Abstract: A novel chemoenzymatic approach for simple and fast site-specific protein labeling is reported. Recombinant tubulin tyrosine ligase (TTL) was repurposed to attach various unnatural tyrosine derivatives as small bioorthogonal handles to proteins containing a short tubulin-derived recognition sequence (Tub-tag). This novel strategy enables a broad range of high-yielding and fast chemoselective C-terminal protein modifications on isolated proteins or in cell lysates for applications in biochemistry, cell biology, and beyond, as demonstrated by the site-specific labeling of nanobodies, GFP, and ubiquitin.

Site-specific functionalization of proteins is crucial for a plethora of applications throughout the life sciences. Fluorescent proteins and self-labeling strategies like SNAP- and HALO-tagging have become indispensable tools for cell biologists to analyze intracellular activity and localize proteins of interest. Genetic fusion with GFP or self-labeling protein tags is straightforward; however, the size and biochemical nature of the attachment may affect the properties and application of the chimeric protein.[3] Protein transsplicing, expressed protein ligation (EPL), and amber suppression and auxotrophic expression in combination with bioorthogonal labeling are prominent tools that allow the placement of small tags and modifications within the protein sequence.[4] However, low expression yields of genetic encoding systems[5] and challenges associated with impaired protein folding with trans-splicing and EPL[6] can be limiting factors when applying these techniques. As an alternative, chemoenzymatic approaches have attracted increasing attention for the site-specific modification of proteins by using short and specific recognition tags. A variety of enzymes, such as trypsin,[7] Sortase A,[8] phosphopantetheinyl transferase (PPTase),[9] biotin ligase,[10] lipoic acid ligase,[11] AnkX,[12] and formylglycine generating enzyme,[13] open up broad possibilities for subsequent chemoselective and site-specific labeling. However, several chemoenzymatic approaches still need to overcome some limitations, including large and hydrophobic substrates for the enzymatic reaction that may affect the protein of interest.[14] Moreover, the reversibility of some enzymatic reactions and product hydrolysis necessitates extensive enzyme engineering and a high excess of catalyst and substrate.[7,14,15]

Herein, we present a novel and fast method for the site-specific labeling of functional proteins that combines the use of small unnatural amino acids as bioorthogonal handles and the technical advantages of chemoenzymatic labeling. The technique, termed Tub-tag labeling, is based on the enzymatic ligation of easy-to-synthesize small tyrosine derivatives to the C terminus of a fourteen amino acid hydrophilic recognition tag (termed Tub-tag) by tubulin tyrosine ligase (TTL, Figure 1a). In nature, TTL catalyzes the post-translational attachment of tyrosine to the C terminus of α-tubulin, which is involved in the regulation of microtubule homeostasis.[16] Interestingly, it has been shown that TTL also utilizes tyrosine derivatives for the C-terminal modification of tubulin.[17]

We started our investigation by probing whether recombinant TTL can conjugate unnatural tyrosine derivatives to the isolated Tub-tag peptide, which mimics the C terminus of tubulin. For initial Tub-tag labeling experiments, we used 3-N-tyrosine (1) and 3-formyl-L-tyrosine (2) as substrates and synthesized a 5,6-carboxylfluorescein-labeled Tub-tag peptide (CF-Tub-tag) by standard solid-phase peptide synthesis (SPPS). The reaction process was analyzed by isocratic HPLC (Figure 1b for 1, Figure 1c for 2, and Figure S1 in the Supporting Information). After 120 min of incubation (TTL/peptide 1:200) at 37°C, we observed a conversion of 90% and 63% with 1 and 2, respectively. The initial reaction rate of azide 1 surpasses that of aldehyde 2, resulting in an approximately 2.6 times faster formation of the ligation product. However, extending the reaction in the case of aldehyde 2 delivers equal amounts of labeled peptide, thus demonstrating the broad substrate acceptance of tubulin tyrosine ligase.

Encouraged by these results, we tested whether Tub-tag labeling can be applied to the site-specific modification of proteins and whether the required C-terminal recognition motif influences the protein function. At the outset of our studies, we focused on camel-derived single-domain nano-

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Versatile and Efficient Site-Specific Protein Functionalization by Tubulin Tyrosine Ligase

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bodies, which are used as analytical tools in biochemistry as well as for the intracellular recognition and manipulation of antigens in cell biology. In this context, it was our particular goal to access a site-specifically fluorescently-labeled nanobody, since these approximately 15 kDa antigen-recognizing proteins display superior properties in superresolution microscopy owing to their exceptionally small size.

We fused the Tub-tag sequence to the C terminus of a GFP-specific nanobody (GBP4) and performed TTL-mediated labeling with a TTL/GBP4 ratio of 1:5 at 37°C for 24 h. Tryptic digest followed by HPLC–MS/MS experiments showed the successful C-terminal addition of tyrosine, 3-NH$_2$-tyrosine (1), 3-formyl-L-tyrosine (2), 3-NO$_2$-L-tyrosine (3), and 3-NO$_2$-L-tyrosine (4; Figure S3 in the Supporting Information). Next, we combined the incorporation of I with subsequent strain-promoted azide–alkyne cycloaddition (SPAAC) for the conjugation of a dibenzocyclooctyne (DBCO)-biotin derivative (Figure 2a). For this, ligation reactions were performed using different ratios of TTL/GBP4 and the reactions stopped by cooling down fast to 4°C after one or three hours. A subsequent dialysis step was used to remove excess tyrosine derivative 1, followed by SPAAC to DBCO-biotin. The yields were determined by SDS-PAGE analysis. Using a TTL/GBP4 ratio of 1:5 at 37°C, we found that 82% of the GBP4 was converted after one hour, whereas a TTL/GBP4 ratio of 1:10 delivered 71% C-terminally modified GBP4. Extending the ligation time to three hours resulted in 99% and 88% conversions at TTL/GBP4 ratios of 1:5 and 1:10, respectively. The incorporation of 3-formyl-L-tyrosine (2) could be achieved with similar efficiency. To further validate the modularity of the Tub-tag labeling concept, we performed fluorescence labeling by SPAAC (Figure S7 in the Supporting Information) and employed a variety of well-established bioorthogonal reactions, including Staudinger ligation (Figure S8 in the Supporting Information) and the Staudinger-phosphite reaction (Figure S9 in the Supporting Information), to 3-NH$_2$-L-tyrosine. In addition, hydrazide- (Figure S10 in the Supporting Information) and oxime- (Figure S11 in the Supporting Information) forming reactions were applied on site-specifically incorporated 3-formyl-L-tyrosine (2). This allowed us to incorporate different functional modules like biotin and fluorophores, and even enabled the branched PEGylation of GBP4 (Figure S9 in the Supporting Information). To demonstrate the broad applicability of this chemoenzymatic modification, we additionally applied it to the site-specific functionalization of GFP, ubiquitin, and another GFP-specific nanobody (GBP1). SDS-PAGE analysis showed similar yields to the modification of GBP4 (Figure S12 and S13 in the Supporting Information). Moreover, the presence of the Tub-tag sequence did not affect the fluorescence and fluorescence-modifying properties of GBP or GFP-specific nanobodies (Figures S4, S5 in the Supporting Information).

