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Synthesis, Structure, and X-Band (9.5 GHz) EPR Characterization of the New Series of pH-Sensitive Spin Probes: *N,N*-Disubstituted 4-Amino-2,2,5,5-tetramethyl-3-imidazoline 1-Oxyls

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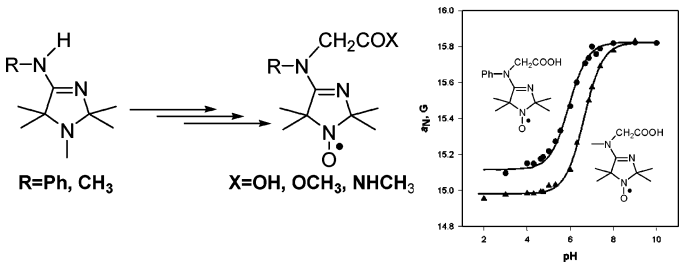

An approach to the synthesis of new imidazoline nitroxides bearing an *N′*,*N′*-disubstituted amidine group is reported. The approach is based on the alkylation of diamagnetic 4-R-amino-1,2,2,5,5-pentamethyl-3-imidazolines with bromoacetic acid ethyl ester; the products of alkylation are further oxidized to the corresponding nitroxides. The approach allows a variety of functional groups to be introduced into the nitroxide molecule structure. Alkylation with bromoacetic acid ethyl ester was found to proceed with high regioselectivity and afford the products of *exo*-alkylation. The regiochemical assignment is made on the basis of ^{13}C NMR spectra and confirmed by X-ray diffraction study. All of the nitroxides synthesized here were shown to have pH-dependent EPR spectra with pK_a ranging from 3.5 to 6.2. For nitroxides **13** bearing the carboxylic group remote to the nitroxide moiety, the changes in isotropic magnetic parameters of EPR spectra due to reversible deprotonation of the carboxylic group were found to be small. For these nitroxides, we demonstrate an alternative approach for pK_a determination that is based on measuring the peak-to-peak line width of the EPR spectrum in the presence of the paramagnetic broadening agent potassium ferricyanide. The partition coefficients of nitroxides in octanol/H_2O and octanol/phosphate buffer solution mixtures were measured to reveal a range of their lipophilicities.

Introduction

An EPR method for pH value determination is based on exquisite dependence of EPR spectra of some nitroxides on the reversible protonation of functional groups adjacent to the nitroxide moiety.[1a,d,2] Typically, pH-induced changes in EPR spectra are assessed from measurements of the isotropic hyperfine coupling constant for nitrogen, a_N, or, sometimes, for phosphorus, a_P,[1d] although the electronic *g*-factor is also affected by proton

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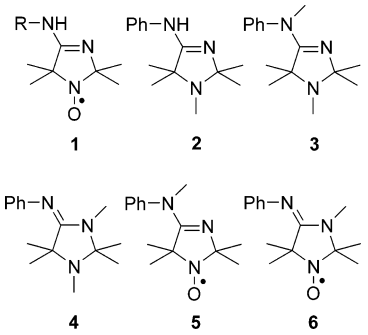
SCHEME 1



exchange reactions. The method was initially reported in the early eighties.[1] Since then, pH-sensitive spin probes have found numerous applications in studying proton-related phenomena, such as transmembrane proton transport in model systems,[3] surface potential and polarity of membranes and proteins,[4] and acidity in the interior ionite grains and at the solid−liquid interface.[5] Recently, with development of low-frequency EPR spectroscopy and imaging, the method was extended to noninvasive pH measurements in living organisms.[6] Other experiments, such as site-directed pH determination of proteins and membrane−protein complexes using high-field EPR, have been recently described.[7] Further advances in these applications call for a series of new spin probes that not only have EPR spectra sensitive to pH changes over a wide range but also have functional groups suitable for residue-specific modification of complex biological molecules.

Among all the pH-sensitive spin probes the 3-imidazoline 1-oxyl series of nitroxides **1** (Scheme 1) containing protonatable amidine functionalities as a part of the heterocycle are, perhaps, the most widely used in EPR studies. These probes exhibit an exceptional sensitivity of EPR spectra to protonation (e.g., $\Delta a_N \approx 0.7-0.9$ G) and a biologically important range of pK values from ca. 4.0 to 7.2 units.[8a,b] 4-R-amino-2,2,5,5-tetramethyl-3-imidazoline 1-oxyls **1** are readily obtained through 1,3-dipolar cycloaddition reaction of isocyanates to aldonitrone 2,2,5,5-tetramethyl-3-imidazoline 1-oxyl.[8a,b] However, current synthetic procedures described for such nitroxides impose certain restrictions on the structure of the compounds. Specifically, only *N*′-monosubstituted derivatives, such as compound **1**, can be synthesized by this method. Meanwhile, an introduction of a second substituent, e.g., bearing a functional group, to the *exo*-cyclic nitrogen of the amidine moiety would allow one to manipulate the properties of the spin probe (Δa_N, pK_a, lipophilicity, chemical binding capabilities) to a greater extent, thus facilitating the further progress of the method. Recently, an elegant approach to the synthesis of *N*,*N*-disubstituted 4-amino-3-imidazoline-1-oxyls via substitution of the cyano group in 4*H*-imidazole-5-carbonitrile 3-oxides with amines has been suggested.[9] However, it appears that the necessity of using organometallic reagents in the final step of the synthesis imposes certain limitations on the structure of the amine to be used. Specifically, this synthesis might not be suitable for the amines bearing functional groups sensitive toward Grignard reagents. We speculate that modification of the amidine function of the nitroxides **1** by means of an alkylation reaction appears to be one of the most attractive solutions. Though the example of alkylation of *N*′-unsubstituted amidine nitroxide **1** (R = H) with a highly reactive enone Mannich base methiodide has been reported,[10] the scope of application of this particular approach is apparently yet restricted by the synthesis of aryl derivatives. Our previous attempts to directly alkylate both *N*′-unsubstituted (R = H) and *N*′-monosubstituted amidines **1** (R = Ph, CH_3) with methyl iodide, acrylonitrile, bromoacetic acid ethyl ester, and dimethyl sulfate failed. An apparent reason for decreased reactivity of the amidine function is a strong electron-withdrawing influence of the nitroxide moiety. As shown earlier, the treatment of the diamagnetic amidine **2** with dimethyl sulfate readily afforded the alkylation products **3** and **4** with subsequent oxidation yielding the new nitroxides **5** and **6** with pH-dependent EPR spectra.[11]

Here we report an approach to the synthesis of *N*,*N*-disubstituted 4-amino-2,2,5,5-tetramethyl-3-imidazoline 1-oxyls via alkylation of the amidine function in the diamagnetic 4-R-amino-1,2,2,5,5-pentamethyl-3-imidazolines **2**.

All the nitroxides synthesized in the present work were characterized by X-band (9−10 GHz) EPR and were shown to have pH-dependent EPR spectra with pK_a ranging from 3.5 to 6.2. For nitroxides with the carboxylic group remote to the nitroxide moiety, we demonstrate an alternative approach for the carboxylic group pK_a determination that is based on measurements of EPR line width as a function of pH in the presence of the paramagnetic broadening agent potassium ferricyanide. We also report the partition coefficients of nitroxides in octanol/H_2O and octanol/phosphate buffer solution mixtures.

Results and Discussion

We chose the readily available bromoacetic acid ethyl ester as an alkylation reagent in the synthesis shown in Scheme 2.

