Ribosome-Inactivating Protein Small Molecule Conjugates can Selectively Inhibit Target-Specific Tumor Cell Growth

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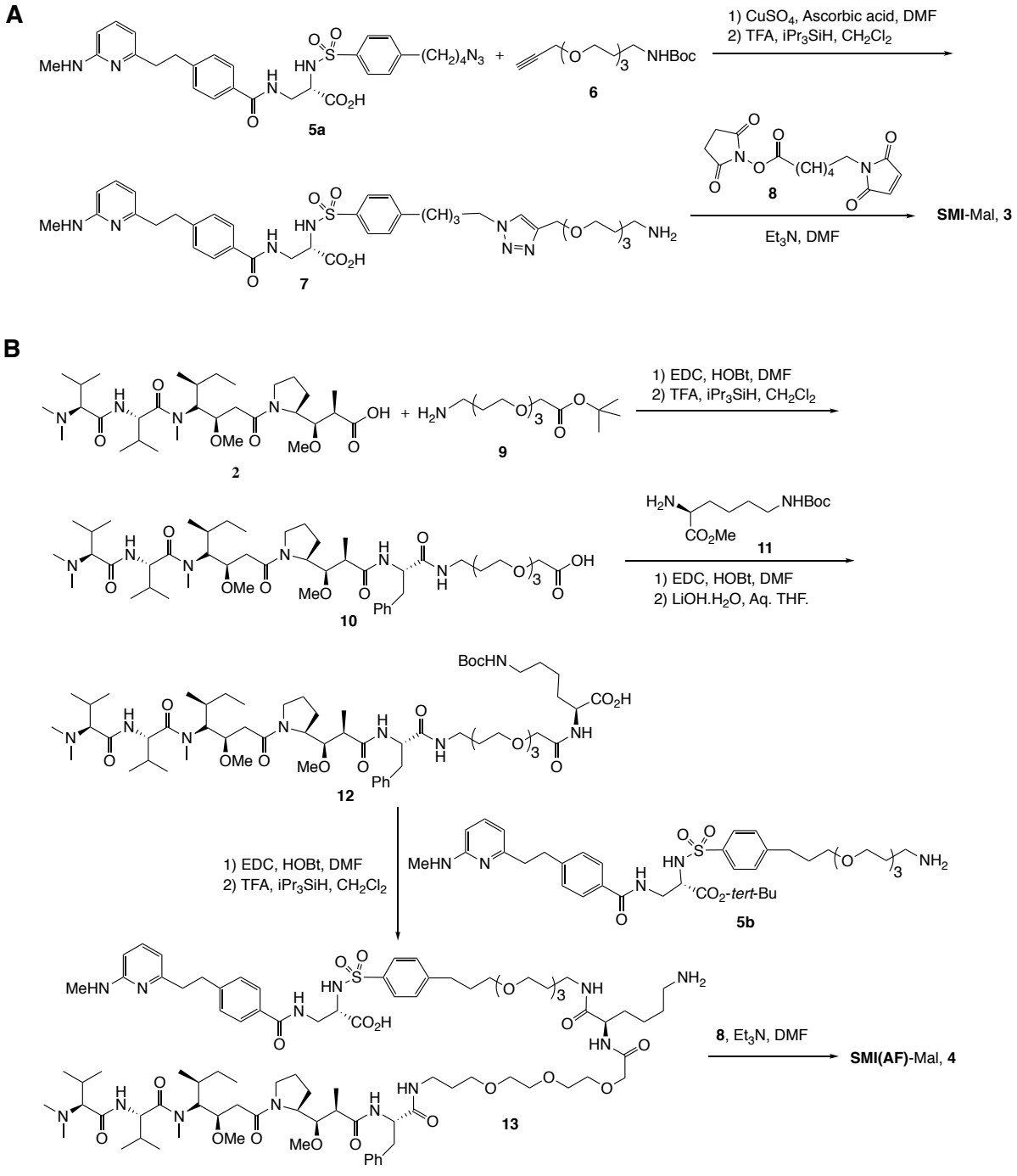
1. Synthesis of compounds 3 and 4.

A) Compound **3** prepared as shown in Scheme 1A using the readily available compounds **5a**, **6** and **8** via intermediate **7**.



Scheme S-1. Synthesis of the (A) SMI-Mal and (B) SMI(AF)-Mal intermediates using the readily available small molecule inhibitor (SMI) of $\alpha v\beta 3/\alpha v\beta 5$ integrins (and Auristatin F (AF)).

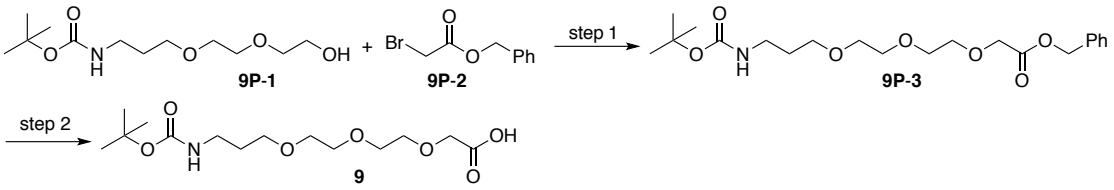
1.1. Compound 7. Cu powder (2 mg) and Aq. $CuSO_4$ (2 µl) were added to a solution of compound **5a**[1] (21 mg, 0. 0.036mmol) and alkyne **6**[2] (13 mg, 0. 0.045 mmol) in CH_3CN (1 ml), and reaction mixture was stirred at 50˚C overnight. Solvents were removed and residues were re-dissolved in a mixture of CH_2Cl_2-MeOH (9:1), filtered to remove insoluble materials, and purified by a preparative Silica gel TLC (CH_2Cl_2-MeOH) to get the corresponding alkyne-azide coupling product (18 mg, 58%) as a liquid

The above- coupling product (18 mg, 0.023 mmol) was dissolved in CH_2Cl_2-TFA (1:1, 1 ml), and the reaction mixture was stirred at room temperature overnight. Solvents were removed to afford the title compound **7** as a TFA salt that was taken to next step without purification and characterization.

1.2. Compound 3 (SMI-Mal). To a solution of compounds **7**.TFA salt (0.023 mmol) and **8** (8.1 mg, 0.0276 mmol) in CH_2Cl_2 (1 ml), Et_3N (0.32 ml, 0.23 mmol) was added, and mixture was stirred overnight. Solvents were removed under vacuum, and the residues were purified by a preparative Silica gel TLC (CH_2Cl_2-MeOH) to yield the title product **3** (16 mg, 74%) as a liquid. HRMS (ESI) *m/z* calcd for $C_{46}H_{59}N_9O_{11}S$ 945.4055, found 945.4057.

