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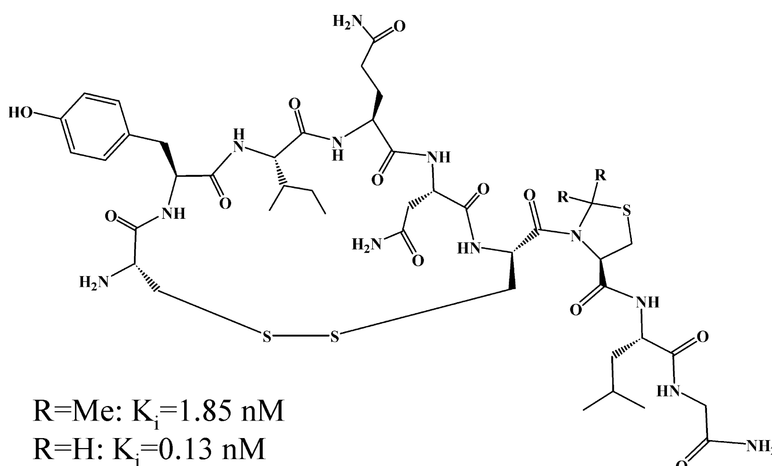

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Articles

Introduction of a *cis*-Prolyl Mimic in Position 7 of the Peptide Hormone Oxytocin Does Not Result in Antagonistic Activity

Angela Wittelsberger,*,† Luc Patiny,† Jirina Slaninova,‡ Claude Barberis,§ and Manfred Mutter†

Institute of Chemical Sciences and Engineering (ISIC), Swiss Federal Institute of Technology, EPFL-BCH, CH-1015 Lausanne, Switzerland, Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, CZ-16610 Prague, Czechoslovakia, and INSERM U469, 141 Rue de la Cardonille, F-34094 Montpellier Cedex 5, France

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New insights into the structure−activity relationship of the peptide hormone oxytocin are presented. Incorporation of the novel *cis*-prolyl mimic 2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid (pseudoproline, ΨPro) at position 7 of the hormone yielded the analogue [Cys($\Psi^{Me,Me}$pro)]^{7}oxytocin (**1**) that showed a 92−95% induction of the cis peptide bond conformation between Cys^6 and ΨPro^7, as determined by one- and two-dimensional NMR spectra in water and in DMSO-d_6. The impact of the dimethyl moiety regarding conformation and bioactivity was investigated by the synthesis of the corresponding dihydro compound, [Cys($\Psi^{H,H}$pro)]^{7}oxytocin (**2**). Biological tests of the uterotonic activity, the pressor activity, and the binding affinity to the rat and human oxytocin receptors were carried out. As a most significant result, no antagonistic activities were found for both the cis-constrained analogue **1** and analogue **2**, suggesting that a cis conformation between residues 6 and 7 of the molecule does not result in antagonistic activity. However, the about 10-fold reduction in agonistic activity of **1** as compared to oxytocin is consistent with the reduction of the trans conformation from 90% for oxytocin to 5−8% for compound **1**. Compound **1** retained a high binding affinity for the oxytocin receptor, with K_i values of 8.0 and 1.9 nM for the rat and the human receptor, respectively. The correlation between the biological activities and the cis contents obtained from NMR analysis for compounds **1**, **2**, and oxytocin leads to the hypothesis that a cis/trans conformational change plays an important role in oxytocin receptor binding and activation.

Introduction

The neurohypophyseal peptide hormone oxytocin (OT) was first isolated from the posterior pituitary and is used clinically for the initiation and maintenance of uterine contractions of labor. OT is a heterodetic cyclic nonapeptide with a disulfide linkage between two cysteine residues at positions 1 and 6. The six amino acid ring with the sequence Cys-Tyr-Ile-Gln-Asn-Cys contains a free N-terminal amino group. The C terminus in the three amino acid tail with the sequence Pro-Leu-Gly is amidated.

Much interest was caused by the presence of the proline residue at position 7 of OT and the structurally related vasopressin. Early results of ^{1}H and ^{13}C NMR experiments with OT and arginine vasopressin (AVP) led to the consideration that the Cys^6-Pro^7 bonds of OT and AVP exist exclusively in the trans conformation.[1−4] Later, investigations by Rabenstein and co-workers on the amide isomer equilibrium about the Cys^6-Pro^7 bond in OT by one- and two-dimensional NMR spectroscopy resulted in the detection of a 10% cis isomer population in water.[5] The relative abundance of the cis isomer was decreased when the solvent was changed from water to methanol.[6] Also, a significantly faster rate of rotation about the Cys^6-Pro^7 bond was observed in methanol. This observation is consistent with the recently proposed mechanism for the cis/trans interconversion of Xaa-Pro peptide bonds, which is a rotation around the peptide bond involving a twisted amide bond in the transition state, with no nucleophilic participation of the solvent. According to this mechanism, no polar resonance structures are possible in the transition state, and the rate of cis/trans interconversion is expected to be faster in nonaqueous solvents.[7]

Cis peptide bonds were also observed in sarcosyl^7- and N-methylalanyl^7-OT.[8] These two analogues showed cis isomer populations of around 25 and 15%, respectively, and they exhibited similar and about 7-fold reduced uterotonic activity relative to the parent compound.[8,9] Also, replacement of the proline at position 7 by a glycine leads to a decrease in activity; but an increase in selectivity,[10−13] i.e. the Gly^7 analogues had substantial oxytocic potency, but their pressor and antidiuretic activities (the vasopressin-like activities) were effectively reduced. Taken together, these results emphasize the role of the proline at position 7 in inducing a

* To whom correspondence should be addressed. Current address: Department of Physiology, M&V 7, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Tel: 617-636 3882. Fax: 617-636 0445. E-mail: Angela.Wittelsberger@tufts.edu.
† Swiss Federal Institute of Technology.
‡ Czech Academy of Sciences.
§ INSERM U469.

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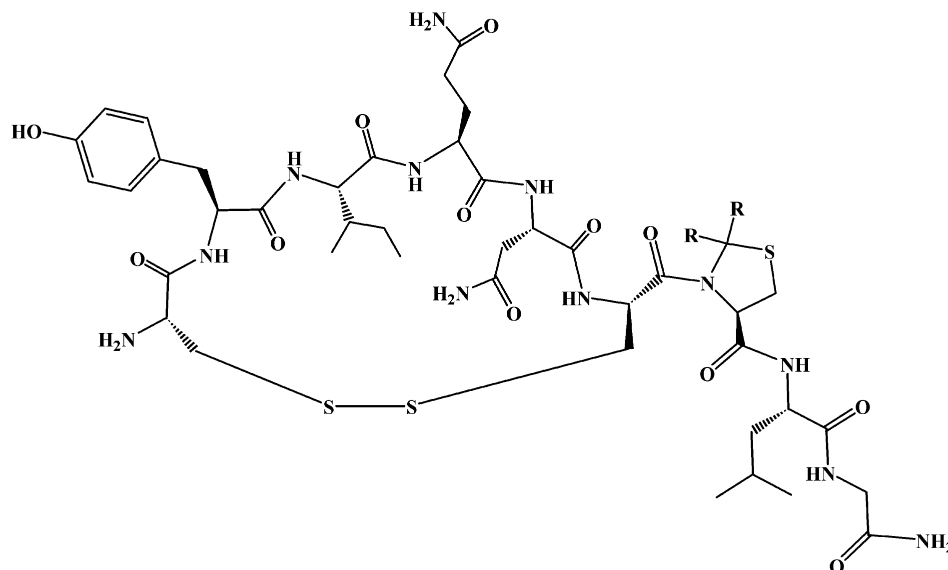