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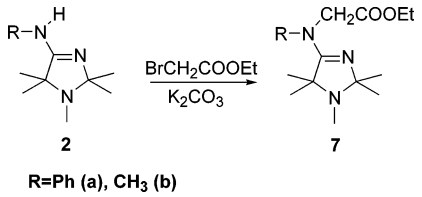
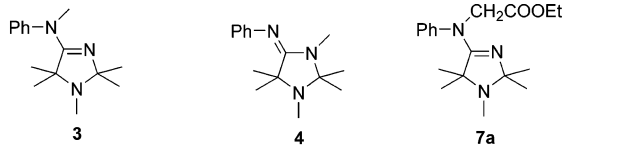
SCHEME 2



SCHEME 3



UV (in EtOH): λ_{max}= 246 nm
lg ε = 3.64
^{13}C NMR (C_i-Ph in $CDCl_3$):
δ = 145.72 ppm

UV (in EtOH): λ_{max}= 238 nm
lg ε = 4.08
^{13}C NMR (C_i-Ph in $CDCl_3$):
δ = 150.31 ppm

UV (in EtOH): λ_{max}= 247 nm
lg ε = 3.68
^{13}C NMR (C_i-Ph in $CDCl_3$):
δ = 139.93 ppm

Refluxing a mixture of 4-phenylamino-1,2,2,5,5-pentamethyl-3-imidazoline **2a**, $BrCH_2COOEt$, and K_2CO_3 in acetonitrile afforded a compound whose IR spectrum revealed an intense absorption at 1750 cm^{-1} that is characteristic for ester carbonyl. ^{1}H NMR spectrum of the resulting compound showed, in addition to resonances of methyl groups in the 1, 2, and 5 positions of the imidazoline ring, ethyl group multiplets at δ 1.30 and 4.30 ppm, and a N$-CH_2$ moiety signal at δ 5.00 ppm. To elucidate the position of the new alkyl group, ^{13}C NMR and UV spectra of the compound synthesized were compared with those of *endo*- and *exo*-derivatives **3** and **4** previously described[11,12] (Scheme 3).

The chemical shift of a phenyl group *ipso*-carbon atom in *N*-phenyl-substituted amidines has been previously shown to be a reliable criterion for regiochemical assignment in a series of isomeric *N*-alkylated amidine derivatives.[12] If the C=N bond is in a direct conjugation with the phenyl group, as in compound **4** (*endo*-alkylation), the resonance of the *ipso*-carbon atom of the phenyl group will be observed at a lower field (δ 150.31 ppm) than for a compound with no conjugation, such as compound **3** (δ 145.72 ppm, *exo*-alkylation). The ^{13}C NMR spectrum of the compound synthesized showed the *ipso*-carbon atom resonance at δ 139.93 ppm, which allowed us to assign to the compound the structure of *exo*-isomeric *N*-(1,2,2,5,5-pentamethyl-3-imidazoline-4-yl)-*N*-phenylamino acetic acid ethyl ester **7a**. The close resemblance of an UV spectrum of compound **7a** to that of 4-methylphenylamino-1,2,2,5,5-pentamethyl-3-imidazoline **3**[11] as compared with the spectrum of *endo*-compound **4** (Scheme 3) is an additional evidence in favor of the assignment made. None of the products of *endo*-alkylation were detected in the reaction mixture. Thus, the alkylation of 4-phenylamino-1,2,2,5,5-pentamethyl-3-imidazoline **2a** with bromoacetic acid ethyl ester proceeds on the *exo*-cyclic nitrogen of the amidine function with high regioselectivity.

In the same manner, the alkylation of 4-methylamino-1,2,2,5,5-pentamethyl-3-imidazoline **2b** was performed. ^{1}H NMR spectrum of the obtained compound displayed,

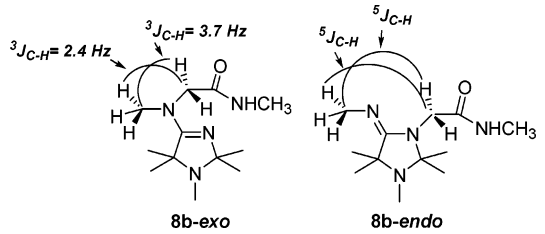


FIGURE 1. Feasible structures for compound **8b** and selected heteronuclear coupling constants for *exo*- and *endo*-forms. See the text for further details.

in addition to the signals of methyl groups of imidazoline ring, a signal at δ 3.29 ppm, assigned to the *exo*-N$-CH_3$ group, ethyl group multiplets at δ 1.22 and 4.12 ppm and the signal of the N$-CH_2$ moiety at δ 4.52 ppm. We failed to obtain this compound in an analytically pure form. The hydrochloride and *p*-toluenesulfonate salts were also prepared but found to be highly hygroscopic and useless for characterization of the compound as well. Finally, the ester obtained was treated with excess of methylamine solution in ethanol, resulting in readily crystallizable *N*-(1,2,2,5,5-pentamethyl-3-imidazoline-4-yl)-*N*-methylamino acetic acid *N*-methyl amide **8b**. The latter compound was characterized and used for further regiochemical assignment. The assignment was made on the basis of a splitting pattern present in the proton-coupled^{13}C NMR spectrum of **8b**. In the spectrum recorded in acetone-d_6 CH_2 group reveals a triplet of quartets at δ 56.0, with $^1J_{C-H}$ = 90.2 Hz and $^3J_{C-H}$ = 2.4 Hz; CH_3 group reveals a quartet of triplets at δ 37.8, with $^1J_{C-H}$ = 109.6 Hz and $^3J_{C-H}$ = 3.7 Hz (see Figure 1).

Assuming that the product of *endo*-alkylation should show negligibly small, if any, $^5J_{C-H}$ coupling constants (Figure 1), the resulting compound was assigned similar to the previous case, as the *exo*-alkylation product **7b**. Basically, examples of *endo*-alkylation of amidines have been described earlier.[13] Obviously, in the case of compounds **2a**,**b** the reason for regioselective *exo*-alkylation is the steric hindrance of the *endo*-cyclic nitrogen atom with bulky methyl groups in the position 2 of the imidazoline ring.

The ester group in the compounds **7a**,**b** allows one to obtain various functional derivatives. For example, treatment of **7a** with ammonia afforded the amide **9** (Scheme 4). Alkaline hydrolysis of the latter as well as of ester **7a** led to the corresponding *N*-phenylamino acetic acid derivative **10**. Because of the high hygroscopicity of the latter, its hydrochloric salt was obtained and used for characterization.

Attempts to prepare the corresponding nitroxides by oxidation of **9**, **7a**, and **10** in $H_2O_2/Na_2WO_4/CH_3OH$ have been made, but did not yield satisfactory results. Ester **7a** and amino acid **10** undergo destruction under the oxidation conditions, and in the case of amide **9** the only product isolated was the nitroxide amino acid **13a** formed in a negligible (6%) yield (Scheme 4). As we discovered, the secondary amide function is much more

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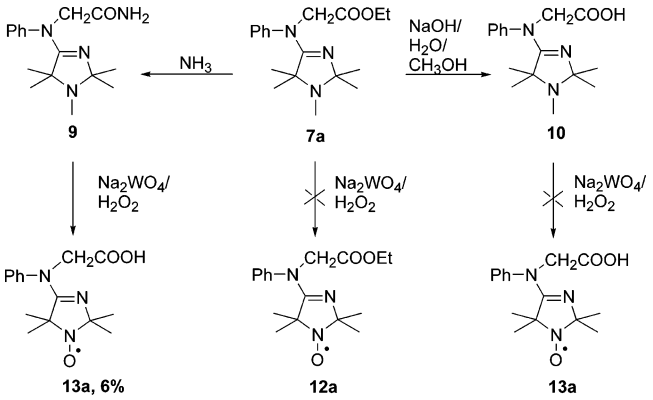
SCHEME 4



