nitrite in dilute acetic acid; at pH 7.0, α_{NO} 10.00; α_N 4.85; $\alpha_{\beta CH}$ 2.35; $\alpha_{\beta CH'}$ 1.45 G. The catalytic hydrogenation of compounds **11** and **13** over 10% palladium on carbon in ethyl acetate gave colorless solutions. After removal of the catalyst by filtration the ethyl acetate solution was stirred over an excess of manganese dioxide and extracted with water to give a solution of a compound which gave the same esr and electrophoresis data as mentioned above. This compound decomposed before elemental analyses could be obtained and was not sufficiently volatile to give a mass spectrum.

N-Acetyl-β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-DL-alanine (10). A solution of 0.5 g of the ethyl ester **9** in 50 ml of methylene chloride was stirred with 50 ml of 0.5 *N* sodium hydroxide for 30 min at 25°. The aqueous layer was separated and washed with methylene chloride. The pH was lowered to 2.5 with hydrochloric acid and the acid **10** was extracted with methylene chloride. Chromatography on silica gel with 5% formic acid in ethyl acetate gave a pure sample. *Anal.* Calcd for $C_{12}H_{22}N_3O_5 \cdot CH_2O_2$: N, 12.66. Found: N, 12.38.

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Angiotensin II Analogs. VIII.[1] The Use of Free Radical Containing Peptides to Indicate the Conformation of the Carboxyl Terminal Region of Angiotensin II

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Abstract: Nitronyl nitroxide amino acid analogs of histidine were used to prepare free radical containing peptides in the angiotensin series. The esr spectra of the nitronylnitroxylalanylprolylphenylalanine derivatives change with pH in a way which indicates that an ion–dipole bond exists between the phenylalanine carboxylate anion and the nitronyl nitroxide ring system. This interaction is facilitated by the presence of the proline ring in the tripeptide and possibly by an intramolecular hydrogen bond between the phenylalanine NH and the amide carbonyl of the free radical amino acid. The nitronyl nitroxide amino acid and peptide derivatives capable of forming this ion–dipole bond also show temperature-dependent changes in esr line widths. The activation energy of conformer interconversion ranges from 2.2 to 5.0 kcal/mole.

The elucidation of intramolecular interactions and tertiary structure of peptides in solution has been the subject of a number of different studies in recent years. Nuclear magnetic resonance and electron spin resonance spectroscopy have proven to be the most useful techniques in this area. The most significant recent results have been from the use of 220-MHz nmr.[2] The utility of this tool has been extended by the syntheses of deuterated amino acids and peptides where one or two amino acids are in the protonated form.[3,4] Similar advances are indicated by recent reports of ^{13}C enriched amino acids.[5] Schwyzer has reported some success using peptides containing reporter groups which produce observable chemical shift changes of interacting functional groups.[6] Electron spin resonance techniques have recently been applied to larger proteins and enzymes by the use of stable free radical spin labels.[7]

Each of these techniques is designed to simplify the spectral data derived from the complex peptide molecule. Electron spin resonance spectroscopy is ideal in this respect as the radical-containing function may be observed without interfering signals. In the preceding paper[8] we have described a free radical analog of histidine and have shown how its esr spectral characteristics can be correlated with properties of the natural amino acid. Incorporation of this amino acid into a peptide enables one to observe the environment of a single functional group resulting from the tertiary structure of the molecule.

In conjunction with our continuing interest in angiotensin II analogs[9] we have replaced the histidine residue of the His-Pro-Phe fragment of angiotensin with the nitronyl nitroxide amino acid. Relatively little is known about the tertiary structure of this octapeptide although a great deal of work has been reported concerning the relation of substituent modifications to biological activity.[10] Results obtained from optical rotatory dispersion curves of [β-Asp[1]-Val[5]]angiotensin II[11] and [Ile[5]]angiotensin II[12] indicate that the octapeptide has a preferred time-averaged structure. Large

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changes were observed in the β-aspartic acid derivative with pH. Deep minima were observed at 217 and 235 nm at pH 10.95 relative to the curves taken at pH 5.67. In other work[13] [Asn[1],Val[5]]angiotensin II was interpreted to have a random coil configuration.