B) Compound **4** was prepared using compounds **2**, **5b**, **8**, **9**, and **11**, as shown in Scheme 1B via intermediates **10**, **12**, and **13**.

1.3. Compound 9. Prepared in 2 steps using the readily available alcohol **9p**-**1** and ester **9P**-**2** *via* **9p**-**2**.



Step 1. To an ice cold solution of compound **9p-1** (502 mg, 2.0 mmol) in THF (8 ml) NaH (96 mg, 2.4 mmol, 60% adsorbed in oil) was added. After stirring for 0.5h, **9P**-**2** (504 mg, 2.2 mmol, dissolved in 2 ml of THF) and TBAI (74 mg, 0.2 mmol) were added sequentially. The reaction mixture was stirred for 6h, prior to work-up using EtOAc-water, and purified using silica gel column (CH_2Cl_2-MeOH) to afford **9P**-**3** (516 mg, 65%).

Step 2. A mixture of **9P**-**3** (230 mg, 0.58 mmol) was hydrogenated using hydrogen balloon and Pd/C (10% w/w, 23 mg) in EtOH (3 ml), and filtered using Celite to afford **9** (152 mg, 85%).

1.4. Compound 10. EDC (15 mg, 0.078 mmol) and HOBt (10 mg, 0.078 mmol) were added sequentially to a solution of AF (**2**, 39 mg, 0.052 mmol) in DMF (2 ml). The reaction mixture was stirred for 20 min, followed by addition of compound **9** (21.6 mg, 0.078 mmol) and DIPEA (0.03 ml, 0.153 ml). After the reaction mixture was stirred overnight, it was worked-up using CH_2Cl_2 and water, and purified over Silica gel to afford the compound **10**-*tert*-Butyl ester (24 mg, 0.024 mmol). LCMS (ESI) *m/z* calcd for $C_{53}H_{92}N_6O_{12}$ 1004.68, found 1005.8 $(M+H)^+$.

tert-Butyl ester in the resulting amide product (18mg, 0.018 mmol) was deprotected using CH_2Cl_2-TFA (1:1, 1ml), as described above for compound **7** to afford **10** (16 mg, 0.017 mmol, 33% in 2 steps from **2**). LCMS (ESI) *m/z* calcd for $C_{49}H_{84}N_6O_{12}$ 948.61, found 949.8 $(M+H)^+$.

1.5. Compound 12. Acid **10** (46 mg, 0.049 mmol) was coupled to amine **11** (22 mg, 0.075 mmol) using DEPC (0.008 ml, 0.059 mmol) and DIPEA (0.024 ml, 0.147 mmol) in DMF (0.5 ml) as described above for compound **10** to afford the corresponding amide product (50 mg, 0.042mol, 86% yield). LCMS (ESI) *m/z* calcd for $C_{61}H_{106}N_8O_{15}$ 1190.78, found 1191.8 $(M+H)^+$

LiOH (5.3 mg, 0.126 mmol) and H_2O (1ml) were added to a solution of the above-described amide (50 mg, 0.042 mmol) in MeOH-THF (1:1, 0.6 ml). After the mixture was stirred at a room temperature for 6 hrs, it was acidified using acetic acid. Solvents were removed under vacuum, and the residue was purified by preparative TLC using CH_2Cl_2-MeOH to afford compound **12** (42 mg, 0.035 mmol, 85% yield) LCMS (ESI) *m/z* calcd for $C_{60}H_{104}N_8O_{15}$ 1177.76, found 1178.8 $(M+H)^+$

1.6. Compound 13. Acid **12** (42 mg, 0.035 mmol) and amine **5b** (36 mg, 0.035 mmol) were coupled together using DEPC (0.008 ml, 0.05 mmol) and DIPEA (0.02 ml, 0.126 mmol) in DMF (0.5 ml) as described above for compounds **10** or **12** to afford the corresponding amide product (29 mg, 0.015 mmol, 44% yield). LCMS (ESI) *m/z* calcd for $C_{94}H_{149}N_{13}O_{22}S$ 1844.07, found 1845.3 $(M+H)^+$

tert-Butyl ester in the resulting amide product (29 mg, 0.015 mmol) was deprotected using CH_2Cl_2-TFA (1:1, 1ml), as described above for compound **7** or **10** to afford **13** as TFA salt (15 mg, 0.007 mmol, 47 % yield). LCMS (ESI) *m/z* calcd for $C_{89}H_{141}N_{13}O_{20}S$ 1744.01, found 1745.2 $(M+H)^+$

1.7. Compound 4. Compound **13**.TFA salt (15 mg, 0.008 mmol) and **8** (5.0 mg, 0.016mmol) were reacted together in CH_2Cl_2 (0.5ml) in the presence of Et_3N (0.01 ml, 0.08 mmol) overnight and the residues were purified by a preparative Silica gel TLC (CH_2Cl_2-MeOH), as described for compound **3**, to afford the title product **4** (10 mg, 0.005 mmol, 63 % yield). LCMS (ESI) *m/z* calcd for $C_{99}H_{152}N_{14}O_{23}S$ 1937.09, found 969.8 $(M+2H)^{+2}$, 647.1 $(M+3H)^{+3}$

2. Production of the SMI-Sap and SMI(AF)-Sap conjugates. Sap-C was produced and purified as described in Reference 3.

>Translation\of\pET-22b\Sap06\A157C+met
MVTSITLDLVNPTAGQYSSFVDKIRNNVKDPNLKYGGTDIAVIGPPSKEKFLRINFQSSRGTVSLGLKRD
NLYVVAYLAMDNTNVNRAYYFKSEITSAELTALFPEATTANQKALEYTEDYQSIEKNAQITQGDKSRKEL
GLGIDLLLTFMEAVNKK**C**RVVKNEARFLLIAIQMTAEVARFRYIQNLVTKNFPNKFDSDNKVIQFEVSWR
KISTAIYGDAKNGVFNKDYDFGFGKVRQVKDLQMGLLMYLGKPK

Sap-C (65 µl, 1.5 mg/ml) was incubated with TCEP.HCl in PBS buffer (8 µl, 50mM, pH 7.5) for 3 h at room temperature, and subsequently incubated with compound **3** (10 µl, 3 mM solution in 20%DMSO in PBS (pH 7.4)). Unreacted small molecule was removed using Superdex 75 (size exclusion chromatography) and eluting with PBS (pH 7.4) buffer to afford **SMI-Sap** (0.1 mg/ml) after purification.

Similarly, Sap-C (65 µl, 1.5 mg/ml), TCEP.HCl in PBS buffer (8 µl, 50mM, pH 7.5), and compound **4** (10 µl, 3 mM solution in 20%DMSO in PBS (pH 7.4)) were used, and crude mixture was purified using Superdex 75 and PBS buffer (pH 7.4) to afford **SMI(AF)-Sap** (0.16 mg/ml).

