Organic and Biological Chemistry

Nuclear Magnetic Resonance Evidence for *cis*-Peptide Bonds in Proline Oligomers

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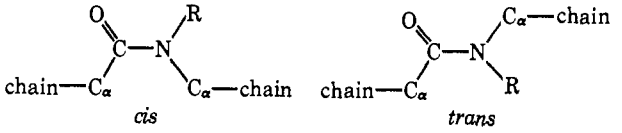
Received March 16, 1970

Abstract: By use of 220-MHz nmr spectroscopy, it has been found that α-hydrogen atoms of proline residues in an oligo- or poly-L-proline chain give rise to separate resonances for *cis*- and *trans*-peptide bonds. Direct observation of the populations and ratios of these bonds in various peptide chains has been achieved by this method. The isomerization of poly-L-proline form I (all-*cis* peptide bonds) to form II (all-*trans* bonds) was monitored by noting the change with time in relative areas of α-hydrogen resonances. Chemical shifts of these resonances were found to be solvent dependent, and correlations between α-hydrogens in glycyl–proline bonds, poly-L-proline, and a model amide were established to ensure unequivocal assignments of *cis* and *trans* resonances in all systems examined. The peptide bond of glycyl–prolyl oligomers was found to be 20–30% *cis* in chloroform and in dimethyl sulfoxide. A series of proline oligomers (*t*-butyloxycarbonyl-(L-proline)$_n$ benzyl esters, n = 2, 3, 4, 5, and 6) was synthesized and their nmr spectra in chloroform were analyzed; it was found that these oligomers contained nearly random distributions of *cis*- and *trans*-peptide bonds when n = 2, 3, and 4, but abruptly assumed an all-*trans*-poly-L-proline II helical structure when n = 5 and 6.

Although peptide bonds involving α-amino acid residues have been shown to be in the *trans* conformation in linear peptides, those comprising the imino acid peptide residue, such as occurs in proline and N-methylglycine peptides, may be *cis* or *trans* at the peptide bond.[2] The definitions of these conformations are in terms of the disposition of the α-carbon atoms on either side of the peptide linkage.



cis-Peptide bonds are known to occur only in a limited number of compounds, *e.g.*, in small cyclic peptides such as the diketopiperazines ("cyclo(dipeptides)") and cyclo(tri-L-prolyl),[3,4] where steric constraints allow only *cis* bonds. Poly-L-proline, as prepared by polymerization of the N-carboxyanhydride,[5] exists as a compact right-handed helix (designated form I) which has been shown to have *cis*-peptide bonds, as determined by X-ray diffraction on the solid.[6] The mutarotation of poly-L-proline I to a left-handed, highly extended helix (form II) with *trans*-peptide bonds, also determined by X-ray diffraction on the solid,[7,8] has been extensively studied.[9-11] Evidence for *cis*-peptide bonds in other cyclic systems has been obtained from an analysis of certain infrared bands in a series of isomeric cyclo(Phe-Leu-Gly)$_2$ cyclohexapeptides,[12] and from an X-ray diffraction study on a cyclotetradepsipeptide.[13] Multiple resonances for N-methyl groups in the nuclear magnetic resonance spectra of polysarcosine[14] and a series of cyclosarcosine peptides[15] led to the suggestion that conformations containing *cis* bonds are present. In proteins, *cis*-peptide bonds involving proline have been postulated to account for certain conformational features of subtilisin BPN'[16] and ribonuclease S.[17]

In this investigation, we demonstrate, by means of 220-MHz nmr, the presence of *cis*-peptide bonds in individual residues of small linear (six residues or less) proline and glycine–proline oligomers. We are also able to use this technique to monitor the *cis–trans* isomerization of poly-L-proline, as well as to follow the onset of the form II helix conformation with increasing proline oligomer chain length.[18]

(1) (a) Harvard Medical School; (b) Bell Laboratories; (c) University of Toronto, Toronto, Ontario, Canada.

(2) For a review on proline conformations, see J. P. Carver and E. R. Blout in "Treatise on Collagen," Vol. 1, G. Ramachandran, Ed., Academic Press, New York, N. Y., 1967, pp 441–526.

(3) C. M. Venkatachalam, *Biochim. Biophys. Acta*, **168**, 397 (1968).

(4) C. M. Deber, A. Scatturin, V. M. Vaidya, and E. R. Blout in "Peptides: Chemistry and Biochemistry," B. Weinstein and S. Lande, Ed., Marcel Dekker, New York, N. Y., 1970, pp 163–173.

(5) G. D. Fasman and E. R. Blout, *Biopolymers*, **1**, 3 (1963).

(6) W. Traub and V. Shmueli, *Nature (London)*, **198**, 1165 (1963).

(7) P. M. Cowan and S. McGavin, *ibid.*, **176**, 501 (1964).

(8) V. Sasisekharan, *Acta Crystallogr.*, **12**, 897 (1959).

(9) J. Kurtz, A. Berger, and E. Katchalski, *Nature (London)*, **178**, 1066 (1956).

(10) I. Z. Steinberg, W. F. Harrington, A. Berger, M. Sela, and E. Katchalski, *J. Amer. Chem. Soc.*, **82**, 5263 (1960).

(11) F. A. Bovey and F. P. Hood, *Biopolymers*, **5**, 325 (1967).

(12) K. Blaha, J. Smilikova, and A. Vitek, *Collect. Czech. Chem. Commun.*, **31**, 4296 (1966).

(13) J. Konnert and I. L. Karle, *J. Amer. Chem. Soc.*, **91**, 4888 (1969).

(14) F. A. Bovey, J. J. Ryan, and F. P. Hood, *Macromolecules*, **1**, 305 (1968).

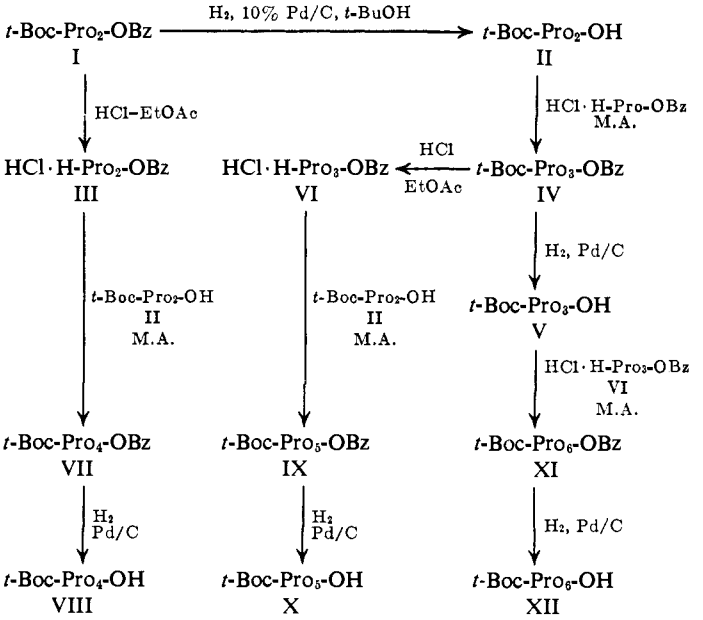
(15) J. Dale and K. Titlestad, *Chem. Commun.*, 656 (1969).

