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Hypersensitive azobenzenes: facile synthesis of clickable and cleavable azo linkers with tunable and high reducibility†

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The aim of this work is to show that by increasing the number of donor substituents in a donor/acceptor system, the sensitivity of the azobenzene linkage towards a reductive cleavage reaction can be enhanced to unprecedented high levels. For instance, in a triple-donor system, less than a second constitutes the half-life of the azo (N=N) bond. Synthetic access to such redox active scaffolds is highly practical and requires only 1–2 synthetic steps. The fundamental molecular design is also adaptable. This is demonstrated through scaffold functionalization by azide, tetraethylene glycol, and biotin groups. The availability of the azide group is shown in a copper-free 'click' reaction suitable in context with protein conjugation and proteomics application. Finally, the clean nature of the scission process is demonstrated with the help of liquid chromatography coupled with mass analysis. This work, therefore, describes development of cleavable azobenzene linkers that can be accessed with synthetic ease, can be multiply functionalized, and show a clean and rapid response to mild reducing conditions.

In chemical proteomics, one of the major goals is to develop strategies to capture and enrich a specific protein from a complex mixture of a cell lysate.[1,2] One approach to achieve this goal is through selective conjugation of the protein onto a solid support, separation of the solid support from rest of the cell lysate, and release of the captured protein for analytical purposes. The conjugation and release processes often involve the formation and disruption of biotin–streptavidin interactions. These interactions are, however, one of the strongest non-covalent interactions found in nature. Therefore, while protein-capture benefits from this trait, harsh conditions are required for the release step. To avoid this, a linker is installed between the biotin and the protein-capture sites (Fig. 1).[3–5] This linker should ideally be cleaved under mild conditions in an efficient and clean manner so that the protein remains in its native state, released quantitatively, and uncontaminated with byproducts to aid the final analysis.

In 2007, Bogyo and coworkers established the utility of an azobenzene group as a cleavable linker in chemical proteomics.[6,7] They used sodium dithionite (25 mM) as the reducing reagent to cleave the azo bond through 3 wash cycles of 15 minutes each. The cleavage efficiency was reported to be 90%. This pioneering study was critical in establishing the design concept of a cleavable linker applicable for chemical proteomics based on the reductive cleavage of the azobenzene scaffold. However, the need of high concentrations of the reducing agent, long reaction times, and non-quantitative release efficiency represent drawbacks of the system. Such attributes

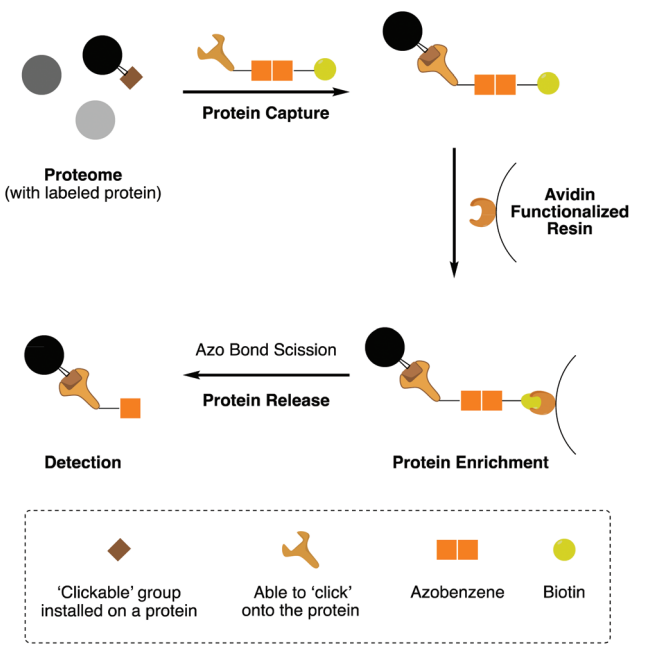

Fig. 1 Schematic representation of the protein capture and release through use of an azobenzene linker.

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may compromise the native protein structure, result into strong background signals from excess reagent, and limit the overall efficacy of the system.

To overcome these issues, Wagner and coworkers designed an azobenzene linker with much higher sensitivity by substituting the aromatic nucleus with oxygen-based donors and an acid-based acceptor.[8,9] The synthesis of the active scaffold was accomplished in 15 steps. With this new molecular design, the dithionite concentration can be decreased to 6 mM and less than 10 seconds is required for full cleavage. This design represents the current state-of-the-art in terms of sensitivity in cleavable azobenzene linkers.

