Supporting information

Exo-Cleavable Linkers: A Paradigm Shift for Enhanced Stability and Therapeutic Efficacy in Antibody-Drug Conjugates

Tomohiro Watanabe[†1], Naoko Arashida, Tomohiro Fujii[†]*, Natsuki Shikida[†], Kenichiro Ito[†], Kazutaka Shimbo[†], Takuya Seki[†], Yusuke Iwai[†], Ryusuke Hirama[†], Noriko Hatada[†], Akira Nakayama[†], Tatsuya Okuzumi[†], Yutaka Matsuda*[‡1]

[1] These authors contributed equally.

* Corresponding authors

TF: tomohiro.fujii.3m2@asv.ajinomoto.com

YM: Yutaka.Matsuda@US.AjiBio-Pharma.com

[1] These authors contributed equally.

[†]Ajinomoto Co., Inc., 1-1, Suzuki-Cho, Kawasaki-Ku, Kawasaki-Shi, Kanagawa 210-8681, Japan.

[‡]Ajinomoto Bio-Pharma Services, 11040 Roselle Street, San Diego, CA 92121, United States.

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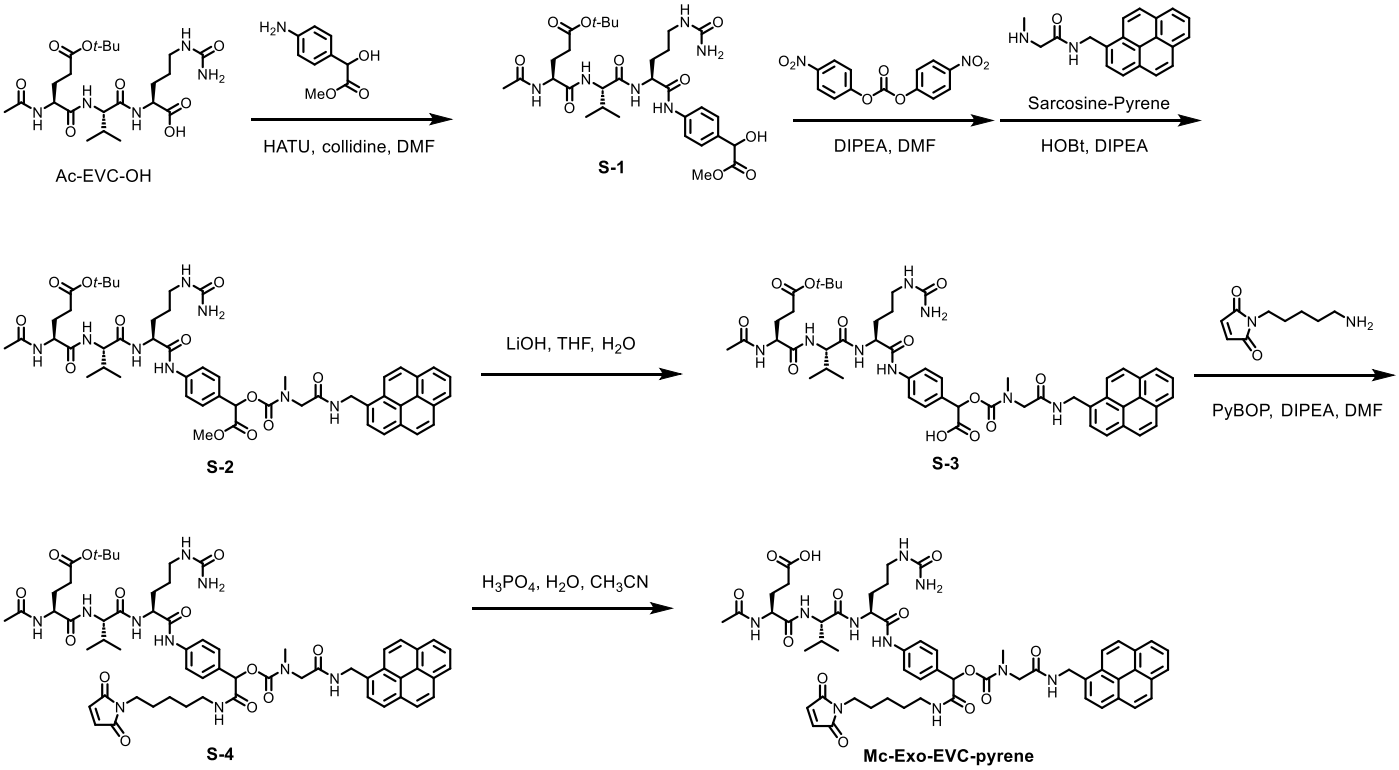
1. Experimental procedure

1-1 Materials

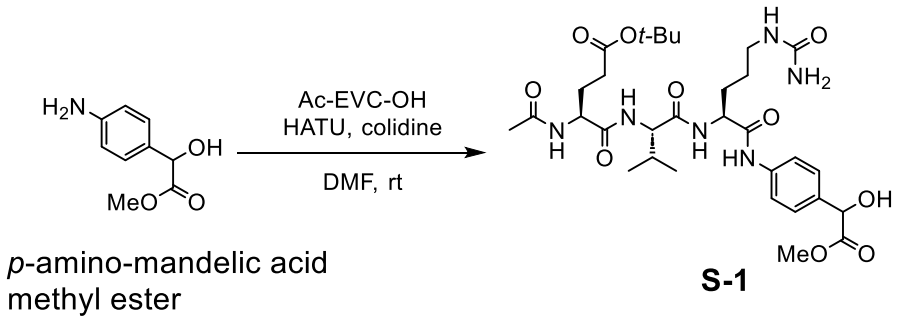
Human IgG1 trastuzumab (Herceptin®) was purchased from the Roche Pharmaceutical Company (Switzerland). Trastuzumab-emutansine (Kadcyla) and trastuzumab deruxtecan (Enhertu) were purchased from WEP clinical (USA). Tri- or tetra peptides for exo-linker were prepared by solid-phase peptide synthesizer based on the previous report [1]. AJICAP peptide reagent was prepared based on the previous report [2]. Trastuzumab-stochastic-MMAE (ADC 9) was synthesized in previous report [3]. All other chemical reagents were purchased from Sigma-Aldrich (USA).

1-2 Payload-linker synthesis

1-2-1 Mc-Exo-EVC-pyrene synthesis

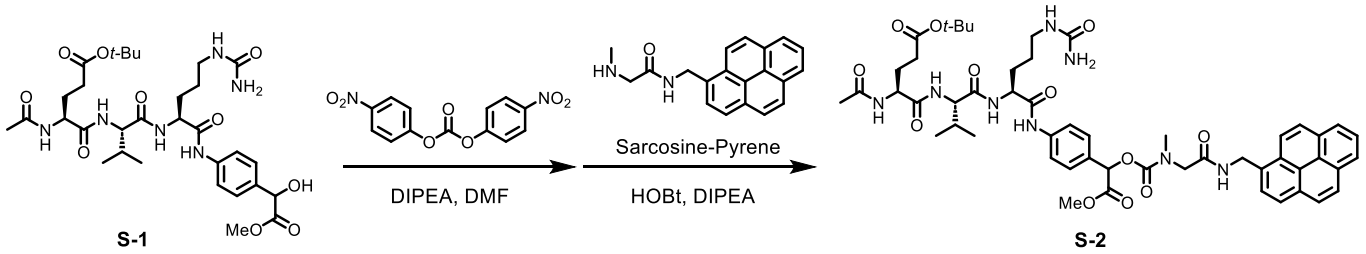


Scheme S-1. Synthesis of Mc-Exo-EVC-pyrene

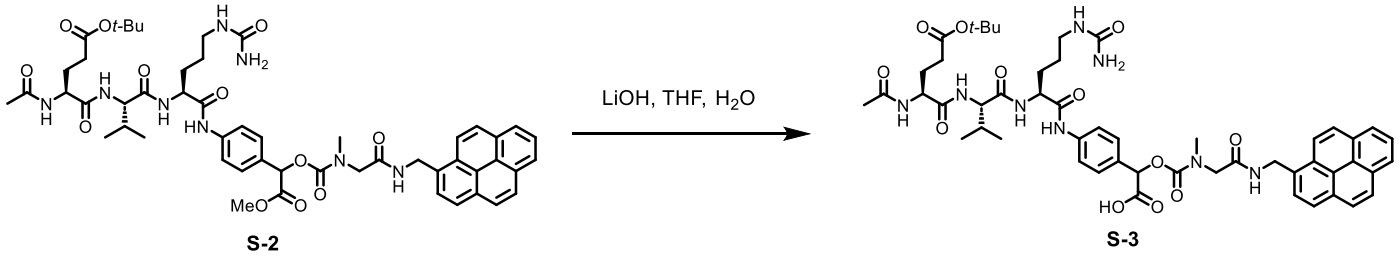


Ac-Glu(OtBu)-Val-Cit-OH (19.9 mg, 39.7 μmol) was dissolved in DMF (400 μL) and 1-[bis(dimethylamino)methylene]-1H-1, 2,3,triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (18.1 mg, 47.6 μmol) and 2,4,6-trimethylpyridine (6.27 μL, 47.6 μmol) were added, Stir at room temperature for 10 minutes. Subsequently, *p*-amino-mandelic acid methyl ester [4] (8.63 mg, 47.6 μmol) was added, and the mixture was stirred at room temperature for 21.5 h, and then purified by reverse phase preparative chromatography.

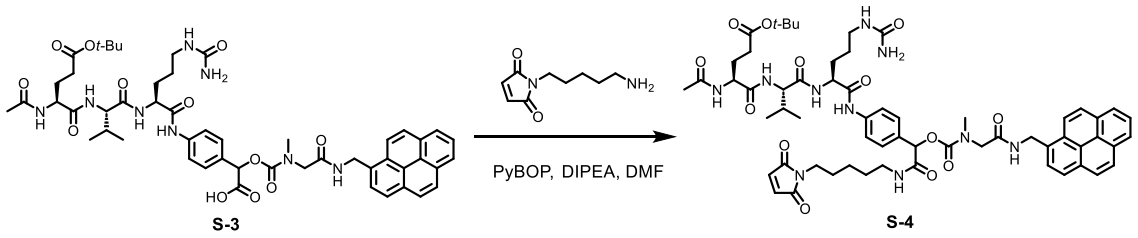
The fraction containing the product was collected, concentrated under reduced pressure to remove acetonitrile, and freeze-dried to obtain the compound **S-1** (28.5 mg, quant).
MS (ESI) m/z: 665.30 $[M+H]^+$



The compound **S-1** (28.5 mg) was dissolved in DMF (430 μL), stirred for 5 min at rt , and then bis(4-nitrophenyl carbonate (26.6 mg, 85.7 μmol) and DIPEA (11.1 μL, 64.4 μmol) were added, and the mixture was stirred at rt for 5 h. Then, after cooling with ice, sarcosine-pyrene [5] (64.9 mg, 215 μmol), 1-hydroxybenzotriazole (8.7 mg, 64 μmol) and DIPEA (57.2 μL, 333 μmol) were added. Stirred for 12 h. After the reaction, the crude product was purified by reverse phase preparative chromatography. Fractions containing the product were collected, concentrated under reduced pressure to remove acetonitrile, and freeze-dried to obtain pyrene **S-2** (26.1 mg, 66%).
MS (ESI) m/z: 993.40 $[M+H]^+$



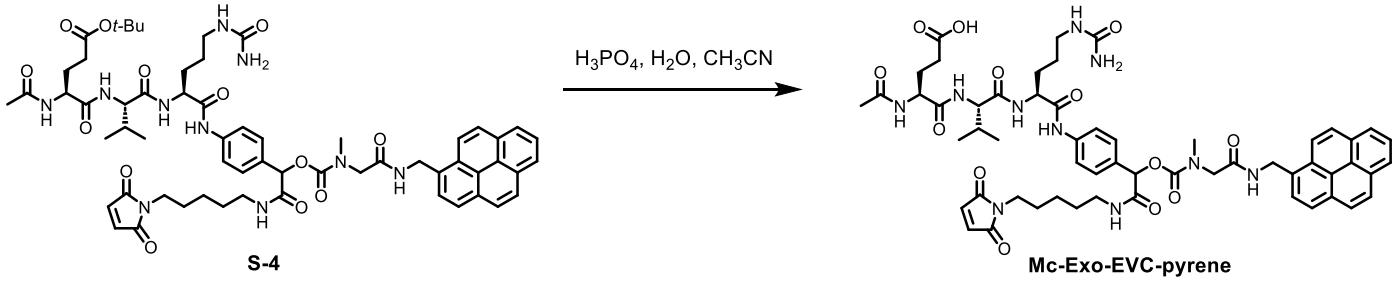
Pyrene **S-2** (10.8 mg, 10.9 μmol) was dissolved in THF (700 μL) and water (400 μL), stirred for 5 minutes under ice-cooling, 1 M lithium hydroxide aqueous solution (109 μL, 109 μmol) was added, and the mixture was stirred at rt for 1 h. After completion of the reaction, the pH was adjusted to about 6.0 using 0.1 M hydrochloric acid, and the product was purified by reverse phase preparative chromatography. Fractions containing the product were collected, concentrated under reduced pressure to remove acetonitrile, and freeze-dried to obtain pyrene **S-3** (4.5 mg, 42%).
MS (ESI) m/z: 979.40 $[M+H]^+$



S-3 S-4

Pyrene **S-3** (3.7 mg, 3.8 μmol) was dissolved in DMF (400 μL), cooled with ice, and DIPEA (1.9 μL, 11 μmol) and 1H-benzotriazole -1-yloxytripyrrolidinophosphonium hexafluorophosphate (2.9 mg, 5.6 μmol) was added. Next, N-(5-aminopentyl)maleimide hydrochloride (1.3 mg, 5.7 μmol) was added and the mixture was warmed to room temperature and stirred for 2 h. After completion of the reaction, the product was purified by reverse phase preparative chromatography. Fractions containing the product were collected, concentrated under reduced pressure to remove acetonitrile, and freeze-dried to obtain pyrene **S-4** (1.3 mg, 29%).

MS (ESI) m/z: 1143.45 $[M+H]^+$

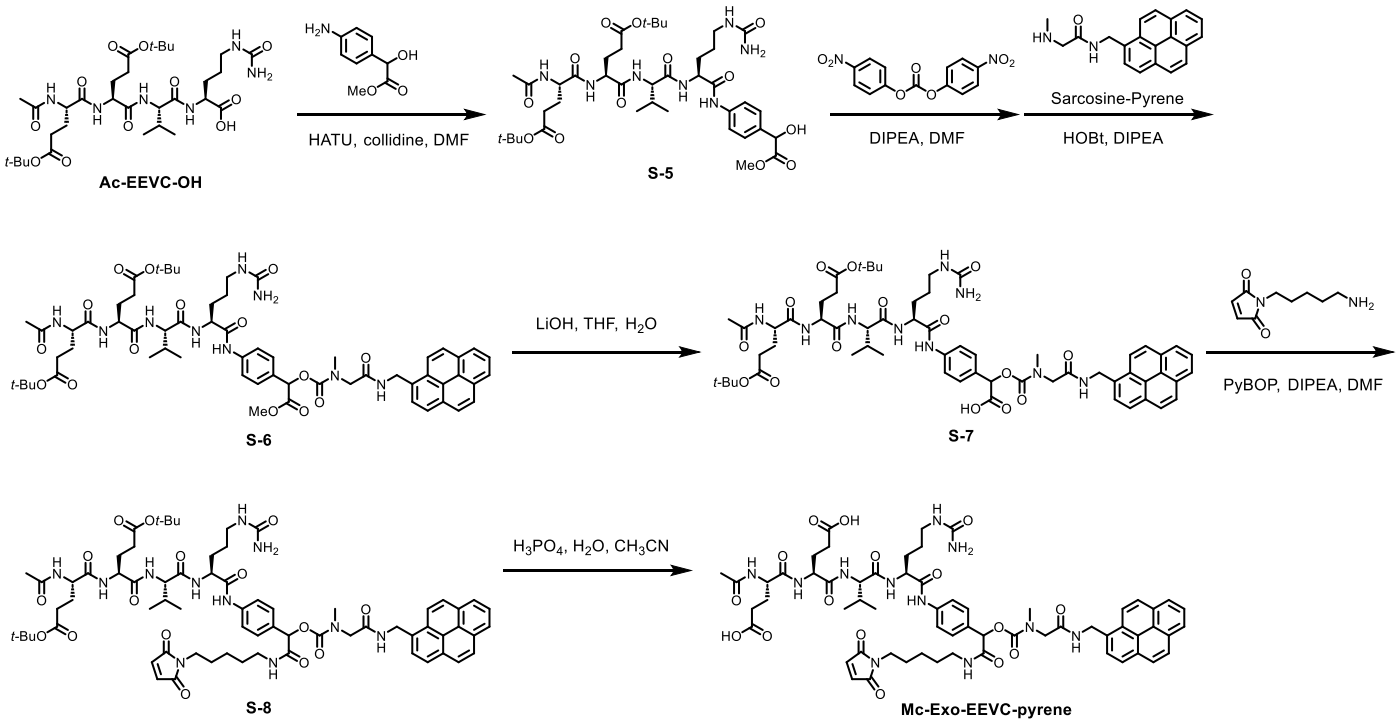


S-4 Mc-Exo-EVC-pyrene

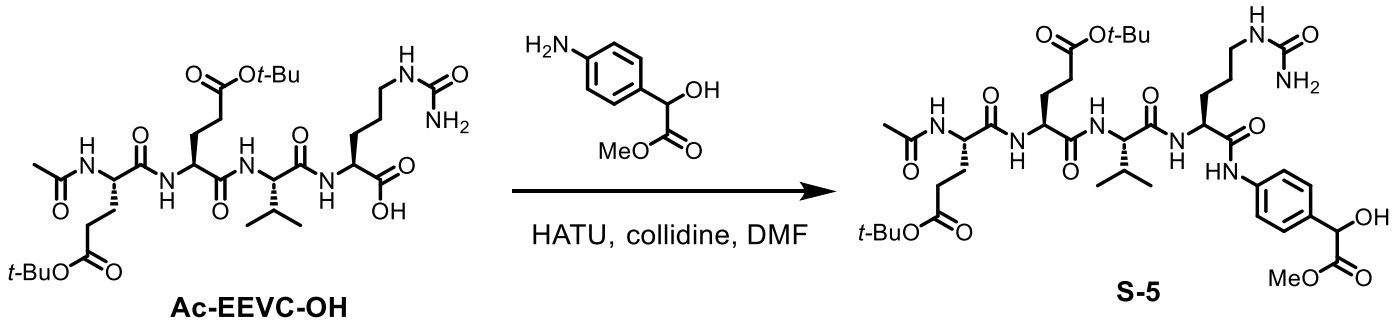
1,4-dioxane (380 μL) and 85% aqueous phosphoric acid (95 μL, 380 μmol) were sequentially added to pyrene **S-4** (2.2 mg, 1.9 μmol), and the mixture was stirred at room temperature for 4 h. After cooling with ice, DIPEA (71.8 μL, 418 μmol) was added and stirred at room temperature for 10 min. The reaction solution was purified by reversed-phase preparative chromatography, the fraction containing the product was collected and concentrated under reduced pressure to remove acetonitrile, and then lyophilized to obtain Mc-Exo-EVC-pyrene (2.1 mg, 99%).

MS (ESI) m/z: 1087.45 $[M+H]^+$

1-2-2 Mc-Exo-EEVC-pyrene synthesis

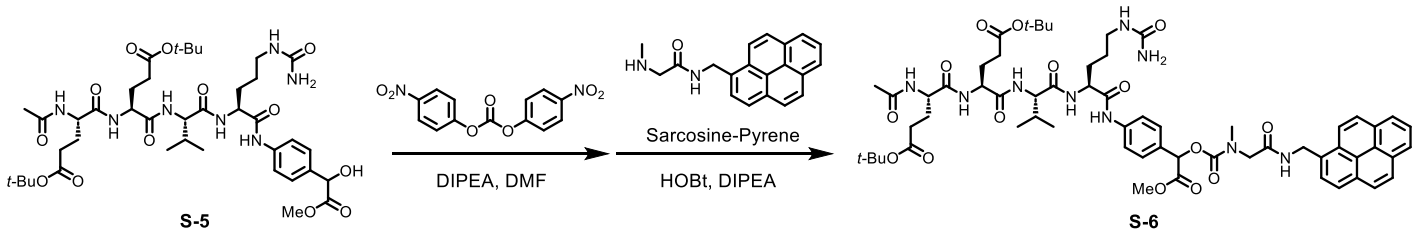


Scheme S-2. Synthesis of Mc-Exo-EEVC-pyrene



Ac-EEVC-OH (50.0 mg, 72.8 μmol) was dissolved in DMF (800 μL) and 1-[bis(dimethyl amino)methylene]-1H-1,2,3,triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (33.2 mg, 87.4 μmol), 2,4,6-trimethylpyridine (11. 5 μL, 87.4 μmol) was added and stirred at room temperature for 10 min. Subsequently, *p*-amino-mandelic acid methyl ester [4] (15.8 mg, 87.4 μmol) was added and the mixture was stirred at rt for 16 h, followed by purification by reverse phase preparative chromatography. Fractions containing the product were collected, concentrated under reduced pressure to remove acetonitrile, and lyophilized to obtain alcohol **S-5** (54.0 mg, 87%).

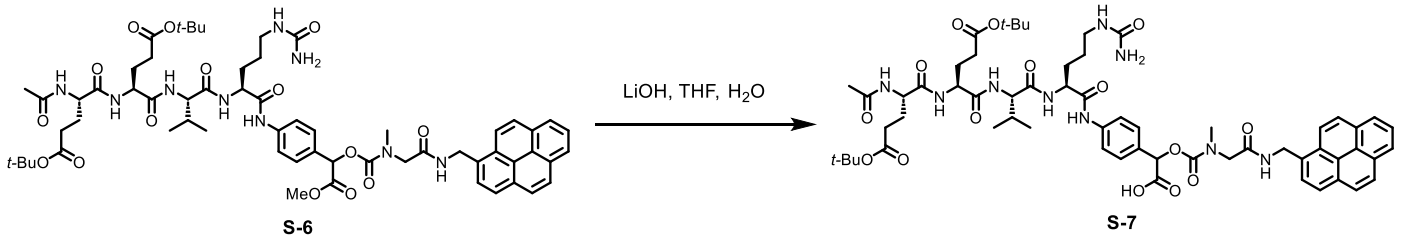
MS (ESI) m/z: 850.40 $[M+H]^+$



S-5 S-6

Alcohol **S-5** (50.3 mg, 59.2 μmol) was dissolved in DMF (650 μL), stirred for 5 min under ice-cooling, and then bis(4-nitrophenyl) carbonate (54.0 mg, 178 μmol) and DIPEA (22.7 μL, 133 μmol) were added, and the mixture was stirred at rt for 5 h. Then, after ice cooling, Sarcosin-Pyrene[5] (89.5 mg, 296 μmol), 1-hydroxybenzotriazole (12.0 mg, 88.8 μmol) and DIPEA (78.1 μL, 459 μmol) were added, and the mixture was brought to rt. The mixture was stirred for 18 hours. After the reaction, the product was purified by reverse phase preparative chromatography. Fractions containing the product were collected, concentrated under reduced pressure to remove acetonitrile, and freeze-dried to obtain pyrene **S-6** (34.0 mg, 57%).

