Efficient photocaging of a tight-binding bisubstrate inhibitor of cAMP-dependent protein kinase

Electronic Supplementary Information

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S1. Experimental section

Materials and methods

Equipment and software

^{1}H NMR (700 MHz) and ^{13}C NMR (176 MHz) spectra were obtained with Bruker Avance-III 700 MHz NMR spectrometer (magnetic field 16.4 T). Tetramethylsilane (TMS) was used as the internal standard.

HPLC analysis and purification was performed with Shimadzu Prominence LC Solution HPLC system with SPD M20A PDA and LCMS-2020 detectors. Purification was performed with the reverse phase column Luna C18 (250 x 4.6 mm, particle size 5 um) that was eluted with ACN/H_2O gradient (0.1% TFA) at flow rate of 1 ml/min. The purification of photocaged substances was carried out with switched-off UV-Vis detector.

High resolution mass spectra (HRMS) were obtained on Thermo Electron LTQ Orbitrap (ESI HRMS) in positive ion mode or with combined Varian 910-FT-ICR and Varian J- 320 3Q spectrometers in positive ion mode.

UV-Vis spectra were obtained with NanoDrop 2000c (Thermo Scientific). Concentrations were calculated using the Lambert-Beer`s law using the following molar extinction coefficients: 15 000 $M^{-1}cm^{-1}$ for ARC-902 at 260 nm; 80 000 $M^{-1}cm^{-1}$ for ARC-583 at 558 nm (absorbance maximum of fluorescent label TAMRA); 250 000 $M^{-1}cm^{-1}$ for ARC-1182 at 652 nm (absorbance maximum of fluorescent label PF-647); 16 000 $M^{-1}cm^{-1}$ for ARC-1408 and ARC-1411 at 285 nm. The extinction coefficients of ARC-2116 ($\varepsilon = 12\ 000\ M^{-1}\,cm^{-1}$ at 286 nm) and substance **6** (ε = 12 000 M^{-1} cm^{-1} at 350 nm) were determined by measuring absorption of solutions prepared from known mass of the compounds.

Procedures with light-sensitive substances were performed under red light (Paulmann RGB LED GSL, 5W, emission peak at 630 nm).

The synthesis on solid phase was carried out in PP SPE tubes equipped with 70-µm porocity PP filters.

Light-sensitive substances were stored in amber tubes (Axygen #MCT-150-X). Stock solutions of other components were prepared in low binding centrifuge tubes (Protein LoBind, Eppendorf AG). The biochemical displacement assay was performed on black non-binding-surface 384-well polystyrene microplates (Corning #4514).

Eppendorf Research (Eppendorf) pipettes with low retention pipet tips (RPT, Starlab GmbH) and 12-channel electronic pipettes with original tips (E1 ClipTip, Thermo Scientific) were used for liquid handling in biochemical assays.

The fluorescence anisotropy (FA), time gated luminescence (TGL) and FRET measurements were performed with microplate reader PHERAstar (BMG Labtech).

The fluorescence spectra of cell lysates were measured on 384-well plate in 20 μL volume with Synergy NEO (BioTek) plate reader at 30 °C with the following settings: excitation at 420 (30) nm, emission recorded from 460 to 700 nm with 5 nm detection intervals.

Data were processed with Graphpad Prism software (v 6.04, GraphPad Software, La Jolla California USA) and Microsoft Excel (2013).

Photolysis was performed with the following irradiation systems:

1) Mercury vapour lamp (300 W, Ultra-Vitalux, OSRAM). The lamp was switched on 30 min before the experiment to achieve stable working regime. The distance between the light source and the sample was 40 cm. The samples were held in closed quartz cuvettes or in open eppendorfs (1.5 mL) cooled in ice bath during the irradiation.

2) Custom-made LED array accommodating 16 LEDs [150 mcd, λ_{max} = 398 nm (Figure S1), light output 15 mW per one LED; code S300JOUV4G] designed for irradiation of one row of wells at a time in 384-well microtiter plate. Irradiation experiments were performed in black 384-well polystyrene microplates at 5 mm distance between the sample and the light source. The irradiation was carried out at room temperature.

Reagents and solutions

Chemicals from the following commercial sources were used: AIBN (Bapex); Fmoc-[D-Arg(Pbf)]$_6$-Rink amide resin (0.18 mmol/g; Caslo ApS); deuterated solvents (Deutero GmbH); 2-bromodibenzo[d,b]furan (Fluorochem); Fmoc-protected amino acids and resins (Iris Biotech GmbH); 4-chloro-7*H*-pyrrolo[2,3-d]pyrimidine (Pharnorcia); and HEPES sodium hemisalt (Santa Cruz). Other chemicals were obtained from Acros, Alfa Aesar, Bachem, Fisher, Fluka, Lachner, Mallinckrodt, Merck, Reachim, Riedel-de Haën and Sigma-Aldrich. Silica gel and TLC plates were obtained from Macherey-Nagel. ARC-902, ARC-1408, ARC-1411, ARC-1182 and ARC-583 had been previously synthesized in the same laboratory.[1,3,4]

THF was distilled from sodium benzophenone ketyl. 4-chloro-7*H*-pyrrolo[2,3-d]pyrimidine was passed through short pad of silica gel in etherous solution to get white powder instead the

initial faint solid. Other chemicals were used as provided by the manufacturer. Human recombinant full-length PKAc α-isoform had been produced previously.[1]

Semi-stable cell line C9H6 (Chinese hamster ovary, CHO) transfected with CFP-fused PKA regulatory subunit (PKArIIβ-L20-CFP) and YFP-fused PKA catalytic subunit (PKAcα-YFP) was a kind gift from Prof. Manuela Zaccolo (University of Oxford, UK).

The following buffers were used in biochemical measurements:

Buffer A – [50 mM HEPES (pH = 7.5); 150 mM NaCl; 0.005 % (vol) Tween®20 (P20); 5 mM DTT; 0,5 mg/mL BSA],

Buffer B – [50 mM HEPES (pH = 7.5); 150 mM NaCl; 0.005 % (vol) P20; 5 mM DTT],

Buffer C – [50 mM HEPES (pH = 7.5); 150 mM NaCl; 0.005 % (vol) P20].

Biochemical displacement assay

The biochemical displacement assay was performed in a final volume of 20 μL per one well. The concentration of the active form of PKAcα was determined by titration of a fixed concentration of fluorescent probe ARC-1182 (10 nM; K_D = 0.02 nM)[2] or ARC-583 (20 nM; K_D = 0.5 nM)[3] with 2-fold dilutions of the enzyme. Formation of the complex was followed by observation of the FA change of the solution and the concentration of PKAcα was calculated by non-linear regression analysis as previously described.[3]

The equilibrium dissociation constants (K_D) of the complexes between novel inhibitors and PKACα were established by FA-based[2,3] and TGL-based[1,4] displacement assays according to the published procedures. The FA assay was run in two configurations.

The first configuration was used for determination of K_D values of inhibitors with moderate and weak affinity: ARC-2112 (before and after irradiation), ARC-2113 and ARC-2116. ARC-902 (K_D = 0.5 nM)[3] was used as a reference compound. The solutions contained the competing ligand (3-fold dilutions), PKAcα (3 nM active catalytic subunit) and fluorescent probe ARC-583 (2 nM; K_D = 0.5 nM for the complex with PKAc) in buffer A.[3]

The second configuration of the assay was used for determination of K_D values of inhibitors with high affinity: ARC-2114 and ARC-2115, also ARC-2113 after irradiation. ARC-1408 (K_D = 0.18 nM)[1] was used as reference compound. The solutions contained the competing ligand (3-fold dilutions), PKAcα (1.5 nM active catalytic subunit) and fluorescent probe ARC-1182 (1 nM; K_D = 0.02 nM for the complex with PKAc) in buffer A.[2]

The TGL-based assay was used for determination of K_D-value of an inhibitor with very high affinity, ARC-2123, at the following concentrations of the components: competing ligand (3-

fold dilutions), PKACα (1 nM active catalytic subunit) and fluorescent probe ARC-1182 (50 nM) in buffer B.[1,4] ARC-1411 was used as the reference compound.[1]

The apparent affinities* (K_D^{app}) of photolyzed inhibitors were determined after irradiation of the solutions containing the photocaged inhibitors ARC-2112 or ARC-2113 (3-fold dilutions) in buffer B for up to 64 min under Hg vapour lamp. Aliquots of the solutions collected at fixed time points were mixed with the solution of the fluorescent probe and PKAcα to yield assay mixtures with compositions as described above. In addition, the aliquots were analyzed by HPLC-MS (Figure S6) and UV-Vis (Figure S7).

The microplates were incubated for 30 min at 30 °C before measurement. In the case of the TGL assay, the measurement was repeated after 60 min incubation at 30 °C and it gave practically coinciding data (not shown).

The FA measurements were performed with optical modules [EX 590(50) nm, EM 675(50) nm] for the fluorescent probe ARC-1182 and [EX 540(50) nm, EM 590(50) nm] for the fluorescent probe ARC-583. The detector was calibrated with the solution of the fluorescent probe in the absence of PKAcα.

The TGL measurements were performed with HTRF optical module [EX 330(50) nm, EM 675(50) nm]. The TGL signal was recorded for 200 μs after 50 μs time delay from the start of the excitation pulse.

To make the results obtained at different assay conditions comparable, K_D values of competing ligands were calculated by fitting the data to the one-site competitive binding model, as described previously.[1]

All experiments were carried out in minimum of at least two independent experiments ($n \geq 2$) and the reported errors are the standard errors of the mean. The displacement curves are given in Figures S2, S3, and S4 and the calculated IC_{50} and K_D values are given in Table S3.

Cell lysate experiments

The C9H6 cells (Chinese hamster ovary, CHO) transfected with CFP-fused PKA regulatory subunit (PKArII*β*-CFP) and YFP-fused PKA catalytic subunit (PKAc*α*-YFP) were seeded on a 10 mm Petri dish (ThermoFisher BioLite) and grown in Dulbecco's Modified Eagle's Medium and Ham's-F12 mixture supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 800 μg/ml G418, and 300 μg/ml

* With 100% photoconversion, K_D^{app} of photolyzed ARC-2112 and ARC-2113 would correspond to K_D of ARC-1408 and ARC-2123, respectively.

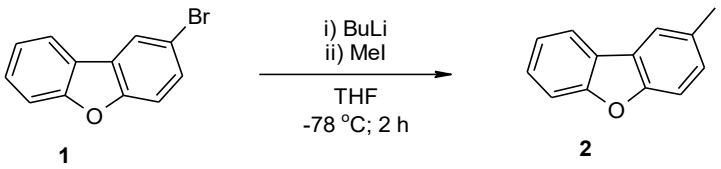
Zeocin™ at 37° C in 5% CO_2 atmosphere as previously described.[5] After achieving confluency, cells were trypsinized, counted using TC10™ Automated Cell Counter (Bio-Rad Laboratories), pelleted, rinsed with PBS and pelleted again (800 rpm, 5 min). Next, the pellet was lysed using NP-40 cell lysis buffer (FNN0021, Life Technologies), 1x protease inhibitor cocktail (Complete EDTA free, Roche), 1% Triton X, 0.5 mM DTT, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF). The volume of lysis buffer used was 40 pL per cell, typically about 500 µL lysate was produced by lysis of 12.5 million cells. The protein content in the lysate was 2300 µg/mL (according to Bradford assay).

FRET experiments with C9H6 cell lysate containing fused proteins PKArIIβ-CFP and PKAcα-YFP were carried out as follows. 3-fold diluted series of cAMP, ARC-2113 and ARC-1411 were made in assay buffer C. C9H6 cell lysate was diluted in buffer C containing $(CH_3COO)_2Mg$, and ATP; the diluted lysate was added to each well containing cAMP or inhibitor yielding final concentrations of 16% lysate (by volume), 10 mM Mg^{2+} and 1 µM ATP in 20 µL volume. FRET was measured with optical module [excitation at 427(10) nm, emission at 530(10) nm and 480(10) nm] after 1 h incubation at 30 °C. The irradiation of cell lysate was performed with LED array for 1 minute, followed by monitoring the FRET change within 1 h.

Chemical synthesis

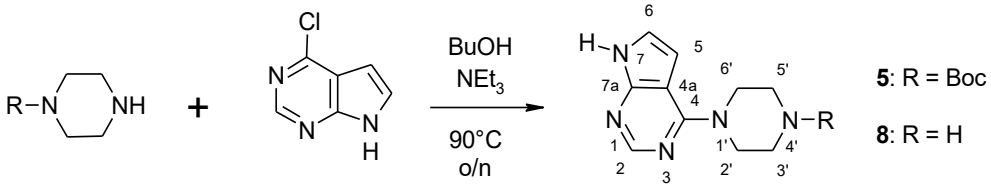
Compounds **3** and **4** were synthesized according to published procedures.[6] Novel ARCs (full structures are given in Table S1) were made according to standard solid phase Fmoc-peptide synthesis protocols. Pbf was used as the protecting group for D-Arg. Triisopropylsilane (TIPS) was used as scavenger in cleavage coctail. Coupling of 7DP-Pip **8** and its derivatives **7** and **10** to resin-attached peptide was carried out as described by Ivan *et al.*[1] Procedures with light sensitive compounds were carried out under red light. The NMR spectra of synthesized compounds are given in Chapter S4; the UV-Vis and HPLC-MS data in Table S2 and in Chapter S5.

2-methyldibenzo[*b*,*d*]furan (2)



2 M solution of *n*-BuLi (80 µl, 0.971 mmol, 1.2 eq) in cyclohexane was added to **1** dissolved in dried THF at −78 °C under Ar atmosphere and stirred for 30 min. MeI (100 µl, 1.619 mmol, 2 eq) was added dropwise at −78 °C over 10 min. Then the reaction mixture was warmed to rt. Reaction was monitored with TLC (Hexane:DCM, 10:1 v:v; starting material R_f = 0.44; product R_f = 0.33). After 2 h the reaction mixture was diluted with diethyl ether (2 ml), quenched with 5% NH_4Cl (1 ml), washed with 1 M NaOH (1 ml), H_2O (1 ml), and brine (1 ml), dried over Na_2SO_4 and concentrated in vacuum. Obtained yellow oil (1080 mg) was analyzed with NMR, indicating a 90% conversion to **2** (989 mg), but also a DBF(dibenzo[b,d]furan) side product (9%). NMR spectrum corresponds to that of the previously published compound.[7] Substance **2**: **NMR δ_{1H}**(700 MHz; $CDCl_3$; Me_4Si) 7.91 (1H, d, *J* = 7.7 Hz, H_{ar}); 7.74 (1H, s, H_{ar}); 7.54 (1H, d, *J* = 7.7 Hz, H_{ar}); 7.47-7.42 (2H, m, H_{ar}); 7.34-7.31 (1H, m, H_{ar}); 7.25 (2H, d, *J* = 8.4 Hz, H_{ar}); 2.51 (3H, s, CH_3). **NMR δ_{13C}**(176 MHz; $CDCl_3$; Me_4Si) 156.5, 154.5, 132.2, 128.2, 127.2, 124.3, 124.2, 122.5, 120.6, 120.5, 111.7, 111.2, 21.4. **DBF**: **NMR δ_{1H}**(700 MHz; $CDCl_3$; Me_4Si) 7.94 (2H, d, *J* = 7.7 Hz, H_{ar}); 7.56 (2H, d, *J* = 7.7 Hz, H_{ar}); 7.46-7.41 (2H, m, H_{ar}), 7.34-7.32 (2H, m, H_{ar}). **NMR δ_{13C}**(176 MHz; $CDCl_3$; Me_4Si) 156.2, 126.9, 124.2, 122.5, 120.6, 111.6.

7*H*-pyrrolo[2,3-*d*]pyrimidin-piperazine adducts (5, 8)

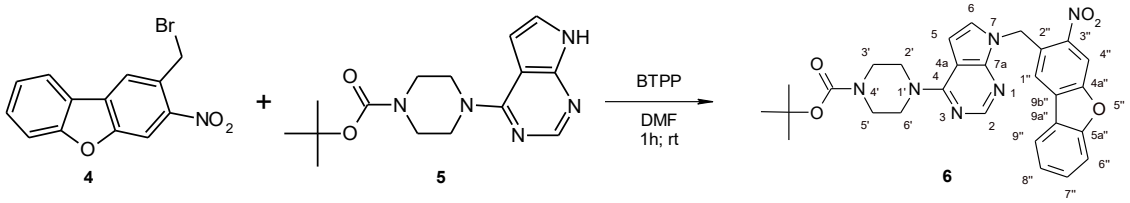


Compounds **5** and **8** were made by procedures described for the preparation of adducts of purine with derivatives of piperidine.[8] Briefly, 4-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine was heated at 90 °C in the solution of piperazine (4 eq) and TEA (5 eq) or Boc-piperazine (1.2 eq) and TEA (2 eq) in *n*-BuOH (5-10 ml/mmol/eq) overnight. The product was isolated as described below.

***tert*-butyl 4-(7*H*-pyrrolo[2,3-d]pyrimidin-4-yl) piperazine-1-carboxylate, 7DP-Pip-Boc (5)** was made in 0.4 mmol scale and the product was purified by silica gel column chromatography ($CHCl_3$:MeOH 95:5 v:v).[9] **5** was obtained as off-white solid (90 mg, 74%). **NMR δ_{1H}**(700 MHz; $CDCl_3$; Me_4Si) 11.70 (1H, s, 7-H), 8.37 (1H, s, 2-H), 7.15 (1H, d, J = 3.5 Hz, 5-H or 6-H), 6.51 (1H, d, J = 3.5 Hz, 5-H or 6-H), 3.99 (4H, m, 2×CH_2), 3.61 (4H, m, 2×CH_2), 1.50 (9H, s, 3×CH_3); **NMR δ_{13C}**(176 MHz; $CDCl_3$; Me_4Si) 157.1, 154.8, 152.3, 150.8, 121.0, 103.1, 101.1, 80.2, 45.3 (br s), 43.9 (br s), 42.8 (br s), 28.5.