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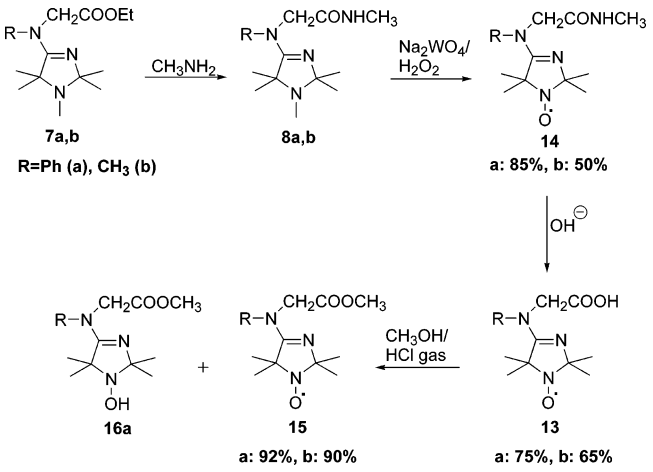

stable under the oxidation conditions as compared to the primary amide. Thus, the oxidation of *N*-methylamide derivative **8a** in the system $H_2O_2/Na_2WO_4/CH_3OH$ at a slightly elevated temperature led to the paramagnetic *N*-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl)-*N*-phenylamino acetic acid *N*-methyl amide **14a** in a good yield (Scheme 5). Using similar conditions, *N*-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl)-*N*-methylamino acetic acid *N*-methyl amide **14b** was synthesized in a satisfactory yield (see the Experimental Section). Paramagnetic amino acids **13a**,**b** were obtained by alkaline hydrolysis of *N*-methyl amides **14a**,**b**. Esterification of amino acid **13a** was performed using the standard CH_3OH/HCl procedure. Along with methyl ester of *N*-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl)-*N*-phenylamino acetic acid **15a**, the formation of another product with a lower R_f value was observed by TLC. This product was not isolated in a pure form and characterized. It was found that it could be easily oxidized to ester **15a** with PbO_2 in chloroform solution or by air on aluminum oxide TLC plate. This low R_f product was assumed to be the hydroxylamino derivative **16a**, which results from the disproportionation of the nitroxide **15a** in an acidic medium.[14]

The crystal structure of methyl ester **15a** was determined by a single-crystal X-ray diffraction analysis. The X-ray data (see the Supporting Information) confirm the

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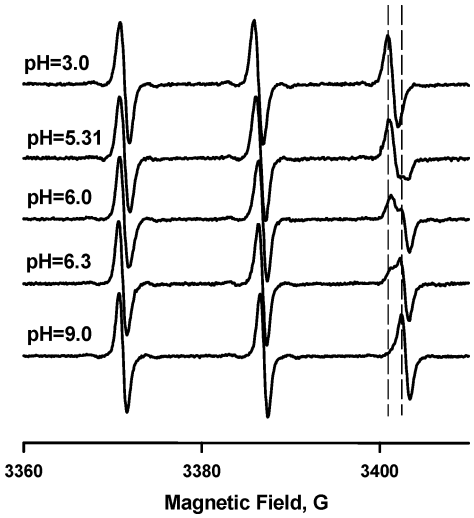

FIGURE 2. Representative room temperature 9.5 GHz (X-band) EPR spectra of the carboxylic acid nitroxide **13a** taken in 50 mM buffer solutions at various pH (concentration of nitroxide ca. 0.1 mM). Approximate positions of the peak height of the high-field nitrogen hyperfine components for the protonated (pH = 3.0) and deprotonated (pH = 9.0) forms of the nitroxide are shown by dashed lines.

unambiguity of regiochemical assignment made on the basis of ^{13}C NMR spectra.

The X-band (9.5 GHz) EPR spectra of the nitroxides synthesized were studied as a function of pH. All new nitroxides were found to show reversible effects of pH on EPR spectra. Figure 2 shows a series of EPR spectra of the carboxylic derivative **13a** in aqueous buffers with pH ranging from 3.0 to 9.0 units. It was found that each individual EPR spectrum in such a series could be approximated as a superposition of spectra from protonated, $R^{\bullet}H^+$, and nonprotonated, $R^{\bullet}$, forms of the nitroxide probe. These forms differ in their isotropic nitrogen hyperfine coupling constants a_N and *g*-factors.[1b] The two-component spectra shown in Figure 2 are indicative of a slow, on the EPR time scale, $R^{\bullet} \rightleftarrows R^{\bullet}H^+$ chemical exchange[1b] and were observed for all nitroxides studied in this work.

We have utilized two approaches to derive pK_a's from the nitroxide EPR spectra. The first approach is based on calibrating experimentally measured splitting between the low field and the central nitrogen hyperfine components of the first-derivative experimental EPR spectra as a function of pH (see the Experimental Section and Table 1, method A). If nitroxide EPR spectra fall into a regime of a fast tumbling and the chemical exchange $R^{\bullet} \rightleftarrows R^{\bullet}H^+$ is fast on the EPR time scale, then all anisotropies and the contributions from the two nitroxide forms will be averaged out. As result the experimental splitting between the nitroxide hyperfine components will be approximately equal to a weighted average of the isotropic nitrogen hyperfine coupling constant of the two forms, $R^{\bullet}$ and $R^{\bullet}H^+$, with the weights proportional to the fraction of the radical in each of the forms. For nitroxides in a regime of intermediate and slow exchange this condition is not satisfied. However, at X-band (9.5 GHz) no splitting of low field and central nitrogen hyperfine components is observed unless the nitroxide is per-deuterated. That allowed many researchers to use ex-

TABLE 1. Δa_N Values, pK_a's, and Partition Coefficients (P) for Nitroxides 13−15

no.	a_N, G R•H$^+$	a_N, G R•	Δa_N, G	pK_a	P^a A	P^a B	P^a C
13a	15.09	15.82	0.73	5.5[b]	1.1		0.12
				5.94[cb]			
				5.97[d]			
13b	14.95	15.83	0.88	6.2[b]	0.015		
				6.63[c]			
				6.60[d]			
14a	14.95	15.83	0.88	4.2[b]	5.0		5.1
				4.33[c]			
14b	14.91	15.80	0.89	5.2[c]	0.4		0.6
15a	15.00	15.85	0.85	3.5[b]	27.7		30.6
15b	14.89	15.81	0.92	5.0[c]			
1 (R = Ph)				4.95[8d]	8.8		118.0
1 (R = α-$C_{10}H_7$)				5.0[8d]	11.5		197.0
1 (R = CH_3)				6.2[11]	0.57		2.24
ATI			0.79	6.2[c]	0.14	2.8	1.8
HMI			1.27	4.7[c]	2.0	13.4	6.5

[a] A: measured in octanol−H_2O mixture. B: measured in octanol−NaOH (0.1 M) mixture, ref 9. C: measured in octanol−phosphate buffer solution (0.1 M) mixture, pH = 7.5. [b] Method A (see the Experimental Section). [c] Method B, 50 mM buffer solutions. [d] Method B, 5 mM buffer solutions.

perimentally observed splittings as an approximation of the weighted average of the isotropic nitrogen hyperfine coupling constants even when the fast-exchange condition is not satisfied.[8c]

In the second approach (see the Experimental Section and Table 1, method B), we have modeled experimental spectra as a superposition of two EPR spectra from nitroxides in a fast motion limit. The line shape of each of the nitrogen hyperfine components is approximated by a Voigt function, which is a convolution of Gaussian and Lorentzian shapes.[15] Parameters of each of the two nitroxide spectra as well as the weight of the components were adjusted during Levenberg−Marquardt optimization. Figure 3 shows a representative spectrum of the nitroxide **13a** taken at pH = 6.0 together with results of least-squares simulation with the use of software described earlier.[15,16] A residual of the fit—the difference between experimental and best-fit spectrum (bottom line in the Figure 3)—demonstrates that two nitroxide model fits the experimental spectrum rather well.

