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Direct Detection of Nitrotyrosine-Containing Proteins Using an Aniline-Based Oxidative Coupling Strategy†

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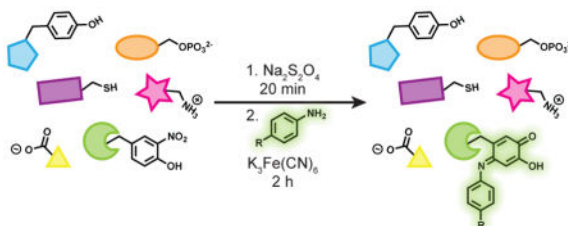
Abstract

A convenient two-step method is described for the detection of nitrotyrosine-containing proteins. First, nitrotyrosines are reduced to aminophenols using sodium dithionite. Following this, an oxidative coupling reaction is used to attach anilines bearing fluorescence reporters or affinity probes. Features of this approach include fast reaction times, pmol-level sensitivity, and excellent chemoselectivity.

Graphical Abstract



Tyrosine nitration is a covalent post-translational protein modification associated with oxidative stress.[1–3] The formation of this group typically involves peroxynitrite generated by the reaction between superoxide anions (O_2^-) and nitric oxide (NO).[4] Other reactive nitrogen species, such as NO_2^+, NO_2Cl, and NO_2, are also suggested as possible sources of tyrosine nitration in living cells.[5,6] This modification is of current interest due to its potential to modulate changes in protein function and turn over.[7] Recently, it was shown that 3-nitrotyrosine (Y-NO_2) has been implicated in multiple disorders, such as Parkinson's disease, Alzheimer's disease, and over 50 others.[7]

The continued study of this posttranslational modification creates a need for reliable analytical methods that can be used to study protein nitration, and, indeed, several

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approaches have been developed for this purpose.[8] These include Western blotting with anti-Y-NO_2 antibodies,[9] direct analysis using mass spectrometry,[10] and chemical detection methods based on biotinylation,[11] resin capture,[12,13] and fluorophore formation.[14–16]. In the latter cases,[11–16] the Y-NO_2 groups are reduced to aminophenols (Y-NH_2) with dithionite[17] prior to probe attachment. In our lab, aminophenols have been central to a series of chemoselective oxidative coupling (OC) strategies,[18,19] which have involved the facile addition of anilines to iminoquinone intermediates resulting from oxidation with periodate[18,20] or ferricyanide, Figure 1, step 2.[21] A closely related strategy has been reported for Y-NO_2 detection by the Schöneich group,[15,16] in which benzylamine derivatives are coupled to the same intermediate. Their strategy leads to the formation of a new fluorophore and the simultaneous attachment of a diol handle for use in boron-based affinity chromatography. However, these nucleophiles require pH 9 for the addition to occur, and are in competition with other amines in solution and on the protein itself. This can lead to multiple products and potential crosslinking that can complicate sample analysis.

Based on our experience with the OC strategy for the preparation of complex bioconjugates,[21] we hypothesized that suitably-functionalized anilines would offer higher coupling yields at lower pH values, and the formation of single products rather than mixtures. The reaction exhibits very high rates and chemoselectivities at pH 6.0 to 6.5, and is compatible with glycoproteins,[22] nucleic acids,[20] and complex molecular structures.[19] However, the chemoselectivity of the aniline-based OC strategy has not been tested for use in protein mixtures, and the concentration limits of the reaction have not yet been determined. In this report, we show that this aniline-based OC method is sufficiently selective to label proteins with Y-NH_2 groups in crude lysates with no additional protecting group strategies, leading to pmol-level detection limits.

