Supporting information for

Kinetic and structural characterization of the self-labeling protein tags HaloTag7, SNAP-tag and CLIP-tag

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Chemical Synthesis

General information

All chemical reagents and (anhydrous) solvents for synthesis were purchased from commercial suppliers (Merck KGaA, Darmstadt, Germany; Honeywell, Charlotte, NC, USA; TCI, Tokyo, Japan; Thermo Fisher Scientific, Waltham, MA, USA; SiChem, Bremen, Germany) and were used without further purification or distillation. Anhydrous solvents were handled under argon atmosphere. SLP substrates were purchased from commercial sources, synthesized according to published procedures or gifts from colleagues. Details are given in Material Table.

$^1$H- and $^{13}$C-NMR spectra were recorded in deuterated solvents on a Bruker (Bruker Corp., Billerica, MA, USA) DPX400 (400 MHz for $^1$H, 101 MHz for $^{13}$C, respectively) or on a Bruker AVANCE III HD 400 (400 MHz for $^1$H, 101 MHz for $^{13}$C, respectively) equipped with a CryoProbe. Chemical shifts (δ) are reported in ppm referenced to the residual solvent peaks of DMSO-$d_6$ (δ$_H$ = 2.50 ppm, δ$_C$ = 39.52 ppm), acetone-$d_6$ (δ$_H$ = 2.05 ppm, δ$_C$(CH$_3$) = 29.84 ppm, δ$_C$(CO) = 206.26 ppm) or CDCl$_3$ (δ$_H$ = 7.26 ppm, δ$_C$ = 77.16 ppm). Coupling constants $J$ are reported in Hz and corresponding multiplicities are abbreviates as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet and br = broad.

Reaction progress was monitored by thin layer chromatography (TLC) (Silica gel 60G F$_{254}$ on TLC glass plates) in appropriate solvents. Reaction spots were visualized under UV lamp (254 nm or 366 nm) and/or by staining solutions. LC-MS was performed on a Shimadzu MS2020 (Shimadzu Corp., Kyoto, Japan) connected to a Nexera UHPLC system equipped with a Waters (Waters Corp., Milford, MA, USA) ACQUITY UPLC BEH C18 (1.7 μm, 2.1x50 mm) column. Buffer A: 0.1% formic acid in H$_2$O, Buffer B: acetonitrile. Measurements were done with an analytical gradient from 10% to 90% B over 6 min or from 1% to 90% B over 10 min.

Normal phase flash chromatography was performed on self-packed silica gel (60 M, 0.04 - 0.063 mm, Macherey-Nagel GmbH & Co. KG, Düren, Germany) columns or by using an Isolera One system (Biotage Sweden AB, Uppsala, Sweden) using pre-packed silica gel columns (ultra pure silica gel 12 g or 25 g). Solvent compositions are reported individually in parentheses.

Preparative reversed phase high-performance liquid chromatography (RP-HPLC) was conducted using a Waters SunFire™ Prep C18 OBDTM column (10 × 150 mm, 5 μm pore size, 4 mL/min. flow rate) or an Ascentis (Merck KGaA, Darmstadt, Germany) C18 column (10 × 250 mm, 5 μm pore size, 8 mL/min. flow rate) on either a Waters Alliance e2695 separation module connected to a 2998 PDA detector or a Dionex system equipped with an UVD (170 U, UV-Vis detector). Solvent A: 0.1% TFA in H$_2$O, Solvent B: acetonitrile.

High resolution mass spectra (HRMS) were measured by the MS-service of the EPF Lausanne (SSMI) on a Waters Xevo® G2-S Q-Tof spectrometer (Waters, Milford, MA, USA) with electron spray ionization (ESI) or by the MS-facility of the Max Planck Institute for Medical Research on a Bruker maXis IITM ETD.
**Material Table:** Substrate and chemical source used in the study

| Substrate         | Source / reference                     |
|-------------------|----------------------------------------|
| CPY-6-COOH        | Butkevich et al., (2016)               |
| CPY-5-COOH        | Butkevich et al., (2016)               |
| TMR-5-COOH        | Mudd et al., (2015)                    |
| TMR-6-COOH        | Mudd et al., (2015)                    |
| Cy3-COOH          | Ueno et al., (2011)                    |
| Cy5-COOH          | Ueno et al., (2011)                    |
| SiR-COOH          | Lukinavicius et al., (2013)            |
| meAm-6-TMR        | this study                             |
| meAm-5-TMR        | this study                             |
| meAm-6-CPY        | this study                             |
| meAm-5-CPY        | this study                             |
| CA-TMR            | Purchased from Promega, Madison, WI, USA |
| CA-Alexa488       | Purchased from Promega, Madison, WI, USA |
| CA-Fluorescein    | Purchased from Promega, Madison, WI, USA |
| CA-Oregon green   | Purchased from Promega, Madison, WI, USA |
| CA-JF549          | Gift from Dr. Luke Lavis, HHMI, Ashburn, VA, USA |
| CA-JF503          | Gift from Dr. Luke Lavis, HHMI, Ashburn, VA, USA |
| CA-JF525          | Gift from Dr. Luke Lavis, HHMI, Ashburn, VA, USA |
| CA-JF608          | Gift from Dr. Luke Lavis, HHMI, Ashburn, VA, USA |
| CA-JF669          | Gift from Dr. Luke Lavis, HHMI, Ashburn, VA, USA |
| CA-TMR-az-F4      | Gift from Dr. Luke Lavis, HHMI, Ashburn, VA, USA |
| CA-TMR-CN         | Wang et al., (2020)                    |
| CA-TMR-SCH3       | Wang et al., (2020)                    |
| CA-TMR-SNH2       | Wang et al., (2020)                    |
| CA-MaP555         | Wang et al., (2020)                    |
| CA-CPY            | Butkevich et al., (2016)               |
| CA-500R           | Butkevich et al., (2016)               |
| CA-510R           | Purchased from Abberior GmbH, Göttingen, Germany |
| CA-515R           | Purchased from Abberior GmbH, Göttingen, Germany |
| CA-580CP          | Gift from Dr. Alexey N. Butkevich, MPI-MF, Heidelberg, Germany |
| CA-LIVE580        | Purchased from Abberior GmbH, Göttingen, Germany |
| CA-Cy3            | this study                             |
| CA-Cy5            | this study                             |
| CA-TMR-biotin     | this study                             |
| CA-PEG-biotin     | Purchased from Promega, Madison, WI, USA |
| CA-Ac             | this study                             |
| CA-N3             | this study                             |
| CA-Nor1           | this study                             |
| CA-Nor2           | this study                             |
| CA-Tz             | this study                             |
| SNAP substrates         | Source                                                                 |
|-------------------------|------------------------------------------------------------------------|
| CA-PhN$_3$              | this study                                                             |
| CA-Vbn                  | this study                                                             |
| CA-BCN                  | this study                                                             |
| CA-SCO                  | this study                                                             |
| BG                      | Purchased from Santa Cruz Biotechnology, Dallas, TX, USA               |
| CP                      | this study                                                             |
| BG-NH$_2$               | Keppler et al., (2003)                                                 |
| CP-NH$_2$               | Srikun et al., (2010)                                                 |
| BG-TMR                  | Keppler et al., (2004)                                                 |
| CP-TMR                  | Correa et al., (2013)                                                 |
| BG-Alexa488             | Purchased from NEB as SNAP-Surface® Alexa Fluor® 488, Ipswitch, MA, USA |
| CP-Alexa488             | this study                                                             |
| BG-Fluorescein          | Keppler et al., (2003)                                                 |
| CP-Fluorescein          | this study                                                             |
| BG-CPY                  | Hiblot et al., (2017)                                                 |
| CP-CPY                  | this study                                                             |
| BG-5-TMR                | this study                                                             |
| BG-5-CPY                | this study                                                             |
| BG-MaP555               | Wang et al., (2020)                                                   |
| BG-SiR                  | Lukinavicius et al., (2013)                                           |
| CP-SiR                  | this study                                                             |
| BG-JF549                | Grimm et al., (2015)                                                  |
| BG-JF646                | Grimm et al., (2015)                                                  |
| BG-Cy3                  | this study                                                             |
| BG-sulfo-Cy3            | Gautier et al., (2008)                                                |
| BG-Cy5                  | this study                                                             |
| BG-sulfo-Cy5            | Gautier et al., (2008)                                                |
| BG-Atto565              | Correa et al., (2013)                                                 |
| BG-Atto590              | Bottanelli et al., (2016)                                             |
| BG-N$_3$                | this study                                                             |
| CP-N$_3$                | this study                                                             |
| BG-Nor2                 | this study                                                             |
| CP-Nor2                 | this study                                                             |
| BG-Tz                   | this study                                                             |
| CP-Tz                   | this study                                                             |
| BG-PhN$_3$              | this study                                                             |
| CP-PhN$_3$              | this study                                                             |
| BG-Vbn                  | this study                                                             |
| CP-Vbn                  | this study                                                             |
| BG-BCN                  | this study                                                             |
| CP-BCN                  | this study                                                             |
| BG-Ac                   | this study                                                             |
| CP-Ac                   | this study                                                             |
| CLIP substrates | BG-SCO | this study |
|----------------|--------|------------|
| CP-SCO         |        | this study |
| BC-NH₂         | Gautier et al., (2008) |
| BC-TMR         | Gautier et al., (2008) |
| BC-Alexa488    | Purchased from NEB as CLIP-Surface® Alexa Fluor® 488, Ipswitch, MA, USA |
| BC-Fluorescein | Gautier et al., (2008) |
| BC-CPY         |        | this study |
Chemical Synthesis

1.1 Synthesis of substrate amines

1.1.1 2-((2-(6-chlorohexyl)oxy)ethoxy)ethan-1-amine (CA-NH₂)

CA-NH₂ was synthesized according to the procedure from Zhang et al. 2006.

1.1.2 6-((4-(aminomethyl)benzyl)oxy)-9H-purin-2-amine (BG-NH₂)

BG-NH₂ was synthesized according to the procedure from Keppler et al. 2003.

1.1.3 4-((4-(aminomethyl)benzyl)oxy)-6-chloropyrimidin-2-amine (CP-NH₂)

CP-NH₂ was synthesized according to the procedure from Srikun et al. 2010.

1.1.4 2-((4-(aminomethyl)benzyl)oxy)pyrimidin-4-amine (BC-NH₂)

BC-NH₂ was synthesized according to the procedure from Gautier et al. 2008.
1.2 General procedure A for peptide coupling reactions

To a solution of TSTU (1.2 equiv.) in dry DMSO (0.3 mL), DIPEA (10.2 equiv. for Halo-tag, 5.0 equiv. for SNAP-substrates) and different carboxylic acids (1.1 equiv.) were added. After 5 min., a solution of 10 mg of corresponding amine (1.0 equiv.) in dry DMSO (0.1 mL) was added and the reaction mixture was stirred at r.t. for 2 hours. The reaction mixture was quenched by addition of water (100 μL) and acidified with acetic acid (50 μL), then purified by semi-preparative HPLC, eluted with a gradient of MeCN/H₂O + 0.1% TFA (equilibration at 15% MeCN for 5 min, then gradient of 15-100% MeCN over 25 min, followed by 100% MeCN for 10 min.). Fractions containing the desired product were combined and lyophilized. Final compounds were stored as DMSO stocks for biochemical testing.

1.3 HT7 substrates

1.3.1 2-azido-N-(2-(6-chlorohexyloxy)ethoxy)ethylacetamide (CA-N₃)

Reaction was conducted according to general procedure A using CA-NH₂ and 2-azidoacetic acid (4.6 μL, 32.6 μmol). The desired product (4.6 mg, 15.0 μmol) was obtained as a yellowish oil in 51% yield.

¹H NMR (400 MHz, DMSO-d₆) δ [ppm] = 8.15 (t, J = 5.8 Hz, 1H), 3.81 (s, 2H), 3.62 (t, J = 6.6 Hz, 2H), 3.53 – 3.40 (m, 6H), 3.37 (t, J = 6.6 Hz, 2H), 3.24 (dd, J = 5.7 Hz, J = 5.8 Hz, 2H), 1.75 – 1.65 (m, 2H), 1.54 – 1.43 (m, 2H), 1.43 – 1.25 (m, 4H).

¹³C NMR (101 MHz, DMSO-d₆) δ [ppm] = 167.31, 70.17, 69.56, 69.40, 68.83, 50.69, 45.36, 38.67, 32.00, 29.04, 26.10, 24.91.

HRMS (ESI): calc. for C₁₂H₂₃ClN₄O₄ [M+Na]+: 329.1351; found 329.1354.
1.3.2 (1R,4R)-N-(2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (CA-Nor1)

Reaction was conducted according to general procedure A using CA-NH₂ and (1R,4R)-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid (6.2 µL, 32.6 µmol). The desired endo-isomer (5.6 mg, 16.3 µmol) of was obtained in 55% yield.

$^1$H NMR (400 MHz, DMSO-δ6) δ [ppm] = 7.59 (t, J = 5.7 Hz, 1H), 6.08 (dd, J = 5.8, 3.1 Hz, 1H), 5.80 (dd, J = 5.8, 3.0 Hz, 1H), 3.62 (t, J = 6.6 Hz, 2H), 3.54 – 3.30 (m, 8H), 2.32 – 3.02 (m, 3H), 2.84 – 2.71 (m, 2H), 1.77 – 1.63 (m, 3H), 1.55 – 1.43 (m, 2H), 1.42 – 1.18 (m, 7H).

$^{13}$C NMR (101 MHz, DMSO-δ6) δ [ppm] = 172.86, 136.76, 132.18, 70.18, 69.58, 69.45, 69.09, 45.59, 45.37, 43.25, 42.08, 38.55, 32.02, 29.09, 28.35, 26.13, 24.94.

HRMS (ESI) calc. for C₁₉H₂₁ClNO₅ $^{1+}$: 344.1987; found 344.1989.

1.3.3 2-((1S,4S)-bicyclo[2.2.1]hept-5-ene-2-yl)-N-(2-((6-chlorohexyl)oxy)ethoxy)ethyl]acetamide (CA-Nor2)

Reaction was conducted according to general procedure A using CA-NH₂ and 2-((1S,4S)-bicyclo[2.2.1]hept-5-ene-2-yl)acetic acid (5.6 µL, 32.6 µmol) yielding 6.4 mg (17.9 µmol) of the desired product as a colorless oil in 60% yield.

$^1$H NMR (400 MHz, DMSO-δ6) δ [ppm] = 7.73 (t, J = 5.7 Hz, 1H), 6.15 (dd, J = 5.8, 3.0 Hz, 1H), 5.95 (dd, J = 5.8, 2.9 Hz, 1H), 3.62 (t, J = 6.6 Hz, 2H), 3.47 – 3.36 (m, 8H), 3.23 – 3.09 (m, 2H), 2.76 – 2.68 (m, 2H), 2.40 – 2.29 (m, 1H), 1.89 – 1.74 (m, 3H), 1.74 – 1.65 (m, 2H), 1.53 – 1.43 (m, 2H), 1.39 – 1.17 (m, 6H), 0.47 (m, J = 11.5, 4.4, 2.6 Hz, 1H).

