Electronic Supporting Information

for

“Chemoselective triazole-phosphonamidate conjugates suitable for photorelease”

1) General information 2

2) Synthesis of central building block 1
   2.1 Synthesis route via PCl₃
   2.2 Synthesis route via bis(diisopropylamino)chlorophosphine 3

3) Synthesis of model azides 2a-2c, 2e 4

4) Synthesis of biotinol S1 and biotin azide 2d 5

5) Peptide synthesis
   5.1 Peptide 2f 7
   5.2 Peptide 2g
   5.3 Peptide 2h
   5.4 Peptide 2i

6) General synthesis of borane-protected triazole-phosphonites
   6.1 Compound 3a
   6.2 Compound 3b
   6.3 Compound 3c
   6.4 Compound 3d
   6.5 Compound 3e 10

7) Synthesis of phosphoramidates
   7.1 Compound 4a
   7.2 Compound 4b
   7.3 Compound 4c
   7.4 Compound 4d
   7.5 Compound 4e
   7.6 Compound 4f 13

8) Irradiation of phosphonamidate 4f
   8.1 Release of amine 5 via phosphonamidate acid 6
   8.2 Release of amine 5 in 0.1% TFA/H₂O
   8.3 Stability of phosphonamidate 4f in 0.1% TFA/H₂O
   8.3 Standard curve for quantification
   8.4 Quantification of amine release 18

9) Immobilization & irradiation experiments
   9.1 General procedure
   9.2 Phosphonamidate 4f
   9.3 pH evaluation for release of peptide
   9.4 Streptavidin beads degradation in 0.1% TFA/H₂O 21

10) NMR spectra 24
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 (Em = 495 nm, Ex = 517 nm). The flow rate was 1 mL/min.

**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 or QDaTM detector with an Acquity UPLC-BEH C18 1.7 μm, 2.1 x 50 mm RP column with a flow rate of 0.6 mL/min (Water Corp., USA). 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 H Tec Spum column (Macherey-Nagel GmbH & Co. Kg, Germany). The following gradient was used: (A = H2O + 0.1% TFA, B = MeCN + 0.1% TFA) flow rate 30 mL/min, 10% B 0-5 min, 10-100% B 5-55 min, 100 % B 35-40 min.

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

**NMR** spectra were either recorded with a Bruker Ultrashield 300 MHz spectrometer or a Bruker Ultrashield 600 MHz (both Bruker Corp., USA) at ambient temperature. The chemical shifts are reported in ppm relative to the residual solvent peak.

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

**UV irradiation** was carried out with a LOT Hg (Xe) arc lamp (LOT-QuantumDesign GmbH, D-64293 Darmstadt, Germany) using a 297 nm filter from the Andover Incorporation with 15 % transmission. Probes were positioned in 20 cm distance and irradiated while stirring.

**Streptavidin beads** (“Streptavidin Sepharose High Performance”) were purchased from GE Healthcare and have a binding capacity of 300 nmol/mL.
2) Synthesis of central building block 1

Route A via PCl₃:

PCl₃ (0.41 g, 3 mmol) was cooled to -96 °C and triethylamine (0.83 mL, 6 mmol, 2 eq.) was added dropwise. 2-Nitrobenzylalcohol (0.92 g, 6 mmol, 2 eq., dissolved in 1:9 THF:Toluene, 36 mL) was added, stirred for 10 mins at -96 °C and was then warmed to room temperature overnight. The reaction was then cooled to 0 °C and triethylamine (0.11 mL, 0.75 mmol, 0.25 eq.) and 2-nitrobenzylalcohol (0.23 g, 1.5 mmol, 0.5 eq., dissolved in 1:9 THF:Toluene, 9 mL) was slowly added. The reaction was warmed to room temperature and stirred for 2 hrs. After cooling down to -96 °C again, ethynylmagnesium bromide (9 mL, 4.5 mmol, 1.5 eq., 0.5 M in THF) was added dropwise and the reaction was stirred under cooling for 10 mins before it was warmed to room temperature and allowed to react for 2 hrs. BH₃·THF (4.5 mL, 4.5 mmol, 1.5 eq., 1 M in THF) was then added under cooling at 0 °C and stirred for 2 hrs at room temperature. The solvent was removed in vacuo and the compound was purified by silica gel column chromatography (Hex 5: EtOAc 1) to yield the desired product 1 as white powder (0.45 g, 1.2 mmol, 40 %).

Route B via bis(diisopropylamino)chlorophosphine:

Bis(diisopropylamino)chlorophosphine (0.2 g, 0.79 mmol) was suspended in dry THF (1 mL) and ethynylmagnesium bromide (1.6 mol, 0.8 mmol, 1.01 eq., 0.5 M in THF) was added dropwise at 0 °C, before the reaction was stirred for 2 hrs at room temperature. 2-Nitrobenzylalcohol (0.3 g, 1.98 mmol, 2.5 eq., dissolved in 2 mL dry MeCN) and tetrazole (9 mL, 4 mmol, 6.4 eq., 0.45 M in MeCN) was added at 0 °C and stirred for further 2 hrs at room temperature. BH₃·THF (1.1 mL, 1.1 mmol, 1.4 eq., 1 M in THF) was then added at 0°C and reacted for 2 hrs at room temperature. The solvent was removed in vacuo and the compound was purified by silica gel column chromatography (Hex 5: EtOAc 1) to yield the desired product 1 as white powder (0.190 mmol, 0.51 mmol, 65 %).

