro[2]-Phe[3]-Pro[4]-NH_2) was developed by taking account of accessible conformations around rotatable bonds which define relative spatial arrangements of opioid pharmacophores, the amine and phenolic groups of tyrosine and the aromatic ring of phenylalanine, necessary for receptor recognition. For this purpose, ^{1}H-NMR measurements and computer simulations were extensively carried out on 10 stereoisomeric analogs related to morphiceptin: Tyr-Pro-(L and D)-Phe-(L and D)-Pro-NH_2; Tyr-Pro-(L and D)-(NMe)Phe-(L and D)-Pro-NH_2; Tyr-(NMe)Ala-Phe-D-Pro-NH_2; and Tyr-Ala-Phe-D-Pro-NH_2. These analogs are structurally close to one another but display various opiate potencies from highly active to inactive. The conformation of each rotatable bond has been specifically identified by measuring accessible space for the analogs, in which the difference in composition is observed in the specific site affecting only the conformation around the target bond. The most interesting characteristic of the model is a requirement of a cis amide bond linking residues 1 and 2. The model also requires the side chains in a trans conformation (χ_1 = 180°) for the Tyr and Phe residues. The distances between the three pharmacophores, d_1 (Tyr N to Tyr OH), d_2 (Tyr N to the center of the aromatic ring of the third residue), and d_3 (Tyr OH to the center of the aromatic ring of the third residue), were found to be ~8, ~7, and ~11-13 Å, respectively. This model should aid in pharmaceutical design of peptide and nonpeptide ligands with opioid potencies.

Introduction

The structures of naturally occurring peptide opioids such as enkephalin (Tyr-Gly-Gly-Phe-Leu/Met-OH), β-casomorphin (Tyr-Pro-Phe-Gly-Pro-Ile-OH), dermorphin (Tyr-D-Ala-Phe-Gly-Pro-Tyr-Pro-Ser-NH_2), and deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) can be divided into two components,[1] the biologically important N-terminal tri- or tetrapeptide fragment (message sequence) and the remaining C-terminal fragment (address sequence) as shown in Table I. The N-terminal message sequence is composed of two pharmacophoric amino acid residues, Tyr and Phe, in which the amine and phenolic groups of Tyr and the aromatic group of Phe are required for opioid receptor recognition. This sequence also includes a spacer residue(s), which join(s) the pharmacophoric residues of the message sequence.

It has been suggested that there are at least three different opioid receptors, μ, δ, and κ.[2-4] Each receptor requires different three-dimensional structures, so-called "bioactive conformations", with specific arrays of the pharmacophoric groups within the N-terminal message sequence. In this sense, it is no exaggeration to say that the biological activity of opioid peptides is determined by the conformation of the N-terminal sequence. The "bioactive conformation" must be one of the accessible conformations for the molecule. The C-terminal address sequence may stabilize a specific conformation among various conformations accessible to the N-terminal message sequence. This would alter receptor selectivity as suggested by the fact that dermorphin and deltorphin I display the μ-receptor and the δ-receptor selectivities, respectively, although both of the peptides possess the same N-terminal sequence, Tyr-D-Ala-Phe.[5,6]

Table I. Structural Components of Peptide Opioids

	message sequence			
opioid	pharmacophoric residue	spacer residue(s)	pharmacophoric residue	address sequence: tail residues
β-casomorphin	Tyr	Pro	Phe	Pro-Gly-Pro-Ile-OH
dermorphin	Tyr	D-Ala	Phe	Gly-Tyr-Pro-Ser-NH_2
deltorphin I	Tyr	D-Ala	Phe	Asp-Val-Val-Gly-NH_2
enkephalin	Tyr	Gly-Gly	Phe	Leu/Met-OH

In terms of molecular conformations, spacer residues play a significant role in orienting the biologically important Tyr and Phe residues in the correct arrays necessary for opioid activity. Each class of peptide opioids shows distinct chiral requirements for the spacer residues. In enkephalin, the glycine at the third position cannot be readily substituted with a D- or an L-amino acid, whereas the glycine at the second position can be replaced with most D-amino acids without significantly affecting bioactivity.[7] Incorporation of an L-amino acid at position 2 of dermorphin and deltorphin I results in a remarkable reduction in bioactivity.[8,9] The β-casomorphin requires an L chirality for proline at the second position.[10,11] In this respect, β-casomorphin is clearly distinguished from the other classes of peptide opioids.

A tetrapeptide amide, Tyr-Pro-Phe-Pro-NH_2 (morphiceptin), is a potent and selective agonist at the μ-opiate receptor. This peptide was originally synthesized as an analog possessing the N-terminal tetrapeptide fragment of β-casomorphin[11] and recently was isolated from an enzymatic digest of bovine β-casein[12] similar to β-casomorphin.[13,14] Morphiceptin is about 50-100 times more active than β-casomorphin in receptor binding assays and in the guinea pig ileum (GPI) test.[11]

Structures of morphiceptin analogs are characterized by a wealth of *N*-alkyl amino acid residues. Morphiceptin

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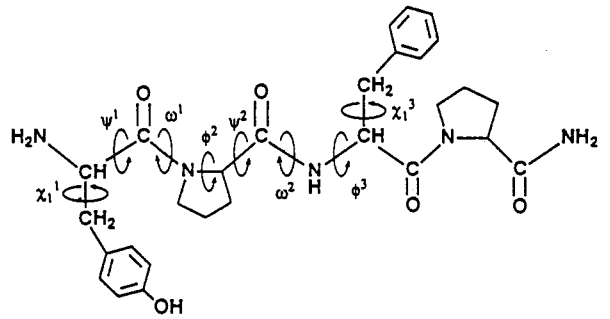

Figure 1. A schematic representation of morphiceptin structure. Torsions given in the figure define spatial orientations of opioid pharmacophores, the amine and phenolic groups of tyrosine and the aromatic group of phenylalanine, required for receptor recognition.

has two prolines (Figure 1). One of the most potent analogs, Tyr-Pro-(NMe)Phe-D-Pro-NH_2, contains an *N*-methylphenylalanine in addition to two prolines and has no secondary amides.[15] The N-alkylation of an amide of an amino acid residue reduces allowed conformational space as compared to that for the corresponding unsubstituted residue.[16] Furthermore, the incorporation of an *N*-alkyl amino acid restricts conformations accessible to the Cα–C(O) bond (ψ) of the preceding residue.[16,17] Therefore, morphiceptin analogs are expected to exhibit clear conformational preferences. However, a consistent explanation for morphiceptin bioactivity in terms of molecular conformation has not been established in spite of a wealth of related analogs and a constrained nature of molecules. One of the reasons may arise from experimental difficulties caused by the coexistence of different configurational isomers from the cis/trans isomerization around the amide bonds preceding the *N*-alkyl amino acid residues. Our ^{1}H- and ^{13}C-NMR spectroscopic studies of Tyr-Pro-Phe-Pro-NH_2 and Tyr-Pro-(NMe)Phe-D-Pro-NH_2 demonstrate that both of the analogs exhibit four discernible configurational isomers in dimethylsulfoxide (DMSO) with an approximate ratio of 55:25:15:5 and 65:25:5:5, respectively.[18] The major isomer, 55% for the former and 65% for the latter, has been assigned the all-trans structure. The second largest isomer accounting for 25% in both of the molecules adopts a cis configuration around the Tyr–Pro amide bond.

The cis/trans configuration around the Tyr–Pro amide bond is of particular significance for morphiceptin bioactivity because this bond is located within the biologically important N-terminal tripeptide message sequence. In previous papers,[19,20] we reported biological and conformational studies of morphiceptin-related analogs containing 2-aminocyclopentanecarboxylic acid (2-Ac^{5}c) as the second residue, Tyr-2-Ac^{5}c-Phe-X^4-NH_2 [X^4 = Pro, (L and D)-Val]. The 2-Ac^{5}c containing analogs were designed to eliminate cis/trans isomerization around the amide bond linking residues 1 and 2 and to define proper spatial arrangements of the pharmacophoric groups within the Tyr and Phe residues necessary for biological activity. The peptidomimetic 2-Ac^{5}c is a β-amino acid with two chiral centers; thus four isomers are probable, i.e. two trans [(1*S*,2*S*)-2-Ac^{5}c and (1*R*,2*R*)-2-Ac^{5}c] and two cis [(1*R*,2*S*)-2-Ac^{5}c and (1*S*,2*R*)-2-Ac^{5}c]. Among the four stereoisomers, only the morphiceptin analogs containing *cis*-(1*S*,2*R*)-2-Ac^{5}c are potent opioids displaying approximately the same bioactivity profiles as the parent compounds which have a proline at position 2. The structure of residue 4 does not affect recognition at the receptor site. Although the 2-Ac^{5}c analogs can only adopt a trans configuration around the Tyr–2-Ac^{5}c amide bond, the bioactive analogs, Tyr-*cis*-(1*S*,2*R*)-2-Ac^{5}c-Phe-X^4-NH_2, are topochemically similar to morphiceptin with the Tyr-Pro amide bond in a cis configuration. In addition, the topological structures of the inactive analogs, Tyr-*cis*-(1*R*,2*S*)-2-Ac^{5}c-Phe-X^4-NH_2, are similar to morphiceptin with the Tyr–Pro amide bond in a trans configuration. We therefore proposed that a cis configuration around the Tyr–Pro amide bond is required for bioactivity of the morphiceptin analogs containing a proline as the second residue.[20]

