Enhanced cytotoxicity through conjugation of a "clickable" luminescent Re(I) complex to a cell-penetrating lipopeptide

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Experimental section

Materials

Chemicals and solvents were of reagent grade or better and purchased from commercial suppliers and were used without further purification unless otherwise specified. (S)-2-(Fmoc-amino)-6-azidohexanoic acid (Fmoc-Lys(N_3)-OH) was purchased from AAPTEC Reagents used in peptide synthesis were from AusPep. All other chemicals were of reagent-grade and sourced from Sigma-Aldrich. *Dried solvents* were obtained with the purification system MB-SPS 800 from M. Braun. The human plasma was provided by the Blutspendezentrum, Zurich, Switzerland.

Instrumentation and methods

NMR spectra were recorded on an Avance III Nanobay 400 MHz Bruker spectrometer coupled to the BACS 60 automatic sample changer. Data acquisition and processing was managed using MestReNova version 8.0.0-10524 and iNMR version 3.1.4. Abbreviations used to describe ^{1}H NMR spectra are: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet) or b (broad). *High resolution-mass spectrometry (HRMS), method A* (for **[Re(CO)$_3$(bipy)(py-alkyne)](BF$_4$)** and **myr-Tat**): measurements were run on a Waters LCT TOF LC/MS Mass Spectrometer coupled to a 2795 Alliance Separations module. All data were acquired and mass-corrected via a dual-spray Leucine Enkephaline reference sample. The mass spectrum was created by averaging the scans across each peak and subtracting the background of the TIC. Acquisition and analysis was performed using the Masslynx software version 4.1. *High resolution-mass spectrometry (HRMS), method B* (for **Re-myr-Tat**): mass spectra were performed on a Bruker maXis QTOF high-resolution mass spectrometer (Bruker Daltonics, Bremen, Germany). *Analytical high-performance liquid chromatography* (HPLC) was carried out on the Waters 2690 Separation Module coupled with a Waters 996 Photodiode Array Detector with a Phenomenex Luna C-8 column (100 Å, 5µm, 150 × 4.6 mm). *UPLC-MS* was carried out on an AcquityTM from Waters system equipped with a PDA detector and an auto sampler and connected to a Bruker Daltonics HCT 6000 mass spectrometer. Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm) was run (flow rate: 0.6 mL min^{-1}) with a linear gradient of buffer A (double distilled water containing 0.1% v/v formic acid) and B (acetonitrile containing 0.1% v/v formic acid); **[Re(CO)$_3$(bipy)(py-alkyne)](BF$_4$)** was analyzed with the following linear gradient: t = 0 min, 20% B; t = 0.25 min, 20% B; t = 4.5 min, 100% B; t = 5.5 min, 100% B. The peptide conjugate **Re-myr-Tat** was analyzed using a different linear gradient: t = 0 min, 5% B; t = 0.25 min, 5% B; t = 1.5 min, 100% B; t = 2.5 min, 100% B. *The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry* (MALDI-TOF) mass spectra were recorded on a Bruker Daltonics Autoflex. The experiments were performed in reflector mode with positive polarity using α-cyano-4-hydroxy-cinnamic acid (4-HCCA) on a Prespotted AnchorChip as the matrix. *Preparative HPLC, method A* (for **myr-Tat**): separation was performed on a Waters Prep LC controller with a Waters 486 Tunable Absorbance detector using a Phenomenex Luna C-8 column (100 Å, 5µm, 150 × 4.6 mm; flow rate: 10 mL min^{-1}) using a 60-min gradient from 100% buffer A (double distilled water containing 0.1% v/v TFA) to 80% buffer B (79.9% v/v acetonitrile/20% v/v water/0.01% v/v TFA). *Preparative HPLC purification, method B* (for **Re-myr-Tat**) was carried out on Merck Hitachi system (interface D-7000, diode array detector L-7455, pump L-7100) and an Agilent Zorbax 300 SB-C18 prep column (5 µm particle size, 300 Å pore size, 250 × 9.4 mm; flow rate: 4 mL min^{-1}). The run was performed with a linear gradient of A (double distilled water containing 0.1% v/v TFA) and B (acetonitrile containing 0.1% v/v TFA, Sigma–Aldrich

HPLC- grade). Preparative run: t = 0 min, 5% B; t = 3 min, 15% B; t = 27 min, 45% B; t = 30 min, 100% B; t = 35 min, 100% B. UV spectra were recorded using a Carry 50 Scan Varian spectrophotometer. Luminescence lifetimes were determined using Edingburgh LP-920 setup equipped with a Continuum Surelite laser (355 nm). UV spectra were recorded on Carry 50 Scan Varian spectrophotometer. Emission spectra were recordered on Perkin Elmer LS50B luminescence spectrophotometer. *Cytotoxicity* of the compounds towards HeLa cells was assessed by resazurin assay using a SpectraMax M5 microplate reader. *Confocal fluorescence microscopic imaging* of the compounds inside HeLa cells was carried out on a CLSM Leica SP5 microscope.

Synthesis and characterization

[Re(2,2'-bipyridine)(3-ethynylpyridine)(CO)$_3$](BF$_4$) (**[Re(CO)$_3$(bipy)(py-alkyne)](BF$_4$)**)

[Re(2,2'-bipyridine)(CO)$_3$(CH$_3$CN)](BF$_4$)[1] (300 mg, 0.541 mmol) and 3-ethynylpyridine (558 mg, 0.541 mmol) were dissolved in dry THF (40 mL) and the solution refluxed in the dark under a nitrogen atmosphere for 2 h. The yellow solution was cooled, filtered and then hexane (150 mL) added. After cooling in the fridge for 3 h, the yellow precipitate that formed was collected via vacuum filtration, washed with hexane (2 x 10 mL) and Et$_2$O (3 x 10 mL), then dried in a vacuum desiccator. Yield: 255 mg (76%). Water solubility 0.79 ± 0.13 mmol/L (25 °C); logD(octanol/PBS pH 7.01) = -0.36 ± 0.05; UV absorbtion (PBS buffer, pH 7.4): ε_{307} = 14832 ± 312 M^{-1}cm^{-1}; ε_{319} = 15419 ± 373 M^{-1}cm^{-1}. Anal. Calcd for $C_{20}H_{13}BF_4N_3O_3Re$: C, 38.97; H, 2.13; N, 6.82. Found: C, 38.83; H, 2.05; N, 6.70. HRMS (ESI) calcd (found): [M – BF$_4$]$^+$ 530.0515 (530.0514). ^{1}H NMR (400 MHz, CD$_3$CN): δ 9.23 (ddd, *J* = 5.5, 1.5, 0.8 Hz, 2H), 8.40 – 8.36 (m, 2H), 8.35 – 8.33 (m, 1H), 8.31 – 8.23 (m, 2H), 8.18 (dd, *J* = 5.7, 1.1 Hz, 1H), 7.92 (dt, *J* = 8.0, 1.6 Hz, 1H), 7.79 (ddd, *J* = 7.6, 5.5, 1.3 Hz, 2H), 7.28 (ddd, *J* = 8.0, 5.7, 0.8 Hz, 1H), 3.68 (s, 1H). ^{13}C NMR (101 MHz, CD$_3$CN): δ 156.88, 155.35, 154.98, 152.50, 143.67, 142.19, 129.78, 127.38, 125.76, 123.16, 84.95, 78.39.

Myr-Tat peptide.

The Lys(N$_3$)-bearing peptide was synthesized by automated solid-phase peptide synthesis (SPPS) on a PS3 Automated Peptide Synthesiser (Protein Technologies Inc.) using a Rink amide resin. The peptide sequence, Lys(N$_3$)-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Glu-Arg-Arg-Arg, was firstly constructed on a Rink amide resin (0.1 mmol) pre-swelled in DMF, using Fmoc-protected L-amino acids (0.3 mmol) and standard automated coupling and deprotection protocols with HCTU as the activating agent (0.3 mmol) and 20% piperidine in DMF for Fmoc deprotections. The resin was then *N*-myristylated by performing a standard coupling cycle with myristic acid (1.5 mmol). The peptide-resin was then transferred to 5 mL-syringes and washed with DMF (3 × 3 mL), MeOH (3 × 3 mL) and Et$_2$O (3 × 3 mL). Cleavage of the peptide from the resin was achieved by suspending the resin in a 90% TFA, 5% H$_2$O, 2.5% TIPS, 2.5% DMB cleavage cocktail (2 mL) and rocking gently for 2 h. The resin was then filtered off, the filtrate concentrated using a gentle stream of nitrogen, and Et$_2$O (10 mL) added to initiate precipitation of the crude peptide. It was pelleted by centrifugation, washed with Et$_2$O (3 x 5 mL) and dried under reduced pressure, before being purified to >95% purity by preparative HPLC. HRMS (ESI) calcd (found): [M + 3H]$^{3+}$ 641.7574 (641.7601), [M + 2H]$^{2+}$ 962.1324 (962.1332).

