

# Protein Conjugation with Triazolinediones: Switching from a General Tyrosine-Selective Labeling Method to a Highly Specific Tryptophan Bioconjugation Strategy

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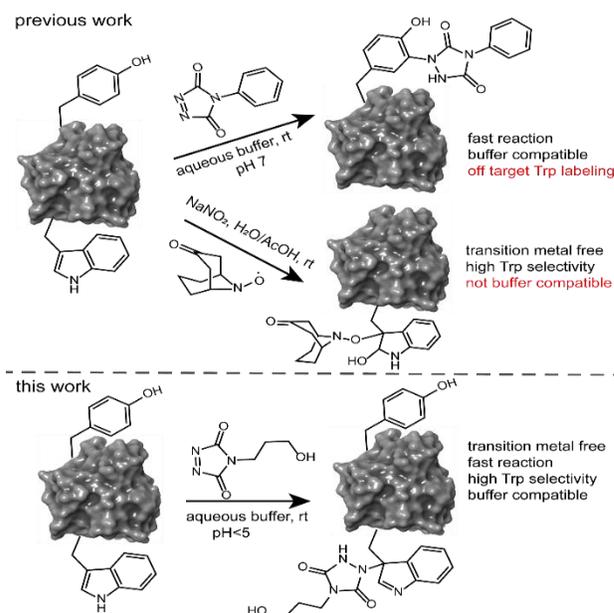
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**ABSTRACT:** Selective labeling of tyrosine residues in peptides and proteins can be achieved via a 'tyrosine-click' reaction with triazolinedione reagents (TAD). We have found that tryptophan residues are in fact often also labeled with this reagent. This off-target labeling is only observed at very low levels in protein bioconjugation but remains under the radar due to the low relative abundance of tryptophan compared to tyrosines in natural proteins, and because of the low availability and accessibility of their nucleophilic positions at the solvent-exposed protein surface. Moreover, because TAD-Trp adducts are known to be readily thermoreversible, it can be challenging to detect these physiologically stable but thermally labile modifications using several MS/MS techniques. We have found that fully solvent-exposed tryptophan side chains are kinetically favored over tyrosines under almost all conditions, and this selectivity can even be further enhanced by modifying the pH of the aqueous buffer to effect selective Trp-labeling. This new site-selective bioconjugation method does not rely on unnatural amino acids and has been demonstrated for peptides and for recombinant proteins. Thus, the TAD-Tyr click reaction can be turned into a highly site-specific labeling method for tryptophans.

Site-selective protein modification is of utmost importance for many applications from fundamental biology (fluorescent tagging) to therapeutic development (antibody-drug conjugates).<sup>1,2,3,4</sup> While amino acid selectivity can be achieved by exploiting the nucleophilic functionalities of e.g. lysines and cysteines,<sup>5,6</sup> genuine site selectivity depends on their representation density on the protein surface. In this regard, tryptophan (Trp) is an interesting target for native conjugation strategies, with an abundance of just over 1% in proteins.<sup>7</sup> Despite the indole side chain not being the most chemically tractable target, several groups have reported methodologies for selective modification of tryptophan in peptides and proteins.<sup>8,9,10,11</sup> Many of these strategies employ transition metal catalyzed reactions and/or conditions limiting downstream biochemical applications. These reactions are typically alkynylations and C-H arylations of the indole.<sup>12,13,14,15,16</sup> Also, Trp sulfonylation was demonstrated for peptide ligation.<sup>17</sup> While Francis and coworkers showed rhodium carbenoid-based Trp labeling at mild pH,<sup>18</sup> this method is dependent on transition metal catalysis and requires long reaction times. An organoradical Trp conjugation was demonstrated on peptides and proteins<sup>19</sup> and even if the method is devoid of transition metals, it requires acidic conditions and is not compatible with buffers. Very recently, a novel biomimetic approach for the selective conjugation of tryptophan was developed, this method however employs UV irradiation and needs to be performed in absence of oxygen.<sup>20</sup>



Scheme 1. Prototype reactions for the TAD-Y click, organo-radical tryptophan modification (previous work) and TAD tryptophan labeling (this work).