Since three-dimensional structures of a molecule are determined by a combination of torsional angles around the rotatable bonds, it is necessary for a full explication of structure–activity relationships of biologically active peptides to estimate conformations around all the rotatable bonds linking the pharmacophoric groups. In the case of morphiceptin, the relative spatial arrangements of the pharmacophoric groups, the amine and phenolic groups of the Tyr^1 residue and the aromatic group of the Phe^3 residue, are defined by a set of eight bond conformations: ψ^1, $\chi_1{}^1$, and ω^1 of Tyr^1; ϕ^2, ψ^2, and ω^2 of Pro^2; and ϕ^3 and $\chi_1{}^3$ of Phe^3 (Figure 1). In the present investigation, we identified the torsional angles around these eight bonds required for the morphiceptin (μ-receptor) bioactivity based on accessible conformations of stereoisomeric analogs related to morphiceptin, i.e. Tyr-Pro-(L and D)-X^3-(L and D)-Pro-NH_2 [X^3 = Phe and (NMe)Phe], Tyr-(NMe)Ala-Phe-D-Pro-NH_2, and Tyr-Ala-Phe-D-Pro-NH_2. Among these analogs, Tyr-(NMe)Ala-Phe-D-Pro-NH_2 and Tyr-Pro-D-(NMe)Phe-(L and D)-Pro-NH_2 were newly synthesized by our laboratories. The (NMe)Ala^2 analog was designed to confirm the requirement of a cis configuration around the amide bond linking residues 1 and 2 ($\omega^1 = 0°$) for bioactivity.[21] The D-(NMe)Phe^3 analogs were designed to estimate rotational states around the Cα–C(O) bond (ψ^2) of the Pro^2 residue. Accessible conformations of the morphiceptin analogs were analyzed by considering the restriction of the conformational space from the alkylation of the amide groups. In addition, conformational preferences of the Tyr^1 and Phe^3/(NMe)Phe^3 side chains were elucidated by ^{1}H-NMR spectroscopy. These studies allowed us to propose specific three-dimensional structures necessary for morphiceptin bioactivity.

Design of Tyr-L-(NMe)Ala-Phe-D-Pro-NH_2

In an attempt to confirm the requirement of a cis amide bond linking residues 1 and 2 for morphiceptin bioactivity, we designed a morphiceptin analog containing *N*-methyl-L-alanine as the second residue: Tyr-(NMe)Ala-Phe-D-Pro-NH_2.[21] The parent compound, Tyr-Pro-Phe-D-Pro-NH_2, has been reported to be one of the most potent analogs among the β-casomorphins.[15] To our knowledge, no biological activity has been reported for opioid peptides to incorporate *N*-alkyl-L-amino acids at position 2, with the exception of proline homologs. One of the reasons may be the fact that peptides containing an L-amino acid at the second position generally have low opiate potencies. However, the incorporation of L-(NMe)Ala into the second position of the morphiceptin sequence leads to possible cis/trans isomerization around the Tyr–(NMe)Ala amide bond as in a similar situation around the Tyr–Pro amide bond. In order to explain the topochemical effects of the second residue on bioactivity, we synthesized Tyr-Ala-Phe-D-Pro-NH_2 as a control molecule which we expected to be inactive.[21]

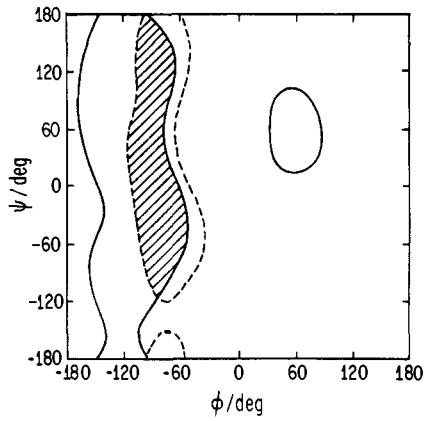

Figure 2. The (ϕ, ψ) regions allowed for cis configurational isomers of Ac-(NMe)Ala-NHMe (solid) and Ac-Pro-NHMe (dashed). The inside of the regions represents 5 kcal mol^{-1} or less in energy above the global minimum for each molecule. The shaded region designates conformations common to both molecules and therefore defines common structures which can be recognized at the μ-receptor for the second residues of the morphiceptin analogs, Tyr-(NMe)Ala-Phe-D-Pro-NH_2 and Tyr-Pro-Phe-D-Pro-NH_2.

Since the analogs Tyr-X^2-Phe-D-Pro-NH_2 [X^2 = Pro, (NMe)Ala, and Ala] differ only at the second residue, differences in bioactivities can be interpreted in terms of the conformations of residue 2. In order to measure accessible conformational space for the second residues of the above analogs, energy calculations were carried out for three *N*-acetyl *N'*-methylamide derivatives, Ac-X^2-NHMe [X^2 = Pro, (NMe)Ala, and Ala]. The derivatives, Ac-X^2-NHMe, display the largest accessible space because they do not have neighboring residues which might restrict conformations for the X^2 residues in a long peptide sequence. The backbone conformation of such model compounds is characterized by a set of angles, ϕ and ψ, respectively, representing the rotational states around the skeletal single bonds, N–C^α and C^α–C(O) of the X^2 residues, in addition to a cis/trans configuration around the amide bond preceding the X^2 residues.

The allowed (ϕ, ψ) regions which have 5 kcal mol^{-1} or less energy for the cis isomers of Ac-(NMe)Ala-NHMe and Ac-Pro-NHMe are shown in Figure 2. For Ac-Pro-NHMe, there are two major puckerings for the five-membered pyrrolidine ring. One has an angle, χ_1, which represents the rotational state around the C^α–C^β bond, ca. −30° (form I), and the other has an angle χ_1 ca. +30° (form II). Energy of form II ($\chi_1 \sim +30°$) was plotted in Figure 2 because this form was calculated to be lower in energy than form I ($\chi_1 \sim -30°$) at any given (ϕ, ψ) values. This is in agreement with experimental observation that form II has been found to dominate for the cis proline. This is verified from the vicinal ^{1}H–^{1}H coupling constants, 2.6 and 8.7 Hz, for the H-C^α-C^β-H groupings of the morphiceptin analogs containing prolines in conjunction with a Karplus-like relationship.[22] The shaded region in Figure 2, which is common to both the molecules, spreads out to almost the entire space allowed for Ac-Pro-NHMe. These results suggest that the designed analog, Tyr-(NMe)Ala-Phe-D-Pro-NH_2, with the Tyr–(NMe)Ala amide bond in a cis configuration, can assume the same overall topochemistry as the parent analog, Tyr-Pro-Phe-D-Pro-NH_2, with a cis amide bond about Tyr–Pro. The Ala^2 analog cannot assume this topochemistry because the Tyr–Ala amide bond can only adopt a trans configuration. Therefore, we predict that the designed analog, Tyr-(NMe)Ala-Phe-D-Pro-NH_2, would be bioactive whereas Tyr-Ala-Phe-D-Pro-NH_2 would be inactive. We also postulate that morphiceptin bioactivity requires a cis configuration around the amide bond linking residues 1 and 2.

Design of Tyr-Pro-D-(NMe)Phe-(L and D)-Pro-NH_2

There are two minimum energy conformations in the accessible (ϕ, ψ) space for the cis isomer of Ac-Pro-NHMe. One is in an F domain which is defined by torsions ϕ = −110° to −40° and ψ = 130–220° and the other is in an A domain which is dfined by torsions ϕ = −110° to −40° and ψ = −90° to −40° (Figure 2). The definition of the F and A domains follows the convention of Zimmerman et al.[23] It may be fair to say that high μ-receptor activities of the morphiceptin analogs are attributed to structures adopting either one of these conformations for the second residue in addition to the cis amide bond linking residues 1 and 2. The F and A conformations have the same values for the ϕ angle but are different for the ψ angle. To elucidate the (ϕ, ψ) conformational requirements for morphiceptin bioactivity, we designed new analogs incorporating *N*-methyl-D-phenylalanine as the third residue, Tyr-Pro-D-(NMe)Phe-(L and D)-Pro-NH_2, after due consideration of the restriction of accessible conformations as induced by the stereochemistry and N-methylation of phenylalanine at position 3.

Since morphiceptin, Tyr-Pro-Phe-Pro-NH_2, and the corresponding D-Phe^3 analog, Tyr-Pro-D-Phe-Pro-NH_2, display μ-receptor activities,[24] both of the molecules assume topochemically similar structures in which all the pharmacophoric groups orient themselves in the correct array necessary for the μ-receptor recognition. All of the pharmacophoric groups of the morphiceptin analogs, the amine and phenolic groups of Tyr^1 and the aromatic group of Phe^3, are located within the N-terminal tripeptide sequences. Therefore, we searched through accessible conformations of L-tyrosyl-L-prolyl-(L and D)-phenylalanine dimethylamides [Tyr-Pro-(L and D)-Phe-N(Me)$_2$] for the common spatial arrangements of these functional groups. These tripeptide dimethylamides possess the structural characteristics of the N-terminal tripeptide sequences of the morphiceptin analogs, Tyr-Pro-(L and D)-Phe-(L and D)-Pro-NH_2. There are advantages in the use of such model compounds. The molecules display greater accessible space than the tetrapeptides without being restricted by the fourth residue. In addition, the effects of the chirality of the fourth residue on conformational preferences and on bioactivity can be eliminated.

