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Supporting Information

Immune and Anticancer Responses Elicited by Fully Synthetic Aberrantly Glycosylated MUC1 Tripartite Vaccines Modified by a TLR2 or TLR9 Agonist

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Contents:

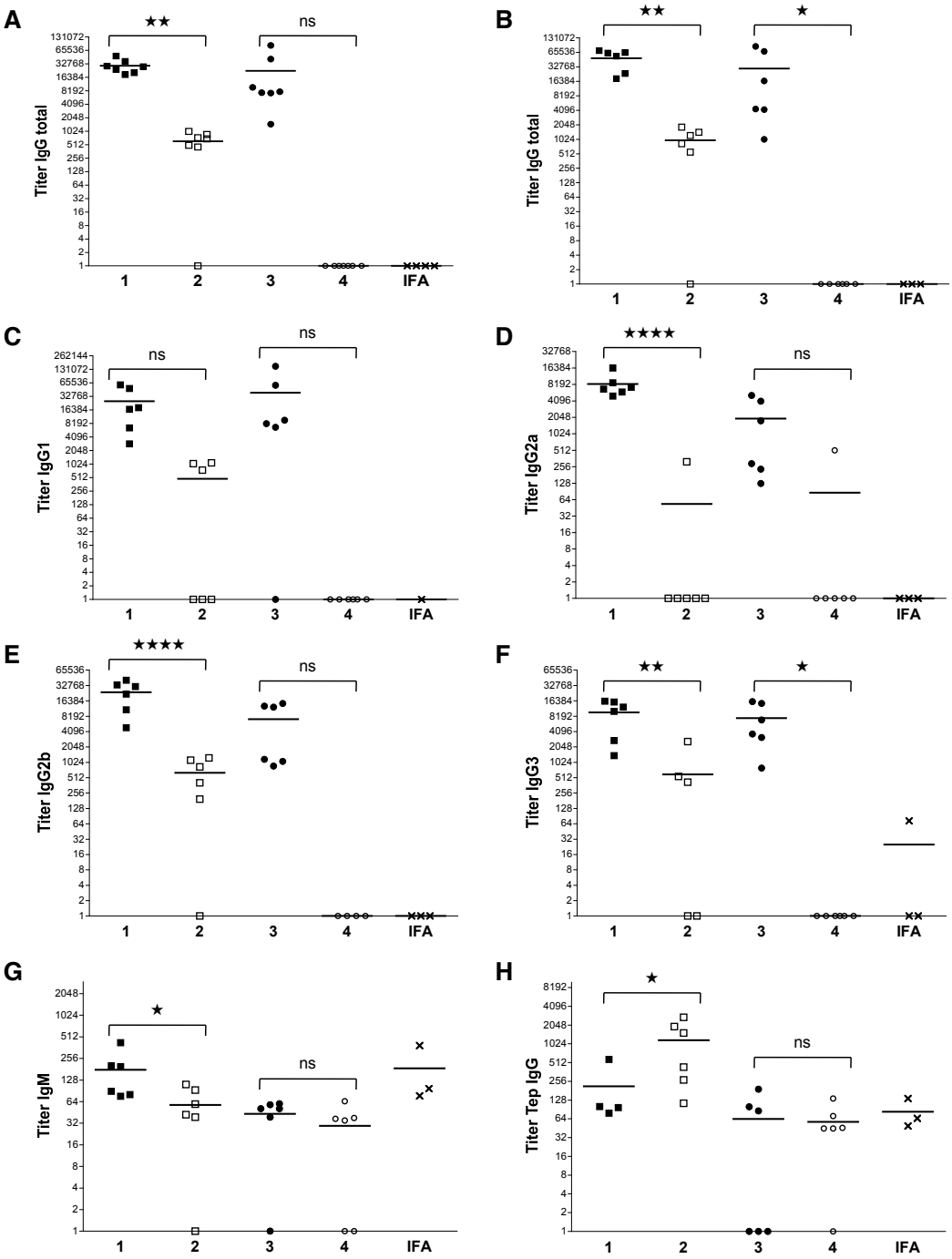


Figure S1. ELISA anti-MUC1 and anti-T_{helper} antibody titers after (A) 3 or (B-H) 4 immunizations with IFA, **1** and **2** in liposomes, or **3** and **4** in the presence of IFA as indicated. ELISA plates were coated with (A-G) BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate or (H) NeutrAvidin-biotin-T_{helper} and titers were determined by linear regression analysis, plotting dilution *vs.* absorbance. Titers were defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera. Each data point represents the titer for an individual mouse and the horizontal lines indicate the mean for the group of mice. Asterisks indicate statistically significant difference (★★★★ $P<0.0001$, ★★★ $P<0.001$, ★★ $P<0.01$, ★ $P<0.05$) and ns indicates no significant difference.

