ELSEVIER

Dyes and Pigments 51 (2001) 41–49

DYES and PIGMENTS

www.elsevier.com/locate/dyepig

Proteins and cyanine dyes. Part III. Synthesis and spectroscopic studies of benzothiazolo-4-[1,2,6-trimethylpyridinium] monomethine cyanine dyes for fluorescent detection of bovine serum albumin in solutions

Sergiy M. Yarmoluk*, Dmytro V. Kryvorotenko, Anatoliy O. Balanda, Mykhaylo Yu. Losytskyy, Vladyslava B. Kovalska

Institute of Molecular Biology and Genetics, Ukrainian National Academy of Sciences, 150, vul. Zabolotnoho, Kyiv, 03143, Ukraine

Received 20 March 2001; received in revised form 5 July 2001; accepted 8 August 2001

Abstract

The spectral-luminescent properties of a series of new cyanine dyes as possible probes for homogeneous assay of proteins have been studied. The fluorescent cyanine dyes development was based on the principle of "affinity-modifying group" with the use of benzothiazolo-4-[1,2,6-trimethylpyridinium] monomethine as template. It was shown that the cyanine dye **P-5** can be used as a sensitive and specific fluorescent probe for the detection of BSA. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Cyanine dyes; Fluorescent probes; Protein detection; Homogeneous assay

1. Introduction

The spectral changes observed on the binding of fluorophores with proteins are an important tool for the investigations of the topology of binding sites, conformational changes and characterization of substrate to ligand binding [1]. Besides, determination of protein quantity in biological liquids is of great importance in biology and medicine [2] and fluorescent probes are successfully applied for this approach [3].

Albumins, especially bovine (BSA) and human (HSA) sera albumins labeled with fluorescent probes are commonly used as the model systems for the investigations of surface-induced conformational changes in protein interfaces [1]. From biopharmaceutical point of view one of the most important biological functions of albumins is their ability to carry drugs as well as endogenous and exogenous substances, and numerous experiments with the aim to characterize the binding capacity and sites of albumins have been carried out [1,2].

Only a few cyanine dyes are used for the homogeneous assay of proteins. The best of the proposed probes are Nano Orange and Albumin Blue [3], besides, anionic cyanine dyes AB633 and AB620 are described as probes for HSA detection

* Corresponding author. Tel.: +38-44-2522389; fax: +38-44-2522458.

E-mail address: otava@carrier.kiev.ua (S.M. Yarmoluk).

PII: S0143-7208(01)00055-9

[2]. Also, it was shown [4] that symmetrical carbocyanine dye 3,3′-dipropylthiacarbocyanine interact with BSA with significant increase of fluorescence intensity. Recently we proposed new carbocyanine dye **14K** for BSA and lectine detection [5].

Earlier [6] we developed the principle of the "affinity-modifying groups" in the design of fluorescent probes for homogeneous assay of nucleic acids. Such groups slightly distort chromophore π-electron system but influence on the dye binding with biopolymers and thus on fluorescent properties of dye-biopolymer complexes. This principle of the "affinity-modifying groups" for the designing of fluorescent probes in the protein study was applied in our research. Previously [6] it was shown that the **D-8** dye (Scheme 1) that contains the indole fragment, contrarily to **Cyan 40** (Scheme 1) increases significantly the fluorescence intensity in the presence of BSA. Thus, we considered that incorporation of a polycyclic aromatic fragment into a cyanine molecule could increase the dye affinity to proteins. The purpose of this paper is to present some novel cyanine dyes based on **Cyan 40** and modified with various aromatic polycyclic templates. Spectral-luminescent properties of the dyes in the presence of proteins and nucleic acids were studied.

