2,2-Disubstituted-4,4-dimethylimidazolidinyl-3-oxy Nitroxides: Indicators of Aqueous Acidity through Variation of a_N with pH

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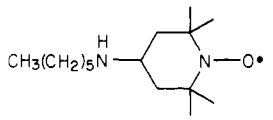
Abstract: The synthesis and properties of two members, **17** and **18**, of a new series of nitroxide absolute pH indicators are reported. Imidazolidine nitroxide **17** is prepared from cyclohexanone and 1,2-diamino-2-methylpropane via the route **11** → **13** → **14** → **17**. Amino imidazolidine nitroxide **18** is prepared similarly from *N*-benzoyl-4-piperidone via the route **12** → **15** → **16** → **18**. The ESR hyperfine splitting constant, a_N, diminishes as the pH is reduced in the region of the pK_a of the other nitrogen atom of the imidazolidine ring. Typical values for a_N (pH) in gauss for **17** are 16.20 (5.90), 15.80 (4.95), 15.33 (4.03), and 14.98 (3.00) with p$K_a \simeq 4.5$. Values for **18** are 15.55 (3.15), 15.27 (2.27), 14.68 (1.53), and 14.42 (1.07) with p$K_a \simeq 2.0$. Egg yolk lecithin vesicles (pH 2.4) containing entrapped probe **18** were suspended in pH 6.9 buffer. The ESR spectrum of the vesicles was only partially affected by addition of the nitroxide reducing agent sodium ascorbate and showed a_N = 15.33 G. This value corresponds to the expected pH 2.4 and demonstrates the ability of the probe to report intravesicle pH.

The measurement of pH is fundamental to much of aqueous solution chemistry.[1] Biological research requirements in this area include measurement of gradients of pH across lipid membranes and, ideally, measurement of absolute values of pH inside vesicles and cells.[2] Electron spin resonance (ESR) methods, in general, have the advantage of high sensitivity ($\simeq 10^{-5}$ M probe) without the requirement of opitcal transparency associated with conventional optical probes. Recently, Cafisco and Hubbell[3] and Quintanilha and Mehlhorn[4] have described two different ESR methods for measuring pH gradients across membranes. In the first ESR method[3] the ESR spectra of the probe (e.g., **1**) reflect



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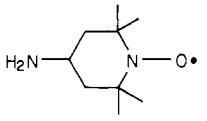

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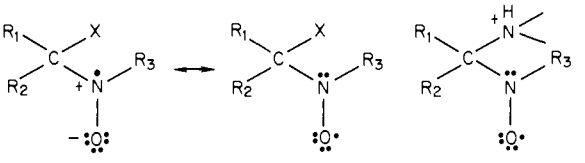
changes in the ratio of membrane-associated to free aqueous probe as a transmembrane pH gradient is established. As the gradient increases, inside acidic, the amount of membrane-associated probe increases, with attendant broadening of the ESR spectral lines. In the second ESR method[4] the membrane permeable (in its uncharged form) nitroxide amine **2** is allowed to partition between inside and outside regions of the (thylakoid) membrane while the membrane impermeable ESR line broadening agent $K_3Fe(CN)_6$[5] is present only in the outside region. Thus, as the inside becomes more acidic upon illumination, more amine diffuses inside where the ESR signal is not broadened by the $K_3Fe(CN)_6$. Thus, ESR signal height increases as the proton gradient increases.

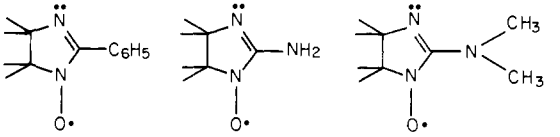
We became interested in the development of an ESR method capable of measuring *absolute values* of aqueous pH. We sought a series of water-soluble stable nitroxide free radicals which have simple three-line ESR spectra, the hyperfine splitting constants a_N of which were conveniently sensitive toward pH. The rationale for our approach was based on earlier work of de Boer et al.,[6] who showed that the magnitude of a_N for a series of α-substituted nitroxides **3**–**5** depends on the electronegativity of the substituent. As the electron-withdrawing tendency of X increases, the contribution of resonance structure A to the overall nitroxide electronic structure diminishes. This is accompanied by a decrease in a_N since unpaired spin density is on the oxygen atom in contributing structure B. We reasoned that the positive charge generated upon protonation of an amino group in a position α to the nitroxide group (cf. salt **6**) should likewise cause a decrease in a_N, the magnitude of which may prove sufficient to be a convenient indicator of pH in the region of the pK_a of the amino group.



