Supplementary Information

Table of contents

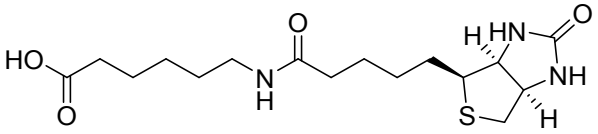
Supporting Information....1
Table of contents....1
Materials and Methods....2
Synthetic procedures....2
Automation device....9
ENACT procedure....20
Post-modification of trastuzumab-9 with 10a and 10b....22
Native mass spectrometry....23
Peptide mapping....23
References....24

Materials and Methods

All reagents were obtained from commercial sources and used without prior purifications. Dry solvents were obtained from Sigma-Aldrich. ^{1}H and ^{13}C NMR spectra were recorded at 23°C on Bruker 400 spectrometer. Recorded shifts are reported in parts per million (δ) and calibrated using residual non-deuterated solvent. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad), coupling constant (*J*, Hz) and integration. The LCMS analysis was carried out on Waters 2695 separations module equipped with Waters 2487 UV detector, Waters Acquity QDa mass detector and CORTECS, 2.7 µm, C18, 50 x 4.6 mm column. The flow rate was 1 ml/min. Solvent A: 0.05% HCOOH in water. Solvent B: 0.05% HCOOH in acetonitrile. Gradient run: 0-5 min – 5% to 95% B; 5-6 min – 95% B; 6-7.8 min – 5% B. Mass detector was operated in positive MS Scan mode with 600°C probe temperature, 1.5 kV capillary voltage and 10 V cone voltage. Column chromatography was carried out as "Flash Chromatography" using silica gel G-25 (40-63 µm) from Macherey-Nagel. Protein concentration of protein solutions was determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Illkirch, France). 3D printing was carried out on Tevo Tarantula - Prusa i3 3D printer using 1.75 mm PLA filament. Preparative HPLC was carried out on a Shimadzu system consisting of two LC-8A pumps, an SPD-10A VP detector, an SCL-10A VP controller, an SIL-10A autosampler, a 2 mL sample loop and a SunFire C18 column (150 mm × 19 mm i.d., 5 µm, Waters).

Synthetic procedures

6-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanoic acid (S1, Biotin-Caproyl-OH)

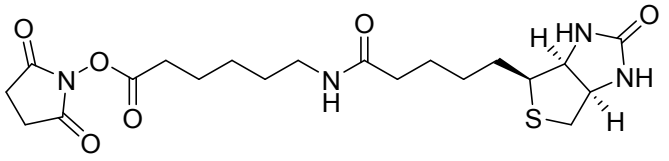


(+)-Biotin N-hydroxysuccinimide ester (1 eq., 388 mg, 1.14 mmol) was dissolved in the anhydrous DMF (8.54 mL). To the resulting solution was added 6-aminocaproic acid (1 eq., 149 mg, 1.14 mmol) and the mixture was stirred at r.t. overnight and then concentrated to yield a mixture of the desired product and NHS. To the residue was then added a 95:1:4 mixture of EtOH-AcOH-H_2O. The resulting suspension was heated to boiling temperature, filtered, and the filtrate was cooled to r.t. to allow the crystallization of the product, which was isolated by filtration and dried under vacuum to afford compound **S1** (305 mg, 0.854 mmol, 75 %) as a white solid.

^{1}H NMR (DMSO-d_6) δ: 7.72 (t, *J* = 4.9 Hz, 1H), 6.40 (s, 1H), 6.34 (s, 1H), 4.25 - 4.39 (m, 1H), 4.00 - 4.25 (m, 1H), 3.06 - 3.17 (m, 1H), 2.92 - 3.06 (m, 2H), 2.83 (dd, J = 12.4, 5.0 Hz, 1H), 2.58 (d, *J* = 12.4 Hz, 1H), 2.19 (t, *J* = 7.4 Hz, 2H), 2.05 (t, *J* = 7.4 Hz, 2H), 1.18 - 1.71 (m, 12H).

^{13}C NMR (DMSO-d_6) δ: 174.9, 172.3, 163.2, 61.6, 59.7, 55.9, 38.7, 35.7, 34.1, 29.4, 28.7, 28.5, 26.5, 25.8, 24.7.

2,5-dioxopyrrolidin-1-yl 6-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanoate (S2, Biotin-Caproyl-NHS)

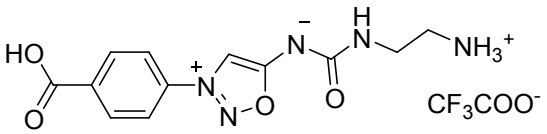


To a suspension of **S1** (1 eq., 520 mg, 1.45 mmol) in DMF (25.5 mL) were added *N,N'*-disuccinimidyl carbonate (2.5 eq., 931 mg, 3.64 mmol) and TEA (2 eq., 294 mg, 0.404 mL, 2.91 mmol). After 15 minutes the mixture became clear and stirring was continued for 2h at room temperature. 250 mL of Et_2O was added and the obtained precipitate was filtered to yield crude product, which was recrystallized from a minimum amount of acetonitrile to yield compound **S2** (443 mg, 0.975 mmol, 67%) as a white solid.

^{1}H NMR (DMSO-d_6) δ: 7.71 - 7.85 (m, 1H), 6.46 (br. s., 1H), 6.40 (br. s., 1H), 4.33 - 4.44 (m, 1H), 4.14 - 4.26 (m, 1H), 3.13 - 3.22 (m, 1H), 3.03 - 3.13 (m, 2H), 2.85 - 2.95 (m, 1H), 2.60 - 2.76 (m, 5H), 2.26 (t, *J* = 7.3 Hz, 2H), 2.11 (t, *J* = 7.0 Hz, 2H), 1.26 - 1.74 (m, 12H).

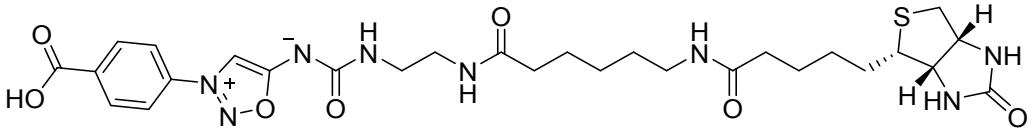
^{13}C NMR (DMSO-d_6) δ: 174.9, 173.2, 172.3, 170.7, 163.2, 61.6, 59.7, 55.9, 38.7, 35.7, 34.1, 29.4, 29.1, 28.7, 28.5, 26.5, 25.9, 25.8, 25.7, 24.7.

((2-ammonioethyl)carbamoyl)(3-(4-carboxyphenyl)-1,3,4-oxadiazol-3-ium-5-yl)amide 2,2,2-trifluoroacetate (S3)



Compound **S3** was prepared as previously described.[1]

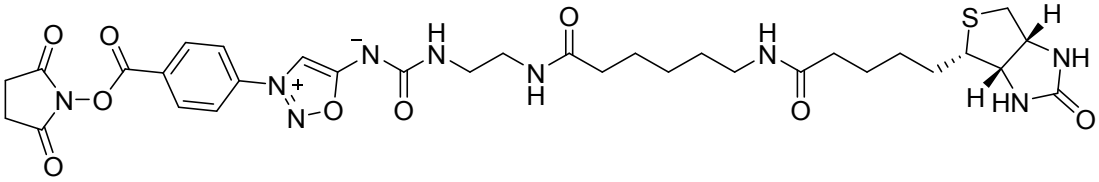
(3-(4-carboxyphenyl)-1,3,4-oxadiazol-3-ium-5-yl)((2-(6-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanamido)ethyl)carbamoyl)amide (S4)



To the solution of **S3** (1 eq., 333 mg, 0.822 mmol) in DMF (5 mL) was added TEA (4 eq., 0.457 mL, 3.287 mmol) followed by a solution of **S2** (1 eq., 374 mg, 0.822 mmol) in DMF (10 mL) and the mixture was stirred overnight. Et_2O (150 mL) was added to precipitate TEA salt of the product. The precipitate was filtered suspended in EtOH (15 mL), then TFA was added (5 mL). Stirring continued until complete dissolution of the precipitate. The solution was concentrated on rotatory evaporator, dried under vacuum to eliminate the excess of TFA, resolubilized with slight heating in minimum amount of DMF (3 mL), precipitated with warm (30˚C) acetonitrile and filtered to yield compound **S4** (440 mg, 0.698 mmol, 85 %) as a yellow solid. Crude **S4** was used in the next step without further purification.

