Pseudoproline-Containing Analogues of Morphiceptin and Endomorphin-2: Evidence for a *Cis* Tyr−Pro Amide Bond in the Bioactive Conformation

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Analogues of the opioid peptides [D-Phe^3]morphiceptin (H-Tyr-Pro-D-Phe-Pro-NH_2) and endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2) containing the pseudoproline (ΨPro) (4*R*)-thiazolidine-4-carboxylic acid (Cys[$\Psi^{R1,R2}$pro]) or (4*S*)-oxazolidine-4-carboxylic acid (Ser[$\Psi^{R1,R2}$pro]) in place of Pro^2 were synthesized. The pseudoproline ring in these compounds was either unsubstituted (R^1, R^2 = H) or dimethylated (R^1, R^2 = CH_3) at the 2-C position. 2-C-dimethylated pseudoprolines are known to be quantitative or nearly quantitative inducers of the *cis* conformation around the Xaa_{i-1}−Xaa_i[Ψ^{CH_3,CH_3}pro] imide bond. All dihydropseudoproline-containing analogues (R^1, R^2 = H) showed good μ opioid agonist potency in the guinea pig ileum (GPI) assay, high μ receptor binding affinity in the rat brain membrane binding assay, and, like their parent peptides, excellent μ receptor binding selectivity. ^{1}H NMR spectroscopic analysis of the Cys[$\Psi^{H,H}$pro]^2- and Ser[$\Psi^{H,H}$pro]^2-containing analogues in DMSO-d_6 revealed that they existed in a conformational equilibrium around the Tyr−Xaa[$\Psi^{H,H}$pro] peptide bond with *cis*/*trans* ratios of 40:60 and 45:55, respectively. The dimethylated thiazolidine- and oxazolidine-containing [D-Phe^3]morphiceptin- and endomorphin-2 analogues (R^1, R^2 = CH_3) all retained full μ agonist potency in the GPI assay and displayed μ receptor binding affinities in the nanomolar range and high μ receptor selectivity. As expected, no conformers of the latter analogues with a *trans* conformation around the Tyr−Xaa[Ψ^{CH_3,CH_3}pro] imide bond were detected by ^{1}H NMR spectral analysis, indicating that in these compounds the *cis* conformation is highly predominant (>98%). These results represent the most direct evidence obtained so far to indicate that morphiceptin and endomorphin-2 have the *cis* conformation around the Tyr−Pro peptide bond in their bioactive conformations.

Introduction

Morphiceptin (H-Tyr-Pro-Phe-Pro-NH_2)[2] and the endomorphins [endomorphin-1 (H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2)][3] are opioid peptide agonists with high selectivity for μ opioid receptors. These peptides contain a Pro residue in the 2-position of the peptide sequence and, consequently, *cis*−*trans* isomerization occurs at the Tyr^1−Pro^2 peptide bond.[4,5] The question arises whether morphiceptin and the endomorphins adopt the *cis* or the *trans* configuration at this peptide bond when bound to the receptor. The results of ^{1}H NMR spectroscopic studies performed with morphiceptin[6] and endomorphin-1[5,7] in aqueous solution indicated the existence of a *cis*/*trans* equilibrium with a predominance of the *trans* isomer in both cases. However, conformational studies of these peptides free in solution do not permit any definite conclusions with regard to their receptor-bound conformation, even if they indicate that one isomer is predominant. *Cis*/*trans* isomerization around the peptide bond preceding a Pro residue in a peptide is a dynamic process,[8] and the two isomers cannot be isolated at room temperature. The difference in energy ($\Delta G°$) between the *cis* and *trans* conformers of a Tyr−Pro dipeptide unit is quite low, typically ∼2−4 kcal/mol. Therefore, it is quite possible that the minor, *cis* conformer might bind to the receptor and that its relatively higher energy might be paid for by the ligand−receptor interaction energy. After binding of the *cis* conformers initially present in solution, the *cis*/*trans* equilibrium would be continuously re-established, thus permitting the binding of newly generated *cis* conformers to the receptor in a relatively slow binding process. Therefore, even when the *trans*/*cis* ratio is quite high in solution, such as reported for endomorphin-1 (*trans*/*cis* ratio of 75:25 in DMSO[5]), the receptor-bound peptide might nonetheless have the *cis* conformation at the Tyr^1−Pro^2 peptide bond.

Direct experimental determinations of the conformation of a receptor-bound peptide are not yet feasible. However, it is possible to rule out the *trans* isomer of a Pro-containing peptide as the receptor-bound species through substitution of the proline with an appropriate proline analogue capable of forcing the peptide bond in question into the *cis* conformation. 2-C-dimethylated pseudoproline[9,10] or proline[11] analogues have previously been shown to be quantitative or nearly quantitative *cis* inducers. The pseudoprolines are structurally derived from cysteine, threonine, or serine and contain a

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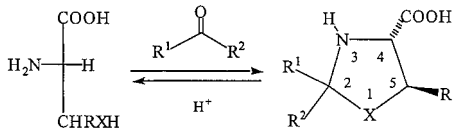

Figure 1. Pseudoprolines Xaa($\Psi^{R1,R2}$pro) are obtained by cyclocondensation reaction of aldehydes or ketones with Xaa = Cys, Ser, or Thr. X = S, R = H: Cys-derived thiazolidines. X = O, R = H: Ser-derived oxazolidines. X = O, R = CH_3: Thr-derived oxazolidines. R^1, R^2 = H, CH_3, aryl.

sulfur or oxygen atom in place of the $-CH_2-$ moïety in the 1-C position of the proline ring structure (Figure 1). Pseudoprolines were originally introduced for use in a novel, orthogonal protection strategy for Cys, Thr, and Ser in peptide synthesis.[10] In addition, they were successfully used to prevent secondary structure formation during solid-phase peptide synthesis of difficult peptides.[12] In a number of studies with model peptides, the incorporation of pseudoprolines was shown to lower the transition-state barrier ($\Delta G^{\ddagger}$) of the *cis* → *trans* isomerization of the peptide bond preceding the pseudoproline residue,[13] to quantitatively fixate a *cis* peptide bond in short peptides,[14] to induce an *all-cis* conformation of a cyclic tripeptide,[15] and to lock in oligopeptides in an *all-cis* polyproline I helix.[16] Furthermore, the use of 2-C-substituted pseudoprolines permitted the modulation of the physicochemical and biological properties of the cyclic peptide cyclosporin C,[17] as well as the production of a monoclonal antibody recognizing the consensus loop tip GPGR of HIV glycoprotein 120 in its *cis*-Gly-Pro conformation.[18]

In the present paper, we describe analogues of endomorphin-2 and of the potent D-Phe^3-analogue of morphiceptin[6,19] in which the Pro^2 residue was replaced with 2-C-dimethylated pseudoprolines known to be quantitative or nearly quantitative *cis*-inducers[9,13] (compounds **5**, **7**, **9**, and **11**). In case these peptides turned out to retain opioid activity, this would constitute strong evidence to indicate that the receptor-bound conformation of [D-Phe^3]morphiceptin and endomorphin-2 has the *cis* conformation at the Tyr$^1-$Pro^2 peptide bond. In addition, we prepared analogues of these two peptides, in which the sterically demanding methyl substituents at the 2-C position of the pseudoproline ring were replaced by two hydrogen atoms (Figure 1) (compounds **4**, **6**, **8**, and **10**). 2-C-dihydro-oxazolidine and thiazolidine differ from native proline by the exchange of the γ-methylene group in the proline ring with an oxygen or a sulfur atom, respectively. They have previously been shown to induce substantial structural flexibility around the amide bond to the preceding amino acid due to decreased *cis*−*trans* activation barriers.[13] Therefore, it was expected that the conformational adaptation of these analogues to the μ receptor would be facilitated.