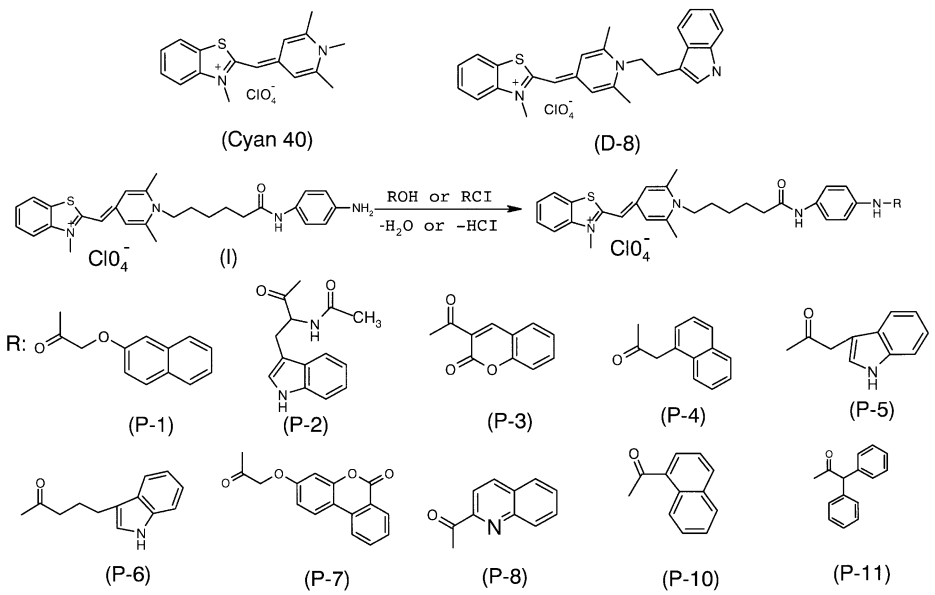
2. Results and discussion

Methods of the incorporation of "affinity-modifying groups" into the cyanine template described in literature are less numerous. The reaction of halogenalkyl derivatives of cyanines and activated esters of cyanines containing carboxyl group with different nucleophiles was used [7]. Recently, we proposed some novel suitable procedures of cyanine dye modification for the development of probes with "affinity-modifying groups" [7]. In this paper we present the method of cyanine dye modification with aminogroup. The structures of the dyes obtained using this method are presented in Scheme 1.

To incorporate an "affinity-modifying groups" into chromophore, the dye (**I**) was acylated by acid chloroanhydrides in the presence of pyridine or by free acids in the presence of carbonyldiimidasole (CDI) (Scheme 1). The proposed procedure allows preparation of the dyes with high yields (Appendix).

2.1. Spectral properties of free dyes

Characteristics of absorption and fluorescent spectra of free dyes and their nucleic acids com-



Scheme 1. The scheme of the reaction and structures of the novel dyes.

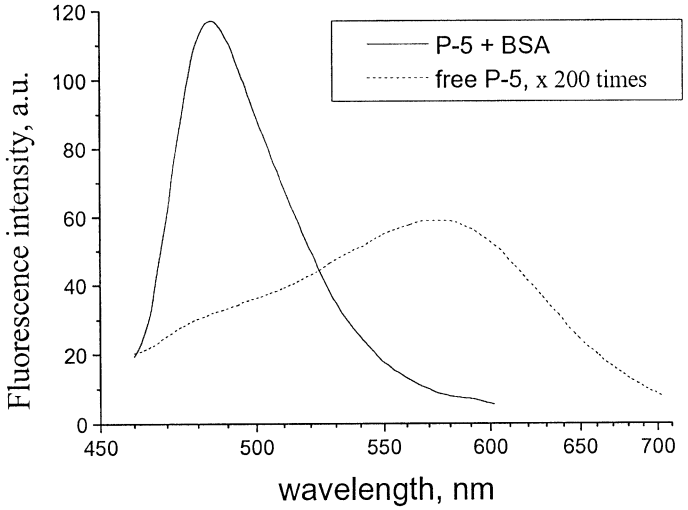
plexes are presented in Table 1. The maximum in the absorption spectra of dyes in dimethylformamide (DMF) located at 443–446 nm is observed. The values of molar extinction coefficients are in the range from $0.83 \cdot 10^5$ M^{-1} cm^{-1} to $1.1 \cdot 10^5$ M^{-1} cm^{-1}. In the aqueous buffer absorption maxima of dyes are hypsochromically shifted on 3–18 nm and coefficients of molar extinction values decrease in 1.5–4 times as compared with the corresponding DMF solutions. For all cyanines also the widening of absorption bands in comparison with DMF is observed. For some of the dyes (**P-1**, **P-5**, **P-7**) a new shoulder close to the main band arises. We consider such behavior of the dyes in polar aqueous solution to be the evidence of the dye aggregate formation [8].

The emission maxima of most dyes in buffer are located in the 560–581 nm range except the **P-2** and **D-8** dyes, fluorescence maxima of which are situated at 486 and 484 nm, respectively. The **P-5** dye fluorescence spectrum is presented in Scheme 2. The decrease of the dye concentration causes the vanishing of long-wave fluorescence maximum and appearance of a new maximum near 485 nm. We consider that the long-wave band corresponds to the emission of the dye aggregates and short-wave one—to the monomer emission of its molecule [8]. This suggestion is supported by the excitation

Table 1
The characteristics of absorption and fluorescence spectra of the dyes ($pH_{buffer} = 7.9$)

