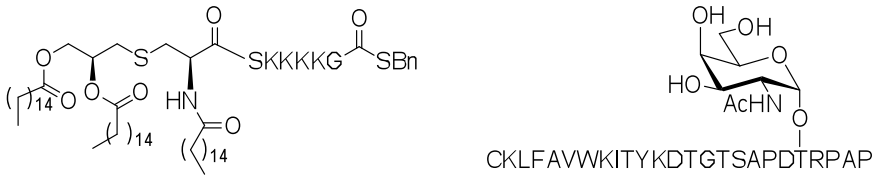
Supporting Information

Immune recognition of tumor-associated MUC1 is achieved by aberrantly glycosylated MUC1 tripartite vaccine

Vani Lakshminarayanan[a], Pamela Thompson[c], Margreet A. Wolfert[c], Therese Buskas[c], Judy M. Bradley[b], Latha B. Pathangey[b], Cathy S. Madsen[a], Peter A. Cohen[b], Sandra J. Gendler[a], and Geert-Jan Boons[c]

Departments of [a]Biochemistry and Molecular Biology and [b]Hematology and Oncology, Mayo Clinic College of Medicine and Mayo Clinic Comprehensive Cancer Center, Scottsdale, AZ 85259; [c]Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602



10 11

Fig. S1. Chemical structures of compounds **10** and **11**.

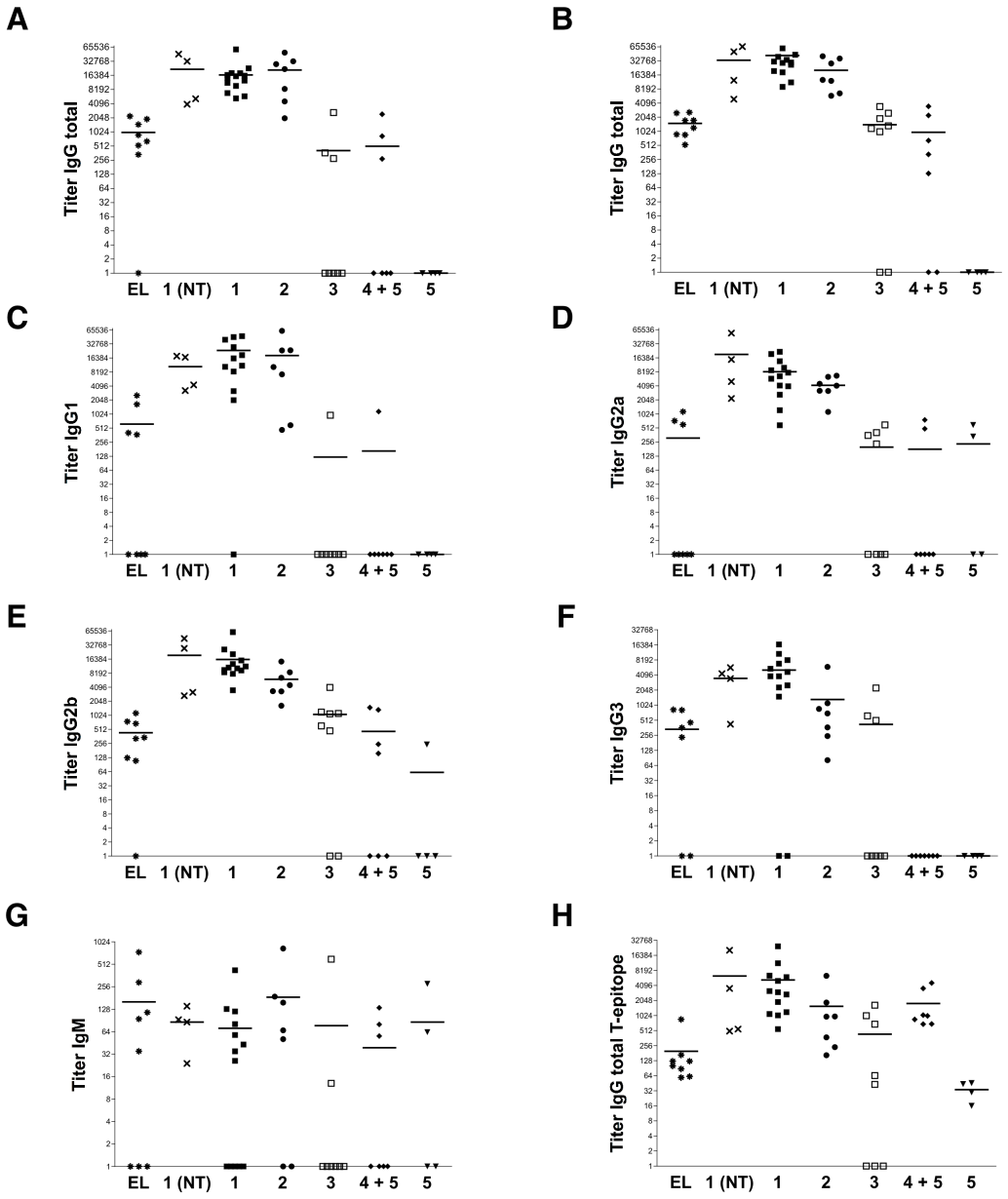


Fig. S2. ELISA anti-MUC1 and anti-T_{helper} antibody titers after 3 (*A*) or 4 (*B*-*H*) immunizations with **1**, **2**, **3**, **4 + 5**, or **5** with or without (NT) tumor induction as indicated. ELISA plates were coated with BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate (*A*-*G*) or NeutrAvidin-biotin-T_{helper} (*H*) 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 after 4 immunizations and the horizontal lines indicate the mean for the group of mice.

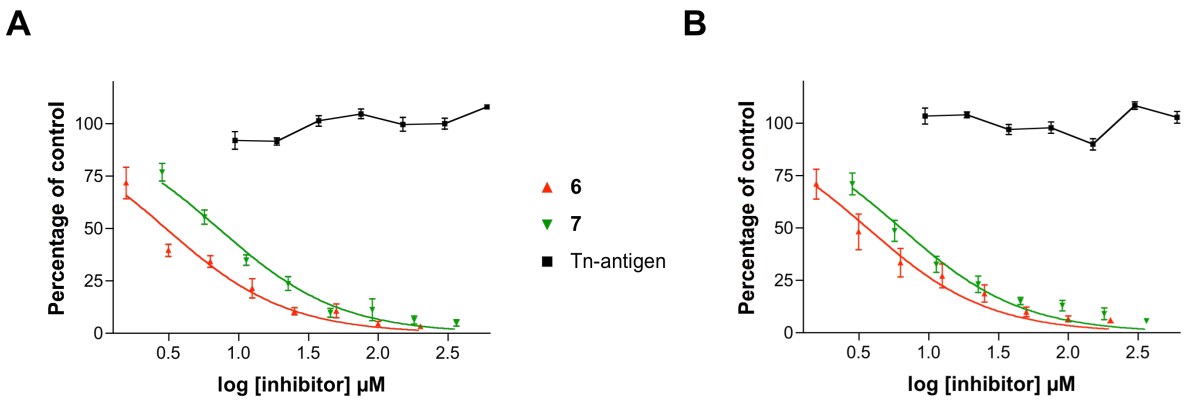


Fig. S3. Competitive inhibition of antibody binding to BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate by glycopeptide **6** (SAPDT(αGalNAc)RPAP), peptide **7** (SAPDTRPAP), and Tn-antigen (α-O-GalNAc-Thr). ELISA plates were coated with BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate. Serum samples after immunizations with (*A*) **1** and (*B*) **2**, diluted to obtain in the absence of an inhibitor an OD of approximately 1 in the ELISA, were first mixed with **6**, **7**, or Tn-antigen (0-500 μM final concentration) and then applied to the coated microtiter plate. Optical density values were normalized for the optical density values obtained with serum alone (0 μM inhibitor, 100%). The data are reported as the means ± SEM of groups of mice (n=7).