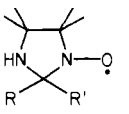
A B 6

3, X = H, a_N = 14–16 G
4, X = Cl, a_N = 11.8–13.0 G
5, X = NO_2, a_N = 12.6 G

A few nitroxides have been synthesized which show changes in the ESR spectra as the pH of the aqueous medium is varied. Nitroxide **7**[7] has a complex spectrum which shows a change in a_N with pH of 3.9 G (pK_a = 1.9). More interesting in this regard are nitroxides **8** and **9**,[7] though the ESR spectra are also quite



7, pK_a = 1.9 8, pK_a = 6.4 9, pK_a = 6.8



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complex, owing to hyperfine splitting from three nonequivalent nitrogen atoms. Helbert et al.[8] observed similar spectral changes in a related series of nitroxides.

Recently, we introduce a new series of amino nitroxides **10** derived by oxidation of 2,2,4,4,5,5-hexasubstituted imidazolidines with *m*-chloroperoxybenzoic acid (MCPA).[9] These stable ni-

(1) Wescott, C. C. "pH Measurements"; Academic Press: New York, 1978.
(2) For leading references see: Thomas, J. A.; Buchsbaum, R. N.; Zimniak, A.; Racker, E *Biochemistry* **1979**, *18*, 2210–2218.
(3) Cafiso, D. S.; Hubbell, W. L. *Biochemistry* **1978**, *17*, 3871–3877.
(4) Quintanilha, A. T.; Mehlhorn, R. J. *FEBS Lett.* **1978**, *91*, 104–108.
(5) Chromium oxalate is reported to be a superior spin broadening agent with thylakoids. See: Berg, S. P.; Nesbitt, D. M. *Biochim. Biophys. Acta* **1979**, *548*, 608–615.
(6) Kayen, A. H. M.; Bolsman, Th. A. B. M.; De Boer, Th. J. *Recl. Trav. Chim. Pays-Bas* **1976**, *95*, 14–20.
(7) Ullman, E. F.; Call, L.; Osiecki, J. H. *J. Org. Chem.* **1970**, *35*, 3623–3631.
(8) Helbert, J. N.; Kopf, P. W.; Poindexter, E. H.; Wagner, B. E. *J. Chem. Soc., Dalton Trans.* **1975**, 998–1006.

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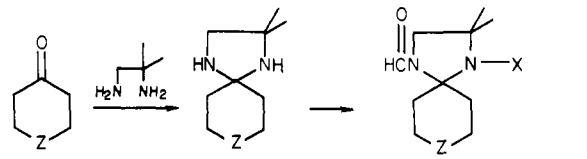
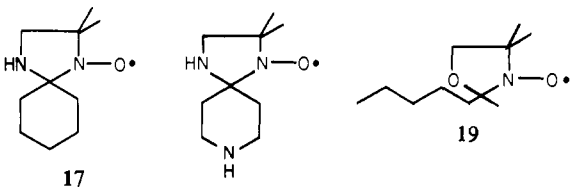
troxides, precursors for the corresponding novel dinitroxides, exhibit essentially a three-line ESR spectrum with small additional splittings due to the protons on the cyclohexane ring and the other ^{14}N atom.[10]

While the desired juxtaposition of amine and nitroxide groups is present in **10**, the two methyl groups at C-4 would be best deleted for purposes of this present study. Not only do they increase the hydrophobicity for the probes, but also they may interfere with solvation of the protonated species. We have therefore chosen to synthesize and investigate the properties of two representative C-4-unsubstituted imidazolidine nitroxides, namely, **17** and its aza analogue, **18**.

Synthesis of pH Probes 17 and 18. Acid-catalyzed condensation of cyclohexanone with 1,2-diamino-2-methylpropane in benzene gave imidazolidine **11** which was immediately formylated selec-



11, Z = CH_2 13, X = H, Z = CH_2
12, Z = $NCOC_6H_5$ 14, X = O·, Z = CH_2
15, X = H, Z = $NCOC_6H_5$
16, X = O·, Z = $NCOC_6H_6$



17 18 19

tively on the less hindered nitrogen atom with formic acetic mixed anhydride[11] to give **13** and then oxidized with MCPA[12] to nitroxide formamide **14**. This substance existed in two interconvertible crystalline forms, mp 84–85 °C and mp 88–89 °C. Hydrolysis of **14** with methanolic aqueous sodium hydroxide afforded the desired amino nitroxide **17** as yellow crystals, mp 64–65 °C.[10] Similarly, crystalline diamino nitroxide **18** (mp 85–87 °C) was prepared from *N*-benzoyl-4-piperidone via intermediates **12, 15** (mp 149–150 °C), and **16** (mp 120–121 °C).

