Electronic Supplementary Material (ESI) for ChemComm.

Antibody drug conjugates with hydroxamic acid cargoes for histone deacetylase (HDAC) inhibition

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Supporting Information

General methods 2
Synthesis of compound **7** 2
Synthesis of compound **8** 3
Synthesis of compound **10** 4
Synthesis of compound **11** 4
Synthesis of compound **12** 5
Synthesis of compound **15** 5
General procedure for the preparation of ADCs 6
General Procedure for plasma stability assay 8
General biology procedures and in vivo experiments 9
Figure S1 (MALDI of conjugate **17**) 11
Figure S2 (MALDI of conjugate **18**) 13
Figure S3 (MALDI of conjugate **19**) 15
Figure S4 (MALDI of conjugate **20**) 17
Figure S5 (Binding of ADCs to tumor cells) 19
Figure S6 (Internalization of ADCs by tumor cells) 20
Figure S7 (Acetylation of HDAC-target proteins in A549 cells through Western Blot analysis) 21
NMR spectra, HPLC-MS and MS-ESI for product characterizations 22

General methods

All reagents were used as purchased from commercial suppliers without further purification. The reactions were carried out in oven dried or flamed vessels. Solvents were dried and purified by conventional methods prior use or, if available, purchased in anhydrous form. Flash column chromatography was performed with Merck silica gel 60, 0.040-0.063 mm (230-400 mesh). Merck aluminum backed plates pre-coated with silica gel 60 (UV254) were used for analytical thin layer chromatography and were visualized by staining with a KMnO4 solution. NMR spectra were recorded at 25 °C and 400 or 600 MHz for 1H and 100 or 150 MHz for ^{13}C. The solvent is specified for each spectrum. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Chemical shifts (d) are given in ppm relative to the resonance of their respective residual solvent peaks. High and low resolution mass spectroscopy analyses were recorded by electrospray ionization with a mass spectrometer Q-exactive Plus. Melting points were determined in open capillary tubes and are uncorrected. HPLC/MS analysis were performed with the chromatographic LC/MSD system Agilent 1100 series, connected with UV detector (254 nm) using an Intersil ODS-3V C18 column (5 μm, 4.6 x 250mm), flow 0.8 mL/min, MeCN (0.1% HCOOH)/H_2O (0.1% HCOOH) gradient from 1:9 to 9:1 in 10 minutes. ESI ionization, flow of the drying gas (N_2) 9L/min, temperature 350 °C, atomizing pressure 40 PSI, fragmentation.

6-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-(prop-2-yn-1-yl)hexanamide (5)

To a solution of 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoic acid (500 mg, 2.37 mmol) in CH_2Cl_2 dry (25 mL) in a round-bottom flask under magnetic stirring and atmosphere of N_2 at 0 °C in an ice bath were added in sequence propargylamine (162 μL, 2.37 mmol), 1-hydroxybenzotriazole hydrate (HOBt, 580 mg, 3.79 mmol), HBTU (1437 mg, 3.79 mmol) and DIPEA (1.65 mL, 9.48 mmol). The clear solution was warmed to room temperature and stirred overnight. The reaction mixture was then diluted with EtOAc (35 mL) and washed with $NaHCO_3$ ss (3x15 mL), HCl 1 N (3x15mL), water (3x15 mL) and brine (2x15 mL), dried over anhydrous sodium sulfate, filtered, concentrated in vacuo and purified by silica gel flash chromatography (PE:EtOAc 1:1) to provide compound **5** (330 mg, 1.33 mmol) as a white solid (yield 56%).). Spectral data are consistent with reported values[1].

[1] S. Köhling, G. Künze, K. Lemmnitzer, M. Bermudez, G. Wolber, J. Schiller, D. Huster, J. Rademann, *Chem. Eur. J.* 2016, **22**, 5563–5574

(4-Mercaptophenyl)methanol (6)

The product was prepared according to literature[2]

In a 250 mL two-neck round bottom flask provided with an addition funnel was added a solution of $LiAlH_4$ 1 M in THF (38.9 mL, 38.9 mmol) under an atmosphere of N_2; the solution was cooled to 0 °C in an ice bath and a solution of 4-mercaptobenzoic acid (2 g, 12.97 mmol) in THF dry (26 mL) was added dropwise in 30' through the addition funnel. The resulting mixture was vigorously stirred at room temperature for 16 h. The reaction mixture was cooled to 0°C, quenched with water (3 mL), acidified to pH 2 with HCl 1 N and extracted with EtOAc (3x30 mL); the organic phase was washed with water (3x40 mL) and brine (2x40 mL), dried over anhydrous sodium sulfate, filtered and concentrated in vacuo. The crude reaction mixture was purified by silica gel flash chromatography (PE:EtOAc 4:1) to provide compound **6** (1.204 g, 8.60 mmol) as a white solid (yield 74%).

6-(3-((4-(hydroxymethyl)phenyl)thio)-2,5-dioxopyrrolidin-1-yl)-N-(prop-2-yn-1-yl)hexanamide (7)

Freshly prepared thiol **6** (135 mg, 0.96 mmol) was added to a solution of compound **5** (200 mg, 0.80 mmol) in CH_3CN (8 mL) in a round bottom flask under an atmosphere of N_2. The resulting solution was stirred at room temperature overnight and concentrated *in vacuo*. The crude reaction mixture was purified by silica gel flash chromatography (PE:EtOAc 1:4) to provide the desired alcohol **7** (242 mg, 0.62 mmol) as a yellow oil (yield 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 – 7.34 (m, 2H), 7.24 (d, *J* = 6.1 Hz, 2H), 6.57 (s, 1H), 4.58 (s, 2H), 4.08 – 3.79 (m, 3H), 3.27 (t, *J* = 6.8 Hz, 2H), 3.19 – 2.98 (m, 1H), 2.77 – 2.53 (m, 1H), 2.18 (s, 1H), 2.01 (t, J = 7.6 Hz, 2H), 1.55 – 1.38 (m, 2H), 1.25-1.23 (m, 2H), 1.02-0.96 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.08, 174.32, 172.63, 142.93, 134.66, 127.61, 126.99, 79.35, 71.03, 63.37, 43.49, 38.38, 35.62, 35.47, 28.60, 26.70, 25.66, 24.58. Elemental Analysis calcd for $C_{20}H_{24}N_2O_4S$: C, 61.84; H, 6.23; N, 7.21; O, 16.47; S, 8.25 found C, 61.82; H, 6.25; N, 7.20; O, 16.50; S, 8.23.

N1-((4-((2,5-dioxo-1-(6-oxo-6-(prop-2-yn-1-ylamino)hexyl)pyrrolidin-3-yl)thio)benzyl)oxy)-N8-phenyloctanediamide (8)

[2] Q. Xie, C. Ni, R. Zhang, L. Li, J. Rong, J. Hu, Angew. Chemie Int. Ed. 2017, **56**, 3206–3210.).

The alcohol **7** (246 mg, 0.63 mmol) solubilized in dry THF (15 mL) was converted into the corresponding bromide by adding PBr_3 (87 µL, 0.96 mmol) at 0 °C and stirring the solution at 0 °C for 3 h. The crude reaction mixture was concentrated *in vacuo* and filtered through a silica gel path with EtOAc to provide the corresponding bromide in 71% yield as a bright orange oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47 (d, *J* = 8.0 Hz, 2H), 7.34 (d, *J* = 8.0 Hz, 2H), 4.47 (s, 2H), 4.14 (dd, *J* = 9.2, 2.8 Hz, 1H), 3.94 (s, 2H), 3.38 (t, *J* = 7.2 Hz, 2H), 3.14 (dd, *J* = 18.8, 9.2 Hz, 1H), 2.66 (dd, *J* = 18.8, 2.8 Hz, 1H), 2.21 (s, 1H), 2.15 (d, *J* = 7.2 Hz, 2H), 1.64-1.48 (m, 2H), 1.48- 1.42 (m, 2H), 1.20-1.16 (m, 2H); MS-ESI: m/z 473-475 $[M + Na]^+$. Vorinostat (**1**, 96 mg, 0.36 mmol) was dissolved in MeOH (6 mL) and NaOH 40% solution (76 µL, 0.76 mmol) was added. The resulting mixture was stirred for 10' and then added to the bromide (201 mg, 0.44 mmol). Immediately the solution turned purple, after 10' the base was neutralized with HCl 1 N, diluted with CH_2Cl_2, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo.* The crude reaction mixture was purified by silica gel flash chromatography (PE:EtOAc 1:4) to provide compound **8** (150 mg, 0.24 mmol) as a white solid (yield 67%). ^{1}H NMR (600 MHz, CD_3OD): δ 7.54-7.52 (m, 4H), 7.42 (d, J = 7.6 Hz, 2H), 7.30-7.28 (m, 2H), 7.08 (t, *J* = 7.3 Hz, 1H), 4.86 (s, 2H), 4.22 (d, J = 8.0 Hz, 1H), 3.93 (s, 2H), 3.38 (t, *J* = 7.1 Hz, 2H), 3.21 (dd, *J* = 18.5, 9.0 Hz, 1H), 2.63 (dd, *J* = 18.6, 3.3 Hz, 1H), 2.57 (s, 1H), 2.36 (t, *J* = 7.2 Hz, 2H), 2.16 (t, *J* = 7.3 Hz, 2H), 2.06 (t, *J* = 7.0 Hz, 2H), 1.69-1.68 (m, 2H), 1.60-1.57 (m, 4H), 1.43-1.29 (m, 6H) , 1.21-1.19 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD): δ 175.56, 174.68, 173.13, 172.09, 138.39, 137.20, 134.74, 129.68, 129.01, 124.20, 120.06, 79.84, 71.57, 43.82, 38.98, 37.40, 36.25, 36.01, 29.78, 29.19, 28.70, 27.31, 26.31, 25.44, 25.03. Anal calcd for $C_{34}H_{42}N_4O_6S$: C, 64.33; H, 6.67; N, 8.83; O, 15.12; S, 5.05 found C, 64.35; H, 6.69; N, 8.82; O, 15.15; S, 4.99.

6-(1-((6-(2,5-dioxo-3-((4-(((8-oxo-8-(phenylamino)octanamido)oxy)methyl)phenyl)thio)pyrrolidin-1-yl)hexanamido)methyl)-1H-1,2,3-triazol-4-yl)hexanoic acid (10)

Compounds **8** (90 mg, 0.14 mmol) and **9** (17 mg, 0.11 mmol) were dissolved in DMF dry (10 mL) in a round-bottom flask under magnetic stirring and atmosphere of argon. The solution was degassed with three cycles of argon/*vacuum*. To this solution, a freshly prepared aqueous mixture (5.5 mL) of $Cu(OAc)_2$ (7 mg, 0.03 mmol) and sodium ascorbate (13 mg, 0.07 mmol), previously degassed by argon/*vacuum* cycles, was added dropwise. The reaction mixture was degassed and left to stir under argon at room temperature for 72 h. The solvent was evaporated and the crude reaction mixture was purified by silica gel flash chromatography (CH_2Cl_2:MeOH 9:1) to provide compound **10** (57 mg, 0.09 mmol) as a white solid (yield 65%). ^{1}H NMR (600 MHz, CD_3OD): δ 7.85 (s, 1H), 7.54-7.51 (m, 4H), 7.41 (d, *J* = 7.9 Hz,

2H), 7.29 (t, *J* = 7.8 Hz, 2H), 7.07 (t, *J* = 7.3 Hz, 1H), 4.85 (s, 2H), 4.42 (s, 2H), 4.38 (t, *J* = 7.3 Hz, 2H), 4.24 (dd, *J* = 9.0, 3.7 Hz, 1H), 3.38 (t, *J* = 7.0 Hz, 2H), 3.22 (dd, *J* = 18.7, 9.1 Hz, 1H), 2.63 (dd, *J* = 18.6, 3.8 Hz, 1H), 2.36 (t, *J* = 7.4 Hz, 2H), 2.26 (m, 2H), 2.21 (t, *J* = 7.2 Hz, 2H), 2.06 (t, *J* = 7.2 Hz, 2H), 1.94 – 1.83 (m, 2H), 1.69 – 1.59 (m, 8H), 1.38 – 1.33 (m, 8H), 1.22-1.18 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD): δ 177.81, 176.74, 176.54, 176.29, 174.77, 141.06, 139.74, 134.94, 133.09, 131.02, 129.64, 125.47, 121.27, 78.14, 74.94, 51.49, 44.91, 39.76, 37.86, 37.11, 33.71, 30.89, 29.86, 28.28, 27.29, 26.68, 26.40, 25.58. Anal calcd for $C_{40}H_{53}N_7O_8S$: C, 60.66; H, 6.75; N, 12.38; O, 16.16; S, 4.05 found C, 60.65; H, 6.73; N, 12.38; O, 16.15; S, 4.09.

(E)-6-(3-((4-(((3-(4-(((2-(1H-indol-3-yl)ethyl)(2-hydroxyethyl)amino)methyl)phenyl)acrylamido)oxy)methyl)phenyl)thio)-2,5-dioxopyrrolidin-1-yl)-N-(prop-2-yn-1-yl)hexanamide (11)

The alcohol **7** (246 mg, 0.63 mmol) solubilized in dry THF (15 mL) was then converted into the corresponding bromide by adding PBr_3 (87 µL, 0.96 mmol) at 0 °C and stirring the solution at 0 °C for 3 h. The crude reaction mixture was concentrated *in vacuo* and filtered through a silica gel path with EtOAc to provide the corresponding bromide in 71% yield as a bright orange oil. ^{1}H NMR (400 MHz, $CDCl_3$,): δ 7.47 (d, *J* = 8.0 Hz, 2H), 7.34 (d, *J* = 8.0 Hz, 2H), 4.47 (s, 2H), 4.14 (dd, *J* = 9.2, 2.8 Hz, 1H), 3.94 (s, 2H), 3.38 (t, *J* = 7.2 Hz, 2H), 3.14 (dd, *J* = 18.8, 9.2 Hz, 1H), 2.66 (dd, *J* = 18.8, 2.8 Hz, 1H), 2.21 (s, 1H), 2.15 (d, *J* = 7.2 Hz, 2H), 1.64-1.48 (m, 2H), 1.48- 1.42 (m, 2H), 1.20-1.16 (m, 2H); MS-ESI: m/z 473-475 $[M + Na]^+$. Dacinostat (**4**, 136 mg, 0.36 mmol) was dissolved in MeOH (6 mL) and NaOH 40% solution (76 µL, 0.76 mmol) was added. The resulting mixture was stirred for 10' and then added to the bromide (201 mg, 0.44 mmol). Immediately the solution turned purple, after 10' the base was neutralized with HCl 1 N, diluted with CH_2Cl_2, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo.* The crude reaction mixture was purified by silica gel flash chromatography (PE:EtOAc 1:4) to provide compound **11** (208 mg, 0.28 mmol) as a white solid (yield 77%). ^{1}H NMR (600 MHz, CD_3OD) δ 7.59 – 7.33 (m, 11H), 7.17(s, 1H), 7.08 (t, *J* = 7.4 Hz, 1H), 6.95 (t, *J* = 7.3 Hz, 1H), 6.46 (d, *J* = 16.0 Hz, 1H), 4.96 (s, 2H), 4.60 (d, *J* = 12.9 Hz, 1H), 4.45 (d, *J* = 12.9 Hz, 1H), 4.23 (m, 1H), 3.97-3.93 (m, 4H), 3.65 – 3.31 (m, 8H), 3.25-3.20 (m, 1H), 2.65 (d, *J* = 18.1 Hz, 1H), 2.56 (s, 1H), 2.21 – 2.14 (m, 2H), 1.64-1.55 (m, 2H), 1.39-1.29 (m, 2H), 1.19-1.18 (m 2H).

^{13}C NMR (100 MHz, CD_3OD): δ 177.33, 176.37, 175.21, 141.09, 137.83, 134.57, 132.31, 130.79, 130.72, 129.44, 127.48, 127.32, 124.34, 122.69, 120.01, 119.67, 118.72, 112.51, 109.16, 80.92, 58.16, 56.40, 55.58, 54.39, 51.54, 49.64, 49.43, 49.21, 44.77, 44.62, 39.57, 36.92, 36.34, 29.32, 27.97, 27.01,

25.93, 21.15. Anal calcd for $C_{42}H_{47}N_5O_6S$: C, 67.27; H, 6.32; N, 9.34; O, 12.80; S, 4.28 found C, 67.25; H, 6.33; N, 9.37; O, 12.79; S, 4.26.

