Supporting Information for

Design, Synthesis and Evaluation of a Neurokinin-1 Receptor-Targeted Near IR Dye for Fluorescence Guided Surgery of Neuroendocrine Cancers

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EXPERIMENTAL PROCEDURES

General. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} TLC plates. The TLC plates were visualized with a 254 nm UV lamp and TLC staining solutions were ceric ammonium molybdate, *p*-anisaldehyde and iodine. Silica gel column chromatography was performed using silica gel (60-120 µm particle size). Preparative reverse-phase high performance liquid chromatography (RP-HPLC) was performed on a Waters, XBridge™ Prep C18, 5 µm; 19 × 100 mm column, mobile phase A = 20 mM ammonium acetate buffer, pH 5 or 7, B = acetonitrile, system with gradients in 30 min, 13 mL/min, λ = 254/280 nm. ^{1}H and ^{13}C NMR spectra were recorded on 400-500 MHz Bruker spectrometers in $CDCl_3$/D_2O/DMSO solutions. Chemical shifts were reported in parts per million (ppm) on the δ scale from an internal standard. The LRMS-ESI (LC-MS) was recorded on Agilent LCMS 1220 system, with Waters, XBridge™ RP18, 3.5 µm; 3 × 50 mm column, mobile phase A = 20 mM ammonium bicarbonate buffer, pH 5 or 7, B = acetonitrile, system with gradients in 12-15 min, 0.75 mL/min, λ = 254/280 nm. The high resolution mass measurements were recorded on a LTQ Orbitrap XL mass spectrometer utilizing electrospray ionization (ESI).

Procedure for Synthesis of Ester 2: The ligand, (*2S,3S*)-3-((3,5-bis(trifluoromethyl)benzyl)-oxy)-2-phenylpiperidine (L-733, 060, **1**) was synthesized according to the published procedure.[1] To the compound, **1** (0.065 g, 0.16 mol) in dry THF (1.5 mL) were added triethylamine (0.056 mL, 0.4 mmol, 2.5 eq.) followed by tert-butyl-2-bromo acetate (0.035 mL, 0.24 mmol, 1.5 eq.) under N_2 and reaction mixture was stirred for 16 h at r.t. The reaction was quenched with water and 2% HCl solution and extracted with EtOAc (3x5 mL) and combined organic layers were washed

[1] Mizuta, S., and Onomura, O. (2012) Diastereoselective addition to N-acyliminium ions with aryl- and alkenyl boronic acids via a Petasis-type reaction. *RSC Advances 2*, 2266-2269.

with brine, dried (Na_2SO_4), filtered and concentrated. The crude residue was purified by silica-gel column chromatography (hexane:EtOAc, 4:1) to yield product, **2** (0.075 g, 92%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.63 (br, s, 1H), 7.60 (br, s, 2H), 7.55-7.45 (m, 1H), 7.30-7.25 (m, 4H), 4.63 (d, J = 12.8 Hz, 2H), 4.60-4.55 (m, 1H), 4.22 (d, J = 12.8 Hz, 1H), 3.68 (d, J = 3.5 Hz, 1H), 3.53 (d, J = 18.0 Hz, 1H), 3.43-3.33 (m, 1H), 2.20 (d, J = 15.8 Hz, 1H), 2.06 (s, 1H), 1.96 (s, 1H), 1.71-1.60 (m, 1H), 1.41 (s, 1H), 1.40 (s, 1H), 1.36-1.32 (m, 1H), 1.28 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.8, 140.2, 131.4, 131.1, 129.4, 128.9, 128.5, 127.5, 124.4, 121.7, 121.2, 82.8, 82.2, 75.7, 69.7, 68.1, 61.0, 53.9, 52.8, 27.8, 27.7, 26.4, 18.8; ^{19}F NMR (376 MHz, $CDCl_3$): δ -64.4; ESI m/z Anal. Calcd for $C_{26}H_{29}F_6NO_3$, 517.20; found $[M+H]^+$ 518.19.

Hydrolysis of *tert*-Bu Ester, 2: To the ester (**2**, 0.075 g, 0.14 mmol) in dry CH_2Cl_2 was added trifluoroacetic acid (TFA, 20 eq.) and reaction mixture was stirred for 4 h at r.t. The excess of TFA was removed, diluted with water and extracted using CH_2Cl_2 (3x5 mL). The combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated. The residue obtained was purified by flash silica-gel column chromatography (hexane:EtOAc, 3:7) to give acid, **3** (0.06 g, 90%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71 (br, s, 1H), 7.55 (br, s, 2H), 7.44-7.35 (m, 2H), 7.32-7.24 (m, 3H), 4.56 (d, *J* = 12.5 Hz 1H), 4.45 (br, s, 1H), 4.17 (d, *J* = 12.6 Hz, 1H), 3.72 (br, s, 1H), 3.39 (br, d, *J* = 12.2 Hz, 2H), 3.10-3.20 (m, 2H), 2.18 (d, *J* = 12.5 Hz, 2H), 1.71 (t, 2H), 1.25 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.7, 140.0, 135.0, 131.6, 131.2, 128.8, 128.7, 128.6, 127.5, 124.4, 121.7, 121.4, 76.2, 69.9, 68.8, 55.7, 54.0, 26.4, 18.9; ^{19}F NMR (376 MHz, $CDCl_3$): δ -64.3; HRMS (ESI) m/z Anal. Calcd for $C_{22}H_{21}F_6NO_3$: 461.1426; found $[M+H]^+$ 462.1494.

Synthesis of NK1RL-Lys (4). H-Lys (Boc)-2-Cl-Trt resin (80 mg, 0.75 mmol) was swollen in dichloromethane (2 × 5 mL) and DMF (2 x 3 mL) while bubbling under argon. A solution of Fmoc-Asp(O^{t}Bu)-OH (2.5 equiv), PyBOP (2.5 equiv), and DIPEA (5 equiv) in DMF was added. The resulting solution was bubbled under argon for 3 h and drained, and the resin was washed with DMF (3 × 5 mL) and i-PrOH (3 × 5 mL). Fmoc deprotection was carried out using 20% piperidine in DMF (3 × 5 mL). The deprotection solution was removed, and the resin was washed again with DMF (3 × 5 mL) and i-PrOH (3 × 5 mL). Kaiser tests were conducted to assess coupling and

deprotection steps. The same procedure were followed for Fmoc-Arg(pbf)-OH, Fmoc-PEG_1-CO_2H and NK1RL couplings. But, reaction was bubbled overnight in the case of ligand coupling step. The resin was washed with DMF (3 × 5 mL) and i-PrOH (3 ×5 mL) and allowed to dry under nitrogen. The NK1RL-Lys linker was then cleaved from the resin using a mixture of 95% TFA, 2.5% H_2O and 2.5% triisopropylsilane. The solution was bubbled thrice under nitrogen for 15 min, drained, concentrated, and then precipitated by addition of cold diethyl ether. Crude product was collected by centrifugation, washed three more times with cold diethyl ether, dried under vacuum, and then purified by preparative reverse-phase HPLC (Waters, XBridge™ Prep C18, 5 µm; 19 × 100 mm column, mobile phase A = 20 mM ammonium acetate buffer, pH 7, B = acetonitrile, gradient 0−80% B in 30 min, 13 mL/min, λ = 254 nm). Pure fractions were analyzed by LC-MS (XBridge™ RP18, 3.5 µm; 3.0 × 50 mm column) and were pooled and lyophilized to furnish NK1RL-Lys, **4** (Scheme 2), yield 16 mg (26%).

^{1}H NMR (500 MHz, D_2O): δ 7.40 (s, 1H), 7.25 (s, 2H), 7.08 (s, 2H), 6.95 (s, 2H), 6.85-6.90 (t, 1H), 4.68, 4.50-4.47 (t, 1H), 4.33 (d, *J* = 12.7 Hz, 1H), 4.27-4.24 (t, 1H), 4.04-4.01 (t, 1H), 3.86 (d, *J* = 9.8 Hz, 1H), 3.60-3.45 (m, 3H), 3.38 (s, 4H), 3.22 (br, 1H), 3.15-3.05 (m, 1H), 2.99 (br, 1H), 2.89-2.86 (t, 1H), 2.64-2.56 (m, 2H), 2.55-2.54-2.45 (m, 1H), 2.51, 2.45-2.35 (m, 2H), 2.30 (br, 1H), 1.93 (br, 1H), 1.86 (s, 2H), 1.78-1.68 (m, 1H), 1.66-1.59 (m, 1H), 1.56-1.52 (m, 2H), 1.32-1.23 (m, 4H), 1.15 (br, 2H); ^{13}C NMR (126 MHz, D_2O): δ 179.6, 178.2, 177.2, 173.5, 173.3, 172.2, 171.1, 156.7, 140.4, 138.0, 130.9, 130.6, 128.4, 128.0, 127.7, 126.3, 124.1, 122.0, 120.8, 119.80, 76.4, 70.0, 69.5, 69.4, 68.7, 66.6, 57.6, 54.8, 53.2, 51.6, 40.6, 39.2, 38.3, 35.6, 31.0, 28.5, 26.3, 24.4, 22.2, 21.8, 19.2; ^{19}F NMR (376 MHz, $CDCl_3$): δ -64.2; HRMS (ESI) m/z Ana. Calcd for $C_{45}H_{63}F_6N_9O_{11}$: 1019.4551; found $[M+H]^+$ 1020.4621.

Synthesis of NK1RL-Lys-Rhodamine (5). The purified NK1RL-Lys linker **4** was coupled with NHS-rhodamine by stirring 1:1.2 ratios of linker **4** and NHS-rhodamine in dry DMSO, DIPEA under argon for 12 h at r.t. The crude reaction mass was purified by RP-HPLC, mobile phase A = 20 mM ammonium acetate buffer, pH 7, B = acetonitrile, gradient 0−80% B in 30 min, 13 mL/min, λ = 280 nm). Pure fractions were combined, concentrated under vacuum, and lyophilized to yield the product, NK1RL-Lys-Rhodamine (**5)**, which was analyzed by LC-MS and LR-ESIMS, yield was 65%.

