O(2), the explanation for the difference in rates of hydrolysis between the various substrates must be based on the variation of the substrate position and orientation. Since the K_m values for 2′,3′-cCMP and 2′,3′-cUMP hydrolysis by RNase A (3.4 and 5.0 mM, respectively[13]) and their hydrolysis rates, k_2 (5.55 and 2.25 s^{-1} at 27 °C and pH 7.0, respectively[13]), are so similar, it seems reasonable these differences can arise from the different C(4) substituents and pK_a values of N(3) for 2′,3′-cCMP and 2′,3′-cUMP. While the NH_2 and C=O groups may be hydrogen bonded to the same group in RNase, and N(3) of each cyclic nucleotide may interact with the same residue, the strength of these interactions would not be expected to be the same. It would be interesting to obtain activation parameters for the enzymatic hydrolysis so that more light could be shed on the intimate mechanism.

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^{13}C NMR Conformational Studies of Oxytocin Analogues with a Prolyl Residue in Position 3

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Abstract: Carbon-13 NMR chemical shifts and spin–lattice relaxation times (T_1) of [Pro³,Gly⁴]-oxytocin and oxytocin are compared. Assignments were also made for [Pro³]-oxytocin and [Leu²,Pro³,[α,α-$^2H_2$]Gly⁴]-oxytocin. These studies have shown that introduction of a prolyl residue in position 3 of the cyclic portion of oxytocin causes conformational heterogeneity in the peptide backbone of the 20-membered covalent ring moiety as a consequence of cis–trans isomerism about the Tyr-Pro peptide bond. The amount of cis isomer is larger in the more sterically crowded [Pro³]-oxytocin (45%) when compared with [Pro³,Gly⁴]-oxytocin (33%). Introduction of the Pro-Gly sequence in positions 3 and 4 of oxytocin, which is thought to favor formation of a β turn, results in shorter T_1 values for the α carbons in [Pro³,Gly⁴]-oxytocin than for the same carbons in oxytocin under analogous experimental conditions, but it does not eliminate the intrinsic flexibility of the α carbon of the glycyl-4 residue. The rotational correlation times for the linear portion of the peptide backbone (residues 7 through 9) of [Pro³,Gly⁴]-oxytocin and oxytocin are similar, which may be due to dominance of the T_1 behavior by segmental motion of the tripeptide.

In continuing studies dedicated to understanding the physico-chemical properties of peptides and peptide hormones, we have been using carbon-13 nuclear magnetic resonance (^{13}C NMR) to elucidate structural and conformational characteristics of these compounds.[2] The chemical shifts of carbon-13 are sensitive to the time-averaged conformation of peptides. If conformers are interconverting slowly on the NMR time scale, separate resonances for each conformer can be observed. Carbon-13 spin–lattice relaxation times (T_1) can provide information on the relative conformational flexibility of back-

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bones and side chains in cyclic and linear peptides.[3]

Using ^{13}C NMR techniques we have shown that the cyclic portion of the oxytocin backbone is more restricted than the acyclic terminal tripeptide, the latter experiencing increasingly greater motional freedom from the prolyl residue in position 7 toward the terminal glycinamide.[4] A major problem in the analysis of the conformational flexibility of peptides using ^{13}C T_1 values resides in determining the relative contributions of overall molecular motion and internal or segmental motion to the observed relaxation times. Even in the case of α-amino acids with cyclic side chains and cyclic peptides, internal motions or pseudorotations can occur which are reflected in the observed T_1 values. Proline and proline-containing peptides provide good examples of internal flexion occurring at a rate which is sufficiently rapid to affect the observed T_1 values.[5] In the case of oxytocin, the cyclic portion of the peptide backbone could possibly be undergoing a conformational averaging process in aqueous solution at a rate sufficiently rapid to contribute to the observed T_1 values. In order to explore this possibility, we have investigated the ^{13}C NMR characteristics of an oxytocin analogue in which substitutions were performed which could be expected to favor steric restriction of the peptide backbone. We chose to investigate [Pro^3,Gly^4]-oxytocin, for which a type-II β turn has been found in dimethyl sulfoxide solution by ^1H NMR spectroscopy[6] in agreement with theoretical predictions for this type of peptide sequence.[7,8]

Herein we report that in [Pro^3,Gly^4]-oxytocin the α carbons located in the 20-membered ring exhibit NT_1 values (where N is the number of protons directly bonded to the carbon under study) which are shorter than those reported previously for oxytocin. Furthermore, it was found that this analogue exhibits an *increased* rate of motion for the α carbon of the glycyl residue located in the 20-membered ring (position 4 in oxytocin) relative to the other α carbons of residues in the cyclic moiety. It was also observed that the introduction of a prolyl residue into position 3 of the ring portion of oxytocin produces conformational heterogeneity as manifest by the presence of both cis and trans isomers about the Tyr-Pro peptide bond. In view of these results, [Pro^3]-oxytocin[9] was also investigated and found to contain an even higher percentage of cis isomer about the Tyr-Pro peptide bond.

Experimental Section[10]

[Pro^3]-oxytocin was from the same batch for which the synthesis and biological activities were described previously.[9] Melting points of peptides synthesized for this study were determined in open capillary tubes and are reported uncorrected. Optical rotations were measured in a Zeiss circle polarimeter, 0.01°. For thin-layer chromatography (TLC) up to 50 μg was applied to precoated plates of silica gel 60 F-254 (E. Merck, Darmstadt, West Germany). Chromatograms were developed for 100–150 mm with solvent mixture ratios given in volume by volume. Visualization of peptides after chromatography was achieved by treatment with Cl_2 followed by toluidine spray.[11] Amino acid analyses[12] were performed on a Durrum D-500 amino acid analyzer in duplicate, following hydrolysis of the peptides for 24 h in constant boiling 6 N HCl at 110 °C in vacuo.