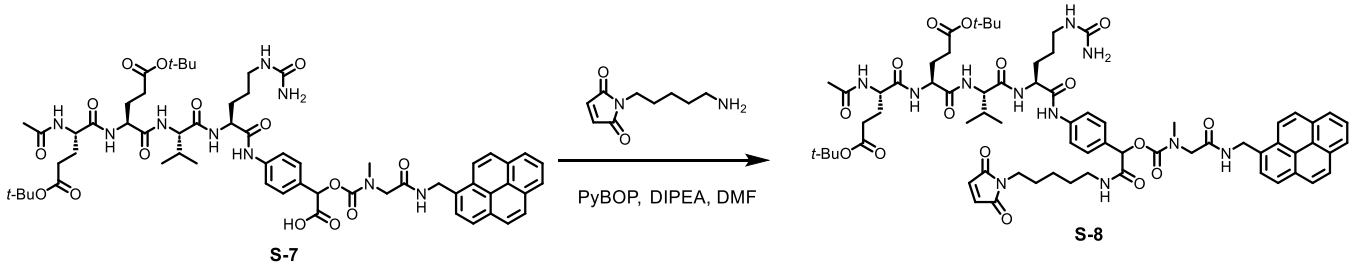
MS (ESI) m/z: 1178.50 $[M+H]^+$



S-6 S-7

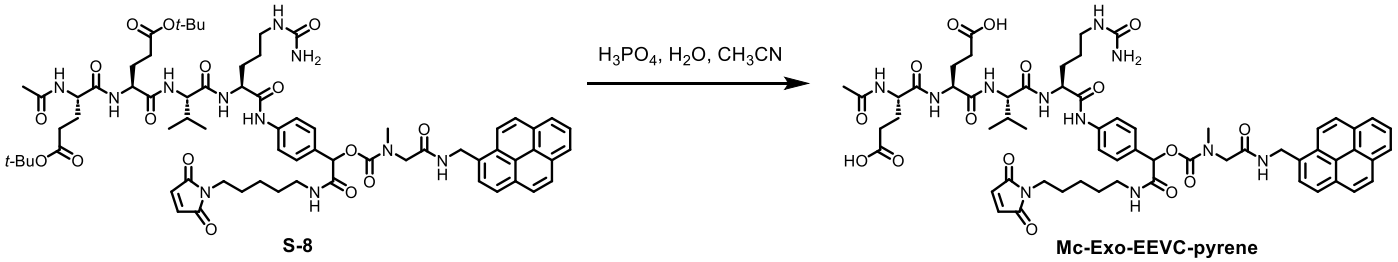
Pyrene **S-6** (30.7 mg, 26.1 μmol) was dissolved in THF (2.25 mL) and water (0.75 mL), stirred for 5 min under ice-cooling, and then lithium hydroxide monohydrate (5. 5 mg, 0.13 mmol) was added, and the mixture was stirred at rt for 4 h. After completion of the reaction, the pH was adjusted to about 6.0 using 0.1 M hydrochloric acid, and the product was purified by reverse phase preparative chromatography. Fractions containing the product were collected, concentrated under reduced pressure to remove acetonitrile, and freeze-dried to obtain the above pyrene **S-7** (17.2 mg, 57%).

MS (ESI) m/z: 1164.55 $[M+H]^+$



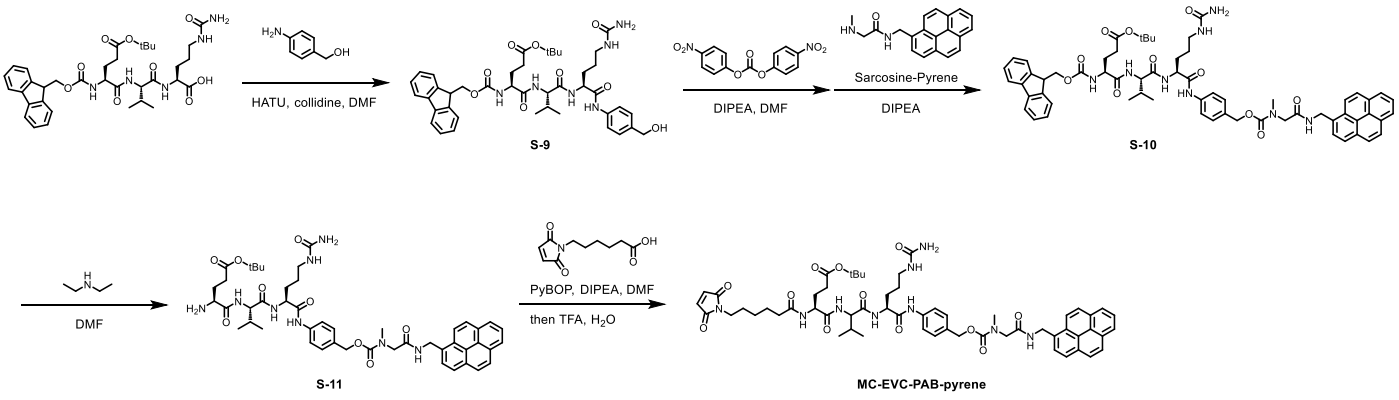
S-7 S-8

Pyrene **S-7** (14.7 mg, 12.6 μmol) was dissolved in DMF (1.0 mL), cooled with ice, DIPEA (4.29 μL, 25.2 μmol), 1H-Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (9.8 mg, 19 μmol) was added. Next, N-(5-aminopentyl)maleimide hydrochloride (4.1 mg, 19 μmol) was added and the mixture was warmed to rt and stirred for 3.5 h. After completion of the reaction, the product was purified by reverse phase preparative chromatography. Fractions containing the product were collected, concentrated under reduced pressure to remove acetonitrile, and freeze-dried to obtain pyrene **S-8** (7.0 mg, 73%).
MS (ESI) m/z: 1328.60 $[M+H]^+$

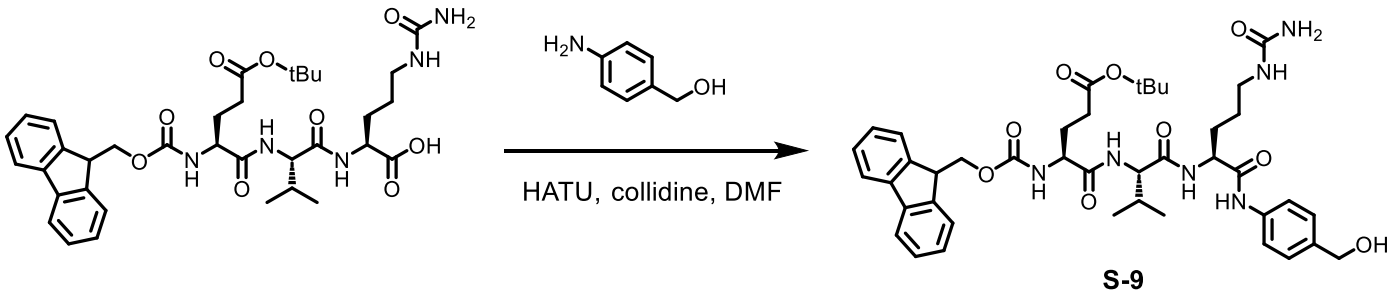


1,4-dioxane (920 μL) and 4 M hydrogen chloride/dioxane solution (230 μL, 918 μmol) were sequentially added to pyrene **S-8** (6.1 mg, 4.6 μmol), and the mixture was stirred at rt for 4 h. After cooling with ice, DIPEA (172 μL, 1.10 mmol) was added and stirred at rt for 10 min. The reaction solution was purified by reversed-phase preparative chromatography, the fraction containing the product was collected, concentrated under reduced pressure to remove acetonitrile, and then lyophilized to obtain Mc-Exo-EEVC-pyrene (3.7 mg, 65%).
MS (ESI) m/z: 1216.45 $[M+H]^+$

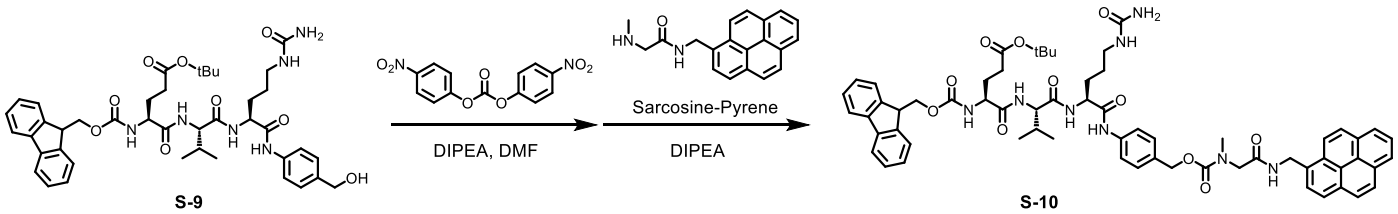
1-2-3 Mc-EVC-PAB-pyrene synthesis



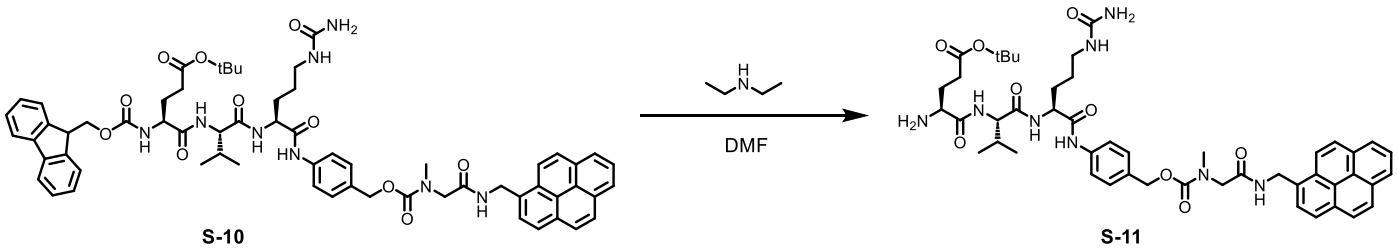
Scheme S-3. Synthesis of Mc-EVC-PAB-pyrene



Fmoc-Glu(OtBu)-Val-Cit-OH (100 mg, 147 µmol) was dissolved in N,N-dimethylformamide (733 µL). To this solution, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (66.9 mg, 176 µmol) and 2,4,6-trimethylpyridine (23.0 µL, 176 µmol) were added, and the mixture was stirred at room temperature for 10 minutes. Subsequently, (4-aminophenyl)methanol (21.7 mg, 176 µmol) was added, and the reaction was allowed to stir at room temperature for an additional 3 hours. The mixture was then purified using reverse-phase column chromatography. Fractions containing the product were collected, solvent was removed under reduced pressure, and the residue was lyophilized to afford the desired alcohol (**S-9**) (51.1 mg, 64.9 µmol).
MS (ESI) m/z: 787.45 $[M+H]^+$

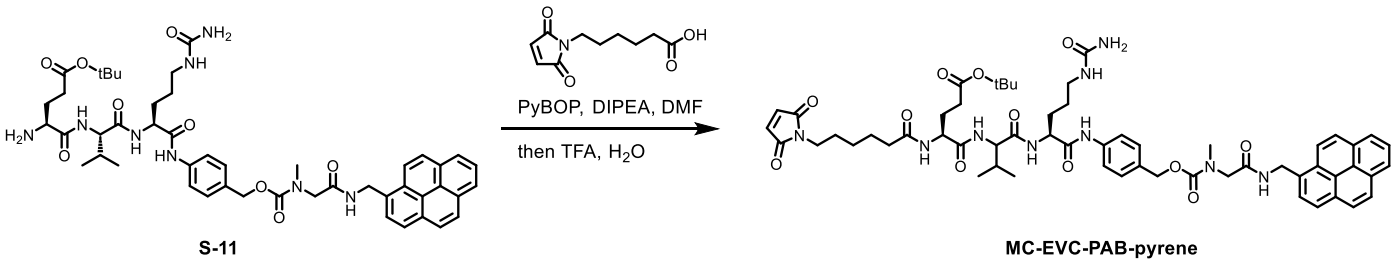


Alcohol (S-9) (20.0 mg, 25.4 µmol) was dissolved in N,N-dimethylformamide (254 µL). Subsequently, bis(4-nitrophenyl) carbonate (23.2 mg, 76.2 µmol) and N,N-diisopropylethylamine (DIPEA) (9.70 µL, 57.2 µmol) were added, and the mixture was stirred at room temperature for 2 hours. The reaction mixture was then cooled on ice, and Sarcosine-Pyrene (23.1 mg, 76.2 µmol) and N,N-diisopropylethylamine (DIPEA) (16.0 µL, 95.3 µmol) were added, followed by stirring at room temperature for 1 hour. The reaction was purified using normal-phase column chromatography. Fractions containing the product were collected, the solvent was removed under reduced pressure, and the residue was lyophilized to obtain the described pyrene (3) (16.2 mg, 14.5 µmol).
MS (ESI) m/z: 1115.550 [M+H]+



Pyrene (**S-10**) (15.0 mg, 13.4 μmol) was dissolved in N,N-dimethylformamide (134 μL). Diethylamine (40.0 μL, 403 μmol) was then added, and the mixture was stirred at room temperature for 3 hours. After completion of the reaction, it was purified using reverse-phase column chromatography. Fractions containing the product were collected, the solvent was removed under reduced pressure, and the residue was lyophilized to obtain the described pyrene (**S-11**) (9.00 mg, 10.1 μmol).

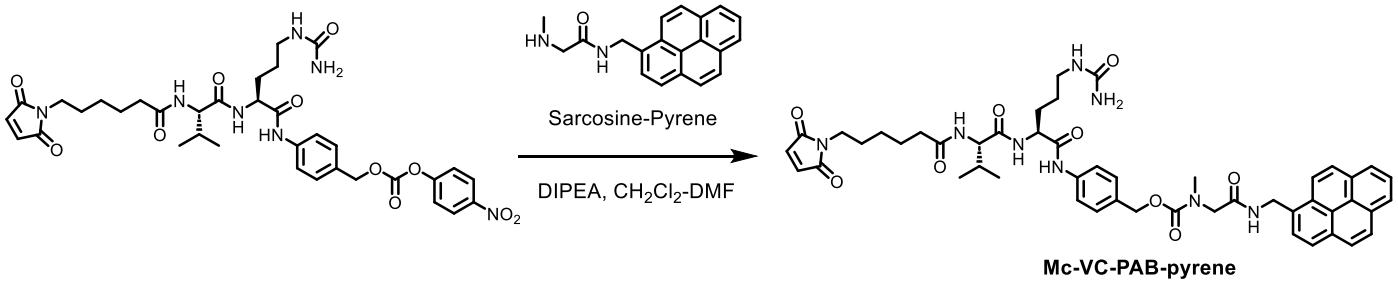
MS (ESI) m/z: 893.450 [M+H]+



Pyrene (**S-11**) (8.00 mg, 8.96 μmol) was dissolved in N, N-dimethylformamide (89.6 μL). N, N-diisopropylethylamine (3.00 μL, 17.9 μmol) and 1H-benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (6.99 mg, 13.4 μmol) were then added. Subsequently, 6-maleimidohexanoic acid (2.84 mg, 13.4 μmol) was added and the mixture was stirred at room temperature for 3 hours. Acetonitrile (276 μL) was added followed by the addition of TFA (11.3 μL, 1.6 mmol) under ice cooling, and the mixture was stirred at room temperature for 0.5 hours. After completion of the reaction, it was purified using reverse-phase column chromatography. The fractions containing the product were collected and concentrated under reduced pressure. After removing acetonitrile, lyophilization yielded the described Mc-EVC-PAB-pyrene (0.94 mg, 0.91 μmol).

MS (ESI) m/z: 1030.450 [M+H]+

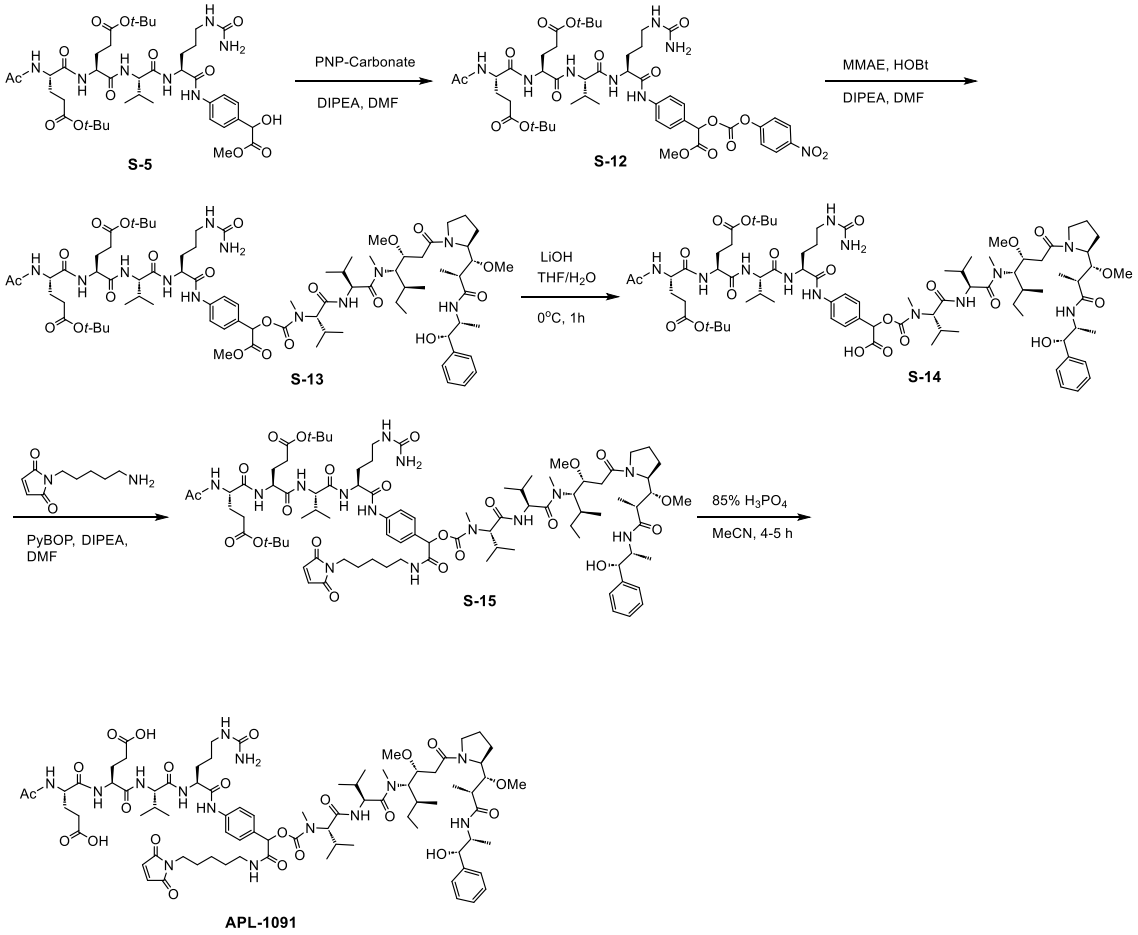
1-2-4 Mc-VC-PAB-pyrene synthesis



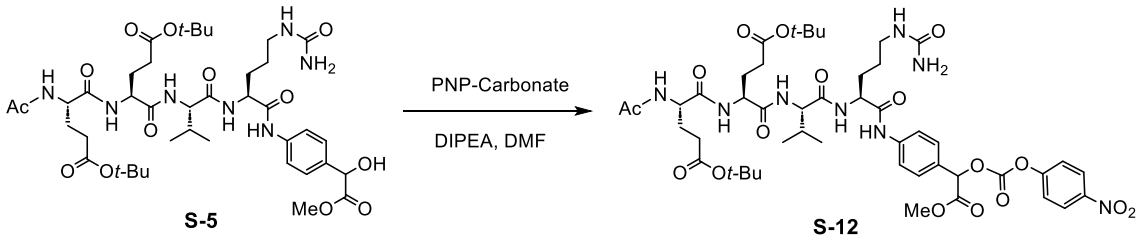
Scheme S-4. Synthesis of Mc-VC-PAB-pyrene

Mc-VC-PAB-PNP (15.5 mg, 0.021 mmol) was dissolved in dichloromethane (1 mL), and DMF (0.025 mL, 0.142 mmol), Sarcosine-Pyrene {Anami, 2018 #454} (7.6 mg, 0.025 mmol), and a DMF solution (0.5 mL) were added and stirred for 17 h. After purification by reversed-phase preparative chromatography, the fraction containing the product is collected, concentrated under reduced pressure to remove acetonitrile, and lyophilized to obtain a Mc-VC-PAB-pyrene (7.3 mg, 38%).
MS (ESI) m/z: 901.45 $[M+H]^+$

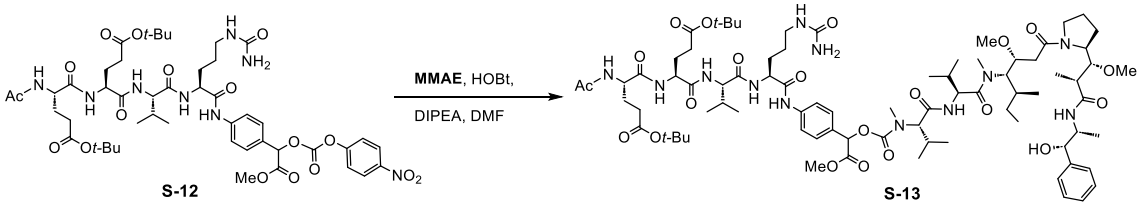
1-2-5 APL-1091 (Mc-Exo-EEVC-MMAE) synthesis



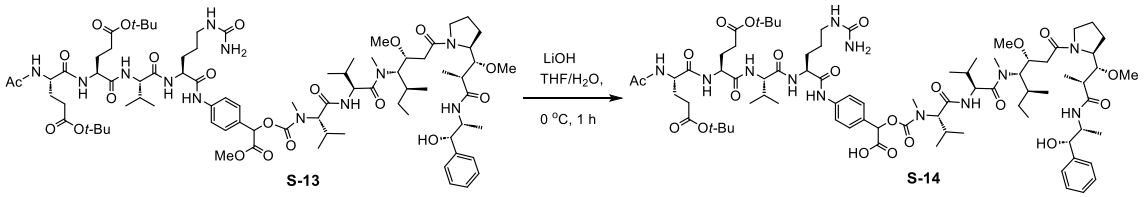
Scheme S-5. Synthesis of APL-1091



Compound **S-5** (140 mg, 0.165 mmol) and bis(4-nitrophenyl) carbonate (108 mg, 0.355 mmol) were weighed to a dry flask, dissolved in anhydrous DMF (4 mL), and treated with DIPEA (100 mL, 0.574 mmol). The flask was sealed with a rubber septum under nitrogen balloon and the reaction stirred at room temperature for 18 hours. The DMF was evaporated off *in vacuo* and the reaction was dissolved in 1:1 MeCN:H_2O with a trace of formic acid. The mixture was purified by reverse-phase flash chromatography on a C18 silica gel column (gradient elution, 20 – 90% MeCN in H_2O, 0.05% FA both phases), and the cleanest product fractions combined, partially concentrated *in vacuo*, frozen on dry ice, and lyophilized to yield compound **S-12** as a white solid (130 mg, 0.128 mmol, 78% yield).
MS (ESI) m/z: 1015.6 $[M+H]^+$

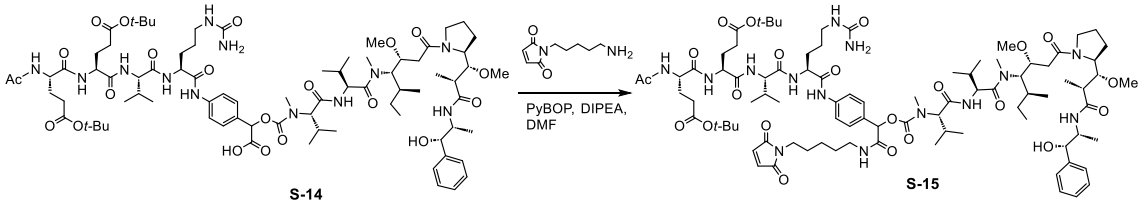


S-12 (85 mg, 0.084 mmol) and HOBT(20 mg, 0.13 mmol) were dissolved in anhydrous DMF (2 mL) then treated with a solution of monomethylauristatin E (MMAE, 63 mg, 0.088 mmol) in anhydrous DMF (2 mL). The reaction was then treated with DIPEA (75 mL, 0.43 mmol), the flask purged with nitrogen, sealed with a rubber septum under nitrogen balloon, and stirred at room temperature overnight. After 23 h, the reaction mixture was concentrated *in vacuo*, dissolved in 1:1 MeCN:H_2O with trace FA, and the crude material purified by reverse-phase flash chromatography on a C18 silica gel column (gradient elution, 20 – 80% MeCN in H_2O, 0.05% FA both phases). The cleanest product fractions by LCMS were combined, partially concentrated *in vacuo*, frozen on dry ice, and lyophilized to yield **S-13** as a white solid (94 mg, 0.059 mmol, 71% yield).
MS (ESI) m/z: 1593.6 $[M+H]^+$



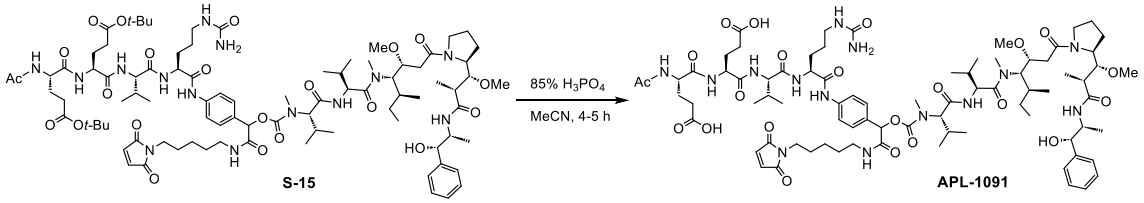
A solution of **S-13** (94 mg, 0.059 mmol) in tetrahydrofuran (THF, 5 mL) and water (2 mL) was cooled to 0 °C under nitrogen balloon. After a few minutes, 1.0 M aqueous lithium hydroxide (0.60 mL, 0.60 mmol) was added and the reaction stirred at 0 °C for 50 minutes. The reaction was acidified to ca. pH 5 with 5% aqueous hydrochloric acid and saturated sodium bicarbonate, then briefly concentrated in vacuo to remove the THF. MeCN (ca. 2 mL) was added and the mixture was purified by reverse-phase flash chromatography on a C18 silica gel column (gradient elution, 20 – 90% MeCN in H_2O, 0.05% FA both phases). The cleanest product fractions were combined, partially concentrated *in vacuo*, frozen on dry ice, and lyophilized to yield **S-14** as a white solid (77 mg, 0.049 mmol, 83% yield).