(16) C. S. Wright, R. A. Alden, and J. Kraut, *Nature (London)*, **221**, 235 (1969).

(17) H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, L. M. Johnson, and F. M. Richards, *J. Biol. Chem.*, **242**, 3984 (1967).

(18) For a preliminary account of this work, see C. M. Deber, F. A. Bovey, J. P. Carver, and E. R. Blout in Proceedings of the Tenth European Peptide Symposium, Abano Terme, Italy, 1969, E. Scoffone, Ed., North-Holland Publishing Co., Netherlands, in press.

Chart I. Scheme of Synthesis of L-Proline Oligomers[a]



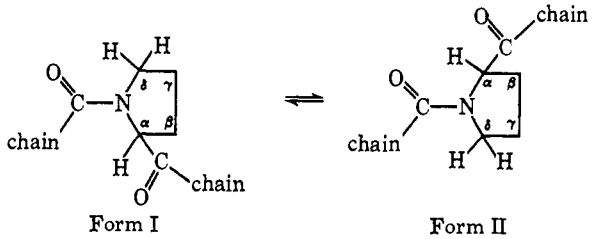
[a] Abbreviations: OBz = benzyl ester; Pd/C = 10% palladium–charcoal; *t*-Boc = *t*-butyloxycarbonyl; M.A. = mixed anhydride formed with isobutyl chloroformate and N-methylmorpholine.

Synthesis of Materials

A series of proline oligomers of general formula *t*-Boc-$(Pro)_n$-OR, where R = H or benzyl, and n = 2, 3, 4, 5, and 6 has been synthesized *via* the method of mixed anhydride coupling with isobutyl chloroformate and N-methylmorpholine.[19] Appropriate fragments were joined according to the outline shown in Chart I. *t*-Boc-Gly-Pro-OR compounds were prepared in a similar manner. Poly-L-proline was synthesized by polymerization of L-proline N-carboxyanhydride in acetonitrile using sodium methoxide as the initiator.[5] All proline residues in this work were of the L configuration. See the Experimental Section for details.

Results and Discussion

1. Polyproline. When isomerization of a proline–proline peptide bond occurs, the α and δ protons of the proline ring change positions with respect to the carbonyl group.



Form I (*cis*) ⇌ Form II (*trans*)

The *cis* conformation of prolyl residues is considerably more probable than the corresponding one in poly(α-amino acids), where the *cis* form places the two chains in steric proximity while the *trans* form allows maximum separation. In proline and other N-alkylated imino acids, there is significantly less energy difference between the two forms, since the presence of the N—C_δ bond causes either alignment to give rise to *cis* carbon–carbon interactions.

Poly-L-proline I is a compact structure with close intramolecular contacts, and is stable in relatively poor solvents such as ethyl or benzyl alcohol in which polymer–polymer contacts are preferred to polymer–solvent contacts.[20] When form I is dissolved[21] in a solvent which favors form II, such as water, organic acids, and certain organic solvents such as trifluoroethanol, the extent of conformational isomerization to form II can be observed optically by measuring the rate of mutarotation or the rate of change of the circular dichroism (CD) spectrum.[11] We have been able to follow the transformation of I to II by means of 220-MHz nmr spectra, where we find a pair of resonances near τ 5.20 and 5.45 in the region of the α-protons (Figure 1), which change in relative area as the isomerization proceeds, the process being relatively rapid in CD_3CO_2D (Figure 1) (a few hours) and very much slower in chloroform (Figure 2). By correlating the nmr spectra with optical rotation changes, it is apparent that the lower field peak (in $CDCl_3$) at τ 5.25 corresponds to the *cis* form. Our results substantiate those of Conti, *et al.*,[22] who studied similar systems using 100-MHz nmr. Using 220-MHz spectra, changes are visible also in the δ-proton region (*ca.* τ 6.2) and in the more shielded β- and γ-proton regions (*ca.* 7.6–8.0) (see Figure 1).

2. Glycylproline. From a large number of investigations of amides by nmr it is known[23] that protons in positions which simulate the α- and δ-protons of the proline ring (see Figure 3) experience different shieldings, being commonly more shielded when on the same

(19) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **89**, 5012 (1967).

(20) P. R. Schimmel and P. J. Flory, *Proc. Nat. Acad. Sci. U. S.*, **58**, 52 (1967).

(21) It is not clear whether form I "dissolves" in form II solvents, or whether it is "dispersed" until solvent-induced I → II isomerization occurs, after which true solvation takes place.

(22) F. Conti, M. Piatelli, and P. Viglino, *Biopolymers*, 7, 411 (1969).

(23) M. B. Robin, F. A. Bovey, and H. Basch in "Chemistry of the Amides," J. Zabicky, Ed., Wiley, New York, N. Y., 1970.

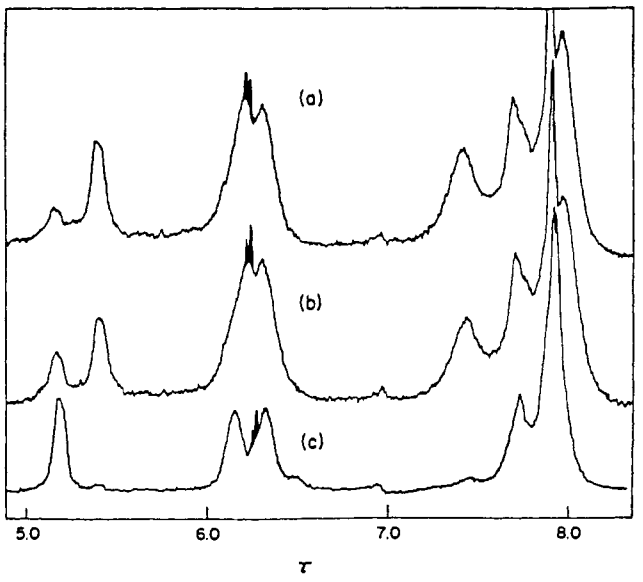


Figure 1. 220-MHz nmr spectra of freshly polymerized poly-L-proline I in CD_3CO_2D. Time after dissolving: (a) 10 min; (b) 4 hr; (c) 100 hr.

