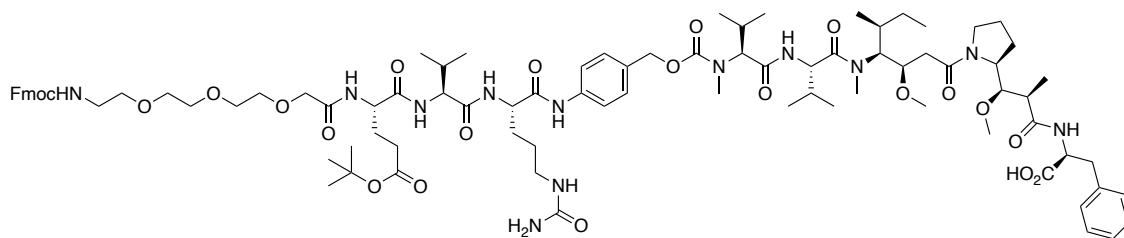
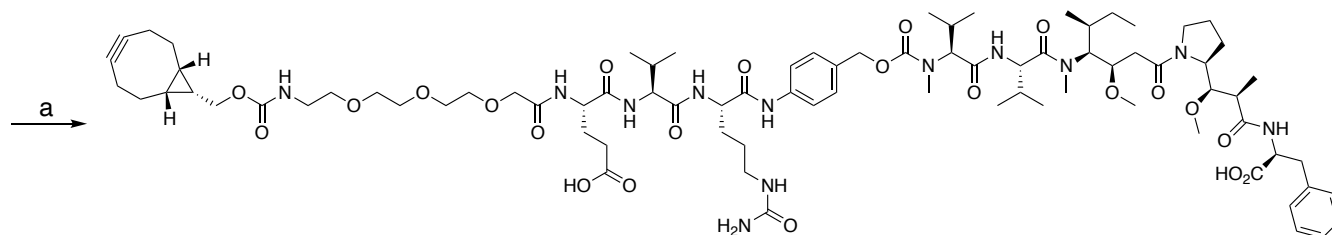


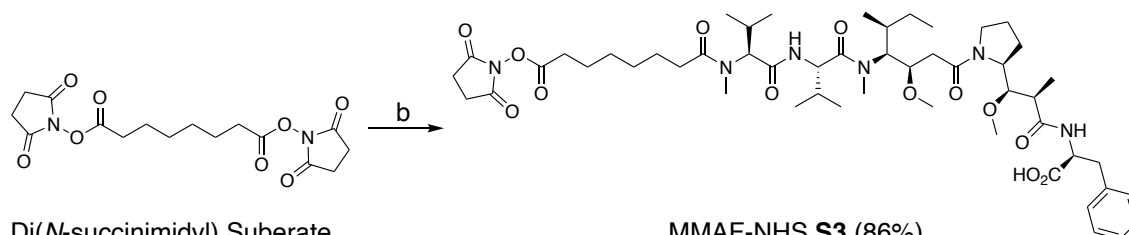
Supplemental Data S1 Synthetic schemes and characterization of chemical compounds and Cy5.5 labeled antibody–drug conjugates. Related to the STAR Methods.



S1



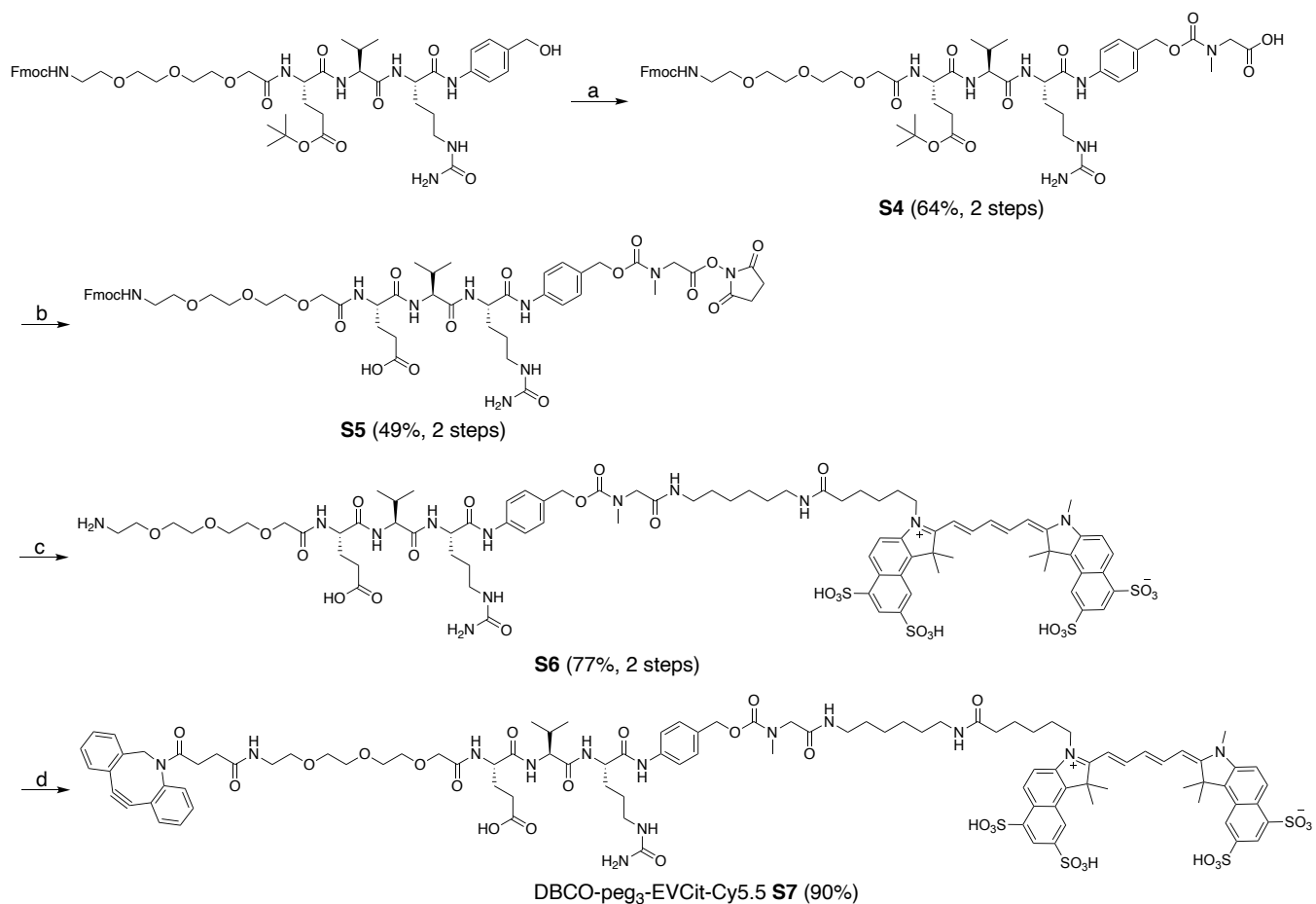
BCN-peg₃-EVCit-MMAF **S2** (36%, 3 steps)



