FLUORESCENCE REAGENTS FOR LABELLING OF BIOMOLECULES. PART III. STUDY OF THE REACTIONS OF 2- AND 4-SUBSTITUTED 9-ISOTHIOCYANATOACRIDINES WITH GLYCINE

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Kinetics of nucleophilic addition reaction of 2- and 4-substituted 9-isothiocyanatoacridines *I – VII* with glycine in buffered aqueous dimethylformamide has been studied. The addition products, *N*-(9-acridinylthiocarbamoyl)glycines *VIII – XIV*, were characterized by IR, UV, ^{1}H NMR, mass and fluorescence spectra. Derivatives *VIII*, *X* and *XII* exhibited higher fluorescence intensity than the starting isothiocyanates; the highest fluorescence was found for the unsubstituted compound *X*. The reaction mechanism is discussed on the basis of properties of the reaction products and kinetic characteristics.

In our previous communications[1,2] we described the synthesis and physicochemical studies of substituted 9-isothiocyanatoacridines *I – VII* and investigated the reaction of unsubstituted acridinyl isothiocyanate *III* with various amino acids. We found that isothiocyanatoacridines and *N*-(9-acridinylthiocarbamoyl)amino acids derived from them exhibited marked fluorescence intensity. Our kinetic studies showed that with various amino acids 9-isothiocyanatoacridine reacts 6 – 22 times faster than phenyl isothiocyanate[2]. In the light of the possible utilization of 9-isothiocyanatoacridines as sequence peptide reagents, it was of interest to determine how substituents on the acridine nucleus affect the rate of addition of amino acids and the fluorescence intensity of the reaction products. In the present communication we study the kinetics of reaction of substituted 9-isothiocyanatoacridines with glycine and describe the properties of their addition products.

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EXPERIMENTAL

9-Isothiocyanatoacridines were prepared by reaction of the corresponding 9-chloroacridines with AgSCN in anhydrous toluene[1,3].

N-(9-Acridinylthiocarbamoyl) Amino Acids *VIII – XIV*

The described[2] method of preparation was modified as follows. A solution of glycine (120 mg, 1.6 mmol) in water (15 ml), adjusted to pH 9 with 1 M NaOH, was added to a solution of the substituted 9-isothiocyanatoacridine (2 mmol) in pyridine (25 ml). If the isothiocyanatoacridine precipitated, it was dissolved again by addition of a small amount of 1 M NaOH. The reaction mixture was heated under stirring at 60 °C for about 2 h. The excess isothiocyanatoacridine was removed by extraction with benzene, the layers were separated and air was introduced into the aqueous phase to remove traces of benzene. Addition of an equivalent amount of hydrochloric acid to the aqueous phase (to pH 3) precipitated *N*-(9-acridinylthiocarbamoyl)glycine which was collected, washed with hot methanol and dried. Because of low solubility in organic solvents, the obtained products were not crystallized.

Spectral and Kinetic Measurements

Proton NMR spectra were measured on a Tesla BS 587A instrument (80 MHz) at room temperature in $(CD_3)_2SO$ (internal standard tetramethylsilane) and in 0.5 M NaOH in D_2O (internal standard sodium 3-(trimethylsilyl)propanesulfonate). Infrared spectra were obtained with a double-beam spectrometer IR-75 (Zeiss, Jena) in KBr pellets in the region 400 – 4 000 cm^{-1}. Electronic spectra were taken on a UV-3000 Shimadzu spectrophotometer, concentration 8.0 . 10^{-6} mol l^{-1}, and fluorescence spectra on an RF 5000 Shimadzu spectrofluorimeter in 50 mmol l^{-1} HEPES* (pH 7.4); concentration of thiourea 1.6 . 10^{-6} mol l^{-1} at 25 °C (excitation wavelength 395 nm). Mass spectra were measured on an MS 902S (AEI, Manchester) spectrometer, direct inlet at 70 eV, ionization chamber temperature 180 °C.