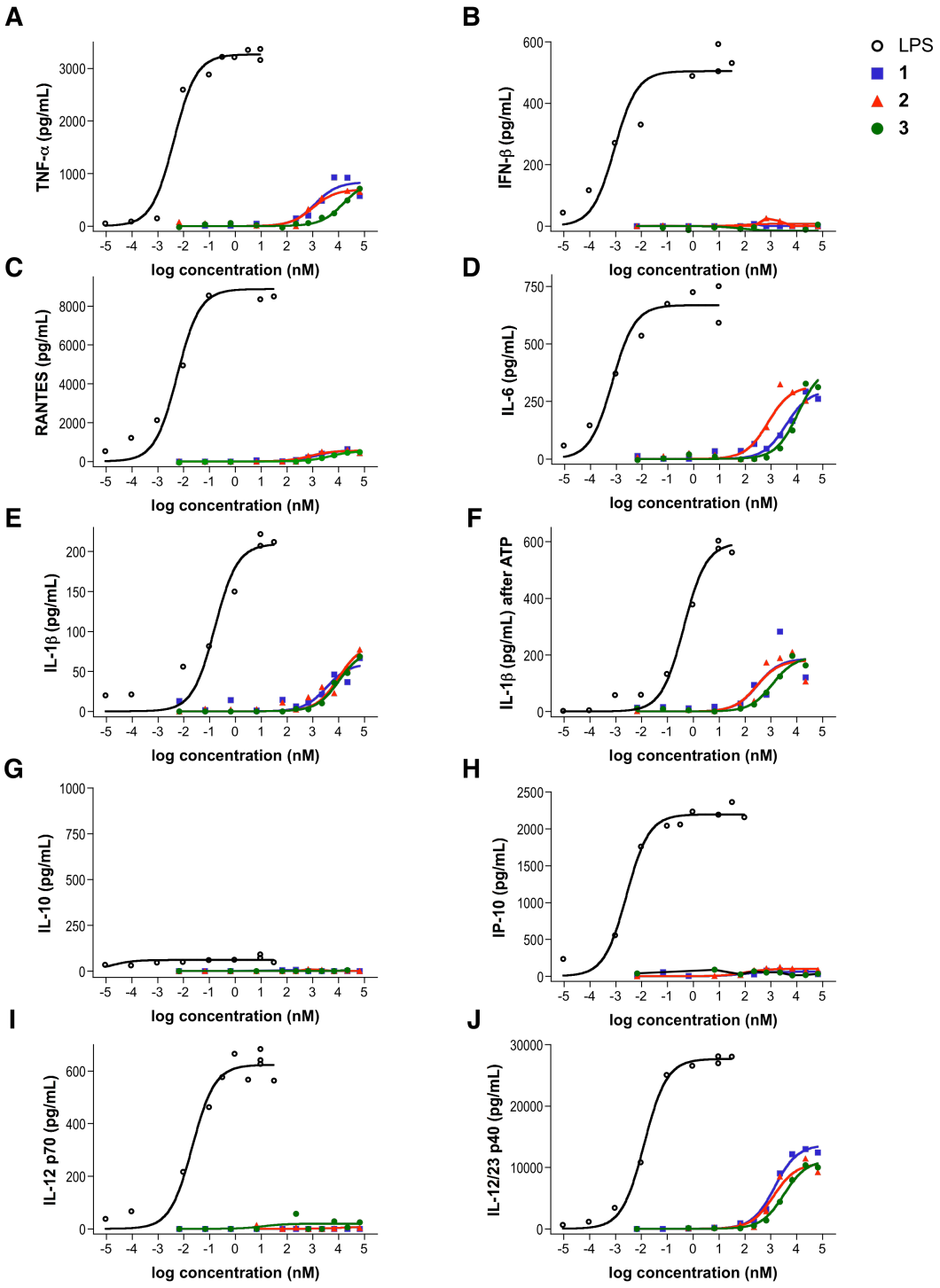


Fig. S4. Cytokine production by dendritic cells (DCs) after stimulation with liposome preparations loaded with compound **1**, **2**, or **3**, or *E. coli* LPS for 24 h. Primary mouse DCs were incubated for 24 h with increasing concentrations of liposome preparations loaded with compound **1**, **2**, or **3**, or *E. coli* LPS as indicated. (*A*) TNF-α, (*B*) IFN-β, (*C*) RANTES, (*D*) IL-6,

(*E* and *F*) extracellular IL-1β, (*G*) IL-10, (*H*) IP-10, (*I*) IL-12 p70, and (*J*) IL-12/23 p40 in cell supernatants were measured using ELISAs. For estimation of IL-1β secretion after ATP treatment, cells were incubated with ATP (5 mM) for 30 min subsequent to the 24 h incubation with inducers. The data are reported as the means ± SD of triplicate treatments.

Table S1. Competitive inhibition ELISA[a].

Immunization	IC_{50} inhibitors (μM)	
	SAPDT(αGalNAc)RPAP (**6**)	SAPDTRPAP (**7**)
1	3.01	7.19
	(2.54 to 3.59)	(6.23 to 8.29)
2	3.63	6.30
	(2.88 to 4.56)	(5.36 to 7.41)

[a] ELISA plates were coated with BSA-MI-CTSAPDT(αGalNAc)RPAP conjugate. Serum samples of groups of 7 mice after immunizations with **1** or **2**, diluted to obtain in the absence of an inhibitor an OD of approximately 1 in the ELISA, were first mixed with glycopeptide **6** (SAPDT(αGalNAc)RPAP) or peptide **7** (SAPDTRPAP) (0-500 μM final concentration) and then applied to the coated microtiter plate. Optical density values were normalized for the optical density values obtained with serum alone (0 μM inhibitor, 100%). Inhibition data were fit with the following logistic equation: Y = Bottom + (Top – Bottom) / $(1 + 10^{(X - \text{Log } IC50)})$, where Y is the normalized optical density, X is the logarithm of the concentration of the inhibitor and IC_{50} is the concentration of the inhibitor that reduces the response by half. The IC_{50} values are reported as best-fit values and as 95% confidence intervals.

Table S2. Cytokine plateau values[a] (pg/mL) of dose-response curves of liposome preparations loaded with compound **1**, **2**, or **3** and *E. coli* LPS obtained after incubation of primary DCs for 24 h.

Cytokine (pg/mL)	**1**	**2**	**3**	LPS
TNF-alpha	836 ± 103	695 ± 50	854 ± 67	3,265 ± 96
IFN-beta	nd[b]	nd	nd	505 ± 34
RANTES	584 ± 59	553 ± 54	536 ± 28	8,869 ± 416
IL-6	298 ± 28	316 ± 40	401 ± 43	668 ± 34
IL-1beta	60 ± 10	84 ± 13	77 ± 4	209 ± 15
IL-1beta/ATP	187 ± 50	181 ± 26	194 ± 14	596 ± 24
IL-10	nd	nd	nd	91 ± 6
IP-10	nd	nd	nd	2,196 ± 44
IL-12 p70	nd	nd	nd	623 ± 19
IL-12/23 p40	13,668 ± 496	10,692 ± 853	11,192 ± 382	27,679 ± 460

[a] Plateau values as reported by Prism as best-fit values ± SEM using non-linear least squares curve fitting as picogram of cytokine per μg of total protein.
[b] nd indicates not detected.

Table S3. Cytokine log EC_{50} values[a] (nM) of liposome preparations loaded with compound **1**, **2**, or **3** and *E. coli* LPS in primary DCs.

Cytokine (pg/mL)	**1**	**2**	**3**	LPS
TNF-alpha	3.08 ± 0.25	2.99 ± 0.14	4.17 ± 0.10	-2.38 ± 0.12
IFN-beta	nd[b]	nd	nd	-3.04 ± 0.24
RANTES	3.12 ± 0.17	2.88 ± 0.19	3.66 ± 0.09	-2.25 ± 0.16
IL-6	3.58 ± 0.16	2.88 ± 0.23	4.05 ± 0.14	-3.15 ± 0.18
IL-1beta	3.52 ± 0.28	3.99 ± 0.21	4.01 ± 0.08	-0.80 ± 0.22
IL-1beta/ATP	2.48 ± 0.48	2.44 ± 0.31	3.06 ± 0.13	-0.37 ± 0.12
IL-10	nd	nd	nd	nd
IP-10	nd	nd	nd	-2.59 ± 0.09
IL-12 p70	nd	nd	nd	-1.67 ± 0.14
IL-12/23 p40	3.15 ± 0.07	3.10 ± 0.16	3.51 ± 0.06	-1.89 ± 0.06

[a] Log EC_{50} values as reported by Prism as best-fit values ± SEM using non-linear least squares curve fitting.
[b] nd indicates not detected at levels for accurate EC_{50} determination.

Synthesis of Compound 1: Pam_3CysSK_4 thioester **10** (S1) (1.1 mg, 0.674 μmol), glycopeptide **11** (1.0 mg, 0.337 μmol), and dodecylphosphocholine (1.5 mg, 4.38 μmol) were dissolved in a mixture of $CHCl_3$/trifluoroethanol (1/1 v/v, 5 mL). The solvents were removed under reduced pressure to give a thin film, which was hydrated for 4 h at 41 ^oC using a sodium phosphate (200 mM) buffer containing TCEP (2 mM) and EDTA (0.3%). The mixture was sonicated and the suspension was extruded through polycarbonate membranes (1.0 μm Whatman, Nucleopore, Track-Etch Membrane) at 50 ^oC to obtain uniform vesicles. To the vesicle suspension was added sodium 2-mercaptoethane sulfonate (1 mM) to initiate the reaction (1.5 mM final peptide concentration). The reaction was incubated for 2 h at 37 ^oC. The reaction mixture was then purified by RP-HPLC on an Phenomenex Jupiter analytical C-4 reversed phase column using a gradient of 0 – 100% B in A over a period of 40 min. Lyophilization of the appropriate fractions afforded **1** (0.76 mg, 50%). $C_{217}H_{367}N_{45}O_{53}S_2$ HR MALDI-ToF MS: observed 4516.9668 [M+H]; calculated 4516.685 [M+H].

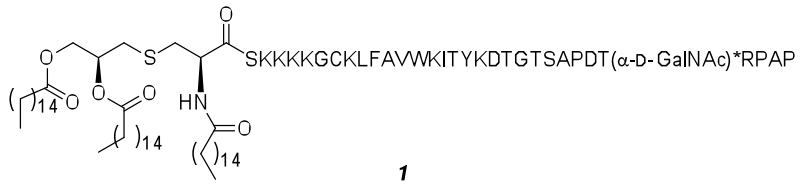
S1. Ingale S, Buskas T, & Boons GJ (2006) Synthesis of glyco(lipo) peptides by liposome-mediated native chemical ligation. *Org. Lett.* 8:5785-5788.

Synthesis of Compound 2: Lipopeptide **2** was synthesized by solid phase peptide synthesis (SPPS) following the general protocol on Rink Amide AM resin (0.1 mmol). After the assembly of the peptide, the remaining steps were performed manually. *N*-α-Fmoc-*R*-(2,3-bis (palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine (180 mg, 0.2 mmol) was dissolved in DMF (5 mL), and 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (76 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) were premixed for 2 min and added to the resin. The coupling reaction was monitored by the Kaiser test and was complete. Upon completion of the coupling, the *N*-Fmoc group was cleaved using 20% 4-methyl piperidine in DMF (6 mL). Palmitic acid (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol) and DIPEA (67 μL, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2%; 10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume.

