Traceless Purification and Desulfurization of Tau Protein Ligation Products**

Oliver Reimann, Caroline Smet-Nocca, and Christian P. R. Hackenberger*

anie_201408674_sm_misellaneous_information.pdf
1. General information

**Analytical HPLC** was conducted on a Waters™ 600S controller system (Waters Corporation, Milford, Massachusetts, USA) with a 717 plus autosampler, 2 pumps 616 and a 2489 UV/Visible detector connected to a 3100 mass detector using a Kromasil C18 5 μm, 250 x 4.6 mm RP-HPLC-column with a flow rate of 1.0 mL/min. The following gradient of solvents was used: Method A: (A = H₂O + 0.1% TFA, B= MeCN + 0.1% TFA) 5 min at 10% B, 10 – 90% B from 5-36 min, 90% B from 36-45 min, 90-10% B in 45-50 min. HPLC chromatograms were recorded at 220 nm.

**Analytical UPLC:** UPLC-UV traces were obtained using a Waters H-class instrument, equipped with a Quaternary Solvent Manager, a Waters autosampler, a Waters TUV detector connected to a 3100 mass detector using an Acquity UPLC-BEH C18 1.7 μM 2.1 x 50 mm RP column with a flow rate of 0.6 mL/min. The following solvents and gradients were applied for all peptides if not further mentioned: Method B: (A = H₂O + 0.1% TFA, B= MeCN + 0.1% TFA) 5% B 0-5 min, 5-95% B 5-15 min, 95% B 15-17 min. Method C: (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.

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

**Fluorescence** spectra were recorded using a FP-6500 fluorescence spectrometer (Jasco, Tokyo, Japan).

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

**MALDI-TOF** analysis was carried out AB SCIEX 5800 TOF/TOF System (Applied Biosystems) with nanoLC (Dionex) and robotic system for spotting (Probot, Dionex) and 4700 Proteomics Analyzer (Applied Biosystems).

**NMR** spectra were recorded on a Jeol ECX-400 400 MHz spectrometer (JEOL corporation, Akishima, Tokyo, Japan) or Bruker Ultrashield 300 MHz spectrometer (Bruker Corp. Billerica, Mass., USA) at ambient temperature in CDCl₃. The chemical shifts are reported in ppm relatively to the residual solvent peak.

**Preparative HPLC** was carried out on a JASCO LC-2000 Plus system (JASCO, Inc., Easton, Maryland, USA) using a reversed phase C18 column (Kromasil material) at a constant flow of 32 ml/min using water and acetonitrile with 0.1% TFA. This system was equipped with a Smartline Manager 5000 with interface module, two Smartline Pump 1000 HPLC pumps, a 6-port-3-channel injection valve with 1.0 mL loop, a UV detector (UV-2077) and a high pressure gradient mixer (All Knauer, Berlin, Germany). Purification was carried out after Method D: (A = H₂O + 0.1% TFA, B= MeCN + 0.1% TFA) 10% B 0-5 min, 10-70% B 5-70 min.

**Reagents and solvents** were, unless stated otherwise, commercially available as reagent grade and did not require further purification. All resins or Fmoc-protected amino acids were purchased from IRIS BioTech or Novabiochem.

**Semi-preparative HPLC purification** was carried out on a Dionex 580 HPLC system using a reversed phase Nucleodur C18 HTec column (10 x 250 mm) at a flow rate of 2 ml/min. Used was a gradient referred to as Method D: (A = H₂O + 0.1% TFA, B= MeCN + 0.1% TFA) 10% B 0-5 min, 10-70% B 5-70 min.

**SPPS** was carried out manually for peptides 2, 2*, 6, 7, and 9, via standard Fmoc-based conditions (Fast-moc protocol with HOBT/HBTU conditions). Peptide 4 was synthesized on an Activo-P11 automated peptide synthesizer (Activotec) under Fmoc-based conditions.

**UV-irradiation** was carried out with a LOT Hg (Xe) arc lamp (LOT-QuantumDesign GmbH, D-64293 Darmstadt, Germany) at 297 nm. Probes were positioned in 20 cm distance and irradiated while stirring.
Synthesis of compound 1 was achieved in an overall yield of 32% over four steps. All spectra were in accordance to literature\(^1\) (for \(^1\)H-NMR and HRMS of compound 1 see figures S25 and S26).

Scheme S1. Synthetic route of photolinker 1 according to the literature known procedure.\(^1\)

