Amino Acids (2005) 29: 151–160
DOI 10.1007/s00726-005-0183-z

Amino Acids
Printed in Austria

Synthesis and utilization of ^{13}C and ^{15}N backbone-labeled proline: NMR study of synthesized oxytocin with backbone-labeled C-terminal tripeptide amide

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Received October 6, 2004
Accepted January 21, 2005
Published online March 30, 2005;

Summary. The ^{13}C and ^{15}N backbone-labeled proline was prepared using Oppolzer's method based on application of a sultam as chiral auxiliary. This isotopomer was used in the synthesis of the ^{13}C, ^{15}N backbone-labeled C-terminal tripeptide amide fragment of neurohypophyseal hormone oxytocin. Finally, this tripeptide amide was coupled by segment condensation with N-Boc- or N-Fmoc-tocinoic acid, followed by N-deprotection with TFA or piperidine. The labeled oxytocin exhibited biological activity identical with that of natural oxytocin. A detailed ^{1}H, ^{13}C and ^{15}N NMR study confirmed the assigned oxytocin conformation containing a β-turn in the cyclic part of the molecule, stabilized by H-bond(s) that can be perturbed by the C-terminal tripeptide amide moiety as indicated by comparison of NMR data for both the tocine ring in oxytocin and tocinoic acid.

Keywords: Labeled proline – Oxytocin isotopomer – Peptide synthesis – Segment condensation – Protected tocinoic acid – Bioassay – NMR study

Abbreviations: The nomenclature and symbols of amino acids follow Recommendations of IUPAC/IUB Joint Commission on Biochemical Nomenclature. Eur J Biochem (1984) 138: 9–37. Additional abbreviations include: AAA, amino acid analysis; AcOH, acetic acid; ACN, acetonitrile; Boc, *tert*-butoxycarbonyl; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EC_{50}, effective concentration; EDC, N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide; EtOAc, ethyl acetate; Et_2O, diethyl ether; FAB MS, fast atom bombardment mass spectrometry; Fmoc, (fluoren-1-yl-methoxy)carbonyl; HMPA, hexamethylphosphoramide; HOBt, 1-hydroxybenzotriazole; *t*Bu, tert-butyl; TBTU, O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; THF, tetrahydrofurane

1 Introduction

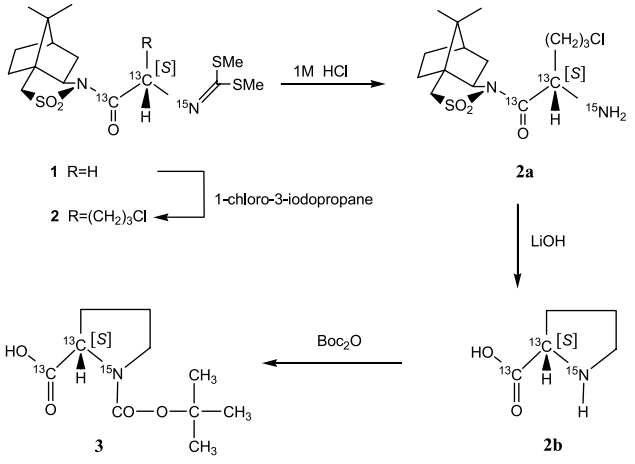
The amino acid proline has a unique structure that imposes many structural restrictions on peptides and proteins containing this residue. Proline is only amino acid where the side chain is connected to the peptide or protein backbone "twice", forming a five-membered nitrogen-containing ring. This difference is very important as it means that proline is unable to occupy many of the main chain conformations easily adopted by all other natural amino acids. For this reason, proline can often be found in very tight turns in peptide and protein structures (i.e. where the polypeptide chain must change direction). It can also function to introduce "kinks" into α-helices, since it is unable to adopt a normal helical conformation. This amino acid displays a frequent occurrence in molecules of many biologically active polypeptides, e.g. collagen, elastin, enterostatin, casomorphin, neurohypophyseal hormones or insect peptides, to mention some of them.

In the frame of our program on isotopically labeled amino acids, we have found the proline to be a useful object for labeling and utilization of corresponding topoisomer in a construction of larger peptide molecules. For this purpose, we have chosen the neurohypophyseal hormone oxytocin containing this amino acid in the C-terminal tripeptide amide part. Firstly, backbone-labeled proline was prepared and consequently used with the other labeled amino acids for a construction of the Pro-Leu-Gly-NH_2 moiety, backbone-fully labeled with ^{13}C and ^{15}N. This peptide amide was finally incorporated into the molecule of oxytocin in order to study the structural aspects of the C-terminal part of this hormone. This approach was based on access to the corresponding, properly protected isotope-labeled amino acids (Ragnarsson, 1995).

A series of studies on the three-dimensional structure of oxytocin have been published since the seventies (Hruby et al., 1987; Hruby et al., 1990), suggesting the different

conformational models and dynamics of the molecule in solution as a base for better understanding of the hormone receptor interactions. A further improvement of the insight into these processes might be obtained by NMR studies on oxytocin just using various isotopomers.

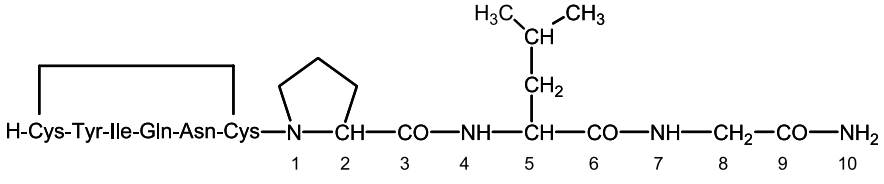
Besides proline itself, other required labeled derivatives of glycine and leucine needed for the synthesis of the tripeptide amide have been already available. In the labeled proline preparation (Scheme 1), we have chosen Oppolzer's method based on application of the sultam as chiral auxiliary (Oppolzer et al., 1994). Alkylation of glycine protected derivative **1** with 1-chloro-3-iodopropane furnished **2** which, on removal of its N^{α}-protecting group by 1 M HCl, afforded intermediate **2a**. This compound, treated with LiOH, spontaneously cyclized to the corresponding proline isotopomer **2b** with a simultaneous cleavage of the sultam auxiliary. Finally, the reaction with Boc_2O gave the required derivative **3**.



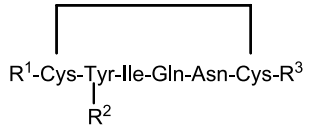
Scheme 1

Deprotection of Boc-[1,2-$^{13}C_2$,^{15}N]glycine-[$^{15}N_2$]amide gave [1,2-$^{13}C_2$,^{15}N]glycine-[$^{15}N_2$]amide hydrochloride (**4**) which, on coupling with Boc-(*S*)-[1,2-$^{13}C_2$,^{15}N]leucine, gave Boc-(*S*)-[1,2-$^{13}C_2$,^{15}N]leucine-[1,2-$^{13}C_2$,^{15}N]glycine-[$^{15}N_2$]amide (**5**). Cleavage of Boc from **5** furnished (*S*)-[1,2-$^{13}C_2$,^{15}N]leucine-[1,2-$^{13}C_2$,^{15}N]glycine-[$^{15}N_2$]amide hydrochloride (**6**), which finally was coupled with **3** to give Boc-(*S*)-[1,2-$^{13}C_2$,^{15}N]proline-(*S*)-[1,2-$^{13}C_2$,^{15}N]leucine-[1,2-$^{13}C_2$,^{15}N]glycine-[$^{15}N_2$]amide (**7**).

