Photochromism

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Azobenzene Photoswitches for Staudinger–Bertozzi Ligation**

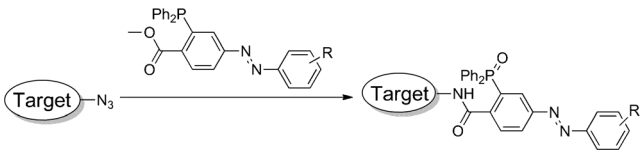
*Wiktor Szymański, Bian Wu, Claudia Poloni, Dick B. Janssen, and Ben L. Feringa**

Azobenzenes, which exist in the form of *trans* and *cis* isomers and can be isomerized by irradiation with UV/Vis light, form one of the largest and most widely studied classes of photochromic molecules,[1,2] and have been used for photochemical control over important physical, chemical, and biological events. For example, the introduction of azobenzene moieties onto surfaces allows control of cell adhesion[3] and light-driven motion of liquids.[4] In biosystems[1,5] azobenzenes have been used to obtain highly spatiotemporal control over a plethora of processes, such as changes in peptide[6] and DNA[7] conformation and function, enzyme activity,[8] receptor affinity,[9a–d] and pore function.[9e] Recently, an azobenzene-based affinity label was used to partially restore vision in blind mice.[10]

For any given application, the properties of the azobenzene chromophore, such as the absorption maximum and thermal stability of the *cis* state have to be adjusted to fit the requirements. For instance,[1] short-lived *cis* azobenzenes are required for photomodulating fast biological events, where rapid, reversible changes are desired. In contrast, application of azobenzenes with higher thermal stability is beneficial for studying longer-lasting processes, without the need for constant irradiation.

In the search for a new, generic way of incorporating molecular photoswitches into biomolecules, we turned our attention to the selective functionalization of the azide group, which exploits azide's high and bioorthogonal reactivity.[11] In recent years, the development of methods for the site-selective incorporation of azides into proteins[12] turned this moiety into a very attractive handle which upon functionalization allows the introduction of a large variety of chemical entities. Among these functionalization methods, the most prominently used are the copper-catalyzed azide–alkyne cycloaddition (CuAAC),[13] strain-promoted copper-free azide–alkyne cycloaddition (SPAAC),[14] and Staudinger–Bertozzi ligation.[15]

The ideal reaction for the ligation of molecular photoswitches to azide groups present on the biomolecule should proceed fast and with high conversion, without any adducts (reagents or catalysts) and should have high selectivity towards the azide function, thus avoiding the formation of side products. Furthermore, the reactants for such conversion should be easy to prepare and soluble in water, as well as in organic solvents. Finally, the reaction with the azide group should yield a rigid bond to ensure the maximal translation of photoinduced changes in the molecular photoswitch to the functional properties of the biomolecule. With these requirements in mind, we decided to study the possibility of using the Staudinger–Bertozzi ligation, thus making the aromatic moiety used in this reaction a part of the chromophore system of the molecular photoswitch (Scheme 1). This



Scheme 1. Staudinger–Bertozzi ligation of molecular photoswitches to azide-bearing targets.

ligation reaction is known[15] to be fast and bioorthogonal, does not need any adducts (in contrast to CuAAC), and results in the formation of a rigid amide bond, which distinguishes it from the reactions based upon strained alkyne systems.

Herein, we present the preparation of two phosphine azobenzene molecules, **1a** and **1b** (Scheme 2), for the Staudinger–Bertozzi ligation. Furthermore, we report the use of **1b** for the introduction of azobenzenes onto azide-decorated surfaces and proteins.

The compound **1a** was designed as the simplest phosphine azobenzene, which upon Staudinger–Bertozzi ligation would introduce a functional switch to a target molecule. The structure of its analogue, **1b**, contains a short polyethylene glycol (PEG) chain at the 4′-position with the aim of increasing the solubility in water and organic solvents, and exploring additional possibilities offered by the formation of a push-pull-type azobenzene. These additional properties include the red-shift of the UV absorption maximum[16] and the shorter lifetime of *cis* isomer in polar solvents.[17]

The compounds **1a** and **1b** can be conveniently synthesized in two to three steps from the common precursor **2**[18] (Scheme 2). The reaction of **2** with nitrosobenzene under acidic conditions yields the diazobenzene **3**, and subsequent palladium-catalyzed cross-coupling with diphenylphosphine

