

A new series of lipophilic pH-sensitive spin probes

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A series of new lipophilic spin probes with pH-dependent ESR spectra was synthesized: cyano-substituted enamino ketones, *viz.*, derivatives of imidazolidine nitroxyl radicals. The use of these compounds makes it possible to measure the acidity of the medium in the pH 6–12 range.

Key words: nitroxyl radicals, nitroxides, enamino ketones, nucleophilic substitution, spin probes, pH measurement using ESR spectroscopy.

The use of stable nitroxyl radicals with ESR spectra dependent on pH of the medium has become a powerful method for studying acidity of the medium and proton transfer in various biological systems using ESR spectroscopy.^{1–4} A nitroxyl radical can be used as a pH-sensitive probe when its molecule contains a fragment (at a sufficiently short distance from the nitroxyl group) capable of reversible protonation–deprotonation with the *pK* value close to the measured pH interval of the medium. Presently, many pH-sensitive spin probes, which enable the determination of acidity of the medium in the pH 0–7 range, have been synthesized. However, only some of them are appropriate^{3,4} for using in the area most interesting for biology: near pH 7.0. Such spin probes are, first, the 4-R-amino-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-imidazole-1-oxyl derivatives.^{3,4} The syntheses of similar compounds are problematic, because the closely arranged nitroxyl group decreases substantially (by two–three units) the *pK* value of the fragment capable of reversible protonation–deprotonation.⁵ Therefore, a nitroxyl radical molecule should contain a strongly basic group to measure acidity in the pH 6–8 range, which is of greatest interest for the molecular biology and biophysics. This makes it possible to determine⁵ the acidity of the medium to pH 7. It is noteworthy that higher pH values are inaccessible, as a rule, even for measuring by paramagnetic amidines. In addition, the amidine group itself imparts significant hydrophilicity to the spin probe, while some

biological applications are based on the use of lipophilic probes (for example, studies of the surface properties of proteins and membranes²). Another necessary property of a spin probe is its sufficiently high solubility for the incorporation into the object under study, although the working concentration of the probe is rather low.

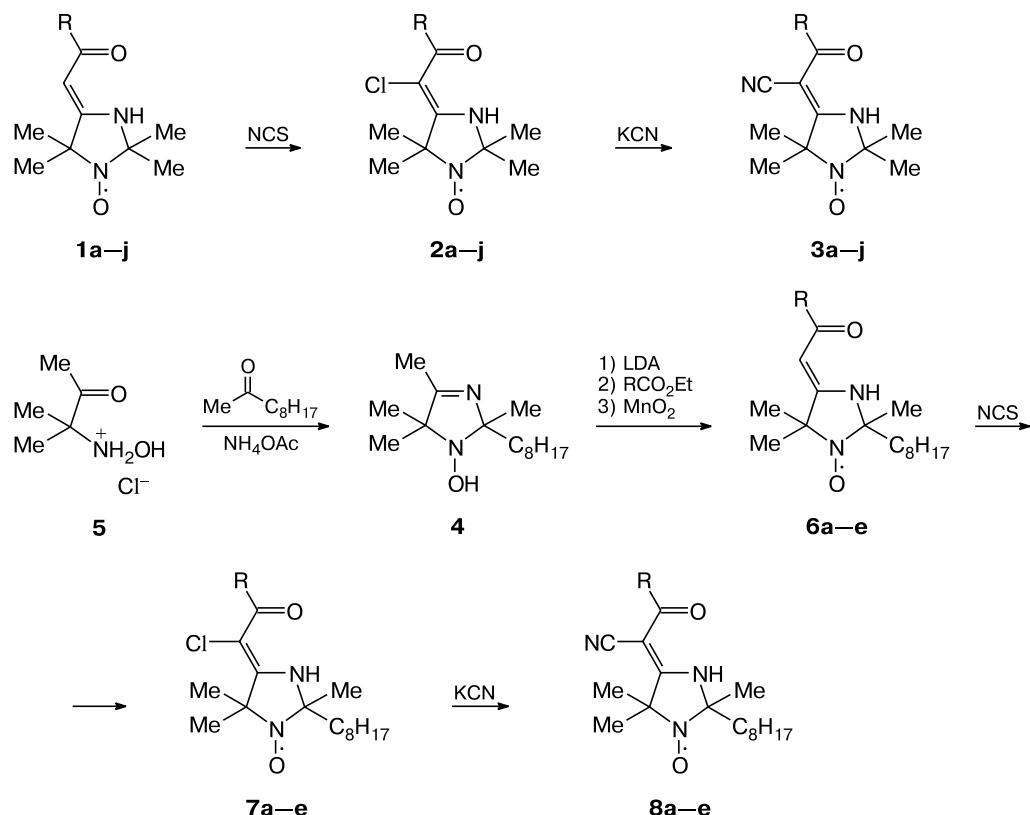
The aim of this work is to synthesize new spin probes with pH-dependent ESR spectra, which can be used for measuring acidity of the medium in the region of pH > 7 and are simultaneously characterized by high lipophilicity.