Experimental data:

¹H-NMR (CDCl₃, 300 MHz) δ 8.16 (d, J = 8.1 Hz, 2H, C5-H), 7.79-7.70 (m, 2H), 7.56-7.51 (m, 1H, C4-H), 5.66-5.53 (m, 4H, C7-2H), 3.32 (d, J = 7.35, 1H, C9-H)

¹³C-NMR (CDCl₃, 75 MHz) δ 146.7 (s, C6), 134.4 (s, C2), 132.0 (d, J = 8 Hz, C8), 129.2 (s, C3), 128.6 (s, C4), 125.3 (s, C5), 94.9 (d, J = 25 Hz, C9), 66.5 (d, J = 5 Hz, C7)

³¹P-NMR (CDCl₃, 121 MHz) δ 108.9 (dd, J = 360, 123 Hz)

HRMS (ESI, m/z) calcd for C₁₆H₁₆BN₂O₆PNa [M+Na]⁺: 397.0737, found: 397.0736
3) Synthesis of model azides

3.1 3-Phenylpropyl azide 2a

3-Phenylpropyl bromide (2 g, 10 mmol) was diluted in dry DMSO (20 mL) and sodium azide (1.2 g, 18 mmol, 1.8 eq.) was added. The reaction was stirred overnight at room temperature. The reaction mixture was diluted with water (50 mL) and extracted with Et<sub>2</sub>O (5 x 50 mL). The organic layer was washed with H<sub>2</sub>O (3 x 50 mL) and brine. It was dried with MgSO<sub>4</sub>, filtered and the solvent was removed in vacuo. Product 2a was isolated as oil (1.66g, 10 mmol, 100 %).

<sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 7.31 (m, 2H), 7.23 – 7.16 (m, 3H), 3.30 (t, <i>J</i> = 6.8 Hz, 2H), 2.72 (t, <i>J</i> = 7.6 Hz, 2H), 2.01 – 1.83 (m, 2H).

<sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ 140.75, 128.43, 128.37, 126.05, 50.56, 32.68, 30.36.

All values were according to literature<sup>1</sup>.

3.2 Benzyl azide 2b

Benzyl bromide (1.71 g, 10 mmol) was dissolved in 4:1 acetone:H<sub>2</sub>O (20 mL) and sodium azide (1.9 g, 30 mmol, 3 eq.) was added. The reaction was stirred overnight at room temperature. The reaction mixture was extracted with Et<sub>2</sub>O (3 x 50 mL) and the organic phase was washed with H<sub>2</sub>O (50 mL) and brine (50 mL). The organic phase was dried with MgSO<sub>4</sub>, filtered and the solvent was removed in vacuo to yield the desired product 2b as oil (1.3 g, 9.8 mmol, 98 %).

<sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 7.48 – 7.29 (m, 5H), 4.35 (s, 2H).

<sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ 135.29, 128.77, 128.25, 128.16, 54.73.

All values were according to literature<sup>2</sup>.

3.3 Phenyl azide 2c

Aniline (3.1 g, 33 mmol) was dissolved in H<sub>2</sub>O (25 mL) and conc. H<sub>2</sub>SO<sub>4</sub> (10.5 mL) was added at 0 °C. NaNO<sub>2</sub> (2.6 g, 38 mmol, 1.15 eq.) dissolved in H<sub>2</sub>O (15 mL), and added dropwise. Hexane (50 mL), followed by sodium azide (2.6 g, 40 mmol, 1.2 eq.) in H<sub>2</sub>O (10 mL) was added and the reaction
was stirred for 3 hrs at room temperature. The organic phase was separated, washed with H₂O (3 x 50 mL), dried with MgSO₄ and filtered. The solvent was removed in vacuo, yielding compound 3c as oil (2.75 g, 23.1 mmol, 70 %).

¹H NMR (CDCl₃, 300 MHz) δ 7.41 – 7.32 (m, 2H), 7.20 – 7.11 (m, 1H), 7.08 – 7.01 (m, 2H).

¹³C NMR (CDCl₃, 75 MHz) δ 140.10, 129.88, 124.99, 119.14.

All values were according to literature³.

3.4 OH-PEG-N₃ 2e

![OH-PEG-N₃ 2e](image)

Compound 2e was synthesized starting from commercially available Cl-TEG-N₃ using a previously published protocol.

¹H NMR (CDCl₃, 300 MHz) δ 3.75 – 3.70 (m, 2H), 3.70 – 3.64 (m, 6H), 3.63 – 3.58 (m, 2H), 3.39 (t, J = 5.0 Hz, 2H).

¹³C NMR (CDCl₃, 75 MHz) δ 72.59, 70.74, 70.46, 70.14, 61.84, 50.74.

All values were according to literature⁴.

4) Synthesis of biotin azide 2d

![Synthesis of biotin azide 2d](image)

4.1 Synthesis of biotinol S1

Biotin (5 g, 20.5 mmol) was suspended in dry MeOH (60 mL) and thionyl chloride (7.4 g, 4.5 mL, 62 mmol (3 eq.) was added dropwise at 0 °C. The reaction was stirred overnight at room temperature and the solvent was removed in vacuo. The biotin methyl ester was used without further purification. It was suspended in dry THF (300 mL) and a suspension of LiAlH₄ (3.1 g, 82 mmol, 4 eq.) in dry THF
(120 mL) was added dropwise at 0 °C. The reaction was stirred at room temperature overnight, before it was quenched with MeOH (50 mL) and H₂O (50 mL) at 0 °C. Afterwards a solution of conc. Na₂SO₄ (50 mL) was added and the reaction mixture was stirred for another 20 mins. The solvent was removed in vacuo and a 1:4 MeOH:DCM (20 mL) mixture was added and stirred for 1 hr. The white solid was filtered off over celite and washed with 1:4 MeOH:DCM and the filtrate was reduced in vacuo. Product S1 was isolated by silica gel column chromatography (DCM: 10% MeOH) as white powder (2.91 g, 12.6 mmol, 62 %, over two steps).

¹H NMR (DMSO, 300 MHz) δ 6.44 (s, 1H, NH), 6.36 (s, 1H, NH), 4.35-4.28 (m, 2H, OH, C9-H), 4.17 – 4.06 (m, 1H, C7-1H), 3.37 (m, 2H, C1-2H), 3.10 (m, 1H, C6-H), 2.82 (dd, J = 12.4, 5.0 Hz, 1H, C10-1H), 2.57 (d, J = 12.4 Hz, 1H, C10-1H), 1.68-1.52 (br s, 1H, OH), 1.52 – 1.20 (m, 8H, C2-5).

¹³C NMR (DMSO, 75 MHz) δ 162.74, 61.10, 60.67, 59.21, 55.59, 32.36, 28.57, 28.37, 25.58. Carbon C6 is hidden underneath the signal of the deuterated solvent.