[*] Dr. W. Szymański, C. Poloni, Prof. Dr. B. L. Feringa
Center for Systems Chemistry, Stratingh Institute for Chemistry
University of Groningen
Nijenborgh 4, 9747 AG, Groningen (The Netherlands)
E-mail: b.l.feringa@rug.nl
Dr. B. Wu, Prof. Dr. D. B. Janssen
Department of Biochemistry, University of Groningen
Nijenborgh 4, 9747 AG, Groningen (The Netherlands)

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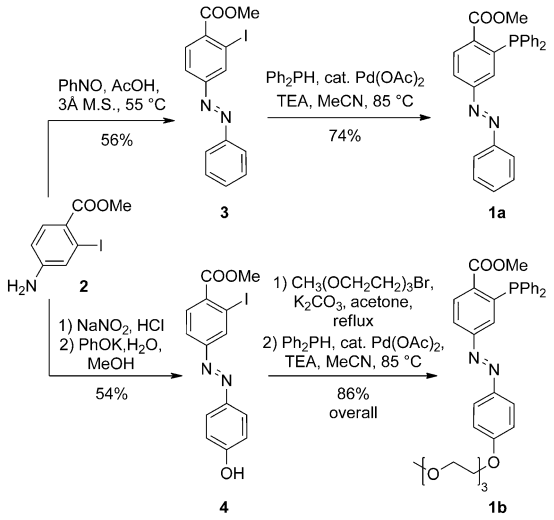


Scheme 2. Synthesis of azobenzene-phosphines **1a** and **1b** for Staudinger–Bertozzi ligation. M.S = molecular sieves, TEA = triethylamine.

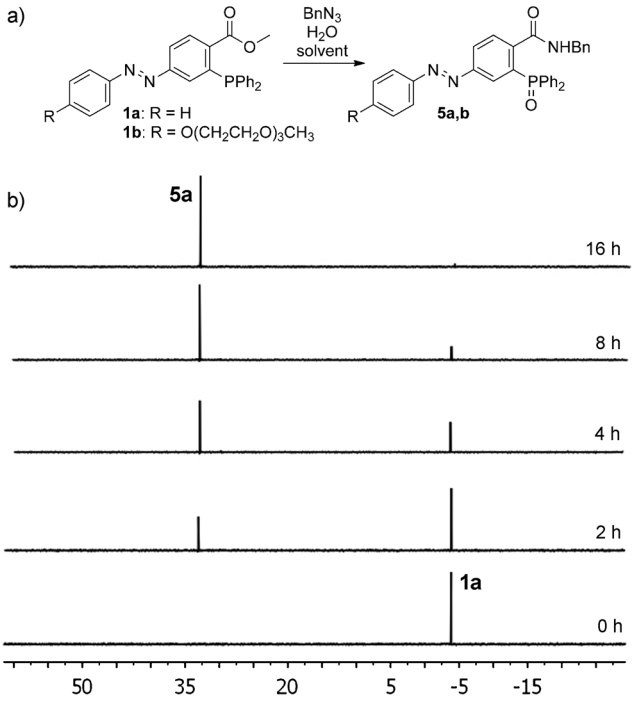


Figure 1. a) Staudinger–Bertozzi ligation of molecular photoswitches **1a** and **1b** with benzyl azide at RT; b) ^{31}P NMR spectra obtained from monitoring the reaction of **1a** with 2 equiv of benzyl azide (see the Supporting Information for details).

leads to **1a**. Diazotization of **2** and its subsequent reaction with potassium phenolate results in the formation of the azobenzene **4**, which undergoes alkylation with commercially available PEG-bromide and then cross-coupling with Ph_2PH to provide **1b** in good overall yield. Although the reagents for Staudinger ligation are known to be prone to degradation through phosphine oxidation,[19] we have observed that **1a** and **1b** are relatively stable[20] when stored.

To study the efficiency and selectivity of the ligation of compounds **1** with azide-bearing molecules, we investigated their reactions with benzyl azide as a model substrate (Figure 1a). Inspired by the seminal study on the mechanism of the Staudinger ligation by Bertozzi et al.,[21] we used ^{31}P NMR spectroscopy to analyze the progress of the reaction at given points in time (Figure 1b). A clean conversion of **1a** into **5a** was observed, and it reaches completion in 16 h at room temperature.

