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Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Oxytocin, Related Oligopeptides, and Selected Analogs†

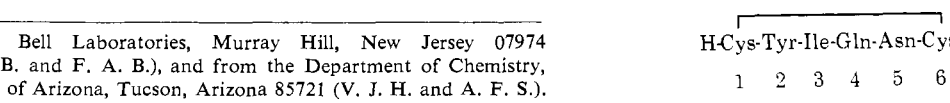
A. I. Richard Brewster,* V. J. Hruby, A. F. Spatola, and F. A. Bovey

ABSTRACT: The C-13 nuclear magnetic resonance (nmr) spectra of oxytocin and several analogs in dimethyl sulfoxide solution have been studied. Assignments of all 43 carbon resonances have been made by reference to the spectra of the linear precursors and through the use of partially deuterated peptides and oxytocin derivatives. The closing of the disulfide bond yielding oxytocin from the linear nonapeptide precursor results in a number of significant chemical shift changes, particularly of the α and β carbons, which cannot at present be interpreted in detail. Significant chemical shift differences are observed for some oxytocin analogs which may be related to conformational changes.

In the past few years there has been a marked upsurge of interest in the conformations of cyclic polypeptides. Their study by proton nuclear magnetic resonanse (nmr) has been particularly rewarding and has been recently reviewed (Urry and Ohnishi, 1970; Bovey *et al.*, 1972; Hassall and Thomas, 1971; Thomas, 1973). Very recently, ^{13}C nmr has been applied to this problem. Investigations of gramicidin S (Gibbons *et al.*, 1970), of valinomycin and its K^+ complex (Ohnishi *et al.*, 1972; Patel, 1973a), of antamanide and its Na^+ complex (Patel, 1973b,c), and of oxytocin (Smith *et al.*, 1972; Deslauriers *et al.*, 1972; Bovey, 1972) have been reported. As yet, ^{13}C nmr data, *i.e.*, chemical shifts and in some studies T_1 values, cannot be as directly interpreted in terms of conformation as ^1H nmr data. One of the most useful and unambiguous conformation correlations is that of the chemical shift of the γ carbon of proline with the conformation, cis or trans, of the preceding peptide bond (Dorman and Bovey, 1973; Thomas and Williams, 1972; Wüthrich *et al.*, 1972; Deslauriers *et al.*, 1972). As we shall see, other marked variations in carbon shielding can occur which seem to be primarily related to alterations of conformation.

In this paper, we present assignments for the ^{13}C spectra of the neurohypophyseal hormone, oxytocin, its analogs, [7-D-proline]-oxytocin and [4-glycine]-oxytocin, and deamino-oxytocin, as well as for seven precursors of oxytocin. These assignments are presented in more detail and with more supporting evidence than in the preliminary report (Bovey, 1972). They differ at some points from those of Deslauriers *et al.* (1972) for oxytocin.

Oxytocin is a nonapeptide with a hexapeptide cyclic portion closed by a disulfide bond and a tripeptide linear segment terminated by a carboxamide group.



Various aspects of the conformation of oxytocin and related peptides have been studied by proton magnetic resonance (pmr) spectroscopy (Johnson *et al.*, 1969; Urry *et al.*, 1970;

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Hruby *et al.*, 1971; Urry and Walter, 1971; Feeney *et al.*, 1971; Brewster *et al.*, 1972).

Experimental Section

Materials

The precursor peptides to oxytocin, Pro-Leu-Gly-NH_2 (I), *S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (II), Z-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (III), Z-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (IV), Z-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (V), Z-*O*-Bzl-Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (VI), and Z-*S*-Bzl-Cys-Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (VII), and oxytocin (VIII) were prepared by the methods of Bodanszky and du Vigneaud (1959). Oxytocin was purified by partition chromatography on Sephadex G-25 (Yamashiro *et al.*, 1966) and by gel filtration on Sephadex G-25 (Porath and Flodin, 1959). The [4-glycine]-oxytocin (IX) was a generous gift from Professor V. du Vigneaud. Deamino-oxytocin (X) was prepared by published procedures (Ferrier *et al.*, 1965). [7-D-Proline]-oxytocin (XI) was prepared by methods similar to those previously reported (Ferraro and du Vigneaud, 1966).