Re-myr-Tat.

Myr-Tat peptide (10 mg, 5.2 μmol), **[Re(CO)$_3$(bipy)(py-alkyne)](BF$_4$)** (6.41 mg, 10.4 μmol), $CuSO_4$ (0.830 mg, 5.2 μmol), TBTA (2.76 mg, 5.2 μmol) and sodium ascorbate (3.1 mg, 15.6 μmol) were dissolved in 3.5 mL of water:acetonitrile (1:1) and left to react for 14 h at room temperature. The solvent was then removed by lyophilization and the brownish solid was then purified by semi-preparative HPLC to afford **Re-myr-Tat** as pale-yellow powder. logD(octanol/PBS pH 7.01) = 0.86 ± 0.15; ESI-MS m/z 338.9 $[M-(Re(bipy)(CO)_3)+6H]^{6+}$, 406.4 $[M-(Re(bipy)(CO)_3)+5H]^{5+}$, 491.6 $[M+5H]^{5+}$, 507.8 $[M-(Re(bipy)(CO)_3)+4H]^{4+}$, 614.2 $[M+4H]^{4+}$, 818.7 $[M+3H]^{3+}$ MALDI-TOF m/z 2026.4 $[M-(Re(bipy)(CO)_3)+H]^{+}$, 2048.6 $[M-(Re(bipy)(CO)_3)+Na]^{+}$. HR ESI-MS 338.7231 (calc. 338.7562), 406.2668 (calc. 406.3077), 491.4673 (calc. 491.4683), 507.5816 (calc. 507.6303), 613.8314 (calc. 614.0834), 818.4394 (calc. 818.4419).

Luminesce quantum yield

[Re(CO)$_3$(bipy)(py-alkyne)](BF$_4$) was disolved in PBS (pH 7.4) to obtain a series of solutions of various concentrations adjusted so that their absorbance never exceeded 0.1 at and above the excitation wavelenght (≥ 355 nm). Reference solutions of quinine hemi-sulfate in 0.1 M H_2SO_4 were prepared in the same manner. Emission spectra of all solutions excited at 355 nm were then recordered. Integrated fluorescence intensity of each solution was plotted vs its absorbance and fitted with linear function. The slopes were then used to calculate the luminesce quantum yields according to the following equation:

$$\Phi_{sample} = \Phi_{reference}\left(\frac{S_{sample}}{S_{reference}}\right)\left(\frac{\eta^2_{sample}}{\eta^2_{reference}}\right)$$

where Φ denotes quantum yield ($\Phi_{reference}$ = 0.54), S denotes slope and η is the refractive index of the solvent.

Water and plasma stability

[Re(CO)$_3$(bipy)(py-alkyne)](BF$_4$) (1 μL, 20 mM DMSO stock solution) and diazepam (1 μL, 20 mM DMSO stock solution) were mixed with water (998 μL, double distilled) or human plasma (998 μL) and incubated on a thermoshaker at 37 °C for 0, 24, 48, or 72 h (water) and for 0, 5, 10, 24, 48 and 72 h (human blood plasma). The compounds were then extracted with CH_2Cl_2 (6 mL) by shaking the samples for 15 min and then centrifuging them for 10 min. The organic phase was collected and dried under a nitrogen flow. The resulting residue was dissolved in acetonitrile (100 μL) and analyzed by UPLC-MS. The area of the $[Re(CO)_3(bipy)(py\text{-}alkyne)]^+$ peak was normalized to the peak area of diazepam (internal standard), which is known to remain stable in blood plasma.

Distribution coefficients

The distribution coefficients (logD) for **[Re(CO)$_3$(bipy)(py-alkyne)](BF$_4$)** and **Re-myr-Tat** were measured using the "shake-flask" method similar to that previouly reported by our group.[2] In brief, each compound was dissolved in a 0.5 mL of 10 mM phosphate buffer (pH 7.01) pre-saturated with n-octanol to obtain a solution with a concentration of 20 μM. The same volume of octanol (pre-saturated with 10 mM phosphate buffer) was then added and the solution was

manually shaken for 2 min, equilibrated for 1 h and centrifuged for 5 min. The concentrations of the compound in the aqueous and organic phases were determined by UV-vis absorbtion (the absorbtion of the aqueous phase was compared to that of the initial phosphate buffer solution prior to octanol addition), Measurements were repeated 3 times for each compound.

Cell culture

Human cervical carcinoma cells (HeLa) were maintained in DMEM (Gibco) with 5% fetal calf serum (FCS, Gibco), 100 U mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin at 37 °C and 5% CO_2.

Cytotoxicity studies

The cytotoxicity of the rhenium complex and its bioconjugate to HeLa cells was assayed by a fluorometric cell viability assay using resazurin (Promocell GmbH). Cells were seeded in triplicates in 96-well plates at a density of 4×10^3 cells per well in 100 µL 24 h prior to treatment. To evaluate the cytotoxicity, cells were treated with increasing concentrations of compounds (DMSO stock solutions of compounds diluted in cell culture medium, final content of DMSO <1%) for 48 h. After incubation, the medium was replaced by 100 µL complete medium with resazurin (0.2 mg mL^{-1} final concentration). Upon 4 h of incubation at 37 °C, the fluorescence of the highly red fluorescent resorufin product was quantified at 590 nm emission with 540 nm excitation wavelength.

***In vitro* fluorescence evaluation**

Cellular localization of the luminescent rhenium complex and bioconjugate was visualized by fluorescence microscopy. HeLa cells were grown on 18 mm Menzel-Glaser coverslips in 2 mL complete medium at a density of 1×10^5 cells per mL and incubated for 2 h with rhenium complex (at 100 µM) and its bioconjugate (at 20 µM). Cells were fixed in a 4% formaldehyde solution in PBS and mounted on slides for viewing by confocal microscopy. The compounds were excited at 405 nm and the emission above 420 nm was recorded.

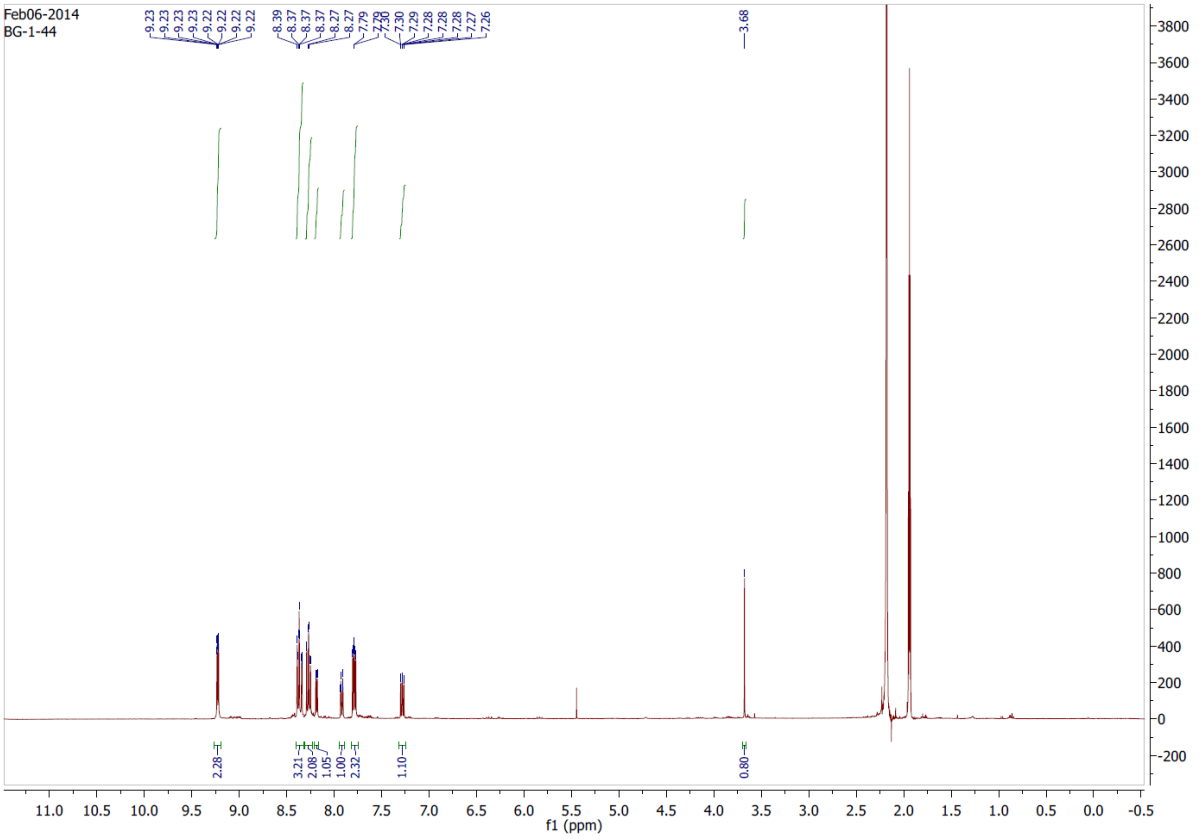


Figure S1. ^{1}H NMR spectrum of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$.

