Supporting Information

Towards ^{99m}Tc-based Imaging Agents with Effective Doxorubicin Mimetics: A Molecular and Cellular Study

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Materials

All commercially available reagents were used without further purification. Water was doubly distilled before use. Doxorubicin·HCl (≥95%) and *N,N,N′,N′*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (≥99%) were purchased from I²CNS LLC (Switzerland). *N,N*-dimethylformamide (99.8%, extra dry, AcroSeal®) was purchased from ACROS Organics (Switzerland). Silica gel (230-400 mesh) was purchased from Merck KGaA. Fmoc-Lys-OH was purchased from Novabiochem, Switzerland. Sodium triacetoxyborohydride (97%), 2-pyridinecarboyaldehyde (99%), methanesulfonyl chloride (>99.7%), *N,N*-diisopropylethylamine (≥99%), piperidine (99%), triethylamine (>99%), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 98%), sodium bicarbonate (99%), potassium carbonate (99%), magnesium sulfate (97%), potassium nitrate (≥99%), sodium L-tartrate dibasic dihydrate (≥99%), sodium tetraborate decahydrate (≥99%), dichloromethane, dichloroethane, chloroform, methanol, deoxyribonucleic acid sodium salt from calf thymus (at 1 mg per vial), Sephadex G10 and HPLC solvents were purchased from Sigma Aldrich (Switzerland). Trifluoroacetic acid (>99.5%, HPLC grade) was purchased from Alfa Aesar. Purified hTopoII α was purchased from Affimetrix (Santa Clara, CA). hTopoII β was received from Austin C.A. and purified as published previously.[1] High purity supercoiled plasmid NTC7485E-U6-shRNA containing triplex forming insert[2] was purchased from Nature Technology Corp. (Lincoln, NE). The probe oligonucleotide 5'-BODIPY-TMR-TTCTTCTTCT was purchased from Trilink Biotechnologies (San Diego, CA). Potassium chloride, dithiothreitol, glycerol, magnesium sulfate, bovine serum albumin (BSA), ATP and DMSO were the highest purity grades available from Sigma-Aldrich (St. Louis, MO). Brij-35 was obtained from Thermo Fisher scientific/Pierce protein research (Rockford, IL). 1 M Tris-HCl (pH 7.5) stock solution was purchased from Life Technoligues/InvitrogenTM (Grand Island, NY). Deuterated NMR solvents were obtained from Armar Chemicals (Switzerland). Sodium boranocarbonate was a gift from Mallinckrodt Medical B.V. (The Netherlands). Na[$^{99m}TcO_4$] in 0.9% saline was eluted from a $^{99}Mo/^{99m}Tc$ UTK FM generator purchased from Mallinckrodt Medical B.V. (The Netherlands)

Characterization

Fluorescence spectra were recorded on a PerkinElmer Luminescence Spectrophotometer LS50B. IR spectra were recorded on a PerkinElmer Spectrum Two FT-IR spectrometer, equipped with a SPECAC Golden Gate™ ATR. ^{1}H-NMR and ^{13}C-NMR were recorded on a Bruker DRX 500 MHz spectrometer. ^{1}H and ^{13}C chemical shifts were referenced with the residual solvent resonances relative to TMS. Spectra were fully assigned to structures with the help of various experiments (1D NOE, 1H-COSY, C,H-Correlation and 13C-DEPT). Electrospray-ionization mass spectrometry (ESI-MS) was performed on a Bruker HCT™ spectrometer. High-resolution mass spectrometry (HR-MS) was performed on a Thermo DFS double-focusing system (ThermoFisher Scientific, Germany). Values are reported for the ^{187}Re isotope. Inductively-coupled plasma mass spectrometry (ICP-MS) measurements were performed on an Agilent QQQ 8800 Triple quad, equipped with a standard x-lens setting, nickel cones and a "micro-

mist" quartz nebulizer. Microwave reactions were perfomed using a Biotage Initiator+ Robot Eight instrument. Fluorescence anisotropy measurements were made using a Pherastar plate reader (BMG Labtech, Cary, NC) equipped with a fluorescence polarization optics module with a 540 nm excitation filter and 590 nm filters for parallel and perpendicular emission. All filters had 20 nm bandwiths.

Preparative HPLC was performed on a Varian ProStar 320 system, using a Dr. Maisch Reprosil C18 100-7 (40 x 250 mm) column. HPLC solvents were 0.1% trifluoroacetic acid and acetonitrile, HPLC grade.

Analytical HPLC was performed on a Merck Hitachi L7000 system, equipped with a L-7400 UV-detector and an in-line radio-detector Berthold FlowStar LB513, using an analytical Macherey-Nagel Nucleosil C18 5 μm (4.6 x 250 mm) column. HPLC solvents were 0.1% trifluoroacetic acid (solvent A) and acetonitrile, HPLC grade (solvent B).

Gradient A1:

Time:	A:	B:
0.0	100	0
3.0	100	0
3.1	75	25
9.0	75	25
9.1	66	34
19.0	7	93
19.1	0	100
25.0	0	100
25.1	100	0
30	100	0

Flow rate: 0.5 ml/min

Gradient A2:

Time:	A:	B:
0.0	100	0
3.0	100	0
3.1	75	25
9.0	75	25
9.1	66	34
20	0	100
25.0	0	100
25.1	100	0
30	100	0

0.5 ml/min

Detector: 250 nm

UPLC-ESI-MS was performed on a Waters Acquity UPLC System coupled to a Bruker HCT^{TM}, using an Acquity UPLC BEH C18 1.7 μm (2.1 x 50 mm) column. UPLC solvents were formic acid (0.1% in millipore water) (solvent A) and acetonitrile HPLC grade (solvent B).

Gradient U1:

Time:	A:	B:
0.0	95	5
0.25	95	5
1.25	0	100
2.5	0	100

Flow rate: 0.6 ml/min

Detector: DAD, monitoring at 250 nm and 480 nm

Methods

Distribution coefficient

The distribution coefficient ($\log D_{\text{octanol/PBS}}$) was determined according to a modification of a method published previously.[3] In short, purified compounds were dissolved in phosphate buffer (10 mM, pH 7.4), previously saturated with *n*-octanol, to a volume of about 1 ml (activity concentration of *ca*. 1 MBq/ml). An equal volume of *n*-octanol (presaturated with 10 mM phosphate buffer) was then added and the solution was vortexed for 1 min. After an equilibration time of 5 h, the radioactivity in the two phases was evaluated by a gamma counter. The $\log D_{\text{octanol/PBS}}$ is calculated according to equation (1) and expressed as the mean ± standard deviation of five individual measurements.

$$\log D_{\text{octanol/PBS}} = \log\left(\frac{[analyte]_{\text{octanol}}}{[analyte]_{\text{PBS}}}\right) \qquad (1)$$

DNA-binding affinity

DNA solutions were prepared by dissolving 1 mg ctDNA (sodium salt, Sigma Aldrich) in PBS buffer (10 mM, pH 7.4). The exact concentration was determined by UV/VIS by using ε_{260}= 6600 $M^{-1}cm^{-1}$ per nucleotide. Test compounds were dissolved in PBS buffer (10 mM, pH 7.4) with NaCl (50 mM) at a constant concentration in a 3 ml cuvette and the afore mentioned solution of ctDNA (sodium salt) was then added in aliquots of max 2 μl. After every addition, samples were left to equilibrate at room temperature for 5-10 min and the emission spectrum (510 - 700 nm) was recorded (excitation at 488 nm, excitation slit 5 nm, emission slit 20 nm, scan speed 100 nm/min) to give F_{obs}= F_{593nm}. Additions of DNA were carried out until changes in the spectra were incremental, at that point the minimum fluorescence (F_{min}) was determined by using an excess of 50-100 equivalents of DNA/intercalator. F_{bound} was then calculated by the following equation:

$$F_{bound} = \frac{F_{obs} - F_{\max}}{F_{\min} - F_{\max}} \qquad (2)$$

where F_{obs} is the fluorescence of intercalator at a given DNA concentration, F_{max} is the fluorescence of the intercalator in the absence of DNA and F_{min} is the fluorescence of the intercalator when completely bound to DNA. The DNA binding constant (K_b) was determined by fitting F_{bound} to the "Bard equation":[4,5]

$$\frac{(F_{obs} - F_{\max})}{(F_{\min} - F_{\max})} = \frac{\sqrt{b - \left(b^2 - 2K_b^2 C\frac{[DNA]}{s}\right)}}{2K_b[DNA]} \qquad (3)$$

$$b = 1 + K_b C + K_b\frac{[DNA]}{2s} \qquad (4)$$

where [*DNA*] is the molar concentration of ctDNA per nucleotide, *C* is the total concentration of the intercalator and *s* is the binding site size in base pairs. From plots of F_{bound} versus [*DNA*], K_b values were calculated from fitting with OriginLab 8.6.

Cell culture

The HeLa cervical cancer cell line was cultivated in DMEM medium (Gibco) supplemented with 5 % fetal calf serum (FCS, Gibco) 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C and 6% CO_2.

Chemotoxicity

Cytotoxicity of cisplatin, ADR, **4** and **6** on HeLa cells was assessed by a fluorometric cell viability assay using Resazurin (Promocell GmbH). Cells were plated in triplicates in 96-well plates at a density of 4×10^3 cells/well in 100 μl, 24 h prior to treatment. Cells were then treated with increasing concentrations of test samples for 48 h. The medium was replaced by complete medium containing resazurin (0.2 mg/ml final concentration) and incubated for 4 h at 37 °C. Finally, the fluorescence of the highly red fluorescent resorufin product was quantified (590 nm emission with 540 nm excitation wavelength) in a SpectraMax M5 microplate Reader.

Fluorescence microscopy

Cellular localization of ADR, **4** and **6** was performed by confocal fluorescence microscopy. HeLa cells were grown on 18 mm Menzel-gläser coverslips in 2 ml complete medium at a density of 1×10^5 cells per ml and incubated for 2 h with the compounds (**4**: 20 μM, **6**: 20 μM, ADR: 1 μM). Cells were fixed in formaldehyde solution (4% formaldehyde in PBS) and mounted on slides in Vectashield® solution containing DAPI prior to viewing by confocal microscopy on a CLSM Leica SP5 microscope. ADR and its derivatives were excited at 488 nm and emission above 600 nm was recorded.

Human topoisomerase II α and β inhibition assay

Sample preparation

Inhibitors **4** and **6** were dissolved at 20 mM in DMSO. Serial 2-fold dilutions were prepared and transferred to a black polystyrene 384-well assay plate (Thermo Fisher Scientific/Matrix Technology Corp., Hudson, NH) at 2% of the final assay volume (0.6 μl). ATP-dependent DNA relaxation assays in 30 μl contained either 3 U/ml hTOPOII α or 90 *n*g/ml hTOPOII β, 85 μg/ml (10 nM) supercoiled plasmid and 1 mM ATP in 50 mM Tris-HCl (pH 7.5), 150 mM KCl, 5% (v/v) glycerol, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.002% (v/v) Brij-35, and 200 nM bovine serum albumin (BSA). To allow for correction for interference in fluorescence measurements by compounds using the method of Shapiro *et al*.,[6] a duplicate plate was prepared in the same way except for the omission of the enzyme. Reactions, conducted at room temperature, were quenched after 1 h by addition of 10 μl of a solution containing 40

nM 5'-BODIPY-TMR-TTCTTCTTCT oligodeoxyribonuleotide in 300 mM NaCl and 300 mM sodium acetate, adjusted to pH 3.5 with acetic acid.

Fluorescence anisotropy measurements

Parallel and perpendicular fluorescence intensities were measured 1 h after addition of the quencher using a Pherastar plate reader (BMG Labtech, Cary, NC). The focal height was 7.0 mm and each measurement was the average of 50 flashes. The gains on parallel and perpendicular emission detectors were set to 1530 and 1558, respectively. After correcting parallel and perpendicular fluorescence measurements separately for interference by compounds[6], the anisotropy was calculated. The %-inhibition at each compound concentration was calculated by:

$$\%\text{-inhibition} = 100\left[1 - \frac{(r - r_{\min})}{(r_{\max} - r_{\min})}\right] \qquad (5)$$

where r is the anisotropy measurement in the presence of the inhibitor, $r_{\min}$ is the anisotropy measurement in the absence of the enzyme, and $r_{\max}$ is the anisotropy of the uninhibited enzyme. The IC_{50} was calculated by nonlinear least-squares regression of the %-inhibition data to equation (6):

$$\%\text{-inhibition} = \frac{100[I]^b}{(IC_{50} + [I]^b} \qquad (6)$$

where $[I]$ is the concentration of inhibitor and b is the Hill slope.

ICP-MS quantification

Sample preparation

To assess the cellular distribution of ^{99m}Tc-complexes **3**/**5** and Re-complexes **4**/**6**, compartmental fractionation studies were performed in HeLa cells. Adherent and confluent cells (T75 culture flask) were incubated with ^{99m}Tc-complexes **3** and **5** (~12 MBq in culture medium) or Re-complex **4** and **6** (3 ml of a 20 μM solution in culture medium) for 2 h at 37ºC and 5% CO_2. The harvested cell suspension with 10-20 × 10^6 cells was split in 3 fractions (2 ml each), centrifuged at 850 *g* for 2 min at 4ºC (Centrifuge 5804R, Eppendorf) and cells washed twice with cold PBS to remove the unbound complex. An aliquot of 0.1 ml of cells suspension (whole-cell fraction) was separated for the cellular uptake determination. To obtain nuclear and mitochondrial fractions, the remaining cells suspension (1.9 ml) was treated with the "Mitochondria Isolation Kit for Cultured cells" (Thermo Scientific) according the manufacturer's protocol. Briefly, the pellet (about one third of total pellet: 2 × 10^7 cells) was resuspended with reagent A (0.8 ml/2 × 10^7 cells) supplemented with a cocktail of protease inhibitors (Roche, Basel, Switzerland), vortexed at medium speed for 5 s and incubated on ice for exactly 2 min. Reagent B (10 μl / 2 × 10^7 cells) was added and the samples were vortexed at maximum speed for 5 seconds, incubated on ice and vortexed every minute for 5 min. Then, reagent C (0.8 ml/2 × 10^7 cells), supplemented with a cocktail of protease inhibitors, was added, and the cells suspension

centrifuged for 10 min at 700 *g* at 4ºC (Centrifuge 5417R, Eppendorf). The nucleus isolation steps were monitored by trypan blue staining under a phase contrast microscope. The resulting pellet corresponded to the nuclear fraction. The supernatant, transferred to a new tube, was centrifuged for 15 min at 3'000 *g* at 4 ºC to remove the lysosomal and peroxisomal contaminants. The pellet, containing the isolated mitochondria, was again treated with reagent C (0.5 ml/2 × 10^7 cells) and centrifuged for 5 min at 12'000 *g*. The resulting mitochondria pellet (mitochondrial fraction) as well as the whole-cell fraction and the nuclear fraction were directly counted in a dose calibrator (Berthold, LB2111) in the case of **3** and **5** or lyophilized and stored until determination of Re content by ICP-MS.

ICP-MS measurements

Rhenium was measured against a Re single element standard (Merck 170344.100) and verified by a control (Agilent5188-6524 PA Tuning 2). Rhenium content of the samples was determined by means of a 7-step serial dilution in the range between 0 and 300 ppb in Re (R> 0.99) with a background equivalent concentration of BEC: 9.4 ppt and a detection limit of DL: 2.6 ppt. The isotope Re^{185} (37.4% abundance) Re^{187} (62.6% abundance) was evaluated in "no-gas" mode and He-gas mode. Spiking the samples with untreated negative controls (to account for potential carbon content from the biological samples) resulted in equivalent values within error ranges. A solution of indium (500 ppb) and tungsten (500 ppb) was used as internal standard. The results are expressed as ng Re / mg protein (correction due to the different masses of the observed cellular compartments), as means ± standard deviations of three experiments.