Results and Discussion

We have recently shown⁶ that the chlorination of paramagnetic enamino ketones **1a–h** affords monochloro derivatives **2a–h**, which can readily be transformed into nitriles **3a–h** (Scheme 1). The latter are good complex-forming agents^{7,8} partially due to the easiness of deprotonation of compounds **3** because of the electron-withdrawing nitrile group. Thus, the electron-withdrawing effect of the nitroxyl group, in this case, also decreases the *pK* value, bringing it to the neutral region.

On the one hand, the delocalization of a positive charge in the protonated group or a negative charge in the deprotonated group stabilizes them (increases basicity or acidity, respectively). On the other hand, it decreases the difference in the ESR spectra of the protonated and deprotonated forms, although the value of this effect can-

Scheme 1



1–3: R = H (**a**), Me (**b**), Bu^t (**c**), CO₂Me (**d**), CF₃ (**e**), OEt (**f**), Ph (**g**), 4-pyridyl (**h**), n-C₁₇H₃₅ (**i**), (CH₂)₈CO₂H (**j**)
6–8: R = H (**a**), Me (**b**), Bu^t (**c**), CF₃ (**d**), Ph (**e**)

not be predicted quantitatively. Evidently, when this difference in the ESR spectra is low, the use of these nitroxides as pH-spin probes becomes difficult or impossible, especially when real biological objects are studied (due to an increase in the linewidth in the ESR spectra). However, all pH-spin probes, which can work in the pH 5–8 interval, necessarily contain groups with charge delocalization, and the difference in HFC constants of the protonated and deprotonated forms ($\Delta\alpha_N$) of these compounds rarely exceed 1 G. Radicals with $\Delta\alpha_N > 0.4$ G are considered³ as appropriate for practical use. The measurement of pK values of some synthesized enamino ketones **3a–h** (Table 1) showed that they can be used as pH-spin probes in the pH 7–11 range, which is inaccessible for the previously synthesized pH probes.

A disadvantage of these compounds is a very low solubility in both water and usual organic solvents. Lipophilicity of such compounds can be increased by the modification of the structure, introducing a lipophilic substituent into their molecules. The simplest approach to the solution of this problem is to use enamino ketones **1** (containing the long alkyl chain as the substituent at the carbonyl group) as precursors of nitriles **3**. In fact, the reac-

tion of compounds **1i,j** with *N*-chlorosuccinimide (NCS) affords chloro derivatives **2i,j**, which are further transformed into the corresponding nitriles **3i,j** by the reaction with KCN (see Scheme 1).