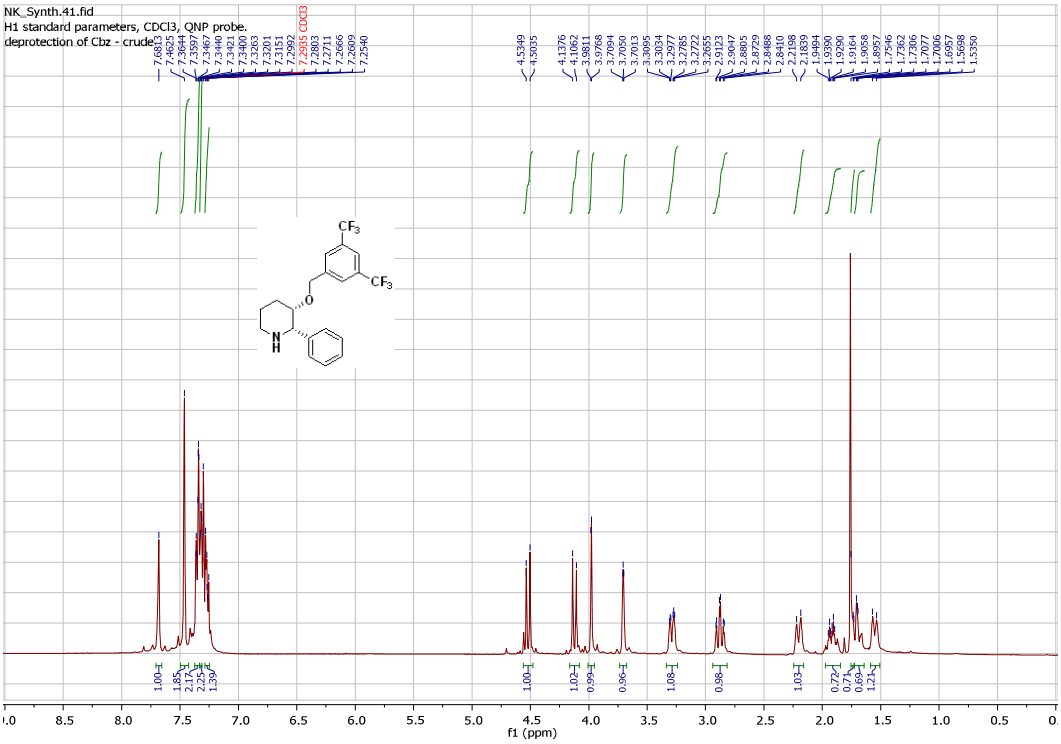
^{1}H NMR (500 MHz, DMSO): δ 9.41 (br, s, 1H), 9.25 (br, s, 1H), 8.83 (br, s, 1H), 8.69 (br, s, 1H), 8.52 (s, 1H), 8.42 (s, 1H), 8.36 (br, s, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.14 (d, *J* = 7.9 Hz, 1H), 8.08-7.96 (m, 2H), 7.90 (br, s, 2H), 7.84-7.70 (m, 2H), 7.65 (br, 4H), 7.39 (br, 3H), 7.23 (br, s, 6H), 6.49 (br, s, 12H), 4.62 (t, 1H), 4.52 (br, s, 1H), 4.25 (br, s, 2H), 4.12 (d, *J* = 12.6 Hz, 2H), 4.04 (br, 1H), 3.09-2.97 (m, 1H), 2.5 (s, 2H), 2.44-2.39 (m, 1H), 2.36-2.25 (m, 1H), 2.22-2.10 (m, 1H), 1.99 (br, s, 1H), 1.90 (br, 2H), 1.66 (br, s, 13H), 1.55-1.36 (m, 9H), 1.22 (br, s, 1H); ^{13}C NMR (126 MHz, DMSO): δ 175.2, 170.1, 152.6, 152.4, 142.7, 140.9, 130.5, 130.3, 129.2, 128.9, 128.1, 127.5, 124.8, 122.7, 121.3, 109.5, 106.1, 98.4, 77.1, 72.7, 69.9, 69.6, 68.7, 67.2, 60.7, 59.0, 54.1, 40.8, 38.6, 27.3, 25.0, 20.2; ^{19}F NMR (471 MHz, DMSO): δ -62.4; HRMS (ESI) m/z Anal. Calcd for $C_{70}H_{83}F_6N_{11}O_{15:}$ 1431.5974; found $[M+H]^+$ 1432.6072.

Solid Phase Synthesis of NK1RL-Peptide (6). H-Cys(Trt)-Wang resin (150 mg, 0.64 mmol) was swollen in dichloromethane (2 × 5 mL) and DMF (2 x 3 mL) while bubbling under argon. After swelling the resin in DMF, a solution of Fmoc-Asp(O^{t}Bu)-OH (2.5 equiv), PyBOP (2.5 equiv), and DIPEA (5 equiv) in DMF were added. The resulting solution was bubbled under argon for 3 h and drained, and the resin was washed with DMF (3 × 5 mL) and i-PrOH (3 × 5 mL). Fmoc deprotection was carried out using 20% piperidine in DMF (3 × 5 mL) and the resin was washed with DMF (3 × 5 mL) and i-PrOH (3 × 5 mL). Kaiser tests were conducted to assess coupling and deprotection steps. The above sequence was repeated for 5 more coupling steps as shown in scheme 4. Final coupling was done by NK1RL (**3**, 1.5 eq.) under same conditions for 12 h. The resin was washed with DMF (3 × 5 mL) and i-PrOH (3 ×5 mL) and allowed to dry under nitrogen. Finally, NK1RL-Peptide linker was then cleaved from the resin using a mixture of trifluoroacetic acid (TFA):H_2O: triisopropylsilane: ethanedithiol cocktail (92.5:2.5:2.5: 2.5). The solution was bubbled three times under nitrogen for 15 min, drained, concentrated, and then precipitated by addition of cold diethyl ether. Crude product was collected by centrifugation, washed three more times with cold diethyl ether, dried under vacuum, and then purified by preparative reverse-phase HPLC (Waters, XBridge^{TM} Prep C18, 5 µm; 19 × 100 mm column, mobile phase A = 20 mM ammonium acetate buffer, pH 5, B = acetonitrile, gradient 10−100% B in 30 min, 13 mL/min, λ = 280 nm). Pure fractions were analyzed by LC-MS and lyophilized to furnish **6**. Further, confirmed the products by mass spectrometry analysis.

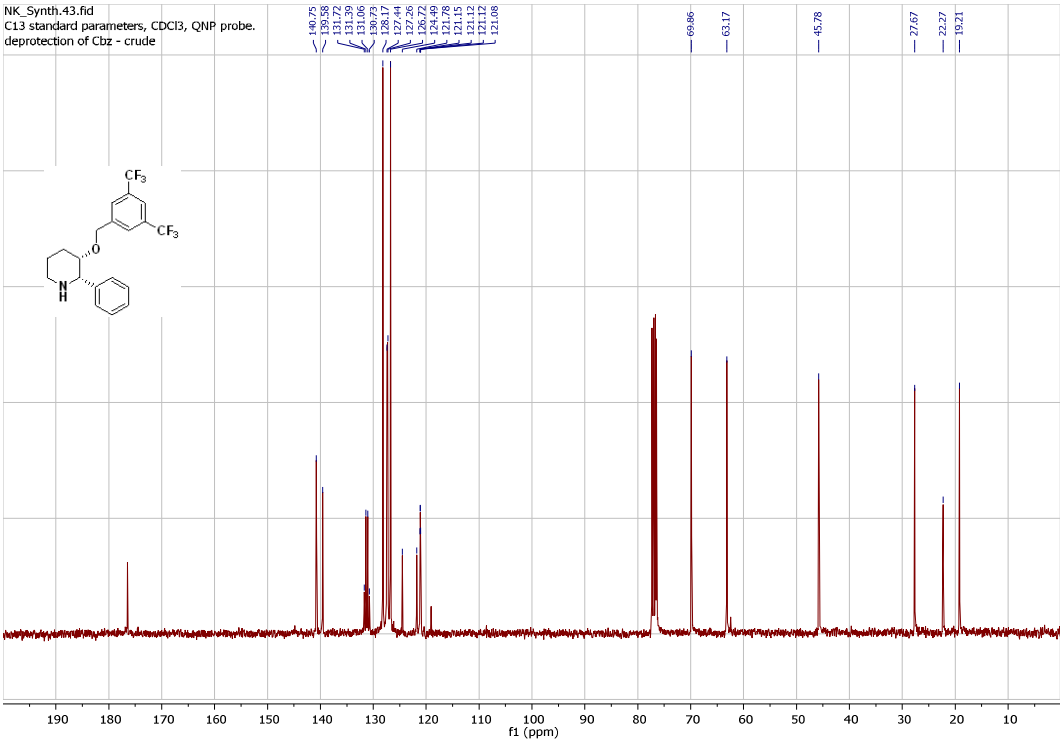
^{1}H NMR (500 MHz, DMSO): δ 9.29 (br, 1H), 9.21 (br, 1H), 8.51 (s, 1H), 8.28, 8.01 (s, 1H), 7.90 (s, 1H), 7.85 (s, 1H), 7.72, 7.71, 7.66 (s, 2H), 7.47 (d, *J* = 10 Hz, 1H), 7.40 (d, *J* = 5 Hz, 2H), 7.24-7.21 (m, 3H), 4.65 (d, *J* = 15 Hz, 1H), 4.19-4.12 (m, 2H), 3.99 (s, 1H), 3.58 (s, 2H), 3.55 (s, 1H), 3.49 (t, 1H), 3.45-3.34 (m, 7H), 3.28-3.22 (m, 1H), 3.21-3.14 (m, 1H), 3.06 (br, 1H), 2.96-2.91 (m, 1H), 2.89-2.84 (m, 1H), 2.83-2.79 (m, 1H), 2.75-2.66 (m, 1H), 2.67-2.61 (m, 1H), 2.46-2.38 (m, 3H), 2.25-2.21 (t, 1H), 2.18-2.13 (m, 1H), 1.89 (s, 4H), 1.67 (s, 2H), 1.58-1.39 (m, 8H), 1.11 (s, 1H); ^{13}C NMR (126 MHz, D_2O): δ 174.2, 173.8, 173.5, 172.4, 172.1, 171.6, 171.4, 171.2, 170.3, 169.6, 157.3, 142.3, 140.4, 130.1, 129.8, 129.5, 128.8, 127.7, 127.6, 127.0, 124.4, 122.2, 120.8, 76.7, 72.3, 69.4, 69.1, 68.3, 66.5, 60.2, 58.5, 54.7, 53.6, 40.0, 39.8, 39.7, 39.3, 39.2, 39.0, 38.2, 38.1, 35.9, 30.6, 28.5, 27.3, 26.8, 24.6, 24.1, 21.1, 19.7; HRMS (ESI) m/z Anal. Calcd for $C_{55}H_{79}F_6N_{13}O_{16}S$: 1323.5393; found $[M+H]^+$ 1324.0487.

Synthesis of NK1RL-Peptide-LS288 Conjugate (8). To the NK1RL-Peptide linker (**6**) in dry DMSO were added LS288-maleimide (**7**, 1 eq.) followed by diisopropylethylamine (5 eq.) under argon atmosphere and the reaction mixture was stirred at r.t for overnight. The progress of the reaction was monitored through LC-MS as conditions shown above and crude product was purified by reverse phase-HPLC using a mobile phase of A = 20 mM ammonium acetate buffer, pH 7; B = acetonitrile; gradient 0−50% B in 30 min, 13 mL/min, λ = 280 nm. Pure fractions were combined and concentrated under vacuum and lyophilized to yield the product, **8** and analyzed by LC-MS and LR-ESIMS. ESI m/z Anal. Calcd for $C_{106}H_{137}F_6N_{17}O_{31}S_5$: 2417.8174; found $[M+H]^+$ 1210.1 (half mass).