$^{13}$C NMR (101 MHz, DMSO-δ6) δ [ppm] = 171.61, 136.88, 132.47, 70.15, 69.52, 69.42, 69.08, 49.03, 45.32, 45.13, 42.02, 40.58, 40.14, 39.93, 39.73, 39.51, 39.31, 39.10, 38.89, 38.35, 35.06, 31.99, 31.37, 29.04, 26.08, 24.89.

HRMS (ESI) calc. for C₁₉H₂₁ClNaO₅ $^{1+}$: 380.1963; found 380.1963.

1.3.4 N-(2-((6-chlorohexyl)oxy)ethoxy)ethyl-2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetamide (CA-Tz)

Reaction was conducted according to general procedure A using CA-NH₂ and 2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetic acid (7.5 mg, 32.6 µmol) yielding 7.4 mg (17.0 µmol) of the desired product as a rose solid in 57% yield.

$^1$H NMR (400 MHz, DMSO-δ6) δ [ppm] = 8.44 – 8.36 (m, 2H), 8.23 (t, J = 5.6 Hz, 1H), 7.58 – 7.50 (m, 2H), 3.61 (t, J = 6.6 Hz, 2H), 3.56 (s, 2H), 3.53 – 3.40 (m, 6H), 3.36 (t, J = 6.6 Hz, 2H), 3.23 (q, J = 5.7 Hz, 2H), 2.99 (s, 3H), 1.75 – 1.62 (m, 2H), 1.53 – 1.42 (m, 2H), 1.42 – 1.25 (m, 4H).

$^{13}$C NMR (101 MHz, DMSO-δ6) δ [ppm] = 169.58, 167.05, 163.22, 141.29, 130.05, 130.00, 127.28, 70.20, 69.60, 69.45, 69.05, 45.37, 42.19, 38.79, 32.02, 29.07, 26.12, 24.93, 20.83.

HRMS (ESI) calc. for C₂₁H₂₃ClN₅O₂ $^{1+}$: 436.2110; found 436.2113.
1.3.5 4-azido-N-(2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)benzamide (CA-PhN₃)

\[
\text{Cl} \quad \text{O} \quad \text{O} \quad \text{N} \quad \text{H} \quad \text{O} \\
\text{O} \quad \text{N}_3
\]

Reaction was conducted according to general procedure A using CA-NH₂ and 4-azidobenzoic acid (5.3 mg, 32.6 µmol) to obtain 6.1 mg (15.5 µmol) of the desired product as a colorless oil in 56% yield.

\(^1\)H NMR (400 MHz, DMSO-d₆) \(\delta\) [ppm] = 8.52 (t, \(J = 5.6\) Hz, 1H), 7.90 (d, \(J = 8.6\) Hz, 2H), 7.20 (d, \(J = 8.6\) Hz, 2H), 3.60 (t, \(J = 6.6\) Hz, 2H), 3.56 – 3.49 (m, 4H), 3.45 – 3.30 (m, 4H), 1.74 – 1.61 (m, 2H), 1.51 – 1.39 (m, 2H), 1.40 – 1.20 (m, 4H).

\(^{13}\)C NMR (101 MHz, DMSO-d₆) \(\delta\) [ppm] = 165.23, 142.19, 130.95, 129.06, 118.85, 70.17, 69.62, 69.40, 68.84, 45.35, 39.21, 32.00, 29.07, 26.12, 24.91.

HRMS (ESI) calc. for C₁₇H₂₅ClN₄NaO₃ \([\text{M+Na}]^+\): 391.1507; found 391.1511.

1.3.6.1 Cyclooct-2-yn-1-yl (2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamate (CA-SCO)

\[
\text{Cl} \quad \text{O} \quad \text{O} \quad \text{N} \quad \text{O} \\
\text{O} \quad \text{N}
\]

BCN-NHS (14.0 mg, 47.6 µmol, 1.1 eq) was dissolved in 500 µL DMSO. DIPEA (71.4 µL, 432 µmol, 10 equiv.) was added followed by CA-NH₂ (14.0 mg, 43.2 µmol, 1.0 equiv.) solubilized in DMSO. The solution was stirred for 30 min. The crude product was purified by preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (50% - 90% MeCN over 60 min) to obtain 11.9 mg (29.8 µmol) of the product as a clear oil in 69% yield after lyophilization.

\(^1\)H NMR (400 MHz, DMSO-d₆): \(\delta\) = 7.07 (t, \(J = 5.7\) Hz, 1H), 4.03 (d, \(J = 8.0\) Hz, 2H), 3.62 (t, \(J = 6.6\) Hz, 2H), 3.52 – 3.44 (m, 4H), 3.38 (dt, \(J = 11.3, 6.3, 4\) Hz), 3.11 (q, \(J = 6.0\) Hz, 2H), 2.30 – 2.06 (m, 6H), 1.78 – 1.64 (m, 2H), 1.59 – 1.42 (m, 4H), 1.41 – 1.19 (m, 4H), 0.95 – 0.78 (m, 2H).

\(^{13}\)C NMR (100 MHz, DMSO-d₆): \(\delta\) = 156.4, 99.0, 70.2, 69.5, 69.4, 69.1, 61.3, 45.4, 40.1, 32.0, 29.1, 28.6, 26.1, 24.9, 20.8, 19.5, 17.6.

HRMS (ESI) calc. for [M+H]^+: 400.2249, found 400.2250.
CA-NH₂ (15 mg, 44.4 μmol, 1.3 equiv.) was dissolved in dry DMSO (0.15 mL) and a solution of cyclooct-2-yn-1-yl (4-nitropheryl) carbonate (10 mg, 34.2 μmol, 1.0 equiv.) in dry DMF (0.4 mL) was added followed by DIPEA (58 μL, 348 μmol: 10.2 equiv.). The reaction mixture was stirred at r.t. for 1 h. The resulted mixture was acidified with 50 μL of acetic acid and afterwards purified by semi-preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (15%) MeCN for 2 min., then 15 - 100% MeCN over 25 min., followed by 100% MeCN for 15 min.) to give 8.7 mg (23.3 μmol) of the desired product as a colorless oil in 68% yield after lyophilization.

1H NMR (400 MHz, CDCl₃) δ [ppm] = 7.18 (t, J = 5.9 Hz, 1H), 5.18 – 5.09 (m, 1H), 3.62 (t, J = 6.6 Hz, 2H), 3.50 – 3.43 (m, 4H), 3.40 – 3.34 (m, 4H), 3.09 (q, J = 5.9 Hz, 2H), 2.30 – 2.00 (m, 3H), 1.93 – 1.78 (m, 3H), 1.76 – 1.65 (m, 3H), 1.64 – 1.54 (m, 2H), 1.53 – 1.43 (m, 3H), 1.42 – 1.25 (m, 4H).

13C NMR (101 MHz, CDCl₃) δ [ppm] = 155.29, 100.82, 91.79, 70.19, 69.53, 69.42, 68.99, 65.70, 45.38, 41.59, 40.07, 33.85, 32.03, 29.21, 29.06, 26.13, 25.78, 24.94, 19.95.

HRMS (ESI) calc. for C₁₁H₈ClINaO₄⁺ [M+Na⁺]: 396.1912; found 396.1923.

1.3.7 N-(2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)acetamide (CA-Ac)

Tert-butyl (2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamate (301 mg, 0.93 μmol, 1.0 equiv.) was deprotected by addition of TFA (2 mL) and afterwards dried under a stream of pressured air for 15 min. DIPEA (307 μL, 1.86 mmol, 2.0 equiv.) and DMSO (333 μL) were added followed by dropwise addition of acetic anhydride (131 μL, 1.39 mmol, 1.5 equiv.) while stirring. The reaction was stirred at r.t for 1 h. The mixture was quenched with saturated solution of NaHCO₃ (20 mL) and extracted with DCM (3 × 20 mL). The combined organic layers were washed with brine and dried over MgSO₄. All volatiles were evaporated and the crude product was purified over normal phase flash chromatography (MeOH: DCM = 2% : 98% to 3% : 97%). The fractions containing the product were combined to give 238 mg (896 μmol) of the desired product as a colorless oil in 97% yield after evaporation.

1H NMR (400 MHz, CDCl₃) δ [ppm] = 6.05 (s, 1H), 3.67 – 3.38 (m, 12H), 1.98 (s, 3H), 1.83 – 1.71 (m, 2H), 1.61 (p, J = 6.8 Hz, 2H), 1.52 – 1.31 (m, 4H).

13C NMR (101 MHz, CDCl₃) δ [ppm] = 169.92, 71.09, 70.07, 69.83, 69.60, 44.84, 39.10, 32.32, 29.28, 26.49, 25.24, 23.10.

HRMS (ESI) calc. for C₁₁H₈ClINaO₄⁺ [M+H⁺]: 266.1517; found 266.1518.

1.3.1 5-(2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate (CA-5-TMR)

To a solution of TMR-5-COOH (2.5 mg, 5.81 μmol, 1.0 equiv.) in dry DMSO (500 μL), benzotriazolylxytris(dimethylamino)-phosphonium hexafluorophosphat (BOP) (0.5 M in DMSO, 16.4 μL, 8.21 μmol, 1.5 equiv.) was added and the reaction was shaken at 500 rpm and r.t. for 5 min. DIPEA (3.84 μL, 23.2 μmol, 4.0 equiv.) and CA-NH₂ (1 M in DMSO, 8.71 μL, 8.71 μmol, 1.5 equiv.) were added and the reaction was shaken at 500 rpm and r.t. for 4 h. The crude product was acidified with acetic acid and purified over preparative HPLC eluted with MeCN / H₂O (0.1% FA) (10% - 90% MeCN over 50 min) to give 1.2 mg (1.89 μmol) of the desired product in 33% yield after lyophilization.

HRMS (ESI): calc. for C₉₆H₆₂N₄O₁₀Cl⁺ [M+H⁺]: 635.2887; found 635.2882.
1.3.2 5-((2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-10,10-dimethyl-3,10-dihydroantracen-9-yl)benzoate (CA-5-CPY)

To a solution of CPY-5-COOH (2.5 mg, 5.48 μmol, 1.0 equiv.) in dry DMSO (1 mL), BOP (0.5 M in DMSO, 16.4 μL, 8.21 μmol, 1.5 equiv.) was added and the reaction was shaken at 500 rpm and r.t. for 5 min. DIPEA (3.62 μL, 21.9 μmol, 4.0 equiv.) and CA-NH₂ (1 M in DMSO, 8.21 μL, 8.21 μmol, 1.5 equiv.) were added and the reaction was shaken at 500 rpm and r.t. for 4 h. The crude product was acidified with acetic acid and purified over preparative HPLC eluted with MeCN / H₂O (0.1% FA) (10% - 90% MeCN over 50 min) to give 0.38 mg (0.57 μmol) of the desired product in 10% yield after lyophilization.

HRMS (ESI): calc. for C₃₈H₄₉N₃O₅Cl+ [M+H]+: 662.3360; found 662.3349.

1.3.3 1-(6-((2-((6-chlorohexyl)oxy)ethoxy)ethyl)amino)-6-oxohexyl)-3,3-dimethyl-2-((E)-3-((Z)-1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium (CA-Cy3)

Cy3-COOH was synthesized according to Ueno et al. 2010 ². To a solution of Cy3-COOH (100 mg, 219 μmol, 1.0 equiv.) in dry DMSO (2 mL), DIPEA (217 μL, 1.3 mmol, 6.0 equiv.) and TSTU (92.1 mg, 306 μmol, 1.4 equiv.) were added and the reaction mixture was stirred for 10 min. at r.t. CA-NH₂ (58 mg, 262 μmol, 1.2 equiv.) in 0.5 mL DMSO was added and the reaction was stirred for 30 min, at r.t. The reaction was quenched by addition of acetic acid (230 μL) and 10% H₂O, followed by purification over preparative HPLC eluted with MeCN / H₂O (0.1% FA) (10% - 90% MeCN over 60 min) to give 102 mg (154 μmol) of the desired product in 70% yield after lyophilization.

HRMS (ESI): calc. for C₄₀H₅₇N₃O₃Cl[⁺+M]: 662.4083; found 662.4084.
1.3.4 1-((2-((2-((6-chlorohexyl)oxy)ethoxy)ethyl)amino)-6-oxohexyl)-3,3-dimethyl-2-((1E,3E)-5-((Z)-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium (CA-Cy5)

Cy5-COOH was synthesized according to Ueno et al. 2010. To a solution of Cy5-COOH (100 mg, 207 μmol, 1.0 equiv.) in dry DMSO (2 mL), DIPEA (205 μL, 1.24 mmol, 6.0 equiv.) and TSTU (87.1 mg, 289 μmol, 1.4 equiv.) were added and the reaction mixture was stirred for 10 min. at r.t. CA-NH₂ (55.5 mg, 248 μmol, 1.2 equiv.) in 0.5 mL DMSO was added and the reaction was stirred for 30 min, at r.t. The reaction was quenched by addition of acetic acid (291 μL) and 10% H₂O, followed by purification over preparative HPLC eluted with MeCN / H₂O (0.1% FA) (10% - 90% MeCN over 60 min) to give 98 mg (142 μmol) of the desired product in 69% yield after lyophilization.

HRMS (ESI): calc. for C₄₂H₅₉N₃O₃Cl⁺ [M⁺] : 688.4239; found 688.4239.

1.3.5 4-carboxy-2-((3-dimethyliminio)-6-((4-methoxy-4-oxobutyl)(methyl)amino)-3H-xanthen-9-yl)benzoate (CA-TMR-biotin-1)

The compound was synthesized according to the procedure from Masharina et al. 2012.

1.3.6 4-((2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)-2-(3-dimethyliminio)-6-((4-methoxy-4-oxobutyl)(methyl)amino)-3H-xanthen-9-yl)benzoate (CA-TMR-biotin-2)

To a solution of CA-TMR-biotin-1 (17.0 mg, 32.9 μmol, 1.0 equiv.) in dry DMF, TSTU (11.9 mg, 39.5 μmol, 1.2 equiv.) and DIPEA (32.6 μL, 197 μmol, 6.0 equiv.) were added and the reaction was stirred at r.t. for 5 min. CA-NH₂ (14.7 mg, 65.8 μmol, 2.0 equiv.) was added and the reaction was stirred at r.t. for 2 h. The crude product was acidified with acetic acid and purified via preparative eluted with MeCN / H₂O (0.1% TFA) (10% - 90% MeCN over 50 min) to give 10 mg (13.8 μmol) of the desired product in 42% yield after lyophilization.
1.3.7 2-(6-((3-carboxypropyl)(methyl)amino)-3-(dimethyliminio)-3H-xanthen-9-yl)-4-((2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)benzoate (CA-TMR-biotin-3)

To a solution of CA-TMR-biotin-2 (8.0 mg, 11.1 μmol, 1.0 equiv.) in THF: H2O (4:1), lithium hydroxide (1 M in H2O, 22.2 μL, 22.2 μmol, 2.0 equiv.) was added and the reaction was stirred at r.t. for 6 h. The crude product was acidified with acetic acid and purified via preparative HPLC eluted with MeCN / H2O (0.1% TFA) (10% - 90% MeCN over 50 min) to give 6.3 mg (8.9 μmol) of the desired product in 80% yield after lyophilization.

