ture of amino acids was then subjected to chromatography on several thick sheets of filter paper, developing with *n*-BuOH–HOAc–H_2O (8:2:1). The zone corresponding to phenylalanine was extracted with hot H_2O. Evaporation and crystallization of the residue from aqueous ethanol afforded phenylalanine (58 mg) having an activity of only 1.6×10^4 dpm/mmol.

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Studies of Peptide Conformation. Evidence for β Structures in Solutions of Linear Tetrapeptides Containing Proline[1]

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Abstract: Carbobenzyloxy tetrapeptide *tert*-butoxycarbonyl hydrazides containing proline were investigated by study of their proton magnetic resonances in methanol and chloroform and their infrared absorption in chloroform. The infrared spectra indicate that internally hydrogen bonded structures are important. Studies of the resonances of nitrogen-bound protons included observation of differential line broadening effects produced by an added nitroxyl, used as an indication of solvent exposure, and determination of the concentration dependence of chemical shift (in chloroform), used as an indication of participation in intermolecular hydrogen bonding. For derivatives with the sequence Gly-L-Pro-D-Xxx-Gly the data support a major contribution, in both solvents, of a β structure with two intramolecular hydrogen bonds and a type II turn at Pro-Xxx. For the sequence D-Val-L-Pro-Gly-Xxx, evidence is less convincing, but a structure with a type I turn at Pro-Gly provides an explanation of the observations.

Nuclear magnetic resonance studies of oligopeptide conformation have dealt largely with cyclic peptides, because the information contained in the NMR spectra relates to only a fraction of the conformational variables. The cyclic constraint reduces the field of conformational possibilities for the molecule itself and limits the number of models that must be tested against the observable data. The conformation space available to linear, noncyclic peptides is much greater, and it is also less likely that there will be small regions of minimum conformational energy separated by bar-

Table I. Peptides Used in This Study

Abbr	Peptide	Formula	C, calcd (found)	H, calcd (found)	N, calcd (found)
GPGG	Z-Gly-d_2-L-Pro-Gly-Gly-NHNHBoc	$C_{24}H_{32}D_2N_6O_8 \cdot H_2O$	53.20 (53.38)	6.18 (6.40)	15.51 (15.77)
GP-D-AG	Z-Gly-d_2-L-Pro-D-Ala-Gly-NHNHBoc	$C_{25}H_{34}D_2N_6O_8$	54.91 (54.58)	6.31 (6.56)	15.37 (15.43)
GP-D-VG	Z-Gly-d_2-L-Pro-D-Val-Gly-NHNHBoc	$C_{27}H_{38}D_2N_6O_8 \cdot H_2O$	54.69 (54.72)	6.84 (6.89)	14.18 (14.15)
L-VPGG	Z-L-Val-L-Pro-Gly-Gly-NHNHBoc	$C_{27}H_{40}N_6O_8 \cdot 0.5H_2O$	55.37 (55.25)	7.06 (7.07)	14.35 (14.47)
D-VPGG	Z-D-Val-L-Pro-Gly-Gly-NHNHBoc	$C_{27}H_{40}N_6O_8 \cdot 0.5H_2O$	55.37 (55.10)	7.06 (7.23)	14.35 (14.02)
D-VPG-D-Ph	Z-D-Val-L-Pro-Gly-D-Phe-NHNHBoc	$C_{34}H_{46}N_6O_8$	61.25 (61.05)	6.95 (6.95)	12.60 (12.43)

riers sufficient to make for slow exchange among them. Thus the convenient assumption that NMR observations are mean values for a molecule moving in a very limited region of conformation space, i.e., reflect a single favored conformation, is less likely to be valid than it is for cyclic oligopeptides. There need not be predominant conformations at all. In addition, the conformational distribution of a linear oligopeptide will be more sensitive to environmental changes, such as those of solvent and temperature, than that of a cyclic peptide of comparable complexity. Finally, if there is a favored conformation, the increased range of possibilities that must be tested increases the difficulty of the model-building process.

Nevertheless, it is important to study the conformations of linear peptides, precisely because constraints are fewer and one can, if successful, obtain a better idea of the relation between sequence and conformation. However, in exploring linear peptides, it seemed wise to begin with a conformational feature that is potentially identifiable, the β turn, to begin with peptides in which it has a high probability of occurrence, and to avoid techniques in which major environmental perturbations are employed to obtain data.

The β turn is an arrangement of four amino acid residues, in which the peptide backbone reverses direction in the second and third residues and there is, or almost is, a ten-membered ring formed by a hydrogen bond between the N-H of the fourth residue and the C=O of the first. This backbone structure, which has been the subject of empirical conformational energy calculations,[2,3] has appeared frequently in cyclic oligopeptide structures deduced from NMR[4-9] and crystallographic studies.[10-14] It also has been identified in numerous examples in the backbones of globular proteins[15-18] and has been suggested as a regular feature of certain fibrous proteins.[19] In addition, X-ray crystallographic studies of linear tetra- and pentapeptides closely related to those to be described below have revealed β-turn structures,[20,21] and the conformation has also been suggested for some di- and tripeptide derivatives in the solid state.[22a] Through infrared studies, β turns have been identified among the conformations of dipeptide derivatives of the type RCO-L-Pro-Xxx-$NHCH_3$ dissolved in carbon tetrachloride or tetrachloroethylene.[22b]

Initially, we have examined a series of tetrapeptide derivatives containing a central L-Pro-Xxx sequence to determine whether an identifiable β turn will persist in solution in molecules so small. Because ϕ_{Pro} is restricted to $-60°$ by the pyrrolidine ring, proline in position 2 is expected to enhance the probability of β-turn formation.[2,3] Confirming this expectation, the protein data compiled by Fasman and Chou[16] show that proline is the most likely of all amino acid residues to appear in position 2 of a β turn. The most probable sequences, they found, were Pro^2-Asn^3 and Pro^2-Gly^3. Urry et al. have already reported NMR studies indicating the likely existence of β turns in L-Val-L-Pro-Gly-L-Val-Gly, its dimer and trimer, and related peptides.[23,24] A priori empirical calculations indicate also that the sequence L-Pro^2-D-Xxx^3 should provide a particularly favorable β turn,[2,3] and this is supported by studies of cyclic hexapeptides.[6,7c] The peptides we prepared and studied are listed in Table I. They were prepared, with a view to further elongation, as *N*-carbobenzyloxy *tert*-butoxycarbonyl hydrazides, and examined as such in this work. In potential for intramolecular hydrogen bonding these derivatives are the equivalent of linear hexapeptides.

