FLUORESCENCE REAGENTS FOR LABELLING OF BIOMOLECULES. PART I. SYNTHESIS AND SPECTRAL CHARACTERIZATION OF 2- AND 4-SUBSTITUTED 9-ISOTHIOCYANATOACRIDINES

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9-Isothiocyanatoacridines *VIII – XIV* were prepared from the corresponding 9-chloroacridines *I – VII*. The IR, ^{1}H NMR, ^{13}C NMR and fluorescence spectra of the products are given. The ^{13}C NMR chemical shifts of the C-9 ipso carbon atom exhibit a trend that is in accord with the Hammett constants of substituents bonded to the C-2 carbon. Effect of these substituents on the chemical shift of C–NCS was only small. The dependence of hydrolysis of isothiocyanates *VIII – XIV* on pH of the medium was studied. It was found that 9-isothiocyanatoacridines do not undergo hydrolysis at pH 7 – 10. The relative fluorescence intensities (F/F_0) of compounds *VIII – XIV* at pH 7.4 have been determined in comparison with that of 9-aminoacridine. No direct dependence between the fluorescence intensity and the polar character of substituents has been found.

The high chemical reactivity of isothiocyanates has become attractive for organic chemists as well as biochemists. The polar multiple bonds N=C=S easily enter addition and cyclization reactions under formation of various types of organic compounds[1]. From the biochemical viewpoint, isothiocyanates are utilized in the determination of structure of proteins and nucleic acids[2,3].

Recently, great attention has been paid to compounds suitable for fluorescence labelling of biomolecules[4,5]. As such fluorescent reagents may be regarded also acridine derivatives[6,7] and 9-isothiocyanatoacridine[7,8], commercially available since 1992 (Serva). This derivative was for the first time prepared in our Laboratory[9] as early as 1961, by heating of 9-chloroacridine with silver thiocyanate in toluene. Later on, the preparation of several other derivatives of 9-isothiocyanatoacridine has been repor-

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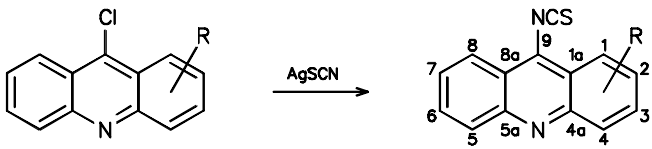
ted[10 – 12]. In the present communication we focussed on the study of spectral, structural and physicochemical characteristics of 2- and 4-substituted 9-isothiocyanatoacridines with particular stress on the effect of substituents on the relative fluorescence intensity.

EXPERIMENTAL

Preparation of 2- and 4-Substituted 9-Isothiocyanatoacridines *VIII – XIV*. General Procedure

A mixture of 9-chloroacridine *I – III*, *V – VII* (0.024 mol), AgSCN (0.025 mol) and dry toluene (250 ml) was refluxed for 5 – 6 h under exclusion of air moisture. The reaction mixture was filtered while hot to remove silver chloride and the unreacted silver thiocyanate. After evaporation of toluene in vacuo, the crude product was extracted with light petroleum in a Soxhlet apparatus for about 14 h. The solvent was evaporated in vacuo and the obtained product was crystallized from dry acetone or toluene (Scheme 1).



I–VII *VIII–XIV*

For R see Tables II and III

SCHEME 1

2-Chloro-9-isothiocyanatoacridine (*XI*) was prepared by reaction of 2,9-dichloroacridine (*IV*) with potassium thiocyanate in a dichloromethane–water mixture in the presence of tetrabutylammonium iodide at room temperature[12].

Purity of the compounds was checked by thin-layer chromatography on Silufol sheets (Kavalier, Votice, The Czech Republic) in benzene–acetone (7 : 1); UV-detection at 366 nm.

