FLUORESCENCE REAGENTS FOR LABELLING OF BIOMOLECULES. PART II. REACTIONS OF 9-ISOTHIOCYANATOACRIDINE WITH AMINO ACIDS

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N-(9-Acridinylthiocarbamoyl)amino acids (ATC-AA) *II* – *VII* were synthesized by reaction of amino acids with 9-isothiocyanatoacridine *I*, a new fluorescence labelling agent. The amino acid derivatives *II* – *VII* show high relative fluorescence, which is suitable for the determination of nanomolar amounts of ATC-AA. The kinetic measurements show that reaction of *I* with amino acids is 6 to 22 times faster than analogous reaction of phenyl isothiocyanate. The possibility of using 9-isothiocyanatoacridine for structure determination of proteins is discussed.

In contemporary biochemistry there is a steady interest in highly sensitive reagents for quantitative determination of biological compounds containing an amino group, particularly amino acids and peptides. The fluorescence detection, applied especially after HPLC separation, often enables lowering the detection limits below the femtomolar level[1]. So far, in spite of their high fluorescence yield, acridine derivatives have not belong to the many fluorescent reagents used for the covalent labelling of biological preparations. On the other hand, isothiocyanates are used for such labelling very often[2].

Isothiocyanates react with the NH_2 group of amino acids under formation of *N*-thioureidocarboxylic acids. This reaction is utilized e.g. in the Edman degradation of peptides[3]. Nucleophilic additions, typical for isothiocyanates, are conditioned by the presence of a strong electrophilic center at the NCS carbon atom. The kinetics of reactions of glycine[4], amines[5 – 8], hydroxyl ions[9] and thiols[7,10] with aliphatic and aromatic isothiocyanates was studied by many authors but no kinetic investigations are known on the reaction of 9-isothiocyanatoacridine with amino acids. In spite of the fact that the synthesis of asymmetric thioureas by reaction of isothiocyanates with amines is generally known, there is an only one publication describing the preparation of such thioureas from 9-isothiocyanatoacridine[11].

In the present paper we describe the preparation of thioureas by reaction of 9-isothiocyanatoacridine with a series of amino acids. At the same time, we try to compare

kinetically the reactivity of 9-isothiocyanatoacridine with that of phenyl isothiocyanate employed in the Edman degradation of peptides. We intend to make use of the obtained results in the proposed complex study of acridine isothiocyanates as fluorescence reagents in the chemistry of peptides, proteins and nucleic acids.

EXPERIMENTAL

The melting points are uncorrected. ^{1}H NMR spectra were measured on a Tesla BS 487A (80 MHz) spectrometer in hexadeuteriodimethyl sulfoxide solutions with tetramethylsilane as internal standard, UV-VIS spectra were obtained with a SuperScan 3 (Varian Techtron, Australia) spectrophotometer.

Chemicals

9-Isothiocyanatoacridine was prepared according to Kristian[12] by reaction of 9-chloroacridine with AgSCN in anhydrous toluene and subsequent crystallization of the crude product from anhydrous acetone. All the D,L-amino acids used were commercial products (Calbiochem). The solvents and buffer components were of spectral or analytical purity.

N-(9-Acridinylthiocarbamoyl)amino acids II – VII were obtained using the following modified procedure[11]. A solution of 9-isothiocyanatoacridine (236 mg, 1 mmol) in pyridine (10 ml) was mixed with a solution of the corresponding amino acid (0.8 mmol) in water (3 ml) which had been adjusted to pH 9 with 1 M NaOH. The stirred mixture was heated at 60 °C for 1 h, the pH being maintained at 8 – 9 by dropwise addition of NaOH solution (about 1 ml). The excess isothiocyanatoacridine was extracted with benzene and the traces of benzene left in the aqueous layer were removed by evaporation under diminished pressure. Addition of an equivalent amount of hydrochloric acid liberated the crude thiourea which was dried and crystallized from dry methanol. The physico-chemical and spectral parameters of the synthesized thioureas are given in Table I.