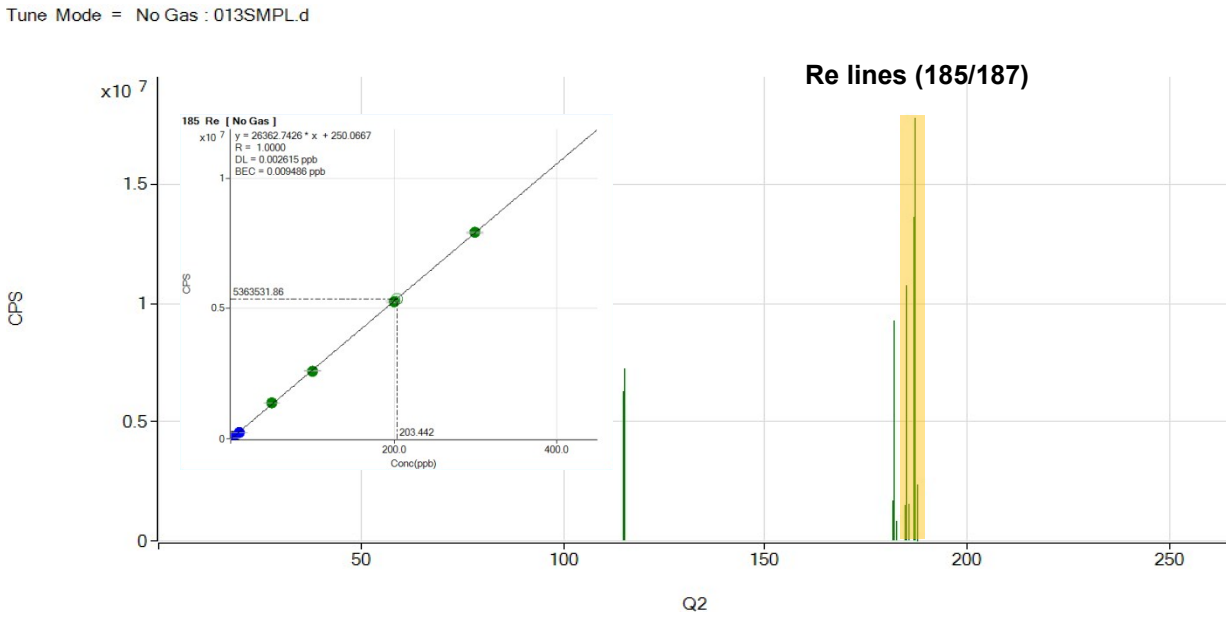
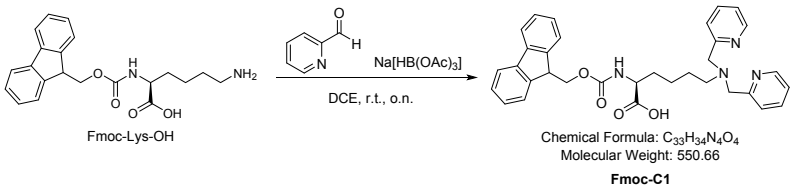


Figure 1. Example of the rhenium lines observed during ICP-MS measurements. Inset: Calibration curve of the actual measurements. Detection limit (DL): 2.6 ppt, background equivalent concentration (BEC): 9.4 ppt.

Synthesis

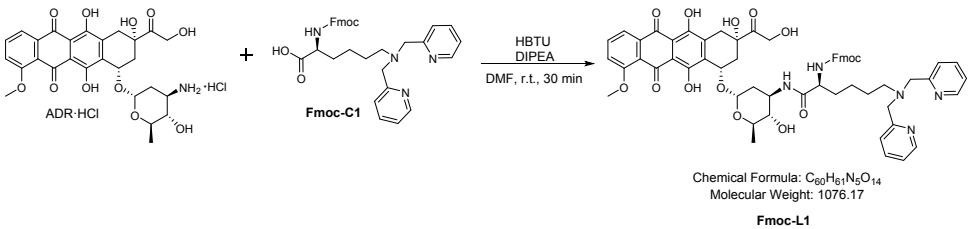
Chart S 1 – Experimental data of **L1**·2TFA



Fmoc-C1 *as in Levadala et al.[7], Armstrong et al.[8]*

Fmoc-Lys-OH (0.5 g, 1.35 mmol) was suspended in 10 ml of 1,2-dichloroethane. Upon addition of picolinaldehyde (269 μl, 2.84 mmol, 2.1 eq.) the solution was stirred for about 30 min. In this time the solution turned orange and a precipitate formed. Thereafter, $Na[HB(OAc)_3]$ (858 mg, 4.05 mmol, 3 eq.) was added in portions. Stirring this mixture overnight resulted in a bright orange solution. The solvent was evaporated under reduced pressure and the remaining $Na[HB(OAc)_3]$ quenched by a sat. $NaHCO_3$ solution until no further effervescence was observed. After concentration to a yellowish gum, the mixture was dissolved in $CHCl_3$ and washed with fresh H_2O and sat. NaCl. The combined organic phases were dried over $MgSO_4$ and after evaporation of the solvent under reduced pressure a brownish oil remained. This oil was eluted through a plug of silica gel (2 × 4 cm) with a mixture of CH_2Cl_2/MeOH (6:1) and collected in fractions. The product-containing fractions were combined and reduced *in vacuo* to an off-white foam. Yield: 462 mg (0.84 mmol, 62%).

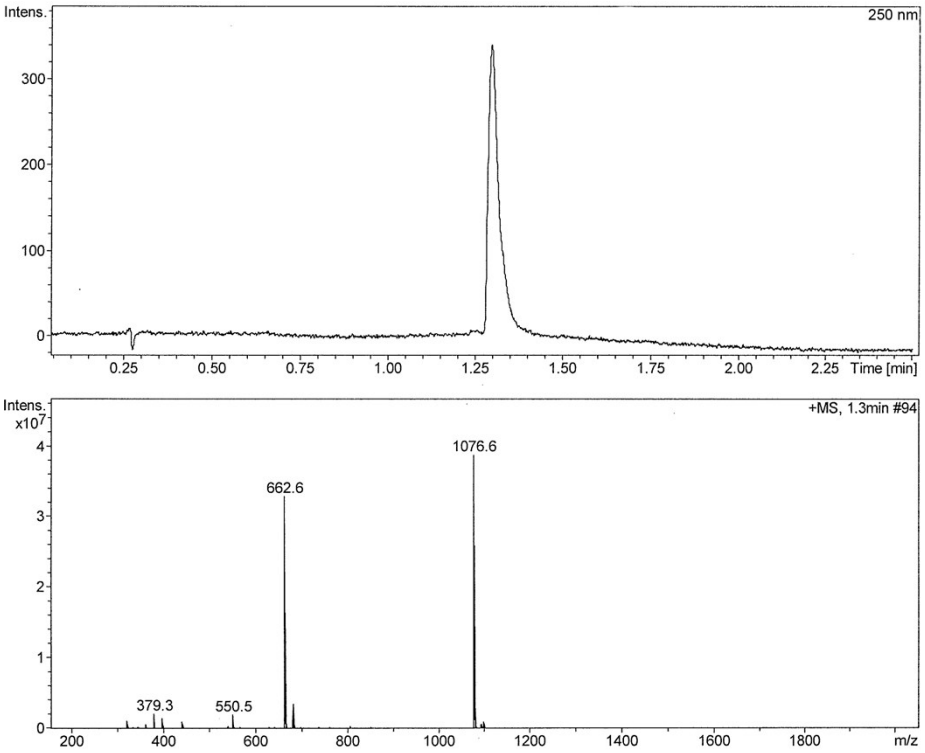
ESI^+-MS: m/z = 551.2 $[M]^+$, 573.3 $[M+Na]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 10.85 (bs, 1H), 8.50 (d, *J* = 5.1 Hz, 2H), 7.70 (d, *J* = 7.24 Hz, 2H), 7.55 (m, 4H), 7.46 (m, 2H), 7.32 (t, *J* = 7.7 Hz , 2H), 7.22 (t, *J* = 7.5 Hz, 2H), 7.09 (m, *J* = 6.2 Hz, 2H), 6.0 (bs, 1H), 4.29 (m, 3H), 4.17 (t, *J* = 6.2 Hz, 1H), 3.86 (bs, 4H), 2.57 (t, 2H), 1.90–1.20 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): d = 175.67, 157.13, 155.93, 148.03, 143.75, 143.56, 140.87, 137.04, 127.36, 126.76, 124.92, 123.44, 122.35, 119.63, 66.40, 58.66, 54.34, 53.68, 46.85, 32.21, 25.70, 22.71.



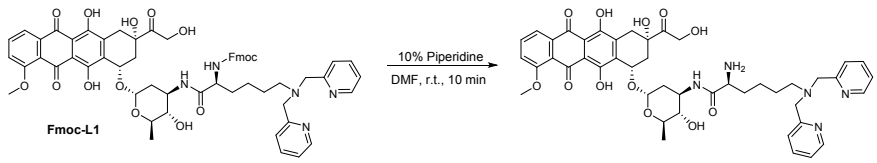
Fmoc-L1. ADR·HCl (25 mg, 43 μmol, 1.2 eq.), HBTU (41 mg, 0.109 mmol, 3 eq.) and **Fmoc-C1** (20 mg, 36 μmol, 1.1 eq) were added to a 10 ml flask under a nitrogen atmosphere. Anhydrous DMF (2 ml) was then added. To this suspension, DIPEA (19 μl, 0.111 mmol, 3 eq.) was added upon which the mixture became a homogenous and dark red solution. After 30 min, UPLC control (gradient U1) indicated complete consumption of ADR·HCl. DMF was

blown off in a nitrogen stream to yield a dark red oil. Upon addition of water (5 ml), a red precipitate formed. After sonication of the resulting suspension, the precipitate was collected with a glass sintered frit and washed thoroughly with 3 × 10 ml of water to remove remainders of unreacted ADR·HCl. This red powder was dried *in vacuo* and then washed out of the frit with CH_2Cl_2 (4 ml). Removing the solvent yielded in a dark red solid. The crude material was directly purified by preparative HPLC and lyophilized to a red foam. Yield (·TFA salt): 23.5 mg (19.7 μmol, 55%).

UPLC-ESI$^+$: R_t= 1.30 min, area ≥97%, m/z= 1076.6 $[M+H]^+$. HR-MS (ESI$^+$) calcd. for $[C_{59}H_{62}N_5O_{14}+H]^+$: 1076.4288, found: 1076.4272, calcd. for $[C_{60}H_{61}N_5O_{14}+Na]^+$: 1098.4107, found: 1098.4095.



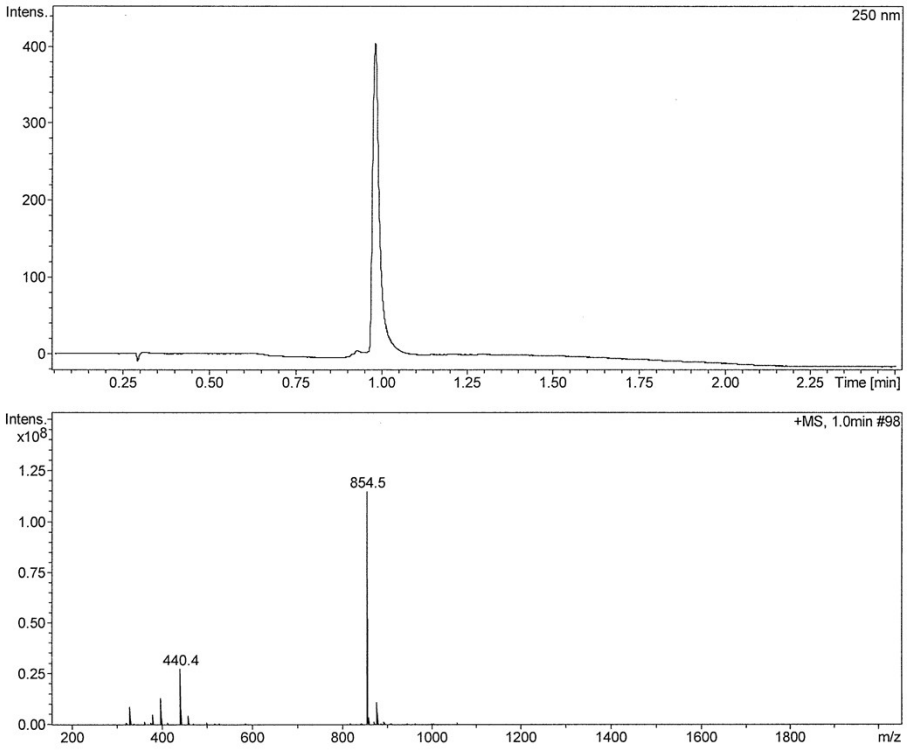
Compound	Retention Time [min]	Area [%]	Molecular Formula	Exact Mass [m/z]	Mass Found [m/z]
Fmoc-L1	1.30	≥97	$C_{60}H_{61}N_5O_{14}$	1075.42	1076.6 $[M+H]^+$



L1. Deprotection of the Fmoc-group was accomplished either by dissolving **Fmoc-L2** (20 mg, 12 μmol) in a solution of 10% piperidine in DMF or 2% DBU in DMF and stirring at room temperature for 10 min. Piperidine in DMF proved somewhat superior. The reaction was followed by UPLC (gradient U1) and upon complete deprotection, the reaction was quenchend by adding a solution of TFA (2% in H_2O)/MeCN (50:50) and immediate purification by preparative HPLC. CAUTION: Prolonged deprotection under the conditions indicated above results in product decomposition! Yield (·2TFA salt): 10 mg (7.4 μmol, 62%).

UPLC-ESI$^+$: R_t= 0.98 min, area ≥97%. m/z= 854.5 $[M+H]^+$. ^{1}H-NMR (500 MHz, CD_3OD) δ = 8.64-8.63 (m, 2H), 7.90 (dt, 2H, *J* = 7.5 Hz, *J* = 1.5 Hz), 7.85-7.80 (m, 1H), 7.80-7.75 (m, 1H), 7.53-7.48 (m, 3H), 7.46 (dd, 2H, *J* = 8 Hz, *J* = 5 Hz), 5.39 (bs, 1H), 5.04 (bs, 1H), 4.72 (d, 2H, *J* = 2.5 Hz), 4.56 (s, 4H), 4.26 (q, 1H, *J* = 6.5 Hz), 4.18-4.16 (m, 1H), 4.00 (s, 3H), 3.83 (t, 1H, *J* = 6.5 Hz), 3.56 (bs, 1H), 3.26 (t, 2H, *J* = 8 Hz), 3.00 (d, 1H, *J* = 18 Hz), 2.86-2.82 (m, 1H), 2.34 (d, 1H, *J* = 15 Hz), 2.13 (dd, *J* = 15 Hz, *J* = 4.5 Hz), 1.99 (dt, 1H, *J* = 8 Hz, *J* = 4.5 Hz), 1.93-1.74 (m, 5H), 1.43 (qi, 2H, *J* = 7.5 Hz), 1.24 (d, 3H, *J* = 6.5 Hz) ppm. ^{13}C-NMR (125 MHz, CD_3OD) δ = 214.83, 188.16, 187.80, 169.47, 162.54, 157.43, 156.28, 152.07, 150.31, 139.89, 137.43, 136.37, 135.80, 135.33, 125.75, 125.71, 121.49, 120.67, 120.45, 102.10, 77.39, 71.41, 70.00, 68.64, 65.80, 58.64, 57.27, 55.99, 54.13, 47.65, 37.37, 34.01, 32.27, 30.55, 25.17, 23.06, 17.38 ppm. HR-MS (ESI$^+$) calcd. for $[C_{45}H_{50}N_5O_{12}+H]^+$: 854.3607, found: 854.3592, calcd. for $[C_{45}H_{51}N_5O_{12}+Na]^+$: 876.3426, found: 876.3413.