After preparation of the backbone fully labeled C-terminal tripeptide amide **7**, the synthesis of partially labeled oxytocin **8b** was carried out. A modified segment condensation (Mühlemann et al., 1972; Hlaváček et al., 1979; Hlaváček et al., 1984; Hlaváček et al., 1987) of the **7** with the protected tocinoic acids **9a**–**9c** (Hlaváček et al., 2001) in solution was chosen as the method of choice in the preparation of **8b**. After the Boc deprotection of the **7** with TFA, the (*S*)-H-[1,2-$^{13}C_2$,^{15}N]proline-(*S*)-[1,2-$^{13}C_2$,^{15}N]leucine-[1,2-$^{13}C_2$,^{15}N]glycine-[$^{15}N_2$]amide trifluoroacetate was condensed with N^{α}-Boc-tocinoic acid (**9a**), N^{α}-Boc-[Tyr(t-Bu)^2]-tocinoic acid (**9b**) or N^{α}-Fmoc-tocinoic acid (**9c**) using a TBTU – HOBt mixture in DMF, in the presence of DIEA with yield of the corresponding oxytocin derivatives **9d**–**9f**. The final deprotection of the **9d** and **9e** with TFA and the **9f** with 20% piperidine in DMF afforded the labeled oxytocin **8b**.



8a: oxytocin
8b: labeled oxytocin (1, 4, 7, 10 = ^{15}N; 2, 3, 5, 6, 8, 9 = ^{13}C)



	R^1	R^2	R^3
9a:	Boc	H	OH
9b:	Boc	t-Bu	OH
9c:	Fmoc	H	OH
9d:	Boc	H	Pro-Leu-Gly-NH_2
9e:	Boc	t-Bu	Pro-Leu-Gly-NH_2
9f:	Fmoc	H	Pro-Leu-Gly-NH_2
10:	H	H	OH

2 Materials and methods

A. General methodology

Optical rotations were measured on a Perkin-Elmer 141 MCA polarimeter at 22°C. Solvents were evaporated *in vacuo* on a rotary evaporator (bath temperature 30°C); DMF was evaporated at 30°C and 150 Pa. The samples for amino acid analysis were hydrolyzed with 6 M HCl at 110°C for 20 h in the presence of phenol. The amino acid analyses were performed on Biochrom 20 instrument (Pharmacia, Sweden). The molecular weights of the peptides were determined using mass spectroscopy with FAB technique (Micromass, Manchester, England). For HPLC a Spectra-Physics instrument with an SP 8800 pump, an SP 4290 integrator and Thermo Separation Products Spectra 100 UV detector was used. The peptides were purified by preparative HPLC on a 25×2.2 cm column, 10 μm Vydac, RP-18 (The Separations Group, Hesperia CA, U.S.A), flow rate 6 ml/min, detection at 280 nm using 0–100% gradient of ACN in 0.05% aqueous TFA, 60 min. The analytical HPLC was carried out with the same gradient using a 25×0.4 cm column, 5 μm LiChrospher WP-300, RP-18 (Merck, Darmstadt, Germany), flow rate 1 ml/min, detection at 220 nm, unless otherwise is stated. The ^{1}H-, ^{13}C- and ^{15}N-NMR spectra of compounds **3**, **5** and **7** were recorded for ~5% solutions in $CDCl_3$ or DMSO-d_6 at 25°C on a JEOL JMN EX 400 spectrometer at 400, 100.4 and 40.4 MHz, respectively. Assignments were made tentatively by comparing chemical shifts and peak multiplicities with those for various reference compounds. The ^{1}H-, ^{13}C- and ^{15}N-NMR spectra of labeled oxytocin **8b** were measured in DMSO-d_6 at 20°C on a Varian UNITY-500 spectrometer (^{1}H at 500 MHz, ^{13}C at 125.7 MHz, ^{15}N at 50.7 MHz). In addition to 1D-spectra also 2D-homonuclear (^{1}H, ^{1}H-COSY, TOCSY and ROESY) and indirectly detected 2D-heteronuclear correlated spectra (^{1}H, ^{13}C-HMQC, ^{1}H, ^{13}C-HMBC, ^{1}H, ^{15}N-HMQC and ^{1}H, ^{15}N-HMBC) were measured and used for the structural assignments. All chemical shifts are given in ppm using $\delta_H(TMS) = 0$, $\delta_C(CDCl_3) = 77.00$, $\delta_C(DMSO\text{-}d_6) = 39.70$ and $\delta_N(HCO\text{–}^{15}NH_2) = 113.20$ or $\delta_N(CH_3{}^{15}NO_2) = 380$.

B. Backbone labeled tripeptide amide synthesis

Boc-[1,2-$^{13}C_2$,^{15}N]proline (**3**)

(a) Alkylation step. To a stirred solution of (2*R*)-*N*-{bis(methylsulfanyl)-methylene[1′,2′-$^{13}C_2$,^{15}N]glycyl}-bornane-10,2-sultam (**1**) (Lankiewicz et al., 1994), (1.04 g; 2.74 mmol) in THF (19 ml; freshly distilled from Na/Ph_2CO) a BuLi (1.6 M in hexane; 1.90 ml; 3.04 mmol) was slowly added under argon at −78°C for 45 min. The resulting solution was left under the same conditions for 1 h, whereupon HMPA (3.3 ml, dried over 4 Å molecular sieves) was added drop wise, followed, after 15 min, by drop wise introduction of 1-chloro-3-iodopropane (1.68 g; 8.23 mmol). After 1 h the reaction mixture was gradually allowed to attain −30°C and was left under stirring for 18 h at this temperature. Then, saturated NaCl solution (3 ml) was added and the mixture was partitioned between EtOAc (120 ml), water (3 ml) and additional saturated NaCl solution (3 ml). The aqueous phase was extracted with EtOAc (2×15 ml), the combined extracts were washed with saturated NaCl solution, dried over Na_2SO_4 and evaporated. The residue was purified by fast chromatography on silica in a light petroleum-Et_2O (1:1) mixture to afford a pure fraction. This was triturated with cold pentane to give a solid, which was collected by filtration and dried to give an essentially pure product **2** (1.09 g, 87%). Crystallization from dry Et_2O (20 ml/g) at −20°C afforded **2** (0.976 g, 78%), m.p. 118.5–119°C; $[\alpha]^{25}_D$ −71.3° (*c* 1, $CHCl_3$) {(for non-labeled see Oppolzer et al., 1994, m.p. 115–117°C; $[\alpha]^{20}_D$ −72.4° (*c* 2.14, $CHCl_3$)}. ^{1}H NMR: δ_H (400 MHz, $CDCl_3$) the spectrum was essentially identical with that for the unlabelled compound except that the α-^{13}CH signal at δ 4.93 was further split by coupling to ^{13}C ($^1J_{HC} = 136.3$); δ_C (100.4 MHz, $CDCl_3$): the signals differed by less than 0.2 ppm and the *J* values by less than 0.2 Hz from those described in literature (Lodwig et al., 1998) except for the doublet at δ 44.66 ($^3J_{CC} = 4.6$ instead of 3.76 Hz). Furthermore, an additional splitting of the carbonyl signal at δ 171.11 ($^2J_{CN} = 1.2$ Hz); δ_N (40.4 MHz, $CDCl_3$): 287.6, was observed.