Figure 4 shows weighted, according to the double-integrated intensities of the species present, a_N for nitroxides **13a**,**b** as a function of pH as determined from least-squares simulation of slow-exchange X-band EPR spectra. The entire a_N titration curve is fitted exceptionally well to the Henderson−Hasselbalch equation[17]

$$a = \frac{a_b 10^{(pH - pK_a)} + a_a}{1 + 10^{(pH - pK_a)}} \quad (1)$$

where a_a and a_b represent nitrogen hyperfine coupling constants for the acidic and the basic form of the nitroxide, respectively. Upon increasing the pH from 3.0 to 9.0 the hyperfine splitting increased from 15.09 to 15.82 G for **13a** and from 14.94 to 15.83 G for **13b**. pK_a values for nitroxides **13**−**15** determined using eq 1 are summarized in Table 1.

Table 1 shows that pK_a values derived using these two methods are somewhat different (see, for instance, **13a** and **13b**). It is worthwhile to note here that these deviations arise not from the difference in experimental conditions such buffer type and/or buffer concentration (see the Experimental Section, X-Band EPR Titration of Nitroxides **13**−**15**) but rather from approximations during the analysis. It is likely that differential line width

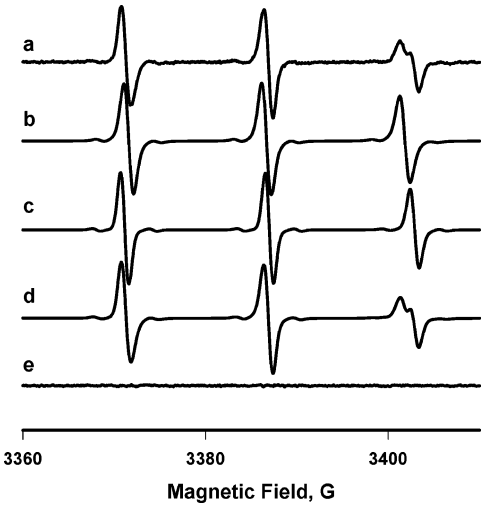

FIGURE 3. (a) Experimental room-temperature 9.5 GHz EPR spectrum of the nitroxide **13a** taken in 50 mM phthalate buffer at pH = 6.0; least-squares simulated spectra of the protonated (b) and nonprotonated (c) forms of the nitroxide; (d) least-squares simulated spectrum of the nitroxide **13a** at pH = 6.0; (e) residual of the fit—the difference between experimental and simulated spectra.

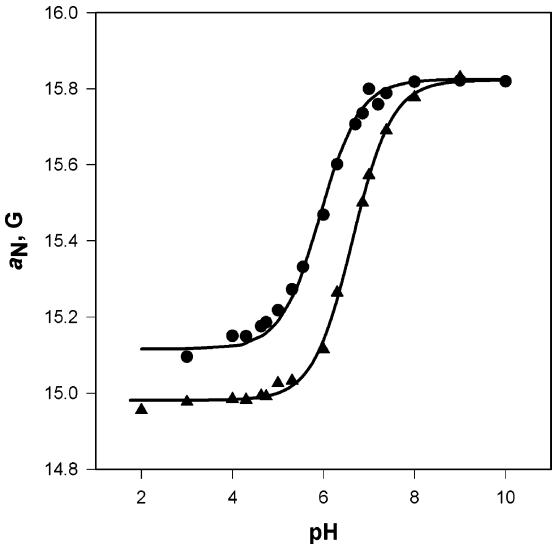

FIGURE 4. Weighted average a_N from least-squares simulation of the two-component X-band EPR spectra as a function of pH. Spectra were taken at room temperature in 50 mM VWR buffer solutions. Corresponding least-squares Henderson−Hasselbalch titration curves are shown as solid lines: filled circles, compound **13a**; filled triangles, **13b**.

(15) Smirnov, A. I.; Belford, R. L. *J. Magn. Reson. A* **1995**, *98*, 65−73.

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(17) Khramtsov, V. V.; Weiner, L. M. In *Imidazoline Nitroxides; Vol. II, Applications*; Volodarsky, L. B., Ed.; CRC Press: Boca Raton, FL, 1988; Chapter 2, p 42.

broadening effects are responsible for some systematic errors in the first method, which is based on measurements of experimental splitting between the low field and the central nitrogen hyperfine components.

For all nitroxides studied in this work the differences between a_a and a_b were found to be $\Delta a_N \approx 0.9$ G, which is similar to Δa_N observed for other spin probes bearing the amidine function.[8b] There are appreciable differences in the a_N values of radicals **13a** and **13b** (~0.14 G, see Figure 4 and Table 1) and radicals **15a** and **15b** (~0.11 G, see Table 1) at pH = 3.0. This indicates that the spin density on the nitrogen atom of the nitroxyl group for these nitroxides is somewhat different for protonated forms but is essentially the same for nonprotonated molecules. We speculate that one of the likely reasons for the difference in hfs's of protonated forms of nitroxides is the interaction of the lone pair of electrons of *exo*-cyclic nitrogen atom of amidine function with delocalized π orbitals of the aromatic ring. For the protonated form of the nitroxide this interaction could result in a partial delocalization of the positive charge over the aromatic π system. This would decrease the electron-withdrawing effect of N3 atom of imidazoline heterocycle and, thus, increase nitrogen hyperfine coupling constant. Other effects such as a conformation of phenylamino moiety, steric perturbation and solvation may contribute to the differences in hfs's observed for the protonated form of nitroxides. We believe that detailed consideration and modeling of these fine effects lay outside the scope of this study.

pK_a values of the nitroxides listed in the Table 1 show a clear trend with the character of the substituents at the *exo*-cyclic nitrogen of the amidine moiety. pK_a values of compounds **13a**,**b** are noticeably higher than those of **14a**,**b** and **15a**,**b** because of a strong electron-donating effect of COO− group that increases the amidine function basicity. Relatively high Δa_N (~ 0.9 G) values for the compounds **13**−**15** demonstrate the high sensitivity of their EPR spectra toward the changes in acidity of the medium. The pK_a values of the compounds **13**−**15** spread from 3.5 to 6.2 pH units, making them attractive pH-sensitive spin probes for EPR application in vivo, including imaging. pH-sensitive unnatural amino acid derivatives **13a**,**b** could be covalently attached to either terminal or side chain amino groups of peptides and, hence, are potential candidates for investigating peptide structure and polarity using SDSL (site-directed spin labeling) EPR methods.[4,18,19]

It is worthwhile to note here that although nitroxides **13a**,**b** are ampholytes, like amino acids, their EPR titration curves do not display the plateau characteristic for zwitterion region of the latter. The EPR titration curves of these nitroxides have only one transition corresponding to the pK_a value of the amidine function (Figure 4, Table 1). The ionization of the carboxylic group does not affect the hfs because this group is located further away from the nitroxide moiety than the amidine group. However, the pK_a of the carboxylic group still could be determined from the EPR spectra if the nitroxide line width measured as a function of pH in the presence of $K_3[Fe(CN)_6]$. Paramagnetic $K_3[Fe(CN)_6]$ is an effective broadening agent for nitroxide radicals in solution.[20] The leading mechanism of magnetic interactions of paramagnetic anions with nitroxides is a spin exchange during bimolecular collisions. Such collisions are affected by a charge on the nitroxide and this leads to different broadening of the EPR spectra from nitroxides in acidic, neutral, and basic forms. Thus, one would expect to observe a progressive change in the line width of EPR spectra when a nitroxide containing both the carboxylic and the amidine groups, such as **13a** or **b**, is titrated in the presence of $K_3[Fe(CN)_6]$. The maximum spectral line width should correspond to a nitroxide form with both carboxylic and amidine groups protonated (low pH, a positively charged spin probe). Figure 5 (filled triangles) shows the peak-to-peak line width of the central EPR spectral component of the nitroxide **13b** measured in the presence of 32 mM of $K_3[Fe(CN)_6]$, as a function of pH. As seen in Figure 5, the line width titration curve reveals a plateau in the pH range from 3 to 4 pH units that is characteristic for zwitterions. Because the experimental EPR spectra line width arises from a superposition of the two nitroxide forms, the pK value cannot be determined from the line width plot directly. Instead, the fraction f