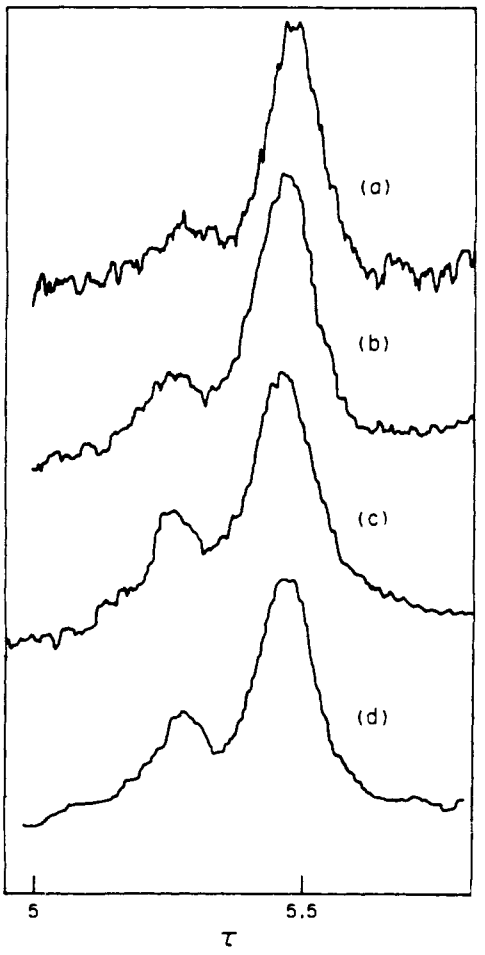


Figure 2. 220-MHz nmr spectra of freshly polymerized poly-L-proline I in $CDCl_3$. Time after dissolving: (a) 10 min; (b) 1.5 hr; (c) 5 hr; (d) 168 hr.

side as the carbonyl oxygen. For the two methyl groups in dimethylformamide (DMF), the nmr spectrum (recorded "neat") reveals two separate resonances,

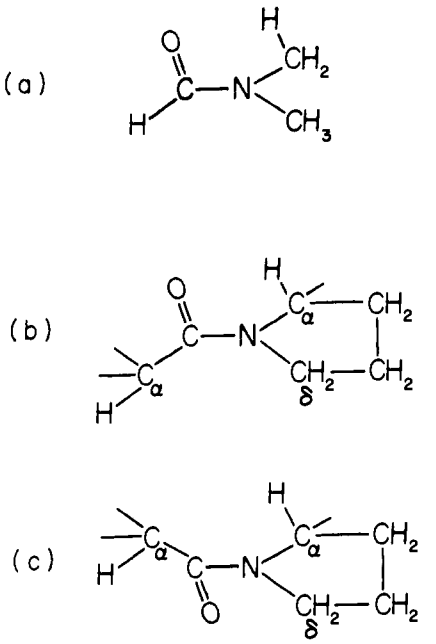
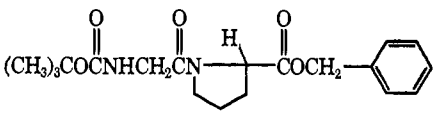


Figure 3. Relationship of protons in model amide to those in X-Pro peptide bonds, where X is any amino acid: (a) N,N-dimethylformamide; (b) *trans*-X-Pro bond; (c) *cis*-X-Pro bond.

presumably a consequence of the relatively slow rate of rotation about the C—N bond, which has considerable double bond character. The relative shielding of the methyl groups of DMF has been established by nuclear Overhauser measurements[24] to be as stated, *i.e.*, the methyl group nearer the oxygen is the more highly shielded (upfield) one. In order to establish the relationship between these findings and the situation in proline peptides, we have examined the conformation of the glycine–proline peptide bond in *t*-Boc-Gly-L-Pro-OBz (XIII). This compound is a better model



XIII

than simple amides for observation of separate resonances which may arise due to *cis*- and *trans*-peptide bond conformations, since the proline residue is in a peptide environment next to the amino acid (glycine) with the minimum possible steric restrictions. The partial 220-MHz nmr spectrum of this material (Figure 4) shows only the α-proton and benzylic proton resonances.[25] The Pro α-H appears in chloroform as *two* four-line resonances at τ 5.43 and 5.58 with an approximate area ratio of 4:1 favoring the downfield signal; thus, two distinct conformations about the peptide bond are present.

While it seemed quite likely that the *trans* conformation was the predominant one, the situation was

(24) F. A. L. Anet and A. J. R. Bourn, *J. Amer. Chem. Soc.*, 87, 5290 (1965).

(25) The *t*-Boc group, joined to N-terminal proline through a urethan linkage, is similar to a peptide bond in its ability to assume a *cis* or *trans* conformation with respect to the C–N bond. In this compound (XIII) the *t*-Boc resonance (not shown in Figure 4) is a singlet (at τ 9.0) which does not reflect the conformational state of the Pro residue, the latter apparently being too far away. The Gly NH peak is masked by the aromatic resonance.

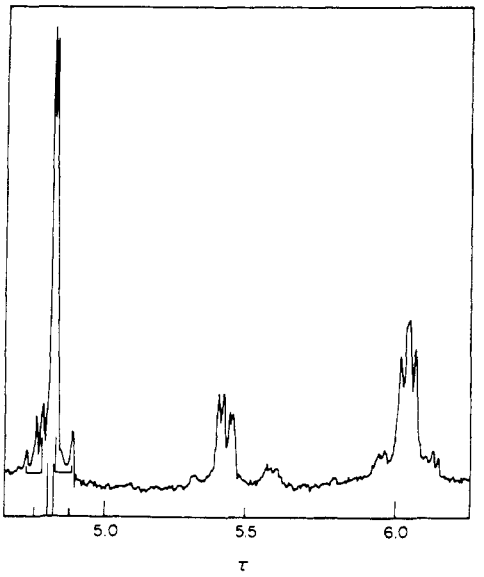


Figure 4. Partial 220-MHz nmr spectrum of *t*-Boc-Gly-L-Pro-OBz (XIII), in $CDCl_3$. Bars drawn in near τ 4.75 indicate positions of overlapping AB quartets.

obviously somewhat more complex than it seemed at first glance. When we attempted to make correlations between Pro α-H chemical shifts with those expected from DMF methyl group chemical shifts (see relationships of protons in Figure 3), it was apparent that a reversal of peaks had occurred. The experiments described below demonstrate that the *trans* conformation *does* correspond to the major peak at τ 5.43 in chloroform, and that positions of chemical shifts of Pro α-H's are quite sensitive to solvent.

When the nmr spectrum of *t*-Boc-Gly-L-Pro-OBz is recorded in dimethyl sulfoxide-d_6 (DMSO-d_6), a solvent more closely resembling DMF than chloroform in its polarity and physical properties, a reversal of the resonances due to Pro α-H's occurs. Now the higher field portion at 5.64 predominates in area by about 4:1 over the lower field peak at 5.25. Proof that this represents a solvent-induced alteration in chemical shifts (rather than in molecular conformation) was obtained by recording the spectra in $CDCl_3$–DMSO-d_6 mixtures, in which the two resonances can be seen to "cross over" each other while maintaining the same relative areas. Even as little as 25 vol % DMSO-d_6 moves the downfield peak strongly upfield and beyond the smaller peak. This phenomenon of chemical shift crossover is illustrated for the related *t*-Boc-Gly-Pro-OH system (XIV) (Figure 5), which displayed nmr behavior similar to the benzyl ester.