The N-H proton of the fourth residue in a β turn is directed to the interior of the turn, and indications that this proton is sequestered from the solvent have been the major single NMR clue to the existence of β turns in oligopeptides. (Alone, of course, such indications do not certify the existence of the feature, and supporting evidence from the spectra, e.g., coupling constants, ^{13}C spectra,[24] and/or the model building process is required.) In this work the means we chose to identify an internally directed peptide proton excluded methods that could result in serious conformational changes in flexible peptide chains. We did not consider spectral changes produced by temperature and major solvent perturbations, or shift reagents, which coordinate with basic centers and can thereby influence conformation.[39]

We studied our peptides in methanol and chloroform solutions; a later report will deal with water-soluble derivatives. In both solvents we looked for differences in the broadening of peptide proton (N-H) resonances that are produced by small additions (up to 3% by volume) of a stable free radical, 3-oxyl-2,2,4,4-tetramethyloxazolidine.[25,26] In chloroform, a very weak hydrogen bonding solvent, these line width differences can be expected to reflect differences in the ability of peptide protons to form external hydrogen bonds. In methanol, where hydrogen bonding to solvent is dominant, the differences reflect differences in solvent exposure, not specifically hydrogen bond formation. We consider that the perturbation produced by the radical is minor, since the interaction that produces the line broadening is a weak one and the mole fraction of the perturbant in the solvent is quite low. Spectral overlaps produced by the broadening of lines make this method difficult to use quantitatively; a better adaptation would utilize direct measurement of changes in proton spin-lattice relaxation times under the influence of radical. Preliminary indications are that useful effects are produced at 0.2% added radical in hydrogen bonding solvents; at these concentrations the lines are much less broadened.[27]

Using the chloroform solutions, we examined the limiting N-H proton chemical shifts as peptide concentration is reduced, interpreting concentration-dependent chemical shifts

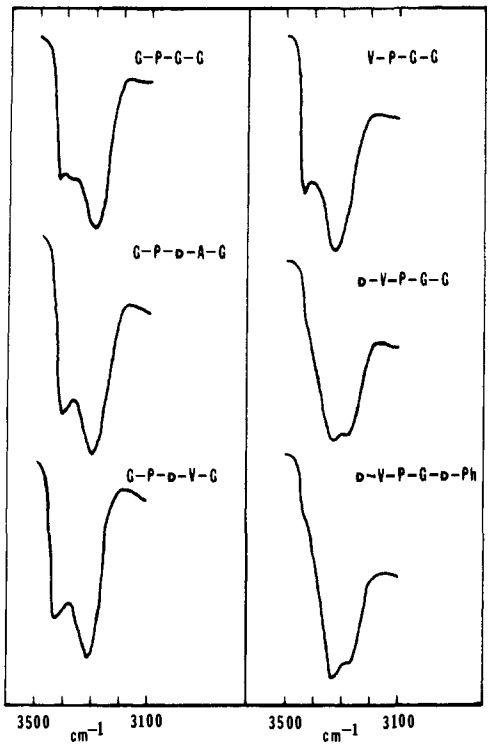

Figure 1. Infrared N-H stretching absorptions of the carbobenzyloxy tetrapeptide hydrazides at about 10^{-3} M in chloroform. The identifying abbreviations are defined in Table I.

as an indication of intermolecular hydrogen bonding, and using the limiting chemical shift as a guide to the involvement of particular peptide protons in intramolecular hydrogen bonds. We also examined the infrared spectra of the peptides in chloroform at concentrations down to 10^{-4} M, although the insolubility and complexity of these molecules precluded the sort of detailed study made by Boussard, Marraud, and Neel[22b] on dipeptide derivatives.

Experimental Section

Peptides. The carbobenzyloxy tetrapeptide *tert*-butoxycarbonyl hydrazides listed in Table I were prepared by stepwise elongation from C-terminal Gly-NHNHBoc or D-Phe-NHNHBoc, using carbobenzyloxy amino acid *N*-hydroxysuccinimide esters. Couplings were performed at about 0.4 M in purified dimethylformamide. Carbobenzyloxy groups were removed by hydrogenolysis over 10% palladium on carbon in 95% ethanol. Yields in each step were 80–95%, except for coupling with N-terminal proline, when the yield dropped in each case to 50–60%. Intermediates were not purified for analysis but were shown to be homogeneous on thin-layer chromatography before use in the next step. The final products were crystallized from 95% ethanol or ethanol–ether. They were dried at 100° (0.05 mm) for 48 hr before analysis and use in the infrared studies. Analyses are given in Table I.

NMR Spectra. Proton magnetic resonance spectra at 250 MHz were obtained using the spectrometer of the NMR Facility for Biomedical Studies of the Carnegie-Mellon University.[1] Spectra of the peptides were obtained from solutions in chloroform-*d* and methanol and of the deuterium-exchanged peptides in methanol-d_4. The spectra of chloroform solutions were measured at varying concentrations between 1–2 and 40–60 mg/ml, except for GPGG (the abbreviations used for the peptides are identified in Table I) which was only soluble to the extent of about 1 mg/ml. The spectra in methanol were obtained at 40–60 mg/ml, and about 10% acetic acid-d_4 was added in some cases to minimize line broadening by proton exchange with solvent. Chemical shift data, however, are reported for solutions containing no acetic acid. By shifting the hydroxyl line downfield, addition of acetic acid made more of the α-proton region directly observable. Spectra were obtained in the continuous-wave mode, except for the lowest concentration, where the rapid scan correlation technique[28] was employed. For the dilute solutions, the nominal 100.0 atom % D chloroform-*d* supplied by Aldrich was used. Resonance assignments were made by the usual double irradiation techniques. Sample temperature was constant at a nominal 30°, and internal tetramethylsilane was used as reference.

Infrared Spectra. Chloroform for the infrared studies was freed of ethanol by stirring with concentrated sulfuric acid and distilling from the mixture; it was kept in the dark under nitrogen and used within 1 week. The concentration of water in this solvent, estimated from the O-H absorption at 3690 cm^{-1}, was 0.6×10^{-4} M. Spectra were measured using a Perkin-Elmer Model 257 grating infrared spectrophotometer at its normal slit width, corresponding to a resolution of about 5 cm^{-1}, in the region of interest. A pair of matched 1-cm sample cells was used, and measurements were made in the concentration range 5×10^{-3}–1×10^{-4} M. The ratio of absorbance at the maximum of the peak near 3300 cm^{-1} to that of the maximum (or inflection) corresponding to the band near 3400 cm^{-1} was determined at 7–10 different concentrations. For none of the peptides was the concentration dependence of the 3300/3400 ratio significant over that concentration range. Examples of the spectra are reproduced in Figure 1. The samples used in these measurements were the analytical samples, which had been dried at 100° and about 20 μ of pressure; according to the analytical data (Table I), most of them contain 0.5–1 molecule of water, but the N-H region infrared absorption of GP-D-AG, which did not retain water, is the same, within the accuracy of the measurements, as that of the closely analogous GP-D-VG, which retained one molecule. The same was true of the pair D-VPG-D-Ph (no water) and D-VPGG (0.5 H_2O). In one experiment the N-H region of 5.5×10^{-4} M solutions of D-VPG-D-Ph was examined at increasing water concentrations from 0.6×10^{-4} (stock) to 6×10^{-4} M. No change was noted.

