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CycloOctaTetraene as a Photostabilizer of Fast Protein Fluorogen

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Abstract—We report two new analogs of the N871b fluorogen that contain CycloOctaTetraene. We have studied the photostability of fluorescent complexes of the FAST protein with new compounds by confocal microscopy when staining living HeLa Kyoto cells. One of the new analogs exhibits a higher photostability in the complex with FAST than the previously published N871b fluorogen. The results indicate that cyclooctatetraene is a highly promising compound for the modification of dyes based on arylidene imidazolones.

Keywords: imidazolones, fluorogens, fluorescence-activating proteins, fluorescence, triplet-state quenchers
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INTRODUCTION

Fluorescent dyes, which are used in modern fluorescence microscopy are characterized by long-wave absorption and emission and have a small size and low toxicity. The other important properties of a fluorescent label include the brightness and photostability of the dye. The latter is of particular importance for use in ultrahigh resolution microscopy because of the intense irradiation of an object under study.

In this regard, the recently proposed so-called self-healing dyes have become a promising new object for research [1–4]. These dyes consist of two fragments, i.e., a fluorescent dye and a photostabilizer. The latter prevents the formation of an excited triplet state of the fluorophore, which leads to photobleaching. An example of a photostabilizer is cyclooctatetraene (COT) (Fig. 1). At the moment, the COT group is used in most cases for the modification of cyanine dyes [5–8]. Cyclooctatetraene, as a rule, is bound to a dye molecule through either an alkyl linker or the amide or ester group, which increases the π-conjugation system of COT (Fig. 1). Moreover, one or another type of linkage with COT has a different photostabilizing effect depending on the structure of the cyanine dye (e.g., the number of double bonds) [5, 6].

In this paper, we studied the effect of cyclooctatetraene on the photostability of arylidene-imidazolone fluorescent dyes. The previously synthesized N 871b fluorogen was selected as a model compound, which is successfully used in combination with the FAST fluorogen-activating protein for genetically encoded fluorescent labeling (Fig. 2) [9].

The goal of this work was to synthesize analogs of N871b fluorogen that contains the COT group and to study the photostability of these compounds when staining living HeLa Kyoto cells using confocal microscopy.

RESULTS AND DISCUSSION

An important condition for the chemical modification of a fluorogen is the preservation of its ability to form a fluorescent complex with a protein. Therefore, the prerequisite for the introduction of a COT group is the choice of the fragment of the molecule, which is least involved in the formation of a fluorogen/activating protein complex. We modified the N871b fluorogen by the nitrogen atom of imidazolone because our previous data on the structure of the fluorescent complex of the N871b fluorogen with the FAST protein demonstrated that the modified fragment was located outside the protein pocket and this modification probably did not affect the formation of the complex (Fig. 2) [10].

At the first stage of the work, we synthesized an analog of the N871b fluorogen that contained the ester group in the first position of imidazolone (**II**) (Scheme 1). Hydrolysis of the latter made it possible to obtain the derivative of N871b (**III**) with the carboxyl group. After the interaction of acid (**III**) with a protected diamine, followed by the removal of the protective group, we obtained amine (**V**) (Scheme 1). In addition, we synthesized the necessary carboxyl-containing derivatives of cyclooctatetraene (**VI**) and (**IX**). At the final stage, the target compounds (**X**) and (**XI**) were synthesized by the reaction of amine (**V**) with acids (**VI**) and (**IX**) (Scheme 1).

