Novel CHK1 inhibitor MU380 exhibits significant single-agent activity in TP53-mutated chronic lymphocytic leukemia cells

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Supplementary Data

Content:

Supplementary Methods

Additional agents

Gemcitabine, fludarabine, and cytarabine were purchased from Sigma-Aldrich (St. Louis, MO, USA), ATR inhibitor VE-821 from APIs Chemical Co., Ltd (Shanghai, China), and CHK1 inhibitor CHIR-124 and STAT3 inhibitor WP1066 from Selleckchem (Munich, Germany).

All working solutions were prepared fresh by dissolving compounds in appropriate solvents. Final concentration of DMSO was maintained at less than 0.2 % in all experiments; this concentration was found non-cytotoxic in control viability measurements.

Additional cell lines and characterization of primary CLL cells

The following non-cancerous cell lines were used: RPE-1, human epithelial cells immortalized with hTERT obtained from the *American Type Tissue Collection* (ATCC) (CRL-4000); HS-5, human bone marrow/stromal fibroblasts transformed with a human papilloma virus 16 obtained from the ATCC (CRL-11882); primary human skin fibroblasts established "in-house" from a biopsy ("Fibroblasts normal" in Table 1); primary skin fibroblasts from a patient with *ataxia-telangiectasia* (cell line GM02052 obtained from *Coriell Institute*; NJ, USA; *ATM* gene mutation: c.103C>T, p.R35X) ("Fibroblasts AT" in Table 1). Additionally, three cultures of peripheral blood mononuclear cells (PBMNC) from healthy donors were used; these cultures were used either fresh or vitally frozen.

Mutation status of *TP53*, *ATM*, *NOTCH1*, and *SF3B1* genes in CLL samples was assessed by next-generation sequencing using MiSeq (Illumina, San Diego, CA, USA); details are available upon request. Cytogenetic aberrations were detected by FISH using probes from MetaSystems (Altussheim, Germany). Complex karyotype (CK) was assessed by

conventional karyotyping by analyzing at least 15 mitoses. The CK was defined as „three or more aberrations in two or more mitoses“. Mutation status of the immunoglobulin heavy chain variable region (*IGHV*) was determined by PCR and sequencing; homology ≥ 98% to the germ-line sequence was considered as “unmutated”.

Antibodies for immunoblotting

Antibodies detecting total CHK1, pS296 CHK1, pS317 CHK1, pS345 CHK1, β-actin, total H2AX, pS139 H2AX, total PARP, cleaved-PARP, total caspase-3, cleaved-caspase-3, MYC, pS15 p53, MCL1, BCL2, CDC25A, CDC25C, pS216CDC25C, CDK1, pY15 CDK1, cyclin B1 and cyclin E1 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-NFκB antibody was from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-p53 antibody (DO-1) was a gift from Dr. Borivoj Vojtesek (Masaryk Memorial Cancer Institute, Brno, Czech Republic). The secondary anti-rabbit and anti-mouse antibodies were purchased from DakoCytomation (currently Agilent Technologis, Santa Clara, CA, USA) and Cell Signaling Technology.

Cell viability measurement

In WST-1 assay, the cells were seeded in 96-well plates in quadruplicates using the following count per well: SU-DHL-4 cell line: 2.5×10^4 cells/well; all other leukemia and lymphoma cell lines: 5×10^4 cells/well; primary fibroblasts, the fibroblast cell lines and RPE-1 cell line 10^4 cells/well; unstimulated primary CLL cells: 5×10^5 cells/well; healthy PBMNC: 2.5×10^5 cells/well. The cells were treated with a tested agent or DMSO (mock control) for 72 h. Final cell viability after the treatments was assessed using a WST-1 Cell Proliferation Reagent (Roche, Basel, Switzerland) according to the manufacturer´s

instructions; the spectrophotometers 1420 Multilabel Counter Victor (PerkinElmer, Waltham, MA, USA) or Sunrise™ Absorbance Reader (Tecan, Männedorf, Switzerland) were used.

In viability measurement with DiOC6 dye (Thermo Fisher Scientific, Waltham, MA, USA), cells were seeded in 96-well plates in triplicates at the density of 5×10^4 cells/well. Treatments with MU380 or DMSO (mock control) were done for 48 h. Thereafter, the cells were incubated with DiOC6 and propidium iodide (PI) for 15 min at 37°C, and cell viability was measured using flow-cytometer Accuri C6 (BD Biosciencies, San Jose, CA, USA); viable cells were DiOC6-positive and PI-negative.

Immunoblotting and real time PCR

Cells were seeded in six-well plates at the following density: cell lines 5×10^6/5 ml; primary CLL cells 1.25×10^7/5 ml. After respective treatments, western blotting and *real time* PCR procedures were performed as we previously described[1]. In case of γH2AX (and total H2AX) detection, histone lysates were prepared using EpiQuik™ Total Histone Extraction Kit (Epigentek, Farmingdale, NY, USA). CHK1 protein level in primary CLL cells was detected using a sensitive kit (Clarity Western ECL Substrate; Bio-Rad, Hercules, CA, USA).

Tissue samples (tumors from immunodeficient mice) were frozen in liquid nitrogen. The tissue was mechanically homogenized and lysed in 200 µl of cold RIPA buffer. Samples were agitated for 2 h at 4°C and centrifuged at 18 000 rcf for 30 min. Supernatant was collected and the procedure of western blotting was performed.

Analysis of cell cycle

Cells were treated in 6-well plates at the density of 1.25×10^6/5 ml. For cell cycle analysis, cells were fixed in 70% ice-cold ethanol, and DNA content was analyzed using PI staining and flow cytometer BD FACSVerse (BD Biosciencies).

Multi-parametric flow-cytometry analysis

Analysis was performed as we previously published[2] with slight modifications. Cells were treated with 10 μM 5-ethynyl-2'-deoxyuridine (EdU) for 1 h, harvested, counted using CASY system and total number of 2×10^6 cells was used per one sample. Staining and washing steps were done with staining buffer (PBS containing 1% BSA) at 4°C or room temperature (RT) and in the dark. The cells were stained for viability with LIVE/DEAD Red dye (Thermo Fisher Scientific), fixed with 4% paraformaldehyde and permeabilized by 0.25% Triton-X100. Clic-iT reaction (A647, Thermo Fisher Scientific) was performed according to the manufacture protocol. Next, the cells were incubated overnight with anti-cleaved-PARP antibody (Cell Signaling Technology, 1:400) at 4°C, washed with PBS and incubated with anti-rabbit A488 antibody (Thermo Fisher Scientific, 1:1000). Finally, DNA was stained by FxCycle Violet (Thermo Fisher Scientific, 1:1000) in the presence of RNase A (Sigma-Aldrich, 100 μg/ml) for 1 h at RT. Fluorescence was analyzed using BD FACSVerse system and compensation values for multicolor analyses were calculated according to single-conjugate stained UltraComp eBeads (Thermo Fisher Scientific) assay setup within BD FACS Suite software. Cell aggregates and debris were excluded from analysis based on a dual-parameter dot plot (FSC-H vs. FSC-A). Acquired data were analyzed using FlowJo v10.4.2 software.

Analysis of mitotic cells

MEC-1 cells (1.25×10^5/5 ml) were cultivated for 24 h with MU380 (200 or 400 nM), VE-821 (1 or 2 μM) or combination of aforementioned lower and higher concentrations of the inhibitors. Thereafter, cells were arrested in mitosis using additional 7 h culture in the presence of 0.01% colchicine. Mitotic index was calculated from ~1 000 cells per sample, and

detected mitoses were further evaluated for the presence of chromosome damage. The procedures were as we previously described[1].

Xenograft experiments

The mice strain NOD-*scid IL2Rγ*^{null} exhibits the following immunologic defects: absent mature B-cells, mature T-cells, natural killer cells and complement, and defective macrophages and dendritic cells. Animals were housed in a pathogen-free facility, given autoclaved food, and maintained on UV-sterilized water with 12 h light/dark cycles. MEC-1 cells (5×10^6 in 200 µl of PBS) were transplanted subcutaneously into eight- to ten-week-old mice (of both genders). After tumors became readily visible (at day +14 post-transplant), mice were randomized into treated and control groups. In experiment I, treatment consisted of seven doses of MU380 (each 20 mg/kg) administered via intraperitoneal injection between days +14 to +28 post-transplant. In experiment II, treatment involved ten doses of MU380 provided between days +14 to +25 post-transplant. The size of the tumors was measured successively using caliper, and tumor volume was calculated as "(length x width^2)/2"[3]. The animals were sacrificed when the tumor volume reached 2000 mm^3 in the control group.

Immunohistochemistry

After incision of tumors, tissues were placed into 10% neutral-buffered formalin for overnight fixation, then processed and paraffin embedded. For routine histopathological analysis, 5 µm thick paraffin sections were stained with hematoxylin and antibodies against cleaved-caspase-3 (Cell Signaling Technology), pS139 H2AX (Cell Signaling Technology), CD20 (Abcam, Cambridge, UK), and Ki-67 (DCS-diagnostics, Hamburg, Germany). Incubation with primary antibody was performed at 4 °C overnight after heat induced antigen

retrieval. Specifically bound primary antibody was visualized with a biotinylated goat antibodies followed by addition of Envision+ reagent (DakoCytomation) and 3,3-diaminobenzidine. Slides were scanned using TissueFAX system (TissueGnostics, Vienna, Austria).

Transfection of CLL cells

CLL cells were thawed and cultured in RPMI-1640 (20% FBS) medium without antibiotics for at least 3 hours. Then, 12.5×10^6 cells were transfected by electroporation using Neon Transfection System (ThermoFisher Scientific) (1900 V, 30 ms, 1 pulse) with specific siRNA targeting *CHEK1* (200 nM; ON-TARGETplus Human CHEK1 siRNA – SMARTpool; Dharmacon Lafayette, CO, USA) or the negative control (200 nM; ON-TARGETplus Non-targeting Pool; Dharmacon). The cells were cultured in RPMI-1640 (20% FBS) medium without antibiotics for 48 h. Then, the viability was measured by flow cytometry using DiOC6 (3,3'-dihexyloxacarbocyanine iodide; Thermo Fisher Scientific) and propidium iodide (Sigma-Aldrich).

Statistical analyses

Synergy between gemcitabine and MU380 in individual cell lines was evaluated by Chou-Talalay test using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). Differences between the two groups (gemcitabine alone vs. gemcitabine with MU380; leukemia vs. lymphoma cell lines; flow-cytometry and immunohistochemical analyses) were evaluated by one-way ANOVA test followed by Scheffé comparison. Differences in the sensitivity to MU380 between the genetic groups (in cell lines and primary CLL cells), *real time* PCR analyses and xenograft data were evaluated by two-way ANOVA test followed by Scheffé comparison.

Changes in the cell cycle distribution were assessed by chi-squared test. Half-maximal inhibitory concentration values (IC_{50}) were assessed using mycurvefit.com website. Correlation between pCHK1, total CHK1, pH2AX expression and IC_{50} values of individual cell lines was done using regression analysis. Semi-quantitative analyses of western blots were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The values were calculated in relation to loading control (internal standard). Quantification of immunohistochemical images was performed by ImageJ software using at least 10 representative images for each sample.

Statistical evaluation and graphs were done using Statistica 13 program (StatSoft, Tulsa, OK, USA) and GraphPad Prism (GraphPad Software, San Diego, CA, USA).

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Supplementary Tables

Supplementary Table S1: Evaluation of the combined effect of gemcitabine (Gem) with MU380 using Chou-Talalay test.

Cell line	Gem (ng/ml)	Combination index (CI)	Effect
MEC-1	2.5	0.49	+++
	5	**0.39**	**+++**
	10	0.49	+++
	20	0.77	++
MEC-2	2.5	1.56	-
	5	0.58	+++
	10	**0.40**	**+++**
	20	0.77	++
BL-41	2.5	0.78	++
	5	0.74	++
	10	**0.42**	**+++**
	20	0.66	+++
SU-DHL-4	2.5	17.11	-
	5	31.70	-
	10	0.12	++++
	20	**0.06**	**+++++**
JEKO-1	0.625	2.50	-
	1.25	1.54	-
	2.5	0.36	+++
	5	**0.16**	**++++**
NALM-16	0.625	0.33	+++
	1.25	**0.27**	**++++**
	2.5	0.34	+++
	5	0.48	+++
RAJI	4	0.50	+++
	8	**0.37**	**+++**
	16	0.52	+++
	32	0.92	+-
REC-1	2.5	1.35	-
	5	**0.52**	**+++**
	10	0.64	+++
	20	1.15	-
MAVER-1	0.4	2.49	-
	1.6	0.55	+++
	6.25	**0.27**	**++++**
	25	0.67	+++

Cell line	Gem (ng/ml)	Combination index (CI)	Effect
MINO	1.25	1.77	-
	2.5	0.74	++
	5	**0.36**	**+++**
	10	0.52	+++
OSU-CLL	0.625	4.89	-
	1.25	1.21	-
	2.5	**0.48**	**+++**
	5	0.91	+-
JVM-2	0.625	0.70	+++
	1.25	**0.67**	**+++**
	2.5	0.75	++
	5	1.08	+-
JVM-3	0.625	1.65	-
	1.25	0.53	+++
	2.5	**0.48**	**+++**
	5	0.89	+
WSU-NHL	0.125	1.50	-
	0.25	1.30	-
	0.5	0.66	+++
	1	**0.13**	**++++**
DOHH-2	0.625	1.23	-
	1.25	**0.57**	**+++**
	2.5	0.61	+++
	5	1.03	+-
NALM-6	0.625	5.08	-
	1.25	0.88	+
	2.5	0.55	+++
	5	**0.51**	**+++**
Granta-519	2.5	0.48	+++
	5	**0.42**	**+++**
	10	0.54	+++
	20	1.06	+-

CI values are the following: <0.1 very strong synergism (+++++); 0.1-0.3 strong synergism (++++); 0.3-0.7 synergim (+++); 0.7-0.85 moderate synergism (++); 0.85-0.90 slight synergism (+); 0.9-1.1 nearly additive (+-); >1.20 antagonism (-).

Supplementary Table S2: CLL samples pre-treated with the pro-proliferative stimuli.

Sample (category)	*TP53* mut (%)	17p- (%)	*ATM* mut (%)	11q- (%)	Complex karyotype	*ATM*/*TP53* mutation	Mutation analysis
CLL-1 (wt)	no	no	no	no	yes	none	NGS
CLL-56 (wt)	no	no	no	no	ND	none	NGS
CLL-93 (wt)	no	no	no	no	yes	none	NGS
CLL-34 (*ATM*-mut)	no	no	98	96	ND	p.H2125R	NGS
CLL-62 (*ATM*-mut)	no	no	96	99	ND	p.I323K	NGS
CLL-75 (*ATM*-mut)	no	no	99	97	yes	p.L2452R	NGS
CLL-98 (*TP53*-mut)	39	no	ND	no	ND	p.H214R	NGS
CLL-15 (*TP53*-mut)	62	no	no	no	ND	c.626_627del	NGS
CLL-16 (*TP53*-mut)	99	95	ND	no	ND	R248Q	FASAY
CLL-99 (*TP53*-mut)	20	no	ND	38	ND	M237I	FASAY
CLL-55 (*TP53*-mut)	99	UPD	no	no	yes	R282W	NGS
CLL-86 (*TP53*-mut)	50	33	ND	no	ND	L265R	FASAY
CLL-88 (*TP53*-mut)	99	87	ND	no	ND	c.741_742 delinsTT	FASAY

NGS: next-generation sequencing; FASAY: functional analysis of separated alleles in yeast; specific details for CLL cells´ analysis can be found in Malcikova et al., *Blood*. 2009;114(26):5307-5314. UPD: copy-neutral loss of heterozygosity involving *TP53* gene detected by Cytoscan Affymetrics Arrays; wt: samples *ATM*-wt/*TP53*-wt. NA: not applicable; ND: not determined.

Supplementary Table S3. CLL samples used in the study.

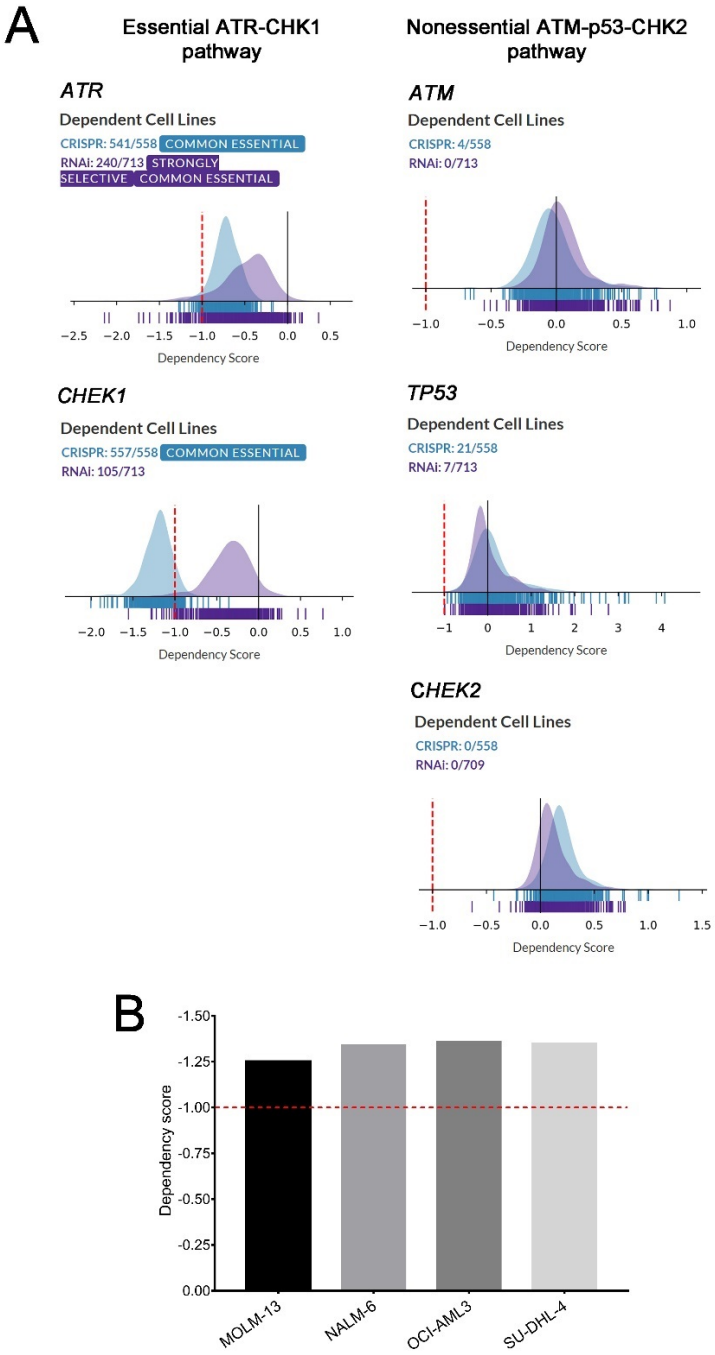
Sample ID	Status	c (MU380) [nM]				*SF3B1* mutation	*NOTCH1* mutation	*IGHV* status	Complex karyotype	Therapy
		100	200	300	400					
CLL-1	wt	90	89	85	74	no	no	unmut	yes	no
CLL-2	wt	100	94	87	85	no	yes	unmut	no	yes
CLL-3	*ATM*-mut	88	76	63	56	no	no	unmut	ND	yes
CLL-4	*ATM*-mut	66	58	41	27	no	no	unmut	yes	no
CLL-5	wt	81	69	62	56	no	yes	unmut	no	no
CLL-6	wt	100	95	87	77	no	no	unmut	no	no
CLL-7	*TP53*-mut	49	48	38	34	ND	ND	unmut	ND	no
CLL-8	*TP53*-mut	93	79	71	68	no	no	unmut	yes	yes
CLL-9	wt	89	82	73	63	no	yes	unmut	ND	no
CLL-10	*ATM*-mut	77	73	66	59	no	no	unmut	ND	yes
CLL-11	11q-	71	68	52	35	no	no	unmut	ND	no
CLL-12	11q-	78	67	51	40	no	no	unmut	ND	no
CLL-13	wt	86	80	71	63	yes	yes	unmut	ND	no
CLL-14	*TP53*-mut	87	79	71	68	yes	no	unmut	ND	no
CLL-15	*TP53*-mut	75	66	65	52	no	no	mut	no	no
CLL-16	*TP53*-mut	79	56	38	32	ND	ND	unmut	yes	no
CLL-17	11q-	58	44	42	34	no	no	unmut	ND	no
CLL-18	11q-	68	65	62	57	ND	ND	unmut	ND	yes
CLL-19	*TP53*-mut	77	59	58	40	no	no	mut	ND	yes
CLL-20	*ATM*-mut	91	94	100	99	no	no	unmut	ND	no
CLL-21	wt	84	67	51	42	no	no	unmut	no	no
CLL-22	11q-	72	68	66	66	no	no	unmut	ND	no
CLL-23	11q-	87	79	71	69	yes	yes	unmut	ND	yes
CLL-24	11q-	98	88	72	53	no	yes	unmut	ND	no
CLL-25	*ATM*-mut	60	49	39	31	no	no	unmut	no	no
CLL-26	wt	93	83	79	71	no	no	mut	ND	yes
CLL-27	*ATM*-mut	77	56	43	28	no	no	unmut	ND	no
CLL-28	wt	81	70	60	52	no	no	unmut	no	no
CLL-29	wt	74	62	51	47	no	no	mut	no	no
CLL-30	wt	83	86	85	80	no	no	mut	ND	yes
CLL-31	wt	75	60	50	44	no	no	mut	ND	no
CLL-32	*TP53*-mut	99	86	71	62	no	yes	unmut	no	no
CLL-33	wt	86	82	74	67	no	no	unmut	yes	yes
CLL-34	*ATM*-mut	75	64	53	42	no	no	mut	yes	no
CLL-35	*ATM*-mut	57	51	38	31	no	no	unmut	no	no
CLL-36	wt	51	46	43	36	no	no	mut	ND	yes
CLL-37	wt	86	77	74	67	no	no	mut	yes	no
CLL-38	*ATM*-mut	96	91	85	85	no	no	unmut	yes	yes
CLL-39	wt	65	56	46	32	no	no	mut	ND	no
CLL-40	wt	88	75	64	56	no	no	unmut	ND	no
CLL-41	wt	62	57	47	39	no	yes	unmut	no	no
CLL-42	*TP53*-mut	55	43	31	32	ND	ND	unmut	yes	yes
CLL-43	*ATM*-mut	72	59	38	29	no	no	unmut	ND	no
CLL-44	wt	84	65	60	50	no	no	mut	no	yes
CLL-45	wt	81	67	54	45	yes	no	unmut	ND	no
CLL-46	*TP53*-mut	77	72	62	54	yes	no	mut	ND	yes
CLL-47	wt	80	27	20	14	ND	yes	unmut	ND	yes
CLL-48	wt	69	59	48	41	ND	ND	unmut	ND	yes
CLL-49	*TP53*-mut	91	83	73	60	ND	ND	unmut	ND	yes
CLL-50	wt	74	60	51	51	no	no	unmut	no	no
CLL-51	wt	82	69	58	46	no	yes	unmut	no	yes
CLL-52	*ATM*-mut	76	57	56	56	yes	no	mut	no	no
CLL-53	wt	79	69	63	50	no	no	mut	no	yes
CLL-54	*TP53*-mut	47	30	16	10	no	yes	unmut	yes	no

Sample ID	Status	c (MU380) [nM]				SF3B1 mutation	NOTCH1 mutation	IGHV status	Complex karyotype	Therapy
		100	200	300	400					
CLL-55	*TP53*-mut	28	18	19	13	no	no	unmut	yes	no
CLL-56	wt	76	65	60	62	yes	no	unmut	no	no
CLL-57	*ATM*-mut	80	66	54	38	no	no	unmut	no	no
CLL-58	*ATM*-mut	80	64	56	44	yes	no	unmut	ND	yes
CLL-59	11q-	96	96	66	35	ND	ND	unmut	no	no
CLL-60	wt	58	43	28	25	no	no	unmut	ND	no
CLL-61	11q-	47	37	31	26	no	no	unmut	no	no
CLL-62	*ATM*-mut	60	52	44	32	yes	no	unmut	no	no
CLL-63	11q-	91	73	58	46	no	no	unmut	no	no
CLL-64	*ATM*-mut	93	81	74	60	no	yes	unmut	yes	no
CLL-65	wt	78	60	53	47	no	yes	unmut	ND	yes
CLL-66	wt	94	86	79	67	no	no	unmut	no	no
CLL-67	11q-	89	79	69	58	no	no	unmut	ND	no
CLL-68	*TP53*-mut	50	32	31	24	yes	no	unmut	ND	yes
CLL-69	*TP53*-mut	82	76	67	64	no	yes	mut	ND	yes
CLL-70	*ATM*-mut	76	63	51	42	no	no	unmut	yes	yes
CLL-71	*TP53*-mut	92	84	72	58	no	no	unmut	yes	yes
CLL-72	wt	72	68	58	46	yes	no	mut	no	no
CLL-73	*TP53*-mut	81	64	51	37	no	no	unmut	yes	no
CLL-74	wt	73	71	67	61	no	no	mut	ND	no
CLL-75	*ATM*-mut	98	92	93	90	no	no	unmut	yes	no
CLL-76	*TP53*-mut	100	82	79	68	no	no	unmut	yes	no
CLL-77	11q-	84	74	64	54	yes	ND	unmut	ND	no
CLL-78	*TP53*-mut	81	80	79	74	yes	no	unmut	yes	no
CLL-79	*TP53*-mut	100	81	55	46	no	no	unmut	yes	no
CLL-80	11q-	47	46	26	15	yes	no	unmut	ND	no
CLL-81	11q-	86	70	63	53	no	no	unmut	yes	no
CLL-82	*TP53*-mut	72	60	50	42	yes	no	mut	no	yes
CLL-83	wt	69	51	42	33	no	yes	unmut	no	no
CLL-84	11q-	80	54	48	42	no	no	unmut	no	no
CLL-85	*ATM*-mut	63	49	34	25	no	no	unmut	ND	yes
CLL-86	*TP53*-mut	73	51	37	28	yes	ND	unmut	yes	yes
CLL-87	11q-	82	58	46	33	no	yes	unmut	no	no
CLL-88	*TP53*-mut	73	63	46	33	ND	ND	unmut	yes	no
CLL-89	*ATM*-mut	73	67	63	58	no	no	unmut	yes	yes
CLL-90	wt	71	58	48	31	yes	no	mut	ND	yes
CLL-91	11q-	81	71	63	49	no	no	unmut	ND	yes
CLL-92	wt	44	30	17	10	no	no	unmut	ND	no
CLL-93	wt	85	81	67	56	no	no	unmut	yes	no
CLL-94	wt	96	74	63	49	yes	no	unmut	no	no
CLL-95	*ATM*-mut	79	70	60	36	no	yes	unmut	ND	yes
CLL-96	*TP53*-mut	59	47	37	27	ND	ND	unmut	yes	no
CLL-97	*ATM*-mut	ND				yes	no	unmut	yes	yes
CLL-98	*TP53*-mut	ND				ND	ND	mut	ND	ND
CLL-99	*TP53*-mut	ND				ND	ND	unmut	ND	ND
CLL-100	*TP53*-mut	ND				ND	ND	unmut	yes	yes
CLL-101	*TP53*-mut	ND				ND	ND	unmut	yes	yes
CLL-102	wt	ND				no	no	mut	ND	no
CLL-103	11q-	ND				no	no	unmut	ND	no

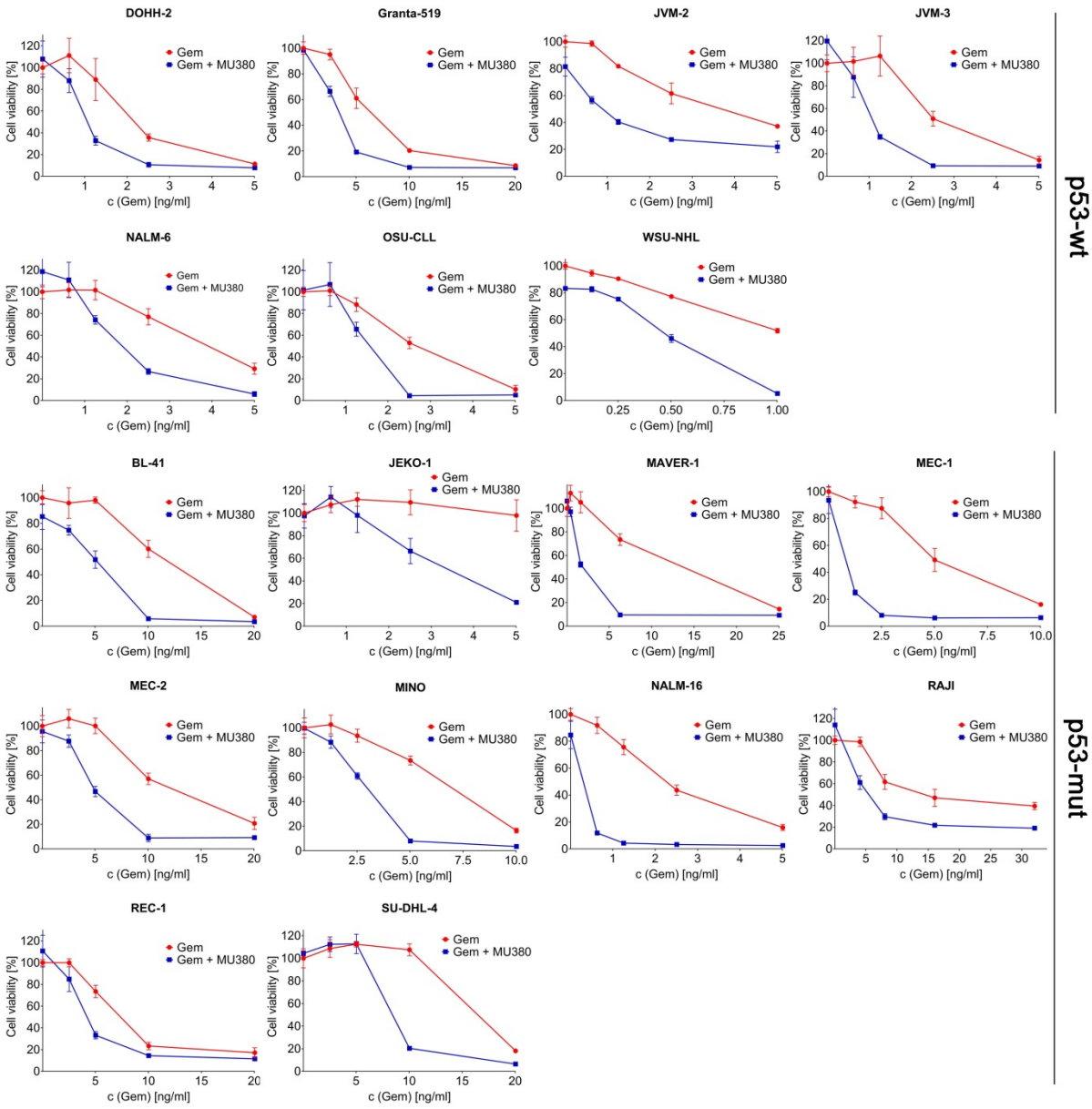
The samples CLL-1 to CLL-96 were used in the viability testing of non-stimulated CLL cultures; the final viability related to untreated control is listed; in all figures, tables and text of the manuscript, samples´ ID correspond to this Table S3. *TP53*-mut: mutation in the *TP53*

gene regadless of other factors; *ATM*-mut: mutation in the *ATM* gene, *TP53*-wt; 11q-: samples with sole deletion 11q (the other *ATM*-allele wt, *TP53*-wt); wt: samples *ATM*-wt/*TP53*-wt. Column “Therapy”, no: samples from untreated patients; yes: samples from previously treated patients. NA: not applicable; ND: not determined.

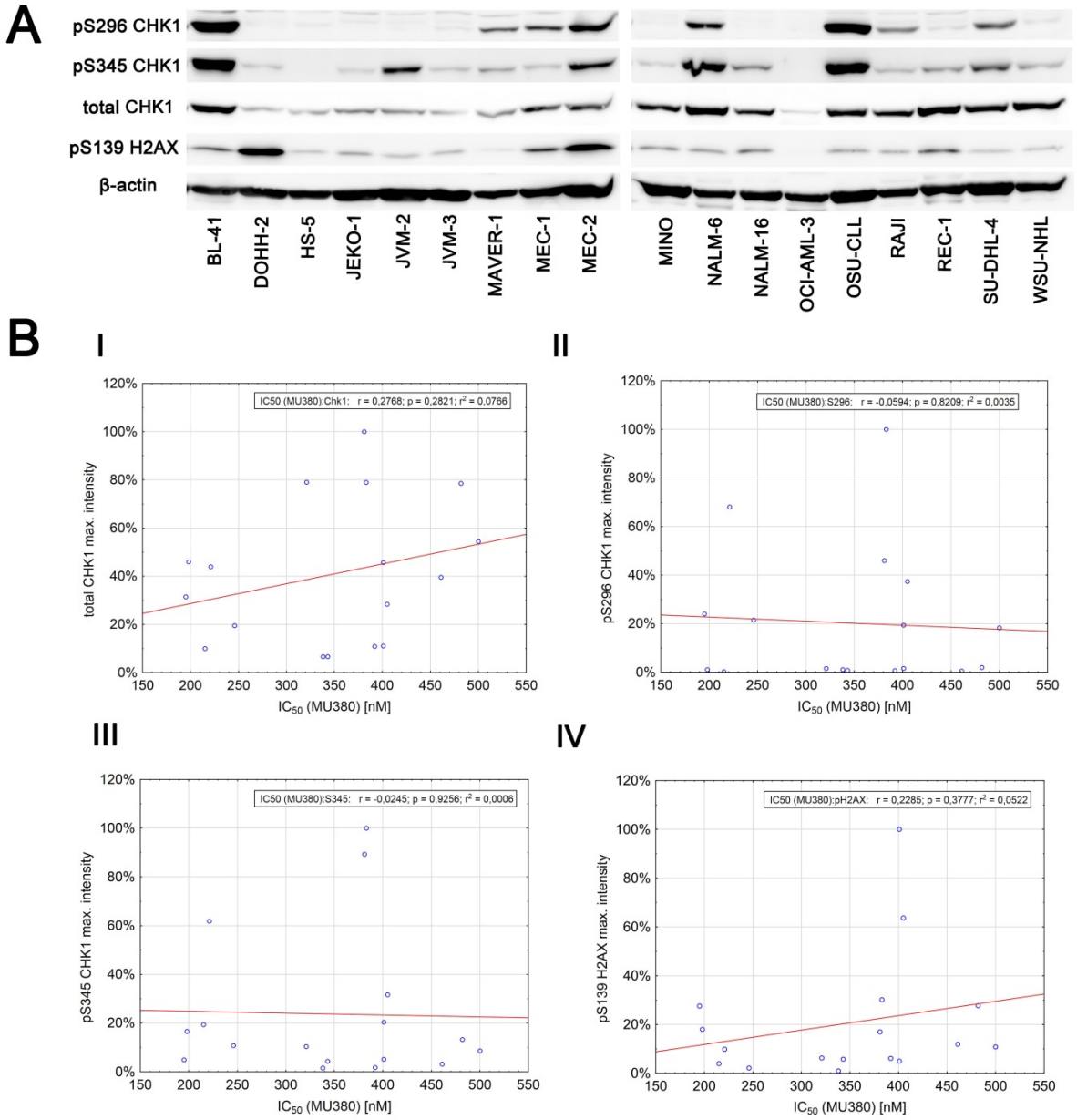
Supplementary Figures



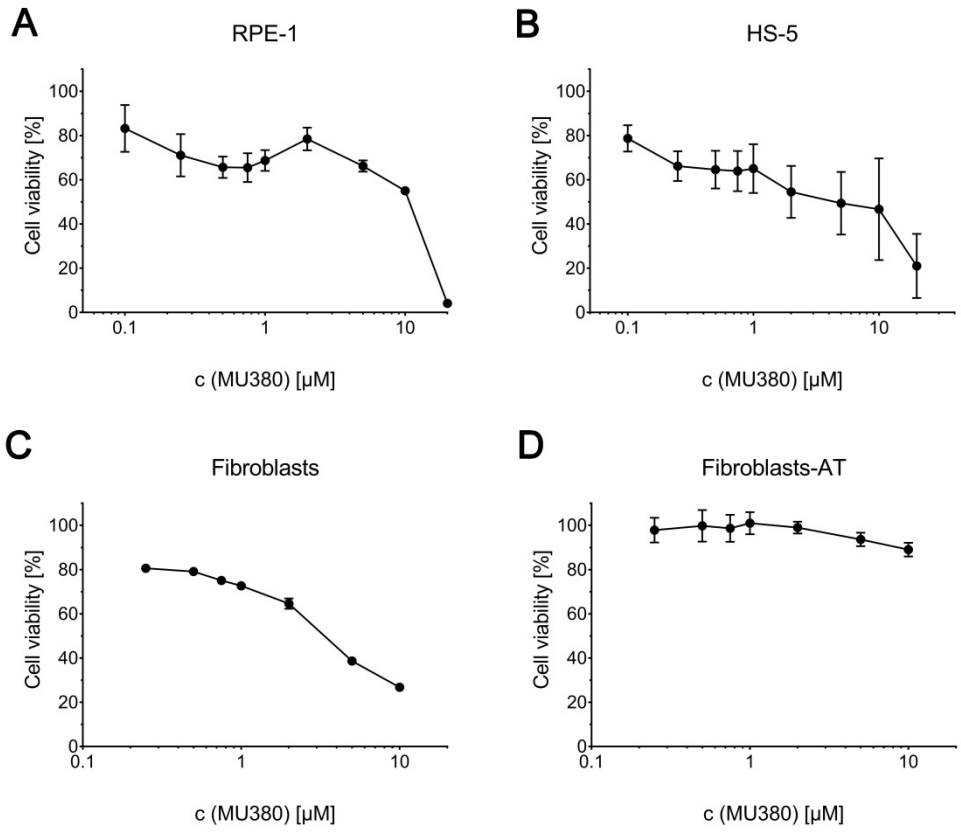
Supplementary Figure S1. Data adopted from DepMap database (The Cancer Dependency Map Project at Broad Institute). (A) Dependency score of genes involved in essential ATR-CHK1 and nonessential ATM-p53-CHK2 pathways. (B) Dependency score of our tested cell lines. Dependency score: a lower score means that the gene is more likely to be dependent for survival in a given cell line. A score of 0 is equivalent to a gene that is not essential whereas a score of -1 corresponds to the median of all common essential genes.



