Supporting Information

Exploiting intracellular oncogenic proteins to release cytotoxins

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Contents

1 Abbreviations and definitions

ACN	Acetonitrile (CAS: 75-05-8)
ADC	Antibody-drug conjugate
Afa-Tz	Afatinib-tetrazine
Afa	Afatinib (CAS: 850140-72-6)
APS	Ammonium persulfate (CAS: 7727-54-0)
Ara-Tz	Aryl-alkyl-tetrazine (CAS: 1345955-28-3)
Boc	*tert*-Butyloxycarbonyl
Cy5.5-nrTCO	Cy5.5-non-releasing *trans*-cyclooctene conjugate
Cy5.5	Cyanine 5.5 (fluorescent dye, CAS: 2097714-45-7, amine derivative)
DCM	Dichloromethane (CAS: 75-09-2)
DIPEA	*N,N*-Diisopropylethylamine (CAS: 7087-68-5)
DMAP	4-Dimethylaminopyridine (CAS: 1122-58-3)
DMEM	Dulbecco's modified Eagle's medium
DMF	*N,N*-Dimethylformamide (CAS: 68-12-2)
DMSO	Dimethyl sulfoxide (CAS: 67-68-5)
Dox-TCO	Doxorubicin-*trans*-cyclooctene conjugate
Dox	Doxorubicin (CAS: 23214-92-8)
DTT	Dithiothreitol (CAS: 3483-12-3)
EDTA	Ethylenediaminetetraacetic acid (CAS: 60-00-4)
EGFR	Epidermal growth factor receptor, P00533
EISA	Enzyme-instructed supramolecular self-assembly
ERBB	Erythroblastic oncogene B, receptor family of four receptor tyrosine kinases, comprising EGFR (HER1), HER2, HER3, and HER4
ESI-MS	Electrospray ionization-mass spectrometry
ESI	Electronic Supplementary Information
EtOAc	Ethyl acetate (CAS: 141-78-6)
FCS	Fetal calf serum
FDA	Food and Drug Administration (United States)
HATU	Hexafluorophosphate azabenzotriazole tetramethyl uronium (CAS: 148893-10-1)
HCl	Hydrochloric acid (CAS: 7647-01-0)
HER2	Human epidermal growth factor receptor 2, receptor tyrosine-protein kinase erbB-2 (ERBB2), P04626
HER3	Human epidermal growth factor receptor 3, receptor tyrosine-protein kinase erbB-3 (ERBB3), P21860
HER4	Human epidermal growth factor receptor 4, receptor tyrosine-protein kinase erbB-4 (ERBB4), Q15303
HOBt	Hydroxybenzotriazole (CAS: 123333-53-9)
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
IC_{50}	Half maximal inhibitory concentration
IEDDA	Inverse-electron-demand Diels-Alder
KRAS	Kirsten rat sarcoma viral oncogene homolog, P01116
krcf	Kilo relative centrifugal force (1000 × rcf)
LCMS	Liquid chromatography-mass spectrometry
MeOH	Methanol (CAS: 67-56-1)
milliQ	Ultrapure water (≥18 MΩ cm) (CAS: 7732-18-5)
MMAE-TCO	Monomethyl auristatin E-*trans*-cyclooctene conjugate
MMAE	Monomethyl auristatin E (CAS: 474645-27-7)

(m/v)	Mass per volume
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
nrTCO	Non-releasing *trans*-cyclooctene
p-NPE	*para*-Nitrophenyl ester
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PET	Positron emission tomography
ppm	Parts per million
PROTAC	Proteolysis targeting chimera
Q-TOF	Quadrupole time-of-flight
rcf	Relative centrifugal force (× g)
RIPA	Radioimmunoprecipitation assay buffer
RT	Room temperature
SDS	Sodium dodecyl sulfate (CAS: 151-21-3)
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBST	Tris-buffered saline with Tween 20
TCO	*trans*-Cyclooctene
TEMED	*N,N,N',N'*-Tetramethylethylenediamine (CAS: 110-18-9)
TFA	Trifluoroacetic acid (CAS: 76-05-1)
THF	Tetrahydrofuran (CAS: 109-99-9)
TLC	Thin-layer chromatography
TMS	Tetramethylsilane (CAS: 993-07-7)
TPS	Total protein stain
Tz	Tetrazine
UFLC	Ultra-fast liquid chromatography
UHPLC-MS	Ultra-high performance liquid chromatography-mass spectrometry
UV	Ultraviolet
(v/v)	Volume per volume

2 Synthesis

General All solvents and reagents were purchased from commercial suppliers and used without further purification unless noted otherwise. Dry solvents were purchased from Thermo Fisher and Sigma-Aldrich as their respective *AcroSeal* or *Sure/Seal* brands. Reactions were generally conducted under nitrogen atmosphere with stirring or shaking unless noted otherwise. Work-up procedures were performed under ambient atmosphere. *trans*-Cyclooctenes were bought as activated esters from Sirius Fine Chemicals SiChem GmbH (Bremen, Germany): TCO*A - active ester (p-NPE) (SC-8007, CAS: 1580501-97-8) and non-release (E)-cyclooct-4-en active ester / TCO4 / A- active ester (p-NPE) / axial (SC-8019, CAS: 1438415-89-4). Reactions were monitored using UHPLC-MS (see below) or TLC (TLC Silica gel 60 F_{254} aluminum sheets, Sigma-Aldrich/Merck) either detected under UV at 254 nm or by $KMnO_4$ stain. Yields were not optimized. Systematic names were generated using ChemDraw (version 23.1.1). Stock solutions of compounds were made in biograde DMSO (D8418, Sigma-Aldrich) by serial dilution.

UHPLC-MS Reaction control was performed on an Agilent 1290 Infinity ultrahigh performance liquid chromatography (UHPLC) system coupled to an Agilent 6130 Quadrupole ESI-MS. Mass spectra were generally recorded in positive ion mode with the notable exception of doxorubicin-TCO, which could only be detected in negative ion mode. Column used was an Agilent ZORBAX RRHD Eclipse Plus C18 2.1 mm – 50 mm, 1.8 µm, 95 Å, running an Agilent ZORBAX RRHD Eclipse Plus C18 2.1 mm – 5 mm, 1.8 µm, 300 Å guard column in front. Standard gradient was 3 % to 95 % B over 2.3 min, 95 % B for 0.45 min at 0.8 ml min^{-1}. Solvent A was ultrapure water with 1 % ACN and 0.1 % formic acid. Solvent B was ACN with 1 % ultrapure water and 0.1 % formic acid.

Preparative HPLC Preparative HPLC was performed on a Shimadzu UFLC system using a Phenomenex (Aschaffenburg, Germany) Gemini 5 µm NX-C18 110 Å, 250 mm × 21.5 mm column. The following gradient was used for all purifications: 5 min 100 % A, gradient over 25 min to 100 % solvent B, 5 min 100 % B at 20 ml min^{-1}. Solvent A was ultrapure water with 0.1 % *v/v* TFA, solvent B HPLC-grade ACN with 0.1 % *v/v* TFA.

Column chromatography For normal phase flash column chromatography SiliaFlash P60 40 µm – 63 µm/230-400 mesh (SiliCycle, Quebec, Canada) was used, for reverse phase separations LiChroprep RP-18 40 µm – 63 µm (Merck). Reverse phase columns were reused until performance degradation could be observed. Chromatography was performed on an Isolera Four (Biotage) or a CombiFlash Rf+ (Teledyne ISCO). Normal phase separations were conducted using gradients of hexane-EtOAc or DCM-MeOH, reverse phase chromatography using ultrapure water with 0.1 % *v/v* TFA and HPLC-grade ACN with 0.1 % *v/v* TFA.

NMR NMR spectra were recorded on a Bruker Avance III at 500 MHz (^{1}H) and 126 MHz (^{13}C) at ambient temperature unless noted otherwise. NMR solvents were bought from Apollo Scientific or Eurisotop. Chemical shifts are reported in ppm relative to residual solvent signals relative to TMS or to TMS directly where possible. Coupling constants are given in hertz (Hz). Abbreviations used: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad.

HRMS High resolution mass spectrometry of small molecules was performed on a Bruker maXis 4G ESI-Q-TOF with direct injection using methanol with 0.1 % formic acid. Compounds were prepared in ACN:water 1:1 at approximately 10 µM. Analysis was performed by Michael Pfeffer of the analytics team at University of Basel.

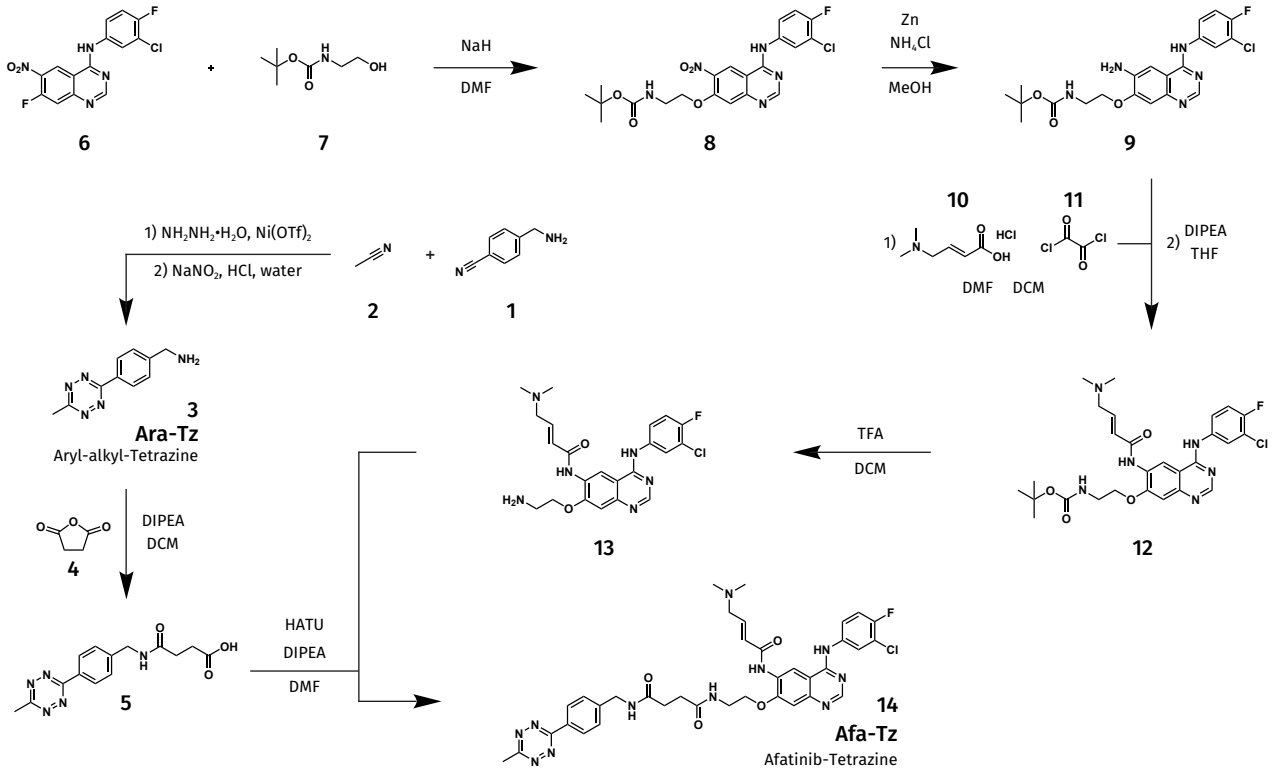
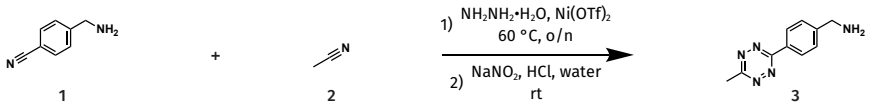


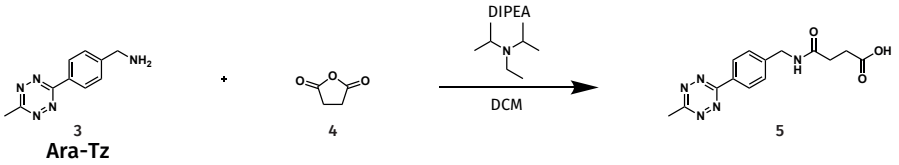
Figure S1: Synthesis overview of **Afa-Tz** (afatinib-tetrazine) and **Ara-Tz** (aryl-alkyl-tetrazine).

Ara-Tz/Aryl-alkyl-Tetrazine (3)
(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine



Metal-catalyzed tetrazine synthesis was performed following a modified procedure by Yang et al.[1] and Fan et al.[2]. Under a nitrogen atmosphere, nitrile **1** (1 g, 7.6 mmol) and $Ni(OTf)_2$ (1.35 g, 3.8 mmol) were dissolved in ACN (**2**, 4 ml, 76 mmol) and the reaction was put into an ice bath. Hydrazine hydrate (20 ml, 248 mmol) was slowly added and the reaction was stirred for another hour at 0 °C. The reaction was then heated to 60 °C and stirred overnight. The reaction was allowed to cool down. $NaNO_2$ (12 g dissolved in 50 ml water) was added and the reaction was acidified with 1 M HCl until no further color change and fume development could be observed. The reaction was extracted with DCM and the combined organic layers were washed with brine and dried over Na_2SO_4. Solvents were removed under reduced pressure. The crude reaction product was purified using normal phase column chromatography to give the title compound in 41 % yield (619 mg) as a red solid.
^{1}H-NMR (500 MHz, MeOD): δ 8.63 (d, J = 8.4 Hz, 2 H), 7.72 (d, J = 8.8 Hz, 2 H), 4.26 (s, 2 H), 3.06 (s, 3 H). ^{13}C-NMR (126 MHz, MeOD): δ 167.7, 163.5, 137.4, 132.9, 129.4 (2 C), 128.1 (2 C), 42.5, 19.7. HRMS: $[M+H]^+/C_{10}H_{12}N_5^+$ calculated 202.1088 *m/z*, found 202.1085 *m/z*.

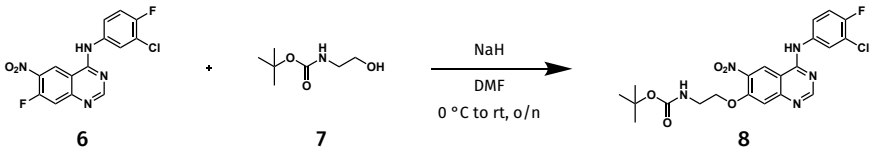
(5)
4-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-4-oxobutanoic acid



Ara-Tz (**3**, 50 mg, 0.248 mmol) was dissolved in DCM. Succinic anhydride (**4**, 124 mg, 1.24 mmol) and DIPEA (346 µl, 2.48 mmol) were added and the reaction was stirred at room temperature overnight. Solvents were removed under reduced pressure and the crude reaction product was purified by preparative HPLC to yield title compound **5** as a red solid in 31 % yield (23 mg).
^{1}H-NMR (500 MHz, DMSO): δ 12.09 (s, br, 1 H), 8.51 (t, J = 6.0 Hz, 1 H), 8.40 (d, J = 8.4 Hz, 2 H), 7.53 (d, J = 8.6 Hz, 2 H), 4.40 (d, J = 6.0 Hz, 2 H), 2.99 (s, 3 H), 2.50 – 2.46 (m, 2 H), 2.46 – 2.41 (m, 2 H). ^{13}C-NMR (126 MHz, DMSO): δ 174.3, 171.7, 167.5, 163.7, 145.0, 130.8, 128.4 (2 C), 127.8 (2 C), 42.3, 30.5, 29.6, 21.3. HRMS: $[M+H]^+/C_{14}H_{16}N_5O_3^+$ calculated 302.1248 *m/z*, found 302.1251 *m/z*.

(8)

***tert*-butyl (2-((4-((3-chloro-4-fluorophenyl)amino)-6-nitroquinazolin-7-yl)oxy)ethyl)carbamate**



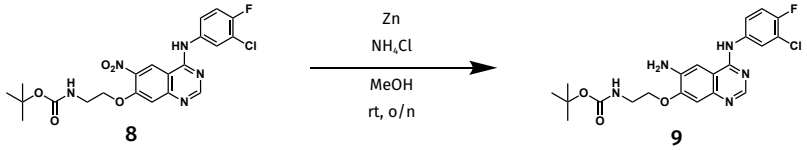
6 **7** **8**

Sodium hydride (60 % suspension in mineral oil, 689 mg, 17.2 mmol) was suspended in DMF on ice. *tert*-butyl (2-hydroxyethyl)carbamate (**7**, 2.87 g, 17.8 mmol, dissolved in DMF) was added dropwise. The reaction was stirred for 30 min at 0 °C. Afatinib core **6** (2 g, 5.94 mmol) was added. The deep red solution was stirred overnight allowing it to slowly reach room temperature. The reaction was quenched with 1 M HCl and extracted with DCM. Solvents were removed under reduced pressure and the crude reaction product was purified by reverse phase column chromatography to give title compound **8** as a strongly yellow powder in 95 % yield (2.7 g).

^{1}H-NMR (500 MHz, DMSO): δ 12.28 (s, 1 H), 9.85 (s, 1 H), 8.95 (s, 1 H), 8.06 (dd, J = 6.8, 2.6 Hz, 1 H), 7.79 (ddd, J = 9.0, 4.3, 2.6 Hz, 1 H), 7.75 (s, 1 H), 7.52 (t, J = 9.0 Hz, 1 H), 7.04 (t, J = 5.7 Hz, 1 H), 4.30 (t, J = 5.5 Hz, 2 H), 3.40 (q, J = 5.4 Hz, 2 H), 1.37 (s, 9 H). ^{13}C-NMR (126 MHz, DMSO): δ 159.4, 155.7, 155.2, 155.2 (d, J(FC) = 246.7 Hz), 153.3, 143.7, 139.7, 133.9 (d, J(FC) = 3.3 Hz), 126.3, 125.1 (d, J(FC) = 7.4 Hz), 123.6, 119.2 (d, J(FC) = 18.7 Hz), 116.9 (d, J(FC) = 22.0 Hz), 106.7, 103.8, 78.0, 69.1, 38.7, 28.2 (3 C). HRMS: $[M+H]^+/C_{21}H_{22}ClFN_5O_5^+$ calculated 478.1288 *m/z*, found 478.1295 *m/z*.

(9)

***tert*-butyl (2-((6-amino-4-((3-chloro-4-fluorophenyl)amino)quinazolin-7-yl)oxy)ethyl)carbamate**



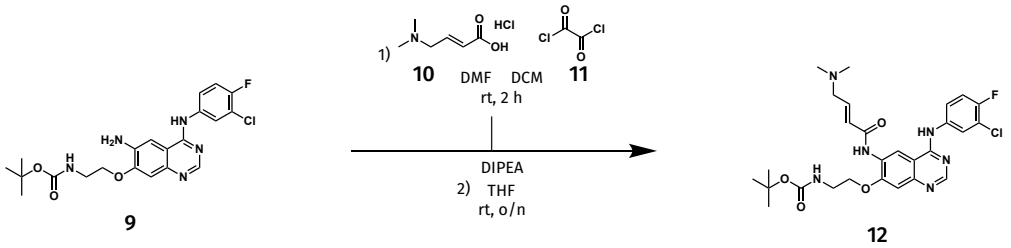
8 **9**

Afatinib-tetrazine precursor **8** (148 mg, 0.31 mmol), zinc powder (101 mg, 1.55 mmol), and ammonium chloride (99 mg, 1.86 mmol) were suspended in 10 ml methanol. The reaction was stirred overnight under a nitrogen atmosphere at room temperature. Solids were removed by filtration through a pad of celite on a glass filter. Solvents were removed under reduced pressure to yield title compound **9** in 96 % yield (133 mg) as a yellow powder.

^{1}H-NMR (500 MHz, DMSO): δ 10.8 (s, 1 H), 8.7 (s, 1 H), 8.0 (dd, J = 6.8, 2.6 Hz, 1 H), 7.7 (ddd, J = 9.0, 4.4, 2.6 Hz, 1 H), 7.6 (s, 1 H), 7.5 (t, J = 9.1 Hz, 1 H), 7.3 (m, 1 H), 7.2 (s, 1 H), 4.1 (t, J = 5.0 Hz, 2 H), 3.5 (q, J = 5.4 Hz, 2 H), 1.4 (s, 9 H). ^{13}C-NMR (126 MHz, DMSO): δ 157.0, 155.7, 154.8 (d, J(FC) = 254.6 Hz), 153.5, 146.3, 140.8, 134.8 (d, J(FC) = 3.3 Hz), 132.9, 126.0, 124.7 (d, J(FC) = 7.3 Hz), 119.2 (d, J(FC) = 18.6 Hz), 116.8 (d, J(FC) = 21.9 Hz), 108.9, 100.8, 99.1, 78.0, 68.7, 39.1, 28.3 (3 C). HRMS: $[M+H]^+/C_{21}H_{24}ClFN_5O_3^+$ calculated 448.1547 *m/z*, found 448.1556 *m/z*.

(12)

tert-butyl (*E*)-(2-((4-((3-chloro-4-fluorophenyl)amino)-6-(4-(dimethylamino)but-2-enamido)quinazolin-7-yl)oxy)ethyl)carbamate

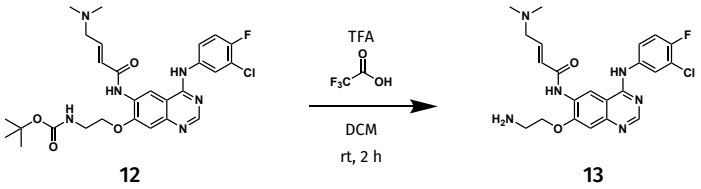


(*E*)-4-(dimethylamino)but-2-enoic acid hydrochloride (**10**, 41 mg, 0.25 mmol) was dissolved in 1 ml DCM and a few drops of DMF were added. Oxalyl chloride (**11**, 140 µl of a 2 M solution in DCM, 0.28 mmol) was added dropwise and the reaction was stirred for 2 h at room temperature under nitrogen. Solvents were removed under reduced pressure. Afatinib-tetrazine precursor **9** (60 mg, 0.13 mmol) was dissolved in 2 ml THF and DIPEA (210 µl, 1.2 mmol) was added. The THF solution was added to the previously prepared acyl chloride and the reaction was stirred at room temperature overnight. Solvents were removed under reduced pressure and the crude reaction product was directly purified by reverse phase column chromatography. Title compound **12** was obtained as a yellowish powder in 39 % yield (29 mg).

^{1}H-NMR (500 MHz, DMSO): δ 10.81 (s, br, 1 H), 10.21 (s, br, 1 H), 9.78 (s, 1 H), 9.19 (s, 1 H), 8.76 (s, 1 H), 8.01 (dd, J = 6.8, 2.6 Hz, 1 H), 7.70 (ddd, J = 9.0, 4.3, 2.7 Hz, 1 H), 7.50 (t, J = 9.1 Hz, 1 H), 7.36 (s, 1 H), 7.26 (t, J = 6.1 Hz, 1 H), 6.91 – 6.81 (m, 1 H), 6.83 – 6.75 (m, 1 H), 4.24 (t, J = 5.2 Hz, 2 H), 4.00 (d, J = 6.8 Hz, 2 H), 3.49 (q, J = 5.4 Hz, 2 H), 2.82 (s, 6 H), 1.40 (s, 9 H). ^{13}C-NMR (126 MHz, DMSO): δ 162.2, 158.3, 158.1, 156.1, 154.7, 154.4 (d, J(FC) = 250 Hz, estimated from HMBC),151.6, 135.2, 133.1, 131.3, 128.1, 125.6, 124.4, 119.0 (d, J(FC) = 18.4 Hz), 116.7 (d, J(FC) = 22.0 Hz), 114.6, 107.9, 78.2, 69.5, 56.8, 42.1 (2 C), 39.0, 28.2 (3 C), HRMS: $[M+H]^+/C_{27}H_{33}ClFN_6O_4^+$ calculated 559.2231 *m/z*, found 559.2233 *m/z*.

(13)

(*E*)-*N*-(7-(2-aminoethoxy)-4-((3-chloro-4-fluorophenyl)amino)quinazolin-6-yl)-4-(dimethylamino)but-2-enamide

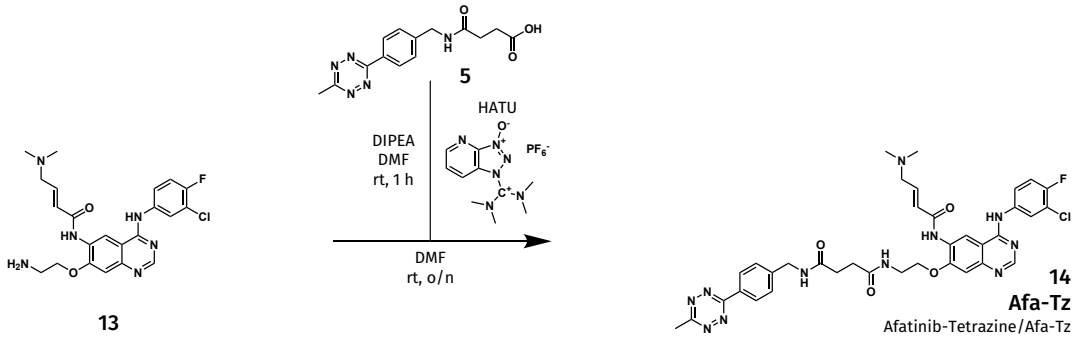


Afatinib-tetrazine precursor **12** (10 mg, 18 µmol) was dissolved in 0.5 ml DCM. 0.5 ml TFA was added and the reaction was stirred for one hour at room temperature until LCMS showed complete deprotection. Solvents were removed under reduced pressure. The reaction product **13** was used without further purification.

