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New Azobenzene Derivatives for Directed Modification of Proteins

Le Thi Hien[a], B. Schierling[b], A. Yu. Ryazanova[a], T. S. Zatsepin[a], E. M. Volkov[a], E. A. Kubareva[a], T. I. Velichko[a], A. Pingoud[b], and T. S. Oretskaya[a,1]

[a] *Chemistry Department, Faculty of Bioengineering and Bioinformatics, A.N. Belozersky Institute of Physico-Chemical Biology, M.V. Lomonosov Moscow State University, Moscow, 119991 Russia*

[b] *Institute of Biochemistry, Justus-Liebig University, Giessen, Germany*

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Abstract—Derivatives of azobenzene which contained a maleimide group in one of the benzene rings (for binding to a protein cysteine residue) and maleimide, hydroxyl, or carboxyl substitutes in another benzene ring were synthesized. The reactivity of these compounds towards a cysteine residue of a protein and their optical properties in a free form and after their attachment to the mutant forms of the SsoII restriction endonuclease were studied.

Key words: maleimidoazobenzene; the SsoII restriction endonuclease; proteins, modification of cysteine residues

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INTRODUCTION

The chemical modification of proteins plays an important role both for the investigation of their functioning and for the creation of protein constructions with novel properties.[2] Such constructions are often used as instruments in molecular biology, in particular, for the induction of protein activity in a desired moment. For example, DNA polymerase from *Thermus aquaticus* can be reversibly inactivated by treatment with formaldehyde, resulting in the blocking of the lysine residue in the binding site. The regeneration of the native enzyme proceeds through hydrolysis of the Schiff base upon heating to 95°C [1].

Azobenzene undergoes *cis-trans*-(*syn-anti*)-isomerization induced by illumination with light of appropriate wavelengths (Fig. 1a). The isomerization proceeds quickly and effectively and is practically independent of the environment and polarity of the solvent. Azobenzene derivatives have been frequently introduced into proteins for modulation of their activity [2–5]. Such reagents are of interest because azobenzene is isomerized by illumination with light with a wavelength of more than 300 nm and, thus, allows for protein activation/deactivation *in vivo* changes of the protein activity either due to the direct alteration of the conformation of the azobenzene derivatives with subsequent steric hindrances [6, 7] or due to a change in the protein conformation because of *cis-trans*-isomerization of the azobenzene derivatives [8, 9]. As a rule, cysteine residues of a protein are modified by both the symmetrical azobenzene derivatives bis(chloroacetamidosulfo)azobenzene [4] and bis(iodoacetamido)azobenzene [10] and its asymmetrical derivatives (4-maleimidobenzophenone [11] and maleimidoazobenzeneglutamic acid [12]). It is important to note that the compounds with a maleimide group are more reactive towards sulfhydryl groups than the chloroacetamide and iodoacetamide derivatives.

We proposed to use only the asymmetrical derivatives of azobenzene for the regulation of the accessibility of the active site of enzyme and synthesized conformationally flexible compounds with hydroxyl and carboxyl substitutes. Further, in case the desirable effect would be achieved, the other derivatives will be synthesized.

In this study, we prepared previously unknown derivatives of azobenzene with a maleimide group (for the binding to a protein cysteine) in one of the benzene rings and with a maleimide, hydroxyl, or carboxyl substitute in another ring. The reactivity of the obtained derivatives was confirmed with the model proteins, mutant forms of the SsoII restriction endonuclease (R.SsoII). SH groups of cysteine residues of the protein were modified, and the photoisomerization of the protein-bound azobenzene derivatives was also demonstrated (Fig. 1b). Such derivatives were proposed for modulation of the DNA binding to the following proteins: restriction endonucleases, DNA-methyltransferases, and transcription factors. The possibility of the intracellular activation/deactivation of these enzymes will give novel information on their functioning and allow regulating their activity in cells.

[1] Corresponding author; phone: (495) 939-5411; fax: (495) 939-3181; e-mail: oretskaya@belozersky.msu.ru.

[2] Abbreviations: NBS, N-bromosuccinimide; PEG-Mal, (methyl-polyethylenglycol$_{12}$)$_3$-polyethylenglycol$_4$-maleimide; R.SsoII, the SsoII restriction endonuclease; SDS, sodium dodecylsulfate.