Having established the modular and high-yielding TTL-based modification method, we investigated the selectivity of the tyrosine transfer reaction within complex protein mixtures. *E. coli* cell lysate containing overexpressed GFP-Tub-tag was incubated with 3-NH$_2$-L-tyrosine (1) and TTL for four hours. A subsequent dialysis was used to remove excess azide, and SPAAC to DBCO-biotin showed selective C-terminal functionalization of GFP (Figure 3a, b and Figure S14 in the Supporting Information). Next, we probed the specificity and applicability of the site-specifically modified nanobody GBP1 for the enrichment and isolation of overexpressed GFP from
cell lysate. Following 3-N$_2$-tyrosine (1) incorporation via TTL, GBP1 was biotinylated with DBCO-biotin as described above and immobilized on Streptavidin-coated magnetic beads (Figure 3c). These beads were then used for the immunoprecipitation of GFP from HEK cell lysates. Subsequent western blot analysis demonstrated specific GFP
pulldown compared to controls with mock-transfected cell lysate and non-functionalized beads (Figure 3d).

We subsequently studied whether site-specifically TTL-labeled GBP1 can be used to immunostain cellular structures for superresolution microscopy. The higher resolution of superresolution microscopy imposes new requirements on detection reagents, and using the smallest possible immunofluorescent binding reagents is thus important for unleashing the full potential of superresolution microscopy.[206]

Following 3-formyl-l-tyrosine (2) incorporation via TTL, GBP1 was labeled with the Alexa594 dye by using oxime formation (Figure 3e). In addition to the microscopy application, it was shown that both the azide and the aldehyde tyrosine derivatives could be used to obtain functional protein conjugates. HeLa cells expressing GFP-LaminB1, which localizes at the interior of the nuclear envelope and forms the nuclear lamina, were fixed and stained with GBP1-Alexa594 (ca. 1 µg per object slide). 3D-SIM superresolution microscopy revealed laminar colocalization of the GBP1 staining reagent and the GFP/LaminB1 at high resolution, thereby indicating functional binding to GFP in this cellular context (Figure 3f–h). Similar results were obtained with GFP-PCNA and the detection of subnuclear DNA replication sites (Figure S15 in the Supporting Information).

These studies demonstrate the applicability of Tub-tag-labeled nanobodies for functional studies in biochemistry and cell biology.

In summary, we introduce Tub-tag labeling for the simple, site-specific modification of functional and antigen-recognizing proteins. The TTL-mediated chemoenzymatic ligation of unnatural tyrosine derivatives like 3-N$_2$-l-tyrosine (1) and 3-formyl-l-tyrosine (2) enabled bioorthogonal functionalization in a total yield of up to 99% when using moderate enzyme concentrations and short reaction times. These modified tyrosine residues then serve as bioorthogonal handles for a variety of well-established chemoselective labeling reactions. The overall labeling efficiency under mild reaction conditions yields homogeneously modified and functional proteins, as demonstrated by the site-specific labeling of nanobodies for immunoprecipitation and super-resolution microscopy. In summary, Tub-tag labeling endows recombinant antibodies—and proteins in general—with novel properties for exploring and manipulating cellular functions, with possible applications in biotechnology and medicine.

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**Keywords:** antibodies · chemoenzymatic labeling · nanobodies · site-specific functionalization · tubulin tyrosine ligase

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Versatile and Efficient Site-Specific Protein Functionalization by Tubulin Tyrosine Ligase

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1. SUPPLEMENTARY RESULTS

1.1. Fluorescence-HPLC analysis of the C-terminal addition of 1 and 2

Figure S1. C-terminal addition of (a) 3-\(N_3\)-L-tyrosine (1) and (b) 3-formyl-L-tyrosine (2) to carboxyfluorescein labeled peptide (CF–Tub-tag) catalysed by TTL. HPLC-fluorescence traces were taken at different time points of the TTL reaction.
1.2. Dependency of the initial velocity towards substrate concentration of 3-N$_3$-L-tyrosine

Figure S2. TTL displays Michaelis-Menten kinetics for the ligation of N$_3$-L-tyrosine to carboxyfluorescein labeled peptide. The $v_0$ values were determined using fluorescence HPLC analysis. Substrate and product were quantitated by peak integration. The mean value and standard deviation (SD) of three replicate reactions is shown. One should note that due to limited solubility of the tyrosine derivative, substrate saturation couldn’t be reached. Using this data, $V_{max}$ and $K_m$ have been determined by Michaelis-Menten fit (Prism) to be 0.232 µM • s$^{-1}$ ± 0.007 and 2222 µM, respectively.
1.3. ESI-MS/MS spectra

Figure S3. ESI-MS/MS spectra of C-terminal modified nanobodies. Different tyrosine derivatives have been added to the C-terminus of the nanobody GBP4 using Tub-tag labeling. Tryptic digest followed by HPLC-MS/MS experiments revealed successful incorporation of (a) L-tyrosine, (b) 3-N3-L-tyrosine, (c) 3-formyl-L-tyrosine, (d) 3-NO2-L-tyrosine and (e) 3-NH2-L-tyrosine. The N-terminal b-ions and the C-terminal y-ions confirm the sequence of the C-terminally modified tryptic peptides. In particular, the mass of the modified y4 ions (b-e) in combination with the unmodified b15-, b22- ions prove the presence of the tyrosine modification at the very C-terminus.
1.4. GBP1–Tub-tag and GBP1 induced modulation of GFP spectral properties

Figure S4. GBP1 induced modulation of GFP spectral properties. (a) GFP was incubated with equimolar amounts of GBP1 and GBP1–Tub-tag as previously described.\(^1\) Similar increase in GFP fluorescence indicates comparable GFP-binding capacities of GBP1 and GBP1–Tub-tag. The mean value and standard deviation (SD) of three replicate reactions is shown. (b) GFP emission spectra upon titration of GBP1–Tub-tag from 0.0–0.5 µM on 0.5 µM purified GFP.

1.5. Fluorescence spectra of GFP and GFP–Tub-tag

Figure S5. Fluorescence spectra of GFP and GFP–Tub-tag. Similar intensities show conserved protein functionality upon C-terminal addition of the Tub-tag sequence.
1.6. Labeling reagents

Figure S6. Molecules used for bioorthogonal addition to C-terminal modified nanobodies
1.7. SPAAC labeling of GBP4-N\textsubscript{3} with sulfo-Cy5-DBCO

Figure S7. SPAAC labeling of GBP4-N\textsubscript{3} with sulfo-Cy5-DBCO. Shown is the fluorescent labeling of GBP4 with sulfo-Cy5-DBCO. 3-N\textsubscript{3}-L-tyrosine was enzymatically incorporated to the C-terminus of GBP4 using TTL. A following incubation with 30 eq. sulfo-Cy5-DBCO shows selective labeling of 3-N\textsubscript{3}-L-tyrosine containing nanobody by strain promoted azide-alkyne click reaction (SPAAC).
1.8. Staudinger-Ligation with biotin-phosphine

Figure S8. Staudinger-Ligation with biotin-phosphine. Shown is the labeling of GBP4 with biotin-phosphine using Staudinger-Ligation. 3-N$_3$-L-tyrosine was enzymatically incorporated to the C-terminus of GBP4 using TTL. A following incubation with 40 eq. Biotin-phosphine shows selective labeling of 3-N$_3$-L-tyrosine containing nanobody by Staudinger-Ligation.
1.9. Staudinger-Phosphite reaction with tris(PEG750)phosphite S1