(b) Deprotection on nitrogen, cleavage from auxiliary with simultaneous cyclization and final Boc-protection. Compound **2** (0.91 g; 2.0 mmol), dissolved in THF (30 ml) was deprotected with 1 M HCl (20 ml) under argon at RT. After 20 h the clear solution of **2a** was concentrated to ~10 ml, water (30 ml) was added, the solution washed with Et_2O (2×10 ml) and evaporated to dryness. Then, the cleavage from auxiliary and cyclization was performed in a THF-water 4:1 (50 ml) mixture with 1 M LiOH (10 ml) under stirring at RT to obtain intermediate **2b**. After 20 h most of the solvents was evaporated at RT, the semisolid dissolved in water (25 ml) and the solution neutralized with 1 M HCl to pH 5. The auxiliary was removed by extraction with DCM (3×10 ml), the aqueous phase was concentrated to ~25 ml and treated first with 1 M LiOH (8 ml) and then with Boc_2O (438 mg; 2.0 mmol) in Et_2O (10 ml) in 20 min. After 24 h, the alkaline solution was washed with Et_2O (3×10 ml), acidified to pH 2 with 1 M $KHSO_4$ and again washed with Et_2O (3×20 ml). The combined ether solutions were washed with 1 M $KHSO_4$, saturated NaCl solution (2×15 ml) and dried over Na_2SO_4. Evaporation yielded a white solid **3** (0.38 g; 87% based on **2**). Crystallization from an EtOAc-hexane mixture (1:4; 50 ml/g) afforded an analytical sample, m.p. 135–135.5°C; $[\alpha]^{25}_D$ −59.9° (*c* 1.03; AcOH). {For non-labeled compound see Anderson et al., 1957, m.p. 136–137°C; $[\alpha]^{25}_D$ −60.2° (*c* 2.01, AcOH)}. ^{1}H NMR spectra contain two sets of signals due to the presence of *cis*- and *trans*-isomers at >CO–N< bond. ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.48 s and 1.42 s, 9H (Boc); 1.93 m, 2.08 m and 2.27 m, 4H (β-H_2 and γ-H_2 (Pro)); 3.37 m, 3.46 m and 3.55 m, 2H (δ-H_2 (Pro)); 4.36 m and 4.24 m, 1H (α-H (Pro), $^1J_{HC} = 146$). ^{13}C NMR (100.4 MHz, $CDCl_3$): δ_C 24.26 s and 23.60 s (γ-*C*H_2 (Pro)); 28.33 s and 28.22 s ((*C*H_3)$_3$ (Boc)); 28.56 d, $^1J_{CC} \sim 31$ and 30.77 d, $^1J_{CC} \sim 31$ (β-*C*H_2 (Pro)); 46.92 dd, $^1J_{CN} = 9$, $^2J_{CC} = 5$ and 46.26 dd, $^1J_{CN} = 10$, $^2J_{CC} = 4$ (δ-*C*H_2 (Pro)); 59.07 dd, $^1J_{CC} = 58.0$, $^1J_{CN} = 11.7$ and 58.85 dd, $^1J_{CC} = 60.7$, $^1J_{CN} = 11.7$ (α-^{13}*C*H (Pro)); 81.32 s and 80.28 s (>*C*< (Boc)); 156.33 d, $^1J_{CN} = 26.3$ and 153.83 d, $^1J_{CN} = 26.8$ (C=O (Boc)); 175.11 d, $^1J_{CC} = 58.0$ and 178.75 d, $^1J_{CC} = 60.7$ (^{13}C=O (Pro); ^{15}N NMR (40.4 MHz, $CDCl_3$): δ_N 100.3 d, $^1J_{NC} = 11.7$ and 98.3 d, $^1J_{NC} = 11.7$ (^{15}N (Pro)).

Backbone-labeled Boc-Leu-Gly-NH_2 (**5**)

(a) Synthesis of $^{15}NH_2$–$^{13}CH_2$–^{13}CO–$^{15}NH_2 \cdot HCl$ (**4**). Boc–^{15}NH–$^{13}CH_2$–^{13}CO–$^{15}NH_2$ (Grehn et al., 1993) (0.89 g; 5 mmol) was treated with 4 M HCl in dioxane (20 ml) under nitrogen for 1 h. Then the product was precipitated as hydrochloride with Et_2O to give solid 4 (0.55 g; 95%); m.p. 198–201°C; pure by a TLC (n-BuOH–AcOH–H_2O 4:1:1 mixture).

(b) Subsequent coupling of **4** *with Boc-L-[1,2-*$^{13}C_2$,^{15}*N]leucine*. To a pre-cooled solution of labeled Boc–Leu–OH (Lankiewicz et al., 1994) (0.50 g; 2 mmol) and **4** (0.23 g; 2 mmol) in DMF (10 ml), HOBt (0.31 g; 2 mmol) and Et_3N (0.28 ml; 2.0 mmol) were added followed by EDC (0.41 g; 2.1 mmol) in portions during 0.5 h. After another 2 h at 0°C and 16 h at RT, the DMF was evaporated and the residue partitioned between EtOAc (30 ml) and brine (10 ml). The organic layer was washed with 5% $NaHCO_3$, brine, 1 M $KHSO_4$-brine, 5% $NaHCO_3$ and brine (10 ml each) and dried over $MgSO_4$. Evaporation left a viscous oil which was purified using silica with EtOAc to give **5** as an oil (0.54 g, 95%); pure by TLC; $[\alpha]^{25}_D$ −11.4° (*c* 1, MeOH) {for non-labeled see Thorsen et al., 1983: $[\alpha]^{22}_D$ −12.3° (*c* 1, MeOH)}. ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.93 d, 3H, $J = 7.0$ and 0.95 d, 3H, $J = 7.0$ ($2 \times CH_3$ (Leu)); 1.42 s, 9H (Boc); 1.52 m, 1H (γ-H(Leu)); 1.64 m, 2H (β-H_2 (Leu)); 3.84 dm, 1H, $^1J_{HC} \sim 140$ (α-H (Leu)); 4.07 d, 2H, $^1J_{HC} \sim 139$ (α-H_2 (Gly)); 5.35 dd, 1H, $^1J_{HN} = 91.1$, $^2J_{HC} = 6$ (NH (Leu)); 6.19 d, 1H, $^1J_{HN} = 89.6$ and 6.87 d, 1H, $^1J_{HN} = 90.3$ ($CONH_2$ (Gly)); 7.44 dd, 1H, $^1J_{HN} = 92.8$, $^2J_{HC} = 5.4$ (NH (Gly)); ^{13}C NMR (100.4 MHz, $CDCl_3$): δ_C 21.86 s and 22.86 s ($2 \times CH_3$ (Leu)); 24.69 d, $^2J_{CC} = 1.5$ (γ-*C*H (Leu)); 28.28 s ((*C*H_3)$_3$ (Boc)); 40.79 d, $^1J_{CC} = 35.1$ (β-*C*H_2 (Leu)); 42.70 ddd, $^1J_{CC} = 51.4$, $^1J_{CN} = 11.7$, $^2J_{CN} = 8.3$ (α-*C*H_2