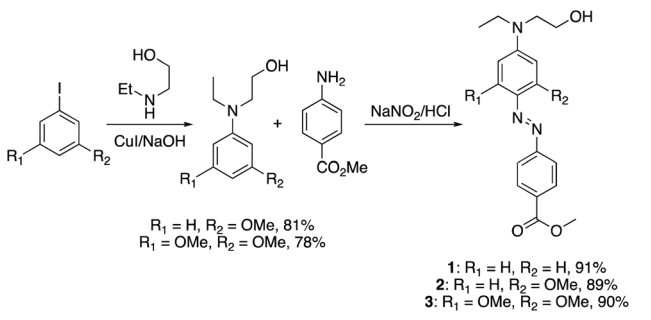
Besides proteomics, the azobenzene cleavage reaction finds applicability in various other disciplines[10] ranging from hypoxia sensing[11] and drug delivery[12] to self-assembly[13] and disassembly of synthetic constructs.[14] In most of these applications, highly sensitive azobenzene scaffolds are required. For instance, in hypoxia sensing, high sensitivity translates to reliable detection under mild conditions.[11] In colon-specific drug delivery, a rapid response under reducing conditions means higher efficacy of the delivery vehicle.[15]

The design and synthesis of highly sensitive azobenzene linkers, therefore, is a valuable goal with implications in various research fields. Toward this end, with the help of three molecular designs, we show that an increase in the number of donor substituents in an amine/ester-based donor/acceptor system can systematically enhance the sensitivity of the azo (N═N) bond towards a reductive cleavage reaction. To our knowledge, the sensitivities described here are the highest reported thus far. In addition, the synthesis is concise and efficient and allows for tailored molecular-level adaptations in order to meet structural requirements of a certain application.

Our previous work on the development of polymeric drug delivery and imaging vehicles indicated that amine- and ester-based donor/acceptor (DA) azobenzenes were more sensitive to the reductive cleavage reaction than are donor/donor (DD), acceptor alone (A), and non-substituted systems.[16] Encouraged by these results, we explored easily accessible synthetic avenues that would allow further increase in the electron density of the azobenzene nucleus. This is achieved by placing electron-donating methoxy-substituent(s) in *ortho*-position to the azo bond (Scheme 1). Initially, three molecules were prepared (Fig. S1–S18†). **1** having dialkyl amine and ester groups which serve as the control DA system. The synthesis in this case is a single-step diazotization reaction between commercially available ethylanilinoethanol and 4-amino methylbenzoate.[17] **2** and **3** carry one and two methoxy substituents and represent two-donor/one acceptor (DDA) and three-donor/one acceptor (DDDA) systems, respectively. In **2** and **3**, the copper-catalyzed amination provided the alkyl aniline segments with donor substituents that could be joined by the acceptor fragment through the diazotization reaction. Typically the isolated yields in these synthetic transformations ranged from 78–91%.

Having efficient access to the three compounds, the N═N bond scission reaction was studied with the help of UV-Vis spectroscopy. As shown in Fig. 2 and Fig. S19–S21,† the π–π* absorption band of the azobenzene chromophore located at 400–550 nm gradually decreases in intensity as a function of exposure time to sodium dithionite. Based on this data (Fig. S22†), it became clear that an increase in the number of the donor groups enhances the reducibility of the azo bond (Table 1). **2** and **3** were found to be more sensitive than **1**. The most sensitive system, compound **3**, cleaves quantitatively even at a 0.5 mM dithionite concentration. In comparison to the state-of-the-art,[8,9] compound **3** has similar half-life (<1 s) and total cleavage time (<5 s). However, compound **3** achieves this with one-twelfth of the concentration of the reducing agent. A direct comparison would require operating at a 6 mM dithionite concentration. However, **2** and **3** fully cleave instantly over 2 mM concentration of the reducing agent without allowing for accumulation of reaction kinetics data to be compared.

Having confirmed superior sensitivities of **2** and **3**, adaptation of the most potent molecular design was considered for applications. For instance, if the fundamental design concept of compound **3** is to be translated to chemical proteomics



Scheme 1 Synthesis of **1**, **2**, and **3**.

