Supporting Information for:

Dual optical control and mechanistic insights into photoswitchable group II and III metabotropic glutamate receptors

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Supplementary Figures

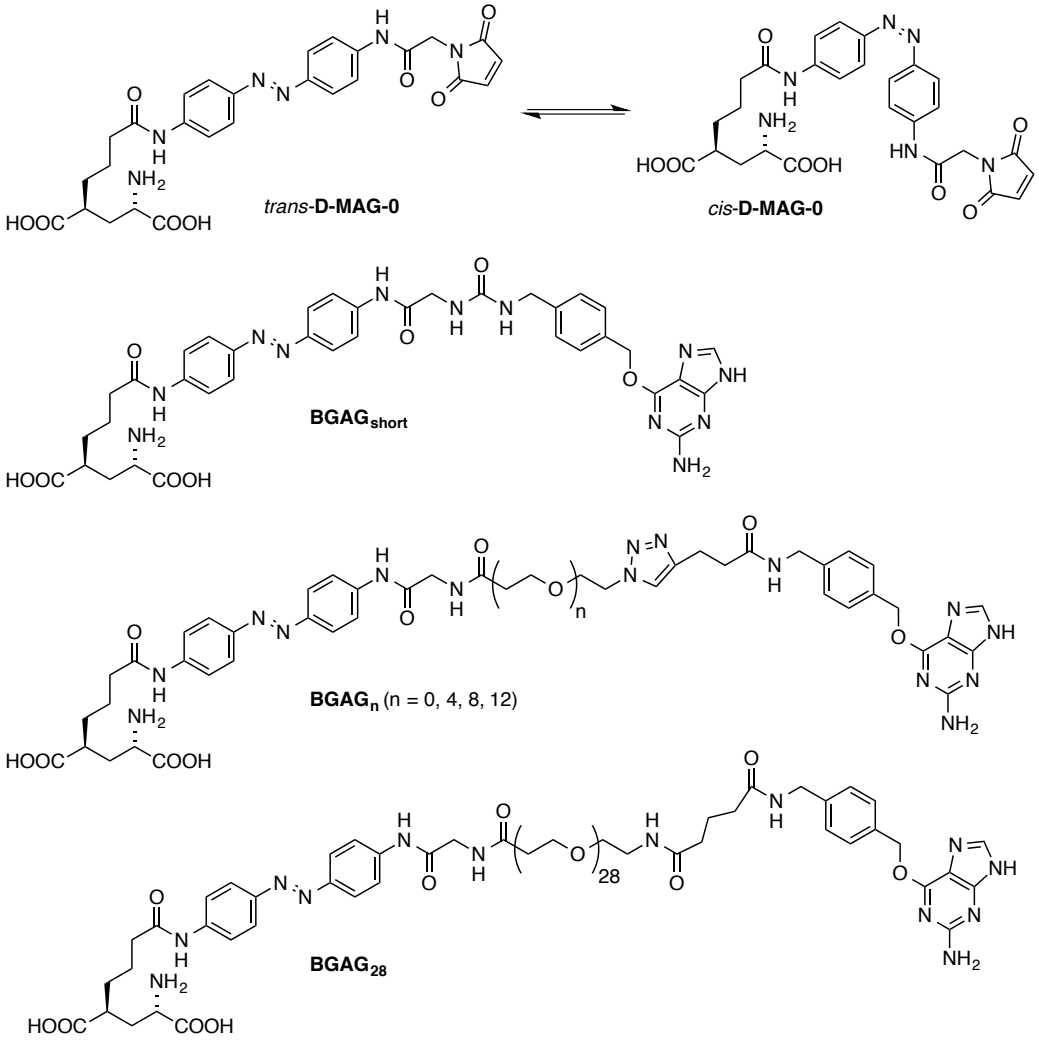
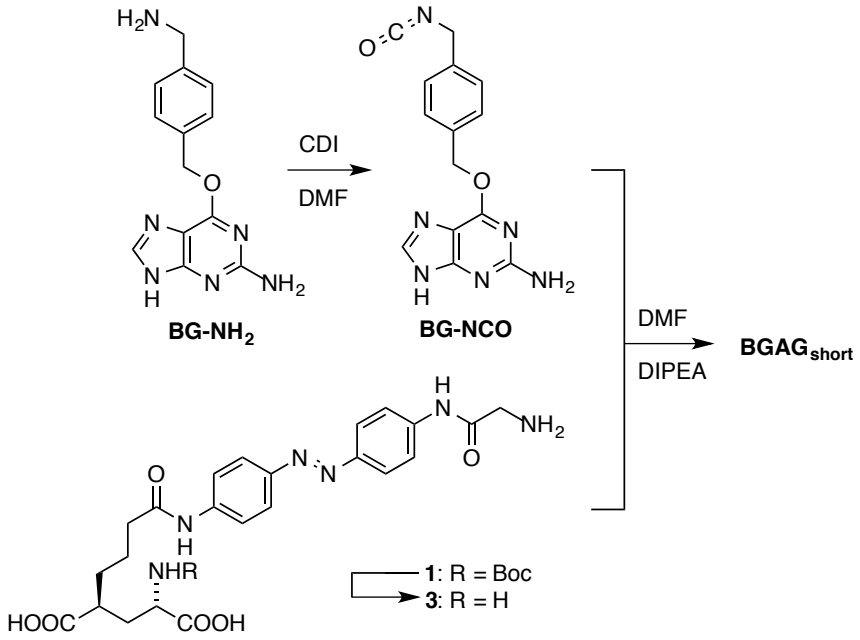
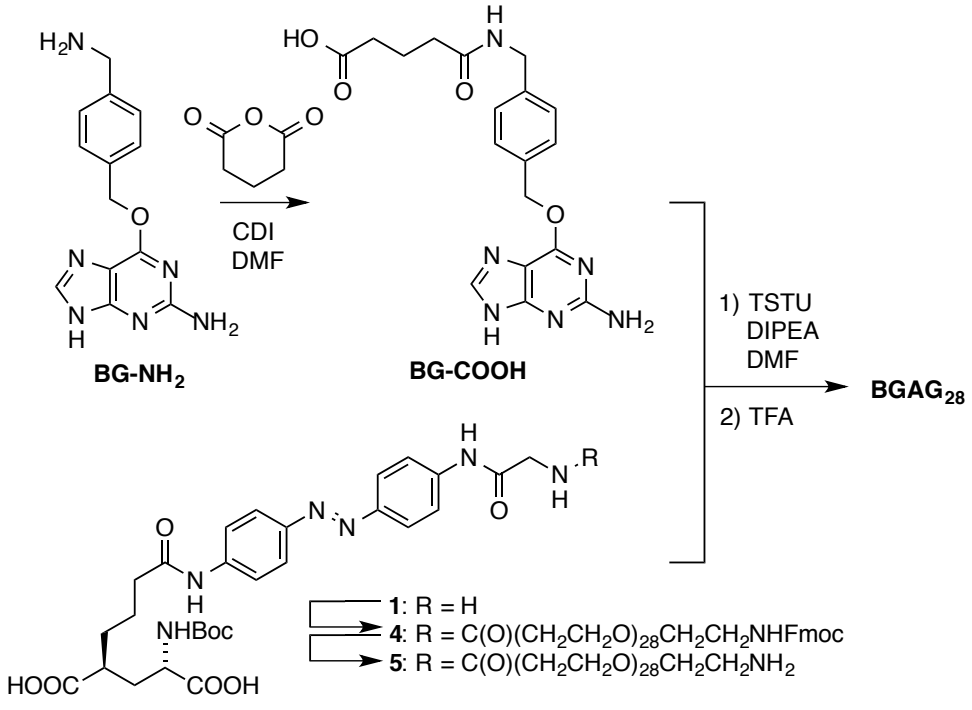


Fig. S1. Chemical structures of MAG and BGAG photoswitches.



Scheme S1. Synthesis of $BGAG_{short}$.



Scheme S2. Synthesis of $BGAG_{28}$.