Sap-C, MS: 28622 (M), 28753 (M+Met)

SMI-Sap, MS: 29596 (M), 29727 (M+Met)

SMI(AF)-Sap, MS: 30560 (M), 30691 (M+Met)

3. Construction of cpAb 38C2-1, and evaluation of the cpAb binding to tumor cells expressing αvβ3, αvβ5, and/or αvβ6 integrin.

CpAb 38C2-1 was prepared using Ab38C2 and compound **1a**, as described.[2]

Flow cytometry. Cells were detached by brief trypsinization with 0.25% (w/v) trypsin, 1 mM EDTA, washed with PBS, and resuspended at $2x10^6$ cells/mL in ice cold flow cytometry buffer (for anti integrin binding: 1% BSA in TBS pH 7.4; for chemically programmed Abs (cpAbs): 1% BSA, 100 µM $MnCl_2$, TBS pH 7.4). Aliquots of 50 mL containing 10^5 cells were distributed into tubes for indirect immunofluorescence staining in the presence of 20 µg/mL of cpAbs or 10 µg/mL of anti-integrin mAbs. After incubation for 45 mins on ice and washing, cells were incubated with FITC or APC conjugated goat anti-mouse polyclonal antibodies (at a 1:100 dilution, *i.e.*, 10 mg/mL in FACS buffer) for 45 min on ice. After a final wash, cells were analyzed using flow cytometry using a FACScalibur (Becton-Dickinson) as described earlier.[20] All binding experiments were repeated at least three times at different time points using independent cell batches to determine the consistency of the results. Representative analyses are shown.

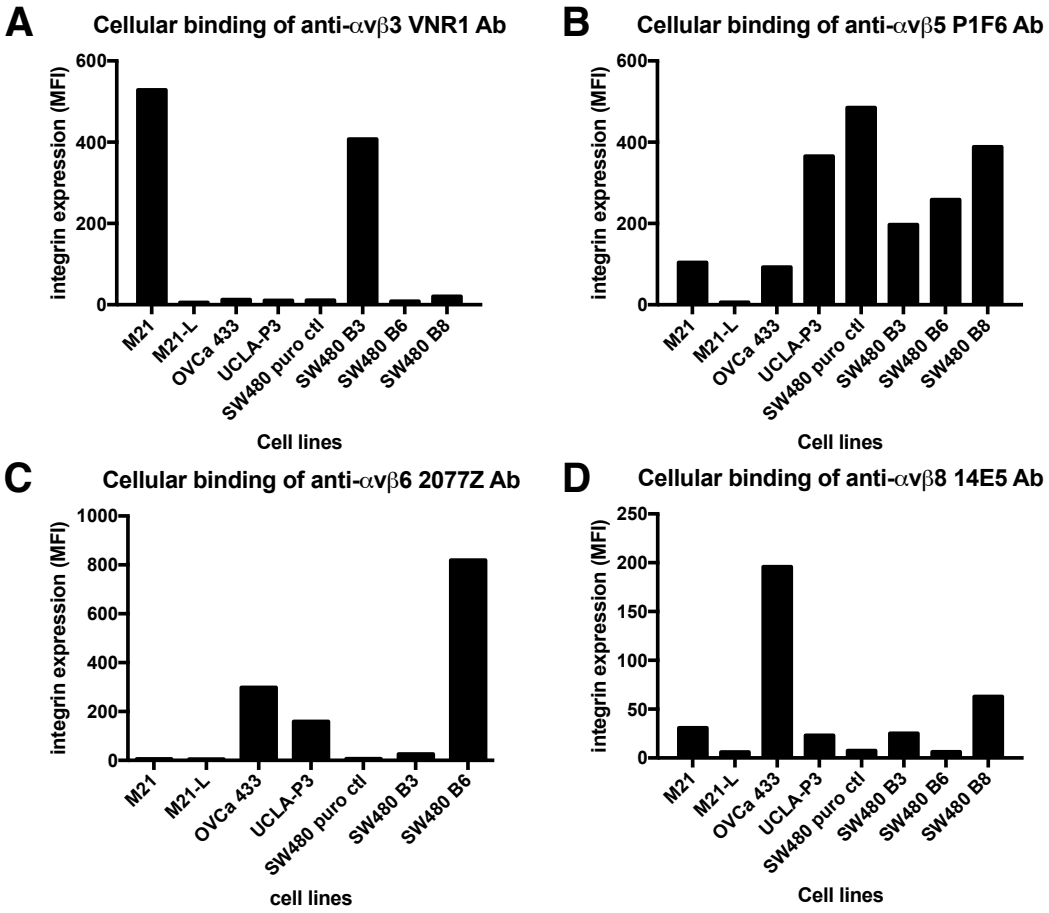


Figure S-1. Flow cytometry analysis showing expression level of (A) αvβ3, (B) αvβ5, (C) αvβ6, or (D) αvβ8 integrin in M21, M21-L, OVCa 433, UCLA-P3, SW480 puro ctl, SW480 B3, and SW480 B6 cells. Polyclonal mouse Ab VNR1 (for αvβ3), P1F6 (αvβ5), 2077Z (αvβ6), and 14E5 (αvβ8), were used at 10 μg/mL and APC labeled anti-mouse polyclonal Ab were used at a 1:100 dilution, i.e., 10 μg/mL in FACS buffer. The y-axis gives the relative mean fluorescence intensity in linear scale, and the x-axis describes cell line name.

