nm (ε 14); nmr (D_2O) 8.55 (s, 1 H), 4.18 (t, 2 H), 3.63 (singlet methyl superimposed on multiplet, 4 H), 1.7–2.8 (m, 2 H), 1.33 (d, 3 H); tlc [I_2 detection, EtOH–0.1 *N* HCl, 2:1] one spot, R_f 0.43; tlc [I_2 detection, *n*-BuOH–HOAc–H_2O, 4:1:5] one spot, R_f 0.31 after two elutions; mass spectrum (70 eV) *m/e* (rel intensity) 97 (54, M^+ − HCl), 96 (100), 82 (34). *Molecular Formula:* Calcd for $C_6H_{11}N$ (M^+ − HCl): 97.0891. Found: 97.0887.

1,3,3-Trimethyl-2-pyrrolidinone-*3,3-dimethyl*-^{14}C (18). To 500 ml of anhydrous diethyl ether and 220 mmole (31 ml) of diisopropylamine (freshly distilled from BaO), cooled to −70°, was added 131 ml (210 mmol) of *n*-butyllithium (1.6 *M* in hexane). Then 4.95 g (50 mmol; 4.85 ml) of 1-methyl-2-pyrrolidinone was added, the solution was stirred for 15 min at −70°, and 13.8 ml (220 mmol) of CH_3I-^{14}C[8] was added. Stirring was continued at room temperature for 16 hr, 150 ml of H_2O was added, the ether layer was removed and evaporated *in vacuo*, and the residue was dissolved in 100 ml of H_2O. The combined aqueous solutions were continuously extracted with methylene chloride for 24 hr. Removal of the solvent and distillation of the residue at 95–97° (27 mm) gave 4.79 g (78%) of **18**: nmr ($CDCl_3$) 3.29 (t, 2 H), 2.79 (s, 3 H), 1.81 (t, 2 H), 1.04 (s, 6 H); glpc on 30% QF-1 on Chromosorb P (168°; 100 ml/min; 10 ft × 0.25 in.) one peak, 9.0 min. *Anal.* Calcd for $C_7H_{13}NO$: C, 66.1; H, 10.3; N, 11.0. Found: C, 65.9; H, 10.2; N, 10.9.

2-Acetonyl-1,3-dimethylpyrrolidine (17). To 6 mmol of 1,3-dimethyl-1-pyrrolinium chloride and 6 ml of H_2O were added 35 ml of 1 *N* NaOH, 10 ml of H_2O, 20 ml of ethanol, and 15 ml of ethyl acetoacetate. After stirring in the dark under nitrogen for 17 days, 50 ml of concentrated HCl was added. The reaction mixture was warmed on a steam bath for 5 hr, and concentrated to 5 ml *in vacuo*. The residue was dissolved in 50 ml of water and made strongly alkaline with 6 *N* NaOH; the resulting aqueous solution was continuously extracted with methylene chloride for 4 days. Removal of the solvent gave 440 mg (47%) of **17**: ir (thin film) 1730 cm^{-1} (C=O); nmr ($CDCl_3$) 2.7–3.7 (m, 5 H), 2.69 (s, 3 H), 1.4–2.5 (m, 3 H), 2.30 (s, 3 H), 1.12 (d, 3 H); mass spectrum (70 eV) *m/e* (rel intensity) 155 (4, M^+), 140 (2), 124 (28), 109 (21), 98 (100).

Warming **17** with a saturated ethanolic solution of picric acid gave the picrate which was recrystallized from absolute ethanol: mp 147–151 dec; nmr (pyridine-d_5) 8.97 (s, 2 H), 3.1–4.0 (m, 5 H), 3.08 (s, 3 H), 1.6–2.6 (m, 3 H), 2.25 (s, 3 H), 1.01 (overlapping doublets, 3 H). *Anal.* Calcd for $C_{15}H_{20}N_4O_8$: C, 46.9; H, 5.3; N, 14.6; O, 33.3. Found: C, 47.1; H, 5.3; N, 14.7.