In 2010, Barbas and co-workers reported a click like reaction for the more abundant tyrosine (Tyr, 3.3% abundance<sup>7</sup>) using triazolinedione chemistry,<sup>21</sup> and several ap-

lications for protein conjugation followed.<sup>22,23,24,25,26</sup> Interestingly, when exploring this powerful Tyr click reaction on Trp-containing peptides, we observed a high degree of off-target labeling on Trp residues, even in aqueous buffers. While the swift reaction of indoles with triazolinediones was reported by Baran, Guerrero and Corey in 2003,<sup>27,28</sup> Barbas and co-workers demonstrated that tyrosine labeling is kinetically favored in buffers. However, we now surmise that this competitive Trp-labeling in protein bioconjugation remained under the radar, likely due to a combination of the low abundance and low solvent accessibility of Trp residues. Moreover, in line with observations of Baran and coworkers, we found that indole-TAD modifications have limited thermal stability and can reverse under MS/MS conditions rendering their detection more tedious. We thus decided to more closely examine the competition between Trp and Tyr labeling by TADs in order to probe the potential of TAD reagents for selective Trp-bio-conjugation (scheme 1). For that purpose, tetrapeptides NWA **1a** and NYAS **1b** were tested in intermolecular competition experiments with phenyltriazolinedione (PTAD **2a**) in PBS-buffer at two different pH values, allowing for head to head comparison between Tyr and Trp side chains embedded in the exact same chemical environment (figure 1). Signals for peptide conjugates **2aa** and **2ba** overlap on the HPLC UV chromatogram, therefore extracted ion chromatograms (XIC's) were used for the analysis.

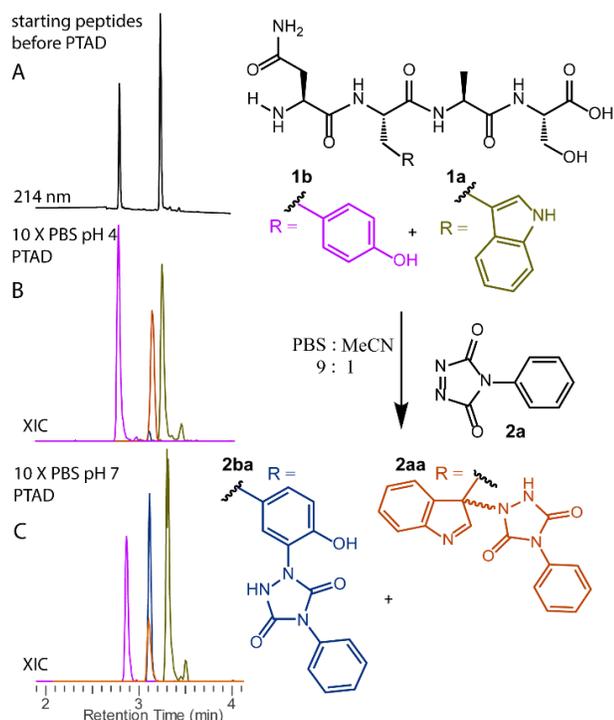


Figure 1. Competition experiment between NWA (**1a**, 0.3 mM) and NYAS (**1b**, 0.3 mM) peptides for PTAD (**2a**, 0.4 mM) in 10x PBS buffers at pH 4 and pH 7. HPLC chromatogram before reaction (A). Extracted ion chromatograms (XIC) of both starting peptide ions **1a** (green) and **1b** (pink) and conjugated peptide ions **2aa** (orange) and **2ba** (blue) for reaction in 10x PBS at pH 4 (B) or pH 7 (C).