HRMS (ESI): calc. for C39H49N3O8Cl+ [M+H]+: 722.3208; found 722.3202.

1.3.8 4-((2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamoyl)-2-(3-(dimethyliminio)-6-((4,18-dioxo-22-((3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-8,11,14-trioxa-5,17-diazadocosyl)(methyl)amino)-3H-xanthen-9-yl)benzoate (CA-TMR-biotin)

To a solution of CA-TMR-biotin-3 (6.0 mg, 8.47 μmol, 1.0 equiv.) in dry DMF, TSTU (3.06 mg, 10.2 μmol, 1.2 equiv.) and DIPEA (8.4 μL, 50.8 μmol, 6.0 equiv.) were added and the reaction was stirred at r.t. for 5 min. Biotin-PEG3-NH2 (7.09 mg, 16.9 μmol, 2.0 equiv.) was added and the reaction was stirred at r.t. for another 2 h. The crude product was acidified with acetic acid and purified via preparative HPLC eluted with MeCN / H2O (0.1% TFA) (10% - 90% MeCN over 50 min) to give 6.2 mg (5.6 μmol) of the desired product in 66% yield after lyophilization.

HRMS (ESI): calc. for C56H80N7O12ClS2+ [M+2H]2+: 554.7628; found 554.7632.

1.4 SNAP substrates based on benzylguanine (BG)

1.4.1 N-4-(((2-amino-9H-purin-6-yl)oxy)benzyl)-2-azidoacetamide (BG-N3)
Reaction was conducted according to general procedure A using BG-NH₂ and 2-azidoacetic acid (40.7 µmol; 5.7 µL) and 11.1 mg (23.8 µmol) of the desired product were obtained as a colorless TFA-salt in 64% yield.

\(^1^H\) NMR (400 MHz, DMSO-d₆) δ [ppm] = 8.65 (t, J = 5.9 Hz, 1H), 8.34 (s, 1H), 7.55 – 7.44 (m, 2H), 7.36 – 7.28 (m, 2H), 5.52 (s, 2H), 4.31 (d, J = 5.9 Hz, 2H), 3.89 (s, 2H).

HRMS (ESI) calc. for C₁₅H₁₆N₉O₂⁺ [M+H]⁺: 354.1421; found 354.1423.

1.4.2  
\(N\)-(4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)-2-((1S,4S)-bicyclo[2.2.1]hept-5-en-2-yl)acetamide (BG-Nor2)

\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\end{align*}
\]

Reaction was conducted according to general procedure A with a reduced reaction time of 15 min. using BG-NH₂ and 2-((1S,4S)-bicyclo[2.2.1]hept-5-en-2-yl)acetic acid (40.7 µmol; 7.0 µL) resulting in 15.9 mg (30.7 µmol) of the desired product as a colorless TFA-salt in 83% yield.

\(^1^H\) NMR (400 MHz, DMSO-d₆) δ [ppm] = 8.47 (s, 1H), 8.29 (t, J = 6.0 Hz, 1H), 7.49 (d, J = 8.1 Hz, 2H), 7.27 (d, J = 8.1 Hz, 2H), 6.16 (dd, J = 5.7, 3.0 Hz, 1H), 5.96 (dd, J = 5.7, 2.9 Hz, 1H), 5.53 (s, 2H), 4.25 (d, J = 6.0 Hz, 2H), 2.77 – 2.69 (m, 2H), 2.45 – 2.34 (m, 1H), 1.95 (dd, J = 13.8, 7.6 Hz, 1H), 1.90 – 1.77 (m, 2H), 1.33 – 1.26 (m, 1H), 1.25 – 1.19 (m, 1H), 0.50 (m, J = 11.4, 4.5, 2.5 Hz, 1H).

\(^{13}C\) NMR (101 MHz, DMSO-d₆) δ [ppm] = 171.71, 158.83, 158.03, 153.44, 140.89, 140.30, 137.07, 133.90, 132.45, 128.84, 127.13, 68.12, 49.10, 45.26, 42.09, 41.71, 40.67, 35.15, 31.47.

HRMS (ESI) calc. for C₂₂H₂₅N₆O₂⁺ [M+H]⁺: 405.2034; found 405.2034.

1.4.3  
\(N\)-(4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)-2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetamide (BG-Tz)

\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\end{align*}
\]

Reaction was conducted according to general procedure A using BG-NH₂ and 2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetic acid (40.7 µmol, 9.4 mg) to give 12.4 mg (17.0 µmol) of the desired product as a rose TFA-salt in 56% yield.

\(^1^H\) NMR (400 MHz, DMSO-d₆) δ [ppm] = 8.70 (t, J = 5.9 Hz, 1H), 8.45 – 8.39 (m, 2H), 8.37 (s, 1H), 7.60 – 7.53 (m, 2H), 7.52 – 7.44 (m, 2H), 7.33 – 7.26 (m, 2H), 5.51 (s, 2H), 4.31 (d, J = 5.9 Hz, 2H), 3.64 (s, 2H), 2.99 (s, 2H), 2.54 (s, 3H).

\(^{13}C\) NMR (101 MHz, DMSO-d₆) δ [ppm] = 170.04, 167.53, 163.69, 159.37, 158.37, 158.03, 153.44, 140.89, 140.30, 137.07, 133.90, 132.45, 128.84, 127.13, 134.78, 130.62, 130.61, 129.34, 127.81, 68.28, 42.69, 40.90, 21.31.

HRMS (ESI) calc. for C₂₄H₂₃N₁₀O₂⁺ [M+H]⁺: 483.2000; found 483.2006.

1.4.4  
\(N\)-(4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)-4-azidobenzamide (BG-PhN₃)

\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\end{align*}
\]

Reaction was conducted according to general procedure A with a reduced reaction time of 15 min. using BG-NH₂ and 4-azidobenzoic acid (6.6 mg, 40.7 µmol) to obtain 15.5 mg (29.3 µmol) of the desired product as a colorless TFA-salt in 79% yield.
1H NMR (400 MHz, DMSO-d6) δ [ppm] = 9.10 (t, J = 5.9 Hz, 1H), 8.38 (s, 1H), 7.99 – 7.89 (m, 2H), 7.55 – 7.46 (m, 2H), 7.38 – 7.33 (m, 2H), 7.25 – 7.16 (m, 2H), 5.52 (s, 2H), 4.48 (d, J = 5.9 Hz, 2H).

13C NMR (101 MHz, DMSO-d6) δ [ppm] = 165.27, 158.91, 158.61, 158.26, 153.77, 142.36, 140.57, 140.02, 134.19, 130.81, 129.15, 128.89, 127.36, 118.96, 67.94, 42.48.

HRMS (ESI) calc. for C_{30}H_{33}N_{10}O_{2}^+ [M+H]^+: 416.1578; found 416.1577.

1.4.5  N-(4-(((2-amino-9H-purin-6-yloxy)methyl)benzyl)-4- vinylbenzamide (BG-VBn)

Reaction was conducted according to general procedure A with a reduced reaction time of 15 min. using BG-NH2 and 4-vinylbenzoic acid (40.7 μmol; 6.5 mg) to obtain 14.7 mg (28.6 μmol) of the desired product as a colorless TFA-salt in 77% yield.

1H NMR (400 MHz, DMSO-d6) δ [ppm] = 9.09 (t, J = 6.0 Hz, 1H), 8.44 (s, 1H), 7.87 (d, J = 8.3 Hz, 2H), 7.57 (d, J = 8.3 Hz, 2H), 7.51 (d, J = 7.9 Hz, 2H), 7.36 (d, J = 7.9 Hz, 2H), 6.79 (dd, J = 17.1, 11.0 Hz, 1H), 5.95 (d, J = 17.7 Hz, 1H), 5.53 (s, 2H), 5.37 (d, J = 11.0 Hz, 1H), 4.49 (d, J = 6.0 Hz, 2H).

13C NMR (101 MHz, DMSO-d6) δ [ppm] = 165.81, 158.95, 158.62, 158.28, 153.55, 140.79, 140.13, 139.84, 135.91, 134.19, 130.81, 129.15, 128.92, 127.61, 127.35, 126.03, 116.24, 67.94, 42.46.

HRMS (ESI) calc. for C_{30}H_{33}N_{10}O_{2}^+ [M+H]^+: 401.1721; found 401.1707.

1.4.6  (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (4-(((2-amino-9H-purin-6-yloxy)methyl)benzyl)carbamate (BG-BCN)

A solution of ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (10 mg, 34.3 μmol, 1.0 equiv.) in dry DMSO (0.4 mL) was added to a solution of 10.2 μmol (0.2 mmol) of ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl)benzamide (BG-VBn) in dry DMSO (0.1 mL) followed by 28.4 μL of DIPEA (172 μmol, 5 equiv.). The reaction was stirred at r.t. for 30 min. The resulting mixture was acidified with acetic acid (3 μL) and H$_2$O (53 μL), then purified by semi-preparative HPLC eluted with MeCN / H$_2$O (0.1% TFA) (10% MeCN for 10 min., then 10 - 90% MeCN over 55 min. followed by 99% MeCN for 5 min.) to give 14.0 mg (31.4 μmol) of the desired product as a colorless solid in 91% yield after lyophilization.

1H NMR (400 MHz, DMSO-d6) δ [ppm] = 8.36 (s, 1H), 7.70 (t, J = 6.2 Hz, 1H), 7.52 – 7.46 (m, 2H), 7.28 (d, J = 8.0 Hz, 2H), 5.31 (s, 2H), 4.18 (d, J = 6.0 Hz, 2H), 4.06 (d, J = 8.0 Hz, 2H), 2.28 – 2.07 (m, 6H), 1.52 (d, J = 12.4 Hz, 2H), 1.28 (dt, J = 18.3, 9.1 Hz, 1H), 0.86 (t, J = 9.8 Hz, 2H).

HRMS (ESI) calc. for C_{34}H_{33}N_{10}O_{3}^+ [M+H]^+: 447.2139; found 447.2135.

1.4.6.1  N-(4-(((2-amino-9H-purin-6-yloxy)methyl)benzyl)acetamide (BG-Ac)

BG-NH$_2$ (300 mg, 1.11 mmol, 1.0 equiv.) was dissolved in dry DMSO (2.5 mL) and 367 μL of DIPEA (2.22 mmol, 2.0 equiv.) was added followed by dropwise addition of acetic anhydride (156 μL, 1.66 mmol, 1.5 equiv.) while stirring. The reaction mixture was stirred at r.t.
for 1 h. Afterwards, the reaction was quenched with acetic acid (387 μL) and H₂O (341 μL) followed by centrifugation at 3'000 rpm for 3 min. The pellet was washed twice with H₂O and afterwards lyophilized to obtain 190 mg (608 μmol) of the desired product as a colorless solid in 55% yield.

**¹H NMR** (400 MHz, DMSO-d₆) δ [ppm] = 8.30 (s, 1H), 7.45 (d, J = 8.1 Hz, 2H), 7.27 (d, J = 7.9 Hz, 2H), 6.82 (s, 2H), 5.48 (s, 2H), 4.24 (d, J = 5.9 Hz, 2H), 1.86 (s, 3H).  

**HRMS** (ESI) calc. for C₁₅H₁₇N₆O₂ [M+H]+: 313.1408; found 313.1406.

**1.4.8 5-(((4-amino-9H-purin-6-yl)oxy)methyl)benzyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-2,3,4,4a-tetrahydro-1H-xanthen-9-yl)benzoate (BG-5-TMR)**

TSTU (1.45 mg, 4.82 μmol, 1.2 equiv.) was dissolved in dry DMSO-d₆ (500 μL). TMR-5-COOH (1.15 mg, 2.68 μmol, 1.0 equiv.) was dissolved in the TSTU solution and DIPEA (1.77 μL, 10.7 μmol, 4.0 equiv.) was added. The mixture was stirred at r.t. for 10 min. BG-NH₂ (1.08 mg, 4.01 μmol, 1.5 equiv.) was dissolved in dry DMSO-d₆ (200 μL) and added to the reaction. The reaction mixture was
stirred at r.t. for 1 h. The compound was purified over preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (10% MeCN for 10 min., then 10 - 90% MeCN over 40 min., followed by 90% MeCN for 5 min.) to give after lyophilization 378 μg (554 nmol) of the desired product in 21% yield.

HRMS (ESI): calc. for C₃₈H₃₇N₈O₅ [M+2H]²⁺: 342.1399; found 342.1394.

¹H NMR (TMR-5-COOH) (400 MHz, DMSO-d₆) δ [ppm] = 8.39 (s, J = 1.5 Hz, 1H), 8.28 (dd, J = 8.1, 1.5 Hz, 1H), 7.33 (d, J = 8.0 Hz, 1H), 6.58 – 6.45 (m, 6H), 2.95 (s, 12H).

¹³C NMR (TMR-5-COOH) (101 MHz, DMSO-d₆) δ [ppm] = 168.31, 166.09, 152.03, 135.96, 132.76, 128.50, 109.05, 97.95, 40.15, 39.99, 39.79.

1.4.9 5-((4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-10,10-dimethyl-1,2,3,4a,10-hexahydroanthracen-9-yl)benzoate (BG-5-CPY)

1.4.9.1 1-(6-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)amino)-6-oxohexyl)-3,3-dimethyl-2-((E)-3-((Z)-1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium (BG-Cy3)

TSTU (1.44 mg, 4.78 μmol, 1.2 equiv.) was dissolved in dry DMSO-d₆ (500 μL). CPY-5-COOH (2.0 mg, 4.38 μmol, 1.1 equiv.) was dissolved in the TSTU solution and DIPEA (2.63 μL, 15.9 μmol, 4 equiv.) was added. The mixture was stirred at r.t. for 10 min. BG-NH₂ (1.08 mg, 3.98 μmol, 1.5 equiv.) was dissolved in dry DMSO-d₆ (200 μL) and added to the reaction. The reaction mixture was stirred at r.t. for 1 h. The compound was purified over preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (10% MeCN for 10 min., then 10 - 90% MeCN over 40 min., followed by 90% MeCN for 5 min.) to give 346 μg (488 nmol) of the desired product in 18% yield after lyophilization.

HRMS (ESI): calc. for C₄₁H₄₂N₈O₄ [M+2H]²⁺: 355.1659; found 355.1659.

