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# Optimization of the Azobenzene Scaffold for Reductive Cleavage by Dithionite; Development of an Azobenzene Cleavable Linker for Proteomic Applications

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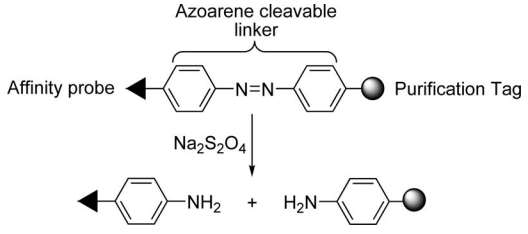
In this paper we conducted an extensive reactivity study to determine the key structural features that favour the dithionite-triggered reductive cleavage of the azo–arene group. Our stepwise investigation allowed identification of a highly reactive azo–arene structure **25** bearing a carboxylic acid at the *ortho* position of the electron-poor arene and an *ortho*-*O*-alkyl-resorcinol as the electron-rich arene. Based on this 2-(2′-alkoxy-4′-hydroxyphenylazo)benzoic acid (HAZA) scaffold, the orthogonally protected difunctional azo–arene cleavable linker **26** was designed and synthesized. Selective linker deprotection and derivatization was performed by introducing an alkyne reactive group and a biotin affinity tag. This optimized azo–arene cleavable linker led to a total cleavage in less than 10 s with only 1 mM dithionite. Similar results were obtained in biological media.

## Introduction

Since the discovery of "aniline yellow" in 1861 by Mene,[1] azo–arenes have become the largest class of synthetic dyes. More recently, these compounds attracted much attention for the development of molecular switches and cleavable linkers in biological applications. The specific and reversible photo-induced (*Z*)/(E) isomerization of the N=N motif enabled the control of peptide conformations,[2] remote control of ion channels in excitable cells[3] and the switch-cell adhesion on surfaces coated with arginine–glycine–aspartic acid (RGD) peptides.[4] Because of its ability to undergo smooth cleavage upon treatment with dithionite, a mild and potentially bio-orthogonal reducing agent,[5] azo–arene compounds are used as cleavable linkers. Azo–arene reduction found innovative applications in protein cross linking[6] and very recently in functional proteomics.[7a] In the latter application, the incorporation of an azo–arene cleavable linker between a protein's affinity probe and a purification tag, allows specific elution of captured proteins (Scheme 1).[7] Noteworthy, azo–arene reductions require multiple washings and high concentrations of the reducing agent, which can involve denaturation of the proteins and presence of strong background signals.




Scheme 1. Cleavage of the azo–arene probe with sodium dithionite.

With the continuous need for improved tools for modern proteomics, the search of mild cleavable linkers is a very active field of research. Vinyl sulfide,[8] acylhydrazone,[9] diaryl hydrazone[10] or azo–arene[7] based cleavable linkers are therefore object of recent research. In this paper we report our study to fine-tune the structure of azo–arenes toward dithionite reduction. This study led to the design of a new orthogonally diprotected cleavable linker, which can be derivatized by many tags.

## Results and Discussion

Many studies have been published dealing with the sensitivity of azo–arene dyes under reducing biological conditions.[11] However, only scarce data concerning the reactivity of these compounds toward dithionite were reported.[12] In order to optimize the sensitivity of the azo–arene motif toward dithionite reduction, we therefore decided to carry out an extensive structure/reactivity study. A set of substituted azo–arene compounds was synthesized by using classical procedures.[13] Cleavage assays were performed under standardized condition at room temperature with 47.5 μM azo–arene and 6 mM sodium dithionite in phosphate buffer

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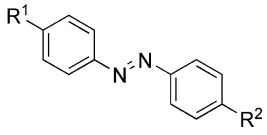
(pH = 7.4). Azo bond cleavage was monitored by UV/Vis spectrophotometry at 376 nm (measurement every second) and the half-life was calculated for each compound (Tables 1, 2, 3, 4, and 5). This wavelength was chosen, because below 376 nm dithionite perturbations were observed in the UV spectra.[14]

As a first set, substrates bearing combinations of electron-withdrawing and electron-donating groups (EWGs and EDGs) at the *para* position of each aromatic ring were assayed (Table 1). Introducing a hydroxy group on one aromatic ring (**2**) resulted in a clear acceleration of the reduction compared to unsubstituted compound **1** (Table 1, Entries 1 and 2). Introducing a carboxylic acid (**3**) decreased the kinetics of cleavage compared to the phenol derivative (Table 1, Entry 3). The reduction kinetics of symmetric compounds **4** and **5** were less effective than those of **2**, and the best result was obtained with 4-(4′-hydroxyphenylazo)benzoic acid (**6**) (Table 1, Entries 4–6). These observations confirm the literature results stating that azo–arenes substituted with both EDGs and EWGs are reduced faster than their analogues bearing only EDGs.[11a] Due to their reduction efficiency, azo compounds possessing an electron-poor arene (A) and an electron-rich arene (B) will therefore be used for further investigations (Figure 1).