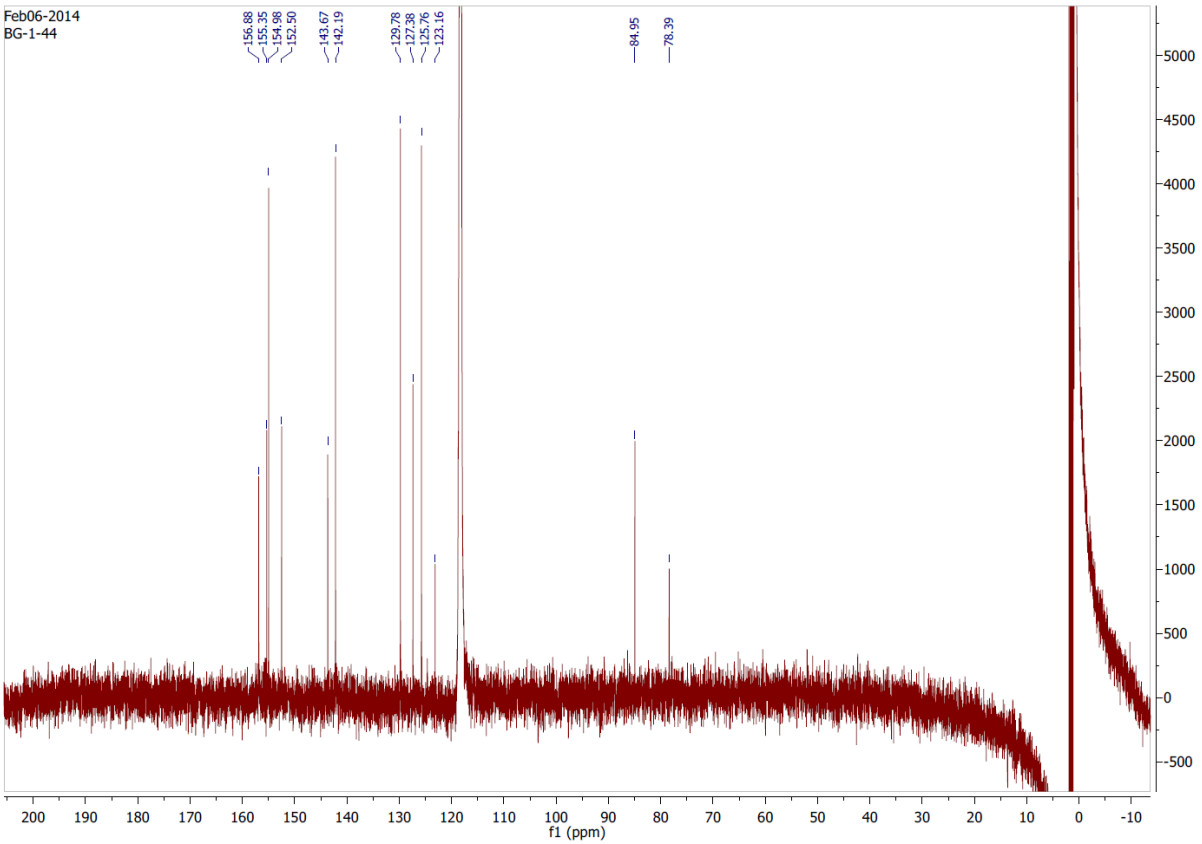


Figure S2. ^{13}C NMR spectrum of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$.

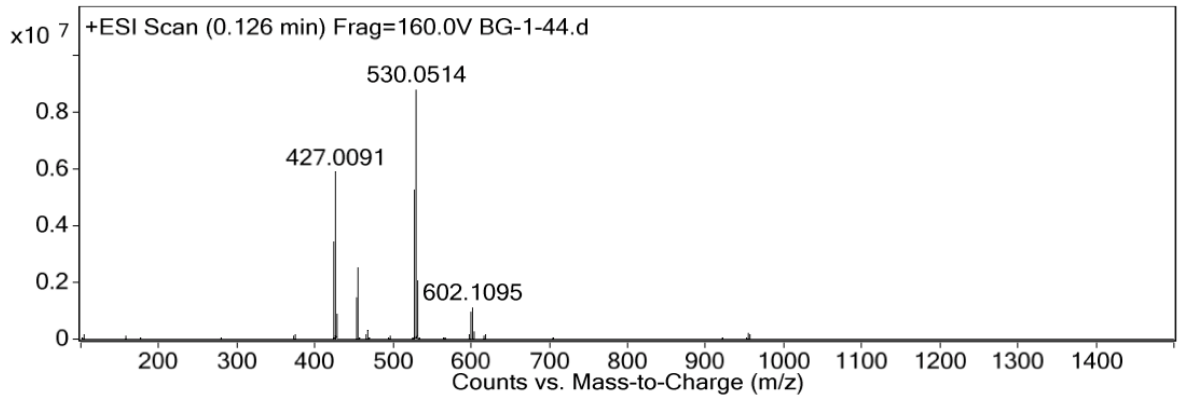


Figure S3. HRMS spectrum of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$.

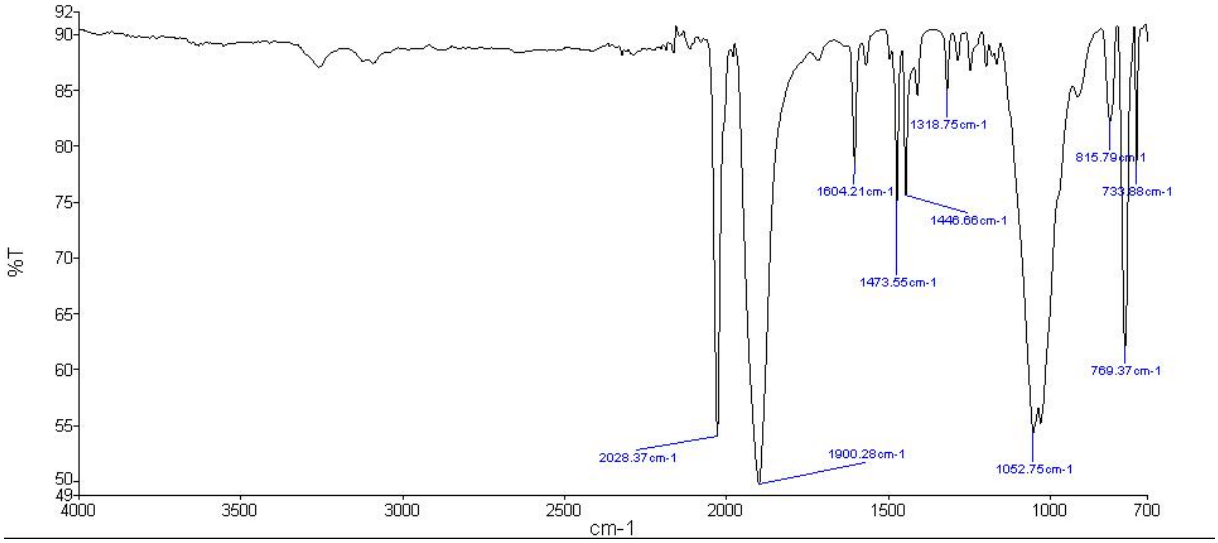


Figure S4. IR spectrum of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$.