The peptide was precipitated using diethyl ether (0 ^{o}C; 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude lipopeptide was purified a Phenomenex Jupiter C-4 semi preparative column using a linear gradient of 0-100% solvent B in A over a period of 40 min, and the appropriate fractions were lyophilized to afford **2**. $C_{206}H_{349}N_{43}O_{47}S$ HR MALDI-ToF MS: observed, [M+2H] 4211.0464; calculated [M+2H] 4211.5962.

Synthesis of Compound 3: Lipopeptide **3** was synthesized on Rink Amide AM resin (0.1 mmol) following the general protocol for peptide synthesis and the procedure for lipidation described for compound **2**. The resulting crude lipopeptide was purified by HPLC on a Phenomenex Jupiter C4 semi preparative column using a linear gradient of 0-100% solvent B in A over a period of 40 min, and the appropriate fractions were lyophilized to afford **3.** $C_{162}H_{279}N_{29}O_{31}S$, MALDI-ToF MS: observed, [M+H] 3160.237; calculated, [M+H] 3156.087.

Analytical Data of Compounds 1-11.



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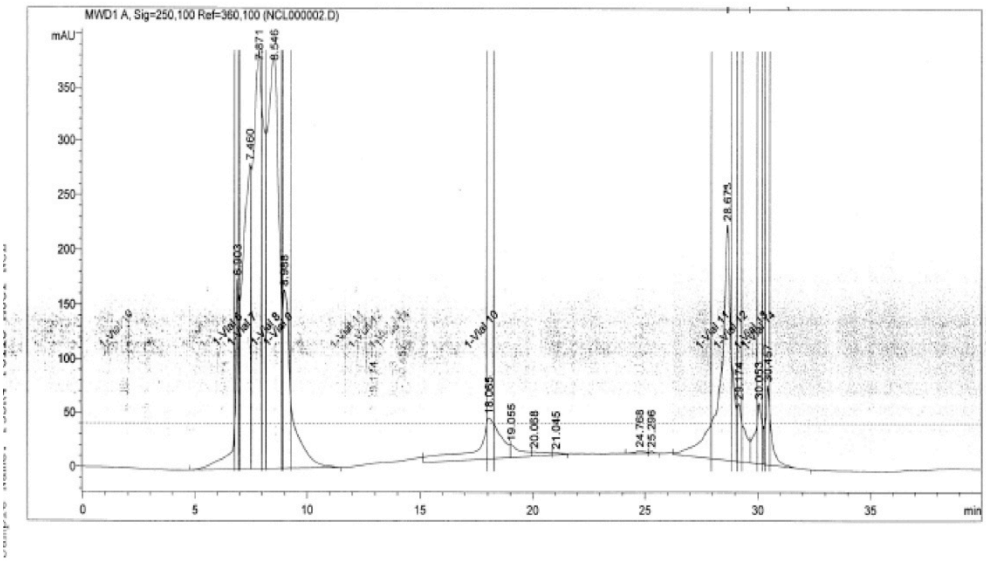