HRMS (ESI, m/z) calcd for C₁₀H₁₉N₂O₂S [M+H]^+: 231.1167, found: 231.1161

Synthesis of biotin azide 2d

Biotinol (2.91 g, 12.6 mmol) was dissolved in dry pyridine (50 mL) and tosyl chloride (5.76 g, 30.2 mmol, 2.4 eq.) was added in two portions at 0 °C. The reaction was stirred overnight at room temperature. The reaction was diluted with DCM (100 mL) and the organic phase was extracted with 1 M H₂SO₄ (3 x 75 mL), brine (50 mL) and was dried with MgSO₄. After filtration, the solvent was removed in vacuo and biotin tosylated was used without further purification. The residue was dissolved in dry DMF (50 mL) and biotin azide (3.81 g, 60 mmol, 5 eq.) was added. The reaction was stirred at 80 °C overnight and after removal of the solvent in vacuo, the reaction was purified by silica gel column chromatography (DCM: 5 % MeOH). Compound 3d was isolated as white powder (1.07 g, 4.19 mmol, 33 % in two steps).

¹H NMR (CDCl₃, 300 MHz) δ 5.33 (s, 1H, NH), 5.07 (s, 1H, NH), 4.54 (dd, J = 7.7, 5.1 Hz, 1H, C7-H), 4.33 (dd, J = 8.1, 4.7 Hz, 1H, C9-H), 3.29 (t, J = 6.8 Hz, 2H, C1-2H), 3.21-3.15 (m, 1H, C6-H), 2.95 (dd, J = 12.8, 5.0 Hz, 1H, C10-1H), 2.75 (d, J = 12.8 Hz, 1H, C10-1H), 1.79 – 1.46 (m, 8H, C2-5).

¹³C NMR (CDCl₃, 75 MHz) δ 163.57, 61.97, 60.01, 55.51, 51.29, 40.52, 28.53, 28.44, 26.57. Due to overlapping carbon resonances in the aliphatic chain, one peak is missing.

HRMS (ESI, m/z) calcd for C₁₀H₁₉N₂O₂S [M+H]^+: 256.1232, found: 256.1223
5) Peptide synthesis

Peptides were either synthesised manually or using the automated PTI Tribute (Gyros Protein Technologies) via standard Fmoc-based conditions (fast-Fmoc protocol with HBTU/HOBt/NMM). Fmoc deprotection was carried out using 20% piperidine in DMF (3 x 3 min). Single couplings were performed for all standard amino acids (aa), using 5 eq. aa, 5 eq. HBTU, 5 eq. HOBt and 5 eq. DIPEA. Unnatural amino acids were incorporated using 2 eq. aa, 2 eq. HATU and 4 eq. DIPEA. Peptides were synthesised on Rink Amide resin and, if not specified otherwise, in 0.1 mmol scale. All peptides were cleaved using 95:2.5:2.5 TFA:TIS:H₂O for 2 hrs. Before purification, all peptides were precipitated in cold diethylether. The crude peptides were all purified by preparative HPLC using a gradient from 10-90% in 50 mins (Solvent A: H₂O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA). All peptides were characterized either by analytical HPLC or by UPLC-MS.

Peptide 2f:

Column: Agilent Eclipse XDB C18, 4.6 x 250 mm (analytical HPLC)
Gradient: 5-95 % B in 30 mins (Solvent A: H₂O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)
Retention time: 13.2 min
Yield: 61.9 mg, 0.0544 mmol, 54.4%

HRMS (ESI, m/z) calcd for C₅₅H₇₈N₁₈O₁₃ [M+2H]²⁺: 569.2992, found: 569.3021
Peptide 2g:

![Peptide 2g](image)

Column: Agilent Eclipse XDB C18, 4.6 x 250 mm (analytical HPLC)
Gradient: 5-95 % B in 30 mins (Solvent A: H$_2$O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)
Retention time: 15.4 min
Yield: 59.8 mg, 0.0498 mmol, 49.8 %

HRMS (ESI, m/z) calcld for C$_{50}$H$_{76}$N$_{18}$O$_{13}$ [M+2H]$^{2+}$: 600.3071, found: 600.3097

Peptide 2h:

5,6-Carboxyfluorescein was installed via Lys(ivdee), which was selectively deprotected on-resin after capping of the peptide with azidobenzoic acid using hydrazine solution (2 % in DMF, 5 x 2 min). 5,6-Carboxyfluorescein was coupled by using 2 eq. of fluorophore, 2 eq. of HATU, 2 eq. of HOBT.H$_2$O and 4 eq. of DIPEA.
Column: Phenomenex Synergi 4u Hydro-RP, 4.6 x 250 mm (analytical HPLC)
Gradient: 0-60 % B in 25 mins (Solvent A: H₂O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)
Retention time: 24.7 min
Yield: 26 mg, 0.017 mmol, 8.5 % (0.2 mmol scale)

HRMS (ESI, m/z) calcd for C₅₅H₇₈N₁₈O₁₃ [M+2H]⁺: 779.3309, found: 779.3339

Peptide 2i:
Peptide 2i was synthesized by reduction of peptide 2h (4 mg, 2.6 µmol) with TCEP (1.5 mg, 5.2 µmol, 2 eq.) for 1 hr in 20 mM NH₄HCO₃ with 10 % MeCN (1 mL, 2.6 mM). The peptide was purified over a SepPak column (1 g) and the product was isolated as a yellow powder in quantitative yield.

Column: Phenomenex Synergi 4u Hydro-RP, 4.6 x 250 mm (analytical HPLC)
Gradient: 0-60 % B in 25 mins (Solvent A: H₂O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)
Retention time: 22.4 min (Remark: The double peak arises from the two diastereoisomers of the 5,6-carboxyfluorescein. They both have the same mass.)

HRMS (ESI, m/z) calcd for C₅₅H₇₈N₁₈O₁₃ [M+2H]²⁺: 766.3357, found: 766.3388

6) General procedure for the synthesis of triazole-phosphonites:

To a solution of phosphonite 1 in degassed MeCN (0.03 M), the corresponding azide (1 eq.), 2,6-lutidine (3 eq.), DIPEA (3 eq.) and CuI (30 %) were added. The reaction was stirred overnight at room temperature and purified by silica gel column chromatography (3 Hex: 1 EtOAc), if not otherwise specified.