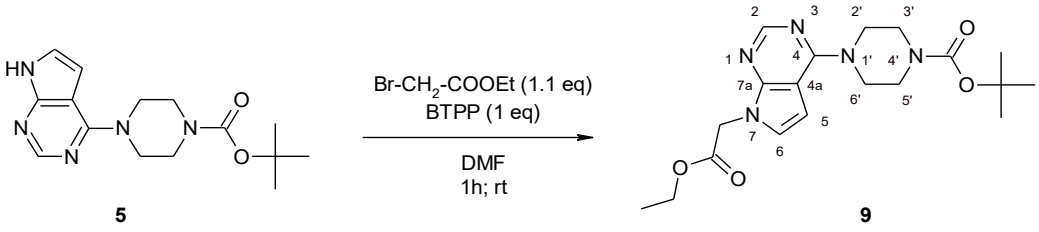
4-(piperazin-1-yl)-7*H*-pyrrolo[2,3-d]pyrimidine (8) was made in 1.3 mmol scale. The reaction was monitored with TLC ($CHCl_3$:MeOH, 10:1, v:v; starting material R_f = 0.8; product R_f = 0.0). Precipitate was formed that was filtered, washed with small amount of EtOH and dried in vacuum to yield 170 mg of **8** as white powder (65%). **NMR δ_{1H}**(700 MHz; DMSO-d6; Me_4Si) 11.82 (1H, s, 7-NH); 8.20 (1H, s, 2-H); 7.25 (1H, dd, J = 2.1 Hz, 3.5 Hz, 5-H or 6-H); 6.65 (1H, dd, J = 1.4 Hz, 3.5 Hz, 5-H or 6-H); 4.00 (4H, m, CH_2-3`, $CH_2$-5`), 3.12 (4H, m, CH_2-2`, $CH_2$-6`). **NMR δ_{13C}**(176 MHz; DMSO-d6; Me_4Si) 156.0, 151.9, 150.3, 121.9, 102.3, 100.5, 43.2, 43.0.

tert-butyl 4-(7-[(3-nitrodibenzo[*b*,*d*]furan-2-yl)methyl]-7*H*-pyrrolo[2,3-*d*]-pyrimidin-4-yl)piperazine-1-carboxylate, NDBF-7DP-Pip-Boc (6)



Synthesis was performed inside an aluminium foil-covered flask placed under fume hood illuminated with red light when necessary. **5** (7.42 mg, 0.025 mmol, 1.5 eq) and phosphazene base P_1-t-Bu-tris(tetramethyl) (BTPP; 7.50 µl, 0.025 mmol, 1.5 eq) were dissolved in DMF (400 µl) and stirred for 30 min. Solution of **4** (5.18 mg, 0.017 mmol, 1 eq) in DMF (170 µl) was added and the mixture was stirred for 2 h. Reaction was monitored with TLC (Hexane:EtOAc, 1:1, v:v; substance **5** R_f = 0.41; starting material **4** R_f = 0.05; product R_f = 0.43). Reaction mixture was diluted with $CHCl_3$, washed twice with H_2O, and purified by column chromatography (Hexane:EtOAc; 1:1, v:v). 4.9 mg of product **6** was obtained in a 55% yield. **NMR δ_{1H}**(700 MHz; $CDCl_3$; Me_4Si) 8.38 (1H, s, H-4′′); 8.37 (1H, s, H-2); 7.81 (1H, d, *J* = 7.7 Hz, H-6′′ or H-9’’); 7.60 (1H, d, *J* = 8.4 Hz, H-6′′ or H-9’’); 7.55 (1H, td, *J* = 7.7 Hz, *J* = 0.7 Hz, H-7′′ or H-8′′); 7.40 (1H, s, H-1′′), 7.34 (1H, td, *J* = 7.7 Hz, *J* = 0.7 Hz, H-7′′ or H-8′′); 7.10 (1H, d, *J* = 3.5 Hz, H-5 or H-5); 6.62 (1H, d, *J* = 3.5 Hz, H-5 or H-6), 5.94 (2H, s, CH_2-7-N); 4.02 (4H, t, *J* = 4.9 Hz, CH_2-3′, CH_2-5′); 3.64 (4H, s, CH_2-2′, CH_2-6′); 1.51 (9H, s, 3×CH_3). **NMR δ_{13C}**(176 MHz; $CDCl_3$; Me_4Si) 158.4, 157.2, 154.8, 154.2, 151.8, 151.6, 146.0, 129.7, 129.5, 128.5, 124.2, 123.7, 122.2, 122.0, 120.8, 112.2, 109.4, 103.3, 101.7, 80.3, 45.8, 44.0 (2 C), 42.7 (2 C), 28.4 (3 C).

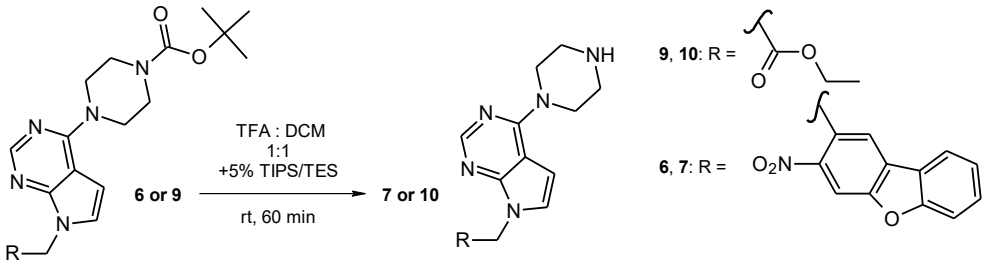
tert-butyl 4-[7-(2-ethoxy-2-oxoethyl)-7*H*-pyrrolo [2,3-*d*]pyrimidin-4-yl] piperazine-1-carboxylate, EtOCOCH$_2$-7DP-Pip-Boc (9)



6-(piperazine-4-(tert-buthylcarbonyl)-1-yl)-7-deazapurine **(5)** (10.02 mg, 0.033 mmol, 1 eq) and BTPP (10.08 µl, 0.033 mmol, 1 eq) were dissolved in DMF (400 µl) and stirred for 30

min. Ethylbromoacetate (Br-CH_2-COOEt, 4 µl, 0.036 mmol, 1.1 eq) was added and mixture stirred for 2 h. The reaction was monitored with TLC (Hexane:EtOAc, 1:2 v:v, starting material $R_f = 0.1$; product $R_f = 0.4$). The reaction mixture was diluted with H_2O, extraxted with $CHCl_3$ (2x), and the product was purified with column chromatography (Hexane:EtOAc, 1:1 v:v). The product **9** yielded 90% and was analysed with NMR. Substance **9**: **NMR δ_{1H}** (700 MHz; $CDCl_3$; Me_4Si) 8.35 (1H, s, 2-H); 7.00 (1H, d, J = 2.1 Hz, 5-H or 6-H); 6.55 (1H, d, J = 2.1 Hz, 5-H or 6-H); 4.97 (2H, s, N-CH_2-CO); 4.23 (2H, q, J = 7.1 Hz, CH_3-CH_2-); 3.96 (4H, s, CH_2-3′, CH_2-5′); 3.60 (4H, s, CH_2-2′, CH_2-6′); 1.50 (9H, s, 3×CH_3); 1.28 (3H, t, J = 7.0 Hz, CH_3). **NMR δ_{13C}**(176 MHz; $CDCl_3$; Me_4Si) 168.4, 156.9, 154.8, 151.6, 151.2, 124.4, 103.1, 101.5, 80.2, 61.8, 45.5, 44.0 (2 C), 42.7 (2 C), 28.4 (3 C), 14.1.

Boc removal (synthesis of 10 and 11)



Substance **6** (11.36 mg, 0.03 mmol, 1 eq) or **9** (4.8 mg, 0.009 mmol, 1 eq) was dissolved in DCM:TFA:triethylsilane (TES; 1:1:0.1; 200 µl: 200 µl: 20 µl) and stirred 1h at rt. The reaction mixture was diluted with hexane (1.5 ml) and the solvents were removed *in vacuo* under gentle heating (40 °C). The products were used in the following synthesis step without further characterization.

S2. Supplementary Figures

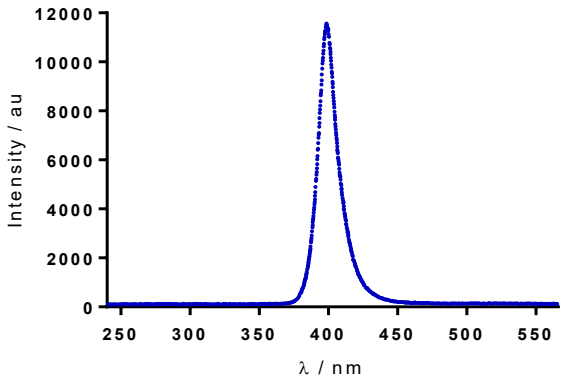


Figure S1. Irradiation spectrum of the LED S300JOUV4G, measured in the Institute of Physics, University of Tartu.

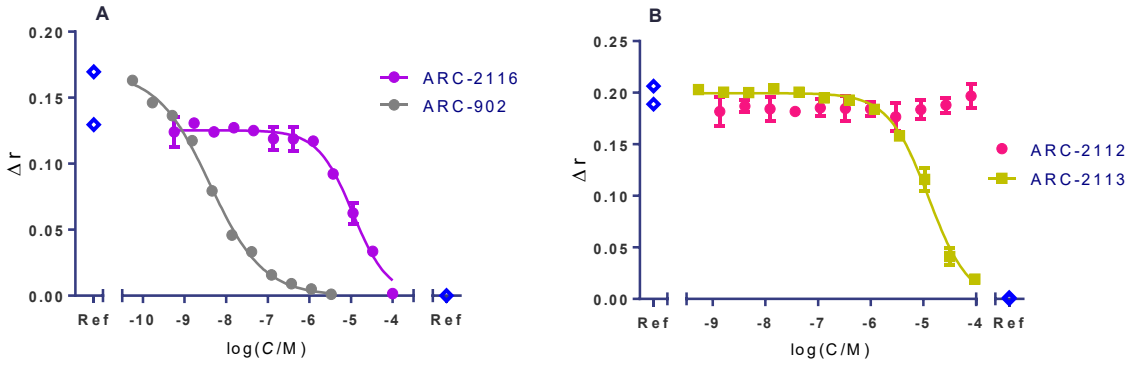


Figure S2. Results of FA assay: displacement of the fluorescent probe ARC-583 (2.0 nM) from complex with PKAcα (3 nM) by competing ligands ARC-2116 and ARC-902 (reference compound; **A**), and ARC-2112 and ARC-2113 (**B**). Reference datapoints (Ref) indicate the relative FA values of the complex of ARC-583 with PKAc (defining upper plateau) and the free ARC-583 (defining the lower plateau).

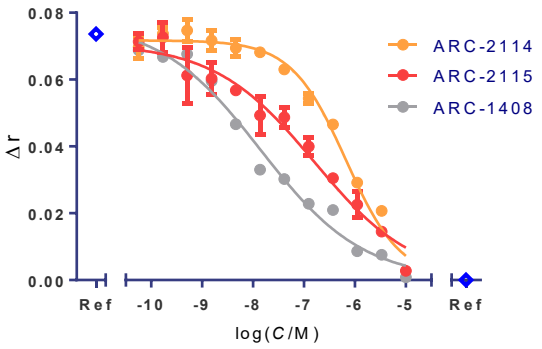


Figure S3. Results of FA assay: displacement of the fluorescent probe ARC-1182 (1.0 nM) from complex with PKAcα (1.5 nM) by competing ligands ARC-2114, ARC-2115 and ARC-1408 (reference compound). Reference datapoints (Ref) indicate the relative FA values of the complex of ARC-1182 with PKAc (defining upper plateau) and free ARC-1182 (defining the lower plateau).

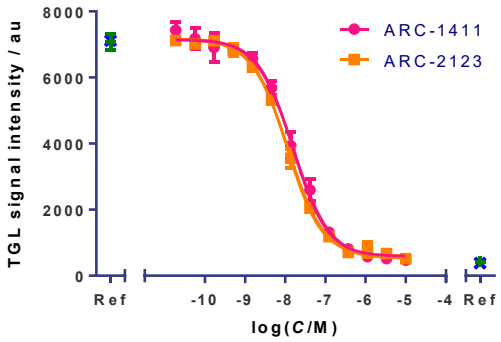


Figure S4. Results of TGL assay after 30 min incubation: displacement of the fluorescent probe ARC-1182 (50 nM) from complex with PKAcα (1 nM) by competing ligands ARC-2123 and ARC-1411 (reference compound). Reference datapoints (Ref) indicate the average TGL signal of the complex of ARC-1182 with PKAc (defining upper plateau) and free ARC-1182 (defining the lower plateau).

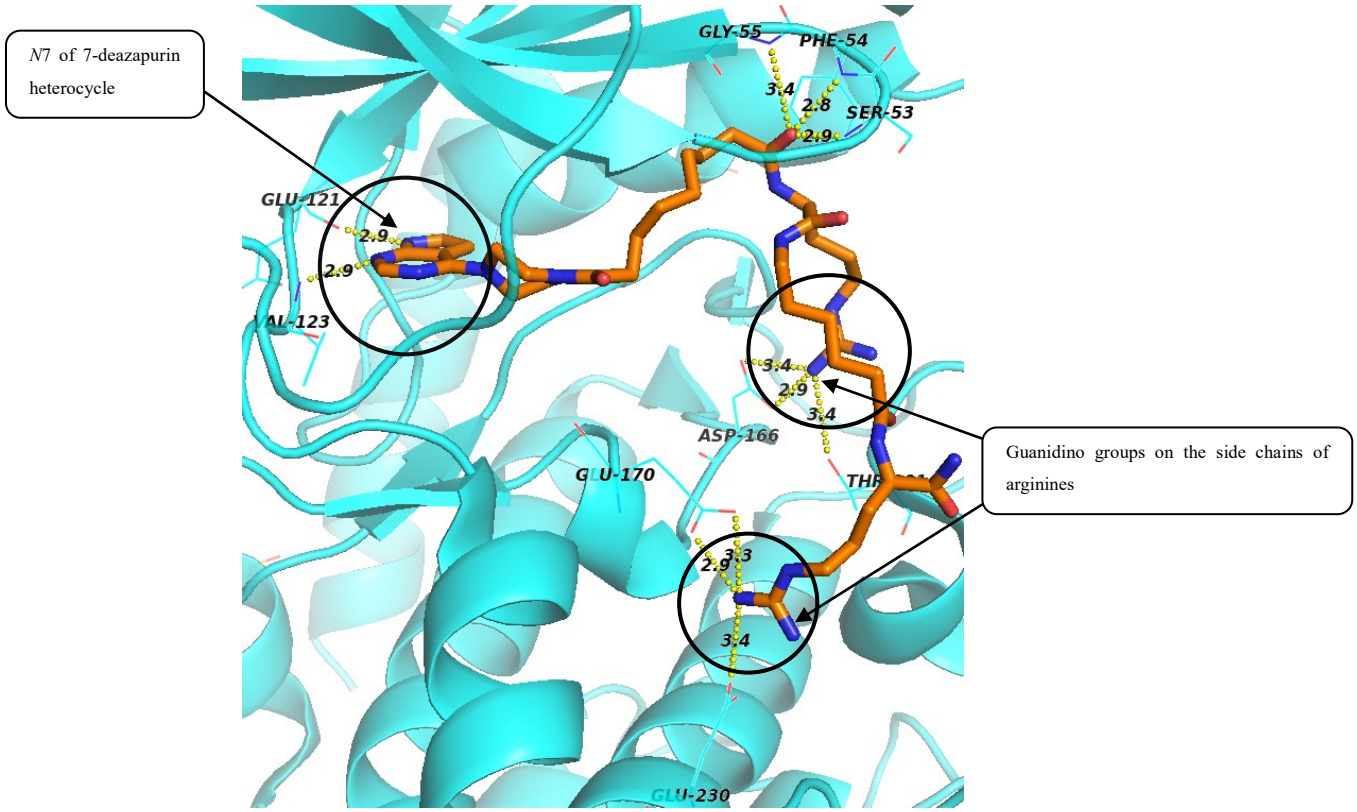


Figure S5. ARC-1408 (shown as coloured sticks: C atoms orange, N atoms blue, O atoms red) and PKAc (teal cartoon) complex to illustrate the ARC-1408 positioning inside the PKAcα catalytic pocket (PDB: 5IZF).[10]

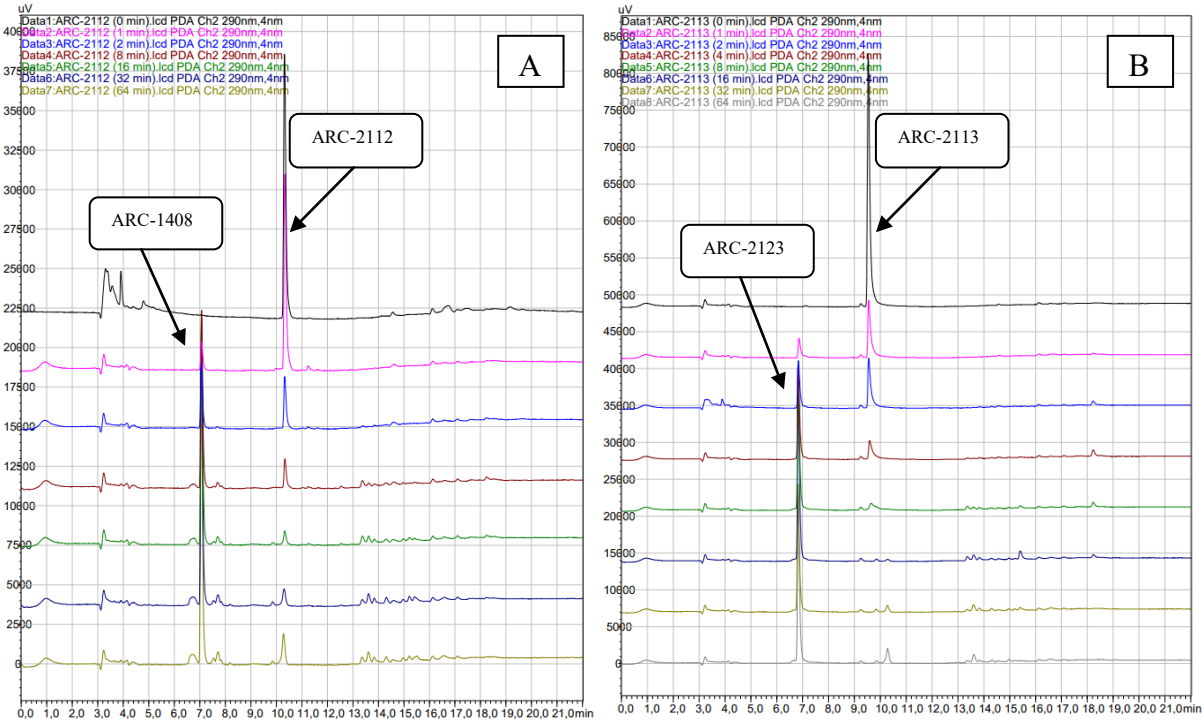


Figure S6. HPLC chromatograms after 0, 1, 2, 4, 8, 16, 32, 64 min irradiation of ARC-2112 (A) and ARC-2113 (B) under mercury vapour lamp. Gradient of 5-50% ACN/H_2O in 9 min and 50-95% ACN/H_2O in 6 min, 95% ACN/H_2O for 4 min, 5% ACN/H_2O for 3 min was used. Eluents contained 0.1% TFA.

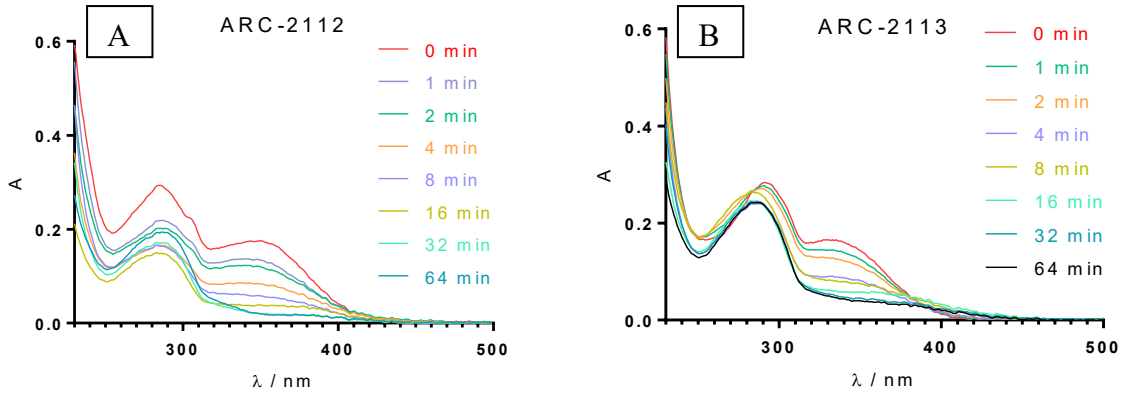


Figure S7. Photolysis of (A) ARC-2112 and (B) ARC-2113 monitored by UV-Vis spectroscopy. Irradiation (1 to 64 min) was carried out under mercury vapour lamp.

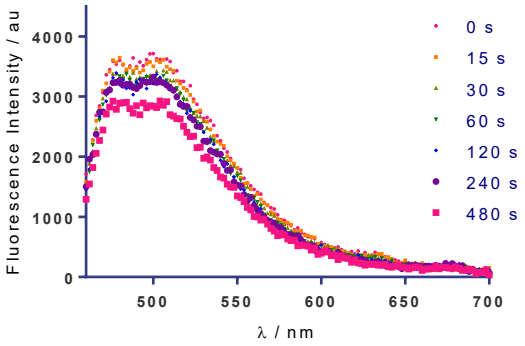


Figure S8. Fluorescence emission spectra of C9H6 cell lysate containing fused proteins PKArIIβ-CFP and PKAcα-YFP before and after illumination for 15 to 480 s under 398 nm LED. The final concentrations were 16% lysate, 10 mM Mg^{2+} and 10 µM ATP in assay buffer C. The excitation was at 420 (30) nm.