When analyzing the XIC's of the starting peptide-ions NWA **1a** (green) and NYAS **1b** (pink) and conjugated peptide-ions NWA-PTAD **2aa** (orange) and NYAS-PTAD **2ba** (blue), a pronounced difference can be observed between the reaction at pH 4 and pH 7. Indeed, at pH 4 Trp conjugate **2aa** was detected nearly exclusively while at pH 7 a mixture of conjugates was obtained. This observed pH-dependent reactivity of TADs with Tyr is in accord with previous mechanistic studies of the tyrosine-TAD click reaction, which indicate the phenolate species as the prevalent nucleophile.<sup>29</sup> Lowering the pH will effectively decrease the amount of tyrosine-phenolate form and thus decrease the extent of reaction of Tyr with TAD. This was further confirmed using additional peptides (**1a-1h**, table 1) and TAD-propanol **2b**, PTAD-alkyne **2c** and fluorescent DMEQ-TAD **2d** (Section S2.2.2). It was also observed that, even without competing Trp-peptide present, lowering of pH causes a significant reduction in Tyr-conjugate formation (Section S2.2.1). These findings at peptide level prompted us to look in more detail to earlier reports on the tyrosine click protein modification. Indeed, off-target Trp-labeling was observed earlier at protein level. In the initial study of Ban *et al.* modification on tryptophan was observed upon myoglobin labeling albeit in a very low amount.<sup>21,25</sup> Furthermore, careful reinterpretation of the MALDI-TOF MS spectra (kindly provided by the authors) of Vandewalle *et al.*,<sup>23</sup> who labeled BSA with butyl-TAD, showed that Trp-modification was indeed noticeable (Section S3.1). These findings demonstrate that researchers can incorrectly assume that tryptophan will not react with TAD-reagents in protein conjugation reactions, possibly leading to flawed interpretation of data.

**Table 1. Peptide sequences used in this study, structures of TAD reagents **2b**, **2c** and **2d**.**

| Entry     | Sequence   | Structure |
|-----------|--|-----------|
| <b>1a</b> | Asn-Trp-Ala-Ser-OH   |           |
| <b>1b</b> | Asn-Tyr-Ala-Ser-OH   |           |
| <b>1c</b> | Asn-Ser-Ala-Trp-OH   |           |
| <b>1d</b> | Asn-Ser-Ala-Tyr-OH   |           |
| <b>1e</b> | Trp-Ser-Ala-Asn-OH   |           |
| <b>1f</b> | Tyr-Ser-Ala-Asn-OH   |           |
| <b>1g</b> | Lys-Lys-Ser-Tyr-Leu-Ser-Pro-Arg-Thr-Ala-Leu-Ile-Asn-Phe-Leu-Val-OH |           |
| <b>1h</b> | Lys-Lys-Ser-Trp-Leu-Ser-Pro-Arg-Thr-Ala-Leu-Ile-Asn-Phe-Leu-Val-OH |           |
| <b>1i</b> | Val-Trp-Ser-Asn-Arg-His-Phe-Tyr-OH                                 |           |
| <b>1j</b> | Val-Tyr-Ser-Asn-Arg-His-Phe-Trp-OH                                 |           |
| <b>1k</b> | Val-Trp-Ser-Gln-Lys-Arg-His-Phe-Gly-Tyr-OH                         |           |
| <b>1l</b> | Lys-Asp-Tyr-Trp-Glu-Cys-Ala-OH                                     |           |

Triggered by these findings, indicating that a completely Trp-selective modification can be possible, we examined TAD-Trp conjugation in peptides by investigating the influence of the relative amino acid positioning on the outcome of the reaction. Competition experiments between tetrapeptides NWA **1a** and NWA **1c** and TAD-propanol **2b** showed a remarkable difference in reactivity (figure 2).