Accessible conformations were elucidated in two steps. The mainchain conformations were first estimated by carrying out energy minimizations for L-alanyl-L-prolyl-(L and D)-alanine dimethylamides [Ala-Pro-(L and D)-Ala-N(Me)$_2$] with a cis configuration around the Ala–Pro amide bond. The approach of concentrating on the mainchain structures was used in order to allow the greatest number of solutions for the conformational search. In this manner, accessible space for the mainchain is not excluded because of the presence of the Tyr and Phe side chains. Energy minimizations were then carried out for Tyr-Pro-(L and D)-Phe-N(Me)$_2$. Molecular structures were optimized from initial structures generated by considering the accessible conformations of Ala-Pro-(L and D)-Ala-N(Me)$_2$ estimated from the above treatments and by placing the Tyr and Phe side chains in the regularly staggered confor-

Table II. Comformations of the Second (Pro^2) and Third (X^3) Residues for Topologies I–IV Commonly Accessible for Morphiceptin Analogs, Tyr^1-Pro^2-X^3-$N(Me)_2$, with the Tyr^1–Pro^2 Amide Bond in a Cis Configuration[a]

analog Tyr^1-Pro^2-X^3-$N(Me)_2$, X^3 =		topology I	topology II	topology III	topology IV
L-Phe	Pro^2 (ϕ^2, ψ^2)	(−76°, 129°)	(−79°, 153°)	(−72°, −52°)	(−75°, −47°)
	X^3 (ϕ^3, ψ^3, χ_1^3)	(−147°, 130°, −168°)	(−109°, 102°, −63°)	(−127°, 93°, −169°)	(−124°, 88°, −67°)
	ΔE/kcal mol^{-1}	0.660	0.000	0.054	0.667
D-Phe	Pro^2 (ϕ^2, ψ^2)	(−74°, −39°)	(−78°, −61°)	(−74°, 136°)	(−74°, 134°)
	X^3 (ϕ^3, ψ^3, χ_1^3)	(111°, −104°, 169°)	(108°, −105°, 67°)	(111°, −98°, 171°)	(116°, −94°, 69°)
	ΔE/kcal mol^{-1}	1.937	1.479	0.000	1.194
L-(NMe)Phe	Pro^2 (ϕ^2, ψ^2)	(−77°, 147°)	(−77°, 159°)	(−75°, 144°)	(−75°, 147°)
	X^3 (ϕ^3, ψ^3, χ_1^3)	(−119°, 92°, −164°)	(−114°, 91°, −60°)	(55°, 73°, −158°)	(57°, 57°, −62°)
	ΔE/kcal mol^{-1}	2.588	0.000	10.146	9.102
D-(NMe)Phe	Pro^2 (ϕ^2, ψ^2)	(−78°, 143°)	(−78°, 162°)	(−77°, 143°)	(−77°, 146°)
	X^3 (ϕ^3, ψ^3, χ_1^3)	(−80°, −121°, 158°)	(−73°, −139°, 57°)	(118°, −91°, 165°)	(122°, −86°, 62°)
	ΔE/kcal mol^{-1}	10.858	9.314	0.000	0.515

[a] The values of ΔE represent energies of the molecular conformations with the Tyr^1 side chain in a trans conformation ($\chi_1 \sim 180°$) relative to the lowest energy conformation of each analog.

mations, i.e. χ_1 = 60° (g^+), 180° (t), −60° (g^-). The geometries of the resulting accessible structures were characterized by three distances between the three pharmacophoric groups, d_1 (Tyr N–Tyr OH), d_2 (Tyr N–the center of the Phe aromatic ring), and d_3 (Tyr OH–the center of the Phe aromatic ring), so that each of the structures was represented by a triangle. Among these three distances, the value of d1 was determined by only the Tyr side chain χ_1 conformation.

Four topologies commonly accessible to both Tyr-Pro-Phe-$N(Me)_2$ and Tyr-Pro-D-Phe-$N(Me)_2$ were obtained when the Tyr moiety adopted similar conformations around the ψ^1 and χ_1^1 angles (Table II). These four topologies correspond to the four lowest energy conformations of each molecular system with the Tyr–Pro amide bond in a cis configuration. It should be mentioned that the relative spatial arrangements of the three pharmacophores are largely different from one another in these four topologies. In topologies I and II, the Pro^2 residue in the L-Phe^3 analog adopts the conformation in the F domain while the same residue in the D-Phe^3 analog adopts the conformation in the A domain (Figure 2). Topologies III and IV require the opposite conformations for the Pro^2 residues from topologies I and II, i.e. the A conformation for the L-Phe^3 analog and the F conformation for the D-Phe^3 analog. These results indicate that the chirality change of the Phe^3 residue can be compensated by a ca. 180° rotation around the C^α–C(O) bond (ψ^2) of the Pro^2 residue.

Similar calculations were carried out for the *N*-methyl-(L and D)-phenylalanine containing compounds, Tyr-Pro-(L and D)-(NMe)Phe-$N(Me)_2$, to determine topochemical requirements of the Pro^2 residue for morphiceptin bioactivity. The N-methylation of the (L and D)-Phe^3 residues restricts the ψ angle of the preceding Pro^2 residue to positive values, 60° < ψ < 180°. Therefore, only the F conformation was calculated for the Pro^2 residue (Table II). Conformations with −180° < ψ < 0° are precluded because of steric overlap between the pyrrolidine ring and the *N*-methyl group of the (L or D)-(NMe)Phe residue. In fact, it has been reported that poly-L-proline assumes either the polyproline I structure with (ϕ, ψ) = (−83°, 158°) and a cis amide bond[25] or the polyproline II structure with (−75°, 149°) and a trans amide bond.[26]

The L-(NMe)Phe^3 analog, Tyr-Pro-(NMe)Phe-$N(Me)_2$, prefers topologies I and II over topologies III and IV by ca. 9 kcal mol^{-1}. On the contrary, the D-(NMe)Phe^3 analog prefers topologies III and IV over I and II by ca. 9 kcal mol^{-1}. It may be fair to say that the L-(NMe)Phe^3 analog assumes only topologies I and II while the D-(NMe)Phe^3 analog assumes only topologies III and IV to a substantial extent. It has been reported that the L-(NMe)Phe^3 morphiceptin analogs, Tyr-Pro-(NMe)Phe-Pro-NH_2 and Tyr-Pro-(NMe)Phe-D-Pro-NH_2, display comparable or slightly increased bioactivities as compared with the corresponding L-Phe^3 analogs.[15,24] We, therefore, predict that morphiceptin bioactivity requires either topology I or II. According to the above considerations, the designed D-(NMe)Phe^3 morphiceptin analogs, Tyr-Pro-D-(NMe)-Phe-Pro-NH_2 and Tyr-Pro-D-(NMe)Phe-D-Pro-NH_2, are predicted to be inactive because the analogs cannot assume topologies I and II.

Results

Cis/Trans Configurational Isomers of Morphiceptin Analogs. Cis/trans configurational isomers of morphiceptin analogs, Tyr-Pro-(L and D)-X^3-(L and D)-Pro-NH_2 [X^3 = Phe and (NMe)Phe], Tyr-(NMe)Ala-Phe-D-Pro-NH_2, and Tyr-Ala-Phe-D-Pro-NH_2, were studied by ^{1}H-NMR spectroscopy. Two proline residues at positions 2 and 4 within Tyr-Pro-(L and D)-Phe-(L and D)-Pro-NH_2 allow for the possibility of cis/trans isomerization around the amide bonds between residues 1 and 2 and between residues 3 and 4, resulting in four configurational isomers, $trans^{1-2}$-$trans^{3-4}$, $trans^{1-2}$-cis^{3-4}, cis^{1-2}-$trans^{3-4}$, and cis^{1-2}-cis^{3-4}. These isomers were characterized by use of ^{1}H-NMR spectroscopy. All of the proton resonances were assigned using two-dimensional homonuclear Hartman–Hahn (HOHAHA)[27] and rotating frame nuclear Overhauser enhancement (ROESY)[28] experiments. The assignment of a cis amide bond was achieved by a strong sequential nuclear Overhauser effect (NOE) between α-protons of adjacent residues resulting from a close contact of these nuclear spins (~2 Å). Populations of individual isomers, determined from an integration of the one-dimensional spectra, are summarized in Table III.