Cy3-COOH was synthesized according to Ueno et al. 2010. To a solution of Cy3-COOH (100 mg, 219 μmol, 1.0 equiv.) in dry DMSO (1.5 mL), DIPEA (217 μL, 1.3 mmol, 6.0 equiv.) and TSTU (92.1 mg, 306 μmol, 1.4 equiv.) were added and the reaction mixture was stirred for 10 min. at r.t. BG-NH₂ (70.9 mg, 262 μmol, 1.2 equiv.) was added and the reaction was stirred for 30 min. at r.t. The reaction
was quenched by addition of acetic acid (230 μL) and 10% H$_2$O, followed by purification over preparative HPLC eluted with MeCN / H$_2$O (0.1% FA) (10% - 90% MeCN over 60 min.) to give. 28.5 mg (40.1 μmol) of the desired product in 18% yield after lyophilization. 

HRMS (ESI): calc. for C$_{40}$H$_{38}$N$_{2}$O$_{8}$ $^{35}$M+H$^{+}$= 355.2023; found 355.2022.

1.4.10 1-((4-(((2-amino-9H-purin-6-yl)oxy)methyl)(benzyl)(amino)-6-oxohexyl)-3,3-dimethyl-2-(((1E,3E)-5-((Z)-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium (BG-Cy5)

Cy5-COOH was synthesized according to Ueno et al. 2010$^2$. To a solution of Cy5-COOH (50.0 mg, 103 μmol, 1.0 equiv.) in dry DMSO (1.5 mL), DIPEA (103 μL, 620 μmol, 6.0 equiv.) and TSTU (43.6 mg, 145 μmol, 1.4 equiv.) were added and the reaction mixture was stirred for 10 min. at r.t. BG-NH$_2$ (33.5 mg, 124 μmol, 1.2 equiv.) was added and the reaction was stirred for 30 min. at r.t. The reaction was quenched by addition of acetic acid (109 μL) and 10% H$_2$O, followed by purification over preparative HPLC eluted with MeCN / H$_2$O (0.1% FA) (10% - 90% MeCN over 60 min.) to give 45 mg (61.1 μmol) of the desired product in 59% yield after lyophilization. 

HRMS (ESI): calc. for C$_{40}$H$_{38}$N$_{2}$O$_{8}$ $^{35}$M+H$^{+}$= 368.2101; found 368.2102.

1.5 SNAP substrates based on chloropyrimidine (CP)

1.5.1 $^{N}$-4-(((2-amino-6-chloropyrimidin-4-yl)oxy)methyl)(benzyl)-2-azidoacetamide (CP-N$_3$

Reaction was conducted according to general procedure A using CP-NH$_2$ and 2-azidoacetic acid (5.8 μL, 41.6 μmol) to obtain 10.1 mg (21.9 μmol) of the desired product as a colorless TFA-salt in 58% yield.

$^1$H NMR (400 MHz, DMSO-d$_6$) δ [ppm] = 8.62 (t, J = 5.8 Hz, 1H), 7.40 (d, J = 7.7 Hz, 2H), 7.28 (d, J = 7.7 Hz, 2H), 6.13 (s, 1H), 5.29 (s, 2H), 4.30 (d, J = 5.8 Hz, 2H), 3.88 (s, 2H).

$^{13}$C NMR (101 MHz, DMSO-d$_6$) δ [ppm] = 170.28, 167.32, 162.77, 160.01, 138.90, 134.90, 128.44, 127.46, 94.42, 67.21, 50.78, 42.01.

HRMS (ESI) calc. for C$_{19}$H$_{22}$ClN$_{2}$O$_{2}^{-}$ [M+H]$^{+}$= 348.0970; found 348.0971.

1.5.2 $^{N}$-4-(((2-amino-6-chloropyrimidin-4-yl)oxy)methyl)(benzyl)-2-((1S,4S)-bicyclo[2.2.1]hept-5-en-2-yl)acetamide (CP-Nor2)

Reaction was conducted according to general procedure A with a reduced reaction time of 15 min. using CP-NH$_2$ and 2-((1S,4S)-bicyclo[2.2.1]hept-5-en-2-yl)acetid acid (7.1 μL, 41.6 μmol) resulting in 14.5 mg (28.3 μmol) of the desired product as a colorless TFA-salt in 75% yield.

$^1$H NMR (400 MHz, DMSO-d$_6$) δ [ppm] = 8.25 (t, J = 5.9 Hz, 1H), 7.38 (d, J = 8.1 Hz, 2H), 7.23 (d, J = 8.1 Hz, 2H), 7.10 (brs, 2H), 6.16 (dd, J = 5.7, 3.0 Hz, 1H), 6.13 (s, 1H), 5.96 (dd, J = 5.7, 2.9 Hz, 1H), 5.28 (s, 2H), 4.24 (d, J = 5.9 Hz, 2H), 2.77 – 2.69 (m, 2H), 2.46 –
2.35 (m, 1H), 1.94 (dd, J = 13.8, 7.6 Hz, 1H), 1.90 – 1.76 (m, 2H), 1.35 – 1.26 (m, 1H), 1.26 – 1.18 (m, 1H), 0.50 (m, J = 11.4, 4.3, 2.5 Hz, 1H).

\(^{13}\text{C} \text{ NMR} \text{ (101 MHz, DMSO-}d_6\) \(\delta \text{ [ppm]} = 171.61, 170.28, 162.75, 159.97, 139.81, 137.01, 134.53, 132.42, 128.31, 127.11, 94.39, 67.23, 49.07, 45.24, 42.06, 41.69, 40.64, 35.11, 31.45.

HRMS (ESI) calc. for C\(_{21}\)H\(_{23}\)ClN\(_4\)O\(_2\) \([\text{M+Na}^+]^+\): 421.1402; found 421.1403.

1.5.3 \(N\)-(4-(((2-amino-6-chloropyrimidin-4-yl)oxy)methyl)benzyl)-2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetamide (CP-Tz)

Reaction was conducted according to general procedure A using CP-NH\(_2\) and 2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetic acid (9.6 mg, 41.6 \(\mu\)mol). The product was purified by preparative HPLC eluted with MeCN / H\(_2\)O (0.1\% TFA) (10\% MeCN for 10 min., then 10 - 90\% MeCN over 40 min., followed by 90\% MeCN for 10 min.) to give 2.6 mg (4.4 \(\mu\)mol) of the desired product as a rose TFA-salt in 12\% yield after lyophilization.

\(^1\text{H} \text{ NMR} \text{ (400 MHz, DMSO-}d_6\) \(\delta \text{ [ppm]} = 8.66 (t, J = 5.9 Hz, 1H), 8.41 (d, J = 8.3 Hz, 2H), 7.56 (d, J = 8.3 Hz, 2H), 7.38 (d, J = 7.9 Hz, 2H), 7.26 (d, J = 7.9 Hz, 2H), 7.10 (s, 2H), 6.13 (s, 1H), 5.28 (s, 2H), 4.29 (d, J = 5.9 Hz, 2H), 3.63 (s, 2H), 2.99 (s, 3H).

\(^{13}\text{C} \text{ NMR} \text{ (101 MHz, DMSO-}d_6\) \(\delta \text{ [ppm]} = 170.28, 169.51, 167.04, 163.21, 162.76, 159.99, 141.13, 139.32, 134.75, 130.14, 130.07, 128.42, 127.34, 94.40, 67.22, 42.21, 42.09, 20.83.

HRMS (ESI) calc. for C\(_{23}\)H\(_{22}\)ClN\(_8\)O\(_2\) \([\text{M+H}^+]^+\): 477.1549; found 477.1553.

1.5.4 \(N\)-(4-(((2-amino-6-chloropyrimidin-4-yl)oxy)methyl)benzyl)-4-azidobenzamide (CP-PhN\(_3\))

Reaction was conducted according to general procedure A with a reduced reaction time of 15 min. using BG-NH\(_2\) and 4-azidobenzoic acid (6.8 mg, 41.6 \(\mu\)mol) to obtain 12.0 mg (22.9 \(\mu\)mol) of the desired product as a colorless TFA-salt in 61\% yield.

\(^1\text{H} \text{ NMR} \text{ (400 MHz, DMSO-}d_6\) \(\delta \text{ [ppm]} = 9.06 (t, J = 5.9 Hz, 1H), 7.94 (d, J = 8.4 Hz, 2H), 7.39 (d, J = 7.9 Hz, 2H), 7.32 (d, J = 8.4 Hz, 2H), 7.10 (s, 2H), 5.29 (s, 2H), 4.47 (d, J = 5.9 Hz, 2H).

\(^{13}\text{C} \text{ NMR} \text{ (101 MHz, DMSO-}d_6\) \(\delta \text{ [ppm]} = 170.28, 169.51, 167.04, 163.21, 162.76, 159.99, 141.13, 139.32, 134.75, 130.14, 130.07, 128.42, 127.34, 94.40, 67.22, 42.21, 42.09, 20.83.

HRMS (ESI) calc. for C\(_{19}\)H\(_{16}\)ClN\(_7\)O\(_2\) \([\text{M+Na}^+]^+\): 432.0946; found 432.0942.

1.5.5 \(N\)-(4-(((2-amino-6-chloropyrimidin-4-yl)oxy)methyl)benzyl)-4-vinylbenzamide (CP-Vbn)

Reaction was conducted according to general procedure A with a reduced reaction time of 15 min. using CP-NH\(_2\) and 4-vinylbenzoic acid (6.8 mg, 41.6 \(\mu\)mol) to obtain 11.6 mg (22.8 \(\mu\)mol) of the desired product as a colorless TFA-salt in 60\% yield.
$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ [ppm] = 9.04 (t, $J$ = 6.0 Hz, 1H), 7.87 (d, $J$ = 8.3 Hz, 2H), 7.57 (d, $J$ = 8.3 Hz, 2H), 7.40 (d, $J$ = 8.1 Hz, 2H), 7.34 (d, $J$ = 8.1 Hz, 2H), 6.79 (dd, $J$ = 17.7, 10.9 Hz, 1H), 7.10 (brs, 2H), 6.12 (s, 1H), 5.95 (d, $J$ = 17.7 Hz, 1H), 5.37 (d, $J$ = 10.9 Hz, 1H), 5.29 (s, 2H), 4.47 (d, $J$ = 6.0 Hz, 2H).

$^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ [ppm] = 170.29, 165.77, 162.77, 159.99, 139.80, 139.68, 135.91, 134.66, 133.45, 128.41, 127.61, 127.31, 126.00, 116.20, 94.40, 67.27, 42.43.

HRMS (ESI) calc. for C$_{21}$H$_{20}$ClN$_4$O$_2$ [M+H]$^+$: 395.1269; found 395.1258.

1.5.6 4-(Benzyloxy)-6-chloropyrimidin-2-amine (CP)

2-Amino-4,6-dichloropyrimidine (200 mg, 1.22 mmol, 1.0 equiv.) was dissolved in dry DMF (2 mL). Benzyl alcohol (63 μL, 1.22 mmol, 1.0 equiv.), KOtBu (342.2 mg, 3.04 mmol, 2.5 equiv.) and KI (20.2 mg, 0.122 mmol, 0.1 equiv.) were added and the reaction mixture was stirred at room temperature for 4 h. Afterwards, the reaction was quenched with water and extracted with EtOAc (3 ×). The combined organic layers were washed with brine and dried over MgSO$_4$. The volatiles were evaporated and the crude product was purified over normal phase flash chromatography (hexane:DCM = 50% : 50% to 100% DCM). The fractions containing the product were combined, volatiles were evaporated and 134 mg (0.569 mmol) of the desired product was obtained as a yellowish solid in 47% yield.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ [ppm] = 7.43 – 7.30 (m, 5H), 6.01 (d, $J$ = 0.7 Hz, 1H), 5.31 (s, 2H), 2.26 (s, 3H).

$^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ [ppm] = 170.6, 168.4, 162.6, 136.7, 128.7, 128.5, 128.0, 127.4, 97.2, 93.0, 77.4, 77.1, 76.7, 67.5, 123.7 ppm.

HRMS (ESI) calc. for C$_{11}$H$_{11}$ClN$_3$O$_2$ [M+H]$^+$: 236.0585; found 236.0583.

1.5.7 ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl(4-(((2-amino-6-chloropyrimidin-4-yl)oxy)methyl)benzyl)carbamate (CP-BCN)

(1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (10.0 mg, 34.3 μmol; 1.0 equiv.) was dissolved in dry DMSO (0.5 mL) and DIPEA (28.4 μL, 172 μmol, 5 equiv.) followed by CP-NH$_2$ (10.0 mg, 37.8 μmol, 1.1 equiv.) were added. The reaction was stirred at r.t. for 30 min. The resulted mixture was acidified with acetic acid (3 μL) and H$_2$O (53 μL), then purified by preparative HPLC eluted with MeCN / H$_2$O (0.1% TFA) (10% MeCN for 10 min., then 10 - 90% MeCN over 55 min., followed by 99% MeCN for 5 min.) to give 1.4 mg (3.11 μmol) of the desired product as a colorless solid in 9% yield after lyophilization.

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ [ppm] = 7.68 (q, $J$ = 6.4 Hz, 1H), 7.38 (d, $J$ = 8.0 Hz, 2H), 7.25 (d, $J$ = 7.8 Hz, 2H), 6.12 (s, 1H), 5.28 (s, 2H), 4.17 (d, $J$ = 6.1 Hz, 2H), 4.06 (d, $J$ = 8.0 Hz, 2H), 2.29 – 1.72 (m, 6H), 1.71 – 1.38 (m, 2H), 1.35 – 0.60 (m, 3H).

HRMS (ESI) calc. for C$_{23}$H$_{26}$ClN$_4$O$_3$ [M+H]$^+$: 441.1688; found 441.1688.

1.5.8 N-(4-(((2-amino-6-chloropyrimidin-4-yl)oxy)methyl)benzyl)acetamide (CP-Ac)
CP-NH₂ (300 mg, 1.13 mmol, 1.0 equiv.) was dissolved in dry DMSO (1.5 mL) and DIPEA (375 μL, 2.27 mmol, 2.0 equiv.) was added followed by dropwise addition of acetic anhydride (160 μL, 1.70 mmol, 1.5 equiv.) while stirring. The reaction mixture was stirred at r.t. for 1 h. Afterwards, the reaction was quenched with acetic acid (387 μL) and H₂O (341 μL) followed by purification over preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (30% MeCN for 10 min., then 30 - 90% MeCN over 55 min., followed by 99% MeCN for 5 min.) to give 201 mg (655 μmol) of the desired product as a colorless solid in 58% yield after lyophilization.

¹H NMR (400 MHz, DMSO-d₆) δ [ppm] = 8.33 (t, J = 6.0 Hz, 1H), 7.41 – 7.35 (m, 2H), 7.28 – 7.22 (m, 2H), 7.09 (s, 2H), 6.13 (s, 1H), 5.29 (s, 2H), 4.24 (d, J = 5.9 Hz, 2H), 1.86 (s, 3H).

HRMS (ESI) calc. for C₁₃H₁₄ClN₂O₇⁺ [M+H]: 307.0956; found 307.0957.