Abbreviations: COT, CycloOctaTetraene

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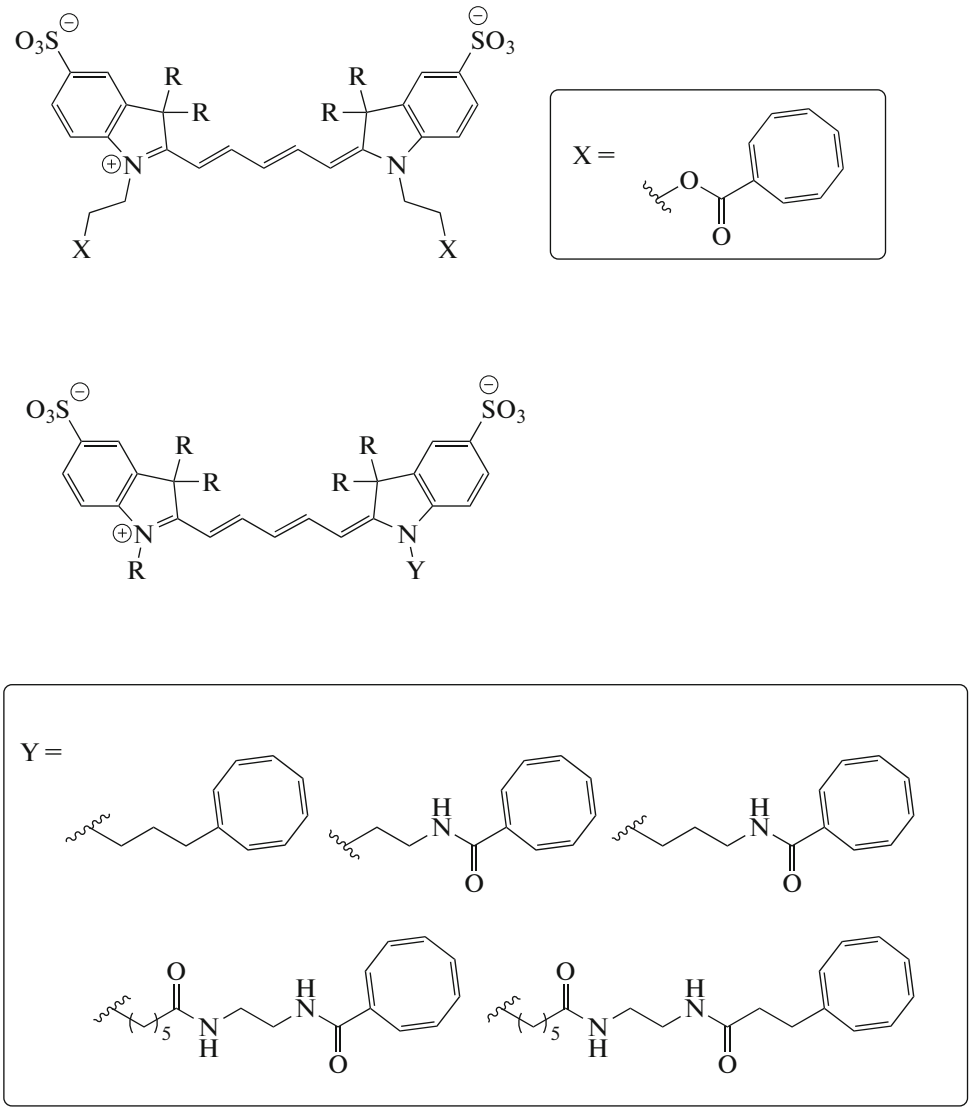


Fig. 1. Modification with the COT group with an example of cyanine dye Cy5.