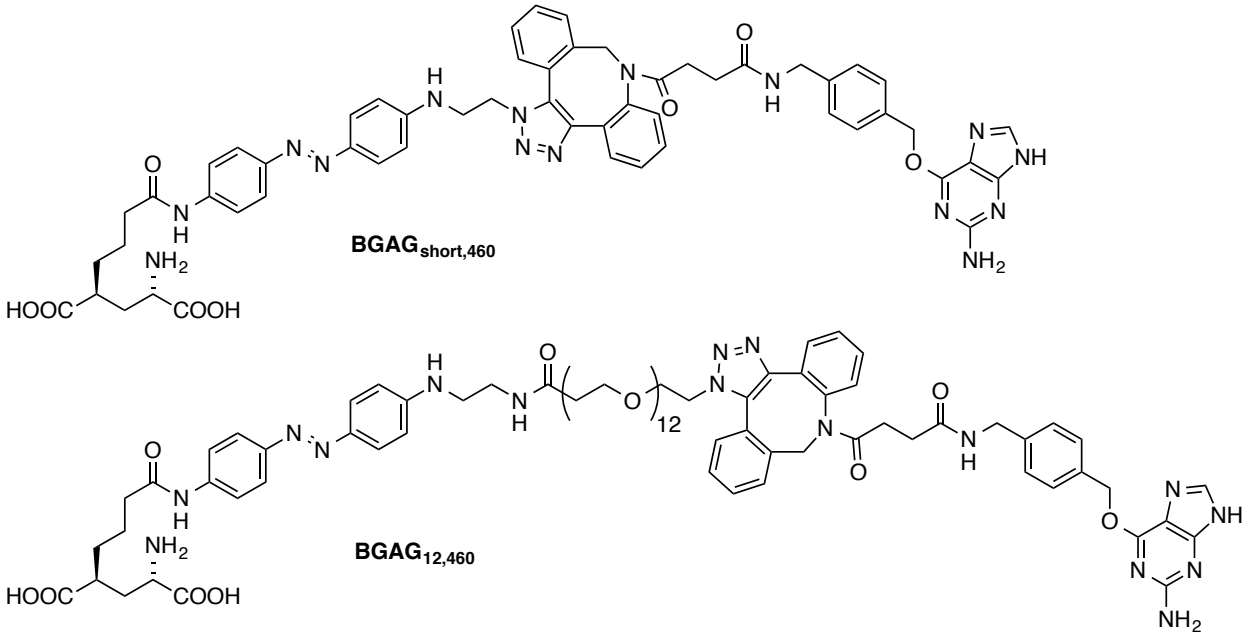
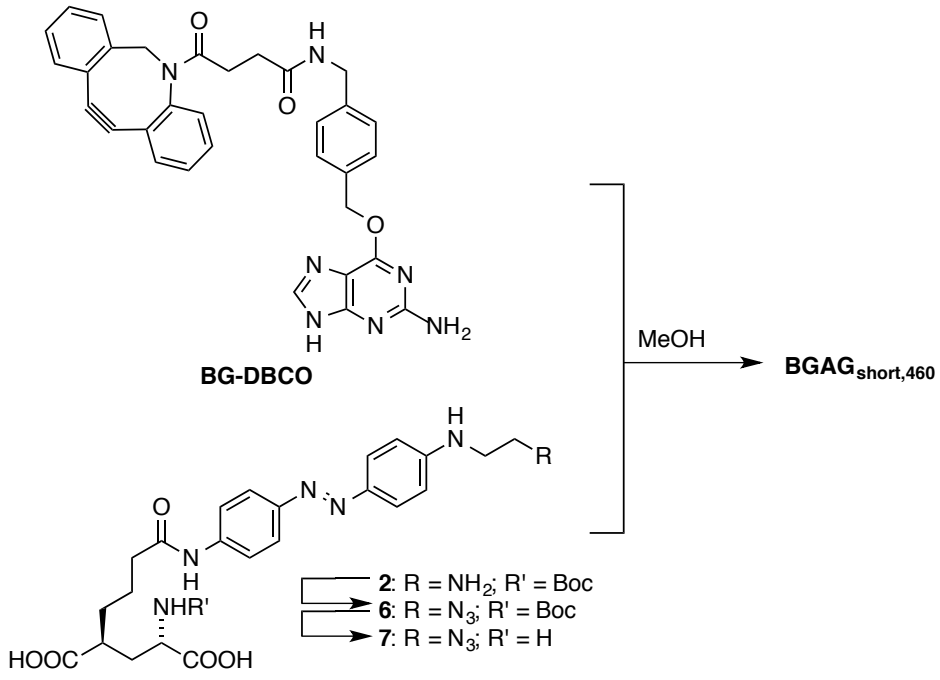


Fig. S2. Chemical structures of $BGAG_{460}$ photoswitches



Scheme S3. Synthesis of $BGAG_{short,460}$

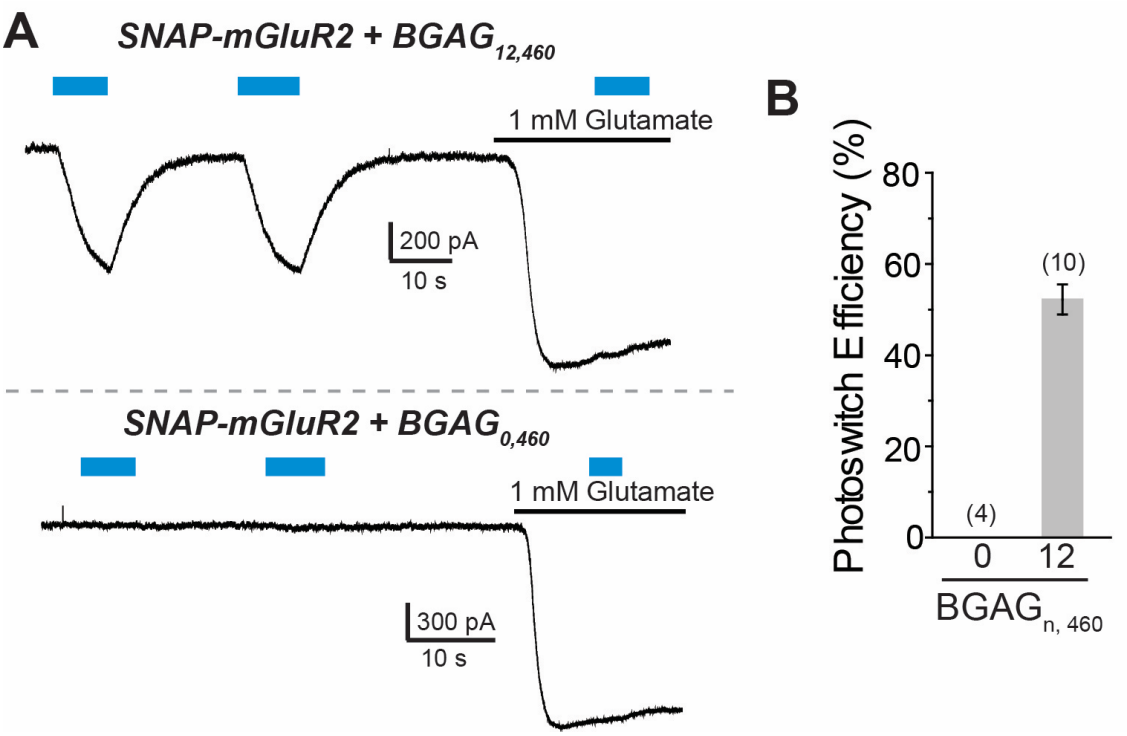


Fig. S3. Length-dependence of $BGAG_{460}$ photoswitching. (A) Representative traces showing light responses (blue bar, 460 nm) for SNAP-mGluR2 labeled with $BGAG_{12,460}$ (top) or $BGAG_{0,460}$ (bottom). (B) Summary bar graph showing robust photoswitching for $BGAG_{12,460}$ but not $BGAG_{0,460}$. The numbers of cells tested are shown in parentheses.