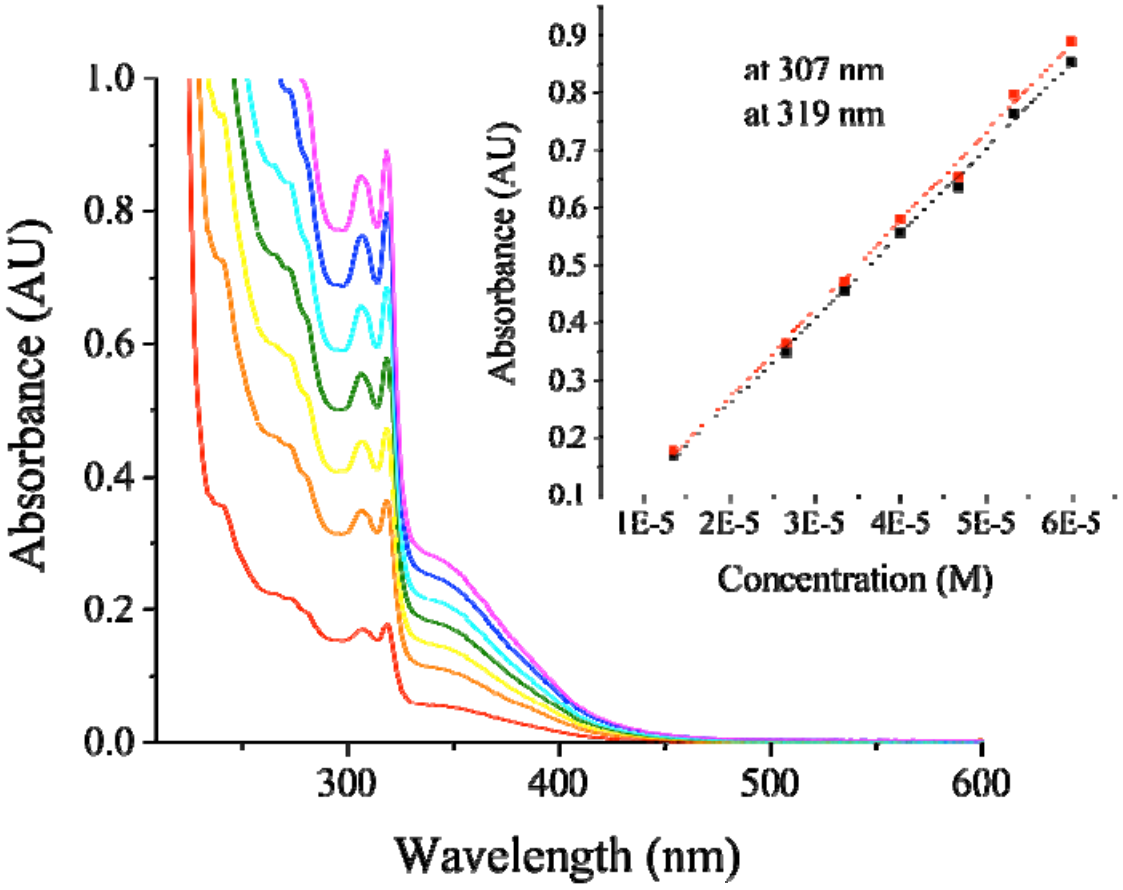


Figure S5. UV absorption spectrum of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$.

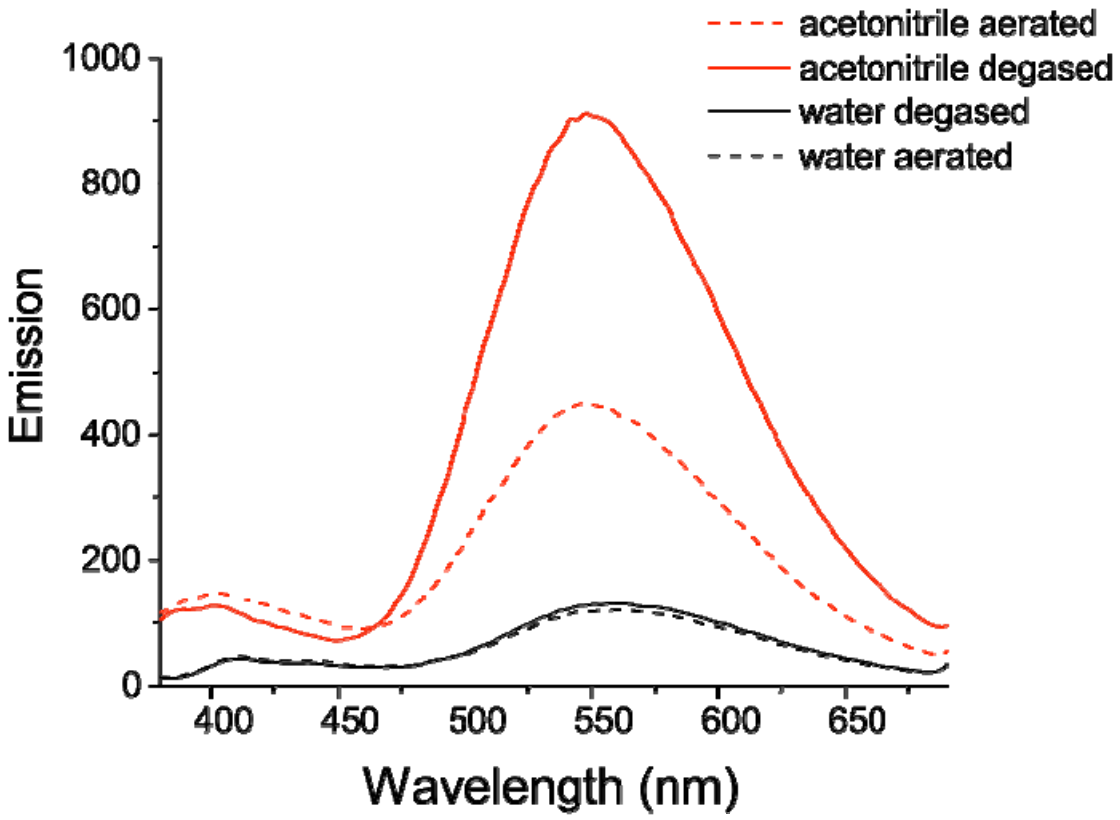


Figure S6. Emission spectrum of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$.

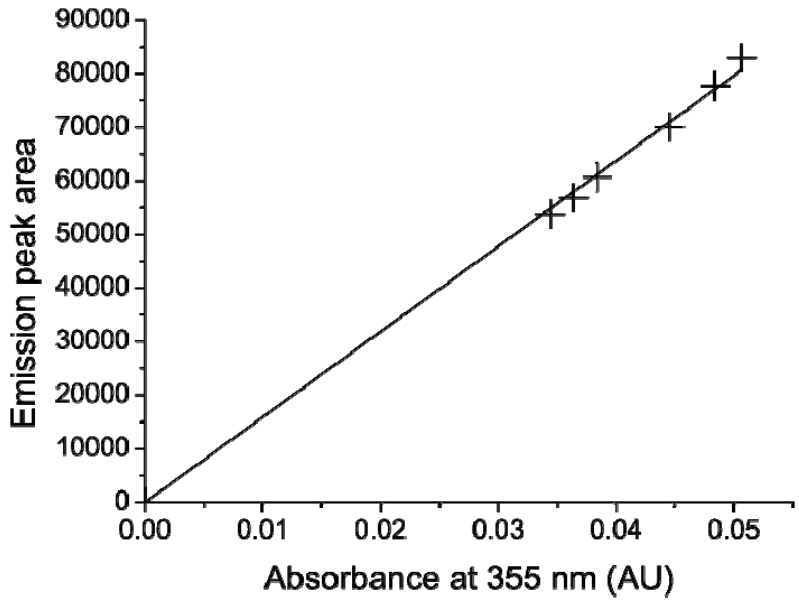


Figure S7. Determination of emission quantum yield: emission of the reference compound quinine hemisulfate vs. its absorbtion at the excitation wavelength (355 nm).

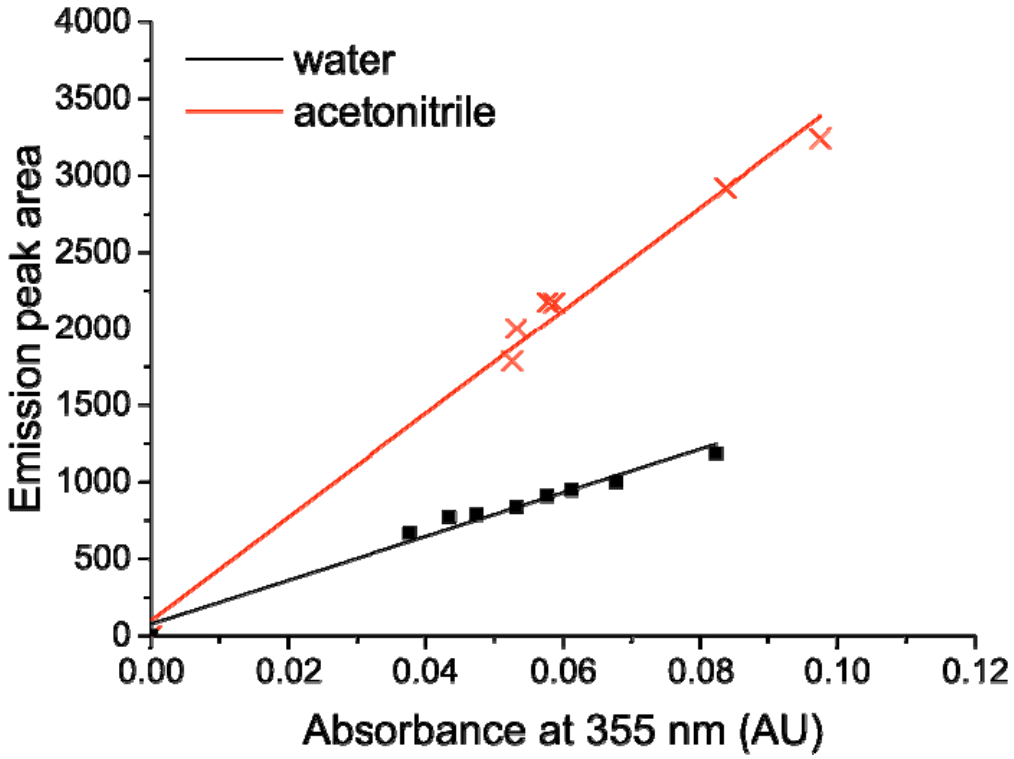


Figure S8. Determination of emission quantum yield: emission of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$ vs. its absorbtion at the excitation wavelength (355 nm).

