The Synthesis of Amidine Derivatives of Imidazoline Nitroxides – A New Series of pH-Sensitive Spin Probes and Labels

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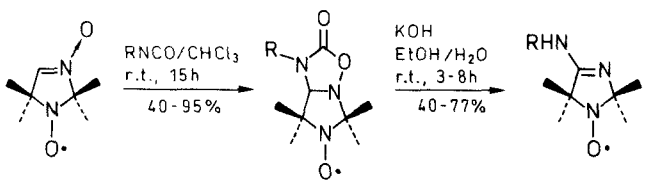
A synthesis of amidine derivatives, 4-alkyl[aryl]amino-2,5-dihydro-2,2,5,5-tetramethylimidazol-1-yl oxides is described. The first step is a 1,3-dipolar cycloaddition of 2,5-dihydro-2,2,5,5-tetramethylimidazol-1-yl 1,3-dioxide with isocyanates or diisocyanates. Alkaline hydrolysis of the resulting 3-substituted 2,3,3a,4,5,6-hexahydro-4,4,6,6-tetramethyl-2-oxoimidazo[1,5-*b*][1,2,4]oxadiazol-5-yl oxides affords the corresponding amidines. The resulting spin probes and labels may be used for measuring the local pH values in the range 3.3–7.8 in biological systems.

A new application of nitroxyl radicals for electron paramagnetic resonance (EPR) spectroscopy was developed on the basis of spectral sensitivity of these radicals to the pH of the medium.[1] The pH-sensitive nitroxyl radicals are most promising for pH studies in biological systems including membranes,[2,3] proteins,[4] cells and cell organelles.[5] Among them dihydro imidazole and imidazolidine radicals have the most pH-sensitive EPR spectrum parameters[6] due to protonation of the N3 atom of a radical heterocycle.

Because the basicity of the N3 atom is 3 or 5 orders lower[7] due to proximity of the nitroxyl group, the radicals are applicable for a limited range of pH from 2 to 5.5. The fact that the most vital biological processes take place at the neutral pH encouraged us to develop a novel approach to synthesis of dihydroimidazole probes sensitive to physiological pH values.

We wish here to report the method to increase pK values of radicals by incorporating N3 atom into the heterocyclic amidine group. The method is based on the reaction of 1,3-dipolar cycloaddition of paramagnetic aldonitrone 2,5-dihydro-2,2,5,5-tetramethylimidazol-1-yl 1,3-dioxide (**1**) to the isocyanates, followed by alkaline hydrolysis of the cycloadduct **2** (Scheme 1).

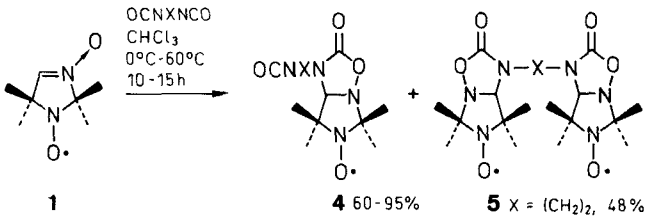


1 **2** **3**

2, 3	R	2, 3	R
a	Bu	d	4-MeC_6H_4
b	$ClCH_2CH_2$	e	4-ClC_6H_4
c	*c*-C_6H_{11}		

Scheme 1

pK Values of the resulting amidines, **3**, range from 4.5 to 6.6, so they are useful to the pH-measurement from 3.3 to 7.8 pH units. The failure of the substitute R (Scheme 1) to contain amino, hydroxy or other functional groups due to its reactivity towards isocyanates was a limiting factor in the synthesis. To solve this problem, the cycloaddition of radical nitrone **1** to diisocyanates has been effected yielding the adducts **4** with a free isocyanate group (Scheme 2).