Deuterated analogs of oxytocin and related precursors were prepared *via* solid-phase peptide synthesis and/or classical routes. The following compounds were prepared: *S*-PMB[1]-Cys-*O*-Bzl-Tyr-Ile-Gln-Asn-S-MeOBzl-Cys-Pro-Leu-[α,α-$^2H_2$]-Gly-NH_2 (XII), *S*-Bzl-[α,β,β-$^2H_3$]-DL-Cys-*O*-Bzl-Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 (XIII), *S*-Bzl-Cys-*O*-Bzl-Tyr-Ile-Gln-Asn-*S*-Bzl-[α,β,β-$^2H_3$]-DL-Cys-Pro-Leu-Gly-NH_2 (XIV), [1-[α,β,β-$^2H_3$]hemi-L-cystine]-oxytocin (XV), and [6-[α,β,β-$^2H_3$]hemi-L-cystine]-oxytocin (XVI). [1-[$\alpha,\alpha,\beta,\beta$-$^2H_4$]-$\beta$-Mercaptopropionic acid]-oxytocin (XVII) was a gift from Professor V. du Vigneaud. The experimental procedure which follows for [9-[α,α-$^2H_2$]glycine]-oxytocin (XVIII) illustrates the general solid-phase methods used.

Samples of [α,α-$^2H_2$]glycine were generous gifts from Dr. Jay Glasel and Professor A. T. Blomquist. The synthesis of Boc-[α,α-$^2H_2$]glycine (XIX) was carried out according to the method of Schnabel (1967). The purity of the product was confirmed by melting point and thin-layer chromatography. Nuclear magnetic resonance spectroscopy established the product as $\geq 96\%$ deuterated in the α position. Attachment of XIX to a chloromethylated styrene resin (2% cross-linked with divinylbenzene, chlorine substitution = 2.07 mmol/g) was accomplished in dimethylformamide according to the method of Marglin (1971) to give a glycine substitution of 0.39 mmol/g.

The solid-phase synthesis of protected nonapeptide XII was based on the synthesis of deamino-oxytocin (Takashima *et al.*, 1968) following the methodology of Hruby *et al.*, (1972). The peptide XII was cleaved from the resin by ammonolysis (Takashima *et al.*, 1968) and deprotected with sodium in $NH_{3(l)}$ (Sifferd and du Vigneaud, 1935). Oxidation to [9-[α,α-$^2H_2$]glycine]-oxytocin was effected with potassium ferricyanide (Hope *et al.*, 1962). The product was purified using partition chromatography on Sephadex G-25 (Yamashiro *et al.*, 1966) followed by gel filtration on Sephadex G-25 (Porath and Flodin, 1959). The lyophilized white powder gave only a single spot on silica gel thin-layer chromatography, identical with that of authentic oxytocin in the solvent systems 1-butanol–H_2O–HOAc (4:1:1) and 1-butanol–HOAc–H_2O–pyridine (15:3:12:10).

The syntheses of [1-[α,β,β-$^2H_3$]hemi-L-cystine]-oxytocin (XV) and [6-[α,β,β-$^2H_3$]hemi-L-cystine]-oxytocin (XVI) were also performed by the solid-phase technique. Details of this work including the synthesis of *S*-benzyl-[α,β,β-$^2H_3$]-DL-cysteine in Professor Blomquist's laboratory will be published elsewhere (Cornelius *et al.*, 1973).

Methods

^{13}C nmr spectra were obtained using a Varian XL-100 spectrometer which has been modified for pulse Fourier transform spectroscopy and interfaced with a Nicolet Model 1080 computer (Sternlicht and Zuckerman, 1972). The free induction decay was stored in 8K computer locations, using a dwell time of 100 μsec. The pulse was located at 25.16512 MHz, *i.e.*, beyond the high field end of the spectrum. Repetition intervals varied between 1 and 8 sec. Between 4000 and 8000 spectra were accumulated for all samples except oxytocin, for which 16,000 spectra were employed. Tetramethylsilane was used as internal standard. Chemical shifts are reported as parts per million from $^{13}CS_2$. All observations were made at 29°.

The compounds were each dissolved in deuterated dimethyl sulfoxide (99.5% ^2H), $(C^2H_3)_2SO$, to a concentration of about 7% (w/v). A microcell surrounded by water in a 12-mm diameter nmr tube held the sample.

