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Torsion Angles in the Cystine Bridge of Oxytocin in Aqueous Solution. Measurements of Circumjacent Vicinal Couplings between ^1H, ^{13}C, and ^{15}N[1a]

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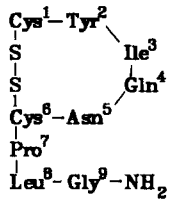
Abstract: The six couplings from $^1H^\alpha$, $^{13}C'$, and $^{15}N'$ to the two β protons have been measured for the two half-cystyl residues in oxytocin, using a series of specifically designed and synthesized isotopic isomers. In the case of half-cystyl 1, the observed couplings strongly suggest that the torsion angle χ^1 (N'-C^α-C^β-S) has the eclipsed value of $-120°$. The value for this angle in the half-cystyl residue 6 is less clearly defined, but is probably also fixed. Using the same analysis as for $\chi_1{}^1$, $\chi_6{}^1$ is approximately $+120°$. The stereochemical assignments of the β protons of the two residues have been confirmed by stereoselective deuteration.

A substantial number of observations suggest that the conformation of the peptide hormone oxytocin is relatively flexible in aqueous solution and that the number of conformers contributing to this dynamic equilibrium is small.[2] Such



flexibility may be significant in activation of the hormonal receptor upon binding, and it has been suggested that inhibition by binding without activation, as, for example, by [1-penicillamine]oxytocin, is a result of the more rigid conformation of such an inhibitor.[3]

In an effort to understand this dynamic conformation we are currently investigating torsion angles in the molecule by measurement of homo- and heteronuclear vicinal coupling constants in specifically designed and synthesized isotopic isomers of oxytocin.[4] As part of this program we have been interested in the torsion angles about the α and β carbons of half-cystyl residues 1 and 6, $\chi_1{}^1$ and $\chi_6{}^1$. These angles are important since, along with the other angles of the disulfide bridge of cystine ($\chi_1{}^2$, χ^{SS}, $\chi_6{}^2$), they are torsion angles of the 20-atom ring of oxytocin and thus are involved in the stereoisomerism of this ring in solution.

In the synthetic isotopic isomers described here, deuterium substitution of protons has been used to remove overlapping resonance patterns of similar chemical shifts, to simplify spin systems in which the participating nuclei are either so numerous or so close in chemical shift that analysis of the spectra of the natural material is impossible, or to identify stereospecifically particular protons. In addition, substitutions of ^{13}C for ^{12}C and of ^{15}N for ^{14}N have been used so that the vicinal heteronuclear coupling constants between ^{13}C or ^{15}N and protons can be directly observed in the proton spectrum of the molecule.[5]

It has previously been demonstrated[6] that in a free amino acid it is feasible using similar strategies of isotopic substitution to determine the six homo- and heteronuclear couplings between two β protons and the three α-carbon substituents $^1H^\alpha$, $^{13}C'$, and $^{15}N'$. In that case,[6] it was shown that those couplings were consistent with averaging among staggered rotamers about the C^α-C^β bond, that stereochemical assignments could be made, and that it might be possible to describe the torsion angle and its distribution with time by the use of these multiple vicinal couplings without resort to the usual assumption either of the exclusive presence of rotamers or of a single fixed angle.

This report is concerned with NMR studies of a series of isotopic isomers of oxytocin designed to permit measurements of the heteronuclear coupling constants about the C^α-C^β bonds of the two half-cystyl residues in order to determine unequivocally the torsion angle and its distribution with time. The homonuclear vicinal proton coupling constants have been previously reported.[2]

Experimental Section

Five isotopic isomers of oxytocin, from a larger set, were used in deriving the results described here. The methods of peptide synthesis and characterization used have been previously described.[4] The composition of some of the isotopic isomers[4,7] and outlines of some syntheses of amino acids have been presented elsewhere.[6,7] The isotopic substitutions in these peptides are summarized in Table I.

Syntheses of Amino Acids. All amino acids described are the L stereoisomer, unless otherwise indicated. Syntheses described here are for the L stereoisomers of the *S*-*p*-methylbenzyl (MeBzl) derivative of [$^{15}N'$,α-^2H], [$^{13}C'$,α-^2H], [$\beta2$,$\beta3$-$^2H_2$], and [α,$\beta3$-$^2H_2$] isotopic isomers of cysteine.

S-*p*-Methylbenzyl[$\beta2$,$\beta3$-$^2H_2$]cysteine (**1**)[8] was synthesized by the addition of *S*-*p*-methylbenzyl chloro[$^2H_2$]methyl sulfide (**2**)[9] to diethyl α-acetamidomalonate.[10]

***S*-*p*-Methylbenzyl Chloro[$^2H_2$]methyl Sulfide (2).** A 48.7-g portion of *p*-methylbenzyl mercaptan (Parrish) was placed in a flask with 15.0 g of [$^2H_2$]paraformaldehyde (Merck Sharp and Dohme). The mixture was cooled in an ice bath and saturated with dry hydrogen chloride; during the saturation process the mixture became quite viscous and 10 mL of dry benzene was added. A 9.0-g portion of anhydrous calcium chloride was then added and the mixture stirred at room temperature for 24 h. The solid material was removed by filtration and washed thoroughly with anhydrous ether. The resulting solution was freed of solvent and the residual liquid distilled at reduced pressure

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Table I. Isotopic Isomers of Oxytocin Used in This Report

isomer	residue no.	isotopically substituted residue
	1	[^{15}N′,α-^{2}H]Cys
	2	[β2,β3-$^2H_2$]Tyr
1	6	[β2,β3-$^2H_2$]Cys
	8	[γ,δ-$^2H_2$]Leu
	1	[β2,β3-$^2H_2$]Cys
	2	[β2,β3-$^2H_2$]Tyr
2	6	[^{15}N′,α-^{2}H]Cys
	8	[^{15}N′]Leu
	1	[^{13}C′,α-^{2}H]Cys
	2	[β2,β3-$^2H_2$]Tyr
	4	[γ2,γ3-$^2H_2$]Gln
3	5	[β2-^{2}H]Asn
	6	[β2,β3-$^2H_2$]Cys
	9	[^{15}N′]Gly
	1	[β2,β3-$^2H_2$]Cys
	2	[β2,β3-$^2H_2$]Tyr
4	5	[^{15}N′,β2-^{2}H]Asn
	6	[^{13}C′,α-^{2}H]Cys
	8	[^{15}N′,α,γ,δ-$^2H_3$]Leu
	1	[α,β3-$^2H_2$]Cys
	2	[β2,β3-$^2H_2$]Tyr
5	6	[α,β3-$^2H_2$]Cys
	9	[α2-^{2}H]Gly

(115 °C, 2 Torr): yield 52.0 g (60%); NMR (CCl_4) δ 7.12 (q, 4 H), 3.78 (s, 2 H), 2.31 (s, 3 H), $ClCH_2$ undetectable.