(E)-6-(4-((6-(3-((4-(((3-(4-(((2-(1H-indol-3-yl)ethyl)(2-hydroxyethyl)amino)methyl)phenyl)acrylamido)oxy)methyl)phenyl)thio)-2,5-dioxopyrrolidin-1-yl)hexanamido)methyl)-1H-1,2,3-triazol-1-yl)hexanoic acid (12)

Compounds **11** (105 mg, 0.14 mmol) and **9** (17 mg, 0.11 mmol) were dissolved in DMF dry (10 mL) in a round-bottom flask under magnetic stirring and atmosphere of argon. The solution was degassed with three cycles of argon/*vacuum*. To this solution, a freshly prepared aqueous mixture (5.5 mL) of $Cu(OAc)_2$ (7 mg, 0.03 mmol) and sodium ascorbate (13 mg, 0.07 mmol), previously degassed by argon/*vacuum* cycles, was added dropwise. The reaction mixture was degassed and left to stir under argon at room temperature for 72 h. The solvent was evaporated and the crude was purified by silica gel flash chromatography (CH_2Cl_2:MeOH 9:1) to provide compound **12** (62 mg, 0.068 mmol) as a white solid (yield 62%). 1H NMR (600 MHz, CD_3OD) δ 7.81 (s, 1H), 7.53 – 7.44 (m, 9H), 7.35 (d, *J* = 7.8 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 7.08-7-06 (m, 2H), 6.93 (t, *J* = 7.4 Hz, 1H), 6.44 (d, *J* = 15.7 Hz, 2H), 4.95 (s, 2H), 4.42 (s, 2H), 4.35 – 4.32 (m, 3H), 4.15 (s, 2H), 3.81 (m, 2H), 3.37 – 3.34 (m, 2H), 3.24 – 3.11 (m, 7H), 2.64 (d, *J* = 18.6 Hz, 1H), 2.24 – 2.17 (m, 4H), 1.88 – 1.84 (m, 2H), 1.62 – 1.57 (m, 4H), 1.38-1.37 (m, 4H), 1.16-1.14 (m, 2H). . ^{13}C NMR (100 MHz, CD_3OD) 177.68, 176.76, 176.73, 175.85, 175.62, 141.48, 141.23, 138.25, 137.26, 134.99, 133.91, 132.33, 131.01, 129.84, 129.52, 127.99, 124.34, 124.15, 122.75, 120.04, 119.00, 112.56, 110.25, 69.32, 58.56, 57.24, 56.07, 54.86, 51.17, 45.02, 40.17, 39.76, 37.10, 36.62, 35.66, 32.47, 31.58, 30.95, 30.11, 28.26, 27.27, 27.02, 26.23, 25.61, 24.99, 24.88, 23.98, 21.66, 14.30, 11.34.. Anal calcd for $C_{48}H_{58}N_8O_8S$: C, 63.56; H, 6.44; N, 12.35; O, 14.11; S, 3.53 found C, 63.55; H, 6.45; N, 12.37; O, 14.14; S, 3.49.

4-((S)-2-((S)-2-(7-ethoxy-7-oxoheptanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl 8-oxo-8-(phenylamino)octanoate (15)

Vorinostat (**1**, 162 mg, 0.61 mmol) was dissolved in MeOH (6 mL) and NaOH 40% solution (730 µL, 0.73 mmol) was added. The resulting mixture was stirred for 10' and then added to the bromide **14** (559 mg, 0.92 mmol) prepared as described in reference 18. Immediately the solution turned purple, after 10' the base was neutralized with HCl 1 N and the solvent was evaporated. The crude reaction mixture was purified by silica gel flash chromatography (CH_2Cl_2:MeOH 9:1) to provide compound **15** (214 mg, 0.27 mmol) as a yellow oil (yield 46%). ^1H NMR (400 MHz, CD_3OD): δ ^1H NMR (400 MHz, CD3OD) δ

7.65-7-56 (m, 4H), 7.40-7-29 (m, 4H), 7.10 (t, *J* = 7.4 Hz, 1H), 4.82 (s, 2H), 4.58-4.55 (m,2H), 4.21 (d, *J* = 7.2 Hz, 1H), 4.13 (q, *J* = 7.1 Hz, 2H), 3.92 (dd, *J* = 13.3, 6.4 Hz, 1H), 3.51 (t, *J* = 6.7 Hz, 1H), 3.28 – 3.08 (m, 2H), 2.42 – 2.39 (m, 2H), 2.34-2.30 (m, 4H), 2.15-1-93 (m, 3H), 1.96-1.93 (m, 2H), 1.82-1.60 (m, 10H), 1.42-1.31 (m, 6H), 1.26 (t, *J* = 7.2 Hz, 3H), 1.01-0.97 (m, 6H). ^{13}C NMR (100 MHz, CD3OD): 176.44, 175.34, 174.53, 174.10, 172.45, 167.51, 162.01, 139.72, 138.43, 131.02, 129.64, 128.47, 125.00, 121.43, 121.18, 64.77, 61.32, 60.59, 60.50, 55.07, 45.39, 37.81, 36.53, 36.42, 34.84, 33.72, 33.62, 31.76, 31.51, 30.44, 30.33, 30.26, 29.73, 29.59, 27.62, 26.60, 26.49, 26.40, 26.33, 25.60, 20.17, 19.98, 19.94, 19.81, 18.92, 14.55. Anal calcd for $C_{41}H_{60}N_6O_9$: C, 61.94; H, 7.86; N, 14.10; O, 16.10 found C, 61.96; H, 7.85; N, 14.11; O, 16.08.

General procedure for the preparation of ADCs through conjugation via ε-amino groups of lysine residues. 17 (Ctx-NH-10), 18 (Trast-NH-10), 19 (Ctx-NH-12), 20 (Trast-NH-16).

The proper carboxylic acid (**10**, **12** or **16,** obtained from ester **15** through hydrolysis using 3 equivalents of LiOH in a 1:1:1 mixture of THF/H_2O/EtOH for 2 h, 0.020 mmol) was dissolved in anhydrous DMF (0.5 mL) in a round-bottom flask under magnetic stirring and atmosphere of N_2. DCC (7 mg, 0.035 mmol) and N-hydroxysuccinimide (3.4 mg, 0.030 mmol) were then added to the resulting solution and the mixture was stirred at room temperature for 16 hours. The white solid was removed by filtration and the solvent removed under vacuum, to provide a white solid (MS(ESI): m/z 911 $[M+Na]^+$ for activated **10**, 1026 $[M+Na]^+$ for activated **12** and 887 $[M+Na]^+$ for activated **16)** that was dissolved in DMSO in order to obtain a solution of 10 mM.

Contemporary, a solution of the proper antibody (Trastuzumab or Cetuximab) was buffer exchanged using a 10 kDa cutoff dialysis membrane to obtain antibodies dissolved in PBS pH 7.4 and to remove interfering preservative (glycine). The concentration of the antibodies after dialysis was determined measuring the OD280 and the observed absorbance was divided by 1.35. A 40 fold molar excess of the various NHS ester 10 mM solution was the added to the dialyzed antibody solution.

The reaction was incubated at room temperature with gentle continuous mixing and after 1 hour quenched with a 20 mM glycine aqueous solution. The final product was dialyzed in PBS overnight at 4 °C using a 10 kDa cutoff membrane in order to remove the excess of unreacted payload.

The DAR was determined by MALDI mass spectrometry, using an Ultraflex III mass spectrometer (Bruker, GmbH), operating in positive linear mode. Briefly, 100 µL of unconjugated antibodies and products **17**, **18**, **19** and **20** was desalted using PD spin trap G25 (GEHealthcare) eluting in water. A 10 mg/ml s-DHB MALDI matrix solution was prepared in 0.1%TFA dissolved in a mixture of water and

acetonitrile (50:50, v/v). The sample solution (2 µL) was deposited on MALDI target using a double layer sample deposition method. The mass spectra were acquired in a mass range from 50 kDa to 180 kDa. The average DAR was calculated (over three experiments) dividing mass difference between unconjugated and conjugated antibodies by the MW of the linker-payload.

Compound **17** DAR = 4 ± 0.5. Compound **18** DAR = 5 ± 0.5. Compound **19** DAR 3 ± 0.5. Compound **20** 6 ± 0.5.

General Procedure for plasma stability assay [3].

A solution containing **10**, **12** or **15** (4 µL, 10 mM) in methanol was added to mouse plasma (0.5 mL) in sodium phosphate buffer (0.5 mL) and incubated at 37 °C. Aliquots (50 µL) were withdrawn at different time. The sample was extracted with acetonitrile (0.2 mL) and centrifugated for 10 minutes at 10000 rpm. The supernatant (0.15 mL) was evaporated, solubilized in methanol (1 mL) and subjected to HPLC/MS analysis analysis. Intersil ODS-3V C18 column (5 µm, 4.6 x 250mm), flow 0.8 mL/min, MeCN (0.1% HCOOH)/H_2O (0.1% HCOOH) gradient from 1:9 to 9:1 in 10 minutes. ESI ionization, flow of the drying gas (N_2) 9L/min, temperature 350 °C, atomizing pressure 40 PSI, fragmentation. Analysis done in triplicate.

Quantisation (single ion current) was done based on a calibration curve done with compounds alone in concentration range 1-20 mM in MeOH/PBS extracted with acetonitrile and submitted to the same workup.

For compounds **10** and **12** we observed 85±8% remaining after 12 h incubation. For compound **15** we observed 45±10% remaining after 12 of incubation with formation acid **16 (**25±5%).

[3] G. M. Dubowchik, R. A. Firestone, L. Padilla, D. Willner, S. J. Hofstead, K. Mosure, J. O. Knipe, S. J. Lasch and P. A. Trail, *Bioconjug. Chem.*, 2002, **13**, 855–869

General biology procedures

Cell lines

A549 (human non-small cell lung carcinoma) cell line was from DSMZ, Capan-1 (human pancreas carcinoma) cell line was from ATCC, and SKBR3 (human breast carcinoma) cell line was kindly provided by Istituto Superiore di Sanità of Rome. Capan-1 and SKBR3 cells were cultivated in RPMI-1640 medium supplemented with 10% FBS, 2 m; L-glutamine (L-Glu) and 1% non-essential amino acids (NEAA), whereas A549 cells were cultivated in DMEM medium supplemented with 10% FBS and 2 mM L-Glu.

ADC binding (FACS Analysis)

Pellets of A549, Capan-1 or SKBR3 cells were incubated 1 h at 4°C with ADCs or related antibodies, Cetuximab (Ctx) and Trastuzumab (Trast), all at 5 μg/mL in 100 μL. After washings, cells were incubated with mouse anti-human FITC-conjugated Ig (BD) and propidium iodide. Cytofluorimetry was performed with FACScalibur (BD).

High content screening (HCS) fluorescence imaging

Cells were seeded in 96-well microtiter plates in complete medium and then incubated with Ctx, Trast or ADCs (5 μg/mL), for the indicated times. After cell fixation with 4% formaldehyde in PBS, permeabilization with 0.2% Tween-20 in PBS (PBS-T) and blocking with 2% BSA in PBS-T, Ctx, Trast or ADCs were detected by FITC conjugated mouse anti-human Ig (BD).

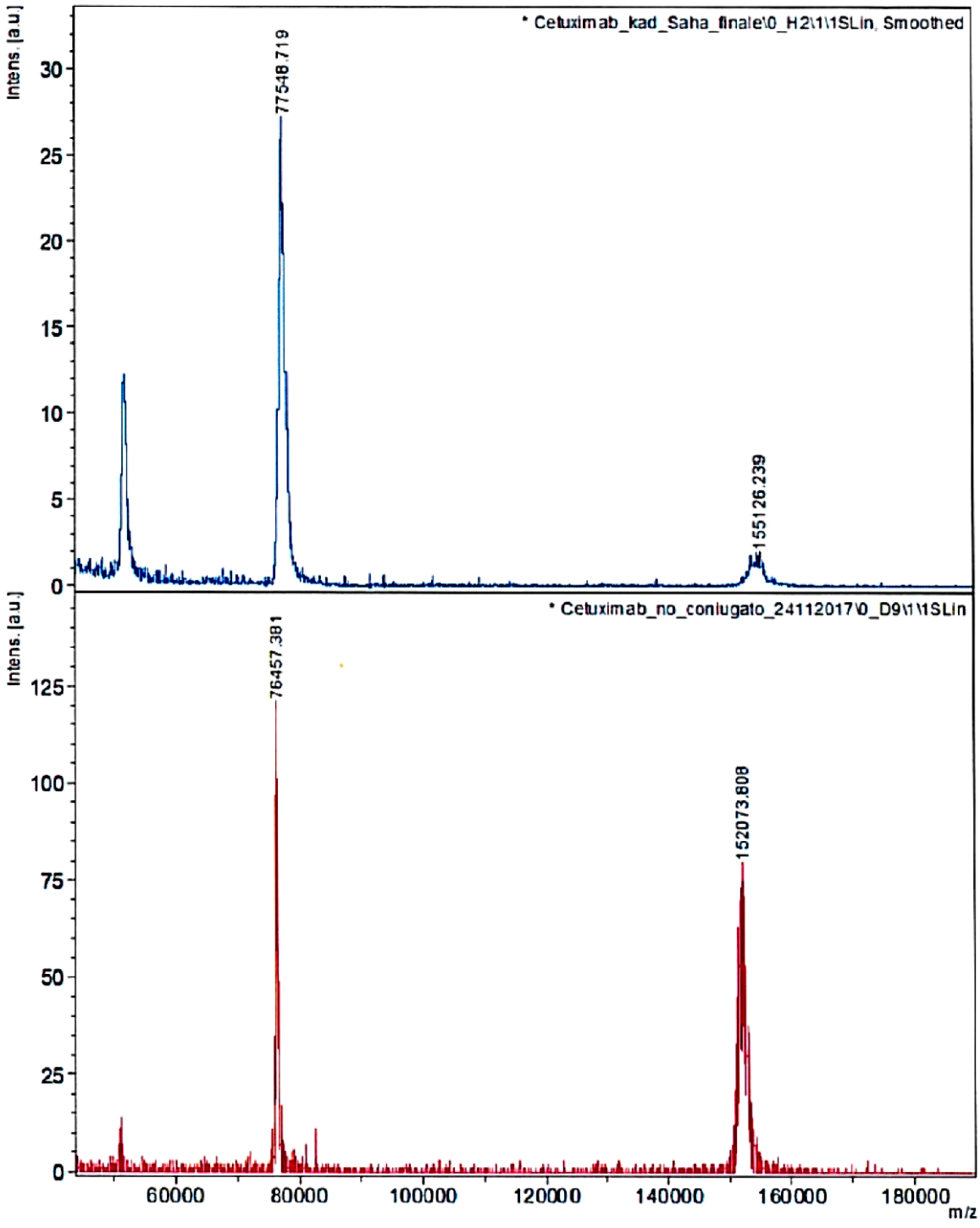
Expression of protein targets after cell fixation, permeabilization and blocking as described above, was evaluated by adding the following specific primary antibodies: rabbit anti-acetyl-Histone H3 (Lys9/Lys14) (Cell Signaling) or mouse anti acetyl-tubulin (6-11B-1) (Santa Cruz). FITC-conjugated goat anti-rabbit or goat anti-mouse IgG (BD) were then added, according to the primary antibody used. Cells were counterstained with Draq5 dye (Cell Signaling). Fluorescence signals were acquired by the High Content Screening (HCS) system Operetta (Perkin Elmer) and images analyzed through Harmony software (Perkin Elmer).

Western blotting

Tumor cells were seeded in 10-cm culture plates in complete medium, and then cultivated with 5 µg/mL Ctx, Trast or ADCs, or with 50 nM reference HDAC inhibitors (HDACis), for 3 hours. Whole cell lysates were then prepared and protein content was determined by Bradford method. Equal amounts of soluble proteins were separated on SDS-PAGE and then transferred to nitrocellulose membrane (Amersham Hybond-ECL; GE Healthcare). Membranes were blocked 3 hours with 5% non-fat dry milk in PBS 0.05% Tween-20 (PBS-T) before overnight incubation, at 4°C, with one of the following primary antibodies: rabbit anti- acetyl-Histone H4 (Ser1/Lys5/Lys8/Lys12) (Santa Cruz) or mouse anti acetyl-tubulin (6-11B-1) (Santa Cruz). Immunoblotting with mouse anti-β-actin antibody (Sigma Aldrich) was performed to normalize protein loading. After washings with PBS-T, membranes were incubated 1 hour with the appropriate secondary HRP-conjugated anti-rabbit or anti-mouse IgGs (Sigma Aldrich and Amersham GE-Healthcare, respectively). Immunoreactive bands were visualized by enhanced chemiluminescence detection and analyzed through phosphoimaging (STORM, Molecular Dynamics) or by exposure to X-ray film (Amersham Hyperfilm ECL; GE-Healthcare).