Table 1. Half-lives of azo–arenes substituted by a combination of EDGs and EWGs at the 4- and 4′-positions.




| Entry | $R^1$ | $R^2$ | Azo–arene | Half-life [s] |
|---|---|---|---|---|
| 1 | H | –H | **1** | 455[a] |
| 2 | H | –OH | **2** | 39 |
| 3 | COOH | –H | **3** | 121 |
| 4 | COOH | –COOH | **4** | 110 |
| 5 | OH | –OH | **5** | 185 |
| 6 | COOH | –OH | **6** | 20 |

[a] Cleavage in a mixture of acetonitrile/phosphate buffer (2:8).

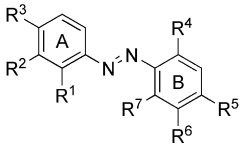



Figure 1. General structure of azo–arene compounds synthesized for the structure/reactivity study.

The influence of the structure of ring A was first evaluated. In the following set of experiments, the phenol group was fixed in position $R^5$ and different EWGs in position $R^3$ were introduced (Table 2).

For this series, comparable half-lives were observed (Table 2, Entries 1–4) except for the nitro compound **10**, which cleaved in more than 500 s (Table 2, Entry 5). This surprising result could be ascribed to a possible competitive reduction of the nitro group, giving an unfavorable electron-rich azo–arene substrate.[15] A carboxylic acid group was chosen as the most convenient EWG to ease post-functionalizations of the scaffold in order to obtain our difunctional cleavable linker. We next investigated the influence of carboxylic acid positions on the reduction kinetic (Table 3).

Table 2. Half-lives of azo–arenes substituted with different EWGs at the $R^3$ position ($R^1$, $R^2$, $R^4$, $R^6$, $R^7$ = H; $R^5$ = OH).

| Entry | $R^3$ | Azo–arene | Half-life [s] |
|---|---|---|---|
| 1 | COOH | **6** | 20 |
| 2 | $PO(OEt)_2$ | **7b** | 9 |
| 3 | CN | **8** | 12 |
| 4 | $SO_3H$ | **9** | 16 |
| 5 | $NO_2$ | **10** | 576 |

Table 3. Half-lives of azo–arenes substituted with carboxylic acid in different positions of ring A ($R^4$, $R^6$, $R^7$ = H; $R^5$ = OH).

| Entry | $R^1$ | $R^2$ | $R^3$ | Azo–arene | Half-life [s] |
|---|---|---|---|---|---|
| 1 | H | H | COOH | **6** | 20 |
| 2 | H | COOH | H | **11** | 70 |
| 3 | COOH | H | H | **12** | <1 |

As anticipated, due to mesomeric effects, substrate **11** with the COOH group in position $R^2$ showed a longer half-life than the previous compound **6** bearing the acid group in position $R^3$ (Table 3, Entries 1 and 2). With the acid group in position $R^1$, a significant decrease in half-life was observed with a cleavage in less than 1 s (Table 3, Entry 3). Assuming that the carboxylic acid might be used as a straightforward conjugation point to introduce a reactive linker, we decided to study the effect of acid modifications on the half-life (Table 4).

Table 4. Half-lives of azo–arenes substituted with carboxylic acid analogues at the $R^1$ position ($R^2$, $R^3$, $R^4$, $R^6$, $R^7$ = H; $R^5$ = OH).

| Entry | $R^1$ | Azo–arene | Half-life [s][a] | Half-life [s][b] |
|---|---|---|---|---|
| 1 | COOH | **12** | <1 | <1 |
| 2 | COOMe | **13** | <1 | 13 |
| 3 | $CONH(CH_2C{\equiv}CH)$ | **14** | 40 | 193 |

[a] 6 mM dithionite. [b] 1 mM dithionite.

