Russian Chemical Reviews **82** (10) 942 – 963 (2013) © 2013 Russian Academy of Sciences and Turpion Ltd

DOI 10.1070/RC2013v082n10ABEH004355

Design of photocontrolled biomolecules based on azobenzene derivatives †

T S Zatsepin, L A Abrosimova, M V Monakhova, Le Thi Hien, A Pingoud, E A Kubareva, T S Oretskaya

Contents

I.	Introduction	942
II.	Synthesis and application of nucleic acids and their analogues containing azobenzene fragments	943
III.	Incorporation of azobenzene moiety into peptides and proteins	947
IV.	Photoregulation of protein activity	950
V.	Use of photocontrolled substrates, inhibitors and ligands for protein activity regulation	954
VI.	Conclusion	960

Abstract. This review focuses on methods of designing photocontrolled proteins and nucleic acids. Data on preparation and modification of proteins and nucleic acids with azobenzene derivatives are summarized. Examples of using photoswitchable proteins, their substrates, inhibitors and ligands containing azobenzene, as well as azobenzene derivatives of nucleic acids, for design of nanomachines are considered. The bibliography includes 122 references.

I. Introduction

Light plays a key role in many processes in animate and inanimate nature. During evolution, all organisms — from bacteria to mammals — have acquired a variety of mechanisms in order to detect light and respond to it. As a rule, they have specialized organs and tissues; however, there are also examples of intracellular structures. Proteins are one of the major biopolymers in the cell, and they are very different in structure and function. The protein activity can be tailored at the stage of synthesis (*i.e.*, at the stage of transcription and translation), as well as by regulating its degradation and using inhibitors or activators. If the protein has certain structural elements, its activity can be regulated by light. In nature, such proteins are rare (*e.g.*, bacteriorhodopsin); however, it is possible to introduce, into proteins, so-called 'photoswitches' — fragments that change their structure under the influence of light. This can be accomplished by chemical reactions: either by incorporation of unnatural amino acids into proteins or by modification of a substrate, inhibitor or cofactor. A major property of nucleic acids (NAs) is the formation of double and triple helix complexes. The ability of photoswitches introduced into nucleic acids to modify the stability of these complexes will influence the processes in which they are involved and extend the use of NA derivatives.

Currently, substituted azobenzenes, spiropyrans, sterically hindered stilbenes, thioindigo derivatives and other compounds are used as photoswitches. Reversible transitions of these compounds are accompanied by one of the following processes: *cis* – *trans* or *syn* – *anti* isomerization, photocyclization and the establishment of keto – enol tautomer equilibrium.[1–5] Azobenzene derivative are the most common photoswitches. Although azobenzenes have long been used as dyes in industry, their application as molecular photoswitches has begun relatively recently. Numerous papers have dealt with the use of azobenzene derivatives in various fields of chemistry — polymer, organic and bioorganic chemistry[6,7] and materials science.[8,9] Considerable interest in using such reagents in bioorganic chemistry is due to the fact that the isomerization of azobenzene occurs under the action of light with a wavelength larger than 300 nm and therefore can activate/deactivate the proteins *in vivo*, at least, in cell culture. Azobenzene derivatives enable

T S Zatsepin, L A Abrosimova, M V Monakhova, E A Kubareva, T S Oretskaya Department of Chemistry, Department of Bioengineering and Bioinformatics, A N Belozersky Institute of Physico-Chemical Biology, M V Lomonosov Moscow State University, 119991 Moscow, Leninskie Gory 1, Russian Federation. Tel. (7-495) 939 31 48,
e-mail: tsz@yandex.ru (T S Zatsepin),
abrludmila@rambler.ru (L A Abrosimova),
monakhovam@gmail.com (M V Monakhova);
tel. (7-495) 939 54 11,
e-mail: kubareva@belozersky.msu.ru (E A Kubareva),
oretskaya@belozersky.msu.ru (T S Oretskaya);
Le Thi Hien Department of Engineering Physics and Nanotechnology, University of Engineering and Technology, Vietnam National University, Xuan Thuy 144, Cau Giay, Hanoi, Vietnam.
Tel. (84-43754) 94 29, e-mail: lehien1411@gmail.com;
A Pingoud Insitute for Biochemistry, Justus-Liebig University, Heinrich-Buff-Ring 58, D-35392 Giessen, Germany. Tel. (49-641) 993 54 00,
e-mail: Alfred.M.Pingoud@chemie.bio.uni-giessen.de

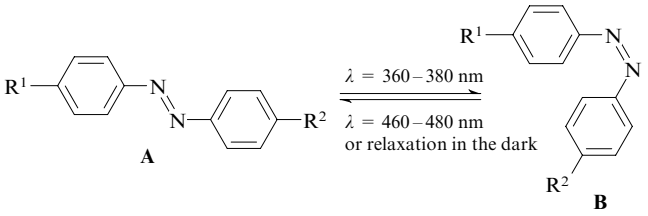
Received 12 October 2012
Uspekhi Khimii **81** (10) 942 – 963 (2013); translated by G A Kirakosyan

† This review is dedicated to the memory of Professor Har Gobind Khorana, a Nobel Prize winner, an outstanding scientist who determined the development of nucleic acid chemistry.

the most efficient regulation of the protein activity as compared with other reagents.

Azobenzene with the double bond in the *anti* configuration‡ (**A**) is converted into the *syn* form (**B**) upon illumination with near-UV light (~380 nm), and inverse transformation occurs upon illumination with blue light (470 nm) or upon incubation in the dark (Scheme 1).[10–12]

Scheme 1



Azobenzene derivatives are widely used for photoregulation of protein activity owing to the following unique properties:

— minimal effect of the dipole moment of a medium on the absorption spectrum and isomerization process,

— slow fluorophore bleaching,

— high yield of the *syn*–*anti* transition,

— significant difference in geometry between the *syn* and *anti* isomers (a large difference in distances and angles between two ends of the molecule in the *syn* and *anti* configuration),

— short isomerization time (1–10 ps),

— chemical stability of azobenzene in the absence of strong reducing agents.

The isomerization of the central double bond leads to a change in the geometry of the molecule and its dipole moment.[13–15] The *syn*/*anti* ratio for azobenzene derivatives is usually determined by HPLC[16] or ^{1}H NMR.[17] The singlet of the proton in the *ortho* position of *anti*-azobenzene is converted into a doublet in the *syn* isomer. The reversible *syn*–*anti* isomerization mechanism of azobenzene remains unclear despite a large number of experimental and theoretical studies addressing this issue.[18–21] The following *syn*–*anti* isomerization mechanism of azobenzene has been proposed: a combination of rotation of one of the benzene rings out of the molecule plane (Scheme 2, path *a*) and its inversion in the molecule plane (Scheme 2, path *b*).

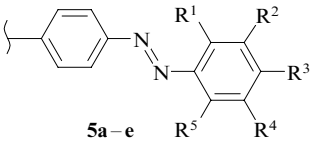
‡ Hereinafter, prefixes *syn* and *anti* are used to denote the *Z*- and *E*-configurations of isomers as most common in this field of chemistry.

This review focuses on incorporation of azobenzene derivatives into nucleic acids and their analogues, as well as peptides, proteins, their substrates and ligands, aimed at changing the structure and regulating the activity of these biologically active molecules.[22–26]

II. Synthesis and application of nucleic acids and their analogues containing azobenzene fragments

To regulate the activity of NA-binding proteins, analogues of oligonucleotide substrates that change their properties upon irradiation can be used. An azobenzene derivative based on D-threoninol (compound **1**) is most commonly used for this purpose.[27] It has been shown that the D-threoninol-tethered azobenzene moiety protrudes toward the minor groove of the DNA duplex, while the azobenzene moiety of an L-threoninol residue (**2**) protrudes toward the major groove. A structural change of the azobenzene moiety in the narrow minor groove has a significant effect on the structure and stability of the NA duplex, whereas the effect is smaller in the wide major groove. The introduction of one or two azobenzene moieties into oligonucleotide induces a difference of 15–20 °C between the thermal stabilities of the DNA duplexes for the *syn* and *anti* forms. To synthesize such oligonucleotides (X_D and X_L), phosphoramidite derivatives containing *N*-(4-phenylazobenzoyl)-D-threoninol and *N*-(4-phenylazobenzoyl)-L-threoninol residues (**3** and **4**, respectively) have been used (Scheme 3).[27]

Structures **5**

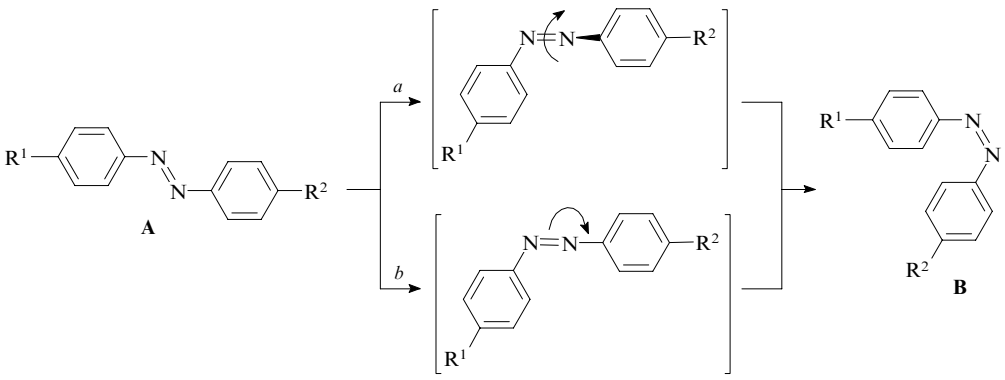


R^1 = Me, R^2 = R^3 = R^4 = R^5 = H (**a**); R^2 = Me, R^1 = R^3 = R^4 = R^5 = H (**b**); R^3 = Me, R^1 = R^2 = R^4 = R^5 = H (**c**); R^1 = R^5 = Me, R^2 = R^3 = R^4 = H (**d**); R^1 = R^4 = Me, R^2 = R^3 = R^5 = H (**e**)

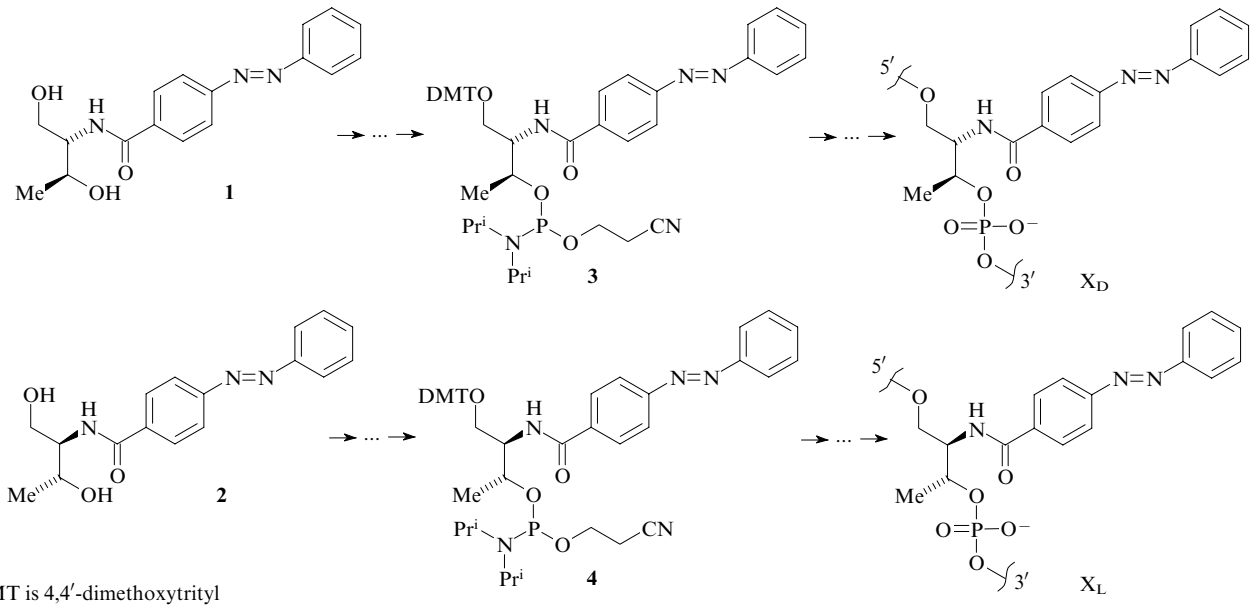
As photoswitches for changing the duplex stability, different D-threoninol-tethered substituted azobenzenes **5a**–**e** incorporated into oligonucleotides have been used.[28]

An azobenzene moiety in the *anti* configuration is able to stabilize the DNA duplex. On irradiation with UV light, the double bond configuration changes to give the *syn* form, which destabilizes the DNA duplex. It has been shown that the introduction of one methyl group into the azobenzene moiety leads to a larger change in the melting point of the DNA duplex after UV irradiation (~380 nm) as compared

Scheme 2



Scheme 3



DMT is 4,4′-dimethoxytrityl

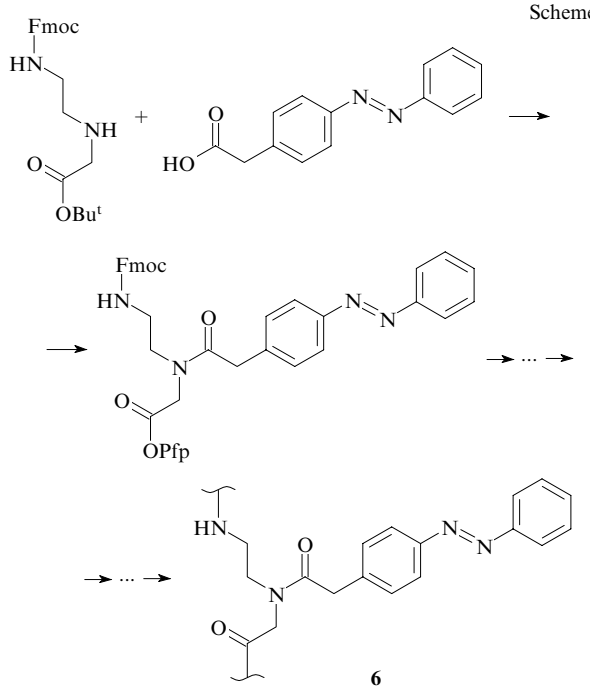
to the duplexes containing unmodified azobenzene. The use of disubstituted azobenzenes leads to an even larger change in the melting point of corresponding DNA duplexes. However, it should be taken into account that the thermal *syn*–*anti* relaxation of substituted azobenzenes incorporated into the oligonucleotide is 10 times slower than that of the unsubstituted compound.

In the case of hybridization of DNA containing an azobenzene moiety with its complementary RNA, there is also a significant difference in the duplex stability for *syn* and *anti* configurations.[29] Unlike the DNA/DNA duplex, DNA/RNA duplexes in some sequences are destabilized even by azobenzene in the *anti* configuration. This is probably due to the difference in the duplex structure between DNA/DNA (B-form) and DNA/RNA (A-form).

Azobenzene-modified DNA/RNA duplexes have been studied by the circular dichroism (CD).[29] The weak negative Cotton effect at ~360 nm ($\pi-\pi^*$ transition of azobenzene) indicates the intercalation of *anti*-azobenzene between adjacent base pairs of the duplex. In addition, the absorption band of azobenzene in the *anti* configuration exhibits a bathochromic shift upon duplex formation. In the case of the *anti* configuration, the induced CD is much weaker. These spectral changes are almost the same as those observed for the DNA/DNA duplex.[29] The RNA/DNA duplex incorporating an azobenzene moiety[30] is also characterized by a significant difference in thermal stability between the two forms, which is favourable for its photoregulation. In addition, an azobenzene-tethered oligonucleotide is able to form a triplex, which can be used for blocking the binding of transcription factors (activators) and RNA polymerase.[31] For example, in the case of a 13-mer triplex containing *m*-amidoazobenzene tethered to the 5′-end of one of the chains, the difference in thermal stability caused by the *anti*–*syn* isomerization of *m*-amidoazobenzene is 19.2 °C. It is worth noting that the introduction of *m*-amidoazobenzene in the *anti* configuration into one of the triplex strands increases the melting point (T_m) by 6.3 °C as compared to the unmodified triplex. Upon introduction of *p*-amidoazobenzene into an analogous triplex, the change in T_m caused by the *anti*–*syn* isomerization is 14.3 °C. This difference strongly depends on the position of the azobenzene moiety: when *p*-amidoazobenzene is introduced into the middle of the oligonucleotide chain, this difference in more than 30 °C.