Methods

Reagents and general analytical procedures: Amino acid derivatives and resins were purchased from NovaBioChem and Applied Biosystems. *N*,*N*-dimethylformamide (DMF) was obtained from EM Science, *N*-methylpyrrolidone (NMP) was from Applied Biosystems. The 3′-disulfide functionalized ODN (**5)** was purchased from the Midland Certified Reagent Co. All other chemical reagents were purchased from Aldrich, Acros, Alfa Aesar, and Fischer and used without further purification. All solvents employed were reagent grade. Reversed phase high performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 series system equipped with an autosampler, UV-detector, and fraction-collector. Analytical samples were run using a Zorbax Eclipse C18 analytical column (5 μm, 4.6 x 150 mm) at a flow rate of 0.5 mL/min while purification was achieved using a semi-preparative C18 column (5 μm, 25 x 250 mm) at a flow rate of 2 mL/min. Elution was performed using linear gradients of 0-30% solvent B for 15 minute followed by 5 min gradient of 30-100% solvent B. Two methods were used to run samples. In method 1, samples were eluted using 0.1% ammonium acetate buffered solution (pH=7.4) as solvent A and 90% acetonitrile in 0.1% ammonium acetate buffered solution as solvent B. In method 2, samples were eluted using acidic solvents where solvent A was 0.1% trifluouroacetic acid in water and solvent B was 90% acetonitrile in 0.1% trifluouroacetic acid in water. Method 1 was applied to analyze and purify compounds **3**-**6** (CpG and CpG conjugated compounds). In contrast bromo-acetylated (glyco)peptides **7**-**8** were found difficult to elute using the ammonium acetate buffered system and therefore were purified using method 2. UV detection of eluted peaks was at 260 nm (for CpG and CpG-conjugated compounds) and at 214 nm for bromo-acetylated (glyco)peptides. MALDI-ToF mass spectra were recorded on an ABI 4700 or ABI 4800 proteomic analyzer using linear positive acquisition mode. In this work, we found that 2',4',6'-trihydroxyacetophenone (THAP) is a good matrix for analysis of the conjugates of oligonucleotides with (glyco)peptides. MALDI matrix solution consists of 50 mg of THAP and 25 mg ammonium citrate in 50% acetonitrile in water.

General methods for solid-phase peptide synthesis (SPPS): Bromoacylated peptides (**7** and **8**) were assembled on Rink amide MBHA resin (0.1 mmol) by microwave-assisted solid-phase peptide synthesis (SPPS) using N^{α}-Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-

1,1,3,3-tetramethyl hexafluorophosphate (HBTU)/*N*-hydroxybenzotriazole (HOBt) as the activating reagents. SPPS was carried out at 40 watt and 75 ^{o}C using a SPS mode CEM Discovery reactor (CME Corporation, Matthews, NC, USA). Single coupling steps were performed with conditional capping. The following protected amino acids were employed: N^{α}-Fmoc-Arg(Pbf)-OH, N^{α}-Fmoc-Asp(OtBu)-OH, N^{α}-Fmoc-Lys(Boc)-OH, N^{α}-Fmoc-Ser(tBu)-OH, N^{α}-Fmoc-Thr(tBu)-OH, N^{α}-Fmoc-Tyr(tBu)-OH. N^{α}-Fmoc groups were cleaved by treatment with 20% piperidine/DMF (1×2 min) then (1×5 min) utilizing the microwave radiation. The coupling of glycosylated amino acid N^{α}-Fmoc-Thr-(AcO_3)-α-D-GalNAc[1] was carried out manually using *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyl-uronium hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt) as coupling reagents. Progress of the manual couplings was monitored by standard Kaiser test. *N*-bromoacetylation was performed using bromoacetic acid *N*-hydroxysuccinimide ester in DMF (5.0 eq). After completion of the glycopeptide synthesis, the resin was thoroughly washed with DMF (6 mL), DCM (6 mL), and methanol (MeOH; 6 mL) and dried *in vacuo* to a constant weight. Cleavage of the peptide from the resin was achieved by stirring the resin with a cleavage cocktail composed of trifluoracetic acid (TFA) (4.25 mL), triisopropylsilane (TIS) (0.25 mL), and water (0.25 mL) for 2.5 h. The resin was washed with TFA (4×2 mL) and the combined solutions were concentrated *in vacuo*. The crude product was precipitated with ice-cold diethyl ether and filtered. The crude precipitate was then dissolved in acetonitrile/water/1% TFA and lyophilized prior to purification by HPLC on a C18 column using a gradient of solvents A and B (see above). The fractions were analyzed by MALDI-ToF and analytical RP-HPLC and, when appropriate, combined to give pure product.

Compound 8: Peptide assembly was performed on Rink amide AM resin (0.1 mmol) according to the general method for SPPS. Yield: 44%; HPLC: *t*R = 18.288 (method 1, C8 column), *t*R = 3.610 min (method 2, C18 column). MALDI-ToF MS: observed 1847 Da, 1867 Da, 1880 Da, 1886 Da,1902 Da, 1918 Da; calculated $[M+H]^{+1}$ ($C_{85}H_{130}BrN_{20}O_{21}^{+1}$, 1846 Da), $[M+Na]^{+1}$ ($C_{85}H_{129}BrN_{20}NaO_{21}^{+1}$, 1868 Da), $[M+K]^{+1}$ ($C_{85}H_{129}BrKN_{20}O_{21}^{+1}$, 1884 Da), $[M+2H_2O+H]^{+1}$ ($C_{85}H_{134}BrN_{20}O_{23}^{+1}$, 1882 Da), $[M+2H_2O+Na]^{+1}$ ($C_{85}H_{133}BrN_{20}NaO_{23}^{+1}$, 1904 Da), $[M+2H_2O+K]^{+1}$ ($C_{85}H_{133}BrKN_{20}O_{23}^{+1}$, 1920 Da).

Compound 7: The first four amino acids were coupled to the Rink amide AM resin (0.1 mmol) using the previously described SPPS protocols followed by a double manual coupling of N^{α}-Fmoc-Thr-(AcO_3)-α-D-GalNAc)[1] (0.4 mmol, 268 mg) using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyl-uronium hexafluorophosphate (HATU; 0.4 mmol, 152 mg), 1-hydroxy-7-azabenzotriazole (HOAt; 0.4 mmol, 55 mg), and diisopropylethylamine (DIPEA; 0.4 mmol, 70 μL) in DMF for 6 h. The resulting glycopeptide was further elongated using the standard SPPS protocol. The acetyl ester of the glycoside residue were cleaved while on the solid support using a solution of hydrazine/MeOH (4:1, v:v) for 6 h followed by *N*-bromoacetylation and cleavage from the resin using the standard procedure to afford compound **7**. Yield: 33%; HPLC: *t*R = 3.149 (method 2, C18 column). MALDI-ToF MS: observed 3136 Da; calculated $[M+K]^{+1}$ ($C_{137}H_{212}BrKN_{35}O_{42}{}^{+1}$, 3137 Da).