1 **4** 60-95% **5** X = $(CH_2)_2$, 48%

4	X	4	X
a	$(CH_2)_2$		
b	$(CH_2)_4$	d	
c	$(CH_2)_6$		
		e	

Scheme 2

Table 1. Cycloadducts Prepared

Product	Yield (%)	mp (°C)	Molecular Formula[a]	IR (cm^{-1}) $\nu_{C=O}$
2a	42	oil	$C_{12}H_{22}N_3O_3$ (256.3)	1780
2b	81	117–120	$C_{10}H_{17}N_3O_3$ (227.2)	1750
2c	40	88–91	$C_{14}H_{24}N_3O_3$ (282.3)	1750
2d	81	164–167	$C_{15}H_{20}N_3O_3$ (290.3)	1750
2e	89	175–176	$C_{14}H_{17}N_3O_3Cl$ (310.7)	1780
4d	95	197–199	$C_{16}H_{19}N_4O_4$ (331.3)	1750, 2290 (N=C=O)
5	48	185–186	$C_{18}H_{30}N_6O_6$ (426.4)	1755
7aa	75	73–76	$C_{13}H_{23}N_4O_5$ (315.3)	1760, 1710
7ab	64	oil	$C_{18}H_{25}N_4O_5$ (377.4)	1765, 1710
7ba	72	oil	$C_{15}H_{27}N_4O_5$ (343.4)	1760, 1710
7bb	60	oil	$C_{20}H_{29}N_4O_5$ (385.3)	1765, 1710
7da	58	158–186	$C_{18}H_{25}N_4O_5$ (377.4)	1760, 1720
7db	24	117–119	$C_{26}H_{41}N_4O_5$ (489.6)	1765, 1730
7dc	32	138–140	$C_{43}H_{65}N_4O_5$ (717.9)	1780, 1710
9aa	60	oil	$C_{14}H_{24}N_5O_6$ (358.3)	1765, 1700, 1665
9da	25	95–98	$C_{19}H_{26}N_5O_6$ (420.4)	1770, 1695, 1670
9db	33	118–119	$C_{20}H_{24}N_7O_4$ (426.4)	1780, 1705
9dc	26	149–150	$C_{27}H_{32}N_7O_5$ (534.5)	1770, 1705

[a] Satisfactory microanalyses obtained: C, H, N ±0.35.

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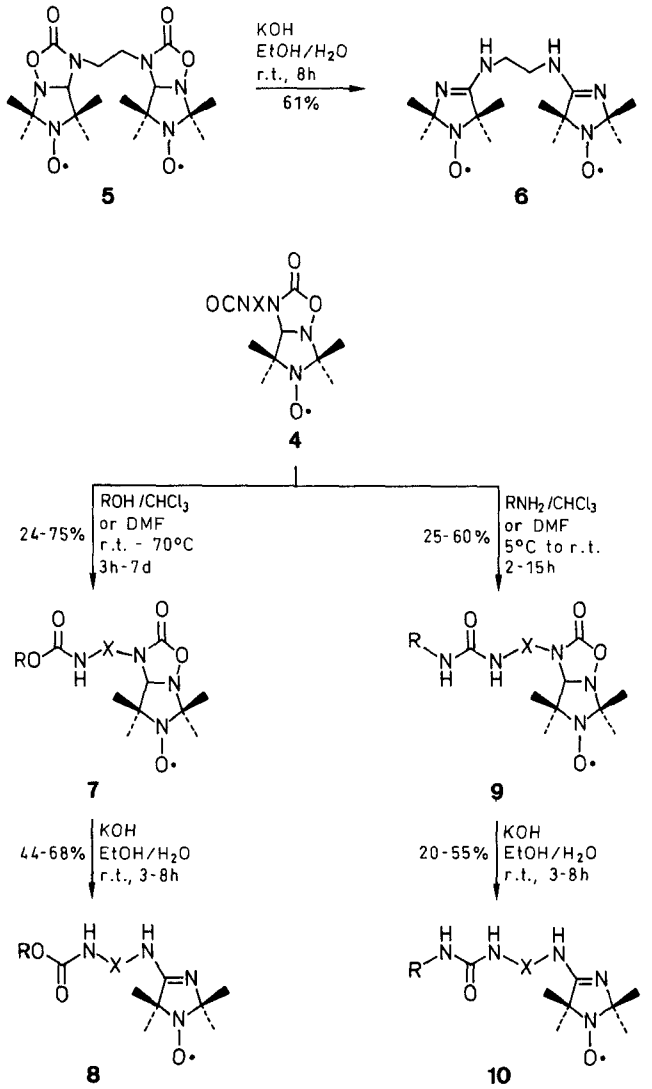
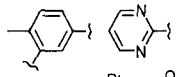
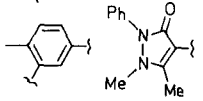
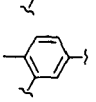
These compounds are capable of modifying different chemical functions and of binding to biological molecules (Scheme 3). Following alkaline hydrolysis of the perhydrooxadiazole cycle of modified products results in pH-sensitive amidine group formation (Scheme 3). These compounds are applicable as pH-probes in the physiological range of pH and seem to be very useful for biochemical studies. Recently, spin-labelled dextran derivative **8ca** has been used in a kinetic investigation of proton transport across model phospholipid membranes.[3]