Spectral Data:



^{1}H NMR (400 MHz) spectra in $CDCl_3$ of L-733 060, 1



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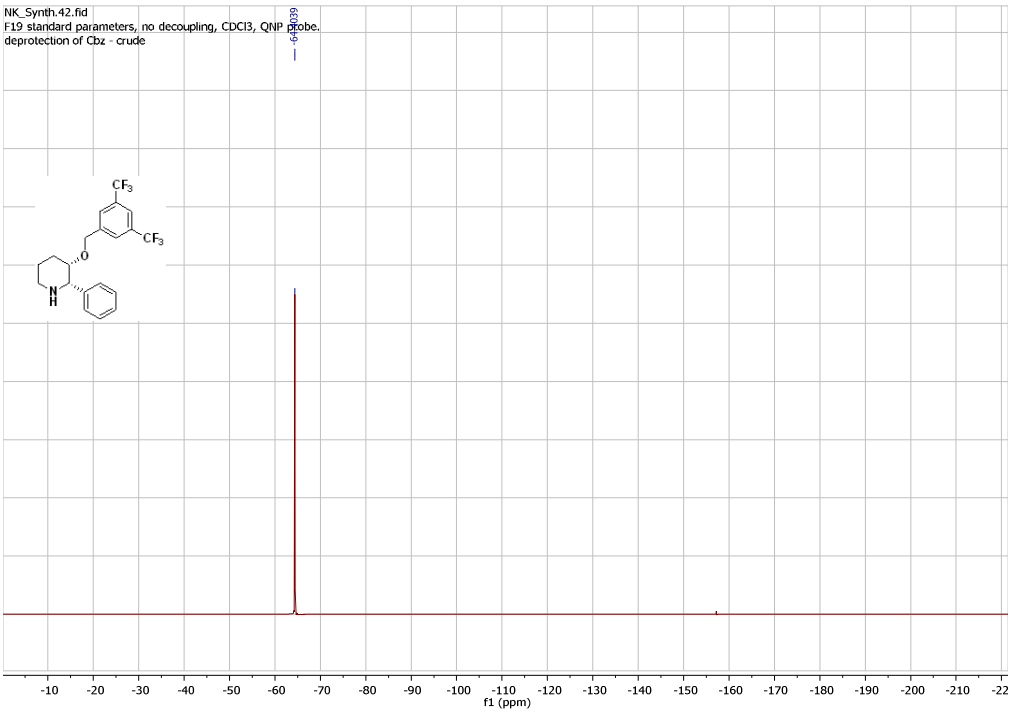
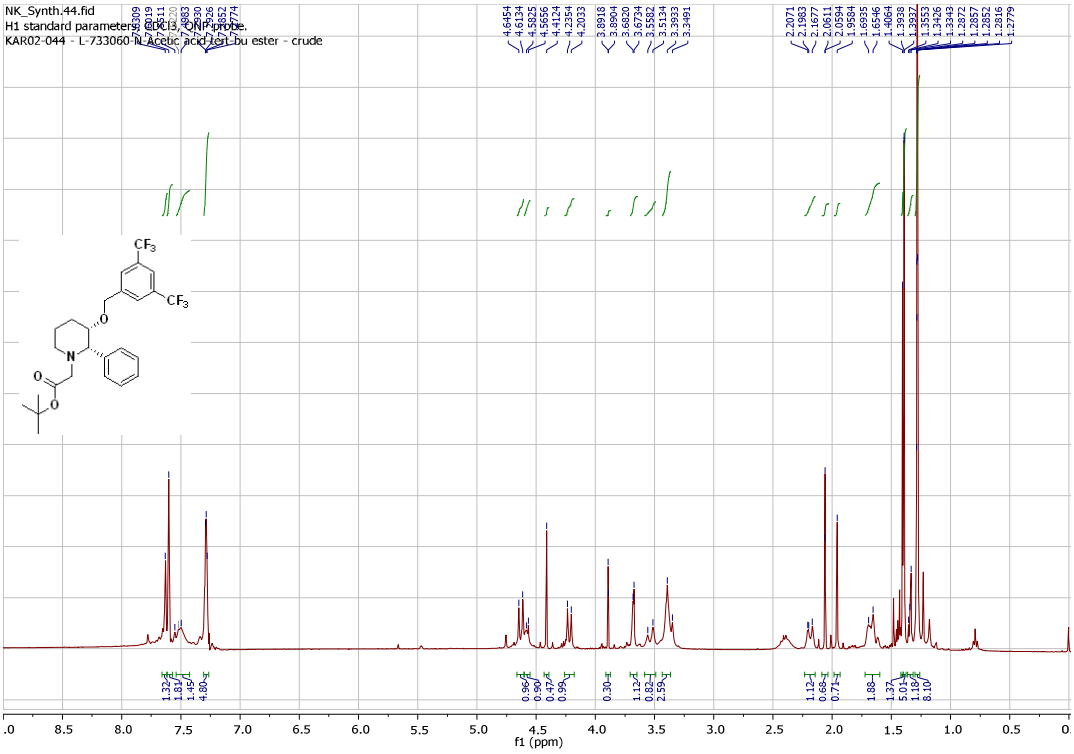
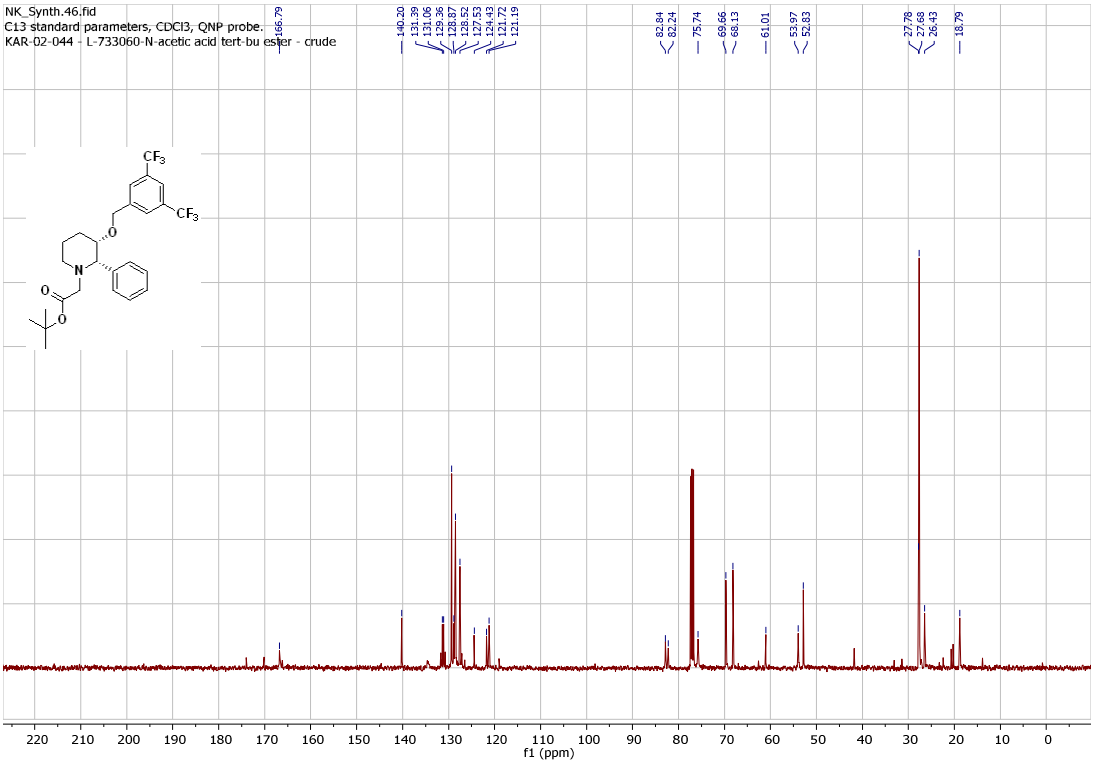


^{19}F NMR (376 MHz) spectra in $CDCl_3$ of L-733060, 1



^{1}H NMR (400 MHz) spectra in $CDCl_3$ of NK1RL-tert-Bu ester, 2



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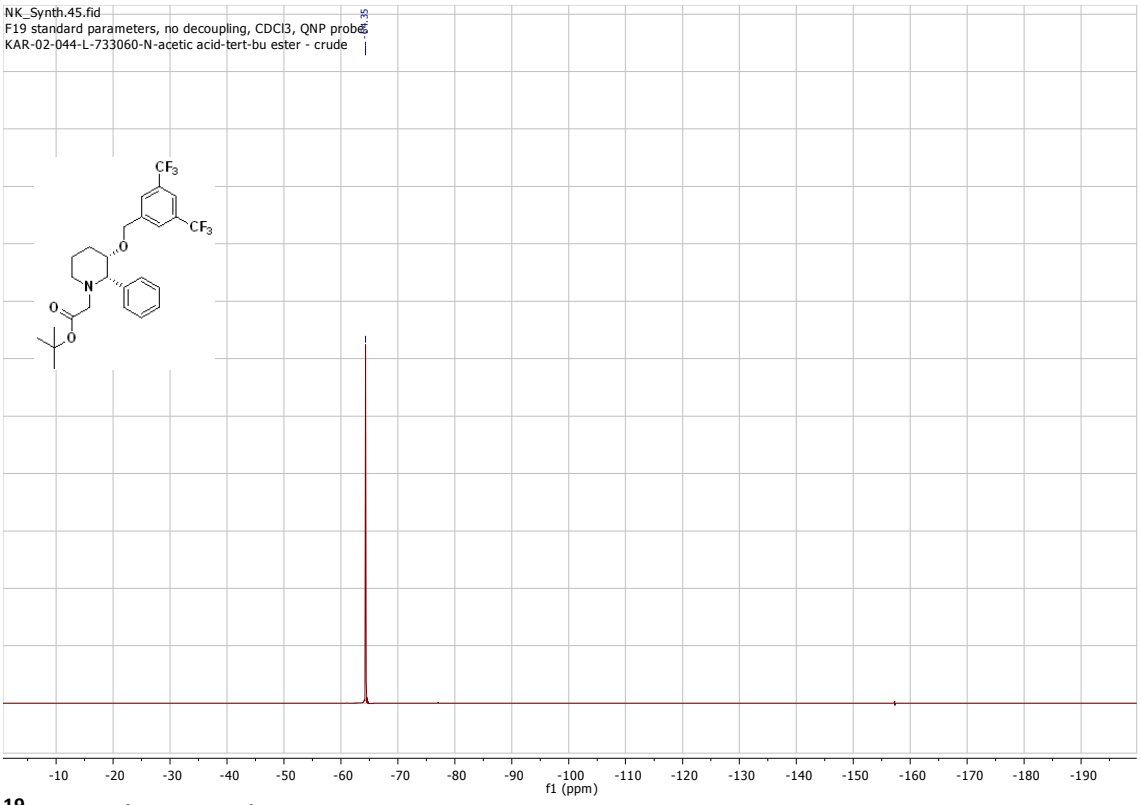
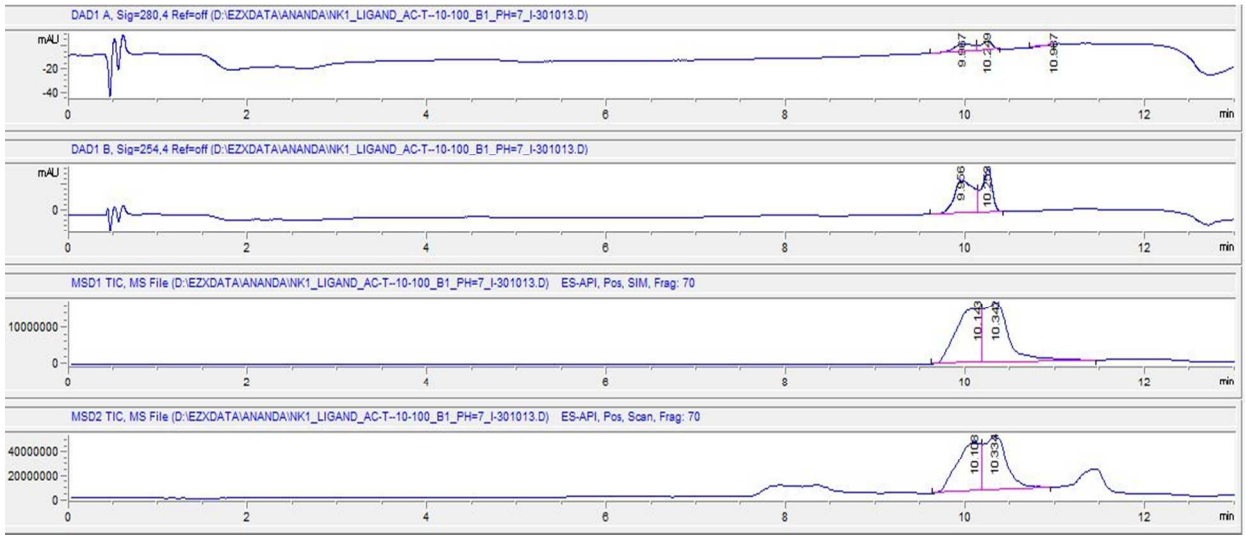
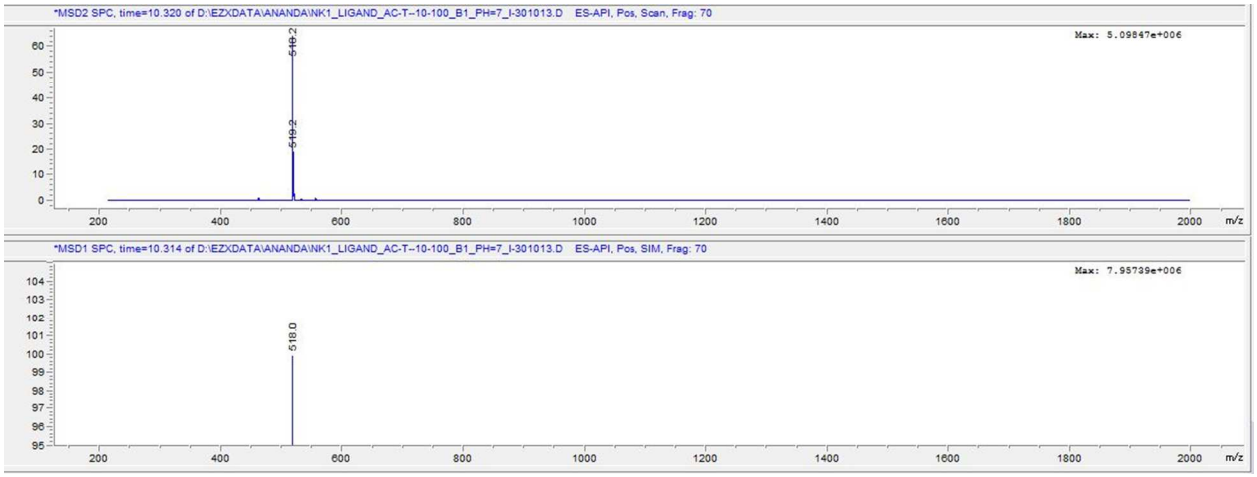