Preparation of free thiol-functionalized ODNs 6: The 3′-disulfide functionalized ODN **5** (5.45 mg, 0.823 μM) and the reducing reagent Reductaryl (37.4 mg, 2.47 μM) were mixed under Argon in 0.1 M sodium phosphate, 1.0 mM EDTA, pH 8.0 buffer (5 mL) at room temperature. The mixture was vortexed and the reaction progress monitored by MALDI/ToF MS . The reaction was completed after 10 h. The resin was removed by centrifugation for 5 min at 15,000 rpm at 5 ºC. Analytical RP-HPLC samples indicate α quantitative reduction. Thiol **6** was immediately used for preparation of compounds **3** and **4**. Yield: 96%; HPLC: *t*R = 3.060 min (method 1, C8 column). MALDI-ToF MS: observed 6510 Da, 6531 Da, 6551 Da; calculated $[M-H_2O+H]^{+1}$ ($C_{197}H_{254}N_{67}O_{104}P_{20}S_{21}{}^{+1}$, 6513 Da), $[M+H]^{+1}$ ($C_{197}H_{256}N_{67}O_{105}P_{20}S_{21}{}^{+1}$, 6531 Da), $[M+Na]^{+1}$($C_{197}H_{255}N_{67}NaO_{105}P_{20}S_{21}{}^{+1}$, 6553 Da).

Preparation of CpG ODN conjugates 3 and 4: Bromoacetylated (glyco)peptides (**8**, 619 μg or **7**, 369 μg, 2.0 eq) were added to a solution of 3′-thiol functionalized ODN (**6**, 653 μg, 0.1 μmol) dissolved in acetonitrile (0.5 mL) and 0.1 M sodium phosphate, 1.0 mM EDTA, pH 8.0 (2.0 mL) buffer under an atmosphere of Argon. The reaction mixture was incubated at 37 ^{o}C and the progress of the reaction monitored with RP-HPLC, MALDI-MS, and standard Ellman Test[2]. After 48 h, the reaction mixture was desalted using a PD-10 desalting column and lyophilized. Further purification was achieved by RP-HPLC on a semi-preparative C18 column using linear gradient of 0-30% of acetonitrile in 0.1% ammonium acetate buffered solution. Fractions

containing pure product were combined and lyophilized (3 times) to give a white fluffy solid. The purity of the compound was confirmed by RP-HPLC using an analytical C18 column. The product was reconstituted in distilled water and its concentration was determined by measuring the UV absorbance at 260 nm.

Compound 3: Yield: 42%; HPLC: *t*R = 5.478 min (method 1, C18 column). MALDI-ToF MS: observed 3139 Da, 3158 Da, 3180 Da, 3202 Da; calculated $[M\text{-}A+3H]^{+3}$ ($C_{329}H_{465}N_{97}O_{147}P_{20}S_{21}^{3+}$, 3139 Da), $[M\text{-}T\text{-}H_2O+3Na]^{+3}$ ($C_{329}H_{459}N_{100}Na_3O_{144}P_{20}S_{21}^{3+}$, 3158 Da), $[M\text{-}C+Na+2K]^{+3}$ ($C_{330}H_{462}N_{99}NaK_2O_{146}P_{20}S_{21}^{3+}$, 3180 Da), $[M\text{-}H_2O+H+2K]^{+3}$ ($C_{334}H_{465}K_2N_{102}O_{146}P_{20}S_{21}^{3+}$, 3203 Da).
A=Adenine; C=Cytosine; T=Thymine

Compound 4: Yield: 36%; HPLC: *t*R = 5.515 min (method 1, C18 column). MALDI-ToF MS: observed 1660 Da, 1682 Da; calculated $[M+5H]^{+5}$ ($C_{282}H_{388}N_{87}O_{126}P_{20}S_{21}^{5+}$, 1660 Da), $[M+5Na]^{+5}$ ($C_{282}H_{383}N_{87}Na_5O_{126}P_{20}S_{21}^{5+}$, 1682 Da).

UV Spectroscopy: UV spectroscopy scan (220-300 nm) and determination of A260/280 ration are routinely used to differentiate between peptides/proteins and nucleic acids irrespective of their content of amino acid or nucleic bases. UV spectroscopy of the vaccine candidate (Figure S2) indicated that it combines characteristics of both peptides and nucleic acids. A_{260}/A_{280} of compound **3** was 1.48, for the 3′-disulfide functionalized ODN **5** A_{260}/A_{280} = 1.65 and for glycopeptide **8** A_{260}/A_{280} = 0.81.