Thin-film dialysis studies have resulted in conflicting reports. Craig, *et al.*,[14] have reported rate changes with pH which indicate conformational variations of [β-Asp[1],Val[5]]angiotensin II. In acidic solution (pH <6) a loose association due to electrostatic interactions between the positive and negative charges along the peptide (β-Asp-COO^-, β-Asp-NH_3^+, His-Im^+, Phe-COO^-) results in a compact structure. In basic solution another more extended conformation which may have a substantial degree of stability was indicated. Ferreira and coworkers,[15] in contrast, report pH-independent dialysis and gel filtration data between pH 2 and 11 for [Asn[1],Ile[5]]angiotensin II. They indicate that two slowly interconverting conformers of different shapes are present in relative concentrations dependent on the ionic strength of the solution. Fernandez, *et al.*,[16] report rates of dialysis for [Asn[1],Ile[5]]angiotensin II which are pH independent between 5 and 9 but which increase on ionization of the phenolic hydroxyl group at pH 10.

These results may be interpreted as an indication that there is conformational stability of angiotensin II in solution but they do not offer an indication of the mechanism of conformational stability. There have been working models proposed to account for structure–activity relationships such as the close juxtaposition of the aromatic residues[17] or a partial α helix.[12] The use of a free radical analog of angiotensin can provide information on a variety of conformational models as it is sensitive to steric hindrance, solvation changes, and electrostatic interactions.

Peptide Synthesis. The peptides used in this study were prepared from the *N*-carbobenzyloxy (**1**) and *N*-acetyl (**2**) free radical amino acids using the solid phase method of synthesis.[18] Coupling of the amino acids was carried out with dicyclohexylcarbodiimide in methylene chloride. No alteration of the standard coupling procedure was necessary to accommodate the free radical system. Some deoxygenation of the nitronyl nitroxide ring to the imino nitroxide was observed on prolonged treatment (>20 hr) with excess dicyclohexylcarbodiimide.[8]

Deprotection of the *tert*-butyloxycarbonyl free radical peptide with hydrochloric acid in glacial acetic acid resulted in decomposition of the free radical. Under strongly acidic conditions the nitronyl nitroxide ring is protonated and then disproportionates to give two diamagnetic compounds.[8,9] One of these has been identified as a strong oxidizing agent.[19] Decomposition of the disproportionated radical may occur during the process of cleaving the *tert*-butyloxycarbonyl group, by reaction with the resin, or during the washing procedure before neutralization. The protonated radical, however, does not disproportionate in anhydrous trifluoroacetic acid.[19] Attempts to avoid radical decomposition by using anhydrous trifluoroacetic acid to cleave the protecting group followed by washing with methylene chloride and neutralization with 10% triethylamine in dimethylformamide also failed. The susceptibility of the radical to decomposition by deprotection techniques apparently limits its utility in solid phase syntheses which requires repeated coupling–deprotection steps to increase chain length.

Cleavage of the radical-containing peptide from the resin may be carried out with the trifluoroacetic–hydrobromic acid reagent usually used.[18] When the radical amino acid is protected with a carbobenzyloxy group the cleavage procedure gives the deprotected peptide radical after neutralization.

Results

The esr spectra of the nitronylnitroxylalanine derivative, as shown in Table I, vary with pH in a manner which could be correlated to the ionic species in solution.[8] In acidic media a small coupling constant difference of 0.1 to 0.3 G results from the magnetic nonequivalence of the methylene hydrogens. The *N*-acetyl ethyl ester **7** and the free amino acid **8** show this small difference throughout the pH range. The *N*-carbobenzyloxy (**1**) and *N*-acetyl (**2**) derivatives show larger differences of 0.8 to 1.0 G at pH values where the carboxyl group is ionized. The *N*-acetyl di- and tripeptides **4** and **5** do not show pH-dependent esr spectra. The coupling constant range from 0.2 to 0.5 G is slightly larger than that observed for the amino acid derivatives **7** and **8**. This may be due to the nonequivalence of the methylene hydrogens or to a small sterically induced shift in rotamer equilibria. These differences are, however, significantly less than those of the *N*-carbobenzyloxy (**1**) and *N*-acetyl (**2**) amino acids. The angiotensin II analog **3** which has the proline unit between the *N*-acetyl radical and the phenylalanine amino acids shows a coupling constant difference of 0.5 G in acid and a difference of 0.9 at pH levels above four. This change in coupling constants with pH parallels those amino acids which show ion–dipole bonding between the carboxylate anion and the radical ring. A plot of the methylene coupling constants against pH

Table I. Coupling Constants[a] of Nitronyl Nitroxide Amino Acids and Peptides at pH 7

