Articles

A Study of the Relationship between Biological Activity and Prolyl Amide Isomer Geometry in Oxytocin Using 5-*tert*-Butylproline To Augment the Cys[6]-Pro[7] Amide *Cis*-Isomer Population

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Three [5-*t*-BuPro[7]]oxytocin analogues were synthesized by substituting (2*S*,5*R*)-5-*tert*-butylproline for proline in oxytocin, [Mpa[1]]oxytocin, and [dPen[1]]oxytocin. Relative to oxytocin, [5-*t*-BuPro[7]]oxytocin and [Mpa[1],5-*t*-BuPro[7]]oxytocin exhibited strongly reduced binding affinity to the receptor; however, both peptides maintained the pharmacophore characteristics responsible for signal transfer evoking the same maximal response as oxytocin in the single-dose procedure and exhibiting partial agonistic activity in the cumulative dose−response procedure. Although [dPen[1]]oxytocin exhibited inhibitory as well as partial agonistic activity, [dPen[1],5-*t*-BuPro[7]]-oxytocin exhibited only inhibitory potency with a similar in vitro pA_2 value of 7.50 in the absence of magnesium. In the presence of magnesium, [dPen[1],5-*t*-BuPro[7]]oxytocin exhibited stronger inhibitory potency than [dPen[1]]oxytocin and no partial agonism. Assignment of the proton signals for the 5-*tert*-butylprolyl amide *cis*- and *trans*-isomers by two-dimensional NMR experiments in water indicated that the Cys[6]-Pro[7] peptide bond *cis*-isomer population was augmented relative to the prolyl peptides and measured respectively at 35%, 33%, and 20% in the 5-*tert*-butylproline[7] analogues of oxytocin, [Mpa[1]]oxytocin and [dPen[1]]oxytocin. Although caution must be taken when relating the increase in *cis*-isomer population with an influence on biological activity in [5-*t*-BuPro[7]]oxytocin analogues, the synthesis and evaluation of analogues **1**−**3** have provided additional evidence that can be used to support the hypothesis that the prolyl amide *cis*-isomer may favor antagonism and the *trans*-isomer is necessary for agonist activity.

Introduction

Isomerization about the amide N-terminal to prolyl residues can produce multiple conformers in biologically active peptides which can complicate their characterization. Because the rational design of therapeutics based on peptide lead structures requires detailed knowledge of their spatial requirements for biological activity, conformationally rigid prolyl amide surrogates have emerged as important tools for probing the relationship between amide geometry and peptide bioactivity.[1] We report now the first employment of 5-*tert*-butylproline to explore the conformational requirements for prolyl peptide bioactivity. Specifically, (2*S*,5*R*)-5-*tert*-butylproline has been used to augment the Cys[6]-Pro[7] peptide bond *cis*-isomer population in analogues of the neurohypophyseal peptide hormone oxytocin.

Oxytocin is a nonapeptide composed of a six-amino acid macrocyclic disulfide body appended to a three-amino acid tail by the Cys[6]-Pro[7] peptide bond (Figure 1).[2,3] As the most potent stimulator of uterine contractions and milk ejection, oxytocin is used clinically to stimulate rhythmic contractions of the uterus, to increase the frequency of existing contractions, and to ease breast feeding.[4] On the other hand, potent peptide and non-peptide inhibitors of oxytocin action have been synthesized with the promise of using such antagonists for arresting preterm labor.[5,6] Since the Nobel Prize distinguished studies of du Vigneaud,[7,8] numerous oxytocin analogues have been studied in an important effort to elucidate its biological function and to understand

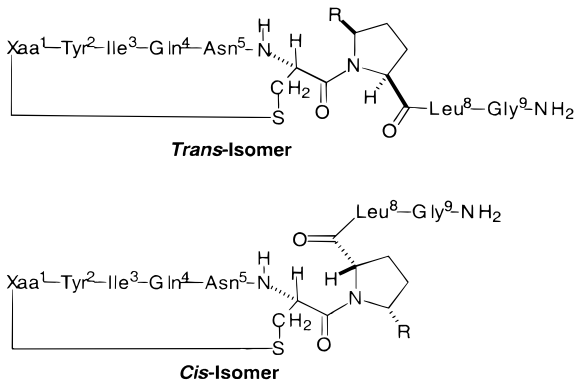

Figure 1. Prolyl amide isomers of oxytocin (Xaa = Cys, R = H), as well as analogues **1** (Xaa = Cys, R = *t*-Bu), **2** (Xaa = Mpa, R = *t*-Bu), and **3** (Xaa = dPen, R = *t*-Bu).

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the requirements for peptide−receptor recognition and signal transduction.[9]

The existence of an amide isomer equilibrium about the Cys6-Pro7 peptide bond in oxytocin was first demonstrated by one and two-dimensional NMR experiments which detected a 10% *cis*-isomer population in water that was somewhat less in methanol.[10,11] A significantly faster rate for rotation about the Cys6-Pro7 peptide bond was also observed in methanol,[11] suggesting that isomerization proceeded via a less polar twisted amide transition state.[12,13] Furthermore, a comparison with the kinetic and thermodynamic parameters for prolyl amide isomerization in other proline-containing peptides led to the conclusion that the amide *trans*-isomer in oxytocin was not stabilized by intramolecular interactions, such as hydrogen bonding between the tripeptide tail and macrocyclic hexapeptide ring.[11] Prior to these studies, the amide *cis*-isomer had not been observed in related proline7-oxytocin analogues; however, 14% and 25% *cis*-isomer populations were respectively observed in sarcosyl7- and *N*-methylalanyl7-oxytocin which exhibited similar and 7-fold reduced uterotonic activity relative to the parent peptide.[14,15] In addition, ^{1}H NMR spectroscopic studies showed that the amide *cis*- and *trans*-isomer populations were nearly equal in the inactive C-terminal tetrapeptide, *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide in DMSO.[16]