Chart S 2 – Chromatographic data and purity of **L1**·2TFA



Compound	Retention Time [min]	Area [%]	Molecular Formula	Exact Mass [m/z]	Mass Found [m/z]
L1	0.98	≥97	$C_{45}H_{51}N_5O_{12}$	853.93	854.5 $[M+H]^+$

Chart S 3 – Nummerical assignement for NMR **L1**·2TFA

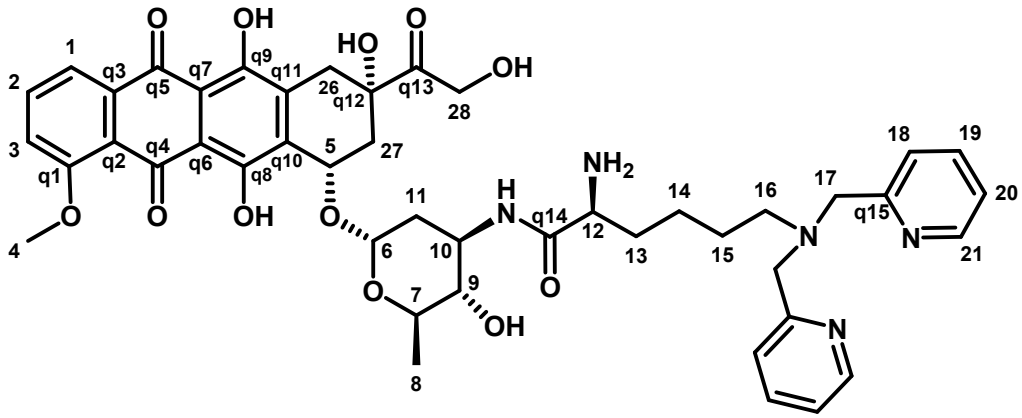


Chart S 4 – ^{1}H-NMR of **L1**·2TFA, 500 MHz, CD_3OD

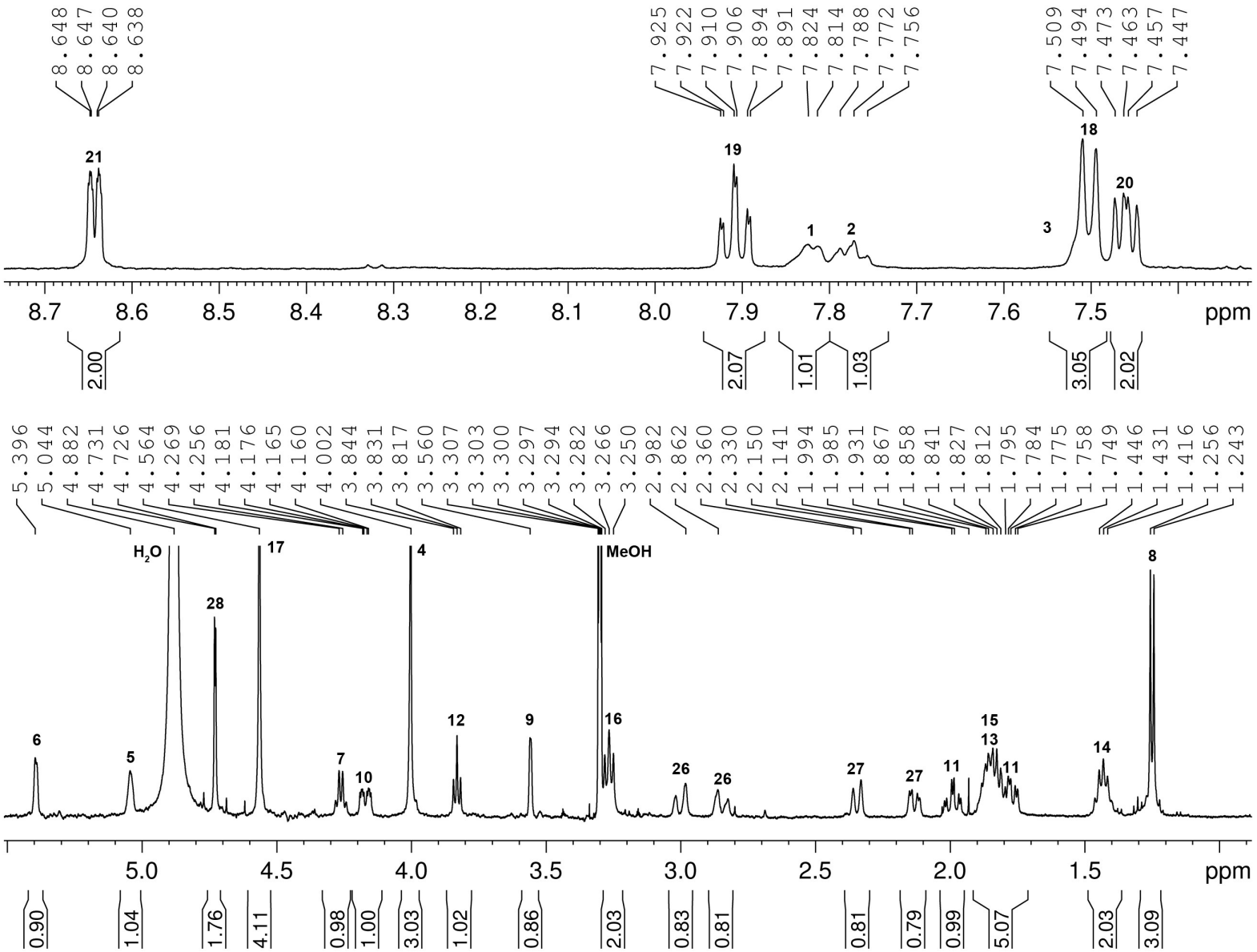


Chart S 5 – ^{13}C-NMR of **L1**·2TFA, 125 MHz, CD_3OD

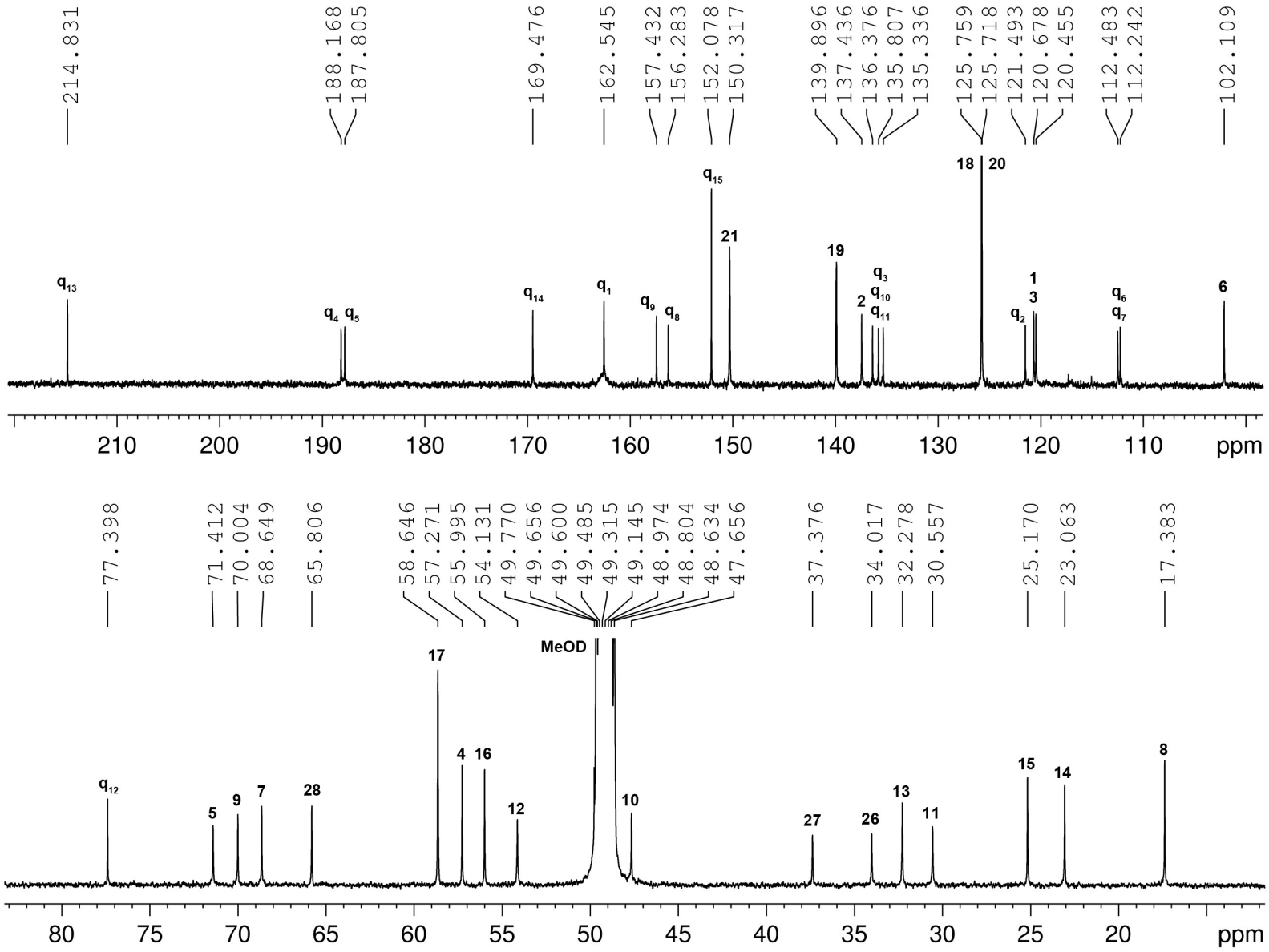


Chart S 6 – C,H-Correlation of **L1**·2TFA

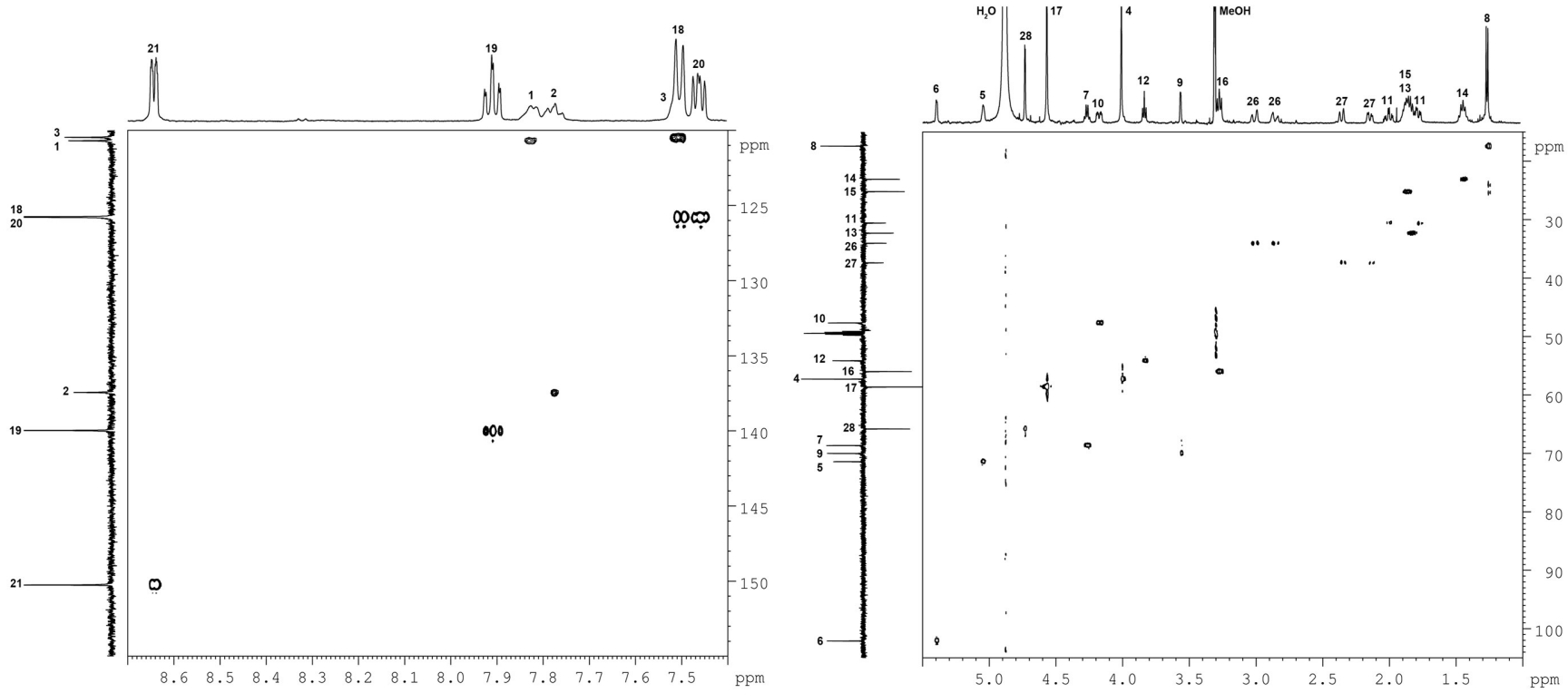
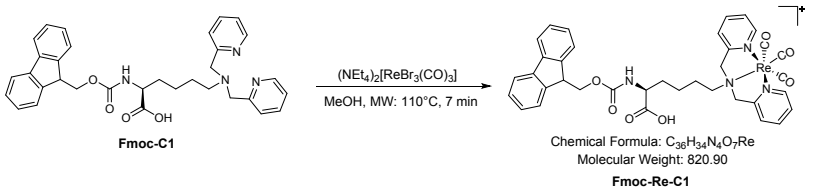


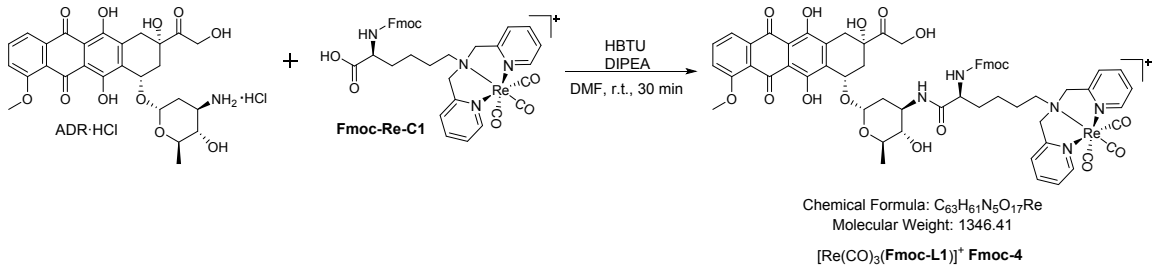
Chart S 7 – Experimental data of **4**·2TFA



Fmoc-Re-C1 *as in Levadala et al.[7], Armstrong et al.[8]*

$(NEt_4)_2[ReBr_3(CO)_3]$ was synthesized according to a literature procedure.[9] A 2-ml microwave vial was charged with **Fmoc-C1** (10 mg, 18 μmol) and $(NEt_4)_2[ReBr_3(CO)_3]$ (28 mg, 36 μmol, 2 eq.). MeOH (1 ml) was added to dissolve the solids and the solution was heated to 110 °C for 8 min in a microwave oven. UPLC (gradient U1) showed quantitative conversion to one main product and a small side product (OMe-ester, 5-10%). MeOH was blown off in a stream of nitrogen to yield a yellowish gum. This residue was triturated with 1 ml H_2O upon which a white precipitate formed. After centrifugation and decanting of the water, the remaining grey solid was dried *in vacuo*. This product was used in the next step without further purification. Yield (Br^- salt): 14.2 mg (15.7 μmol, 87%).