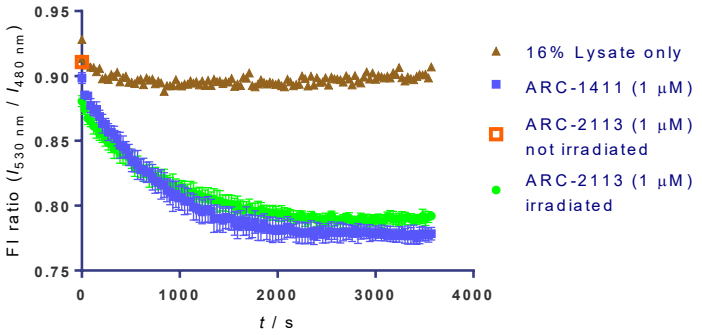
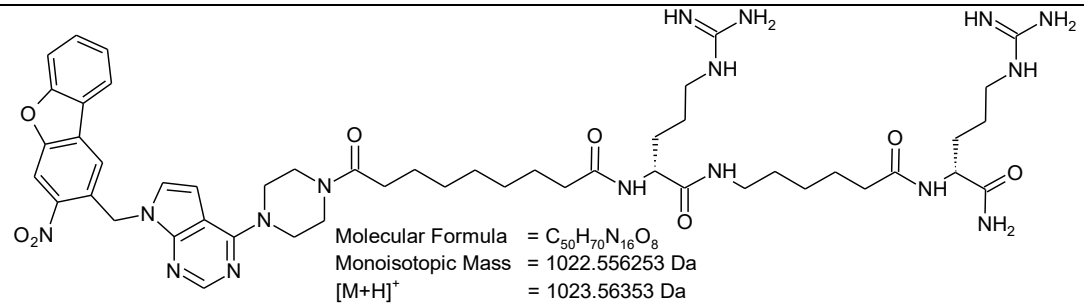
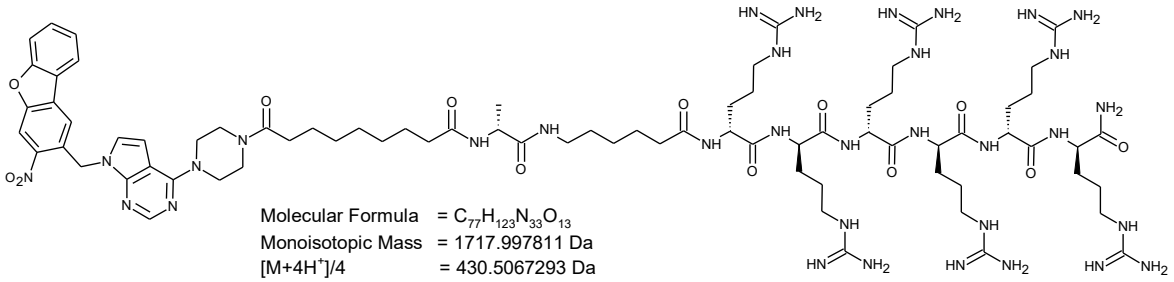
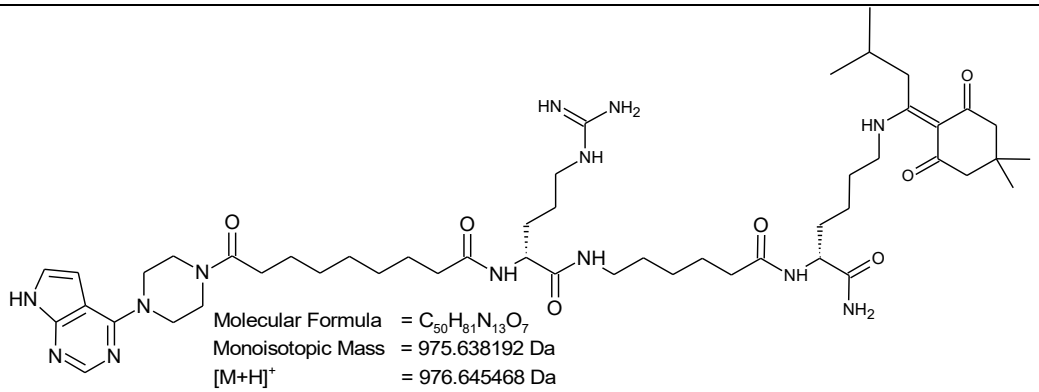


Figure S9. Monitoring the FRET change (ex at 427 nm) during dissociation of holoenzyme composed of PKArIIβ-CFP and PKAcα-YFP in lysate of C9H6 cells (16% dilution in assay buffer C containing 10 mM Mg^{2+} and 10 µM ATP). The dissociation was induced by addition of ARC-1411 (1 µM final concentration) or by addition of ARC-2113 (1 µM final concentration) followed by irradiation under 398 nm LED for 1 min.

S3. Supplementary Tables

Table S1. The structures of the unionized forms of the novel ARCs presented in this study, as well as ARC-1411 and ARC-1408

Compound code	Structure and calculated molecular mass
ARC-2112	
ARC-2113	
ARC-2114	

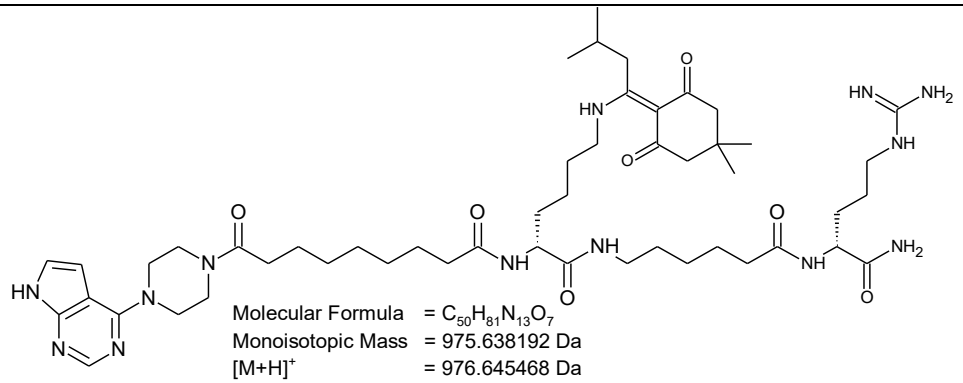
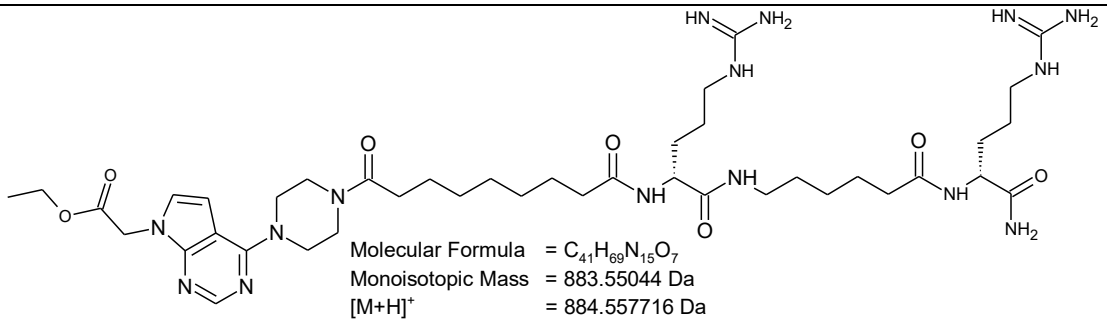
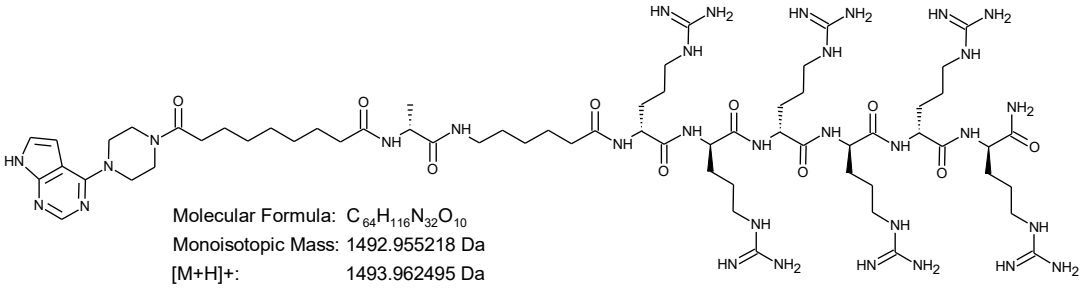
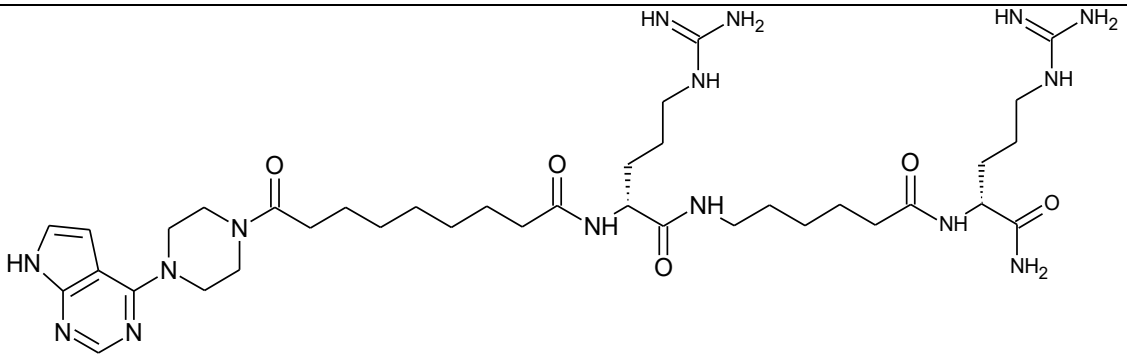
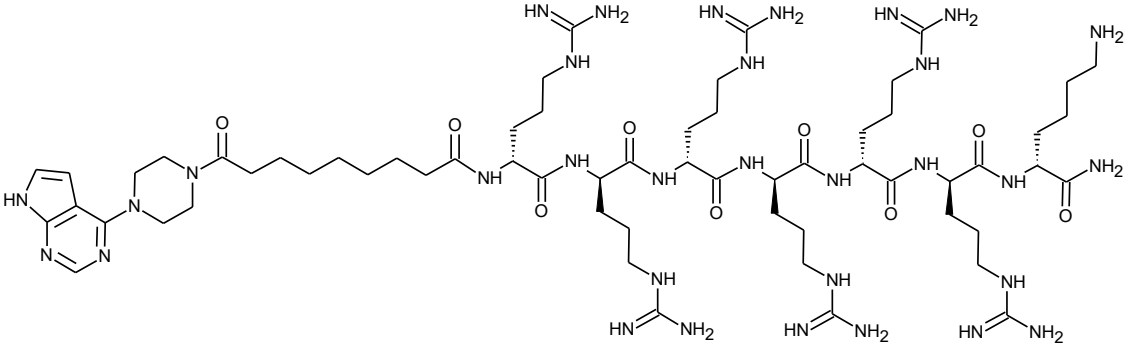
ARC-2115	 Molecular Formula = $C_{50}H_{81}N_{13}O_7$ Monoisotopic Mass = 975.638192 Da $[M+H]^+$ = 976.645468 Da
ARC-2116	 Molecular Formula = $C_{41}H_{69}N_{15}O_7$ Monoisotopic Mass = 883.55044 Da $[M+H]^+$ = 884.557716 Da
ARC-2123	 Molecular Formula: $C_{64}H_{116}N_{32}O_{10}$ Monoisotopic Mass: 1492.955218 Da [M+H]+: 1493.962495 Da
ARC-1408[1]	
ARC-1411[1]	

Table S2. Analytical data for novel ARCs

Compound code	Molecular formula	MS Data				UV-Vis data		HPLC data	
		Calculated MW	Calculated *m/z* $[M+H^+]$	Observed *m/z* $[M+H^+]$	Δ*m*/ppm	λ_{max} / nm	ε / $M^{-1}cm^{-1}$ (λ / nm)	t_R / min	Peak Area % (220 nm)
ARC-2114	$C_{50}H_{81}N_{13}O_7$	975.638192	976.645468	976.64356	-1.954	294	35 000 (286)	10.2 [a]	96.6
ARC-2115	$C_{50}H_{81}N_{13}O_7$	975.638192	976.645468	976.64320	-2.322	295	35 000 (286)	10.3 [a]	98.9
ARC-2116	$C_{41}H_{69}N_{15}O_7$	883.55044	884.557716	884.55530	-2.731	286	12 000 (286)	7.9 [a]	96.8
ARC-2112	$C_{50}H_{70}N_{16}O_8$	1022.556253	1023.56353	1023.56220	-1.299	290; 350	12 000 (350)	12.5 [b]	99.3
ARC-2113	$C_{77}H_{123}N_{33}O_{13}$	1717.997811	430.5067293 $[M+4H^+]/4$	430.50561 (z = 4)	-2.600	290; 350	12 000 (350)	11.8 [b]	95.5
ARC-2123	$C_{64}H_{116}N_{32}O_{10}$	1492.955218	747.48489 $[M+2H^+]/2$	747.48492 (z = 2)	0.040	286	16 000 (286)	7.7 [b]	97.1

[a] gradient of 5-50% ACN/H_2O in 9 min and 50-95% ACN/H_2O in 6 min. Eluents contained 0.1% TFA

[b] gradient of 5-50% ACN/H_2O in 12 min and 50-95% ACN/H_2O in 8 min. Eluents contained 0.1% TFA

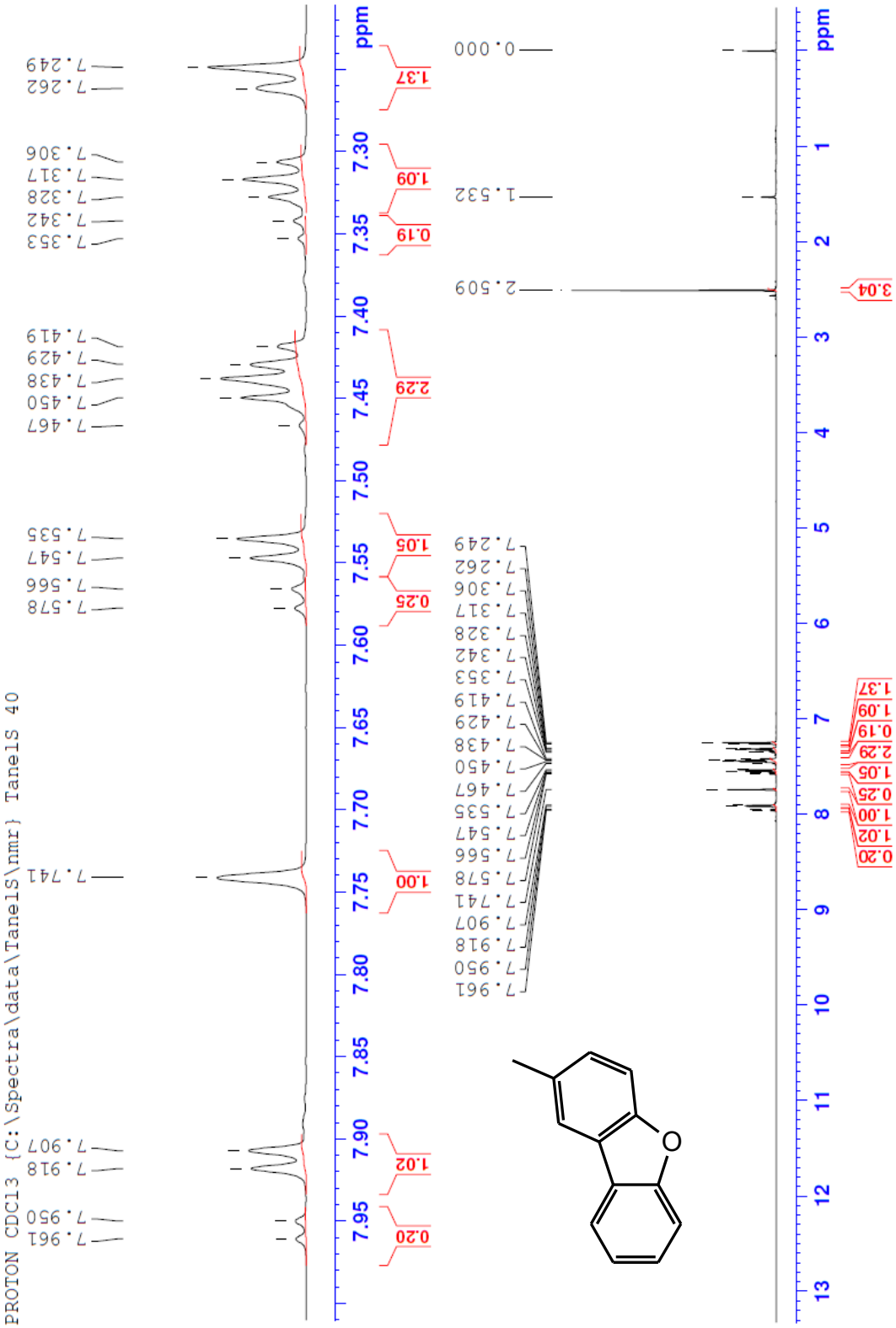
Table S3. Results of displacement experiments

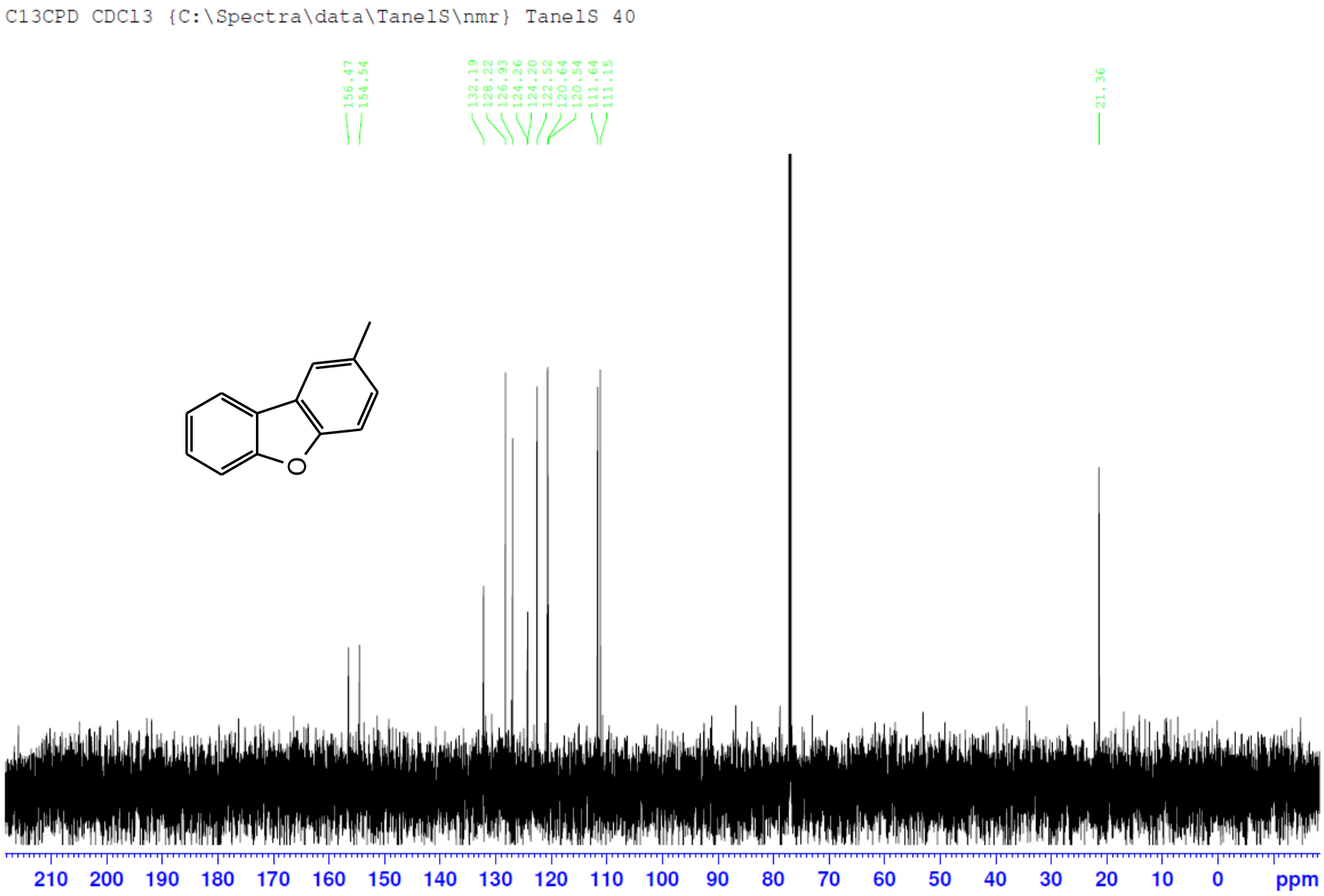
Competing ligand	$t_{irradiation}$ / min[a]	Assay conditions[b]	IC_{50} / M	K_D / nM or K_D^{app} / nM
ARC-2114	-	A	6.7E-07 ± 6.0E-08	12 ± 1.1
ARC-2115	-	A	1.6E-07 ± 1.1E-08	2.9 ± 0.2
ARC-2116	-	B	1.1E-05 ± 1.5E-06	1700 ± 230
ARC-2112	-	B	> 1.0E-04	> 15 000
	2 min	A	1.6E-06 ± 1.5E-07	24 ± 2.3 [c]
	4 min	A	8.9E-07 ± 3.9E-07	13 ± 5.9 [c]
	8 min	A	2.3E-07 ± 1.6E-08	3.4 ± 0.24 [c]
	64 min	A	5.2E-08 ± 1.7E-08	0.76 ± 0.25 [c]
ARC-2113	-	B	1.8E-05 ± 5.1E-06	2800 ± 780
	2 min	A	3.8E-08 ± 1.1E-08	0.56 ± 0.16 [c]
	4 min	A	2.2E-08 ± 7.4E-09	0.32 ± 0.11 [c]
	8 min	A	2.1E-09 ± 1.8E-09	< 0.1 [c]
	64 min	A	< 1.0E-09	< 0.1 [c]
ARC-2123	-	C	1.20E-08 ± 2.2E-09	0.005 ± 0.001

[a] Irradiation was performed with Hg vapour lamp; [b] A: ARC-1182 (1 nM), PKAcα (1.5 nM), B: ARC-583 (2 nM), PKAcα (3 nM), C: ARC-1182 (50 nM), PKAc (1 nM); [c] K_D^{app}

S4. NMR spectra

S4.1. Substance **2**; 2-methyldibenzo[*b*,*d*]furan ^{1}H and ^{13}C spectra.