The *N*-methyl amino acids also allow for possible cis/trans isomerization around the N-methylated amide bonds in a similar manner as proline. The (NMe)Ala^2 analog, Tyr-(NMe)Ala-Phe-D-Pro-NH_2, exhibits all of the four configurational isomers possible for the molecule. Although eight configurational isomers can be envisioned for the (NMe)Phe containing morphiceptin analogs, Tyr-Pro-(L and D)-(NMe)Phe-(L and D)-Pro-NH_2, only four isomers have been experimentally observed. The four

Table III. Populations[a] (%) of Configurational Isomers of Morphiceptin Analogs

analog	configurational isomers[b]				total[c] of cis[1-2]
	trans[1-2]-trans[3-4]	trans[1-2]-cis[3-4]	cis[1-2]-trans[3-4]	cis[1-2]-cis[3-4]	
Tyr-Pro-Phe-Pro-NH_2	55	15	25	5	30
Tyr-Pro-Phe-D-Pro-NH_2	56	14	24	6	30
Tyr-Pro-D-Phe-Pro-NH_2	33	28	21	18	39
Tyr-Pro-D-Phe-D-Pro-NH_2	57	7	30	6	36
Tyr-Pro(NMe)Phe-Pro-NH_2[d]	58	6	32	4	36
Tyr-Pro-(NMe)Phe-D-Pro-NH_2[d]	65	5	25	5	30
Tyr-Pro-D-(NMe)Phe-Pro-NH_2[d]	47	15	30	8	38
Tyr-Pro-D-(NMe)Phe-D-Pro-NH_2[d]	68		32		32
Tyr-(NMe)Ala-Phe-D-Pro-NH_2	43	28	19	10	29
Tyr-Ala-Phe-D-Pro-NH_2	68	32			

[a] The populations were estimated by ^{1}H-NMR experiments carried out in DMSO-d_6 at 25 °C. [b] The notations trans[1-2], cis[1-2], trans[3-4], and cis[3-4] denote configurations of the amide bonds linking residues of superscript numbers, i.e., cis[1-2]-trans[3-4] represents a configurational isomer in which the amide bond linking residues 1 and 2 is in a cis configuration and the amide bond linking residues 3 and 4 is in a trans configuration. [c] The value represents a total population of isomers which contain the amide bond linking residues 1 and 2 in a cis configuration, i.e. cis[1-2]-trans[3-4] and cis[1-2]-cis[3-4]. [d] Only a trans configuration has been observed for the amide bond linking residues 2 and 3, although incorporation of N-methylated phenylalanine at position 3 allows for the possibility of cis/trans isomerization around this amide bond.

Table IV. Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays of Morphiceptin Analogs

analog	X^3 = Phe			X^3 = (NMe)Phe		
	IC_{50}/nM		IC_{50} ratio	IC_{50}/nM		IC_{50} ratio
	GPI	MVD	MVD/GPI	GPI	MVD	MVD/GPI
Tyr-Pro-L-X^3-Pro-NH_2	552 ± 151	3690 ± 740	6.68	170 ± 25	1790 ± 50	10.5
Tyr-Pro-L-X^3-D-Pro-NH_2	28.7 ± 2.4	1508 ± 180	52.5	20.7 ± 2.4	1250 ± 220	60.4
Tyr-Pro-D-X^3-Pro-NH_2	109 ± 16	594 ± 77	5.45	>100000	>1000000	
Tyr-Pro-D-X^3-D-Pro-NH_2	557 ± 58	4810 ± 320	8.34	2660 ± 120	564800	20.6
Tyr-(NMe)Ala-L-X^3-D-Pro-NH_2	30.6 ± 6.1	202 ± 28	6.60			
Tyr-Ala-L-X^3-D-Pro-NH_2	26900 ± 7200	>100000				

isomers for each analog contain a trans amide bond between the Pro[2] and (NMe)Phe[3] residues.

From a total population of the cis[1-2]-trans[3-4] and cis[1-2]-cis[3-4] isomers, a configurational energy of the cis state relative to the trans state around the amide bond linking residues 1 and 2 was estimated to be 0.26–0.53 kcal mol^{-1}.

Biological Activity. The in vitro biological activities of the 10 analogs measured in the guinea pig ileum (GPI)[29] and mouse vas deferens (MVD)[30] assays are summarized in Table IV. The GPI and MVD assays were used for determining the bioactivities at the μ- and δ-opioid receptors, respectively. As expected, Tyr-Ala-Phe-D-Pro-NH_2 is inactive in both the GPI and MVD tests. The N-methylation of the L-Ala[2] residue of this inactive analog results in a highly potent analog, Tyr-(NMe)Ala-Phe-D-Pro-NH_2, displaying approximately the same potency at the μ-receptor and 7 times higher potency at the δ-receptor as compared with the parent Pro[2] analog, Tyr-Pro-Phe-D-Pro-NH_2. The observed high μ-receptor activity of Tyr-(NMe)Ala-Phe-D-Pro-NH_2 and inactivity of Tyr-Ala-Phe-D-Pro-NH_2 are in agreement with our predictions based on accessible conformational space which is induced by the cis/trans configuration at the second residues.

As reported in the literature,[15,24] the N-methylation of the L-Phe[3] residues of Tyr-Pro-Phe-(L and D)-Pro-NH_2 does not appreciably affect the activity at the μ-receptor. In contrast, the same modification of the D-Phe[3] residues of the biologically active analogs, Tyr-Pro-D-Phe-(L and D)-Pro-NH_2, results in a remarkable reduction in potency at both the μ- and the δ-receptor. These observations are again in agreement with our predictions derived from inspection of the accessible conformations for the model compounds, Tyr-Pro-(L and D)-X^3-$N(Me)_2$ [X^3 = Phe and (NMe)Phe].

As revealed by the comparison of bioactivities between Tyr-Pro-Phe-Pro-NH_2 and Tyr-Pro-Phe-D-Pro-NH_2 and between Tyr-Pro-D-Phe-Pro-NH_2 and Tyr-Pro-D-Phe-D-Pro-NH_2, the relative potency at the μ-receptor is strongly dependent upon the chirality sequence of residues 3 and 4. The analogs with a heterochiral sequence at positions 3 and 4, Tyr-Pro-Phe-D-Pro-NH_2 and Tyr-Pro-D-Phe-Pro-NH_2, display higher μ-receptor activities than the corresponding analogs with a homochiral sequence at the same positions, Tyr-Pro-Phe-Pro-NH_2 and Tyr-Pro-D-Phe-D-Pro-NH_2, respectively. The chirality effects of the fourth residue on bioactivity are discussed below with the conformational requirements of the L-Phe[3] and D-Phe[3] side chains.

Discussion

Structural studies of receptor–ligand complexes should provide the "bioactive conformations" directly if a purified receptor were available. However, since no opioid receptor has been isolated in pure form, the "bioactive conformations" of opioid peptides must be estimated indirectly from conformational studies of peptides. We believe that the "bioactive conformation" must be one of the accessible conformations for the isolated molecule because receptors cannot convert inaccessible structures to allowed conformations by interaction of the receptor with a ligand. In addition, the biological potency of a molecule depends upon the relative stability of the "bioactive conformation" as compared to the other accessible conformations. Therefore, it might be possible to estimate "bioactive conformations" by searching common spatial arrangements of pharmacophoric groups through all conformations accessible for a family of peptides with different biological potencies. However, such a search is time consuming and costly because the number of accessible conformers rises rapidly as the number of amino acid residues increases. In addition, this method will probably lead to multiple candidates of "bioactive conformations" with different spatial arrays of the pharmacophores.

Since the three-dimensional array of the pharmacophoric groups are defined by a set of conformational states around rotatable bonds, it is also possible to estimate the "bioactive conformations" by identifying the specific conformational states for all of these rotatable bonds. For this purpose, we have developed a new methodology. A combined use of a stereoisomeric approach and incorporation of peptidomimetic structures allows us to restrict accessible conformational space about a specific bond to particular regions. We compare accessible conformations with biological potencies for a series of peptide analogs whose compositions vary from one another at a specific site affecting only the conformation around a specific bond. The "bioactive conformations" could be estimated by repeating the same process for all of the rotatable bonds connecting the pharmacophores. Since we deal with only one or two specific bond conformations at a time, our methodology can be applied to bioactive peptide systems of various sizes from small to relatively large molecules. Below we present an application of this methodology to opioid peptides of the morphiceptin family.

Morphiceptin and related analogs have sequences containing conformationally constrained amino acids such as proline and *N*-methyl amino acids. These amino acids also restrict accessible space for the preceding residues.[16,17] Thus, a limited number of conformations are allowed for the morphiceptin analogs. As shown in Figure 1, the relative spatial arrangements of the pharmacophoric groups of morphiceptin, the amine and phenolic groups in the Tyr^1 residue and the aromatic group in the Phe^3 residue, are defined by a set of eight torsional angles: ψ^1, $\chi_1{}^1$, and ω^1 of Tyr^1; ϕ^2, ψ^2, and ω^2 of Pro^2; ϕ^3 and $\chi_1{}^3$ of Phe^3. Among these eight angles, the ϕ^2 angle is restricted to ca. −75° because of the constrained nature of the five-membered pyrrolidine ring of Pro^2. The ω^2 angle is 180° (a trans configuration) because of the normal amide bond linking the Pro^2 and Phe^3 residues. Although the methylation of the amide group of Phe^3 allows for the possibility of a cis/trans isomerization around the Pro^2–(NMe)Phe^3 amide bond, only a trans configuration ($\omega^2 \sim 180°$) has been observed for the bioactive analogs, Tyr-Pro-(NMe)-Phe-(L and D)-Pro-NH_2, through the ^{1}H-NMR studies (Table III). Therefore, the remaining six angles define spatial arrays of pharmacophoric groups. Here, we identify a specific topochemical geometry necessary for the bioactivity of morphiceptin analogs by considering accessible rotational states for these six angles. A portion of the following results and investigations has been reported in a brief communication.[21]

Structural Factors of Topochemistry for Morphiceptin Bioactivity. (1) Configuration of the Tyr^1–Pro^2 Amide Bond (ω^1). Because of a proline at position 2, two configurations, cis ($\omega^1 \sim 0°$) and trans ($\omega^1 \sim 180°$), are probable for the Tyr^1–Pro^2 amide bond of the Pro^2 morphiceptin analogs. The configuration of the Tyr^1–Pro^2 amide bond plays a significant role in the overall structures of the molecule. To examine whether a cis or trans configuration is responsible for morphiceptin bioactivity, we synthesized two analogs, Tyr-(NMe)Ala-Phe-D-Pro-NH_2 and Tyr-Ala-Phe-D-Pro-NH_2, which contain the L-amino acids at the second position as in the parent compound, Tyr-Pro-Phe-D-Pro-NH_2. As revealed by the NMR studies, the biologically active (NMe)Ala^2 analog exhibits cis and trans forms around the amide bond linking residues 1 and 2 (29:71) similar to the Pro^2 analog (30:70),

