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Communication

Bioorthogonal Cross-Linkers

Synthesis of Hetero-bifunctional Azobenzene Glycoconjugates for Bioorthogonal Cross-Linking of Proteins

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Abstract: Modification of proteins with azobenzene derivatives allows their form and function to be controlled by photochemical *E*/*Z* isomerization of the azobenzene N=N bond. Because azobenzene glycoconjugates (ABGs) are particularly promising cross-linkers for the modification of peptides and proteins, we advanced the collection of so far known homo-bifunctional ABGs with the synthesis of hitherto unknown hetero-bifunctionalized ABGs. Alkyne and alkene, alkyne and sulfhydryl, and azido and alkene functions were combined in one molecule to serve as bioorthogonal reaction pairs. With this, access to a multitude of photosensitive proteins is provided.

Introduction

Currently there is tremendous interest in the development of functional molecules for the specific modification of peptides and proteins. Important fields of application are related to the labeling of proteins in biological environments, identification of peptides in complex mixtures, and changing and control of structure and function of proteins.[1]

Lately, azobenzene derivatives have been employed for intramolecular protein modification; this has resulted in photosensitive conjugates that can be reversibly manipulated by light. Hence, irradiation of azobenzene-modified proteins at appropriate wavelengths effects *E*/*Z* isomerization of the azobenzene unit, and concomitantly, the whole protein structure can be switched between two different states.[2] This approach allows modification of protein function with spatiotemporal resolution under minimally invasive conditions.

Ideally, the azobenzene derivatives used for protein modification should be water soluble and biocompatible to allow biological studies in a natural environment. Thus, we recently introduced bifunctional azobenzene glycoconjugates for cysteine-based cross-linking of peptides.[3] As azobenzene glycosides have favorable photochromic properties, glycoazobenzene cross-linkers are promising candidates for photoswitching of protein function. In addition, carbohydrates offer a number of advantages in protein modification. They are nontoxic, improve water solubility, and their derivatization can be easily adjusted to control distances between functional groups and their orientation. This was exemplified in our work by the synthesis of dichloroacetamido-functionalized and di-*O*-allylated azobenzene glycoconjugates.[3] However, the application of such homo-difunctionalized cross-linkers has limitations. Especially, the possibility of controlled consecutive cross-linking is excluded. Also, the orthogonal functionalization of peptides, modified with unnatural amino acids, cannot be fully addressed. Therefore, it has become our goal to advance the existing collection of homo-bifunctional glycoazobenzene cross-linkers by the synthesis of orthogonally bifunctionalized examples. On the basis of state-of-the-art approaches in the field of bioorthogonal ligations,[4] we focused on sulfhydryl, alkene, alkyne, and azido functional groups as complementary reaction pairs (Figure 1). Sulfhydryl groups can ligate with maleimides or alkene functionalities according to thiol–ene coupling.[5] The thiol–ene reaction is orthogonal to cross-linking between azido and alkyne functional groups, which can be ligated in a Cu^{I}-catalyzed[6] and copper-free click reaction,[7] respectively. Also, Staudinger–Bertozzi ligation[8] can be envisaged to achieve bioorthogonal peptide conjugation with, for example, an azido–alkene hetero-bifunctional cross-linker.

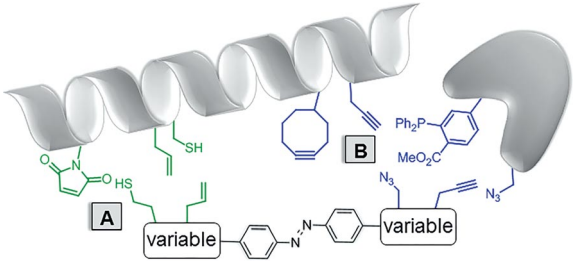

Figure 1. Hetero-bifunctional azobenzene derivatives enable the orthogonal inter- and intramolecular cross-linking of (modified) proteins. For cross-linking we aimed at thiol–ene coupling between thiols and alkenes or maleimides (a). This is compatible with a click reaction between azido and alkyne functional groups as well as Staudinger–Bertozzi ligation (b). As variable, carbohydrates were chosen and functionalized appropriately.