The overall strategy for Y-NO_2 detection involved the development of the two-step protocol outlined in Figure 1. In the first step, Y-NO_2 groups are reduced to Y-NH_2 in the presence of sodium dithionite. This is then followed by an OC with a suitably functionalized aniline probe: either a fluoresceinamine (**2a**) or a biotinylated aniline (**2b**). In much of our previous work, sodium periodate was used as an oxidant for the OC reaction due to its high coupling rates. However, ferricyanide was used for this application because (1) it forms a single reaction product, while periodate generates a mixture of two products, and (2) it does not oxidize thiols, 1,2-diols, tryptophans, N-termini, or other functionalities on protein surfaces.[21] For the aniline component, fluoresceinamine was selected due to the convenience of its commercial availability. In addition, the corresponding biotin aniline (**2b**) was synthesized in one step (SI, synthetic procedures). This substrate can be detected using Western blotting techniques, and it can also be used to enrich samples for proteomic analysis. To verify the anticipated products of the OC reaction, the coupling reactions were performed using a small molecule Y-NH_2 mimic (2-amino-*p*-cresol, **1**). After 7 min, both of the aniline probes cleanly coupled to **1** in the presence of ferricyanide, as determined by ESI-TOF MS (Figure 2).

After confirming the reactivity patterns of the two aniline probes, their ability to label nitrated protein substrates was investigated. First, Y-NO_2 was incorporated site-selectively into the protein monomers of MS2 viral capsids (MS2-T19(Y-NO_2)) using amber stop codon

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suppression.[20,23–25] Interestingly, ESI-TOF MS analysis of the initially obtained protein indicated that some Y-NO_2 groups had been reduced during expression (Figure 3b,iv). To convert the remaining Y-NO_2 groups to Y-NH_2 moieties, the sample was treated with 10 mM sodium dithionite[17] for 20 min and then purified by ultrafiltration. ESI-TOF MS analysis revealed complete reduction at this point (Figure 3b, v).

The specificity of the OC was then evaluated by comparing wild-type (wt) MS2 coat proteins with those bearing Y-NH_2 groups. Following exposure of the samples to **2a** or **2b** and ferricyanide at room temperature for 2 h, the reaction mixtures were analyzed using ESI-TOF MS (Figure 3). Coupling products were obtained only for the Y-NH_2 cases, with comparable results being obtained for both anilines (Figure 3b, vi, vii). No reactivity was observed for wt MS2 with either aniline (Figure 3b, ii, iii).

As a second, non-site-selective incorporation method, tyrosines in other proteins (bovine serum albumin (BSA), chymotrypsinogen, and lysozyme) were nitrated with tetranitromethane and then reduced with dithionite equivalently (SI Figures S1–S2 for mass spectra and details). Samples of unmodified protein, Y-NO_2 containing proteins, purified Y-NH_2 containing proteins, and Y-NH_2 containing proteins that still contained the dithionite reductant were exposed to both aniline probes (**2a** and **2b**) in the presence of ferricyanide. The reaction mixtures were then purified using ultrafiltration to remove the excess, unreacted anilines. The reaction products were analyzed by SDS-PAGE, and the labeling was detected by fluorescence imaging or immunoblotting, as appropriate (Figure 4). Only the Y-NH_2 modified proteins were successfully labeled with the anilines, with no significant differences being observed between the purified and unpurified samples. These results demonstrate the chemoselectivity of this two-step process. They also indicate that the dithionite reductant need not be removed from the samples before performing the OC step so long as excess ferricyanide is used.

Although the OC reactions were found to be specific to Y-NH_2 containing proteins, the initial reactions were run using single-component samples and relatively high concentrations of protein (10 μM). In biological samples, Y-NO_2 containing proteins are only observed at nanomolar concentrations,[26] and always in the presence of many other species. In order to adapt the method for use in cell lysates, the reaction conditions were optimized to maximize detection sensitivity (SI Figures S3–S4 for details). In these experiments, Y-NO_2 modified chymo-trypsinogen (0.8 μM) was added to 20 μg of centrifuged Ramos cell lysate, and the mixture was subsequently reacted with excess $Na_2S_2O_4$ (0.25–4 mM, 1.25–20 equiv relative to **2a**). Next, 25–1000 μM of aniline **2a** (31–500 equiv relative to Y-NO_2 protein) was added in the presence of excess ferricyanide (5–80 mM, 25–400 equiv relative to **2a**). The effects of the dithionite reduction and OC reaction times were also explored. To quench the reactions, the samples were precipitated two times by adding a $CHCl_3$-MeOH mixture.[27]