Peptide nucleic acids (PNAs) are synthetic analogues of nucleic acids and consist of heterocyclic bases attached to *N*-(2-aminoethyl)glycine polyamide.[32] Although they are not natural compounds, they can form standard Watson–Crick and Hoogsteen pairs with DNA and with each other. Owing to the high affinity of PNAs for DNA, as well as to their physiological stability, PNAs are often superior in hybridization efficiency to other DNA analogues when

Scheme 4



Fmoc is 9-fluorenylmethyloxycarbonyl; Pfp is pentafluorophenyl

acting on different biochemical processes, such as transcription and translation. For photoregulation of these processes, an azobenzene moiety is introduced into PNA (Scheme 4). The photochromic properties of the resulting compound **6** are very similar to those of analogous nucleic acid derivatives.[33, 34]

An interesting approach to stabilization of mismatch pairs in the DNA duplex is the use of low-molecular-weight compounds binding to heterocyclic bases. Dimeric *N*-(7-methyl-1,8-naphthyridin-2-yl)carbamate (NC) selectively binds to the double-stranded 5′-Y**G**G-3′/3′-G**G**Y-5′ sequence involving a G·G mismatch through hydrogen bonds with guanine bases (structure **7**). If Y = T, the stabilizing effect of NC is maximal (Fig. 1).[35]

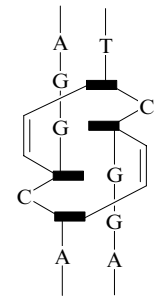
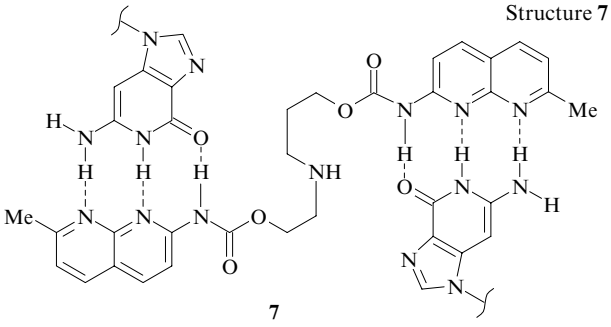


Figure 1. Schematic representation of binding of two DNA strands through the NC_2Az dimer (**8**).[35]

Structure **7**



7

In particular, 11-mer 5′-CCTT**TGG**TCAG-3′ DNA at room temperature is a single-stranded structure, whereas in the presence of 100 μmol litre^{-1} of dimer **7,** a duplex with a melting temperature of 58.8 °C is formed. The introduction of azobenzene into the linker between the naphthyridine moieties (NC_2Az, **8**) permits control of duplex formation.

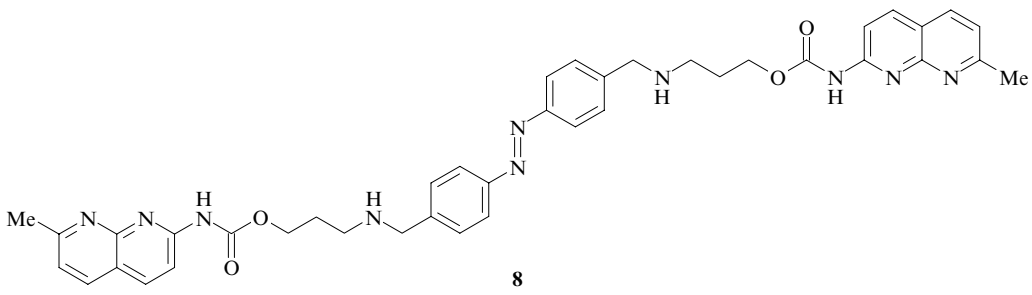
When azobenzene is in the *anti* form, the melting temperature of DNA duplex 5′-CTAA**CGG**AATG-3′/3′-GATT**GGC**TTAC-5′ was 32.7 °C. Exposure to 360 nm light increased the T_m value by 15.2 °C ($T_m \approx 48.0$ °C). This effect indicates that the *syn* configuration of NC_2Az has a higher binding affinity to the G·G-mismatch-containing 5′-**CGG**-3′/3′-**GGC**-5′ sequence as compared to the canonical sequence. The nonplanar linker with *syn*-azobenzene allows two naphthyridine rings to be placed in the appropriate position for binding to guanine residues in the mismatch pair. The duplex stabilization is fully reversible.

Nucleic acids are prone to self-assembly based on hybridization of complementary strands, which enables the preparation of photonic wires, enzyme ensembles and DNA probes.[36–43] On the basis of DNA, 'nanomachines' are developed that can change the efficiency in response to an external trigger. In the last decade, many nanomachines of different construction have been built.[36, 44] In most cases, such 'molecular motors' are fueled by the energy released during DNA hybridization. For each working cycle, hybridized DNAs have to be removed and replaced by single-stranded complementary DNAs. It is natural that the working efficiency of nanomachines strongly decreases because of similar operations. The use of DNA photoswitchable by means of azobenzene derivatives made it possible to overcome these drawbacks. Liang *et al.*[45] developed a 'nanopincette' (tweezers) whose operation efficiency did not change during 10 cycles of opening and closing. Such tweezers are composed of three DNA strands (strands A, B, C) and have the structure shown in Fig. 2.

Segments B1 and C1 hybridize with oligonucleotide A to form 22-bp DNA duplexes as two arms of the tweezers. Strand B also contains segment B2 into which the D-threoninol residue modified by azobenzene (**3**) or *p*-isopropylazobenzene is introduced. Single-stranded segment B2 at the 3′-end of strand B is able to hybridize with its complementary single-stranded segment in strand C (C4). Upon formation of such a 10-bp duplex, the pincette is closed (the *anti* configuration of azobenzene promotes duplex formation). Upon irradiation with UV light, the duplex between segments B2 and C4 dissociates because of the *anti*–*syn* isomerization of the azobenzene moiety, and the pincette is opened. The operation of the pincette was monitored by measuring fluorescence. To do this, a fluorophore and a 'quencher' were attached to the two ends of oligonucleotide A, and opening and closing of the tweezers was monitored by measuring the change in fluorescence intensity. Interestingly the use of a *p*-isopropylazobenzene moiety as the photoswitch led to the opposite effect: irradiation with visible light opens the pincette, whereas UV light irradiation closes it.

Photoresponsive nanostructures have been constructed on the basis of azobenzene-modified DNA.[46] Recently, a molecular motor controlled by azobenzene-modified DNA has been designed. It operates owing to exchange between many DNA strands. Further improvement of the optical

Structure **8**



8

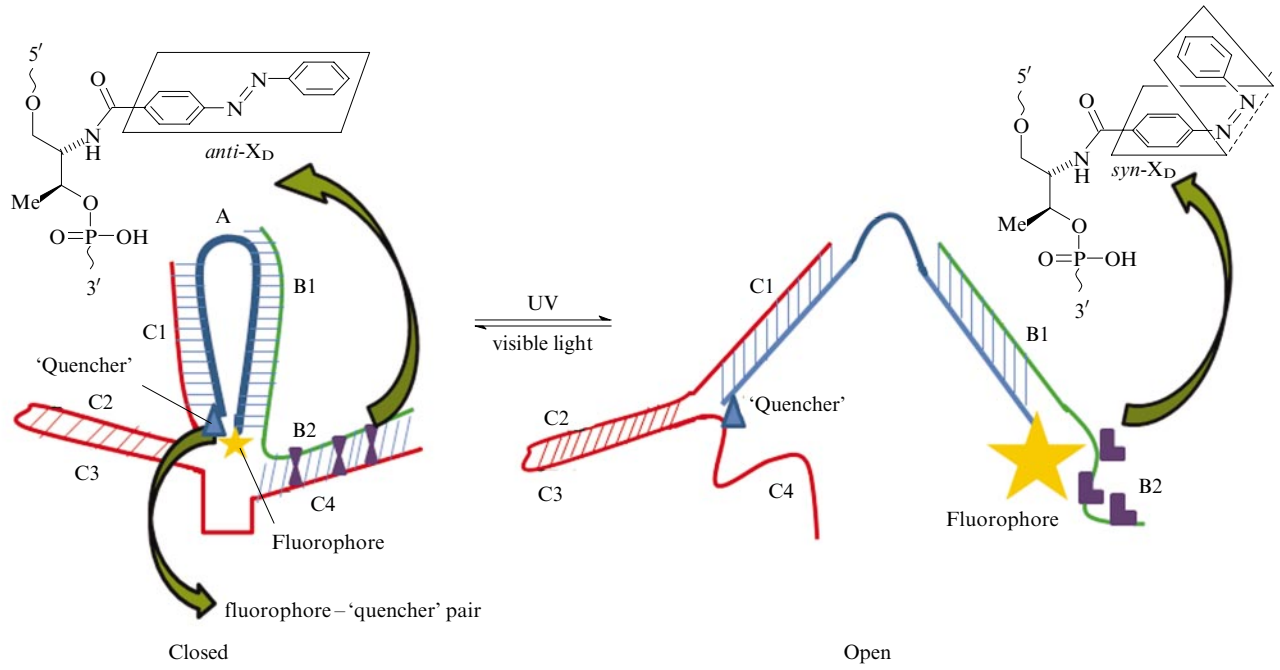


Figure 2. Structure and the scheme of action of a pincette constructed from DNA fragments tethering azobenzenes.[45]

properties of a photoswitch led to the creation of a single-molecule DNA nanomotor.[47] The principle of operation of the single-molecule DNA motor is based on controllable dehybridization (in the open state) and hybridization (in the closed state) of the hairpin DNA structure with incorporated azobenzene moieties. Due to the bilateral movement (expansion or contraction), such a molecule is a motor, and its motion can be characterized by the change in fluorescence with a change in the distance between the fluorophore and 'quencher' at the DNA ends (Fig. 3).

As compared to the previous DNA motors in which the working cycles of the engine involve bimolecular or multimolecular interactions between several independent DNA strands, the hairpin-structured single-molecule DNA motor has a much simpler operation principle owing to its unique structure. Because of its simplicity and intramolecular interactions driven by UV and visible light, the motor displays 40%–50% close–open conversion efficiency. This type of nanomotor exhibits well-regulated activity under mild conditions without loss of matter. In contrast

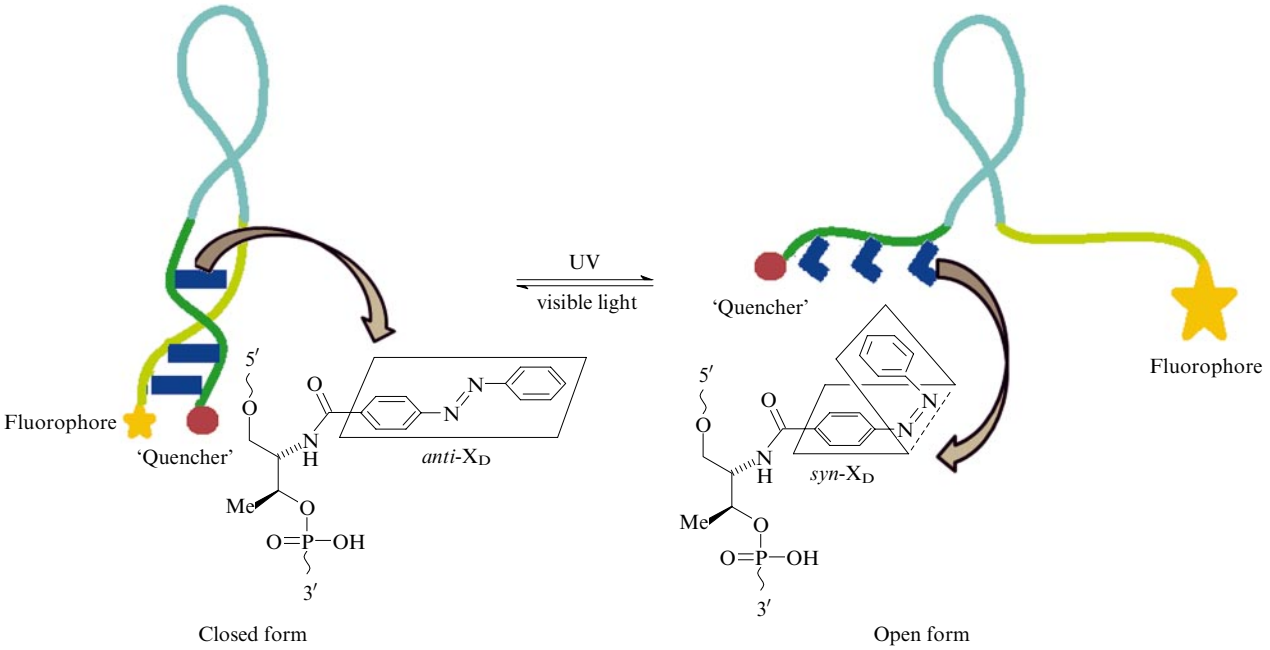


Figure 3. Principle of operation of the DNA nanomotor containing three azobenzene moieties X_D in the hairpin structure.[47]

to multicomponent DNA machines, the suggested system offers unique concentration-independent motor functionality. Moreover, the hairpin structures of the motor backbone significantly enhance the efficiency of light-to-movement energy conversion.[47]

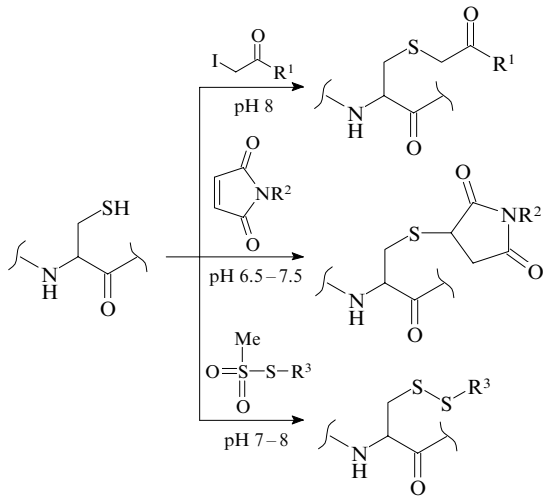
For protein activity regulation, nucleic acid aptamers are widely used, which are able to efficiently and selectively bind to the active site of a definite protein or allosterically regulate its activity. A photoswitchable thrombin aptamer with three functional domains — inhibitory, regulatory and linking — has been synthesized.[48] This aptamer makes it possible to reversibly regulate the thrombin activity depending on the irradiation wavelength.

III. Incorporation of azobenzene moiety into peptides and proteins

The following approaches are available for incorporation of azobenzene into peptides and proteins: chemical modification of peptides and proteins,[49–51] incorporation during peptide synthesis[16, 23, 52, 53] or during *in vitro* translation[54–56] and *in vivo*.[57] Depending on the method selected for the introduction of the azobenzene moiety into a peptide or protein, different compounds are used.