The importance of the Cys6-Pro7 amide *cis*-isomer to the biological activity of oxytocin was not seriously considered until the synthesis and conformational analysis of the bicyclic analogue [Mpa1,cyclo(Glu4,Lys8)]oxytocin.[17−19] This peptide and its related analogue [dPen1,-cyclo(Glu4,Lys8)]oxytocin[19] both acted as potent oxytocin antagonists and both possessed a Cys6-Pro7 amide *cis*-isomer due to constraints created by the additional macrocyclic lactam. Furthermore, the antagonist activity of the bicyclic analogue was in sharp contrast to its monocyclic counterpart, [Mpa1,Glu4,Lys8]oxytocin, which was a very weak agonist. In addition to the Cys6-Pro7 amide *cis*-isomer, the bicyclic antagonists possessed β-turns centered at Tyr2 and Ile3 which differed from the β-turn geometry centered at residues 3 and 4 that had been previously ascribed to the conformation of oxytocin agonists.[20] Despite this and other geometrical features present in the potent bicyclic antagonists, the conformation of the Cys6-Pro7 amide has raised the intriguing possibility that the *cis*-isomer may favor antagonism and the *trans*-isomer is necessary for agonist activity.[20]

Although the rigid bicyclic analogues support the importance of the Cys6-Pro7 amide *cis*-isomer for antagonism, additional approaches to generate constrained amide isomers are needed in order to probe the relationship between prolyl peptide geometry and activity at the oxytocin receptor. In model studies with *N*-(acetyl)dipeptide *N*′-methylamides, we showed that the *cis*-amide isomer was preferably adopted up to 90% in (2*S*,5*R*)-5-*tert*-butylprolyl peptides in contrast to the natural proline analogues which preferred the *trans*-amide isomer in solution.[21,22] We have thus begun to investigate the relationship between prolyl amide geometry and bioactivity at the oxytocin receptor by the synthesis of a series of 5-*tert*-butylproline7-oxytocin analogues, because the steric interactions between the 5-*tert*-butyl substituent and the N-terminal residue should disfavor the *trans*-isomer and increase the *cis*-isomer population.[1] Proline was replaced by (2*S*,5*R*)-5-*tert*-butylproline in the native peptide, the potent agonist [Mpa1]oxytocin, and the potent antagonist [dPen1]-oxytocin (Figure 1).[23,24] We report now the synthesis of these three oxytocin analogues, their biological activities, and their Cys6-Pro7 amide isomer equilibria in water.

Experimental Section

General Methods. Unless otherwise noted, all reactions were run under nitrogen atmosphere and distilled solvents were transferred by syringe. Acetonitrile and dichloromethane were distilled from CaH_2; diisopropylethylamine (DIEA) was distilled first from ninhydrin then from CaH_2; piperidine was distilled from KOH. Final reaction mixture solutions were dried over Na_2SO_4. Chromatography was performed on 230−400 mesh silica gel and TLC on aluminum-backed silica plates. Mass spectral data, LRMS and HRMS (EI and FAB), were obtained by the Université de Montréal Mass Spectroscopy facility. Amino acid analysis was performed on samples, after hydrolysis with 6 M HCl at 105 °C for 20 h, on a D-500 Durrum Analyzer. Analytical reverse-phase HPLC was performed on a Vydac C_{18}, 10-μm × 25-cm × 4.6-mm analytical column using a gradient of B = CH_3CN containing 0.06% TFA in A = 0.06% TFA in H_2O, and retention times (t_R) are reported in minutes. Preparative reversed-phase HPLC was performed on a Waters Delta Prep 3000 RP-HPLC (Waters PrepPAK C18 cartridge 33 × 6 cm, particle size 15 μm): solvent A, H_2O (0.06% TFA); solvent B, 75% CH_3CN/H_2O (0.06% TFA).

NMR Measurements. ^{1}H and ^{13}C NMR experiments were performed on Bruker DMX600 and ARX400 spectrometers. The chemical shifts are reported in ppm (δ units) downfield of the internal tetramethylsilane ($(CH_3)_4Si$). Coupling constants are in Hz. The chemical shifts for the carbons and protons of minor isomers are respectively reported in parentheses and brackets. COSY, TOCSY and ROESY spectra were obtained on 3.0 mM samples in 9:1 H_2O:D_2O and 3.5 mM samples in D_2O at pH 6.5 without added buffer. Spectra were measured at 30 °C with 2048 by either 512 or 1024 data points with mixing times of 70 and 107 ms for TOCSY spectra and 250 and 500 ms for ROESY spectra.[25] At 15 °C in D_2O, ROESY spectra were obtained with 2048 by either 320 or 400 data points and a 250-ms mixing time.

***N*-Fmoc-*S*-trityl-L-cysteinyl-(2*S*,5*R*)-5-*tert*-butylproline Allyl Ester (5).** A solution of *N*-Fmoc-*S*-trityl-L-cysteine (2.60 g, 4.43 mmol, 130 mol %) in CH_2Cl_2 (15 mL) at 0 °C was treated with BOP-Cl[26−28] (1.13 g, 4.43 mmol, 130 mol %) and DIEA (1.32 mL, 7.50 mmol, 220 mol %) followed by a solution of (2*S*,5*R*)-5-*tert*-butylproline allyl ester hydrochloride (**4**; 846 mg, 3.41 mmol, prepared according to ref 21) in CH_2Cl_2 (15 mL). The mixture was stirred for 12 h maintaining the reaction temperature between 0 and 4 °C. The volatiles were removed by evaporation on a rotary evaporator and the residue was partitioned between EtOAc (30 mL) and brine (30 mL). The aqueous phase was extracted with EtOAc (2 × 30 mL), and the organic phases were combined and washed with HCl (2 × 20 mL, 1 N), $NaHCO_3$ (2 × 20 mL, 5% solution) and brine (1 × 20 mL), dried, filtered and evaporated to a residue that was purified by chromatography on silica gel using a gradient of 0−20% EtOAc in hexanes. Evaporation of the collected fractions gave 2.02 g (76% yield) of *N*-Fmoc-*S*-trityl-L-cysteinyl-(2*S*,5*R*)-5-*tert*-butylproline allyl ester (**5**) as an oil: $[\alpha]^{20}_D$ −28.3 (*c* 1.2, MeOH); TLC R_f = 0.3 (1:4 EtOAc:hexane); ^{1}H NMR δ ($CDCl_3$) 0.86 (s, 5.8 H), [0.94 (s, 3.2 H)], 1.69−2.30 (m, 4 H), 2.49−2.70 (m, 2H), [4.08−4.16 (m, 0.46 H)], 4.20 (t, 2 H, 7.1), 4.28 (dd, 2 H, 10.7, 18.1), 4.45 (dd, 1 H, 7.7, 16.5), 4.52−4.61 (m, 2 H), 4.62−4.72 (m, 0.54 H), 5.08−5.32 (m, 2 H), 5.46 (d, 0.56 H, 8.5), [4.82 (d, 0.44 H, 9.2)], 5.79−5.91 (m, 1 H), 7.20 (t, 3 H, 5.4), 7.26 (dd, 8 H, 9.0, 17.0), 7.38 (m, 8 H), 7.56 (m, 2 H), 7.73 (m, 2 H); ^{13}C NMR δ ($CDCl_3$) 5.8, (28.4), 27.4, (27.5), 35.1, (33.9), 36.1, (35.7), 47.0, 51.0, (51.7), 60.0, (60.5), (65.3),