Free Radical Analogs of Histidine

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Abstract: Some stable free radical analogs of histidine have been prepared which contain a nitronyl nitroxide ring system in place of the imidazole function. The esr spectra of these amino acids show magnetically nonequivalent methylene protons as shown by different hyperfine coupling constants of the β-methylene hydrogens. The spectra also show coupling constant changes with pH which have been correlated with an ion–dipole interaction between the nitronyl nitroxyl ring and the carboxylate anion in a manner which is analogous to histidine. The analogs which show this interaction also show esr line broadening effects consistent with a model involving hindered interconversion of conformers.

Stable free radicals have recently been used in the investigation of a number of biologically important systems. This method utilizes spin labels[1,2] or paramagnetic ions[3,4] to obtain information about the environment surrounding the paramagnetic species. Electron spin resonance is the major spectroscopic tool applied to these systems but the nmr,[5] visible, ultraviolet,[6] and fluorescence[7] spectra of the host system may also be affected by the free radical.

Where the molecule or biological system is not normally paramagnetic the free radical function may be added as a spin label. Ideally the addition is carried out in such a way that the label, usually a nitroxide radical, does not significantly alter the molecular structure or biological activity. The nitroxide radical can reflect large changes in solvent polarity[8] and losses of rotational freedom.[9,10] Since the nitroxide group is not coupled to a proton, large spectral changes are, in general, observed only when the radical has a low rate of rotation relative to the magnetic field ($\tau > 5 \times 10^{-11}$ sec). This effectively limits the use of spin labels to larger rigid molecules or to systems within viscous oriented media such as crystals or membranes.

These limitations precluded the use of the spin label technique in our investigation of conformations and intramolecular interactions in smaller peptides. In order to retain the unique advantages that electron spin resonance spectroscopy offers in the study of complex molecules we have attempted to construct a system which uses the free radical as a functioning portion of the amino acid rather than as a reporter group. For such a system to have significant utility the free radical amino acid must have a size, shape, and polarity similar to its analog.

We would like to report such a free radical analog of histidine. This model, β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanine (**1**), contains the nitronyl nitroxide ring system in place of the im-

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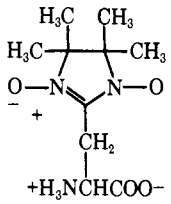
idazole ring of histidine (**2**). The nitronylnitroxylalanine (NNAla) has several properties which allow it to be a versatile indicator of molecular and solvation changes. As reported by Ullman and coworkers[11-16] the free radical is stable under a variety of conditions so that it may be used in biological systems. The unpaired spin resonates through the nitronyl nitroxide system so that the nitrogens are equivalent.[11] Some



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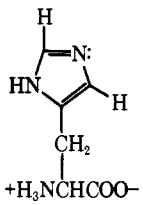

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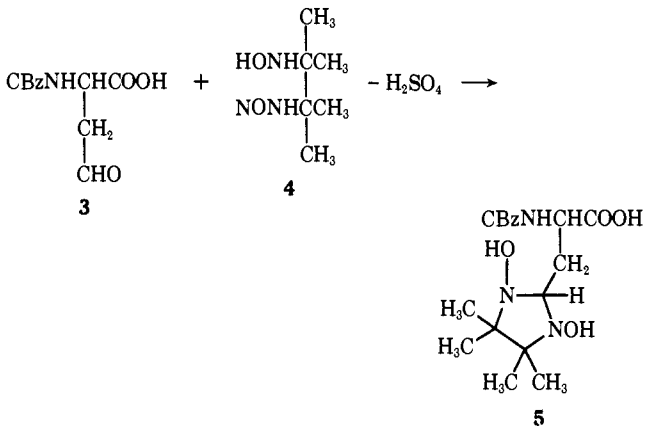
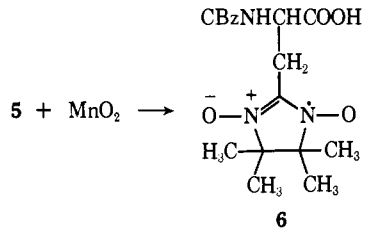
spin density resides on the sp^2 carbon and this is coupled to the β-methylene hydrogens. The spin density distribution (ρ eq 1) is dependent on the solvent polarity so that the nitrogen and hydrogen coupling constants reflect the solvating environment. The magnitude of the methylene coupling constants depends on the alignment of the carbon–hydrogen bond with the $p\pi$ orbit of the ring[16] so changes in these parameters occur with changes in rotamer population and with conformer interconversion rates. The nitronylnitroxyl ring system is also capable of forming an ion–dipole bond with the carboxylate anion of the amino acid in a manner which is analogous to histidine.[17]