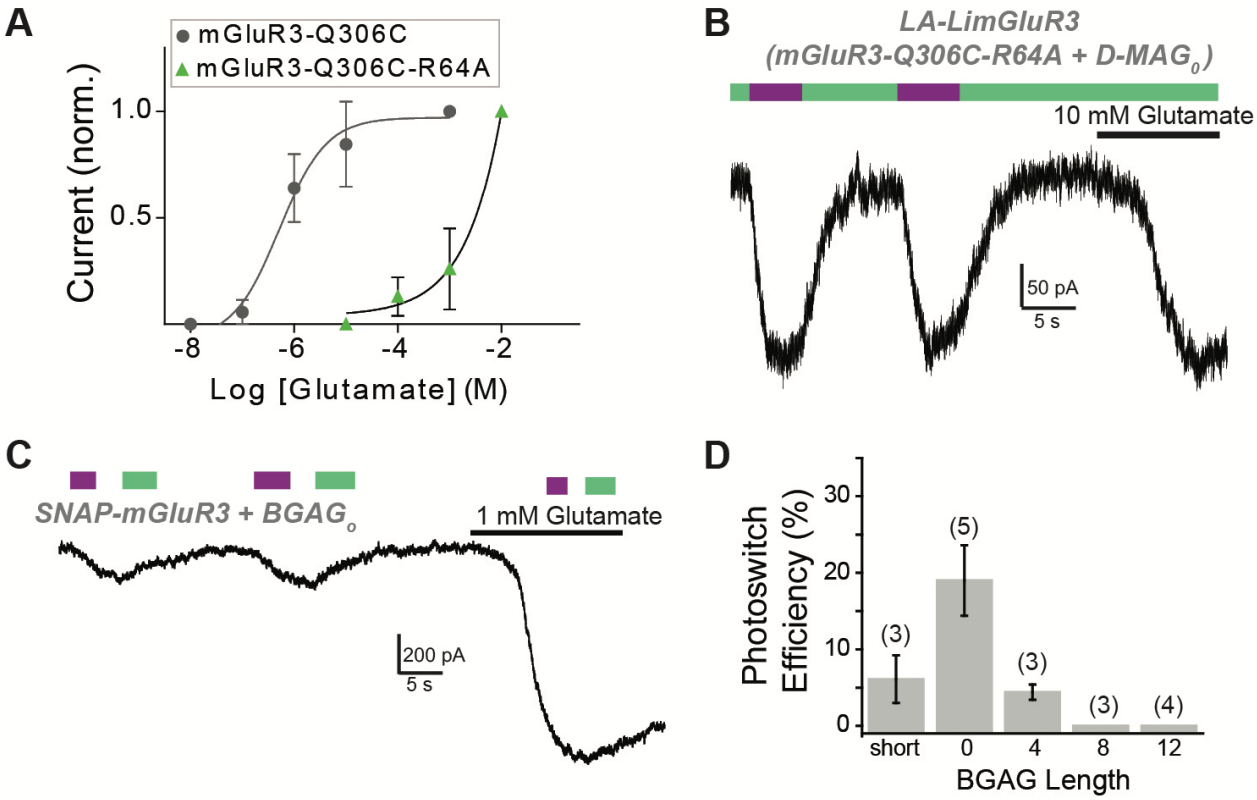


Fig. S4. Optical control of mGluR3 reveals length-dependent photoswitching. (A) Glutamate dose-response curves for LimGluR3 (mGluR3-Q306C + D-MAG-0) and LA-LimGluR3 (mGluR3-Q306C-R64A + D-MAG-0). (B) Representative trace showing photoactivation of LA-LimGluR3. (C) Representative trace showing photoactivaiton of SNAP-mGluR3 by $BGAG_0$. (D) Summary of photoswitch efficiency for SNAP-mGluR3 plus BGAGs of various lengths. The numbers of cells tested are shown in parentheses.

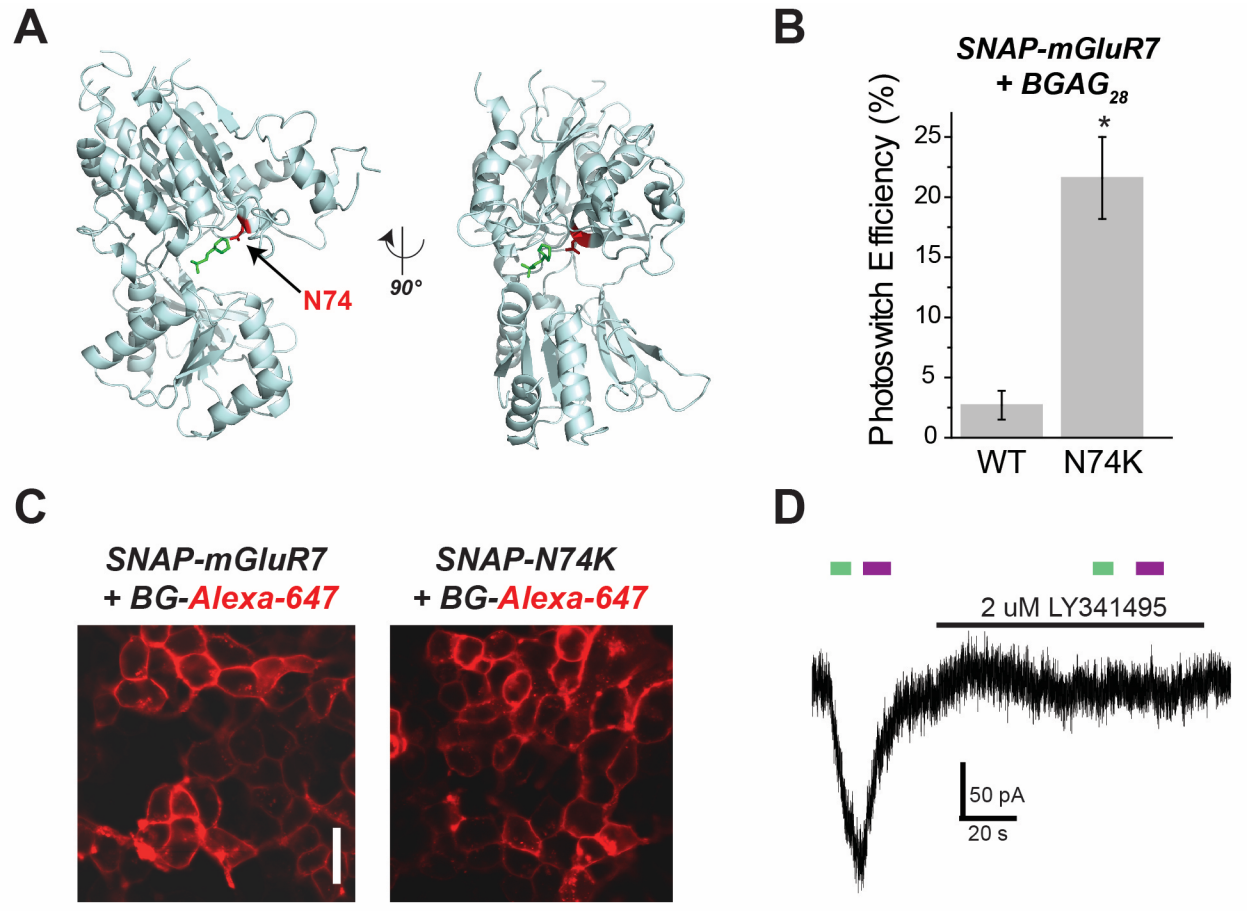


Fig. S5. Further characterization of SNAP-mGluR7 photo-agonism. (A) mGluR7 ligand binding domain crystal structure (PDB:3KS9) showing location of N74 residue (red) which is mutated to lysine to increase glutamate affinity and permit efficient $BGAG_{12}$ photoagonism. (B) Summary of BGAG28 photoactivation efficiency for SNAP-mGluR7 and SNAP-mGluR7-N74K. * indicates statistical significance (Unpaired, two-tailed T test; p=0.046). (C) Images showing surface expression of SNAP-mGluR7 and SNAP-mGluR7-N74K via labeling of SNAP with a membrane-impermeable dye. (D) SNAG-mGluR7 (SNAP-mGluR7-N74K + $BGAG_{12}$) photoactivation is blocked by the competitive antagonists LY341495.