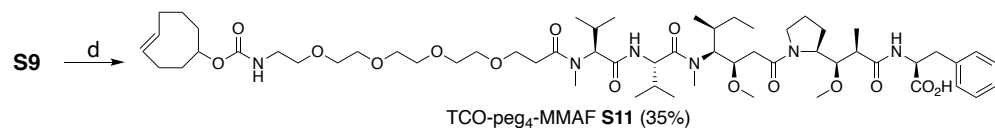
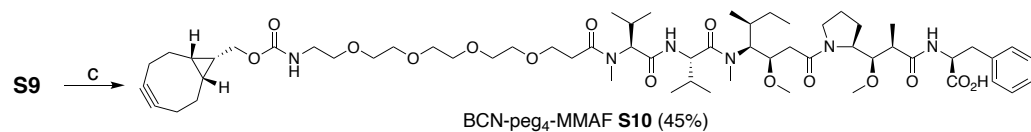
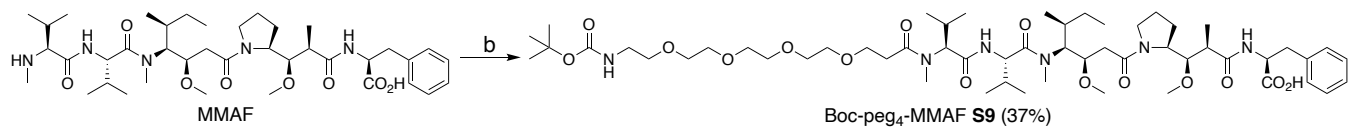
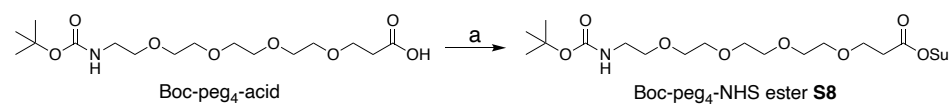
Di(*N*-succinimidyl) Suberate

MMAF-NHS **S3** (86%)

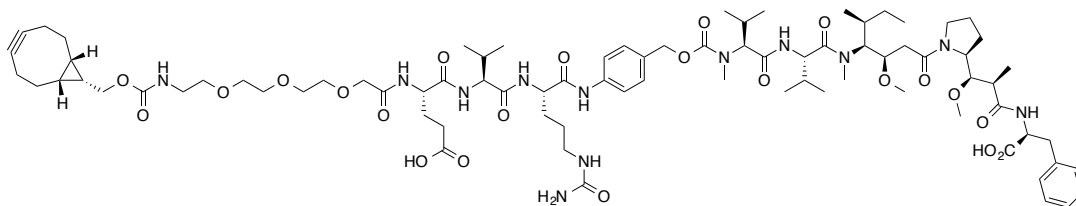
Synthesis of BCN-EVCit-MMAF module S2 and MMAF-NHS ester (S3). Reagents and conditions: (a) 50% diethylamine/DMF, rt, 1 h, and 50% TFA/DCM, rt, 1 h, then MMAF, DIPEA, HOAt, DMF, rt, overnight. (b) MMAF, DIPEA, HOAt, di(*N*-succinimidyl) suberate, 37 °C, overnight.



Synthesis of DBCO-EVCit-Cy5.5 module S7. Reagents and conditions: (a) bisPNP, DMAP, DMF, rt, 2 h, then sarcosine, rt, 1h. (b) NHS, EDC·HCl, DCM, then 10% TFA/DCM. (c) Cy5.5-amine, DIPEA, rt, overnight, then diethylamine. (d) DBCO-NHS, DIPEA, DMF, DMSO, rt, overnight.

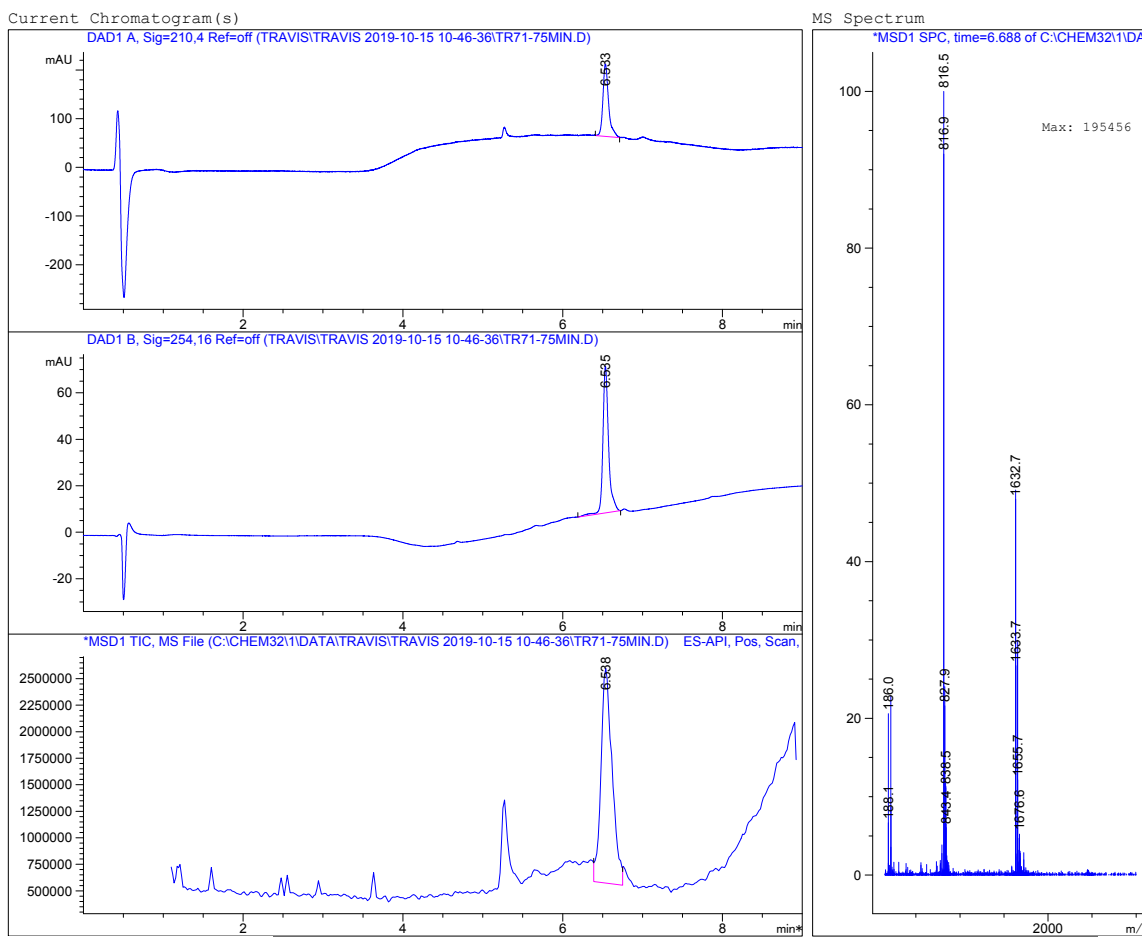


Synthesis of non-cleavable MMAF modules S10 and S11. Reagents and conditions: (a) NHS, EDC·HCl, DCM, rt, overnight. (b) **S8**, HOAt, DIPEA, DMF, 37 °C, overnight. (c) 50% TFA/DCM, rt, 30 min, then BCN-NHS, DIPEA, DMF, rt, 3 h. (d) 50% TFA/DCM, rt, 30 min, then TCO-NHS, DIPEA, DMF, rt, 3 h.