ESR Spectra of 17 and 18 in Aqueous Solution as a Function of pH. ESR spectra (25 °C) of **17** were recorded at several pH values by using three buffer systems. Under these conditions proton-exchange rates involving the amino group are fast on the ESR time scale.[7] Each observed spectrum therefore is a weighted average between that of the protonated and that of the unprotonated forms. Figure 1 shows that as the pH decreases from 5.90 to 3.00, a_N decreases from 16.20 to 14.98 G. Since a_N can be readily measured to within ±0.05 G, this translated into an uncertainty of about ±0.1 pH unit in the region of the pK_a of the amino nitroxide. The observed changes in a_N as a function of pH are essentially independent of the nature of the buffers used or the presence in the buffer of Na^+ and K^+ ions at concentrations approximately those in extracellular plasma. For purposes of comparison doxyl nitroxide **19** shows a_N = 15.13 G over the entire pH range investigated (pH 1.1–11.1).

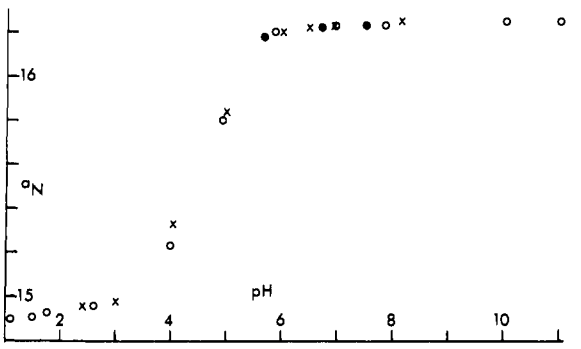


Figure 1. pH dependence of a_N for imidazolidine nitroxide **17**. Values of a_N are ±0.05 G. Values were obtained in buffer system I (○), values in buffer system II (×), and values in buffer system III (●). The apparent pK_a is about 4.5. Representative measured values of a_N (pH) are as follows: 14.98 (3.00); 15.23 (4.00); 15.33 (4.03); 15.80 (4.95); 15.84 (5.01); 16.18 (5.70); 16.20 (5.90); 16.20 (6.00); 16.22 (6.52).

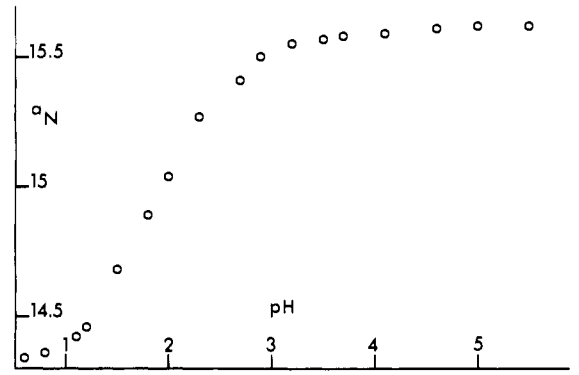


Figure 2. pH dependence of a_N for amino imidazolidine nitroxide **18**. Values of a_N are ±0.05 G. The apparent pK_a is about 2.0. Measured values of a_N (pH) are as follows: 14.36 (0.80); 14.42 (1.07); 14.46 (1.22); 14.68 (1.53); 14.89 (1.79); 15.04 (1.99); 15.27 (2.27); 15.41 (2.66); 15.50 (2.92); 15.55 (3.15); 15.57 (3.48); 15.58 (3.68); 15.59 (4.09); 15.61 (4.55); 15.62 (5.03); 15.62 (5.54); 15.64 (7.87).

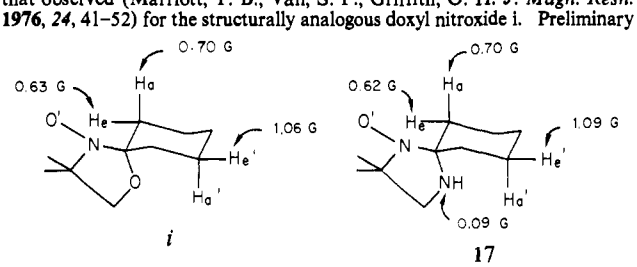
The pK_a of amino nitroxide **17** is about 4.5. Apparently the presence of the proximally located nitroxide group causes a dramatic (nearly 10^6-fold) decrease in basicity of the secondary amino group in **17** (cf. pyrrolidine, pK_a = 11.3). The partial positive charge carried by the nitroxyl nitrogen atom (see Structure A above) likely is responsible for this diminished basicity.