Dye	Absorption of dyes						Fluorescence of dyes					
	Free				In the presence of 1 mg/ml of BSA		Free in the buffer			In the presence of 0.1 mg/ml of BSA		
	In DMF		In the buffer									
	λ_{abs} (nm)	$\varepsilon \cdot 10^{-5}$ (M^{-1} cm^{-1})	λ_{abs} (nm)	$\varepsilon \cdot 10^{-5}$ (M^{-1} cm^{-1})	λ_{abs} (nm)	$\varepsilon \cdot 10^{-5}$ (M^{-1} cm^{-1})	λ_{fl} (nm)	ΔS^{fd} (nm)	I_0 (a.u.[a])	λ_{fl} (nm)	I^{BSA} (a.u.[a])	ΔQ
P-1	443	0.94	429 452[a]	0.42	428	0.46	579	150	0.72 0.12[b]	483	1.2	10
P-2	443	0.85	440	0.51	440	0.57	486	46	0.47[b]	482	1.57	3.3
P-3	445	0.92	441	0.37	444	0.43	~560	~119	0.055 0.025[b]	481	0.083	3.3
P-4	444	0.88	430	0.22	449 437[a]	0.44	581	151	0.40 0.12[b]	487	11.7	97.5
P-5	444	1.12	427 448[a]	0.38	451 431[a]	0.45	575	141	0.29 0.16[b]	484	18.4	115
P-6	444	0.97	437	0.61	439	0.59	575	138	0.36 0.2[b]	482	1.18	5.9
P-7	444	0.88	426 455[a]	0.36	427 454[a]	0.35	581	155	0.6 0.12[b]	587	0.6 0.32[b]	2.7
P-8	444	0.97	438	0.38	446 431[a]	0.41	575	137	0.12 0.088[b]	483	0.8	9.1
D-8	446	0.74	443	0.7	444	0.59	484	41	0.32	486	2.95	9.2
P-10	444	0.98	432	0.38	428 446[a]	0.52	580	148	0.175 0.15[b]	482	1.55	10.3
P-11	444	0.83	427	0.35	442 427[a]	0.47	571	144	0.38 0.17[b]	486	8.5	50

[a] Shoulder.
[b] Fluorescence intensity of a free dye at the λ_{max} of its fluorescence in the presence of the protein.



Scheme 2. Profiles of fluorescence spectra of dye **P-5** (10^{-5} M) free (- - - -) and in the presence of 1 mg/ml of BSA (—). The low-intensity spectrum of free dye is multiplied 200 times (a.u., arbitrary units).

spectra of dyes (data not presented). Intrinsic fluorescence intensities of all the dyes are rather low (0.055–0.72 arbitrary units (a.u.)) (Table 1).

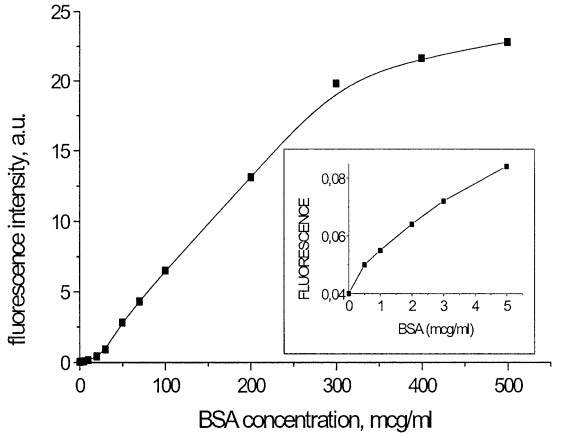
2.2. Spectral properties of the dyes in the presence of BSA

Maxima of the dyes absorption in the presence of BSA are situated over the range 427–451 nm (Table 1), extinction coefficients for all dyes except **P-6** and **P-7** enhance relative to the free dyes in buffer. Fluorescence maxima of the dyes are spaced in the range from 481 to 487 nm (except **P-7**, fluorescence maximum of which is situated at 587 nm). In Scheme 2 the fluorescence spectrum of the **P-5** dye in the presence of BSA is shown.

For most of the studied dyes binding with proteins takes place with a significant blue shift of fluorescence maximum, that provides possibility of the easy separation of the fluorescent contribution from the free and bound probe (Scheme 2). Similar effect observed for Prodan–albumin complexes was caused by the sensitivity of the dye to the polarity of the environment [9]. For the cyanine such behavior is connected with their ability to form aggregates in the water solutions. As usual, the distance between position of fluorescent red-shifted maximum of aggregates and "monomer" band maximum is near to 100 nm. Thus the formation of aggregates decreases the intensity of monomer fluorescence. The interaction with biopolymers leads to the breaking of aggregates and to the fixation of "monomer" form of the dye and this way to the significant increasing of monomer fluorescence intensity.

The fluorescence intensity enhancement (ΔQ) is defined as the ratio between the bound to protein dye fluorescence intensity and intensity of a free dye fluorescence measured on the wavelength of dye–protein complex emission maximum ($\Delta Q = I^{BSA}/I_0$). At the 0.1 mg/mL BSA concentration for studied dyes fluorescence intensity enhancement (ΔQ) in 3.3–115 times was observed. Thus we consider that the ability of the dyes to aggregate in free state can increase signal/noise ratio and this way the sensitivity of detection (Table 1). We believe that incorporation of the groups, which can increase the ability of dye to aggregate in the free state, can be used as one of the ways of obtaining the high-fluorescent probes for proteins detection.

The titration of the fixed 10^{-6} M concentration of the dye **P-5** with the protein has shown that the fluorescence intensity enhancement relates linearly to the BSA concentration over the range from 5 to 300 mcg/ml. It corresponds approximately to the



Scheme 3. The plot of fluorescence titration of 10^{-6} M of the **P-5** dye with BSA.

range from $8{\cdot}10^{-8}$ to $4.5{\cdot}10^{-6}$ M concentration of the protein (Scheme 3). So, in this range accurate determination of the protein concentration is possible.