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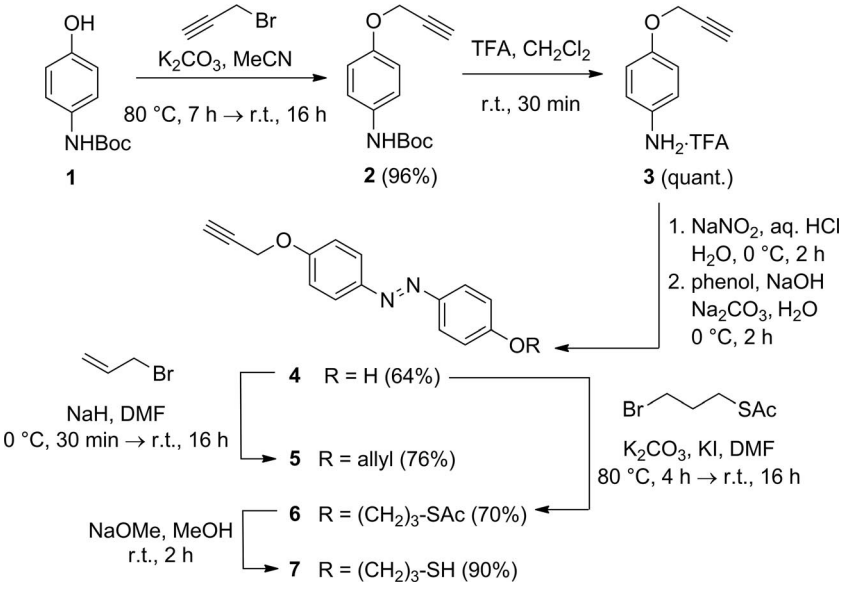
Results and Discussion

We report on the synthesis of hetero-bifunctional (glyco)azobenzene derivatives for bioorthogonal cross-linking of proteins with unnatural amino acid residues.

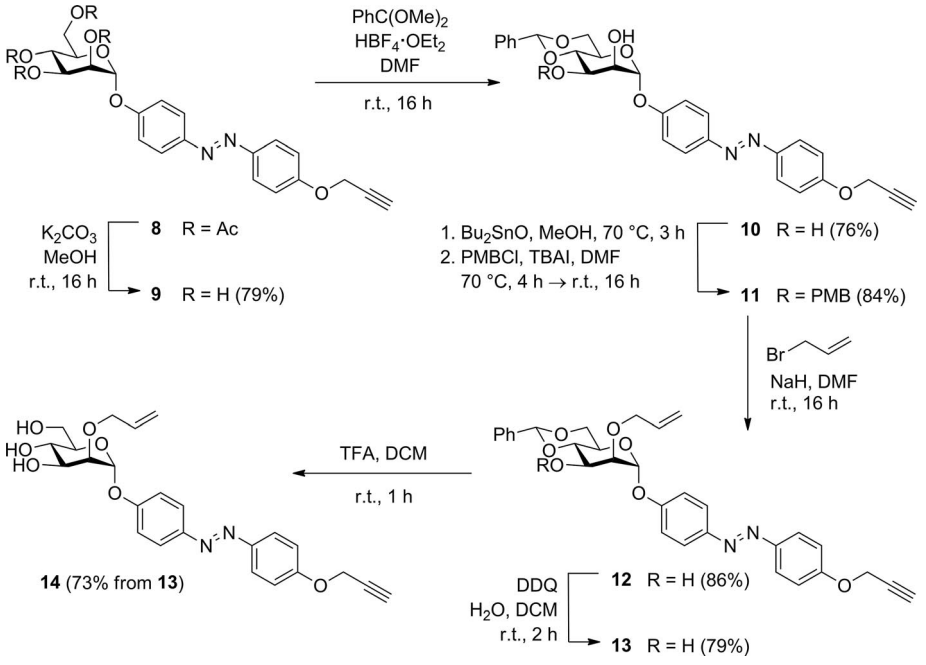
Quite clearly, even azobenzene as such can be converted into many variations of bifunctional derivatives.[9,10] For example, *p,p'*-dihydroxyazobenzene has been used for the synthesis of symmetrical azobenzene glycosides;[3,10,11] however, it does not offer entry into hetero-bifunctional azobenzene derivatives. Thus, we employed propargyloxy-functionalized azobenzene alcohol **4** in our earlier work,[11] but here we thought of a more rational approach to *p,p'*-hetero-bifunctionalized azobenzene derivatives (Scheme 1). The synthesis started from commercially available *N*-*tert*-butoxycarbonyl (Boc)-aminophenol **1**. A synthetic sequence of propargylation to **2**,[12] removal of the Boc protecting group, and subsequent azo coupling of **3**[13] with phenol furnished azobenzene alcohol **4**[11] in 61 % overall yield. Then, allylation under classical Williamson etherification conditions gave hetero-bifunctionalized azobenzene **5**. Propargyl alcohol **4** could also be converted into thioacetate **6** with thioacetic acid *S*-(3-bromopropyl) ester under basic conditions to provide azobenzene derivative **7** after deacetylation with sodium methoxide in high yield. Azobenzene derivatives **5** and **7** allow bioorthogonal cross-linking according to click chemistry[6,7] on the one hand and to thiol–ene coupling[5] on the other hand. Cross-linker **7** also enables maleimide–sulfhydryl coupling, which has been widely used in protein conjugation and labeling.[4,14]