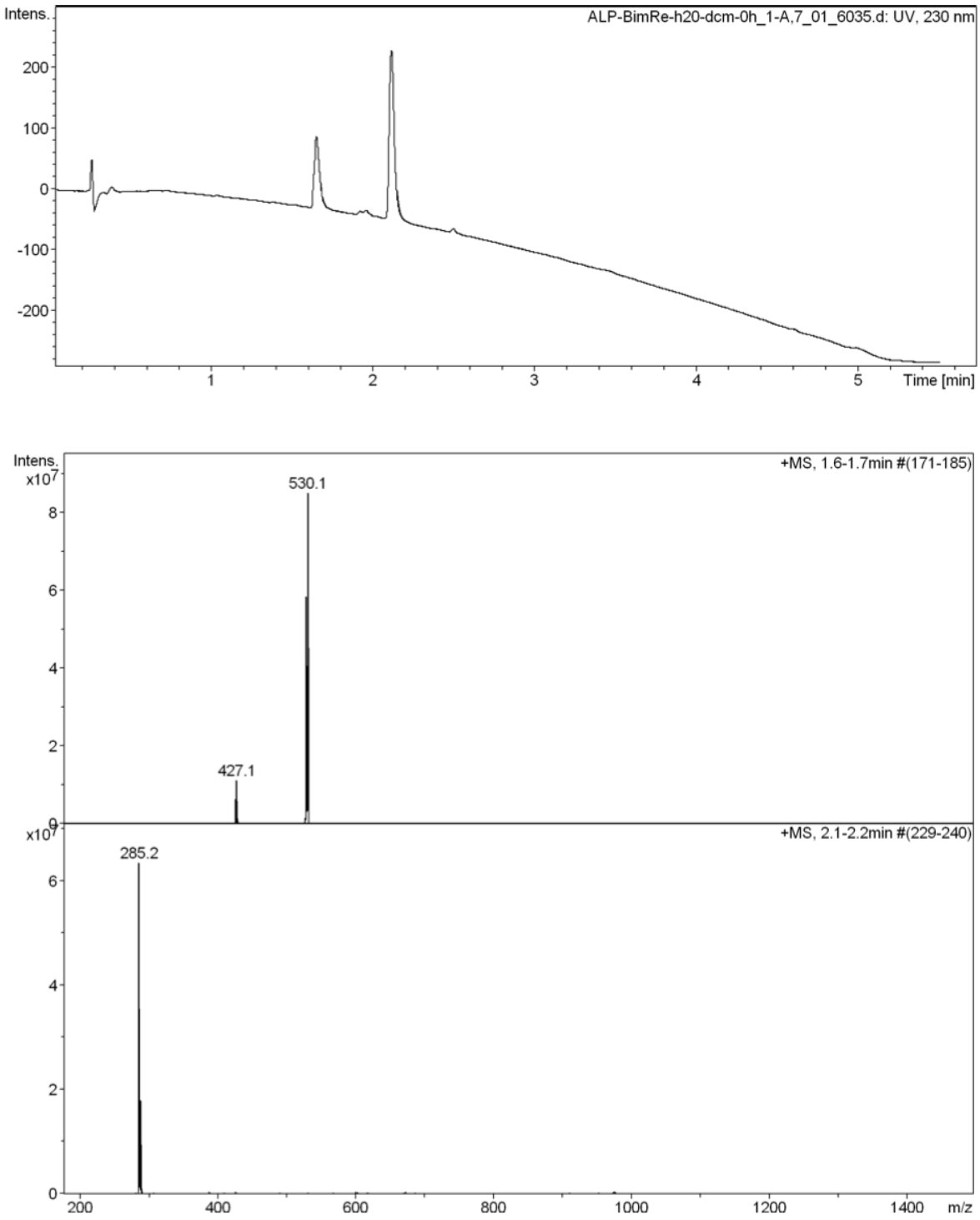


Figure S9. UPLC-MS of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$ and diazepam incubated in water for 0 h (DCM extract).

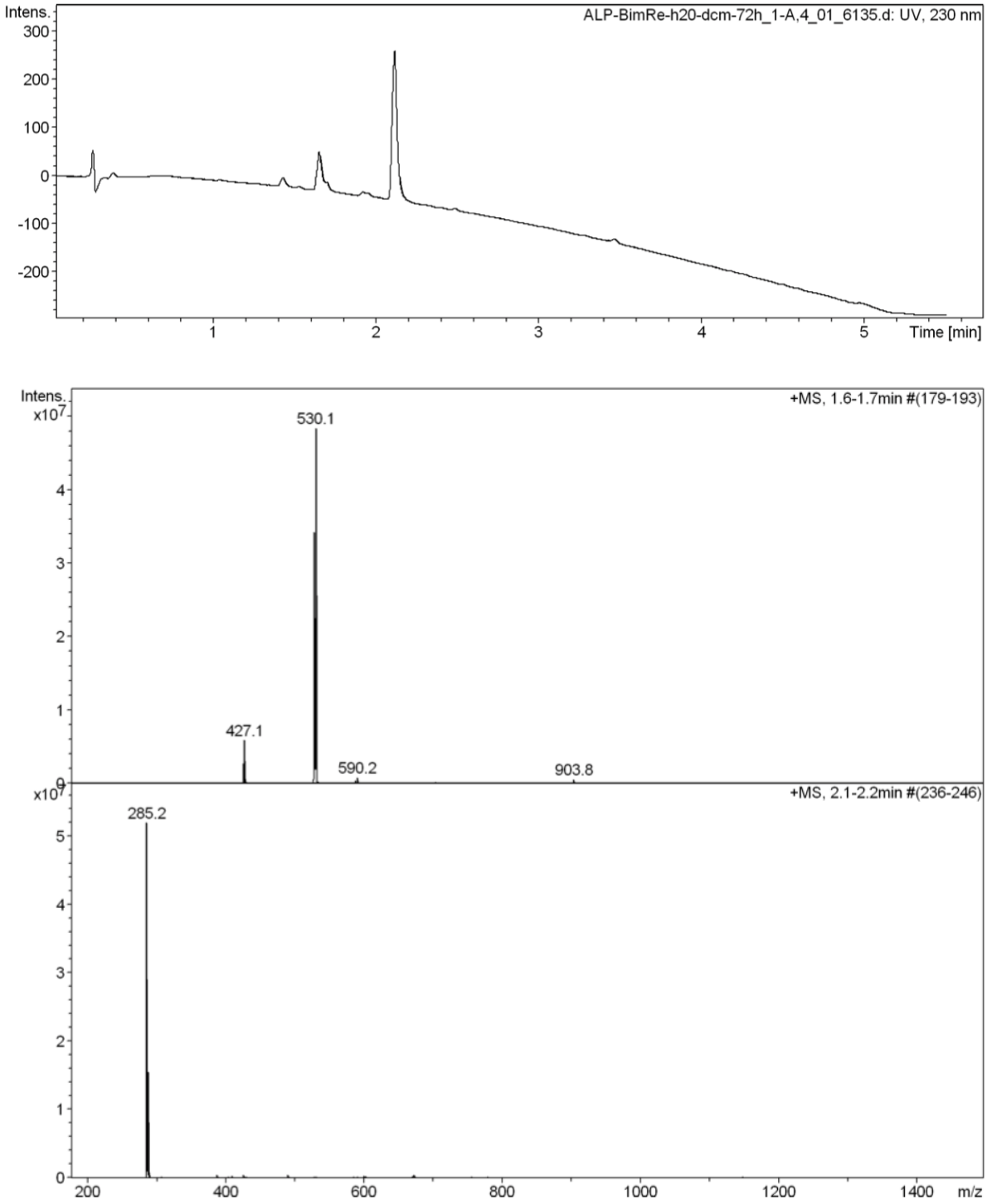


Figure S10. UPLC-MS of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$ and diazepam incubated in water for 72 h (DCM extract).