Viewing these results in conjunction with the well-defined DMF experiments, it becomes reasonably certain that in chloroform, the downfield resonance corresponds to a *trans*-Gly-Pro bond, while the upfield resonance corresponds to a *cis*-Gly-Pro peptide bond. In the more polar solvent DMSO-d_6, these assignments are reversed.[26] The *trans*/*cis* ratio in the –OH dipeptide XIV is qualitatively the same as that in the –OBz dipeptide (XIII) (both lie between 70/30 and 80/20); in the –OH compound, this ratio can be obtained also from the areas of a correspondingly

(26) Studies on the model compound N-acetyl-L-proline methyl ester also revealed the "crossover" in going from chloroform to DMSO-d_6.

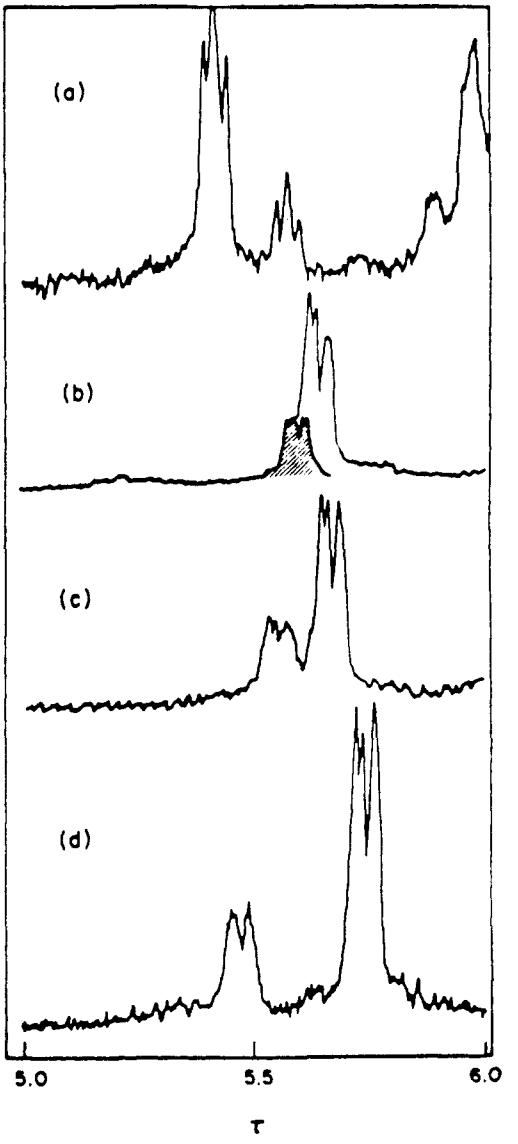


Figure 5. Partial 220-MHz spectrum of *t*-Boc-Gly-L-Pro-OH (XIV), showing "crossover" of chemical shifts of *cis*- and *trans*-Pro α-H resonances in $CDCl_3$–DMSO-d_6 solvent mixtures: (a) $CDCl_3$; (b) 75 $CDCl_3$–25 DMSO-d_6; (c) 50 $CDCl_3$–50 DMSO-d_6; (d) DMSO-d_6.

unequal pair of resonances for the Gly NH proton centered at τ 4.3 (not shown in Figure 5).

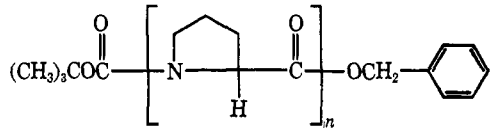
The conformation of the Pro residue in *t*-Boc-Gly-Pro-OBz is also clearly signaled by the benzylic resonance. In $CDCl_3$, the dominant *trans* conformation appears as an AB quartet for the CH_2 benzylic protons, overlapping somewhat with the small *cis* quartet (Figure 4). The nonequivalence of these protons apparently disappears in DMSO-d_6, but the resulting collapse of the quartets to a pair of singlets of different areas (not shown in Figure 4) still provides an accurate measure of the *trans*/*cis* ratio. Changes in coupling constants upon changing solvent are apparent throughout these spectra, suggesting some alterations in bond angles, but not in gross conformation.

It is significant that in DMF and in the two Gly-Pro compounds, the medium *surrounding* the molecule seems to have a more profound effect upon the chemical shifts of certain protons than do local shielding situations predicted from neighboring atoms *within* the

molecule itself. This effect does not appear to exist in poly-L-proline, in which the position of the form I α-H resonance is determined mainly by the relatively nonpolar internal environment of the polymer backbone (see ref 21). Although the positions of the *cis* and *trans* resonances of the α-protons of the polymer in chloroform fit those predicted from the "crossover" analysis just presented, firm correlations between dipeptides and polymer may not be valid. Note, for example, that in the acidic solvent CD_3COOH (Figure 1), the nmr spectrum of the polymer does not show the "crossover" expected in "polar" solvents.

Thus, in various solvent systems, despite widely divergent solvent polarities, the Gly–Pro peptide bond has been found to be a mixture of conformers, with the *trans* bond always predominating to the extent of 70–80%. We believe, however, that these results constitute the first unequivocal evidence for the existence of *cis*-peptide bonds in simple linear oligomeric peptides, although *cis*-amide bonds have been previously found in N-acetylsarcosine methyl ester,[14] and in N-acetyl-N-methylalanine methyl ester.[27]

3. Proline Oligomers. A series of L-proline oligomers has been synthesized which have the general structure



I, $n = 2$
IV, $n = 3$
VII, $n = 4$
IX, $n = 5$
XI, $n = 6$

with n equal to 2, 3, 4, 5, and 6. We have carried out an analysis of their peptide bond conformations using 220-MHz nmr. The partial spectra containing the α-protons, the benzylic methylene group, and the *t*-Boc group are shown in Figure 6.

Because the N-terminal urethan linkage is capable of assuming a *cis* or *trans* conformation with respect to the C–N bond, the resonance for the nine methyl protons of the *t*-Boc group appears not as one singlet, but as two or more singlets near τ 8.5, indicating the presence of distinct conformations about the N-terminal *t*-Boc-Pro bond and a measure of their ratio.[28] The resonances for the α-protons appear at 5.2–5.6, and are sensitive to both the positions in the chain and the conformation of the proline residues. The α-protons of the *cis*- and *trans*-N-terminal residues show well-separated resonances in the most shielded α-H region at τ 5.60 and 5.48, respectively, the assignments being correlated with the above-described studies in $CDCl_3$. The C-terminal α-H appears at τ 5.38, the *cis* and *trans* resonances being nearly overlapping. The α-protons of the central residues (for $n \geq 3$) are at lowest field; the *trans* units appear at 5.25, as in form *II* poly-L-proline, and the *cis* units at slightly higher field. All the α-proton resonances are four-line spectra (not always clearly resolved), reflecting J coupling of differing magnitude to the two nonequivalent β-protons. Their similar form suggests that the C_α—C_β bond, and probably the entire proline ring, has about the same geometry irrespective of the conformation of the main chain.