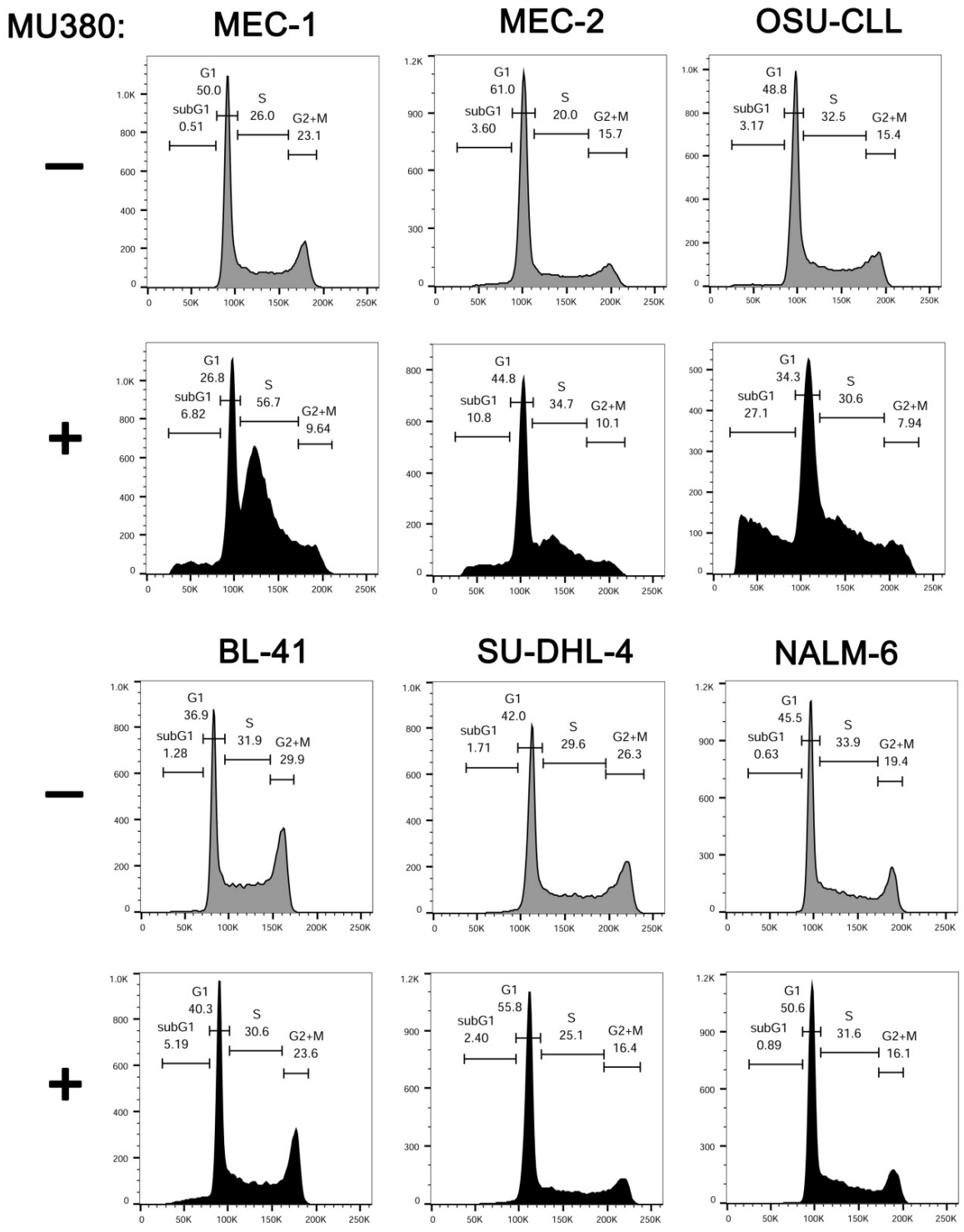
Supplementary Figure S2. MU380 shows synergy with gemcitabine in lymphoid cancer cell lines. The leukemia and lymphoma cell lines were treated for 72 h with the indicated concentrations of gemcitabine (Gem) +/- 100 nM MU380. Cell viability was measured by WST-1 assay. Synergy between gemcitabine and MU380 was evaluated using Chou-Talalay test; the results are summarized in Table 1 and Supplementary Table S1.



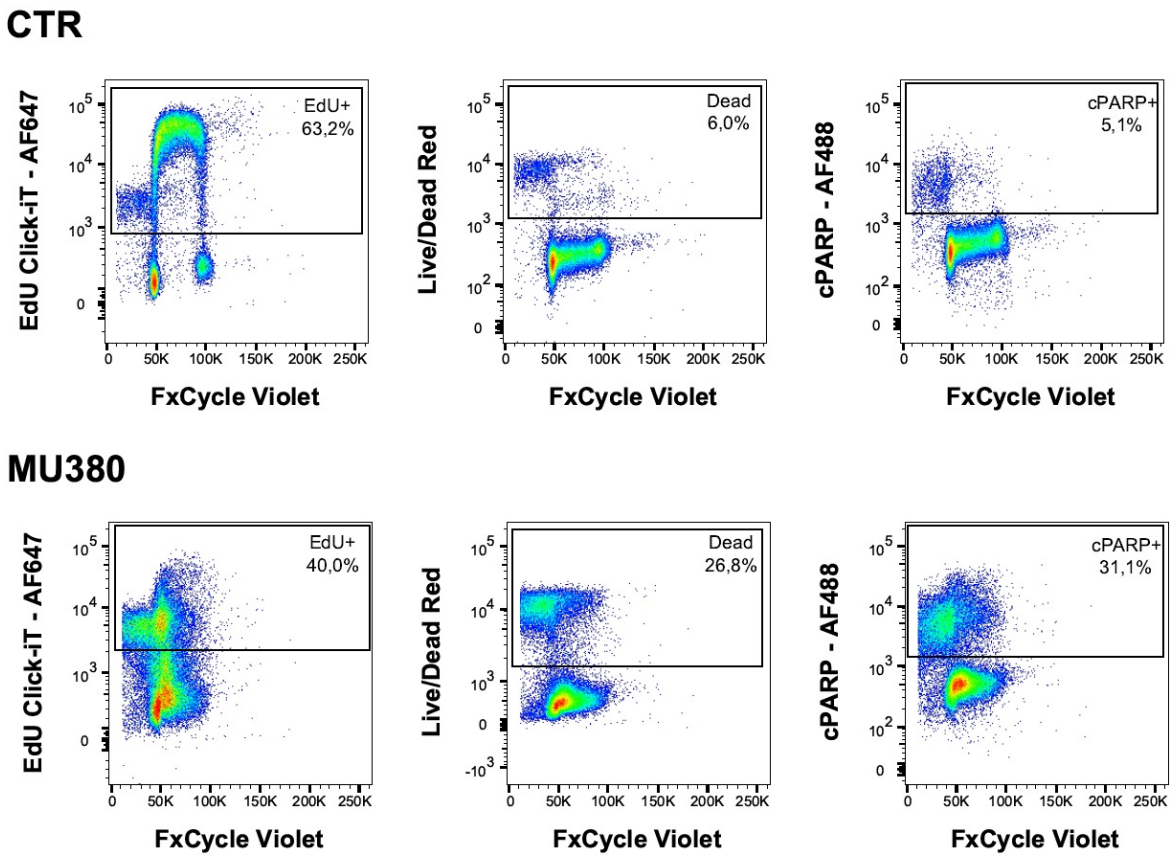
Supplementary Figure S3. Baseline replication stress in the leukemia and lymphoma cell lines. (A) Baseline level of CHK1 isoforms and γH2AX. (B) Correlation to MU380 effects. (I) Total CHK1. (II) pS296 CHK1. (III) pS345 CHK1. (IV) pS139 H2AX. No studied factor correlated with the viability decrease observed after treatment with MU380.



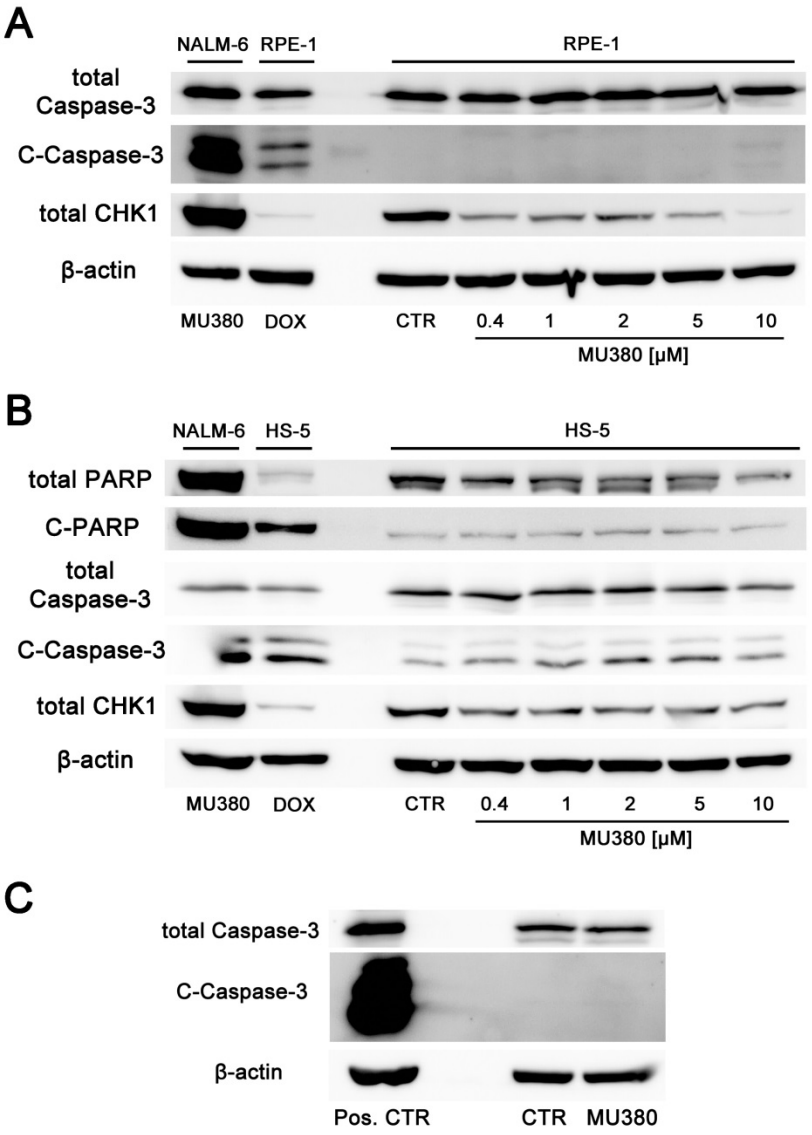
Supplementary Figure S4. Effects of MU380 on viability of non-cancerous human cells. The cells were treated with indicated concentrations of the inhibitor for 72 h and viability was measured by WST-1 reagent. (A) RPE-1 cell line (immortalized epithelial cells; IC_{50} > 10 µM). (B) HS-5 cell line (immortalized bone marrow/stromal fibroblasts; IC_{50} = 3.7 µM). (C) Primary skin fibroblasts (IC_{50} = 3.7 µM). (D) Primary skin fibroblasts from the patient with *ataxia-telangiectasia* harboring complete *ATM* gene inactivation (IC_{50} > 10 µM). Origin and characterization of the cell lines is provided in Supplemental Methods.



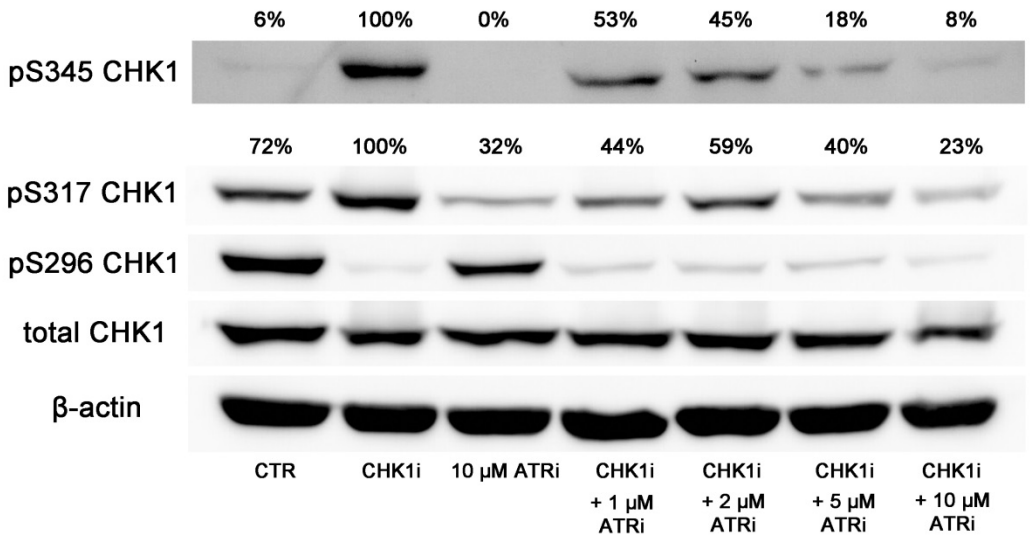
Supplementary Figure S5. Histograms from the flow-cytometry cell cycle analysis. Treatment time: 24 h; MU380 concentration: 400 nM.



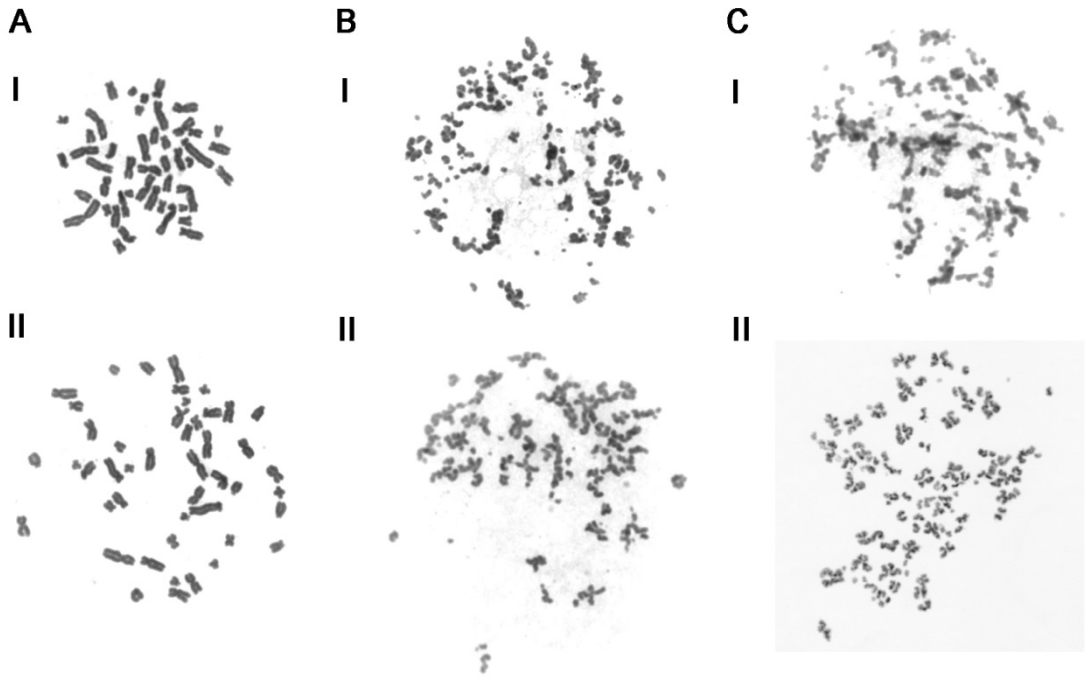
Supplementary Figure S6. Flow-cytometry analysis of DNA synthesis rate and apoptosis induction in MEC-1 cell line. (A) Cells treated with MU380 (400 nM; 24 h) exhibited significantly reduced DNA synthesis rate compared to control untreated cells (lower EdU incorporation, $P = 0.001$) and consequently manifested extensive apoptosis as evidenced by the PARP protein cleavage ($P = 0.001$). The cell death was also confirmed using labelling with Live/Dead Red agent ($P = 0.002$).



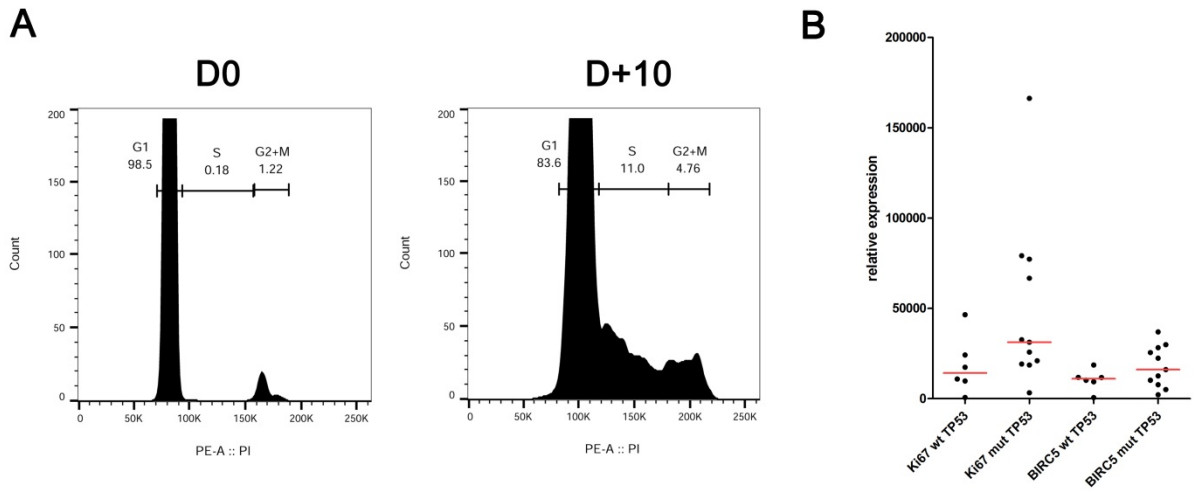
Supplementary Figure S7. MU380 does not induce apoptosis in non-cancerous human cells. Treatment time: 48 h. (A) Human epithelial cell line RPE-1. The rise of cleaved caspase-3 (C-Caspase-3) is negligible compared to untreated control. Positive control: RPE-1 treated with doxorubicin (DOX; 1 μg/ml); NALM-6: additional positive control for western blotting (treated with 400 nM MU380). (B) Stromal/bone marrow fibroblast cell line HS-5. The rise of cleaved PARP (C-PARP) and C-Caspase-3 is negligible compared to the untreated control. The positive controls are similar to Figure A. (C) Primary skin fibroblasts. MU380 concentration: 400 nM. Positive control: MEC-1 cells treated with STAT-3 inhibitor WP1066 (5 μM; 4 h).



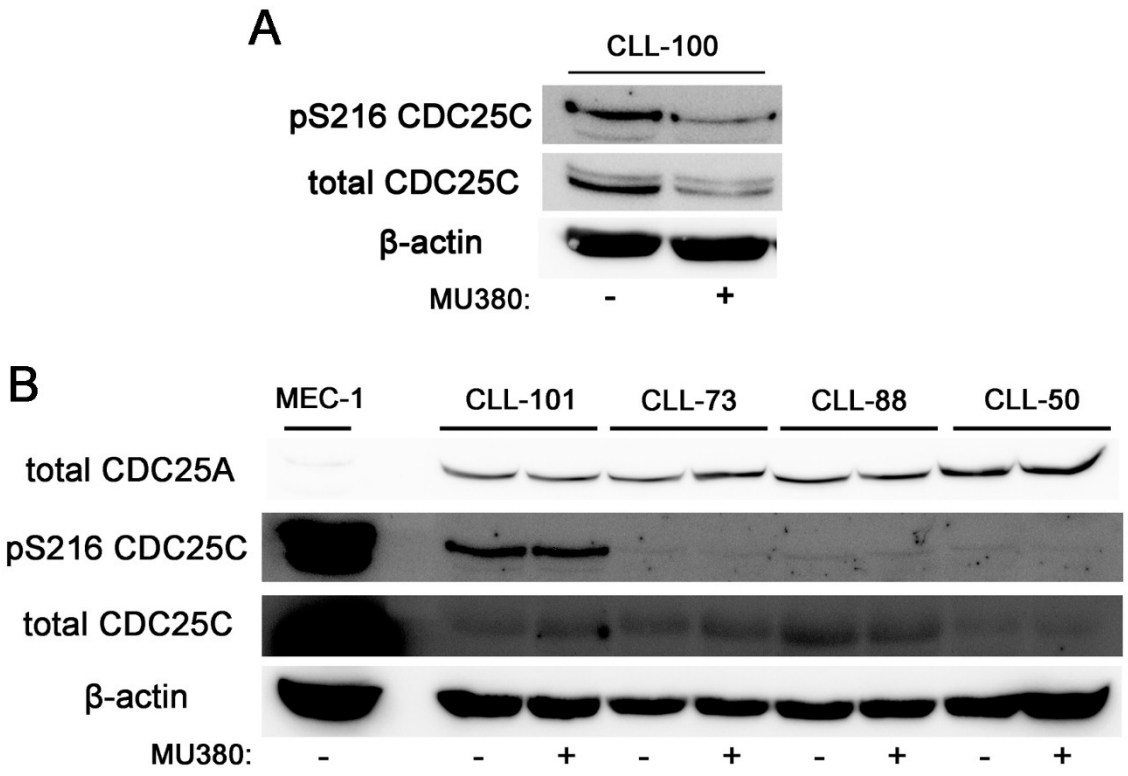
Supplementary Figure S8. Inhibitor VE-821 suppresses ATR-mediated signaling towards CHK1 protein in MEC-1 cell line. Cells were treated for 6 h with 400 nM MU380 (CHK1i) and indicated concentrations of VE-821 (ATRi). ATR-mediated phosphorylations pS345 and pS317 of the CHK1 protein were reduced by VE-821 in a concentration-dependent manner; the values (%) are related to the sole CHK1 inhibition when the signalling from ATR towards the CHK1 is at maximum. Densitometric analysis was done using ImageJ software.



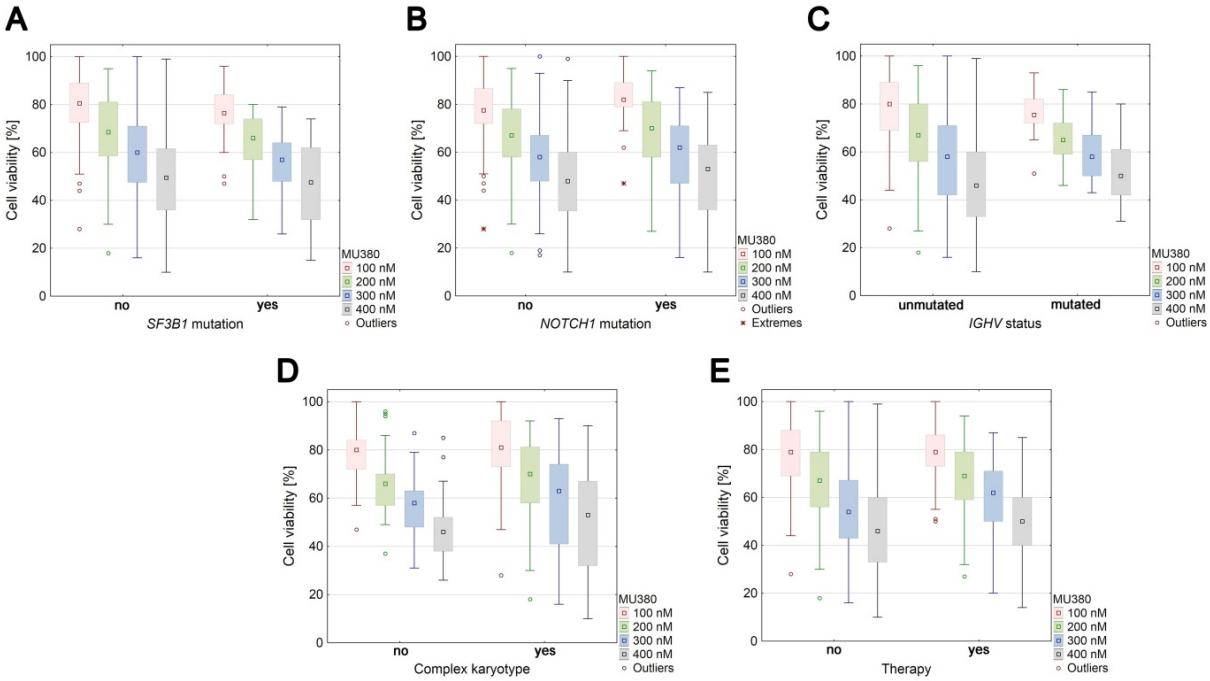
Supplementary Figure S9. Cytogenetic analysis of mitotic chromosomes in MEC-1 cell line. (A) Control untreated cells. Two mitoses (I and II) are shown. Intact chromosomes were present in all analyzed mitoses. (B) 400 nM MU380. (C) 400 nM MU380 + 2 μM VE-821. Both types of treatments resulted in the chromosome breaks or complete chromosome pulverization (shown in the figures).



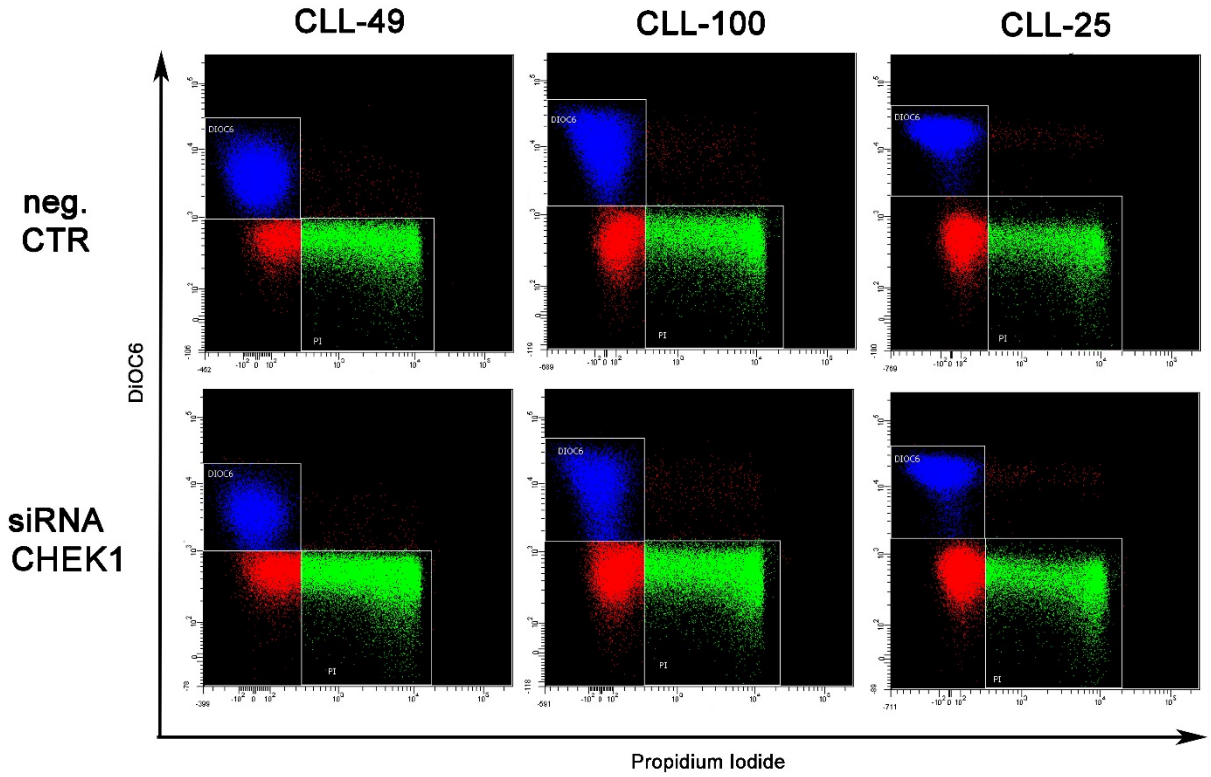
Supplementary Figure S10. Pro-proliferative stimulation of CLL cells. (A) DNA content analysis. Non-stimulated primary CLL cells (D0) (sample CLL-8) are arrested in G_0/G_1 phase of the cell cycle. The 10-day co-culture involving pro-proliferative stimuli (D+10) (see Methods) resulted in cell cycle entry in a proportion of the cells. (B) Real time PCR analysis of proliferation markers. Graph shows relative expression of *MKi67* and *BIRC5* genes (encoding proteins Ki67 and survivin, respectively) in stimulated cultures (samples withdrawn at D+7) compared to the non-stimulated counterparts (D0). *MKi67* expression was stimulated more distinctively in *TP53*-mutated samples compared to *TP53*-wt ones ($P = 0.063$; Mann-Whitney test).



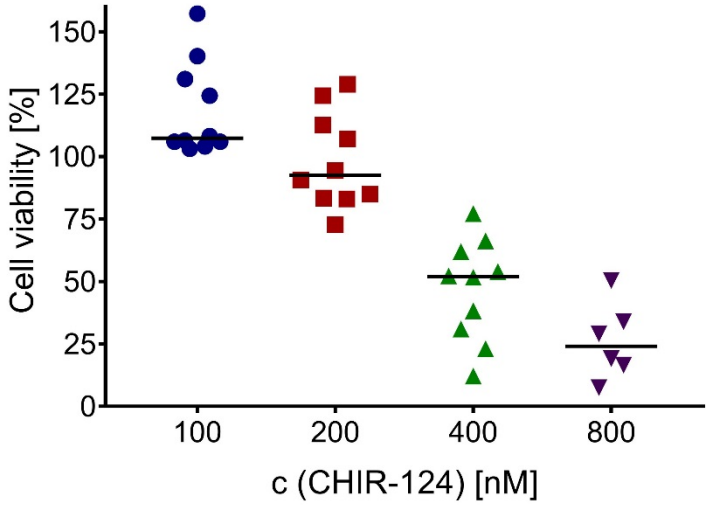
Supplementary Figure S11. Level of CDC25A and CDC25C in CLL cells. (A) Decrease of total and phosphorylated level of CDC25C in a CLL sample. (B) Variable levels of CDC25A and CDC25C in other CLL samples.



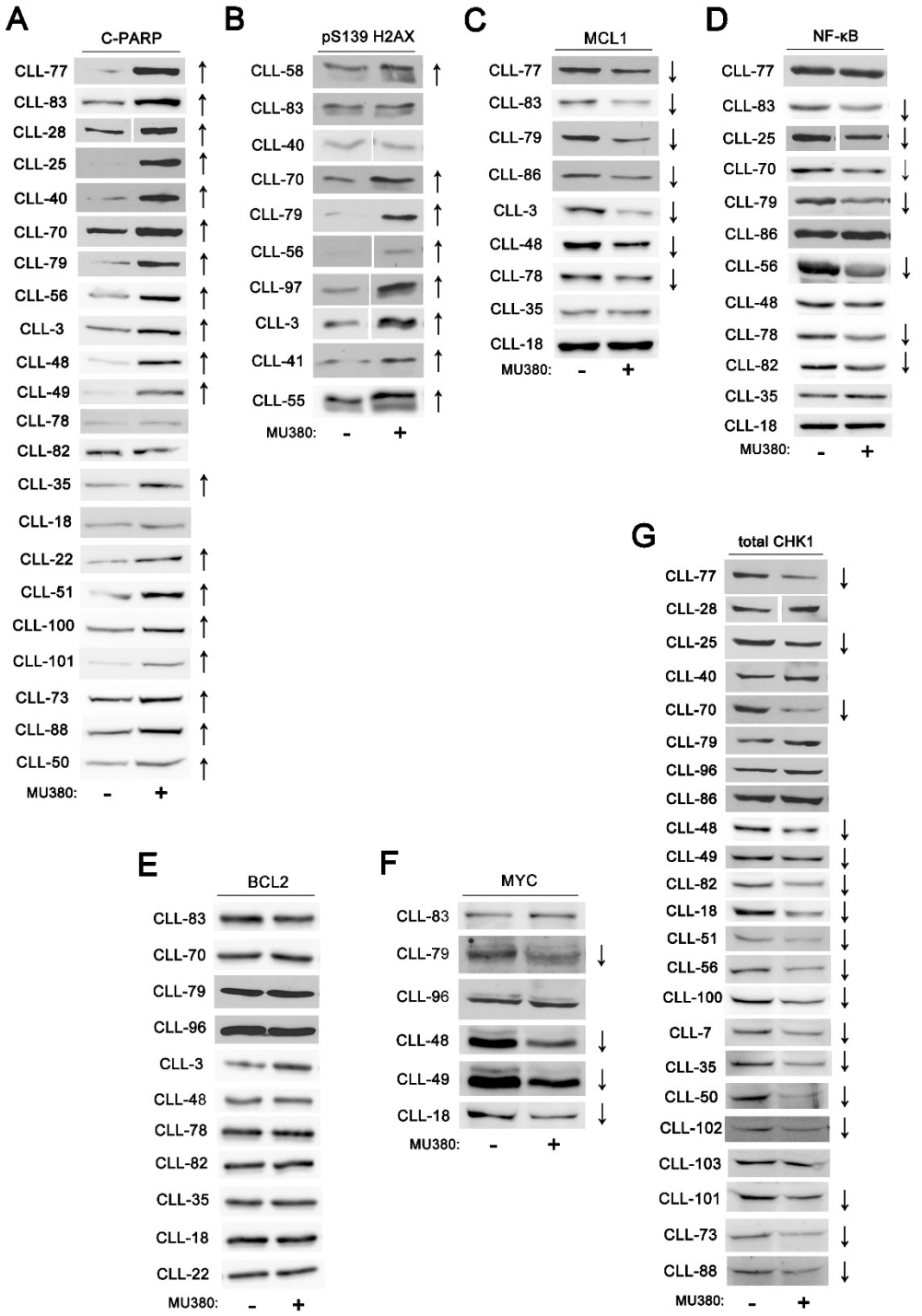
Supplementary Figure S12. Effect of MU380 on viability of non-stimulated CLL cells stratified according to additional categories. (A) *SF3B1* mutations (P = 0.191). (B) *NOTCH1* mutations (P = 0.387). (C) *IGHV* status (P = 0.506). (D) Complex karyotype (P = 0.566). (E) Therapy status (P = 0.145).



Supplementary Figure S13. The effect of transfection on viability of CLL cells. CLL cells were transfected with siRNA targeting *CHEK1*, or siRNA negative control (neg. CTR) and analysed after 48 h incubation. The percentage of viable cells was determined by gating on PI-negative and DiOC6-positive cells.



Supplementary Figure S14. Impact of structurally different CHK1 inhibitor CHIR-124 on viability of non-stimulated CLL cultures. Treatment time: 72 h; concentration range: 100-800 nM. Cell viability was measured by WST-1 reagent. CHIR-124 was tested in 10 cultures with the following genotypes: ATM-wt/TP53-wt (n = 5), TP53-mutation (n = 4) and ATM-mutation (n = 1).



Supplementary Figure S15. Effect of MU380 on the level of selected proteins. Treatment time: 24 h; MU380 concentration: 400 nM. (A) Cleaved PARP. (B) γH2AX. (C) MCL1. (D) NF-κB. (E) BCL2. (F) MYC. (G) Total CHK1. Arrows mark a shift from a baseline level. The level of β-actin showed equal loading in all samples (data not shown). All paired samples are visualized from the same blot (image); in some cases, these samples were not immediately adjacent to each other and were therefore compiled together.

Appendix – Enantioselective Synthesis of MU380

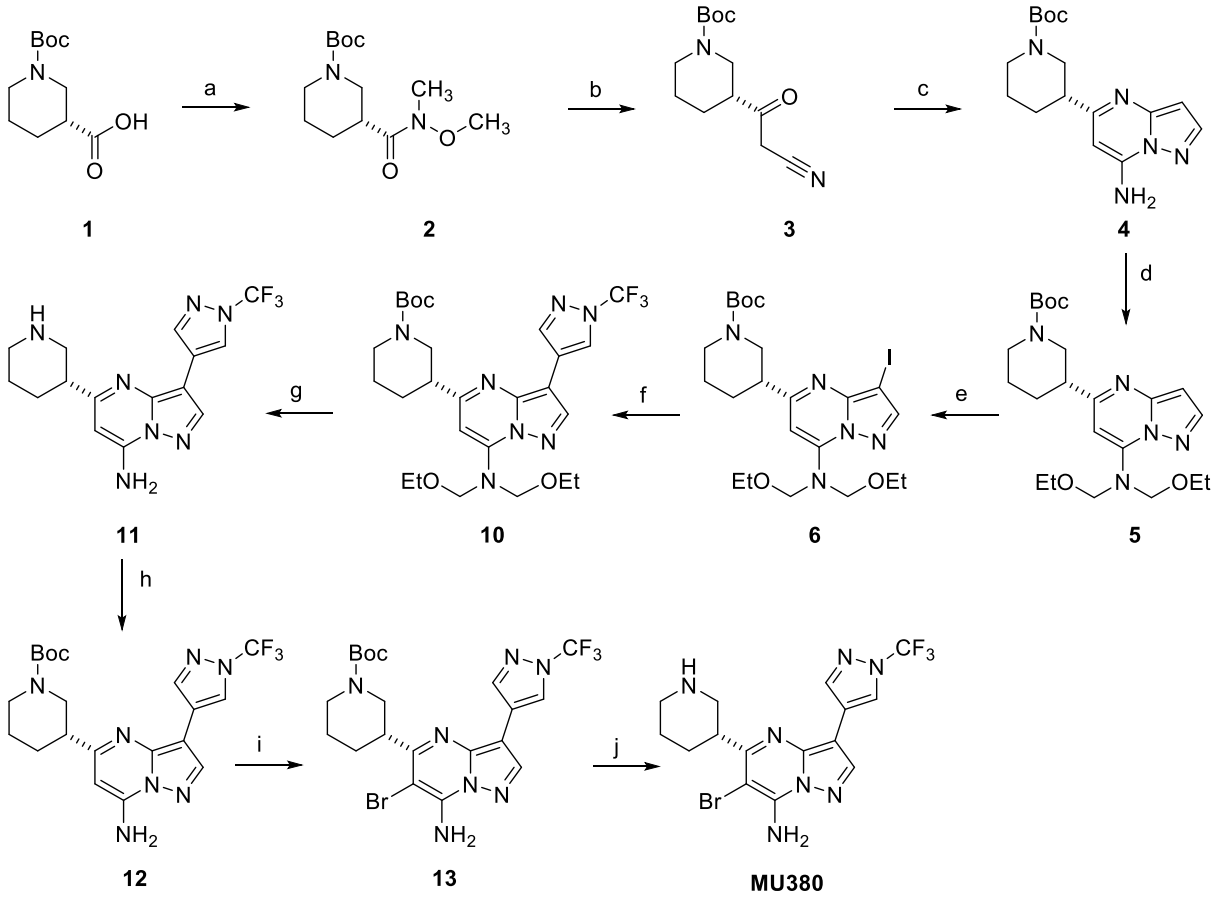
Experimental Section

General

All chemicals were purchased from commercial suppliers and were used without further purification. The solvents were purchased as extra dry and stored over 4Å molecular sieves under nitrogen. Unless noted otherwise, the reactions were carried out in oven-dried glassware under nitrogen atmosphere.

