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References

Baddiley, J., Buchanan, J. G., and Carss, B. (1957), *J. Chem. Soc. (London) 3,* 4058–4063.

Bullock, F. J., and Jardetzky, O. (1965), *J. Org. Chem. 30,* 2056–2057.

Edmondson, D. E. (1974), *Biochemistry 13,* 2817–2821.

Edmondson, D. E., and Singer, T. P. (1973), *J. Biol. Chem. 248,* 8144–8149.

Kainosho, M., and Kyogoku, Y. (1972), *Biochemistry 11,* 741–752.

Kenney, W. C., Edmondson, D. E., and Singer, T. P. (1974a), *Biochem. Biophys. Res. Comm. 57,* 106–111.

Kenney, W. C., Edmondson, D. E., Singer, T. P., Steenkamp, D. J., and Schabort, J. C. (1974b), *FEBS Lett. 41,* 111–114.

Koziol, J. (1971), *Methods Enzymol. 18B,* 276–285.

McCormick, D. B. (1970), *J. Heterocycl. Chem. 7,* 447–450.

Möhler, H., Brühmüller, M., and Decker, K. (1972), *Eur. J. Biochem. 29,* 152–155.

Müller, F., Hemmerich, P., and Ehrenberg, A. (1971), in Flavins and Flavoproteins, Kamin, H., Ed., University Park Press, Baltimore, Md., pp 107–120.

Müller, F., Massey, V., Heizmann, C., Hemmerich, P., Lhoste, J. M., and Gould, D. C. (1969), *Eur. J. Biochem. 9,* 392–401.

Nakagawa, H., Osano, A., and Sato, R. (1975), *J. Biochem. 77,* 221–232.

Pinto, J. T., and Frisell, W. R. (1975), *Arch. Biochem. Biophys. 169,* 483–491.

Salach, J., Walker, W. H., Singer, T. P., Ehrenberg, A., Hemmerich, P., Ghisla, S., and Hartmann, U. (1972), *Eur. J. Biochem. 26,* 267–278.

Singer, T. P., and Edmondson, D. E. (1974), *FEBS Lett. 42,* 1–14.

Singer, T. P., and Kenney, W. C. (1974), *Vitam. Horm. (N.Y.) 32,* 1–45.

Walker, W. H., Kenney, W. C., Edmondson, D. E., Singer, T. P., Cronin, J. R., and Hendricks, R. (1974), *Eur. J. Biochem. 48,* 439–448.

Walker, W. H., Salach, J., Gutman, M., Singer, T. P., Hyde, J. S., and Ehrenberg, A. (1969), *FEBS Lett. 5,* 237–240.

Walker, W. H., Singer, T. P., Ghisla, S., and Hemmerich, P. (1972), *Eur. J. Biochem. 26,* 279–289.

Solution Conformation of a Retro-D Analogue of Tocinamide†

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ABSTRACT: The solution conformation of a retro-D analogue of tocinamide

H-D-Cys-D-Asn-D-Gln-D-aIle-D-Tyr-NHCH$_2$CH$_2$S

was examined using proton magnetic resonance and circular dichroism spectroscopy. The observations support major contributions to the conformational distribution from structures with a type I β turn in the sequence D-Asp-D-Gln-D-aIle-D-Tyr. This is topologically similar to the β turn proposed for oxytocin, L-Tyr-L-Ile-L-Gln-L-Asn, but with the polarity of the CONH groups reversed along the chain; the peptide is, however, hormonally inert. In conjunction with nuclear magnetic resonance data, the circular dichroism spectra are interpreted to indicate that the region of the peptide ring near the disulfide occurs in at least two different conformations. One of the side-chain carboxamides, probably that of asparagine, appears to be intramolecularly associated rather than freely exposed to solvent.

Incorporation of D-amino acid residues has been one tool in the study of structure–activity relationships of biologically active peptides. Enantiomers of several peptide hormones and active fragments have been found to be essentially inactive (Rudinger, 1971), but examination of peptides made of enantiomeric amino acids coupled in reverse sequence has been more hopeful. Shemyakin and his collaborators (1969) suggested that there might be prepared enzymatically resistant retro-D analogues of peptide hormones (or active fragments) in those cases where the parent molecules are devoid of N- and C-terminal charges and of proline residues. Supporting examples include retro-D-[Gly^{5,10}]gramicidin S (Shemyakin et al., 1967), which has antibiotic activity equal to that of the parent L-antibiotic. Similarly, the synthetic retro-D analogue of [Tyr^6]antamanide sulfate ester was found substantially to retain the activity of the L-compound in antagonizing the lethal effect on mice of the toxin phalloidin (Wieland et al., 1972). However, Vogler et al. (1966) found retro-D-bradykinin to be inactive as an agonist or antagonist of bradykinin. Because bradykinin bears charged termini and contains proline residues, its retro-D analogue would not be expected to be sufficiently

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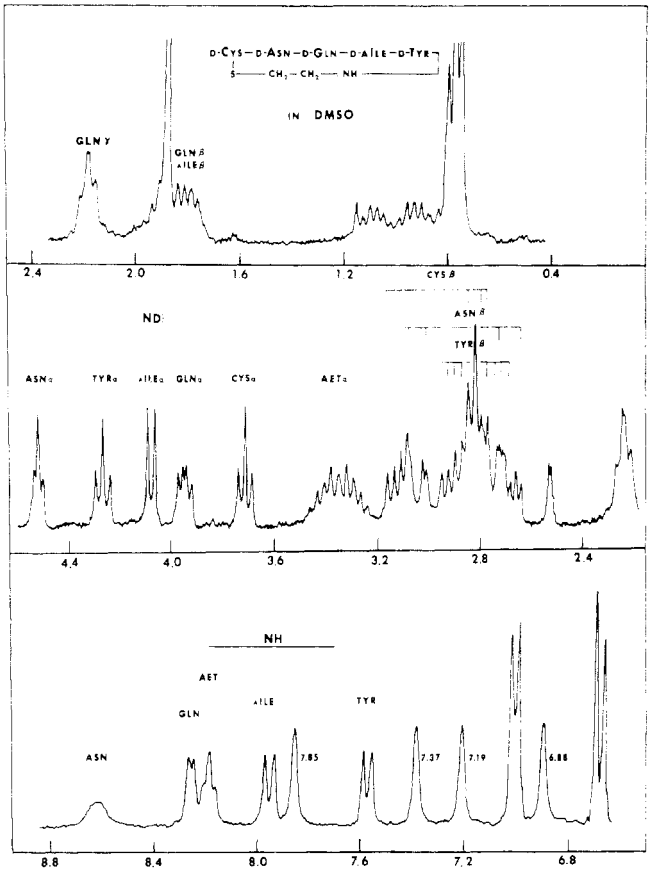