Figure 2 shows the dependence of a_N on pH for nitroxide diamine **18**. Interestingly, the pK_a of the imidazolidine nitrogen atom in **18** is depressed to a value of about 2. This is a reflection of the presence of both the nitroxide group and of the nearby positively charged nitrogen atom of the piperidine ring.

Vesicle Experiments with pH Probe 18. The ability of **18** to report the absolute value of the pH of intravesicular solutions was next investigated. Following the general method of Kornberg and McConnell,[13] a mixture of citrate buffered solution of **18** and egg yolk lecithin (EYL) was sonicated for about 30 min. at 0 °C (pH of sonicate 2.4). The vesicles containing the trapped probe were

(9) Keana, J. F. W.; Norton, R. S.; Morello, M.; Van Engen, D.; Clardy, J. *J. Am. Chem. Soc.* **1978**, *100*, 934–937.

(10) A degassed $CHCl_3$ solution (10^{-4} M) of **17** showed a ESR hyperfine splitting pattern (sweep width = 10 G) similar to but slightly broader than that observed (Marriott, T. B.; Van, S. P.; Griffith, O. H. *J. Magn. Resn.* **1976**, *24*, 41–52) for the structurally analogous doxyl nitroxide i. Preliminary



ESR spectral simulation experiments taken together with the reported splitting constants for nitroxide *i* gave rise to the set of tentative splitting assignments for **17** shown below. With the reasonable assumption that **i** and **17** both exist in solution predominantly in the conformation shown, the increased broadening observed in the spectra of **17** over that in **i** is likely due to the additional unresolved splitting (0.09 G) due to the second ^{14}N atom. Proton-exchange rates involving the amino group are expected to be fast on the ESR time scale.[7]

(11) Büchi, G.; Gould, S. J.; Näf, F. *J. Am. Chem. Soc.* **1971**, *93*, 2492–2501.

(12) Keana, J. F. W.; Keana, S. B.; Beetham, D. *J. Am. Chem. Soc.* **1967**, *89*, 3055–3056.

(13) Kornberg, R. D.; McConnell, H. M. *Biochemistry* **1971**, *10*, 1111–1120.

separated from untrapped probe by gel filtration over Sephadex G-25 coarse which had been preequilibrated with phosphate buffer (pH 6.9). The vesicle fraction (pH 6.9) was eluted immediately after the column void volume. An ESR spectrum of the vesicle fraction at 0 °C showed a_N = 15.33 G. It was gratifying to note that this value corresponds (Figure 1) to pH 2.4 for the intravesicular solution. Continued elution of the Sephadex column with pH 6.9 buffer gave fractions containing the untrapped probe. The ESR signal was rapidly and completely eliminated by addition of sodium ascorbate (see below).

Further evidence of the intravesicular location of **18** in the vesicle fractions was obtained from a series of experiments utilizing the reducing agent sodium ascorbate and the spin broadening agent $K_3Fe(CN)_6$. The ascorbate reduction technique is widely used in nitroxide spin labeling studies for the quantitative reduction of nitroxides which are accessible to the aqueous ascorbate solution.[14] Excess sodium ascorbate (pH 6.8) was added to the above vesicle suspension at 0 °C, and the ESR spectrum was recorded at 0 °C within 50 s of mixing. The peak height was diminished to about 33% of the original value. This in part was due to the dilution of the sample by the ascorbate solution and to the reduction of any probe molecules which had gained access to the ascorbate, possibly as a result of the mixing procedure within a small capillary tube. The remainder of the signal decayed at a much slower rate ($t_{1/2}$ > 4 h). In a separate experiment the peak height was reduced to 68% of the original value by dilutions with the appropriate amount pH 6.9 buffer not containing ascorbate. At 0 °C EYL vesicles are essentially impermeable toward ascorbate on this time scale.[15] Thus, the persistence of the nitroxide ESR signal in the vesicle fraction in the presence of excess ascorbate confirms the intravesicular location of **18** and the ability to report intravesicle pH.