^{1}H-NMR (500 MHz, DMSO): δ 10.73 (s, 1 H), 10.44 (s, 1 H), 9.93 (s, 1 H), 9.19 (s, 1 H), 8.76 (s, 1 H), 8.33 (t, J = 5.7 Hz, 3 H), 8.04 (dd, J = 6.8, 2.6 Hz, 1 H), 7.73 (ddd, J = 9.0, 4.3, 2.6 Hz, 1 H), 7.54 – 7.45 (m, 2 H), 6.86 (d, J = 3.5 Hz, 2 H), 4.46 (t, J = 4.5 Hz, 2 H), 4.01 – 3.97 (m, 2 H), 3.46 – 3.39 (m, 2 H), 2.82 (s, 6 H). HRMS: $[M+H]^+/C_{22}H_{25}ClFN_6O_2^+$ calculated 459.1707 *m/z*, found 459.1702 *m/z*.

Afa-Tz/Afatinib-Tetrazine (14)

(*E*)-*N1*-(2-((4-((3-chloro-4-fluorophenyl)amino)-6-(4-(dimethylamino)but-2-enamido) quinazolin-7-yl)oxy)ethyl)-*N4*-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)succinamide

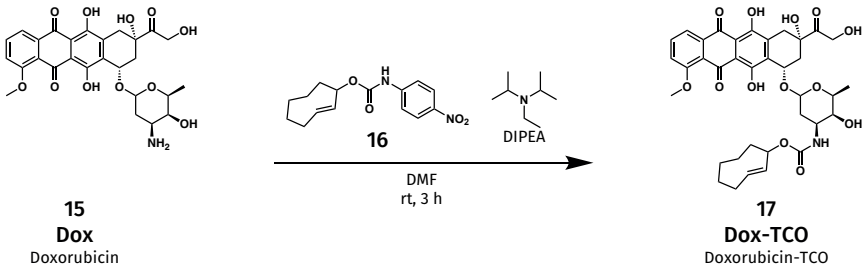


Tetrazine **5** (4 mg, 13 µmol) was dissolved in 500 µl DMF. HATU (10.1 mg, 26 µmol) and DIPEA (11 µl, 60 µmol) were added and the reaction was stirred at room temperature for one hour. Afatinib derivative **13** (5.5 mg, 12 µmol) was dissolved separately in 500 µl DMF and added to the activated acid. The reaction was stirred overnight at room temperature. Solvents were removed under reduced pressure and the crude reaction product was redissolved in DMSO and purified by preparative HPLC. After lyophilization a red powder was obtained in 45 % yield (Afa-Tz/afatinib-tetrazine, **14**, 4 mg).

^{1}H-NMR (500 MHz, DMSO): δ 11.02 (s, br, 1 H), 10.86 (s, br, 1 H), 9.87 (s, 1 H), 9.18 (s, 1 H), 8.78 (s, 1 H), 8.57 (t, J = 6.0 Hz, 1 H), 8.52 (t, J = 5.9 Hz, 1 H), 8.35 (d, J = 8.2 Hz, 2 H), 7.98 (dd, J = 6.8, 2.6 Hz, 1 H), 7.69 (ddd, J = 9.0, 4.2, 2.5 Hz, 1 H), 7.52 (t, J = 9.0 Hz, 1 H), 7.47 (d, J = 8.1 Hz, 2 H), 7.35 (s, 1 H), 7.03 – 6.97 (m, 1 H), 6.97 – 6.88 (m, 1 H), 4.35 (d, J = 5.9 Hz, 2 H), 4.24 (t, J = 5.2 Hz, 2 H), 3.98 (d, J = 6.7 Hz, 2 H), 3.62 (q, J = 5.4 Hz, 2 H), 2.97 (s, 3 H), 2.78 (s, 6 H), 2.52 – 2.46 (m, 4 H). ^{13}C-NMR (126 MHz, DMSO): δ 172.5, 171.6, 167.0, 163.1, 162.6, 158.3, 155.2, 154.8 (d, J(FC) = 250 Hz, estimated from HMBC),150.3, 144.4, 134.8, 133.0, 131.4, 130.2, 128.6, 127.8 (2 C), 127.3 (2 C), 126.1, 124.8, 119.0 (d, J(FC) = 18.2 Hz), 116.8 (d, J(FC) = 21.8 Hz), 115.0, 107.5, 101.3, 69.2, 56.8, 41.9 (2 C), 41.8, 37.7, 30.9, 30.7, 20.8. HRMS: $[M+H]^+/C_{36}H_{38}ClFN_{11}O_4^+$ calculated 742.2775 *m/z*, found 742.2778 *m/z*.

Doxorubicin-TCO (17)

(*E*)-cyclooct-2-en-1-yl ((2S,3S,4S)-3-hydroxy-2-methyl-6-(((1S,3S)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-10-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-1-yl)oxy)-tetrahydro-2H-pyran-4-yl)carbamate

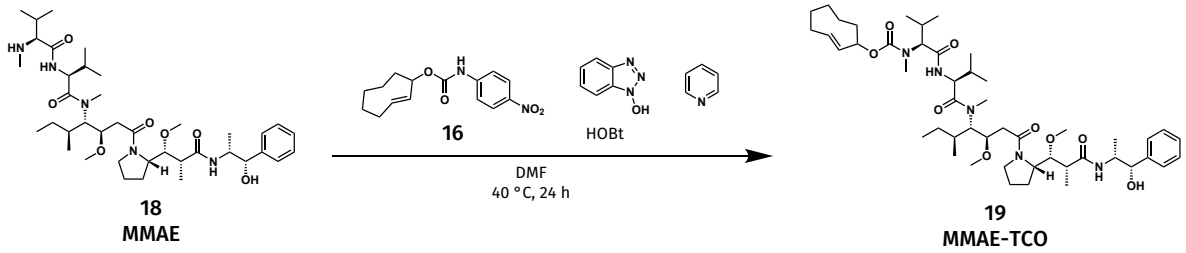


Doxorubicin (**15**, 40 mg, 69 µmol) and activated *trans*-cyclooctene **16** (18 mg, 62 µmol) were combined and dissolved in 3 ml DMF. While stirring at room temperature, 120 µl DIPEA (690 µmol) was added. After 3 h solvents were removed under reduced pressure. Reaction products were redissolved in DMSO and purified by preparative HPLC. After lyophilization Dox-TCO/Doxorubicin-TCO (**17**) was obtained as a red powder (30 mg, 69 %). Synthesis was performed following previously described procedures.[3]

^{1}H-NMR (500 MHz, $CDCl_3$): δ 13.97 (s, 1 H), 13.23 (s, 1 H), 8.03 (dt, J = 7.7, 1.0 Hz, 1 H), 7.82 – 7.75 (m, 1 H), 7.39 (dd, J = 8.6, 3.3 Hz, 1 H), 5.83 – 5.69 (m, 1 H), 5.56 – 5.42 (m, 2 H), 5.29 (s, 1 H), 5.24 (s, 1 H), 5.09 (s, 1 H), 4.80 – 4.71 (m, 2 H), 4.52 (s, 1 H), 4.18 – 4.10 (m, 1 H), 4.10 – 4.02 (m, 3 H), 3.90 – 3.82 (m, 1 H), 3.69 (s, 1 H), 3.27 (d, J = 18.8 Hz, 1 H), 3.04 – 2.98 (m, 2 H), 2.47 – 2.40 (m, 1 H), 2.33 (dt, J = 14.6, 2.2 Hz, 1 H), 2.20 – 2.14 (m, 1 H), 2.05 – 1.75 (m, 6 H), 1.61 (s, 2 H), 1.47 (s, 1 H), 1.30 (d, J = 6.5 Hz, 3 H), 1.08 – 0.98 (m, 1 H), 0.84 – 0.70 (m, 1 H). HRMS: $[M-H]^-/C_{36}H_{40}NO_{13}^-$ calculated 694.2505 *m/z*, found 694.2513 *m/z*.

MMAE-TCO (19)

(*E*)-cyclooct-2-en-1-yl ((*S*)-1-(((S)-1-(((3*R*,4*S*,5*S*)-1-((*S*)-2-((1*R*,2*R*)-3-(((1*S*,2*R*)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)(methyl)amino)-3-methyl-1-oxobutan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)(methyl)carbamate



18
MMAE

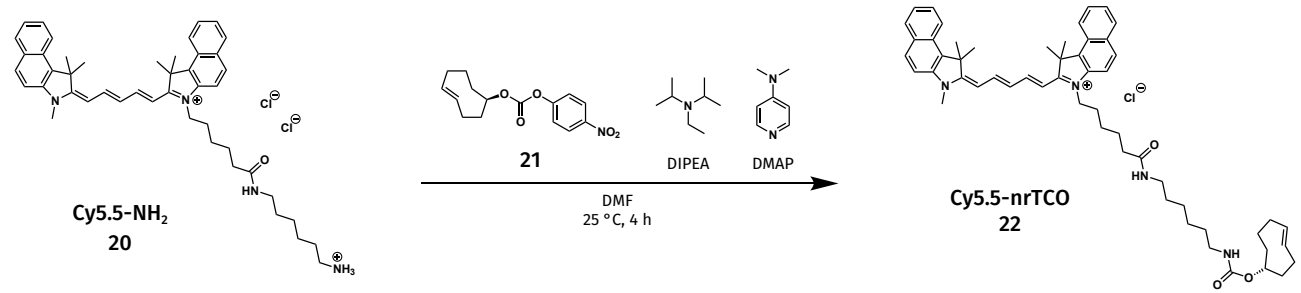
19
MMAE-TCO

MMAE (**18**, 32 mg, 45 µmol), activated *trans*-cyclooctene **16** (15 mg, 51 µmol) and HOBt (12 mg, 76 µmol) were combined in a 2 ml glass vial. 200 µl DMF and 800 µl pyridine were added and the solution was stirred for 24 h at 40 °C. Solvents were removed under reduced pressure. The resulting oil was dissolved in DMSO and purified by preparative HPLC. After lyophilization MMAE-TCO (**19**) was obtained as a white powder (17 mg, 44 %). Synthesis was performed following previously described procedures.[4]

^{1}H-NMR (500 MHz, DMSO): δ 8.33 – 7.90 (m, 1 H), 7.90 – 7.58 (m, 1 H), 7.33 – 7.28 (m, 2 H), 7.28 – 7.23 (m, 2 H), 7.20 – 7.13 (m, 1 H), 6.00 – 5.60 (m, 1 H), 5.60 – 5.47 (m, 1 H), 5.42 – 5.31 (m, 1 H), 5.27 (s, 1 H), 4.80 – 4.51 (m, 1 H), 4.51 – 4.38 (m, 2 H), 4.28 – 4.20 (m, 1 H), 4.06 – 3.75 (m, 3 H), 3.64 – 3.52 (m, 1 H), 3.47 (s, 1 H), 3.32 (s, 1 H), 3.26 – 3.2 (m, 4 H), 3.22 – 3.1 (m, 3 H), 3.16 – 3.0 (m, 2 H), 3.08 – 2.9 (m, 1 H), 2.98 – 2.8 (m, 4 H), 2.46 – 2.3 (m, 2 H), 2.33 – 2.2 (m, 1 H), 2.19 – 2.0 (m, 2 H), 2.04 – 1.8 (m, 3 H), 1.86 – 1.6 (m, 5 H), 1.62 – 1.4 (m, 3 H), 1.49 – 1.2 (m, 3 H), 1.08 – 0.9 (m, 7 H), 0.92 – 0.6 (m, 18 H). HRMS: $[M+Na]^+/C_{48}H_{79}N_5NaO_9^+$ calculated 892.5770 *m/z*, found 892.5783 *m/z*.

Cy5.5-nrTCO (22)

3-(6-((6-(((((*R*,*E*)-cyclooct-4-en-1-yl)oxy)carbonyl)amino)hexyl)amino)-6-oxohexyl)-1,1-dimethyl-2-((1E,3E,5E)-5-(1,1,3-trimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)-penta-1,3-dien-1-yl)-1H-benzo[e]indol-3-ium chloride



Cy5.5-NH$_2$
20

Cy5.5-nrTCO
22

5 mg Cyanine5.5-Amine (Cy5.5-NH$_2$, **20**, CAS: 2097714-45-7, lumiprobe, Artikel-Nr. 270C0) was dissolved as received in 1 ml ACN to be volumetrically aliquoted into 5x equal 1 mg parts into 1.5 ml Eppendorf tubes. ACN was removed on a SpeedVac. A 1 mg aliquot of Cy5.5-NH$_2$ (1.3 µmol) in a 1.5 ml Eppendorf tube was redissolved in 500 µl DMF. Non-release activated *trans*-cyclooctene **21** (1.2 mg, 4.1 µmol) was dissolved in a separate 1.5 ml Eppendorf tube in 200 µl DMF and added to the Cy5.5-NH$_2$. 3 µl of DIPEA (17 µmol) and 7 µl of a 100 mM stock solution of DMAP (0.7 µmol) in DMF were added and the reaction was shaken for 4 h at 25 °C on a thermo shaker. Solvents were removed under reduced pressure and the reaction products were redissolved in 1.5 ml ACN:DMSO 2:1, filtered and purified by preparative HPLC. Product fractions were lyophilized to obtain Cy5.5-nrTCO (**22**) as a deep blue residue. Stock solutions of Cy5.5-nrTCO were prepared in DMSO, with concentration determined by comparing the UV trace from LCMS analysis to that of a Cy5.5-NH$_2$ stock solution of known concentration.

HRMS: $[M]^+/C_{55}H_{69}N_4O_3^+$ calculated 833.5364 *m/z*, found 833.5375 *m/z*.

3 Cell culture

All cells were cultured at 37 °C and 5 % CO_2 in humidified incubators. Cells were monthly tested for mycoplasma contamination using the MycoStrip detection kit (InvivoGen, Toulouse, France). The following list summarizes the cell lines used and their respective media and passaging conditions.

3.1 Cell lines

A431 RRID:CVCL_0037, kind gift from Michael Walser, University of Zürich. Medium: **A**; passaging: **b**, trypsinization time approximately 8 min

MDA-MB-468 RRID:CVCL_0419; kind gift from Cora-Ann Schöneberger/Palivan-Group, University of Basel. Medium: **A**; passaging: **b**, trypsinization time approximately 6 min

MCF-7 RRID:CVCL_0031; ACC 115, DSMZ, Braunschweig, Germany. Medium: **A**; passaging: **b**, trypsinization time approximately 5 min

SW620 RRID:CVCL_0547; CCL-227, ATCC, Manassas, USA. Medium: **A**; passaging: **a**, trypsinization time approximately 4 min

A Medium A consists of Dulbecco's Modified Eagle's Medium - high glucose (DMEM, D5796-500ML, Merck/Sigma-Aldrich, Darmstadt, Germany) supplemented with 10 % fetal calf serum (FCS, 2-01F00-I, BioConcept, Allschwil, Switzerland) and 1 % penicillin-streptomycin (4-01F00-H, BioConcept, Allschwil, Switzerland).

a Cells were detached using 0.05 % trypsin-EDTA (25300-054, Gibco/ThermoFisher Scientific, Waltham, MA, USA).

b Cells were detached using 0.25 % trypsin-EDTA (25200-056, Gibco/ThermoFisher Scientific, Waltham, MA, USA).

3.2 Typical seeding conditions

A431 500 k, 2 ml, 6-well plates, 24 h/overnight until treatment

MCF-7 600 k, 2 ml, 6-well plates, 24 h/overnight until treatment

SW620 800 k, 1 ml, 12-well plates, 24 h/overnight until treatment

MDA-MB-468 500 k, 2 ml, 6-well plates, 24 h/overnight until treatment

4 SDS-PAGE

Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a Mini-PROTEAN Tetra system (Bio-Rad, Hercules, CA, USA) using 1.5 mm hand-cast discontinuous 15-well (nominal well volumes of 40 µl) mini-gels. The resolving/running gel mixture (8 %) for one gel was prepared by combining 2 ml of a 40 % acrylamide/bis-acrylamide solution (CAS: 79-06-1 and 110-26-9, 37.5:1, Cat. 330225000, Thermo Scientific, Waltham, MA, USA); 5.3 ml milliQ; 2.5 ml tris buffer (1.5 M, pH 8.8 at room temperature, prepared from tris base, (CAS: 77-86-1, Cat. A1086, PanReac/AppliChem/ITW Reagents, Darmstadt, Germany), adjusted with HCl (25 % for analysis EMSURE, CAS: 7647-01-0, Cat. 1.00316, Supelco/Sigma-Aldrich, St. Louis, MO, USA)); and 100 µl SDS 10 % (w/v) (prepared from 10 g SDS (CAS: 151-21-3, Cat. A2262, PanReac/AppliChem/ITW Reagents, Darmstadt, Germany) dissolved in a total volume of 100 ml milliQ). The mixture was homogenized by gentle pipetting, followed by the immediate addition of 5 µl TEMED (*N,N,N',N'*-tetramethylethylenediamine, CAS: 110-18-9, Cat. 17919, Thermo Scientific, Waltham, MA, USA) and 50 µl of a 10 % (w/v) APS (Ammonium persulfate, CAS: 7727-54-0, Cat. A3678, Sigma-Aldrich, St. Louis, MO, USA) solution in milliQ. After thorough but gentle mixing with a serological pipette, the solution was promptly poured between the glass plates to approximately 2 cm below the top edge. The top of the gel was carefully leveled using ethanol and left to polymerize at room temperature.

The stacking gel was prepared similarly to the resolving gel by combining 0.25 ml acrylamide/bis-acrylamide solution; 1.6 ml milliQ; 0.65 ml Tris-HCl (1.0 M, pH 6.8 at room temperature); 25 µl 10 % (w/v) SDS; 2.5 µl TEMED; and 12.5 µl 10 % (w/v) APS. The ethanol overlay was completely removed from the polymerized resolving gel by aspiration with a blunt needle connected to a vacuum line. The stacking gel mixture was poured on top of the resolving gel, and a 15-well comb was carefully inserted while avoiding bubble formation. The gel was allowed to polymerize for 30 min at room temperature.

Fully polymerized gels were removed from the casting apparatus and placed into the electrode assembly. Combs were gently removed, and wells were flushed with tris-glycine running buffer (10x stock prepared from 30 g tris base; 144 g glycine (CAS: 56-40-6, Cat. #1023, GERBU Biotechnik GmbH, Heidelberg, Germany); and 10 g SDS, dissolved in a final volume of 1 l milliQ. Diluted to 1x for use by combining 0.1 l of 10x stock with 0.9 l milliQ) for final concentrations of 25 mM Tris, 192 mM glycine, and 0.1 % SDS at pH 8.3 (left unadjusted). The electrode chamber was filled with sufficient running buffer to ensure complete submersion of all wells.

The loading dye used was either 5x homemade (300 mM Tris-HCl pH 6.8 at room temperature; 10 % SDS (w/v); 50 % glycerol; 0.05 % bromophenol blue (w/v) (CAS: 115-39-9, Cat. B0631, TCI, Tokyo, Japan), 500 mM DTT (CAS: 3483-12-3, Cat. A1101, PanReac/AppliChem/ITW Reagents, Darmstadt, Germany), used from single-use frozen aliquots), or 3x from a commercial vendor (Blue Protein Loading Dye, B7703S, New England BioLabs, Ipswich, MA, USA; prepared from 1 part 3x SDS Blue Loading Buffer (187.6 mM Tris-HCl pH 7.0; 6 % SDS (w/v); 30 % glycerol; 0.03 % bromophenol blue (w/v)) and 1/10 part 30x Reducing Agent (1.25 M DTT). Protein aliquots were combined with loading dye (for 5x: 4 parts sample, 1 part loading dye; for 3x: 2 parts sample, 1 part loading dye) and in either case heated at 98 °C for 5 min while shaking at 300 rpm, then immediately placed on ice to cool and briefly spun down.

Samples were carefully loaded into wells. Prestained molecular weight marker (AcuteBand Pre-Stained Protein Ladder, LU5001-2500, LubioScience GmbH, Zürich, Switzerland) was loaded (commonly 2 µl), typically in both outer lanes plus an additional asymmetrically placed lane to facilitate gel orientation during subsequent analysis. The loaded gel assembly was placed in the electrophoresis tank filled with running buffer. Electrophoresis was performed using a programmable power supply (PowerPac Basic, Bio-Rad, Hercules, CA, USA) at constant voltage, initially at 80 V until the tracking dye reached the resolving gel interface (approximately 15 min-20 min), then at 120 V-140 V until the loading dye front approached within approximately 0.5 cm of the gel bottom (approximately 60 min-90 min depending on the protein size range of interest). Following electrophoretic separation, gels were removed from the glass plates and processed for either in-gel fluorescence detection or western blotting analysis.

5 In-gel fluorescence

Cells were seeded as described in section 3.2. For blocking experiments, cells were treated with afatinib at indicated concentrations and incubated for indicated duration, otherwise this step was skipped. Cells were treated with afatinib-tetrazine from DMSO stocks at indicated concentrations with final DMSO concentrations kept below 0.1 %. Cells were incubated for indicated times after which medium was removed and the cells were washed twice with 2 ml PBS (Cat. D8537, Sigma-Aldrich, St. Louis, MO, USA) and then harvested in 1 ml PBS by scraping (Cell Scrapers, Cat. 83.3951, Sarstedt, Nümbrecht, Germany). Cells were collected by centrifugation (15 min, 8.8 krcf, 4 °C) and the supernatant was removed by suction and discarded. Cell pellets were stored at −80 °C until further processing.

Cell pellets were resuspended in RIPA lysis buffer (50 mM Tris-HCl pH 8 at room temperature, 150 mM NaCl (CAS: 7647-14-5, Cat. A2942, PanReac/AppliChem/ITW Reagents, Darmstadt, Germany), 1 % Triton X-100 (CAS: 9002-93-1, Cat. T8787, Sigma-Aldrich, St. Louis, MO, USA), 0.25 % sodium deoxycholate (CAS: 302-95-4, Cat. 30970, Sigma-Aldrich, St. Louis, MO, USA), 0.1 % SDS, supplemented with cOmplete Mini Protease Inhibitor (Cat. 11836153001, Roche, Basel, Switzerland) and 1 mM sodium orthovanadate (CAS: 13721-39-6, Cat. 450243, Sigma-Aldrich, St. Louis, MO, USA)), left on ice for 30 min and centrifuged at 16 krcf and 4 °C for 15 min. The supernatant was collected, and protein concentration was measured (DC Protein Assay, Cat. #5000116, Bio-Rad, Hercules, CA, USA). Protein concentration was normalized to typically around 1 µg µl^{-1}. Cy5.5-nrTCO was added from a DMSO stock solution to a final concentration of 1 µM. Samples were spun down and put on a thermoshaker for 1 h at 37 °C, shaking at 300 rpm. Samples were spun down and loading dye (see section 4) was added. Samples were heated to 98 °C for 4 min, shaking at 300 rpm. If not loaded immediately, samples were stored at −20 °C until use for which they were reheated to 98 °C again under the same conditions as before. Proteins were separated on hand-cast SDS-PAGE gels (again, see section 4). Gels were then imaged either on a Bio-Rad ChemiDoc MP using the Cy5.5 protocol (Light: Red Epi illumination; Filter: 695/55 (Filter 2)) or an Odyssey XF in the 700 channel. For protein loading control, gels were stained using a commercial Coomassie stain (QuickBlue Protein Stain, Cat. LU001000, LubioScience GmbH, Zürich, Switzerland). Gels were covered with QuickBlue solution and shaken at room temperature for at least 30 min before imaging. Images were acquired again on either a ChemiDoc or an Odyssey XF.

6 Western blotting

Cells were seeded as described in section 3.2. After cell growth, medium was removed and the cells were washed twice with 2 ml PBS (Cat. D8537, Sigma-Aldrich, St. Louis, MO, USA) and then harvested in 1 ml PBS by scraping (Cell Scrapers, Cat. 83.3951, Sarstedt, Nümbrecht, Germany). Cells were collected by centrifugation (15 min, 8.8 krcf, 4 °C) and the supernatant was removed by suction and discarded. Cell pellets were stored at −80 °C until further processing.