Figure 1. Structure of the OT analogues cyclo[Cys-Tyr-Ile-Gln-Asn-Cys]-Cys($\Psi^{Me,Me}$pro)-Leu-Gly-NH2 (**1**) and cyclo[Cys-Tyr-Ile-Gln-Asn-Cys]-Cys($\Psi^{H,H}$pro)-Leu-Gly-NH2 (**2**). Compound **1**, R = Me; **2**, R = H.

conformational restriction. When the proline residue in position 7 was replaced by isosteric substitutions, such as thiazolidine-4-carboxylic acid[14] or 3,4-didehydroproline,[15] OT analogues with doubled oxytocic activity were obtained.

Other observations of a cis peptide bond preceding proline in the side chain of OT were made by Hruby et al. during investigations on the cis/trans isomerism in the inactive tri- and tetrapeptide derivatives of the side chain of OT.[1−3]

Structure−activity studies regarding agonism/antagonism of OT analogues prompted investigations on the hypothesis that the cis Cys^6-Pro^7 isomer of OT favors antagonism, whereas the trans isomer would result in agonistic activity.[16] For example, a cis Cys-Pro peptide bond was observed in the potent bicyclic antagonists of OT, [dPen^1,cyclo(Glu^4,Lys^8)]OT and [Mpa^1,cyclo(Glu^4, Lys^8)]OT, by NMR spectroscopy and computational analysis.[17] The structural analysis revealed β-turns around residues Tyr^2 and Ile^3, which differed from the previously discussed β-turn geometry around residues 3 and 4 that was ascribed to the conformation of OT agonists.[18,19] The X-ray structure of the potent OT agonist, [Mpa^1]OT (deaminooxytocin), showed the Cys-prolyl imide bond in the trans conformation.[20] Motivated by these observations, Lubell and co-workers synthesized analogues of OT, [Mpa^1]OT, and [dPen^1]OT, replacing Pro^7 by (2*S*,5*R*)-5-*tert*-butylproline, a proline analogue inducing up to 90% of the cis amide conformation in N-(acetyl)dipeptide-N′-methylamides as shown previously.[21,22] However, NMR investigations of the corresponding OT compounds showed a maximal induction of the cis geometry of 35%,[16] and efforts to increase the cis content further by replacing Cys^6 with the bulky penicillamin residue yielded the same maximal cis content (35%). Although augmentation of cis isomer population was shown to result in decreased agonism, no decisive conclusions could be drawn regarding the hypothesis of an antagonistic cis conformation.

With the goal of determining the role of a cis Cys^6-Pro^7 conformation in antagonistic activity, we report here on the synthesis and the conformational and biological properties of pseudoproline-containing OT analogues. Pseudoprolines (ΨPro) are thiazolidine or oxazolidine derivatives that serve as proline analogues with increased proline specific properties.[23−25] For example, the population of the cis conformation of a ΨPro-preceding peptide bond can be modulated depending on the C2 substituents of the cyclic system. Most notably, the dimethyl-substituted analogues 2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid and 2,2-dimethyl-1,3-oxazolidine-4-carboxylic acid induce up to 100% of the cis conformation in model compounds as well as in several target-derived bioactive peptide sequences.[24−26] Incorporation of the novel cis mimic 2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid at position 7 of OT ([Cys($\Psi^{Me,Me}$pro)]^{7}OT, **1**) yielded a 95% induction of the cis conformation, as determined by one- and two-dimensional NMR spectroscopy in DMSO and in water. The impact of the dimethyl moiety at 2-C was assessed by comparison with the corresponding dihydro compound, [Cys($\Psi^{H,H}$pro)]^{7}OT (**2**). The biological activities of the two analogues, the uterotonic activity as well as the binding affinity to the human and the rat OT receptor, were related to the cis contents and provided novel insights into the role of a *cis*-Pro at position 7 of OT.

Results and Discussion

The synthesis of OT analogues **1** and **2** containing 2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid and 1,3-thiazolidine-4-carboxylic acid, respectively, in position 7 is outlined in Figure 2. Because of the low nucleophilicity of its secondary amine in addition to the steric hindrance imposed by the two methyl groups in the case of 2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid, pseudoprolines are generally introduced in the solid phase synthesis as preformed dipeptide building blocks.[27,28] A convenient way to synthesize the dipeptides is by formation of the Fmoc and side chain-protected cysteine fluoride with (diethylamino)sulfur trifluoride (DAST)[29] and subsequent coupling to the pseudoproline. Because of the acidic conditions during the formation of the dipeptide building block, the acid stable orthogonal

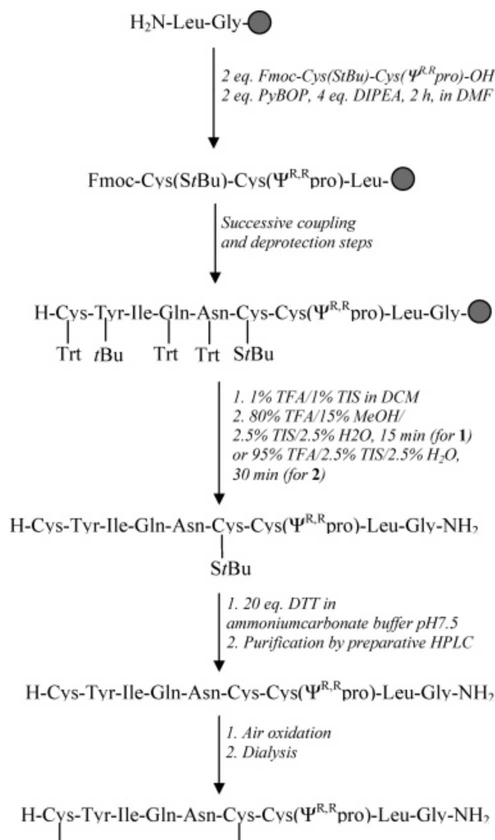

Figure 2. Scheme of the solid phase synthesis of the ΨPro-containing OT analogues.

protecting group S-*tert*-butyl was used for the cysteine in position 6 of the hormone analogue. The peptide hormone OT is amidated it its C-terminal. Therefore, the chemical synthesis of the two analogues was carried out on Sieber amide resin, which allows one to obtain the C-terminal amide upon cleavage of the peptide from the resin with 1% TFA. The synthesis was carried out using Fmoc/*t*Bu chemistry.[41] The peptide containing the dimethyl-substituted thiazolidine was deprotected with a mixture of 80% TFA, 15% of MeOH, 2.5% TIS, and 2.5% water. The *t*Bu protecting group on Tyr and the Trt protecting groups on Cys and Asn were effectively cleaved after 10−15 min, as judged by HPLC analysis, which allowed us to reduce the acid-induced cleavage of the thiazolidine ring to a minimum. The C(2)-dihydro-pseudoproline is known to be stable toward TFA,[23] and the corresponding peptide was therefore treated with a mixture of 95% TFA, 2.5% TIS, and 2.5% water for deprotection. After cleavage of the acid labile protecting groups and removal of S*t*Bu by the reducing agent dithiothreitol, the reduced precursors of **1** and **2** were purified at this stage by preparative HPLC. The final products were then obtained in high purity by air oxidation followed by dialysis against water, requiring no additional purification step.