2. Experimental details for peptides

2.1 Synthesis of peptide 2

A Fmoc-protected Rink Amide resin (0.05 mmol scale, Fmoc-Rink-Amide AM resin, 100-200 mesh (75-150 μm), 0.14 mmol/g loading, Iris) was deprotected by treatment with 4 mL DMF containing 20% piperidine for 20 min. In the following couplings, the orthogonally protected amino acids Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Lys(ivDde)-OH and Boc-L-thiazolidine-4-carboxylic acid were coupled using 5 eq of each amino acid, HOBt, HBTU and DIPEA over a time span of 12 h each. Standard Fmoc-protected amino acids were coupled with 10 eq amino acid, HOBt, HBTU and DIPEA over 1 h. Alloc deprotection was carried out by bubbling N\(_2\) through a solution containing the resin and Pd(PPh\(_3\))\(_4\) (173 mg, 0.15 mmol, 3 eq) in 4 mL CHCl\(_3\)/AcOH/NMM (37/2/1) for 2 h. Full deprotection was confirmed by test-cleavage and an LC-UV/MS measurement. Coupling of 5(6)-Carboxyfluorescein (470 mg, 1.25 mmol, 2.5 eq) was achieved with HOBt (19 mg, 0.125 mmol, 2.5 eq) and DIC (19 μl, 0.125 mmol, 2.5 eq) in 4 mL DMF overnight, followed by trityl-protection
with Trt-Cl (335 mg, 1.2 mmol, 24 eq) and DIPEA (210 µL, 1.18 mmol, 24 eq) in CH₂Cl₂, which was performed twice for 16 h. Next, the deprotection of ivDde was carried out by treating the resin with DMF containing 2% hydrazine (4 mL) four times for 2-3 min. Full deprotection was again confirmed by test-cleavage and an LC-UV/MS measurement. Linker 1 was attached to the free Lys moiety by incubating the peptide with the reactive linker 1 (40 mg, 0.11 mmol, 2.1 eq) in 4 mL DMF upon the addition of DIPEA (50 µL, 0.28 mmol, 5.6 eq) in an overnight reaction. In an one-pot procedure, the azide was reduced by Staudinger reduction with tri-n-butylphosphine (63 µL, 0.25 mmol, 5 eq) and biotin (53 mg, 0.2 mmol, 4 eq) was coupled to the peptide with DIC (31 µL, 0.2 mmol, 4 eq) and HOBT (27 mg, 0.2 mmol, 4 eq) in 3 mL NMP, which was chosen as a solvent due to low solubility of biotin in DMF. The combined TFA fractions were concentrated by strong nitrogen flow, followed by the addition of ice-cold diethyl ether. The precipitated peptide was dissolved in water and lyophilized, before it was submitted for purification, carried out by preparative HPLC (Method D, see general information). The yield of peptide 2 was 7% (5 mg, 3 µmol) of a yellow product in good purity; molar mass peptide = 1740.7 Da. HRMS: m/z: 581.2427 [M+3H]^3+ (calcd. m/z: 581.2429).

Figure S1. HPLC-UV of peptide 2 (Method A).

Figure S2. HRMS of Peptide 2: m/z: 581.2427 [M+3H]^3+ (calcd. m/z: 581.2429).
2.2 Synthesis of peptide 2*

Peptide 2* was obtained from peptide precursor A (see Scheme S2) by cleavage from the resin using TFA/TIS/H$_2$O (95/2.5/2.5) for 3 h.

\[
\text{peptide precursor A}
\]

Scheme S3. Reaction conditions for the synthesis of peptide 2*: a) TFA/TIS/H$_2$O (95/2.5/2.5).

Peptide 2* was purified by preparative HPLC (Method D) and was isolated with a yield of 16% (12 mg, 8 µmol) as yellow powder in high purity; molar mass peptide = 1306.5 Da; HRMS: m/z: 436.5350 [M+3H]$^{3+}$ (calcd. m/z: 436.5341).

[UV absorbance graph]

Figure S3. HPLC-UV of peptide 2* (Method A).

[HRMS graph]

Figure S4. HRMS of Peptide 2*: m/z: 436.5350 [M+3H]$^{3+}$ (calcd. m/z: 436.5341).
2.3 Synthesis of peptide 4

Peptide 4 (tau[390-441] (Ala390/Cys)) was synthesized by standard Fmoc-based chemistry in a linear synthesis on an Activotec peptide synthesizer. The resin used was H-Leu-HMPB NovaPEG (subst: 0.49 mmol/g). The scale of the reaction was 0.1 mmol. Amino acids were used in five fold excess. Coupling was achieved by HOBt/HBTU/DIPEA addition. Lys438 was introduced with Alloc protection of the side-chain ε-NH₂-group (Fmoc-Lys(Alloc)-OH). N-terminal Cys was coupled as Boc-protected building block Boc-Cys(Trt)-OH. After amino acid coupling, Alloc was deprotected by addition of Pd(PPh₃)₄ (12 mg, 0.001 mmol, 0.1 eq) and phenylsilane (300 µL, 2.4 mmol, 24 eq) in CH₂Cl₂ and shaking for 10 min. This deprotection step was repeated. Hereafter, the photolinker was introduced by addition of 1 (80 mg, 0.22 mmol, 2.2 eq) in 4 mL DMF with DIPEA (120 µL, 0.68 mmol, 6.8 eq). The mixture was shaken overnight and a test cleavage confirmed the coupling of 1 to the peptide. Biotin (106 mg, 0.4 mmol, 4 eq), DIC (62 µL, 0.4 mmol, 4 eq), tri-n-butylphosphine (120 µL, 0.5 mmol, 5 eq) and HOBt (54 mg, 0.4 mmol, 4 eq) were added to the resin in 4 mL NMP, in which the resin was shaken for 2 h. The peptide was cleaved off the resin by addition of TFA/DTT/TIS/thioanisol (95:2:1:1) in 4 h. Subsequently, the cleavage cocktail was evaporated by N₂-flow and the peptide precipitated by the addition of ice-cold diethyl ether. The precipitate was spun down, dissolved in water and acetonitrile and purified by preparative HPLC (Method D). The peptide was obtained with a yield of 3% (18 mg, 3 µmol); molar mass peptide = 5792.7 Da; HRMS: m/z: 1159.5482 [M+5H]⁵⁺ (calcd. m/z: 1159.5643).

![Scheme S4. Synthesis of peptide 4.](image)

![Figure S5. UPLC-UV of peptide 4 (Method B).](image)
2.4 Synthesis of peptide 6

The synthesis of peptide 6 was performed on a Dawson Dbz NovaSynTGR resin (subst: 0.24 mmol/g, Novabiochem, 0.1 mmol scale). The preswollen resin was treated with a solution of allyl chloroformate (50 eq) and DIPEA (2eq) in CH₂Cl₂ for 6 h. In a next step, the Fmoc group was removed from the resin by shaking it with 20% piperidine in DMF for 20 min. Hereafter, amino acids were coupled in a double coupling procedure with a first coupling using 10 eq amino acid, 10 eq HATU and 20 eq DIPEA for 45 min, followed by a second coupling with 10 eq HOBt, 10 eq HBTU and 20 eq DIPEA. The N-terminal Fmoc-group from aspartic acid was removed and a capping step was carried out with 50 eq of acetic anhydride and 50 eq DIPEA in DMF for 30 minutes.