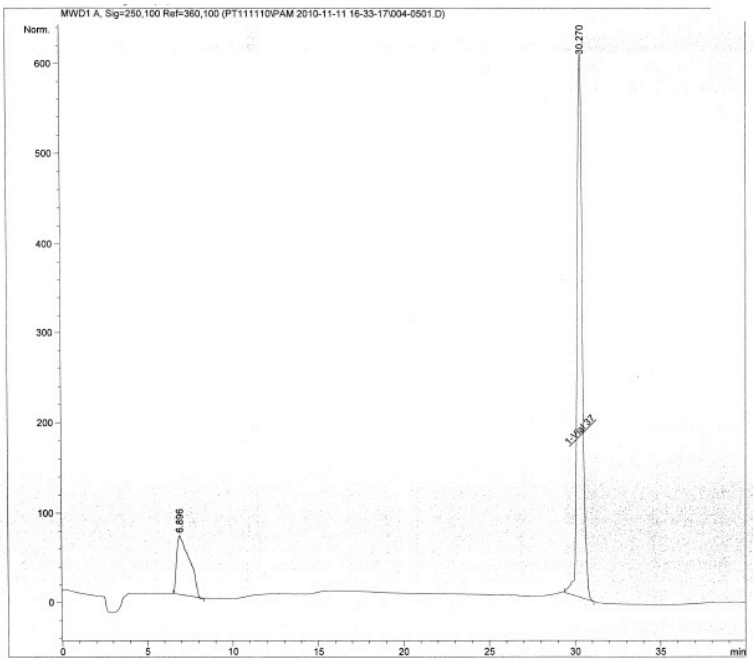

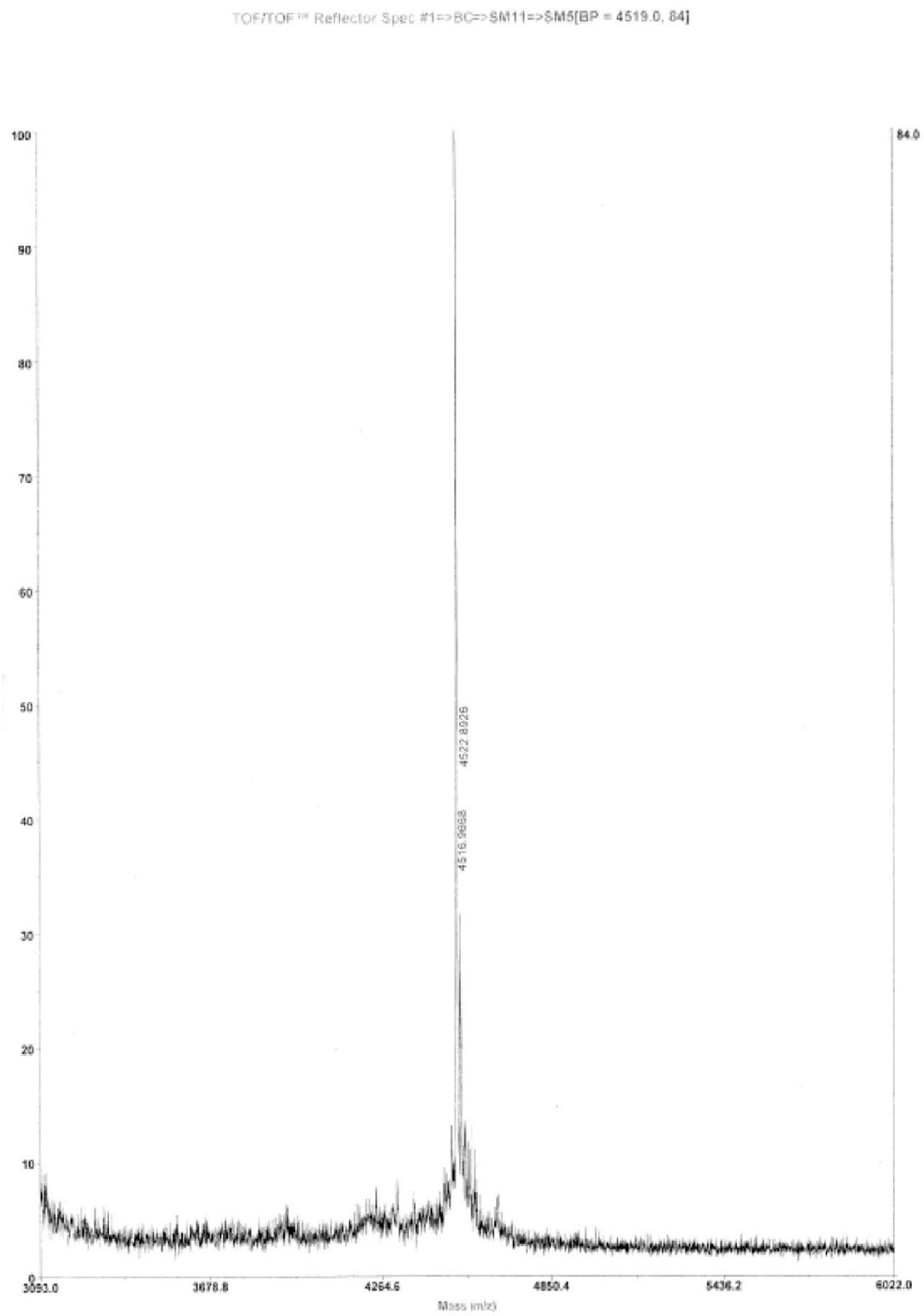
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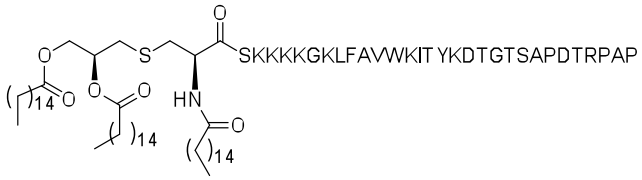
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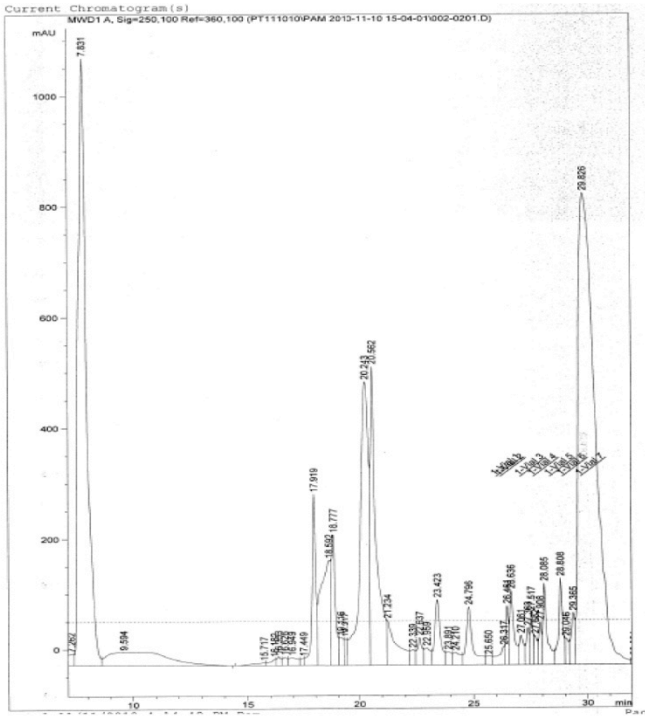
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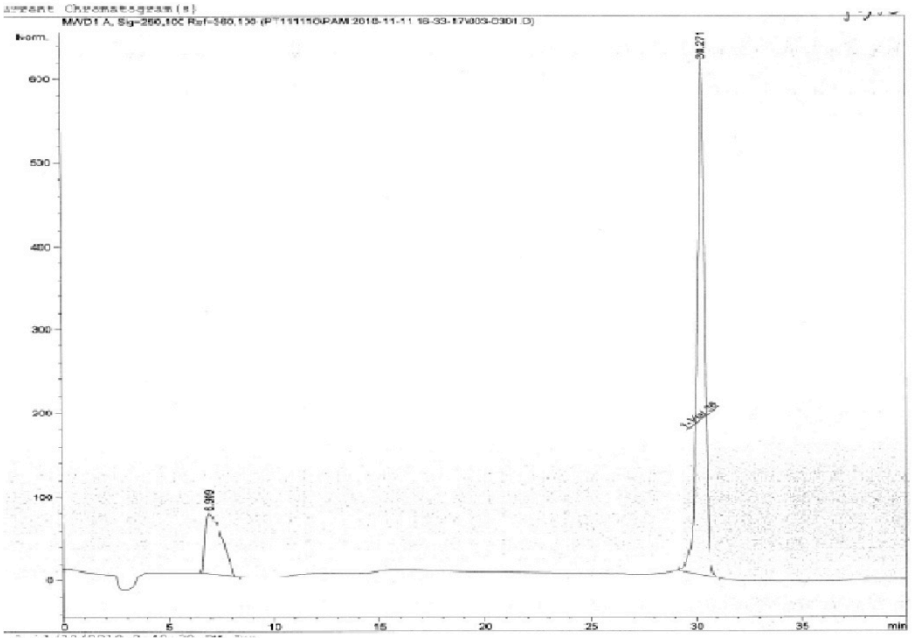
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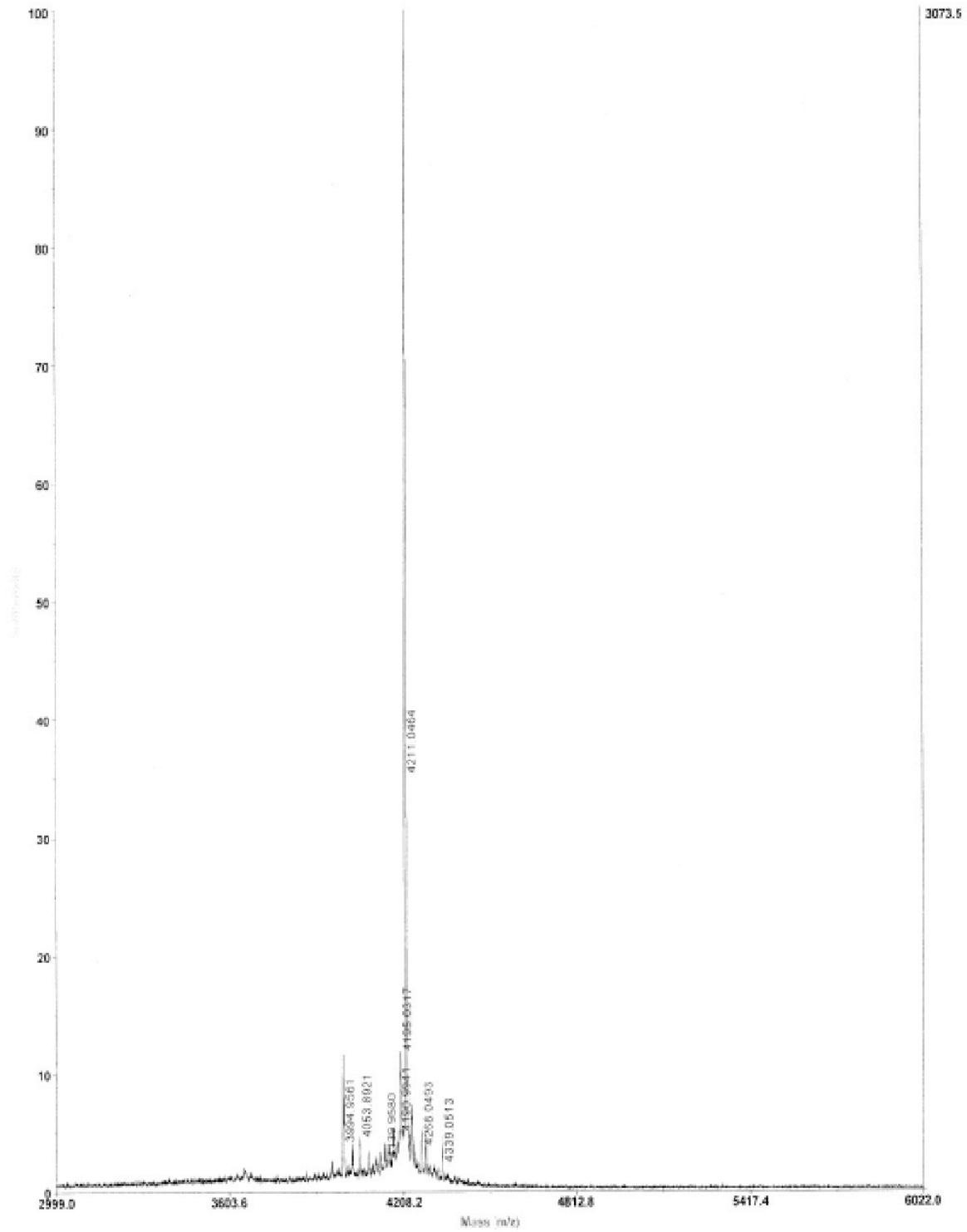
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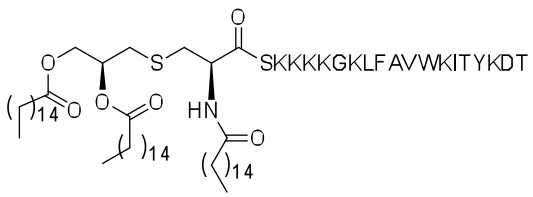
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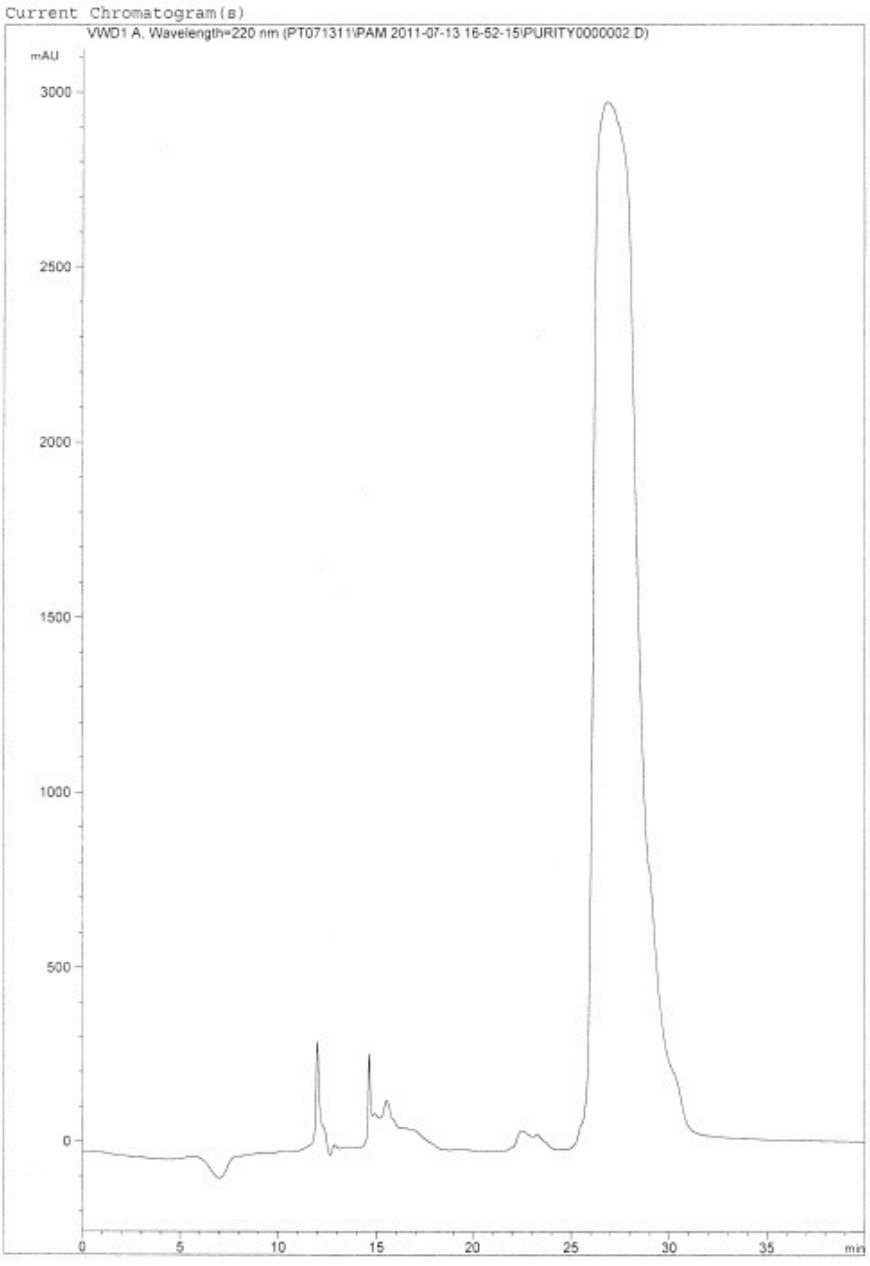
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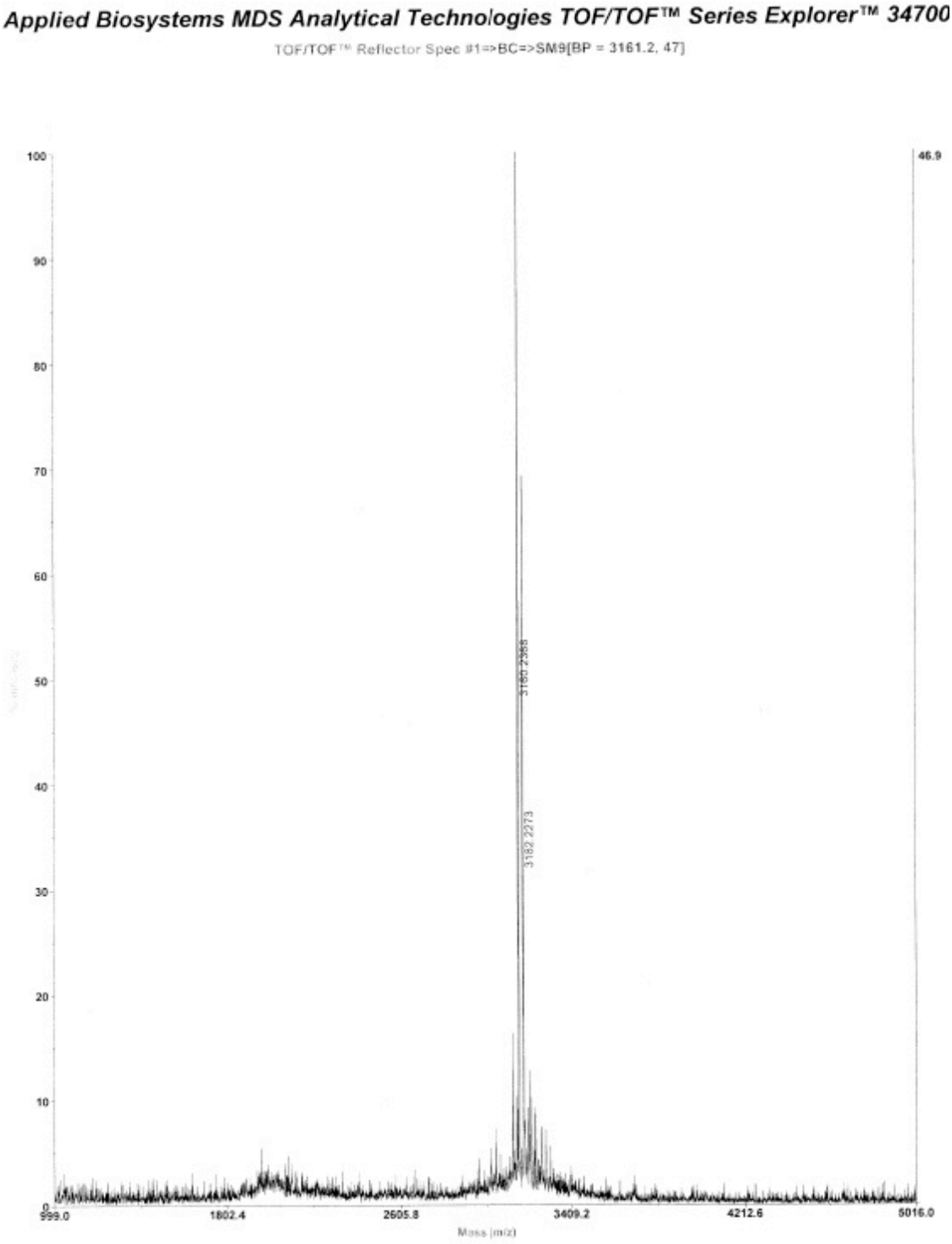
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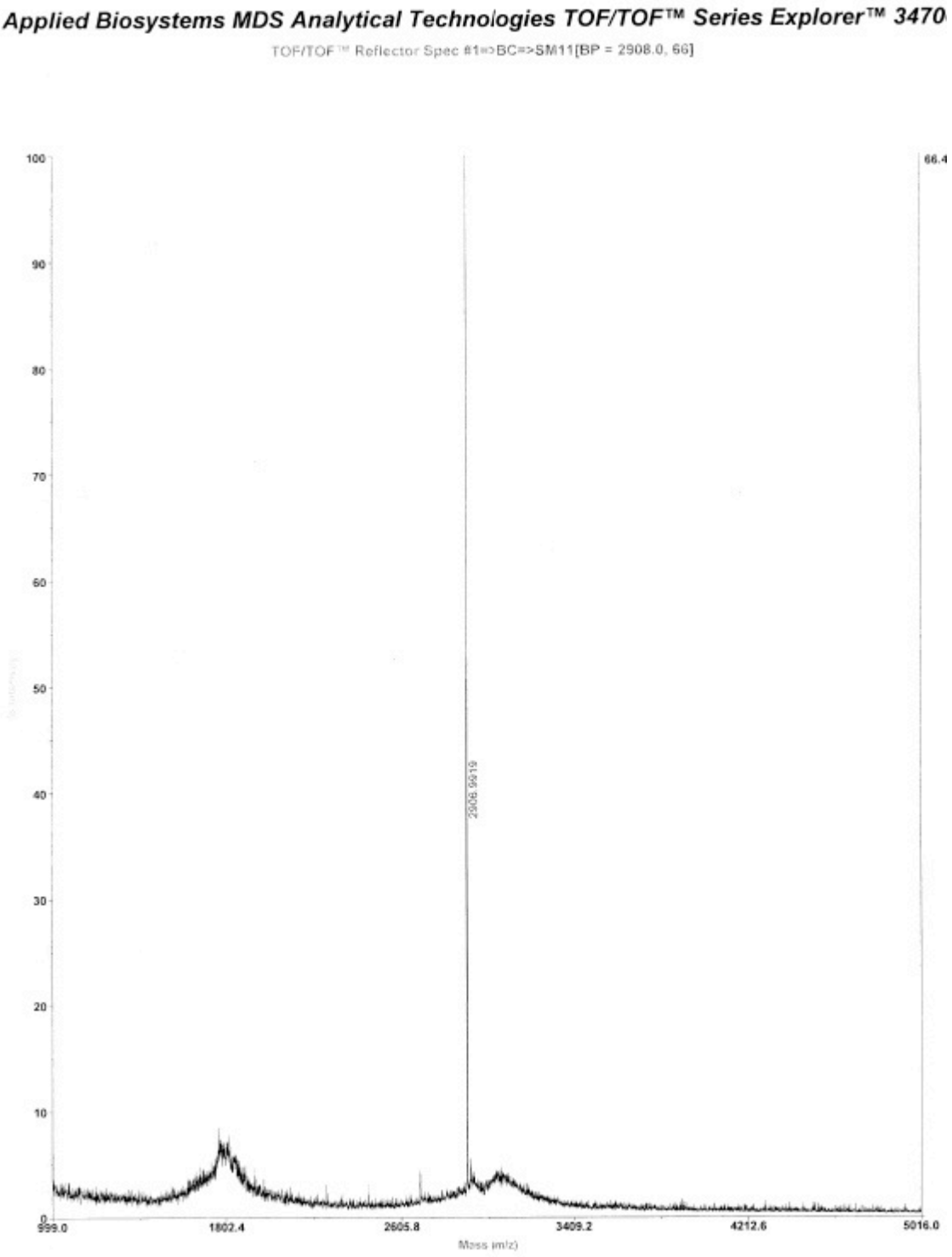
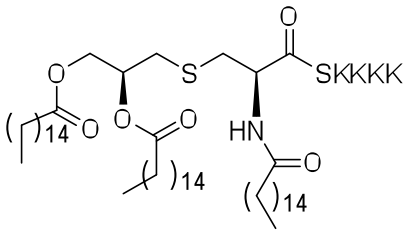
4