^{1}H NMR (DMSO-d_6) δ: 8.50 (s, 1H), 8.12 - 8.26 (m, 4H), 7.79 (br. s., 1H), 7.66 - 7.74 (m, 1H), 6.99 (br. s., 1H), 6.40 (br. s., 1H), 6.35 (br. s., 1H), 4.25 - 4.37 (m, 1H), 4.07 - 4.19 (m, 1H), 3.06 - 3.18 (m, 5H), 2.94 - 3.06 (m, 2H), 2.83 (dd, *J* = 12.5, 5.0 Hz, 1H), 2.60 (s, 1H), 2.05 (d, *J* = 6.5 Hz, 4H), 1.14 - 1.68 (m, 12H).

(3-(4-(((2,5-dioxopyrrolidin-1-yl)oxy)carbonyl)phenyl)-1,3,4-oxadiazol-3-ium-5-yl)((2-(6-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanamido) ethyl)carbamoyl)amide (1)



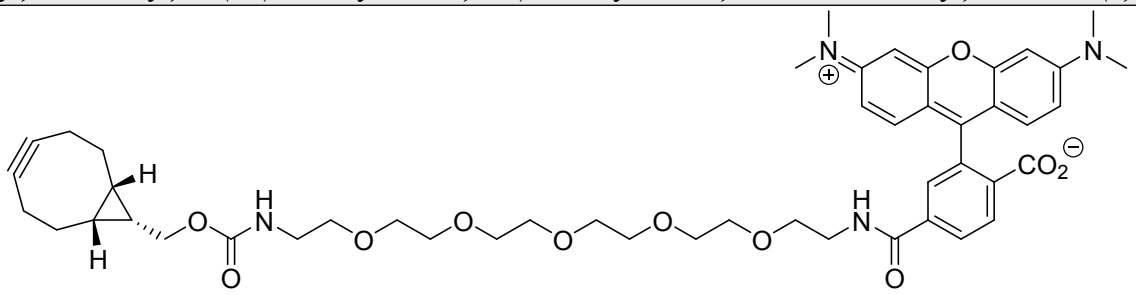
A solution of **S4** (1 eq., 78 mg, 0.124 mmol), NHS (5 eq., 71.2 mg, 0.618 mmol) and DCC (2 eq., 51 mg, 0.247 mmol) in DMSO (3 mL) was stirred overnight at room temperature. The resulting mixture was purified by preparative HPLC (40 min run, detection at 254 nm; buffer A: H_2O miliQ + 0.05% of TFA; buffer B: ACN; 40 min – from 5% to 95% B) and promptly lyophilized to yield **1** (82.8 mg, 0.114 mmol, 92 %) as a yellow solid.

^{1}H NMR (DMSO-d_6) δ: 8.83 (br. s., 1H), 8.29 - 8.48 (m, 4H), 7.82 (br. s., 1H), 7.71 (t, *J* = 5.3 Hz, 1H), 7.16 (br. s., 1H), 6.40 (br. s., 1H), 4.31 (dd, *J* = 7.4, 5.1 Hz, 1H), 4.13 (dd, *J* = 7.5, 4.5 Hz, 1H), 3.13 - 3.22 (m, 4H), 3.07 - 3.13 (m, 1H), 2.97 - 3.05 (m, 2H), 2.94 (s, 4H), 2.83 (dd, *J* = 12.4, 5.1 Hz, 1H), 2.56 - 2.62 (m, 2H), 2.05 (q, *J* = 7.6 Hz, 4H), 1.18 - 1.67 (m, 12H).

^{13}C NMR (DMSO-d_6) δ: 172.7, 172.3, 170.5, 163.2, 161.2, 132.4, 124.2, 61.6, 59.7, 55.9, 41.0, 38.8, 35.9, 35.7, 29.5, 28.7, 28.5, 26.6, 26.1, 25.8, 25.7, 25.5.

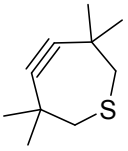
MS (ESI) *m/z*: 728.43 $[M+H]^+$.

4-((1-(bicyclo[6.1.0]non-4-yn-9-yl)-3-oxo-2,7,10,13,16,19-hexaoxa-4-azahenicosan-21-yl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate (2)



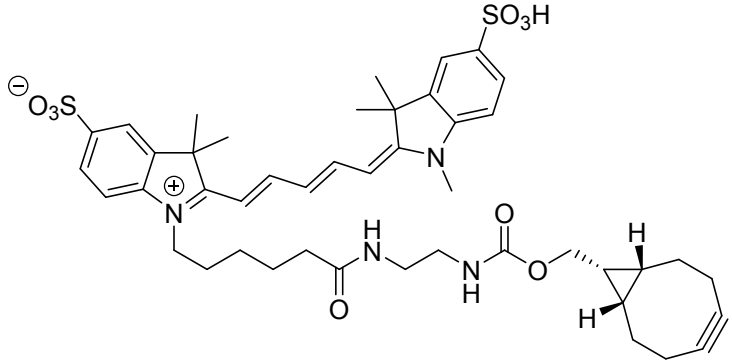
Compound **2** was prepared as previously described.[2]

3,3,6,6-Tetramethylthiacycloheptyne (5)



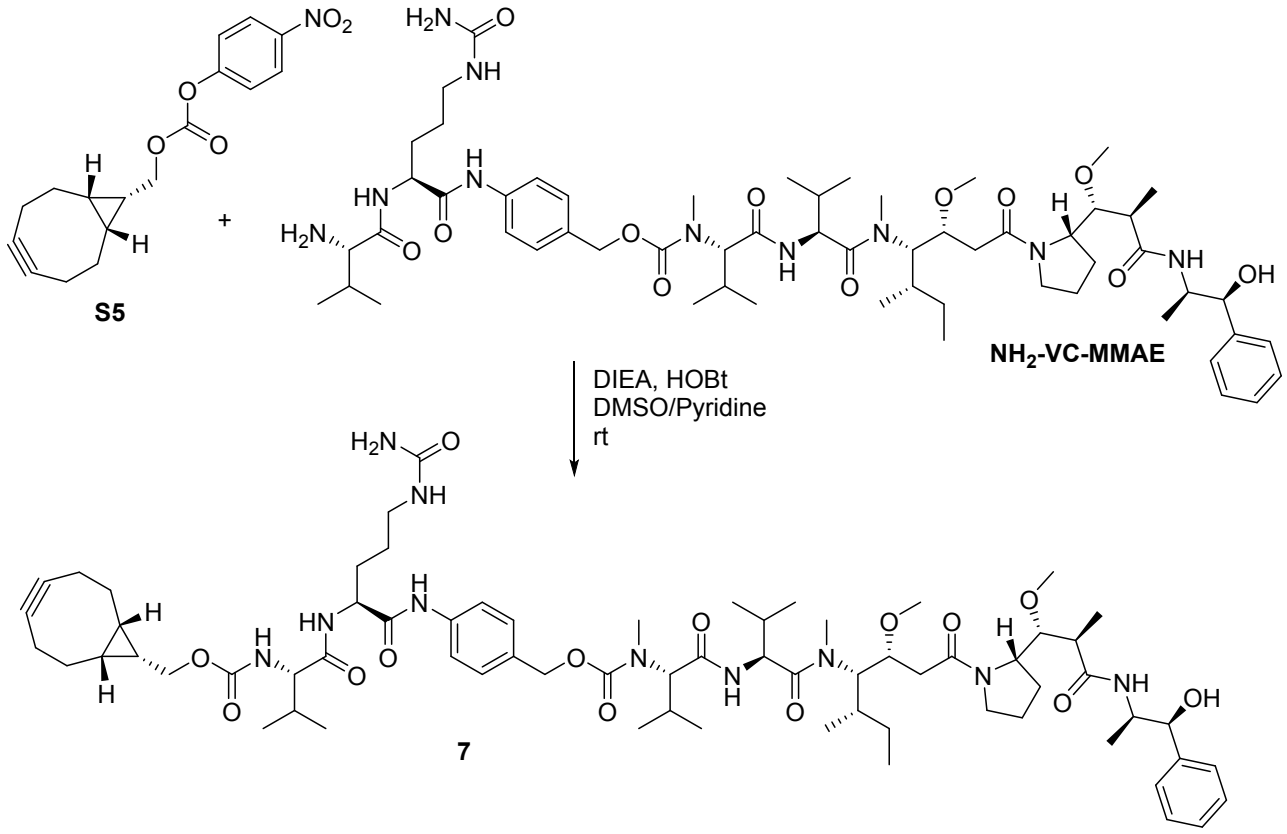
Compound **5** was prepared as previously described.[3]