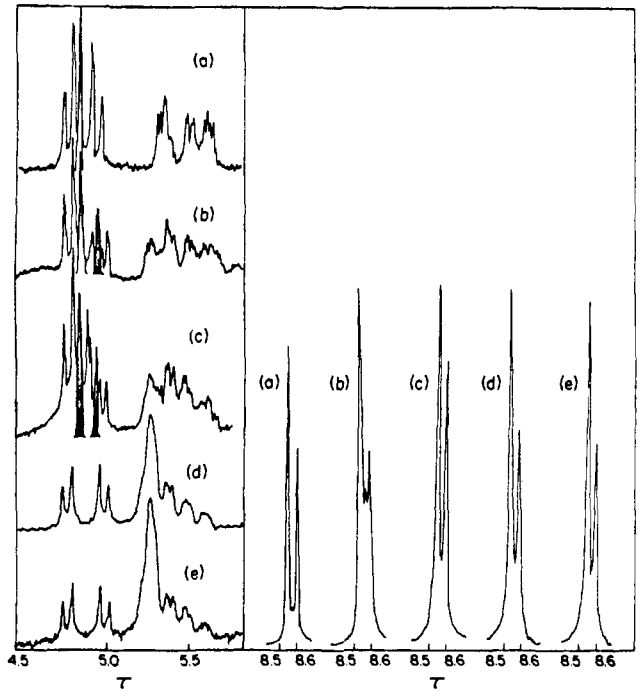


Figure 6. Partial 220-MHz spectra of *t*-Boc-$(Pro)_n$-OBz oligomers in $CDCl_3$. Left, benzyl ester resonances (τ 4.6–5.1) and α-H resonances (τ 5.1–5.8); right, the *t*-Boc resonances (τ 8.5–8.6). Cross-hatched peaks near τ 4.8 in a, b, and c, and τ 4.9 in b and c indicate the singlet resonances attributable to *cis*-C-terminal residues: a, $n = 2$; b, $n = 3$; c, $n = 4$; d, $n = 5$; e, $n = 6$.

When $n = 2$, there are four peptide bond isomers possible: *cis–cis*, *cis–trans*, *trans–cis*, and *trans–trans*. An analysis of spectrum a (Figure 6) reveals that three of the four conformational isomers of *t*-Boc-Pro-Pro-OBz (I) are being observed. In this spectrum (and in all of the higher oligomers we studied), the benzylic protons signal the state of the C-terminal residue; when it is *cis*, the benzylic group is fortuitously homosteric (*i.e.*, the two protons, although in an asymmetric situation, have the same chemical shift) and gives a singlet resonance; when the C-terminal residue is *trans*, these protons appear as an AB quartet. (Note that this situation differs somewhat from the spectrum of *t*-Boc-Gly-Pro-OBz in $CDCl_3$, where the benzylic protons of both *cis*- and *trans*-C-terminal Pro benzyl ester residues appear as quartets.) Furthermore, the conformational state of the residue *next* to the C-terminal residue is signaled by a marked splitting of these singlet and/or quartet resonances. For example, when $n = 2$, the members of the quartet related to the C-terminal *trans* residue are split because the neighboring residue (in this case the N terminus) can be *cis* or *trans*, the splitting thus being conclusive evidence that both *cis*- and *trans*-N-terminal residues are indeed present.

We can rule out the possibility that we are dealing with mixtures in the $n = 2$ oligomer of *only* all-*cis* and all-*trans* isomers because (1) while the areas of the N-

(27) M. Goodman and M. Fried, *J. Amer. Chem. Soc.*, **89**, 1264 (1967).

(28) Similar effects in the model compound benzyloxycarbonyl-L-proline nitrophenyl ester were observed by R. Garner and W. B. Watkins, *Chem. Commun.*, 386 (1969).

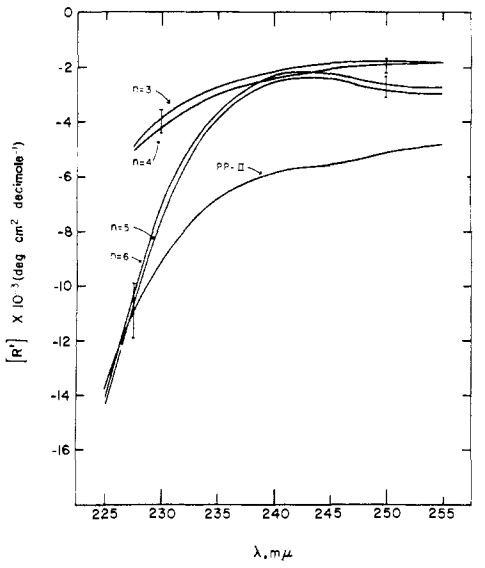


Figure 7. Optical rotatory dispersion curves of proline oligomers (t-Boc-(L-Pro)$_n$-OBz, n = 3–6) in chloroform and poly-L-proline II (PP-II) in water, recorded on a Cary 60 spectropolarimeter at 24°. Concentrations 0.05–0.1% wt/v. Vertical lines indicate estimated error limits of the measurements.

terminal *cis* and *trans* resonances (at τ 5.6 and 5.4, respectively) are approximately equal, the area ratio of the benzylic *cis*-singlet/*trans*-quartet is about 1:3, and (2) splitting of the *trans*-C-terminal quartet would not occur if the adjacent residue were only *trans*.

A comparable analysis can be applied to the nmr spectra of n = 3 oligomer (b in Figure 6). In this instance, multiple benzylic quartets *and* singlets at τ 4.7–5.0 indicate conformational mixtures of the C-terminal residue and its neighboring residues; the N-terminal at τ 5.4 and 5.6 is still a 50:50 mixture, and in the spectrum of this oligomer one can discern three singlets in the t-Boc region between τ 8.5 and 8.6. Careful analysis of an expanded-scale spectrum of τ 4.5–5.0 reveals that five of the eight possible conformational isomers are present.

An analysis of the increasingly complex benzylic resonance pattern at τ 4.6–5.0 in the n = 4 oligomer (Figure 6, spectrum c) reveals that out of the 16 possible peptide bond conformational isomers, eight are apparently present. Only the conformational state of the residue next to the N-terminal residue cannot be clearly ascertained; however, it seems highly probable that most of the possible conformers are present in appreciable proportions, excluding perhaps the all or mostly *cis* isomers. The peptide bond conformations along the chain are thus thoroughly "scrambled" although not strictly random, for throughout, the *trans* conformation is somewhat preferred. This situation is similar to that of polysarcosine (poly-N-methylglycine) for which a scrambled structure has been established[14] in a similar way by nmr.

When one more unit is added to the proline oligomer chain, however (at n = 5), there is a dramatic simplification of the benzyl resonance pattern (Figure 6, d), which now appears as a single AB quartet centered near τ 4.8 with no indication of any other conformation. All residues except the N terminus now appear to be entirely *trans* (at τ 5.25), strongly suggesting that the chain has assumed a form II helical conformation, into which only the t-Boc N-terminal unit is not firmly incorporated.