ADC tumor cell proliferation

The effect of test items on cell proliferation was evaluated on the human non-small cell lung carcinoma A549 cell line. Cells were seeded (at 5.000 cells/well) into 96-well plates in complete culture medium and then incubated for 6 days, in quadruplicate, with scalar concentrations of ADCs, ranging from 500 to 100 nM, or of reference HDACis (from 2000 to 100 nM range of doses). Inhibition of cell proliferation was measured by CellTiter-Glo Luminescent Cell Viability Assay (Promega), through a Veritas luminometer (Promega). Data were expressed as the average (± SE) of percentage inhibition of two independent experiments. The IC50 values were ultimately calculated by using the GraphPad Prism 5.02 software.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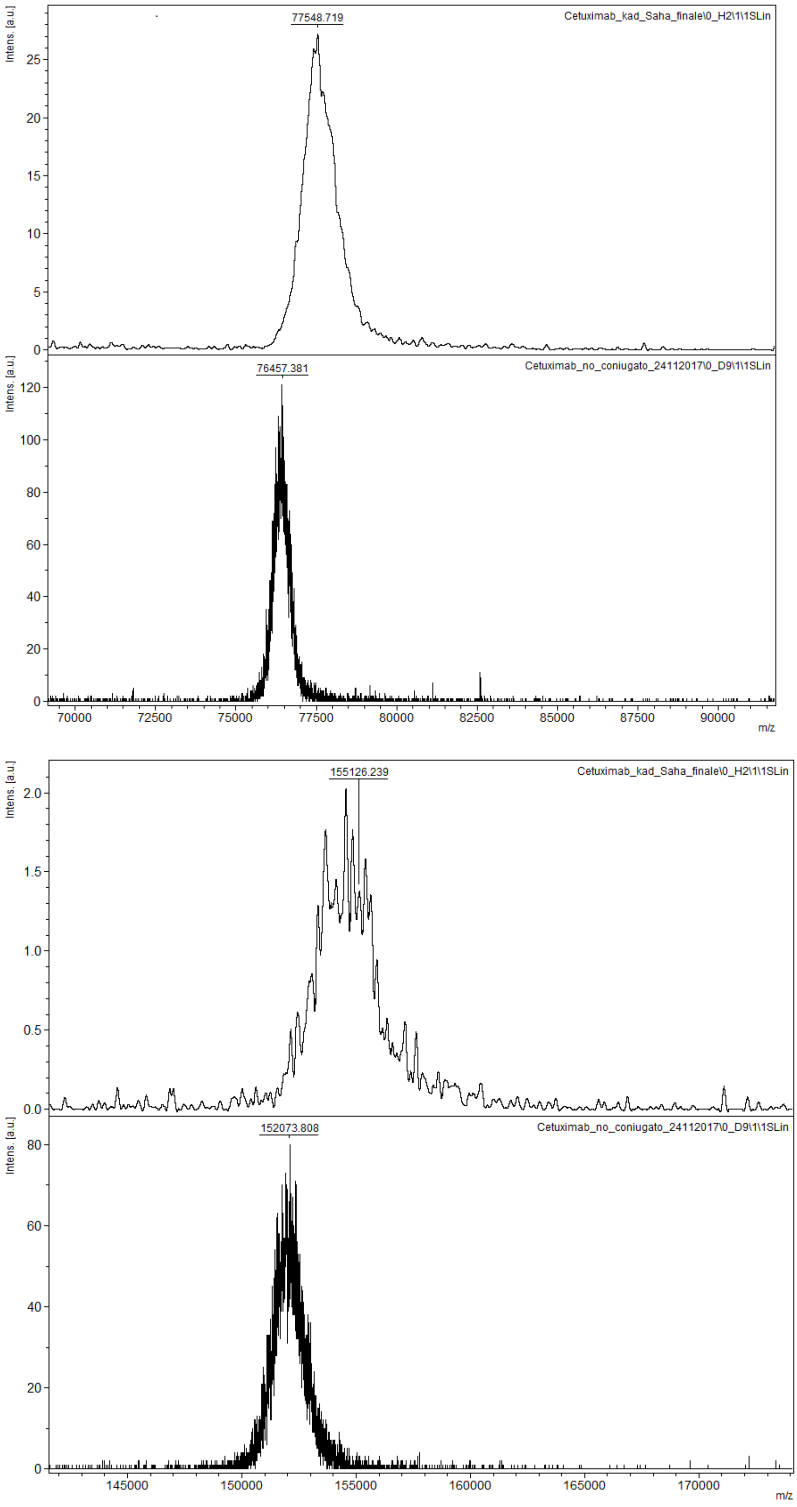
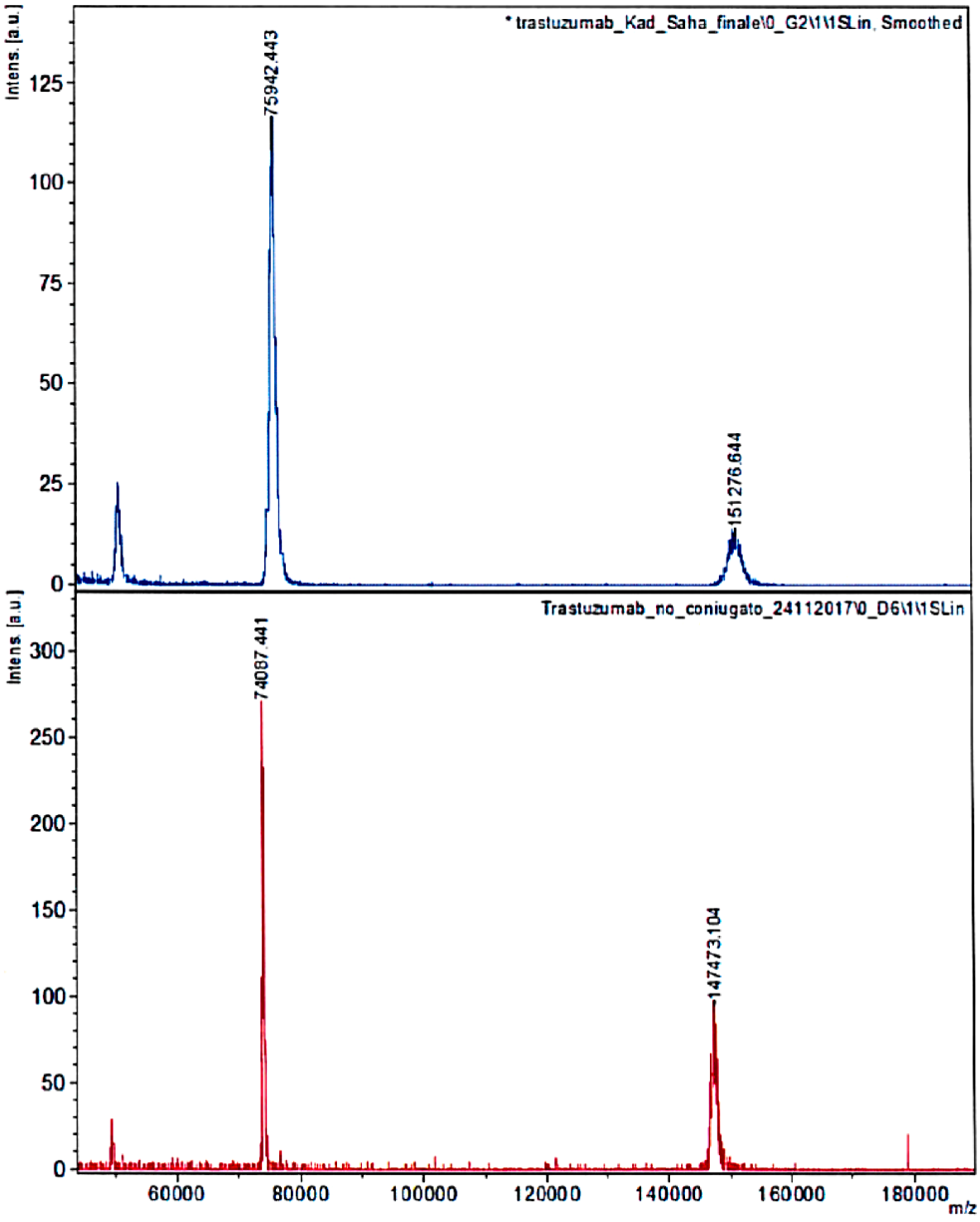
m/z

A



B

FIGURE SI1 **A** Comparison of MALDI spectra of ADC **17** (blue line, up) and cetuximab alone (red line, down). **B** Expansion of di- and mono-charged peak mass. The images are representative of one over 3 experiments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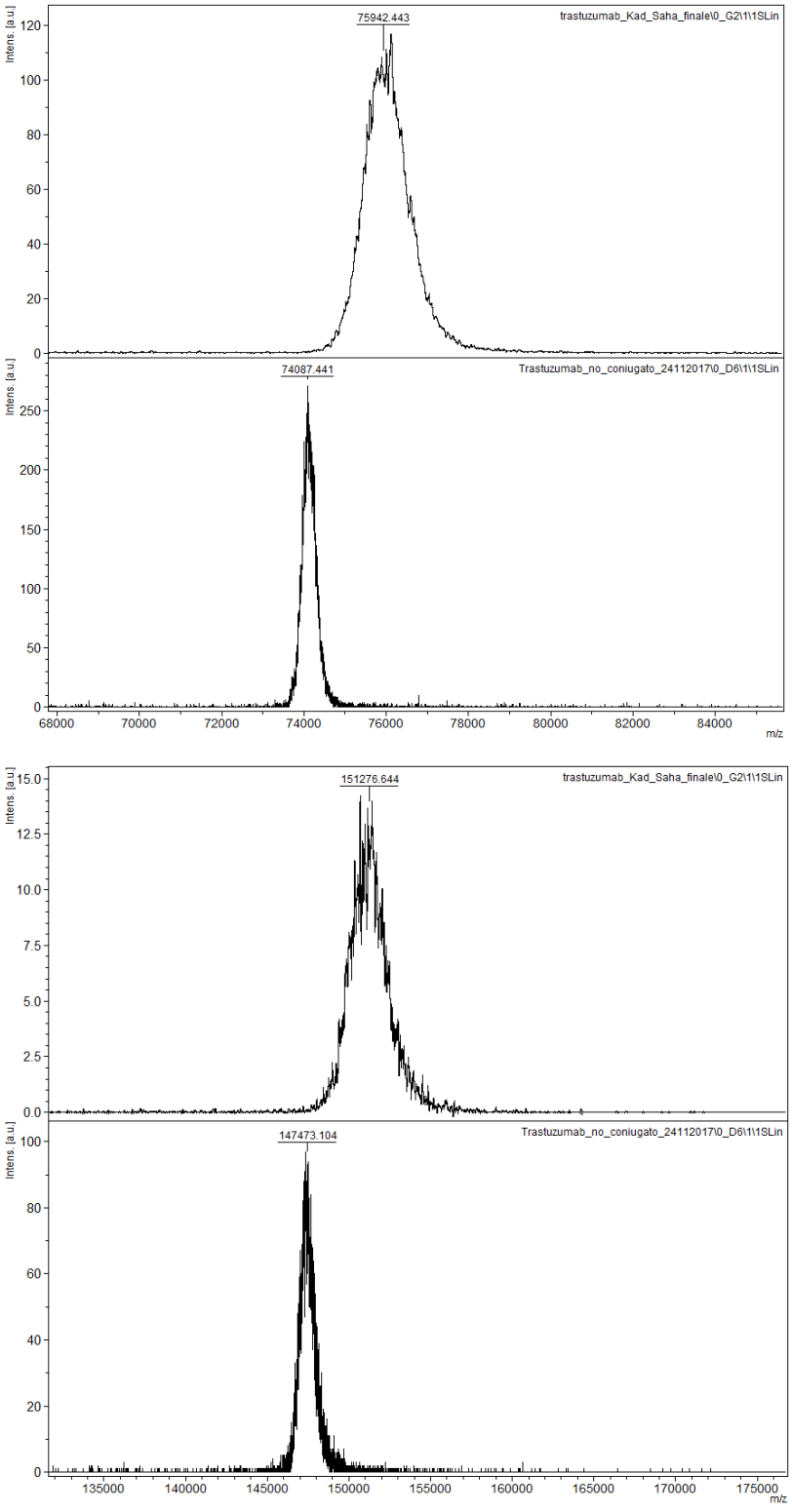
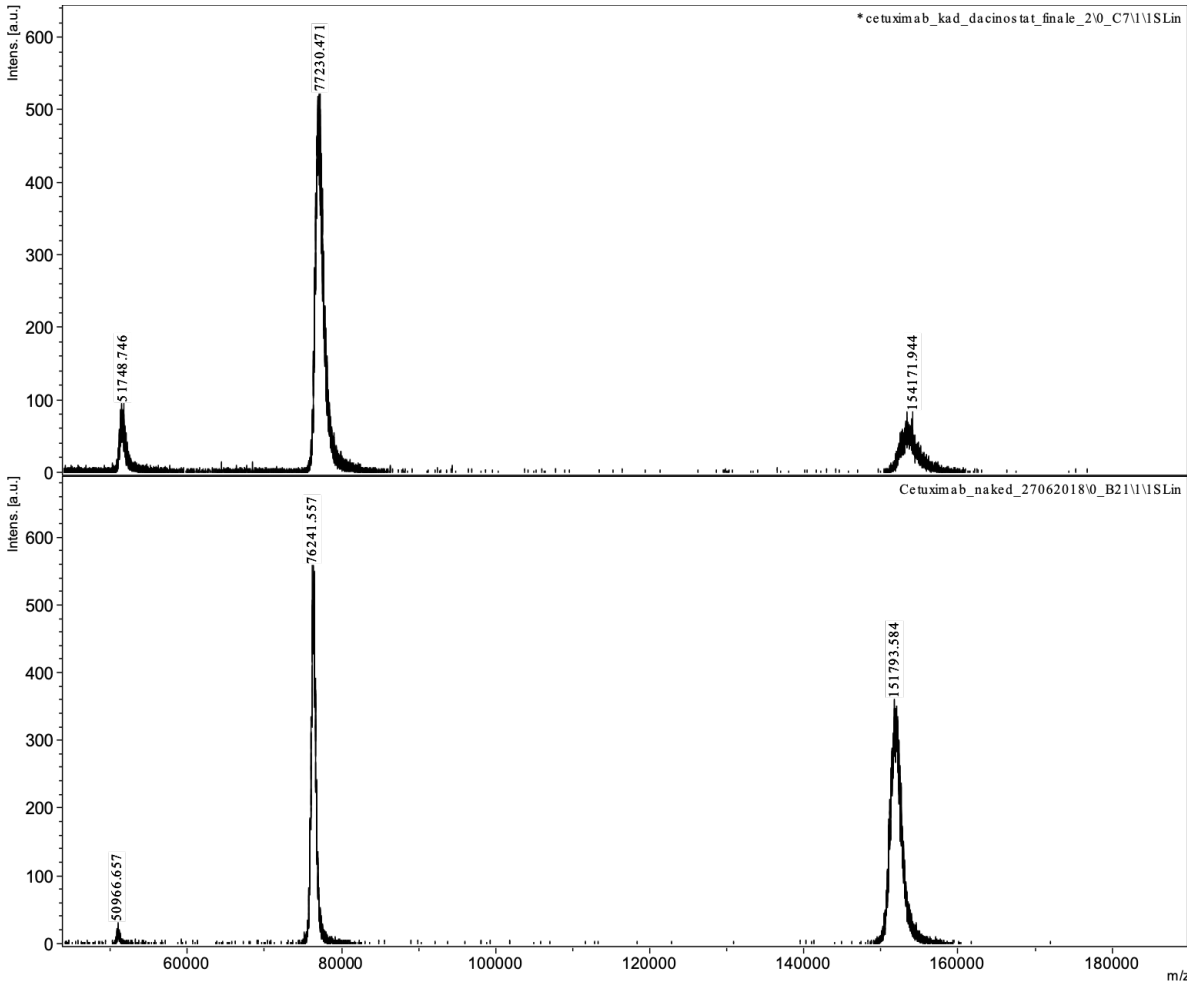
m/z

A



B

FIGURE SI 2 A Comparison of MALDI spectra of conjugate **18** (blue line, up) and trastuzumab alone (red line, down). **B** Expansion of di- and mono-charged peak mass. The image is representative of one over 3 experiments.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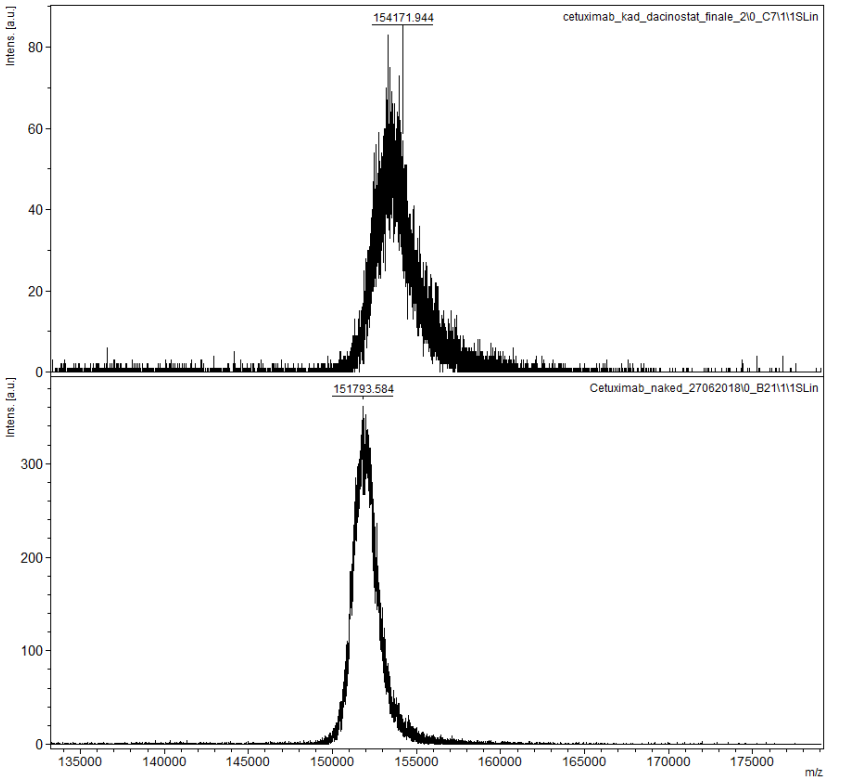
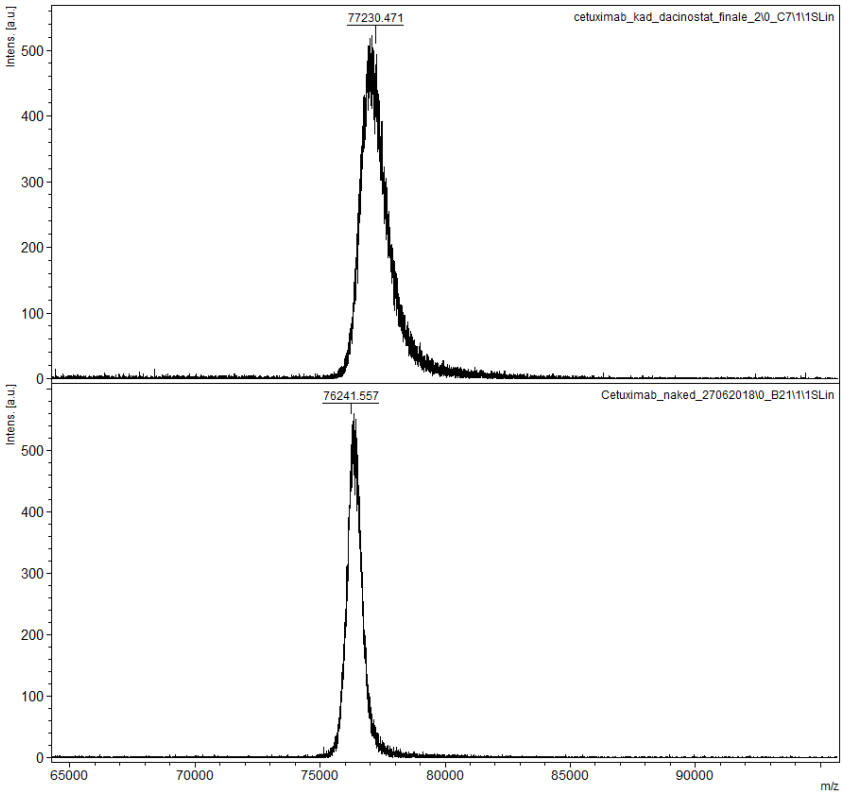
120000
140000
160000
180000