UPLC-ESI$^+$: R_t= 1.25 min, area ≥80%. m/z= 820.9 $[M]^+$.

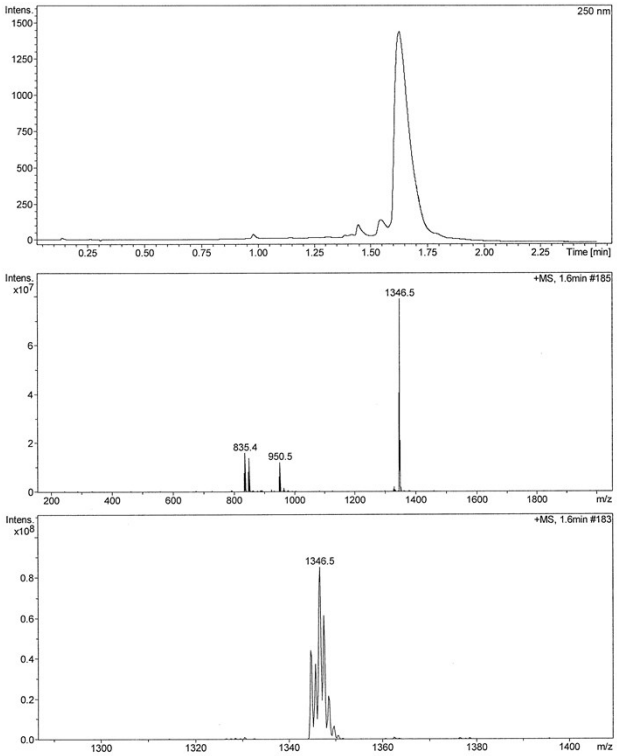


$[Re(CO)_3$(Fmoc-L1)$]^+$ (Fmoc-4). ADR·HCl (11 mg, 19 μmol, 1.2 eq.), HBTU (22 mg, 57 μmol, 3 eq.) and [**Fmoc-Re-C1]Br** (14.2 mg, 15.7 μmol) were added to a 10 ml flask under a nitrogen atmosphere. Anhydrous DMF (2 ml) was then added. To this suspension, DIPEA (10 μl, 60 μmol, 3 eq.) was added upon which the mixture became a homogenous and dark red solution. After 30 min, UPLC control (gradient U1) indicated complete consumption of ADR·HCl. DMF was blown off in a nitrogen stream to yield a dark red oil. Upon addition of water (5 ml), a red precipitate formed. After sonication of the resulting suspension, the precipitate was collected with a glass sintered frit and washed thoroughly with 3 × 10 ml of water to remove remainders of unreacted ADR·HCl.

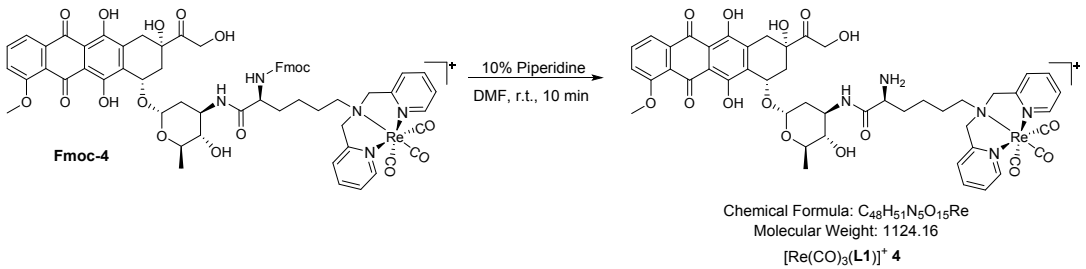
This red powder was dried *in vacuo* and then washed out of the frit with CH_2Cl_2 (4 ml). Removing the solvent yielded in a dark red solid.

Note: The Fmoc-protected complex is an intermediate and was normally used in crude for the next step. Purification (for analysis purposes): The crude material was subjected to preparative TLC (SiO_2, MeCN/H_2O/1.3M KNO_3, 100:15:15) and the product band extracted with the eluent. To remove excess KNO_3, the remainder was passed through a column loaded with Sephadex G10, the product collected in fractions and dried *in vacuo* to a red solid. Yield (NO_3^- salt): 14.7 mg (10.5 μmol, 55%).

UPLC-ESI$^+$: R_t= 1.62 min, area ≥90%, m/z= 1346.5 $[M]^+$. IR (ATR): ν= 2030 (s, $\nu_{sym.}CO$), 1914 (s, $\nu_{asym.}CO$) cm^{-1}. ESI-MS (MeOH): m/z = 1346.5 M^+. HR-MS (ESI$^+$) calcd. for $[C_{63}H_{61}N_5O_{17}Re_1]^+$: 1346.3620, found: 1346.3631.



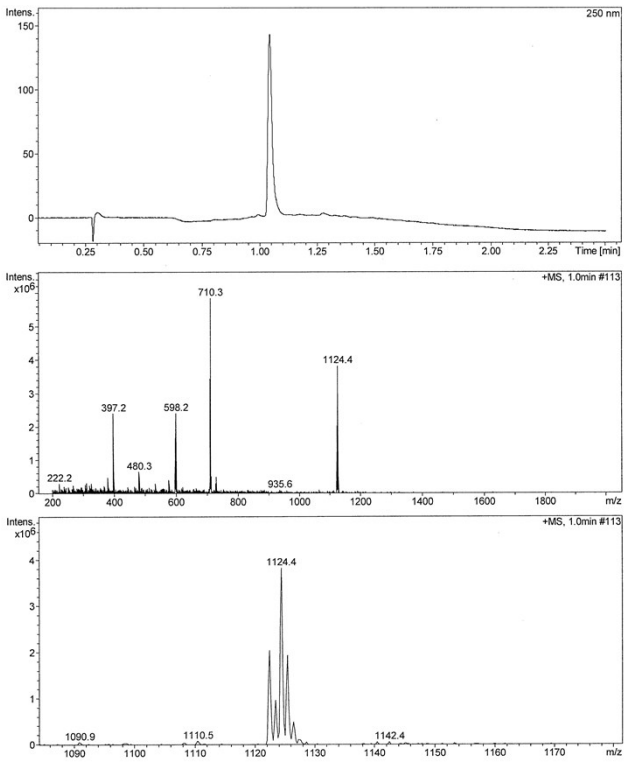
Compound	Retention Time [min]	Area [%]	Molecular Formula	Exact Mass [m/z]	Mass Found [m/z]
Fmoc-4	1.62	≥90	$C_{63}H_{61}N_5O_{17}Re$	1346.36	1346.5 $[M]^+$



[Re(CO)$_3$(L1)]$^+$ (4). Deprotection of the Fmoc-group was accomplished either by dissolving **[Fmoc-4]NO$_3$** (14.7 mg, 10.5 μmol) in a solution of 10% piperidine in DMF or 2% DBU in DMF and stirring at room temperature for 10 min. Piperidine in DMF proved somewhat superior. The reaction was followed by UPLC (gradient U1) and upon complete deprotection, the reaction was quenched by adding a solution of TFA (0.1% in H_2O)/MeCN (50:50) and immediate purification by preparative HPLC. CAUTION: Prolonged deprotection under the conditions indicated above results in product decomposition! Yield (·2TFA): 10 mg (7.4 μmol, 70%).

UPLC-ESI$^+$: R_t= 1.04 min, area ≥97%, m/z= 1124.4 [M]$^+$. ^{1}H-NMR (500 MHz, CD_3OD) δ = 8.84 (d, 2H, *J* = 5.5 Hz), 7.94 (m, 3H), 7.83 (t, 1H, *J* = 8.5 Hz), 7.56 (d, 1H, *J* = 8.5 Hz), 7.51 (d, 2H, *J* = 8 Hz), 7.36 (t, 2H, *J* = 6.5 Hz), 5.43 (m, 1H), 5.14 (m, 1H), 4.71 (d, 2H, *J* = 3.5 Hz), 4.28 (q, 1H, *J* = 6.5 Hz), 4.21 (m, 1H), 4.03 (s, 3H), 3.89 (t, 1H, *J* = 6.5 Hz), 3.81 (t, 2H, *J* = 8.5 Hz), 3.61 (bs, 1H), 3.09 (d, 1H, *J* = 18.5 Hz), 2.99 (d, 1H, *J* = 18.5 Hz), 2.37 (bd, 1H, *J* = 12.5 Hz), 2.18 (dd, 1H, *J* = 14.5 Hz, *J* = 5 Hz), 2.03-1.93 (m, 5H), 1.78 (dd, 1H, *J* = 13 Hz, *J* = 4.7 Hz), 1.52 (m, 2H), 1.24 (d, 3H, *J* = 7 Hz) ppm. ^{13}C-NMR (125 MHz, CD_3OD) δ = 214.71, 197.40, 196.39, 188.58, 188.21, 169.49, 162.73, 162.15, 157.56, 156.48, 153.39, 141.85, 137.44, 136.72, 135.96, 135.40, 127.12, 124.69, 121.88, 120.73, 120.50, 112.75, 112.48, 102.14, 77.46, 71.60, 71.53, 70.14, 68.94/68.89, 68.67, 65.78, 57.28, 54.29, 47.75, 37.59, 34.13, 32.61, 30.65, 25.99, 24.35, 23.33, 17.37 ppm. IR (ATR): ν= 2030 (s, $\nu_{sym.}$CO), 1925 (s, $\nu_{asym.}$CO) cm^{-1}. ESI-MS (MeOH): m/z = 1124.5 M$^+$. HR-MS (ESI$^+$) calcd. for $C_{48}H_{51}N_5O_{15}Re_1$: 1124.2934, found: 1124.2935.

Chart S 8 – Chromatographic data and purity of **4**·2TFA



Compound	Retention Time [min]	Area [%]	Molecular Formula	Exact Mass [m/z]	Mass Found [m/z]
4	1.04	≥97	$C_{48}H_{51}N_5O_{15}Re$	1124.29	1124.4 $[M]^+$