Chemistry. The N-terminal dipeptide segments of analogues **4**−**11** were synthesized in solution, and the final tetrapeptide amides were prepared by using the solid-phase technique. The protected, thiazolidine-containing dipeptides Fmoc-Tyr(All)-Cys($\Psi^{H,H}$pro)-OH and Fmoc-Tyr(All)-Cys($\Psi^{Me,Me}$ pro)-OH were prepared by coupling Fmoc-Tyr(All)-F with Cys($\Psi^{H,H}$pro)-OH and Cys($\Psi^{Me,Me}$pro)-OH, respectively. The dihydro-oxazolidine-containing dipeptide Fmoc-Tyr(All)-Ser($\Psi^{H,H}$pro)-OH was prepared by reacting Fmoc-Tyr(All)-F with serine in the presence of formaldehyde. For the preparation of the dimethyloxazolidine-containing dipeptide Fmoc-Tyr(All)-Ser($\Psi^{Me,Me}$pro)-OH, Fmoc-Tyr(All)-F was coupled to serine benzyl ester and the resulting dipeptide was then reacted with dimethoxypropane in the presence of pyridinium *p*-toluenesulfonate (PPTS). Subsequent catalytic hydrogenation yielded the desired product. For the solid-phase syntheses of the final tetrapeptide amides the C-terminal dipeptide segments were first assembled on a *Sieber*-resin[20] using 9-fluorenylmethyloxycarbonyl-protected amino acids and benzotriazol-1-yloxytris(pyrrolidinophosphonium-hexafluorophosphate) (PyBOP) as coupling agent. The N-terminal dipeptide segments were then coupled to the resin-bound dipeptides using the same coupling agent. After removal of the Tyr^1 allyl protecting group with Pd-[PPh_3]$_4$, the peptides were cleaved from the resin with 2% TFA in CH_2Cl_2 and were purified by reversed-phase HPLC.

Opioid Receptor Binding Assays and in Vitro Bioassays. Binding affinities of compounds for μ and δ opioid receptors were determined by displacing, respectively, [^{3}H]DAMGO and [^{3}H]DSLET from rat brain membrane binding sites, and κ opioid receptor binding affinities were measured by displacement of [^{3}H]U69,593 from guinea pig brain membrane binding sites. For the determination of their in vitro opioid activities, analogues were tested in bioassays based on inhibition of electrically evoked contractions of the GPI and MVD. The GPI assay is usually considered as being representative for μ receptor interactions, even though the ileum does also contain κ receptors. In the MVD assay opioid effects are primarily mediated by δ receptors, but μ and κ receptors also exist in this tissue.

Results and Discussion

In the GPI assay the dihydrothiazolidine-containing [D-Phe^3]morphiceptin analogue **4** showed slightly higher potency than the parent peptide [D-Phe^3]morphiceptin and was 3 times as potent as [Leu^5]enkephalin (Table 1). In agreement with these functional assay data, analogue **4** displayed ~4-fold higher μ receptor affinity in the rat brain membrane binding assay than the parent compound (Table 2). It bound very weakly to δ opioid receptors (K_i^{δ} = 2450 nM) and, thus, retained high μ versus δ receptor selectivity (K_i^{δ}/K_i^{μ} = 286), similar to that of [D-Phe^3]morphiceptin. Analysis of the ^{1}H NMR spectrum of **4** in DMSO-d_6 solution revealed that this compound exists in a conformational equilibrium around the Tyr−Pro amide bond with a *cis*/*trans* ratio of 40:60 (Table 3). This ratio is similar to the *cis*/*trans* ratio (39:61) around that same peptide bond observed in the parent peptide [D-Phe^3]morphiceptin.[6] Introduction of two methyl substituents at the 2-C position of the Pro^2 ring in peptide **4** resulted in a compound, H-Tyr-Cys($\Psi^{Me,Me}$pro)-D-Phe-Pro-NH_2 (**5**), that showed only ~3−4-fold lower μ agonist potency and μ receptor binding affinity than **4**. Inspection of the ^{1}H NMR resonances of the 2-C dimethyl groups of the Cys-($\Psi^{Me,Me}$pro) residue in DMSO-d_6 revealed that this compound assumed almost exclusively the *cis* conformation (>98%) around the Tyr−Cys($\Psi^{Me,Me}$pro) peptide bond. This result together with the observed high opioid activity clearly indicates that analogue **5** must have the

Table 1. GPI and MVD Assay of Opioid Peptide Analogues

		GPI		MVD		MVD/GPI
no.	compd	IC_{50}[a] (nM)	rel potency	IC_{50}[a] (nM)	rel potency	IC_{50} ratio
	H-Tyr-Pro-D-Phe-Pro-NH_2	109 ± 16	2.26 ± 0.33	594 ± 77	0.0192 ± 0.0025	5.45
4	H-Tyr-Cys($\Psi^{H,H}$pro)-D-Phe-Pro-NH_2	74.5 ± 9.3	3.30 ± 0.41	190 ± 14	0.0600 ± 0.0044	2.55
5	H-Tyr-Cys($\Psi^{Me,Me}$pro)-D-Phe-Pro-NH_2	246 ± 12	1.00 ± 0.05	1420 ± 4	0.00803 ± 0.00002	5.77
6	H-Tyr-Ser($\Psi^{H,H}$pro)-D-Phe-Pro-NH_2	470 ± 41	0.523 ± 0.046	1020 ± 80	0.0112 ± 0.0009	2.17
7	H-Tyr-Ser($\Psi^{Me,Me}$pro)-D-Phe-Pro-NH_2	860 ± 135	0.286 ± 0.045	851 ± 178	0.0134 ± 0.0028	0.990
	H-Tyr-Pro-Phe-Phe-NH_2	7.71 ± 1.47	31.9 ± 6.1	15.3 ± 1.8	0.745 ± 0.088	1.98
8	H-Tyr-Cys($\Psi^{H,H}$pro)-Phe-Phe-NH_2	22.2 ± 2.6	11.1 ± 1.3	138 ± 40	0.0826 ± 0.0239	6.22
9	H-Tyr-Cys($\Psi^{Me,Me}$pro)-Phe-Phe-NH_2	502 ± 27	0.490 ± 0.026	PA (26%)[b]		
10	H-Tyr-Ser($\Psi^{H,H}$pro)-Phe-Phe-NH_2	41.7 ± 1.7	5.9 ± 0.24	151 ± 37	0.0755 ± 0.0185	3.62
11	H-Tyr-Ser($\Psi^{Me,Me}$pro)-Phe-Phe-NH_2	305 ± 37	0.807 ± 0.098	606 ± 65	0.0188 ± 0.0020	1.99
	[Leu^5]enkephalin	246 ± 39	1	11.4 ± 1.1	1	0.0463

[a] Mean of three determinations ± SEM. [b] Partial agonist (maximal inhibition of electrically evoked contractions = 26%).