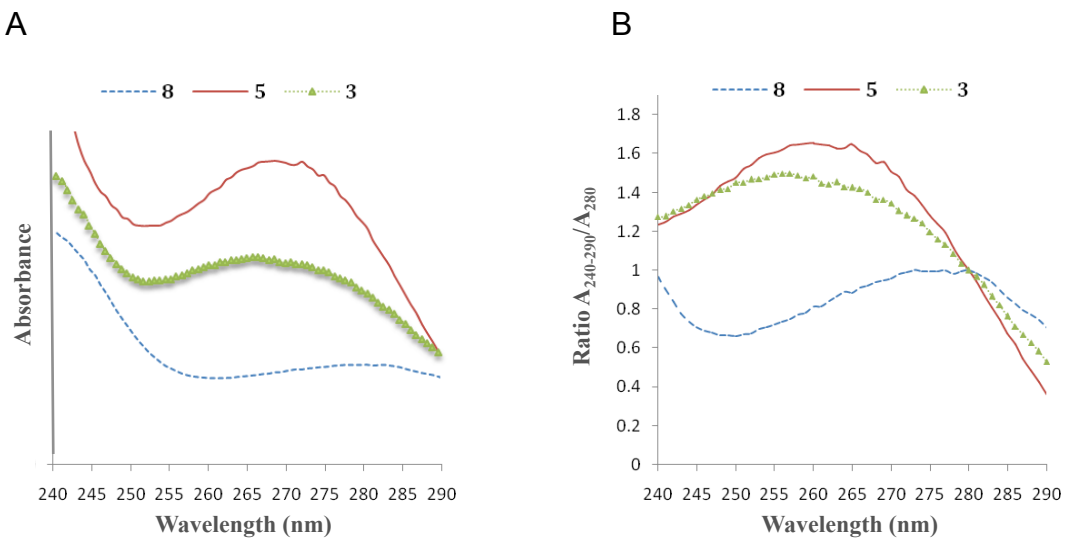


Figure S2. (A) Absorbance profiles and (B) A_{280} ratios of CpG-conjugated vaccine (**3**), CpG (**5**), and glycopeptide alone (**8**). Absorbance measurements were performed at wavelengths from 240 to 290 nm. Absorbances of aqueous samples were determined in 1 nm increments using Beckman Coulter DU 800 UV/Vis Spectrophotometer. For each sample the A_{280} ratios were calculated by dividing the absorbance determined from 240 to 290 nm by the absorbance at 280 nm. A_{260}/A_{280} ratios of compounds **3**, **5**, and **8** were 1.48, 1.65, and 0.81, respectively.

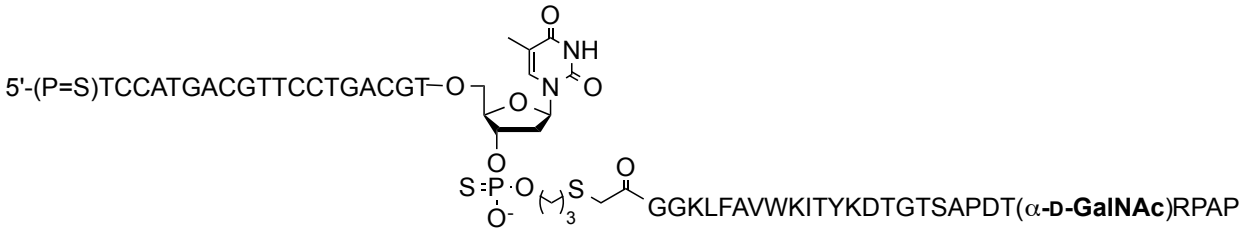
Liposome preparation for immunizations with 1 and 2: Each glycolipopeptide was incorporated into phospholipid-based small unilamellar vesicles (SUVs) by hydration of a thin film of the synthetic compounds, egg phosphatidylcholine, phosphatidylglycerol, and cholesterol in a HEPES buffer (10 mM, pH 7.4) containing NaCl (145 mM) followed by extrusion through a 0.1 μm Nucleopore® polycarbonate membrane.

References

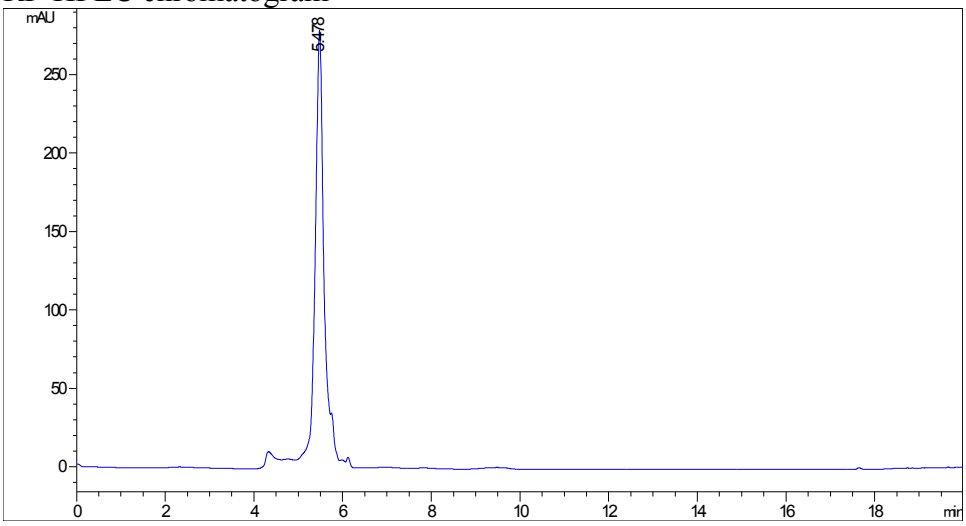
[1] D. Cato, T. Buskas, G. J. Boons. *J. Carbohydr. Chem.* **2005**, *24*, 503-516.
[2] P. W. Riddles, R. L. Blakeley, B. Zerner. *Methods Enzymol.* **1983**, *91*, 49-60.