7, 8	X	R	9, 10	X	R
aa	$(CH_2)_2$	Et	**aa**	$(CH_2)_2$	CH_2CO_2Me
ab	$(CH_2)_2$	Bn			CH_2CO_2H[a]
ba	$(CH_2)_4$	Et	**ca**	$(CH_2)_6$	polylysine
bb	$(CH_2)_4$	Bn			
ca	$(CH_2)_6$	dextran	**da**		CH_2CO_2Me
					CH_2CO_2H[a]
da		Et			
			db		
db		n-$C_{10}H_{21}$			
			dc		
dc		cholesteryl			

[a] The compounds **10aa** and **10da** are obtained as their carboxylic acids due to hydrolysis of the esters in the compounds **9aa** and **9da**.

Scheme 3

Table 2. Amidines Prepared

Product	Yield (%)	mp (°C)	Molecular Formula[a]	IR (cm^{-1}) ν	pK^b (± 0.1)
3a	40	28–32	$C_{11}H_{22}N_3O$ (212.3)	1620 (C=N)	6.60
3b	50	178–182	$C_9H_{17}N_3OCl$ (218.7)	1640 (C=N)	5.60
3c	61	223–224	$C_{13}H_{24}N_3O$ (238.3)	1620 (C=N)	6.45
3d	53	184–186	$C_{14}H_{20}N_3O$ (246.3)	1640 (C=N)	5.10
3e	77	221–223	$C_{13}H_{17}N_3OCl$ (266.7)	1640 (C=N)	4.50
6	61	212–213	$C_{16}H_{30}N_6O_2$ (338.4)	1630 (C=N)	5.55
8aa	78	159–161	$C_{12}H_{23}N_4O_3$ (271.3)	1615 (C=N), 1705 (C=O)	5.85
8ab	54	81–84	$C_{17}H_{25}N_4O_3$ (333.4)	1630 (C=N), 1705 (C=O)	5.90
8ba	68	110–112	$C_{14}H_{27}N_4O_3$ (299.4)	1620 (C=N), 1705 (C=O)	6.30
8bb	49	76–78	$C_{19}H_{29}N_4O_3$ (361.4)	1625 (C=N), 1705 (C=O)	6.25
8da	40	180–181	$C_{17}H_{25}N_4O_3$ (333.4)	1640 (C=N), 1705 (C=O)	5.15
8db	44	120–121	$C_{25}H_{41}N_4O_3$ (445.6)	1630 (C=N), 1705 (C=O)	5.00
8dc	42	182–183	$C_{42}H_{65}N_4O_3$ (673.9)	1630 (C=N), 1695 (C=O)	4.80
10aa	39	208–210	$C_{12}H_{22}N_5O_4$ (300.3)	1615 (C=N), 1670, 1720 (C=O)	5.86
10da	27	237–240	$C_{17}H_{24}N_5O_4$ (362.4)	1610 (C=N), 1630, 1720 (C=O)	4.75
10db	55	225–227	$C_{19}H_{24}N_7O_2$ (382.4)	1620 (C=N), 1700 (C=O)	4.95
10dc	20	120–121	$C_{26}H_{32}N_7O_3$ (490.5)	1620 (C=N), 1700 (C=O)	4.90

[a] Satisfactory microanalyses obtained: C, H, N ± 0.35.

[b] pK Values of the radicals were determined by EPR from the pH-dependence of their isotropic hyperfine splitting, a_N, according to Ref. 6. The limited values of a_N were:
$a_N(pH < pK - 2) = (15.05 \pm 0.1)G$;
$a_N(pH > pK + 2) = (15.85 \pm 0.1)G$.

IR spectra were run on a UR-20 spectrophotometer (Germany) in KBr tablets or in CCl_4 solution. Aqueous solutions of pH-sensitive radicals (concentrations 10^{-4} M or less) were titrated with KOH or HCl solutions to the required pH which was measured with an accuracy of up to 0.05 pH units using an OP-205/I pH meter (Hungary). EPR spectra were recorded on a Bruker ER-200D-SRC spectrometer in a flat tube or a 1 mm quartz capillary. pK Values of the radicals were determined by EPR spectroscopy from pH-dependence of their isotropic hyperfine splitting, a_N, according to Ref. 6. The degrees of modification of spin-labelled dextran derivatives **7ca**, **8ca** and polylysine derivatives **9ca**, **10ca** were determined from the double integral of the EPR spectrum. Column chromatography was performed on a silica gel L 40/100 um column (Chemapol), the eluent was $CHCl_3$ or $CHCl_3$/MeOH mixture. $CHCl_3$ used as solvent

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in the reactions with isocyanates was purified by filtration on a column with activated Al_2O_3.