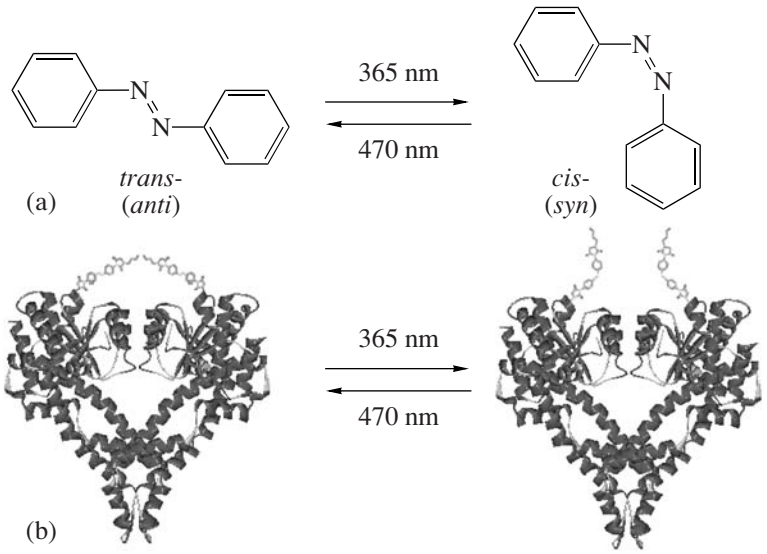


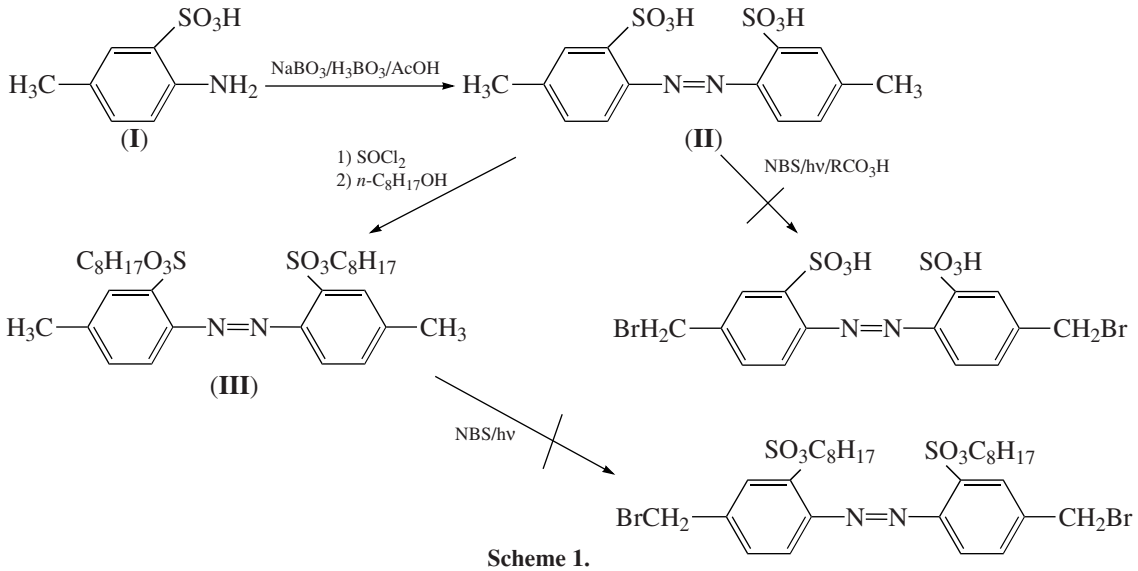
Fig. 1. The *cis-trans*-isomerization of azobenzene (a) in its free form and (b) its derivative attached to the mutant form of the R.SsoII(C33S/C60S/R174C) induced by illumination with light with certain wavelengths.