Diethyl 2-Acetamido-2-*S*-*p*-methylbenzylthio[$^2H_2$]methylmalonate (3). An 8.65-g portion of sodium hydride (50% in oil) was placed in a dry nitrogen-filled reaction flask, covered with dry *N,N*-dimethylformamide (DMF), and cooled at 0 °C. A solution of 39.15 g of diethyl α-acetamidomalonate in 250 mL of DMF was added to the cold hydride mixture over 20 min. Stirring was continued until hydrogen evolution stopped (~1 h). A 33.61-g portion of **2** was added and the flask placed in a 90–100 °C oil bath for 60 h. The mixture was cooled, filtered, and concentrated in vacuo to a brown oil. Trituration with 80 mL of water was followed by three extractions with $CHCl_3$. The combined organic phase was dried over K_2CO_3 and filtered. The filtrate was concentrated in vacuo to a brown solid which was treated eight times with 100-mL portions of boiling hexane. Each time the clear hexane layer was decanted from the oily lower layer. As the hexane cooled white needles were deposited: yield 35.0 g (52%); mp 87.9–88.2 °C; NMR ($CDCl_3$) δ 7.18 (q, 4 H), 7.07 (s, 1 H), 4.24 (m, 4 H), 3.67 (s, 2 H), 2.33 (s, 3 H), 1.24 (t, 6 H), 3.52 (undetectable).

***S*-*p*-Methylbenzyl-DL-[β2,β3-$^2H_2$]cysteine (4).** A 6.0-g portion of **3** was treated with concentrated HCl (35 mL) at reflux for 6 h. After the solution was cooled slightly, Norite was added and the mixture was brought briefly to boiling. The mixture was rapidly filtered and the filtrate was evaporated to dryness. The residue was suspended in water, and concentrated ammonium hydroxide was added to pH 5.5. The resulting crystals were washed with water, ethanol, and ether and dried in vacuo: yield 3.5 g (95%); mp 209–214 °C dec (lit.[11] for protium compound 209–211 °C dec); NMR (2 M NaOD) δ 7.21–6.98 (q, 4 H), 3.65 (s, 2 H), 3.44 (s, 1 H), 2.13 (s, 3 H), (β2, β3) H undetectable.

***S*-*p*-Methylbenzyl-L-[β2,β3-$^2H_2$]cysteine (1).** *N*-Acetyl-*S*-*p*-methylbenzyl-DL-[β2,β3-$^2H_2$]cysteine was obtained by adding 2.3 g of **4** to 5.5 mL of water and treating the slurry with 1.6 g of Na_2CO_3 and 2 mL of acetic anhydride. After 20 min, the solution was acidified with concentrated HCl (to pH 1) and filtered. The solid was washed with 1 M HCl and dried in vacuo, yield 2.47 g (90%). The acetylated amino acid was dissolved in 300 mL of water (solution occurred as the pH was adjusted to 7.5). A 450-mg portion of acylase I (Sigma grade I) was added and the solution incubated at 38 °C for 2 days. At the end of this period 500 mg of acylase I (Sigma grade II) was added and the solution incubated for an additional 2 days. The pH of the digest was adjusted to 10.5 with 2 M NaOH and the mixture stirred until most of the solid dissolved. The solution was filtered and the filtrate passed through a 400-mL Amicon cell containing a Diaflow UM10 filter. The cell was run until all but 10 mL of liquid had passed through. The ultrafiltrate was then concentrated to ~50 mL; a white solid separated. This mixture was adjusted to pH 6 and filtered, and the precipitate washed successively with water, ethanol, and ether and dried in vacuo, yield 660 mg (64% of L); Manning–Moore analysis[12] established the product to be >99.9% L.

***N*′-*tert*-Butoxycarbonyl-*S*-*p*-methylbenzyl-L-[β2,β3-$^2H_2$]cysteine (5).** The title compound was prepared from **1** using *tert*-butoxycarbonyl azide,[13] or by the following procedure using di-*tert*-butyl dicarbonate (Boc anhydride). A solution of **1** was made by dissolving 0.72 g in 12 mL of H_2O–*tert*-butyl alcohol (2:1) under argon by adding a small amount of 10 M NaOH. Di-*tert*-butyl dicarbonate (1.0 mL) was added by drop to the stirred solution. A further 10 mL of *tert*-butyl alcohol was added, and the alcohol removed by three successive extractions with 30-mL portions of pentane. The aqueous phase was adjusted to pH 2 by the addition of 1 M HCl and extracted with four 30-mL portions of ethyl acetate. The organic phases were combined, dried over magnesium sulfate, and filtered. The solvent was removed by rotary evaporation, yield 98%. The product gave a single uniform spot in two TLC systems [1-butanol–acetic acid–water (4:1:5, upper phase only) and chloroform–methanol–acetic acid (85:10:5)].

S-*p*-Methylbenzyl[^{15}N′,α-^{2}H]cysteine (**6**) was prepared similarly by way of the reaction of *S*-*p*-methylbenzyl chloromethyl sulfide with the sodium salt of diethyl [^{15}N]phthalimidomalonate.[10]

***S*-*p*-Methylbenzyl Chloromethyl Sulfide (7).** A 6.25-g portion of paraformaldehyde was treated as described for compound **2**: yield 21.0 g (54%); bp 115 °C (2 Torr); NMR δ 7.03 (q, 4 H), 4.29 (s, 2 H), 3.67 (s, 2 H), 2.19 (s, 3 H).

Diethyl 2-*S*-*p*-Methylbenzylthiomethyl-2-[^{15}N]phthalimidomalonate (8). A mixture of 6.49 g of **7**, 14.0 g of the sodium salt of diethyl [^{15}N]phthalimidomalonate, and 40 mL of anhydrous toluene was refluxed for 2.5 h. The precipitated sodium chloride was filtered and washed thoroughly with toluene. The combined filtrate was concentrated in vacuo to give **8** as an oil.