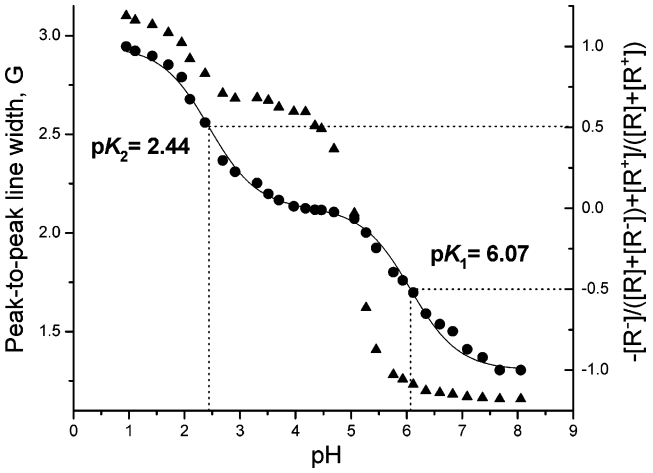

FIGURE 5. Experimental pH dependence of the line width of the EPR spectrum central nitrogen hyperfine component of the nitroxide **13b** in 1 mM Na-phosphate buffer solution in the presence of 32 mM $K_3[Fe(CN)_6]$ (▲) and corresponding calculated values of the term $(-[R^-]/([R] + [R^-]) + [R^+]/([R] + [R^+]))$ (●), where R^- and R^+ represent deprotonated and protonated forms of the nitroxide, respectively, and R represents uncharged zwitterion form. At pH below 4.0, the above term is equal to the fraction f of the radical in protonated form ($f(R^+) = [R^+]/([R] + [R^+])$ and $[R^-] \approx 0$). At pH above 4.0, the term is equal to the fraction of the radical in deprotonated form ($f(R^-) = [R^-]/([R] + [R^-])$ and $[R^+] \approx 0$) taken with sign minus, $-f(R^-)$, to keep similar graphic representation for the experimental (▲) and calculated (●) dependencies. The solid line represents the nonlinear least-squares fit of the data to the Henderson−Hasselbalch equation, yielding pK_1 = 6.07 and pK_2 = 2.44.

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of the nitroxide in the certain ionization state has to be calculated and plotted as a function of pH.

To derive a fraction f of a radical in protonated ($f = [R^+]/([R] + [R^+])$) and deprotonated ($f = [R^-]/([R] + [R^-])$) states from the peak-to-peak EPR line width of the central nitrogen hyperfine component measured in the presence of $K_3[Fe(CN)_6]$, we assumed that: (1) the proton exchange between all the radical forms is slow, (2) the line shape of individual components of nitroxide spectra is well approximated by a Lorentzian function, and (3) the changes in the magnetic field position of the central nitrogen hyperfine component due to different g-factors for the nitroxide forms are negligible compared with the line width. Then, at any pH value the first-derivative shape $I'(H)$ of nitroxide central component can be modeled as a superposition of two Lorentzian lines with two different peak-to-peak widths W_a and W_b

$$I'(H) = A\left[\frac{fW_a(H_0 - H)}{\left(\frac{3}{4}W_a^2 + (H_0 - H)^2\right)^2} + \frac{(1-f)W_b(H_0 - H)}{\left(\frac{3}{4}W_b^2 + (H_0 - H)^2\right)^2}\right] \quad (2)$$

where A is a constant proportional to the total quantity of the nitroxide and H_0 is the common center of the lines. The peak-to-peak width ΔH_{p-p} of such a superposition spectrum could be used to derive the fraction f of the radical in a particular form.

Indeed, when $H = H_0 \pm \Delta H_{p-p}/2$ the function $I'(H)$ is either maximal or minimal and

$$\left.\frac{\partial I'(H)}{\partial H}\right|_{H=H_0\pm\Delta H_{p-p}/2} = 48A\left[\frac{fW_a(\Delta H_{p-p}^2 - W_a^2)}{(3W_a^2 + \Delta H_{p-p}^2)^3} + \frac{(1-f)W_b(\Delta H_{p-p}^2 - W_b^2)}{(3W_b^2 + \Delta H_{p-p}^2)^3}\right] = 0 \quad (3)$$

From this equation f can be easily determined. The experimentally measured line widths of the particular forms $R^{\bullet}H^+$, $R^{\bullet}$ and $R^{\bullet -}$ were used in the calculations for the individual Lorentzian lines. We assumed that the line width observed at pH = 1.0 (3.1 G) corresponds to the $R^{\bullet}H^+$ form with both carboxylic and amidine functions protonated, while the line width at pH = 8.0 (1.16 G) corresponds to the deprotonated form, $R^{\bullet -}$. The peak-to-peak line width at pH = 4.0 (2.64 G) was assigned to the zwitterion form, $R^{\bullet}$ (pH region from 3 to 4 pH units). Using these values for W_a and W_b, the fraction f was calculated from the line width data using eq 3 and plotted in Figure 5 (filled circles) as a function of pH. Titration plots, depicted in Figure 5, similar to conventional amino acids titration curves, show the regions corresponding ionization of carboxylic and amidine groups as well as the plateau of the zwitterion form. pK_a values of carboxylic and amidine groups were determined from nonlinear least-squares fit of the data to the Henderson–Hasselbalch equation. From this data, the pK_a value for the amidine function (pK_a = 6.07) is rather close to pK_a = 6.60 obtained from a_N titration plot (see Table 1). The difference is likely due to approximation of the EPR line shape by a Lorentzian function. The pK_a value for carboxylic group determined using this approach (pK_a = 2.4) agrees well with the literature data for terminal carboxylic groups of most amino acids (typically, pK_a = 1.77–2.58 units).[cf.21]

Partition coefficients (P) are important characteristics of a compound lipophilicity.[22] Partition coefficients of new nitroxides were determined and are listed in the Table 1 along with the corresponding data for widely used pH-sensitive spin probes **1** (R = Ph, α-naphthyl, CH_3), ATI (4-amino-2,2,5,5-tetramethyl-3-imidazoline-1-oxyl), and HMI (2,2,3,4,5,5-hexamethylimidazolidine 1-oxyl). The compounds synthesized in this work reveal a wide range of lipophilicities (viz. from 0.015 to 27) that makes them suitable pH-probes in varieties of heterogeneous environments, particularly in biological systems (e.g., blood, cell membranes).

Conclusion

We have synthesized a series of new nitroxides using the approach based on the alkylation of diamagnetic 4-R-amino-1,2,2,5,5-pentamethyl-3-imidazolines. The approach allows one to overcome difficulties resulting from the reduced reactivity of the amidine function in nitroxides toward alkylation. In contrast to nitroxide amidine **1**, diamagnetic amidines **2** are available using both isocyanates and isothiocyanates as addends in 1,3-dipolar cycloaddition reactions.[11] All of the nitroxides synthesized were characterized with X-band EPR spectroscopy and were shown to have pH-dependent slow-exchange two-component EPR spectra. The pK_a values of the nitroxides determined from EPR spectra were found to lie in the range from 3.5 to 6.6 pH units. This pK_a range is particularly suitable for studies of biological systems. The approach for the determination of pK_a's of groups remote from the nitroxide moiety based on the nitroxide spectral line width broadening by paramagnetic $K_3[Fe(CN)_6]$, measured as a function of pH, is proposed. Keeping in mind the possible areas of application, the partition coefficients of new nitroxides were determined as a measure of their lipophilicity. The synthetic approach described here noticeably extends the conceivable set of nitroxides with pH-dependent EPR spectra and tunable variable properties (Δa_N, pK_a, lipophilicity, binding capabilities), thus providing the basis for further advances in application of pH-sensitive spin probes.

