Synthesis and Peptide Bond Orientation in Tetrapeptides Containing L-Azetidine-2-Carboxylic Acid and L-Proline

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SYNOPSIS

The role of the amino acid proline in influencing the secondary and tertiary structure of proteins and polypeptides has been an area of active study for many years. We have investigated this problem by incorporating the four-membered ring amino acid, azetidine-2-carboxylic acid, into some proline polypeptides. An adjunct to the synthesis of the peptides was the synthesis of azetidine-2-carboxylic acid and its resolution. We developed an improved synthesis of N-benzhydryl-2-carbobenzyloxy azetidine, an essential intermediate required for the synthesis of L-azetidine-2-carboxylic acid. This amino acid was subsequently obtained via the partial hydrogenation of the N-benzhydryl compound, under mild conditions. Our ability to isolate the intermediate N-benzhydryl-2-carboxylic acid demonstrated that the rate of cleavage of the *O*-benzyl ester group in this molecule is faster than the cleavage of the N-benzhydryl group.

The tetrapeptides, Boc-(L-Pro)$_3$-L-Aze-Opcp, and Boc-(L-Aze-L-Pro)$_2$-Opcp (Boc: *t*-butoxycarbonyl; Pro: proline; Aze: azetidine-2-carboxyl acid; Opcp: pentachlorophenyl), were prepared using traditional solution peptide synthesis. They were characterized by direct chemical ionization–mass spectrometry, CD spectra, and ^{13}C- and ^{1}H-nmr spectroscopy. The assessment of the secondary structure of the two peptides using the methods noted above has led us to conclude that the compound Boc-(L-Aze-L-Pro)$_2$-Opcp, in trifluoroethanol, has an all-*cis* peptide bond conformation with ϕ and ψ torsion angles compatible with a left-handed helix. The secondary structure assessment of the peptide Boc-(L-Pro)$_3$-L-Aze-Opcp, in chloroform or trifluoroethanol, leads to an assignment of both *cis* and *trans* peptide bonds as being present in the peptide. We have interpreted this latter finding as indicating that the introduction of the azetidine group into a peptide containing three consecutive proline residues in a linear sequence perturbs the normal proline peptide secondary structure in this tetrapeptide.

INTRODUCTION

Three amino acids derived from 4-, 5-, and 6-membered nitrogen-containing rings occur in nature. The 5-membered ring amino acid, proline, occurs in proteins, and exerts a major influence on the secondary structure and folding of the protein. The synthetic polypeptide, polyproline, has been extensively studied as a model system for those proteins such as collagen that contain high levels of this amino acid.[1] Because this cyclic amino acid exerts such a strong influence on the secondary structure of proteins, many model systems have been developed that incorporate proline as probes to perturb the secondary structure in synthetic polypeptides as well as proteins. The use of alkyl-substituted prolines in synthetic peptides has proven to be a useful tool for examining the influence of steric factors on the orientation of the peptide bond. The influence of alkyl substituents at the 2 and 5 positions of the pyrrolidine ring on the structure of oligomeric peptides has previously been evaluated in this laboratory.[2,3] The 4-membered ring amino acid, azetidine-2-carboxylic acid, occurs naturally in plants, but is not

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incorporated into proteins. It has not received much attention as a probe to determine factors that affect protein conformation. The x-ray crystal structure of L-azetidine-2-carboxylic acid has been reported,[4] as has the structure of L-proline.[5,6] The crystal structures of the dipeptides benzyloxycarbonylprolylproline and N-benzyloxycarbonylprolyl-L-azetidine-2-carboxylic acid have also been reported.[7] For these dipeptides the structures of the two molecules show no significant differences. The ϕ and ψ angles at the C-terminal azetidine do not differ from those at the C-terminal proline residue by more than the differences between the equivalent N-prolyl residues. The dipeptide structures are virtually superimposable except for the C-terminal rings. This demonstrates that substitution of azetidine-2-carboxylic acid for proline might be expected to affect the macromolecular structure largely through steric effects and not via effects on the conformation of the main chain. Only various di- and tripeptides of azetidine-2-carboxylic acid or azetidine-2-carboxylic acid derivatives have received much attention. The crystal structure of N-(*t*-butyloxycarbonyl)-L-azetidine-2-carboxylic acid was reported by Cesari et al.,[8] who used the data to carry out a conformational analysis of poly(L-azetidine-2-carboxylic acid), and were able to demonstrate that the polymer formed helices in which there is a hydrophilic spiral of oxygen atoms and a hydrophobic spiral formed by the azetidine rings. The *trans* form was more extended than the *cis* form, and had its oxygen atoms more accessible to the solvent, which could facilitate hydrogen-bond formation. The structures of the cyclic trimers of azetidine[9] and of proline[10] have also been reported. Reports of conformational or structural studies of linear peptides containing proline and azetidine larger than the dipeptide have been limited. We have now examined the influence of azetidine-2-carboxylic acid on the peptide bond orientation of two proline-containing tetrapeptides.

MATERIALS AND METHODS

N-Benzhydryl-2-Carbobenzyloxyazetidine

Benzyl 2,4-dibromobutyrate (13.46 g, 0.04 mol), benzhydrylamine (7.64 g, 0.04 mol), and N,N-diisopropylethylamine (10.64 g, 0.08 mol) were added to 250 mL of acetonitrile and the solution heated under reflux for 20 h. After the reaction was cooled to room temperature, the acetonitrile was removed on a rotary evaporator and ether was added to dissolve the product leaving a residue of N,N-diisopropylethylamine hydrobromide. This salt was then removed by filtration and subsequent addition of gaseous HCl to the mother liquor yielded a precipitate. This precipitate was dissolved in 150 mL of chloroform and the solution treated with triethylamine (12.48 g, 0.08 mol). Removal of the chloroform on the rotary evaporator, and addition of ether dissolved the product and left a residue of triethylamine hydrochloride. The triethylamine hydrochloride was removed by filtration and the ether by evaporation. The residue was dissolved in a mixture of ethyl acetate/*n*-hexane (1 : 3 v/v) and filtered through a 20 g pad of Florisil. Evaporation of the ethyl acetate/*n*-hexane mixture gave an oil that could be crystallized from an ether/pentane solution (3 : 1 v/v). The yield of product was 11.78 g, 82.5% yield, mp 61–63°C, lit.[11] mp 61–63°C.

N-Benzhydryl-Azetidine-2-Carboxylic Acid

A solution of N-benzhydryl-2-carbobenzyloxyazetidine (6.6 g, 0.018 mol) in 150 mL of methanol was mixed, under a nitrogen atmosphere, with 0.2 g of 1% palladized charcoal (Pd/C) catalyst. The hydrogenator was charged with hydrogen at 45 psi and shaken at this pressure for 2 h. The catalyst was filtered off on a bed of Celite and, after concentrating the solution to a small volume, water was added with stirring to obtain a white precipitate. Recrystallization of the precipitate from hot ethyl acetate gave the desired product, 4.52 g, 94% yield, mp 138–139°C.

