

Supplementary data

A new smoothened antagonist bearing the purine scaffold shows antitumor activity *in vitro* and *in vivo*

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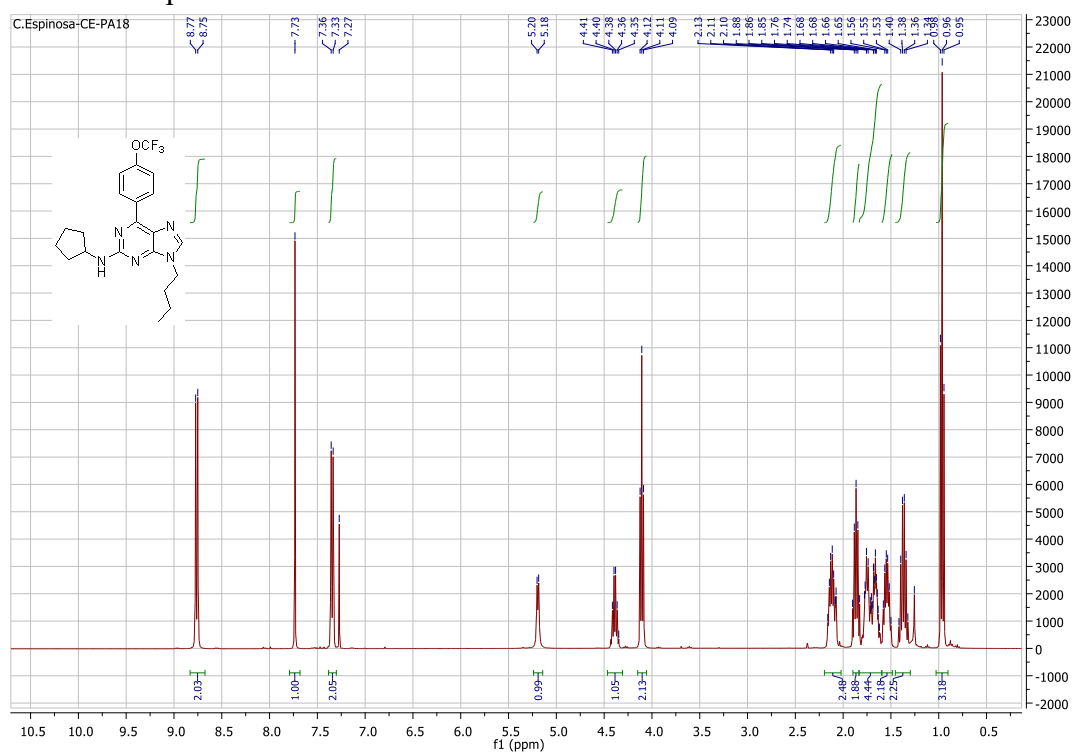
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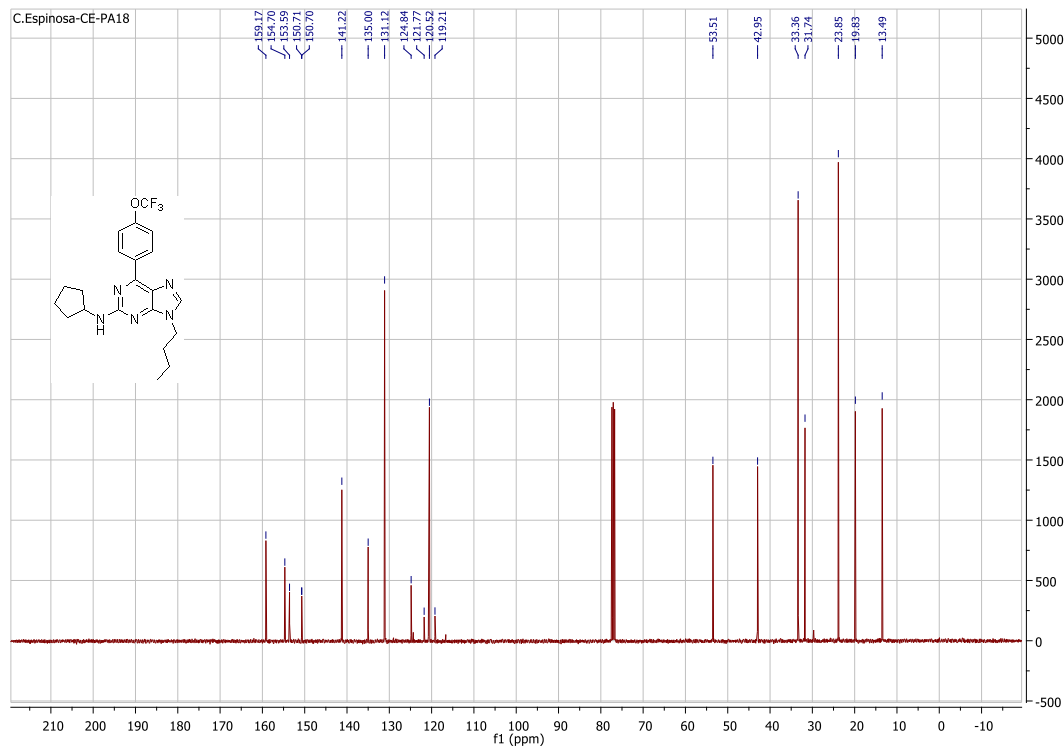
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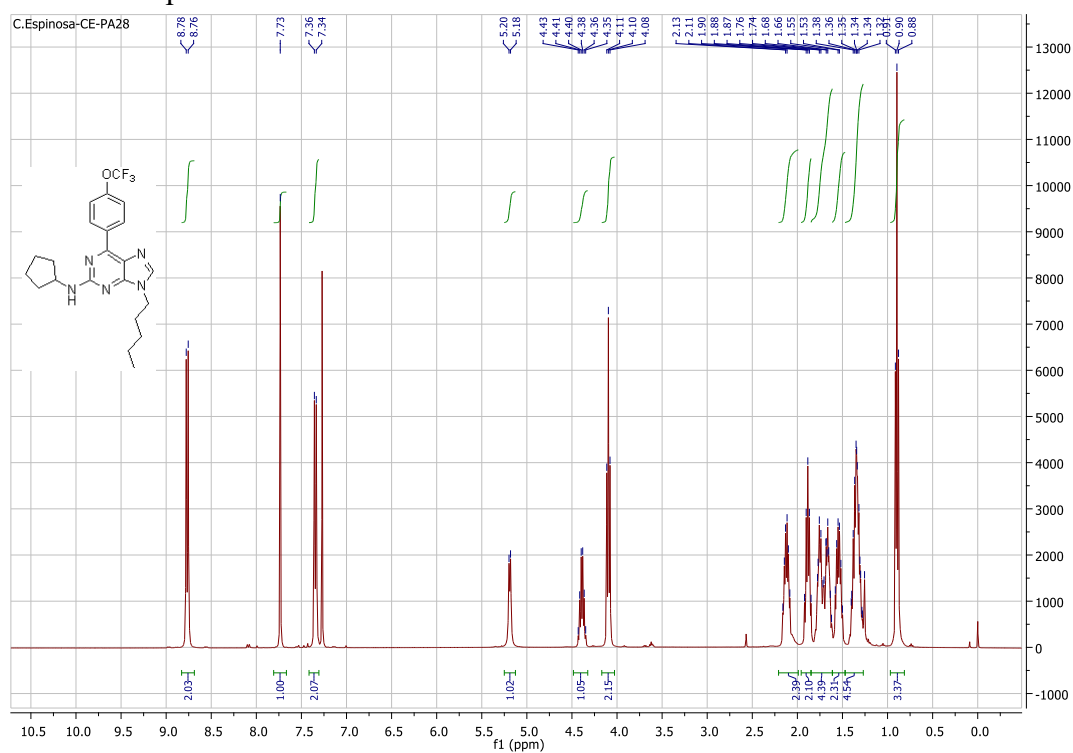
¹H NMR of compound **4a**



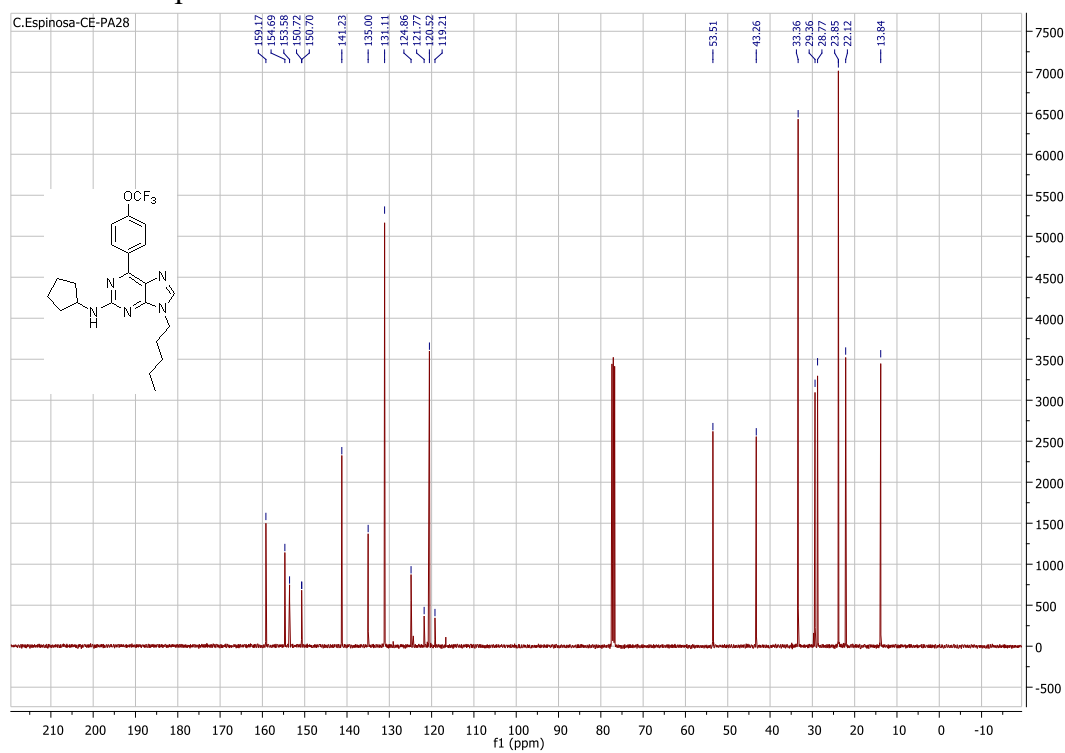
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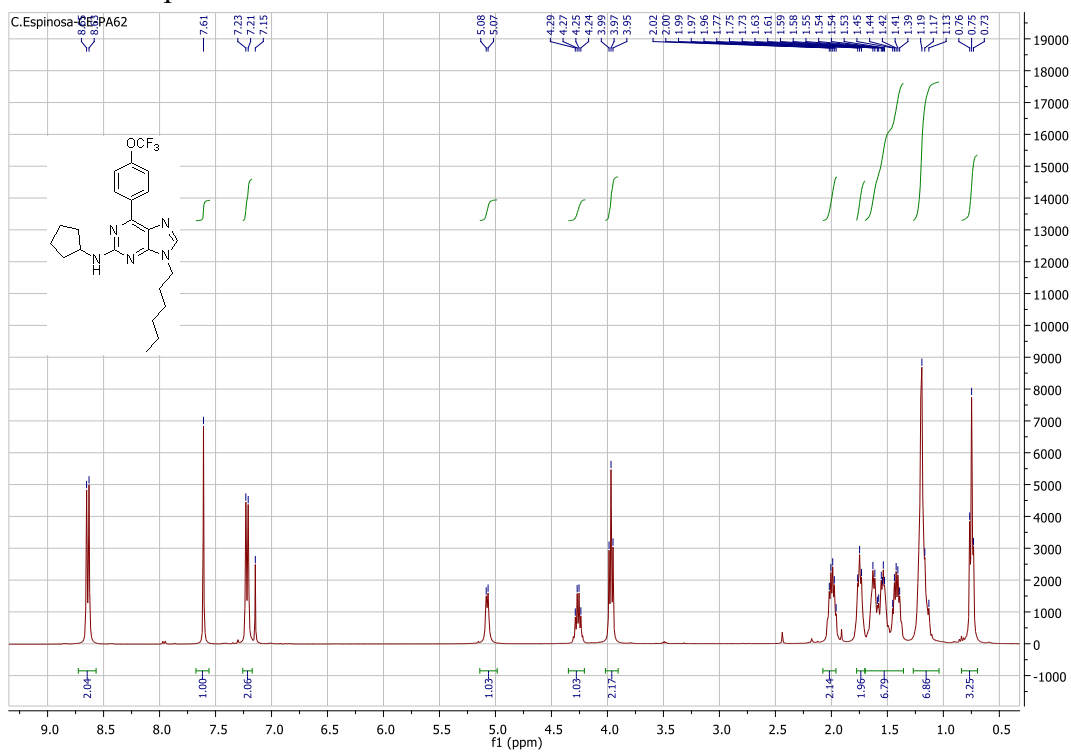
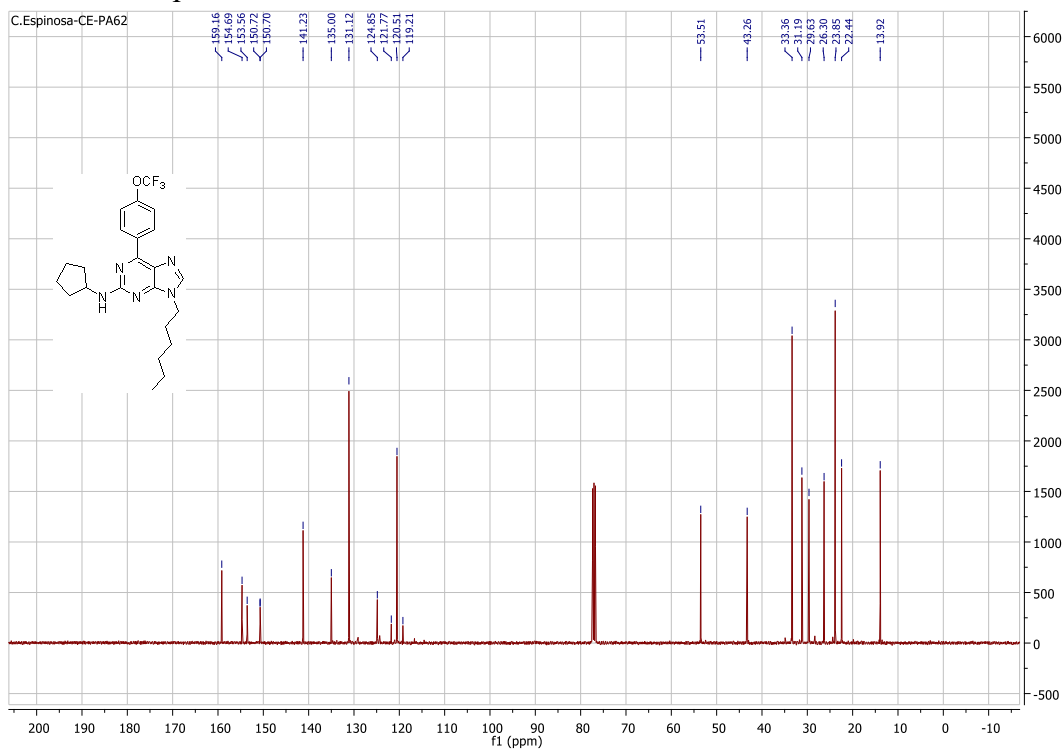