2.3. Spectral properties of the dyes in the presence of other proteins

The spectral-luminescent properties of the dyes were studied in the presence of HSA, lysozyme, trypsin, hemoglobin and cytochrome C (Table 2). In the presence of HSA the fluorescence intensity enhancement of the **P-4**, **P-7**, **P-8**, **P-10** and **D-8** dyes runs up to 10–17 times. The HSA presence does not change the fluorescence intensity of the **P-1**, **P-2**, **P-3** and **P-6** dyes. The presence of the other proteins insignificantly increases the emission intensity of all the studied dyes (no more than by two times). So, the **P-8**, **P-10** and **D-8** dyes enhance fluorescence intensity in the presence of BSA and HSA by one order, while the **P-4** and **P-5** show a significant specificity to BSA.

2.4. Dependence of the dye fluorescence in the presence of a protein on the environment pH value

It is known that changes in the pH value can affect on electrostatic charge of a protein molecule and thus on the ability of protein to bind with a charged probe. Therefore we investigated the dependence of the emission intensity of the dye–protein complex on the pH value for the **P-5** dye (Table 3). For the dye complexes with BSA and HSA the ΔQ values increase at the basic pH. At acidic environment the decrease of ΔQ values is observed. Particularly, in the pH = 11.5 buffer the **P-5** dye in the presence of the 1 mg/ml BSA concentration enhances the intensity of its fluorescence at 1060 times. Therefore, we consider that the pH value influences the interaction between the **P-5** dye and albumins. In the presence of the other proteins enhancement of the dye fluorescence intensity with changing of pH is slight (no more than 1.2 times) (Table 3).

2.5. The fluorescence intensity of dyes in the presence of denatured proteins

To establish the difference between the interaction modes of the **P-5** and **D-8** dye the behavior of the dyes in the presence of native and denatured proteins was studied.

The BSA denaturation leads to the decreasing of the **P-5** dye ΔQ value from 131 to 34.8, and in the same time the denaturation of HSA increased ΔQ

Table 2
The change of fluorescence intensity ΔQ of dyes in the presence of 0.1 mg/ml of proteins

Dye	BSA		HSA		Trypsin		Lysozyme		Hemoglobin		Cytochrome	
	I	ΔQ	I	ΔQ	I	ΔQ	I	ΔQ	I	ΔQ	I	ΔQ
P-4	11.7	97.5	1.38	11.5	0.13	1.1	0.15	1.3	0.1	0.83	0.11	0.9
P-5	18.4	131	1.86	13.2	0.16	1.1	0.17	1.2	0.13	0.9	0.14	1.0
P-7	0.32	2.7	1.23	10.3	0.1	0.83	0.14	1.2	0.08	0.67	0.11	0.9
P-8	0.8	9.1	1.48	16.8	0.11	1.25	0.1	1.14	0.08	0.91	0.07	0.8
D-8	2.95	8.94	4.17	12.6	0.4	1.21	0.41	1.24	0.34	1.03	0.31	0.94
P-10	1.55	10.3	2.5	16.7	0.18	1.2	0.19	1.27	0.14	0.92	0.15	1.0

Table 3
Dependence of fluorescence intensity increase ΔQ of the **P-5** dye in the presence of native (n) and denatured (dn) proteins on the buffer pH value

pH	I_0 (a.u.)	ΔQ, the dye in the presence of 0.1 mg/ml of a protein										
		BSA		HSA		Trypsin		Lysozyme		Hemoglobin	Cytochrome C	
		n	dn	n	dn	n	dn	n	dn	n	n	dn
4.8	0.3	16.8	18.3	2.73	13.22	1	–	1.05	–	0.8	0.9	–
7.9	0.14	131	34.8	13.2	33.9	1.1	1.4	1.2	7.6	0.9	1.0	1.1
11.5	0.15	211	–	40.7	–	1.1	–	1.2	–	0.6	1.0	–

Table 4
The fluorescence intensity enhancement (ΔQ) of dyes in the presence of DNA and RNA

	P-1	**P-2**	**P-3**	**P-4**	**P-5**	**P-6**	**P-7**	**P-8**	**D-8**	**P-10**	**P-11**
RNA	16.3	14.6	20.4	28.9	23.1	85.0	40.3	98.9	256.3	134.0	88.2
DNA	34.2	43.8	53.6	51.7	68.8	174.0	84.2	177.3	107.5	238.7	223.5

value from 13.2 to 33.9 (Table 3). It could be inferred that the dye interacts with the BSA globule at a specific site, which is formed at the level of the protein tertiary structure. Denaturaton destroys this site and results in decrease of the ΔQ value. By contrast, with the HSA molecule the **P-5** dye interacts unspecifically and binds with the negatively charged surface of protein via electrostatic, hydrophobic and van der Waals forces. The denaturation unfolds the polypeptide chain and thus the number of the "binding sites" increases. The increasing of the viscosity under denaturation can also partially cause the increasing of fluorescent intensity value.