1-(6-((2-(((bicyclo[6.1.0]non-4-yn-9-ylmethoxy)carbonyl)amino)ethyl)amino)-6-oxohexyl)-3,3-dimethyl-2-((1E,3E)-5-((E)-1,3,3-trimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium-5-sulfonate (6)



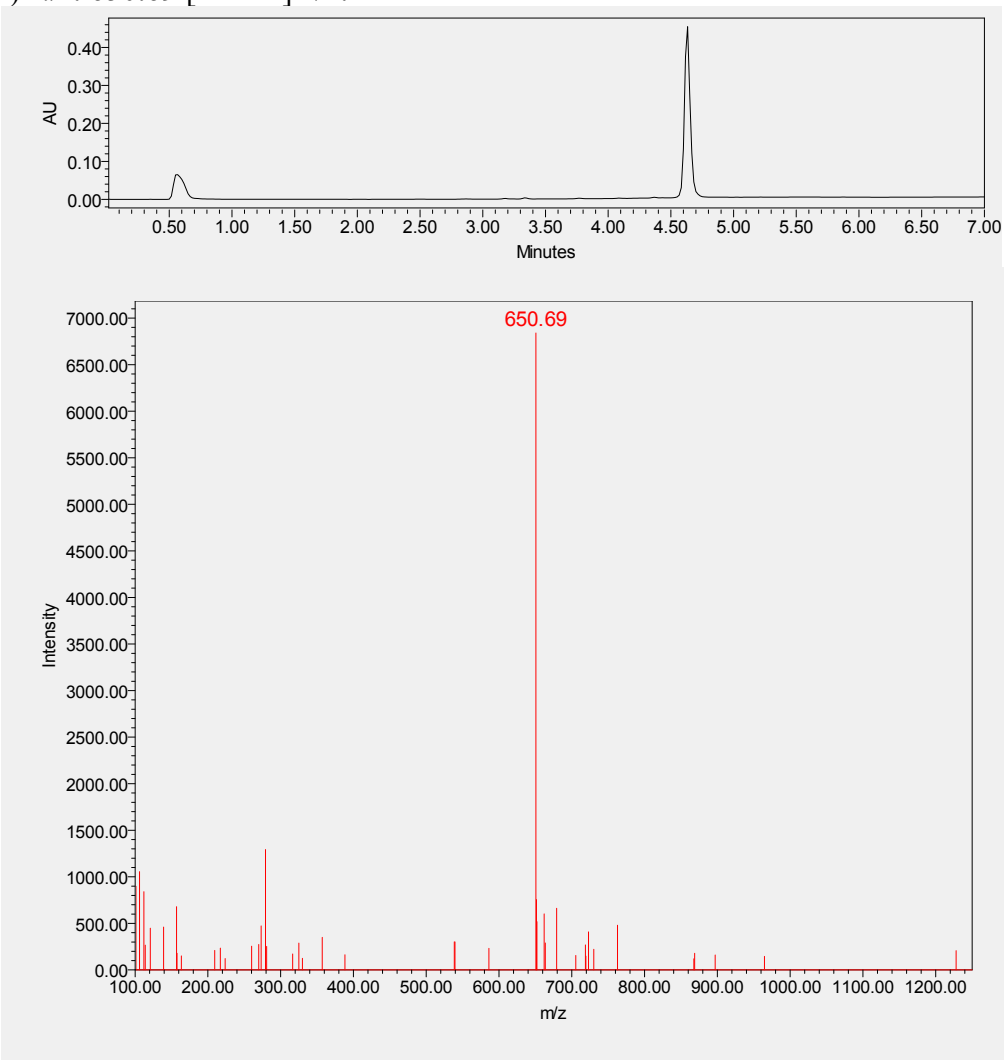
Compound **6** was prepared as previously described.[2]

4-((2R)-2-((2R)-2-(((bicyclo[6.1.0]non-4-yn-9-ylmethoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl ((S)-1-(((S)-1-(((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)(methyl)amino)-3-methyl-1-oxobutan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)(methyl)carbamate (7, BCN-Val-Cit-PAB-MMAE)

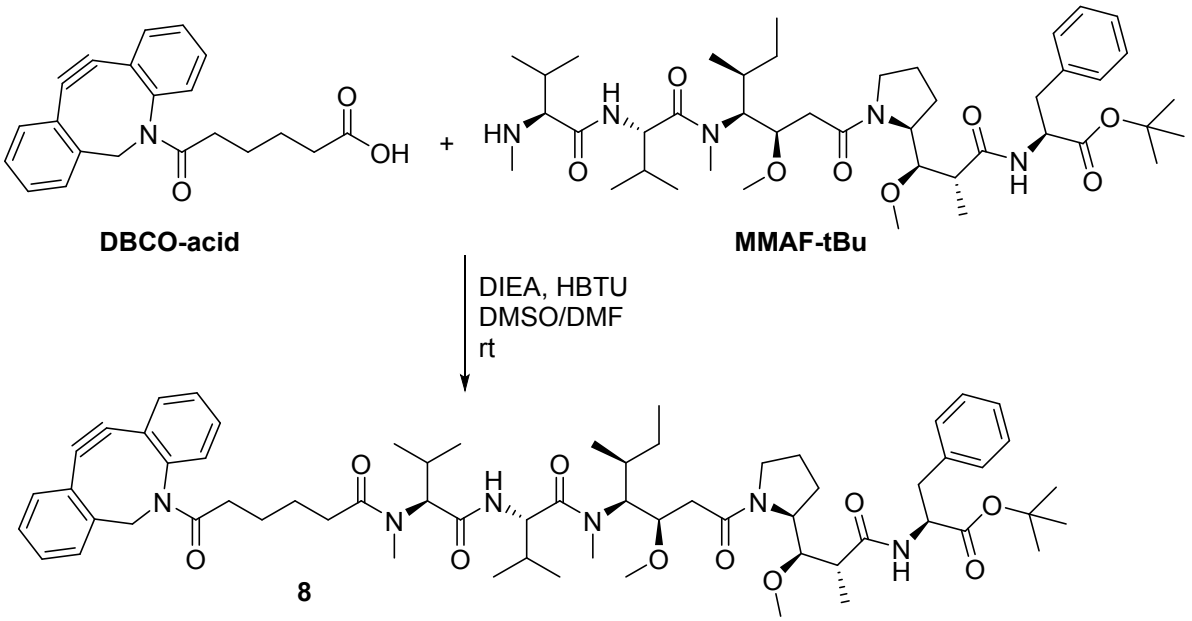


Compound **S5** was prepared as previously described.[4] NH_2-VC-MMAE was purchased from ACES Pharma (Princeton, NJ). To the solution of NH_2-VC-MMAE (1 eq., 0.1 M in DMSO, 50 µL, 5 µmol) was added **S5** (2 eq., 0.1 M in DMSO, 100 µL, 10 µmol) and a mixture of DIEA (10 eq., 0.5 M in pyridine, 100 µL, 50 µmol) and HOBt (4 eq., 0.2 M in pyridine, 100 µL, 20