FIGURE 1: 250-MHz proton magnetic resonance spectra of the cyclic peptide in dimethyl-d_6 sulfoxide 30 °C, about 45 mg/ml. Chemical shifts are in ppm from internal tetramethylsilane. The spectrum in the 0–4.5 ppm range is of a peptide sample in which exchangeable protons have been replaced by deuterium. The intense singlet at 1.9 ppm is assigned to an equivalent of acetic acid present in the peptide sample; the peak at 2.5 ppm is from residual protons on the Me_2SO.

similar in distribution of side chains and charge to bind to the bradykinin receptor.

Recently the Shemyakin suggestion was tested by the synthesis and biological evaluation of retro-D analogues of deaminotocinamide (Nakagawa et al., 1976). Deaminotocinamide is the peptide ring of oxytocin, and is devoid of N or C termini and proline residues; it retains about 7% of the oxytocic activity of the parent hormone. In contrast, the peptide

H-D-Cys-D-Gln-D-aIle-D-Tyr-NHCH$_2$CH$_2$-S

called [D-aIle^3]retro-D-deaminotocinamide and its *N*-formyl derivative are only weak competitive inhibitors of oxytocin in the oxytocic assay, at doses 10^6–10^7 larger than that of the hormone. Since similarities in the topochemical arrangement of side chains in [D-aIle^3]retro-D-deaminotocinamide and deaminotocinamide are possible, it was suggested by Hechter et al. (1975) that one reason for the lack of activity of the retro-D compound might be alteration of the sense of the backbone amide units, which might have an essential role in transmitting the hormonal message.

We thought it feasible to shed light on this problem by studying the solution conformation of [D-aIle^3]retro-D-deaminotocinamide, to establish whether there are similarities to oxytocin or deaminooxytocin.

Experimental Section

Proton Magnetic Resonance. Proton magnetic resonance spectra were measured with the 250-MHz instrument of the Carnegie-Mellon NMR Facility for Biomedical Research, operated either in the slow scan continuous wave mode or in the rapid scan correlation mode. (The spectra shown in the figures are single, slow-scan spectra.) Double resonance experiments on peptide and deuterium-exchanged peptide were employed to identify the protons of the amino acid residues. Sample temperature was about 30 °C, and peptide concentration was about 45 mg/ml.

Circular Dichroism and Absorption Spectra. Circular dichroism (CD)[1] spectra of the peptide in aqueous solution were measured on a Cary 60 spectropolarimeter with a 6003 circular dichroism accessory. Peptide concentrations used were adjusted so that the absorbance was never greater than 1.5 in the region being examined. Circular dichroism data were recorded on a strip chart and also on magnetic tape. The raw data were smoothed by multiscan averaging and use of a Fourier transform filtering program (Bush, 1974). Absorption spectra were obtained with a Cary 15 spectrophotometer, which was flushed with nitrogen for measurements below 200 nm. All spectra were measured at about 25 °C. The pH of the solutions used was 5.3.

The processed circular dichroism data were subjected to decomposition using a digital computer program that matches the observed spectrum to a selected number of Gaussian curves by an iterative nonlinear least-squares procedure (Dickinson and Bush, 1975).

Results

Proton Magnetic Resonance Assignments. The assignments are shown in Figure 1, and the details of chemical shift and coupling constants are given in Table I.

Because of the quality of the spectra obtained and the fortunate, overlap-free placement of N–H and H^α resonances, it was not difficult to assign sets of resonances to single residues by double resonance experiments. It proved possible to identify all of the β-proton lines of the asparagine, half-cystine, and tyrosine residues that occur in the complex region of the spectrum between 2.6 and 3.2 ppm, a region which also includes the S–CH_2 of the aminoethanethiol unit. Identification of the residues themselves utilized the following guideposts: the half-cystine H^α was identified as that not coupled to any observed N–H resonance; the half-cystine nitrogen, present as amino or ammonium ion, is broadened to invisibility by exchange and/or quadrupole relaxation. The isoleucine H^α is a doublet in the spectrum of a deuterium-exchanged sample. An ambiguity in the assignments to asparagine and tyrosine, which occurs because both H^α are coupled to otherwise uncoupled β-CH_2 groups in the 3 ppm region, was resolved by noting that the N–H resonance at 8.63 ppm is very broad (ca. 25 Hz). The broadened resonance is assigned to the asparagine N–H, subject to proton exchange catalyzed by the proximate amino function of the half-cystine. The N–H line of the *N*-penultimate residue of a sequence has been observed to be broadened in other neurohypophyseal hormone derivatives that bear a terminal free amino group (Glickson et al., 1972; Von Dreele et al., 1971a; Johnson et al., 1969). This assignment is corroborated by the two-bond proton–proton couplings of the corresponding β-methylene groups. For the CH_2 assigned to the tyrosine residue the coupling is 13 Hz, and for that assigned to asparagine it is 16.5 Hz, a significant difference consistent with observations in other asparagine and tyrosine derivatives (Kopple and Ohnishi, 1969; Ziauddin, 1972).

[1] Abbreviation used: CD, circular dichroism.