Column chromatography was carried on silica gel (230-400 mesh). TLC plates (silica gel 60 F_{254} or silica gel-RP18 60 F_{254}) were visualized under UV and/or with phosphomolybdic acid, $KMnO_4$, CAM or H_2SO_4 in MeOH. Determination of enantiomeric excess (ee) were performed on HPLC Ultimate 3000 LC Systems (Thermo Scientific) with UV detection or Corona charged aerosol detection (CAD) using analytical columns with chiral stationary phase Chiralpak® AD™, Chiralpak® IA™ and Chiralpak® OJ™.

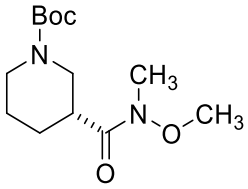
HRMS spectra were measured on Agilent 6224 Accurate-Mass TOF LC-MS with dual electrospray/chemical ionization mode with mass accuracy greater than 2 ppm. NMR spectra were recorded on Bruker Avance 300 MHz and 500 MHz spectrometers. Chemical shifts (δ in ppm) of ^{1}H NMR and ^{13}C NMR were referenced to the residual signals of solvents: $CDCl_3$ [7.26 (^{1}H) and 77.16 (^{13}C)] and DMSO-d_6 [2.50 (^{1}H) and 39.52 (^{13}C)], chemical shifts of ^{19}F to the signal of α,α,α-trifluorotoluene [-63.72]. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and/or multiple resonances), coupling constant (*J*) in Hertz, integration. IR spectra (4000-400 cm^{-1}) were collected on ALPHA ATR spectrometer (BRUKER); solid samples were measured neat and oily samples as films.



1 **2** **3** **4** **5** **6** **10** **11** **12** **13** **MU380**

Reagents and conditions: a) CDI, THF, 0 °C, 1 h then $HN(OCH_3)CH_3 \cdot HCl$, Et_3N, THF, 0 °C to rt, 16 h, 99 %; b) CH_3CN, LiHMDS (1M in THF), THF, -78 °C, 1h then 20 min, 99 %, 99% ee.; c) 3-aminopyrazole, CH_3COOH, 40 °C, 16 h, 95 %, 96% ee.; d) $CH_3CH_2OCH_2Cl$, DIPEA, $(CH_2Cl)_2$, 80 °C, 16h, 84 %; e) NIS, CH_3CN, rt, 16 h, 95 %; f) 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-(trifluoromethyl)-*1H*-pyrazole, K_3PO_4, $Pd(dppf)Cl_2$, DME, H_2O, 100 °C, 3 h, 62 %; g) EtOH, aq. 3 M HCl sol., 80 °C, 3h then 7 M NH_3 in MeOH, rt, 2 h, 80 %. 96% ee.; h) $(Boc)_2O$, Et_3N, CH_2Cl_2, rt, 4 h, 97 %; i) Br_2, *t*-$BuNH_2$, CH_2Cl_2, rt, 16 h, 96 %; j) (i) CF_3COOH, CH_2Cl_2, rt, 2 h then 7.0 M NH_3 in MeOH, rt, 2 h, 97 %, 96% ee.; (ii) recrystallization from CH_3CN, 75 %, 99% ee.

***tert*-butyl (*R*)-3-(methoxy(methyl)carbamoyl)piperidine-1-carboxylate (2):**



1, 1′-Carbonyldiimidazole (3.96 g, 24.33 mmol) was added to a solution of (*R*)-1-(*tert*-butoxycarbonyl)piperidine-3-carboxylic acid (4.65 g, 20.28 mmol) in anhydrous THF (60 mL) and the resulting mixture was stirred at 0 °C for 1 hour. Then, a suspension of *N*, *O*-dimethylhydroxylamine hydrochloride (2.334 g, 23.94 mmol) and triethylamine (3.39 mL, 24.33 mmol) in THF (60 mL) were added at 0 °C and the resulting mixture was stirred at 25 °C for 16 hours. The reaction mixture was quenched with water (300 mL), and extracted with EtOAc (3 × 600 mL). The organic extracts were washed with 0.1M aqueous HCl (300 mL), brine (300 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 75:25) to afford **2** (5.49 g, 99%) as a colorless oil.

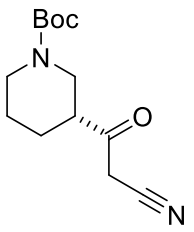
^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.22 – 3.99 (m, 2H), 3.72 (s, 3H), 3.17 (s, 3H), 2.91 – 2.75 (m, 2H), 2.70 (t, J = 12.4 Hz, 1H), 1.91 (d, J = 13.5 Hz, 1H), 1.75 – 1.60 (m, 2H), 1.53 – 1.46 (m, 1H), 1.45 (s, 9H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 174.0, 154.8, 79.6, 61.8, 46.4, 44.3, 38.7, 32.3, 28.6, 27.5, 24.7 ppm.

HRMS (APCI-TOF) m/z: calculated for $C_{13}H_{25}N_2O_4$ $[M+H]^+$: 273.1809, found 273.1809.

IR ($\tilde{\nu}_{max,}$ neat) = 2971, 2936, 2865, 1688, 1656, 1466, 1411, 1389, 1364, 1254, 1237, 1175, 1143, 986, 942, 886, 856, 818, 799, 768, 62, 534 cm^{-1}.

***tert*-butyl (*R*)-3-(2-cyanoacetyl)piperidine-1-carboxylate (3):**



CH_3CN (2.106 mL, 40.29 mmol) was added dropwise to a solution of LiHMDS (1.0 M in THF, 40.29 mL, 40.29 mmol) in anhydrous THF (60 mL) at -78 °C and the resulting mixture was stirred for 1 hour. Then, a solution of **2** (5.49 g, 20.16 mmol) in THF (15 mL) was added dropwise over 15 minutes at -78 °C and the resulting mixture was stirred for 5 minutes at -78 °C. The reaction mixture was quenched with saturated NH_4Cl (300 mL) at -78 °C, and extracted with Et_2O (2 × 600 mL). The organic extracts were washed with brine (300 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 60:40) to afford **3** (5.04 g, 99%) as a pale yellow oil with 99% ee.

^1H NMR (500 MHz, $CDCl_3$): δ = 3.96 – 3.82 (m, 1H), 3.80 – 3.48 (m, 3H), 3.40 – 2.86 (m, 2H), 2.74 – 2.63 (m, 1H), 1.98 – 1.82 (m, 1H), 1.80 – 1.61 (m, 2H), 1.50 – 1.44 (m, 1H), 1.42 (s, 9H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 198.5, 154.6, 113.7, 80.3, 47.6, 44.9, 31.0, 28.4, 26.3, 23.7 ppm.

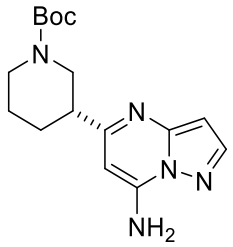
HRMS (APCI-TOF) m/z: calculated for $C_8H_{13}N_2O$ $[M\text{-}Boc + H]^+$: 153.1022, found 153.1021.

IR ($\tilde{\nu}_{max,}$ neat) = 2975, 2935, 2863, 2257, 1722, 1677, 1468, 1420, 1365, 1324, 1293, 1263, 1240, 1142, 1066, 990, 944, 915, 858, 768, 731, 647, 533 cm^{-1}.

Determination of enantiomeric excess (ee) of compound (3):

Column	: Daicel-Chiralpak-IA Analytical column (particle size 4.6 mm × 250 mm)
Injection Volume	: 20 μL (a solution of 2 mg of compound **3** in 1 mL of EtOH)
Flow Rate	: 1 mL/min
Mobile Phase	: n-hexane/EtOH 90 : 10
Wavelength	: 246 nm
Retention times	: Enantiomer - A (10.95 min) + Enantiomer - B (13.00 min, required)

***tert*-butyl (*R*)-3-(7-aminopyrazolo[1,5-a]pyrimidin-5-yl)piperidine-1-carboxylate (4):**



A mixture of **3** (4.05 g, 16.05 mmol), 3-aminopyrazole (1.332 g, 16.05 mmol) and glacial acetic acid (5.4 mL, 94.38 mmol) were stirred at 40 °C for 16 hours. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (CH_2Cl_2/EtOAc = 50:50) to afford **4** (4.80 g, 95 %) as a white solid with 97% ee.

^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.07 (d, J = 2.3 Hz, 1H), 8.01 (brs, 2H), 6.33 (d, J = 2.3 Hz, 1H), 6.05 (s, 1H), 4.08 (brs, 1H), 4.00 – 3.88 (m, 1H), 2.83 – 2.72 (m, 1H), 2.71 – 2.61 (m, 1H), 2.52 – 2.48 (m, 1H), 2.03 – 1.90 (m, 1H), 1.77 – 1.65 (m, 2H), 1.50 – 1.41 (m, 1H), 1.39 (s, 9H) ppm.

^{13}C NMR (126 MHz, DMSO-d_6): δ = 161.4, 153.8, 148.5, 147.3, 144.1, 93.0, 86.0, 78.7, 47.8, 42.9, 29.6, 28.0, 24.6 ppm.

HRMS (APCI-TOF) m/z: calculated for $C_{16}H_{24}N_5O_2$ $[M+H]^+$: 318.1925, found 318.1925.

IR ($\tilde{\nu}_{max,}$ neat) = 3361, 3308, 3218, 3181, 2974, 2931, 1647, 1596, 1561, 1468, 1426, 1365, 1266, 1240, 1144, 1065, 963, 853, 769, 628 cm^{-1}.

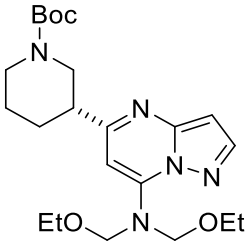
m.p. = 182 °C (dec.)

Determination of enantiomeric excess (ee) of compound (4):

Column	: Daicel-Chiralpak-IA Analytical column (particle size 4.6 mm × 250 mm)
Injection Volume	: 20 μL (a solution of 1 mg of compound **4** in 1 mL of EtOH)
Flow Rate	: 1 mL/min
Mobile Phase	: n-hexane/EtOH 98 : 02 + 0.5% diethylamine

Retention times : Enantiomer - A (26.38 min) + Enantiomer - B (28.87 min, required)

***tert*-butyl (*R*)-3-(7-(bis(ethoxymethyl)amino)pyrazolo[1,5-a]pyrimidin-5-yl)piperidine-1-carboxylate (5):**



Ethoxymethyl chloride (3.99 mL, 42.99 mmol) was added dropwise to a solution of **4** (5.46 g, 17.19 mmol) and DIPEA (9.00 mL, 51.60 mmol) in 1,2-dichloroethane (120 mL) and the mixture was stirred at 70 °C for 16 hours. The reaction mixture was cooled to 25 °C, quenched with water (300 mL), and extracted with CH_2Cl_2 (3 × 600 mL). The organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2/EtOAc = 70:30) to afford **5** (6.264 g, 84%) as a pale yellow wax.

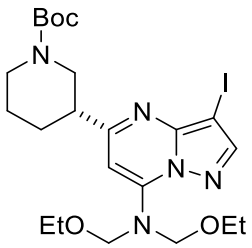
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.02 (d, J = 2.3 Hz, 1H), 6.53 (d, J = 2.4 Hz, 1H), 6.41 (s, 1H), 5.26 (m, 1H), 4.40 – 4.25 (m, 1H), 4.20 – 4.00 (m, 1H), 3.60 (q, J = 7.1 Hz, 4H), 3.16 – 2.94 (m, 1H), 2.88 – 2.70 (m, 1H), 2.13 – 2.05 (m, 1H), 1.90 – 1.72 (m, 2H), 1.64 – 1.54 (m, 1H), 1.47 (s, 9H), 1.20 (t, J = 7.0 Hz, 6H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 163.2, 154.7, 150.8, 148.2, 143.7, 95.1, 94.1, 79.8, 79.4, 63.9, 48.3, 44.3, 30.3, 28.4, 25.0, 15.0 ppm.

HRMS (APCI-TOF) m/z: calculated for $C_{22}H_{36}N_5O_4$ $[M+H]^+$: 434.2762, found 434.2762.

IR ($\tilde{\nu}_{max,}$ neat) = 2974, 2931, 2897, 2867, 1687, 1604, 1542, 1460, 1415, 1390, 1363, 1300, 1289, 1265, 1254, 1238, 1171, 1147, 1065, 1005, 966, 905, 854, 823, 766, 740 cm^{-1}.

***tert*-butyl (*R*)-3-(7-(bis(ethoxymethyl)amino)-3-iodopyrazolo[1,5-a]pyrimidin-5-yl) piperidine-1-carboxylate (6):**



A solution of N-iodosuccinimide (3.15 g, 14.04 mmol) acetonitrile (60 mL) was added dropwise to a solution of **5** (6.09 g, 14.04 mmol) in acetonitrile (60 mL). The reaction mixture was stirred at 25 °C for 16 hours, and then concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 70:30) to afford **6** (7.44 g, 95 %) as a colorless wax.

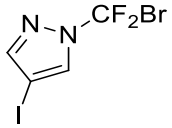
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.01 (s, 1H), 6.45 (s, 1H), 5.25 – 5.19 (m, 4H), 4.45 – 4.22 (m, 1H), 4.22 – 4.00 (m, 1H), 3.57 (q, J = 7.0 Hz, 4H), 3.14 – 3.00 (m, 1H), 2.91 – 2.70 (m, 2H), 2.14 – 2.05 (m, 1H), 1.94 – 1.72 (m, 2H), 1.64 – 1.54 (m, 1H), 1.47 (s, 9H), 1.18 (t, J = 7.1 Hz, 6H), ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 164.9, 155.0, 149.8, 148.6, 147.9, 94.9, 80.1, 79.6, 64.2, 48.2, 44.5, 30.5, 28.6, 25.2, 15.2 ppm.

HRMS (APCI-TOF) m/z: calculated for $C_{22}H_{35}IN_5O_4$ $[M+H]^+$: 560.1728, found 560.1731.

IR ($\tilde{\nu}_{max,}$ neat) = 2925, 2854, 2248, 1732, 1679, 1603, 1541, 1465, 1453, 1421, 1390, 1380, 1365, 1302, 1256, 1171, 1036, 1011, 727, 647 cm^{-1}.

1-(bromodifluoromethyl)-4-iodo-1*H*-pyrazole (7):



500 mL (with a magnetic stirring bar) and 100 mL round-bottom flasks (both fitted with silicon septa) were evacuated and then filled with argon. Then, a solution of 4-iodopyrazole (15.00 g, 77.3 mmol) in 70 mL of anhydrous DMF (Merck) was added to the 500 mL flask.

The solution was cooled to -30 °C (ethanol/dry ice bath) and a solution of NaHMDS in THF (1M, 81.2 mL, 81.2 mmol) was added dropwise over the period of 50 min while the temperature was maintained below -20 °C. During the formation of sodium salt, the solution became green.

This solution was stirred at 0 °C for 15 minutes while 15 mL of anhydrous DMF was added to the 100 mL flask. The solvent was cooled to -30 °C (ethanol/dry ice bath) and CBr_2F_2 (8.48 mL, 92.8 mmol) was added quickly. This solution was then added to a solution of the sodium pyrazole salt dropwise at -10 °C over the period of 30 minutes.

Then, the cooling bath was removed and the mixture was stirred under argon for 18 hours. Then, the mixture was slowly poured into water (200 mL) and extracted with Et_2O (5 × 100 mL). The combined organic extracts were washed with water (100 mL) and brine (100 mL), dried over Na_2SO_4, and filtered. Et_2O ether and THF were evaporated at 30 mbar and the residue (ca. 23 g) was distilled under reduced pressure (20 mbar). The following fractions were collected:

1. 75-95 °C
2. 95-105 °C
3. > 105 °C

Then, the first and second fractions (ca. 13 g) were combined and purified by flash column chromatography (hexane/CH_2Cl_2 = 10:1 to 5:1) to afford the product **7** as a colorless oil (8.80 g, 35%).

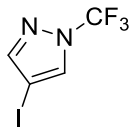
^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.90 (s, 1H), 7.78 (s, 1H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 148.5, 132.1, 109.5 (t, $^1J_{C\text{-}F}$ = 306.3 Hz), 61.3, ppm.

^{19}F NMR (282 MHz, $CDCl_3$): δ = −33.7 (s) ppm.

HRMS (APCI-TOF) m/z: calculated for $C_4H_3BrF_2IN_2$ $[M+H]^+$: 322.8487/324.8468, found 322.8485/324.8465.

4-iodo-1-(trifluoromethyl)-1*H*-pyrazole (8):



A mixture of compound **7** (8.80 g, 27.27 mmol) and AgF (4.50 g, 135.45 mmol) in pentane (100 mL) was stirred in a 150 mL pressure tube at 25 °C for 9 days. Then, additional AgF (1.73 g, 13.63 mmol) was added and the mixture was stirred for additional 5 days. Then,

additional AgF (0.69 g, 5.45 mmol) was added and the mixture was stirred for additional 1 day. The mixture was quickly filtered and the solvent from the filtrate was evaporated at 25 °C under reduced pressure (570 mbar). The product **8** (5.11 g, 71%) was obtained as colorless oil.

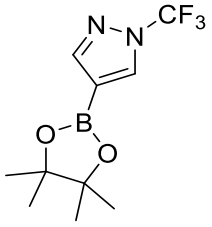
^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.88 (s, 1H), 7.76 (s, 1H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 148.7, 132.6, 117.6 (q, $^{1}J_{C\text{-}F}$ = 264.0 Hz), 60.9 ppm.

^{19}F NMR (282 MHz, $CDCl_3$): δ = −61.6 (s) ppm.

HRMS (APCI-TOF) m/z: calculated for $C_4H_3F_3IN_2$ $[M+H]^+$: 262.9288, found 262.9285.

4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-(trifluoromethyl)-1*H*-pyrazole (9):



A mixture of compound **8** (4.50 g, 17.16 mmol), $Pd(dppf)Cl_2$ (1.257 g, 1.72 mmol), bis(pinacolato)diboron (4.59 g, 18.03 mmol) and potassium acetate (6.75 g, 68.64 mmol) in DMF (60 mL) was stirred in a pressure tube at 90 °C for 18 hours. The mixture was cooled to 25 °C, mixed with water (300 mL), and extracted with Et_2O (2 × 350 mL). The combined organic extracts were washed with brine (2 × 300 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to yield the crude product **9** as a brown solid (5.40 g), which was directly used in the next step without further purification.

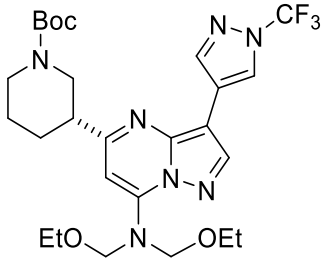
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.10 (s, 1H), 7.95 (s, 1H), 1.31 (s, 12H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 148.8, 135.3, 118.2 (q, $^{1}J_{C\text{-}F}$ = 263.9 Hz), 110.6 (HMBC), 84.2, 25.0 ppm.

^{11}B NMR (160 MHz, $CDCl_3$): δ = 29.22 ppm.

^{19}F NMR (282 MHz, $CDCl_3$): δ = -61.25 (s) ppm.

***tert*-butyl (*R*)-3-(7-(bis(ethoxymethyl)amino)-3-(1-(trifluoromethyl)-1*H*-pyrazol-4-yl) pyrazolo[1,5-a]pyrimidin-5-yl)piperidine-1-carboxylate (10):**



A solution of **6** (7.23 g, 12.90 mmol) in DME/H_2O (150+36 mL) was added to a mixture of 4-(4, 4, 5, 5-tetramethyl-1, 3, 2-dioxaborolan-2-yl)-1-(trifluoromethyl)-1*H*-pyrazole (5.07 g, 19.38 mmol), K_3PO_4 (10.98 g, 51.69 mmol) and Pd(dppf)Cl_2 (0.945 g, 1.29 mmol). The reaction mixture was stirred at 90 °C for 3 hours, then diluted with water (300 mL), and extracted with EtOAc (2 × 900 mL). The combined organic extracts were washed with brine (300 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 70:30) to afford **10** (4.56 g, 62 %) as a light brown solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.37 (s, 1H), 8.29 (s, 1H), 8.21 (s, 1H), 6.48 (s, 1H), 5.38 – 5.18 (m, 4H), 4.46 – 4.28 (m, 1H), 4.26 – 4.07 (m, 1H), 3.61 (q, J = 7.0 Hz, 4H), 3.19 – 2.96 (m, 1H), 2.94 – 2.72 (m, 2H), 2.21 – 2.07 (m, 1H), 1.95 – 1.75 (m, 2H), 1.70 – 1.55 (m, 1H), 1.48 (s, 9H), 1.21 (t, J = 7.0 Hz, 6H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 164.0, 155.0, 148.5, 147.2, 141.7, 140.8, 123.7, 119.5 (q, $^1J_{C\text{-}F}$ = 262.0 Hz), 116.7, 99.8, 94.9, 80.1, 79.8, 64.3, 48.7, 44.3, 30.6, 28.6, 25.2, 15.2 ppm.

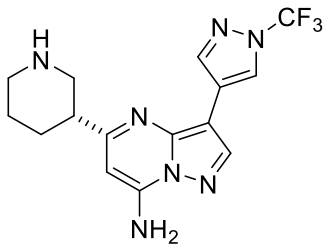
^{19}F NMR (282 MHz, $CDCl_3$): δ = -61.14 (s) ppm.

HRMS (APCI-TOF) m/z: calculated for $[M+H]^+$: 568.2854, found 568.2851.

IR ($\tilde{\nu}_{max,}$ neat) = 3142, 3095, 2972, 2937, 2889, 2859, 1681, 1590, 1542, 1488, 1424, 1410, 1382, 1365, 1308, 1283, 1270, 1221, 1195, 1165, 1142, 1106, 1088, 1074, 1034, 1000, 969, 908, 868, 663, 628, 466 cm^{-1}.

m.p. = 122 – 124 °C

(*R*)-5-(piperidin-3-yl)-3-(1-(trifluoromethyl)-1*H*-pyrazol-4-yl)pyrazolo[1,5-a]pyrimidin-7-amine (11):



Aqueous 3 M HCl (90 mL) was added to a solution of **10** (4.56 g, 3.00 mmol) in ethanol (90 mL) and the reaction mixture was stirred at 80 °C for 3 hours. The solvents were evaporated, the residue was dissolved in 7 M NH_3 in MeOH (90 mL) and the mixture was stirred at 25 °C for 2 hours. The solvents were evaporated under reduced pressure and the residue was purified by flash column chromatography (CH_2Cl_2/7 M NH_3 in MeOH = 90:10) to afford **11** (2.25 g, 80 %) as a white solid with 96% ee.

^{1}H NMR (500 MHz, DMSO-*d6*): δ = 8.70 (s, 1H), 8.55 (s, 1H), 8.48 (s, 1H), 7.74 (s, 2H), 6.07 (s, 1H), 3.24 – 3.15 (m, 1H), 3.05 – 2.96 (m, 1H), 2.83 – 2.72 (m, 2H), 2.63 – 2.55 (m, 1H), 2.07 – 1.97 (m, 1H), 1.81 – 1.67 (m, 2H), 1.61 –1.49 (m, 1H) ppm.

^{13}C NMR (126 MHz, DMSO-*d6*): δ = 164.9, 148.0, 145.3, 141.9, 141.2, 123.4, 119.2 (q, $^1J_{C\text{-}F}$ = 261.0 Hz), 117.4, 97.2, 86.4, 51.7, 46.1, 45.1, 30.3, 26.1 ppm.

^{19}F NMR (282 MHz, DMSO-*d6*): δ = -61.71 (s) ppm.

HRMS (APCI-TOF) m/z: calculated for $C_{15}H_{17}F_3N_7$ $[M+H]^+$: 352.1492, found 352.1492.

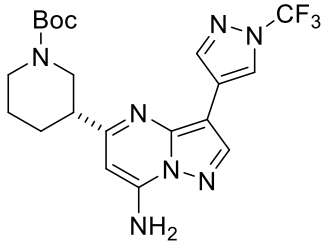
IR ($\tilde{\nu}_{max,}$ neat) = 3336, 3305, 3162, 2957, 2937, 2854, 2827, 1654, 1567, 1483, 1421, 1397, 1316, 1292, 1260, 1209, 1161, 1133, 981, 941, 915, 800, 763, 640, 627, 620, 501 cm^{-1}.

m.p. = 221 – 224 °C

Determination of enantiomeric excess (ee) of compound (11):

Column : Daicel-Chiralpak-AD Analytical column (particle size 4.6 mm×250 mm)
Injection Volume : 20 μL (a solution of 1 mg of compound **11** in 1 mL of IPA)
Flow Rate : 1 mL/min
Mobile Phase : n-hexane/IPA 90 :10+ 0.5% diethylamine
Retention times : Enantiomer - A (16.58 min) + Enantiomer - B (21.90 min, required)

***tert*-butyl (*R*)-3-(7-amino-3-(1-(trifluoromethyl)-1*H*-pyrazol-4-yl)pyrazolo[1,5-a] pyrimidin-5-yl)piperidine-1-carboxylate (12):**



Di-tert-butyl dicarbonate (1.518 g, 6.96 mmol) was added to a mixture of **11** (2.220 g, 6.33 mmol) and triethylamine (4.41 mL, 31.59 mmol) in CH_2Cl_2 (90 mL) and the mixture was stirred at 25 °C for 4 hours. The resulting solution was quenched with aqueous saturated $NaHCO_3$ (150 mL) and the mixture was extracted with CH_2Cl_2 (3 × 600 mL). The organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH = 95:05) to afford **12** (2.760 g, 97 %) as a pale yellow solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.36 (s, 1H), 8.27 (s, 1H), 8.19 (s, 1H), 6.07 (s, 1H), 5.67 (s, 2H), 4.41 – 4.26 (m, 1H), 4.22 – 3.96 (m, 1H), 3.18 – 2.99 (m, 1H), 2.92 – 2.67 (m, 2H), 2.20 – 2.05 (m, 1H), 1.96 – 1.74 (m, 2H), 1.70 – 1.62 (m, 1H), 1.48 (s, 9H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 164.2, 155.1, 146.7, 145.6, 141.6, 141.3, 123.6, 119.5 (q, $^1J_{C\text{-}F}$ = 262.1 Hz), 116.8, 99.7, 87.8, 79.8, 48.7, 44.4, 30.4, 28.6, 25.2 ppm.

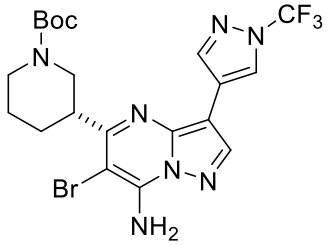
^{19}F NMR (282 MHz, CDCl3): δ = -61.13 (s) ppm.

HRMS (APCI-TOF) m/z: calculated for $C_{20}H_{25}F_3N_7O_2$ $[M+H]^+$: 452.2016, found 452.2015.

IR ($\tilde{\nu}_{max,}$ neat) = 3453, 3312, 3179, 2977, 2934, 2859, 1668, 1645, 1624, 1591, 1563, 1526, 1477, 1417, 1366, 1319, 1288, 1170, 917, 883, 803, 733, 634 cm^{-1}

m.p. = 124 – 127 °C

***tert*-butyl (*R*)-3-(7-amino-6-bromo-3-(1-(trifluoromethyl)-1*H*-pyrazol-4-yl)pyrazolo[1,5-a] pyrimidin-5-yl)piperidine-1-carboxylate (13):**



To a solution of **12** (2.760 g, 6.09 mmol) in CH_2Cl_2 (60 mL) was added *t*-$BuNH_2$ (45 mL). Then, a solution of Br_2 (0.315 mL, 6.09 mmol) in CH_2Cl_2 (15 mL) was added dropwise and the resulting mixture was stirred at 25 °C for 16 hours. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (CH_2Cl_2/MeOH = 97:03) to afford **13** (3.105 g, 96 %) as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.30 (s, 1H), 8.28 (s, 1H), 8.16 (s, 1H), 6.11 (s, 2H), 4.47 – 4.27 (m, 1H), 4.25 – 4.05 (m, 1H), 3.34 – 3.18 (m, 1H), 3.17 – 3.00 (m, 1H), 2.94 – 2.71 (m, 1H), 2.18 – 2.07 (m, 1H), 1.94 – 1.76 (m, 2H), 1.74 – 1.63 (m, 1H), 1.48 (s, 9H) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ = 160.6, 154.9, 144.5, 143.5, 141.6, 141.6, 123.7, 119.5 (q, $^1J_{C\text{-}F}$ = 263.0 Hz), 116.3, 100.4, 85.5, 79.7, 48.1, 44.3, 42.8, 29.8, 28.7, 25.2 ppm.

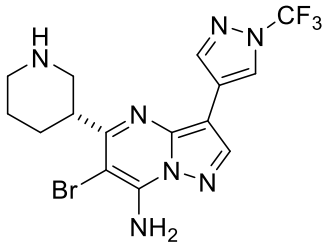
^{19}F NMR (282 MHz, $CDCl_3$): δ = -61.16 (s) ppm.

HRMS (APCI-TOF) m/z: calculated for $C_{20}H_{24}BrF_3N_7O_2$ $[M+H]^+$: 530.1121, found: 530.1119.

IR ($\tilde{\nu}_{max,}$ neat) = 3435, 3297, 3235, 3198, 2976, 2928, 2854, 1676, 1642, 1618, 1588, 1540, 1468, 1417, 1397, 1365, 1317, 1288, 1245, 1174, 1150, 1065, 941, 802, 626 cm^{-1}.

m.p. = 110 – 113 °C

(*R*)-6-bromo-5-(piperidin-3-yl)-3-(1-(trifluoromethyl)-1*H*-pyrazol-4-yl)pyrazolo[1,5-a]pyrimidin-7-amine (MU380):



Trifluoroacetic acid (17.85 mL, 233.16 mmol) was slowly added to a solution of **10** (3.09 g, 5.82 mmol) in CH_2Cl_2 (75 mL). The reaction mixture was stirred at 25 °C for 2 hours, then the solvents were evaporated. The residue was dissolved in 7 M NH_3 in MeOH (45 mL) and the solution was stirred at 25 °C for 2 hours. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (CH_2Cl_2/7 M NH_3 in MeOH = 95:05) to afford **MU380** (2.430 g, 97 %) as a white solid with 96% ee.

Determination of enantiomeric excess (ee) of MU380:

Column : Daicel-Chiralpak-OJ Analytical column (particle size 4.6 mm×250 mm)

Injection Volume : 20 μL (a solution of 1 mg of compound **MU380** in 1 mL of EtOH)

Flow Rate : 1 mL/min

Mobile Phase : n-hexane/EtOH = 95:05+ 0.5% diethylamine

Retention times : Enantiomer - A (30.07 min, required) + Enantiomer - B (35.76 min)

Recrystallization of MU380:

MU380 (2.430 g, 96% ee) in acetonitrile (33 mL) was refluxed for 1 hour, then the solution was allowed to cool to 25 °C and stirred for 1 hour. The precipitated white solid was filtered, washed with acetonitrile (6 mL) and dried under vacuum to afford enantiomerically pure **MU380** (1.815 g, 75%) with >99% ee.

Determination of enantiomeric excess (ee) of MU380:

Column : Daicel-Chiralpak-OJ Analytical column (particle size 4.6 mm×250 mm)
Injection Volume : 20 μL (a solution of 1 mg of compound **MU380** in 1 mL of EtOH)
Flow Rate : 1 mL/min
Mobile Phase : n-hexane/EtOH = 95:05 + 0.5% diethylamine
Retention times : Enantiomer - A (29.82 min, required)

^{1}H NMR (500 MHz, DMSO-*d6*): δ = 8.72 (s, 1H), 8.54 (s, 1H), 8.51 (s, 1H), 3.23 – 3.15 (m, 2H), 3.14 – 3.09 (m, 1H), 2.99 – 2.93 (m, 1H), 2.80 – 2.72 (m, 1H), 2.58 – 2.52 (m, 1H), 2.04 – 1.94 (m, 1H), 1.82 – 1.68 (m, 2H), 1.57 – 1.46 (m, 1H) ppm.

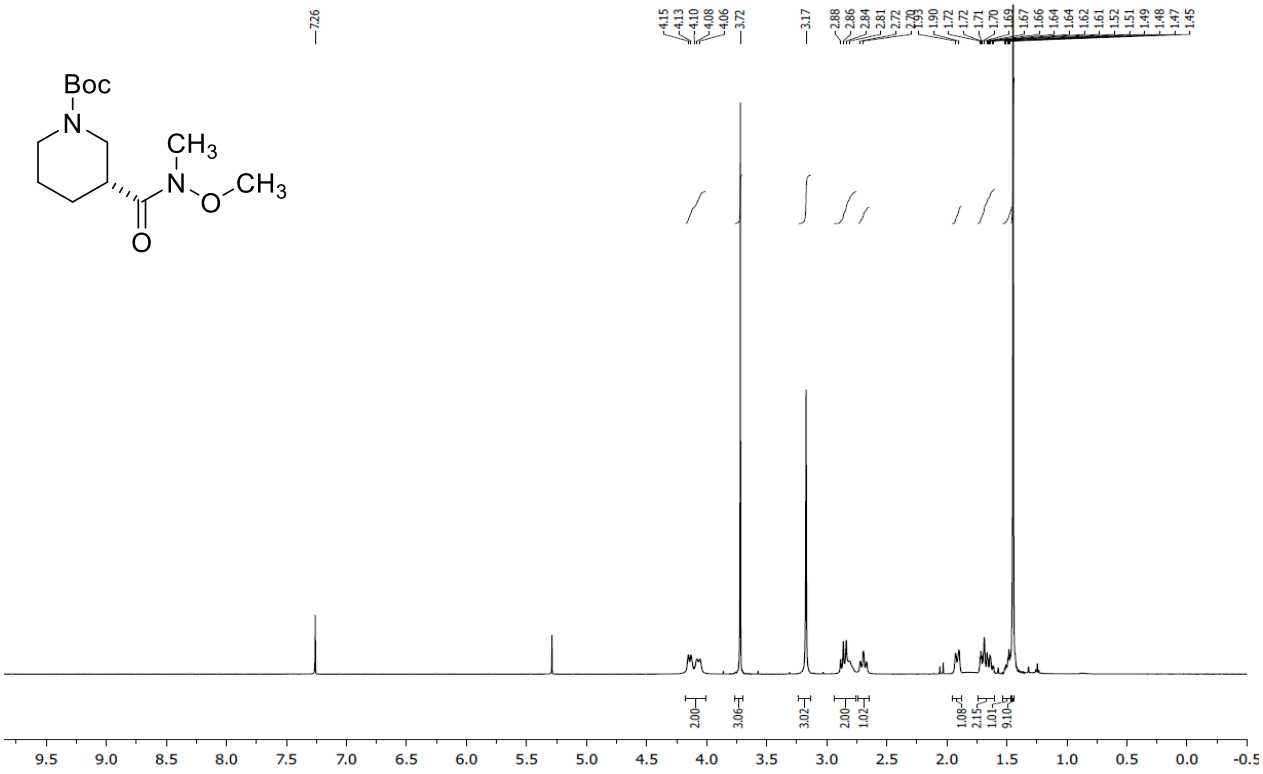
^{13}C NMR (126 MHz, DMSO-*d6*): δ = 161.2, 145.5, 143.2, 141.9, 141.7, 123.8, 119.1 (q, $^{1}J_{C\text{-}F}$ = 261.1 Hz), 116.8, 98.2, 83.9, 50.4, 45.9, 43.4, 29.5, 26.0 ppm.

^{19}F NMR (282 MHz, DMSO-*d6*): δ = -61.76 (s) ppm.

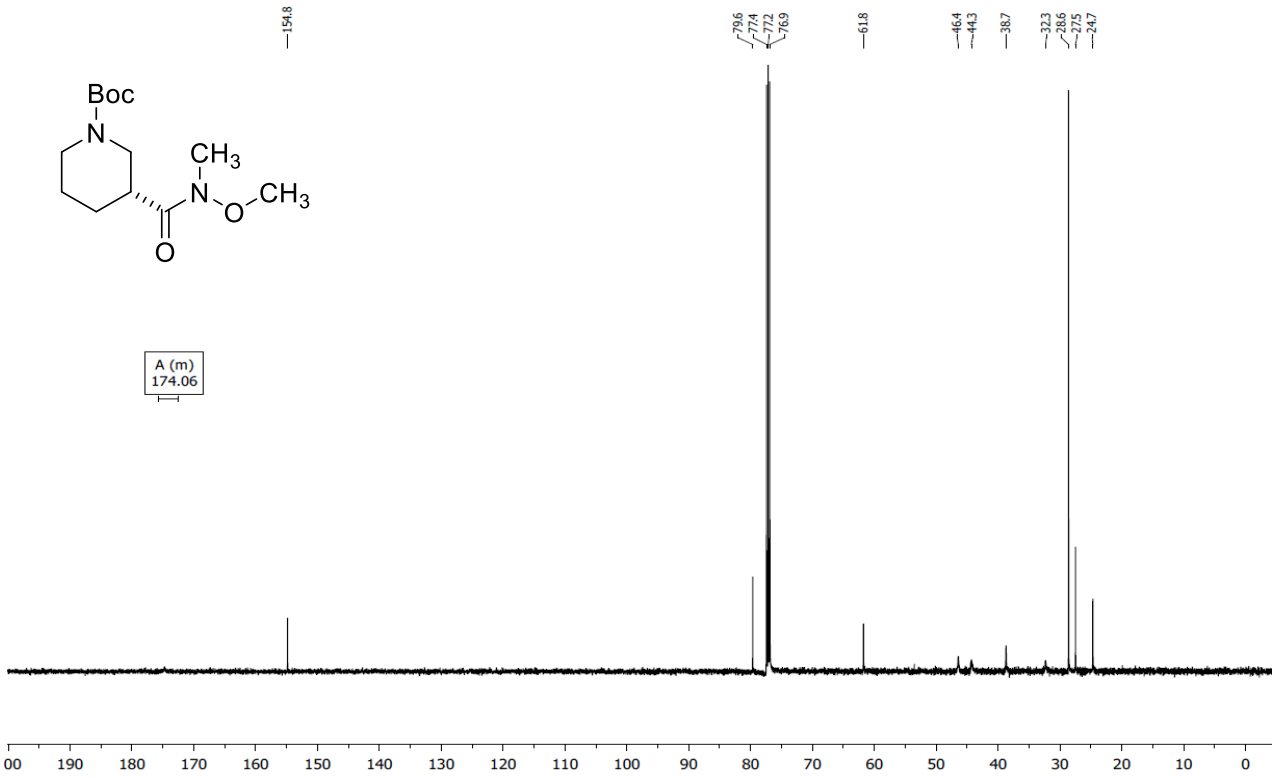
HRMS (APCI-TOF) m/z: calculated for $C_{15}H_{16}BrF_3N_7$ $[M+H]^+$: 430.0597, found 430.0595.