Boc-Gly-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (1). The Boc group was removed from Boc-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2[13] (2.58 g, 3.74 mmol) by treatment with CF_3COOH (25 mL) for 60 min at 25 °C. The peptide salt was precipitated by the addition of anhydrous Et_2O, washed with Et_2O by decantation, and dried in vacuo. A solution of Boc-Gly (0.79 g, 4.5 mmol) and HBT·H_2O[14] (1.0 g, 6.5 mmol) in DMF (5 mL) and EtOAc (15 mL) was activated with DCC (0.93 g, 4.5 mmol). After 1 h the preactivation mixture was filtered into a solution of the pentapeptide trifluoroacetate in DMF (10 mL); the pH had been adjusted to 8 (Fisher Indicator Solution) with N-methylmorpholine. After 15 min, 1.5 mmol of N-methylmorpholine was added. After 45 min, the precipitated product was filtered, washed with EtOAc (3 × 50 mL), 5% $NaHCO_3$ (3 × 25 mL), and H_2O (3 × 50 mL), and dried in vacuo to give 1.56 g (55%): mp 196–198 °C; $[\alpha]^{25}_D$ −49° (c 1, DMF); homogeneous upon TLC in n-BuOH/AcOH/H_2O (4:1:1); R_f 0.57. Anal. Calcd for $C_{34}H_{52}N_8O_9S$: C, 54.5; H, 7.00; N, 15.0. Found: C, 54.3; H, 7.01; N, 15.0.

Boc-[α,α-$^2H_2$]Gly-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (2). This compound was synthesized, using Boc-[α,α-$^2H_2$]Gly prepared in our laboratory, in an identical manner to **1** from 2 g (2.9 mmol) of the protected pentapeptide to give 1.55 g (70%): mp 195.5–197.5 °C; $[\alpha]^{25}$ −50° (c 1, DMF); homogeneous by TLC in n-BuOH/AcOH/H_2O (4:1:1); R_f 0.57. Anal. Calcd for $C_{34}H_{50}D_2N_8O_9S$: C, 54.4; (H, D), 7.25; N, 14.9. Found: C, 54.1; (H, D), 7.22; N, 14.9.

Boc-Pro-Gly-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (3). The Boc group was removed from hexapeptide **1** (1.52 g, 2.03 mmol) by treatment with CF_3COOH as described above. Boc-Pro (0.54 g, 2.5 mmol) was coupled with DCC (0.52 g, 2.5 mmol) mediated with HBT·H_2O (0.68 g, 4.4 mmol) using the preactivation procedure described for the synthesis of **1**. After 1 h the reaction solution was diluted with EtOAc; the product was filtered, washed with EtOAc, and dried in vacuo to give 1.61 g (93%): mp 193–195 °C; $[\alpha]^{25}_D$ −80° (c 1, EtOH); homogeneous by TLC in n-BuOH/AcOH/H_2O (4:1:1); R_f 0.44. Anal. Calcd for $C_{39}H_{59}N_9O_{10}S$: C, 55.4; H, 7.03; N, 14.9. Found: C, 55.3; H, 7.10; N, 15.1.

Boc-Pro-[α,α-$^2H_2$]Gly-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (4). This compound was prepared in the same manner and using the same quantities of reactants as described for **3** to give 1.68 g (96%): mp 203–205 °C; $[\alpha]^{25}_D$ −82° (c 1, EtOH); homogeneous by TLC in n-BuOH/AcOH/H_2O (4:1:1); R_f 0.44. Anal. Calcd for $C_{39}H_{57}D_2N_9O_{10}S$: C, 55.2; (H, D), 7.25; N, 14.9. Found: C, 54.8; (H, D), 7.26; N, 14.5.

Boc-Tyr(Bzl)-Pro-Gly-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (5). The Boc group was removed from heptapeptide **3** (1.51 g, 1.78 mmol) with CF_3COOH as described above. The heptapeptide trifluoroacetate was dissolved in EtOH (15 mL) and DMF (15 mL), and the pH adjusted to 8.5 with (i-Pr)$_2$NEt (0.75 mL). Boc-Tyr(Bzl)-OSu (1.17 g, 2.5 mmol) was added, and the reaction mixture allowed to stand for 3 days. The solvents were removed by evaporation, and the residual oil was dissolved in $CHCl_3$ (50 mL). The $CHCl_3$ solution was extracted with H_2O, 5% $NaHCO_3$, and H_2O and dried over Na_2SO_4. The $CHCl_3$ was evaporated, and the product twice precipitated from EtOAc/hexane to give 1.25 g (64%): mp 152–154 °C; $[\alpha]^{24}_D$ −38° (c, 1, DMF); homogeneous by TLC in $CHCl_3$/CH_3OH/AcOH (15:5:1); R_f 0.73. Anal. Calcd for $C_{55}H_{74}N_{10}O_{12}S$: C, 60.1; H, 6.79; N, 12.7. Found: C, 59.9; H, 6.66; N, 12.6.

Z-Cys(Bzl)-Tyr(Bzl)-Pro-Gly-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (6). The protected octapeptide **5** (1.08 g, 0.98 mmol) was converted to the trifluoracetate in the manner previously described and, after neutralization with (i-Pr)$_2$NEt, coupled to Z-Cys(Bzl) (0.78 g, 2.25 mmol) using DCC (0.47 g, 2.25 mmol) employing the preactivation procedure. After 72 h the product, which had precipitated from solution, was filtered and washed with Et_2O. The product was reprecipitated with Et_2O from EtOH to give 944 mg (74%): mp 154–156 °C (in a second preparation, mp 128–134 °C); $[\alpha]^{25}_D$ −53° (c 1, DMF); homogeneous by TLC in $CHCl_3$/CH_3OH/AcOH (8:2:0.05). Anal. Calcd for $C_{68}H_{83}N_{11}O_{13}S_2$: C, 61.6; H, 6.31; N, 11.6. Found: C, 61.3; H, 6.12; N, 11.8.