It was found that the conversion was highest using 2 mM $Na_2S_2O_4$ for 20 min, followed by the reaction with 200 μM **2a** and 80 mM $K_3Fe(CN)_6$ for 2 h. Increasing the reduction time beyond 30 min was not beneficial. It is possible that with extended reduction times sodium dithionite decomposition products add to the Y-NH_2 group and prevent the desired reaction with the aniline probe. Using more than 250 equiv of **2a** resulted in higher levels of

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background labeling. We hypothesized that this was largely due to non-specific associations between the dye and the proteins. To address this issue, several methods of purification were tested, including ultrafiltration by spin concentration, protein precipitation, and a combination of the two. The use of ultrafiltration purification helped minimize the background signal (especially for high molecular weight proteins, SI Figures S6–S7).

To explore the compatibility with other fluorophores, additional aniline dyes based on Oregon Green 488 and TAMRA were synthesized. Both of these anilines reacted successfully with Y-NO_2 modified proteins, but they exhibited nonspecific background signals in the analysis of cell lysates even in the absence of ferricyanide. Nonetheless, the simple synthesis of the fluorescent-aniline probes suggests that this method could be generalized to accommodate many different detection strategies. Altogether, the fluoresceinamine probe was chosen for further studies, and the ultrafiltration purification step was utilized before gel analysis. Using these conditions, the amount of Ramos lysate could be increased to 72 μg for a 100 μL reaction volume while still maintaining Y-NO_2 selectivity.

To investigate the sensitivity of the detection strategy in complex cellular mixtures, known concentrations of Y-NO_2 modified proteins were added to Ramos cell lysates. The two-step procedure was then performed in the same reaction tube using the optimized conditions described above. The excess small molecules were removed by ultrafiltration after the OC step, and the reaction mixtures were analyzed by SDS-PAGE. For fluorescence detection with **2a**, 5–16 pmol of Y-NO_2 modified protein could be detected, depending on the target (Figure 5 and SI Figure S5). The limit for unambiguous detection for biotinylated aniline **2b** was established at 20 pmol after visualization with an anti-biotin-HRP Western blotting probe. The specificity for Y-NO_2 modified proteins was further verified by evaluating lysate samples that contained native versions of the protein targets. Only the Y-NO_2 versions of the proteins were detected by fluorescence and anti-biotin antibodies. For comparison purposes, an anti-Y-NO_2 antibody was used with a HRP-secondary antibody detection probe. This system showed visible bands as low as 2 pmol (Figure 5). Although the direct antibody detection method can achieve lower detection limits than probe **2a**, the OC labelling method still offers advantages of fewer steps, lower costs, and significantly shorter times. The ability to attach biotin probes also opens avenues for proteomic studies and immunohistochemical staining.

In summary, we have demonstrated the use of a fast aniline-based OC bioconjugation reaction for the detection of nitrotyrosine-containing proteins in cell lysates. The tandem reaction sequence occurs in a single sample vessel in less than 2.5 h, and is likely to be compatible with a variety of different anilines. Fluoresceinamine used in this study is commercially available, and the easily-synthesized biotin tagged substrate offers both detection and the potential for enrichment of tagged proteins. This OC methodology therefore provides a valuable advance for monitoring changes in tyrosine nitration due to oxidative stress, and will likely facilitate future studies of the biological role of this posttranslational modification.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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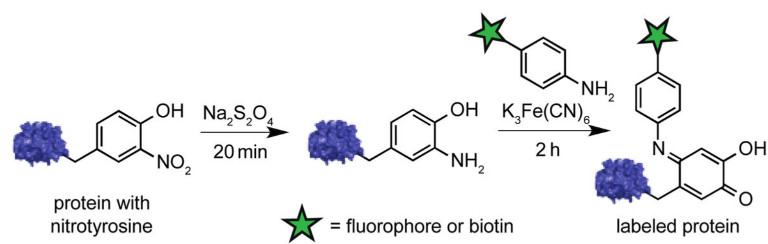

Fig. 1.

Detection of nitrotyrosine-containing proteins using a two-step protocol.