6.1 Compound 3a

Isolated yield: 93 %

¹H NMR (CDCl₃, 300 MHz) δ 8.10 (dd, J = 8.2, 1.3 Hz, 2H, C4-H), 8.01 (s, 1H, C9-H), 7.80 (dd, J = 7.8, 1.4 Hz, 2H, C7-H), 7.66 (td, J = 7.6, 1.3 Hz, 2H, C6-H), 7.47 (td, J = 7.8, 1.5, 2H, C5-H), 7.35 – 7.28 (m, 2H, C14-H, C18-H), 7.25 – 7.15 (m, 3H, C15-C17-H), 5.68 – 5.50 (m, 4H, C1-2H), 4.43 (t, J = 7.2 Hz, 2H, C10-2H), 2.69 (t, J = 7.4 Hz, 2H, C12-2H), 2.39 – 2.25 (m, 2H, C11-2H).

¹³C NMR (CDCl₃, 75 MHz) δ 146.83 (C3), 139.74 (C13), 134.29 (C6), 132.43 (d, J = 11 Hz, C9), 130.92 (d, J = 34 Hz, C8), 129.09 (C7), 128.89 (C14), 128.87 (C15), 128.56 (C5), 126.70 (C16), 125.13 (C4), 66.34 (d, J = 6 Hz, C1), 49.98 (C12), 32.55 (C11), 31.51 (C10).

³¹P-NMR (CDCl₃, 121 MHz) δ 122.32 (dd, J = 153.2, 58.1)

HRMS (ESI, m/z) calcd for C₂₅H₂₄BN₅O₆P [M+H]+: 536.1871, found: 536.1858
6.2 Compound 3b

Isolated yield: 87 %

$^1$H-NMR (CDCl$_3$, 300 MHz) $\delta$ 8.09 (dd, $J$ = 8.2, 1.3 Hz, 2H, C4-H), 7.96 (s, 1H, C9-H), 7.78 (dd, $J$ = 7.9, 1.3 Hz, 2H, C7-H), 7.64 (td, $J$ = 7.6, 1.3 Hz, 2H, C6-H), 7.51 – 7.46 (m, 2H, C5-H), 7.44 – 7.39 (m, 3H, arom. H), 7.35 – 7.27 (m, 2H, arom. H), 5.66 – 5.48 (m, 6H, C1-2H, C10-2H).

$^{13}$C-NMR (CDCl$_3$, 75 MHz) $\delta$ 146.76 (C3), 139.52 (d, $J$ = 103.5, C2), 134.28 (C6), 133.52 (C11), 132.43 (d, $J$ = 6.75 Hz, C8), 130.72 (d, $J$ = 33.8 Hz, C9), 129.52 (C5), 129.39 (C13), 129.05 (C7), 128.81 (C12), 128.51 (C14), 125.10 (C4), 66.32 (d, $J$ = 3.75 Hz, C1), 54.59 (C10).

$^{31}$P-NMR (CDCl$_3$, 121 MHz) $\delta$ 122.5 – 121.4 (m)

HRMS (ESI, $m/z$) calcd for C$_{23}$H$_{23}$BN$_5$O$_6$PNa [M+Na]$^+$: 530.1377, found: 530.1379

6.3 Compound 3c

Isolated yield: 71 %

$^1$H-NMR (CDCl$_3$, 300 MHz) $\delta$ 8.48 (s, 1H, C9), 8.10 (dd, $J$ = 8.2, 1.3 Hz, 2H, C4-H), 7.83 (dd, $J$ = 7.8, 1.4 Hz, 2H, C7-H), 7.80 – 7.73 (m, 2H, C11-H), 7.68 (td, $J$ = 7.6, 1.3 Hz, 2H, C6-H), 7.61 – 7.45 (m, 5H, C12-2H, C13-H, C5-2H), 5.64 (m, 4H, C1-2H).

$^{13}$C-NMR (CDCl$_3$, 75 MHz) $\delta$ 146.84 (C3), 139.96 (d, $J$ = 103.5 Hz, C2), 136.23 (C10), 134.32 (C6), 132.31 (d, $J$ = 7.5 Hz, C8), 130.13 (C12), 129.83 (C5), 129.16 (C7), 129.03 (d, $J$ = 33.75 Hz, C9), 128.91 (C13), 125.14 (C4), 121.12 (C11), 66.50 (d, $J$ = 3.75 Hz, C1).

$^{31}$P-NMR (CDCl$_3$, 121 MHz) $\delta$ 123.43-121.12 (m)

HRMS (ESI, $m/z$) calcd for C$_{22}$H$_{21}$BN$_5$O$_6$PNa [M+Na]$^+$: 516.1221, found: 516.1216
6.4 Compound 3d

Compound 3d was isolated by silica gel column chromatography using 5 % MeOH in DCM as eluent.

Isolated yield: 81 %

$^1$H NMR (DMSO, 300 MHz) $\delta$ 8.84 (s, 1H, C9-H), 8.12 (dd, $J = 8.2, 1.2$ Hz, 2H, C6-H), 7.85 – 7.70 (m, 4H, C4-H, C3-H), 7.62 (dd, $J = 8.1, 1.7$ Hz, 2H, C5-H), 5.60 – 5.42 (m, 4H, C1-2H), 4.47 (t, $J = 7.1$ Hz, 2H, C10-2H), 4.29 (dd, $J = 7.7, 4.8$ Hz, 1H, C18-1H), 4.12 (dd, $J = 7.8, 4.4$ Hz, 1H, C16-1H), 3.38 – 3.05 (m, 9H, C12-2H, C13-H, C14-2H, OH), 3.59 – 3.53 (m, 2H, C15-2H).