[Pro^3,Gly^4]-oxytocin (7). All the protecting groups from the nonapeptide **6** (450 mg, 0.339 mmol) were simultaneously removed by treatment with sodium in anhydrous liquid ammonia.[15,16] The residue, after evaporation of the ammonia by a stream of N_2, was dissolved in 500 mL of CH_3OH/H_2O (1:1, v/v), and the disulfide bond formed by oxidation with diiodoethane (100 mg, 0.5 mmol).[17] Disappearance of sulfhydryl groups was monitored by the method of Ellman,[18] and the reaction was complete in 5 min at pH 8. The pH was adjusted to 4 with AcOH, and the volume of solvent reduced to 2 mL by evaporation. AcOH (2 mL) was added, and the product subjected to gel filtration on a 2.15 × 115 cm column of Sephadex G-15. The column was eluted with 50% AcOH[19] at 7 mL/h. Fractions (3 mL volume) comprising the main peak area (56–71 as determined by the method of Lowry[20]) were pooled to give 191 mg after lyophilization. The entire product was further purified by partition chromatography[21] in the system n-BuOH/H_2O containing 3.5% AcOH and 1.5% pyridine (1:1, v/v) on a 2.82 × 67 cm column of Sephadex G-25 (block polymerisate, 100–200 mesh). The column was eluted with the upper phase of the solvent mixture at 20 mL/h, and peptide material was detected by the method of Lowry. The product appeared as a symmetrical peak with a maximum at R_f 0.16. Fractions (5.9 mL volume) comprising the peak area (105–132) were pooled, the organic solvent was evaporated, and the aqueous phase was lyophilized to give 117 mg (37%): $[\alpha]^{25}_D$

+41° (*c* 0.41, 1 N AcOH); homogeneous by TLC in *n*-BuOH/AcOH/H_2O (4:1:5, upper phase), R_f 0.38; in *n*-BuOH/AcOH/pyridine/H_2O (15:3:10:6), R_f 0.66; in EtOH/pyridine/AcOH/H_2O (5:5:1:3), R_f 0.64. For elementary analysis a sample was dried for 18 h over P_2O_5 in a vacuum with a weight loss of 11%. Anal. Calcd for $C_{39}H_{57}N_{11}O_{11}S_2 \cdot CH_3COOH$: C, 50.2; H, 6.27; N, 15.7. Found: C, 50.3, H, 6.34; N, 15.7.

A sample was hydrolyzed in 6 N HCl at 110 °C for 28 h, and the hydrolysate subjected to amino acid analysis. The following molar ratios were found: Asp, 1.00; Pro, 2.06; Gly, 2.04; half-Cys, 1.97; Leu, 1.05; Tyr, 0.96; and NH_3, 2.11.

Boc-Leu-Pro-[α,α-$^2H_2$]Gly-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (8). The heptapeptide **4** (1.50 g, 1.66 mmol) was treated with CF_3COOH (15 mL) as previously described. The heptapeptide trifluoroacetate was dissolved in DMF (15 mL), the solution was diluted with EtOAc (15 mL), and *i*-Pr_2NEt (0.60 mL, 3.5 mmol) as well as Boc-Leu-OSu (1.32 g, 2 mmol) were added. After stirring for 24 h the solvents were evaporated, and the residual oil was dissolved in $CHCl_3$ (50 mL). The $CHCl_3$ solution was extracted with saturated aqueous NaCl (30 mL), 5% $NaHCO_3$ (2 × 40 mL), H_2O (50 mL), 1 N H_2SO_4 (50 mL), and H_2O (2 × 40 mL) and then dried over Na_2SO_4. After removal of the $CHCl_3$ the product was precipitated from $CHCl_3/Et_2O$ to give 1.50 g (88%): mp 144–148 °C; $[\alpha]^{26}_D$ −58° (*c* 1, EtOH), homogeneous upon TLC in *n*-BuOH/AcOH/H_2O (4:1:1); R_f 0.49. Anal. Calcd for $C_{45}H_{68}D_2N_{10}O_{11}S \cdot 0.5CHCl_3$: C, 53.5; H, 7.16; N, 13.7. Found: C, 53.4; H, 7.25; N, 14.1.

Z-Cys(Bzl)-Leu-Pro-[α,α-$^2H_2$]Gly-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (9). The octapeptide **8** (1.60 g, 1.57 mmol) from two batches was treated with CF_3COOH at 25 °C for 1 h, and the product worked up as before. Z-Cys(Bzl) (0.78 g, 2.25 mmol) was preactivated as described for **1** with HBT·H_2O (0.74 g, 4.8 mmol) and DCC (0.47 g, 2.25 mmol) in DMF (2 mL) plus EtOAc (10 mL). The mixture was filtered into a solution of the deprotected octapeptide and *i*-Pr_2NEt (0.50 mL, 2.90 mmol) in DMF (2 mL) and EtOAc (10 mL). After 30 min an aliquot of *i*-Pr_2NEt (0.25 mL, 1.45 mmol) was added, and the mixture stirred at 25 °C for 16 h. Evaporation of the solvents gave an oil which was dissolved in $CHCl_3$ (50 mL); the solution was extracted with 5% $NaHCO_3$ (2 × 35 mL) and ice water (2 × 50 mL) and the organic phase dried over Na_2SO_4. After evaporation of the $CHCl_3$ the product was precipitated from $CHCl_3/Et_2O$ to give 1.72 g (89%). The crude material was reprecipitated from tetrahydrofuran/Et_2O to give 1.63 g (84%): mp 135–140 °C; $[\alpha]^{27}_D$ −67° (*c* 1, *p*-dioxane); homogeneous upon TLC in *n*-BuOH/AcOH/H_2O (4:1:1); R_f 0.53. Anal. Calcd for $C_{58}H_{77}D_2N_{11}O_{12}S_2 \cdot 0.5H_2O$: C, 58.2; H, 6.90; N, 12.9. Found: C, 58.1; (H, D), 6.86; N, 12.5. Amino acid analysis gave the following molar ratios: Asp, 1.00; Pro, 2.01; Gly, 2.01; Leu, 2.04; Cys(Bzl), 1.86; NH_3, 2.05.