In the next step, we wished to include carbohydrate moieties in the azobenzene cross-linkers to improve their water solubility. In addition, if functional groups are installed at various positions of a sugar ring, the distance between two orthogonal functional groups can be adjusted. As we recently optimized access to 2-*O*-allylmannosyloxy azobenzene derivatives,[3] we utilized this synthetic pathway to access alkene–alkyne-bifunctional azobenzene glycoconjugate **14** (Scheme 2). Hence, known azobenzene mannoside **8**,[11] which can be obtained from D-mannose in four steps, was deprotected with potassium carbonate to afford OH-free azobenzene mannoside **9**[15] in 79 % yield. This was then subjected to benzylidenation under catalysis of tetrafluoroboric acid in the presence of benzaldehyde dimethyl acetal to provide 4,6-*O*-benzylidene acetal **10** in 76 % yield. To selectively protect the hydroxy group at C3 of diol **10**, dibutyltin oxide was employed in refluxing methanol to form a stannylidene acetal intermediate. After exchanging the solvent to dimethylformamide the stannylidene acetal was subsequently treated with *p*-methoxybenzyl chloride (PMBCl) and tetrabutylammonium iodide to afford exclusively 3-*O*-PMB-protected ether **11** in 84 % yield over two steps. Reaction with sodium hydride and propargyl bromide then gave fully protected azobenzene mannoside **12** in 86 % yield. Oxidative removal of the PMB group in **12** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and subsequent cleavage of the benzylidene acetal with TFA in dichloromethane furnished desired 2-*O*-propargylated azobenzene glycoconjugate **14**.

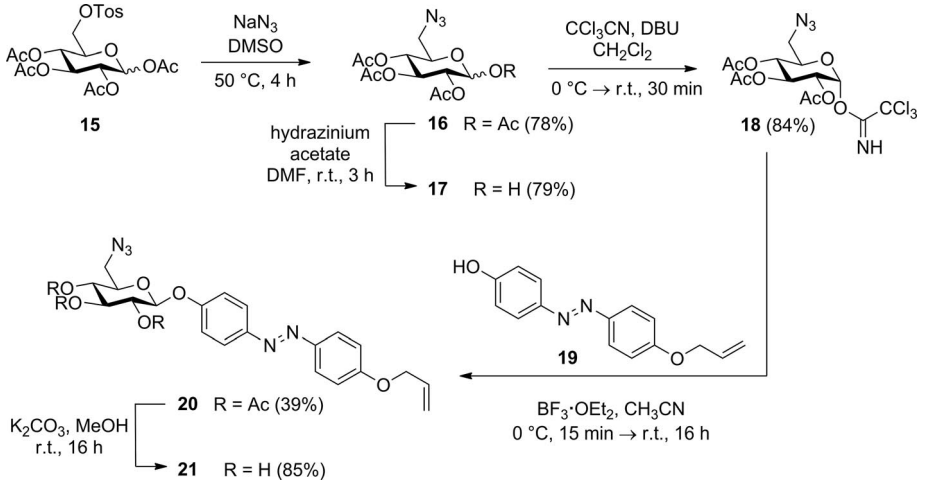
Carbohydrate chemistry also provides facile access to hetero-bifunctional azobenzene glycosides that combine an azido functional group with an orthogonal alkene functionality. For this, 6-azido-6-deoxyglucosyl donor **18**[16] can be utilized, which is available in five steps from D-glucose.[17] Tosylate **15** was obtained after tosylation and acetylation in one pot and undergoes nucleophilic substitution to yield azide **16** and after anomeric modification functionalized glucosyl donor **18** (Scheme 3). Then, BF_3·OEt_2-catalyzed glycosylation of recently described allylated azobenzene derivative **19**[3] was performed in acetonitrile owing to the limited solubility of **19**. This glycosylation reaction led to bifunctional azobenzene glycoside **20** in moderate yield (Scheme 3). Final deprotection gave pure **21** in excellent yield. The azido group of azobenzene glycoside **21**



Scheme 1. Synthesis of hetero-bifunctional allyl–alkyne and alkyne–sulfhydryl azobenzene derivatives **5** and **7**, respectively (TFA = trifluoroacetic acid).