This conclusion was supported by experiments with the membrane impermeable spin broadening agent $K_3Fe(CN)_6$.[4] When aqueous $K_3Fe(CN)_6$ was added to an aliquot of the vesicle fraction at 0 °C such that the final concentration of the broadening agent was 27 mM, the peak height of the ESR signal was reduced to about 60% of the original value, owing largely to the dilution effect. Under these conditions, the signal due to **18** inside the vesicles is observed since the ferricyanide ion cannot enter the vesicle.

Probe **18** (and presumably other analogues as well) is most applicable as an *intravesicle* pH indicator when the inside pH is lower than that of the outside medium. Under these conditions the probe tends to remain inside the vesicles over longer periods of time, as indicated by studies with ascorbate and other combinations of inside–outside pH. This is not unexpected in view of the known ready membrane permeability of the uncharged form of other amines (e.g., **1**[3] and **2**[4]). We are currently pursuing the synthesis of analogues of **17** and **18** which bear permanent positive or negative charges. Owing to a much diminished membrane permeability, such probes should be useful under a broad range of pH conditions and may well display a range of pK_a values (cf. pK_a of **18** vs. **17**).

Experimental Section[16]

2,2-Pentamethylene-4,4-dimethylimidazolidine (11). To a stirred solution of 1.072 g (12.2 mmol) of 1,2-diamino-2-methylpropane (Aldrich Co.) in 33 mL of benzene was added 1.213 g (12.4 mmol) of cyclohexanone and 18 mg of *p*-toluenesulfonic acid monohydrate. The flask was fitted with a Dean–Stark water separator containing anhydrous potassium carbonate, and the mixture was gently refluxed for 48 h. After removal of the benzene vacuum distillation at 95 °C and 15 torr gave 1.75 g (86%) of **11** as a clear colorless hygroscopic oil which was immediately used for the next step; NMR δ 1.18 (s, 6), 2.78 (s, 2).

1-Formyl-2,2-pentamethylene-4,4-dimethylimidazolidine (13). Formic acetic mixed anhydride[11] was generated by combining 327 mg (3.21 mmol) of acetic anhydride and 173 mg (3.31 mmol) of 99% formic acid in 2 mL of ether and stirring at 55 °C for 2 h. The resulting solution was cooled and added slowly to a stirred ice-cold solution of 536 mg (3.19 mmol) of **11** in 4 mL of ether. After 12 h at 25 °C the solution was concentrated to a tacky yellow oil which was dissolved in $CHCl_3$, washed twice with chilled 10% Na_2CO_3 solution, and dried (anhydrous K_2CO_3). Evaporation of the solvent afforded 538 mg (86%) of crude **13** as a pale yellow oil which was used immediately for the next experiment: NMR δ 1.26 (s, 6), 3.42 (s, 2), 8.3 (s, 1), IR ($CHCl_3$) 1650 cm^{-1}.

1-Formyl-2,2-pentamethylene-4,4-dimethylimidazolidinyl-3-oxy (14). To a stirred solution of 259 mg (1.32 mmol) of **13** in 15 mL of ether in a 4 °C cold room was added slowly dropwide a chilled solution of 403 mg (1.5 × 1.32 mmol) of 85% MCPA in 10 mL of ether. After 20 h at 4 °C the solution was twice washed wtih 10% Na_2CO_3 and then twice with water. The aqueous layers were back-washed with ether. The combined ether solutions were dried (Na_2SO_4) and concentrated, affording a semicrystalline yellow oil. The product was dissolved in 7 mL of hexane and cooled, giving 161 mg (58%) of **14** as yellow crystals: mp 84–85 °C and 88–89 °C; NMR ($CDCl_3$ + phenylhydrazine[17]) δ 1.26 (s, 6), 3.45 (s, 2), 8.39 (s, 1); IR ($CHCl_3$) 1660 cm^{-1}; ESR ($CHCl_3$) three lines, a_N = 14.33 G. Anal. Calcd for $C_{11}H_{19}N_2O_2$: C, 62.53; H, 9.06; N, 13.26. Found: C, 62.53; H, 8.91; N, 12.99.