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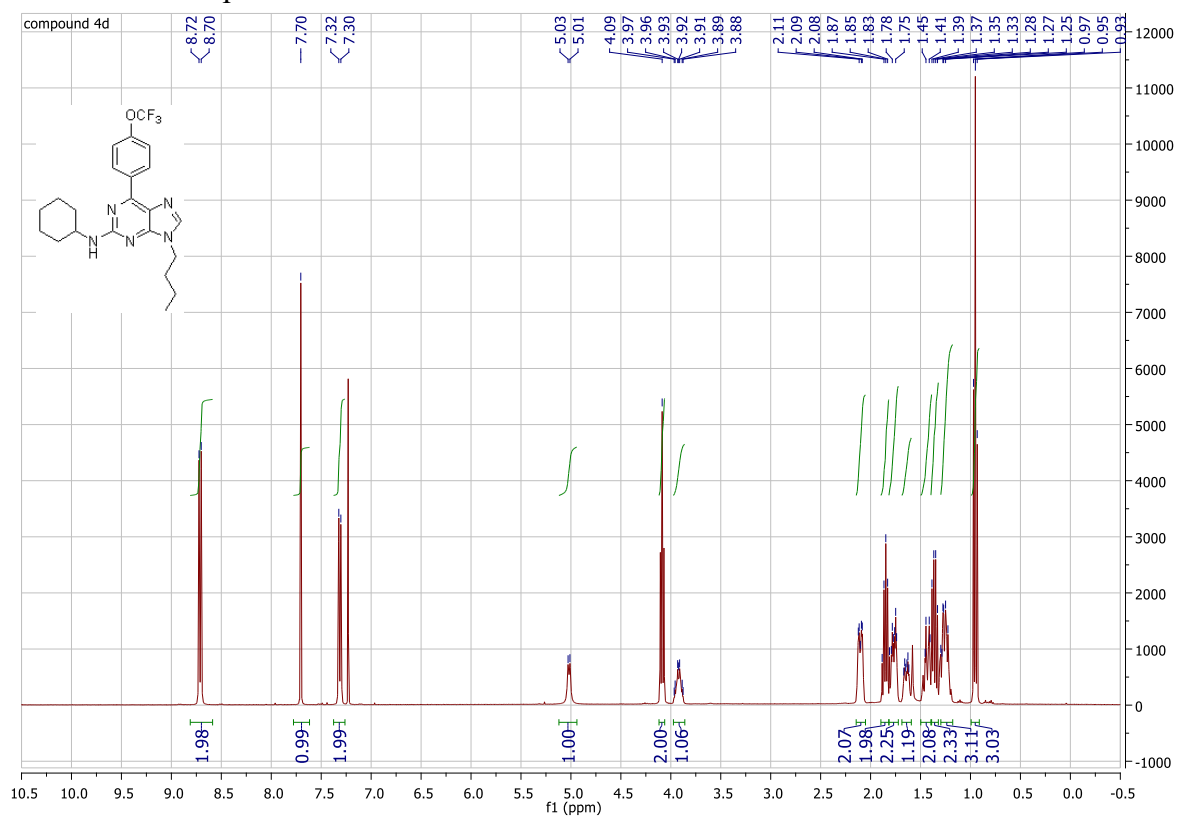


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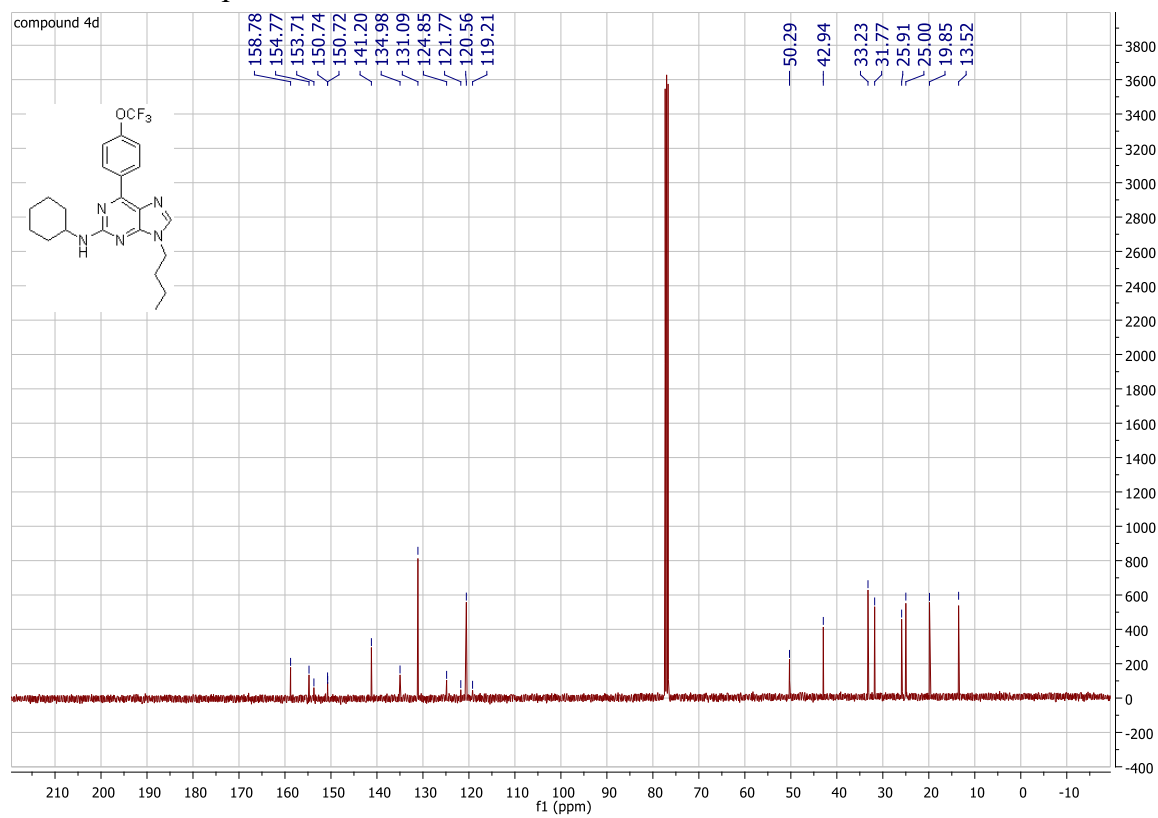