Anal. Theoretical $C_{17}H_{17}O_2N$: C, 76.38; H, 6.41; N, 5.24.
Found: C, 75.87; H, 6.56; N, 5.56.
δ(hot DMSO-d_6): 2.20–2.38; 2.49–2.50 (2H, m, βCH_2), 2.82–2.88 (1H, dd, γCH_2); 3.24–3.20 (1H, m, γCH_2), 3.61–3.67 (1H, t, αCH), 4.60 [1H, s, $CH(C_6H_5)_2$], 7.12–7.44 [10H, m, $(C_6H_5)_2$]. ν_{max} (KBr): 1616 cm^{-1} (ionic carboxylate).
$(M + 1)^+$. Calcd. for $C_{17}H_{17}O_2N$: m/e 268.1338.
Found: 268.1339; (relative intensity): 268 (31.96), 222 (5.37), 195 (9.94) 190 (4.31), 167 (100), 91 (5.69).

L-Azetidine-2-Carboxylic Acid

This amino acid and its resolution was prepared using the procedure of Rodebaugh and Cromwell.[12] Recrystallization from 90% methanol gave an analytically pure crystalline solid.

$[\alpha]^{25}_D = -108°$ (c = 3.6 in water). lit.[12] $[\alpha]^{25}_D = -109°$ (c = 3.6 in water).
$\delta(D_2O)$: 2.49–2.55, 2.74–2.79 (2H, m, βCH_2), 3.88–3.93, (1H, m, γCH_2), 4.03–4.10 (1H, dd, γCH_2), 4.79 (1H, s, αCH) (COOH and NH exchanged with H_2O in 96% D_2O).

Boc-(L-Pro)$_3$-OH

A stirred solution of Boc-L-Pro-OH (Boc: *t*-butoxycarbonyl; Pro: proline; 2.15 g, 0.01 mol) in 100 mL of chloroform was chilled to −23°C; *N*-methylmorpholine (1.12 mL, 0.01 mol) and isobutylchloroformate (1.4 mL, 0.01 mol) were added successively. After 0.5 h, HCl-(L-Pro)$_2$-Obn (bn: benzyl; see Ref. 13; 3.39 g 0.01 mol) was added, followed by the addition of N-methylmorpholine (1.12 mL, 0.01 mol). The reaction mixture was warmed slowly to room temperature and allowed to stir overnight. The solution was then washed successively with water, 5% sodium bicarbonate, and saturated sodium chloride; lastly, the chloroform solution was dried over anhydrous magnesium sulfate, filtered, and the chloroform evaporated. The residual oil was taken up in 100 mL of methanol and then hydrogenated at 45 psi with shaking, for 6 h, with 10% Pd/C (0.2 g) as a catalyst. The catalyst was removed by filtration through a bed of Celite and the solvent evaporated. The residual oil was crystallized from hot ethyl acetate; mp 209–211°C, reported[14] mp 214–215°C, 3.07 g, yield 75%.

$\delta(CDCl_3)$: 1.40–1.50 (9H, d, Boc), 1.78–2.38 (12H, m, βCH_2Pro, γCH_2Pro), 3.35–3.95 (6H, m, δCH_2Pro), 4.35–4.45 (0.4H, dd, αCHPro), 4.48–4.52 (0.6H, dd, αCHPro), 4.54–4.62 (1H, m, αCHPro), 4.62–4.72 (0.4H, dd, αCHPro), 4.72–4.80 (0.6H, dd, αCHPro).

Boc-(L-Pro)$_3$-L-Aze-Opcp

Boc-(L-Pro)$_3$-OH, 1 g, 2.44 mmol, was added to 26 mL of chloroform, and the solution was stirred and cooled to −23°C. N-methylmorpholine (0.28 mL, 2.44 mmol) and isobutylchloroformate (0.29 mL, 2.44 mmol) were added in succession. After 0.5 h, L-Aze-Opcp HCl (Aze: azetidine-2-carboxylic acid; pcp: pentachlorophenyl; see Ref. 15; 1.02 g, 2.44 mmol) was added. Lastly, an additional equivalent of N-methylmorpholine (0.28 ml, 2.44 mmol) in 10 mL of chloroform was added dropwise. The cooling bath was removed and the reaction was stirred at room temperature for an additional 18 h. The chloroform solution was washed with water, saturated with sodium chloride solution, and then dried over anhydrous sodium sulfate. After drying, the solution was filtered and the chloroform removed. The residue was triturated with anhydrous ether and the resulting precipitate was then recrystallized from ethyl acetate/*n*-hexane (10 : 1 v/v), mp 208–209°C, yield 73.3%.

Anal. Theoretical ($C_{30}H_{35}O_7N_4Cl_5$): C, 48.63; H, 4.76; N, 7.56.
Found: C, 48.66; H, 4.77; N, 7.46 $[\alpha]^{25}_{573} = -121.8°$ ($c = 0.5$ in $CHCl_3$).
$\delta(CDCl_3)$: 1.40–1.50 (9H, d, Boc), 1.72–2.26 (12H, m, βCH_2Pro, γCH_2Pro), 2.52–2.62; 2.75–2.90 (2H, m, βCH_2Aze), 3.35–3.80 (6H, m, δCH_2Pro), 4.26–4.36 (1H, m, γCH_2Aze), 4.38–4.55 (3H, m, γCH_2Aze, αCHPro), 4.65–4.75 (1H, m, αCHPro), 5.08–5.14 (1H, dd, αCHAze).
ν_{max} (KBr): 1784, 1694, 1690, 1653, 1647 cm^{-1}; λ_{max}: 225, ϵ_{max}: 1.4×10^4 ($c = 0.05$ mg/mL in trifluoroethanol).
$(M + 1)^+$ Calc'd for ($C_{30}H_{35}O_7N_4^{35}Cl_5H$): m/e 739.1027.
Found: 739.1019; m/e (relative intensity): 739 (1.5), 492 (18.6), 392 (8.4), 366 (7.1), 293 (5.5), 266 (5.3), 226 (8.5), 212 (26.4), 195 (100), 181 (35.3), 167 (22.0), 146 (12.9), 131 (12.2), 113 (15.5), 101 (20.1).
Found for (C_6HOCl_5): m/e 264 (64.1), 265 (7.4), 266 (100), 267 (10.6), 268 (66), 269 (7).