IR ($\tilde{\nu}_{max,}$ neat) = 3292, 3201, 3153, 2935, 2855, 1642, 1628, 1591, 1541, 1419, 1397, 1316, 1289, 1263, 1207, 1169, 1133, 1104, 940, 921, 868, 802, 747, 664, 622, 560 cm^{-1}.

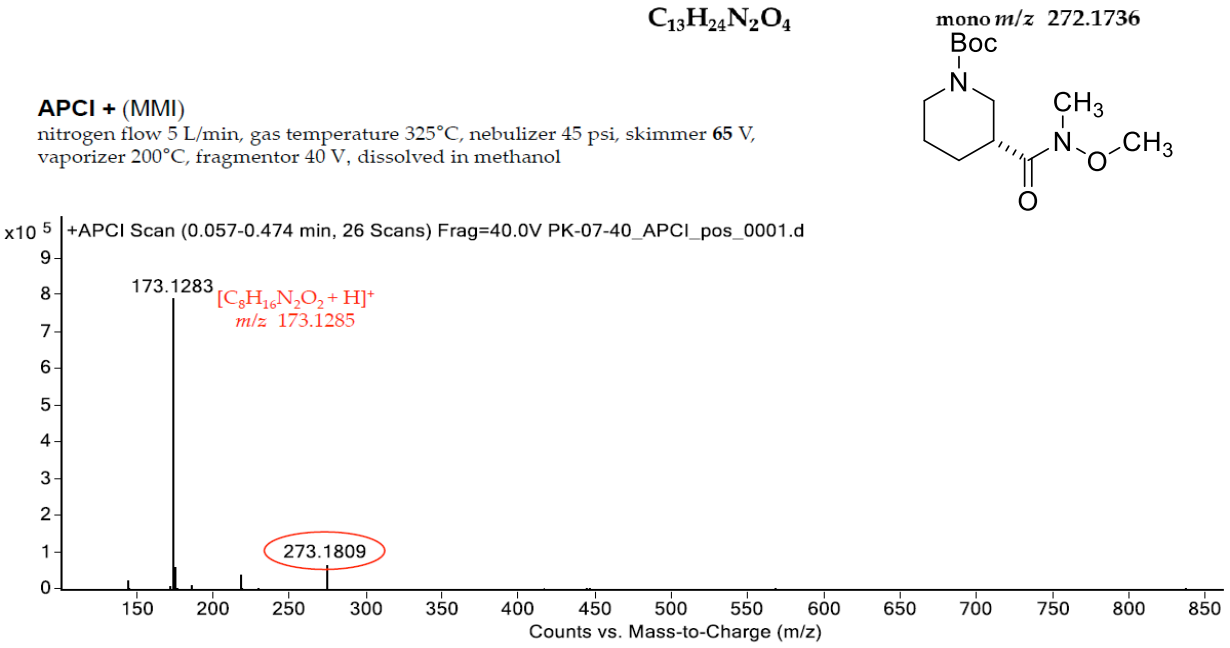
m.p. = 147 – 148 °C



^{1}H NMR (500MHz) spectrum of compound **2** in $CDCl_3$

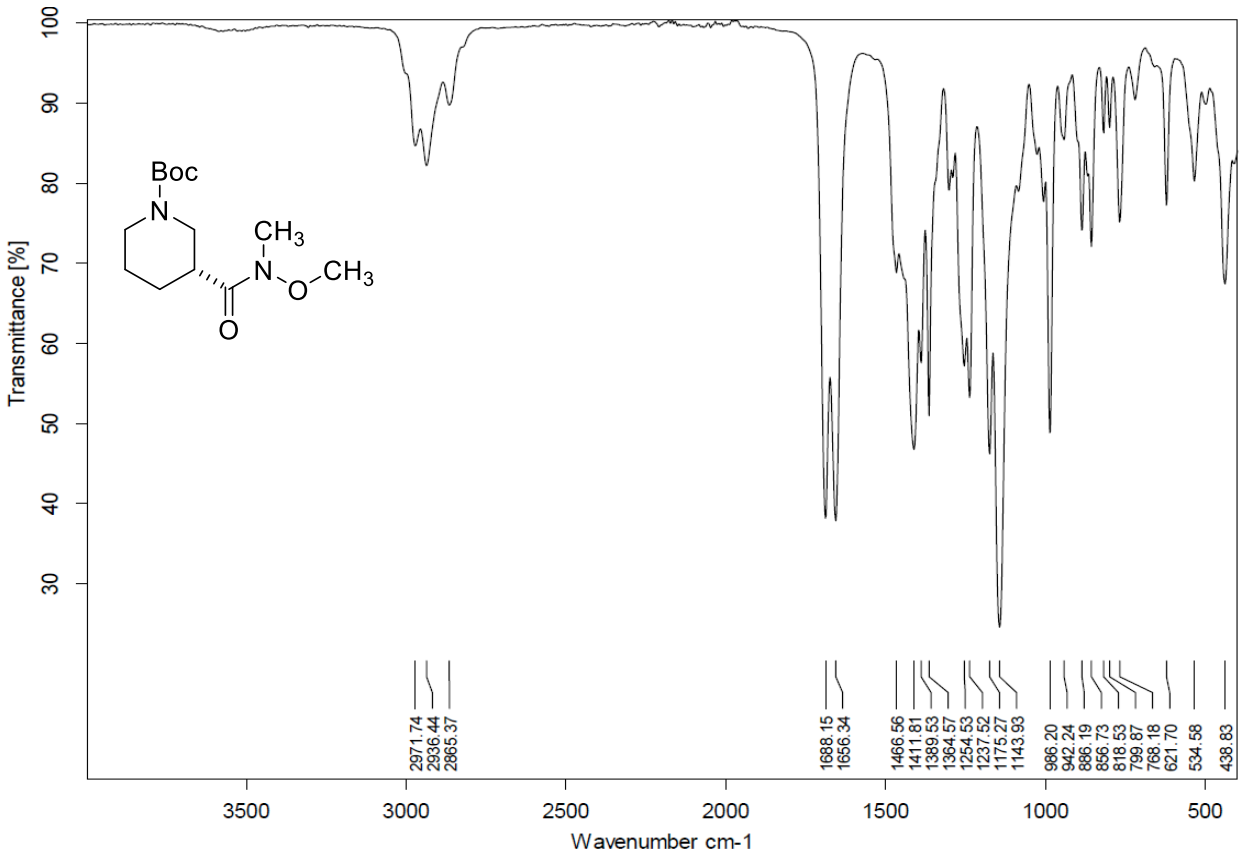


^{13}C NMR (126 MHz) spectrum of compound **2** in $CDCl_3$

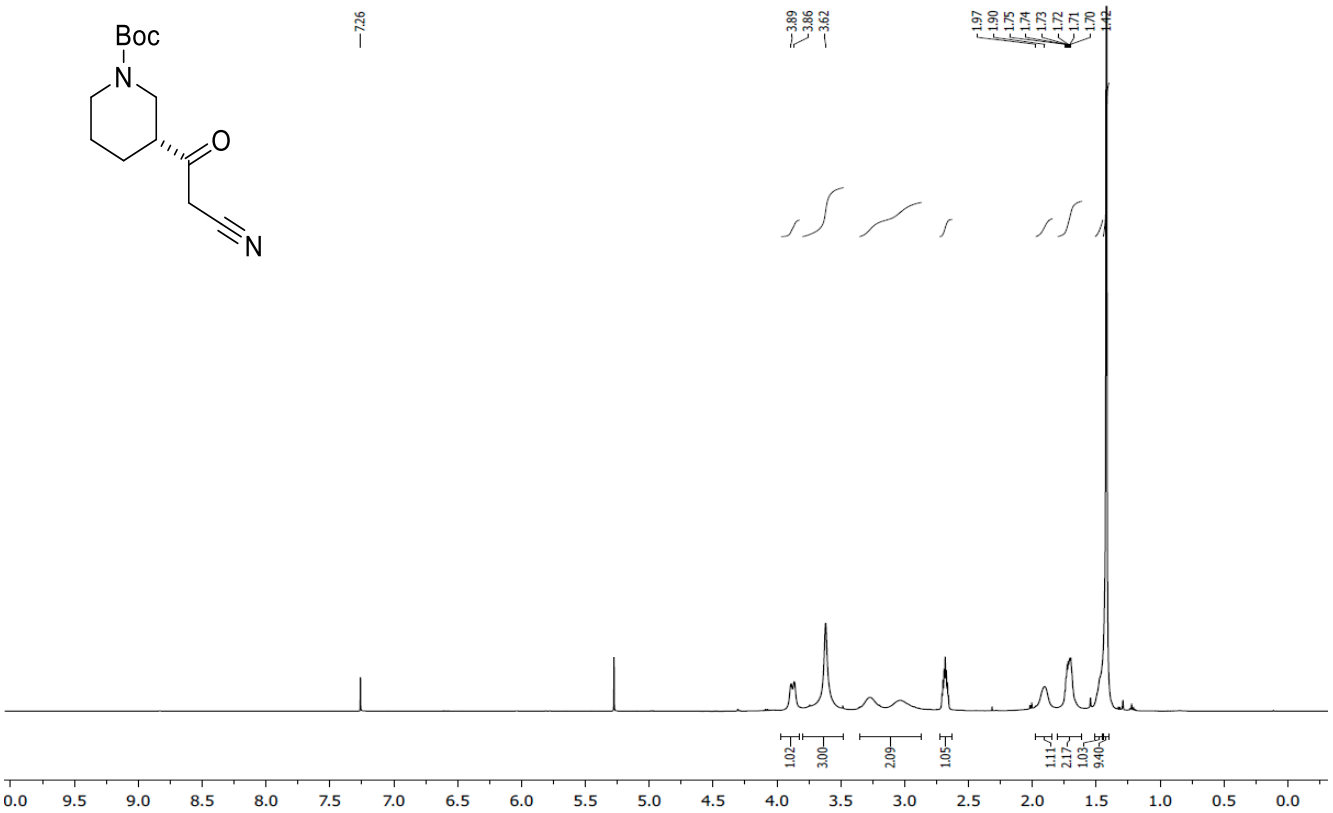


calculated mass: $[M+H]^+ = 273.1809$ observed: $[M+H]^+ = 273.1809$ mass accuracy < 0.1 ppm

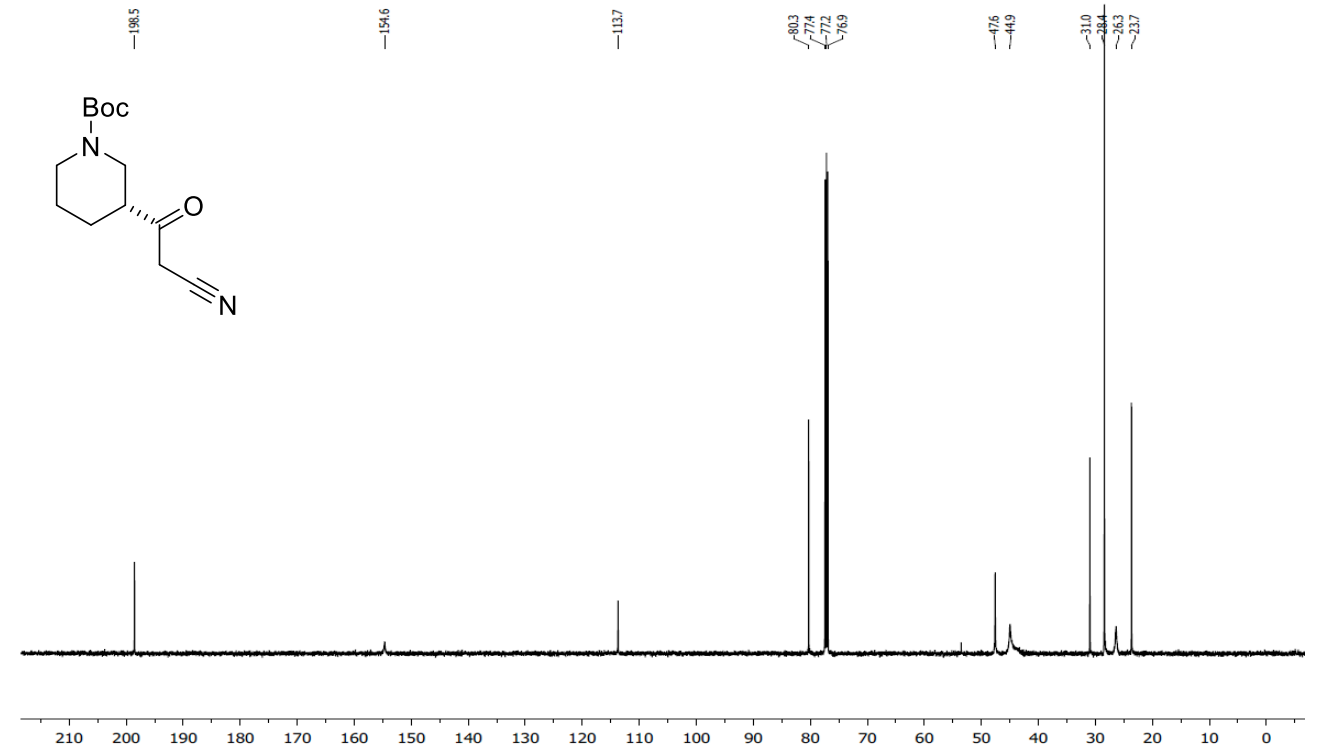
HRMS of compound **2**



IR spectrum of compound **2**

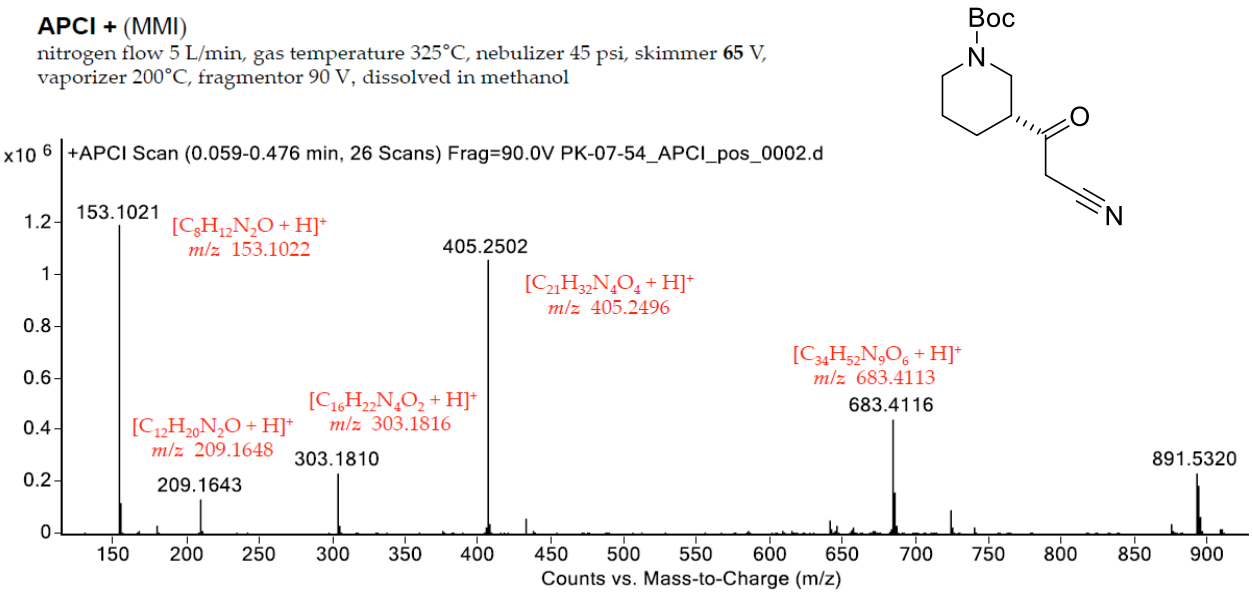


^{1}H NMR (500MHz) spectrum of compound **3** in $CDCl_3$



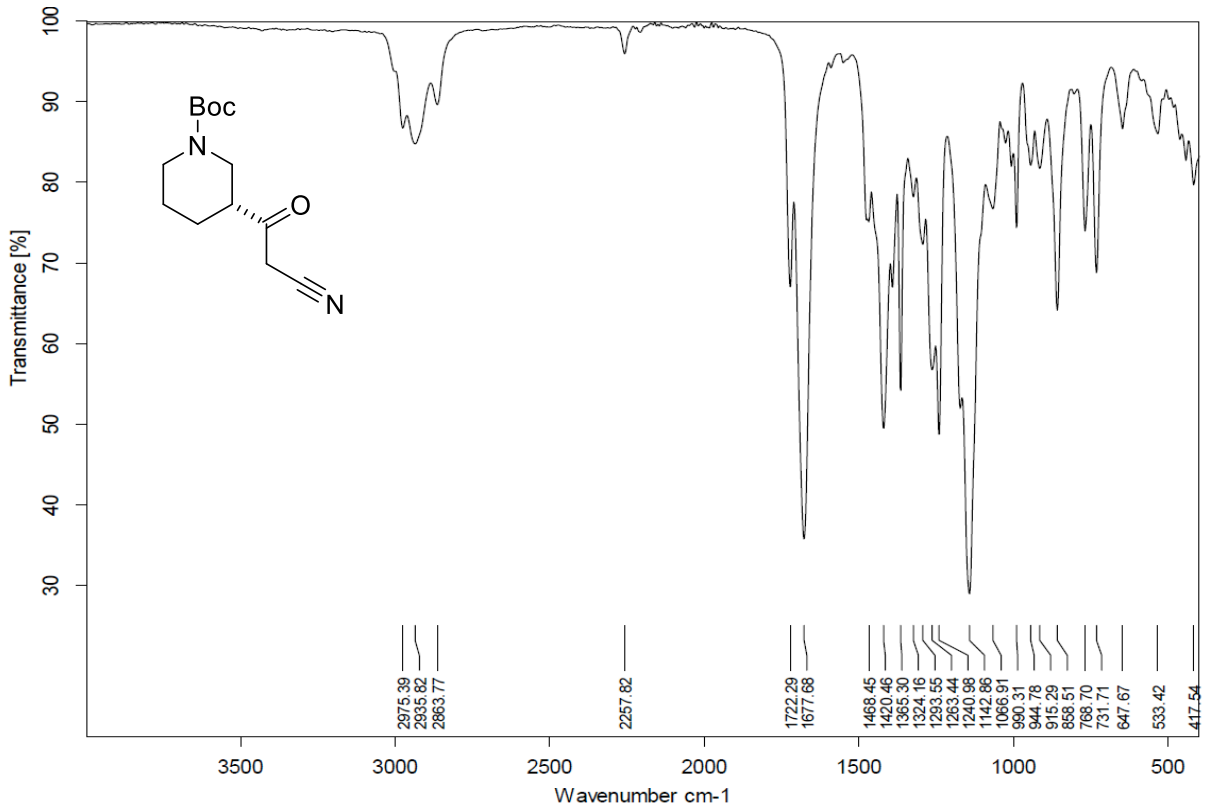
^{13}C NMR (126 MHz) spectrum of compound **3** in $CDCl_3$

$C_{13}H_{20}N_2O_3$ mono *m/z* **252.1474**



calculated mass: $[M\text{-Boc}]^+ = 153.1022$ observed: $[M\text{-Boc}]^+ = 153.1021$ mass accuracy < 0.1 ppm

HRMS of compound **3**



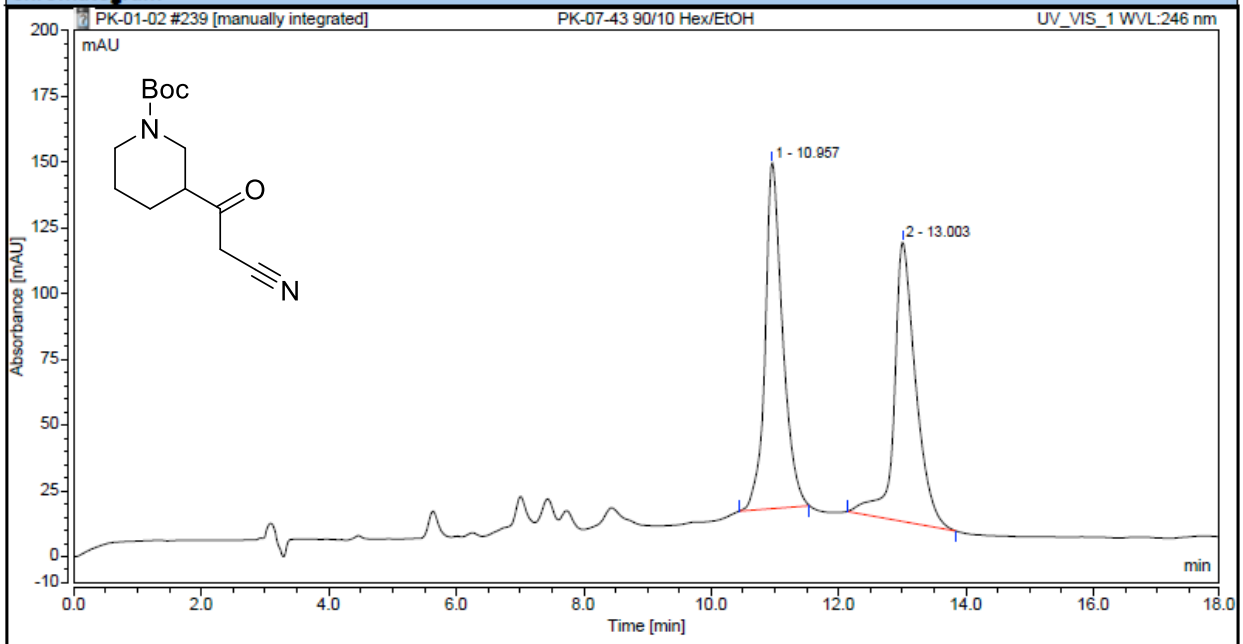
IR spectrum of compound **3**

Chromatogram and Results

Injection Details

Injection Name:	**PK-07-43**	*Run Time (min):*	**17.97**
Vial Number:	**244**	*Injection Volume:*	**20.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Flow Rate	**1 mL/min**	*Wavelength:*	**246 nm**
Instrument Method:	**PK_Analytical Method**	*Bandwidth:*	**10**
Processing Method:	**New Processing Method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**14.9.16 14:37**	*Sample Weight:*	**1.0000**
Mobile phase	**Hexane/EtOH 90/10**	*Column*	**CHIRALPAK IA**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		10.957	41.823	131.807	50.04	55.37	n.a.
2		13.003	41.762	106.259	49.96	44.63	n.a.
Total:			**83.584**	**238.066**	**100.00**	**100.00**	

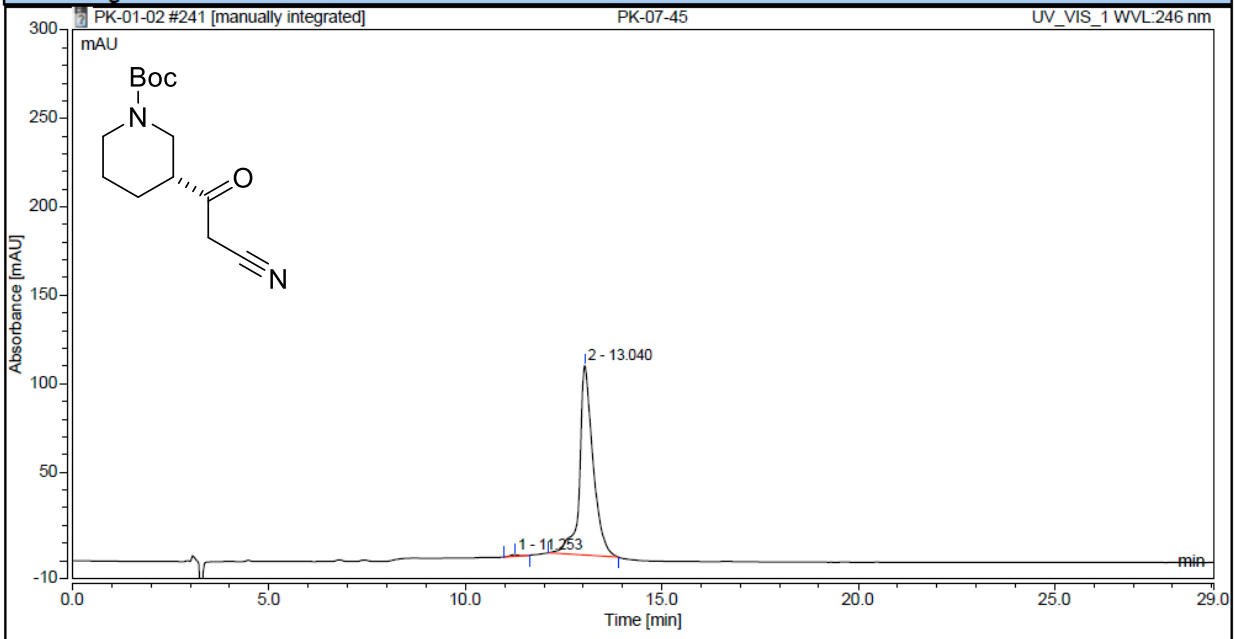
HPLC chromatogram of compound **3** (racemic)

Chromatogram and Results

Injection Details

Injection Name:	**PK-07-45**	*Run Time (min):*	**29.04**
Vial Number:	**246**	*Injection Volume:*	**20.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Flow Rate	**1 mL/min**	*Wavelength:*	**246 nm**
Instrument Method:	**PK_Analytical Method**	*Bandwidth:*	**10**
Processing Method:	**New Processing Method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**14.9.16 15:26**	*Sample Weight:*	**1.0000**
Mobile phase	**Hexane/EtOH 90/10**	*Column*	**CHIRALPAK IA**

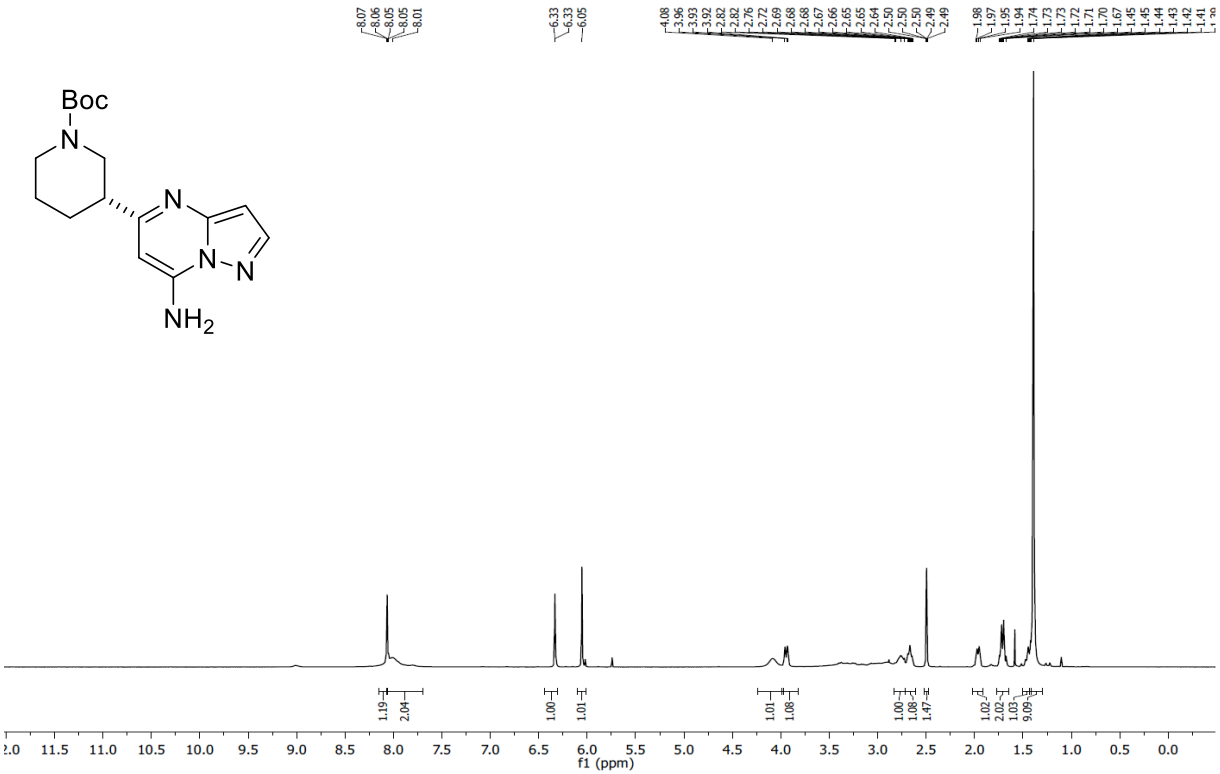
Chromatogram



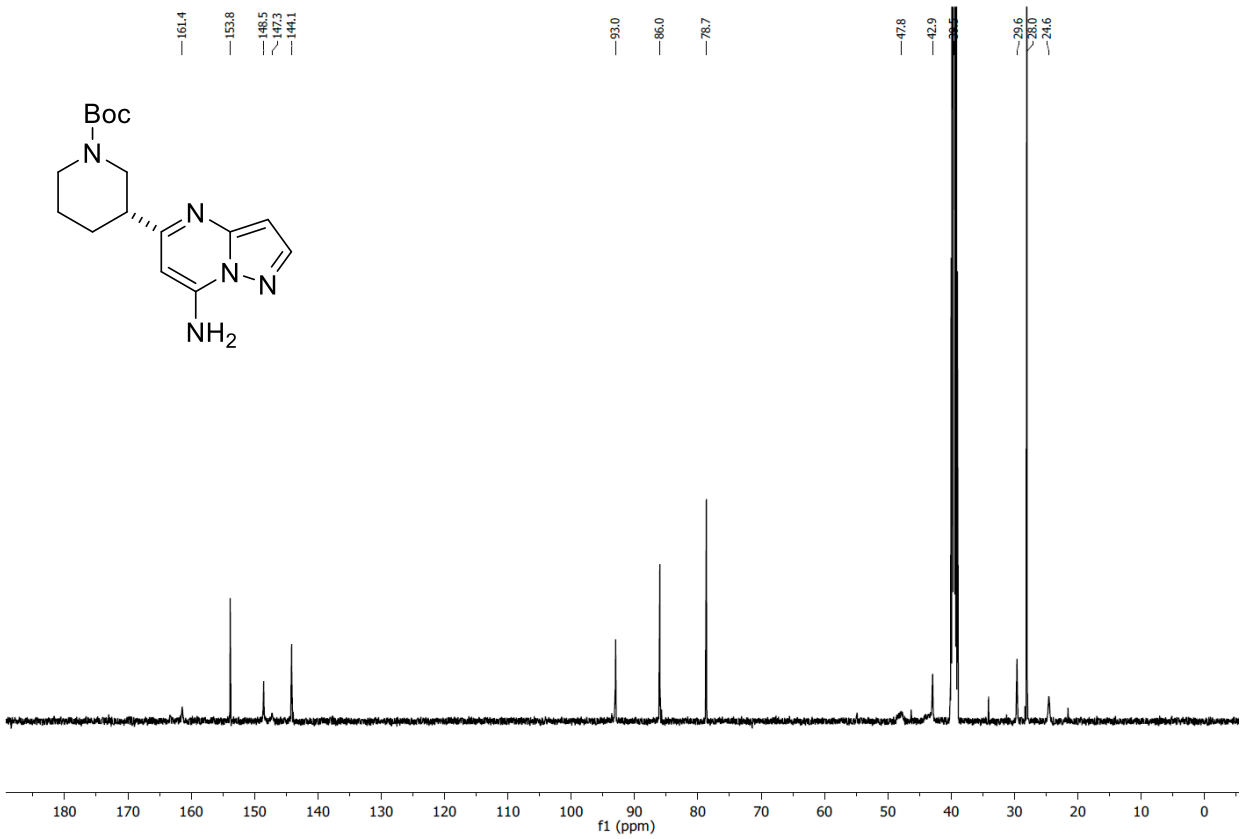
Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		11.253	0.203	0.846	0.47	0.78	n.a.
2		13.040	42.675	107.162	99.53	99.22	n.a.
Total:			**42.878**	**108.008**	**100.00**	**100.00**	

HPLC chromatogram of compound **3** (non-racemic)



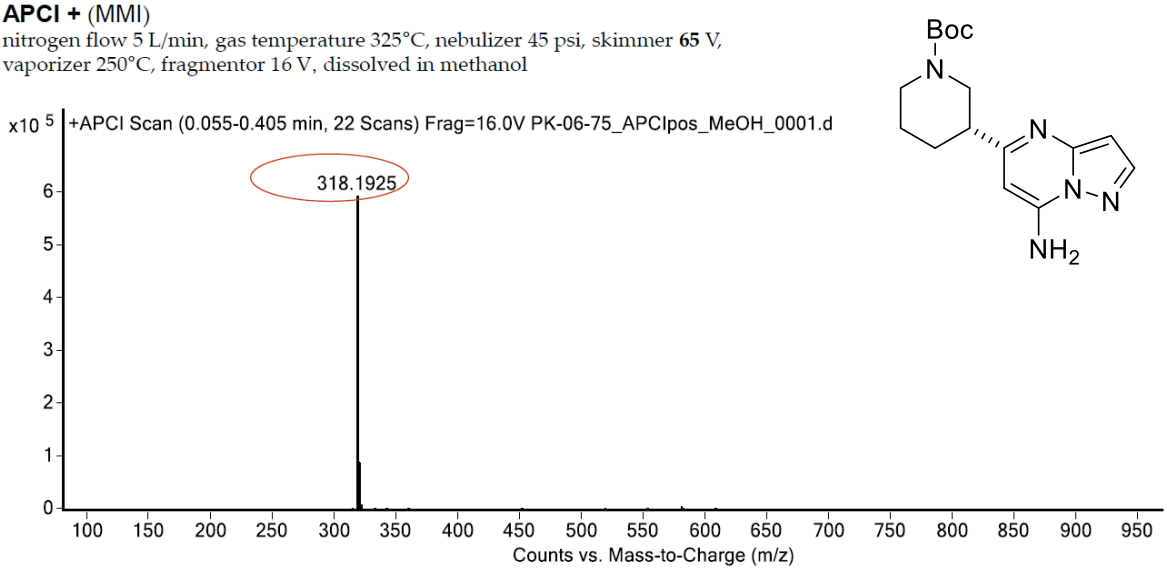
^{1}H NMR (500MHz) spectrum of compound **4** in DMSO-*d6*



^{13}C NMR (126 MHz) spectrum of compound **4** in DMSO-*d6*

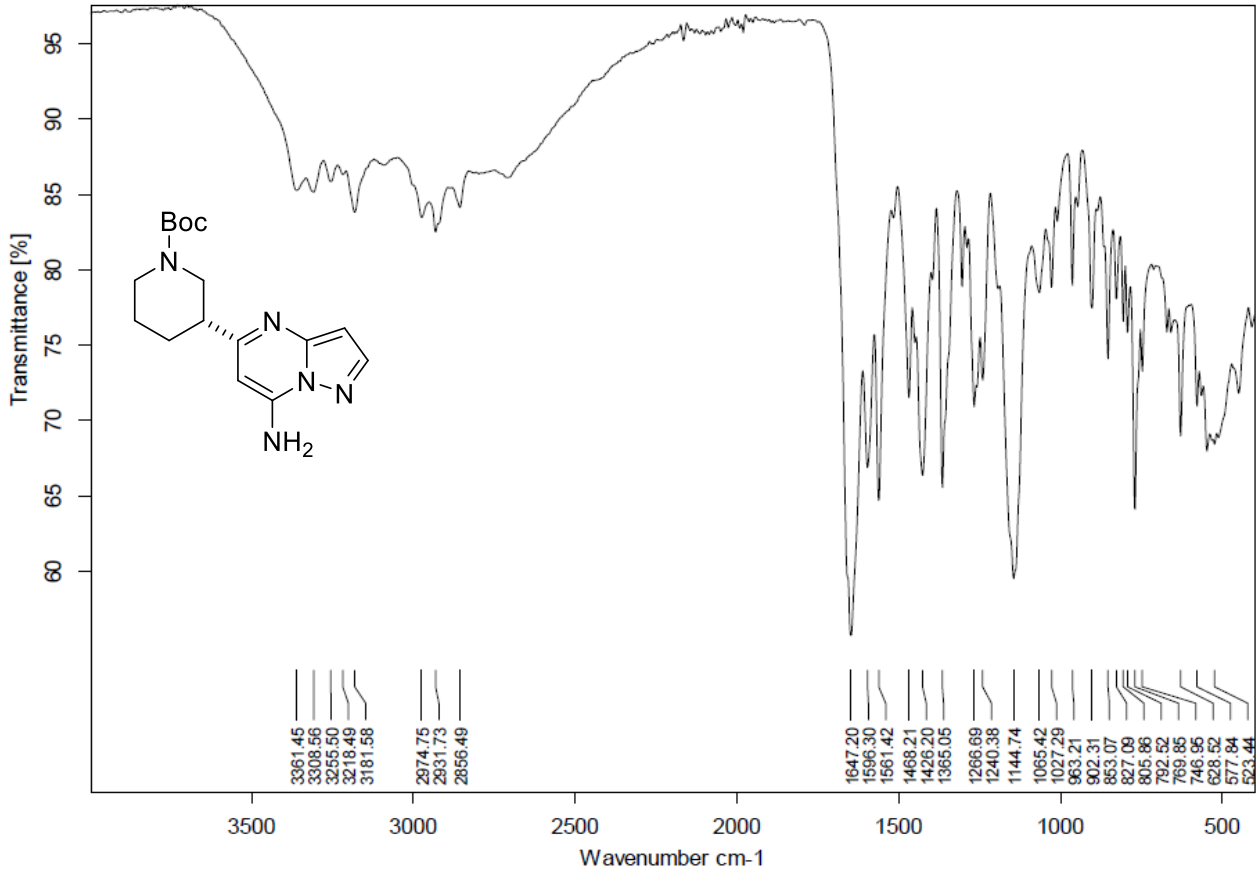
$C_{16}H_{23}N_5O_2$ mono *m/z* 317.1852