On resin Alloc deprotection and NCL precursor formation: Alloc removal was performed on the solid support twice with PhSiH₃ (24 eq with respect to the initial loading of the resin) and Pd(PPh₃)₄ (0.1 eq) in CH₂Cl₂, shaking for 10 min at RT. The resin was carefully washed with CH₂Cl₂ and a solution of p-nitrophenyl chloroformate (5 eq) in 4 mL of dry CH₂Cl₂ was added and shaken for 1 h at RT, followed by thorough washing with CH₂Cl₂ (5x), DMF (5x) and CH₂Cl₂ (5x). This step was repeated for two more times, before the resin was washed with DMF in the end. The resin was reacted 2x 30 min with a 0.5 M solution of DIPEA in DMF (4 mL) to perform the cyclization. In a next step, the resin was washed with DMF (5x), methanol (3x) and CH₂Cl₂ (3x) and then dried under vacuum.

Cleavage from the resin: The peptide was cleaved in 4 h from the resin using TFA/DCM/TIS/H₂O (90/5/2.5/2.5). The cleavage solution was filtered and added to a solution of ice-cold diethyl ether. The precipitated peptide was lyophilized and purified by preparative HPLC (Method D). Peptide 6 was obtained with a yield of 8% (6.1 mg, 8.03 µmol) as a colorless product in high purity; molar mass peptide = 759.3 Da; HRMS: m/z: 760.3267 [M+1H]⁺ (calcd. m/z: 760.3260).
**Figure S7.** UPLC-UV of peptide 6 (Method B).

**Figure S8.** HRMS of Peptide 6: m/z: 760.3267 [M+H]^+ (calcld. m/z: 760.3260).
Peptide 7 was synthesized on H-Leu-HMPB Nova PEG resin (loading of 0.49 mmol/g). The scale of the synthesis was 0.1 mmol. Standard Fmoc-based conditions (Fast-moc protocol with HOObt/ HBTU conditions) were applied using 10 eq of Fmoc-L-amino acids and coupling reagents. Alloc-protected Lys13 was introduced by addition of 10 eq of Fmoc-Lys(Alloc)-OH, HOAt, HATU and DIPEA. Cys1 was introduced as Boc-Cys(Trt)-OH under standard conditions. Hereafter, the Alloc-protecting group was removed by the addition of Pd(PPh₃)₄ (0.1 eq) and Phenylsilane (24 eq) in CH₂Cl₂. The resin was gently shaken 2x for 10 minutes in this mixture followed by extensive washing of the resin with CH₂Cl₂ and DMF. Thereafter, the photolinker was introduced by incubation of the resin with a mixture of 1 (80 mg, 0.22 mmol, 2.2 eq) and DIPEA (100 µL, 0.5 mmol, 5 eq) o.n.[1] In an one pot reaction, the azide was reduced to the amine and coupled to biotin by the addition of tri-n-butylphosphine (126 µL, 0.5 mmol, 5 eq), biotin (106 mg, 0.4 mmol, 4 eq), DIC (62 µL, 0.4 mmol, 4 eq) and HOBt (54 mg, 0.4 mmol, 4 eq) in NMP. The resin was left shaking in this mixture for 2 h. The peptide was cleaved from the resin by the addition of 3 mL TFA/DTT/TIS/thioanisol (95/2/2/1) for 3 h, whereas 15 min before the end of the cleavage time ethanedithiol (16.8 μL/ml cleavage cocktail; 0.2 mol/L) and trimethylsilyl bromide (13 μL/mL cleavage cocktail; 0.1 mol/L) were added to the mixture to reduce observed oxidation.[3] The final biotinylated peptide was purified by preparative HPLC (Method D) and obtained with a yield of 4% (8 mg, 3.8 μmol); molar mass peptide = 2038.9 Da; HRMS: m/z: 680.6509 [M+3H]⁺ (calcd. m/z: 680.6519).

Figure S9. HPLC-UV of peptide 7 (Method A).
2.6 Synthesis of peptide 9

Peptide 9 was synthesized on Dawson Dbz NovaSyn TGR resin (subst: 0.24 mmol/g) applying an Alloc protection strategy. The scale of the synthesis was 0.05 mmoles. The resin was swollen in DMF/CH₂Cl₂ (1:1) for 30 min and subsequently treated with a solution of allyl chloroformate (50 eq) and DIPEA (2 eq) in CH₂Cl₂ for 12 h. Hereafter, Fmoc removal was accomplished by treating the resin with 20% piperidine in DMF for 20 min. Amino acids were coupled by a double coupling procedure. First coupling was carried out with 10 eq of amino acid, 10 eq of HATU, and 20 eq of DIPEA in 4 mL DMF for 40 min and the second coupling with 10 eq of amino acid, 10 eq of HCTU, and 20 eq of DIPEA in 4 mL DMF for 1 h. Occasionally, the Kaiser test was applied to verify quantitative coupling. Leu⁴⁰⁸-Ser⁴⁰⁹, Ser⁴¹²-Ser⁴¹³, and Gly⁴¹⁵-Ser⁴¹⁶ junctions were introduced as pseudoprolines (Fmoc-Leu-Ser(VMe,Mepro)-OH, Fmoc-Ser(tBu)-Ser(VMe,Mepro)-OH, Fmoc-Gly-Ser(VMe,Mepro)-OH). Phosphoserine (Fmoc-Ser(PO(OBzl))OH)-OH) was introduced by using double coupling with 5 eq amino acid, HATU in the first and HCTU in the second coupling with 10 eq of DIPEA for 1 h in case of HATU and 2 h in case of HCTU.