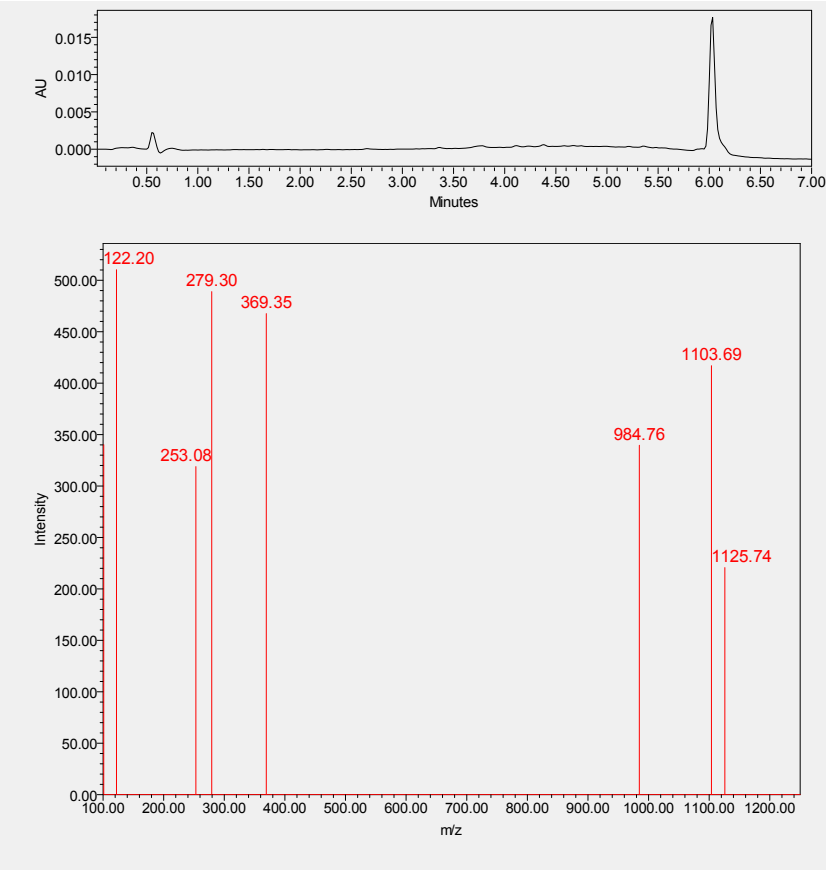
µmol). The mixture was flushed with Ar and incubated at r.t. for 36h. The crude reaction mixture was purified by preparative HPLC (40 min run, detection at 254 nm; buffer A: H_2O miliQ + 0.05% of TFA; buffer B: ACN; 40 min – from 5% to 95% B) and promptly lyophilized to yield **7** as a white solid. The solid was dissolved in DMSO-d6 (1ml) and the concentration of **7** was determined by NMR (ERETIC method). 888 µl of the solution of **7** (0.7 mM in DMSO-d6, 0.622 µmol, 12% yield) were obtained.
MS (ESI) *m/z*: 650.69 $[M+2H]^{2+}/2$.



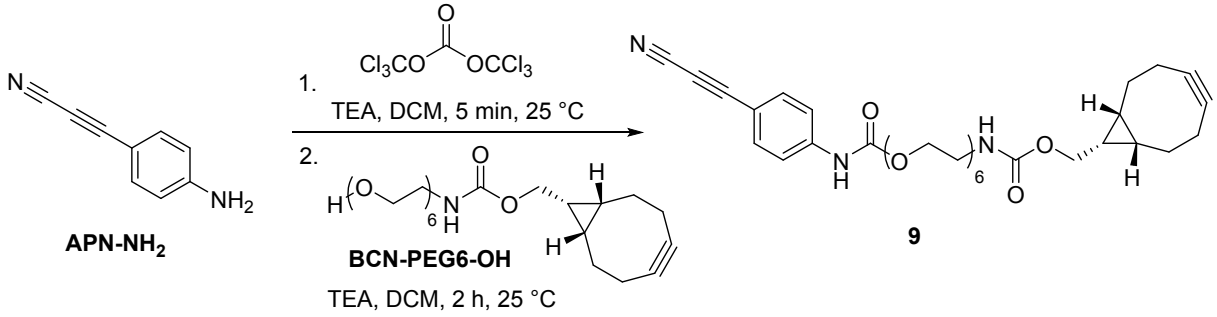
tert-butyl ((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-(6-(dibenzocyclooctynyl)-N-methyl-6-oxohexanamido)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanoyl)-L-phenylalaninate (8, DBCO-MMAF-tBu)



MMAF-tBu was purchased from ACES Pharma (Princeton, NJ). To the solution of DBCO-acid (1 eq., 0.1 M in DMSO, 25 µL, 2.5 µmol) was added a solution of HBTU (4 eq., 0.1 M in DMSO, 100 µL, 10.0 µmol). The mixture was stirred for 5 min and then the solution of MMAF-tBu (0.8 eq., 0.1 M in DMSO, 20 µL, 2.0 µmol) was added, followed by the solution of DIEA (5 eq., 0.5 M in DMF, 25 µL, 12.5 µmol). The mixture was incubated for 1h r.t. and then purified by preparative HPLC (40 min run, detection at 254 nm; buffer A: H_2O miliQ + 0.05% of TFA; buffer B: ACN; 40 min – from 5% to 95% B) to afford **8** as a white solid. DMSO-d6 (1ml) was added and the concentration of **8** was determined by NMR (ERETIC method). 750 µl of the solution of **8** (1.12 mM, in DMSO-d6, 0.840 µmol, 42% yield) were obtained.
MS (ESI) *m/z*: 1103.69 $[M+H]^+$, 1125.74 $[M+Na]^+$.



Bicyclo[6.1.0]non-4-yn-9-ylmethyl (1-((4-(cyanoethynyl)phenyl)amino)-1-oxo-2,5,8,11,14,17-hexaoxanonadecan-19-yl)carbamate (9, BCN-PEG6-APN)

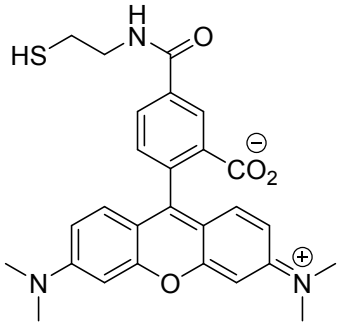


3-(4-aminophenyl)propiolonitrile (APN-NH_2)[5] and bicyclo[6.1.0]non-4-yn-9-ylmethyl N-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)carbamate (BCN-PEG6-OH)[6] were synthesized as previously described. To a solution of triphosgene (0.33 eq., 16.4 mg, 9.24 µL, 0.0554 mmol) in DCM (2 mL) was added a solution of 3-(4-aminophenyl)propiolonitrile (1 eq., 23.9 mg, 0.168 mmol) in DCM (0.5 mL). To the stirring mixture was added dropwise triethylamine (6 eq., 101 mg, 140 µL, 1.01 mmol) and stirring continued for 5 min at room temperature. A solution of BCN-PEG6-OH (0.95 eq., 73 mg, 0.16 mmol) and triethylamine (2 eq., 34 mg, 46.7 µL, 0.336 mmol) in DCM (1 mL) was added and the reaction mixture was stirred at room temperature for 2h. Full conversion was confirmed by LCMS and the mixture was concentrated and purified by flash chromatography (cyclohexane/EtOAc gradient 100/0 to 0/100) to give title product **9** (8 mg, 0.0128 mmol, 23 %) as a transparent oil.

^{1}H NMR (400 MHz, DMSO-d_6) δ**:** 10.30 (s, 1H), 7.74 (d, J = 8.4 Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H), 7.06 (t, J = 5.7 Hz, 1H), 4.24 (t, J = 4.5 Hz , 2H), 4.03 (d, J = 8.0 Hz, 2H), 3.66 (t, J = 4.6

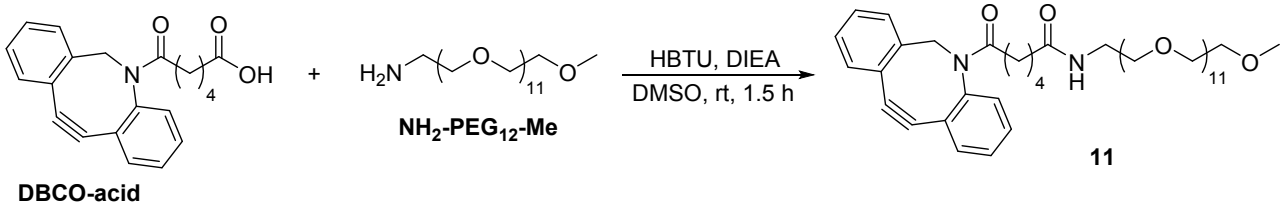
Hz, 2H), 3.57-3.46 (m, 16H), 3.41-3.36 (m, 2H), 3.11 (q, *J* = 6.0 Hz, 2H), 2.26-2.12 (m, 6H), 1.58-1.44 (m, 2H), 1.30-1.20 (m, 1H), 0.90-0.80 (m, 2H).
MS (ESI) *m/z*: 648.37 $[M+Na]^+$.