(Gly)); 53.61 ddd, $^1J_{CC} = 53.5$, $^1J_{CN} = 11.5$, $^2J_{CN} = 8.5$ (α-*C*H (Leu)); 80.43 s (>*C*< (Boc)); 156.30 d, $^1J_{CN} = 24.7$ (*C*=O (Boc)); 172.19 dd, $^1J_{CC} = 51.4$, $^1J_{CN} = 16.0$ (*C*=O (Gly)); 173.58 dd, $^1J_{CC} = 53.5$, $^1J_{CN} = 14.5$ (*C*=O (Leu)); ^{15}N NMR (40.4 MHz, $CDCl_3$): δ_N 90.0 d, $^1J_{NC} = 11.5$ (*N*H (Leu)); 100.5 dd, $^1J_{NC} = 16.0$, $^2J_{NC} = 8.3$ (*N*H$_2$ (Gly)); 105.4 m (*N*H (Gly)).

Backbone-labeled Boc–Pro–Leu–Gly–NH_2 (**7**)

(a) Synthesis of L–$^{15}NH_2$–^{13}CH(i-Bu)–^{13}CO–^{15}NH–$^{13}CH_2$–^{13}CO–$^{15}NH_2$ · HCl (**6**). Compound **6** was prepared as described for **4** above. From **5** (0.54 g; 1.90 mmol) was obtained **6** (0.44 g) as a very hygroscopic solid; pure by TLC (BuOH–HOAc–H_2O 4:1:1 and EtOAc–Me_2CO–AcOH–H_2O 5:3:1:1). It was used as such for the synthesis of **7**.

(b) Coupling of **6** *with* **3** *using EDC*. This experiment was performed with **3** (0.17 g; 0.8 mmol) and **6** (0.19 g; 0.8 mmol) as described for **5**. Work-up and crystallization from an EtOAc-hexane mixture provided **7** (0.25 g; 79%) as a white solid; m.p. 150–152°C (crystallized from a Me_2CO–Et_2O mixture), softens at about 123°C; pure by TLC (EtOAc–MeOH 4:1, CH_2Cl_2–MeOH–AcOH 18:2:1 and EtOAc–Me_2CO–HOAc–H_2O 5:3:1:1); $[\alpha]^{25}_D$ −70.8° (*c* 1.0, MeOH) {for non-labeled compound Mizoguchi et al., 1970: m.p. 137–139°C; $[\alpha]^{25}_D$ −72.3° (*c* 1.8, MeOH)}. ^{1}H NMR (400 MHz, DMSO) spectra contain two sets of signals due to the presence of *cis*- and *trans*- isomers at >CO–N< bond: δ_H 0.89 d, 3H, $J = 6.5$ and 0.86 d, 3H, $J = 6.5$ (2 × CH_3 (Leu)); 1.32 s and 1.40 s, 9H (Boc); 1.50 m and 1.64 m, 3H (β-H_2 and γ-H (Leu)); 1.80 m and 2.09 m, 4H (β-H_2 and γ-H_2 (Pro)); 3.26 m, 2H (δ-H_2 (Pro)); 3.26–3.51 m, $^1J_{HC} \sim 138$ and 3.71–3.95 m, $^1J_{HC} \sim 138$, 2H (α-H_2 (Gly)); 3.95 m, $^1J_{HC} \sim 138$ and 4.32 m, $^1J_{HC} \sim 138$, 1H (α-H (Leu)); 4.06 m, $^1J_{HC} \sim 139$ and 4.41 m, $^1J_{HC} \sim 139$, 1H (α-H (Pro)); 7.09 d, $^1J_{HN} = 88.3$, 7.07 d, $^1J_{HN} = 88.3$, 7.17 d, $^1J_{HN} = 89.0$ and 7.12 d, $^1J_{HN} = 89.0$, 2H ($CONH_2$ (Gly)); 7.85–7.97 m, $^1J_{HN} \sim 91$ and 8.12–8.22 m, $^1J_{HN} \sim 91$, 2H (NH (Leu) and NH (Gly)); ^{13}C NMR (100.4 MHz, DMSO): δ_C 21.71 s and 23.21 s (2 × CH_3 (Leu)); 24.14 s and 23.13 s (γ-CH_2 (Pro)); 24.20 s and 24.24 s (γ-*C*H (Leu)); 28.12 s and 28.30 s (CH_3)$_3$ (Boc)); 31.11 d, $^1J_{CC} = 32$ and 29.80 d, $^1J_{CC} = 32$ (β-CH_2 (Pro)); 40.65 d, $^1J_{CC} = 35.7$ (β-CH_2 (Leu)); 42.02 ddd, $^1J_{CC} = 50.4$, $^1J_{CN} = 11.6$, $^2J_{CN} = 9.0$ and 42.12 ddd, $^1J_{CC} = 50.4$, $^1J_{CN} = 11.6$, $^2J_{CN} = 9.0$ (α-CH_2 (Gly)); 46.65 d, $^1J_{CN} \sim 11$ and 46.86 d, $^1J_{CN} \sim 11$ (δ-CH_2 (Pro)); 51.53 ddd, $^1J_{CC} = 53.1$, $^1J_{CN} = 10.2$, $^2J_{CN} = 9.5$ (α-*C*H (Leu)); 59.29 ddd, $^1J_{CC} = 53.6$, $^1J_{CN} = 11.2$, $^2J_{CN} = 9.0$ and 59.57 ddd, $^1J_{CC} = 53.6$, $^1J_{CN} = 11.2$, $^2J_{CN} = 9.0$ (α-*C*H (Pro)); 78.51 s and 79.02 s (>*C*< (Boc)); 153.47 d, $^1J_{CN} = 26$ and 154.14 d, $^1J_{CN} = 26$ (*C*=O (Boc)); 170.96 dd, $^1J_{CC} = 50.4$, $^1J_{CN} = 15.6$ and 171.02 dd, $^1J_{CC} = 50.4$, $^1J_{CN} = 15.6$ (*C*=O (Gly)); 172.45 dd, $^1J_{CC} = 53.1$, $^1J_{CN} = 14.2$ and 172.39 dd, $^1J_{CC} = 53.1$, $^1J_{CN} = 14.2$ (*C*=O (Leu)); 173.04 dd, $^1J_{CC} = 53.6$, $^1J_{CN} = 14.2$ and 172.68 dd, $^1J_{CC} = 53.6$, $^1J_{CN} = 14.2$ (C=O (Pro)). ^{15}N NMR (40.4 MHz, DMSO): δ_N 98.4 d, $^1J_{NC} = 11.2$ and 99.7 d, $^1J_{NC} = 11.2$ (*N* (Pro)); 103.6 dd, $^1J_{NC} = 15.6$, $^2J_{NC} = 9.0$ and 103.5 dd, $^1J_{NC} = 15.6$, $^2J_{NC} = 9.0$ (*N*H$_2$ (Gly)); 106.5 ddd, $^1J_{NC} = 14.2$, $^1J_{NC} = 11.6$, $^2J_{NC} = 9.5$ (*N*H (Gly)); 117.3 ddd, $^1J_{NC} = 14.2$, $^1J_{NC} = 10.2$, $^2J_{NC} = 9.0$ (*N*H (Leu)).