On-resin Alloc removal and NCL precursor formation: The Alloc-protected resin was washed and swollen in CH₂Cl₂. Hereafter, a solution of Pd(PPh₃)₄ (0.35 eq with respect to the loading of the resin) and PhSiH₃ (20 eq with respect to initial loading of the resin) in CH₂Cl₂ were added to the resin and shaken for 1 h at ambient temperature. Then, the resin was washed thoroughly with CH₂Cl₂ and a solution of p-nitrophenoxy chloroformate (100 mg, 10 eq) in 4 mL CH₂Cl₂ was added. The mixture was allowed to shake for 30 min at RT. This step was repeated in order to complete the cyclization.

Cleavage from the resin: The resin was washed with DMF, methanol and CH₂Cl₂ before it was dried in high vacuum. The cocktail used for peptide cleavage consisted of TFA/CH₂Cl₂/TIS/H₂O (90/5/2.5/2.5). Cleavage was conducted for 3.5 h at RT. Afterward, the TFA cocktail was nearly completely evaporated by nitrogen flow, followed by the addition of ice-cold diethyl ether. The precipitate was spun down and purified by preparative HPLC (Method D).

Peptide 9 was obtained with a yield of 2% (4.7 mg, 1.02 µmol); molar mass peptide = 4214.7 Da; LR-MS: m/z: 1392.03 [M+3H]³⁺ (calcd. m/z: 1391.28).
Figure S11. UPLC-UV of peptide 9 (Method C).

Figure S12. LRMS of Peptide 9: 1392.03 [M+3H]^2+ (calcd. m/z: 1391.28).

3. Determination of cleavage efficiency

3.1 Photocleavage of PC-biotin from peptides in solution

Scheme S8. Photocleavage of biotin from peptide 2, leaving unmodified lysine on irradiated peptide 2*.

Photocleavage in solution was carried out by preparing a stock solution from peptide 2 (0.6 mg, 345 nmol) solubilized in 1 mL water. An aliquot of 218 µL (75 nmol) was diluted to a total volume of 2 mL in 20mM NH₄HCO₃ at pH 7 and transferred to a glass cuvette (Hellma precision cuvettes, 10mm). Upon stirring, the cuvette was irradiated at 297 nm for 5 minutes from a distance of 20 cm. HPLC-UV and LR-MS analysis revealed quantitative cleavage of the sample.
3.2 Fluorescence measurement to determine cleavage efficiency

Fluorescence measurements of different concentrations of the synthetically obtained peptide 2* (see page S4, section S2.2) were performed to ensure the accuracy of the quantification by florescence spectroscopy. It was found, that concentrations of 5- (6)-carboxyfluorescein containing peptide 2* between 0.5 μM and 5 μM delivered fluorescence intensity values with a linear correlation. Concentrations considerably higher than 5 μM led to fluorescent quenching. The probes were dissolved in 1 mL of a 20 mM NH₄HCO₃ buffer with an pH adjusted to 7 and always measured in PS semi-micro cuvettes (12.5 x 12.5 x 45 mm). The maximal emission was determined to be λ_{em} = 517 nm for peptide 2*, which was used for later measurements at a fixed wavelength of λ_{Ex} = 492 nm and λ_{Em} = 517 nm. Spectra were accumulated 3 times and repeated 3 times.
**Figure S15.** Determination of $E_{m_{\max}}$ for peptide $2^*$.

**Table S2.** Measured fluorescence intensities for a dilution series of peptide $2^*$.

<table>
<thead>
<tr>
<th>concentration</th>
<th>0.5 µM</th>
<th>1.0 µM</th>
<th>2.0 µM</th>
<th>3.0 µM</th>
<th>4.0 µM</th>
<th>5.0 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>experiment 1</td>
<td>17.96</td>
<td>80.21</td>
<td>157.00</td>
<td>284.06</td>
<td>358.62</td>
<td>412.15</td>
</tr>
<tr>
<td>experiment 2</td>
<td>30.64</td>
<td>95.82</td>
<td>196.48</td>
<td>313.01</td>
<td>373.43</td>
<td>442.91</td>
</tr>
<tr>
<td>experiment 3</td>
<td>21.87</td>
<td>90.06</td>
<td>180.39</td>
<td>301.33</td>
<td>382.02</td>
<td>455.91</td>
</tr>
<tr>
<td>mean</td>
<td>23.49</td>
<td>88.69</td>
<td>177.95</td>
<td>299.46</td>
<td>371.35</td>
<td>436.99</td>
</tr>
<tr>
<td>standard deviation</td>
<td>6.49</td>
<td>7.89</td>
<td>19.85</td>
<td>14.56</td>
<td>11.83</td>
<td>22.47</td>
</tr>
<tr>
<td>standard error</td>
<td>3.75</td>
<td>4.56</td>
<td>11.46</td>
<td>8.40</td>
<td>6.83</td>
<td>12.97</td>
</tr>
</tbody>
</table>

**Figure S16.** Fluorescence for serial dilution of peptide $2^*$ in 20 mM NH$_4$HCO$_3$ at pH 7 ($y = a + bx$ with $a = -18.07$ and $b = 97.86$).
### 3.3 Immobilization and elution of peptide 2

![Diagram of peptide immobilization and elution](image)

**Scheme S9.** Release of peptide $2^*$ from immobilized peptide 2 upon UV light irradiation.

500 µL of agarose/streptavidin slurry (Streptavidin Agarose Novagen EMD Chemicals; binding capacity $>85$ nmol free biotin/mL) were placed in a 600 µL volume microcentrifuge spin cup with filter to yield after spinning approx. 250 µL of settled resin. Subsequently, peptide 2 (97 µL, 40 nmol) was added in the spin tube and 153 µL of buffer (20 mM NH$_4$HCO$_3$, pH 7) were added. After 1 h of incubation time, the mixture was centrifuged at 14000 rpm for 1 min. Afterward, the beads were washed 5 times with 20 mM NH$_4$HCO$_3$ buffer at pH 7. The flow through (FT) and the wash fractions (W1-5) were filled up to 1 mL volume and the pH was readjusted to 7. The flow through and washes were irradiated by UV light (297 nm) for 5 min. Subsequent fluorescent measurements showed, that 5 washes were enough to approach the baseline of fluorescent intensity. Moles per fraction were calculated according to the dilution by the linear regression for peptide $2^*$.