2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-5-((2-mercaptoethyl)carbamoyl) benzoate (10b, TAMRA-SH)



Compound **10b** was prepared as previously described.[7]

DBCO-PEG12-Me (11)



DBCO-acid + **NH_2-PEG_{12}-Me** → **11**

To the solution of DBCO-acid (1.1 eq., 5.2 mg, 14.8 μmol) in DMSO (350 μL) was added HBTU (1.2 eq., 6.13 mg, 16.2 μmol) and DIEA (1.2 eq., 2.82 μL, 16.2 μmol). The mixture was stirred for 5 min at r.t. and a solution of NH_2-PEG_{12}-Me (1 eq., 7.54 mg, 13.5 μmol) in DMSO (350 μL) was added. The resulting mixture was briefly vortexed, flushed with Ar and incubated at r.t. for 1.5h. Crude reaction mixture was purified by preparative HPLC (40 min run, detection at 254 nm; buffer A: H_2O miliQ + 0.05% of TFA; buffer B: ACN; 40 min – from 5% to 95% B) to afford **11** (7.31 mg, 8.35 μmol, 62% yield) as a pale yellow oil.
^{1}H NMR (DMSO-d_6) δ: 7.67 (t, *J* = 5.4 Hz, 1 H), 7.63 (d, *J* = 7.0 Hz, 1 H), 7.60 - 7.53 (m, 1 H), 7.53 - 7.44 (m, 3 H), 7.44 - 7.26 (m, 3 H), 5.04 (d, *J* = 13.8 Hz, 1 H), 3.61 (d, *J* = 14.1 Hz, 1 H), 3.57 - 3.40 (m, 46 H), 3.24 (s, 3 H), 3.11 (q, *J* = 5.8 Hz, 2 H), 2.24 - 2.09 (m, 1 H), 1.85 (t, *J* = 6.9 Hz, 2 H), 1.80 - 1.68 (m, 1 H), 1.33 - 1.07 (m, 4 H).
MS (ESI) *m/z*: 875.61 $[M+H]^+$.

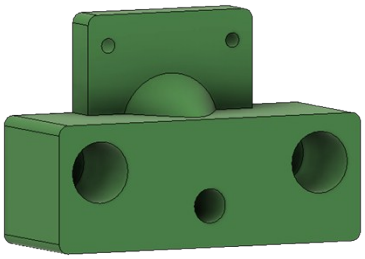
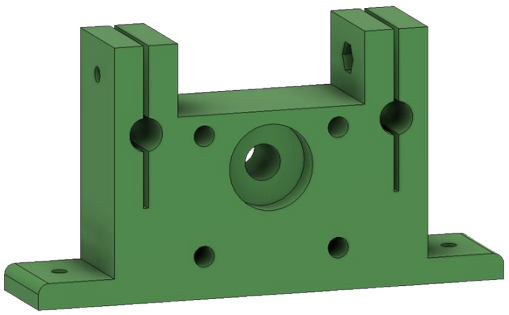
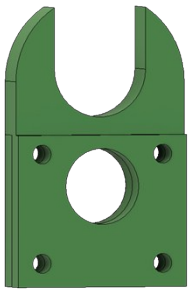
BCN-ON

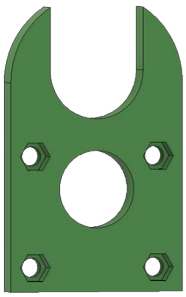
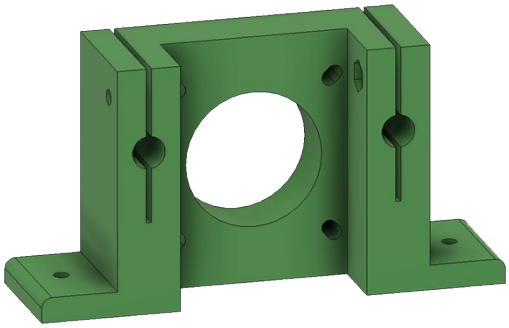
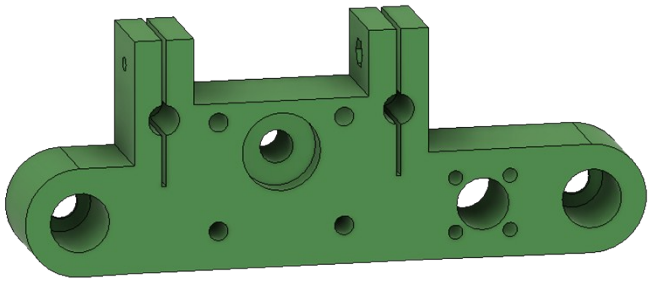
Amino-modified oligonucleotides (ON and cON) were purchased from IDT. BCN-ON was prepared as previously described.[8]

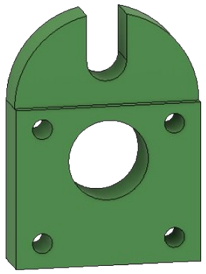
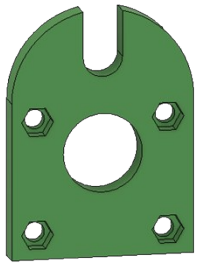
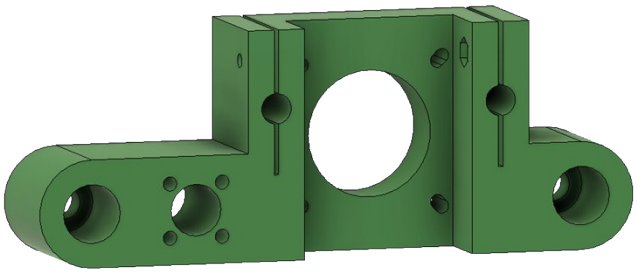
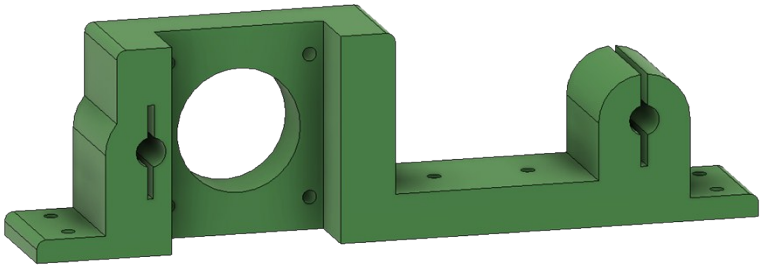
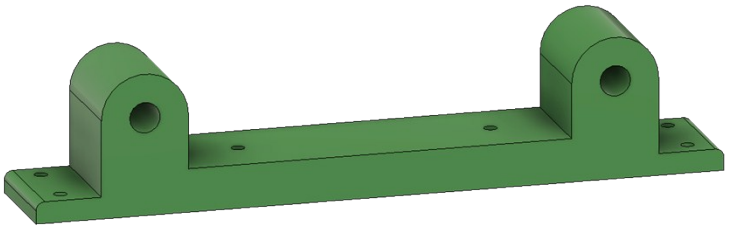
Automation device

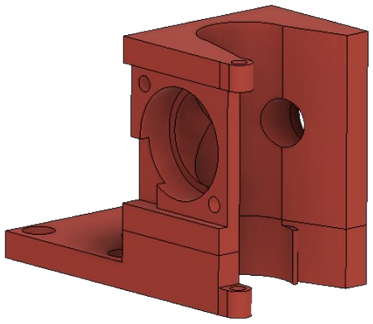
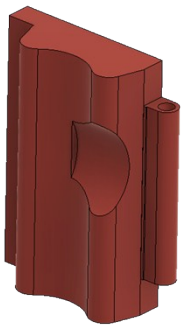
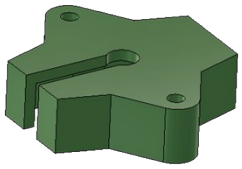
3D printing was carried out on Tevo Tarantula - Prusa i3 3D printer using 1.75 mm PLA filament. The full list of parts can be found in Table S1. 3D CAD models of all printed parts can be accessed throug the following link: http://www.thingiverse.com/thing:3273388
GUI for the automation device can be downloaded using the following link: https://github.com/k0n1ev/darx
Assembly instructions are shown on Figure S1.