Deuterium substitution of the samples was found to be as effective for assignment of carbons as it is for assignment of protons. Because of a combination of slower relaxation, broadening arising from $^{13}C-^2H$ coupling, and reduced Overhauser enhancement, deuterium substituted carbons essentially disappear from the spectrum. This will be illustrated in later discussion.

Results

The spectra of oxytocin and of its seven precursors are shown in Figure 1. The tentative assignments for all the resonances in the spectra of these seven compounds are presented in Figure 2. The large multiplet between 151 and 156 ppm is due to the solvent, Me_2SO-d_6, which appears as a 1:3:6:7:6:3:1 septet from $^{13}C-^2H$ coupling. The use of a sequence of precursors allows one to make assignments with some assurance, a task which would otherwise be nearly impossible. Heteronuclear spin decoupling of the protons removed all multiplicity of the ^{13}C resonances so this could not be used as a means of identification. Reference to the spectra of cysteine, cystine, and [$C_{2,6}$-$^2H_2$]tyrosine and to other ^{13}C studies of amino acids and peptides aided assignments (Horsley *et al.*, 1970; Christl and Roberts, 1972; Voelter *et al.*, 1971; Jung *et al.*, 1970).

Throughout the spectra of the precursors the chemical shift for each peak remains fairly constant. The effect of the carbobenzoxy blocking group is seen in the downfield position of the α carbon of the residue blocked at the N-terminal position. As this blocking group is removed by the addition of another residue, the α carbon moves upfield to its characteristic position in subsequent spectra.

The existence of cis–trans isomerism about the cysteine–proline peptide bond is evident in the spectrum of the *S*-benzyl-tetrapeptide, as was noted in the ^1H nmr spectrum of this compound (Hruby *et al.*, 1971). With the addition of the next amino acid, asparagine-5, one isomer only is present, and, as observed by Deslauriers *et al.* (1972), only one isomer is present in Z-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2. An explanation of this cannot yet be offered. This is inferred to be the trans

[1] Abbreviations used are: MeOBzl, *p*-methoxybenzyl; β-MPA, β-mercaptopropionic acid.