$^{13}$C NMR (DMSO, 75 MHz) $\delta$ 162.71 (C17), 146.62 (C7), 137.15 (d, $J = 109$ Hz, C2), 134.38 (C4), 131.75 (d, $J = 6.8$ Hz, C9), 129.41 (C3), 128.52 (C5), 124.90 (C6), 65.45 (d, $J = 4.1$ Hz, C1), 61.00 (C16), 59.19 (C18), 55.42 (C15), 49.74 (C10), 40.34 (C19), 29.38 (C11), 28.09 (C13), 27.91 (C14), 25.81 (C12).

$^{31}$P-NMR (CDCl$_3$, 121 MHz) $\delta$ 122.85 – 120.90 (s br)

HRMS (ESI, $m/z$) calcd for C$_{10}$H$_{19}$N$_2$O$_2$SNa [M+Na]$^+$: 652.1891, found: 652.1882

6.5 Compound 3e

Isolated yield: 81 %

$^1$H NMR (CDCl$_3$, 300 MHz) $\delta$ 8.31 (s, 1H, C9-H), 8.09 (dd, $J = 8.2, 1.3$ Hz, 2H, C4-H), 7.81 (dd, $J = 7.8, 1.3$ Hz, 2H, C7-H), 7.67 (td, $J = 7.6, 1.3$ Hz, 2H, C6-H), 7.52 – 7.43 (m, 2H, C5-H), 5.58 (qd, $J = 14.9, 7.5$ Hz, 4H, C1-2H), 4.65 (t, $J = 4.9$ Hz, 2H, C10-2H), 3.92 (dd, $J = 5.5, 4.4$ Hz, 2H, C11-2H), 3.78 – 3.60 (m, 9H, C12-2H, C13-H, C14-2H, OH), 3.59 – 3.53 (m, 2H, C15-2H).
$^{13}$C NMR (CDCl$_3$, 75z MHz) δ 146.77 (C3), 134.31 (C6), 132.48 (d, $J = 7.5$ Hz, C9), 132.32 (d, $J = 33.8$ Hz, C8), 129.04 (C7), 128.83 (C5), 125.09 (C4), 72.59, 70.78, 70.40, 69.06 (C11), 66.26 (d, $J = 4.5$ Hz, C1) 61.79, 50.64 (C10).

$^{31}$P-NMR (CDCl$_3$, 121 MHz) δ 123.50 – 121.25 (s br)

HRMS (ESI, $m/z$) calcd for C$_{22}$H$_{29}$BN$_5$O$_9$PNa [M+Na]$^+$: 572.1694, found: 572.1686

7) Small-molecule phosphoramidates

7.1 Compound 4a

Triazole-phosphonite 3a (70 mg, 0.130 mmol) was dissolved in dry, degassed DMF (0.65 mL, 0.2 M) under argon and DABCO (21 mg, 0.195 mmol, 1.5 eq.) was added. The reaction was heated to 80 °C for 4 hrs. The temperature was decreased to 60 °C and azide 2a (63 mg, 0.39 mmol, 3 eq.) was added for 5 hrs. The solvent was removed by lyophilisation in 50:50 H$_2$O:MeCN and the crude was purified by silica gel column chromatography (20 % acetone in hexane $→$ EtOAc). Product 4a was isolated as colourless oil (57 mg, 0.110 mmol, 85 %).

$^1$H NMR (CDCl$_3$, 300 MHz) δ 8.12 (d, $J = 8.2$ Hz, C4-H), 8.03 (s, 1H, C9-1H), 7.88 (d, $J = 7.8$ Hz, 1H, C7-1H), 7.67 (t, $J = 7.6$ Hz, 1H, C6-1H), 7.47 (t, $J = 7.7$ Hz, 1H, C5-1H), 7.36 – 7.10 (m, 10H, C14-C16-5H, C21-C23-5H), 5.62 – 5.42 (m, 2H, C1-2H), 4.42 (t, $J = 7.1$ Hz, 2H, C10-2H), 3.34 (m, 1H, NH), 3.15 (m, 2H, C17-2H), 2.66 (m, C19-2H, C21-2H), 2.29 (m, $J = 7.3$ Hz, 2H, C11-2H), 1.86 (p, $J = 7.3$ Hz, 2H, C18-2H).

$^{13}$C-NMR (CDCl$_3$, 150 MHz): δ 146.74 (C3), 141.39 (C20), 139.85 (C13), 139.30 (d, $J = 436$ Hz, C2) 134.21 (C6), 133.25 (d, $J = 8.25$ Hz, C9), 129.9 (d, $J = 32$ Hz, C8), 129.68 (C7), 128.81 (C5), 128.64 (C14), 128.62 (C15), 128.52 (C21), 128.43 (C22), 126.60 (C16), 126.04 (C23), 124.99 (C4), 63.40 (d, $J = 3.8$ Hz, C1), 49.75 (C12), 40.55 (C19), 33.58 (C18), 33.50 (C17), 32.51 (C11), 31.60 (C10)

$^{31}$P-NMR (CDCl$_3$, 121 MHz): 12.5

HRMS (ESI, $m/z$) calcd for C$_{10}$H$_{10}$N$_2$O$_2$S [M+Na]$^+$: 542.1928, found: 542.1932
7.2 Compound 4b

Triazole-phosphonite 3a (22.5 mg, 0.042 mmol) was dissolved in dry, degassed DMF (0.2 mL, 0.2 M) under argon and DABCO (7 mg, 0.062 mmol, 1.5 eq.) was added. The reaction was heated to 80 °C for 4 hrs. The temperature was decreased to 60 °C and azide 2b (17 mg, 0.13 mmol, 3 eq.) was added for 5 hrs. The solvent was removed by lyophilisation in 50:50 H2O:MeCN and the crude was purified by silica gel column chromatography (20 % acetone in hexane → EtOAc). Product 4b was isolated as colourless oil (18 mg, 0.036 mmol, 86 %).

1H NMR (CDCl3, 600 MHz) δ 8.10 (dd, J = 8.2, 1.3 Hz, 1H, C4-1H), 7.99 (s, 1H, C9-1H), 7.79 (dd, J = 7.9, 1.3 Hz, 1H, C7-1H), 7.63 (dd, J = 7.7, 1.3 Hz, 1H, C6-1H), 7.47 – 7.43 (m, 1H, C5-1H), 7.34 – 7.26 (m, 6H, arom. H), 7.25 – 7.21 (m, 2H, arom. H), 7.19 – 7.16 (m, 2H, arom. H), 5.53 – 5.45 (m, 2H, C1-2H), 4.41 (td, J = 7.1, 2.8 Hz, 2H, C10-2H), 4.33 – 4.28 (m, 2H, C17-2H), 2.68 (t, J = 7.4 Hz, 2H, C12-2H), 2.32 – 2.26 (m, 2H, C11-2H).