RESULTS AND DISCUSSION

Synthesis of Azobenzene Derivatives

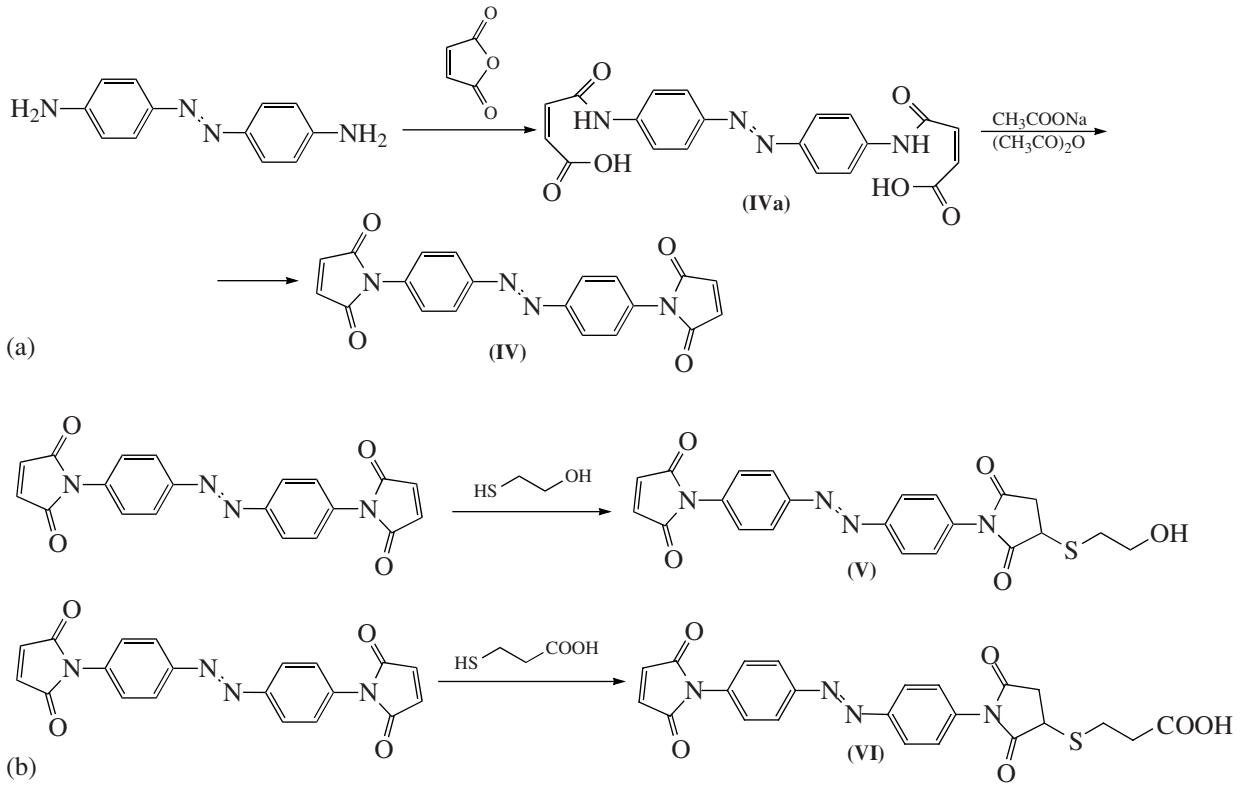
Azobenzene derivatives have often been used for protein modification [7, 10–13]. The goal of this study was the synthesis of new derivatives of azobenzene which contained the maleimide group for binding to a protein cysteine residue in one of the benzene rings and substitutes with different functional groups in the other ring. We also planned to prepare azobenzene derivatives with sulfogroups in the aromatic ring, which would increase the solubility of the modifying agents in water.

In the first stage, we attempted the synthesis of a water-soluble derivative of azobenzene (Scheme 1). The most frequently used methods of the preparation of azobenzenes are the oxidation of anilines by hypochloride [14] or sodium perborate [15] or the reduction of nitrobenzenes by tin (II) chloride [16]. 4-Aminotoluene-3-sulfonic acid (**I**) was oxidized by sodium perborate in the presence of boric acid in glacial acetic acid. As a rule, radical bromination is performed in nonpolar haloid-containing solvents, and the choice of solvents was rather small. Compound (**II**) was practically insoluble in dichloromethane and tetrachloromethane and, thus, the attempts of bromination with *N*-bromosuccinimide during illumination or in the presence of 3-chloroperoxybenzoic acid in the next stage were unsuccessful. Compound (**II**) was converted into its dioctyl ester [17] in order to increase its solubility in low-polar solvents, but diester (**III**) was also insoluble in the aforementioned solvents. As a result, we gave up the preparation of water-soluble sulfoderivatives of azobenzene.



Scheme 1.