Results

The line broadening, chemical shift, and infrared observations described below show that the average structure of the peptides is sequence dependent, and the data constitute evidence for the contribution of folded β-type structures in certain instances.

Table II reports chemical shift and coupling constant data for the peptides. Figures 2–5 show the N-H regions of the proton spectra, and the effect of added radical. From the spectra unperturbed by nitroxyl it can be seen that there is one dominant component; there are no N-H resonances of significant intensity beyond the single set of five expected for one peptide. Cis–trans isomerism about a peptide bond to proline is slow on the NMR time scale, and if both cis and trans Xxx-Pro bonds were present at comparable mole fractions, two sets of N-H resonances would be discernible. If visible at all, additional N-H resonances were in all cases well below 10% of the intensity of the principal absorptions. That the dominant configuration at Xxx-Pro is trans is inferred from the α-proton resonances of the proline residue. These are uniformly triplets or quartets, with $J_{\alpha\beta}$, the separation between the outer peaks, 12–14 Hz. A sum near 8 Hz, the α-proton resonance appearing as a doublet, is characteristic of proline residues with a cis Xxx-Pro peptide bond.[29]

From the spectra shown in the figures, plus spectra measured at other radical concentrations, estimates can be made of the relative broadening of amide proton resonances by nitroxyl. In methanol (Figures 2 and 3), the resonances of NH^1 and NH^4 of GP-D-AG and GP-D-VG are least affected. The estimated order of sensitivity is 5,6 > 3 > 1,4. This is clearly seen for GP-D-AG in Figure 2. (The amide protons are identified by numbering from the amino end the nitrogens that bear them.) GPGG and L-VPGG have undeuterated glycine residues in both position 3 and 4, but one NH triplet is more sensitive than the other, and by analogy it may tentatively be assigned as NH^4. In these peptides

Table II. Proton Resonances of Carbobenzyloxy Tetrapeptide *tert*-Butoxycarbonyl Hydrazides, 30°[a]

Sequence 1	2	3	4	Z,	1				2			3			4		CONH–NHBoc	
				δCH_2	δNH	$J_{\alpha\text{-NH}}$	δ_α	$J_{\alpha\beta}$	δ_α	$J_{\alpha\beta}$[c]	δ_δ	δNH	$J_{\alpha\text{-NH}}$	δ_α	$J_{\alpha\beta}$	δNH	$J_{\alpha\text{-NH}}$	δ_α	δNH	δNH
Gly-d_2-L-Pro-Gly-Gly																				
MeOH				5.09, 5.10	7.07				4.38	q, 12	3.60	8.56	11.5[c]	3.85, 3.97		8.14	11.5[c]	3.83, 3.97	9.46	8.48
$CDCl_3$[b]					6.10		3.94, 4.20[e]		4.31	t, 12	~3.60	7.04	–, 5.5	3.64, 3.76		7.85	6, 6	3.80, 4.06	8.36	6.36
Gly-d_2-L-Pro-D-Ala-Gly																				
MeOH				5.07, 5.13	7.00		3.88, 4.02[e]		4.36	t, 12	3.60	8.76	6.5	4.25	7	8.27	12[c]	3.79, 3.97	9.14	8.47
$CDCl_3$[b]				4.91	5.97		3.88, 4.08[e]		~4.28	*g*	3.54	6.47	5.5	4.25		7.98	4.5, 6.5	3.67, 4.12	8.30	6.25
Gly-d_2-L-Pro-D-Val-Gly																				
MeOH				5.08, 5.12	7.01		3.90, 4.02[e]		4.51	q, 12	3.60	8.34	6.5	4.16	5.5	8.28	12[c]	3.82, 3.94	9.25	8.53
$CDCl_3$[b]				5.06	6.00		3.91, ~4.1[e]		4.41	t, 12	3.54	6.52	7.0	4.18	5	7.90	~4, ~6	3.72, 4.06	8.26	6.26
L-Val-L-Pro-Gly-Gly																				
MeOH				5.07	7.20	8	4.21	8	4.35	t, 13	3.65, 3.93	8.55	5.5, ~6	3.82, 3.94		8.31	11[c]	3.88, 3.96	9.62	8.60
$CDCl_3$[b]				5.00, 5.12	6.17	7.5	4.28	8	4.39	t, 12	3.66, 3.94	7.24	~4.5, 7	3.66, 4.22		7.80	11[c]	3.66, 4.12	9.03	6.88
D-Val-L-Pro-Gly-Gly																				
MeOH				5.05, 5.11	7.32	8	4.20	8	4.39	q, 13	3.69, 3.96	8.28	11[c]	3.78, 3.86		8.10	11[c]	3.88, 3.94	9.56	8.55
$CDCl_3$[b]				4.91, 5.11	7.17[d]		4.11		4.49	q, 12	3.62, ~4.1	~7.35[f]	4.5, 8	3.66, 4.17		7.47	4.5	3.86, 4.14	9.00	6.31
D-Val-L-Pro-Gly-D-Phe																				
MeOH				4.96, 5.06	~7.35[f]	7.5	4.19	8.3	4.65	q, 14	3.69, 3.85	8.31	12[c]	3.62, 3.76		8.04	8	4.37[h]	9.55	8.40
$CDCl_3$[b]				4.85, 5.11	7.08[d]	6.5	3.96	9.5	4.47	q, 12	3.62, 4.15	7.23[f]	4, 8.5	3.45, 4.25		7.23[f]		4.81[i]	9.09	6.22

[a] Concentration is about 40 mg/ml unless otherwise specified. Reference is internal tetramethylsilane. [b] NH resonances are given for about 2 mg/ml concentration. [c] Sum of both coupling constants, ±0.5 Hz. [d] Broad, about 30 Hz wide at half-height. [e] From resonances of residual protons. [f] Under aromatic proton absorption. [g] Overlap precludes measurement. [h] β protons at 3.09 ($J_{\alpha\beta}$ = 10 Hz) and 3.25 ($J_{\alpha\beta}$ = 4 Hz). [i] β protons at 3.06 ($J_{\alpha\beta}$ = 8.5 Hz) and 3.23 ($J_{\alpha\beta}$ = 4.5 Hz).