Information on the peptidyl-prolyl imide bond geometry and amounts of the population of the cis and the trans isomer were obtained by NMR spectroscopy. The two OT analogues **1** and **2** were analyzed by one- and two-dimensional ^{1}H NMR spectroscopy in DMSO as well as in water and in deuterated water. The data were processed with the SwaN-MR software,[30] which allowed for the superposition of ROESY, COSY-DQF, and TOCSY spectra. Thereby, the color of NOE cross-peaks was chosen pink, positive/negative signals in the COSY spectrum were blue/light blue, and TOCSY connectivities were shown in green (see Figure 3). Two-dimensional ^{1}H NMR TOCSY, COSY-DQF, and ROESY data obtained in DMSO-d_6 allowed for a complete attribution of the proton chemical shifts for both molecules (Table 1). The data showed a mixture of one major and one minor conformation. An expanded part from the α-proton region of peptide **1** superposing the TOCSY, the COSY-DQF, and the ROESY spectra is shown in Figure 3, A. It shows a NOE cross-peak (b) between the α-proton of residue 7 (ΨPro, a) and the α-proton of residue 6 (Cys, d) characteristic for a cis peptide bond between the two residues. The exchange cross-peak marked c between the α-protons of Cys^6 of the minor and the major conformations is due to the cis/trans isomerization taking place during the mixing time and constitutes a proof that the minor resonances are from another conformation of the same molecule and not an impurity. A cis content of 92% was calculated by integration of the peaks from the Gln^4 α-protons from the major and the minor conformation, connected by an exchange cross-peak (h). For the calculation, it was considered that the signal from the Gln^4 α-proton of the major conformer (f) was superposed to the signal from the Ile^3 Hα of the minor conformer, as demonstrated by the exchange connectivity between Ile^3 Hα (major)/Hα (minor) (g). The minor conformation was expected to be the corresponding trans isomer, but the characteristic NOE connectivities between the α-proton of Cys^6 and the δ-protons of the pseudoproline could not be observed due to the dimethyl moiety in the δ-position of Cys(Ψ^{Me,Me}pro).

The NMR spectra of **2** in DMSO-d_6 represented a mixture of one major and one minor conformation. A strong exchange cross-peak was observed between the α-protons of ΨPro^7 of the major and the minor conformer, which were separated by 0.54 ppm, indicating an important change in the environment upon isomerization. This is consistent with a cis/trans isomerization around the ΨPro-preceding peptide bond, and the minor conformation was therefore expected to be a conformation with the Cys^6-ΨPro^7 peptide bond in the cis geometry. However, because of the weak population of the minor conformation, the characteristic NOE connectivity between the Cys^6 α-proton and the Ψpro^7 α-proton expected for the cis conformer was not observed. The cis/trans ratio was determined by integration of the ΨPro^7 Hα-signals from the major and the minor conformation, since they were both sufficiently isolated. From the integrals, a cis content of 5.7% was calculated. The dihydro-pseudoproline-containing OT analogue **2** showed therefore a smaller cis content than does OT itself, for which a population of about 10% was reported to exist in the cis geometry.[5]

DMSO is generally accepted as one of the most waterlike organic solvents.[51] However, to have identical solvent conditions as in the biological tests, the cis contents of the OT analogues were also determined in water. The one-dimensional NMR spectra in water were done by excitation sculpting. As a result, the proton signals that are in close proximity to the suppressed water peak were lacking in strength. Therefore, only