Table 2. Binding Affinities of Opioid Peptide Analogues at μ and δ Receptors in Rat Brain Homogenates

		[^{3}H]DAMGO		[^{3}H]DSLET		
no.	compd	K_i^μ (nM)[a]	rel potency	K_i^δ (nM)[a]	rel potency	K_i^δ/K_i^μ
	H-Tyr-Pro-D-Phe-Pro-NH_2	35.2 ± 6.3	0.268 ± 0.048	>10000	<0.000253	>284
4	H-Tyr-Cys($\Psi^{H,H}$pro)-D-Phe-Pro-NH_2	8.57 ± 0.70	1.10 ± 0.09	2450 ± 200	0.00103 ± 0.00008	286
5	H-Tyr-Cys($\Psi^{Me,Me}$pro)-D-Phe-Pro-NH_2	37.7 ± 4.5	0.250 ± 0.030	>10000	<0.000253	>265
6	H-Tyr-Ser($\Psi^{H,H}$pro)-D-Phe-Pro-NH_2	58.9 ± 5.9	0.160 ± 0.016	>10000	<0.000253	>170
7	H-Tyr-Ser($\Psi^{Me,Me}$pro)-D-Phe-Pro-NH_2	96.5 ± 16.1	0.0977 ± 0.0163	>10000	<0.000253	>104
	H-Tyr-Pro-Phe-Phe-NH_2	2.06 ± 0.19	4.58 ± 0.42	3940 ± 460	0.000642 ± 0.000075	1910
8	H-Tyr-Cys($\Psi^{H,H}$pro)-Phe-Phe-NH_2	6.41 ± 0.22	1.47 ± 0.05	1980 ± 590	0.00128 ± 0.00038	309
9	H-Tyr-Cys($\Psi^{Me,Me}$pro)-Phe-Phe-NH_2	50.4 ± 2.2	0.187 ± 0.008	>10000	<0.000253	>198
10	H-Tyr-Ser($\Psi^{H,H}$pro)-Phe-Phe-NH_2	7.86 ± 1.11	1.20 ± 0.17	>4000	<0.000632	>509
11	H-Tyr-Ser($\Psi^{Me,Me}$pro)-Phe-Phe-NH_2	46.9 ± 5.8	0.201 ± 0.025	>10000	<0.000253	>241
	[Leu^5]enkephalin	9.43 ± 2.07	1	2.53 ± 0.35	1	0.268

[a] Mean of three determinations ± SEM.

Table 3. Analytical Data of Morphiceptin and Endomorphin-2 Analogues 4−11

no.	compd	*cis*/*trans*[a] (%)	$\Delta G°$ [b] (kcal/mol)
4	H-Tyr-Cys($\Psi^{H,H}$pro)-D-Phe-Pro-NH_2	40:60	−0.242
5	H-Tyr-Cys($\Psi^{Me,Me}$pro)-D-Phe-Pro-NH_2	>98	2.32
6	H-Tyr-Ser($\Psi^{H,H}$pro)-D-Phe-Pro-NH_2	45:55	−0.120
7	H-Tyr-Ser($\Psi^{Me,Me}$pro)-D-Phe-Pro-NH_2	>98	2.32
8	H-Tyr-Cys($\Psi^{H,H}$pro)-Phe-Phe-NH_2	40:60	−0.242
9	H-Tyr-Cys($\Psi^{Me,Me}$pro)-Phe-Phe-NH_2	>98	2.32
10	H-Tyr-Ser($\Psi^{H,H}$pro)-Phe-Phe-NH_2	45:55	−0.120
11	H-Tyr-Ser($\Psi^{Me,Me}$pro)-Phe-Phe-NH_2	>98	2.32

[a] Determined by integration of the ^{1}H NMR resonances of the 2-C protons (compounds **4**, **6**, **8**, and **10**). In the case of compounds **5**, **7**, **9**, and **11** inspection of the resonances of the 2C-dimethyl groups of the pseudoproline residue indicated a high predominance of the cis conformation (>98%) in all cases. [b] Calculated from equation $\Delta G°_{cis-trans} = -\ln K \times RT$, where R is the universal gas constant, T the temperature (300 K), and K the equilibrium constant *cis*/*trans*.

cis conformation around the amide bond between the first and second residues when bound to the μ receptor. As was the case with the parent peptide, compound **5** showed no δ receptor binding affinity at concentrations up to 10 μM and, thus, also was μ receptor-selective (K_i^δ/K_i^μ > 265, Table 2).

In comparison with the parent peptide [D-Phe^3]-morphiceptin, the dihydrooxazolidine-containing analogue (**6**) was a ∼3 times less potent μ agonist in the GPI assay, had about half the affinity for μ receptors in the binding assay, and showed a similarly high preference for μ receptors over δ receptors. Thus, compound **6** was a ∼6 times less potent μ agonist than the corresponding Cys($\Psi^{H,H}$pro)[2] analogue. For the Tyr−Ser($\Psi^{H,H}$pro) peptide bond in compound **6** a *cis*/*trans* ratio of 45:55 was determined in DMSO-d_6, similar to the ratio found for the dihydrothiazolidine-containing analogue (**4**) and for the parent peptide. The dimethyloxazolidine-containing [D-Phe^3]morphiceptin analogue (**7**) displayed somewhat lower μ agonist potency than the corresponding dimethylthiazolidine analogue in the GPI assay but, importantly, was still a full μ agonist. It also showed somewhat lower μ receptor affinity but retained high μ receptor selectivity. As was the case with analogue **5**, the Tyr−Ser($\Psi^{Me,Me}$pro) peptide bond in compound **7** was found to be almost exclusively in the *cis* conformation (>98%,Table 3).

Similar results were obtained with the compounds of the endomorphin-2 series. However, the dihydrooxazolidine-containing analogue (**10**) displayed only 2-fold lower μ agonist potency in the GPI assay than the dihydrothiazolidine-containing analogue (**8**) and almost equal μ receptor binding affinity. The *cis*/*trans* ratios for the Tyr−Pro peptide bond in compounds **8** and **10** in DMSO-d_6 were found to be exactly the same as those observed with the corresponding [D-Phe^3]morphiceptin analogues (**4** and **6**). Interestingly, the dimethyloxazolidine-containing endomorphin-2 analogue (**11**) turned out to have slightly higher μ agonist potency and μ receptor binding affinity than the dimethylthiazolidine-containing analogue (**9**). These results are in contrast to those obtained with the corresponding dimethylated pseudoproline-containing analogues of [D-Phe^3]morphiceptin, because compound **5** is a more potent μ agonist than compound **7**. These differences may be explained with slightly different modes of receptor binding between the [D-Phe^3]morphiceptin- and endomorphin-2 analogues.