66.3, 66.8, 66.9, (118.2), 118.9, 119.8, (119.9), 125.1, (125.2), (126.7), 126.8, 127.0, 127.6, (127.6), (127.9), 128.0, 129.4, (129.5), (131.5), 131.8, (141.1), 141.2, (143.5), 143.9, 144.4, (143.7), 154.8, (155.7), 171.0, (171.4), (171.8), 172.3; HRMS calcd for $C_{49}H_{51}N_2O_5S_1$ (MH^+) 779.3519, found 779.3476.

***N*-Fmoc-*S*-trityl-L-cysteinyl-(2*S*,5*R*)-5-*tert*-butylproline (6).** A solution of *N*-Fmoc-*S*-trityl-L-cysteinyl-(2*S*,5*R*)-5-*tert*-butylproline allyl ester (**5**; 1.79 g, 2.30 mmol) in freshly distilled and degassed CH_2Cl_2 (23 mL) was treated with $Pd(PPh_3)_2Cl_2$ (82 mg, 115 μmol, 5 mol %), followed by a solution of Bu_3SnH (3.75 mL, 13.9 mmol, 600 mol %) in CH_2Cl_2 (2−3 mL) which was added dropwise until analysis by TLC showed complete consumption of the allyl ester (R_f = 0.24, 1:24:75 AcOH:EtOAc:hexanes) after 10−15 min. The mixture was evaporated to a residue that was partitioned between hexanes (25 mL) and CH_3CN (25 mL) containing acetic acid (1.2 mL). The CH_3CN phase was washed with hexanes (4 × 25 mL), evaporated and purified by chromatography on silica gel using a gradient of 0−2% ethanol in $CHCl_3$. Evaporation of the collected fractions gave 1.69 g (96% yield) of **6** as a foam: $[\alpha]^{20}_D$ −26.2 (*c* 0.6, MeOH); TLC R_f = 0.3 (1:24:75 AcOH:EtOAc:hexanes); ^{1}H NMR δ ($CDCl_3$) 0.88 (s, 9 H), 1.53−1.90 (m, 2 H), 1.93−2.24 (m, 2 H), 2.39−2.67 (m, 2 H), 4.02−4.40 (m, 4.5H), 4.45−4.57 (m, 1 H), 4.62 (dd, 0.5 H, 7.8, 13.2), 4.87 (d, 0.57 H, 8), [5,63 (s, 0.43 H)], 7.23 (m, 3 H), 7.28 (m, 8H), 7.40 (m, 8H), 7.55 (m, 2 H), 7.75 (m, 2H); ^{13}C NMR δ ($CDCl_3$) 25.4, (25.5), 26.1, (26.7), (27.2), 27.3, 27.7, (29.6), 33.4, (34.0), 35.2, (35.9), (46.8), 46.9, 51.0, (52.3), (60.5), 62.0, 66.9, (67.2), (67.4), 68.0, (119.8), 120.0, 124.8, 125.0, (125.0), (125.2), (126.9), 127.0, 127.7, (127.8), 128.0, 129.4, 141.2, (141.3), (143.3), 144.1, 155.6, 171.3, 175.3; LRMS calcd for $C_{46}H_{47}N_2O_5S_1$ (M^+) 738.3, found 761.1 (MNa^+) and 777.1 (MK^+); HRMS calcd for $C_{46}H_{47}N_2O_5S_1$-Na (MNa^+) 761.3025, found 761.3021.

***S*-(Trityl)mercaptopropionic acid (Trt-Mpa)** was prepared according to the literature procedure for the tritylation of cysteine.[29] A solution of mercaptopropionic acid (0.3 mL, 3.46 mmol, 100 mol %), triphenylmethanol (900 mg, 3.46 mmol, 100 mol %; recrystallized from Et_2O:hexanes), glacial acetic acid (3.36 mL, 58.8 mmol, 1700 mol %) and boron trifluoride etherate (0.48 mL, 3.8 mmol, 110 mol %) was stirred for 1 h at 100 °C and for an additional hour at 22 °C. The mixture was treated with Et_2O (6 mL) and water (5 mL) followed by sodium acetate (1.5 g) and additional water (20 mL). A white precipitate was formed that was filtered and rinsed with water to give 464 mg (1.33 mmol, 39% yield) of *S*-(trityl)mercaptopropionic acid: ^{1}H NMR δ ($CDCl_3$) 2.24 (t, 2 H, 7.3), 2.47 (t, 2 H, 6.9), 7.20−7.44 (m, 15 H); ^{13}C NMR δ ($CDCl_3$) 26.4, 32.9, 67.0, 126.6, 127.8, 129.4, 144.4, 175.5.

***S*-(*p*-Methoxybenzyl)-*β*,*β*-dimethylmercaptopropionic acid (*p*-MeOBn-dPen)** was prepared by a modification of the literature procedure for the synthesis of *S*-(*p*-methylbenzyl)-*β*,*β*-dimethylmercaptopropionic acid.[18] A solution of 3,3-dimethylacrylic acid (1.4 g, 14 mmol, 100 mol %) and 4-methoxy-α-toluenethiol (1.8 mL, 14 mmol, 100 mol %) in piperidine (2.1 mL, 21 mmol, 150 mol %) was heated at a reflux for 16 h, cooled to room temperature and treated with 2 N HCl (20 mL). The mixture was extracted with Et_2O (3 × 20 mL) and the combined Et_2O fractions were extracted with saturated $NaHCO_3$ (3 × 20 mL). The combined aqueous fractions were acidified to pH ≈ 2 with 2 N HCl (25 mL) and extracted with Et_2O (3 × 15 mL). The organic phases were combined, dried and evaporated to a residue that was purified by crystallization in Et_2O:hexanes (1:1) to give 1.36 g (5.3 mmol, 38% yield) of *S*-(*p*-methoxybenzyl)-*β*,*β*-dimethylmercaptopropionic acid: ^{1}H NMR δ ($CDCl_3$) 1.50 (s, 6H), 2.67 (s, 2 H), 3.79 (s, 3 H), 6.84 (d, 2 H, 8.5), 7.27 (d, 2H, 8.4); ^{13}C NMR δ ($CDCl_3$) 28.6, 32.6, 43.7, 46.8, 55.1, 113.9, 129.3, 130.0, 158.5, 176.9; HRMS calcd for $C_{13}H_{17}O_3S_1$ (M^+ − H) 253.0898, found 253.0908.