In this paper we will describe the synthesis and some chemical reactions characteristic of the free radical amino acid. Esr spectroscopic changes which occur in solutions at different pH values are correlated to changes in the nmr spectra of histidine derivatives.

Syntheses and Reactions. The *N*-carbobenzyloxy amino acid free radical **6** was prepared from the previously known *N*-carbobenzyloxy-L-aspartic-β-semialdehyde (**3**)[18] by condensation with *N*,*N*′-dihydroxy-2,3-diamino-2,3-dimethylbutane monosulfate (**4**)[19] and subsequent oxidation of the adduct **5** with active manganese dioxide. The red radical **6** [λ_{max}^{EtOH} 522 (ϵ 1370), 555 nm (1400)] is stable in solution and in the solid state for several weeks when pure. In a corresponding series of reactions, *N*-acetylaspartic-β-semialdehyde ethyl ester (**7**) was prepared from α-acetamidoallylmalonate diethyl ester (**8**) and then condensed with the bis(hydroxylamine) **4**. This adduct was oxidized with manganese dioxide to give the *N*-acetyl ethyl ester radical **9**. The ester function was hydrolyzed in dilute base to give the *N*-acetyl nitronyl nitroxide free radical **10**.



3 **4** **5**



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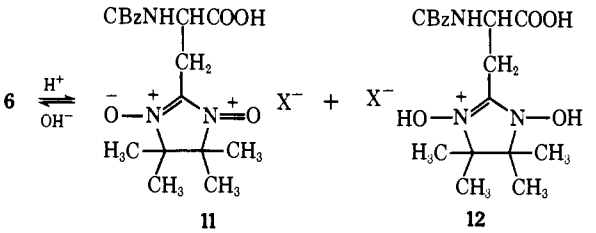
The carbobenzyloxy derivative **6** was converted to the amino acid **1** by dissolving in a 50% mixture of dry benzene and anhydrous trifluoroacetic acid into which hydrogen bromide was bubbled for 15 min.[20] Under these conditions the radical was protonated,[11] and the solution became orange. The protonated radical slowly decomposes so the solvent was evaporated quickly and the residue was taken up in a basic aqueous solution. The characteristic red color slowly returns on stirring under basic conditions. These results suggested that disproportionation had occurred in the trifluoroacetic acid–hydrogen bromide mixture in contrast to the simple protonation observed in dry trifluoroacetic acid.[11] This was further supported as the orange solution gave no esr signal.

The disproportionation product **11** has been found to be a strong oxidizing agent.[11] Although the disproportionation is readily reversible with base, the



11 **12**

reactive nature of the compound (**11**) leads to irreversible reactions in the presence of reducible compounds such as water and ethanol[11] but not trifluoroacetic acid. This led to some limitation in the usefulness of this radical in solid phase peptide synthesis.[21]

Partial reduction of the nitronyl nitroxide radical to the imino nitroxide **13** results from treatment with sodium nitrite in dilute acid solutions.[15] The imino nitroxide ring system has also been formed from the adduct **5** which is easily dehydrated in acid or with dicyclohexylcarbodiimide in methylene chloride. The

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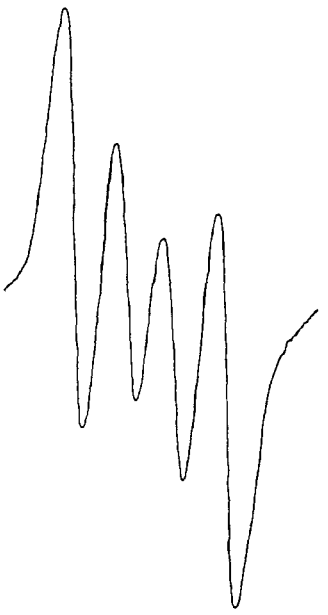
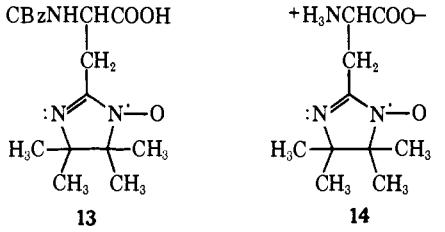