***S*-*p*-Methylbenzyl-DL-[^{15}N′-α-^{2}H]cysteine (9).** The oil **8** was suspended in 75 mL of 50% [O-^{2}H]ethanol–D_2O and 6 mL of dioxane. One drop of phenolphthalein (1% in [O-^{2}H]ethanol) was added and the mixture was heated to 50 °C. Sodium deuteroxide (5 M) was added dropwise with swirling, until the solution remained basic to the indicator for 10 min (the temperature remained at between 55 and 60 °C throughout the addition of base). The temperature of the mixture was then raised to 70 °C; the flask was removed from the bath and the mixture stirred until the temperature dropped to 40 °C (ca. 20 min). Concentrated DCl was then added until the mixture was acid to the indicator. The mixture was then evaporated to 50% of its original volume. The volume was adjusted to 120 mL with D_2O, 15 mL of concentrated DCl was added, and the mixture was refluxed for 1.5 h. Concentrated DCl (75 mL) was then added and the solution refluxed for an additional 2.5 h. The solution was evaporated to dryness and the residue suspended in 50 mL of water. The slurry was adjusted to pH 5.5 with concentrated ammonia, stirred overnight, and readjusted to pH 5.5. The solid was filtered, washed with water (thrice), warm ethanol (thrice), cold ethanol (thrice), and ether (five times), and dried in vacuo: yield 2.8 g (35%); mp 209–213 °C dec (lit.[11] for unenriched compound 209–211 °C dec); NMR (2 M NaOD) δ 7.23–6.99 (q, 4 H), 3.65 (s, 2 H), 2.97 (q, 1 H), 2.66 (q, 1 H), 2.14 (s, 3 H).

S-*p*-Methylbenzyl[^{13}C′,α-^{2}H]-L-cysteine (**10**) was prepared via a modified Strecker reaction from the suitable aldehyde.[14]

1,1-Diethoxy-2-*S*-*p*-methylbenzylthioethane (11). A 4.6-g portion of sodium was dissolved in 200 mL of absolute ethanol and the resulting solution cooled to 0 °C. A 27.6-g portion of *p*-methylbenzyl mercaptan was added (slowly with continued chilling) followed by 39.4 g of bromoacetal. The mixture was refluxed for 2 h, cooled, and poured into 500 mL of ice water. The product was extracted with ether, and the extract dried over anhydrous sodium sulfate. The solvent was removed, and the residual liquid distilled in vacuo (185 °C, 15 Torr): yield 41 g (80%); NMR δ 7.07 (q, 4 H), 4.49 (t, 1 H), 3.67 (s, 2 H), 3.59–3.31 (m, 4 H), 2.50 (d, 2 H), 2.20 (s, 3 H), 1.11 (t, 6 H). Anal. ($C_{14}H_{22}O_2S$) C, H.

***S*-*p*-Methylbenzyl-DL-[^{13}C′]cysteine (12).** A mixture of **11** (6.35 g) and 5 mL of 5 M hydrochloric acid was shaken at room temperature for 24 h. The resulting mixture was extracted exhaustively with ether, and the aqueous phase was neutralized with sodium carbonate and reextracted with ether. The combined organic phase was treated in

the cold with 3.5 g of sodium sulfite (in 5 mL of H_2O) and 2.6 g of sodium acid sulfite (in 10 mL of H_2O). The mixture was shaken and allowed to stand for 15 min. The solid bisulfite adduct was filtered, washed with ether, and air dried, yield 2.6 g (40%).

A 2.6-g portion of the adduct was suspended in 10 mL of H_2O containing 660 mg of $K^{13}CN$. The mixture was shaken for 2 h, extracted exhaustively with ether, and evaporated to give an oil containing 3-*S*-*p*-methylbenzylthio-2-hydroxy[1-^{13}C]propanonitrile. The oil was suspended in 150 mL of ammonia-saturated 70% ethanol. One gram of ammonium chloride was added and the mixture again saturated with ammonia. The mixture was then stirred for 12 h and evaporated to an oil containing 3-*S*-*p*-methylbenzylthio-2-amino[1-^{13}C]propanonitrile (**13**). The oil was suspended in 50 mL of concentrated HCl and refluxed for 5 h. The resulting solution was treated with Norite, filtered while still hot, and adjusted to pH 5.5 with NH_4OH. The crystals were filtered, washed with H_2O (thrice), cold ethanol (thrice), and ether (five times), and dried in vacuo: yield 1.0 g (40%); mp 210–213 °C (lit.[11] for unenriched compound 209–211 °C); NMR δ 7.20–7.98 (q, 4 H), 3.63 (s, 2 H), 3.45 (m, 1 H), 2.96 (m, 1 H), 2.64 (m, 1 H), 2.15 (s, 3 H).

***N*-Acetyl-*S*-*p*-methylbenzyl-DL-[^{13}C′,α-^{2}H]cysteine (14).** The title compound was obtained by adding 2.3 g of **13** to 25 mL of D_2O and treating the slurry with 6.4 g of Na_2CO_3 and 8 mL of acetic anhydride. The solution was stirred for 12 h, acidified to pH 1 with concentrated DCl, and filtered. The solid was washed with 1 M HCl and dried in vacuo: yield 2.4 g (99%); mp 158.5–159.4 °C; NMR ([$^2H_6$]Me_2SO) δ 8.35 (s, 1 H), 7.19 (q, 4 H), 3.71 (s, 2 H), 2.69 (m, 2 H), 2.31 (s, 3 H), 1.90 (s, 3 H), (α-H undetectable).

***S*-*p*-Methylbenzyl[^{13}C′,α-^{2}H]cysteine (10).** Compound **10** was resolved with hog renal acylase I as described for **1**, yield 690 mg (68%). Manning–Moore analysis[12] established the product to be >99.9% L; NMR (2 M NaOD) δ 7.21–6.99 (q, 4 H), 3.65 (s, 2 H), 2.95 (q, 1 H), 2.65 (q, 1 H), 2.13 (s, 3 H) (α-CH undetectable).

The synthesis of stereospecifically β-deuterated cysteine was accomplished by the procedure of Young et al.,[15] using deuterium instead of hydrogen gas, and employing a larger scale.