Table I: Proton Resonances of H-D-Cys-D-Asn-D-Gln-D-alle-D-Tyr-NHCH$_2$CH$_2$S– in Dimethyl Sulfoxide, 30 °C.[a]

Residue	δ_{NH}	$J_{H\alpha H}N$	δ_α	$J_{H\alpha B\beta}$	δ_β	$J_{H\alpha H\beta}$	Other
Cys			3.72	6.2	2.80	14	
				6.8	3.12		
Asn	8.63	(*b*)	4.54	4.8	2.67	16.5	$CONH_2$: 7.19, 7.85[c]
				4.2	3.05		
Gln	8.26	4	3.96	Σ = 12.8[d]	~1.85		$C\gamma H_2$: 2.20
							$CONH_2$: 6.88, 7.37
Allo-Ile	7.95	9	4.09	7	~1.85		$C\gamma H_2$: 0.8–1.2
							CH_3: 0.76, 0.77
Tyr	7.56	8	4.28	8.0	2.72	13.2	
				6.6	2.90		
Aet[e]	8.20	Σ = 11[d]	~3.35		2.8[f]		

[a] Chemical shifts in ppm downfield from internal tetramethylsilane, coupling constants in Hz. [b] Broad line, width at half-height 25 Hz. [c] Uncertain assignment; see discussion in text. [d] Sum of two coupling constants. [e] Aminoethanethiol. [f] Under higher field Cys β.

The principal challenge in assigning the resonances lies in the singlets arising from the carboxamido protons of the glutamine and asparagine side chains. These resonances have been reported to lie in narrow regions, 6.7–7.0 ppm for a carboxamido proton cis to the carbonyl oxygen and 7.3–7.5 ppm for one trans, in all spectra of oxytocin, vasopressins, and their analogues in dimethyl sulfoxide near 30° (Von Dreele et al., 1971b; Richard Brewster et al., 1973; Brewster et al., 1972). Assignments to asparagine or glutamine have been on the basis of small differences within these ranges in precursors, model compounds, and analogues of the hormones with asparagine or glutamine replaced. Considering the large range of chemical shifts that an amide proton may exhibit depending on its average state of hydrogen bonding, this use of analogy to assign resonances when asparagine and glutamine are present in the same molecule must be somewhat doubtful, but nothing more definite has appeared to replace it. Fortunately, in most of the derivatives studied so far, the uncertainty has been of little consequence for building models of preferred conformations. Indeed, one may take the constancy of the chemical shifts as an indication that the carboxamide protons are only associated with the solvent, i.e., their environment does not change from compound to compound when the solvent is kept constant. In the present peptide, however, one carboxamide proton has a chemical shift well outside of the usual ranges, at 7.85 ppm. By the hypothesis just stated, this is probably useful conformational information, which makes it of interest to have assignments specifically to asparagine or glutamine.

Upon irradiation of the carboxamido proton resonance at 6.88 ppm there is a slight decrease, readily observable in difference spectra, in the intensity of another singlet at 7.37 ppm. This effect can be ascribed to transfer of saturation by rotation about the C–N bond of a carboxamido group (Von Dreele et al., 1971a, 1972). Thus, the 6.88 and 7.37 ppm resonances can be taken, respectively, as those of the cis and trans protons of one carboxamido group, occurring in the usual positions. The remaining pair of singlet resonances at 7.85 and 7.19 ppm then correspond to the other carboxamide. It is not possible to define with confidence cis and trans in this second set; either both resonances are shifted downfield about 0.5 ppm, or one is unchanged and the other shifted about 1 ppm. Irradiation of the 7.85 ppm resonance does not produce a decrease in the 7.19 ppm intensity, presumably because of more hindered rotation about the corresponding C–N bond.

We have not been successful in assigning these pairs to asparagine or glutamine. Using difference spectroscopy we looked for effects of irradiation into the asparagine β-proton resonances around 3.05 and 2.67 ppm and the glutamine γ-proton resonances at 2.2 ppm, searching for indication of residual spin–spin coupling or a nuclear Overhauser effect. Any effects were too small to be reproducible. These experiments did incidentally reveal transfer of saturation from residual water in the solvent specifically to the exchange-broadened asparagine peptide N–H (Von Dreele et al., 1972; Glickson et al., 1976).

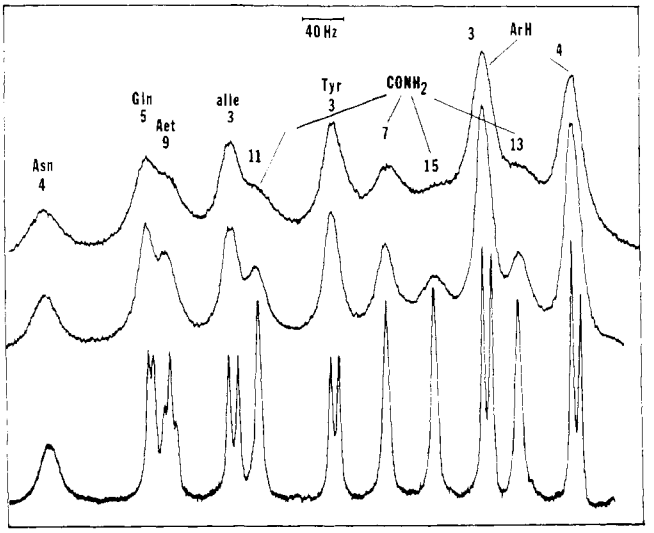


FIGURE 2: Low-field region of the spectrum, peptide concentration about 40 mg/ml, 30 °C in dimethyl-d_6 sulfoxide containing 0, 2, and 4.5 volume percent 2,2,4,4-tetramethyloxazolidinyl-3-oxy. The numbers given with the assignments are estimates of the observed line width increments in units of Hz at half-height percent added nitroxyl. The spectra were measured using an internal Me_4Si lock.

Solvent Exposure. Figure 2 shows the effect of 2 and 4.5 volume % 2,2,4,4-tetramethyloxazolidinyl-3-oxy on the low-field region of the spectrum. Greater broadening of a resonance indicates closer average approach by the unpaired electron spin to the proton giving rise to the resonance. The acidic N–H protons of peptide and amide units may associate specifically with the N–O group of the nitroxyl by hydrogen bonding, but for the nonacidic C–H protons of the aromatic ring only collision complexes are likely. In general, then, one expects greater sensitivity of the N–H resonances to radical (Kopple and Schamper, 1972a,b).