APCI + (MMI)
nitrogen flow 5 L/min, gas temperature 325°C, nebulizer 45 psi, skimmer **65** V, vaporizer 250°C, fragmentor 16 V, dissolved in methanol



calculated mass: $[M+H]^+ = 318.1925$ observed: $[M+H]^+ = 318.1925$ mass accuracy < 0.1 ppm

HRMS of compound **4**



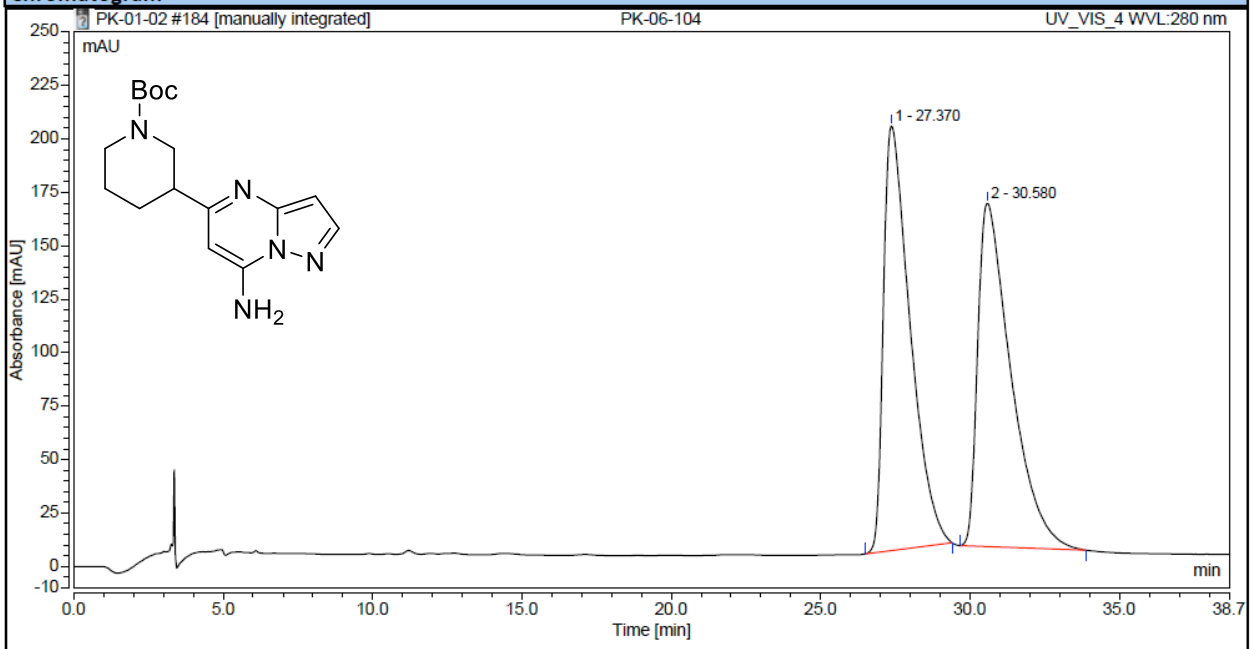
IR spectrum of compound **4**

Chromatogram and Results

Injection Details

Injection Name:	**PK-06-104**	*Run Time (min):*	**38.67**
Vial Number:	**184**	*Injection Volume:*	**20.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_4**
Flow Rate	**1 mL/min**	*Wavelength:*	**280 nm**
Instrument Method:	**PK_Analytical Method**	*Bandwidth:*	**10**
Processing Method:	**New Processing Method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**18.4.16 18:05**	*Sample Weight:*	**1.0000**
Mobile phase	**Hexane/EtOH 98/02 + DEA 0.5%**	*Column*	**CHIRALPAK IA**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		27.370	205.129	198.636	49.81	55.28	n.a.
2		30.580	206.730	160.722	50.19	44.72	n.a.
Total:			**411.859**	**359.358**	**100.00**	**100.00**	

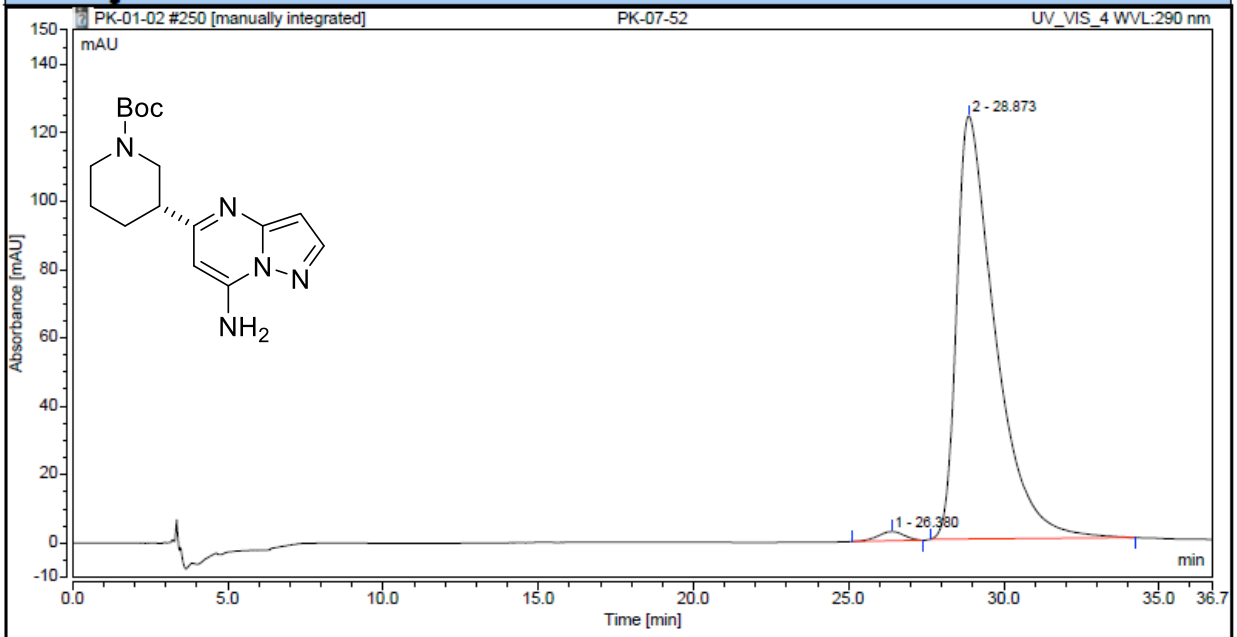
HPLC chromatogram of compound **4** (racemic)

Chromatogram and Results

Injection Details

Injection Name:	**PK-07-52**	*Run Time (min):*	**36.74**
Vial Number:	**255**	*Injection Volume:*	**20.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_4**
Flow Rate	**1 mL/min**	*Wavelength:*	**290 nm**
Instrument Method:	**PK_Analytical Method**	*Bandwidth:*	**10**
Processing Method:	**New Processing Method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**19.9.16 17:48**	*Sample Weight:*	**1.0000**
Mobile phase	**Hexane/EtOH 98/02 + DEA 0.5%**	*Column*	**CHIRALPAK IA**

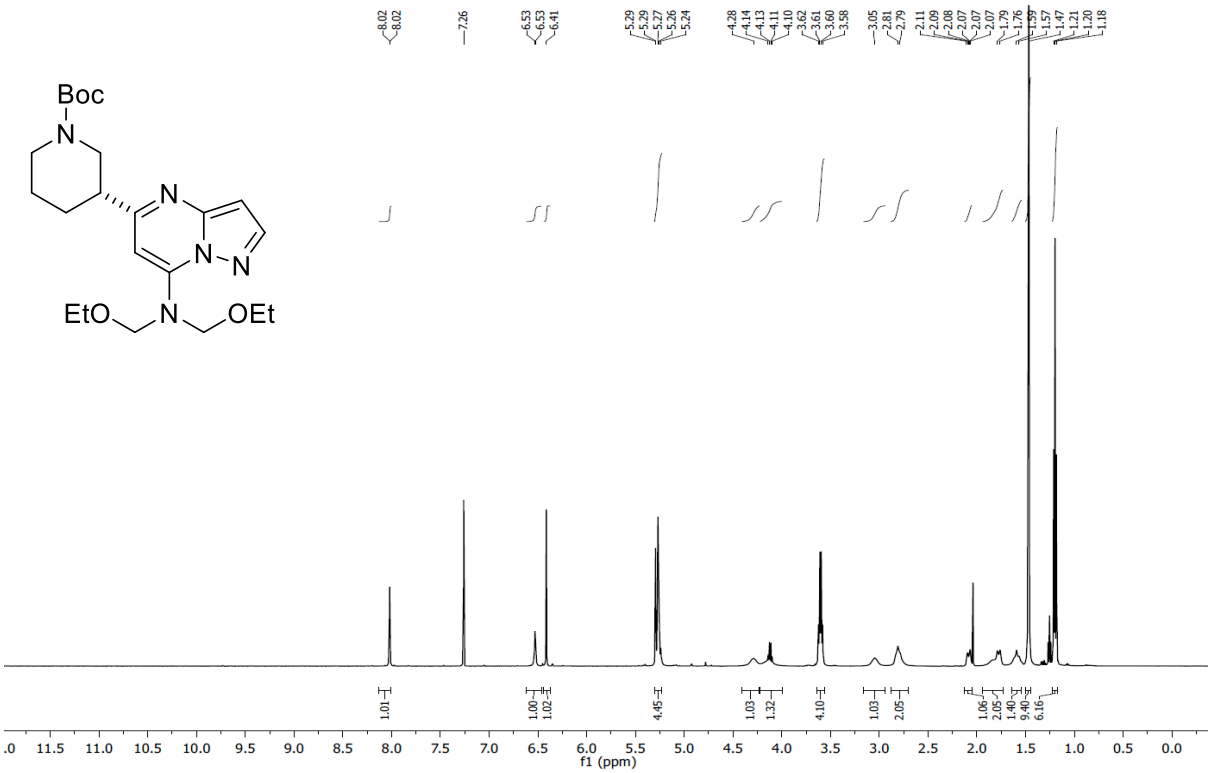
Chromatogram



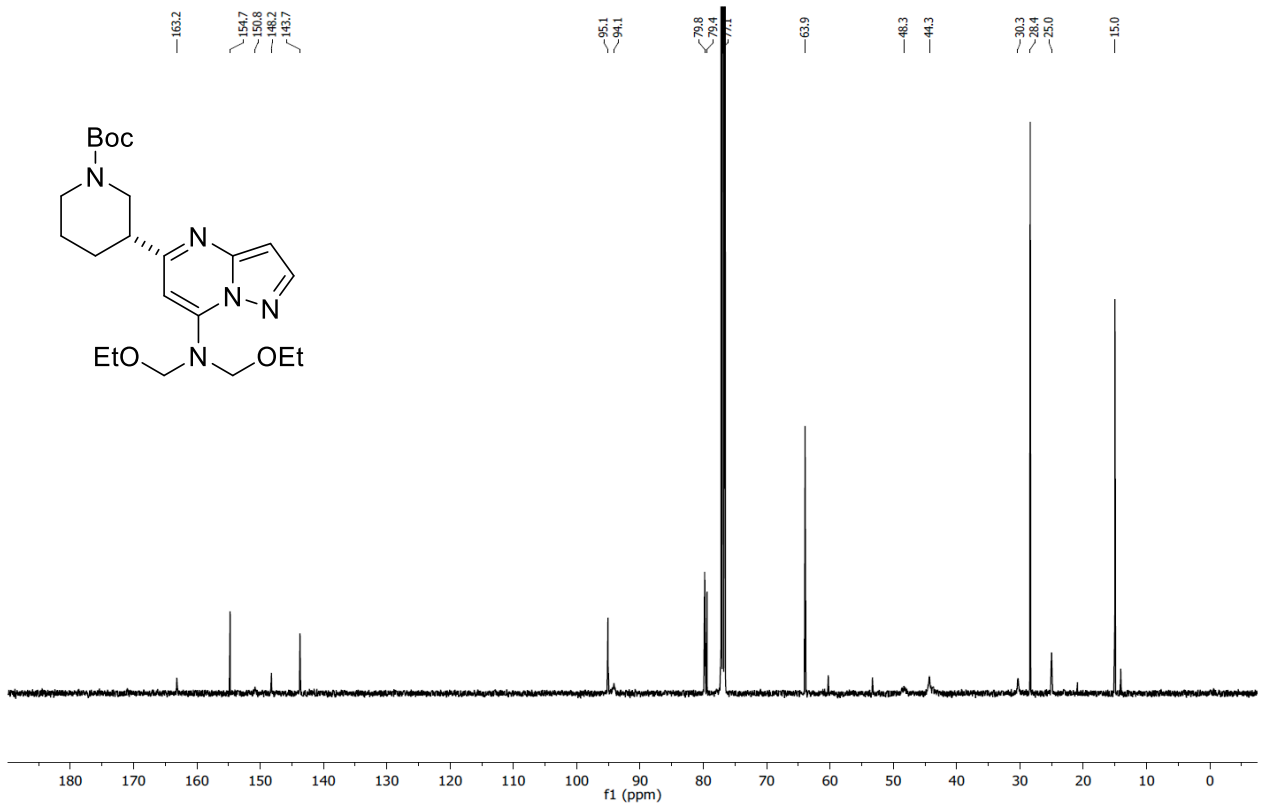
Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		26.380	2.596	2.702	1.44	2.14	n.a.
2		28.873	178.213	123.768	98.56	97.86	n.a.
Total:			**180.809**	**126.470**	**100.00**	**100.00**	

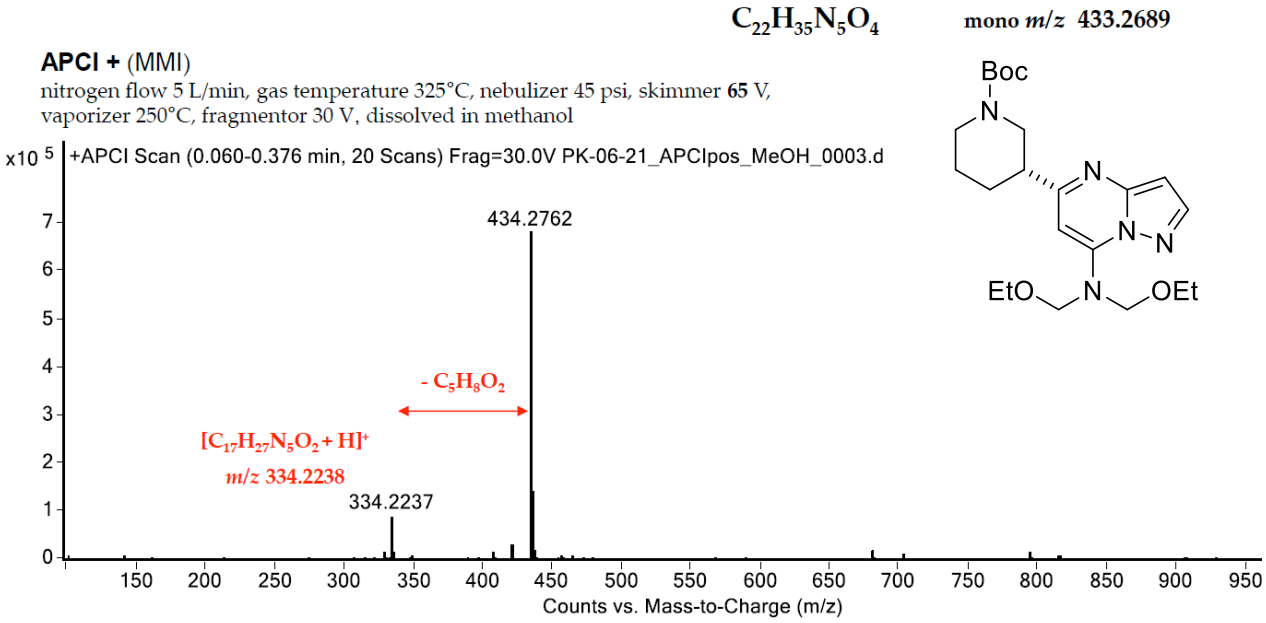
HPLC chromatogram of compound **4** (non-racemic)



^{1}H NMR (500MHz) spectrum of compound **5** in $CDCl_3$

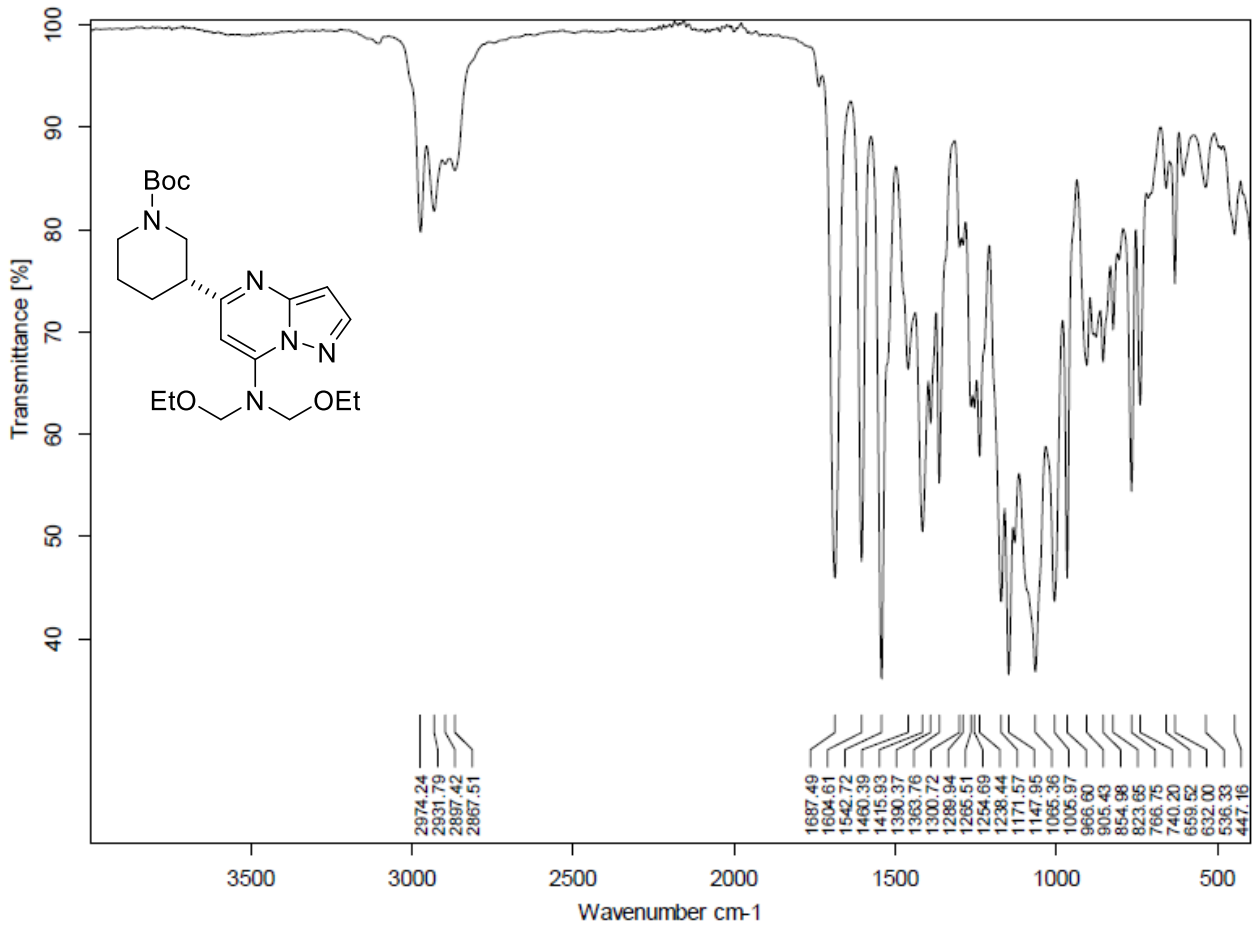


^{13}C NMR (126 MHz) spectrum of compound **5** in $CDCl_3$

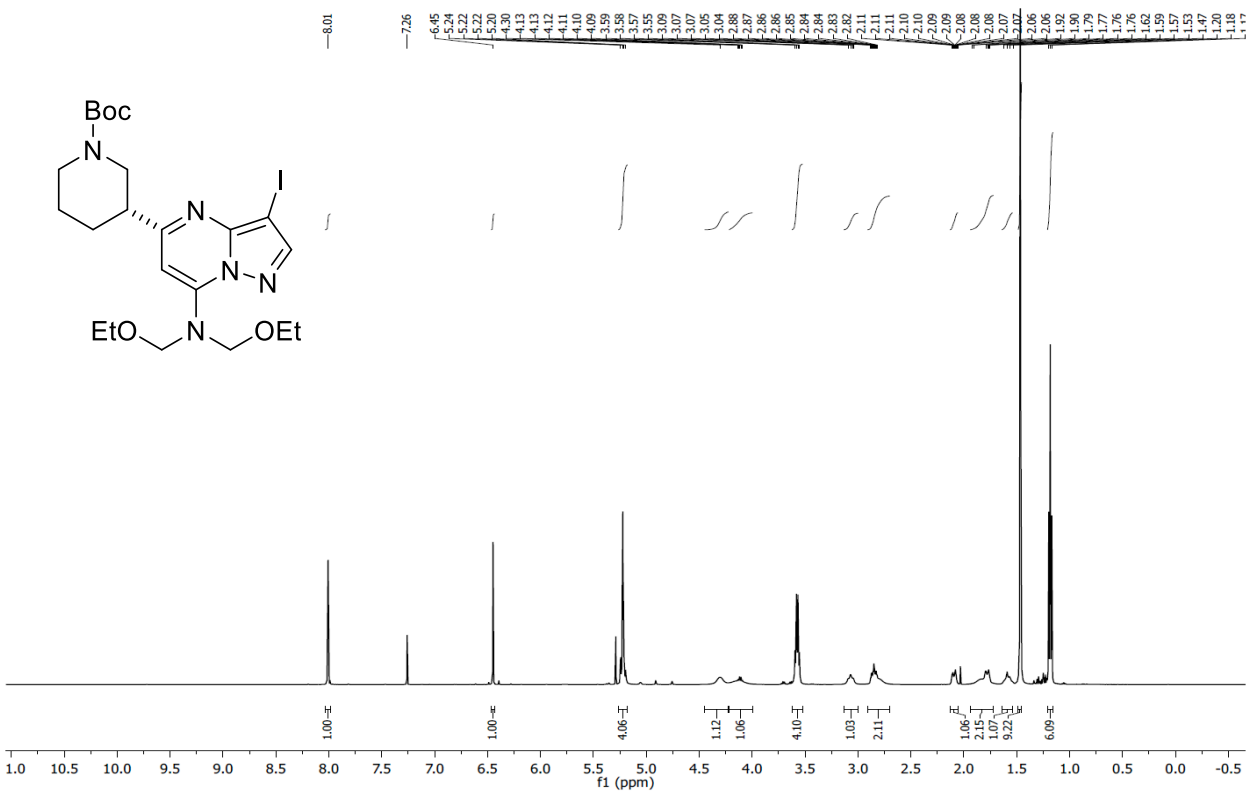


calculated mass: $[M+H]^+ = 434.2762$ observed: $[M+H]^+ = 434.2762$ mass accuracy < 0.1 ppm

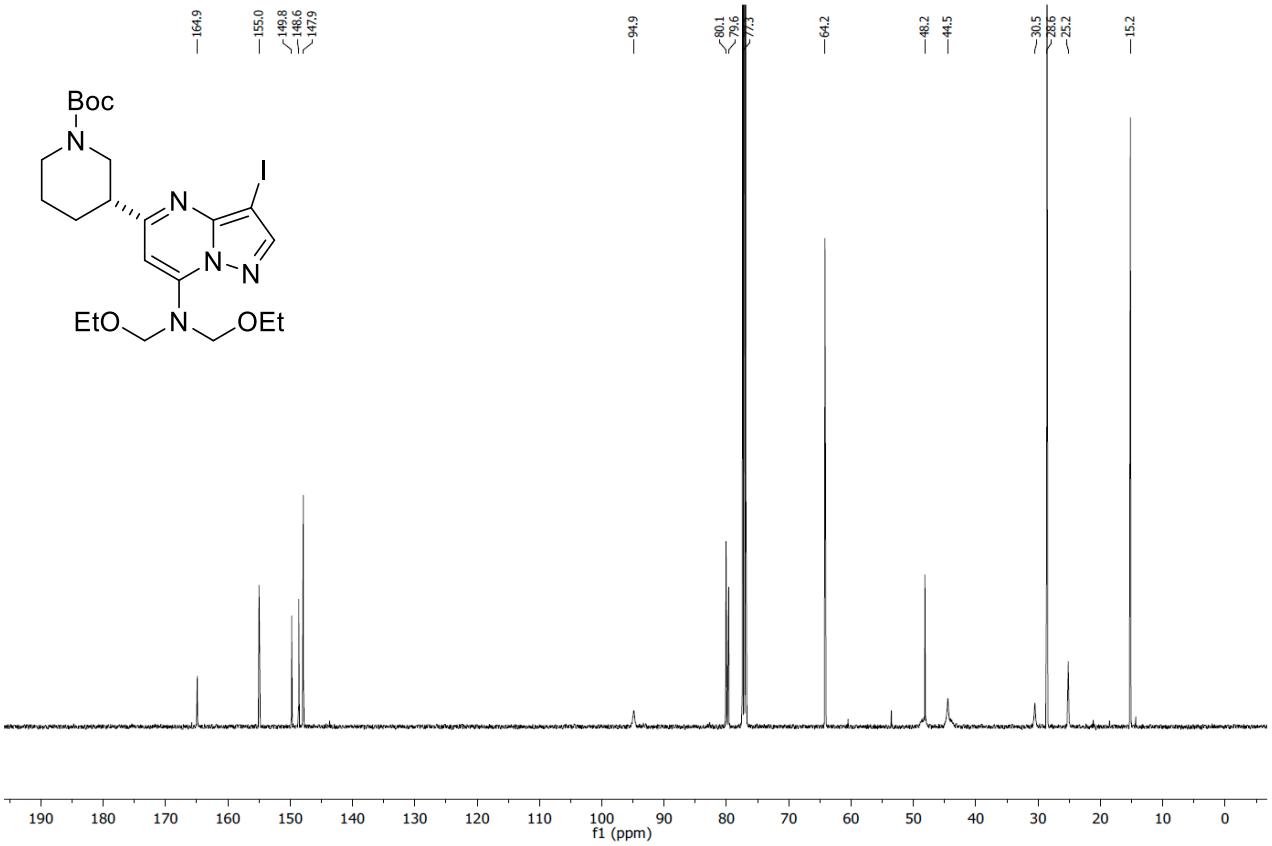
HRMS of compound **5**



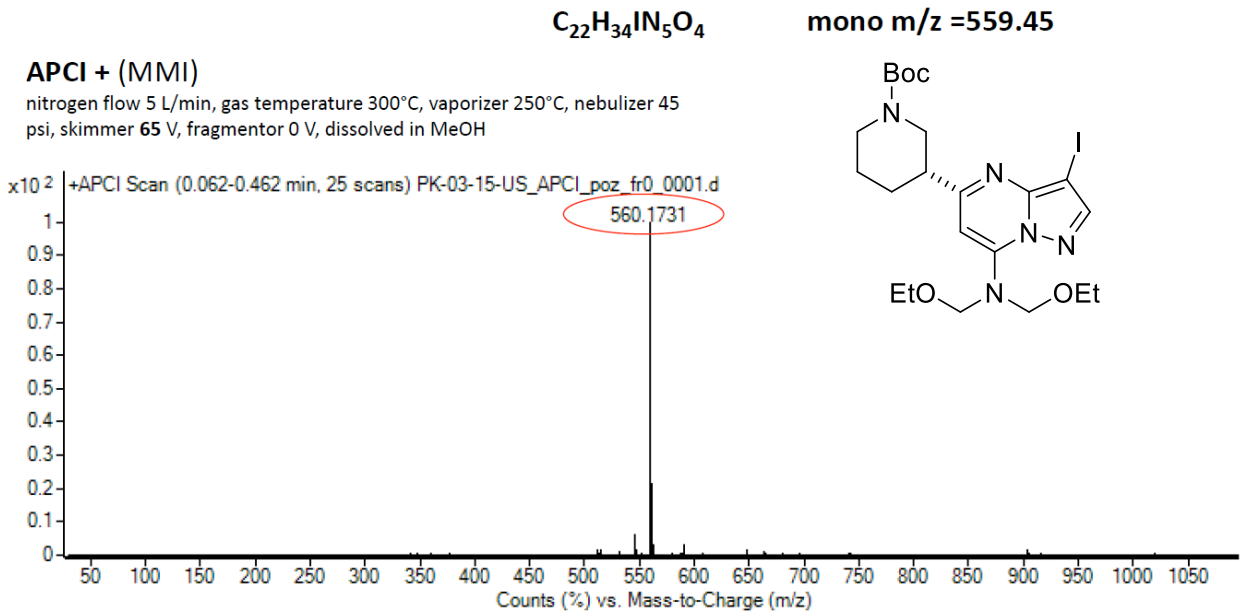
IR spectrum of compound **5**



^{1}H NMR (500MHz) spectrum of compound **6** in $CDCl_3$

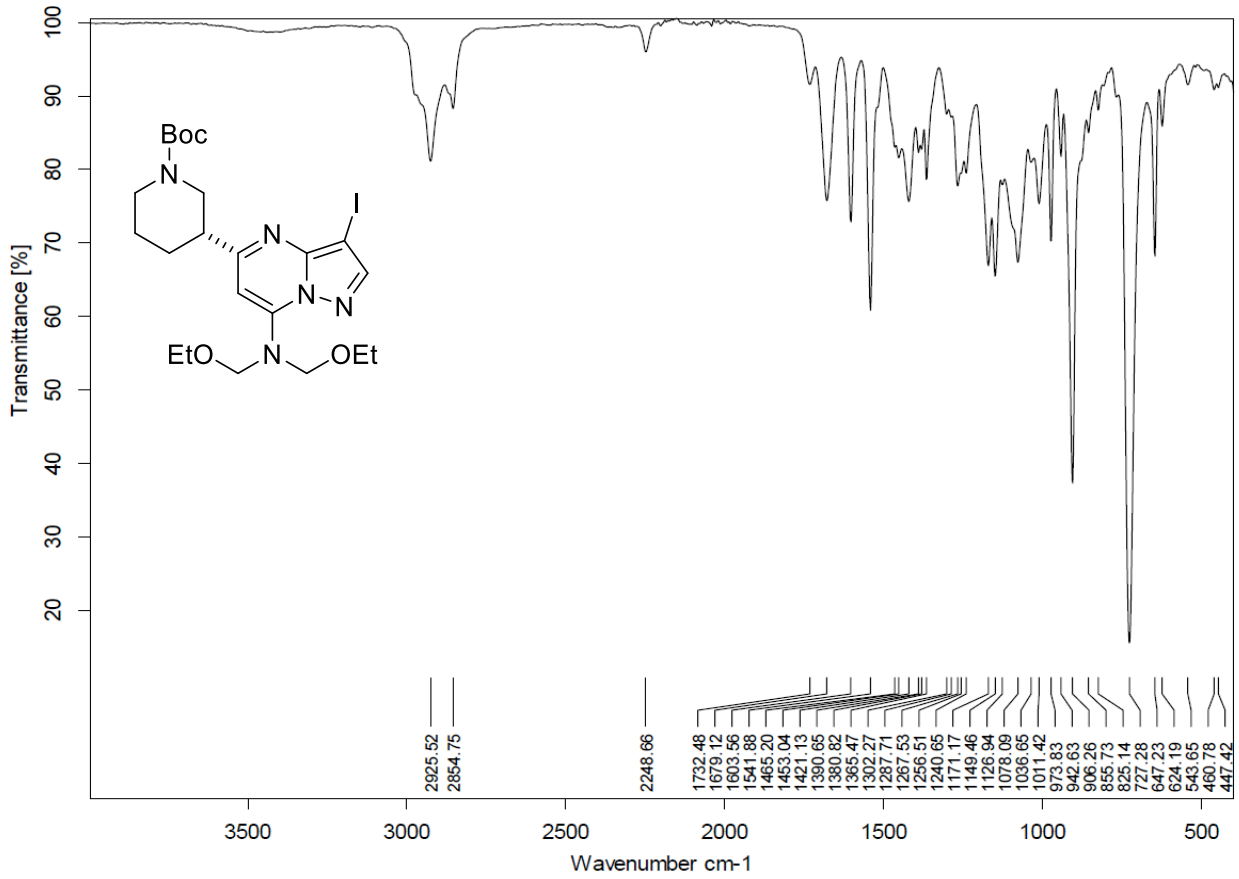


^{13}C NMR (126 MHz) spectrum of compound **6** in $CDCl_3$

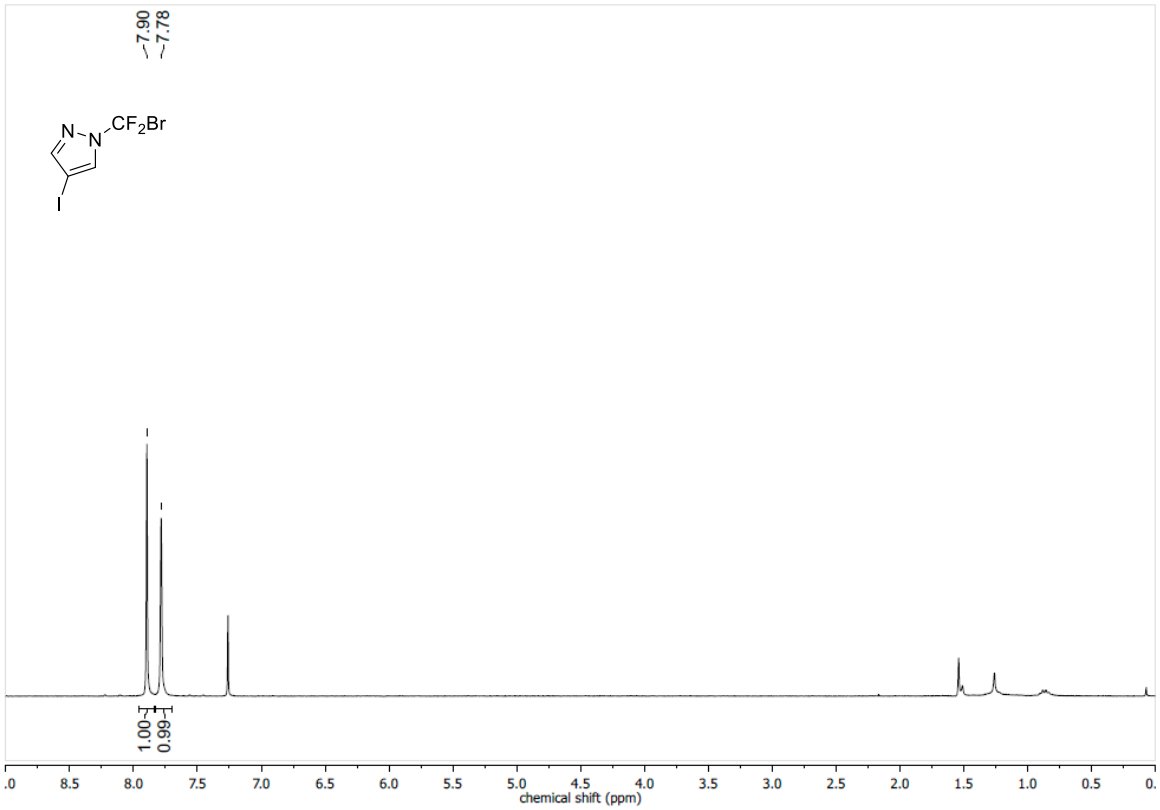


calculated mass: [M+H]+ = 560.1728 observed: [M+H]+ = 560.1731 max. mass error = 0.5 ppm