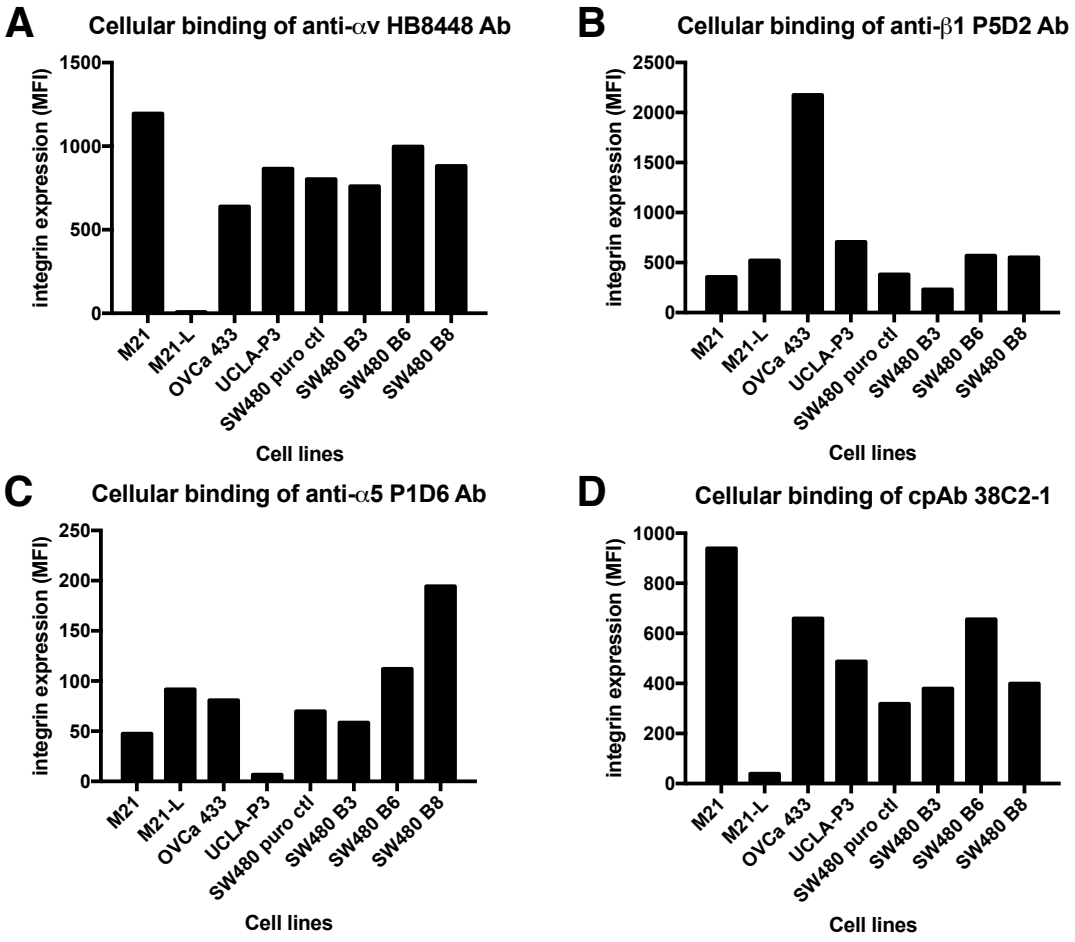


Figure S-2. Flow cytometry analysis showing (1) expression level of (A) αv, (B) β1, or (C) α5 integrin in M21, M21-L, OVCa 433, UCLA-P3, SW480 puro ctl, SW480 B3, and SW480 B6 cells, and (2) binding of (D) 38C2-**1** to these cells. Polyclonal mouse Ab HB8448 (for αv), P5D2 (β1), and P1D6 (α5) were used at 10 μg/mL, cpAb 38C2-**1** at 20 10 μg/mL, and APC labeled anti-mouse polyclonal Ab at a 1:100 dilution, i.e., 10 μg/mL in FACS buffer. The y-axis gives the relative mean fluorescence intensity in linear scale, and the x-axis describes cell line name.

4. Evaluation of SMI-Sap and SMI(AF)-Sap conjugates. M21, M21-L, MDA-MB-435 b3- and MDA-MB-435 ScrB3 cells were treated with SMI-Sap or SMI(AF)-Sap conjugates (0.156-20nM) in 24 well plates (5000 cells/well) for 72 hrs. Sap, SMI **1**, and compound **13** were also used as controls in some experiments.

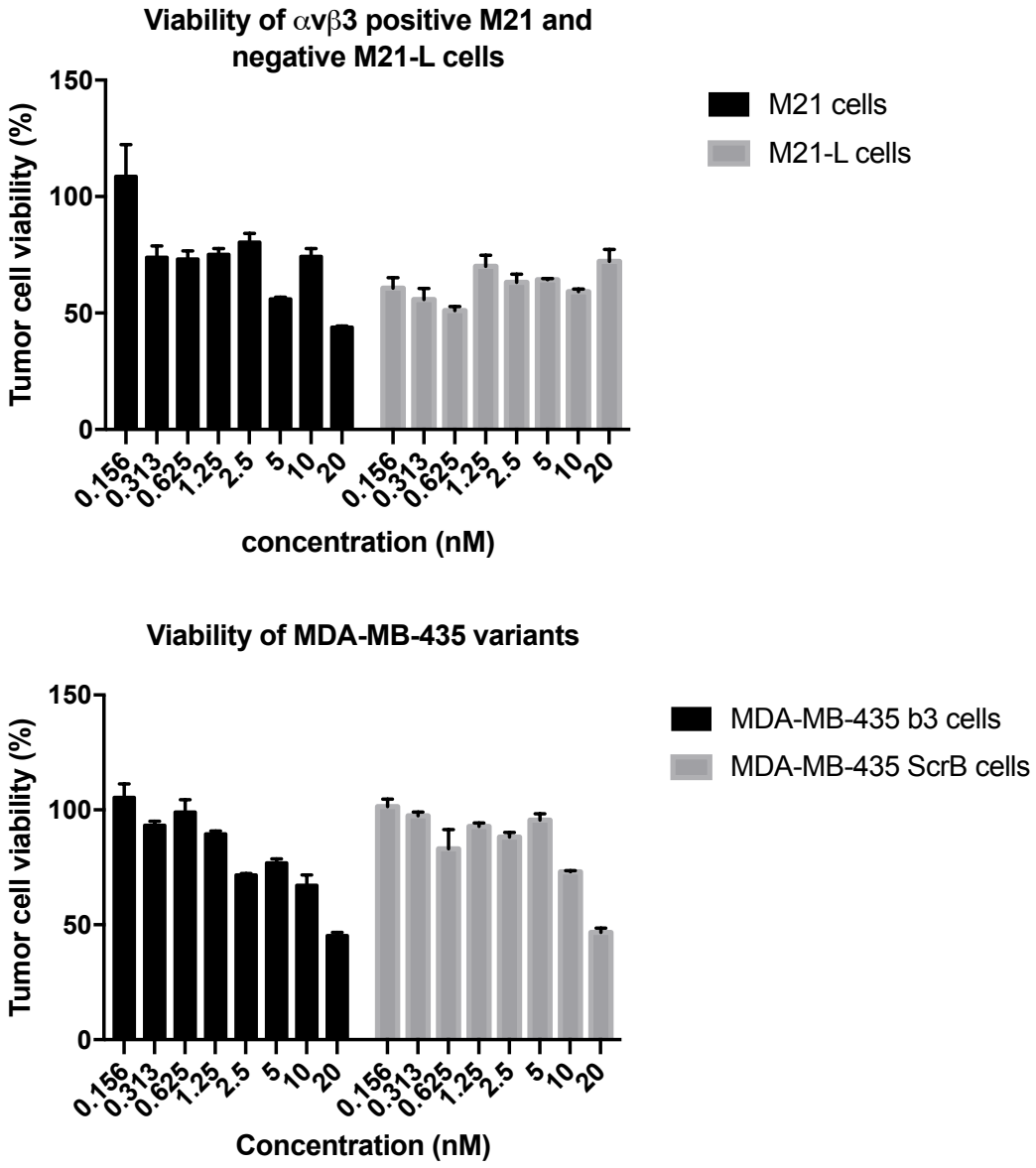


Figure S-3. Effect of compound **13** on viability and proliferation of M21 vs. M21-L cells (Upper) and of MDA-MB-435 b3- vs. ScrB cells. To measure effects of the compounds and conjugates on tumor cell viability and proliferation, $5x10^3$ cells were plated into 24 well plates and incubated with or without compounds and conjugates at various concentration. After 72 hrs, cells were harvested and counted. Live cells were identified and counted based on trypan blue exclusion. Cell-survival assay was performed once, and there were three replicates.