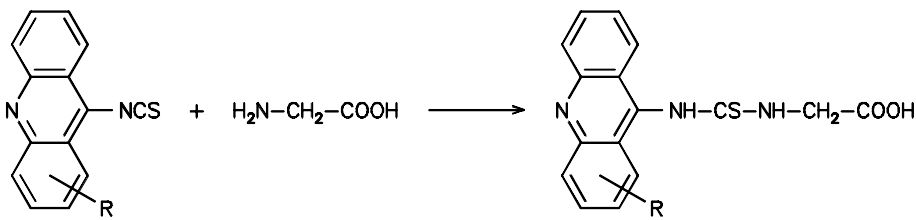
The kinetic measurements were performed at 25 °C using a SuperScan 3 spectrophotometer (Varian) in Clark–Lubs buffer adjusted by means of a digital pH-meter (OP-208, Radelkis); deviation ±0.1. Concentration of isothiocyanates *II – VII* was 6.4 . 10^{-5} mol l^{-1} and of glycine 6.4 . 10^{-3} mol l^{-1} which ensured a pseudo-first order reaction. In the case of the nitro derivative which exhibited high reactivity and strong absorption, the concentration was reduced to 3.2 . 10^{-5} mol l^{-1} and that of glycine to 1.6 . 10^{-3} mol l^{-1}. The measurements were carried out in a mixture of Clark–Lubs buffer (pH 11.1) and DMF (7 : 3) at 367 nm, except for derivative *I* which was measured at 305 nm. The apparent rate constants k were determined from the slope of the linear relationship log [log (A_∞/A_t)] against time t. The value of rate constant k (l mol^{-1} s^{-1}) was obtained by dividing the rate constant by concentration of the amino acid in dissociated form.

* Abbreviations used: HEPES, [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)]; DMF, dimethylformamide.

RESULTS AND DISCUSSION

The reaction of 9-isothiocyanates with glycine can be depicted by Scheme 1. Characteristics of the synthesized *N*-(9-acridinylthiocarbamoyl)glycines are given in Table I. In accord with their structure, all the products *VIII – XIV* exhibit IR absorption bands due to NH groups at 3 263 – 3 368 cm^{-1} and the so-called "thiureido" bands of NHCS in the region 1 510 – 1 520 cm^{-1} that are characteristic of a thiourea residue[4]. Absorption bands due to stretching vibrations of the acridine skeleton[5] (C–C, C–N) were observed at 1 635 cm^{-1} (Table II).

Because of low solubility in deuteriochloroform, the products were measured in $(CD_3)_2SO$; however, in this solvent they gradually decomposed. Therefore, we performed the measurements also in a solution of NaOH in D_2O, in which the compounds were well soluble and stable. The observed chemical shifts are given in Table II. Whereas in $(CD_3)_2SO$ the CH_2-signal of the glycine moiety was split into a doublet as the result of interaction with the N–H proton, in deuterated hydroxide this signal appeared as a singlet because of chemical exchange at the NH group. The differences between chemical shifts of CH_2 protons in the zwitterion form of derivatives *IX – XIV* (in $(CD_3)_2SO$: 4.20 – 4.26 ppm) were very similar to the values for their sodium salts (in $NaOH/D_2O$: 4.13 – 4.16 ppm). In $(CD_3)_2SO$, the N–H proton in the $NH–CH_2$ grouping appeared as a triplet in the region 8.70 – 10.56 ppm.



I – VII *VIII – XIV*

	R
I, VIII	2-NO_2
II, IX	2-Cl
III, X	H
IV, XI	4-OCH_3

	R
V, XII	2-CH_3
VI, XIII	2-OCH_3
VII, XIV	4-CH_3

SCHEME 1

In order to confirm the structure of the synthesized thiocarbamoylglycines we measured mass spectrum of compound *X*. There is no molecular ion in the spectrum, the base peak being the fragment ion of *m*/*z* 194 due to 9-aminoacridine. The thiocarbamide structure of adduct *X* is confirmed by the ion $M^{+\bullet} - 34$ (*m*/*z* 277) arising by elimination of H_2S.

Typical for the UV spectra of compounds *VIII – XIV* are very strong bands at 233 nm ($\log \varepsilon \approx 5$) as well as bands of medium intensity at 460 nm ($\log \varepsilon \approx 4$; refs[3,6]) which are bathochromically shifted relative to those of isothiocyanates (Fig. 1). Whereas the position of the former band in compounds *VIII – XIV* is not influenced by substitution, the long-wave maxima show a strong bathochromic shift, particularly in derivatives with auxochromic CH_3O and NO_2 groups (Table I). In all cases, the UV-VIS spectra of addition products isolated from the kinetic measurements were identical with those of the corresponding standards prepared independently.