[Leu^2,Pro^3,[α,α-$^2H_2$]Gly^4]-oxytocin (10). This analogue was prepared from nonapeptide **9** (300 mg, 0.34 mmol) by treatment with Na/NH_3 and oxidation with diiodoethane in CH_3OH/H_2O as described for **7**. The crude product was purified by gel filtration on Sephadex G-15 in 50% AcOH and by partition chromatography on Sephadex G-25 in the system *n*-BuOH/EtOH/H_2O containing 3.5% AcOH and 1.5% pyridine (5:1:6) with an R_f of 0.31 using the same columns and conditions as described for **7** to give 152 mg (52%): $[\alpha]^{24}_D$ +35° (*c* 1, AcOH); TLC in *n*-BuOH/AcOH/H_2O (4:1:1), R_f 0.38; in *n*-BuOH/AcOH/pyridine/H_2O (15:3:10:6), R_f 0.70; in EtOH/pyridine/AcOH/H_2O (5:5:1:3), R_f 0.65. Anal. Calcd for $C_{36}H_{57}D_2N_{11}O_{12}S_2 \cdot 2AcOH \cdot H_2O$: C, 47.6; H, 7.08; N, 15.3. Found: C, 47.6; (H, D), 6.97; N, 15.4. Amino acid analysis gave the following molar ratios: Asp, 1.00; Cys(O_3H),[22] 2.05; Pro, 1.93; Gly, 2.03; Leu, 1.99; NH_3, 2.12.

Bioassay Methods. At least three animals were used for the determination of activities in each assay system. Because of the exceedingly low potency of [Pro^3,Gly^4]-oxytocin and [Leu^2,Pro^3,[α,α-$^2H_2$]-Gly^4]-oxytocin, attempts were made to assess their activities initially by a "match" design, in which the U.S.P. Posterior Pituitary Reference Standard is diluted to an activity similar to that of the sample. Not less than ten matches were carried out. In addition, single doses of 0.6 to 2.4 mg of peptide were tested. Oxytocic activity was measured on six isolated uterine horns from rats in natural estrus, determined on the morning of the assay by vaginal smear, by the method of Holton, as modified by Munsick utilizing Mg^{2+}-free Van Dyke–Hastings solution.[23] Avian vasodepressor assays were performed on conscious chickens[24] according to the procedure of Coon.[25] The antidiuretic activity was determined in inactin- and ethanol-anesthetized, hydrated male Sprague-Dawley rats according to the method of Jeffers et al.[26] with minor modifications. Pressor activity was determined in atropinized, urethane-anesthetized male rats following the procedure in the U.S. Pharmacopeia.[27]

Carbon-13 NMR Techniques. Preliminary ^{13}C NMR spectra were run at 25 MHz on a Varian XL-100-15 spectrometer using a Varian 620-L computer with 16K memory. Spectra obtained at 68 MHz were obtained on a Bruker HX-270 spectrometer interfaced to a Nicolet 1089 36K computer having a 600K word auxiliary disk memory. Investigations were carried out mainly at 68 MHz because of the greater dispersion and sensitivity at this frequency. For molecules the size of the neurohypophyseal hormones, the extreme narrowing condition[28] may not be fulfilled, and the relative values of the measured spin-lattice relaxation times, NT_1, may not reflect the relative mobilities of the peptide moieties. We have performed measurements at both 25 and 68 MHz to justify the use of relative T_1 values as indicators of relative degrees of mobility.

Spin-lattice relaxation times were obtained at 68 MHz using a (T_∞–180°–τ–90°–) pulse sequence,[29] where τ is a variable delay time and T_∞ is at least four times longer than the longest T_1 to be measured. The 90° pulse widths are 14 μs (XL-100) and 20 μs (HX-270). T_1 values were determined from a semilogarithmic plot using

$$M(\tau) = M(\infty)[1 - \exp(-\tau/T_1)]$$

where $M(\infty)$ is the equilibrium value of the magnetization and $M(\tau)$ is the value of the magnetization resulting from a given value of τ. T_1 measurements were performed in duplicate at 68 MHz using 10–15 values of τ. The sample of oxytocin used to measure T_1 at 68 MHz was rerun, in duplicate, at 25 MHz using 10 values of τ. The reproducibility of the measured values at each frequency was better than ±10%. At 68 MHz 2000 scans/spectrum were obtained, and 10 000 scans/spectrum were obtained at 25 MHz. Samples were studied in 12-mm (XL-100) and 10-mm tubes (HX-270). Chemical shifts are reported downfield from external tetramethylsilane (TMS, $(CH_3)_4Si$) at 32 °C. Spectra were obtained on 1.5-mL samples containing 100 mg of peptide in D_2O. pH was adjusted using CD_3COOD and is reported as the uncorrected pH-meter reading.

Results

Biological Properties of [Pro^3,Gly^4]-oxytocin and [Leu^2,-Pro^3,[α,α-$^2H_2$]Gly^4]-oxytocin. [Pro^3,Gly^4]-oxytocin showed an extremely weak rat pressor activity (0.04 ± 0.001 unit/mg), while the second title compound gave no response when tested at single doses as high as 1.0 mg. Neither of the analogues had any detectable rat antidiuretic activity when single injections of 0.6 mg of peptide were tested. Moreover, doses of 0.72 mg demonstrated no rat uterotonic activity in vitro, as was the case with doses as high as 2.4 mg in the avian vasodepressor assay. Neither of the analogues inhibited the action of oxytocin in these assays.