MS (ESI) m/z: 1579.7 $[M+H]^+$



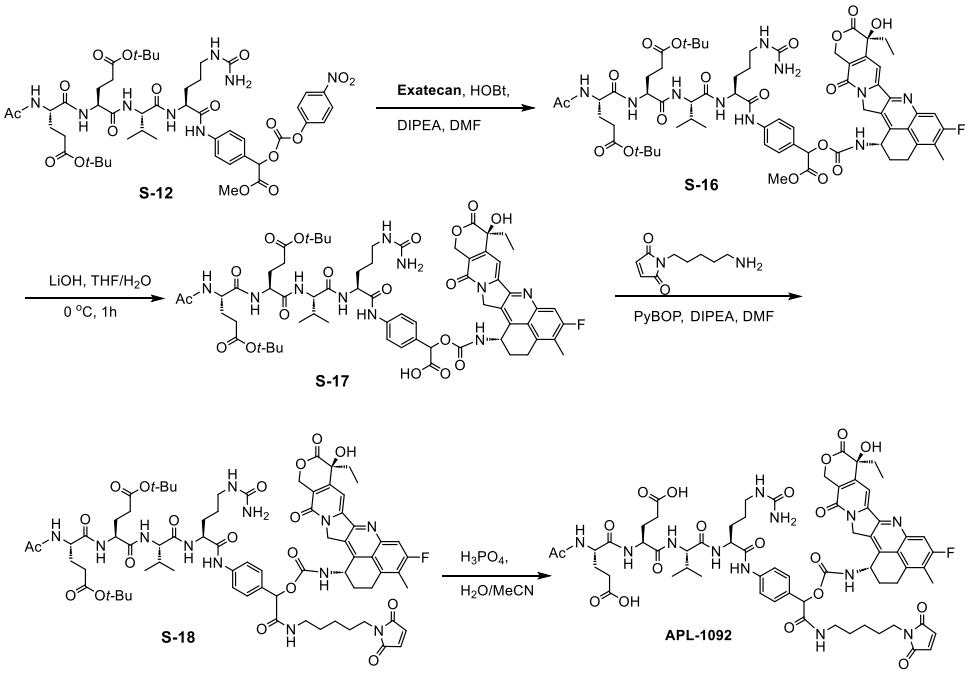
1*H*-Benzotriazol-1-yloxytripyrrolidinophosphonium Hexafluorophosphate (PyBOP, 87 mg, 0.17 mmol) and N-(5-aminopentyl)maleimide HCl salt (35 mg, 0.16 mmol) were weighed to a flask containing **S-14** (77 mg, 0.049 mmol), and all were dissolved in anhydrous DMF (3 mL). DIPEA (50 mL, 0.29 mmol) was added, the flask was sealed with a rubber septum under nitrogen balloon, and the reaction was stirred at room temperature for 6 hours. DMF was evaporated *in vacuo* and the reaction was dissolved in 1:1 MeCN:H_2O with a trace of FA. The mixture was purified by reverse-phase flash chromatography on a C18 silica gel column (gradient elution, 20 – 80% MeCN in H_2O, 0.05% FA both phases), and the cleanest product fractions combined, partially concentrated *in vacuo*, frozen on dry ice, and lyophilized to yield **S-15** as a white solid (65 mg, 0.037 mmol, 76%).

MS (ESI) m/z: 1744.7 $[M+H]^+$

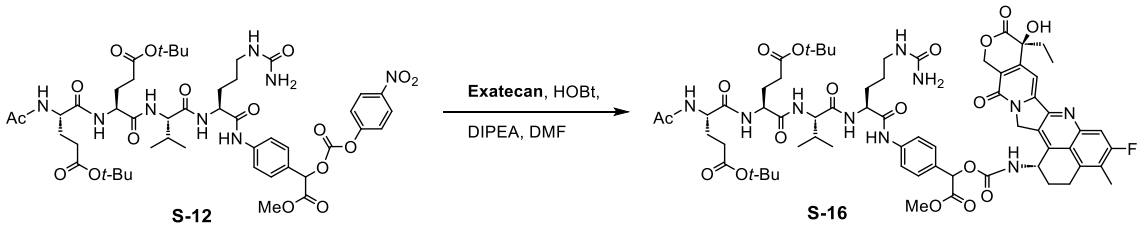


S-15 (65 mg, 0.037 mmol) was treated with MeCN (2 mL) and 85% aqueous phosphoric acid (1.00 mL, 14.6 mmol), the flask sealed with rubber septum under nitrogen balloon, and the reaction stirred at ambient temperature. After 5 hours, water (2 mL) was added and the mixture was purified by reverse-phase flash chromatography on a C18 silica gel column (gradient elution, 20 – 80% MeCN in H_2O, 0.05% FA both phases). The cleanest product fractions were combined, partially concentrated *in vacuo*, frozen on dry ice, and lyophilized to yield APL-1091 as a white solid (49 mg, 0.030 mmol, 80% yield).
MS (ESI) m/z: 1631.6 $[M+H]^+$

1-2-6 APL-1092 (Mc-Exo-EEVC-Exatecan) synthesis

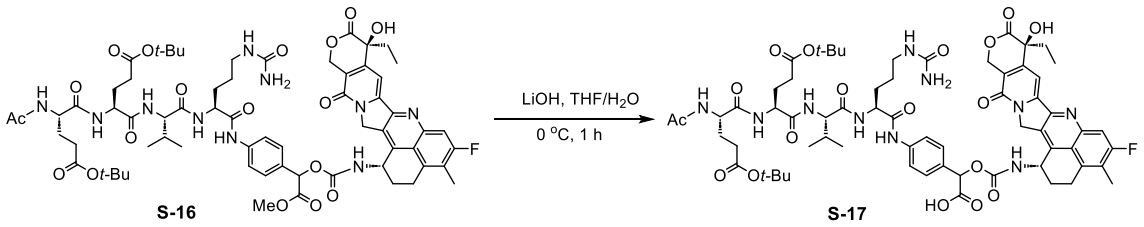


Scheme S-6. Synthesis of APL-1092

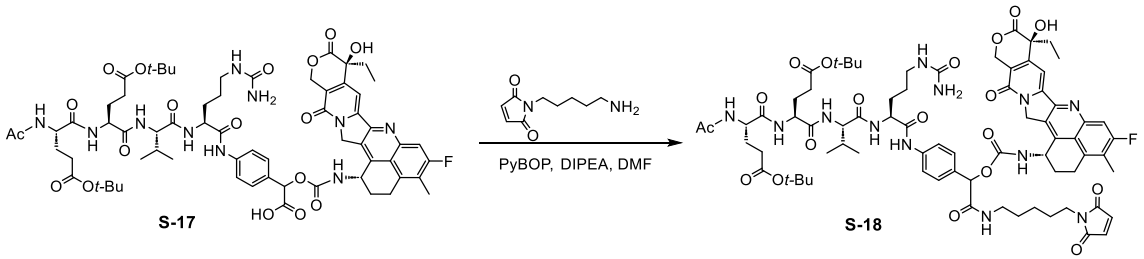


S-12 (67 mg, 0.066 mmol) and HOBt (17 mg, 0.11 mmol) were dissolved in anhydrous DMF (2 mL) then treated with a suspenion of Exatecan mesylate (35 mg, 0.066 mmol) in anhydrous DMF (2 mL). The reaction was then treated with DIPEA (50 mL, 0.29 mmol), the flask purged with nitrogen, sealed with a

rubber septum under nitrogen balloon, and stirred at room temperature. After 4 hours the reaction mixture was concentrated *in vacuo*, dissolved in 1:1 MeCN:H_2O with trace FA, and the crude material purified by reverse-phase flash chromatography on a C18 silica gel column (gradient elution, 20 – 80% MeCN in H_2O, 0.05% FA both phases). The cleanest product fractions by LCMS were combined, partially concentrated *in vacuo*, frozen on dry ice, and lyophilized to yield **S-16** as a white solid (57 mg, 0.043 mmol, 66% yield).
MS (ESI) m/z: 1311.7 $[M+H]^+$

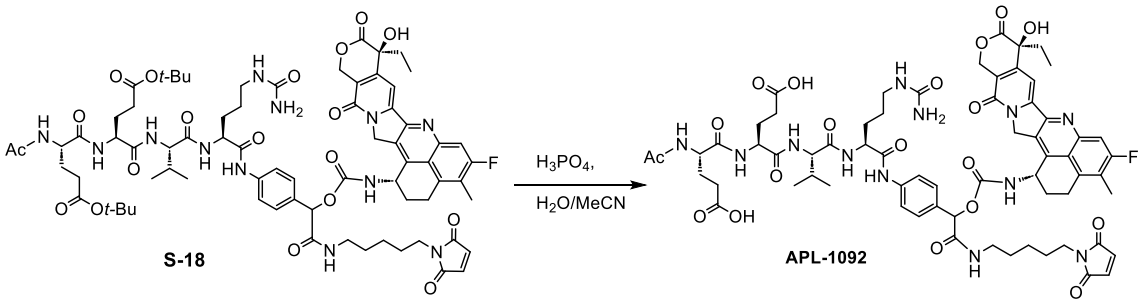


A solution of **S-16** (57 mg, 0.043 mmol) in THF (3 mL) and water (1.5 mL) was cooled to 0 °C under nitrogen balloon. After a few minutes, 1.0 M aqueous lithium hydroxide (0.50 mL, 0.50 mmol) was added and the reaction stirred at 0°C for ca. 1 hour. The reaction was acidified to ca. pH 5 with 5% aqueous hydrochloric acid and saturated sodium bicarbonate, then briefly concentrated *in vacuo* to remove the THF. MeCN (ca. 2 mL) was added and the mixture was briefly sonicated to dissolve the solids, then purified by reverse-phase flash chromatography on an oversized C18 silica gel column (gradient elution, 20 – 90% MeCN in H_2O, 0.05% FA both phases). The two products did not separate well, so all product fractions were combined, partially concentrated *in vacuo*, frozen on dry ice, and lyophilized to yield **S-17** as an impure white solid (45 mg, 0.035 mmol, 80% yield).
MS (ESI) m/z: 1297.6 $[M+H]^+$



1*H*-Benzotriazol-1-yloxytripyrrolidinophosphonium Hexafluorophosphate (PyBOP, 55 mg, 0.11 mmol) and N-(5-aminopentyl)maleimide HCl salt (22 mg, 0.10 mmol) were weighed to a flask containing **S-17** (45 mg, 0.035 mmol), and all were dissolved in anhydrous DMF (3 mL). DIPEA (30 mL, 0.17 mmol) was added, the flask was sealed with a rubber septum under nitrogen balloon, and the reaction was stirred at room temperature for 18 hours. DMF was evaporated *in vacuo* and the reaction was dissolved in 1:1 MeCN:H_2O with a trace of FA. The mixture was purified by reverse-phase flash chromatography on a C18 silica gel

column (gradient elution, 20 – 80% MeCN in H_2O, 0.05% FA both phases), and the cleanest product fractions combined, partially concentrated *in vacuo*, frozen on dry ice, and lyophilized to yield **S-18** as a white solid (22 mg, 0.015 mmol, 43%).
MS (ESI) m/z: 1462.7 $[M+H]^+$



S-18 (22 mg, 0.015 mmol) was treated with MeCN (1 mL) and 85% aqueous phosphoric acid (1.00 mL, 14.6 mmol), the flask sealed with rubber septum under nitrogen balloon, and the reaction stirred at ambient temperature. After 1.5 hours water (ca. 1 mL) was added and the mixture was purified by reverse-phase flash chromatography on a C18 silica gel column (gradient elution, 10 – 70% MeCN in H_2O, 0.05% FA both phases). The cleanest product fractions were combined, partially concentrated *in vacuo*, frozen on dry ice, and lyophilized to yield **APL-1092** as a white solid (18.8 mg, 0.0139 mmol, 92% yield), which appeared to be a mixture of stereoisomers by LCMS.
MS (ESI) m/z: 1349.2 $[M+H]^+$

1-3 ADC synthesis

1-3-1 General procedure for DAR = 8 ADC synthesis

The trastuzumab (1 mg) was dissolved in water then buffer exchanged into conjugation buffer (0.2 mL, pH 7.5, 50 mM PBS, 10 mM EDTA) to prepare for the conjugation process. The reduction reaction began with the addition of 5 eq. tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP-HCl) to the antibody and stirred mildly for 1.5 h at 37 ºC. The resulting reaction mixture, DMA (8% v/v, not needed for APL-1091 or APL-1092) and 10 eq. of payload-linker were sequentially added and stirred mildly for 1 h at 20 ºC. Unreacted drug linker was quenched with the addition of 25 eq of *N*-Acetyl-L-cysteine (NAC) and mixed for 25 min at 20 ºC. The final mixture was purified using NAP-5 desalting columns and eluted with pH 5.2, 20 mM histidine, 5.5% trehalose.

1-3-2 General procedure for DAR = 2 AJICAP-ADC synthesis

2.5 equivalent of AJICAP peptide reagent (20 mM in DMF) was added to trastuzumab (10 mg/mL, 20 mM acetate buffer, pH 5.5), and then it was allowed to incubate for 1 h at 20 °C. After 1 h had passed, excess NH_2OH HCl was introduced, and the mixture was left to be stirred for an additional 1 h. The reaction mixture was then purified using a NAP-25 desalting column and was eluted with a 20 mM acetate buffer at pH 5.5. The resulting trastuzumab-Lys248 thiol, 2.7 eq. of APL-1091 or APL-1092 were sequentially added and stirred mildly for 1 h at 20 ºC. Unreacted drug linker was quenched with the addition of 25 eq of *N*-

Acetyl-L-cysteine (NAC) and mixed for 25 min at 20 ºC. The final mixture was purified using NAP-5 desalting columns and eluted with pH 5.2, 20 mM histidine, 5.5% trehalose.

1-3-3 Instruments and analytical methods

The ADC concentration and recovery were measured using the Slope Spectroscopy® method with a Solo-VPE system. [6]

Hydrophobic interaction chromatography-HPLC analysis was performed as previously reported [2].

SEC-HPLC analysis of ADCs was performed using Waters ACQUITY UPLC Protein BEH SEC column (200 Å, 4.6 x 300 mm, 1.7 μm) as previously reported [7].

1-4 in vivo xenograft study

Cells

NCI-N87 cells (Cat # CRL-5822) were purchased through ATCC. Cells were cultured by a previously established procedure [8].

Animals

NOD.CB17 homozygous mice were procured, fed, and housed by a previously established procedure [8].

Implantation

Implantation was performed as previously reported. [8]

Study Arms and Treatments for NCI-N87

Tumor volumes were monitored, and on first day (when mean tumor volume reached ~120mm^3), mice were stratified and placed into 3 treatment groups of (10) mice as outlined in Table S1, S2.

Table S1. Study arms in NCI-N87 first run.

Group	N	Agent	mg/kg
1	10	Vehicle	
2	10	Trastuzumab-emtansine (Kadcyla)	5
3	10	Trastuzumab-emtansine (Kadcyla)	2.5
4	10	ADC 5 (AJICAP, APL-1091)	2.5
5	10	ADC 5 (AJICAP, APL-1091)	1.25
6	10	ADC 9 (interchainbreak, Mc-VC-PAB-MMAE)	2.5
7	10	ADC 9 (interchainbreak, Mc-VC-PAB-MMAE)	1.25
8	10	ADC 10 (AJICAP, Mc-VC-PAB-MMAE)	2.5
9	10	ADC 10 (AJICAP, Mc-VC-PAB-MMAE)	1.25

Table S2. Study arms in NCI-N87 second run.

Group	N	Agent	mg/kg
1	10	Vehicle	
2	10	Trastuzumab-deruxtecan (Enhertu)	2.5
3	10	Trastuzumab-deruxtecan (Enhertu)	1.25
4	10	ADC 6 (AJICAP, APL-1092)	10
5	10	ADC 6 (AJICAP, APL-1092)	5.0
6	10	ADC 6 (AJICAP, APL-1092)	2.5
7	10	ADC 8 (interchainbreak, APL-1092)	2.5
8	10	ADC 8 (interchainbreak, APL-1092)	1.25

Treatments were administered by tail vein injection (100 μL volumes). Doses were administered on day 0, 4, 7, 11 for a total of 4 doses for the study. Animal weights and tumor volumes were measured.

1-5 Rat PK study using ligand-binding assay

Animal experiments

Animal experiments were cultured by a previously established procedure [8].

Clinical observations

The animals were observed once daily for clinical signs. Individual body weight were measured on Days 0, 7, 14, and 21 with the first day of administration defined as Day 0.

Total antibody analysis

The blood samples were collected on 6 time points (immediately after administration, after administration and 1, 3, 7, 14, and 21 after administration) via caudal vein. The concentrations of total antibody were measured by double sandwich ELISA method.

Ligand-binding assay for total ADC analysis

25 μL of Dynabeads™ M-280 Streptavidin was washed with PBS buffer (pH 7.4). Then, 50 μL of human IgG-Fc fragment antibody (1 mg/mL, PBS buffer, Bethyl Laboratories, Inc, USA) was added. After shaking at room temperature for 2 h, the beads were washed with PBS buffer (pH 7.4) five times. The blood sample was added to the beads and shaken for additional 2 h. The beads were then washed with PBS buffer (pH 7.4) five times and elution solution (25 μL, 10% ACN, 1% formic acid v/v in water) was added to each sample. After shaking for 30 min, the eluent was injected for LC-MS analysis.

The resulting sample was analyzed using a NexeraBio system (Shimadzu, Kyoto, Japan) coupled to a timsTOF Pro mass spectrometer (Bruker Daltonics, Bremen, Germany). A PLRP-S column (5 μm, 2.1 × 50 mm, 1000 Å (Agilent Technologies, Santa Clara, CA)) was used for the analytical column with a column temperature of 80 °C. The autosampler temperature was set at 4 °C and an injection volume was 25 μL. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The gradient conditions of B% were: 0 - 0.5 min hold at 10% and 0.5 - 1.0 min linear gradient from 10% to 25%. After 3 min hold at 25%, a linear gradient from 25% to 90% in 6 min was performed. A wash step was conducted as 0.1 min linear gradient from 90% to 98%, holding for 5 min at 98%. Subsequently, mobile phase B was reduced to 10% within 0.1 min and maintained at 10% for 4.8 min. The flow rate was 400 μL/min. Mass spectrometry analysis was conducted in the positive ion mode with scan range of *m/z* 500 - 6,000 and TIMS was set to unable. The end plate offset and the capillary voltage were set to 500 V and 4,500 V, respectively. The nebulizer gas and the dry gas was 1.4 bar and 5.5 L/min and the dry gas temperature was 200 °C. All sample was analyzed with in-source collision-induced dissociation (isCID) energy of 100 eV.

The resulting data were processed and analyzed with Genedata Expressionist® software verion 16. 5. 1 (Genedata, Basel, Switzerland). The spectrum of retention time 5.1 - 6.1 min and *m/z* 2,300 - 4,400 were extracted and analyzed with using a time-resolved deconvolution and the Maximum Entropy (MaxEnt) algorithm. Output of minimum and maximum masses were respectively set to 145 and 160 kDa with 1.0 Da

mass step. Appropriate smoothing was conducted before peak detection node. After valid feature filtration of intensity and repetition of detection, protein mapping was conducted with mass tolerance of 500 ppm and each linker and payload was set for fixed modifications or conjugates. Each peak height was provided and the sum of top 3 glycan variant (2 × A2G0F, A2G0F/A2G1F, 2 × A2G1F) was used for drug to antibody ratio (DAR) calculation.

1-6 in vitro human neutrophil assay

Neutrophil elastase treatment

Neutrophil elastase treatment was performed according to previously reported method [9] [10] with slight modifications. 7.5 μL of ADC solution (2.66 μM, PBS, pH 7.4) was mixed with 92.5 μL of tris-buffered saline (TBS, pH 7.5) and human neutrophil elastase 100 μL (20 μg/mL, TBS, pH 7.5) (Enzo Life Sciences, USA). The mixture was incubated at 37 °C at 24 h. Then, this mixture was employed *in vitro* cytotoxicity assay.

Cell culture

MCF-7 cell line was obtained from Japanese Collection of Research Bioresources Cell Bank (Japan). SKBR-3 cell line was obtained from Memorial Sloan Kettering Cancer Center (USA). Both cells were cultured on Collagen I-coated cell culture dish (IWAKI, Japan) in the culture medium (D-MEM supplemented with 10% FBS and 1% penicillin-streptomycin) in 5% CO_2 at 37 °C.

Cytotoxicity assay

In vitro cytotoxicity assay was performed according to previously reported method [11] with slight modifications. 5 μl of 100 nM NE-treated or untreated ADCs or MMAE in NE-reaction buffer were mixed with 45 μl of culture medium and serially 3 or 4-fold diluted by the culture medium. 1,000 cells of MCF-7 and SKBR-3 cells were seeded onto Collagen I-coated 96-well cell culture plate (IWAKI, Japan) with 50 μl of culture

medium and cultured for 24 h in 5% CO_2 at 37 °C. Diluted ADCs were added to each cells and cultured for 6 days. Relative cell numbers were evaluated using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) following to manufacturer's protocol. Luminescence was analyzed using Nivo plate reader (Perkin Elmer).