Analytical Data

Compound 3



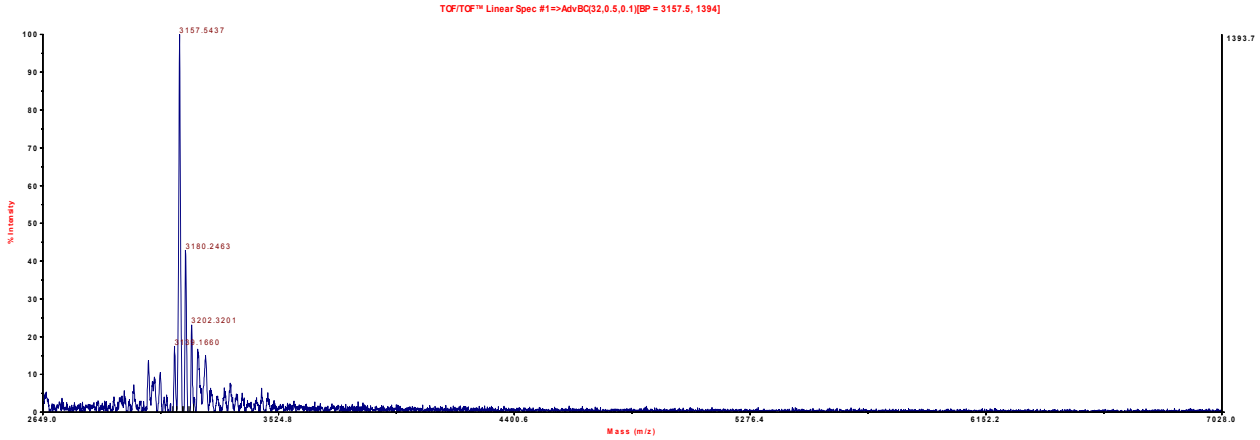
RP-HPLC chromatogram



Column
Zorbax Eclipse C18 analytical RP-HPLC column

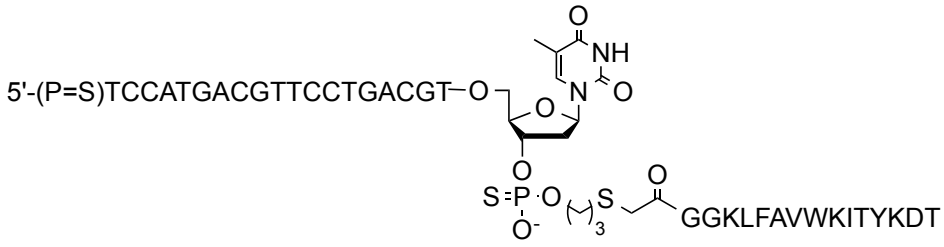
Eluent
0-30% of 0.1% ammonium acetate solution (pH=7.4) as solvent A in 90% acetonitrile/0.1% ammonium acetate solution as solvent B

MALDI-ToF MS spectra

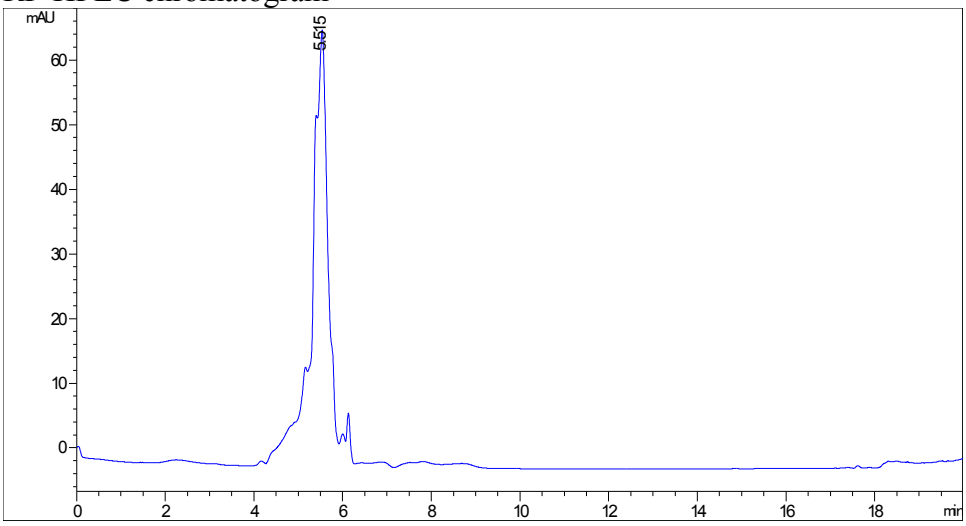


Observed 3139 Da, 3158 Da, 3180 Da, 3202 Da
Calculated $[M\text{-}A+3H]^{+3}$ (3139 Da), $[M\text{-}T\text{-}H_2O+3Na]^{+3}$ (3158 Da), $[M\text{-}C+Na+2K]^{+3}$ (3180 Da), $[M\text{-}H_2O+H+2K]^{+3}$ (3203 Da)

Compound 4



RP-HPLC chromatogram



Column
Zorbax Eclipse C18 analytical RP-HPLC column

Eluent
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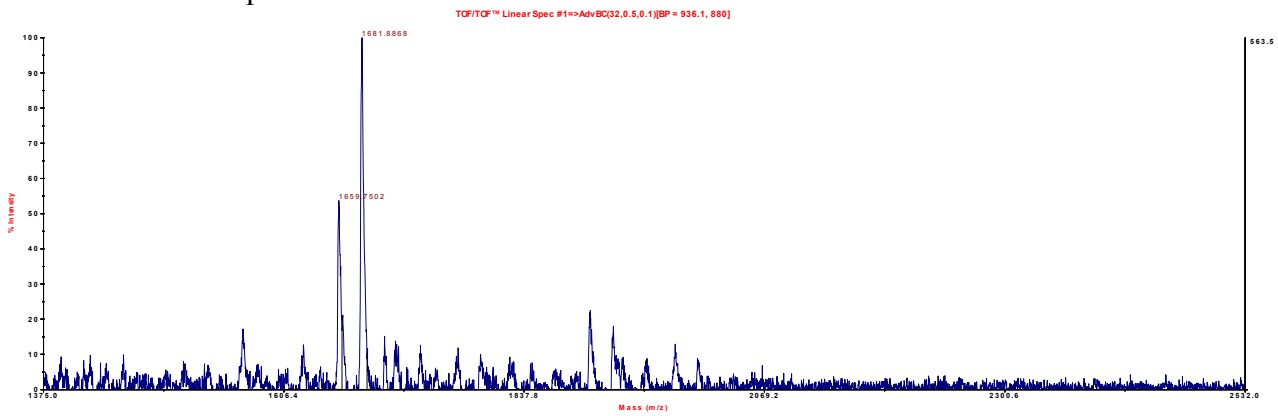
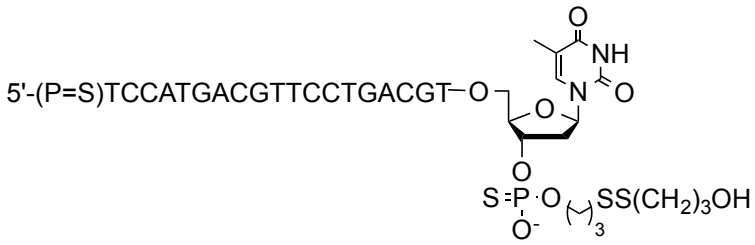
MALDI-ToF MS spectra