Preparation of Solid Support. Benzhydrylamine resin hydrochloride (2 g, loading ≈ 0.51 mmol/g, 1% DVB, 100−200 mesh) was washed for 1 min three times with 10 mL/g of each of the following reagents: 5% DIEA/CH_2Cl_2; CH_2Cl_2; DMF. The resin was treated with a solution of *N*-(Fmoc)aminocaproic acid (400 mg, 100 mol %), TBTU (980 mg, 300 mol %), DIEA (1 mL, 600 mol %), and HOBt (410 mg, 300 mol %) in DMF (20 mL, 10 mL/g of resin) and agitated for 1 h when a negative ninhydrin test was observed.[30] The resin was sequentially washed with 10 mL/g of the following solutions: DMF (2 × 2 min), piperidine in DMF (20% v/v, 1 × 2 min, 1 × 3 min and 1 × 10 min) and DMF (4 × 2 min). The resin was agitated with a solution of 4-[(*R*,*S*)-α-[1-(9*H*-fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl]phenoxyacetic acid (Knorr linker;[31] 826 mg, 150 mol %), TBTU (600 mg, 150 mol %), HOBt (300 mg, 150 mol %) and DIEA (600 μL, 300 mol %) in DMF (10 mL/g of resin) for 1 h, rinsed with DMF (10 mL/g of resin, 3 × 2 min) and capped by agitating with Ac_2O (30 μL) and DIEA (30 μL) in DMF (10 mL/g of resin) for 30 min.

Peptide Synthesis. Synthesis was conducted, after resin deprotection using piperidine in DMF (10 mL/g of resin, 20% v/v, 1 × 2 min, 1 × 3 min and 1 × 10 min) followed by washing with DMF (10 mL/g of resin, 4 × 2 min), by agitation with the Fmoc-protected amino acid (150 mol %), TBTU (150 mol %), HOBt (100 mol %, except in the case of Gly) and DIEA (300 mol %) in DMF for 1 h. The resin was agitated with N_2 bubbles during coupling, rinsing and deprotection sequences. Elongation of the linear peptides was performed on a semiautomatic peptide synthesizer. Ninhydrin tests were performed after each coupling reaction.[30] In cases where a ninhydrin test of the resin showed incomplete coupling, the resin was resubmitted to the same conditions for an additional 0.5 h. This process was used with *N*-Fmoc-Gly, *N*-Fmoc-Leu, dipeptide **6**, *N*-Fmoc-Asn(Trt), *N*-Fmoc-Pro, *N*-Fmoc-Gln(Trt), *N*-Fmoc-Ile, *N*-Fmoc-Tyr(O-*t*-Bu), *N*-Fmoc-*S*-(Trt)Cys, *S*-(Trt)Mpa, and *S*-(4-MeOBn)dPen.

[Cys(H)^1,Cys(H)^6,5-*t*-BuPro^7]oxytocin. The *N*-Fmoc group was removed from the resin-bound nonapeptide with piperidine (as described above). Cleavage from the resin (1.97 g, 0.26 mmol/g) with simultaneous side chain deprotection was conducted by treating the resin with 10 mL/g of a cocktail containing TFA (90%), thioanisole (5%), ethanedithiol (3%) and anisole (2%) and agitating with a mechanical shaker for 2 h at room temperature. Subsequent filtration, rinsing with TFA (2 × 2 mL) and precipitation in Et_2O (100 mL/g of resin) at 0 °C, followed by filtration through a membrane filter afforded the unprotected linear nonapeptide. Lyophilization gave 210 mg of a white powder that was shown to be 72% pure by RP-HPLC (t_R = 8.3) using an eluant of 20−60% B in A over 20 min with a flow rate of 1.5 mL/min and the detector centered at λ = 230 nm; LRMS calcd for $C_{47}H_{76}N_{12}O_{12}S_2$ (MH^+) 1065, found 1065. Half of the material was taken to the next step.

[5-*t*-BuPro^7]oxytocin (1). A solution of Cys-Tyr-Ile-Gln-Asn-Cys-5-*t*-BuPro-Leu-Gly-NH_2 (104 mg, of crude post-cleavage material) in DMSO (40 mL) was adjusted to a 0.2 mg/mL concentration with a pH 6 buffer (7% CH_3CO_2H and $(NH_4)_2CO_3$ in H_2O)[32] and agitated for 20 h. On the observation of a negative Ellman test,[33] the maximum quantity of DMSO was removed by vacuum distillation and the peptide was precipitated on the addition of CH_3CN and Et_2O and collected on a membrane filter. Purification on preparative C_{18} RP-HPLC, pooling of purest fractions and subsequent lyophilization gave **1** as a white powder (30.5 mg, 29 μmol, 11% overall yield from initial loading on resin; an additional 12 mg of 87% purity was also obtained from the less pure fractions): RP-HPLC (t_R = 13.06) using an eluant of 20−40% B in A over 20 min and a flow rate of 1.0 mL/min with the detector centered at λ = 230 nm; LRMS calcd for $C_{47}H_{75}N_{12}O_{12}S_2$ (MH^+) 1063, found 1063; amino acid composition Asp 1.02 (1), Glu 1.01 (1), Gly 0.98 (1), Ile 1.03 (1), Leu 1.00 (1), Tyr 0.96 (1).

[Mpa(H)^1,Cys(H)^6,5-*t*-BuPro^7]oxytocin. The nonapeptide was cleaved from the resin (500 mg, 0.26 mmol/g) according to the procedure described above to afford 26 mg of linear peptide that was shown to be 63% pure by RP-HPLC (t_R = 13.4) using an eluant of 5−65% B in A over 15 min and a flow rate of 1.5 mL/min with detection centered at λ = 220 nm; LRMS calcd for $C_{47}H_{76}N_{11}O_{12}S_2$ (MH^+) 1050.5, found 1050.4.