1,2-Ethylene diisocyanate and tetramethylene diisocyanate were prepared by Curtius rearrangement of respective diacyl azides according to Ref. 8. All other isocyanates were purchased from Fluka. 2,5-Dihydro-2,2,5,5-tetramethylimidazol-1-yl 1,3-dioxide (**1**) was synthesized according to Ref. 9.

3-Alkyl-2,3,3a,4,5,6-hexahydro-4,4,6,6-tetramethyl-2-oxoimidazo[1,5-*b*][1,2,4]oxadiazol-5-yl Oxides (Cycloadducts) 2a–e; General Procedure:

A solution of respective isocyanate (15 mmol) in dry $CHCl_3$ (5 mL) was added to stirred solution of aldonitrone **1** (1.57 g, 10 mmol) in dry $CHCl_3$ (6 mL) at r.t. The mixture was allowed to stand overnight (15 h), then $CHCl_3$ was evaporated and the residue was washed with hexane (3 × 20 mL). The cycloadduct **2a–e** was purified by column chromatography (silica gel, $CHCl_3$). Recrystallization from hexane/EtOAc (3:1) gave analytical samples (Table 1).

4-Alkylamino-2,5-dihydro-2,2,5,5-tetramethylimidazol-1-yl Oxides (Amidines) 3a–e; General Procedure:

A solution of adduct **2a–e** (10 mmol) in EtOH (5 mL) was added to a solution of KOH (1.12 g, 20 mmol) in EtOH/H_2O (9:1, 10 mL). The reaction was continued for 3–8 h at r.t., then the mixture was neutralized to pH 7 with a solution of HCl in EtOH. The solution was evaporated to dryness, the residue was suspended in $CHCl_3$ and filtered. The filtrate was evaporated and chromatographed (silica gel, $CHCl_3$, MeOH, 98:2). Recrystallization from hexane/EtOAc (3:1) gave analytical samples (Table 2).

2,3,3a,4,5,6-Hexahydro-3-isocyanatoalkyl-4,4,6,6-tetramethyl-2-oxoimidazo[1,5-*b*][1,2,4]oxadiazol-5-yl Oxides 4a–c; General Procedure:

A mixture of diisocyanate (50 mmol) with dry $CHCl_3$ (5 mL) was added dropwise to stirred solution of aldonitrone **1** (1.57 g, 10 mmol) in dry $CHCl_3$ (6 mL) at 0 °C. The reaction took 10 h for 1,2-ethylene diisocyanate, 15 h for tetramethylene diisocyanate at r.t. and 15 h for hexamethylene diisocyanate at 60 °C. Then $CHCl_3$ was evaporated at reduced pressure and the excess of diisocyanate was removed by extraction with hexane (5 × 100 mL). The residue was kept in vacuum until constant weight. Cycloadducts **4a–c** are obtained in the form of reddish powder, **4a**, or red oil, **4b, 4c**. The structure of compounds **4a–c** was confirmed by the data of IR spectra (the NCO bond at $\nu = 2280\ cm^{-1}$ and the CO band of the oxadiazolidine at $\nu = 1765\ cm^{-1}$), and by the IR and elemental analysis data (Table 1) for the products **5**, **7**, **9** of their reactions (Scheme 3). Due to high lability of the isocyanate group, the compounds **4a–c** cannot be prepared of analytical grade and are used directly in the next step without purification. The yields of radicals **4a–c** were about 60%.

2,3,3a,4,5,6-Hexahydro-3-(3-isocyanato-4[and 6]-methylphenyl)-4,4,6,6-tetramethyl-2-oxoimidazo[1,5-*b*][1,2,4]oxadiazol-5-yl Oxide (4d) [and (4e)]:

2,4-Diisocyanatotoluene (2.60 g, 15 mmol) was added to a stirred solution of aldonitrone **1** (1.57 g, 10 mmol) in dry $CHCl_3$ (10 mL) at r.t. The mixture was allowed to stand overnight (15 h) and the precipitate of two compounds **4d** and **4e** was filtered. Cycloadduct **4d** was obtained of analytical grade by crystallization from EtOAc (Table 1).