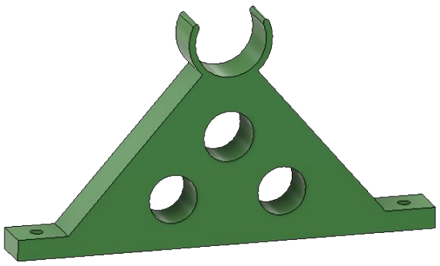
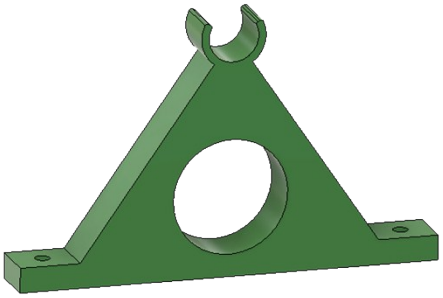
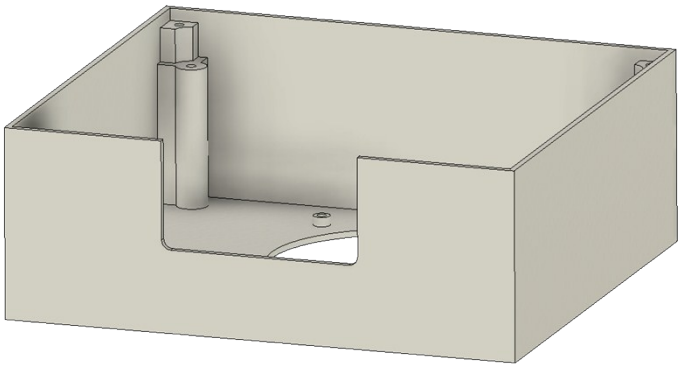
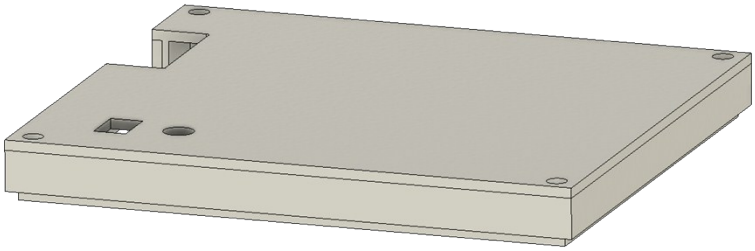
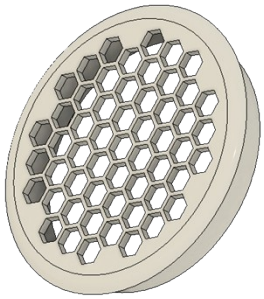
Table S1. List of parts for the assembly of the automation device

Printed parts (3D CAD models available in STL.zip)		
10 mL syringe pump		
Carriage	1	
Syringe plunger clamp	1	
Idler End	1	
Syringe barrel clamp: front	1	

Syringe barrel clamp: back	1	
Motor end	1	
1 mL syringe pump		
Carriage	1	
Idler End	1	

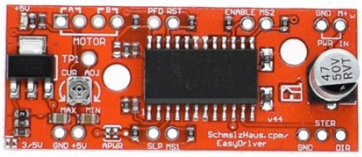
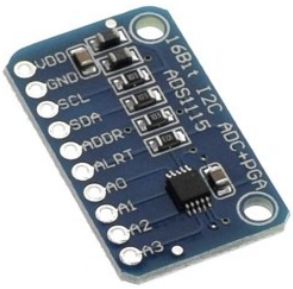
Syringe barrel clamp: front	1	
Syringe barrel clamp: back	1	
Motor end	1	
Platform top	1	
Platform bottom	1	
UV Detector		

UV Detector body	1	
UV Detector door	1	
Miscellaneous parts		
Reactor cap	1	
Stirring motor holder	1	

Reactor clamp holder	1	
Column clamp holder	1	
Control box	1	
Control box cover	1	
Fan grid	1	

Hardware		
NEMA 17 motor	3	
DC geared electric motor (1000 rpm)	1	
5mm x 5mm motor coupler	2	
5mm x 8mm motor coupler	1	
3mm x 3mm motor coupler	1	
T8 lead screw threaded rod (200 mm) with brass nut	1	
M5 threaded rod 200mm	2	
6mm A2 tool steel 400 mm	2	

6mm A2 tool steel 200 mm	4	
625Z ball bearing	2	
LM6UU linear bearing	6	
Connector 1/16" Male/Luer Female	2	
Union 1/16" female-1/16" female	1	
Connector 1/16" Male	1	
PEEK tubing OD 1/16" x ID 0.15 mm x L 150 mm	1	
M5 hex nut	2	
M3 hex nut	24	
M3 x 15 mm socket head cap screw	16	
M3 x 20 mm socket head cap screw	8	
M3 x 40 mm socket head cap screw	8	
Self-tapping screw	16	

4mm polypropylene (PP) stirring bar	1	
10 mL PP syringe	2	
1 mL PP syringe	1	
Electronic parts		
Raspberry Pi Zero W	1	
A3967 B02 EasyDriver 4.4	3	
ADS1115 ADC	1	
280 nm UV LED	1	
TOCON_B5 UV detector	1	

DC-DC step down module	2	
1 Channel Relay Module	2	

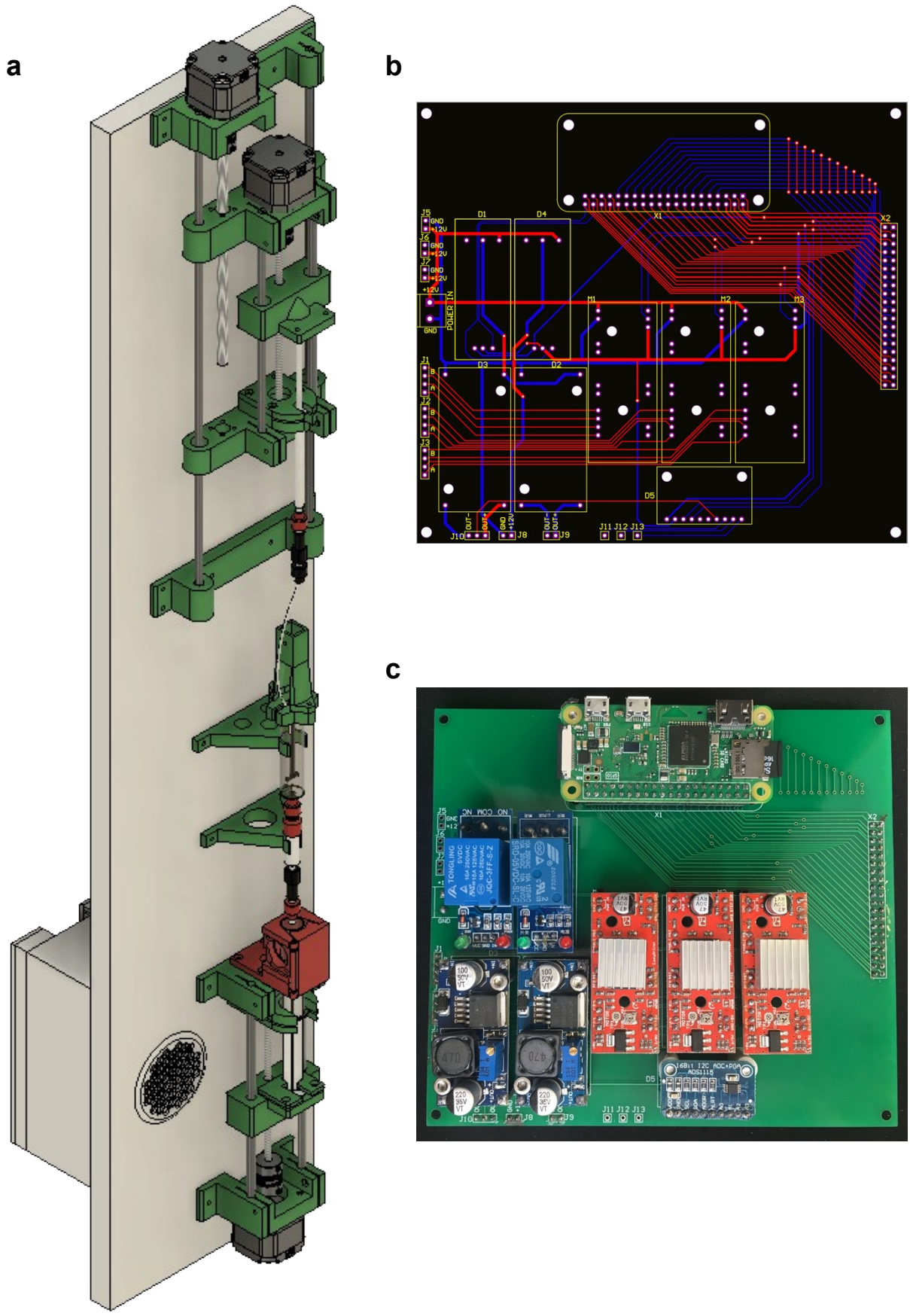


Figure S1. Assembed instructions for the automation device. a) View of the assembled automation device. b) PCB Scheme of the CPU board. c) View of the assembled CPU.