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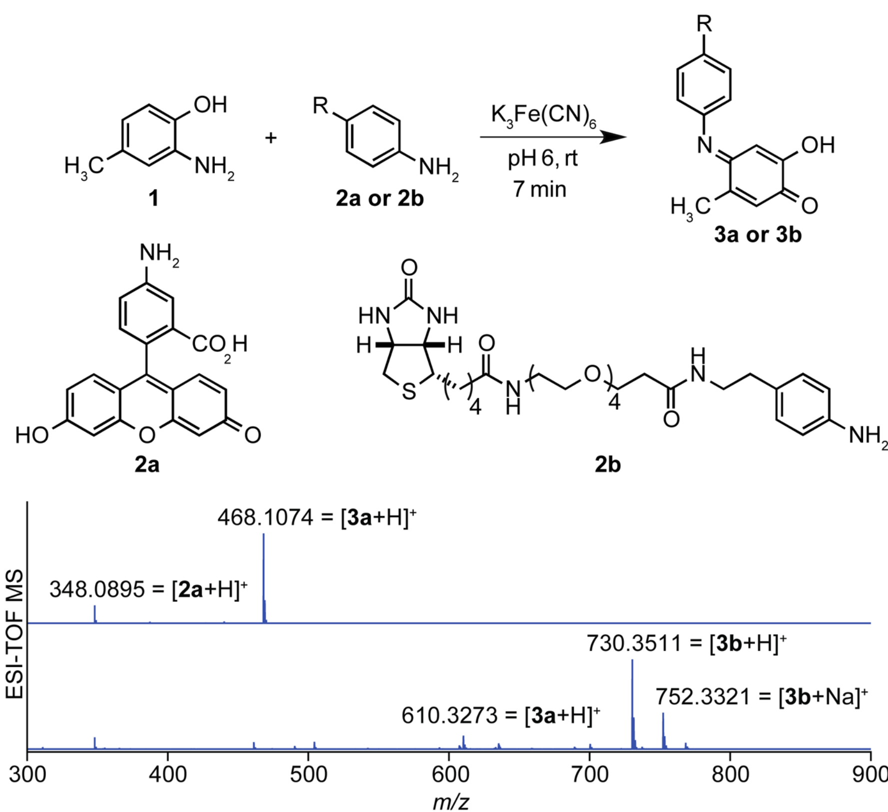

Fig. 2.

Oxidative coupling reactions between *o*-aminophenols and functionalized anilines. The ESI-TOF MS analysis of the reactions of *o*-aminophenol **1** with fluoresceinamine (**2a**) or biotin aniline (**2b**) shows clean conversion to the respective oxidative coupling products (expected $[M+H]^+$ m/z = 468.1078 for **3a** and 730.3480 for **3b**). The reactions were run with 200 μM functionalized anilines, 3 μM **1**, and 80 mM ferricyanide in 100 mM phosphate buffer, pH 6.

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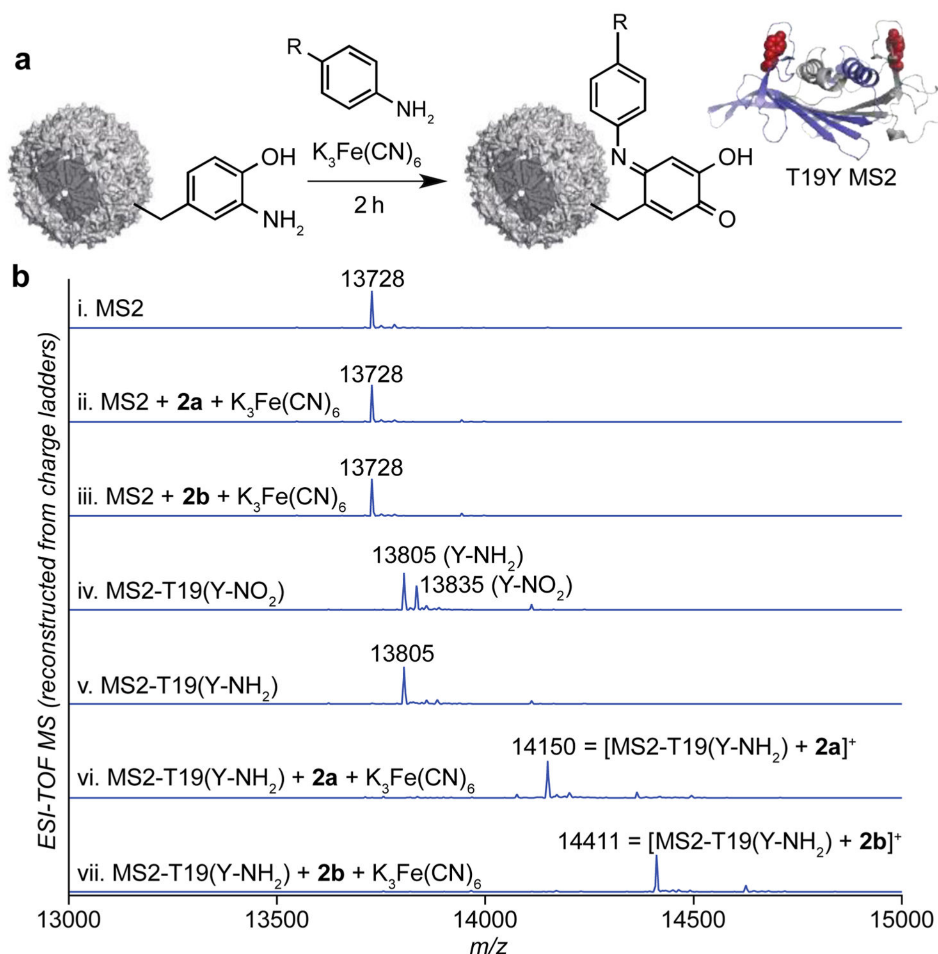