1-7 other assays

1-7-1 Mouse plasma stability assay

Mouse plasma treatment

A solution was prepared by spiking ADC into 500 μL of the mouse plasma (Charles River Laboratories) until a definitive concentration of 0.1mg/mL was reached. Subsequently, sterile filtration of the mixture was performed. The prepared solution was then carefully aliquoted into six separate Eppendorf tubes, with 50 μL being dispensed into each. Three of these aliquots were incubated at a controlled temperature of 37 °C for a duration of 4 days. Concurrently, the remaining three tubes were stored at -80 °C for the same period (these samples were designated as the Day 0 samples.). After these periods, 100 μL acetonitrile was added to each sample. Once thoroughly vortex-mixed, the samples were centrifuged and the pellet was isolated. The resulting supernatants from each tube were then collected and were subsequently subjected to an advanced LC-MS analysis.

LC-MS analysis

The quantification of the released payload from the ADC was conducted using RP-HPLC. The fluorescence of the detached payload relatives detected from the Day 4 and Day 0 samples was respectively determined using the extracted ion chromatogram. The difference between these values was analysed to deduce payload release. Separately, using free pyrene, a correlation fluorescence wavelength area on HPLC and concentration was established. Using this correlation equation, the fluorescent strength of each ADC sample

was converted into concentration. The percentage difference in the ion chromatogram, using Day 0 concentration as the 100% benchmark, was calculated to determine the payload release rate.

1-7-2 Cathepsin B cleavage assay for ADCs

Cathepsin B cleavage assay was cultured by a previously reported procedure [5].

2. Analytical results

2-1 Payload-linker synthesis: HPLC and LC-MS

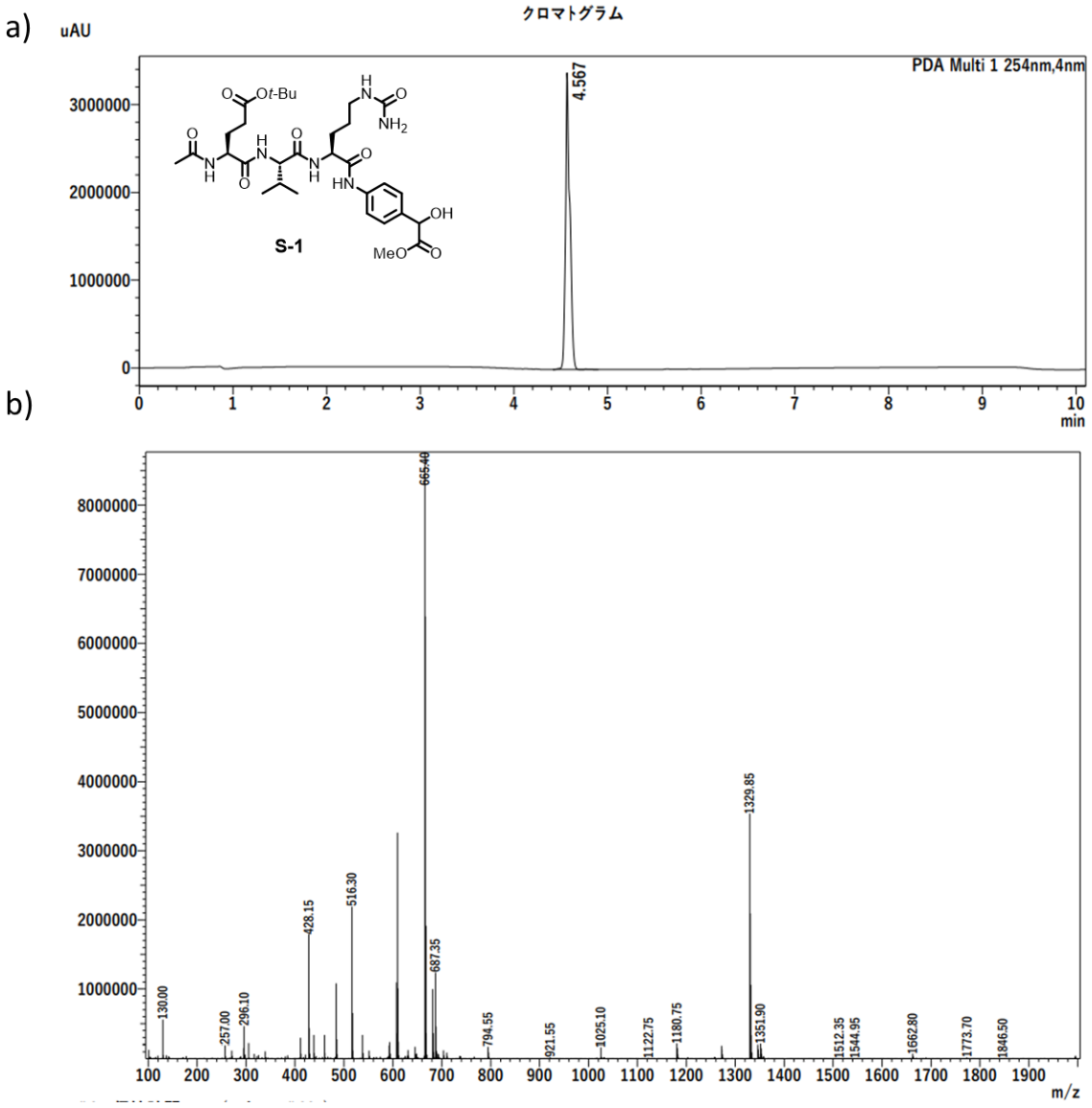


Figure S1. Analysis of compound **S-1**, a) HPLC analysis, b) MS analysis

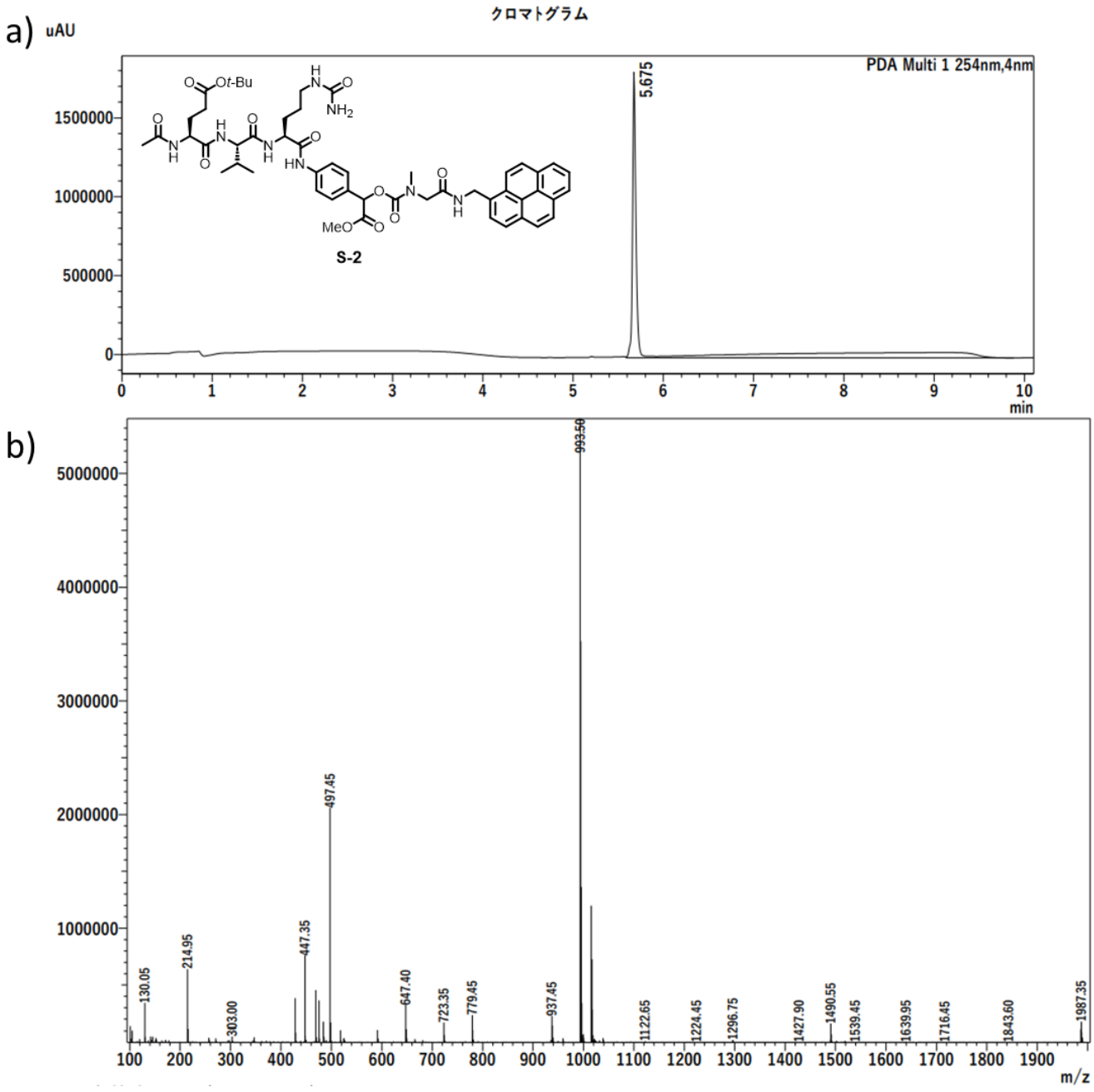


Figure S2. Analysis of compound **S-2**, a) HPLC analysis, b) MS analysis

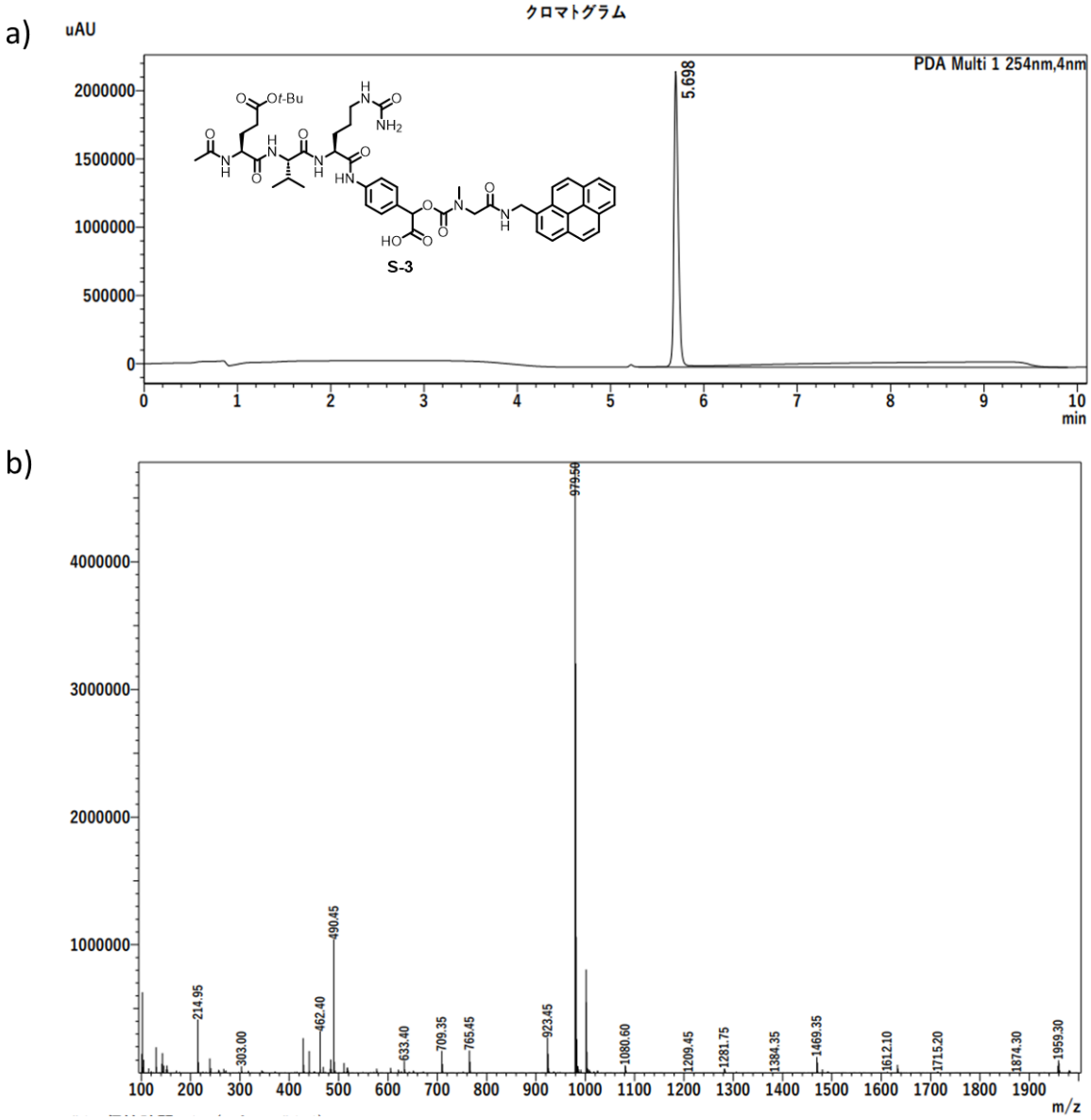


Figure S3. Analysis of compound **S-3**, a) HPLC analysis, b) MS analysis

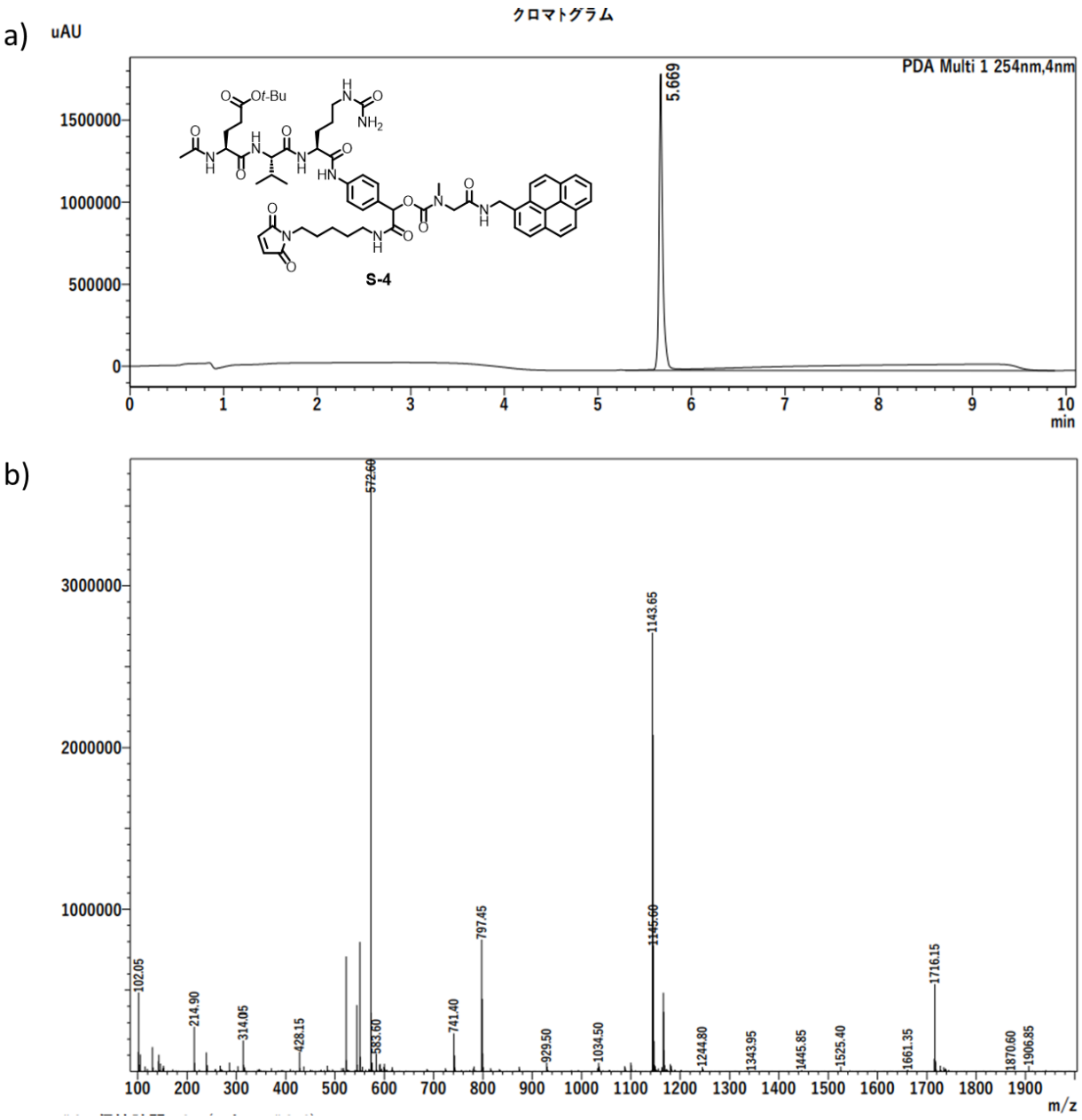


Figure S4. Analysis of compound **S-4**, a) HPLC analysis, b) MS analysis

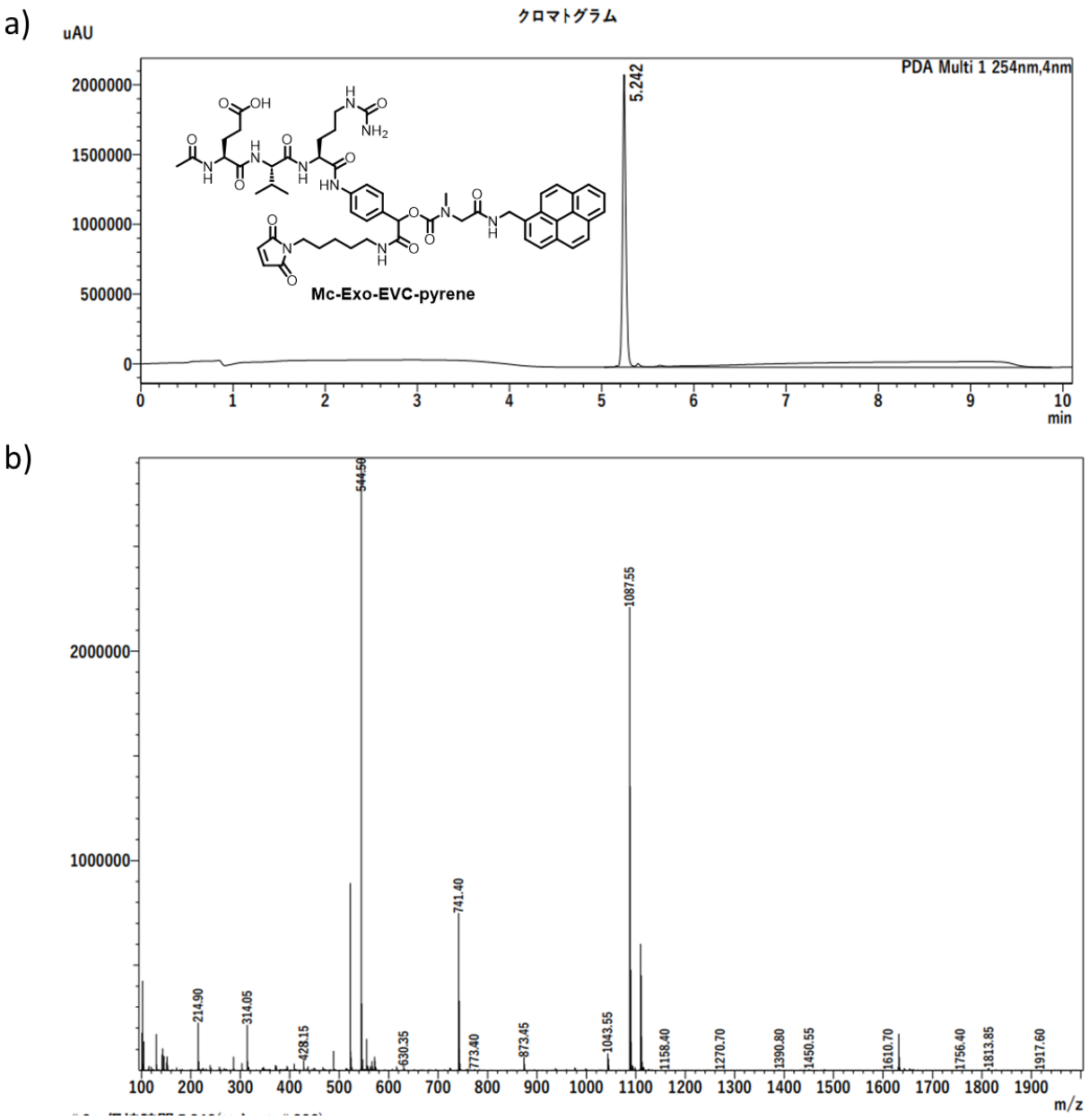


Figure S5. Analysis of compound Mc-Exo-EVC-pyrene, a) HPLC analysis, b) MS analysis