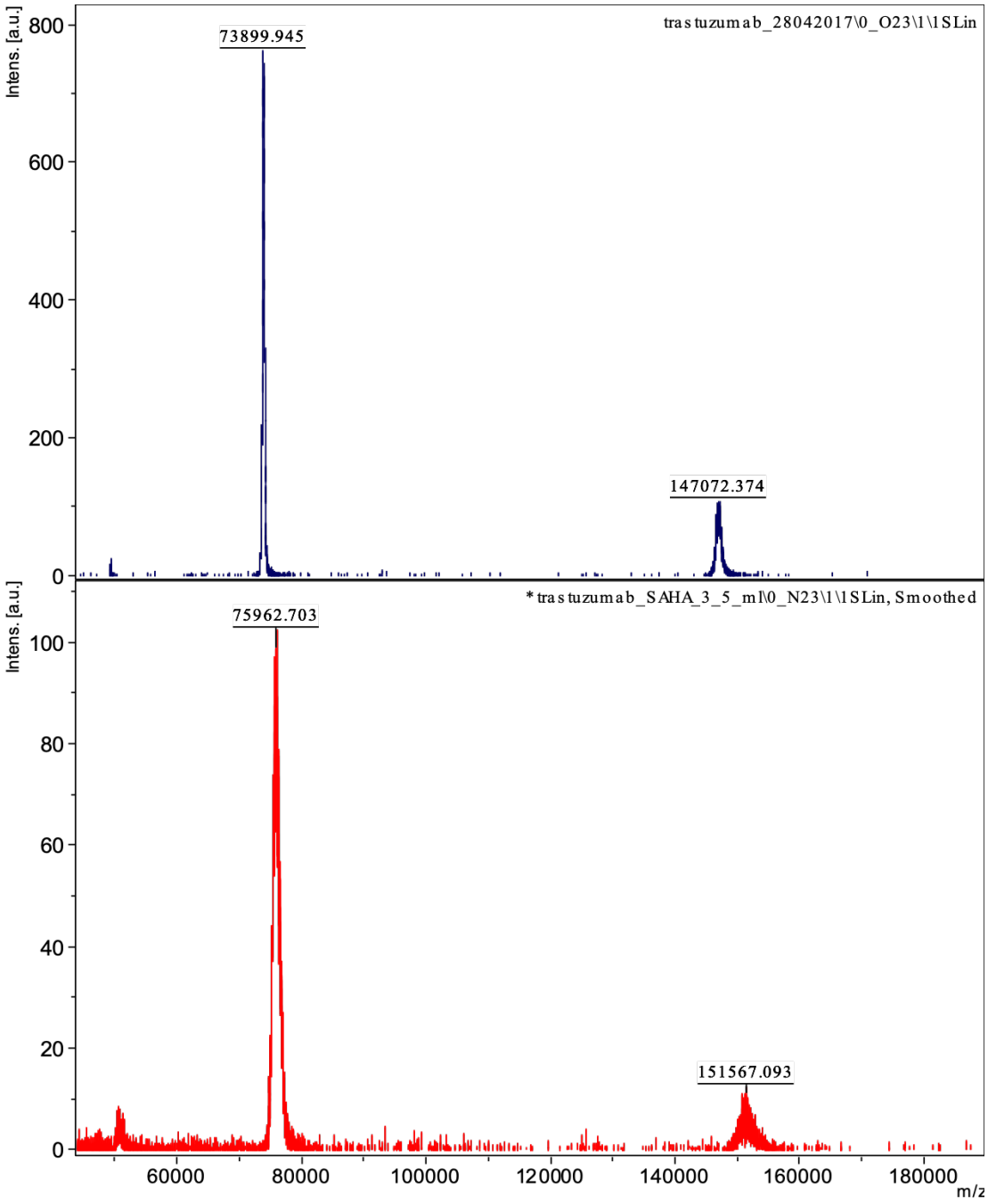
A





B

FIGURE SI 3 A Comparison of MALDI spectra of conjugate **19** (up) and cetuximab alone (down). **B** Expansion of di- and mono-charged peak mass. The image is representative of one over 3 experiments.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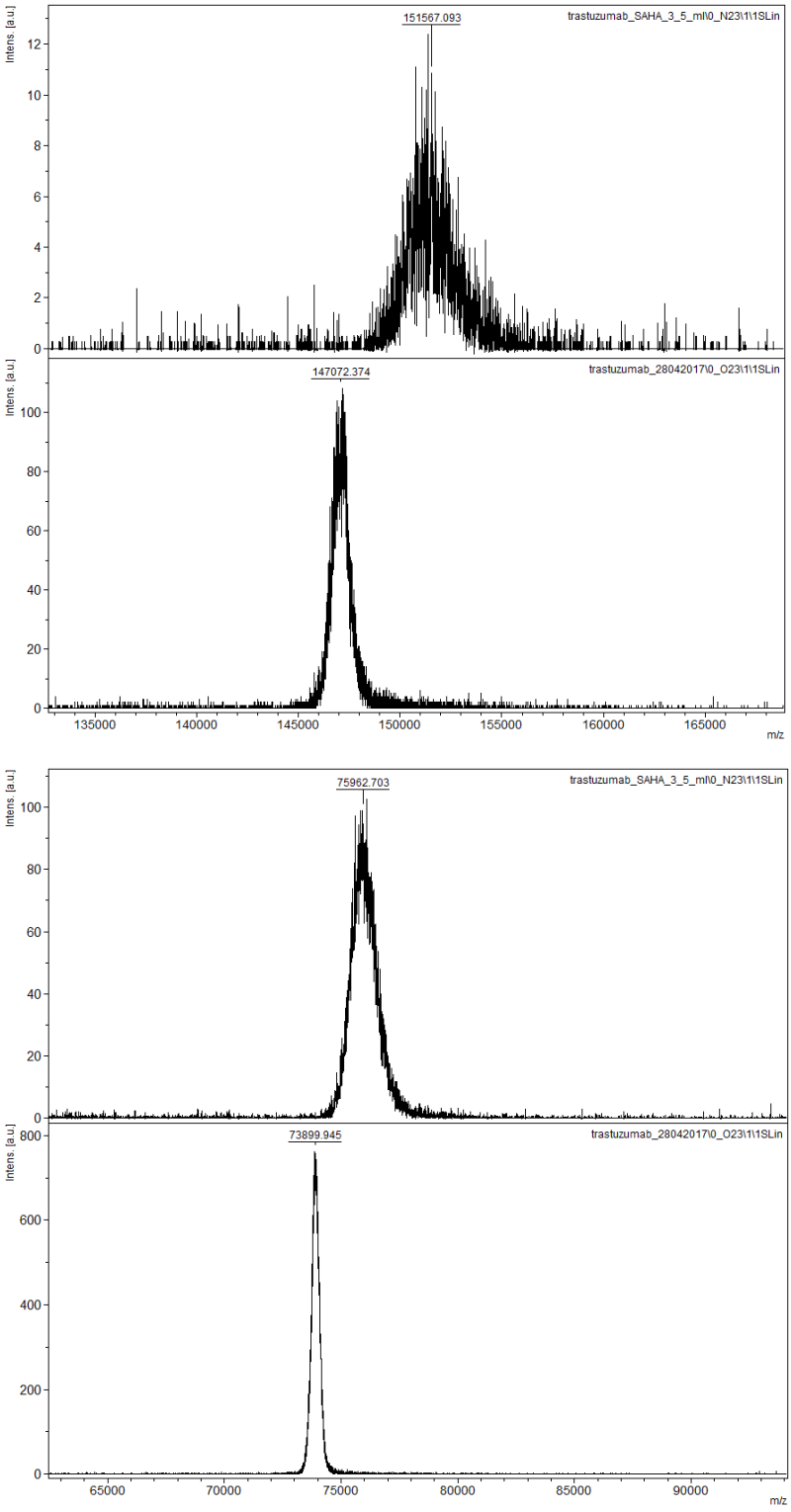
m/z

A



B

FIGURE SI 4 A Comparison of MALDI spectra of trastuzumab alone (blue line, up) and conjugate **20** (red line); down). B Expansion of di- and mono-charged peak mass, conjugate **20** up and trastuzumab alone down . The image is representative of one over 3 experiments

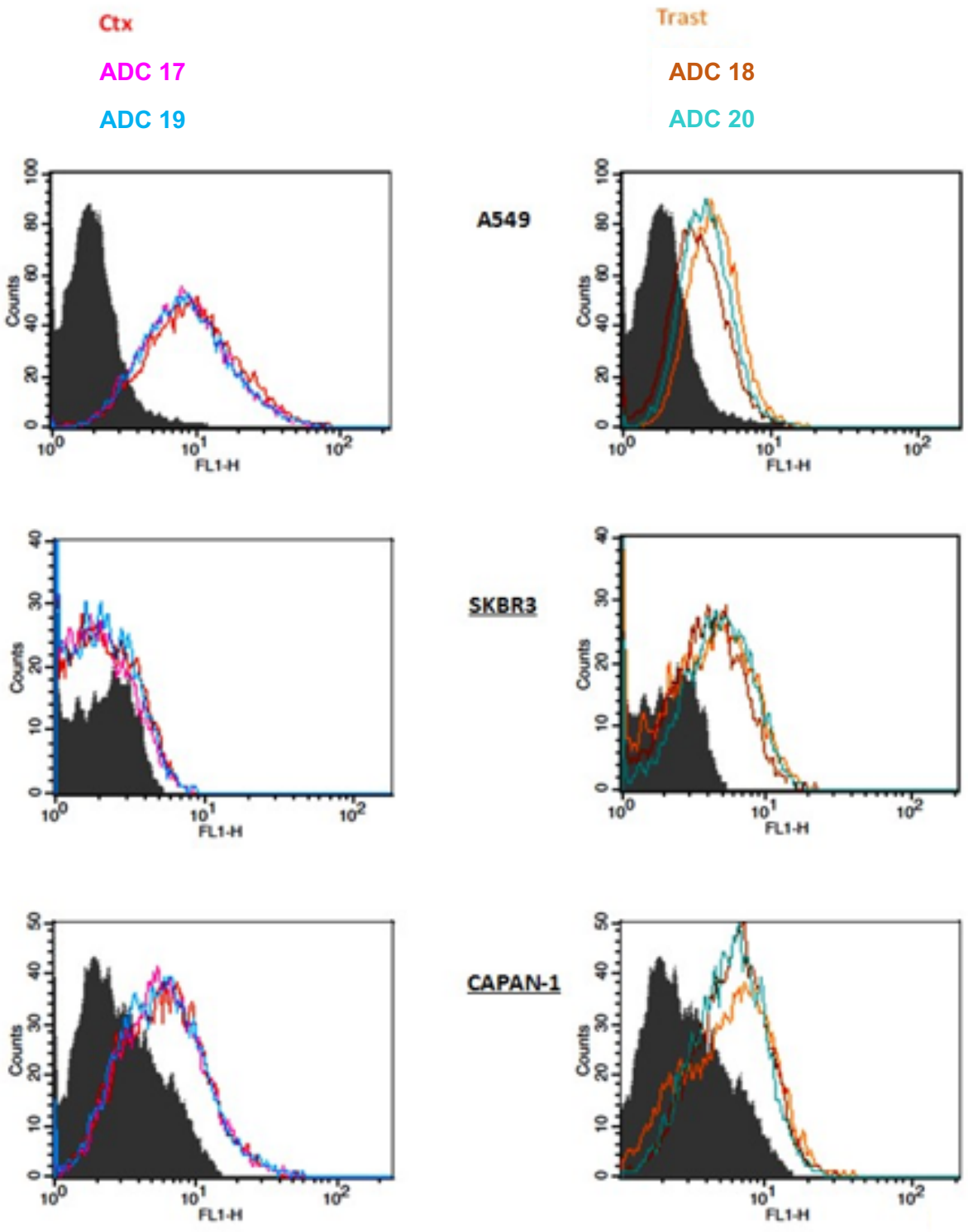


FIGURE SI 5 | Receptor binding of new ADCs. Binding of ADCs **17** (pink line) and **19** (light blue line) compared to cetuximab (Ctx; red line) (left panel), and of ADCs **18** (brown line) and **20** (cyan line) compared to trastuzumab (Trast; orange line) (right panel), by cytofluorimetry analysis on human lung (A549), breast (SKBR3) and pancreas (Capan-1) carcinoma cell lines. Cell pellets were incubated with test items and then stained with FITC-conjugated mouse anti-human Ig and propidium iodide. Dark grey peaks refer to cells without primary antibody.

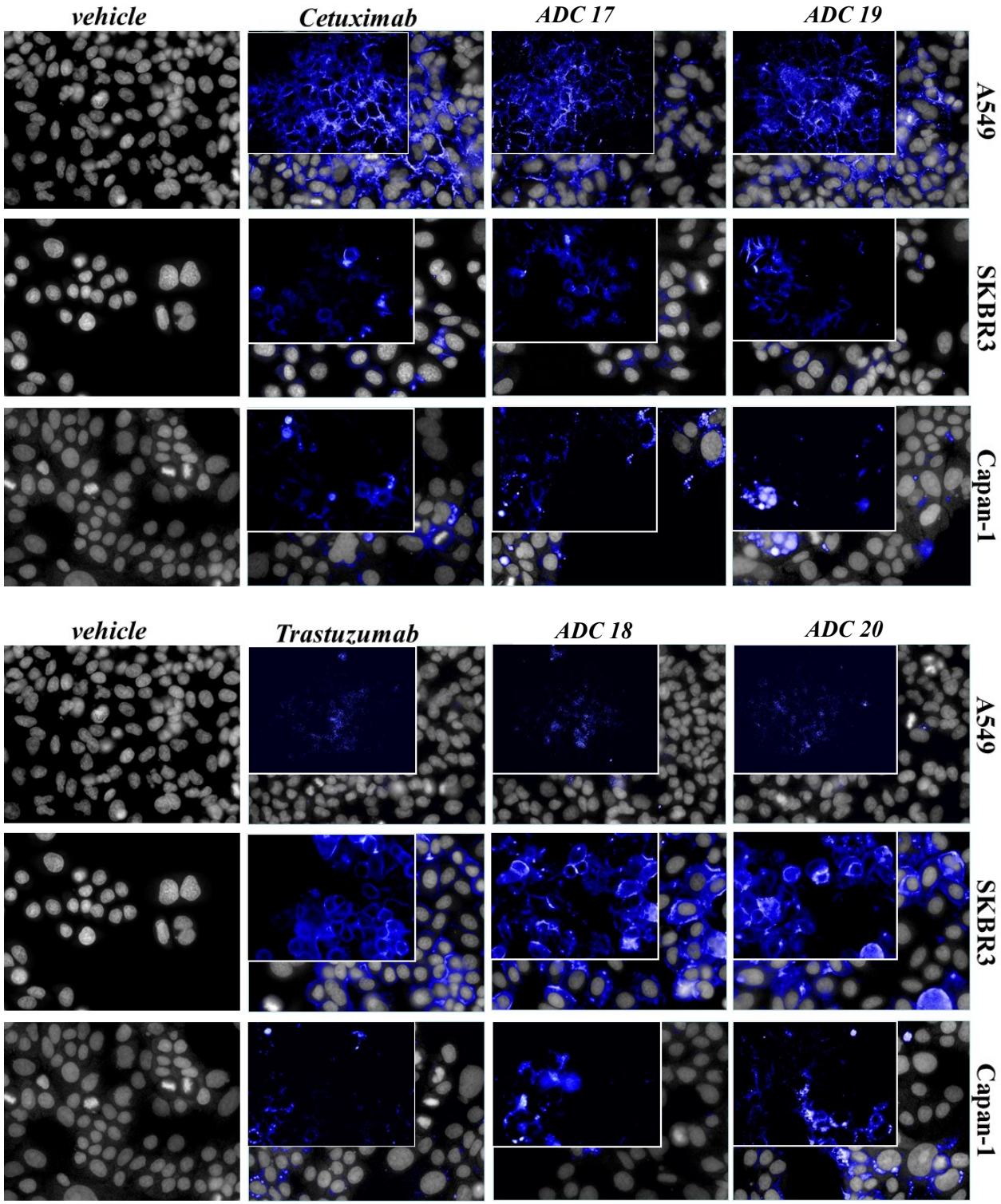


FIGURE SI 6 | Internalization of new ADCs, as compared to Cetuximab or Trastuzumab, (all at 5 µg/mL) by tumor cells, as measured by HCS fluorescence imaging after 1 hour incubation. Insets show specific (blue) fluorescence signals within the cells. Draq5 dye staining of nucleus (grey). Each image is representative of at least 5 fields of duplicate wells. Magnification 60X. Data are from one representative experiment out of two.