Compd no.	Species in solution[b]	$\alpha_{\beta CH}$, G	$\alpha_{\beta CH'}$, G	Δ-$\alpha_{\beta CH,\beta CH'}$, G
1	CBzNH-NNAla-O^-	1.60	2.60	1.00
2	AcNH-NNAla-O^-	1.70	2.50	0.80
3	AcNH-NNAla-Pro-Phe-O^-	1.60	2.50	0.90
4	AcNH-NNAla-Phe-O^-	1.90	2.20	0.30
5	AcNH-NNAla-Phe-Phe-O^-	1.80	2.30	0.50
6	H_3N^+-NNAla-Pro-Phe-O^-	2.10	2.50	0.40
7	AcNH-NNAla-OCH_2CH_3	2.05	2.20	0.15
8	H_3N^+-NNAla-O^-	2.15	2.45	0.30

[a] ±0.05 G in water. [b] NNAla refers to the nitronylnitroxylalanine fragment $CH(CH_2C_7H_{12}N_2O_2)CO$.

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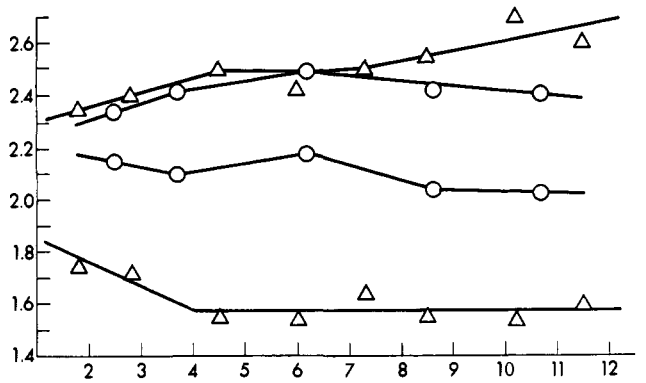
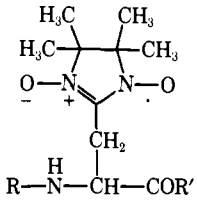


Figure 1. A plot of β-methylene coupling constants against pH for AcNH-NNAla-Pro-Phe-OH (**3**), Δ, and for H_3^+N-NNAla-Pro-Phe-OH (**6**), ○; (y axis) β-methylene coupling constants, $\alpha_{\beta CH}$ and $\alpha_{\beta CH'}$ (gauss), (x-axis) pH.

for AcNH-NNAla-Pro-Phe-OH (**3**) and H_2N-NNAla-Pro-Phe-OH (**6**) is shown in Figure 1.



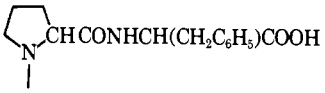
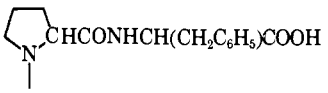
1, R = $C_6H_5CH_2OCO$; R′ = OH
2, R = CH_3CO; R′ = OH
3, R = CH_3CO; R′ =  $CHCONHCH(CH_2C_6H_5)COOH$
4, R = CH_3CO; R′ = $NHCH(CH_2C_6H_5)COOH$
5, R = CH_3CO; R′ = $NHCH(CH_2C_6H_5)CONHCH(CH_2C_6H_5)COOH$
6, R = H; R′ =  $CHCONHCH(CH_2C_6H_5)COOH$
7, R = CH_3CO; R′ = OC_2H_5
8, R = H; R′ = OH

Table II gives the esr line widths of the two outer and two inner lines that make up the sets of doublets ob-

Table II. Esr Line Widths[a] for Amino Acid and Peptide Radicals at pH 2.5 and 8.5

Compd no.	pH 2.5		pH 8.5	
	w_o	w_i	w_o	w_i
1	0.81	0.86	0.78	0.96
2	0.88	0.88	0.85	0.95
3	0.86	0.88	0.88	1.08
4	0.86	0.88	0.86	0.86
5	0.86	0.88	0.86	0.85
6	0.85	0.89	0.85	1.12
7	0.88	0.81	0.88	0.87
8	0.85	0.87	0.85	0.94

[a] In gauss. $w_o \pm 0.03$ G; $w_i \pm 0.06$ G at pH 2.5 and for compounds **4**, **5**, **7** and **8** at pH 8.5; $w_i \pm 0.03$ G for compounds **1**, **2**, **3**, and **6** at pH 8.5

served in each spectrum (Figure 2). The relation between the line width and intensity is given by

$$w_i = \left[\frac{I_o w_o^2}{I_i}\right]^{1/2} \quad (1)$$

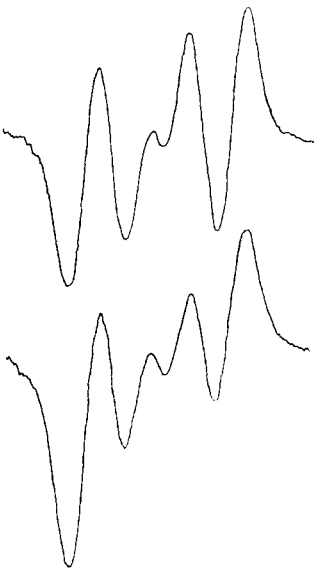


Figure 2. The esr spectra of the center pair of doublets from tripeptide radical **3** in water at pH 8.5 and at 1° (bottom) and 53° (top).