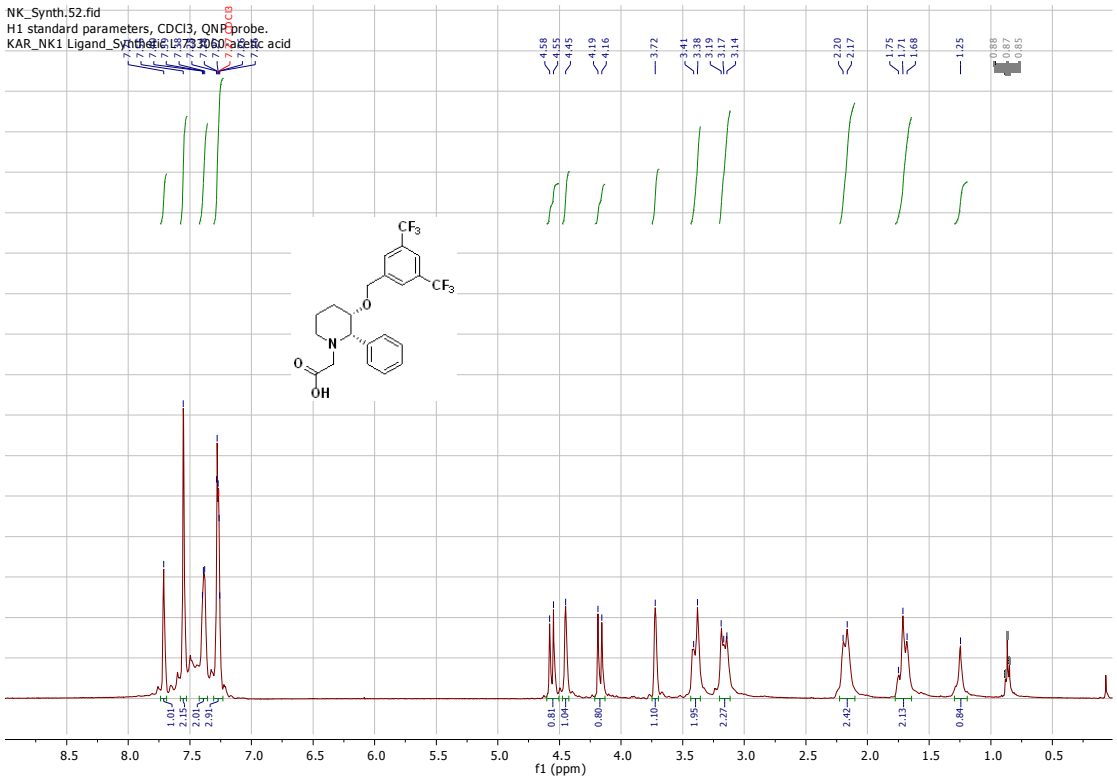
^{19}F NMR (376 MHz) spectra in $CDCl_3$ of NK1RL-tert-Bu ester, 2



LC-MS of NK1RL-*tert*-Bu ester (2)



Mass spectrum of NK1RL-*tert*-Bu ester (2)



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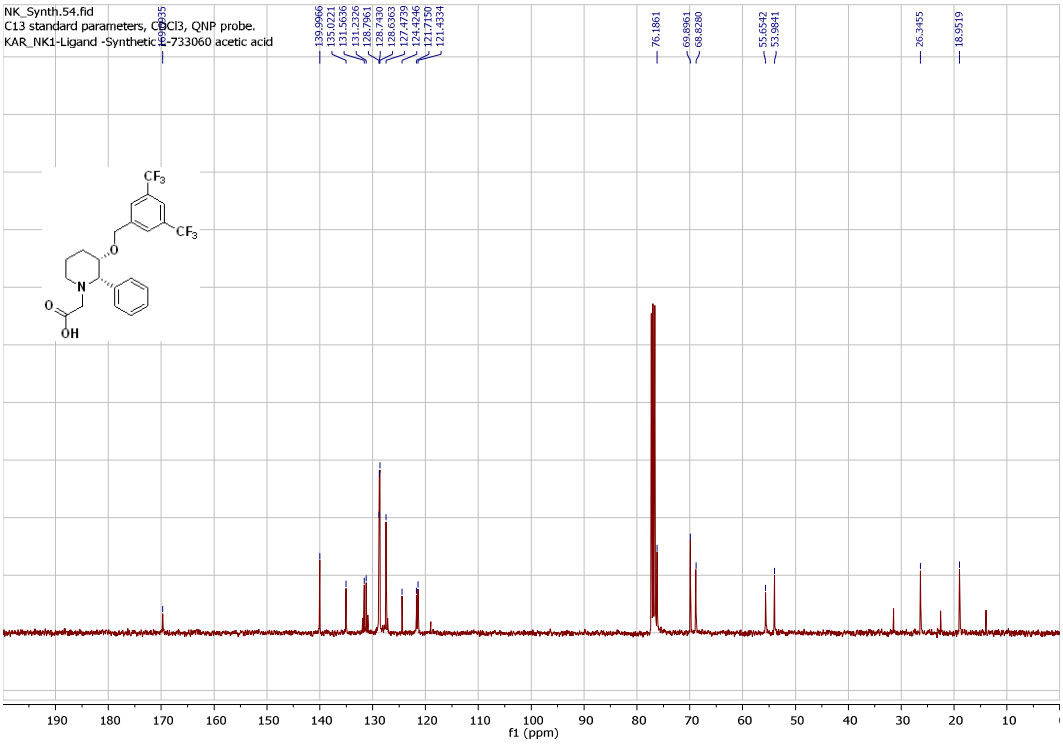
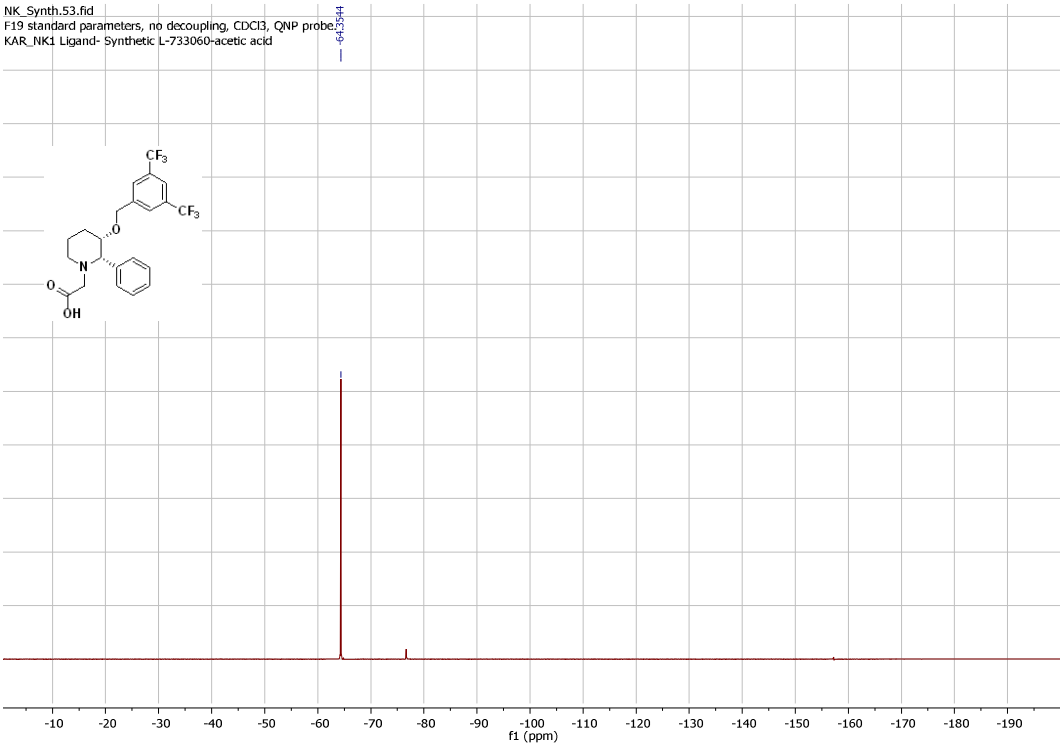