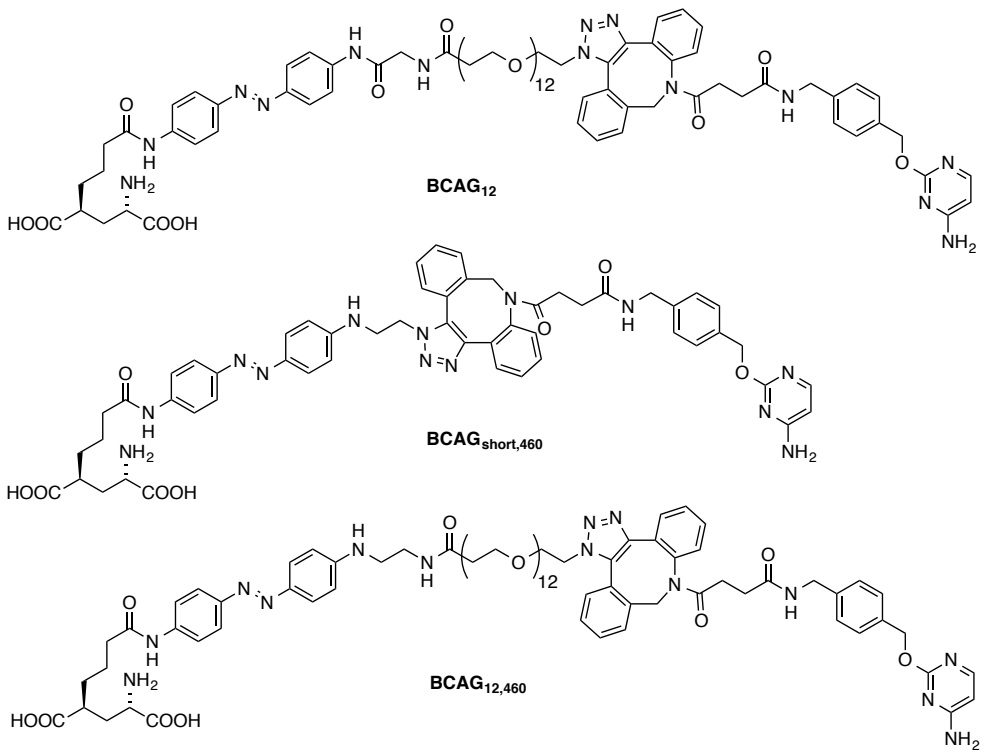
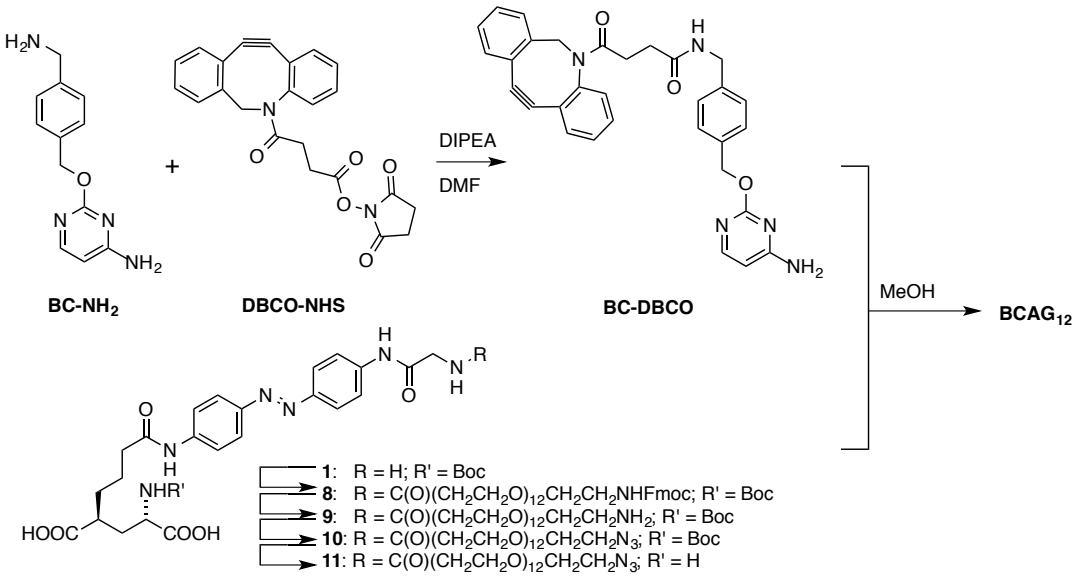
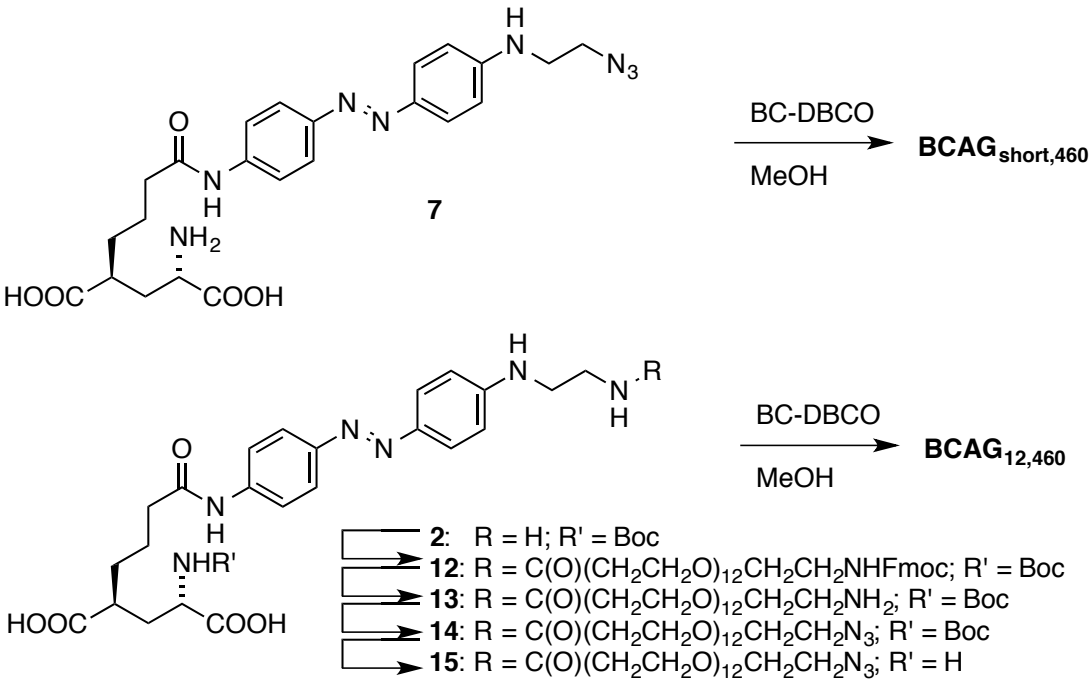


Fig. S6. Chemical structures of BCAG photoswitches.



Scheme S4. Synthesis of $BCAG_{12}$.



Scheme S5. Synthesis of $BCAG_{460}$.

Table S1, Methods for manipulation of GPCRs

Method	Receptors Targeted	Spatial Precision?	Genetic Targeting?	Multi-plexable?	Temporal Control?	Maintain Native Signaling/Regulation?	Key Publications
Pharmacology	all GPCRs (Agonists, Antagonists, Allosteric Modulators)	Limited by diffusion	No	Yes (w/ off-target effects)	Limited by drug application	Yes	
Visual Opsins	Rhodopsin; Cone Opsins (vSWO, vLWO)	Yes	Yes	Yes; (w/ vSWO & vLWO)	Fast ON Variable OFF	N/A	1, 2
Non-Visual Opsins	Melanopsin, Jellyfish Opsin, others	Yes	Yes	No	Fast ON Variable OFF	N/A	3, 4
Opsin-based Chimeras (OptoXRs)	OptoXRs (a1AR, ß2AR); OMOR (MOR); 5-HT1a, 2c; mGluR6	Yes	Yes	No	Fast ON Variable OFF	Partially (Not Fully Characterized)	5,6,7
DREADDs	hM3Dq (based on M3R); hMD4i (based on M4R); rM3Ds (chimeras); KORD (based on kappa-OR)	Limited by diffusion	Yes	Yes; (see SI ref(9))	Limited by drug application	Partially (Not Fully Characterized)	8, 9
Photoswitchable mGluRs	mGluR2, mGluR3, mGluR6, mGluR7, mGluR8	Yes	Yes	Yes (w/ SNAP and CLIP)	Fast ON Fast OFF	Yes	10, 11 This paper
[12]Caged & Photochromic Ligands	many caged compounds; mGluR4 (opto-Glu NAM"); MOR (photofentanyl-2); GLP-1R (PhotoETP)	Yes, but still limited by diffusion	No	~380 nm: Antag. ~500 nm: OFF	Fast ON Variable OFF	Yes	12,13,14

Supplementary Materials and Methods

1. *Chemical Synthesis*

1.1. General

Solvents for chromatography and reactions were purchased dry over molecular sieves or in HPLC grade. Unless otherwise stated, all other reagents were used without further purification from commercial sources.

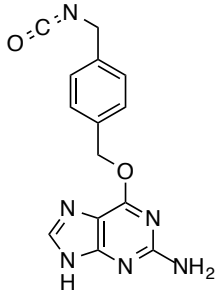
LC-MS was performed on a Shimadzu MS2020 connected to a Nexerra UHPLC system equipped with a Waters ACQUITY UPLC BEH C18 (2.1×50 mm, particle size 1.7 micron) RP column with a constant flow rate of 0.5 mL/min. Retention times (t_R) are given in minutes (min).

High-resolution mass spectra (HRMS) were measured on a Micromass Q-TOF Ultima spectrometer with electrospray ionization (ESI).

Preparative RP-HPLC was performed on a Dionex system equipped with an UVD 170U UV-Vis detector for product visualization on a Waters SunFire^{TM} Prep C18 OBDTM 5 µm 10×150 mm column. Buffer A: 0.1% TFA in H_2O Buffer B: acetonitrile. The typical gradient was from 10% to 90% B within 30 min with 4 mL/min flow.

Compounds **1**, **2** and **BG-DBCO** were previously described in Broichhagen *et al.*, *ACS Cent. Sci.* **2015**, *1*, 383–393. The previously reported synthesis of **BG-DBCO** yielded the compound in 7% yield and was optimized by using dry DMSO (AcroSeal) to yield **BG-DBCO** in 91%. **BG-NH$_2$**, **BC-NH$_2$**, **DBCO-NHS**, FmocPEG$_{11}$COOH and FmocPEG$_{27}$COOH were a kind gift from Prof. Kai Johnsson, EPFL, Switzerland.

1.2. 6-((4-(Isocyanatomethyl)benzyl)oxy)-9*H*-purin-2-amine (BG-NCO)



A round bottom flask was charged with 10 mg (37.0 µmol, 1.0 equiv.) of **BG-NH$_2$** dissolved in 500 µL DMF and 7.7 µL (5.7 mg, 47.5 µmol, 1.2 equiv.) DIPEA was added. CDI (7.7 mg, 47.5 µmol, 1.2 equiv.) was added in one portion and the reaction mixture was stirred for 90 min at r.t. and LCMS analysis

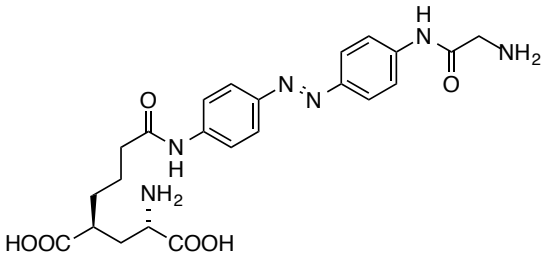
indicated complete conversion. The isocyanate was used as a stock solution (73 mM) without further purification for the next reaction step.