1.5.9 Cyclooct-2-yn-1-yl (4-(((2-amino-6-chloropyrimidin-4-yl)(oxy)methyl)benzyl)carbamate (CP-SCO)

CP-NH₂ (10 mg; 37.8 μmol, 1.3 equiv.) was dissolved in dry DMF (0.3 mL) and a solution of 8.4 mg cyclooct-2-yn-1-yl (4-nitrophenyl) carbonate (29.1 μmol, 1.0 equiv.) in dry DMF (0.2 mL) was added followed by DIPEA (24.0 μL, 145 μmol: 5.0 equiv.). The reaction mixture was stirred at r.t. for 2 h. The resulted mixture was acidified with acetic acid (25 μL) and afterwards purified by semi-preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (15% MeCN for 2 min., then 15 - 100% MeCN over 25 min., followed by 100% MeCN for 15 min.) to give 12.0 mg (22.7 μmol) of the desired product as a colorless TFA-salt in 78% yield after lyophilization.

¹H NMR (400 MHz, DMSO-d₆) δ [ppm] = 7.78 (t, J = 6.2 Hz, 1H), 7.38 (d, J = 8.1 Hz, 2H), 7.24 (d, J = 8.1 Hz, 2H), 6.13 (s, 1H), 5.28 (s, 2H), 5.21 – 5.11 (m, 1H), 4.15 (d, J = 6.2 Hz, 2H), 2.29 – 2.02 (m, 3H), 1.95 – 1.85 (m, 1H), 1.85 – 1.78 (m, 2H), 1.76 – 1.65 (m, 1H), 1.65 – 1.54 (m, 2H), 1.54 – 1.42 (m, 1H).

¹³C NMR (101 MHz, DMSO-d₆) δ [ppm] = 170.30, 162.78, 159.99, 155.51, 139.64, 134.73, 128.40, 127.11, 100.94, 94.41, 91.76, 67.25, 65.93, 43.50, 41.58, 33.84, 29.21, 25.79, 19.96.

HRMS (ESI) calc. for C₂₁H₂₀ClN₂O₇NaO⁺ [M+Na]: 437.1351; found 437.1358.

1.5.10 2-(6-amino-3-iminio-4,5-disulfonato-3H-xanthen-9-yl)-4-(((2-amino-6-chloropyrimidin-4-yI)(oxy)methyl)benzyl)carbamoyl)benzoate (CP-Alexa488)

In an Eppendorf tube, CP-NH₂ (0.34 μg, 1.27 μmol, 2.0 equiv.) was dissolved in dry DMSO (100 μL) followed by addition of DIPEA (885 μL, 5.1 μmol, 8.0 equiv.) and a solution of 2-(6-amino-3-iminio-4,5-disulfonato-3H-xanthen-9-yl)-4-(((2,5-dioxopyrrolidin-1-yI)(oxy)carbonyl)benzoate (0.4 mg, 0.64 μmol, 1.0 equiv.) in dry DMSO (100 μL). The reaction was kept at r.t. for 1 h. The compound was purified over preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (10% MeCN for 10 min., then 10 - 90% MeCN over 40 min., followed by 90% MeCN for 5 min.) to give 195 μg (252 nmol) of the desired product as a yellow solid in 79% yield after lyophilization.

HRMS (ESI) calc. for C₃₀H₂₀ClN₆O₁₁S₂ [M+3H]: 781.0784; found 781.0772.
1.5.11 4-((4-(((2-amino-6-chloropyrimidin-4-yl)oxy)methyl)benzyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoate (CP-Fluorescein)

Fluorescein-6-COOH (25.0 mg, 66.4 μmol, 1.0 equiv.) was dissolved in dry DMSO (1.25 mL) and DIPEA (22.0 μL, 133 μmol, 2.0 equiv.) as well as TSTU (24.0 mg, 79.7 μmol, 1.2 equiv.) were added and the mixture was stirred at r.t. for 30 min. Afterwards, CP-NH₂ (26.4 mg, 99.7 μmol, 1.5 equiv.) was added and the reaction mixture was stirred at r.t. for 1 h. The resulted mixture was quenched with acetic acid (22.0 μL) and 10% H₂O, then the compound was purified over preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (10% MeCN for 10 min., then 10 - 90% MeCN over 40 min., followed by 90% MeCN for 5 min.) to give 31 mg (49.8 μmol) of the desired product in 75% yield after lyophilization.

HRMS (ESI) calc. for C₃₃H₂₄ClN₄O₇+: [M+H]⁺: 623.1328; found 623.1327.

1.5.12 4-(((2-amino-6-chloropyrimidin-4-yl)oxy)methyl)benzyl)carbamoyl)-2-(dimethylamino)-3-(dimethyliminio)-10,10-dimethyl-3,10-dihydroanthracen-9-yl)benzoate (CP-CPY)

CPY-6-COOH (250 mg, 530 μmol, 1.0 equiv.) was dissolved in dry DMSO (2 mL) and DIPEA (362 μL, 2.19 mmol, 4.0 equiv.) as well as TSTU (231 mg, 767 μmol, 1.4 equiv.) were added and the mixture was stirred at r.t. for 5 min. Afterwards, CP-NH₂ (217 mg, 821 μmol, 1.5 equiv.) was added and the reaction mixture was stirred at r.t. for 35 min. The resulted mixture was acidified with acetic acid (362 μL) and H₂O (500 μL), then the compound was purified over preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (10% MeCN for 10 min., then 10 - 90% MeCN over 40 min., followed by 90% MeCN for 5 min.) to give 130 mg (184.9 μmol) of the desired product in 34% yield after lyophilization.

¹H NMR (400 MHz, acetone-d₆) δ [ppm] = 8.51 (t, J = 6.4 Hz, 1H), 8.23 (d, J = 8.1 Hz, 1H), 8.12 (d, J = 8.7 Hz, 1H), 7.67 (s, 1H), 7.39 – 7.30 (m, 4H), 7.11 (s, 2H), 6.67 (s, 4H), 6.36 (s, 1H), 6.07 (m, J = 10.7, 2.5 Hz, 1H), 5.30 (m, J = 11.2, 2.5 Hz, 2H), 4.55 (d, J = 5.9 Hz, 2H), 3.11 (s, 12H), 1.89 (d, J = 2.5 Hz, 3H), 1.76 (d, J = 2.4 Hz, 3H).

¹³C NMR (101 MHz, acetone-d₆) δ [ppm] = 171.72, 165.87, 161.60, 140.12, 136.37, 134.01, 129.34, 129.25, 128.85, 120.23, 113.03, 110.69, 96.16, 68.31, 44.02, 40.62, 35.59, 33.04, 30.42, 30.22, 30.03, 29.84, 29.65, 29.45, 29.26, 26.13.

HRMS (ESI) calc. for C₄₀H₃₉ClN₆O₄+: [M+H]⁺: 703.2794; found 703.2792.
1.5.13 4-(((2-amino-6-chloropyrimidin-4-yl)oxy)methyl)benzyl)carbamoyl)-2-{7-(dimethylamino)-3-(dimethyliminio)-5,5-dimethyl-3,5-dihydrodibenzo[b,e]silin-10-yl}benzoate (CP-SiR)

SiR-6-COOH\* (481 mg, 1.02 mmol, 1.1 equiv.) was dissolved in dry DMSO (4 mL) and DIPEA (919 μL, 5.56 mmol, 6.0 equiv.) was added. The mixture was sonicated until complete solution and TSTU (391 mg, 1.30 mmol, 1.4 equiv.) was added and the mixture was stirred at r.t. for 5 min. Afterwards, CP-NH$_2$ (294 mg, 1.11 mmol, 1.2 equiv.) was added and the reaction mixture was stirred at r.t. for 2h. The resulted mixture was quenched by addition of acetic acid (973 μL) and H$_2$O (500 μL), then compound was purified over preparative HPLC eluted with MeCN / H$_2$O (0.1% TFA) (10% - 90% MeCN over 40 min., then 10% MeCN for 40 min., followed by lyophilization. 355 mg (494 μmol) of the desired product in 53% yield after lyophilization.

HRMS (ESI): calc. for C$_{39}$H$_{39}$N$_6$O$_4$Si$^+$ [M+H]$^+$: 719.2563; found 719.2561.

1.6  CLIP substrates

1.6.1 4-(((4-aminopyrimidin-2-yl)oxy)methyl)benzyl)carbamoyl)-2-{6-(dimethylamino)-3-(dimethyliminio)-10,10-dimethyl-3,10-dihydroanthracen-9-yl}benzoate (BC-CPY)

CPY-6-COOH\* (250 mg, 530 μmol, 1.0 equiv.) was dissolved in dry DMSO (2 mL). DIPEA (362 μL, 2.19 mmol, 4.0 equiv.) and TSTU (231 mg, 767 μmol, 1.4 equiv.) were added and the mixture was stirred at r.t. for 5 min. Afterwards, BC-NH$_2$ (189 mg, 821 μmol, 1.5 equiv.) was added and the reaction mixture was stirred at r.t. for 35 min. The resulted mixture was acidified with acetic acid (362 μL) and H$_2$O (500 μL), then compound was purified over preparative HPLC eluted with MeCN / H$_2$O (0.1% TFA) (10% MeCN for 10 min., then 10% MeCN for 40 min., followed by lyophilization. To give 180 mg (269.1 μmol) of the desired product in 49% yield after lyophilization.

$^1$H NMR (400 MHz, acetone-$_d_6$) $\delta$ [ppm] = 8.51 (t, $J = 6.9$ Hz, 1H), 8.21 (d, $J = 8.1$ Hz, 1H), 8.10 – 8.01 (m, 2H), 7.61 (s, 1H), 7.38 (d, $J = 7.4$ Hz, 2H), 7.32 (d, $J = 7.8$ Hz, 2H), 7.27 (s, 1H), 7.05 (s, 2H), 6.61 (s, 4H), 6.40 (d, $J = 6.6$, 2.4 Hz, 1H), 5.36 (s, 2H), 4.56 – 4.50 (m, 2H), 3.04 (s, 12H), 1.88 (d, $J = 2.5$ Hz, 3H), 1.64 (d, $J = 2.6$ Hz, 3H).

$^{13}$C NMR (101 MHz, acetone-$_d_6$) $\delta$ [ppm] = 169.57, 165.97, 162.80, 152.54, 152.01, 148.78, 141.22, 140.20, 136.01, 130.47, 129.26, 128.83, 126.23, 124.12, 120.13, 112.83, 110.38, 100.42, 69.65, 44.02, 43.89, 40.54, 39.65, 35.58, 33.19, 30.36, 30.23, 30.03, 29.84, 29.65, 29.46, 29.26.

HRMS (ESI): calc. for C$_{39}$H$_{39}$N$_6$O$_4$Si$^+$ [M+2H]$^{2+}$: 335.1628; found 335.1629.

1.7  Additional substrates
1.7.1 2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-4-(methylcarbamoyl)benzoate (meAm-6-TMR)

To a solution of TMR-6-COOH (1.0 mg, 2.32 μmol, 1.1 equiv.) in dry DMSO (500 μL), TSTU (763 µg, 2.53 μmol, 1.2 equiv.) was added and the mixture was stirred at r.t. for 5 min. Afterwards, DIPEA (1.4 μL, 8.45 μmol, 4 equiv.) and methylamine (2 M, 1.06 μL, 2.11 μmol, 1 equiv.) were added and the reaction mixture was stirred at r.t. overnight. The compound was purified over preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (10% MeCN for 10 min., then 10 - 90% MeCN over 40 min., followed by 90% MeCN for 5 min.) to give 91.1 μg (205.4 nmol) of the desired product in 10% yield after lyophilization.

1H NMR (TMR-6-COOH) (400 MHz, DMSO-d₆) δ [ppm] = 8.21 (dd, J = 8.0, 1.4 Hz, 1H), 8.17 – 7.99 (m, 1H), 7.61 – 7.56 (m, 1H), 6.58 – 6.45 (m, 6H), 2.95 (s, 12H).

13C NMR (TMR-6-COOH) (101 MHz, DMSO-d₆) δ [ppm] = 168.56, 166.53, 152.67, 152.47, 131.16, 128.91, 109.56, 105.91, 98.43, 40.46, 40.26.

HRMS (ESI): calc. for C₂₆H₂₆N₃O₄ [M+H]+: 444.1918; found 444.1914.

1.7.2 2-(6-(dimethylamino)-3-(dimethyliminio)-10,10-dimethyl-3,10-dihydroanthracen-9-yl)-4-(methylcarbamoyl)benzoate (meAm-6-CPY)

To a solution of CPY-6-COOH (1.0 mg, 2.19 μmol, 1.1 equiv.) in dry DMSO (500 μL), TSTU (719 µg, 2.39 μmol, 1.2 equiv.) was added and the mixture was stirred at r.t. for 5 min. Afterwards, DIPEA (1.32 μL, 7.97 μmol, 4.0 equiv.) and methylamine (2 M, 0.996 μL, 1.99 μmol, 1.0 equiv.) were added and the reaction mixture was stirred at r.t. overnight. The compound was purified over preparative HPLC eluted with MeCN / H₂O (0.1% TFA) (10% MeCN for 10 min., then 10 - 90% MeCN over 40 min., followed by 90% MeCN for 5 min.) to give 97.7 μg (208.1 nmol) of the desired product in 10% yield after lyophilization.

HRMS (ESI): calc. for C₂₉H₃₂N₃O₃ [M+H]+: 470.2438; found 470.2434.

1.7.3 2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-5-(methylcarbamoyl)benzoate (meAm-5-TMR)
To a solution of TMR-5-COOH (2.5 mg, 5.81 μmol, 1.0 equiv.) in dry DMSO (500 μL), BOP (2.59 mg, 8.71 μmol, 1.5 equiv.) was added and the reaction was shaken at r.t. and 500 rpm for 5 min. DIPEA (3.84 μL, 23.2 μmol, 4.0 equiv.) and methylamine (2M in THF, 4.36 μL, 8.71 μmol, 1.5 equiv.) were added and the reaction was shaken at r.t. and 500 rpm for 4 h. The crude product was acidified with acetic acid and purified over preparative HPLC eluted with MeCN / H₂O (0.1% FA) (10% - 90% MeCN over 50 min) to give 0.97 mg (2.19 μmol) of the desired product in 38% yield after lyophilization.

1H NMR (TMR-5-COOH) (400 MHz, DMSO-d₆) δ [ppm] = 8.39 (s, J = 1.5 Hz, 1H), 8.28 (dd, J = 8.1, 1.5 Hz, 1H), 7.33 (d, J = 8.0 Hz, 1H), 6.58 – 6.45 (m, 6H), 2.95 (s, 12H).

13C NMR (TMR-5-COOH) (101 MHz, DMSO-d₆) δ [ppm] = 168.31, 166.09, 152.03, 135.96, 132.76, 128.50, 109.05, 97.95, 40.15, 39.99, 39.79.

HRMS (ESI): calc. for C₂₆H₂₆N₃O₄+[M+H]+: 444.1923; found 444.1914.