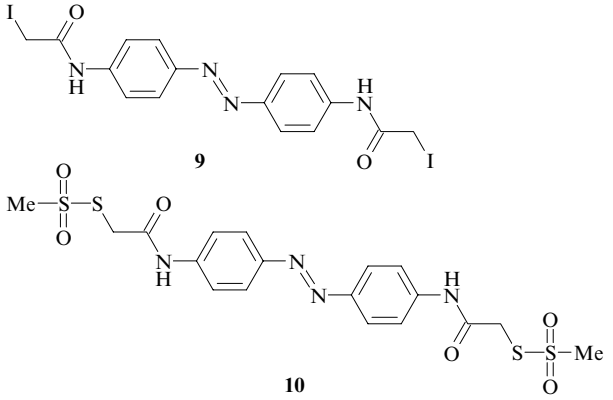
In most cases, peptides and proteins are modified through specific reactions involving thiol groups of cysteine residues or ε-amino groups of lysine residues. The thiol group of cysteine residues is rather reactive in weak alkaline solutions, which makes it possible to introduce this group in the reaction with haloacetamides, maleimides and thioesters (Scheme 5).[58] In the last case, the S–S bond can be broken in the presence of reducing agents to regenerate the initial protein. It should be taken into account that maleimide reacts specifically with the cysteine thiol group only at pH 6.5–7.5, and an increase in pH promotes a side reaction with protein amino groups.[59]

Scheme 5



For modification of cysteine, two symmetric reagents based on diaminoazobenzene — compounds **9** (see [50, 60–62]) and **10**[51, 63] — have been suggested. In the case of the second reagent, the azobenzene moiety can be removed by the action of a reducing agent.

Structures **9**, **10**



9

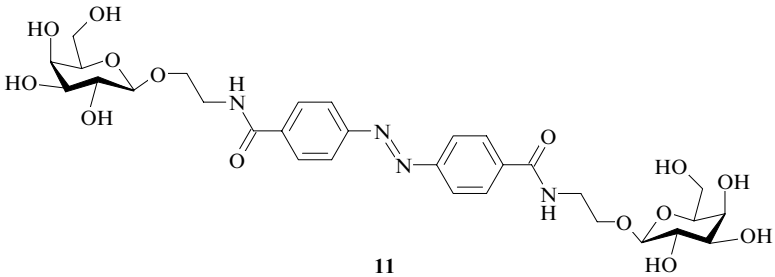
10

An alternative way of introducing photoswitchable moieties implies the use of noncovalent interactions. Among biological recognition processes, specific carbohydrate–protein interaction is relatively weak; however, the use of glycoclusters leads to multiplicative enhancement of binding.[64] The synthesis of a new class of azobenzene derivatives containing several carbohydrate residues has been described.[17] These compounds are synthesized by acylation of 2-aminoethyl glycopyranosides with azobenzene-4,4′-dicarboxylic acid chloride (compound **11**).

Cooperative binding of the lactoside-bearing azobenzene derivative to the corresponding lectin has been revealed. Upon UV irradiation (azobenzene in the *syn* configuration), the lectin binding affinity of glycoside increases. It should be noted that thermal *syn*–*anti* relaxation of such azobenzene derivatives in an aqueous solutions is rather slow (< 5% in 3 h).[17]

To synthesize azobenzene containing several substituents in benzene rings, appropriate aniline derivatives with the corresponding substituents should be first obtained. Subsequent oxidative coupling of the aniline derivatives under the action of sodium hypochlorite,[65] silver oxide[66] or sodium tetrafluoroborate[67] leads to symmetric azobenzenes. Azobenzene derivatives can also be synthesized by the reduction of nitrobenzenes with tin(II) chloride.[68] The poor aqueous solubility of azobenzene derivatives complicates their use for protein modification. To overcome this drawback, compounds with sulfo or amino groups in the aromatic ring have been synthesized. For example, a water-

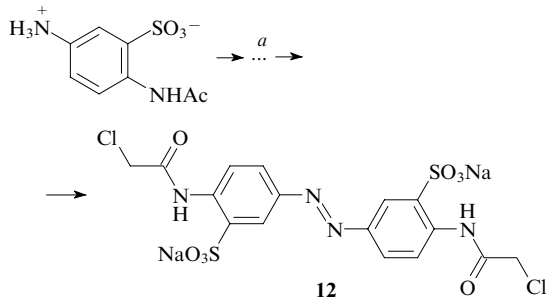
Structure **11**



11

soluble sulfo azobenzene derivative **12** has been synthesized by oxidation of 5-amino-2-acetylaminobenzenesulfonic acid with sodium hypochlorite and subsequent acylation of the aromatic amino group (Scheme 6).[60, 65, 69]

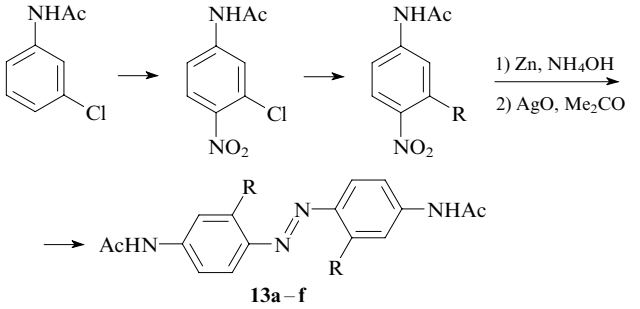
Scheme 6



(*a*) 1) NaClO, Na_2CO_3, H_2O; 2) HCl, H_2O; 3) NaOH, H_2O; 4) $ClCH_2CO_2H$, $(ClCH_2CO)_2O$

4,4′-Diacetamidoazobenzenes **13a–f** with different amino groups in the 2- and 2′-positions have been synthesized in several stages from *N*-(3-chlorophenyl)acetamide. At the final stage, the corresponding substituted acetamidoaniline is oxidized with silver oxide (Scheme 7).[66] It has been shown that hydrophobic interactions between the substituents in the 2- and 2′-positions of azobenzene stabilize its *syn* configuration.

Scheme 7

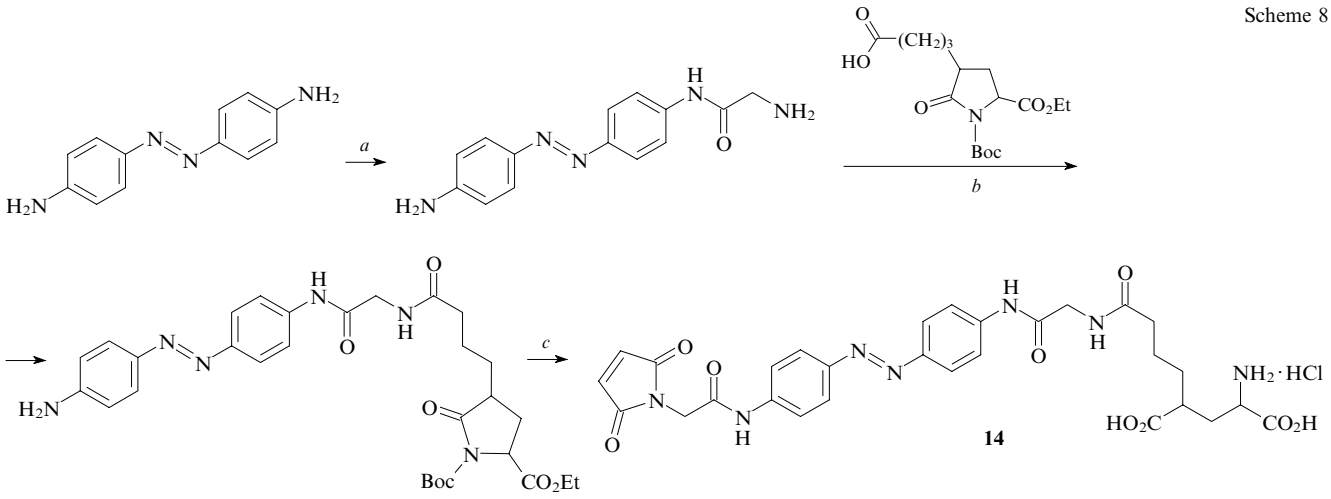


R = Me_2N (**a**), Et_2N (**b**), $(CH_2)_4N$ (**c**), $(CH_2)_5N$ (**d**), $O(CH_2CH_2)_2N$ (**e**), $MeN(CH_2CH_2)_2N$ (**f**)

Symmetric azobenzene derivatives are often used for cross-linking of two spatially close cysteine residues of peptides or proteins. Asymmetric azobenzene derivatives containing a reactive group in one of the benzene rings and various substituents in the other ring are used when it is necessary to modify one amino acid residue. As a rule, to obtain such compounds, diaminoazobenzene is acylated, at the first stage, by one equivalent of a carboxylic acid (Scheme 8). For example, compound **14**, containing the glutamic acid residue, was used for allosteric control of the glutamate receptor activity with light.[49]

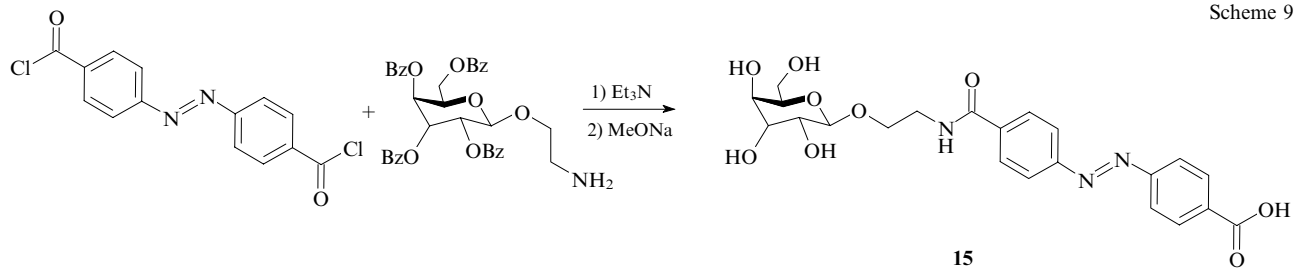
Monosubstituted azobenzenedicarboxylic acid **15** has been synthesized analogously (Scheme 9).[17]

Scheme 8

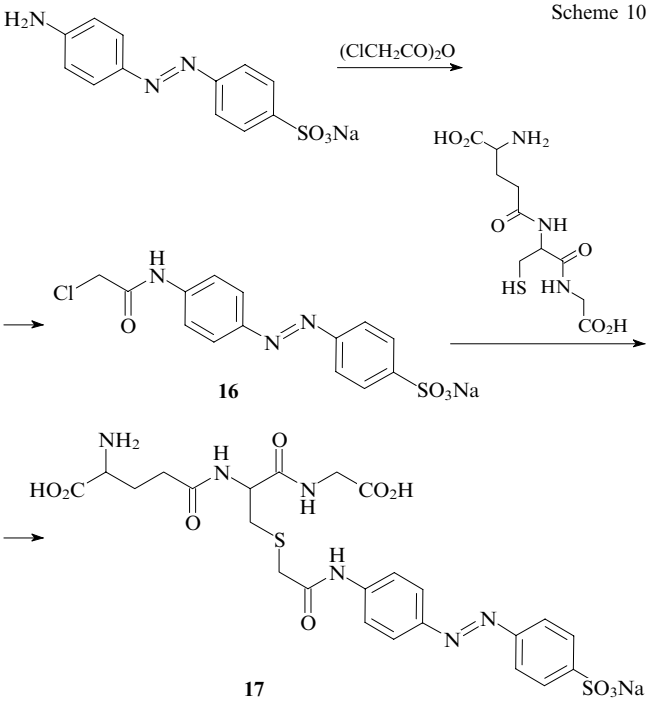


(*a*) 1) Boc-Gly-OH, EDC, HOBt, DIPEA (yield 66%); 2) TFA, CH_2Cl_2 (yield 98%); (*b*) 1) EDC, HOBt, DIPEA (yield 97%); 2) Fmoc-Gly-OH, $(COCl)_2$, DMF; 3) piperidine, DMF (yield 43% as calculated for two stages); (*c*) 1) 1 M LiOH b H_2O, THF, 0 °C (yield 80%); 2) *N*-methoxycarbonylmaleimide, $NaHCO_3$, THF, H_2O (yield 71%); 3) EtOAc, sat. HCl (yield 87%);
Boc is *tert*-butoxycarbonyl, EDC is 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, HOBt is hydroxybenzotriazole, DIPEA is diisopropylethylamine, TFA is trifluoroacetic acid

Scheme 9



Scheme 10

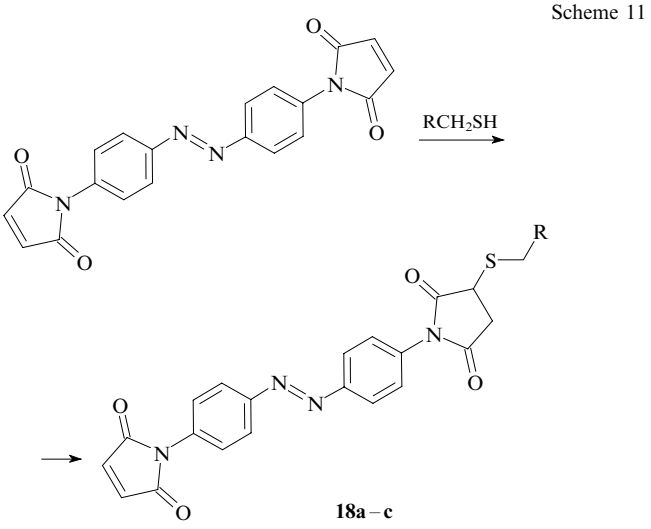


Commercially available asymmetric azobenzene derivatives can also act as initial compounds (Scheme 10).[70] Compound **16** has been used for modifying α-hemolysin. It has been shown that the thermal *anti*–*syn* relaxation rate of azobenzene derivative **16** is 4 times as high as the thermal relaxation rate of compound **17**, containing glutathione.

For regulation of the protein active site, asymmetric azobenzene derivatives with a maleimide moiety have been used.[71] For example, compounds **18a**–**c** prepared for this purpose contain unsubstituted maleimide in one benzene ring for binding to the cysteine residue of the protein and maleimide with a peripheral hydroxyl, carboxyl or amino acid group in the other ring (Scheme 11).

Goodman and Kossoy[72] in the 1960s suggested for the first time to use azobenzene-containing amino acid (4′-phenylazophenylalanine, PAP, **19**) for peptide modification. Photoregulation of the conformation of poly-α-amino acid

Scheme 11



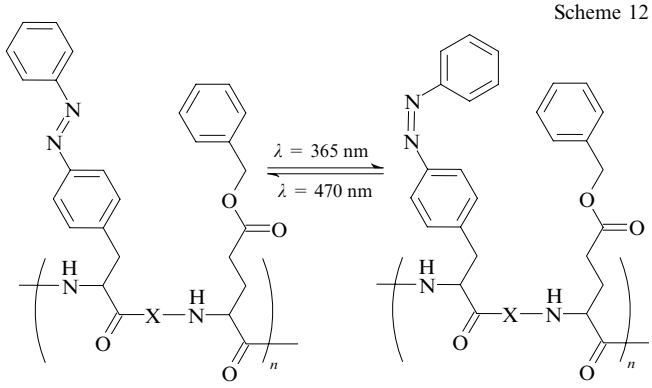
R = CH_2OH (**a**), CH_2CO_2H (**b**), $CH(CO_2H)NH_2$ (**c**)

in a copolymer of 4′-phenylazophenylalanine with γ-benzylglutamic acid was accomplished by light (Scheme 12).

Reagents for solid-phase peptide synthesis — Fmoc-protected 4′-phenylazophenylalanine **20** (Scheme 13)[28] and 4-(aminomethyl)phenylazobenzoic acid **21** (Scheme 14)[73] — have been synthesized by the coupling reaction of the corresponding aniline and nitrobenzene derivatives.