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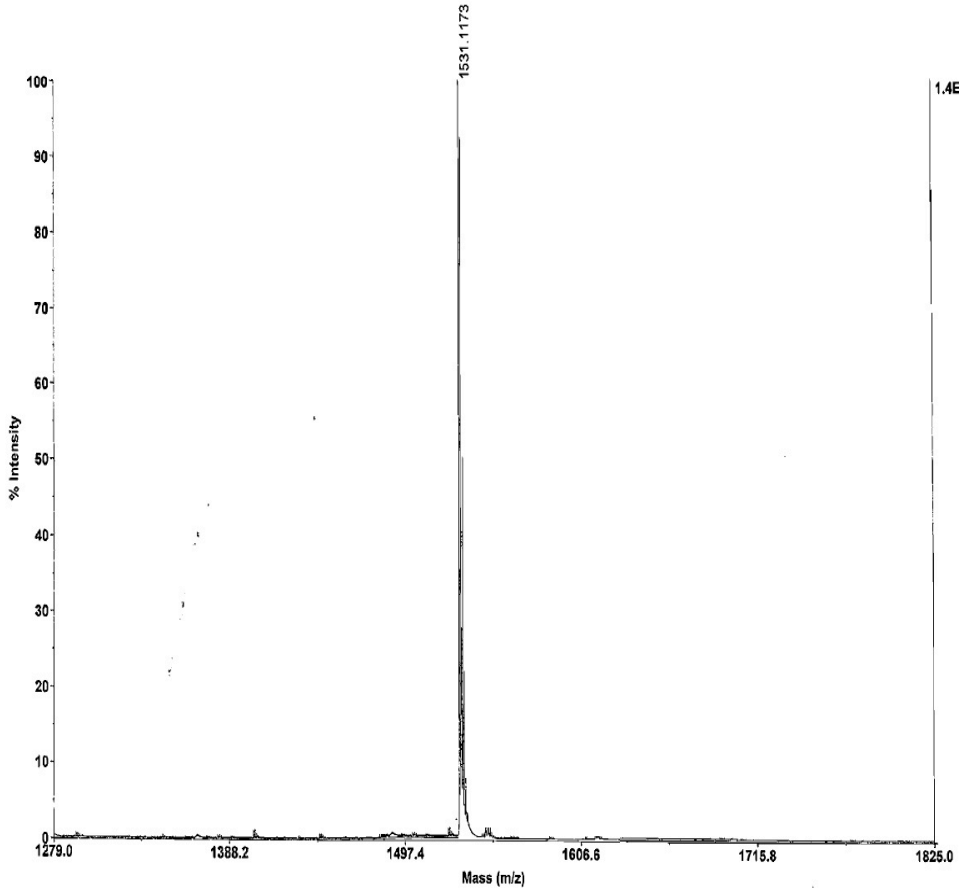
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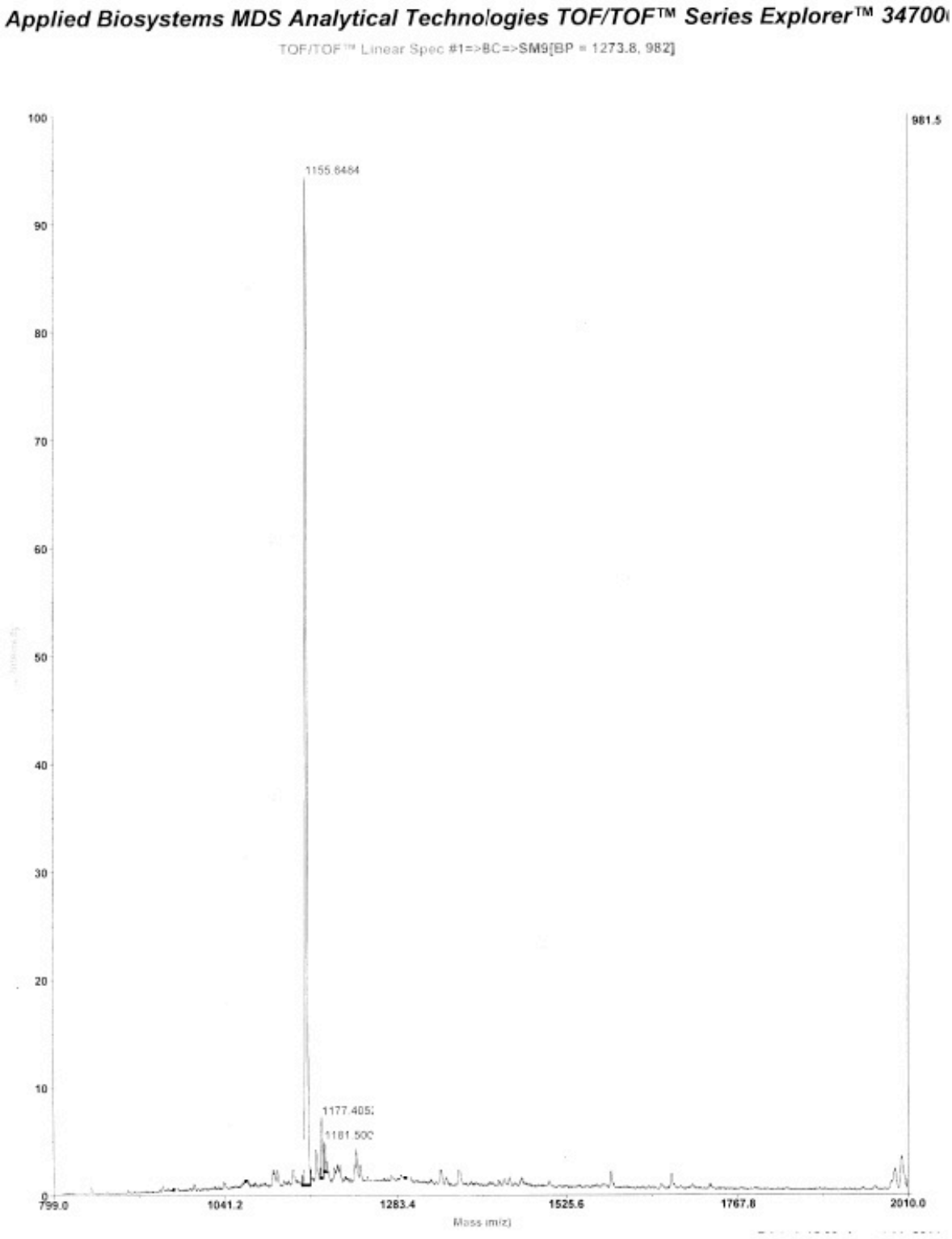