where w_i and w_o are the line widths of the inner and outer lines and I_i and I_o are the respective intensities.[20] The intensity of the inner lines was corrected for distortion caused by overlapping peaks, as previously described.[20] The spectra taken at pH 2.5 do not show line width variation and appear to result from time averaging of rapid rotamer interconversion. Several compounds show increases in the inner lines when measured at pH 8.5. This line broadening results from a slowed rate of isomer interconversion where the spectra of the contributing conformers have different coupling constants. However, since the outer lines are not broadened, the sum of these coupling constants is similar.[20] The compounds **1**, **2**, and **3** which show large coupling constant differences in Table II also show significant broadening of the inner lines in basic solution. The unacylated amino acid **8** and the tripeptide **6** also show line width increases indicating hindered rotation of the C_β–C_γ bond. In the case of compounds **6** and **8** the hindered rotation and necessary rotamer preference do not result in large coupling constant differences.

The widths of the inner lines decreased relative to the outer line width as the temperature was increased. A plot of log $(w_i - w_o) \times 10^2$ against the reciprocal of temperature for compounds **1**, **2**, and **3** is shown in Figure 3. The activation energies of interconversion may be calculated from the rate of line broadening with temperature if the line widths are directly proportional to the rotamer lifetimes. These calculations give values of 5.0 kcal/mole for the N-carbobenzyloxy derivative **1**, 2.2 kcal/mole for the N-acetyl free radical amino acid **2**, and 2.4 kcal/mole for the angiotensin analog AcNH-NNAla-Pro-Phe-OH (**3**).

Discussion

The previous paper[8] established the correlation of binding properties between histidine and the nitronyl nitroxide analogs. The compounds examined in this study are directed toward the possible extension of this

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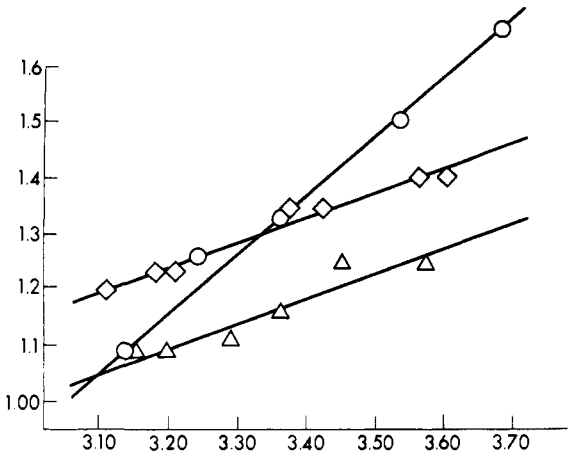

Figure 3. A plot of the line broadening against the reciprocal of temperature for CbzNH-NNAla-O$^-$ (**1**), ○; for AcNH-NNAla-O$^-$ (**2**), △; and for AcNH-NNAla-Pro-Phe-O$^-$, (**3**), ◇; (y axis log $(\omega_i - \omega_0) \times 10^2$ (gauss), (x axis) $1/T \times 10^3$ (°K^{-1}).

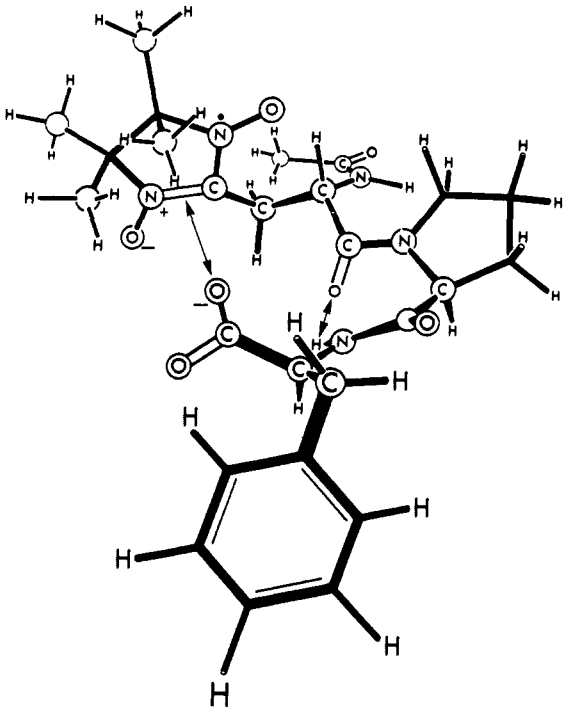

Figure 4. The structure of a preferred conformer of N-acetyl-β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanyl-L-prolyl-L-phenylalanine anion (**3**).