Cell pellets were resuspended in RIPA lysis buffer (50 mM Tris-HCl pH 8 at room temperature, 150 mM NaCl (CAS: 7647-14-5, Cat. A2942, PanReac/AppliChem/ITW Reagents, Darmstadt, Germany), 1 % Triton X-100 (CAS: 9002-93-1, Cat. T8787, Sigma-Aldrich, St. Louis, MO, USA), 0.25 % sodium deoxycholate (CAS: 302-95-4, Cat. 30970, Sigma-Aldrich, St. Louis, MO, USA), 0.1 % SDS, supplemented with cOmplete Mini Protease Inhibitor (Cat. 11836153001, Roche, Basel, Switzerland) and 1 mM sodium orthovanadate (CAS: 13721-39-6, Cat. 450243, Sigma-Aldrich, St. Louis, MO, USA)), left on ice for 30 min and centrifuged at 16 krcf and 4 °C for 15 min. The supernatant was collected, and protein concentration was measured (DC Protein Assay, Cat. #5000116, Bio-Rad, Hercules, CA, USA). Protein concentration was normalized to typically around 1 µg µl^{-1}. Samples were combined with loading dye (see section 4), heated to 98 °C for 4 min, shaking at 300 rpm. If not loaded immediately, samples were stored at −20 °C until use for which they were reheated to 98 °C again under the same conditions as before. Proteins were separated on hand-cast SDS-PAGE gels (again, see section 4). Proteins were transferred onto nitrocellulose membranes using a semi-dry blotting apparatus (Trans-Blot Turbo, Bio-Rad, Hercules, CA, USA) and corresponding transfer packs (Mini Format, 0.2 µm Nitrocellulose, Single Application, Cat. #1704158, Bio-Rad, Hercules, CA, USA) using built-in transfer protocols (StandardSD (25 V, 1 A, 30 min) or 1.5 mm Gel (25 V, 1.3 A, 10 min)). Protein loading was assessed using the Revert 700 Total Protein Stain (TPS) kit (Cat. 926-11016, LI-COR Biotechnology, Lincoln, NE, USA) and imaged on an Odyssey XF (LI-COR Biotechnology, Lincoln, NE, USA). Membranes were then blocked with Intercept (TBS) Blocking Buffer (Cat. 927-60001, LI-COR Biotechnology, Lincoln, NE, USA) for 1 h at room temperature or overnight at 4 °C while shaking. Blots were incubated with primary antibodies (see below) diluted in TBST (20 mM Tris-HCl pH 7.6 adjusted at room temperature, 150 mM NaCl, supplemented with 0.1 % Tween 20 (CAS: 9005-64-5, Cat. 93773, Sigma-Aldrich, St. Louis, MO, USA)) overnight at 4 °C, washed three times for 5 min with 10 ml TBST, incubated with secondary antibodies (see below) for 1 h at room temperature, and washed three times for 5 min with 10 ml TBST. Membrane manipulation steps were typically performed with membranes placed in transparent polypropylene or polycarbonate Jass card boxes (standard Swiss playing card boxes). The protein of interest was preferentially detected using fluorescence at 800 nm. Specific antibody dilutions and additional experimental details are provided in figure legends of full blot images. Images were acquired using a LI-COR Odyssey XF and processed, including integration, using LI-COR EmpiriaStudio (currently at version 3.2) software.

6.1 Primary antibodies

anti-EGFR Anti-EGFR antibody [EP38Y]; Abcam Cat# ab52894, RRID:AB_869579, Host/Isotype: Rabbit/IgG (monoclonal); 1:1 000 dilution in TBST

6.2 Secondary antibodies

R800 IRDye 800CW Goat anti-Rabbit IgG Secondary Antibody, LICORbio Cat# 926-32211, RRID:AB_621843; 1:10 000 dilution in TBST

7 Cell viability

7.1 Resazurin assay (single treatment)

In clear, flat bottom 96-well plates (Cat. 83.3924, Sarstedt, Nümbrecht, Germany), 10 000 cells were seeded in 100 µl of complete medium (see 3.1) and grown for 24 h. Typically, outer wells of the plates were filled with medium but not used for experiments to minimize edge effects and provide evaporation protection. A separate 96-well plate was preloaded with complete medium. Compounds from DMSO stock were added to first rows of the plate and serially diluted (typically in 5-fold dilutions for a total of 8 steps) on an ASSIST PLUS (Integra, Zizers, Switzerland) pipetting robot. For MMAE and MMAE-TCO, tips were automatically changed after every dilution step or serial dilutions were started from concentrations no higher than 100 nM due to otherwise observed carryover. Medium from cell growth plates was removed and replaced with 100 µl of pre-treated medium. Each condition was tested as technical triplicates on the same plate. Cells were grown for 72 h under standard conditions after which 20 µl of a commercial resazurin solution (CellTiter-Blue, Cat. G8081, Promega, Madison, WI, USA) were added and cells were further incubated for 2 h. Fluorescence was measured in *Fluorescence Top Reading* mode on a Spark microplate reader (Tecan, Männedorf, Switzerland) with λ_{ex} = 560 nm and λ_{em} = 590 nm. Data was background subtracted (background = 100 µl medium + 20 µl resazurin) and normalized to full viability wells. A three- or four-parameter log-logistic function was fit using the drc package in R (R version 4.4.0 (2024-04-24))[5,6] to calculate IC_{50} values. If not noted otherwise, bars represent means, and error bars represent standard error of the mean (SEM) as calculated by tidyverse's stat summary function. All data points in graphs represent independent biological experiments.

7.2 Resazurin assay (co-incubation)

Cells were seeded and grown as described above. In a Falcon tube, indicated tetrazine was diluted in growth medium. Of this solution, 50 µl were transferred to cells for co-incubation experiments, leaving out wells for full viability control. Medium in full viability control wells was replaced by fresh medium. Serial dilutions of caged drugs were prepared as indicated above. To the tetrazine treated medium, 50 µl of prepared serial dilutions were added. Indicated concentrations for tetrazines and caged drugs correspond to the concentrations in these final 100 µl of medium. The assay was completed following the regular resazurin assay procedure as described above.

7.3 Resazurin assay (pre-targeting)

Cells were seeded and grown as described above. In a Falcon tube, indicated tetrazine was diluted in growth medium. Of this solution, 50 µl were transferred to cells for pre-targeting experiments, leaving out wells for full viability control. Medium in full viability control wells was replaced by fresh medium. Cells were then incubated/pre-targeted for the indicated time. All wells, including full viability controls, were then washed three times with 100 µl medium. Washes were performed roughly 20 min apart. After the medium of the final wash was removed, 50 µl of untreated medium was added. Serial dilutions of caged drugs were prepared as indicated above. 50 µl of prepared serial dilutions were added to the cells. Indicated concentrations of caged drugs correspond to the concentrations in these final 100 µl of medium. The assay was completed following the regular resazurin assay procedure.

8 Software

Data analysis and visualization were performed in R[6] using the `tidyverse`[7] collection for data processing and `ggplot2`[8] for plotting. Figures were enhanced using `cowplot`[9] for consistent themes and `ggbeeswarm`[10] for data point positioning. Dose-response curves were fitted using 3- or 4-parameter log-logistic models implemented via `LL.3()` and `LL.4()` functions from the `drc` package,[5] from which IC_{50} values were calculated. The 3-parameter model (with lower asymptote constrained to zero) was used when the 4-parameter model failed to converge to a reasonable lower asymptote, typically when insufficient data points were available at high compound concentrations. Color schemes were based on or inspired by Paul Tol's scientific color palettes[11] with additional support from `RColorBrewer`.[12] Figure text was set in Fira Sans (Mozilla Foundation/Telefónica S.A.).

This supporting information was typeset in LaTeX (XeLaTeX) using KOMA-Script and numerous packages. Gratitude to Donald Knuth for TeX, and to Markus Kohm for KOMA-Script (with apologies for any typographical transgressions). Special thanks to the entire LaTeX community for maintaining this exceptional typesetting environment.

The main body text and supporting information were edited using artificial intelligence (AI) language models, including the large language model (LLM) Claude by Anthropic and the writing assistant DeepL Write, for grammar, clarity, and style improvements. These AI tools were used solely for language refinement and did not generate any scientific content, analysis, or conclusions. All AI-suggested changes were manually reviewed and verified for scientific accuracy. The paper also incorporates valuable feedback from human proofreaders Basilius, Angel, Seemon, and Marco.

9 Supporting figures and tables

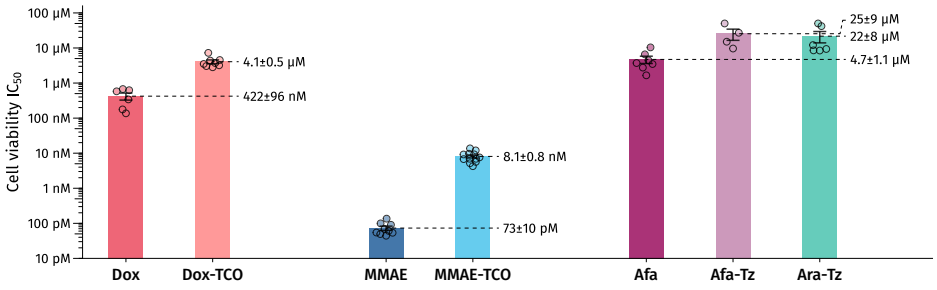


Figure S2: Complete cell viability data (IC_{50} values) of single agent treatments in A431 cells. Cell viability data from Fig. 3A(iii) is included: Dox, Dox-TCO, MMAE, and MMAE-TCO. Additional cell viability data shown for Afa, Afa-Tz, and Ara-Tz as single agents. Cell viability assays were performed using resazurin at 72 h. Data points represent independent biological experiments, bars and error bars indicate mean and SEM, respectively. Individual data points and summary statistics are provided in Tab. S1 and Tab. S7.

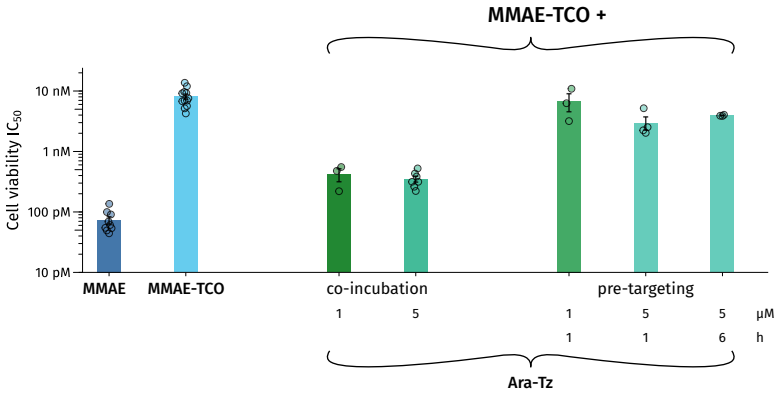


Figure S3: Complete cell viability data (IC_{50} values) using the non-binding control Ara-Tz in A431 cells. Cell viability data from Fig. 3A(iii) and Fig. 4B is included: MMAE, MMAE-TCO, and pre-targeting with Ara-Tz at different time points and concentrations. Additional cell viability data shown for co-incubation of Ara-Tz at 1 µM and 5 µM with MMAE-TCO. Cell viability assays were performed using resazurin at 72 h. Data points represent independent biological experiments, bars and error bars indicate mean and SEM, respectively. Individual data points and summary statistics are provided in Tab. S1 and Tab. S6.

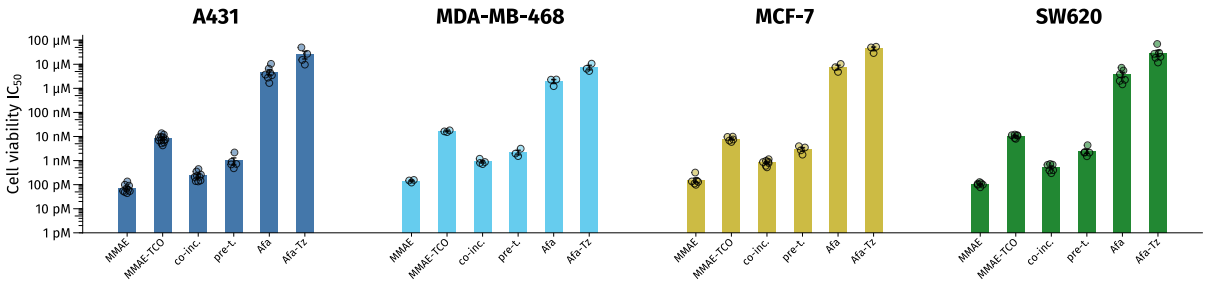


Figure S4: Complete cell viability data (IC_{50} values) for cell lines A431, MDA-MB-468, MCF-7, and SW620 as seen in Fig. 5. Cell viability data from Fig. 5C is included: MMAE, MMAE-TCO, and pre-targeting (pre-t.) with Afa-Tz at 5 µM for 1 h followed by MMAE-TCO. Additional cell viability data shown for co-incubation (co-inc.) of Afa-Tz at 5 µM with MMAE-TCO, as well as Afa and Afa-Tz as single agents. Cell viability assays were performed using resazurin at 72 h. Data points represent independent biological experiments, bars and error bars indicate mean and SEM, respectively. Individual data points and summary statistics are provided in Tab. S7.

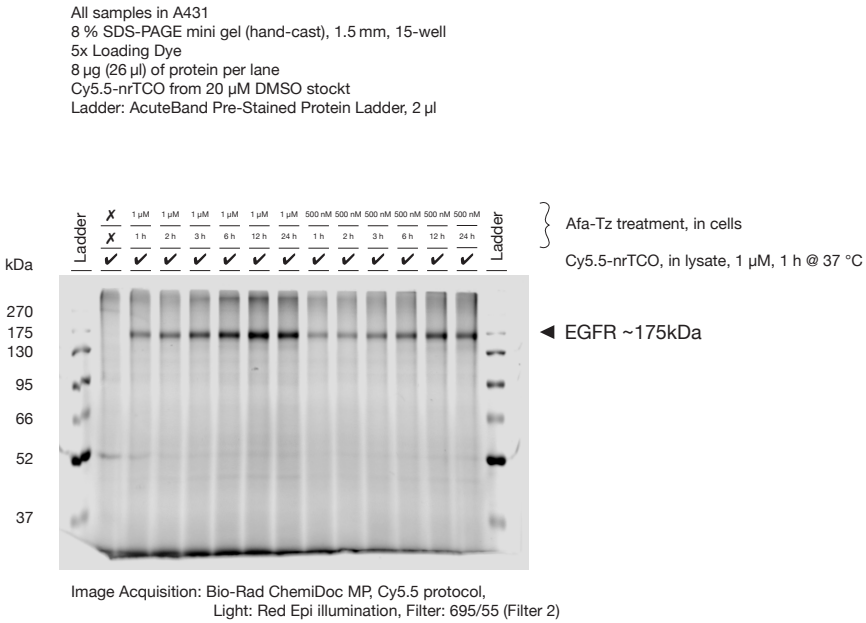


Figure S5: Full in-gel fluorescence gel image of Fig. 2D.

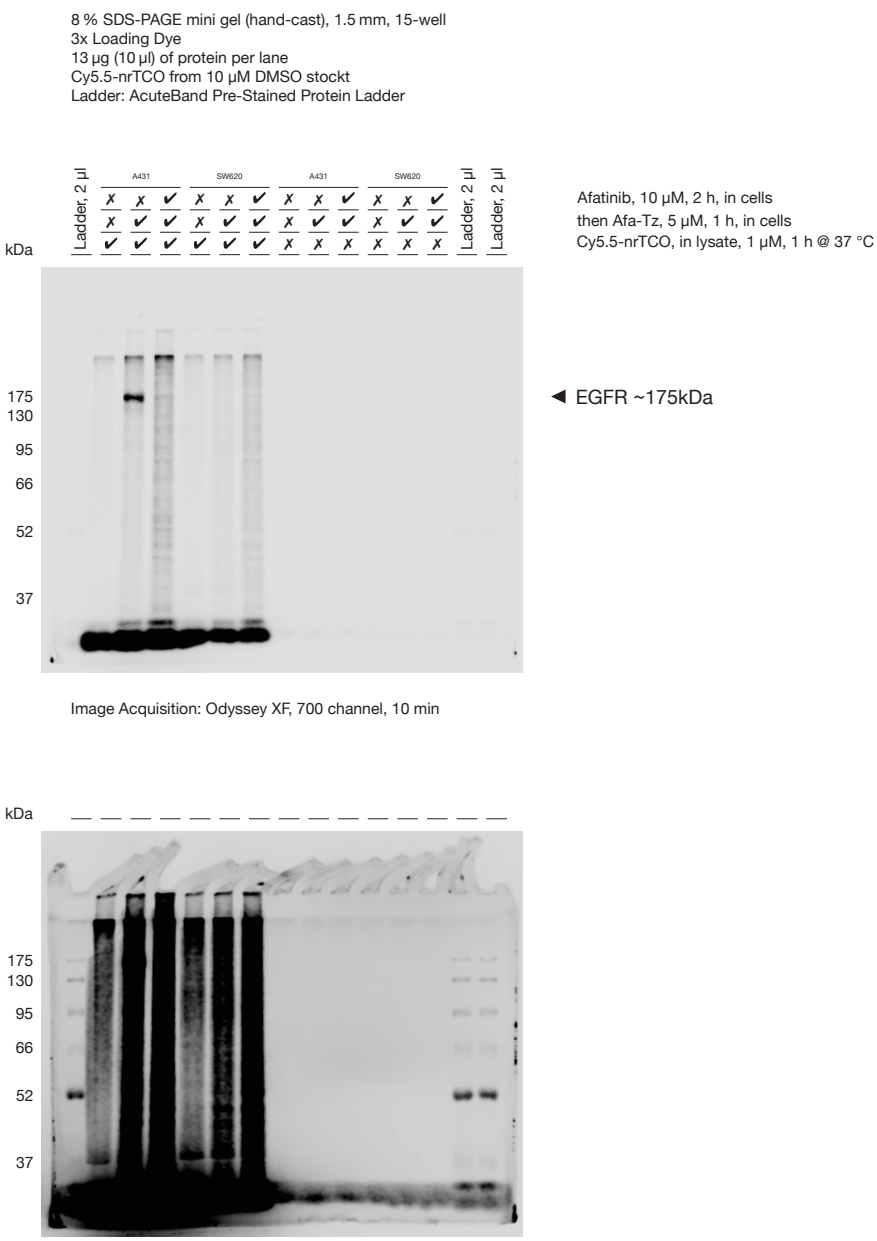


Figure S6: Full in-gel fluorescence gel image of Fig. 2E.

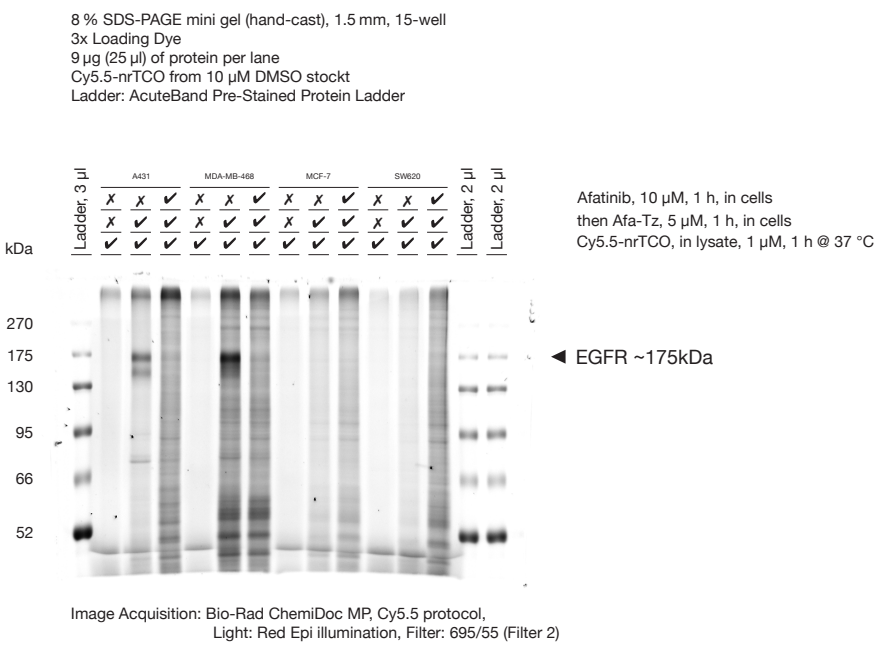


Figure S7: Full in-gel fluorescence gel image of Fig. 5B.

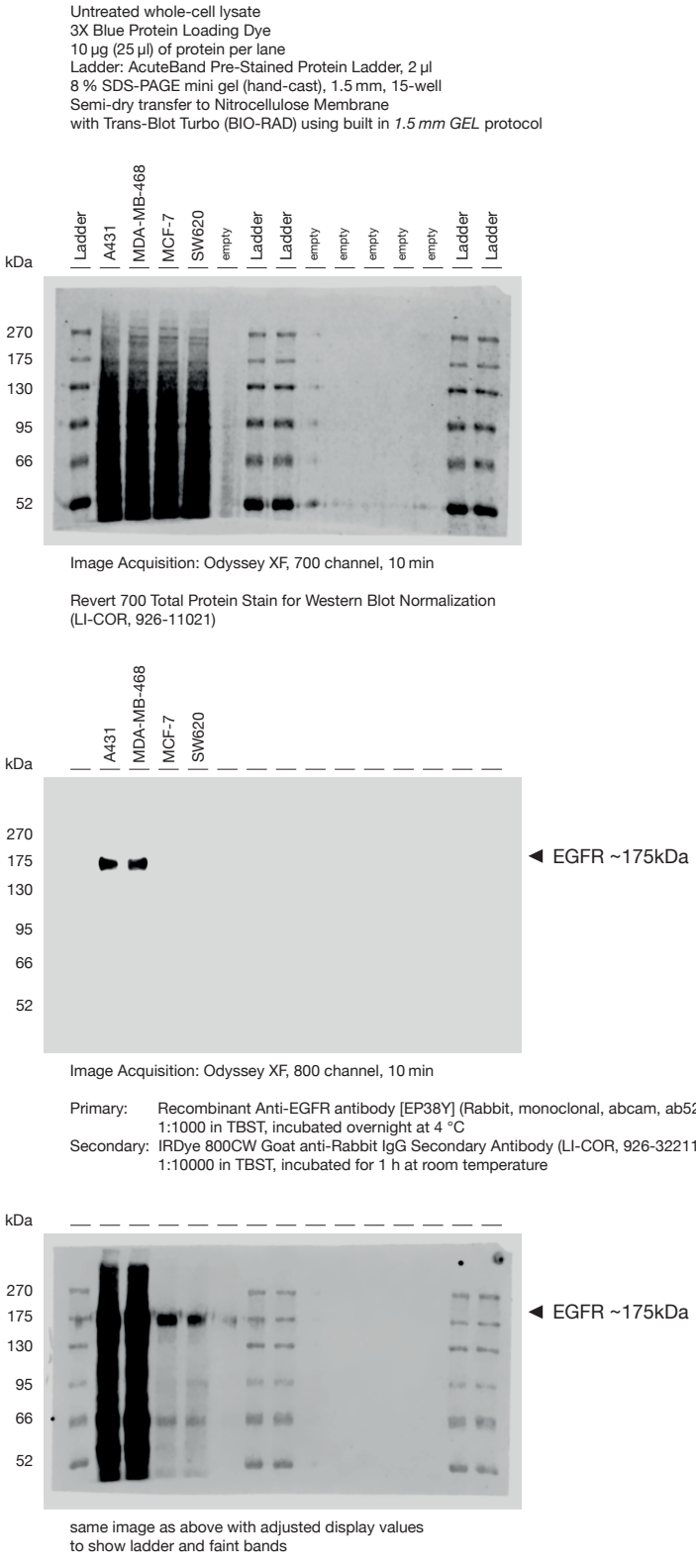


Figure S8: Full membrane image of Fig. 5A.

Table S1: Individual data points and summary statistics used to create Fig. 3A(iii). Bold rows show mean IC_{50} ± SEM in pM calculated from all independent experiments listed below each condition.

Condition	IC_{50} / pM	Experiment
Dox	420 000 ± 100 000	
Dox	580 000	1
Dox	342 000	2
Dox	177 000	3
Dox	625 000	4
Dox	674 000	5
Dox	137 000	6
Dox-TCO	4 100 000 ± 500 000	
Dox-TCO	3 210 000	1
Dox-TCO	4 540 000	2
Dox-TCO	4 370 000	3
Dox-TCO	7 260 000	4
Dox-TCO	3 050 000	5
Dox-TCO	3 720 000	6
Dox-TCO	2 820 000	7
Dox-TCO	3 510 000	8
MMAE	73 ± 10	
MMAE	90.8	1
MMAE	69.2	2
MMAE	99.5	3
MMAE	44.3	4
MMAE	54.6	5
MMAE	53.8	6
MMAE	49.4	7
MMAE	60.7	8
MMAE	136	9
MMAE-TCO	8 100 ± 800	
MMAE-TCO	5 650	1
MMAE-TCO	6 930	2
MMAE-TCO	7 590	3
MMAE-TCO	4 260	4
MMAE-TCO	6 780	5
MMAE-TCO	9 260	6
MMAE-TCO	5 170	7
MMAE-TCO	6 950	8
MMAE-TCO	9 640	9
MMAE-TCO	9 190	10
MMAE-TCO	12 000	11
MMAE-TCO	13 700	12

Table S2: Individual data points and summary statistics used to create Fig. 3B(i). Bold rows show mean IC_{50} ± SEM in pM calculated from all independent experiments listed below each condition.