Figure 1. Esr spectrum of the center pair of doublets from radical **6** in water at pH 6.3 and 25°.

dehydration product, an iminohydroxylamine, gave the radical **13** on oxidation with active manganese dioxide. Hydrogenation of an ethyl acetate solution of the carbobenzyloxynitronylnitroxylalanine (**6**) over



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14

10% palladium on carbon cleaved the benzyloxy group and reduced the free radical ring system. Treatment of the reduced amino acid with manganese dioxide yielded the deprotected imino nitroxide **14**. Cleavage of the carbobenzyloxy group from **13** with anhydrous trifluoroacetic acid yielded the same compound (**14**).

An acylated stable free radical amino acid such as **6** or **10** can be coupled with the amino group of another amino acid or peptide using the solid phase method of peptide synthesis.[20] The peptide bond was formed under the usual conditions employing dicyclohexylcarbodiimide in methylene chloride.[20] Some deoxygenation of the nitronyl nitroxide ring was observed after prolonged treatment (>20 hr) under these conditions to give the imino nitroxide ring system. This side reaction was not detectable when the usual 2-hr coupling reaction time was employed.[21]

Electron Spin Resonance Spectra. The spin density of the imino nitroxide ring is distributed over the nitroxide group and the imine function. The complex esr signal results from splitting of the nitroxide triplet into triplets by the imine nitrogen and further splitting of this signal by the magnetically nonequivalent methylene hydrogens. The carbobenzyloxy derivative **13** gives coupling constants α_{NO} 10.20, α_N 4.55, $\alpha_{\beta CH}$ 1.65, $\alpha_{\beta CH'}$ 1.05 ± 0.05 G in aqueous solution at pH 7.0. The use of the imino nitroxide as a model compound is complicated by the fact that the spin density distribution is strongly affected by the substituents on the amino acid and the pH of the solution. The amino acid **14** gave the coupling constants α_{NO} 10.00, α_N 4.85, $\alpha_{\beta CH}$ 2.20, $\alpha_{\beta CH'}$ 1.25 ± 0.05 G at pH 7.0 but values of α_{NO} 9.85, α_N 5.00, $\alpha_{\beta CH}$ 2.35, $\alpha_{\beta CH'}$ 1.45 ± 0.05 G at pH 3.7. It appears that the asymmetric nature of the amino acid affects the spin density distribution as do solvation changes at the nonequivalent nitrogens. While there may be conformational preference on rotation about the C_β–C_γ bond which would contribute to the coupling constant differences between the β-methylene hydrogens these differences do not appear to be pH dependent. Although the imino nitroxide free radical is also a potentially useful model, the nitronyl nitroxide more closely parallels the properties of histidine and its use has been emphasized in this report.

The nitronyl nitroxide radical gives a five-line pattern caused by splitting of two equivalent nitrogen nuclei. Each of these lines may be coupled to substituents on the 2 position. For the amino acids in this study, each of these five lines were split into a pair of doublets by the β-methylene protons as shown in Figure 1. The inherent asymmetry of the amino acid makes the β-hydrogens magnetically nonequivalent so that small coupling constant differences of 0.15 to 0.30 G are observed in the case of free rotation about the C_β–C_γ bond.

When steric or electronic factors led to an unequal distribution among the possible rotamer conformations, larger coupling constant differences were observed. The magnitude of the coupling constant for each rotamer has been shown to depend on the angle of deviation, θ, of the β-hydrogen from the plane defined by the $p\pi$ orbital containing the free spin and the two carbons.[22] This relation is given by eq 1 and 2,

$$\alpha_{\beta CH} = \rho[B_0 + B\cos^2\theta] \quad (1)$$

$$\alpha_{\beta CH'} = \rho[B_0 + B\cos^2(\theta + 120)] \quad (2)$$

where $\alpha_{\beta CH}$ is the coupling constant of the β-hydrogen in a single rotamer, B_0 and B are constants estimated to be 3 and 45 G, and ρ is the spin density of the 2p orbital. The contribution of each conformation is proportional to its relative residence time.