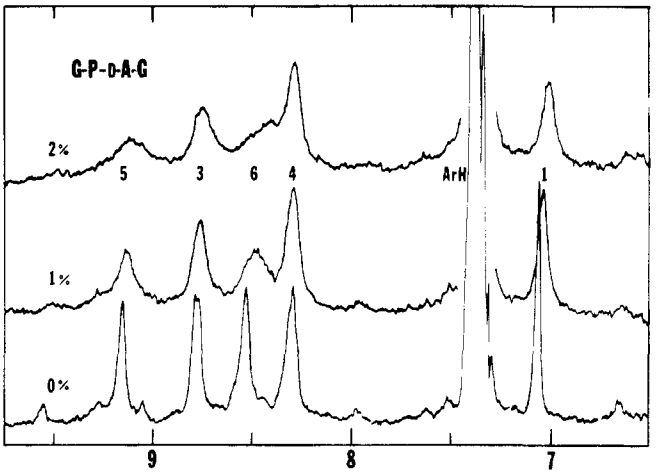

Figure 2. Peptide proton region (7–9 ppm below internal Me_4Si) of Z-Gly-d_2-L-Pro-D-Ala-Gly-NHNHBoc(GP-D-AG) in methanol, without added acetic acid, 30°, 60 mg/ml. The differential line broadening produced by 1 and 2% added 3-oxyl-2,2,4,4-tetramethyloxazolidine is shown. The identifying numbers refer to nitrogens numbered from the amino end of the peptide, as indicated in Figure 6.

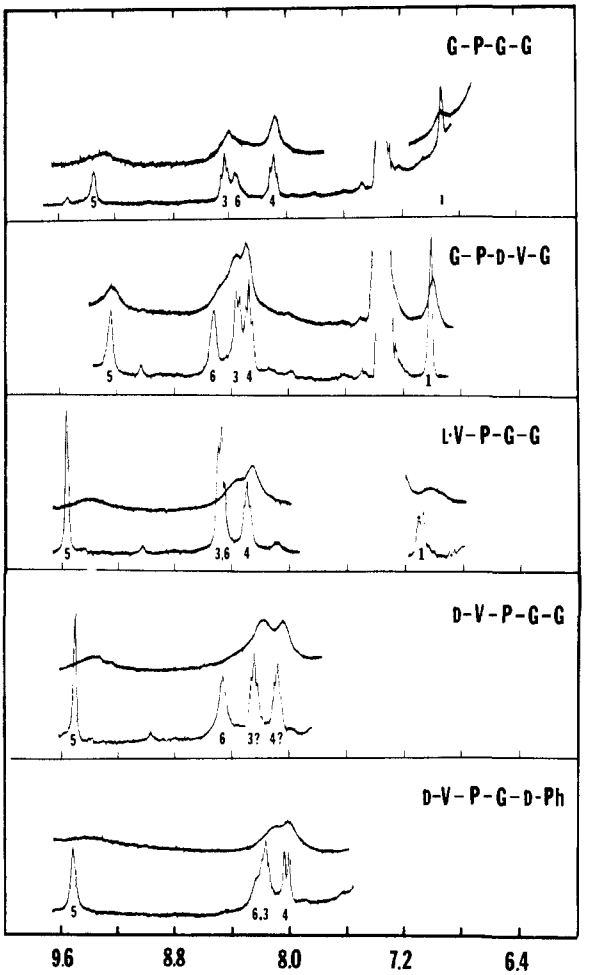

Figure 3. Peptide proton spectra (6–10 ppm below internal Me_4Si) of carbobenzyloxy tetrapeptide *tert*-butoxycarbonyl hydrazides, 30°, 40–60 mg/ml in methanol containing 10% acetic acid-d_3, showing line broadening effects of 1–2% 3-oxyl-2,2,4,4-tetramethyloxazolidine. (The solvent for GP-D-VG is methanol alone.) Aromatic proton resonances are defined in Table I; protons are identified by the nitrogens to which they are bonded, numbering from the amino end.

NH^1 is not shielded from nitroxyl. In D-VPG-D-Ph, NH^4 is again distinctly less affected by nitroxyl than the other amide protons. For D-VPGG and D-VPG-D-Ph the reso-

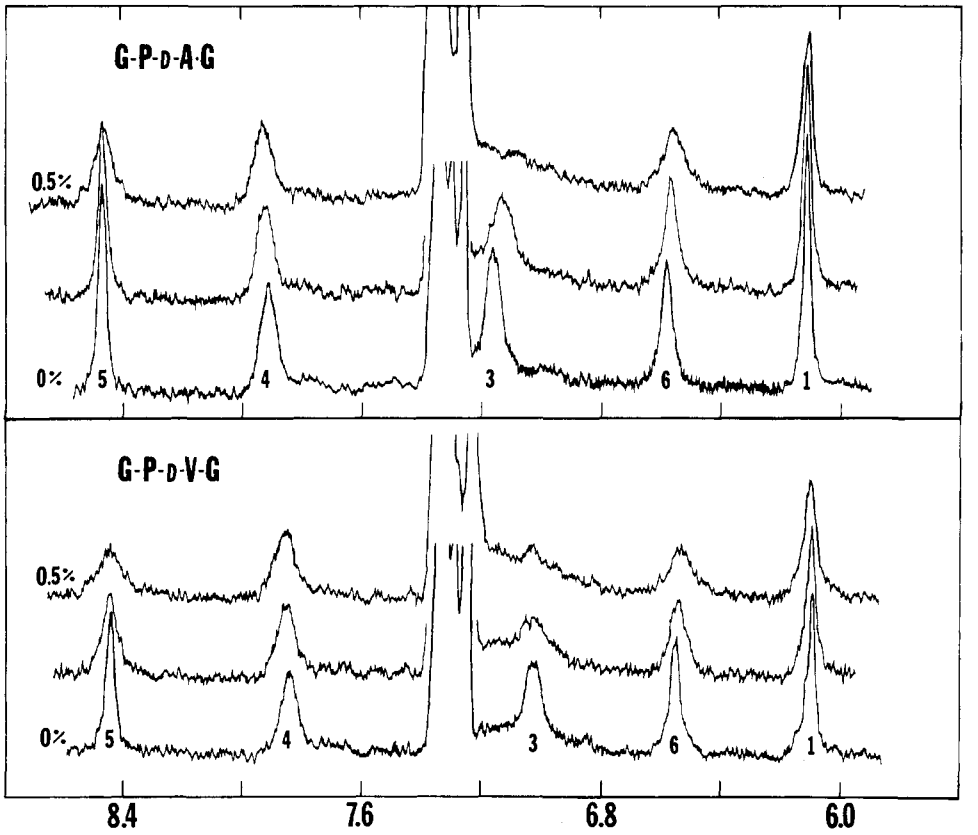

Figure 4. Peptide proton spectra (6–8.5 ppm below internal Me_4Si) of Z-Gly-d_2-L-Pro-D-Ala-Gly-NHNHBoc and Z-Gly-d_2-L-Pro-D-Val-Gly-NHNHBoc in chloroform, 30°, 14 mg/ml, showing the effect of added 3-oxyl-2,2,4,4-tetramethyloxazolidine at 0.25–0.5% by volume. Identification of resonances is as in previous figures.