Kinetic Measurements

The kinetic measurements were performed on a SuperScan 3 spectrophotometer. Clark–Lubs buffer was adjusted using a digital pH-meter (OP-208 Radelkis); deviation ±0.01. The concentration of isothiocyanate *I* was $6.4 \cdot 10^{-5}$ mol l^{-1}, concentration of the amino acids $6.4 \cdot 10^{-3}$ mol l^{-1}. Because of low solubility of the compound *I*, the measurements were carried out in a 7 : 3 mixture of Clark–Lubs buffer (pH 9.8) and acetonitrile, the analytical wavelength being 435 nm. The apparent rate constants k' were obtained from slope of the linear dependence of $\log (A_\infty/A_t)$ against time t, the value of rate constant k [l mol^{-1} s^{-1}] was calculated by dividing k' with concentration of dissociated form of the amino acid.

Fluorescence Measurements

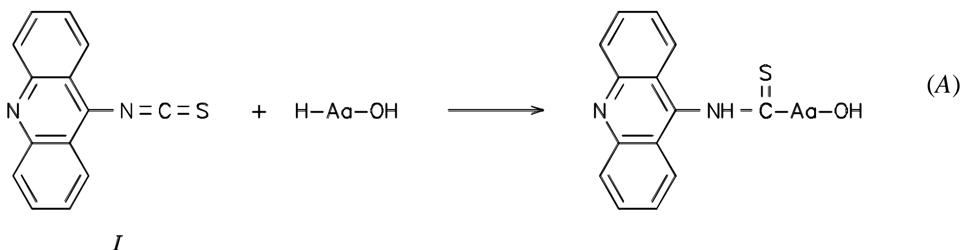
The fluorescence spectra of thioureas *II* – *V* and *VII* were measured on an RF 5000 Shimadzu spectrofluorimeter in 50 mM HEPES buffer (pH 7.4), the concentration of the thiourea being $1.6 \cdot 10^{-6}$ mol l^{-1}, with exception of compound *III* whose concentration was $1.6 \cdot 10^{-7}$ mol l^{-1}. The fluorescence spectrum of 9-isothiocyanatoacridine *I* was taken in a 7 : 3 mixture of 50 mM HEPES buffer (pH 7.4) and acetonitrile. The fluorescence emission spectra were measured at excitation wavelength $\lambda_{ex} \approx 396$ nm whereas the excitation spectra at the emission wavelength $\lambda_{em} \approx 456$.

TABLE I
Analytical data on N-(9-acridinylthiocarbamoyl)amino acids *II* – *VII*

	Formula M.w.	M.p., °C Yield, %	Calculated/Found			λ_{max}, nm log ε	^{1}H NMR, ppm
			% C	% H	% N		
II	$C_{16}H_{13}N_3O_2S$ 311.4	185 – 187 87	61.72 61.79	4.21 4.33	13.49 13.61	426 4.12	9.54 bs , 1 H (NH) ; 8.69 – 7.35 m, 8 H (arom. H); 4.59 s, 2 H (CH_2); 4.17 s, 1 H (NH)
III	$C_{17}H_{15}N_3O_2S$ 325.4	203 – 206 65	62.75 62.84	4.65 4.76	12.91 13.05	426 407 4.01 4.0	8.92 – 7.42 m, 8 H (arom. H); 4.70 m, 1 H (CH); 1.74 d, 3 H (CH_3)
IV	$C_{23}H_{19}N_3O_2S$ 401.5	175 – 176 59	68.81 68.52	4.77 4.53	10.47 10.31	426 4.09	9.26 bs, 1 H (NH); 8.63 – 6.88 m, 13 H (arom. H); 4.98 t, 1 H (CH); 3.18 d, 2 H (CH_2)
V	$C_{20}H_{17}N_5O_2S$ 391.5	171 – 174 53	61.36 61.65	4.38 4.61	17.89 18.08	424 4.07	8.80 – 7.44 , 8 H (arom. H); 7.50 s, 1 H (CH); 6.76 s, 1 H (CH); 5.01 m, 1 H (CH); 3.03 d, (CH); 2 H (CH_2)
VI	$C_{20}H_{21}N_3O_2S$ 367.5	149 – 151 57	65.37 65.49	5.76 5.69	11.43 11.22	426 4.15	8.88 – 7.40 m, 8 H (arom. H); 5.02 m, 1 H (CH); 1.63 m, 3 H (CH and CH_2); 0.95 d, 6 H (2 × CH_3)
VII	$C_{19}H_{17}N_3O_2S$ 351.3	163 – 165 50	64.97 64.79	4.88 4.71	11.96 12.08	428 4.21	8.70 – 7.49 m, 8 H (arom. H); 3.88 m, 1 H (CH); 2.18 – 1.44 m, 6 H (3 × CH_2)