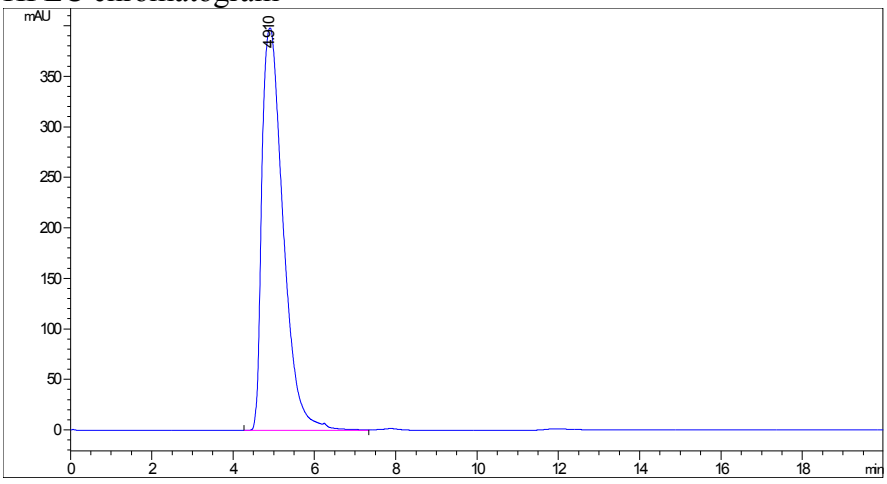


Observed 1660 Da, 1682 Da
Calculated $[M+5H]^{+5}$ (1660 Da), $[M+5Na]^{+5}$ (1682 Da)

Compound 5



HPLC chromatogram



Column
Zorbax Eclipse C18 analytical RP-HPLC column

Eluent
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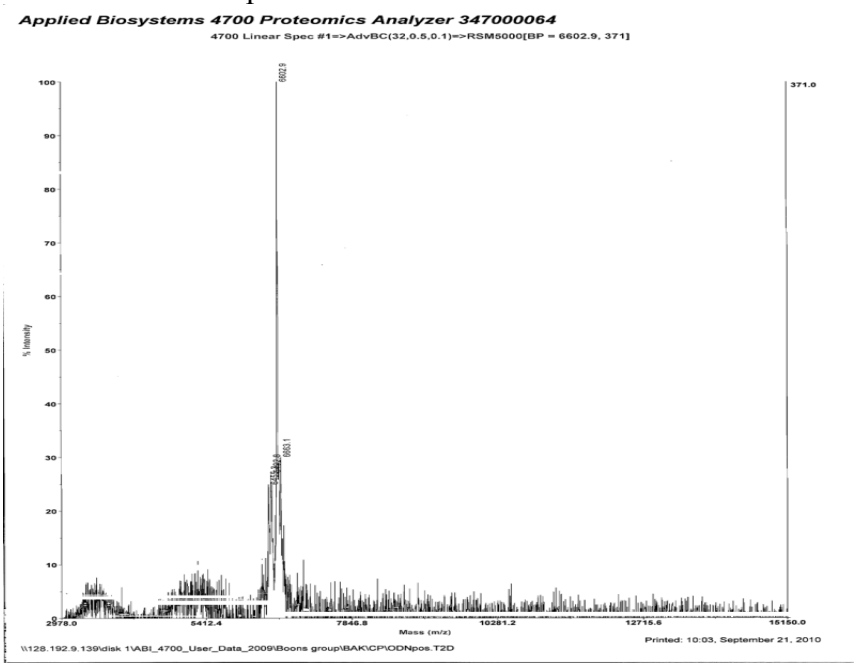
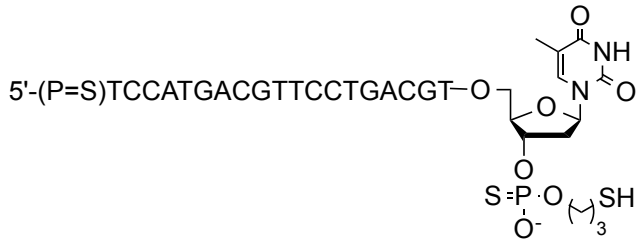
MALDI-ToF MS spectra