Obviously, this approach enables one to enhance significantly the lipophilicity of the probe. However, it cannot provide the synthesis of compounds that differ substantially in pK. Therefore, to increase the lipophilicity of the target cyano-substituted enamino ketones, the lipophilic fragment can be introduced into a molecule of the starting 3-imidazoline derivative **4**. Compound **4** was synthesized by the reaction of hydroxylamino ketone hydrochloride **5** with methyl octyl ketone in the presence of ammonium acetate (*cf.* Ref. 9). Then imidazoline **4** was introduced into reactions with a broad scope of esters in the presence of LDA, and enamino ketones **6a–e** were obtained after oxidation (*cf.* Refs. 10 and 11). Compounds **6a–e** were further transformed into the corresponding nitriles **8a–e** (*cf.* Ref. 6). It is of interest that in the IR spectrum of compound **8d** both the nitrile (2201 and 2220 cm⁻¹) and carbonyl group (1650 and 1634 cm⁻¹) exhibit two bands of vibrations, which is likely related to E–Z-isomerism relatively to the exocyclic C=C bond.

Table 1. Data of titration of enamino ketones **3b,d–f,h–j** and **8a–e**

Compound	pK ^a (solvent)	Approximation of pK to water	Δa _N /G
3b	9.7 (H ₂ O)	—	0.71
3d	6.5 (H ₂ O)	—	0.69
3e	6.6 (H ₂ O)	—	0.80
3f	10.1 (H ₂ O)	—	0.72
3h	8.1 (H ₂ O)	—	0.75
3i	9.1 (acetone (50%)+H ₂ O (50%))	9.6 ^b	0.82
3j	10.1 (H ₂ O)	—	0.85
8a	7.2 (H ₂ O), 6.85 (DMSO (10%)+H ₂ O (90%))	6.9 ^c	0.73
8b	8.6 (acetone (50%)+H ₂ O (50%))	9.1 ^b	0.86
8c	10.8 (acetone (50%)+H ₂ O (50%))	11.3 ^b	1.06
8d	7.7 (H ₂ O), 7.45 (DMSO (10%)+H ₂ O (90%))	7.5 ^c	0.40
8e	8.8 (acetone (50%)+H ₂ O (50%)), 9.25 (DMSO (10%)+H ₂ O (90%))	9.3 ^{b,c}	0.90

^a The measurement error for aqueous solutions, 10% solutions of DMSO, and 50% solutions of acetone is 0.1, 0.2, and 0.3 pH units, respectively.

^b According to the data of titration in aqueous acetone.

^c According to the data of titration in aqueous DMSO.

We have previously⁶ made similar observations for compounds **2e,h**.

The results of measuring the pK values of compounds **8** are presented in Table 1 and show that it is difficult to predict the pK value on the basis of the character of the substituent. It is noteworthy that all synthesized cyano-substituted enamino ketones are highly soluble in nonpolar organic solvents. This provides their use as lipophilic spin probes with pH-dependent ESR spectra. The solubility of some of them in water is so low that they can be titrated only in the water-organic phase.

Thus, a series of new lipophilic pH-spin probes, which enable measuring acidity of the medium using ESR spectroscopy in the pH 6–12 range inaccessible for measuring by the previously synthesized pH probes, was synthesized.

Experimental

IR spectra were recorded in KBr pellets (concentration 0.25%, thickness 1 mm) on a Bruker IFS-66 spectrometer. UV spectra were obtained on a Specord M-40 spectrometer for solutions in EtOH. Enamino ketones **1i,j** were synthesized accord-

ing to a previously described procedure.¹⁰ Compounds **2a–h** and **3a–h** were synthesized using a known procedure.⁶ Medical grade ether (Et₂O), used for the synthesis of LDA, was dried successively over CaCl₂ and sodium metal. Chloroform (technical grade) and CCl₄ were dried over CaCl₂ and distilled. Dimethyl sulfoxide was dried over NaOH and distilled *in vacuo* over NaOH. Hexane (pure grade) was used without additional purification. Chlorination was carried out using NCS (Fluka). Manganese(IV) oxide (catalyst, TU 6-09-01-718-87 trade mark) was used. Alumina (neutral, II activity grade) was used for chromatographic purification of the synthesized compounds. In all cases, solutions were concentrated in a vacuum of a water-aspirator pump. The yields and characteristics of the synthesized compounds are presented in Table 2.