binding mechanism to biologically important systems. Angiotensin II contains the peptide fragment His-Pro-Phe-O$^-$ which may exist in an intramolecularly ion–dipole bound conformer. The spectral results obtained from the nitronyl nitroxide containing peptides (**3**–**6**) suggest that this type of interaction is possible in angiotensin II.

All esr spectra show a minimum coupling constant difference of 0.1 to 0.3 G due to coupling of the magnetically nonequivalent β-methylene hydrogens to the unpaired spin of the nitronyl nitroxide ring. Larger coupling constants may be observed when the equilibrium of rotamer conformations is shifted from the equal populations present in the freely rotating system. Such increases have been noted in the N-carbobenzyloxy (**1**) and N-acetyl (**2**) amino acid anions where the carboxylate anion binds to the polar nitronyl nitroxide ring to give a conformer where these functions are in close proximity.[8] A comparable increase in the coupling constant differences was observed in the angiotensin analog AcNH-NNAla-Pro-Phe-OH (**3**) peptide but not in the AcNH-NNAla-Phe-OH (**4**) and AcNH-NNAla-Phe-Phe-OH (**5**) peptides, suggesting that the terminal phenylalanine carboxyl anion in **3** may be held in an orientation where binding to the polar nitronyl nitroxide ring may occur. The formation of an intramolecular carboxylate–radical bond requires the 11-membered ring system as shown in Figure 4. There are seven bonds where rotation could cause disruption of this cyclic conformation (from the Phe C_α to the NNAla β-CH_2). Three of these bonds are fixed by the trans CONH units and the proline ring. Two other bonds have large steric requirements (NNAla CO–C_α and NNAla C_α–C_β) which have sterically favorable conformations in the cyclic structure shown. The cyclic system could be further stabilized by a hydrogen bond between the Phe NH and NNAla carbonyl group. The presence of the L-proline residue is crucial as indicated by the loss of the cyclic conformation in the tripeptide **5**.

The presence of the intramolecular bond between the radical and the carboxylate anion slows the rate of isomer interconversion and rotation about the C_β–C_γ bond. This results in the inner line width increase shown in Table II. The amino acids having a free carboxylate ion **1**, **2**, and **8** and the peptides having the NNAla-Pro-Phe-O$^-$ structure (**3** and **6**) show such increases. The amino acids and peptides which have protonated or esterified carboxyl functions or which lack the proline-containing tripeptide sequence do not form this cyclic interaction and do not show line width increases or large coupling constant differences.

It may be noted that the intramolecularly bound compounds (**6** and **8**) show relatively small coupling constant differences (Table I). This would occur if the preferred conformation held the β-CH and β-CH′ hydrogens at equal dihedral angles (30 or 60°) from the $p\pi$ orbital of the radical and if the amino substituent produces a steric influence on the radical ring when the molecule is held in the cyclic configuration.

The steric and electronic properties displayed in these compounds are relevant to the angiotensin system as the structural features involved in the AcNH-NNAla-Pro-Phe-OH (**3**) peptide are identical with those in the angiotensin octapeptide H_2N-Asn-Arg-Val-Tyr-Ile-His-Pro-Phe-OH. It indicates that the preference for the cyclic intramolecular ion–dipole bond in angiotensin would be at least as great as the conformational preference in N-acetylhistidine. The attachment of the five amino acid chain to the histidine amino group could act to further limit the possible conformations in this portion of the cyclic conformer by seeking out the least sterically hindered position. It would also add a significant bulk effect to dampen tendencies to modify this conformation.