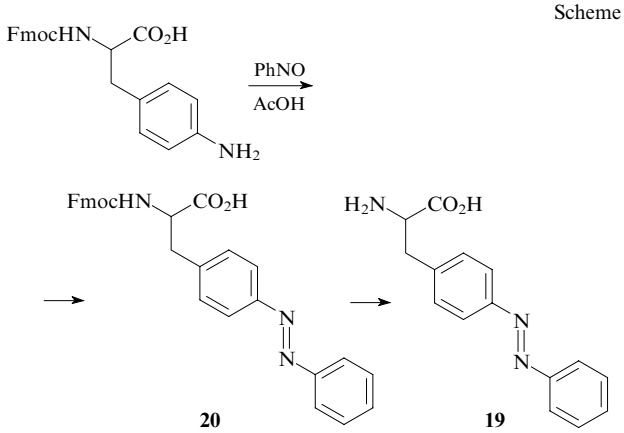
Such Fmoc-protected unnatural amino acids containing a photoswitch either in the side chain (compound **20**) or in the backbone (compound **21**) have been used for incorporation of the azobenzene moiety into peptides. For example, Fmoc-protected amino acid **20** was incorporated into the

Scheme 12

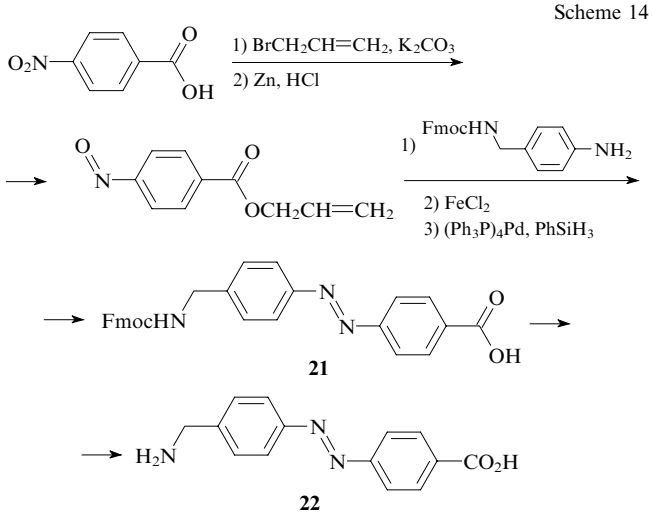


X is hydrocarbon moiety

Scheme 13



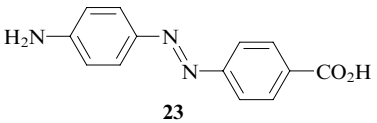
Scheme 14



S-peptide in the course of common solid-phase peptide synthesis.

Ulysse and Chmielewski[74] were the first to develop the procedure of inclusion of 4-(aminomethyl)phenylazobenzoic acid (**22**) into the polypeptide backbone by means of solid-phase peptide synthesis. Then, this amino acid was introduced into γ-ITAM (immunoreceptor tyrosine-based activation motif) for regulation of protein–protein interaction. Renner and co-workers[22,23] have synthesized another chromophore — 4-aminophenylazobenzoic acid (**23**), which, as they believed, should enhance the effect of the azobenzene moiety on the peptide structure.

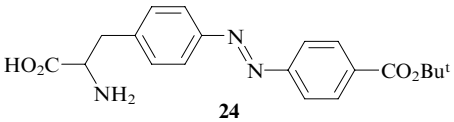
Structure **23**



23

Unnatural amino acids are often introduced into proteins in the course of *in vitro* translation with the use of four-nucleotide codons.[56,75] In particular, compound **24** was incorporated into the protein using aminoacyl $tRNA_{CCCG}$.

Structure **24**



24

Modified tRNA was obtained by the coupling of the pdCpA-**24** conjugate with $tRNA_{CCCG}(-CA)$ (*i.e.*, tRNA with the CCCG anticodon without the CA dinucleotide at the 3′-end) catalyzed by T4 RNA ligase. Translation was accomplished with mutant mRNA containing a CGGG four-nucleotide codon in *in vitro* translation system with the synthetic aminoacyl-**24**-$tRNA_{CCCG}$ and the 30S ribosomal subunit in an *Escherichia coli* cell extract. The incorporation efficiency of unnatural amino acid **24** was about 40%. By this method, compound **24** containing the azobenzene moiety was incorporated into a definite position of the BamHI restriction endonuclease.[54]

The possibility of incorporation of a photoresponsive unnatural amino acid at a definite site of protein located in a living body significantly expands the scope of photoregulation of biological processes. Bose *et al.*[57] have described the preparation of an aminoacyl tRNA synthetase/tRNA pair that allows the incorporation of the photoresponsive amino acid 4′-phenylazophenylalanine (**19**) into proteins in *E. coli*. Since PAP is similar in structure to tyrosine, the authors used the mutant tyrosine tRNA synthetase to obtain PAP tRNA. The resulting PAP tRNA is tyrosine tRNA with the CUA anticodon corresponding to the UAG stop codon. As shown, the PAP tRNA/PAP tRNA synthetase pair can efficiently operate without interfering with other tRNAs or aminoacyl tRNA synthetases in *E. coli* cells.[57]

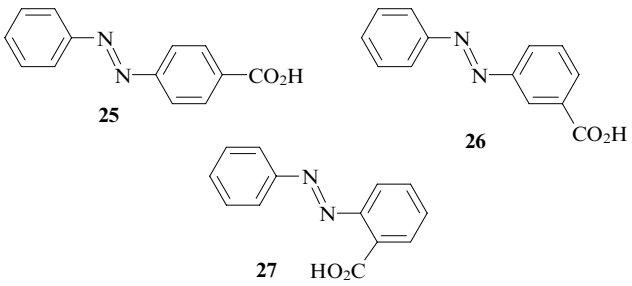
Thus, four strategies are used for introducing the azobenzene moiety into peptides or proteins: in the course of peptide synthesis, by *in vitro* and *in vivo* translations and by chemical modification of biomolecules. It follows from their comparison that chemical modification is the simplest, efficient and widely used method. Reactive azobenzene derivatives used for these purposes are synthesized by two methods — by transformations of simple and available azobenzenes or by coupling of anilines with nitrobenzenes of different structure.

IV. Photoregulation of protein activity

The enzyme activity can be regulated by light in two ways: by the direct method when the photoswitch is attached to the protein, and by the indirect method when an azobenzene moiety is incorporated into the protein substrate, ligand or inhibitor. To achieve the photoswitching effect, a system should be designed in which a minimal change in the geometry of a photochromic compound has a significant effect on the protein structure or activity. As known, azobenzene exists in the dark in the more stable *anti* configuration, which isomerizes to the *syn* form only when exposed to UV light. However, UV irradiation does not lead to pure *syn*-azobenzene. At the same time, the incubation of azobenzene in the dark or irradiation with blue light afford a mixture enriched in the *anti* form and containing a small amount of the *syn* isomer.[76] Hence, for the protein to have the photoregulated activity in the absence of continuous UV irradiation, it is necessary to design a molecule that would be inactive when azobenzene is in the *anti* configuration and would become active after irradiation with UV light when azobenzene has the *syn* configuration. It has been shown that a peptide or protein modified with symmetric azobenzene derivatives can reversibly change its conformation and/or activity with a change in the excitation light wavelength.[61,71,77,78] It is also known that, under the same conditions, the functional activity of proteins modified with asymmetric azobenzene derivatives can change *in vivo*[70,79,80] and *in vitro*.[19,81–84] For enzymes, there are a limited number of examples when modification with azobenzene derivatives makes it possible to regulate their activity upon irradiation.[54,55,85–88]

Examples of non-regioselective protein modification, for example, acylation of lysine residues, have been described. For papain,[86] carboxyl derivatives **25**–**27** have been used. It has been shown that the activity of *anti*-**25**-papain and *anti*-**26**-papain is 80% and 36% of the activity of wild-type papain. The *anti*-**27**-papain completely loses its activity after incorporation of the azobenzene moiety. Is has also been shown that *anti*-**25**-papain is 2.75 times more active than *syn*-**25**-papain.

Structures **25**–**27**



25 **26** **27**

Hence, nonspecific protein modification with azobenzene derivatives can be a simple procedure since it does not require knowledge of the protein structure, and the chem-

ical reaction involves directly a wild-type enzyme. However, it is impossible to predict the behaviour of the enzyme after the addition of several azobenzene moieties to it (as a rule, protein contains a large number of lysine residues) without knowledge of the location of modified units. Design of a photoresponsive enzyme seems to be more rational if azobenzene moieties are attached to the enzyme in a definite manner.

1. Regulation of the α-helix structure

The α-helix is a key structural element in a wide range of peptides and proteins and plays an important role in protein–protein and DNA–protein recognition, and well as in protein binding to ligands. Photoswitching either leads to the destruction of the α-helix or changes its parameters. First, photoregulation of the α-helix was achieved for a peptide.[50] To this end, two cysteine residues were incorporated into the peptide and cross-linked by a bifunctional azobenzene-based linker (Fig. 4). Such a variant of protein structure regulation has been referred to as the 'molecular spring' method.

Symmetric azobenzene derivatives are usually used as cross-linking agents. Two variants have been realized depending on the configuration in which azobenzene stabilizes the α-helix — in the *anti* (see Fig. 4 *a*)[65, 69] or *syn* configuration[24, 50, 51, 87] (see Fig. 4 *b*). To optimize the photoregulation effect, the location of cysteine residues in the α-helix and the length of cross-linking agents have been varied (see Fig. 4 *c*). For a leucine zipper that forms upon dimerization of two parallel leucine-rich α-helices, it has been demonstrated that photoswitching of the structure of α-helices has a strong effect on their dimerization and DNA binding efficiency.[88] This approach has been applied to the DNA-binding domain of the yeast transcription activator bZIP GCN4.[78] In the dark, the α-helix content of the modified protein decreases as compared to the wild type protein; UV irradiation increases the α-helix content and substantially (~20-fold) enhances DNA binding. The change in the DNA-binding activity of the modified protein is fully reversible.

The 'molecular spring' approach has been used for MyoD transcription factor, which is a protein containing the helix–loop–helix domain for DNA binding.[89] Upon irradiation with UV light (azobenzene is in the ground-state *syn* configuration), the α-helix is stabilized, which leads to formation of the DNA–protein complex. It is of interest that the binding affinity of photoMyoD to the control DNA, which does not contain the specific sequence, is the same in the dark and under illumination. The binding efficiency of photoMyoD to the specific DNA ligand increases by two orders of magnitude after UV activation, which is the strongest effect among the hitherto known photoswitching effects for proteins. Presumably, this is caused by a minimal change in the helix–loop–helix domain structure of the MyoD transcription factor leading to a significant change in the DNA ligand binding affinity of the protein.

The 'molecular spring' method also affords control of protein–protein interactions for Syk tyrosine kinase.[74] The Syk protein contains the kinase domain and the tandem SH2 domain (tSH2), which play a key role in binding to a specific receptor containing an intracellular amino acid sequence referred to as ITAM. When two tyrosine residues in the ITAM sequence are phosphorylated, the tandem SH2 domain of the Syk protein binds to ITAM, which leads to the activation of the kinase domain of the Syk protein. Since the distance between two tyrosine phosphate residues in the native γ-ITAM peptide (structure **28**) plays an important role in the binding of the tandem SH2 domain of the Syk protein to the ITAM sequence of the receptor, a photoswitchable ITAM peptide containing an azobenzene moiety (photoITAM, **29**) has been synthesized. It has been shown that the binding efficiency of photoITAM to the Syk protein is 10-fold lower for the *syn*-azobenzene than for the *anti* configuration, which enables the control of signal transmission through the light-induced change of the receptor.

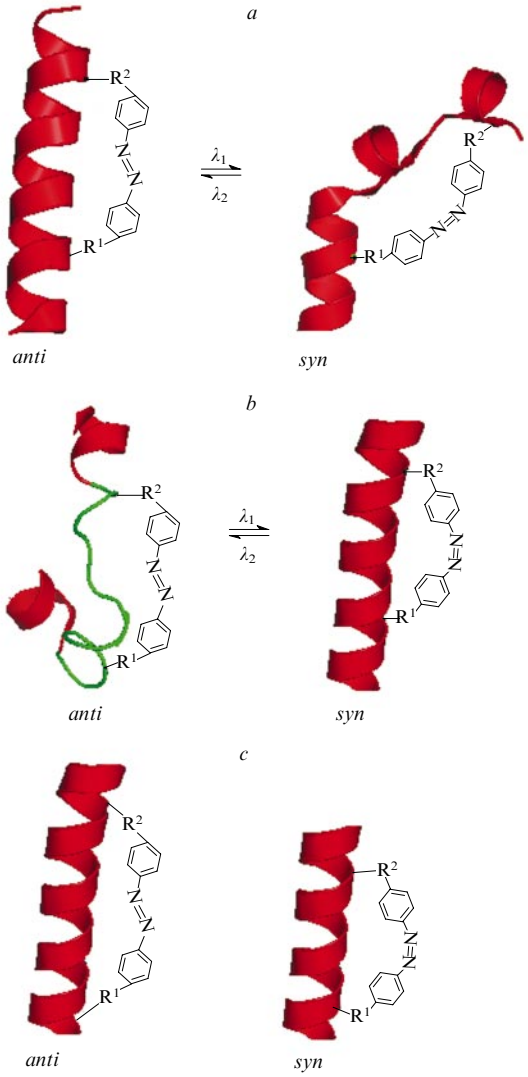
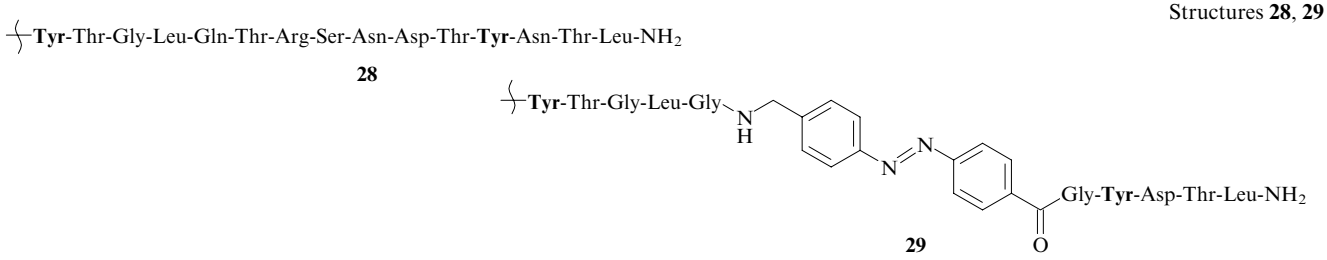


Figure 4. Formation of the α-helix when azobenzene is in the *anti* configuration and its disruption upon azobenzene isomerization to the *syn* configuration (*a*), formation of the α-helix when azobenzene is in the *syn* configuration and its disruption upon azobenzene isomerization to the *anti* configuration (*b*) and formation of α-helices of different structure with the same azobenzene cross-linker in the *anti* or *syn* configuration (*c*).[50]
Hereinafter, λ_1 and λ_2 are wavelengths used for reversible photoswitching.

Structures **28**, **29**



The 'molecular spring' approach has been applied to a specially designed analogue of the PvuII enzyme. In solution, wtR.PvuII exists as a dimer composed of two identical subunits. A mutant form of the enzyme, scPvuII (single chain PvuII), has been used [90] in which the C-terminus of one subunit (N-subunit) is linked with the N-terminus of the other subunit (C-subunit) through a four-amino acid Gly-Ser-Gly-Gly peptide linker. To regulate the activity of the mutant PvuII form, 4,4′-bis(maleimide)azobenzene is used to connect two spatially proximate cysteine residues of the protein. The best result — a 16-fold difference between the DNA hydrolysis rates upon irradiation with UV and blue light — is achieved when two bis(maleimide)azobenzene moieties are located near the catalytic centre and the catalytically important Tyr94 residue in the immediate vicinity of this centre is replaced by Phe to reduce the initial enzyme activity.[90]

2. Change in the β-hairpin structure in peptide

The smallest structural motif comprising a β-sheet is the β-hairpin where two antiparallel β-strands are linked by one turn or a short loop (Fig. 5). β-Hairpins are often involved in protein–protein and DNA–protein interactions; however, the β-hairpin structure is unstable and is difficult to characterize by spectral methods. It has been found that a tryptophan zipper structural motif (trpzip) stabilizes the β-hairpin conformation in short peptides.[91] A photoswitchable analogue of the tryptophan zipper based on azobenzene has been synthesized (structure **30**).[23]
Upon the *syn–anti* transition, the β-hairpin structure is disrupted.