Carbon-13 NMR Spectroscopy. The assignments of the ^{13}C spectra of [Pro^3,Gly^4]-oxytocin and [Pro^3]-oxytocin[9] are based on comparison with the previously assigned spectrum of oxytocin at the same pH[30] as well as with [Leu^2,Pro^3,[α,α-$^2H_2$]Gly^4]-oxytocin (see below) (Table I). The values given in column m for [Pro^3,Gly^4]-oxytocin are "extra" resonances which appear in the spectrum and are assigned to the presence of a cis Tyr-Pro peptide link. This assignment is based on the fact that the γ carbon of a prolyl in the trans conformer resonates at 25.0 ∓ 1.0 ppm and, in the cis conformer, at 22.4 ∓ 0.8 ppm.[31] Furthermore, the *difference* in chemical shifts for the β- and γ-carbon resonances of a prolyl residue in a trans X-Pro bond is 1.3 to 6.0 ppm, whereas for a cis X-Pro bond the difference is 8.3 to 10.0 ppm.[32] We observe chemical shifts at 26.1 and 22.6 ppm, and differences between the chemical shifts of 3.7 and 9.6 ppm for the resonances we have attributed to the trans and cis isomers about the Tyr-Pro peptide link. The ratio of the populations of cis and trans isomers is ca. 1:3. The presence of cis and trans isomers about the Tyr-Pro peptide bond was also manifest on the tyrosyl residue, which demonstrated doubling of the α-, β-, and ε-carbon resonances with extra chemical shifts of 55.0, 37.1, and 116.7 ppm, respectively.

Table I. Carbon-13 Chemical Shifts of [Pro^3]-oxytocin, [Pro^3,Gly^4]-oxytocin, and [Leu^2,Pro^3,[α,α-$^2H_2$]Gly^4]-oxytocin in D_2O[a]

Residue[b]		[Pro^3]-oxytocin, pH 3.5		[Pro^3,Gly^4]-oxytocin pH 3.5		[Pro^3,Gly^4]-oxytocin pH 9.0		[Leu^2,Pro^3, [α,α-$^2H_2$]Gly^4] oxytocin, pH 3.5,
		M[c]	m[d]	M[c]	m[d]	M[c]	m[d]	M[c]
Cys^1	α-CH	52.4		52.9		53.1		52.7
	β-CH_2	41.5		40.9		45.5	46.0	40.7
	C=O	168.6	167.6	167.8		175.4		168.1
Tyr^2	α-CH	54.5	55.0	54.2	55.0	54.1	54.9	
	β-CH_2	36.2	35.7	36.4	37.1	36.6		
	γ-C	128.7	128.0	128.5		128.5	127.9	
	δ-CH	131.2	131.5	131.9		132.0		
	ϵ-CH	116.6		116.5	116.7	116.6	117.0	
	ζ-C	155.7		155.7		156.0		
	C=O	173.0	173.2	172.9		171.6		
Pro^3	α-CH	64.4		63.3		63.2		62.9
	β-CH_2	30.2		29.8	32.2	29.7	31.9	29.7
	γ-CH_2	26.0		26.1	22.6	26.2	22.5	26.0
	δ-CH_2	48.7	48.1	48.9	47.7	48.9	48.1	48.7
	C=O	174.4	174.3	175.1		175.1		N.O.[e]
Gly^4	α-CH_2			43.5	42.5	43.2	42.6	
	C=O			175.1		175.1		
Asn^5	α-CH	51.0	51.4	50.8		50.9	51.2	50.8
	β-CH_2	38.2		37.7		37.2	37.8	38.0
	γC=O	175.7		175.5		175.7		N.O.[e]
	C=O	172.3	172.0	173.1		172.5		173.0
Cys^6	α-CH_2	52.4		52.0		52.2	51.9	51.7
	β-CH_2	39.4		39.3		40.3		40.0
	C=O	170.6	170.7	170.6		170.4		170.4
Pro^7	α-CH	61.7		61.6		61.9		61.6
	β-CH_2	30.2		30.2		30.4		30.1
	γ-CH_2	25.6		25.2		25.8		25.5
	δ-CH_2	48.7		48.9		48.9		48.7
	C=O	175.1		175.3		175.2		175.4
Leu^8	α-CH	53.5		53.6		53.6		53.5
	β-CH_2	40.2		40.6		40.3		40.4
	γ-CH	25.3		25.3		25.3		25.3
	δ-CH_3	23.1		23.1		23.3		23.4
	δ-CH_3	21.6		21.7		21.7		21.7
	C=O	176.0		176.0		176.1		175.7
Gly^9	α-CH_2	43.2		43.1		43.2		43.0
	C=O	174.9		174.9		174.9		174.8
Gln^4	α-CH	56.3						
	β-CH_2	27.2						
	γ-CH_2	32.1						
	δC=O	178.8	178.4					
	C=O	174.4	174.3					
Leu^2	α-CH							51.2
	β-CH_2							40.4
	γ-CH							25.3
	δ-CH_3							23.1
	δ-CH_2							21.3
	C=O							172.7

[a] Chemical shifts are in parts per million downfield from external tetramethylsilane (Me_4Si). Assignments of carbonyl carbons are tentative. [b] Numbers indicate residue position in oxytocin. [c] Major conformer. [d] Minor conformer. [e] Not observed.

Also apparent at 68 MHz is doubling of the α-carbon resonance of the asparaginyl residue. The ratios of the minor and major peaks in all cases are also ca. 1:3. In the carbonyl carbon region at 68 MHz there is definite doubling of the resonances assigned to the cystyl residue, the assignment of which was varified by obtaining spectra at pH 9. Other resonances may be doubled elsewhere in the spectrum but are not detectable due to their weak intensities or to overlap with other resonances in the spectrum of the major component.

The resonances of the two prolyl and two glycyl residues in [Pro^3,Gly^4]-oxytocin are quite distinct. The α carbons of the prolyl-3 and prolyl-7 residues differ by 1.7 ppm; those of the glycyl-4 and glycyl-9 residues differ by 0.4 ppm. The assignment of the glycyl-4 α-carbon resonance was verified by measuring the spectrum of [Leu^2,Pro^3,[α,α-$^2H_2$]Gly^4]-oxytocin, an oxytocin analogue in which the glycyl-4 residue is deuterated. Deuteration at the α carbon of the glycyl residue in position 4 effectively causes the disappearance of this α-carbon resonance due to splitting from coupling with the deuterons and to the long spin-lattice relaxation time of this nonprotonated carbon.