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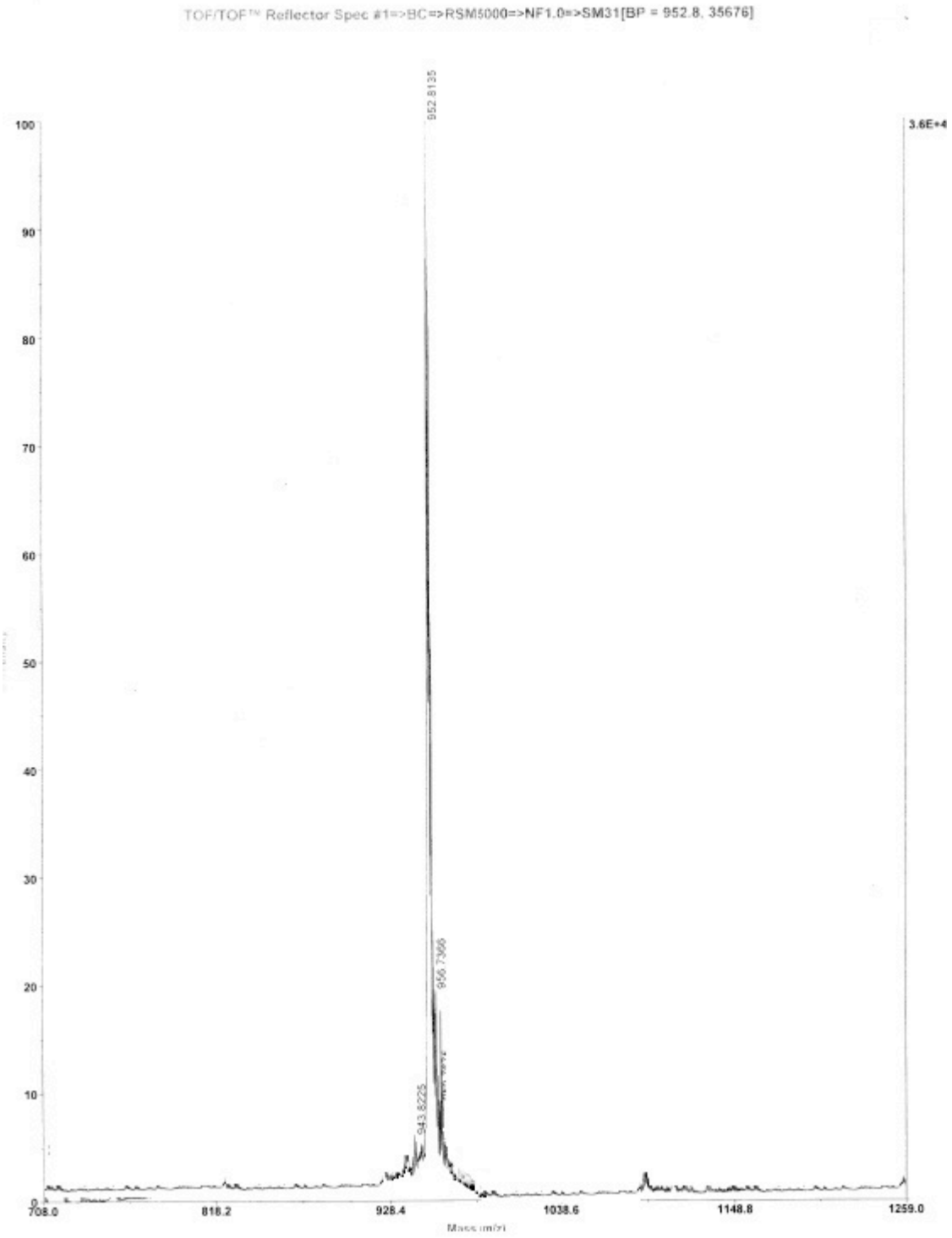
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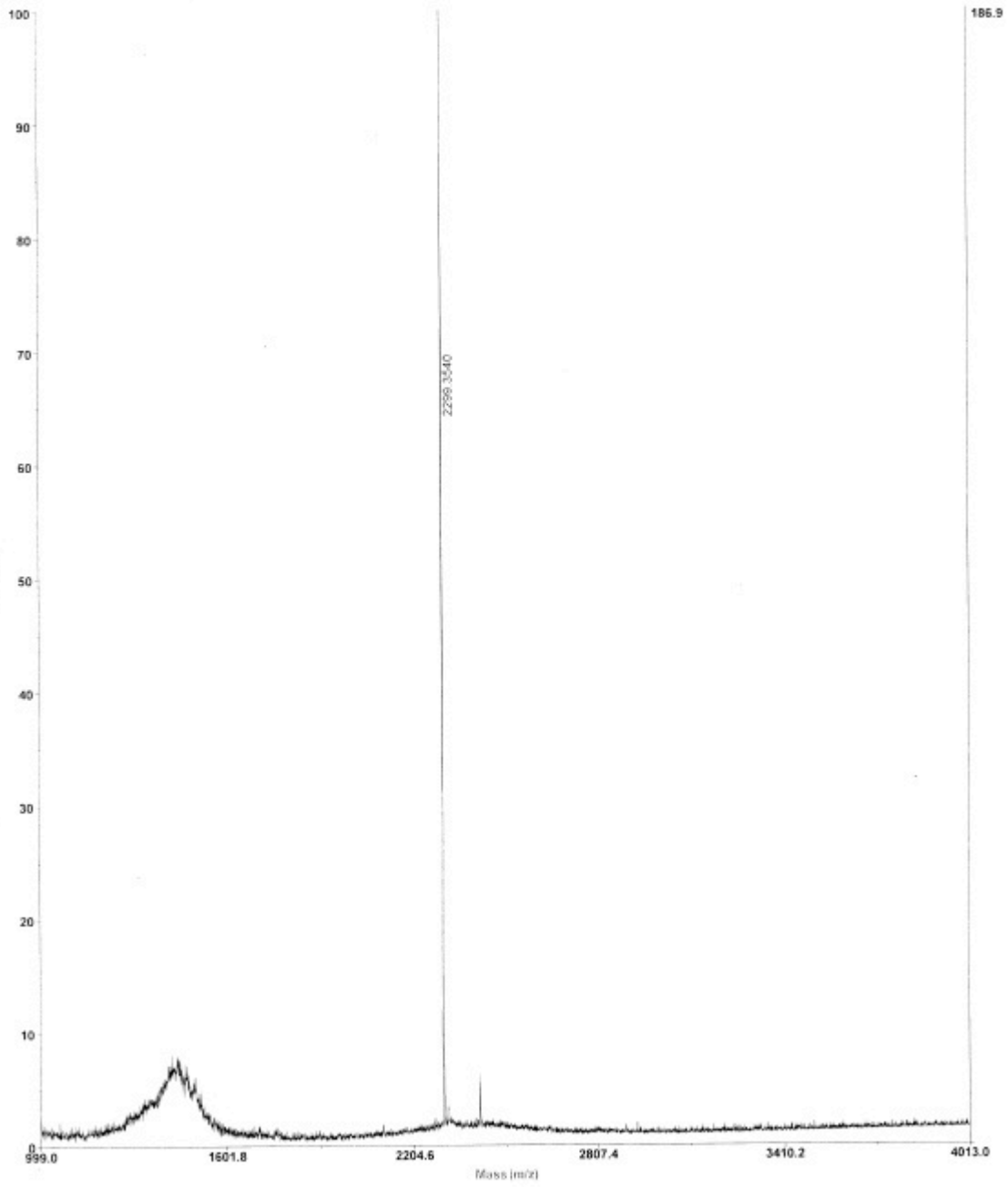

