

SUPPLEMENTARY TABLES

Supplementary Table 1: Immunogenic B16-F10 mutations (CD8 responses)

Mutation	Mutated sequence	Predicted MHC-I epitope	IEDB score	Published CD8 response*		Present study	
				RNA	LP + polyIC	Screened	SNP-7/8a
B16-M34	HLTQQLDITYILKNVAFSRTDKYRQLP	VVAFSRTDKYRQL	0.1			×	
B16-M49	EKFMSDHKTGTIAMQNTTQLRSRYELT	HKTGTIAMQNTTQL	0.1			×	
B16-M05	FVVKAYLPVNESFAFTADLRSTGGQA	LPVNESFAFTADL	0.1			×	×
B16-M39	ELINFKRKRVAAFQKNLIEMSELEIKH	RVAAFQKNLIEM	0.1			×	×
B16-M26	RVDQKTLHNLRLKVVPSFSAEIERLNQ	RKVVPSFSAEIERL	0.2			×	
B16-M44	EFKHIKAFDRTFANNPGPMVVFATPGM	FANNPGPM	0.2			×	
B16-M21	SSPDEVALVEGVQSLGFTYLRKLDNYM	QSLGFTYL	0.25			×	×
B16-M07	TAKSVMCTYSPPLDKLFCQLAKTCPVQ	SVMCTYSPPLDK	0.3			×	×
B16-M37	GPDGLALPNNYCDVCLGDSKINKKTGQ	LALPNNYCDV	0.3			×	
B16-M33	DSGSPFFAAVILRDLALHMARGLYLHQ	AAVILRDAL	0.3	×		×	×
B16-M01	SGCYFMVAVAHVAFLLEDRAVCVERF	VAVAHVA AFL	0.35			×	×
B16-M17	VVDRNPQFLDPVLAYLMKGLCEKPLAS	RNPQFLDPVLAYL	0.4			×	
B16-M36	CGTAFFINFIAIYHHSRAIPFGTMVA	IAIYHHSRAIPF	0.4			×	
B16-M25	STANYNTSHLNNDVWQIFENPVDWKEK	SHLNNDVWQI	0.4			×	×
B16-M40	NREKMRGELGMLLILQNVIQKTTTPGE	MLLILQNV	0.5			×	
B16-M29	IPSGTTILNCFHDVLSGKLSGGSPGVP	GTTILNCFHDVL	0.6			×	
B16-M46	NHSGLVTFQAFIDVMSRETTDTADQ	VTQAFIDVMS	0.65			×	
B16-M47	GRGHLGLRLAAIVGKQVLLGRKVVVVR	AAIVGKQVL	0.8			×	×
B16-M23	LILISTNGSFIRLLDAFKGVVMHTFGG	TNGSFIRLL	0.85			×	
B16-M27	REGVELCPGNKYEMRRHGTTHSLVIHD	GVELCPGNKYEM	1	×	×	×	×
B16-M19	NEVAPLEWLRYPDKKELEMLCGMQEI	EWLRYFDKKE	1.05			×	
B16-M30	PSKPSFQEFVDWENVSPELNSTDQPFL	NVSPELNSTDQPFL	1.1			×	
B16-M08	ANFESGKHKRYQTAMFTATMPPAVERL	AMFTATMPPAVERL	1.1			×	×
B16-M22	PKPDFSQLQRNLLPSNPRVTRFHINWD	DFSQQRNLLPS	1.3			×	
B16-M20	FRKAFLEHWYTGEMDEMEFTEAESNM	KAFLEHWYTGEM	0.4				
B16-M38	SCTEGQISIAKYENCPKDNPMYCNKK	AKYENCPKDNPM	0.6				
B16-M35	LVLSFVGQTRVLMINGEEVEETELMGF	VLMINGEEV	0.7				
B16-M02	NQGAENVEQVLVTSIQGAVDYPDPIAQ	VTSIQGAVDYPDPI	0.8				
B16-M03	MTESEFKKFEQCHNI IKLQNGHTSLA	QHNI IKLQNGHTSL	0.9				
B16-M10	RGQQLFSLNPRGRSLVTAGRIDREEL	QSQFLSLNPRGRSL	0.9				
B16-M11	ASSSLARGPLSEAGLALFDPYSKEEST	SSLARGPLSEAGL	0.9				
B16-M14	AVTYAAQQHETFLTNGDRAGFLIGDGA	VTYAAQQHETFLT	0.9				
B16-M48	SHCHWDLAVIPAGVVHNDWDFEPRKVS	AGVVHNDWDFEPRKV	1				
B16-M45	ECRITSNFVIPSEYWVEEKEKQKLIQ	SNFVIPSEYWVEE	1.4				
B16-M28	NIEGIDKLTQLKKPFLVNNKINKIENI	PFLVNNKINKIENI	1.6				
B16-M31	DNIPALVEEYLERGNFVANDLDWLLAL	GNFVANDLDWLLAL	1.6				
B16-M09	QRAAAPSAASPADVQSLKKAMSSLQN	ADVQSLKKAMSSL	1.9				
B16-M12	TPPPEEAMPFEFNGPAQGDHSQPPLQV	PEEAMPFEFNGPA	1.9				
B16-M32	EATELKACKPNGKRNPFCEVSMGSQSY	NGKRNPFCEV	2.3				
B16-M50	GFSQPLRRLVHVSAQAERLARAE	VLHVSAQAERL	2.5				
B16-M41	HSLSIECKGIDKEINESKNTHLDIPRI	INESKNTHL	3				
B16-M13	KFRLTASNMEGKSWPSEVLVCTTSPDR	KSWPSEVL	4.4				
B16-M24	TAVITPPTTTTKKARVSTPKPATPSTD	ITPPTTTTKKARV	5.9				
B16-M06	PDVHTLLEITEESGAVLVKSDSD	ITEESGAV	7.65				
B16-M16	RVTNCRAGEKHCSSNEAARDFGGAIQ	SSNEAARDFGGAI	8.9				
B16-M04	PRVSALIESLNQKTQSTGDHPQPTDTT	SALIESLNQKTQ	11				
B16-M18	VETLEECERGQEA KLLAERSRFEDEKQ	KLLAERSRF	18.1				
B16-M42	#						
B16-M43	#						
B16-M15	#						

*Kreiter S, et al. Nature (2015); #sequence not found

Supplementary Table 2: Immunogenic B16-F10 mutations (CD4 responses)

Mutation	Mutated sequence	Predicted MHC-II epitope	IEDB score	Published CD4 response*		Present study	
				LP + polyIC	RNA	Screened	SNP-7/8a
B16-M34	HLTQQLDITYILKNVAFSRTDKYRQLP	DTYILKNVAFSRTD	21.37			x	
B16-M49	EKFSMDHKGTIAMQNTTQLRSRYELT	MDHKTGTIAMQNTTQ	32.48			x	
B16-M05	FVVKAYLPVNESFAFTADLRNNTGGQA	VKAYLPVNESFAFTA	10.16	x		x	x
B16-M39	ELINFKRRKRVAAAFQKNLIEMSELEIKH	RKRVAAFQKNLIEMS	21.91			x	
B16-M26	RVDQKTLHNLRLKVVPSFSAEIERLNQ	NLLRKVVPSFSAEIE	6.12			x	
B16-M44	EFKHIKAFDRTFANNPGPMVVFATPGM	FDRTFANNPGPMVVF	6.61	x	x	x	x
B16-M21	SSPDEVALVEGVQSLGFTYLRKLDNYM	EVALVEGVQSLGFTY	47.12		x	x	
B16-M07	TAKSVMCTYSPPLDKLFCQLAKTCPVQ	KSVMCTYSPPLDKLF	16.92			x	
B16-M37	GPDGLALPNNYCDVCLGDSKINKKTGQ	GPDGLALPNNYCDVC	63.43			x	
B16-M33	DSGSPFPAAVILRDLALHMARGLKYLHQ	DSGSPFPAAVILRDA	8.37			x	x
B16-M01	SGCYFMVAHVAAFLLEDRAVCVERF	SGCYFMVAHVAAAF	2.23			x	
B16-M17	VVDRNPQFLDPVLAYLIMKGLCEKPLAS	VDRNPQFLDPVLAYL	19.75	x		x	x
B16-M36	CGTAFFINFIAIYHHASRAIPFGTMVA	FAIYHHASRAIPFG	7.52	x		x	
B16-M25	STANYNTSHLNNDVWQIFENPVWKEK	STANYNTSHLNNDVW	13.05	x	x	x	x
B16-M40	NREKMGELGMLLILQNVIQKTTTPGE	LILQNVIQKTTTPGE	40.53			x	
B16-M29	IPSGTTILNCFHDVLSGKLSGGSPGVP	DVLSGKLSGGSPGVP	10.55	x		x	
B16-M46	NHSGLVTFQAFIDVMSRETTDTADQ	LVTFFQAFIDVMSRET	33.91	x	x	x	x
B16-M47	GRGHLLGRLLAAIVGKQVLLGRKVVVVR	GRGHLLGRLLAAIVGK	35.75		x	x	
B16-M23	LILISTNGSFIRLLDAFKGVVMHTF	IRLLDAFKGVVMHTF	21.82			x	x
B16-M27	REGVELCPGNKYEMRRHGTTHSLVIHD	KYEMRRHGTTHSLVI	31.81			x	
B16-M19	NEVAPLEWLRYFDKKELEMLCGMQEI	LEWLRYFDKKELELM	66.41			x	
B16-M30	PSKPSFQEFVDWENVPELNSTDQPFL	EFVDWENVPELNST	18.92	x	x	x	x
B16-M08	ANFESGKHKRYQTAMFTATMPPAVERL	TAMFTATMPPAVERL	0.14		x	x	x
B16-M22	PKPDFSQLQRNLPNPRVTRFHNWD	LQRNLPNPRVTRF	4.9	x		x	x
B16-M20	FRKAFHLHWYTGAEDEMEFTEAESNM			x			
B16-M38	SCTEGQISIAKYENCPKDNPMYCNKK						
B16-M35	LVLVSVGQTRVLMINGEEVEETELMGF						
B16-M02	NQGAENVEQVLVTSIQGAVDYPDPIAQ						
B16-M03	MTESEFKKFECEQHNIKLNQNGHTSLA						
B16-M10	RGQSOLFSLNPRGRSLVTAGRIDREEL						
B16-M11	ASSSLARGPLSEAGLALDFPYSKEEST						
B16-M14	AVTYAAQQHETFLTNGDRAGFLIGDGA						
B16-M48	SHCHWNDLAVIPAGVVHNDWDFPRKVS			x	x		
B16-M45	ECRITSNFVIPSEYVWVEKEEKQKLIQ			x			
B16-M28	NIEGIDKLTQLKKPFLVNNKINKIENI			x	x		
B16-M31	DNIPALVEEYLERGNFVANDLDWLLAL						
B16-M09	QRAAAPSAASSPADVQSLKKAMSSLQN						
B16-M12	TPPPEEAMPFEFNGPAQGDHSQPPLQV			x			
B16-M32	EATELKACKPNGKRNPFCEVSMGQS						
B16-M50	GFSQPLRRLVLHVVSAAQAERLARAE			x	x		
B16-M41	HSLSIECKGIDKEINESKNTHLDIPRI						
B16-M13	KFRILTASNMEGKSWPSEVLVCTTSPDR						
B16-M24	TAVITPPTTTTKKARVSTPKPATPSTD			x			
B16-M06	PDVHTLLEITEESGAVLVKSDSD						
B16-M16	RVTCNRAGEKHCFSNEAARDFGGAIQ						
B16-M04	PRVSALIESLNQKTQSTGDHPQPTDIT						
B16-M18	VETLECERKQEAKLLAERSRFEDEKQ						
B16-M42	#						
B16-M43	#						
B16-M15	#						

*Kreiter S, et al. Nature (2015); #sequence not found

SUPPLEMENTARY NOTES

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) as reagent grade or higher purity, unless stated otherwise. All peptides, including native and modified antigens were produced by Genscript (Piscataway, NJ) using solid phase synthesis. Dibenzocyclooctyne (DBCO) used for conjugation of peptides to hydrophobic blocks was purchased from Click Chemistry Tools (Scottsdale, AZ). The red fluorescent reactive dye Alexa Fluor® 647 carboxylic acid NHS ester used to track vaccines *in vivo* was purchased from Fisher Scientific (Waltham, MA).