^{13}C NMR (100 MHz) spectra in $CDCl_3$ of NK1 Ligand (NK1RL, 3)



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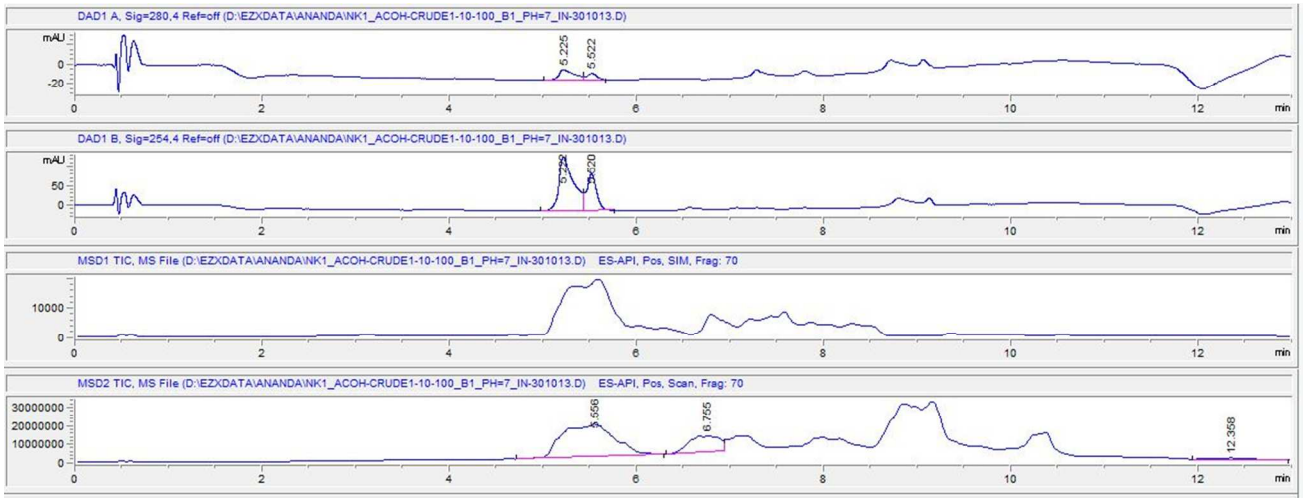
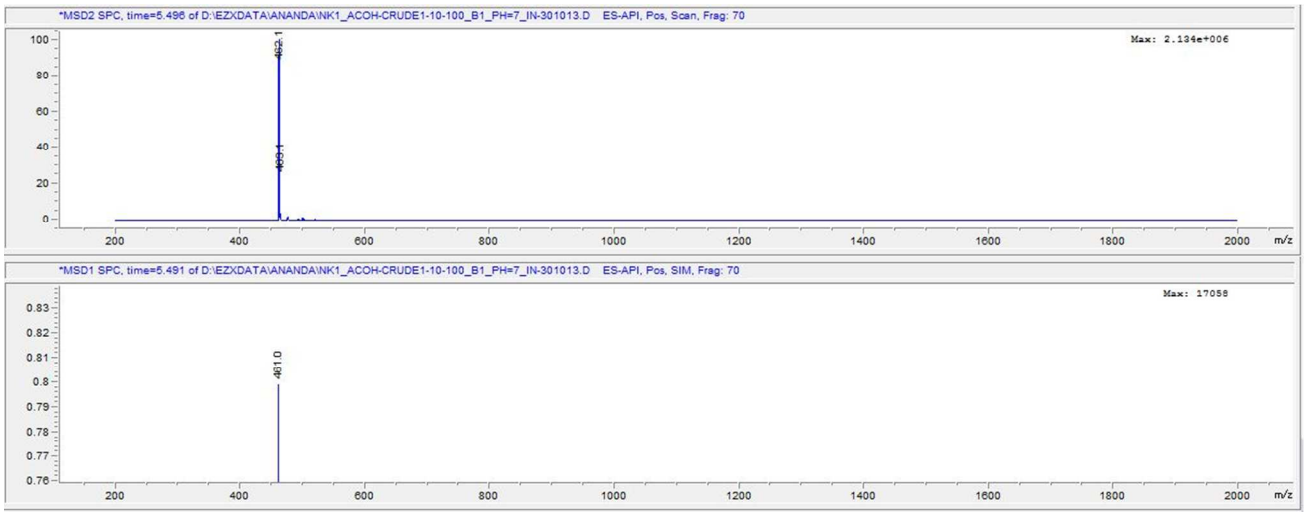
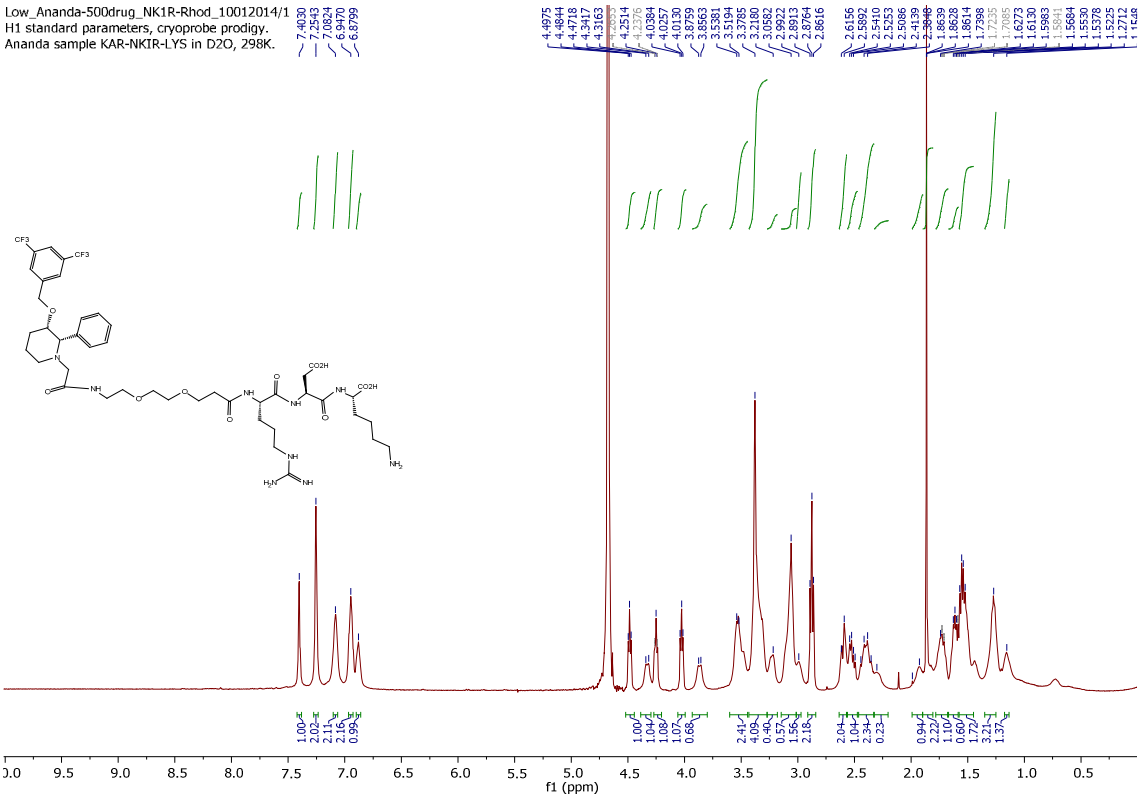


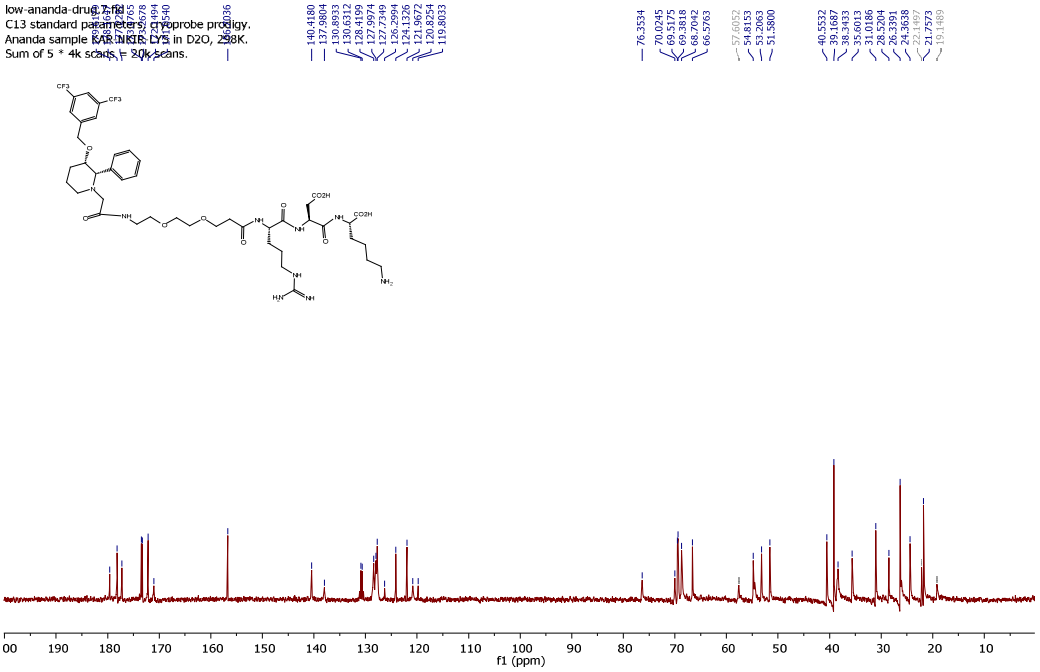
LC-MS of NK1 Ligand (NK1RL, 3)



Mass spectrum of NK1 Ligand (NK1RL, 3)