Figure S9. Staudinger-Phosphite reaction with tris(PEG750)phosphite S1. Shown is the PEGylation of GBP4 with tris(PEG750)phosphite (S1) by Staudinger-Phosphite reaction. 3-N$_3$-L-tyrosine was enzymatically incorporated to the C-terminus of GBP4 using TTL. A following incubation with 40 eq. phosphite shows selective labeling of 3-N$_3$-L-tyrosine containing nanobody by Staudinger Ligation.
1.10. Fluorescent labeling of GBP4 by hydrazone forming reaction

Figure S10. Fluorescent labeling of GBP4 by hydrazone forming reaction. Shown is the labeling of GBP4 with Alexa594-hydrazide using hydrazone forming reaction. 3-formyl-L-tyrosine was enzymatically incorporated to the C-terminus of GBP4 using TTL. A following incubation with 30 eq. Alexa594-hydrazide shows selective labeling of 3-formyl-L-tyrosine containing nanobody by aldehyde condensation.
1.11. Biotin labeling of GBP4 by oxime forming reaction.

Figure S11. Biotin labeling of GBP4 by oxime forming reaction. Shown is the labeling of GBP4 with biotin S2 using oxime forming reaction. 3-formyl-L-tyrosine was enzymatically incorporated to the C-terminus of GBP4 using TTL. A following incubation with 30 eq. S2 shows selective labeling of 3-formyl-L-tyrosine containing nanobody by aldehyde condensation.
1.12. Site-specific functionalization of GBP1

Figure S12. Site-specific functionalization of GBP1. Shown is the site-specific labeling of the GFP specific nanobody GBP1 using Tub-tag labeling. (a) 3-N$_2$-L-tyrosine was enzymatically incorporated to the C-terminus of GBP1 using TTL. A following incubation with 30 eq. sulfo-Cy5-DBCO or 30 eq. biotin-DBCO shows selective labeling of 3-N$_2$-L-tyrosine containing nanobody by strain promoted azide-alkyne click reaction (SPAAC). (b) 3-formyl-L-tyrosine was enzymatically incorporated to the C-terminus of GBP4 using TTL. A following incubation with 30 eq. Alexa594-hydrazone shows selective labeling of 3-formyl-L-tyrosine containing nanobody by aldehyde condensation.
1.13. Site-specific functionalization of GFP and ubiquitin

Figure S13. Site-specific functionalization of GFP and Ubiquitin. (a) 3-N<sub>3</sub>-L-tyrosine was enzymatically incorporated to the C-terminus of GFP using TTL. A following incubation with 30 eq. biotin-DBCO shows selective labeling of 3-N<sub>3</sub>-L-tyrosine containing GFP by strain promoted azide-alkyne click reaction (SPAAC). (b) 3-N<sub>3</sub>-L-tyrosine was enzymatically incorporated to the C-terminus of Ubiquitin (Ub) using TTL. A following incubation with 30 eq. biotin-DBCO shows selective labeling of 3-N<sub>3</sub>-L-tyrosine containing Ub by strain promoted azide-alkyne click reaction (SPAAC).
1.14. Selective labeling of GFP within *E. coli* Cell lysate

Figure S14. Tub-tag labeling of GFP in complex protein mixtures (E.coli lysate). Coomassie staining and western blot analysis show high selectivity of the tyrosine ligation and subsequent biotinylation. 3-N$_3$-L-tyrosine was enzymatically incorporated to the C-terminus of GFP using TTL. A following incubation with 3 eq. biotin-DBCO shows selective labeling of 3-N$_3$-L-tyrosine containing GFP by strain promoted azide-alkyne click reaction (SPAAC).

1.15. Confocal microscopy

Figure S15. Confocal micrographs showing co-localization of GBP1–Tub-tag–Alexa594 and GFP-PCNA fusions. Confocal micrographs of HeLa cells transfected with GFP-PCNA fusions. Cells were labeled with anti-GFP (GBP1) conjugated to the fluorescent dye Alexa594 at 1:25. The DAPI, GFP and Alexa594 channels, as well as the overlay are shown. Scale bar: 5 µm.
2. Material and Methods

2.1. General Information

Analytical HPLC was conducted on a SHIMADZU HPLC system (Shimadzu Corp., Japan) with a SIL-20A autosampler, 2 pumps LC2 AAT, a 2489 UV/Visible detector, a CTO-20A column oven and an RF-10 A X2 fluorescence detector using an Agilent Eclipse C18 5 µm, 250 x 4.6 mm RP-HPLC-column with a flow rate of 0.5 mL/min. The following gradient was used: Method A: (A = H2O + 0.1% TFA, B = MeCN + 0.1% TFA) 35% B, 0-15 min, 10-100% B 15-17 min, 100% B 17-22 min, 100-35% B 22-25 min and 35% B 25-30 min. UV chromatograms were recorded at 220 nm and fluorescence spectra with Ex/Em 495/517 were recorded.

Analytical UPLC: UPLC-UV traces were obtained on a Waters H-class instrument equipped with a Quaternary solvent manager, a Waters autosampler and a Waters TUV detector connected to a 3100 mass detector with an Acuity UPLC-PEH C18 1.7 µm, 2.1 x 50 mm RP column with a flow rate of 0.6 mL/min (Waters Corp., USA). The following gradient was used: Method B: (A = H2O + 0.1% TFA, B = MeCN + 0.1% TFA) 5-95% B 0-3 min, 95% B 3-5 min. UPLC-UV chromatograms were recorded at 220 nm.

Preparative HPLC was performed on a Gilson PLC 2020 system (Gilson Inc., WI, Middleton, USA) using a Macherey-Nagel Nucleodur C18 HTec Spum column (Macherey-Nagel GmbH & Co. Kg, Germany). The following gradient was used: Method C: (A = H2O + 0.1% TFA, B = MeCN + 0.1% TFA) flow rate 32 mL/min, 10% B 0-5 min, 10-100% B 5-35 min, 100% B 35-40 min. Method D: (A = H2O + 0.1% TFA, B = MeCN + 0.1% TFA) 10% B 0-5 min, 10-100% B 5-50 min, 100% B 50-55 min.

Analytical HPLC-MSMS: Peptides were analyzed by a Ultimate 3000 nanoLC system (Thermo Scientific, USA) connected to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, USA). LC separations were performed on a capillary column (Acclaim PepMap100, C18, 3 µm, 100 Å, Thermo Scientific, USA) at an eluent flow rate of 30 nL/min. The following gradient was used: Method D: (A = H2O + 0.1% formic acid, B = MeCN + 0.1% formic acid) 3-50% B 0-50 min Mass spectra were acquired in a data-dependent mode with one MS survey scan with a resolution of 30,000 (LTQ Orbitrap XL) or 60,000 (Orbitrap Elite) and MS/MS scans of the five most intense precursor ions in the linear trap quadrupole, respectively.

Column chromatography was performed on silica gel (Acros Silica gel 60 Å, 0.035-0.070 mm).