In molecules with stronger electronic and steric interactions the rate of isomerism will be slowed. This results in increasing the line width of the central peaks of each set of doublets.[23] At still slower rates of isomerization the spectrum of specific conformers may be observed. Changes in the solvating environment of the radical cause perturbations in the spin density distribution of the resonating unpaired electron. This change in ρ values results in changes of the nitrogen and hydrogen coupling constants. Close association of the radicals in solution is characterized by line broadening throughout the spectrum. Effects analogous to those observed with spin labels may also be observed when the radical has a low tumbling rate.

The esr spectra of several amino acid derivatives were obtained at different pH levels where coupling constants were found to vary with the ionic species in solution. The *N*-acetyl (**10**) and *N*-carbobenzyloxy

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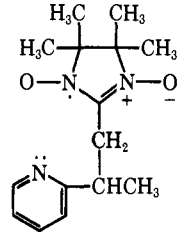
Table I. Nmr Coupling Constants of Histidine and Esr Coupling Constants of Nitronylnitroxylalanine Derivatives

Line no.	Species[c] in solution	Nmr coupling constants[a] $J_{\alpha\beta}$	$J_{\alpha\beta'}$	$\Delta J_{\alpha\beta,\alpha\beta'}$	Species[d] in solution	Esr coupling constants[b] $\alpha_{\beta CH}$	$\alpha_{\beta CH'}$	$\Delta\alpha_{\beta CH,\beta CH'}$
1	$H_3\overset{+}{N}$-$\overset{H}{\underset{\vert}{His^+}}$-OH	7.1	5.7	1.4	$H_3\overset{+}{N}$-NNAla-OH	2.25	2.45	0.20
2	$H_3\overset{+}{N}$-His-O^-	6.5	6.5	0	$H_3\overset{+}{N}$-NNAla-O^-	2.50	2.60	0.10
3	H_2N-His-O^-	4.5	8.7	4.2	H_2N-NNAla-O^-	1.95	2.25	0.30
4	AcNH-$\overset{H}{\underset{\vert}{His^+}}$-OH	5.4	7.8	2.4	AcNH-NNAla-OH	2.05	2.15	0.10
5	AcNH-$\overset{H}{\underset{\vert}{His}}$-$O^-$	4.1	9.7	5.6	AcNH-NNAla-O^-	1.70	2.50	0.80
6	*tert*-BocNH-$\overset{H}{\underset{\vert}{His^+}}$-OH	6.8	6.8	0	CBzNH-NNAla-OH	1.80	2.40	0.60
7	*tert*-BocNH-*im*BzHis-O^-	2.8	10.0	7.2	CBzNH-NNAla-O^-	1.50	2.65	1.15
8	H_2N-His-OCH_3	6.7	6.7	0	AcNH-NNAla-OCH_2CH_3	2.50	2.25	0.25

[a] ±0.1 Hz in D_2O. [b] ±0.05 G in H_2O. [c] His refers to $CH(CH_2C_3H_2N_2)CO$. [d] NNAla refers to the nitronylnitroxylalanine fragment $CH(CH_2C_7H_{12}N_2O_2)CO$.

(**6**) derivatives showed large coupling constant differences of 0.8 to 1.1 G at pH levels above 4. In more acidic solution these values converged. The free amino acid (**1**) and the *N*-acetyl ethyl ester (**9**), in contrast, showed small chemical shift differences through the pH range 2–11. The pH dependence of the *N*-acetyl and *N*-acetyl ethyl ester methylene coupling constants is shown in Figure 2.