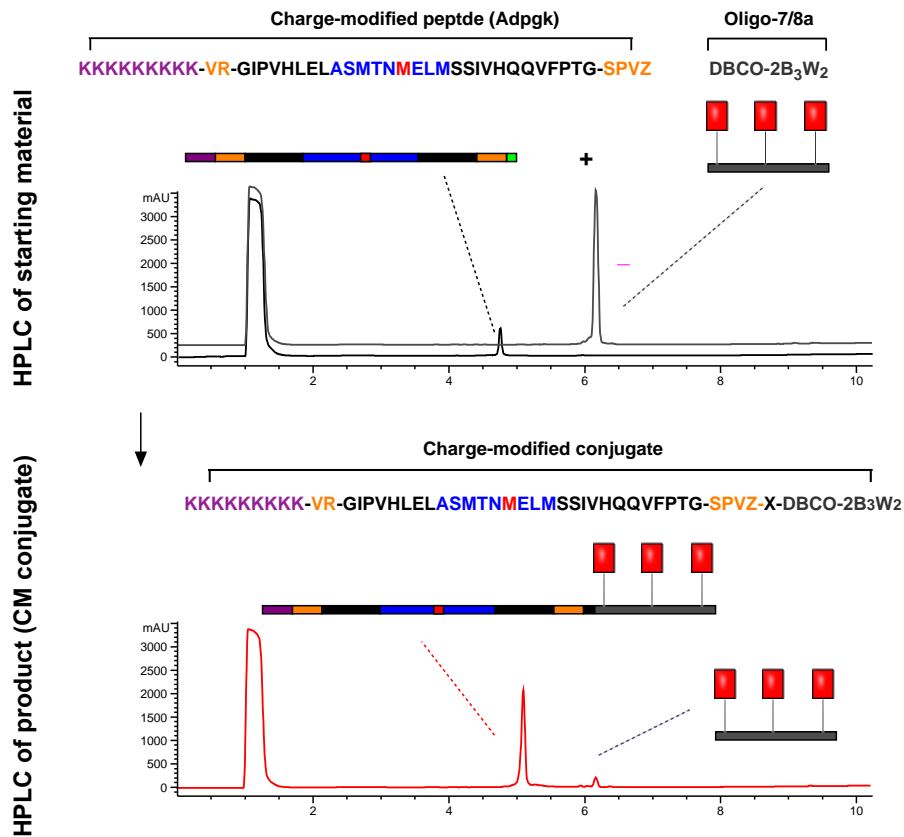
Instrumentation for synthesis, purification and chemical characterization

Conjugate vaccines and charge-modified (CM) conjugate vaccines were purified on an Agilent Technologies (Santa Clara, CA) 1260 series Preparatory HPLC equipped with a C18 prep column (100 x 50 mm, 5.0 μ m) using mobile phase conditions specific to each of the materials being purified as described below. Purity of vaccines was assessed by analytical HPLC analysis using an Agilent 1290 series instrument equipped with multi-wavelength detectors using a reverse-phase Poroshell 120 (Agilent), EC-C18 column (4.6 x 50 mm, 3.5 μ m) with a flow rate of 1.25 mL/min. Solvent A was 0.05% trifluoroacetic acid (TFA) in water (H₂O), solvent B was 0.05% TFA in acetonitrile (ACN), and a linear gradient of 5% B to 95% B over 10 minutes was used. Identity (i.e. mass) of the vaccines was determined using ESI mass spectrometry (MS) on a 6130 Quadrupole LC/MS Agilent Technologies instrument equipped with a diode array detector. ¹H NMR spectra were recorded on a Varian spectrometer operating at 400 MHz. Ultraviolet-Visible (UV-Vis) light spectroscopy was performed on a Lambda25 UV/Vis system from PerkinElmer (Waltham, MA) and fluorescence spectroscopy was carried out on a PerkinElmer brand Fluorescence Spectrometer, model LS 55.

Synthesis and characterization of conjugate vaccines

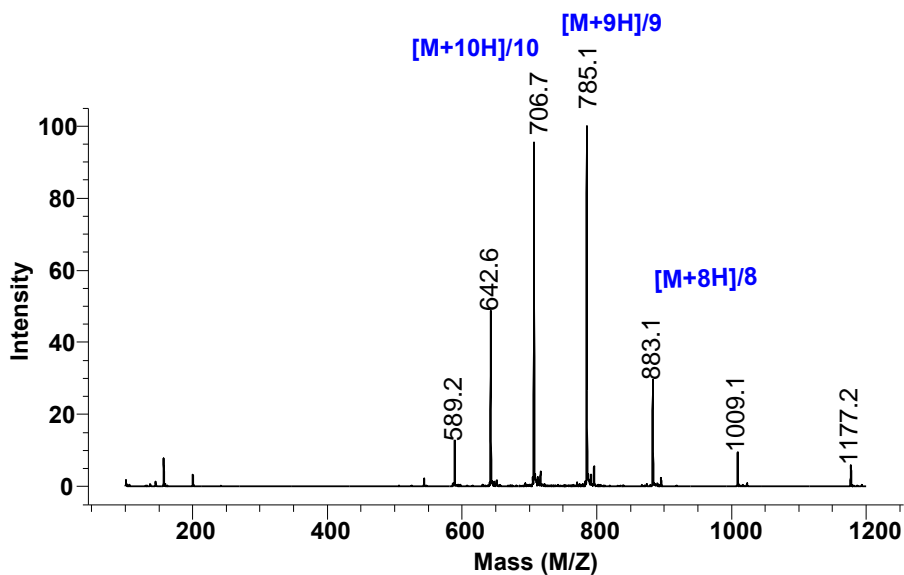
Conjugates and CM conjugates were both produced by linking azide-containing peptide antigens to dibenzocyclooctyne (DBCO)-containing hydrophobic blocks (*e.g.*, oligo-7/8a) using a copper-free click chemistry reaction, strain-promoted azide-alkyne cycloaddition.

As an example, the following procedure was employed for the synthesis of the MC38-derived neoantigen, Adpgk (GIPVHLELASMTNMELMSSIVHQVFPT), as a CM conjugate. Charge-modified Adpgk (KKKKKKKKKVRGIPVHLELASMTNMELMSSIVHQVFPTSPVZX, where Z = citrulline and X = azido-lysine) was produced by solid phase peptide synthesis and then reacted with the DBCO-containing Oligo-7/8a, DBCO-2B₃W₂, that was prepared as described below. Briefly, to a dry 1.5 mL polypropylene tube was aliquoted the charge-modified Adpgk LP in 20 mg/mL DMSO (0.383 mg, 74.9 nmol, 1 equivalent) and 2B₃W₂ in 20 mg/mL DMSO (0.160 mg, 82.3 nmol, 1.1 equivalents). The mixture was stirred and heated to 40 °C. Reaction progress was monitored by HPLC (see below) and showed complete conversion of the peptide (4.7 min) to the CM conjugate (5.1 min) after 16 h. The resulting CM conjugate in clear dimethyl sulfoxide (DMSO) solution was further analyzed as described below.



Characterization of peptide identity by LC-MS

To confirm the identity of conjugates and CM conjugates, samples were diluted to 0.1 mg/mL in DMSO and injected onto an LC instrument equipped with a quadrupole mass spectrometer and ions were analyzed in ESI+ mode. As an example, the mass spectrum of the Adgpk LP CM conjugate (MW = 7056.17 g/mol) conforms with the theoretical as shown below.



PLGA formulations of peptide neoantigens

PLGA particles were synthesized using a double emulsion method as previously described¹⁻⁴. Briefly, an organic phase was prepared consisting of 80 mg of PLGA dissolved in 5 mL dichloromethane. To obtain particles with a target loading of 5% peptide antigen (mass peptide/mass polymer), an inner aqueous phase consisting of 4 mg peptide antigen and 1.33 mg of the TLR-7/8a, Compound 1 (referred to as “2B”), dissolved in 500 μ L 26.7% (v/v) DMSO in water. The inner aqueous phase was added to the inner organic phase and sonicated for 30 seconds at 12 W to form the first emulsion. This emulsion was then added to 40 mL of a 1% w/v PVA solution (89,000 – 98,000 MW) in water and sonicated for 3 minutes at 12 W. Following overnight evaporation with stirring, particles were poured through a 40 μ m cell strainer and collected by centrifugation (5000xg, 5 min, 4°C). Supernatants were removed, and particles were washed two times with 1 mL water.

The mass of peptide antigen loaded in the PLGA particles was determined using the microBCA protein assay kit (ThermoFisher, Waltham, MA, USA) as previously described⁵. An aliquot of the solution of PLGA particles was dried to determine the mass of particles. The dried samples was then dissolved in DMSO at 18 mg/mL for 1 hour. Five volumes of 0.5% SDS (VWR, Radnor, PA, USA) in 0.05 N NaOH was then added, and after 1 hour the BCA assay was run according to the manufacturer’s instructions. Neoantigen encapsulation efficiency was calculated based on the mass of peptide antigen determined by the BCA assay according to the following relationship.

$$\text{Encapsulation efficiency} = \frac{\text{mass peptide antigen}}{\text{mass of particle}} \times \% \text{ target loading}$$

Liposome formulations of peptide neoantigens

Liposomal nanoparticles (NPs) were formulated as previously described⁶. To a lipid film dried under nitrogen consisting of 40 mg of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG) (Avanti Polar Lipids), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Avanti polar lipids) and cholesterol (Avanti polar lipids, Alabaster, AL, USA) at a mass ratio of 5:60:35, respectively, a solution of 1 mg peptide antigen and 0.33 mg of Compound 1 in a solution of 3.34% DMSO in PBS was added to generate liposomal particles with a target loading of 2.5 % peptide antigen (mass peptide/mass lipids). The lipid film was resuspended by repeated pipetting, followed by extrusion through a polycarbonate membrane with 200 nm pore size (Avanti Polar Lipids).

The mass of peptide antigen loaded in the liposomal particles was determined using HPLC equipped with a UV detector based on a standard curve relating peptide antigen mass and absorbance at 220 nm. In brief, an aliquot of the liposomal particle solution was dialyzed against PBS over 16 hours using 100 kDa MWCO cellulose ester membrane (Repligen, Waltham, MA, USA) to remove peptide antigen unassociated with the particles. The dialyzed and non-dialyzed liposomal particle solutions were then suspended in acetonitrile to a final solution composition of 80% acetonitrile/PBS (v/v) with 0.01% TFA, which fully disrupted and solubilized the lipids and peptide antigen cargo. Known volumes of each of the samples were assessed by HPLC and the peaks (UV, 220 nm) corresponding to peptide antigen were integrated to determine the absorbance attributable to the peptide antigen, which was used to calculate the mass of peptide antigen based on a standard curve. Encapsulation efficiency was calculated as described above.

Particle size and turbidity measurements

Native peptide antigens and conjugate vaccines were analyzed in triplicate for turbidity to assess aggregation and by dynamic light scattering (DLS) to assess particle size. Briefly, peptide antigen or conjugate vaccines in DMSO solution were diluted with PBS buffer (1x, pH 7.4) to a final concentration of 0.5 mg/mL. The solutions were vortexed for a few short pulses and placed at room temperature for 1 h prior to analysis.

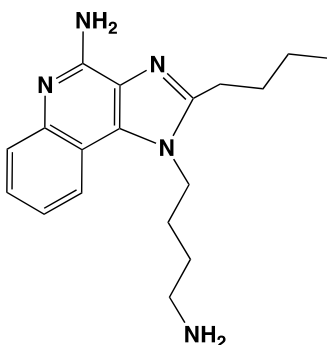
Turbidity was determined by adding 200 μ L of each of the native peptide antigen or conjugate vaccine solutions to a 96-well plate (flat-bottom, Corning) and measuring absorption at 490 nm using a SpectraMax Plus 384 (Molecular devices) spectrophotometer. PBS buffer was used as a control to assess background. Turbidity is reported as optical density (OD), arbitrary units.

Particle size was determined by adding 35 μ L of each of the native peptide antigen or conjugate vaccine solutions to a 384-well plate (black with clear bottom, Corning) and evaluating light scattering using a DynaPro Plate Reader II (Wyatt) with a laser wavelength of 822.5 nm and a scattering angle of 150°.

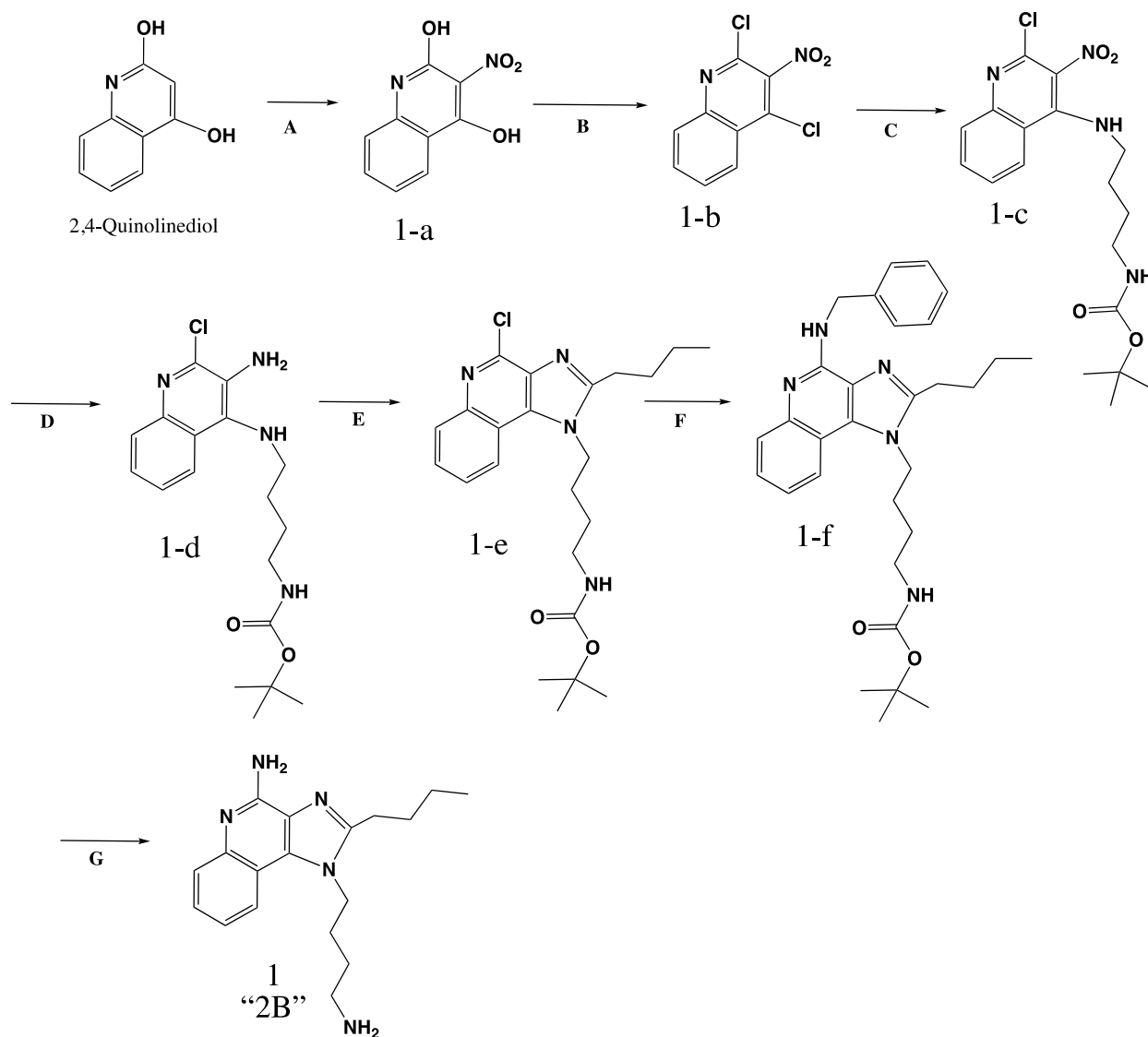
Determination of grand average of hydropathy (GRAVY) and net charge

The Hydropathy scale of Kyte and Doolittle was used to calculate the grand average of hydropathy (GRAVY) of peptide antigens and conjugate vaccines⁷. The GRAVY value is the sum of the Hydropathy values of each of the amino acids comprising the peptide divided by the length (i.e. number of amino acids) of the peptide. The net charge of native peptide antigens and conjugate vaccines was calculated by summing the estimated charge at pH 7.4 of each of the amino acids (R, K = +1; D, E = -1; all other amino acids = 0) comprising the peptide. For conjugate vaccines, the triazole linker and hydrophobic block (*e.g.*, oligo-7/8a) were not considered in the calculations of GRAVY and net charge.

