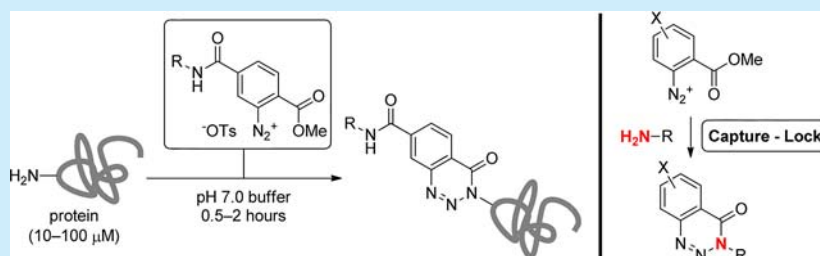# Amine-Selective Bioconjugation Using Arene Diazonium Salts

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S *Supporting Information*

ABSTRACT: A novel bioconjugation strategy is presented that relies on the coupling of diazonium terephthalates with amines in proteins. The diazonium captures the amine while the vicinal ester locks it through cyclization, ensuring no reversibility. The reaction is highly efficient and proceeds under mild conditions and short reaction times. Densely functionalized, complex natural products were directly coupled to proteins using low concentrations of coupling partners.

The chemical modification of biomolecules has gained tremendous attention in the past few years. In particular, the covalent coupling of two or more bioactive components, a process generally termed bioconjugation,[1,2] has allowed for a myriad of applications in biological chemistry and pharmaceutical sciences, including the investigation of native biomolecules or the construction of new functional entities, such as biomolecule-based materials. Despite the development of numerous approaches, various challenges remain to be addressed.[2b,3] In particular, there is a need for the identification of new bioconjugation reactions that combine efficiency along with site-selective targeting and short reaction times as well as the use of low substrate concentration. Herein, we report a new approach for the selective modification of amino groups on native proteins using newly designed diazonium terephthalate ester **1** as a bioconjugation reagent (Scheme 1). The approach allows for the attachment of highly functionalized components to proteins, resulting in the formation of stable benzo[d]-[1,2,3]triazin-4(3H)-one products **2**. The reaction proceeds rapidly under mild conditions at physiological pH and with low substrate concentration.

Scheme 1. Amine-Selective Bioconjugation of Proteins with Diazonium Salt 1
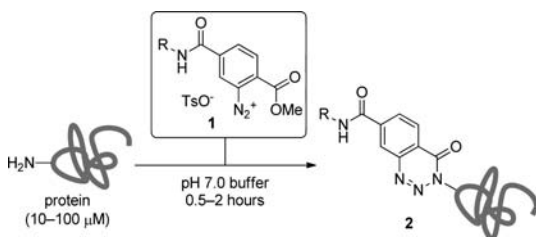
Our interests in developing chemoproteomic reagents to assess ligand–receptor interactions[4] have led us to examine novel bioconjugation strategies for protein modification. In particular, we sought mildly electrophilic reagents that could react with amino groups of proteins in aqueous media.[5] We reasoned that aryl diazonium salts could serve this purpose because of their stability in water and their inherent reactivity profile.[6] The early reports on the reactivity of arene diazoniums (**3**) toward single amino acids highlighted the potential application of these electrophiles for the derivatization of lysine, histidine, tryptophan, and tyrosine residues. However, only their reaction with tyrosines has found application in bioconjugate chemistry on the basis of their ability to form diazo adducts such as **4** by electrophilic aromatic substitution (Scheme 2A).[7]

To the best of our knowledge, the electrophilicity of arene diazoniums toward lysine-ε-NH$_2$ to furnish triazenes has never been exploited for bioconjugation. This can likely be attributed to two key reasons. First, lysine-ε-NH$_2$'s on protein surfaces are largely found as protonated ammonium salts (pK_a 10.5), rendering them poorly reactive under physiological conditions. Second, it has been appreciated for some time that the triazene adducts formed between amines and arene diazoniums are susceptible to a variety of decomposition reactions, most notably substitution by water or other nucleophiles (Scheme 2B).[8] With these challenges in mind, we decided to embark on the design of a diazonium reagent that would serve as a new tool for targeting amines in proteins.