To explain these data it is postulated that the electron-deficient nitronyl nitroxide ring associates with the basic carboxylate anion. The ion–dipole interaction affects the conformational equilibrium causing the observed changes in the β-hydrogen coupling constants. A similar interaction has been observed for the pyridyl derivative **15** where the nonequivalence of



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the methylene hydrogens observed in neutral or basic solutions collapses on protonation of the pyridyl nitrogen.

There is a close parallel between derivatives of the free radical amino acid and corresponding histidine derivatives as shown in Table I. Derivatives of both of these amino acids show an ion–dipole interaction which affects the conformational equilibrium. The basic carboxylate ion binds to the dipolar ring system to give a favored conformation in which the ring and the anion are in close proximity. This interaction is reflected in the C_α–C_β rotation as shown by the nmr parameters of histidine[17] and by the C_β–C_γ rotation as shown by the esr spectra of the radical amino acid derivatives. In each case the molecules which can form an ion–dipole bond have large coupling constant differences.

The dipolar amino acids of line 2, Table I, do not show a preferred conformation as the interaction between the ammonium ion and the carboxylate group supersedes the possible interaction between the ring and the carboxylate anion. The small difference between the coupling constants of the free radical amino acid zwitterion, line 2, and its anion, line 3, suggests either that the anion exists in a preferred conformation where the β-hydrogens have equal dihedral angles (θ = 30 or 60°, eq 1) or that there is little conformer preference by the amino acid **1** due to the low order

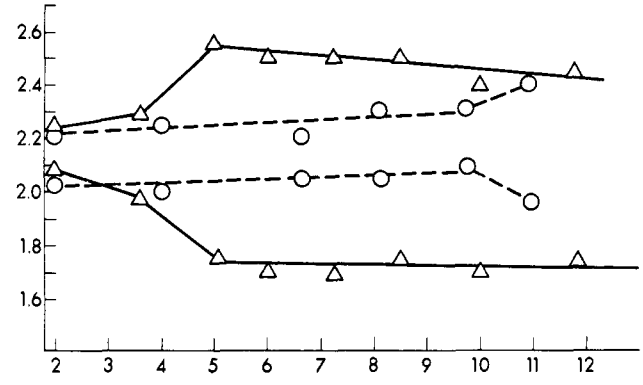

Figure 2. A plot of the β-methylene coupling constants against pH for AcNH-NNAla-OCH_2CH_3 (**9**), ○, and for AcNH-NNAla-OH (**10**), △; (*y* axis) β-methylene coupling constants, $\alpha_{\beta CH}$ and $\alpha_{\beta CH'}$ (gauss), (*x* axis) pH.

of basicity of its anion form. The former conclusion is supported by line broadening data. The relatively large coupling constant difference of the carbobenzyloxy derivative in line 6 may reflect a sterically preferred conformation for this compound or a somewhat larger magnetic nonequivalence of the β-hydrogens than is observed in the other derivatives.

The esr spectra of the free radical amino acid **1** and its *N*-acetyl (**10**) and *N*-carbobenzyloxy (**6**) derivatives at pH 8.5 show unequal line widths. The outer two lines of each set of doublets have a width of 0.85, 0.78, and 0.83 ± 0.03 G while the inner two lines are broadened to 0.96, 0.96, and 0.95 ± 0.03 G respectively. The inner lines of these derivatives in acidic solution show no broadening nor do the other derivatives listed in Table I. This is a strong indication that the ion–

dipole interaction postulated to account for the coupling constant differences not only alters the rotamer equilibrium but is strong enough to slow the rate of conformer interconversion.

Conclusion

The stable free radical nitronyl nitroxide amino acid has been shown to be an effective analog of histidine. It approximates the size and shape of the natural amino acid and parallels the ion–dipole binding observed in histidine derivatives. The spectral characteristics of the nitronyl nitroxide group make it sensitive to variations in its solvation shell and the reasonable stability of the radical should make it a versatile tool in the investigation of histidine reactions and those of histidine-containing peptides.

Experimental Section

Melting points were measured with a Thomas-Hoover capillary melting point apparatus and are uncorrected. 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. Nmr spectra were taken on a Varian A-60-A spectrometer and the esr spectrometer has been previously described.[24] A Varian flat cell with a volume of 0.04 ml within the microwave cavity was used. Visible spectra were taken on a Cary Model 11 spectrometer and mass spectra on an Associated Electrical Industries MS-9 high-resolution mass spectrometer.