HCl-L-Pro-L-Aze-L-Pro-Opcp

A solution of Boc-L-Pro-L-Aze-L-Pro-Opcp[16] (3.86 g, 6 mmol) in 120 mL of anhydrous dioxane cooled to 15°C was treated with dry gaseous hydrogen chloride. After 0.5 h, a white crystalline precipitate was obtained that redissolved after an additional 10 min. The resulting acid solution was concentrated under reduced pressure to obtain a white crystalline product; mp 181–182°C, yield 82%.

Anal. Calcd. for ($C_{20}H_{21}O_4N_3Cl_6 \cdot 2H_2O$), C, 38.98; H, 4.09; N, 6.82.
Found: C, 38.51; H, 4.13; N, 6.38. $[\alpha]^{25}_{573} = -183.6°$ ($c = 0.5$ in $CHCl_3$).
$\delta(CDCl_3)$: 2.15–2.80 (10H, m, βCH_2Aze, βCH_2Pro, γCH_2Pro), 2.44–2.51 (3H, bs, δCH_2Pro), 2.81 (1H, bs, δCH_2Pro), 4.18–4.20 (1H, m, γCH_2Aze), 4.38 (1H, bs, γCH_2Aze), 4.55 (1H, bs, αCHPro), 4.93–4.98 (1H, dd, αCHAze), 5.33 (1H, bs, αCHPro). ν_{max} (KBr): 1766, 1641 cm^{-1}.
$(M + 1) - HCl+$. Calcd. for ($C_{20}H_{20}O_4N_3^{35}Cl_5$): m/e 541.
Found: m/e 541.

Boc-L-Aze-L-Pro-L-Aze-L-Pro-Opcp

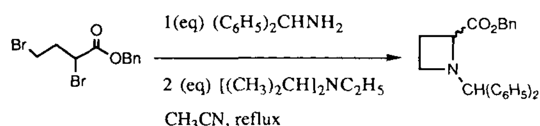
N-methylmorpholine (0.65 mL, 6 mmol) and isobutylchloroformate (0.78 ml, 6 mmol) were added to a stirred solution of Boc-L-Aze-OH[8] (1.21 g, 6 mmol) in 60 mL of chloroform at −15°C. After 0.5 h, 25 mL of a chloroform solution containing 3.48 g, 6 mmol, of freshly lyophilized L-Pro-L-Aze-L-Pro-OpcpHCl was added dropwise. After this addition was complete, a fresh 10 mL solution of chloroform containing 0.65 mL, 2.44 mmol, of N-methylmorpholine was added dropwise. The cooling bath was then removed and the reaction mixture was stirred

for an additional 18 h at room temperature. After washing with water and a saturated sodium chloride solution, the chloroform was dried over anhydrous sodium sulfate and then evaporated. The residual solid was recrystallized from hot ethyl acetate to give a product of mp 188.5–190°C; yield 85.4%.

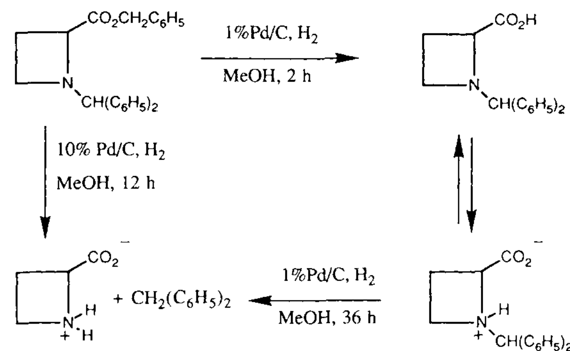
$[\alpha]^{25}_{573} = -160.2°$ (c = 0.5 in $CHCl_3$) $\delta(CDCl_3)$: 1.41 (9H, s, Boc), 1.92–2.50 (12H, m, βCH_2Pro, γCH_2Pro, βCH_2Aze), 3.50–3.55 (1H, m, δCH_2Pro), 3.56–3.57 (1H, dd, δCH_2Pro), 3.58–3.60 (1H, m, δCH_2Pro), 3.83–3.89 (2H, m, δCH_2Pro, γCH_2Aze), 4.00–4.02 (1H, dd, γCH_2Aze), 4.23–4.26 (1H, dd, γCH_2Aze), 4.41–4.43 (1H, dd, αCHPro), 4.45–4.54 (1H, dd, αCHAze), 4.79–4.84 (1H, dd, γCH_2Aze), 4.94–4.98 (1H, dd, αCHAze), 5.00–5.05 (1H, dd, αCHPro). ν_{max} (KBr): 1784, 1708, 1659 cm^{-1}. λ_{max}: 214; ϵ_{max}: 2.5×10^4 (c = 0.02 mg/mL in trifluoroethanol).
$(M + 1)^+$. Calcd. for ($C_{30}H_{35}O_7N_4Cl_5$): m/e 727.0841. Found: 727.0839: m/e (relative intensity): 727 (8.3), 627 (4.4), 547 (12.9), 461 (33.3), 447 (28), 366 (6.8), 361 (8.0), 333 (6.2), 298 (4.6), 266 (7.5), 198 (63.6), 181 (100), 153 (12.0), 101 (4.6).

RESULTS AND DISCUSSION

The chemical literature contains reports of a number of synthetic approaches to the synthesis of L-azetidine-2-carboxylic acid. Fowden used a β-lactone as his starting material.[17] The synthesis was complicated by the partial racemization that occurred when L-α,γ-diaminobutyric acid was diazotized and consequently yielded a significant amount of the D-γ-amino-α-chlorobutyric acid isomer. Ishikara and Yoneda[18] developed a synthesis based upon tosyl-L-homoserine lactone as an intermediate in a three-step synthesis of the desired product. Rodebaugh and Cromwell[11] reported a synthesis that utilized γ-butyrolactone and yielded both the D and L isomers. An advantage of their procedure is that the alkyl-substituted γ-butyrolactone is readily prepared and can be used to prepare alkyl-substituted azetidine-2-carboxylic acids. Unfortunately, the intermediate, N-benzhydryl-2-carbobenzyloxyazetidine, in this synthetic route is difficult to purify. In the present study we have developed an alternative procedure by replacing two of the three equivalents of aminodiphenylmethane with N,N-disopropylethylamine (Scheme 1). Our analysis of the reaction leads us to conclude that this tertiary base circumvents the formation of the benzyl-2,4-diaminobutyrate by-product via a competitive intermolecular interaction to give the desired intramolecular cyclization product exclusively. The N,N-diisopropylethylamine hydrobromide is readily removed since it is not soluble in anhydrous ether. The advantage of our modification is that it now permits the large-scale preparation of the L-azetidine-2-carboxylic acid derivative in pure form.



Scheme 1



Scheme 2

We have developed a reaction sequence in which the partial hydrogenation resulted in the selective cleavage of the benzyl group of N-benzhydryl-2-carbobenzyloxyazetidine leaving the benzhydryl-protecting group intact. The ir spectrum of benzhydrylazetidine-2-carboxylic acid yielded an ir band at 1616 cm^{-1}, which is assigned to the ionic carboxylate group. This provides support for the proposed mechanism in Scheme 2.