Conclusion

An intramolecular ion–dipole bond has been postulated as an important feature in the conformational stability of certain nitronyl nitroxide peptides and amino

acids. These results were used to describe a conformation of angiotensin II which involves an intramolecular ion–dipole bond between the phenylalanine carboxylate anion and the imidazole ring. This cyclic conformation would be stabilized by the proline ring, trans amide bonds, sterically preferred conformations of the His CO–C_α and C_α–C_β bonds, and a hydrogen bond between the Phe NH and His CO groups. The proposed conformation is consistent with optical rotatory dispersion and thin-film dialysis data of Craig and coworkers.[11,14] Their data suggest that angiotensin II could have a somewhat extended stable conformation at basic pH and a randomly oriented compact structure at acidic pH, which structure is held together by electrostatic attraction of the positive and negative charges along the peptide chain. We have recently obtained additional supporting evidence for the proposed structure from 220-MHz nmr spectra of angiotensin analogs.[21]

Experimental Section

General. Melting points were measured with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Amino acid analyses were done on a Spinco 120 B analyzer. Microanalyses were performed by the Microanalytical Department, University of California, Berkeley, Calif. Rotations were measured with a Rudolph photoelectric polarimeter. Precoated plates of silica gel F-254, Brinkmann Instruments, were used for thin-layer chromatography; silica gel powder, J. T. Baker, was used for column chromatography. Electrophoreses were carried out on Whatman No. 1 paper at 5000 V using acetic acid–formic acid buffer, pH 1.85, in a Savant apparatus. E_H indicates the electrophoretic mobility relative to histidine = 1.00. The esr spectrometer has been previously described.[22] A Varian flat cell with a volume of 0.04 ml within the microwave cavity was used.

***N*-Carbobenzyloxy-β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanine (1).** The compound was prepared as the monoformate as previously described.[8] To remove the formic acid the carbobenzyloxy derivative was dissolved in methylene chloride, washed with pH 2 hydrochloric acid, lyophilized, and chromatographed on silica gel with ethyl acetate. The absence of formic acid was confirmed by the mass spectrum of the chromatographed sample. Esr coupling constants in water at pH 2.2, $\alpha_{\beta CH}$ 1.75 ± 0.05 G, $\alpha_{\beta CH'}$ 2.50 ± 0.05 G (lit.[8] $\alpha_{\beta CH}$ 1.80 ± 0.05 G, $\alpha_{\beta CH'}$ 2.40 ± 0.05 G).

***N*-Acetyl-β-(1,3-Dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-DL-alanine (2).** The compound was prepared by hydrolysis of the *N*-acetyl ethyl ester as previously described.[8] Chromatography of the hydrolysis product on silica gel with ethyl acetate gave a pure sample. Esr coupling constants in water at pH 8.5, $\alpha_{\beta CH}$ 1.80 ± 0.05, $\alpha_{\beta CH'}$ 2.45 ± 0.05 G (lit.[8] $\alpha_{\beta CH}$ 1.70 ± 0.05, $\alpha_{\beta CH'}$ 2.50 ± 0.05 G).

Solid Phase Peptide Synthesis. ***tert*-Boc-L-phenylalanyl Resin.** A suspension of 15 g of chloromethylated polystyrene-*co*-2%-divinylbenzene[18] (100 mequiv of chlorine per gram of resin) in 20 ml of absolute ethanol was mixed with 2.65 g (10 mmoles) of *tert*-Boc-L-phenylalanine[23] and 1.01 g (10 mmoles) of triethylamine. The suspension was stirred at 80° for 15 hr. After filtering, the esterified resin was washed with 100 ml of ethanol, then with 100 ml of methanol. Amino acid analyses showed that the resin contained 0.125 mmole of phenylalanine per gram.

***N*-Carbobenzyloxy-β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanyl-L-prolyl-L-phenylalanyl Resin.** The *tert*-Boc-phenylalanyl resin (1.0 g, 0.125 mmole) was coupled successively with *tert*-Boc-L-proline and with the *N*-carbobenzyloxyamino acid free radical **1** by repeating the following series of operations for each residue. The resin was (1) washed for 2 min each with three 20-ml portions of acetic acid; (2) mixed for 15 min each with two 25-ml portions of 1.0 *N* hydrochloric acid in acetic acid; (3) washed with acetic acid (three times with 20 ml); (4) washed with absolute ethanol (three times with 20 ml); (5) washed with dimethylformamide (three times with 20 ml); (6) washed for 10 min with 30 ml of 5% triethylamine in dimethylformamide; (7) washed with dimethylformamide (three times with 25 ml); (8) washed with methylene chloride (three times with 20 ml); (9) mixed for 5 min with 0.40 mmole of *tert*-Boc-amino acid in 20 ml of methylene chloride; (10) added 0.40 mmole of dicyclohexylcarbodiimide in 5 ml of methylene chloride and then mixed continuously for 2 hr; (11) washed with methylene chloride (three times with 20 ml); (12) after the coupling of the free radical amino acid the resin was washed with 5% triethylamine in methylene chloride and then washed with methylene chloride (three times with 20 ml). This series of operations was followed to synthesize the other peptides by using the appropriate N-acetylated amino acids. Characteristic esr spectra of the tripeptide resins suspended in methylene chloride gave the coupling constants α_N 7.95 ± 0.05, $\alpha_{\beta CH}$ 2.1 ± 0.1, $\alpha_{\beta CH'}$ 2.3 ± 0.1 G. Little variation in this splitting pattern was observed for the different peptide derivatives.