1.7.4 2-(6-(dimethylamino)-3-(dimethyliminio)-10,10-dimethyl-3,10-dihydroanthracen-9-yl)-5-(methylcarbamoyl)benzoate (meAm-5-CPY)

To a solution of CPY-5-COOH (2.5 mg, 5.48 μmol, 1.0 equiv.) in dry DMSO (1 mL), BOP (0.5 M in DMSO, 17.4 μL, 8.71 μmol, 1.5 equiv.) was added and the reaction was shaken at r.t and 500 rpm for 5 min. DIPEA (3.62 μL, 21.9 μmol, 4.0 equiv.) and methylamine (2M in THF, 4.11 μL, 8.21 μmol, 1.5 equiv.) were added and the reaction was shaken at 500 rpm, r.t. for 4 h. The crude product was acidified with acetic acid and purified over preparative HPLC eluted with MeCN / H₂O (0.1% FA) (10% - 90% MeCN over 50 min) to give 0.77 mg (1.64 μmol) of the desired product in 30% yield after lyophilization.

HRMS (ESI): calc. for C₂₉H₃₂N₆O₃+[M+H]+: 470.2443; found 470.2437.
Figure S1: Chemical structures of SLP substrates. (continued on the next page)
Figure S1 (continued): Chemical structures of SLP substrates.

A. Chemical structures of HT7 (CA), SNAP (BG and CP) and CLIP (BC) core substrates. B. Chemical structures of fluorescent substituents. C. Chemical structures of non-fluorescent substituents. Colored dots indicate the tested substrates for the corresponding SLPs (grey = CA, blue = BG, green = CP and orange = BC).
Figure S2: Labeling kinetics of HT7 with fluorescent CA substrates.

Full anisotropy traces (points) and predications of fits based on model 2 (lines) along with zoom on the first second are represented on the top panels. Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence anisotropy changes over time using a stopped flow device. All conditions are 1:1 mixtures of protein and substrate at the given concentrations (conc.). For structures of CA substrates see Fig. S1.
Figure S3: Comparison of model 1 and model 2 fitted to HT7 labeling kinetics.

Anisotropy traces (points) and predictions of fits based on either model 1 or model 2 (lines) of the labeling reaction between HT7 and CA-TMR are represented in the top panels. Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence anisotropy changes over time using a stopped flow device. All conditions are 1:1 mixtures of protein and substrate at the given concentrations (conc.). Model 2 describes the data better than the simplified model 1. For structures of CA substrates see Fig. S1.
Figure S4: Modeling of HT7 labeling kinetics using measured parameters to compare the kinetic models 1 and 2.

A. Modeling of the fluorescence anisotropy response at different reactant concentrations using model 1 and 2 with parameters determined for HT7 labeling with CA-TMR. At concentrations below $K_d$ (327 nM for CA-TMR) both models yield a rather similar response. At concentrations higher than $K_d$ (1000 nM) the response for model 2 shows a strong biphasic character as observed in the measured data, which is not matching the monoexponential behavior of model 1. At very high concentrations (10000 nM) the response for model 2 is again close to a monoexponential curve but the kinetic is much faster than the model 1 curve. This happens since the rise in fluorescence anisotropy for model 2 in the first milliseconds is not due to covalent reaction but mostly binding ($k_1$). The binding rate constant $k_1$ is faster than $k_{app}$ if $k_1$ is not zero ($k_{app} = k_1^* k_2 / (k_2 + k_1^*)$). Hence directly estimating $k_{app}$ from fluorescence anisotropy traces by fitting model 1 to the data is only valid for concentrations below $K_d$ or if $k_1 \ll k_2$. B. Modeling the formation of covalently labeled product at different reactant concentrations using model 1 and 2 with parameters determined for HT7 labeling with CA-CPY. At concentrations below $K_d$ (46 nM for CA-CPY) both models yield a rather similar behavior. At higher concentrations model 1 predicts a much faster product formation than model 2 since it does not account for enzyme saturation. C. Plot of the apparent first order reaction rate constant for product formation against substrate concentration for model 1 and 2 with parameters for CA-CPY. In contrast to model 1, model 2 accounts for enzyme saturation leading to a maximum reaction rate of $k_{max} = k_2 = 9.9 \text{ s}^{-1}$. The models start to diverge significantly once the substrate concentration exceeds $K_d$ (46 nM). As a consequence, model 2 should be used for predicting formation of labeled HT7 if labeling is performed at high concentrations.
Figure S5: Labeling kinetics of HT7 and HOB with CA-TMR (A) and CA-Alexa488 (B).

A: Labeling kinetics of HT7 and HOB with CA-TMR. Full anisotropy traces (points) and predictions of fits based on model 2 (lines) along with zoom on the initial part are represented on the top panels. Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence anisotropy changes over time using a stopped flow device. All conditions are 1:1 mixtures of protein and substrate at the given concentrations (conc.).

B: Labeling kinetics of HT7 and HOB with CA-Alexa488. Full fluorescence polarization traces (points) and predictions of fits based on model 1 (lines) along with zoom on the initial part are represented on the top panels. Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence polarization changes over time using a plate reader. All experiments were performed at a fixed substrate concentration of 50 nM with varying protein concentrations. For structures of CA substrates see Fig. S1.
Figure S6: Rate and equilibrium constants of HT7 labeling with various fluorescent CA substrates.

Rate constants $k_1$ (A), $k_1$ (B), $k_2$ (C) and the calculated dissociation constants ($K_d = k_1/k_2$, D) obtained from fitting model 2 to stopped flow labeling experiments of HT7 and HOB. The catalytic rate constant ($k_2$) is rather constant among these substrates, while there are significant differences in the dissociation constant ($K_d$). The $K_d$ variations are due to large differences in $k_1$ and minor differences in $k_2$. As a result, differences in $k_{app}$ can be mostly explained by affinity differences of HT7 towards its substrates. 

**E.** Correlation between the calculated $K_d$ from the stopped flow kinetic experiments and the $K_d$ obtained from titration experiments performed with the dead mutant HT7$^{D106A}$. Log transformed values were fitted to a linear model ($\log(y) = 1.455 * \log(x) - 2.567$; black line, 95% confidence bands in grey, depicting the area in which the true regression line lies with 95% confidence). The linear correlation in logarithmic space suggests that the $K_d$ of CA rhodamine substrates with HT7$^{D106A}$ could represent a valid proxy to estimate their $K_d$ with the native HT7. 

**F.** $K_d$ values of the tested substrates calculated from the kinetics ($k_i/k_{-1}$) and measured by fluorescence polarization titration against the dead mutant HT7$^{D106A}$.
Figure S7: Affinity of the dead mutant HT7D106A to fluorescent CA substrates.

A. Titration curves of fluorescent CA substrates against HT7D106A measured via fluorescence polarization. The FP value of each dye fully bound to native HT7 was added at c = 0.1 M to improve fitting of the upper plateau. (See corresponding methods section for more details.)

B. Table summarizing fitted $K_d$ values with 95% confidence intervals. For structures of CA substrates see Fig. S1.

| Substrate          | $K_d$ [μM] (95% CI) |
|--------------------|---------------------|
| CA–580LIVE         | 0.31 (0.27 to 0.35) |
| CA–CPY             | 0.74 (0.65 to 0.85) |
| CA–580CP           | 2.08 (1.94 to 2.23) |
| CA–JF608           | 2.43 (2.16 to 2.73) |
| CA–Cy3             | 2.44 (2.08 to 2.67) |
| CA–Cy5             | 2.92 (1.70 to 5.08) |
| CA–TMR–CN          | 3.29 (2.98 to 3.62) |
| CA–JF549           | 4.48 (3.61 to 5.55) |
| CA–TMR–SCH3        | 4.94 (4.36 to 5.61) |
| CA–JF669           | 5.16 (4.59 to 5.79) |
| CA–TMR             | 6.24 (5.25 to 7.41) |
| CA–500R            | 6.51 (5.92 to 7.16) |
| CA–TMR–SNH2        | 7.23 (6.29 to 8.43) |
| CA–MaP555          | 9.36 (8.06 to 10.9) |
| CA–TMR–az–F2       | 15.5 (14.8 to 16.3) |
| CA–515R            | 16.7 (13.5 to 20.8) |
| CA–5–CPY           | 17.6 (15.9 to 19.6) |
| CA–JF525           | 21.8 (17.9 to 26.5) |
| CA–510R            | 38.4 (32.1 to 45.9) |
| CA–JF503           | 39.7 (30.1 to 52.0) |
| CA–5–TMR           | 40.9 (36.9 to 45.4) |
| CA–Oregon green    | 866 (697 to 1109)   |
| CA–Alexa488        | 902 (804 to 1109)   |
| CA–Fluorescein     | 1135 (736 to 2098)  |
Figure S8: Labeling kinetics of HT7 with non-fluorescent CA substrates.

Fluorescence polarization traces (points) of kinetic competition assays and predications of fits (lines) based on a simple competitive model (see methods section for details) of HT7 labeling with CA-Alexa488 in the presence of different concentrations of non-fluorescent CA substrates are represented on the top panels. Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence polarization changes over time using a plate reader. For structures of CA substrates see Fig. S1.
**Figure S9**: Additional structural information on HaloTag proteins.

**A.** Alkane-TMR constraints by the crystal packing. Two monomers of HT7-TMR are displayed in grey and light-pink. The alkane-TMR (orange sticks) conformation is constrained by the light-pink monomer that was generated as a symmetry mate. A zoom is shown on the right panel. **B.** Structural comparison between $^{U32}$HT7-TMR (previously published) and $^{Y7A}$HT7-TMR (PDB ID 6Y7A, this study). A zoom into the binding site with hydrogen bonds between $^{Y7A}$HT7-TMR and $^{U32}$HT7-TMR and their respective reacted substrates are represented as black and dark-purple lines, respectively. **C.** Zoom into isolated catalytic aspartate and alkane-TMR substrate from both $^{Y7A}$HT7-TMR and $^{U32}$HT7-TMR crystal structures. **D.** Structural comparison between HT7-TMR and HOB-TMR. Hydrogen bonds between HT7 and HOB and their respective reacted substrates are represented as black and gold dashed lines, respectively. The blue spheres represent differences between HT7 and HOB caused by the mutations.
Figure S10: Biochemical study of the interaction of HT7 with CA-fluorophores.

A. Affinity of the dead mutant HT7<sub>D106A</sub> towards different fluorophore derivatives measured via fluorescence polarization assay. The FP value of each dye as CA substrate fully bound to native HT7 was added at c = 0.1 M or c = 1 M in order to improve fitting of the upper plateau. B. Affinity of HT7<sub>D106A</sub> to CA-Ac measured via fluorescence polarization competition assay against CA-TMR. C. Summary of dissociation constants (K<sub>d</sub>) and calculated free binding energies (∆G) of HT7<sub>D106A</sub> with CA-Ac, meAm-5-CPY and CA-5-CPY. The representation highlights the additive nature of the binding energies from the chloroalkane and the CPY moieties for the binding energy of the full substrate. D. Table summarizing values and confidence intervals (95%) of the fits.

| dye | position | ligand | K<sub>d</sub> [μM] | 95% CI [μM] |
|-----|----------|--------|------------------|-------------|
| TMR | 5 position | CA     | 39.4            | (35.7 to 43.4) |
|     |          | meAm   | 1930            | (1660 to 2291) |
|     | 6 position | CA     | 6.24             | (5.25 to 7.41) |
|     |          | meAm   | 1512            | (1402 to 1640) |
| CPY | 5 position | CA     | 16.8             | (15.2 to 18.6) |
|     |          | meAm   | 1987            | (1688 to 2398) |
|     | 6 position | CA     | 0.74             | (0.65 to 0.85) |
|     |          | meAm   | 707             | (497 to 785) |
Figure S11: Labeling kinetics of SNAP with fluorescent BG and CP substrates.

Full fluorescence polarization traces (points) and predications of fits based on model 1 or 1.2 (lines) along with zoom on the initial 5 minutes are represented on the top panels. Most substrates were fitted to model 1 except CP-Fluorescein and CP-CPY, which showed an additional phase (model 1.2). Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence polarization changes over time using a plate reader. Labeling was performed at different concentrations of SNAP protein. Substrate concentrations were aimed at 20 nM based on the dyes extinction coefficient but fitted in the model since significant deviations from the expected stoichiometry were observed. For structures of BG and CP substrates see Fig. S1.
Figure S12: Labeling kinetics of SNAP measured by stopped flow fluorescence anisotropy.

A. Comparative data analysis of SNAP labeling kinetics with BG-TMR. Anisotropy traces (points) and predictions of fits based on either model 1 or model 2 (lines) of the labeling reaction between SNAP and BG-TMR are represented in the top panels. Residuals from the fits are depicted in the bottom panels. Labeling was performed at different concentrations of SNAP protein and a constant substrate concentration of 1 µM. Model 2 describes the data better than the simplified model 1. (for model description see Fig. 1).

B. Kinetic traces of SNAP labeling with CP-TMR represented as previously explained and fit with model 2. For structures of BG and CP substrates see Fig. S1.

C. \( K_d \) and \( k_{\text{app}} \) values calculated from parameters obtained by fitting model 2 to stopped flow anisotropy data \( (K_d = k_0/k_1, k_{\text{app}} = k_1^2/k_0(k_1+k_2)) \) compared to values directly fitted to fluorescence polarization assay with SNAP\(^{\text{C145A}} \) \( (K_d) \) and plate reader kinetics at lower SNAP concentrations fitted with model 1 \( (k_{\text{app}}) \).
Figure S13: Comparison of fluorophore substrate affinities between the dead mutants SNAP\textsuperscript{C145A} and SNAPf\textsuperscript{C145A}.

A. Titration curves obtained for the dead mutants SNAP\textsuperscript{C145A} and SNAPf\textsuperscript{C145A} measured via fluorescence polarization. The FP value of each dye fully bound to native SNAP/SNAPf was added at \( c = 0.005 \) M to improve fitting of the upper plateau. (See corresponding methods section for more details). B. Table summarizing fitted \( K_d \) values with 95% confidence intervals. For structures of BG and CP substrates see Fig. S1.
Figure S14: Comparison of non-derivatized core substrate affinities with the dead mutant SNAP$^{C145}$.

A. Titration curves obtained for the dead mutant SNAP$^{C145A}$ measured via competitive fluorescence polarization. The FP value of free dye was added at c = 0.1 M to improve fitting of the lower plateau. (See corresponding methods section for more details)

B. Table summarizing fitted Kd values with 95% confidence intervals. For structures of substrates see Fig. S1.
Figure S15: Sequence and additional structural information related to SNAP.

A. Sequence alignment of hAGT, SNAP, SNAPf, CLIP and CLIPf. Differences are highlighted in yellow, red and violet in the hAGT, SNAP(f) and CLIP(f) sequences, respectively.

B. Modeling of the E30R mutation in the SNAP-TMR crystal structure. SNAP is represented as cartoon, the fluorophore substrate and residues as sticks. Putative hydrogen bonds and corresponding distances are indicated by black dashes.