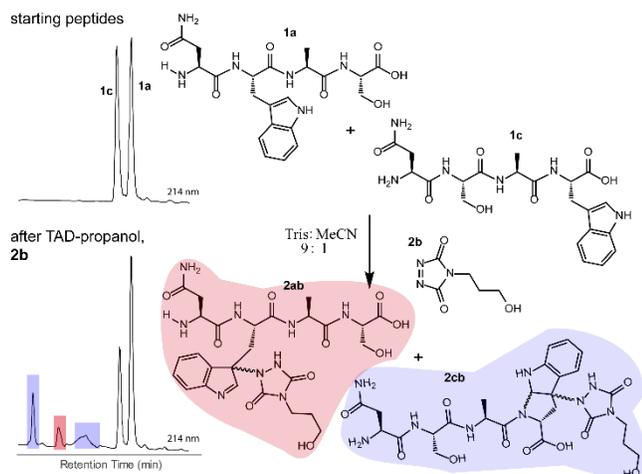


Figure 2. Competition experiment of NWAS (**1a**, 0.3 mM) and NSAW (**1c**, 0.3 mM) peptides with TAD-propanol (**2b**, 0.5 mM) in Tris buffer pH 5.8. Zoom of the HPLC chromatogram at 214 nm of the starting mixture (top left) and after reaction with TAD-propanol **2b** (bottom left).

Intermolecular competition between **1a** and **1c** clearly demonstrates the position-sensitivity of the Trp-TAD reaction: the C-terminal tryptophan in **1c** is labeled to a 3 times higher extent, as calculated via HPLC peak integration at 214 nm, compared to its internal tryptophan **1a** counterpart. This reactivity difference can be attributed to the more exposed reactive center as well as to the presence of the carboxylic acid which can transiently donate a proton to the TAD moiety rendering it even more electrophilic. A second striking difference resides in the nature of the formed adducts. For the C-terminal tryptophan, two peaks for the labeled product **2cb** are observed, indicating the formation of isomers. Indeed, we found this adduct had undergone an additional annulation caused by the reaction of the lone pair on the backbone nitrogen with the indole C2 after reaction of TAD with the indole C3. These findings were confirmed via NMR analysis of Boc-Trp-OH and N-Ac-Trp-OMe adducts with TAD-propanol **2b** (Section S4) and are in agreement with the results reported by Baran *et al.*<sup>27</sup> on non-peptide related TAD-indole reactions.

In a subsequent series of experiments, we investigated if the observed intermolecular selectivity, translates into intramolecular Trp versus Tyr selectivity. To this end, competition experiments were performed with peptides containing both tyrosine and tryptophan (**1i-1l**, table 1). MS/MS analyses were done to determine the modification site. We found that the modification on tryptophan is unstable in all tested MS/MS conditions except for ESI in combination with electron transfer dissociation (ETD). ESI-HCD, ESI-CID as well as MALDI-TOF/TOF all largely lead to the loss of the TAD modification on tryptophan. The TAD modification on tyrosine was found to be stable in all tested conditions. These findings are in agreement with earlier work on the thermoreversibility of indole-TAD reactions.<sup>30</sup>

Peptide VWSQKRHFGY **1k** was labeled using TAD-propanol **2b** at pH 4 to selectively target the tryptophan residue in the peptide, or at pH 7 to mainly target the tyrosine. The conjugated peptide will have the same mass regardless of the site of the modification ( $M^+$ : 1464.71), the triply charged precursor ( $M^{+++}$ : 488.91) was selected for electron transfer

dissociation (ETD) MS/MS analysis. At pH 4 modification only takes place on tryptophan and Trp-modified fragment ions are observed (figure 3, top). If the modification reaction is performed at pH 7 the major product is the tyrosine modified peptide (figure 3 bottom). However upon detailed analysis of the full ion chromatograms (SI section 2.4.1) it can be seen that in contrast to Trp-labeling which occurs cleanly at pH 4, the Tyr-labeling of this peptide at pH 7 is accompanied by formation of Trp-modified side products. Further confirmation for the off-target labeling at pH 7 is provided through MS based screening for doubly modified peptides (SI, Figure 2.4.2.3) showing double TAD modified peptides only at pH 7, implying additional off-target Trp labeling next to the intended Tyr labeling.