References.

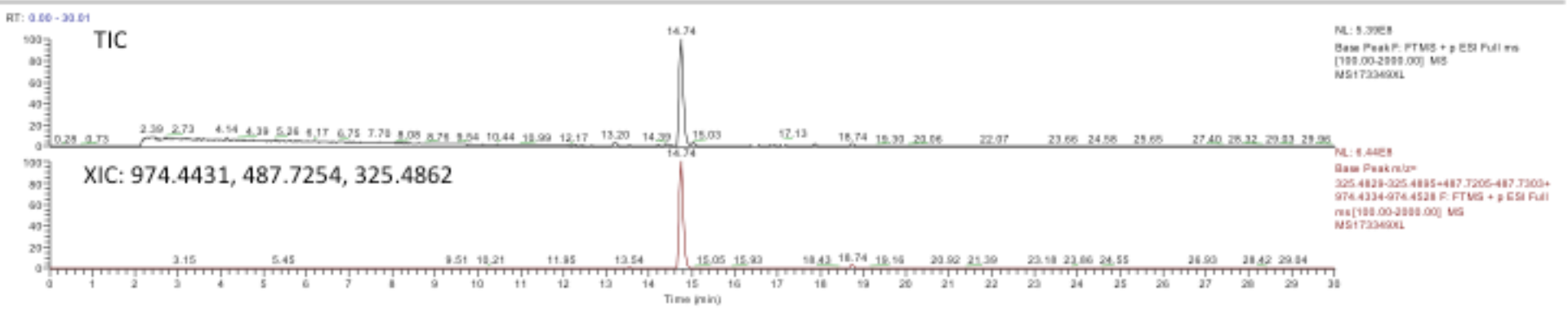
1) Liu, Y.; Bajjuri, K. M.; Liu, C.; Sinha, S. C. Targeting cell surface alpha(v)beta(3) integrin increases therapeutic efficacies of a legumain protease-activated auristatin prodrug. *Mol Pharm*., 2012, **9**, 168-75.

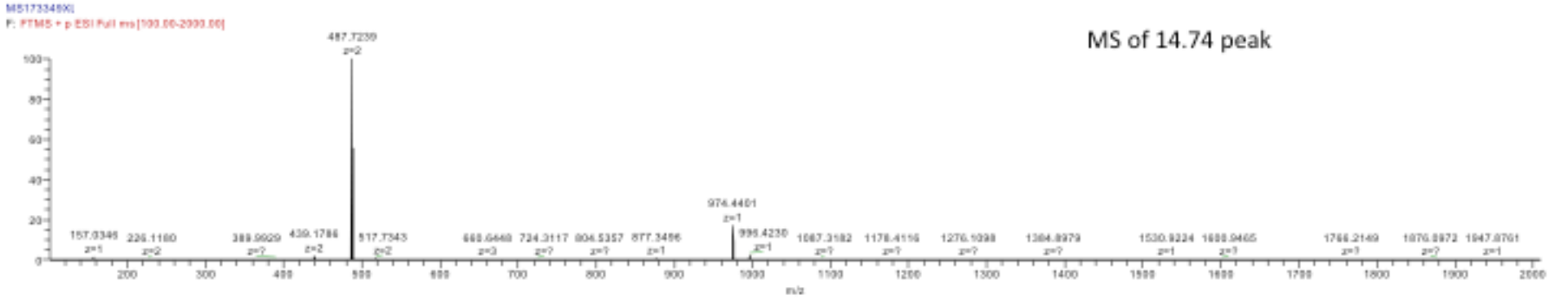
2) Goswami, R. K.; Bajjuri, K. M.; Forsyth, J. S.; Das, S.; Hassenpflug, W.; Huang, Z. Z.; Lerner, R. A.; Felding-Habermann, B.; Sinha, S. C. Chemically Programmed Antibodies Targeting Multiple Alpha(V) Integrins And Their Effects On Tumor-Related Functions In Vitro. *Bioconjug. Chem.* **2011**, *22*, 1535-1544.

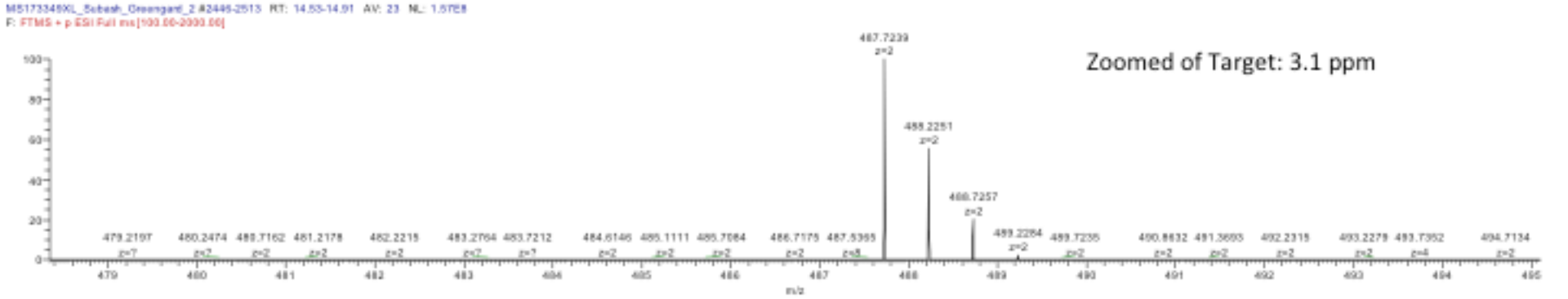
3) Hutchins, B. M.; Kazane, S. A.; Staflin, K.; Forsyth, J. S.; Felding-Habermann, B.; Smider, V. V.; Schultz, P. G. Selective formation of covalent protein heterodimers with an unnatural amino acid. *Chem. Biol.*, 2011, **18**, 299-303.