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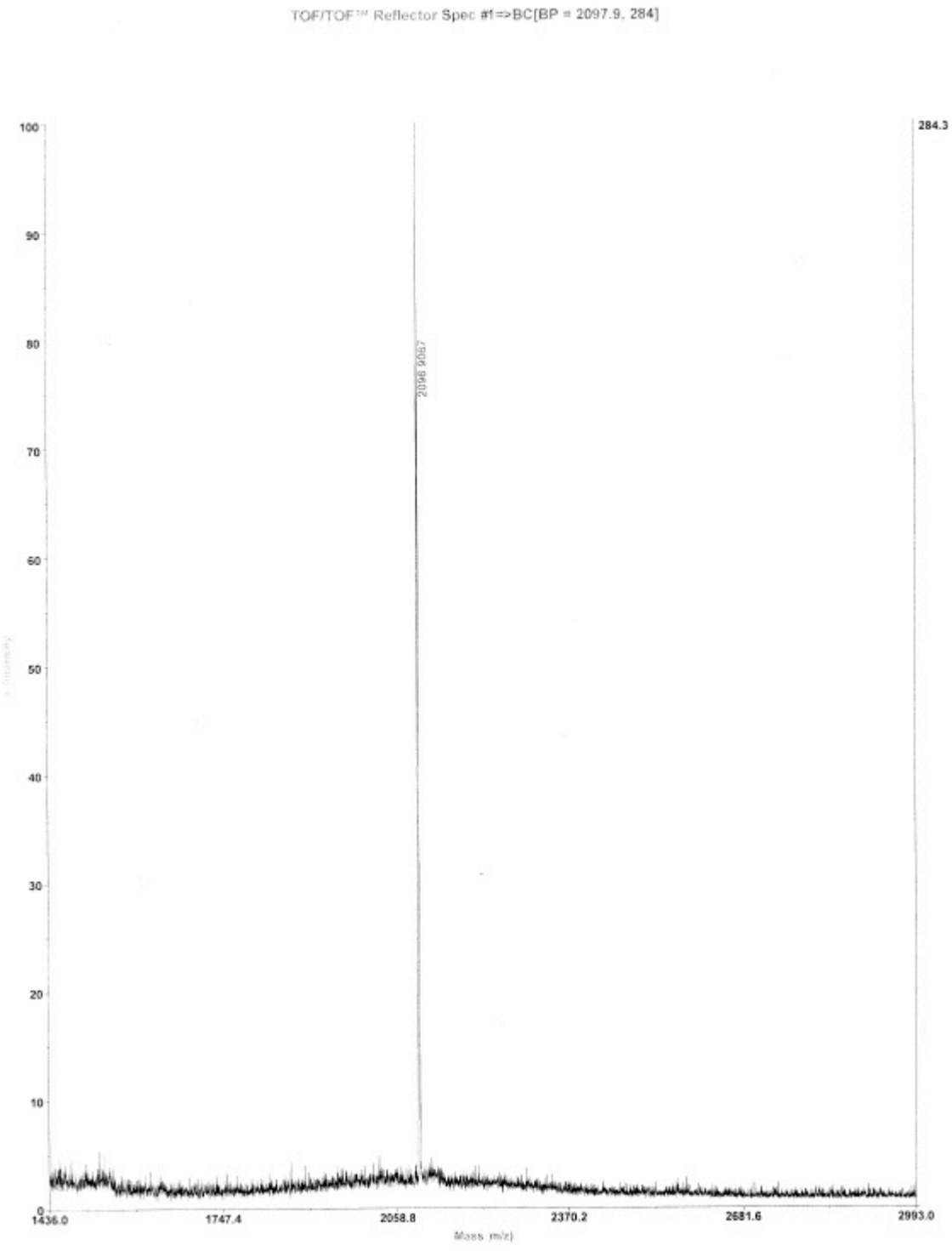