C. Crystal structure of SNAP labeled with TMR with α-carbons of the residues that differ between SNAP and CLIP represented as purple spheres.

D. Benzyl-TMR constraints by the crystal packing. Two monomers of SNAP-TMR are represented as grey and yellow cartoons. The conformation of the benzyl-TMR (orange sticks) of both monomers is constrained by the other monomer. Symmetry mates were generated within 4 Å radius and selected to highlight the packing constraints.

E. Structural alignment of SNAP-TMR with SsOGT-H5-VG structure (PDB ID 6GA0).
Figure S16: Labeling kinetics of SNAPf with fluorescent BG and CP substrates.

Full fluorescence polarization traces (points) and predictions of fits based on model 1 or 1.2 (lines) along with zoom on the initial 5 minutes are represented on the top panels. All substrates were fitted to model 1 except CP-CPY, which showed an additional phase (model 1.2). Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence polarization changes over time using a plate reader. Labeling was performed at different concentrations of SNAPf protein. Substrate concentrations were aimed at 20 nM based on the dyes extinction coefficient but fitted in the model since significant deviations from the expected stoichiometry were observed. For structures of BG and CP substrates see Fig. S1.
Figure S17: Labeling kinetics of CLIP and CLIPf with fluorescent BC substrates.

Full fluorescence polarization traces (points) and predictions of fits based on model 1 or 1.2 (lines) along with zoom on the initial 20 minutes are represented on the top panels. All substrates were fitted to model 1 except BC-CPY, which showed an additional phase (model 1.2). Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence polarization changes over time using a plate reader. Labeling was performed at different concentrations of CLIP and CLIPf protein. Substrate concentrations were aimed at 20 nM based on the dyes extinction coefficient but fitted in the model since significant deviations from the expected stoichiometry were observed. For structures of BC substrates see Fig. S1.
Figure S18: Labeling kinetics of hAGT, SNAP and CLIP with the non-respective BG-, CP- and BC-TMR substrates.

Full fluorescence polarization traces (points) and predications of fits based on model 1 along with zoom on the initial part (except for BC-TMR and hAGT) are represented on the top panels. Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence polarization changes over time using a plate reader. Labeling was performed at different concentrations of hAGT, SNAP and CLIP proteins. Substrate concentrations were aimed at 20 nM based on the dyes extinction coefficient but fitted in the model since significant deviations from the expected stoichiometry were observed. For structures of BG, CP and BC substrates see Fig. S1.
Figure S19: Labeling kinetics of SNAP with non-fluorescent BG and CP substrates.

Fluorescence polarization traces (points) of kinetic competition assays and predications of fits based on a simple competitive model (lines, see methods section for details) of SNAP labeling with BG-Alexa488 in the presence of different concentrations of non-fluorescent BG/CP substrates are represented on the top panels. Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence polarization changes over time using a plate reader. For structures of BG and CP substrates see Fig. S1.
Figure S20: Labeling kinetics of SNAP and SNAPf with BG-5-TMR and BG-5-CPY.

Full fluorescence polarization traces (points) and predictions of fits based on model 1.2 (lines) along with zoom on the initial 10 minutes are represented on the top panels. Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence polarization changes over time using a plate reader. Labeling was performed at different concentrations of SNAP and SNAPf protein. Substrate concentrations were aimed at 20 nM based on the dyes extinction coefficient but fitted in the model since significant deviations from the expected stoichiometry were observed. For structures of BG substrates see Fig. S1.

Figure S21: SNAPf kinetic and affinity correlations.

Correlation between SNAPf labeling kinetics ($k_{app}$) and affinity ($K_a = 1/K_d$) for different fluorophore substrates. Affinities were obtained with the catalytically dead mutant SNAPf$^{C145A}$. Log transformed values were fitted to a linear model (black line, $\log(k_{app}) = 0.2568 + \log(K_a) \times 1.0697$, 95% confidence bands in grey, depicting the area in which the true regression line lies with 95% confidence). The linear correlation in logarithmic space suggests that the $K_d$ of fluorescent SNAP substrates towards SNAPf$^{C145A}$ could represent a valid proxy to estimate their $K_{app}$ towards native SNAPf.
Figure S22: Labeling kinetics of SsOGT-H5 with BG-Alexa488 and BG-TMR.

Full fluorescence polarization traces (points) and predictions of fits based on model 1 (lines) along with zoom on the initial 5 hours are represented on the top panels. Residuals from the fits are depicted in the bottom panels. Kinetics were recorded by following fluorescence polarization changes over time using a plate reader. Labeling was performed at different concentrations of SsOGT-H5 protein. Substrate concentrations were aimed at 20 nM based on the dyes extinction coefficient but fitted in the model since significant deviations from the expected stoichiometry were observed. For structures of BG substrates see Fig. S1.
Table S1: Data collection and refinement statistics the X-ray crystal structures.

| Data collections | SNAP-TMR | HT7-TMR | HT7-CPY | HOB-TMR |
|------------------|----------|---------|---------|---------|
| PDB ID           | 6Y8P     | 6Y7A    | 6Y7B    | 6ZCC    |
| Beamline         | ESRF ID29| PXII-X10SA, SLS | PXII-X10SA, SLS | PXII-X10SA, SLS |
| Wavelength (Å)   | 0.976    | 1.00001 | 1.0006  | 0.99984 |
| Resolution (Å)   | 36.88 - 2.3 | 50-1.40 | 50-3.10 | 50-1.50 |
| (last bin)       | (2.382 - 2.3) | (1.50-1.40) | (3.20-3.10) | (1.60-1.50) |
| Space group      | P32 1     | P12 1   | P32 1   | P2 2 2 2 |
| **Unit cell dimensions** | | | | |
| a (Å)            | 65.5148   | 44.00   | 161.27  | 52.21   |
| b (Å)            | 65.5148   | 78.14   | 161.27  | 64.77   |
| c (Å)            | 97.067    | 45.24   | 124.66  | 78.85   |
| No. observed reflections | 119190 (12210) | 160637 (29978) | 231609 (21528) | 228695 (8515) |
| No. unique reflections | 11152 (1087) | 50448 (9451) | 34294 (3081) | 38699 (3579) |
| Completeness (%) | 99.94 (100.00) | 96.5 (97.1) | 99.8 (99.9) | 88.9 (47.5) |
| Rmerge           | 0.1015 (0.8636) | 0.063 (0.410) | 0.196 (0.596) | 0.042 (0.241) |
| l/σ(I)           | 13.48 (2.82) | 9.59 (2.83) | 8.53 (3.10) | 18.87 (2.39) |
| CC ½ (%)         | 99.9 (19.3) | 99.7 (86.4) | 98.8 (85.7) | 99.9 (93.4) |
| Redundancy       | 10.7 (11.2) | 3.18 (3.17) | 6.75 (6.99) | 5.91 (2.38) |
| Wilson B         | 47.75     | 21.39   | 37.99   | 32.28   |
| **Refinement statistics** | | | | |
| Resolution range (Å) | 36.88-2.3 | 39.19-1.40 | 49.32-3.10 | 43.53-1.52 |
| No. Reflections  | 8878      | 50435   | 34290   | 38697   |
| Rwork (%)        | 0.2385    | 0.1558  | 0.2074  | 0.1887  |
| Rfree (%)        | 0.2694    | 0.1868  | 0.2594  | 0.2238  |
| No. protein atoms| 1231      | 2397    | 11750   | 2348    |
| No. water atoms  | 50        | 348     | 0       | 312     |
| No. ligand atoms | 45        | 51      | 235     | 52      |
| Average B factor (Å²) | 73.06     | 18.93   | 51.23   | 31.74   |
| **RMSD from ideal** | | | | |
| Bond lengths (Å) | 0.007     | 0.013   | 0.004   | 0.009   |
| Bond angles (°)  | 1.24      | 1.247   | 0.788   | 1.014   |
Table S2: Kinetic parameters of HT7 labeling with fluorescent CA substrates.

| Substrate     | $k_1$ (± S.D.) [M$^{-1}$ s$^{-1}$] | $k_1$ (± S.D.) [s$^{-1}$] | $k_2$ (± S.D.) [s$^{-1}$] | $k_{app}$ (± S.D.) [M$^{-1}$ s$^{-1}$] |
|---------------|-----------------------------------|---------------------------|---------------------------|---------------------------------------|
| CA-TMR        | 7.84 (± 0.76) × 10$^7$            | 2.56 (± 0.38) × 10$^7$    | 8.06 (± 0.29)             | 1.88 (± 0.01) × 10$^7$                |
| CA-JF549      | 1.60 (± 0.16) × 10$^8$            | 9.83 (± 2.04) × 10$^1$    | 1.82 (± 0.07) × 10$^1$    | 1.66 (± 0.01) × 10$^7$                |
| CA-JF669      | 2.35 (± 0.75) × 10$^7$            | 3.39 (± 1.49) × 10$^1$    | 6.94 (± 0.47)             | 4.03 (± 0.02) × 10$^6$                |
| CA-CPY        | 1.67 (± 0.067) × 10$^8$           | 7.60 (± 0.98)             | 9.86 (± 0.73)             | 9.44 (± 0.18) × 10$^7$                |
| CA-LIVE580    | 1.74 (± 0.05) × 10$^8$            | 1.75 (± 0.28)             | 6.77 (± 0.77)             | 1.39 (± 0.03) × 10$^8$                |
| CA-TMR-biotin | 3.69 (± 0.25) × 10$^7$            | 2.10 (± 0.25) × 10$^1$    | 8.24 (± 0.28)             | 1.04 (± 0.01) × 10$^7$                |

Data analyzed using model 2.

Table S3: Comparison $k_{app}$ of HT7 labeling kinetics analyzed using models 1 and 2.

| Substrate     | $k_{app}$ (± S.D.) [M$^{-1}$ s$^{-1}$] |
|---------------|---------------------------------------|
|               | Model 1                               | Model 2                       |
| CA-TMR        | 1.79 (± 0.01) × 10$^7$                | 1.88 (± 0.01) × 10$^7$        |
| CA-JF549      | 1.46 (± 0.01) × 10$^7$                | 1.66 (± 0.01) × 10$^7$        |
| CA-JF669      | 3.95 (± 0.02) × 10$^6$                | 4.03 (± 0.02) × 10$^6$        |
| CA-CPY        | 1.10 (± 0.02) × 10$^8$                | 9.44 (± 0.18) × 10$^7$        |
| CA-LIVE580    | 1.58 (± 0.02) × 10$^8$                | 1.39 (± 0.03) × 10$^8$        |
| CA-TMR-biotin | 9.00 (± 0.04) × 10$^6$                | 1.04 (± 0.01) × 10$^7$        |

Table S4: Comparison of HT7 and HOB labeling kinetics with fluorescent CA substrates.

| Protein | Substrate     | $k_1$ (± S.D.) [M$^{-1}$ s$^{-1}$] | $k_1$ (± S.D.) [s$^{-1}$] | $k_2$ (± S.D.) [s$^{-1}$] | $k_{app}$ (± S.D.) [M$^{-1}$ s$^{-1}$] |
|---------|---------------|-----------------------------------|---------------------------|---------------------------|---------------------------------------|
| HT7     | CA-TMR        | 7.84 (± 0.76) × 10$^7$            | 2.56 (± 0.38) × 10$^7$    | 8.06 (± 0.29)             | 1.88 (± 0.01) × 10$^7$                |
|         | CA-Alexa488   | -                                 | -                         | -                         | 2.57 (± 0.01) × 10$^4$                |
| HOB     | CA-TMR        | 4.15 (± 0.26) × 10$^7$            | 1.83 (± 0.17) × 10$^1$    | 5.05 (± 0.13)             | 8.99 (± 0.04) × 10$^6$                |
|         | CA-Alexa488   | -                                 | -                         | -                         | 8.04 (± 0.02) × 10$^4$                |
Table S5: Kinetic parameters of SNAP and CLIP labeling with fluorescent substrates analyzed using model 1.2.

| Substrate     | $k_{app}$ (± S.D.) [s⁻¹] | $k_3$ (± S.D.) [s⁻¹] | $k_{app}$ (± S.D.) [s⁻¹M⁻¹] | $k_3$ (± S.D.) [s⁻¹] |
|---------------|-------------------------|---------------------|-----------------------------|---------------------|
| SNAP CP-Fluorescein | 1.42 (± 0.01) × 10⁴   | 1.61 (± 0.04) × 10⁻³ | -                           | -                   |
| SNAP CP-CPY    | 1.59 (± 0.01) × 10⁴   | 1.26 (± 0.01) × 10⁻² | 3.55 (± 0.02) × 10⁻¹ | 6.22 (± 0.13) × 10⁻³ |
| CLIP BC-CPY    | 1.26 (± 0.01) × 10⁴   | 2.16 (± 0.09) × 10⁻⁴ | 2.65 (± 0.01) × 10⁻¹ | 9.02 (± 0.48) × 10⁻⁷ |

Table S6: Kinetic parameters of SNAP labeling with TMR substrates measured via stopped flow.

| Substrate     | $k_1$ (± S.D.) [M⁻¹ s⁻¹] | $k_{app}$ (± S.D.) [s⁻¹] | $k_3$ (± S.D.) [s⁻¹] | $k_{app}$ (± S.D.) [M⁻¹ s⁻¹] |
|---------------|-------------------------|-------------------------|---------------------|-------------------------------|
| BG-TMR        | 4.93 (± 0.04) × 10⁵     | 1.02 (± 0.03)           | 1.24 (± 0.02)       | 2.71 (± 0.01) × 10⁵           |
| CP-TMR        | 5.36 (± 0.30) × 10⁵     | 8.96 (± 0.71)           | 1.58 (± 0.04)       | 0.81 (± 0.01) × 10⁵           |

Data analyzed using model 2

Table S7: Comparison of SNAP/CLIP with SNAP/CLIP labeling kinetics with fluorescent substrates.

| Substrate     | $k_{app}$ (± S.D.) [s⁻¹] |
|---------------|-------------------------|
| BG-Alexa488   | 1.22 (± 0.01) × 10⁴     |
| BG-Fluorescein| 1.17 (± 0.01) × 10⁴     |
| BG-CPY        | 2.17 (± 0.01) × 10⁴     |
| BG-TMR        | 4.29 (± 0.01) × 10⁵     |
| CP-Alexa488   | 3.12 (± 0.003) × 10⁴    |
| CP-Fluorescein| 1.42 (± 0.01) × 10⁴ (*) |
| CP-CPY*       | 1.59 (± 0.01) × 10⁴ (*) |
| CP-TMR        | 7.69 (± 0.01) × 10⁴     |
| BC-Alexa488   | 1.26 (± 0.01) × 10⁴     |
| BC-Fluorescein| 4.36 (± 0.01) × 10⁣     |
| BC-CPY        | 1.85 (± 0.01) × 10⁴     |
| BC-CPY*       | 1.26 (± 0.01) × 10⁴ (*) |

Data analyzed using model 1 or 1.2 (*) which included an additional phase (see Table S5).