Methyl (4*R*,5*R* and 4*S*,5*S*)-2,2-Dimethyl-3-formyl[4,5-$^2H_2$]thiazolidine-4-carboxylate (15). A solution of 500 mg of methyl 2,2-dimethyl-3-formylthiazolidine-4-carboxylate[15] in 350 mL of redistilled ethyl acetate was deuterated at 2 atm over 4 g of 5% palladized charcoal. After 3 h, the mixture was filtered through Celite, and the solvent was removed in vacuo, yield 300 mg (60%).

(4*R*,5*R* and 4*S*,5*S*)-4-Carboxy-2,2-dimethyl-3-formyl[4,5-$^2H_2$]-thiazolidine (16). A 590-mg portion of **15** was treated as described previously, yield 450 mg (82%), mp 201–206 °C (lit.[15] 200–205 °C).

The racemic mixture of [α,β3-$^2H_2$]-L- and [α,β2-$^2H_2$]-D-cysteine hydrochlorides was prepared from **16** by the standard procedure on a tenfold increased scale, yield 520 mg (94%), amino acid analysis—only cysteine detected. Preparation of the MeBzl derivative of the cysteine followed the procedure of ref 11, and its subsequent resolution proceeded as described above. *S*-*p*-Methylbenzyl[α,β3-$^2H_2$]-L-cysteine so produced was >99.9% L, determined by Manning–Moore analysis. NMR examination of the product revealed that the α and β positions were only 67% deuterated, and preliminary investigation suggested that this incomplete deuteration was due to hydrogen gas initially absorbed to the catalyst. No attempts were made to circumvent this problem, since the product was used solely for the purposes of identification of the β-proton shifts. Examination of the integrals of the NMR spectra of protected amino acid suggested that there was no detectable scrambling of deuterium labeling between the β2 and β3 positions, and that the stereochemical assignment was identical with that derived from analysis using the method of vicinal couplings.[6]

NMR Spectroscopy. Proton spectra were measured on a Varian HR-Nicolet Technology Corp. TT-220 spectrometer. Spectra in D_2O or in organic solvents were recorded by pulse and fast Fourier transform techniques. Sample concentration was in the range 5–30 mg/mL, pH was 4.0, and temperature was 22 °C, unless otherwise indicated.

Data Analysis. Analysis and simulation of spectra were performed using an implementation of LAOCN3.[16,17] All chemical shifts are reported downfield from [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)propionate (TSP), which was used as an internal reference.

Several methods were used to establish line positions accurately from spectra: three-point Lorentzian interpolation,[18] resolution enhancement,[19] or band-shape analysis.[20] Generally, at least two of these methods were used with each spectrum. For coupling constants smaller than 4 Hz, resolution enhancement was used in combination with interpolation in order to circumvent the limitations of resolution between adjacent locations of computer memory. The usual 2000-Hz spectral width occupied 4096 20-bit words of computer memory. In comparisons of the separate analyses of the eight transitions of an AB portion of an ABX spectrum, by band-shape analysis and by resolution enhancement combined with interpolation, the range of differences between values derived by the methods was typically significantly less than 0.15 Hz.

Calculations. The calculations of populations of staggered rotamers, values of standard coupling constants for gauche and trans conformations, and probability of correct assignment for staggered rotamers have been previously presented[6] in a study of the side-chain conformation of the amino acid leucine. The extension of these calculations to any side chain in a peptide containing two β protons is obvious.

The calculations concerning fixed torsion angles were made as follows. It is assumed that, for the coupling of the ith substituent of the α carbon to the jth substituent of the β carbon, the value of the predicted vicinal coupling, K_{ij}, as function of torsion angle, χ, is given by

$$K_{ij}(\chi) = A_{ij}\cos^2(\chi^1 + \alpha_i + \beta_j) + B_{ij}\cos(\chi^1 + \alpha_i + \beta_j) + C_{ij} \quad (1)$$

where A, B, and C are constants for the combination of substituents. In this article, only the two proton substituents of the β carbon are considered, $H^{\beta 2}$ and $H^{\beta 3}$, and it is assumed that the same set of A, B, and C applies to each β proton. In view of the sizable errors associated with such an analysis, deviations from ethane-like staggered values (0, ±120°) of the angles α_i and β_j were not considered.

In general, for any set of fixed parameters (A, B, C, α, β), presumed to be valid for a large set of observations and compounds, standard formulas (in this case eq 1) may be applied to produce a set of calculated values $\mathbf{K}(\chi^1)$ that may be compared to the observed values, $\mathbf{J}$. The value of χ for which $\mathbf{K}(\chi^1)$ and $\mathbf{J}$ best agree is then the derived angle, given the assumption that the method of calculation is correct. The position of best agreement may be obtained using R factor analysis, as described by Hamilton.[21] An R factor was calculated for various values of χ^1 (typically 360 such values at 1° intervals) using the equation

$$R = [\sum\sum W_{ij}(J_{ij} - K_{ij})^2 / \sum\sum (W_{ij}J_{ij}^2)]^{1/2} \quad (2)$$

in which W_{ij} are weights and J_{ij} are observed quantities. It is possible to choose the weights in various ways. Using weights proportional to $|J_{ij}|^x$, with x varying from zero to 2, the results obtained were relatively insensitive to values of x.

The derivation of suitable values of A, B, and C in eq 1 has been the subject of much work.[22–26] Values for these coefficients for $^1H^{\alpha}$ coupled to $^1H^{\beta}$ have been proposed.[26] It is widely accepted that the forms of the carbon–proton[24] and nitrogen–proton[23,25] dependencies conform approximately to eq 1, but the possibilities of substantial deviations as a function of substituent electronegativity and other orientational effects have not been investigated in detail. In Table II, we summarize data from model compounds which have been used to calibrate the dependencies, assuming that the form of eq 1 is adequate. Table III shows the coefficients for eq 1 for ^1H–^1H, ^1H–^{13}C, and ^1H–^{15}N vicinal couplings used here.