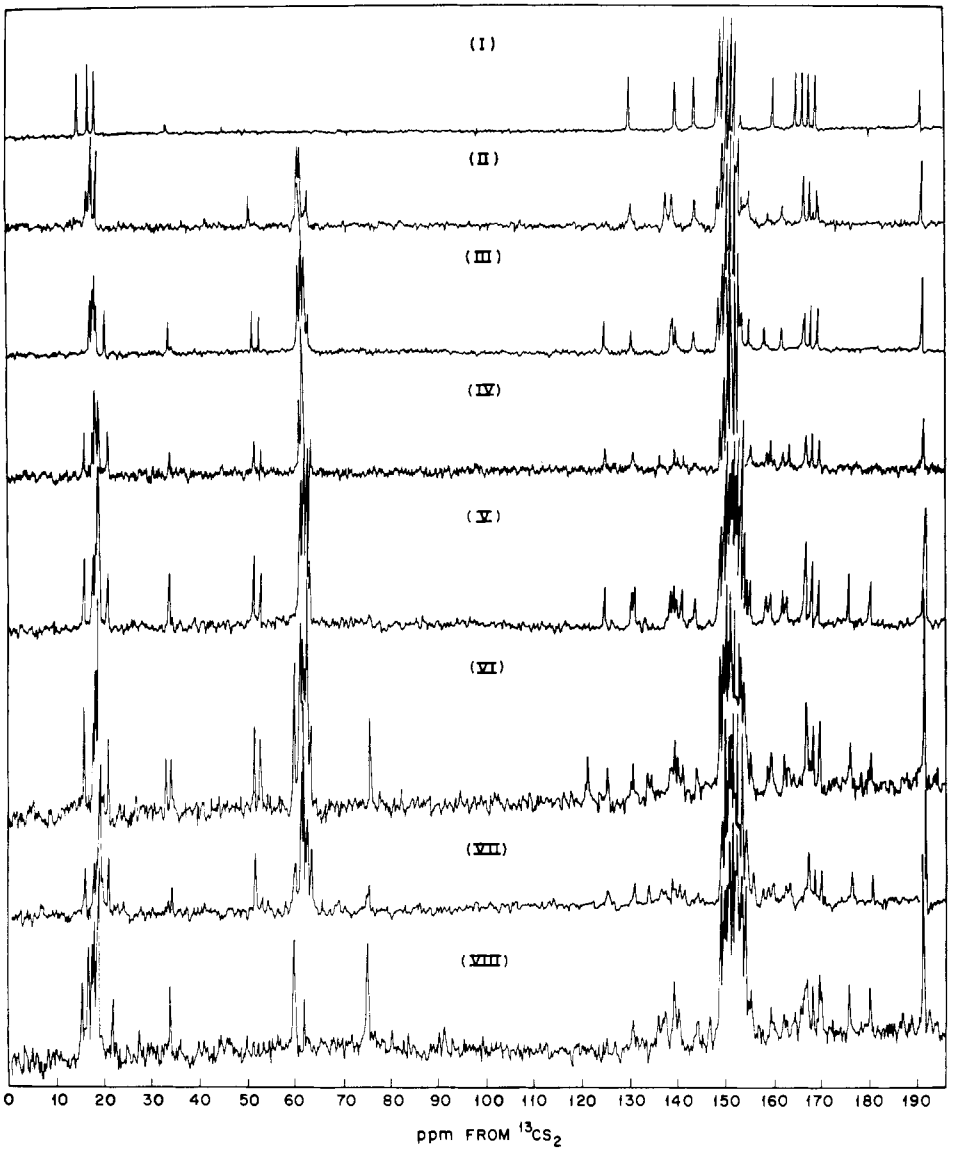


FIGURE 1: Spectra of seven precursors of oxytocin and of oxytocin: I Pro-Leu-Gly-NH_2, II *S*-Bzl-Cys-Pro-Leu-Gly-NH_2, III Z-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2, IV Z-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2, V Z-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2, VI Z-*O*-Bzl-Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2, VII Z-*S*-Bzl-Cys-Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2, VIII oxytocin. Me_2SO-d_6 appears as the large multiplet between 151 ppm and 156 ppm.

isomer not only because trans is commonly the preferred isomer in open-chain peptides but also because the position of the proline γ-carbon resonance corresponds to that of the trans conformation (Dorman and Bovey, 1973; Thomas and Williams, 1972; Wüthrich *et al.*, 1972; Deslauriers *et al.*, 1972).

In the carbonyl region of the spectra, the most shielded peak is attributed to the asparagine-5 carboxamide carbonyl[2] while the lowest field peak is assigned to the glutamine-4 carboxamide carbonyl. Since asparagine-5 is branched two carbons away from its carboxamide carbonyl, the latter experiences an upfield shift (a "B shift") (Dorman, 1972), whereas the glutamine-4 carboxamide carbonyl experiences a downfield "C shift" due to the branching three carbons away. The difference between these two shifts is expected to be at least 4–6 ppm; the observed separation is 5 ppm. Further

[2] At the request of a referee we comment specifically on assignments which are in disagreement with those of Deslauriers *et al.* (1972). (a) Asparagine-5 γ carbonyl: Deslauriers *et al.* do not appear to have taken into account the effect of chain branching (Dorman, 1972), nor since they assign this peak at 25.3 ppm to the half-cystine-6 carbonyl, the downfield shift of cysteine carbonyl upon formation of the cystine disulfide bond, as seen in model compounds. (b) Cystine α carbons: deuteration at these carbons confirmed the assignment of their resonances. The assignment of the α-carbon resonance of half-cystine-1 is further confirmed by the absence of this peak in deamino-oxytocin. (c) Cystine β carbons: we made these assignments largely on the basis of the deuterated compounds, as explained in the text. It is not clear to us how Deslauriers *et al.* were able to distinguish these peaks from the solvent multiplet. Further confirmation of our assignment for the β carbon of half-cystine-1 comes from the absence of its resonance in the spectrum of deamino-oxytocin. (d) Glycine-9 α carbon: in oxytocin, we do not see a peak at 150.9 ppm where Deslauriers *et al.* assign this carbon resonance. However, since the peak at 148.8 ppm, which they assign to the α carbon of half-cystine-1, does not diminish nor disappear in the compounds where deuterium is present at this carbon and does not disappear in the spectrum of deamino-oxytocin, we feel that this peak must be the α carbon of glycine-9. Confirmation of our assignment for the α-carbon resonance of glycine-9 in the linear nonapeptide is provided by the absence of this peak in the linear nonapeptide, where the protons on this carbon have been replaced by deuterons.