Structure **30**

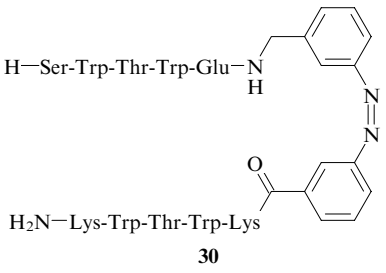




Figure 5. Schematic representation of a β-hairpin.[23]

3. Photoregulation of DNA binding to a peptide containing the 'zinc finger' structural motif

The 'zinc finger' motif exists in the structure of many eukaryotic transcription factors. They allow proteins to site-specifically bind to DNA duplexes. Numerous mutant proteins with the zinc finger motif have been designed for control of gene expression at transcription level, virus replication suppression,[92–95] telomerase inhibition,[96] DNA cleavage and site-directed modification of DNA.[97–100]

Nomura and Okamoto [101] synthesized the peptide conjugate **31** containing a zinc finger motif and an azobenzene moiety at the peptide N-terminus (Fig. 6). Compound **31** recognizes the specific DNA sequence 5′-GGGGCG-3′ after the formation of the zinc finger structure. This peptide binds to DNA through different types of interactions, such as hydrogen bonds between heterocyclic DNA bases and amino acids in the α-helix of the zinc finger and electrostatic contacts between phosphate groups of the DNA and basic amino acids. Modification at the N-terminus of the peptide does not change the zinc finger structure and function; however, azobenzene is located near the DNA binding domain of the peptide, so that the azobenzene isomerization can significantly alter the DNA-binding efficiency of the peptide. After photoisomerization of the azobenzene double

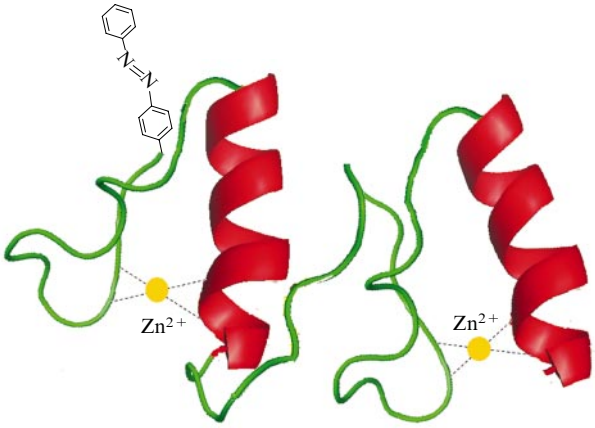
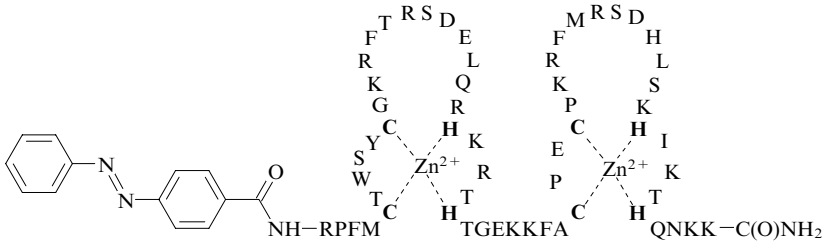


Figure 6. Spatial representation of peptide conjugate **31**, containing the 'zinc finger' motif and modified with azobenzene.[101]

Structure **31**



31

bond, the DNA-binding ability of the peptide considerably decreases, which makes it possible to control the DNA–protein interaction by UV irradiation.[101]

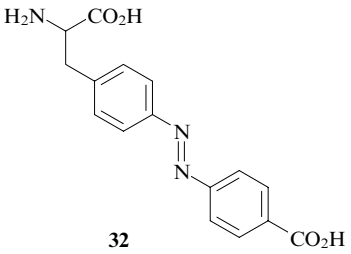
4. Binding of transcription activator CAP to 3′,5′-adenosine cyclophosphate

The *in vivo* incorporation of the photoisomerizable amino acid 4′-phenylazophenylalanine (**19**) into proteins in *E. coli* enables the photoregulation of the binding affinity of transcription factors to their promoters, which has been demonstrated by the example of the catabolite activator protein (CAP), a homodimeric bacterial transcription activator.[57] The binding of CAP to 3′,5′-adenosine cyclophosphate (cAMP) leads to a change in the protein conformation, which enhances its affinity for its promoter, and, thus, the transcription level increases. The crystal structure of the ternary CAP–cAMP–DNA complex shows that Ile71 lies in close proximity to amino acid residues forming an intricate network upon the interaction of CAP with cAMP. The PAP moiety was substituted for the Ile71 amino acid in the catabolite to form the CAP71PAP structure. It has been shown that, before UV irradiation, the binding constant of CAP71PAP to the lactose promoter is 4 times higher than after irradiation at a wavelength of 334 nm.

5. Regulation of protein subunit dimerization

Restriction endonuclease BamHI (R.BamHI) can specifically hydrolyze the DNA duplex only after the formation of the dimeric form of the enzyme.[102] Nakayama *et al.*[54] have suggested the method of regulation of the activity of this enzyme by controlling the dimerization process. Amino acids in the dimer interface that play a key role in the formation of the network of salt bridges necessary for the BamHI dimer conformation being in the active form were replaced by phenylazophenylalanine **19** or its 4-carboxy-containing analogue **32** to afford photoactivated restriction endonucleases **19**-BamHI and **32**-BamHI.

Structure **32**



32

In the dark, the DNA-hydrolyzing activity of the enzyme (*anti*-**19**-BamHI) is suppressed for one of its mutant forms (the percentage of cleaved DNA substrate is only 3%). After irradiation with UV light, the activity of the enzyme (*syn*-PAP1-BamHI) is recovered (the percentage of cleaved DNA substrate is 98%). After irradiation with 436-nm light (for the *syn–anti* isomerization of azobenzene), the decrease in enzyme activity might be expected. However, no change in enzyme activity is observed. This means that, after the formation of the appropriate conformation of R.BamHI dimer (*syn*-azobenzene, active enzyme), the reverse transformation is hindered.

The authors suggested two possible mechanisms of regulation of the enzymatic activity of R.BamHI by means of *anti–syn* isomerization of azobenzene. The first mechanism implies that the azobenzene moiety in the *anti* configuration prevents two R.BamHI monomers from interacting to form the enzyme dimer. The more compact *syn*-azobenzene structure does not hinder the active enzyme formation. According to the second mechanism, the azobenzene moiety changes the local structure of the R.BamHI monomer in the vicinity of the chromophore through the interaction of the aromatic rings with the protein surface. Since the *syn*-azobenzene is more compact as compared to the *anti* isomer, the area of the contact between the protein and the *syn* isomer is smaller than that between the protein and the *anti* isomer. Thus, the *anti*-azobenzene has a stronger impact on the local structure in the dimer interface than the *syn* isomer. The activity of restriction endonuclease **32**-BamHI with the carboxyl group in the azobenzene moiety is recovered after UV irradiation faster than the activity of its analogue **19**-BamHI. This means that the negative charge of the carboxyl group in compound **32** affects the dimerization of restriction endonuclease **32**-BamHI during *anti–syn* isomerization.

6. 'Molecular gate' approach

To control the activity of homodimeric DNA-binding proteins, a 'molecular gate' strategy has been developed, and its efficiency has been demonstrated for restriction endonuclease SsoII.[103] When interacting with the substrate, the restriction endonuclease undergoes conformational changes, which allows DNA to approach the DNA-binding site of the enzyme located in the interface between the two subunits of the homodimer.[104, 105] Three different mutant forms of restriction endonuclease SsoII have been used in which the residues chosen for modification are replaced by cysteine. Modification of the protein by asymmetric azobenzene derivatives near the DNA-binding or catalytic centre can make it inaccessible for DNA because of steric hindrance in the case of the 'extended' *anti* configuration of azobenzene derivatives.[71] Since the *anti* configuration of azobenzene is more stable than the *syn* isomer, the modified

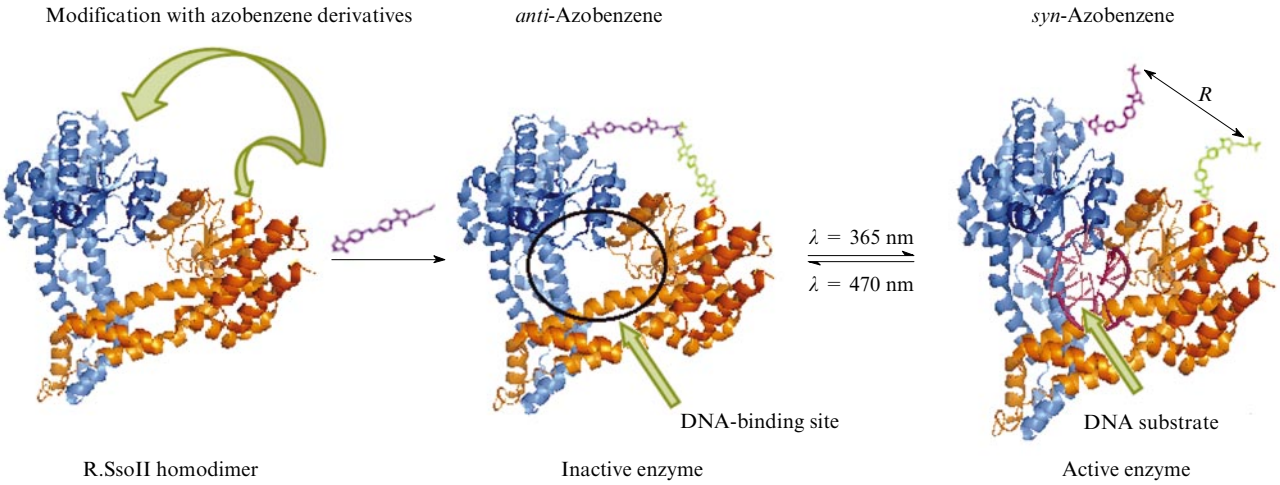


Figure 7. Principle of the 'molecular gate' strategy for restriction endonuclease SsoII.[103]

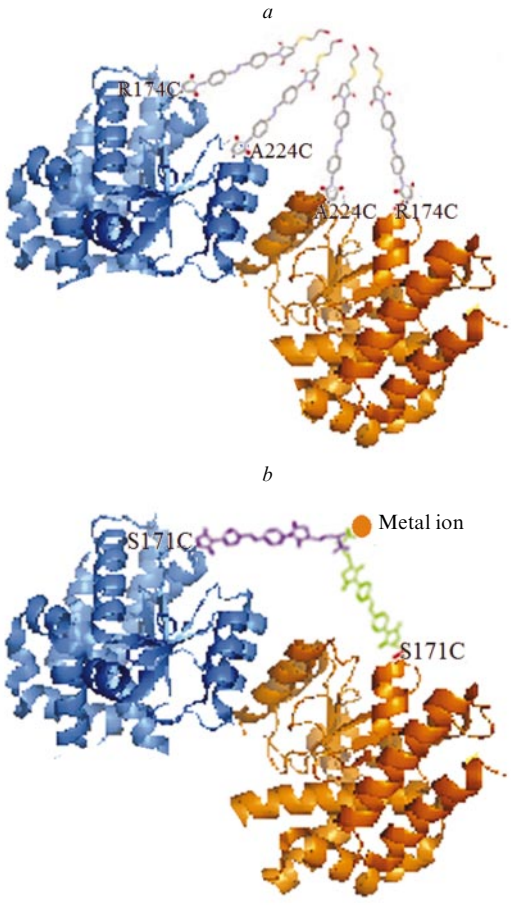


Figure 8. Schematic representation of the molecular gate approach, which shows the reversible blocking of the protein active site by azobenzene derivatives: (*a*) formation of hydrogen bonds involving hydroxyl groups, (*b*) complexation in the presence of a metal ion.[103]

enzyme is unable to bind to the DNA substrate and, therefore, is inactive. Upon UV irradiation, the *anti*-azobenzene transforms to the more compact *syn* configuration, the distance (*R*) between two ends of the azobenzene moieties increases, and the substrate can reach the DNA-binding site of the protein (Fig. 7).

The initial DNA hydrolysis rates obtained upon exposure to light with $\lambda = 365$ and 470 nm differed twofold when, in each R.SsoII subunit, hydroxyl-containing azobenzene derivatives were attached to the Cys residue located into the DNA-binding pocket (C224) and to the Cys residue located on its outer surface (C174) (Fig. 8 *a*). The same effect has been achieved when the chelating groups of azobenzene attached to the Cys171 residue come closer together owing to formation of a complex in the presence of nickel ions (see Fig. 8 *b*).[103]

V. Use of photocontrolled substrates, inhibitors and ligands for protein activity regulation

1. Photocontrolled substrates

To control the activity of NA-binding proteins, oligonucleotide analogues of substrates that change their properties upon illumination can be used. The configuration of the azobenzene moiety has an effect on the DNA duplex stability,[106] which enables the indirect regulation of enzyme activity. RNA polymerases (RNAP) of phages SP6 and T7 are DNA-dependent RNA polymerases and have specific promoter sequences. Both promoters consist of 17 base pairs and include RNAP-binding region (−17 to −5 positions) and TATA region (−4 to −1 positions). In each of these regions of the SP6 promoter, azobenzene moieties were introduced, and the ability of such a promoter to photoregulate transcription has been studied (Fig. 9).[107]

When only one region (RNAP-binding site or TATA box) of the SP6 RNAP promoter was modified, the transcription efficiency upon irradiation did not change; when both regions were modified, a noticeable difference between the amounts of transcription product obtained in the dark and under UV light was observed. In the latter case, RNA was produced 3.5-fold faster.[107] Upon irradiation with UV

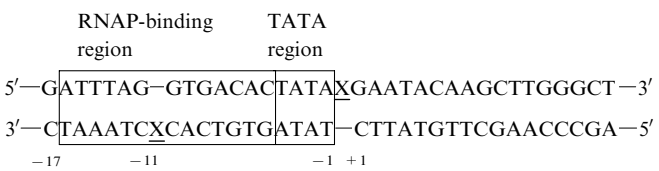


Figure 9. Sequence of modified SP6 promoter.[107] X is *N*-(4-carboxyazobenzoyl)-D-threoninol phosphate (X_D) residue.

light (*anti*-azobenzene isomerization into the *syn* isomer), the duplex in the region of the azobenzene-modified promoter dissociated. This local duplex dissociation promoted the formation of an open complex of RNAP with the promoter, which triggered transcription. With *anti*-azobenzene, the duplex remained stable, and RNAP mainly formed a closed promoter complex.

Photoregulation of T7 RNA polymerase has been achieved by introducing an azobenzene derivative containing D-threoninol (see structure X_D in Scheme 3) into the nontemplate strand of the T7 promoter.[108] As in the case of SP6 RNAP, transcription was strongly impeded in the dark or by visible light irradiation, whereas it proceeded efficiently after UV irradiation (Fig. 10). Thus, with a photoresponsive promoter, on–off switching of transcription by light irradiation can be achieved.

The effect of the modification position in the T7 RNAP promoter on transcription has been studied. It has been shown that, if one azobenzene residue is introduced into the RNA polymerase binding region (−7 to −11 positions) or in the DNA unwinding region (−1 to −4 positions), transcription is suppressed when azobenzene is in the *anti* configuration and is recovered upon its isomerization. The amount of transcript after UV irradiation is 1.5–2 times larger than in the dark. As in the case of SP6 RNAP, the effect strongly increases if two azobenzene moieties are introduced into both regions: after irradiation with UV light, transcription proceeds 7.6 times faster than in the dark, but it is twice as slow as compared to nonmodified T7 promoter.

The effect of the photoregulation of transcription with participation of T7 RNA polymerase on the translation of green fluorescent protein (GFP) has been studied.[109] Measurement of the fluorescence caused by *in vitro* transcription and subsequent translation shows that GFP gene expression can be regulated with light. A maximal difference in gene expression efficiency is as large as twofold.