The products **5a** and **5b** were isolated and characterized to gain more insight into the influence of the structural features, which were introduced to enable the Staudinger–Bertozzi ligation, on the photochemical properties in chloroform and aqueous buffer (Table 1, Figure 2).

Envisioning that the proposed ligation would prove most useful in a biological context, we studied the properties of the molecular photoswitches **5a** and **5b** in aqueous buffer with addition of 2–10 %$_{vol}$ acetonitrile. We found typical azobenzene UV/Vis absorption properties,[16] with the push-pull azobenzene **5b** having the absorption maximum shifted by 31 nm to longer wavelengths[16] (Figure 2a, solid line and see Table 1, entries 1 and 2). Upon incubation at 50 °C in the dark, they can be isomerized fully to the *trans* form (Table 1, entry 3).

Photoirradiation of *trans*-**5** at $\lambda = 365$ nm (Figure 2a, dashed line) results in their isomerization to the *cis* form, and this process is slightly faster for **5b** (Table 1, entry 4). For both photoswitches, this process is reversible upon irradiation with white light, with the reisomerization of **5b** to the *trans* form, again, being faster (Table 1, entry 5). Notably, the switching cycle could be repeated in aqueous environment more than seven times without apparent compound degradation (Figure 2b). The photostationary state (PSS) of **5a** in aqueous buffer at $\lambda = 365$ nm light irradiation comprises 80 % *cis*-**5a** (Table 1, entry 6), while for **5b** it could not be reliably determined because of the low thermal stability of *cis*-**5b** in polar solvents (see below). However, upon irradiation of **5b** in $CDCl_3$ a very high PSS with 95 % of the *cis* isomer was observed (Table 1, entry 7).

Table 1: Properties of the model molecular photoswitches **5a** and **5b**.

Entry	Property	**5a**	**5b**
1	λ_{max} [nm]	330[a]	361[b]
2	molar abs. at λ_{max} [$M^{-1}cm^{-1}$]	24 400[a]	13 200[b]
3	*trans/cis*[c,d]	> 97:3	> 97:3
4	$t_{0.5}$ for *trans*→*cis* ($\lambda = 365$ nm)	38 s[a]	16 s[b]
5	$t_{0.5}$ for *cis*→*trans* (WL)	5.8 s[a]	1.5 s[b]
6	*trans/cis* ($\lambda = 365$ nm, buffer)	20:80[e,f]	n.d.
7	*trans/cis* ($\lambda = 365$ nm, $CDCl_3$)	50:50[c]	5:95[c]
8	$t_{0.5}$ for *cis*→*trans* (dark, $CDCl_3$)	257 min[c,d]	69 min[c,d]
9	$t_{0.5}$ for *cis*→*trans* (dark, buffer)	≫ 41 h[a]	≈ 50 s[b]

[a] Determined at 21 °C for 16×10^{-6} M **5a** in buffer with 10 %$_{vol}$ MeCN. [b] Determined at 21 °C for 30×10^{-6} M **5b** in buffer with 2 %$_{vol}$ MeCN. [c] Determined by ^{1}H NMR spectroscopy at ca. 5 mg mL^{-1} concentration in $CDCl_3$. [d] Incubation at 50 °C. [e] Determined at 21 °C for 19×10^{-6} M **5a** in buffer with 2 %$_{vol}$ MeCN. [f] Determined by HPLC after extraction with ethyl acetate (50 mM Tris·HCl pH 7.5 was used as buffer, see the Supporting Information for further details). WL = white light, n.d. = not determined.