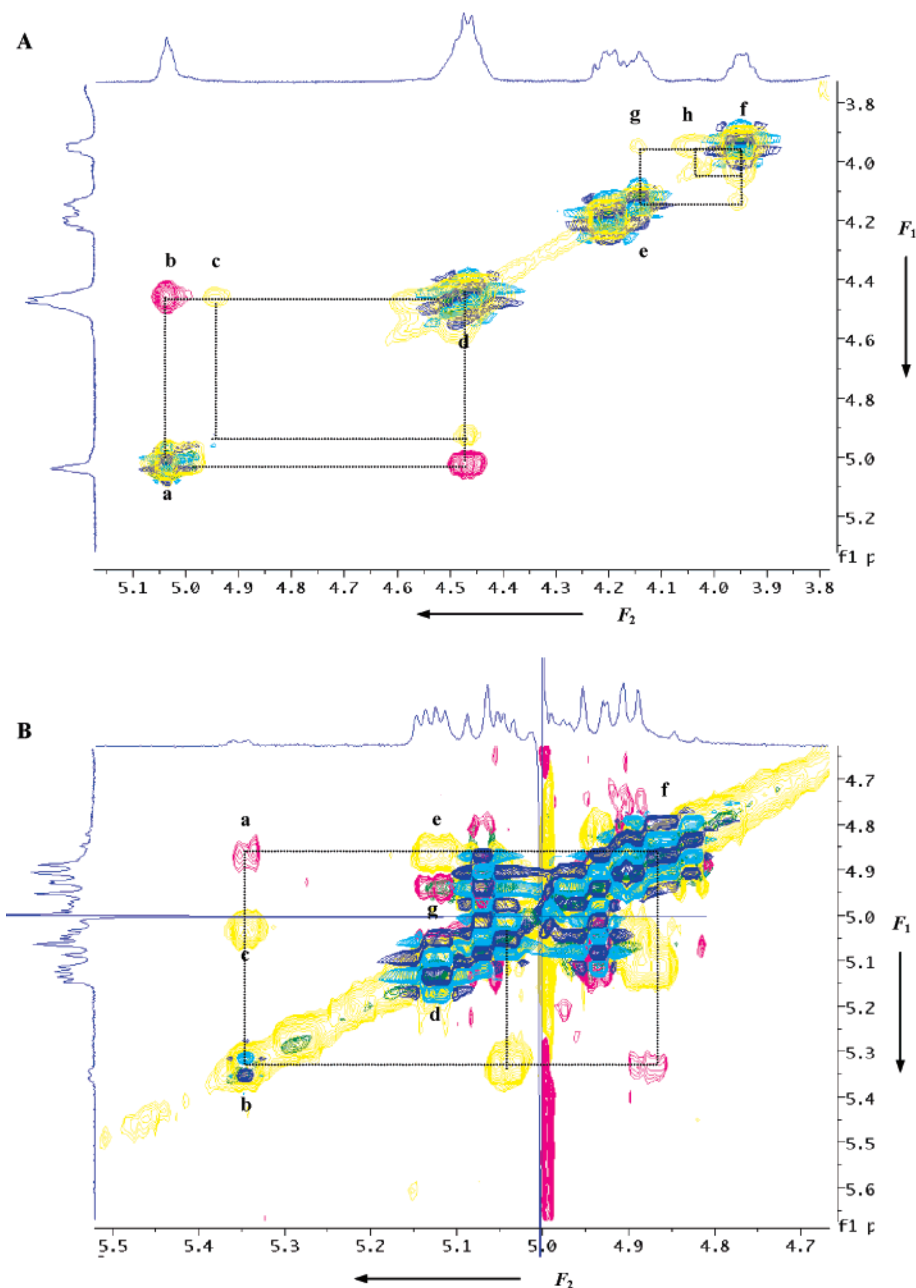

Figure 3. Expanded regions of the 2D ^{1}H NMR spectra obtained for analogues **1** and **2** superposing ROESY (pink), COSY-DQF (blue), and TOCSY (green) spectra. (A) α-Proton region of analogue **1** in DMSO-d_6. Key: a, ΨPro^{7}Hα; b, NOE Cys^{6}Hα-ΨPro^{7}Hα; c, exchange cross-peak Cys^{6}Hα (minor)-Cys^{6}Hα (major); d, Cys^{6}Hα; e, Ile^{3}Hα; f, Gln^{4}Hα (major) + Ile^{3}Hα (minor); g, exchange cross-peak Ile^{3}Hα (major)-Ile^{3}Hα (minor); and h, exchange cross-peak Gln^{4}Hα (minor)-Gln^{4}Hα (major). (B) α-Proton region of analogue **2** in D_2O. Key: a, NOE ΨPro^{7}Hα (minor)-Cys^{6}Hα (minor); b, ΨPro^{7}Hα (minor); c, exchange cross-peak ΨPro^{7}Hα (minor)-ΨPro^{7}Hα (major); d, Cys^{6}Hα (major); e, exchange cross-peak Cys^{6}Hα (major)-Cys^{6}Hα (minor); f, Cys^{6}Hα (minor); and g, Cys^{6}Hα (major)-ΨPro^{7}Hδ′ (major).

the protons from the NH region were attributed from the spectra recorded in water. The attribution from the protons in the α-region as well as the determination of the cis/trans ratio were carried out on spectra obtained in deuterated water. Here, the NH region does not show any signals due to the solvent exchange with D_2O. By combining the attributions from the TOCSY, COSY-DQF, and ROESY spectra in water and in D_2O, a complete attribution of the proton chemical shifts was obtained for both OT analogues **1** and **2**. As in DMSO, the dimethyl-ΨPro containing compound **1** was present as one major and one minor conformer in water. A strong NOE characterictic for a cis peptide bond was observed between the α-protons of residues 7 (ΨPro) and 6 (Cys). In addition, NOE cross-peaks were also observed between ΨPro^7 Hα and the Hβ protons of Cys^6. The cis content was calculated by integration of the signals from the Hα protons of Gln^4 (major) and Ile^3 (minor) that were well-isolated and yielded 95%. This value is slightly higher than the value of 92% obtained for the cis content in DMSO-d_6.

In contrast to the spectra of the dihydro compound **2** recorded in DMSO-d_6, the ROESY spectrum in deuterated water featured the expected NOE (a, in Figure 3B) between the α-proton of Cys^6 (f) and the α-proton of ΨPro^7 (b) for the minor cis conformation. The major

Table 1. ^{1}H and ^{13}C Chemical Shifts (in ppm) in DMSO-d6 for OT Analogues **1** and **2** (major conformers)

	$1H_\alpha$	$1H_\beta$	$1H_\beta'$	2NH	$2H_\alpha$	$2H_\beta$	$2H_\beta'$	$2H_\delta$	$2H_\epsilon$	2OH	$3H_\alpha$	$3H_\beta$	$3Me_\beta$	$3H_\gamma$	$3H_\gamma'$	$3H_\delta$	4NH	$4NH_2$	$4NH_2'$	$4H_\alpha$
1	3.74	3.27	2.87	8.55	4.50	3.07	2.73	7.06	6.66	9.22	4.14	1.90	0.87	1.35	1.10	0.87	8.24	7.28	6.82	3.94
2	3.48	2.90	2.66	8.08	4.58	3.15	2.70	7.09	6.66	9.18	3.88	1.78	0.89	1.50	1.17	0.88	8.12	7.28	6.80	3.98
	$4H_\beta$	$4H_\beta'$	$4H_\gamma$	5NH	$5NH_2$	$5NH_2'$	$5H_\alpha$	$5H_\beta$	$5H_\beta'$	6NH	$6H_\alpha$	$6H_\beta$	$6H_\beta'$	$7H_\alpha$	$7H_\beta$	$7H_\beta'$	7Me	7Me′	$7H_\delta$	$7H_\delta'$
1	1.85	1.85	2.16	8.10	7.34	6.93	4.45	2.52	2.52	7.68	4.46	3.07	3.07	5.04	3.33	3.33	1.79	1.79		
2	1.96	1.85	2.11	7.81	7.37	6.91	4.39	2.57	2.57	8.77	5.13	3.22	2.95	4.66	3.27	3.01			5.07	4.94
	8NH	$8H_\alpha$	$8H_\beta$	$8H_\beta'$	$8H_\gamma$	$8H_\delta$	$8H_\delta'$	9NH	$9NH_2$	$9NH_2'$	$9H_\alpha$	$9H_\alpha'$	$1C_\alpha$	$1C_\beta$	$2C_\alpha$	$2C_\beta$	$2C_\delta$	$2C_\epsilon$	$3C_\alpha$	$3C_\beta$
1	8.32	4.20	1.63	1.48	1.64	0.88	0.83	8.10	7.10	7.07	3.63	3.58	NA[a]	NA	54.7	35.7	129.6	114.4	57.3	36.1
2	8.07	4.24	1.51	1.51	1.64	0.88	0.82	7.86	7.10	7.07	3.65	3.50	54.0	43.7	53.7	36.6	129.8	114.5	58.5	35.1
	$3Me_\beta$	$3C_\gamma$	$3C_\delta$	$4C_\alpha$	$4C_\beta$	$4C_\gamma$	$5C_\alpha$	$5C_\beta$	$6C_\alpha$	$6C_\beta$	$7C_\alpha$	$7C_\beta$	7Me	7Me′	$8C_\alpha$	$8C_\beta$	$8C_\gamma$	$8C_\delta$	$8C_\delta'$	$9C_\alpha$
1	15.0	23.5	10.5	53.6	26.1	30.9	49.5	35.4	51.3	39.5	65.4	30.9	26.8	28.3	52.1	39.5	23.5	22.8	20.9	41.3
2	15.1	24.7	10.6	52.9	26.2	31.0	50.0	35.5	51.4	41.1	62.2	32.5			51.1	39.9	23.6	22.9	21.0	41.4

[a] NA, not available. Signals are hidden in the background noise due to high conformational flexibility of residue **1** indicated by enlarged proton chemical shifts for this residue.