[Mpa^1,5-*t*-BuPro^7]oxytocin (2). A solution of Mpa-Tyr-Ile-Gln-Asn-Cys-5-*t*-BuPro-Leu-Gly-NH_2 (26 mg, of crude post-cleavage material) in DMSO was oxidized to disulfide according to the procedure described above and the reaction mixture

was loaded directly onto a preparative HPLC system. Collection of the purest fractions afforded **2** (8 mg, 7 μmol, 5% overall yield from initial loading on resin): t_R = 13.1 on RP-HPLC using an eluant of 5−65% B in A over 15 min and a flow rate of 1.5 mL/min with the detector centered at λ = 220 nm; LRMS calcd for $C_{47}H_{74}N_{11}O_{12}S_2$ (MH^+) 1048.5, found 1048.3; amino acid composition Asp 1.06 (1), Glu 1.02 (1), Gly 0.98 (1), Ile 1.00 (1), Leu 1.01 (1), Tyr 0.93 (1).

[dPen(H)¹,Cys(H)⁶,5-*t*-BuPro⁷]oxytocin. The nonapeptide was cleaved from the resin (500 mg, 0.26 mmol/g) according to the procedure described above to afford 37 mg of product shown to be of 56% purity by RP-HPLC (t_R = 14.1) using a 5−65% B in A gradient over 15 min with a flow rate of 1.5 mL/min and the detector centered at λ = 220 nm; LRMS calcd for $C_{49}H_{80}N_{11}O_{12}S_2$ (MH^+) 1078.5, found 1078.4.

[dPen¹,5-*t*-BuPro⁷]oxytocin (3). A solution of dPen-Tyr-Ile-Gln-Asn-Cys-5-*t*-BuPro-Leu-Gly-NH_2 (37 mg, of crude post-cleavage material) in DMSO was oxidized to its disulfide according to the procedure described above and the reaction mixture was loaded directly onto a preparative HPLC system. Collection of the purest fractions afforded **3** (8.0 mg, 7 μmol, 6% overall yield from initial loading on resin): t_R = 13.7 by RP-HPLC using a 5−65% B in A gradient over 15 min with a flow rate of 1.5 mL/min and the detector centered at λ = 220 nm; LRMS calcd for $C_{49}H_{78}N_{11}O_{12}S_2$ (MH^+) 1076.5, found 1076.3; amino acid composition Asp 1.01 (1), Glu 1.00 (1), Gly 0.99 (1), Ile 1.00 (1), Leu 1.02 (1), Tyr 0.98 (1).

[Mpa(H)¹,Cys(H)⁶]oxytocin. The nonapeptide was cleaved from the resin (1.62 g, 0.44 mmol/g, calculated at the octapeptide stage from initial loading) according to the procedure described above to afford 217 mg of linear peptide that was shown to be 53% pure by RP-HPLC (t_R = 5.7) using an eluant of 5−65% B in A over 15 min and a flow rate of 3.0 mL/min with detection centered at λ = 220 nm; LRMS calcd for $C_{43}H_{67}N_{11}O_{12}S_2Na$ (MNa^+) 1016.4, found 1016.7, calcd for $C_{43}H_{67}N_{11}O_{12}S_2K$ (MK^+) 1032.4, found 1032.7.

[Mpa¹]oxytocin. A solution of Mpa-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (99 mg, of crude post-cleavage material) in DMSO was oxidized to its disulfide according to the procedure described above and the reaction mixture was loaded directly onto a preparative HPLC system. Collection of the purest fractions afforded [Mpa¹]oxytocin (38 mg, 38 μmol, 19% overall yield from initial loading on resin): t_R = 6.6 on RP-HPLC using an eluant of 5−65% B in A over 15 min and a flow rate of 3.0 mL/min with the detector centered at λ = 220 nm; LRMS calcd for $C_{43}H_{66}N_{11}O_{12}S_2$ (MH^+) 993.4, found 993.1, calcd for $C_{43}H_{65}N_{11}O_{12}S_2Na$ (MNa^+) 1014.4, found 1015.1, calcd for $C_{43}H_{65}N_{11}O_{12}S_2K$ (MK^+) 1030.4, found 1031.1.

[dPen(H)¹,Cys(H)⁶]oxytocin. The nonapeptide was cleaved from the resin (1.8 g, 0.49 mmol/g, calculated at the octapeptide stage from initial loading) according to the procedure described above to afford 278 mg of product shown to be of 63% purity by RP-HPLC (t_R = 6.2) using a 5−65% B in A gradient over 15 min with a flow rate of 3.0 mL/min and the detector centered at λ = 220 nm; LRMS calcd for $C_{45}H_{71}N_{11}O_{12}S_2Na$ (MNa^+) 1044.5, found 1044.6, calcd for $C_{45}H_{71}N_{11}O_{12}S_2K$ (MK^+) 1060.4, found 1060.6.

[dPen¹]oxytocin. A solution of dPen-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (101 mg, of crude post-cleavage material) in DMSO was oxidized to its disulfide according to the procedure described above and the reaction mixture was loaded directly onto a preparative HPLC system. Collection of the purest fractions afforded [dPen¹]oxytocin (40 mg, 39 μmol, 22% overall yield from initial loading on resin): t_R = 5.7 by RP-HPLC using a 5−65% B in A gradient over 15 min with a flow rate of 3.0 mL/min and the detector centered at λ = 220 nm; LRMS calcd for $C_{45}H_{70}N_{11}O_{12}S_2$ (MH^+) 1020.5, found 1021.1, calcd for $C_{45}H_{69}N_{11}O_{12}S_2Na$ (MNa^+) 1042.4, found 1043.1, calcd for $C_{45}H_{69}N_{11}O_{12}S_2K$ (MK^+) 1058.4, found 1059.2.

Biological Evaluations. Peptides were tested for uterotonic activity in vitro in the previously described rat uterotonic test according to Holton[34] in Munsick[35] solution either in the absence of magnesium or in the presence of 1 mM magnesium in the bathing solution. Synthetic oxytocin was used as a standard for the determination of both the agonistic and antagonistic activities. 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. Single dose−response curves were constructed using data from experiments in which different doses of standard or tested compounds were added to the organ bath in random order and the bath fluid was changed immediately after the response. The interval between the doses was minimally 5 min. From the dose−response curve the EC_{50} was determined. The activity in IU/mg was calculated by comparing the threshold doses of the standard and the analogue. In the case of the antagonistic activity, the dose of the antagonist was applied to the organ bath 1 min prior to the standard dose of oxytocin. The antagonistic activity was expressed as EC_{50} or pA_2, i.e., the concentration of the analogue, which reduced the effect of the 2*x* dose of agonist, in our case oxytocin, to the effect of dose *x*, or the negative decadic logarithm of the EC_{50}, respectively. Each analogue was tested on uteri from 3−5 different rats.