This conclusion is greatly strengthened by a very marked increase between n = 4 and n = 5 in the intensity of the onset of the negative Cotton band characteristic of this helix (Figure 7). The hexamer displays similar "form II" character (Figure 6, e). A previous report[29] on the optical rotatory dispersion (ORD) and circular dichroism (CD) spectra of t-amyloxycarbonyl-(L-proline)$_n$-OH oligomers (n = 2–6 and 8) in methanol and water solvents indicated a similar increase in the negative Cotton effect between n = 4 and n = 5, but until the present work, details concerning individual residues, particularly those in oligomers where $n \leq 4$, were not available. Although pure *cis* and *trans* forms of Pro–Pro bonds have differing rotatory characteristics, mixtures of such bonds in the same solution tend to compensate each other and the resulting ORD and CD curves are very difficult to interpret unequivocally.[30] We find then that the onset of the helical state in oligomeric proline benzyl esters takes place abruptly at the pentamer (in chloroform).[31]

It should be noted that the proline oligomer benzyl esters with n = 5 and n = 6 are crystalline materials, while the n = 2, 3, and 4 compounds are syrups which resisted all attempts at crystallization. The crystallinity of the pentamer and hexamer may be a reflection of the ordered form II conformations of these materials, while the noncrystallinity of the lower oligomers is probably a consequence of the extensive conformational mixture of chains present. We did not examine the nmr spectra of the noncrystalline portion of the n = 5 and n = 6 reaction products (the syrupy residue remaining after evaporation of the crystallization solvent from the filtrate), and therefore, we cannot exclude the possibility that these fractions of pentamer and hexamer contained mixed conformations.

Both in experiments with poly-L-proline and with proline oligomers, it appears that the use of chloroform as a solvent produced little or no *cis–trans* or *trans–cis* time-dependent peptide bond isomerizations. This was also true in the Gly-Pro experiments where dimethyl sulfoxide was used as solvent. These results lead one to speculate that the *observed cis–trans* ratio of a given peptide bond in chloroform or DMSO may be a good approximation of that ratio *prior* to dissolving the material (*i.e.*, in the solid or "syrup" state).

Conclusions

We have described the synthesis of a number of proline and glycylproline oligomers, and a method of

(29) H. Okabayashi, T. Isemura, and S. Sakakibara, *Biopolymers*, 6, 307 (1968).

(30) We also studied the oligomer series t-Boc-(Pro)$_n$-OH, n = 1–6, by the same 220-MHz techniques. We found t-Boc-Pro-OH to contain approximately a 50:50 mixture of *cis* and *trans* units at the urethan linkage. The region of the α-protons in this series was not nearly as clearly resolved as in the benzyl ester series, with middle residues overlapping more extensively with C-terminal residues. Nevertheless, we can say with a fair amount of certainty that similar "scrambled" conformations are present also in this series, with the most significant difference between this and the benzyl ester series lying in the fact that when n = 5 in the –OH series, the chain is still mixed, while in the –OBz series, the chain has taken up the poly-L-proline II structure at the pentamer. However, the hexamer in the –OH series, as well as in the –OBz series, does have the poly-L-proline II structure.

(31) In a series of α-amino acid oligomers, the onset of helix occurs *gradually* over the hexamer–decamer range; see M. Goodman, A. S. Verdini, C. Toniolo, W. D. Phillips, and F. A. Bovey, *Proc. Nat. Acad. Sci. U. S.*, 64, 444 (1969).

determining the relative populations of *cis* and *trans* isomers at each of their peptide bonds, by means of 220-MHz nmr spectroscopy. The isomerization of poly-L-proline I → II was also clearly monitored. A study of the nmr spectra of the Gly-Pro oligomers in mixed $CDCl_3$–DMSO-d_6 solvents, correlated with known chemical shifts of protons in the model amide DMF, allowed unequivocal assignments of *cis* and *trans* resonances. In the solvent systems we studied, the *trans*-Gly-Pro bond always predominated over the *cis* by at least 3:1. The proline benzyl ester oligomers were shown to have chains consisting of nearly random mixtures of *cis* and *trans* isomers at all peptide bonds when the chain length was two, three, and four residues. These oligomers assumed a regular poly-L-proline II-type structure when at least five residues were present.

From the experiments reported herein, it is clear that 220-MHz nmr can yield much information of particular relevance to theoretical calculations on the kinetics and thermodynamics of proline peptide bond conformer transitions. For example, a detailed conformational analysis of the populations of various *cis–trans* mixed isomers present in the proline oligomer series is now possible; the results of such an analysis of these experimental data can be applied to test some of the existing hypotheses concerning the cooperativity of the poly-L-proline I–II transition.[32]

Experimental Section

***t*-Butyloxycarbonyl-L-prolyl-L-prolyl Benzyl Ester (*t*-Boc-Pro$_2$-OBz) (I).** A solution of *t*-butyloxycarbonyl-L-proline (12.0 g) in chloroform (100 ml) was cooled to −20° in Dry Ice–CCl_4. N-Methylmorpholine (6.2 ml) and isobutyl chloroformate (7.6 ml) were then added in succession with efficient stirring. After 20 min at −20°, formation of the intermediate mixed anhydride was deemed complete; a flocculent precipitate of N-methylmorpholine hydrochloride was present. Solid L-proline benzyl ester hydrochloride (13.36 g) was stirred into the reaction mixture, followed by an additional equivalent (6.2 ml) of N-methylmorpholine. The reaction mixture was then allowed to warm to room temperature slowly as the Dry Ice evaporated and stirring was continued at room temperature overnight. Work-up was accomplished by dilution with 100 ml of chloroform, then by extraction successively with 200-ml portions of water, 5% aqueous sodium bicarbonate, and saturated sodium chloride. The chloroform layer was separated and dried over sodium sulfate, and the solvent evaporated to yield *t*-butyloxycarbonyl-L-prolyl-L-prolyl benzyl ester (I) (22.0 g, 98% yield) as a pale yellow syrup which could not be crystallized. The material had appropriate ir and nmr spectra and chromatographic (tlc) behavior.

***t*-Butyloxycarbonyl-L-prolyl-L-proline (*t*-Boc-Pro$_2$-OH) (II).** The benzyl ester (I) (21.0 g) was dissolved in 120 ml of *t*-butyl alcohol, treated with a catalytic amount of 10% Pd/C, and hydrogenated at 20 psi for 24 hr at room temperature. The catalyst was then removed by filtration through Celite, and the solvent evaporated. The semisolid residue was taken up in a minimum of boiling ethyl acetate. This solution on standing yielded crystals of *t*-Boc-Pro$_2$-OH (II) (first crop 10.5 g, 64% yield) which were crystallized from ethyl acetate to give 5.2 g of material melting at 186–187°, and displaying appropriate ir and nmr spectra and chromatographic properties.

Anal. Calcd for $C_{14}H_{24}N_2O_5$: C, 57.67; H, 7.74; N, 8.97. Found: C, 57.96; H, 8.06; N, 8.74.

L-Prolyl-L-prolyl Benzyl Ester Hydrochloride (HCl·H-Pro$_2$-OBz) (III). *t*-Boc-Pro$_2$-OBz (18.0 g) was treated with 300 ml of a solution of ethyl acetate which had been saturated with gaseous hydrochloric acid (approximately 4 *N* HCl) for 1 hr with stirring at room temperature. The solvent and excess HCl were evaporated under reduced pressure to yield after several hours under high vacuum a very viscous yellowish syrup (15.8 g, 100%), which displayed ir spectrum and chromatographic behavior expected for the desired hydrochloride.