Chart S 9 – Nummerical assignement for NMR **4**·2TFA

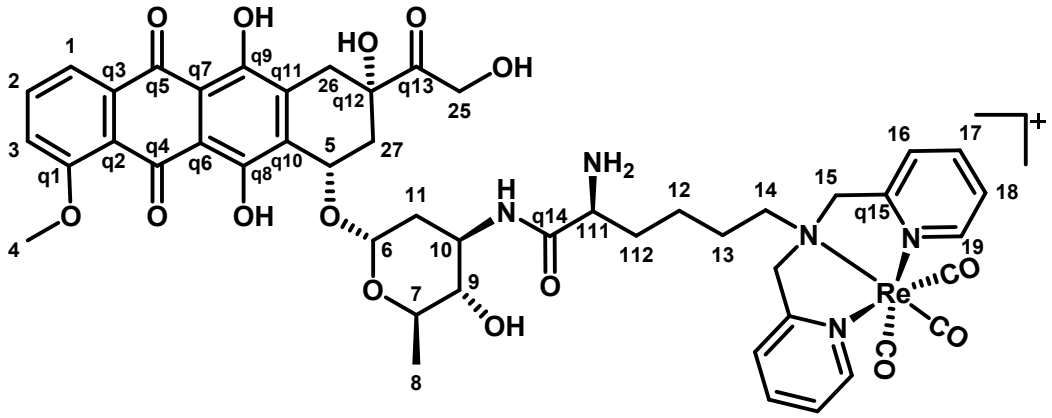


Chart S 10 – ^{1}H-NMR of **4**·2TFA, 500 MHz, CD_3OD

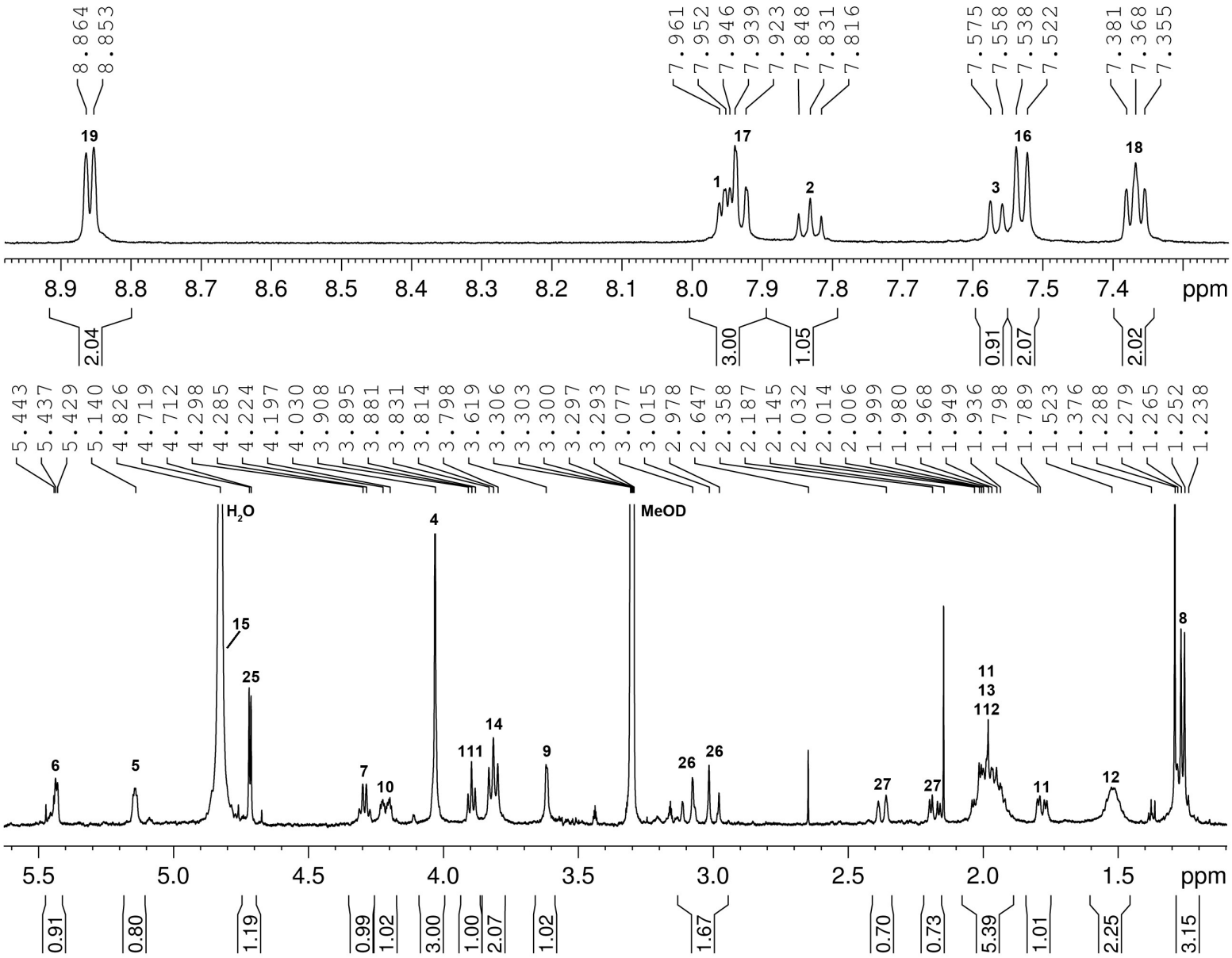


Chart S 11 – ^{13}C-NMR of **4**·2TFA, 125 MHz, CD_3OD

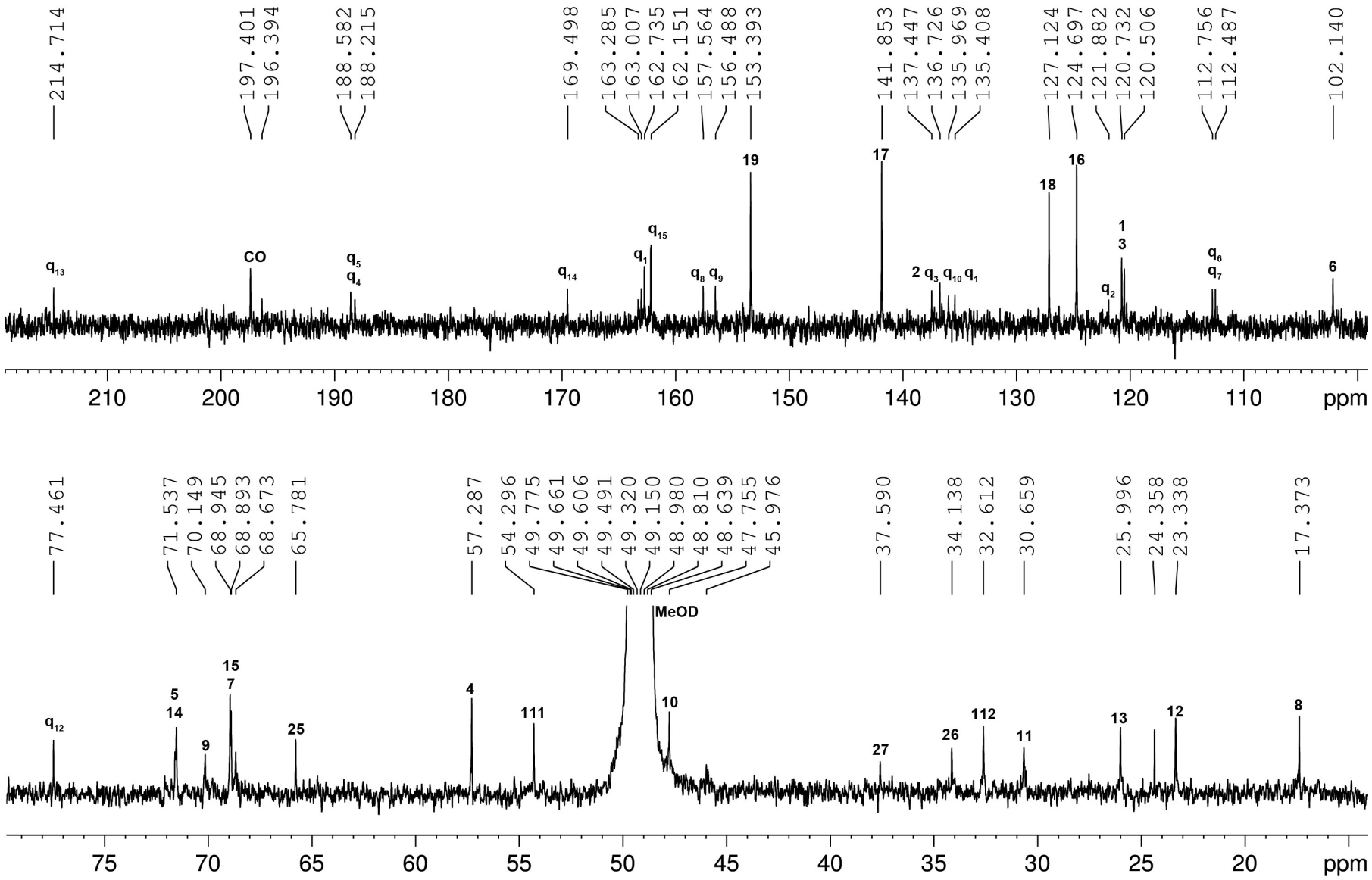


Chart S 12 – C,H-Correlation of **4**·2TFA

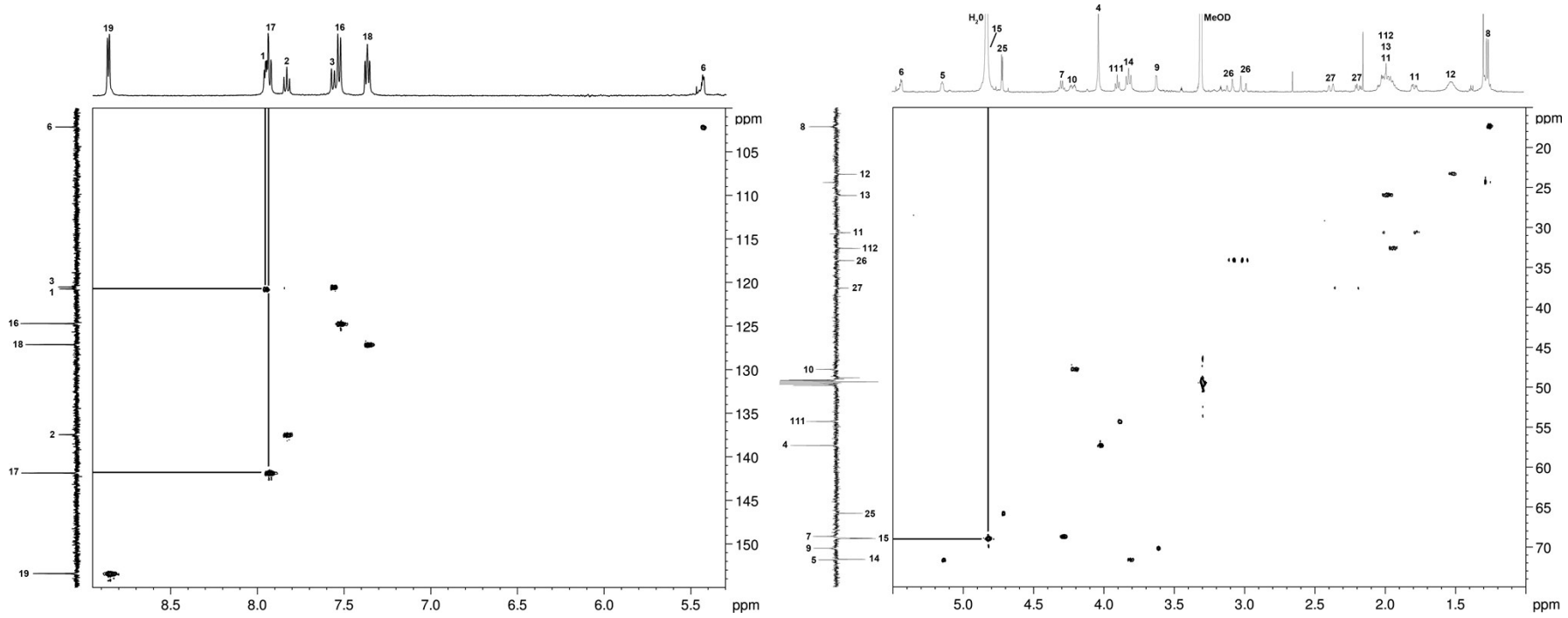
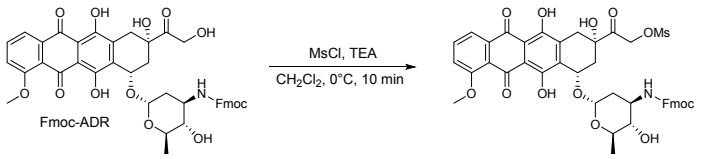


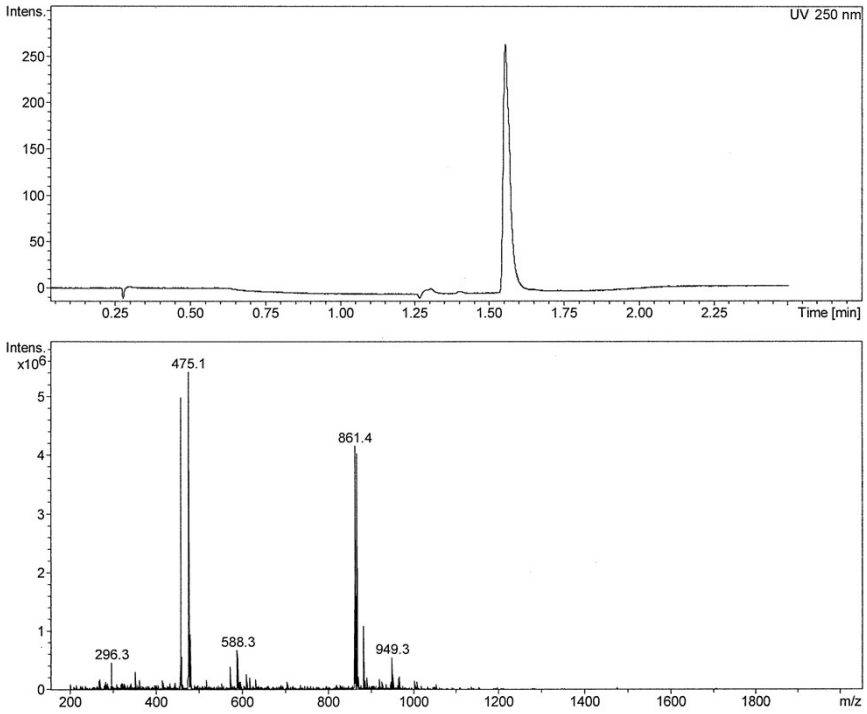
Chart S 13 – Experimental data of **L2**·3TFA



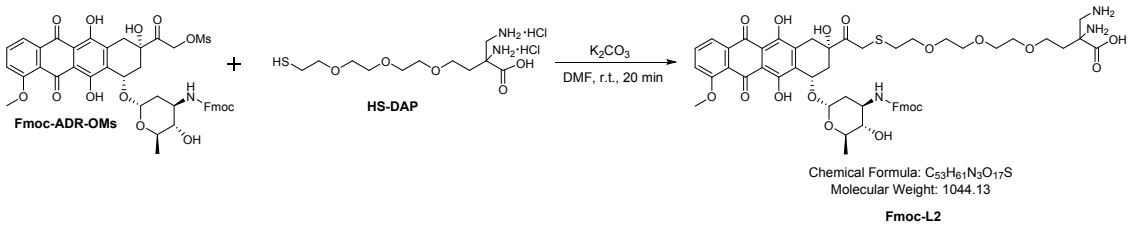
Chemical Formula: $C_{43}H_{41}NO_{15}S$
Molecular Weight: 843.85

Fmoc-ADR-OMs. Fmoc-ADR was prepared according to a literature procedure.[10] Fmoc-ADR (20 mg, 26 μmol) was dissolved in dry CH_2Cl_2 (2 ml) under a N_2-atmosphere. To this solution was added TEA (50 μl, 0.365 mmol, 14 eq.) while it was cooled to 0°C with vigorous stirring to yield in a dark red mixture. Methanesulfonyl chloride (4.82 μl, 70 μmol, 2.7 eq.) was then added in tiny steps of 0.5 - 1 μl per 3 min with a pipette and the progress of the reaction was carefully monitored by UPLC (gradient U1). After 95% consumption of Fmoc-ADR, MeOH (0.1 ml) was added to the reaction mixture to quench excess MsCl and the solvent was blown off in a N_2-stream, yielding in a dark red solid. This solid was either used directly in the next step or purified by preparative HPLC (for analysis purposes) which yielded in a red foam after lyophilization. Yield: 16 mg (18 μmol, 69%).

UPLC-ESI$^+$: R_t= 1.55 min, area ≥98%, m/z= 861.4 $[M+NH_4]^+$. HR-MS (ESI$^+$) calcd. for $[C_{43}H_{41}N_1O_{15}S_1+Na]^+$: 866.2089, found: 866.2099.



Compound	Retention Time [min]	Area [%]	Molecular Formula	Exact Mass [m/z]	Mass Found [m/z]
Fmoc-ADR-OMs	1.55	≥98	$C_{43}H_{41}N_1O_{15}S$	843.22	861.4 $[M+NH_4]^+$

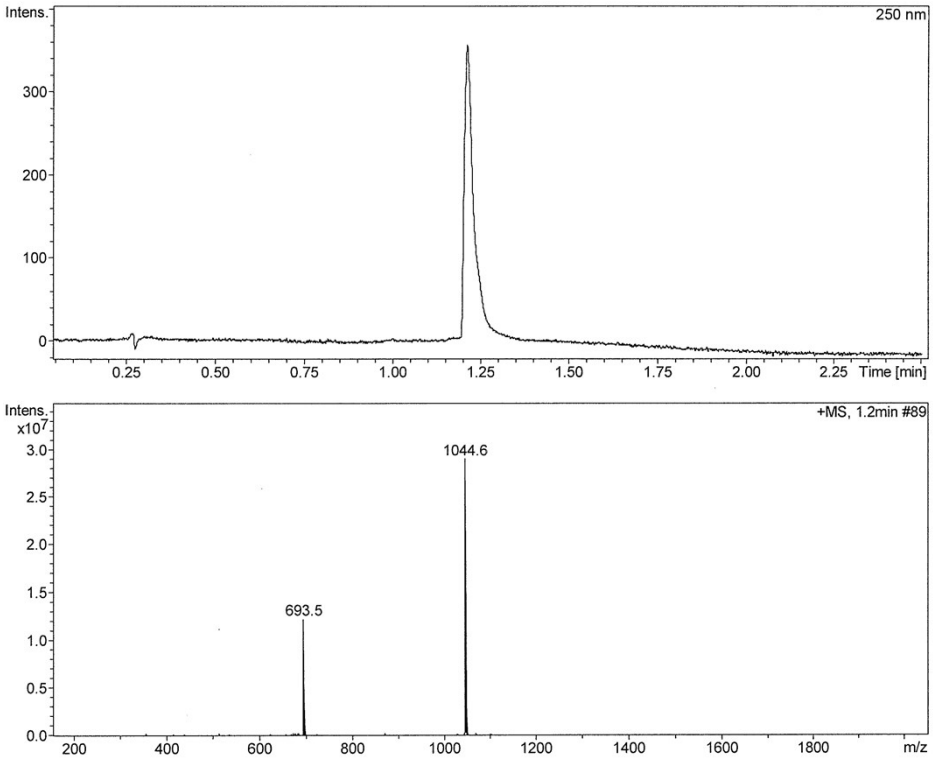


Fmoc-L2 *adapted from Seshadri et al.*[11]

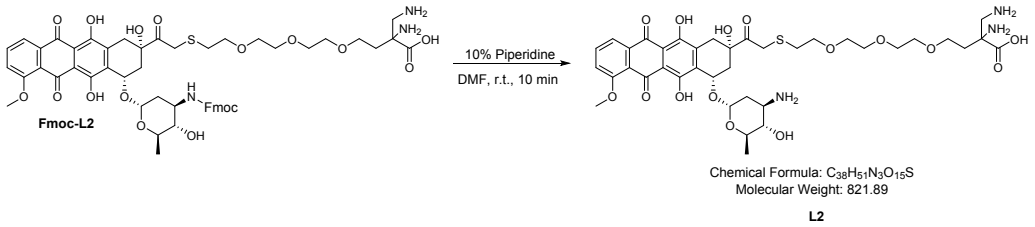
rac-**HS-DAP** was received as a research sample from Felber *et al.* The synthesis is reported elsewhere.[12]

Fmoc-ADR-OMs (9 mg, 10.6 μmol) was dissolved in anhydrous DMF (0.5 ml). **HS-DAP** (6.7 mg, 12.6 μmol, 1.2 eq.) was dissolved separately in anhydrous DMF (0.5 ml) and added to the first solution. Addition of finely ground K_2CO_3 (6 mg, 42.7 μmol, 4 eq.) afforded a deep red solution which turned dark over time. Reaction control *via* UPLC (gradient U1) revealed full consumption of the ADR after 20 min. The reaction solution was then diluted with a mixture of 0.1% TFA in H_2O/MeCN (50:50) and directly subjected to preparative HPLC to afford a dark red foam after lyophilization. Yield (·2TFA): 8.1 mg (6.3 μmol, 60%).