Fig. 3.

Chemoselectivity tests for the oxidative coupling reaction. (a) Two mutants of the MS2 coat protein were used: the wild-type (T19) and a variant containing an aminotyrosine side chain (T19(Y-NH_2), shown). (b) ESI-TOF MS analyses are shown for the MS2 proteins before and after oxidative coupling with functionalized anilines (**2a** and **2b**). Y-NO_2 = nitrotyrosine, Y-NH_2 = aminotyrosine. The reactions were run with 200 μM **2**, 10 μM protein (based on monomer), and 80 mM ferricyanide in 100 mM phosphate, pH 6.

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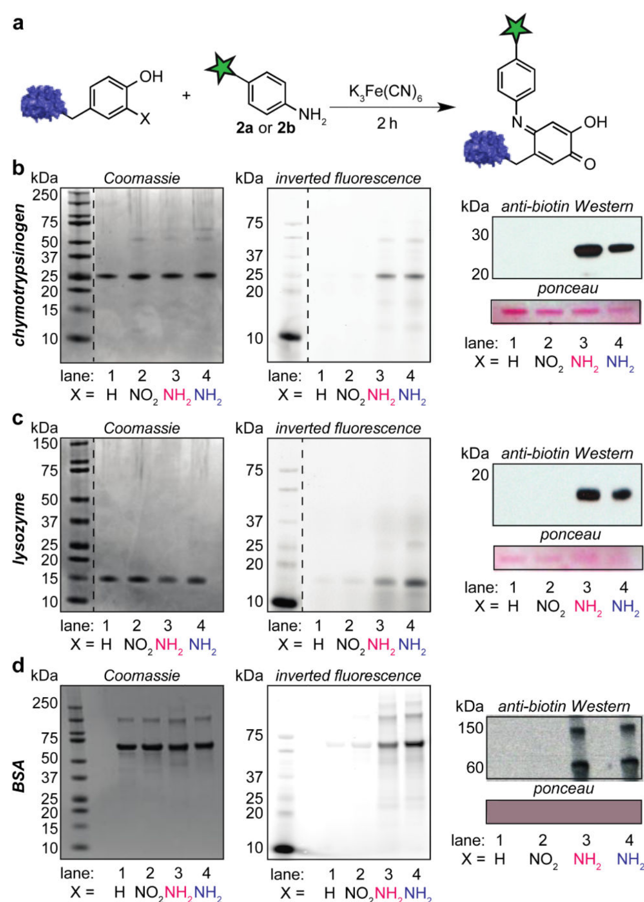

Fig. 4.