Spectral and Kinetic Measurements

The IR spectra ($\tilde{\nu}$, cm^{-1}) of 9-isothiocyanatoacridines *VIII – XIV* were recorded on a double-beam IR-75 spectrometer (Zeiss, Jena) in chloroform. ^1H and ^{13}C NMR spectra (δ, ppm) were measured on Tesla BS 487 (80 MHz) and Tesla BS 567 (25.156 MHz) instruments at room temperature in deuteriochloroform, except for derivative *V* whose ^{13}C NMR spectrum was obtained in dideuterio-1,1,2,2-tetrachloroethane at 105 °C and 9-aminoacridine (Serva) which was measured in hexadeuteriodimethyl sulfoxide. The spectra were referenced to tetramethylsilane as internal standard. Tertiary and quaternary carbon atoms were distinguished by "off resonance" decoupling. Absorption spectra of the isothiocyanates were obtained with a UV-3000 Shimadzu spectrophotometer (concentration $1.6 \cdot 10^{-5}$ mol l^{-1}) and fluorescence spectra on an RF 5000 Shimadzu spectrofluorimeter (concentration $1.6 \cdot 10^{-6}$ mol l^{-1}) in a mixture of HEPES buffer (50 mmol l^{-1}, pH 7.4)–acetonitrile (7 : 3). Fluorescence emission spectra were measured at excitation wavelength λ_{ex} 395 nm, whereas the excitation spectra at emission wavelength λ_{em} 460 nm. Kinetic measurements of decomposition of the acridine isothiocyanates were carried out on a Shimadzu UV-3000 spectrophotometer in 0.1 M HCl–

acetonitrile (7 : 3) mixture. The rate constants were evaluated according to first-order equation. All the measurements were performed at 25 °C.

RESULTS AND DISCUSSION

For the study of relationships between structure and fluorescence properties of 9-isothiocyanatoacridines we have chosen a series of 2-substituted derivatives and also the 4-methyl and 4-methoxy derivatives which were synthetically the best accessible. As intermediates for the synthesis of isothiocyanates we used 9-chloroacridines *I* – *VII*, obtained by cyclization of the corresponding 2′- and 4′-substituted diphenylamino-2-carboxylic acids with phosphorus oxychloride[13 – 15].

The physicochemical characteristics, together with the IR and ^{1}H NMR data, are given in Table I. Although some of the synthesized compounds have already been described previously[10], it was necessary to determine optimal conditions of their synthesis in an analytically pure state. Crude 9-isothiocyanates were strongly contaminated with hydrolysis products (acridone, aminoacridine) as well as with the unreacted 9-chloroacridines whose physical properties were similar to those of the corresponding isothiocyanates, making thus isolation of the pure products difficult. Because purification by chromatography was not successful, we purified the compounds by extraction with light petroleum and subsequent repeated crystallization from toluene or acetone (Table I).

All the isothiocyanates synthesized exhibited in the IR spectra strong broad complex bands of $\tilde{\nu}_{as}$(NCS) in the region 1 950 – 2 150 cm^{-1} and absorption bands due to symmetrical stretching vibrations $\tilde{\nu}_{s}$(NCS) at 907 – 920 cm^{-1} (Table I). There was no correlation between positions of the absorption maxima of $\tilde{\nu}_{as}$(NCS) and Hammett substituent constants. The marked electron-acceptor character of the acridine skeleton is obvious from comparison with the corresponding phenyl isothiocyanates whose absorption bands are shifted about 50 cm^{-1} towards higher wavenumbers[16]. The structure of the obtained compounds *I* – *XIV* was also confirmed by their ^{13}C NMR spectra which for isothiocyanatoacridines have not been hitherto studied. The influence of substituents on the ^{13}C NMR chemical shifts of the carbon atoms of acridine skeleton in compounds *I* – *XIV* was followed using the easily identifiable signal of the quaternary carbon atom C-9 and the NCS signal (Table II and III). As model compounds we used 9-aminoacridine, substituted 9-chloroacridines and their benzene analogues.