The application of mass spectroscopy to the characterization of high molecular weight materials has shown considerable success. The usual method for detecting oligomeric species, whether linear or cyclic, utilizes some chromatographic method such as high performance liquid chromatography or gel permeation chromatography. We have applied the mass spectroscopic method to try to detect the presence of cyclic oligomers present in our peptide samples. In previously described studies, the techniques of electron impact and fast atom bombardment mass spectrometry have been applied to oligomer identification in polyproline and poly(*cis*-3-chloroproline).[19,20] In the present investigation the use of DCI-MS with ammonia as the ion source was used to characterize the two model tetrapeptide systems, Boc-(L-Pro)$_3$-L-Aze-Opcp and Boc-(L-Aze-L-Pro)$_2$-Opcp. The use of ammonium as the ion source takes

the induction factor into account, which simulates the triethylamine used as a catalyst base in the real polycondensation process for polypeptide preparation. Both of these tetrapeptides can be volatilized under high vacuum without undergoing thermal degradation. As shown in the mass spectra (Figures 1 and 2), the breakdown of the two tetrapeptides results in the formation of peaks with m/e ratios of 181 and 195. These base peaks are interpreted as corresponding to the respective monoprotonated Aze-Pro diketopiperazine and Pro-Pro diketopiperazine compounds. Since the intramolecular cyclization results in the formation of the 6-membered diketopiperazine ring, the result is indicative of thermodynamic control in the primary fragmentation process. The peak at m/e 547 in the mass spectrum of Boc-(L-Aze-L-Pro)$_2$-Opcp is assigned to the Boc-(L-Aze-L-Pro)-Opcp (Scheme 3). This spectral finding suggests a back-bite intramolecular rearrangement for the thermal degradation of Boc-(L-Aze-L-Pro)$_2$-Opcp. In contrast to this finding, the absence of the peaks at m/e 547 and 561 indicates that a similar intramolecular rearrangement reaction does not occur for the thermal degradation of Boc-(L-Pro)$_3$-L-Aze-Opcp. It therefore appears that the prolyl group adjacent to the active ester at the C-terminal end of Boc-(L-Aze-L-Pro)$_2$-Opcp has a tendency to undergo intramolecular rearrangement. It seems that in the gaseous state the cyclic oligomers containing azetidine exhibited a tendency to undergo ring chain equilibration reactions. The absence and/or the low relative abundance of cyclic trimers supports this conclusion. The cyclic trimers, which form a 12-membered-ring, favor the open chain structure due to their large angle strain.

The assignment of conformation in the two tetrapeptides was carried out using nmr spectroscopy augmented by CD spectroscopy. Solutions of Boc(L-Aze-L-Pro)$_2$-Opcp in trifluoroethanol gave a CD spectrum (Figure 3) that was similar to but not identical with the mirror image spectrum of the poly(L-proline) form I. The spectrum shows a strong negative band centered at 216 nm, a strong positive band centered at 200 nm, and a weaker positive band at 240 nm. The ^{1}H-nmr of Boc-(L-Aze-L-Pro)$_2$-Opcp in deuterated trifluoroethanol shows two well-resolved resonances at 1.52 and 1.35 ppm, which are assigned to the Boc protons as shown in Figure 4. The major resonance at 1.52 ppm is assigned to the *cis* conformation, whereas the minor peak at 1.35 ppm corresponds to the *trans* conformation. This assessment is based on the resonance assignments of the Boc protons in the ^{1}H-nmr spectra of Boc-(Pro)$_8$-Obn and Boc-(Pro)$_8$-OH.[13] In deuterated trifluoroethanol these peptides show a minor *cis* Boc absorbance peak at 1.30 ppm and a major *trans* Boc absorbance peak at 1.10 ppm.[13] Unlike the small chemical shift change for the *cis* and *trans* Boc group in deuterochloroform, the chemical shift difference (0.2 ppm) between *cis* and *trans* Boc can be attributed to the stronger solvation power of trifluoroethanol on the Boc group. Based on these spectral findings, we conclude that it is possible to use deuterated trifluoroethanol as a solvent to diagnose the *cis-trans* conformation of the Boc group in these peptides. The most downfield resonance at 5.25 ppm (one proton) is assigned to the α proton of the Pro-4 adjacent to the strong electron-withdrawing active

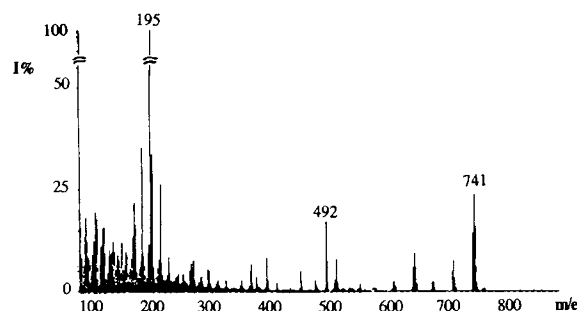


Figure 2. Mass spectrum (DCI) of the products of thermal degradation of Boc-(L-Pro)$_3$-L-Aze-Opcp.

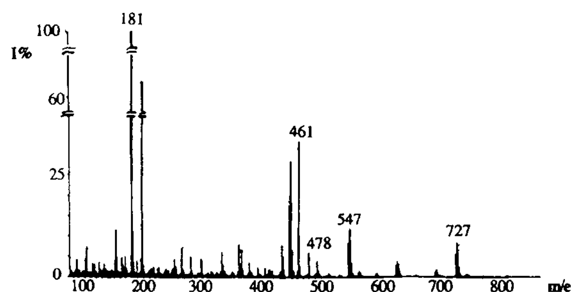
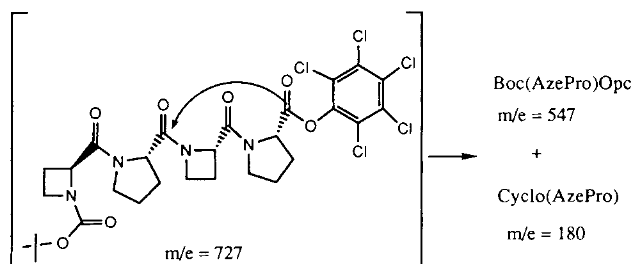


Figure 1. Mass spectrum (DCI) of the products of thermal degradation of Boc-(L-Aze-L-Pro)$_2$-Opcp.



Scheme 3. Fragmentation of Boc-(L-Aze-L-Pro)$_2$-Opcp via intramolecular back-bite rearrangement in the DCI-MS method.