**Table S3.** Fluorescent intensities of probes and moles calculated according to dilution series. Concentrations marked with an * are out of the linear range of measurements in the regression and are therefore not reliable.

<table>
<thead>
<tr>
<th>probe</th>
<th>fl. intensity</th>
<th>moles [nmol]</th>
<th>conc. [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT</td>
<td>62.6</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>W1</td>
<td>10.5</td>
<td>0.3*</td>
<td>0.3*</td>
</tr>
<tr>
<td>W2</td>
<td>3.5</td>
<td>0.2*</td>
<td>0.2*</td>
</tr>
<tr>
<td>W3</td>
<td>1.5</td>
<td>0.2*</td>
<td>0.2*</td>
</tr>
<tr>
<td>W4</td>
<td>1.4</td>
<td>0.2*</td>
<td>0.2*</td>
</tr>
<tr>
<td>W5</td>
<td>0.9</td>
<td>0.2*</td>
<td>0.2*</td>
</tr>
<tr>
<td>$\Sigma = 1.9^*$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure S17.** Fluorescent intensities of flow through (FT) and wash fractions (W1-5) upon incubation of peptide 2 with streptavidin coated agarose beads after irradiation of the flow through for 7 minutes.

In the following, the beads were transferred to a glass cuvette in a volume of 2 mL of 20 mM NH$_4$HCO$_3$ buffer of pH 7. Upon stirring, they were irradiated at 297 nm wavelength from a distance of 20 cm. Every minute, a 200 µL aliquot was taken and resuspended in 800 mL buffer (20 mM NH$_4$HCO$_3$) to monitor the process of the photochemical release of the fluorophore containing peptide. Before every measurement, the pH was readjusted to 7.0. The obtained data were
compared to an aliquot of the same dilution from a standard (97 µL of the same stock solution), that has been irradiated for 7 minutes in solution (complete cleavage as to be seen above). In all of these fluorescence measurements excitation was conducted at $\lambda = 492$ nm, followed by a scan between 505 and 650 nm. All measurements were within the linear range, whereas changes of volume upon withdrawal of aliquots for fluorescence measurements were considered in the calculation.

**Table S4.** Maximal fluorescent intensities of aliquots after 1 to 7 minutes (E1-E7). Also given are moles calculated according to linear regression.

<table>
<thead>
<tr>
<th>probe</th>
<th>fl. intensity (max)</th>
<th>moles [nmol]</th>
<th>conc. [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 – 1 min</td>
<td>111.3</td>
<td>13</td>
<td>1.3</td>
</tr>
<tr>
<td>E2 – 2 min</td>
<td>153.1</td>
<td>18</td>
<td>1.8</td>
</tr>
<tr>
<td>E3 – 3 min</td>
<td>171.3</td>
<td>19</td>
<td>1.9</td>
</tr>
<tr>
<td>E4 – 4 min</td>
<td>190.0</td>
<td>21</td>
<td>2.1</td>
</tr>
<tr>
<td>E5 – 5 min</td>
<td>211.6</td>
<td>24</td>
<td>2.4</td>
</tr>
<tr>
<td>E7 – 7 min</td>
<td>207.8</td>
<td>23</td>
<td>2.3</td>
</tr>
<tr>
<td>Standard</td>
<td>258.2</td>
<td>28</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Figure S18.** Fluorescence intensities measured between 1 and 7 minutes (E1-E7) of irradiated beads in comparison to a standard of the same amount and from the same stock.

These results exhibited a recovery of 82% of unmodified peptide compared to the standard serving as theoretical 100%. To confirm these results, the procedure was repeated under the exact same conditions. The obtained data revealed reproducibility of this procedure and showed a comparable result of 78% recovery (appendix). The release of the correct peptide was confirmed by HPLC-UV and LR-MS.

**Figure S19.** HPLC-UV (Method A) of peptide 2 before (red) and after UV cleavage (black) from agarose/streptavidin beads.
Figure S20. LR-MS of peptide 2 (left) m/z: 871.5 [M+2H]** (calcd: 871.4); LR-MS of cleaved peptide 2* (right) m/z: 654.3 [M+2H]** (calcd: 654.3).

Note, that immobilization on Pierce® Streptavidin Plus UltraLink® resin, an azlactone-activated polyacrylamide resin, and subsequent photocleavage led to the formation of a considerable amount of unidentifiable sideproduct. Polyacrylamide beads are therefore to be circumvented and agarose beads are to be used (see appendix).