13C-NMR (CDCl3, 150 MHz): δ 146.78 (C3), 139.88 (C13), 139.44 (d, J = 876 Hz, C2), 139.31 (d, J = 24 Hz, C18), 134.21 (C6), 133.21 (d, J = 36 Hz, C8), 130.00 (d, J = 126 Hz, C9), 128.87 (C5), 128.75 (C6), 128.65 (C14), 128.56 (C15), 127.60 (C19), 127.56 (C21), 126.67 (C16), 125.00 (C4), 63.65 (d, J = 18 Hz, C1), 49.81 (C10), 44.84 (C17), 32.56 (C12), 31.60 (C11).

31P-NMR (CDCl3, 242 MHz) δ 12.27

HRMS (ESI, m/z) calcd for C25H26N5O4PNa [M+Na]+: 514.1620, found: 514.1625

7.3 Compound 4c

Triazole-phosphonite 3a (22.5 mg, 0.042 mmol) was dissolved in dry, degassed DMF (0.2 mL, 0.2 M) under argon and DABCO (7 mg, 0.062 mmol, 1.5 eq.) was added. The reaction was heated to 80 °C for 4 hrs. The temperature was decreased to 60 °C and azide 2c (15 mg, 0.13 mmol, 3 eq.) was added
for 5 hrs. The solvent was removed by lyophilisation in 50:50 H₂O:MeCN and the crude was purified by silica gel column chromatography (20 % acetone in hexane → EtOAc). Product 4c was isolated as colourless oil (12 mg, 0.021 mmol, 50 %).

³¹H NMR (CDCl₃, 600 MHz) δ 8.11 (dd, J = 8.1, 1.1 Hz, 1H, C4-1H), 8.04 (s, 1H, C9-1H), 7.90 (d, J = 7.9 Hz, 1H, C7-1H), 7.65 (td, J = 7.6, 1.4 Hz, 1H, C6-1H), 7.46 (t, J = 7.6 Hz, 1H, C5-1H), 7.29 (m, 2H, C14-2H), 7.24 − 7.18 (m, 3H, C16-H, C15-2H), 7.16 − 7.11 (m, C18-1H, C19-1H, C20-1H, C21-1H), 6.96 (td, J = 7.3, 1.1 Hz, 1H, C20-H), 5.93 (d, J = 7.8 Hz, 1H, NH), 5.70 − 5.57 (m, 2H, C1-2H), 4.39 (t, J = 7.1 Hz, 2H, C10-2H), 2.63 (td, J = 7.4, 2.4 Hz, 2H, C12-2H), 2.27 (p, J = 7.3 Hz, 2H, C11-2H).

¹³C-NMR (CDCl₃, 150 MHz) δ 146.82 (C3), 139.30 (d, J = 147 Hz, C2), 134.30 (C6), 132.75 (d, J = 9 Hz, C8), 130.63 (d, J = 33 Hz, C9), 129.56 (C7), 128.89 (C5), 128.85 (C19/C21), 128.55 (C15/C14), 126.66 (C16), 125.07 (C4), 122.78 (C20), 118.74 (C18/22), 118.69 (C18/22), 64.13 (d, J = 15 Hz, C1), 49.90 (C10), 32.49 (C11), 31.49 (C12).

³¹P-NMR (CDCl₃, 242 MHz) δ 6.68

HRMS (ESI, m/z) calcd for C₂₄H₂₄N₅O₄PNa [M+Na]: 500.1464, found: 500.1470

7.4 Compound 4d

Triazole-phosphonite 3d (3 mg, 4.8 µmol) was dissolved in dry, degassed DMF (0.8 mL, 7.5 mM) under argon and DABCO (0.8 mg, 7.2 µmol, 1.5 eq.) was added for 4 hrs at 80 °C, after which 0.4 mL of the solution was added to peptide 2f (1.1 mg, 0.96 µmol, 0.2 eq.) and the reaction was stirred for 16 hrs at 50 °C, before the remaining deprotected triazole-phosphonite (0.4 mL) was added and stirred for another 24 hrs. The reaction was monitored by UPLC-MS. Afterwards, the reaction was lyophilized with 50:50 H₂O:MeCN and purified by semi-preparative HPLC. Compound 4d was isolated as white powder (0.84 mg, 0.53 µmol, 55 %).

![Graph showing UPLC retention time](image)

Column: Acquity UPLC-BEH C18 1.7 µm, 2.1x 50 mm RP, (UPLC-MS)
Gradient: 10-95 % B in 8 mins (Solvent A: H₂O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)
Retention time: 2.88 min

LRMS (ESI, m/z): calcd for C₆₉H₁₀₃N₂₂O₁₈PS [M+2H]²⁺: 795.87, found: 795.85
7.5 Compound 4e

Triazole-phosphonite 3d (3 mg, 4.8 µmol) was dissolved in dry, degassed DMF (0.8 mL, 6 mM) under argon and DABCO (0.8 mg, 7.2 µmol, 1.5 eq.) was added for 4 hrs at 80 °C. The solution was cooled to room temperature and peptide 2g (1.2 mg, 0.96 µmol, 0.2 eq.) was added. The reaction was stirred for 24 hrs at room temperature. The reaction was monitored by UPLC-MS. Afterwards, the reaction was lyophilized with 50:50 H$_2$O:MeCN and purified by semi-preparative HPLC. Compound 4d was isolated as white powder (1 mg, 0.61 µmol, 63 %).