Scheme 2. Synthesis of hetero-bifunctional allyl–alkyne azobenzene glycoconjugate **14** (TBAI = tetrabutylammonium iodide).



Scheme 3. Synthesis of hetero-bifunctional allyl–azido azobenzene glycoconjugate **21** (Tos = *para*-tolylsulfonyl, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene).

allows copper-catalyzed and strain-promoted alkyne–azide cycloaddition or Staudinger–Bertzozzi ligation. Consecutive cross-linking experiments with bifunctional peptides by employing allyl–azido-bifunctional azobenzene glycoside **21** are currently in progress.

For any photochemical application, the photochromic properties of hetero-bifunctional azobenzene derivative **5**, **7**, **14**, and **21** have to be known. Ideally, $E \rightarrow Z$ photoisomerization leads to photostationary states (PSSs), in which almost all *E* isomers have been converted. Photoirradiation was performed at room temperature in the dark, and the process was monitored by ^{1}H NMR and UV/Vis spectroscopy. The PSSs of the respective azobenzene derivatives in dimethyl sulfoxide were reached after approximately 10 min by employing a 365 nm light-emitting diode (LED). The resulting *E*/*Z* ratios were determined by integrating the ^{1}H NMR signals of the aromatic protons. The half-lives $\tau_{1/2}$ of the *Z* isomers were determined by ^{1}H NMR spectroscopy. The photochromic data obtained from respective azobenzene derivatives **5**, **7**, **14**, and **21** are collected in Table 1 (see also the Supporting Information).

Table 1. Characterization of the *E* and *Z* isomers of azobenzene derivatives **5**, **7**, **14**, and **21**.

Azobenzene derivative	λ_{max} [nm] *E* isomer	λ_{max} [nm] *Z* isomer	*E*/*Z* (PSS)[a]	$\tau_{1/2}$ [h]
5	360	312, 447	3:97	26.0
7	362	312, 448	2:98	24.5
14	362	309, 447	3:97	37.2
21	360	311, 447	3:97	49.9

[a] According to the integration ratio of the aromatic proton signals of the *E* and *Z* isomers in the ^{1}H NMR spectra.

Azobenzene derivatives **5**, **7**, **14**, and **21** showed favorable photochromic properties. Photoirradiation of the *E* isomers led to almost quantitative isomerization, and all of the *Z* isomers

have half-lives ranging from 1 to 2 days. Thus, the independent investigation of both isomeric states of cross-linked peptides is feasible.

To estimate the effect of azobenzene derivatives **5**, **7**, **14**, and **21** on the conformational properties of cross-linked peptides or proteins, the change in their end-to-end distances upon isomerization were monitored by using molecular dynamics simulations (see the Supporting Information). The end-to-end distance distributions are collected in Table 2. The most probable end-to-end distances of the *E* isomers varies from about 15 to about 18 Å. The respective *Z* isomers show a more complex distribution with the most probable distances varying from around 8 to around 15 Å. The separation of the distance distributions between the *E* and *Z* states is characteristic for the individual cross-linkers.

Table 2. Distance calculations for azobenzene derivatives **5**, **7**, **14**, and **21**.

Azobenzene derivative	Config.	Most probable end-to-end end distance [Å][a]	Range of end-to-end distances [Å][a]
5	*E*	16.7	10.5–17.8
5	*Z*	8.4	3.1–14.6
7	*E*	17.4	10.1–19.5
7	*Z*	14.8	4.6–18.0
14	*E*	17.8	9.7–21.1
14	*Z*	15.5	5.1–19.8
21	*E*	15.1	7.7–19.8
21	*Z*	11.3	3.0–18.8

[a] The distances were calculated between the terminal allyl group carbon atom and the quaternary carbon atom of the propargyl group (for **5** and **14**), the terminal sulfhydryl group and the quaternary carbon atom of the propargyl group (for **7**), and the terminal allyl group carbon atom and the CH_2N group of the azido group (for **21**).

Conclusions

In conclusion, we synthesized bifunctionalized (glyco)azobenzene derivatives in which alkyne–alkene, alkyne–sulfhydryl, and alkene–azido reaction pairs were combined for bioorthogonal cross-linking of peptides and proteins. Hence, we combined variable ligation chemistries and varied the distances between the respective functional groups in both isomeric states (*E*/*Z*). All synthesized representatives of these hetero-bifunctional cross-linker molecules have favorable photochromic properties and should thus be tested in the photoswitching of the form and function of peptides and proteins. We hope to inspire corresponding studies with our work, as the introduced molecules can now be readily accessed and varied according to the synthetic pathways reported by us.

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Keywords: Glycoconjugates · Protein modifications · Bioorthogonality · Photochemistry

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