C13CPD CDCl3 {C:\Spectra\data\TanelS\nmr} TanelS 40
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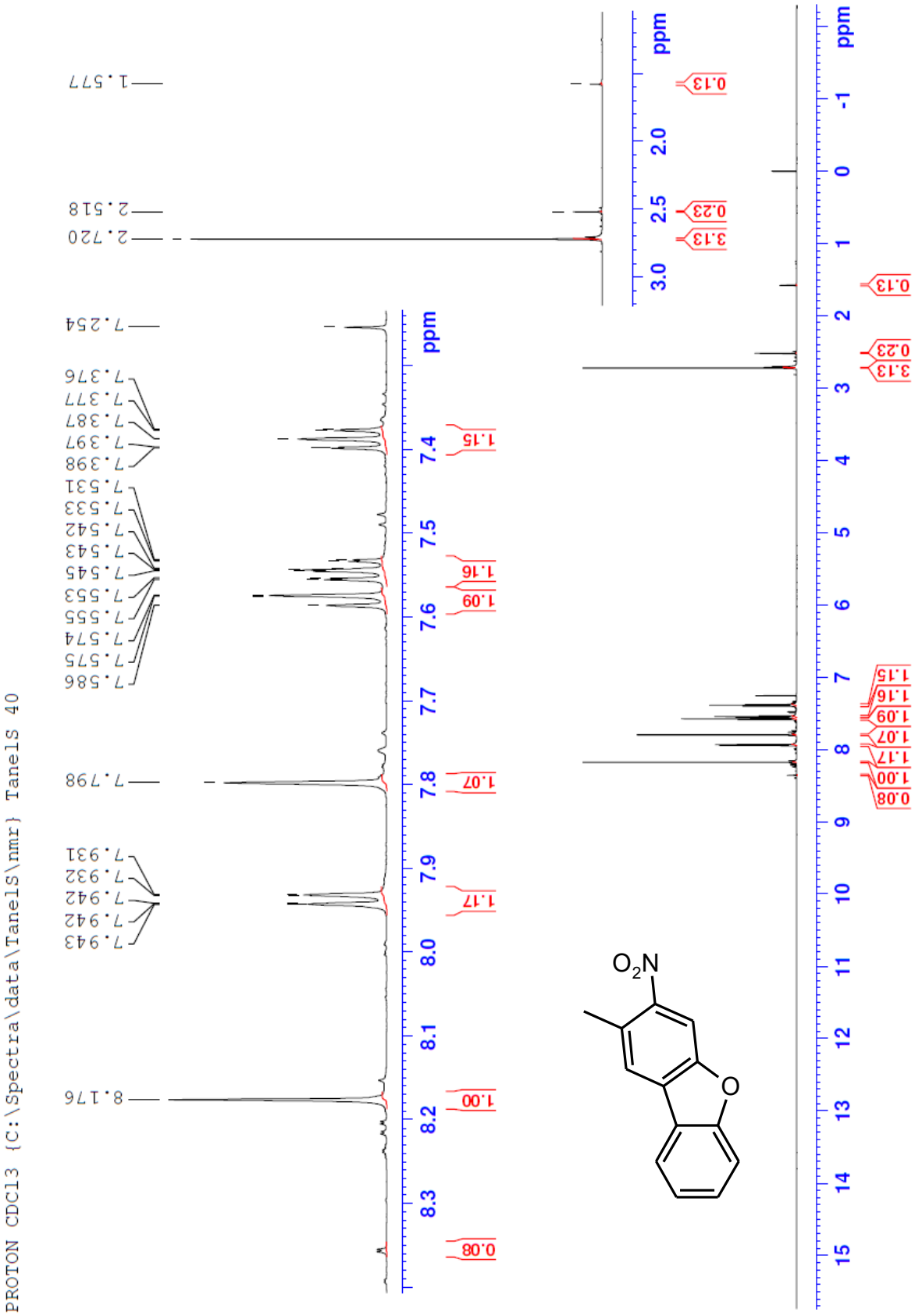
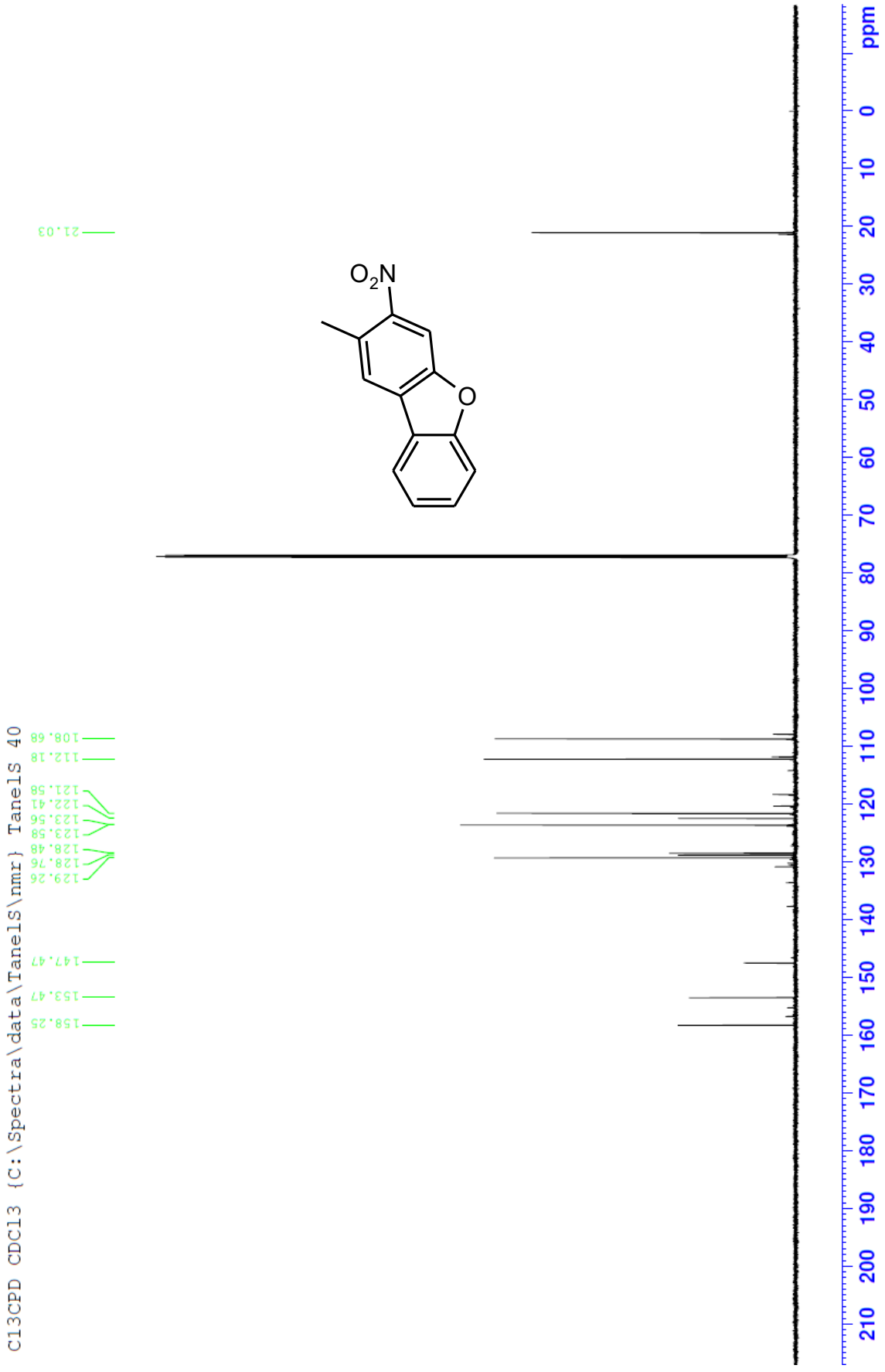
154.54
132.19
128.22
126.93
124.26
124.20
122.52
120.64
120.54
111.64
111.15
21.36
O
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

S4.2. Substance **3**; 2-methyl-3-nitrodibenzo[*b*,*d*]furan ^{1}H and ^{13}C spectra.




C13CPD CDCl3 {C:\Spectra\data\TanelS\nmr} TanelS 40
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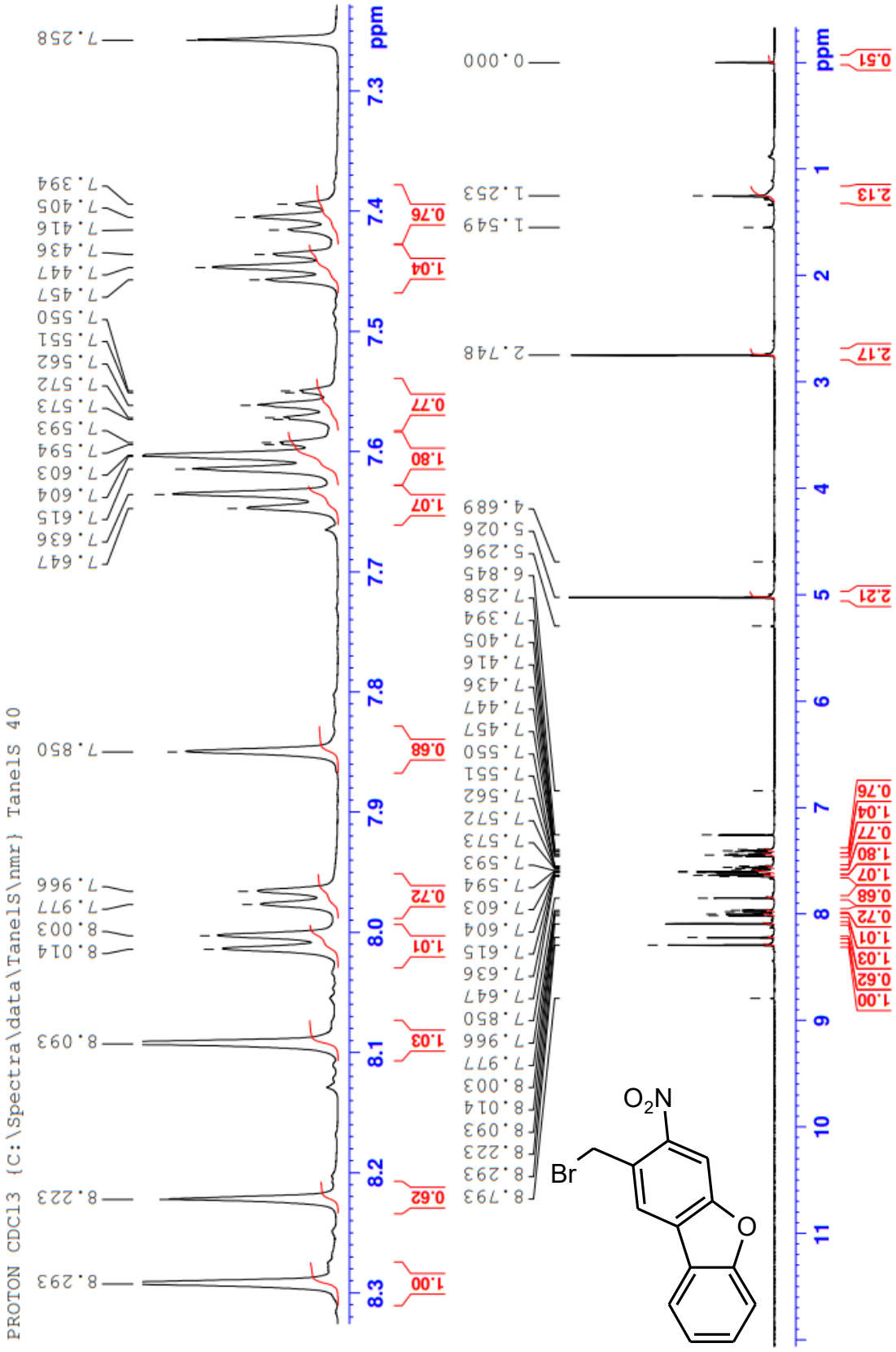
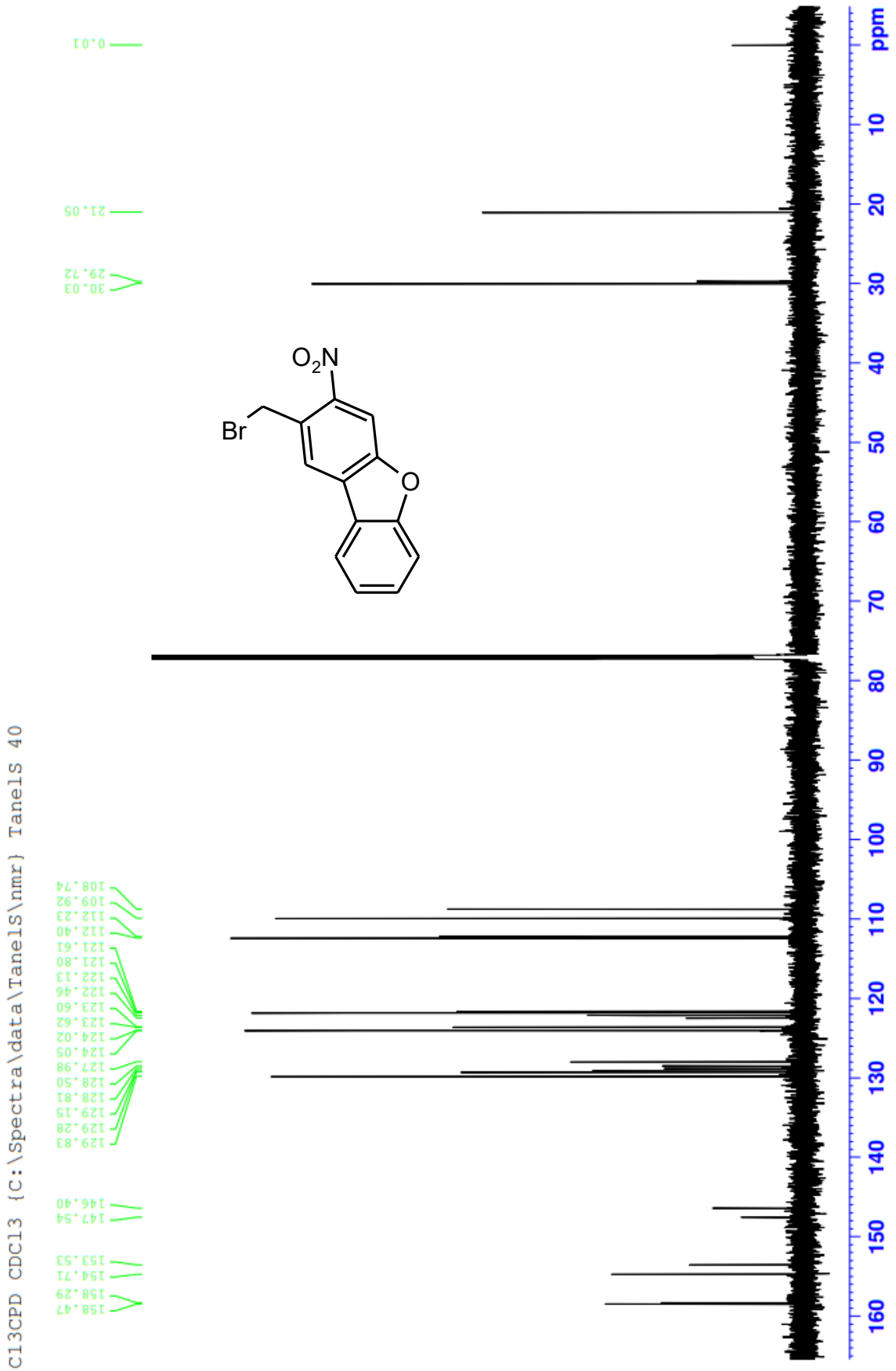
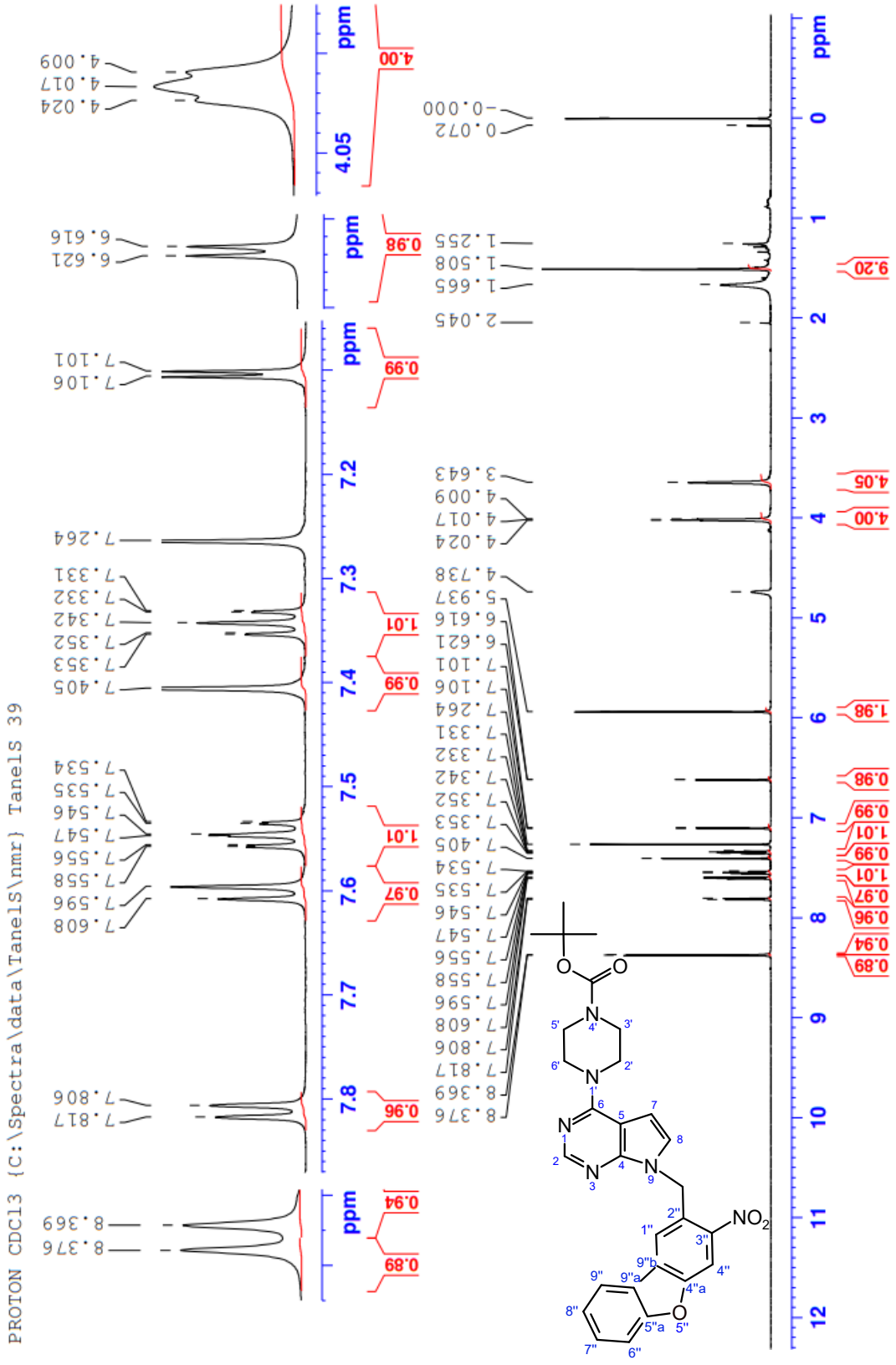
108.68
112.18
121.58
122.41
123.56
123.58
128.48
128.76
129.26
147.47
153.47
158.25
O2N
O
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210