With a 6 mM dithionite solution, ester analogue **13** and free acid **12** were cleaved in less < 1 s, whereas amide analogue **14** displayed a longer half-life (Table 4, Entries 1–3). The dithionite concentration was lowered to 1 mM in order to discriminate compounds **12** and **13**. Under these conditions, the free acid proved to undergo a significantly faster reduction. It thus appeared that for linker development purposes the carboxylic acid had to remain free. The derivatization point, enabling conjugation with the molecular probe or the affinity tag, will be introduced at the $R^3$ position of ring A through C–C linkage.

We then investigated the effect of the substitution pattern of electron-rich arene ring B to further improve the cleavage efficiency. In order to enable accurate half-life measurements, kinetics were slowed down by using unsubstituted ring A. Several azo–arenes with different substituents on ring B were synthesized and assayed (Table 5).

Table 5. Half-lives of azo–arenes substituted on ring B by different EDGs ($R^1$, $R^2$, $R^3$ = H).

| Entry | $R^4$ | $R^5$ | $R^6$ | $R^7$ | Azo–arene | Half-life [s] |
|---|---|---|---|---|---|---|
| 1 | H | $NH_2$ | H | H | **15** | 94 |
| 2 | H | $NEt_2$ | H | H | **16** | 890 |
| 3 | H | NHAc | H | H | **17** | 1839 |
| 4 | H | OH | H | H | **2** | 39 |
| 5 | H | OMe | H | H | **18** | 2694 |
| 6 | OH | H | Et | H | **19** | 46 |
| 7 | OH | OH | H | H | **20** | 121 |
| 8 | OMe | OH | H | H | **21** | 4 |
| 9 | OH | OMe | H | H | **22** | 1540 |
| 10 | OMe | OH | H | OH | **23** | 3354 |
| 11 | OMe | OH | H | OMe | **24** | 7 |

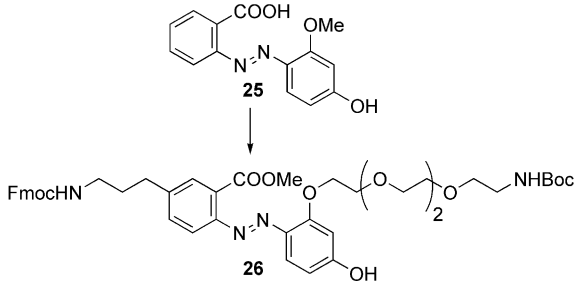



Figure 2. Structure of optimal compound **25** for azo–arene reduction by sodium dithionite and deduced orthogonally protected azo–arene cleavable linker **26**.

Of all $R^5$-monosubstituted arenes, 4-(hydroxyphenylazo)-benzene (**2**) remained the most efficient (Table 5, Entry 4). In fact, aniline **15**, *N*-alkylaniline **16** and *N*-acylaniline **17** were found to undergo much slower cleavages (Table 5, Entries 1–3). The reduction of the *O*-methylated analogue **18** showed a dramatic decrease of the cleavage kinetics compared to those of **2** (Table 5, Entry 5). The hydroxy position was then investigated, and we observed that a hydroxy group in position $R^4$ gives a similar result than a hydroxy group in position $R^5$ (Table 5, Entries 4 and 6). A series of disubstituted phenol substrates were then assayed (Table 5, Entries 7–9). According to the literature, the results obtained with substrates **21** and **22** were quite unexpected (Table 5, Entries 8 and 9).[7a] A hydroxy group in position $R^4$ appears to be not essential for an efficient azo–arene reduction. We found that the combination with a methoxy group in position $R^4$ and a hydroxy group in position $R^5$ (**21**) was more reactive than the reverse combination (**22**). A similar trend was observed in the phloroglucinol series (Table 5, Entries 10 and 11). This set of experiments clearly showed that a hydroxy group in position $R^5$ and an alkoxy group in position $R^4$ is by far the most suitable substitution pattern of ring B. This scaffold offers a significant improvement of the kinetic rate compared with that of a hydroxy group in position $R^4$ already described.[7a–7d] Furthermore, the alkoxy group could be used as a straightforward conjugation point.

With all these prerequisites in hand, the optimal compound **25** bearing an *ortho*-carboxylic acid at the electron-poor arene ring A and an *ortho*-*O*-alkylresorcinol as the electron-rich arene ring B was synthesized (Figure 2). As expected, this optimized compound showed a half-life $< 1$ s and a total cleavage time of $< 5$ s upon treatment with 6 mM sodium dithionite. A decrease of the reducing agent concentration to 1 mM gave the same result and a total cleavage in $< 15$ s.