4. Expression, EPL and purification

4.1 Preparation of the recombinant α-thioester of tau[1-389], EPL and purification

For EPL, a fragment of tau including residues 1 to 389 (3) was expressed in T7 Express competent E. coli cells. As reported previously,[5] the tau gene was cloned into the pTXB1 plasmid (New England Biolabs), which includes the Mxe Gyr A intein-CBD insert. Bacteria were grown at 37°C in LB medium containing 100 mg/L ampicillin. At an OD of 0.7, induction of protein expression induced by the addition of isopropyl 1-thio-β-D-galactoside (IPTG) to a final concentration of 0.25 mM and left shaking overnight at 37°C. Afterwards, bacterial cells were harvested by centrifugation at 10,000 rpm, 4°C for 15 min. The pellet was resuspended in lysis-buffer containing 20 mM Tris, pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1 mM TCEP, complemented with a protease inhibitor cocktail (Complete, Roche). The soluble extract from this culture was obtained by French press and subsequent centrifugation at 25,000 g for 30 min at 4°C. The resulting cell lysate was directly loaded onto 3 ml chitin beads (New England Biolabs) in a PD-10 column and incubated overnight at ambient temperature. The beads were then extensively washed with 10 bed volumes cell-lysis buffer, followed by 10 bed volumes of EPL equilibration buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM TCEP). Subsequently, the beads were quickly flushed three times with cleavage buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.1 mM TCEP, 200 mM MESNa). Afterward, peptide 4 (3 mg, 472 nmol) was added to the chitin beads in 3 ml cleavage buffer. The mixture was protected from light and left gently rocking for 1 day at RT. The mixture was filtered and then concentrated in Amicon Ultra spin-filters (Ultracel-30K, 30 kDa cut-off). This procedure simultaneously allowed buffer exchange to streptavidin binding buffer (20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM DTT) and removal of unligated peptide 4. Subsequently, retained protein was applied in a volume of 1.5 mM streptavidin binding buffer to 500 µL precipitated streptavidin-coated agarose beads in a column (5 mL column volume) and incubated for 2 h at RT. In the next step, a stirring bar was added to the column and the beads were irradiated with UV light (297 nm wavelength) 3x for 10 min each from a distance of 20 cm. The buffer was filtered through the column and protein 5 was obtained with a yield of 0.95 mg, as determined by UV absorption (NanoDrop, ND-1000 spectrophotometer, Thermo Scientific), before the sample was frozen at -20°C until further use.
4.2 Western blots

4.2.1 Tau 46

Proteins were separated by SDS-PAGE (4-20% Mini-Protein TGX precast gel, Bio-Rad) on a Bio-Rad Mini-Protein Tetra System and wet blotted onto a nitrocellulose membrane (0.2 µm, 300 mm x 3 m, 250 mA, 1 h). The membrane was blocked with Roti-Block (Carl Roth) over night at 4°C. The blot was incubated for 1 h with antibody Tau 46 (mouse monoclonal IgG1, Santa Cruz Biotechnology) (1:200) followed by a 1 h incubation at RT with a secondary anti-mouse antibody (1:2000) (Thermo Scientific). The blot was again washed with PBS-T and PBS. Shortly before imaging, the blot was rinsed with WesternBright chemiluminescence solution (WesternBright ECL, advansta). Chemilumiscence detection was carried out on a fluorescence imager Lumi-Imager F1 (Boehringer Mannheim).

4.2.2 Streptavidin-POD conjugate

Proteins were separated by SDS-PAGE (4-20% Mini-Protein TGX precast gel, Biorad) on a Bio-Rad Mini-Protein Tetra System and wet blotted onto a nitrocellulose membrane (0.2 µm, 300 mm x 3 m, 250 mA, 1 h). The membrane was blocked with Roti-Block (Carl Roth) over night at 4°C. The blot was incubated for 1 h with streptavidin peroxidase conjugate (Merck Millipore, 500 U) at RT. After careful washing with PBS-T (0.1% Tween-20) the blot was rinsed with WesternBright chemiluminescence solution (WesternBright ECL, Biozym Scientific). Chemilumiscence detection was carried out on a fluorescence imager Lumi-Imager F1 (Boehringer Mannheim).

4.3 Mass spectrometry of tau proteins

Super-DHB (Fluka) was dissolved in a 30:70 (v/v) mixture of acetonitrile and water containing 0.1% TFA to a final concentration of 50 mg/mL and used as matrix. Samples were prepared by mixing 1 µL of the tau protein solution with 1 µL of water containing 0.1% TFA. Then 1 µL of matrix was added and carefully mixed by pipetting. From this mixture 1 µL was transferred to the MALDI sample plate and dried at ambient temperature. Analysis of the samples was performed in the linear positive ion mode and for each spectrum 5000 consecutive laser shots were accumulated. The spectrometer was calibrated with BSA prior to measurements and analyzed with Data Explorer software (Applied Biosystems). For all spectra baseline corrections were conducted and noise filter smooth applied using Gaussian Smooth with a filter of 51.

![Figure S21. MALDI-TOF spectra of A) EPL-mixture, containing tau[1-389] and semi-synthetic tau[1-441]; B) purified semi-synthetic tau[1-441](Ala390Cys) and C) recombinant tau[1-441](Ala390Cys). The calculated value for tau[1-389] is [M+H]^+ = 40.5 kDa and corresponds to the found 40.6 kDa found in A), while the calculated value for tau[1-441](Ala390Cys) of [M+H]^+ = 45.9 kDa is in accordance with the in A), B) and C) observed 46.2 kDa, 45.9 kDa and 46.0 kDa.](image)

5. NCL, desulfurization and purification

5.1 General procedure for NCL, desulfurization and purification

5.1.1 NCL conditions

Native chemical ligations were carried out in either buffer A (6 M Gln-HCl, 200 mM NaH2PO4 buffer at pH 7.2, 200 mM MPAA concentrations and 100 mM TCEP, pH 7.2) or buffer B (6 M Gln-HCl, 100 mM NaH2PO4 buffer at pH 7.2, 100 mM MPAA concentrations and 100 mM TCEP, pH 7.2). The buffers were degassed and filtered prior to use. Concentrations of peptides during NCL were between 1 and 5 mM. Reactions times were between 90 min and 2 h. The reaction was run until the PC-biotin containing fragment was completely converted as verified by UPLC-UV/MS.

5.1.2 Immobilization on streptavidin coated agarose beads
Due to the high Gn·HCl concentration in the buffer, all NCL reactions were generally diluted 1:10 with PBS prior to incubation with streptavidin coated agarose beads (binding capacity > 85 nmol/mL, Novagen), which simultaneously quenched the NCL reaction. Dilution should be at least 1:1, since guanidine hydrochloride concentrations of 4 M and higher are known to interfere with binding of biotin to streptavidin. The final volume was always about double the volume of streptavidin beads, which were provided in a PD-10 column. Immobilization of biotinylated peptides was usually quantitative after 1 h at RT, before the column was placed on a rocker. The immobilization can be monitored by submitting samples from the flow through from the beads to UPLC-UV/MS analysis. When the immobilization was complete, the beads were thoroughly washed with 15 bed volumes PBS, followed by 5 bed volumes water.