We observed an interesting effect when comparing the ipso SCS increments of the NCS, NH_2 and Cl substituents in 9-acridinyl and phenyl derivatives (Table IV). In contrast to the substituents NH_2 and Cl, the SCS value of NCS group for the C-9 carbon in acridine is negative (–3.3 ppm). This fact is probably connected with different interaction of the NCS group with the acridine hetero atom compared with the chlorine atom or amino group. The electron-acceptor effect of the acridine nitrogen atom manifests itself by an increase of chemical shifts in C–NCS derivatives *VII* – *XIV* (140.4 –

142.4 ppm) as compared[17] with *meta*- and *para*-substituted phenyl isothiocyanates (133.9 – 140.4 ppm). These values also show that the transfer of substitution effect to the NCS carbon atom in phenyl isothiocyanates (Δppm = 6.5) is greater than in isothiocyanatoacridines (Δppm = 2.0). The similar electronic character of the synthesized chloro derivatives (Table II) and isothiocyanates (Table III) is obviously the reason why the chemical shifts of the acridine carbon atoms differ only little, except for the C-9 atom which in chloro derivatives *I* – *VII* resonates at 138.1 – 144.1 ppm and in isothiocyanates *VIII* – *XIV* in the region 130.0 – 136.8 ppm. Moreover, these values

TABLE I
9-Isothiocyanatoacridines *VIII* – *XIV*

Compound R	Yield, %	M.p., °C (Ref.[10] m.p.)	$\tilde{\nu}_{as}$(NCS) $\tilde{\nu}_{s}$(NCS)	^{1}H NMR (CH_3)[a]
VIII 2-OCH_3	67	137 – 138 toluene (137 – 138.5)	2 060 907	4.05 s
IX 2-CH_3	48	124 – 125 acetone (123 – 125)	2 070 913	2.65 s
X H	52	131 – 132 acetone (131 – 132)	2 060 918	–
XI 2-Cl	59	154 – 155 acetone (155 – 156)	2 060 914	–
XII[b] 2-NO_2	47	195 – 197 toluene	2 045 908	–
XIII[c] 4-OCH_3	62	165 – 167 toluene	2 075 920	4.23 s
XIV 4-CH_3	82	135 – 137 acetone (135 – 137)	2 075 920	2.65 s

[a] Other signals: 7.25 – 8.4 m (acridine H). [b] For $C_{14}H_7N_3O_2S$ (281.3) calculated: 59.78% C, 2.51% H, 14.94% N; found: 58.92% C, 2.54% H, 14.81% N. [c] For $C_{15}H_{10}N_2OS$ (266.3) calculated: 67.65% C, 3.78% H, 10.52% N; found: 67.48% C, 3.70% H, 10.41% N.

TABLE II
^{13}C NMR chemical shifts (δ, ppm) of 9-chloroacridines *I* – *VII*

Compound R	C-1a[a] C-8a[a]	C-4a[b] C-5a[b]	C-9	C-2 or C-4	CH	CH_3
I	124.4	146.1	138.1	158.2	99.8; 129.2	55.6
2-OCH_3	125.2	147.3			124.1; 129.8	
					125.8; 131.5	
					127.0	
II	124.2	147.9	139.6	136.9	122.5; 129.8	22.0
2-CH_3	124.3	148.4			124.4; 129.8	
					126.6; 133.2	
					129.5	
III	124.2	148.9	140.9	–	124.4; 129.8	–
H	124.2	148.9			124.4; 129.8	
					126.7; 130.3	
					126.7; 130.3	
IV	124.3	147.0	139.7	133.0	123.0; 130.6	–
2-Cl	124.4	148.9			124.4; 131.6	
					127.4; 131.6	
					130.0	
V	122.6	149.4	144.1	145.8	122.2; 130.4	–
2-NO_2	124.8	151.0			123.0; 132.2	
					124.7; 132.4	
					128.3	
VI	124.6	142.2	141.0	155.4	106.9; 127.4	56.4
4-OCH_3	125.4	147.9			116.5; 130.2	
					124.4; 130.7	
					127.0	
VII	124.0	148.1	140.7	137.8	122.5; 129.7	18.6
4-CH_3	124.2	148.5			124.4; 129.8	
					126.7; 130.4	
					126.7	

[a,b] Assignment may be reversed.