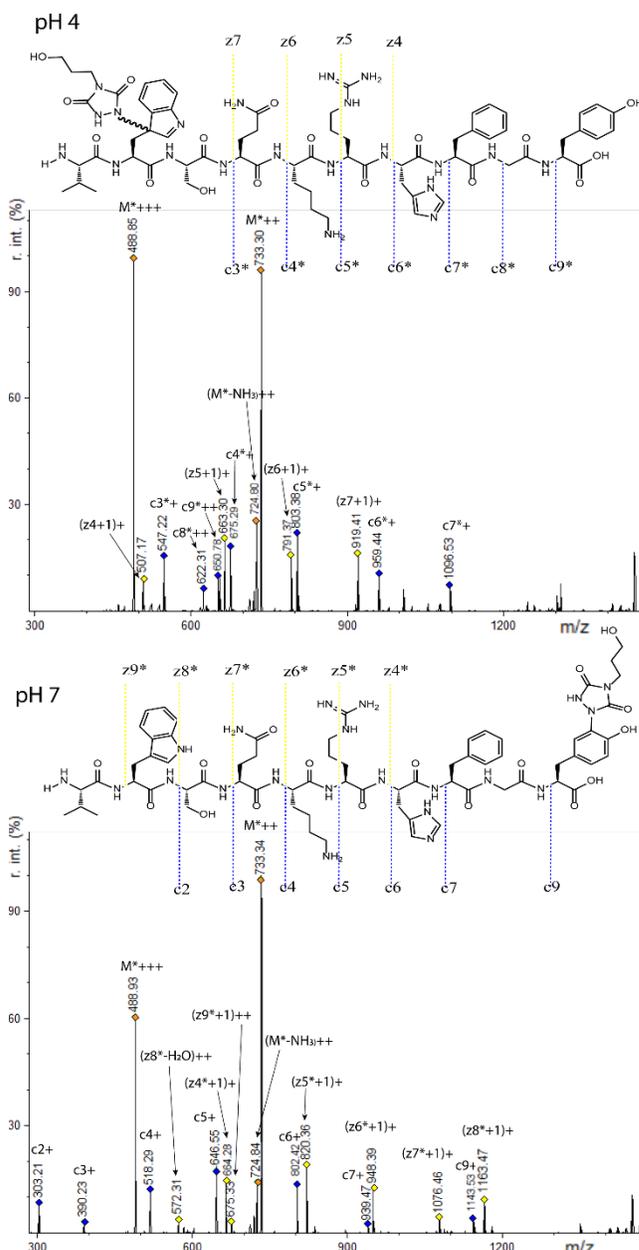


Figure 3. ESI ETD MS/MS of VWSQKRHFGY **1k** after modification with TAD-propanol **2b** in 10 X PBS pH 4 (top) and pH 7 (bottom). In both MS/MS spectra the chemical structure is depicted with the observed fragment ions. Fragment ions

corresponding to TAD-modified peptide fragments are indicated with “\*”.

We next explored TAD-click reactions for Trp-based protein conjugation. Alfabodies have a triple helical coiled coil structure and are developed for intracellular protein interaction targets by Complix N.V.<sup>31</sup> Two recombinant alfabodies were used; the valentine alfabody containing no tryptophans and three tyrosines, and alfabody 586D containing one tryptophan residue next to three tyrosines. A competition experiment between the valentine alfabody and tryptophan containing peptide **1c** (SI3.2) with **2b** at pH4, resulted in 80% conversion of **1c** while alfabody conjugation was absent. Protein conjugation with 586D at pH 4 was carried out using fluorescent DMEQ-TAD (**2d**). MS/MS analysis of the resulting protein conjugate digest (SI3.3) confirms the localization of the TAD modification on tryptophan. Intact protein analysis of the 586D **2d** conjugate (30 eq., pH 4) shows 60 % conjugation. Together, these results indicate that protein TAD modification can be targeted selectively to a Trp side chain, in the presence of tyrosines. As a negative control the valentine alfabody was reacted with 48 eq. of **2b** at pH 4 and no conjugation was observed.