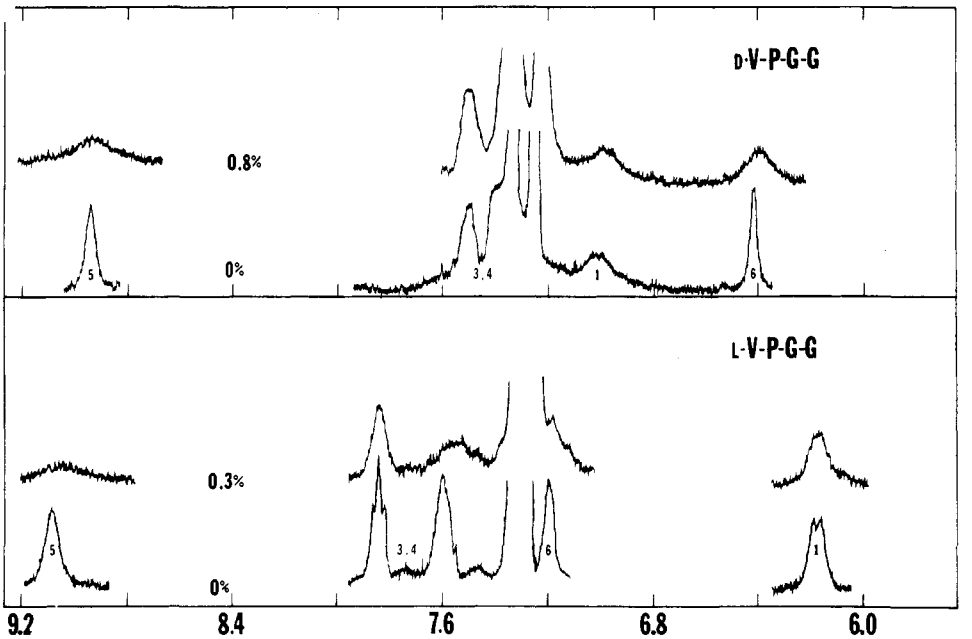

Figure 5. Peptide proton spectra (6–9 ppm below internal Me_4Si) of Z-D-Val-L-Pro-Gly-Gly-NHNHBoc and Z-L-Val-L-Pro-Gly-Gly-NHNHBoc in chloroform, 30°, about 10 mg/ml, showing the effect of added 3-oxyl-2,2,4,4-tetramethyloxazolidine at the indicated volume percent. Identification of resonances is as in previous figures.

nance of NH^1 lies under the resonances of the aromatic protons and no estimate of its sensitivity is possible.

In chloroform, the order of sensitivity for GP-D-AG and GP-D-VG is 3 > 5,6 > 1,4 (Figure 4). As in methanol, NH^1 and NH^4 are least affected by the nitroxyl, although NH^3, rather than NH^5 and NH^6, appears to be most exposed. GPGG was too insoluble in chloroform for study. For L-VPGG the resonance of NH^1 and one of the glycine triplets, NH^3 or NH^4, are least broadened (Figure 5). For D-VPGG the resonance of NH^1, which is broad even in the absence of radical, and the resonance of either NH^3 or NH^4 (one of them is too close to the aromatic proton absorptions for comparison) appear to be least exposed to radical. No comparisons can be made for D-VPG-D-Ph, because the resonances of NH^1, NH^3, and NH^4 are all under, or too close to, the aromatic proton absorption.

In sum, NH^4 of these peptides appears to be relatively shielded from added nitroxyl in both solvents, although there is ambiguity in the case of L-VPGG and D-VPGG. Further, in a number of instances NH^1 is also sequestered. These observations are consistent with dominance of a conformation in which the peptide chain folds sharply at residues 2 and 3, so as to permit internal hydrogen bonding by the N-H group of residue 4, i.e., if there is a β turn at Pro-Xxx as illustrated in Figure 6. If a stable β structure extends one residue beyond the turn, as shown in Figure 6, NH^1 is also sequestered. Evidence for this structure is strongest for GP-D-AG and GP-D-VG. In such a backbone arrangement the exposure of NH^3 will vary depending on its disposition relative to the side chain of residues 2 and 3.

The concentration dependence of peptide proton chemical shift in chloroform, at between 2×10^{-2} and 5×10^{-3} M, was examined for those peptides that were sufficiently soluble. The data are shown in Figure 7.

In GP-D-AG and GP-D-VG the resonance of NH^4 is concentration insensitive, which is consistent with the solvent shielded, internal position suggested by the nitroxyl experiments. The resonance of NH^3, on the other hand, moves 1 ppm upfield on dilution; this indicates that NH^3 is involved in intermolecular hydrogen bonding that is being diluted out in the 2×10^{-2}–5×10^{-3} M range. Comparison of the chemical shift of NH^3 at the highest measured dilution, 6.4–6.5 ppm, with the chemical shift of NH^4, 7.9–8.0 ppm, suggests that the NH^4 resonance is shifted downfield by intramolecular hydrogen bonding. Similar comparisons for the remaining NH's cannot be made, however, since in the other amide bonds the substituents directly attached to the CONH unit vary.

NH^1, which is buried in GP-D-AG and GP-D-VG according to the nitroxyl experiments, shows a relatively small concentration dependence, consistent with it being directed internally most of the time. However, NH^5, which is very much exposed to radical, also shows a smaller concentration dependence than does NH^3. Low concentration dependence is also observed for NH^5 of L-VPGG, D-VPGG, and D-VPG-D-Ph. (The last is not shown in Figure 7.) Association involving NH^5, the most acidic proton present, may persist in chloroform to the lowest concentrations used for the NMR studies. Acidity of NH^5 will also favor its association with nitroxyl.

The concentration dependences of the NH resonances of L-VPGG in chloroform are analogous to those of GP-D-AG and GP-D-VG. One of the two glycine resonances is only weakly concentration dependent; it appears at 7.8–7.9 ppm and is the less sensitive to radical. By analogy it is assigned to NH^4. NH^1 is also only weakly concentration dependent, consistent with its shielding from radical in chloroform, and NH^5, although exposed to radical, is also concentration insensitive. The upfield shift of the resonance assigned to NH^3 on dilution is not so large as in GP-D-AG and GP-D-VG. Since absence of a side chain in residue 3 must lessen steric interference to the involvement of NH^3 in intermolecular hydrogen bonding, association through NH^3 may persist in L-VPGG to lower concentrations than in GP-D-AG and GP-D-VG.