BCN-peg₃-EVCit-PABC-MMAF (S2)

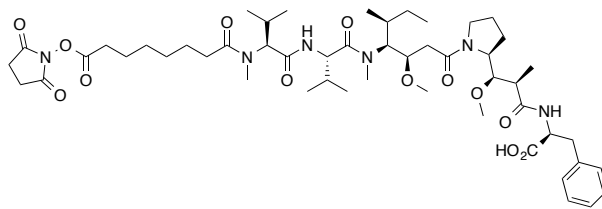
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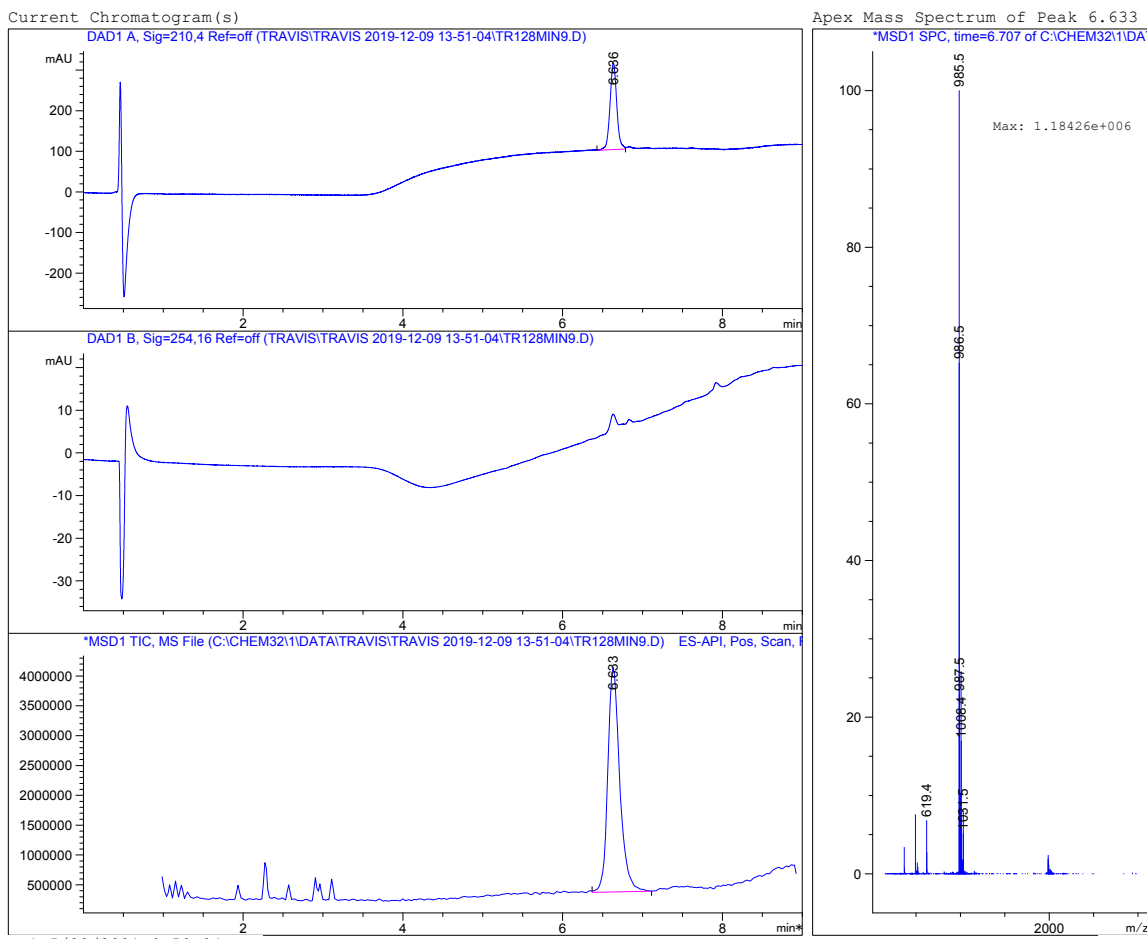
Page 1 of 1

HPLC trace and low-resolution ESI-MS spectrum of pure product S2.



MMAF-NHS ester (**S3**)