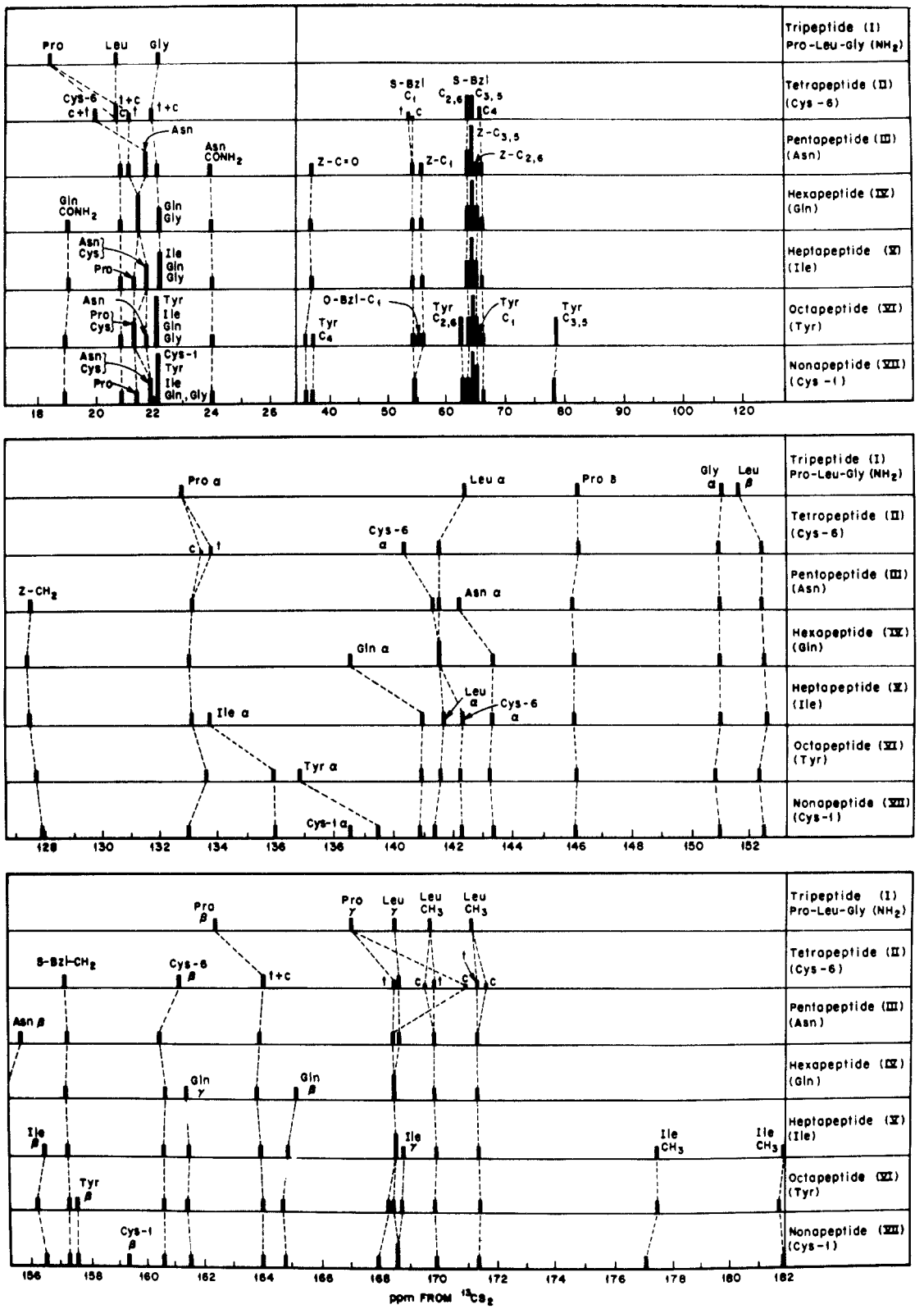


FIGURE 2: Assignment of resonances in seven precursors of oxytocin: I Pro-Leu-Gly-NH_2, II *S*-Bzl-Cys-Pro-Leu-Gly-NH_2, III Z-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2, IV Z-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2, V Z-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2, VI Z-*O*-Bzl-Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2, VII Z-*S*-Bzl-Cys-Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH. Internal Me_4Si appears at 192.8 ppm from the CS_2 resonance.