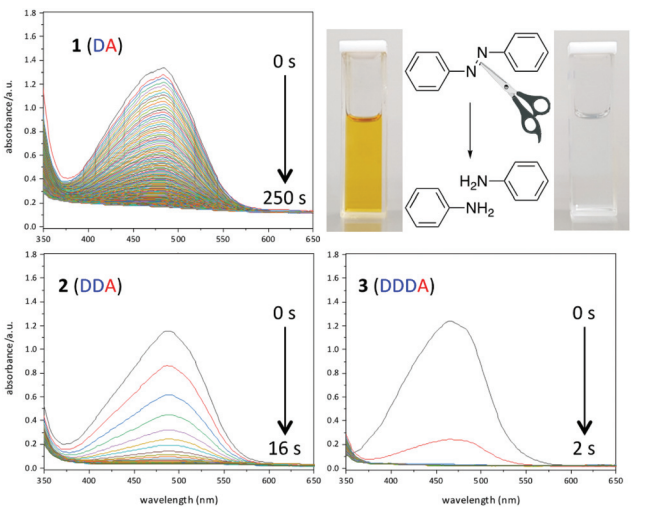


Fig. 2 UV-Vis spectra of compounds **1**–**3** (50 μm) upon exposure to 1 mM sodium dithionite in 2 : 8 DMSO : Tris-HCl buffer solution. The time lapse between each trace is 1 second. The digital picture shows change in color of the azo compound **3** (left) upon reduction (right). The azobenzene scission reaction is shown with the help of chemical structures.

Table 1 Details of the azo cleavage reaction in **1–3**

$[Na_2S_2O_4]$	Compound	Half-life (s)
2 mM	**1**	15
2 mM	**2**	0.7
2 mM	**3**	0.1
1 mM	**1**	35
1 mM	**2**	1.4
1 mM	**3**	0.1
0.75 mM	**1**	—
0.75 mM	**2**	2.2
0.75 mM	**3**	0.3
0.5 mM	**1**	—
0.5 mM	**2**	—
0.5 mM	**3**	0.6

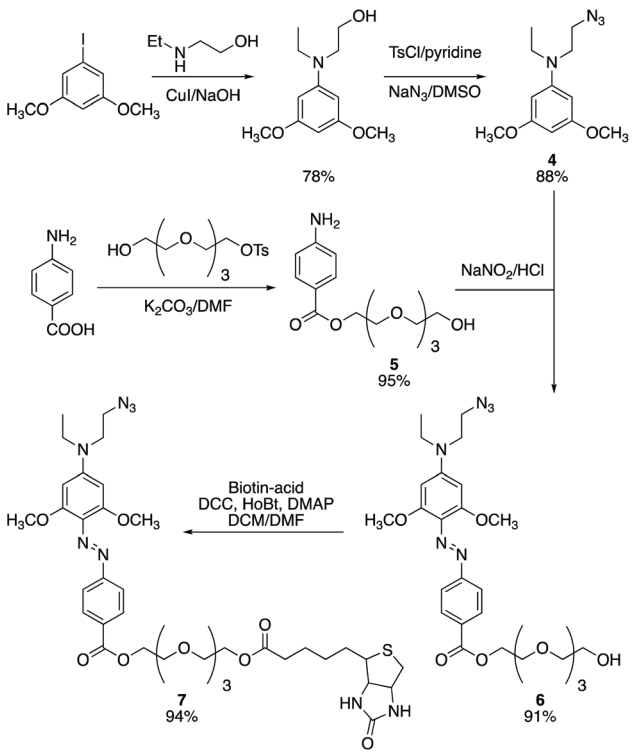
—: The cleavage reaction does not proceed to completion.

applications, it must carry protein capture and immobilization sites. Typically, to aid water solubility, a hydrophilic segment is added to the scaffold as well. In keeping with these established design traits, the hydroxyl group present at the alkyl segment of the amine was transformed into an azide (**4**) so that the known 'click' chemistry protocols can be employed for the capture process (Scheme 2).[18] Separately, the acid group in aminobenzoic acid was used to incorporate a tetraethylene glycol unit. A diazotization reaction between electron-rich **4** and electron-poor **5** afforded the azobenzene **6**. Finally, the terminal hydroxyl group of the ethylene glycol segment could be engaged in an esterification reaction to attach biotin ligand (**7**). These functional group decorations do not alter the fundamental electronic structure of the scaffold **3** and therefore the molecule retains its sensitivity characteristics. The isolated yields in these synthetic transformations range from 78–95%. Overall, compound **7** is decorated with biotin, ethylene glycol, and azide functionalities to meet immobilization and capture requirements under aqueous conditions, has a DDD/A type electronic structure to meet release requirements, and requires only five simple and high-yielding synthetic steps.

Liquid chromatography coupled with mass spectrometry (LC-MS) was then utilized to examine the cleanliness of the cleavage process. Initially, compound **7** was subjected to the analysis (Fig. 3 and Fig. S23†) and the elution chromatograms indicated a clean cleavage to two expected compounds and absence of byproducts. The corresponding mass analyses showed molecular ions of the cleaved aniline segments. No signals were observed at a molecular weight range higher than that of the expected molecular ion. This indicates a clean cleavage to expected products and absence of byproducts.