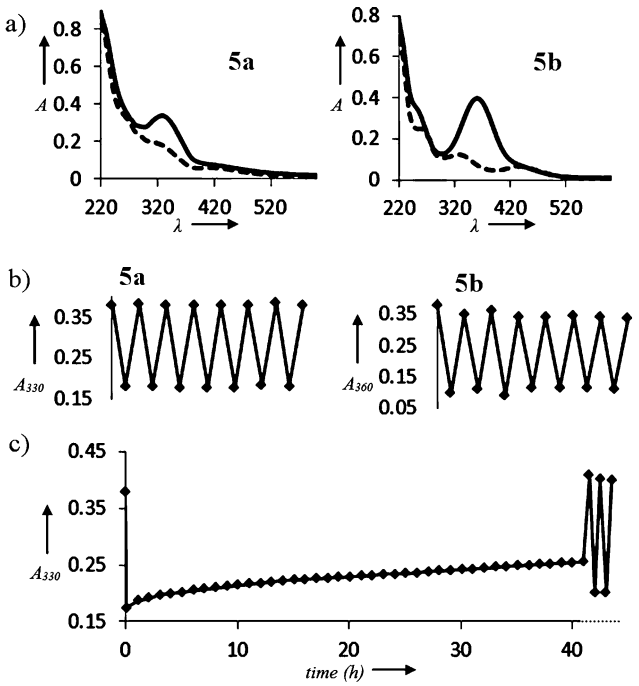


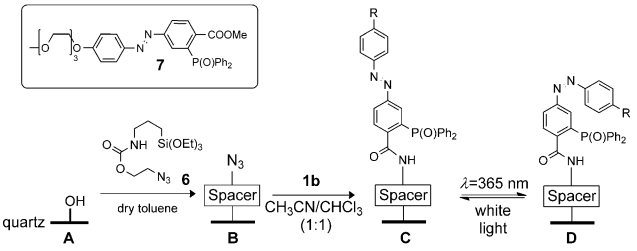
Figure 2. Photochromic properties of compounds **5** in 50 mM Tris·HCl buffer pH 7.5 with MeCN. a) UV/Vis absorption spectra for non-irradiated (solid line) and irradiated (dashed line) solutions of **5a** (left panel, 16×10^{-6} M, 10%$_{vol}$ MeCN) and **5b** (right panel 30×10^{-6} M, 2%$_{vol}$ MeCN). b) Switching cycles of **5a** (16×10^{-6} M, 10%$_{vol}$ MeCN) and **5b** (30×10^{-6} M, 2%$_{vol}$ MeCN). c) Thermal stability of the photo-irradiated state of **5a** (16×10^{-6} M, 10%$_{vol}$ MeCN) in the dark at room temperature. Changes in absorbance at $\lambda = 330$ nm prior to (0–41 h) and after alternating irradiation with white light and $\lambda = 365$ nm light.

The thermal stability of the *cis*-**5** isomers in the dark was studied in $CDCl_3$ at 50 °C and half-lives on the order of hours were measured (Table 1, entry 8). In aqueous buffer, a much shorter half-life of *cis*-**5b** was observed at 21 °C (Table 1, entry 9), and is consistent with the fast *cis–trans* isomerization process observed for push-pull azobenzenes in polar media.[17]

A remarkable effect was observed upon studying the thermal stability of *cis*-**5a** in aqueous medium (Figure 2c, Table 1, entry 9). Upon prolonged incubation (41 h) of the photoirradiated sample in the dark at room temperature we observed a very slow increase in absorbance at $\lambda = 330$ nm (Figure 2c), thus indicating an intriguingly slow thermal reisomerization. After 41 hours the sample was irradiated with white light and resulted in the instantaneous switch back to the *trans* isomer. Following the irradiation with white light, several irradiation cycles were performed to demonstrate that the molecule fully retained its switchable behavior upon prolonged incubation in aqueous buffer (Figure 2c).

The observed unusual thermal stability of *cis*-**5a** in aqueous medium places this molecule among the azobenzenes with highly thermostable photostationary states. Only very few systems are known to have similar stabilities,[22] and are usually ones in which the azobenzene molecules are part of heterocyclic systems[23] or locked in supramolecular cages.[24]

In the next step we conducted model studies on the use of **1b** for the introduction of azobenzene photoswitches on surfaces and proteins.[25] To study the incorporation of **1b** onto an azide-decorated surface, we modified piranha- and plasma-cleaned quartz coverslips (**A**; Scheme 3) with the azide-bearing silane derivative **6**.



Scheme 3. Modification of an azide-decorated quartz surface with **1b**. Inset: Structure of the phosphine oxide **7** used in control studies.