^{1}H NMR (500 MHz) spectra in $CDCl_3$ of NK1RL-Lys, 4



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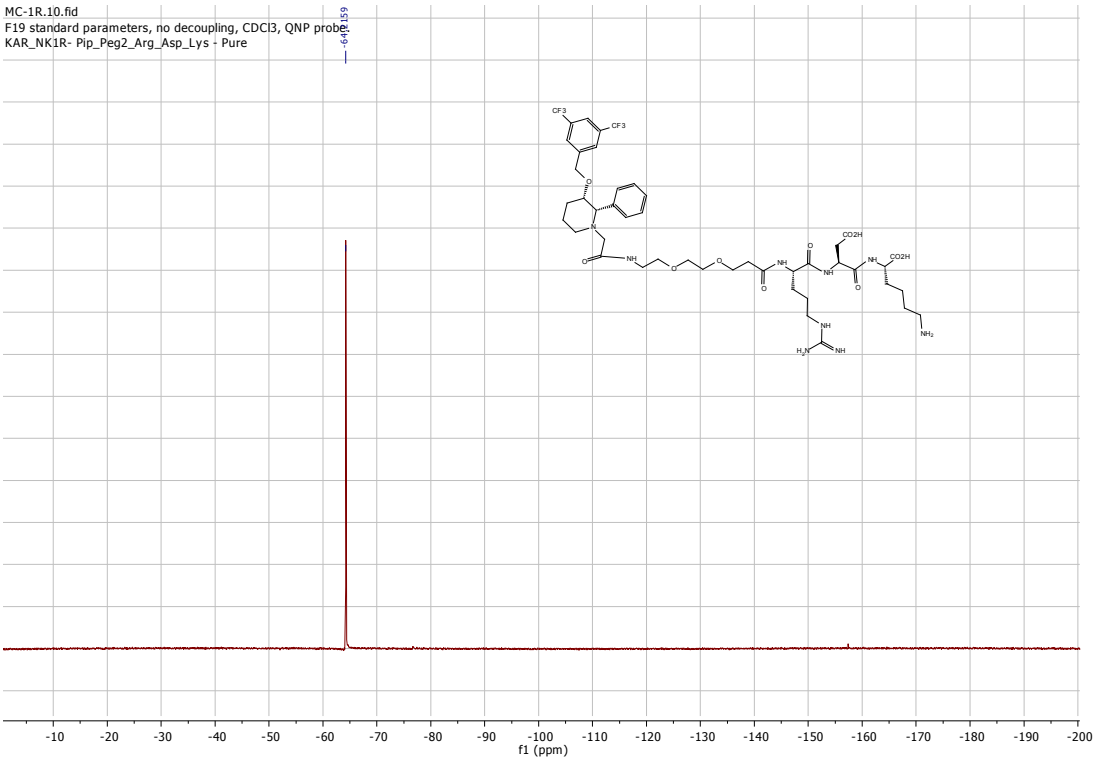
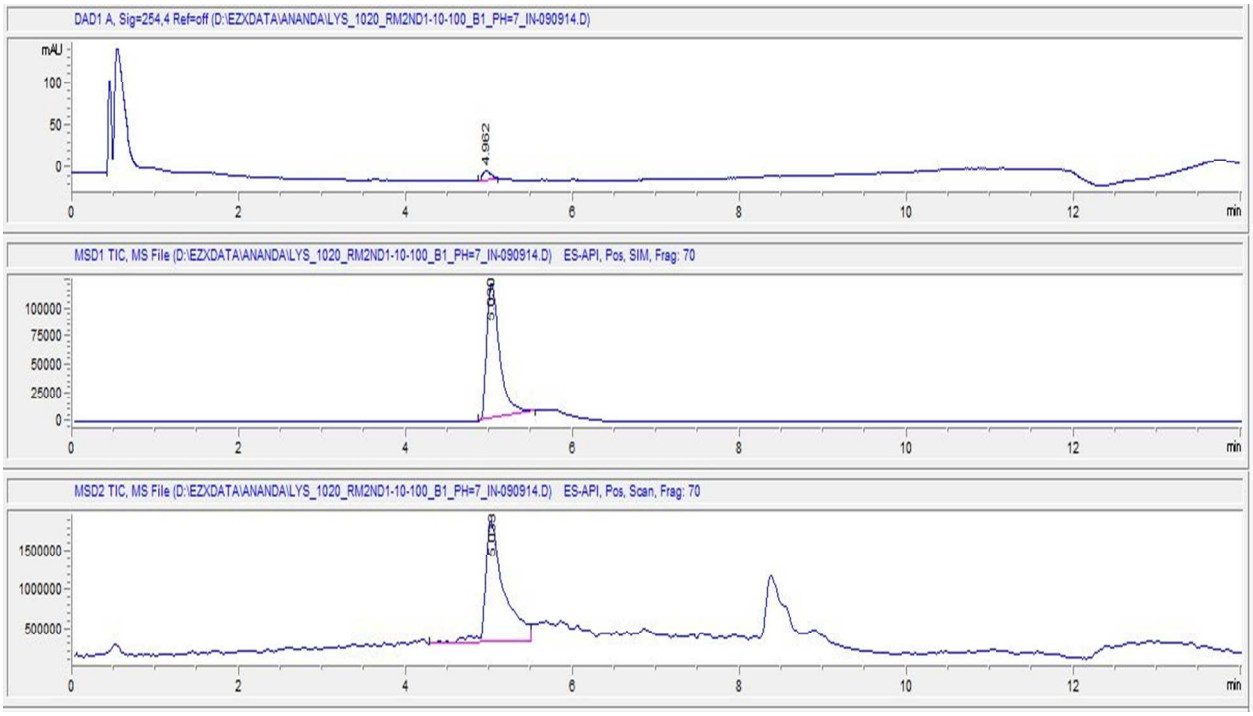
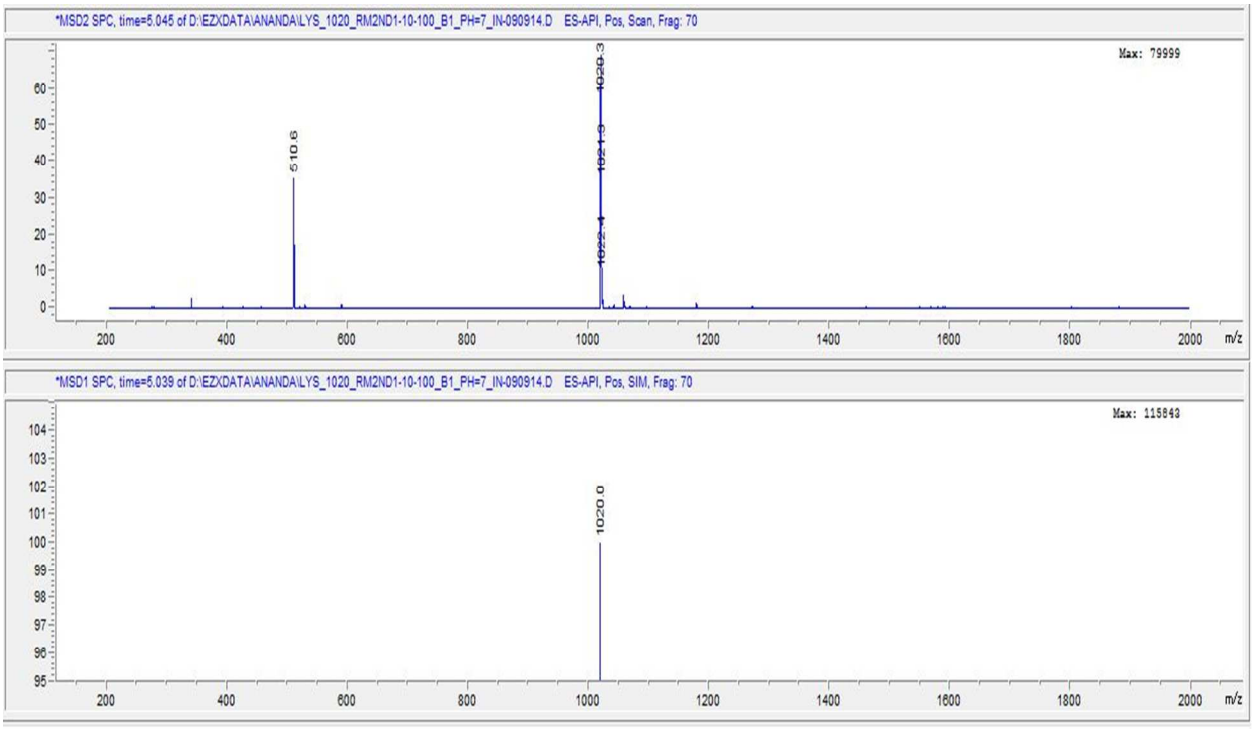


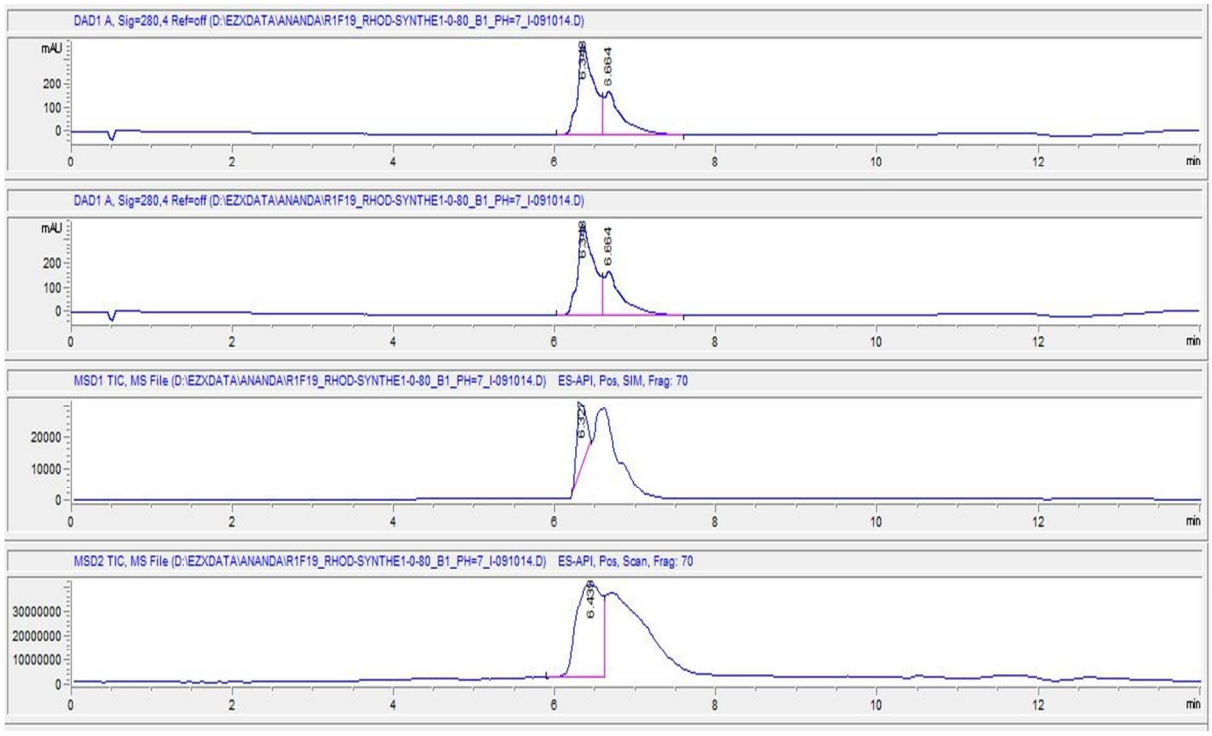
^{19}F NMR (376.49 MHz) spectra in $CDCl_3$ of NK1RL-Lys, 4