The data for D-VPGG are different. Where the NH^1 resonances of GP-D-AG, GP-D-VG, and L-VPGG in chloroform appear at about 6 ppm and move slightly upfield on dilution (0.1–0.2 ppm), in D-VPGG (and D-VPG-D-Ph) the NH^1 resonances are at 7 ppm and move slightly downfield on dilution (0.2 ppm). Further, the NH^1 resonance is quite broad, about 30 Hz at half-height. (See Figure 5.) The chemical shift differences suggest that hydrogen bonding by NH^1 is stronger in the peptides beginning with D-valine. The reverse concentration dependence, downfield on dilution, suggests that interpeptide association may indeed interfere with intramolecular hydrogen bonding. The breadth of the NH^1 line in the D-valine peptides suggests exchange among two or more states at an intermediate rate on the NMR time scale. Another difference is that in D-VPGG the two glycine NH resonances have the same, modest, concentration dependence and almost identical chemical shift. At about 5×10^{-3} M they both appear at about 7.3 ppm. The NH^3 and NH^4 resonances of D-VPG-D-Ph are obscured by the aromatic proton absorptions at 7.2–7.3 ppm, but since they both remain so obscured over the concentration range studied, it can be concluded that they also have similar and small concentration dependences. Probably, therefore, the details of the average conformation of D-VPGG and D-VPG-D-Ph differ from those of the other peptides. In a β turn conformation NH^3 and NH^4 have distinctly different environments, and the nitroxyl line broadening, concentration dependences, and chemical shifts all show this for GP-D-AG, GP-D-VG, and L-VPGG in chloroform. Comparative line broadenings are not available for chloroform solutions of the peptides beginning with D-valine, but the concentration dependences and chemical shifts suggest that in these two peptides NH^3 and NH^4 have similar average environments.

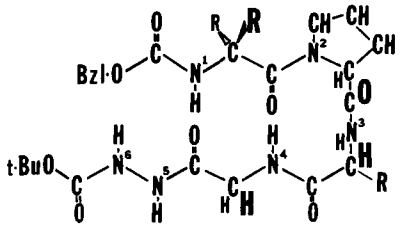

Figure 6. β structure proposed as an important contributor to the average conformation of the carbobenzyloxy tetrapeptide *tert*-butoxycarbonyl hydrazides. A type II β turn is indicated, with the carbonyl oxygen of proline about the average plane of the backbone. The numbering of nitrogens is that used throughout this paper.

Limited conformational information is available from the few observed H-N-C^α-H coupling constants of the tetrapeptides. The D residues of GP-D-AG and GP-D-VG in both solvents have J = 5.5–7.0 Hz, indicating an average dihedral angle of 130–140°;[30] i.e., ϕ_3 is in either the 70–80 or the 160–170° ranges. The former is more consistent with the type II β turn to be expected of an L-Pro-D-Xxx sequence.[3]

The sum of the coupling constants to the α protons of glycine in both positions 3 and 4 of all the peptides is 11–12 Hz, determined from the NH triplets. This matches $\phi \sim \pm 130°$ or $\sim \pm 70°$. For glycine in position 3, the first possibility is consistent with a type I (L-L) β turn and the second with a type II turn.

Some support for the β-turn structure of GP-D-VG exists in the 5-Hz value for the H-C^α-C^β-H coupling of the valine side chain. This low value indicates that the dominant rotamer is one in which H-C^α-C^β-H is gauche.[31] In residues 3 of an L-D β turn, with ψ_3 near 30°,[3] the rotamer that has the β proton rather than a methyl nearest the valine carbonyl ($\chi_1 = -60°$ for a D residue) appears in models to be the least crowded.

For Gly^3 of D-VPG-D-Ph in chloroform, analysis of the α-proton resonances is possible and yields H-N-C^α-H coupling constants near 4 and 8 Hz. One of the glycine residues of D-VPGG shows similar values; these correspond to dihedral angles of 20 and 140°[30] or $\phi \sim \pm 80$%.

The valine residues in L-VPGG, D-VPGG, and D-VPG-D-Ph all have H-N-C^α-H couplings between 6.5 and 8 Hz, indicating $\phi_1 \sim -80$ or $-160°$ for the L residue and $\sim +80$ or $+160°$ for the D residue. For these valine residues the

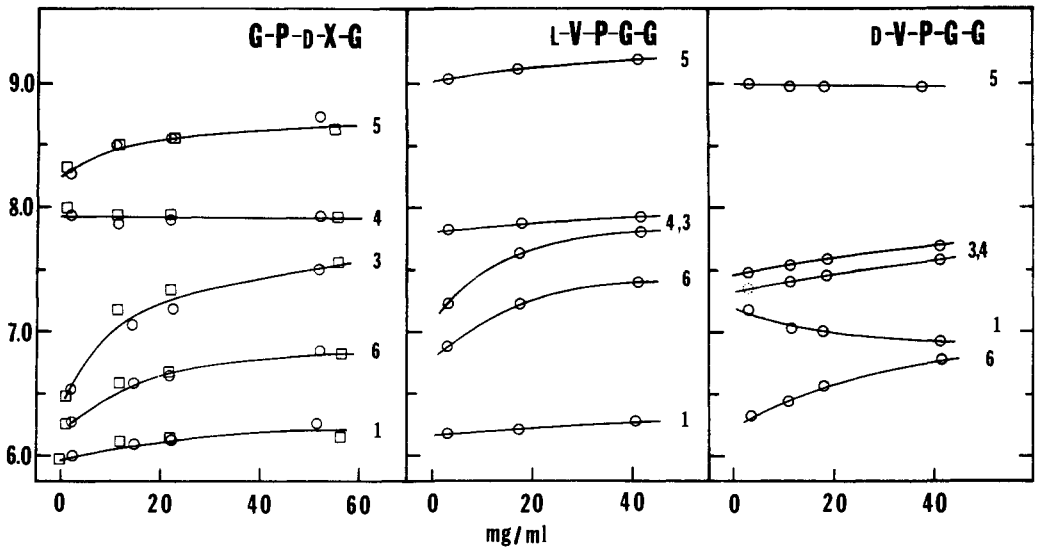

Figure 7. Chemical shift relative to internal Me_4Si, 30°, of carbobenzyloxy tetrapeptide *tert*-butoxycarbonyl hydrazides at varying concentrations in chloroform. In the plot at left, squares are data for GP-D-AG and circles for GP-D-VG.

H_α-H_β coupling is 8–9 Hz, which requires a backbone conformation of such that at least two of the side-chain rotamers, one of them that with trans H-C^α-C^β-H, can be stable.

Turning to the infrared observations, all of the peptides save those with N-terminal D-valine exhibit in chloroform distinct non-hydrogen-bonded N-H stretching absorption near 3430 cm^{-1}, as well as considerably more intense absorption, assigned to hydrogen bonded NH, at 3300 cm^{-1} (Figure 1). The peptides with N-terminal D-valine, again different, exhibit no separate band in the 3430-cm^{-1} region, even at 2×10^{-4} *M*.