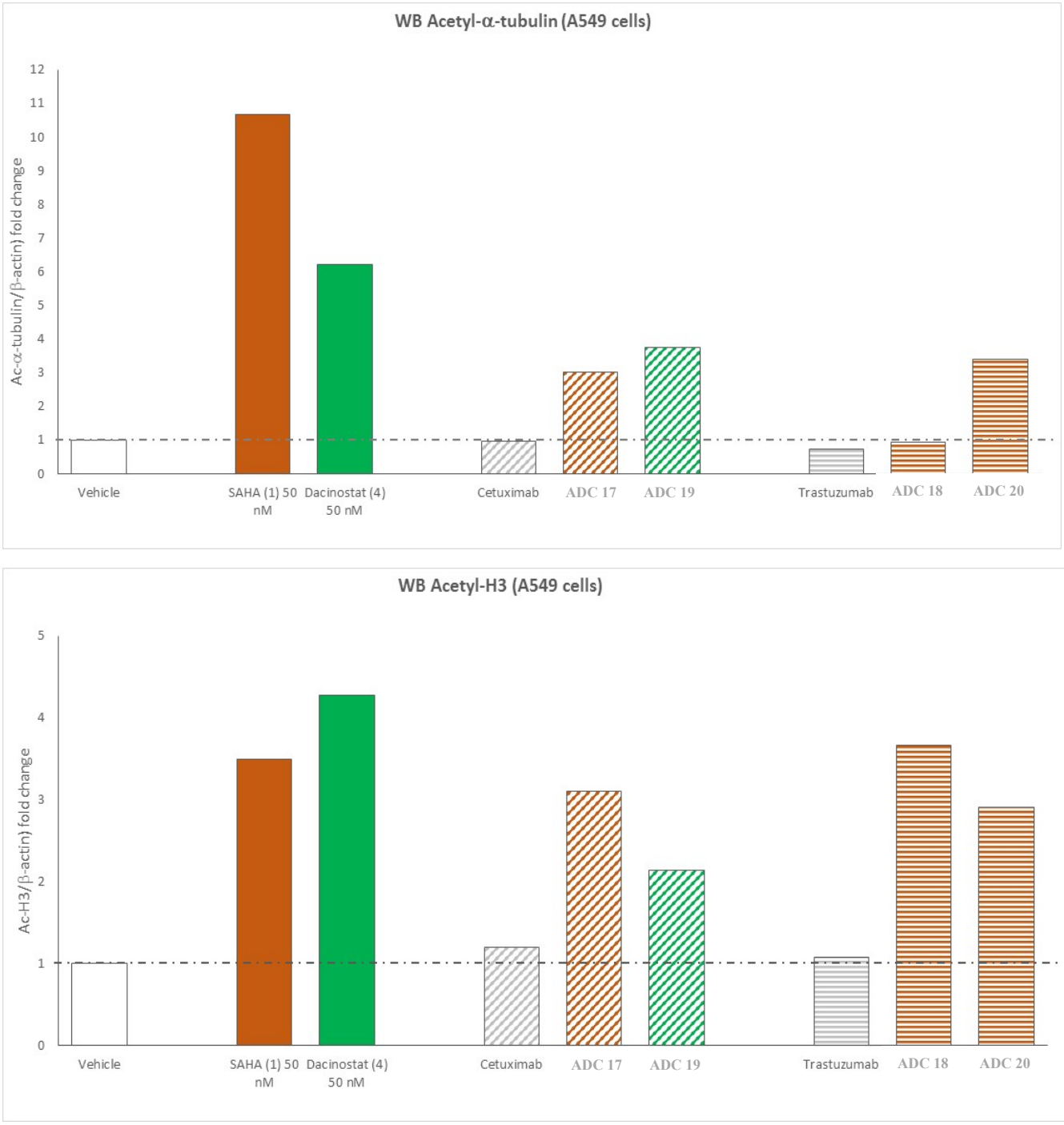
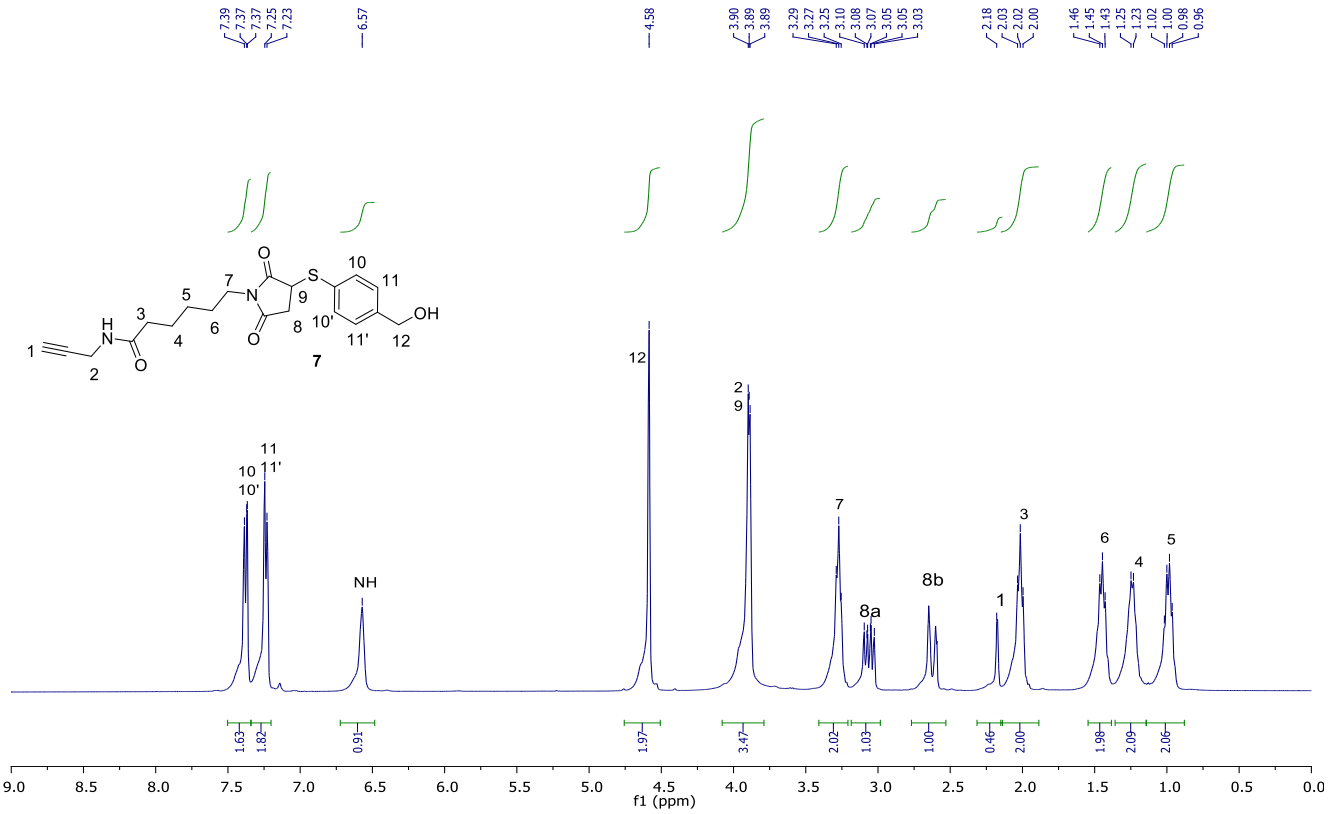
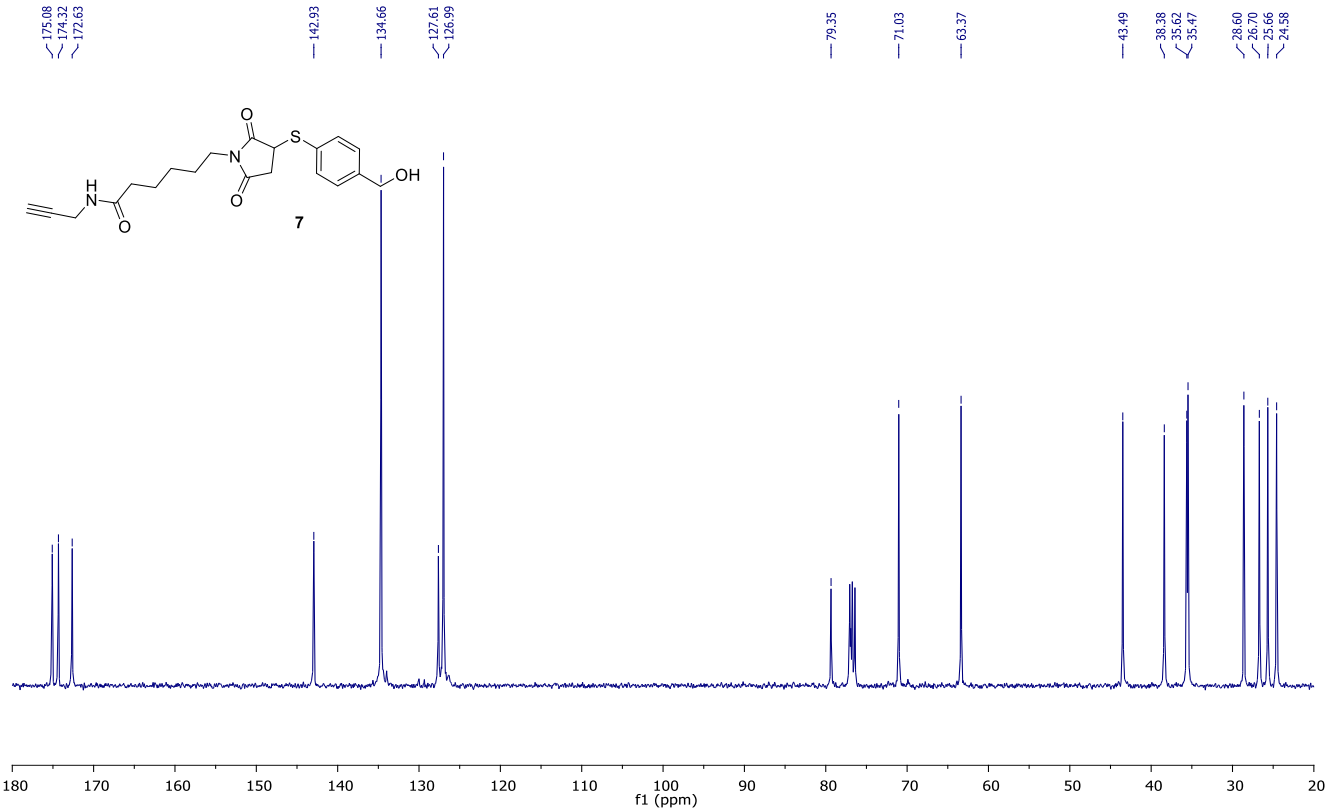


FIGURE SI 7 | Effect of SAHA, Dacinostat, Cetuximab and Trastuzumab, compared to ADCs **17-20**, on acetylation of α-tubulin and histone H3 in A549 (human lung carcinoma) cells. Cells were cultivated 3 hours, at 37 °C, with medium (vehicle), reference HDACi (50 nM) or antibodies (5 μg/mL), and then western blot analysis was carried out on total protein lysates. Densitometric analysis of specific band intensity, after normalization to beta-actin, expressed as fold change, of one representative blot is shown. Data refer to two experiments.