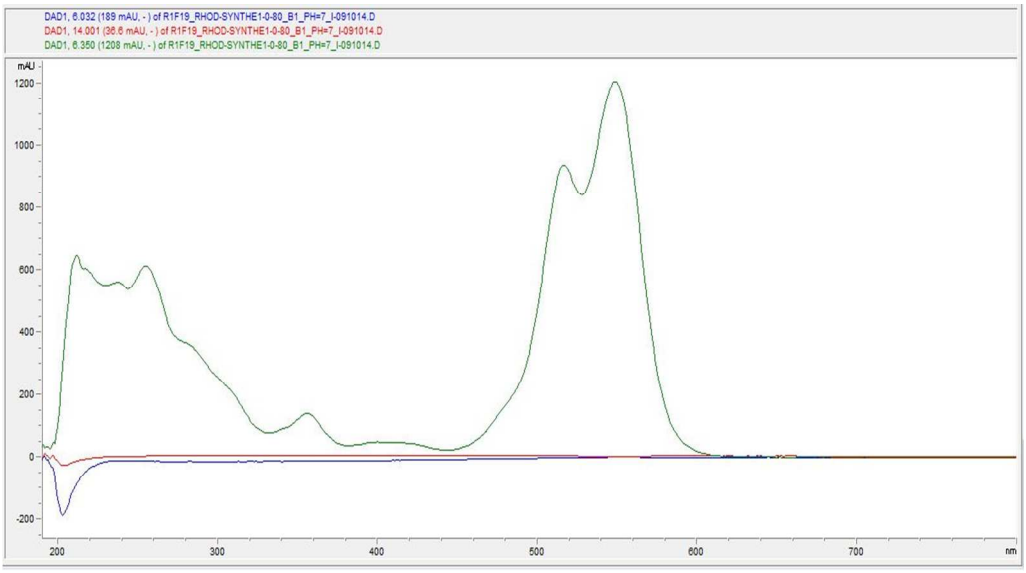
LC-MS profile of NK1RL-Lys, 4



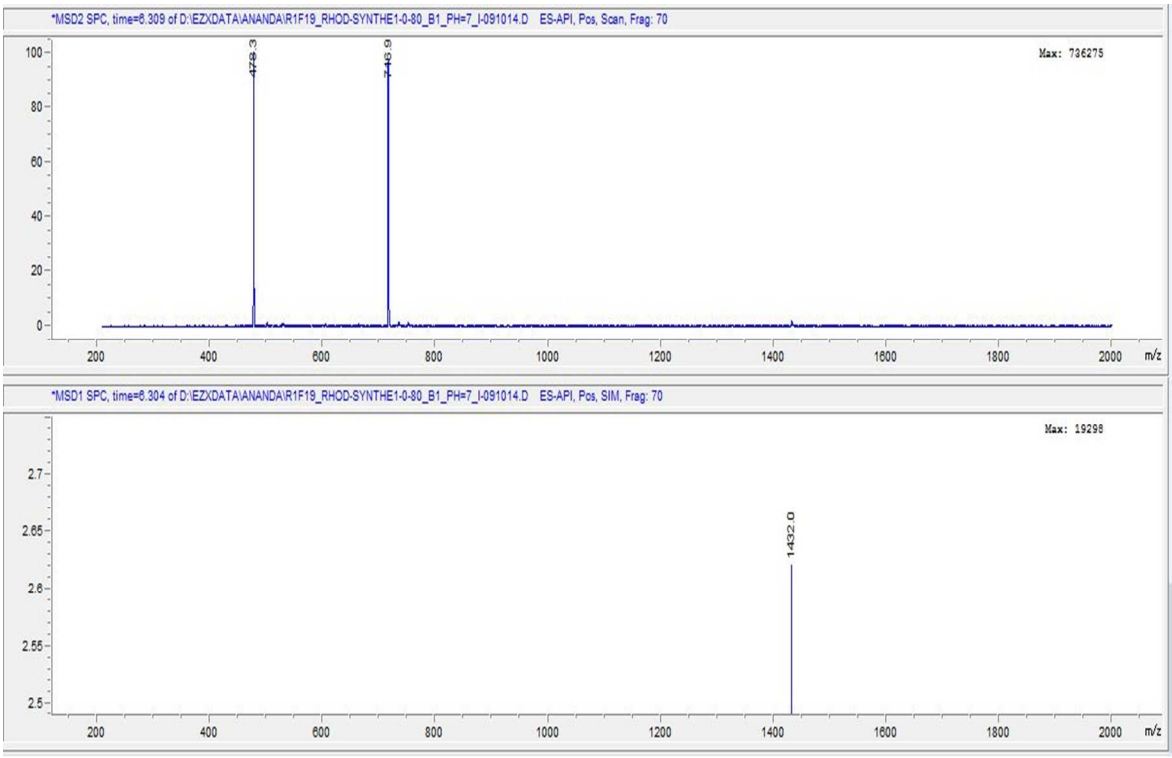
Mass spectrum of NK1RL-Lys, 4



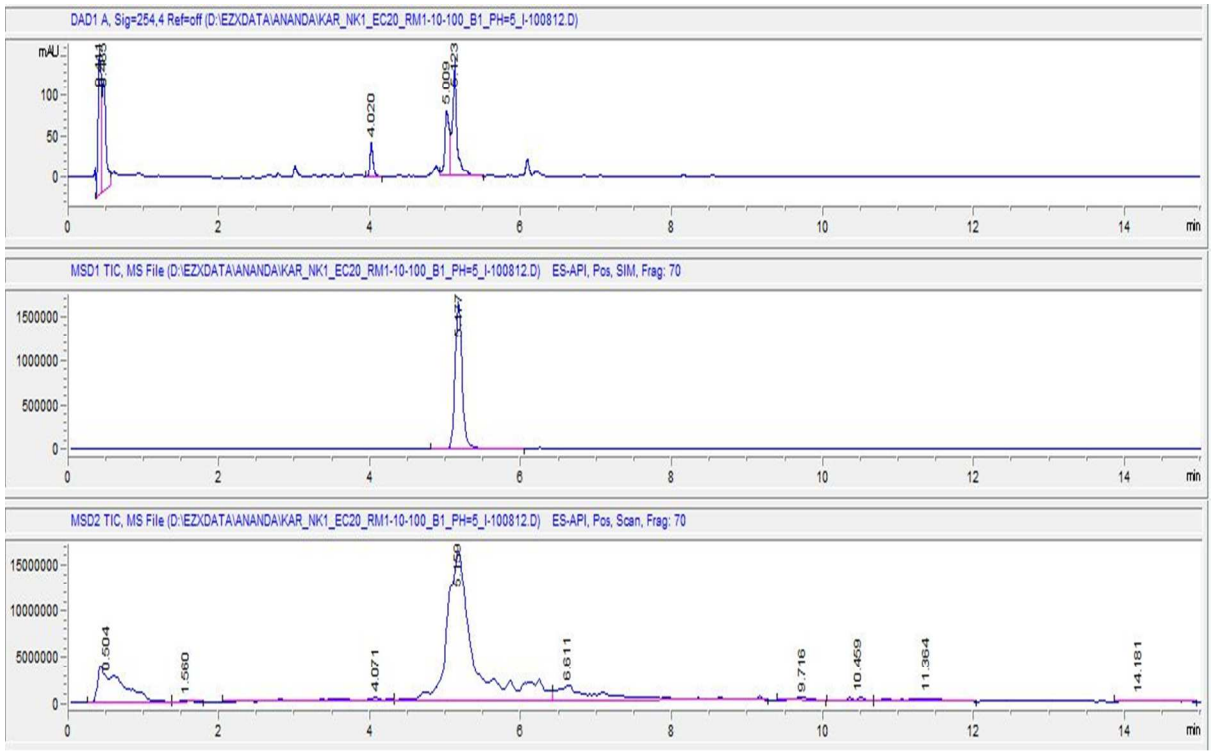
LC-MS profile of NK1RL-Lys-Rhodamine, 5



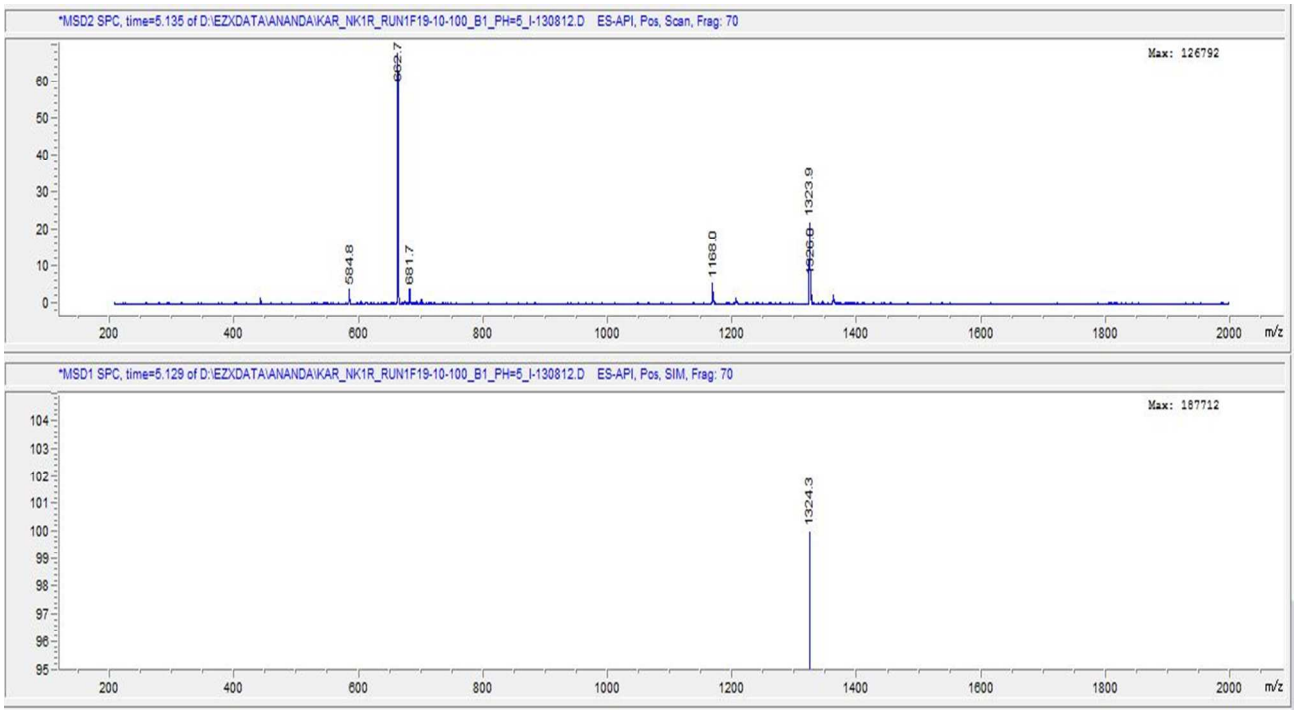
UV profile of NK1RL-Lys-Rhodamine, 5



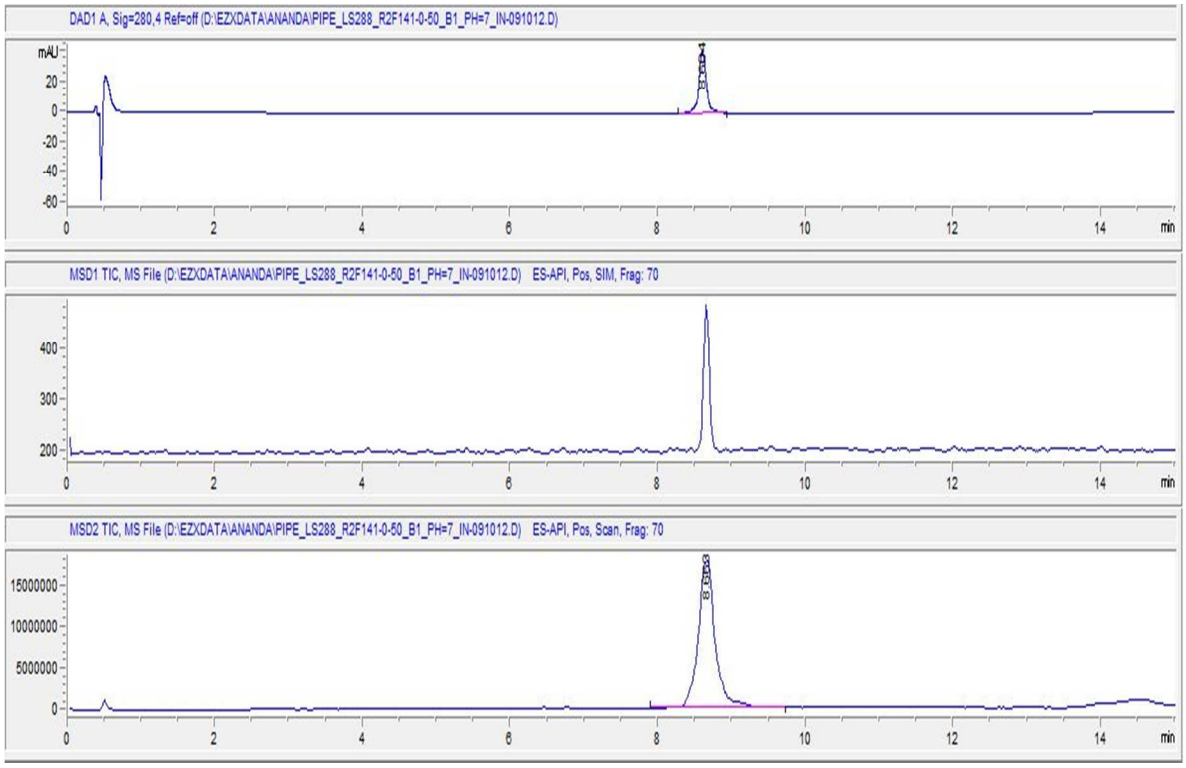
Mass spectrum of NK1RL-Lys-Rhodamine, 5



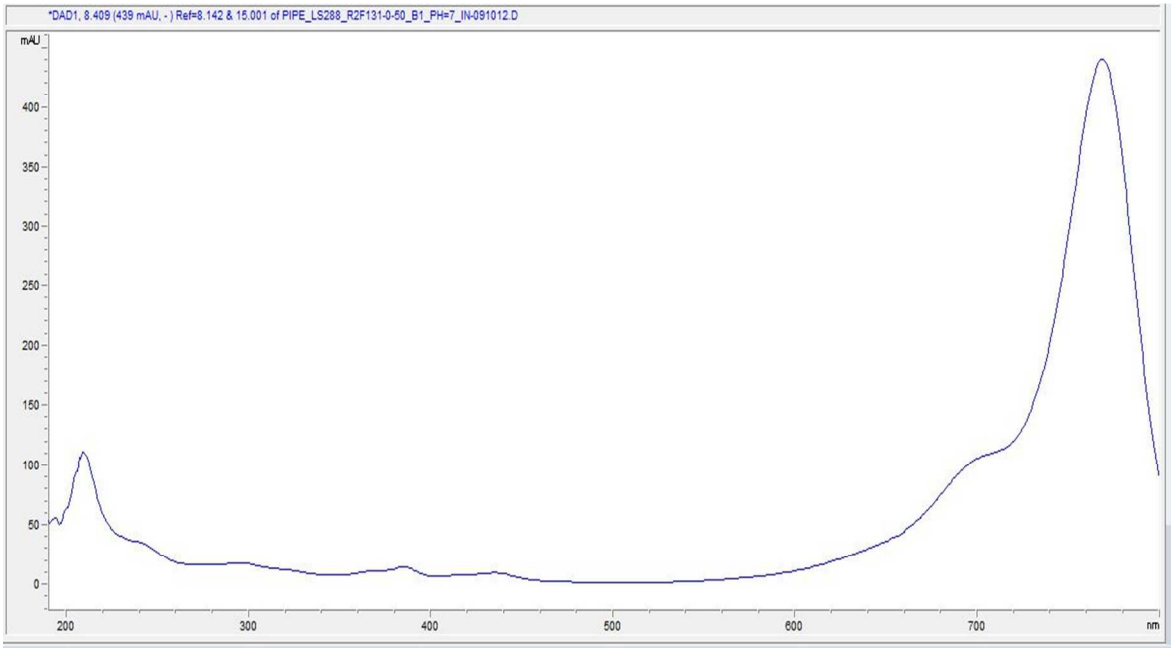
LC-MS of NK1RL-Peptide, 6



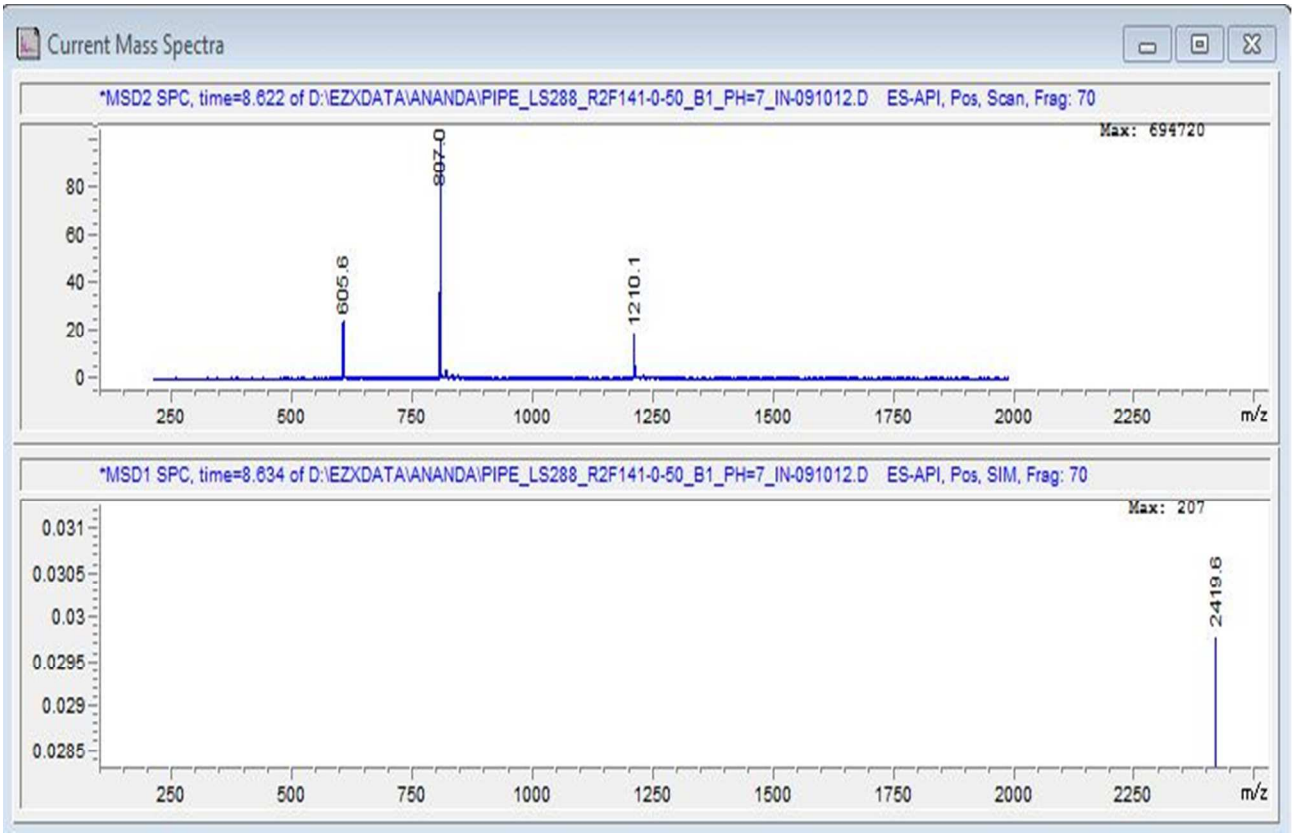