TABLE III
^{13}C NMR chemical shifts (δ, ppm) of 9-isothiocyanatoacridines *VIII – XIV*

Compound R	NCS	C-1a[a] C-8a[a]	C-4a[b] C-5a[b]	C-9	C-2 or C-4	CH	CH_3	NCS[c]	NCS[d]
VIII 2-OCH_3	141.1	122.0 123.2	146.3 147.3	130.0	158.3	98.2; 122.4; 125.7; 127.0; 129.3; 130.0; 131.7	55.7	133.9	135.2
IX 2-CH_3	140.5	121.9 122.0	148.1 148.5	131.3	137.2	120.9; 122.7; 126.8; 129.7; 130.0; 130.0; 133.4	22.1	134.2	–
X H	140.8	121.8 121.8	149.0 149.0	132.5	–	122.7; 122.7; 126.9; 126.9; 130.0; 130.0; 130.5	–	135.2	135.2
XI 2-Cl	141.1	121.8 122.2	147.1 149.0	133.1	133.1	121.3; 122.6; 127.5; 130.1; 130.7; 131.6; 131.8	–	136.5	137.5
XII 2-NO_2	142.4	120.0 123.1	149.8 151.4	136.8	145.5	121.1; 123.1; 123.6; 128.3; 130.6; 132.1; 132.7	–	140.4	139.2
XIII 4-OCH_3	140.98	122.4 123.3	142.4 148.2	132.7	155.7	107.1; 114.7; 122.7; 127.2; 127.5; 130.3; 130.9	56.4	–	–
XIV 4-CH_3	140.4	121.7 121.9	148.2 148.6	132.3	138.1	120.7; 122.6; 126.8; 126.8; 129.9; 130.0; 130.2	18.4	–	–

[a,b] Assignment may be reversed. [c] ^{13}C NMR chemical shifts of NCS carbon of 4-substituted phenyl isothiocyanates. [d] ^{13}C NMR chemical shifts of NCS carbon of 3-substituted phenyl isothiocyanates.

show a trend that corresponds to the Hammett constants of substituents on the C-2 atom. A much weaker interaction was found between substituents in position 2 and the NCS carbon atom; this indicates that outside the acridine skeleton there is no marked conjugation effect.

The electronic absorption spectra of 9-isothiocyanatoacridines in the region 300 – 500 nm have a resolved vibrational structure of the p-band[10] the number and intensity of which depends on the character of the substituent and pH of the medium (Fig. 1). A more detailed study of the position and intensity of the vibrational bands will be the subject of a separate communication.

Protonation of the nitrogen atom in the acridine skeleton destabilizes the NCS group which is hydrolyzed to give an amine (Fig. 2) as confirmed by the spectrum obtained after hydrolysis of 9-isothiocyanatoacridine. On the other hand, phenyl isothiocyanate is stable in an acid medium. The acid hydrolysis of the NCS group was also observed with other 9-isothiocyanatoacridine derivatives. In all cases the reaction was of the first

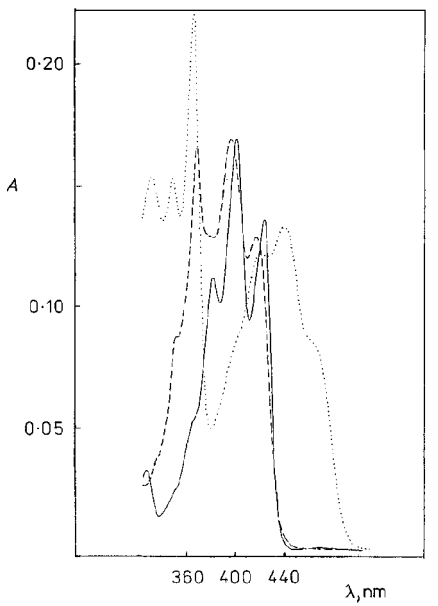


FIG. 1
Electronic absorption spectra of 9-aminoacridine (——) and 9-isothiocyanatoacridine (*X*) (– – – –), measured in 50 mM buffer HEPES (pH 7.4)–acetonitrile (7 : 3). 9-Isothiocyanatoacridine (*X*) (· · · ·) in 0.1 M HCl–acetonitrile (7 : 3). Concentration of all acridine derivatives: $1.6 . 10^{-5}$ mol l^{-1}