Results

In Figure 1, the 220-MHz spectra of the β-proton resonances of the half-cystyls 1 and 6 and tyrosyl 2 are illustrated in unsubstituted oxytocin (A), and in four isomers **1–4** along with a simulated spectrum of unsubstituted oxytocin in line-broadened (B) and in stick (C) form. The small vicinal coupling constants in the spectra of **1–4** often required band fitting or resolution enhancement for their precise determination (Figure 2). Values of R factors[21] for band fitting were typically less than 0.02 for a range of 100–400 for the number of points of observation. Line shapes were found to be moderately close to Lorentzian, with some broadening from deuterium coupling, as expected.[27]

The derived heteronuclear coupling constants are shown in Table IV, along with the homonuclear values. These latter are

Table II, Vicinal Coupling Constants Used to Calibrate Angular Dependencies of $^3J(^{13}C'-H^\beta)$ and $^3J(^{15}N'-H^\beta)$

	dihedral angle, deg[a]	value[b] obsd, Hz	value[c] calcd	compd used[d]
$^{13}C'-H^\beta$	0	5.80	5.76	[$^{13}C'$]-ABCO
	60	1.30	1.38	*e*
	120	3.50	3.42	[$^{13}C'$]-ABCO
	180	9.8	9.84	*e*
$^{15}N'-H^\beta$	0	−3.79[f] −3.80	−3.92	[$^{15}N'$]-ABCO
	60	0[g] −0.42	−1.00	[^{15}N,11-^2H]-DAA [$^{15}N'$]-ABCO
		−1.84		*e*
	120	−1.12 −1.0	−1.38	[$^{15}N'$]-ABCO [^{15}N,11-^2H]-DAA
	180	−4.8	−4.67	*e*

[a] Dihedral angles are assumed to be those of the fully eclipsed (0, ±120°) or staggered (180, ±60°) forms. Expected deviations are of the order of ±5° (see, e.g., Table III, ref 26). [b] D_2O, 25 °C, pD ≃4. [c] From eq 1 and the values of Table III. [d] Abbreviations: ABCO, 2-amino[2.2.2]bicyclooctane-2-carboxylic acid; DAA, 9,10-dihydro-9,10-(11-aminoethano)anthracene. See ref 33 for comparable measurements of $^{14}N-^1H$ coupling. [e] Value from gauche or trans rotamer state. For a review, see ref 6. [f] All $^3J(^{15}N-H')$ are assumed to be negative.[6] [g] Approximate value. Range ±0.4 Hz.

Table III. Coefficients of the Equation for the Angular Dependencies of the Vicinal Coupling Constant between the α Substituent and a β Proton

substituent of C^α	value[a] of coefficient *A*	*B*	*C*
^1H	9.4 (0.3)[b]	−1.4 (0.2)	1.6 (0.3)
$^{15}N'$	−3.75 (0.01)	0.26 (0)	−0.54 (0.50)
$^{13}C'$	7.20 (0)	−2.04 (0)	0.60 (0.11)

[a] Data were fit to the equation $A_{ij}\cos^2\theta + B_{ij}\cos\theta + C_{ij}$ by a least-squares method in which $\cos\theta$ was taken to be the independent variable, and θ, the torsion angle between *i*, the substituent on the C^α atom, and *j*, the vicinal β proton. It is assumed that the same set of coefficients pertains to each β proton. These coefficients correspond to those of eq 1 in the text. Data for $^1H^\alpha-^1H^\beta$ couplings are from ref 26, and those for heteronuclear couplings from Table II. [b] Estimates of the standard deviations are in parentheses. Only a small number of data have been used for the heteronuclear values, and it is possible that the standard deviations are substantially underestimated.

very similar to those previously reported.[2] Studies of the temperature dependencies of the heteronuclear couplings did not reveal significant variations with temperature; the variations of line widths and shifts observed were not correlated with the derived values of coupling constants. It is estimated that the range of 95% confidence limits for the values of Table IV is 0.05–0.2 Hz, with the smaller limits associated with the larger absolute values. More detailed analyses of these errors seems unjustified until a larger number of such constants has been experimentally measured in other peptides.

The assignments of the chemical shifts of the β2 protons of half-cystyls 1 and 6 were made by inspection and by simulation of the observed spectrum of the isotopic isomer (**5**) containing stereospecifically substituted half-cystyls. The chemical shifts of both β2 protons are upfield of their respective β3 protons.

If it is assumed that averaging among three staggered rotamers exists for each half-cystyl, the populations of the three staggered rotamers can be derived from the data of Table IV. These suppositive populations are shown in Table V.

On the other hand, if the conformations of these angles are fixed, then an equivalent set of intersections of the observed

Table IV. Vicinal Coupling Constants about the $C^\alpha-C^\beta$ Bonds of the Half-Cystyl Residues of Oxytocin in D_2O[a]

half-cystyl residues	nucleus coupled to β protons	coupling constant, Hz to β2	to β3[b]	isomer[c] used
1	$^1H^\alpha$	5.0 (4.8)	5.7 (4.8)	*d*
	$^{15}N'$	−3.4 (−4.0)	−2.0[e] (−1.5)	I
	$^{13}C'$	3.1 (3.6)	5.0 (5.8)	III
6	$^1H^\alpha$	9.6 (9.6)	3.7 (4.0)	*d*
	$^{15}N'$	−2.3 (−1.9)	−3.9 (−4.0)	II
	$^{13}C'$	2.0 (2.9)	2.3 (4.0)	IV

[a] Concentration about 20 mg/mL, at 20 °C and pD 3.8. [b] These assignments produce the most consistent interpretation, assuming that fixed angles pertain, and are confirmed by direct stereospecific deuteration. Values in parentheses are those calculated for fixed angles of $\chi_1^1 = -120°$ and $\chi_6^1 = +120°$. [c] Isomers of Figure 1. [d] The proton–proton values were measured with natural material. These values are very close to those reported previously by Wyssbrod et al.,[2] from interpolation of data obtained as a function of temperature. [e] All $^3J(^{15}N'-^1H^\beta)$ couplings are assumed to be negative, ref 13.