Procedure of titration. Solutions of radicals in water (concentration $(1-3) \cdot 10^{-4}$ mol L⁻¹) were titrated with solutions of HCl or KOH at 23 °C until a necessary pH value was achieved. The pH value was measured on an OP-205/1 pH-meter (Hungary) with the 0.05 accuracy. ESR spectra in the X-range were measured on an ER-200D-SRC spectrometer (Bruker) in a flat tube. The HFC constant a_N was determined as a distance between the first and second components of the triplet. The accuracy of a_N measurements depended on the magnetic field stability of the instrument (20 mG h⁻¹), being 0.02 G. Radicals insoluble in water were titrated in aqueous DMSO and aqueous acetone. The pH value in mixtures was determined after the electrode was calibrated. The calibration showed that the calibration curve of the electrode retained linearity in the mixtures studied. In order to correlate the pK values obtained for radicals dissolved in different media, the pK were approximated to water. The approximation accepted that the pK value of any weak acid changes, on going from distilled water to other solvents, by the same value, which is determined as a change in the pK of the second step of H₃PO₄ dissociation. The correction value for the pK of the radical on going from a mixture to water was taken as the difference between the pK values obtained by titration of a phosphate buffer in water and in a mixture under study ($C = 10^{-3}$ mol L⁻¹ with respect to phosphate ion, pH 7.8).

1-Hydroxy-2,4,5,5-tetramethyl-2-octyl-2,5-dihydro-1H-imidazole (4). A suspension of hydroxylamino ketone hydrochloride **5**¹² (5 g, 32.6 mmol), methyl octyl ketone (7.4 g, 39.1 mmol), and NH₄OAc (7.5 g, 97.8 mmol) in MeOH (80 mL) was refluxed for 4 h. Methanol was removed from the reaction mixture, a 5% aqueous solution of Na₂CO₃ (50 mL) was added to the residue, and the resulting mixture was extracted with Et₂O (3×50 mL). The extract was washed with water (2×20 mL) and dried with MgSO₄. The solution was concentrated, the residue was washed with hexane (5–10 mL), and a precipitate of compound **4** was filtered off.

4-(2-Oxoethylidene)- (6a), 4-(2-oxopropylidene)- (6b), 4-(3,3-dimethyl-2-oxobut-1-ylidene)- (6c), 4-(2-oxo-3,3,3-trifluoropropylidene)- (6d), and 4-(benzoylmethylidene)-2,5,5-trimethyl-2-octylimidazoline-1-oxyl (6e) (general procedure). Pr₂NH (5 mL, 35 mmol) was added dropwise with stirring at 20 °C to a solution of PhLi, prepared from Li (0.56 g, 80 mmol) and PhBr (4.2 mL, 40 mmol) in Et₂O (30 mL). The mixture was stirred for 15 min at the same temperature under argon. A solution of imidazoline **5** (2.54 g, 10 mmol) in ether was added to the resulting solution of LDA at such a rate that the solvent boiled slightly. The reaction mixture was