2,2-Pentamethylene-4,4-dimethylimidazolidinyl-3-oxy (17). To a stirred solution (N_2) of 20 mg of **14** in 1 mL of methanol was added 1 mL of deoxygenated 6 N aqueous sodium hydroxide. After 20 h at 23 °C most of the methanol was removed by rotary evaporation at 23 °C to give a slurry which was extracted with ether. Evaporation of the ether gave 24 mg of a yellow solid which was sublimed at 40–50 °C onto an ice-cold finger to give 16 mg (92%) of **17** as yellow crystals (best stored sealed under N_2 at −20 °C): mp 64–65 °C; NMR ($CDCl_3$ + phenylhydrazine[17]) δ 1.27 (s, 6), 2.16 (s, 1, amine), 3.46 (s, 2); ESR three lines with some further splitting (see text), $a_N(CHCl_3)$ = 14.88 G, $a_N(H_2O)$ = 16.30 G; UV (ethanol) max 235 nm (ϵ 2320). Anal. Calcd for $C_{10}H_{19}N_2O$: C, 65.54; H, 10.45; N, 15.28. Found: C, 65.40; H, 10.48; N, 15.47.

Buffer Systems I, II, and III for pH Studies with 17. Buffer system I was prepared as described in ref. 18. The pH range 1.1–1.75 utilized mixtures of 0.2 M KCl and 0.2 M HCl. The pH range 2.6–4.0 utilized mixtures of 0.1 M potassium hydrogen phthalate and 0.1 M HCl. The pH 4.95 utilized 25 mL of 0.1 potassium hydrogen phthalate and 11.3 mL of 0.1 M NaOH. The pH range 5.9–7.9 utilized mixtures of 0.1 M NaH_2PO_4 and 0.1 M NaOH. The pH range 10.1–11.1 utilized mixtures of 0.05 M $NaHCO_3$ and 0.1 M NaOH.

Buffered system II was prepared as described in ref 19 and utilized mixtures of 0.1 M citric acid and 0.2 M Na_2HPO_4 for the pH range 2.4–8.0.

Buffer system III was prepared as follows. For pH 5.70, 1.0 mL of pH 5.9 (buffer **I**) as combined with 79 mg of NaCl and 2 mg of KCl and diluted to 10 mL with water. Final ion concentrations were as follows: Na^+, 145 mM; K^+, 2.7 mM; phosphate buffer, 9 mM. Buffers of pH 6.75 and 7.56 were similarly prepared.

ESR Spectroscopic Measurements in Buffer Systems I, II, and III. A 10.9 mM stock solution of **17** was prepared by dissolving 4 mg of **17** in 2 mL of deoxygenated water. ESR samples ($\sim 10^{-4}$ M) were prepared by combining 0.01 mL of stock solution with 0.99 mL of buffer. Samples were deoxygenated by passing a rapid stream of N_2 through the sample of ~3 min and then sealed.

8-Benzoyl-2,2-dimethyl-1,4,8-triazaspiro[4.5]decane (12). To a stirred solution of 1.28 g (14.5 mMol) of 1,2-diamino-2-methylpropane (Aldrich Co.) in 45 mL of benzene was added 3.00 g (14.8 mmol) of *N*-benzoyl-4-piperidone (Aldrich Co.) and 22 mg of *p*-toluenesulfonic acid monohydrate. The reaction flask was fitted with a Dean-Stark water separator charged with anhydrous K_2CO_3, and the solution was gently refluxed for 14 h. Removal of the benzene afforded 4 g of a yellow oil which was used immediately for the next experiment: NMR δ 1.00 (s,

(14) Keana, J. F. W. In "Spin Labeling: Theory and Applications"; Berliner, L. J., Ed.; Academic Press: New York, 1979. Paleos, C. M.; Dias, P. *J. Chem. Soc., Chem. Commun.* **1977**, *345*–346.

(15) See inter alia: Birrell, G. B.; Boyd, S. A.; Keana, J. F. W.;.Griffith, O. H. *Biochim. Biophys. Acta* **1980**, *603*, 213–219.

(16) Infrared spectra were recorded with either a Beckman IR-5 or IR-7 spectrophotometer. NMR spectra were recorded on a Varian XL-100 high-resolution spectrometer in $CDCl_3$ and only characteristic peaks are reported. Chemical shifts are reported in parts per million (δ) downfield from internal Me_4Si. X-band ESR spectra were recorded at 25 °C unless otherwise stated on a Varian E-line spectrometer interfaced with a 32K Varian 620/L100 computer. All solvents and buffers used for ESR spectra were deoxygenated by bubbling N_2 through the solutions for 3–5 min. Water was doubly glass distilled. Elemental analyses and mass spectra (70 eV) were determined at the University of Oregon by Dr. R. Wielesek. Melting points were determined in an oil bath and are uncorrected. A Beckman Expandomatic SS-2 pH meter with a Sensorex combination pH electrode was used for the pH readings. Preparative thin layer chromatography (TLC) was done on Analtech silica gel GF 20 × 20 cm 1000 μm plates.