ENACT procedure

Materials:

1. Reagent **1**
2. Dry DMSO
3. PBS 1x (pH 7.4)
4. 50 mM potassium phosphate buffer (pH 8.5)
5. Payload containing a strained alkyne function
6. HiTrap Streptavidin HP column (GE Healthcare, cat. no. 17511201)
7. 3x Connectors 1/16" Male/Luer Female (GE Healthcare, cat. no. 18111251)
8. Union 1/16" Female-1/16" Female (GE Healthcare, cat. no. 18385501)
9. Connector 1/16" Male (GE Healthcare, cat. No. 18117263)
10. PEEK tubing OD 1/16" x ID 0.15 mm x L 150 mm
11. 3x 10 mL syringes
12. 2x 1 mL syringes
13. (Optional) Vivaspin 20 ultrafiltration unit (Sartorius)

Equipment:

1. 3D-printed automation device (described above)
2. Syringe pump
3. (Optional) Akta Pure chromatography system (GE Healthcare)
4. (Optional) Superdex 200 Increase 10/300 GL column (GE Healthcare)

General recommendations:

1. Use 5 mg/mL starting protein solution for optimal immobilization.
2. Use 0.16 molar equivalents of reagent **1** to target 5% conversion in each cycle.
3. Select appropriate concentration of reagent **1** in DMSO, in order not to exceed the final DMSO content of 12% in the conjugate solution.
4. Use 1 mL of Streptavidin Sepharose High Performance affinity resin (GE 17511301) per 350 nmol of reagent **1** (cumulative amount).
5. Use 3 molar equivalents of payload to achieve complete trans-tagging of the immobilized protein.

Part I: coupling reaction and protein immobilization

1. Prepare 5 mL of 5 mg/mL (1 eq.) protein solution in 50 mM potassium phosphate buffer (pH 8.5).
   **Note: for sensitive proteins PBS pH 7.4 can be used. In this case incubation time in step 12.4 has to be increased to 1.5 h.*
2. Equilibrate HiTrap Streptavidin HP column with 10 CV of 50 mM potassium phosphate buffer (pH 8.5) at 1 mL/min flow rate using syringe pump.
3. Load protein solution into a 10 mL syringe and remove air bubbles.
4. Attach 1/16" Male/Luer Female connector to the syringe, followed by 1/16" female-1/16" female union, HiTrap Streptavidin HP column, another 1/16" Male/Luer Female connector and a barrel of another 10 mL syringe (to be used as a reactor). The resulting setup is illustrated on Figure S2a. Tight connection of all parts is required in an optimal setup.
5. Push protein solution through the column into the reactor.
6. Install the setup into the automation device (Figure S2b).
7. Equilibrate streptavidin column with protein solution as detailed in the table below.

Step	Action
1	Stir the solution in the reactor for 30 sec
2	Aspirate the solution into the lower syringe at 0.5 mL/min flow rate
3	Measure UV absorbance
4	Push the solution into the reactor at 1 mL/min flow rate
5	Stir the solution in the reactor for 30 sec
6	Repeat steps 2-5 of the table one time

8. Load 1 mL of the solution of reagent **1** (0.5 mM in dry DMSO) into 1 mL syringe. Remove air bubbles.
9. Attach 1/16" Male/Luer Female connector to the 1 mL syringe, followed by 1/16" female-1/16" female union and 1/16" Male connector with an inserted PEEK tubing (OD 1/16" x ID 0.15 mm x L 150 mm).
10. Install the reagent syringe into the automation device.
11. Wipe the end of the PEEK tube and insert it inside of the reactor. The end of the PEEK tube should be 0.5-1 cm above the protein solution level.
12. Perform 20 reaction / immobilization cycles. The procedure for the first reaction / immobilization cycle is detailed in the table below. For each subsequent cycle a multiplier of 0.95 should be applied to the amount of added reagent **1** in order to maintain the reagent/protein molar ratio.

Step	Action
1	Lower the upper syringe pump to immerse the end of the PEEK tube into protein solution
2	Add 54 µL of the reagent **1** solution (0.16 eq.) in DMSO while stirring
3	Stir the solution in the reactor for 30 sec
4	Incubate the solution in the reactor for 15 min at 20°C
5	Aspirate the solution into the lower syringe at 0.5 mL/min flow rate
6	Measure UV absorbance
7	Push the solution into the reactor at 1 mL/min flow rate
8	Stir the solution in the reactor for 30 sec
9	Repeat steps 5-7

13. Remove the solution from the reactor using 10 mL syringe with a long needle.
14. Wash streptavidin column with 10 mL PBS 1x (pH 7.4) at 1 mL/min flow rate using syringe pump. Collect the eluate and combine with the solution obtained in step 13.
    Note: for best results store the column for at least 24h at 2-8°C and repeat the washing step.
15. Measure the concentration of the recovered protein by UV and calculate the amount of immobilized protein.
    Note: loaded streptavidin column can be stored at 2-8°C for weeks and used on-demand in multiple trans-tagging reactions.

Part II: trans-tagging

1. Prepare 1 CV of the solution of desired payload (3 eq.) bearing strained alkyne function in PBS 1x pH 7.4. Organic co-solvents (e.g. up to 10% DMSO) may be used to increase payload solubility. PBS may be replaced with other aqueous buffers.
2. Purge payload solution with argon.
3. Load payload into a syringe.
4. Inject payload into the loaded streptavidin column and incubate the column at 25°C for 14 h.

Part III: elution of the conjugate

Option 1

1. Elute streptavidin column with 10 CV of PBS 1x pH 7.4 using syringe pump.
2. Remove the excess of the payload by performing 3 concentration/dilution cycles in a Vivaspin 20 ultrafiltration unit (Sartorius).

Option 2

1. Connect streptavidin column to the top of the Superdex 200 Increase 10/300 GL column (GE Healthcare) mounted on the Akta Pure chromatography system.
2. Perform isocratic elution with PBS 1x pH 7.4 keeping the pressure below 0.5 MPa.
3. Collect the solution of pure DoC 1 conjugate.

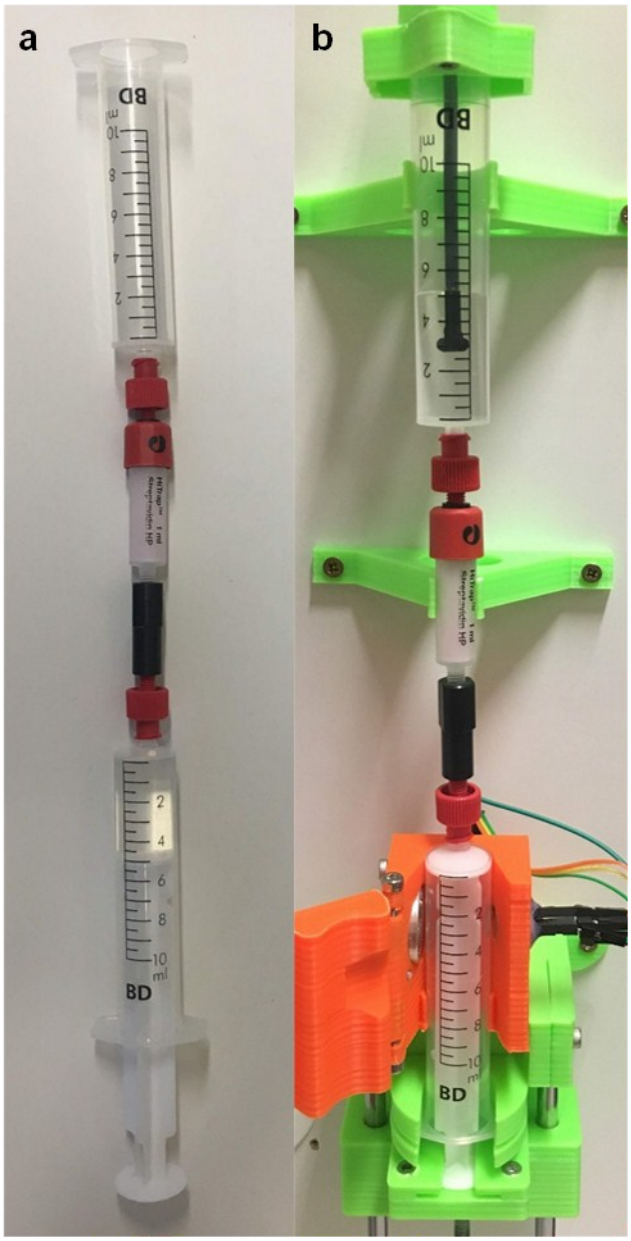


Figure S2. Assembled setup for the ENACT process a) ENACT setup with protein solution loaded into the lower syringe. b) ENACT setup installed into the 3D-printed automation device.