1,2-Bis{2,3,3a,4,5,6-hexahydro-4,4,6,6-tetramethyl-2-oxo-5-oxylimidazo[1,5-*b*][1,2,4]oxadiazol-3-yl}ethane (5):

1,2-Ethylene diisocyanate (1.12 g, 10 mmol) was added dropwise to stirred solution of aldonitrone **1** (3.2 g, 20 mmol) in dry $CHCl_3$ (10 mL) at 0 °C. After 15 h at r.t. $CHCl_3$ was evaporated at reduced pressure and the mixture was separated chromatographically. The biradical fraction was evaporated to dryness and residual product was recrystallized from EtOAc/hexane (1:2) (Table 1).

3-(Alkoxycarbonylaminoalkyl[aryl])-2,3,3a,4,5,6-hexahydro-4,4,6,6-tetramethyl-2-oxoimidazo[1,5-*b*][1,2,4]oxadiazol-5-yl oxides 7 (Reaction of Adducts 4a–d with Alcohols); General Procedure:

Radical **4a–d** (10 mmol) was dissolved in ROH (6 mL) or in a solution of ROH (15 mmol) $CHCl_3$ (6 mL) for the high-boiling alcohols. The reaction took 3–10 h at r.t. or 10–25 h at 70 °C for cholesterol and benzyl alcohol. The mixture is evaporated and chromatographed (silica gel, $CHCl_3$). Recrystallization from hexane/EtOAc (2:1) gave an analytical sample (Table 1).

Spin-Labelled pH-Sensitive Dextran Derivative 8ca:

A mixture of **4c** (6.3 g, 20 mmol) and dextran 40 kD (Fluka) (4 g) in dry DMF (15 mL) was heated for 7 d at 60 °C. The modified dextran **7ca** was precipitated by addition of EtOAc (20 mL), centrifuged, washed with EtOH (2 × 10 mL) and dissolved in a solution of KOH (3 g) in H_2O (5 mL). After 1 d at r.t. the product was precipitated, washed with EtOH (4 × 10 mL) and dried in vacuum. The residue was dissolved in H_2O (3 mL), neutralized with HCl solution to pH 7 and gel filtered on Sefadex G-25. The required fraction was evaporated in vacuum to dryness; yield of **8ca**: 4 g. Modification degree of the compounds **7ca** and **8ca** was about 8 nitroxide moieties per polymer molecule. pK measured by the EPR method is 6.0 ± 0.1. The pH range accessible for EPR measurement is 4.8–7.2.

Spin-Labelled pH-Sensitive Polylysine Derivative 10ca:

A solution of **4c** (6.5 mg, 20 μmol) in dry DMF (10 μl) was added to a solution of polylysine 60 kD (Serva) (30 mg) in dry DMF (100 μl) at 0 °C. The reaction took 1 d at r.t. and gave in result compound **9ca**. Alkaline hydrolysis of **9ca** and purification of labelled product **10ca** were performed as for spin-labelled dextran. Yield of **10ca**: 15 mg. Modification degree of the compounds **9ca** and **10ca** was about 15 nitroxide moieties per polymer molecule. pK measured by the EPR method is 6.4 ± 0.1. The pH range accessible for EPR measurement is 5.2–7.6.

3-{(3-Alkyl[aryl]ureido)alkyl[aryl]}-2,3,3a,4,5,6-hexahydro-4,4,6,6-tetramethyl-2-oxoimidazo[1,5-*b*][1,2,4]oxadiazol-5-yl oxides) (Reaction of Adducts 4a–d with Amines); General Procedure:

A solution of **4a–d** (10 mmol) in dry $CHCl_3$ (6 mL) was added dropwise to a stirred solution of respective amine (10 mmol) in dry $CHCl_3$ (6 mL) at 5 °C. The reaction took 2–15 h at r.t. The mixture was evaporated to dryness in vacuum and chromatographed (silica gel, $CHCl_3$). Recrystallization from hexane/EtOAc (2:3) gave an analytical sample (Table 1).

Amidines **6, 8, 10** were prepared from respective cycloadducts **5, 7, 9** according to procedure described for compounds **3a–e**. Yields and physiochemical data are given in Table 2.

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