Compound-**3** (SMI-Mal)

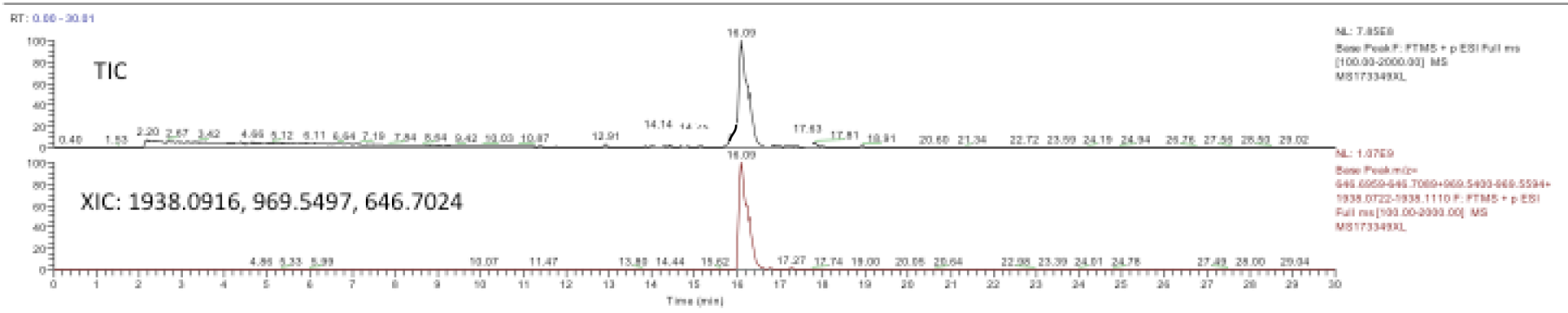
MS173349XL

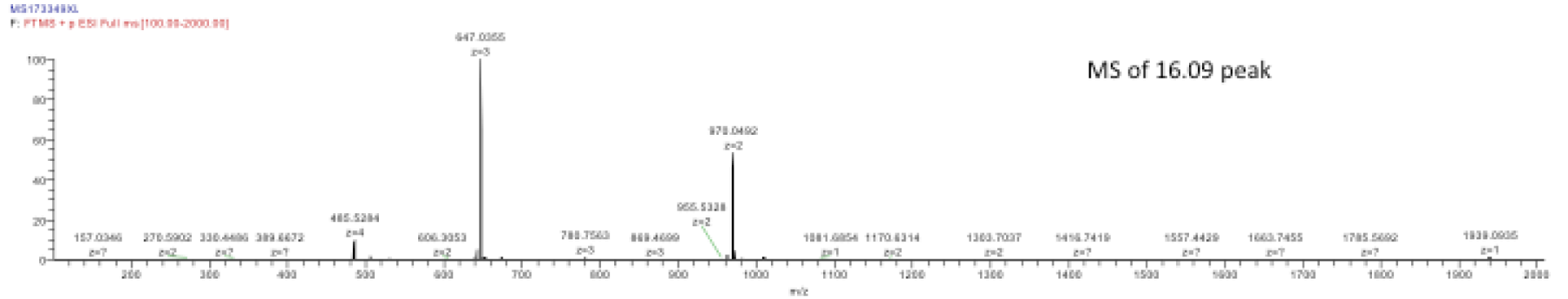


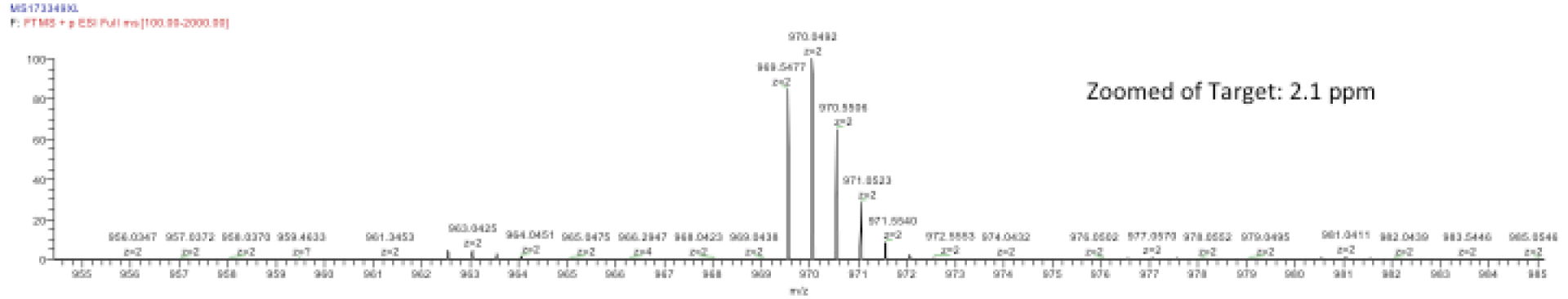




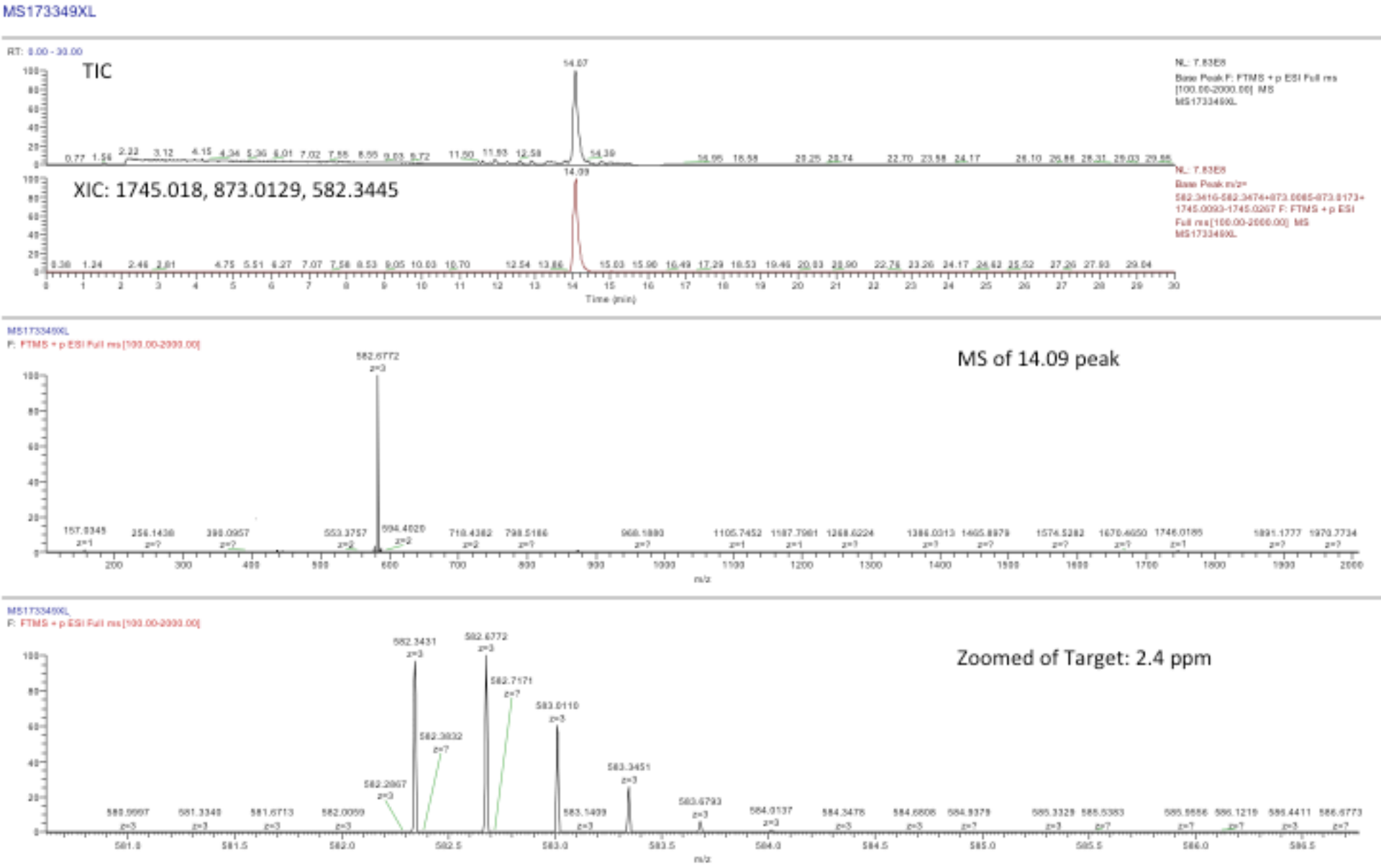
Compound-**4** (SMI(AF)-Mal)







