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Interaction of cyanine dyes with nucleic acids. 6. Synthesis and spectroscopic properties of thiazole orange—amino acids conjugates

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The synthesis of amino acid (L-Trp and L-Tyr) derivatives of thiazole orange, monomethine cyanine dye which significantly increases fluorescence intensity when bound to nucleic acids is described. Hydroxysuccinimide ester of Cyan 6 (2-[4-N-Carboxyethyl-quinoline)-methyl]-3-methylbenzthiazole-1,4-p-toluenesulfonate) was used for the conjugation of dye with amino acids. Interaction of obtained conjugates with nucleic acids was investigated using spectroscopic methods.

Materials and Methods. The TLC was performed on Kieselgel 60 F_{254} plates («Merck», Germany) using $CHCl_3$—MeOH—AcOH (8.5:1.0:0.5) system. FAB MS analyses were carried out in the positive mode using mass spectrometer MI-1201E (PO «Elektron», Ukraine).

Spectroscopic measurements. The absorption spectra were recorded on «Specord M-40» (VEB Carl Zeiss Jena, Germany). Fluorescence spectra were obtained with fluorescence spectrophotometer Hitachi Model 850 (Japan). Fluorescence was excited with 150W Xe-lamp emission and measurements were carried out in thermostatable quartz cell (0.5 × × 0.5 cm). All spectra were corrected by multiplying fluorescence intensities measured over an interval of 5 nm by proper correction factor for corresponding wavelengths. In corrected spectra fluorescence intensity values were proportional to a numbers of photons per unit of wavelength interval.

Preparation of DNA, RNA and dyes stock solutions. Stock solutions of dyes ($2 \cdot 10^{-3}$ M) were prepared by dissolving dyes in DMSO. All dyes were stable under these conditions for several months, whereas in aqueous solutions some dyes gradually lost their fluorescence properties. Working solutions were prepared immediately prior to use. For spectral studies total calf thymus DNA («Sigma») and yeast RNA («Sigma») were used. Nucleic acids stock solutions were prepared in TE buffer (Tris-HCl, 50 mM, EDTA, 10 mM, pH 8.0) in concentration $6 \cdot 10^{-3}$ b. p./ml for DNA and $1.2 \cdot 10^{-2}$ b. p./ml for RNA.

Absorbance and fluorescent emission spectra. For spectral measurements the complexes of dyes with nucleic acids were obtained in Tris-HCl (50 mM, pH 8.0) buffer by mixing of dye stock solution with DNA or RNA solution. The final concentration of NA was 10^{-4} and $2.0 \cdot 10^{-4}$ mM respectively. Final dyes concentrations were 0.02 mM. Dye—nucleic acid complexes were prepared with approxymal ratio 1 dye per 20 b. p. of RNA and per 10 b. p. of DNA. For optical measurement of free cyanines the same dyes concentrations were used.

Fluorescence titration of Cyan 6—Trp and Cyan 6—Tyr were carried out in 0.05 M Tris buffer (pH 8) with total calf thymus DNA and yeast RNA at 540 nm for DNA and 545 nm for RNA. Fixed concentration of DNA $6 \cdot 10^{-3}$ M and $1.2 \cdot 10^{-2}$ M were used for the titration of fixed concentration of dye (Fig. 3). The dye concentration was 10^{-5} M. The DNA and RNA concentration was changed from $2.5 \cdot 10^{-5}$ to 10^{-4} from $5 \cdot 10^{-6}$ to $2 \cdot 10^{-4}$ respectively.

General procedure for the synthesis cyanine dye—amino acids conjugates. The succinimidyl ester of Cyan 6 was prepared by dissolving the dye (5.34 mg, 0.01 mmol) in 1 ml of dry DMFA and then adding N-hydroxysuccinimide (1.15 mg, 0.01 mmol) and

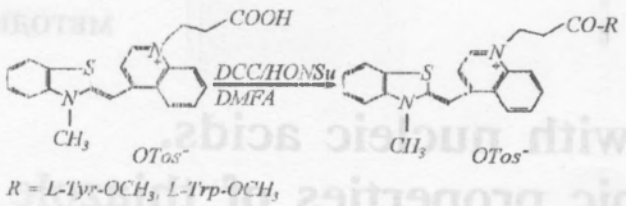


Fig. 1. Synthetic scheme of preparation of dye—amino acid conjugates

DCC (4.12 mg, 0.02 mmol) to stirred solution at 0 °C. Mixture was stirred for 4 h. Then amino acid ester hydrochloride (0.011 mM) and 0.05 ml of dry pyridine were added. Reaction mixture was stirred for 2 h at 0 °C and was kept in refrigerator for 18 h. Purification of amino acid—Cyan 6 conjugates was performed on the prepared C3 stationary phase [1]. Conjugates were recovered from the fractions with a rotary evaporator at 40 °C.