The ΔQ value of the **D-8** dye increases on transition from the native to the denatured protein (BSA and HSA). Therefore, we concluded that the **D-8** dye interacts unspecifically with these two proteins, which accounts for its approximately equal ΔQ values in these two cases (Table 3).

2.6. Influence of DNA and RNA on the dyes fluorescence

The increase of the fluorescence intensity values of the dyes in the presence of 40 mcg/ml of DNA and RNA varies in the range from 14.6 to 257. In particular, for the **P-4** and **P-5** dyes the fluorescence intensity increased up to 51.7 and 68.8 times respectively in the presence of DNA, in 28.9 and 23.1 times in the presence of RNA. It means that nucleic acid admixtures of rather high concentration could substantively influence the results of the quantitative estimation of BSA by these dyes (Table 4).

2.7. What is the correlation between dye structure and its affinity to protein?

The fluorescence enhancement values for the **P-4**, **P-5** and **P-11** in complexes with BSA are significantly higher than for the other dyes. What structural peculiarities of these dyes are responsible for their property? It was suggested that the dye binding with protein is affected by the distance between cyanine nucleus and a cyclic fragment more strongly, than by the nature of this fragment. Dyes **P-4**, **P-5** and **P-11** (Scheme 1) have different cyclic templates of "affinity-modifying groups", but contain the residue of arylacetic acid. We believe that the presence of this fragment in the dye structure can cause the appearance of the affinity to proteins (Table 1).

3. Conclusions

Nine new monomethyne cyanine dyes with polycyclic "affinity-modifying groups" were obtained by the modification of cyanine with primary amino group 2-1-[5-(4-aminophenylcarbamoyl)pentyl]-2,6-dimethyl-1,4-dihydro-4-pyridinylidenmethyl-3-methyl-l,3-benzothiazol-3-ium perchlorate by residues of polycyclic aromatic carboxylic acids.

Two of the dyes, namely **P-4** and **P-5**, were revealed as specific fluorescent probes for the BSA detection. In the presence of BSA the **P-5** dye can enhance its fluorescence intensity up to three orders. In the same time **P-4** and **P-5** slightly increase their emission in the presence of other proteins.

On the basis of the presented results, we suggested that the **P-5** dye interact with BSA via its specific site formed at the level of the protein tertiary structure.

4. Experimental

4.1. Materials

Anhydrous DMF distilled under reduced pressure and anhydrous pyridine distilled over potassium hydride—from "Merck"; CDI, 3-indolylbutanic acid, 1-naphtylacetic acid, 3-indolylacetic acid, 2-quinolinecarbonic acid, 1-naphtoic acid, 2-naphtoxyacetic acid, *N*-acetyltryptophan, Tris(oxymethyl)-aminomethane hydrochloride (Tris)—from "Aldrich" were used.

3-Hydroxy-6*H*-benzo[*c*] chromen-6-on was obtained according to [10]. 2-(6-*Oxo*-6*H*-benzo[*c*]chromen-3-yloxy) acetic acid was obtained by the alkylation with the ethyl chloroacetate in DMF with potassium carbonate. The synthesized ester was hydrolyzed by heating in the mixture with acetic and sulfuric acid (10:1).

Chloranhydrides of 2-naphthoxyacetic acid, 3-coumarincarbonic acid, 1-naphthoic acid were synthesized according to the standard procedure with thionyl chloride used [11].

The **D-8** dye (2-1-[2-(1*H*-3-indolyl)ethyl]-2,6-dimethyl-1,4-dihydro-4-pyridinylidenmethyl-3-methyl-1, 3-benzothiaol-3-ium perchlorate) was obtained in accordance with [4], and 2-1-[5-(4-aminophenylcarbamoyl)pentyl]-2,6-dimethyl-1,4-dihydro-4-pyridinylidenmethyl-3-methyl-1,3-benzothiazol-3-ium perchlorate (I) was synthesized according to [12].

In spectroscopic experiments we used total DNA from chicken erythrocytes, total yeast RNA, BSA, hemoglobin, lysozyme, HSA, trypsin, cytochrome C from "Aldrich", "Sigma". The proteins were denatured by the heating of their solutions at 90 °C during 45 min.

4.2. Spectroscopic measurements

Absorption spectra were recorded on spectrophotometer Specord M-40 (Germany). Fluorescence spectra were registered on spectrofluorimeter Hitachi 850 (Japan). Fluorescence spectra were measured in quartz cell (1×1 cm). The ^{1}H NMR spectra were recorded in DMSO-d_6 using the "Varian" (300 MHz) instrument with TMS as an intrinsic standard, coupling constants are quoted in Hz.