LRMS (ESI): calc. $C_{14}H_{13}N_6O_2^+$ $[M+H]^+$: 297.1, found: 296.9.

UV/Vis (LCMS): λ_{max} = 286 nm.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 1.880 min.

1.3. (2*S*,4*S*)-2-Amino-4-(4-((4-((*E*)-(4-(2-aminoacetamido)phenyl)diazenyl)phenyl)-amino)-4-oxobutyl)pentanedioic acid (3)



A 1 mL vial was charged with 5.6 mg (9.58 µmol) of **1**[15], cooled to 0 °C and 100 µL of TFA were added. The solution turned dark purple and was allowed to stand for 10 minutes before the volatiles were removed with a gentle stream of nitrogen to obtain 6.7 mg of **3** presumably as the double TFA salt that was be used without further purification in the next reaction step.

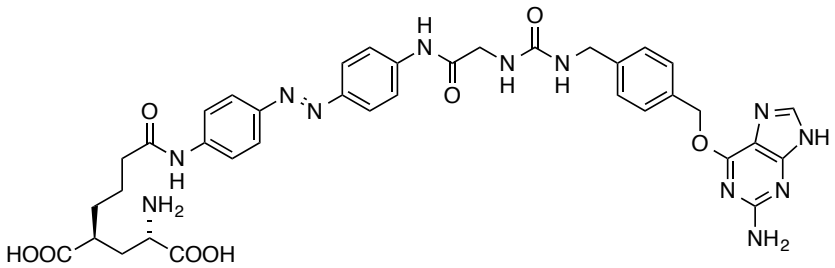
LRMS (ESI): calc. for $C_{23}H_{29}N_6O_6$ $[M+H]^+$: 485.2, found: 485.1.

UV/Vis (LCMS): λ_{max} = 364 nm.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 1.893 min.

HRMS (ESI): calc. for $C_{23}H_{29}N_6O_6$ $[M+H]^+$: 485.2143, found: 485.2148.

1.4. (2*S*,4*S*)-2-Amino-4-(4-((4-((*E*)-(4-(2-(3-(4-(((2-amino-9*H*-purin-6-yl)oxy)methyl)-benzyl)ureido)acetamido)phenyl)diazenyl)phenyl)amino)-4-oxobutyl)-pentanedioic acid ($BGAG_{short}$)



A 1 mL vial was charged with **3** (presumably 9.58 µmol) dissolved in 1 mL DMF and 8.4 µL (47.9 µmol) DIPEA, cooled to 0 °C before 155 µL of a 74 mM solution of **BG-NCO** (11.5 µmol) was added. The reaction mixture was allowed to warm to r.t. o.n. before it was quenched by addition of 10 µL HOAc and subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain 1.7 mg (2.18 µmol) of the desired product as a yellow powder after lyophilisation in 23% yield over 3 steps.

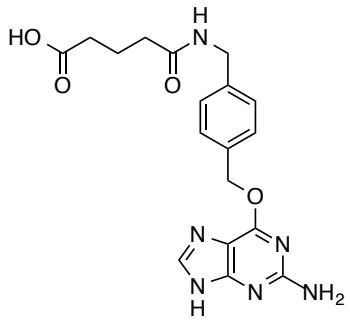
LRMS (ESI): calc. for $C_{37}H_{41}N_{12}O_8$ $[M+H]^+$: 781.3, found: 781.2.

UV/Vis (LCMS): λ_{max} = 366 nm.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 2.089 min.

HRMS (ESI): calc. for $C_{37}H_{41}N_{12}O_8$ $[M+H]^+$: 781.3165, found: 781.3165.

1.5. 5-((4-(((2-Amino-9*H*-purin-6-yl)oxy)methyl)benzyl)amino)-5-oxopentanoic acid (BG-COOH)



A round bottom flask was charged with 50.0 mg (185 µmol, 1.0 equiv.) of **BG-NH_2**, dissolved in 1 mL DMF before addition of 48.5 µL (35.9 mg, 278 µmol, 1.5 equiv.) NEt_3, 32.0 mg (278 µmol, 1.5 equiv.) of glutaric anhydride and 2 grains of DMAP. The reaction mixture was stirred for 5 h at r.t. before it was quenched by addition of 50 µL HOAc and subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 30/70/0.1 over 30 minutes) to obtain 42.9 mg (112 µmol) of the desired product as a white solid after lyophilisation in 61% yield.

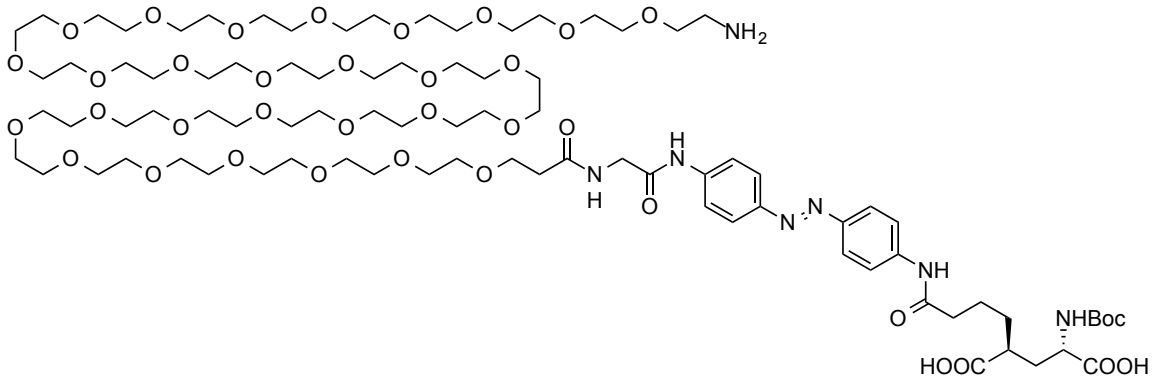
LRMS (ESI): calc. for $C_{18}H_{21}N_6O_4$ $[M+H]^+$: 385.2, found: 384.9.

UV/Vis (LCMS): λ_{max} = 286 nm.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 0.752 min.

HRMS (ESI): calc. for $C_{18}H_{19}N_6O_4$ $[M–H]^-$: 383.1473, found: 383.1468.

1.6. (2*S*,4*S*)-2-(4-((4-((*E*)-(4-(1-Amino-87-oxo-3,6,9,12,15,18,21,24,27,30,33,36,39,42, 45,48,51,54,57,60,63,66,69,72,75,78,81,84-octacosaoxa-88-azanonacontan-90-amido)phenyl)diazenyl)phenyl)amino)-4-oxobutyl)-4-((*tert*-butoxycarbonyl)-amino)pentanedioic acid (5)



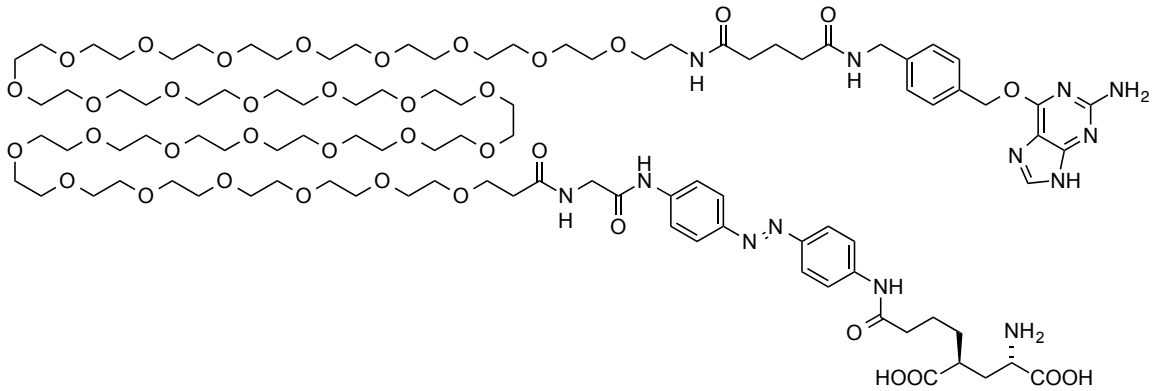
A 1 mL vial was charged with 30 mg (19.4 µmol, 1.0 equiv.) of FmocNH-PEG_{27}-COOH (novabiochem, #A35789) dissolved in 1 mL DMSO and 7.0 µL (5.2 mg, 40.2 µmol, 2.1 equiv.) DIPEA before 7.0 mg (23.3 µmol, 1.2 equiv.) of TSTU was added in one portion. The reaction mixture was stirred at r.t. for 30 min before 13.6 mg (23.3 µmol, 1.2 equiv) of **1**[15] was added in one portion, followed by 14.0 µL (10.4 mg, 80.4 µmol, 4.2 equiv.) of DIPEA and the reaction was stirred for an additional 3 hours until LCMS indicated consumption of all starting material **1** and formation of **4**. The Fmoc group was deprotected *in situ* by the addition of 200 µL piperidine at r.t., continued by an additional hour of stirring. The reaction mixture was quenched by addition of 250 µL HOAc and subjected to RP-HPLC ($MeCN/H_2O$/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain 13.6 mg (7.2 µmol) of the desired product as a yellow powder after lyophilisation in 37% yield over 3 steps.