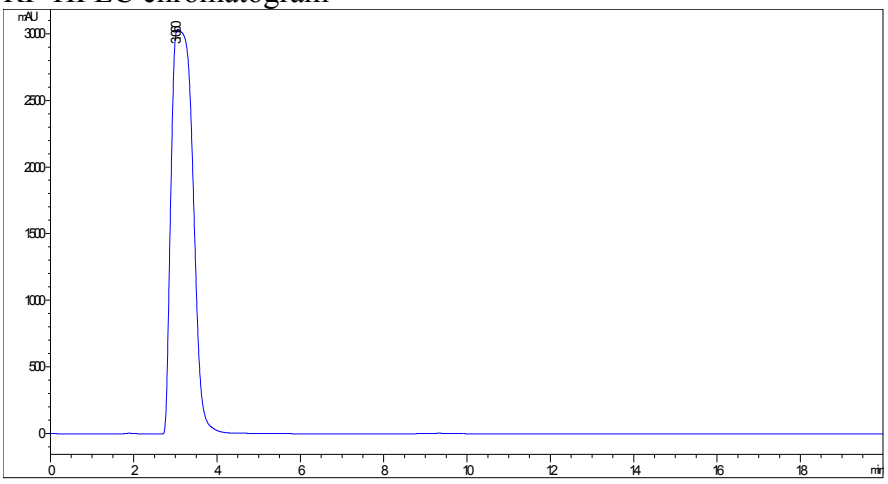


Observed 6603 Da
Calculated $[M\text{-}H_2O+H]^{+1}$ (6603 Da)

Compound 6



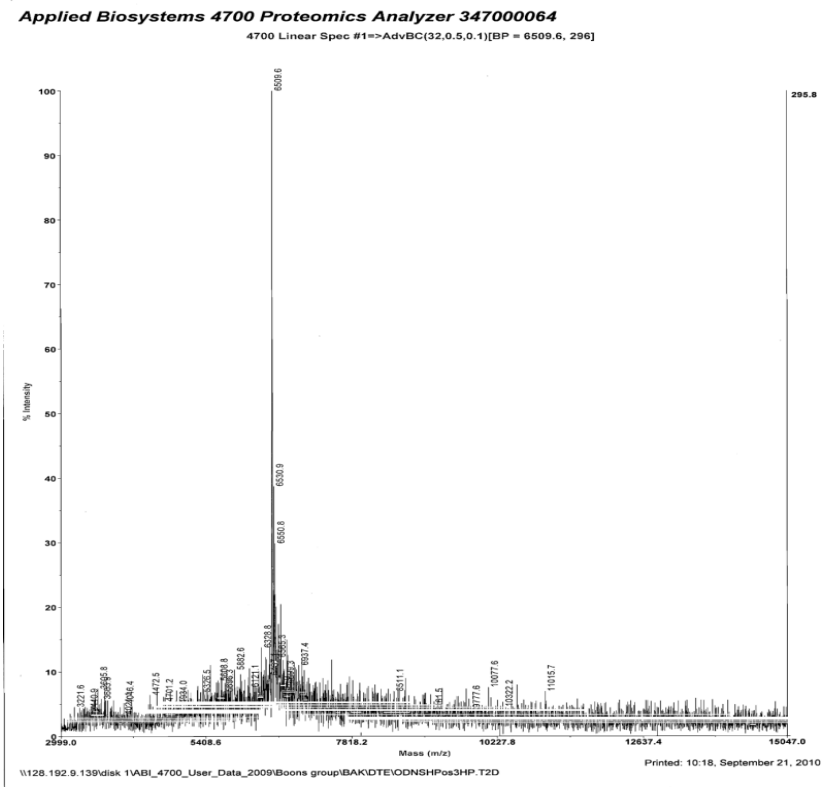
RP-HPLC chromatogram



Column
Zorbax Eclipse C18 analytical RP-HPLC column

Eluent
0-30% of 0.1% ammonium acetate solution (pH=7.4) as solvent A in 90% acetonitrile/0.1% ammonium acetate solution as solvent B

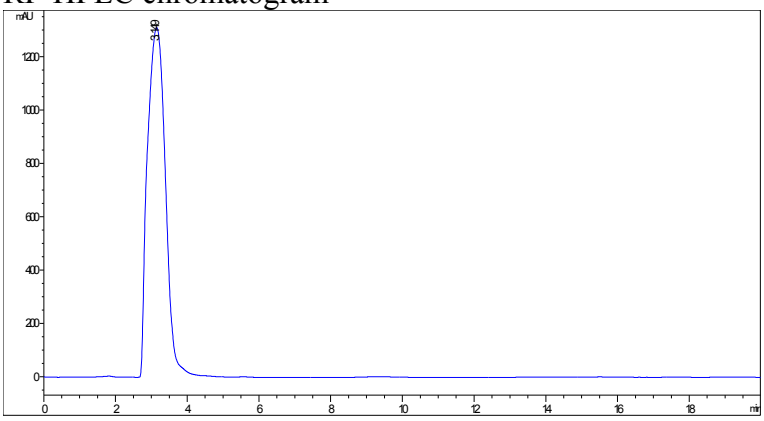
MALDI-ToF MS spectra



Observed 6510 Da, 6531 Da, 6551 Da
Calculated $[M\text{-}H_2O+H]^{+1}$ (6513 Da), $[M+H]^{+1}$ (6531 Da), $[M+Na]^{+1}$ (6553 Da)