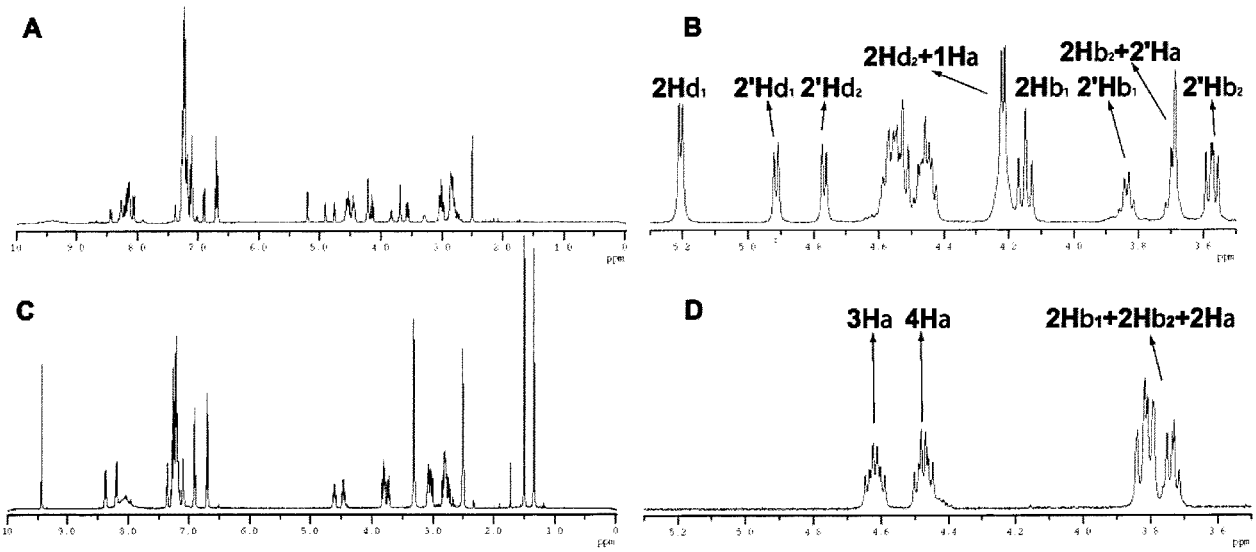

Figure 2. ^{1}H NMR spectra of compounds **10** (A and B) and **11** (C and D) in DMSO at 25 °C. The minor isomer is represented with a prime (′).

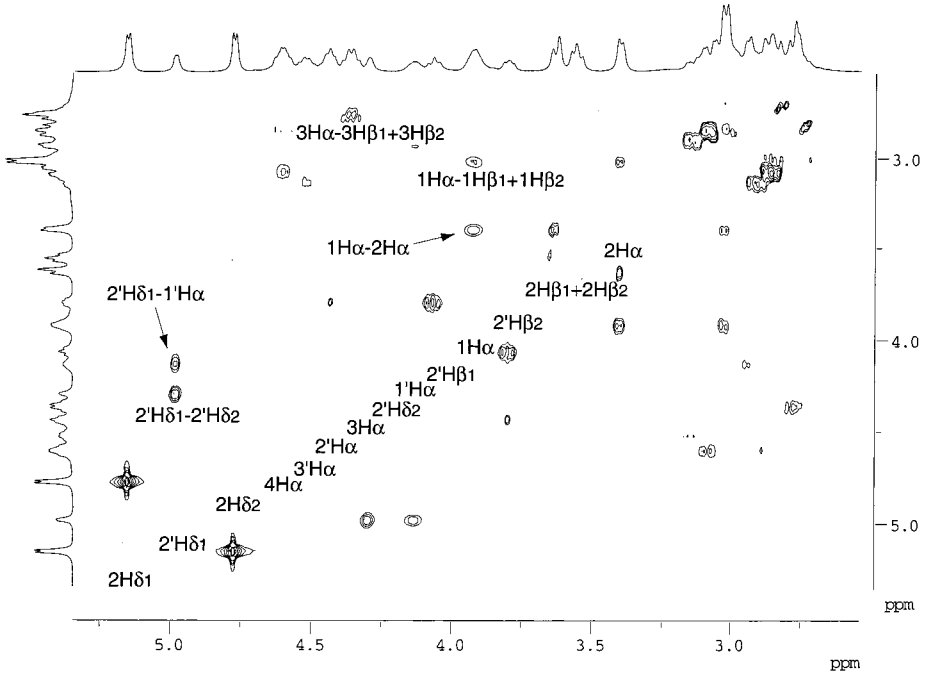

Figure 3. ROESY spectrum of **10** in CD_3CN at −20 °C. The characteristic NOE between 1Hα and 2Hα indicates that the major conformer has a *cis* amide bond between residues 1 and 2. The NOE between 2′Hδ and 1′Hα indicates that in the minor conformer this same amide bond is in the *trans* conformation.

As in the case of the [D-Phe^3]morphiceptin analogues **5** and **7**, the conformation of the peptide bond between Tyr and the dimethylated pseudoproline residue in the endomorphin-2 analogues **9** and **11** was found to be predominantly *cis* (>98%, Table 3). The claim of a highly predominant *cis* conformation was documented further in the case of compound **11** as an illustrative example by presenting 1-D ^{1}H NMR and 2-D ROESY spectra of **11** in comparison with **10**. Whereas a doubling of resonances is clearly evident in the 1-D spectrum of **10** in DMSO-d_6, a single set of resonances is observed with **11** (see Figure 2 and Experimental Section). The ROESY spectra were obtained in CD_3CN at −20 °C to prevent most of the exchange between the *cis* and *trans* conformers that was observed at higher temperature. Under these conditions the *cis*/*trans* ratio of **10** was 70:30. The ROESY spectrum of **10** shows the characteristic NOE between 1Hα and 2Hα, indicating a *cis* amide bond between residues 1 and 2 in the major conformer, and an NOE between 2′Hδ and 1′Hα, indicating a *trans* amide bond in the minor conformer (Figure 3). On the other hand, in the ROESY spectrum of **11** only the NOE between 1Hα and 2Hα is observed (Figure 4). Taken together, these results clearly indicate that the amide bond between the Tyr and Ser($\Psi^{Me,Me}$pro) residues in **11** almost exclusively assumes the *cis* conformation.

Like the [D-Phe^3]morphiceptin analogues (**4**−**7**), all endomorphin-2 analogues (**8**−**11**) displayed high preference for μ receptors over δ receptors. None of the [D-Phe^3]morphiceptin and endomorphin-2 analogues

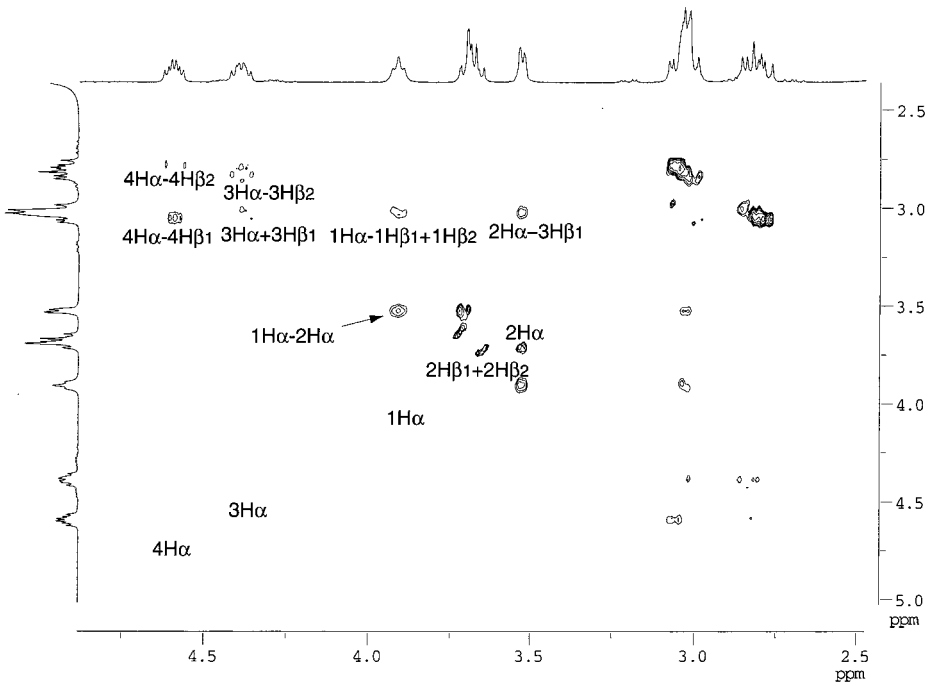

Figure 4. ROESY spectrum of **11** in CD_3CN at −20 °C. The characteristic NOE between 1Hα and 2Hα indicates that the amide bond between residues 1 and 2 is in the *cis* conformation.

described here showed significant binding affinity for κ opioid receptors at concentrations up to 10 μM. Thus, compounds **4**−**11** are all very selective μ opioid agonists.