(c) Coupling of **6** *with* **3** *using TBTU*. To a precooled solution (0°C) of **3** (0.20 g; 0.91 mmol), **6** (0.21 g; 0.91 mmol) and HOBt (0.14 g; 0.91 mmol) in DMF (10 ml), TBTU (0.35 g; 1.1 mmol) was added, followed after 5 min by Et_3N (0.39 ml; 2.76 mmol) in DMF (1 ml). The mixture was stirred for 1 h at 0°C and 3 h at RT and then worked up as described under (b) to give **7** (0.26 g; 83%) with identical physical and spectroscopical properties as described in the experiment with EDC.

C. Labeled oxytocin synthesis

N$^\alpha$-Boc-oxytocin (**9d**), N$^\alpha$-Boc [Tyr(tBu)^2]-oxytocin (**9e**) and N$^\alpha$-Fmoc-oxytocin (**9f**)

The backbone fully isotopically labeled Boc–Pro–Leu–Gly–NH_2 (**7**) (0.15 g, 0.38 mmol) with HPLC peak retention time 16.95 min at the standard gradient, 40 min, was treated with TFA (1 ml) for 30 min at room temperature. TFA was evaporated to dryness, the residue was dissolved in water (10 ml) and the solution was lyophylized. HPLC analysis revealed one peak at 10.09 min under conditions used for Boc-derivative. Trifluoroacetate was dissolved in DMF (0.9 ml), this solution was divided into three portions and each of them (0.3 ml) was added at 0°C to stirred mixtures containing 0.08 mmol of either N$^\alpha$-Boc-tocinoic acid (**9a**, 0.07 g), N$^\alpha$-Boc-[Tyr(tBu)^2]-tocinoic acid (**9b**, 0.072 g) or N$^\alpha$-Fmoc-tocinoic acid (**9c**, 0.083 g) [9] with HOBt (0.1 g) and TBTU (0.032 g) in DMF (0.5 ml), in the presence of DIEA (0.16 ml). Each of the reaction mixtures was stirred for 2 h at 0°C, for 72 h at room temperature and then was evaporated to dryness. The residue was triturated with water (3 × 5 ml), filtered and washed with diethyl ether. The partially protected labeled oxytocin derivatives **9d**–**9f** were purified by preparative HPLC under standard conditions and characterized: **9d** (yield 58 mg), AAA: 1/2 Cys 2.02; Tyr 1.03; Ile 1.00; Glu 1.02; Asp 0.94, Pro 0.98; Leu 0.97; Gly 1.02; HPLC peak retention time was 19.13 min; for $C_{48}H_{74}N_{12}O_{14}S_2$ (1107.3 + 10) FAB MS, m/z: 1117.5 ($M^+ + 1$); **9e** (yield 59 mg), AAA: 1/2 Cys 1.94; Tyr 1.02; Ile 1.00; Glu 1.04; Asp 0.98, Pro 1.03; Leu 0.99; Gly 1.03; HPLC peak retention time was 23.77 min; for $C_{52}H_{82}N_{12}O_{14}S_2$ (1163.3 + 10) FAB MS, m/z: 1173.6 ($M^+ + 1$); **9f** (yield 61 mg), AAA after the N$^\alpha$-Fmoc group removal with 20% piperidine in DMF: 1/2 Cys 1.89; Tyr 1.01; Ile 1.00; Glu 1.02; Asp 1.03, Pro 1.02; Leu 0.98; Gly 1.05; HPLC peak retention time was 31.56 min, for $C_{58}H_{76}N_{12}O_{14}S_2$ (1229.5 + 10), FAB MS, m/z: 1239.8 ($M^+ + 1$).

Final deprotection to labeled oxytocin **8b**

N$^\alpha$-Boc-oxytocin (**9d**, 56 mg, 0.05 mmol) and N$^\alpha$-Boc-[Tyr(tBu)^2]-oxytocin (**9e**, 58 mg, 0.05 mmol) were treated with a TFA-anisole 9:1 mixture (3 ml), 1 h at room temperature. The reaction mixtures were evaporated to dryness, each of the residues was dissolved in 0.05% aqueous TFA – ACN 1:1 mixture (3 ml) and an insoluble material was removed by centrifugation (2500 rpm, 10 min). The solution was applied in several portions on the preparative Vydac column 25 × 2.2 cm and HPLC was performed using the standard conditions. The collected eluates of both the peptides, with identical retention times of HPLC peaks, were freeze dried affording a yield 79 mg (0.78 mmol, 77%) of the pure labeled oxytocin **8b**. The analytical HPLC with a 5–50% gradient of ACN in 0.05% TFA, 60 min revealed the peak at 27.51 min; with the same gradient, 30 min, the peak at 18.52 min; $[\alpha]_D$ −25.9° (*c* 0.3, 1 M AcOH). {Literature gives $[\alpha]_D$ −25.3° (*c* 0.4, 1 M AcOH; Mühlemann et al., 1972); −26.1° (*c* 0.53, water; du Vigneaud et al., 1953) and −23.1° (*c* 0.51, 1 M AcOH; du Vigneaud et al., 1954)}. The AAA found the peptide composition corresponding to that found for the protected peptide **9d**. For $C_{43}H_{66}N_{12}O_{12}S_2$ (1007.2 + 10), FAB MS, m/z: 1017.6 ($M^+ + 1$).

N$^\alpha$-Fmoc-oxytocin (**9f**, 59 mg, 0.047 mmol) was treated with 20% piperidine in DMF (5 ml), 30 min at room temperature. After evaporation of the reaction mixture to dryness and peptide purification the analytical parameters fitted to those described for oxytocin **8b** prepared from precursors **9d** a **9e**, with the yield 36 mg (0.035 mmol, 73%).