Cyan 6—Trp—OCH3. R_f = 0.67 MS:m/e (relative intensity) 563 (MH+,5), 363 (14), 317 (3), 290 (6), 275 (5), 264 (4), 219 (4), 150 (16).

Cyan 6—Tyr—OCH3. R_f = 0.72 MS:m/e (relative intensity) 540 (MH+,14), 526 (6), 407(7), 363 (27), 317 (7), 303 (7), 290 (12), 275 (9), 219 (6), 150 (18).

Results and Discussion. The cyanine monomethine dyes have many of properties presented as ideal for the fluorescent homogeneous detection of nucleic acids. With this determination principle no separation of unbound probe is necessary [2]. A series of new monomeric and dimeric fluorescent cyanines have recently been developed for this purpose [3, 4]. Monomethine cyanine dyes seem to possess all characteristics for quantitation of unknow nucleic acids in solutions, as low intrinsic fluorescence of unbound probe, large fluorescence enhancement upon probe binding, large linear detection range, and sequence non-specific detection [5].

The main research of our laboratory concerns development of sequence specific homogeneous detection of nucleic acids in solution. Recently pyrylium cyanine dye — oligonucleotide conjugate was used for the detection of specific DNA sequences [6]. Here we describe the preparation of cyanine dye—amino acid conjugates for the potential fluorescence sequence specific determination of nucleic acids in solution.

Before some synthetic procedures for the covalent labelling of biomolecules with cyanine dyes were described by Waggoner et al. [7]. Functional groups in proposed cyanine reagents were sulfhydryl [7], isothiocyanate [8] and succinimidyl esters [9]. Procedure using active N-hydroxysuccinimide esters of dye seems to be more convenient for their conjugation with α-amino group of amino acids or peptides. Besides we have shown recently that incorporation of carboxylic acids groups into basic cyanine structure of TO did not influence on fluorescence probe properties [10] (Fig. 1).

Succinimidyl ester of dye carboxyl group and methyl esters of hydrochlorides of amino acids in pyridine were used to synthesize amino acid—dye conjugates. Active esters were prepared with use the DCC and hydroxysuccinimide (HONSu) in DMFA.

Recently published methods for the HPLC of cyanine dyes use C_{18} stationary phase [11]. Unfortunately, used cyanine dyes are very hydrophobic and positively charged compounds. As a result, they have large retention time on HPLC stationary phases. In order to overcome these problems TMS-silica (C_3) stationary phase prepared according to [1] was successfully used (Fig. 2). Chemical structures of synthesized conjugates were confirmed by fast atom bombardment mass spectrometric (FAB MS) analyses.

Spectroscopic data for two synthesized cyanine conjugates and Cyan 6 (TO analog) are presented in Table. 1. Dye Cyan 6 with previously investigated spectral properties was included for the comparison [9]. Absorption spectra of Cyan 6 an its amino acid