![UV spectrum](image)

Column: Acquity UPLC-BEH C18 1.7 µm, 2.1x 50 mm RP (UPLC-MS)
Gradient: 5-95 % B in 2.5 mins (Solvent A: H$_2$O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)
Retention time: 1.97 min

HRMS (ESI, m/z): calcd for C$_{74}$H$_{105}$N$_{22}$O$_{18}$PS [M+2H]$^{2+}$: 826.3712, found: 826.3752

7.6 Compound 4f

Triazole-phosphonite 3d (5 mg, 8 µmol) was dissolved in dry, degassed DMF (0.8 mL, 6 mM) under argon and DABCO (1.1 mg, 12 µmol, 1.5 eq.) was added for 4 hrs at 80 °C. Peptide 2g (2.5 mg, 1.6 µmol, 0.2 eq.) was added at 37 °C and stirred overnight. The reaction was monitored by UPLC-MS. Afterwards, the reaction was lyophilized with 50:50 H$_2$O:MeCN and purified by semi-preparative HPLC. Compound 5 was isolated as yellow powder (1.2 mg, 0.59 µmol, 37 %).
Column: Phenomenex Synergi 4u Hydro-RP, 4.6 x 250 mm (analytical HPLC)
Gradient: 0-60 % B in 25 mins (Solvent A: H$_2$O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)
Retention time: 26.0 min

HRMS (ESI, m/z): calcd for C$_{95}$H$_{116}$N$_{22}$O$_{24}$PS [M+3H]$^{3+}$: 670.9336, found: 670.9361
8. Irradiation of phosphonamidate 4f

Figure S1: Photocleavage of phosphonamidate 4f in two steps. In the first step, 2-nitrosobenzaldehyde is released. In the second step, the reaction solution is acidified and the P-N bond is cleaved to release amine 5.

8.1 Release of amine 5 after irradiation of phosphonamidate 4f via phosphonamidate acid 6

Phosphonamidate 4f (0.11 mg, 0.055 µmol) was dissolved in 50:50 NH₄HCO₃:MeCN (pH 7) to a final concentration of 0.038 mM. The probe was irradiated for 20 min at 297 nm and then analyzed by UPLC-MS. The dominant mass (m/z 938.15 [M+H]⁺) corresponded to phosphonamidate acid 6 (see Figure S2) and only little amine 5 was observed. Similarly, the largest peak observed by fluorescence HPLC did not have the same retention time as our reference probe 2i (see peptide synthesis, page 9). To initiate P-N bond cleavage, the solution was acidified to pH 3 with citric acid, incubated for 16 hrs and analyzed by fluorescence HPLC. Phosphonamidate acid 6 had disappeared and only amine 5 was observed, which had the same retention time as reference peptide 2i.

Figure S2: The irradiation of phosphonamidate 4f at pH 7 (blue line) led to the formation of phosphonamidate acid 6 and little amine 5 (red line). After incubation at pH 3, only amine 5 was observed (green line). It has the same retention time as reference probe 2i (purple line).

Column: Agilent Eclipse XDB C18, 4.6 x 250 mm (analytical HPLC)
Gradient: 5-95 % B in 30 mins (Solvent A: H₂O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)
8.2 Release of amine 5 after irradiation of phosphonamidate 4f in 0.1% TFA/H₂O

Phosphonamidate 4f (0.11 mg, 0.055 µmol) was dissolved in 0.1% TFA/H₂O (pH 2) to a final concentration of 0.038 mM. The probe was irradiated for 20 min at 297 nm and then incubated for 16 hrs at room temperature. It was then analyzed by fluorescence HPLC (see Figure 3) and UPLC-MS. Only amine 5 (m/z 766.46) was observed.

HPLC conditions for Figure 3:
Column: Phenomenex Synergi 4u Hydro-RP, 4.6 x 250 mm (analytical HPLC)
Gradient: 0-60 % B in 25 mins (Solvent A: H₂O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)

8.3 Stability of peptide 4f in 0.1% TFA/H₂O

Phosphonamidate peptide 4f was incubated for 16 hrs in 0.1 % TFA/H₂O (pH 2) and the stability was controlled by fluorescence HPLC. In case of P-N bond cleavage, amine 5 would be formed and have the same retention time as peptide 2i, which was synthesized as a reference for P-N bond cleavage (see peptide synthesis, page 9).

Figure S3: No formation of amine 5 was observed after incubating phosphonamidate 4f in 0.1 % TFA/H₂O (pH 2) for 16 hrs.
No formation of amine 5 was observed after 16 hrs incubation, indicating that the P-N bond of phosphonamidate 4f is stable at pH 2.

Column: Agilent Eclipse XDB C18, 4.6 x 250 mm (analytical HPLC)
Gradient: 5-95 % B in 30 mins (Solvent A: H2O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)

8.4 Standard curve for quantification

To quantify the release, we measured the selected ion recording signal of product 5 by integration and compared the obtained area to a standard curve. Completion of irradiation and P-N bond cleavage was verified by UPLC-MS.

The standard curve was generated by measuring the selection ion recording series of a dilution series of peptide 2i (0.1 mM, 0.05 mM, 0.01 mM, 0.005 mM, 0.001 mM). As ion, the double charged species [M+2H]^{2+} = m/z 766.46 was chosen. 10 µL of each dilution was measured and the SIR area was recorded. In total, 3 dilution series were prepared and measured individually. The average of the three measurements was taken for each concentration and the values were plotted and evaluated using GraphPad Prism.

![Figure S4: Example of SIR integration (m/z 766.46) for 0.01 mM: 2993497](image)

All area values:

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Area m/z 766.46</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0,1</td>
<td>17277860</td>
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<td>1660579</td>
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<tr>
<td>0,001</td>
<td>337230</td>
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</tbody>
</table>

Table T1: All area values from the SIR integration of 2i.

![Calibration curve](image)

Figure S5: All area values were plotted and fitted

Slope: y = 183807092x
8.5 Quantification

Peptide 4f (0.25 mg, 0.124 µmol) was dissolved in 150 µL 2:1 H₂O:MeCN (containing 0.1 % TFA). Concentration of peptide 4f: 0.826 mM.

Three probes were prepared as follows: 15 µL (0.025 mg, 0.0124 µmol) of the stock solution of peptide 4f were diluted to 200 µL 0.1 % TFA/H₂O (pH 2). Final peptide concentration: 0.062 mM.