Among the peptide and amide protons the extent of exposure depends on the degree of substitution at the N–H unit. The resonances of side chain $CONH_2$ groups, primary amides, are

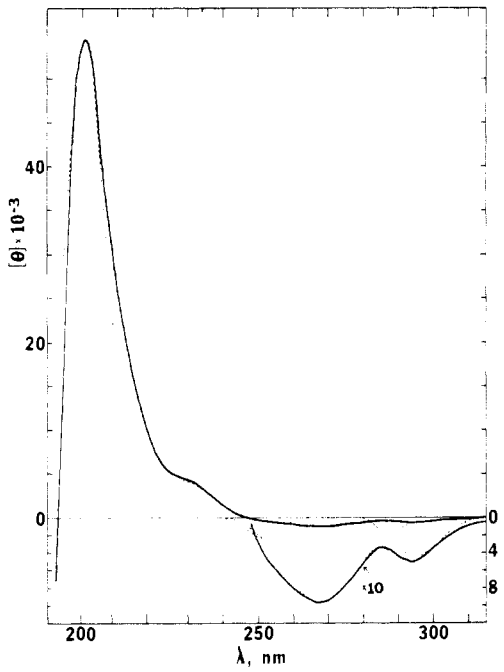


FIGURE 3: Circular dichroism spectrum of the cyclic peptide (solid line) and the Gaussian components (dashed line) described in the text and listed in Table II. The spectrum is shown expanded 10× vertically above 250 nm. Aqueous solution, pH ca. 5, 25 °C.

the most sensitive, although there is considerable variation among them. Of the secondary amide N-H units, the resonance of the aminoethanethiol unit is most sensitive. That N-H is probably readily approached by the nitroxyl, since sensitivity of the same order is exhibited by the resonances of the exposed N-H protons of the ornithine and phenylalanine residues in the cyclic peptide gramicidin S. The N-H resonances of the true amino acid residues are all considerably less sensitive to nitroxyl than a fully exposed proton; this includes the asparagine proton resonance, even though it is exchange broadened. The N-H's of tyrosine and isoleucine are more shielded than the others; they are apparently no more affected by radical than are the aromatic protons.

Tentatively, it might be suggested that the side chain amide proton resonances at 7.19 and 6.88 ppm are more sensitive to radical than the remaining two because they correspond to N-H protons cis to the carbonyl oxygen, thus trans to the side chain and potentially more exposed to solvent. This interpretation would resolve one of the ambiguities with regard to the low-field pair of carboxamide resonances.

Conclusions from NMR Data. Certain of the NMR data suggest a peptide backbone with a β turn of type I (Venkatachalam, 1968) formed by the sequence Asn[1]-Gln[2]-aIle[3]-Tyr[4]. (The superscripts represent the usual numbering of residues in a β turn.) The large proton-proton coupling (9 Hz) of the H-N-C$^{\alpha}$-H unit of the aIle residue indicates an anti arrangement of the two protons, corresponding to $\varphi_3 = 120 \pm 20°$. The 4-5 Hz coupling of the corresponding protons of the Gln residue fits either a dihedral angle of $20 \pm 10°$, corresponding to $\varphi_2 \approx -40$ or $-80°$, or a dihedral angle of close to 120°, corresponding to $\varphi_2 = 60°$. The angles $\varphi_2 = -40$, $\varphi_3 = 120°$ are appropriate for a type I β turn composed of two D residues. In agreement with the β-turn hypothesis is the fact that the peptide proton of the tyrosine residue is least sensitive to the line broadening effects of the added nitroxyl. This result is to be expected if the tyrosine N-H bond is directed to the interior of the turn. The sequestering of the isoleucine N-H is also explained: examination of a space-filling model of this turn shows that approach of radical to this proton is severely hindered by the flanking side chains. In the proposed β turn the N-H bond of the glutamine residue would be directed outwards from the bend backbone; of the N-H protons of the true amino acid residues this is, in fact, the most sensitive to added radical, although not so much so as might be expected for a proton fully exposed to dimethyl sulfoxide.

In addition to this suggestion of a β turn, the NMR data provide several other guides for construction of a model of the peptide. These are enumerated below.

(a) The N-H of the aminoethanethiol unit is fully exposed to the external environment of the peptide, judged by the sensitivity of its resonance to nitroxyl. The other peptide protons are all apparently shielded by backbone and/or side chains.

(b) The peptide N-H of asparagine must be in proximity to the ammonium ion of the half-cystine unit at least some of the time to explain the observed exchange broadening of its resonance.

(c) The dominant rotamer at the C$^{\alpha}$-C$^{\beta}$ bond of asparagine has two gauche H-C-C-H angles, i.e., χ_1 is $-60°$. This is inferred from the relatively low, 4-5 Hz, vicinal couplings.

(d) Rotation about the C$^{\alpha}$-C$^{\beta}$ bond of the half-cystine unit is not severely hindered. The 6-7 Hz couplings observed for the H$^{\alpha}$-H$^{\beta}$ pairs of this residue may be read to suggest that all three of the standard staggered rotamers are equally likely.[2] This is highly unlikely for bonds in the backbone of a peptide ring. A looser, but more likely interpretation is that the approximate equality of the couplings indicates that the two rotamers with one gauche and one anti vicinal proton-proton coupling, $\chi_1 = 60$ and 180°, are present equally. A less likely alternative is that there is averaging over ±30° about the eclipsed state, $\chi_1 = -120°$.

(e) One of the $CONH_2$ groups must be in an unusual environment; this is suggested by its chemical shifts.

Circular Dichroism. The absorbance spectrum of the peptide (not shown) is similar to that of tyrosine; it is dominated above 200 nm by the π-π^* transitions of the aromatic system at 275 and 222.5 nm. The disulfide unit contributes only very weak (electric dipole forbidden) n-π^* absorptions above 230 nm (Carmack and Neubert, 1967), and these are hidden under the tyrosine bands.