Table 2. Physicochemical characteristics of synthesized compounds

Com- ound	Yield (%)	M.p.* /°C	Found Calculated (%)			Molecular formula	IR**, ν/cm ⁻¹	UV, λ _{max} /nm (log ε)
			C	H	N			
2i	95	71–72	68.49 68.46	10.73 10.61	6.14 6.14	C ₂₆ H ₄₈ ClN ₂ O ₂	1631, 1549 (O=C—C=C—N); 3216 (NH)	317 (4.16)
2j	95	112–114	58.10 57.82	8.05 8.09	7.54 7.49	C ₁₈ H ₃₀ ClN ₂ O ₄	1708 (CO ₂ H); 1614, 1544 (O=C—C=C—N); 3239 (NH)	319 (4.16)
3i	70	86–88	72.60 72.60	11.10 10.83	9.35 9.41	C ₂₇ H ₄₈ N ₃ O ₂	1638, 1564 (O=C—C=C—N); 2197 (CN); 3179 (NH)	293 (4.22)
3j	25	95–96	62.38 62.61	8.19 8.30	11.37 11.53	C ₁₉ H ₃₀ N ₃ O ₄	1714 (CO ₂ H); 1644, 1566 (O=C—C=C—N); 2206 (CN); 3242 (NH)	294 (4.19)
4	75	105–107	70.79 70.81	11.81 11.89	10.78 11.01	C ₁₅ H ₃₀ N ₂ O	1660 (C=N); 3580 (OH)	—
6a	50	Oil	68.18 68.29	10.12 10.39	9.94 9.95	C ₁₆ H ₂₉ N ₂ O ₂	1639, 1563 (O=C—C=C—N); 3246 (NH)	304 (4.25)
6b	45	56–57	69.39 69.11	10.74 10.58	9.48 9.48	C ₁₇ H ₃₁ N ₂ O ₂	1636, 1561 (O=C—C=C—N); 3257 (NH)	299 (4.31)
6c	45	Oil	70.93 71.17	10.81 11.05	8.27 8.30	C ₂₀ H ₃₇ N ₂ O ₂	1631, 1548 (O=C—C=C—N); 3230 (NH)	298 (4.31)
6d	55	74–76	58.48 58.44	8.15 8.08	7.81 8.02	C ₁₇ H ₂₈ F ₃ N ₂ O ₂	1634, 1582 (O=C—C=C—N); 3267 (NH)	311 (4.00)
6e	35	88–90	74.29 73.91	9.58 9.30	7.80 7.84	C ₂₂ H ₃₃ N ₂ O ₂	1615, 1582, 1555, 1520 (O=C—C=C—N, Ph); 3230 (NH)	244 (3.98), 329 (4.34)
7c	85	Oil	64.32 64.58	9.57 9.76	7.28 7.53	C ₂₀ H ₃₆ ClN ₂ O ₂	1601, 1535 (O=C—C=C—N); 3183 (NH)	317 (4.09)
7d	75	Oil	52.95 53.19	6.81 7.09	7.05 7.30	C ₁₇ H ₂₇ ClF ₃ N ₂ O ₂	1626, 1544 (O=C—C=C—N); 3188 (NH)	334 (4.02)
7e	75	72–74	67.26 67.41	7.96 8.23	6.91 7.15	C ₂₂ H ₃₂ ClN ₂ O ₂	1597, 1578, 1544, 1520 (O=C—C=C—N, Ph); 3202 (NH)	243 (3.90), 340 (4.21)
8a	65	103–105	66.85 66.63	9.33 9.21	13.55 13.71	C ₁₇ H ₂₈ N ₃ O ₂	1645, 1562 (O=C—C=C—N); 2212 (CN); 3211 (NH)	300 (4.19)
8b	55	Oil	67.21 67.47	9.61 9.44	13.01 13.11	C ₁₈ H ₃₀ N ₃ O ₂	1635, 1562 (O=C—C=C—N); 2207 (CN); 3190 (NH)	297 (4.18)
8c	45	73–75	69.57 69.57	10.28 10.01	11.39 11.59	C ₂₁ H ₃₆ N ₃ O ₂	1625, 1557 (O=C—C=C—N); 2202 (CN); 3183 (NH)	292 (4.22)
8d	50	Oil	57.36 57.74	7.19 7.27	11.01 11.22	C ₁₈ H ₂₇ F ₃ N ₃ O ₂	1650, 1634, 1561 (O=C—C=C—N); 2201, 2220 (CN); 3209 (NH)	305 (4.07)
8e	80	91–92	72.26 72.22	8.16 8.43	10.66 10.99	C ₂₃ H ₃₂ N ₃ O ₂	1605, 1577, 1556 (O=C—C=C—N, Ph); 2207 (CN); 3187 (NH)	236 (4.12), 311 (3.78)

* After recrystallization from hexane (**2i,j, 3i, 4, 6b,e, 7e, 8a,e**), from an AcOEt–hexane mixture (**3j**), from aqueous MeOH (**6d**), and from aqueous EtOH (**8c**) or after chromatography on a column packed with Al₂O₃ (hexane–CHCl₃ (2 : 1) as the eluent) (**6a,c, 7c,d, 8b,d**).