***t*-Butyloxycarbonyl-L-prolyl-L-prolyl-L-prolyl Benzyl Ester (*t*-Boc-Pro$_3$-OBz) (IV).** *t*-Boc-Pro$_2$-OH (5.0 g) was treated with N-methylmorpholine (1.79 ml) and isobutyl chloroformate (2.2 ml) as described above for the preparation of I. After formation of the mixed anhydride, HCl·H-Pro-OBz (3.85 g) and N-methylmorpholine (1.79 ml) were successively added and the mixture was stirred overnight. Work-up as described above gave 7.10 g (86%) of *t*-Boc-Pro$_3$-OBz (IV) as a thick off-white syrup which resisted all attempts at crystallization, but displayed ir and nmr spectra and tlc behavior consistent with that expected for compound IV.

***t*-Butyloxycarbonyl-L-prolyl-L-prolyl-L-proline (*t*-Boc-Pro$_3$-OH) (V).** *t*-Boc-Pro$_3$-OBz (6.5 g) was dissolved in 75 ml of *t*-butyl alcohol, treated with 10% Pd/C, and subjected to the hydrogenation conditions described above. Filtration of catalyst and solvent removal gave crude *t*-Boc-Pro$_3$-OH as a colorless viscous oil which was taken up in a minimum of ethyl acetate. Slow crystallization occurred; a first crop weighed 1.0 g; collection of several crops yielded 4.4 g (total yield 86%) of material, mp 208–209°. A second crystallization of a small amount gave an analytical sample of V, mp 214–215°. The material displayed appropriate ir and nmr spectra and tlc behavior.

Anal. Calcd for $C_{20}H_{31}N_3O_6$: C, 58.66; H, 7.63; N, 10.26. Found: C, 59.07; H, 7.96; N, 10.34.

***t*-Butyloxycarbonyl-L-prolyl-L-prolyl-L-prolyl-L-prolyl Benzyl Ester (*t*-Boc-Pro$_4$-OBz) (VII).** *t*-Boc-Pro$_2$-OH (12.0 g) was treated with N-methylmorpholine (4.32 ml) and isobutyl chloroformate (5.31 ml) as described above for compound I. After formation of the mixed anhydride, HCl·H-Pro$_2$-OBz (13.0 g) and N-methylmorpholine (4.32 ml) were successively added and the mixture was stirred overnight. Work-up as described above provided *t*-Boc-Pro$_4$-OBz (VII) (19.0 g, 83%) as an off-white syrup which could not be crystallized. This product displayed appropriate ir and nmr spectra and tlc behavior.

***t*-Butyloxycarbonyl-L-prolyl-L-prolyl-L-prolyl-L-proline (*t*-Boc-Pro$_4$-OH) (VIII).** *t*-Boc-Pro$_4$-OBz (12.0 g) was dissolved in 100 ml of *t*-butyl alcohol, treated with 10% Pd/C, and subjected to the hydrogenation conditions described above. Filtration of catalyst and solvent removal gave *t*-Boc-Pro$_4$-OH as a viscous colorless syrup; the material appeared to crystallize from ethyl acetate on standing in the refrigerator but became a syrup again at room temperature. When left for 24 hr *in vacuo*, a white amorphous solid foam was formed (9.1 g, 91%), which displayed ir, nmr, and tlc behavior consistent with the expected product. In one small scale preparation, the viscous syrup was dissolved in ether and gave upon standing a short time a small crop of crystalline *t*-Boc-Pro$_4$-OH (VIII), which had mp 122–126°. The elemental analysis on this sample is indicated below.

Anal. Calcd for $C_{25}H_{38}N_4O_7$: C, 59.27; H, 7.56; N, 11.06. Found: C, 59.28; H, 7.89; N, 10.32.

***t*-Butyloxycarbonyl-L-prolyl-L-prolyl-L-prolyl-L-prolyl-L-prolyl Benzyl Ester (*t*-Boc-Pro$_5$-OBz) (IX).** *t*-Boc-Pro$_5$-OH (1.80 g) was treated with N-methylmorpholine (0.65 ml) and isobutyl chloroformate (0.80 ml) as described above for compound I. After formation of the mixed anhydride, HCl·H-Pro$_3$-OBz (2.50 g, prepared by subjecting 2.90 g of *t*-Boc-Pro$_3$-OBz to the action of HCl–ethyl acetate for 1 hr as indicated above) and N-methylmorpholine (0.65 ml) were successively added and the mixture was stirred overnight. Work-up as described above provided *t*-Boc-Pro$_5$-OBz as a thick syrup (2.2 g, 53%), which when taken up in a small amount of ethyl acetate gave crystalline *t*-Boc-Pro$_5$-OBz (IX) (first crop, 0.35 g), mp 196–197°. The material displayed appropriate ir, nmr, and tlc behavior.

Anal. Calcd for $C_{37}H_{51}N_5O_8$: C, 64.05; H, 7.41; N, 10.10. Found: C, 64.18; H, 7.78; N, 10.10.

***t*-Butyloxycarbonyl-L-prolyl-L-prolyl-L-prolyl-L-prolyl-L-proline (*t*-Boc-Pro$_5$-OH) (X).** *t*-Boc-Pro$_5$-OBz (1.2 g) was dissolved in 15 ml of *t*-butyl alcohol, treated with 10% Pd/C, and subjected to the hydrogenation conditions described above. Filtration of catalyst and solvent removal gave *t*-Boc-Pro$_5$-OH (X) as a colorless syrup (1.0 g) which was triturated with ether several times, each time decanting the mother liquor. After treatment of the residue *in vacuo* for 24 hr, there remained an amorphous solid foam (0.70 g,

(32) In another investigation, A. E. Tonelli [*J. Amer. Chem. Soc.*, **92**, 6187 (1970)] has calculated the intramolecular energies of all of the *t*-Boc-proline oligomer benzyl esters for $n = 2$ and $n = 3$, and compared them to quantitative estimates of the conformer populations based on the spectra of this paper. The agreement is in general excellent. However, these calculations, which omit any consideration of solvent interactions, do not provide any explanation of the sudden onset of helicity at $n = 5$.

68%), mp 65–80°, which resisted crystallization. The material displayed appropriate ir and nmr spectra and tlc behavior.

t-Butyloxycarbonyl-L-prolyl-L-prolyl-L-prolyl-L-prolyl-L-prolyl-L-prolyl Benzyl Ester (t-Boc-Pro$_6$-OBz) (XI). t-Boc-Pro$_2$-OH (3.69 g) was treated with N-methylmorpholine (1.33 ml) and isobutyl chloroformate (1.62 ml) as described above. After formation of the mixed anhydride, HCl·H-Pro$_4$-OBz (6.30 g, prepared by subjecting 7.0 g of t-Boc-Pro$_4$-OBz to the action of HCl–ethyl acetate for 1 hr as indicated above) and N-methylmorpholine (1.33 ml) were successively added and the mixture was stirred overnight. Work-up as described above gave t-Boc-Pro$_6$-OBz (XI) as a yellowish syrup (9.2 g, 94%), which when dissolved in a small amount of ethyl acetate gave, on standing, crystalline t-Boc-Pro$_6$-OBz (first crop 1.0 g), mp 221–229°. The material displayed appropriate ir and nmr spectra and tlc behavior.