¹H NMR of compound **4c** ^{13}C NMR of compound **4c**

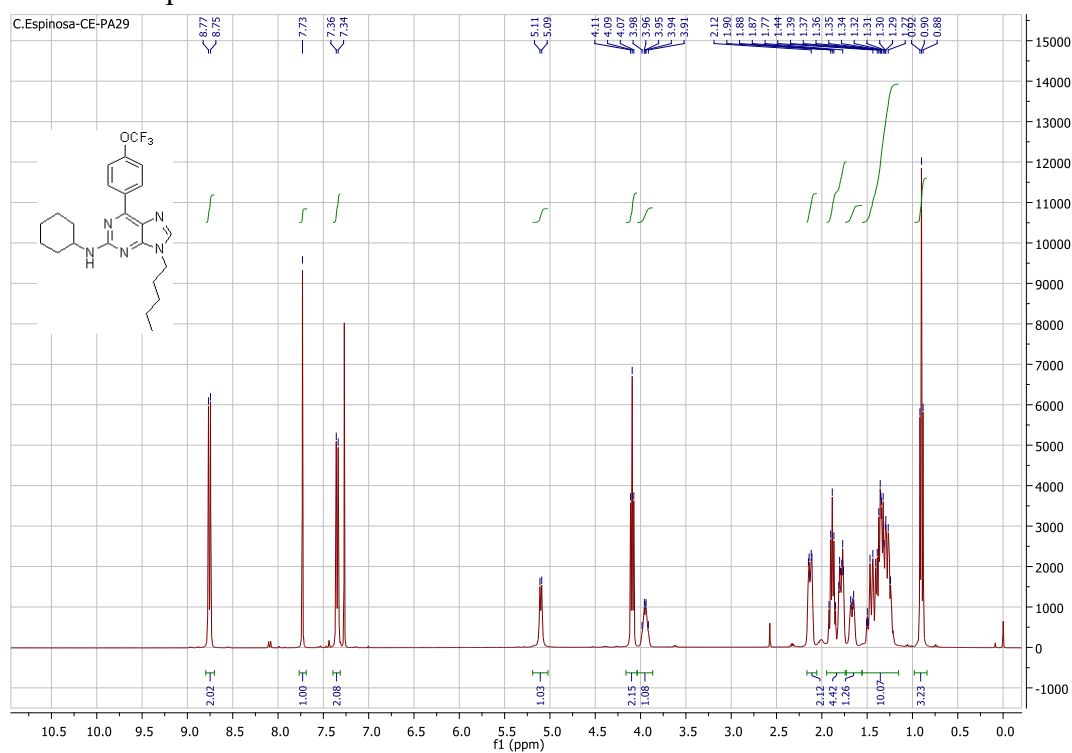
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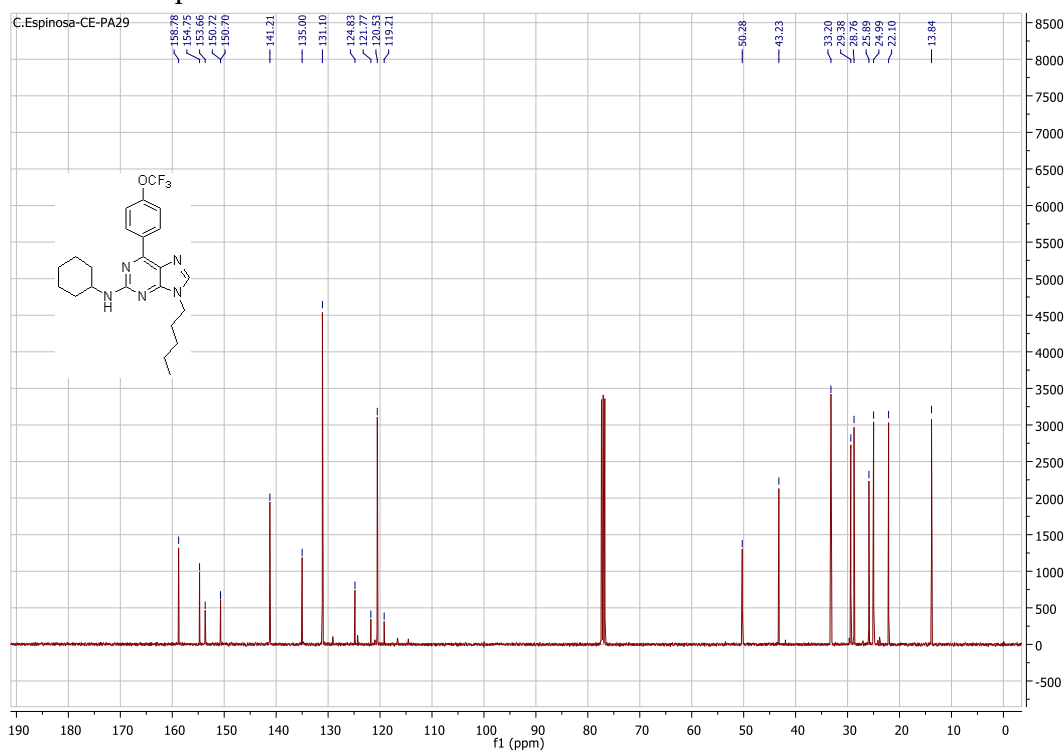
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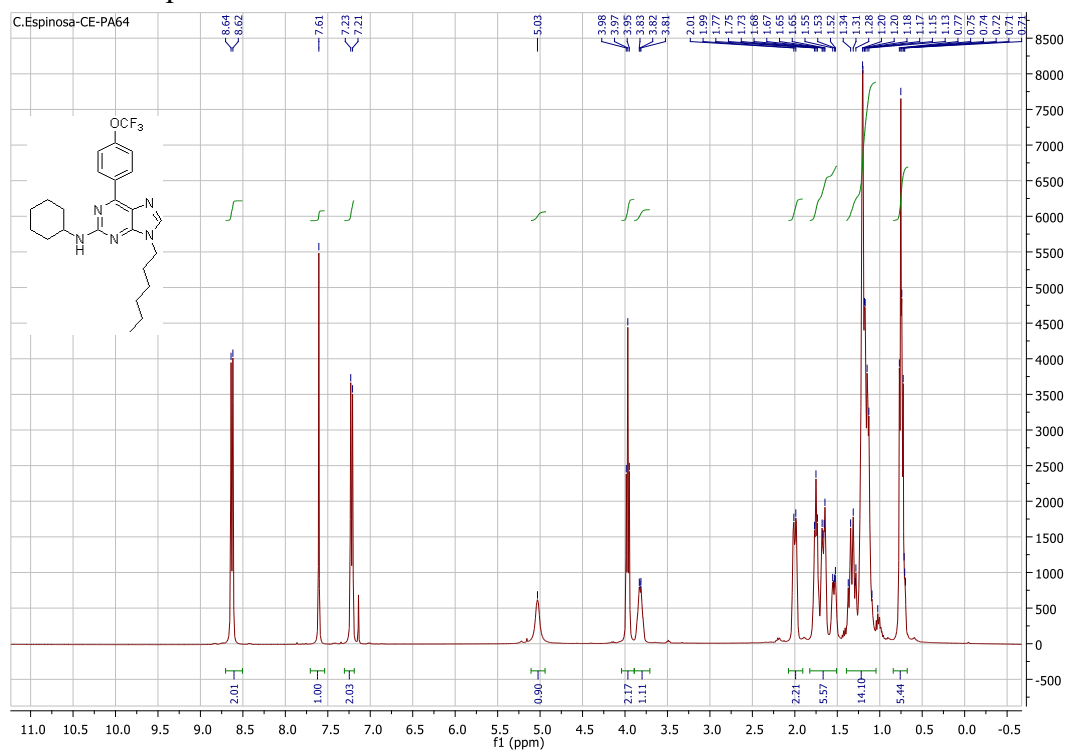
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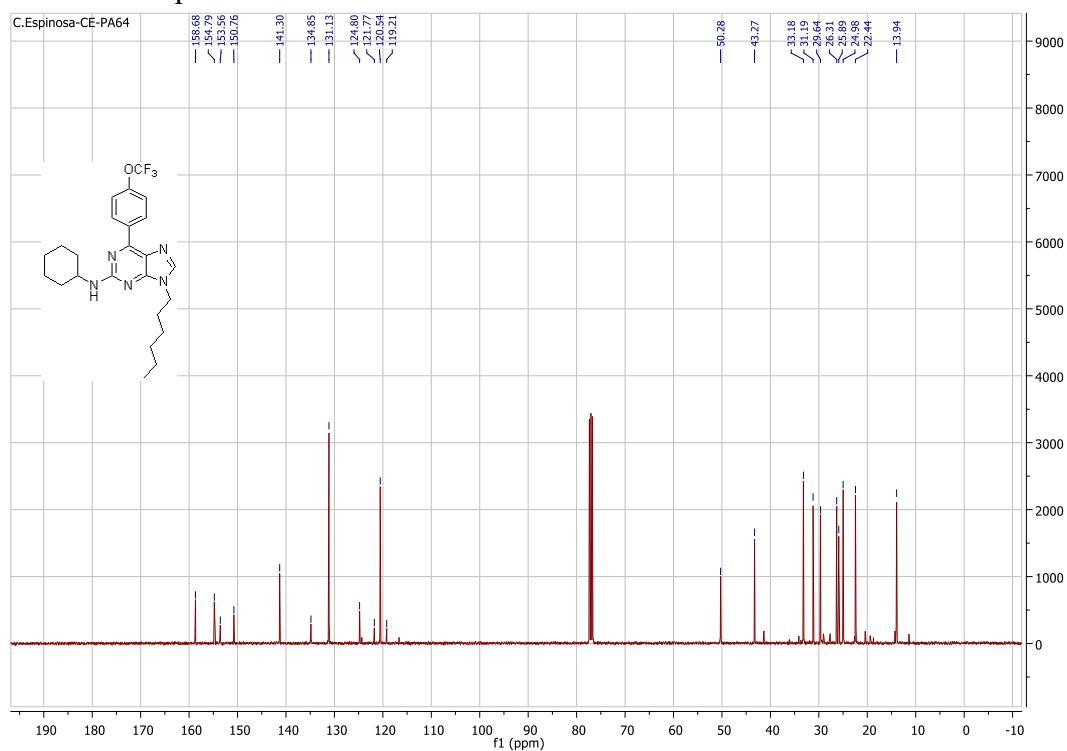
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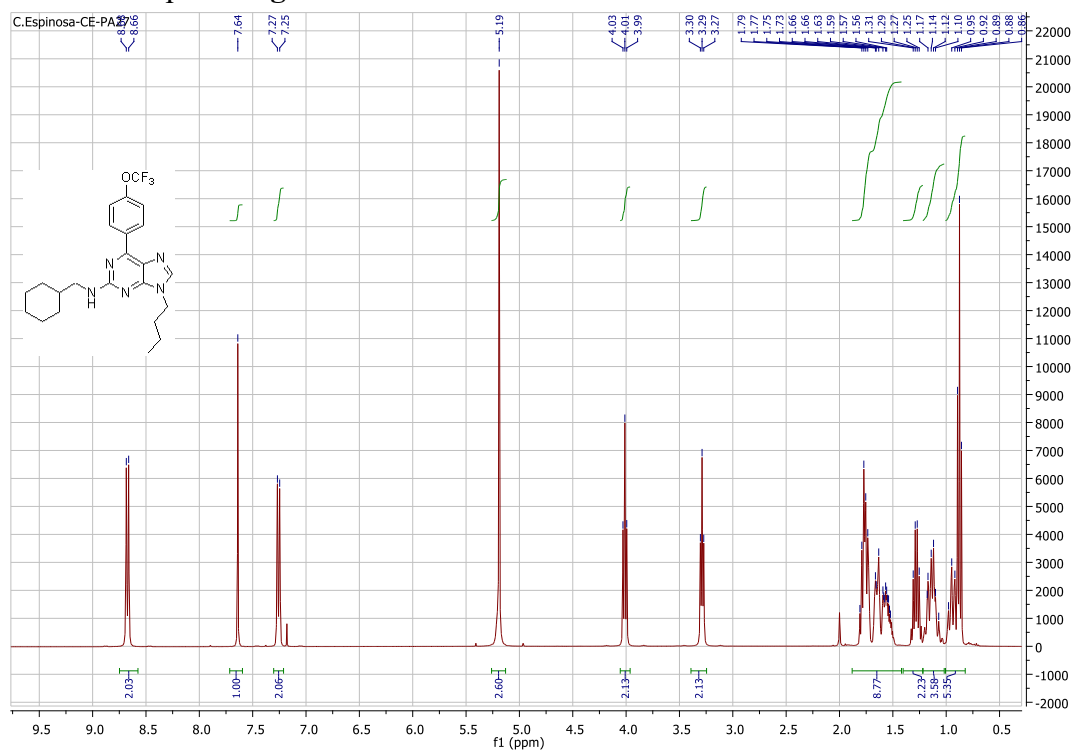
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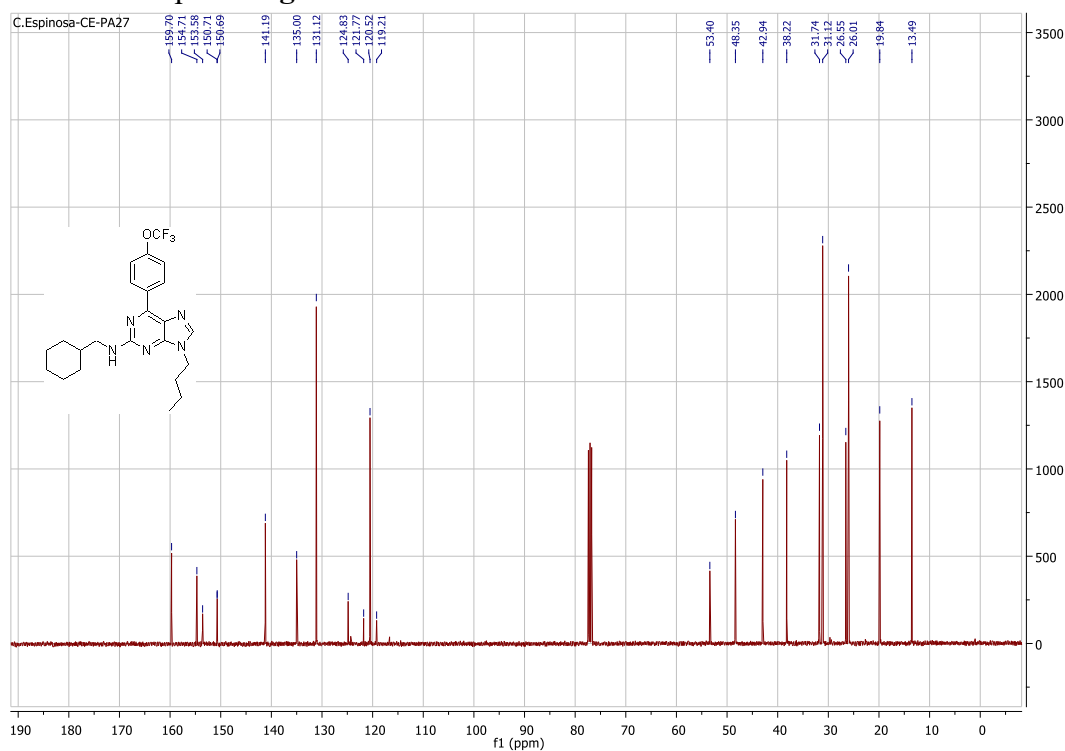
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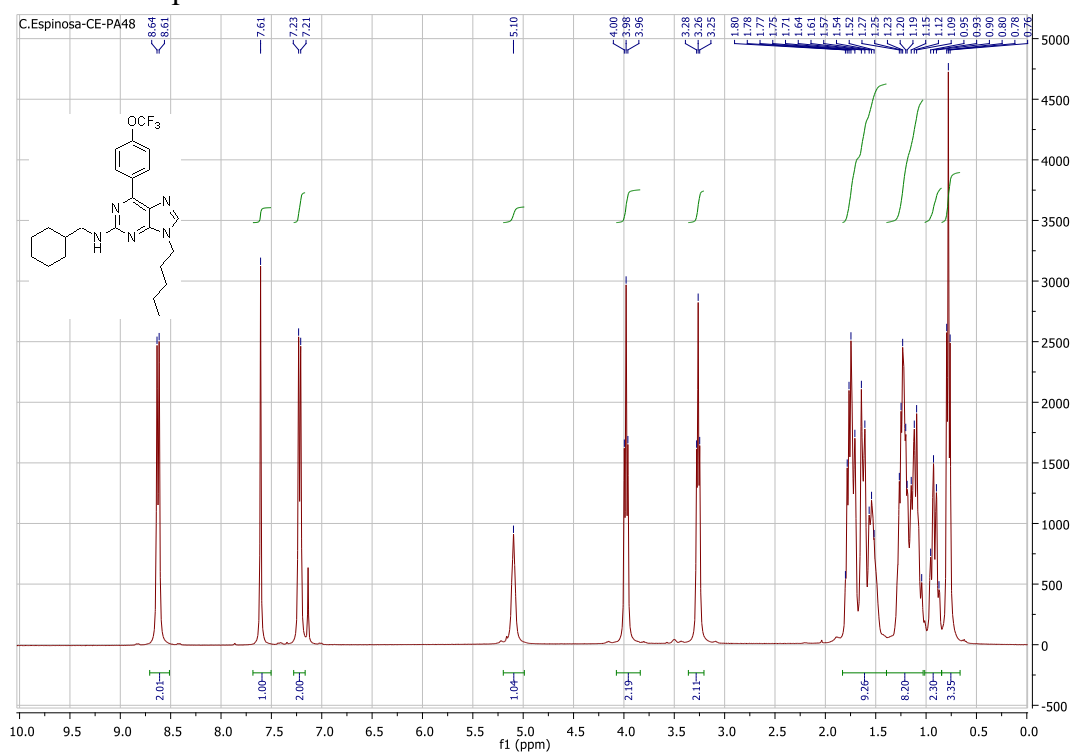
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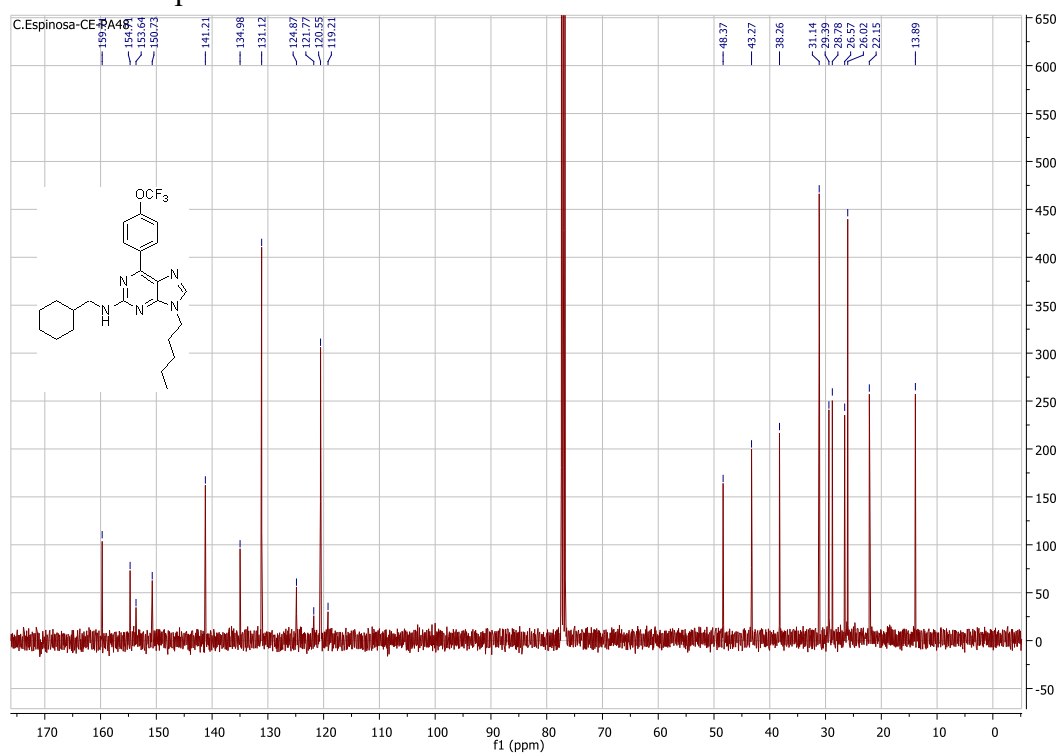
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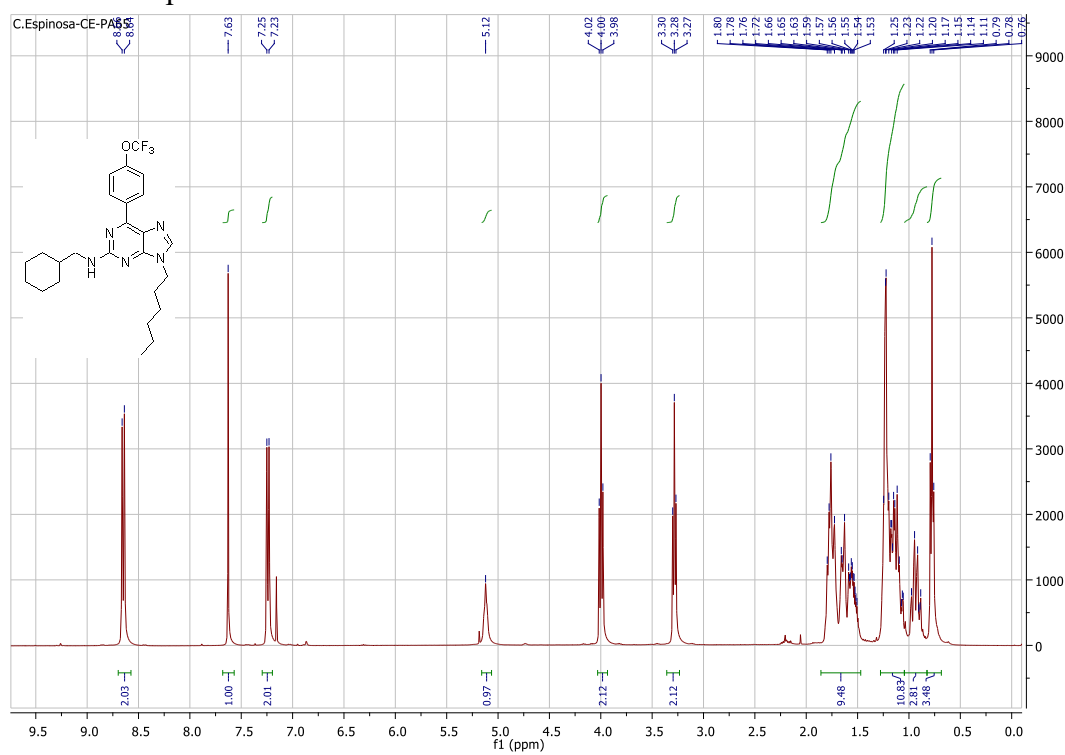
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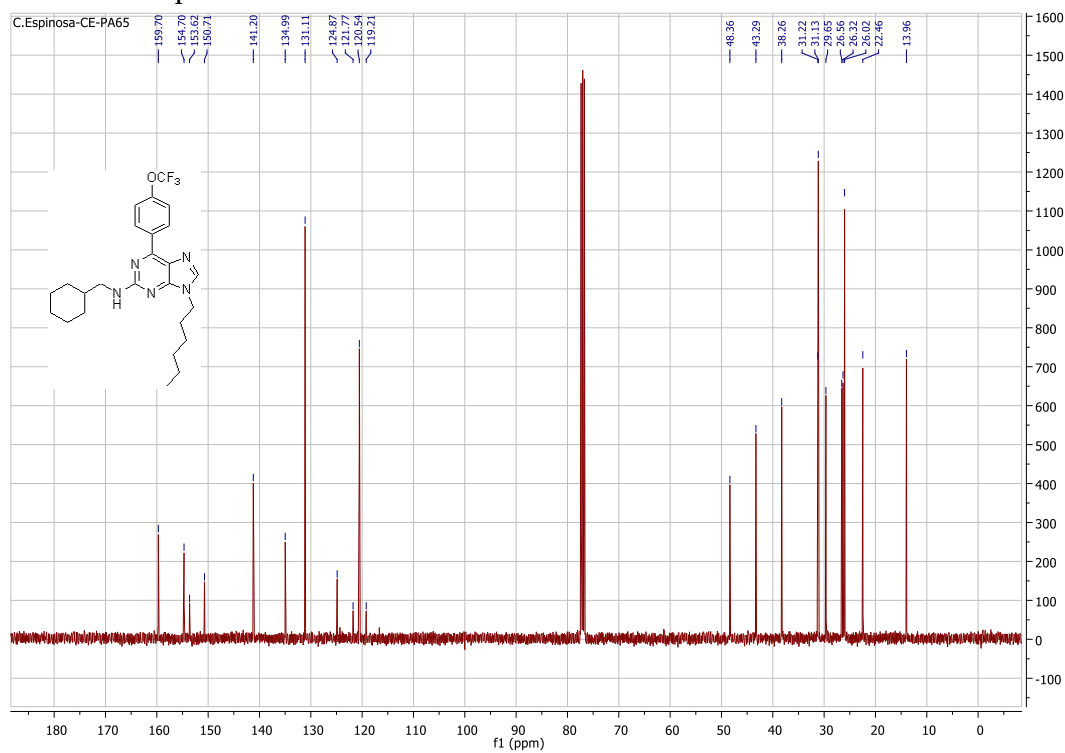
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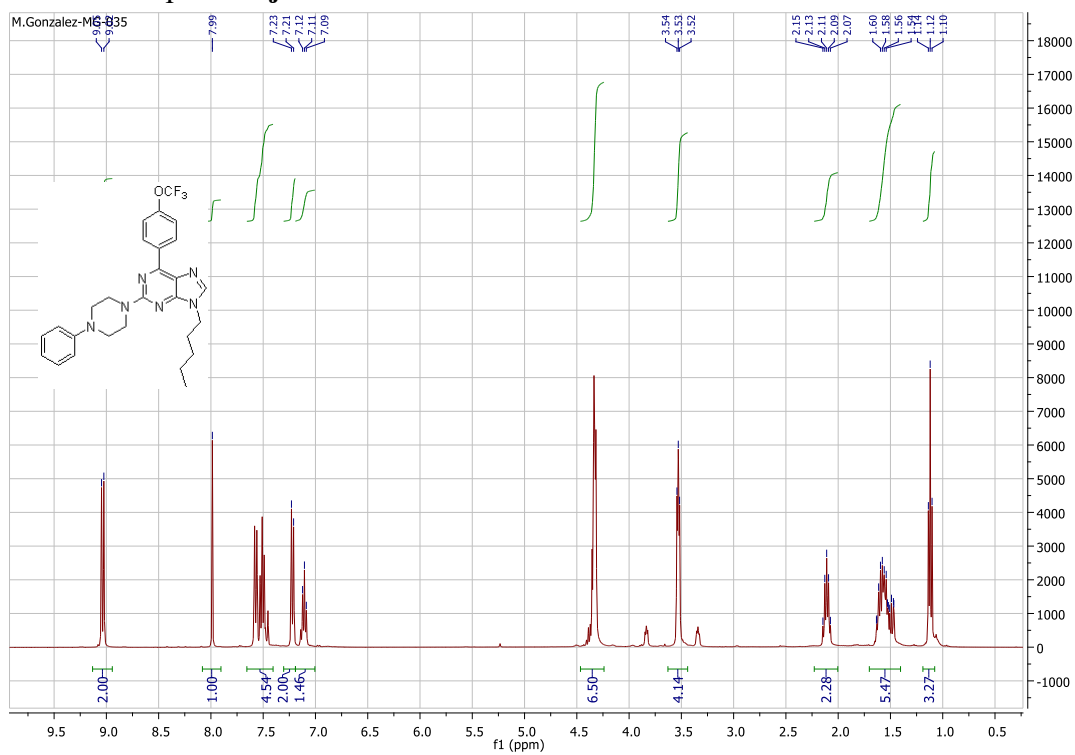
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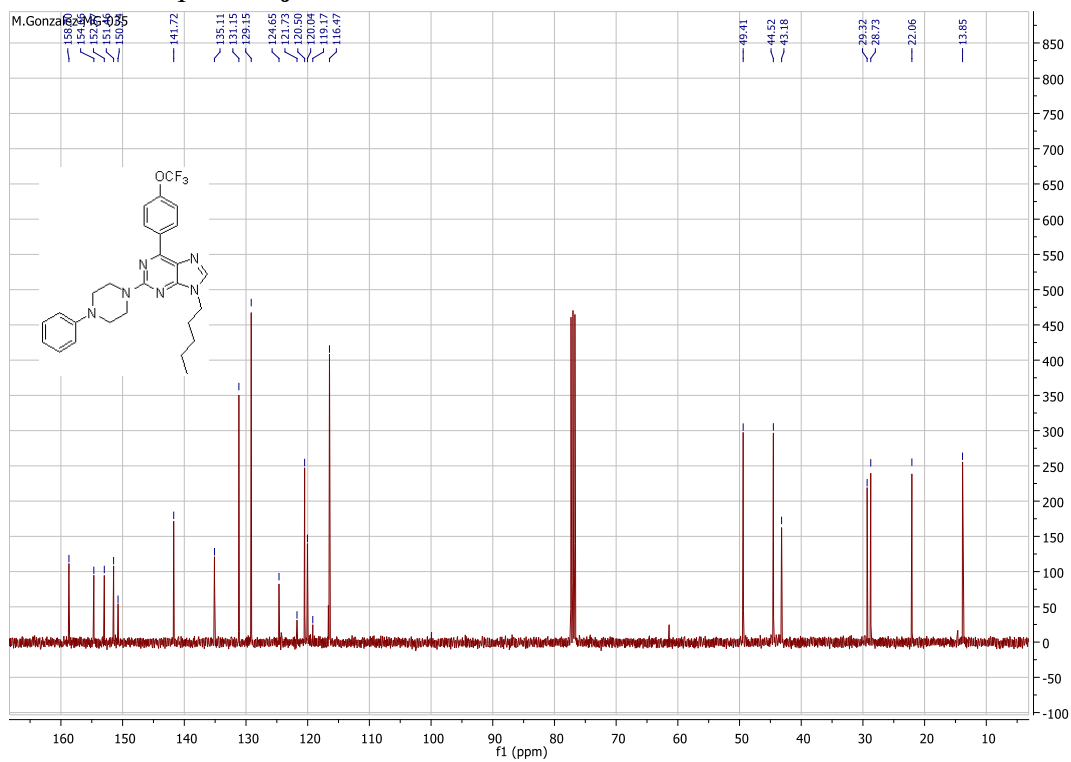
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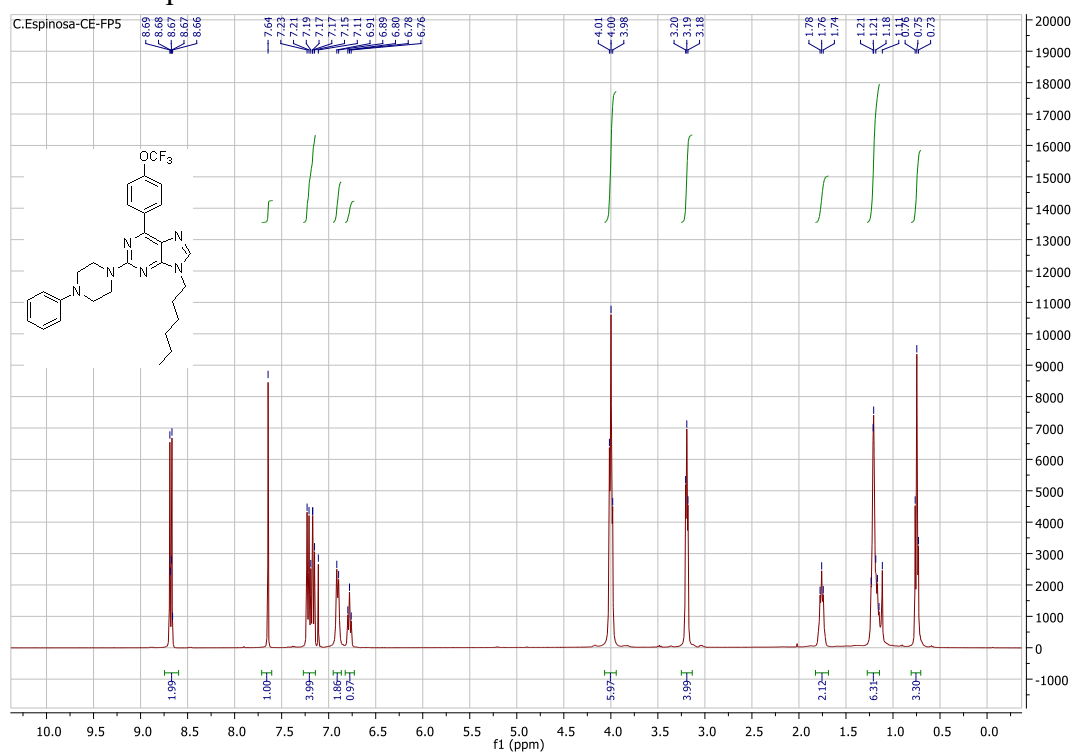
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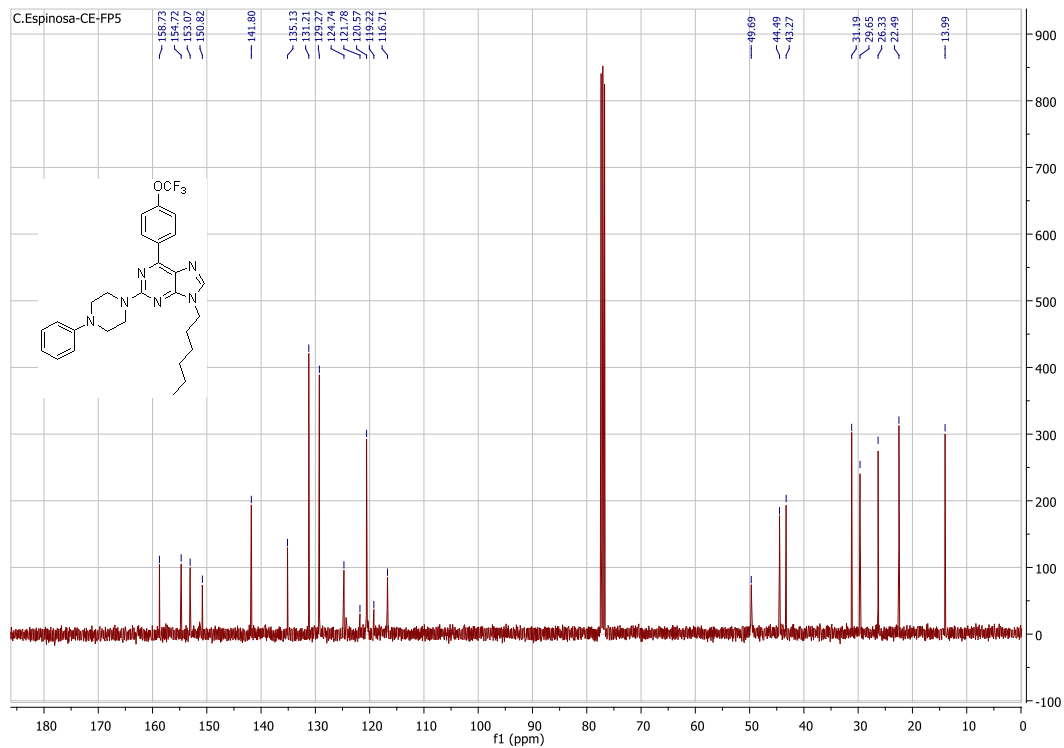
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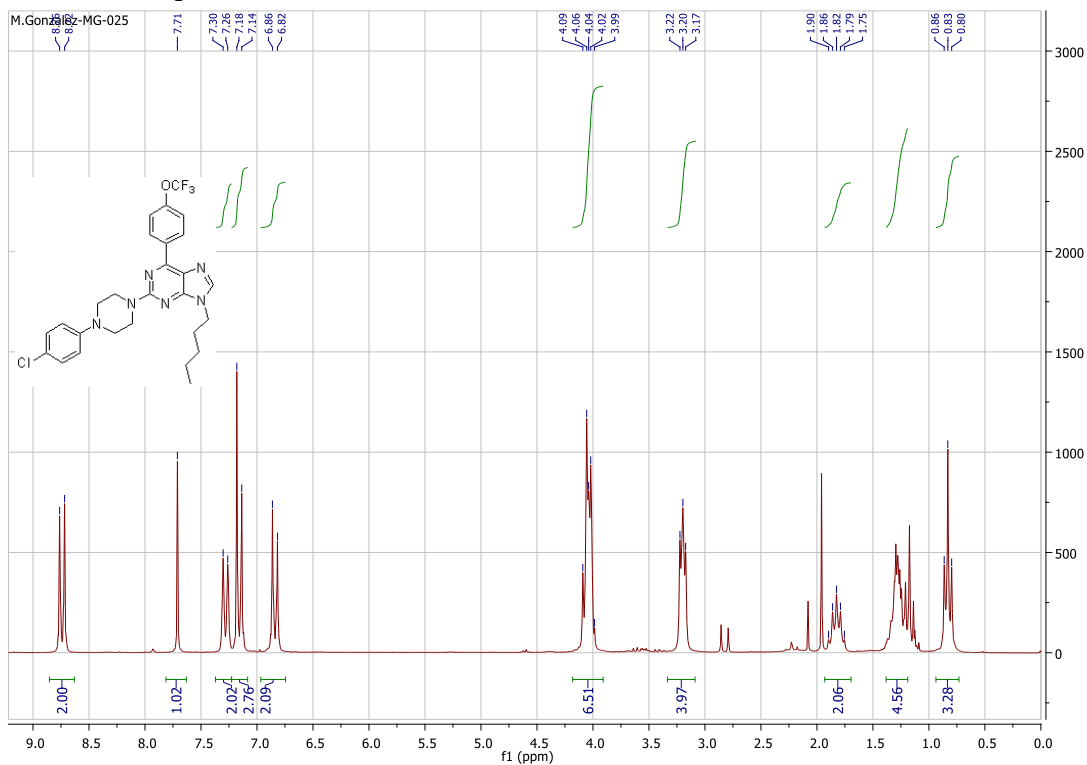
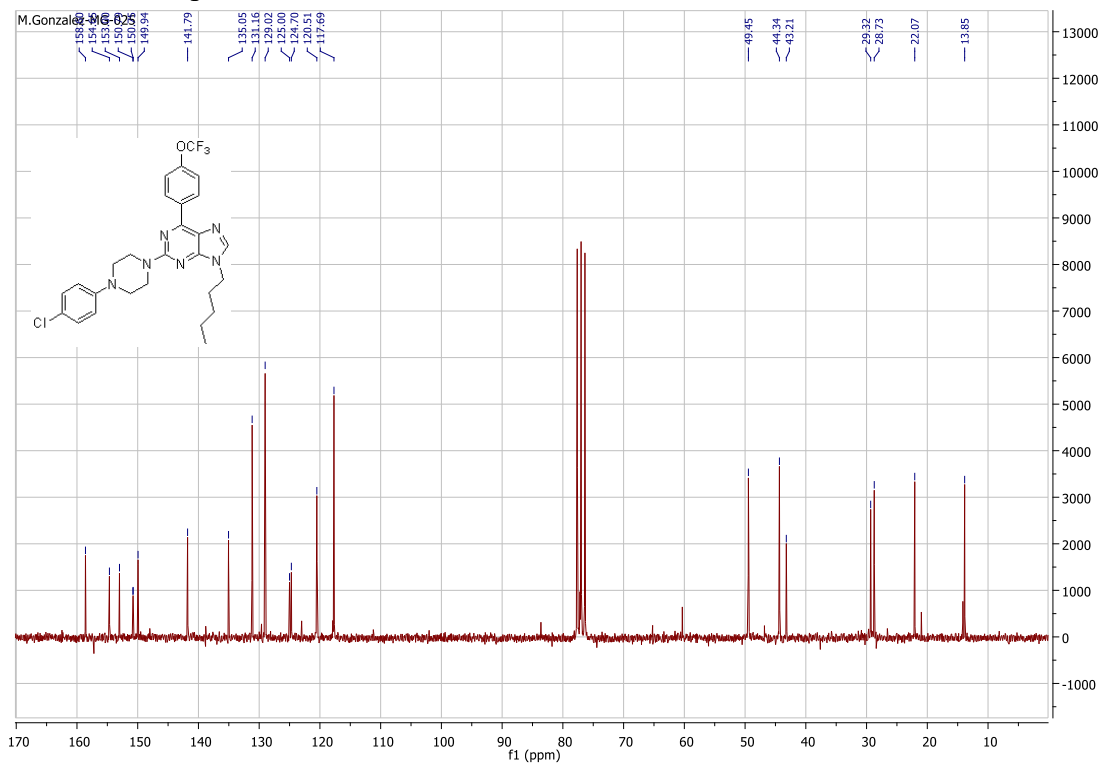