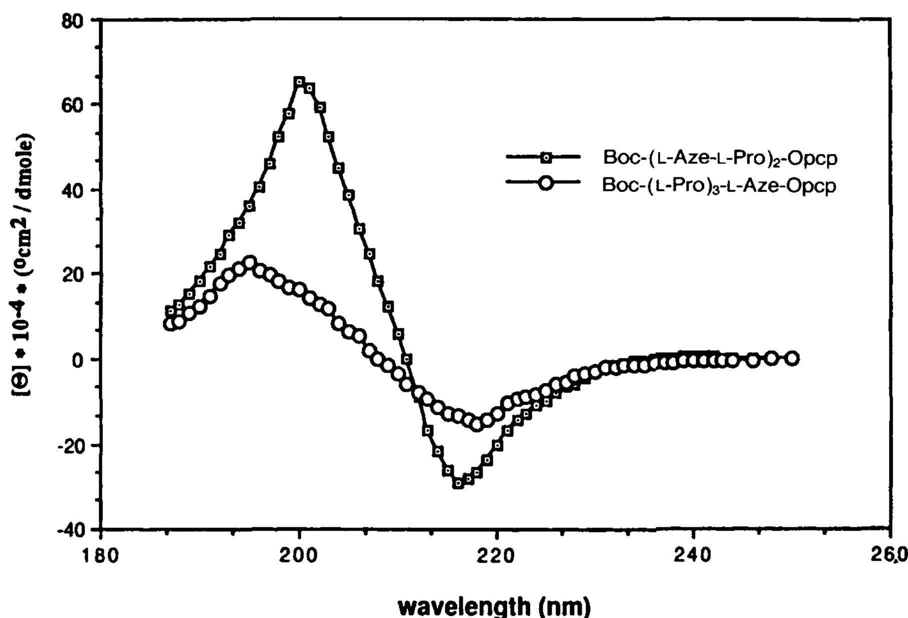


Figure 3. CD spectra of Boc-(L-Pro)$_3$-L-Aze-Opcp, concentration, 0.05 mg in 1 mL of trifluoroethanol. Boc-(L-Aze-L-Pro)$_2$-Opcp concentration, 0.02 mg in 1 mL of trifluoroethanol.

ester group. The resonance at 5.05 ppm (two protons) is attributed to the α protons of Aze-1 and Aze-3 (there is more "s"-orbital character on a 4-membered ring). Based on the molecular model of this peptide, the resonance at 5.05 ppm is assigned to the α proton of Aze-1 adjacent to the Boc group.

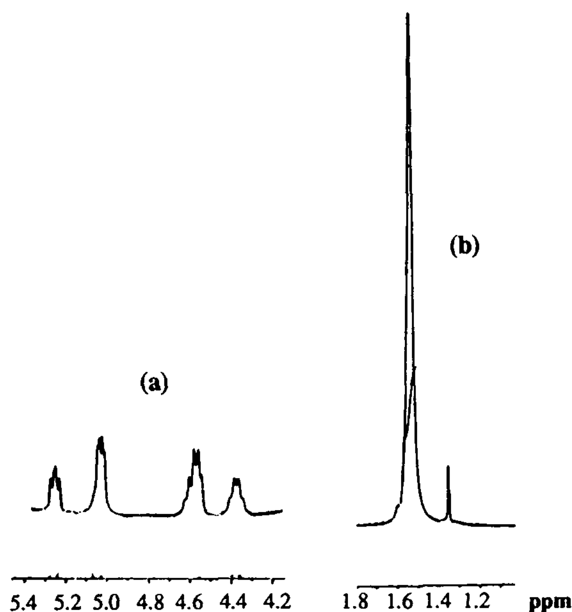


Figure 4. The 360-MHz ^{1}H-nmr spectrum of Boc-(L-Aze-L-Pro)$_2$-Opcp in deuterated trifluoroethanol solvent. (a) The α protons of proline and azetidine and γ protons of azetidine. (b) The Boc protons.

This α proton of Aze-1 is located above the shielding cone of the carbamate carbonyl group. This assessment can be correlated with the polar solvent (trifluoroethanol) since it can provide the H bonding between the carbamate and Aze-1 carbonyl groups. The *cis* conformation allows for the maximum hydrophobic (nonbonding) interaction between the azetidinyl ring and the *t*-butyl group. Therefore, the α-proton resonance at 4.55 ppm is assigned to the α proton of Pro-2. This band overlaps with the γ protons of the azetidine ring, (Figure 4a). The α-proton resonances of Boc-(Pro)$_8$-Obn and Boc-(Pro)$_8$-OH in deuterated trifluoroethanol[13] show the major *trans* conformation peaks at 4.7 ppm. On addition of the less polar isopropanol solvent to the above solution, mutarotation of the octameric peptide residues occurs and show the *cis* conformation, as indicated by the peak at 4.55 ppm in the ^{1}H-nmr spectra. Similarly, the α-proton resonance of Pro-2 in Boc-(L-Aze-Pro)$_2$-Opcp in deuterated trifluoroethanol occurs at 4.55 ppm and we interpret this as indicating that this peptide bond exists in the *cis* conformation. The ^{13}C-nmr spectrum of this peptide in deuterated trifluoroethanol solution (Figure 5), provides additional support for the assignment of one conformation at the N-terminal end, as indicated by a singlet peak at 159 ppm (Figure 5a). The four well-resolved resonances in the range of 26–31 ppm are assigned to the β- and γ-pyrrolidine resonances of this peptide. The singlet at 29.04 ppm is assigned to the Boc carbon (Figure 5b). It should

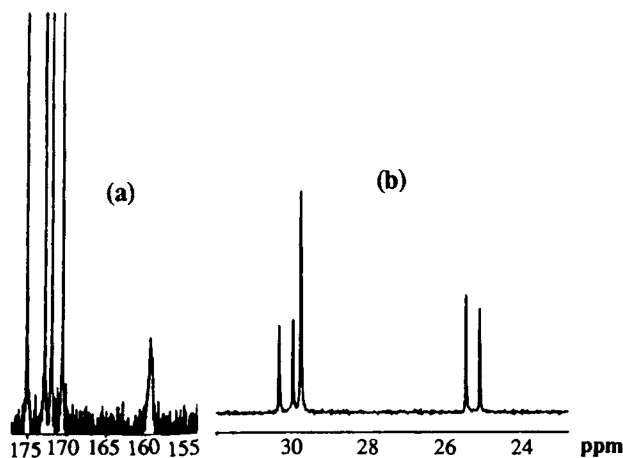


Figure 5. The 75-MHz ^{13}C-nmr spectrum of Boc-(L-Aze-L-Pro)$_2$-Opcp in deuterated trifluoroethanol solvent. (a) Carbonyl carbons, and (b) β and γ carbons of proline.