Compound-**13**


MS173349XL
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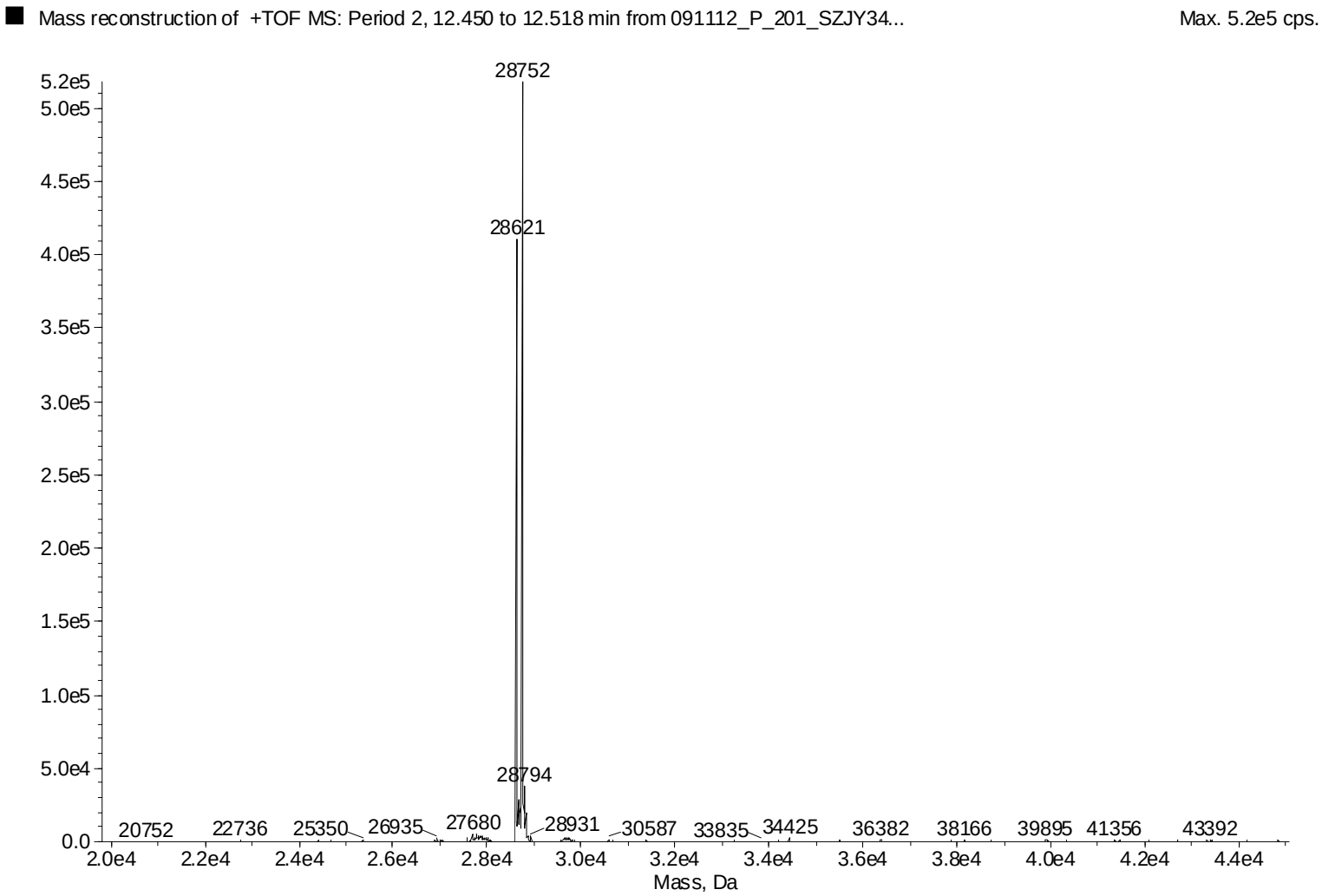
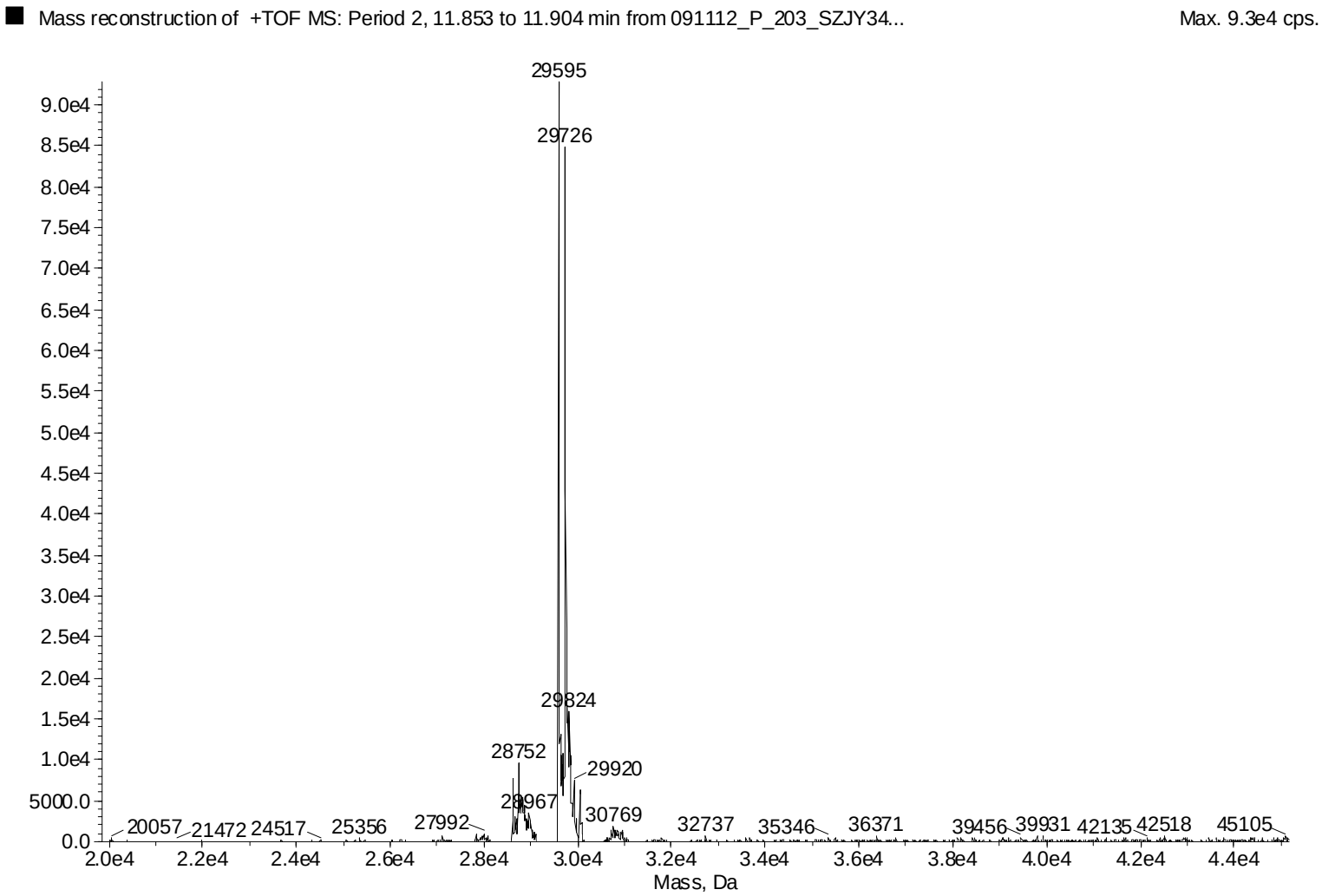