4,4'-Bis(maleimido)azobenzene (**IV**) was prepared from *N*,*N*'-azobenzenedimaleic acid (**IVa**) similarly to the previous synthesis of bisarylmaleimides from bisarylmaleamine acids [18] (Scheme 2a). Acid (**IVa**) was synthesized according to the procedure [19], but in another combination of solvents. The target product (**IV**) was isolated from the reaction mixture by absorption chromatography on a column with silica gel.



Scheme 2.

The preparation of monosubstituted bismaleimides in organic solvents has not been described in literature, although the interaction of maleimides with thiols has been carefully studied, especially in an aqueous medium [20]. We studied the attachment of thiols to azobenzene (**IV**) varying solvent, temperature, and ratio of the initial compounds and chose the optimal conditions for this reaction. The solution of the thiols was slowly dropwise added to the solution of compound (**IV**) in dioxane. Using of other solvents (tetrahydrofurane, dimethylformamide, or methylene chloride) instead of dioxane was possible. The reaction products were maleimidoazobenzenes with one or two thiol molecules incorporated, which were separated by column chromatography. Derivatives of maleimidoazobenzene with 2-mercaptoethanol and 3-mercaptopropionic acid, in particular, compound (**V**) with a hydroxyl group and compound (**VI**) with a carboxyl group, were prepared by this procedure with yields of 25–30% (Scheme 2b).

We have studied the efficacy of the reversible *cis-trans*-isomerization of the prepared compounds. A peak with a maximum at 340–350 nm is characteristic for *trans*-isomers of azobenzene derivatives. The maximum is shifted to 325–330 nm in the case of the *cis*-isomers. All of the synthesized derivatives of azobenzene were quickly and reversibly isomerized under light illumination at 365 and 470 nm, independently of the structure of the substitutes in the benzene rings. The absorption spectra of compound (**IV**) in DMSO are given as an example (Fig. 2).

Modification of Proteins with Azobenzene Derivatives

Mutant forms of the SsoII restriction endonuclease (R.SsoII) were used as model proteins for the attachment of the azobenzene derivatives. These mutant proteins contained one or two cysteine residues: R.SsoII(C33S/C60S/R174C) or R.SsoII(C33S/C60S/S171C/R174C), respectively. Results of the X-ray analysis of the complex of the Ecl18kI (R.Ecl18kI) restriction endonuclease with a substrate [21] served as the basis for the construction of the mutant forms of R.SsoII. R.Ecl18kI is an isoschizomer and the nearest homologue of R.SsoII. These enzymes are distin-

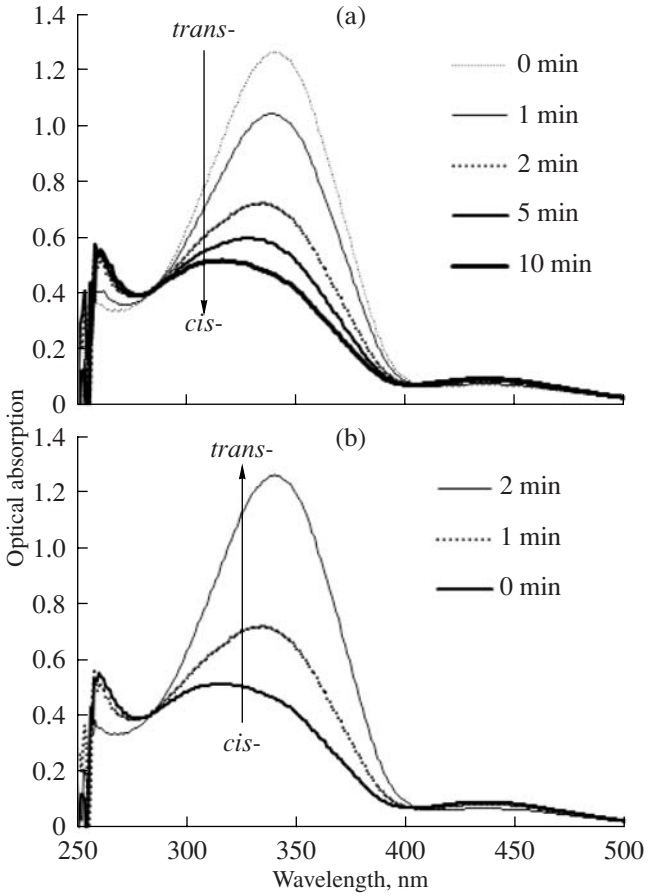


Fig. 2. Absorption spectra of 4,4'-bismaleimidoazobenzene in DMSO in the concentration of 100 μM: (a) *trans*⟶*cis* isomerization at 365 nm and (b) *cis*⟶*trans*-isomerization during the illumination with blue light with a wavelength of 470 nm.

guished by one amino acid residue (Val232 in R.Ecl18kI corresponds to Ile in R.SsoII). R.SsoII has a molecular mass of 37.4 kDa.