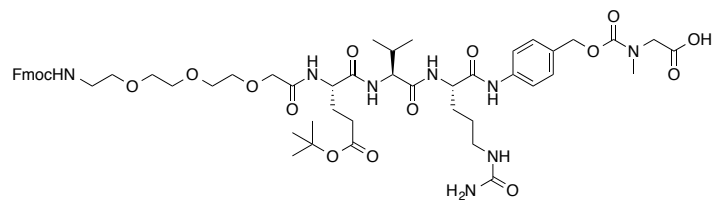
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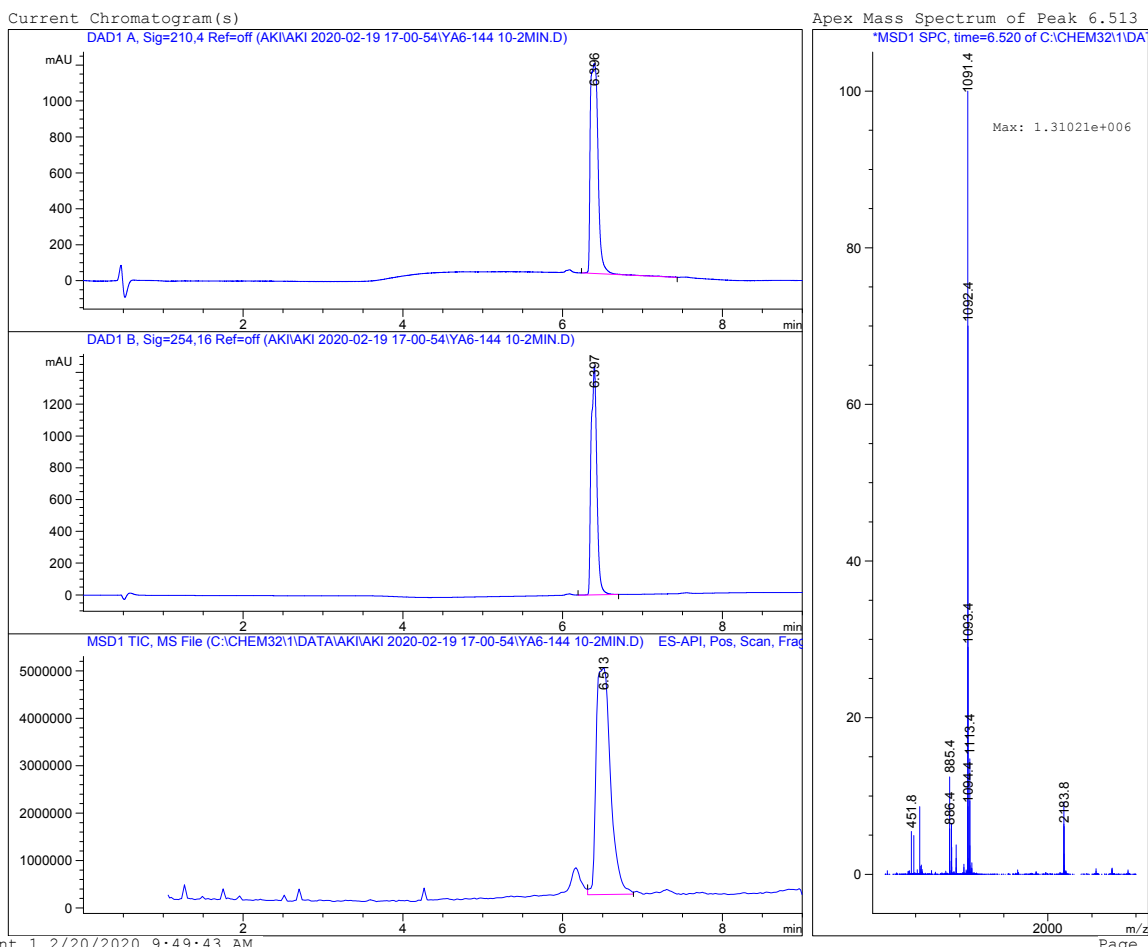
Page 1 of 1

HPLC trace and low-resolution ESI-MS spectrum of pure product S3.

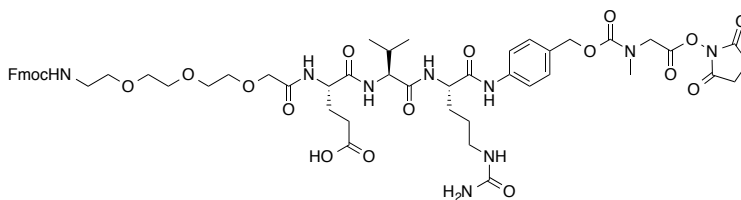


Fmoc-peg₃-E(Ot-Bu)VCit-PABC-sarcosine (**S4**)

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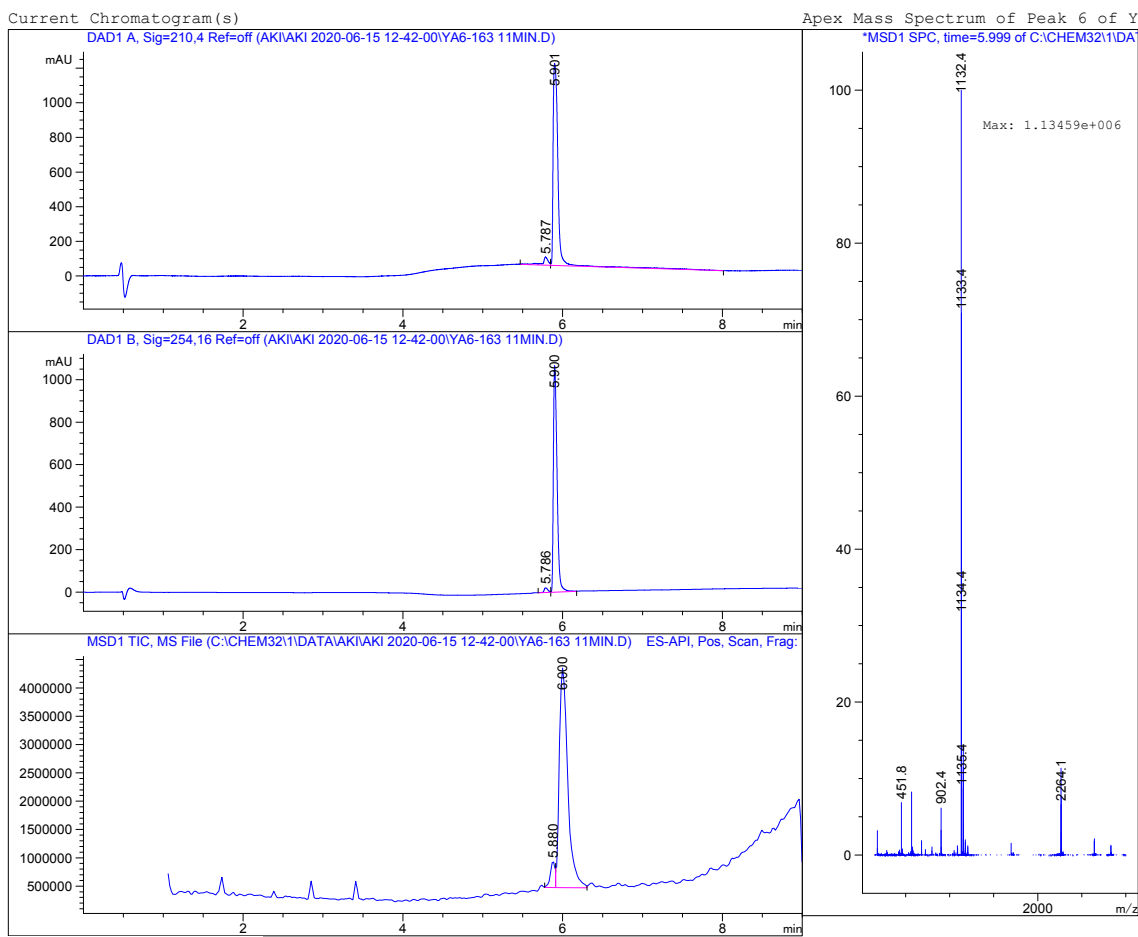


HPLC trace and low-resolution ESI-MS spectrum of pure product **S4**.