High-resolution mass spectra (HRMS) were measured on an Acquity UPLC system and a LCT Premier™ (Waters Corp., USA) time-of-flight mass spectrometer with electrospray ionization using water and acetonitrile (10-90% gradient) with 0.1% formic acid as eluent.

NMR spectra were recorded with a Bruker Ultrashield 300 MHz spectrometer (Bruker Corp., USA) at ambient temperature. The chemical shifts are reported in ppm relative to the residual solvent peak. Product yields were calculated based on 1H-NMR spectra. TFA salt content was determined by 19F-NMR, tetrafluoroethylene as standard and considered in product yield calculation.

GFP fluorescence spectra were recorded with a Jasco FP-6500 spectrometer (Jasco Research Ltd., Canada) using an excitation wavelength of 488 nm.

Reagents and solvents were, unless stated otherwise, commercially available as reagent grade and did not require further purification. Resins and Fmoc-protected amino acids were purchased from IRIS BioTEch (Germany) or Novabiochem (Germany).

SPPS was either carried out manually or with an Activo-P11 automated peptide synthesizer (Activotec, UK) via standard Fmoc-based conditions (Fast-moc protocol with HOOb/HBTU conditions).

2.2. Experimental Procedures

2.2.1. TTL expression and purification

The TTL (Canis lupus) coding sequence was amplified from a mammalian expression vector[2], cloned into a pET28-SUMO3 (EMBL-Heidelberg, Protein Expression Facility) and expressed in E. coli BL21(DE3) as Sumo-TTL fusion protein with an N-terminal His-Tag. Cells were induced with 0.5 mM IPTG and incubated at 18 °C for 18 h. Lysis was performed in presence of Lysozyme (100 µg/mL), DNAse (25 µg/mL) and PMSF (2 mM) followed by sonification (Branson™ Sonifier; 16 x 8sec, 20% amplitude) and debris centrifugation at 20.000 g for 30 min. His-Sumo-TTL was purified using a 5 mL His-Trap. Purified protein was then desalted on a PD10 column (GE Healthcare); buffer was exchanged to MES/K pH 6.8 (20 mM MES, 100 mM KCl, 10 mM MgCl2). Protein aliquots were shock-frozen and stored at -80 °C at 2.7 g/l.
2.2.2. Determination of TTL activity using CF–Tub-tag

Tyrosination reactions were performed in a 250 μL solution consisting of 20 mM MES/K pH 7.0, 100 mM KCl, 10 mM MgCl₂, 2.5 mM ATP, 1 mM tyrosine derivative, 0.2 mM CF–Tub-tag, 1 μM TTL and 5 mM DTT in case of compound 2 or 5 mM reduced glutathione in case of compound 1, respectively. The mixture was incubated at 37 °C and several aliquots (25 μL) were taken, mixed with equal volumes of H₂O + 0.1% TFA and subjected to isocratic analytical HPLC equipped with a fluorescence detector (Method: A = H₂O + 0.1% TFA, B = MeCN + 0.1% TFA; 35% B, 0-15 min, 10-100% B 15-17 min, 100% B 17-22 min, 100-35% B 22-25 min and 35% B 25-30 min.). For v₅ determination of TTL, reactions were performed using different concentrations of 1 (0.5 – 8 μM) and aliquots taken after 3, 6, 9 and 12 minutes. Quantities of substrate and product peptides were estimated from the corresponding peak-area in the fluorescence detection spectrum (Ex/Em: 495/517).

2.2.3. Nanobody–Tub-tag expression and purification

Nanobody–Tub-tag fusion expression constructs (pHen6 bacterial expression vector) were generated by standard molecular biology techniques resulting in nanobodies with an N-terminal 6xHis tag and a C-terminal alpha-Tubulin derived VDSVEGEGEEEGEE peptide (Tub-tag). Proteins were expressed in E. coli (JM109). Cells were induced with 0.5 mM IPTG and incubated at 18 °C for 18 h. Lysis was performed in presence of Lysozyme (100 μg/mL), DNAse (25 μg/mL) and PMSF (2 mM) followed by sonication (Branson® Sonifier; 16 x 8 sec, 20% Amplitude) and debris centrifugation at 20.000 g for 30 min. The protein was purified with an Äkta FPLC system using a 5 mL His-Trap (GE Healthcare, USA) column, peak fractions were concentrated to 2 mL using Amicon filter columns (cut-off 3 kDa; (Merck Millipore, Germany) and subjected to size exclusion chromatography using a Superdex 75 column (GE Healthcare, USA). Peak fractions were pooled and protein aliquots were shock-frozen and stored at -80 °C.

2.2.4. GFP–Tub-tag expression and purification

GFP–Tub-tag fusion expression constructs (pET28(c) bacterial expression vector) were generated by standard molecular biology techniques resulting in nanobodies with an N-terminal 6xHis tag and a C-terminal alpha-Tubulin derived VDSVEGEGEEEGEE peptide (Tub-tag). Proteins were expressed in E. coli BL21(DE3). Cells were induced with 0.5 mM IPTG and incubated at 18 °C for 18 h. Lysis was performed using a high pressure homogenizer (Microfluidics LM10 Microfluidizer) and debris centrifugation at 20.000 g for 30 min. The protein was purified with an Äkta FPLC system using a 5 mL His-Trap (GE Healthcare, USA) column, peak fractions were concentrated to 2 mL using Amicon filter columns (cut-off 10 kDa; (Merck Millipore, Germany) and subjected to size exclusion chromatography using a Superdex 75 column (GE Healthcare, USA). Peak fractions were pooled and protein aliquots were shock-frozen and stored at -80 °C.

2.2.5. Ubiquitin–Tub-tag expression and purification

Ubiquitin–Tub-tag fusion expression constructs (pET28(c) bacterial expression vector) were generated by standard molecular biology techniques resulting in nanobodies with an N-terminal 6xHis tag and a C-terminal alpha-Tubulin derived VDSVEGEGEEEGEE peptide (Tub-tag). Proteins were expressed in E. coli (JM109). Cells were induced with 0.5 mM IPTG and incubated at 18 °C for 18 h. Lysis was performed using a high pressure homogenizer (Microfluidics LM10 Microfluidizer) and debris centrifugation at 20.000 g for 30 min. The protein was purified with an Äkta FPLC system using a 5 mL His-Trap (GE Healthcare, USA) column, peak fractions were concentrated to 2 mL using Amicon filter columns (cut-off 3 kDa; (Merck Millipore, Germany) and subjected to size exclusion chromatography using a Superdex 75 column (GE Healthcare, USA). Peak fractions were pooled and protein aliquots were shock-frozen and stored at -80 °C.