β-(1,3-Dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanyl-L-prolyl-L-phenylalanine (6). Cleavage and deprotection of the carbobenzyloxy tripeptide were carried out by suspending 1.0 g of the resin in 10 ml of anhydrous trifluoroacetic acid and bubbling dry hydrogen bromide through the mixture for 15 min. The resin was removed by filtration and washed with 5 ml of anhydrous trifluoroacetic acid. The solution was evaporated and the yellow residue was taken up in 10% sodium carbonate to give a solution of pH 8.0. The red color of the radical returned slowly with stirring. The identity and homogeneity of the tripeptide were shown by the following data. Chromatography on silica gel with 5% formic acid in 80% ethanol gave a single red spot (R_f 0.30). A single ninhydrin-positive spot was obtained on electrophoresis at pH 1.85 (E_H = 0.62). A sample of the chromatographed peptide was hydrolyzed for 48 hr at 110° in 5.5 *N* hydrochloric acid and contained proline and phenylalanine in the ratios of 1.00 to 0.95. Trace amounts of aspartic acid, a possible hydrolysis product of the radical amino acid, and a compound resembling ornithine in mobility were observed in this analysis and others involving the free radicals. The chromatographed tripeptide could not be removed from the silica gel in a form free of noncombustible material. The esr spectra showed a single radical species with distinctive coupling constants throughout the pH range; at pH 7, α_N 8.46, $\alpha_{\beta CH}$ 2.10, $\alpha_{\beta CH'}$ 2.45 G. Reduction of the radical to the imino-nitroxyl tripeptide H_3N^+-INAla-Pro-Phe-O^- with sodium nitrite in dilute acetic acid gave a sample with coupling constants of α_{NO} 10.02, α_N 4.68, $\alpha_{\beta CH}$ 2.0, $\alpha_{\beta HC'}$ 1.3 in water at pH 7. Chromatography of the imino nitroxide on silica gel with 5% formic acid in ethyl acetate gave a pure sample. *Anal.* Calcd for $C_{24}H_{32}N_5O_5 \cdot 2H_2CO$: C, 55.30; H, 6.78. Found: C, 55.69; H, 6.50.

***N*-Acetyl-β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-DL-alanyl-L-prolyl-L-phenylalanine (3).** The *N*-acetyl tripeptide resin was prepared by the above procedure using the *N*-acetyl free radical amino acid **2**. Cleavage of the protected tripeptide from the resin was achieved by the above procedure without removal of the acetyl group. The tripeptide was purified by chromatography on silica gel with 5% formic acid in ethyl acetate (R_f 0.75). Elution of the red radical and evaporation gave an oil. This compound did not move on electrophoresis and did not give a positive ninhydrin test. Amino acid analyses showed proline and phenylalanine to be present in ratios of 1.00 to 0.92. Elemental analyses of this and the other *N*-acetyl peptides were inconclusive due to decomposition of the radical function before analysis. The esr of the chromatographed material was characteristic of a single nitronyl-nitroxyl radical species with coupling constants of α_N 8.40, $\alpha_{\beta CH}$ 1.64, $\alpha_{\beta CH'}$ 2.50 G in water at pH 7.3.

***N*-Acetyl-β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-DL-alanyl-L-phenylalanyl-L-phenylalanine (5).** The tripeptide was prepared by the procedure described for **2** where *tert*-butyloxycarbonylphenylalanine and the *N*-acetyl nitronyl nitroxide amino acid (**2**) were substituted for the *tert*-Boc-proline and carbobenzyloxy derivative of the free radical amino acid, respectively. Cleavage of the terminal phenylalanyl–resin bond with hydrogen bromide in trifluoroacetic acid and subsequent neutralization gave the red *N*-acetyl tripeptide. Purification by thin-layer chromatography on silica gel with 5% formic acid in ethyl acetate gave a single major spot (R_f 0.65). Elution followed by evaporation yielded a red oil which could not be crystallized, possibly due to the racemic nature of the N-terminal amino acid. The red oil did not show

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electrophoretic mobility or give a positive ninhydrin test. Amino acid analysis showed only phenylalanine to be present with a trace amount of aspartic acid. The esr spectra were characteristic of a single nitronyl nitroxide species and showed distinctive coupling constants in water at pH 6.9, α_N 8.35; $\alpha_{\beta CH}$ 2.34; $\alpha_{\beta CH'}$ 1.80.