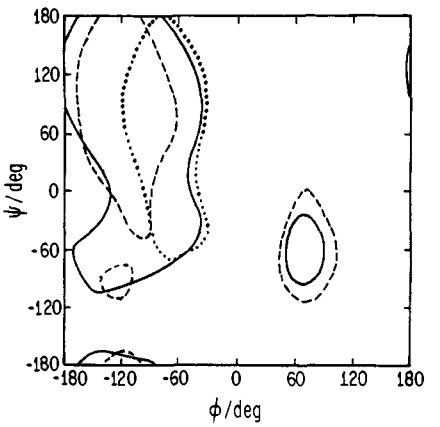

Figure 3. Allowed (ϕ, ψ) regions for Ac-Ala-NHMe (solid) and trans configurational isomers of Ac-(NMe)Ala-NHMe (dashed) and Ac-Pro-NHMe (dotted). The inside of the regions represents 5 kcal mol^{-1} or less in energy above the global minimum for each molecule.

whereas the inactive Ala^2 analog adopts only the trans form (Tables III and IV).

The only difference in the composition of Tyr-X^2-Phe-D-Pro-NH_2 [X^2 = Pro, (NMe)Ala, and Ala] arises from the structure at position 2. Thus, the different bioactivities observed for these analogs must be explained by the conformations of the second residues. Topochemical requirements of the second residues of the Pro^2 and (NMe)-Ala^2 analogs for bioactivities were estimated based on the allowed (ϕ, ψ) space for the model compounds, Ac-X^2-NHMe [X^2 = Pro, (NMe)Ala, and Ala].

The allowed (ϕ, ψ) space calculated for Ac-Ala-NHMe and the trans isomers of Ac-Pro-NHMe and Ac-(NMe)-Ala-NHMe are shown in Figure 3. All of the space accessible for the trans isomers of Ac-Pro-NHMe and Ac-(NMe)Ala-NHMe are also allowed for Ac-Ala-NHMe. Thus, the inactive analog, Tyr-Ala-Phe-D-Pro-NH_2, can assume the same overall topochemistry as Tyr-Pro-Phe-D-Pro-NH_2 and Tyr-(NMe)Ala-Phe-D-Pro-NH_2 with the amide bond linking residues 1 and 2 in a trans configuration. If the "bioactive conformations" of the Pro^2 and (NMe)Ala^2 analogs were to have the trans amide bond, the Ala^2 analog would also be biologically active. However, the GPI and MVD studies show that the Ala^2 analog is inactive at both the μ- and δ-receptors (Table IV). These results indicate that the structures of Tyr-Pro-Phe-D-Pro-NH_2 and Tyr-(NMe)Ala-Phe-D-Pro-NH_2, where a trans amide bond links residues 1 and 2, cannot be responsible for the bioactivities of these molecules. We, therefore, conclude that the high μ-receptor activities of the Pro^2 and (NMe)Ala^2 analogs arise from structures where the amide bonds between residues 1 and 2 are in a cis configuration.

The above postulate could be confirmed by the allowed (ϕ,ψ) regions calculated for the cis configurational isomers of Ac-Pro-NHMe and Ac-(NMe)Ala-NHMe (Figure 2). Because both the Pro^2 and (NMe)Ala^2 opioid analogs display almost the same bioactivities at the μ-receptor, the second residue in each analog most likely assumes similar conformations shown in the shaded region. The shaded region designates conformations common to both analogs and thus defines structures recognized at the μ-receptor for both the opioids. We conclude that high μ-receptor activities of the morphiceptin analogs are attributed to structures where the second residue assumes conformations in the shaded region in Figure 2. In

addition, a cis configuration ($\omega^1 \sim 0°$) is required around the amide bond linking residues 1 and 2. The NMR studies revealed that a cis configuration around the amide bond linking residues 1 and 2 was less favored than a trans configuration for the morphiceptin analogs themselves by energies of 0.26 to 0.53 kcal mol^{-1}. The requirement of a cis configuration for morphiceptin bioactivity suggests that these energies can be compensated for by ligand–receptor interactions.

(2) Backbone Conformation of the First Residue (ψ^1). The rotation of the ψ angle around the C^α–C(O) bond is highly restricted to a range of 60–180° for any L-amino acid residue which is immediately followed by a proline. A range of $-180° < \psi < 0°$ is precluded by a steric overlap between the side chain of the L residue and the $C^\delta H_2$ group attached to the nitrogen of the Pro residue when the Pro residue assumes a trans configuration.[17] The same ψ angle range is also precluded for the L residue which is followed by a cis proline because of a steric overlap between the side chain of the L residue and the $C^\alpha H$ group of the Pro residue. Therefore, the Tyr^1 residue of the morphiceptin analogs adopts only the structures with the ψ^1 angle within a range from 60° to 180°.

(3) Backbone Conformations of the Second and Third Residues (ψ^2 and ϕ^3). Two conformational states, F with $(\phi, \psi) \sim (-75°, 135°)$ and A with $(-75°, -40°)$, are probable for the cis configurational isomer of a proline embedded in a long peptide sequence (Figure 2). Thus, either one of these conformations could be responsible for morphiceptin bioactivity. The ψ angle is the only difference in these states. The Phe^3-modified morphiceptin analogs, Tyr-Pro-(L and D)-X^3-(L and D)-Pro-NH_2 [X^3 = Phe and (NMe)Phe], provide useful insight into the selection of the correct ψ^2 angle. The N-methylation of L-Phe^3 within the active analogs, Tyr-Pro-Phe-(L and D)-Pro-NH_2, maintains the bioactivities and the μ-receptor selectivities. The same modification of D-Phe^3 in the active analogs, Tyr-Pro-Phe-(L and D)-Pro-NH_2, results in a drastic reduction in bioactivity (Table IV). These results demonstrate that the N-terminal tripeptide sequences Tyr-Pro-Phe, Tyr-Pro-(NMe)Phe, and Tyr-Pro-D-Phe can adopt topochemically similar geometries with the correct spatial arrangements of the pharmacophores, which are required for the bioactivity at the μ-receptor. On the other hand, the Tyr-Pro-D-(NMe)Phe sequence cannot assume the geometries for bioactivity.

In order to estimate specific geometries for morphiceptin bioactivity, accessible conformations for the N-terminal tripeptide sequences of the morphiceptin analogs were studied by using model compounds, Tyr-Pro-(L and D)-X^3-N(Me)$_2$ [X^3 = Phe and (NMe)Phe], with the Tyr–Pro amide bond in a cis configuration (see above). As shown in Table II, topologies I and II were found to be commonly accessible to the bioactive sequences, Tyr-Pro-Phe, Tyr-Pro-(NMe)Phe, and Tyr-Pro-D-Phe. In these topologies, the Pro^2 residues within the L-Phe^3 and L-(NMe)Phe^3 analogs adopt the F conformation with $\psi^2 \sim 140°$, while the same residue within the Tyr-Pro-D-Phe sequence adopts the A conformation with $\psi^2 \sim 50°$. In addition, the third residue assumes the lowest energy state around the N–C^α bond (ϕ^3), namely ca. −120° for the L-Phe^3 and L-(NMe)Phe^3 residues and ca. +120° for the D-Phe^3 residue. The inactive Tyr-Pro-D-(NMe)Phe sequence can assume neither topology I nor II. We, therefore, conclude that either topology I or II is responsible for the biological activity of morphiceptin analogs.

(4) Side Chain Conformation of the Third Residue (χ_1^3). Inspection of the accessible conformations for the mainchains of the morphiceptin analogs provides topologies I and II as candidates for the "bioactive conformations". The only difference in these two topologies is the side-chain orientation of the third residue (Table II). Topology I requires the $\chi_1^3 = t$ (180°) conformation for both the L-Phe^3 and D-Phe^3 residues. On the other hand, topology II requires the g^- (−60°) and g^+ (+60°) conformations for the L-Phe^3 and D-Phe residues, respectively. Relative potencies compared between Tyr-Pro-Phe-Pro-NH_2 and Tyr-Pro-Phe-D-Pro-NH_2 and between Tyr-Pro-D-Phe-Pro-NH_2 and Tyr-Pro-D-Phe-D-Pro-NH_2 provide insight into the conformational requirements of the L-Phe^3 and D-Phe^3 side chains (χ_1^3). The analogs with a heterochiral sequence at positions 3 and 4, Tyr-Pro-Phe-D-Pro-NH_2 and Tyr-Pro-D-Phe-Pro-NH_2, display higher μ-receptor activities than the corresponding analogs with a homochiral sequence at the same positions, Tyr-Pro-Phe-Pro-NH_2 and Tyr-Pro-D-Phe-D-Pro-NH_2, respectively (Table IV). The ^{1}H-NMR studies and the conformational energy calculations demonstrate that the main chains of both the heterochiral and homochiral sequence analogs assume quite similar conformations but that the side chains of the third residues show different conformational preferences.