Table V. Suppositive Populations Calculated from the Coupling Constants of Table IV for Three Staggered Rotamers

half-cystyl residue	from values of *J* between β protons and	p^{I}[b]	p^{II}	p^{III}
1[a] (β2 upfield)	$^1H^\alpha$	0.22	0.28	0.50
	$^{15}N'$	0.04	0.38	0.58
	$^{13}C'$	0.35	0.21	0.44
1 (β2 downfield)	$^1H^\alpha$	0.28	0.22	0.50
	$^{15}N'$	0.58	0.38	0.04
	$^{13}C'$	0.35	0.44	0.21
6 (β2 upfield)	$^1H^\alpha$	0.64	0.10	0.27
	$^{15}N'$	0.78	0.05	0.17
	$^{13}C'$	0.80	0.08	0.12
6[c] (β2 downfield)	$^1H^\alpha$	0.10	0.64	0.27
	$^{15}N'$	0.17	0.05	0.78
	$^{13}C'$	0.80	0.12	0.08

[a] In half-cystyl 1, the chemical shifts (δ) of the β protons are 3.29 and 3.47 ppm downfield from TSP. In the upper analysis $\delta_{\beta 2}$ is 3.29 and $\delta_{\beta 3}$ is 3.47 and in the lower vice versa. [b] The populations (*p*) were calculated by standard methods (see ref 2 and 6) and the states I, II, and III represent χ's of −60, 180, and +60°. [c] For half-cystyl 6, the chemical shifts of the β protons are 2.97 and 3.23 ppm downfield from TSP. In the upper analysis $\delta_{\beta 2}$ is 2.97 and $\delta_{\beta 3}$ is 3.23 and in the lower vice versa.

values on the curves showing the angular dependencies of $^3J(^1H^\alpha-^1H^\beta)$, $^3J(^{15}N'-^1H^\beta)$, and $^3J(^{13}C'-^1H^\beta)$ (often called Karplus curves) would be expected. A presentation of such dependencies and intersections is shown in Figure 3, where the range of intersections is restricted for clarity. Additionally, it is possible to calculate for a cycle of rotation the degree of fit between observed and calculated values of the vicinal coupling constants. Figures 4 and 5 show log *R* factor[29] as a function of χ^1 (see methods) for various combinations of the vicinal coupling.

Discussion

It has been proposed[6,22] that use of the six $H^\alpha-H^\beta$, $^{13}C'-H^\beta$, and $^{15}N'-H^\beta$ vicinal coupling constants circumjacent to the angle χ^1 in an amino acid or peptide might provide a general and precise method of investigating that angle, and indeed we

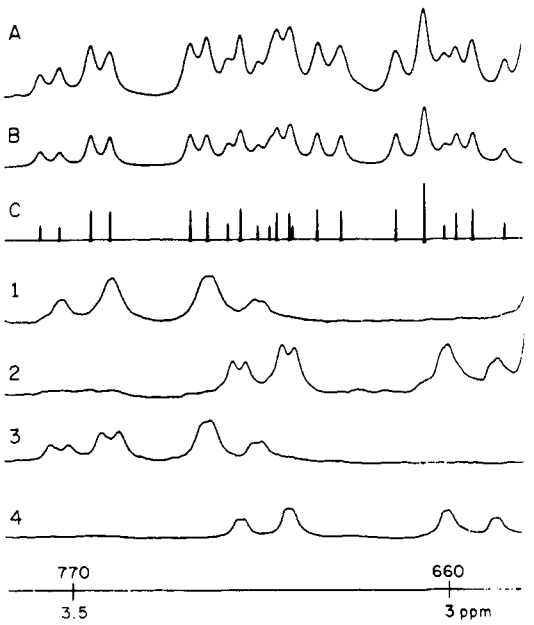

Figure 1. The 220-MHz ^{1}H NMR spectrum of the region between 2.885 and 3.584 ppm for unsubstituted oxytocin (A) and isotopic isomers **1**–**4** in D_2O at 20 °C and pD 3.8. All positions of resonances are downfield with respect to TSP, which was used as an internal standard. A simulation of spectrum A is shown in line-broadened form in B and in stick form in C.

recently demonstrated the experimental feasibility of this approach using isotopic isomers of leucine, where three staggered rotamers about the C^{α}–C^{β} bond exist.[6] In that analysis, good agreement (within ±0.1) was found between populations of rotamers independently derived from the observed $^1H^{\alpha}$–$^1H^{\beta}$, $^{13}C'$–$^1H^{\beta}$, and $^{15}N'$–$^1H^{\beta}$ vicinal coupling constants. Similar agreement has been observed in values from isotopic isomers of the amino acids *S*-*p*-methylbenzylcysteine and cysteine in aqueous solution.[28]

A comparable derivation of suppositive populations for χ_1^1 and χ_6^1 in oxytocin from the values of Table IV is shown in Table V. There is such marked disagreement that we consider that the possibility that averaging occurs among the staggered states about χ_1^1 can be excluded. In the case of χ_6^1, disagreement is less marked, but still substantial, and the large value of one population (I) raises the possibility from this analysis that this angle is fixed, and that the fixed angle might be −60°. We note that in Table V all combinations of assignments of shifts are shown, and that no significant improvements in these calculated values are produced by using the assignments derived from stereospecific substitution of deuterium in the β position.

In considering possible values of fixed angles, *R* factors have been calculated (Figures 4 and 5) for the various couplings. For each of the two half-cystyls, the panels in Figures 4 and 5 represent the calculated log *R* factors for each pair of circumjacent couplings for proton–proton, $^{13}C'$–^1H, and $^{15}N'$–^1H, for the combined values, and for the values derived if the stereochemical assignments are reversed.

For Cys 1, Figure 4 shows that clearly defined absolute minima of log *R* exist for $\chi_1^1 \simeq -120°$ for each circumjacent pair of couplings and for the case where all three sets of couplings are combined. Reversal of assignments of β2 and β3 protons produces a shallower function, with minima close to ±120° and very similar in numerical value.

For Cys 6, Figure 5 shows a less precisely defined situation. A very sharp minimum of log *R* is observed in the proton–proton case at about $\chi_6^1 \sim 120°$, and, although minima are observed at this angle in the heteronuclear sets, these latter sets also contain minima close to −60°. The position of χ_6^1 for the

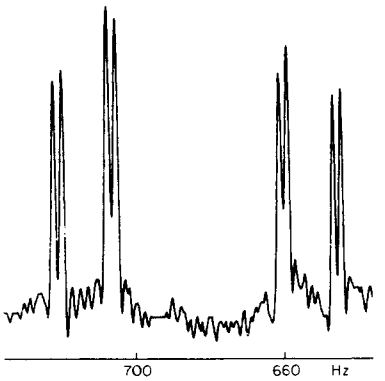

Figure 2. The 220-MHz ^{1}H NMR spectrum of the β-proton region of the half-cystyl 6 residue of isotopic isomer **4** after Fourier transformation of the free-induction decay optimally filtered for resolution enhancement (ref 24). A full width at half-height of 1.5 Hz and a value of *Q* of 10^4 were used. The narrow spacings within the upfield quartet correspond to $^3J(^{13}C'$–$^1H^{\beta 2})$, and those within the downfield quartet to $^3J(^{13}C'$–$^1H^{\beta 3})$. This particular spectrum was chosen for illustration because it was the least favorable case—i.e., of all the paired vicinal couplings, those of this isotopic isomer were the smallest (see Table IV). Compare this spectrum with that of **4** in Figure 1.