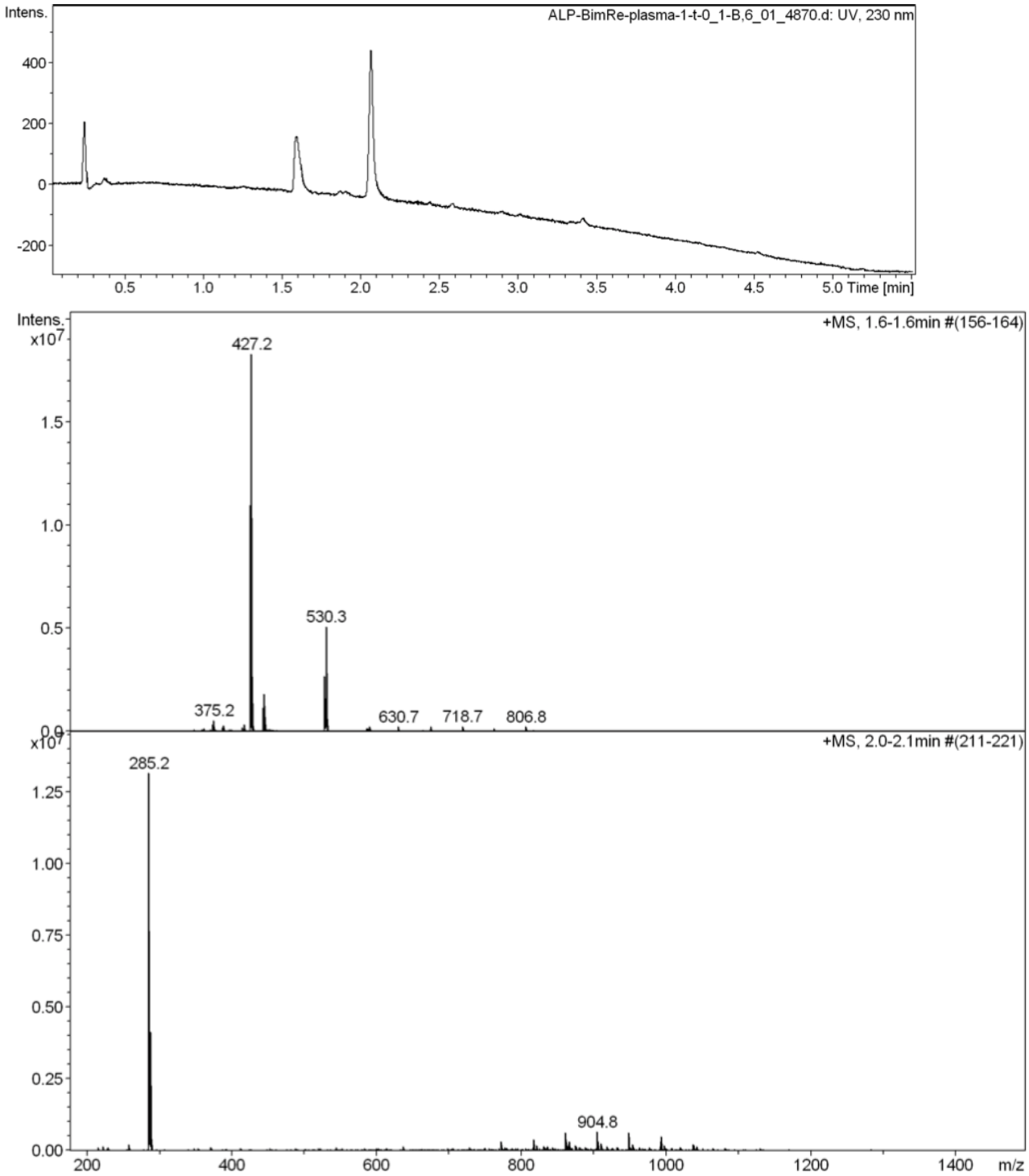


Figure S11. UPLC-MS of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$ and diazepam incubated in human blood plasma for 0 h (DCM extract).

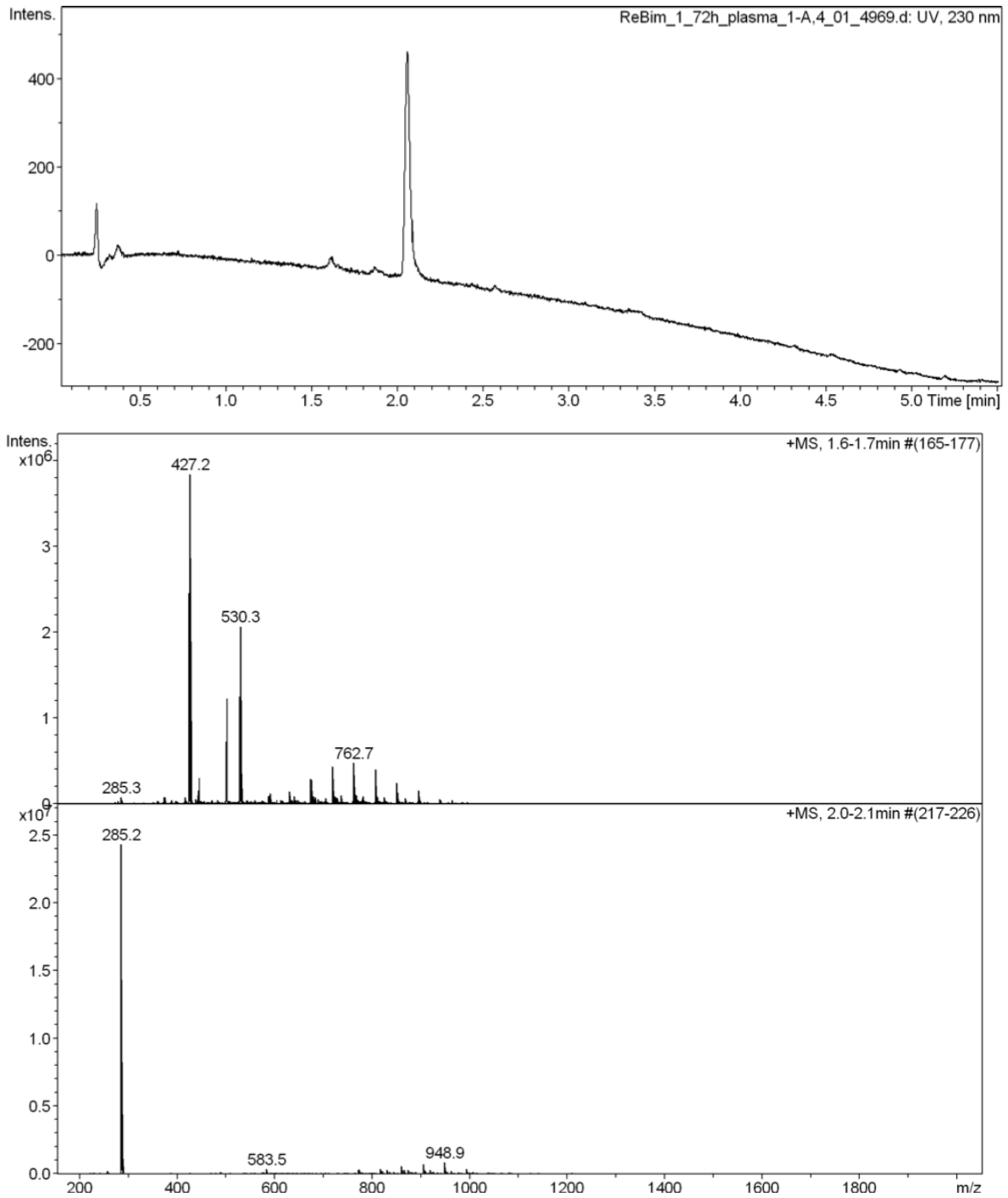


Figure S12. UPLC-MS of $[\mathbf{Re(CO)_3(bipy)(py\text{-}alkyne)}](\mathbf{BF_4})$ and diazepam incubated in human blood plasma for 72 h (DCM extract).