(17) Lee, T. D.; Keana, J. F. W. *J. Org. Chem.* **1975**, *40*, 3145–3147.

(18) "CRC Handbook of Chemistry and Physics", 52nd ed.; CRC Press: Cleveland OH, 1971; p 104.

(19) "CRC Handbook of chemistry and Physics", 39th ed.; CRC Press: Cleveland, OH, 1957–1958; p 1615.

6), 2.82 (s, 2).

8-Benzoyl-2,2-dimethyl-4-formyl-1,4,8-triazaspiro[4.5]decane (15). Formic acetic anhydride[11] was generated by combining 1.52 g (14.9 mmol) of acetic anhydride and 0.705 g (15.2 mmol) of 99% formic acid in 4 mL of ether and stirring the solution at 55 °C for 2 h. The resulting solution was cooled to 0 °C and then slowly added to a stirred solution of 4.04 g (14.8 mmol) of crude **12** in 30 mL of CH_2Cl_2. After a 13-h stir at 25 °C, the reaction mixture was extracted with cold 10% Na_2CO_3 (2 × 15 mL). The aqueous extracts were combined and back-extracted with CH_2Cl_2. The combined CH_2Cl_2 solutions were dried (K_2CO_3) and concentrated to a brownish foam. The foam was dissolved in ethyl acetate and chromatographed over 20 g of silica gel. Elution with ethyl acetate gave crude **15** which was crystallized from ether–hexanes to give 2.84 g (64%) of **15**: mp 149–150 °C; NMR δ 1.29 (s, 6), 3.48 (s, 2), 8.38 (s, 1); IR ($CHCl_3$) 1657, 1628 cm^{-1}. Anal. Calcd for $C_{17}H_{23}N_3O_2 \cdot {}^1/_4H_2O$: C, 66.75; H, 7.74; N, 13.74. Found: C, 67.14; H, 7.61; N, 13.83.

8-Benzoyl-2,2-dimethyl-4-formyl-1,4,8-triazaspiro[4.5]decanyl-1-oxy (16). To a stirred solution of 2.02 g (6.70 mmol) of **15** in 100 mL of CH_2Cl_2 at 0 °C was added dropwise 2.13 g (10.5 mmol) of 85% *m*-chloroperoxybenzoic acid in 100 mL of CH_2Cl_2. The resulting solution began turning orange after 10 min, and the solution was allowed to stir at 25 °C overnight. The deep orange solution was concentrated to dryness, and the residue was dissolved in 1:1 ethyl acetate–hexanes and chromatographed over 60 g of silica gel. Elution with the same solvent gave an orange fraction which was concentrated to dryness, yielding 1.44 g (68%) of crude oily **16**. Crystallization from ethyl acetate–hexanes afforded 1.26 g (59%) of pure orange **16**: mp 118–119 °C; IR ($CHCl_3$) 1665, 1628 cm^{-1}; ESR ($CHCl_3$) three lines, a_N = 14.46 G. Anal. Calcd for $C_{17}H_{22}N_3O_2$: C, 64.54; H, 7.01; N, 13.28. Found: C, 64.21; H, 6.55; N, 12.92.

2,2-Dimethyl-1,4,8-triazaspiro[4.5]decyl-1-oxy (18). To a stirred solution (N_2) of 400 mg of **16** in 16 mL of MeOH was added 16 mL of degassed aqueous 6 N NaOH. The golden solution was stirred in a 47 °C bath for 17 h. The solvent was removed by rotoevaporation, and the resulting yellow slurry was extracted with $CHCl_3$ (3 × 5 mL). The extract was washed with brine and dried (K_2CO_3). Evaporation of the solvent afforded 210 mg (90%) of **18** as a crystalline residue which resisted attempts at recrystallization: mp 85–87 °C; IR ($CHCl_3$) no C=O absorption; ESR ($CHCl_3$) three lines, a_N = 14.83 G; mass spectrum, *m/e* (relative intensity) 185.152 (100) (calcd for $C_9H_{18}N_3O$ 185.153), 112 (26), 111 (32), 87 (28), 75 (47), 74 (37), 73 (26), 72 (32), 64 (47), 61 (21), 60 (58), 48 (32), 47 (42), 46 (63).

pH Studies with 18. Buffer system I,[18] described above, was used for the pH studies with **18**. A 10.9 mM stock solution of **18** was prepared by dissolving 2 mg of **18** in 1 mL of H_2O. ESR samples, $\sim 10^{-4}$ M in **18**, were prepared by combining 10 μL of the stock solution with 990 μL of the appropriate buffer.