Mass Spectrum of NK1RL-Peptide, 6



LC-MS profile of NK1RL-Peptide-LS288, 8



UV profile of NK1RL-Peptide-LS288, 8



Mass spectrum of NK1RL-Peptide-LS288, 8

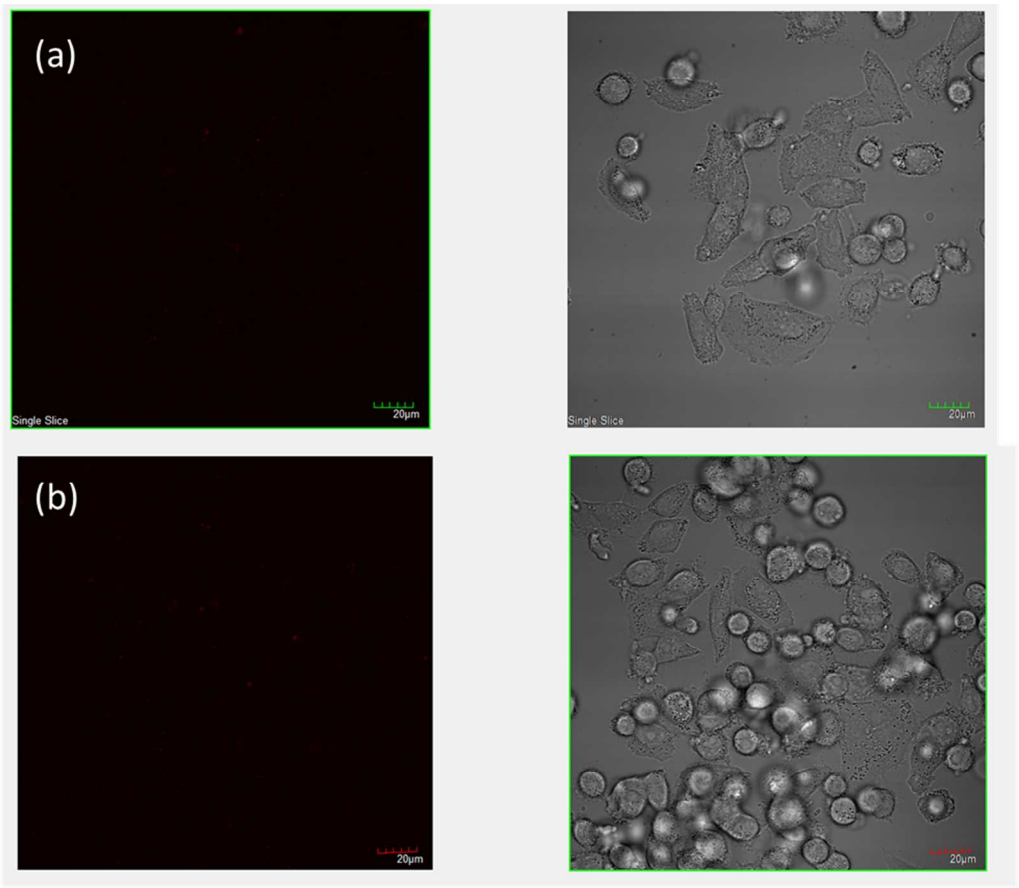


Figure S1. Confocal microscopy images: In vitro binding studies of NK1-Lys-Rhodamine **5** conjugate to KB (NK1R-negative) cells. Incubation of cells for 1 h at 37 ˚C with (a) 25 nM and (b) 50 nM of **5**. The images show no uptake of **5** even at 50 nM and this further supports the specificity of the conjugate for NK1R.