2.2.6. TTL reaction on GBP4 followed by tryptic digest and MSMS analysis

Tyrosination reactions were performed in a 50 μL solution consisting of 20 mM MES/K pH 7.0, 100 mM KCl, 10 mM MgCl₂, 2.5 mM ATP, 1 mM tyrosine derivative, 1 μM TTL, 5 μM nanobody and 5 mM reduced glutathione in case of compound 1 and 5 mM DTT in case of compound 2-4 and tyrosine, respectively. The mixture was incubated at 37 °C for 24 h. Proteins were separated by SDS-PAGE. Protein bands of interest were excised, soaked with 100 μL 50 mM (NH₄)₂CO₃/ACN 1:1 and incubated at 30 °C for 10 min. The supernatant was removed and the gel pieces were incubated in 50 mM (NH₄)₂CO₃ at 30 °C for further 10 min.
The two incubation steps were repeated until the pieces were colorless. Hereafter, the gel pieces were dehydrated by the addition of 25 µL ACN, the supernatant removed and the gels were dried under reduced pressure. In-gel digest was performed in a total volume of 20 µL 50 mM (NH₄)₂CO₃ at 37 °C for 12 h using 0.05 µg trypsin (Thermo Fisher Scientific, USA). 20 µL ACN + 0.5% TFA was added, the mixture incubated in an ultrasonic bath, the supernatant transferred to LC glass vials, the solvent removed under reduced pressure and the residual peptides resuspended in 6 µL 95% H₂O + 0.1% TFA, 5% ACN + 0.1% TFA solution. Peptides were separated by HPLC and analyzed by MSMS experiments.

2.2.7. Chemoenzymatic addition of 1 and 2 to nanobodies, GFP and ubiquitin

Tyrosination reactions were performed in a 150 µL solution consisting of 20 mM MES/K pH 7.0, 100 mM KCl, 10 mM MgCl₂, 2.5 mM ATP, 1 mM 1 or 2, 0.1 - 1 µM TTL, 5 µM nanobody/GFP/ubiquitin and 5 mM reduced glutathione in case of compound 1 or 5 mM DTT in case of compound 2, respectively. The mixture was incubated at 37 °C for 1-3 h.

2.2.8. SPAAC to sulfo-Cy5-DBCO or biotin-DBCO

Tyrosination reactions were performed as described above using compound 1, 5 mM reduced glutathione and a ratio of 10:1 GBP4/TTL for 3 h. The reaction mixtures were rebuffered to Dulbecco’s PBS pH 7.4 and incubated with 30 eq. of Sulfo-Cy5-DBCO (Jena Bioscience GmbH, Germany) or DBCO-PEG₄-biotin (Jena Bioscience GmbH, Germany) at 30 °C for 4 h. Proteins were separated by SDS-PAGE. Biotinylated nanobodies were wet blotted onto a nitrocellulose membrane using a Bio-Rad Mini-Protean Tetra System (250 mA, one hour). A streptavidin peroxidase conjugate (Merck Millipore, Germany) was used for detection. Fluorescently labeled nanobodies were visualized by a Fuji FLA-5000 laser imager (634 nm excitation, LPG-filter) (Fujifilm, Japan).

2.2.9. Staudinger-Ligation

Tyrosination reactions were performed as described above using compound 1, 5 mM reduced glutathione and a ratio of 10:1 GBP4/TTL for 3 h. The reaction mixtures were rebuffered to Dulbecco’s PBS pH 7.4 and incubated with 40 eq. of biotin-phosphine (S₃, BIOMOL GmbH, Germany) at 37 °C for 24 h. Proteins were separated by SDS-PAGE. Biotinylated nanobodies were wet blotted onto a nitrocellulose membrane using a Bio-Rad Mini-Protean Tetra System (250 mA, 1 h). A streptavidin peroxidase conjugate (Merck Millipore, Germany) was used for detection.

2.2.10. Staudinger-Phosphite reaction

Tyrosination reactions were performed as described above using compound 1, 5 mM reduced glutathione and a ratio of 10:1 GBP4/TTL for 3 h. The reaction mixtures were rebuffered to 50 mM Tris pH 8.5, 100 mM KCl and incubated with 40 eq. of tris(PEG750)phosphite (S₂) at 37 °C for 24 h. Proteins were separated by SDS-PAGE. PEGylated nanobodies were wet blotted onto a nitrocellulose membrane using a Bio-Rad Mini-Protean Tetra System (250 mA, 1 h). A monoclonal anti PEG-B-47 antibody (Abcam, UK) and a secondary Goat Anti-Rabbit IgG H&L (HRP) (Abcam, UK) were used for detection.

2.2.11. Hydrazone and oxime forming reactions

Tyrosination reactions were performed as described above with compound 2, 5 mM DTT and a ratio of 10:1 GBP4/TTL for 3 h. The reaction mixtures were rebuffered to 50 mM MES/K pH 6.0, 100 mM KCl and incubated with 30 eq of Alexa594-hydrazide (S₅, Thermo Fisher Scientific, USA) or hydroxylamine-biotin (S₆) at 37 °C for 4 h. Proteins were separated by SDS-PAGE. Biotinylated nanobodies were wet blotted onto a nitrocellulose membrane using a Bio-Rad Mini-Protean Tetra System (250 mA, 1 h). A streptavidin peroxidase conjugate (Merck Millipore, Germany) was used for detection. Fluorescently labeled nanobodies were visualized by a Fuji FLA-5000 laser imager (532 nm excitation, LPG-filter) (Fujifilm, Japan).

2.2.12. Chemoenzymatic addition of 1 to GFP in E. coli lysate.

Tyrosination reactions were performed in 250 µL lysate containing overexpressed GFP–Tub-tag, 2.5 mM ATP, 3 mM 1, 0.2 µM TTL, and 5 mM reduced glutathione. The mixture was incubated at 30 °C for 4 hours. Excess tyrosine was removed using dialysis and SPAAC to 3 eq. DBCO was performed at 30°C for 3 hours.
Proteins were separated by SDS-PAGE. Lysate samples were wet blotted onto a nitrocellulose membrane using a Bio-Rad Mini-Protean Tetra System (250 mA, one hour). A streptavidin peroxidase conjugate (Merck Millipore, Germany) was used for detection.

2.2.13. Streptavidin pulldown assay

HEK 293T cells were maintained in DMEM supplemented with 10% fetal calf serum and gentamycin at 50 µg/mL (PAA, Germany). For pulldown experiments 10⁷ cells were seeded in p100 dishes. Transfection with eGFP encoding plasmid DNA peGFP-C1 (Life Technologies, Germany) was performed with polyethylenimine (PEI, Sigma, USA) with 24 µg DNA per dish. Pulldown reagent was prepared by using biotinylated GBP1 (Tub-tag mediated). For this purpose, 200 µL slurry of Streptavidin-coated, magnetic beads (Dynabeads MyOne Streptavidin T1) were washed with PBS and then loaded with 40 µg biotinylated GBP1 according to the manufacturers’ instructions. Functionalized beads were equilibrated with IP buffer (0.5 mM EDTA, 50 mM Tris/Cl pH 7.0, 150 mM NaCl). Whole cell lysates were prepared using 200 µL lysis buffer (0.5 mM EDTA, 50 mM Tris/Cl pH 7.0, 150 mM NaCl, 1% NP40, 2 mM PMSF, 1x Mammalian Protease Inhibitor Cocktail. 5% of lysate supernatants were collected as input samples. The remaining sample was diluted to 1 mL using IP buffer and incubated with 50 µL bead slurry for 4 h. After magnetic pulldown, 5% supernatant were collected as flowthrough samples. For Coomassie staining and western blotting 2% input, 2% flowthrough and 20% bead fractions were boiled with Laemml buffer at 95 °C and subjected to SDS-PAGE and transfer to a nitrocellulose membrane (Bio-Rad, USA). A monoclonal anti-GFP antibody (Roche, Switzerland) and HRP-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch, USA) was used for detection.