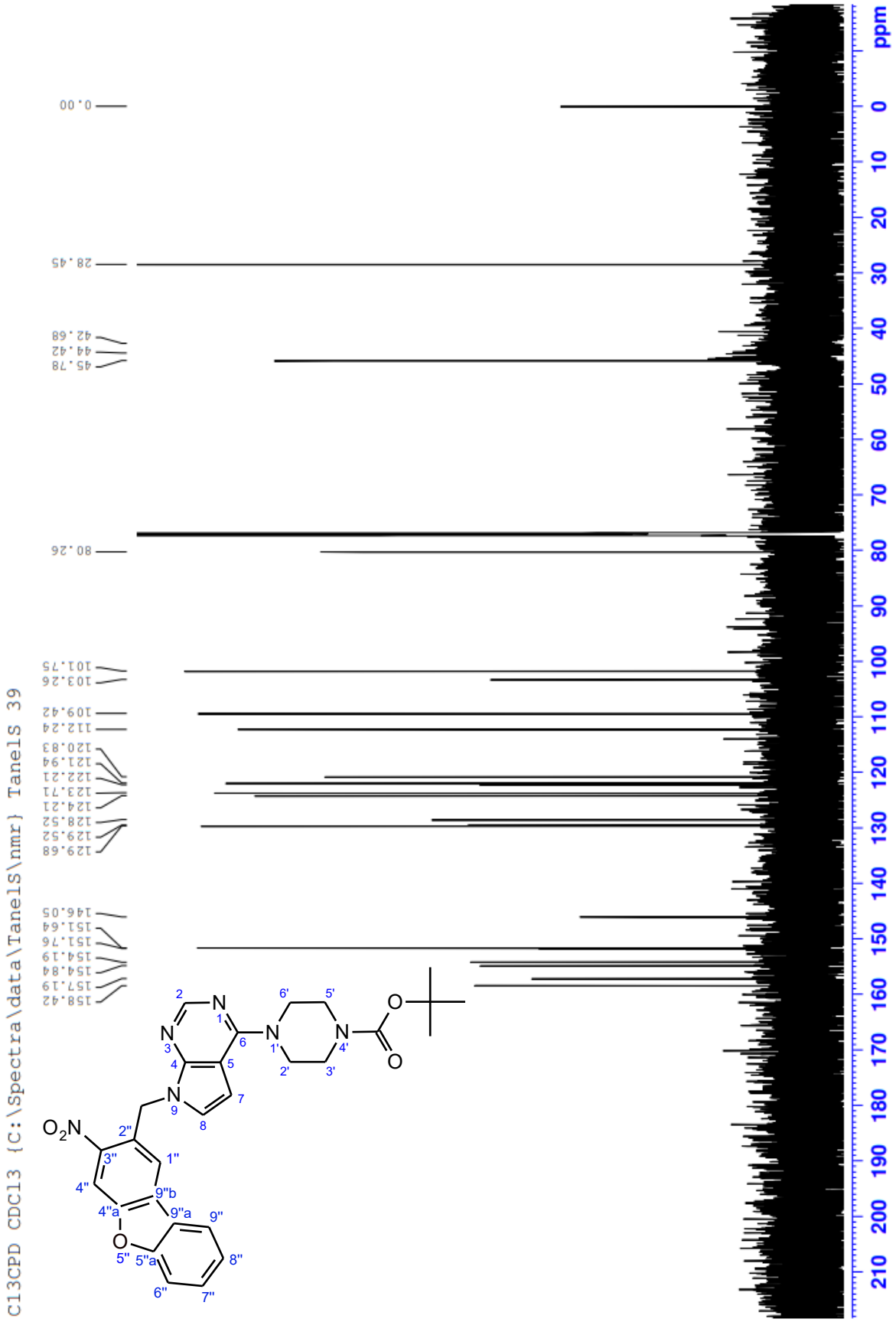
S4.3. Substance **4**; 2-(bromomethyl)-3-nitrobenzo[*b,d*]furan ^{1}H and ^{13}C spectra.




C13CPD CDCl3 {C:\Spectra\data\TanelS\nmr} TanelS 40
ppm
O2N
Br
O

S4.4. Substance **6**; 9-(3-nitro-dibenzo[*b,d*]furan-2-ylmethyl)-6-(piperazine-4-(*tert*-buthyl-carbonyl)-1-yl)-7-deazapurine ^{1}H, ^{13}C, heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple-bond correlation (HMBC) spectra respectively.

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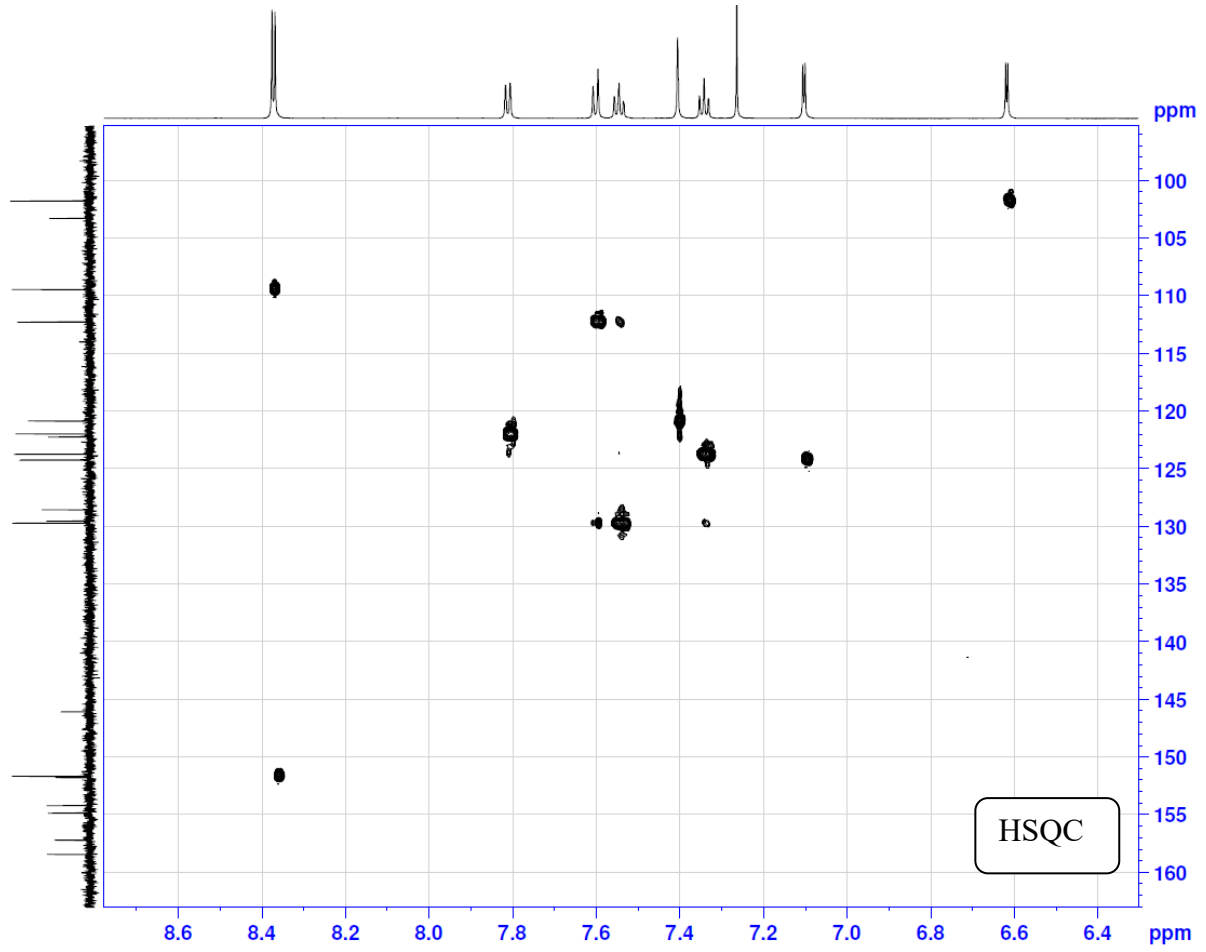
42.68
44.42
45.78
80.26
101.75
103.26
109.42
112.24
120.83
121.94
122.21
123.71
124.21
128.52
129.52
129.68
146.05
151.64
151.76
154.19
154.84
157.19
158.42
ppm
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210


ppm
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105
110
115
120
125
130
135
140
145
150
155
160
8.6
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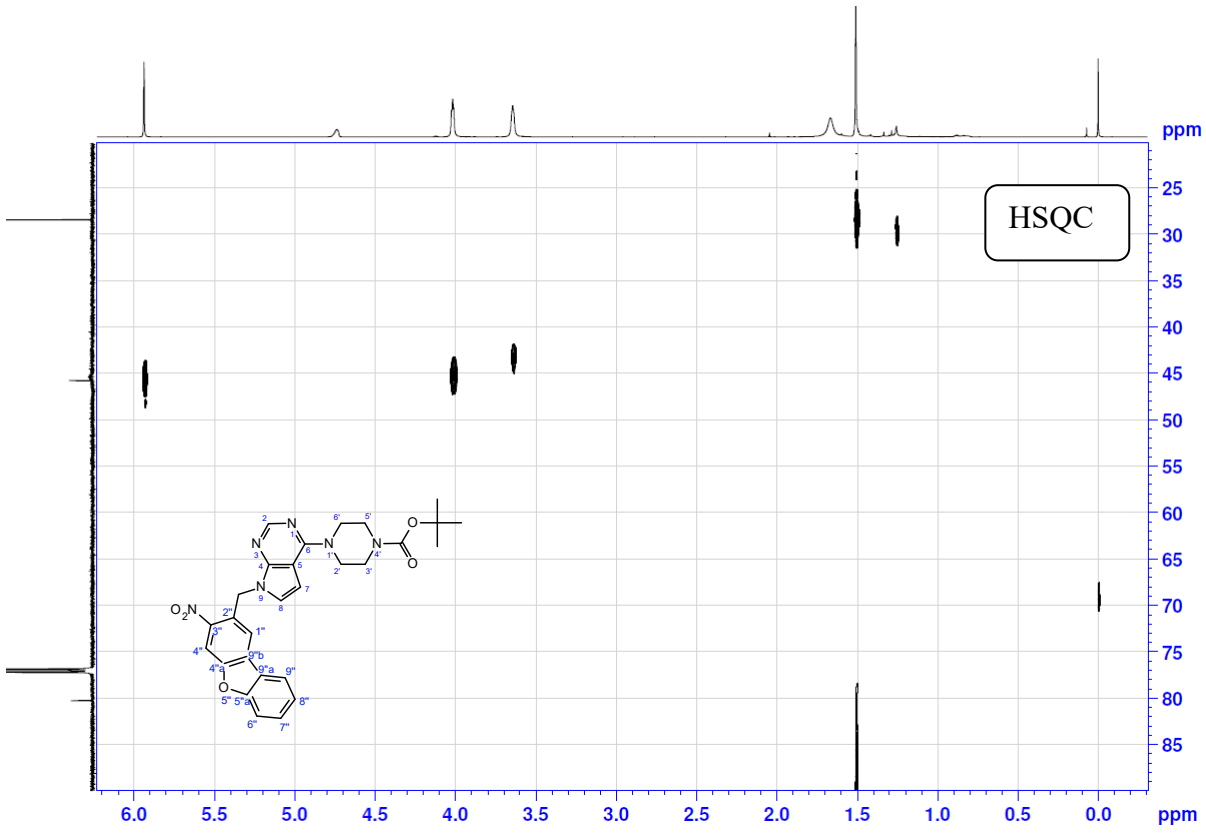
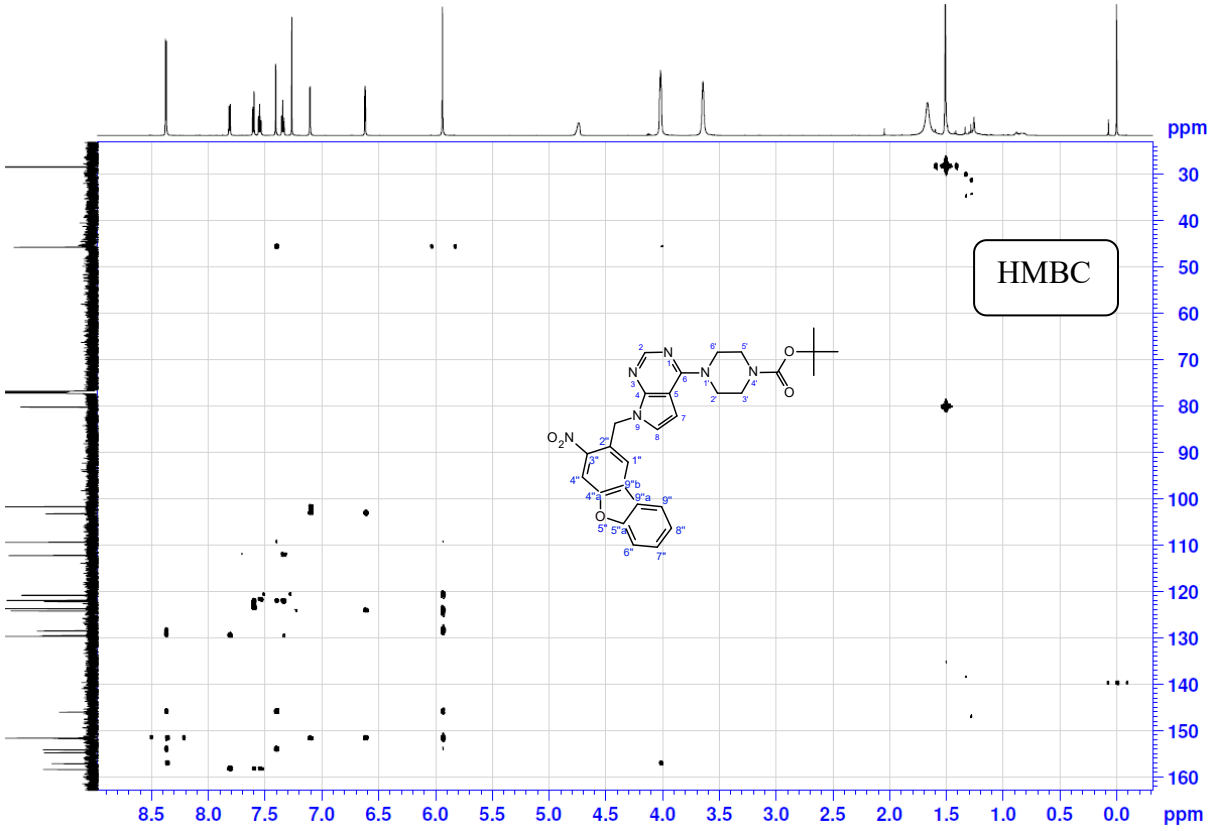
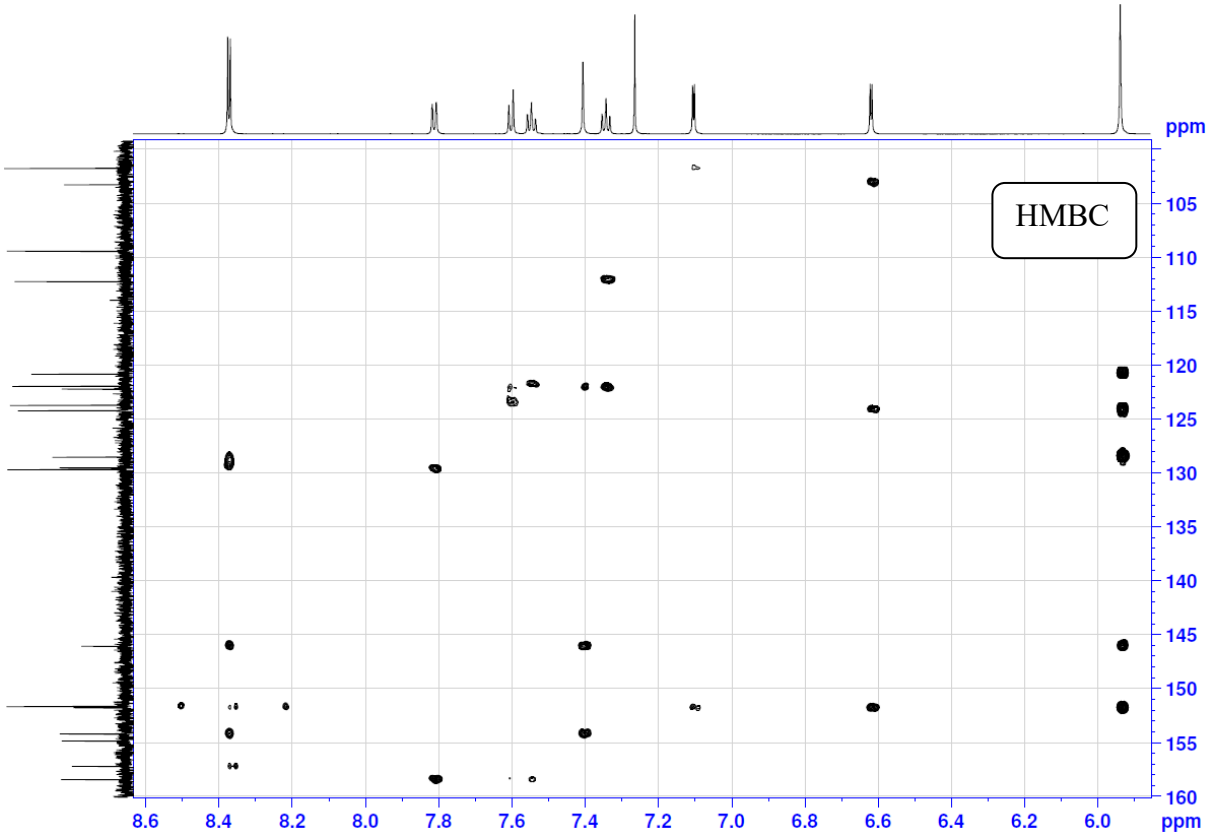
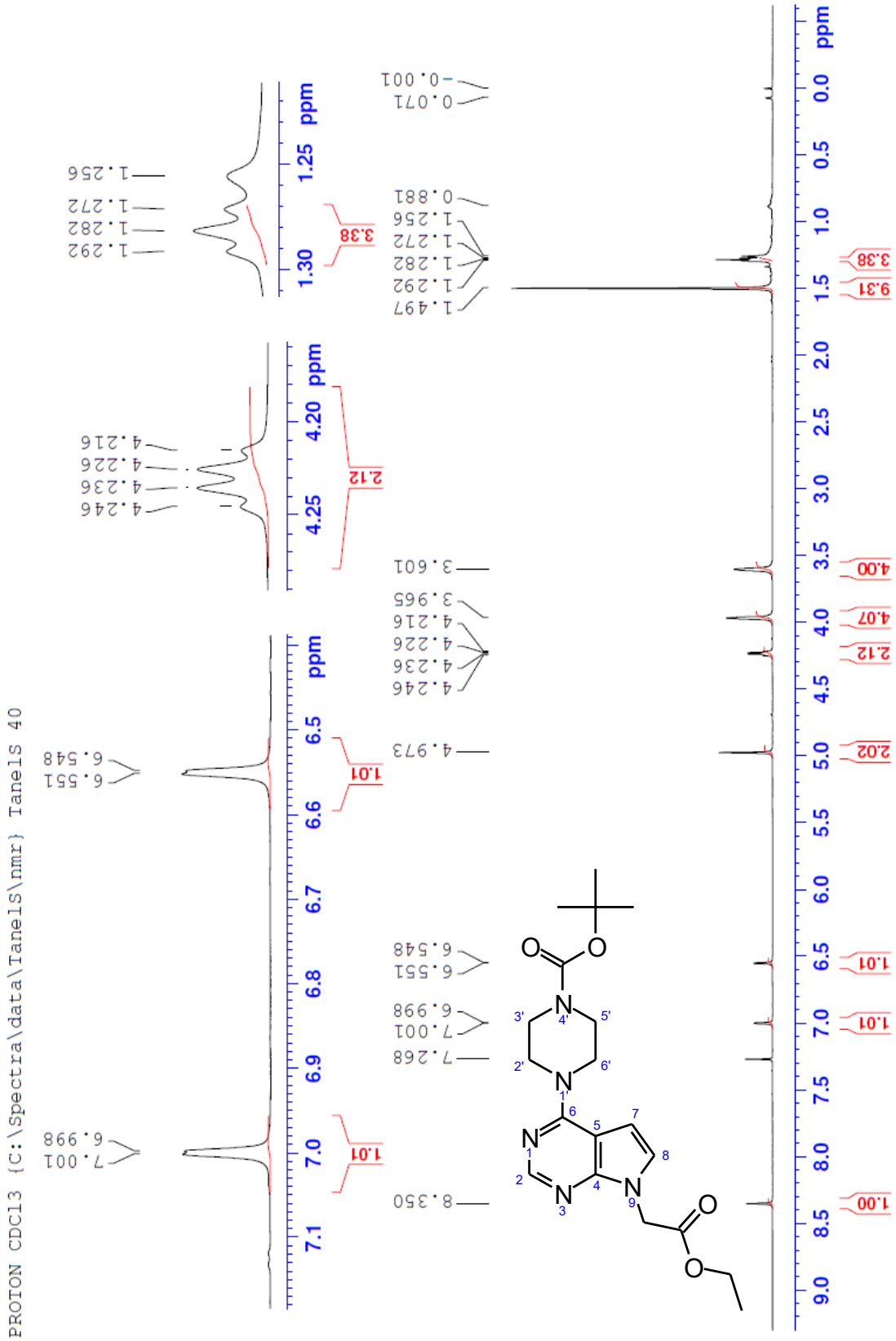
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
ppm
HSQC