Table 2. Overview of the Values Obtained from the Biological Evaluation of the Two OT Analogues in Rat Uterotonic and Pressor Tests and the cis Contents Obtained from NMR Investigations

	uterotonic activity in vitro (IU/mg)			% cis isomer	
compd	no Mg^{2+}	1 mM Mg^{2+}	pressoric activity (IU/mg)	DMSO-d_6	D_2O
[Cys($\Psi^{Me,Me}$pro)[7]]OT (**1**)	31.8 ± 2.0	124 ± 21	0	92	95
[Cys($\Psi^{H,H}$pro)[7]]OT (**2**)	1010 ± 88 (1180 ± 47)[a]	245 ± 43 (ND)[a]	2.4 ± 0.4 (3.46 ± 0.05)[a]	5−6 (ND)[a]	6−7 (ND)[a]
OT	450 (546 ± 18)[a,b]	450	5		10[c]

[a] Ref 14. [b] Ref 50. [c] Ref 5.

conformer showed the NOE cross-peak between the Cys[6] α-proton and the thiazolidine δ-protons characteristic for a trans conformation. The Cys[6]Hα (major)-ΨPro[7]Hδ′ (major) cross-peak is labeled g in Figure 3B. The cis/trans ratio was calculated by integration of the ΨPro[7] Hα-signal of the minor conformer and the Gly[9] Hα-signals of the major conformer, as these were the only well-isolated signals of each conformer. The population with a cis peptide bond around Cys[6]-ΨPro[7] was calculated to represent 6−7%, i.e., a slightly higher value than for the cis content found in DMSO-d_6. The last two columns of Table 2 summarize the cis contents in DMSO and water for **1**, **2**, and the parent OT.

In conclusion, the cis content obtained for the peptide bond between positions 6 and 7 of OT by introduction of 2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid at position 7 reaches about 92% in DMSO and 95% in water. The cis content determined for the dihydro compound varies from 5 to 6% in DMSO to 6−7% in water and is therefore slightly lower than in the parent peptide OT.

The oxytocic activity (uterotonic activity) of the pseudoproline-containing OT analogues was evaluated in the rat uterotonic in vitro test.[31] In this assay, the ability of the analogue to induce contractions of the uterus is measured. The test was carried out in either the absence or the presence of 1 mM magnesium. Cumulative dose−response curves were constructed using data from experiments in which the doses of analogue were added successively in 1 min intervals to the organ bath by doubling the concentration with each addition. As a most important result, the two ΨPro-containing OT analogues showed no inhibitory activity. They both expressed exclusively agonistic behavior, and their dose−response curves were similar to the curves obtained for OT regarding maximal response and slope. The activities in IU/mg of the two OT analogues calculated by comparing the threshold doses of the standard and the analogue are listed in the first two columns of Table 2.

With an activity of 31.8 IU/mg as compared to the value of 450 IU/mg for OT, the dimethyl-pseudoproline containing compound **1** shows a 14-fold decreased activity when no magnesium is present. This corresponds quite exactly to the 14-fold smaller amount of the population with a trans peptide bond between Cys[6] and Pro[7], which is known to be around 90% for OT[5] and which was found to be only 5−8% for **1** (see Table 2). The loss in activity seems therefore to be due to the 92−95% of the population that is forced into the nonactive cis conformation. In contrast, under the same conditions, the dihydro compound **2** shows superagonistic activity with 1010 IU/mg. The increase in activity is in line with an increase in the trans content going from OT to compound **2** (90−93−95%, respectively). However, this increase of the trans content is very subtle. Another reason for the high activity of **2** might be the expected decrease of the energetic barrier of cis/trans isomerization for the Cys[6]-Cys($\Psi^{H,H}$pro)[7] peptide bond,[25] indicating that a cis/trans conformational change may play a role in activation of this ligand−receptor system. The high potency of compound **2** was known from literature. Rosamond et al. reported in 1976 the synthesis of [Thz[7]]OT [Thz = Cys($\Psi^{H,H}$pro)] and an oxytocic activity of 1180 ± 47 IU/mg.[14] Within the same study, the compound [Mpa[1],Thz[7]]OT that combines two activity-increasing substitutions, the Mpa residue at position 1 and the thiazolidine residue at position 7, was shown to feature an even higher agonistic activity of 1538 ± 45 IU/mg. However, no NMR studies concerning the cis/trans isomer equilibrium around positions 6 and 7 were made at that time.

The comparison of the values for the activity in the uterotonic test with the cis/trans ratios calculated from the NMR spectra indicates that the agonistic activity is increasing proportionally with the trans content of the Cys[6]-Pro[7] peptide bond. This result is consistent with the trans conformation observed in the X-ray structure of the potent OT agonist [Mpa[1]]OT (deamino-

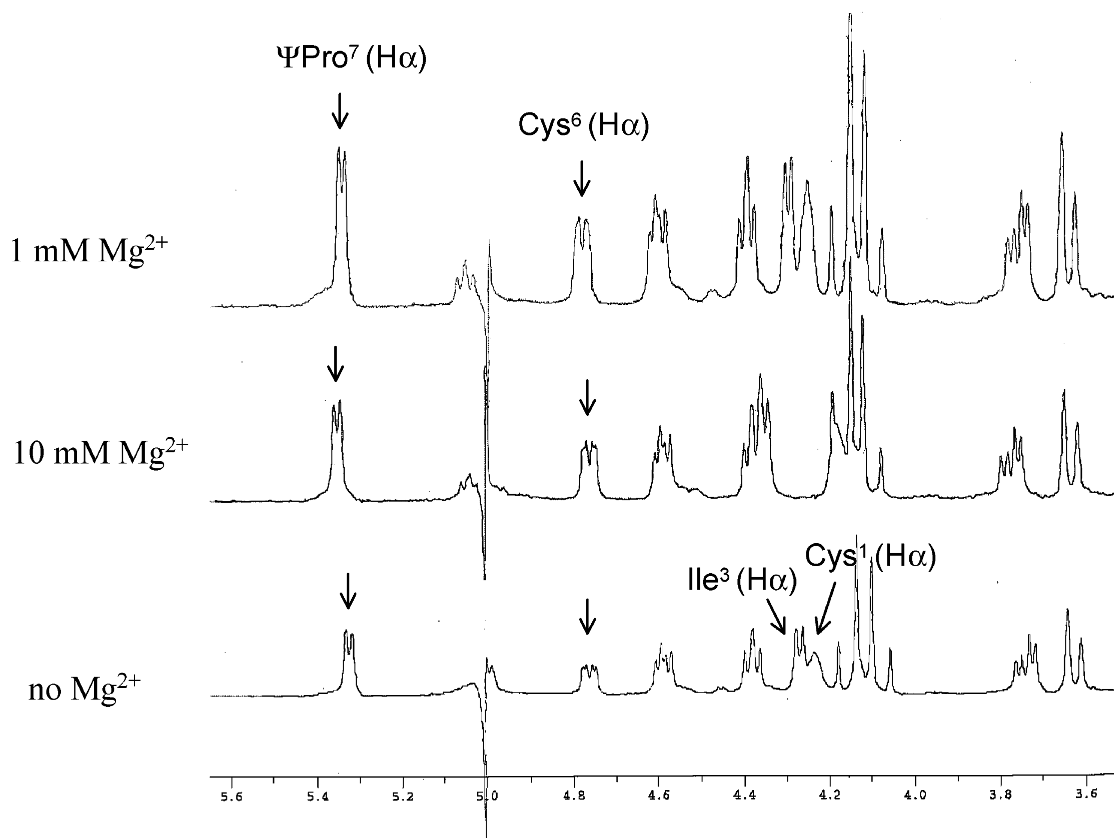

Figure 4. Hα-region of ^{1}H NMR spectra of [Cys($\Psi^{Me,Me}$pro)^7]OT (**1**) recorded in D_2O in the presence of different concentrations of $MgCl_2$. Significant signals discussed in the text are labeled.