4.3. The preparation of stock solutions of nucleic acids and dyes

The $2{\cdot}10^{-3}$ M dyes stock solutions were prepared by dilution of the dye in DMF. Stock solutions of proteins and nucleic acids were prepared by dilution of a biopolymer in a buffer. Their concentrations were 2 mg/ml for proteins and 4 mg/mL for nucleic acids. The Tris·HCl (pH = 7.9), acetate (pH = 4.8) and hydrophosphate (pH = 11.5) buffers were used.

4.4. Preparation of working solutions

Working solutions of the free dyes and dye–protein or dye–nucleic acids complexes were prepared immediately before the experiments by mixing of an aliquot of dye stock solution and an aliquot of respective biopolymer in a buffer. The concentrations of the dye solutions in the cell were 10^{-5} M. The proteins concentrations in working solutions were 0.1 mg/ml and the nucleic acids concentrations were 40 mcg/ml.

4.5. Procedure of *P-1*, *P-3*, *P-10* and *P-11* syntheses

To the solution of 0.287 g (0.5 mM) of dye (I) in 1 ml DMF and 0.5 ml of dry pyridine 0.5 mM of

acid chloroanhydride were added. The mixture was left for 2 h at room temperature, then added 6 ml of water and filtered. The product was crystallized from alcohol (Appendix).

4.6. Procedure of syntheses of the **P-2** and **P-4–P-8** dyes with CDI used

The scheme of the reaction and structures of the compounds obtained are presented in Scheme 1. A mixture of an acid (0.55 mM) and 1 ml of anhydrous DMF at 40 °C was added by CDI (0.55 mM). 0.287 g (0.5 mM) of a dye was added to the mixture in 5 min and the solution was left for 24 h. Then 6 ml of water was added and precipitate was filtered. A product was crystallized from alcohol (Appendix).

Acknowledgements

This work was sponsored by the United States Department of Energy, with funding from the Initiatives for Proliferation Prevention (IPP) Program, and was performed under the terms and conditions of Material Support Agreement No. B507077 between the Institute of Molecular Biology and Genetics, Kyiv, Ukraine, and the University of California, Lawrence Livermore National Laboratory, Livermore, CA, USA.

Appendix. Characteristics of ^{1}H NMR spectra, melting points and yield values of synthesized dyes

P-1 2-(2,6-dimethyl-1-5-[4-(2-napthyloxymethylcarboxamido) phenylcarbamoyl]pentyl-1,4-dihydro-4-pyridinylidenmethyl)-3-methyl-1,3-benzothiazol-3-ium perchlorate. Yield 87%; mp: 123–125 °C; δ_H (DMSO-d_6) 1.45 (2H, *m*), 1.72 (2H, *m*), 1.80 (2H, *m*) 2.34 (2H, *t*, $J=6.3$), 2.68 (6H, *s*), 3.68 (3H, *s*), 4.79 (2H, *s*), 6.03 (1H, *s*), 7.22 (2H, *s*), 7.24–7.39 (4H, *m*), 7.44–7.60 (7H, *m*) 7.78–7.79 (4H. *m*), 9.87 (1H, *s*), 10.07 (1H, *s*).

P-2 2-[1-(5-4-[2-(1*H*-3-indolyl)-1-methylcarboxamidoethylcarboxamido] phenyylcarbamoylpentyl)-2,6-dimethyl-1,4-dihydro-4-pyridinylidenmethyl]-3-methyl-1,3-benzothiazol-3-ium perchlorate. Yield 77%; mp: 145–147 °C; δ_H (DMSO-d_6) 1.43 (2H, *m*), 1.69 (2H, *m*), 1.76 (2H, *m*), 1.82 (3H, *s*), 2.33 (2H, *t*, $J=7.5$), 2.68 (6H, *s*), 3.68 (3H, *s*), 2.99–3.11 (2H, *m*), 4.15 (2H, *t*, $J=7.9$), 4.67 (1H, *q*, $J=6.9$), 6.03 (1H, *s*), 6.97 (1H, *t*, $J=9.2$), 7.06 (1H, *t*, $J=9.2$), 7.16 (1H, *d*, $J=3.5$), 7.22 (2H, *s*), 7.22–7.33 (2H, *m*), 7.5l (6H, *m*), 7.65 (1H, *d*, $J=11$), 7.86 (1H, *d*, $J=9.2$), 8.20 (1H, *d*, $J=11$), 9.82 (1H, *s*), 10.80 (1H, *s*).

P-3 2-(2,6-dimethyl-l-5-[4-(2-oxo-2*H*-3-chromenylcarboxamido)phenylcarbamoyl]pentyl-1,4-dihydro-4-pyridinylidenmethyl)-3-methyl-1, 3-benzothiazol-3-ium perchlorate. Yield 81%; mp: 148–150 °C; δ_H (DMSO-d_6) 1.46 (2H, *m*), 1.73 (4H, *m*), 2.36 (2H, *t*, $J=6.3$), 2.68 (6H, *s*), 3.68 (3H, *s*), 4.19 (2H, *t*, $J=7.2$), 6.02 (1H, *s*), 7.21 (2H, *s*), 7.25 (1H, *t*, $J=7.5$), 7.42–7.56 (4H, *m*), 7.60 (2H, *d*, $J=8.5$), 7.63 (2H, *d*, $J=8.5$), 7.75–7.85 (2H, *m*), 7.99 (1H, *d*, $J=7.5$), 8.88 (1H, *s*), 9.90 (1H, *s*), 10.57 (1H, *s*).