The circular dichroism spectrum (Figure 3) may be compared with the published CD spectrum of oxytocin in water (Walter et al., 1968) and the assignments may be made using the results of the detailed study by Fric et al. (1974a,b, 1975) of the hormone and analogues with the disulfide or tyrosine chromophores replaced. Decomposition of the spectrum into Gaussian bands results in at least seven components (Table II). Transitions calculated to be at 295, 268, 229, and 201 nm, plus one below 198 nm, are obvious in the spectrum itself. A negative band at 251 nm appeared consistently in those decompositions that gave the best fit in the long wavelength region. These bands fit the spectrum (see Figure 3) except between 207 and 225 nm, where the program located one or more positive peaks that could not be defined with precision.

The small negative bands at 295 and 251 nm may be assigned to low energy transitions of the disulfide chromophore, since they are at wavelengths well removed from the 275-nm absorbance of tyrosine. They are discussed further below. The

[2] Casey and Martin (1972) suggest $J_g = 2.5$ and $J_t = 12.8$ Hz; Kopple et al. (1973), $J_g = 3.2$, $J_t = 12.4$ Hz. Averaging over the three staggered rotamers would give $J_{av} \approx 6$ Hz.

Table II: Circular Dichroism Bands of H-D-Cys-D-Asn-D-Gln-D-alle-D-Tyr-$NHCH_2CH_2S$— in Water, pH 5, 25 °C.

Assigned Transition[a]	Wavelength, (nm)[c]	Molar Ellipticity ($[\theta] \times 10^{-3}$)[c]	Half-Width at Half-Maximum (nm)	Rotational Strength (cgs units $\times 10^{40}$)
Disulfide n–σ^*	295	−0.44	8.6	−0.18
Tyrosine π–π^*	268 (281)	−0.97 (−0.3)	12.4	−0.67
Disulfide n–σ^*	251 (250)	−0.21 (0.9)	5.6	−0.07
Tyrosine π–π^*	229 (229)	4.3 (6.6)	8.8	2.4
Amide n–π^*	210–225[b]	4–10		
Amide π–π^*	201 (203)	54 (−54)	6.1	27
Tyrosine π–π^*	198 (195)	Negative (−78)		

[a] See discussion in text. [b] One or more bonds not definitely characterized. [c] Values in parentheses are those given for oxytocin, pH 7.5, by Fric et al. (1974a).

negative band centered at 268 nm is derived from the B_{2u} band of tyrosine. It is the only band that lies under the tyrosine absorption in this region, and its size and position are similar to bands in published spectra of peptides containing tyrosine as the only chromophore absorbing in this region (Chen and Woody, 1971; Bush and Gibbs, 1972; Beychok, 1967). The 228-nm band is under the 223-nm B_{1u} absorption of tyrosine; it is also in the region of amide n–π^* transitions, as observed, for example, in gramicidin S (Urry, 1970). Following Fric et al. (1974a,b, 1975), we may ascribe this band to the tyrosine transition, although it may contain amide n–π^* contributions, and the additional positive ellipticity in the 210–220-nm region may be considered to be amide n–π^* bands. The studies of Fric et al. indicate that the 201-nm band is likely to be an amide π–π^* transition, and the negative band below 198 nm is probably the E_{1u} transition of the tyrosine chromophore.

The bands assigned to the aromatic chromophore of D-tyrosine in the present peptide have the same sign as those assigned to the aromatic system of L-tyrosine in aqueous solutions of oxytocin and its analogues (Fric et al., 1974a,b), including tocinamide

H-L-Cys-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys-NH_2

(Fric et al., 1975). The magnitudes are also similar. This is worthy of remark, and it might suggest that the phenolic ring bears a relation to nearby perturbing groups, such as peptide bonds, that is of similar chirality in both the retro-enantio and natural series. Fric et al. suggest that the Cys^1 amino group of oxytocin has a significant effect on the transitions of the tyrosine chromophore. In the peptide studied here the terminal amino group is probably well removed from the tyrosine.

The 201-nm amide π–π^* band of the retro-enantio peptide has an ellipticity that is opposite in sign (positive) to that of oxytocin. This is consistent with the chain of amide groups going around the peptide ring in the opposite sense.

Published CD spectra of oxytocin (Urry, 1970; Walter et al., 1968) and tocinamide (Fric et al., 1975) show a weak band of negative ellipticity ($[\theta] \approx -100$) at about 300 nm and a somewhat more intense positive band ($[\theta] \approx 1000$) at 250–260 nm. Both of these bands are assigned to the disulfide chromophore. In the present case the 295-nm band is somewhat stronger and also negative, and the 250-nm band is somewhat weaker and negative rather than positive. We suggest that these two bands do not arise from two different transitions of the disulfide unit in one conformation, but correspond instead to the long wavelength transitions of two distinct, slightly more stable conformations of the disulfide region of the peptide ring. Our arguments follow.

In the present study, the two bands at 295 and 251 nm are of the same sign, whereas in cyclic disulfides of fixed conformation the transition of second lowest energy appears below 250 nm and is of opposite sign to the long wavelength band. Also, the intensity of the bands in our peptide (Table II) are less than one-tenth those observed for the rigid compounds *trans*-2,3-dithiodecalin (290 nm, $[\theta] = -17\,000$; 241 nm, $[\theta] = 15\,000$ (Carmack and Neubert, 1967)), and 2,7-cystinegramicidin S (272 nm, $[\theta] = -7600$; 230 nm, $[\theta] = 61\,000$ (Ludescher and Schwyzer, 1971)). Malformin A (280 nm, $[\theta] = -4000$; 240 nm, $[\theta] = 13\,000$ (Bodanszky, et al., 1975)) is probably a further example of a disulfide in a restricted conformation.

The wavelength, as well as the molar ellipticity of the n–σ^* transitions, is a function of the C–S–S–C dihedral angle (Bergson, 1958; Boyd, 1974; Woody, 1973). The lowest energy n–σ^* transition ranges from ca. 250 nm (90°) to near 350 nm (0 or 180°), and its wavelength can be used, therefore, to estimate the departure of the dihedral angle from 90°. If a disulfide unit occurs in many torsional states, the observed CD spectrum will consist of a continuum of bands of varying ellipticities in the 250–350-nm region. In the present peptide much of this continuum would be obscured by the tyrosine band. Any slight preference for a narrow range of C–S–S–C angle would manifest itself as a weak maximum like those we observe.