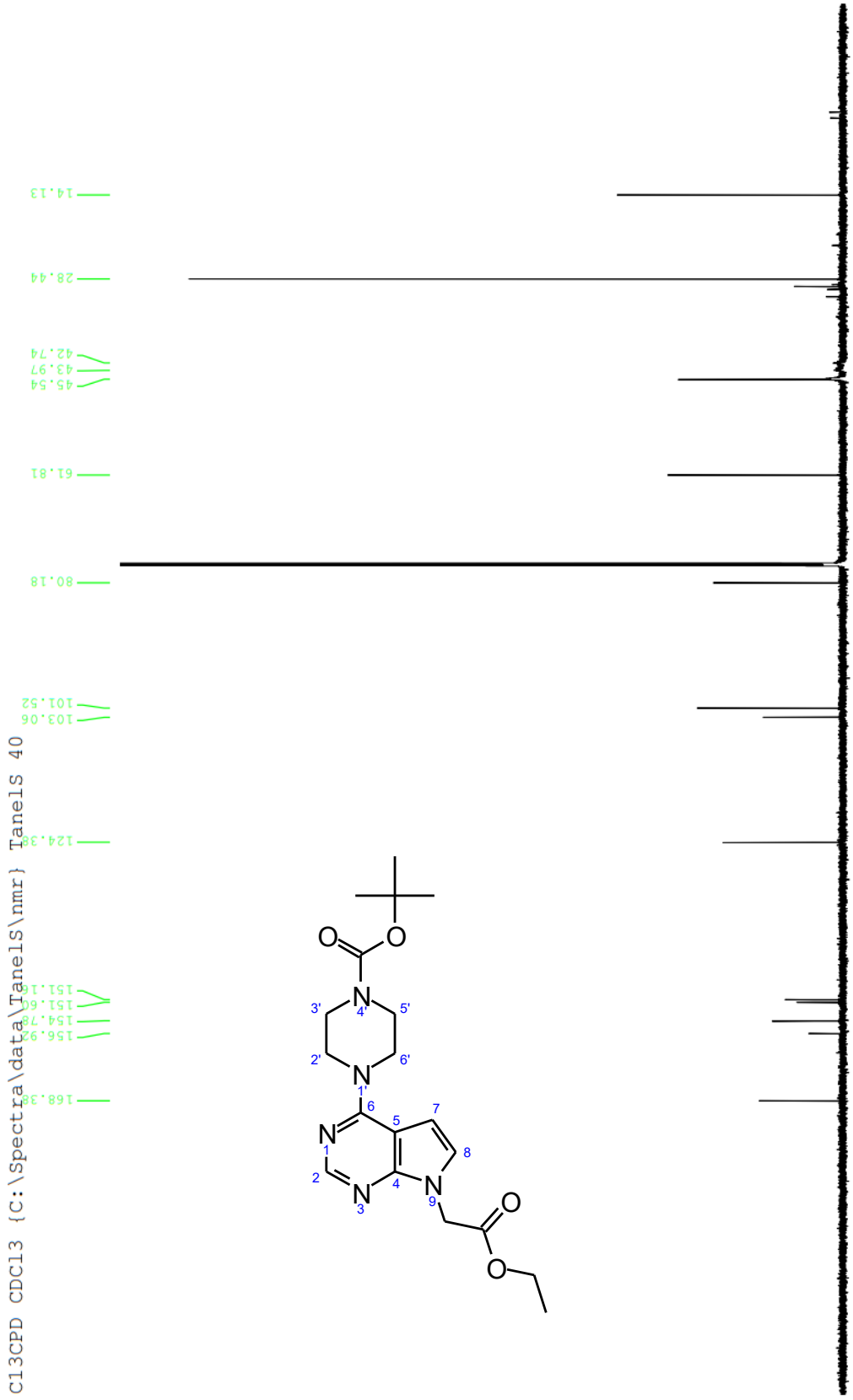

ppm
25
30
35
40
45
50
55
60
65
70
75
80
85
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm
HSQC


HMBC
ppm
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 ppm
30 40 50 60 70 80 90 100 110 120 130 140 150 160


HMBC
ppm
8.6 8.4 8.2 8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 ppm
105 110 115 120 125 130 135 140 145 150 155 160

S4.5. Substance **8**; 9-(ethylacetate)-6-(piperazine-4-(*tert*-buthylcarbonyl)-1-yl)-7-deaza-purine ^{1}H and ^{13}C spectra.

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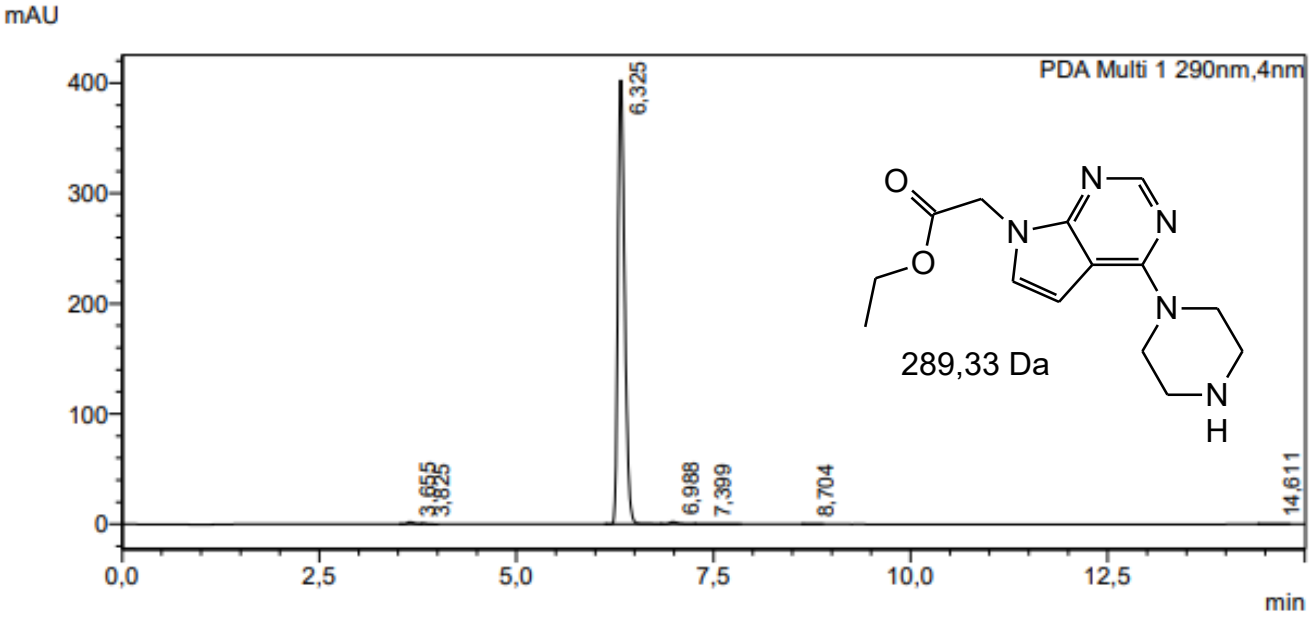
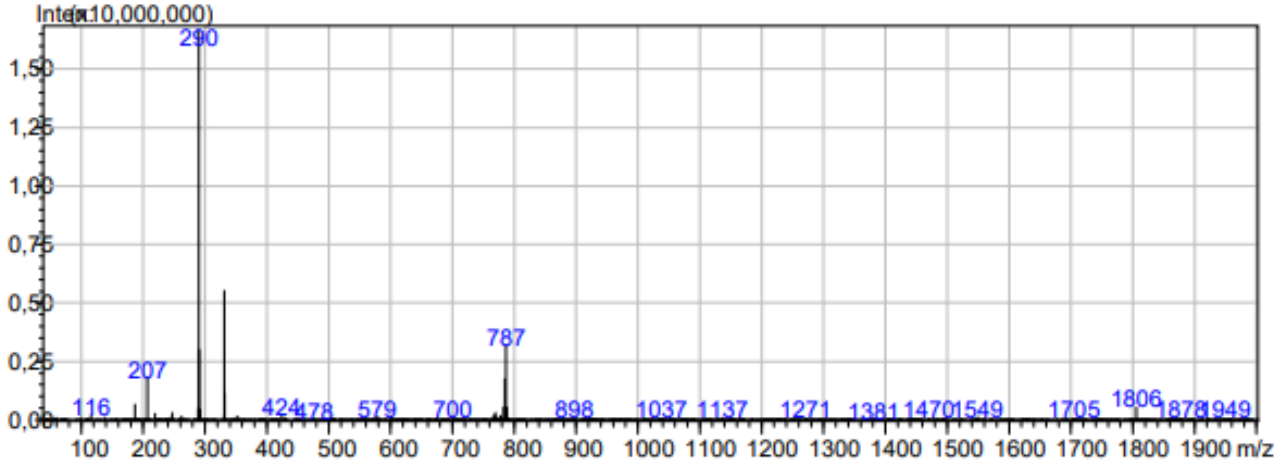
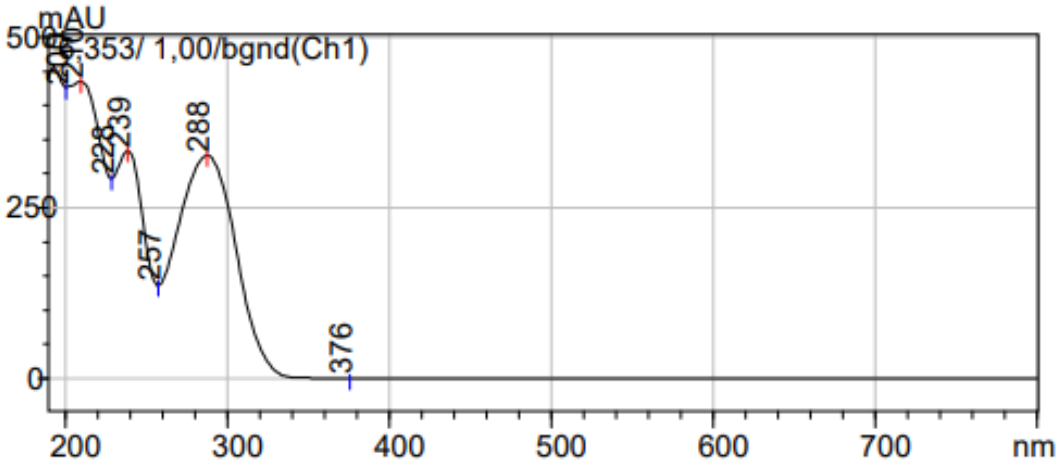
14.13
28.44
42.74
43.97
45.54
61.81
80.18
101.52
103.06
124.38
151.12
151.69
154.78
156.92
168.38
O
O
N
4'
3'
5'
2'
6'
N
1'
6
5
7
N
1
8
2
N
4
N
3
9
O
O

S5. HPLC-MS data and UV-Vis spectra

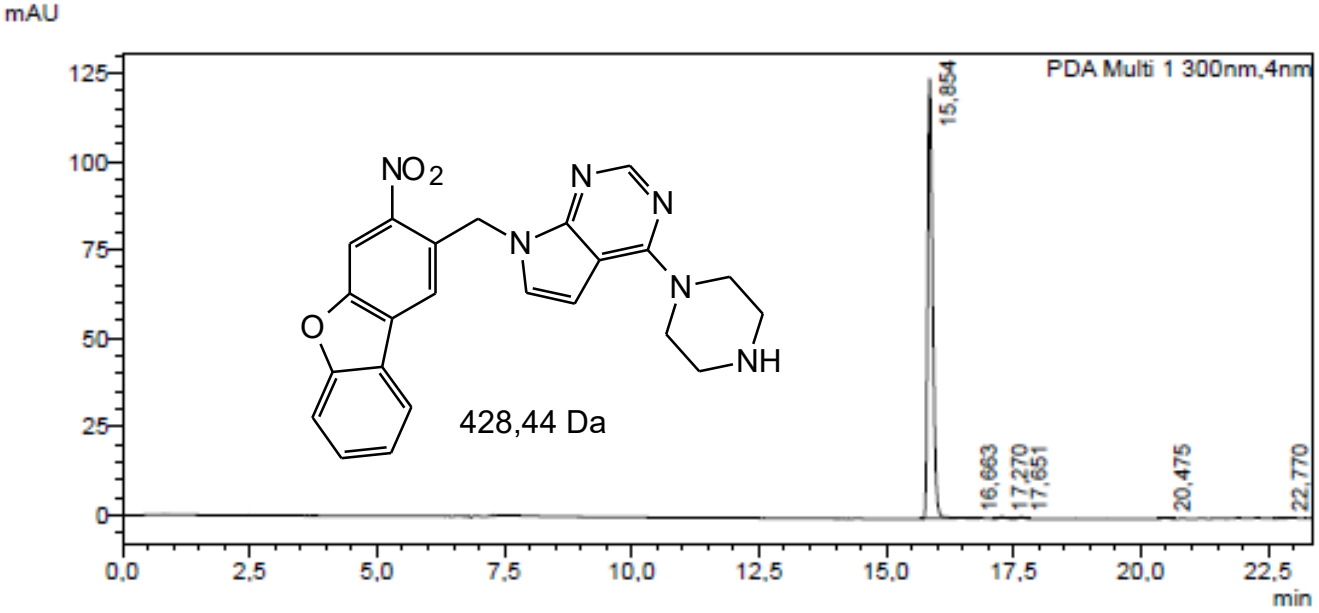
S5.1. Substance **10** HPLC, MS and UV spectra.