oxytocin).[20] A trans conformation between residues 6 and 7 was also found in the crystal structure of the OT-neurophysin II complex.[32]

The presence of magnesium had quite a different effect on the less potent agonist **1** than on the more potent agonist **2**. Whereas the activity of **1** was increased by a factor 4 in the presence of 1 mM magnesium (from 31.8 to 124 IU/mg), the activity of **2** was decreased by the same factor (from 1010 to 245 IU/mg; see first two columns of Table 2). To investigate whether these changes in activities are correlated to the cis/trans ratio of the Cys^6-ΨPro^7 peptide bond geometry, the NMR experiments were repeated in the presence of 1 or 10 mM of magnesium salt. As represented in Figure 4 for the OT analogue **1**, the resonances of the Hα atoms of Cys^6 and ΨPro^7 did not show any change upon increasing the Mg^{2+} concentration from 0 to 10 equivalents. Consequently, the cis content of the Cys^6-ΨPro^7 peptide bond seems not to be altered by the presence of magnesium. However, a considerable change was observed in the pattern of the Hα protons of residues 3 and 1 (see Figure 4), indicating conformational changes that influence activity taking place here and opening up an interesting field for future investigations.

In addition to the uterotonic activity, OT analogues **1** and **2** were also tested in rats for the typical vasopressic activity, the ability to increase blood pressure. The assay was conducted by first carrying out a pretreatment to achieve a stable and low blood pressure and then registrating the increase in blood pressure after substance administration. The results obtained are listed in Table 2. No vasopressic activity was obtained for the dimethyl-pseudoproline containing compound **1**. With 2.4 IU/mg, the dihydro compound **2** showed a 2-fold decreased vasopressic activity as compared to OT. Consequently, both **1** and **2** feature a higher selectivity for the OT receptor than OT itself.

Determination of biological potencies in the rat uterotonic test in vitro was complemented by the measurement of the binding affinities of the OT analogues **1** and **2** to the rat and human OT receptors. Binding to the human OT receptor was determined in inhibition experiments using the well-characterized OT antagonist ^{125}I-d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Tyr-NH$_2$^{9}]OVT as a tracer[49] and membranes from CHO cells stably expressing human OT receptor. The OT antagonist d$(CH_2)_5$-[Tyr(Me)^2,Thr^4,Tyr-NH$_2$^{9}]OVT was used as a control. An example of the displacement binding curves obtained is shown in Figure 5. The K_i values were calculated with the Cheng and Prusoff equation[33] and yielded 1.85 nM for compound **1** and 0.13 nM for compound **2** (Table 3). As compared to a value of 1.4 nM for OT itself, the dimethyl-pseudoproline containing OT analogue **1** showed therefore a similar binding affinity to the human OT receptor, despite the two bulky methyl groups. The binding affinities to the rat OT receptor were obtained from displacement experiments using tritiated OT and membranes isolated from rat uterine tissue. Here, the K_i values obtained were slightly higher than those obtained for the human OT receptor, with 8.0, 1.5, and 3.7 nM for **1**, **2**, and OT, respectively (see Table 3). Species difference (human vs rat), variations in membrane composition (CHO cell membranes vs uterine tissue), and differences in binding mode of the tracers

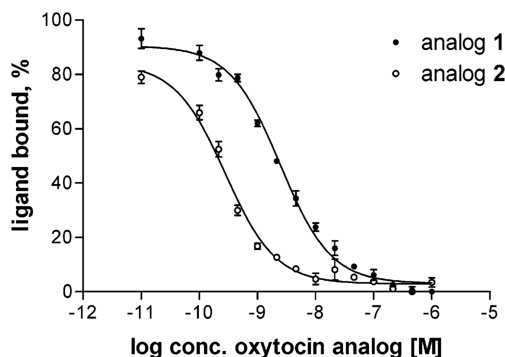

Figure 5. Displacement binding curves obtained with [Cys($\Psi^{Me,Me}$pro)[7]]OT (**1**, ●) and [Cys($\Psi^{H,H}$pro)[7]]OT (**2**, ○). The ligand was ^{125}I-d(CH_2)$_5$[Tyr(Me)[2],Thr[4],Tyr(NH_2)[9]]OVT at a concentration of 262 pM, tested on membranes from CHO cells stably expressing the human OT receptor (3.95 μg protein per assay). Each experimental point was measured in triplicate.

Table 3. Affinities of Analogues **1**, **2**, and OT to the Human and the Rat OT Receptor Obtained from Displacement Binding Assays Expressed as K_i

	affinity, K_i (nM)	
compd	human OT receptor ([^{125}I]-d(CH_2)$_5$[Tyr(Me)[2], Thr[4], Tyr-NH_2[9]]OVT)	rat OT receptor (^{3}HOT)
[Cys($\Psi^{Me,Me}$pro)[7]]OT (**1**)	1.85 ± 1.02	8.0 ± 3.7
[Cys($\Psi^{H,H}$pro)[7]]OT (**2**)	0.13 ± 0.06	1.5 ± 0.5
OT	1.4	3.7

used (antagonist vs agonist) might account for the differences between the two binding assays. However, the order of affinities, 2 > OT > 1, corresponds for both assays. Significantly, compound **1** retains its high binding affinity also for the rat OT receptor. This is in contrast to the 14-fold decreased oxytocic activity in the rat test. Because of the 95% cis content in compound **1**, these results therefore lead to the hypothesis that the cis conformation between Cys[6] and ΨPro[7] is a structural arrangement that plays a role in the initial binding step of the ligand to the receptor. A conformational change might then take place to generate the signal-triggering trans conformation. The reduced activity might be attributed to the steric hindrance introduced by the methyl groups. Such a cis/trans conformational behavior upon receptor binding and activation was proposed previously for analogues of the tripeptide hormone thyroliberin.[34] The dihydro OT analogue **2** showed a 20-fold increased binding affinity to the human OT receptor and an over 2-fold increased binding affinity to the rat OT receptor as compared to OT. This is in line with the increase in activity found for **2**. The energetic barrier for cis/trans isomerization is expected to be decreased in the ΨPro-containing analogues as compared to the proline-containing natural sequence.[25] The increased activity and binding affinity of **2** therefore support the interpretation that the ability to undergo a cis/trans conformational change is important for biological activity.

Conclusion

In conclusion, incorporation of the novel *cis*-Pro mimic 2,2-dimethyl-1,4-thiazolidine caroxylic acid at the Pro position of the hormone yielded an analogue that showed an induction of the cis conformation of 92−95% in water as well as in DMSO-d_6, as determined by one- and two-dimensional NMR spectroscopy. Comparison of the OT activities of the cis-constrained compound **1**, the dihydro-thiazolidine-containing compound **2**, and OT shows that the agonistic potency increases proportionally to the trans content of the Cys[6]-ΨPro[7]/Pro[7] peptide bond. No antagonistic activity was observed for the cis-constrained analogue **1**, restricting the possibility that a cis conformation might be necessary for antagonism and thus resolving a question of long standing in structure−activity relationship studies of this hormone. On the other hand, the cis-constrained analogue **1** retains a high binding affinity to both the human and the rat OT receptors. Analogue **2** shows an even higher binding affinity to the human and rat uterine receptor. Combined with the high activity of compound **2**, the high binding affinities of the pseudoproline-containing OT analogues therefore lead to the hypothesis that a cis/trans conformational change is playing a role in OT receptor binding and activation.