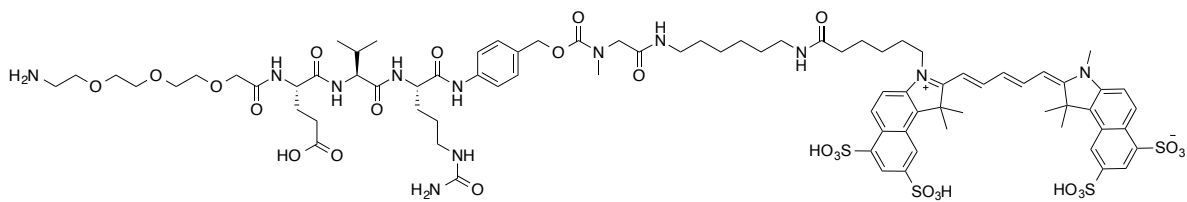


Fmoc-peg₃-EVCit-PABC-sarcosine NHS ester (**S5**)

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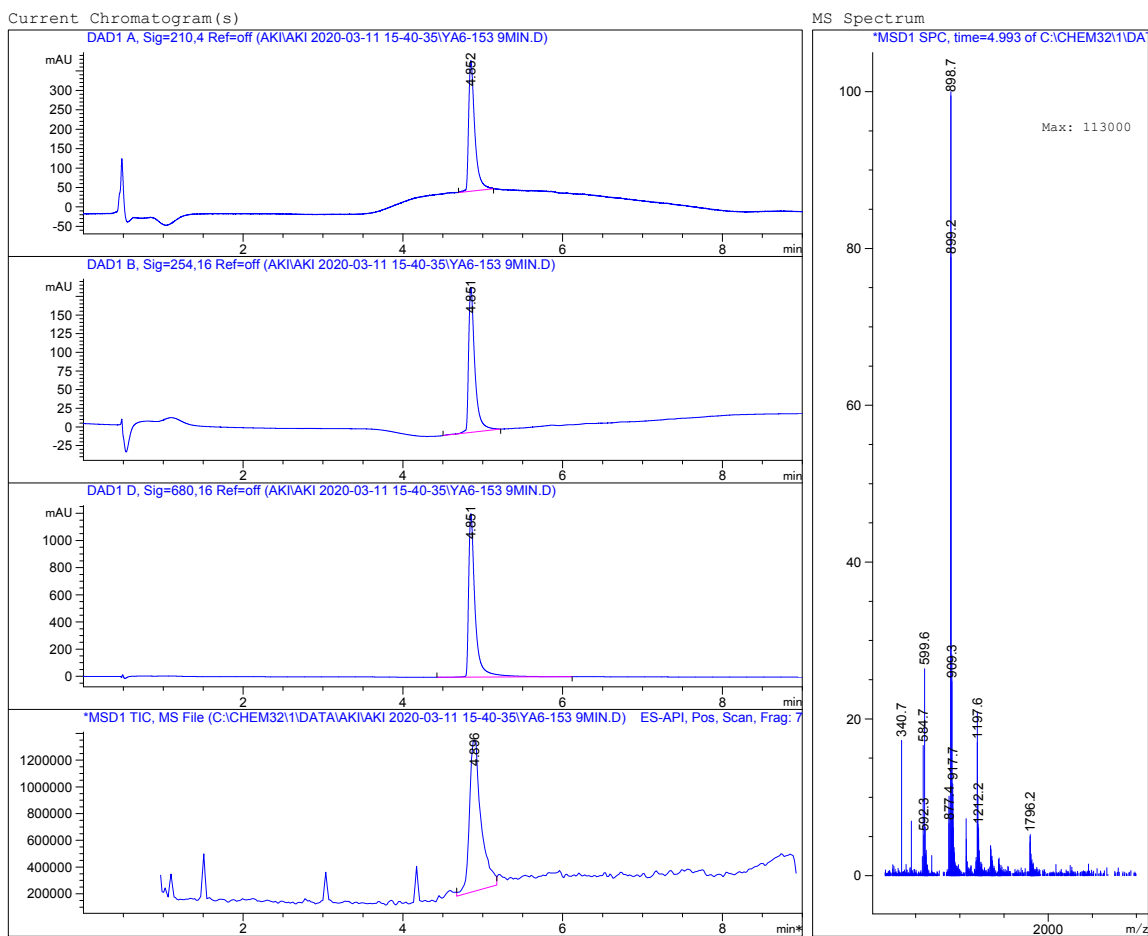


HPLC trace and low-resolution ESI-MS spectrum of pure product S5.

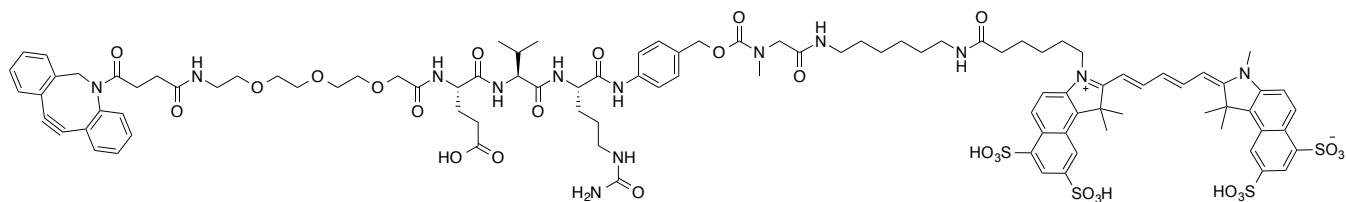


H₂N-peg₃-EVCit-PABC-sarcosine-Cy5.5 (S6)