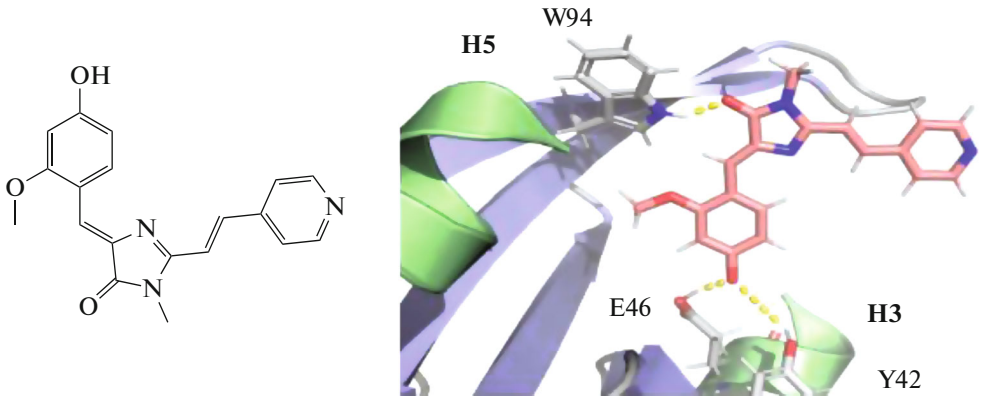
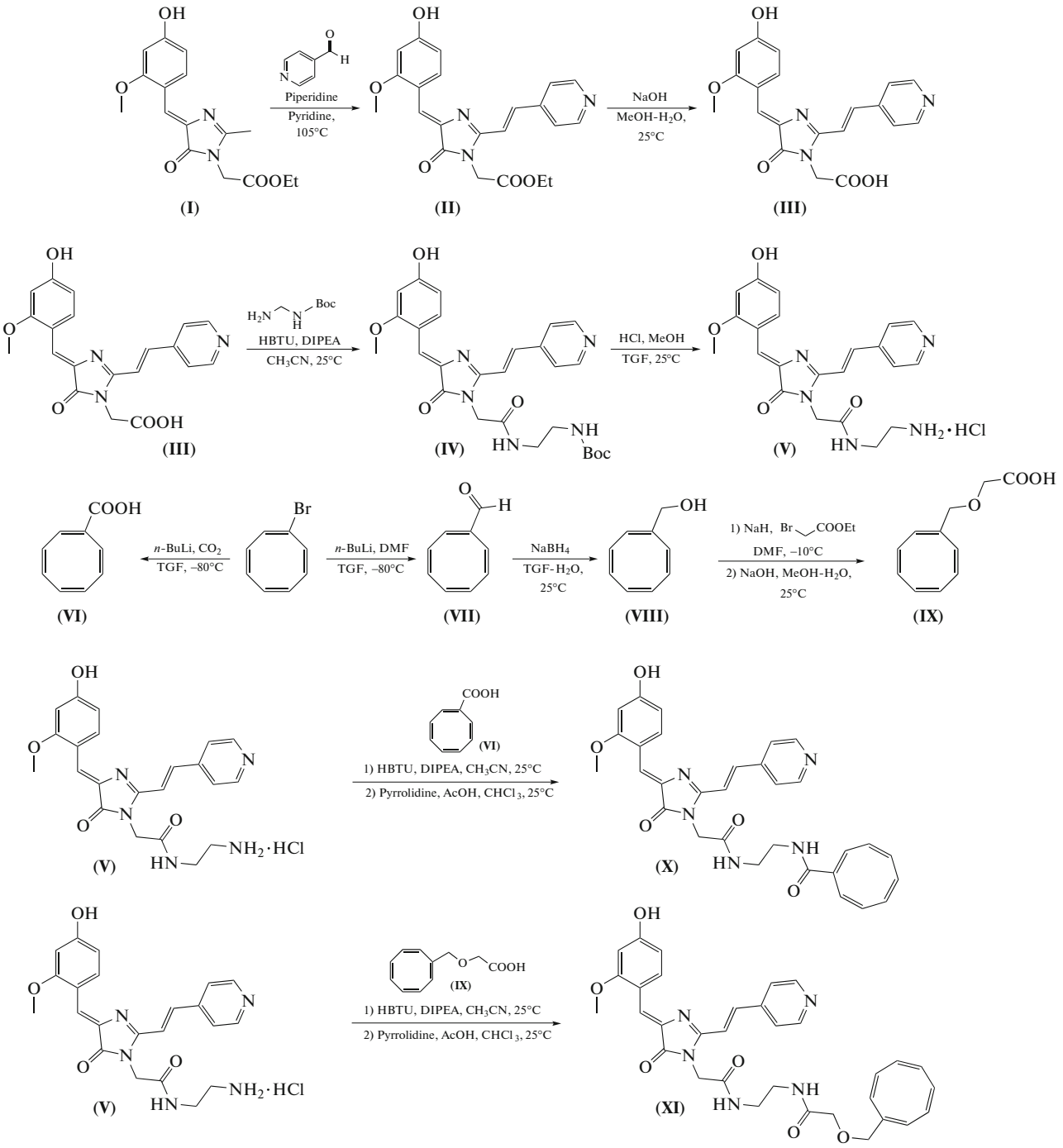