Experimental Section

IR spectra were recorded in KBr pellets (the concentration was 0.25%, the pellet sickness was 1 mm) and CCl_4 solutions. UV spectra were recorded in EtOH solutions. ^{1}H NMR spectra were recorded at 400.136 and 200.132 MHz. ^{13}C NMR spectra were recorded at 100.614 and 50.323 MHz. Solvent signal was used as internal standard. $CDCl_3$ (δ_H = 7.24 ppm, δ_C = 76.69 ppm) was used as a solvent unless otherwise noted. The proton-coupled ^{13}C NMR spectrum of **8b** in acetone-d_6 was acquired at 100.614 MHz. Single crystals of compound **15a** suitable for diffraction study were obtained from slow recrystallization from pentane at ambient temperature over a period of several days. X-band (9.5 GHz) EPR spectra were recorded with samples placed into 100 μL quartz capillaries and in PTFE capillaries (0.81 × 1.12 mm). The PTFE capillaries were

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folded twice and inserted into standard 3 by 4 mm quartz EPR tube. Compounds **2a**,**b** were synthesized according to published procedures.[11] For calibration of pH-sensitive nitroxides the following standard buffer solutions were used: (a) potassium tetraoxalate at pH = 1.68; (b) hydrochloric acid/glycine at pH = 2.0; (c) potassium hydrogen phthalate/hydrochloric acid at pH = 3.0; (d) potassium hydrogen phthalate at pH 4.0; (e) acetic acid/sodium acetate at pH =4 .63; (f) potassium hydrogen phthalate/sodium hydroxide at pH = 5.0; (g) potassium hydrogen phthalate/sodium hydroxide at pH = 6.0; (h) sodium phosphate/potassium phosphate at pH = 6.86, 7.0, 7.38, and 8.0; (i) boric acid/potassium chloride/sodium hydroxide at pH = 9.0; (j) sodium borate at pH = 9.18; (k) sodium bicarbonate/ sodium carbonate at pH = 10. All standard buffer solutions were colorless, contained 0.5% of a biocide Dowicide A (sodium *o*-phenylphenate tetrahydrate), and at 50 mM concentration except the pH 6.86 phthalate buffer which was 25 mM. These reference buffer solutions are specified to be accurate to at least ±0.02 pH at 25 °C and deviate from the specified values by not more than ±0.05 pH at 40 °C. Some measurements were carried out with freshly prepared potassium hydrogen phthalate/sodium hydroxide (pH = 4.3; 4.74; 5.31) and sodium phosphate/sodium hydroxide (pH = 5.0; 6.3) buffer solutions. pH measurements were performed with a digital pH meter.

Alkylation of 4-R-amino-1,2,2,5,5-pentamethyl-3-imidazolines 2a,b with $BrCH_2COOEt$ (General Procedure). The mixture of **2** (0.03 mol), $BrCH_2COOEt$ (0.1 mol), and K_2CO_3 (0.75 mol) in 50 mL of CH_3CN was placed into a round-bottomed flask, fitted with $CaCl_2$ tube and condenser, and refluxed for 80 h in the case of **2a** and 15 h in the case of **2b**. The reaction was monitored by chromatography on TLC plates (**2a**: Al_2O_3, hexane/benzene/ THF, 2:2:1; **2b**: Al_2O_3, hexane/EtOAc, 1:1, treatment in iodine vapors). After the reaction was completed, the inorganic precipitate was filtered off, solvent was removed under reduced pressure, and the residue was separated by column chromatography (Al_2O_3, hexane/ether, 1:1 for **2a**; Al_2O_3, petroleum ether/EtOAc, 10:7 for **2b**) to give **7a,b**. **[(1,2,2,5,5-Pentamethyl-2,5-dihydro-1*H*-imidazol-4-yl)-phenyl-amino]acetic acid ethyl ester 7a** was isolated as a white powder (6.9 g of **2a** afforded 8.0 g of **7a**, 84%): mp 176−178 °C (hexane/EtOAc, 1:3); IR (KBr) 1750 (C=O), 1615 (C=N); UV (C_2H_5OH), λ_{max} (lg ϵ) 210 (4.10), 247 (3.60); ^{1}H NMR ($CDCl_3$), δ = 1.00 (s, 6H, $2CH_3$), 1.29 (t, 3H, CH_2CH_3), 1.59 (s, 6H, $2CH_3$), 2.18 (s, 3H, N-CH_3), 4.29 (q, 2H, CH_2CH_3), 5.01 (s, 2H, CH_2), 7.24−7.45 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$), δ = 14.0, 23.6, 25.9, 25.4, 59.6, 62.7, 66.4, 80.4, 128.4, 129.8, 130.4, 139.9, 166.4, 168.3. Anal. Calcd for $C_{18}H_{27}N_3O_2$·HBr: C, 54.27; H, 7.03; N, 10.55. Found: C, 54.37; H, 7.35; N, 10.37. **[(1,2,2,5,5-Pentamethyl-2,5-dihydro-1*H*-imidazol-4-yl)methylamino]-acetic acid ethyl ester 7b** was isolated as a pale brown viscous oil (5.1 g of **2b** afforded 5.3 g of **7b**, 69%, crude product): IR (CCl_4) 1742 (C=O), 1658 (C=N); ^{1}H NMR ($CDCl_3$), δ = 1.25 (t, 3H, CH_2CH_3), 1.40, 1.48 (both s, 6H, $2CH_3$), 2.24 (s, 3H, N(1)-CH_3), 3.29 (s, 3H, N-CH_3), 4.22 (q, 2H, CH_2CH_3).

Synthesis of *N*-(1,2,2,5,5-Pentamethyl-3-imidazolin-4-yl)-*N*-R-amino Acetic Acid *N*-Methyl Amides 8 (General Procedure). A solution of ester **7** (0.02 mol) in 2 M CH_3NH_2 ethanol solution (50 mL) was allowed to stay at ambient temperature for a few days. After the reaction was complete (control by TLC, **7a**: Al_2O_3, petroleum ether/EtOAc, 1:1, treatment in iodine vapors, R_f = 0.79 for **7a**, R_f = 0.3 for **8a**; **7b**: SiO_2, $CHCl_3$ + 1% CH_3OH), the solvent was removed under reduced pressure, the residue was dissolved in H_2O (25 mL) and extracted with $CHCl_3$ (5 × 20 mL), and the chloroform solution was extracted with 3% HCl (5 × 15 mL). The acidic solution was extracted with $CHCl_3$ (2 × 15 mL, chloroform extract was discarded), neutralized with $NaHCO_3$, and then extracted with $CHCl_3$ (2 × 15 mL), the extract was dried over $MgSO_4$, the solvent was removed under reduced pressure, and the chromatographically pure residue solidified upon standing to give amides **8a**,**b**. ***N*-Methyl-2-[(1,2,2,5,5-pentamethyl-2,5-dihydro-1*H*-imidazol-4-yl)phenylamino]acetamide 8a** was isolated as colorless crystals (6.3 g of **7a** afforded 4.6 g of **8a**, 76%): mp 81−82 °C (hexane); IR (KBr) 1688 (C=O), 1606 (C=N), 3210 (N-H); UV (C_2H_5OH), λ_{max} (lg ϵ) 205 (4.24), 246 (3.64); ^{1}H NMR ($CDCl_3$), δ = 0.86, 1.28 (both s, 6H, $2CH_3$), 2.19 (s, 3H, N(1)-CH_3), 2.79 (d, 3H, NHCH_3), 4.24 (s, 2H, CH_2), 7.18−7.33 (m, 5H, Ph), 8.10 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6), δ = 24.1, 27.1, 25.6, 56.2, 65.7, 83.5. Anal. Calcd for $C_{17}H_{26}N_4O$: C, 67.55; H, 8.61; N, 18.54. Found: C, 67.43; H, 8.55; N, 18.44. ***N*-Methyl-2-[(1,2,2,5,5-pentamethyl-2,5-dihydro-1*H*-imidazol-4-yl)methylamino]acetamide 8b** was isolated as colorless crystals (4.8 g of **7b** afforded 4.4 g of **8b**, 91%): mp 105−107 °C (hexane); IR (KBr) 1650 (C=O), 1601 (C=N), 3401 (N-H); UV (C_2H_5OH), λ_{max} (lg ϵ) 213 (3.86); ^{1}H NMR ($CDCl_3$), δ = 1.22, 1.34 (both s, 6H, $2CH_3$), 2.26 (s, 3H, N(1)-CH_3), 3.61 (s, 3H, N-CH_3), 2.74 (d, 3H, NHCH_3), 3.98 (s, 2H, CH_2), 7.45 (br s, 1H, NH). Anal. Calcd for $C_{12}H_{24}N_4O$·H_2O: C, 55.81; H, 10.08; N, 21.70. Found: C, 55.97; H, 10.10; N, 21.53.