Experimental Section

Abbreviations. COSY-DQF, double quantum filtered correlation spectroscopy; DCM, methylene chloride; DIPEA, N-ethyldiisoprolylamine; DMF, dimethylformamide; DTT, dithiothreitol, 1,4-dimercapto-2,3-butanediol; Fmoc, fluorenylmethyloxycarbonyl; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; PyBOP, benzotriazole-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; ROESY, rotating-frame Overhauser effect spectroscopy; TIS, triisopropylsilane; TFA, trifluoroacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TOCSY, total correlation spectroscopy; Trt, trityl.

Materials and Methods. All reagents employed were of analytical grade and were purchased from Fluka (Switzerland), Sigma-Aldrich Chemie GmbH (Germany), and Acros (Belgium). DCM was distilled over CaH_2, and DMF was degassed with nitrogen before use. Fmoc-protected amino acid derivatives were obtained from Calbiochem-Novabiochem (Switzerland), Bachem Fine Chemicals (Switzerland), Alexis (Switzerland), or Isochem (France). Coupling reagents were purchased from Calbiochem-Novabiochem (Switzerland). Acetonitrile for analytical and preparative HPLC was obtained from Biosolve B. V. (Netherlands). Water used for HPLC was Milli-Q quality, collected after passing through a Milli-Q purification system (MA). Trifluoroacetic acid used for HPLC was purchased from Baker AG (Switzerland), whereas NMR solvents were obtained from Dr. Glaser AG (Switzerland). Sieber amide resin was purchased from Calbiochem-Novabiochem (Switzerland).

Analytical RP-HPLC spectra were recorded on a Waters apparatus consisting of two Waters 600 pumps, a Waters 600 System Controller, and a Waters 486 tunable absorbance detector, used with a Vydac C18218TP54 column (250 mm × 4.6 mm), and connected to a printer Waters 746. Flow rates of 1 mL/min were used, and the UV absorbance was monitored at 214 nm. All gradients were linear in eluent A (0.09% TFA in water) and eluent B (0.09% TFA in 90% aqueous acetonitrile). Semipreparative and preparative HPLC purifications were performed on a Waters Delta Prep 3000 System, with a Waters 600E System Controller and a Waters 484 absorbance detector, and equipped with a Vydac C18218TP1002 column (2.2 cm × 25 cm). Flow rates used were 18 and 80 mL/min, respectively, and the UV absorbance was monitored at 214 nm. The same eluents and gradients as for analytical HPLC were used. ESI mass spectra were recorded on a Finnigan MAT SSQ 710C electrospray mass spectrometer equipped with an IBM PS1295XP486 (software Technivent Vector II) in positive ionization mode.

NMR Measurements. All NMR spectra were recorded on a DRX400 Bruker spectrometer at 30 °C. The products were

characterized by two-dimensional ROESY experiments[35] (mixing time, 200 ms), homonuclear Hartman-Hahn (HOHAHA, TOCSY) experiments[36] (mixing time, 100 ms), and COSY-DQF[37] in the phase sensitive mode using the time proportional phase incrementation method. For the experiments in D_2O, the carrier frequency was set on the HDO signal. The residual water signal was suppressed by presaturation during a relaxation delay of 1.0−3.0 s. For 1D experiments in H_2O, excitation sculpting was used for water suppression.[38] A total of 2K data points were collected in the F_2 dimension with a spectral width of 4000 Hz. In the F_1 dimension, 512 or 1K points were measured. The data were processed using the SwaN-MR software.[39] A zero-filling in the F_1 dimension and a square sine-bell window shifted by 90° in both dimensions were applied prior to the two-dimensional Fourier transformation. In the case of COSY-DQF, a square sine-bell window shifted by 0° was applied in F_1.

Fmoc-Cys(StBu)-Cys($\Psi^{Me,Me}$pro)-OH. To a solution of 2.0 g (10.1 mmol) of 2,2-dimethyl-L-thiazolidine-4-carboxylic acid hydrochloride [H-Cys($\Psi^{Me,Me}$pro)-OH*HCl][40] in 200 mL of dry methylene chloride, 2.90 g (6.7 mmol) of Fmoc-Cys(StBu)-F (prepared following the description in ref 29) and 2.99 g (2.2 eq., 23 mmol) of DIPEA were added. The reaction mixture was stirred during 2 h at room temperature under an argon atmosphere and then washed with citric acid 5% and brine. The organic layer was dried over magnesium sulfate and filtered, and the solvent was removed under reduced pressure at room temperature. The product was used without further purification as a building block in the solid phase synthesis. Yield: 3.39 g (5.9 mmol, 95%). $C_{28}H_{34}N_2O_5S_2$ (542.0). RP-HPLC (C_{18}, 214 nm, gradient 0−100% B in 30 min): R_t = 22.42 min. ESI-MS: m/z = 543.1 $[M + H]^+$.

Fmoc-Cys(StBu)-Cys($\Psi^{H,H}$pro)-OH. L-Thiazolidine-4-carboxylic acid [H-Cys($\Psi^{H,H}$pro)-OH] was purchased from Fluka. To a suspension of 1.34 g (10.1 mmol) of l-thiazolidine-4-carboxylic acid in 200 mL of dry methylene chloride/dry dimethylformamide (1:1), 2.90 g (6.7 mmol) of Fmoc-Cys(StBu)-F and 2.99 g (2.2 eq., 23 mmol) of DIPEA were added. The reaction mixture was heated at 60 °C in order to dissolve the products and stirred during 2 h under an argon atmosphere. After letting the solution cool, the main part of the solvents was evaporated and the residual material was taken up in methylene chloride. The solution was washed four times with citric acid 5% and three times with a saturated solution of sodium chloride. The organic layer was dried over magnesium sulfate and filtered, and the solvent was removed under reduced pressure at room temperature. The product was used without further purification as a building block in the solid phase synthesis. Yield: 2.98 g (5.5 mmol, 82%). $C_{26}H_{30}N_2O_5S_2$ (513.8). RP-HPLC (C_{18}, 214 nm, gradient 0−100% B in 30 min): R_t = 20.02 min. ESI-MS: m/z = 514.3 $[M + H]^+$.