N-Acetyl-β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-DL-alanyl-L-phenylalanine (4). The dipeptide was prepared by the standard procedure where the *N*-acetyl free radical amino acid **2** was coupled to the phenylalanyl resin. After cleavage from the resin the dipeptide was purified by thin-layer chromatography on silica gel using 5% formic acid in ethyl acetate (R_f 0.53). Elution and evaporation of the single red product gave an oil which showed no mobility on electrophoreses nor a positive ninhydrin reaction. Amino acid analysis showed only phenylalanine. The esr spectra indicated that a single nitronyl nitroxide radical species was present and gave the distinctive coupling constants α_N 8.47; $\alpha_{\beta CH}$ 2.18; $\alpha_{\beta CH'}$ 1.92 G at pH 7.3.

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Angiotensin II Analogs. IX.[1] Conformational Studies of Angiotensin II by Proton Magnetic Resonance

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Abstract: Proton magnetic resonance spectra of [Asn[1],Val[5]]angiotensin II and five analogs were determined at 20° and 50° in deuterated dimethyl sulfoxide with a 220-MHz instrument. Spectra of the Gly-Val-Tyr-Ile-His-Pro-Phe analog were taken at 100 MHz in D_2O at different pH values and temperatures. Some observed manifestations of conformational stability included upfield shifts of the Pro β-, γ-, and δ-methylene hydrogens; line broadening of the His C-2 and C-4 hydrogens with increasing temperature; and the presence of a single intramolecular hydrogen bound amide hydrogen. These results have been interpreted to indicate the presence of a stable conformer of angiotensin in solution which includes an intramolecular ion–dipole bond between the Phe carboxylate ion and the imidazole ring of histidine. This conformation holds the imidazole ring in the vicinity of the proline ring causing the observed upfield shifts of the proline methylene hydrogens and line broadening of the imidazole hydrogens. This conformer is stabilized by an intramolecular hydrogen bond between the Phe amide NH and the His carbonyl function and has an activation energy of interconversion of at least 13–15 kcal/mole. Further evidence indicates that the remaining amino acids in the side chain of the intramolecularly bound ring maintain a preferred conformation which is lost on increasing the temperature beyond 40° in D_2O. These results are in agreement with previously indicated conformational properties derived from esr spectra of free radical containing angiotensin analogs.

Angiotensin II is a biologically important octapeptide which has been the subject of numerous studies on the relationship between chemical modification and biological activity.[2] In view of the large number of publications dealing with angiotensin, relatively little is known about its tertiary structure. Optical rotatory dispersion and thin-film dialysis studies indicate that angiotensin has conformational stability, but the studies differ greatly in their description of the structural properties of the peptide in solution. Nmr data concerning angiotensin have not been previously reported.

Three groups have reported different descriptions of the conformational properties of angiotensin derived from thin-film dialyses data. Craig and coworkers[3] reported dialysis rate changes of [β-Asp[1],Val[5]]angiotensin II with pH. A compact structure with high dialysis rates is observed in acidic solution and attributed to a loose association of the positively and negatively charged groups along the peptide chain. A more extended and possibly more rigid conformer was indicated in basic media. Ferreira, *et al.*,[4] reported that two different conformations of [Asn[1],Ile[5]]angiotensin II exist between pH 2 and 11. The relative concentration of these conformations is said to be independent of pH but dependent on the ionic strength of the solution. Fernandez, *et al.*,[5] reported rates of dialysis for [Asn[1],-Ile[5]]angiotensin II which are pH independent between 5 and 9 but which increase at pH 10 where the phenolic hydroxyl group is ionized.

Evidence for conformational stability has also been obtained from optical rotatory dispersion curves. Spectra of [β-Asp[1],Val[5]]angiotensin II[6] and [Ile[5]]angiotensin II[7] show deep minima at 217 and 235 nm at pH 11 but relatively shallow curves when taken at pH 5.67.[6] These data are consistent with a helical model but do not exclude other stable conformers. In other work [Asn[1],Val[5]]angiotensin II was interpreted to have a nonhelical random coil configuration.[8]

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