confirmation of the glutamine-4 carbonyl resonance is provided by the absence of the 18.9-ppm peak and the intensity decrease in the 22.0-ppm peak when glycine-4 replaces glutamine-4 in [4-glycine]-oxytocin. When the ring is closed by cystine disulfide bond formation to form oxytocin, the asparagine-5 carboxamide carbonyl peak moves upfield by 1.4 ppm[3] and two resonances move down to 20.1 ppm. Although in the free amino acids the cystine carbonyl resonates 5 ppm

below that of cysteine, we have assigned the 2-carbon resonance at 20.1 ppm to half-cystine-1 and half-cystine-6 carbonyls on the basis that the 2-ppm downfield shift results from disulfide bond formation, but that the effect is less marked than in the free amino acids because (a) the carbonyls are participating in peptide bonds, and (b) the sulfur atoms were benzyl substituted rather than in the sulfhydryl form. Proton magnetic resonance evidence indicates an intramolecular hydrogen bond involving the asparagine-5 peptide NH (Urry and Walter, 1971; Brewster *et al.*, 1973). If a 1,4 turn ("β turn") is present (Urry and Walter, 1971), the tyrosine-2 carbonyl must be involved. While the tyrosine-2 carbonyl resonance cannot be unambiguously identified, there is no marked shift of any carbonyl resonance (except those of half-cystine-1 and half-cystine-6) upon closing the ring.

By comparison with the spectra of nonapeptides deuterated at the α and β positions of cysteine-1 (XIII) and at the α and β positions of cystine-6 (XIV), respectively, we were able to assign the peaks at 138.5 and 159.4 ppm to the α and β carbons of cysteine-1[2] and the peaks at 142.3 and 160.6 ppm to the α and β carbons of cysteine-6[2] (Figure 2). With a pulse interval of 2 sec, these peaks were absent in the spectra of the deuterated compounds (see Experimental Section). Similarly, comparison of the oxytocin spectrum (see Figure 3) with that of its deuterated forms, XV and XVI, shows that the α carbon of half-cystine-1 gives rise to the peak at 138.7 ppm[2] and that the α carbon of half-cystine-6 gives rise to half of the peak at 141.5 ppm.[2] The β-carbon peaks of half-cystine-1 and of half-cystine-6 are similarly assigned at 152.0 and 154.7 ppm, respectively.[2] These resonances can be assigned from the dissymmetry they produce in the solvent septet when one or the other is deuterated and therefore absent. In the spectrum of oxytocin (undeuterated), the cystine β-carbon resonances are virtually impossible to pick out because they are so placed in relation to the solvent resonance as to cause no observable dissymmetry in it. However, in our continuing investigations of oxytocin, we see peaks in these approximate positions when oxytocin is dissolved in water, *i.e.*, without the obscuring effect of the Me_2SO-d_6. The β carbon of cysteine experiences a downfield shift of 16 ppm upon formation of cystine. Upon closing the ring to oxytocin, the β carbons of the nonapeptide cysteine residues experience a downfield shift of 6.7 ppm for half-cystine-1 and 5.7 ppm for half-cystine-6 (Figure 4).[3]

Comparison with the spectrum of the nonapeptide deuterated at glycine-9, XII, shows that the glycine-9 α carbon resonates at 150.9 ppm.[2] In oxytocin, the peak for the glycine-9 α carbon exhibits a downfield shift of 2.1–148.8 ppm,[2] suggesting ring–side-chain interaction. Of the other α-carbon resonances, those of proline-7, tyrosine-2, and leucine-8 show very little change when the disulfide bond is formed. The β carbons of these residues and the δ carbon of proline-7 also change very little. The α carbons of the other residues all move downfield, isoleucine-3 showing a very marked deshielding of 2 ppm. The β carbons of these residues all move upfield. It is not clear at present whether these compensating shielding effects within each amino acid residue are significant.

The resonances of glutamine-4 in oxytocin were readily assigned by comparison to the spectrum of [4-glycine]-oxytocin. In this spectrum, the α carbons of half-cystine-1, asparagine-5, and glycine-9 and the β-carbon of asparagine-5 have reverted to their open-chain positions.

[3] The deuterated nonapeptides are not Z-blocked at the α-amino group of half-cystine-1. It should be further noted that the first spectrum in Figure 4 represents a composite of XIII and XIV.