LRMS (ESI): calc. for $C_{87}H_{155}N_7O_{37}$ $[M+2H]^{2+}$: 945.0, found: 945.2.

UV/Vis (LCMS): λ_{max} = 367 nm.

t_R (LCMS; $MeCN/H_2O$/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 2.695 min.

1.7. (2*S*,4*S*)-2-Amino-4-(4-((4-((*E*)-(4-(1-(4-(((2-amino-9*H*-purin-6-yl)oxy)methyl)-phenyl)-3,7,95-trioxo-11,14,17,20,23,26,29,32,35,38,41,44,47,50,53,56,59,62,65,68,71,74,77,80, 83,86,89,92-octacosaoxa-2,8,96-triazaoctanonacontan-98-amido)phenyl)diazenyl)-phenyl)amino)-4-oxobutyl)pentanedioic acid ($BGAG_{28}$)



A 1 mL vial was charged with 4.9 mg (12.7 µmol, 1.0 equiv.) of **BG-COOH** dissolved in 500 µL DMSO before the addition of 2.7 µL (2.0 mg, 15.2 µmol, 1.25 equiv.) DIPEA and 4.6 mg (15.2 µmol, 1.25 equiv.) TSTU in one portion. The reaction mixture was stirred at r.t. for 20 min and LCMS analysis indicated full conversion. A separate 1 mL dram vial was charged with 13.6 mg (7.2 µmol, 1.0 equiv.) of **5** dissolved in 500 µL DMSO and 5.0 µL (3.7 mg, 28.8 µmol, 4.0 equiv.) DIPEA. Then, the reaction mixture from the activated ester was added dropwise with the addition of 1 grain of DMAP. The reaction was stirred for additional 2 hours until LCMS indicated consumption of all starting material **5** and was quenched subsequently by addition the of 25 µL HOAc and subjected to RP-HPLC ($MeCN/H_2O$/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain the Boc-protected glutamate after lyophilisation. The material was transferred to a flask, cooled to 0 °C and 500 µL of TFA were added. The solution turned dark purple and was allowed to stand for 10 minutes before the volatiles were removed with a gentle stream of nitrogen and subjected to RP-HPLC ($MeCN/H_2O$/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain 4.2 mg (2.0 µmol) of the desired product in 27% yield over 3 steps.

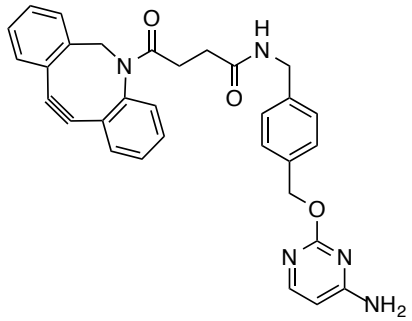
LRMS (ESI): calc. for $C_{100}H_{161}N_{13}O_{38}$ $[M–2H]^{2–}$: 1076.6, found: 1076.0.

UV/Vis (LCMS): λ_{max} = 368 nm.

t_R (LCMS; $MeCN/H_2O$/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 2.348 min.

HRMS (ESI): calc. for $C_{100}H_{165}N_{13}O_{38}$ $[M+2H]^{2+}$: 1078.5701, found: 1078.5729.

1.8. *N*-(4-(((4-Aminopyrimidin-2-yl)oxy)methyl)benzyl)-4-(*11,12*-dehydrodibenzo[*b,f*] azocin-5(6*H*)-yl)-4-oxobutanamide (BC-DBCO)



A round bottom flask was charged with 10.0 mg (24.9 µmol, 1.0 equiv.) DBCO-NHS (Click Chemistry Tools, #A133) and 5.7 mg of **BC-NH$_2$** (24.9 µmol, 1.0 equiv.) dissolved in 1 mL DMSO and 13.0 µL (9.6 mg, 74.6 µmol, 3.0 equiv.) DIPEA. The reaction was stirred for 2 hours until LCMS indicated consumption of all starting material and was quenched subsequently by addition of 13.0 µL HOAc and subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain 11.2 mg (21.7 µmol) of the desired product as a white powder in 87% yield after lyophilisation.

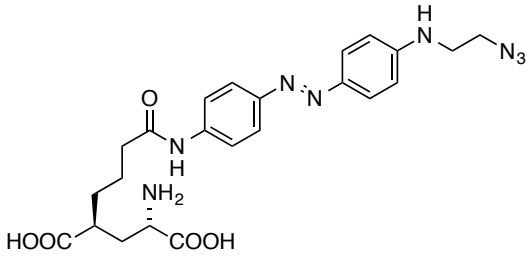
LRMS (ESI): calc. for $C_{31}H_{28}N_5O_3$ $[M+H]^+$: 518.2, found: 518.2.

UV/Vis (LCMS): λ_{max1} = 248, λ_{max2} = 290, λ_{max3} = 307 nm.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 2.825 min.

HRMS (ESI): calc. for $C_{31}H_{28}N_5O_3$ $[M+H]^+$: 518.2187, found: 518.2192.

1.9. (2*S*,4*S*)-2-Amino-4-(4-((4-((*E*)-(4-((2-azidoethyl)amino)phenyl)diazenyl)-phenyl)-amino)-4-oxobutyl)pentanedioic acid (7)



A round bottom flask was charged with 2.2 mg (3.9 µmol, 1.0 equiv.) of **2**[15] dissolved in 1 mL MeOH to which was sequentially added 1.3 mg (9.6 µmol, 2.5 equiv.) of K_2CO_3, 1.0 mg (4.7 µmol, 1.2 equiv.) of $ImSO_2N_3 \times HCl$[16] and 0.4 µL of an aqueous 100 mM stock of $CuSO_4$ (0.39 µmol, 0.1 equiv.). The reaction mixture was stirred for 90 min at r.t. before the volatiles were removed by a gentle stream of nitrogen and

100 µL TFA was added. The dark purple reaction mixture was to let stand at r.t. for 5 min before the volatiles were removed by a gentle stream of nitrogen and the crude subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain 2.6 mg of the desired product presumably as its double TFA salt (3.5 µmol) as a dark red powder in 90% yield after lyophilisation over 2 steps.

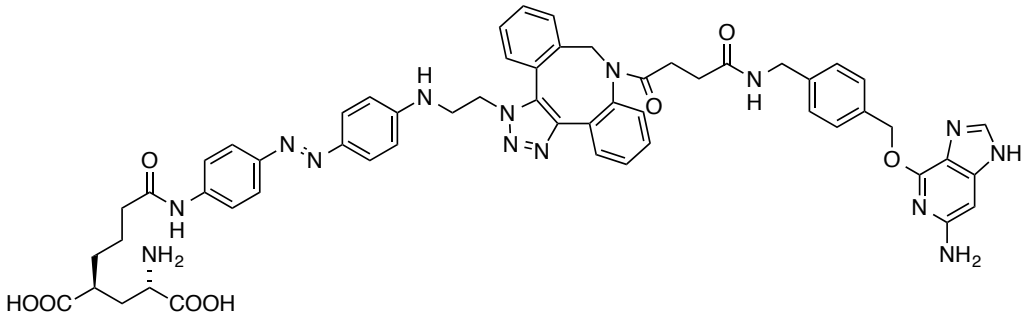
LRMS (ESI): calc. for $C_{23}H_{29}N_8O_5$ $[M+H]^+$: 497.2, found: 497.2.

UV/Vis (LCMS): λ_{max} = 407 nm.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 3.089 min.

HRMS (ESI): calc. for $C_{23}H_{29}N_8O_5$ $[M+H]^+$: 497.2255, found 497.2264.

1.10. (2*S*,4*S*)-2-Amino-4-(4-((4-((*E*)-(4-((2-(8-(4-((4-(((2-amino-9*H*-purin-6-yl)oxy)methyl)-benzyl)amino)-4-oxobutanoyl)-8,9-dihydro-1*H*-dibenzo[*b*,*f*][1,2,3]triazolo[4,5-*d*] azocin-1-yl)ethyl)amino)phenyl)diazenyl)phenyl)amino)-4-oxobutyl)pentanedioic acid ($BGAG_{0,460}$)



A 1 mL vial was charged with 1.3 mg (2.7 µmol, 1.0 equiv.) of **BG-DBCO** and 1.3 mg (2.7 µmol, 1.0 equiv.) of **7** dissolved in 1 mL MeOH. The reaction mixture was stirred for 3 hours before it was subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain 1.0 mg (0.95 µmol) of the desired product in 35% yield after lyophilisation as a red powder.