Encouraged by these results, compound **7** was subjected to a copper-free 'click' reaction with a cyanine dye having a cyclic acetylene to probe the availability and reactivity of the azide group (Scheme 3).[18] The azide–alkyne reaction in this case, therefore, requires only mixing of the two reactants. IR spectroscopy indicated disappearance of the azide signal at 2099 cm^{-1} upon reaction (Fig. S24†). In UV-Vis spectroscopy, the dye-azo conjugate displays signals for three chromophores (Fig. 4). The stilbene-like triazole that results from 'click' conjugation can be observed at 350 nm. The azo and cyanine segments can be seen at 400–550 and 600–750 nm, respectively. As expected, after cleavage, the azo absorption band disappears whereas the stilbene and cyanine bands remain. Compound **8** was also subjected to the LC-MS analysis (Fig. S25†), which again demonstrated a clean cleavage process due to a high sensitivity of the azobenzene scaffold and the need to use only a very dilute solution of the reducing agent.

In summary, facile synthetic access to adaptable azobenzene scaffolds is developed that shows a quantitative and



Scheme 2 Total synthesis of azide, biotin, and tetraethylene glycol-functionalized azo **7**.

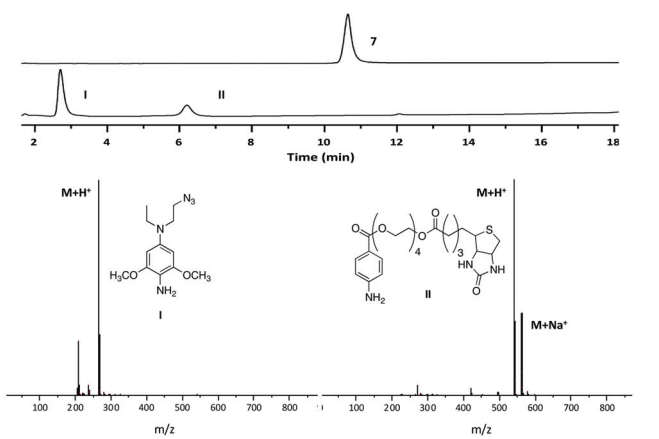
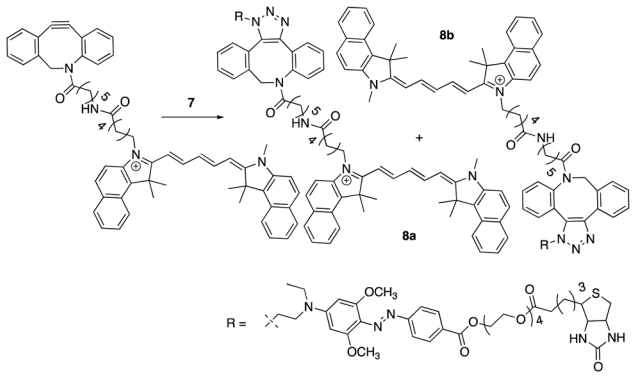


Fig. 3 Liquid chromatography (top) and corresponding mass data (bottom) for compound **7** before and after azo cleavage reaction.



Scheme 3 Strain-promoted copper-free 'click' reaction to examine availability of the azide group in **7** towards conjugation. **8a** and **8b** are positional isomers.

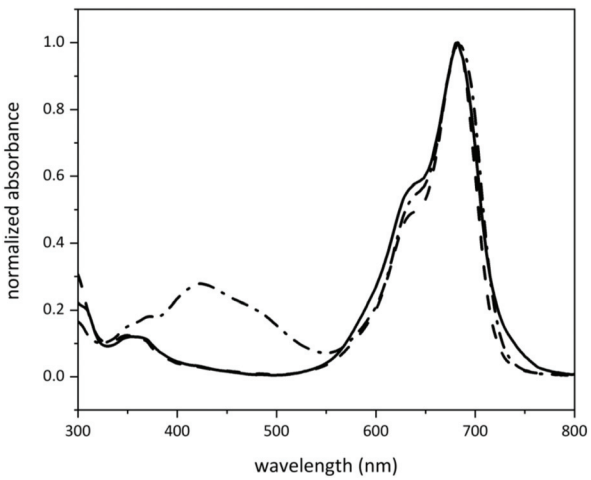


Fig. 4 UV-Vis spectra of cyanine alkyne (solid line) and **8** before (dash dot line) and after (dashed line) azo bond scission in 2 : 8 DMSO : Tris-HCl buffer solution.

clean cleavage of the N=N bond in a few seconds of exposure to low concentrations of a reducing agent. We anticipate that the results presented here would be useful not only for the protein conjugation applications but also in the arena of hypoxia sensing,[11] drug delivery,[12] self-assembly,[13] and self-immolative polymers,[14] in which the triggered cleavage of the azobenzene nucleus is applied for release/activation purposes and a rapid and tunable response time is required.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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