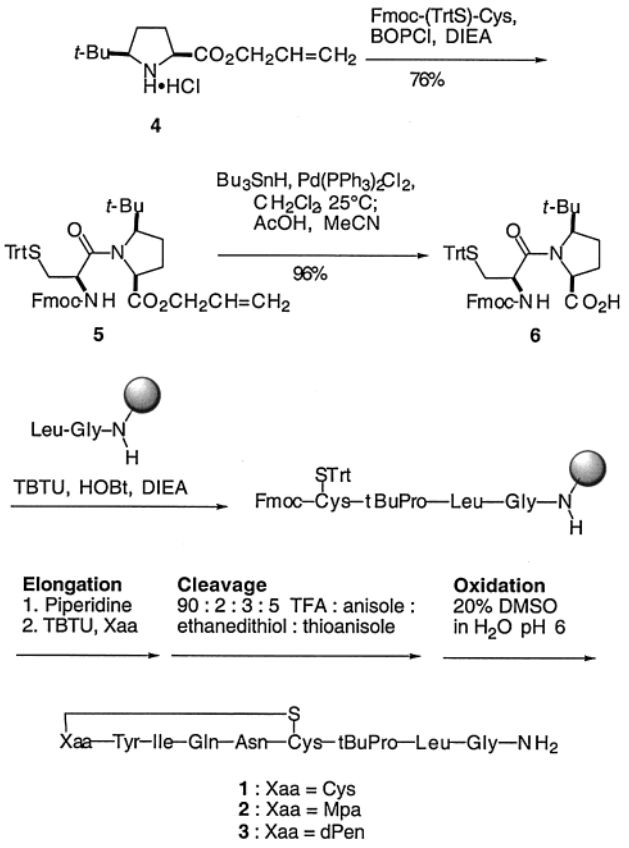
The pressor activity was determined on phenoxybenzamine-treated rats.[36] In short, male rats (220−260 g) were anesthetized using urethane and their vena femoralis and arteria carotis were cannulated for drug administration and blood pressure determination, respectively. Then phenoxybenzamine was administered 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 tested substance. At least two doses of tested compound were always administered: one dose which reduced the effect of 2*x* dose of standard to less than the response to *x* dose of standard and another (lower one) which reduced the response to a low degree. The effective dose was obtained by interpolation on logarithmic scale. Each analogue was tested on 3−4 animals. Detailed descriptions of these protocols can be found in ref 37. The activity values for standard compounds are taken from ref 38.

Binding affinity to uterine membranes was performed basically as described in refs 39 and 40 using tritiated oxytocin from NEN Life Science, Boston, MA. In brief, a crude membrane fraction was incubated with ^{3}HOT (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. Buffer used was 50 mM HEPES at pH 7.6 containing 10 mM $MnCl_2$ and 1 mg/mL bovine serum albumin. The reaction was terminated by quick filtration on a Brandel cell harvester. Binding affinities were expressed as K_i calculated according to the expression: $K_i = IC_{50}/[(c^3_{HOT}/K_{dOT}) + 1]$, where K_{dOT} is taken as 1.8 nM.[41]

Results and Discussion

The syntheses of [5-*t*-BuPro⁷]oxytocin (**1**), [Mpa¹,5-*t*-BuPro⁷]oxytocin (**2**), and [dPen¹,5-*t*-BuPro⁷]oxytocin (**3**) involved the use of a combination of solid- and solution-phase methods (Scheme 1). Automated peptide synthesis was employed for the preparation of the linear peptides on a deprotected Knorr linker attached to benzhydrylamine resin via an aminocaproic acid spacer.[31] Sequential elongation involved couplings of *N*-(Fmoc)-amino acids using the onium salt-based coupling reagent TBTU[42] in DMF and deprotections using piperidine in DMF. Although *N*-protected (2*S*,5*R*)-5-*tert*-butylproline may be introduced into the peptide sequence by solid-phase techniques, preliminary studies indicated that *N*-acylation of the sterically bulky 5-*tert*-butylproline was more effectively achieved in solution. Coupling of 5-*tert*-butylproline allyl ester (**4**)[21,22] with *N*-Fmoc-*S*-

Scheme 1. Synthesis of [5-*t*-BuPro^7]oxytocin Analogues **1**−**3**



trityl-L-cysteine was best accomplished using *N*,*N*′-bis-(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl)[26−28] in CH_2Cl_2 at 5 °C and afforded protected dipeptide **5** in 76% yield after silica gel chromatography. Subsequent palladium-catalyzed removal of the allyl ester then furnished *N*-Fmoc-*S*-trityl-L-cysteinyl-(2*S*,5*R*)-5-*tert*-butylproline (**6**) in 96% yield.[43] Dipeptide **6** was coupled to resin-linked leucylglycinamide using TBTU, and the peptides were elongated using the solid-phase protocol described above. In the cases of analogues **2** and **3**, mercaptopropionic acid (Mpa) was introduced as its *S*-trityl derivative and *β*,*β*-dimethylmercaptopropionic acid (dPen) was incorporated as the *S*-*p*-methoxybenzyl analogue. Resin cleavage with simultaneous side chain deprotections was accomplished using a TFA:thioanisole:ethanedithiol:anisole cocktail to provide the [5-*t*-BuPro^7]dihydrooxytocin derivatives after precipitation and lyophilization. Oxidation to the disulfide was conducted with DMSO in a pH 6 buffer until a negative Ellman test was observed.[32,33] The final [5-*t*-BuPro^7]-oxytocins **1**−**3** were obtained after purification on a reverse-phase C_{18} column by HPLC and lyophilization as described in the Experimental Section. The purity of **1**−**3** was checked by analytical HPLC and amino acid analysis. Their composition was also verified by fast atom bombardment mass spectrometry. Similar synthesis and characterization protocols were used in the preparation of samples of [Mpa^1]oxytocin and [dPen^1]-oxytocin.