Casey and Martin (1972) have shown that in acyclic cystine derivatives the sign and intensity of two circular dichroism bands at ~255 and ~285 nm correlate with the rotamer population about the α–β bond of the cystine estimated from NMR studies. Whatever its intrinsic affect on the rotatory strength of the C–S–S–C group at fixed angle, the α–β rotation should not directly affect the energy of the n–σ^* transition. However, so much as the α–β rotation affects the distribution of C–S–S–C dihedral angles it would correlate with the ellipticity and wavelength of the long wavelength n–σ^* transition.

Because a quadrant rule applies, the negative band at 295 nm observed for the present peptide agrees in sign and wavelength with a C–S–S–C dihedral angle either near −60 or near 120° (Woody, 1973, inter alia). Models of the cyclic peptide can be constructed for these angles and many in between, and so do not resolve the ambiguity. As just noted, the low intensity of the observed band indicates that there is probably no strong preference for a narrow range of C–S–S–C angles. Thus, the circular dichroism data agree with the coupling constants of the half-cystine residue (see above) in suggesting flexibility for the disulfide region of the cyclic peptide.

Likely Conformations. It is possible to incorporate the preceding interpretations of NMR and CD data into the construction of space-filling (CPK) models of the peptide. Models can be generated containing either the −60 or +120° S–S dihedral angles suggested, as slightly favored by the CD measurements. Torsional angles for these models, rounded to the nearest 30°, are given in Table III, and the models are shown in Figure 4. The torsional angles can readily be varied by 30° in at least one and generally in either direction from the angles quoted in Table III. There is certainly no reason to be-

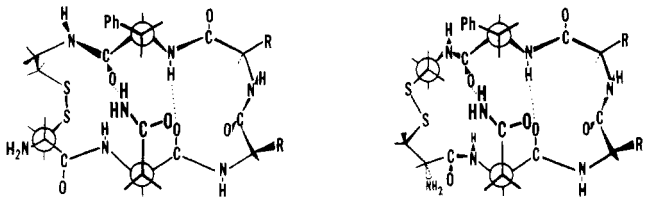


FIGURE 4: Schematic representation of conformations of

cyclo-(D-Cys-D-Asn-D-Gln-D-aIle-D-Tyr-NHCH$_2$CH$_2$S-)

containing the β turn at Asn-Gln-aIle-Tyr with, at left, a C-S-S-C dihedral angle of ca. −60°, and, at right, a C-S-S-C dihedral angle of ca. 120°.

Table III: Possible Conformations of

H-D-Cys-D-Asn-D-Gln-D-aIle-D-Tyr-NH$_2$CH$_2$CH$_2$S-

	Asn	Gln	aIle	Tyr	Aet[b]	Cys
			For θ_{SS} = −60°			
ϕ	150	−30	120	150	−120[b]	
ψ	−150	90	−30	−90		−120
χ_1	−60				60	180
χ_2					150	−30
			For θ_{SS} = 120°			
ϕ	150	−30	120	150	60[b]	
ψ	−120	90	−30	−150		30
χ_1	−60				180	−60
χ_2					90	180

[a] Dihedral angles rounded to nearest multiple of 30°; conventions of IUPAC–IUB (1970). [b] For aminoethanethiol the angle C^β–C^α–N–C′ is given, not ϕ.

lieve that the actual molecule is rigid.

Both models incorporate an Asn-Gln-aIle-Tyr type I β turn; they contain the associated transannular hydrogen bond between the tyrosine peptide N–H and the glutamine peptide carbonyl. In this turn the alloisoleucine N–H is shielded from radical, if not from the less bulky solvent, by the γ-methyl or methylene of its own side chain plus the β-methylene of the glutamine side chain, and the glutamine peptide N–H is somewhat protected by its side chain carboxamide group.

In the model with θ_{SS} = 120°, the peptide N–H of asparagine is directed somewhat to the inside of the peptide ring, and it is adjacent to the half-cystine amino group; this placement can account for the observation that its resonance is broad but not very sensitive to the effect of nitroxyl. In the model with θ_{SS} = −60°, the asparagine N–H is directed entirely to the inside of the ring, toward the tyrosine carbonyl oxygen, and the cystine amino group is not close to it. However, dihedral angle changes that are without large apparent barriers and that do not grossly affect the overall backbone shape can bring the two groups into proximity.

In both models the aminoethanethiol N–H and the half-cystine amino group are readily accessible to solvent and to nitroxyl.

The asparagine side chain is assigned the torsional angle χ_1 = −60° by reason of the low H^α–H^β coupling constants. This places the primary amide group over the backbone and ring and permits in either model a hydrogen bond between side chain N–H and the tyrosine carbonyl. Such an environment for the $CONH_2$ group might explain the downfield position of one set of primary amide proton resonances, which are thereby assigned to the asparagine. A likely situation was earlier proposed for the asparagine residue of the cyclic heptapeptide evolidine. In the proposed evolidine model, as in the present case, an asparagine residue is also in an extended backbone conformation, and the H^α–H^β couplings are also observed to be small. The $CONH_2$ resonances (unambiguously) are at 7.33 and 7.84 ppm (Kopple, 1971).

Returning to the present models, with the asparagine side chain in χ_1 = −60°, the rotation χ_1 = −60° is excluded for the tyrosine side chain; this cannot be confirmed by but is not at variance with the H–H coupling constants observed for the tyrosine. For the aIle residue in the position proposed, χ_1 = 60 and 180° are possible rotamers; again this is not contradicted by the observed coupling constant. The model does not exclude any of the staggered rotamers for the α–β bond of the glutamine residue; there are no coupling constants available to provide guidance.

An average over only the two conformations presented is not consistent with the values of the half-cystine H^α–H^β coupling constants, which have already been discussed. However, model building indicates that there are consistent with the limited data other allowed conformations of the molecular backbone in the region of the disulfide bond, even when the proposed β turn is retained at the other end of the ring. The possibilities are multiplied many fold if the S–S dihedral angle is allowed to vary. The disulfide torsional barrier is not intrinsically high, perhaps 8 kcal (Fraser et al., 1971), and, as has been noted above, the apparent low oscillator and rotational strengths of the disulfide transitions of the peptide are consistent with its existence in a wide range of torsional states. The models presented, therefore, serve only as a rough guide to the average shape of the molecule for use in comparison with oxytocin and analogues composed of amino acids of the L series.