The accessibility of cysteine residues of R.SsoII mutant forms for modification with synthesized reagents (**IV**), (**V**), and (**VI**) was assessed via polyethylene glycol, modified by maleimide group (PEG-Mal with a molecular mass of 2.3 kDa) and the availability of the cysteine residues of the mutant forms of R.SsoII for the modification by the prepared reagents (**IV**), (**V**), and (**VI**) was determined. The use of PEG-Mal had the following advantages:

(1) PEG-Mal contained a maleimide group and was a chemical analogue of the compounds synthesized by us.

(2) PEG-Mal had a rather large molecular mass, and SDS-polyacrylamide gel electrophoresis could be used as a standard method of the analysis of such reaction mixtures. PEG-Mal practically quantitatively interacts with proteins, resulting in the appearance of the corresponding band of the conjugate in polyacrylamide gel (Fig. 3, line *2*).

The tenfold excess of the solution of azobenzene derivative (**IV**), (**V**), or (**VI**) in DMSO was added to the protein solution at room temperature. The efficacy of the reaction with azobenzenes was determined *via* adding an excess of PEG-Mal to an aliquot of the reaction mixture. The yield of the conjugate of the protein with PEG-Mal was determined according to the gel analysis by the ImageQuant program and was no higher than 5% (Fig. 3, line *4*). Hence, the yield of the protein modified by reagents (**IV**), (**V**), and (**VI**) was approximately 95%. Further, the proteins modified by the azobenzene derivatives were separated from the nonreacted reagent by gel filtration. The efficacy of the reaction of the mutant forms of the restriction endonuclease with reagents (**IV**), (**V**), and (**VI**) was evaluated by spectrophotometry as a ratio of the intensity of the peak at 340 nm, which was characteristic of the azobenzene derivatives to the intensity of the protein peak at 280 nm. The extinction coefficients of the R.SsoII mutant form ε_{280} and the azobenzene derivatives were taken to be 42000 M^{-1} cm^{-1} [22] and 22900 M^{-1} cm^{-1} [23], respectively. The yield of the protein modified with derivatives (**IV**), (**V**), or (**VI**) was about 85%. Thus, compounds (**IV**), (**V**), and (**VI**) were shown to be useable for the effective and selective modification of proteins. The substitutes have practically no influence on the reactivity of the maleimide group. A comparison of the optical properties of the azobenzene derivatives with those attached to the cysteine residues of protein revealed no significant differences (Fig. 4).

Further, we plan to perform an intramolecular crosslinking of proteins by the azobenzene derivative (**IV**). Such a crosslinking changes the protein structure by the isomerization of the azobenzene derivative induced by illumination. Compounds (**V**) and (**VI**) will be used for regulation of the activity of R.SsoII during the formation of the enzyme–substrate complex. We propose to modify the enzyme by these derivatives in the immediate vicinity of the DNA-binding site. Changes in the conformation of the protein-attached azobenzene derivatives will allow closing or opening the DNA-binding site of R.SsoII. The proposed scheme of control of the R.SsoII reactivity through the isomerization of the protein-attached compound (**V**) is illustrated in Fig. 1b.

EXPERIMENTAL

The following reagents were used in this study: azodianiline (Acros, Belgium), maleic anhydride, acetic anhydride, thionyl chloride, boric acid (Reakhim, Russia), 2-mercaptoethanol (Amresco, United States), 3-mercaptopropionic acid (Merck, Germany), sodium perborate ($NaBO_3 \cdot 4H_2O$), *N*-bromosuccinimide (Fluka, Germany), 4-aminotoluene-3-sulfonic acid (Aldrich, United States), 3-chloroperoxybenzoic acid (Lancaster, Great Britain), and (methyl-polyethyleneglycol$_{12}$)$_3$-polyethyleneglycol$_4$-maleimide of 2.3-kDa molecular mass (PEG-Mal, Pierce, United