Synthesis of oligopeptide linked TLR-7/8 agonist (oligo-7/8a) based hydrophobic blocks



Compound 1, 1-(4-aminobutyl)-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine, referred to as 2B, was synthesized according to **Scheme 1**, below.



Scheme 1: (A) HNO_3 , 75°C , 1h; (B) PhPOCl_2 , 130°C , 3h; (C) R-NH_2 , DCM , 70°C , 2h (D) 10% pt/c, EtoAc , 55 psi H_2 , 2h (E) (i) Valeroyl chloride, THF , RT , 2h; (ii) CaO(s) , methanol , 100°C ; 5h (F) Benzyl-amine, 110°C , 8h (G) 98% H_2SO_4 , 2h

1-c. Starting from 2,4-quinolinediol, the intermediate, **1-b**, was prepared as previously described⁸. To 21 g of **1-b** (87.8 mmol, 1 eq) in 210 mL of triethylamine (TEA) (10% w/w) was added 16.34 g (87.8 mmol, 1 eq) of N-boc-1,4-butanediylamine while stirring vigorously. The reaction mixture was heated to 70°C and monitored by HPLC, which confirmed that the reaction was complete after 2 hours. The TEA was removed under vacuum and the resulting oil was dissolved in 200 mL of dichloromethane (DCM) and then washed with 3x100 mL DI H_2O . The organic layer was dried with Na_2SO_4 and then removed under vacuum and the resulting oil was triturated with 1:1 (v:v) hexane and diethyl ether to yield 30.7 g of yellow crystals of **intermediate 1-c**. MS (APCI) calculated for $\text{C}_{18}\text{H}_{23}\text{ClN}_4\text{O}_4$, $m/z = 394.1$ (found 394.9)

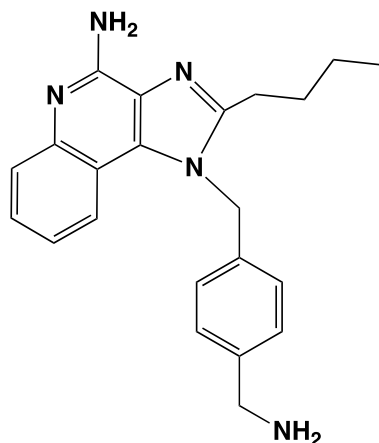
1-d. 30.7 g (76.4 mmol) of intermediate **1-c** was dissolved in 300 mL of ethyl acetate in a Parr Reactor vessel that was bubbled with argon, followed by the addition of 3 g of 10% platinum on carbon. The reaction vessel was kept under argon and then evacuated and pressurized with H₂(g) several times before pressurizing to 55 PSI H₂(g) while shaking vigorously. The H₂(g) was continually added until the pressure stabilized at 55 PSI, at which point the reaction was determined to be complete. The reaction mixture from the Parr Reactor was then filtered through celite and evaporated to dryness to obtain a yellow oil that was triturated with 1:1 hexanes / ether to yield white crystals that were collected by filtration to obtain 27.4 g of spectroscopically pure white crystals of **1-d**. MS (APCI) calculated for C₁₈H₂₅ClN₄O₂, *m/z* = 364.2 (found 365.2).

1-e. To 10 g (27.4 mmol, 1 eq) of **1-d** in 50 mL of tetrahydrofuran (THF) was added 7.7 mL of TEA (54.8 mmol, 2 eq) followed by the drop wise addition of 3.6 g of valeroyl chloride (30.1 mmol, 1.1 eq) in 30 mL of THF while stirring vigorously with the reaction mixture on ice. After 90 minutes, the ice bath was removed and the THF was removed under vacuum, resulting in a yellow oil that was dissolved in 100 mL of DCM that was washed with 3x50 mL of pH 5.5 100 mM acetate buffer. The DCM was removed under vacuum to give an oil that was triturated with ethyl acetate to obtain 10.4 g of a white solid that was dissolved in methanol with 1 g of CaO (s), which was heated at 100°C for 5 hours while stirring vigorously. The reaction mixture was filtered and dried to yield 10.2 g of an off-white solid, intermediate, **1-e**. MS (ESI) calculated for C₂₃H₃₁ClN₄O₂, *m/z* = 430.21 (found 431.2).

1-f. To 10.2 g (23.7 mmol, 1 eq) of **1-e** was added 30.4 g (284 mmol, 12 eq) of benzylamine liquid, which was heated to 110°C while stirring vigorously. The reaction was complete after 10 hours and the reaction mixture was added to 200 mL ethyl acetate and washed 4x100 mL with 1 M HCl. The organic layer was dried with Na₂SO₄ and then removed under vacuum and the resulting oil was recrystallized from ethyl acetate to obtain 10.8 g of spectroscopically pure white crystals of intermediate, **1-f**. MS (ESI) calculated for C₃₀H₃₉N₅O₂, *m/z* = 501.31 (found 502.3).

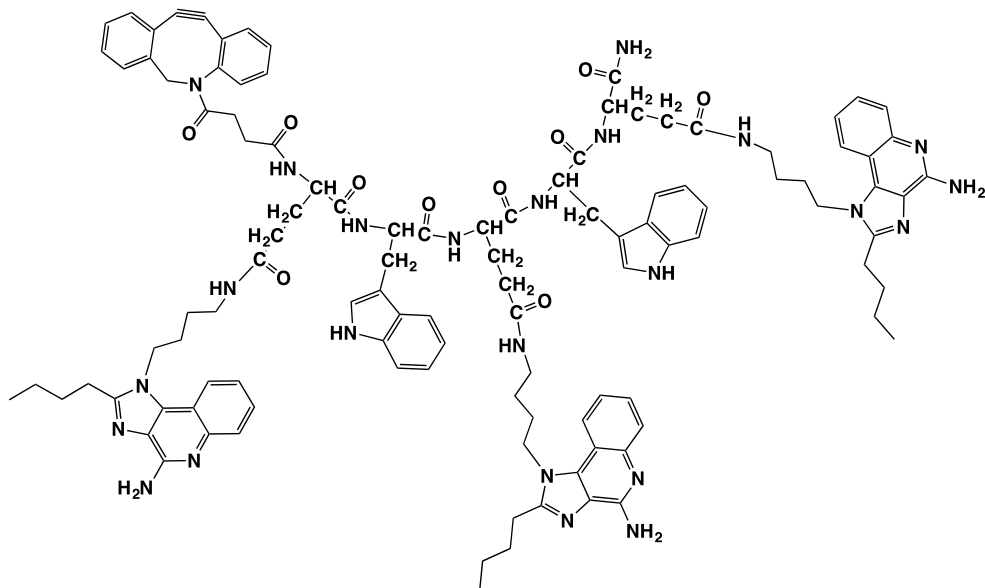
Compound 1. 10.8 g (21.5 mmol) of **1-f** was dissolved in 54 mL of concentrated (>98%) H₂SO₄ and the reaction mixture was stirred vigorously for 3 hours. After 3 hours, the viscous red reaction mixture was slowly added to 500 mL of DI H₂O while stirring vigorously. The reaction mixture was stirred for 30 minutes and then filtered through Celite, followed by the addition of 10 M NaOH until the pH of the solution was ~ pH 10. The aqueous layer was then extracted with 6x200 mL of DCM and the resulting organic layer was dried with Na₂SO₄ and reduced under vacuum to yield a spectroscopically pure white solid. ¹H NMR (400 MHz, DMSO-d₆) δ 8.03 (d, J = 8.1 Hz, 1H), 7.59 (d, J = 8.1 Hz, 1H), 7.41 (t, J = 7.41 Hz, 1H), 7.25 (t, J = 7.4 Hz, 1H), 6.47 (s, 2H), 4.49 (t, J = 7.4 Hz, 2H), 2.91 (t, J = 7.78 Hz, 2H), 2.57 (t, J = 6.64 Hz, 1H), 1.80 (m, 4H), 1.46 (m, J = 7.75 Hz, 4H), 0.96 (t, J = 7.4 Hz, 3H). MS (ESI) calculated for C₁₈H₂₅N₅, *m/z* = 311.21 (found 312.3).

Compound 2



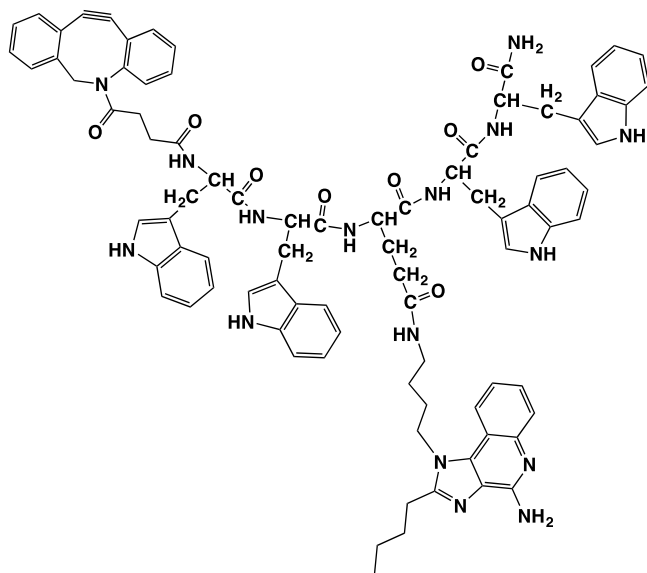
Compound 2, 1-(4-(aminomethyl)benzyl)-2-butyl-1H-imidazo[4,5-c]quinolin-4-amine, referred to as 2BXy, was prepared as previously^{8,9}. ¹H NMR (400 MHz, DMSO-d₆) δ 7.77 (dd, J = 8.4, 1.4 Hz, 1H), 7.55 (dd, J = 8.4, 1.2 Hz, 1H), 7.35 – 7.28 (m, 1H), 7.25 (d, J = 7.9 Hz, 2H), 7.06 – 6.98 (m, 1H), 6.94 (d, J = 7.9 Hz, 2H), 6.50 (s, 2H), 5.81 (s, 2H), 3.64 (s, 2H), 2.92–2.84 (m, 2H), 2.15 (s, 2H), 1.71 (q, J = 7.5 Hz, 2H), 1.36 (q, J = 7.4 Hz, 2H), 0.85 (t, J = 7.4 Hz, 3H). MS (APCI) calculated for C₂₂H₂₅N₅ *m/z* = 359.2 (found 360.3).

Compound 3 (Oligo-7/8a version 2B₃W₂)



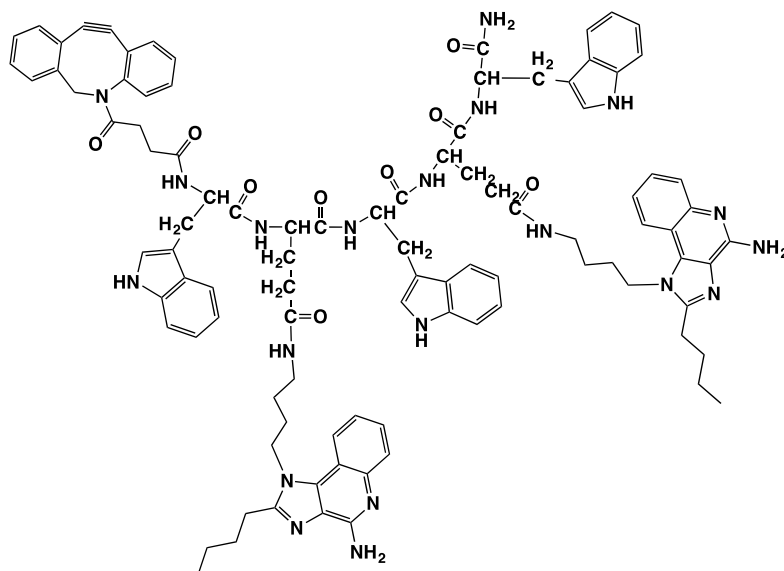
Compound 3, referred to as 2B₃W₂, or DBCO-(Glu(2B)₃(Trp)₂), was prepared starting from a Glu-Trp-Glu-Trp-Glu-NH₂ precursor prepared by solid phase synthesis. 113.9 mg of Glu-Trp-Glu-Trp-Glu-NH₂ (0.147 mmol, 1 eq) was dissolved in 1.5 mL of dry DMSO. 20.4 μ L of TEA (0.147 mmol, 1 eq) was added and the solution was stirred at room temperature for 5 minutes. 177 mg of DBCO-NHS (0.44 mmol, 3 eq) was added and the reaction mixture was stirred at room temperature for 1 hour. The resulting DBCO intermediate was purified on a preparatory HPLC system using a gradient of 32-52% acetonitrile/H₂O (0.05% TFA) over 12 minutes on an Agilent C-18 column, 50x100mm, 5 μ m. The resulting fractions were combined, frozen and lyophilized. 77.9 mg of DBCO-Glu-Trp-Glu-Trp-Glu-NH₂ (0.073 mmol, 1 eq) and 82 mg of **Compound 1** (0.264 mmol, 3.6 eq) were dissolved in 5.8 mL of dry dimethylformamide (DMF). 122.5 μ L of TEA (0.879 mmol, 12 eq) was added and the solution was cooled to 4°C with an ice bath. 91.9 mg of HATU (0.241, 3.3 eq) was added and the reaction mixture was stirred at 4°C for 1 hour. The product in DMF was added to 250 mL of 1M HCl and the precipitate was pelleted by centrifuging at 4000G for 5 minutes. The 1M HCl was decanted and the process was repeated once more with 1M HCl and finally with DI H₂O. The solid was frozen and lyophilized. The dried solid was dissolved in DMSO and purified on a preparatory HPLC system using a gradient of 34-44% acetonitrile/H₂O (0.05% TFA) over 12 minutes on an Agilent C-18 column, 50x100mm, 5 μ m. The resulting fractions were combined, frozen and lyophilized to yield 116.5 mg (60% yield) of DBCO-(Glu(2B)₃(Trp)₂) as a spectroscopically pure (>98% AUC at 254 nm) off-white powder. MS (ESI) calculated for C₁₁₀H₁₂₆N₂₄O₁₀ m/z = 1943 (found 972.3 (m/2)⁺ and 649.0 (m/3)⁺).