Discussion

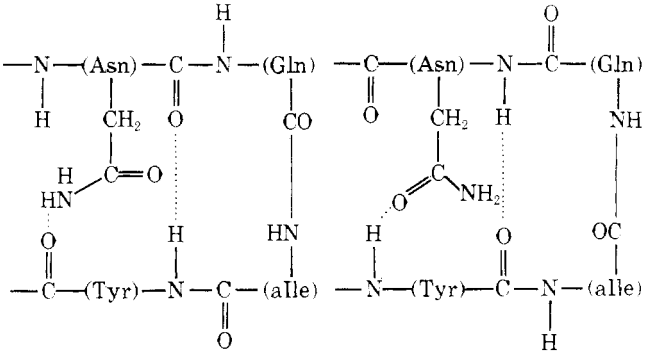
Because the tocinoic acid ring is not homodetic, peptides that bear a retrocyclic relationship will not be functionally equivalent, the amino and carboxyl ends of the peptide backbone being reversed. Thus, there is no exact retro-L analogue of the present peptide. The closest analogues containing amino acids of the natural series are tocinamide

H-L-Cys-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys-NH$_2$

and deaminotocinamide

SCH$_2$CH$_2$CO-L-Tyr-L-Ile-L-Gln-L-Asn-L-Cys-NH$_2$

The proton magnetic resonance spectra of tocinamide and deaminotocinamide in dimethyl sulfoxide have been measured. According to the data these peptides do *not* favor a conformation corresponding to that proposed here for the retro-D compound (Brewster et al., 1972). Such a corresponding conformation would have the sense of the peptide CONH units reversed, but the side chains and α carbons positioned similarly; this is indicated schematically for the suggested β-turn sequence:



proposed β turn in retro D peptide

analogous β turn in normal L sequence

If this β turn, expected to be type I, were to occur in the L-series tocinoic acid derivative, one would anticipate a large value of the H^{α}-H^{β} coupling for the glutamine residue, and a small value for the isoleucine residue. In fact, the glutamine coupling is quite small (1-3 Hz) and the isoleucine coupling quite large (8-9 Hz). One would also expect that the isoleucine peptide N-H be exposed to solvent and that the asparagine peptide N-H would be shielded. In fact, according to the temperature dependence criterion, the isoleucine N-H is highly sequestered in both peptides and the asparagine peptide N-H is exposed in tocinamide, although less so in deaminotocinamide.

Another difference between the L-series peptides and the retro-D peptide is that the side chain carboxamide proton resonances of the L peptides occur in the usual ranges, 6.9-7 and 7.3-7.4 ppm, but one pair of the $CONH_2$ resonances of the retro-D peptide is shifted 0.4-0.5 ppm downfield. This difference does not alone indicate a different placement of side chains however, since the sense of the hydrogen bond between side chain and backbone could be different in the two series even if the backbone foldings did correspond. (See the structures above.)

The structures proposed for tocinamide and deaminotocinamide by Brewster et al. (1972) do not contain a β turn. The observations have been interpreted to suggest that one feature of these molecules is a hydrogen bond between an N-H of the half-cystine carboxamide and the carbonyl of the asparagine side chain. If this interaction is an important conformational determinant, replacement of that C-terminal $CONH_2$ by an N-terminal amino in the retro-D peptide group might well result in an entirely different overall structure.

The β turn proposed for the D-Asn-D-Gln-D-aIle-D-Tyr sequence of our retro-D-decarboxytocinoic acid derivative does correspond, mutatis mutandis, to the L-Tyr-L-Ile-L-Gln-L-Asn β turn of the Urry-Walter conformation for oxytocin itself (Urry et al., 1970; Urry and Walter, 1971). The turn is proposed for the hormone (in dimethyl sulfoxide) largely on the basis of the negligible temperature dependence and high-field position relative to the other peptide N-H (7.9 ppm) of the asparagine peptide N-H resonance. Analogous observations in the present case are the insensitivity to nitroxyl and relatively high-field position (7.56 ppm) of the tyrosine N-H resonance. (Peptide protons sequestered from solvent by the backbone in a β turn do often come into resonance at the high-field end of the peptide N-H range. There can be some argument about the reason for this.)

The coupling constant data, which clearly suggest a type I turn in our peptide, are unfortunately more equivocal in the oxytocin case (Johnson et al., 1969; Richard Brewster et al., 1973). As to the rest of the oxytocin molecule, the published CD spectra suggest to us that it does have stronger preference for a particular range of S-S dihedral angles than our peptide, a consequence, no doubt, of the involvement of the tripeptide tail in determining the structure of the ring. We do not believe, however, that the sense or angle can be unambiguously given in either case.

It would be of interest to compare the side-chain positions in retro-D compound with those in oxytocin and analogues. As mentioned, we believe that the asparagine and glutamine side chains are solvated by dimethyl sulfoxide in all of the derivatives for which data have been published, but that in the retro-D compound one carboxamide, probably the asparagine side chain, is associated intramolecularly. Not many H^{α}-H^{β} coupling constants have been reported. Urry and Walter (1971) report couplings of 3 Hz for the tyrosine of oxytocin in dimethyl sulfoxide. These indicate that only one side chain rotamer, either $\chi_1 = 60$ or 180°, is allowed, which is a greater constraint than present in the substance studied here.

In conclusion, this investigation does not shed any light on the nature of hormone-receptor binding. Had the solution backbone we derive for the retro-D compound

$$\text{H-D-Cys-D-Asn-D-Ile-D-Tyr-NHCH}_2\text{CH}_2\text{S}$$

been entirely different from that generally associated with oxytocin models, it would have been obvious to suggest that the change in backbone conformation led to loss of biological activity. In fact, it seems that the most certain feature of the solution conformation is the same β turn, with carbonyl and N-H units reversed, that is the most certain feature of the Urry-Walter model. This could support the hypothesis that elements of the backbone, as well as of the side chains, are necessary for activity, but hardly proves it. As stated above, we interpret the long wavelength circular dichroism of the disulfide unit to indicate ready accessibility of a range of different conformations for the disulfide regions not only in our compound, but in tocinamide and oxytocin. If this is so, even modest binding energies can significantly affect the shape of the molecule, and detailed models based on estimates of solution conformation can only be speculative.