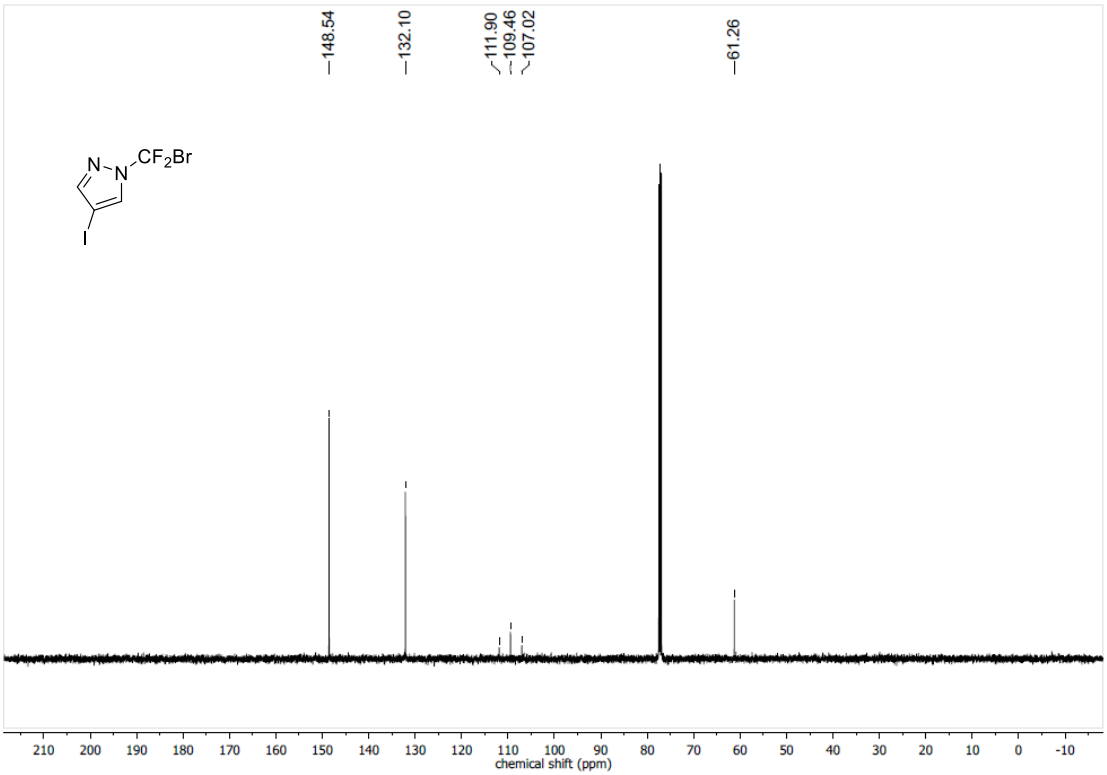
HRMS of compound **6**



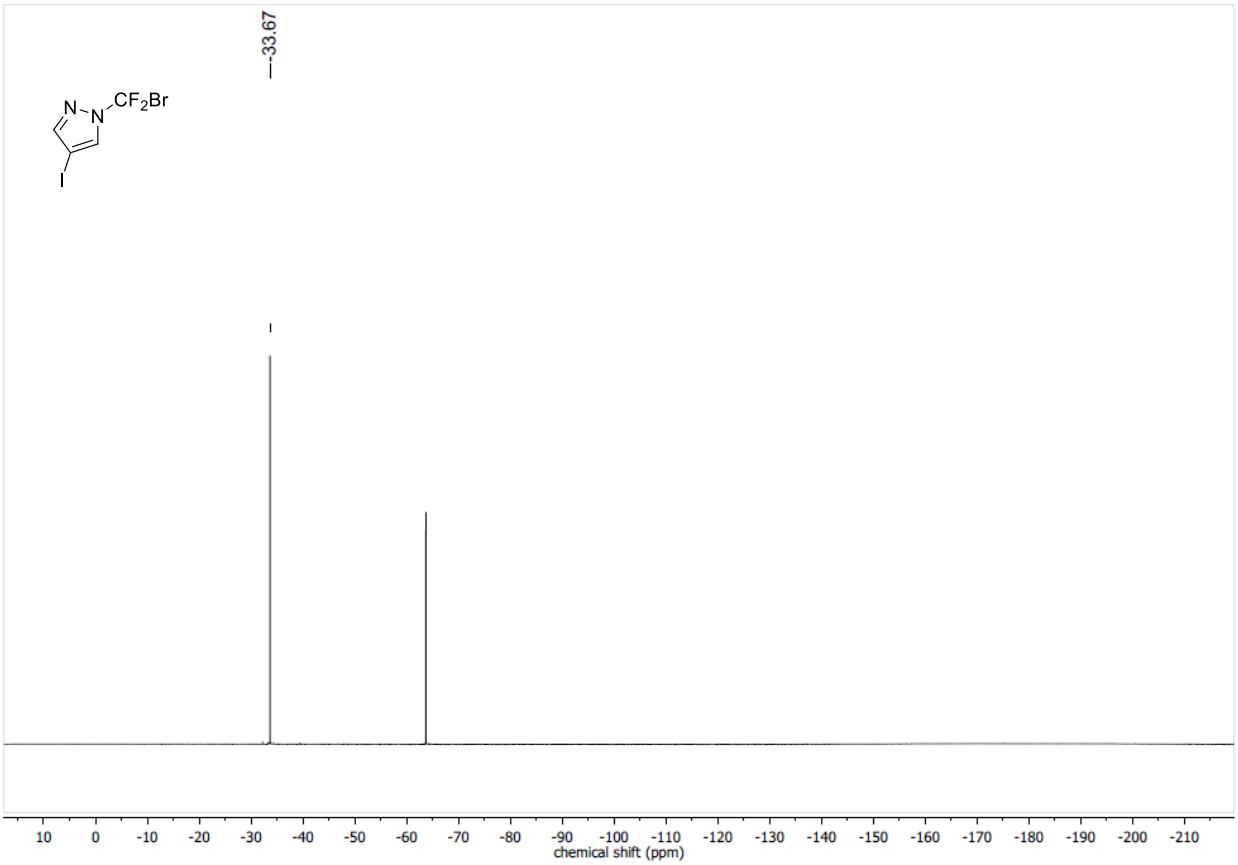
IR spectrum of compound **6**



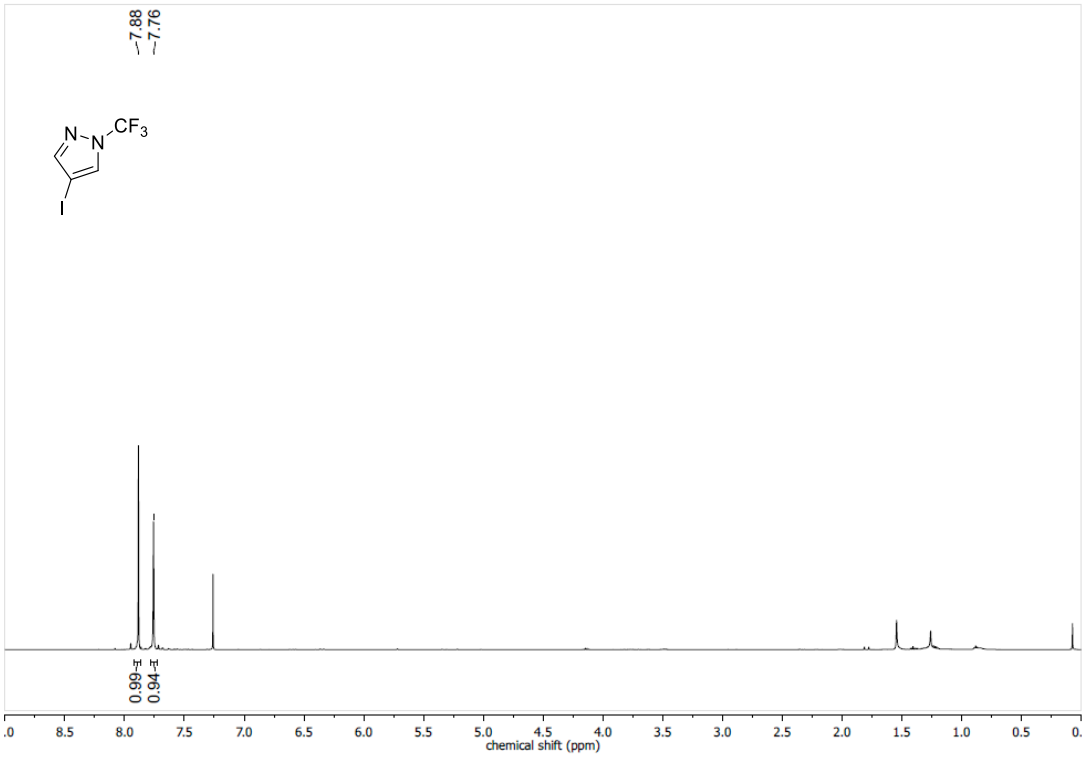
^{1}H NMR (300MHz) spectrum of compound **7** in $CDCl_3$



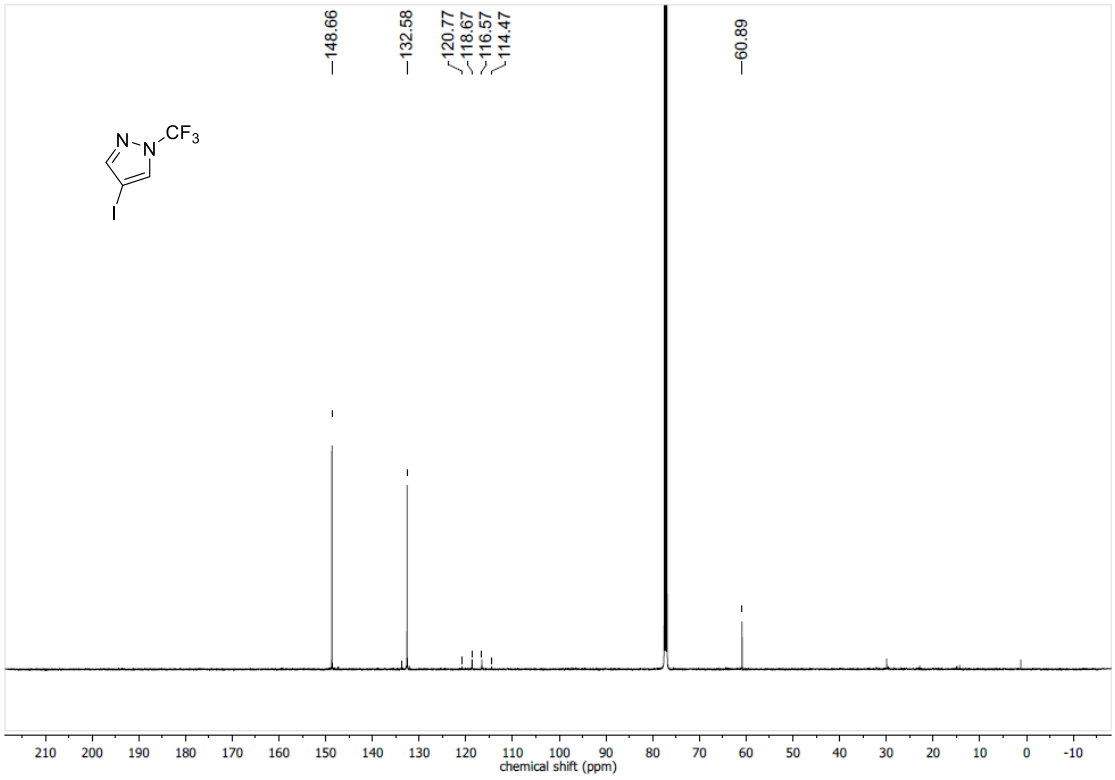
^{13}C NMR (126 MHz) spectrum of compound **7** in $CDCl_3$



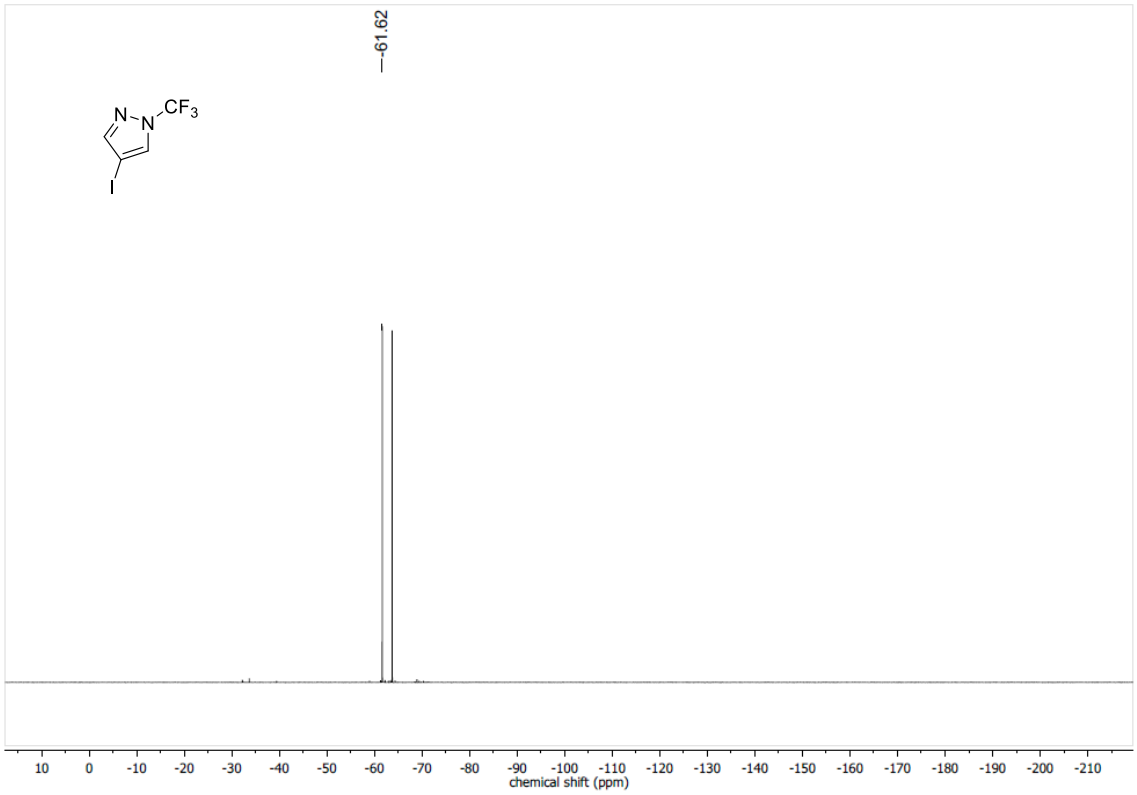
^{19}F NMR (282 MHz) spectrum of compound **7** in $CDCl_3$



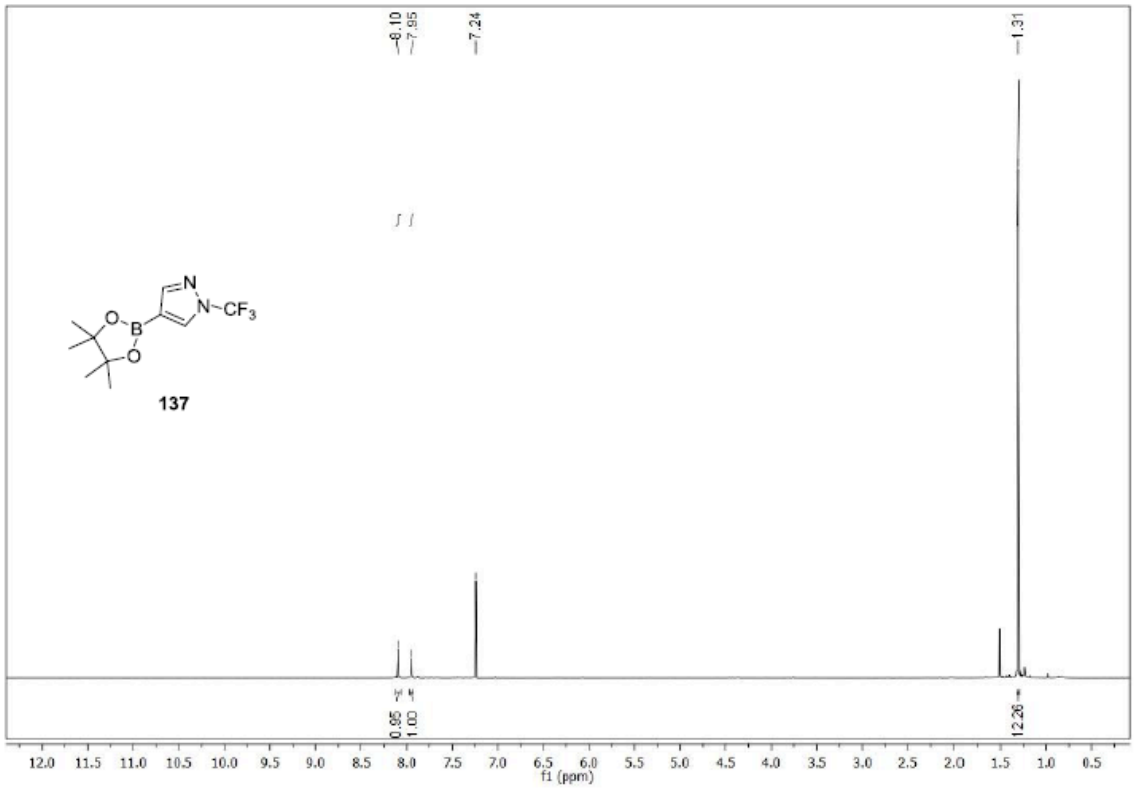
^{1}H NMR (300MHz) spectrum of compound **8** in $CDCl_3$



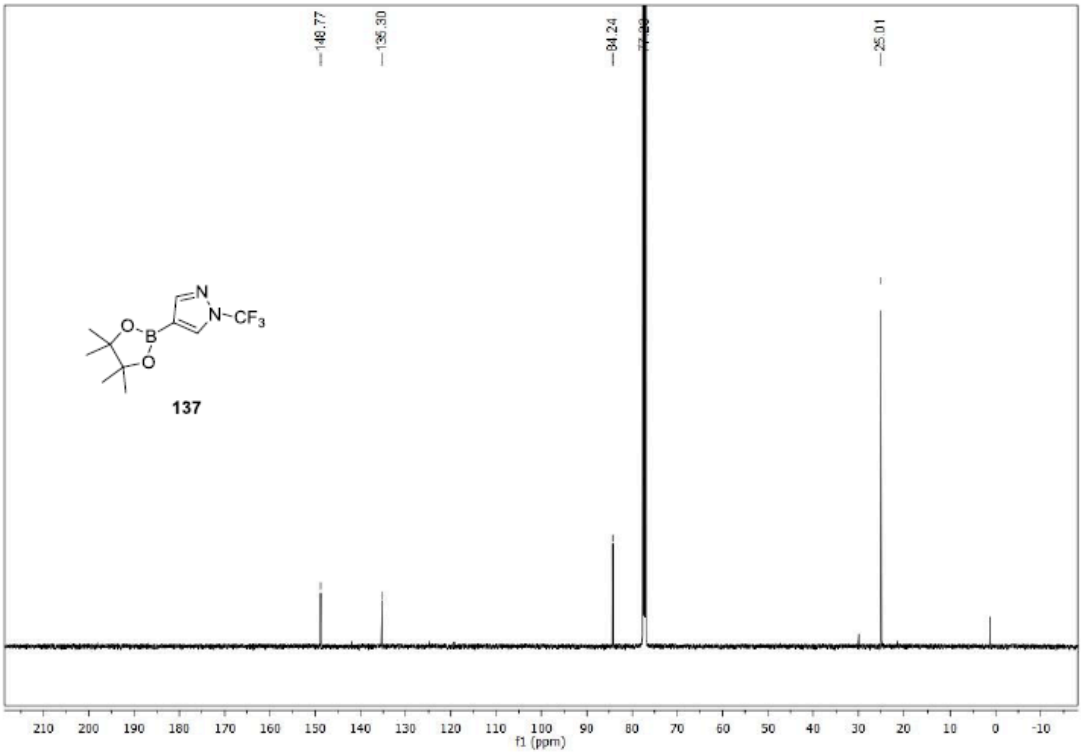
^{13}C NMR (126 MHz) spectrum of compound **8** in $CDCl_3$



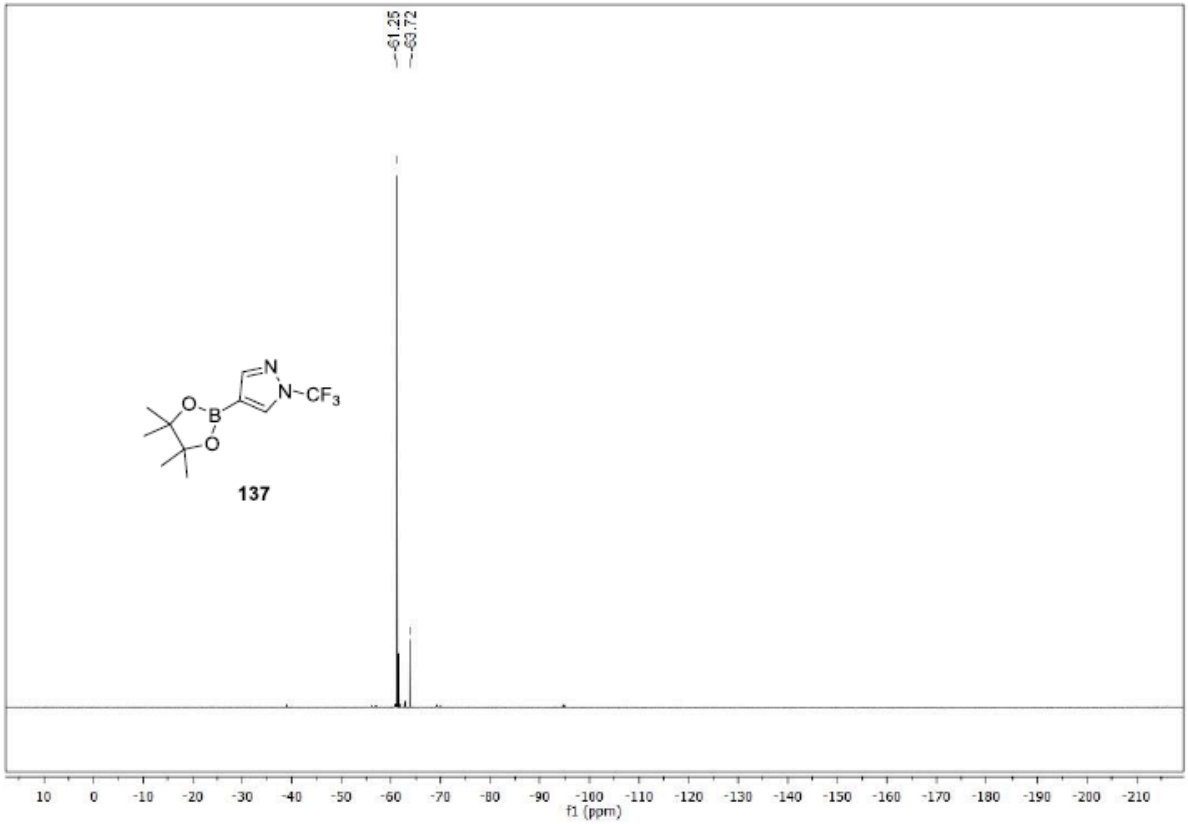
^{19}F NMR (282 MHz) spectrum of compound **8** in $CDCl_3$



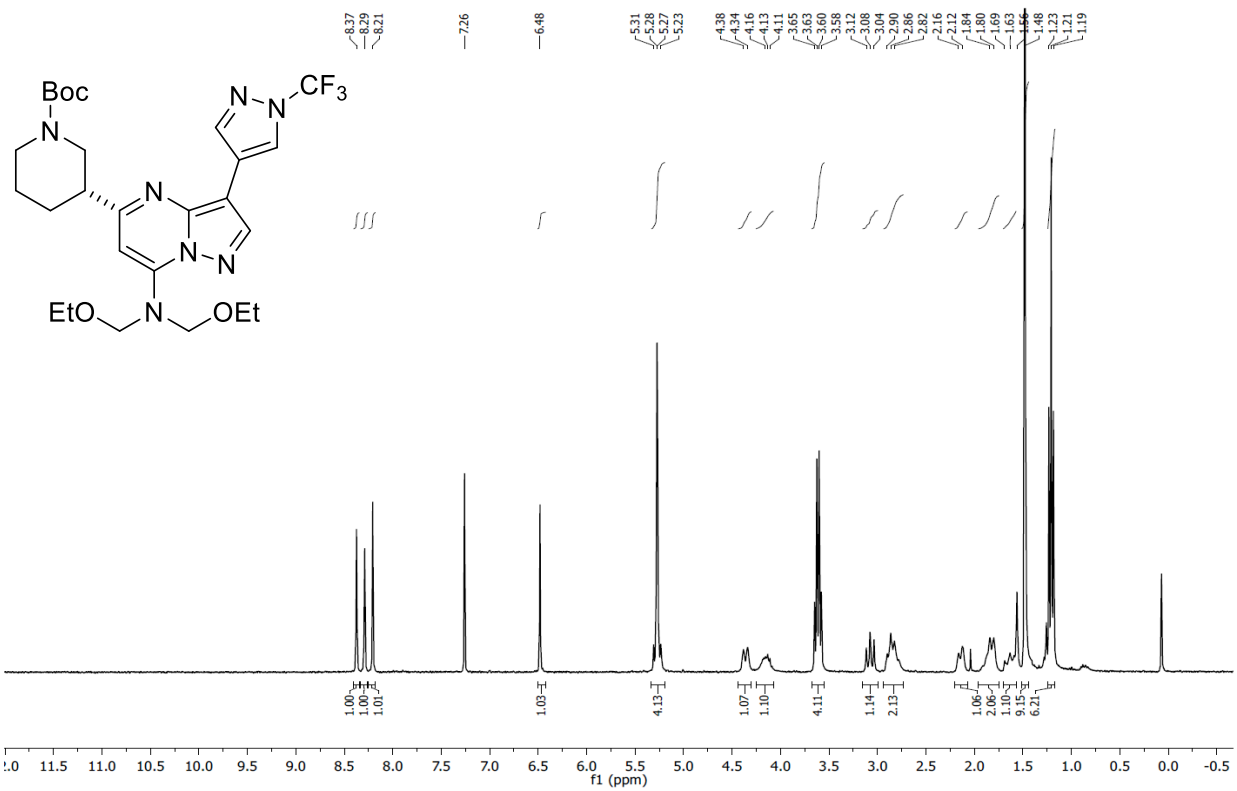
^{1}H NMR (300MHz) spectrum of compound **9** in $CDCl_3$



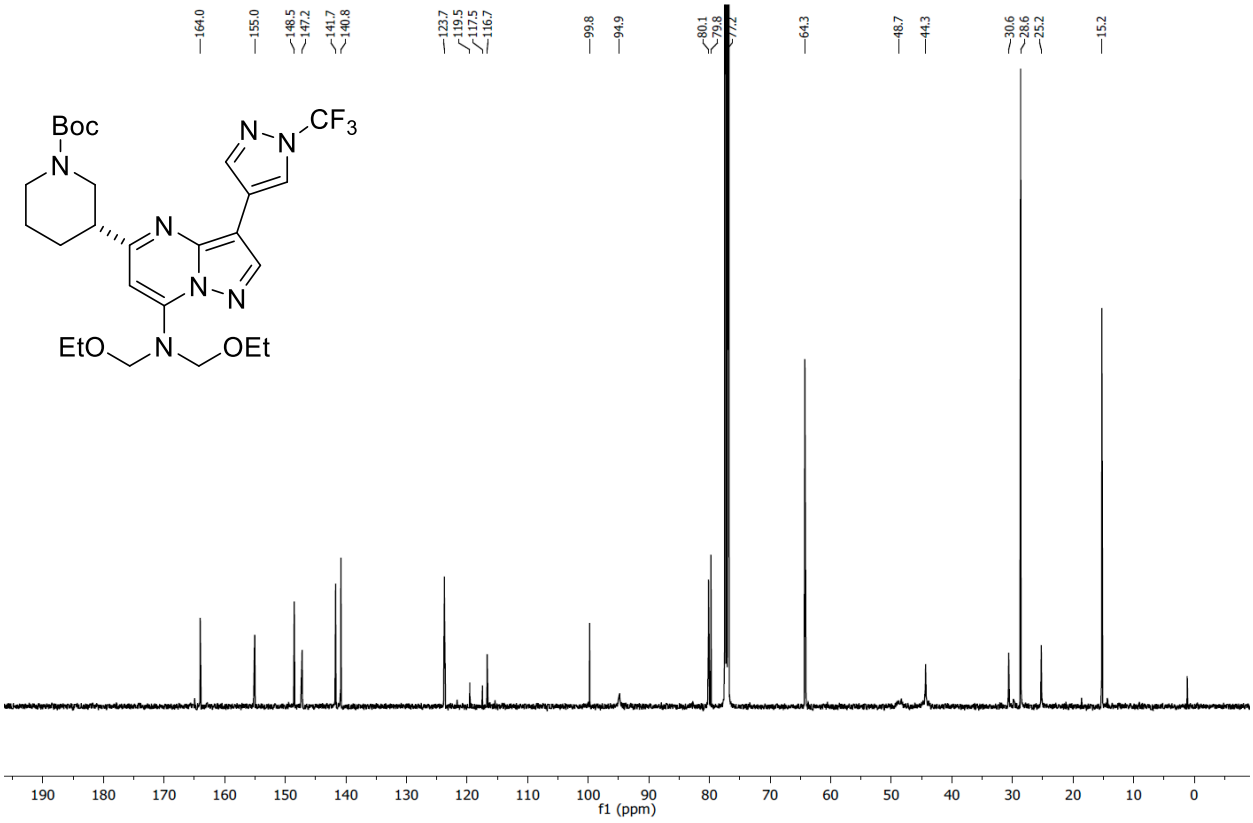
^{13}C NMR (126 MHz) spectrum of compound **9** in $CDCl_3$



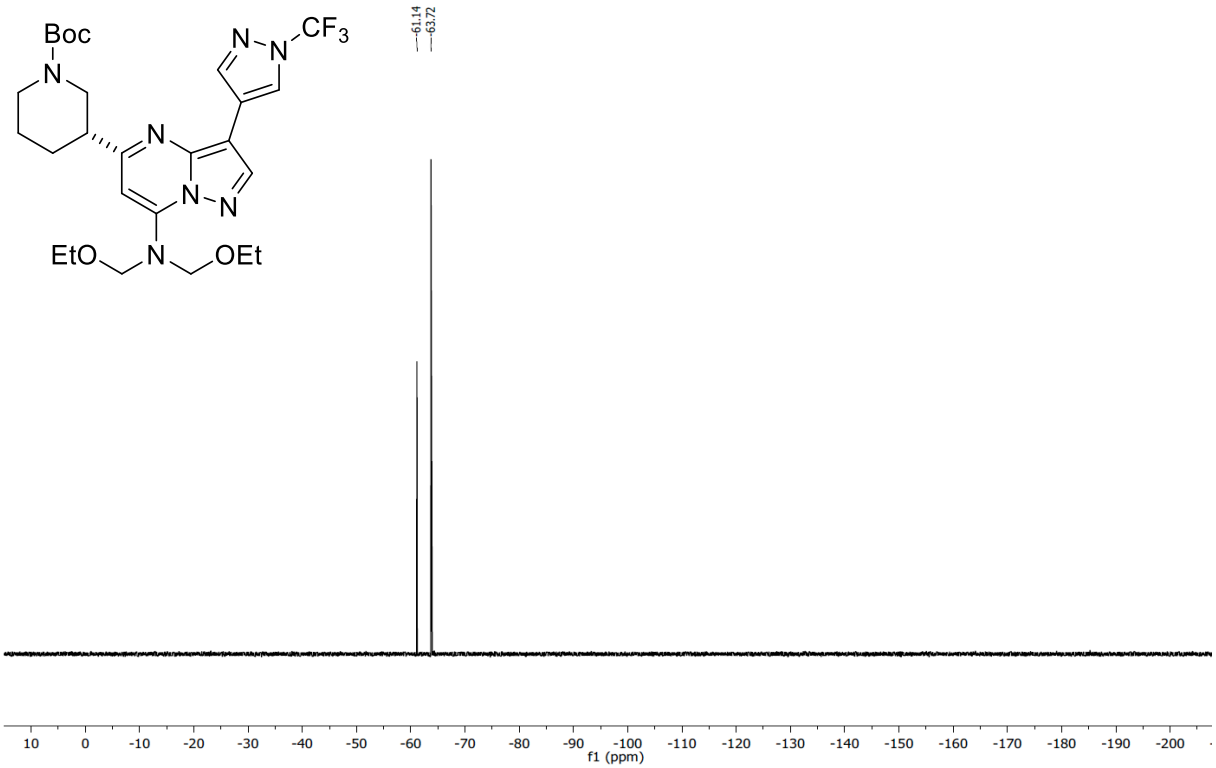
^{19}F NMR (282 MHz) spectrum of compound **9** in $CDCl_3$



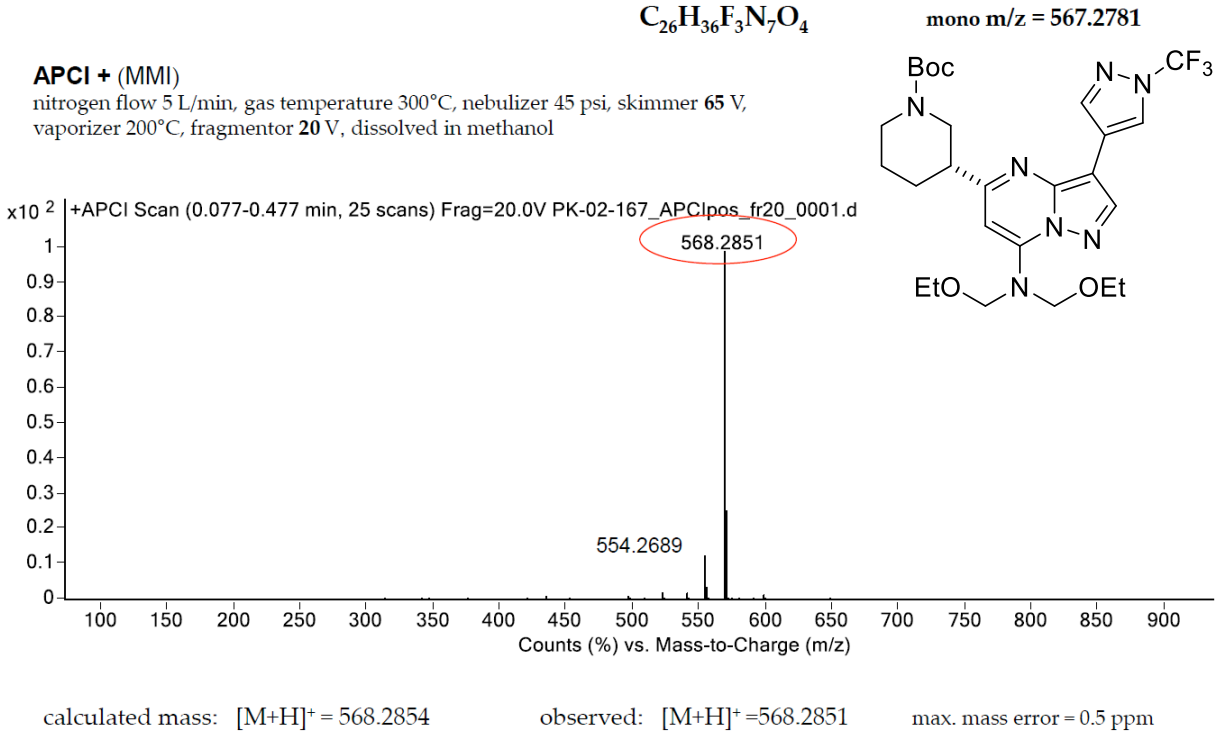
^{1}H NMR (300MHz) spectrum of compound **10** in $CDCl_3$



^{13}C NMR (126 MHz) spectrum of compound **10** in $CDCl_3$

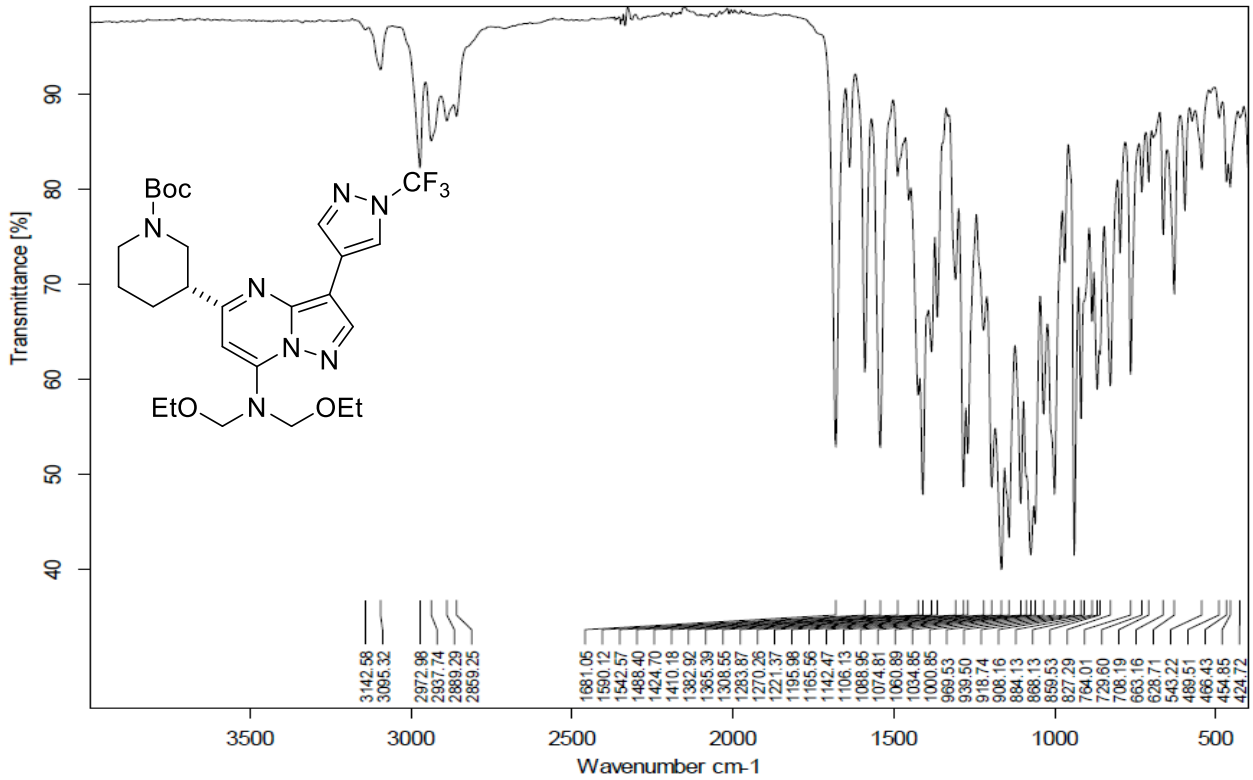


^{19}F NMR (282MHz) spectrum of compound **10** in $CDCl_3$

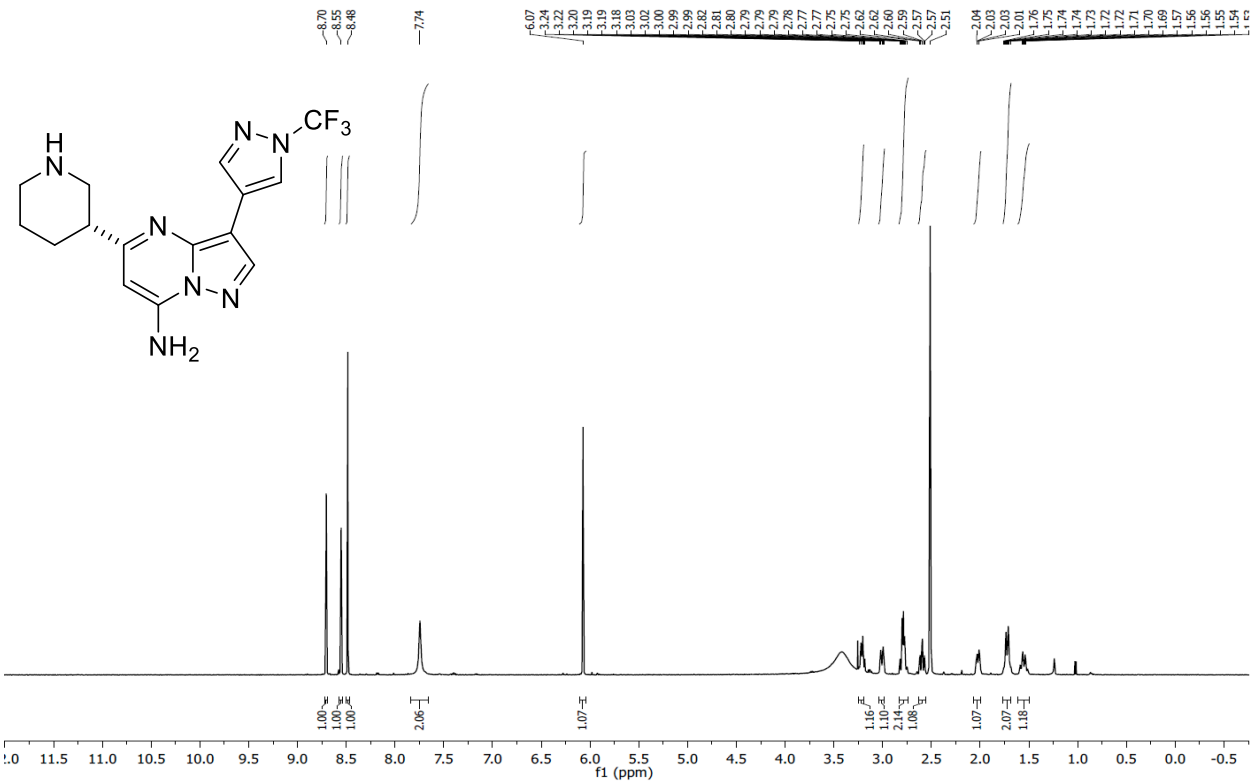


calculated mass: $[M+H]^+ = 568.2854$ observed: $[M+H]^+ = 568.2851$ max. mass error = 0.5 ppm