The modification was confirmed by the change of the water contact angle from $5\pm1°$ (hydrophilic surface **A**) to $62\pm3°$ (hydrophobic surface **B**). Subsequently, the coverslips were immersed in a solution of **1b** for 3 days, after which they were cleaned by sonication in chloroform, acetonitrile, and methanol (1 min each). Although no significant change in the water contact angle was observed, the UV/Vis absorption properties changed significantly and the spectrum of the **1b**-modifed coverslip (**C**; Scheme 3) showed an absorption band in the region of $\lambda = 350$–400 nm (see the Supporting Information). Sequential irradiation at $\lambda = 365$ nm (to obtain **D**; Scheme 3) and with white light resulted in the expected changes of absorbance (Figure 3a), thus indicating the presence of azobenzene moieties on the surface.

To exclude the possibility of noncovalent binding of **1b** to the surface, we followed the same protocol using **7** (Scheme 3, insert), the oxidized analogue of **1b**, which cannot act as a ligation tag. The surface obtained in this way did not show the characteristic UV/Vis absorption properties described above (Figure 3a).

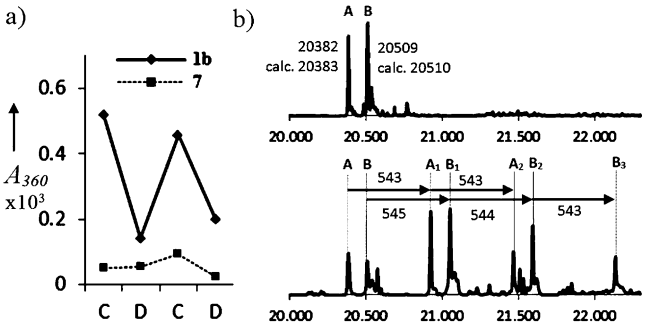


Figure 3. Applications using the molecular photoswitch **1b**. a) Changes in absorbance at $\lambda = 360$ nm for the quartz coverslips modified with **6** and **1b** or **7**, and then irradiated with alternating $\lambda = 365$ nm and white light (see Scheme 3 for the symbols **C** and **D**, and the Supporting Information for the details). b) Deconvoluted ESI/MS spectra of the LipA^{AHA} M135L M138L before (upper panel) and after (lower panel) reaction with **1b**. The introduction of **1b** results in a calculated mass difference of 544 Da.

Next, we studied the modification of a model, azide-bearing protein with **1b**. To this end, Lipase A (LipA) from *Bacillus subtilis* was prepared with azido homoalanine (AHA) residues site-specifically introduced at positions 1, 9, and 79. This was achieved by the incorporation of AHA using a methionine auxotrophic *E. coli* B834(DE3)strain[12a,26] for the expression of LipA mutant in a medium in which AHA was supplemented instead of methionine (for details, see the Supporting Information). Successful incorporation was confirmed by ESI/MS (Figure 3b, upper panel) and CuAAC reaction with a fluorescent, acetylene-modified dansyl tag[27] (see the Supporting Information). A mixture of two proteins was obtained: LipA with N-terminal AHA present (B in Figure 3b, upper panel) and its analogue with AHA removed (A in Figure 3b, upper panel).

The reaction of the thus prepared, azide-bearing model protein with 20 equivalents of **1b** in a buffer/acetonitrile solvent system resulted in the introduction of up to three residues of the azobenzene-based molecular switch onto the structure of the enzyme, as indicated by the emergence of signals in the MS spectrum with the expected mass shift as compared to the signal from the unmodified proteins (Figure 3b, lower panel). In particular, the modification of the truncated protein A, which contains two AHA residues (at positions 9 and 79), gave rise to the singly modified variant A_1, and doubly modified variant A_2 (Figure 3b, lower panel). Modification of the nontruncated protein B, which incorporates three AHA residues (at positions 1, 9, and 79) resulted in singly (B_1), doubly (B_2), and triply (B_3) modified bioconjugates (Figure 3b, lower panel).

In summary, we present herein a novel family of azobenzene switches which can be incorporated into target systems bearing azide functionalities by Staudinger–Bertozzi ligation in aqueous, as well as organic media and without any adducts or catalysts. The molecular photoswitches formed upon this ligation show high fatigue resistance for the reversible switching process in water, photostationary states with up to 95% of the *cis* isomer, and thermal stabilities of the *cis* isomer ranging from seconds to days. These properties, supported by the application in the modification of surfaces and proteins, render this novel family of azobenzene compounds an important addition to our toolbox of photoswitches and is useful for the dynamic and highly spatiotemporal control of events on the molecular scale both in materials and biohybrid systems.

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