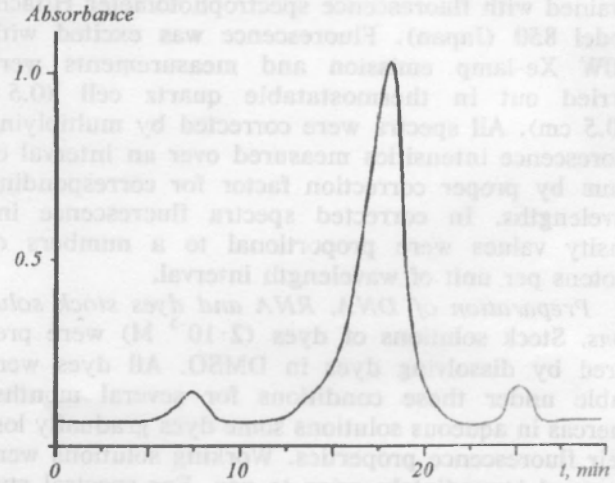
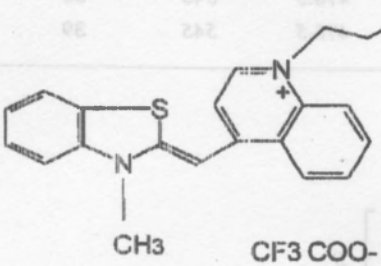
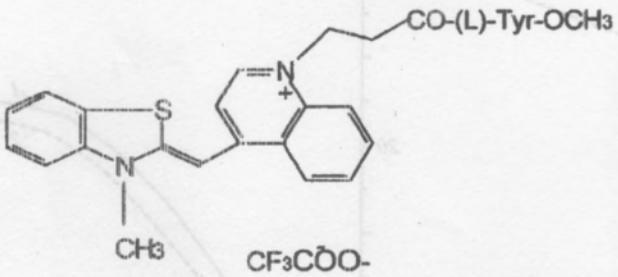
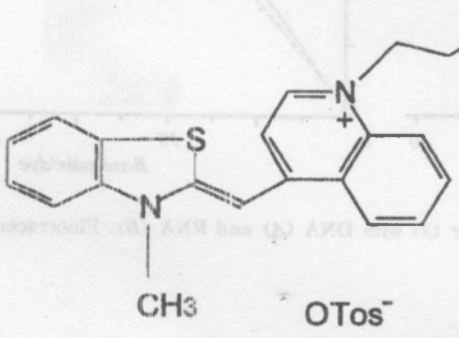


Fig. 2. Chromatogram of reaction mixture of cyanine dye conjugation with L-Trp using C_3 stationary phase. Mobile phase: buffer A 0.05 % TFA, buffer 80 % dioxan. Flow-rate: 5.0 ml/min; λ = 506

Table 1
Chemical structures and spectroscopic properties of cyanine dyes

Dye	*Structure*	$abs_{\lambda max}$ *DMFA [buff.], nm*	$em_{\lambda max}$, *nm*	*ΔS, nm*	*F**
Cyan 6 - Trp		*507 [475, 505]*	**	–	*0.076*
Cyan 6 - Tyr		*506 [470, 506]*	–	–	*0.062*
Cyan 6		*503 [475, 506]*	–	–	*0.03*

*Arbitrary units; **There is no clear maxima.

derivatives were similar. The wavelengths of absorption maxima ($^{abs}\lambda_{max}$) of Cyan 6—Trp and Cyan 6—Tyr slightly depended on solvent. Cyanine dyes showed the red shift (5—7 nm) after going from aqueous buffer to less polar DMSO.

The fluorescence of conjugates and free Cyan 6 were very low and fluorescence spectra had no clear maxima. Fluorescence intensity of amino acids derivatives were slightly higher as compared to free dye.

The data on the absorbance and fluorescence

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Взаємодія ціанінових барвників з нуклеїновими кислотами. 6. Синтез та спектроскопічні властивості кон'югатів тіазолового оранжевого з амінокислотами

Резюме

Описано синтез амінокислотних похідних (L-Trp та L-Tyr) з тіазоловим оранжевим — монометиновим ціаніновим барвником, що значно підвищує інтенсивність власної флюоресценції при зв'язуванні з нуклеїновими кислотами. Гідроксисукцинімідний ефір Суан 6 (2-[-(N-карбоксиетил-хінолін)-метил]-3-метилбензтіазол-1,4-п-толуолсульфата) був використаний для кон'югації. Взаємодію синтезованих кон'югатів з нуклеїновими кислотами досліджено спектроскопічними методами.

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Взаимодействие цианиновых красителей с нуклеиновыми кислотами. 6. Синтез и спектроскопические свойства конъюгатов тиазолового оранжевого с аминокислотами

Резюме

Описан синтез аминокислотных производных (L-Trp и L-Tyr) с тиазоловым оранжевым — монометиновым цианиновым красителем, который значительно увеличивает интенсивность собственной флюоресценции при связывании с нуклеиновыми кислотами. Гидроксисукцинимидный эфир Суан 6 (2-[-(N-карбоксиэтил-хинолин)-метил]-3-метилбензтиазол-1,4 п-толуолсульфата) был использован для конъюгации красителя с аминокислотами. Взаимодействие синтезированных конъюгатов с нуклеиновыми кислотами исследовано спектроскопическими методами.

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