Figure 2 depicts emmision fluorescence spectra of compound *III* and the unsubstituted *N*-(9-acridinylthiocarbamoyl)glycine *X*. The fluorescence spectra of thiocarba-

TABLE I
N-(9-Acridinylthiocarbamoyl)glycines *VIII – XIV*

Compound	Formula M.w.	Calculated/Found			M.p., °C Yield, %	λ_{max}, nm log ε	
		% C	% H	% N			
VIII	$C_{16}H_{12}N_4O_4S$	53.92	3.40	15.73	185 – 190	484	233
	356.1	53.80	3.24	15.62	52	3.53	5.36
IX	$C_{16}H_{12}ClN_3O_2S$	55.57	3.50	12.15	196 – 198	458	233
	345.0	55.62	3.71	12.22	72	3.66	5.37
X	$C_{16}H_{13}N_3O_2S$	61.72	4.21	13.50	187 – 189	450	233
	311.4	61.78	4.32	13.64	66	3.77	5.37
XI	$C_{17}H_{15}N_3O_3S$	59.81	4.43	12.32	188 – 190	458	234
	341.4	59.67	4.18	12.24	82	3.58	5.37
XII	$C_{17}H_{15}N_3O_2S$	62.75	4.65	12.92	185 – 189	453	234
	325.4	62.57	4.56	12.80	74	3.74	5.38
XIII	$C_{17}H_{15}N_3O_3S$	59.81	4.43	12.32	189 – 192	458	233
	341.4	59.98	4.71	12.23	86	3.24	5.37
XIV	$C_{17}H_{15}N_3O_2S$	62.75	4.65	12.92	188 – 190	450	234
	325.4	62.91	4.82	13.03	77	3.82	5.38

moylglycines do not differ substantially in shape from those of the starting isothiocyanates, however, there are differences in the intensity. Table III shows relative fluorescence intensities and position of fluorescence band maxima. The highest fluorescence intensity was observed for the unsubstituted derivative *X*, the lowest for derivatives *VIII* and *IX* with substituents NO_2 and Cl, respectively. Infrared spectra of the isolated thiocarbamoylglycines *VIII – XIV* (Table II) exhibited no carbonyl bands. We assume therefore that the products, isolated after adjusting the reaction mixture to pH 3.0, exist in the form of sparingly soluble inner salts of the carboxy group with the acridine nitrogen atom (pK_a of acridine is about 4 (ref.[7]), pK_a of thiourea about –0.9 (ref.[8])). To confirm this assumption, we measured IR spectrum of the zwitterion form

TABLE II
IR (ν, cm^{-1}) and ^1H NMR (δ, ppm) spectra of substituted *N*-(9-acridinylthiocarbamoyl)glycines *VIII – XIV*

Compound	IR			^1H NMR[a]			
	NH^+	C–C, C–N NHCS	R	CH_2[b]	CH_3	ArH	NH[c]
VIII[d]	3 368	1 635	1 345	–	–	–	–
		1 520		4.30		7.00 – 9.80	10.56
IX	3 300	1 635	–	4.15	–	7.32 – 8.30	–
		1 520		4.26		7.10 – 8.70	9.00
X	3 280	1 635	–	4.13	–	7.30 – 8.35	–
		1 518		4.25		6.98 – 8.82	9.16
XI	3 263	1 635	1 230	4.15	4.08	7.05 – 8.32	–
		1 518		4.23	4.03	6.92 – 8.93	9.12
XII	3 275	1 630	–	4.14	2.52	7.30 – 8.30	–
		1 515		4.23	2.40	7.00 – 8.82	9.03
XIII	3 290	1 635	1 240	4.16	4.00	7.32 – 8.25	–
		1 510		4.20	3.86	6.98 – 8.85	[e]
XIV	3 282	1 630	–	4.13	2.80	7.20 – 8.30	–
		1 520		4.23	2.66	6.96 – 8.35	8.70

[a] Measured in 0.5 M NaOH solution in D_2O (upper value) and $(CD_3)_2SO$ (lower value). [b] Signals of CH_2 protons measured in $(CD_3)_2SO$ (lower value) are doublets with coupling constant in the range 4.4 – 6.0 Hz. [c] 9-NH signal was not detected. [d] Sample decomposes in 0.5 M NaOH solution in D_2O. [e] NH signal is overlapped with signals of aromatic protons.