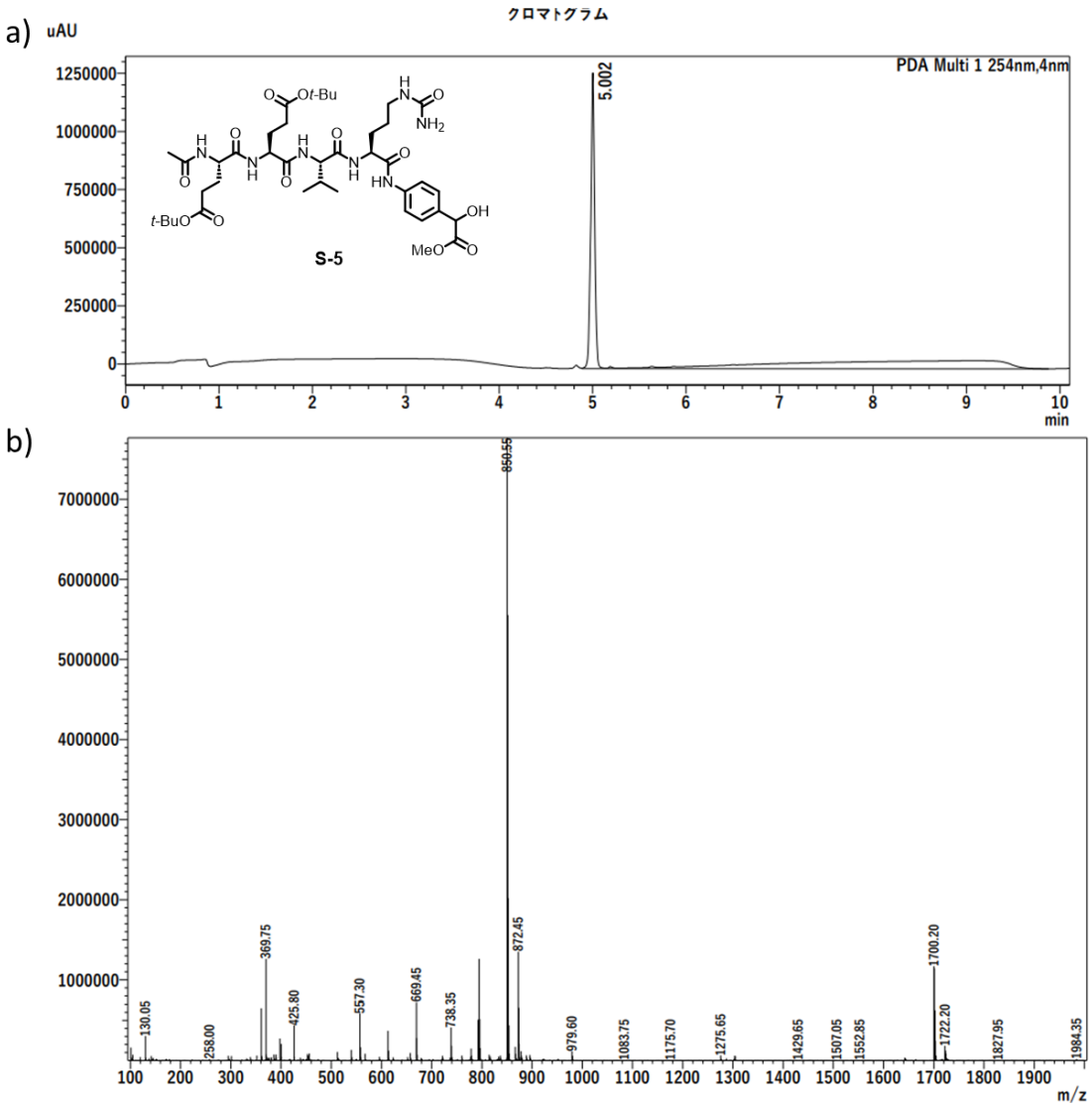


Figure S6. Analysis of compound **S-5**, a) HPLC analysis, b) MS analysis

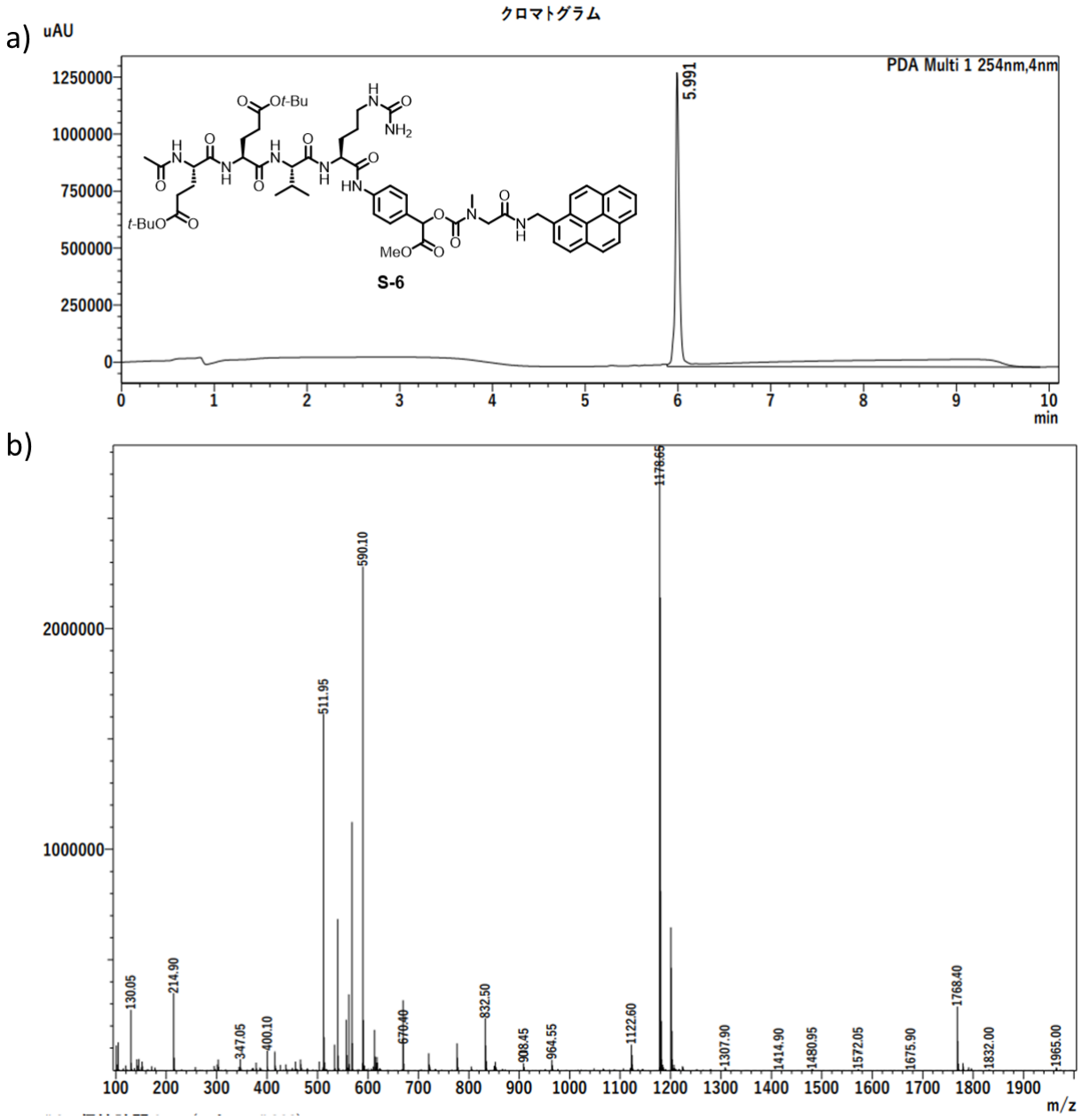


Figure S7. Analysis of compound **S-6**, a) HPLC analysis, b) MS analysis

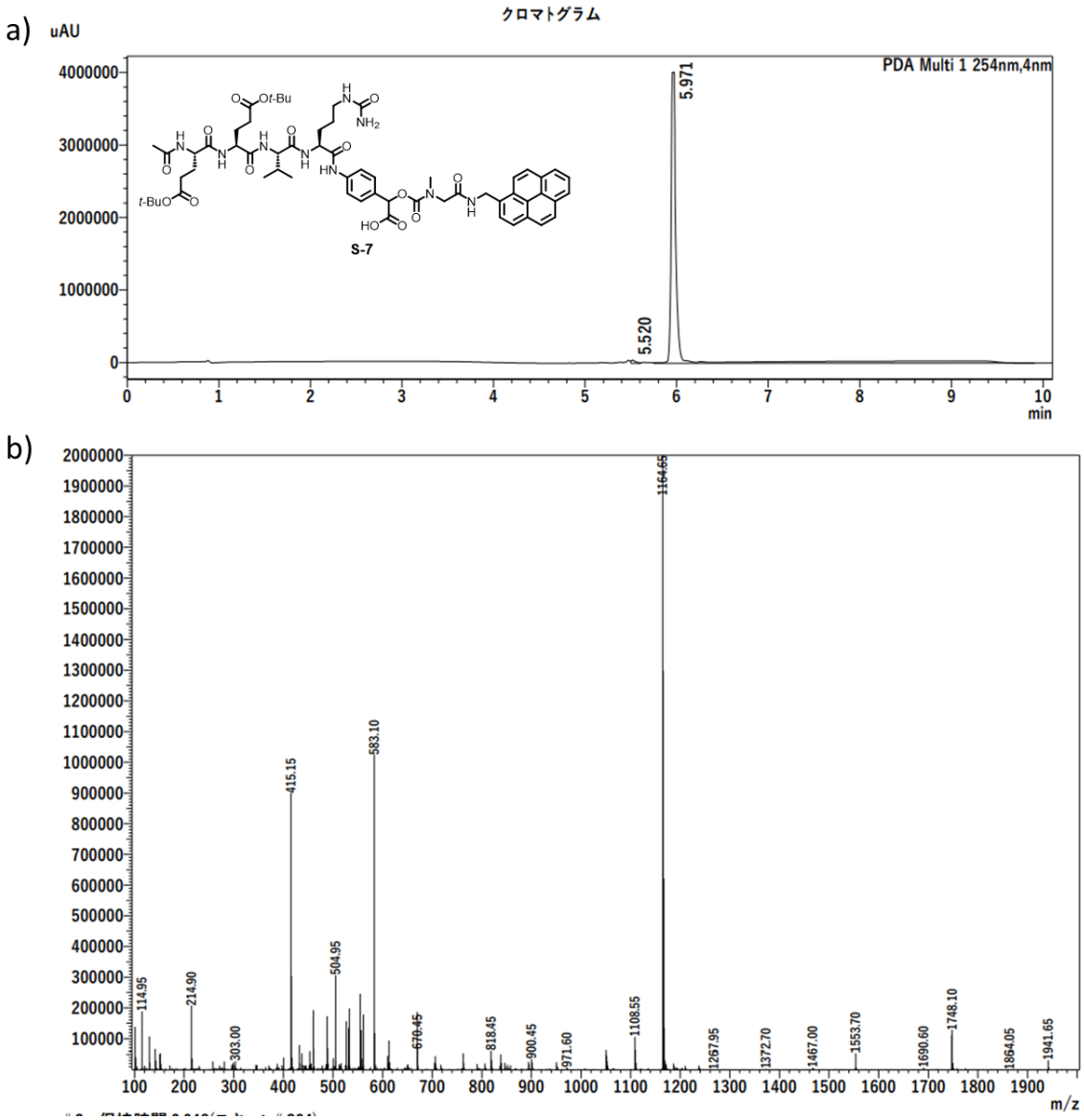


Figure S8. Analysis of compound **S-7**, a) HPLC analysis, b) MS analysis

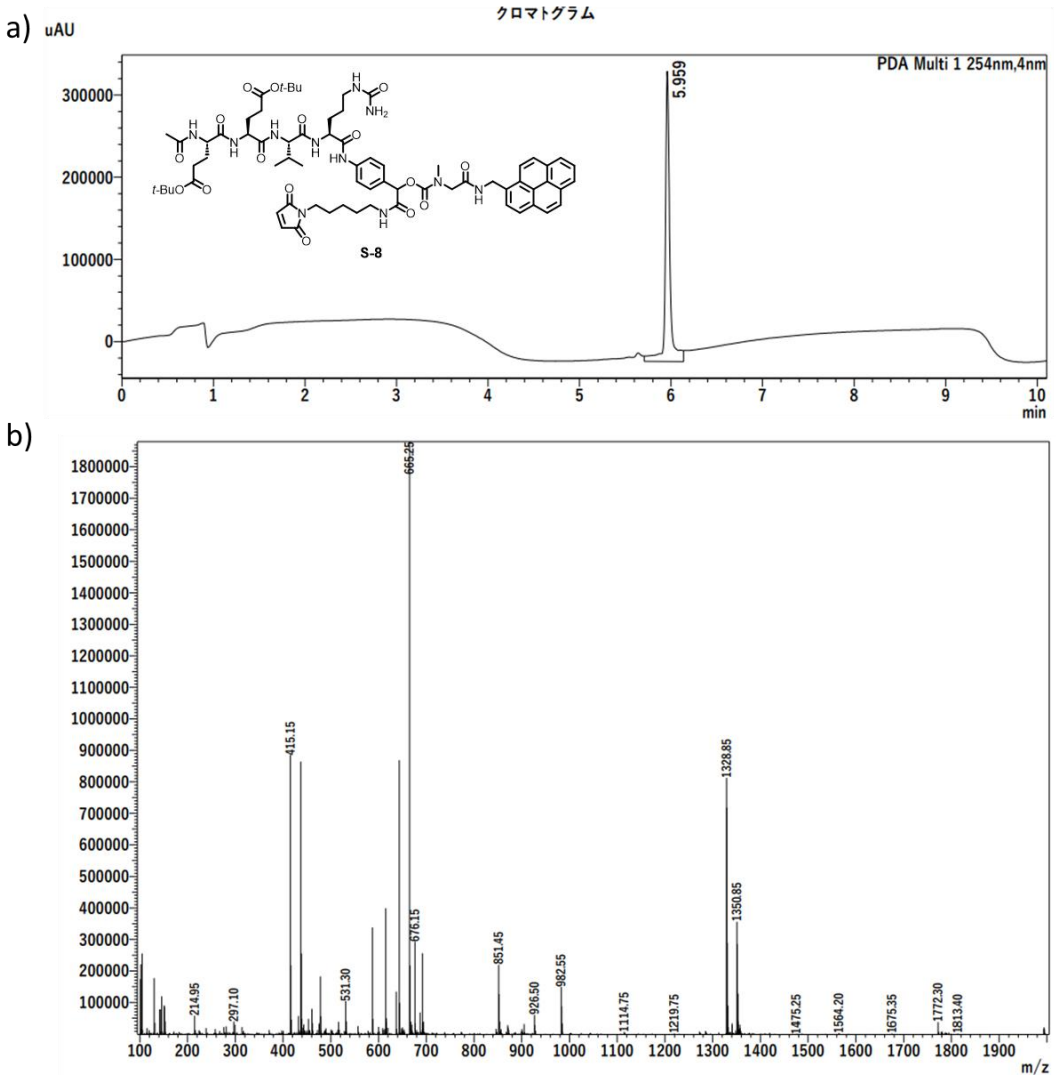


Figure S9. Analysis of compound **S-8**, a) HPLC analysis, b) MS analysis

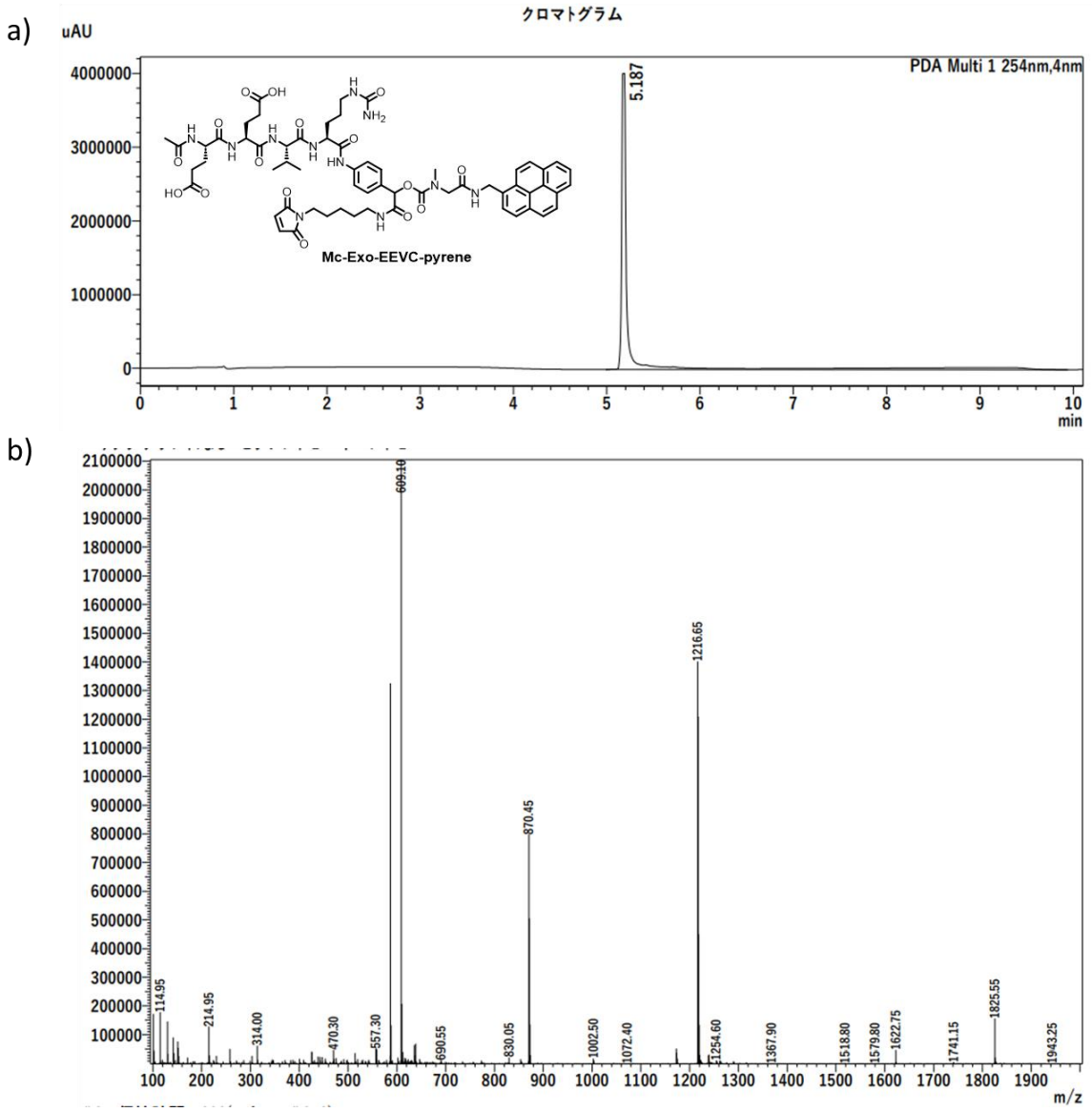


Figure S10. Analysis of compound Mc-Exo-EEVC-pyrene, a) HPLC analysis, b) MS analysis