Condition	IC_{50} / pM	Experiment
Co-incubation 1 µM Afa-Tz + Dox-TCO	**1 500 000 ± 300 000**	
Co-incubation 1 µM Afa-Tz + Dox-TCO	1 600 000	1
Co-incubation 1 µM Afa-Tz + Dox-TCO	1 330 000	2
Co-incubation 1 µM Afa-Tz + Dox-TCO	1 540 000	3
Co-incubation 1 µM Afa-Tz + Dox-TCO	2 660 000	4
Co-incubation 1 µM Afa-Tz + Dox-TCO	579 000	5
Co-incubation 100 nM Afa-Tz + Dox-TCO	**3 400 000 ± 600 000**	
Co-incubation 100 nM Afa-Tz + Dox-TCO	5 060 000	1
Co-incubation 100 nM Afa-Tz + Dox-TCO	2 700 000	2
Co-incubation 100 nM Afa-Tz + Dox-TCO	2 740 000	3
Co-incubation 100 nM Afa-Tz + Dox-TCO	2 980 000	4
Co-incubation 5 µM Afa-Tz + Dox-TCO	**570 000 ± 160 000**	
Co-incubation 5 µM Afa-Tz + Dox-TCO	448 000	1
Co-incubation 5 µM Afa-Tz + Dox-TCO	451 000	2
Co-incubation 5 µM Afa-Tz + Dox-TCO	1 060 000	3
Co-incubation 5 µM Afa-Tz + Dox-TCO	335 000	4
Co-incubation 500 nM Afa-Tz + Dox-TCO	**2 000 000 ± 500 000**	
Co-incubation 500 nM Afa-Tz + Dox-TCO	2 880 000	1
Co-incubation 500 nM Afa-Tz + Dox-TCO	1 110 000	2
Co-incubation 500 nM Afa-Tz + Dox-TCO	1 180 000	3
Co-incubation 500 nM Afa-Tz + Dox-TCO	2 840 000	4
Dox	**420 000 ± 100 000**	
Dox	580 000	1
Dox	342 000	2
Dox	177 000	3
Dox	625 000	4
Dox	674 000	5
Dox	137 000	6
Dox-TCO	**4 100 000 ± 500 000**	
Dox-TCO	3 210 000	1
Dox-TCO	4 540 000	2
Dox-TCO	4 370 000	3
Dox-TCO	7 260 000	4
Dox-TCO	3 050 000	5
Dox-TCO	3 720 000	6
Dox-TCO	2 820 000	7
Dox-TCO	3 510 000	8

Table S3: Individual data points and summary statistics used to create Fig. 3B(ii). Bold rows show mean IC_{50} ± SEM in pM calculated from all independent experiments listed below each condition.

Condition	IC_{50} / pM			Experiment
Co-incubation 1 µM Afa-Tz + MMAE-TCO	**560**	**±**	**170**	
Co-incubation 1 µM Afa-Tz + MMAE-TCO	464			1
Co-incubation 1 µM Afa-Tz + MMAE-TCO	249			2
Co-incubation 1 µM Afa-Tz + MMAE-TCO	1 060			3
Co-incubation 1 µM Afa-Tz + MMAE-TCO	484			4
Co-incubation 10 nM Afa-Tz + MMAE-TCO	**3 300**	**±**	**700**	
Co-incubation 10 nM Afa-Tz + MMAE-TCO	3 710			1
Co-incubation 10 nM Afa-Tz + MMAE-TCO	1 910			2
Co-incubation 10 nM Afa-Tz + MMAE-TCO	2 500			3
Co-incubation 10 nM Afa-Tz + MMAE-TCO	5 030			4
Co-incubation 100 nM Afa-Tz + MMAE-TCO	**380**	**±**	**70**	
Co-incubation 100 nM Afa-Tz + MMAE-TCO	312			1
Co-incubation 100 nM Afa-Tz + MMAE-TCO	356			2
Co-incubation 100 nM Afa-Tz + MMAE-TCO	571			3
Co-incubation 100 nM Afa-Tz + MMAE-TCO	283			4
Co-incubation 5 µM Afa-Tz + MMAE-TCO	**340**	**±**	**60**	
Co-incubation 5 µM Afa-Tz + MMAE-TCO	234			1
Co-incubation 5 µM Afa-Tz + MMAE-TCO	352			2
Co-incubation 5 µM Afa-Tz + MMAE-TCO	445			3
Co-incubation 50 nM Afa-Tz + MMAE-TCO	**1 200**	**±**	**300**	
Co-incubation 50 nM Afa-Tz + MMAE-TCO	870			1
Co-incubation 50 nM Afa-Tz + MMAE-TCO	547			2
Co-incubation 50 nM Afa-Tz + MMAE-TCO	1 820			3
Co-incubation 50 nM Afa-Tz + MMAE-TCO	769			4
Co-incubation 50 nM Afa-Tz + MMAE-TCO	1 140			5
Co-incubation 50 nM Afa-Tz + MMAE-TCO	2 270			6
Co-incubation 500 nM Afa-Tz + MMAE-TCO	**500**	**±**	**200**	
Co-incubation 500 nM Afa-Tz + MMAE-TCO	223			1
Co-incubation 500 nM Afa-Tz + MMAE-TCO	187			2
Co-incubation 500 nM Afa-Tz + MMAE-TCO	464			3
Co-incubation 500 nM Afa-Tz + MMAE-TCO	249			4
Co-incubation 500 nM Afa-Tz + MMAE-TCO	544			5
Co-incubation 500 nM Afa-Tz + MMAE-TCO	1 500			6
MMAE	**73**	**±**	**10**	
MMAE	90.8			1
MMAE	69.2			2
MMAE	99.5			3
MMAE	44.3			4
MMAE	54.6			5
MMAE	53.8			6
MMAE	49.4			7
MMAE	60.7			8
MMAE	136			9
MMAE-TCO	**8 100**	**±**	**800**	
MMAE-TCO	5 650			1
MMAE-TCO	6 930			2
MMAE-TCO	7 590			3
MMAE-TCO	4 260			4
MMAE-TCO	6 780			5
MMAE-TCO	9 260			6
MMAE-TCO	5 170			7
MMAE-TCO	6 950			8
MMAE-TCO	9 640			9
MMAE-TCO	9 190			10
MMAE-TCO	12 000			11
MMAE-TCO	13 700			12

Table S4: Individual data points and summary statistics used to create Fig. 4A(i). Bold rows show mean IC_{50} ± SEM in pM calculated from all independent experiments listed below each condition.

Condition	IC_{50} / pM	Experiment
Pre-targeting 1 µM/1h Afa-Tz + Dox-TCO	5 800 000 ± 1 300 000	
Pre-targeting 1 µM/1h Afa-Tz + Dox-TCO	7 040 000	1
Pre-targeting 1 µM/1h Afa-Tz + Dox-TCO	7 210 000	2
Pre-targeting 1 µM/1h Afa-Tz + Dox-TCO	3 090 000	3
Pre-targeting 1 µM/6h Afa-Tz + Dox-TCO	4 100 000 ± 1 000 000	
Pre-targeting 1 µM/6h Afa-Tz + Dox-TCO	7 140 000	1
Pre-targeting 1 µM/6h Afa-Tz + Dox-TCO	3 570 000	2
Pre-targeting 1 µM/6h Afa-Tz + Dox-TCO	2 740 000	3
Pre-targeting 1 µM/6h Afa-Tz + Dox-TCO	2 920 000	4
Pre-targeting 5 µM/1h Afa-Tz + Dox-TCO	4 800 000 ± 1 000 000	
Pre-targeting 5 µM/1h Afa-Tz + Dox-TCO	6 190 000	1
Pre-targeting 5 µM/1h Afa-Tz + Dox-TCO	6 990 000	2
Pre-targeting 5 µM/1h Afa-Tz + Dox-TCO	3 470 000	3
Pre-targeting 5 µM/1h Afa-Tz + Dox-TCO	2 630 000	4
Pre-targeting 5 µM/6h Afa-Tz + Dox-TCO	3 600 000 ± 800 000	
Pre-targeting 5 µM/6h Afa-Tz + Dox-TCO	2 640 000	1
Pre-targeting 5 µM/6h Afa-Tz + Dox-TCO	6 640 000	2
Pre-targeting 5 µM/6h Afa-Tz + Dox-TCO	3 260 000	3
Pre-targeting 5 µM/6h Afa-Tz + Dox-TCO	2 460 000	4
Pre-targeting 5 µM/6h Afa-Tz + Dox-TCO	2 860 000	5

Table S5: Individual data points and summary statistics used to create Fig. 4A(ii). Bold rows show mean IC_{50} ± SEM in pM calculated from all independent experiments listed below each condition.

Condition	IC_{50} / pM			Experiment
Pre-targeting 0.2 µM/1h Afa-Tz + MMAE-TCO	**6 700**	**±**	**1 500**	
Pre-targeting 0.2 µM/1h Afa-Tz + MMAE-TCO	9 400			1
Pre-targeting 0.2 µM/1h Afa-Tz + MMAE-TCO	6 340			2
Pre-targeting 0.2 µM/1h Afa-Tz + MMAE-TCO	4 380			3
Pre-targeting 0.2 µM/3h Afa-Tz + MMAE-TCO	**2 400**	**±**	**400**	
Pre-targeting 0.2 µM/3h Afa-Tz + MMAE-TCO	2 750			1
Pre-targeting 0.2 µM/3h Afa-Tz + MMAE-TCO	1 720			2
Pre-targeting 0.2 µM/3h Afa-Tz + MMAE-TCO	1 720			3
Pre-targeting 0.2 µM/3h Afa-Tz + MMAE-TCO	3 220			4
Pre-targeting 0.2 µM/6h Afa-Tz + MMAE-TCO	**1 600**	**±**	**200**	
Pre-targeting 0.2 µM/6h Afa-Tz + MMAE-TCO	2 090			1
Pre-targeting 0.2 µM/6h Afa-Tz + MMAE-TCO	1 340			2
Pre-targeting 0.2 µM/6h Afa-Tz + MMAE-TCO	1 340			3
Pre-targeting 1 µM/1h Afa-Tz + MMAE-TCO	**4 000**	**±**	**1 000**	
Pre-targeting 1 µM/1h Afa-Tz + MMAE-TCO	3 180			1
Pre-targeting 1 µM/1h Afa-Tz + MMAE-TCO	1 690			2
Pre-targeting 1 µM/1h Afa-Tz + MMAE-TCO	4 490			3
Pre-targeting 1 µM/1h Afa-Tz + MMAE-TCO	6 620			4
Pre-targeting 1 µM/3h Afa-Tz + MMAE-TCO	**1 300**	**±**	**300**	
Pre-targeting 1 µM/3h Afa-Tz + MMAE-TCO	985			1
Pre-targeting 1 µM/3h Afa-Tz + MMAE-TCO	787			2
Pre-targeting 1 µM/3h Afa-Tz + MMAE-TCO	787			3
Pre-targeting 1 µM/3h Afa-Tz + MMAE-TCO	2 590			4
Pre-targeting 1 µM/3h Afa-Tz + MMAE-TCO	1 320			5
Pre-targeting 1 µM/6h Afa-Tz + MMAE-TCO	**520**	**±**	**70**	
Pre-targeting 1 µM/6h Afa-Tz + MMAE-TCO	662			1
Pre-targeting 1 µM/6h Afa-Tz + MMAE-TCO	448			2
Pre-targeting 1 µM/6h Afa-Tz + MMAE-TCO	448			3
Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	**1 000**	**±**	**300**	
Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	885			1
Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	478			2
Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	692			3
Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	724			4
Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	2 170			5
Pre-targeting 5 µM/3h Afa-Tz + MMAE-TCO	**820**	**±**	**90**	
Pre-targeting 5 µM/3h Afa-Tz + MMAE-TCO	768			1
Pre-targeting 5 µM/3h Afa-Tz + MMAE-TCO	1 090			2
Pre-targeting 5 µM/3h Afa-Tz + MMAE-TCO	748			3
Pre-targeting 5 µM/3h Afa-Tz + MMAE-TCO	670			4
Pre-targeting 5 µM/6h Afa-Tz + MMAE-TCO	**280**	**±**	**50**	
Pre-targeting 5 µM/6h Afa-Tz + MMAE-TCO	318			1
Pre-targeting 5 µM/6h Afa-Tz + MMAE-TCO	179			2
Pre-targeting 5 µM/6h Afa-Tz + MMAE-TCO	355			3

Table S6: Individual data points and summary statistics used to create Fig. 4B and Fig. S3. Bold rows show mean IC_{50} ± SEM in pM calculated from all independent experiments listed below each condition.

Condition	IC_{50} / pM			Experiment
Co-incubation 1 µM Ara-Tz + MMAE-TCO	420	±	100	
Co-incubation 1 µM Ara-Tz + MMAE-TCO	553			1
Co-incubation 1 µM Ara-Tz + MMAE-TCO	478			2
Co-incubation 1 µM Ara-Tz + MMAE-TCO	220			3
Co-incubation 5 µM Ara-Tz + MMAE-TCO	350	±	40	
Co-incubation 5 µM Ara-Tz + MMAE-TCO	385			1
Co-incubation 5 µM Ara-Tz + MMAE-TCO	261			2
Co-incubation 5 µM Ara-Tz + MMAE-TCO	315			3
Co-incubation 5 µM Ara-Tz + MMAE-TCO	524			4
Co-incubation 5 µM Ara-Tz + MMAE-TCO	222			5
Co-incubation 5 µM Ara-Tz + MMAE-TCO	432			6
Co-incubation 5 µM Ara-Tz + MMAE-TCO	315			7
Pre-targeting 1 µM/1h Ara-Tz + MMAE-TCO	7 000	±	2 000	
Pre-targeting 1 µM/1h Ara-Tz + MMAE-TCO	6 280			1
Pre-targeting 1 µM/1h Ara-Tz + MMAE-TCO	3 180			2
Pre-targeting 1 µM/1h Ara-Tz + MMAE-TCO	10 900			3
Pre-targeting 5 µM/1h Ara-Tz + MMAE-TCO	3 000	±	700	
Pre-targeting 5 µM/1h Ara-Tz + MMAE-TCO	5 190			1
Pre-targeting 5 µM/1h Ara-Tz + MMAE-TCO	2 030			2
Pre-targeting 5 µM/1h Ara-Tz + MMAE-TCO	2 240			3
Pre-targeting 5 µM/1h Ara-Tz + MMAE-TCO	2 520			4
Pre-targeting 5 µM/6h Ara-Tz + MMAE-TCO	3 940	±	60	
Pre-targeting 5 µM/6h Ara-Tz + MMAE-TCO	3 900			1
Pre-targeting 5 µM/6h Ara-Tz + MMAE-TCO	3 870			2
Pre-targeting 5 µM/6h Ara-Tz + MMAE-TCO	4 050			3

Table S7: Individual data points and summary statistics used to create Fig. 5C, Fig. S2, and Fig. S4. Bold rows show mean IC_{50} ± SEM in pM calculated from all independent experiments listed below each condition.

Condition	IC_{50} / pM	Experiment
A431, MMAE	**73 ± 10**	
A431, MMAE	90.8	1
A431, MMAE	69.2	2
A431, MMAE	99.5	3
A431, MMAE	44.3	4
A431, MMAE	53.8	5
A431, MMAE	54.6	6
A431, MMAE	49.4	7
A431, MMAE	60.7	8
A431, MMAE	136	9
A431, MMAE-TCO	**8 100 ± 800**	
A431, MMAE-TCO	5 650	1
A431, MMAE-TCO	6 930	2
A431, MMAE-TCO	7 590	3
A431, MMAE-TCO	4 260	4
A431, MMAE-TCO	6 780	5
A431, MMAE-TCO	5 170	6
A431, MMAE-TCO	9 260	7
A431, MMAE-TCO	6 950	8
A431, MMAE-TCO	9 640	9
A431, MMAE-TCO	9 190	10
A431, MMAE-TCO	12 000	11
A431, MMAE-TCO	13 700	12
A431, Co-incubation 5 µM Afa-Tz + MMAE-TCO	**240 ± 30**	
A431, Co-incubation 5 µM Afa-Tz + MMAE-TCO	262	1
A431, Co-incubation 5 µM Afa-Tz + MMAE-TCO	208	2
A431, Co-incubation 5 µM Afa-Tz + MMAE-TCO	136	3
A431, Co-incubation 5 µM Afa-Tz + MMAE-TCO	213	4
A431, Co-incubation 5 µM Afa-Tz + MMAE-TCO	140	5
A431, Co-incubation 5 µM Afa-Tz + MMAE-TCO	149	6
A431, Co-incubation 5 µM Afa-Tz + MMAE-TCO	234	7
A431, Co-incubation 5 µM Afa-Tz + MMAE-TCO	352	8
A431, Co-incubation 5 µM Afa-Tz + MMAE-TCO	445	9
A431, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	**1 000 ± 300**	
A431, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	885	1
A431, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	478	2
A431, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	692	3
A431, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	724	4
A431, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	2 170	5
A431, Afatinib	**4 700 000 ± 1 100 000**	
A431, Afatinib	10 400 000	1
A431, Afatinib	6 580 000	2
A431, Afatinib	4 430 000	3
A431, Afatinib	1 660 000	4
A431, Afatinib	2 760 000	5
A431, Afatinib	3 720 000	6
A431, Afatinib	3 490 000	7
A431, Afa-Tz	**25 000 000 ± 9 000 000**	
A431, Afa-Tz	9 650 000	1
A431, Afa-Tz	27 200 000	2
A431, Afa-Tz	50 000 000	3
A431, Afa-Tz	15 100 000	4

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Table S.7 (continued)

Condition	IC_{50} / pM	Experiment
continued from last page		
MDA-MB-468, MMAE	143	
MDA-MB-468, MMAE	122	1
MDA-MB-468, MMAE	149	2
MDA-MB-468, MMAE	157	3
MDA-MB-468, MMAE-TCO	16 500	
MDA-MB-468, MMAE-TCO	16 300	1
MDA-MB-468, MMAE-TCO	15 200	2
MDA-MB-468, MMAE-TCO	18 000	3
MDA-MB-468, Co-incubation 5 µM Afa-Tz + MMAE-TCO	890	
MDA-MB-468, Co-incubation 5 µM Afa-Tz + MMAE-TCO	1 190	1
MDA-MB-468, Co-incubation 5 µM Afa-Tz + MMAE-TCO	800	2
MDA-MB-468, Co-incubation 5 µM Afa-Tz + MMAE-TCO	714	3
MDA-MB-468, Co-incubation 5 µM Afa-Tz + MMAE-TCO	871	4
MDA-MB-468, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	2 200	
MDA-MB-468, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	3 130	1
MDA-MB-468, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	1 530	2
MDA-MB-468, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	1 950	3
MDA-MB-468, Afatinib	2 000 000	
MDA-MB-468, Afatinib	1 230 000	1
MDA-MB-468, Afatinib	2 330 000	2
MDA-MB-468, Afatinib	2 290 000	3
MDA-MB-468, Afa-Tz	7 400 000	
MDA-MB-468, Afa-Tz	6 470 000	1
MDA-MB-468, Afa-Tz	5 240 000	2
MDA-MB-468, Afa-Tz	10 600 000	3

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Table S7 (continued)

Condition	IC_{50} / pM			Experiment
MCF-7, MMAE	160	±	30	
MCF-7, MMAE	111			1
MCF-7, MMAE	130			2
MCF-7, MMAE	318			3
MCF-7, MMAE	146			4
MCF-7, MMAE	98.6			5
MCF-7, MMAE	136			6
MCF-7, MMAE-TCO	7 900	±	800	
MCF-7, MMAE-TCO	6 780			1
MCF-7, MMAE-TCO	5 920			2
MCF-7, MMAE-TCO	9 880			3
MCF-7, MMAE-TCO	7 510			4
MCF-7, MMAE-TCO	9 620			5
MCF-7, Co-incubation 5 µM Afa-Tz + MMAE-TCO	830	±	80	
MCF-7, Co-incubation 5 µM Afa-Tz + MMAE-TCO	894			1
MCF-7, Co-incubation 5 µM Afa-Tz + MMAE-TCO	869			2
MCF-7, Co-incubation 5 µM Afa-Tz + MMAE-TCO	524			3
MCF-7, Co-incubation 5 µM Afa-Tz + MMAE-TCO	1 140			4
MCF-7, Co-incubation 5 µM Afa-Tz + MMAE-TCO	606			5
MCF-7, Co-incubation 5 µM Afa-Tz + MMAE-TCO	790			6
MCF-7, Co-incubation 5 µM Afa-Tz + MMAE-TCO	978			7
MCF-7, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	3 000	±	500	
MCF-7, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	3 950			1
MCF-7, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	1 760			2
MCF-7, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	3 500			3
MCF-7, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	2 670			4
MCF-7, Afatinib	7 600 000	±	1 600 000	
MCF-7, Afatinib	7 560 000			1
MCF-7, Afatinib	10 300 000			2
MCF-7, Afatinib	4 810 000			3
MCF-7, Afa-Tz	44 000 000	±	8 000 000	
MCF-7, Afa-Tz	53 400 000			1
MCF-7, Afa-Tz	29 200 000			2
MCF-7, Afa-Tz	50 000 000			3

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Table S7 (continued)

Condition	IC_{50} / pM	Experiment
continued from last page		
SW620, MMAE	105 ± 7	
SW620, MMAE	98.6	1
SW620, MMAE	115	2
SW620, MMAE	77.6	3
SW620, MMAE	128	4
SW620, MMAE	99.9	5
SW620, MMAE	109	6
SW620, MMAE-TCO	10 300 ± 700	
SW620, MMAE-TCO	7 980	1
SW620, MMAE-TCO	10 700	2
SW620, MMAE-TCO	8 630	3
SW620, MMAE-TCO	11 400	4
SW620, MMAE-TCO	11 300	5
SW620, MMAE-TCO	12 000	6
SW620, Co-incubation 5 µM Afa-Tz + MMAE-TCO	520 ± 70	
SW620, Co-incubation 5 µM Afa-Tz + MMAE-TCO	299	1
SW620, Co-incubation 5 µM Afa-Tz + MMAE-TCO	400	2
SW620, Co-incubation 5 µM Afa-Tz + MMAE-TCO	387	3
SW620, Co-incubation 5 µM Afa-Tz + MMAE-TCO	661	4
SW620, Co-incubation 5 µM Afa-Tz + MMAE-TCO	724	5
SW620, Co-incubation 5 µM Afa-Tz + MMAE-TCO	674	6
SW620, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	2 500 ± 500	
SW620, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	4 350	1
SW620, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	2 120	2
SW620, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	1 510	3
SW620, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	2 140	4
SW620, Pre-targeting 5 µM/1h Afa-Tz + MMAE-TCO	2 260	5
SW620, Afatinib	3 700 000 ± 900 000	
SW620, Afatinib	3 890 000	1
SW620, Afatinib	7 170 000	2
SW620, Afatinib	5 480 000	3
SW620, Afatinib	1 460 000	4
SW620, Afatinib	2 040 000	5
SW620, Afatinib	2 260 000	6
SW620, Afa-Tz	30 000 000 ± 8 000 000	
SW620, Afa-Tz	20 300 000	1
SW620, Afa-Tz	29 900 000	2
SW620, Afa-Tz	27 100 000	3
SW620, Afa-Tz	11 800 000	4
SW620, Afa-Tz	18 500 000	5
SW620, Afa-Tz	69 600 000	6

10 NMR Spectra

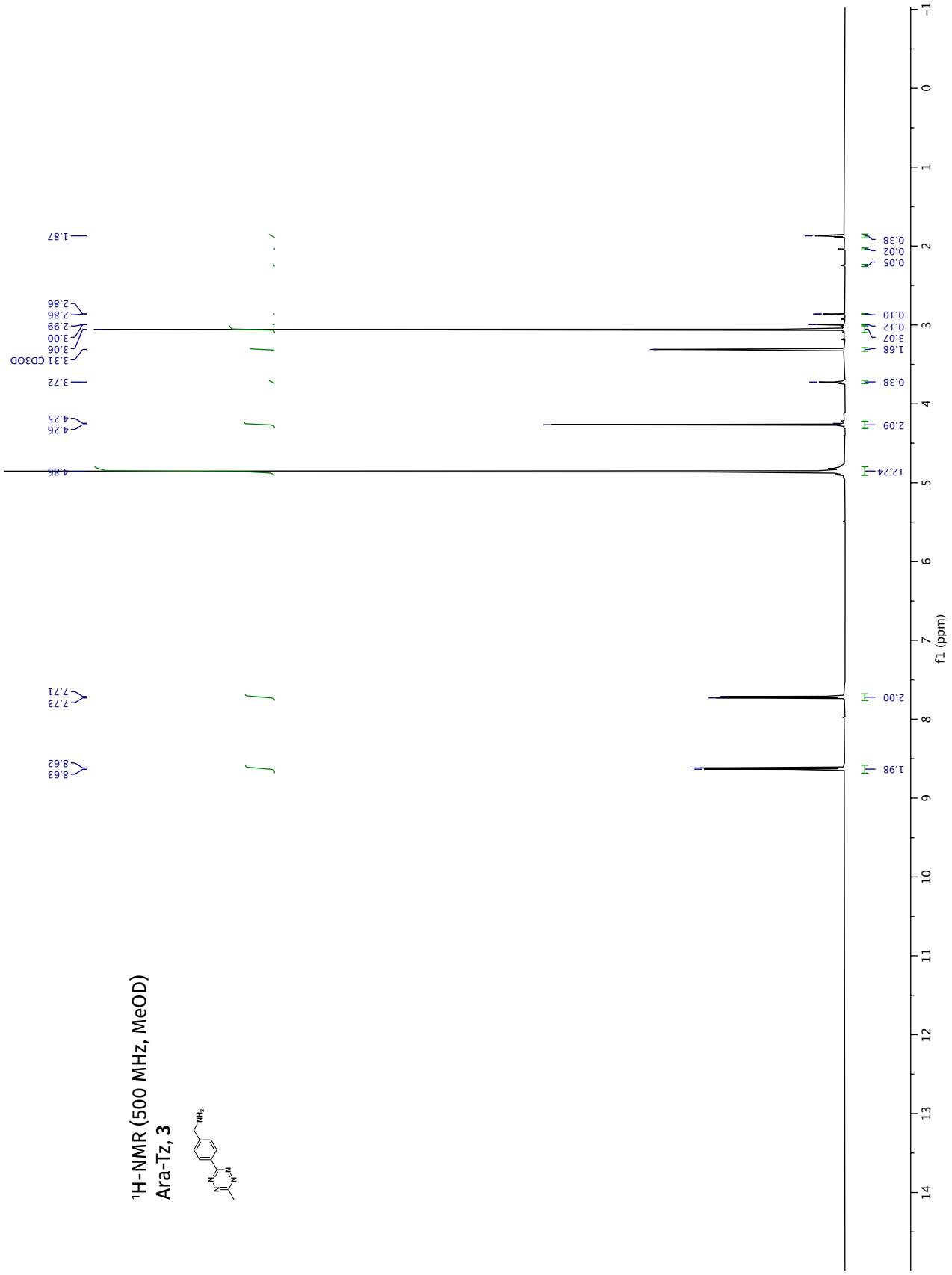


Figure S9: ^{1}H-NMR of compound **3** (Ara-Tz).