In general, the dihydropseudoproline-containing analogues showed slightly lower μ agonist potency and μ receptor binding affinity than the corresponding Pro-containing parent peptides with the exception of compound **4**, which was found to be a more potent μ agonist than its parent. The transition state energies ($\Delta G^{\ddagger}$) for the *cis*/*trans* isomerization of the Xaa-ΨPro unit in peptides containing a dihydropseudoproline are typically 1−3 kcal/mol lower than those of the corresponding peptides with a native proline residue.[13] Because the present results indicate that the [D-Phe^3]morphiceptin and endomorphin-2 analogues have the *cis* conformation around the Tyr^1−ΨPro^2 peptide bond in their bioactive conformation, one would expect that the dihydroproline-containing analogues should display higher potency than the Pro-containing parent peptides because of the lower $\Delta G^{\ddagger}$ for the transition from the *trans* to the *cis* configuration. This turned out to be the case for the dihydrothiazolidine-containing [D-Phe^3]morphiceptin analogue (**4**), but not for the other dihydropseudoproline-containing morphiceptin or endomorphin-2 analogues. Other factors that may be responsible for these differences in potency may include direct effects of the heteroatom in the pseudoproline ring or slightly different receptor binding modes of the various dihydropseudoproline-containing morphiceptin or endomorphin-2 analogues.

All [D-Phe^3]morphiceptin and endomorphin-2 analogues containing a dimethylated pseudoproline residue showed somewhat lower μ agonist potency and lower μ receptor binding affinity than the corresponding dihydropseudoproline-containing analogues and the corresponding parent peptides. These reduced potencies are most likely the result of steric interference caused by the two 2-C-methyl substituents at the receptor binding site. On average, the dimethylated analogues have only a ∼5-fold lower μ receptor binding affinity than the corresponding dihydro analogues. Assuming that the *trans* conformer were the bioactive one, it would be expected that the *cis* conformer should either be inactive or have a μ receptor affinity reduced by several orders of magnitude as compared to that of the *trans* conformer. This expectation is based on the fact that the spatial disposition of the two important pharmacophoric moieties, the Tyr residue and the Phe^3 side chain, in the *cis* conformer is very different from that in the *trans* conformer. Therefore, the obtained results represent strong evidence in favor of a receptor-bound conformation with a *cis* amide bond between the first two residues.

Conclusions

The four [D-Phe^3]morphiceptin and endomorphin-2 analogues containing a dimethylated pseudoproline residue in place of Pro^2 (compounds **5**, **7**, **9**, and **11**) all are full μ opioid agonists showing good potency in the GPI assay and μ receptor binding affinities in the nanomolar range in the binding assay. As expected, ^{1}H NMR spectral analysis revealed that the peptide bond between the tyrosine and the dimethylated pseudoproline residue in these four compounds assumed almost exclusively the *cis* conformation. Taken together, these results represent the most direct evidence obtained so far to indicate that the conformation of the Tyr−Pro peptide bond of [D-Phe^3]morphiceptin and endomorphin-2 in their receptor-bound conformation is *cis*. This finding is in agreement with studies on the pharmacological and conformational properties of morphiceptin analogues containing either *cis*-2-aminocyclopentanecarboxylic acid or N^{α}-methylalanine in place of Pro^2, which had led to the suggestion that the *cis* conformation around the Tyr−Pro amide bond is required for the opioid activity of these morphiceptin analogues.[6,21] The

results of a conformational study recently performed with endomorphin-1 in reversed micelles of bis(2-ethylhexyl)sulfosuccinate sodium salt were also interpreted in favor of a model of the μ receptor-bound conformation with a *cis* Tyr−Pro amide bond.[7]

Experimental Section

General Methods. All solvents were obtained from Fluka, Buchs, Switzerland, and were used without further purification. Assembly of the peptides was carried out by the manual solid-phase method using the Sieber resin (Novabiochem, Läufelfingen, Switzerland). *N*-α-Fmoc amino acids (Alexis Corp., Läufelfingen, Switzerland) were used throughout. 2-C-dihydrothiazolidine was purchased from Fluka. Reversed-phase HPLC was performed on a Waters 600 SL instrument, using columns packed with Vydac Nucleosil 300 Å 5 μm C_{18} particles. Analytical columns (250 × 4.6 mm) were operated at a flow rate of 1 mL/min and preparative columns (250 × 21 mm) at a flow rate of 18 mL/min, with UV monitoring at 214 nm. Solvent A was water (purified on a Milli-Q ion exchange cartridge) containing 0.09% TFA, and solvent B was acetonitrile HPLC-R containing 10% water and 0.09% TFA (Biosolve, Valkenswaard, The Netherlands).

^{1}H NMR spectra were recorded at 400 MHz in DMSO-d_6 at 25 °C or in CD_3CN at −20 °C on a Bruker AM 400 MHz spectrometer using tetramethylsilane as internal standard at a concentration of 10 mg/mL. The assignment of all resonances and the determination of the conformation of the Tyr−ΨPro peptide bond were based on DQF-COSY, HOHAHA, and ROESY experiments. DQF-COSY experiments were done using the phase cycle described by Derma et al.[22] Two-dimensional HOHAHA experiments[23] were carried out using MLEV-17[24,25] and a mixing time of 50 ms. ROESY experiments[26] were performed using a mixing time of 200 ms. All two-dimensional spectra were obtained using 4K data points in the f_2 domain and 512 points in the f_1 domain. The data were processed using SwaN-MR software.[27] A zero-filling in the f_1 dimension and a square sine-bell window shifted by 90° in both dimensions (0° for f_2 in the case of the DQF-COSY) were applied prior to two-dimensional Fourier transformation. The protons were not assigned diastereoselectively. The assignment corresponds to the residue number followed by H and the position in the side chain (α, β, γ, δ, ϵ, ...). For ΨPro, a nomenclature similar to that generally used for proline was employed. In the case of dimethylpseudoproline the methyl groups on the δ carbon were named $Me_{\delta 1}$ and $Me_{\delta 2}$. Coupling constants are given in hertz. Mass spectra were obtained by electron spray ionization (MS-ESI) on a Finnigan LC 710 mass spectrometer.

Synthesis of Thiazolidine-Containing Dipeptides. (A) Fmoc-Tyr(All)-F. Fmoc-Tyr(All)-OH (4.00 g, 8.98 mmol) and pyridine (0.8 mL, 1 equiv) were dissolved in 80 mL of CH_2Cl_2. Addition of cyanuric fluoride (6.5 mL, 8 equiv) resulted in a white suspension. After completion of this reaction, the reaction flask was cooled in an ice bath, and then water (50 mL) was added dropwise to destroy the excess of activating reagent. The suspension was filtered over Celite and the organic layer dried with $MgSO_4$. After evaporation of the solvent, the product was obtained as a white powder (3.2 g, 80%). The purity of the product was assessed by analytical HPLC after transformation of the fluoride to the methyl ester using MeOH/pyridine (10 min): HPLC (C_{18}, 0−100% B, 30 min) t_R = 29.1 min; MS-ESI, (*m*/*z*) 444.2 $[M + H]^+$.