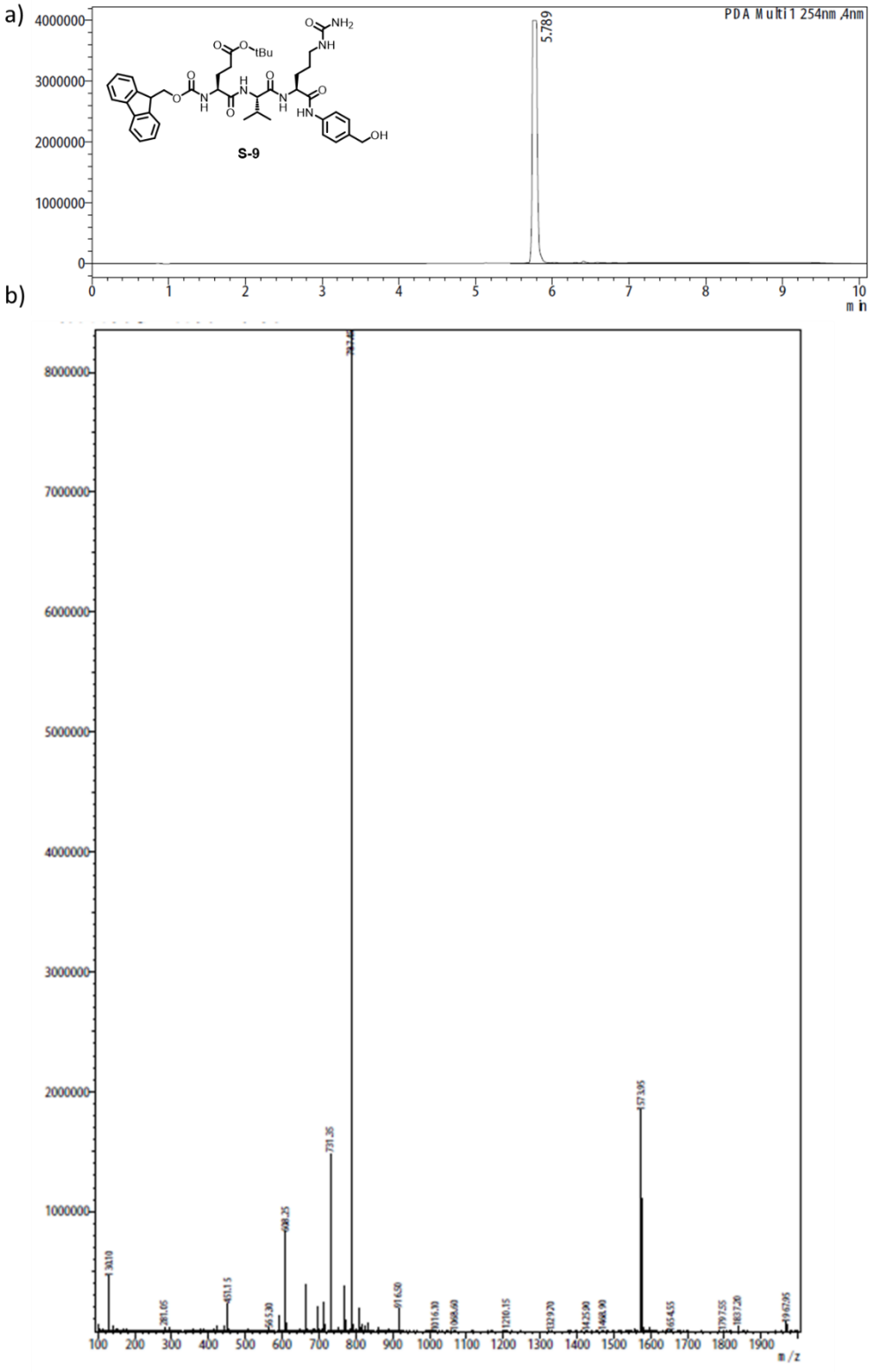


Figure S11. Analysis of compound **S-9**, a) HPLC analysis, b) MS analysis

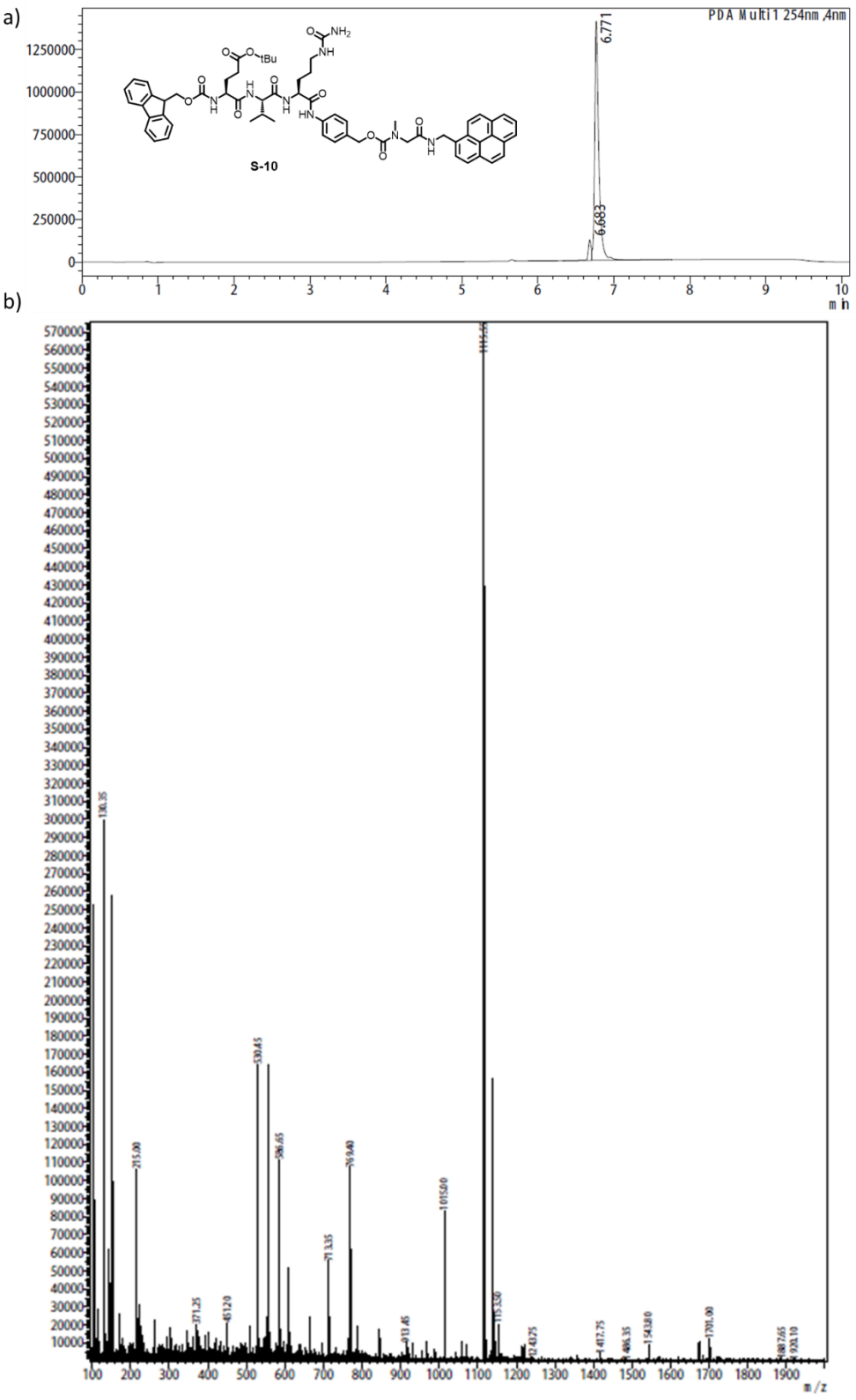


Figure S12. Analysis of compound **S-10**, a) HPLC analysis, b) MS analysis

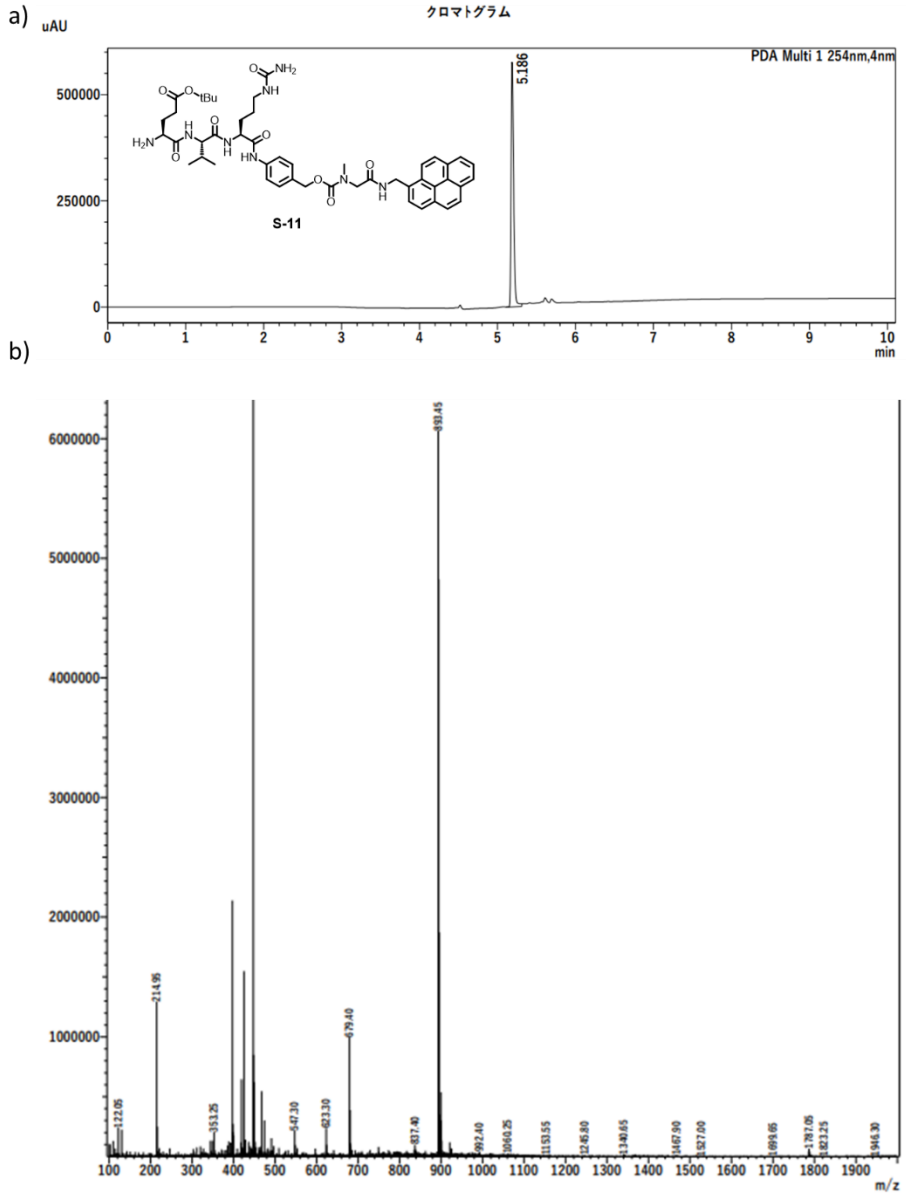


Figure S13. Analysis of compound **S-11**, a) HPLC analysis, b) MS analysis

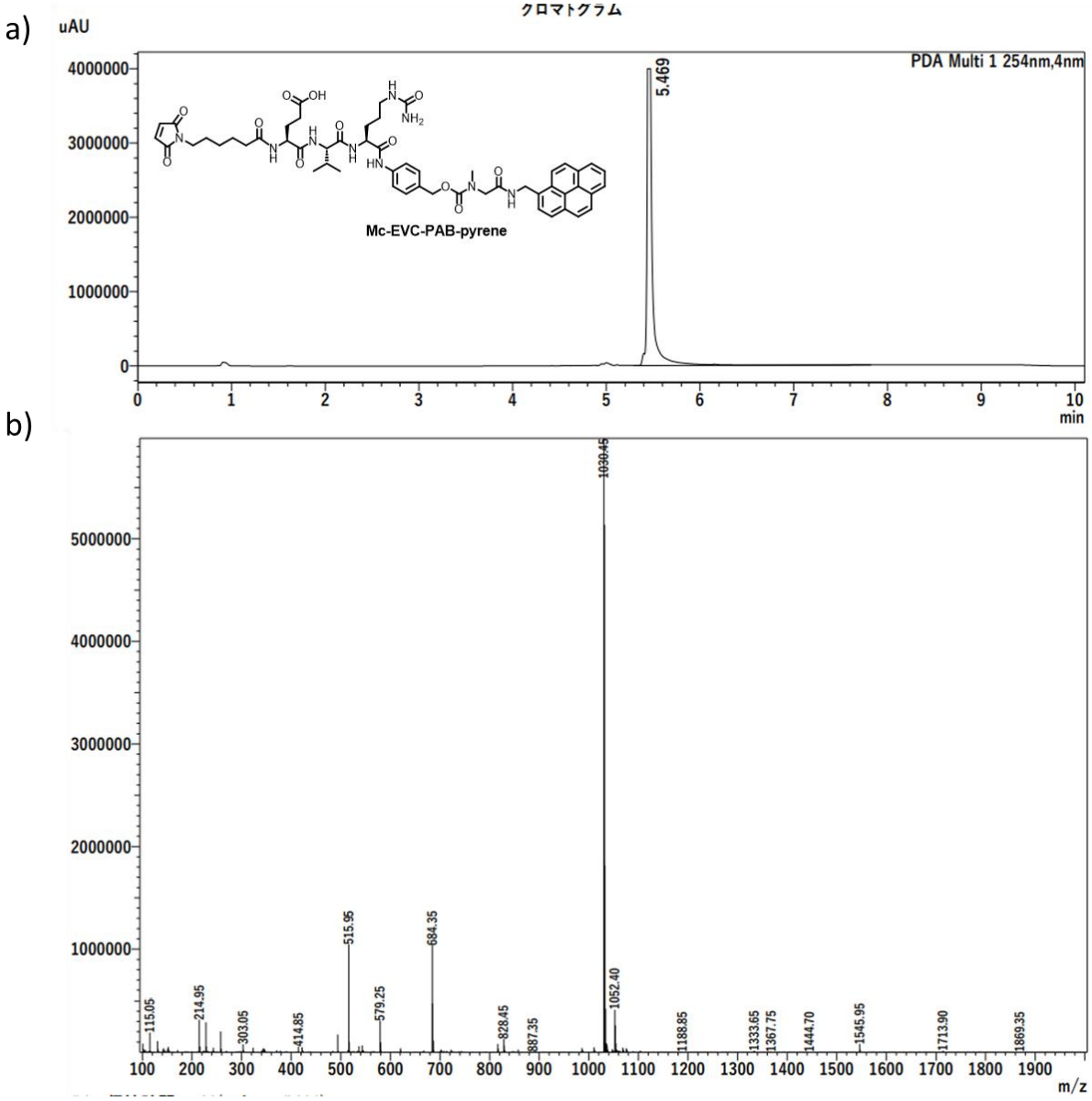


Figure S14. Analysis of compound Mc-EVC-PAB-pyrene, a) HPLC analysis, b) MS analysis

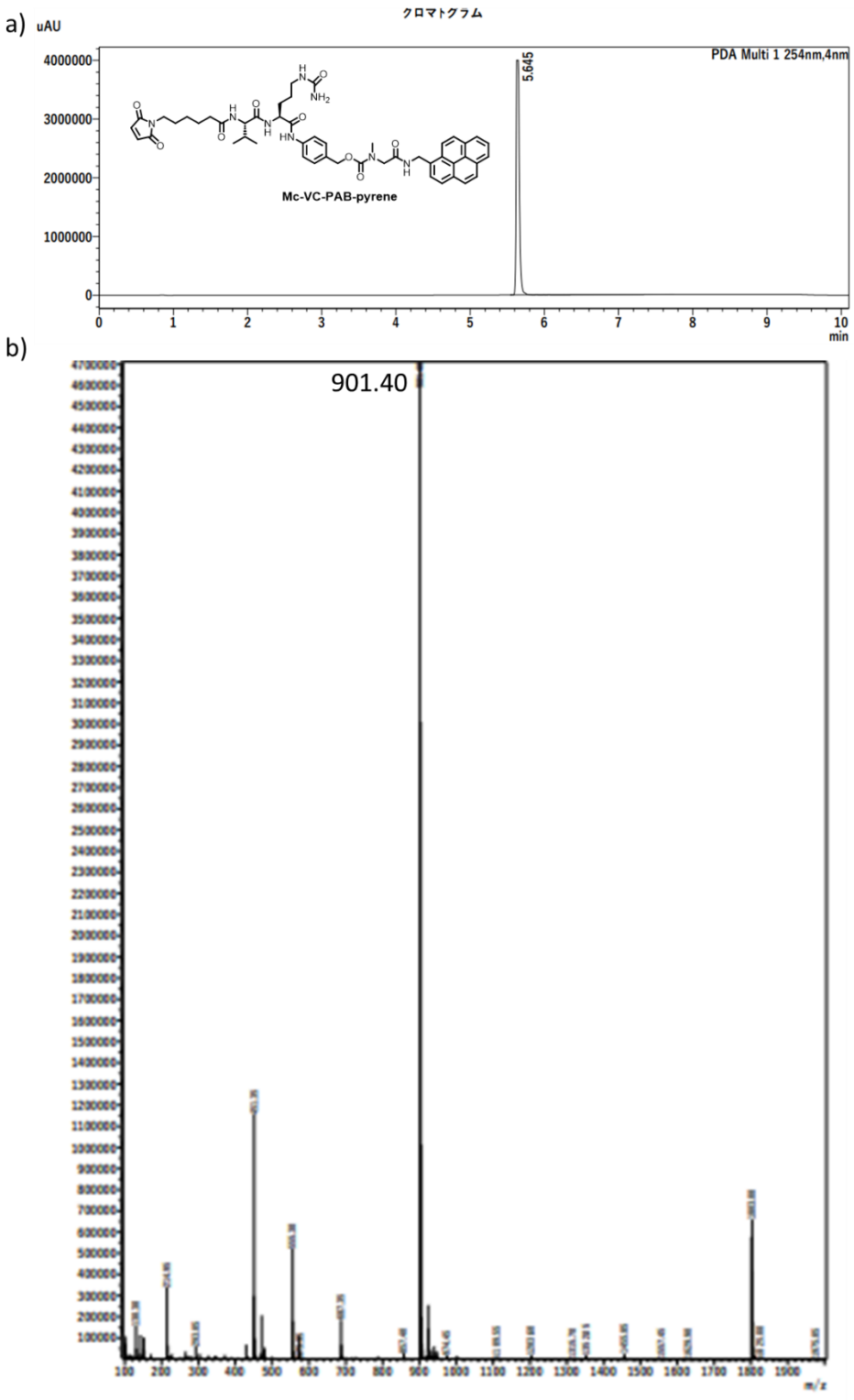
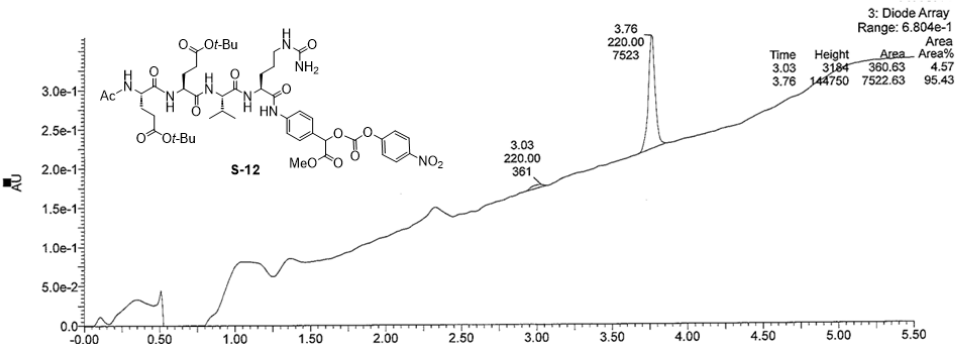


Figure S15. Analysis of compound Mc-VC-PAB-pyrene, a) HPLC analysis, b) MS analysis

a)



b)