Compound 7

RP-HPLC chromatogram



Column
Zorbax Eclipse C18 analytical RP-HPLC column

Eluent
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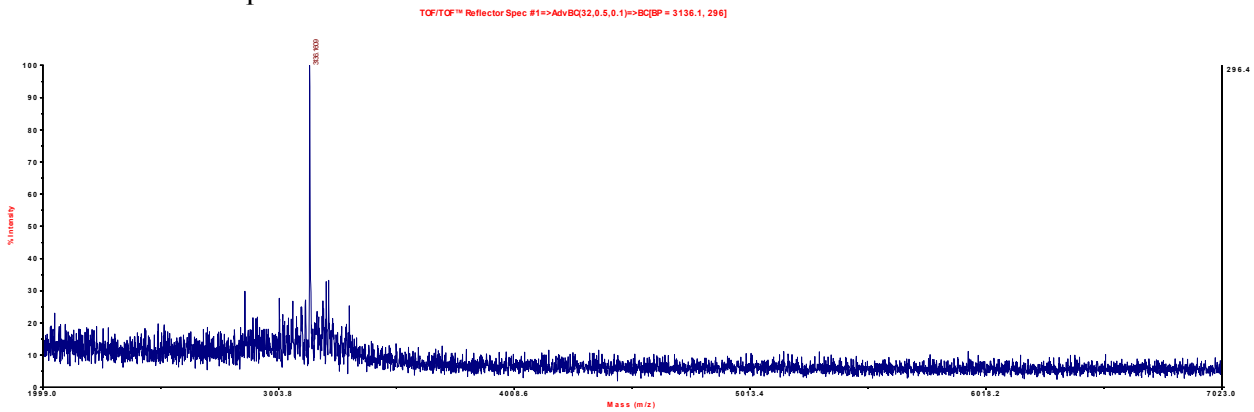
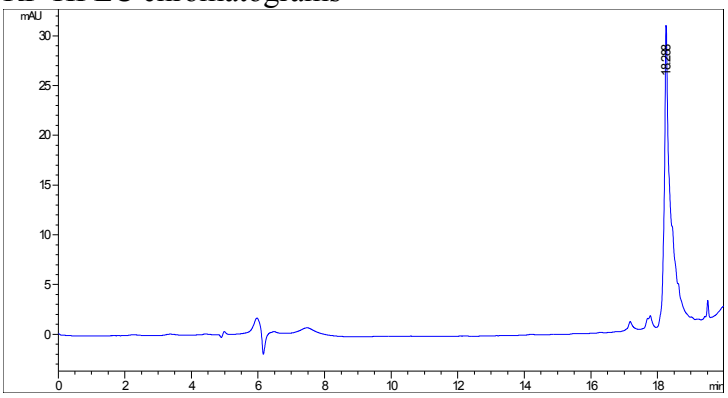
MALDI-ToF MS spectra



Observed 3136 Da
Calculated $[M+K]^{+1}$ (3137 Da)

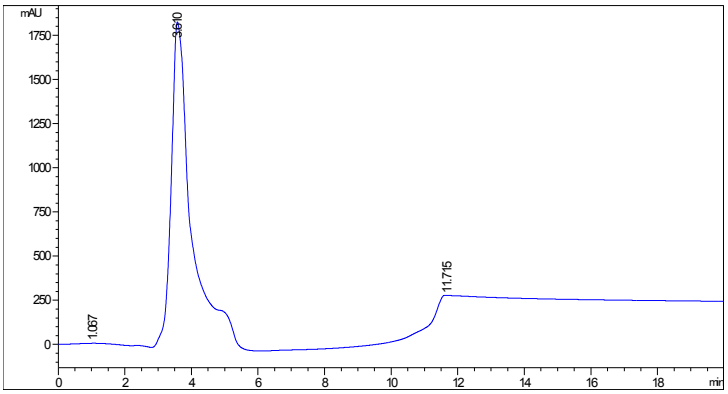
Compound 8

RP-HPLC chromatograms



Column
Zorbax Eclipse C18 analytical RP-HPLC column

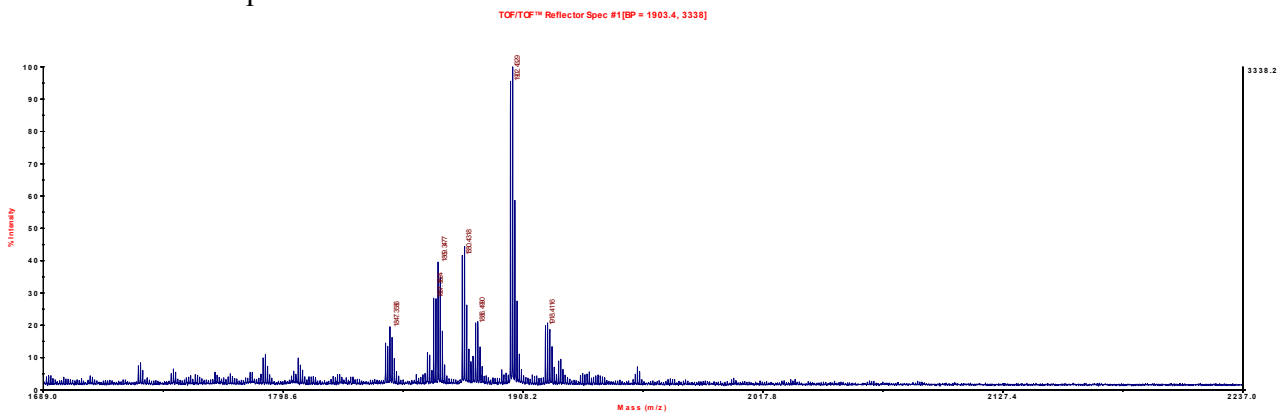
Eluent
0-30% of 0.1% ammonium acetate solution (pH=7.4) as solvent A in 90% acetonitrile/0.1% ammonium acetate solution as solvent B



Column
Zorbax Eclipse C18 analytical RP-HPLC column

Eluent
0-30% of 0.1% trifluoroacetic acid solution as solvent A in 90% acetonitrile/0.1% trifluoroacetic acid solution as solvent B

MALDI-ToF MS spectrum



Observed 1847 Da, 1867 Da, 1880 Da, 1886 Da, 1902 Da, 1918 Da
Calculated BromoAcetyl-Polio: $[M+H]^{+1}$ (1846 Da), $[M+Na]^{+1}$ (1868 Da), $[M+K]^{+1}$ (1884 Da)**;** Hydrolyzed TFA adduct: $[M+H]^{+}$ (1882 Da), $[M+Na]^{+}$ (1904 Da), $[M+K]^{+}$ (1920 Da)