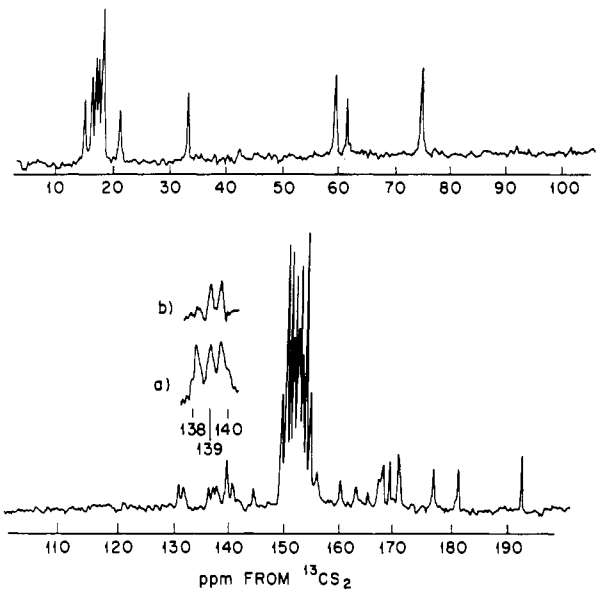
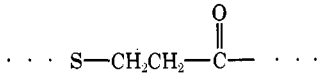


FIGURE 3: Spectrum of oxytocin (a) undeuterated (b) deuterated at α and β of half-cystine-1. Me_2SO-d_6 appears as the large multiplet between 151 ppm and 156 ppm.

The spectrum of [7-D-proline]-oxytocin showed some movements of the proline ring carbons compared to oxytocin, as might be expected, but they are not large. There is, however, a very marked deshielding (3.5 ppm) of the half-cystine-6 β carbon relative to that of oxytocin.

In deamino-oxytocin, the half-cystine-1 unit has been replaced by



i.e., a β-mercaptopropionic acid (β-MPA) unit. It was studied in conjunction with the deuterated analog, [1-[α,α,β,β-$^2H_4$]β-MPA]oxytocin (XVII), in which the four protons on the mercaptopropionic acid portion are replaced by deuterons. Additional evidence for our assignments of the half-cystine-1 α-carbon in oxytocin was gained from these spectra.[2] The MPA α-carbon peak is undetectable and we assume it to be under the $(C^2H_3)_2SO$ resonances, while the β-carbon peak, absent in the spectrum of XVII, appears at 158.1 ppm. In the carbonyl region, it is expected that the half-cystine-1 resonance will be shifted upfield by 3–4 ppm upon removal of the α-amino group; a new peak at 23.2 ppm is accordingly assigned to the β-MPA carbonyl.

Discussion

It is clear from these results that there are substantial variations of chemical shift, particularly for α and β carbons, which cannot be due to primary structural changes only but must be attributed to alterations of the conformation of the main chain, and no doubt to conformational changes of the amino acid side chains as well. The glycine-9 α carbon, although at the end of the chain and seemingly least susceptible to structural influences, is nevertheless sensitive to such alterations as ring closure, substitution of glycine-4 for glutamine-4, inversion of the α carbon of proline-7, and removal of the half-cystine-1 α-amino group. It is an attractive speculation that