RESULTS AND DISCUSSION

The reaction of 9-isothiocyanatoacridine with amino acids proceeds according to the following equation:



	Aa		Aa
II	Gly	*V*	His
III	Ala	*VI*	Leu
IV	Phe	*VII*	Pro

In this nucleophilic addition reaction the lone electron pair of the amino group attacks the electrophilic carbon atom of the NCS group, giving rise to *N*,*N′*-disubstituted thioureas. The physicochemical properties of the obtained products are given in Table I. The electronic absorption spectra of the thioureas *II* – *VII* measured in aqueous solutions exhibit a broad absorption band of high resolution in the region 320 – 450 nm. As seen from comparison of absorption maxima (Table I and Fig. 1), the spectrum is significantly influenced by the permittivity of solvent. The different shape of the spectra indicates a high sensitivity of the electronic absorption spectra of the acridine skeleton to changes in its environment, which can be of value in investigations on isothiocyanatoacridine-modified proteins. Having in mind the potential use of 9-isothiocyanatoacridine (*I*) as fluorescence reagent, we compared its fluorescence with the values for the thiourea derivatives *II* – *V* and *VII* (Table II). In the region 430 – 460 nm, the fluorescence emission spectra exhibited two intense maxima, λ_{em} (max. 1) and λ_{em} (max. 2). In a slightly alkaline medium (pH 7.4) all the thioureas displayed a higher fluorescence than the isothiocyanate *I*. A particularly pronounced fluorescence was observed with *N*-(9-acridinylthiocarbamoyl)alanine (*III*): it is about 16 times higher than that for *I* (see Fig. 2) and corresponds to the fluorescence yield of 9-aminoacridine[11]. This would enable determination od alanine at concentrations as low as $1 \cdot 10^{-9}$ mol l^{-1}.

Because of low solubility if the isothiocyanate *I*, kinetic measurements of its reaction with amino acids were performed in a 7 : 3 mixture of Clark–Lubs buffer (pH 9.8) and acetonitrile. In a medium of pH 9.8 there is practically no concurrent reaction of OH^- ions with the NCS group of *I* ($k'_{OH} = 1.12 \cdot 10^{-5}$ s^{-1}). The kinetics were measured at

435 nm; at this wavelength we observed the highest absorbancy increase of the measured thioureas relative to isothiocyanate *I*. In Fig. 3 we can see the dependence of the apparent rate constant k' on glycine concentration at 26 °C. This dependence is

TABLE II
Fluorescence properties of compounds *I* – *V* and *VII*

Compound	λ_{ex}, nm	λ_{em}, nm	F/F_0 [a]
I	395	438, 459	1
II	396	432, 458	2.62
III [b]	397	430, 458	16.1
IV	396	430, 456	1.67
V	397	430, 456	2.25
VII	397	430, 457	0.53

[a] Relative fluorescence, where F_0 = 1 for 1.6 . 10^{-6} mol l^{-1} solution of 9-isothiocyanatoacridine; [b] compound *III* was measured at concentration 1.6 . 10^{-7} mol l^{-1}.

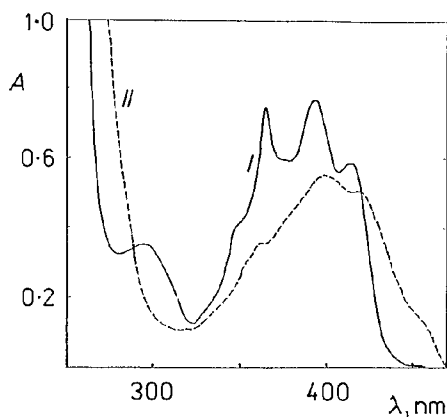


FIG. 1
UV-VIS spectra of compounds *I* (———) and *II* (– – – –). Spectrum of *I* in Clark–Lubs buffer (pH 9.8)–acetonitrile (7 : 3 (v/v)); concentration of *I*: 6.4 . 10^{-5} mol l^{-1}, 26 °C. Spectrum of *II* after 12 h under the same conditions, starting concentrations: 6.4 . 10^{-3} mol l^{-1}, *I*: 6.4 . 10^{-5} mol l^{-1}