2.2.14. Immunofluorescence for superresolution microscopy

HeLa-Kyoto cells were maintained in DMEM supplemented with 10% fetal calf serum and gentamycin at 50 µg/mL (PAA, Germany). For immunofluorescence experiments 10⁶ cells were seeded on gridded 18×18 mm coverslips in a 6-well plate. Transfection with LaminB1-GFP encoding plasmid DNA (kind gift from Jan Ellenberg) was performed with Lipofectamine 3000 (Life Technologies, USA) according to the manufacturers’ instructions. 18 h post transfection, cells were washed with PBST, fixed with 2% formaldehyde/PBS, and permeabilized with 0.5% Triton X-100/PBS. Next, cells were blocked with 2% BSA/PBST. Cells were then incubated with Alexa594 Tub-tag labeled GBP1 (stock solution 1 µg/µL; dilution 1:50) for 1 h, washed with PBST, DAPI stained (Life Technologies, USA) and mounted in Vectashield anti-fading reagent (Vector Laboratories, USA) on object slides. High-resolution microscopy was performed with an DeltaVision OMX v3 (Applied Precision, Slovakia) equipped with 405, 488 and 593 nm laser diodes, a 100×/1.4 NA Plan-Apochromat oil objective lens (Olympus, Japan) and Cascade II:512 EM CCD cameras (Photometrics, USA) as described previously[1]. Line scan fluorescence intensity analysis was performed with ImageJ.

2.2.15 Transfection for confocal microscopy

HeLa cells were seeded at sub-confluent concentration on glass coverslips (Carl Roth, Germany) the day before PEI transfection (Sigma-Aldrich, USA) with 2 µg pENeGFP-PCNA[4]. After transfection, cells were incubated for 18-24 h at 37 °C, 5% CO₂.

2.2.16. Immunofluorescence for confocal microscopy

Cells were fixed in 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 (neoLab Migge Laborbedarf-Vertriebs, Germany) for 10 min, and blocked in 2% bovine serum albumin (Sigma-Aldrich, UK) for 60 min. For GFP labeling, cells were incubated for 60 min with GBP1–Tub-tag–Alexa594 (1:25 or 1:50) prior to extensive washing and DNA counterstain with 1 µg/mL DAPI for 10 min. All steps except fixation were carried out in PBS supplemented with 0.02% Tween 20 (PBST, Carl Roth, 9127.1) at room temperature. Glass coverslips were then mounted with anti-fade Mowiol mounting medium (Sigma-Aldrich, UK).
2.2.17. Confocal Microscopy and Image Analysis

Imaging was carried out with a Leica SP5 II confocal point scanner (Leica Microsystems, Germany) equipped with two Hyd hybrid detectors. Image acquisition was performed with a ×60/1.4-0.6 NA Planapochromat oil immersion objective lens. To visualize DAPI, GFP and GBP1–Tub-tag–Alexa594, the 405, 488 and 561 nm excitation lasers were used, respectively. 16bit images were collected and analyzed with Fiji.

2.3. Chemical Synthesis

2.3.1. Synthesis of 3-nitro-L-tyrosine (4), 3-amino-L-tyrosine (3) and 3-azido-L-tyrosine (1)

![Scheme 1. Synthesis of tyrosine derivatives 1, 3 and 4.](image)

3-nitro-L-tyrosine (4)

L-tyrosine (2.00 g, 11 mmol) was added to 10 mL HOAc, the suspension cooled to 0 °C and HNO₃ (1.47 mL, 11 mmol, 7.5 N) was slowly added. After 4 h the reaction was diluted with H₂O (2.5 mL) followed by neutralization with NH₃ solution (25%). The resultant solution was filtrated, the filtrate lyophilized and subjected to HPLC purification (method C) to give compound 4 as TFA salt (1.38 g, 44%). Analytical data matched the literature.

\[ ^1H-NMR\ (300 \text{ MHz}, \text{D}_2\text{O}): \delta 7.89\ (d, J = 2.3 \text{ Hz}, 1H, \text{CH}_2\text{phenyl}), 7.43\ (dd, J = 8.7, 2.3 \text{ Hz}, 1H, \text{CH}_2\text{phenyl}), 7.03\ (d, J = 8.7 \text{ Hz}, 1H, \text{CH}_2\text{phenyl}), 4.18\ (t, J = 6.6 \text{ Hz}, 1H, \text{CH})\]

\[ ^13C-NMR\ (75 \text{ MHz}, \text{D}_2\text{O}): \delta 171.11, 152.74, 138.21, 133.85, 126.40, 135.76, 120.19, 53.68, 34.23; \text{ESI-HRMS (m/z)}:[M]^+ \text{ calcd. for C}_9\text{H}_{12}\text{N}_2\text{O}_4, 227.0660; \text{found} 227.0674. \]

3-amino-L-tyrosine (3)

Compound 4 (1.38 g, 4.86 mmol) was dissolved in H₂O (100 mL) and conc. HCl (500 µL). The solution was supplemented with Pd/BaSO₄ (40 mg, 5% catalyst loading) and the mixture incubated at ambient temperature for 12 h under H₂ atmosphere. After filtration of the catalyst and removal of the solvent in vacuo, the product 3 was obtained in quantitative yield as TFA salt. Analytical data matched the literature.

\[ ^1H-NMR\ (300 \text{ MHz}, \text{D}_2\text{O}): \delta 7.42-7.15\ (m, 2H, \text{CH}_2\text{phenyl}), 7.05-6.89\ (m, 1H, \text{CH}_2\text{phenyl}), 4.11\ (t, J = 6.5 \text{ Hz}, 1H, \text{CH})\]

\[ ^13C-NMR\ (75 \text{ MHz}, \text{D}_2\text{O}): \delta = 171.91, 149.37, 131.23, 126.32, 124.68, 117.84, 116.79, 54.47, 34.61; \text{ESI-HRMS (m/z)}:[M]^+ \text{ calcd. for C}_9\text{H}_{13}\text{N}_2\text{O}_3, 197.0918; \text{found} 197.0910. \]
3-azido-L-tyrosine (1)

3-amino-L-tyrosine (3, 0.696 g, 3.21 mmol) was dissolved in 0.5 M HCl (6 mL) and a solution of NaNO₂ (0.221 g, 3.21 mmol) in ice-cold H₂O (1 mL) was slowly added at 0 °C. After 20 min, a solution of NaN₃ (0.560 g, 8.62 mmol) in H₂O (3 mL) was added within 30 min and stirred at 0 °C for another 8 h. The grey precipitate was isolated and purified by preparative HPLC (method C) to give pure compound 1 (0.290 g, 41%).