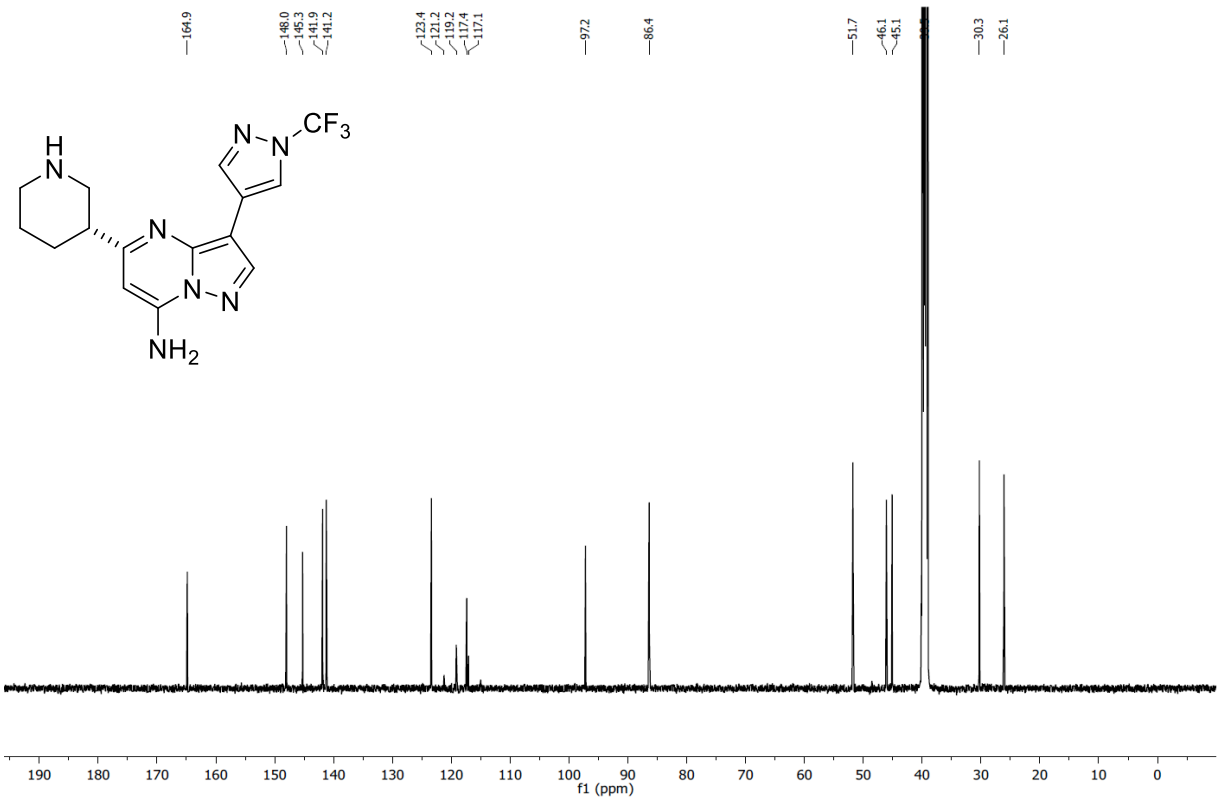
HRMS of compound **7**



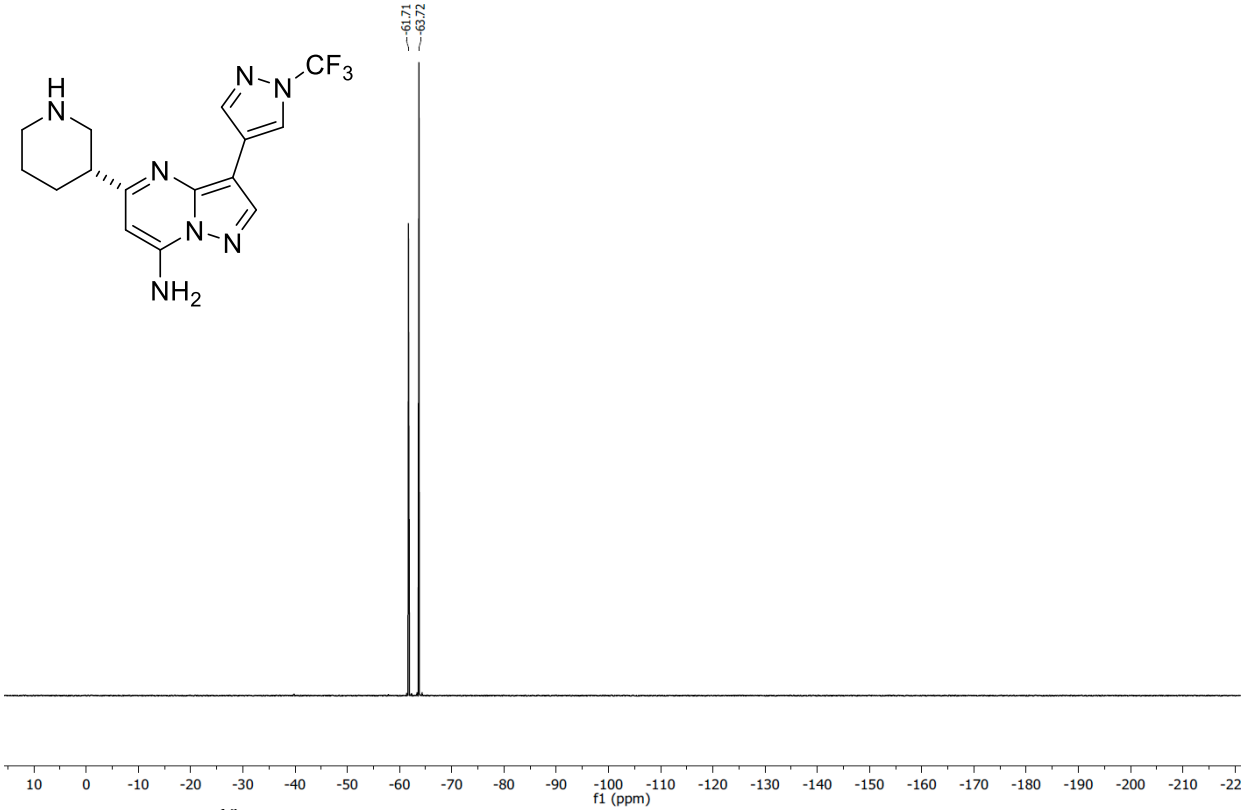
IR spectrum of compound **10**



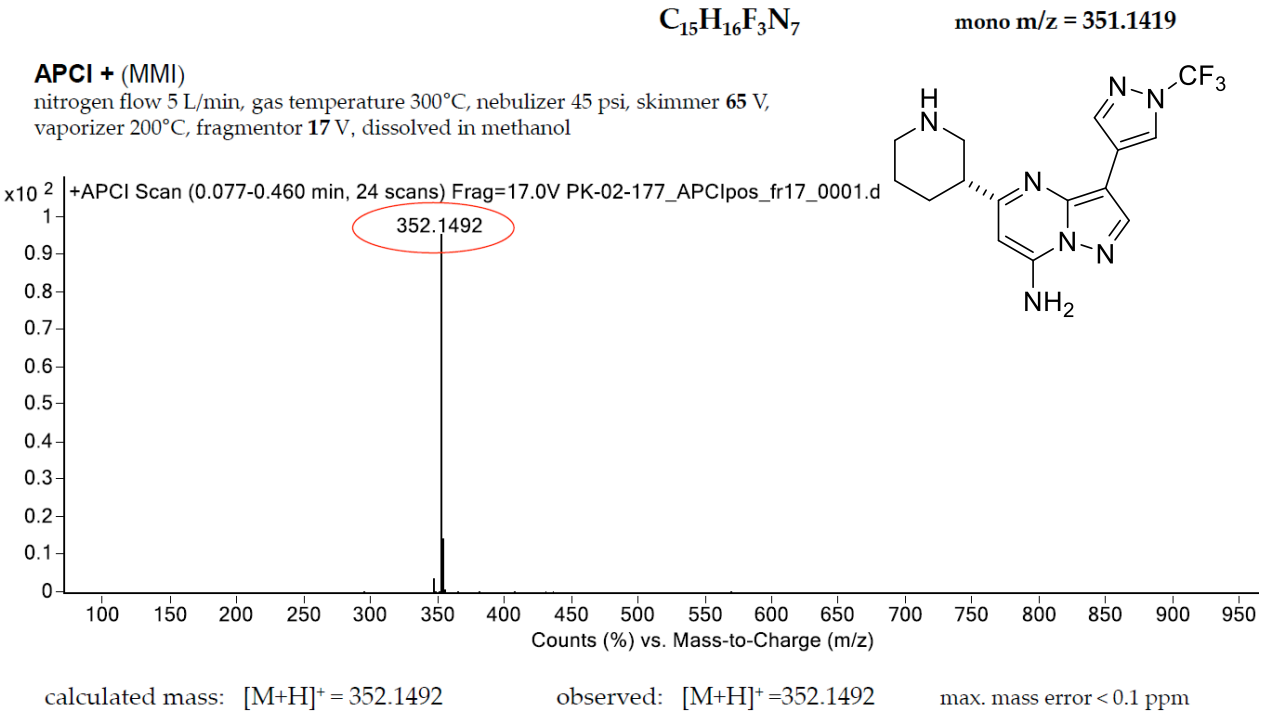
^{1}H NMR (500MHz) spectrum of compound **11** in DMSO-*d6*



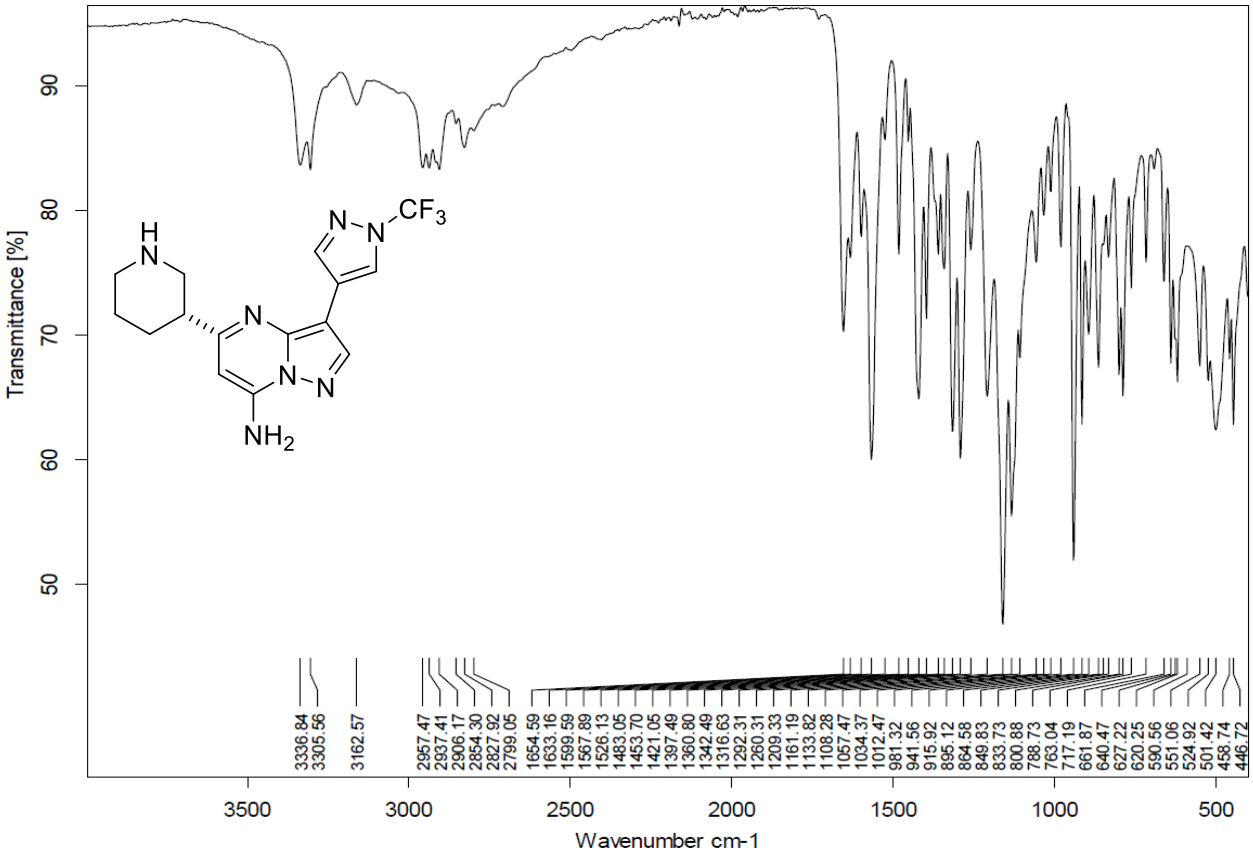
^{13}C NMR (126 MHz) spectrum of compound **11** in DMSO-*d6*



^{19}F NMR (282MHz) spectrum of compound **11** in DMSO-*d6*



HRMS of compound **11**



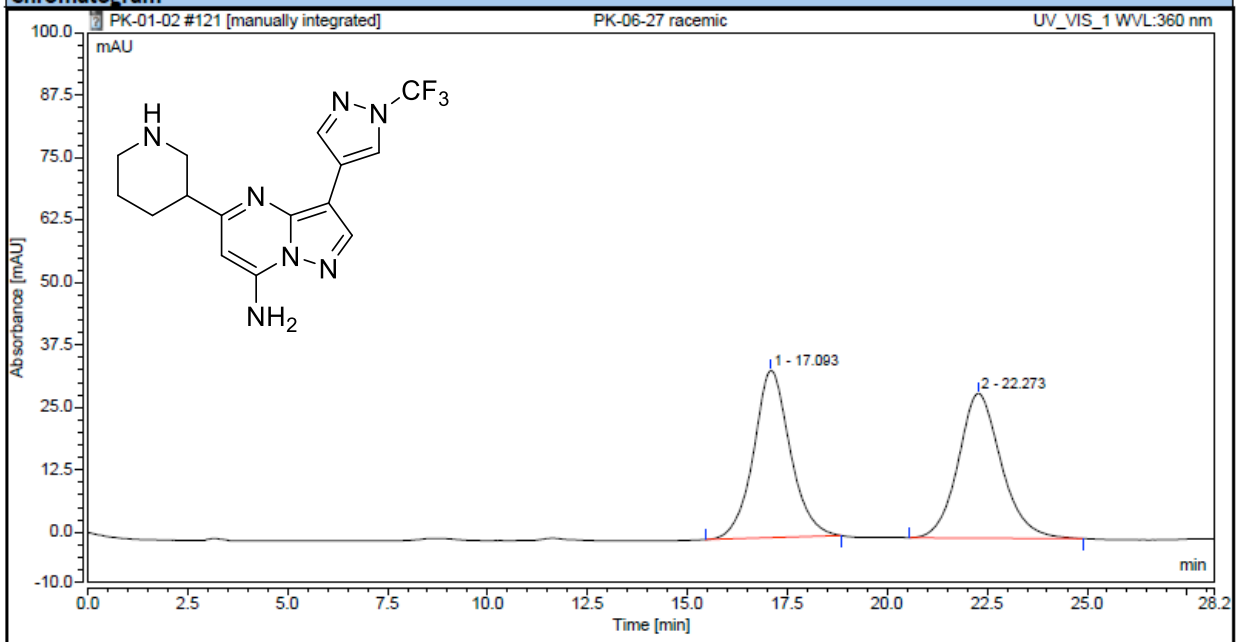
IR spectrum of compound **11**

Chromatogram and Results

Injection Details

Injection Name:	**PK-06-27 racemic**	*Run Time (min):*	**28.20**
Vial Number:	**121**	*Injection Volume:*	**20.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Flow Rate	**1 mL/min**	*Wavelength:*	**360 nm**
Instrument Method:	**PK_Analytical Method**	*Bandwidth:*	**10**
Processing Method:	**New Processing Method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**19.2.16 17:20**	*Sample Weight:*	**1.0000**
Mobile phase	**Hexane/IPA 90/10 + DEA 0.5 %**	*Column*	**CHIRALPAK AD**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		17.093	35.579	33.468	49.59	53.62	n.a.
2		22.273	36.163	28.950	50.41	46.38	n.a.
Total:			**71.741**	**62.418**	**100.00**	**100.00**	

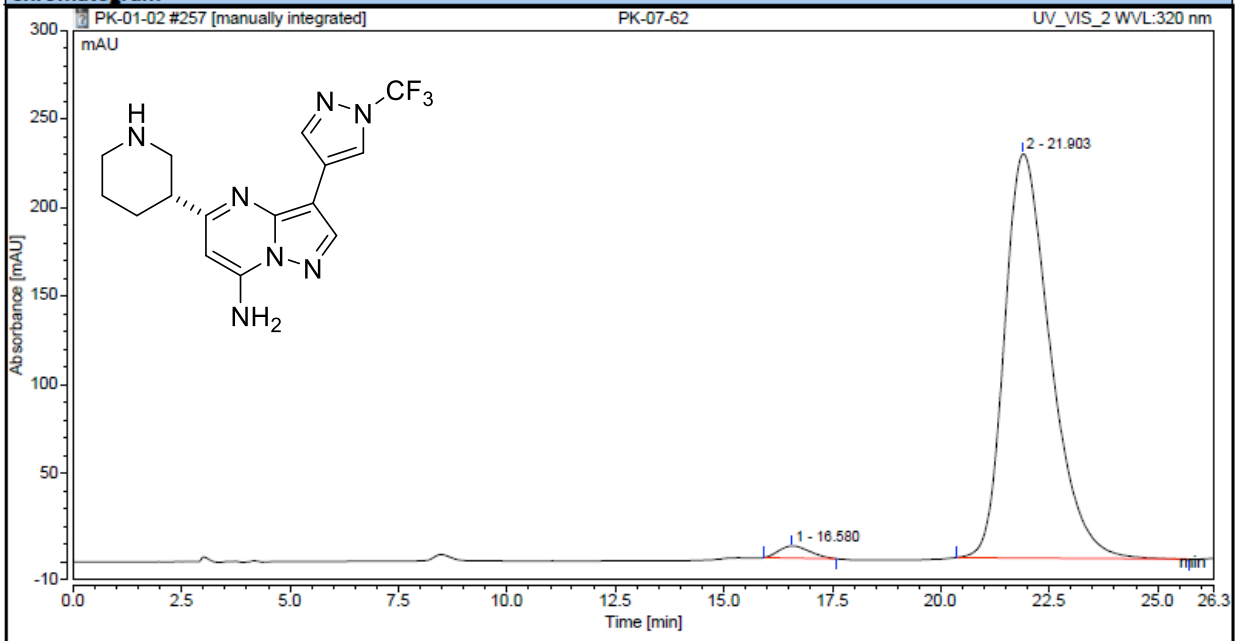
HPLC chromatogram of compound **11** (racemic)

Chromatogram and Results

Injection Details

Injection Name:	**PK-07-62**	*Run Time (min):*	**26.31**
Vial Number:	**262**	*Injection Volume:*	**20.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_2**
Flow Rate	**1 mL/min**	*Wavelength:*	**360 nm**
Instrument Method:	**PK_Analytical Method**	*Bandwidth:*	**10**
Processing Method:	**New Processing Method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**29.9.16 15:07**	*Sample Weight:*	**1.0000**
Mobile phase	**Hexane/IPA 90/10 + DEA 0.5 %**	*Column*	**CHIRALPAK AD**

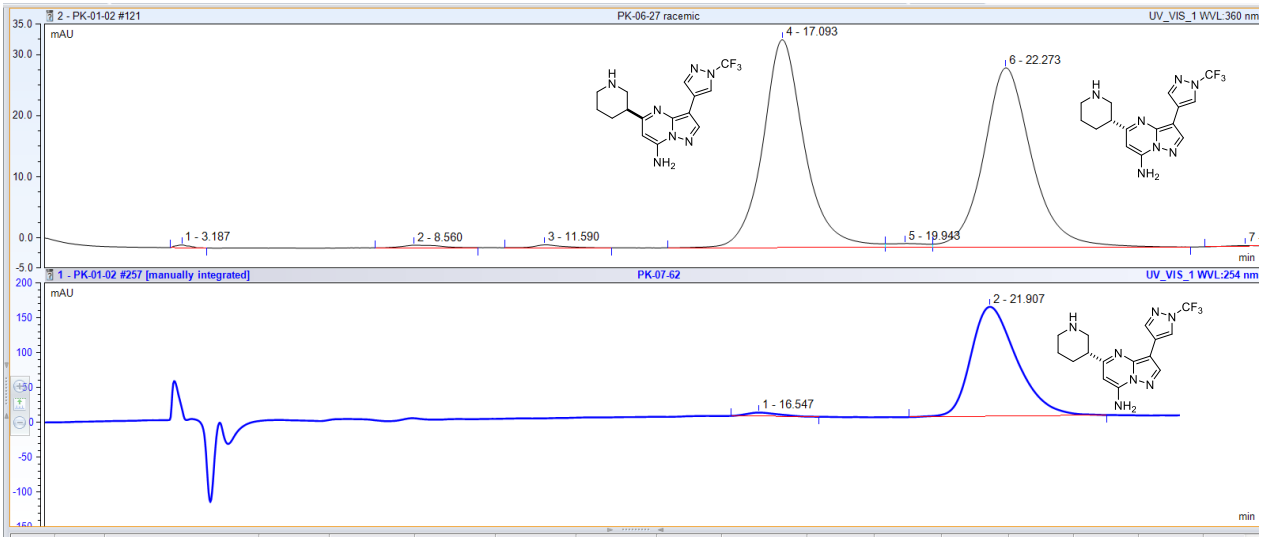
Chromatogram



Integration Results

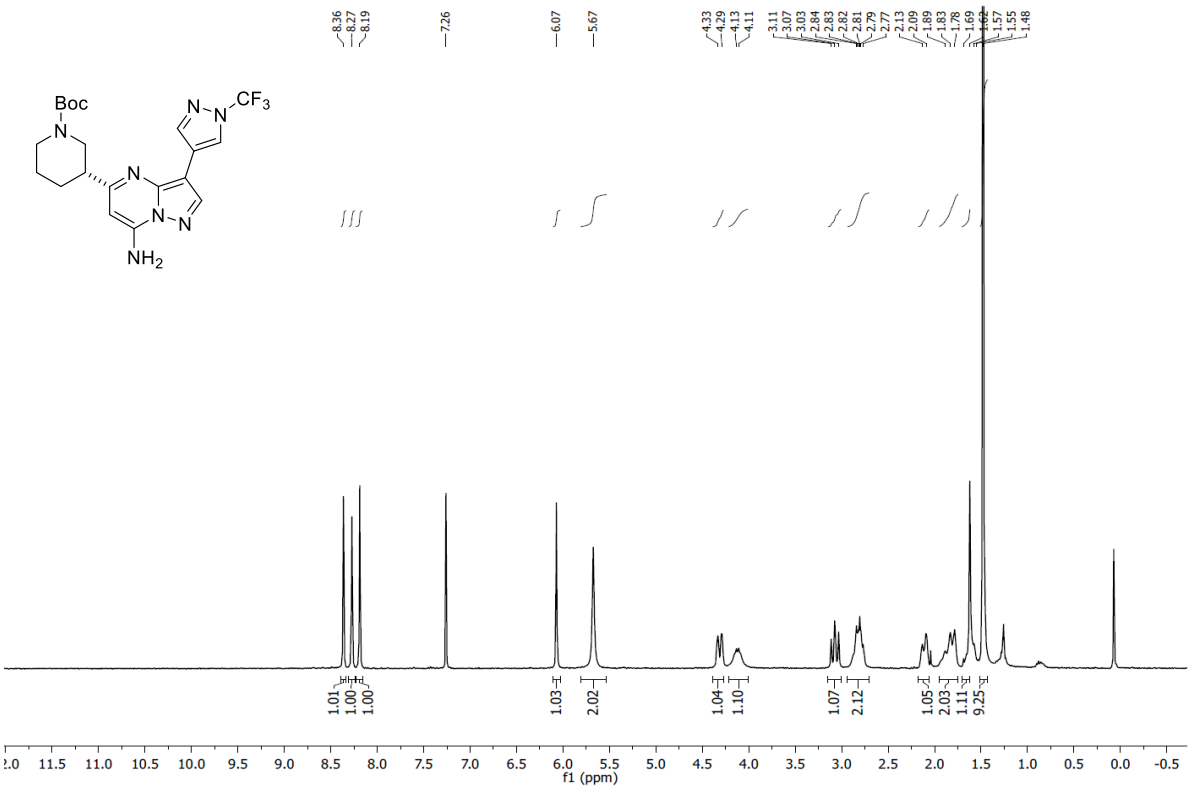
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		16.580	5.554	6.803	1.95	2.90	n.a.
2		21.903	279.679	228.116	98.05	97.10	n.a.
Total:			**285.233**	**234.919**	**100.00**	**100.00**	

HPLC chromatogram of compound **11** (non-racemic)

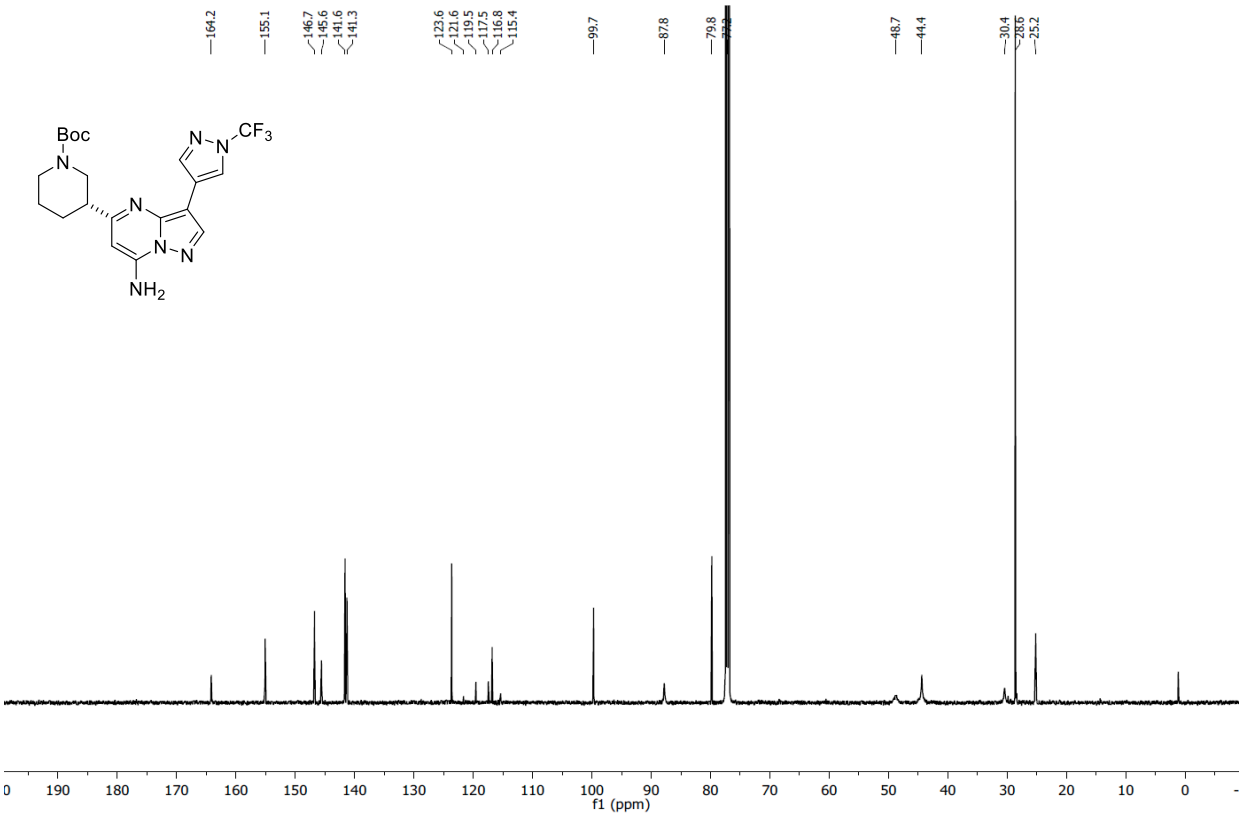


	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP				
4	1		16.547	n.a.	2.21	4.3605	5.08	BMB*	0.821	1.39	3.26	2251				
5	2		21.907	n.a.	97.79	192.8760	156.84	BMB*	1.119	1.36	n.a.	2124				
6	Maximum			0.0000	97.79	192.8760	156.84		1.119	1.39	3.26	2251				
7	Minimum			0.0000	2.21	4.3605	5.08		0.821	1.36	3.26	2124				
8	Sum			0.0000	100.00	197.2366	161.92									

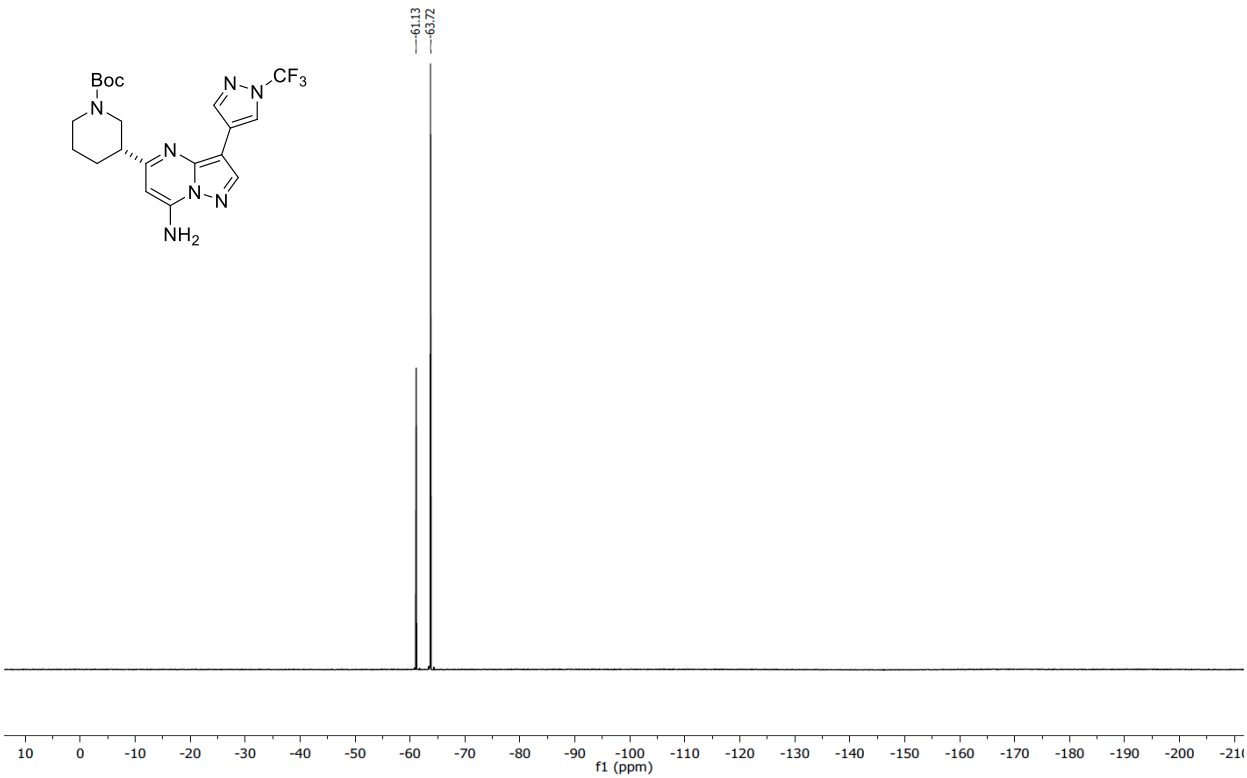
HPLC chromatogram of compound **11**



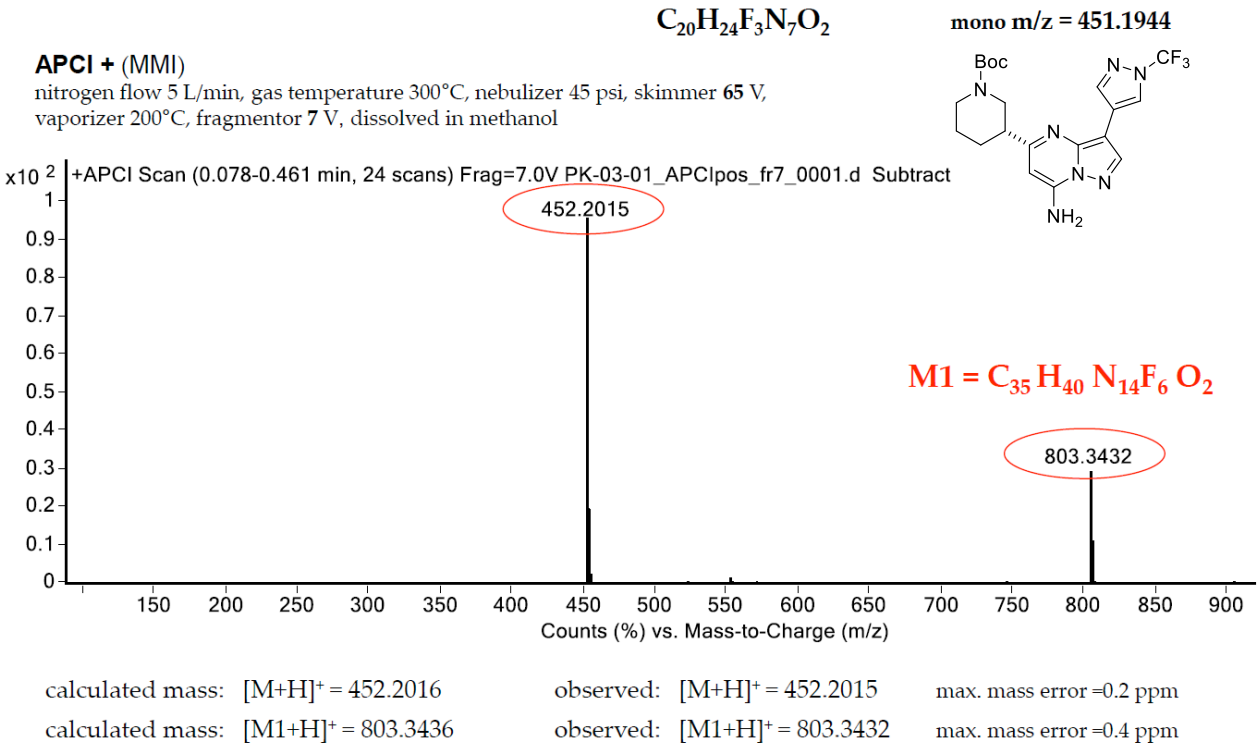
^{1}H NMR (300MHz) spectrum of compound **12** in $CDCl_3$



^{13}C NMR (126 MHz) spectrum of compound **12** in $CDCl_3$



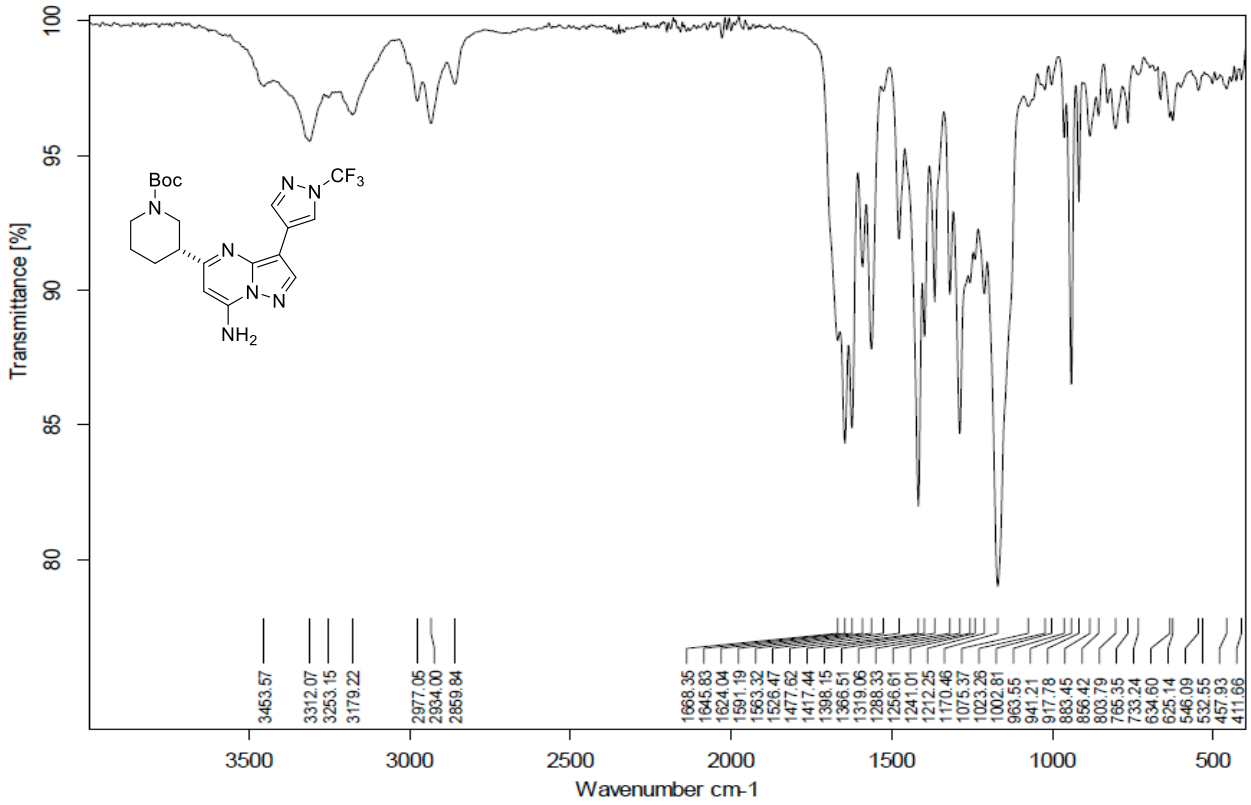
^{19}F NMR (282MHz) spectrum of compound **12** in $CDCl_3$