be stressed that these four singlet peaks cannot result from a mixture of *cis* and *trans* conformations. Goodman[21] has reported that the energy difference between the *cis* and *trans* forms of poly(S-thiazolidine-4-carboxylic acid) as well as poly(S-oxazolidine-4-carboxylic acid) may be changed by varying the ω angle. However, these differences can hardly be related to the ready mutarotation of these peptide bonds. Torchia[22] studied the conversion of polyproline in D_2O from one form to another, which is mainly dependent on the activation energy (approximate 20 kcal for *cis-trans* barrier). Therefore, the mutarotation of peptide bonds of Boc-(L-Aze-Pro)$_2$-Opcp at room temperature should be slow enough within the nmr technique responding time scale. Consequently, the four well-resolved peaks of the β-Pro and γ-pyrrolidine resonances of this peptide strongly suggest that Boc-(L-Aze-Pro)$_2$-Opcp in trifluoroethanol has only one conformation. The spectral result obtained from the CD study for this peptide in trifluoroethanol, which exhibits a Cotton effect opposite in sign to that exhibited by all *cis* polyproline form I, could be due to the influence of the N-terminal and C-terminal groups on this small peptide. The nmr data strongly support an assignment of all *cis* peptide bonds for Boc-(L-Aze-L-Pro)$_2$-Opcp in trifluoroethanol.

The ^{13}C-nmr spectrum of Boc-(L-Aze-L-Pro)$_2$-Opcp in deuterated chloroform solution shows five carbonyl group resonances in the range of 156–173 ppm (Figure 6a). The singlet carbamate carbonyl

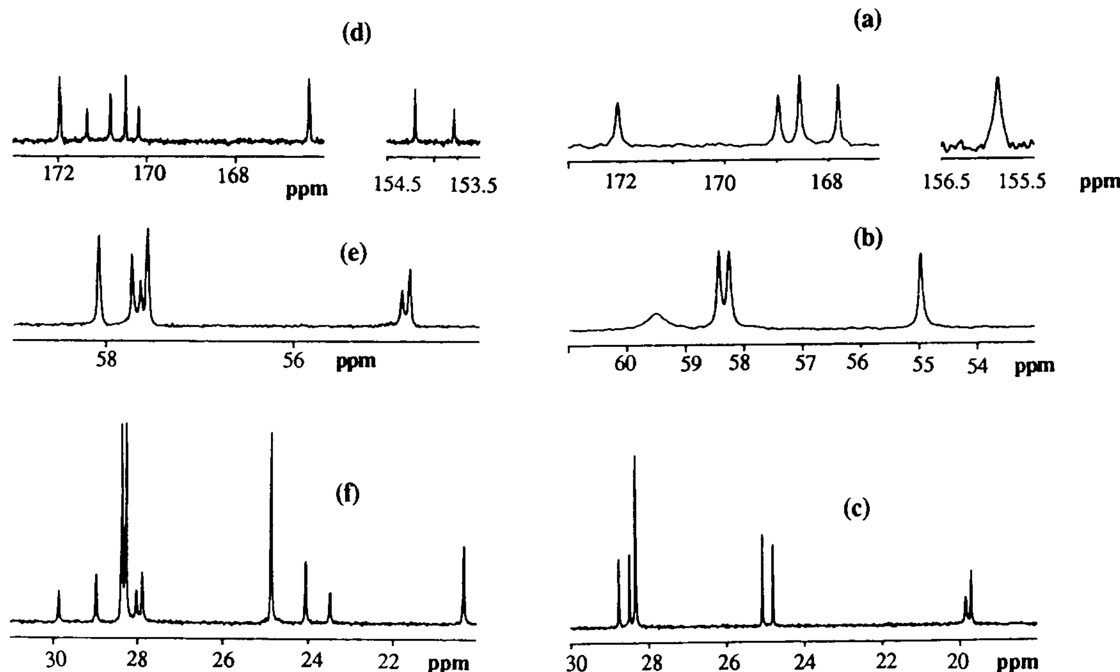


Figure 6. The 75-MHz ^{13}C-nmr spectrum of Boc-(L-Aze-L-Pro)$_2$Opcp and Boc-(L-Pro)$_3$-L-Aze-Opcp in deuterated chloroform. (a) Spectra of carbonyl carbons of Boc-(L-Aze-L-Pro)$_2$-Opcp in deuterated chloroform; (b) α-carbon atoms of Boc-(L-Aze-L-Pro)$_2$-Opcp in deuterated chloroform; (c) β carbons of Aze, and β and γ carbons of proline, in Boc-(L-Aze-L-Pro)$_2$-Opcp plus the methyl carbon of the Boc group in deuterated chloroform. (d–f) Similar spectra obtained on the peptide Boc-(L-Pro)$_3$-L-Aze-Opcp in deuterated chloroform.

group peak at 156 ppm indicates that the Aze-1 can restrict the Boc group at the N-terminal end (Figure 6a). This assessment is further supported by the singlet peak of the $OC(CH_3)_3$ group at 28.2 ppm, and the singlet peaks of the β-carbon resonances of Aze-1 at 19.6 and 19.8 ppm, respectively (Figure 6c). A molecular model of this peptide reveals that the conformation of the *cis* Boc group is more restricted and compacted than that of the *trans* Boc group. The ^{1}H-nmr spectrum of Boc-(L-Aze-L-Pro)$_2$-Opcp in deuterated chloroform shows a singlet Boc resonance at 1.46 ppm and provides additional support for the restriction of conformation by Aze-1 at the N-terminal end. The four well-resolved neat doublet–doublet α-proton resonances in the 4.5–5.1 ppm region (Figure 7) provides additional support for the assignment that Boc-(L-Aze-L-Pro)$_2$-Opcp exists as only one conformation in chloroform. The two resonances most downfield are attributed to either the α protons of Aze-3 with more s-orbital character or to the Pro-4 adjacent to the active ester group. Based on the molecular model of this peptide, the resonance at 4.85 ppm is assigned to the α proton of *cis* Aze-1 adjacent to the Boc group, because this α proton of Aze-1 is located above the shielding cone of the carbamate carbonyl group. Therefore, the resonance at 4.55 ppm is assigned to the α proton of Pro-2, indicative of a *cis* peptide bond.[14] Based on these arguments, we conclude that Boc-(L-Aze-L-Pro)$_2$-Opcp favors an all-*cis* peptide bond geometry either in chloroform or trifluoroethanol solutions.