In laying the groundwork, several challenges would have to be addressed, including the well-known potential for cross-reactivity of the reagent with tyrosines (Scheme 2A) as well as
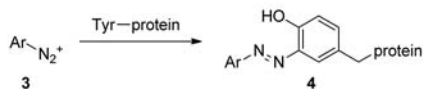
Scheme 2. Arene Diazonium Salts for Bioconjugation
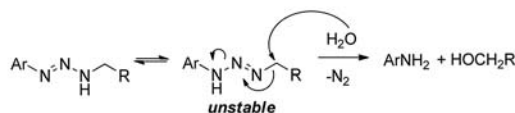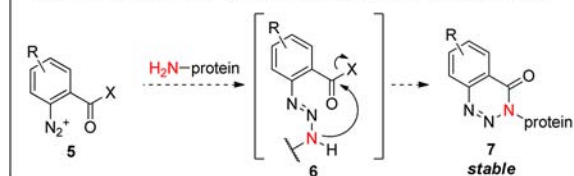
the instability of the triazene adducts formed from amines. We hypothesized that the first of these issues might be circumvented by the careful selection of conjugation conditions and reagent design. The problem of triazene instability could be addressed by incorporating a suitably positioned reactive entity proximal to the diazonium that would capture the triazene intermediate and thereby preclude adduct decomposition (Scheme 2C). We hypothesized that an electrophilic group such as an ester could trap an unstable, initially formed triazene intermediate **6** as the corresponding benzotriazinone **7**.[9]

The study commenced with the preparation of an aniline as a diazonium precursor incorporating a linker component (Scheme 3). As outlined in Scheme 3, we chose commercially

Scheme 3. Preparation of Diazonium Terephthalate 13
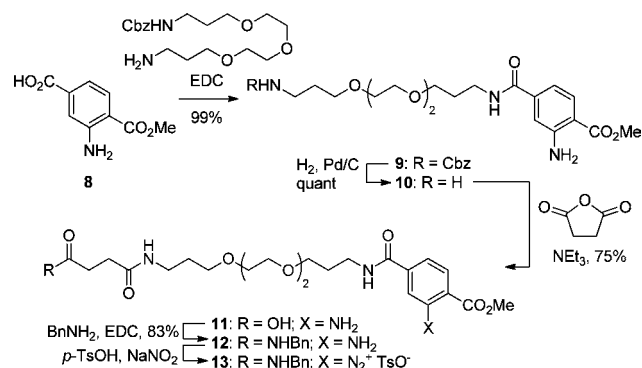
available anthrinilate ester derivative **8** as a starting point.[10] Attachment of a hydrophilic spacer component was carried out by peptide coupling of the carboxylic acid **8** with mono-Cbz protected diethylene glycol bis(3-aminopropyl) ether, producing amide **9** in 99% yield. Hydrogenolytic cleavage of the benzyl carbamate delivered amine **10**. Its subsequent condensation with succinic anhydride furnished carboxylic acid **11** in 75% yield. Both, amine **10** and acid **11**, could serve as versatile platforms for the introduction of functional components to the linker system. As a test substrate, we prepared benzyl amide **12** in 83% yield by peptide bond

formation between acid **11** and N-benzyl amine. **12** was subjected to diazotization using $NaNO_2$ in the presence of p-toluenesulfonic acid at ambient temperature (15 min) providing a solution of diazonium salt **13**, which was used directly for bioconjugation.

In order to evaluate the chemistry of the aryl diazonium salt **13** as a reagent for amine-selective bioconjugation, we first set out to conduct model studies using two tripeptides substrates **14** and **15** (H_2N-VGS-CO_2H and H_2N-AYF-CO_2H; Figure 1).
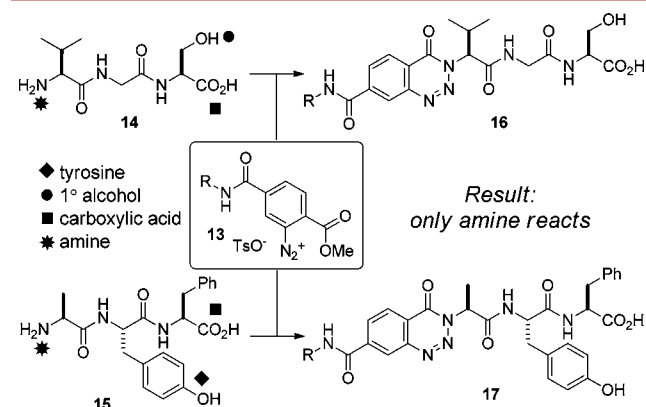

Figure 1. Conjugation of tripeptide **14** (1.0 equiv) with **13** (2.0 equiv) and tripeptide **15** (1.0 equiv) with **13** (0.9 equiv).

Diazonium tosylate **13** was allowed to react with tripeptide H_2N-VGS-CO_2H (**14**) in pH 7.0 phosphate buffer (100 mM NaH_2PO_4).[11] After 2 h the desired benzotriazinone **16** could be isolated in 56% yield after esterification, and its structure was confirmed by 2D NMR analysis. Similarly, tyrosine containing tripeptide H_2N-AYF-CO_2H (**15**) was reacted with 0.9 equiv of **13**. Analysis of this reaction by LC-MS followed by NMR-spectroscopic characterization of the isolated product **17** indicated selective coupling of the N-terminus of the peptide substrate (see Supporting Information). Only a small amount of tyrosine coupled product could be detected by LC-MS.