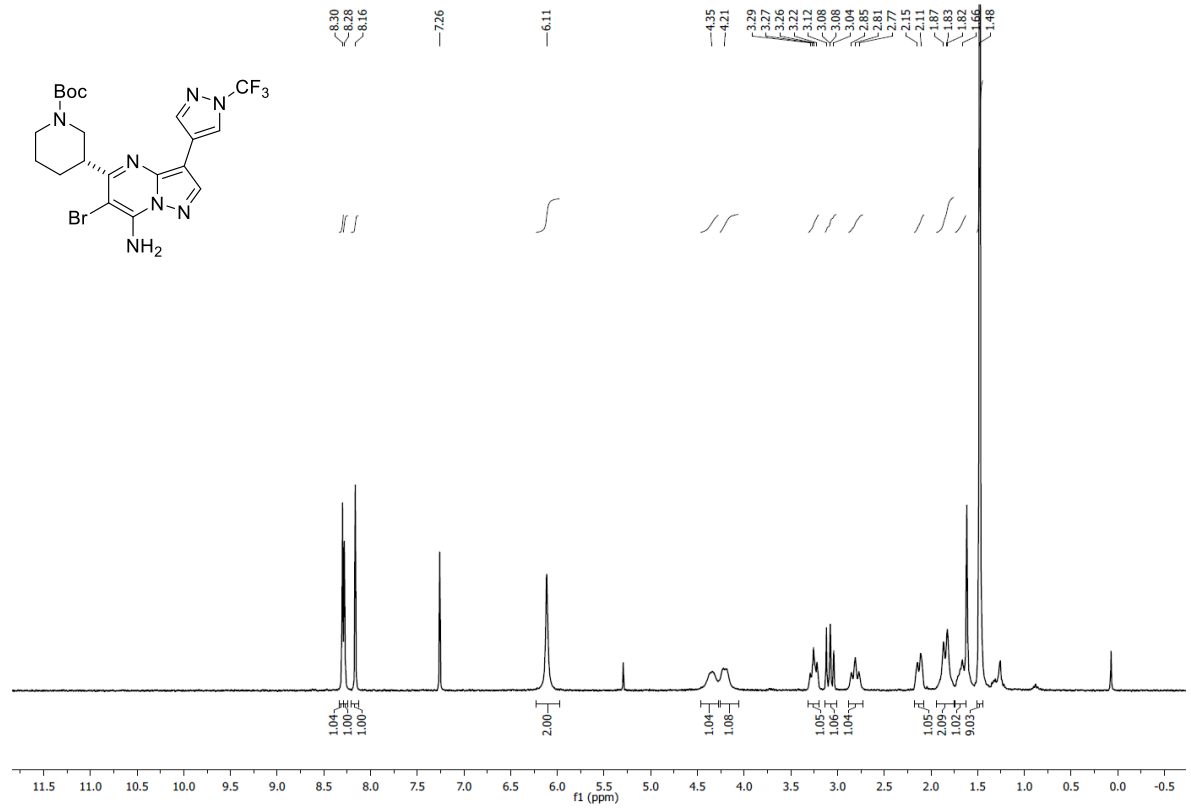
calculated mass: $[M+H]^+$ = 452.2016 observed: $[M+H]^+$ = 452.2015 max. mass error =0.2 ppm

calculated mass: $[M1+H]^+$ = 803.3436 observed: $[M1+H]^+$ = 803.3432 max. mass error =0.4 ppm

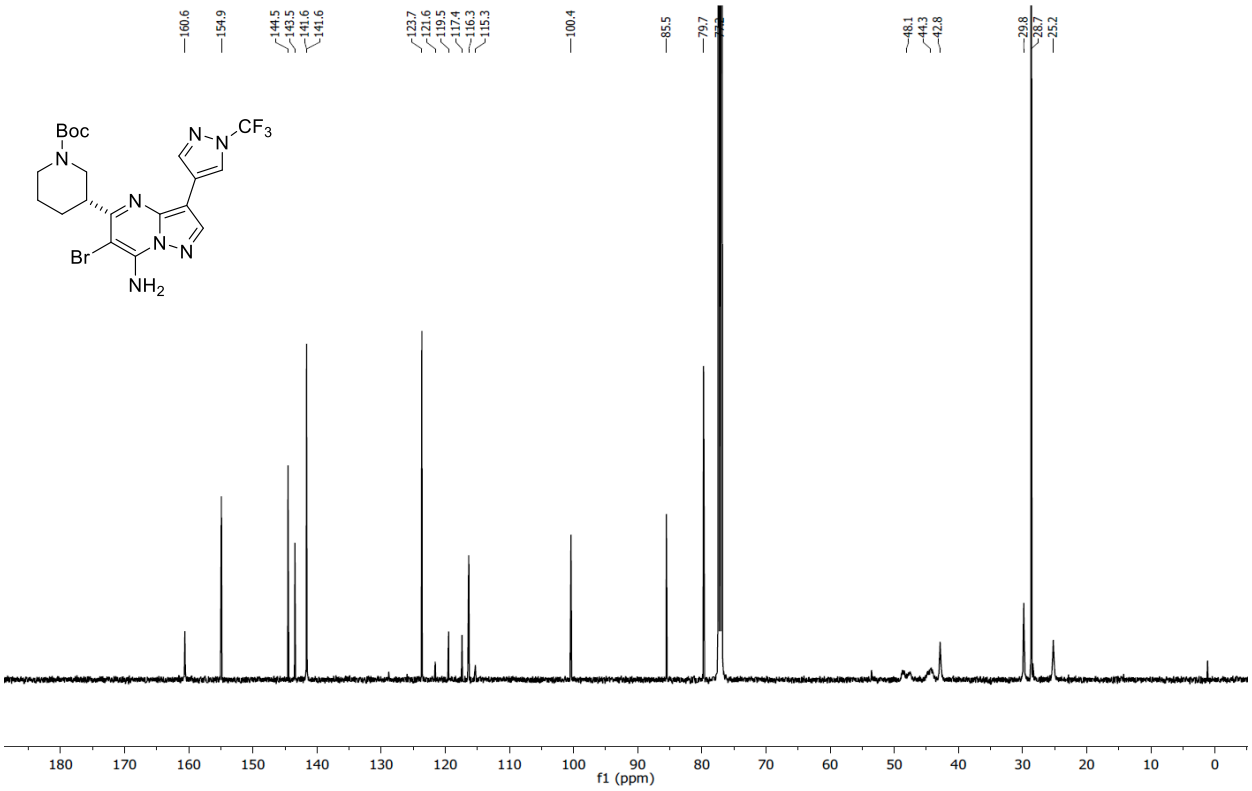
HRMS of compound **12**



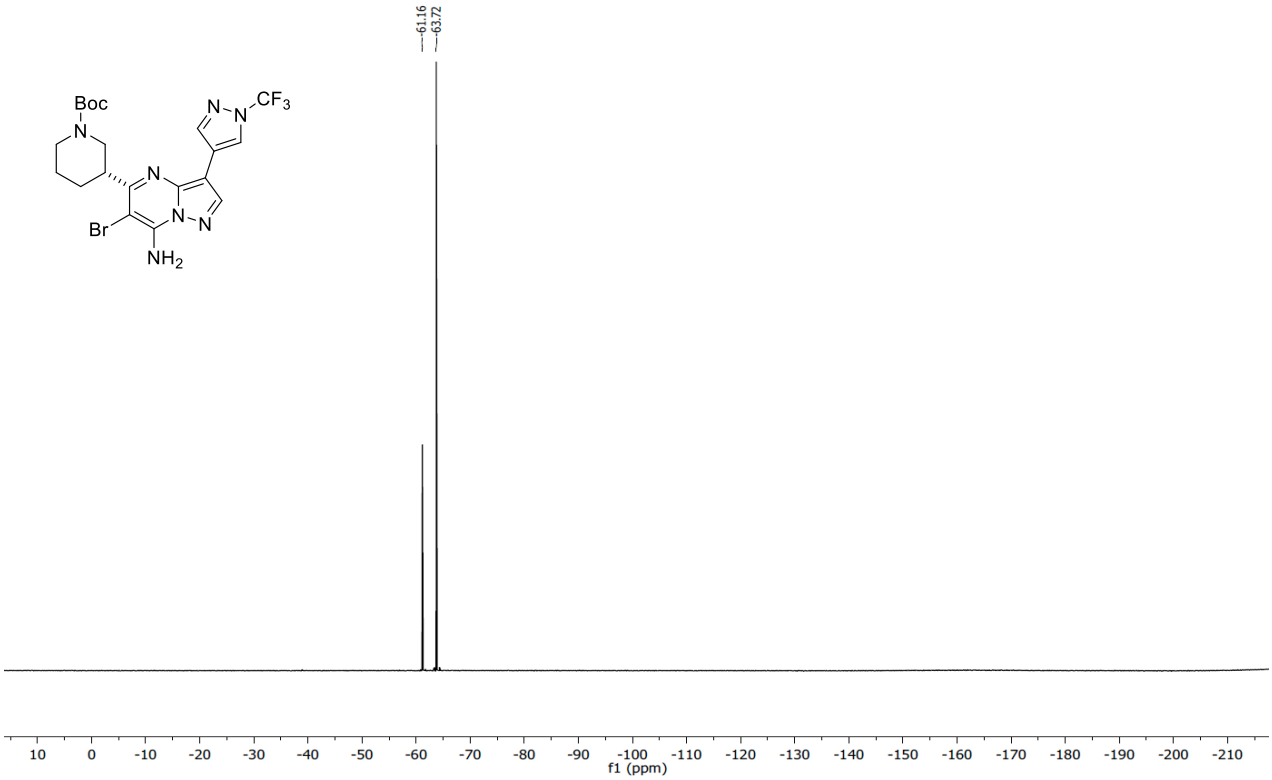
IR spectrum of compound **12**



^{1}H NMR (300MHz) spectrum of compound **13** in $CDCl_3$



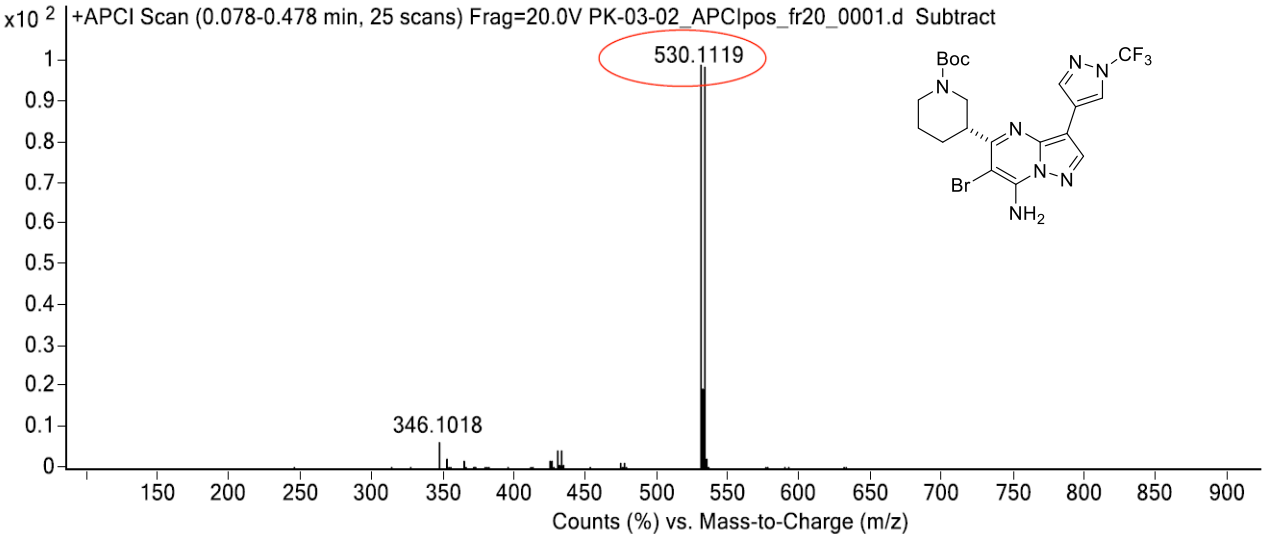
^{13}C NMR (126 MHz) spectrum of compound **13** in $CDCl_3$



^{19}F NMR (282MHz) spectrum of compound **13** in $CDCl_3$

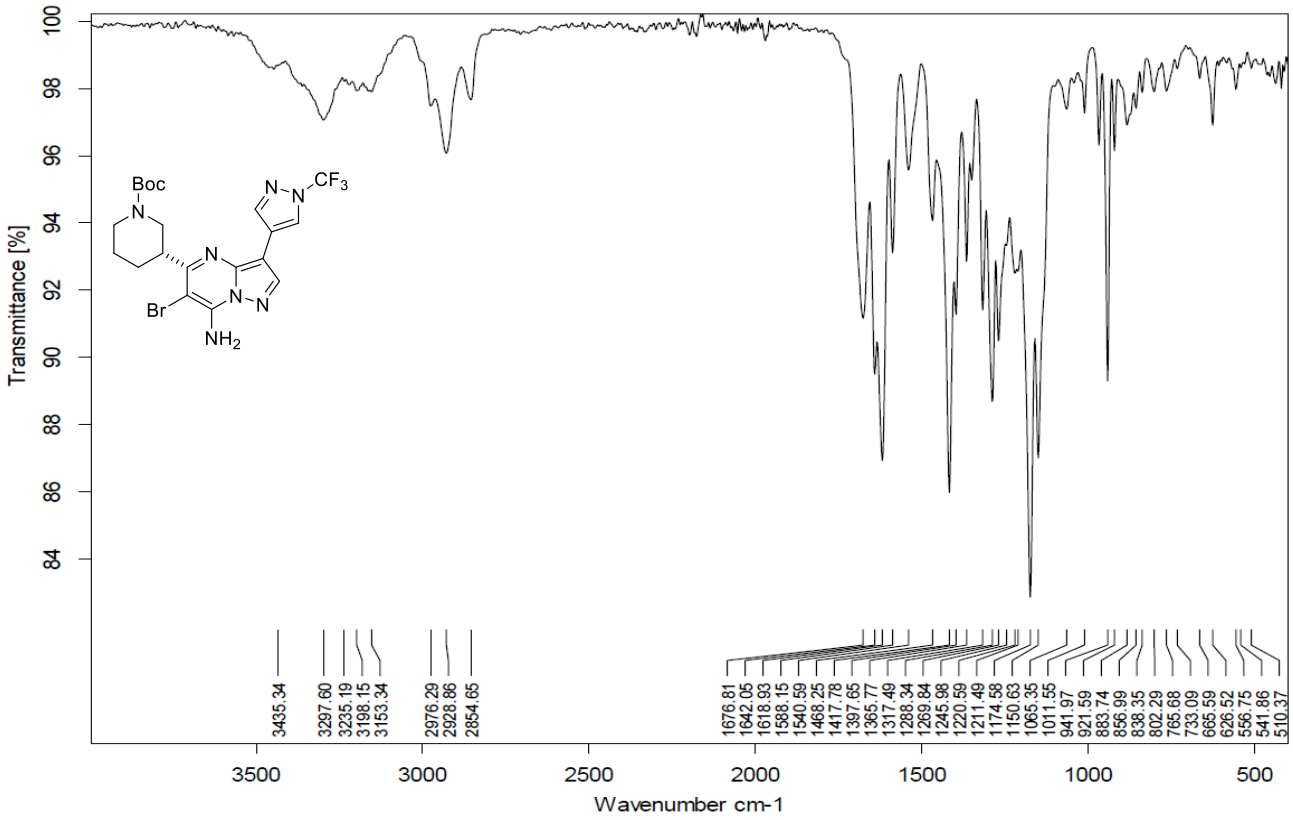
$C_{20}H_{23}BrF_3N_7O_2$ **mono m/z = 529.1049**

APCI + (MMI)
nitrogen flow 5 L/min, gas temperature 300°C, nebulizer 45 psi, skimmer **65** V,
vaporizer 200°C, fragmentor **20** V, dissolved in methanol

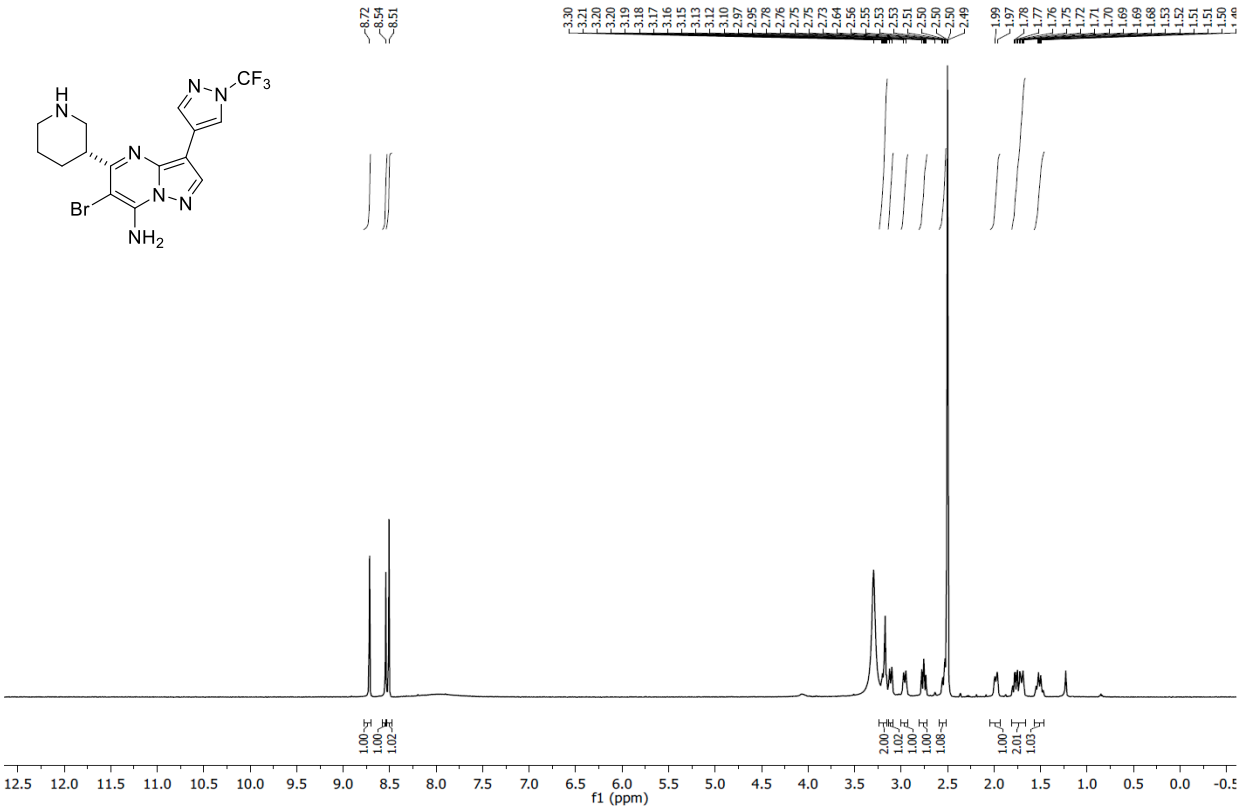


calculated mass: $[M+H]^+ = 530.1121$ observed: $[M+H]^+ = 530.1119$ max. mass error =0.3 ppm

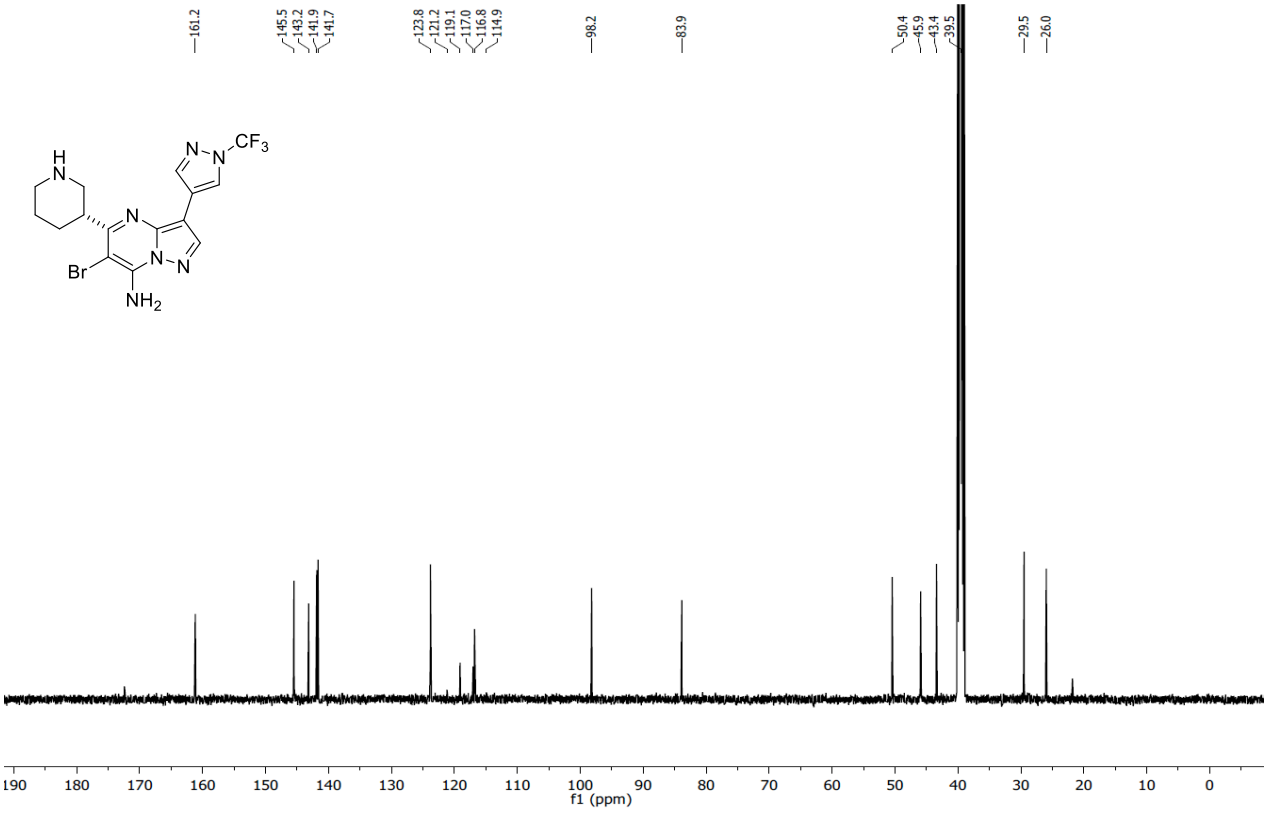
HRMS of compound **13**



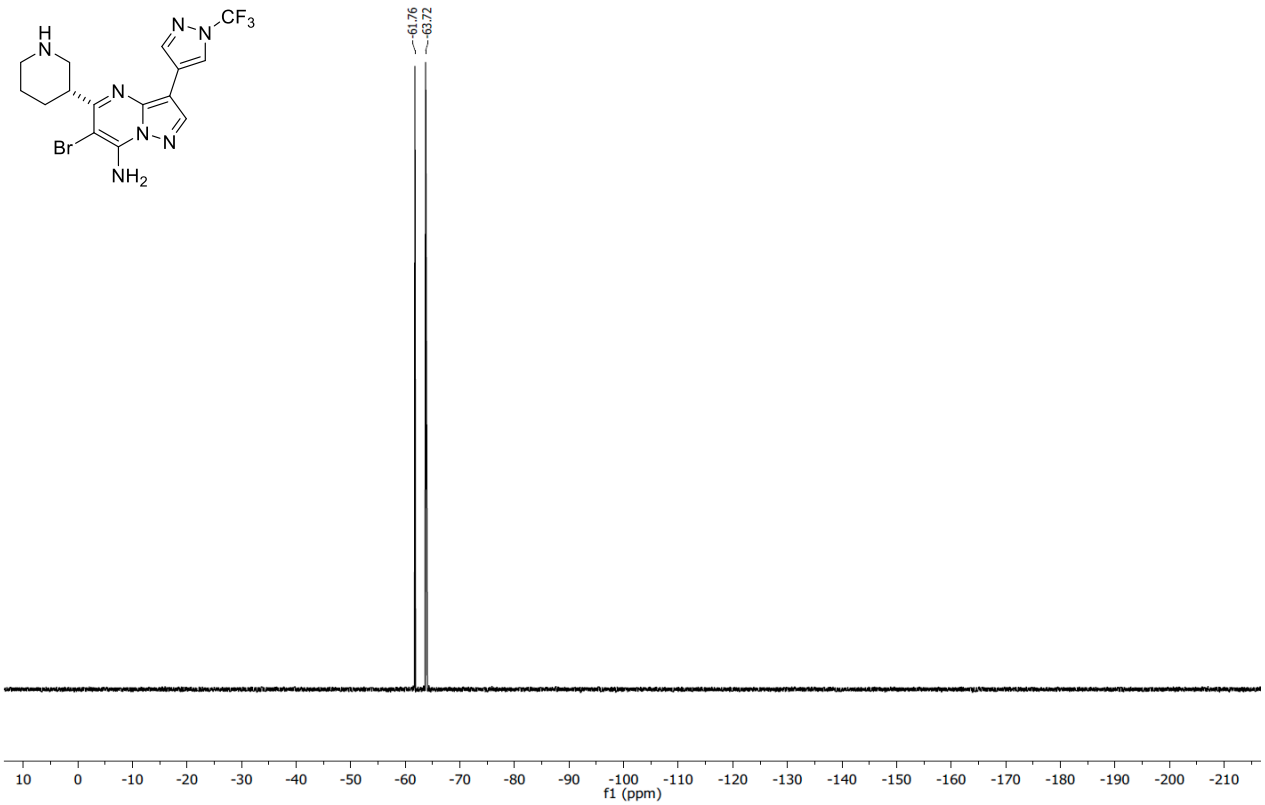
IR spectrum of compound **13**



^{1}H NMR (500MHz) spectrum of **MU380** in DMSO-*d6*



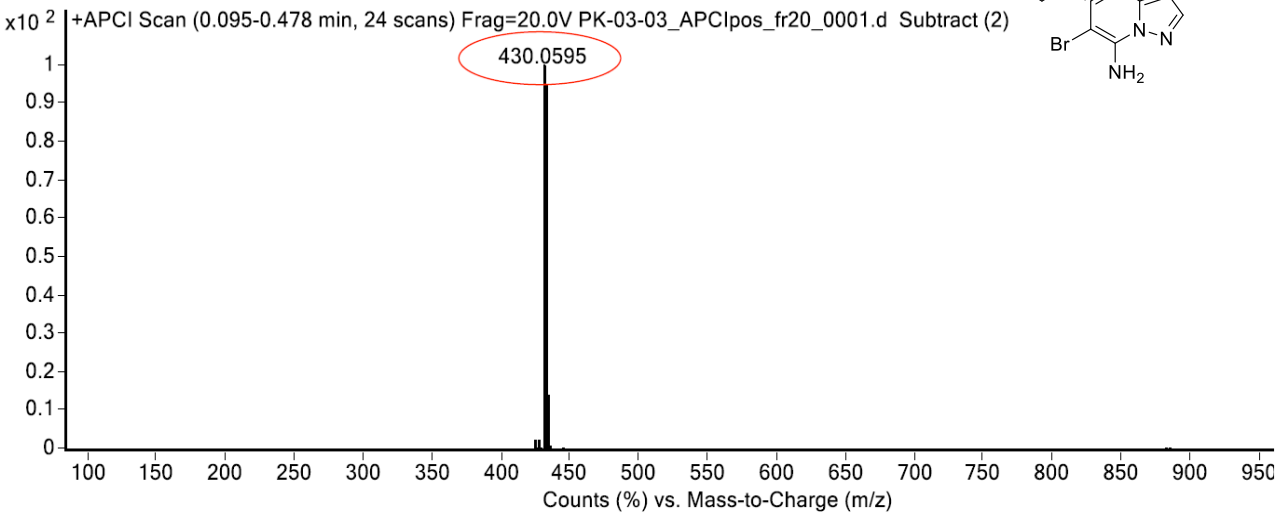
^{13}C NMR (126 MHz) spectrum of **MU380** in DMSO-*d6*



^{19}F NMR (282MHz) spectrum of **MU380** in DMSO-*d6*

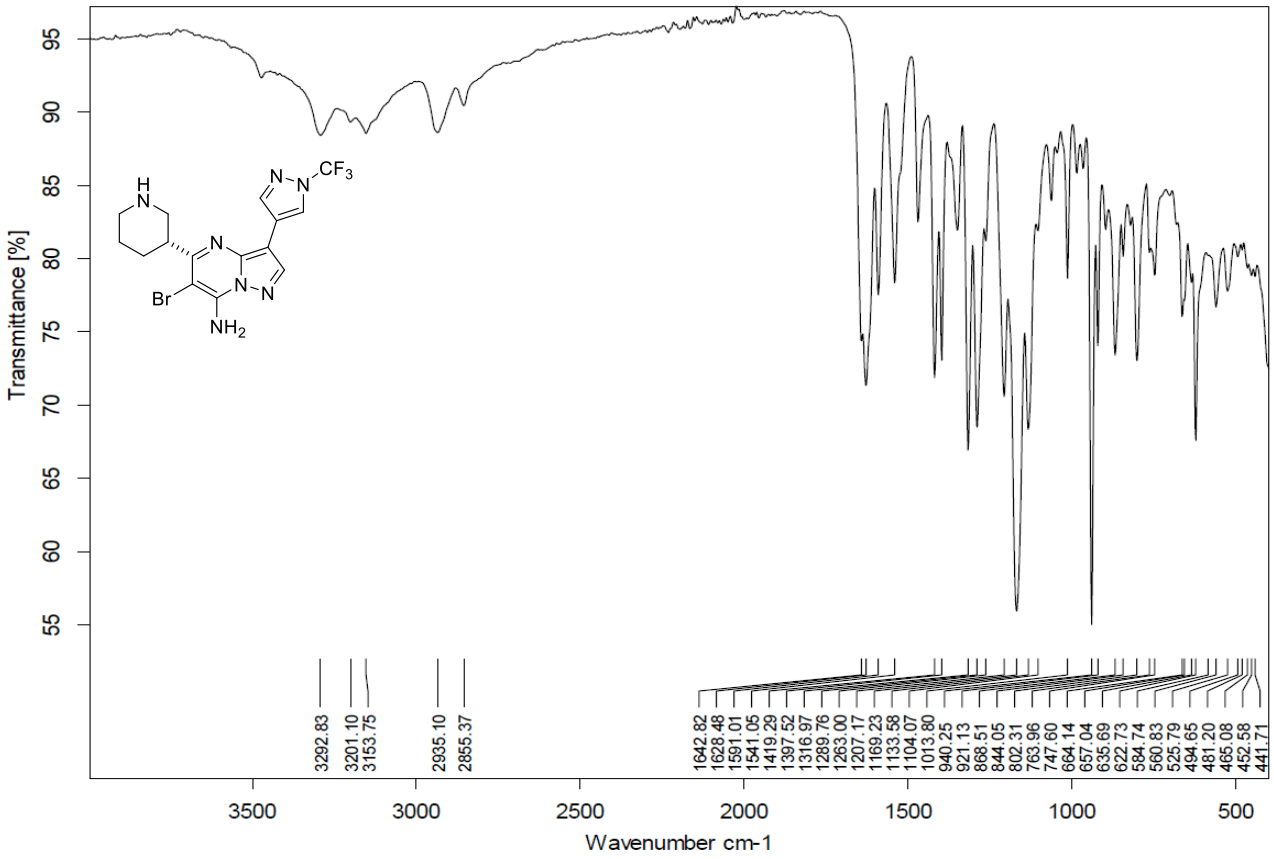
$C_{15}H_{15}BrF_3N_7$ **mono m/z = 429.0524**

APCI + (MMI)
nitrogen flow 5 L/min, gas temperature 300°C, nebulizer 45 psi, skimmer **65** V,
vaporizer 200°C, fragmentor **20** V, dissolved in methanol



calculated mass: $[M+H]^+ = 430.0597$ observed: $[M+H]^+ = 430.0595$ max. mass error =0.4 ppm

HRMS of **MU380**



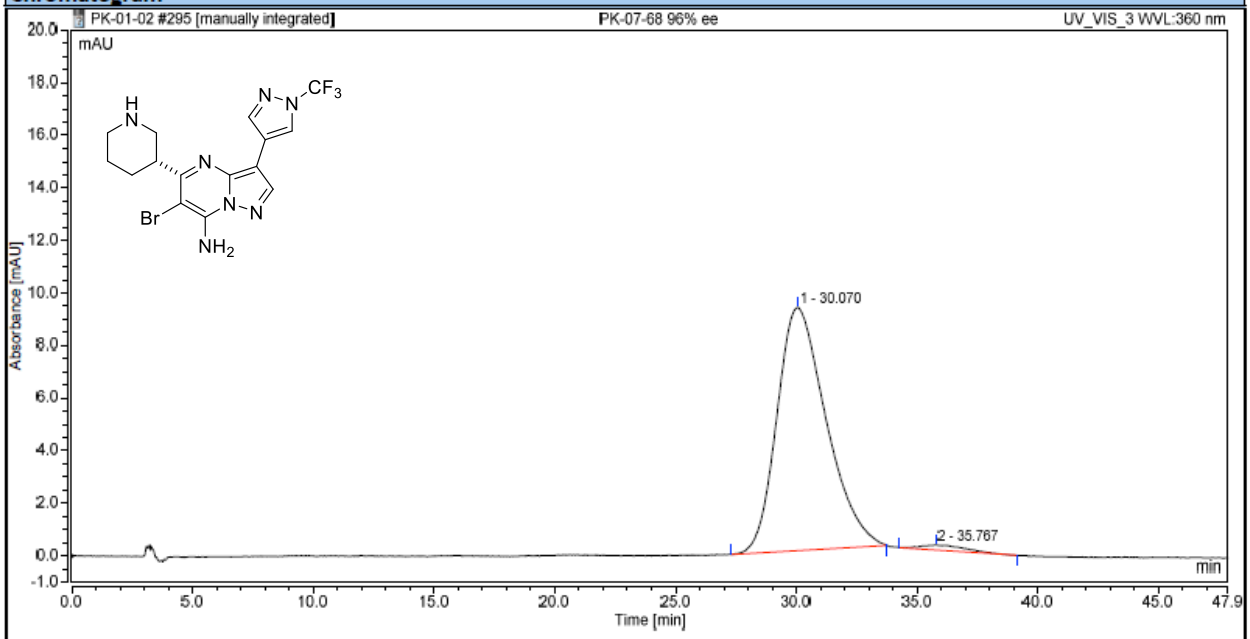
IR spectrum of **MU380**

Chromatogram and Results

Injection Details

Injection Name:	**PK-07-68 Without crystallization**	*Run Time (min):*	**47.88**
Vial Number:	**300**	*Injection Volume:*	**20.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_3**
Flow Rate	**1 mL/min**	*Wavelength:*	**360 nm**
Instrument Method:	**PK_Analytical Method**	*Bandwidth:*	**4**
Processing Method:	**New Processing Method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**06.10.16 19:41**	*Sample Weight:*	**1.0000**
Mobile phase	**Hexane/EtOH 95/05 + DEA 0.5 %**	*Column*	**CHIRALPAK OJ**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		30.070	21.835	9.257	98.12	97.94	n.a.
2		35.767	0.419	0.195	1.88	2.06	n.a.
Total:			**22.254**	**9.452**	**100.00**	**100.00**	

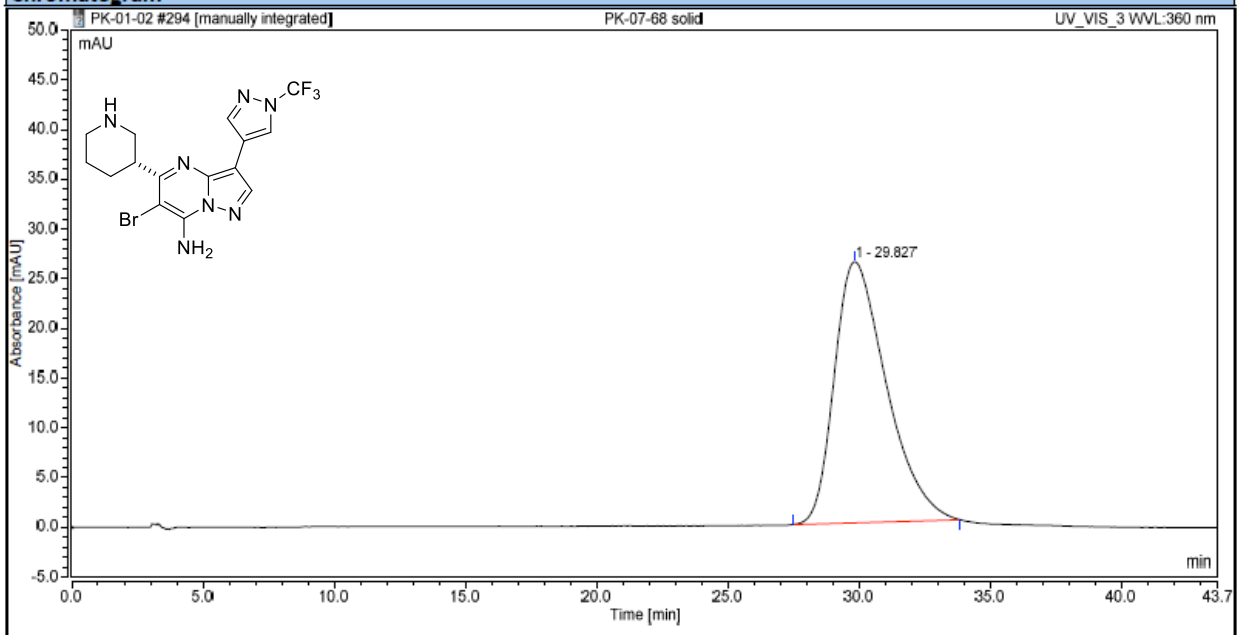
HPLC chromatogram of compound **MU380** (96% ee, before recrystallization)

Chromatogram and Results

Injection Details

Injection Name:	**PK-07-68 Solid**	*Run Time (min):*	**43.69**
Vial Number:	**299**	*Injection Volume:*	**20.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_3**
Flow Rate	**1 mL/min**	*Wavelength:*	**360 nm**
Instrument Method:	**PK_Analytical Method**	*Bandwidth:*	**4**
Processing Method:	**New Processing Method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**06.10.16 18:56**	*Sample Weight:*	**1.0000**
Mobile phase	**Hexane/EtOH 95/05 + DEA 0.5 %**	*Column*	**CHIRALPAK OJ**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		29.827	60.387	26.268	100.00	100.00	n.a.
Total:			**60.387**	**26.268**	**100.00**	**100.00**	

HPLC chromatogram of compound **MU380** (after recrystallization)