Fig. 2. Structure of N871b fluorogen and its binding to fluorogen-activating FAST protein. Hydrogen bonds of N871b fluorogen with tryptophan (W94), glutamic acid (E46), and tyrosine (Y42) residues were noted.



Scheme 1. Synthesis of compounds (**III**–**XI**).

We studied the efficiency of the staining of living cells with compounds (**X**) and (**XI**) by confocal microscopy in comparison with using the original N871b fluorogen. Fluorogen N871b and compounds (**X**) and (**XI**) were added to the HeLa Kyoto cells transfected by a plasmid that contained the gene encoding the FAST protein fused with the cytoskeleton protein. The addition of newly synthesized compounds (**X**) and (**XI**) and fluorogen N871b led to a significant increase in fluorescence. This result suggests that the introduction of the COT group into the N871b molecule did not lead to a loss of fluorogenic properties, and the new compounds can form a fluorescent complex with the FAST protein. It was also found that under prolonged laser irradiation at 543 nm, the FAST protein complexes with compound (**X**) and original N871b fluorogen were discolored at the same rate (Fig. 3), while the complex with compound (**XI**) demonstrated noticeably greater photostability under similar conditions (Fig. 3).

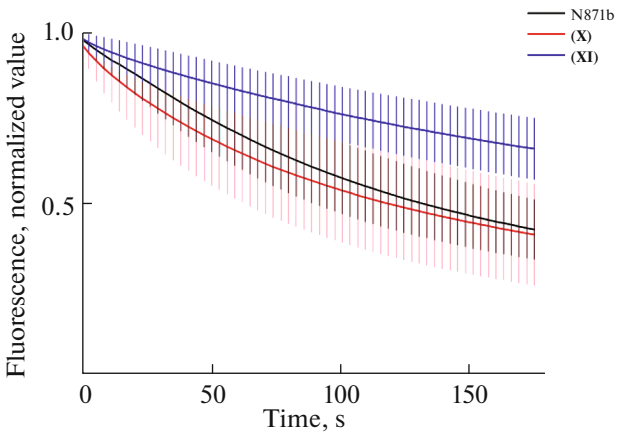


Fig. 3. The photobleaching curves of N871b fluorogen and compounds (**X**) and (**XI**) in the complex with the FAST protein in HeLa Kyoto cell lines, which were obtained using laser scanning microscopy (SD is displayed by a colored area, $n = 9–10$). The compounds were diluted from the 1-mM solution in DMSO to a final concentration of 5–10 μM.

Thus, we have shown that the photostability of the fluorescent complex of the FAST protein with the N871b fluorogen can be increased by introducing cyclooctatetraene into the fluorogen molecule. Probably, the proposed modification can also be used for the synthesis of other photostable fluorescent arylidene-imidazolone dyes.

EXPERIMENTAL

Equipment. The NMR spectra (δ, ppm; J, Hz) were recorded on an Avance III NMR spectrometer (700 MHz; Bruker, United States) at 303 K in DMSO-d_6 (internal standard, Me_4Si). Melting temperatures were measured on an SMP30 device (Stuart Scientific, United Kingdom) and were not corrected. High-resolution mass spectra were recorded on a micrOTOF II spectrometer (Bruker, Germany; electrospray ionization).