![Structure of 3-azido-L-tyrosine (1)](image)

¹H-NMR (300 MHz, D₂O): δ 6.95 (d, J = 2.0 Hz, 1H, CH₆ phenyl), 6.89-6.79 (m, 2H, CH₆ phenyl), 4.06 (t, J = 6.5 Hz, 1H, CH₃), 3.19-2.95 (m, 2H, CH₂); ¹³C-NMR (75 MHz, D₂O): δ 172.04, 146.44, 127.24, 127.07, 126.58, 120.38, 116.86, 54.51, 34.83; ESI-HRMS (m/z): [M]+ calcd. for C₉H₁₁N₄O₃, 223.0823; found 223.0830; FT-IR: \( \nu_{\text{max}} \) (neat, cm⁻¹): 3074 (br), 2116, 1731, 1665, 1597, 1515, 1435, 1299, 1185, 1135, 1095, 813, 721, 655.
2.3.2. Synthesis of 3-formyl-L-tyrosine (2)

The synthesis of 1 was performed according to a known procedure in literature.[7]

Scheme 2. Synthesis of 3-formyl-L-tyrosine (2).

N-[(1,1-dimethylethoxy)carbonyl]-L-tyrosine (S3)

To a solution of L-tyrosine (1 g, 5.5 mmol) in 1/1 dioxane/water (50 mL), triethylamine (1.16 mL, 8.28 mmol) was slowly added. The reaction was cooled to 0°C with an ice/water bath and di-tert-butyl dicarbonate (1.32 g, 6.07 mmol) was added in two steps. After 1 h at 0°C, the temperature was slowly increased to ambient temperature and the mixture was stirred for further 24 h. Dioxane was removed under reduced pressure and the aqueous solution mixed with saturated NaHCO₃ (25 mL), washed with ethyl acetate, acidified to pH 1 with 1 N HCl, extracted with ethyl acetate and the organic extracts were washed with brine, dried over MgSO₄ and evaporated to give Boc protected tyrosine S3 as a white foam (1.471 g, 95%) which was used in the next step without further purification. Analytical data matched the literature[7a].

\[ ^1{H}\text{-NMR} (300 \text{ MHz, CDCl}_3): \delta 7.50-7.22 (m, 2H, CH phenyl), 7.42 (dd, J = 8.6, 2.3 Hz, 1H, CH phenyl), 6.92 (d, J = 8.4 Hz, 1H, CH phenyl), 5.11 (br, 1H, NH), 4.73-4.28 (m, 1H, CH), 3.32-2.90 (m, 2H, CH₂), 1.42 (s, 9H, CH₃). \]

N-[(1,1-dimethylethoxy)carbonyl]-3-(3-formyl-4-hydroxyphenyl)-L-alanine (S4)

To a suspension of S3 (2.00 g, 7.12 mmol) in chloroform (30 mL) and H₂O (0.256 mL, 14.13 mmol) powdered sodium hydroxide (1.71 g, 42.72 mmol) was added and the mixture was refluxed for 4 h. Two additional portions of powdered sodium hydroxide (each 0.42 g, 10.68 mmol) were added after 1 and 2 h. After 8 h at reflux, the reaction was cooled to ambient temperature, diluted with water and ethyl acetate (15 mL each), the organic layer discharged, the aqueous layer acidified to pH 1 with 1 N HCl and back-extracted with ethyl acetate. The organic layers were washed with brine, dried over MgSO₄ and concentrated. Flash column chromatography (silica gel, 12/1 CHCl₃/MeOH, 1% acetic acid) gave compound S4 (0.49 g, 23%). Analytical data matched the literature[7a].

\[ ^1{H}\text{-NMR} (300 \text{ MHz, CDCl}_3): \delta 9.85 (s, 1H, CHO), 7.49-7.21 (m, 2H, CH phenyl), 7.40 (dd, J = 8.6, 2.3 Hz, 1H, CH phenyl), 6.94 (d, J = 8.4 Hz, 1H, CH phenyl), 5.10 (br, 1H, NH), 4.73-4.27 (m, 1H, CH), 3.30-2.89 (m, 2H,CH₂), 1.40 (s, 9H, CH₃). \]
3-formyl-L-tyrosine (1)
Compound S4 (0.49 g, 1.6 mmol) was dissolved in CH₂Cl₂. TFA (4 mL) was added slowly at 0°C and the mixture was warmed to ambient temperature within 2 h. The solvent was removed at high vacuum. Preparative HPLC (method C) gave compound 2 as TFA salt (0.29 g, 80%). Analytical data matched the literature⁹ᵃ.

1H-NMR (300 MHz, D₂O): δ 9.81 (s, 1H, CHO), 7.52 (d, J = 2.4 Hz, 1H, CH₆phenyl), 7.40 (dd, J = 8.6, 2.3 Hz, 1H, CH₆phenyl), 6.90 (d, J = 8.6 Hz, 1H, CH), 3.15 (m, 2H, CH₂), 3.12 (s, 3H, CH₃) ppm. 13C-NMR (75 MHz, D₂O): δ 197.18, 171.68, 159.21, 138.07, 134.02, 126.03, 120.97, 117.73, 54.18, 34.48 ppm. ESI-HRMS (m/z): [M]+ calcd. for C₁₀H₁₂NO₄, 210.0758; found 210.0760.

2.3.3. Synthesis of tris(PEG750)phosphite S1
S4 was synthesized based on a protocol by Nischan et al⁸. Polyethylene glycomethylether was carefully dried at 70°C under high vacuum. Hexamethylphosphortriamide (1 eq., 0.135 mmol, 24.5 µL) was added to dry polyethylene glycomethylether (3 eq., 0.406 mmol, 0.314 g) at 110°C and stirred under N₂ stream for 72 h. The product was recovered as a white paraffinic solid (0.133 mmol, 0.311 g). In order to avoid hydrolysis of the product, no purification was done. Due to the chemoselective character of the Staudinger-phosphite reaction, impurities do not interfere in the reaction and can be removed easily after the Staudinger-phosphite reaction.

1H-NMR (400 MHz, [D]-acetonitrile): δ 3.58-3.57 (m, 19 8.9H, OCH₂CH₂O-), 3.32 (s, 9H, OCH₃); 31P-NMR (400 MHz, [D]-acetonitrile): δ 140.

2.3.4. Synthesis of hydroxylamine-biotin S5


D-biotin N-hydroxysuccinimide ester (S5)
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (184 mg, 0.96 mmol) was added to a solution of D-biotin (200 mg, 0.82 mmol) and N-hydroxysuccinimide (102 mg, 0.89 mmol) in dry DMF (10 mL). The solution was stirred for 12 h at ambient temperature, concentrated and the product crystallized from 2-propanol to give succinimide ester S5 (261mg, 80). The product was used without further purification and analytical data are in accordance with those reported in the literature⁹.
2-Amino-3’[(tert-butoxycarbonyl)amino]ethylene glycol diethyl ether (S6)

To a solution of 2,2’-(ethylenedioxy)-bis(ethyamine) (4.07 g, 27.46 mmol) and N,N-Diisopropylethylamine (1.56 mL, 9.17 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (50 mL) at ambient temperature a solution of di-tert-butyl dicarbonate (2 g, 9.16 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (20 mL) was added dropwise within 20 min. After additional stirring for 1 h at ambient temperature, the mixture was concentrated, redissolved in 20 mL water and extracted four times with CH<sub>2</sub>Cl<sub>2</sub> (10 mL). The organic layers were combined, washed three times with brine, dried with MgSO<sub>4</sub> and concentrated to give 2.02 g of a colorless oil of S6 in an overall yield of 88.8% containing some impurities of double protected species (20% determined from <sup>1</sup>H-NMR). The analytical data are in accordance with those reported in the literature<sup>[10]</sup>.