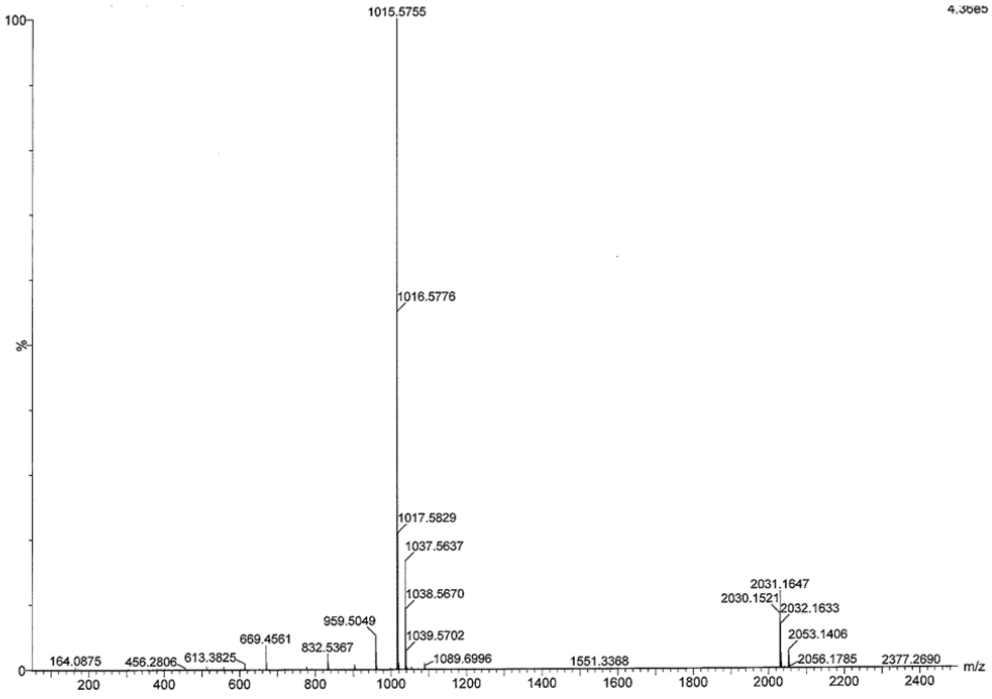


Figure S16. Analysis of compound **S-12**, a) HPLC analysis, b) MS analysis

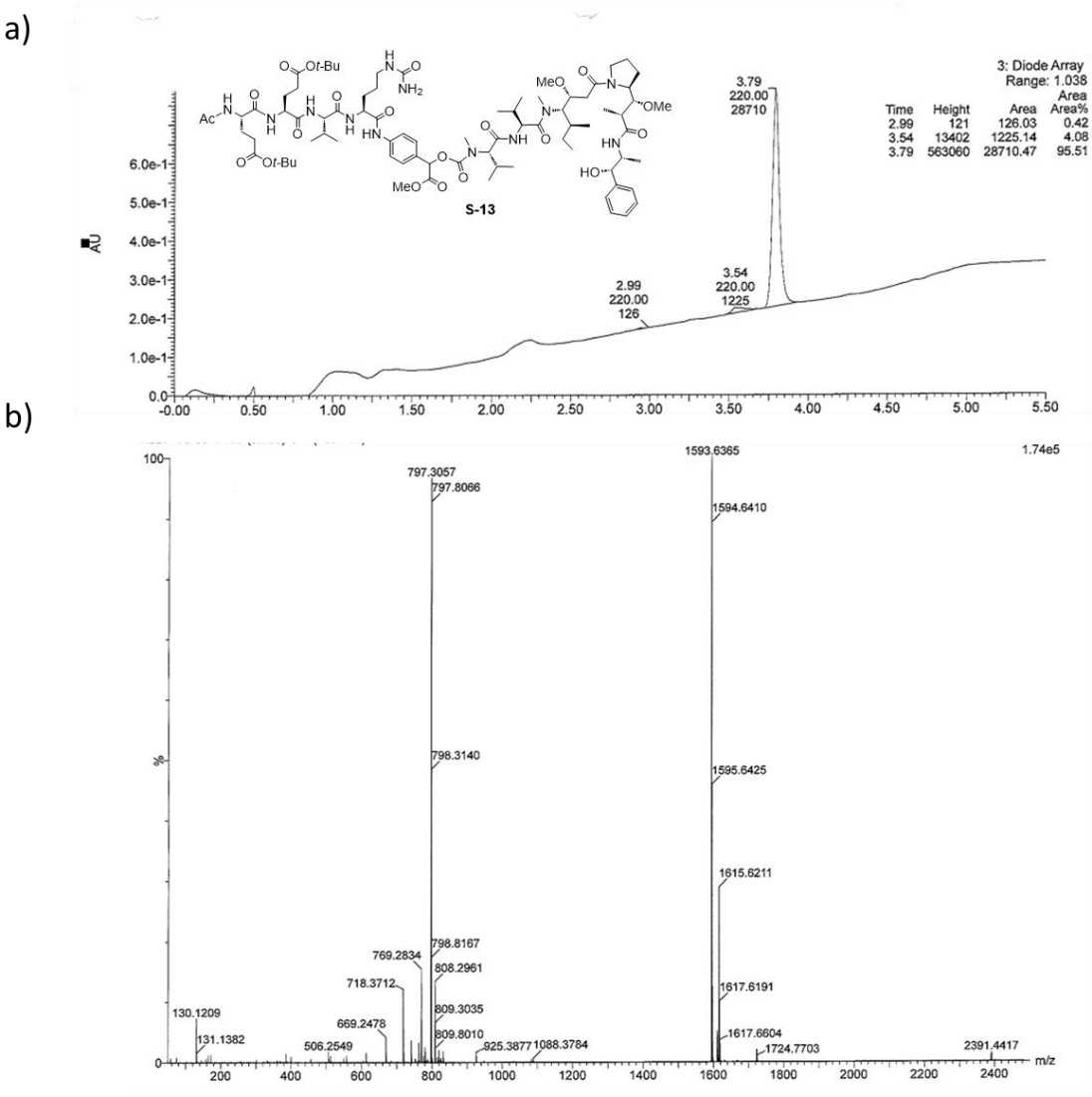


Figure S17. Analysis of compound **S-13**, a) HPLC analysis, b) MS analysis

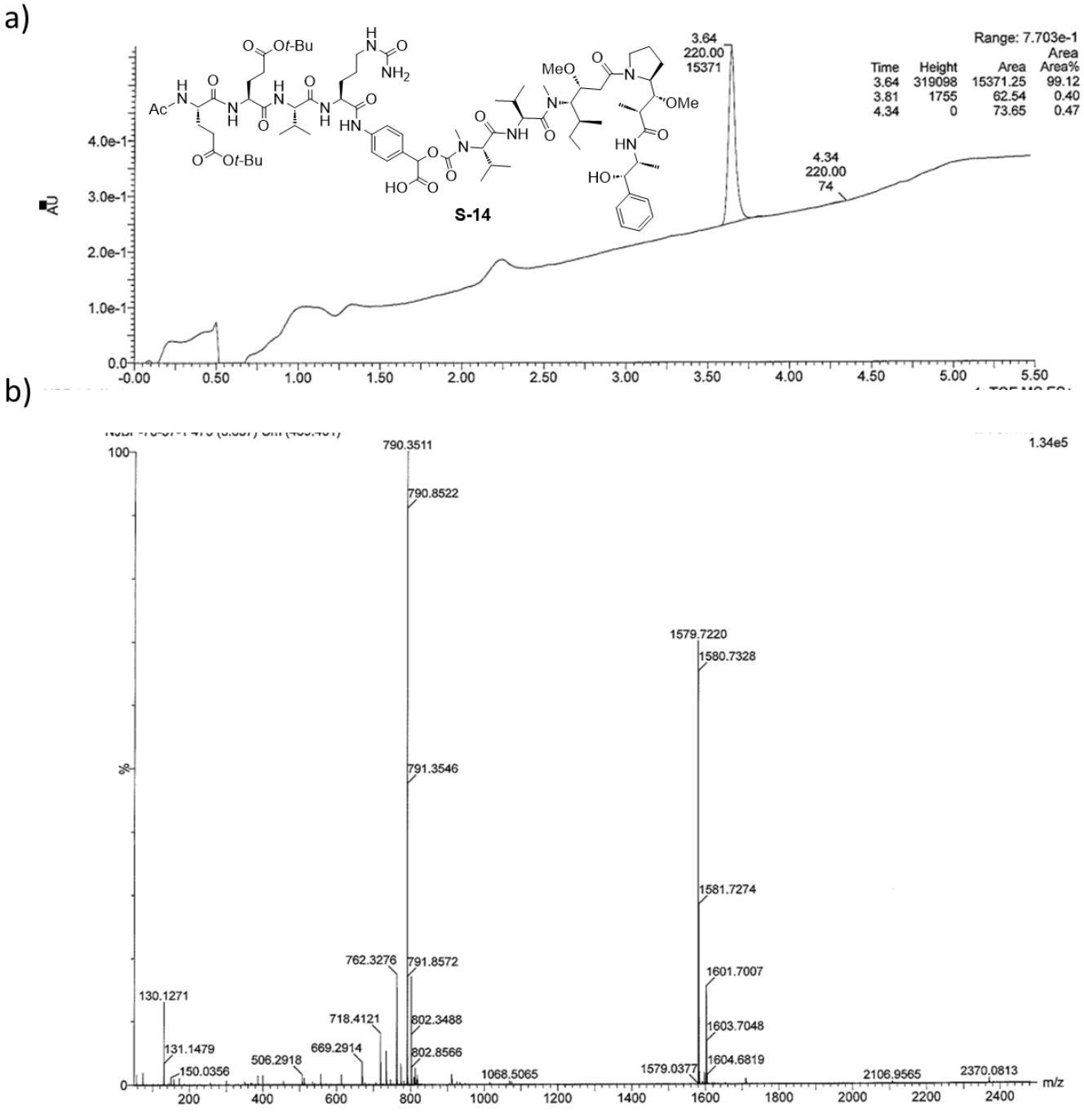


Figure S18. Analysis of compound **S-14**, a) HPLC analysis, b) MS analysis

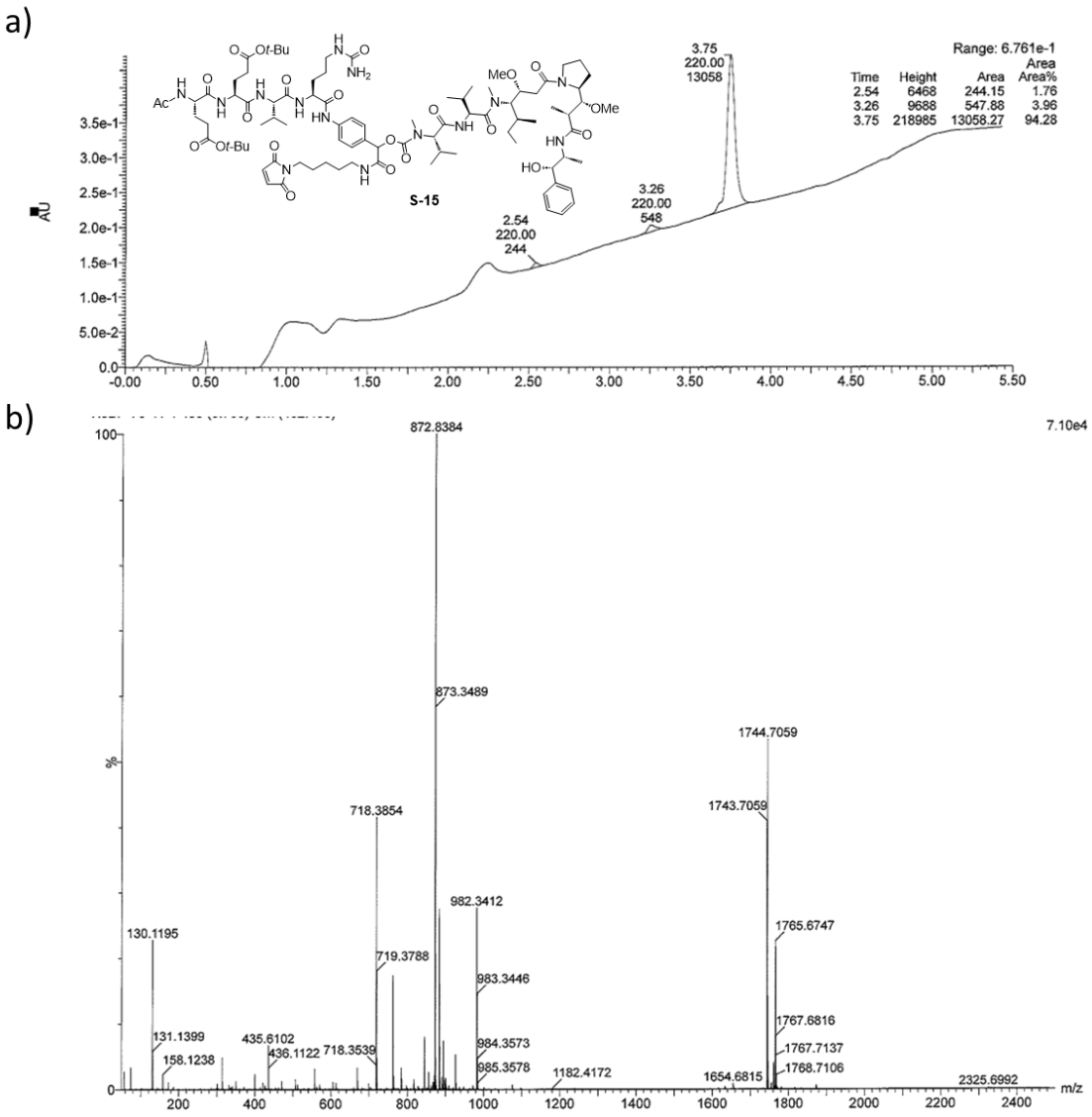


Figure S20. Analysis of compound **S-15**, a) HPLC analysis, b) MS analysis

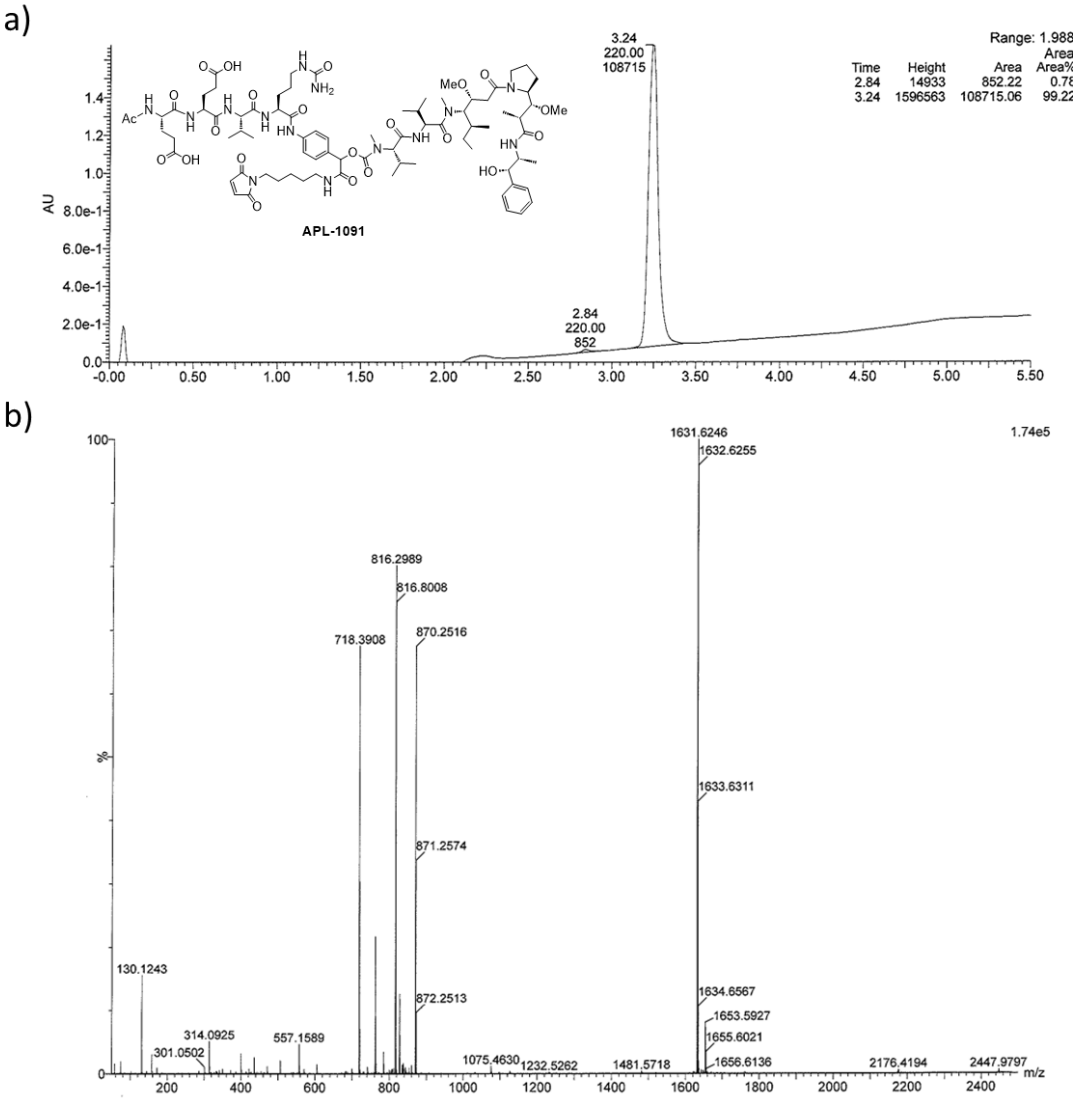


Figure S21. Analysis of compound APL-1091, a) HPLC analysis, b) MS analysis

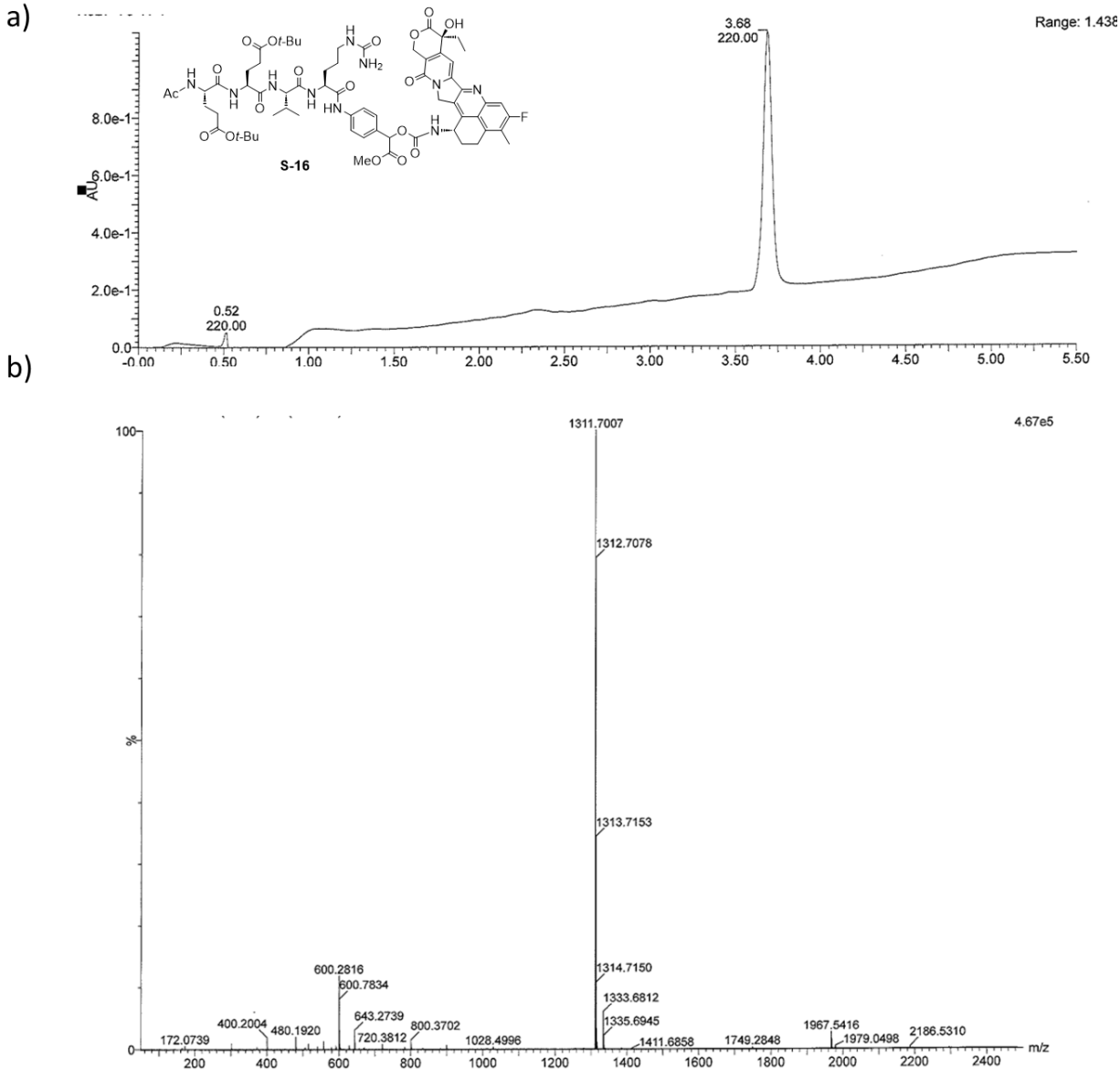


Figure S22. Analysis of compound **S-16**, a) HPLC analysis, b) MS analysis

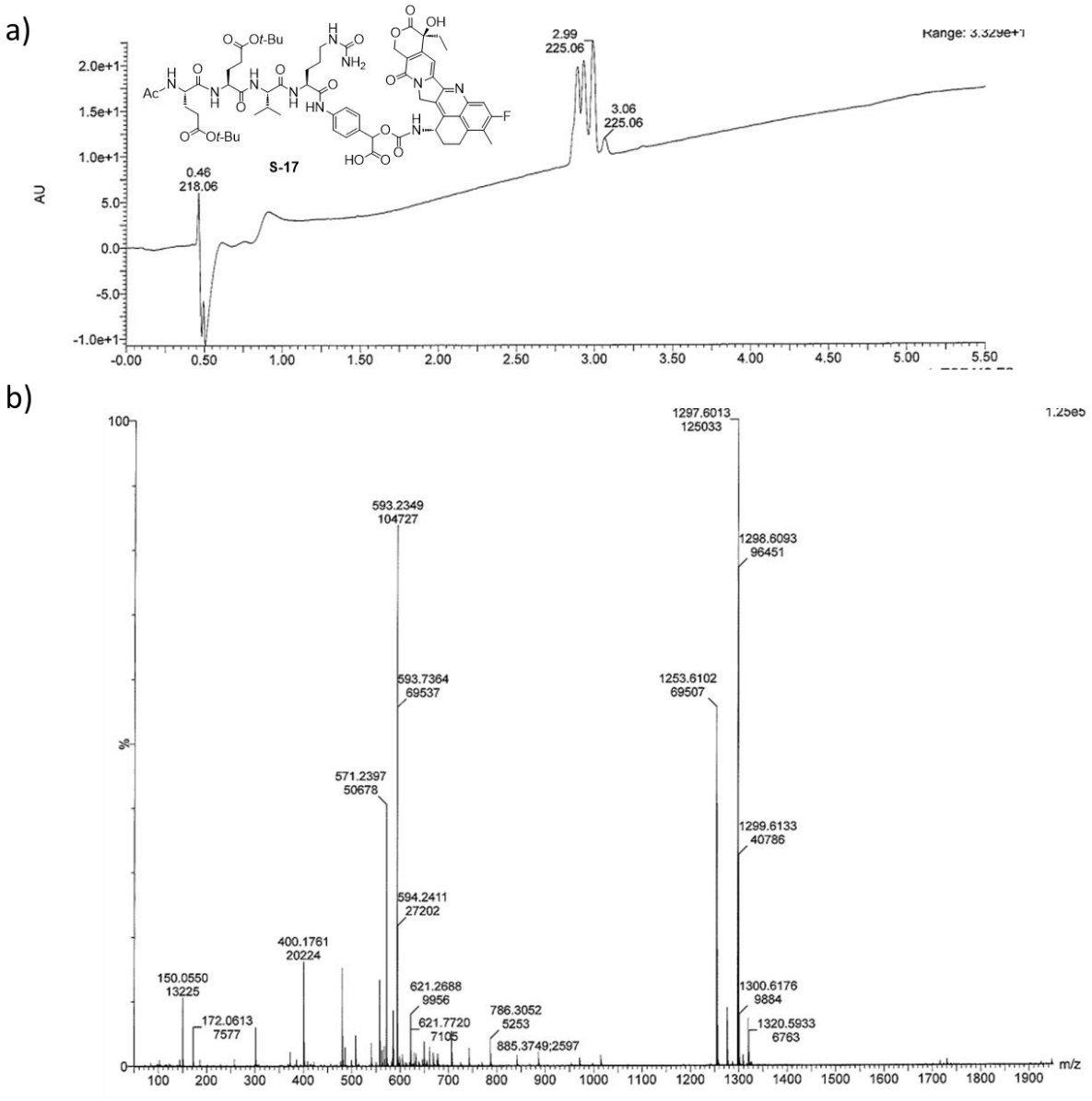


Figure S23. Analysis of compound **S-17**, a) HPLC analysis, b) MS analysis

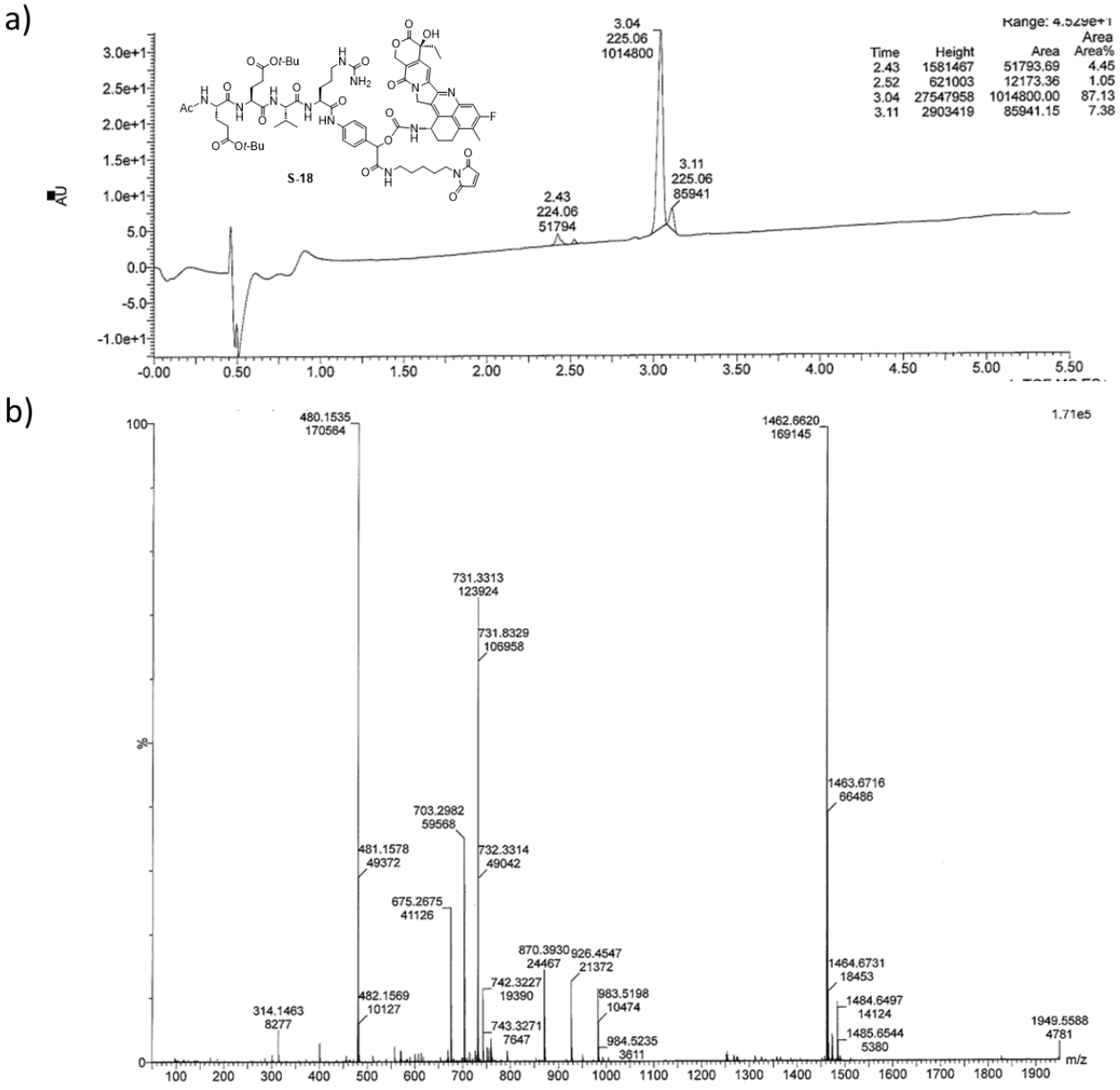


Figure S24. Analysis of compound **S-18**, a) HPLC analysis, b) MS analysis

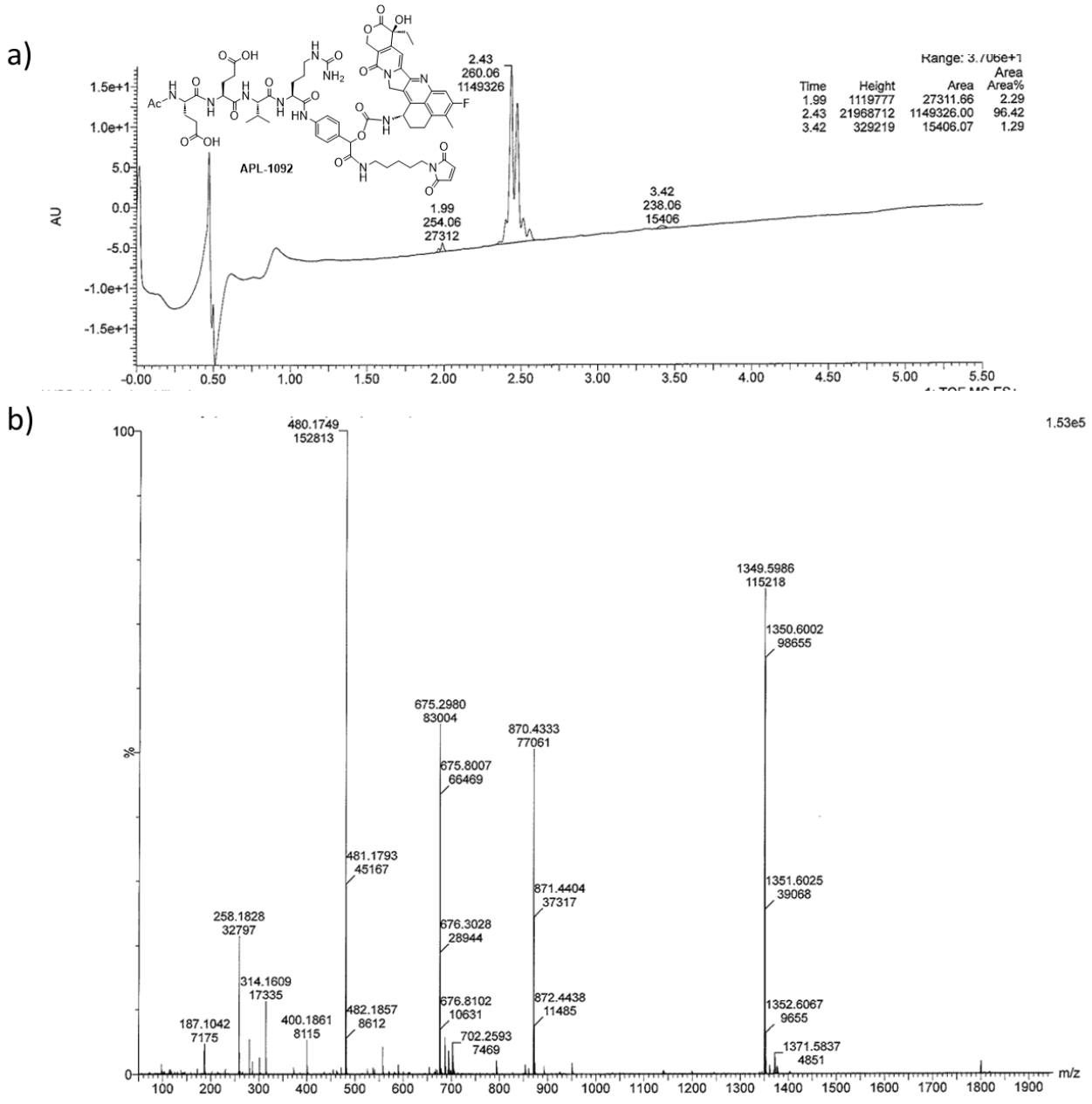


Figure S25. Analysis of compound APL-1092, a) HPLC analysis, b) MS analysis

2-2 ADC synthesis: HIC and SEC analysis

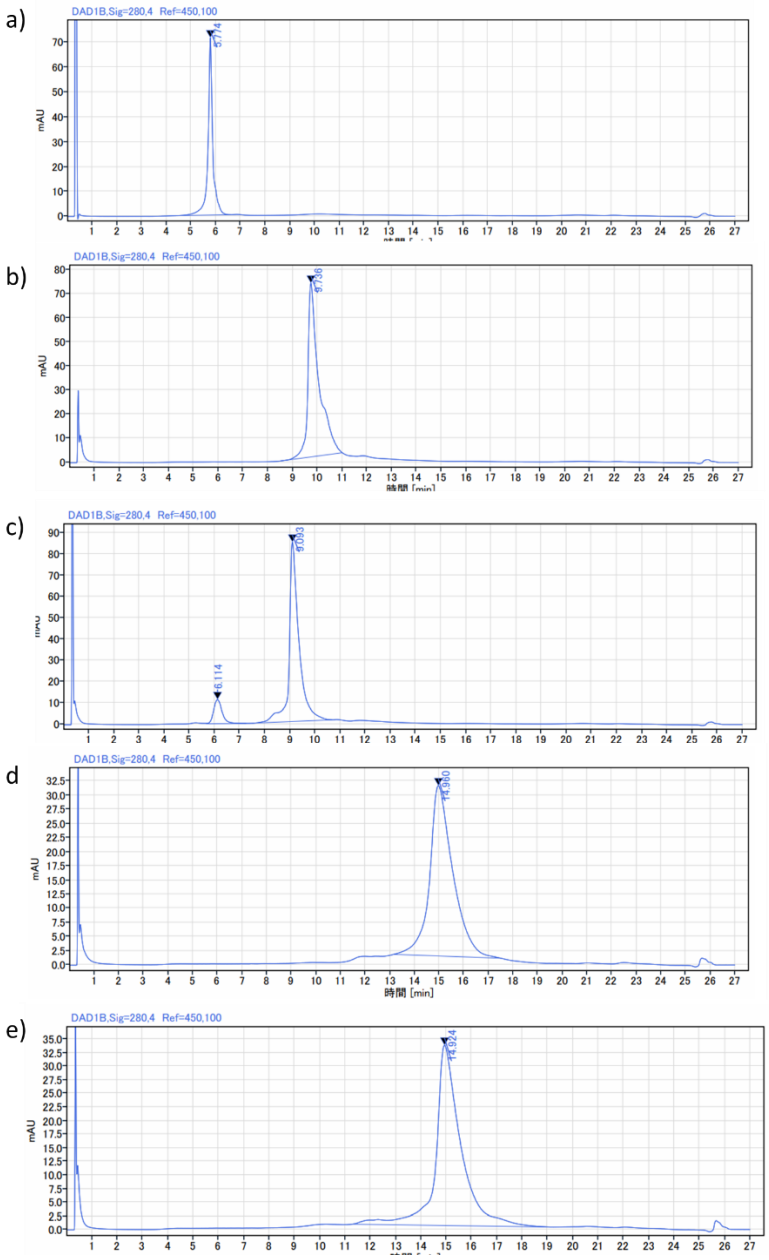


Figure S26. HIC-HPLC analysis of pyrene-based ADCs, a) trastuzumab, b) ADC (1) (Mc-Exo-EVC-pyrene), c) ADC (2) (Mc-Exo-EEVC-pyrene), d) ADC (3) (Mc-VC-PAB-pyrene), e) ADC (4) (Mc-EVC-PAB-pyrene)