(B) Fmoc-Tyr(All)-Cys($\Psi^{H,H}$pro)-OH. Fmoc-Tyr(All)-F (1.89 g, 4.25 mmol) and *N*-methylmorpholine (1.4 mL, 3 equiv) were dissolved in 15 mL of DMF. After addition of Cys($\Psi^{H,H}$pro)-OH (1.7 g, 4.78 mmol), the solution was stirred for 16 h. At the end of the reaction the DMF was evaporated and the residual was taken up in 3 mL of EtOAc. Purification of the dipeptide was achieved by flash chromatography over silica using EtOAc/MeOH/AcOH (100:10:0.5, v/v) as eluent. Three hundred milligrams (13%) of pure Fmoc-Tyr(All)-Cys($\Psi^{H,H}$pro)-OH was obtained: HPLC (C_{18}, 0−100% B, 20 min) t_R = 12.32 min; MS-ESI, (*m*/*z*) 559.2 $[M + H]^+$.

(C) Fmoc-Tyr(All)-Cys($\Psi^{Me,Me}$pro)-OH. Fmoc-Tyr(All)-F (1 g, 2.25 mmol) and *N*-methylmorpholine (700 μL, 3 equiv) were dissolved in 15 mL of DMF. After addition of Cys($\Psi^{Me,Me}$-pro)-OH[28] (1.32 g, 2.25 mmol), the solution was stirred for 16 h. At the end of the reaction, 50 mL of EtOAc was added and the solution was washed with citric acid (5%, 25 mL, two times) and water (25 mL, two times). The organic phase was dried over $MgSO_4$, and after filtration, the solvent was removed under reduced pressure. Purification of the dipeptide was achieved by flash chromatography over silica using $CHCl_3$/MeOH/AcOH (100:10:1, v/v) as eluent. Five hundred and ten milligrams (39%) of pure Fmoc-Tyr(All)-Cys($\Psi^{Me,Me}$pro)-OH was obtained: HPLC (C_{18}, 50−100% B, 20 min) t_R = 15.02 min; MS-ESI, (*m*/*z*) 587.7 $[M + H]^+$.

Synthesis of Oxazolidine-Containing Dipeptides. (A) **Fmoc-Tyr(All)-Ser($\Psi^{H,H}$pro)-OH.** Serine (11.05 g, 105 mmol) was dissolved in aqueous Na_2CO_3 (2.5 N, 32 mL), and the solution was cooled in an ice bath. A solution of formaldehyde (37%, 19.3 mL) was added dropwise, and the solution was kept at 4 °C for 16 h. Fmoc-Tyr(All)-F (2.7 g, 6.1 mmol) dissolved in 84 mL of acetone was then added to the solution. After 3 h of intensive stirring at room temperature, the product was extracted twice with EtOAc (100 mL). The organic layer was dried over $MgSO_4$ and the solvent evaporated in vacuo. The resulting solid was purified by flash chromatography over silica ($CHCl_3$/MeOH/AcOH, 100:5:0.5) to afford 2.4 g (75%) of pure, white product in solid form: HPLC (C_{18}, 0−100% B, 30 min) t_R = 25.7 min; MS-ESI, (*m*/*z*) 543.2 $[M + H]^+$.

(B) Fmoc-Tyr(All)-OpF. Fmoc-Tyr(All)-OH (7 g, 15.8 mmol) was dissolved in a mixture of 30 mL of CH_2Cl_2 and 4 mL of THF. Dicyclohexylcarbodiimide (DCC; 3.58 g, 1.1 equiv) and 2,3,4,5,6-pentafluorophenol (3.2 g, 1.1 equiv) were then added to the solution. A heavy precipitate formed. After completion of the reaction, the suspension was filtered over Celite and the solvent evaporated in vacuo to afford 6.9 g (0.011 mol, 72%) of a pure, white solid: HPLC (C_{18}, 0−100% B, 30 min) t_R = 33.4 min; MS-ESI, (*m*/*z*) 610.2 $[M + H]^+$.

(C) Fmoc-Tyr(All)-Ser-OBzl. To a solution of Fmoc-Tyr-(All)-OpF (5.6 g, 9.18 mmol) in 30 mL of CH_2Cl_2 were added 3.61 g (1.7 equiv) of H-Ser-OBzl and 1.77 mL (1.7 equiv) of *N*-methylmorpholine. The solution was stirred at room temperature for 16 h. After completion of the reaction, citric acid (5%, 2 × 30 mL) was added. The organic layer was dried over $MgSO_4$ and evaporated in vacuo. The resulting solid was dissolved in EtOAc, and the dipeptide was precipitated by the addition of pentane. After filtration, a 95% pure product was obtained as a white solid (5.41 g, 35%): HPLC (C_{18}, 0−100% B, 30 min) t_R = 28.4 min; MS-ESI, (*m*/*z*) 621.4 $[M + H]^+$.

(D) Fmoc-Tyr(All)-Ser($\Psi^{Me,Me}$pro)-OBzl. Fmoc-Tyr(All)-Ser-OBzl (2.9 g, 4.67 mmol) was dissolved in THF/CH_2Cl_2 (1:1, v/v, 60 mL), and 2,2-dimethoxypropane (4.1 mL, 7.1 equiv) was added to the solution. *p*-Toluene pyridinium sulfonic acid (PPTS; 0.59 g, 0.5 equiv) was added, and the solution was then heated under reflux for 16 h. After completion of the reaction, all solvent was evaporated and the residue was taken up in MeOH and purified by flash chromatography over silica ($CHCl_3$/MeOH/AcOH, 100:0.5:0.5). Pure, solid product (3.53 g, 70%) was obtained: HPLC (C_{18}, 0−100% B, 30 min) t_R = 20.2 min; MS-ESI, (*m*/*z*) 661.2 $[M + H]^+$.

(E) Fmoc-Tyr-Ser($\Psi^{Me,Me}$pro)-OBzl. Fmoc-Tyr(All)-Ser-($\Psi^{Me,Me}$pro)-OBzl (3.1 g, 4.67 mmol) was dissolved in a mixture of 30 mL of CH_2Cl_2 and 9 mL of THF, and $SiPhH_3$ (4.1 mL, 7.1 equiv) was added to the solution. The reaction mixture was stirred under N_2 for 10 min, and $Pd(PPh_3)_4$ was then added still under N_2. Deprotection was complete after 16 h of stirring under nitrogen. After removal of all solvent, the remaining black oil was purified by flash chromatography over silica (MeOH) to afford 1.2 g (44%) of a white solid: HPLC (C_{18}, 0−100% B, 30 min) t_R = 15.7 min; MS-ESI, (*m*/*z*) 621.5 $[M + H]^+$.

(F) Fmoc-Tyr-Ser($\Psi^{Me,Me}$pro)-OH. Fmoc-Tyr-Ser($\Psi^{Me,Me}$-pro)-OBzl (1.2 g, 1.93 mmol) was dissolved in 20 mL of MeOH, and 250 mg of Pd/C catalyst and two drops of AcOH were added to the solution under N_2. The solution was then stirred

for 2 h under an H_2 atmosphere. After completion of the reaction, the solution was filtered over Celite and the solvent was evaporated in vacuo. Pure product (0.95 g, 93%) was obtained in solid form: HPLC (C_{18}, 0−100% B, 30 min) t_R = 7.8 min; MS-ESI, (*m*/*z*) 531.1 $[M + H]^+$.