5.1.3 On-bead homogeneous desulfurization

1 mL EtSH in H2O (2% vol/vol) was added to 1.5 mL streptavidin coated agarose beads, followed by 1.5 mL of a 250 mM TCEP in PBS (pH preset to 7.4). Hereafter, 100 µL of tBuSH and 50 µL of a 0.1 M solution of the water soluble radical initiator 2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) in H2O were added. A stirring bar was added to the PD-10 column and the desulfurization reaction was stirred for 1 h at RT or at 37°C. Afterward, the liquid containing the chemicals for desulfurization was filtered off and the beads were carefully washed with 10 bed volumes of PBS.

5.1.4 UV cleavage

UV cleavage was conducted by resuspending the beads in 10 mL H2O in a glass vial and irradiating them with a wavelength of 297 nm, 3x for 5 minutes from a distance of 20 cm while stirring. The solution was filtered, mixed once with cold diethyl ether and concentrated by lyophilization. The lyophilized product was dissolved in H2O containing 0.1% TFA and analyzed by UPLC-UV/MS.

5.2 Synthesis of tau[421-441] by NCL by ligation, purification and desulfurization

![Scheme S11](image)

**Scheme S11.** Native chemical ligation of peptides 6 and 7 yielded peptide 8 upon an immobilization, desulfurization and purification procedure.

**Procedure for NCL:** Peptide 7 (0.75 mg, 348 nmol, 1 eq.) was dissolved in buffer A and the pH was readjusted to 7.4, before peptide 6 (0.38 mg, 495 nmol, 1.5 eq) was added to the ligation mixture to give a final pH of 7.2 at a final volume of 100 µL that gave a concentration of peptide 6 of 5 mM. The mixture was left standing at RT for 90 minutes and was monitored by UPLC-UV/MS. After 90 min peptide 7 completely underwent ligation and the reaction was quenched by the addition of 900 µL H2O. Immobilization, desulfurization and UV cleavage were conducted as described in SI 5.1.2, 5.1.3 and 5.1.4, whereas the desulfurization was carried out at RT. Peptide 8 was isolated with a final yield of 97% (0.85 mg, 341 nmol) in excellent purity (>95%) without HPLC.
Peptide masses of 6, 7, 8, b and c (See Figure 3) were confirmed by LR-MS: 6 m/z: 760.55 [M+H]$^+$ (calcd. m/z: 760.32); 7 m/z: 1021.06 [M+2H]$^{2+}$ (calcd. m/z: 1020.47); b m/z: 751.57 [M+H]$^+$ (calcd. m/z: 752.30); c m/z: 1312.30 [M+2H]$^{2+}$ (calcd. m/z: 1311.60); 8 (left) m/z: 1079.15 [M+2H]$^{2+}$ (calcd. m/z: 1078.55). Peptide 8 was further analyzed by HRMS (right bottom): m/z: 1078.5393 [M+2H]$^{2+}$ (calcd. m/z: 1078.5576).
5.3 Synthesis of tau[390-441](pSer396/400/404) by NCL and desulfurization

Scheme S12. Native chemical ligation of peptides 9 and 7 followed by desulfurization and purification furnishing peptide 10.

Procedure for NCL: Peptide 7 (0.5 mg, 220 nmol, 1 eq) was dissolved in degassed and filtrated buffer B. After dissolving peptide 7, the pH was readjusted to 7.4 and peptide 9 (1.5 mg, 330 nmol, 1.5 eq) was added to the ligation mixture to give a final pH of 7.2 at a final volume of 300 µL that gave a concentration of peptide 9 of 1 mM. The reaction was left standing at RT for 120 min and was monitored by UPLC-UV/MS. After 120 min, peptide 7 completely underwent ligation and the reaction was quenched by the addition of 700 µL H2O. Immobilization, desulfurization and UV cleavage were conducted as described in SI 5.1.2, 5.1.3 and 5.1.4, whereas the desulfurization was carried out at 37°C. The lyophilized peptide 10 (1.0 mg, 163 nmol, 74%) was dissolved in H2O containing 0.1% TFA and analyzed by UPLC-UV/MS, showing the desired product with a purity of approx. 70% according to UV integration. A final semi-preparative HPLC (Method D) rendered the desired product (0.6 mg, 98 nmol, 45%) in high purity.

Figure S24. The mass of peptide 10 was determined by LR-MS (left) m/z: 1393.63 [M+4H]4+ (calc. m/z: 1392.65); m/z 1857.62 [M+3H]3+ (calcd. m/z: 1856.5361). The mass was also confirmed by HRMS (right): m/z: 1392.6270 [M+5H]5+ (calcd. m/z: 1392.6539).
6. Appendix

6.1 $^1$H- NMR and HRMS of compound 1

Figure S25. $^1$H NMR of compound 1.

$^1$H NMR: (400 MHz, CDCl$_3$) $\delta = 2.81$ (s, 4H, CH$_2$), 3.76 (dd, $J = 13.6, 6.4$ Hz, 1H, CH$_2$), 3.96 (dd, $J = 13.6, 2.9$ Hz, 1H, CH$_2$), 6.47 (dd, $J = 6.4, 2.9$ Hz, 1H, CH), 7.58 (m, 1H, CH$_2$phenyl), 7.79 (d, $J = 4.2$ Hz, 2H, CH$_2$phenyl), 8.12 (d, $J = 8.2$ Hz, 1H, CH$_2$phenyl).