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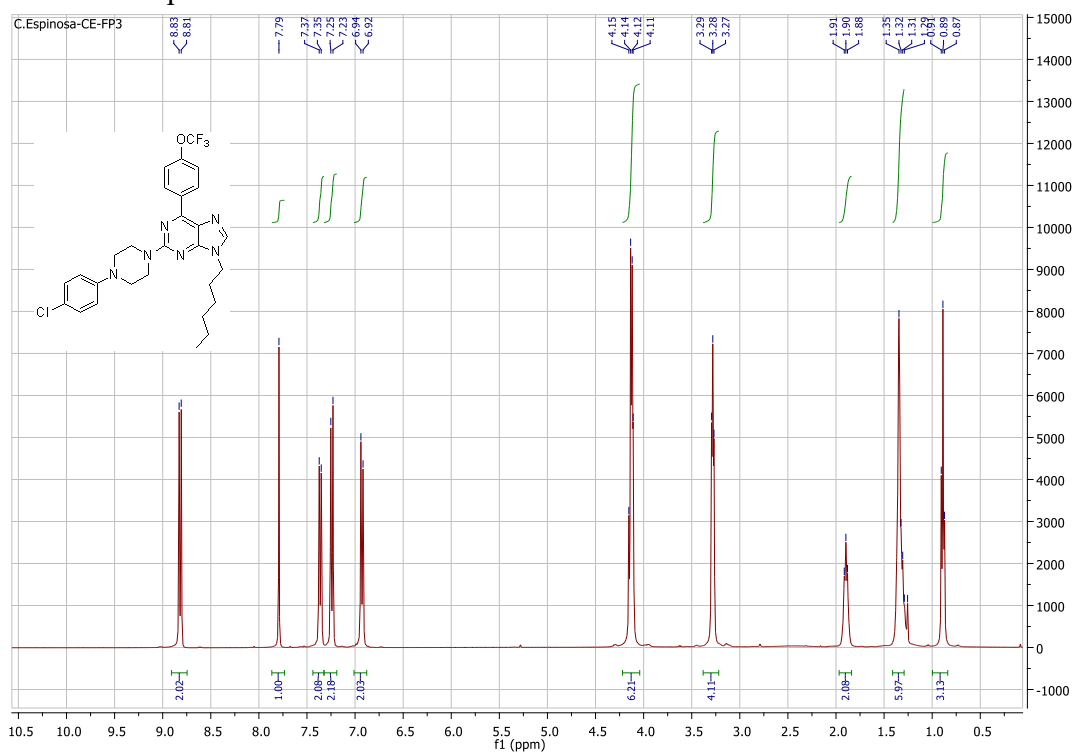