Print of all graphic windows

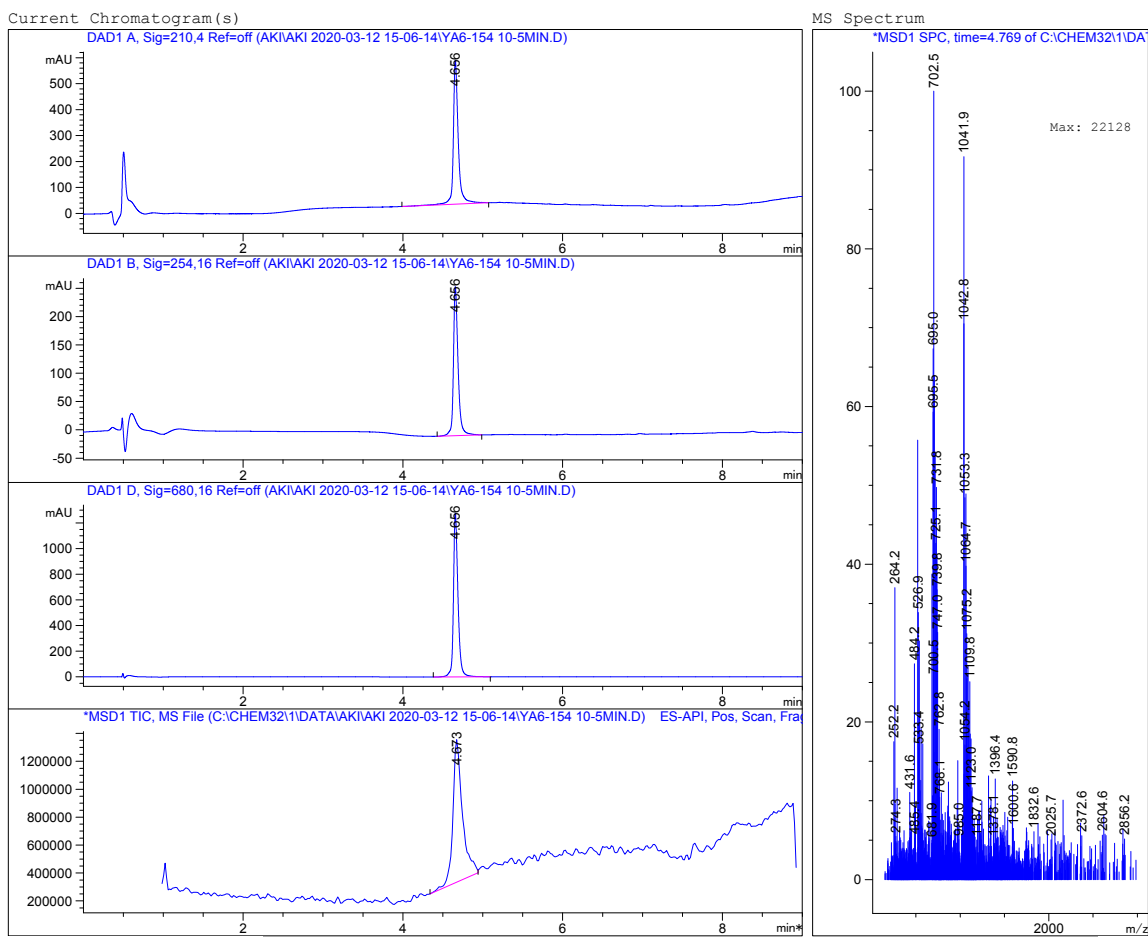


HPLC trace and low-resolution ESI-MS spectrum of pure product S6.



DBCO-peg₃-EVCit-PABC-sarcosine-Cy5.5 (**S7**)

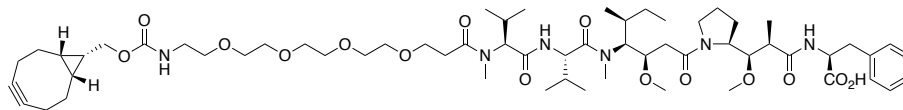
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Page 1 of 1

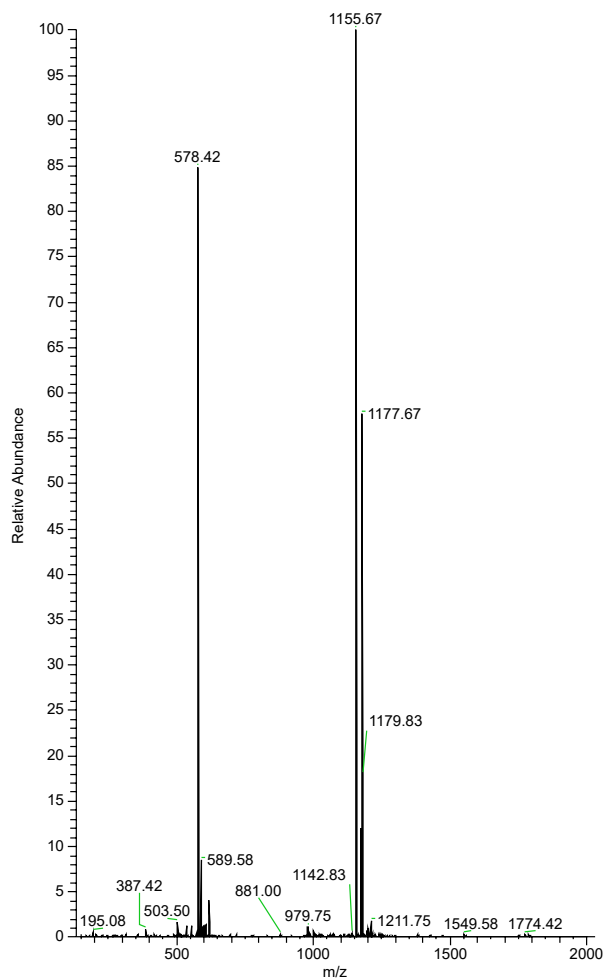
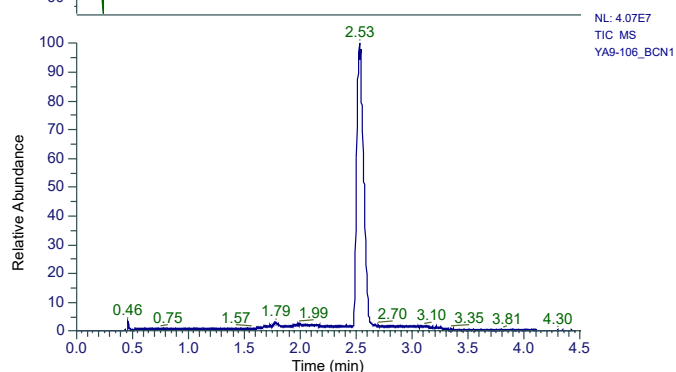
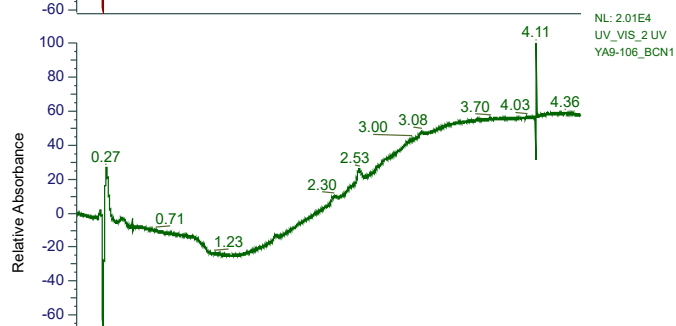
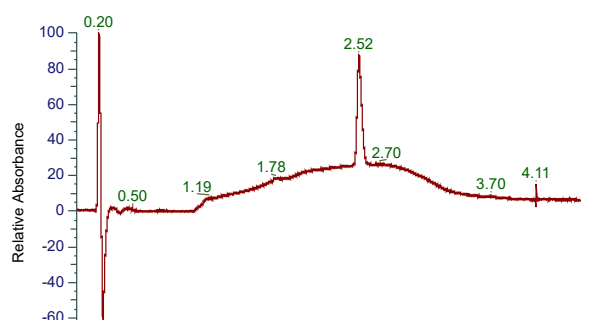
HPLC trace and low-resolution ESI-MS spectrum of pure product **S7**.