N-**Benzyloxycarbonyl-L-aspartic-β-semialdehyde (3)** was prepared by the method of Neuberger and Tait.[18] On crystallization a white solid was obtained, mp 129–131° (lit.[18] mp 135–137°) and $[\alpha]^{22}D$ −21.4° (*c* 1.9, ethyl acetate) [lit.[18] $[\alpha]^{21}D$ −19.8° (*c* 1.7, ethyl acetate)].

α-Acetamidoallylmalonate diethyl ester (8) was prepared by the method of Albertson.[25] The residue from the evaporation of solvent was purified by distillation at 110–114° (0.2 mm) for a 78% yield. The distillate solidified on standing to give a mp of 107° (lit.[25] mp 109°). The nmr spectra in deuteriochloroform showed $COCH_3$, 1.92; NH, 7.33; OCH_2CH_3, 4.09 and 1.17; allylic CH_2, 2.92; vinyl CH's, 4.85, 5.10, and 5.42 ppm downfield from TMS.

α-Acetamidoallylglycine Ethyl Ester (16). A solution of 135 g (0.59 mole) of **8** in 300 ml of 95% ethanol was mixed with 1 equiv (23.6 g) of sodium hydroxide in 200 ml of water. After stirring at 25° for 2 hr no diester remained (tlc on silica gel with benzene). The pH was adjusted to 1.8 with sulfuric acid and the half-ester was extracted with methylene chloride. This extract was washed with 1.3 equiv of sodium carbonate in 400 ml of water. The aqueous solution was then acidified to pH 2.0 with hydrochloric acid and extracted with methylene chloride. After drying over sodium sulfate the solution was filtered and evaporated to give 80 g, 65%, of a white solid, mp 122–123 dec (lit.[18] mp 126–126.5°). This solid half-ester was transferred to a vacuum distillation apparatus, and the distilling pot was placed in an oil bath which was heated to 135°. Heating was continued until gas evolution slowed, at which time the pressure was lowered and the *N*-acetylallylglycine ethyl ester was distilled at 90° (0.2 mm). An 84% yield was obtained from the half-ester. *Anal.* Calcd for $C_9H_{15}NO_3$: N, 7.63. Found: N, 8.03. The nmr spectrum in deuteriochloroform showed $COCH_3$, 1.93; NH, 7.67; α-CH, 4.29; OCH_2CH_3, 3.93 and 1.20; allylic CH_2, 2.26; vinyl CH's, 4.74, 4.92, and 5.43 ppm downfield from TMS.

N-**Acetyl-DL-aspartic-β-semialdehyde Ethyl Ester (7).** A solution of 58 g (0.32 mole) of **16** in 100 ml of 98% formic acid was cooled to 0°, and 30 ml of 30% hydrogen peroxide was added with stirring. The mixture was allowed to warm slowly to 35° and was maintained at this temperature. The reaction was followed by nmr for 5 days as the peaks in the vinyl CH and allylic CH_2 regions decreased. The solvent was then evaporated to give 16 g of a viscous mixture of the diol ethyl ester. This reaction time was considerably longer than that of the carbobenzyloxy derivative[18] which gave the α-amino-5-hydroxy-γ-valerolactone in 16 hr at 40°.

The residue from evaporation (0.09 mole) was taken up in 200 ml of 50% ethanol–water. Dilute sulfuric acid was added to give a pH of 6.5 and 43 g (0.18 mole) of sodium metaperiodate was added. The pH was changed to 8.5 with potassium carbonate, and the mixture was stirred for 20 hr at 25°. The pH was lowered to 3.0 with sulfuric acid, and the mixture was extracted with ethyl acetate. Very little aldehyde was extracted from the aqueous phase which gave a positive Fuchsin aldehyde test. The aqueous solution was evaporated to dryness. Of the residue, 14 g was dissolved by 500 ml of 80% ethanol. Repeated attempts to purify the aldehyde failed to give a sample free of noncombustible material. The ethanol-soluble material can be used in subsequent reactions without further purification.