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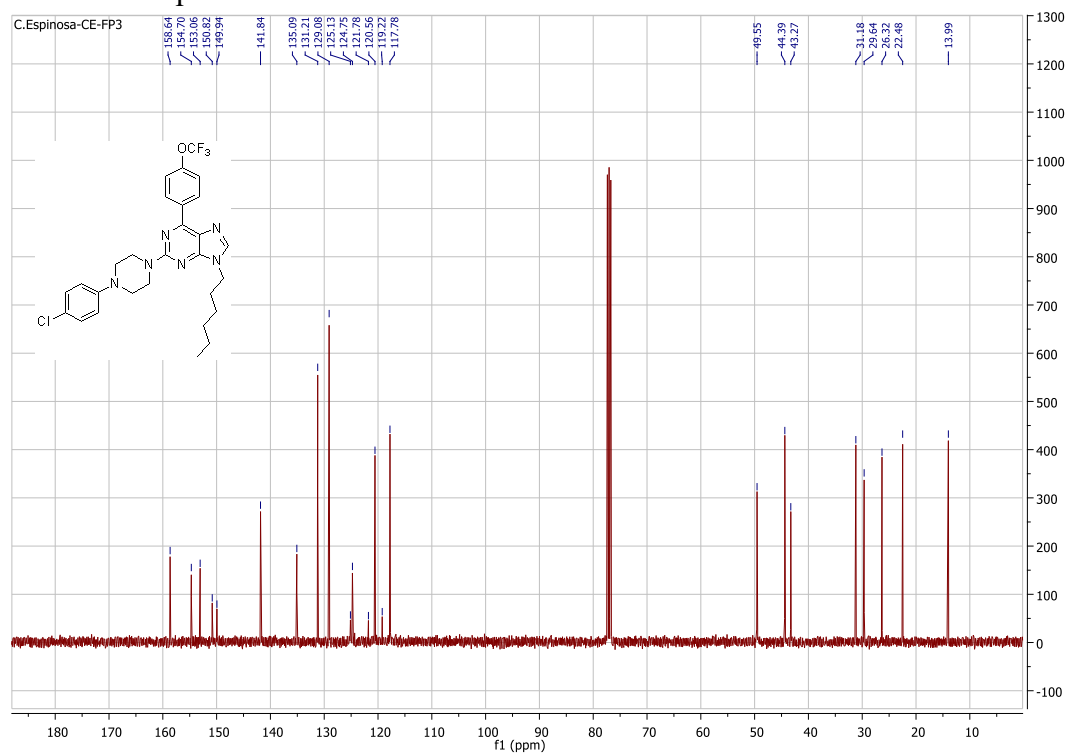