We obtained a spectrum of [Pro^3]-oxytocin in order to determine whether cis-trans isomerism was also manifest, and, if so, to what extent the neighboring glycyl-4 residue influenced this ratio in [Pro^3,Gly^4]-oxytocin. At 25 MHz, there is clear evidence of two molecular species. We cannot detect β- and γ-carbon resonances of a prolyl-3 residue which would be attributable to a cis Tyr-Pro peptide bond because the leucyl γ

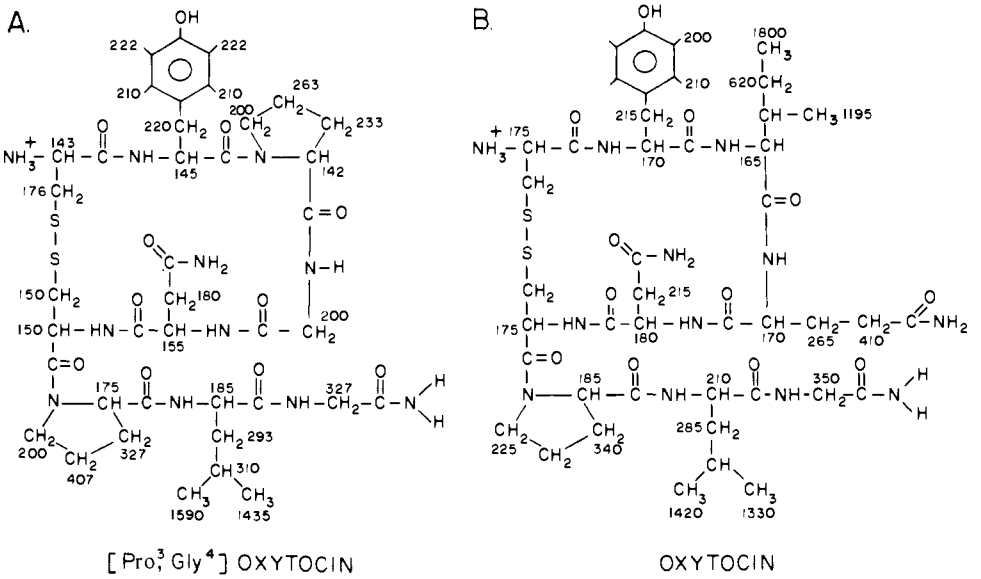

Figure 1. Carbon-13 NT_1 values in milliseconds observed at 68 MHz for [Pro³,Gly⁴]-oxytocin (panel A) and oxytocin (panel B). Spectra were obtained using 65 mg of peptide/mL of D_2O at a pH meter reading of 3.5, 27 °C. Differences greater than 10% are considered significant.

carbon and glutaminyl γ-carbon resonance occur in the same region. We do observe, however, a prolyl δ-carbon resonance which occurs in the same position as that attributed to the δ carbon of the cis isomer about the Tyr-Pro bond in [Pro³,Gly⁴]-oxytocin. Furthermore, very clear doubling occurs on the α-, β-, γ-, and δ-carbon resonances of the tyrosyl residue. The asparaginyl α-carbon resonance is also doubled. Further doubling of other resonances in the spectral region of the proton bearing carbons may be masked by the resonances of the major (trans) isomer. It is worth noting that for [Pro³]-oxytocin the ratio of minor to major species is close to 1:1 (45:55). The carbonyl carbons are particularly interesting; doubling of resonances of the half-cystyl-1, half-cystyl-6, and asparaginyl peptide carbonyl carbons and of the glutaminyl δ-carbonyl carbon can quite clearly be identified. In total, 16 resolved resonances attributable to carbonyl carbons in the peptide are detected (doubling of all the carbonyl carbon resonances of amino acids in the cyclic portion of the peptide with only single resonances for the residues in the acyclic terminal tripeptide would lead to a maximum of 19 resonances). Thus, extensive doubling occurs over the entire cyclic portion. Doubling is detected on the residues farthest removed from the point of cis-trans isomerism, i.e., on the half-cystyl-6 as well as on the side chain of the glutaminyl residue. The sensitivities of the chemical shifts of the half-cystyl-1 (1.0 ppm) and half-cystyl-6 (0.1 ppm) carbonyl carbons, due to the presence of cis and trans isomers about the Tyr-Pro bond, differ significantly.

Discussion

Positions 3 and 4 in neurohypophyseal hormones show considerable biological tolerance to modifications (e.g., see reference 33), which is thought[34] to be due to their location in corner positions of the β turns (residues in positions $i + 1$ and $i + 2$ of a β turn) identified in these hormones.[35] Even during evolution amino acid changes have occurred only at these corner positions.[36] Functionally, the residue in position 3 has been identified as important for the binding of the hormone to its receptor (e.g., see reference 37 and references therein), while that in position 4 has been suggested to be part of the "active center" of the hormone.[34,38] The specific biological activities found for [Pro³,Gly⁴]-oxytocin and [Leu²,-Pro³,[α,α-$^2H_2$]Gly⁴]-oxytocin are even lower than those reported for the weak agonist [Pro³]-oxytocin, which exhibited a rat pressor activity of 0.005 unit/mg, antidiuretic activity of 0.003 unit/mg and a uterotonic activity of <0.02 unit/mg.[9] The prolyl residue has a relatively fixed side-chain conformation since its carbon chain forms a pyrrolidine ring with the peptide backbone. This amino acid residue in position 3 is apparently not complementary to the neurohypophyseal hormone receptors, endowing [Pro³]-oxytocin and its analogue with little or no receptor affinity. Moreover, the substitution of the glutaminyl residue in position 4 of neurohypophyseal hormones by a glycyl residue leads also to a detrimental reduction in potency as revealed by the study of [Gly⁴]-oxytocin.[39] Hence, the lack of significant biological activities of the two oxytocin analogues prepared for this study is not surprising. This does not detract, however, from the value of these analogues as peptide models for probing important conformational features of neurohypophyseal hormones.