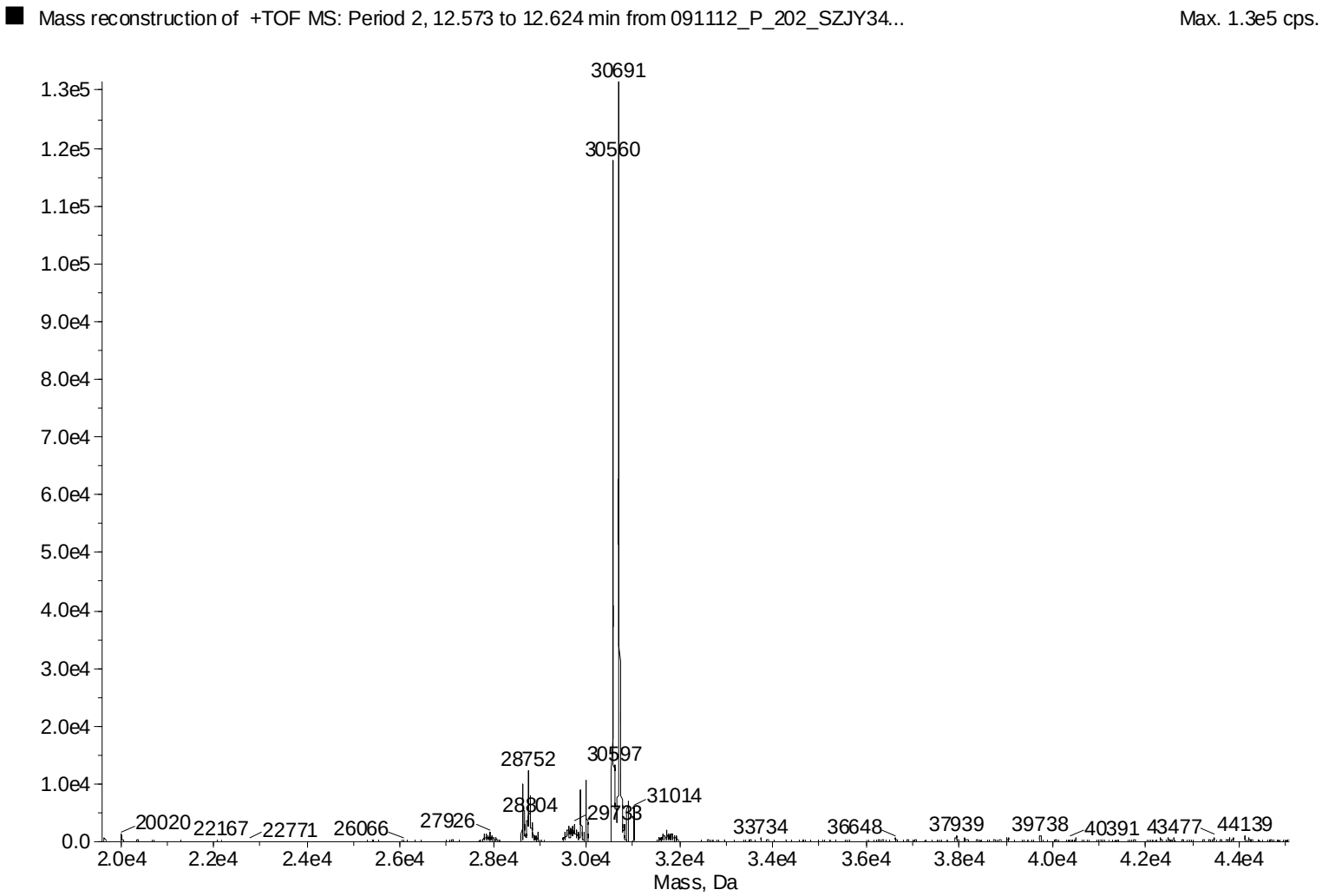
MS of 14.09 peak
Zoomed of Target: 2.4 ppm
m/z



Mass Spectrum of Sap *A157C*



Mass Spectrum of SMI-Sap



Mass Spectrum of SMI(AF)-Sap