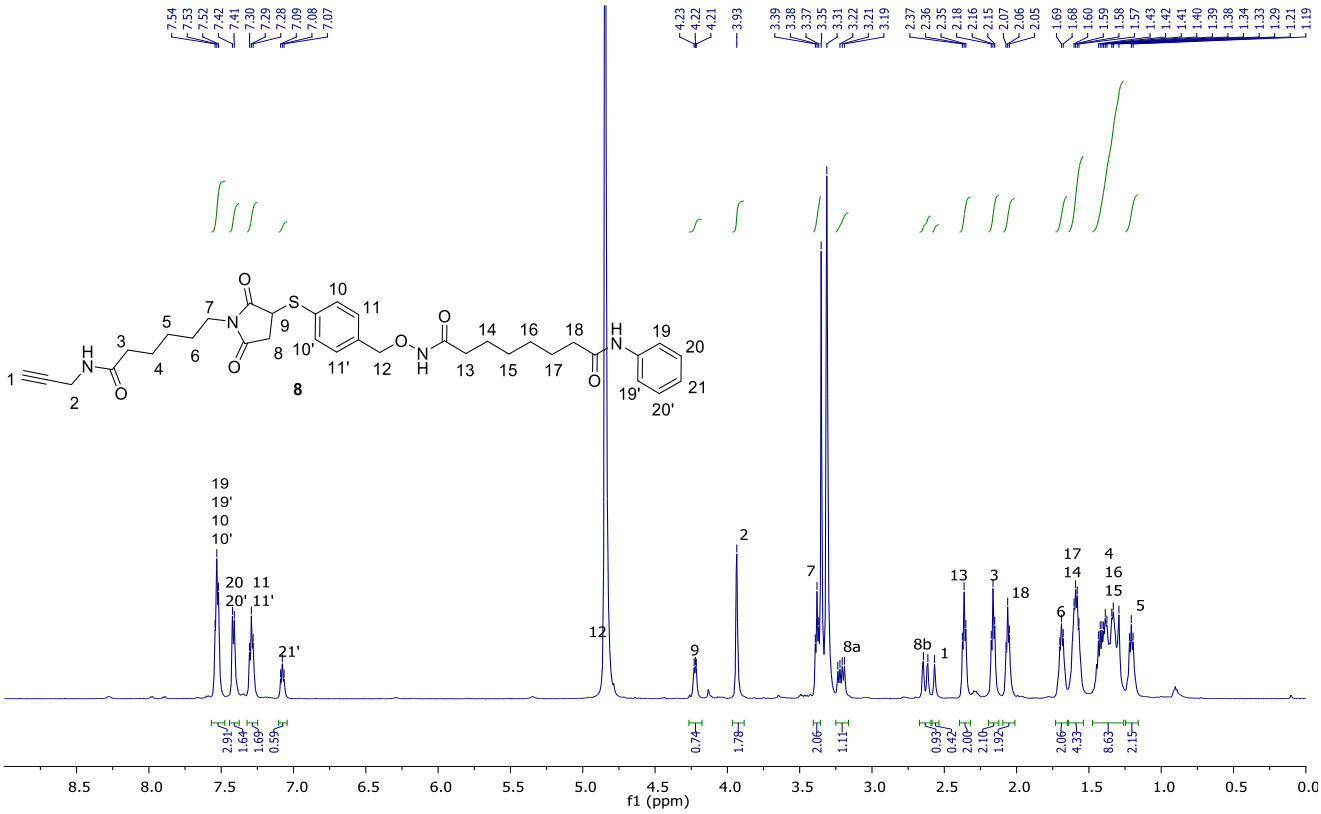
^{1}H-NMR spectrum of **7**



^{13}C-NMR spectrum of **7**



^{1}H-NMR spectrum of **8**



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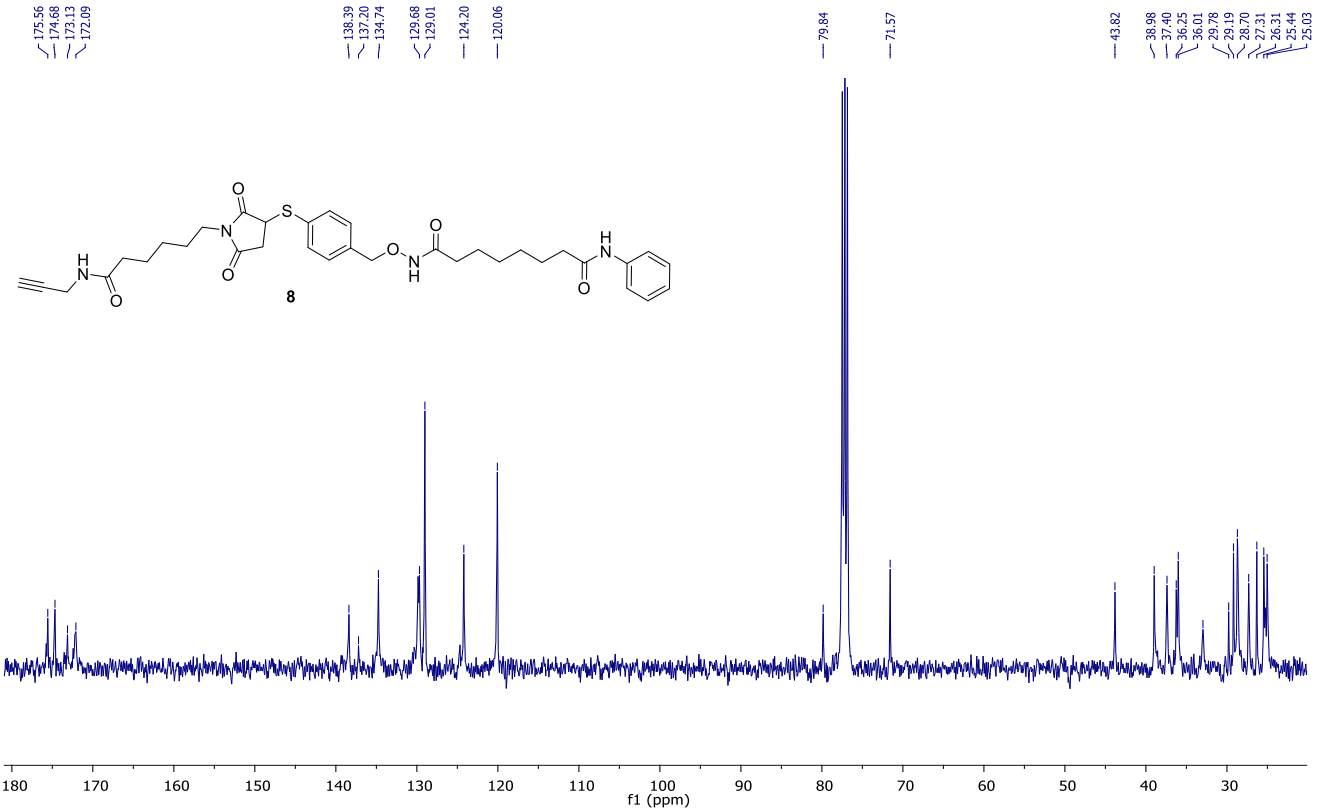
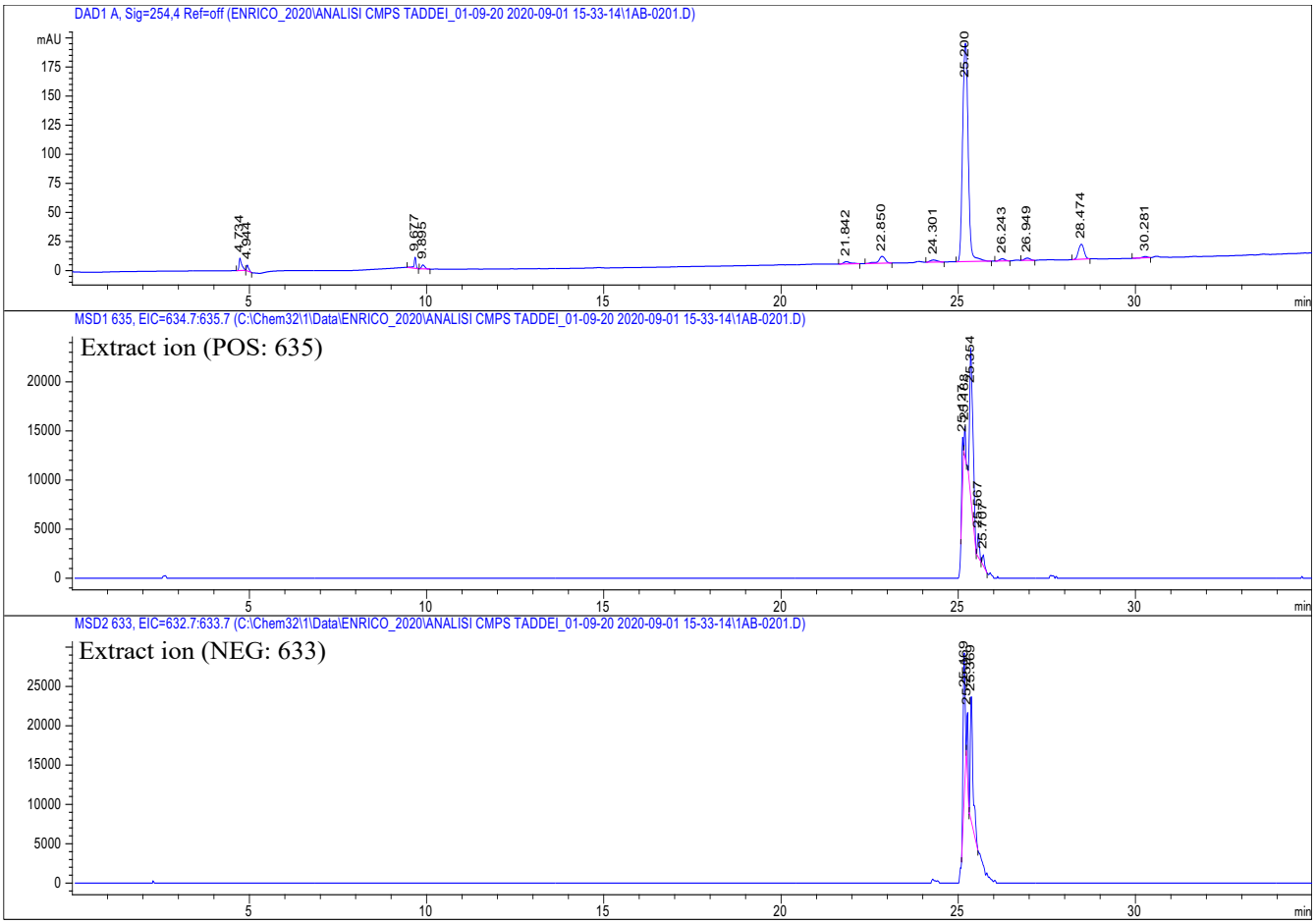
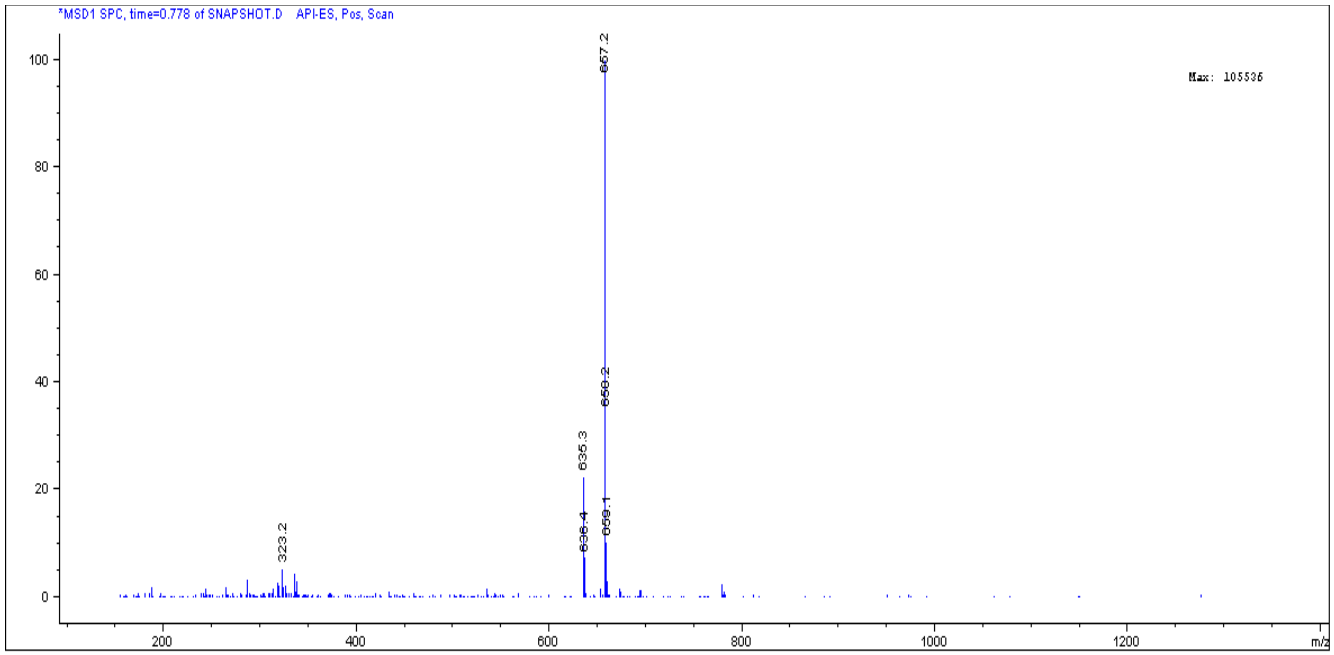
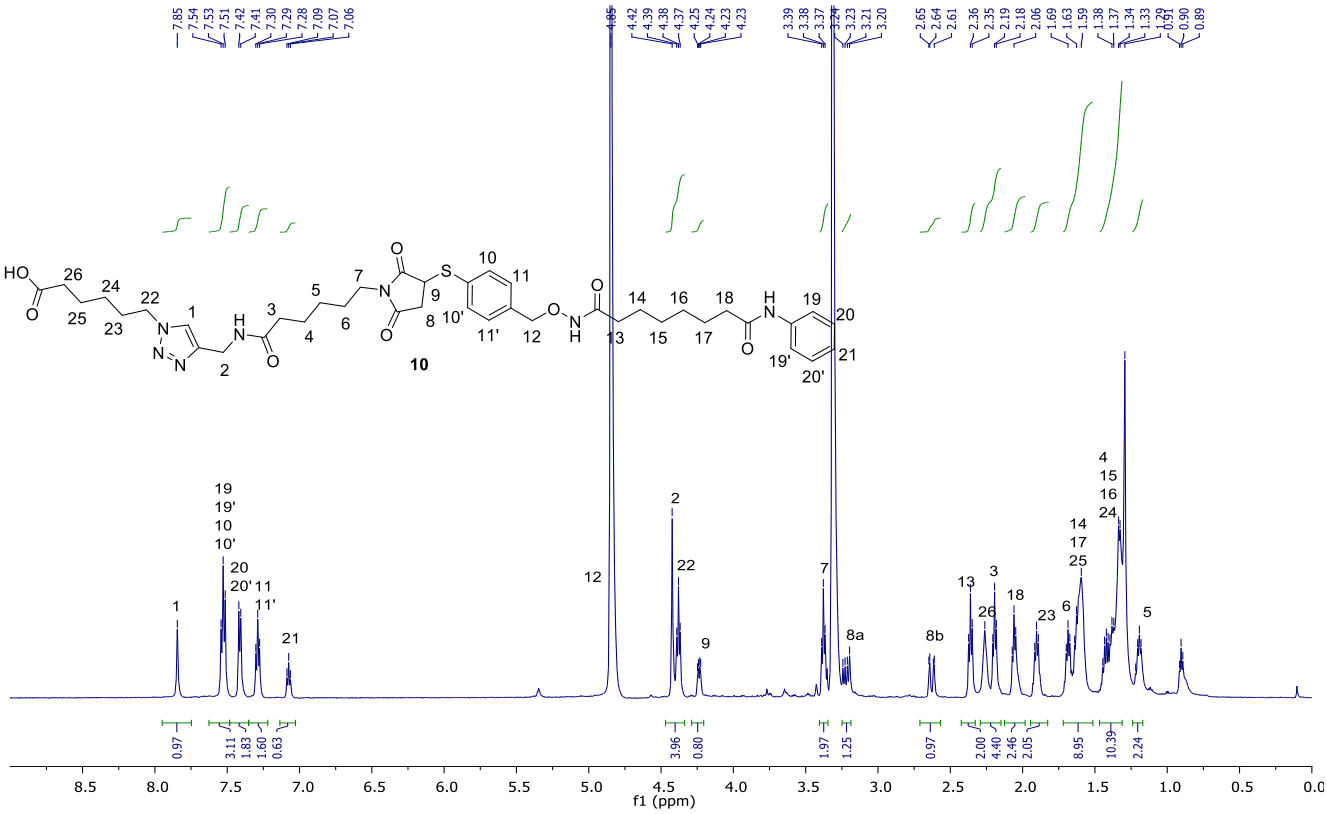


HPLC-MS and MS-ESI **8** (MW: 634)





^{1}H-NMR spectrum of **10**



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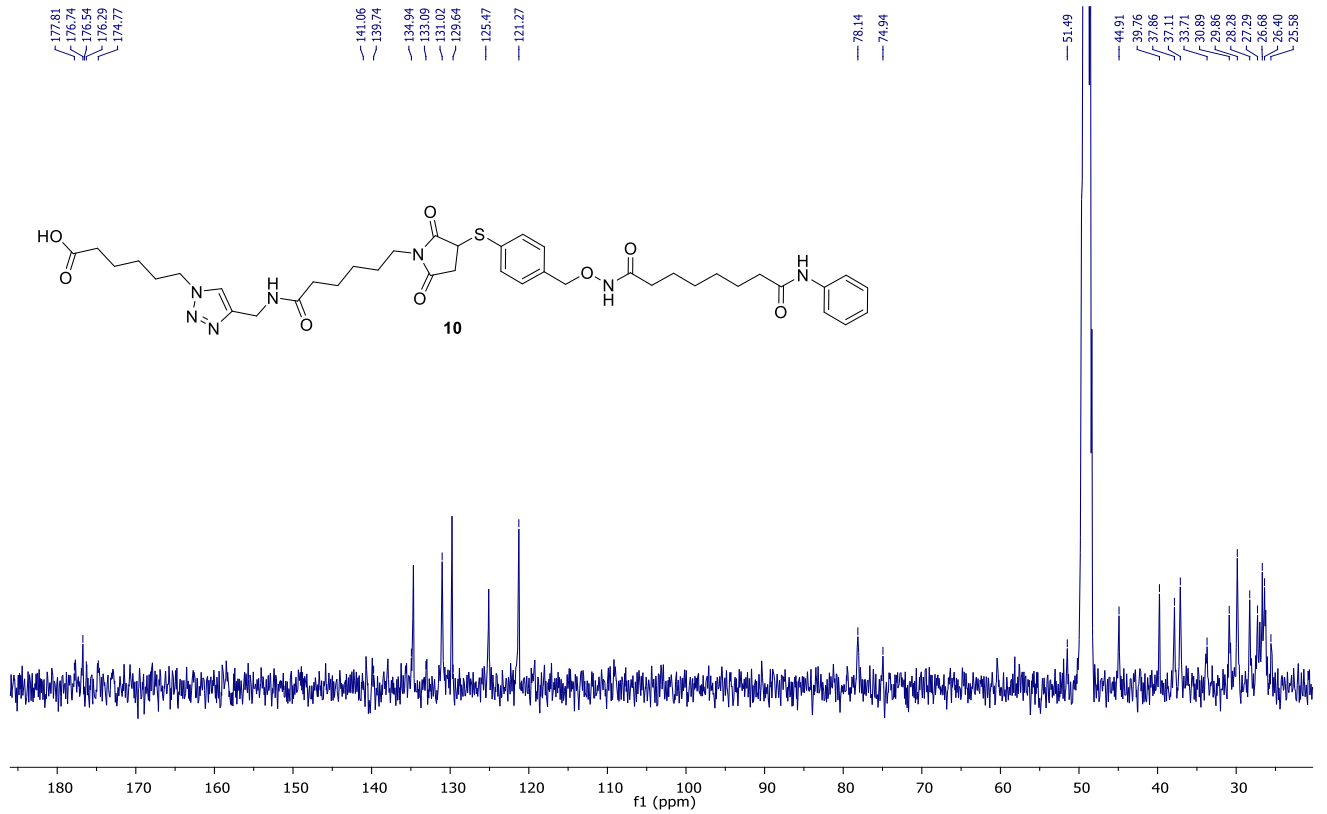

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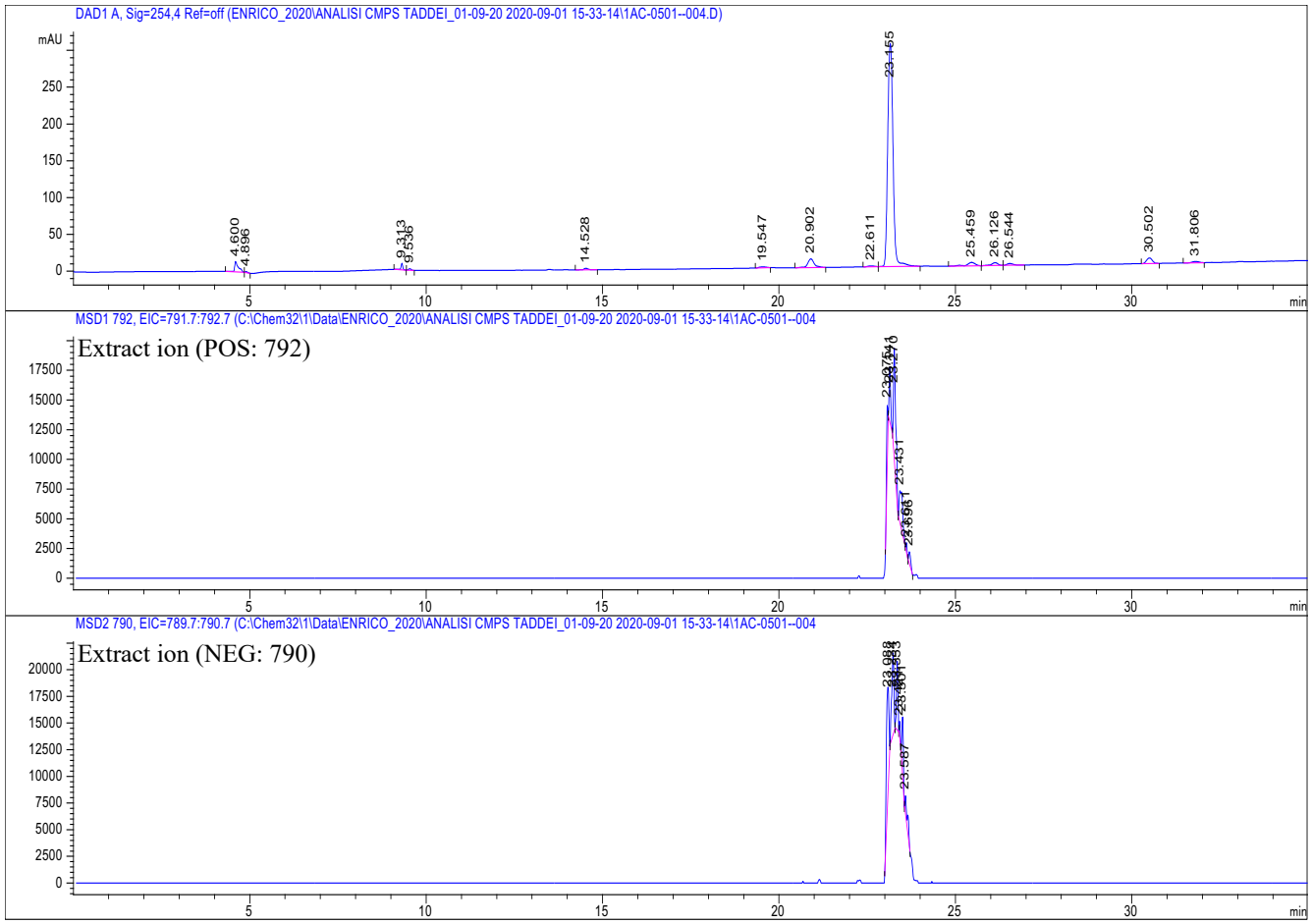