Finally, human galectin-7,<sup>32</sup> containing one tryptophan and one tyrosine residue was treated with **2b**, **2c** and **2d** at pH 4. The conjugated proteins were observed for all TAD reagents. Intact protein analysis of the galectin-7 **2d** conjugate (10 eq., pH 4, SI3.4) shows over 50 % conjugation. Analyses of the galectin-7 TAD propanol conjugate digests confirm the localization of the TAD moiety on tryptophan. Furthermore the MS/MS analyses of conjugation experiments with **2b** (20 eq.; pH 4 and 7) demonstrate that at pH 4 the tryptophan has almost exclusively reacted with TAD while at pH 7 both the tyrosine and the tryptophan had reacted. Additionally, in accord with the findings on the peptide level we found the TAD modification on tryptophan to be labile under the HCD MS/MS conditions used in these experiments.

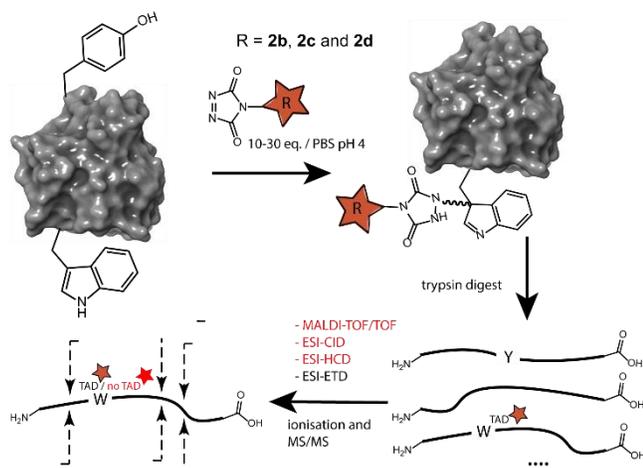


Figure 4. Schematic representation of protein conjugation reaction of alfabody 586D and galectin-7 with **2b**, **2c** and **2d**, subsequent trypsin digest followed by different ionization and MS/MS fragmentation of the modified tryptic fragment. Depending on the applied technique the TAD modification on W can be observed or not.

In conclusion, we report that competitive tryptophan labeling is liable to have so far been systematically overlooked in

the current use of triazolinedione (TAD) chemistry for putative tyrosine-selective protein conjugation, a technique which is growing in popularity. The reversibility of the TAD-tryptophan in MS/MS analysis, in combination with the low abundance and low accessibility of tryptophan side chains likely caused this off-target effect to have remained under the radar. We have found that an exposed tryptophan is in fact kinetically favored over tyrosine in most conditions. Lowering the buffer pH further enhanced the selectivity resulting in a transition metal free, buffer-compatible amino acid specific labeling method for the least abundant natural amino acid tryptophan. Thus, in addition to a better understanding of the factors that govern the click-like TAD-based protein conjugation, its scope has been expanded, and a very interesting new option for native amino acid selective modification has been revealed. The implementation of Trp-substitutions at protein surfaces or loops can thus be an interesting rational design strategy for fully site-selective labeling of native proteins.

## ASSOCIATED CONTENT

Supporting information: this material is available free of charge via the Internet.

- Supplementary methods and figures S1.2.1 – S4.2.1
- Synthesis and characterization of TAD derivatives, peptides and peptide conjugates.
- Synthesis and characterization of protein conjugates.
- NMR spectra

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### Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

## Notes

The authors declare no competing financial interests.

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