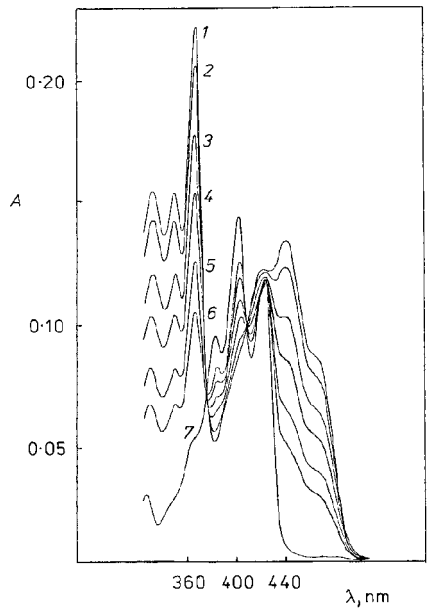


FIG. 2
Kinetics of hydrolysis of 9-isothiocyanatoacridine (*X*). Spectra taken at the rate 200 nm min^{-1} in 0.1 M HCl–acetonitrile (7 : 3); intervals: *1* 1 min, *2* 5 min, *3* 15 min, *4* 25 min, *5* 40 min, *6* 60 min, *7* 70 min

order. The rate constants observed for the individual derivatives are listed in Table V. The stability of 9-isothiocyanatoacridines against hydrolysis increases with increasing pH and in the region above pH 6 there is practically no reaction at all. Further increase

TABLE IV
Substituent chemical shifts (ppm, $CDCl_3$) of the Cl, NH_2 and NCS substituents on ipso carbon in phenyl[19,20] and acridinyl derivatives

Compound	Cl	NH_2	NCS
Benzene (C = 128.5)	5.9	19.2	2.4
Acridine (C-9 = 135.9)	5.1	13.8[a]	–3.3

[a] $(CD_3)_2SO$.

TABLE V
Parameters of the absorption and fluorescence emission spectra and the observed rate constants (k) of hydrolysis of 9-isothiocyanatoacridines *VIII – XIV*

Compound	λ_{max}, nm[a] log ε	λ_{max}, nm[b]	F/F_0[c]	$k \cdot 10^4$ s^{-1}
VIII	399 4.00	450	0.062	1.41
IX	395 4.40	440; 460	0.020	2.12
X	393 4.00	435; 460	0.028	3.16
XI	394 3.87	435; 460	0.036	3.50
XII	421 3.87	417	0.0011	5.1
XIII	414 4.00	508	0.043	3.25
XIV	397 4.09	460	0.032	3.5

[a] Electronic absorption. [b] Fluorescence emission. [c] Relative fluorescence F/F_0, where $F_0 = 1$ for $1.6 \cdot 10^{-6}$ mol l^{-1} solution of 9-aminoacridine.

in concentration of hydroxyl ions (pH > 10) results again in hydrolysis under formation of the corresponding aminoacridines. The determination of the region of pH in which the isothiocyanates *VIII – XIV* are stable was very important, not only for the study of reactivity of isothiocyanates with nucleophiles[18] but also from the viewpoint of characterization of the fluorescence spectra because 9-aminoacridine belongs to fluorescence reagents with a high fluorescence yield. These facts have not been taken into account by authors of a previous study[8] who compared relative fluorescence yields of various acridine derivatives in strongly acidic media. Therefore, in the case of 9-isothiocyanatoacridine their results were affected by the fluorescence contribution of 9-aminoacridine. For this reason we measured the fluorescence spectra of isothiocyanatoacridines *VII – XIV* at pH 7.4 (HEPES–acetonitrile, 7 : 3). Acetonitrile was used to increase the solubility of isothiocyanates which are only sparingly soluble in water.

Figure 3 shows excitation and emission spectra of 9-isothiocyanatoacridine referenced to the maximum fluorescence intensity of 9-aminoacridine. The relative fluorescence intensities (F/F_0; referenced to 9-aminoacridine) at the emission maxima for compounds *VIII – XIV* are given in Table V. As seen, all the 9-isothiocyanatoacridines investigated have lower fluorescence yield than 9-aminoacridine, the differences in the fluorescence intensities being much more pronounced than the differences in the ^{13}C NMR or electronic absorption spectra. The highest fluorescence intensity was found for the 2-OCH_3 derivative *VIII* whereas the lowest for the 2-NO_2 derivative *XII*.