** The spectra were recorded in KBr pellets (for compounds **6a,e, 7c,d**, and **8b,d**, in CCl₄ solutions).

stirred for 15 min at 20 °C and then cooled to 0 °C. A solution of ester (35 mmol) in an equal volume of Et₂O was added as one portion to the cooled mixture. Stirring was continued for 15 min at 0 °C and for 1 h at 20 °C. Then water (10 mL) was added to the reaction mixture. The organic layer was separated, and the aqueous layer was extracted with CHCl₃ (25 mL). The combined extracts were dried with MgSO₄ and concentrated. Chlo-

roform (30 mL) and MnO₂ (5 g, 57 mmol) were added to the residue. The suspension was stirred for 20 min at 20 °C and filtered, and the filtrate was concentrated. Enamino ketones **6a–e** were isolated by chromatography on a column packed with Al₂O₃, eluting successively with hexane and a hexane–CHCl₃ (1 : 1) mixture and collecting the colored zone (cf. Refs. 10 and 11).

4-(1-Chloro-2-oxononadec-1-ylidene)- (2i), 4-(10-carboxy-1-chloro-2-oxodec-1-ylidene)-2,2,5,5-tetramethylimidazolidine-1-oxyl (2j), (1-chloro-2-oxoethylidene)- (7a), 4-(1-chloro-2-oxopropylidene)- (7b), 4-(3,3-dimethyl-1-chloro-2-oxobut-1-ylidene)- (7c), 4-(1-chloro-2-oxo-3,3,3-trifluoropropylidene)- (7d), and 4-(1-chloro-2-oxo-2-phenylethylidene)-2-octyl-2,5,5-trimethylimidazolidine-1-oxyl (7e) (general procedure). NCS (0.14 g, 1 mmol) was added to a solution of enamino ketone **1i,j** or **6a–e** (1 mmol) in CCl_4 (30 mL), and the resulting suspension was stirred for 20–60 min until chlorination was completed (TLC monitoring on Silufol plates eluting with CHCl_3). A succinimide precipitate was filtered off, and the solution was concentrated to obtain a residue of chloro derivatives **7**, which can be used for the syntheses of the corresponding nitriles without additional purification (*cf.* Ref. 6). Compounds **7a,b** were used without purification. Compound **6e** was chlorinated with NCS in refluxing CCl_4 for 5 h.

4-(1-Cyano-2-oxononadec-1-ylidene)- (3i), 4-(10-carboxy-1-cyano-2-oxodec-1-ylidene)-2,2,5,5-tetramethylimidazolidine-1-oxyl (3j), (1-cyano-2-oxoethylidene)- (8a), 4-(1-cyano-2-oxopropylidene)- (8b), 4-(3,3-dimethyl-1-cyano-2-oxobut-1-ylidene)- (8c), 4-(1-cyano-2-oxo-3,3,3-trifluoropropylidene)- (8d), and 4-(1-cyano-2-oxo-2-phenylethylidene)-2-octyl-2,5,5-trimethylimidazolidine-1-oxyl (8e) (general procedure). Chloro-substituted enamino ketone **2i,j** or **7a–e** (1 mmol) was added to a solution of KCN (0.2 g, 3 mmol) in DMSO (10 mL). The suspension was stirred for 10 h at 20 °C, then brine (20 mL) was added, and the resulting solution was neutralized with 5% HCl. The solution was extracted with CHCl_3 (3×15 mL). The combined extracts were washed with brine (3×20 mL) and water (4×15 mL), dried with MgSO_4 , and concentrated. Nitriles **3i,j** and **8a–e** were purified by filtering their solutions through an Al_2O_3 layer ($l = 10$ cm) (*cf.* Ref. 6).

This work was financially supported by the Russian Foundation for Basic Research (Project No. 01-03-32452).

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Received December 9, 2002;
in revised form May 15, 2003