References

Bergson, G. (1958), *Ark. Kemi 12,* 233-237.

Beychok, S. (1967), in Poly-α-amino Acids, Fasman, G., Ed., New York, N.Y., Marcel Dekker, pp 293-337.

Bodanszky, M., Stahl, G. L., and Curtis, R. W. (1975), *J. Am. Chem. Soc. 97,* 2857-2859.

Boyd, D. E. (1974), *J. Phys. Chem. 78,* 1554-1563.

Brewster, A. I., Glasel, J. A., and Hruby, V. J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1470-1474.

Bush, C. A. (1974), *Anal. Chem. 46,* 890-895.

Bush, C. A., and Gibbs, D. E. (1972), *Biochemistry 11,* 2421-2427.

Carmack, M., and Neubert, L. A. (1967), *J. Am. Chem. Soc. 89,* 7134-7136.

Casey, J. P., and Martin, R. B. (1972), *J. Am. Chem. Soc. 94,* 6141-6151.

Chen, A. K., and Woody, R. W. (1971), *J. Am. Chem. Soc. 93,* 29-37.

Coleman, D. L., and Blout, E. R. (1968), *J. Am. Chem. Soc. 90,* 2405-2416.

Dickinson, H. R., and Bush, C. A. (1975), *Biochemistry 14,* 2299-2304.

Fraser, R. R., Boussard, G., Saunders, J. K., Lambert, J. B., and Mixan, C. (1971), *J. Am. Chem. Soc. 93,* 3822-3823.

Frič, I., Kodiček, M., Flegel, M., and Zaoral, M. (1975), *Eur. J. Biochem. 56,* 493-502.

Frič, I., Kodiček, M., Jost, J., and Blaha, K. (1974a), *Collect. Czech. Chem. Commun. 39,* 1271-1289.

Frič, I., Kodiček, M., Prochazka, Z., Jost, K., and Blaha, K. (1974b), *Collect. Czech. Chem. Commun. 39,* 1290-1302.

Glickson, J. D., Rowan, R., Pitner, T. P., Dadok, J., Bothner-by, A. A., and Walter, R. (1976), *Biochemistry 15,* 1111-1119.

Glickson, J. D., Urry, D. W., and Walter, R. (1972), *Proc. Natl. Acad. Sci. U.S.A., 69,* 2566-2569.

Hechter, O., Kato, T., Nakagawa, S. H., Yang, F., and Flouret, G. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,*

563–566.
IUPAC-IUB Commission on Biochemical Nomenclature (1970), *Biochemistry 9,* 3471–3479.
Johnson, L. F., Schwartz, I. L., and Walter, R. (1969), *Proc. Natl. Acad. Sci. U.S.A. 64,* 1269–1275.
Kopple, K. D. (1971), *Biopolymers 10,* 1139–1152.
Kopple, K. D., and Ohnishi, M. (1969), *J. Am. Chem. Soc. 91,* 962–970.
Kopple, K. D., and Schamper, T. J. (1972a), *J. Am. Chem. Soc. 94,* 3644–3646.
Kopple, K. D., and Schamper, T. J. (1972b), in Chemistry and Biology of Peptides, Meinehofer, J., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, pp 75–80.
Kopple, K. D., Wiley, G. R., and Tauke, R. (1973), *Biopolymers 12,* 627–636.
Ludescher, U., and Schwyzer, R. (1971), *Helv. Chim. Acta. 54,* 1637–1644.
Nakagawa, S. H., Yang, F., Kato, T., Flouret, G., and Hechter, O. (1976), *Int. J. Pept. Protein Res.* (in press).
Richard Brewster, A. I., Hruby, V. J., Glasel, J. A., and Tonelli, A. E. (1973), *Biochemistry, 12,* 5294–5304.
Rudinger, J. (1971), in Drug Design, Vol. II, Ariens, E. J., Ed., New York, N.Y., Academic Press, pp 319–419.
Shemyakin, M. M., Ovchinnikov, Yu. A., and Ivanov, V. T. (1969), *Angew. Chem. Int. Ed. Engl. 8,* 492–499.
Shemyakin, M. M., Ovchinnikov, Yu. A., Ivanov, V. T., and Ryabova, I. R. (1967), *Experientia 23,* 326.
Urry, D. W. (1970), in Spectroscopic Approaches to Biomolecular Conformation, Urry, D. W., Ed., Chicago, Ill., American Medical Association, pp 33–119.
Urry, D. W., Ohnishi, M., and Walter, R. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 111–116.
Urry, D. W., and Walter, R. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 956–958.
Venkatachalam, C. M. (1968), *Biopolymers 6,* 1425–1436.
Vogler, K., Lanz, P., Lergier, W., and Hafely, W. (1966), *Helv. Chim. Acta 49,* 390–403.
Von Dreele, P. H., Brewster, A. I., Scheraga, H. A., Ferger, M. F., and du Vigneaud, V. (1971a), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1028–1031.
Von Dreele, P. H., Brewster, A. I., Bovey, F. A., Scheraga, H. A., Ferger, M. F., and du Vigneaud, V. (1971b), *Proc. Natl. Acad. Sci. U.S.A., 68,* 3088–3091.
Von Dreele, P. H., Scheraga, H. A., Dykes, D. F., Ferger, M. F., and du Vigneaud, V. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3322–3326 (1972).
Walter, R., Gordon, W., Schwartz, I. L., Quadrifoglio, F., and Urry, D. W. (1968), in Peptides, 1968, Bricas, E., Ed., New York, N.Y., Wiley, pp 50–55.
Wieland, T., Penke, B., and Birr, C. (1972), *Justus Liebigs Ann. Chem. 759,* 71–75.
Woody, R. W. (1973), *Tetrahedron 29,* 1273–1283.
Ziauddin, Kopple, K. D., and Bush, C. A. (1972), *Tetrahedron Lett.* 483–486.