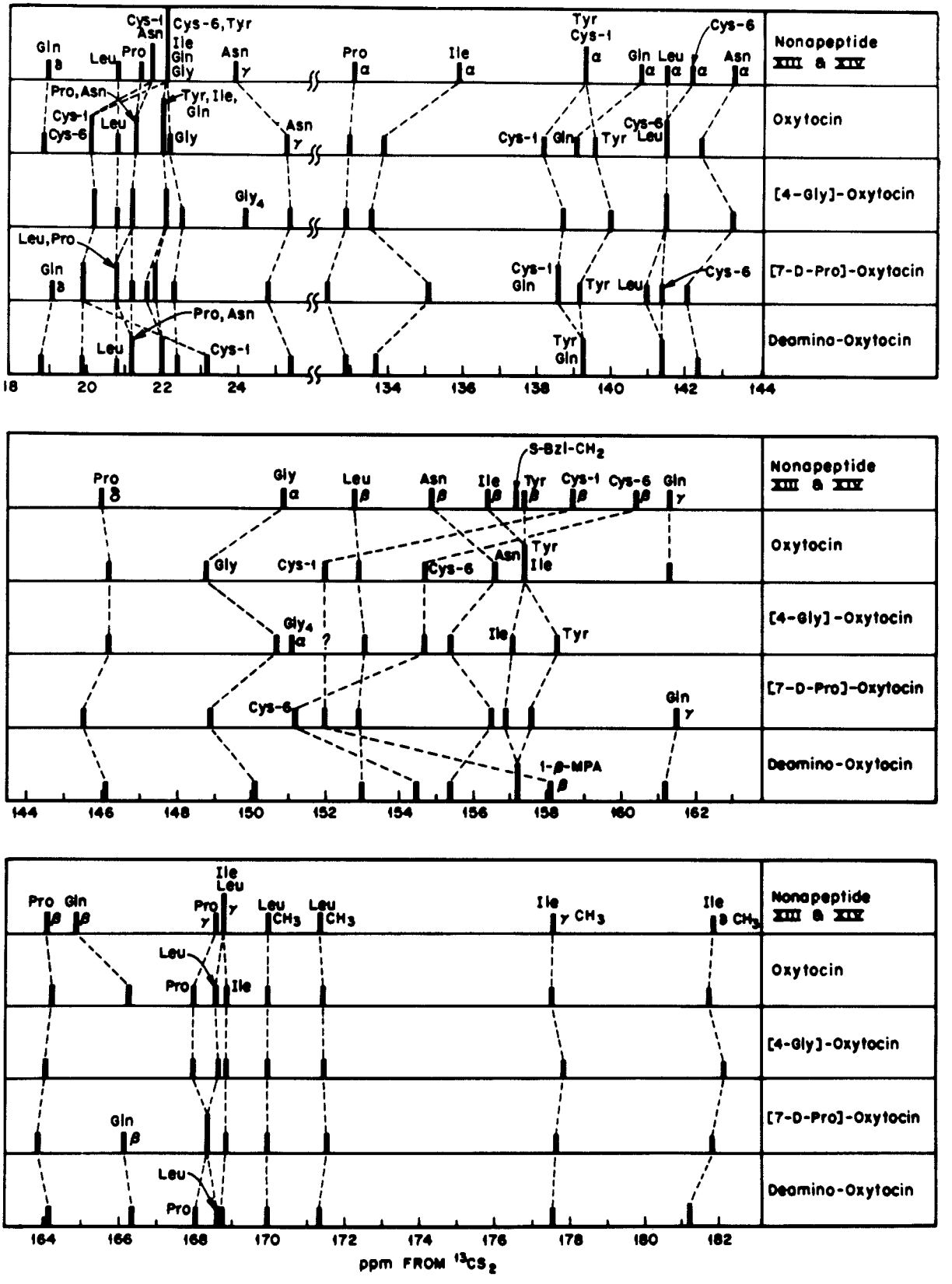


FIGURE 4: Assignment of resonances in linear nonapeptides: XIII *S*-Bzl-[α,β,β-$^2H_3$]-DL-Cys-*O*-Bzl-Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-Gly-NH_2 and XIV *S*-Bzl-Cys-*O*-Bzl-Tyr-Ile-Gln-Asn-*S*-Bzl-[α,β,β-$^2H_3$]-DL-Cys-Pro-Leu-Gly-NH_2 (a composite spectrum), oxytocin, its analogs, and deamino-oxytocin. Internal Me_4Si appears at 192.8 ppm from the CS_2 resonance.

these changes of chemical shift reflect the folded or unfolded state of the side chain. If this is the case, the side-chain conformation must be sensitive to the ring conformation, although at present it is not obvious why the 1–4 turn ("β turn") proposed by Urry (Urry *et al.*, 1971) for the side chain should display such sensitivity. It does seem clear from the

^{13}C data that the cystinyl–proline peptide bond, which is at the point of attachment of the side chain, is trans in oxytocin and remains trans in all the oxytocin analogs reported here.

The use of partial deuteration, carefully selected analogs, and related precursor peptides has allowed us to assign most of the ^{13}C resonances for these compounds with considerable certainty and to note various chemical shift phenomena which are likely to be related to conformational effects. We are now in a position to correlate this ^{13}C nuclear magnetic resonance data with the proton spectra and with the conclusions of theoretical conformational energy calculations.

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