D. Uterotonic activity assessment

Peptides were tested for uterotonic activity *in vitro* in the previously described rat uterotonic test for oxytocin (Holton, 1948; Munsick, 1960), either in the absence of Mg^{2+} or in the presence of 1 mM Mg^{2+} in the bathing solution. Synthetic non-labeled oxytocin **8a** was used as a standard for the determination of both the agonistic and antagonistic activities of labeled oxytocin **8b**. Female rats were estrogenized 24–48 h before the experiment. Cumulative does response curves were constructed using data from experiments in which doses were added successively to the organ bath in doubling concentration and in 1 min intervals without the fluid being changed until the maximal response was obtained. The activity was determined by comparing the threshold doses

of the standard and the analog (IU/mg or EC_{50}). In the case of the antagonistic activity assay, the dose of the analogue 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 $2\times$ dose of agonist, in our case oxytocin, to the effect of dose $\times$, or the negative decadic logarithm of the EC_{50}, respectively. The labeled oxytocin was tested on uteri from 3–5 different Wistar rats.

3 Results and discussion

A biological activity assay found the same agonistic uterotonic activity "*in vitro*" for both the labeled (**8b**) and non-labeled (**8a**) oxytocin (*e.g.* 530 IU/mg), and no antagonistic activity for labeled one. It results from it, that the isotopic labeling of the C-terminal tripeptide backbone in the oxytocin molecule exerts no effect toward the typical biological activity of the oxytocin "*in vitro*".

Isotope labeling with ^{15}N, ^{13}C and/or ^{2}H has found an extensive application in the 3D-structure analysis of small proteins using modern NMR 2D- and 3-D-methods mainly for the complete structural assignment of signals (Le Master, 1994). Syntheses of specifically ^{15}N and ^{13}C labeled oxytocin isotopomers and their NMR spectra were described in literature (Live et al., 1979; Cowburn et al., 1983).

In our paper we describe the preparation of two backbone- ^{13}C,^{15}N-labeled peptides – oxytocin **8b** and its linear C-terminal tripeptide **7**. Our intention to study in details an oxytocin space structure using ^{1}H and ^{13}C NMR spectroscopy was limited by the fact that only C-terminal tripeptide amide part of the oxytocin molecule was labeled isotopically. This isotope-labeling allowed us: (a) to assign unequivocally the signals of N, C^{α} and C′ atoms in Pro–Leu–Gly fragment; (b) observe the heteronuclear couplings (J(C,C) and J(C,N)) between labeled atoms and some of their couplings to side-chain non-labeled carbons (on their signals in ^{13}C and ^{15}N NMR proton-decoupled spectra); (c) determine some of heteronuclear couplings (J(C,H) and J(N,H)) in proton-coupled ^{13}C and ^{15}N NMR spectra; d) compare those J-values in the C-terminal tripeptide **7** and oxytocin **8b**. Thus a correlation between the NMR data for the sole tripeptide amide and for that built in the oxytocin molecule could be correctly performed.

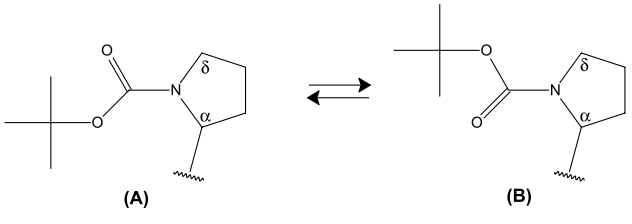


Fig. 1. The *cis*- and *trans*-isomers (**A** and **B**) around urethane bond in Boc–Pro peptides

Backbone-labeled tripeptide Boc–Pro–Leu–Gly–NH_2 **7** and its precursor Boc–Pro–OH **3** showed two sets of signals in the ratio approximately 7:3 in ^{1}H, ^{13}C and ^{15}N NMR spectra. It is obvious that similarly to the peptide bond in X–Pro also the urethane bond in Boc–Pro peptides can exist in solution as an equilibrium mixture of *cis*- and *trans*-isomers (A⇔B, Fig. 1). Consistent with this, the ^{1}H NMR spectra of tripeptide **7** measured in the temperature range 20–80°C showed a coalescence of two singlets of *tert*-butyl protons (δ 1.32 and 1.39 at 20°C) to one broad peak around 60°C. While with X-Pro peptides the signals of the *cis*- and *trans*-isomers can be easily assigned from the NOE contacts between X (Hα) and Pro (Hα) observed in the *cis*- and between X (Hα) and Pro (Hδ) in the *trans*-isomer, the absence of suitable hydrogen atoms makes such assignment in Boc–Pro peptides practically impossible. Since the energy calculation made for Boc–Pro–NH_2 model gives a low energy for *trans*-isomer (B), we suppose that also in our compounds **7** and **3** the *trans*-isomer B is prevailing. ^{1}H, ^{13}C, and ^{15}N chemical shifts and interproton coupling constants in both isomers of tripeptide **7** are given in Tables 1–3.

The oxytocin NMR data shows only one set of signals indicating the presence of only one isomer with Cys–Pro amide bond having *trans*-configuration in accordance with the observation of NOE contact between Cys-6 (Hα) and Pro-7 (H-δ) hydrogens. The observed inter-residual NOE contacts are shown in Fig. 2. The ^{1}H, ^{13}C, and ^{15}N NMR data are given in Tables 1–3.

Heteronuclear coupling constants J(C,C), J(C,N) between isotopically labeled ^{13}C and ^{15}N atoms in tripeptide **7** and oxytocin **8b** are shown in Fig. 3. Unfortunately only one-bond ^{1}J(C,C), one-bond ^{1}J(N,C) and two-bond ^{2}J(N,C) coupling over amide bond (C^{α}–CO–N) could be observed in **7** and **8b**. All vicinal couplings – including those closely related to the peptide torsion angles φ and ψ ($^{3}J(C'_i, C'_{i+1})$ and $^{3}J(N_i, N_{i+1})$, respectively), are obviously too small to give observable splitting. Similar situation was observed in other backbone-^{13}C,^{15}N-labeled peptides, *e.g.* in Leu-enkephalin (Nyassé et al., 1994).

Couplings between labeled ^{13}C or ^{15}N atoms and hydrogens (J(C,H) and J(N,H)) can be detected in principle either in ^{1}H NMR spectra and/or in 1D or 2D spectra of ^{13}C or ^{15}N without proton decoupling. In 1D-^{1}H NMR spectra practically only large one-bond couplings ^{1}J(H,C) and ^{1}J(H,N) can be determined – the others small couplings are usually obscured in complex multiplets or broadened signals (NH).