Table S8: Comparison of SNAP labeling kinetics with 5- and 6-fluorophores.

| Substrate     | $k_{app}$ (± S.D.) [s⁻¹M⁻¹] | $k_3$ (± S.D.) [s⁻¹] | $k_{app}$ (± S.D.) [s⁻¹M⁻¹] | $k_3$ (± S.D.) [s⁻¹] |
|---------------|-----------------------------|---------------------|-----------------------------|---------------------|
| BG-6-TMR      | 4.29 (± 0.01) × 10⁴         | -                   | 3.94 (± 0.01) × 10⁴         | -                   |
| BG-5-TMR (*)  | 2.67 (± 0.01) × 10⁴         | 1.53 (± 0.12) × 10⁻³| 3.23 (± 0.01) × 10⁻¹ | 2.18 (± 0.18) × 10⁻³ |
| BG-6-CPY      | 2.17 (± 0.01) × 10⁴         | -                   | 2.17 (± 0.02) × 10⁻¹        | -                   |
| BG-5-CPY (*)  | 2.51 (± 0.01) × 10⁴         | 2.11 (± 0.04) × 10⁻²| 3.28 (± 0.01) × 10⁻¹ | 1.42 (± 0.03) × 10⁻² |

Data analyzed using model 1 or 1.2 (*) which included an additional phase.

Table S9: Kinetic parameters of SsOGT-H⁶ labeling.

| Substrate     | $k_{app}$ (± S.D.) [s⁻¹M⁻¹] |
|---------------|-----------------------------|
| BG-6-TMR      | 6.78 (± 0.67) × 10⁴         |
| BG-5-TMR      | 1.45 (± 0.92) × 10⁵         |
| BG-6-Alexa488 | 1.24 (± 0.01) × 10⁴         |

Data analyzed using model 1.
Protein sequences:
General color code: **Hisx10-tag** – TEV cleavage site – Protein sequence – **Fast mutation** – **Catalytic residue**

>`HT7
MHMMHHHHHHHLENLYFO|GIGTGFDFPHYVEVLGERMNYVDVGPRLDTQVFLHGNPTSSYWWRIHPHAPTHRCIAPDLIMGKSDKPDLCYFFFFDHHFMDAIELAGLEELREVILHDWSAGLGFHWAKPNPKVGFIAFMFIRPIPITPDWEPFARFFRTTDDVRKLIIDQNVFIEGTPLMGVVRPLTEVMHYREPLNVPDRELPNLPIAGEPANIVALVEEYMDWHLQSPVPKLLFWGTGPGVLIPEAARLAKSLPNCKAVDGPGLNLQEDNPDIGSEIARWLSLEI

>`HOB
MHMMHHHHHHHLENLYFO|GIGTGFDFPHYVEVLGERMNYVDVGPRLDTQVFLHGNPTSSYWWRIHPHAPTHRCIAPDLIMGKSDKPDLCYFFFFDHHFMDAIELAGLEELREVILHDWSAGLGFHWAKPNPKVGFIAFMFIRPIPITPDWEPFARFFRTTDDVRKLIIDQNVFIEGTPLMGVVRPLTEVMHYREPLNVPDRELPNLPIAGEPANIVALVEEYMDWHLQSPVPKLLFWGTGPGVLIPEAARLAKSLPNCKAVDGPGLNLQEDNPDIGSEIARWLSLEISG

Color code: **mutations as compared to HT7**

>`SNAP
MHMMHHHHHHHLENLYFO|GMKDCEMKRTTLDSPGLKLELSGECQGLHEIIFLGKGTSAADAVEVPAAPAVLGPEPLMQALTMLNAYFHOPEAIEEPFPALHHHPVFQESFRQVWLKLKVKKVKGFEVISYSHLAAAGNPATAAVALTAALSNGNPVIPILPCHRVVQGDLDVGGYEGGLAVKEWLLAHEHRLGKPGLG

>`SNAP!
MHMMHHHHHHHLENLYFO|GMKDCEMKRTTLDSPGLKLELSGECQGLHEIIFLGKGTSAADAVEVPAAPAVLGPEPLMQALTMLNAYFHOPEAIEEPFPALHHHPVFQESFRQVWLKLKVKKVKGFEVISYSHLAAAGNPATAAVALTAALSNGNPVIPILPCHRVVQGDLDVGGYEGGLAVKEWLLAHEHRLGKPGLG

>`SNAPf
MHMMHHHHHHHLENLYFO|GMKDCEMKRTTLDSPGLKLELSGECQGLHEIIFLGKGTSAADAVEVPAAPAVLGPEPLMQALTMLNAYFHOPEAIEEPFPALHHHPVFQESFRQVWLKLKVKKVKGFEVISYSHLAAAGNPATAAVALTAALSNGNPVIPILPCHRVVQGDLDVGGYEGGLAVKEWLLAHEHRLGKPGLG

>`CLIP
MHMMHHHHHHHLENLYFO|GMKDCEMKRTTLDSPGLKLELSGECQGLHEIIFLGKGTSAADAVEVPAAPAVLGPEPLMQALTMLNAYFHOPEAIEEPFPALHHHPVFQESFRQVWLKLKVKKVKGFEVISYSHLAAAGNPATAAVALTAALSNGNPVIPILPCHRVVQGDLDVGGYEGGLAVKEWLLAHEHRLGKPGLG

>`CLIPf
MHMMHHHHHHHLENLYFO|GMKDCEMKRTTLDSPGLKLELSGECQGLHEIIFLGKGTSAADAVEVPAAPAVLGPEPLMQALTMLNAYFHOPEAIEEPFPALHHHPVFQESFRQVWLKLKVKKVKGFEVISYSHLAAAGNPATAAVALTAALSNGNPVIPILPCHRVVQGDLDVGGYEGGLAVKEWLLAHEHRLGKPGLG

>`hAGT
MASWSSHPQFEKGAADDDDVPHQTMKDCEMKRTTLDSPGLKLELSGECQGLHEIIFLGKGTSAADAVEVPAAPAVLGPEPLMQALTMLNAYFHOPEAIEEPFPALHHHPVFQESFRQVWLKLKVKKVKGFEVISYSHLAAAGNPATAAVALTAALSNGNPVIPILPCHRVVQGDLDVGGYEGGLAVKEWLLAHEHRLGKPGLG

Color code: **Strep-Tag II**, **Enterokinase cleavage site**, **linkers**

S53
>SsOGT-H5
MASWHPOFEKGADDDKKVPMLVGLYKSPLYTVAKDDKGFIMLDFCDVCVGSRSRDSFTEFFHKLDLYFEGKPINLREPINLK
TYPFRSLVFKEVMKIPWGKVMTYKQIADSLGTAPAAYTALSENPILLIIPCHRIVIAENGIGGYERGVKLARALLELGKIPELAPGFSS
S

Color code: Strep-Tag II, Enterokinase cleavage site, linkers
Example DynaFit scripts:

**HT7 stopped flow labeling kinetics model 2**

```latex
[task]
data = progress
task = fit
confidence = monte-carlo

[mechanism]
P + S <==> P.S : k1 k-1
P.S ----> Z : k2

[constants] ; units: uM, sec
k1 = 10 ?
k-1 = 10 ?
k2 = 10 ?

[parameters]
R = 0.2 ?

[data]
Delay 0.022
offset 0.0262
directory path/to/data
sheet data.csv
response P.S = 1 * R | label c=1
  column 6 | conc P = 1 | conc S = 1 | response Z = 1 * R |
response P.S = 1.333 * R | label c=0.75
  column 5 | conc P = 0.75 | conc S = 0.75 | response Z = 1.333 * R |
response P.S = 2 * R | label c=0.5
  column 4 | conc P = 0.5 | conc S = 0.5 | response Z = 2 * R |
response P.S = 4 * R | label c=0.25
  column 3 | conc P = 0.25 | conc S = 0.25 | response Z = 4 * R |
response P.S = 10 * R | label c=0.1
  column 2 | conc P = 0.1 | conc S = 0.1 | response Z = 10 * R |

[output]
directory path/to/output/folder

[settings]
(ConfidenceIntervals)
LevelPercent = 95
(Output)
  XAxisLabel = time [s]
  YAxisLabel = anisotropy

[end]
```
SNAP stopped flow labeling kinetics model 2

[task]
  data = progress
  task = fit
  confidence = monte-carlo

[mechanism]
  P + S <===> P.S     ;     k1     k-1
  P.S ----> Z         ;     k2

[constants] ; units: uM, sec
  k1 = 1 ?
  k-1 = 1 ?
  k2 = 1 ?

[concentrations] ; units: uM
  S = 2 ?

[responses]
  Z = 0.07 ?
  P.S = 1 * Z

[data]
  delay = 0.022
  offset = 0
  directory = path/to/data
  sheet = data.csv
  column 2 | conc P = 50 | label c=50
  column 3 | conc P = 37.5 | label c=37.5
  column 4 | conc P = 25 | label c=25
  column 5 | conc P = 12.5 | label c=12.5
  column 6 | conc P = 5 | label c=5
  column 7 | conc P = 2.5 | label c=2.5
  column 8 | conc P = 1.25 | label c=1.25

[output]
  directory = path/to/output/folder

[settings]
  {ConfidenceIntervals}
  LevelPercent = 95

  {Output}
  XAxisLabel = time [s]
  YAxisLabel = anisotropy

[end]
HT7 microplate reader labeling kinetics model 1

(Time series of each condition were not averaged before DynaFit analysis since the TECAN plate reader has small inconsistencies in measurement intervals)

```
[task]
data = progress
task = fit
confidence = monte-carlo

[mechanism]
P + S ----> Z : k_app

[constants]; units: uM, sec
k_app = 1 ?
d = 0.05

[concentrations] ; units: uM
S = 0.05

Z = 4000 ?

[data]
delay 1
offset 57,118
directory path/to/data
sheet data.csv

column 2 | conc P = 0 | label 0
column 3 | conc P = 0 | label 0
column 4 | conc P = 0 | label 0

column 5 | conc P = 0.4 | label 400
column 6 | conc P = 0.4 | label 400
column 7 | conc P = 0.4 | label 400

column 8 | conc P = 0.8 | label 800
column 9 | conc P = 0.8 | label 800
column 10 | conc P = 0.8 | label 800

column 11 | conc P = 1.6 | label 1600
column 12 | conc P = 1.6 | label 1600
column 13 | conc P = 1.6 | label 1600

column 14 | conc P = 3.2 | label 3200
column 15 | conc P = 3.2 | label 3200
column 16 | conc P = 3.2 | label 3200

column 17 | conc P = 6.4 | label 6400
column 18 | conc P = 6.4 | label 6400
column 19 | conc P = 6.4 | label 6400

column 20 | conc P = 12.8 | label 12800
column 21 | conc P = 12.8 | label 12800
column 22 | conc P = 12.8 | label 12800

column 23 | conc P = 25.6 | label 25600
column 24 | conc P = 25.6 | label 25600
column 25 | conc P = 25.6 | label 25600

column 26 | conc P = 51.2 | label 51200
column 27 | conc P = 51.2 | label 51200
column 28 | conc P = 51.2 | label 51200

[output]
directory path/to/output/folder

[settings]
(ConfidenceIntervals)
LevelPercent = 95
(Output)
XAxisLabel = time [s]
YAxisLabel = anisotropy
```

S57
SNAP-CLIP microplate reader labeling kinetics model 1

(Time series of each condition were not averaged before DynaFit analysis since the TECAN plate reader has small inconsistencies in measurement intervals)

[task]
data = progress
task = fit
confidence = monte-carlo

[mechanism]
P + S ----> Z       : $k_{app}$

[constants]
$P$; units: nM, sec
$k_{app} = 0.0001$ ?

[concentrations]; units: nM
$S = 50$ ?

[responses]
$Z = 2$ ?

[data]
delay   2.7
offset   73.46
directory path/to/data
sheet    data.csv

column 2 | conc P = 52 | label 52
column 3 | conc P = 52 | label 52
column 4 | conc P = 52 | label 52
column 5 | conc P = 79 | label 79
column 6 | conc P = 79 | label 79
column 7 | conc P = 79 | label 79
column 8 | conc P = 118.5 | label 118.5
column 9 | conc P = 118.5 | label 118.5
column 10 | conc P = 118.5 | label 118.5
column 11 | conc P = 177.7 | label 177.7
column 12 | conc P = 177.7 | label 177.7
column 13 | conc P = 177.7 | label 177.7
column 14 | conc P = 266.6 | label 266.6
column 15 | conc P = 266.6 | label 266.6
column 16 | conc P = 266.6 | label 266.6
column 17 | conc P = 400 | label 400
column 18 | conc P = 400 | label 400
column 19 | conc P = 400 | label 400
column 20 | conc P = 600 | label 600
column 21 | conc P = 600 | label 600
column 22 | conc P = 600 | label 600
column 23 | conc P = 900 | label 900
column 24 | conc P = 900 | label 900
column 25 | conc P = 900 | label 900

[output]
directory path/to/output/folder

[settings]
(ConfidenceIntervals)
LevelPercent = 95
(Output)
XAxisLabel = time [s]
YAxisLabel = anisotropy

[end]
SNAP-CLIP microplate reader labeling kinetics model 1.2

(Time series of each condition were not averaged before DynaFit analysis since the TECAN plate reader has small inconsistencies in measurement intervals)

```
[task]
data  = progress
task  = fit
confidence = monte-carlo

[mechanism]
P + S ----> Z       : k_app
Z ----> Z2       : k_app_2

[constants] ; units: nM, sec
k_app  = 0.0001 ?
k_app_2 = 0.0001 ?

[concentrations] ; units: nM
S = 50 ?
Z = 2 ?
Z2 = 2 ?

[data]
delay 2.7
offset 73.46
directory path/to/data
sheet data.csv

column 2 | conc P = 52 | label 52
column 3 | conc P = 52 | label 52
column 4 | conc P = 52 | label 52
column 5 | conc P = 79 | label 79
column 6 | conc P = 79 | label 79
column 7 | conc P = 79 | label 79
column 8 | conc P = 118.5 | label 118.5
column 9 | conc P = 118.5 | label 118.5
column 10 | conc P = 118.5 | label 118.5
column 11 | conc P = 177.7 | label 177.7
column 12 | conc P = 177.7 | label 177.7
column 13 | conc P = 177.7 | label 177.7
column 14 | conc P = 266.6 | label 266.6
column 15 | conc P = 266.6 | label 266.6
column 16 | conc P = 266.6 | label 266.6
column 17 | conc P = 400 | label 400
column 18 | conc P = 400 | label 400
column 19 | conc P = 400 | label 400
column 20 | conc P = 600 | label 600
column 21 | conc P = 600 | label 600
column 22 | conc P = 600 | label 600
column 23 | conc P = 900 | label 900
column 24 | conc P = 900 | label 900
column 25 | conc P = 900 | label 900

[output]
directory path/to/output/folder

[settings]
(ConfidenceIntervals)
LevelPercent = 95
(Outp

[XAxisLabel = time [s]
YAxisLabel = anisotropy

[end]
```
References:

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