2-[(1,2,2,5,5-Pentamethyl-2,5-dihydro-1*H*-imidazol-4-yl)phenylamino]acetamide 9. NH_4OH (30%, 25 mL) was added to the solution of ester **7a** (3.17 g, 0.01 mol) in CH_3OH (10 mL) and allowed to stay at ambient temperature for a few days. The course of reaction was monitored by chromatography on TLC plates (Al_2O_3, hexane/ether, 1:1). The further treatment was performed as in the case of amide **8**. The residue, obtained after solvent evaporation, solidified after trituration with hexane/ether (1:1) mixture to give product **9** as a white powder (2.6 g, 92%): mp 138−140 °C (hexane/EtOAc, 1:1); IR (KBr) 1700 (C=O), 1578 (C=N), 3320, 3125 (NH_2); UV (C_2H_5OH), λ_{max} (lg ϵ) 208 (4.22), 245 (3.79); ^{1}H NMR ($CDCl_3$), δ = 0.69, 1.29 (both s, 6H, $2CH_3$), 2.21 (s, 3H, N(1)−CH_3), 4.28 (s, 2H, CH_2), 7.25−7.32 (m, 5H, Ph), 5.42 (br s, 1H, NH, $CONH_2$), 8.00 (br s, 1H, NH, $CONH_2$). Anal. Calcd for $C_{16}H_{24}N_4O$: C, 66.67; H, 8.33; N, 19.45. Found: C, 66.86; H, 8.42; N, 19.17.

[(1,2,2,5,5-Pentamethyl-2,5-dihydro-1*H*-imidazol-4-yl)-phenylamino]acetic Acid 10 Hydrochloride. A solution of ester **7a** (0.29 g, 0.0009 mol) and NaOH (0.036 g, 0.0009 mol) in 5 mL of 1/10 H_2O/CH_3OH mixture was heated on a water bath at 45 °C. The course of the reaction was monitored by chromatography on TLC plates (Al_2O_3, hexane/ether, 1:1). The reaction mixture was diluted with H_2O (10 mL) and extracted with $CHCl_3$ (1 × 5 mL, chloroform extract was discarded). The water phase, neutralized with 1% H_2SO_4, was extracted with $CHCl_3$ (4 × 5 mL), the extract was dried over $MgSO_4$, the solvent was removed in a vacuum, and the residue solidified to give chromatographically pure **10** in 70% yield. The stream of dry HCl gas was passed through the solution of amino acetic acid **10** (0,58 g, 0,002 mol) in the ether/CH_2Cl_2 (5:1) mixture. The light yellow precipitate of the hydrochloric salt was collected on the filter; the mother solution was evaporated under reduced pressure to give, after trituration with ether, an additional amount of hydrochloric salt. The crude product was recrystallized from CH_3OH/ether (1:4) to give **10** hydrochloride as white crystals: mp 133−134 °C; IR (KBr) 1747 (C=O), 1658 (C=N); UV (C_2H_5OH), λ_{max} (lg ϵ) 207 (4.17); ^{1}H NMR (DMSO-d_6), δ = 1.06 (br s, 6H, $2CH_3$), 1.41 (s, 6H, $2CH_3$), 2.24 (s, 3H, N−CH_3), 4.60 (s, 2H, CH_2), 7.53 (s, 5H, Ph); ^{13}C NMR (DMSO-d_6), δ = 23.1, 25.5, 25.8, 57.5, 66.7, 80.4, 128.7, 129.6, 130.0, 139.9, 167.1, 167.8. Anal. Calcd for $C_{16}H_{23}N_3O_2$·2HCl·$2H_2O$: C, 48.25; H, 7.28; N, 10.55; Cl, 17.81. Found: C, 48.32; H, 7.22; N, 10.60; Cl, 17.60.

Synthesis of Nitroxides 14a,b (General Procedure). Na_2WO_4 (0.1 g, 0.00034 mol) and H_2O_2 (3 mL) were added to the solution of *N*-methylamide **8** (0.002 mol) in CH_3OH (8 mL). The resulting mixture was heated on a water bath at temperatures not exceeding 50 °C. The course of reaction was monitored by chromatography on TLC plates (**14a**: SiO_2, $CHCl_3$ + 1% CH_3OH; **14b**: Al_2O_3, petroleum ether/EtOAc, 1:1). The reaction mixture was diluted with H_2O (15 mL) and

extracted with $CHCl_3$ (3 × 10 mL). The chloroform extract was dried over $MgSO_4$, the solvent was removed under reduced pressure, and the chromatographically pure **14** solidified after evaporation. **2-[(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-4-yl)phenylamino]-*N*-methylacetamide 14a** was isolated as canary crystals (0.6 g of **8a** affords 0.5 g of **14a**, 85%): mp 148−150 °C (EtOAc/hexane, 1:15); IR (KBr) 1680 (C=O), 1580 (C=N), 3245 (NH); UV (C_2H_5OH), λ_{max} (lg ϵ) 209 (4.21), 243 (3.88). Anal. Calcd for $C_{16}H_{23}N_4O_2$: C, 63.36; H, 7.59; N, 18.48. Found: C, 62.96; H, 7.99; N, 18.35. **2-[(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-4-yl)-methylamino]-*N*-methylacetamide 14b** was isolated as yellow plates (0.48 g of **8b** affords 0.24 g of **14b**, 50%): mp 59−61 °C (hexane/EtOAc, 1:2); IR (KBr) 1671 (C=O), 1595 (C=N), 3451 (NH); UV (C_2H_5OH), λ_{max} (lg ϵ) 217 (4.09). Anal. Calcd for $C_{11}H_{21}N_4O_2 \cdot H_2O$: C, 50.96; H, 8.88; N, 21.62. Found: C, 50.88; H, 8.90; N, 21.81.