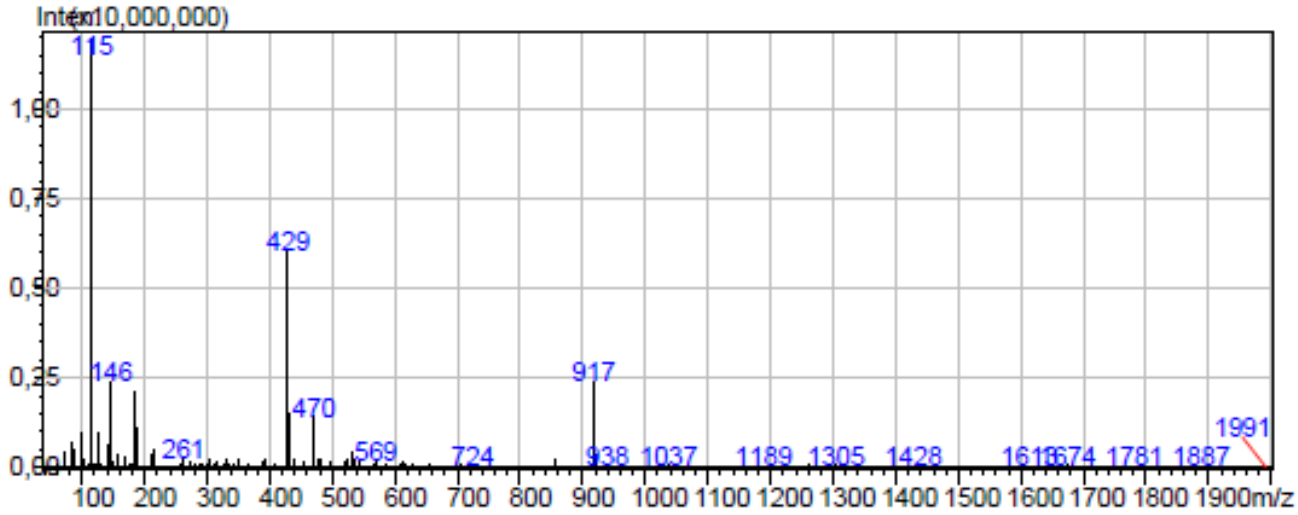


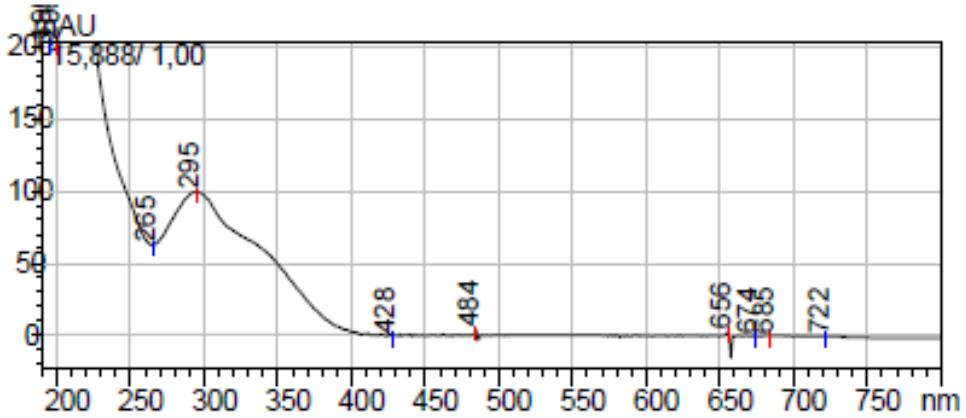




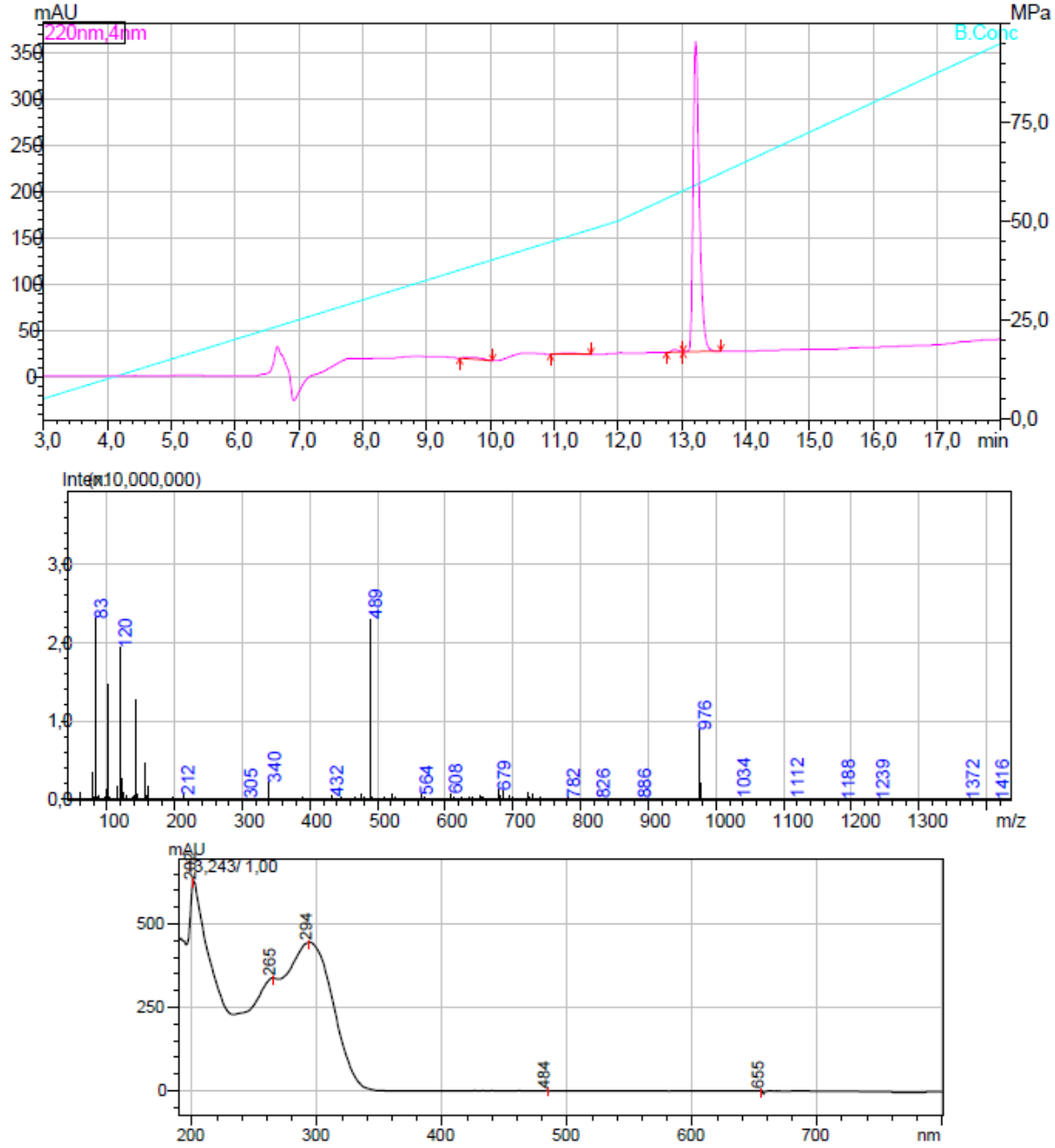
S5.2. Substance **11** HPLC, MS and UV spectra.



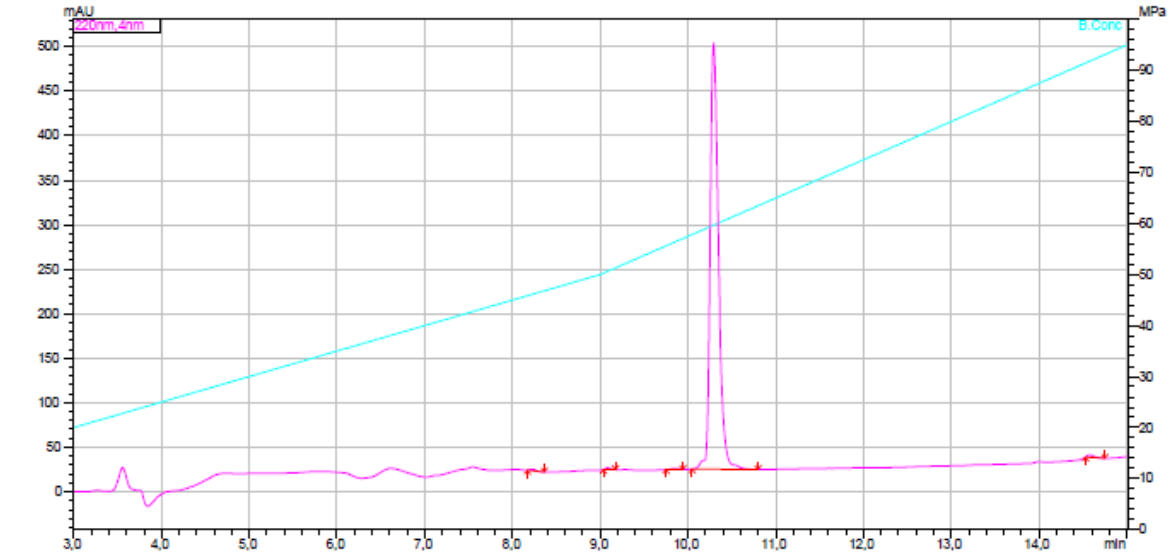


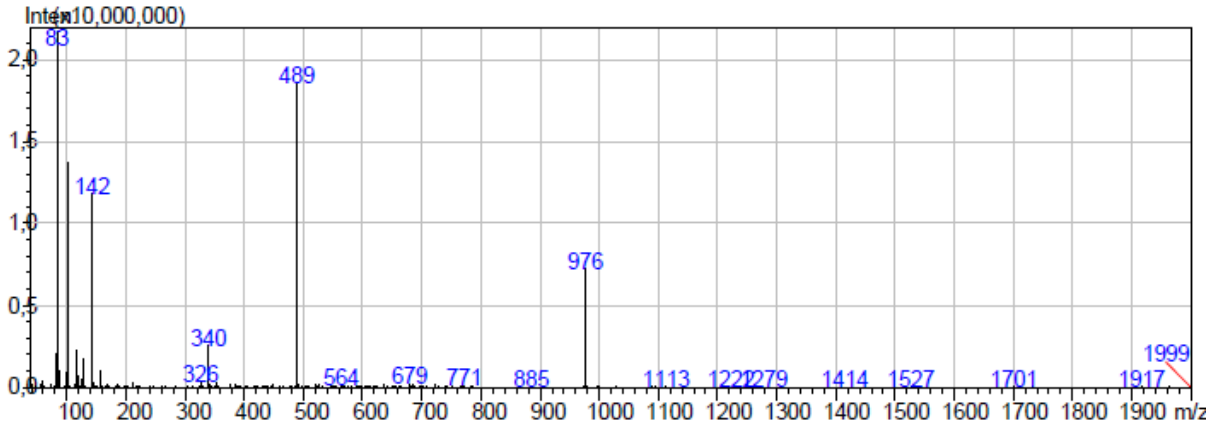


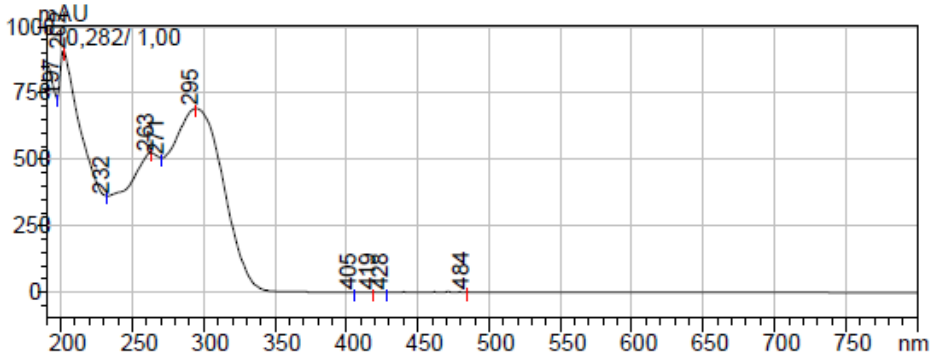
S5.3. ARC-2114 purity HPLC, MS and UV spectra at 220 nm; injection at 3 min.



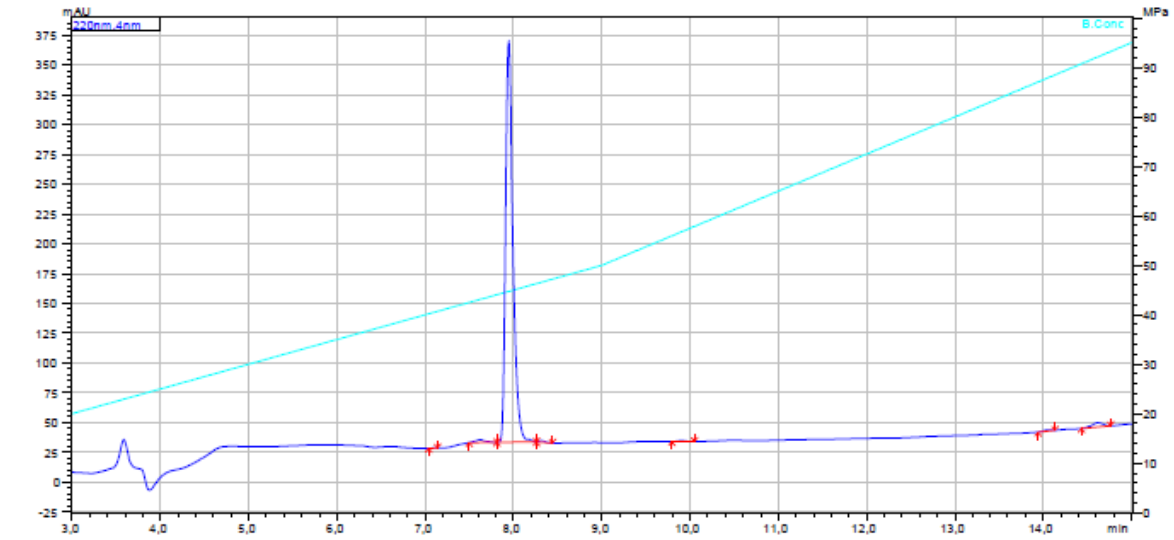
S5.4. ARC-2115 purity HPLC, MS and UV spectra at 220 nm; injection at 0 min.

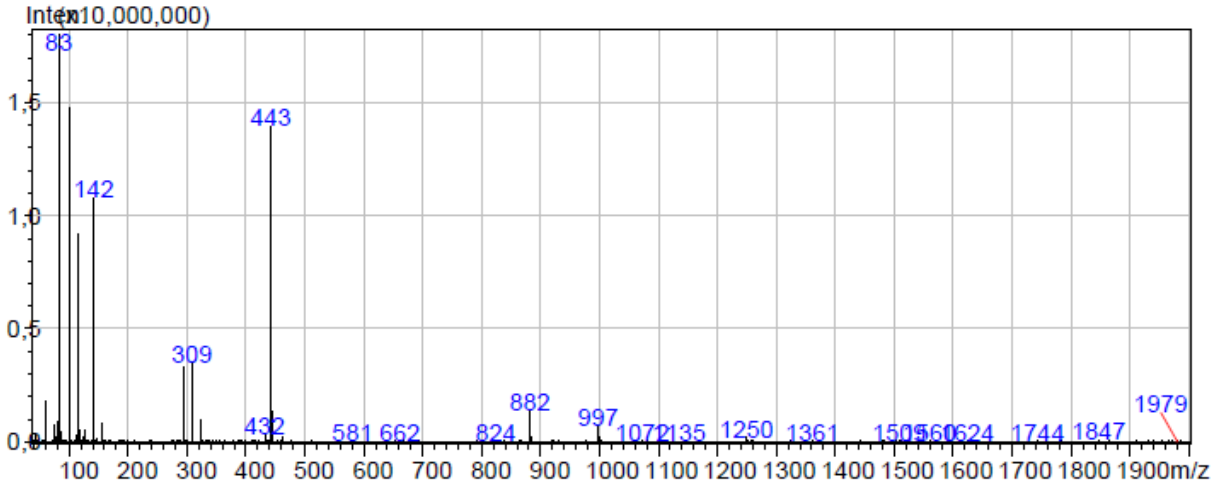


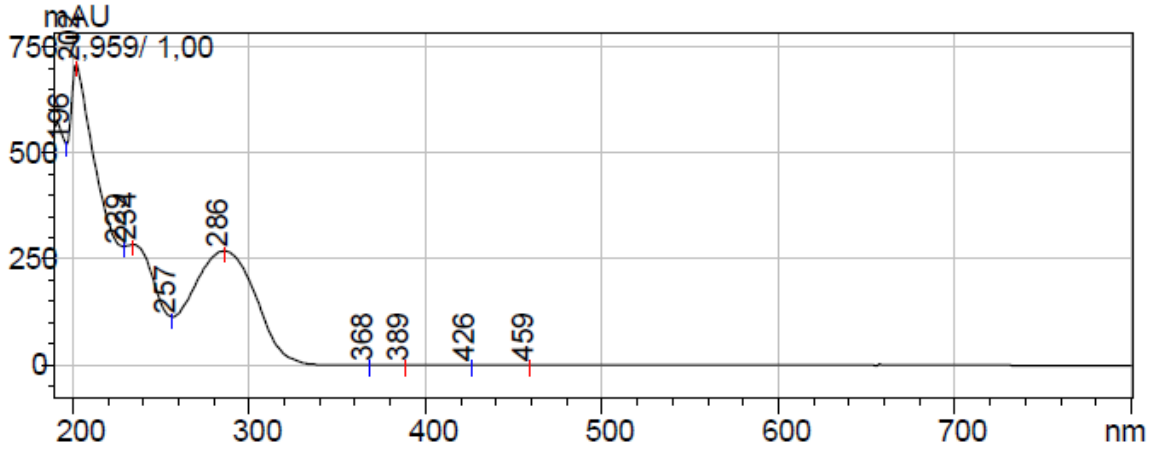




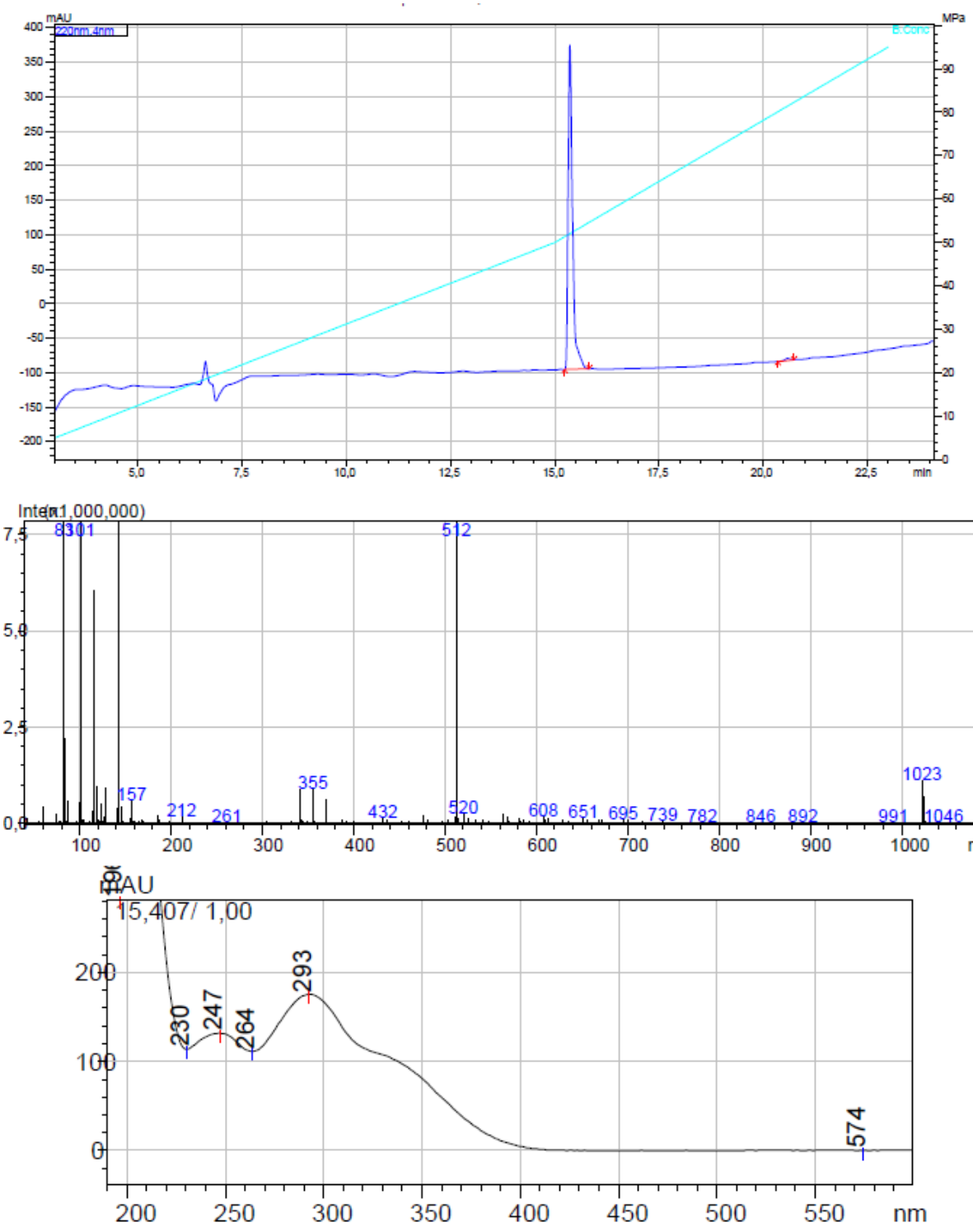
S5.5. ARC-2116 purity HPLC, MS and UV spectra at 220 nm; injection at 0 min.