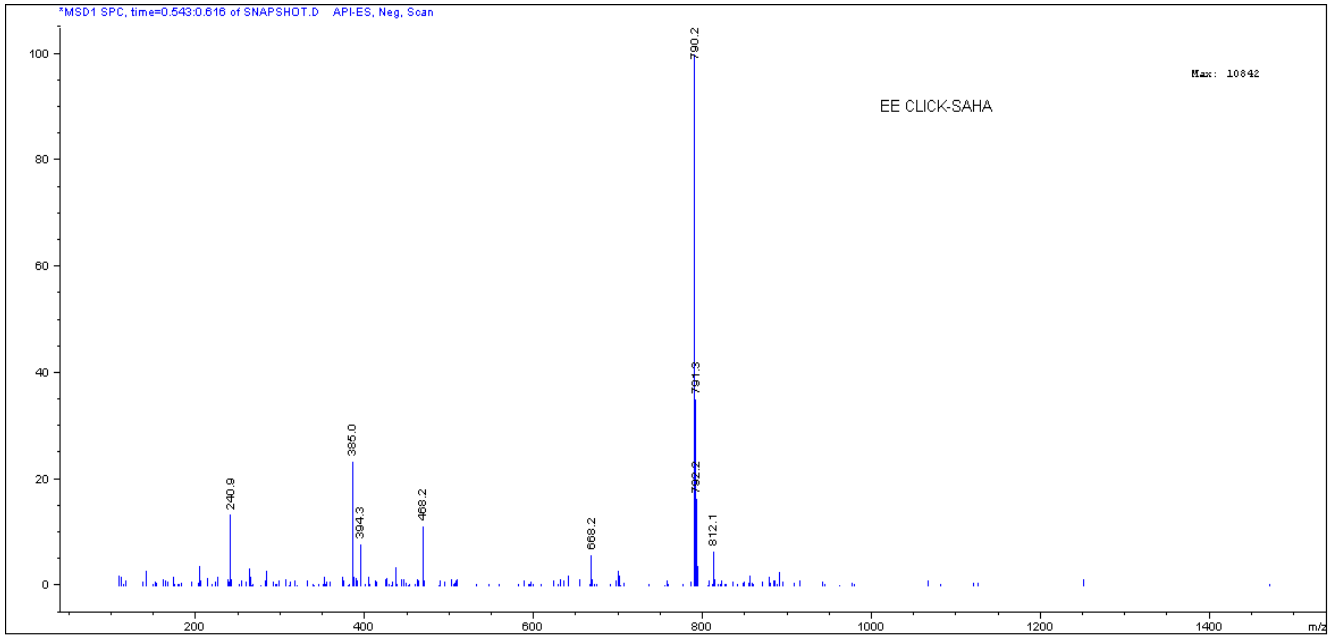

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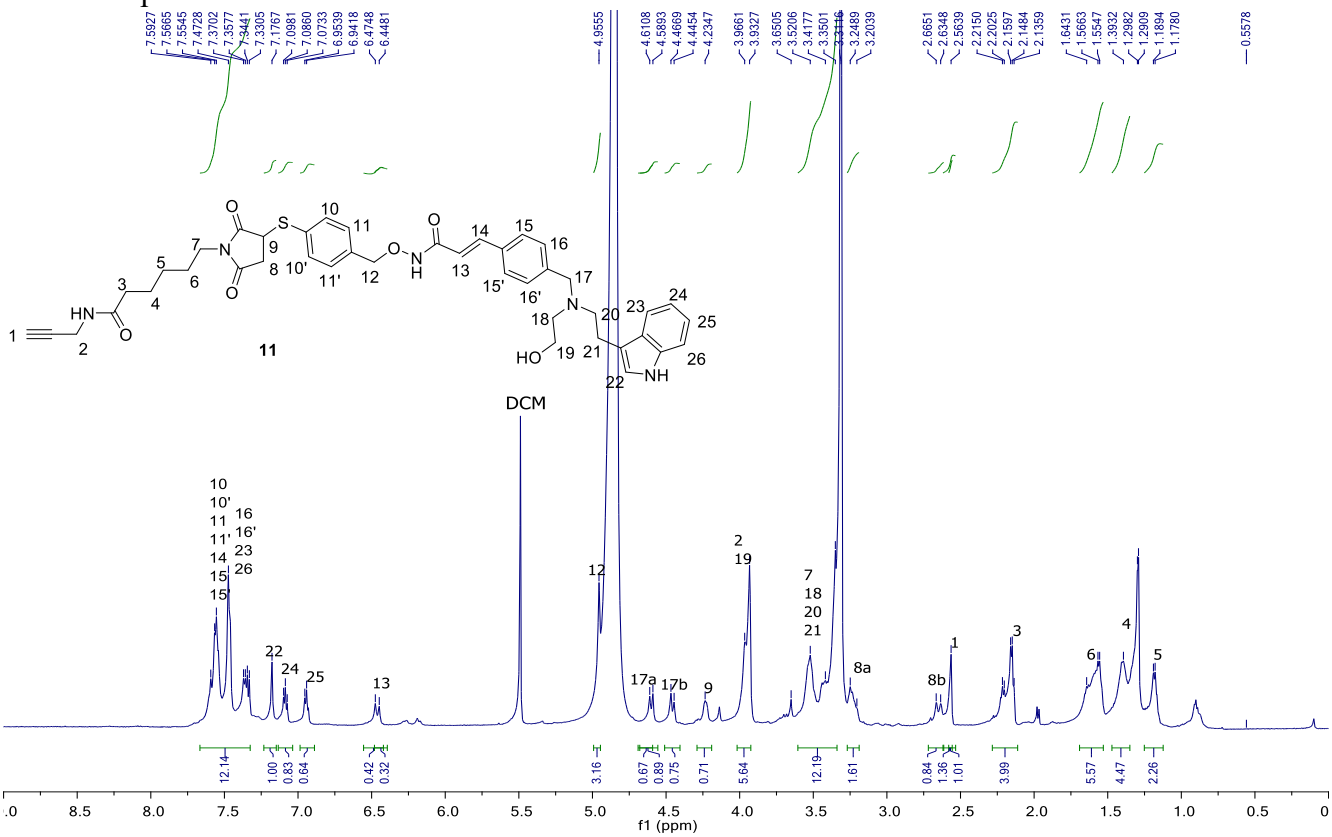

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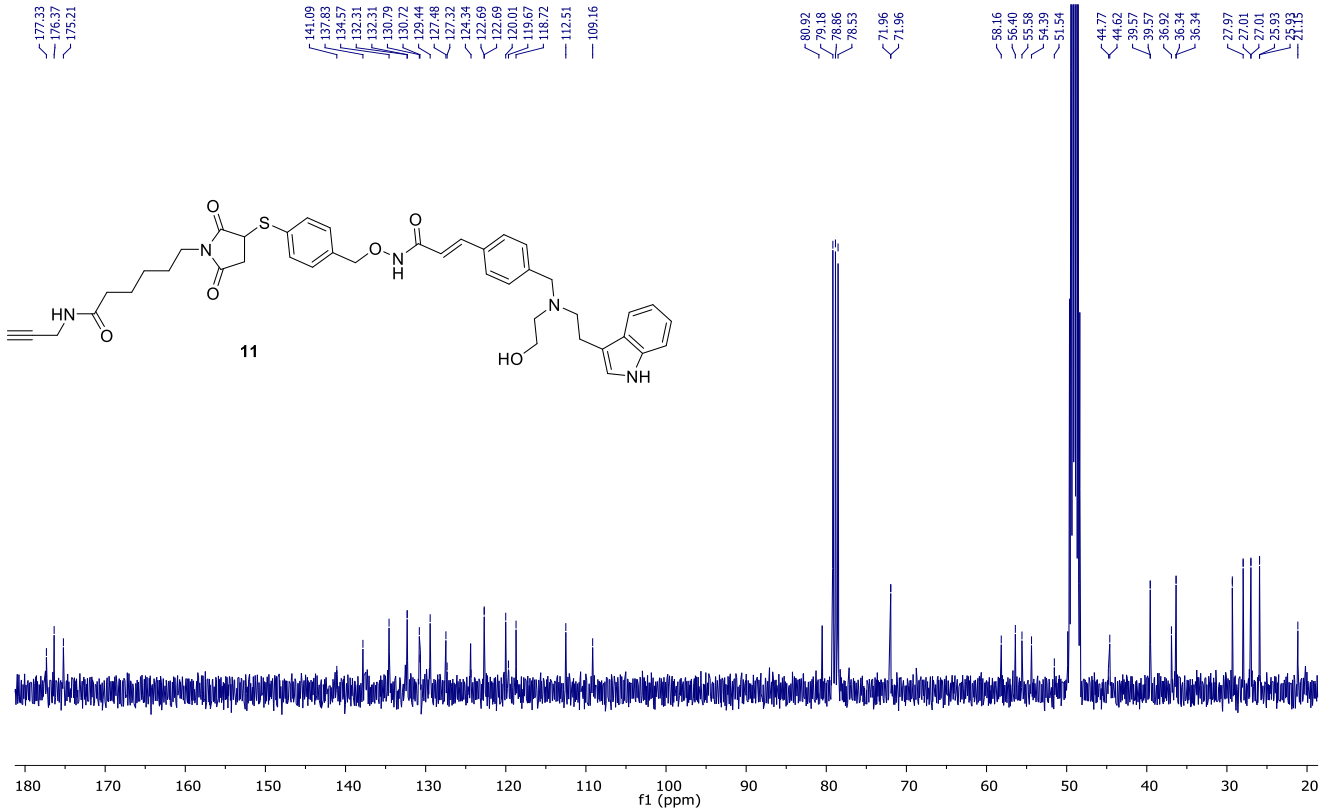

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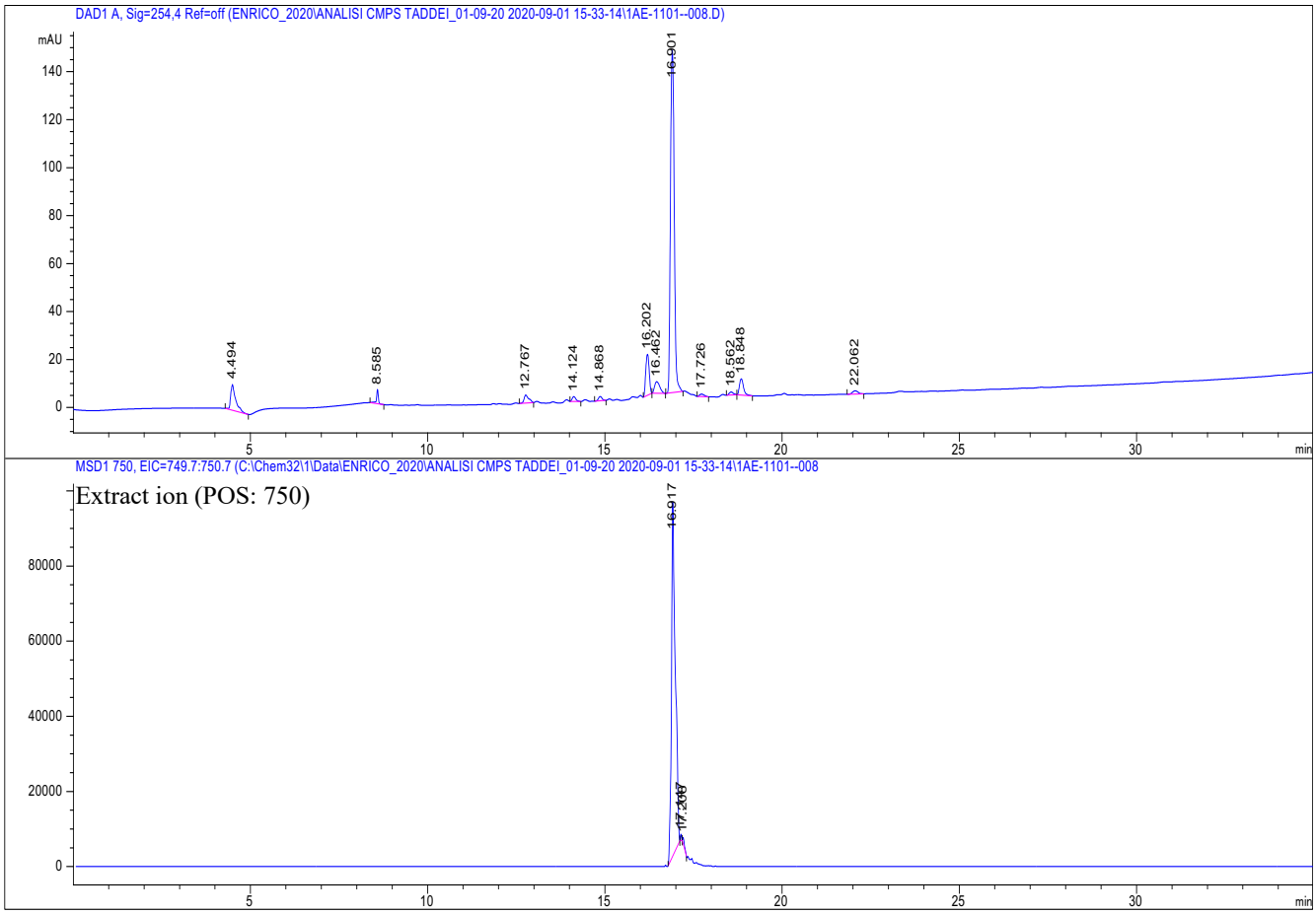

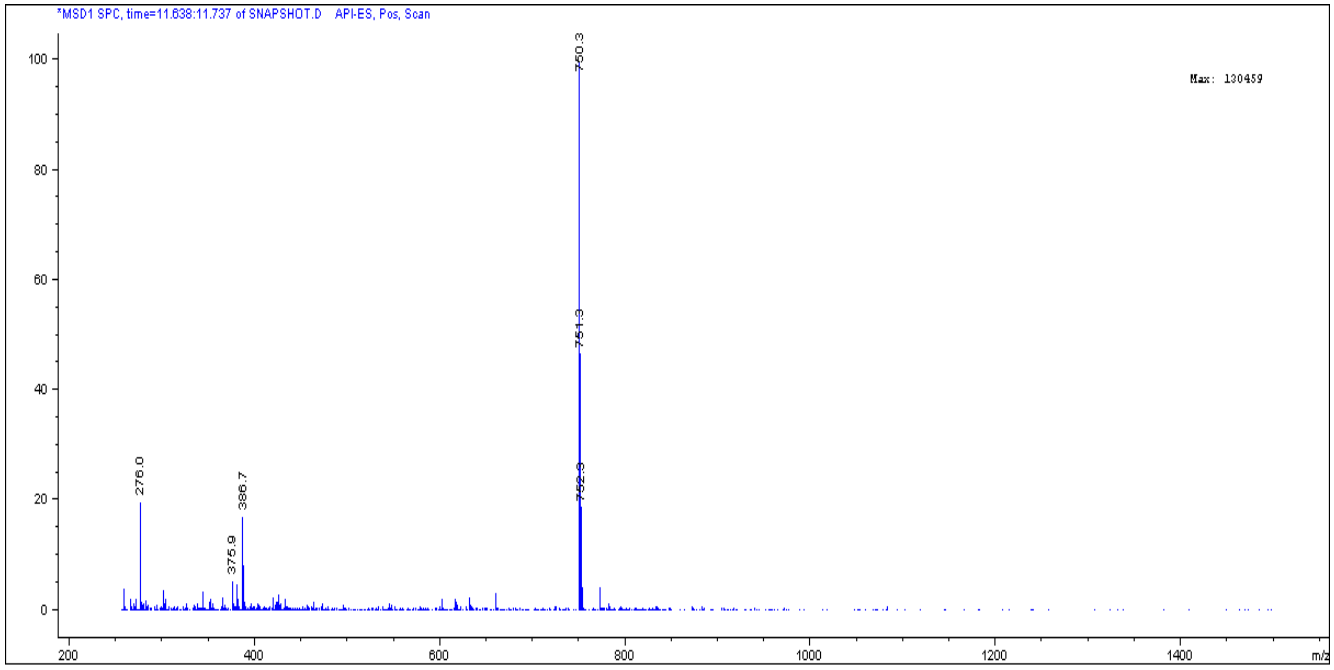

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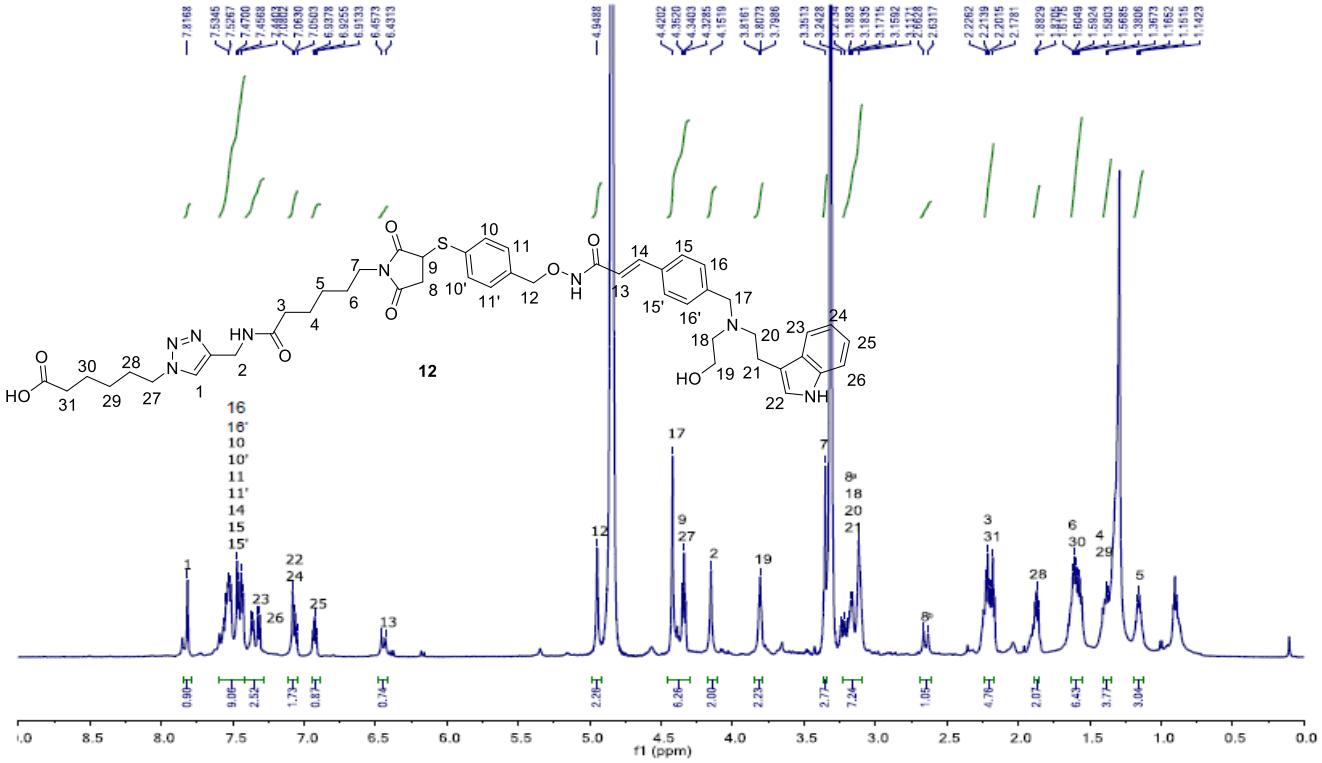