N,*N*′-**Dihydroxy-2,3-diamino-2,3-dimethylbutane Monosulfate (4).** The salt was prepared by the method of Lamchen and Mittag.[19] A 99% yield was obtained and gave a compound with mp 194–195° (lit.[19] 172–174°). The higher melting point was observed on repeating the synthesis. *Anal.* Calcd for $C_6H_{18}N_2O_2 \cdot H_2SO_4$: SO_4^{2-}, 39.40. Found: SO_4^{2-}, 39.68. The use of the monosulfate was found to be more convenient than that of the volatile hygroscopic bishydroxylamine.[19]

N-**Acetyl-β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-DL-alanine Ethyl Ester (9).** The above ethanol solution of **7** was concentrated to 100 ml and 24.6 g (0.10 mole) of *N*,*N*′-dihydroxy-2,3-diamino-2,3-dimethylbutane monosulfate (**4**) and 0.15 mole of potassium carbonate were added. After 2 hr the mixture no longer gave a positive Fuchsin aldehyde test. The ethanol was removed, and the residue was taken up in ethyl acetate. After drying over sodium sulfate and filtering, 40 g of activated manganese dioxide was added, and the mixture was stirred for 45 min. The deep red mixture was filtered, and the solvent was evaporated in the dark. Chromatography of the residue on silica gel with 5% formic acid in ethyl acetate gave 5 g, 15%, of the radical amino acid as the monoformate. *Anal.* Calcd for $C_{14}H_{24}N_3O_5 \cdot CH_2O_2$: C, 50.02; H, 7.24; N, 11.65. Found: C, 49.65; H, 6.91; N, 11.35. The mass spectrum showed the molecular ion to have a molecular weight of 314.171584 characteristic of a $C_{14}H_{24}N_3O_5$ unit.

N-**(Carbobenzyloxy-β-(1,3-dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanine (6).** The general procedure used in the preparation of **9** was applied to this synthesis in which *N*-benzyloxycarbonyl-L-aspartic-β-semialdehyde (**3**)[18] was condensed with **4**, followed by oxidation with manganese dioxide. Chromatography on silica gel with 5% formic acid in ethyl acetate gave an analytically pure sample in 77% yield from the aldehyde. *Anal.* Calcd for $C_{18}H_{23}N_3O_6 \cdot CH_2O_2$: C, 53.50; H, 6.17; N, 9.89. Found: C, 53.26; H, 6.01; N, 9.78.

N-**Carbobenzyloxy-β-(1-oxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanine (13).** A sample of **6** in ethanol was stirred over sodium nitrite. Acetic acid was added slowly until the red color disappeared, in about 10 min. The ethanol solution was chromatographed on silica gel with 5% formic acid in ethyl acetate. *Anal.* Calcd for $C_{18}H_{24}N_3O_5 \cdot CH_2O_2$: C, 55.93; H, 6.45; N, 10.30. Found: C, 55.81; H, 6.17; N, 10.62.

β-(1,3-Dioxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanine (1). A sample of **6** was dissolved in 1 ml of dry benzene and 2 ml of anhydrous trifluoroacetic acid. Hydrogen bromide was bubbled into the mixture for 15 min. The solvent was evaporated under reduced pressure, and the residue was washed with benzene. The remaining material was dissolved in 2 ml of 0.5 *N* sodium hydroxide. The characteristic red color of the radical chromophore returned on standing. The amino acid **1** gave a single ninhydrin-positive spot on electrophoreses (E_H = 0.61). The amino acid zwitterion was soluble in water but insoluble in organic solvents. Structural assignment was based on the fact that the amino acid **1** was converted to the carbobenzyloxy derivative **6** by the method of Neuberger and Tait.[18] This derivative was identified by its mass spectrum.

β-(1-Oxy-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanine (14). A sample of the carbobenzyloxy amino acid **13** was deprotected using the procedure described for **1**. The product amino acid **4** gave a single ninhydrin-positive spot on electrophoreses (E_H = 0.69). An amino acid with the same esr spectrum and electrophoretic mobility was obtained from the reduction of **1** with sodium

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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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