Fractions of three side chain conformers, $\chi_1^3 = g^-$, t, g^+, of the third residue were estimated from the observed vicinal ^{1}H–^{1}H coupling constants ($J_{\alpha-\beta}$'s) for the H-C^α-C_β-H groupings by employing the rotational isomeric state approximation. The trans (J_T) and gauche (J_G) coupling constants required for the analysis were set to 13.85 and 3.55 Hz following Cung et al.[31] The results are summarized in Table V. The *pro*chiralities of the β-protons of the $C^\beta H_2$ groups were assigned by a combination of the observed $J_{\alpha-\beta}$ values and NOEs including the β-protons. Compared to the corresponding homochiral sequence analog, Tyr-Pro-Phe-Pro-NH_2, a larger fraction of t conformer and a smaller g^- conformer fraction were observed for the L-Phe^3 residue of the more potent heterochiral analog, Tyr-Pro-Phe-D-Pro-NH_2. Similarly, Tyr-Pro-D-Phe-Pro-NH_2 displays a larger χ_1^3 = t conformer fraction and a smaller g^+ fraction than Tyr-Pro-D-Phe-D-Pro-NH_2. When the amide bond linking residues 3 and 4 adopts a trans configuration (cis$^{1-2}$-trans$^{3-4}$ isomer), the χ_1^3 = t conformer in a homochiral sequence includes short contacts between the aromatic ring of a phenylalanine and the C=O group of a proline at position 4. When the same amide bond adopts a cis configuration (cis$^{1-2}$-cis$^{3-4}$ isomer), similar short contacts are observed for the t conformer in a heterochiral sequence. However, the cis$^{1-2}$-cis$^{3-4}$ isomer would contribute less to the molecular bioactivity than the cis$^{1-2}$-trans$^{3-4}$ isomer because the population of the former isomer is smaller than that of the latter (Table III). Therefore, higher potencies observed for Tyr-Pro-Phe-D-Pro-NH_2 and Tyr-Pro-D-Phe-Pro-NH_2 are attributed to larger fractions of the χ_1^3 = t conformers of the L-Phe^3 and D-Phe^3 residues as compared to the corresponding diastereomers, Tyr-Pro-Phe-Pro-Pro-NH_2 and Tyr-Pro-D-Phe-D-Pro-NH_2, respectively. We conclude that morphiceptin bioactivity requires topology I, in which the third residue has an aromatic side chain in the χ_1^3 = t (~180°) conformation.

Table V. Conformer Fractions of Tyr[1] and (L or D)-Phe[3]/(NMe)Phe[3] Side Chains (χ_1) of Morphiceptin Analogs[a]

configuration			Tyr[1]			(L or D)-Phe[3]/(NMe)Phe[3]		
Tyr[1]-Pro[2]	X[3]-(L or D)-Pro[4]	population/%	g⁻	t	g⁺	g⁻	t	g⁺
			(A) Tyr-Pro-Phe-Pro-NH_2					
cis	trans	25	0.37	0.49	0.14	0.72	0.09	0.19
cis	cis	5	*b*	*b*	*b*	*b*	*b*	*b*
			(B) Tyr-Pro-Phe-D-Pro-NH_2					
cis	trans	26	*b*	*b*	*b*	0.50	0.41	0.09
cis	cis	4	*b*	*b*	*b*	*b*	*b*	*b*
			(C) Tyr-Pro-D-Phe-Pro-NH_2					
cis	trans	21	0.33	0.49	0.18	0.06	0.45	0.49
cis	cis	18	0.42	0.42	0.16	0.15	0.07	0.78
			(D) Tyr-Pro-D-Phe-D-Pro-NH_2					
cis	trans	30	0.41	0.40	0.19	0.15	0.16	0.69
cis	cis	6	*b*	*b*	*b*	0.11	0.37	0.52
			(E) Tyr-Pro-(NMe)Phe-Pro-NH_2[c]					
cis	trans	32	*b*	*b*	*b*	0.66	0.16	0.18
cis	cis	4	*b*	*b*	*b*	*b*	*b*	*b*
			(F) Tyr-Pro-(NMe)Phe-D-Pro-NH_2[c]					
cis	trans	30	0.36	0.46	0.18	0.41	0.51	0.09
cis	cis	6	*b*	*b*	*b*	*b*	*b*	*b*

[a] Fractions were evaluated from vicinal ^1H-^1H coupling constants for H-C^α-C^β-H groupings observed in DMSO-d_6 at 25 °C. [b] The values could not be obtained because of the severe overlap of the α- and β-proton signals with the other peaks. [c] Only a trans configuration has been observed for the Pro[2]–(NMe)Phe[3] amide bond.

Table VI. Minimum Energy Conformations of (A) Tyr-Pro-(NMe)Phe-D-Pro-NH_2 and (B) Tyr-Pro-D-Phe-Pro-NH_2 Which Assume Topology I

residue	torsion angle/deg	(A) Tyr-Pro-(NMe)Phe-D-Pro-NH_2 1	2	3	(B) Tyr-Pro-D-Phe-Pro-NH_2 1	2	3
Tyr[1]	ψ^1	131	139	144	131	146	140
	χ_1^1	−163	−89	72	−167	−75	71
	ω_1	5	10	3	5	7	2
Pro[2]	ϕ^2	−77	−71	−77	−72	−74	−74
	ψ^2	148	151	139	−38	−34	−36
	ω^2	−177	−159	−177	−179	179	−178
X[3]	ϕ^3	−118	−122	−117	102	103	101
	ψ^3	88	86	88	−106	−106	−107
	χ_1^3	−165	−161	−165	169	169	169
	ω^3	177	177	178	−177	−177	−177
Pro[4]	ϕ^4	78	78	78	−79	−78	−79
	ψ^4	−84	−83	−85	82	82	82
ΔE[a]/kcal mol^{-1}		0.000	2.532	7.389	0.000	3.930	6.417
distances[b]/Å	d_1	7.8	6.9	6.7	7.9	6.7	6.7
	d_2	7.1	6.2	7.2	7.1	7.2	7.4
	d_3	11.0	5.0	13.7	12.5	9.6	14.0

[a] The values of ΔE represent relative energies of the three conformations reported for each analog. [b] A topochemical structure of the minimum energy conformation is characterized by a triangle defined by three distances between three pharmocophoric groups, d_1 (Tyr[1] N–Tyr[1] OH), d_2 (Tyr[1] N–the center of the aromatic ring of the third residue), and d_3 (Tyr[1] OH–the center of the aromatic ring of the third residue).

(5) Side Chain Conformation of the First Residue (χ_1^1). As mentioned above, topology I requires different structures for the second residues (ψ^2) depending upon the chirality at position 3. In this respect, the bioactive morphiceptin analogs investigated here are divided into two classes, one incorporates L-Phe or L-(NMe)Phe at the third position and the other incorporates D-Phe at the same position. Among the L-Phe[3] and L-(NMe)Phe[3] containing analogs, Tyr-Pro-(NMe)Phe-D-Pro-NH_2 is the most potent. The analog, Tyr-Pro-D-Phe-Pro-NH_2, exhibits higher activity than Tyr-Pro-D-Phe-D-Pro-NH_2.

The minimum energy conformations of the most potent analogs, Tyr-Pro-(NMe)Phe-D-Pro-NH_2 and Tyr-Pro-D-Phe-Pro-NH_2, with topology I, are given in Table VI. For each analog, three structures with different Tyr[1] side chain χ_1^1 states are shown. The structures 1, 2, and 3 assume the χ_1^1 = t (ca. 180°), g⁻ (ca. −60°), and g⁺ (ca. 60°), respectively. In the table, the characteristic distances among the three pharmacophoric groups, d_1 (Tyr N–Tyr OH), d_2 (Tyr N–the center of the aromatic ring of the third residue), and d_3 (Tyr OH–the center of the aromatic ring of the third residue) are also reported. A reasonable degree of fit is obtained for the conformational pairs of the two analogs with χ_1^1 = t (ca. 180°) and g⁺ (ca. +60°). However, the deviation in the d_3 values is quite large for the conformations with χ_1^1 = g⁻ (ca. −60°). Thus, the structures where the Tyr[1] residue adopts the χ_1^1 = g⁻ state may be excluded for the "bioactive conformations" of the morphiceptin analogs.