UPLC-ESI$^+$: R_t= 1.21 min, area ≥97%, m/z= 1044.6 $[M+H]^+$. HR-MS (ESI$^+$) calcd. for $[C_{53}H_{61}N_3O_{17}S_1+H]^+$: 1044.3799, found: 1044.3795.



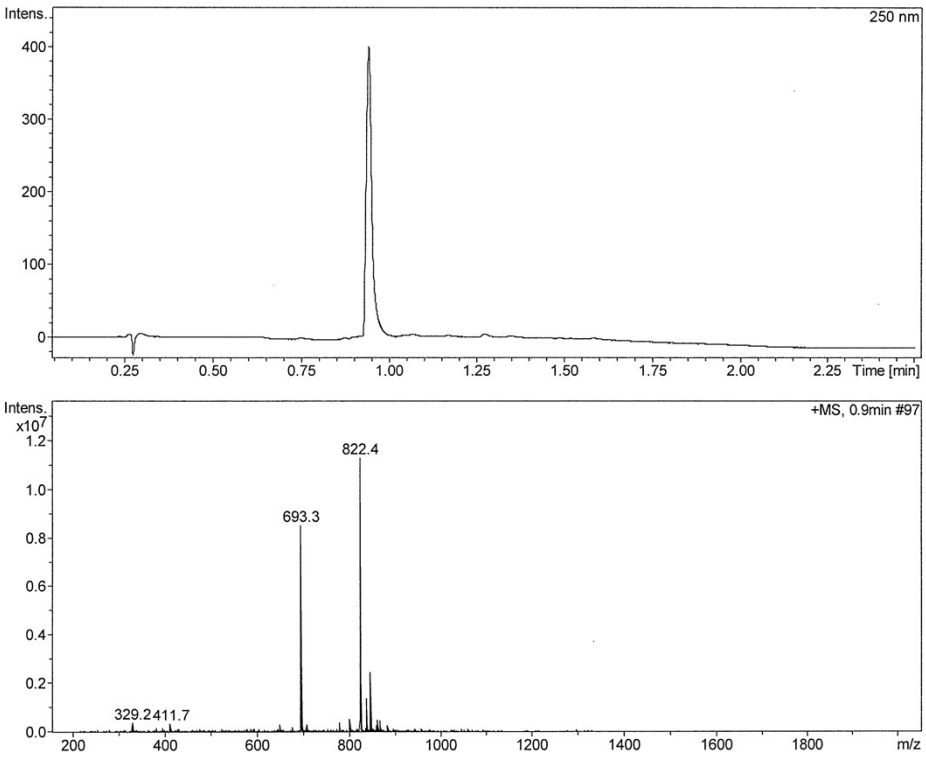
Compound	Retention Time [min]	Area [%]	Molecular Formula	Exact Mass [m/z]	Mass Found [m/z]
Fmoc-L2	1.21	≥97	$C_{53}H_{61}N_3O_{17}S$	1043.37	1044.6 $[M+H]^+$



L2. Deprotection of the Fmoc-group was accomplished either by dissolving **Fmoc-L2** (17 mg, 12 μmol) in a solution of 10% piperidine in DMF or 2% DBU in DMF and stirring at room temperature for 10 min. Piperidine in DMF proved somewhat superior. The reaction was followed by UPLC (gradient U1) and upon complete deprotection, the reaction was quenchend by adding a solution of 0.1% TFA in H_2O/MeCN (50:50) and immediate purification by preparative HPLC. CAUTION: Prolonged deprotection under the conditions indicated above yields to product decomposition! Yield (·3TFA, mixture of diastereomers): 9.3 mg (8 μmol, 67%).

UPLC-ESI$^+$: R_t= 0.94 min, area ≥97%, m/z= 822.4 $[M+H]^+$. ^{1}H-NMR (500 MHz, CD_3OD) δ = 7.88 (d, 1H, *J* = 8 Hz), 7.80 (t, 1H, *J* = 8.5 Hz), 7.54 (d, 1H, *J* = 8.5 Hz), 5.45 (bd, 1H, *J* = 3 Hz), 5.04 (bs, 1H), 4.29 (q, 1H, *J* = 6.5 Hz), 4.01 (s, 3H), 3.91-3.55 (m, 16H), 3.38 (d, 1H, *J* = 13 Hz) , 3.33 (d, 1H, *J* = 12.5 Hz), 3.09 (d, 1H, *J* = 18.5 Hz), 2.93 (dd, 1H, *J* = 18.5 Hz, *J* = 3.5 Hz), 2.80 (t, *J* = 6 Hz), 2.43 (bd, 1H, *J* = 14.5 Hz), 2.27-2.14 (m, 3H), 2.03 (td, 1H, *J* = 13 Hz, *J* = 3.5 Hz), 1.88 (dd, 1H, *J* = 12.5 Hz, *J* = 4.5 Hz), 1.28 (d, 3H, *J* = 6.5 Hz) ppm. ^{13}C-NMR (125 MHz, CD_3OD) δ = 210.99, 188.35, 188.06, 173.19, 162.96, 162.69, 157.55, 156.34, 137.47, 136.53, 135.91, 135.70, 121.61, 120.68, 120.53, 112.61, 112.35, 101.22, 77.63, 71.92, 71.39, 71.33, 71.25, 71.15, 70.76, 68.19, 68.01, 67.44, 67.41, 57.29, 48.64, 43.42, 38.40, 36.94, 34.54, 34.49, 32.66, 29.57, 17.36 ppm. HR-MS (ESI$^+$) calcd. for $[C_{38}H_{51}N_3O_{15}S_1+H]^+$: 822.3114, found: 822.3112.

Chart S 14 – Chromatographic data and purity of **L2**·3TFA



Compound	Retention Time [min]	Area [%]	Molecular Formula	Exact Mass [m/z]	Mass Found [m/z]
L2	0.94	≥97	$C_{38}H_{51}N_3O_{15}S$	821.30	822.4 $[M+H]^+$