Anal. Calcd for $C_{42}H_{58}N_6O_9 \cdot H_2O$: C, 62.35; H, 7.47; N, 10.39. Found: C, 62.45; H, 7.44; N, 10.29.

In another run, t-Boc-Pro$_3$-OH (1.0 g) was treated with N-methylmorpholine (0.28 ml) and isobutyl chloroformate (0.29 ml) as described. After mixed anhydride formation, HCl·H-Pro$_3$-OBz (1.07 g) and N-methylmorpholine (0.28 ml) were added. Overnight stirring and work-up as usual gave crude t-Boc-Pro$_6$-OBz (1.0 g, 60%), which when crystallized from ethyl acetate gave a first crop of crystalline material (0.36 g), mp 221–224°, which was identical with the "2 + 4" t-Boc-Pro$_6$-OBz.

t-Butyloxycarbonyl-L-prolyl-L-prolyl-L-prolyl-L-prolyl-L-prolyl-L-proline (t-Boc-Pro$_6$-OH) (XII). t-Boc-Pro$_6$-OBz (0.9 g) was dissolved in 15 ml of t-butyl alcohol, treated with 10% Pd/C, and subjected to the hydrogenation conditions described above. Filtration of catalyst and solvent removal gave 0.8 g (100%) of an amorphous foam which dissolved slowly in boiling ethyl acetate, and yielded, on standing, crystals of t-Boc-Pro$_6$-OH (XII) (first crop 0.46 g) which had mp 229–231°, and displayed ir and nmr spectra and tlc behavior appropriate for the expected compound.

Anal. Calcd for $C_{35}H_{52}N_6O_9$: C, 59.98; H, 7.48; N, 11.99. Found: C, 59.71; H, 7.75; N, 11.70.

t-Butyloxycarbonylglycyl-L-prolyl Benzyl Ester (t-Boc-Gly-L-Pro-OBz) (XIII). t-Butyloxycarbonylglycine (19.6 g) was dissolved in 270 ml of chloroform and cooled to −20° in a Dry Ice–CCl_4 cooling bath. Triethylamine (15.5 ml) and isobutyl chloroformate (14.4 ml) were added with stirring. After 30 min, L-proline benzyl ester hydrochloride (26.7 g) was added, followed by an additional equivalent of triethylamine (15.5 ml). After overnight stirring, work-up was accomplished by extractions of the reaction mixture successively with 200-ml portions of water, 5% aqueous sodium bicarbonate, and saturated sodium chloride. The chloroform layer was dried over sodium sulfate and evaporated to give a colorless syrup (41.0 g, 100%). When 10 g of this syrup was dissolved in a minimum of ethyl acetate and hexane was added to the cloud point, shiny crystals of t-Boc-Gly-L-Pro-OBz (XIII) formed (first crop 5.4 g). The crystals had mp 76–77°, and showed appropriate ir and nmr spectra and tlc behavior.

Anal. Calcd for $C_{19}H_{26}N_2O_5$: C, 62.96; H, 7.23; N, 7.73. Found: C, 62.96; H, 7.14; N, 7.71.

t-Butyloxycarbonylglycyl-L-proline (t-Boc-Gly-L-Pro-OH) (XIV). A solution of t-Boc-Gly-L-Pro-OBz (30 g) in 150 ml of t-butyl alcohol was treated with a catalytic amount of 10% Pd/C and hydrogenated at 20 psi for 30 hr at room temperature. Filtration through a pad of Celite to remove the catalyst followed by evaporation of the solvent gave a syrup. Crystallization from ethyl acetate–ether gave t-Boc-Gly-L-Pro-OH (XIV), first crop 10.0 g (45%), mp 126–135°, ir and nmr spectra and tlc behavior appropriate for the expected compound. A small amount of material which was crystallized in a similar fashion for analytical data had the same melting point.

Anal. Calcd for $C_{12}H_{20}N_2O_5$: C, 52.8; H, 7.4; N, 10.2. Found: C, 53.1; H, 7.6; N, 10.2.

Poly-L-proline. The sample of this polymer employed for these studies was prepared according to the method of Fasman and Blout.[5] The anhydride-initiator (sodium methoxide) ratio was 10, and the acetonitrile-insoluble fraction of the polymer was used. The reduced specific viscosity of this sample, determined on a solution of 15.0 mg of polymer in 10 ml of dichloroacetic acid, was 0.0296. From these data, the molecular weight of the sample, while not determined directly, was less than 5000. Higher polymers of proline were not sufficiently soluble in chloroform to allow nmr studies.

Acknowledgment. We are pleased to acknowledge support in part of this work by U.S. Public Health Service Grants No. AM-07300 and AM-10794. One of us (C. M. D.) held a Public Health Service Postdoctoral Fellowship (No. AM-20628) in the years 1967–1969. We thank Mr. J. J. Ryan and Mrs. A. I. Brewster for expert technical assistance in recording nmr spectra.

The Organic Solid State. III. Spectroscopic and Electrical Properties of Biferrocene[Fe(II)Fe(III)] Picrate[1]

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Abstract: The mixed valence compound biferrocene[Fe(II)Fe(III)] picrate (**1**) was prepared by the benzoquinone oxidation of biferrocene in the presence of picric acid. The ultraviolet, visible, and near-infrared spectra of **1** indicate transitions characteristic of the picrate anion, of ferrocene, and of ferrocenium ion. In addition a new mixed valence transition is observed (solution and solid) in the near-infrared [1900 mμ (ε 551)]. This new transition is discussed along with the observed shifts in metal–ligand stretching frequencies (far-infrared) which are known to be strongly dependent on the oxidation state of the central iron atom. This mixed valence compound was found to be six orders of magnitude more conducting than either ferrocene or ferrocenium picrate [$\sigma(298) = 2.3 \times 10^{-8}$ ohm^{-1} cm^{-1}].

There are a large number of inorganic compounds which contain metal ions of the same element in two different oxidation states.[4] Many of these mixed valence compounds have physical properties that are strikingly different from the materials containing just one of the oxidation states. For example, Fe_2O_3 and Fe_3O_4 have almost identical iron–iron distances (3 Å)

(1) (a) The Organic Solid State. Part II: D. O. Cowan and F. Kaufman, submitted for publication. (b) Part I: D. O. Cowan and F. Kaufman, *J. Amer. Chem. Soc.*, **92**, 219 (1970).

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(4) (a) M. B. Robin and P. Day, *Advan. Inorg. Chem. Radiochem.*, **10**, 247 (1967); (b) G. C. Allen and N. S. Hush, *Progr. Inorg. Chem.*, **8**, 357 (1967).