¹H NMR of compound **4l** ^{13}C NMR of compound **4l**

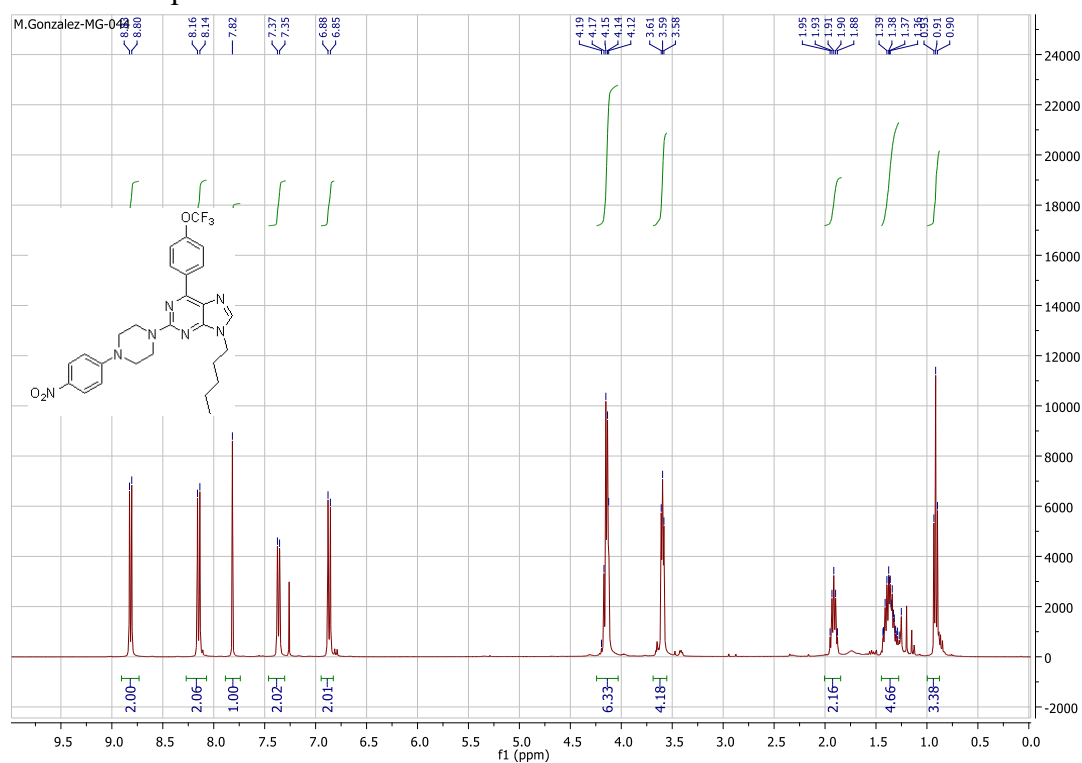
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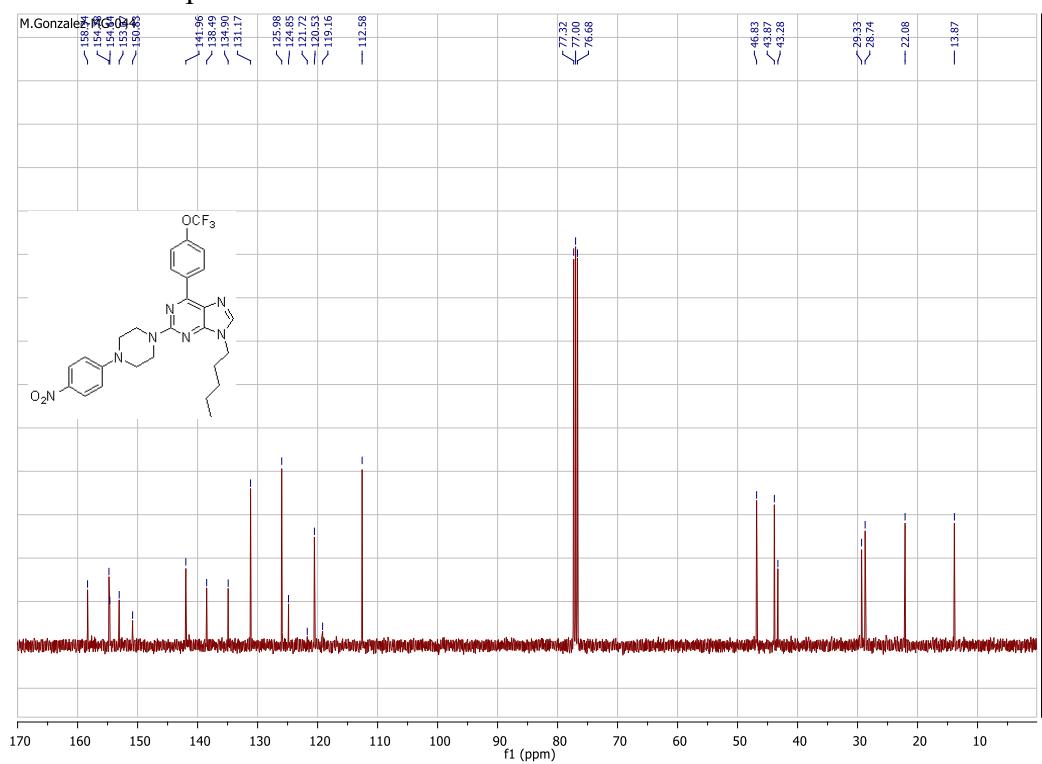
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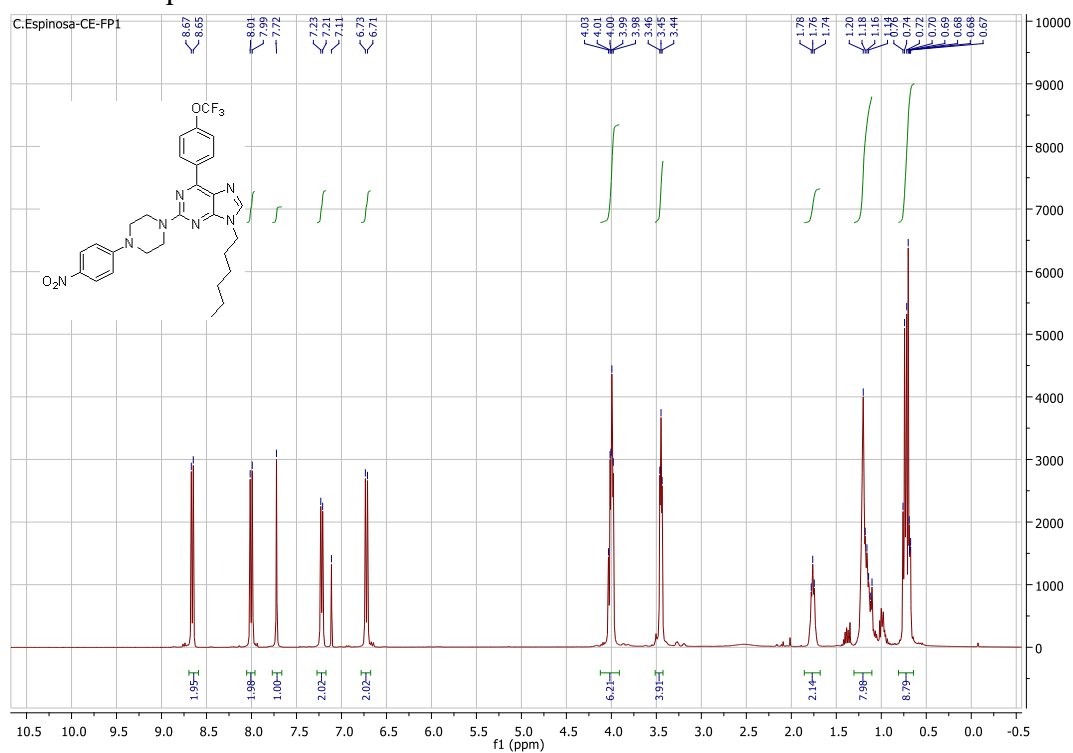
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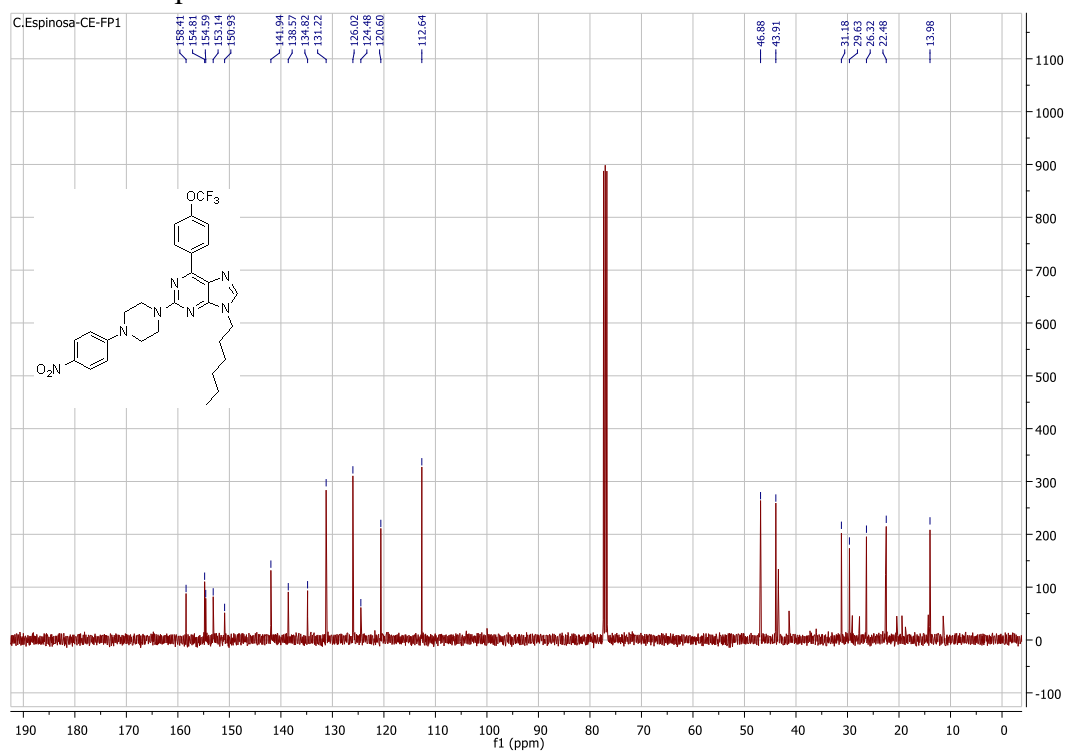
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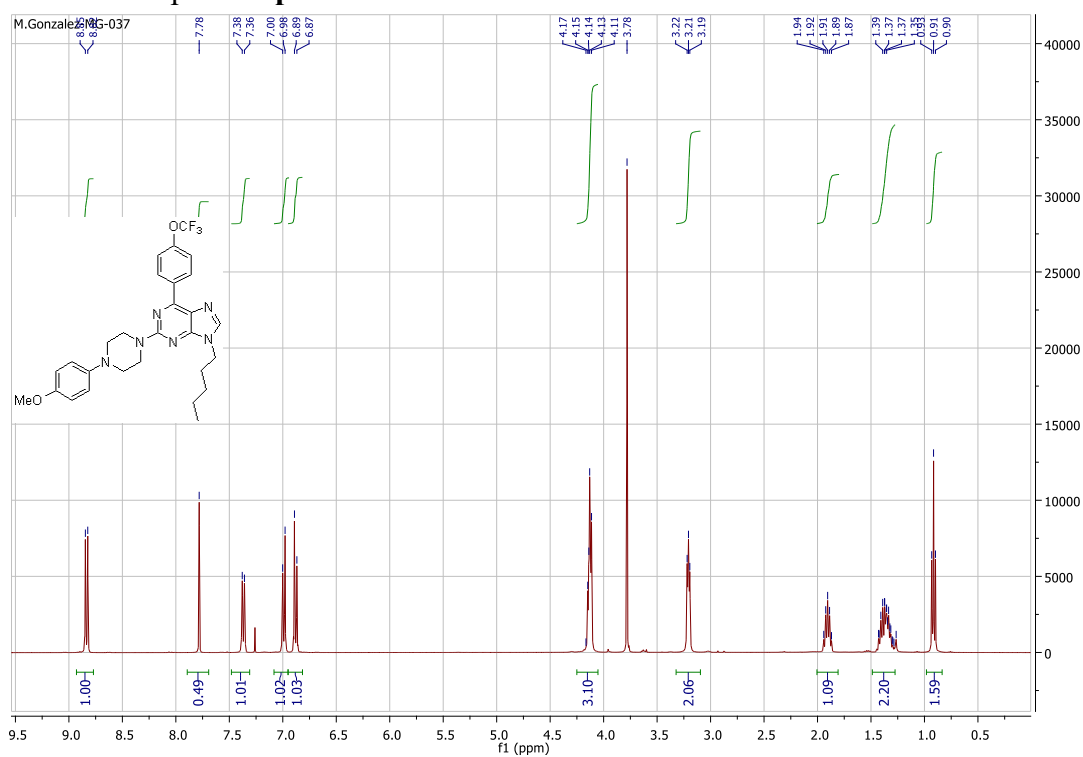
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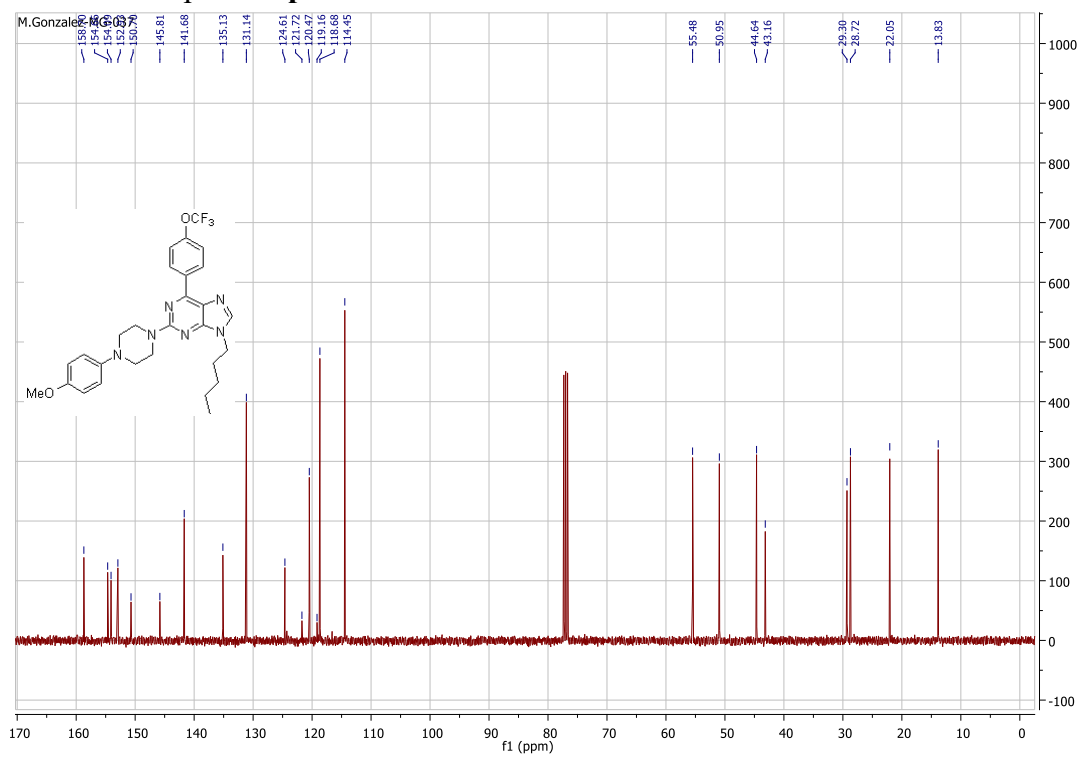
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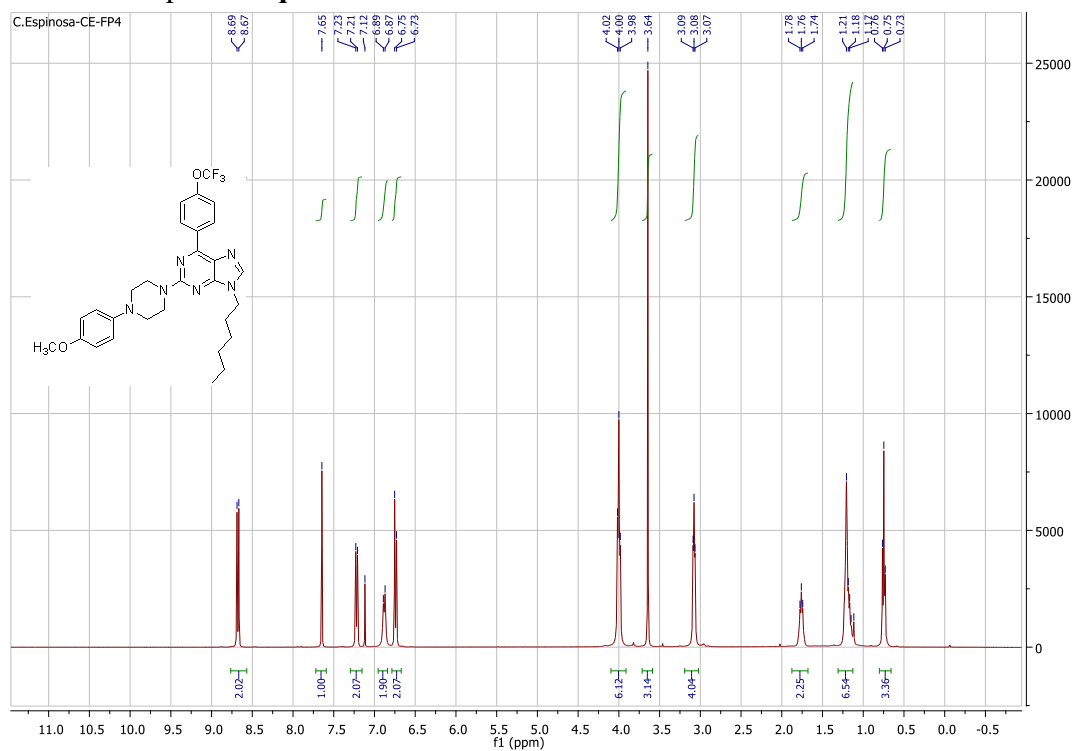
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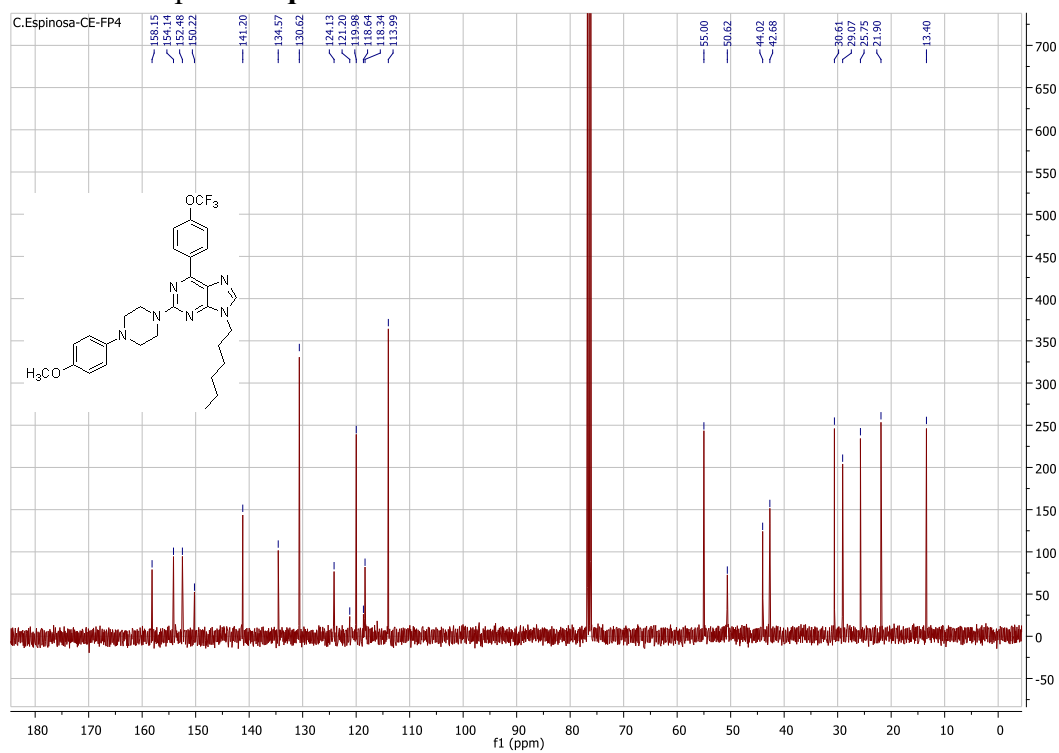
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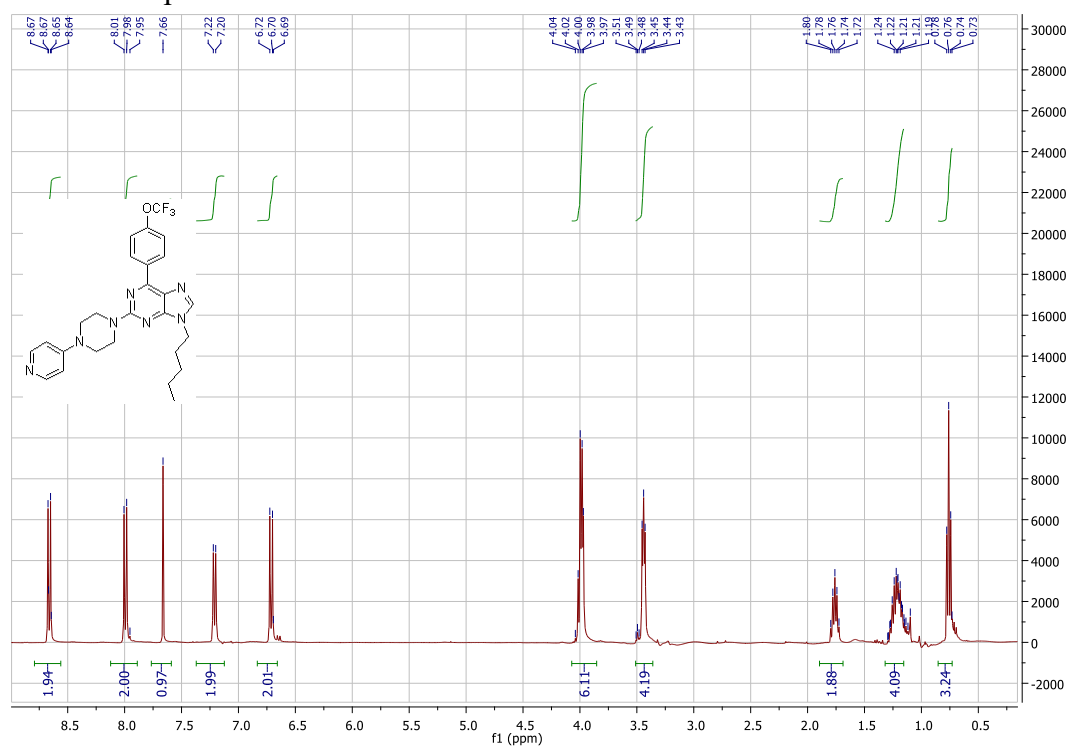
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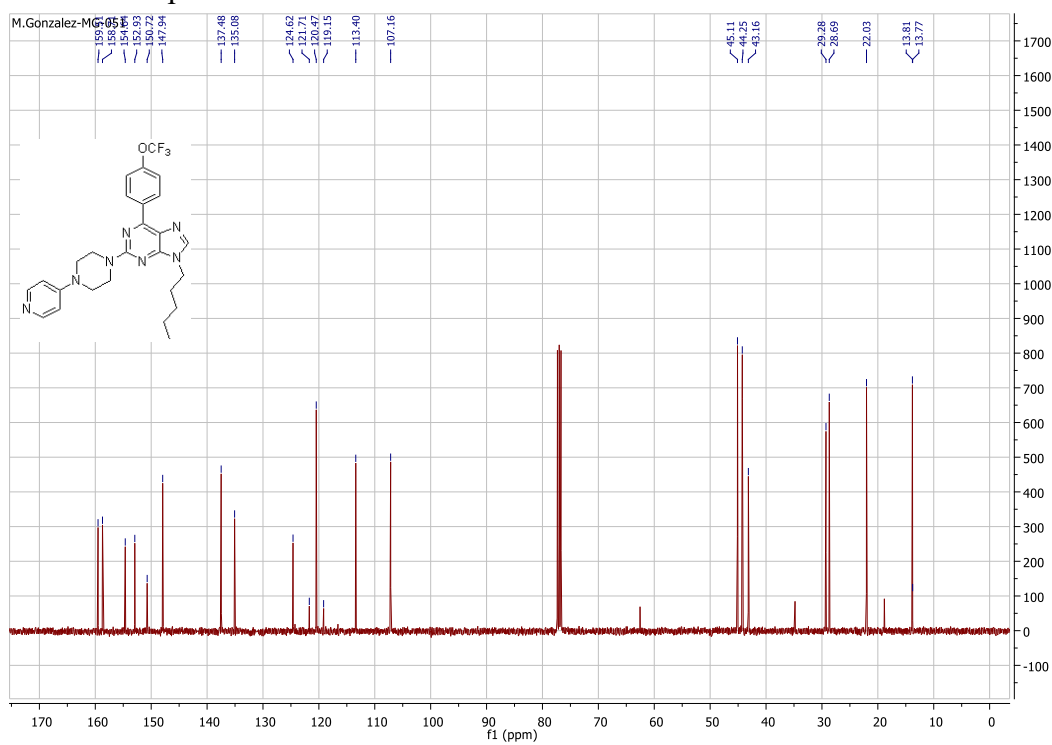
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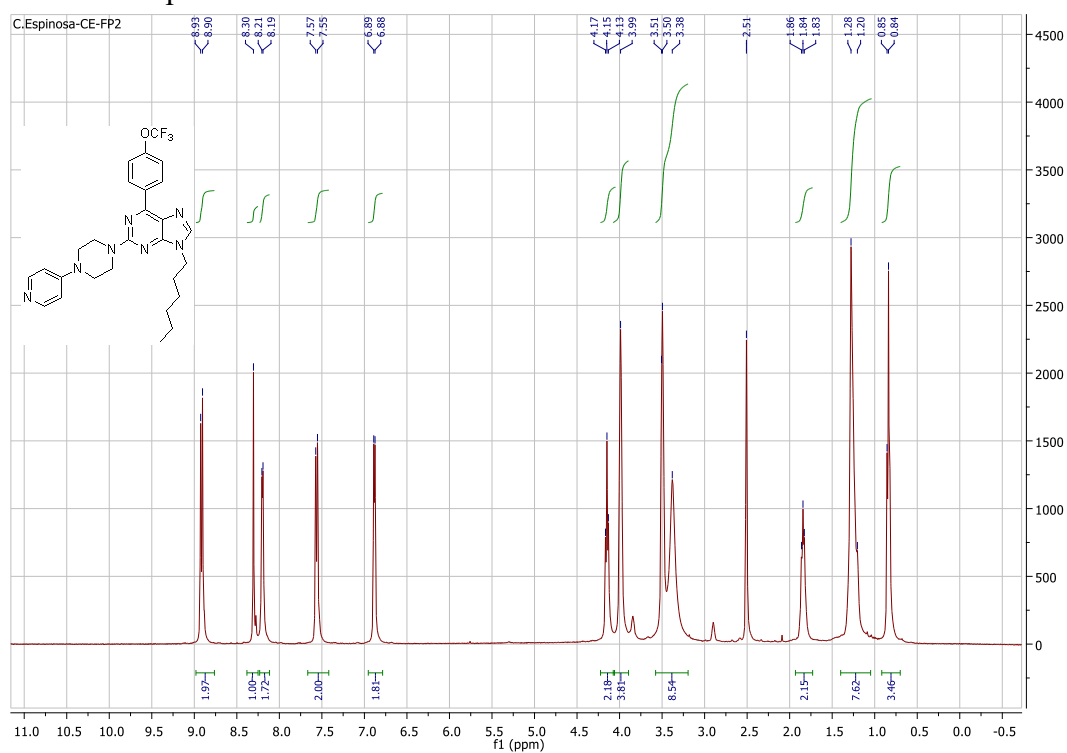
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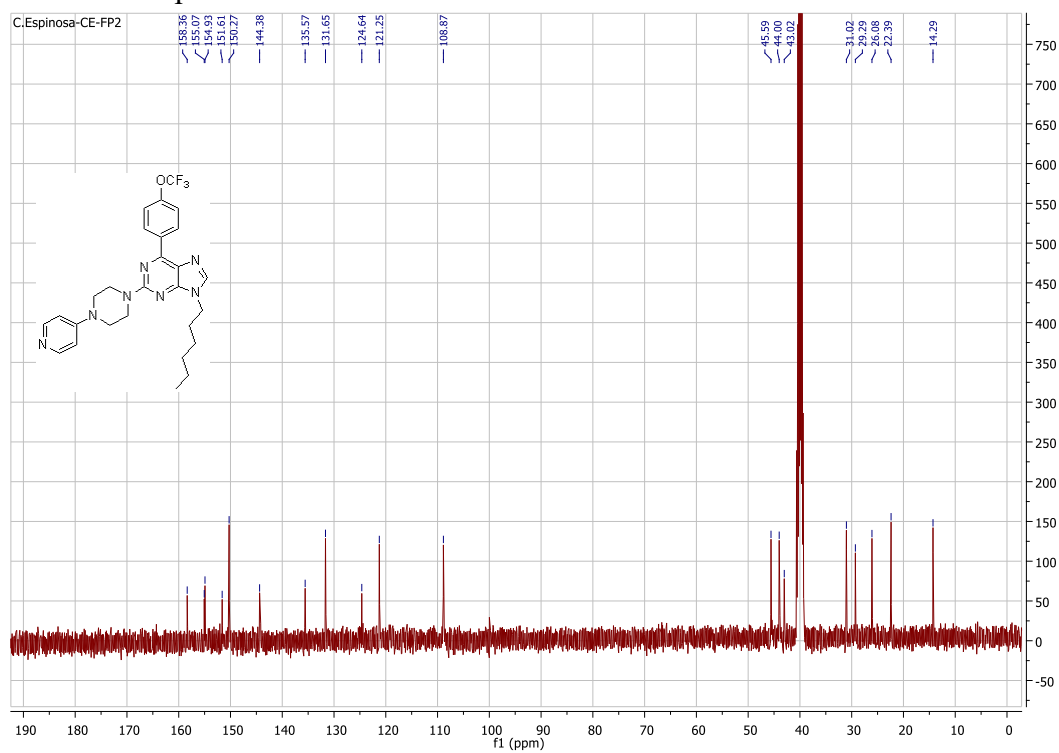
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¹H NMR of compound 4s