Chart S 15 – Nummerical assignement for NMR **L2**·3TFA

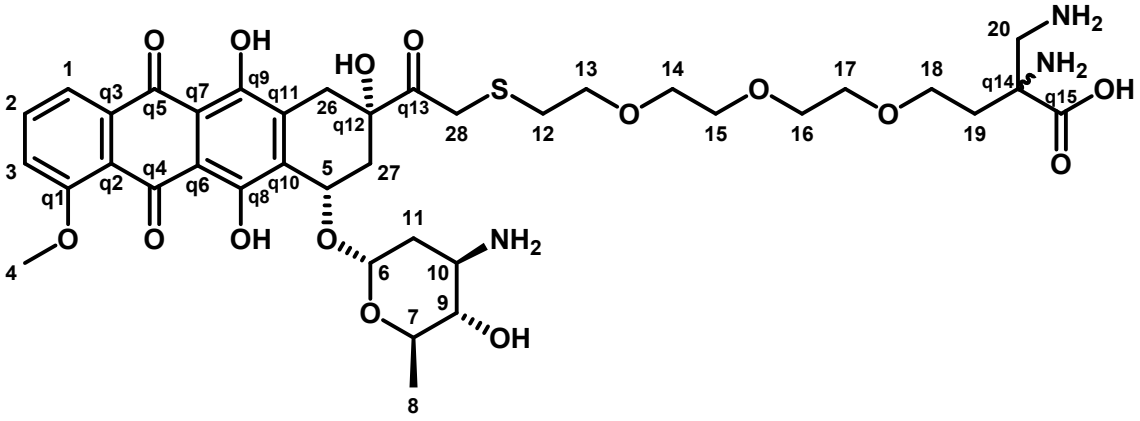


Chart S 16 – ^{1}H-NMR of **L2**·3TFA, 500 MHz, CD_3OD

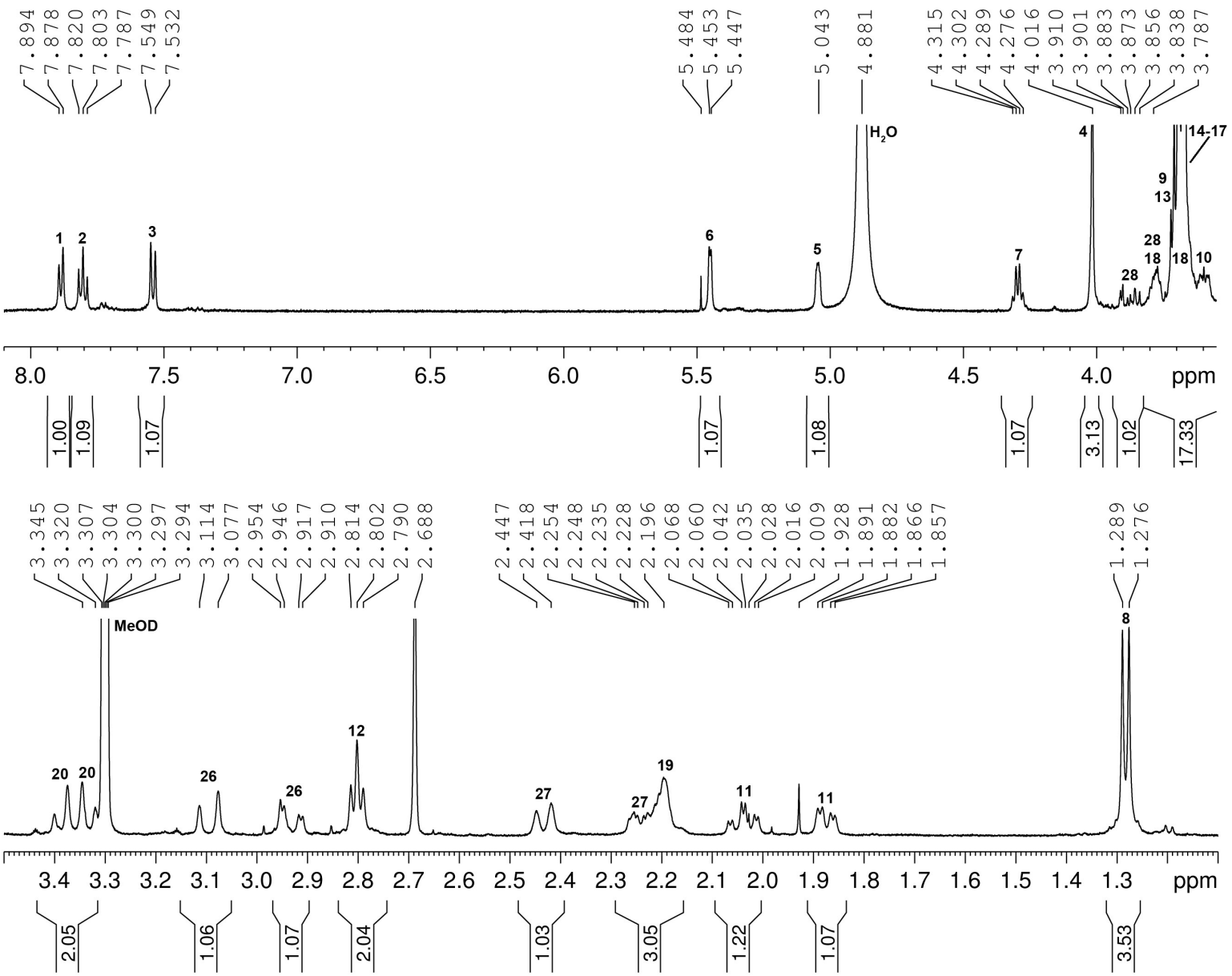


Chart S 17 – ^{13}C-NMR of **L2**·3TFA, 125 MHz, CD_3OD

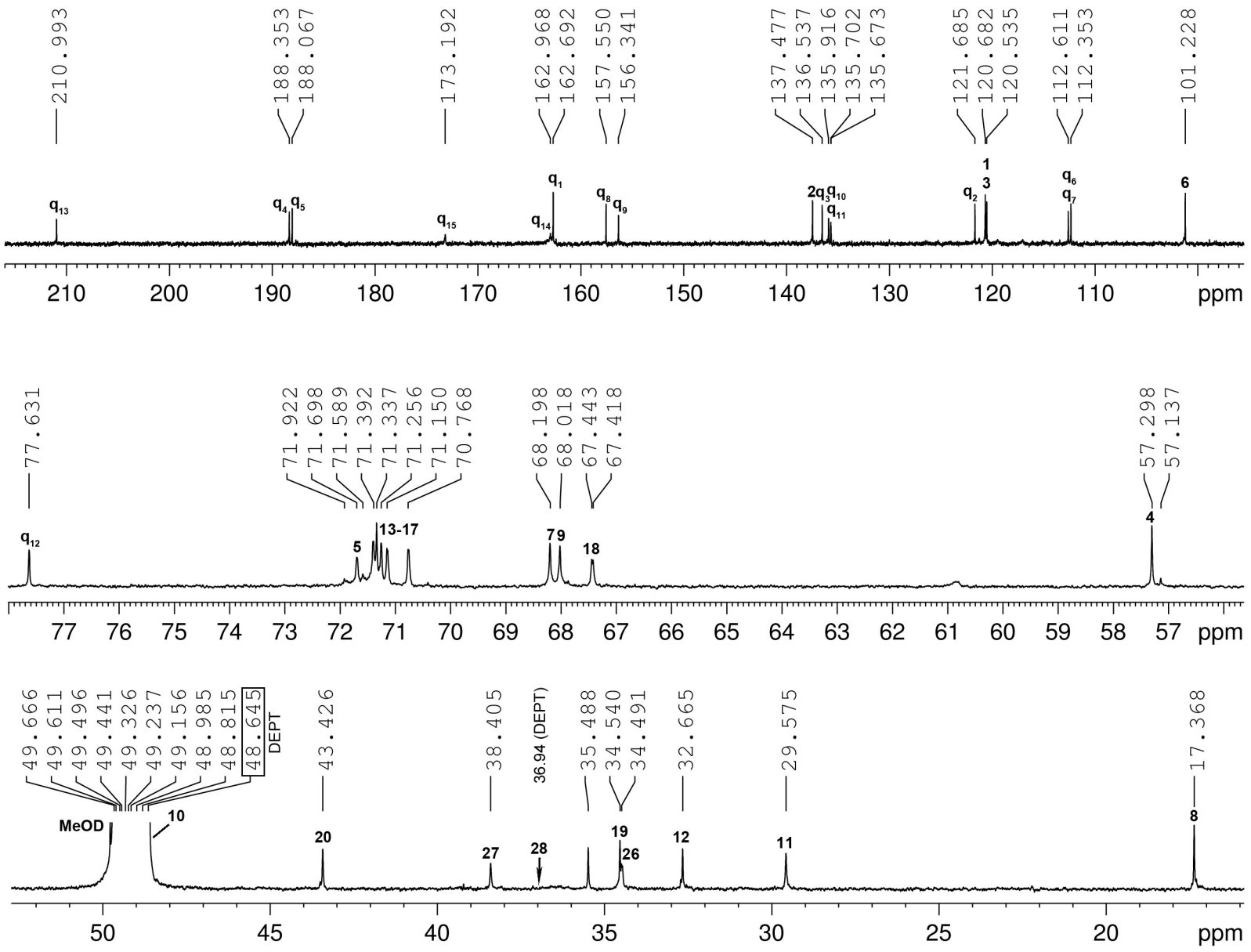


Chart S 18 – C,H-Correlation of **L2**·3TFA

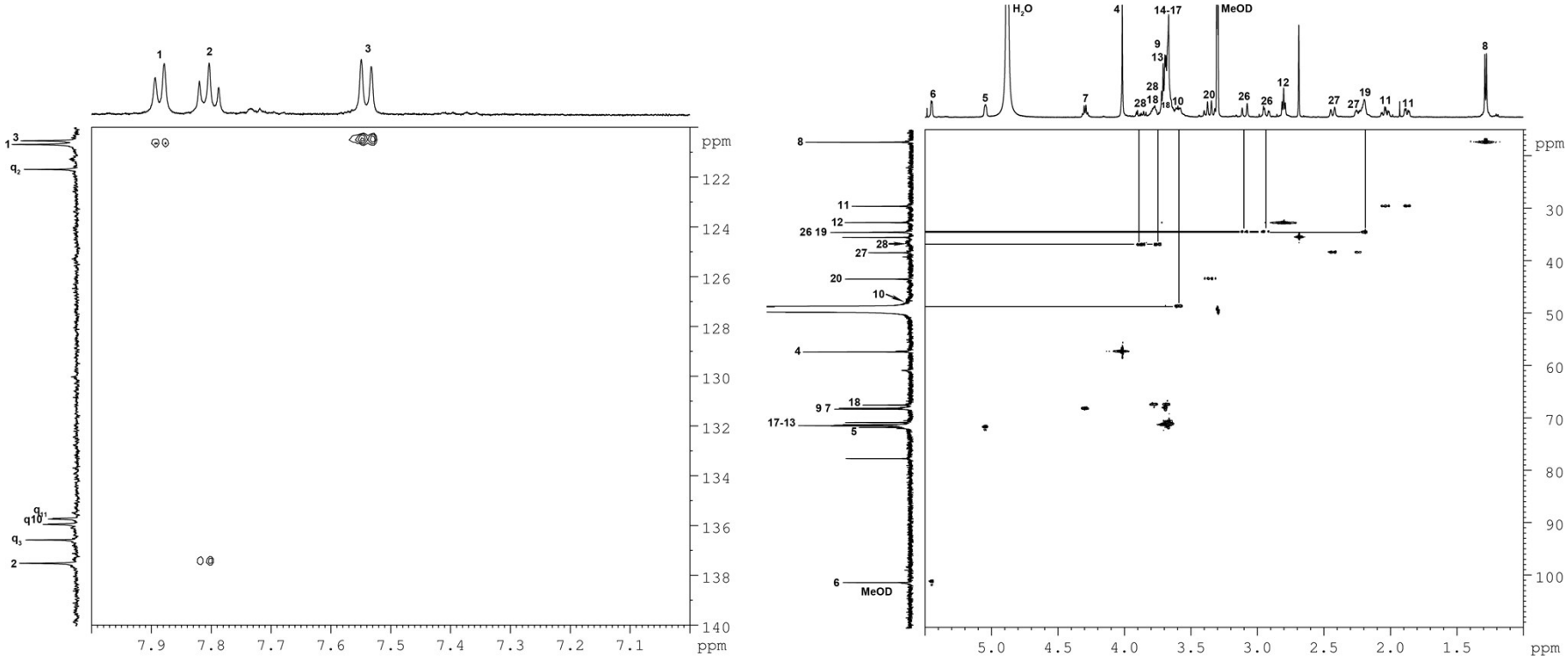
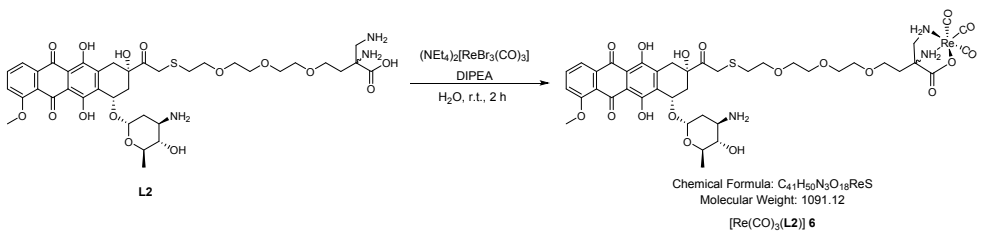


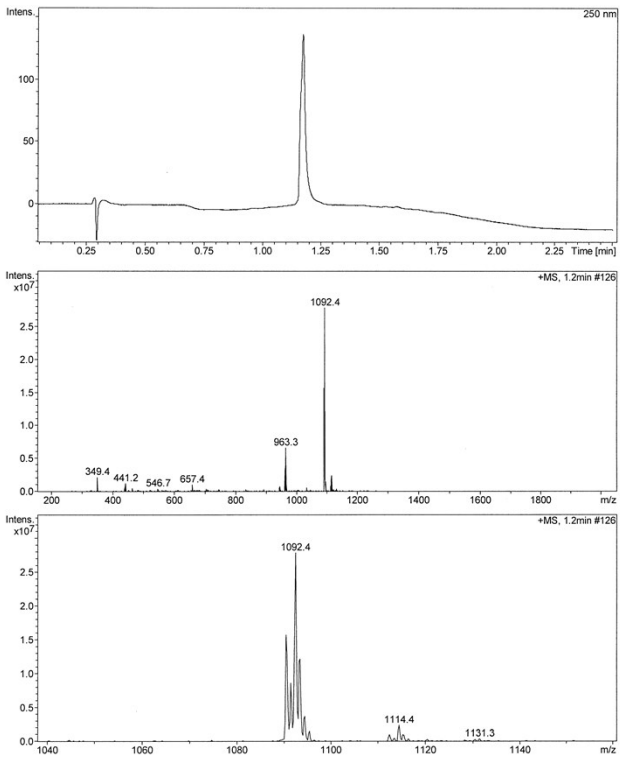
Chart S 19 – Experimental data of **6**·TFA



[Re(CO)$_3$(L2)] (6). L2 (10 mg, 8.6 μmol) was dissolved in pure H_2O (1 ml). To this orange solution was added $(NEt_4)_2[ReBr_3(CO)_3]$ (10 mg, 13 μmol, 1.5 eq.) and DIPEA (4.5 μl, 3.1 eq.) and the mixture was stirred at room temperature upon which the solution turned dark orange. Over the course of 2h a brownish-red precipitate formed and reaction control *via* UPLC (gradient U1) indicated complete consumption of **L2** into a more hydrophobic product. The reaction solution was then diluted with a mixture of 0.1% TFA in H_2O/MeCN (1:1) and the product separated by preparative HPLC. Product containing fractions were combined and lyophilized to yield **6** as an orange powder. Yield (·TFA, mixture of diastereomers): 5.71 mg (4.7 μmol, 55%).

UPLC-ESI$^+$: R_t= 1.17 min, area ≥97%, m/z= 1092.4 $[M+H]^+$. ^{1}H-NMR (500 MHz, CD_3OD) δ = 7.92 (m, 1H), 7.82 (bt, 1H, *J* = 7.5 Hz), 7.56 (d, 1H, *J* = 8 Hz), 5.46 (bs, 1H), 5.14-5.10 (m, 1H, NH), 5.07 (bs, 1H), 4.95-4.91 (m, 1H, NH), 4.71 (bd, 1H, *J* = 11 Hz), 4.64 (m, 1H), 4.29 (q, 1H, *J* = 6.5 Hz), 4.03 (s, 3H), 3.85 (bd, 1H, *J* = 14.5 Hz), 3.79-3.76 (m, 1H), 3.72-3.61 (m, 12H), 3.58-3.55 (m, 2H), 3.10 (d, 1H, *J* = 15 Hz), 2.94 (d, 1H, *J* = 15 Hz), 2.79-2.77 (m, 3H), 2.71-2.68 (m, 1H), 2.45, (bd, 1H, *J* = 14.5 Hz), 2.26 (bd, 1H, *J* = 15 Hz), 2.06-1.85 (m, 4H), 1.28 (d, 3H, *J* = 6.5 Hz) ppm. ^{13}C-NMR (125 MHz, CD_3OD) δ = 210.67, 198.47, 197.30, 188.44, 188.09, 181.22, 162.69, 157.59, 156.39, 137.48, 136.60, 135.88, 135.75, 121.71, 120.71, 120.52, 112.61, 112.35, 101.33, 77.62/77.59, 71.94, 71.61, 71.56, 71.51, 71.06, 68.19, 68.15, 68.06, 67.01, 57.29, 47.7, 46.64, 38.45, 36.84/36.75, 34.46, 34.33, 32.69/32.65, 29.58, 17.37 ppm. IR (ATR): ν= 2021 (s, $\nu_{sym.}$CO), 1892 (s, $\nu_{asym.}$CO) cm^{-1}. ESI-MS (MeOH): m/z = 1092.4 $[M+H]^+$. HR-MS (ESI$^+$) calcd. for $[C_{41}H_{50}N_3O_{18}Re_1S_1+H]^+$: 1092.2446, found: 1092.2449.

Chart S 20 – Chromatographic data and purity of **6**·TFA



Compound	Retention Time [min]	Area [%]	Molecular Formula	Exact Mass [m/z]	Mass Found [m/z]
6	1.17	≥97	$C_{41}H_{50}N_3O_{18}ReS$	1091.24	1092.4 $[M+H]^+$