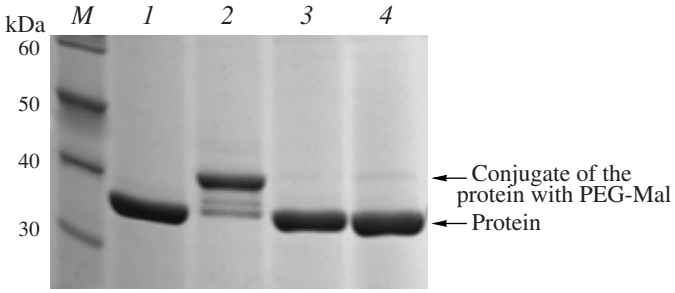


Fig. 3. Analysis of the interaction of the mutant form of R.SsoII(C33S/C60S/R174C) (5 μM) with PEG-Mal (500 μM) and (or) (**V**) (50 μM) by Laemmli gel electrophoresis. The gel was stained with the Coomassie G250 solution. The protein was incubated with (line *2*) PEG-Mal, (line *3*) compound (**V**), and (line *4*) initially with compound (**V**) and, then, with PEG-Mal. Line *1*: protein control. M are the marker proteins. The molecular masses are indicated on the left.

States). The absolute solvents were prepared by the standard methods.

Mass spectra were recorded on a system that consisted of an ACQUITY UPLC liquid chromatograph and a TQD tandem quadrupole mass-spectrometric detector (Waters, United States).

NMR spectra were recorded on the Avance 400 spectrometer (Bruker, Germany) at 25°C. $CHCl_3$ served as an internal standard.

TLC was performed by an ascending method on Kieselgel 60 F254 plates (Merck, Germany). The substances with absorption in the UV area were detected at 254 nm.

The derivatives of azobenzene were isolated by chromatography on a column with silica gel (Kieselgel 60, 0.040–0.063, Merck, Germany).

Synthesis of sulfoderivatives of azobenzene. 4,4'-Azotoluene-3,3'-disulfonic acid (II). A mixture of 4-aminotoluene-3-sulfonic acid (**I**) (4 g, 0.02 mol), sodium perborate ($NaBO_3 \cdot 4H_2O$) (2.9 g, 0.028 mol), and boric acid (1.05 g, 0.017 mol) in glacial acetic acid (100 ml) was stirred at 60°C for 6 h. The mixture became dark orange in one hour. White precipitate and dark resins (different oxidation products) were filtered off, and the dark orange filtrate was evaporated to dryness and dissolved in water. The crystals of target product (**II**) were precipitated via cooling the solution to 4°C. The yield of compound (**II**) was 1.4 g (35%); MS: $C_{14}H_{14}N_2O_6S_2$ (M^-); calcd/found 369.03/369.11.

Dioctyl ester of 4,4'-azotoluene-3,3'-disulfonic acid (III). Thionyl chloride ($SOCl_2$) (4 ml) was dropwise added to compound (**II**) (1 g). Another 4 ml of $SOCl_2$ and anhydrous dioxane (4 ml) were added one hour later, and the reaction mixture was kept for 1 h until stopping of the liberation of hydrogen chloride and sulfur dioxide. The mixture was evaporated to dryness, reevaporated with dioxane (3 × 5 ml), and stirred for 2 h at 40°C in octanol (7 ml). The obtained compound was insoluble in most of the organic solvents. Our attempts to isolate it and to confirm its structure failed.