Schiller et al.[32,33] have reported opioid receptor affinities of cyclic tetrapeptide dermorphin analogs in which the Tyr[1] side chain is constrained by incorporating 6-hydroxy-2-aminotetralin-2-carboxylic acid (Hat) with an L and a D configuration at position 1. Two optically pure diastereomers have been obtained from a diastereomeric mixture of the peptide by reversed-phase HPLC, although the chirality of the Hat residue in each diastereomer is not specified. Both of the diastereomers containing L-Hat and D-Hat display relatively high affinities at both the μ- and δ- receptors; one is about 20 times more potent than the

other. The replacement of Tyr^1 with Hat is also tolerated for enkephalin as seen by the fact that a diastereomeric mixture of [Leu^5]enkephalin analogs containing (L and D)-Hat in place of Tyr^1 exhibits 7–8 times higher agonist potency at the μ-receptor on GPI and is about 30 times less active at the δ-receptor on MVD preparations as compared to [Leu^5]enkephalin.[35] According to the constrained nature of the six-membered tetralin ring structure, the accessible side-chain χ_1 conformations for the Hat residue are restricted to two states, i.e. χ_1 = t (~180°) and g$^-$ (~–60°) for L-Hat and χ_1 = t (~180°) and g$^+$ (~+60°) for D-Hat. Here, it is necessary to call attention to the fact that the χ_1 = g$^+$ (~+60°) state of the D-Hat residue, in which the aromatic ring is anti to C(O) and syn to N, corresponds to the χ_1^1 = g$^-$ (~–60°) conformation of the Tyr^1 residue, in which the aromatic ring is anti to C(O) and syn to N, because of the chirality inversion between these two residues. The side-chain conformer χ_1 = g$^-$ (~–60°) of the L-Hat residue, in which the aromatic ring is anti to C(O) and syn to N, directly corresponds to the χ_1^1 = g$^-$ (~–60°) conformation of the Tyr^1 residue since both the residues have the same L configuration. The χ_1 = t (~180°) conformation of the L-Hat and D-Hat residues, in which the aromatic ring is anti to N and syn to C(O), corresponds to the χ_1^1 = t (~180°) of the Tyr^1 residue. These results suggest that the χ_1^1 = g$^+$ (~+60°) conformation of the Tyr^1 side chain, in which the aromatic ring is syn to both of N and C(O) simultaneously, does not explain the opioid activities of the cyclic dermorphin and [Leu^5]enkephalin. Although similar substitution studies of Tyr^1 with Hat have not been carried out, the above results could be applicable to the morphiceptin family since the Tyr^1 moieties of the peptide opioids such as enkephalin, dermorphin, and morphiceptin may interact with the μ-receptor in a similar manner. Therefore, the structures where the Tyr^1 residue adopts the χ_1^1 = g$^+$ conformer could be excluded from the candidates for the "bioactive conformations" of the peptide opioids. As a consequence, the molecular recognition of the morphiceptin analogs at the μ-receptor most likely requires the χ_1^1 = t (ca. 180°) conformation of the Tyr^1 side chain in addition to topology I. The argument in favor of the χ_1^1 = t (~180°) conformation for the Tyr^1 side chain is further strengthened by the observation that cyclic dermorphin analogs containing 7-hydroxytetrahydroisoquinoline-3-carboxylic acid (Htc) in place of Tyr^1 show low affinites at both the μ- and δ-opioid receptors.[33] Neither L- nor D-Htc can assume the χ_1 = t (~180°) side-chain conformation. Interestingly, the recent substitution studies of Tyr^1 with β-methylated tyrosine and Hat suggest that the χ_1^1 = t (~180°) conformation of the Tyr^1 side chain is also responsible for the recognition of a cyclic pentapeptide enkephalin analog, Tyr-c[D-Pen-Gly-D-Pen]-OH, at the δ-receptor.[35]

The Topochemical Structure for Morphiceptin Bioactivity. Figure 4 shows the structures of the most potent analogs, Tyr-Pro-(NMe)Phe-D-Pro-NH$_2$ [(A)-1 in Table VI] and Tyr-Pro-D-Phe-Pro-NH$_2$[(B)-1 in Table VI], which are consistent with all the topochemical requirements for morphiceptin bioactivity discussed above. These structures represent the most preferred conformation for each molecule with the amide bond linking residues 1 and 2 in a cis configuration as elucidated by ^{1}H-NMR spectroscopy and molecular modeling studies. The topochemical equivalency of the two structures is appreciated in the superposition shown in Figure 4C.

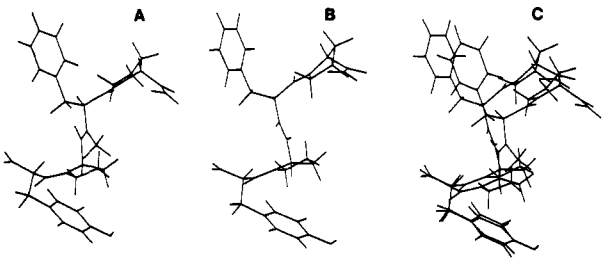

Figure 4. The topochemical array for the μ-receptor bioactivity of (A) Tyr-Pro-(NMe)Phe-D-Pro-NH$_2$ and (B) Tyr-Pro-D-Phe-Pro-NH$_2$. This array requires a cis configuration for the Tyr–Pro amide bond, along with the side chains in a trans conformation (χ_1 ~ 180°) for the Tyr and (NMe)Phe/D-Phe residues. The structures A and B are topochemically equivalent as shown in the superposition C.

It may be interesting for justification of our model to discuss highly constrained morphiceptin analogs reported by Nelson et al.[24] These analogs are Tyr-2-MePro-Phe-Pro-NH$_2$ where 2-MePro represents 2-methylproline (proline with a methyl substituent at the α-carbon) and Tyr-Pro-3-PhPro-N(Me)$_2$ where 3-PhPro represents *trans*-3-phenylproline (proline with a phenyl substituent at the β-carbon). The observation that the former analog is inactive at the μ-receptor supports the requirement of the cis amide bond linking residues 1 and 2. As reported by Denaley and Madison,[36] Ac-2-MePro-NHMe adopts only the trans form since the cis form is destabilized by steric interactions between the 2-methyl and the acetyl methyl groups. According to Madison et al.'s results, Tyr-2-MePro-Phe-Pro-NH$_2$ may assume only the trans configuration around the Tyr-2-MePro amide bond because of steric repulsions between the 2-methyl group of the 2-MePro residue and the C$^\alpha$H group of the Tyr residue, resulting in no opioid activity.

The analog, Tyr-Pro-3-PhPro-N(Me)$_2$, has a similar structure to the tripeptide model compounds, Tyr-Pro-(L and D)-(NMe)Phe-N(Me)$_2$, used to estimate topochemical requirements in this study. One diastereomer of Tyr-Pro-3-PhPro-N(Me)$_2$ is more potent in the GPI test (IC$_{50}$ = 40.7 nM) than Tyr-Pro-Phe-Pro-NH$_2$, while the other diastereomer is inactive in the same test (IC$_{50}$ = 171 00 nM).[24] Although Nelson et al.[24] have not assigned the chiralities of the 3-PhPro residues, the bioactive analog must possess (2*S*,3*R*)-3-PhPro because the D-(NMe)Phe^3 containing morphiceptin analogs are inactive (Table IV). Our molecular modeling studies of the two diastereomers of Tyr-Pro-3-PhPro-N(Me)$_2$ suggest that incorporation of (2*S*,3*R*)-3-PhPro at position 3 restricts the ϕ^3 angle to ca. –75° and the χ_1^3 angle to ca. –150° (t). In addition, the F conformation with (ϕ^2, ψ^2) ~ (–75°, 148°) is only allowed for the Pro^2 residue and the ψ^1 angle of the Tyr^1 residue is restricted to values of 60–180°. Thus, only one structure, with the exception of the three states for the Tyr^1 side chain, is accessible to Tyr-Pro-(2*S*,3*R*)-3-PhPro-N(Me)$_2$ with the Tyr–Pro amide bond in a cis configuration. All of these bond conformations are consistent with our topology I. Therefore, the three pharmacophoric groups of this highly constrained bioactive morphiceptin analog are exactly superimposable with those of the "bioactive conformations" shown in Figure 4.

In a previous paper,[20] we reported preferred structures of the morphiceptin analogs which incorporate 2-ami-

nocyclopentanecarboxylic acid (2-Ac^{5}c) as the second residue in place of proline. It is worthwhile mentioning that the lowest energy conformations reported for the biologically active analogs, Tyr-*cis*-(1*S*,2*R*)-2-Ac^{5}c-Phe-X^4-NH$_2$ (X^4 = Pro and Val: srp1 and srl1 in Figure 9 of ref 20), are topochemically equivalent to topology I, even though the 2-Ac^{5}c analogs can only adopt a trans amide bond linking residues 1 and 2.

Conclusions

Opioid receptor recognition requires specific three-dimensional structures of the ligand molecules, so-called "bioactive conformations", with the correct array of three pharmacophoric groups. These groups are the amine and phenolic groups of the N-terminal tyrosine and the aromatic ring of the third or fourth residue. The spatial arrangements of the pharmacophoric groups are defined by a set of conformational states around rotatable bonds connecting them. For a full understanding of opioid activity, it is necessary to identify the specific conformational states for all of these rotatable bonds. For this purpose, we have developed a new methodology which includes a stereoisomeric approach and incorporation of peptidomimetic structures. This methodology could be applied to most linear bioactive peptide systems. In this paper, we estimated "bioactive conformations" of morphiceptin analogs at the μ-opioid receptor by use of our new approach.

The morphiceptin analogs have quite unique structures characterized by a wealth of *N*-alkyl amino acids such as proline. Incorporation of an *N*-alkyl amino acid into the peptide sequence restricts accessible conformational space for the C$^\alpha$-C(O) bond (ψ) of the preceding residue as well as for the conformation of the amino acid itself. Thus, the morphiceptin analogs show clear conformational preferences and are well suitable to build the "bioactive conformations" based on the rotational states around the bonds. The spatial arrangements of the three pharmacophoric groups within the morphiceptin analogs are defined by a set of eight torsion angles, ψ^1, $\chi_1{}^1$, ω^1, ϕ^2, ψ^2, ω^2, ϕ^3, and $\chi_1{}^3$ where the superscripts represent the residue number in the sequence. In order to elucidate the topochemical structure to explain morphiceptin bioactivity, we studied accessible conformations of stereoisomeric analogs, Tyr-Pro-(L and D)-X^3-(L and D)-Pro-NH$_2$ [X^3 = Phe and (NMe)Phe], Tyr-(NMe)Ala-Phe-D-Pro-NH$_2$, and Tyr-Ala-Phe-D-Pro-NH$_2$, which are structurally close to one another. The analogs, Tyr-Ala-Phe-D-Pro-NH$_2$ and Tyr-Pro-D-(NMe)Phe-(L and D)-Pro-NH$_2$, are inactive in both the GPI and MVD tests whereas the remaining analogs are biologically active, displaying selectivities for the μ-receptor over the δ-receptor. The conformation of each rotatable bond has been specifically identified by measuring accessible space of the analogs with various opioid potencies from highly active to inactive, where the difference in composition is observed in the specific site affecting only the conformation around the target bond.