Analogues **1**−**3** were tested for their potency in uterotonic tests in vitro and in pressor tests, see Experimental Section. In addition, their affinity to the receptors in the uterine membrane preparation was determined (Table 1). Both [5-*t*-BuPro^7]oxytocin (**1**) and [Mpa^1,5-*t*-BuPro^7]oxytocin (**2**) exhibited strongly reduced binding affinity to the receptors. Relative to oxytocin, the receptor affinity of [5-*t*-BuPro^7]oxytocin (**1**) was about 440 times weaker and that of [Mpa^1,5-*t*-BuPro^7]-oxytocin (**2**) was about 70 times weaker. Both analogues **1** and **2** maintained the pharmacophore characteristics responsible for the transfer of signal, and their decreased agonist activity corresponded roughly to their reduced affinity. Furthermore, both **1** and **2** were able to reach the same maximal response as evoked by oxytocin when single doses were tested (Figure 2).

The behavior of analogues **1** and **2** in the uterotonic test in vitro differed when the cumulative dose−response procedure was used to determine activity.

Table 1. Biological Activities of Oxytocin and [5-*t*-BuPro^7]oxytocin Analogues

entry	uterotonic activity in vitro (IU/mg or pA_2 and EC_{50})		pressor activity[b]	binding affinity K_i (nM)
	no Mg	1 mM Mg		
oxytocin	450 EC_{50} = 0.6 nM	450 EC_{50} = 0.5 nM	3.1−5 *d*	3.2
[5-*t*-BuPro^7]oxytocin (**1**)	1.8[a] EC_{50} = 150 nM	0.5 EC_{50} = 450 nM	0[c]	1430
[Mpa^1]oxytocin	599−837	760 high	1.1−1.5 *d*	3.2[e]
[Mpa^1,5-*t*-BuPro^7]oxytocin (**2**)	6.1[a] EC_{50} = 50 nM	3.0 EC_{50} = 75 nM	0[c]	240
[dPen^1]oxytocin	pA_2 = 7.4[f] EC_{50} = 40 nM	pA_2 = 5.6−6.4[f] EC_{50} = 2500−450 nM	pA_2 = 6.5[g] ED_{50} = 20 nmol/kg	540
[dPen^1,5-*t*-BuPro^7]oxytocin (**3**)	pA_2 = 7.50 EC_{50} = 32 nM	pA_2 = 7.0 EC_{50} = 100 nM	pA_2 = 5.9[g] ED_{50} = 80 nmol/kg	170

[a] The compounds were partial agonists when tested using a cumulative dose−response procedure and pure agonists when tested using a single-dose procedure (see Figure 2); however, both procedures gave the same activity value. [b] In the pressor test, the standard curve was constructed in the dose range $2 \times 10^{-5} - 2 \times 10^{-4}$ mg/kg of rat, i.e., roughly 20−200 nmol/kg of rat. The standard dose used for determination of antagonistic activity was usually 100 nmol. [c] Inactive until a dose of 200 *μ*mol/kg of rat. [d] Doses of 20−100 nmol/kg gave a dose−response curve comparable to that of oxytocin. [e] According to ref 37, the affinity of deaminooxytocin to the rat uterine receptors is the same as that of oxytocin. [f] Compound displays also partial agonistic qualities corresponding to 0.33 and 4.5 IU/mg in the absence and presence of 1 mM magnesium, respectively. [g] Estimated in vivo pA_2 values represent the negative logarithm of the effective concentration which represents the effective dose divided by the estimated volume of distribution (67 mL/kg).

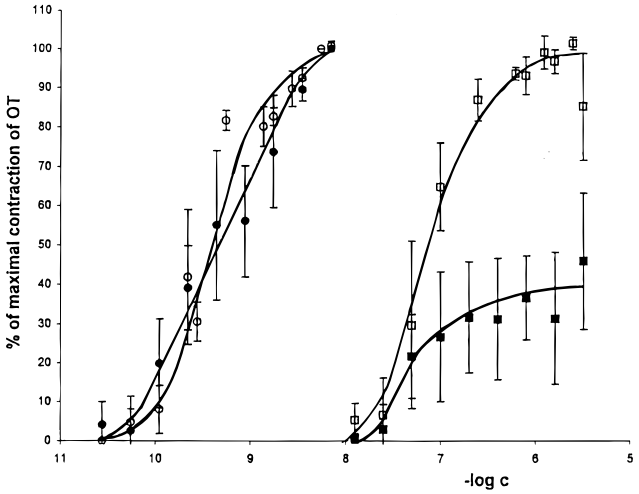


Figure 2. Comparison of the dose−response curves of the uterine effect of oxytocin (open and closed circles) and [5-*t*-BuPro^7]oxytocin (open and closed squares) on the concentration of the substance in the organ bath (M) in the cumulative dose (closed symbols) and single dose (open symbols) arrangements of the uterus in vitro test. The data are averages with standard deviations from 3−5 independent determinations. The effect is expressed as a percentage of the maximal contraction induced by oxytocin. Similar curves were obtained for [Mpa^1,5-*t*-BuPro^7]oxytocin (not shown).

During this procedure, the response of the uterus on increasing doses of oxytocin or analogue was followed without washings until the maximal response was obtained. The compound was in contact with the uterine tissue for up to 10 min, and analogues **1** and **2** behaved in this arrangement as partial agonists and were not able to reach the maximal response that was evoked by oxytocin. Their behavior suggests the presence of two conformers: one that may initially bind quickly and transfer signal and the other isomer that may bind more slowly and not be able to transfer the signal thus functioning as an inhibitor.

Relative to [dPen^1]oxytocin, which in our hands displayed in the absence of magnesium inhibitory activity with a pA_2 value of 7.4 and partial agonistic activity in 80% of the experiments performed, [dPen^1,5-*t*-BuPro^7]-oxytocin (**3**) exhibited only inhibitory activity with a similarly high pA_2 value. The binding affinity of **3** was about 50 times lower than that of oxytocin, yet higher than that of **1** and **2**. Furthermore, [dPen^1,5-*t*-BuPro^7]-oxytocin exhibited about a 3-fold better binding affinity than [dPen^1]oxytocin. In the uterotonic test in the presence of 1 mM magnesium, [dPen^1,5-*t*-BuPro^7]-oxytocin exhibited inhibitory potency that was notably higher than that of [dPen^1]oxytocin, which exhibited enhanced agonistic potency and decreased antagonism. Introduction of the *tert*-butyl substituent at the 5-position of proline^7 in [dPen^1]oxytocin may have thus improved the binding affinity of a conformation that was unable to transfer signal.