Post-modification of trastuzumab-9 with 10a and 10b

Trastuzumab-9 was prepared by trans-tagging immobilized trastuzumab with **9** (20 µM in PBS 1x, pH 6.0 containing 10 % DMSO) for 16 h at 25 °C. The conjugate was eluted from the streptavidin column using PBS 1x, pH 6.0 and was purified using Vivaspin 2k unit (MWCO 30kDa, GE Healthcare). 3 concentration/dilution cycles were performed with PBS 1x, pH 6.0 and one cycle with PBS 1/20x, pH 6.0. Trastuzumab-9 in PBS (1/20x, pH 6.0) was diluted with PBS (1x, pH 7.5, 5 mM EDTA) to 100 µL at 1 mg/mL and **10a** or **10b** (50 eq., 3.42 µL, 10 mM in DMSO) was added at room temperature. The reaction mixture was incubated for 18 h at 25 °C and then purified using BioSpin 30 (Bio-Rad) column to afford trastuzumab-10a or trastuzumab-10b.

Native mass spectrometry

Native MS analyses were carried-out in positive mode, on an ESI-TOF (LCT, Micromass, Altrincham, UK) upgraded by MS Vision (MS Vision, Almere, Netherlands) coupled with an automated chip-based nanoESI infusion source (Triversa Nanomate, Advion Ithaca, USA). Instrumental parameters were tuned to ensure transmission of high molecular weight species and preservation of potential non-covalent interactions. The acceleration voltage was set to 120 V and the pressure in the interface region of the mass spectrometer was 6.0 mbar. Acquisitions were performed during 2 min with a scan time of 4 s after external calibration with cesium iodide 2 mg/mL. MS data interpretations were performed using Mass Lynx V4.1 (Waters, Manchester, UK).

Peptide mapping

Sample preparation for peptide mapping analysis. Fifteen micrograms of deglycosylated antibody conjugates was solubilized in 150 mM NH_4HCO_3, 0.1% RapiGest™ (Waters, Milford, USA) at pH 7.4. Disulfide reduction was performed by incubating the conjugate solution with 5 mM DTT for 30 min at 60°C. Alkylation was performed with 15 mM IAA for 30 min in the dark. After these steps, the samples were split in two for enzymatic digestion using trypsin or pepsin. Digestion was performed by adding trypsin (Promega, Madison, USA) at a 1:50 enzyme:substrate ratio. Samples were incubated overnight at 37°C. The reaction was quenched by adding 1% of TFA. RapiGest™ was eliminated by centrifugation at 10 000 g for 5 min. For pepsin digestion, pH was decreased to 2.0 prior to pepsin (Promega, Madison, USA). Digestion was performed by adding pepsin at a 1:50 enzyme:substrate ratio. Samples were incubated at 37°C for 3h. The reaction was stopped by heating at 95°C for 10 min. RapiGest™ was eliminated by a centrifugation at 10 000 g for 5 min.

Peptide mapping analysis. NanoLC-MS/MS analysis was performed using a nanoAcquity Ulta-Performance-LC (Waters, Milford, USA) coupled to the Q-Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) with a nanoSpray source. The sample were trapped on a nanoACQUITY UPLC precolumn (C18, 180µm x 20mm, 5 µm particle size), and the peptides were separated on a nanoACQUITY UPLC column (C18, 75µm x 250 mm with 1.7 µm particle size, Waters, Milford, USA) maintained at 60°C. Mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. A gradient (1-8% B for 2min, 8-35% B for 58min, 35-90% B for 1min, 90% B for 5min, 90-1% B for 1min and maintained 1% B for 20min) was used at a flow rate of 450 nL/min. The Q-Exactive Plus Orbitrap source temperature was set to 250°C and spray voltage to 1.8kV. Full scan MS spectra (300-1800 m/z) were acquired in positive mode at a resolution of 140 000, a maximum injection time of 50 ms and an AGC target value of 3×10^6 charges, with lock-mass option being enabled (polysiloxane ion from ambient air at 445.12 m/z). The 10 most intense

multiple-charged peptides per full scan were isolated using a 2 m/z window and fragmented using higher energy collisional dissociation (normalised collision energy of 27). MS/MS spectra were acquired with a resolution of 17 500, a maximum injection time of 100 ms and an AGC target value of 1 x 10^5, and dynamic exclusion was set to 60 sec. The system was fully controlled by XCalibur software v3.0.63, 2013 (Thermo Scientific) and NanoAcquity UPLC console v1.51.3347 (Waters). Each digested sample was injected in triplicate.

Identification of conjugation sites. Raw data collected were processed and converted in .mgf format. The mgf files of the trypsin and pepsin digestion were merged using Mass Spectrometry Data Analysis 2.7.3 (MSDA).[9] The MS/MS data were interpreted using a local Mascot server with MASCOT 2.5.0 algorithm (Matrix Science, London, UK). Spectra were searched with a mass tolerance of 5 ppm for MS and 0.07 Da for MS/MS data, using none as enzyme. Carbamidomethylation of cysteine residues, oxidation of methionine residues and linker (+477 Da) were specified as variable modifications. Protein identifications were validated with Mascot ion score above 25. Each conjugation site, observed on the three merged files, was manually validated based on the presence of y-ion and b-ion series and the peak intensity observed on the MS/MS spectra, using Proline 1.5 software.[10]

References

1 S. Bernard, D. Audisio, M. Riomet, S. Bregant, A. Sallustrau, L. Plougastel, E. Decuypere, S. Gabillet, R. A. Kumar, J. Elyian, M. N. Trinh, O. Koniev, A. Wagner, S. Kolodych and F. Taran, *Angew. Chemie Int. Ed.*, 2017, **56**, 15612–15616.

2 I. Dovgan, S. Ursuegui, S. Erb, C. Michel, S. Kolodych, S. Cianférani and A. Wagner, *Bioconjugate Chem.*, 2017, **28**, 1452–1457.

3 G. de Almeida, E. M. Sletten, H. Nakamura, K. K. Palaniappan and C. R. Bertozzi, *Angew. Chemie Int. Ed.*, 2012, **51**, 2443–2447.

4 J. Dommerholt, S. Schmidt, R. Temming, L. J. A. Hendriks, F. P. J. T. Rutjes, J. C. M. van Hest, D. J. Lefeber, P. Friedl and F. L. van Delft, *Angew. Chemie*, 2010, **122**, 9612–9615.

5 O. Koniev, G. Leriche, M. Nothisen, J.-S. Remy, J.-M. Strub, C. Schaeffer-Reiss, A. Van Dorsselaer, R. Baati and A. Wagner, *Bioconjugate Chem.*, 2014, **25**, 202–206.

6 S. Ursuegui, M. Recher, W. Krężel and A. Wagner, *Nat. Commun.*, 2017, **8**, 15242.

7 WO2015001117, 2015, 128.

8 I. Dovgan, S. Erb, S. Hessmann, S. Ursuegui, C. Michel, C. Muller, G. Chaubet, S. Cianférani and A. Wagner, *Org. Biomol. Chem.*, 2018, **16**, 1305–1311.

9 C. Carapito, A. Burel, P. Guterl, A. Walter, F. Varrier, F. Bertile and A. Van Dorsselaer, *Proteomics*, 2014, **14**, 1014–1019.

10 C. Carapito, L. Lane, M. Benama, A. Opsomer, E. Mouton-Barbosa, L. Garrigues, A. Gonzalez de Peredo, A. Burel, C. Bruley, A. Gateau, D. Bouyssié, M. Jaquinod, S. Cianferani, O. Burlet-Schiltz, A. Van Dorsselaer, J. Garin and Y. Vandenbrouck, *J. Proteome Res.*, 2015, **14**, 3621–3634.