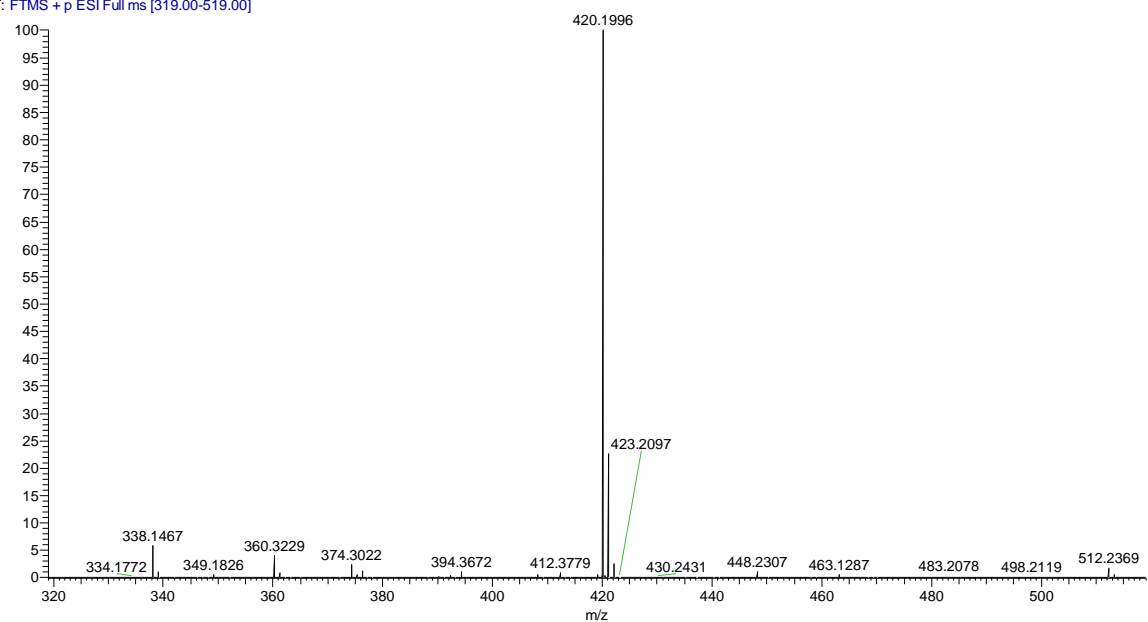
¹³C NMR of compound 4s



Mass spectrum of compound **4a**

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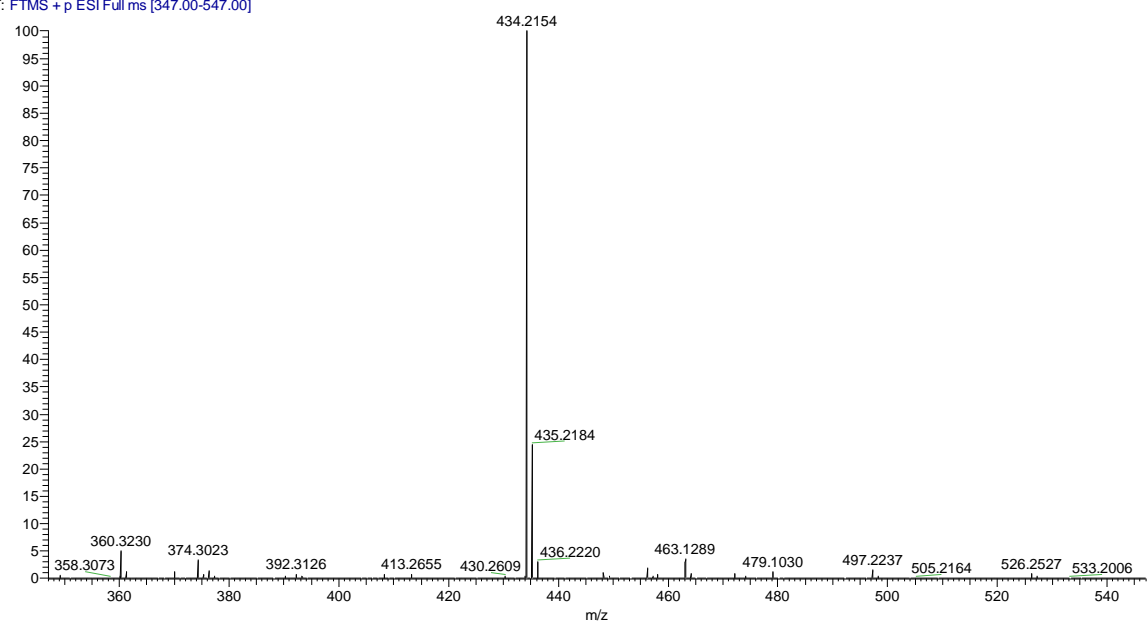
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Mass spectrum of compound **4b**

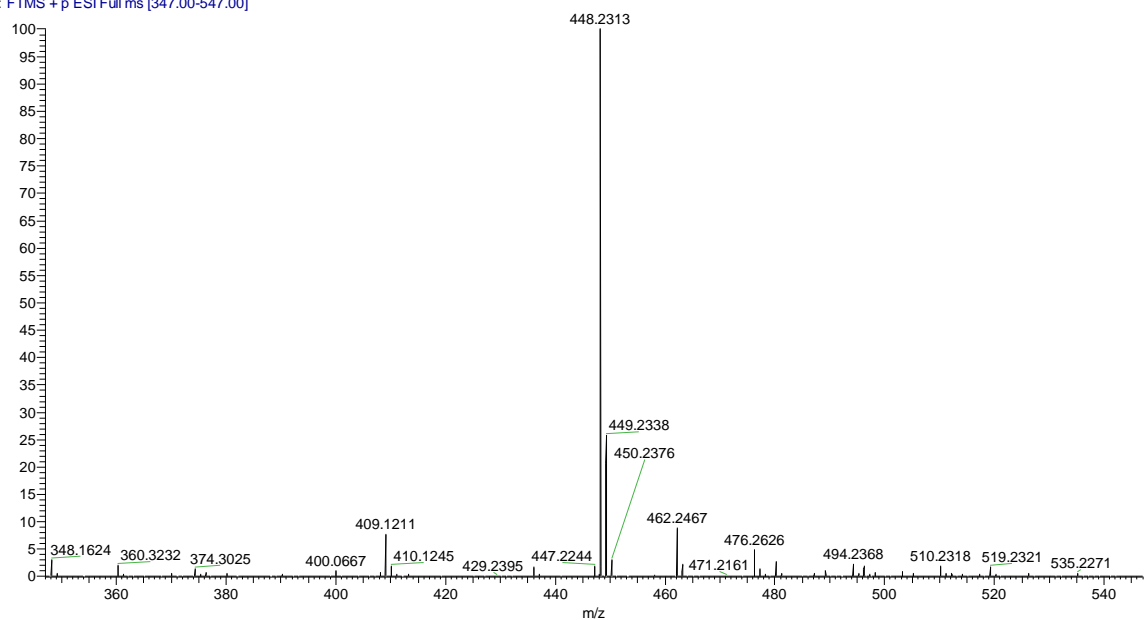
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T: FTMS + p ESI Full ms [347.00-547.00]