Compound 4 (Oligo-7/8a version 2B₁W₄)



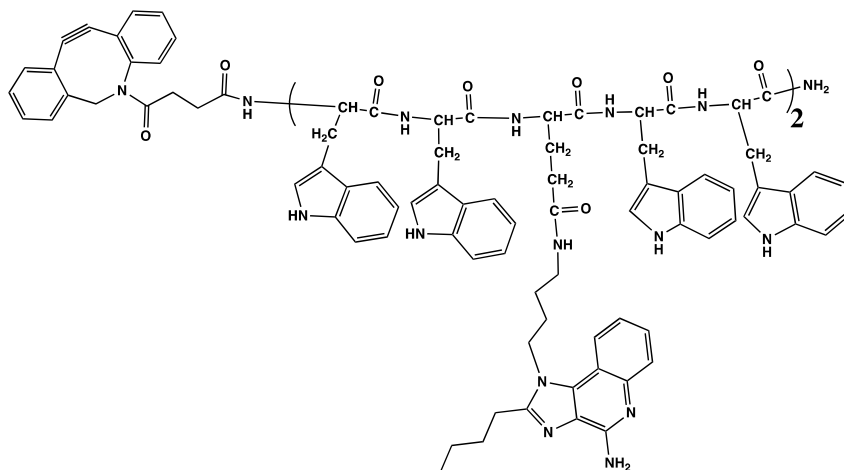
Compound 4, referred to as 2B₁W₄ was synthesized using a similar procedure as describe for **Compound 3**, except Trp-Trp-Glu-Trp-Trp-NH₂ was used as the peptide precursor. **Compound 4** was purified on a preparatory HPLC system using a gradient of 50-55% acetonitrile/H₂O (0.05% TFA) over 12 minutes on an Agilent Prep-C18 column, 50x100mm, 5 μm. The product eluted at 8.9 minutes and the resulting fractions were collected, frozen and then lyophilized to obtain 9.7 mg (55.4% yield) of a spectroscopically pure (> 95% AUC at 254 nm) white powder. MS (ESI) calculated for C₈₆H₈₆N₁₆O₈ m/z = 1470.68 (found 736.6 (M/2)⁺).

Compound 5 (Oligo-7/8a version 2B₂W₃)



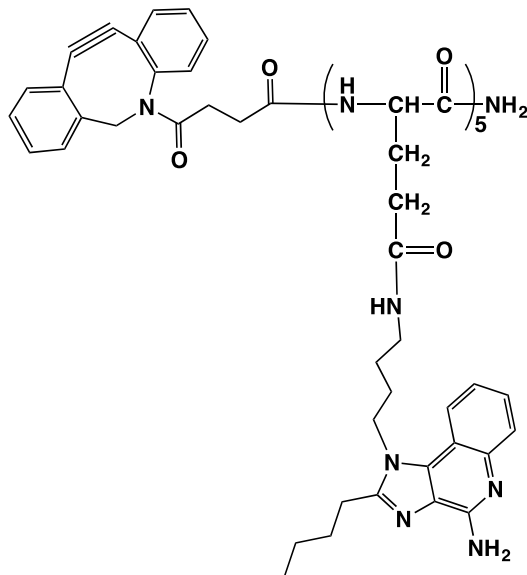
Compound 5, referred to as 2B₂W₃ or DBCO-(Glu(2B)₂(Trp)₃), was synthesized using a similar procedure as described for **Compound 3**, except Trp-Glu-Trp-Glu-Trp-NH₂ was used as the starting material. **Compound 5** was purified on a preparatory HPLC system using a gradient of 35-65% acetonitrile/H₂O (0.05% TFA) over 12 minutes on an Agilent Prep-C18 column, 50x100 mm, 5 μm. The product eluted at ~ 9 minutes and the resulting fractions were collected, frozen and then lyophilized to obtain 11.6 mg (62.5% yield) of a spectroscopically pure (> 95% AUC at 254 nm) white powder. MS (ESI) calculated for C₉₈H₁₀₆N₂₀O₉ m/z = 1706.85 (found 854.9 (M/2)⁺).

Compound 6 (Oligo-7/8a version 2B₂W₈)



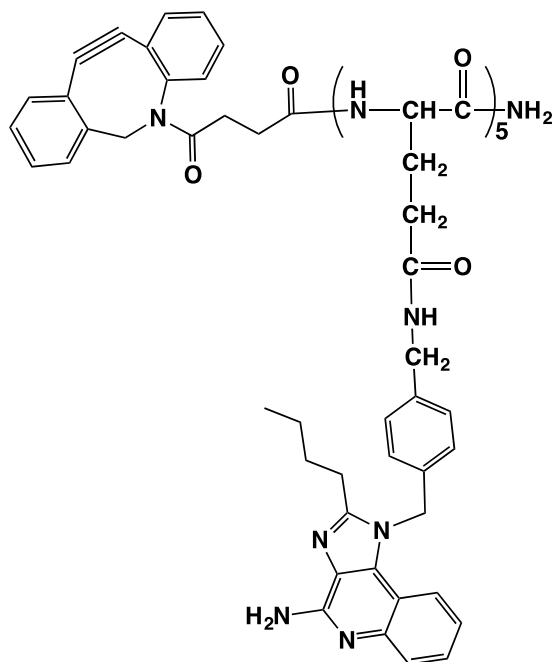
Compound 6, referred to as DBCO-2B₂W₈ or DBCO-(Glu(2B)₂(Trp)₈), was synthesized using a similar procedure as described for **Compound 3**, except Trp-Trp-Glu-Trp-Trp-Trp-Trp-Glu-Trp-Trp-NH₂ was used as starting material. **Compound 6** was purified on a preparatory HPLC system using a gradient of 35-85% acetonitrile/H₂O (0.05% TFA) over 12 minutes on an Agilent Prep-C18 column, 50x100mm, 5 μm. The product eluted at ~ 8.0 minutes and the resulting fractions were collected, frozen and then lyophilized to obtain 3.3 mg (16.3% yield) of a spectroscopically pure (> 95% AUC at 254 nm) white powder. MS (ESI) calculated for C₁₅₃H₁₅₆N₃₀O₁₄ m/z = 2637.24 (found 1320.2 (M/2)⁺).

Compound 7 (Oligo-7/8a version 2B₅)



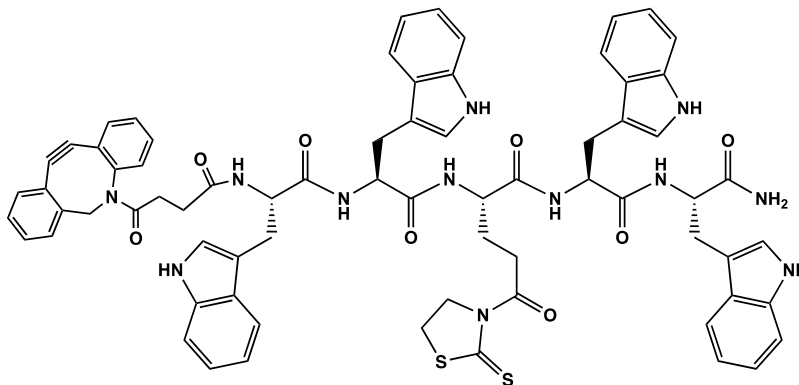
Compound 7, referred to as 2B₅ or DBCO-(Glu(2B)₅), was synthesized using a similar procedure as described for **Compound 3**, except (Glu)₅-NH₂ was used as the starting material. **Compound 7** was purified on a preparatory HPLC system using a gradient of 33-45% acetonitrile/H₂O (0.05% TFA) over 12 minutes on an Agilent Prep-C18 column, 50x100mm, 5 μm. The product eluted at ~ 10.0 minutes and the resulting fractions were collected, frozen and then lyophilized to obtain 25.2 mg (62.6% yield) of a spectroscopically pure (> 95% AUC at 254 nm) white powder. MS (ESI) calculated for C₁₃₄H₁₆₆N₃₂O₁₂ m/z = 2415.34 (found 1209.3 (M/2)⁺).

Compound 8 (Oligo-7/8a version 2BXy₅)



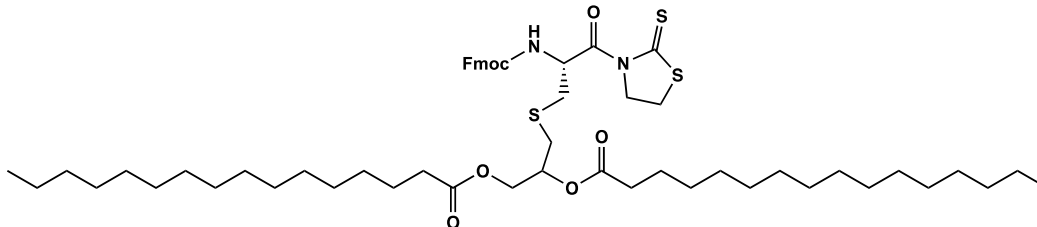
Compound 8, referred to as 2BXy₅ or DBCO-(Glu(2BXy)₅), was synthesized using a similar procedure as described for **Compound 3**, except (Glu)₅-NH₂ was used as the starting material for conjugation of **Compound 2**. **Compound 8** was purified on a preparatory HPLC system using a gradient of 38-48% acetonitrile/H₂O (0.05% TFA) over 12 minutes on an Agilent Prep-C18 column, 50x100mm, 5 μm. The product eluted at 8.0 minutes and the resulting fractions were collected, frozen and then lyophilized to obtain 45.9 mg (63.4% yield) of a spectroscopically pure (> 95% AUC at 254 nm) white powder. MS (ESI) calculated for C₁₅₄H₁₆₆N₃₂O₁₂ *m/z* = 2655.34 (found 886.6 (M/3)⁺).

Compound 9



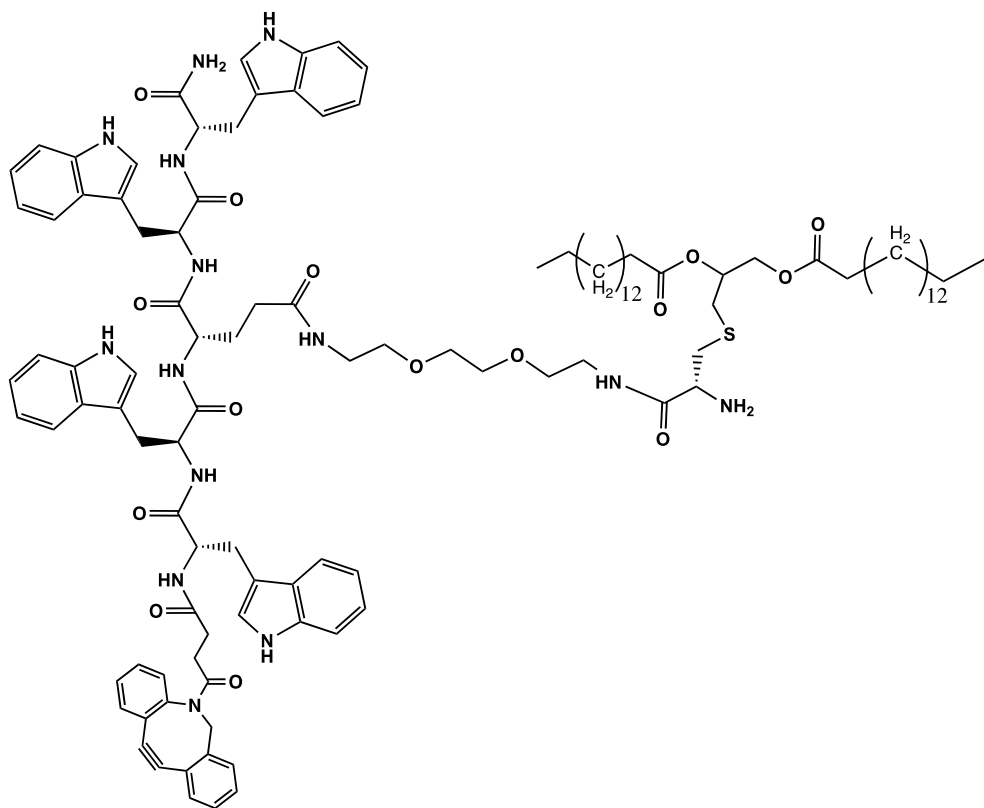
Compound 9, referred to as DBCO-WWTTWW or W₄TT, was prepared starting from a Trp-Trp-Glu-Trp-Trp-NH₂ precursor prepared by solid phase peptide synthesis. 32.8 mg of Trp-Trp-Glu-Trp-Trp-NH₂ (0.04 mmol, 1 eq) was dissolved in 0.5 mL of dry DMSO and 15.5 mg (0.04 mmol, 1 eq) of DBCO-NHS was added followed by addition of TEA (0.04 mmol, 1 eq). The reaction mixture was stirred at room temperature for 1 hour. The resulting DBCO intermediate was purified on a preparatory HPLC system using a gradient of 40-70% acetonitrile/H₂O (0.05% TFA) over 16 minutes on an Agilent Prep C-18 column, 30x100mm, 5 μm. The resulting fractions were combined, frozen and lyophilized. To TT activate the DBCO-peptide, 6.1 mg of DBCO-Trp-Trp-Glu-Trp-Trp-NH₂ (0.005 mmol, 1 eq), 0.68 mg of TT (0.006 mmol, 1.1 eq) and 1.26 mg of EDC (0.007 mmol, 1.3 eq) were dissolved in 0.2 mL of a 1:1 solution of DMSO and DCM. To the reaction mixture was added 0.06 mg of DMAP (0.001 mmol, 0.1 eq) while stirring vigorously at room temperature. After 2 hours, an additional 3.3 equivalents of TT, 4.0 equivalents of EDC and 0.1 equivalents of DMAP were added and reaction was complete after 3 hours total. The DBCO-WWTTWW was used as an intermediate in subsequent steps.