Ribonuclease H (RNase H) hydrolyzes RNA in the hybrid RNA/DNA duplex. Regulation of its activity is based on the considerable difference in thermal stability of the DNA/DNA duplex containing azobenzene in the *syn* and *anti* configurations.[110] The photoswitch based on azobenzene binding to D-threoninol (X_D) has been introduced into the sense DNA strand as an additional unit. In the dark, azobenzene is in the *anti* configuration, which enables the formation of the stable DNA duplex, and RNA thereby exists in the single-stranded form. On UV irradiation, azobenzene adopts the *syn* configuration, the thermal stability of the DNA duplex decreases, and the duplex dissociates. As a result, a more stable DNA/RNA duplex is formed, and the RNA strand is digested by RNase H. It has been shown that the photoswitching efficiency increases with an increase in the number of tethering azobenzene moieties: the difference is as large as twofold for three azobenzene moieties and as large as fourfold for five azobenzene moieties.[110]

To assess the action of PNA as a photoswitch of transcription, an azobenzene derivative has been introduced at the N- or C-terminus of two PNAs. Upon the formation of the triplex, the PNA sequence forms the stable right-handed helix of the PNA_2/DNA composition. The presence of the

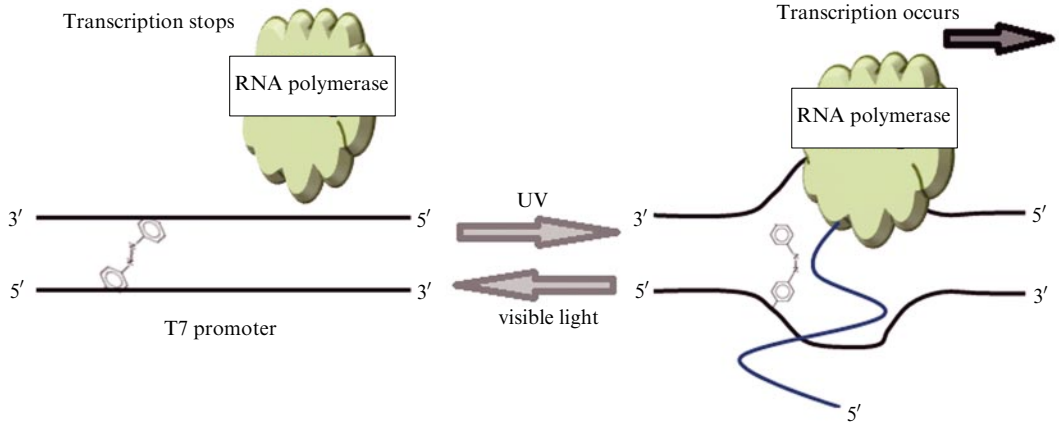


Figure 10. Photoregulation of the transcription activity of T7 RNA polymerase.[108]

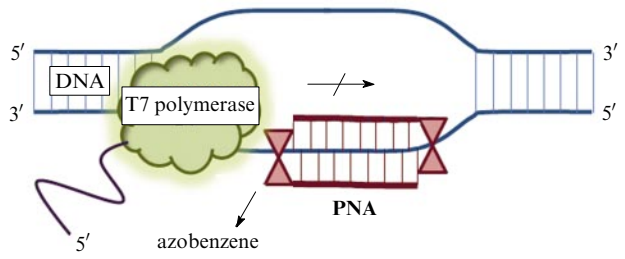


Figure 11. Mechanism of transcription inhibition by means of azobenzene-tethered PNA.[34]

anti-azobenzene moiety considerably stabilizes the triplex, whereas the *syn* isomer can either stabilize or destabilize the triplex depending on the position of modification. In particular, the introduction of azobenzene at the C-terminus of the PNA sequence stabilizes the triplex, while the N-terminal azobenzene group exhibits a destabilizing effect as compared to the nonmodified triplex. Azobenzene in the *anti* configuration tethering the PNA stabilizes more strongly the PNA_2/DNA triplex as compared to *anti*-azobenzene in the analogous DNA triplex.[34] This property of PNA makes it possible to use considerably lower concentrations of oligonucleotides and to decrease the Mg^{2+} concentration for recognition of the target DNA sequence. For studying possible photoregulation of transcription, EGFP (enhanced green fluorescent protein) gene transcription in the presence of a short complementary PNA with or without the azobenzene moiety has been considered as a model (Fig. 11).[34]

PNA containing the *anti*-azobenzene moiety forms the most stable triplex structure with the target single-stranded segment of gene (T_m = 58 °C) and is a transcription inhibitor. The PNA concentration at which the transcription efficiency decreases twofold (IC_{50}) is 35 μmol litre^{-1}. An analogous PNA with *syn*-azobenzene (T_m = 45 °C) is less efficiently involved in triplex formation and twice less efficiently inhibits transcription (IC_{50} = 90 μmol litre^{-1}). In the absence of azobenzene, PNA forms the most unstable triplex structure (T_m = 40 °C), and its ability to inhibit transcription is even lower (IC_{50} > 125 μmol litre^{-1}). Nucleotide substitutions leading to formation of mismatch pairs in the DNA-binding segment of PNA (5′-GAAAAAG-3′ → 5′-GAACGCG-3′) considerably reduce the inhibiting activity of PNA. In the case of such substitutions, transcription is inhibited only at high PNA concentrations (> 100 μmol litre^{-1}) by means of nonspecific binding of PNA to DNA. Thus, these results show that PNA derivatives site-specifically interact with target single-stranded DNA and, being modified with azobenzene derivatives, can regulate gene transcription.

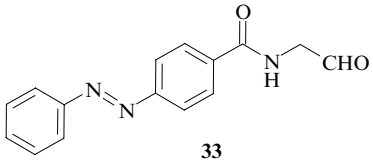
2. Photocontrolled inhibitors and ligands

Many studies have focused on the synthesis of photoswitchable inhibitors and their ability to reversibly inhibit the enzymatic reaction. The general structure of photoregulated inhibitors consists of two parts: the binding functional group at the active site of the enzyme and the photoswitch whose structure changes with a change in the excitation light wavelength. For one of the two photoswitch configurations, the geometry of the inhibitor corresponds to the structure of the active site of the enzyme and can impede the function of the latter. Upon irradiation with light of definite wavelength, the structure of the photoswitch and, hence, of the inhibitor molecule, changes, and the enzyme activity is restored.

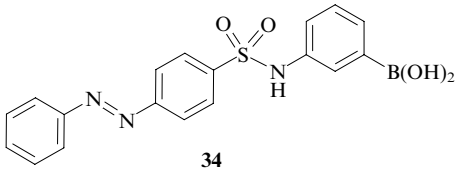
Ribonuclease A is specifically cleaved by the subtilisin into two parts referred to as S-peptide and S-protein. Taken separately, they are inactive. When S-peptide and S-protein form a complex, the enzyme activity is restored. Chemical synthesis of an S-peptide analogue makes it possible to introduce unnatural amino acids at desired positions of the peptide chain. According to HPLC, under experimental conditions, a solution of '*syn*'-S-peptide contains 90% *syn* and 10% *anti* isomers of the PAP moiety, and a solution of '*anti*'-S-peptide has 96% *anti* and 4% *syn* forms.[16] It has been shown that *syn*–*anti* isomerization of substituted azobenzene **19** mainly does not lead to a significant change in enzyme activity. It is likely that such a moiety, being located in the side chain of the polypeptide, has considerable conformational mobility, and its isomerization effect is levelled.

Photochromic inhibitors of cysteine proteases[111,112] and serine proteases[113,114] have been synthesized, and their ability to reversibly inhibit these enzymes under irradiation has been studied. For cysteine proteases (papain and calpain), the functional group of the inhibitor is aldehyde **33**, which reversibly binds to the thiol group of the cysteine residue of the enzyme to form hemithioacetal in the active site. For serine proteases (α-chymotrypsin and subtilisin), the inhibitor functional group is arylboronic acid residue **34**, which can reversibly bind to the serine residue in the active site of the enzyme.[112] In both cases, azobenzene derivatives act as the photoswitch of inhibitor molecules. It has been shown that the papain inhibition efficiency by compound **33** in the *anti* configuration is six times higher than in the *syn* configuration, and this switching is reversible. In the case of azobenzene derivative **34**, the α-chymotrypsin inhibition activity differs only by a factor of 1.3 upon the *anti*–*syn* isomerization of the photoswitch.[112]

Structures **33**, **34**



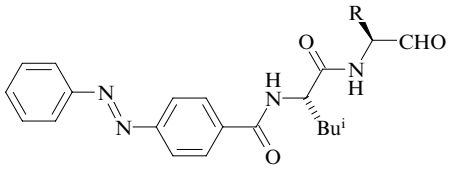
33



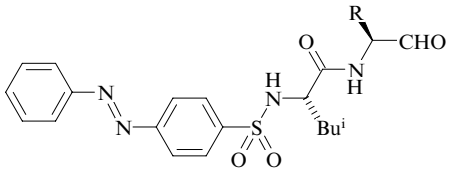
34

Calpain is a calcium-dependent cysteine protease. For inhibiting this enzyme, photoregulated dipeptides **35a**,**b**, **36a**,**b** have been created.[111] They contain a photoresponsive azobenzene moiety at the N-terminus located in the substrate-binding pocket of the enzyme and an aldehyde group for reversible binding to the cysteine residue of the active site. In addition, photoresponsive dipeptides **36a**,**b** contain a

Structures **35**–**39**

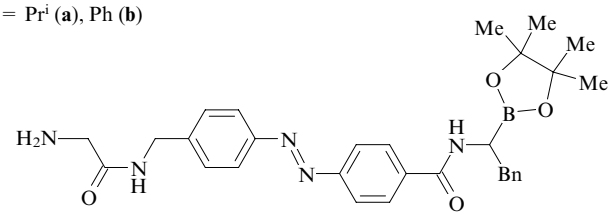


35a,**b**

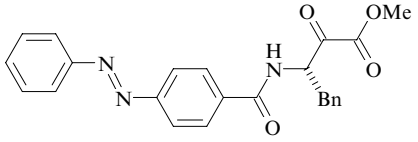


36a,**b**

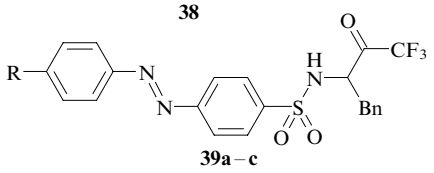
R = Pr^i (**a**), Ph (**b**)



37



38



39a–**c**

R = H (**a**), Br (**b**), I (**c**)

sulfamide bond rather than a common amide bond, which increases the resistance of the inhibitor to hydrolysis. After irradiation, the inhibiting activity of these compounds toward calpain is four times lower than before irradiation.[111]

In later studies, for α-chymotrypsin inhibition, peptide mimetics containing different functional groups — boronate ester **37**,[113] α-ketoester **38** and trifluoromethyl ketones **39a**–**c** [114] — have been synthesized.

Compound **37** decomposes when exposed to UV and blue light, so that it cannot be used. α-Ketoester **38** in the *syn* form inhibits α-chymotrypsin two times more efficiently than in the *anti* form. In trifluoromethyl ketones **39b**,**c** a halogen atom is in the *para* position of azobenzene. It was expected that the introduction of a halogen atom, due to its larger size as compared to the hydrogen atom, should enhance the difference in geometry between the *syn* and *anti* isomers of the photoswitch. It has been shown that *syn*-azobenzene derivatives containing a trifluoromethyl moiety inhibit α-chymotrypsin 5 times more efficiently than *anti* isomers. Protein activity regulation is fully reversible, but incomplete switching does not allow for the maximal effect. An interesting application of such inhibitors has been presented by Pearson *et al.*[114] The authors immobilized an inhibitor at the surface, which made it possible to photoregulate the α-chymotrypsin adsorption.

For practical use, it is necessary to develop an efficient approach to photoregulated binding of biomolecules on the surface the result of which could be predicted and controlled. Reversible photoregulation of adsorption on the gold surface coated with an inhibitor has been studied.[115] Alternate irradiation with UV and blue light reversibly changes the azobenzene geometry, which leads to a change in the enzyme-binding affinity of the inhibitor and, thus, enables the control of protease binding to the surface. However, whereas the experiments in solution showed that the inhibitor in the *syn* configuration suppresses the α-chymotrypsin activity by a factor of 3.6 as compared to the *anti* form, the activity in a heterophase system differed only by a factor of 1.7.

A similar approach has been used for photoregulating the sorption of cells containing integrin on the surface that selectively interact with the RGD-peptide (Arg-Gly-Asp).[116] The RGD peptide was immobilized on a solid substrate through an azobenzene-containing linker. With *anti*-azobenzene, the degree of cell adhesion was about 1.5 times higher than with the *syn* isomer. This effect is explained by the decrease in the distance between the substrate and RGD peptide, which decreases the conformational mobility and cell integrin binding efficiency of the latter.[116]

In studies dealing with photoregulation of ion channels, azobenzene-containing ligands have been used for chemical modification of proteins. The ligands either reversibly block the channel pore, without inducing a noticeable conformational change of the protein structure, or can interact with

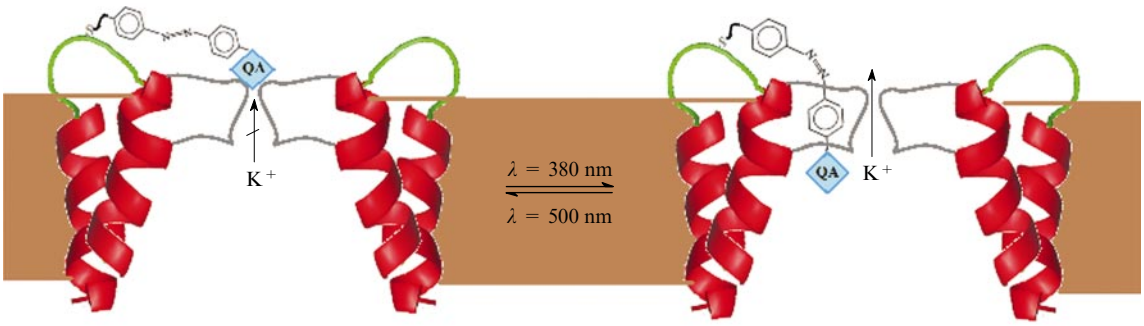
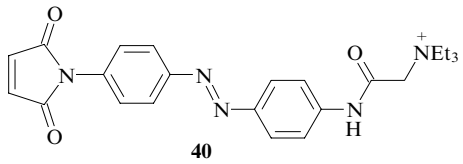


Figure 12. Photoregulation of exogenous potassium channel (SPARK).[81]

the allosteric binding site, which leads to such a change of the protein structure.

Banghart *et al.*[81] created a light-activated potassium channel (Fig. 12). As known, the potassium channel can be blocked by a quaternary ammonium ion (QA), for example, tetraethylammonium.[117] An additional cysteine residue was introduced at the channel surface and tethered, through some linkers,[118] to maleimidobenzene **40** containing tetraalkylammonium cation.

Structure **40**



40

In the case of the E442C mutant form of the potassium channel, with the Cys residue tethered to compound **40**, the electrical conductance for *anti*-azobenzene is 20-fold lower than for the *syn* isomer. This is due to the fact that when azobenzene is in the *anti* configuration, the tetraethylammonium ligand reaches the channel and binds to it, whereas *syn*-azobenzene retracts the ligand from the channel, which restores its functioning. This regulation is reversible, which can be used in therapy of neural damage.

In addition to chemical modification of mutant proteins, modification of native protein is also possible. A concept of photoswitchable affinity labelling, schematically shown in Fig. 13, has been reported.[119,120] For affinity labelling of proteins, azobenzene derivatives have been synthesized containing an electrophilic group in one benzene ring and a ligand in the other ring. The choice of the ligand is dictated by the protein nature. Then, the protein has been affinity labelled with the azobenzene derivative (see Fig. 13).