Each probe was irradiated for 30 mins at 297 nm, then stirred for 2 hrs at room temperature to ensure P-N bond cleavage. The probes were then analyzed by SIR integration (m/z 766.46):

<table>
<thead>
<tr>
<th>Area measurement</th>
<th>Concentration (mM)</th>
<th>Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9970971</td>
<td>0.054</td>
</tr>
<tr>
<td>2</td>
<td>9539093</td>
<td>0.052</td>
</tr>
<tr>
<td>3</td>
<td>9404347</td>
<td>0.051</td>
</tr>
</tbody>
</table>

This results in an overall release of 84 % (± 2.5 %) of amine 5 after irradiation of peptide 4f.

9. Immobilization and irradiation of beads

9.1 General procedure

For the immobilization of the biotin-phosphonamidates, the beads were first thoroughly washed with 20 mM NH₄HCO₃ (pH 7) buffer to remove the storage buffer. The biotin-phosphonamidates were dissolved in 20 mM NH₄HCO₃ (pH 7) with 5 % MeCN and added to the beads for 2 hrs at room temperature. The beads were then washed 3 times with 20 mM NH₄HCO₃ (pH 7) and 2 times with 0.1 % TFA/H₂O (pH 2). The beads were then transferred to a UV cuvette with a stirrer and were irradiated for 1 hr at 297 nm. Afterwards, the beads were incubated for further 16 hrs at room temperature, before the beads were filtered. The elution was lyophilized and analyzed by UPLC-MS or fluorescence HPLC.

9.2 Phosphonamidate 4f

Phosphonamidate 4f (0.19 mg, 0.094 µmol) was dissolved in 400 µL 20 mM NH₄HCO₃ (pH 7) with 5 % MeCN and added to 200 µL beads (dead volume) of GE Healthcare Sepharose Streptavidin beads. The beads were shaken for 2 hrs at room temperature and were then washed three times with 500 µL 20 mM NH₄HCO₃ (pH 7) and two times with 500 µL 0.1 % TFA/H₂O (pH 2). The beads were suspended in 500 µL 0.1 % TFA/H₂O (pH 2), transferred into an UV Quartz cuvette and irradiated for 1 hr at room temperature at 297 nm, followed by 16 hrs incubation. The elute was lyophilized, re-dissolved in 50 µL 50:50 H₂O:MeCN each and analyzed by fluorescence HPLC (see Figure 4).

HPLC conditions for Figure 4:

Column: Agilent Eclipse XDB C18, 4.6 x 250 mm (analytical HPLC)
Gradient: 5-95 % B in 30 mins (Solvent A: H₂O + 0.1% TFA, solvent B: MeCN + 0.1 % TFA)
9.3 Evaluation of different buffers for release of peptide

Streptavidin beads only have a limited stability below pH 3. We therefore wanted to evaluate whether the release of phosphonamidate 4f from streptavidin beads could be carried out at pH 3 instead of pH 2. To do so, the phosphonamidate-conjugate was immobilized at pH 7 and the irradiation and incubation was carried out in a citric acid-phosphate buffer at pH 3. To check if all the peptide had eluted, the beads were additionally incubated at pH 2 to see whether any more fluorescent peptide was released.

Phosphonamidate 4f (0.1 mg, 0.049 µmol) was dissolved in 300 µL 20 mM NH₄HCO₃ (pH 7) with 5% MeCN and added to 150 µL streptavidin beads (dead volume). The beads were shaken for 2 hrs at room temperature and then washed three times with 300 µL NH₄HCO₃ (pH 7) and two times with 300 µL 0.1% TFA/H₂O (pH 2). The beads were suspended in 300 µL citric acid-phosphate buffer (pH 3), transferred into an UV Quartz cuvette and irradiated for 1 hr at room temperature at 297 nm, followed by 16 hrs incubation. The beads were washed 3 times with 300 µL citric acid-phosphate buffer (pH 3) and once quickly with 300 µL 0.1% TFA/H₂O (pH 2), before they were suspended in 300 µL 0.1% TFA/H₂O (pH 2) for 2 hrs. The elutes were lyophilized, re-dissolved in 50 µL 50:50 H₂O:MeCN each and analyzed by fluorescence HPLC (see Figure S6) and UPLC-MS.

As shown in Figure S6, pH 3 could initiate P-N bond cleavage. However, not all of amine 5 was released. Incubation at pH 2 led to additional elution of amine 5, suggesting that pH 3 is not sufficient to achieve full P-N bond cleavage of phosphonamidate acid 6 on beads.

As control, beads were loaded with phosphonamidate 4f, but the beads were only incubated at pH 2 without irradiation. The elute was analyzed by UPLC-MS and no release could be observed.

![Figure S6: Phosphonamidate 4f was immobilised on streptavidin beads (blue), then irradiated and eluted at pH 3 (red) and pH 2 (green) to release amine 5.](image-url)
9.4 Streptavidin beads degradation in 0.1% TFA/H$_2$O

To probe the stability of streptavidin beads in 0.1% TFA/H$_2$O, a sample of streptavidin beads was incubated in 0.1% TFA/H$_2$O overnight and the elute was analyzed by UPLC-MS. The UV trace was measured at 220 nm and 272 nm. A broad elution peak could be seen, suggesting that the beads only have limited stability in 0.1% TFA/H$_2$O.

\[ \text{Figure S7: Streptavidin beads were incubated for 16 hrs and the elute was analyzed by UPLC-MS. The UV traces for 220 and 272 nm are shown.} \]

Literature:

KS.6-631.170731.13.fid

The image contains a nuclear magnetic resonance (NMR) spectrum with peaks labeled with their corresponding chemical shifts in ppm. The molecule structure is also shown, labeled as 3b, with specific atoms and functional groups indicated.

Chemical Shifts:
- 146.76
- 140.21
- 138.83
- 134.28
- 132.81
- 132.47
- 130.94
- 130.87
- 129.95
- 129.39
- 129.81
- 128.81
- 128.51
- 125.10

Functional Groups:
- CDCl3
- O2N
- N=N
- BH3
- NO2

Peak Intensities:
- 54.59
- 66.35
- 66.30
- 74.30
- 77.38