***N*-(2,2,5,5-Tetramethyl-3-imidazolin-1-oxyl-4-yl)-*N*-R-amino Acetic Acids 13a,b (General Procedure).** KOH (0.06 g) was added to the solution of *N*-methylamide **14** (0.005 mol) in 50% aqueous methanol (6 mL), and the resulting mixture was heated on the oil bath at 80 °C. The reaction was monitored by chromatography on TLC plates (**13a**: SiO_2, $CHCl_3$ + 5% CH_3OH; **13b**: Al_2O_3, petroleum ether/EtOAc, 1:1). CH_3OH was removed under reduced pressure, and the residue was neutralized with 1% H_2SO_4 and extracted with $CHCl_3$ (7 × 10 mL). The chloroform extract was dried over $MgSO_4$, and amino acetic acids **13a**,**b** were obtained after the solvent evaporation as the solid products. **[(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-4-yl)phenylamino]acetic acid 13a** was isolated as pale yellow crystals (1.52 g of **14a** afforded 0.9 g of **13a**, 68%): mp 148−150 °C dec (hexane/EtOAc, 4:3); IR (KBr) 1740 (C=O), 1591 (COO^-), 1649 (C=N); UV (C_2H_5OH), λ_{max} (lg ϵ) 206 (4.22), 243 (3.81); Anal. Calcd for $C_{15}H_{20}N_3O_3$: C, 62.07; H, 6.90; N, 14.48. Found: C, 62.20; H, 7.11; N, 14.27. **[(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-4-yl)-methyl-amino]-acetic acid 13b** is isolated as pale yellow crystals (1.2 g of **14b** affords 0.74 g of **13b**, 65%). mp 136−137 °C (hexane/EtOAc, 1:5); IR (KBr): 1598 (COO^-), 1667 (C=N); UV (C_2H_5OH), λ_{max} (lg ϵ): 215 (4.06); Anal. Calcd for $C_{10}H_{18}N_3O_3$: C, 52.63; H, 7.89; N, 18.42. Found: C, 52.77; H, 7.55; N, 18.32.

***N*-(2,2,5,5-Tetramethyl-3-imidazolin-1-oxyl-4-yl)-*N*-R-amino Acetic Acid Methyl Ester 15a,b (General Procedure).** A stream of the dry HCl gas was passed into the methanol solution of amino acetic acid **13** (0.0034 mol) for 15−20 min. The course of the reaction was monitored by chromatography on TLC plates (Al_2O_3, petroleum ether/EtOAc, 1:1). After the reaction was completed, the solvent was removed under vacuum, the solid residue, containing, along with nitroxide **15**, the corresponding hydroxylamine derivative **16a** was dissolved in $CHCl_3$, and PbO_2 (0.007 mol) was added with vigorous stirring. After the hydroxylamine derivative vanished (control by TLC analysis), the lead oxides were filtered off, the solvent was removed under reduced pressure, and the residue was separated on preparative TLC plate (Al_2O_3, petroleum ether/EtOAc, 1:1). **[(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-4-yl)phenylamino]acetic acid methyl ester 15a** was isolated as yellow crystals (0.98 g of **13a** afforded 0.9 g of **15a**, 92%): mp 106−107 °C (pentane); IR (KBr) 1750 (C=O), 1588 (C=N); UV (C_2H_5OH), λ_{max} (lg ϵ) 203 (4.18), 242 (3.78). Anal. Calcd for $C_{16}H_{22}N_3O_3 \cdot 1.5H_2O$: C, 58.01; H, 7.55; N, 12.68. Found: C, 58.28; H, 7.78; N, 12.60. **[(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazol-4-yl)-methyl-amino]-acetic acid methyl ester 15b** is isolated as light yellow crystals (0.77 g of **13b** affords 0.74 g of **15b**, 90%). mp 89−91 °C (hexane/EtOAc, 5:1); IR (KBr): 1750 (C=O), 1588 (C=N); Anal. Calcd for $C_{11}H_{20}N_3O_3$: C, 54.54; H, 8.26; N, 17.35. Found: C, 54.28; H, 7.97; N, 17.24.

X-Band EPR Titration of Nitroxides 13−15. Method A. Titration of nitroxides was performed similarly to the published procedure.[8b] Solutions of nitroxides in a mixture of 5 mM sodium phosphate buffer and 0.1 mM DTPA with the final concentration of the nitroxides of ca. 0.1 mM were prepared. The resulting solutions were titrated with HCl or NaOH solutions to the required pH using a digital pH-meter equipped with a glass electrode. The accuracy of pH value measurements was ±0.05 pH units. The EPR spectra of the samples were recorded in a 200 μL quartz EPR flat cell. Spectrometer settings were as follows: modulation amplitude, 0.8 G; scan width, 50 G; sweep time, 200 s; time constant, 200 ms; incident microwave power, 10 mW. Hyperfine splitting constants were measured as a distance between the low field and the central nitrogen hyperfine components of the EPR spectra and are accurate to 0.02 G. To determine the pK_a values of the nitroxides, the experimental curves of the hfs were fitted to the Henderson−Hasselbalch eq 1.

Method B. A series of the nitroxide solutions in standard 50 mM buffer solutions in a pH range from 2.0 to 10.0 with the final concentration of nitroxide ca. 0.1 mM were prepared. The room-temperature X-band EPR spectra were measured. Hfs constants of the nitroxides (a_N) were determined from least-squares simulation of slow-exchange EPR spectra with the software described.[15,16] To determine the pK_a values the experimental a_N were fitted to the Henderson−Hasselbalch eq 1. Typical spectrometer settings were as follows: modulation amplitude, 1 G; time constant, 32 ms; microwave power, 3.1 mW; sweep time, 60 s; scan width, 80 G. To be assured that the spectrometer settings did not affect the results of titration, the experiments (for nitroxide **14a**) were repeated with the following combinations of modulation amplitude and microwave power (pK_a values obtained are given in parentheses): 1 G, 3.1 mW (4.33); 0.2 G, 3.1 mW (4.36); 1 G, 0.4 mW (4.37); 0.8 G, 0.4 mW (4.37); 0.5 G, 0.4 mW (4.34); 0.2 G, 0.4 mW (4.37).

To elucidate the role of molarity of the buffer solutions, the titrations of nitroxides **13a** and **13b** were repeated in 5 mM buffer solutions. Deionized water (pH=4.66) was used as a dilutant. The final pH values of the samples were measured with three-point calibrated digital pH-meter and corrected values were used in an a_N−pH plot (see Table 1).

X-Band EPR Titration of the Nitroxide 13b in the Presence of $K_3[Fe(CN)_6]$. $K_3[Fe(CN)_6]$ (32 mM) was added to 0.5 mM solution of the nitroxide **13b** in 1 mM phosphate buffer. The resulting solution was titrated with solutions of HCl or KOH to the required pH value. The peak-to-peak line width of the central nitrogen hyperfine component of the spectrum was measured as a function of pH. Spectrometer settings were the same as in X-band EPR titration experiments, method A.

Partition Coefficients. The partition coefficients were determined with a "shake flask" method. To ensure accuracy, the measurements were repeated twice with two different nitroxide concentrations, 0.5 and 0.7 mM. Preliminary tests showed that shaking the solution for more than 5 min did not influence the reproducibility of the results. Typically, a solid sample of a nitroxide was placed in a vial containing 1 mL of H_2O (or phosphate buffer solution at pH = 7.5) and octanol (1 mL). The mixture was shaken vigorously for 5 min and allowed to stand for 4 h until separation of the phases was completed. The partition coefficients were determined from EPR spectra of the nitroxides as octanol/water phase integral intensities ratio. The spectrometer settings were as follows: time constant, 100 ms; sweep time, 160 s; modulation amplitude, 0.8 G; microwave power, 12.5 mW. The solutions of 2,2,6,6-tetramethylpiperidine 1-oxyl (Tempo) and N-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl)-*N*-phenylamino acetic acid **13a** of known concentration in phosphate buffer solution, water, and octanol, were used as the references. Error in the partition coefficients was estimated up to 5%.

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Supporting Information Available: Crystallographic information file (CIF) and ORTEP for compound **15a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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