Vesicle Experiments with 18. A vial was charged with 83 mg of egg yolk lecithin, 2.5 mL of pH 2.2 citric acid buffer,[19] and 5 mg of **18**. The resulting mixture, 11 mM in **18**, was sonicated (N_2) in an ice bath for 30 min (40-W setting). The pH of the sonicate was 2.4. Half of the sonicate was placed (5 °C) on a 0.8 × 22 cm Sephadex G-25 coarse column which had been preequilibrated with pH 6.9 buffer. Elution with pH 6.9 buffer afforded, after about a 3-mL void volume, the turbid vesicle fraction (1.9 mL, pH 6.9). Continued elution with the same buffer afforded the pale yellow fractions containing untrapped **18**. The vesicle fraction was divided into several aliquots and stored on ice for the ESR experiments involving sodium ascorbate and $K_3Fe(CN)_6$. The ESR spectrum (see text) of a 40-μL aliquot was recorded at 0 °C, and then the sample was immediately treated with 10 μL of chilled sodium ascorbate stock solution (pH 6.8) (250 mg/mL of phosphate buffer). A similar series of experiments was performed by utilizing a stock solution of $K_3Fe(CN)_6$ (99 mg/mL of phosphate buffer).

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Registry No. 11, 80028-49-5; **12**, 80028-50-8; **13**, 80028-51-9; **14**, 80028-52-0; **15**, 80028-53-1; **16**, 80028-54-2; **17**, 80028-55-3; **18**, 80028-56-4; 1,2-diamino-2-methylpropane, 811-93-8; cyclohexanone, 108-94-1; *N*-benzoyl-4-piperidone, 24686-78-0.

Studies on Spin-Trapped Radicals in γ-Irradiated Aqueous Solutions of L-Alanylglycine and L-Alanyl-L-alanine by High-Performance Liquid Chromatography and ESR Spectroscopy

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Abstract: Aqueous solutions of L-alanylglycine and L-alanyl-L-alanine were γ irradiated in the presence of a spin trap, 2-methyl-2-nitrosopropane. Stable spin adducts produced in the γ-irradiated solutions were analyzed by high-performance liquid chromatography and ESR spectroscopy. The following spin adducts were found and identified: *t*-BuN(O·)CH-(CH_3)CONHCH$_2$COO$^-$ (I), NH_3^+-*CH(CH_3)CONH-*CH(COO$^-$)N(O·)-*t*-Bu (II), and *t*-BuN(O·)CH$_2$CH(NH_3^+)-CONHCH$_2$COO$^-$ (III) from L-alanylglycine; *t*-BuN(O·)-*CH(CH_3)CONH-*CH(CH_3)COO$^-$ (IV), NH_3^+-*CH(CH_3)-CONH-*C(CH_3)(COO$^-$)N(O·)-*t*-Bu (V), NH_3^+CH(CH_3)CONHCH(COO$^-$)CH$_2$N(O·)-*t*-Bu (VI), and *t*-Bu-N(O·)-CH$_2$CH(NH_3^+)CONHCH(CH_3)COO$^-$ (VII) from L-alanyl-L-alanine. Each of spin adducts II, IV, and V was found as a pair of diastereomeric radicals, which revealed mutually different hyperfine splitting constants. The two diastereomeric pairs of spin adducts II and V were individually separated. It was demonstrated that ESR spectra of spin adducts II, III, and VI changed remarkably with pH through the acid-dissociation equilibria of the carboxyl or amino groups. The pK_a values for the dissociation have been determined to be 1.8 and 1.6 for the carboxyl groups of the pair of diastereomeric spin adducts II. The pK_{COOH} values are lower than that of alanylglycine because of the electron-withdrawing character of the nitroxyl group.

The spin-trapping method is one of the useful techniques for detection and identification of short-lived radicals in various reaction systems, by which short-lived radicals are converted into the fairly stable nitroxyl radicals (spin-trapped radicals or spin adducts) through reactions with spin traps such as nitroso or nitrone compounds.[1-3] For example, when 2-methyl-2-nitroso-

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