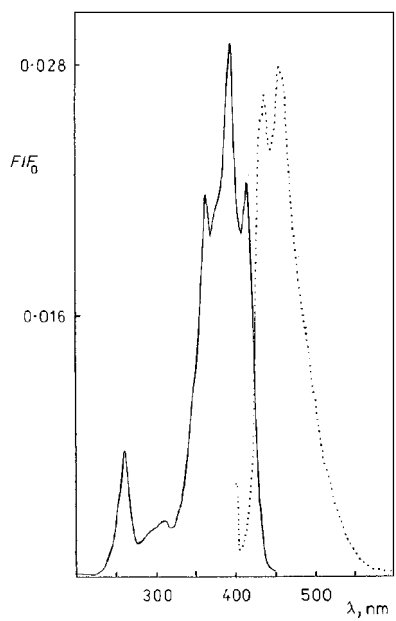


FIG. 3
Excitation and emission spectra of 9-isothiocyanatoacridine (*X*). Concentration 1.6 . 10^{-6} mol l^{-1} in 50 mM buffer HEPES (pH 7.4)–acetonitrile (7 : 3). Excitation spectrum at $\lambda_{em} \approx 460$ nm (——), emission spectrum at $\lambda_{ex} \approx 395$ nm (· · · ·); spectra referenced to that of 9-aminoacridine measured under the same conditions

The importance of isothiocyanatoacridines for the detection of amino acids or other biomolecules containing the NH_2 group consists in the fact that the fluorescence intensity of the addition products (the corresponding thioureas) is comparable with that of 9-aminoacridine[18].

REFERENCES

1. Drobnica L., Kristian P., Augustín J. in: *The Chemistry of Cyanates and Their Thio Derivatives* (S. Patai, Ed.), Part 2, p. 1003. Wiley, New York 1977.
2. Edman P.: Acta Chem. Scand. *4*, 283 (1950).
3. Cabantchik Z. I., Rothstein A.: J. Membr. Biol. *15*, 227 (1974).
4. Jin S. W., Chen G. X., Palacz Z., Wittmann Liebold B.: FEBS Lett. *198*, 150 (1986).
5. Tsugita A., Kamo M.: *Methods in Protein Sequence Analysis*. Birkhammer, Basel 1991.
6. Thuong N. T., Chassignol M.: Tetrahedron Lett. *29*, 5905 (1988).
7. Sinsheimer J. E., Jagodic V., Polak L. J., Hong D. D., Burckhalter J. H.: J. Pharm. Sci. *64*, 925 (1975).
8. Sinsheimer J. E., Hong D. D., Burckhalter J. H.: J. Pharm. Sci. *58*, 1041 (1969).
9. Kristian P.: Chem. Zvesti *15*, 164 (1961).
10. Kristian P.: Chem. Zvesti *23*, 371 (1969).
11. De Leenheer A., Sinsheimer J. E., Burckhalter J. H.: J. Pharm. Sci. *61*, 273 (1972).
12. Vlassa M., Kezdi M.: J. Prakt. Chem. *327*, 1010 (1985).
13. Albert A., Ritchie B.: Org. Synth., Coll. Vol. 3, p. 53. Wiley, New York 1946.
14. Ullmann F.: Justus Liebigs Ann. Chem. *355*, 312 (1907).
15. Magidson O. J., Grigorowsky A. M.: Ber. Dtsch. Chem. Ges. *69*, 396 (1936).
16. Kovac S., Kristian P., Antos K.: Collect. Czech. Chem. Commun. *30*, 3664 (1965).
17. Danihel I., Kristian P., Bohm S., Kuthan J.: Chem. Zvesti *38*, 539 (1984).
18. Podhradsky D., Oravec P., Antalik M., Kristian P.: Collect. Czech. Chem. Commun. *59*, 213 (1994).
19. Bremser W., Ernst L., Franke B., Gerhards R., Hardt A.: *Carbon-13 NMR Spectral Data*. Verlag Chemie, Weinheim 1981.
20. Kristian P., Danihel I., Burger A., Polomska A.: Z. Chem. *21*, 363 (1981).

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