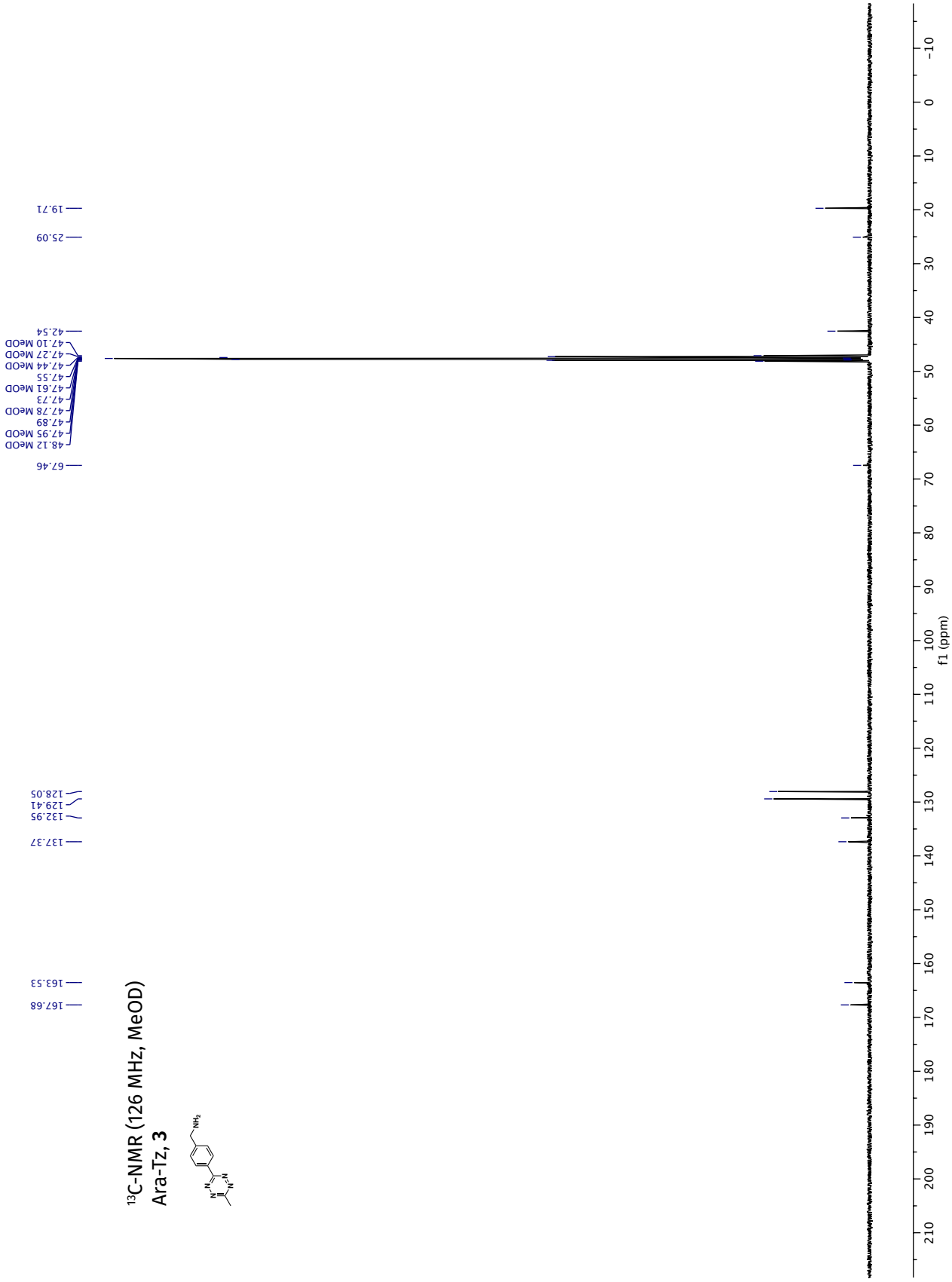


Figure S10: ^{13}C-NMR of compound **3** (Ara-Tz).

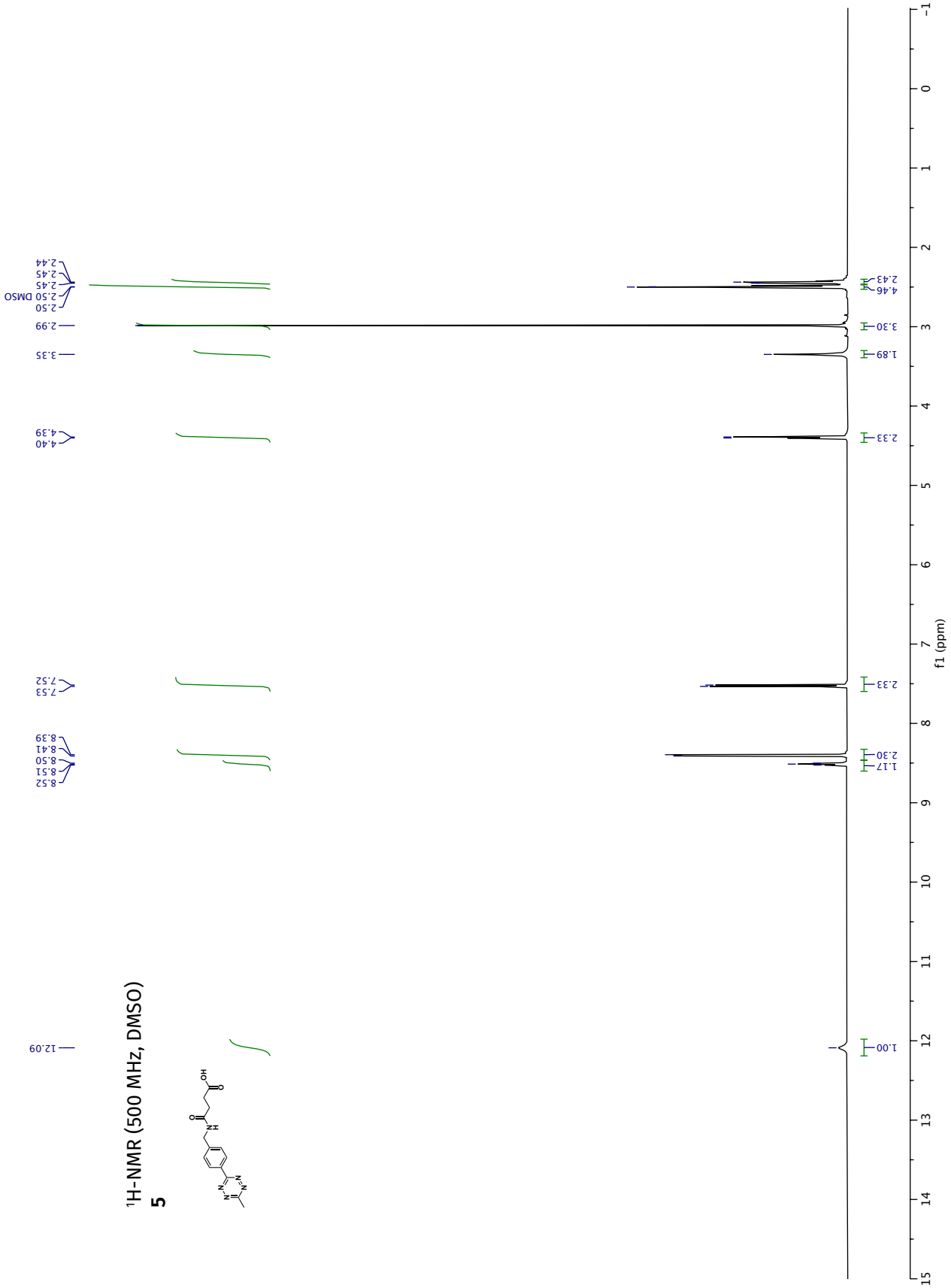


Figure S11: ^{1}H-NMR of compound **5**.

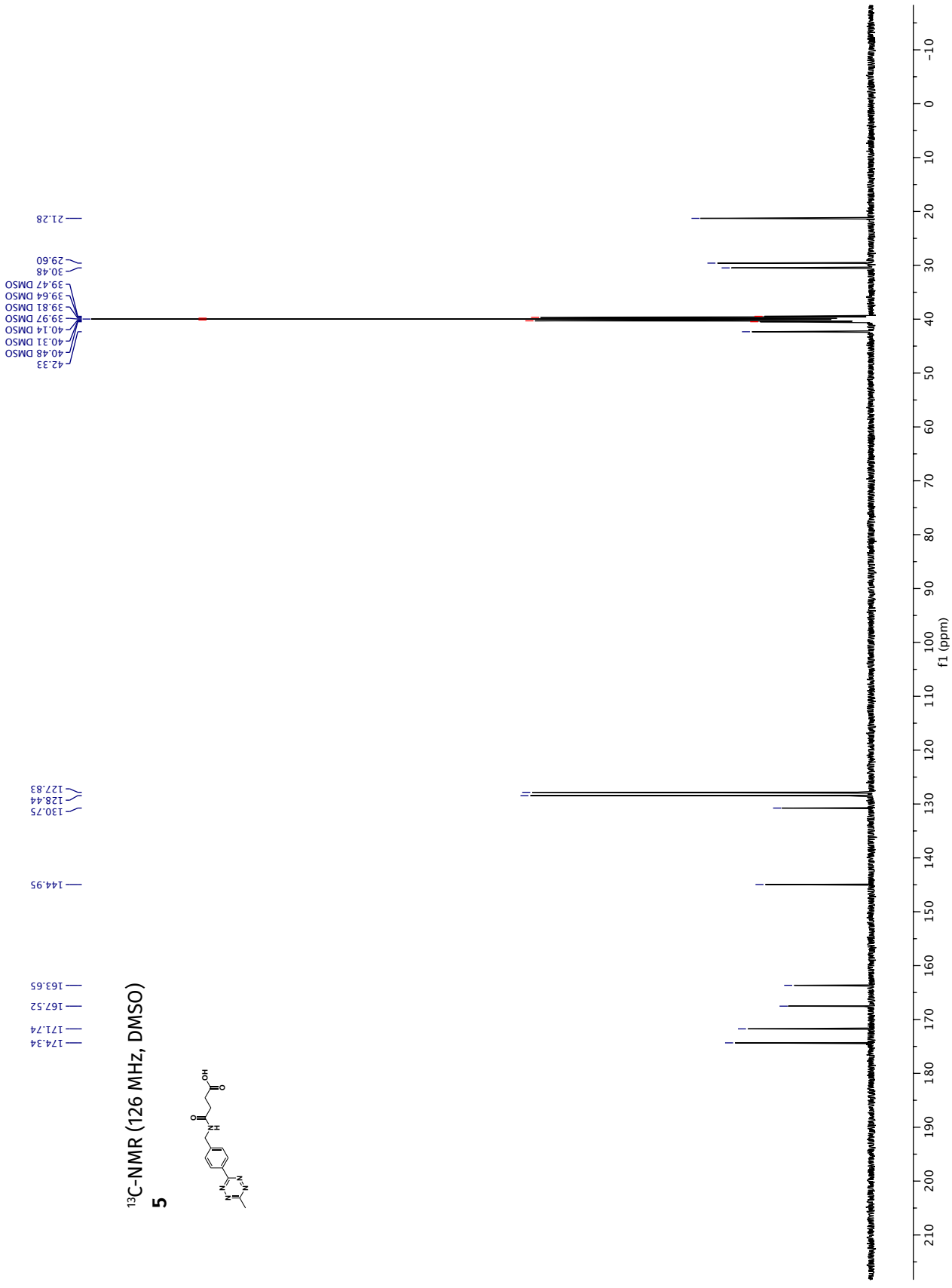


Figure S12: ^{13}C-NMR of compound **5**.

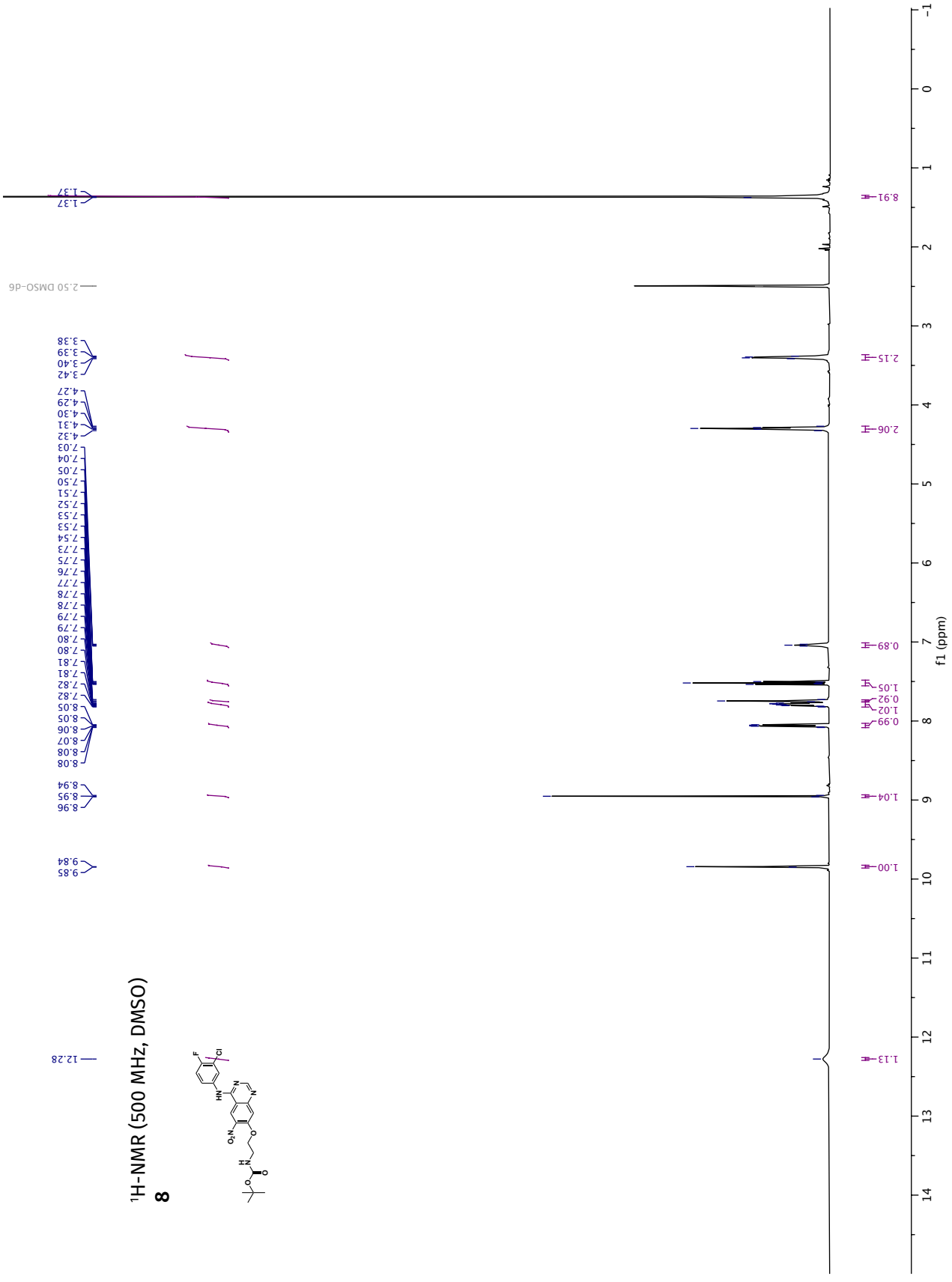


Figure S13: ^{1}H-NMR of compound **8**.

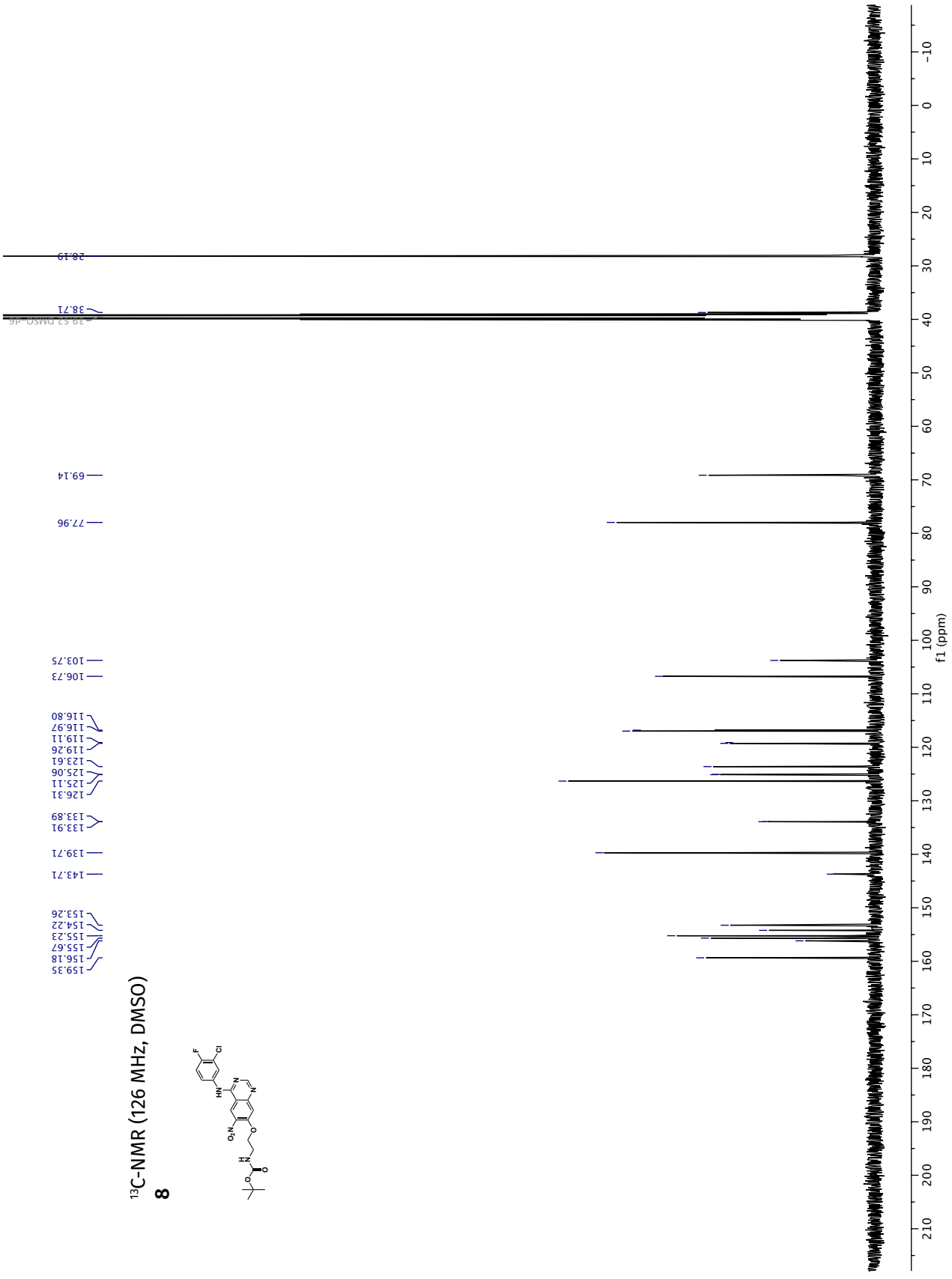


Figure S14: ^{13}C-NMR of compound **8**.

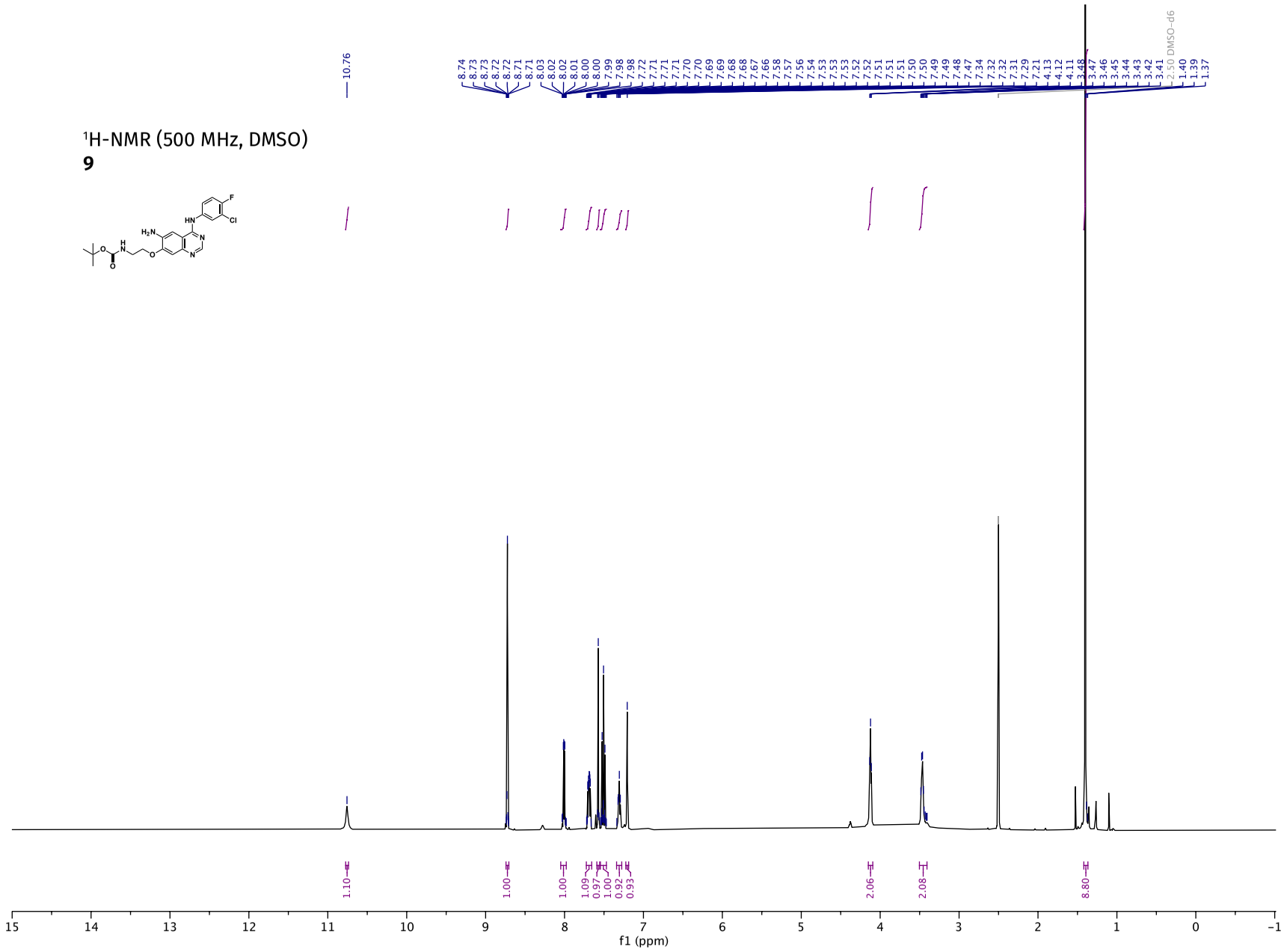


Figure S15: ^{1}H-NMR of compound **9**.