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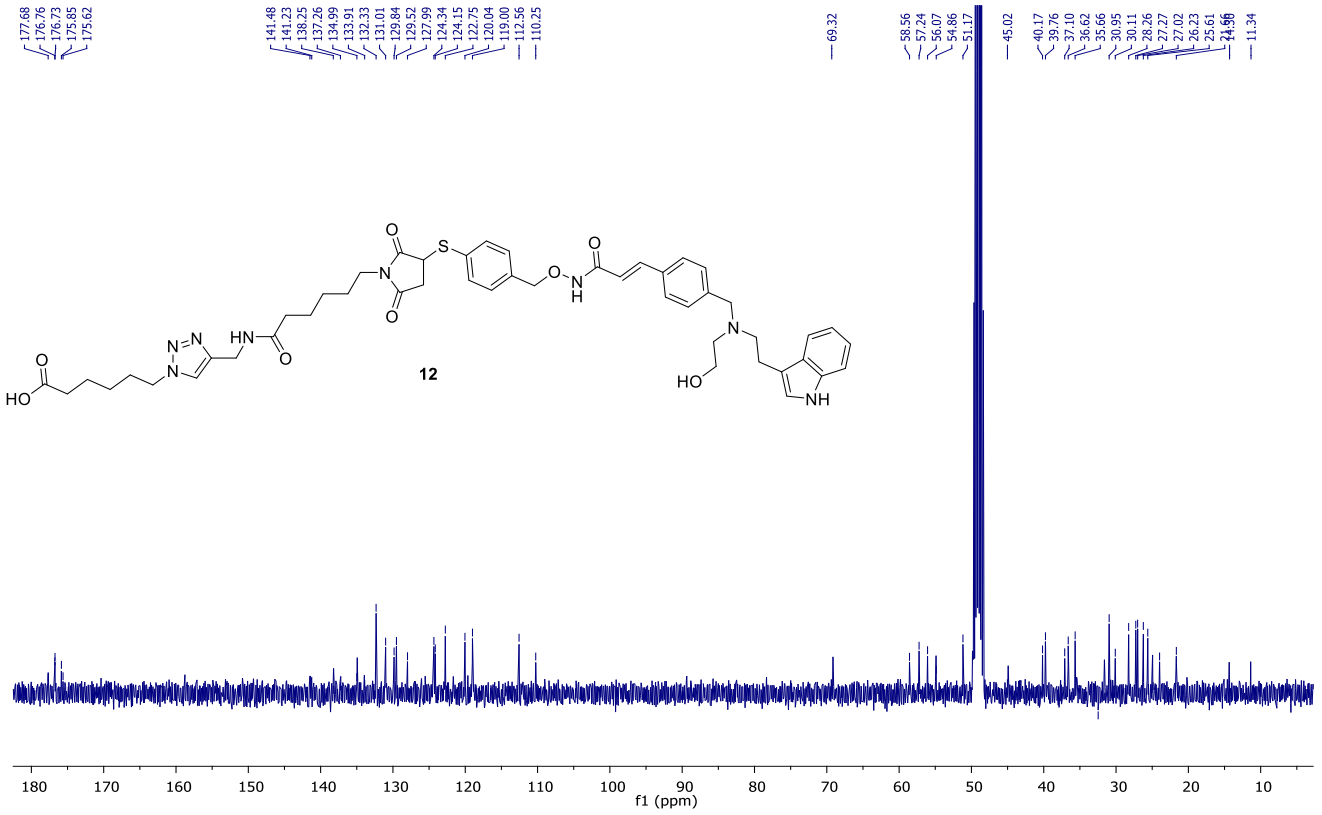

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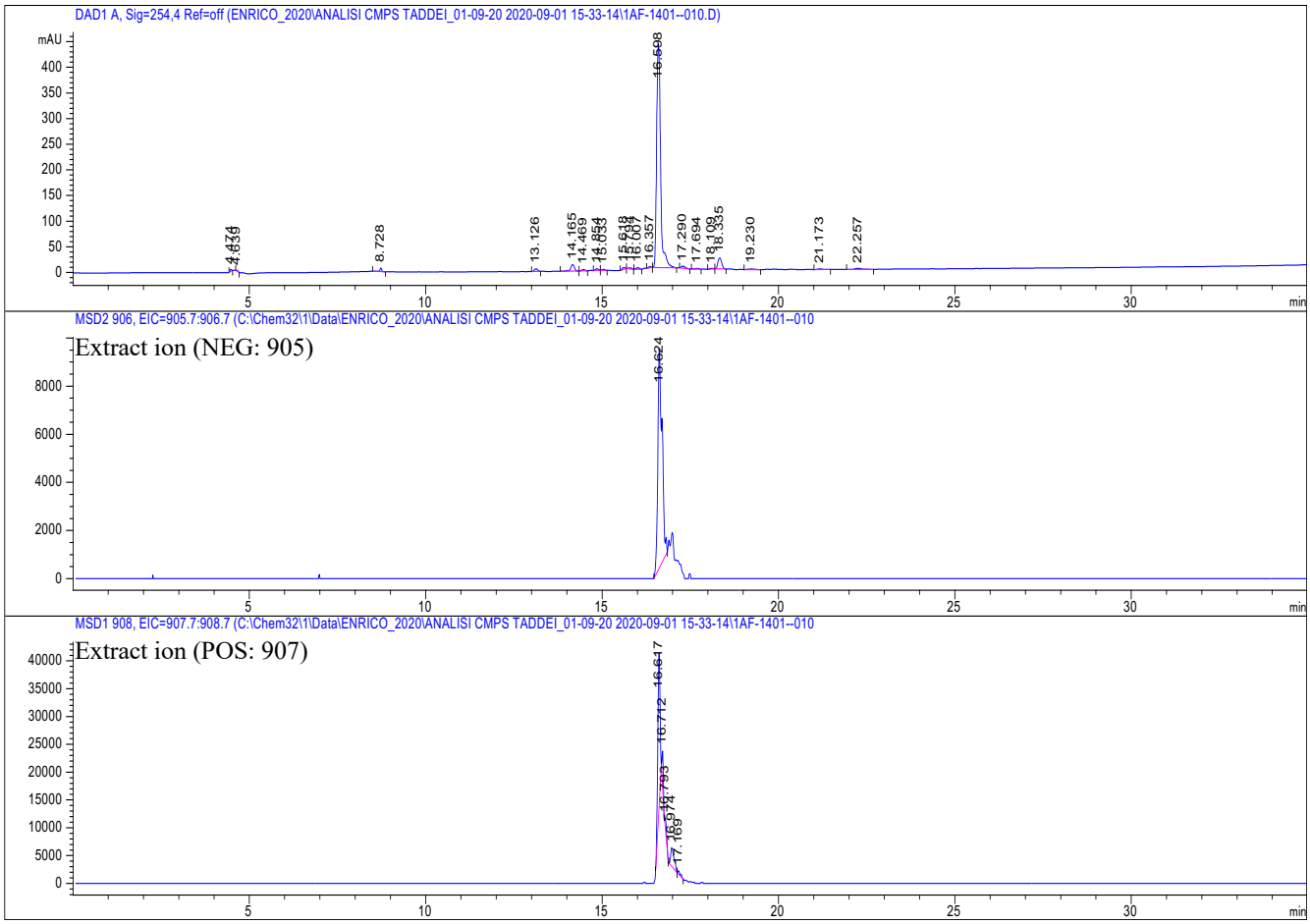
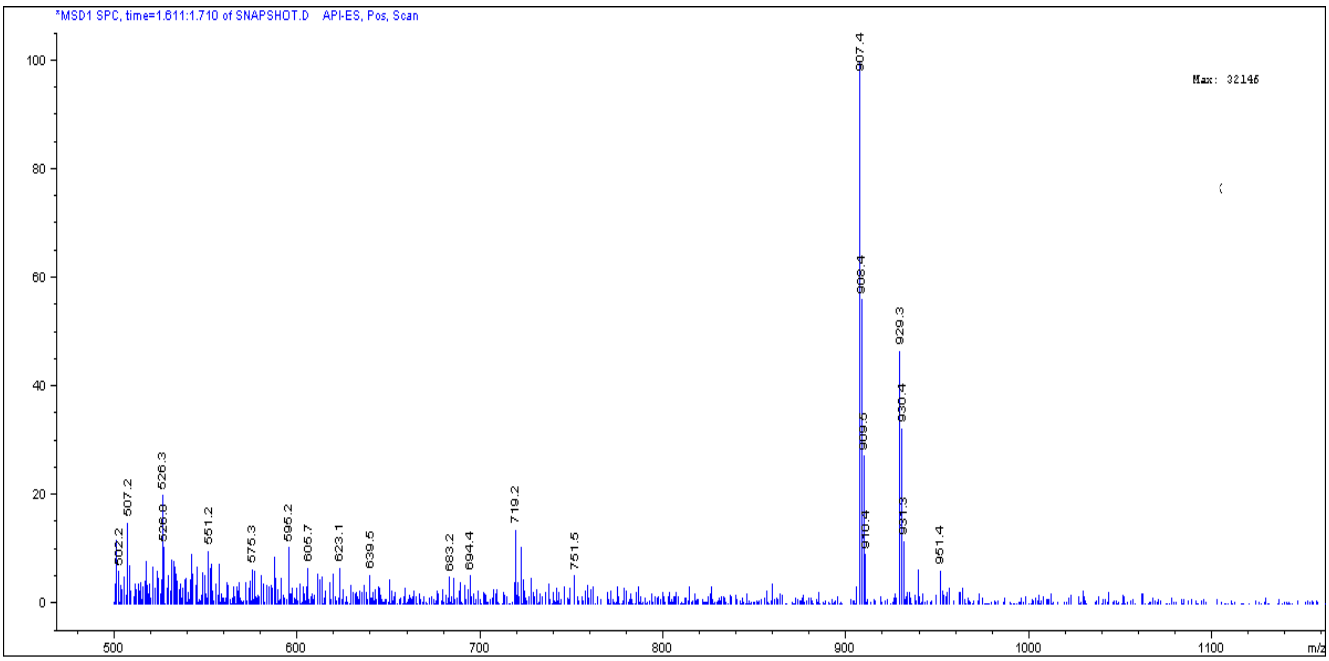
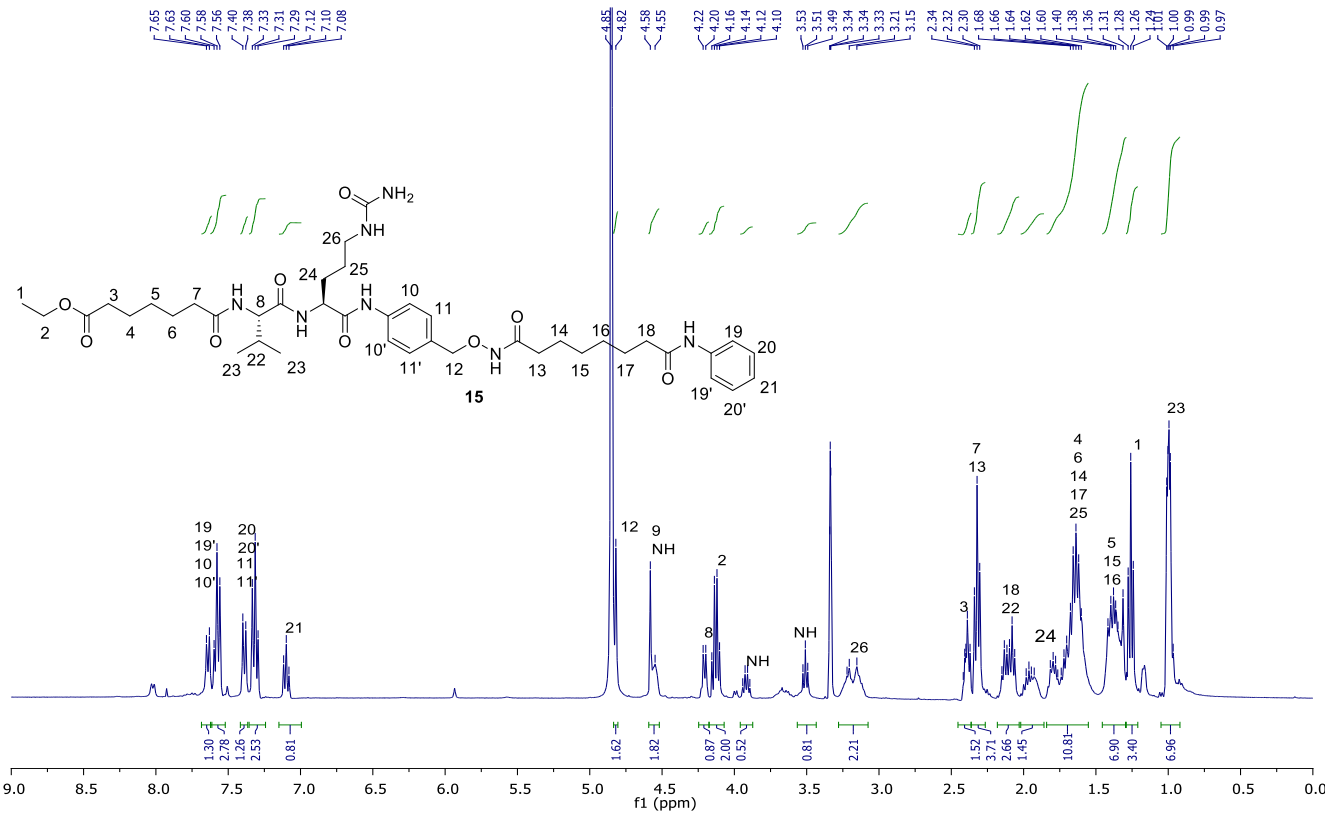




^{1}H-NMR spectrum of **15**



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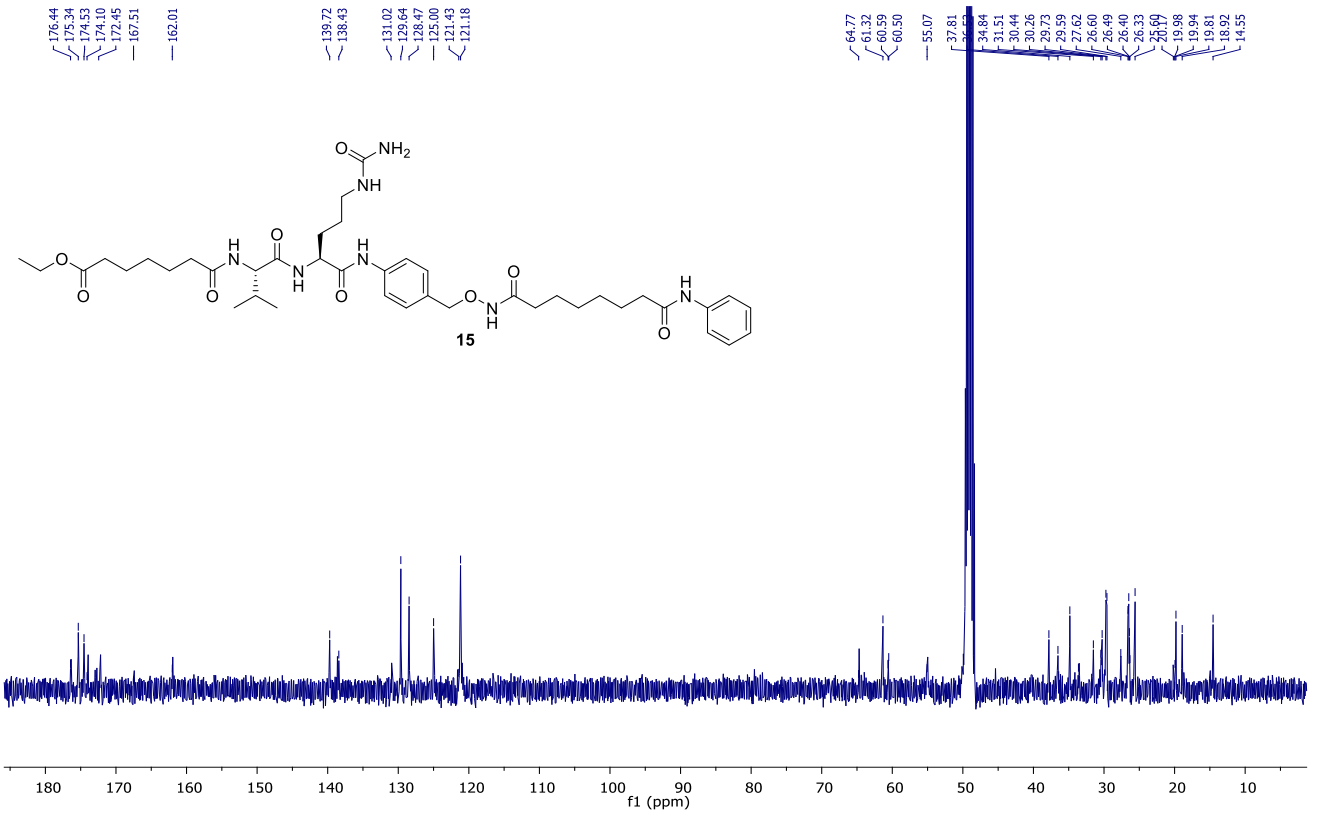

HPLC-MS and MS-ESI **15** (MW: 795)