Chart S 21 – Nummerical assignement for NMR **6**·TFA

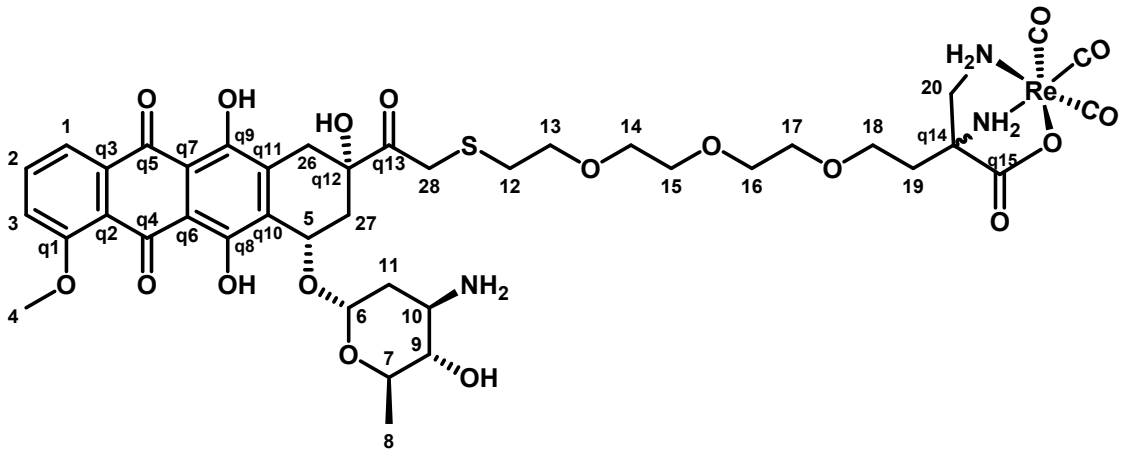


Chart S 22 – ^{1}H-NMR of **6**·TFA (mixture of diastereomers), 500 MHz, CD_3OD

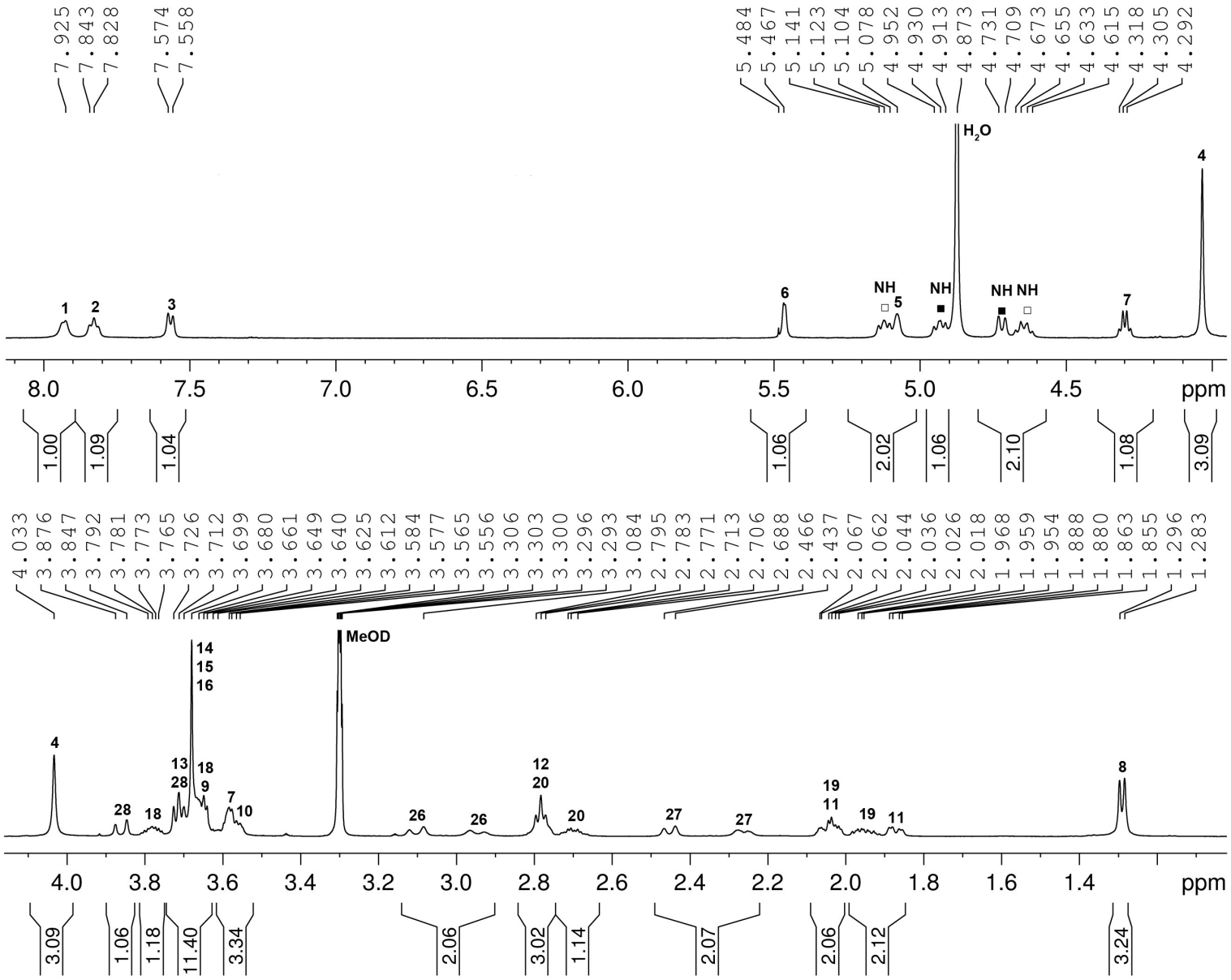


Chart S 23 – ^{13}C-NMR of **6**·TFA (mixture of diastereomers), 500 MHz, CD_3OD

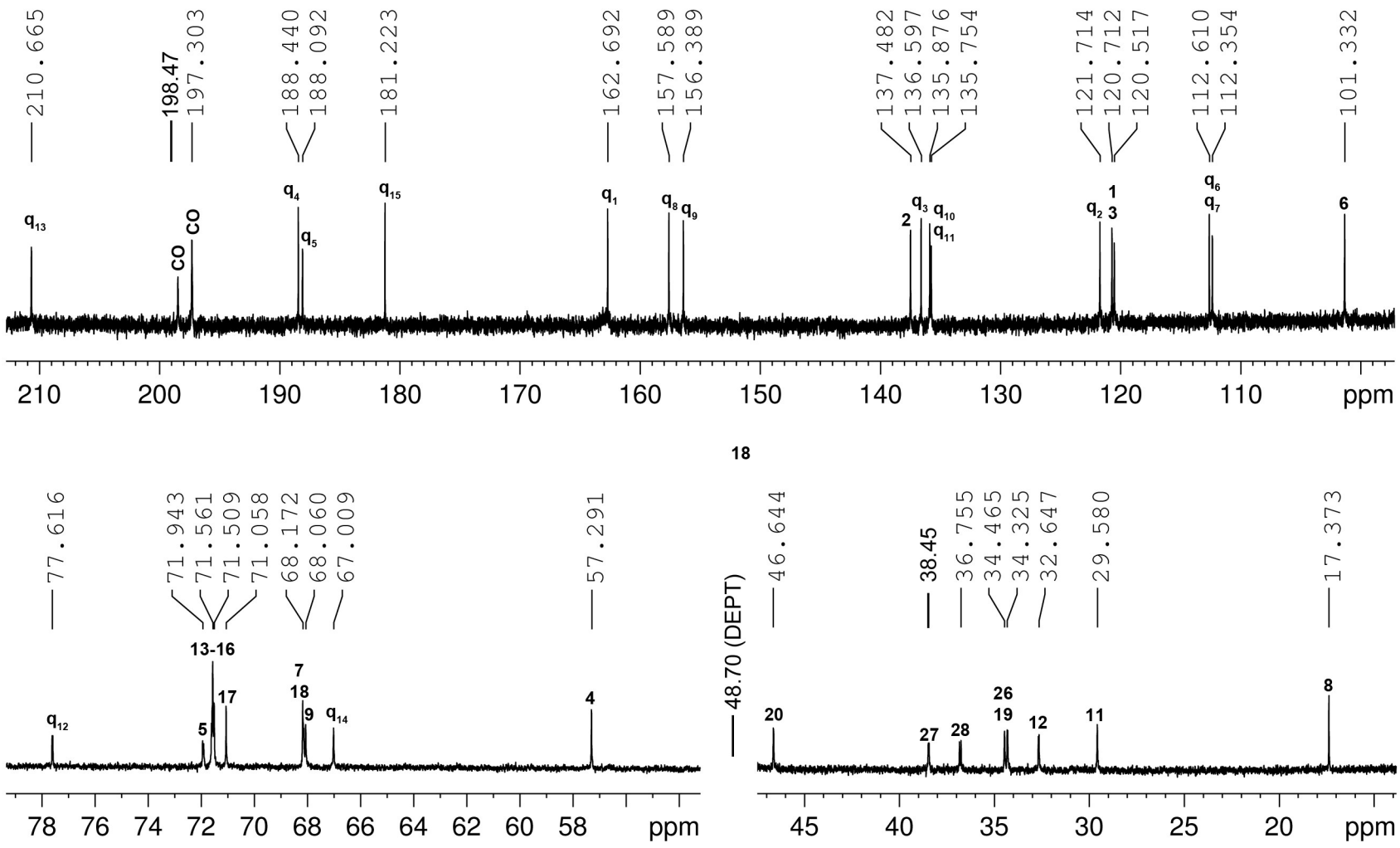


Chart S 24 – C,H-Correlation of **6**·TFA (mixture of diastereomers)

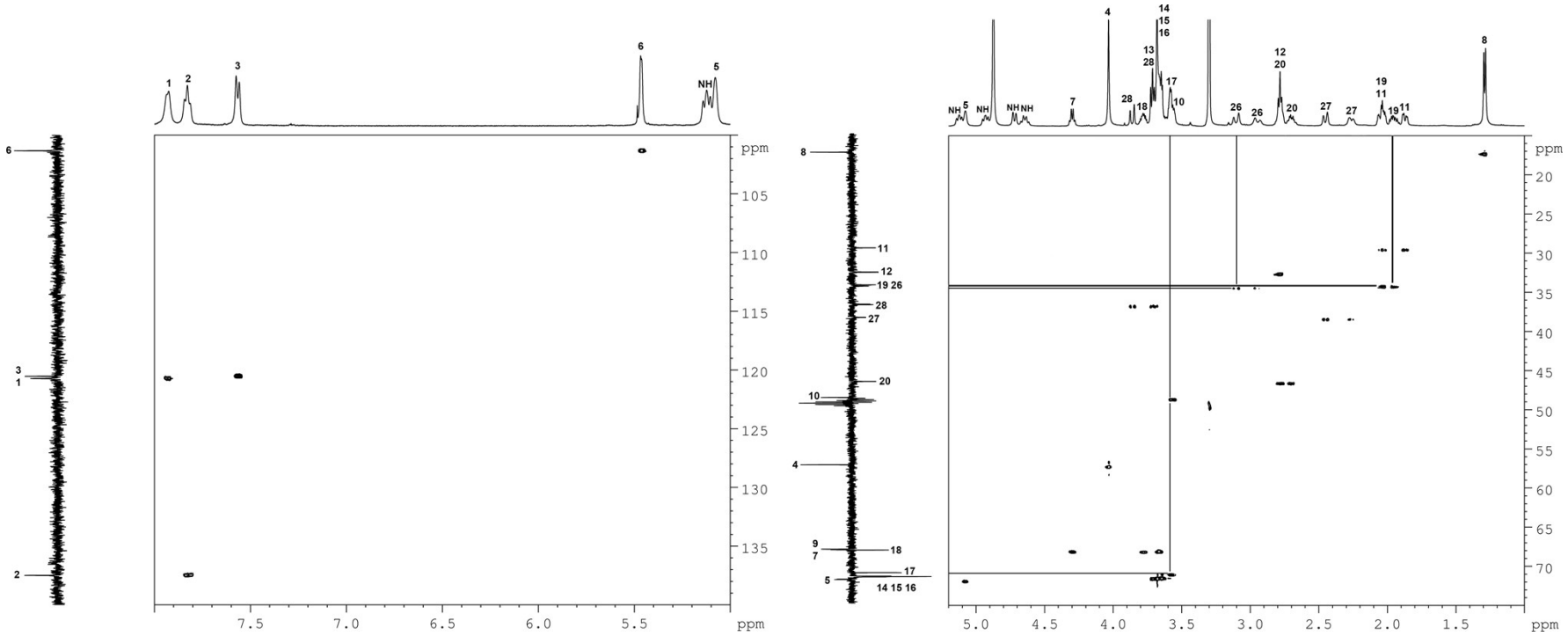


Chart S 25 – Radiolabeling and synthesis of **3** and **5**

Caution! ^{99m}Tc is a γ-emitter (E= 140 keV, $t_{1/2}$= 6.02 h) which should only be handled in a licensed and appropriately shielded facility. Sodium boranocarbonate releases CO gas which is highly toxic, it is recommended to be handled only in ventilated hoods.

$[^{99m}Tc(OH_2)_3(CO)_3]^+$ *modified from Alberto et al.*[13,14]

A crimp sealable glass vial was charged with sodium boranocarbonate (4 mg, 38.5 μmol), sodium tartrate dihydrate (4 mg, 30.4 μmol) and sodium tetraborate decahydrate (4 mg, 18.5 μmol). Upon crimp sealing, this mixture was flushed with N_2 for 5 min before adding $^{99m}TcO_4^-$ eluate (1 ml, ~ 10 - 100 MBq) from a commercial generator. The resulting solution was heated at 95 °C for 20 min without stirring and thereafter cooled in a water bath. In order to normalize the overpressure, evolving gases were removed with a 1 ml disposable syringe. Excess sodium boranocarbonate was quenched *via* dropwise addition of 1M HCl to pH 2 and subsequent neutralizing by addition of 1M NaOH to pH 6-7.

$[^{99m}Tc(CO)_3(Ln)]^+$ (L1= **3**, L2= **5**). A crimp sealable glass vial was pre-charged with **Ln** (0.5 mg, ~0.4 μmol) and flushed with N_2 for 5 min. The neutralized $[^{99m}Tc(OH_2)_3(CO)_3]^+$ solution (1 ml) was directly injected into this vial and the orange reaction mixture heated at 60 °C for 30 min. After that time, the vial was cooled in a water bath. γ-HPLC (gradient A2) revealed quantitative turnover and a RCP 80-90%. After purification (gradient A1), identity of the product was determined by co-injection with rhenium surrogates **4** or **6** respectively.

Supplementary Data

Chart S 26 – Fluorescence quenching of complexes **4** and **6** in the presence of ctDNA

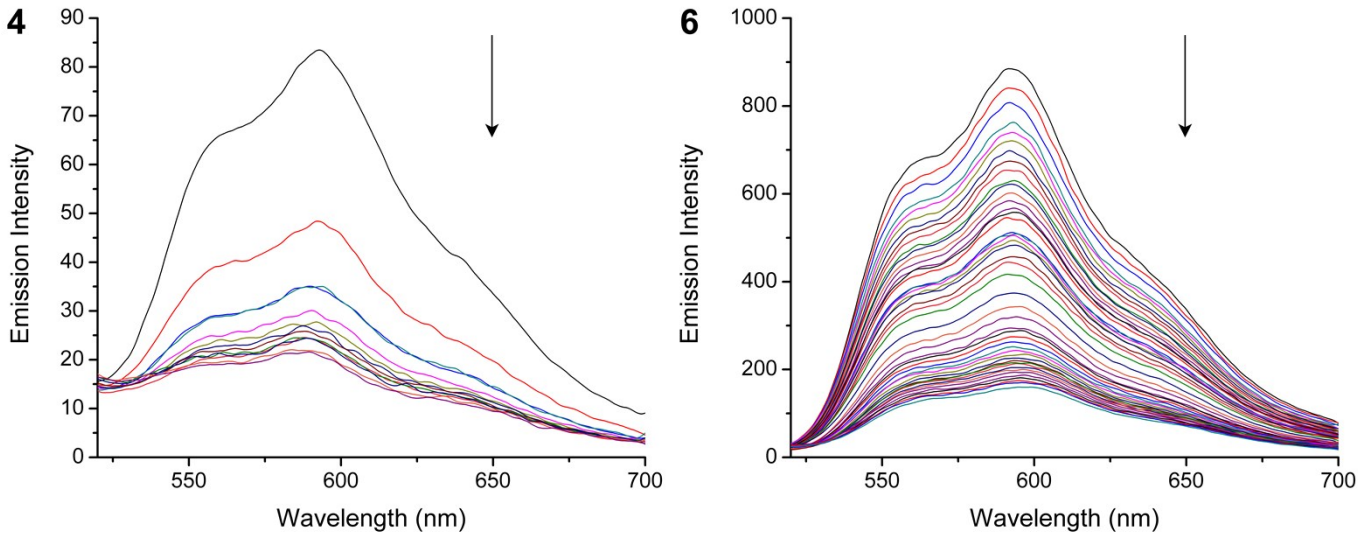


Figure 2. Emission spectra (510-700 nm) of **4** at 0.7 μM and **6** at 2.8 μM in PBS buffer (10 mM, pH 7.48) with NaCl (50 mM). The initial fluorescence is quenched upon incremental addition of ctDNA.

Chart S 27 – Binding curves of ADR and complexes **4** and **6** with ctDNA

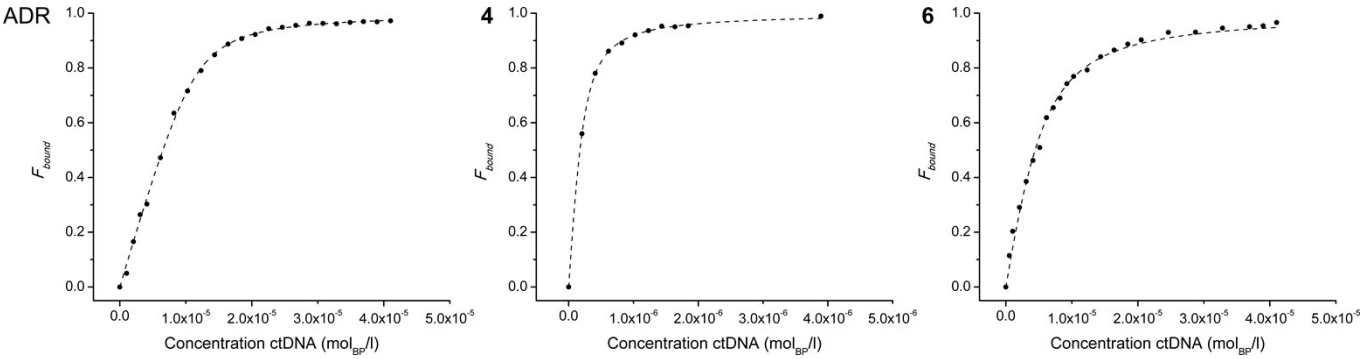


Figure 3. Binding curves of ADR and complexes **4** and **6** to ctDNA. Symbols indicate F_{bound} calculated by equation (2) and the dashed line represents the non-linear least squares fit to equation (3).

Table 1. Parameters extracted from ctDNA titration of ADR and complexes **4/6**.[a]

Compound ([μM])	K_b [M^{-1} per nucl] × 10^6	***s*** [bp]	R^2
ADR (2.8)	4.98 ± 0.45	2.00 ± 0.04	0.998
4 (0.68)	0.23 ± 0.03	0.29 ± 0.01	0.999
6 (3.8)	0.63 ± 0.11	1.26 ± 0.15	0.994

[a]K_b: affinity constant, *s*: binding site size, R^2: goodness of fit of the experimentally determined data to equation (3).

Chart S 28 – Human Topoisomerase II α and β inhibition of ADR and complexes **4** and **6**

According to Shapiro et al.[15,16]

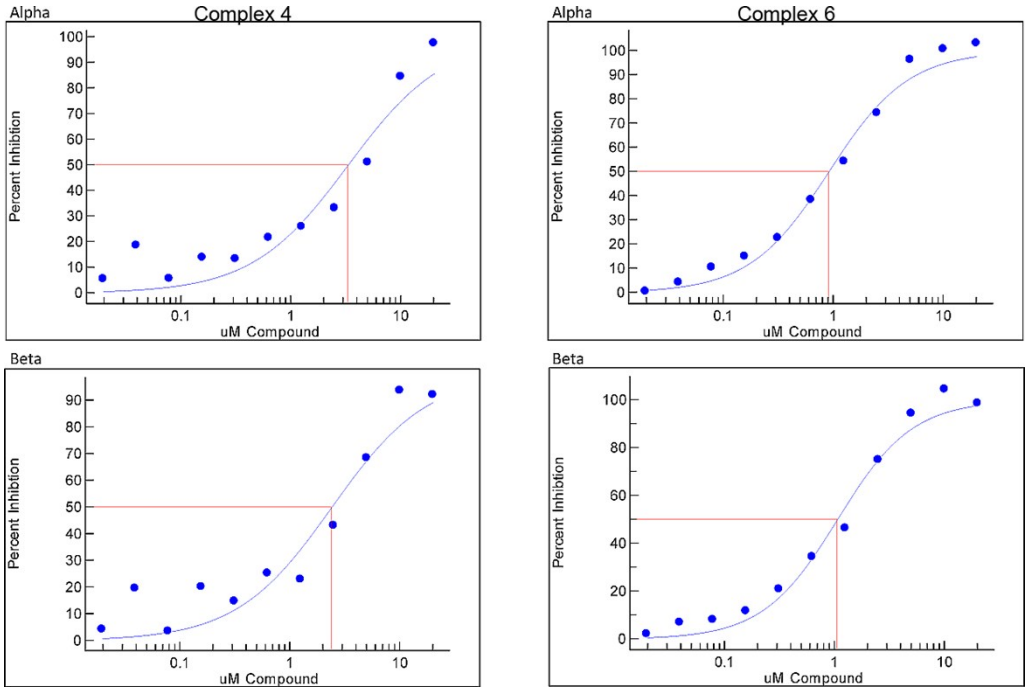


Figure 4. Concentration-dependent inhibition of hTOPOII α and β by complexes **4** (left) and **6** (right). Blue dots represent measured values according to equation (5), the blue line represents a non-linear least squares fit to equation (6) and the red drop-lines indicate the IC_{50} values.

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