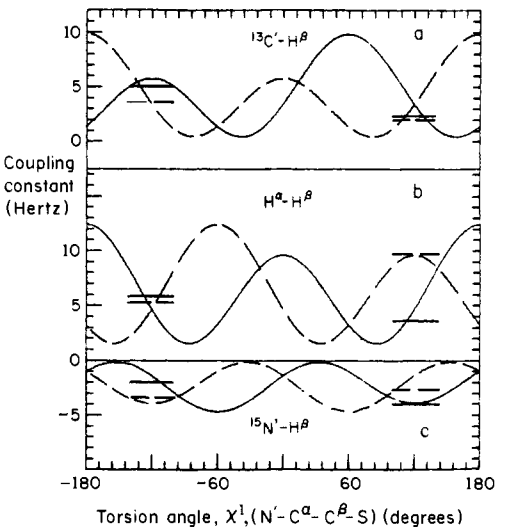

Figure 3. Dependencies on χ^1 of predicted circumjacent vicinal coupling constants between the C^{α} substituents $^1H^{\alpha}$, $^{15}N'$, and $^{13}C'$ and the β protons superimposed on the observed couplings (short horizontal lines) in the half-cystyl residues of oxytocin. Curves associated with β2 protons are dashed. The dependencies have the form of eq 1 in the text, with the constants of Table III. Observed couplings associated with the half-cystyl 1 residue are on the left, and those with the half-cystyl 6 residue on the right.

minimum value of *R* in the set for $^{15}N'$–^1H (curve c, Figure 5) is not well defined; only a small increase in $^3J(^{15}N'$–$^1H^{\beta 3})$ from the observed value of −3.94 Hz results in a shift of the absolute minimum of *R* from +120° to the −80° region. The locus of log *R* for the $^{13}C'$–$^1H^{\beta}$ pair is quite shallow, and has its minimum at −62° (curve b, Figure 5). The observed values (2.0 and 2.3 Hz) of the $^{13}C'$–H^{β} couplings for Cys 6 are quite small considered as a pair compared to any expected values for either a fixed angle or for interconverting conformers. In the combined set, the absolute minimum is at 120°, with another shallow minimum in the region of −100 to −50°. The difference in log *R* between these minima is 0.12. Using standard assumptions,[6,21] the hypothesis that the two minima are indistinguishable may be rejected at greater than the 90% level.[31] Although considerable caution is reasonable in the interpretation of our results, the weight of evidence places χ_6^1 in the region of +120°.

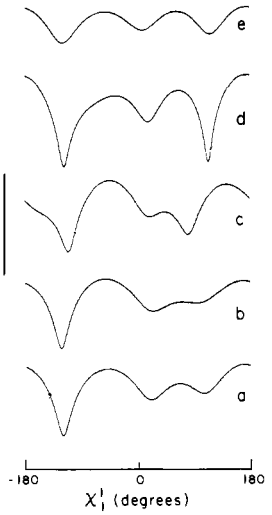

Figure 4. Loci of log R as a function of χ^1 for various combinations of the couplings of the two β protons of the half-cystyl 1 residue of oxytocin to the C^α substituents. R was computed from eq 1 and 2 in the text. In each panel, the value of log R is computed for the calculated couplings as a function of χ^1 for (a) all the six circumjacent couplings of Table IV, (b) the pair to $^1H^\alpha$, (c) the pair to $^{15}N'$, (d) the pair to $^{13}C'$, and (e) the same set as (a) except with the assignments of $\beta 2$ and $\beta 3$ reversed. The factor x used in weighting was chosen to be 1.0 (see Materials and Methods for details). The solid vertical bar on the left is one base-ten logarithmic unit (i.e., a decade of R).

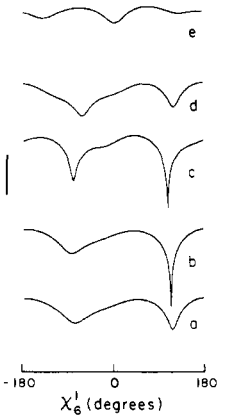

Figure 5. Loci of log R as a function of χ^1 for various combinations of the couplings of the two β protons of the half-cystyl 6 residue of oxytocin to the C^α substituents. See the legend of Figure 4 for additional details.

In our discussion we have first considered the possibility that there might be rotation about the χ^1's of the half-cystyls between staggered rotamers and have rejected that hypothesis in the case of $\chi_1{}^1$ because of the poor agreement between the observed and calculated circumjacent couplings. Secondly, the observed couplings have been compared to those expected if fixed angles pertain to the C^α-C^β bonds of the half-cystyls, and reasonable agreements have been found. We have not considered cases in which there is averaging over nonstaggered rotamers or libration about a fixed angle. It is probable that a more detailed interpretation involving these additional considerations may be feasible when a larger body of consistent data for homo- and heteronuclear circumjacent couplings has been gathered, and these problems are under investigation in this laboratory.

An important factor in extending such interpretations would be more precise representations of the angular dependencies of the various vicinal coupling constants. The dependencies for both heteronuclear couplings are less well defined than for the homonuclear proton–proton case. Among other uncertainties concerning the $^{15}N'$–$^1H^\beta$ coupling, there appears to be substantial uncertainty concerning the value around a dihedral

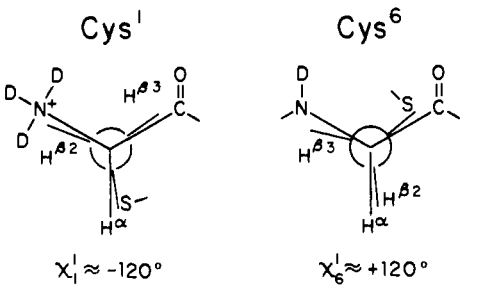

Figure 6. Probable fixed conformations about the C^α-C^β bonds in the half-cystyl 1 and 6 residues of oxytocin in D_2O at pD 3.8 and 20 °C. Small deviations of these conformations from the eclipsed conformation are shown for the sake of clarity. The direction of deviation was chosen to correspond to that which most likely might pertain.