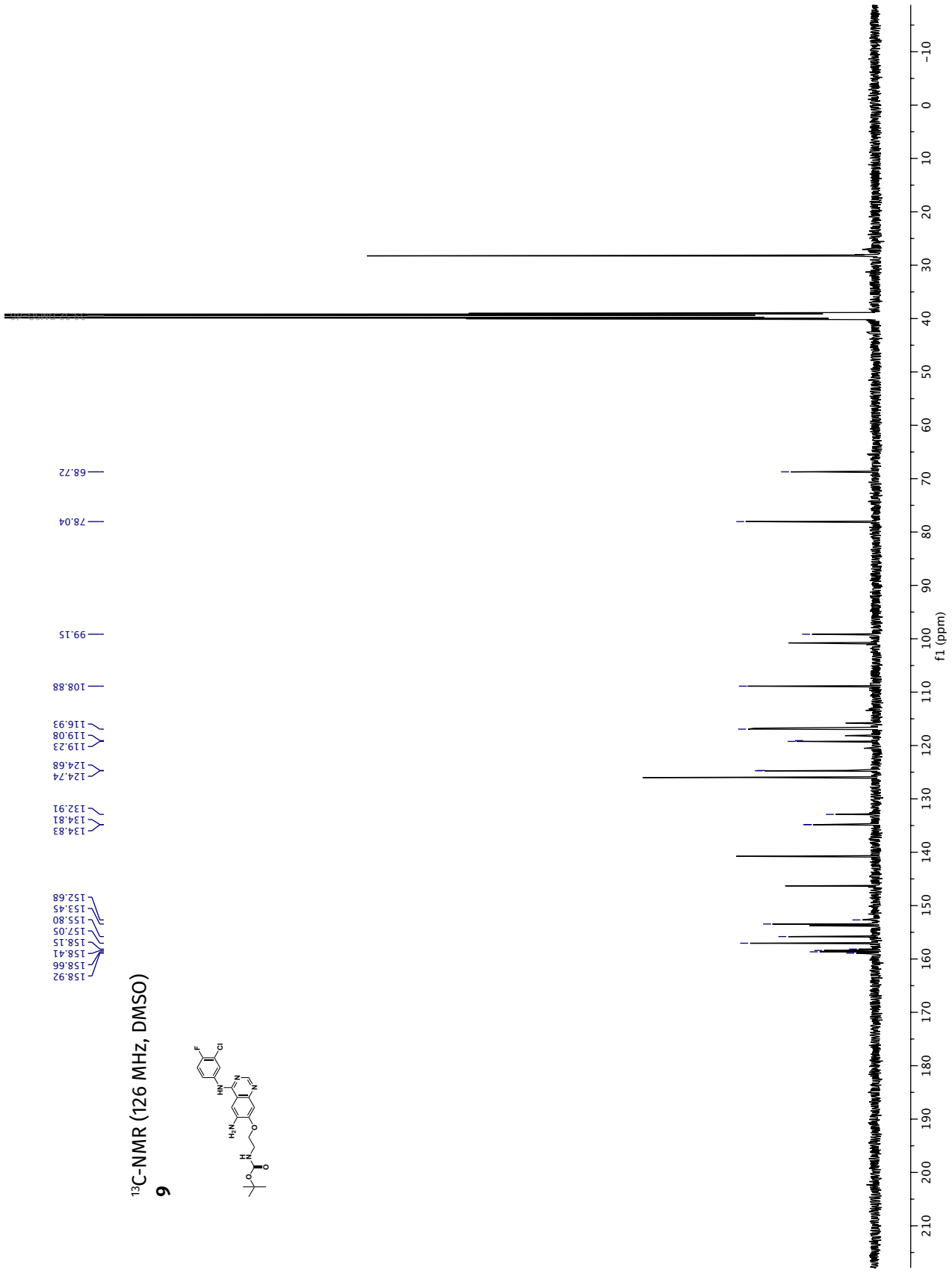


Figure S16: ^{13}C-NMR of compound **9**.

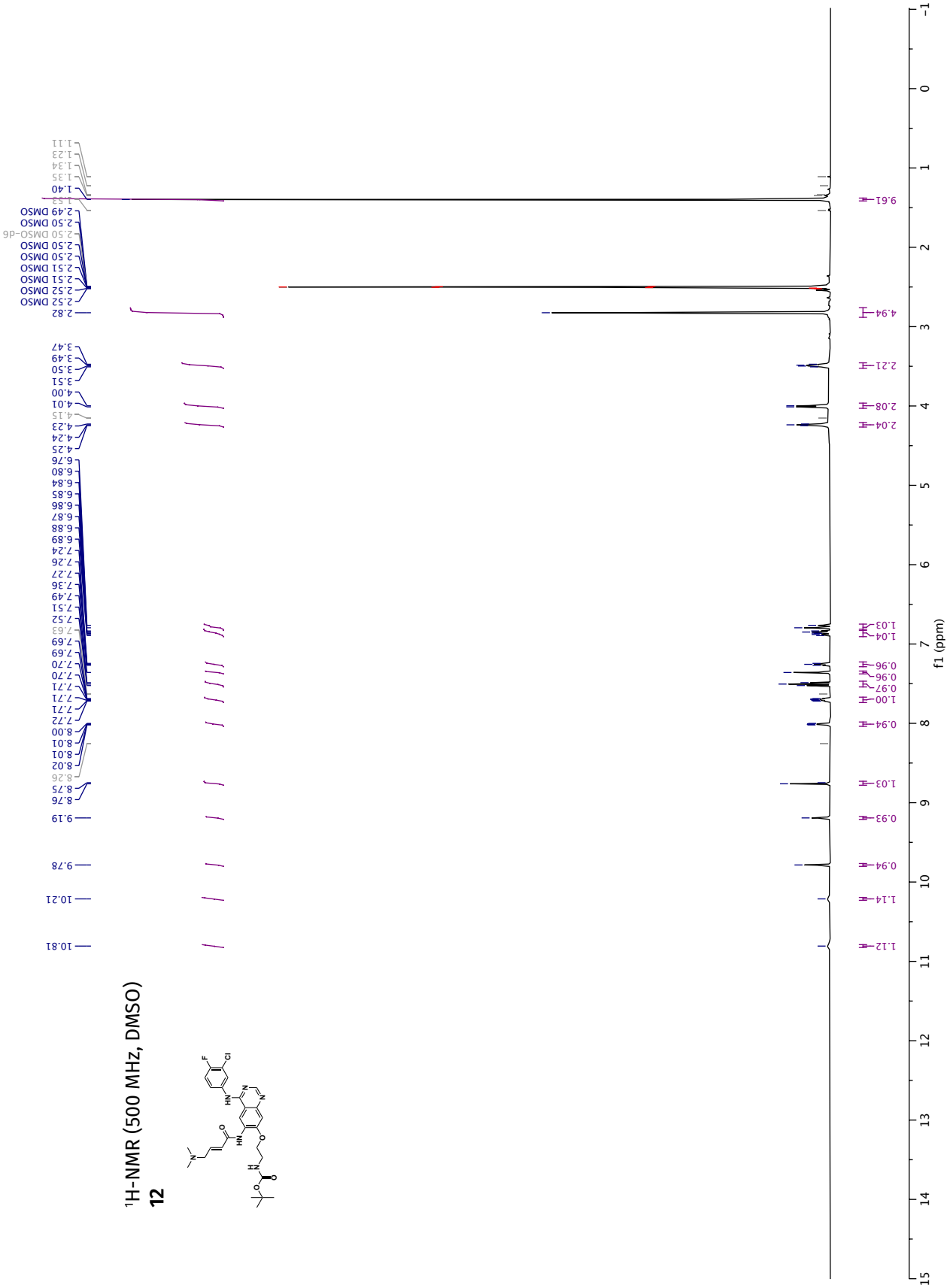


Figure S17: ^{1}H-NMR of compound **12**.

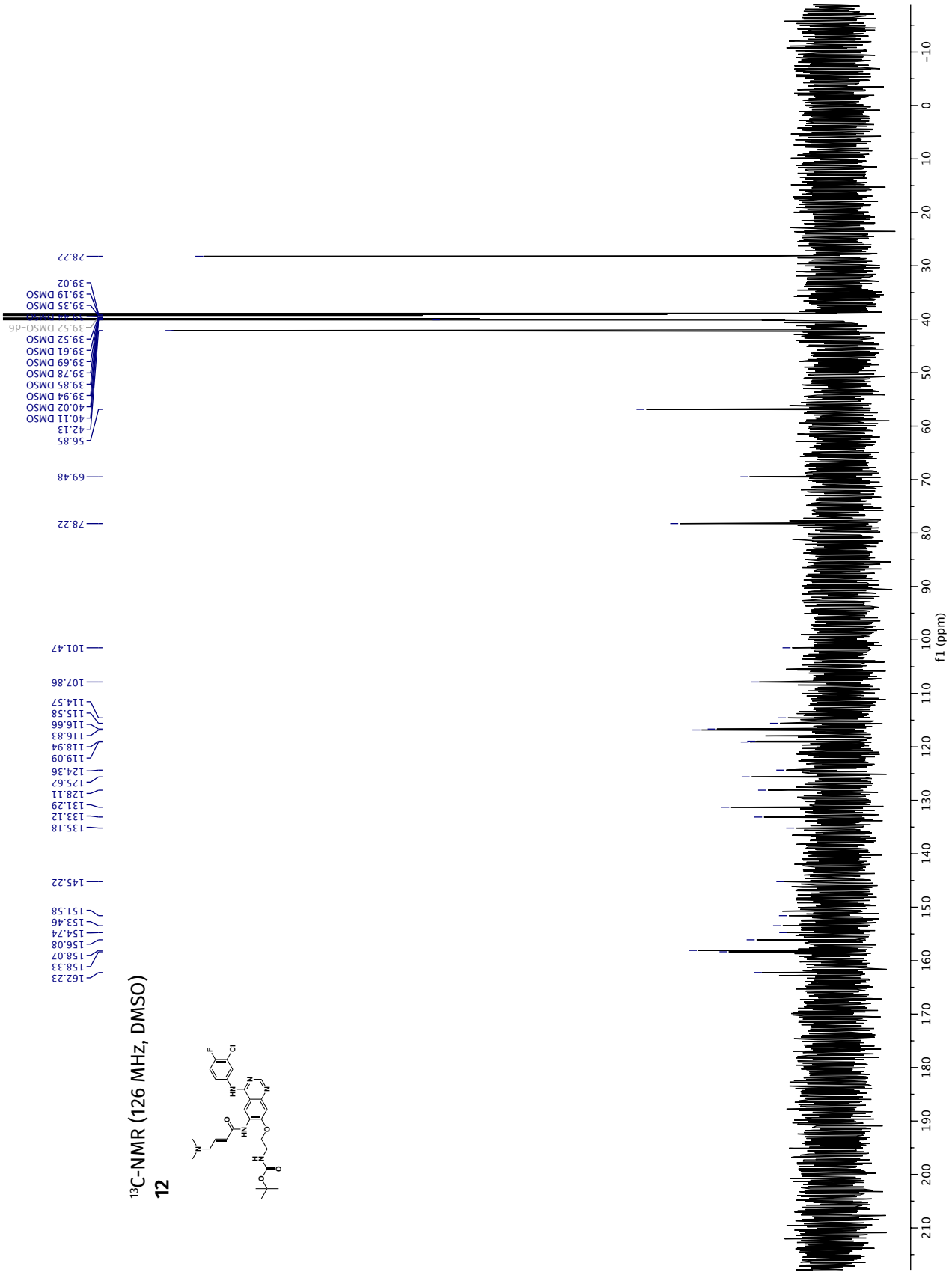


Figure S18: ^{13}C-NMR of compound **12**.

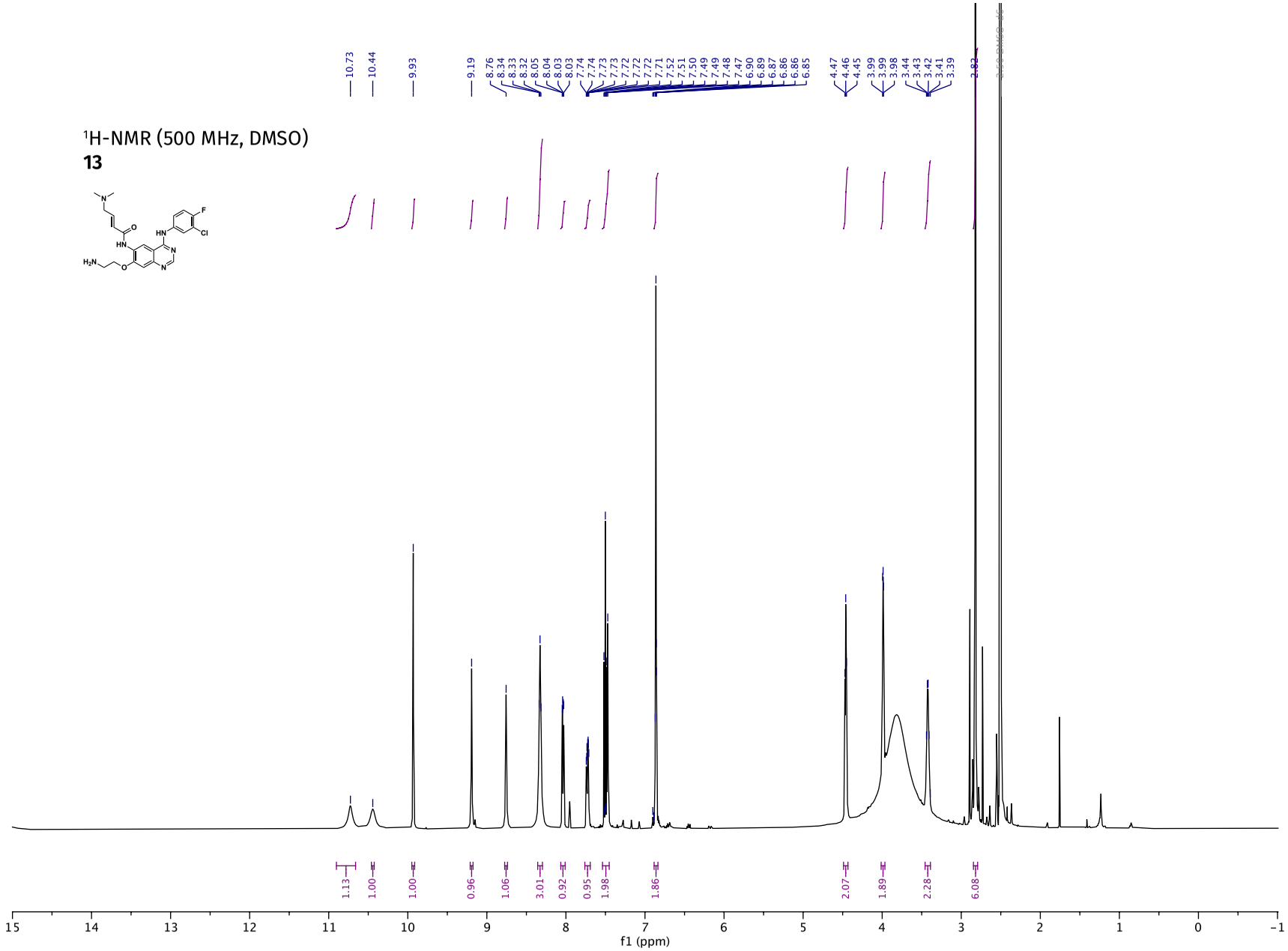


Figure S19: ^{1}H-NMR of compound **13**.

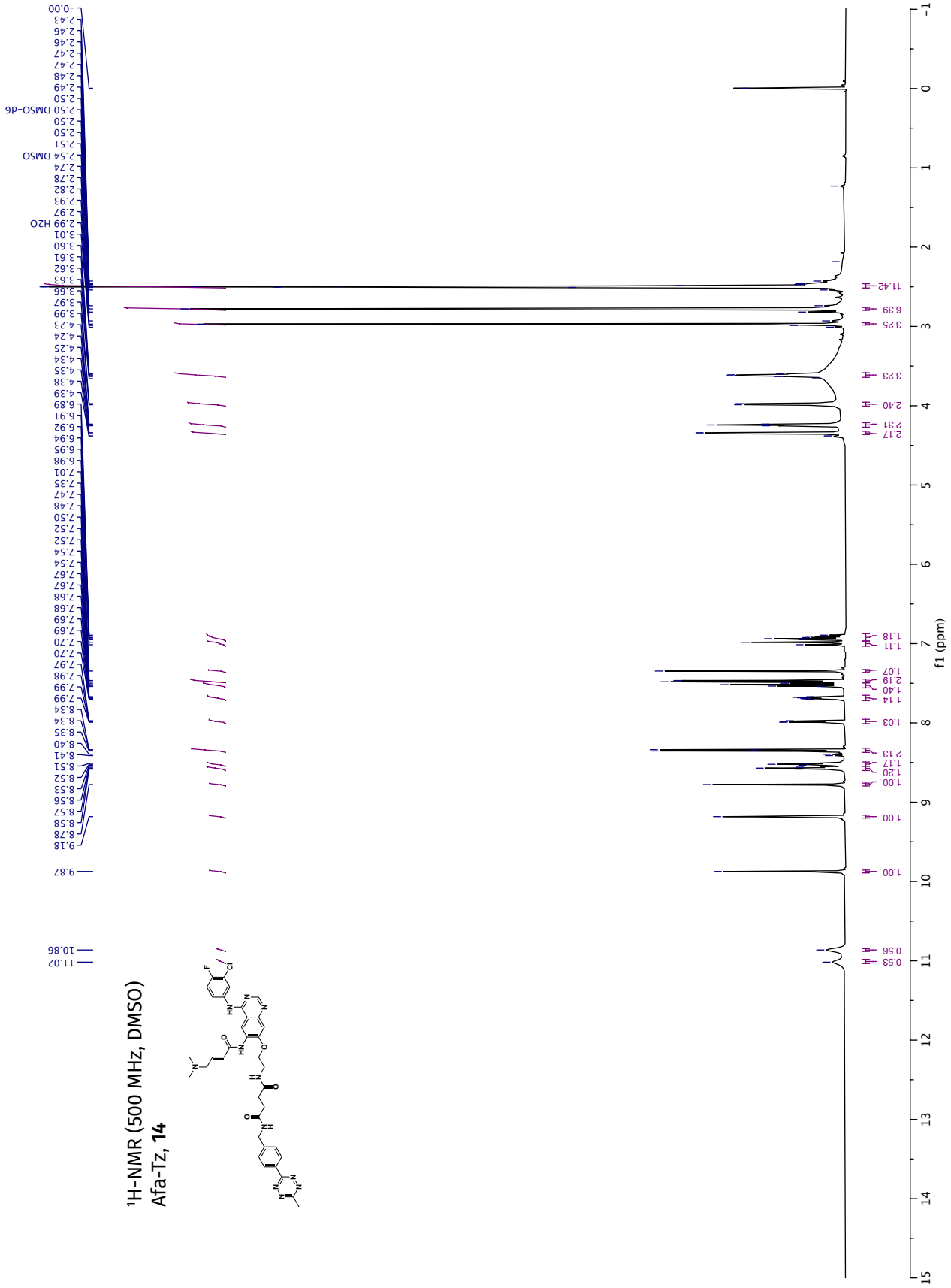


Figure S20: ^{1}H-NMR of compound **14** (Afa-Tz).

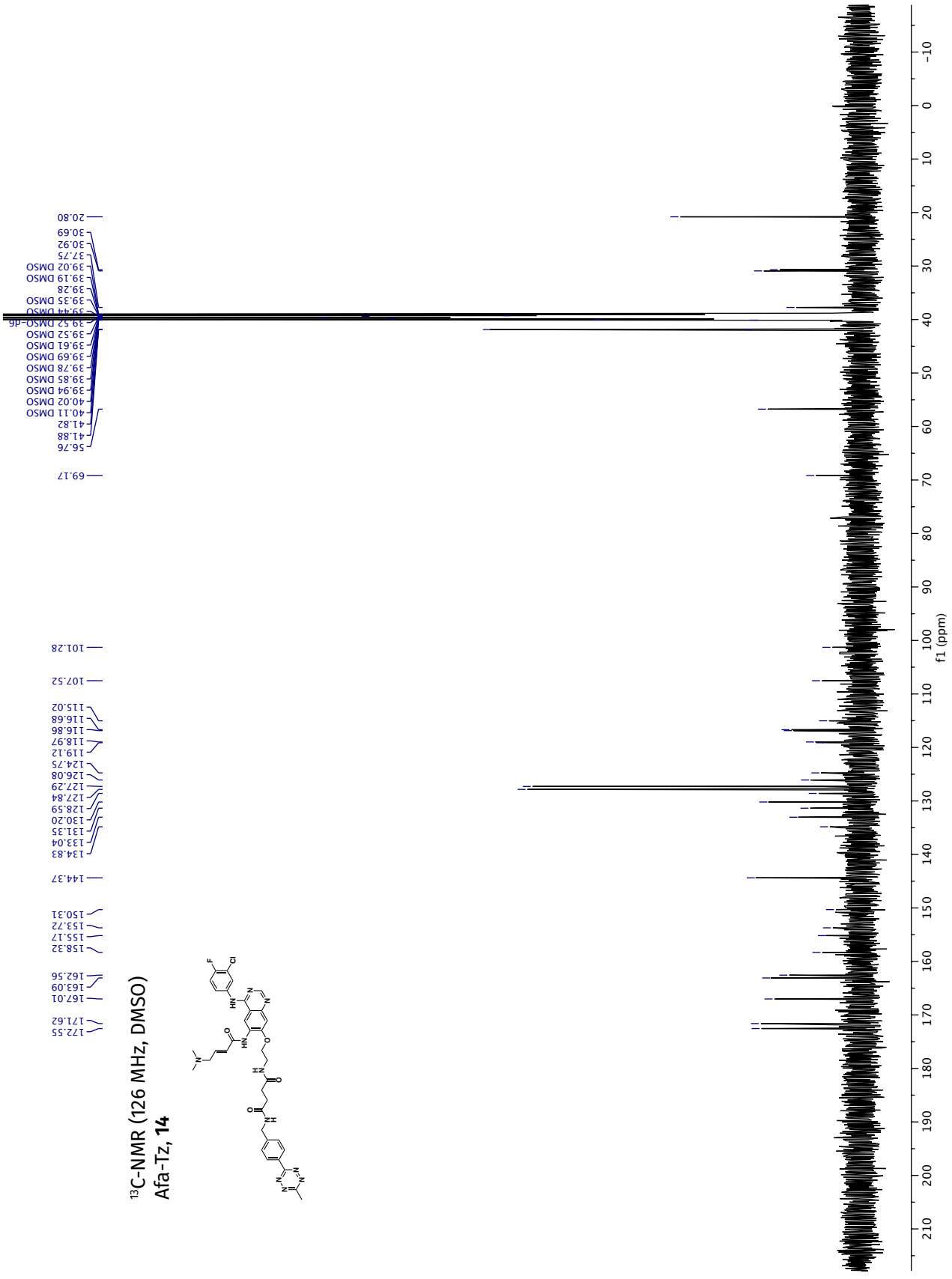


Figure S21: ^{13}C-NMR of compound **14** (Afa-Tz).