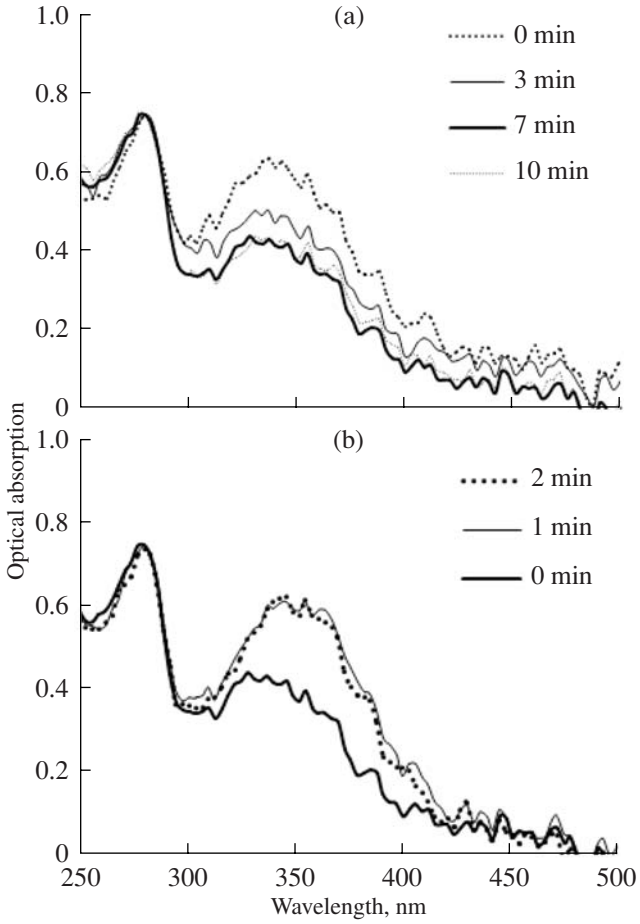


Fig. 4. Absorption spectrum of the conjugate of R.SsoII(C33S/C60S/S171C/R174C) with compound (**VI**): (a) *trans*⟶*cis*-isomerization during illumination with UV light with a wavelength of 365 nm and (b) *cis*⟶*trans*-isomerization during illumination with blue light with a wavelength of 470 nm.

4,4'-Bis(maleimido)azobenzene (IV). Maleic anhydride (1.8 g, 17.7 mmol) was added to the solution of diazoaniline (1.5 g, 7.1 mmol) in DMF (20 ml). The reaction mixture was stirred for 1 h and diluted with dioxane (20 ml). The precipitate was filtered and dried. Sodium acetate (1.8 g, 21.3 mmol) and acetic anhydride (13.4 ml, 142 mmol) were added to the precipitate. The reaction mixture was heated to boiling and kept for 45 min. The precipitate was completely dissolved. The reaction was monitored by TLC in a mixture of chloroform and ethanol (95 : 5). R_f of the product was 0.83. The excess of acetic anhydride was hydrolyzed by the addition of cool water. The precipitate was filtered, washed with a 10% solution of Na_2CO_3 and with water to pH 7, and dried. Product (**IV**) was isolated by chromatography on silica gel in chloroform with a yield of

1.69 g (64%). ^{1}H NMR ($CDCl_3$, δ, ppm): 8.0 (4 H, d), 7.7 (4 H, d), 7.3 (4 H, d).

4-Maleimido-4'-[2-(2-hydroxyethylthio)succinimido]azobenzene (V). The solution of 2-mercaptoethanol (0.4 mmol) in dioxane (2 ml) was added to the solution of compound (**IV**) (150 mg, 0.4 mmol) in dioxane (20 ml) within 30 min during stirring. The reaction was monitored by TLC in the mixture of chloroform and ethanol (95 : 5). Product (**V**) (R_f 0.23) was isolated by chromatography on silica gel in the ethanol gradient in chloroform (0–5%) with a yield of 45 mg (25%). ^{1}H NMR ($CDCl_3$, δ, ppm): 8.0 (4 H, d), 7.7 (4 H, d), 7.3 (2 H, s), 4.2 (1 H, s), 3.7 (2 H, m), 3.0 (2 H, m), 2.7 (2 H, m); MS: $C_{22}H_{18}N_4O_5S$ $(M\mathrm{H})^+$; calcd/found: 451.10/451.24.

4-Maleimido-4'-[2-(2-carboxyethylthio)succinimido]azobenzene (VI). The solution of 3-mercaptopropionic acid (35 μl, 0.4 mmol) in dioxane (2 ml) was added to the solution of compound (**IV**) (150 mg, 0.4 mmol) in dioxane (20 ml) within 30 min during stirring. The reaction was monitored by TLC in the mixture of chloroform and ethanol (95 : 5). Product (**VI**) with R_f 0.11 was isolated by chromatography on silica gel in the gradient of ethanol in chloroform (from 0 to 10%) with a yield of 55.5 mg (29%); ^{1}H NMR ($CDCl_3$, δ, ppm): 12.4 (1 H, s), 8.0 (4 H, d), 7.7 (4 H, m), 7.3 (2 H, s), 4.2 (1 H, s), 3.7 (2 H, m), 3.0 (2 H, m), 2.7 (2 H, m); MS: $C_{23}H_{18}N_4O_6S$ $(M\mathrm{H})^+$; calcd/found: 479.09/478.94.