(G) Solid-Phase Peptide Synthesis. Sieber resin[20] (0.26 g; 0.56 mmol/g loading) was successively washed for 10 min with MeOH (10 mL), toluene (10 mL), and CH_2Cl_2 (10 mL) before swelling in DMF (1 h). Couplings were carried out in DMF, using Fmoc-amino acids (3 equiv), DIEA (5 equiv), and PyBOP (3 equiv) as coupling agent. Coupling time was 2 h. The ninhidrin test[29] was used to check for completion of the coupling reactions. Fmoc deprotection was performed with piperidine (20% in DMF, 3 × 10 min). After assembly of the C-terminal dipeptide H-Xaa-Yaa- (Xaa = Phe, D-Phe; Yaa = Phe, Pro) on the resin, coupling of the N-terminal dipeptide (3 equiv) was carried out in DMF with PyBOP (230 mg, 3 equiv) as coupling agent in the presence of DIEA for 2 h. The Tyr side chain in the dipeptides was protected with the allyl group, except in the case of the 2-C-dimethyloxazolidine-containing dipeptide, where the Tyr side chain was left unprotected. The allyl group of the resin-bound tetrapeptides was removed by reaction with $Pd[P(Ph)_3]_4$ (22.5 mg, 0.1 equiv) and SiH_3Ph (430 μL, 24 equiv) in CH_2Cl_2 under N_2 for 15 min. After removal of the final Fmoc group, peptides were cleaved from the resin by treatment with 5% (v/v) TFA in CH_2Cl_2 (3 × 10 min). To the resulting, slightly red solution was added 10 mL of toluene, and then all solvent was evaporated. Purification of the tetrapeptides was achieved by reversed-phase HPLC, using a gradient of 10−100% B (30 min).

(H) H-Tyr-Cys($\Psi^{H,H}$pro)-D-Phe-Pro-NH_2 (4): HPLC (C_{18}, 10−100% B, 40 min) t_R = 16.93 min; MS-ESI, (*m*/*z*) 524.0 $[M + H]^+$; ^{1}H NMR (DMSO-d_6) a detailed attribution of all signals was not possible due to overlap of the signals of the *cis* and *trans* ω-Tyr−ΨPro conformers. However, certain resonances such as the *ortho*/*meta* protons of the phenyl ring of tyrosine were nicely resolved in both conformations and served as a basis for the calculation of the *cis*/*trans* ratio.

(I) H-Tyr-Cys($\Psi^{Me,Me}$pro)-D-Phe-Pro-NH_2 (5): HPLC (C_{18}, 10−100%, 20 min) t_R = 13.0 min; MS-ESI, (*m*/*z*) 568.6 $[M + H]^+$; ^{1}H NMR (DMSO-d_6) one set of signals, attributed to the *cis* conformation of the Tyr−ΨPro peptide bond, 7.48 (*d*, 1H, 3HN), 7.27−7.35 (*m*, 5H, $3H_\delta+H_\epsilon+H_\zeta$), 7.12 (*d*, 2H, *J* = 8.29, $1H_\delta$), 6.83 (*d*, 2H, *J* = 8.33, $1H_\epsilon$), 6.75 (*s*, 1H, $CONH_2$ *syn*), 6.53 (*s*, $CONH_2$ *anti*), 4.67 (*d*×*d*, 1H, *J* = 7.56/7.58, $3H_\alpha$), 4.18 (*m*, 1H, $2H_\alpha$), 3.64 (*m*, 2H, $1H_\alpha+4H_\alpha$), 3.00 (*m*, 6H, $4H_\beta+4H_\delta+2H_\beta$), 2.95 (m, 2H, $1H_\beta$), 2.93 (*d*×*d*, 2H, $3H_\beta$), 1.8 (*m*, 2H, $4H_\gamma$), 1.76 (*s*, 3H, $2Me_{\delta1}$), 1.68 (*s*, 3H, $2Me_{\delta2}$).

(J) H-Tyr-Ser($\Psi^{H,H}$pro)-D-Phe-Pro-NH_2 (6): HPLC (C_{18}, 0−100% B, 30 min) t_R = 15.5 min; MS-ESI, (*m*/*z*) 524.0 $[M + H]^+$; ^{1}H NMR (DMSO-d_6) a detailed attribution of all signals was not possible due to overlap of the signals of the *cis* and *trans* ω-Tyr−ΨPro conformers. However, certain resonances such as the *ortho*/*meta* protons of the phenyl ring of tyrosine were well resolved in both conformations and served as a basis for the calculation of the *cis*/*trans* ratio.

(K) H-Tyr-Ser($\Psi^{Me,Me}$pro)-D-Phe-Pro-NH_2 (7): HPLC (C_{18}, 0−100% B, 30 min) t_R = 16.9 min; MS-ESI, (*m*/*z*) 551.9 $[M + H]^+$; ^{1}H NMR (CD_3CN) one set of signals, attributed to the *cis* conformation of the Tyr−ΨPro peptide bond, 7.47 (*d*, 1H, 3HN), 7.27−7.35 (*m*, 5H, $3H_\delta+H_\epsilon+H_\zeta$), 7.14 (*d*, 2H, $1H_\delta$), 6.83 (*d*, 2H, $1H_\epsilon$), 6.78 (large resonance, 2H, $CONH_2$), 4.68 (*q*, 1H, $3H_\alpha$), 4.19 (*d*×*d*, 1H, $4H_\alpha$), 3.92 (*d*×*d*, 1H, $2H_{\beta1}$), 3.81 (*m*, 2H, $2H_\alpha + 2H_{\beta2}$), 3.68 (*m*, 1H, $1H_\alpha$), 3.65 (*m*, 1H, $4H_\delta$), 3.14 (*m*, 1H, $4H_{\beta1}$), 2.98 (*m*, 1H, $1H_\beta$), 2.01 (*m*, 1H, $4H_{\beta2}$), 1.86 (*m*, 2H, $4H_{\beta1}+4H_{\gamma2}$), 1.63 (*m*, 1H, $4H_{\gamma1}$), 1.55 (*s*, 3H, $2Me_{\delta1}$), 1.41 (*s*, 3H, $2Me_{\delta2}$).

(L) H-Tyr-Cys($\Psi^{H,H}$pro)-Phe-Phe-NH_2 (8): HPLC (C_{18}, 0−100% B, 30 min) t_R = 15.5 min; MS-ESI, (*m*/*z*) 590.0 $[M + H]^+$; ^{1}H NMR (DMSO-d_6) a detailed attribution of all signals was not possible due to overlap of the signals of the *cis* and *trans* ω-Tyr−ΨPro conformers. However, certain resonances such as the *ortho*/*meta* protons of the phenyl ring of tyrosine were resolved in both conformations and served as a basis for the calculation of the cis/trans ratio.

(M) H-Tyr-Cys($\Psi^{Me,Me}$pro)-Phe-Phe-NH_2 (9): HPLC (C_{18}, 10−100%, 20 min) t_R = 15.02 min; MS-ESI, (*m*/*z*) 618.6 $[M + H]^+$; ^{1}H NMR (DMSO-d_6) one set of signals, attributed to the *cis* conformation of the Tyr−ΨPro peptide bond, 7.60 (*d*, 1H, *J* = 8, 3 or 4HN), 7.52 (*d*, 1H, *J* = 8, 4 or 3HN), 7.15−7.22 (*m*, 10H, $3H_\delta+3H_\epsilon+3H_\zeta+4H_\delta+4H_\epsilon+4H_\zeta$), 7.08 (*d*, 2H, *J* = 8.4, $1H_\delta$), *d*, 2H, *J* = 7.3, $1H_\epsilon$), 6.67 (*s*, 1H, $CONH_2$ *syn*), 6.05 (*s*, 1H, $CONH_2$ *anti*), 4.64 (*d*×*d*, 1H, *J* = 5.27, $1H_\alpha$), 4.53 (*d*×*d*, 1H, *J* = 5.32, $4H_\alpha$), 4.01 (*m*, 1H, $3H_\alpha$), 3.94 (*d*×*d*, 1H, *J* = 6.92, $2H_\alpha$), 3.05 (*m*, 4H, $1H_\beta+2H_\beta$), 2.86 (*m*, 4H, $3H_\beta+4H_\beta$), 1.74 (*s*, 3H, $2Me_{\delta1}$), 1.71 (*s*, 3H, $2Me_{\delta2}$).