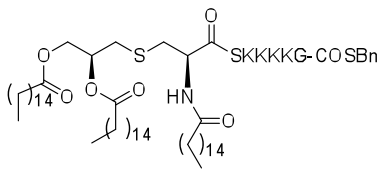
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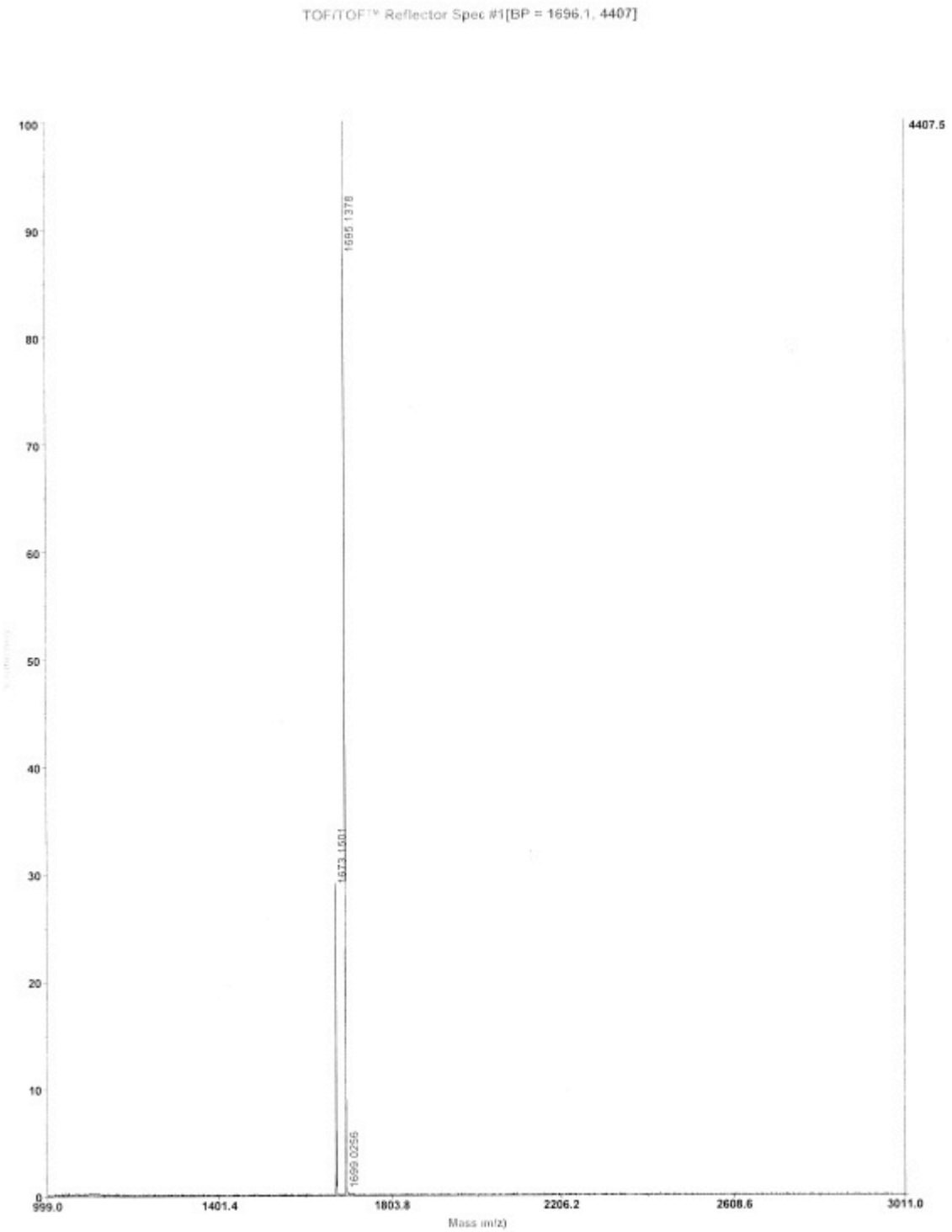
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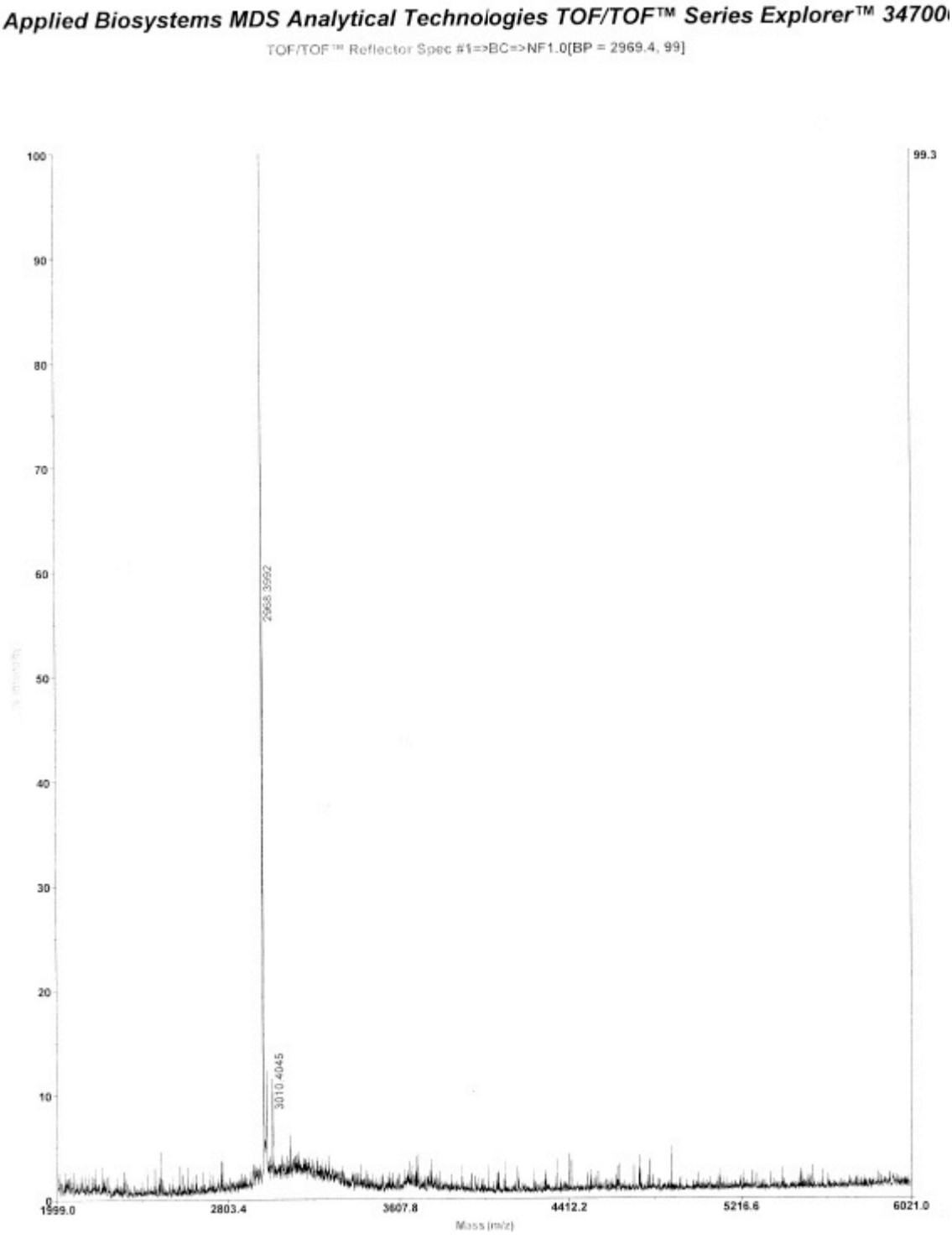

Cell Culture and Transfection

Cell lines used in these studies include MMT mammary gland tumor cells derived from MUC1.Tg mice crossed with MMTV-PyV MT mice (S2) and C57mg.MUC1 mammary gland tumor cells (S3).

Yac-1 lymphoma cells (5 x 10^6 cells), which are sensitive to NK cells, were electroporated with pCI.MUC1 (10 μg) to generate cells (Yac-1.MUC1) that express MUC1 as a relevant target for antibody-dependent cell-mediated cytotoxicity (ADCC). MUC1 cDNA (tagged with the FLAG epitope (S3)) was cloned into the Not I site of pCI.neo vector (Promega; E1841) and the construct was linearized with BspH1 prior to electroporation (300 mV, 500 uF) using a Gene Pulse II with the Capacitance Extender II (BioRad) with 0.4 cm cuvettes. Neomycin-resistant cells were selected (using G418 at 600 μg/mL) and MUC1 expression was determined by flow cytometry using anti-MUC1 antibody CD227 (BD Pharmingen) conjugated to FITC (Fig. S5). Yac-1.neo cells were made similarly with the pCI.neo vector.

Cells were maintained in DMEM supplemented with FCS (5%), penicillin (100 U/mL), streptomycin (0.1 μg/mL), L-glutamax (2 mM), and G418 (300 μg/mL for Yac-1.MUC1 and Yac-1.neo cells and 150 μg/mL for C57mg.MUC1 cells). All cells are derived originally from C57BL/6 mice.

S2. Mukherjee P, et al. (2003) Mucin 1-specific immunotherapy in a mouse model of spontaneous breast cancer. *J. Immunother.* 26:47-62.

S3. Mukherjee P, Ginardi AR, Tinder TL, Sterner CJ, & Gendler SJ (2001) MUC1-specific cytotoxic T lymphocytes eradicate tumors when adoptively transferred *in vivo*. *Clin. Cancer Res.* 7:848s-855s.

S4. Burdick MD, Harris A, Reid CJ, Iwamura T, & Hollingsworth MA (1997) Oligosaccharides expressed on MUC1 produced by pancreatic and colon tumor cell lines. *J. Biol. Chem.* 272:24198-24202.

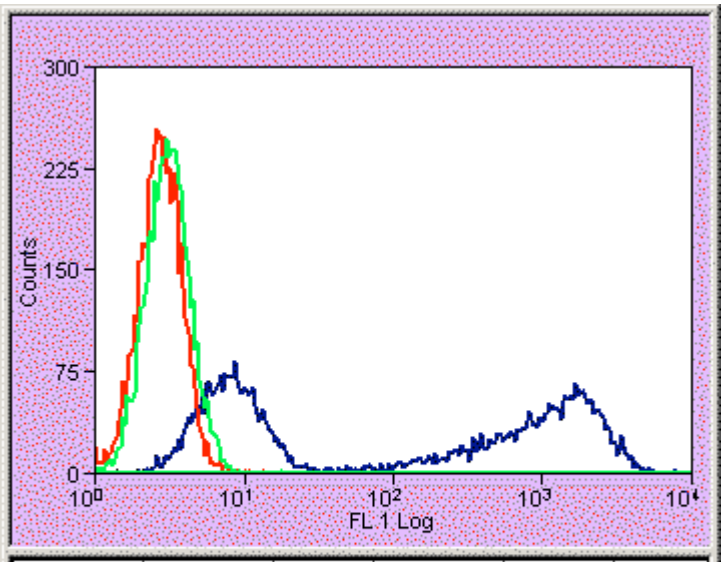

Fig. S5. Flow cytometry analysis of Yac-1.neo and Yac-1.MUC1 cells for MUC1 expression. A subtraction analysis of the area under the MUC1-specific histogram that was confluent with the Yac-1.neo control histogram indicated that 88.3% of the Yac-1.MUC1 cells expressed surface MUC1, albeit at two distinct densities. A similar analysis subtracting the isotype control on Yac-1.MUC1 cells gave 87.0% MUC1 positivity. Green indicates the isotype control, red indicates Yac-1.neo cells stained with anti-MUC1, and blue indicates Yac-1.MUC1 cells stained with anti-MUC1.