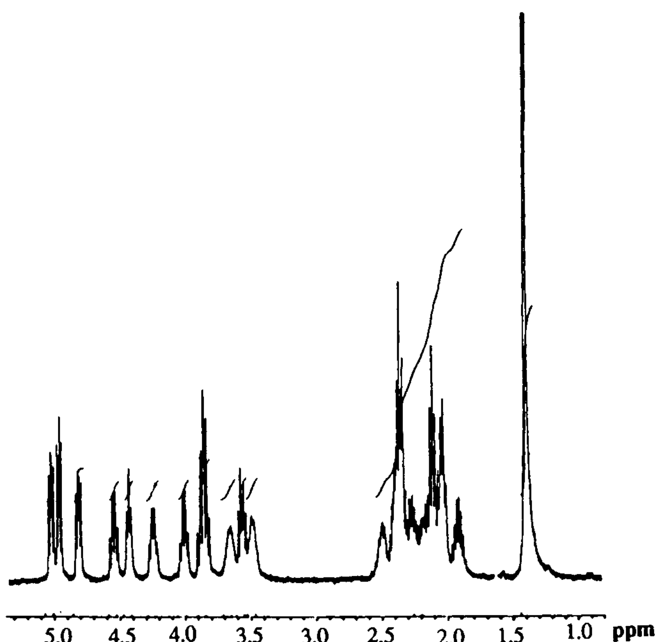


Figure 7. The 360-MHz ^{1}H-nmr spectrum of Boc-(L-Aze-L-Pro)$_2$-Opcp in deuterochloroform.

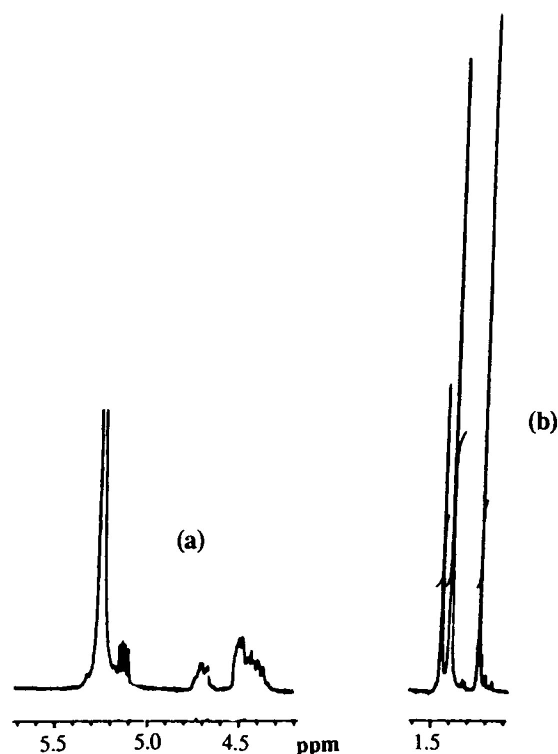


Figure 8. The 360-MHz ^{1}H-nmr spectrum of Boc-(L-Pro)$_3$-L-Aze-Opcp in deuterotrifluoroethanol. (a) The α protons of azetidine and proline, and γ protons of azetidine. The peak at 5.25 ppm is from the hydroxyl group of the solvent. The methylene protons at 3.86 ppm of trifluoroethanol were taken as the internal reference. (b) The Boc protons.

In contrast to the spectral characteristics for Boc-(L-Aze-L-Pro)$_2$-Opcp, the CD spectrum for the peptide Boc-(L-Pro)$_3$-L-Aze-Opcp in trifluoroethanol solvent shows a positive band at 195 nm and a negative band at 218 nm. No positive band was detected in the 230–240-nm region. The intensity of each Cotton effect for this peptide is about one third that observed for the previous peptide. Also, in the present instance the Cotton effect maximum is blue shifted by 5 nm and the negative Cotton effect appears to have its minimum red shifted by 2 nm. The ^{1}H-nmr spectrum of Boc-(L-Pro)$_3$-L-Aze-Opcp, in deuterotrifluoroethanol, shows a doublet-doublet peak at 5.15 ppm, which is assigned to the α proton of Aze-4 adjacent to the active ester. The resonance at 4.7 ppm (one proton) is assigned to the *trans* α proton of pyrrolidine, whereas the complex absorp-

tion pattern at 4.45 ppm (four protons) is attributed to the *cis* α protons of pyrrolidine and the γ protons of the azetidinyl ring (Figure 8a). These results indicate that Boc-(L-Pro)$_3$-L-Aze-Opcp has a mixture of *cis* and *trans* peptide bond conformations in trifluoroethanol. The ratio of *cis* to *trans* peptide bond conformation, estimated by the ratio of the areas between 4.45 and 4.7 ppm, is approximate 2 : 1. This assessment is given additional support by the three Boc resonances. The two resonances at 1.40 and 1.48 ppm are assigned to the *cis* conformation, whereas the peak at 1.31 ppm is attributed to the *trans* conformation (Figure 8b). The ^{13}C spectrum of this peptide in deuterochloroform shows a complex pattern in the range of 18.0–30.0 ppm (Figure 6f). The split two peaks with strong intensities at 28.3 and 28.4 ppm are assigned to the *cis* Boc resonance, whereas the singlet resonance at 24.9 is assigned to the *trans* Boc group (Figure 6f). This assessment is supported by the carbamate resonances at 154.4 and 153.6 ppm, which indicate the *cis–trans* isomers of the Boc group (Figure 6d). Detailed assignments of all three pyrrolidine ring protons in this peptide as the correct resonances would require the presence of an isotope label on each different position of the pyrrolidine ring. However, the ^{13}C-nmr spectrum revealed that all of the carbonyl carbon atoms adjacent to the proline rings were in a mixture of *cis* and *trans* peptide bonds (Figure 6d). The α-pyrrolidine resonances in this peptide (i.e., 54.7, 54.8, 57.6, and 57.7 ppm) show a back-to-back pattern,[23] which further supports the assignment that peptide bonds within this peptide exist as a mixture of *cis* and *trans* conformations (Figure 6e). By using the positions of the carbamate carbonyl carbon absorptions in the ^{13}C-nmr spectrum (Figure 6d) and the Boc carbon peak (i.e., 24.9 ppm, *trans,* 28.3 and 28.4 ppm, *cis*) in the ^{13}C nmr spectrum, it was possible to estimate the ratio of *cis* to *trans* conformations as roughly 2 : 1 (Figure 6f). The Boc proton peak in the ^{1}H-nmr spectrum (Figure 8b) is in accord with this assessment. Again, the most downfield doublet-doublet absorption pattern at 5.05 ppm (Figure 8a) indicates that the Aze-4 has a tendency to restrict the conformation of this peptide at the C-terminal end. The complex pattern at 4.65 ppm is assigned to the *trans* α protons of the Pro residues, whereas the resonances in the range of 4.2–4.5 ppm are attributed to the *cis* α proton of the Pro residues and the γ proton of Aze-4, respectively. By comparing the areas at *cis* and *trans* resonances, the ratio of *cis* to *trans* is approximate 2 : 1. These spectral observations are interpreted as indicating that the peptide Boc-(L-Pro)$_3$-L-Aze-Opcp exists in a conformation that has greater flexibility and permits a greater degree of disorder relative to Boc-(L-Aze-L-Pro)$_2$-Opcp.