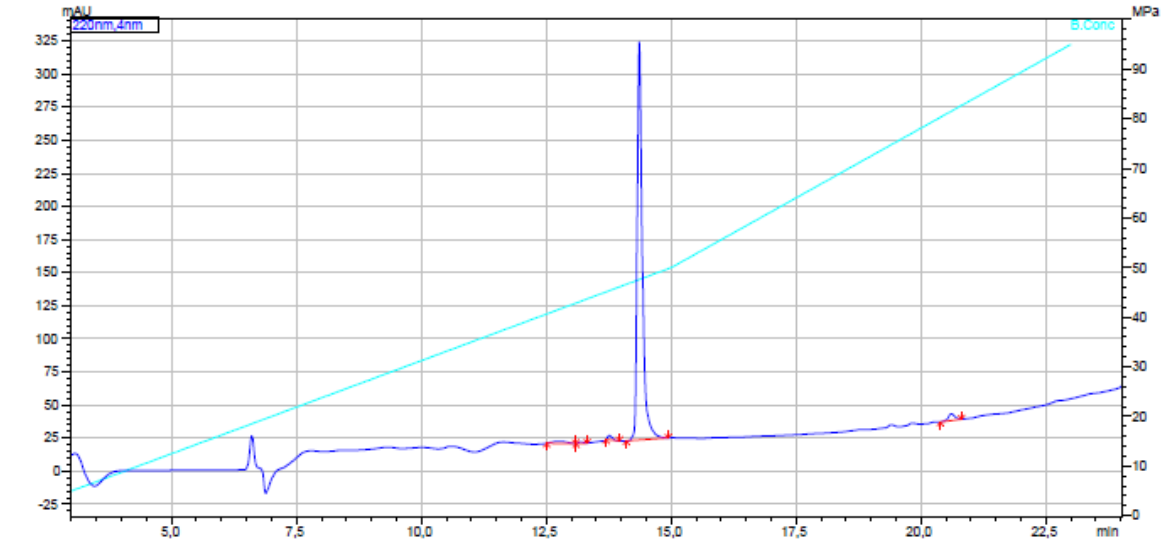


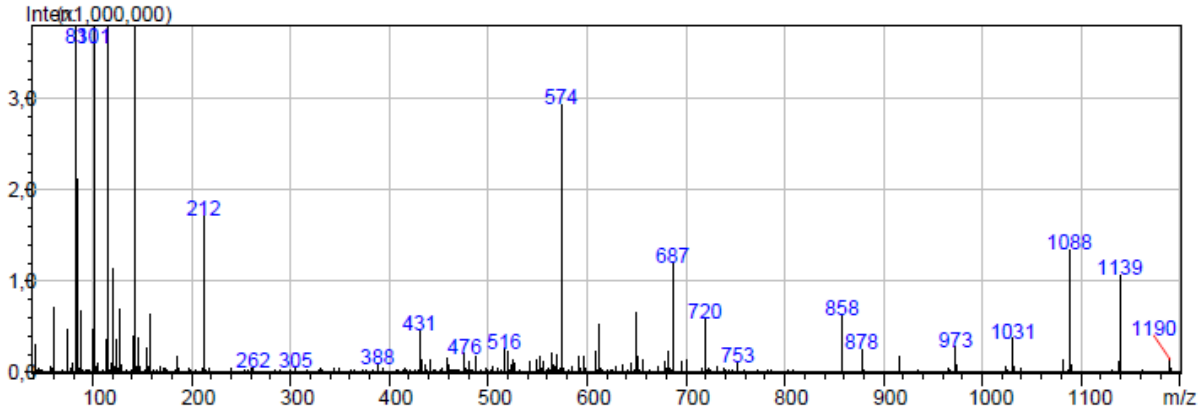


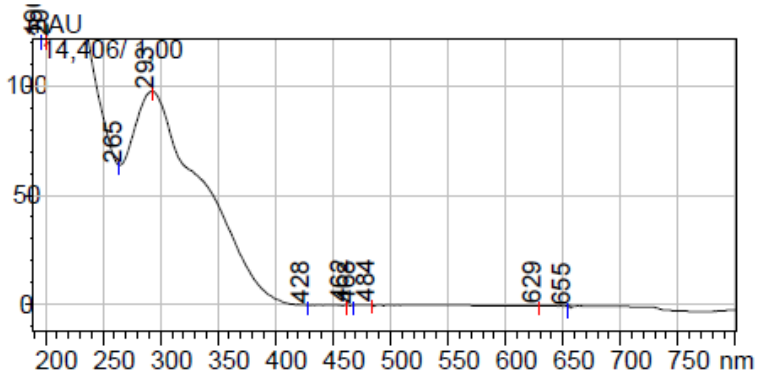
S5.6. ARC-2112 purity HPLC, MS and UV spectra at 220 nm; injection at 3 min.



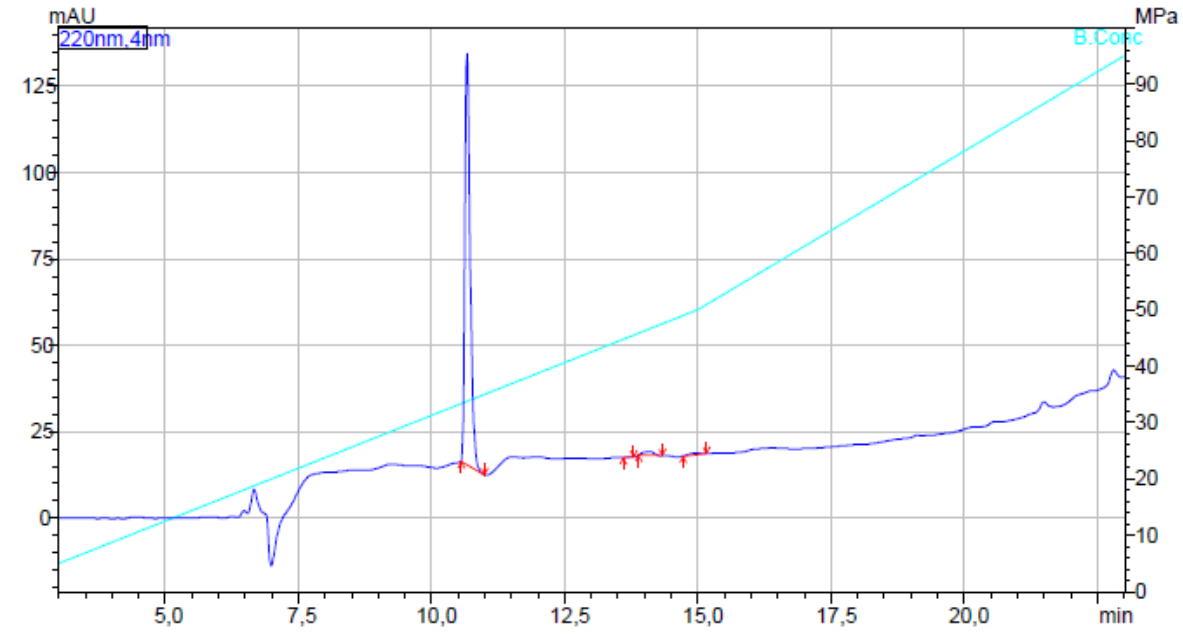
S5.7. ARC-2113 purity HPLC, MS and UV spectra at 220 nm; injection at 3 min.

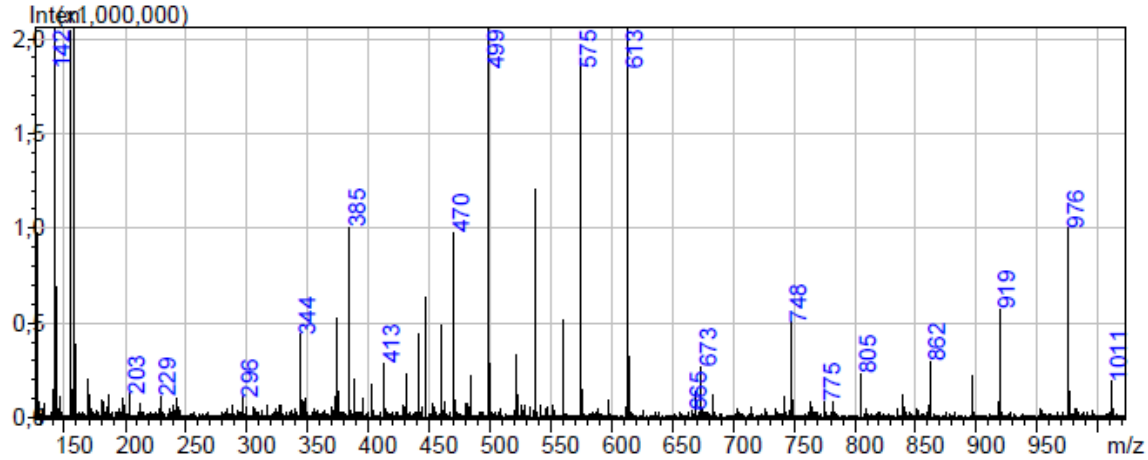


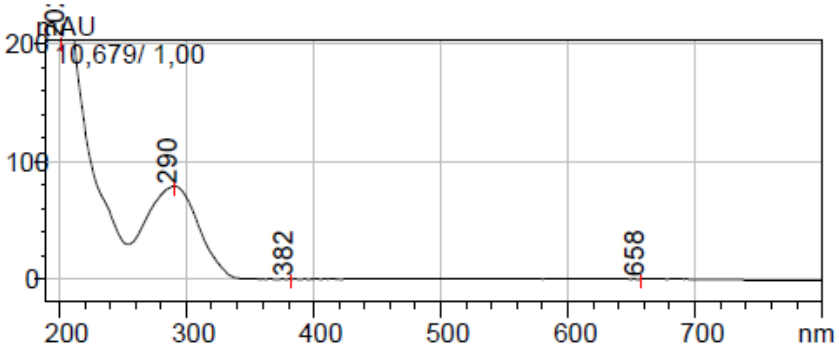




S5.8. ARC-2123 purity HPLC, MS and UV spectra at 220 nm; injection at 3 min.

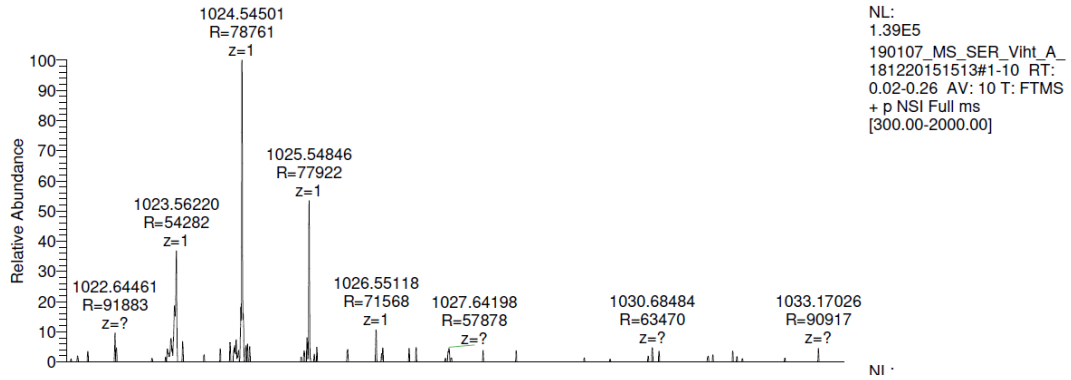




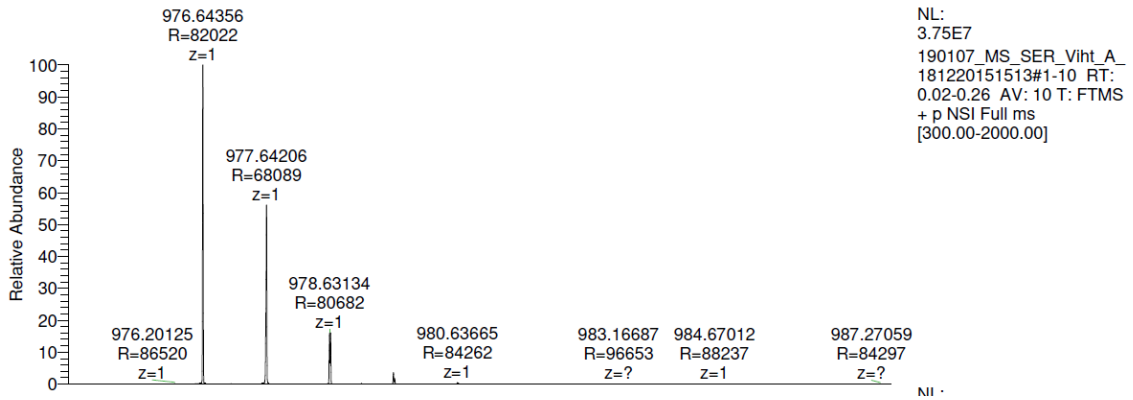


S6. HRMS data

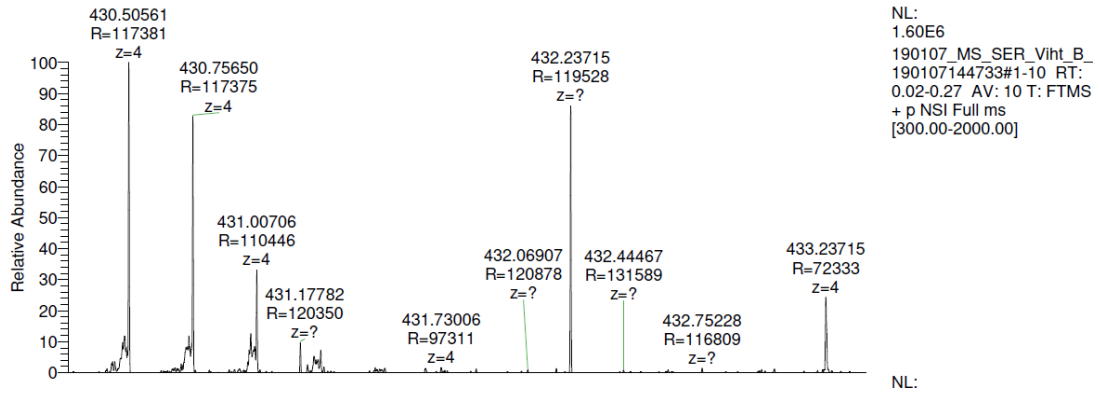
S6.1. ARC-2112 HRMS data measured with Thermo Electron LTQ Orbitrap (ESI HRMS) in positive ion mode.



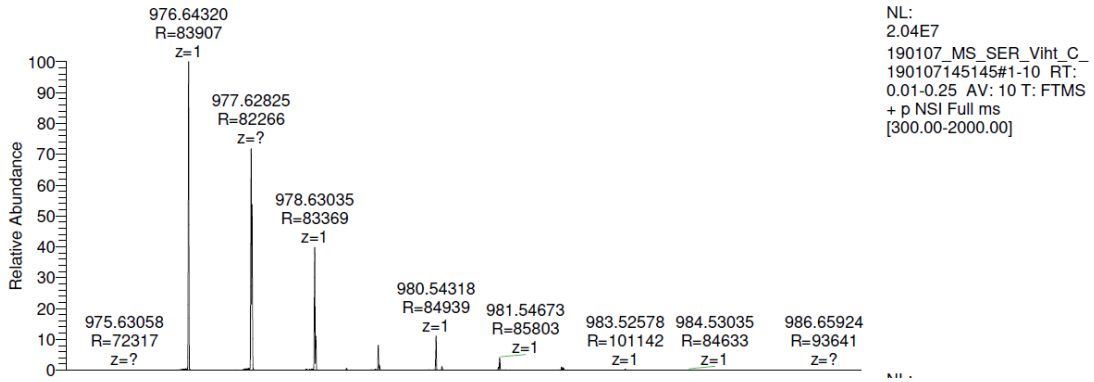
S6.2. ARC-2114 HRMS data measured with Thermo Electron LTQ Orbitrap (ESI HRMS) in positive ion mode.



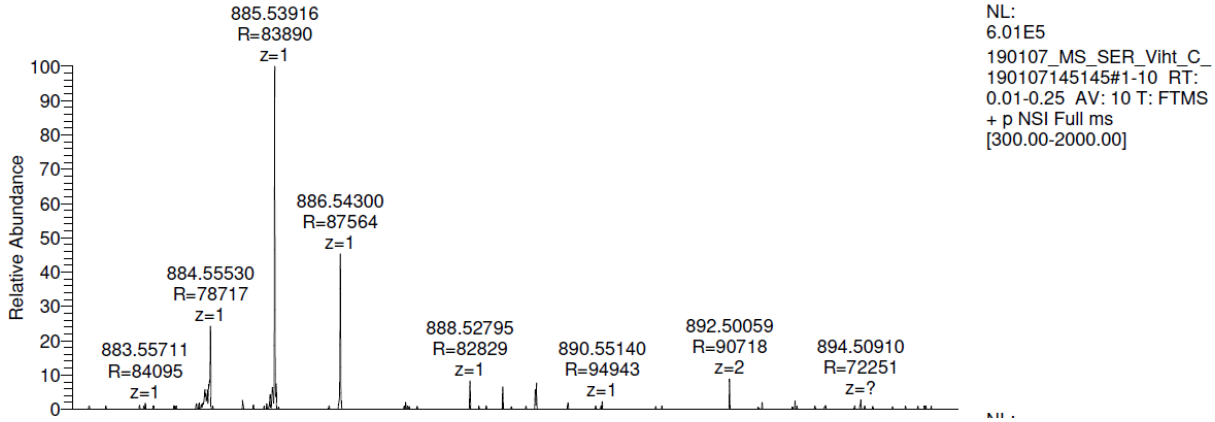
S6.3. ARC-2113 HRMS measured with Thermo Electron LTQ Orbitrap (ESI HRMS) in positive ion mode.



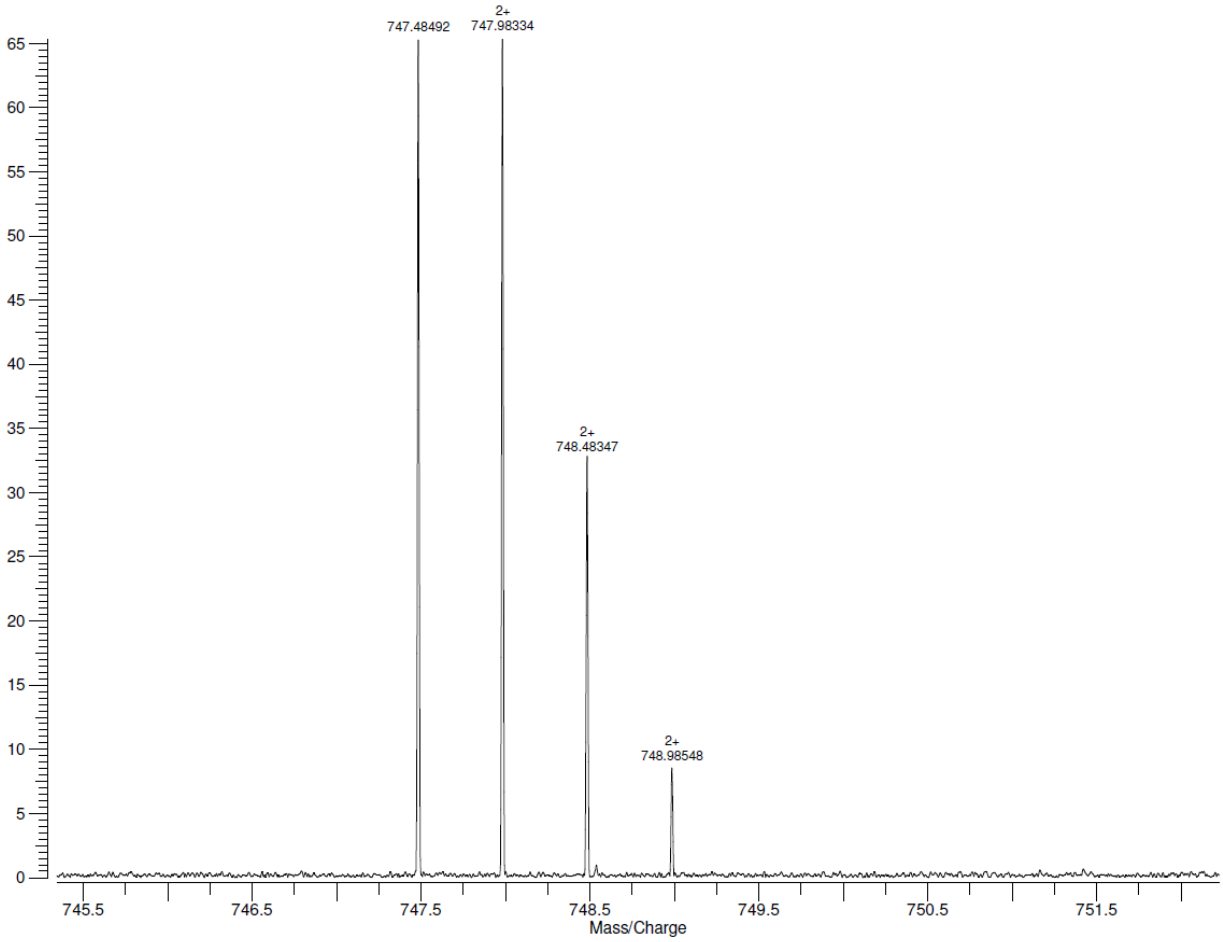
S6.4. ARC-2115 HRMS data measured with Thermo Electron LTQ Orbitrap (ESI HRMS) in positive ion mode.



S6.5. ARC-2116 HRMS data measured with Thermo Electron LTQ Orbitrap (ESI HRMS) in positive ion mode.



S6.6. ARC-2123 HRMS data measured with combined Varian 910-FT-ICR and Varian J- 320 3Q spectrometers in positive ion mode.



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10. Crystal Structure from RCSB Protein Data Bank, PDB ID 5IZF, authors: Pflug, A.; Enkvist, E.; Uri, A.; Engh, R.A.