Initially, the ligand interacts with the binding site of the protein, which fixes the photoswitch and allows the electrophile to form a covalent bond with a limited set of amino acid residues located near the binding site when the photoswitch is in the *anti* configuration. Upon irradiation with UV light, the photoswitch is converted to the *syn* form, which retracts the ligand from the binding site. Thus, the protein can be activated or inactivated through an increase/decrease in the distance between the ligand and the binding site. An advantage of this concept is that it allows making native proteins in cells photoresponsive. This approach is

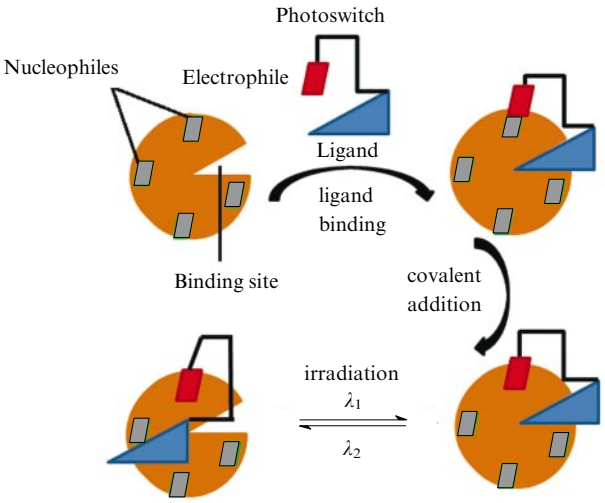
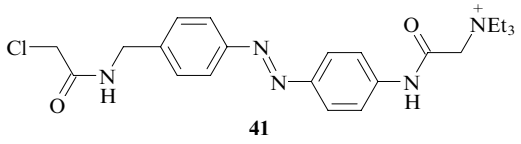


Figure 13. Photoswitchable affinity labelling of protein.[119]

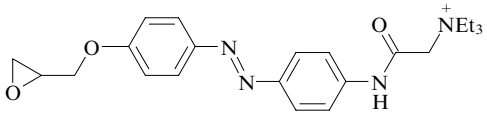
especially useful for cellular systems into which mutant protein genes are difficult to introduce.

The above technique has been used for creation of light-activated potassium channel.[119] Reagents for photoswitchable affinity labelling of cation channels are azobenzene derivatives **41** and **42** containing a quaternary ammonium ion in one benzene ring as the ligand of potassium channels, and different electrophilic groups in the other ring for

Structures **41**, **42**



41



42

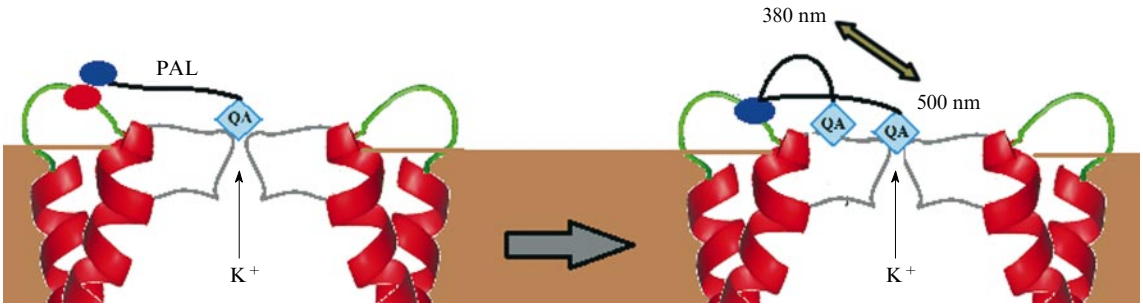


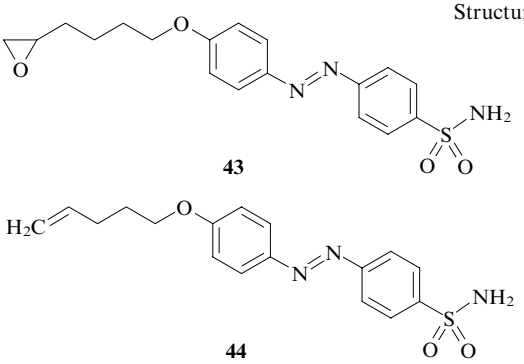
Figure 14. Formation of a photoswitchable potassium channel.[119]
PAL is a reagent for affinity labelling; quaternary ammonium ion binds to the channel pore.

modification of amino acid residues of the protein, mainly, lysine and cysteine.

Ligand binding in the potassium channel increases the local concentration of electrophilic groups, which is favourable for more selective covalent addition of the reagents and makes it possible to obtain photoswitchable potassium channels referred to as PAL channels (Fig. 14). It has been shown that the electrical conductance of the PAL channel upon irradiation with 500 nm light is 10-fold lower than that upon UV irradiation. This process is reversible; however, long-term irradiation leads to the decrease in activity of the PAL channel.[119] Since quaternary ammonium ion is not a ligand for sodium or potassium channels, the suggested strategy is not universal.

The photoswitchable affinity label approach has also been used to control the carbonic anhydrase activity by means of covalent labelling with photoswitchable inhibitors.[119] Carbonic anhydrase catalyzes the reversible reaction of carbon dioxide hydration for maintaining the physiological pH. Azobenzene derivative **43** containing the oxirane ring has been synthesized on the basis of sulfanilamide, a well-known inhibitor of carbonic anhydrase. Incubation of the enzyme with the inhibitor in the dark leads to a significant reduction in enzyme activity. Upon irradiation of the sample with 380 nm UV light, the rate of hydrolysis of the substrate increases twofold. Switching is reversible. When unreactive analogue **44** is used, irradiation wavelength has no effect on the enzyme activity.

Structures **43**, **44**



43

44

The allosteric control of an active site by a remote regulatory binding site is a mechanism of regulation found across protein classes, from enzymes to motor and signalling proteins.

An early version of a photoregulated system has been described for the nicotinic acetylcholine receptor that could be photoactivated *via* a tethered choline analogue.[121] The results of this work lay dormant for many years because of the difficulty of extending the approach to other proteins in the absence of crystal structures and modern approaches to protein engineering. The aforedescribed potassium channel is an attractive target for binding a photoswitchable ligand since the channel pore is symmetric and located in the centre of a wide platform. However, these features are inherent only in the potassium channel. Most proteins have active sites or allosteric sites that bind ligands inside the protein globule through the formation of a specific 'lock and key' complex.

Volgraf *et al.*[49] have described allosteric photoregulation of receptor activity for an ionotropic glutamate receptor (iGluR), which is a membrane receptor of neuronal cells. The interaction of the ligand-binding domain of iGluR with glutamate neuromediator (ligand) leads to conformational change of the transmembrane, which results in the opening of the channel for the K^+, Na^+ and Ca^{2+} flow (Fig. 15 *a*). On the basis of iGluR, the authors have developed its light-activated variant — LiGluR (Fig. 15 *b*). For this purpose, the Cys residues of mutant forms of the LiGluR protein were affinity labelled with azobenzene derivative **45** ($n = 0$) containing the maleimide group in one benzene ring and the glutamic acid residue in the other ring (Scheme 15).

The Cys residues were introduced into the ligand-binding domain at a definite distance from the ligand-binding site of LiGluR. Photoregulation of binding of the ligand attached to the protein in the ligand-binding domain of LiGluR leads to channel opening/closing for the cation flow.

The length of the linker in the compound **45** ($n = 1, 2$), *i.e.*, the position of the Cys residue in the ligand-binding domain of an ionotropic glutamate receptor, has been varied, and the wavelengths for irradiation of LiGluR that afford the largest difference in protein activity have been determined.[82] It has been shown that the largest switching effect for LiGluR functioning is achieved upon irradiation with UV light (380 nm) and with 500 nm light.[82] The largest difference in protein functioning (120 times) upon alternat-

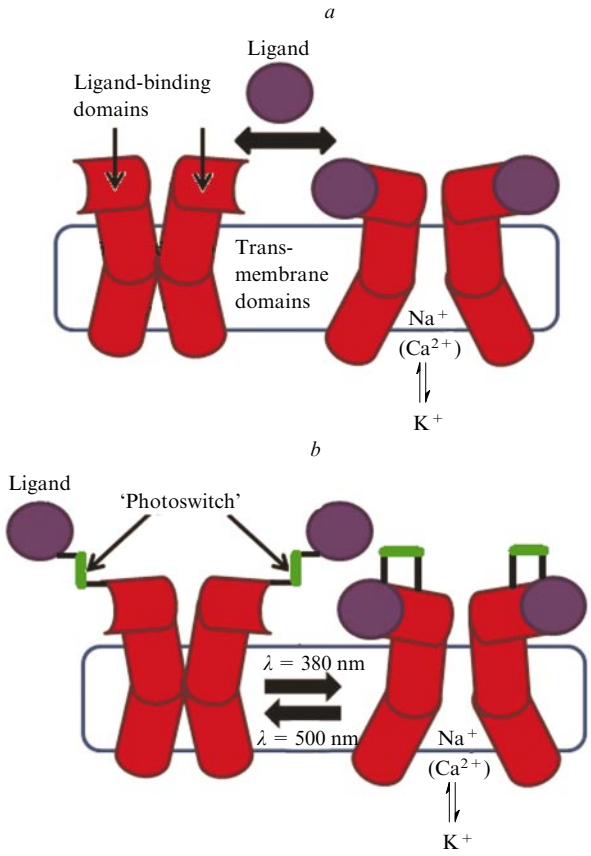
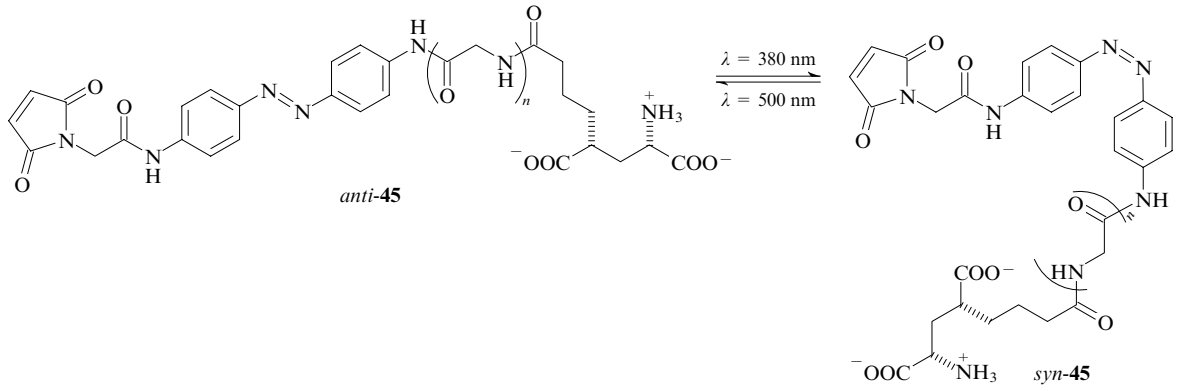


Figure 15. Design of an allosteric switch: operation of iGluR (*a*) and LiGluR (*b*).[49]

Scheme 15

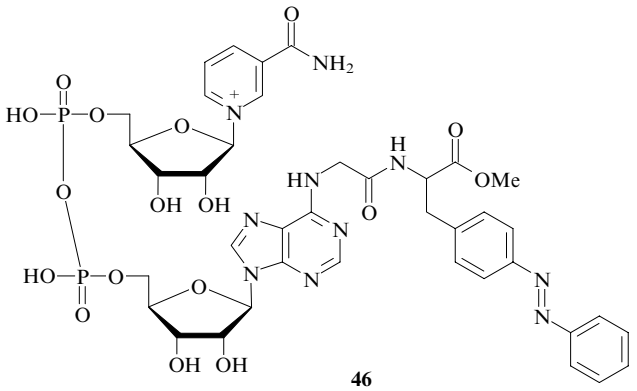


ing irradiation (380 and 500 nm) was achieved for LiGluR(L439C).[84] In addition, it has been demonstrated that irradiation at wavelengths within the range from 380 to 500 nm results in a lower effect of protein activity regulation.[82] Photoswitching of LiGluR through alternate irradiation can be repeated for 30 min with the same amplitude of the detected signal after each cycle of irradiation. In addition, upon irradiation of LiGluR (*syn*-**45**) with UV light, the signal is smaller than upon LiGluR activation with free glutamate ion in its saturated solution. It is possible that modification of the receptor with photoswitch **45** has been incomplete. It is also likely that only a limited amount of glutamate in the photoswitch can bind to the allosteric ligand-binding segment of LiGluR.

Since structures like the ligand binding domain of iGluR are abundant in nature, the above approach can be applied to other transmembrane proteins, for example, to metabotropic glutamate receptors (mGluRs). Inasmuch as the structures of the ligands for other types of ligand-binding domains of proteins are known, this strategy can also be used for other proteins. In each case, this method is attractive because it allows not only switching the protein activity but also regulating the degree of protein activation as a function of the selected irradiation wavelength.

The strategy of regulation of biological reactions with the use of azobenzene derivatives and antibodies recognizing *anti*-azobenzene has been described.[75, 76, 122] To this end, monoclonal antibodies to *anti*-L-4′-phenylazophenylalanine have been obtained that can bind only to azobenzene in the *anti* configuration and dissociate when it isomerizes to the *syn* form. The principle of the strategy has been demonstrated for regulation of alcohol dehydrogenase. The nicotinamide adenine dinucleotide ion (NAD^+) is a coenzyme. The azobenzene derivative of nicotinamide adenine dinucleotide (Ab-NAD^+, **46**) has been synthesized.

Structure **46**



It has been shown that the reduction of Ab-NAD^+ is almost stopped by addition of an antibody binding *anti*-azobenzene in the dark. Upon irradiation with UV light, the reaction rate is recovered to 72% of the initial value. The incomplete recovery of the reaction rate is presumably due to partial isomerization of the *anti* isomer to the *syn* form. The reaction rate also increases at high concentrations of the *anti* configuration of Ab-NAD^+. The activity switching is reversible, and the wide occurrence of NAD^+ as the coenzyme for various oxidoreductases renders this approach applicable to different systems.

VI. Conclusion

There are three strategies of regulation of protein activity by azobenzene-based photoswitches: (1) direct modification of proteins with azobenzene derivatives, (2) creation of photoregulated enzyme inhibitors or substrates and (3) site-specific protein modification with azobenzene derivatives containing simultaneously a protein-binding ligand and a reactive group. A decisive factor in choosing the method is the aim of protein activity regulation, the structure of the protein itself and its substrate, cofactor and/or inhibitor. When choosing a particular method, one should take into account not only the final result (the difference in protein activity upon specific irradiation) but also a combination of factors that lead to this effect. For applying the site-specific protein modification strategy, it is necessary to know the enzyme structure. Azobenzene derivatives are usually introduced into structural elements of peptides and proteins that play an important role in protein–protein and/or DNA–protein recognition and protein–ligand binding. Design of photoregulated enzyme inhibitors or substrates is used in practice, for example, in the field of enzyme purification for simplifying the technique of affinity chromatography of proteins.

In affinity binding, enzyme inhibitors can be used as ligands that, upon irradiation, will convert from the configuration with the high affinity for the target enzyme into the low-affinity configuration. This procedure can be considered a new approach to enzyme purification under mild conditions. In addition, the possibility of light-driven regu-

lation of interactions of biomolecules with the surface contributes to the design of new reversible biosensors, advanced medical implants, *etc*.

Site-specific protein modification with azobenzene derivatives containing a ligand for binding to a given protein is mainly used for proteins forming ion channels and for receptors with known ligands. Incorporation of an appropriate group into photoswitches makes it possible to modify different types of ion channels and receptors so that various physiological functions of proteins can be light-controlled. Varying the nature of photoswitchable reagents for binding to protein allows one to modify different amino acids, which provides a tool for photoregulation of endogenous proteins. For example, a reactive group can be chosen to selectively react with individual amino acids of the protein, and a photoresponsive residue can be modified to modulate its spectral characteristics and, hence, photoswitching kinetics. Incorporation of an appropriate group as a ligand into these reagents enables the modification of different types of native ion channels and receptors, so that various physiological functions of proteins can be light-controlled.

The above examples of protein activity regulation demonstrate the efficiency of azobenzene derivatives incorporated into both enzymes and their substrates. There are prospects for using azobenzene-containing nucleic acids in bio- and nanoengineering. However, incomplete *syn–anti* isomerization upon irradiation, in some cases, limits the use of azobenzene-based structures. Therefore, an important task is to search for photochromic compounds suitable for regulation of the activity of biologically active molecules.

This review was prepared with financial support of the RFBR-DFG International Graduate Research Programme (Project No. 11-04-91338).