TABLE III
Fluorescence properties and rate constants of compounds *VIII – XIV*

Compound	λ_{em}, nm[a]	F/F_0[b]	k, l mol^{-1} s^{-1}
VIII	420, 460	0.13	9.4
IX	430, 461	0.15	2.9
X	430, 461	1.00	1.7
XI	462, 480	0.42	1.6
XII	420, 464	0.64	1.4
XIII	460	0.31	1.3
XIV	421, 460	0.20	1.2

[a] Excitation wavelength λ_{ex} = 395 nm. [b] Relative fluorescence, where $F_0 = F$ for 1.6 . 10^{-6} mol l^{-1} solution of *N*-(9-acridinylthiocarbamoyl)glycine at the higher wavelength maximum.

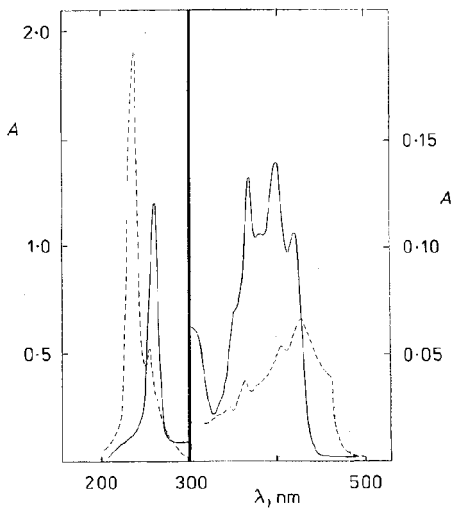


FIG. 1
UV-VIS spectra of compounds *III* (——) and *X* (— — —) measured in 7 : 3 mixture of HEPES buffer (50 mmol l^{-1}; pH 7.4) and DMF at 25 °C; concentration of compounds 8.0 . 10^{-6} mol l^{-1}

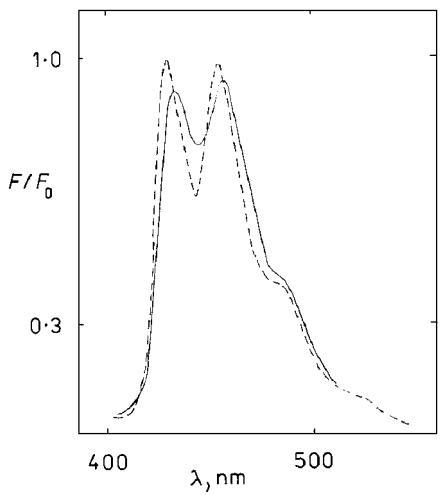


FIG. 2
Fluorescence emission spectra of compounds *III* (——) and *X* (— — —), measured in 7 : 3 mixture of HEPES buffer (50 mmol l^{-1}; pH 7.4) and DMF at excitation wavelength $\lambda_{ex} \approx$ 395 nm; concentration of compounds 1.6 . 10^{-6} mol l^{-1}

of glycine in KBr: similarly as in the case of the addition products *VIII – XIV*, there was no CO band in the spectrum.

In our preceding communication we described kinetic studies of reaction of 9-isothiocyanatoacridines with fifteen amino acids and showed it to be nucleophilic addition. We have found a correlation between rate constants for reaction of isothiocyanatoacridine and phenyl isothiocyanate with amino acids[2]. In the present study we investigate how the reaction rate is affected by various substituents on the acridine moiety. Because od different solubilities of compounds *I – VII*, we chose dimethylformamide as solvent. Figure 3 depicts the dependence of rate constant for the reaction of derivative *III* with glycine on the concentration of dimethylformamide. In order to eliminate the strong solvation effect of DMF on the rate constant and simultaneously to ensure sufficient solubility of the reactants, we performed the measurements in 30% DMF. Because in the region pH 7 – 11 the second-order rate constants of the studied reaction are independent of pH (Fig. 4), there is no protonation of the heteroaromatic ring nitrogen, and thus no change of the reaction mechanism. This fact is also confirmed by data in Fig. 5, depicting the dependence of rate constants on the ionic strength of the medium. At pH 11.1, the ionic strength does not affect the reaction rate and we can thus assume that the reaction takes place between nonionized molecules[9]. The results of the kinetic measurements are summarized in Table III. As expected, compounds with electron-accepting substituents are the most reactive ones.