(N) H-Tyr-Ser($\Psi^{H,H}$pro)-Phe-Phe-NH_2 (10): HPLC (C_{18}, 0−100% B, 30 min) t_R = 16.2 min; MS-ESI, (*m*/*z*) 574.2 $[M + H]^+$; ^{1}H NMR (CD_3CN, −20 °C) *trans*/*cis* 30:70 (major isomer) 8.21 (3HN), 7.84 (4HN), 7.63 (1HN), 7.03 ($1H_\delta$), 6.89 ($CONH_2$), 6.78 ($1H_\epsilon$), 6.36 ($CONH_2$), 5.15 ($2H_{\delta1}$), 4.77 ($2H_{\delta2}$), 4.60 ($4H_\alpha$), 4.36 ($3H_\alpha$), 3.92 ($1H_\alpha$), 3.61 ($2H_{\beta1}$), 3.55 ($2H_{\beta2}$), 3.39 ($2H_\alpha$), 3.07 ($4H_{\beta1}$), 3.02 ($1H_\beta$), 2.86 ($4H_{\beta2}$), 2.77 ($3H_\beta$); (minor isomer) 7.41 (3HN), 7.35 (4HN), 7.28 (1HN), 7.01 ($1H_\delta$), 6.75 ($1H_\epsilon$), 6.63 ($CONH_2$), 6.32 ($CONH_2$), 4.98 ($2H_{\delta1}$), 4.51 ($4H_\alpha$), 4.44 ($3H_\alpha$), 4.43 ($2H_\alpha$), 4.29 ($2H_{\delta2}$), 4.13 ($1H_\alpha$), 4.06 ($2H_{\beta1}$), 3.80 ($2H_{\beta2}$), 3.13 ($4H_{\beta1}$), 3.00 ($3H_{\beta1}$), 2.94 ($1H_\beta$), 2.91 ($4H_{\beta2}$), 2.85 ($3H_{\beta2}$); important peaks in DMSO-d_6 at 25 °C *trans*/*cis* 64:36 (major isomer) 5.20 ($2H_{\delta1}$), 4.52 ($2H_\alpha$), 4.21 ($1H_\alpha$), 4.21 ($2H_{\delta2}$), 4.14 ($2H_{\beta1}$), 3.57 ($2H_{\beta2}$), 2.85 ($1H_\beta$); (minor isomer) 4.91 ($2H_{\delta1}$), 4.76 ($2H_{\delta2}$), 3.83 ($2H_{\beta1}$), 3.69 ($2H_\alpha$), 3.68 ($2H_{\beta2}$), 3.29 ($1H_\alpha$), 2.84 ($1H_\beta$).

(O) H-Tyr-Ser($\Psi^{Me,Me}$pro)-Phe-Phe-NH_2 (11): HPLC (C_{18}, 0−100% B, 30 min) t_R = 17.1 min; MS-ESI, (*m*/*z*) 602.1 $[M + H]^+$; ^{1}H NMR (DMSO-d_6) one set of signals, attributed to the *cis* conformation of the Tyr−ΨPro peptide bond, 8.36 (*d*, 1H, 3HN), 8.20 (*d*, 1H, 4HN), 7.37 (*s*, 1H, $CONH_2$), 7.15−7.30 (*m*, 10H, $3H_\delta+3H_\epsilon+3H_\zeta+4H_\delta+4H_\epsilon+4H_\zeta$), 7.11 (*s*, 1H, $CONH_2$), 6.92 (*d*, 2H, $1H_\delta$), 6.71 (*d*, 2H, $1H_\epsilon$), 4.62 (*m*, 1H, $3H_\alpha$), 4.48 (*m*, 1H, $4H_\alpha$), 3.81 (*m*, 2H, $2H_{\beta2}+2H_\alpha$), 3.73 (*d*×*d*, 1H, $2H_{\beta1}$), 3.07 (*m*, 1H, $1H_\alpha$), 3.04 (*m*, 2H, $3H_{\beta2}+4H_{\beta2}$), 2.83 (*m*, 2H, $1H_\beta$), 1.86 (*m*, 2H, $3H_{\beta1}+4H_{\beta1}$), 1.50 (*s*, 3H, $2Me_{\delta2}$), 1.34 (*s*, 3H, $2Me_{\delta1}$); ^{1}H NMR (CD_3CN, −20 °C) 7.93 (1HN), 7.76 (4HN), 7.74 (3HN), 7.06 ($1H_\delta$), 6.80 ($1H_\epsilon$), 6.80 & 6.21 ($CONH_2$), 4.59 ($4H_\alpha$), 4.39 ($3H_\alpha$), 3.89 ($1H_\alpha$), 3.68 ($2H_\beta$), 3.52 (d, *J* = 5.0, $2H_\alpha$), 3.05 ($4H_{\beta1}$), 3.01 ($1H_\beta$), 3.01 ($3H_{\beta1}$), 2.81 ($3H_{\beta2}$), 2.77 ($4H_{\beta2}$), 1.51 (s, 3H, $2Me_{\delta1}$), 1.43 (s, 3H, $2Me_{\delta2}$).

(P) In Vitro Bioassays and Opioid Receptor Binding Assays. The GPI[30] and MVD[31] bioassays were carried out as reported in detail elsewhere.[32,33] A log dose−response curve was determined with [Leu^5]enkephalin as standard for each ileum and vas preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure.[34]

Opioid receptor binding studies were performed as described in detail elsewhere.[32] Binding affinities for μ and δ receptors were determined by displacing, respectively, [^{3}H]DAMGO (Multiple Peptide Systems, San Diego, CA) and [^{3}H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor affinities were measured by displacement of [^{3}H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0 °C with [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593 at respective concentrations of 0.72, 0.78, and 0.80 nM. IC_{50} values were determined from log dose−displacement curves, and K_i values were calculated from the obtained IC_{50} values by means of the equation of Cheng and Prusoff,[35] using values of 1.3, 2.6, and 2.9 nM for the dissociation constants of [^{3}H]-DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593, respectively.

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References

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC−IUB Joint Commission on Biochemical Nomenclature: Nomenclature and Symbolism for Amino Acids

and Peptides *Biochem. J.* **1984**, *219*, 345−373. The other abbreviations are as follows: All, allyl; DAMGO, H-Tyr-D-Ala-Gly-N$^{\alpha}$MePhe-Gly-ol; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; Fmoc, 9-fluorenylmethyloxycarbonyl; GPI, guinea pig ileum; MS-ESI, electron spray ionization mass spectrometry; MVD, mouse vas deferens; PPTS, pyridinium *p*-toluenesulfonate; PyBOP, benzotriazol-1-yloxytris(pyrrolidinophosphonium-hexafluorophosphate); TFA, tetrafluoroacetic acid; THF, tetrahydrofuran; U69,593, (5α,7α,8β)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro-[4.5]dec-8-yl]benzeneacetamide.

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