Starting from this scaffold, 2-(2′-alkoxy-4′-hydroxyphenylazo)benzoic acid (HAZA) **26** was designed with one orthogonal protecting group on each side of the cleavable bond. *tert*-Butoxycarbonyl (Boc) and (fluorenylmethoxy)-carbonyl (Fmoc) groups were chosen because of their versatile well-established chemistry. Selective deprotection will allow the subsequent introduction of a bioorthogonal chemical hook (e.g. alkyne, phosphane, azide, ...) at one end and the introduction of different tags such as biotin or a fluorescent dye at the other end. In addition, the carboxylic acid will be kept protected to avoid side-reaction and purification problems. On the phenol side, three units of an ethylene glycol spacer will be introduced to increase the water solubility (Figure 2).

A convergent approach was used to synthesize protected azo–arene **26** by a diazonium coupling reaction between aniline **28** and resorcinol **30** as the key-step (Figure 3). On the one hand, the amine derivative **28** was obtained from 2-amino-5-iodobenzoic acid. After amine and carboxylic acid protection, *N*-Fmoc-propargylamine was introduced into the protected iodide derivative by Sonogashira–Hagihara coupling reaction. The corresponding aniline **28** was obtained by deprotection and reduction by hydrogen (4 steps, 17% overall yield). On the other hand, a triethylene glycol spacer terminated by the Boc-protected amine **29**, was obtained in 5 steps with 53% overall yield.[13] Briefly, phenol derivative **30** was easily prepared by alkylation of resorcinol with the tosylated spacer **29**. Diazotation of aniline **28** and reaction with phenol **30** gave the orthogonally protected HAZA **26** with 56% of yield. The desired *ortho*-alkylated compound was confirmed by NOESY NMR spectroscopy.

Alkyne as reactive group and biotin as affinity tag have been chosen to exemplify the chemoselective deprotection and derivatization sequence of HAZA **26**. Piperidine treatment and further coupling with *N*-succinimidyl-5-hexynoate gave HAZA **31** with 78% yield. Under acidic conditions, the Boc group was removed, and the linker derivatization was achieved by coupling with biotin in 78% yield. Final hydrolysis of the methyl ester provided the water-soluble functionalized HAZA **32** in good yield.

The reduction of the final alkyne–HAZA–biotin **32** was monitored by UV spectroscopy at 463 nm, which showed a half-life $< 1$ s and a total cleavage time of $< 10$ s with 1 mM dithionite (Figure 4). Under these conditions, no side products were observed and the total cleavage was confirmed by mass spectrometry.[13] The reaction of **32** with 1 mM dithionite, in the presence of *E coli* cell lysate, lead to a total cleavage time of $< 10$ s (Figure 4). This result confirmed that dithionite is a potent bioorthogonal reagent and dem-