Where two separate absorption maxima are observed, the ratio of absorbance at the maximum near 3300 cm^{-1} to that near 3430 cm^{-1} is 1.3–1.5 and is independent of concentration between 10^{-3} and 10^{-4} *M* (Figure 2). This ratio does not bear a direct relation to the fraction of peptide protons hydrogen bonded, of course, and in addition it may be that more intense free N-H absorption would appear at concentrations lower than we were able to use. However, the observations may be compared with those of Shields et al.[32] on some proline-free peptides. For Boc-L-Val-L-Val-L-Ala-Gly-OEt, Boc-(L-Ala)$_4$-OMe, Boc-(L-Leu)$_4$-OMe, and Boc-L-Ala-L-Val-L-Ala-L-Val-OMe they observed both bonded and nonbonded N-H absorption at 10^{-3}–10^{-4} *M* in chloroform. The absorbance ratios A_{3330}/A_{3430} were 0.5–0.8, not strongly concentration dependent, and they interpreted them as indicating some intramolecularly hydrogen bonded structures. For other tetrapeptides, e.g., Boc-(L-Val)$_4$-OMe and Boc-L-Val-L-Ala-L-Ala-OMe, the ratios were lower and very dependent on concentration in the 10^{-3}–10^{-4} *M* range.

The ratios of bonded to nonbonded N-H absorbance that we observe for all of the peptides of the present study at 10^{-3}–10^{-4} *M*, taken with the apparent concentration independence of this ratio, are only qualitative indications, but they do show that intramolecular hydrogen bonding is an important feature of the conformations present. In dilute solution in chloroform considerable internal hydrogen bonding is expected if it is possible, and a β structure such as that of Figure 7 would accomplish this. Not only can NH^1 and NH^4 be intramolecularly associated with carbonyls, but NH^6 may also be associated with the ether oxygen of the carbobenzyloxy group. NH^3 and NH^5 would, in the model, still be available for intermolecular association, some of which could persist to lower concentrations than those examined.

The appearance of the carbonyl stretching regions of the peptide infrared spectra, Figure 8, agrees with the other observations in dividing the peptides into three classes, depending on whether glycine, L-valine, or D-valine precedes proline. The band in the spectra of the Gly^1-Pro^2 peptides at 1710–1720 cm^{-1}, assigned to one of the urethane carbonyls, most likely that of the carbobenzyloxy group,[33] moves to below 1700 cm^{-1} when Gly^1 is replaced by D- or L-valine. The band at 1640 cm^{-1} assigned to the carbonyl of Gly^1 [22] also moves 15–25 cm^{-1} to lower frequency upon substitution for Gly^1. These changes might reflect changes in hydrogen bonding of the corresponding carbonyls. However, in the case of CO^1 they should be paralleled by a corresponding change in the (NMR) chemical shift of NH^4, which is not observed. It is more likely that the changes result from changes in ϕ_1 and ψ_1 that affect the orientations of nearby bond dipoles. By analogy with the well-established effects in α-halo ketones,[34] one may expect ν_{CO} to decrease as ψ_1 departs from 180°, i.e., as the C^α-N bond rotates away from the C=O bond.

Discussion

Evidence for the stability of an antiparallel β structure such as that shown in Figure 6 is strongest for Z-Gly-d_2-L-Pro-D-Ala-Gly-NHNHBoc (GP-D-AG) and Z-Gly-d_2-L-Pro-D-Val-Gly-NHNHBoc (GP-D-VG). In these peptides NH^1 and NH^4 are both sequestered in methanol and in chloroform according to the line broadening effects of added nitroxyl, and they are only weakly if at all involved in intermolecular hydrogen bonding in chloroform, according the the concentration dependence of the NH^1 and NH^4 chemical shifts. The H-N-C^α-H couplings of Val^3 and Ala^3 in these peptides also support the β-turn conformation of the expected type II variety. The observations that could be made on Z-Gly-d_2-L-Pro-Gly-Gly-NHNHBoc are analogous. Likely dihedral angles for the proposed backbone structure, chosen to match the observed coupling constants and optimize the $NH^4 \cdots OC^1$ and $NH^1 \cdots OC^4$ interactions, are ϕ_1 180°, ψ_1 180°, ϕ_2 ~−60°, ψ_2 ~100°, ϕ_3 ~80°, ψ_3 ~40° (ϕ_3 ~160°, ψ_3 ~−30° is an alternative), ϕ_4 ~−130°, ψ_4 ~150°. The structure is undoubtedly flexible, however, and likely to be even more so toward the ends of the chain, so that NH^1 would be less hydrogen bonded to OC^4, on the average, than NH^4 is to OC^1. Any association of NH^6 with either of the carbobenzyloxy oxygens suggested by Figure 6

need not be sufficiently important to evidence itself in our NMR experiments.

A model for these substances in which there is a 1-3 hydrogen bond between NH^4 and OC^2 can be constructed in the $\phi_3 = 80°$ model, by rotation of ψ_3 to $-60°$. Other accommodations could be made to retain an $NH^1 \cdots OC^4$ hydrogen bond. An $NH^4 \cdots OC^2$ hydrogen bond would be consistent with the concentration dependence of the NH^4 chemical shift in chloroform. However, it is our belief, based on examination of space-filling models, that this model does not provide the solvent shielding of NH^4 indicated by the experiments with added free radical. Further, a conformation near 80°, −60° for a D residue is close to the region of severe interference between side chain and carbonyl oxygen. On the other hand, a γ turn[35] that adds to the internal $NH^4 \cdots OC^1$ interaction a seven-membered $NH^3 \cdots OC^1$ hydrogen bond could be a significant contributor. To the extent that this intramolecular hydrogen bonding of NH^3 occurs one expects a reduced concentration dependence of the NH^3 chemical shift and of the NH^3 contribution to the infrared absorption, although NH^3 would not be shielded from the radical.

Because the backbone conformation average may vary with solvent, chemical shift differences between the methanol and chloroform solutions are not good evidence for any particular conformation of these peptides, but it is worth noting that in GPGG, GP-D-AG, and GP-D-VG the change for NH^4 is only 0.3-0.4 ppm. Contrasted with values of 1.5-2.3 ppm for NH^3 and about 2.1 ppm for NH^6, this small difference does indicate the environment of NH^4 is relatively independent of solvent, and this supports the involvement of NH^4 in stable β turn.

Related conformations of crystalline peptides have been found. One such is the structure found for crystalline Z-Gly^1-L-Pro^2-L-Leu^3-Gly^4-L-Pro^5 by Ueki et al.[20] In this open chain carbobenzyloxy pentapeptide there are hydrogen bonds between $N\text{-}H_{Gly_1}$ and $C\text{-}O_{Gly_4}$ and between $N\text{-}H_{Gly^4}$ and $C\text{-}O_{Gly^1}$. The structure differs from the likely solution conformation of Z-Gly-L-Pro-D-Xxx-Gly-NHNHBoc at least in the orientation of the plane of the peptide bond between Pro and Leu. In the crystal, the β turn is type I (L-L), and in the derivatives just discussed it is more likely to be type II.[2,3]

A cyclic hexapeptide structure analogous to the present case is that of $(L\text{-Ala-L-Pro-D-Phe})_2$. The crystal structure recently determined by Brown[14] is in agreement with deductions from NMR data about one of the two solution conformations,[7a] in that there are two type II L-Pro-D-Phe β turns connected by two extended Ala residues which form mutually two good transannular hydrogen bonds. The sequence L-Ala-L-Pro-D-Phe-L-Ala in this compound is thus similar to the Gly-L-Pro-D-Xxx-Gly sequence in the tetrapeptide derivatives.