Compound 10



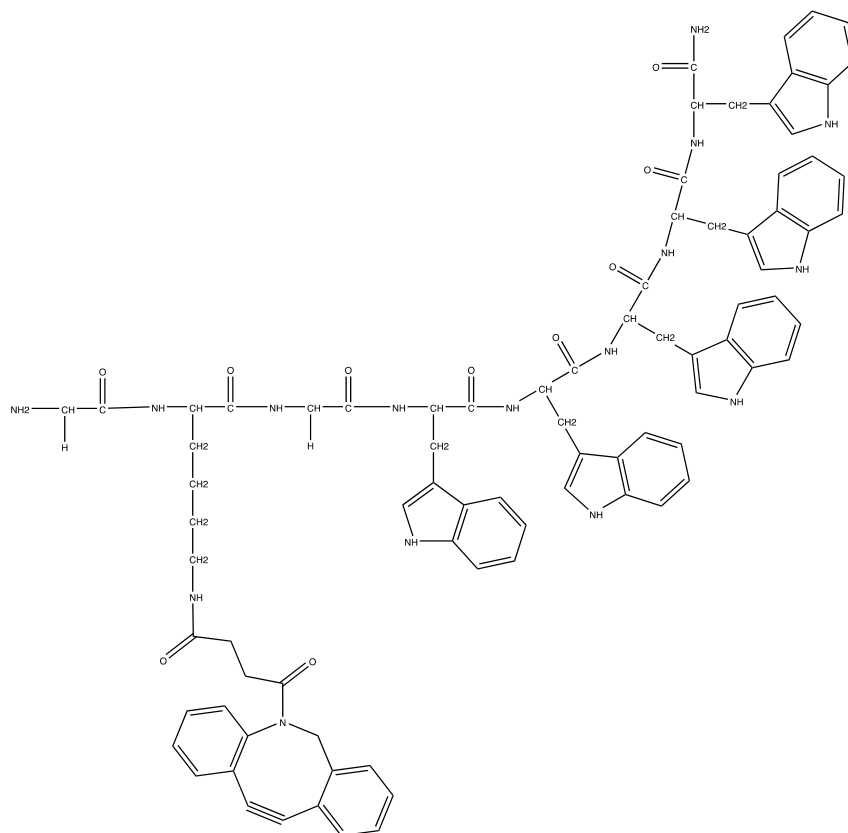
Compound 10, referred to as Pam2Cys-TT or P2C-TT, which is a TLR-2/6 agonist, was synthesized using Fmoc-Pam2Cys (Bachem, Torrance, CA) as the starting material. 50 mg of Fmoc-Pam2Cys-Acid (0.056 mmol, 1 eq), 7.33 mg of TT (0.06 mmol, 1.1 eq), and 13.93 mg of EDC (0.07 mmol, 1.3 eq) were dissolved in 1 mL of DCM. 0.68 mg of DMAP (0.01 mmol, 0.1 eq) was added. After 1 hour TLC in DCM showed that the reaction was complete. The reaction mixture was diluted with DCM and washed twice with 1M HCl and once with DI H₂O. The organic layer was collected, dried with Na₂SO₄ and dried to provide Pam2Cys-TT as a yellow solid in quantitative yield.

Compound 11 (“Oligo-TLR-2/6a”)



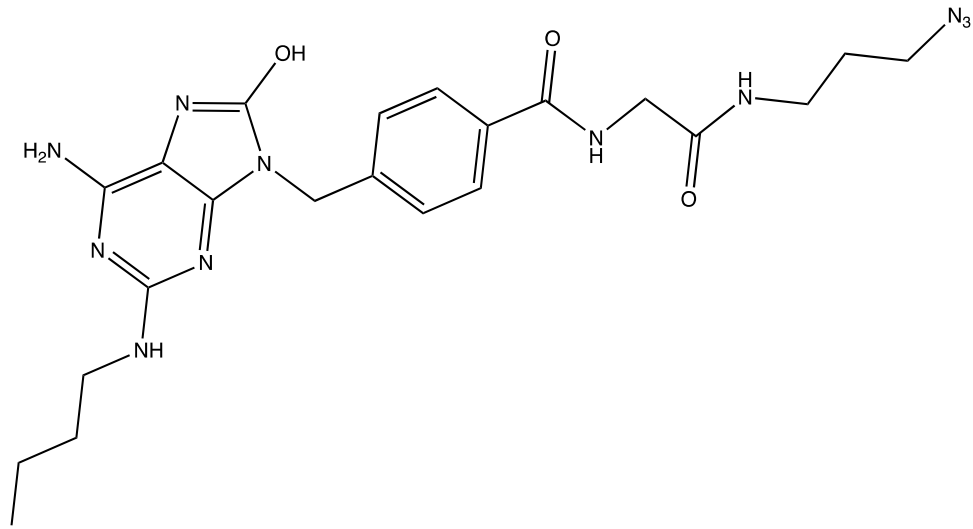
Compound 11, referred to as DBCO-WWPam2CysWW, WW(P2C)WW or P2C(W)₄, was prepared using **Compound 9**, PEG₂-diamine, and **Compound 10** as starting materials. First, 1.15 mg of PEG₂-diamine (0.008 mmol, 3 eq) was added to a crude reaction mixture containing 3.31 mg of **Compound 9** (0.0026 mmol, 1 eq). The reaction mixture was stirred for 1 hour at room temperature then added to 15 mL of DI H₂O and centrifuged. The pellet was collected and further washed three times with DI H₂O and lyophilized. The intermediate (DBCO-WWPeg2NH₂WW) was taken up in 0.1 mL of dry DMSO and 2.58 mg of **Compound 10** (0.0026 mmol, 1 eq) was added as a 100 mg/mL stock solution in DCM. The reaction mixture was stirred for 1 hour at room temperature and then DCM was removed under vacuum followed by the addition of 0.2 mL of 20% piperidine in DMF. The reaction mixture was stirred for 30 minutes at room temperature then diluted with DCM and washed three times with DI H₂O. The organic layer was dried with Na₂SO₄ and evaporated. The resulting oil was dissolved in DMSO and purified on a preparatory HPLC system using a gradient of 70-100% Isopropanol/H₂O (0.05% TFA) over 16 minutes on an Agilent Prep C-18 column, 9.4x100mm, 5 μm. The resulting fractions were combined and evaporated to provide the product, DBCO-WWPam2CysWW. Product molecular weight verified based on the MS (ESI⁺) for peptide antigen conjugates of **Compound 11**. Briefly, DBCO-WWPam2CysWW was dissolved in DMSO and reacted with KKKKKKVRGIPVHLELASMTNMELMSSIVHQQVFPTGSGVZX, MW 4745.65 g/mol). After 1 hour, LC-MS was run on the conjugate. Expected MW = 6707.73 (found 1118.8 (m/6)⁺).

Compound 12



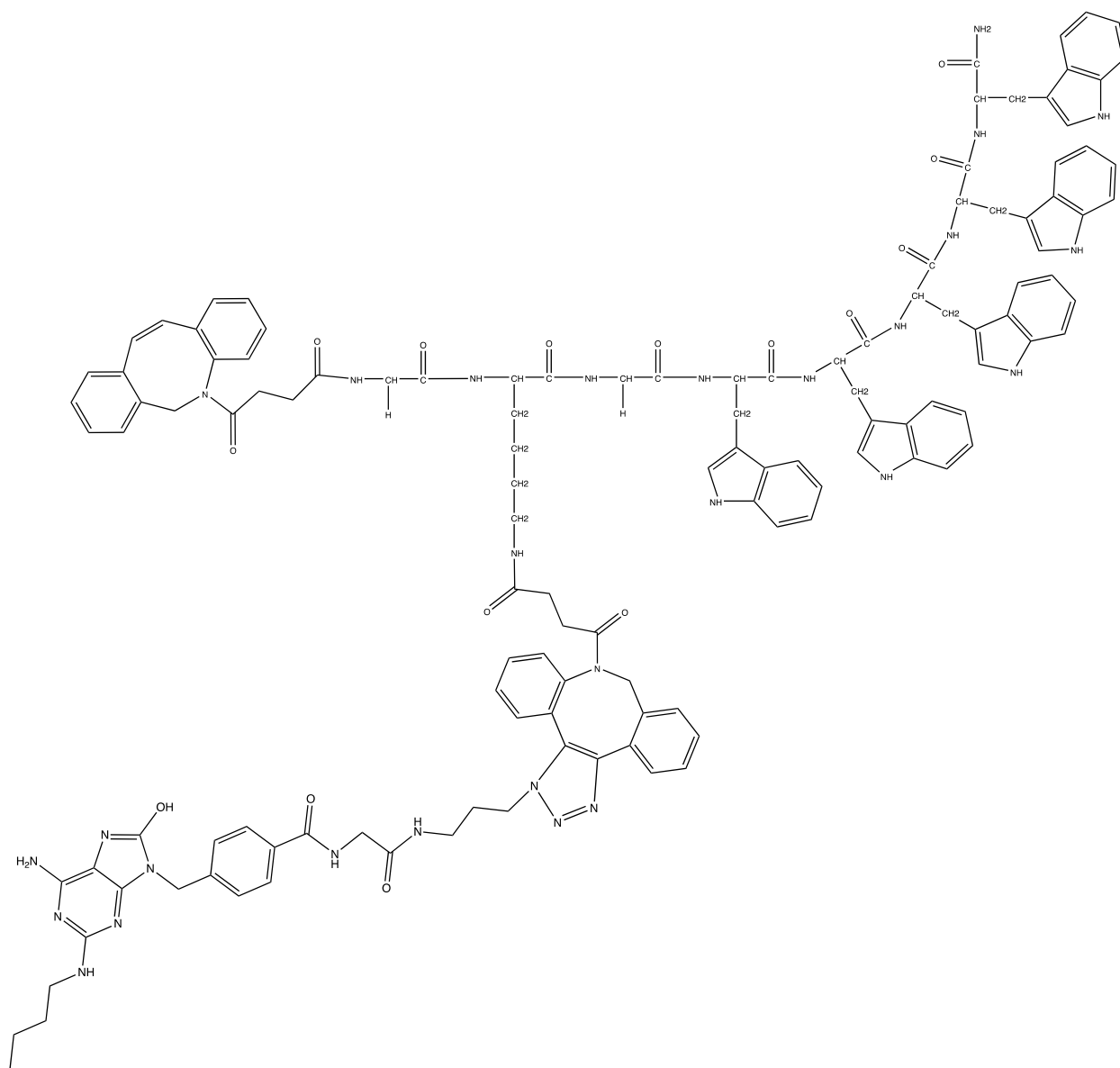
Compound 12, referred to as NH₂-GK(DBCO)GW₅ or NH₂-Gly-Lys(DBCO)-Gly-(Trp)₅-NH₂ was synthesized using Gly-Lys-Gly-(Trp)₅-NH₂ prepared by solid phase peptide synthesis and DBCO-NHS as the starting materials. 50 mg of Gly-Lys-Gly-(Trp)₅-NH₂ (0.04 mmol, 1 eq) was dissolved in 0.25 mL of dry DMSO. TEA (0.04 mmol, 1 eq) was added and the solution was stirred at room temperature for 5 minutes. 14.24 mg of DBCO-NHS (0.04 mmol, 1 eq) was added and the reaction mixture was stirred for 1 hour at room temperature. The reaction was quenched with amino-2-propanol (0.04 mmol, 1 eq) and 0.5 mL of 20% piperidine in DMF was added. The reaction mixture was stirred at room temperature for 30 minutes. The product was then precipitated from 50 mL of ether and centrifuged at 3000g at 4°C for 10 minutes. The product was collected as a solid pellet and then washed twice more with ether, followed by drying under vacuum. The product was purified on a preparatory HPLC system using a gradient of 25-45% acetonitrile/H₂O (0.05% TFA) over 10 minutes on an Agilent Prep C-18 column, 30x100mm, 5 μm. The product eluted at 9.4 minutes and the resulting fractions were combined, frozen and lyophilized to obtain a spectroscopically pure off-white solid. MS (ESI) calculated for C₈₄H₈₄N₁₆O₁₀ m/z = 1477.64 (found = 739.6 (m/2)⁺).

Compound 13



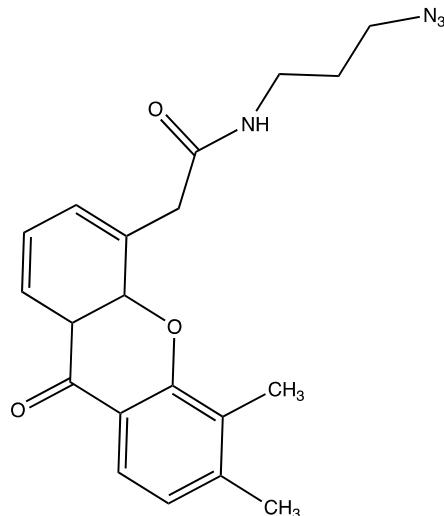
Compound 13, referred to as CL264-Azide, hydroxyadenine-Azide or TLR-7-azide, which is a TLR-7 agonist, was synthesized using CL264 (Invigogen, San Diego, CA, USA) and Azido-propyl-amine as starting materials. 5 mg of CL264 (0.012 mmol, 1 eq) and 6.1 mg of Azido-propyl-amine (0.061 mmol, 5 eq) were dissolved in dry DMF. TEA (0.073 mmol, 6 eq) was added and the solution was cooled to 0°C with an ice bath. 27.6 mg of HATU (0.073 mmol, 6 eq) was added and the reaction was stirred at 0°C for 1 hour. **Compound 29** was purified on a preparatory HPLC system using a gradient of 20-40% acetonitrile/H₂O (0.05% TFA) over 10 minutes on an Agilent Prep C-18 column, 30x100mm, 5 μm. The product eluted at 5 minutes and the resulting fractions were combined, frozen and lyophilized. MS (ESI) calculated for C₂₂H₂₉N₁₁O₃ m/z = 495.25 (found 496.3 (m+H)⁺).