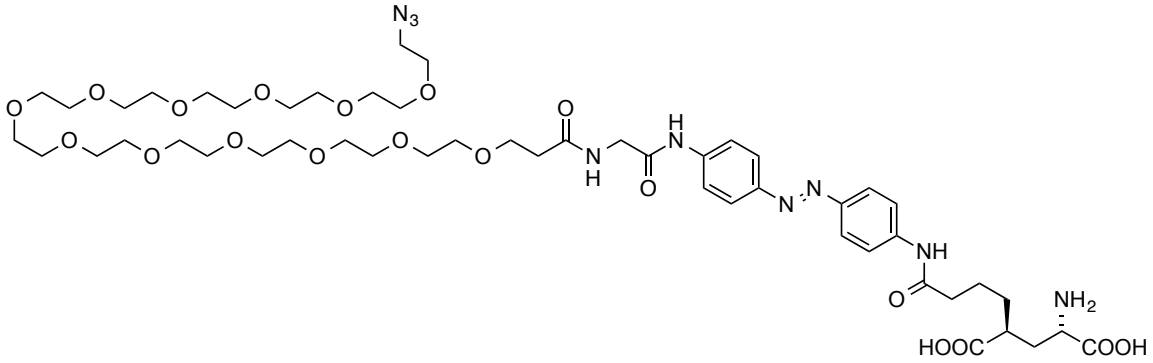
LRMS (ESI): calc. for $C_{55}H_{57}N_{15}O_8$ $[M+2H]^{2+}$: 527.7, found: 527.9.

UV/Vis (LCMS): λ_{max} = 410 nm.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 2.655 min.

HRMS (ESI): calc. for $C_{55}H_{56}N_{15}O_8$ $[M+H]^+$: 1054.4431, found 1054.4419.

1.11. (2*S*,4*S*)-2-Amino-4-(4-((4-((*E*)-(4-(1-azido-39-oxo-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxa-40-azadotetracontan-42-amido)phenyl)diazenyl)phenyl)-amino)-4-oxobutyl)pentanedioic acid (11)



A 1 mL vial was charged with 12.0 mg (14.3 µmol, 1.2 equiv.) of FmocNH-PEG_{11}-COOH (novabiochem, #8510240001) dissolved in 1 mL DMSO and 12.5 µL (9.2 mg, 71.5 µmol, 5.0 equiv.) DIPEA before 4.7 mg (15.7 µmol, 1.1 equiv.) of TSTU was added in one portion. The reaction mixture was stirred at r.t. for 10 min before 8.4 mg (14.3 µmol, 1.0 equiv) of **1**[15] was added in one portion. The reaction was stirred for additional 3 hours until LCMS indicated complete consumption of **1** and formation of **8**, which was obtained after RP-HPLC purification (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) and removal of all volatiles *in vacuo*. Subsequently, Fmoc was deprotected by the addition of 630 µL DMF and 70 µL piperidine at r.t. continued by an additional hour of stirring. The reaction mixture was quenched by addition of 100 µL HOAc and subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain **9** as a yellow powder after lyophilisation, which was transferred to a 1 mL dram vial, dissolved in 1 mL MeOH to which was sequentially added 8.9 mg (64.4 µmol, 4.5 equiv.) of K_2CO_3, 3.6 mg (17.2 µmol, 1.2 equiv.) of $ImSO_2N_3 \times HCl$[16] and 0.4 mg (1.43 µmol, 0.1 equiv.) of $CuSO_4 \times 5\ H_2O$. The reaction mixture was stirred for 90 min at r.t. before it was quenched by addition of 20 µL HOAc and subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain azide **10** after removal of all solvents *in vacuo*. The product was transferred to a vial, cooled to 0 °C and 200 µL of TFA were added. The solution turned dark purple and was allowed to stand for 5 minutes before the volatiles were removed with a gentle stream of nitrogen to obtain 6.0 mg (5.41 µmol) of the desired product in 38% yield over 4 steps, which was clicked in the next reaction step without further purification.

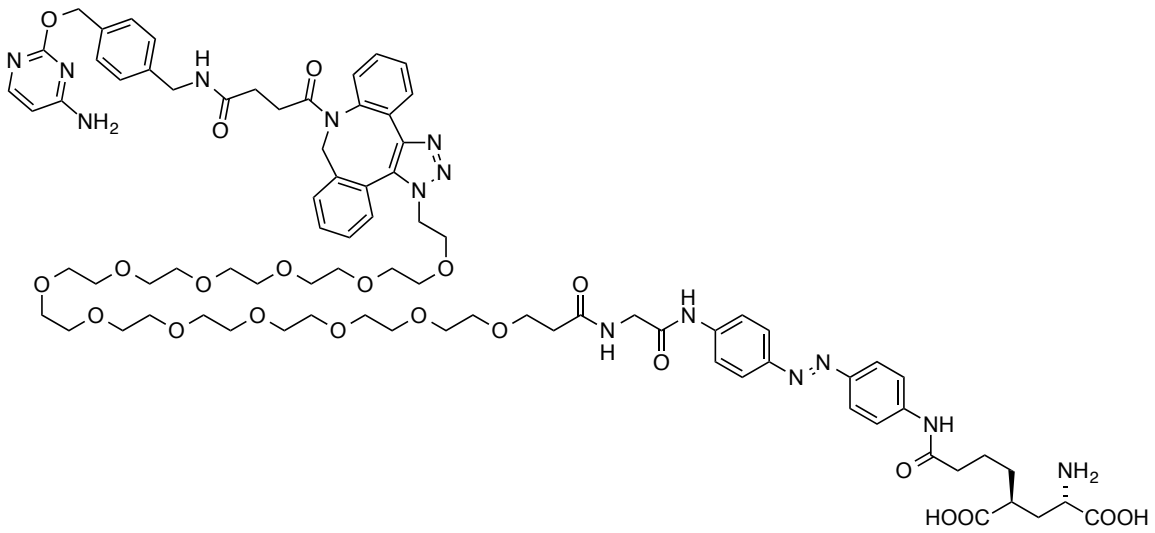
LRMS (ESI): calc. for $C_{50}H_{81}N_9O_{19}$ $[M+2H]^{2+}$: 555.8, found: 555.9.

UV/Vis (LCMS): λ_{max} = 367 nm.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 2.461 min.

HRMS (ESI): calc. for $C_{50}H_{82}N_9O_{19}$ $[M+H]^+$: 1110.5655, found 1110.5620.

1.12. (2*S*,4*S*)-2-Amino-4-(4-((4-((*E*)-(4-(1-(8-(4-((4-(((4-aminopyrimidin-2-yl)oxy)methyl)-benzyl)amino)-4-oxobutanoyl)-8,9-dihydro-1*H*-dibenzo[*b*,*f*][1,2,3]triazolo[4,5-*d*] azocin-1-yl)-39-oxo-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxa-40-azadotetracontan-42-amido)phenyl)diazenyl)phenyl)amino)-4-oxobutyl)pentanedioic acid ($BCAG_{12}$)



A 1 mL vial was charged with 2.8 mg (5.4 µmol, 1.0 equiv.) of **BC-DBCO** and 6.0 mg (5.4 µmol, 1.0 equiv.) of **11** dissolved in 1 mL MeOH. The reaction mixture was stirred for 3 hours before it was subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain 4.9 mg (3.0 µmol) of the desired product in 56% yield after lyophilisation as a red powder.

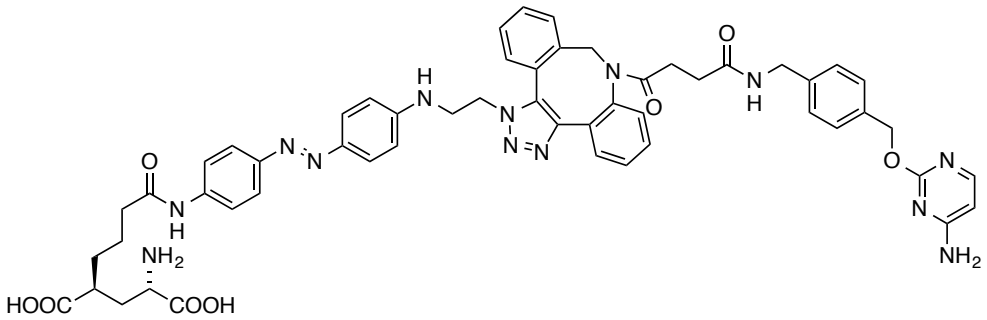
LRMS (ESI): calc. for $C_{81}H_{108}N_{14}O_{22}$ $[M+2H]^{2+}$: 814.4, found: 814.7.

UV/Vis (LCMS): λ_{max} = 367 nm.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 2.473 min.

HRMS (ESI): calc. for $C_{81}H_{107}N_{14}O_{22}$ $[M+H]^+$: 1627.7679, found: 1627.7684.