We next examined the use of aryl diazonium salt **13** for amine-selective bioconjugation of proteins. Treatment of a 100 μM solution of myoglobin with diazonium salt **13** (1 mM) at pH 7.0 led to modification of the protein substrate (Figure 2).[12] ESI-MS analysis of the product after 30 min of reaction time indicated the conjugation of up to six linker molecules to myoglobin (Figure 2A).[13] Most importantly, no byproducts
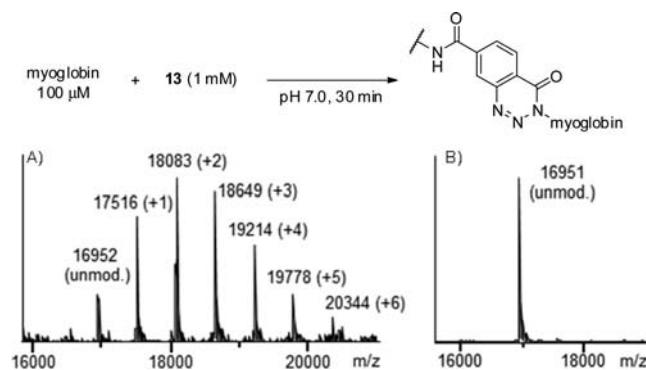

Figure 2. Diazonium bioconjugation of myoglobin. ESI-MS spectrum (deconvoluted) of the product mixture (A) and unmodified myoglobin (B).

resulting from the reaction of electron-rich aromatic rings of tyrosine, tryptophan, or histidine residues could be detected by mass spectrometric methods.[14] Moreover, no uncyclized triazene intermediates (**6**) were observed.

We next examined various reaction parameters including substrate concentration and reaction time (see Supporting Information for more details). A prolonged reaction time of 2 h led to a slight increase in the conversion to modified products with up to nine linker molecules attached to myoglobin. When 5 equiv of **13** relative to protein were used, the conversion slightly decreased (Table 1, entry 1). Using a more dilute

Table 1. Protein Bioconjugation Using Diazonium Reagent 13[a]

entry	protein[b]	unmod. (%)[c]	+1 (%)	+2 (%)	+3 (%)	+4 (%)	+5 (%)	+6 (%)
1[d]	100 μM **A**	16	29	26	17	8	3	0
2[d,e]	10 μM **A**	0	6	12	18	21	18	14
3	100 μM **B**	6	20	39	29	7	0	0
4[e]	100 μM **C**	4	10	12	22	21	13	8
5	100 μM **D**	0	12	21	27	21	13	6
6[e]	100 μM **E**	0	4	13	21	25	19	12

[a]Reagents and conditions: protein, diazonium reagent **13** (1 mM), pH 7.0 buffer (100 mM NaH$_2$PO$_4$), 23 °C, 2 h. [b]**A**: myoglobin; **B**: lysozyme; **C**: cytochrome c; **D**: ribonuclease a; **E**: α-chymotrypsinogen. [c]Determined through the relative ratio of peak intensities in the ESI-MS spectra of the product mixture. unmod. = unmodified; +1 = singly modified protein, etc. [d]Only 500 μM of **13** employed. [e]Minor amounts of +7 and +8 conjugates were also observed.

solution of protein substrate (10 μM) full conversion to modified products could still be achieved using 500 μM of **13** (entry 2). We next tested various other protein substrates for the reaction with the arene diazonium **13**. As outlined in Table 1, lysozyme (**B**), cytochrome c (**C**), ribonuclease a (**D**), and α-chymotrypsinogen (**E**) were all modified with excellent efficiency (entries 3−6).

Having an efficient bioconjugation protocol in hand, we next turned to evaluating its compatibility with a range of conjugation partners (Figure 3). We ultimately opted for the direct attachment of highly functionalized components to proteins. In particular, bioactive natural products would represent interesting targets for the conjugation to proteins, thereby creating novel molecules with potentially interesting bioactivities.[15,16] Accordingly, various linkers incorporating secondary metabolites of different substance classes were prepared starting from either amine **10** or acid **11**.[17] Conjugates containing carboxylic acid derivatives such as pantothenic acid (**18**), biotin (**19**), the terpenoid plant hormones abscisic acid (**20**) or gibberellic acid (**21**), and the steroid cholic acid (**22**) were synthesized in a single step from the natural products by peptide coupling to amine **10**. Moreover, the antibiotic lincomycin was coupled to **11**, furnishing **23**. Finally, we synthesized deacetoxy colchicine conjugate **24**.