Replacement of glycine preceding proline by L-valine, as in L-VPGG, introduces the possibility of interference between the valine side chain and the δ-methylene of proline when ψ_{Val} is near 180°. (Limitations to the conformations of L-alanine before L-proline have been discussed.[36,37]) Models indicate that the interference is eliminated for lower values of $\psi_{Val} \leq 150°$. ϕ_{Val} also must depart from the fully extended position possible for glycine to allow free motion of the isopropyl side chain. The coupling constant data do agree with $\phi_{Val} = -160°$. These changes from the model just suggested tend to expose NH^1, and this may be a part of the explanation for the observed exposure of NH^1 to radical in methanol. In chloroform, where stabilization by association of NH^1 and CO^4 may be more important, accommodations may be made to hold NH^1 near OC^4.

A related situation is found in the cyclic hexapeptides

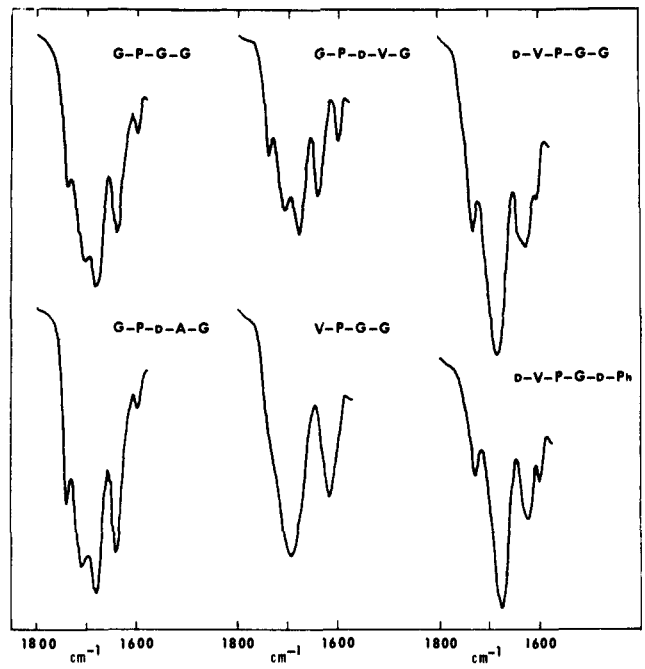

Figure 8. Infrared spectra in the carbonyl stretching region of the carbobenzyloxy tetrapeptide *tert*-butoxycarbonyl hydrazides in chloroform at $(5 \pm 2) \times 10^{-4}$ *M*. The identifying abbreviations are defined in Table I.

cyclo-$(Gly\text{-L-Pro-Gly})_2$ and *cyclo*-$(L\text{-Val-L-Pro-Gly})_2$. The former takes a conformation with β turns in which the Gly-Pro peptide bonds are trans, and the glycine preceding proline is extended and involved in two transannular hydrogen bonds.[38] In the valine analog, the β-turn structure is unfavorable, and the dominant conformation of the cyclic peptide is one in which both L-Val-L-Pro peptide bonds are cis.[6] In the L-VPGG case there is no cyclic constraint, so that the Val-Pro stress can be relieved without a change in stereochemistry of the L-Val-L-Pro peptide bond.

The peptides D-VPGG and D-VPG-D-Ph can also be folded into a type II β structure that sequesters NH^1 and NH^4 while avoiding interference between the valine side chain and the proline δ-methylene, but we can present no evidence that a β conformation is dominant. Although NH protons assignable to residue 4 have been shown to be relatively unexposed to radical, for one reason or another (overlaps, ambiguities) we have no example in which NH^1 and NH^4 are shown simultaneously to be sequestered. More important, there are distinct differences between the NMR observations of these peptides and of those with Gly^1-L-Pro^2 or L-Val^1-L-Pro^2. In chloroform, the resonance of NH^1 is broad and shifted 1 ppm downfield; also there is little distinction in chemical shift or concentration dependence between NH^3 and NH^4. In methanol the NH^1 resonance is shifted sufficiently downfield to lie under the aromatic proton resonances. There are also the infrared observations, which cannot definitively be interpreted, but show a significant decrease in the amount of absorption by non-hydrogen-bonded N-H, relative to the other peptides at the same concentrations in chloroform. A major contribution by another structure to the average state seems indicated. A tentative suggestion is a type I β turn, although this does not explain the infrared observations (see below).

Interference between the D-valine side chain and the L-proline ring can be eliminated in a β structure with a turn close to type I, the turn that accommodates L residues in both positions 2 and 3.[2,3] Dihedral angles for this structure consistent with the observed couplings are $\phi_1 \sim 160°$, $\psi_1 \sim -130°$, $\phi_2 \sim -60°$, $\psi_2 \sim -60°$, $\phi_3 \sim -80°$, $\psi_3 \sim 30°$, ϕ_4

$\sim 160°$, and $\psi_4 \sim 180°$. The turn in this structure does not form an optimal $NH^4 \cdots OC^1$ hydrogen bond, although the NH^4 bond is directed internally. NH^3 in this conformation is somewhat shielded by the pyrrolidine ring, which is not the case in the type II structure. The chemical shifts and concentration dependences of the NH^4 and NH^3 resonances in chloroform thus could coincidentally be similar. A good hydrogen bond is formed by $NH^1 \cdots OC^4$ in this structure, and an explanation suggests itself for the breadth of the NH^1 resonance in chloroform: there may be exchange between a slightly more stable (1-1.5 kcal) type I structure and one or more others in which the NH^1 resonance is shifted upfield because of weaker hydrogen bonding. If the shift difference is 1 ppm (250 Hz), the barrier for the interconversion of conformers is estimated from the line width to be in the 10-15 kcal range, somewhat less than that for cis-trans amide interconversion.

The type I β-turn hypothesis does not offer an explanation for the greater hydrogen bonding evidenced by the N-H stretching absorption of D-VPGG and D-VPG-D-Ph in chloroform. These peptides may be more strongly *inter*molecularly associated than the others, and this might in turn suggest that they are less stable in an *intra*molecularly hydrogen bonded, folded form. If the association involves NH^1 it might be invoked in an alternative explanation of the NH^1 line width.

A measure of the stabilities of the folded structures that are suggested by this work may perhaps be obtained from study of the spin-lattice relaxation times of the ^{13}C resonances of the α-carbons. We expect to make such studies.

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References and Notes

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