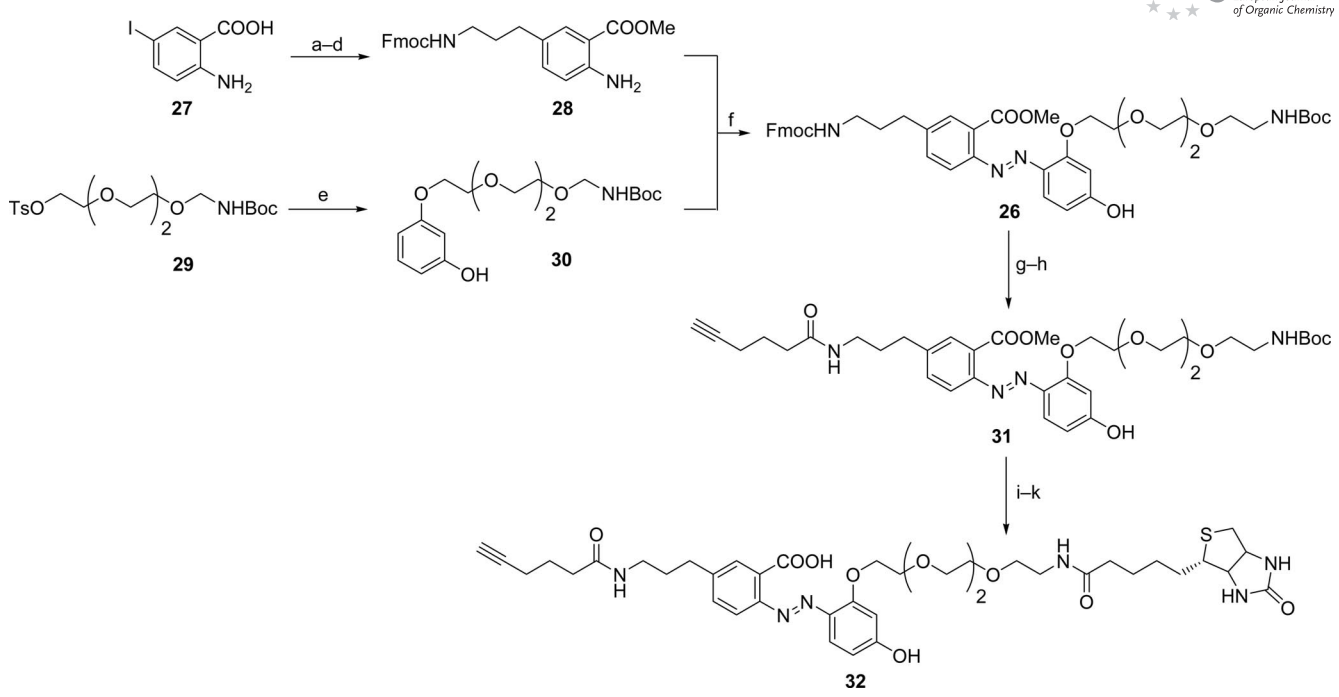



Figure 3. Reaction pathway for the formation of alkyne–HAZA–biotin **32**: (a) benzyl chloroformate, 0.1 mol-% β-CD, $H_2O$, room temp., 16 h, 71%; (b) dimethyl sulfate, $K_2CO_3$, acetone, 40 °C, microwaves, 20 min, 63%; (c) *N*-Fmoc-propargylamine, $PdCl_2(PPh_3)_2$, CuI, TEA, DMF, room temp., 16 h, 60%; (d) $H_2$, Pd/C, DMF/EtOAc, room temp., 16 h, 65%; (e) $K_2CO_3$, DMF, microwaves, 120 °C, 20 min, 57%; (f) $NaNO_2$, HCl, $H_2O$/acetone (1:1), 0 °C, 45 min, then $Na_2CO_3$, NaOH, $H_2O$/acetone (1:1), 0 °C to room temp., 10 min, 54%; (g) piperidine, DCM, room temp., 3 h; (h) 5-hexynoic acid–NHS, TEA, DMF, room temp., 16 h, 78% (for two steps); (i) TFA, DCM, room temp., 16 h; (j) biotin, HBTU, TEA, DMF, room temp., 16 h 78% (for two steps); (k) LiOH, MeOH/$H_2O$ (4:1), 40 °C, 16 h, 61%.

onstrates the potency of alkyne–HAZA–biotin **32** as a highly efficient cleavable linker for future proteomic studies.

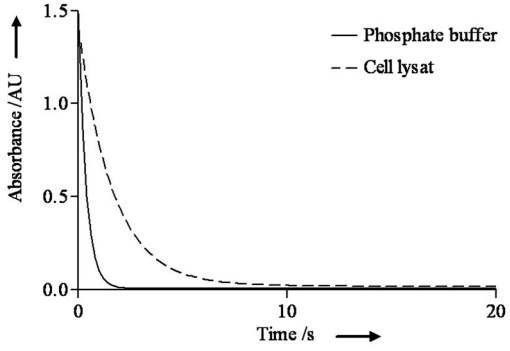



Figure 4. Reduction kinetics of alkyne–HAZA–biotin **32** with 1 mM sodium dithionite in two different medias: phosphate buffer (solid line) and cell lysate (dashed line).

## Conclusions

Structural optimization of the azobenzene scaffold enabled the development of an efficient HAZA linker. This difunctional orthogonally protected linker can be conveniently conjugated with various molecular tags and affinity probes. Most interestingly, instead of using elution with 100 mM dithionite or several elutions with 25 mM dithionite to reduce N=N bond as it was needed with previous generations of linkers,[7] alkyne–HAZA–biotin **32** can be reduced in $< 10$ s with 1 mM reducing agent, and the reactivity was 500-fold increased with respect to our reference.[13] Furthermore, the presence of a carboxylic acid group and the triethylene glycol spacer provided a very good water solubility. The application of the HAZA linker **26** for protein complex enrichment is currently under investigation and will be reported in due course.

**Supporting Information** (see footnote on the first page of this article): Experimental procedures and spectroscopic data for all compounds.

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