Table 1. ^{1}H-NMR parameters of oxytocin (**8b**), tocinoic acid (**10**) and Boc–Pro–Leu–Gly–NH_2 (**7**) containing C-terminal tripeptide amide backbone labeled with ^{13}C and ^{15}N (in d_6DMSO)

Residue/Parameter[a]	Oxytocin (**8b**)	Tocinoic acid (**10**)	Boc–Pro–Leu–Gly–NH_2 (**7**)	
			trans-(73%)	*cis*-(27%)
Cys-1				
NH_2 ($\Delta\delta NH_2/\Delta T$)	8.28 (−1.8)	[b]	–	–
αH	4.00	3.57	–	–
βH_2 ($J\alpha\beta$)	3.37 (5.7); 3.11 (7.1)	2.45	–	–
Tyr-2				
NH ($\Delta\delta NH/\Delta T$)	8.49 (−1.2)	8.36 (−6.3)	–	–
αH ($JN\alpha$)	4.62 (8.5)	4.62 (8.1)	–	–
βH_2 ($J\alpha\beta$)	3.17 (3.8); 2.76 (10.8)	2.87 (4.5); 2.71 (8.2)	–	–
ortho-H	7.12	6.97	–	–
meta-H	6.68	6.61	–	–
OH ($\Delta\delta OH/\Delta T$)	9.25 (−4.8)	9.18 (−6.4)	–	–
Ile-3				
NH ($\Delta\delta NH/\Delta T$)	8.07 (−5.7)	8.14 (−8.2)	–	–
αH ($JN\alpha$)	3.88 (b)	4.22 (8.8)	–	–
βH ($J\alpha\beta$)	1.79 (5.9)	1.69	–	–
γH_2	1.45; 1.12	1.36; 0.98	–	–
β-CH_3	0.90	0.75	–	–
γ-CH_3	0.86	0.66	–	–
Gln-4				
NH ($\Delta\delta NH/\Delta T$)	8.28 (−3.1)	8.05 (−4.9)	–	–
αH ($JN\alpha$)	3.95 (5.4)	4.29 (7.8)	–	–
βH_2 ($J\alpha\beta$)	1.83–1.90	1.81; 1.91	–	–
γH_2	2.16	2.14	–	–
$CONH_2$	7.29 (−3.4)	7.20 (−6.0)	–	–
($\Delta\delta NH/\Delta T$)	6.78 (−4.4)	6.79 (−6.4)		
Asn-5				
NH ($\Delta\delta NH/\Delta T$)	7.78 (−1.5)	8.05 (−5.7)	–	–
αH ($JN\alpha$)	4.49 (7.4)	4.61	–	–
βH_2 ($J\alpha\beta$)	2.63 (6.7); 2.59 (6.7)	2.50; 2.38	–	–
$CONH_2$	7.37 (−3.4)	7.31 (−3.0)	–	–
($\Delta\delta NH/\Delta T$)	6.90 (−4.5)	6.92 (−6.0)		
Cys-6				
NH ($\Delta\delta NH/\Delta T$)	8.34 (−6.2)	8.17 (−5.4)	–	–
αH ($JN\alpha$)	4.72 (7.6)	4.10 (7.7)	–	–
βH_2 ($J\alpha\beta$)	3.18 (6.5); 3.03 (6.7)	2.41; 2.51	–	–
Pro-7				
αH	4.32	–	4.13	4.12
βH_2 ($J\alpha\beta$)	2.03 (3.0)	–	2.08	2.04
	1.83 (9.0)		1.70–1.83	1.70–1.83
γH_2	1.80–1.90	–	1.70–1.83	1.70–1.83
δH_2	3.59; 3.44	–	3.35; 3.24	3.35; 3.28
(Boc)	–	–	1.32	1.39
Leu-8				
NH ($\Delta\delta NH/\Delta T$)	8.01 (−4.4)	–	8.07 (−6.7)	8.08 (−7.1)
αH ($JN\alpha$)	4.18 (7.6)	–	4.23 (7.3)	4.18 (∼7.5)
βH_2 ($J\alpha\beta$)	1.51 (b)	–	1.43–1.52	1.43–1.52
γH	1.63	–	1.63	1.63
δ-CH_3	0.89; 0.83	–	0.88	0.85
Gly-9				
NH ($\Delta\delta NH/\Delta T$)	7.85 (−4.0)	–	8.11 (−8.0)	8.02 (−6.0)
αH_2 ($JN\alpha$)	3.65 (6.0); 3.56 (5.6)	–	3.65 (6.2); 3.55 (5.4)	3.65 (6.2); 3.50 (5.5)
$CONH_2$	7.10 (−2.7)	–	7.17 (−3.9)	7.11 (−3.6)
($\Delta\delta NH/\Delta T$)	7.03 (−5.0)		7.08 (−6.3)	7.08 (−6.3)

[a] The temperature coefficients $\Delta\delta NH/\Delta T$ are given in ppb deg^{-1}; [b] the value of parameter was not determined

Table 2. ^{13}C-NMR parameters of oxytocin (**8b**), tocinoic acid (**10**) and Boc–Pro–Leu–Gly–NH_2 (**7**) containing C-terminal tripeptide amide backbone labeled with ^{13}C and ^{15}N (in d_6DMSO)

Residue/Parameter	Oxytocin (**8b**)	Tocinoic acid (**10**)	Boc–Pro–Leu–Gly–NH_2 (**7**)	
			trans-(73%)	*cis*-(27%)
Cys-1				
CO	166.85	170.34[a]	–	–
Cα	51.95	51.82	–	–
Cβ	40.35	[b]	–	–
Tyr-2				
CO	171.98	170.85[a]	–	–
Cα	54.95	54.09	–	–
Cβ	36.73	37.58	–	–
C-*ipso*	127.71	127.41	–	–
C-*ortho*	130.15	130.47	–	–
C-*meta*	115.29	115.05	–	–
C-*para*	156.10	156.02	–	–
Ile-3				
CO	171.54	171.00[a]	–	–
Cα	59.56	57.00	–	–
Cβ	35.81	36.83	–	–
Cγ	25.06	24.50	–	–
β-CH_3	15.60	15.57	–	–
γ-CH_3	11.16	11.14	–	–
Gln-4				
CO	171.25	171.17[a]	–	–
Cα	54.42	52.28	–	–
Cβ	26.46	28.52	–	–
Cγ	31.61	31.69	–	–
$CONH_2$	171.10	171.47[a]	–	–
Asn-5				
CO	170.84	171.63[a]	–	–
Cα	50.38	49.47	–	–
Cβ	36.37	37.09	–	–
$CONH_2$	171.89	172.22[a]	–	–
Cys-6				
CO	167.91	174.23	–	–
Cα	51.64	51.23	–	–
Cβ	41.20	[b]		
Pro-7				
CO	172.03	–	173.05	172.70
Cα	60.29	–	59.30	59.59
Cβ	29.05	–	31.12	29.82
Cγ	24.59	–	23.14	24.15
Cδ	46.94	–	46.67	46.90
Boc (CO)		–	153.47	154.12
Boc (>C<)		–	78.50	79.02
Boc $(CH_3)_3$		–	28.12	28.31
Leu-8				
CO	172.34	–	172.46	172.40
Cα	51.53	–	51.55	51.55
Cβ	40.10	–	40.68	[b]
Cγ	24.36	–	24.22	24.27
Cδ	23.18; 21.66	–	23.24; 21.72	23.24; 21.72
Gly-9				
$CONH_2$	174.48	–	170.96	171.02
Cα	42.17	–	42.03	42.17

[a] The assignment of carbonyl carbon atoms may be mutually interchanged; [b] overlapped with a signal of solvent (DMSO)