General method for synthesis of compounds (X) and (XI). Compound (**V**) (137 mg, 0.3 mmol), corresponding acid (**VI**) or (**IX**) (0.66 mmol), HBTU (318 mg, 0.84 mmol), and DIPEA (0.21 mL, 1.23 mmol) were suspended in acetonitrile (10 mL). The resulting mixture was stirred at room temperature for 12 h in an inert atmosphere. The reaction mixture was evaporated, and the product was purified using flash chromatography (eluent, chloroform/methanol, 100 : 7). The resulting product was dissolved in chloroform (10 mL), followed by the addition of pyrrolidine (37 mL, 0.45 mmol). The resulting mixture was stirred at room temperature for 30 min, followed by the addition of acetic acid (50 mL), and stirring the mixture at room temperature for 20 min. The reaction mixture was diluted with chloroform (20 mL) and washed with an aqueous solution of NaH_2PO_4 (30 mL, pH 6.0). Organic extracts were dried over anhydrous sodium sulfate and evaporated. The resulting product was additionally purified by column chromatography (gradient chloroform/methanol from 100 : 3 to 100 : 7).

The method of the synthesis of the initial compounds, reaction yields, melting temperatures, and spectral characteristics of the synthesized compounds (**I–XI**) are given in supplementary materials.

Photobleaching under the conditions of fluorescence microscopy. The experiments were carried out on an immortalized (human cervical cancer) HeLa Kyoto (ATCC) cell line. The cells were sown on plates (35 mm in diameter) with a glass bottom (SPL Life Sciences, Korea). The cells were cultivated in the DMEM medium (PanEco, Russia) that contained penicillin (100 U/mL), streptomycin (100 U/mL) (PanEco, Russia), and 10% embryonic calf serum (PanEco, Russia). Transfection was performed when confluence reached 80% of the maximum. For transfection, the cell medium was replaced by 500 μL of Opti-MEM (Gibco, United States), and the cells were incubated for another 2 h, followed by the addition of Opti-MEM (500 μL) that contained a mixture of a Transfectin agent (Bio-Rad, United States) (3.75 μL) and the plasmid encoding the FAST protein gene fused with the cytoskeleton protein vimentin (https://pubmed.ncbi.nlm.nih.gov/33336838/) (1.5 μg). After 4–6 h, the medium was replaced with DMEM that contained penicillin–streptomycin and embryonic calf serum at the above concentrations. The next day after transfection, the medium was replaced with a Hanks solution that contained 20 mM HEPES. Compounds (**X**) and (**XI**) at a concentration of 10 μM and compound N871b at a concentration of 5 μM were added to HeLa Kyoto cells (the compounds were diluted from 1-mM DMSO stock solution (PanEco, Russia)). The cells were examined under a confocal microscope DM IRE2 (Leica, Germany) equipped with an immersion oil lens HCX PL APO Lbd.BL 63 × 1.40 and a helium–neon (HeNe) laser (543 nm). In cells expressing the vimentin-FAST construct, regions of interest (7.5 × 7.5 μm) were selected, which were continuously scanned with a HeNe laser (100%) for 175 s ($n = 9–10$ cells). The resulting images were processed and analyzed using the Fiji ImageJ program. The photobleaching curves of the fluorescent signal were obtained using the OriginPro8.1 program.

CONCLUSIONS

Two new derivatives of cyclooctatetraene-containing fluorogen N871b were synthesized. It was found by confocal microscopy when staining living cells that the new compounds form the fluorescent complexes with the FAST fluorogen-activating protein. Under prolonged irradiation, compound (**XI**) demonstrated greater photostability in comparison with the original fluorogen N871b. Therefore, the study of the effect of

cyclooctatetraene on the photostability of other arylidene-imidazolone fluorescent dyes seems to be an relevant direction for further research.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors state that there is no conflict of interest.

This article does not contain any studies on the use of animals as objects of research.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1134/S1068162022060231.

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