Two sets of NMR signals corresponding to the two conformers for the Cys^6-5-*t*-BuPro^7 amide *cis*- and *trans*-isomers were observed in the proton spectra of peptides **1**−**3** in 9:1 H_2O:D_2O at 30 °C with presaturation of the water peak. The assignments of the proton signals as well as the relative populations of the amide *cis*- and *trans*-isomers N-terminal to the prolyl residues of peptides **1**−**3** were ascertained by two-dimensional NMR experiments. Initially, COSY and TOCSY experiments were performed to ascertain the through bond couplings for each of the amino acid residues in the major and minor conformers (Figure 3 in Supporting Information). Assignments of the sequential connectivities of the different residues were subsequently supported by cross-peaks in the ROESY spectra between amino acid α-protons and the amide protons of their C-terminal residue. For example, the assignment of Cys^1 in 5-*tert*-butylproline^7-oxytocin (**1**) was made on observation of the cross-peak between the cysteine α-hydrogen and tyrosine amide proton.

Table 2. Percent *cis*-Isomer in Oxytocin Analogues[a]

compd	% *cis*-isomer
oxytocin	10[b]
[5-*t*-BuPro^7]oxytocin	35
[Mpa^1]oxytocin	13
[Mpa^1,5-*t*-BuPro^7]oxytocin	33
[dPen^1]oxytocin	9
[dPen^1,5-*t*-BuPro^7]oxytocin	20

[a] Measured at 30 °C in 9:1 H_2O:D_2O. [b] See ref 10.

The Cys^6-5-*t*-BuPro^7 amide *trans*-isomer was assigned based on observation of the cross-peak arising from the nuclear Overhauser effect between the cysteine α-hydrogen and the 5-*tert*-butylproline δ-hydrogen in the ROESY spectra (Figure 4 in Supporting Information). The assignment of the Cys^6-5-*t*-BuPro^7 amide *cis*-isomer was complicated due to the overlap of the water signal (≈4.7 ppm) with the cysteine and proline α-hydrogens at 30 °C. The desired signals were later resolved by switching to D_2O as solvent and recording the spectra at various temperatures. At 15 °C, the populations of the amide isomers as well as the general appearance of the spectra were unaffected; however, the water and α-hydrogen signals were separated and the cross-peak arising from the nuclear Overhauser effect between the cysteine and the 5-*tert*-butylproline α-hydrogens was observed for the amide *cis*-isomer in the ROESY spectra. The populations of the amide isomers were measured by integration of the tyrosine amide signals of **1** and the tyrosine aromatic signals of **2** and **3** in their respective ^{1}H NMR spectra. In addition, the *tert*-butyl singlet of the amide *trans*-isomer appeared always downfield from that of the *cis*-isomer.[21,22] The percentages of amide *trans*- and *cis*-isomers are listed in Table 2. The assignments of the proton signals of each conformer in **1**−**3** as well as those for [Mpa^1]oxytocin and [dPen^1]oxytocin, which were assigned in a similar manner, are listed in Tables 3 and 4 in Supporting Information. In the ROESY spectra of [Mpa^1]oxytocin and [dPen^1]oxytocin, a significant NOE was observed between the δ-proton of proline^7 and the α-proton of cysteine^6. Integrations of the clearly resolved resonances for the major and minor isomeric amide protons of Asn^5 and Cys^6 were respectively used to measure the amide populations for [Mpa^1]oxytocin and [dPen^1]oxytocin.

A modest yet significant increase in the *cis*-isomer population was obtained on incorporation of 5-*tert*-butylproline into the oxytocin analogues. For example, the steric effects of the 5-*tert*-butyl substituent in **1** gave a 25% augmentation of *cis*-isomer relative to oxytocin. Since the presence of a prolyl amide *cis*-isomer has not been reported for [Mpa^1]oxytocin nor for [dPen^1]oxytocin, both peptides were synthesized and their *cis*-isomer

populations were measured for comparison with their 5-*t*-BuPro[7] counterparts **2** and **3**. Introduction of the *tert*-butyl substituent onto the proline residues of [Mpa[1]]-oxytocin and [dPen[1]]oxytocin augmented similarly, albeit to a lesser extent, the *cis*-isomer populations in **2** and **3** by 20% and 11%, respectively. Similar to comparisons between oxytocin and other prolyl peptides,[11] we have observed less *cis*-isomer in 5-*tert*-butylproline[7]-oxytocin analogues **1**−**3** relative to *N*-acetyldipeptide *N*′-methylamides possessing 5-*tert*-butylproline at the C-terminal residue. The steric effects of the 5-*tert*-butyl substituent may thus be modulated in the oxytocin analogues by similar factors to those previously suggested to favor the *trans*-isomer population in the parent peptide.[11]

Caution must be taken when relating the increase in *cis*-isomer population with an influence on biological activity in 5-*tert*-butylproline[7]-oxytocin analogues **1**−**3**, because the bulky *tert*-butyl substituent may interact with the receptor or the peptide backbone conformation in ways that modify normal binding and signal transduction. However, analogues **1**−**3** do provide more evidence to support the hypothesis that the *cis*-isomer may favor antagonism. For example, the partial agonistic activity exhibited by **1** and **2** in the cumulative dose−response procedure could be explained by the *cis*-isomer being incapable of transferring signal yet able to inhibit binding of the *trans*-isomer and its transfer of signal. Furthermore, because [dPen[1],5-*t*-BuPro[7]]-oxytocin (**3**) exhibited only inhibitory activity (3-fold increased activity in the presence of magnesium) in the same uterotonic tests where [dPen[1]]oxytocin exhibited partial agonist activity, the *tert*-butyl substituent may have reduced the population of conformers able to transfer signal by decreasing the *trans*-isomer population. The synthesis and evaluation of additional analogues possessing alternative amide *cis*-isomer isosteres are necessary to confirm these hypotheses.[44] Nevertheless, effective methodology has been developed for replacing proline with (2*S*,5*R*)-5-*tert*-butylproline in biologically active peptides to explore the relationship between prolyl amide isomer geometry and peptide bioactivity. This strategy has led to the synthesis of two new partial agonists and a novel inhibitor of oxytocin action on the uterus.

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Supporting Information Available: Tables 3 and 4, Figures 3 and 4, ^{1}H and ^{13}C NMR spectra of **5** and **6**, as well as ^{1}H NMR, TOCSY, and ROESY spectra for **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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