Figure S26. HRMS of compound 1, m/z: 350.0749 [M+H]$^+$ (calcd. m/z: 350.0731).
6.2 PCB cleavage from azlactone-activated polyacrylamide resin

Figure S27. HPLC-UV trace of photo cleavage of peptide 2 from streptavidin coated azlactone-activated polyacrylamide beads (Method A).

Figure S28. LR-MS spectra of unknown sideproduct A (left) m/z: 664.4 [M+2H]^2+ (calcd. m/z: 654.3, Δ = 20.2) and a mixture of the desired product (m/z: 654.3, calcd. m/z: 654.3) and other unknown sideproducts B (right).

6.2 Additional material of PCB characterization

Table S4. Fluorescent intensities of probes and moles calculated according to dilution series. Concentrations marked with an * are out of the linear range of measurements in the regression and are therefore not reliable.

<table>
<thead>
<tr>
<th>probe</th>
<th>fl. intensity (max)</th>
<th>moles [nmol]</th>
<th>conc. [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT b</td>
<td>101.84</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>W1 b</td>
<td>20.61</td>
<td>0.4</td>
<td>0.4*</td>
</tr>
<tr>
<td>W2 b</td>
<td>8.92</td>
<td>0.3</td>
<td>0.3*</td>
</tr>
<tr>
<td>W3 b</td>
<td>4.19</td>
<td>0.2</td>
<td>0.2*</td>
</tr>
<tr>
<td>W4 b</td>
<td>3.30</td>
<td>0.2</td>
<td>0.2*</td>
</tr>
<tr>
<td>W5 b</td>
<td>2.21</td>
<td>0.2</td>
<td>0.2*</td>
</tr>
<tr>
<td>Σ = 2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure S29. Fluorescent intensities of flow through (FT) and wash fractions (W1-5) upon incubation of peptide 2 with streptavidin coated agarose beads.

Table S5. Maximal fluorescent intensities of aliquots from 1 to 7 minutes (every minute one aliquot) from E1 b to E7 b with a new standard. Also given are moles calculated according to linear regression. All measurements were within the linear range.

<table>
<thead>
<tr>
<th>probe</th>
<th>fl. intensity (max)</th>
<th>moles [nmol]</th>
<th>conc. [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 b – 1 min</td>
<td>101.22</td>
<td>12</td>
<td>1.2</td>
</tr>
<tr>
<td>E2 b – 2 min</td>
<td>158.90</td>
<td>18</td>
<td>1.8</td>
</tr>
<tr>
<td>E3 b – 3 min</td>
<td>172.23</td>
<td>20</td>
<td>2.0</td>
</tr>
<tr>
<td>E4 b – 4 min</td>
<td>177.69</td>
<td>20</td>
<td>2.0</td>
</tr>
<tr>
<td>E5 b – 5 min</td>
<td>203.22</td>
<td>23</td>
<td>2.3</td>
</tr>
<tr>
<td>E7 b – 7 min</td>
<td>203.62</td>
<td>23</td>
<td>2.3</td>
</tr>
<tr>
<td>standard b</td>
<td>259.67</td>
<td>28</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Figure S30. Repetition of fluorescence measurements between 1 and 7 minutes of irradiated beads in comparison to a standard of the same amount and from the same stock.

7. Abbreviations

AcOH       acetic acid
Alloc      allyloxycarbonyl
BSA        bovine serum albumin
Conc.      concentration
Da         Dalton
DIC        diisopropylcarbodiimide
DIPEA      diisopropylethylamine
DMF        N,N-dimethylformamide
DTT        dithiotreitol
EDTA       ethylenediaminetetraacetic acid,
EPL        expressed protein ligation
eq         equivalents
ESI        electron spray ionization
Fmoc  fluorenylmethyloxycarbonyl
5,6-FAM  5,6-carboxyfluorescein
Gn  guanidine
h  hour
HATU  1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxido
hexafluorophosphonate
HBTU  N,N,N',N'-Tetramethyl-O-(1H-benzo[d]imidazol-1-yl)uronium hexafluorophosphonate, O-
(benzo[d]imidazol-1-yl)-N,N,N',N'-tetramethylyuronium hexafluorophosphonate
HCTU  1-[bis(dimethylamino)methylene]-5-chlorobenzotriazol-3-oxido hexafluorophosphonate,
N,N,N',N'-Tetramethyl-O-(6-chloro-1H-benzo[d]imidazol-1-yl)uronium hexafluorophosphonate
HOAt  1-hydroxy-7-azabenzotriazole
HOBt  hydroxybenzotriazole
HPLC  high performance liquid chromatography
HRMS  high resolution mass spectrometry
IPTG  isopropyl β-D-1-thiogalactoside
ivDde  (1-(4,4-Dimethyl-2,6-dioxocyclohex-1-ylidene)-3-ethyl)(Dde) and 1-(4,4-Dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl
k  kilo
LC  liquid chromatography
LR-MS  low-resolution mass spectrometry
MALDI  matrix-assisted laser desorption/ionization
MESNa  2-mercaptoethane sulfonate Na
MeCN  acetonitrile
MHz  megahertz
min  minutes
MPAA  4-mercaptophenylacetic acid
MS  mass spectrometry
NCL  native chemical ligation
NMM  N-methylmorpholine
NMR  nuclear magnetic resonance
NMP  N-methyl-2-pyrrolidone
PBS  phosphate buffered saline
PBS-T  phosphate buffered saline - tween
PC  peroxodase
POD  peroxidase
RP  reversed phase
RT  room temperature
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
SI  supporting information
TCEP  tris(2-carboxyethyl)phosphine
TFA  trifluoroacetic acid
TGR  tentagel resin
TIS  tris(2-carboxyethyl)phosphine
TOF  time of flight
Trt  trityl
TUV  tropospheric ultraviolet
U  units
UPLC  ultra performance liquid chromatography
UV  Ultraviolet
VA-044  2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride

8. References