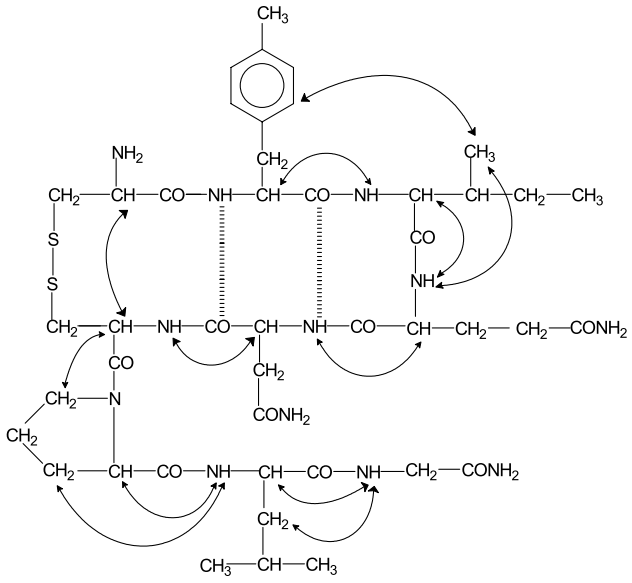


Fig. 2. The observed inter-residual NOE contacts (indicated with arrows) and hydrogen bridges (dashed lines) in oxytocin

The same *J*-values have been found for both the isomers of tripeptide **7**. Also the comparison of corresponding *J*'s in tripeptide **7** and oxytocin **8b** shows very similar values in both the compounds except of those for proline nitrogen, obviously due to the structural difference in its neighborhood. The isotopic shifts – the effect of the presence of ^{13}C and ^{15}N in the vicinity of the observed carbon atom – obtained from comparison of backbone-^{13}C,^{15}N-labeled and non labeled tripeptide **7** are much smaller (0–0.03 ppm) than deuterium isotopic shifts and in general also to the higher field.

Table 3. ^{15}N-chemical shifts (in d_6DMSO) of C-terminal tripeptide amide in oxytocin (**8b**) and Boc–Pro–Leu–Gly–NH_2 (**7**) containing amide backbone labeling with ^{13}C and ^{15}N

Residue	Type of nitrogen	Oxytocin (**8b**)	Boc–Pro–Leu–Gly–NH_2 (**7**)	
Pro	NH	131.1	98.4	99.7
Leu	NH	116.3	117.3	
Gly	NH	104.9	106.5	
	$CONH_2$	103.0	103.6	103.5

To estimate the effect of C-terminal tripeptide on the conformation of oxytocin we have synthesized tocinoic acid **10** from its protected precursor (Hlaváček et al., 2001) and their ^1H and ^{13}C NMR spectra in DMSO were measured. The obtained data are summarized in Tables 1 and 2. A comparison of ^1H and ^{13}C NMR parameters of oxytocin **8b** and tocinoic acid **10** indicates substantial differences in their conformational features. Substantially higher negative temperature shifts of NH protons ($\Delta\delta NH/\Delta T$ – see Table 1) in tocinoic acid **10** indicate its higher flexibility

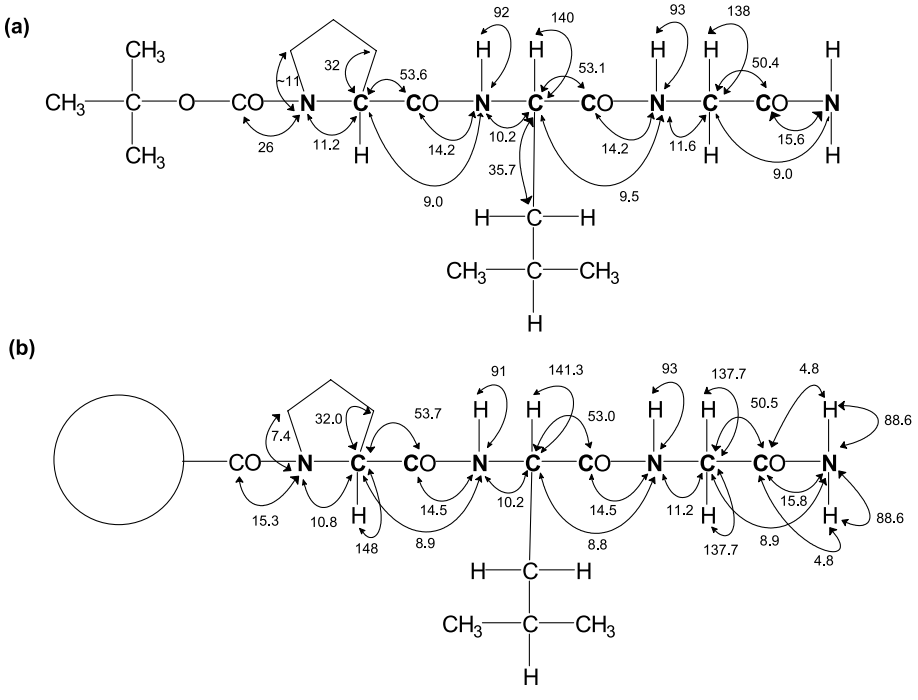


Fig. 3. Heteronuclear coupling constants determined in backbone ^{13}C and ^{15}N-labeled (**a**) Boc–Pro–Leu–Gly–NH_2 **7** and (**b**) oxytocin **8b**

and there is no evidence for hydrogen bonding. This is in contrast to the corresponding tocine ring of oxytocin **8b**, where conformation is stabilized by two H-bridges between Tyr-2 and Asn-5 (see Fig. 2) as indicated by low negative temperature shifts ($\Delta\delta NH/\Delta T = -1.2$ and -1.5 ppb/deg, respectively) of the amide protons of Tyr-2 and Asn-5. This conformation, designed to be a β-turn structure (Kotelchuk et al., 1972), was also confirmed by our study on isosteric analogs of oxytocin and vasopressin lacking the β-turn stabilizing H-bond in this position of the ring (Mařík et al., 2001). Consequently, the biological activities of these analogs were eliminated. Another turn-stabilizing factor can be represented by weak hydrogen bonding between one of $CONH_2$ amide protons of Gly-9 (with a lower $\Delta\delta NH/\Delta T = -2.7$ ppb/deg) and probably C=O of Cys-6. A similar type of H-bonding between C=O of Cys-6 and amide NH of Gly-9 was suggested for oxytocin solution in DMSO (Baccarat et al., 1992). Although there are not any significant NOE contacts observed between the C-terminal tripeptide and the cyclic part of the oxytocin molecule, the presence of the C-terminal tripeptide contributes to the conformation behavior of oxytocin substantially.

The above NMR results are also in agreement with those described in our older study on CD spectra measurements, which has shown relationships between structures of non coded amino acids placed in position 8, in the C-terminal tripeptide amide of corresponding oxytocin analogs and the CD complex band of tyrosine in position 2, in the cyclic part of oxytocin (Hlaváček et al., 1989).

It is known that the proline residue often plays an important role in very tight turns in peptide and protein structures, as well as in changing the polypeptide chain direction. Therefore, this amino acid, placed in position 7 in oxytocin, seems also to affect significantly the correct spatial arrangement and direction of the above tripeptide amide as a whole and consequently contributes to hormone conformation significantly.

Acknowledgement

This study was supported by Grants of the Grant Agency of the Czech Republic, No. 203/98/1330, 203/00/0955 and research project Z4055 905.

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