1.13. (2*S*,4*S*)-2-amino-4-(4-((4-((*E*)-(4-((2-(8-(4-((4-(((4-aminopyrimidin-2-yl)oxy)-methyl)-benzyl)amino)-4-oxobutanoyl)-8,9-dihydro-1*H*-dibenzo[*b*,*f*][1,2,3]triazolo[4,5-*d*] azocin-1-yl)ethyl)amino)phenyl)diazenyl)phenyl)amino)-4-oxobutyl)pentanedioic acid ($BCAG_{0,460}$)



A 1 mL vial was charged with 1.3 mg (2.7 µmol, 1.0 equiv.) of **BC-DBCO** and 1.3 mg (2.7 µmol, 1.0 equiv.) of **7** dissolved in 1 mL MeOH. The reaction mixture was stirred for 3 hours before it was subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain 1.2 mg (1.2 µmol) of the desired product in 44% yield after lyophilisation as a red powder.

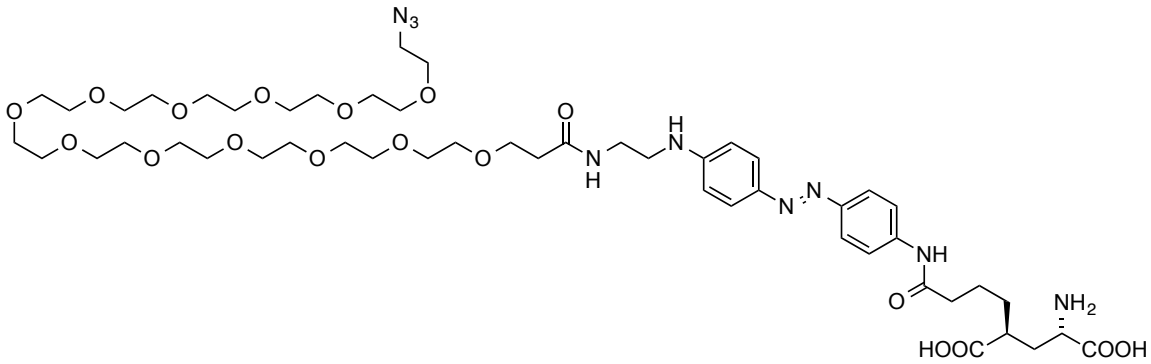
LRMS (ESI): calc. for $C_{54}H_{57}N_{13}O_8$ $[M+2H]^{2+}$: 507.7, found: 507.9.

UV/Vis (LCMS): λ_{max} = 410.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 2.994 min.

HRMS (ESI): calc. for $C_{54}H_{56}N_{13}O_8$ $[M+H]^+$: 1014.4369, found 1014.4366.

1.14. (2*S*,4*S*)-2-amino-4-(4-((4-((*E*)-(4-((1-azido-39-oxo-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxa-40-azadotetracontan-42-yl)amino)phenyl)diazenyl)phenyl)amino)-4-oxobutyl)pentanedioic acid (15)



A 1 mL vial was charged with 52.2 mg (62.2 µmol, 1.8 equiv.) of FmocNH-PEG_{12}-COOH dissolved in 1 mL DMSO and 18.1 µL (13.4 mg, 104 µmol, 3.0 equiv.) DIPEA before 21.8 mg (72.6 µmol, 2.1 equiv.) of TSTU was added in one portion. The reaction mixture was stirred at r.t. for 30 min before 20.0 mg

(35.1 µmol, 1.0 equiv) of **2**[15] was added in one portion, followed by 18.1 µL (13.4 mg, 104 µmol, 3.0 equiv.) of DIPEA and the reaction was stirred for additional 3 hours until LCMS indicated consumption of all starting material. Finally, Fmoc was deprotected in situ by the addition of 200 µL piperidine at r.t. continued by an additional hour of stirring. The reaction mixture was quenched by addition of 250 µL HOAc and subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain **13** as a yellow powder after lyophilisation, which was transferred to a 1 mL dram vial, dissolved in 1 mL MeOH to which was sequentially added 12.4 mg (89.6 µmol, 2.6 equiv.) of K_2CO_3, 8.0 mg (38.4 µmol, 1.1 equiv.) of $ImSO_2N_3 \times HCl$[16] and 3.5 µL of an aqueous 100 mM $CuSO_4$ stock solution (3.5 µmol, 0.1 equiv.). The reaction mixture was stirred for 90 min at r.t. before it was quenched by addition of 20 µL HOAc and subjected to RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain azide **14** after removal of all solvents *in vacuo*. The product was transferred to a vial, cooled to 0 °C and 100 µL of TFA were added. The solution turned dark purple and was allowed to stand for 5 minutes before the volatiles were removed with a gentle stream of nitrogen to obtain 8.2 mg (7.5 µmol) of the desired product in 21% yield over 4 steps after final RP-HPLC (MeCN/H_2O/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) purification and lyophilisation.

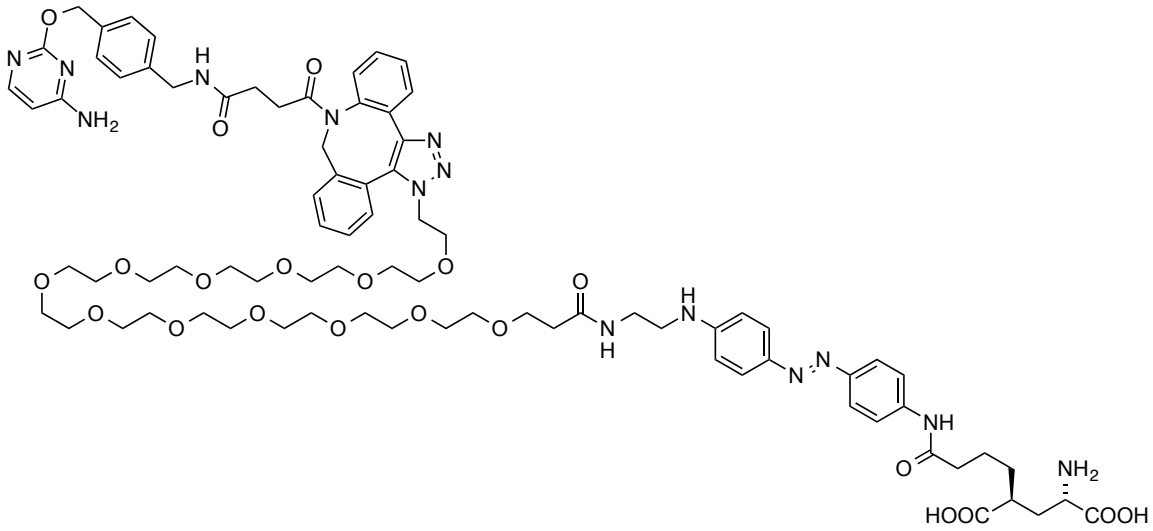
LRMS (ESI): calc. for $C_{50}H_{83}N_9O_{18}$ $[M+2H]^{2+}$: 548.8, found: 548.9.

UV/Vis (LCMS): λ_{max} = 413 nm.

t_R (LCMS; MeCN/H_2O/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 2.554 min.

HRMS (ESI): calc. for $C_{50}H_{82}N_9O_{18}$ $[M+H]^+$: 1096.5772, found 1096.5781.

1.15. (2*S*,4*S*)-2-amino-4-(4-((4-((*E*)-(4-((1-(8-(4-((4-(((4-aminopyrimidin-2-yl)oxy)-methyl)-benzyl)amino)-4-oxobutanoyl)-8,9-dihydro-1*H*-dibenzo[*b*,*f*][1,2,3]triazolo[4,5-*d*] azo-cin-1-yl)-39-oxo-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxa-40-azadotetracontan-42-yl)amino)phenyl)diazenyl)phenyl)amino)-4-oxobutyl)-pentanedioic acid ($BCAG_{12,460}$)



A 1 mL vial was charged with 0.4 mg (0.75 µmol, 1.0 equiv.) of **BC-DBCO** and 0.8 mg (0.75 µmol, 1.0 equiv.) of **15** dissolved in 200 µL MeOH. The reaction mixture was stirred for 2 hours at r.t. before it was subjected to RP-HPLC ($MeCN/H_2O$/TFA = 10/90/0.1 → 90/10/0.1 over 30 minutes) to obtain 0.7 mg (0.50 µmol) of the desired product in 67% yield after lyophilisation as a red powder.

LRMS (ESI): calc. for $C_{81}H_{110}N_{14}O_{21}$ $[M+2H]^{2+}$: 807.4, found: 807.8.

UV/Vis (LCMS): λ_{max} = 413 nm.

t_R (LCMS; $MeCN/H_2O$/formic acid = 10/90/0.1 → 100/0/0.1 over 6 min) = 2.564 min.

HRMS (ESI): calc. for $C_{81}H_{110}N_{14}O_{21}$ $[M+2H]^{2+}$: 807.3980, found 807.3990.

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