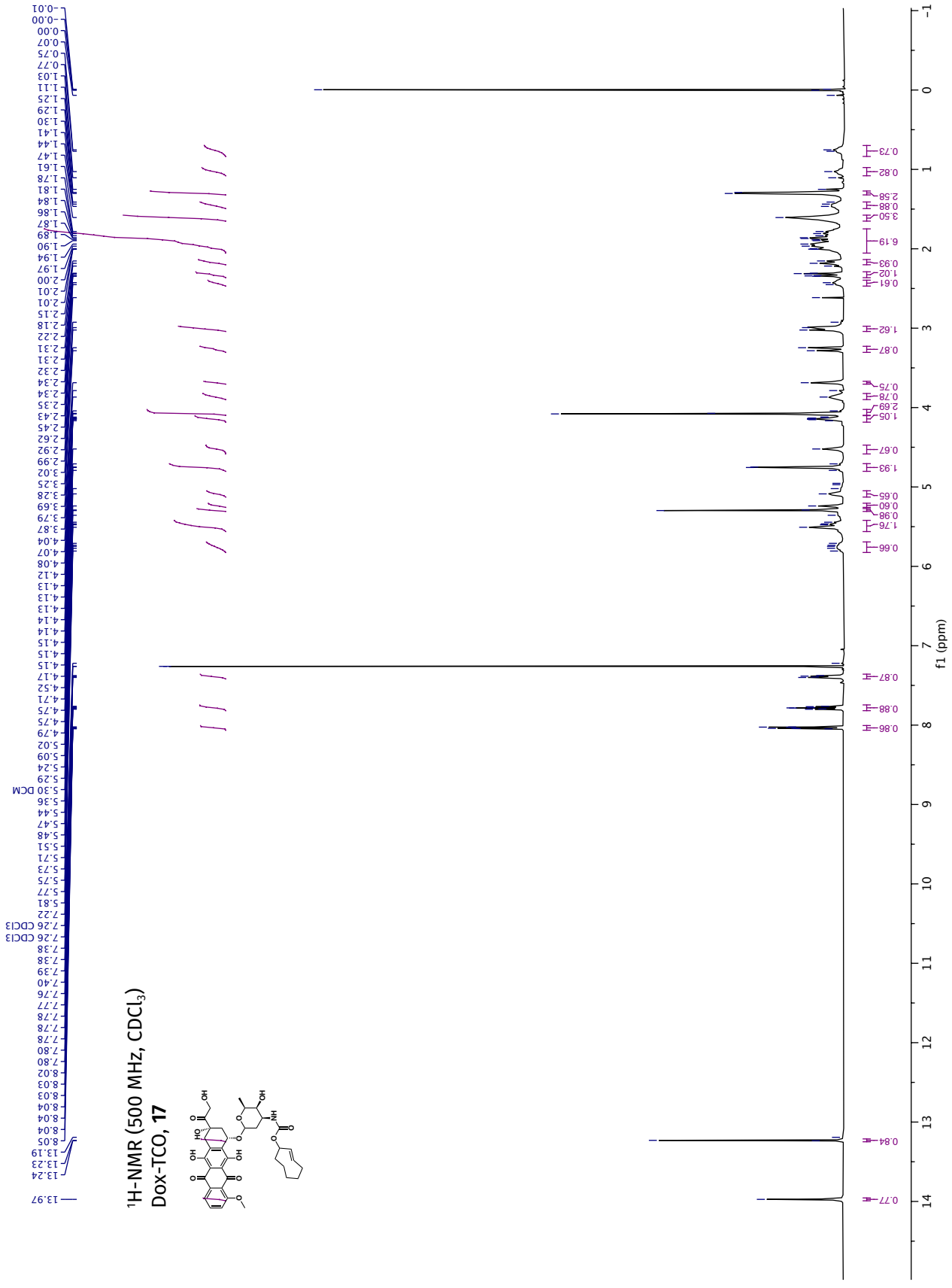


Figure S22: ^{1}H-NMR of compound **17** (Dox-TCO).

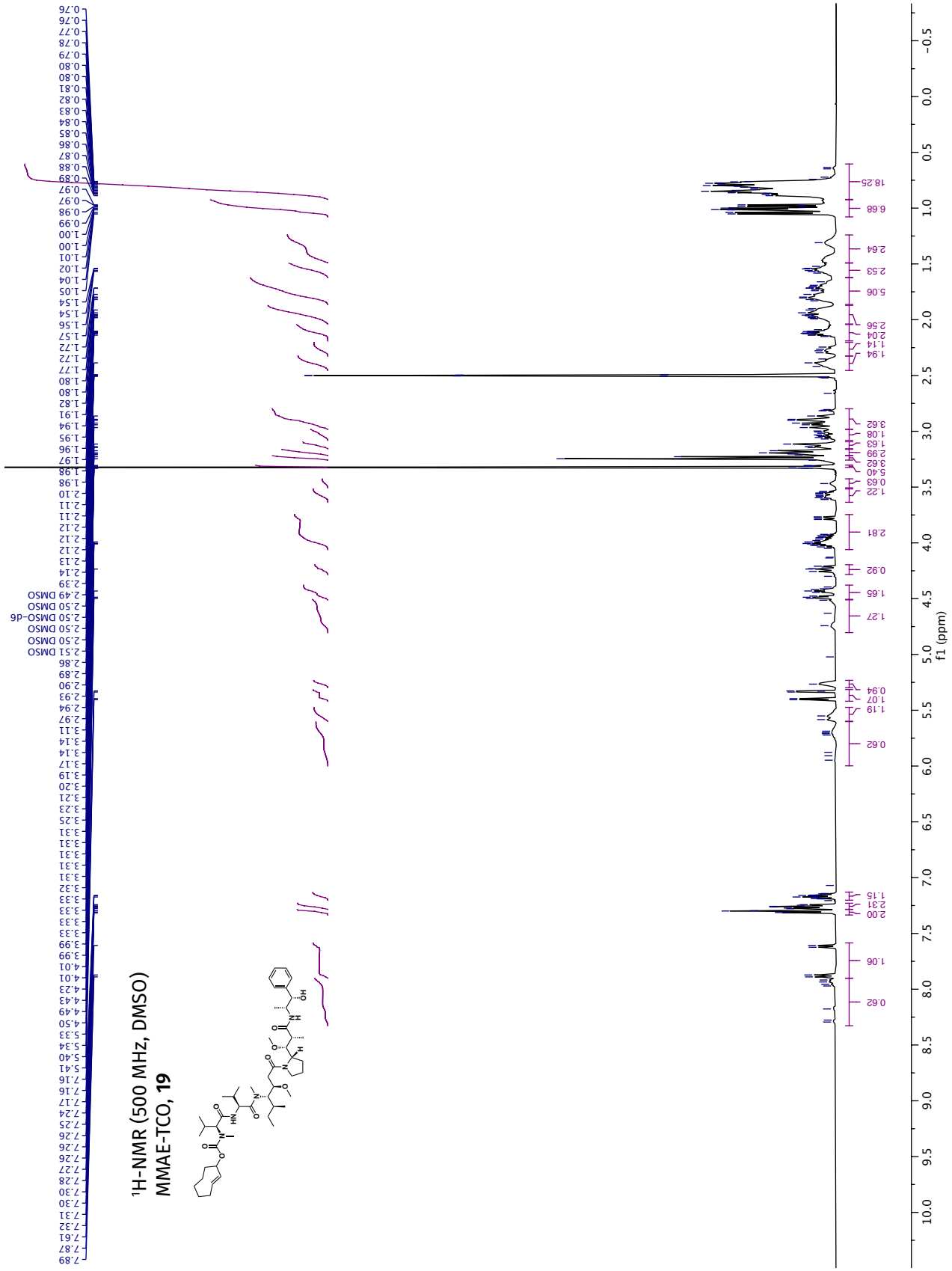


Figure S23: ^{1}H-NMR of compound **19** (MMAE-TCO).

11 High Resolution Mass Spectra

HRMS
Ara-Tz, **3**
Exact Mass: 201.10145

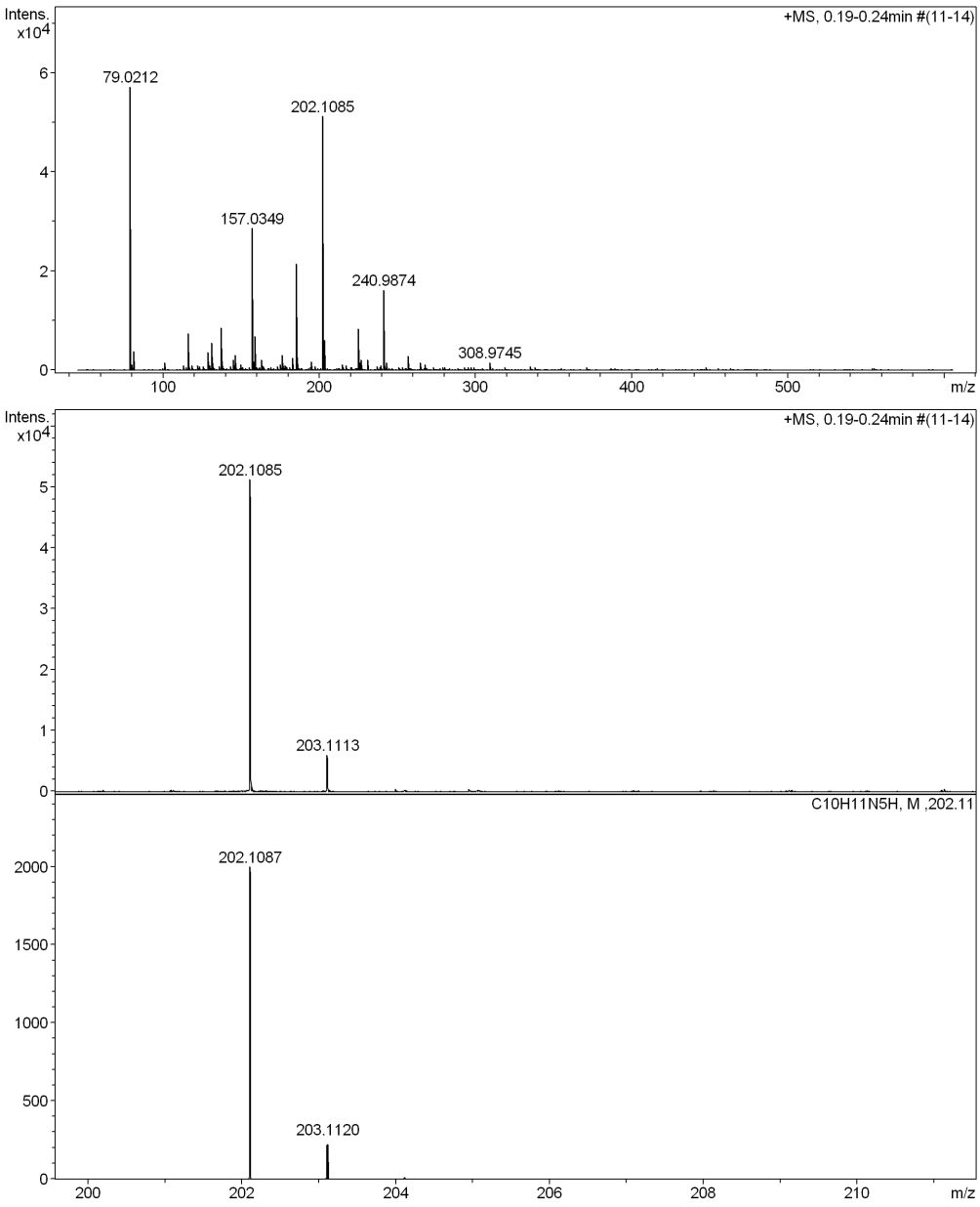
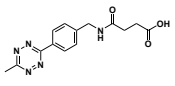


Figure S24: HRMS spectrum of compound **3** (Ara-Tz) (ESI+).

HRMS

5

Exact Mass: 301.11749



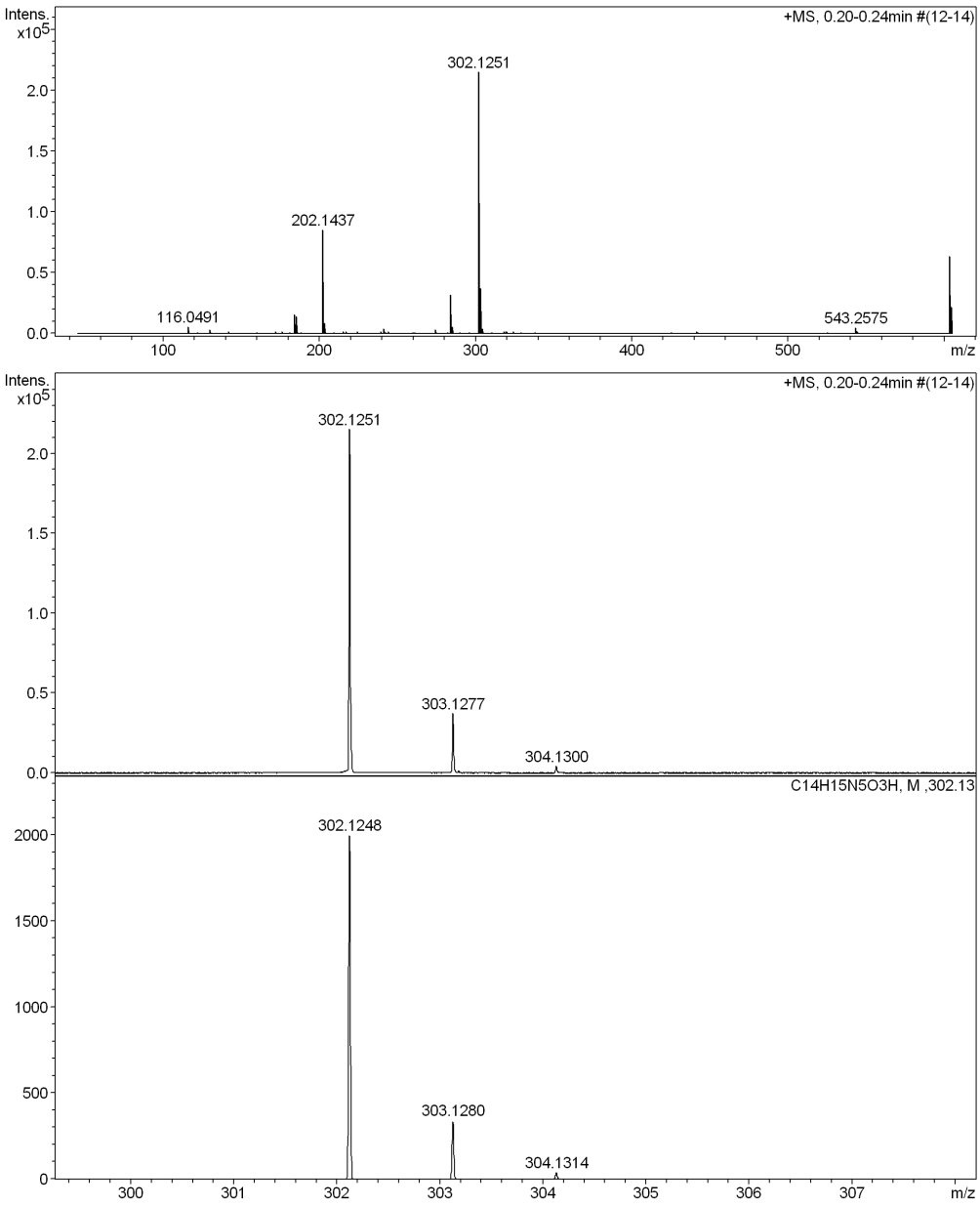
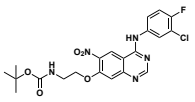


Figure S25: HRMS spectrum of compound **5** (ESI+).

HRMS

8

Exact Mass: 477.12152



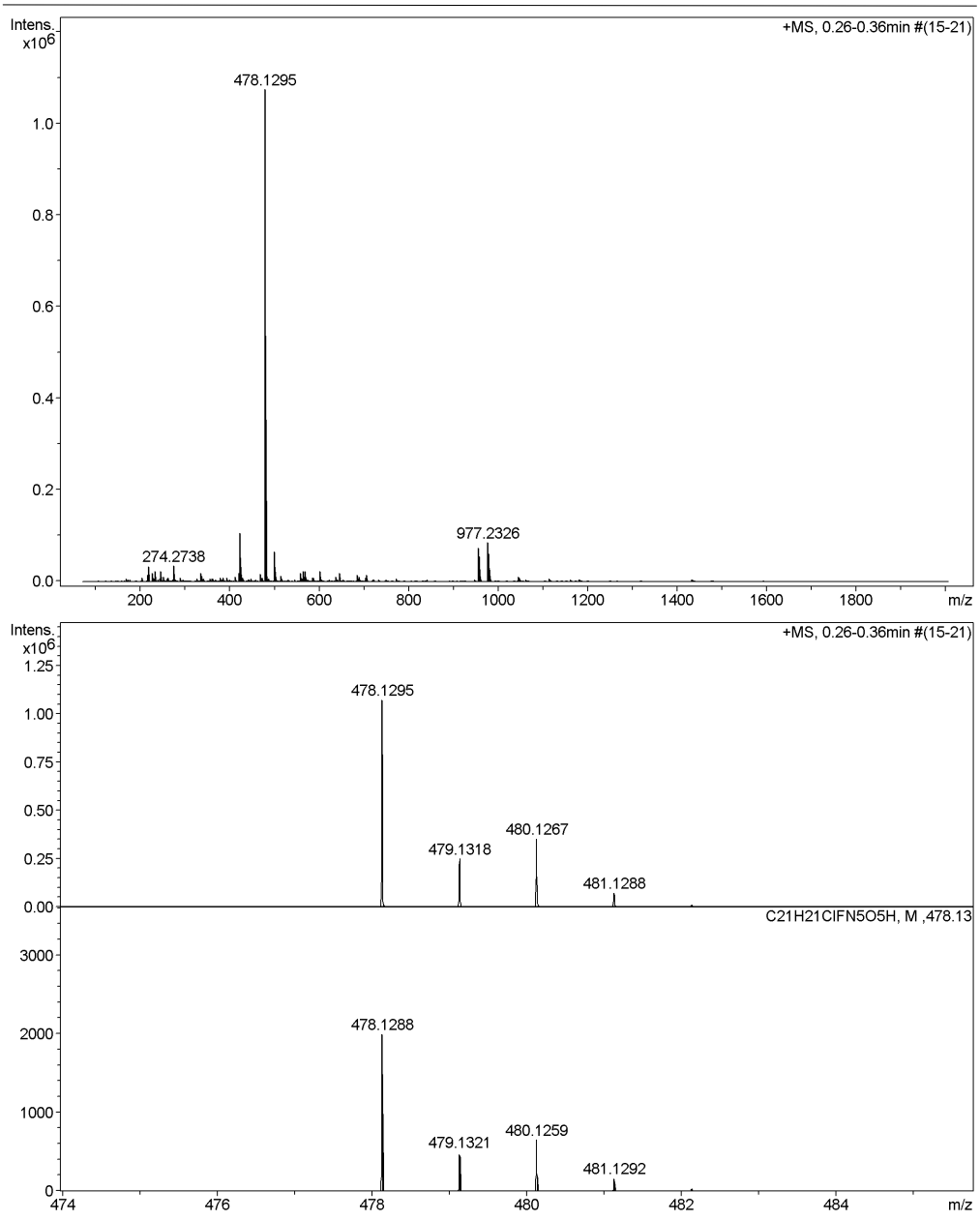
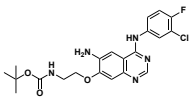


Figure S26: HRMS spectrum of compound **8** (ESI+).

HRMS

9

Exact Mass: 447.14735



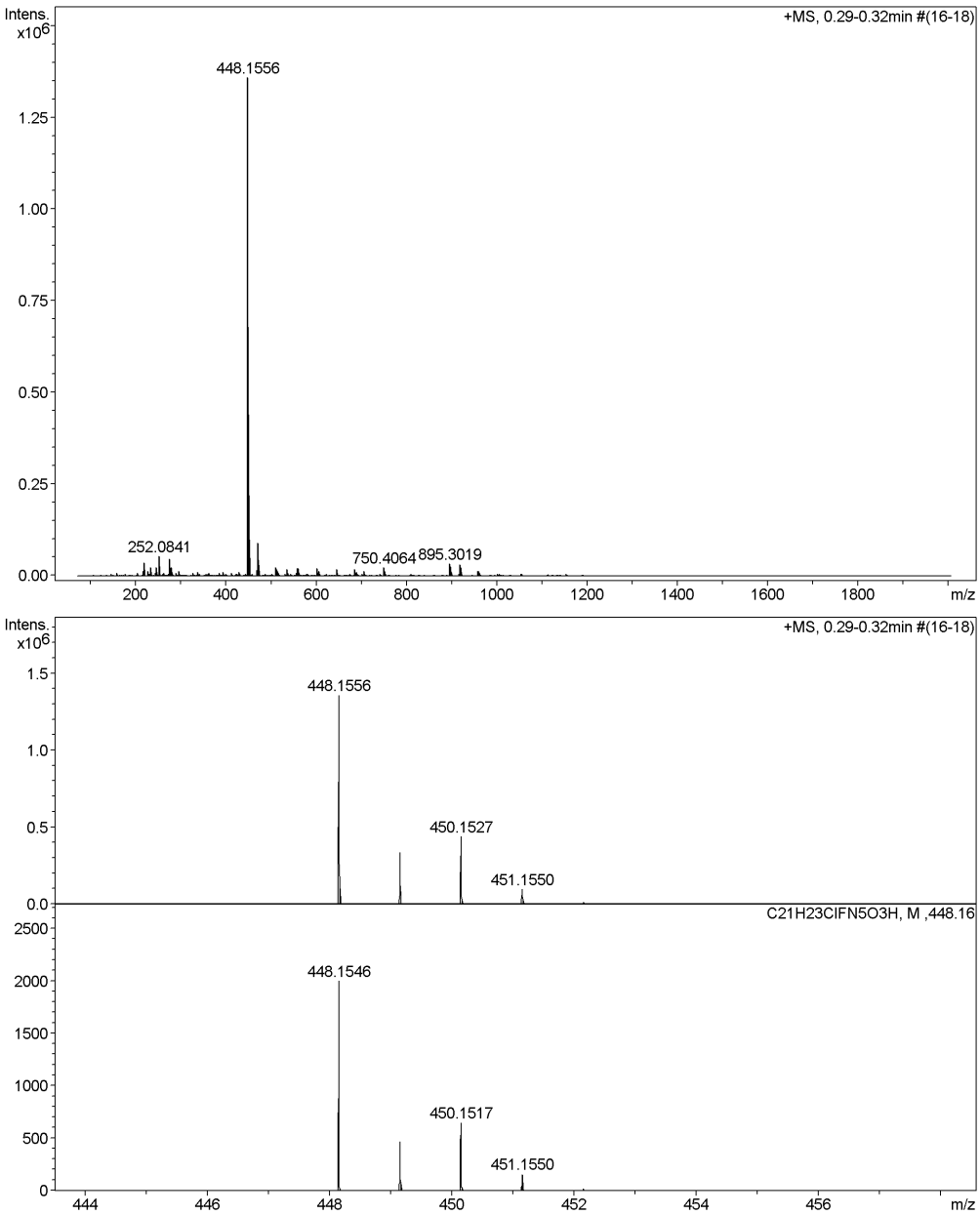
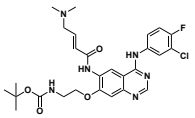


Figure S27: HRMS spectrum of compound **9** (ESI+).

HRMS

12

Exact Mass: 558.21576



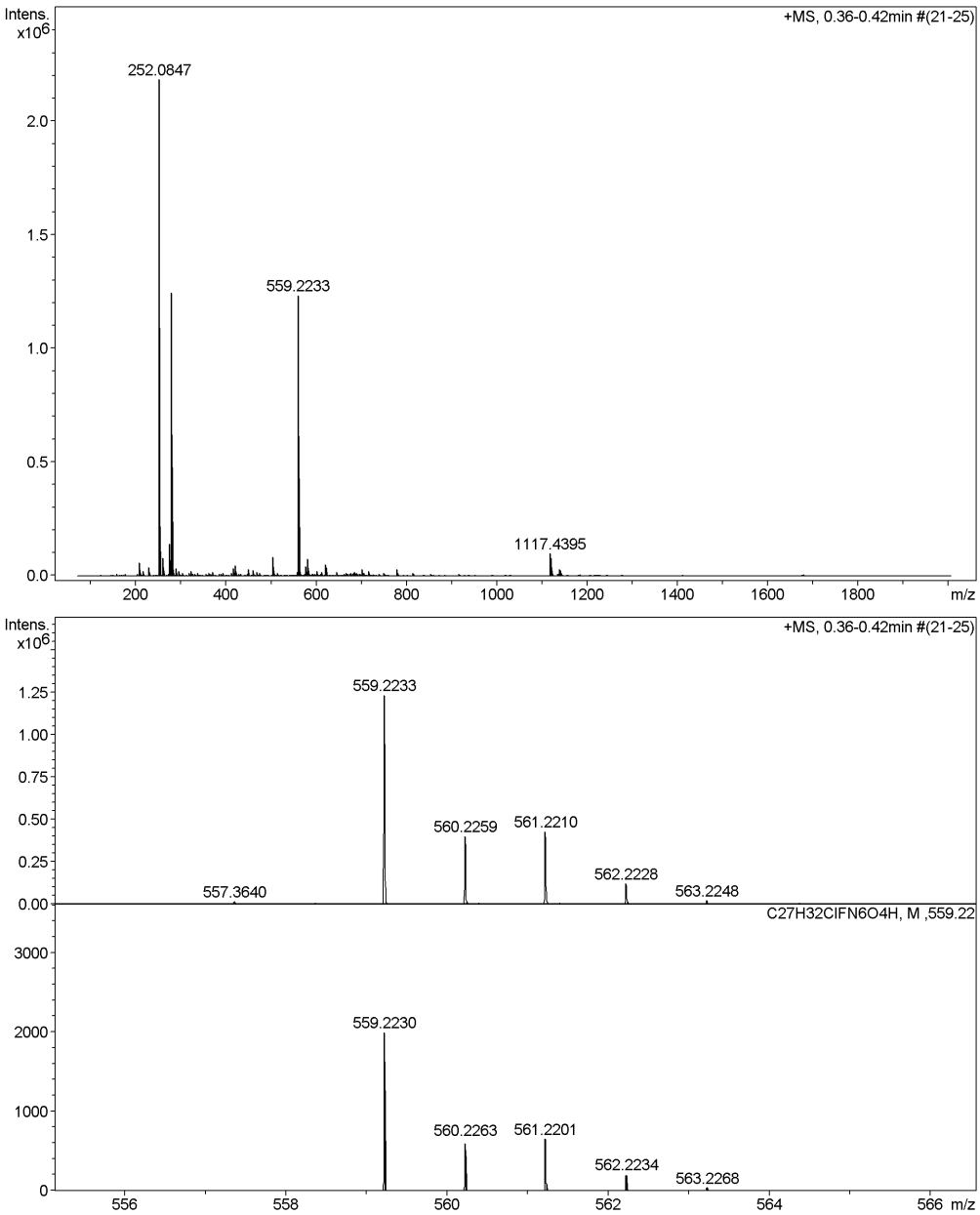
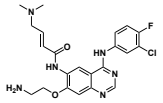


Figure S28: HRMS spectrum of compound **12** (ESI+).