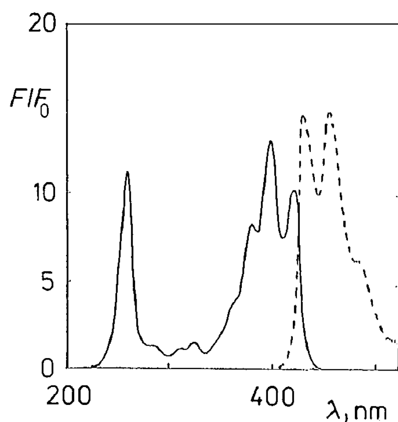


FIG. 2
Excitation (———) and emission (– – – –) spectrum of compound *III* (50 mM HEPES, pH 7.4, concentration of *III* 1.6 . 10^{-7} mol l^{-1}, at excitation wavelength λ_{ex} 398 nm and emission wavelength λ_{em} 456 nm)

linear, proving thus that with a 100-fold excess of amino acid the reaction is of pseudo-first order. The results of the kinetic measurements are given in Table III. The rate

TABLE III
Rate constants for the reaction of 9-isothiocyanatoacridine (*I*) with amino acids

Amino acid	pK_a [a]	k', s^{-1}	$t_{1/2}$, s	k, $l\ mol^{-1}\ s^{-1}$	k_x [b], $l\ mol^{-1}\ s^{-1}$	k/k_x
Gly	9.60	$1.08 . 10^{-2}$	64	2.7	0.123 [c]	21.9
Phe	9.13	$1.27 . 10^{-2}$	54	2.4	0.146 [d]	16.4
Ala	9.69	$0.60 . 10^{-2}$	115	1.6	0.130 [d]	12.3
Val	9.62	$0.79 . 10^{-2}$	87	2.0	0.155 [d]	12.9
Leu	9.60	$1.23 . 10^{-2}$	56	3.1	0.160 [d]	19.4
Ile	9.68	$0.95 . 10^{-2}$	72	2.6	0.142 [c]	18.3
Trp	9.39	$2.75 . 10^{-2}$	25	5.9	–	–
Ser	9.15	$0.39 . 10^{-2}$	174	0.7	0.083 [c]	8.4
Met	9.21	$0.74 . 10^{-2}$	93	1.4	0.087 [c]	16.1
Glu	9.67	$0.25 . 10^{-2}$	271	0.7	0.11 [d]	6.4
Arg	9.04	$0.53 . 10^{-2}$	130	0.9	0.069 [c]	13.0
His	9.17	$0.56 . 10^{-2}$	123	1.1	0.086 [c]	12.8
Thr	10.43	$0.32 . 10^{-2}$	215	2.6	0.121 [c]	21.5
Asn	8.80	$0.31 . 10^{-2}$	220	0.5	0.078 [c]	6.4
Asp	9.82	$0.25 . 10^{-2}$	274	0.8	0.138 [d]	5.8

[a] Taken from ref.[13]; [b] rate constant for reaction of phenyl isothiocyanate with the amino acid; [c] measurement conditions according to ref.[10]; [d] taken from ref.[10].

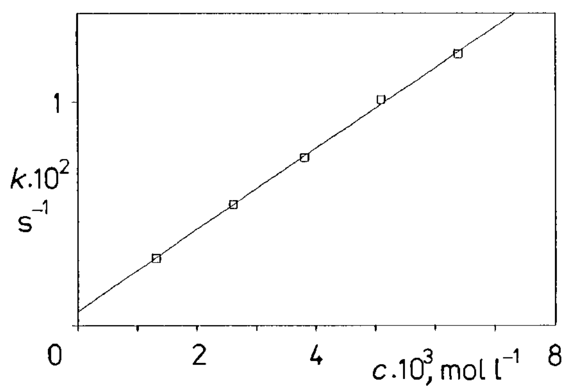


FIG. 3
Effect of glycine concentration (c) on apparent rate constant k' of its reaction with *I*. $k' = A + B\ c$; $A = 0.0673$, $B = 0.1807$, $r = 0.9994$