P-4 2-(2,6-dimethyl-1-5-[4-(1-naphthylmethylcarboxamido) phenylcarbamoyl]pentyl-1,4-dihydro-4-pyridinylidenmethyl)-3-methyl-1,3-benzothiazol-3-ium perchlorate. Yield 84%; mp: 133–135 °C; δ_H (DMSO-d_6) l.44 (2H, *m*), 1.70 (4H, *m*), 2.33 (2H, *t*, $J=5.6$), 2.66 (6H, *s*) 3.66 (3H, *s*), 4.12 (2H, *s*), 4.16 (2H, *m*), 6.01 (1H, *c*), 7.19 (2H, *s*), 7.27 (1H, *t*, $J=9.2$), 7.49–7.52 (10H, *m*), 7.85 (2H, *m*), 7.95 (1H, *d*, $J=9.1$), 8.13 (1H, *d*, $J=9.1$), 8.86 (1H, *s*), 10.27 (1H, *s*).

P-5 2-(1-5-[4-(1*H*-3-indolylmethylcarboxamido) phenylcarbamoyl]pentyl-2, 6-dimethyl-1, 4-dihydro-4-pyridinylidenmethyl)-3-methyl-1,3-benzothiazol-3-ium perchlorate. Yield 79%; mp: 147–149 °C; δ_H (DMSO-d_6) 1.43 (2H, *m*), 1.65 (4H, *m*), 2.32 (2H, *t*, $J=4.4$), 2.66 (6H, *s*), 3.66 (3H, *s*), 3.70 (2H, *s*), 4.17 (2H, *m*), 6.01 (1H, *s*), 6.98 (1H, *t*, $J=6.3$), 7.07 (1H, *t*, $J=6.3$), 7.20–7.36 (5H, *m*), 7.51–62 (7H, *m*), 7.86 (1H, *d*, $J=7.5$), 9.84 (1H, *s*), 10.02 (1H, *s*), 10.91 (1H, *s*).

P-6 2-[1-(5-4-[3-(1*H*-3-indolyl)propylcarboxamido]phenylcarbamoylpentyl) - 2,6 - dimethyl - 1,4-dihydro-4-pyridinylidenmethyl]-3-methyl-1,3-benzothiazol-3-ium perchlorate. Yield 76%; mp: 120–122 °C; δ_H (DMSO-d_6) 1.47 (2H, *m*), 1.69 (4H, *m*) 1.98 (2H, *m*), 2.33 (6H, *m*), 2.67 (6H, *s*), 3.68 (3H, *s*) 4.17 (2H, *t*, $J=6.9$), 6.02 (1H, *s*), 6.98 (1H, *t*, $J=6.3$), 7.07 (1H, *t*, $J=6.3$), 7.20–7.36 (5H, *m*),

7.51–62 (7H, *m*), 7.86 (1H, *d*, $J=7.5$), 9.84 (1H, *s*), 10.02 (1H, *s*), 10.91 (1H, *s*).

P-7 2-(2,6-dimethyl-1-5-[4-(6-oxo-6*H*-benzo[*c*]-chromen-3-yloxymethylcarboxamido)phenylcarbamoyl]pentyl-1,4-dihydro-4-pyridinylidenmethyl)-3-methyl-1, 3-benzothiazol-3-ium perchlorate. Yield 82%; mp: 185–187 °C; δ_H (DMSO-d_6) 1.42 (2H, *m*), 1.73 (4H, *m*), 2.37 (2H, *t*, $J=5.2$), 2.67 (6H, *s*) 3.67 (3H, *s*), 4.18 (2H, *s*), 6.01 (1H, *s*), 7.20 (2H, *s*), 7.27 (1H, *t*, $J=8.3$), 7.49–7.61 (7H, *m*), 7.83 (1H, *d*, $J=8.7$), 7.92 (1H, *t*, $J=8.3$), 8.20 (1H, *d*, $J=8.7$), 9.94 (1H, *s*), 10.18 (1H, *s*).