HRMS

13

Exact Mass: 458.16333



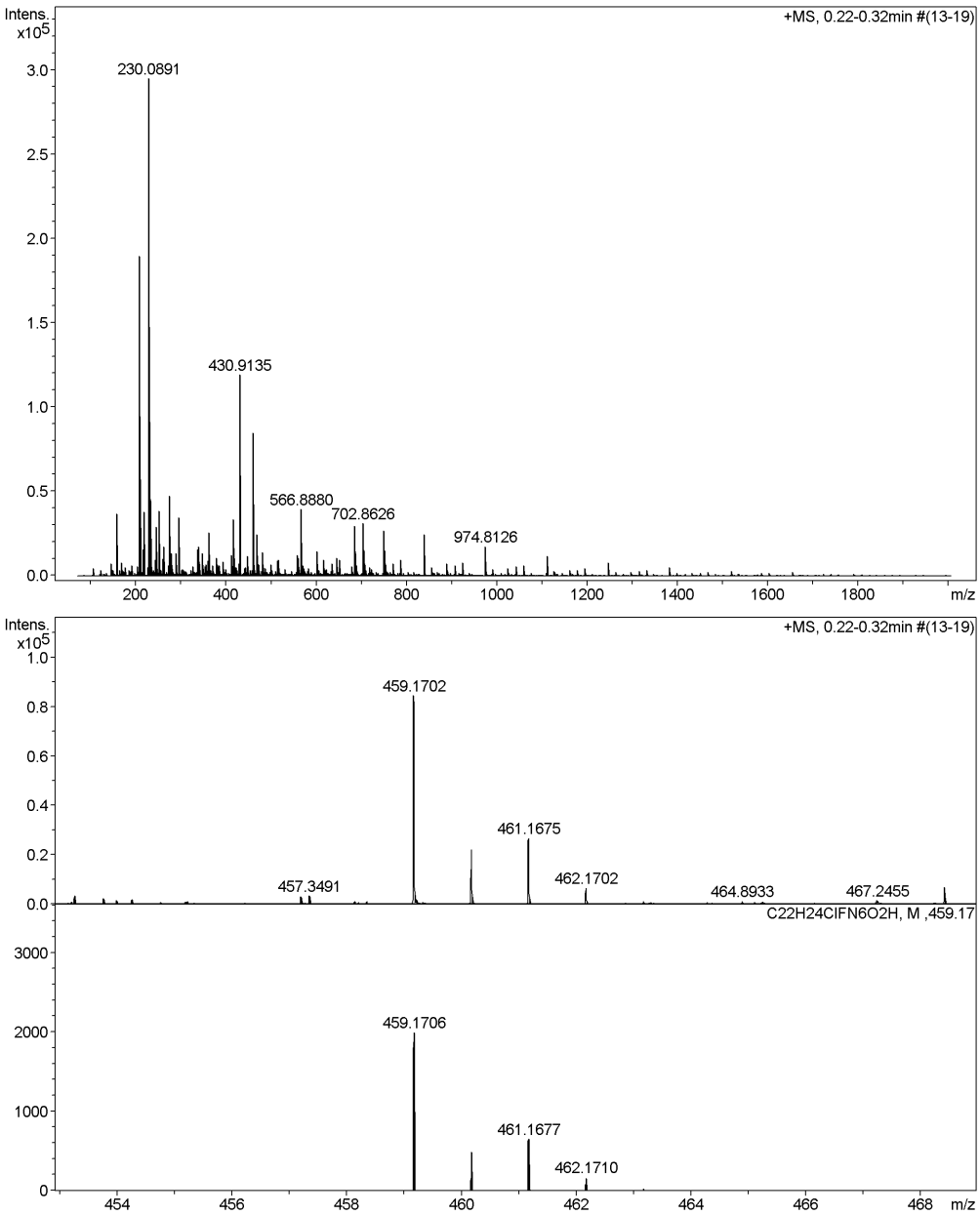


Figure S29: HRMS spectrum of compound **13** (ESI+).

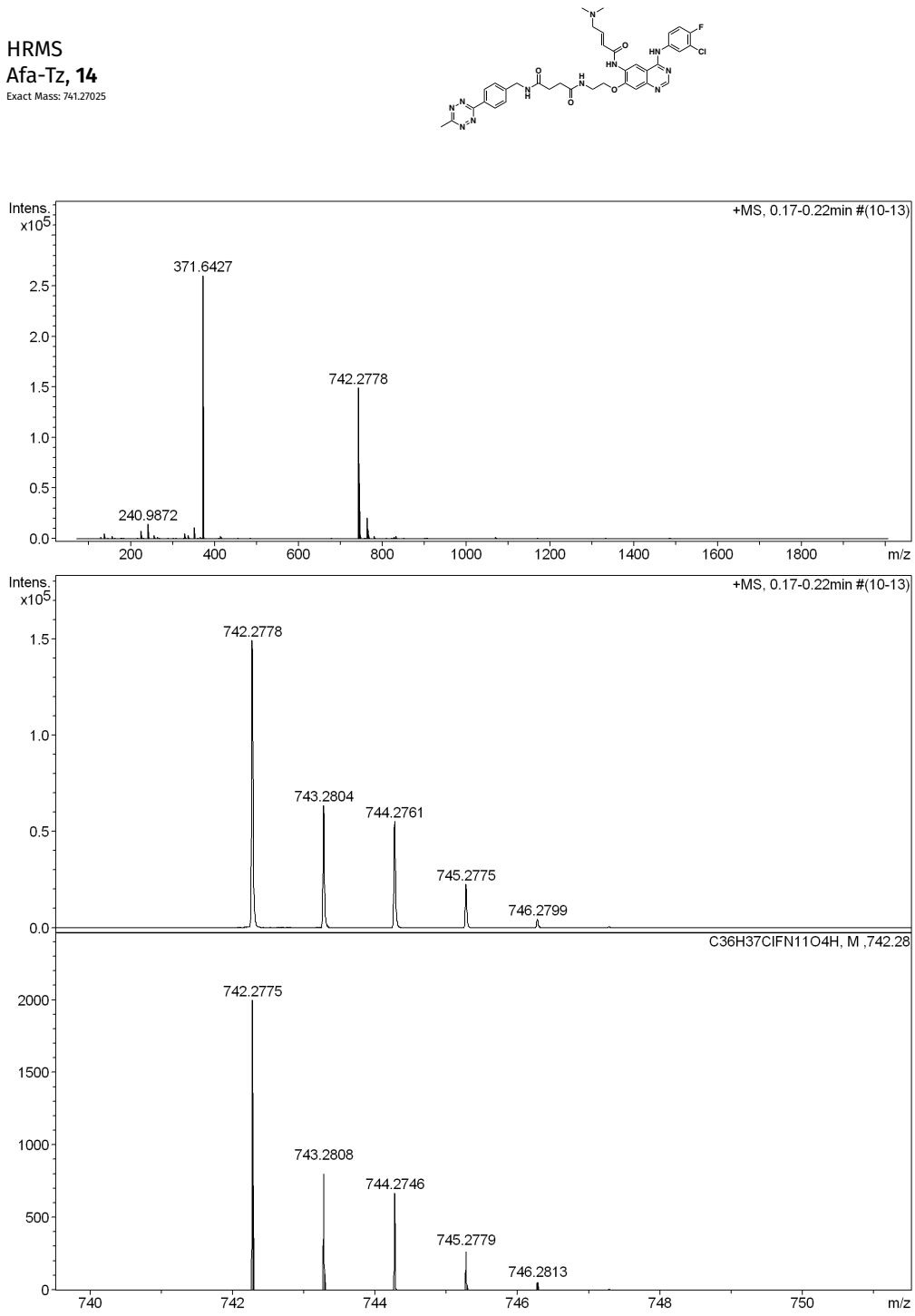
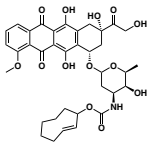


Figure S30: HRMS spectrum of compound **14** (Afa-Tz) (ESI+).

HRMS
Dox-TCO, **17**
Exact Mass: 695.25779



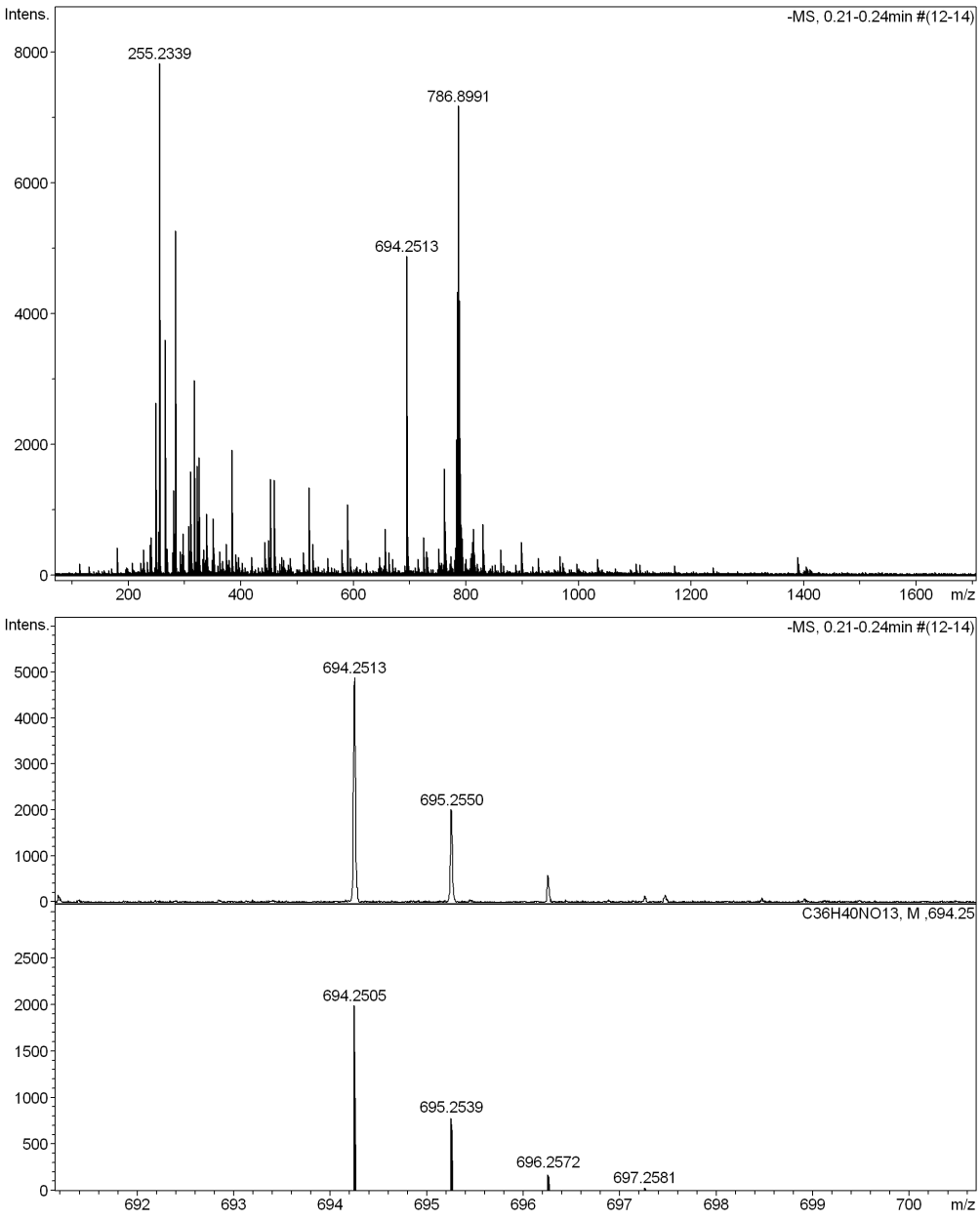


Figure S31: HRMS spectrum of compound **17** (Dox-TCO) (ESI-).

HRMS
MMAE-TCO, **19**
Exact Mass: 869.58778

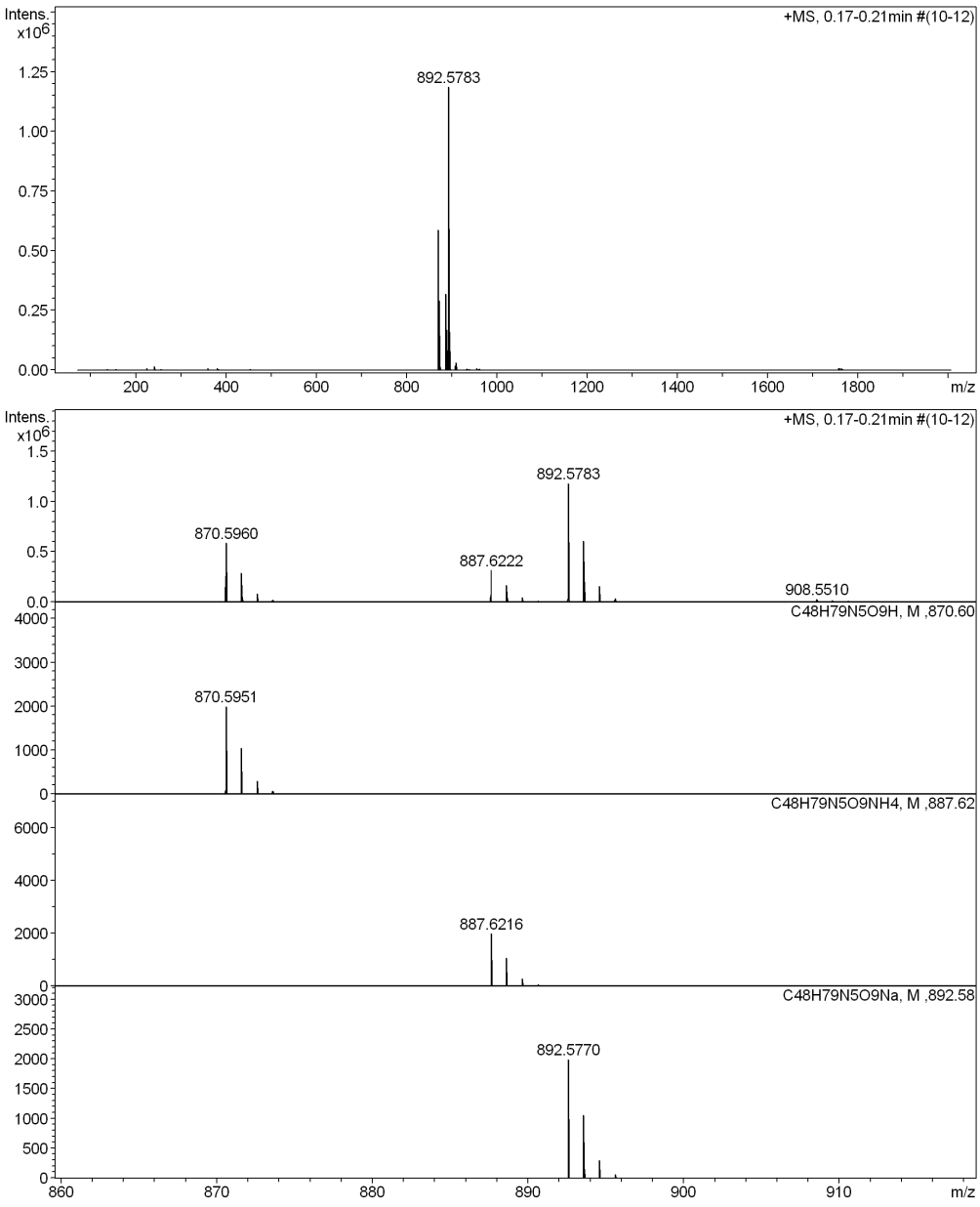
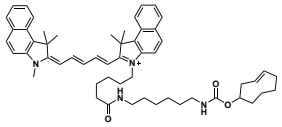


Figure S32: HRMS spectrum of compound **19** (MMAE-TCO) (ESI+).

HRMS
Cy5.5-nrTCO, **22**
Exact Mass: 833.53642



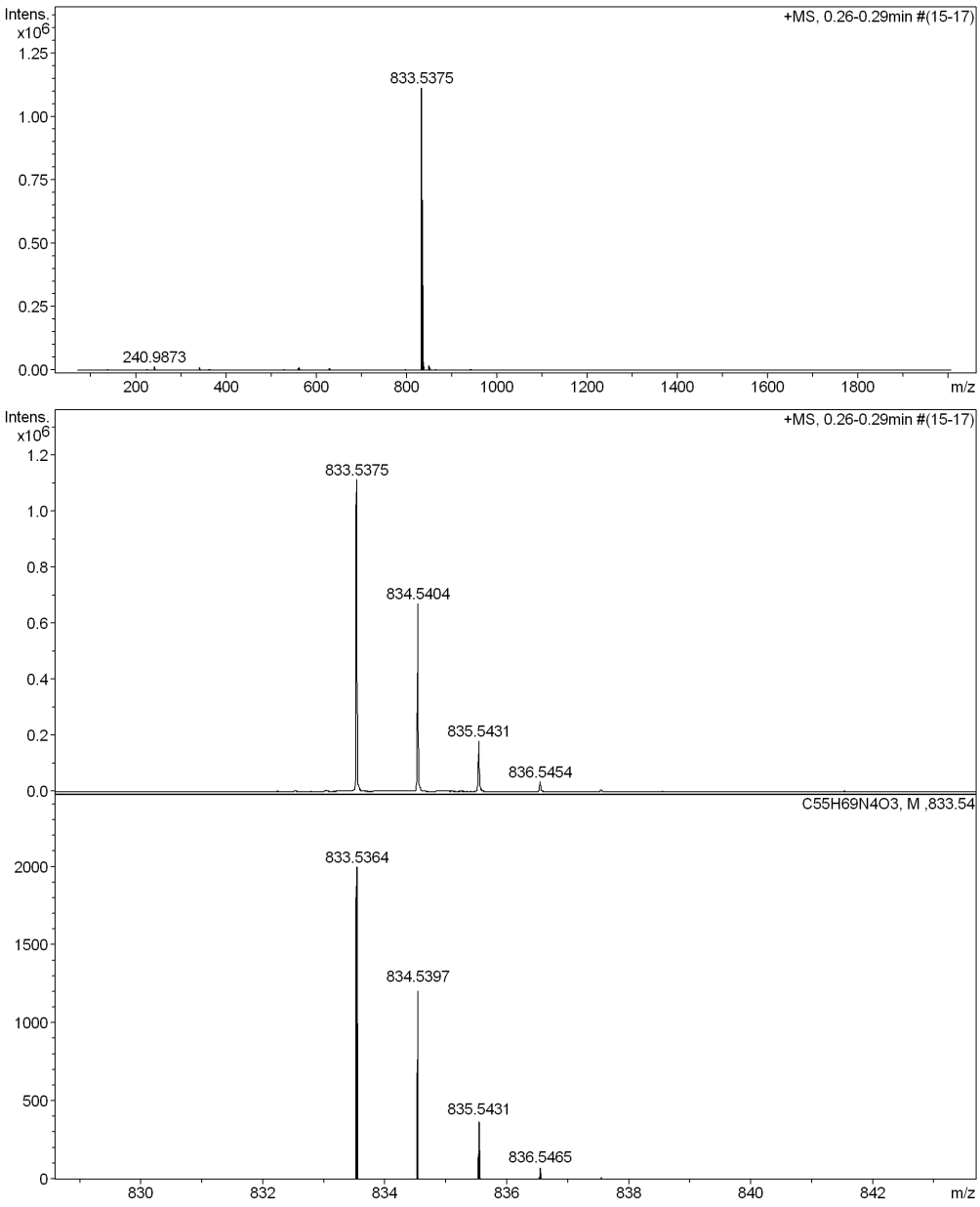


Figure S33: HRMS spectrum of compound **22** (Cy5.5-nrTCO) (ESI+).

Bibliography

[1] Jun Yang, Mark R. Karver, Weilong Li, Swagat Sahu, and Neal K. Devaraj. "Metal-Catalyzed One-Pot Synthesis of Tetrazines Directly from Aliphatic Nitriles and Hydrazine." In: *Angewandte Chemie International Edition* 51.21 (2012), pp. 5222–5225. DOI: 10.1002/anie.201201117.

[2] Xinyuan Fan, Yun Ge, Feng Lin, Yi Yang, Gong Zhang, William Shu Ching Ngai, Zhi Lin, Siqi Zheng, Jie Wang, Jingyi Zhao, Jie Li, and Peng R. Chen. "Optimized Tetrazine Derivatives for Rapid Bioorthogonal Decaging in Living Cells." In: *Angewandte Chemie International Edition* 55.45 (2016), pp. 14046–14050. DOI: 10.1002/anie.201608009.

[3] Ron M. Versteegen, Raffaella Rossin, Wolter ten Hoeve, Henk M. Janssen, and Marc S. Robillard. "Click to Release: Instantaneous Doxorubicin Elimination upon Tetrazine Ligation." In: *Angewandte Chemie International Edition* 52.52 (2013), pp. 14112–14116. DOI: 10.1002/anie.201305969.

[4] Samer Gnaim, Anna Scomparin, Sayantan Das, Rachel Blau, Ronit Satchi-Fainaro, and Doron Shabat. "Direct Real-Time Monitoring of Prodrug Activation by Chemiluminescence." In: *Angewandte Chemie* 130.29 (2018), pp. 9171–9175. DOI: 10.1002/ange.201804816.

[5] Christian Ritz, Florent Baty, Jens C. Streibig, and Daniel Gerhard. "Dose-Response Analysis Using R." In: *PLoS ONE* 10.12 (2015), e0146021. DOI: 10.1371/journal.pone.0146021. (R package version 3.0.1).

[6] R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. Vienna, Austria, 2024. URL: https://www.R-project.org. (R version 4.4.2).

[7] Hadley Wickham, Mara Averick, Jennifer Bryan, Winston Chang, Lucy McGowan, Romain François, Garrett Grolemund, Alex Hayes, Lionel Henry, Jim Hester, Max Kuhn, Thomas Pedersen, Evan Miller, Stephan Bache, Kirill Müller, Jeroen Ooms, David Robinson, Dana Seidel, Vitalie Spinu, Kohske Takahashi, Davis Vaughan, Claus Wilke, Kara Woo, and Hiroaki Yutani. "Welcome to the Tidyverse." In: *Journal of Open Source Software* 4.43 (2019), p. 1686. DOI: 10.21105/joss.01686. (R package version 2.0.2).

[8] Hadley Wickham. "ggplot2, Elegant Graphics for Data Analysis." In: *Use R!* (2016). DOI: 10.1007/978-3-319-24277-4. (R package version 3.5.1).

[9] Claus O. Wilke. *cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'*. 2024. URL: https://wilkelab.org/cowplot/. (R package version 1.1.3).

[10] Erik Clarke, Scott Sherrill-Mix, and Charlotte Dawson. *ggbeeswarm: Categorical Scatter (Violin Point) Plots*. 2023. URL: https://github.com/eclarke/ggbeeswarm. (R package version 0.7.2).

[11] Paul Tol. *Colour Schmes, Technical note SRON/EPS/TN/09-002 3.2*. 2021. URL: https://sronpersonalpages.nl/~pault.

[12] Erich Neuwirth. *RColorBrewer: ColorBrewer Palettes*. 2022. URL: https://CRAN.R-project.org/package=RColorBrewer. (R package version 1.1.3).