Compound 14 (“Oligo-TLR-7a”)



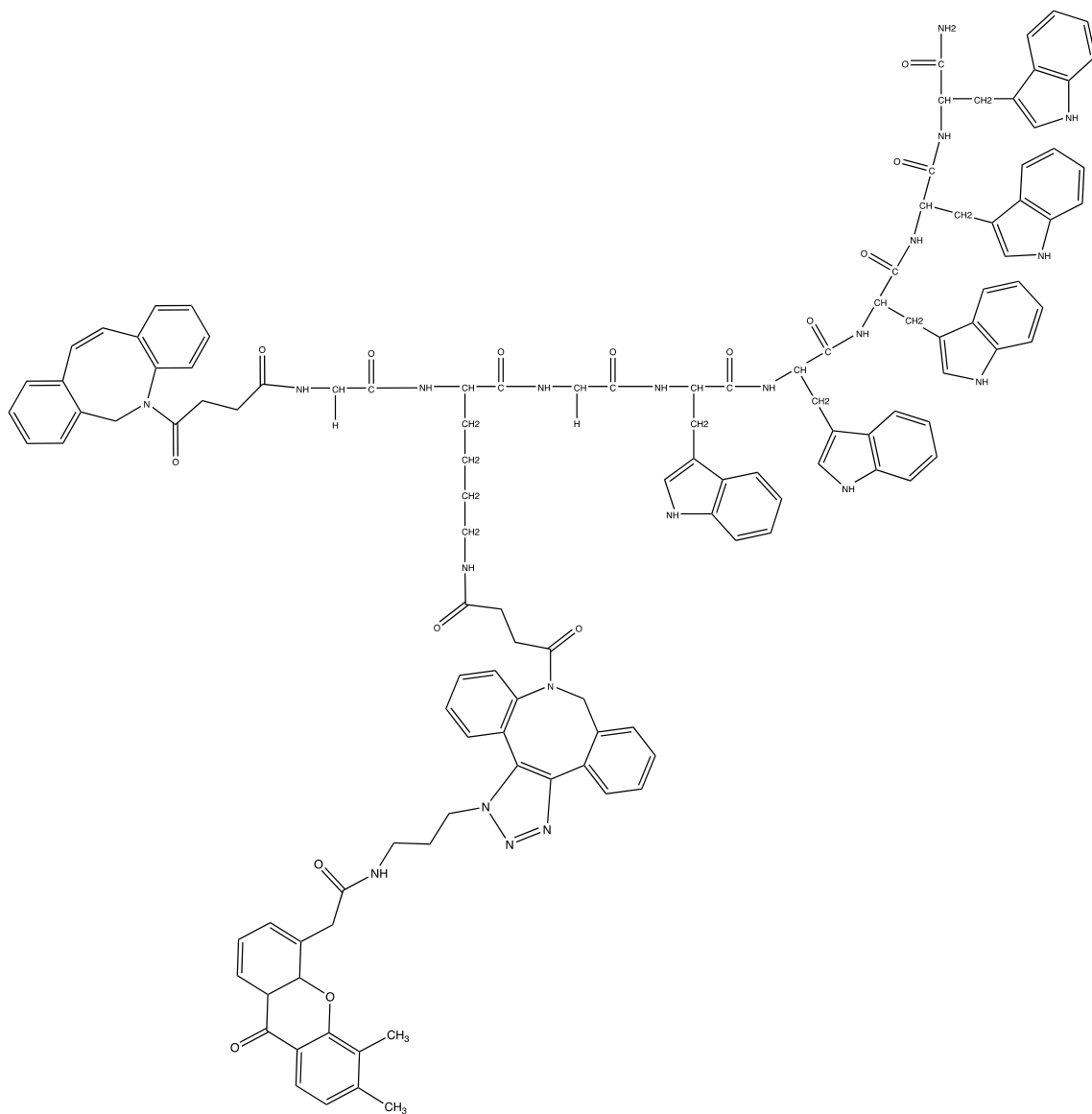
Compound 14, referred to as Oligo-TLR-7a was synthesized using **Compounds 12 and 13** as the starting materials. 5 mg of **Compound 12** (0.0034 mmol, 1 eq) was dissolved in 0.25 mL of dry DMSO and 1.68 mg of **Compound 13** (0.0034 mmol, 1 eq) was added and the reaction mixture was stirred for 2 hours, followed by the addition of 1.5 mg of DBCO-NHS (0.0037 mmol, 1.1 eq) and 0.5 eq of TEA. The reaction mixture was stirred overnight at room temperature and then the product was purified on a preparatory HPLC system using a gradient of 25-55% acetonitrile/H₂O (0.05% TFA) over 10 minutes on an Agilent Prep C-18 column, 30x100mm, 5 μ m. The product eluted at 9.4 minutes and the resulting fractions were combined, frozen and lyophilized to obtain a spectroscopically pure white solid. MS (ESI) calculated for C₁₂₅H₁₂₈N₂₈O₁₅ m/z = 2261.01 (found 1132.2 ($m/2$)⁺).

Compound 15



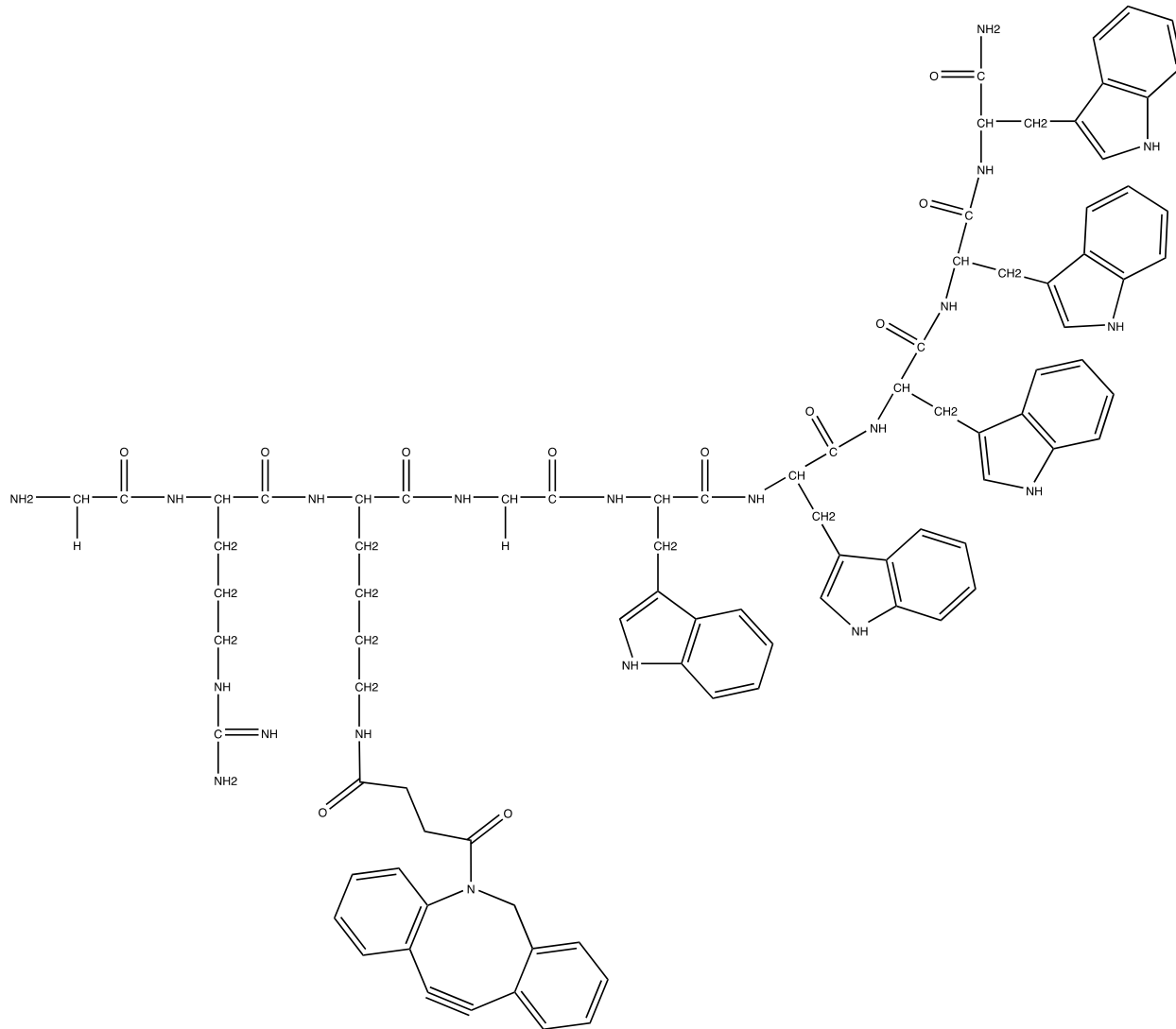
Compound 15, referred to as DMXAA-Azide, which is a STING agonist, was synthesized using the same procedure as **Compound 13**, except DMXAA (Invivogen) was used as the starting material. **Compound 15** was purified on a preparatory HPLC system using a gradient of 35-65% acetonitrile/H₂O (0.05% TFA) over 10 minutes on an Agilent Prep C-18 column, 30x100mm, 5 μm. The product eluted at 6 minutes and the resulting fractions were combined, frozen and lyophilized to obtain a spectroscopically pure white solid. MS (ESI) calculated for C₂₀H₂₂N₄O₃ m/z = 366.42 (found 365.2).

Compound 16 (“Oligo-STINGa”)



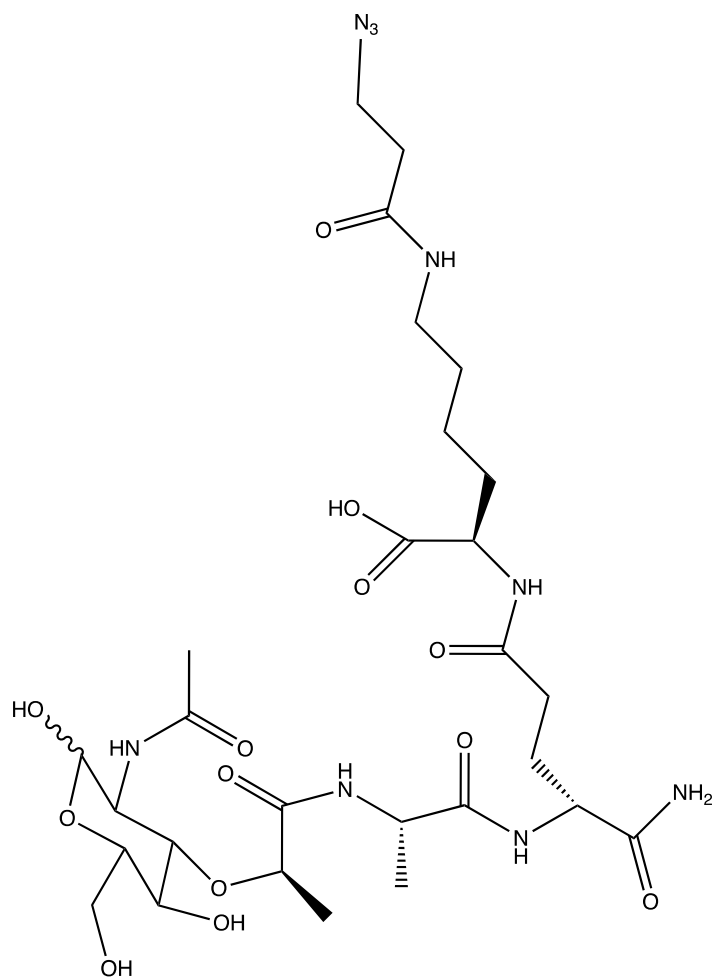
Compound 16, referred to as DBCO-GK(DMXAA)-G-W₅ or DBCO-Gly-Lys(DBCO-DMXAA)-Gly-(Trp)₅-NH₂ was synthesized using a similar procedure as that used to prepare **Compound 14**, except **Compound 15** was used as the agonist cargo. **Compound 16** was purified on a preparatory HPLC system using a gradient of 45-85% acetonitrile/H₂O (0.05% TFA) over 10 minutes on an Agilent Prep C-18 column, 30x100mm, 5 μm. MS (ESI) calculated for C₁₂₃H₁₂₁N₂₁O₁₅ $m/z = 2131.94$ (found 1067.6 ($m/2$)⁺).