Similarly as in our previous study[2], we compared the reactivity of 9-isothiocyanatoacridines with that of phenylisothiocyanates, with glycine as the second component. As

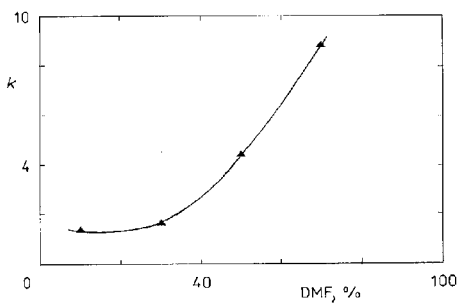


FIG. 3
Dependence of rate constant k (l mol^{-1} s^{-1}) for reaction of compound *III* with glycine on the percentage of DMF (measured at pH 11.1)

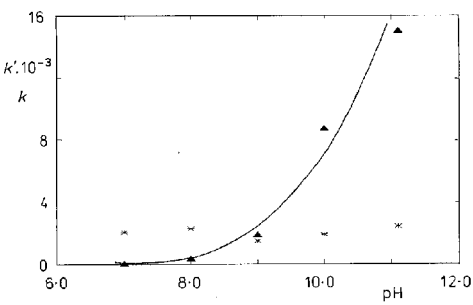


FIG. 4
Effect of pH of the medium on rate constants k' (s^{-1}) (▲) and k (l mol^{-1} s^{-1}) (✳) for reaction of compound *III* with glycine in 7 : 3 mixture of Clark–Lubs buffer and DMF

seen from Fig. 6, there is a good correlation between the experimental rate constants of 2-substituted isothiocyanatoacridines and the corresponding *para*-substituted phenyl isothiocyanates[10] whereas no correlation was observed for the analogous *meta*-derivatives. It follows that, analogously to *para*-substituted phenylisothiocyanates, both the inductive and mesomeric effects operate in the reaction of 9-isothiocyanatoacridines.

Thus, of the 9-isothiocyanatoacridines studied in this paper, the unsubstituted derivative *III* has the best properties for possible use as fluorescent reagent for labelling macromolecules.

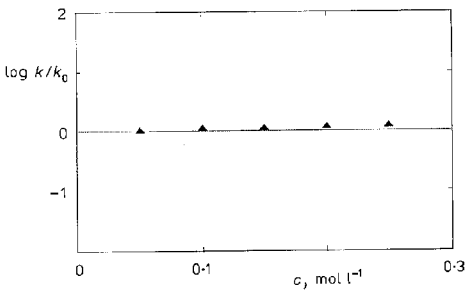


FIG. 5
Dependence of rate constant k (l mol^{-1} s^{-1}) on ionic strength of the medium at pH 11.1 in 30% DMF. Linear dependence log $k/k_0 = A + B\,c$, where A = 0.38, B = 0.005 and r = 0.98

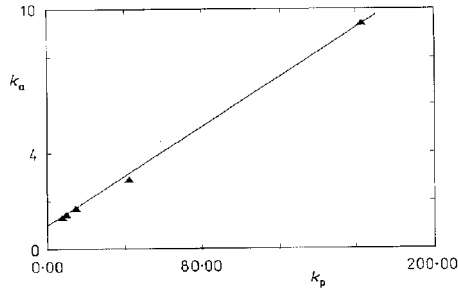


FIG. 6
Correlation of rate constants for reaction of 9-isothiocyanatoacridine *III*, k_a (l mol^{-1} s^{-1}), and phenyl isothiocyanate[8], k_p (l mol^{-1} s^{-1}), measured at pH 11.1. Linear dependence $k_a = A + B\,k_p$, where A = 0.05, B = 0.86 and r = 0.99

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