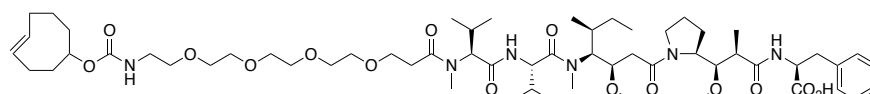
BCN-peg₄-MMAF (S10)

RT :0.00-4.50

YA9-106_BCN1 #620 RT: 2.52 AV: 1 NL: 1.11E+006
T: ITMS + p ESI Full ms [150.00-2000.00]



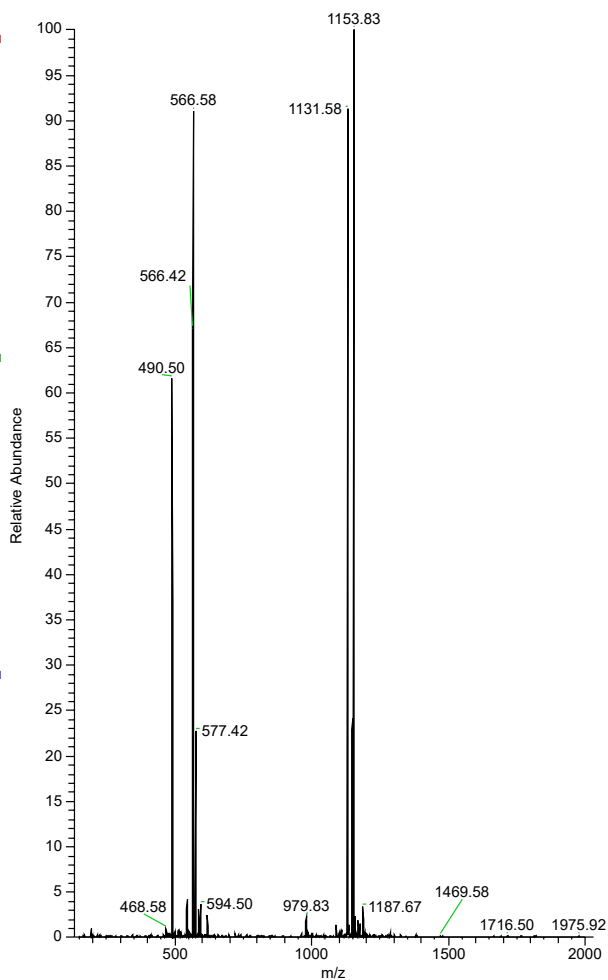
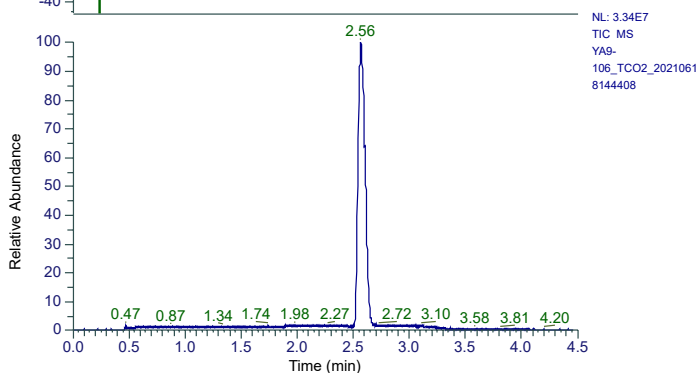
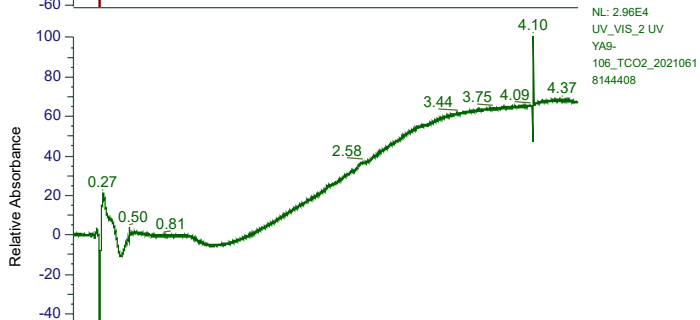
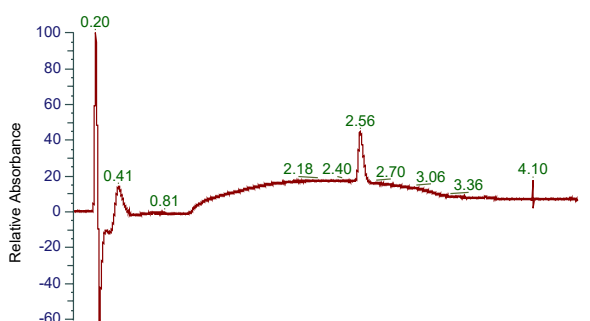
HPLC trace and low-resolution ESI-MS spectrum of pure product S10.



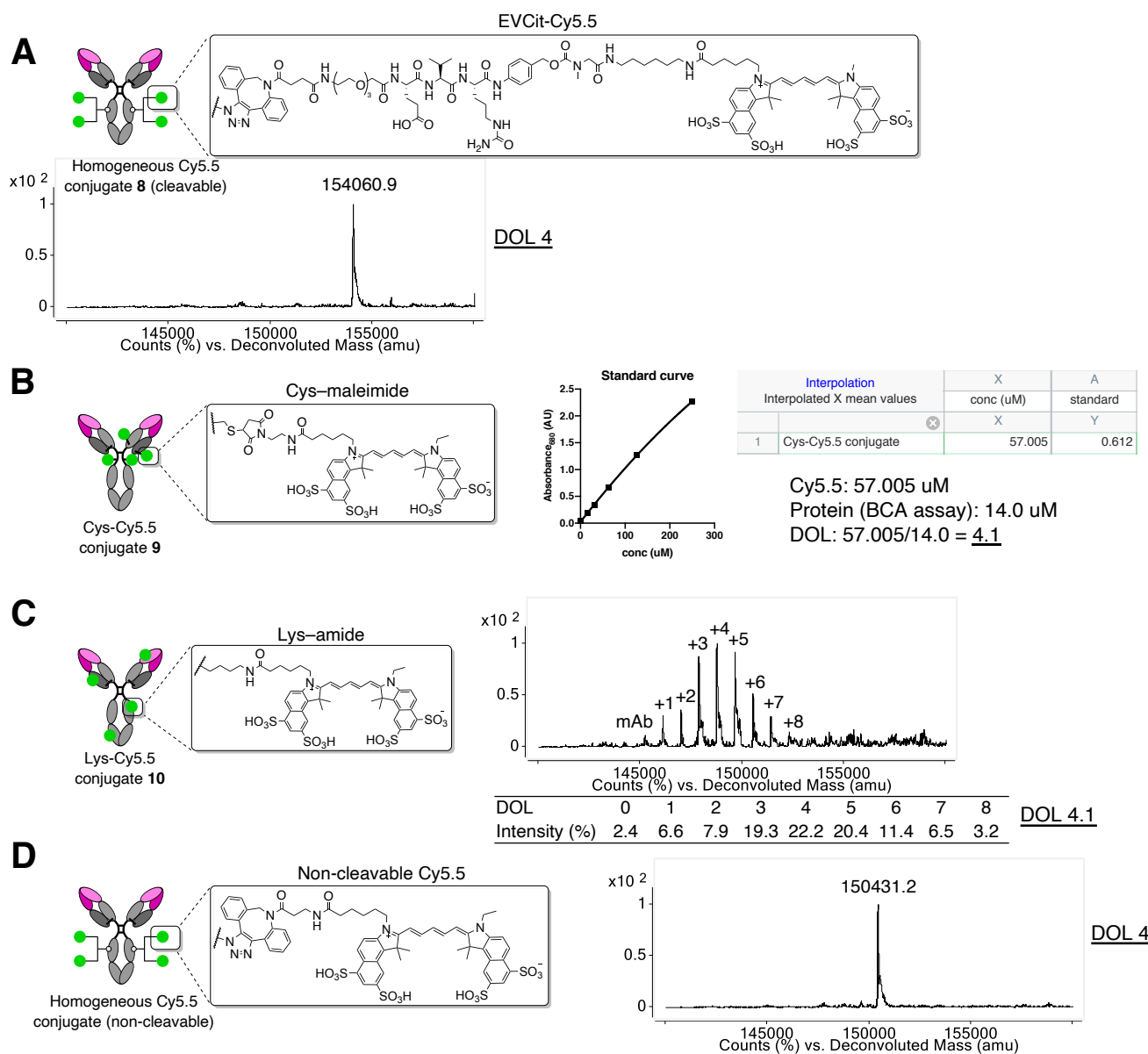
TCO-peg₄-MMAF (S11)