The evaluated torsional angles are summarized as follows: $\psi^1 = 60$–$180°$, $\chi_1{}^1 \sim 180°$, $\omega^1 \sim 0°$ (a cis configuration around the amide bond linking residues 1 and 2), $\phi^2 \sim -75°$, $\omega^2 \sim 180°$ (a trans configuration around the amide bond linking residues 2 and 3), $\chi_1{}^3 \sim 180°$. The angles ψ^2 and ϕ^3 are $\sim 150°$ and $\sim -120°$ for the analogs incorporating either L-Phe or L-(NMe)Phe as the third residue. On the other hand, the angles of $\psi^2 \sim -40°$ and $\phi^3 \sim +120°$ are evaluated for the analogs with D-Phe in the same position. The chirality change of the Phe residue is compensated by a ca. 180° rotation around the C$^\alpha$–C(O) bond (ψ^2). Interestingly, the molecular structures which are generated from the above results represent one of the most preferred conformations for each analog with the amide bond linking residues 1 and 2 in a cis configuration as elucidated by ^{1}H-NMR spectroscopy. The characteristic distances with the three pharmacophoric groups, d_1 (Tyr N–Tyr OH), d_2 (Tyr N–the center of the aromatic ring of the third residue), and d_3 (Tyr OH–the center of the aromatic ring of the third residue), are found to be $\sim$8, $\sim$7, and $\sim$11–13 Å, respectively. The biological activities of the vast majority of morphiceptin analogs reported in the literature can be explained by our topochemical model.

One of the most important characteristics of this model is a requirement of a cis configuration around the amide bond linking residues 1 and 2. As revealed by NMR studies, a cis configuration is less favored than a trans configuration for the morphiceptin analogs themselves. However, the energy difference between these two states is within a range from 0.26 to 0.53 kcal mol^{-1} and is small enough to be paid for by ligand–receptor interactions. These results as well as the molecular model for morphiceptin bioactivity should provide better understanding of opioid peptide action and aid in pharmaceutical design of peptide and nonpeptide ligands with opioid activities.

Experimental Section

Spectroscopy Measurements. The ^{1}H-NMR spectra were recorded on a General Electric GN-500 spectrometer operating at 500 MHz. The samples were prepared in DMSO-d_6 (MSD Isotope) at a concentration of 10 mM. The one-dimensional spectra contain 16 K data points in 5000 Hz. The two-dimensional homonuclear Hartman–Hahn (HOHAHA) experiments[27] were carried out using the MLEV-17[37,38] and the time proportional phase increment.[39] A mixing time of 100 ms (48 cycles of MLEV sequence) with a spin locking field of 10.2 kHz was employed. The rotating frame nuclear Overhauser enhancement (ROESY) experiments[28] were carried out by varying mixing time from 50 to 250 ms with a spin locking field of 2.5 kHz. All of the two-dimensional spectra were obtained using 2 K data points in the f_2 domain and 256 points in the f_1 domain. Applying the zero-filling procedure to the f_1 domain resulted in a final matrix of 2K × 2K data points. Multiplication with either a phase-shifted sine or a Gaussian function was used to enhance the spectra.

Computer Simulations. Conformational energy calculations were carried out with the Newton–Raphson method until the maximum derivative was less than 0.001 kcal mol^{-1} Å^{-1} by employing the DISCOVER flexible geometry program.[40] Conformational energies were estimated as the sum of nonbonded van der Waals interactions, Coulombic interactions, intrinsic torsional potentials, and energies of deformation of bond lengths and bond angles. Parameters required for the description of the torsional potentials for the internal bond rotation are provided in the DISCOVER program and used without modification. Various force constants defined in the force field scheme were also adopted as specified in the program. The default dielectric constant of 1.0 was used for all calculations in vacuo. The (ϕ, ψ) energy contour maps of *N*-acetyl *N'*-methylamide derivatives of amino acids were produced by constraining the angles ϕ and ψ to a particular value with a force constant of 100 kcal mol^{-1} and minimizing the energy with respect to all the other coordinates in the molecule.

Biological Assays. Measurements of opioid activities in the guinea pig ileum (GPI)[29] and mouse vas deferens (MVD)[30] bioassays were carried out as reported in detail elsewhere.[41,42] A long dose–response curve was determined with [Leu^5]enkephalin as the standard for each ileum and vas preparation. The IC$_{50}$ values for the compounds being tested were normalized according to a published procedure.[43]

Table VII. Characteristics of Synthesized Morphiceptin Analogs

analog	TLC[a] R_f	FAB-MS (MH^+)	amino acid analysis ratio
Tyr-(NMe)Ala-Phe-D-Pro-NH_2	0.30	510	Tyr (1.00) Phe (0.98) Pro (1.03)
Tyr-Ala-Phe-D-Pro-NH_2	0.45	496	Tyr (1.00) Ala (1.01) Phe (0.98) Pro (1.02)
Tyr-Pro-D-Phe-Pro-NH_2	0.35	522	Tyr (1.00) Pro (2.07) Phe (1.05)
Tyr-Pro-D-Phe-D-Pro-NH_2	0.26	522	Tyr (1.00) Pro (2.01) Phe (1.00)
Tyr-Pro-D-(NMe)Phe-Pro-NH_2	0.48	536	Tyr (1.00) Pro (2.01)
Tyr-Pro-D-(NMe)Phe-D-Pro-NH_2	0.40	536	Tyr (1.00) Pro (2.10)
Tyr-Pro-(NMe)Phe-Pro-NH_2	0.43	536	Tyr (1.00) Pro (1.98)

[a] The R_f values were measured in a mixture of butanol, water, and acetic acid with a ratio of 4:1:1.

Synthesis. The designed molecules were synthesized in our laboratories except for Tyr-Pro-Phe-Pro-NH_2, Tyr-Pro-Phe-D-Pro-NH_2, and Tyr-Pro-(NMe)Phe-D-Pro-NH_2, which are commercially available (BACHEM Inc. and Peninsula Laboratories Inc.). Amino acid derivatives used for the synthesis of the designed molecules were purchased from BACHEM Inc. All materials and solvents were of reagent grade and used without further purification with the following exceptions. Dimethylformamide was distilled from ninhydrin under reduced pressure and stored over molecular sieves. HPLC grade water and acetonitrile were purchased from Fisher Scientific Co.

All of the synthesis were carried out in solution. The *tert*-butyloxycarbonyl (Boc) group was employed for the protection of the amino group of each amino acid. Water-soluble 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) and *N*-hydroxybenzotriazole (HOBt) were used as coupling reagents for all of the coupling reactions. The amino acid derivative, Boc-(NMe)-Ala-OH, was prepared by N-methylation of Boc-Ala-OH with sodium hydride and methyl iodide in tetrahydrofuran employing the method reported by Benoiton et al.[44,45] The amidation of the D-Pro residue was accomplished by the reaction between Boc-D-Pro-OH and ammonium chloride using the EDC/HOBt method.

The tetrapeptide analogs related to morphiceptin, Tyr-(NMe)-Ala-Phe-D-Pro-NH_2, Tyr-Ala-Phe-Pro-NH_2, Tyr-Pro-(NMe)Phe-Pro-NH_2, and Tyr-Pro-D-(NMe)Phe-Pro-NH_2, were synthesized by stepwise elongation from the carboxyl terminus to the amino terminus. The other tetrapeptide analogs, Tyr-Pro-D-Phe-Pro-NH_2, Tyr-Pro-D-Phe-D-Pro-NH_2, and Tyr-Pro-D-(NMe)Phe-D-Pro-NH_2, were prepared by the fragment condensation method between the amino terminal and the carboxyl terminal dipeptide units. The common amino terminal dipeptide unit, Boc-Tyr-(*t*Bu)-Pro-OH, was obtained from the coupling between Boc-Tyr(*t*Bu)-OH and HCl*H-Pro-OBzl and the hydrogenolysis of the benzyl protecting group using a palladium on a carbon catalyst. The other dipeptide units were synthesized through similar reactions after the acidolytic deprotection of the Boc protecting group.

The assembled protected tetrapeptides from the fragment condensation and sequential couplings were purified by flash column chromatography using a silica gel. Final deprotection with thioanisole produced target compounds. These crude products were purified by column chromatography using LH-20 (Sigma) and CHP20P (MCI GEL). The purity of the synthesized compounds was examined by analytical HPLC, and the structure of the products were confirmed by fast atom bombardment mass spectroscopy (FAB-MS), amino acid analysis, and 500-MHz ^{1}H-NMR spectroscopy. The characterizations of the analogs synthesized are summarized in Table VII.

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Supplementary Material Available: Details for the synthesis of peptides (9 pages). Ordering information is given on any current masthead page.

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