Compound 17



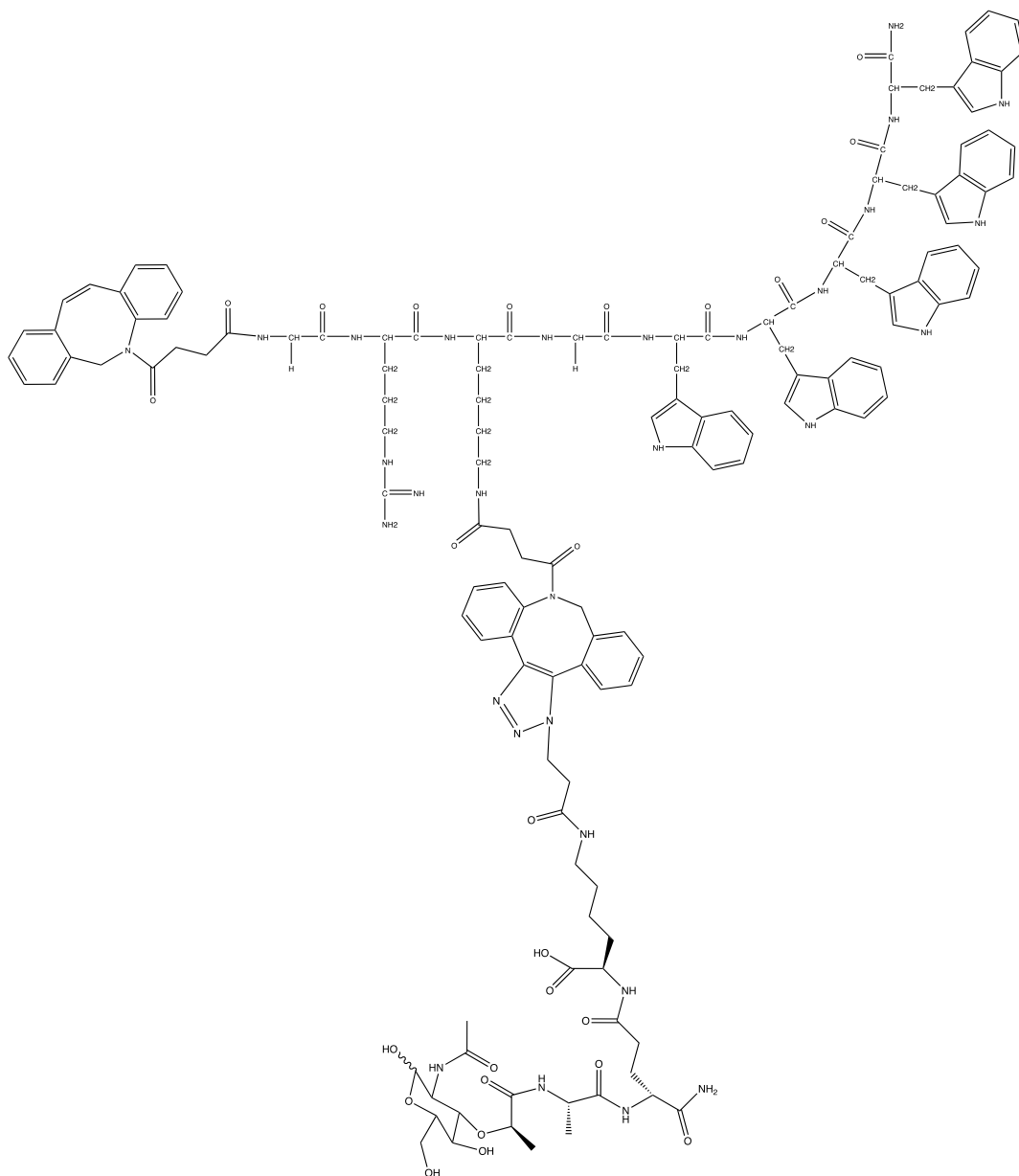
Compound 17, referred to as NH₂-GRK(DBCO)GW₅ or NH₂-Gly-Arg-Lys(DBCO)-Gly-(Trp)₅-NH₂ was synthesized using a similar procedure as that used to prepare **Compound 12**, except Gly-Arg-Lys-Gly-(Trp)₅-NH₂ was used as the starting material. **Compound 17** was purified on a preparatory HPLC system using a gradient of 25-55% acetonitrile/H₂O (0.05% TFA) over 10 minutes on an Agilent Prep C-18 column, 30x100mm, 5 μm. The product eluted at 7.5 minutes and the resulting fractions were combined, frozen and lyophilized. MS (ESI) calculated for C₉₀H₉₆N₂₀O₁₁ $m/z = 1633.74$ (found 817.5 (m/2)⁺).

Compound 18 (NOD2a)



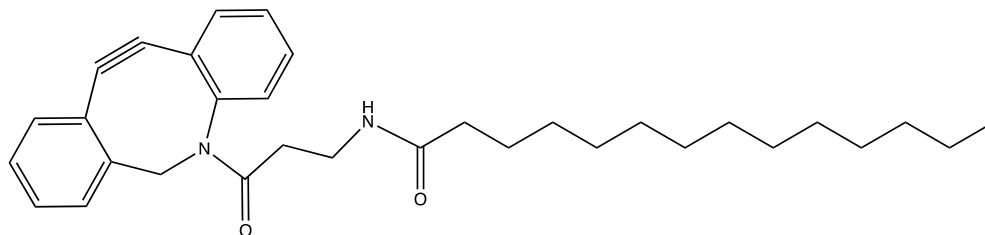
Compound 18, Muramyl Azide, which is a NOD2a, was synthesized using Muramyl tri-lysine (M-TriLys, Invivogen) and Azido propionic acid sulfo NHS as starting materials. 5 mg of Muramyl tri-lysine (0.008 mmol, 1 eq) was dissolved in 0.5 mL of dry DMSO with TEA (0.02 mmol, 2.5 eq) and 2.2 mg of Azido propionic acid sulfo NHS (0.008 mmol, 1 eq) was added. The reaction mixture was stirred at room temperature for 2 hours. The reaction mixture was quenched with amino-2-propanol (0.008 mmol, 1 eq) and the intermediate was used immediately for reaction.

Compound 19 (oligo-NOD2a)



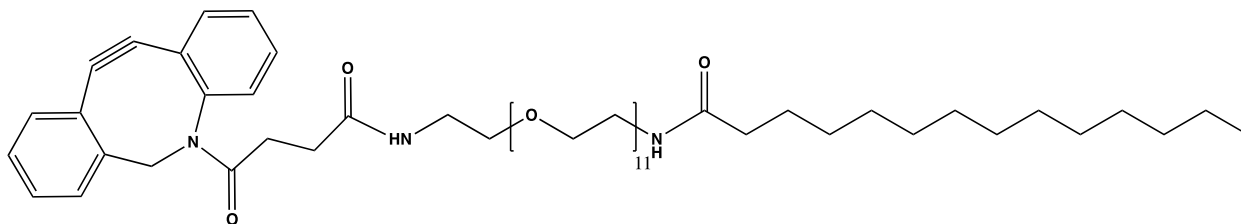
Compound 19, referred to as DBCO-GRK(Muramyl)GW₅, or Oligo-NOD2a was synthesized using the same procedure **Compound 14**, except **Compounds 17 and 18** were used as starting material. **Compound 19** was purified on a preparatory HPLC system using a gradient of 35-65% acetonitrile/H₂O (0.05% TFA) over 10 minutes on an Agilent Prep C-18 column, 30x100mm, 5 μm. The product eluted at 4.6 minutes and the resulting fractions were combined, frozen and lyophilized. MS (ESI) calculated for C₁₃₇H₁₅₈N₃₀O₂₆ m/z = 2639.2 (found 1319.5 ($m/2$)⁺).

Compound 20 (C14)



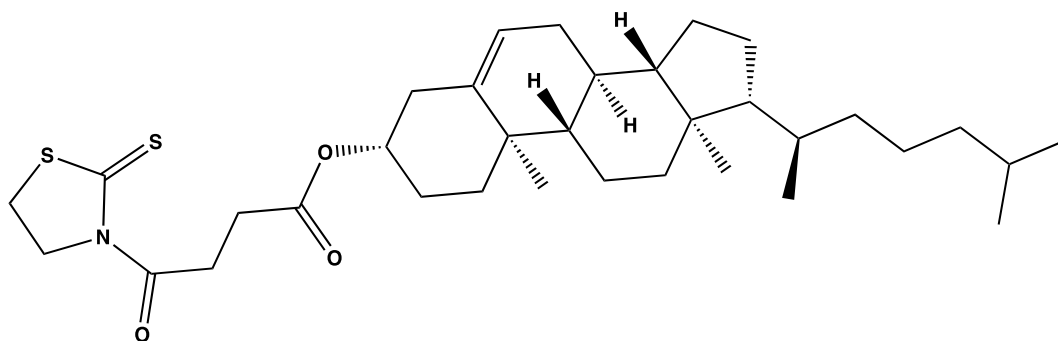
Compound 20, referred to as Myr-DBCO or C14 was prepared from Myristoyl Chloride and DBCO-Amine. 50 mg of DBCO-Amine (0.18 mmol, 1 eq) was dissolved in 0.5 mL of DCM. TEA (0.22 mmol, 1.2 eq) was added and the solution was stirred for 5 minutes at room temperature. Myristoyl Chloride (0.16 mmol, 0.9 eq) was added and the reaction mixture was stirred for 1 hour at room temperature. TLC (2.5% methanol in DCM) showed a new spot with R_f of 0.5 for Myr-DBCO. The reaction mixture was injected on a flash chromatography column and purified using a gradient of 0-3% methanol in DCM over 12 CVs. The fractions were collected and dried to provide 86 mg of Myr-DBCO in quantitative yield. MS (ESI) calculated for $C_{32}H_{42}N_2O_2$ $m/z = 486.32$ (found 487.3 ($m+H$)⁺).

Compound 21 (PEG-C14)



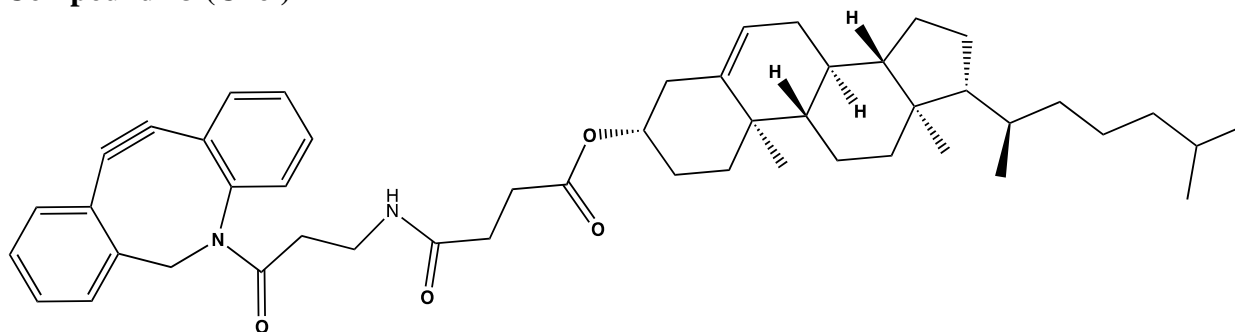
Compound 21, referred to as Myr-Peg₁₁-DBCO or C14-PEG was synthesized using Myristoyl Chloride, Boc-Peg₁₁-Amine and DBCO-NHS as starting materials. 116.06 mg Boc-Peg₁₁-Amine (0.18 mmol, 1 eq) was dissolved in 0.5 mL of DCM. TEA (0.22 mmol, 1.2 eq) was added and the reaction mixture was stirred for 5 minutes at room temperature. Myristoyl Chloride was added and the reaction was stirred at room temperature for 1 hour. The reaction mixture was diluted with DCM and washed twice with 1 M HCl and once with DI H₂O. The organic layer was dried with Na₂SO₄ and evaporated. The intermediate Myr-Peg₁₁-Boc was dissolved in 0.35 mL of DCM and 0.15 mL of TFA was added. The reaction mixture was stirred for 30 minutes at room temperature then dried by blowing with air and further dried under high vacuum. 122.5 mg of Myr-Peg₁₁-NH₂ as an oil (0.162 mmol, 1 eq) was dissolved in 0.5 mL of DCM. TEA (0.49 mmol, 3 eq) was added and the solution was stirred for 5 minutes at room temperature. 65.19 mg of DBCO-NHS (0.162 mmol, 1 eq) was added and the reaction mixture was stirred for 1 hour at room temperature. The reaction mixture was injected on a flash chromatography column and purified using a gradient of 0-15% methanol in DCM over 15 CVs. The fractions were collected and dried to provide Myr-Peg₁₁-DBCO, which was reacted with the charge modified peptide, KKKKKKVRGIPVHLELASMTNMELMSSIVHQVFPVSGVZX; wherein X = azido-lysine; MW = 4745.65 g/mol. Calculated conjugate MW = 5787.3 (found 827 ($m/7$)⁺).

Compound 22



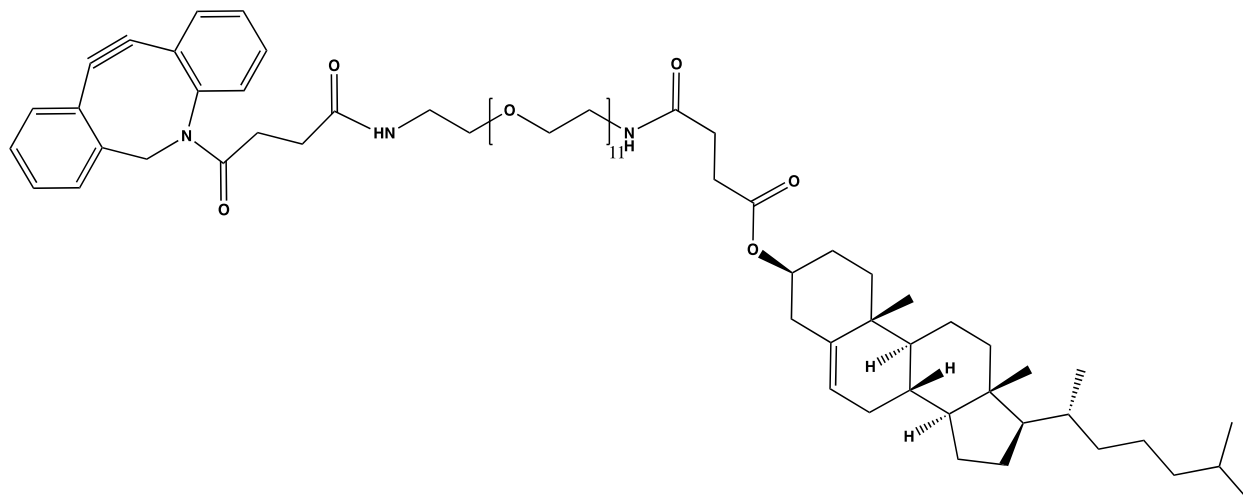
Compound 22, referred to as Chol-TT, was prepared using the same procedure as **Compound 10** except Cholesteryl hemisuccinate was used as the starting material. This procedure provided 120 mg of Chol-TT as a yellow solid in quantitative yield. **Compound 22** was used as an intermediate to prepare **Compound 23** and **Compound 24** without any further work-up.