constants and reaction half-times show that, similarly as observed for phenyl isothiocyanate[14], there is no linear relationship between the reaction rates (k) and basicities of the amino acids (pH) throughout the whole series studied. The lowest reactivity has asparagine, the highest tryptophan which is twelve times more reactive. Because of low solubility of tryptophan in Clark–Lubs buffer, the reaction of tryptophan with isothiocyanate *I* was followed kinetically in concentrations 6.4 . 10^{-4} mol l^{-1} of tryptophan and 6.4 . 10^{-6} mol l^{-1} of *I*. In the reaction of *I* with arginine whose molecule contains two amino groups of different pK_a ($pK_a[\alpha\text{-}NH_2]$ 9.04 and $pK_a[\omega\text{-}NH_2]$ 12.48) we assumed that only the α-NH_2 group reacts, in analogy with our previous finding for the reaction of phenyl isothiocyanate with lysine[15].

From the viewpoint of utilization of 9-isothiocyanatoacridine as a sequence reagent, its reactivity compared with other isothiocyanates plays an important role. For comparison, Table III also includes the results of kinetical measurements with phenyl isothiocyanate[10] used in the Edman degradation of peptides. We supplemented the missing values of rate constants for this reaction by kinetical measurements in a 98 : 2 buffer (pH 9.8)–dioxane mixture at 244 nm and 26 °C according to the literature[10]. As seen from Fig. 4, there is a correlation between the rate constants for the reaction of 9-isothiocyanatoacridine (*I*) and phenyl isothiocyanate (k and k_x, respectively) with amino acids, the compound *I* being 6 – 22 times more reactive than phenyl isothiocyanate. Derivatives of glutamic and aspartic acid are not included in the comparison because their rate constants are outside the correlation, probably due to the presence of the second carboxyl group.

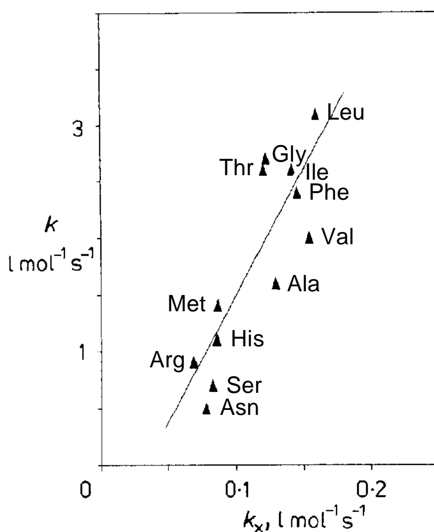


FIG. 4
Correlation of rate constants for reaction of 9-isothiocyanatoacridine (*I*) (k) and phenyl isothiocyanate (k_x) with amino acids. $k = A + B\, k_x$; $A = -0.855$, $B = 23.093$, $r = 0.855$

From the temperature dependence of reaction rates we calculated the activation energy and frequency factor for the reaction of isothiocyanate *I* with alanine (Table IV, Eq. (*1*)). Comparison of these values with the activation energy for reaction of phenyl isothiocyanate[14] or of 2-naphthyl isothiocyanate[14] with, e.g., glycine (E_A = 72.1 and 72.6 kJ mol^{-1}, respectively) shows that the reactions of amino acids with isothiocyanate *I* are faster and energetically less demanding than their reactions with phenyl isothiocyanate or 2-naphthyl isothiocyanate.

TABLE IV
Temperature dependence of rate of reaction of 9-isothiocyanatoacridine (*I*) (6.4 . 10^{-5} mol l^{-1}) with alanine (6.4 . 10^{-4} mol l^{-1}) in Clark–Lubs buffer (pH 9.8)–CH_3CN mixture (7 : 3)

T, K	k', s^{-1}	k, l mol^{-1} s^{-1}
294.15	8.29 . 10^{-4}	2.3
299.15	11.28 . 10^{-4}	3.1
304.15	16.58 . 10^{-4}	4.6
309.15	18.19 . 10^{-4}	5.0
314.15	24.41 . 10^{-4}	6.8

$$\log k = \log A - E_A/19.15\,T \qquad (1)$$

$$\log A = 7.594 \text{ l mol}^{-1} \text{ s}^{-1},\ E_A = 40.6 \text{ kJ mol}^{-1},\ r = 0.987$$

The results of our kinetic and fluorescence measurements promise that 9-isothiocyanatoacridine might be used as a new fluorescence reagent in the determination of peptide and protein structures.

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