RT: 0.00-4.50

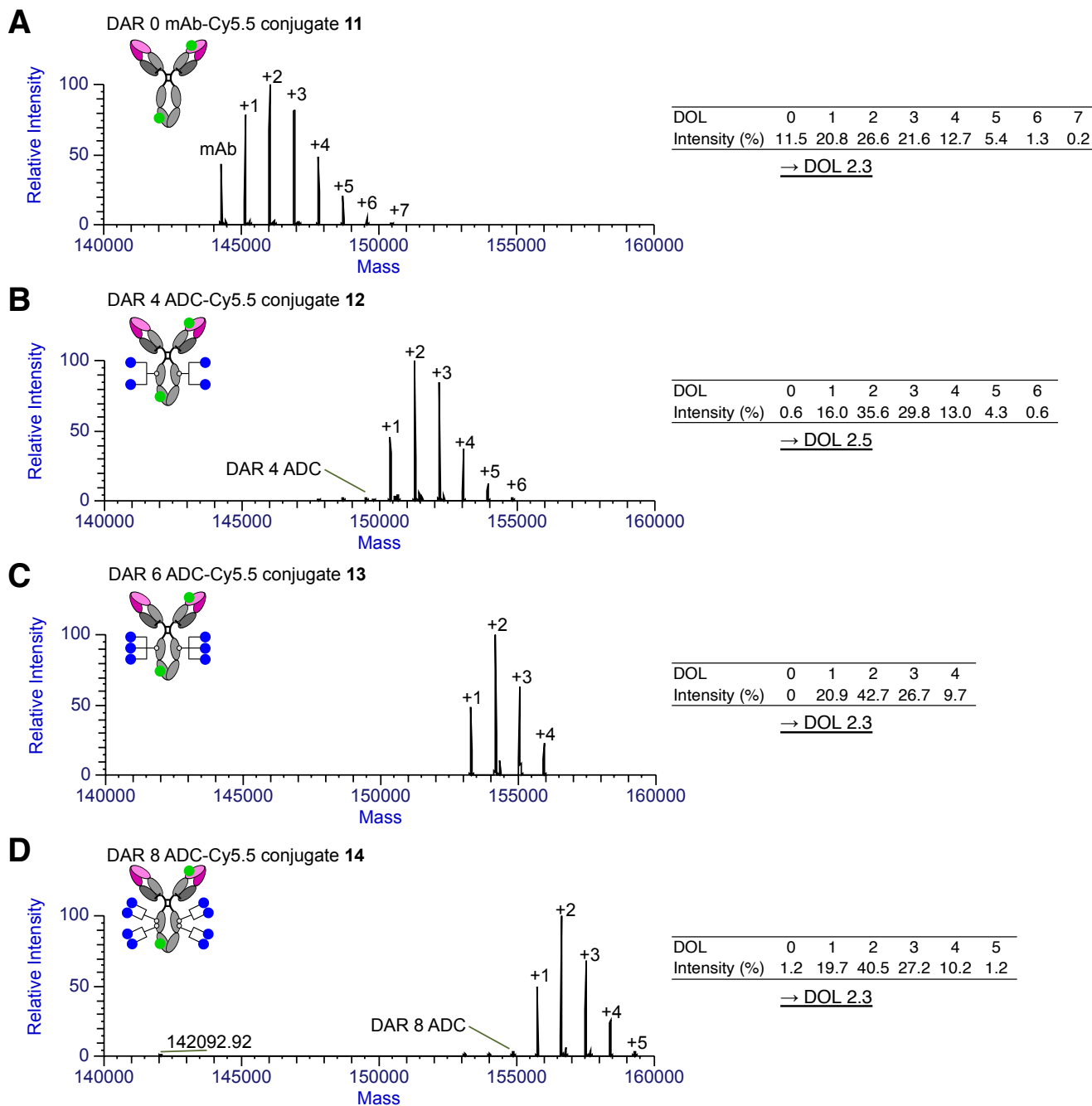
YA9-106_TCO2_20210618144408 #621 RT: 2.57 AV: 1 NL: 6.43E+005
T: ITMS + p ESI Full ms [150.00-2000.00]



HPLC trace and low-resolution ESI-MS spectrum of pure product S11.



Characterization of Cy5.5 conjugates 8–10. (A) ESI-MS analysis of homogeneous cleavable Cy5.5 conjugate **8** with a DOL of 4. (B) Determination of DOL of Cys-Cy5.5 conjugate **9**. Protein concentration was determined by BCA assay and the molar amount of conjugated Cy5.5 was calculated using a free Cy5.5-based standard curve. The average DOL was then determined to be 4.1 based on the ratio of those values. (C) ESI-MS analysis of Lys-Cy5.5 conjugate **10**. The average DOL was determined to be 4.1 based on the ion intensity of each DOL species. (D) ESI-MS analysis of the homogeneous non-cleavable Cy5.5 conjugate with a DOL of 4.



Characterization of Cy5.5-labeled MMAF conjugates 11–14. (A) ESI-MS spectra of anti-EGFRvIII mAb-Cy5.5 conjugate **11** with an average DOL of 2.3, (B) DAR 4 MMAF-Cy5.5 conjugate **12** with an average DOL of 2.5, (C) DAR 6 MMAF-Cy5.5 conjugate **13** with an average DOL of 2.3, and (D) DAR 8 MMAF-Cy5.5 conjugate **14** with an average DOL of 2.3.