Testing the specificity of the oxidative coupling reaction for *o*-aminophenol residues on additional proteins. (a) Reactions were run with 200 μM **2a** or **2b**, 10 μM protein, and 80 mM ferricyanide in 100 mM phosphate buffer, pH 6, for 2 h. (b–d) Following SDS-PAGE, samples labeled with **2a** were visualized using fluorescence imaging (shown as an inverted image) and Coomassie stain. Samples labeled with **2b** were visualized through Western blotting with anti-biotin-HRP probes. The **X** labels refer to the tyrosine substituents, as defined in the structure in (a). NH_2 groups in pink indicate protein samples that were purified after dithionite reduction. NH_2 groups in blue indicate samples that were not purified between the steps. The vertical dashed lines indicate where two portions of the same gel were juxtaposed.

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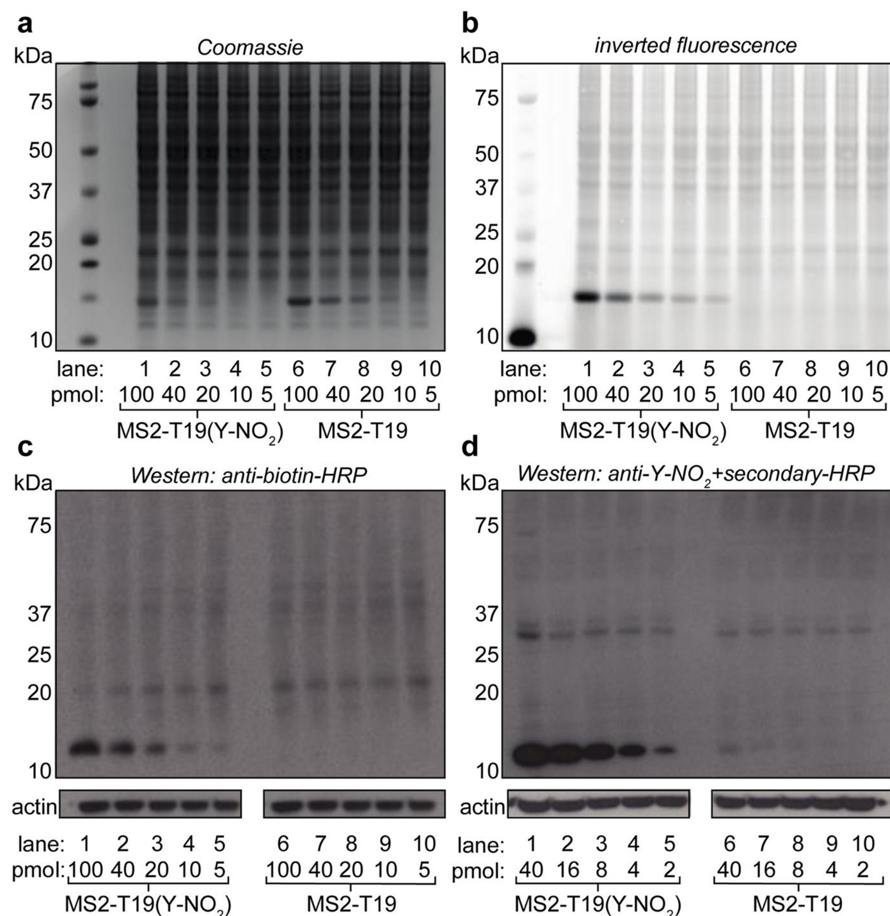

Fig. 5.

Detection of MS2-T19(Y-NO_2) in cell lysates (24 μg Ramos lysate loaded per lane). Portions of MS2-T19(Y-NO_2) were added to 72 μg of cell lysate in 100 μL of phosphate buffer, pH 6. The samples were reduced with 2 mM sodium dithionite for 20 min, and then treated with 200 μM **2a** and 80 mM ferricyanide for 2 h. After ultrafiltration, the reactions were analzed by SDS-PAGE with (a) Coomassie staining and (b) fluorescence detection (inverted image shown). (c) The same procedure was used with **2b**, and the samples were visualized via Western blotting with an anti-biotin-HRP probe. (d) For comparison pusposes, MS2-T19Y-NO_2 was also added to lysates and visualized via Western blotting with anti-nitrotyrosine and secondary antibody-HRP.