Compound 23 (Chol)



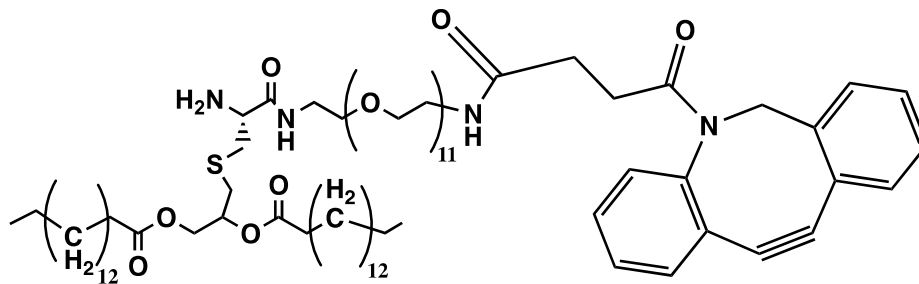
Compound 23, referred to as Chol-DBCO or Chol, was prepared from **Compound 22** and DBCO-Amine. 106.28 mg of **Compound 22** (0.18 mmol, 1 eq) and 50 mg of DBCO-Amine (0.18 mmol, 1 eq) were dissolved in 0.5 mL of DCM and stirred at room temperature. Over the course of an hour the bright yellow color of the solution faded and TLC in DCM indicated complete absence of **Compound 22**. The reaction mixture was injected on a flash chromatography column and purified using a gradient of 0-3% methanol in DCM over 12 CVs. The fractions were collected and dried to provide 139 mg of Chol-DBCO in quantitative yield. **Compound 23**, Chol-DBCO, was dissolved in DMSO and reacted with the charge modified peptide, KKKKKKVRGIPVHLELASMTNMELMSSIVHQVFP TGSGVZX; wherein X = azido-lysine; MW: 4745.65. Calculated conjugate MW = 5490.71 (found 1098.9 (m/5)⁺).

Compound 24 (PEG-Chol)



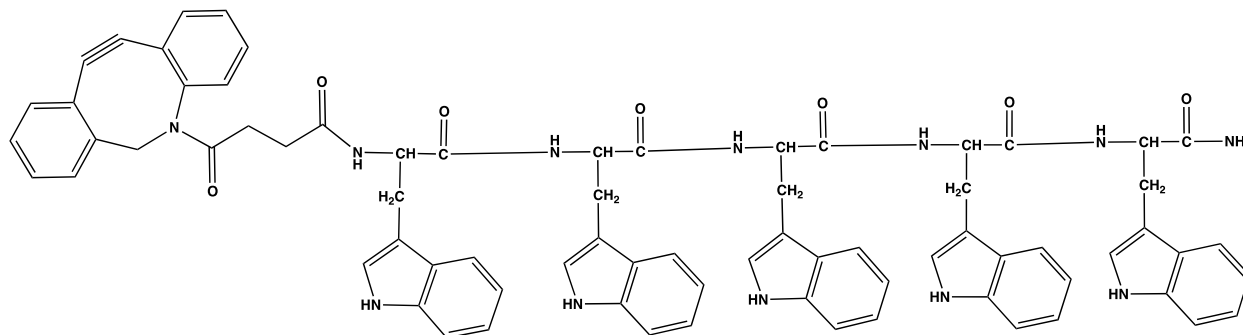
Compound 24, referred to as Chol-Peg₁₁-DBCO, was prepared from **Compound 22**, Boc-Peg₁₁-amine and DBCO-NHS. 50 mg of **Compound 22** (0.085 mmol, 1 eq) was dissolved in 0.5 mL of DCM. 60.38 mg of Boc-Peg₁₁-amine (0.09 mmol, 1.1 eq) was added and the reaction mixture was stirred at room temperature. Over the course of an hour the bright yellow color of the solution faded and TLC in DCM showed complete absence of **Compound 22**. To the reaction mixture was added 150 μ L of TFA to make a 30% solution in DCM. The reaction mixture was stirred for 30 minutes at room temperature then dried by blowing with air and further dried under high vacuum. 86.25 mg of Chol-Peg₁₁-NH₂ as an oil (0.085 mmol, 1 eq) was dissolved in 0.5 mL of DCM. TEA (0.26 mmol, 3 eq) was added and the solution was stirred for 5 minutes at room temperature. 34.26 mg of DBCO-NHS (0.085 mmol, 1 eq) was added and the reaction mixture was stirred for 1 hour at room temperature. The reaction mixture was injected on a flash chromatography column and purified using a gradient of 0-15% methanol in DCM over 15 CVs. The fractions were collected and dried to provide Chol-Peg₁₁-DBCO. **Compound 24**, Chol-Peg₁₁-DBCO was dissolved in DMSO and reacted with the charge modified peptide, KKKKKKVRGIPVHLELASMTNMELMSSIVHQVFP TGS GVZX; wherein X = azido-lysine; MW: 4745.65 g/mol. Calculated conjugate MW = 6052.5 (found 1210 (m/5)⁺).

Compound 25 (PEG-Lipid)



Compound 25, referred to as PEG-Lipid was synthesized using **Compound 10**, Boc-Peg₁₁-Amine, and DBCO NHS as starting materials. 60 mg of **Compound 10** (0.06 mmol, 1 eq) was dissolved in 0.6 mL of DCM. 38.9 mg of Boc-Peg₁₁-Amine (0.06 mmol, 1 eq) was added and the reaction mixture was stirred at room temperature for 30 minutes. The DCM was removed under vacuum and the oil was taken up in DMSO and added to DI H₂O. The water was centrifuged at 3000g for 5 minutes and the pellet was collected and dried under vacuum. The PEGylated intermediate was dissolved in 0.35 mL of DCM and 0.15 mL of TFA was added. The reaction mixture was stirred at room temperature for 30 minutes then blown dry with air and further dried under high vacuum. 71.76 mg of Lipid-Peg₁₁-NH₂ as an oil (0.05 mmol, 1 eq) was dissolved in 1 mL of DMSO and TEA (0.1 mmol, 2eq) was added followed by 20.3 mg of DBCO-NHS (0.05 mmol, 1 eq). The reaction mixture was stirred for 1 hour at room temperature. 0.5 mL of 20% piperidine in DMF was added and the reaction mixture was stirred for 30 minutes at room temperature. The reaction mixture was diluted with DCM and washed three times with pH 9.5 Sodium Bicarbonate. The organic layer was dried with Na₂SO₄ and evaporated. The solid was taken up in 0.5 mL of DCM and injected on a flash chromatography column and purified. The gradient used was 0-10% methanol in DCM over 30 CVs. This provided the product in 6.7% overall yield. **Compound 25**, PEG-Lipid was dissolved in DMSO and reacted with the charge modified peptide, KKKKKKVRGIPVHLELASMTNMELMSSIVHQVVFPTGSGVZX; wherein X = azido-lysine; MW 4745.65 g/mol. Calculated conjugate MW = 6230.73 (found 779.8 (m/8)⁺).

Compound 26, DBCO-WWWWW



Compound 26, referred to as DBCO-W₅, W₅ or DBCO-(Trp)₅ was synthesized by reacting 137.6 mg (0.15 mmol, 1 eq) of the precursor NH₂-(Trp)₅-NH₂ prepared by solid phase peptide synthesis with 146.1 mg of DBCO-NHS (0.057 mmol, 2.5 eq) and 14.7 mg of triethylamine (0.15 mmol, 1.1 eq) in 3.0 mL of DMSO. Compound 26 was purified by preparatory HPLC using a gradient of 52-72% acetonitrile/H₂O (0.05% TFA) over 12 minutes on an Agilent Prep-C18 column, 50x100 mm, 5 μm. The product eluted at ~ 10 minutes and the resulting fractions were collected, frozen and then lyophilized to obtain 75.1 mg (42% yield) of a spectroscopically pure (> 95% AUC at 254 nm) white powder. MS (ESI) calculated for C₇₄H₆₆N₁₂O₇ *m/z* = 1234.52 (found 1235.6 (M+H)⁺).

Synthesis of charge-modified conjugates covalently linked to Pam2Cys (TLR-2/6a)

CM conjugates linked to the TLR-2/6a, Pam2Cys, were produced using a similar method as for the preparation of CM conjugates of TLR-7/8a as described above, i.e. the hydrophobic block comprising the TLR-2/6a was linked to the peptide neoantigen at the C-terminus through an azide group using copper-free click chemistry. In short, the MC38-derived neoantigen, Cpne1 (DFTGSNGDPSSPYSLHYLSPTGVNEY) was prepared by solid phase peptide synthesis as charge-modified Cpne1, **KKKKKKKKKVRDFTGSNGDPSSPYSLHYLSPTGVNEYSPVZX**, where Z = citrulline and X = azido-lysine, and then reacted with the DBCO-containing TLR-2/6a, Compound 25. The reaction was monitored by HPLC and the resulting conjugate was characterized for identity and purity by UPLC-MS. Calculated MW: 6292.69; found 1049 (m/6)⁺.

Synthesis of charge-modified conjugates of CpG (TLR-9a) and CDN (STINGa)

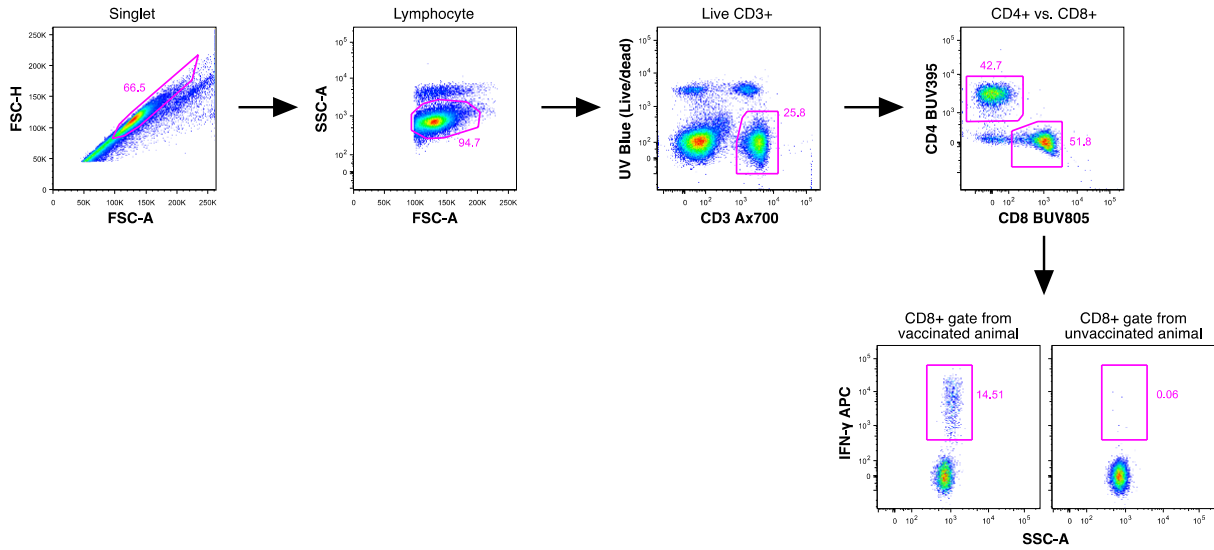
CpG- and CDN-based agonists of TLR-9 and STING, respectively, carry net charge at physiologic pH and were therefore placed at the N-terminus and used as the charge-modifying group of CM conjugates.

To enable site-selective attachment to the N-terminus of modified peptide antigens, azide-modified CpG- and CDN-based agonists were prepared. Briefly, azide-modified CpG ODN 1826 of formula **/5AzideN//iSp9/G*G*T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T** was custom synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA), wherein **/5AzideN//iSp9/** is an azide-terminated PEG3 spacer linked at the 5'-OH of the DNA sequence **G*G*T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T** with a phosphorothioate backbone. An azide-modified CDN based on a bisphosphorothioate analog of cyclic adenosine monophosphate (c-di-AMP) was prepared by Aduro Biotech (Berkeley, CA, USA).

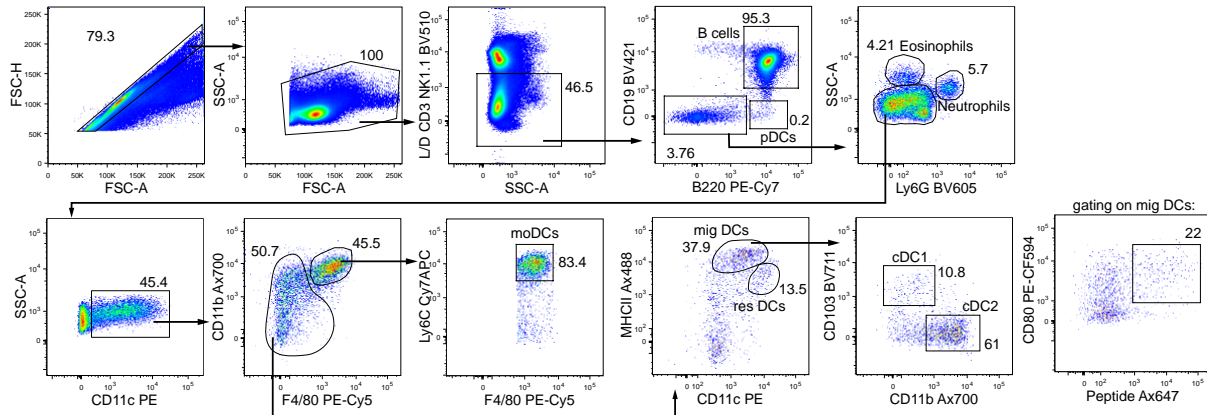
A similar procedure was used for the preparation of CM conjugates bearing CpG or CDN. For example, a hydrophobic oligopeptide, **Compound 26**, was linked at the C-terminus to a modified peptide neoantigen, Cpne1, **VRDFTGSNGDPSSPYSLHYLSPTGVNEYSPVZX**, to generate a conjugate, **VRDFTGSNGDPSSPYSLHYLSPTGVNEYSPVZX(DBCO-W5)** with a hydrophobic C-terminal oligopeptide. The resulting conjugate was then modified at the N-terminus with BCN-PEG4-NHS (BroadPharm, San Diego, CA, USA) to introduce an azide-reactive strained-alkyne. The BCN modified conjugate was purified by preparatory HPLC to yield spectroscopically pure (> 95% at 254 nm) product (MW = 5593.75). BCN-modified peptide was then reacted with azide-modified CpG in 50% (v/v) DMSO/PBS. Reactions were determined to be complete following disappearance of starting material and identity of CM conjugates was characterized by matrix assisted laser desorption ionization (MALDI-TOF) mass spectrometry.

Flow cytometry gating trees:

Representative gating tree for T cell analyses



Representative gating tree for DC analyses



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