N-Boc-N’-D-biotinyl-3,6-dioxaoctane,1,8-diamine (S7)

To a solution of Boc-diamine S6 (109 mg, 0.44 mmol) and NEt<sub>3</sub> (81 µL, 0.59 mmol) in dry DMF (5 mL), D-biotin N-hydroxysuccinimide ester (S5, 100 mg, 0.29 mmol) was added and stirred for 12 h. The solvent was removed, the residue resolved in CH<sub>2</sub>Cl<sub>2</sub> (40 mL), washed with 20 mL brine, dried over MgSO<sub>4</sub> and concentrated. Flash column chromatography (silica gel, CH<sub>2</sub>Cl<sub>2</sub> / MeOH: 99 / 1) gave compound S7 (0.128 g, 93%). Analytical data matched the literature<sup>[11]</sup>.

N’-Boc-aminoxyacetyl-N-hydroxysuccinimide ester (S8)

1-ethy-1-3-(3-dimethylaminopropyl)carbodiimide (227 mg, 1.8 mmol) and N-hydroxysuccinimide (190 mg, 1.65 mmol) was added to a solution of N-Boc-aminoxyacetic acid (287 mg, 1.5 mmol) in dry DMF (10 mL) and stirred at ambient temperature for 12 h. The mixture was diluted by the addition of H<sub>2</sub>O (10 mL), extracted twice with EtOAc, the organic phase dried over MgSO<sub>4</sub> and concentrated under vacuum. The yellowish liquid was used without further purification (352 mg, 81%). Analytical data matched the literature<sup>[12]</sup>.
\[ \text{Boc protected diamine S7 (127 mg, 0.32 mmol) was dissolved in CH}_2\text{Cl}_2 (4 mL), TFA (1 mL) was added and the solution stirred at ambient temperature for 2 h. TFA was removed and the remaining solid was dried using high vacuum. The deprotected diamine was dissolved in a mixture of dry DMF (3 mL) and NEt}_3 (89 µL, 0.64 mmol). Hydroxysuccinimide ester S8 (152 mg, 0.52 mmol) was dissolved in dry DMF (0.5 mL), slowly added to the diamine and the resulting mixture was stirred at ambient temperature for 12 h. The solvent was removed and flash column chromatography (silica gel, CH}_2\text{Cl}_2:MeOH, 99:1 to 93:7) gave boc protected hydroxylamine S2 (TLC = [CH}_2\text{Cl}_2:MeOH, 90:10 v/v]: Rf = 0.3). A final deprotection in 25% TFA solution (CH}_2\text{Cl}_2) followed by TFA removal gave deprotected hydroxylamine S2 (102.2 mg, 71%).} \\

\[ \text{1H-NMR (300 MHz, D}_2\text{O):} \delta 4.50 \text{ (s, 2H, COCH}_2\text{O), 4.49-4.43 (m, 1H, CH), 4.24-4.31 (m, 1H,CH), 3.54 (s, 4H, OCH}_2\text{CH}_2\text{O), 3.52-3.45 (m, 4H, CH}_2\text{O), 3.33 (dt, J}_1 = J}_2 = 5.3 Hz, 2H, CH}_2\text{NH}, 3.24 (dt, J}_1 = J}_2 = 5.4 Hz, 2H, CH}_2\text{NHboc), 3.21-3.14 (m, 1H, CH), 2.85 (dd, J}_1 = 13, J}_2 = 4.9, Hz 1H, CHH}_\text{exc}, 2.62 (d, J = 13 Hz, 1H, CHH}_\text{endo}, 2.13 (t, J = 7.2 Hz, 2H, CH}_2\text{CO), 1.65-1.36 (m, 4H, CH}_2\text{), 1.32-1.20 (m, 2H, CH}_2\text{);} \text{13C-NMR (151 MHz, D}_2\text{O)} \delta 176.79, 168.56, 165.14, 71.52, 69.26, 69.23, 68.70, 68.44, 61.93, 60.09, 55.20, 39.52, 38.67, 38.50, 35.26, 27.68, 27.52, 24.97; \text{ESI-MS (m/z):}[M]^+ \text{ calcd. for C}_10\text{H}_34\text{N}_5\text{O}_6\text{S, 448.22; found 448.21.} \\

\text{2.4. Synthesis of CF–Tub-tag peptide} \\

\[ \text{CF–Tub-tag peptide was synthesized by standard Fmoc-based chemistry in a linear synthesis on an Activotec peptide synthesizer followed by manual coupling of 5(6)-carboxyfluorescein. 0.1 mmol of Fmoc-L-Glu(tBu)-Wang resin (subst: 0.58 mmol/g) was added to a reaction vessel and synthesis was performed with five fold amino acid excess. Coupling was achieved by HOBT/HBTU/DIPEA addition. After the final amino acid coupling, the fluorophore was coupled in a double coupling procedure with 5 eq of 5(6)-carboxyfluorescein, HOBT, HBTU and DIPEA in DMF for 1 h. The peptide was cleaved off the resin by addition of TFA/DTT/Tis/thioanisol (95/2/2/1) within 4 h. Subsequently, the cleavage cocktail was evaporated by N}_2-flow and the peptide was precipitated by the addition of ice-cold diethyl ether. The precipitate was spun down, dissolved in water and purified by preparative HPLC (method D). The peptide was obtained with a yield of 8% (16 mg, 8 µmol); molar mass peptide = 1850.6 Da; ESI-HRMS (m/z): [M+2H]^{2+} \text{ calcd. 926.3165; found 926.3065.} \\

S23
Figure S16. LC-UV at 220 nm, 10 to 100% of acetonitrile in water containing 0.1% TFA on a RP-C18 column.
3. NMR Spectra of 1, 2, 3, 4 and S2
3.1 3-N$_3$-L-tyrosine (1)

Figure S17. $^1$H-NMR (300 MHz, D$_2$O) of 3-N$_3$-L-tyrosine (1)

Figure S18. $^{13}$C-NMR (75 MHz, D$_2$O) of 3-N$_3$-L-tyrosine (1)
3.2 3-formyl-L-tyrosine (2)

Figure S19. $^1$H-NMR (300 MHz, D$_2$O) of 3-formyl-L-tyrosine (2)

Figure S20. $^{13}$C-NMR (75 MHz, D$_2$O) of 3-formyl-L-tyrosine (2)
3.3 3-NH$_2$-L-tyrosine (3)

Figure S21. $^1$H-NMR (300 MHz, D$_2$O) of 3-NH$_2$-L-tyrosine (3)

Figure S22. $^{13}$C-NMR (75 MHz, D$_2$O) of 3-NH$_2$-L-tyrosine (3)
3.4 3-NO$_2$-L-tyrosine (4)

Figure S23. $^1$H-NMR (300 MHz, D$_2$O) of 3-NO$_2$-L-tyrosine (4)

Figure S24. $^{13}$C-NMR (75 MHz, D$_2$O) of 3-NO$_2$-L-tyrosine (4)
3.5 Hydroxylamine biotin S2

Figure S25. $^1$H-NMR (300 MHz, D$_2$O) of hydroxylamine biotin S2

Figure S26. $^{13}$C-NMR (75 MHz, D$_2$O) of hydroxylamine biotin S2
4. References