As presented in Table 2, the aniline substrates depicted in Figure 3 were subjected to the standard diazotization conditions (NaNO$_2$/p-TsOH) and the resulting aryl diazonium tosylates were reacted with myoglobin as the coupling partner. Notably, in all cases excellent conversion to modified products was detected. In particular, various unprotected functional groups were well tolerated under the reaction conditions including alcohols (entries 1, 3−6), tertiary amines (entry 6), thioethers (entries 2 and 6), and sugar derivatives (entry 6).
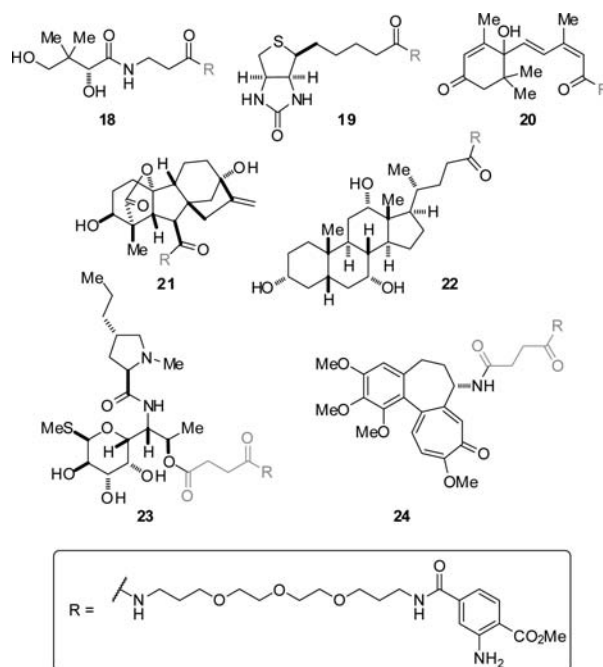

Figure 3. Natural product derivatives tested in the bioconjugation of myoglobin.

Table 2. Diazonium Bioconjugation of Myoglobin (100 μM) Using Different Natural Product Incorporating Aniline Substrates[a]

entry	substrate	unmod (%)[b]	+1 (%)	+2 (%)	+3 (%)	+4 (%)	+5 (%)	+6 (%)
1[c]	1 mM **18**	5	11	20	23	19	13	6
2[c]	1 mM **19**	7	18	23	22	16	9	4
3[c]	1 mM **20**	2	9	16	21	22	14	8
4[c]	1 mM **21**	2	8	16	20	20	13	11
5	2 mM **22**	20	28	27	19	5	1	0
6	2 mM **23**	7	18	24	23	15	9	4
7[c]	1 mM **24**	21	21	21	15	12	7	3

[a]Reagents and conditions: myoglobin (100 μM), diazonium reagent (1 mM), pH 7.0 buffer (100 mM NaH$_2$PO$_4$), 23 °C, 2 h. [b]Determined through the relative ratio of peak intensities in the ESI-MS spectra of the product mixture. unmod. = unmodified; +1 = singly modified protein, etc. [c]Minor amounts of +7 and +8 conjugates were also detected.

Additionally, electron-rich and -deficient olefins were compatible with the reagent system (entries 3 and 4), and even the highly electron-rich aromatic ring of colchicine derivative **24** was not affected (entry 7). In all cases conjugates resulting from the selective coupling of the protein's amino groups were the only products observed. As before, no side reactions involving other functional groups could be detected by MS-analysis.

In summary, we have developed an efficient bioconjugation protocol relying on the coupling of o-ester substituted diazonium salts with amino groups on proteins and peptides. The design of the reagent relies on the initial capture of amines by an electrophilic diazonium salt followed by a locking mechanism to form stable benzotriazinone adducts. The reaction proceeds smoothly at physiological pH with short reaction times. Highly functionalized substrates could be attached to proteins using small concentrations of both coupling partners. Moreover, the reported protocol is

characterized by preparative ease and is free of transition metal additives. The reagent system offers further opportunities for fine-tuning and enhancing its reactivity through substitution of the aryl ring in **25**.[18] Investigations toward this end are currently underway in our laboratory and will be reported in due course.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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(11) **13** can be employed in concentrations of 50 mM or more in a 100 mM phosphate buffer.

(12) Myoglobin harbors a total of 19 lysine residues, all of which are surface exposed.

(13) When the reaction was allowed to run for 2 h a slightly better degree of protein modification could be observed (see Supporting Information for detail).

(14) Such byproducts would be characterized by a mass difference of 32 Da with respect to the desired ring closed product resulting from amine-selective reaction.

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