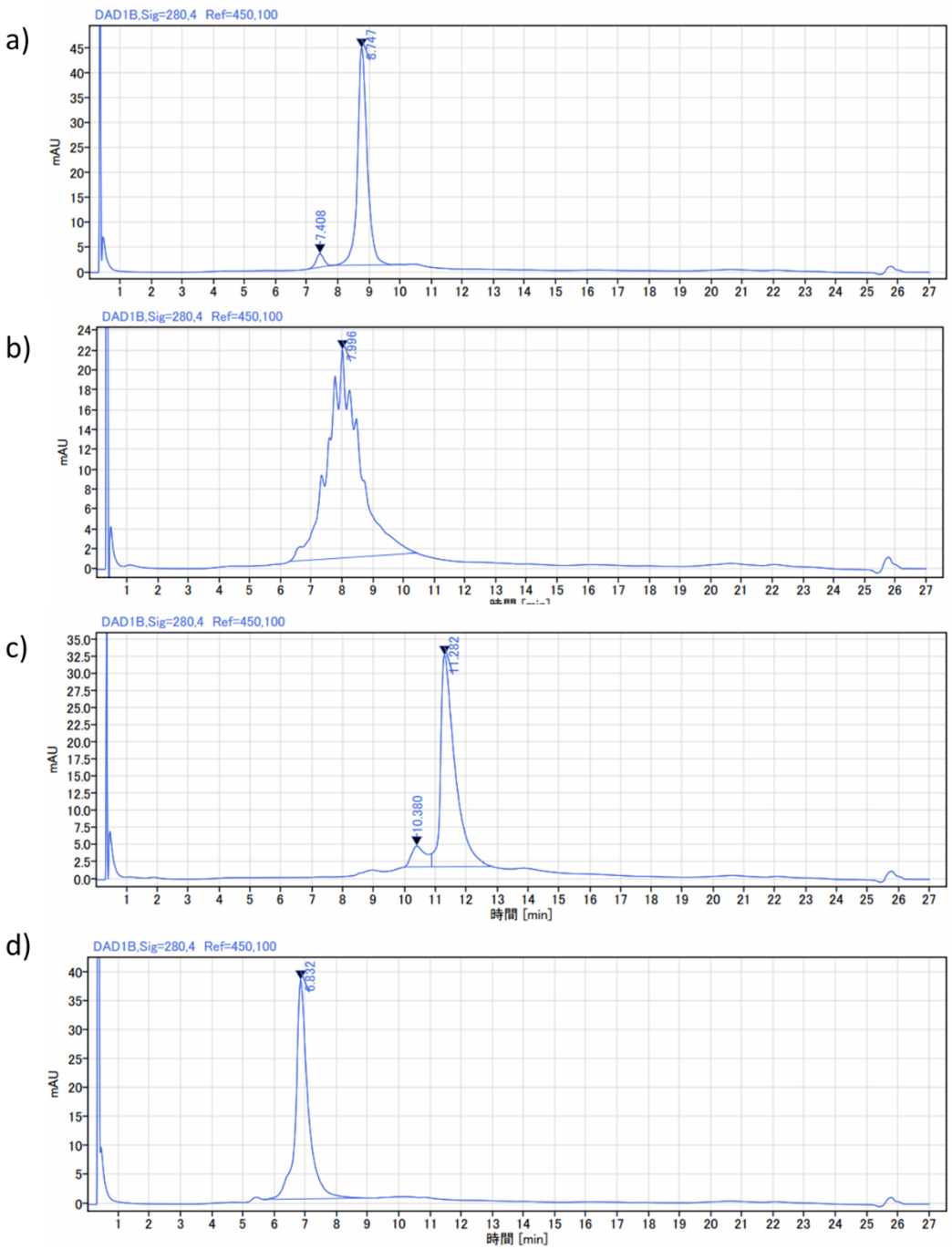


Figure S27. HIC-HPLC analysis of cytotoxic payload-based ADCs, a) ADC (5) (APL-1091, DAR=2), b) ADC (6) (APL-1092, DAR=2), c) ADC (7) (APL-1091, DAR=8), d) ADC (8) (APL-1092, DAR=8)

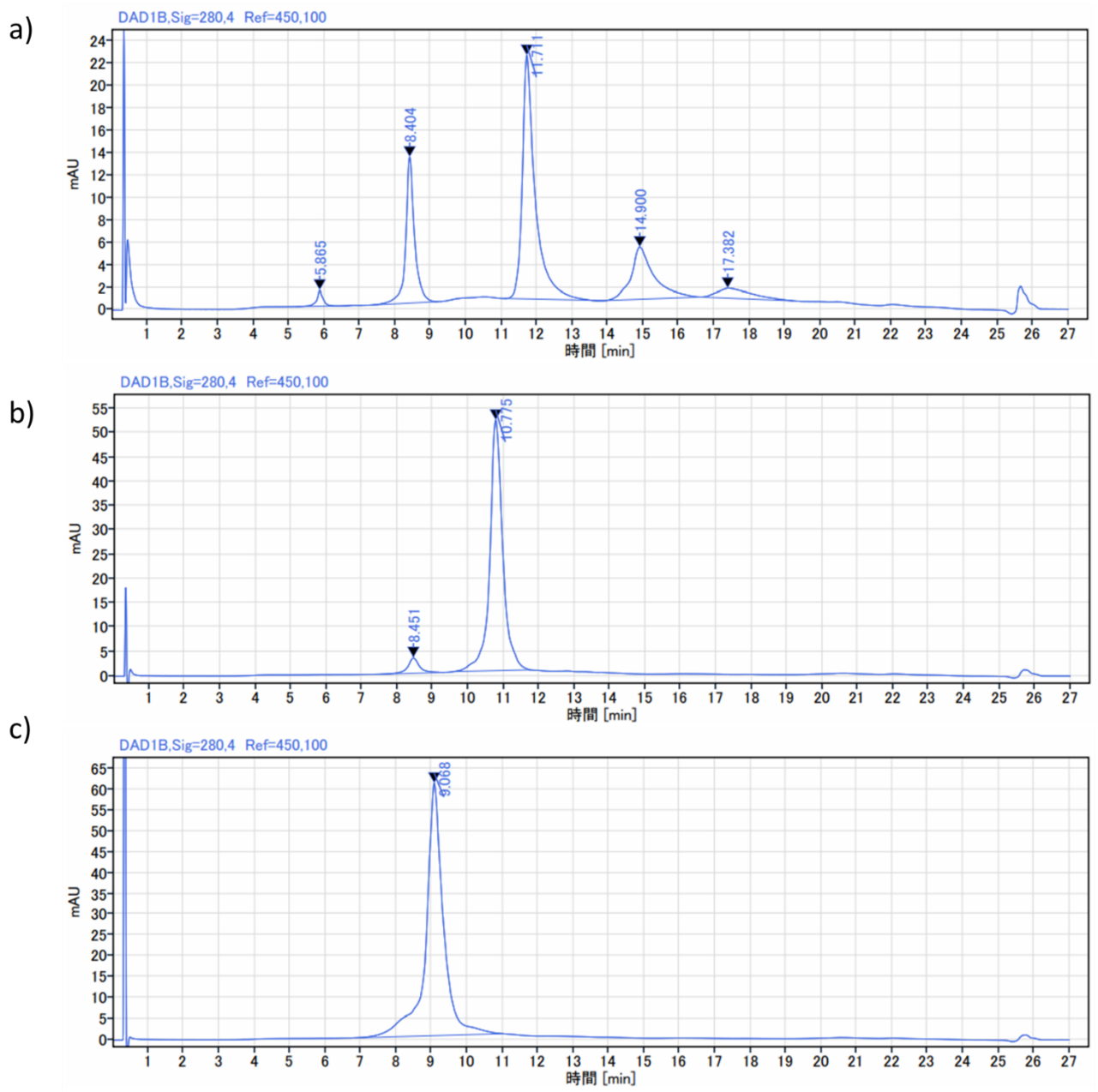


Figure S28. HIC-HPLC analysis of benchmark ADCs, a) ADC (9) (Mc-VC-PAB-MMAE, DAR=4), b) ADC (10) (Mc-VC-PAB-MMAE, DAR=2), c) T-Dxd (deruxtecan, DAR=8),

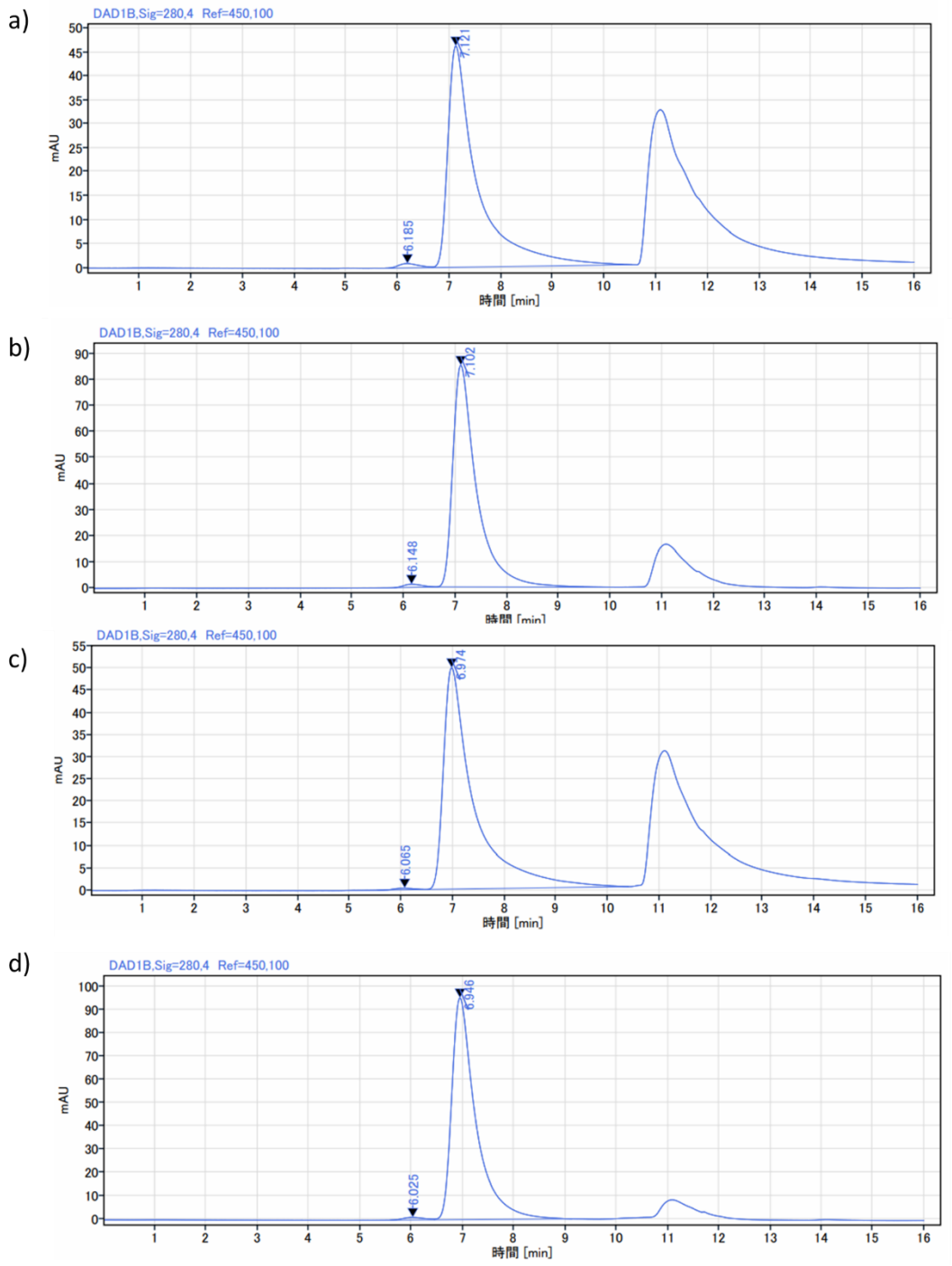


Figure S29. SEC-HPLC analysis of cytotoxic payload-based ADCs, a) ADC (5) (APL-1091, DAR=2), b) ADC (6) (APL-1092, DAR=2), c) ADC (7) (APL-1091, DAR=8), d) ADC (8) (APL-1092, DAR=8)

2-3 Rat PK study

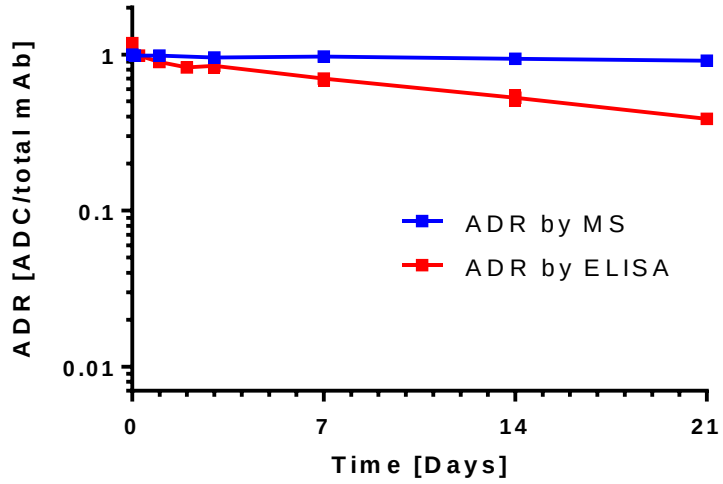


Figure S30. Comparison of ELISA assay and LBA assay of ADC 10

2-3 In vitro human neutrophil assay

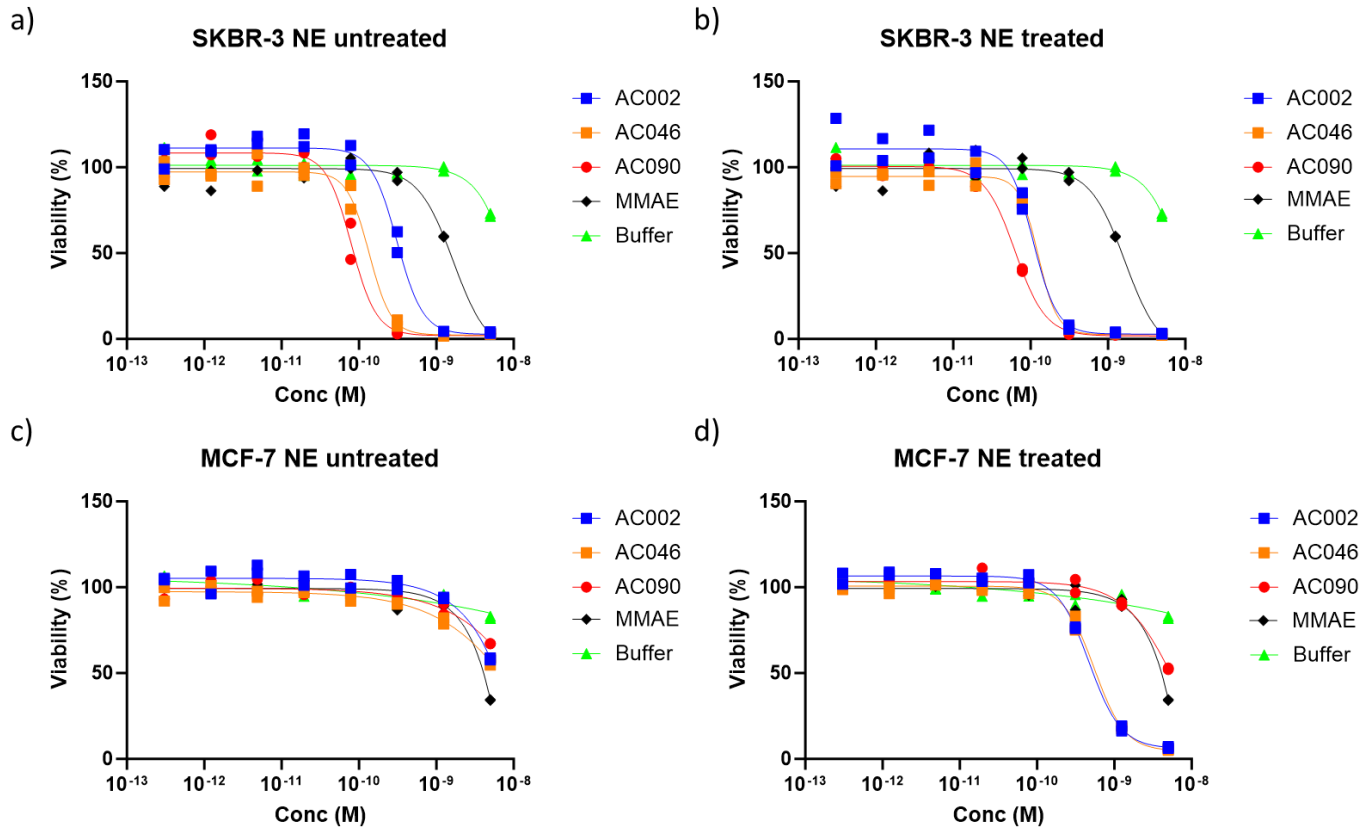


Figure S-31 Evaluation of ADCs by *in vitro* cytotoxicity assay. a, b) HER2-high expressing SKBR-3 cells and c,d) HER2-low expressing MCF-7 cells were incubated with 0.31 pM-5 nM of a, c) NE-untreated and b,c) NE-treated ADCs for 6 days. AC002 (blue squares) and AC046 (orange squares) were used as Val-Cit linker ADCs and AC090 (red circles) was used as an exo-linker ADC. As controls, MMAE (black rhombuses) and blank buffer (green triangles) were incubated with the cells. The individual values and fitted curves are shown from the results of duplicated experiments.

1. Salomon, P. L.; Reid, E. E.; Archer, K. E.; Harris, L.; Maloney, E. K.; Wilhelm, A. J.; Miller, M. L.; Chari, R. V. J.; Keating, T. A.; Singh, R., Optimizing Lysosomal Activation of Antibody-Drug Conjugates (ADCs) by Incorporation of Novel Cleavable Dipeptide Linkers. *Mol Pharm* **2019,** *16* (12), 4817-4825.

2. Fujii, T.; Matsuda, Y.; Seki, T.; Shikida, N.; Iwai, Y.; Ooba, Y.; Takahashi, K.; Isokawa, M.; Kawaguchi, S.; Hatada, N.; Watanabe, T.; Takasugi, R.; Nakayama, A.; Shimbo, K.; Mendelsohn, B. A.; Okuzumi, T.; Yamada, K., AJICAP Second Generation: Improved Chemical Site-Specific Conjugation Technology for Antibody-Drug Conjugate Production. *Bioconjug Chem* **2023,** *34* (4), 728-38.

3. Matsuda, Y.; Leung, M.; Tawfiq, Z.; Fujii, T.; Mendelsohn, B. A., In-situ Reverse Phased HPLC Analysis of Intact Antibody-Drug Conjugates. *Anal Sci* **2021,** *37* (8), 1171-1176.

4. Zhu, Q.; Girish, A.; Chattopadhaya, S.; Yao, S. Q., Developing novel activity-based fluorescent probes that target different classes of proteases. *Chem Commun (Camb)* **2004,** (13), 1512-3.

5. Anami, Y.; Yamazaki, C. M.; Xiong, W.; Gui, X.; Zhang, N.; An, Z.; Tsuchikama, K., Glutamic acid-valine-citrulline linkers ensure stability and efficacy of antibody-drug conjugates in mice. *Nat Commun* **2018,** *9* (1), 2512.

6. Yamazaki, S.; Shikida, N.; Takahashi, K.; Matsuda, Y.; Inoue, K.; Shimbo, K.; Mihara, Y., Lipoate-acid ligase a modification of native antibody: Synthesis and conjugation site analysis. *Bioorg Med Chem Lett* **2021,** *51*, 128360.

7. Nakahara, Y.; Mendelsohn, B. A.; Matsuda, Y., Antibody–Drug Conjugate Synthesis Using Continuous Flow Microreactor Technology. *Organic Process Research & Development* **2022,** *26* (9), 2766-2770.

8. Matsuda, Y.; Seki, T.; Yamada, K.; Ooba, Y.; Takahashi, K.; Fujii, T.; Kawaguchi, S.; Narita, T.; Nakayama, A.; Kitahara, Y.; Mendelsohn, B. A.; Okuzumi, T., Chemical Site-Specific Conjugation Platform to Improve the Pharmacokinetics and Therapeutic Index of Antibody-Drug Conjugates. *Mol Pharm* **2021,** *18* (11), 4058-4066.

9. Zhao, H.; Gulesserian, S.; Malinao, M. C.; Ganesan, S. K.; Song, J.; Chang, M. S.; Williams, M. M.; Zeng, Z.; Mattie, M.; Mendelsohn, B. A.; Stover, D. R.; Donate, F., A Potential Mechanism for ADC-Induced Neutropenia: Role of Neutrophils in Their Own Demise. *Mol Cancer Ther* **2017,** *16* (9), 1866-1876.

10. Ha, S. Y. Y.; Anami, Y.; Yamazaki, C. M.; Xiong, W.; Haase, C. M.; Olson, S. D.; Lee, J.; Ueno, N. T.; Zhang, N.; An, Z.; Tsuchikama, K., An Enzymatically Cleavable Tripeptide Linker for Maximizing the Therapeutic Index of Antibody-Drug Conjugates. *Mol Cancer Ther* **2022,** *21* (9), 1449-1461.

11. Ogitani, Y.; Aida, T.; Hagihara, K.; Yamaguchi, J.; Ishii, C.; Harada, N.; Soma, M.; Okamoto, H.; Oitate, M.; Arakawa, S.; Hirai, T.; Atsumi, R.; Nakada, T.; Hayakawa, I.; Abe, Y.; Agatsuma, T., DS-8201a, A Novel HER2-Targeting ADC with a Novel DNA Topoisomerase I Inhibitor, Demonstrates a Promising Antitumor Efficacy with Differentiation from T-DM1. *Clin Cancer Res* **2016,** *22* (20), 5097-5108.