Peptide Synthesis. The solid phase peptide synthesis was conducted using the Fmoc/tBu strategy,[41] working on Sieber amide resin.[42] The peptide synthesis reaction vessels were silylated with toluene/20% dichlorodimethylsilane for 1 h or overnight, then washed once with toluene, once with methanol, and several times with DCM before adding the resin. The resin was swollen twice during 15 min in DCM before starting the synthesis. In a typical coupling step, 2 equivalents of the Fmoc-protected amino acid derivative were reacted with 2 equivalents of PyBOP and 4 equivalents of DIPEA in DMF (10 mL/g of resin) for 1 h. After each coupling step, the resin was washed twice with DMF and a TNBS test was carried out. Fmoc deprotection was achieved with 20% piperidine in DMF (20 mL/g of resin, 1 × 5 min, 2 × 10 min), and then, the resin was washed three times with DMF. The pseudoprolines were introduced as preformed dipeptide building blocks. The reaction time for the coupling of the dipeptide building blocks, Fmoc-Cys(StBu)-Cys($\Psi^{Me,Me}$pro)-OH and Fmoc-Cys(StBu)-Cys($\Psi^{H,H}$pro)-OH, was increased to 2 h. The Trt protecting group was chosen for the N-terminal cysteine, as well as for the asparagine and for the glutamine. None of the coupling reactions had to be repeated, as indicated by negative TNBS tests. The peptides were cleaved from the resin by treatment with a solution of 1% TFA/1% TIS in DCM.

[Cys($\Psi^{Me,Me}$pro)]^{7}OT (1). After cleavage from the resin, acid labile protecting groups were removed by treatment of the crude material with 50 mL of a mixture of 80% TFA, 15% MeOH, 2.5% TIS, and 2.5% water. The solution was stirred for 15 min, and then, the solvents were evaporated, and the product was washed with diethyl ether and dried. The crude product contained a majority of the desired product, as well as a minor part of the product with the thiazolidine ring cleaved. For cleavage of the S*t*Bu protecting group, 200 mg (0.175 mmol) of the crude product was dissolved in 3 mL of acetonitrile/ammoniumcarbonate buffer, pH 7.5 (3:2, v/v), and the solution was degassed with argon for 10 min. Then, 20 equivalents of dithiothreitol (DTT, 3.498 mmol, 540 mg) was added in 1 mL of ammoniumcarbonate buffer, pH 7.5, and the solution was stirred under an argon atmosphere. The reduction was followed by HPLC analysis and mass spectrometry and was complete after 1 h. The solution was acidified to a pH of 5−6 with acetic acid and lyophilized. From purification by preparative RP-HPLC (C_{18}, gradient 30−60% B in 30 min) and after lyophilization, 63 mg of the desired product as well as 25 mg of the product with the thiazolidine ring cleaved, but still S*t*Bu-protected in position 6, could be isolated. Yield (after S*t*Bu deprotection and purification): 63 mg (60.7 μmol, 87%). Air oxidation of the linear pure peptide was achieved by stirring 20 mg (19.0 μmol) in 100 mL of ammoniumhydrogencarbonate buffer 0.1 M (c = 0.2 mg/mL) overnight at open atmosphere and room temperature. The oxidation was followed by HPLC analysis and mass spectrometry. The solution was then lyophilized, and the salt-containing product was dialyzed in a Spectra/Por CE (Cellulose Ester) sterile DispoDialyzer (MWCO 500, diameter 10 mm, sample volume 5 mL) by Spectrum against bidistilled water (3 × 1 h, 1 × 12 h). Lyophilization yielded 12 mg (11.4 μmol, 60%) of **1**. $C_{44}H_{68}N_{12}O_{12}S_3$ (1052.4). Overall yield: 36%. RP-HPLC (214 nm, gradient 5−100% B in 30 min): R_t = 15.64 min (C_{18}), 9.44 (C_4). ESI-MS: m/z = 1053.3 $[M + H]^+$, 527.4 $[M + 2H]^{2+}$. ^1H and ^{13}C NMR spectroscopy (1D, TOCSY, COSY-DQF, ROESY, HSQC) at 400 MHz in DMSO-d_6: see Table 1.

[Cys($\Psi^{H,H}$pro)]^{7}OT (2). The material obtained after cleavage from the resin was reacted with a mixture of 95% TFA, 2.5% of TIS, and 2.5% of water for 30 min, washed with ether, and lyophilized. Subsequently, the peptide was treated as described above for removal of the S*t*Bu protecting group, purification, and air oxidation. $C_{42}H_{64}N_{12}O_{12}S_3$ (1024.4). Overall yield: 15%. RP-HPLC (214 nm, gradient 5−100% B in 30 min): R_t = 13.99 min (C_{18}), 7.93 (C_4). ESI-MS: m/z = 1025.9 $[M + H]^+$, 513.8 $[M + 2H]^{2+}$. ^1H and ^{13}C NMR spectroscopy (1D, TOCSY, COSY-DQF, ROESY, HSQC) at 400 MHz in DMSO-d_6: see Table 1.

Biological Evaluations. Peptides were tested for uterotonic activity in vitro in the rat uterotonic test according to Holton[31] in Munsick[43] solution either in the absence of magnesium or in the presence of 1 mM magnesium in the bathing solution. Synthetic OT was used as a standard. Female rats were estrogenized 24−48 h before the experiment. Cumulative dose−response curves were constructed using data from experiments in which doses were added successively to the organ bath in doubling concentrations and in 1 min intervals without the fluid being changed until the maximal response was obtained. The activity in IU/mg was calculated by comparing the threshold doses of the standard and the analogue. Each analogue was tested on uteri from 4 to 5 different rats.

The pressor activity was determined on phenoxybenzamine-treated rats.[44] In short, male rats (220−260 g) were anaesthetized using urethane and their vena femoralis and arteria carotis were cannulated for drug administration and blood pressure determination, respectively. Then, phenoxybenzamine was administered in two doses. After pressure stabilization, usually after 30 min, bolus doses of standard or tested substance were administered in random order. When antagonistic activity was expected, a standard dose of vasopressin

was administered 1 min after the administration of the test substance. Each analogue was tested on 3−4 animals. A more detailed description of these protocols can be found in ref 45.

Binding affinities to the rat OT receptor were determined as described in refs 46 and 47 using tritiated OT from NEN Life Science (Boston, MA). In brief, a crude membrane fraction from rat uteri was incubated with $[^3H]OT$ (2 nM) and various concentrations of peptides (0.1−10000 nM) for 30 min at 35°C. The total volume of the reaction mixture was 0.25 mL. Incubation medium consisted of HEPES (50 mM, pH 7.6), $MnCl_2$ (10 mM), and bovine serum albumin (BSA, 1 mg/mL). The reaction was terminated by quick filtration on a Brandel cell harvester. OT was used as a control and for determination of nonspecific binding. Binding affinities were expressed as K_i values calculated according to the expression $K_i = IC_{50}/[(c_{3HOT}/K_{dOT}) + 1]$, where K_{dOT} is taken as 1.8 nM.[48]

Binding affinities to the human OT receptor were obtained using membranes of CHO cells stably expressing the human OT receptor and the radioiodinated OT antagonist ^{125}I-d$(CH_2)_5$-[Tyr(Me)^2,Thr^4,Tyr$(NH_2)^9$]OVT as a tracer.[49] The assay was carried out in a medium constituted of Tris-HCl (50 mM), $MgCl_2$ (5 mM), and BSA (1 mg/mL) at 30 °C for 60 min in borosilicate tubes in a total volume of 200 μL. In a typical assay, 3.95 μg of protein, 262 pM radioligand, and the analogue at variable concentrations were used. After the incubation, the samples were filtered on a glass fiber filter and the filter was counted. Each concentration was made in triplicate. The OT antagonist d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Tyr$(NH_2)^9$]OVT was used as a control and for the determination of nonspecific binding. The K_i values were calculated as described above.

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