Preparation of the mutant forms of R.SsoII. We performed site-specific mutagenesis of the R.SsoII gene by the Kirsch method [24]. The Arg(AGA)174 codon in the R.SsoII(C33S/C60S/R174C) gene was exchanged for Cys(TGT), and the Ser(TCT)171 and Arg(AGA)174 codons in the R.SsoII(C33S/C60S/S171C/R174C) gene were exchanged for Cys(TGC) and Cys(TGT) codons, respectively. The pACMS7/pQESso9 C33S/C60S plasmids were used as starting plasmids, where pQESso9 C33S/C60S contained the R.SsoII gene without cysteine residues. These plasmids were kindly presented by Doctor V. Pingoud (Germany). DNA sequencing over the entire coding region confirmed the presence of the corresponding codon substitutions in them. The mutant forms of the R.SsoII recombinant proteins with the HiS_6-tag on their N-termini were produced in the JM109 *Escherichia coli* cells and isolated by affinity chromatography of Ni-NTA-agarose as described in [22].

Laemmli gel electrophoresis [25] was performed in flat PAAG (10 × 8 × 0.1 cm) in a buffer containing 25 mM Tris, 250 mM glycine, 0.1% SDS (m/v) at pH 8.3, and a field strength of 14 V/cm. The separating gel consisted of 0.375 M Tris-HCl (pH 8.8), 14.25% acrylamide, 0.75% *N*,*N*'-methylenebisacrylamide, and 0.1% SDS. The concentrating gel contained 0.125 M Tris-HCl (pH 6.8), 3.9% acrylamide, 0.1% *N*,*N*'-methylenebisacrylamide, and 0.1% SDS. Samples were heated for 5 min at 95°C and applied to the gel in 10–20 μl of a buffer containing 62 mM Tris-HCl (pH 6.8), 2% SDS (m/v), 0.7 M 2-mercaptoethanol, 10% glycerol (v/v), and 0.1% Bromophenol Blue (m/v). Protein markers of molecular mass (10 μl, Fermentas, Lithuania) were applied to one of the lines. The gel was stained with a Coomassie R250 solution (Fermentas, Lithuania) or Coomassie G250 (Serva, Germany).

The protein modification by derivatives (IV), (V), or (VI). 1 μl of 50 mM solution of azobenzene derivative (**IV**), (**V**), or (**VI**) in DMSO was added to a 1 ml of 5 μM solution of a protein in a 20 mM potassium phosphate buffer pH 7.4, 150 mM KCl. The reaction mixture was kept for 15 min at room temperature and centrifuged for 5 min at 12000 *g*. The proteins modified with the azobenzene derivatives were separated from the nonreacted reagent by gel filtration on an Akta Purifier chromatograph on a HiTrap column with a volume of 5 ml with Sephadex G-25 Superfine (GE Healthcare, United States). The proteins were eluted with a 20 mM Tris-HCl buffer (pH 7.5) containing 20 mM KCl.

Modification of the proteins with PEG-Mal. 2 μl of 5 mM solution of PEG-Mal (2.3 kDa) in DMSO was added to 20 μl of 5 μM solution of the protein in a 20 mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl. The reaction mixture was kept for 15 min at room temperature.

The photoisomerization of the free azobenzene derivatives was performed on a Cary Eclipse spectrofluorimeter (Varian, United States) in a quartz cuvette with an optical path of 1 cm. The absorption spectra of these solutions were recorded on a Cary 50 UV-Vis spectrophotometer (Varian, United States). The solution volume was 200 μl. The isomerization degree was determined according to the height of the peak at 325–350 nm. The solution of the azobenzene derivatives before the illumination contained a mixture of *cis*- and *trans*-isomers, and the *trans*-isomer predominated. The solution was illuminated with light with a wavelength of 470 nm for 1 min in order to convert the compound into the *trans*-conformation. The height of the peak maximum was not changed with further illumination. The solution was subsequently illuminated at 365 nm for 1, 2, 5, and 10 min for the preparation of the *cis*-isomer. After that, reverse isomerization was performed by the subsequent illumination of the solution at 470 nm for 1 and 2 min, and the height of the maximum achieved the starting value.

Photoisomerization of the proteins modified with derivatives of azobenzene was carried out using a UV lamp with a wavelength of 365 nm and a lamp of blue light with a wavelength of 470 nm (Bachofer, Germany). The absorption spectra of these solutions were recorded on a NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnologie GmbH, Germany).

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