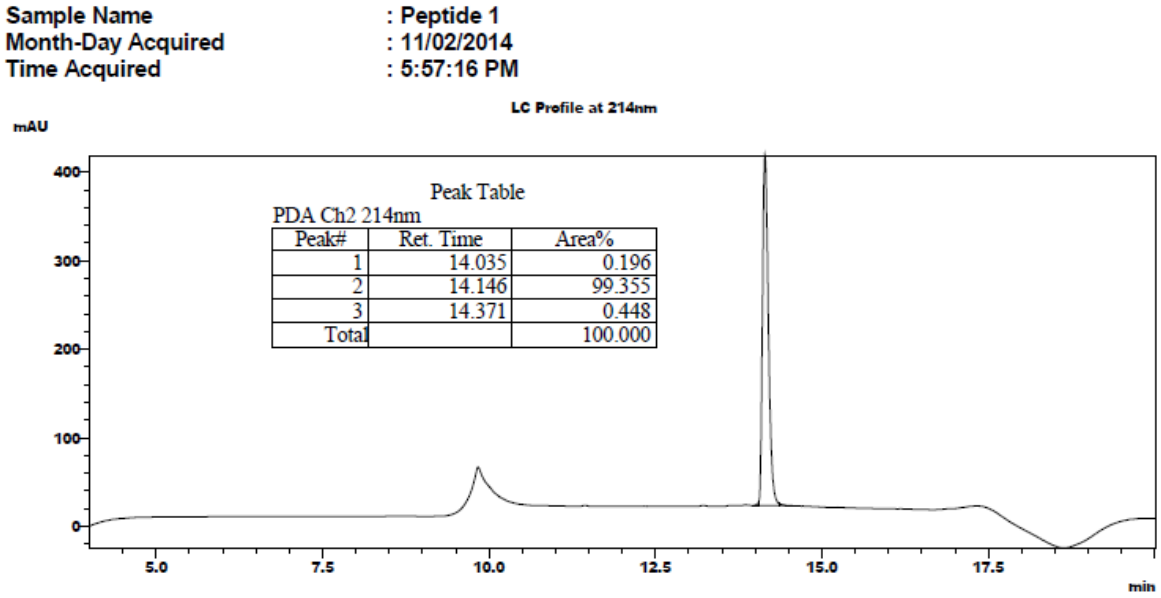
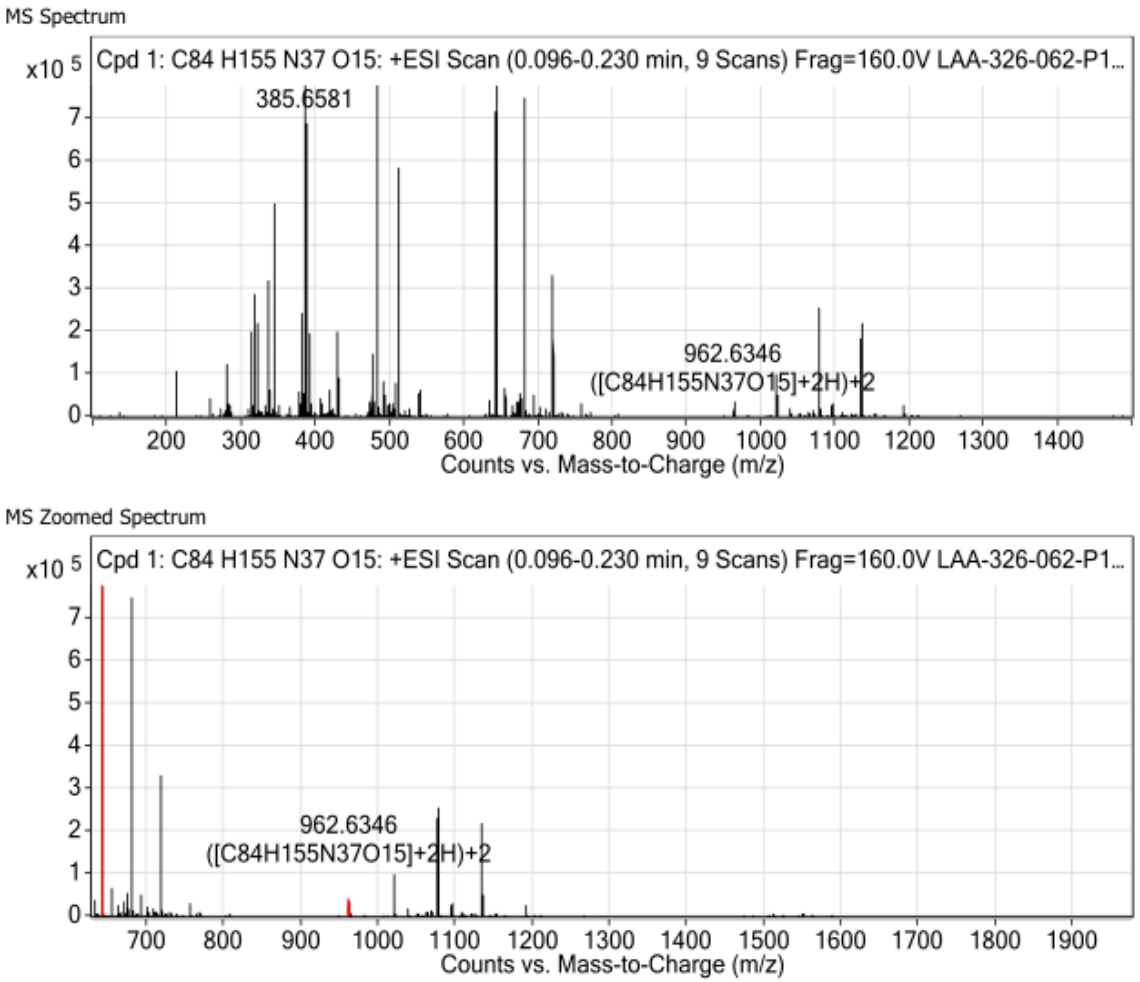


Figure S13. Analytical HPLC chromatogram for **myr-Tat**.



MS Spectrum Peak List

m/z	Calc m/z	Diff(ppm)	z	Abund	Formula	Ion
640.7495	640.7496	0.04	3	1367.14	C84H155N37O15	M+3
641.7601	641.7574	-4.25	3	715334.85	C84H155N37O15	(M+3H)+3
962.1332	962.1324	-0.81	2	34771.5	C84H155N37O15	(M+2H)+2

Figure S14. HRMS spectrum of **myr-Tat**.

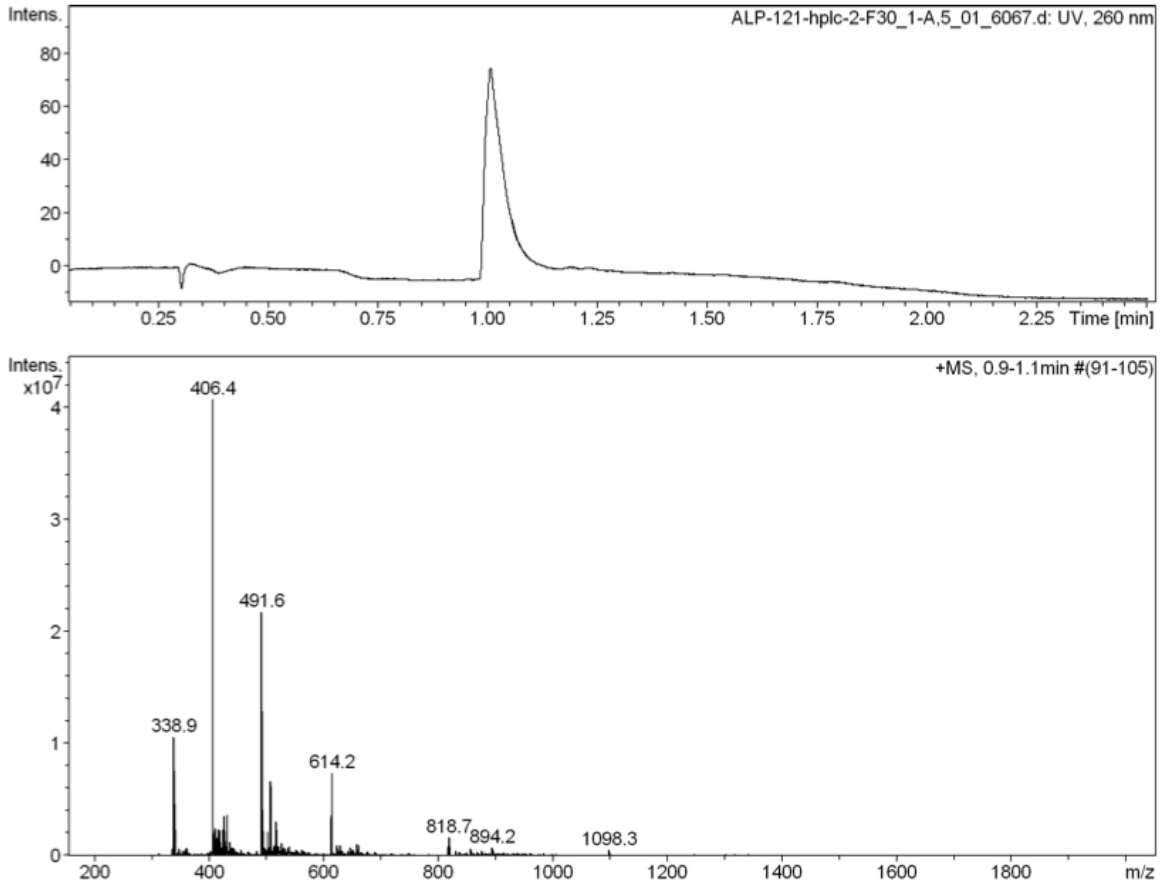


Figure S15. LC-MS spectrum of **Re-myr-Tat**.