Spectroscopic analysis of [Pro³,Gly⁴]-oxytocin reveals doubling of carbon-13 resonances attributable to cis-trans isomerism about an X-Pro peptide bond in the cyclic hormone analogue. Such phenomena are not uncommon in cyclic peptides,[3] and coexistence of cis and trans isomers about the X-Pro bond has been reported for linear peptide hormones, such as angiotensin-II at basic pH[40] and in the analogue [Pro³,Pro⁵]-angiotensin at pH 6.0.[41] A particularly relevant example in cyclic peptides is that of *cyclo*-(Gly-Ala-Gly-Gly-Pro) in dimethyl sulfoxide.[42] This peptide shows two species, one (65% of total) containing a trans Gly-Pro linkage and the other (35%), a cis Gly-Pro linkage. There are five distinct carbonyl carbon resonances for each conformer in this peptide. In addition, there are two resonances for every carbon in each type of amino acid in the peptide. Thus, the effect of cis-trans isomerism about the Gly-Pro peptide bond is effectively transmitted throughout the ring. The presence of 16 distinct resonances in the carbonyl carbon region of [Pro³]-oxytocin at 25 MHz suggests that a similar long-range effect occurs in the 20-membered cyclic portion of this analogue. Comparison of the relative populations of cis-trans isomers in [Pro³,Gly⁴]-oxytocin (33% cis isomer) and [Pro³]-oxytocin (45% cis isomer) indicates a greater percentage of cis isomer in the sterically more crowded [Pro³]-oxytocin, which has a glutaminyl instead of a glycyl residue in position 4. The observation of distinct resonances for the two conformations of the prolyl residues in the above peptides indicates that exchange between these conformations takes place more slowly than 20 s^{-1}. This exchange is very slow on the time scale of

motions contributing to spin-lattice relaxation (5×10^7 s^{-1}). Therefore, the observed T_1 values of one conformation will be unaffected by those of the other or by the rate of exchange between the two.

The results of the spin-lattice relaxation time (T_1) measurements performed at 68 MHz on the major (trans) isomer of [Pro^3,Gly^4]-oxytocin are given in Figure 1. The general trend of the relaxation times follows what has been observed in oxytocin itself:[4] a restricted backbone in the cyclic portion of the peptide and increasing segmental motion of the acyclic terminal tripeptide. One point, however, is worthy of interest in the cyclic portion of [Pro^3,Gly^4]-oxytocin, namely the longer NT_1 value observed for the α carbon of the glycyl residue when compared with the α carbons of the other amino acids in the 20-membered ring. This has also been observed for the glycyl-6 residue of the linear peptide luliberin (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) and in des-Gly^{10}-luliberin-*N*-ethylamide.[43] Comparing the NT_1 values observed for similar residues in the oxytocin analogues, the prolyl-3 and glycyl-4 residues are more restricted than their cogeners in positions 7 and 9 of the flexible acyclic tripeptide. From the relative ratios of T_1 values within the two prolyl residues, it is apparent that the prolyl-3 residue shows less intracyclic mobility, possibly as a result of constraint from the neighboring tyrosyl residue and/or the peptide backbone of the 20-membered ring moiety.[5]

It would be of interest to compare the results obtained on [Pro^3,Gly^4]-oxytocin with those obtained on oxytocin under identical conditions. Such a stringent comparison, however, is difficult to perform in a meaningful manner unless the compounds were dissolved in the same solution, which may not be feasible because of resonance overlap. Numerous factors can influence observed T_1 values when samples are studied separately, e.g., variations in temperature and concentration, but even more seriously, variations in macroscopic (and microscopic) viscosities which can occur in spite of careful control of the previous two parameters. Recognizing these limitations, we did, however, examine the *relative* NT_1 values observed at 68 MHz in samples of oxytocin and [Pro^3,Gly^4]-oxytocin recorded under similar experimental conditions. It can be seen from Figures 1A and 1B that the NT_1 values in the cyclic portion of [Pro^3,Gly^4]-oxytocin are shorter than those observed for oxytocin. The terminal tripeptide α-carbon NT_1 values for [Pro^3,Gly^4]-oxytocin and oxytocin, however, are rather similar. The cyclic portions of the two peptides definitely have different correlation times; whether this be due to a more restricted internal flexibility within the peptide backbone of [Pro^3,Gly^4]-oxytocin compared to oxytocin or to a short-lived, aggregated species in [Pro^3,Gly^4]-oxytocin is not directly testable by ^{13}C NMR spectroscopy. The similarity in T_1 values of the terminal tripeptide could imply that segmental motion effectively "decouples" these motions from the overall molecular motion.[44] The relative NT_1 values of the side-chain carbons in these peptides are indicative of varying degrees of segmental motion. The conclusions regarding these mobilities are consistent with those already reported for oxytocin itself.[2-4]

These studies on [Pro^3,Gly^4]-oxytocin and related proline-containing peptides have shown that introduction into the hormone of a prolyl residue in position 3 results in conformational heterogeneity within the 20-membered covalent peptide backbone as a result of cis-trans isomerism about the Tyr-Pro peptide bond. Comparing the relative ratios of cis and trans isomers in [Pro^3,Gly^4]-oxytocin and [Pro^3]-oxytocin, we find a larger proportion of cis isomer in [Pro^3]-oxytocin (45%) as compared to the sterically less crowded [Pro^3,Gly^4]-oxytocin (33%). Spin-lattice relaxation time measurements have yielded shorter NT_1 values for the α carbons located in the 20-membered ring of [Pro^3,Gly^4]-oxytocin than for oxytocin observed under similar conditions. The NT_1 values of the linear tripeptide portions, which undergo segmental motion with respect to the cyclic portions, are more similar in oxytocin and its analogue. The glycyl residue in position 4 of [Pro^3,Gly^4]-oxytocin shows increased local mobility within the 20-membered ring when compared with the other, optically active residues, in the ring.