The uv spectra of poly(L-proline) I and II in aqueous solution show absorption maxima at 208 and 203 nm, respectively,[24] while in trifluoroethanol they display absorbance maxima at 210 and 202 nm.[20] The absorption spectrum of Boc-(L-Aze-L-Pro)$_2$-Opcp in trifluoroethanol shows a maximum at 214 nm (Figure 9). This red shift of 4 nm from the maximum of poly(L-proline) I can be the consequence of a solvent effect, or a small structural difference in the ϕ and ψ angles in the helix. It should be stressed that the introduction of a pentachlorophenyl group into our model tetrapeptides helps purify the products via recrystallization, whereas the introduction of para-nitrophenyl and benzyl groups into the model tetrapeptides did not facilitate the purification. The pentachlorophenyl group is helpful for the preparation of poly(L-Aze-L-Pro) and poly[(L-Pro)$_3$-L-Aze], which will be reported shortly. Although the introduction of the pentachlorophenyl group may contribute to the molar absorptivity at the maximum absorbance for these peptides in the uv studies, the molar absorptivities of the amide bond chromophore in our model tetrapeptides {$\epsilon = 2.5 \times 10^4$ for Boc-(L-Aze-L-Pro)$_2$-Opcp and 1.4×10^4 for Boc-(L-Pro)$_3$-L-Aze-Opcp are two times larger than that of the pentachlorophenyl group ($\epsilon = 7.0 \times 10^3$). Therefore, the maximum absorbances for our model tetramers in the far-uv spectra should reflect the true maximum position even if the pentachlorophenyl group contributes interference.

Neither the CD spectrum nor the ^{1}H-nmr spectrum of the oligopeptides Boc-(Pro)$_8$-Obn, Boc-(Pro)$_8$-OH and H-(Pro)$_8$-Obn showed any time-dependent spectral changes indicative of conformational transitions.[13] Based on the above results, the stability of the peptide bond geometry in these proline oligomers can be assumed to be independent of the end group. Analysis of the ^{13}C-nmr spectra of the peptides Boc(L-Aze-L-Pro)$_2$-Opcp and Boc-(L-Pro)$_3$-L-Aze-Opcp in chloroform solvent permitted an assessment of the influence of the azetidine ring on the helical character of the peptide. Comparing the carbonyl carbon positions in the ^{13}C nmr spectra of both peptides shows the presence of a single absorption by the carbamate group adjacent to azetidine in Boc-(L-Aze-L-Pro)$_2$-Opcp and two peaks attributed to proline in Boc-(L-Pro)$_3$-L-Aze-Opcp. Also, the amide group adjacent to the third peptide

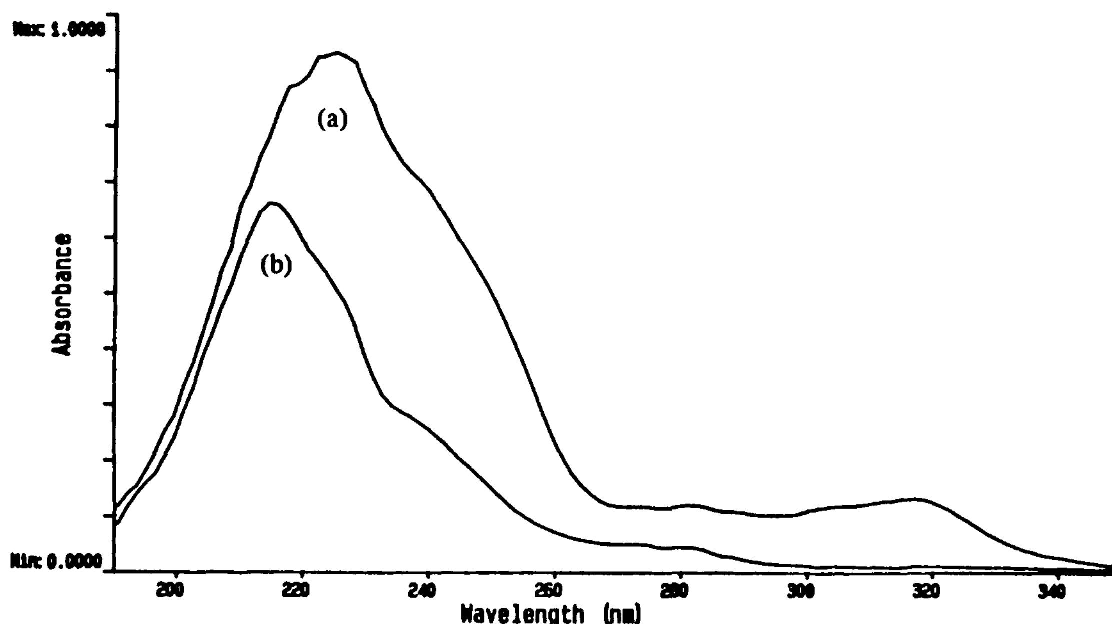


Figure 9. For the uv spectra of Boc-(L-Pro)$_3$-L-Aze-Opcp, concentration was 0.05 mg in 1 mL of trifluoroethanol. For Boc-(L-Aze-L-Pro)$_2$Opcp, the concentration was 0.02 mg in 1 mL of trifluoroethanol.

of Aze in Boc-(L-Aze-L-Pro)$_2$-Opcp shows a single peak, whereas two peaks attributed to proline are present in the Boc-(L-Pro)$_3$-L-Aze-Opcp spectrum. Only a single carbonyl carbon absorption of the active ester carbon adjacent to either Pro in Boc-(L-Aze-L-Pro)$_2$-Opcp or azetidine in Boc-(L-Pro)$_3$-L-Aze-Opcp is found. These results suggest that azetidine is capable of restricting the flexibility of the N-terminal carbamate, whereas proline is less restricting. Azetidine located at distances from both the N- and C-terminal ends in Boc-(L-Aze-L-Pro)$_2$-Opcp was capable of acting as a bridge to further lock this peptide into one conformation, but proline was not effective in causing such restrictions. This is reasonably correlated with the greater flexibility of the 5-membered proline ring and the more extensive pyramidyl character of the nitrogen atom in this ring.[25] This is in contrast to the greater rigidity of the 4-membered azetidine ring and lesser pyramidyl character associated with the nitrogen of this ring.[8] It is therefore highly probable that the conformation of the tetramer, in chloroform or trifluoroethanol solution, is influenced more effectively by the rigid conformation of the azetidine, and that both the N-terminal carbamate and C-terminal active ester did not influence the tetrapeptide conformation in any significant manner.

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