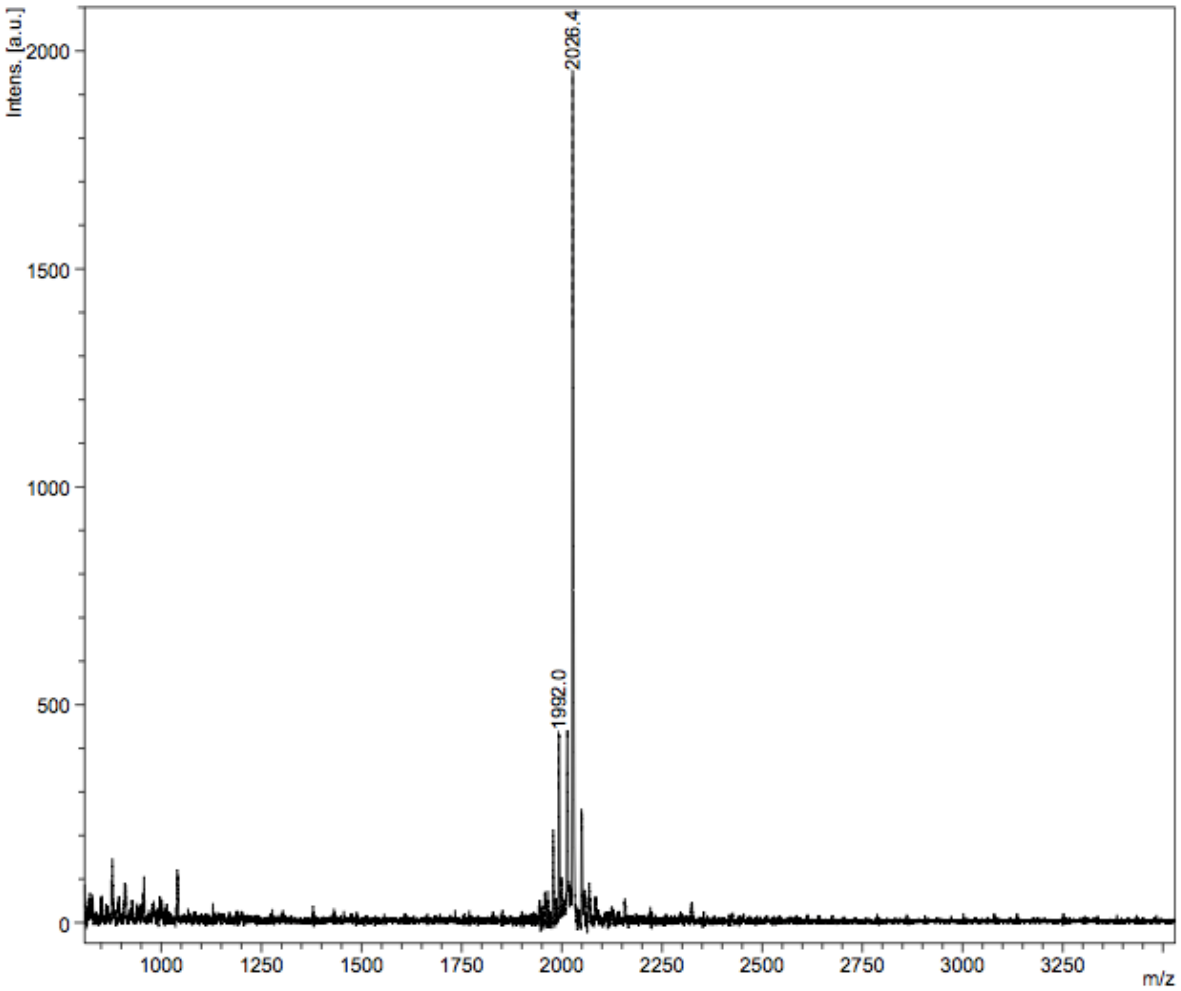


Figure 16. MALDI-TOF spectrum of **Re-myr-Tat**.

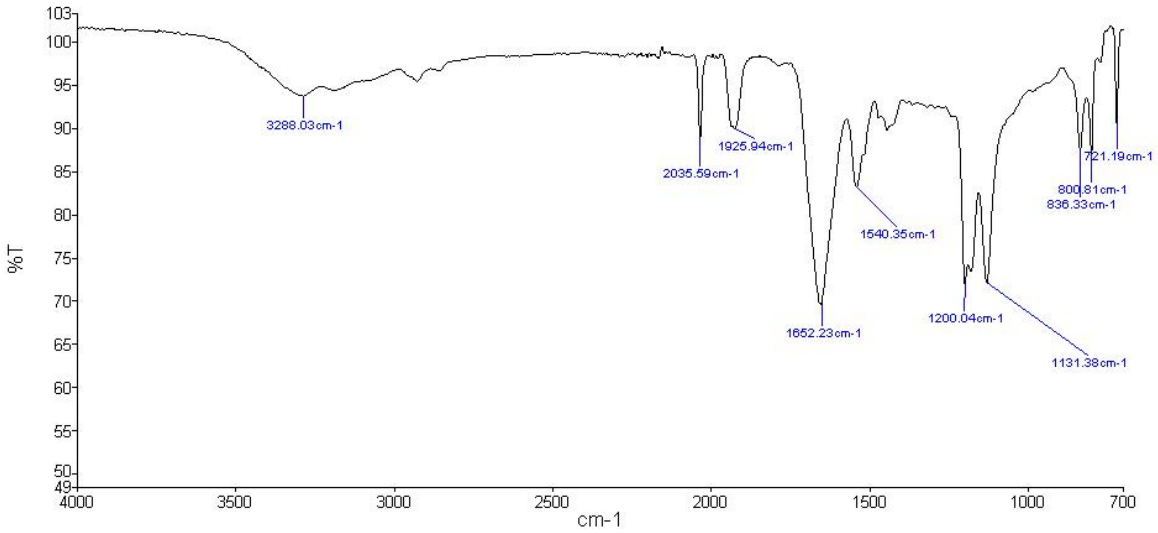


Figure 17. IR spectrum of **Re-myr-Tat**.

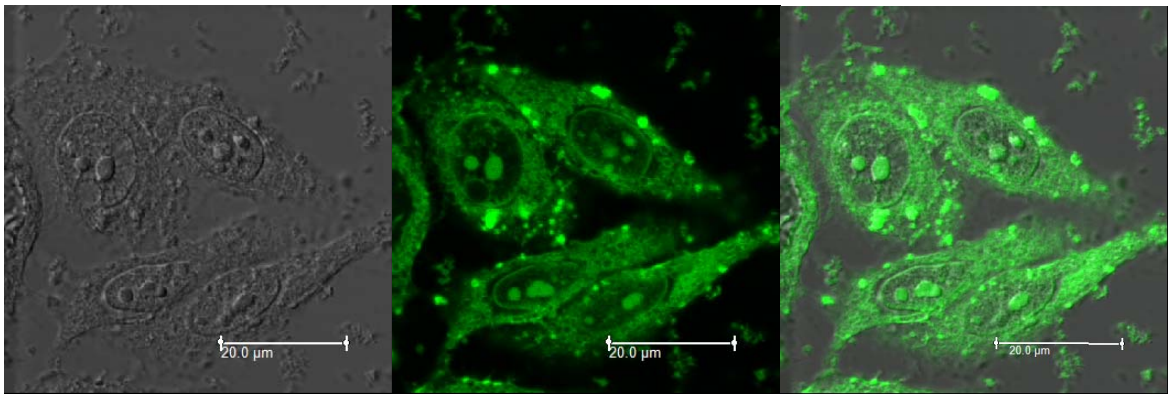


Figure 18. Fluorescence microscopy images of HeLa cells treated with **Re-myr-Tat** at 50 µM and fixed after 2 h.

References

(1) Amoroso, A. J.; Coogan, M. P.; Dunne, J. E.; Fernandez-Moreira, V.; Hess, J. B.; Hayes, A. J.; Lloyd, D.; Millet, C.; Pope, S. J. A.; Williams, C. Rhenium fac tricarbonyl bisimine complexes: biologically useful fluorochromes for cell imaging applications. *Chem. Commun.* **2007**, 2007.

(2) Pierroz, V.; Joshi, T.; Leonidova, A.; Mari, C.; Schur, J.; Ott, I.; Spiccia, L.; Ferrari, S.; Gasser, G. Molecular and Cellular Characterization of the Biological Effects of Ruthenium(II) Complexes Incorporating 2-Pyridyl-2-pyrimidine-4-carboxylic Acid. *J. Am. Chem. Soc.* **2012**, *134*, 2012.