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(10) Abbreviations follow the IUPAC–IUB Tentative Rules on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972). Optically active amino acids are of the L configuration. The following additional abbreviations are used: EtOH, ethanol; DMF, *N,N*-dimethylformamide; AcOH, acetic acid; *n*-BuOH, *n*-butyl alcohol; HBT, 1-hydroxybenzotriazole; EtOAc, ethyl acetate; Et_2O, ethyl ether; $(i\text{-Pr})_2NEt$, diisopropylethylamine; DCC, dicyclohexylcarbodiimide.
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Models for Metal Binding Sites in Zinc Enzymes. Syntheses of Tris[4(5)-imidazolyl]carbinol (4-TIC), Tris(2-imidazolyl)carbinol (2-TIC), and Related Ligands, and Studies on Metal Complex Binding Constants and Spectra

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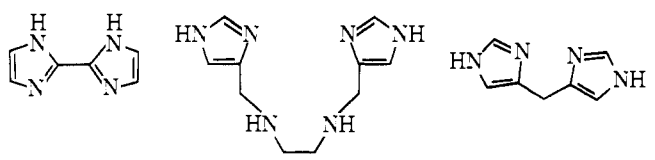
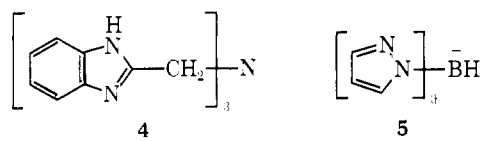
Abstract: Tris[4(5)imidazolyl]carbinol (4-TIC) and tris(2-imidazolyl)carbinol (2-TIC) have been synthesized as models for the zinc binding site of carbonic anhydrase and of alkaline phosphatase. Bis[4(5)imidazolyl]glycolic acid (4-BIG) has been synthesized to mimic the zinc binding site of carboxypeptidases and of thermolysin; bis[4(5)imidazolyl]carbinol (4-BIC) has also been synthesized. Basicities and metal binding constants have been determined for 4-TIC, 2-TIC, 4-BIG, and 4-BIC, and as well for the known bis(2-imidazolyl)methane (2-BIM) and 3-[bis(2-imidazolyl)]propionic acid (2-BIP). The data are compared with those reported for bis[4(5)imidazolyl]methane (4-BIM) and for human carbonic anhydrase B and carboxypeptidase A. 4-TIC and 2-TIC are tridentate ligands using three imidazole groups, but 4-TIC is more basic and a stronger metal complexing agent. The binding constants of 4-TIC are comparable to those of the enzymes for cobalt, nickel, and copper dications but not for zinc dication. Spectral and binding studies suggest that the geometry of 4-TIC is not quite right for a good mimic of carbonic anhydrase.

Extensive studies of various carbonic anhydrases[1] and alkaline phosphatases[2] indicate the presence of a catalytic Zn^{2+} bound to three imidazole residues of enzyme histidines. In the carboxypeptidases[3] and in thermolysin[4], the critical Zn^{2+} is bound to two imidazoles and a carboxylate group of the enzyme. In spite of the obvious interest such systems would have, few chelating ligands using imidazole rings have been made so far, and none which combine three simple imidazole rings as models for the metal binding sites of carbonic anhydrase.

Holmes et al.[5] have investigated metal binding by 2,2′-bis(imidazole) (**1**) while Gruenwedel[6] has studied Zn^{2+} and Co^{2+} binding by the tetradentate ligand **2.** An important study by Fruton[7] led to the synthesis and metal-binding constants for bis[4(5)-imidazolyl]methane (**3**), which we call 4-BIM. Fruton's synthesis, from histidine, is not adaptable for the preparation of related tris(imidazoles). Very recently,



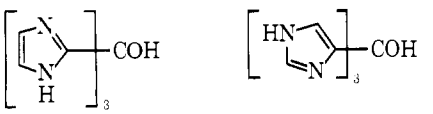
1 2 4-BIM (3)



4 5

Thompson et al.[8] have described some metal binding properties of a tris(benzimidazole) ligand system (**4**). Finally, the tris(pyrazolyl)borohydride ligand **5,** first reported by Trofimenko[9] but recently studied by Marks and Ibers,[10] is relevent to our studies.

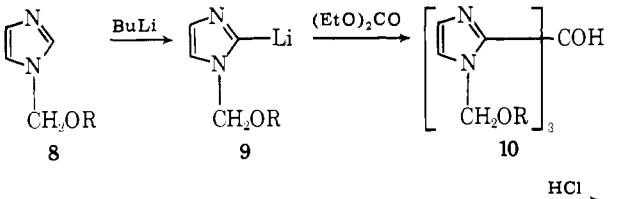
The x-ray studies[11] on carbonic anhydrase show that the three imidazole ligands have distorted tetrahedral coordination to the Zn^{2+}. Molecular models suggested that a similar geometry could be attained with a tris(imidazolyl)methane derivative. We now wish to report the synthesis of two isomeric chelating ligands, tris(2-imidazolyl)carbinol (2-TIC) (**6**) and



2-TIC (6) 4-TIC (7)

tris[4(5)-imidazolyl]carbinol (4-TIC) (**7**), and their metal binding properties.

Syntheses. Roe[12] and Shirley and Alley[13] have reported the metallation of *N*-benzylimidazole at C-2 with *n*-butyllithium, but in our hands significant benzylic lithiation also occurred. However, *N*-methoxymethylimidazole[14] (**8a**) and *N*-ethoxymethylimidazole (**8b**) were smoothly metallated at C-2; re-



8 9 10

a, R = CH_3
b, R = C_2H_5

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