P-8 2-(2,6-dimethyl-1-5-[4-(2-quinolylcarboxamido)phenylcarbamoyl]pentyl-1,4-dihydro-4-pyridinylidenmethyl)-3-methyl-1,3-benzothiazol-3-ium perchlorate. Yield 79%; mp: 250–252 °C; δ_H (DMSO-d_6) 1.49 (2H, *m*), 1.74 (3H, *m*), 2.37 (2H, *t*, $J=5.2$), 2.68 (6H, *s*), 3.67 (3H, *s*), 4.18 (2H, *t*, $J=7.5$), 6.01 (1H, *s*), 7.20 (2H, *s*), 7.25 (1H, *t*, $J=8.0$), 7.50 (2H, *m*), 7.64 (2H, *d*, $J=9.0$), 7.76 (1H, *t*, $J=8.0$), 7.82–7.96 (4H, *m*), 8.11 (1H, *d*, $J=9.0$), 8.20 (1H, *d*, $J=9.3$), 8.24 (2H, *m*), 8.61 (1H, *d*, $J=9.3$), 9.94 (1H, *s*), 10.66 (1H, *s*)

P-10 2-(2,6-dimthyl-1-5-[4-(1-naphthylcarboxamido)phenylcarbamoyl]pentyl-1,4-dihydro-4-pyridinylideninmethyl)-3-methyl-1,3-benzothiazol-3-ium perchlorate. Yield 88%; mp: 136–138 °C; δ_H (DMSO-d_6) 1.47 (2H, *m*), 1.76 (4H, *m*), 2.39 (2H, *t*, $J=4.9$), 2.68 (6H, *s*), 3.67 (3H, *s*), 4.18 (2H, *m*), 6.01 (1H, *s*), 7.20 (2H, *s*), 7.27 (1H, *t*, $J=8.0$), 7.49–7.61 (7H, *m*), 7.72 (3H, *m*), 7.83 (1H, *d*, $J=8.3$), 8.00–8.09 (2H, *m*), 8.20 (1H, *m*), 9.91 (1H, *s*), 10.50 (1H, *s*).

P-11 2-1-[5-(4-benzhydrylcarboxamidophenylcarbamoyl)pentyl]-2,6-dimethyl-1,4-dihydro-4-pyridinylidenmethyl-3-methyl-1,3-benzothiazol-3-ium perchlorate. Yield 85%; mp: 139–141 °C; δ_H (DMSO-d_6) 1.44 (2H, *m*), 1.68 (4H, *m*), 2.33 (2H, *m*), 2.67 (6H, *s*), 3.67 (3H, *s*), 4.17 (2H, *m*), 5.15 (1H, *s*), 6.02 (1H, *s*), 7.2–7.55 (19H, *m*), 7.84 (1H, *d*, $J=8.3$), 9.86 (1H, *s*), 10.36 (1H, *c*).

References

[1] Moreno F, Cortijo M, Gonzalez-Jimenez J. Interaction of acrylodan with human serum albumin. A fluorescence spectroscopic study. Photochemistry and Photobiology 1999;70:695–700.

[2] Kessler M, Wolfbeis O. Laser-induced fluorometric determination of albumin using longwave absorbing mohecular probes. Anal Biochem 1992;200:254–9.

[3] Haughland R. Molecular probes. Handbook of fluorescent probes and research chemicals. Molecular Probes, Inc.. Eugene, OR, 1996:679.

[4] Mishra A, Behera R, Behera P, Mishra B, Behera G. Cyanines during the 1990s: a review. Chemical Review 2000;100:1973–2011.

[5] Yarmoluk S. Asymmetric carbocyanine 14K dye—a new fluorescent probe for a homogeneous detection of proteins in solution. Biopolymers and Cell 2000;16:236–9 (in Ukrainian).

[6] Kryvorotenko D, Kovalska V, Yarmoluk S. Interaction of cyanine dyes with nucleic acids. 10. Synthesis and spectral properties of new benzothiazol-4-[2,6-dimethylpyridinium] monomethine cyanine dyes. Biopolymers and Cell 2000; 16:145–52 (in Ukrainian).

[7] Yarmuoluk S, Kryvorotenko D, Kovalska V. Interaction of cyanine dyes with nucleic acids. XX. New methods of preparation of fluorescent probes based on benzothiazol-4-[2,6-dimethylpyridinium]cyanine dyes. Dyes and Pigments 2001;48:165–72.

[8] Ogul'chansky T, Yarmoluk S, Yashchuk V, Losytskyy M. The spectral response on DNA interaction with some monomethyne cyanine dyes. In: Greve J, Puppels G, Otto C, editors. Spectroscopy of biological molecules: new directions. Dordrecht/Boston, London: Kluwer Academic Publishers, 1999. p. 309–10.

[9] Moreno F, Cortijo M, Gonzalez-Jimenez J. The fluorescent probe prodan characterizes the warfarin binding site on human serum albumin. Photochemistry and Photobiology 1999;69:8–15.

[10] Adams R, Pease D, Clark J, Baker B. Structure of cannabinol. I. Preparation of an isomer 3-hydroxy-l-*n*-amyl-6,6,9-trimethyl-6-dibenzopyran. JACS 1940;62:2197.

[11] Becker H, Berger W, Domschke G. Organicum VEB. Berlin: Deutscher Verlag der Wissenschaften, 1990.

[12] Kryvorotenko D, Kovalska V, Yarmoluk S. Interaction of cyanine dyes with nucleic acids. 16. New fluorescent benzothiazole-4-[2,6-dimethylpyridinium] monomethyne cyanine dyes. Biopolymers and Cell 2000;16:320–6.