angle of +60°. It is possible to derive a value from compounds of fixed conformation around this angle by scaling data from ^{14}N studies[25,33] or by direct ^{15}N measurements on similar enriched compounds (Table II). The value derived in this way, about −0.3 Hz, is much smaller in absolute magnitude than that derived from the assumption that the gauche coupling obtained from rotamers, −1.8 Hz, is equivalent to that at +60°. Similar uncertainties concern the value at the dihedral angle around 180°.[25]

The semiquantitative uses of eq 1 are well known. Various corrections and possible limitations have been recognized.[23,24,34] Deviation from the analysis would be expected for a large range of substituent electronegativities or for deformed bond lengths or bond angles. In the particular case of the χ^1 angle of peptides it is expected that this type of analysis should be more reliable than in some other kinds of molecular fragment. In peptides the range of electronegativities of substituents is small with the exception of threonine and serine,[26] and in those peptides and proteins that are internally moderately flexible[2,35] it should be expected that distortion of bond angles and bonds lengths and energetically unfavorable steric interactions would be relatively unusual.

Serious discrepancies from observed values of $^3J(H-^{15}N)$ and $^3J(H-^{13}C)$ and those calculated via eq 1 might arise from substituent effects which have an orientational dependence,[36,37] although such effects for couplings involving protons are thought to be usually small.[38] The compounds used for calibration in Table II do not all contain the same pattern of β[37] or γ[36] substituents, and the derived constants of Table III are not therefore free of these possible orientational effects.

It has previously been suggested on the basis of $^3J(H^\alpha-H^\beta)$ values[2,39] and from a study of lanthanide binding to tocinoic acid[39] that $\chi_1{}^1$ and $\chi_6{}^1$ are fixed. Our present report excludes the possibility[2] that $\chi_6{}^1$ is about 0°.[40] The lanthanide binding study[39] suggested a value of 73°, as defined here, for $\chi_6{}^1$ in tocinoic acid. The torsion angles in that study[39] are defined differently, and are converted to those of this discussion by addition of 120° and reversal of sign. This value of $\chi_6{}^1$ seems excluded in oxytocin by the approximate equality of the observed $^{13}C'$–H^β coupling for Cys 6, implying a similar dihedral angle between C′ and the two β protons. The determination of $\chi_1{}^1$ for tocinoic acid from lanthanide binding[39] was less precise (−20 to +80°) than that of $\chi_6{}^1$, and disagrees with the value of −120° reported here. It is possible that the observed conformation of tocinoic acid is different from that of oxytocin, or that the assumptions made in analysis of data on the lanthanide-induced shifts[41] are not completely fulfilled, or that neglect of libration and interconversions between nonstaggered rotamers in either or both studies leads to these inconsistencies.

The balance of evidence then strongly suggests that in oxytocin in aqueous solution $\chi_1{}^1$ is fixed and has a single value

in the neighborhood of −120°, and that χ_6^1 probably also has either a single fixed value of ~+120° in combination with some unusual factors affecting the heteronuclear couplings or is possibly librating about the +120° value.[42] A further, more remote, possibility is that χ_6^1 interconverts between various values, one or more of which is not staggered. This last case seems hard to fit qualitatively to a situation in which small, approximately equal couplings for $^{13}C'$–H^β are combined with pairs of one large and one small value for $^{15}N'$–H^β and H^α–H^β couplings.[34]

Figure 6 shows projections of the χ_1^1 and χ_6^1 derived. It is obvious that these eclipsed conformations are expected to be energetically somewhat unfavorable, particularly that of χ_6^1 in which the C_6' and S_6^γ atoms are at their closest approach distance for their 1–4 interaction, and their torsion energy is at its most unfavorable.[43] The relative rigidity of these two angles in the disulfide bridge and previous suggestions that backbone torsion angles in the vicinity of the fourth and fifth residue of the ring (glutaminyl 4 and asparaginyl 5) are restricted in conformational interconversion[3,7,44] suggest that the main site of conformational flexibility in the tocin ring is around the second and third residues (tyrosyl 2 and isoleucyl 3). Complete definition of these stereoisomeric structures is expected to add significantly to our knowledge of the conformation–function relation of oxytocin, and we are actively pursuing that goal in this laboratory.

This study has shown that measurement of the complete set of couplings to β protons circumjacent to the C^α–C^β bond in peptides can be readily accomplished in a series of synthetic isotopic isomers. The stereochemical assignment of the β protons was also derived from the couplings and by direct stereospecific replacement with deuterium. The two χ^1's of cysteine in oxytocin are probably not averaged among staggered rotamers. In one case, χ_1^1, a single fixed eclipsed conformation is strongly suggested. In the case of χ_6^1, a more complex situation pertains, though it seems likely that it too possesses a single fixed eclipsed conformation.

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References and Notes

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(29) The use of the logarithm seems appropriate, because it is the difference in the ratio of *R* factors, $\mathcal{R}$, that is of significance (ref 21). Log $\mathcal{R}$ then is simply represented by log R_1 − log R_2.
(30) The form of *R* as a function of χ^1 is a relatively simple serial summation of circular functions which may be stated explicitly. We have chosen to use numerical methods since it is then easier to obtain intermediate results and to vary arguments in the calculations.
(31) Probabilities for values of the ratio of *R* factors are tabulated in ref 21, from which log $\mathcal{R}_{2,4,0.1} = 0.25$.
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(42) Such libration could be responsible for the moderately large temperature dependencies of $^3J(H^\alpha$–$H^\beta)$'s for cys[6] reported in ref 2.
(43) An approximate estimate of the additional energies of the eclipsed conformers compared to the staggered is available from empirical calculation. Using a program specially designed for peptides (Browman, M. J., et al. *QCPE* **1975**, *11*, 286) a difference of about 4.5 kcal/mol is calculated, which consists of contributions from torsional (~60%), nonbonded (~35%), and electrostatic components. The −120 and +120° conformers were almost equally disfavored in this empirical calculation.
(44) For a review see: Glickson, J. D. "Peptides: Chemistry, Structure and Biology", Walter, R., Meienhofer, J., Eds.; Ann Arbor Science Publishers: Ann Arbor, Mich., 1975; pp 787–802.