References

1. B L Feringa, R A Delden, N Koumura, E M Geertsema *Chem. Rev*. **100** 1789 (2000)
2. M Erdélyi, A Karl, A Gogol *Chem. – Eur. J*. **12** 403 (2006)
3. T Cordes, D Weinrich, S Kempa, K Riesselmann, S Herre, C Hoppmann, K Rück-Braun, W Zinth *Chem. Phys. Lett*. **42** 167 (2006)
4. T Cordes, C Elsner, T Herzog, C Hoppmann, T Schadendorf, W Summerer, K Rück-Braun, W Zinth *Chem. Phys*. **358** 103 (2009)
5. T Schadendorf, C Hoppmann, K Rück-Braun *Tetrahedron Lett*. **48** 9044 (2007)
6. A Natansohn, P Rochon *Chem. Rev*. **102** 4139 (2002)
7. V Shibaev, A Bobrovsky, N Boiko *Prog. Polym. Sci*. **28** 729 (2003)
8. I Willner, S Rubin *Angew. Chem., Int. Ed. Engl*. **35** 367 (1996)
9. I Willner *Acc. Chem. Res*. **30** 347 (1997)
10. G S Kumar, D C Neckers *Chem. Rev*. **89** 1915 (1989)
11. H Rau, in *Molecules and Systems* (Eds H Dürr, H Bonas-Laurent) (Amsterdam: Elsevier, 1990) p. 165
12. H Rau, in *Photoreactive Organic Thin Films* (Eds Z Sekkat, W Knoll) (New York: Academic Press, 2002) p. 3
13. I K Lednev, T Q Ye, P Matousek, M Towrie, P Foggi, F V R Neuwahl, S Umapathy, R E Hester, J N Moore *Chem. Phys. Lett*. **290** 68 (1998)
14. T Fujino, Y S Arzhantsev, T Tahara *J. Phys. Chem. A* **105** 8123 (2001)
15. R Nägele, R Hoche, W Zinth, J Wachtveitl *Chem. Phys. Lett*. **272** 489 (1997)
16. D A James, D C Burns, G A Woolley *Protein Engin*. **14** 983 (2001)
17. O Srinivas, N Mitra, A Surolia, N Jayaraman *J. Am. Chem. Soc*. **124** 2124 (2002)
18. C R Crecca, A E Roitberg *J. Phys. Chem. A* **110** 8188 (2006)
19. W Fuss, C Kosmidis, W E Schmid, S A Trushin *Angew. Chem., Int. Ed*. **43** 4178 (2004)
20. M L Tiago, S Ismail-Beigi, S G Louie *J. Chem. Phys*. **122** 094311 (2005)
21. A Cembran, F Bernardi, M Garavelli, L Gagliardi, G Orlandi *J. Am. Chem. Soc*. **126** 3234 (2004)
22. C Renner, L Moroder *ChemBioChem* **7** 868 (2006)
23. S Dong, M Leweneck, T E Schrader, W J Schreier, W Zinth, L Moroder, C A Renner *Chem. – Eur. J*. **12** 1114 (2006)
24. D C Burns, D G Flint, J R Kumita, H J Feldman, L Serrano, Z Zhang, O S Smart, G A Woolley *Biochemistry* **43** 15329 (2004)
25. Beharry, G A Woolley *Chem. Soc. Rev*. **40** 4422 (2011)
26. C Brieke, F Rohrbach, A Gottschalk, G Mayer, A Heckel *Angew. Chem., Int. Ed*. **51** 8446 (2012)
27. H Asanuma, T Takarada, T Yoshida, D Tamaru, X Liang, M Komiyama *Angew. Chem., Int. Ed*. **40** 2671 (2001)
28. H Nishioka, H Kashida, M Komiyama, X Liang, H Asanuma *Nucleic Acids Symp. Ser*. **50** 85 (2006)
29. X Liang, T Yoshida, H Asanuma, M Komiyama *Nucleic Acids Symp. Ser*. **44** 277 (2000)
30. H Hayashi, H Nishioka, X Liang, H Asanuma *Nucleic Acids Symp. Ser*. **51** 171 (2007)
31. X Liang, H Asanuma, M Komiyama *J. Am. Chem. Soc*. **124** 1877 (2002)
32. P E Nielsen, M Egholm, R H Berg, O Buchardt *Science* **254** 1497 (1991)
33. P E Nielsen *Mol. Biotechnol*. **26** 233 (2004)
34. T Stafforst, D Hilvert *Angew. Chem., Int. Ed*. **49** 9998 (2010)
35. C Dohno, S Uno, K Nakatani *Nucleic Acids Symp. Ser*. **50** 87 (2006)
36. C Seeman *J. Biomol. Struct. Dyn*. **8** 573 (1990)
37. C M Niemeyer, J Koehler, C Wuerdermann *ChemBioChem* **3** 242 (2002)
38. X Yang, A V Vologodskii, B Liu, B Kemper, N C Seeman *Biopolymers* **45** 69 (1998)
39. J S Shin, N A Pierce *J. Am. Chem. Soc*. **126** 10834 (2004)
40. J H Chen, N C Seeman *Nature (London)* **350** 631 (1991)
41. W M Shih, J D Quispe, G F Joyce *Nature (London)* **427** 618 (2004)
42. R P Goodman, I A Schaap, C F Tardin, C M Erben, R M Berry, C F Schmidt, A J Turberfield *Science* **310** 1661 (2005)
43. S Higashi-Fujime, R Ishikawa, H Iwasawa, O Kagami, E Kurimoto, K Kohama, T Hozumi *FEBS Lett*. **375** 151 (1995)
44. Y W Zhang, N C Seeman *J. Am. Chem. Soc*. **116** 1661 (1994)
45. X Liang, N Takemaka, H Nishioka, H Asanuma *Nucleic Acids Symp. Ser*. **52** 697 (2008)
46. X Liang, H Nishioka, N Takenaka, H Asanuma *ChemBioChem* **9** 702 (2008)
47. H Kang, H Liu, J A Phillips, Z Cao, Y Kim, Y Chen, Z Yang, J Li, W Tan *Nano Lett*. **9** 2690 (2009)
48. Y Kim, J A Phillips, H Liu, H Kang, W Tan *Proc. Natl. Acad. Sci. USA* **106** 6489 (2009)
49. M Volgraf, P Gorostiza, R Numano, R Kramer, E Isacoff, D Trauner *Nat. Chem. Biol*. **2** 47 (2006)

50. J R Kumita, O S Smart, G A Woolley *Proc. Natl. Acad. Sci. USA* **97** 3803 (2000)
51. N Pozhidaeva, M Cormier, A Chaudhari, G A Woolley *Bioconjug. Chem.* **15** 1297 (2004)
52. D Liu, J Karanicolas, C Yu, Z Zhang, G A Woolley *Bioorg. Med. Chem. Lett.* **7** 2677 (1997)
53. T Ueda, K Murayama, T Yamamoto, S Kimura, Y Imanishi *J. Chem. Soc., Perkin Trans. 1* 225 (1994)
54. K Nakayama, M Endo, T Majima *Chem. Commun.* 2386 (2004)
55. N Muranaka, T Hohsaka, M Sisido *FEBS Lett.* **510** 10 (2002)
56. T Hohsaka, D Kajihara, Y Ashizuka, H Murakami, M Sisido *J. Am. Chem. Soc.* **121** 34 (1999)
57. M Bose, D Groff, J Xie, E Brustad, P Schultz *J. Am. Chem. Soc.* **128** 388 (2006)
58. D G Smyth, A Nagamatsu, J S Fruton *J. Am. Chem. Soc.* **82** 4600 (1960)
59. C F Brewer, J P Riehmn *Anal. Biochem.* **18** 248 (1967)
60. D C Burns, F Zhang, G A Woolley *Nat. Protoc.* **2** 251 (2007)
61. D G Flint, J R Kumita, O S Smart, G A Woolley *Chem. Biol.* **9** 391 (2002)
62. G A Woolley *Acc. Chem. Res.* **38** 486 (2005)
63. L Chi, O Sadovski, G A Woolley *Bioconjug. Chem.* **17** 670 (2006)
64. Y C Lee, R T Lee *Acc. Chem. Res.* **28** 321 (1995)
65. Z Zhang, D C Burns, J R Kumita, O S Smart, G A Woolley *Bioconjug. Chem.* **14** 824 (2003)
66. O Sadovski, A A Beharry, F Zhang, G A Woolley *Angew. Chem., Int. Ed.* **48** 1484 (2009)
67. S M Menta, M V Vakilwala *J. Am. Chem. Soc.* **74** 563 (1952)
68. A H Cook *J. Chem. Soc.* 876 (1938)
69. F Zhang, O Sadovski, G A Woolley *ChemBioChem.* **9** 2147 (2008)
70. S Loudwig, H Bayley *J. Am. Chem. Soc.* **128** 12404 (2006)
71. Le Thi Hien, B Shirling, A Yu Ryazanova, T S Zatsepin, E M Volkov, E A Kubareva, T I Velichko, A Pingoud, T S Oretskaya *Russ. J. Bioorg. Chem.* **35** 549 (2009)
72. M Goodman, A Kossoy *J. Am. Chem. Soc.* **88** 5010 (1966)
73. J Kuil, L T M Wandelen, N J Mol, R M J Liskamp *Bioorg. Med. Chem.* **16** 1393 (2008)
74. L Ulysse, J Chmielewski *Bioorg. Med. Chem. Lett.* **4** 2145 (1994)
75. T Hohsaka, Y Ashizuka, H Murakami, M Sisido *J. Am. Chem. Soc.* **118** 9778 (1996)
76. T Hohsaka, K Kawashima, M Sisido *J. Am. Chem. Soc.* **116** 413 (1994)
77. U Kusebauch, S A Cadamuro, H J Musiol, M O Lenz, J Wachtveitl, L Moroder, C Renner *Angew. Chem., Int. Ed.* **45** 7015 (2006)
78. G A Woolley, A S I Jaikaran, M Berezovski, J P Calarco, S N Krylov, O S Smart, J R Kumita *Biochemistry* **45** 6075 (2006)
79. P V Jog, M S Gin *Org. Lett.* **10** 3693 (2008)
80. S Muramatsu, K Kinbara, H Taguchi, N Ishii, T Aida *J. Am. Chem. Soc.* **128** 3764 (2006)
81. M Banghart, K Borges, E Isacoff, D Trauner, R Kramer *Nat. Neurosci.* **7** 1381 (2004)
82. P Gorostiza, M Volgraf, R Numano, S Szobota, D Trauner, E Y Isacoff *Proc. Natl. Acad. Sci. USA* **104** 10865 (2007)
83. S Szobota *Neuron* **54** 535 (2007)
84. R Numano *Proc. Natl. Acad. Sci. USA* **106** 6814 (2009)
85. M D Yamada, Y Nakajima, H Maeda, S Maruta *J. Biochem.* **142** 691 (2007)
86. I Willner, S Rubin, A Riklin *J. Am. Chem. Soc.* **113** 3321 (1991)
87. I Hamachi, Y Yamada, R Eboshi, T Hiraoka, S Shinkai *Bioorg. Med. Chem. Lett.* **9** 1215 (1999)
88. J R Kumita, D G Flint, G A Woolley, O S Smart *Faraday Discuss.* **122** 89 (2002)
89. L Guerrero, O S Smart, C J Weston, D C Burns, G A Woolley, R K Allemann *Angew. Chem., Int. Ed.* **44** 7778 (2005)
90. B Schierling, A J Noël, W Wende, L T Hien, E Volkov, E Kubareva, T Oretskaya, M Kokkinidis, A Römpp, B Spengler, A Pingoud *Proc. Natl. Acad. Sci. USA* **107** 1361 (2010)
91. A G Cochran, N J Skelton, M A Starovasnik *Proc. Natl. Acad. Sci. USA* **9**8 5578 (2001)
92. K Tachikawa, O Schröder, G Frey, S P Briggs, T Sera *Proc. Natl. Acad. Sci. USA* **101** 15225 (2004)
93. L Reynolds, C Ullman, M Moore, M Isalan, M J West, P Clapham, A Klug, Y Choo *Proc. Natl. Acad. Sci. USA* **100** 1615 (2003)
94. D J Segal, J Goncëalves, S Eberhardy, C H Swan, B E Torbett, X Li, C F Barbas III *J. Biol. Chem.* **279** 14509 (2004)
95. T Sera *J. Virol.* **79** 2614 (2005)
96. S D Patel, M Isalan, G Gavory, S Ladame, Y Choo, S Balasubramanian *Biochemistry* **43** 13452 (2004)
97. Y G Kim, J Cha, S Chandrasegaran *Proc. Natl. Acad. Sci. USA* **93** 1156 (1996)
98. F D Urnov, J C Miller, Y L Lee, C M Beausejour, J M Rock, S Augustus, A C Jamieson, M H Porteus, P D Gregory, M C Holmes *Nature (London)* **435** 646 (2005)
99. E A Moehle, J M Rock, Y L Lee, Y Jouvenot, R C Kelver, P D Gregory, F D Urnov, M C Holmes *Proc. Natl. Acad. Sci. USA* **104** 3055 (2007)
100. W Nomura, C F Barbas III *J. Am. Chem. Soc.* **129** 8676 (2007)
101. A Nomura, A Okamoto *Chem. Commun.* 1906 (2009)
102. M Newman, T Strzelecka, L F Dorner, I Schildkraut, A K Aggarwal *Nature (London)* **368** 660 (1994)
103. L T Hien, T S Zatsepin, B Schierling, E M Volkov, W Wende, A Pingoud, E A Kubareva, T S Oretskaya *Bioconjug. Chem.* **22** 1366 (2011)
104. D Golovenko, E Manakova, G Tamulaitiene, S Grazulis, V Siksnys *Nucleic Acids Res.* **37** 6613 (2009)
105. C Schulze, A Jeltsch, I Franke, C Urbanke, A Pingoud *EMBO J.* **17** 6757 (1998)
106. H Asanuma, X Liang, T Yoshida, M Komiyama *ChemBioChem* **2** 39 (2001)
107. M Liu, H Asanuma, M Komiyama *Nucleic Acids Res.* (Suppl. 3) 265 (2003)
108. H Asanuma, D Tamaru, A Yamazawa, M Liu, M Komiyama *ChemBioChem* **3** 786 (2002)
109. J Zhao, J Zhou, M Liu, H Asanuma, M Komiyama *Nucleic Acids Symp. Ser.* **48** 199 (2008)
110. D Matsunaga, H Asanuma, M Komiyama *J. Am. Chem. Soc.* **126** 11452 (2004)
111. A D Abell, M A Jones, A T Neffe, S G Aitken, T P Cain, R J Payne, S B McNabb, J M Coxon, B G Stuart, D Pearson, H Y Lee, J D Morton *J. Med. Chem.* **50** 2916 (2007)
112. P R Westmark, J P Kelly, B D Smith *J. Am. Chem. Soc.* **115** 3416 (1993)
113. D Pearson, A D Abell *Org. Biomol. Chem.* **4** 3618 (2006)
114. D Pearson, N Alexander, A D Abell *Chem.–Eur. J.* **14** 7358 (2008)

115. D Pearson, A J Downard, A Muscroft-Taylor, A D Abell *J. Am. Chem. Soc.* **129** 14862 (2007)
116. J Auernheimer, C Dahmen, U Hersel, A Bausch, H Kessler *J. Am. Chem. Soc.* **127** 16107 (2005)
117. R MacKinnon, G Yellen *Science* **250** 276 (1990)
118. R O Blaustein, P A Cole, C Williams, C Miller *Nat. Struct. Biol.* **7** 309 (2000)
119. D L Fortin, M R Banghart, T W Dunn, K Borges, D A Wagenaar, Q Gaudry, M H Karakossian, T S Otis, W B Kristan, D Trauner, R H Kramer *Nat. Methods* **5** 331 (2008)
120. J H Harvey, D Trauner *ChemBioChem* **9** 191 (2008)
121. E Bartels, N H Wassermann, B F Erlanger *Proc. Natl. Acad. Sci. USA* **68** 1820 (1971)
122. J Parisot, K Kurz, F Hilbrig, R Freitag *J. Sep. Sci.* **32** 1613 (2009)