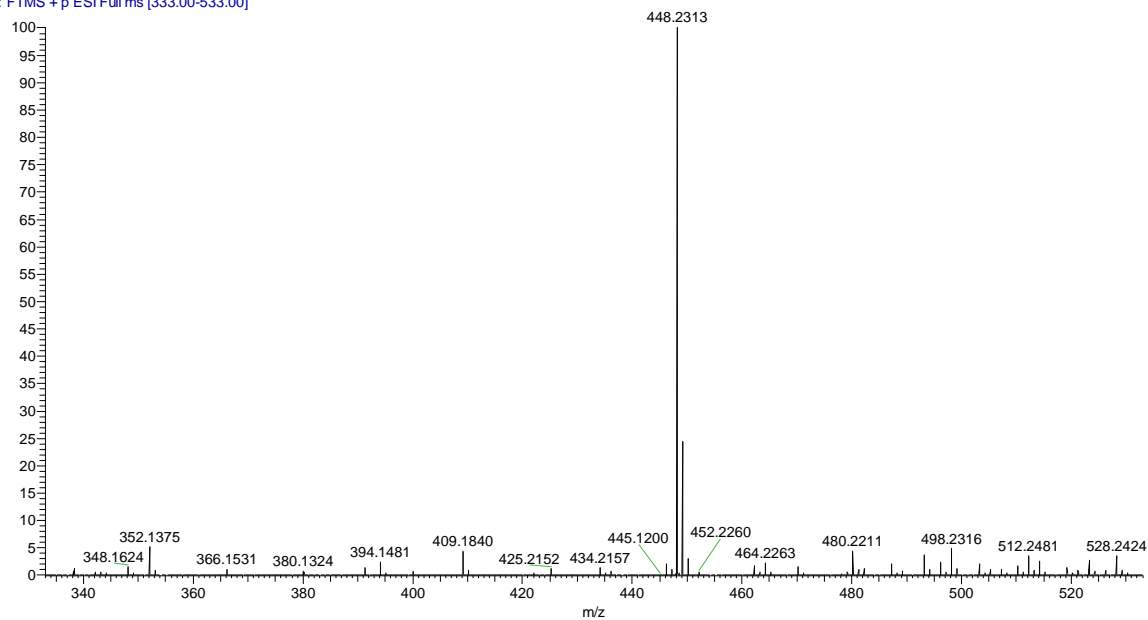
Mass spectrum of compound 4c

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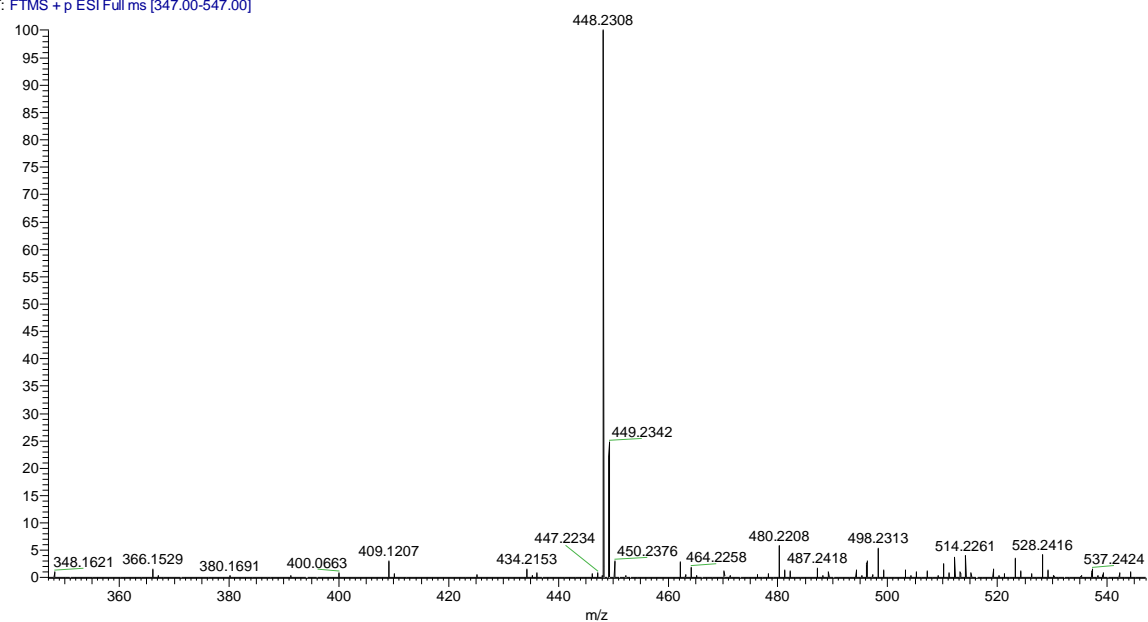
Mass spectrum of compound 4d

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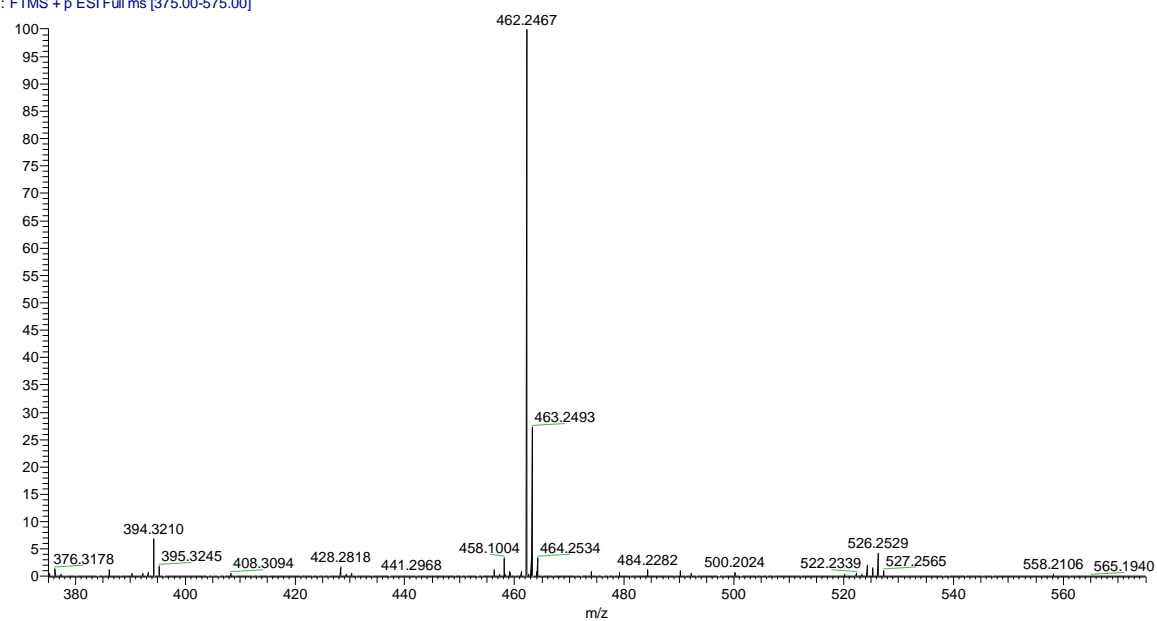
Mass spectrum of compound 4e

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Mass spectrum of compound 4f

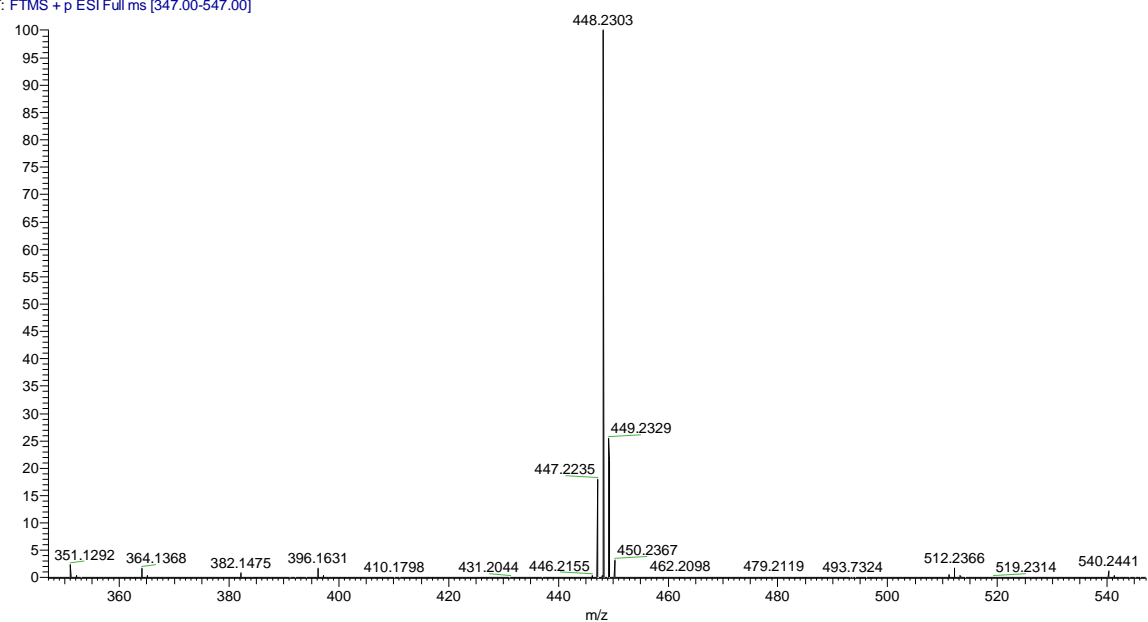
CE_PA64 #1 RT: 0.00 AV: 1 NL: 1.31E8
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Mass spectrum of compound 4g

5K #1 RT: 0.00 AV: 1 NL: 4.25E8

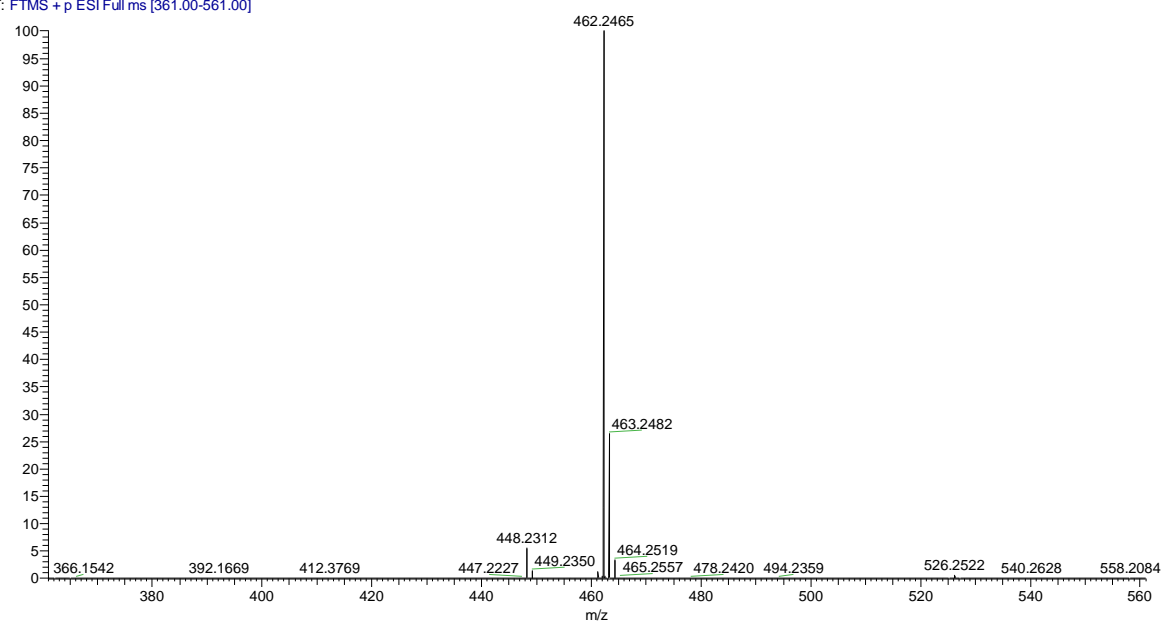
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Mass spectrum of compound 4h

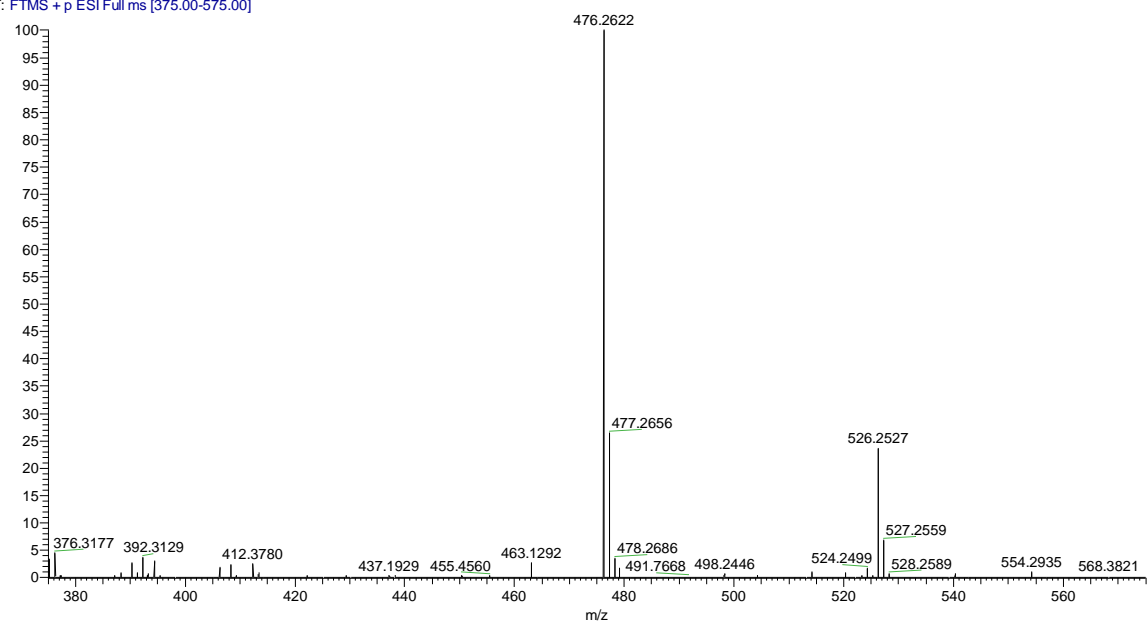
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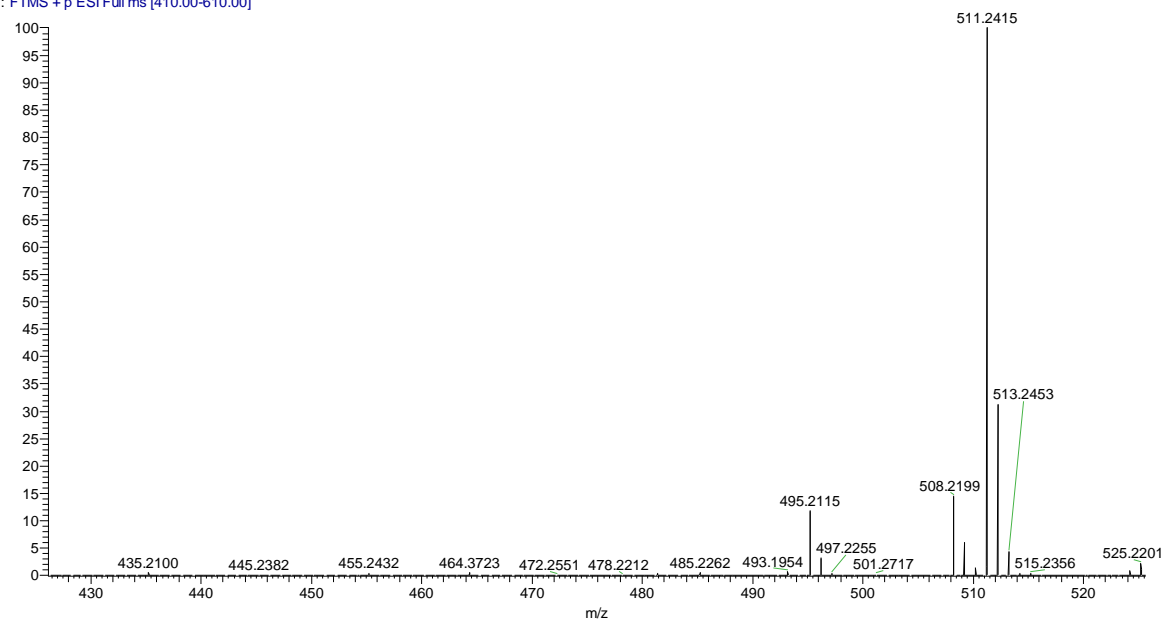
Mass spectrum of compound 4i

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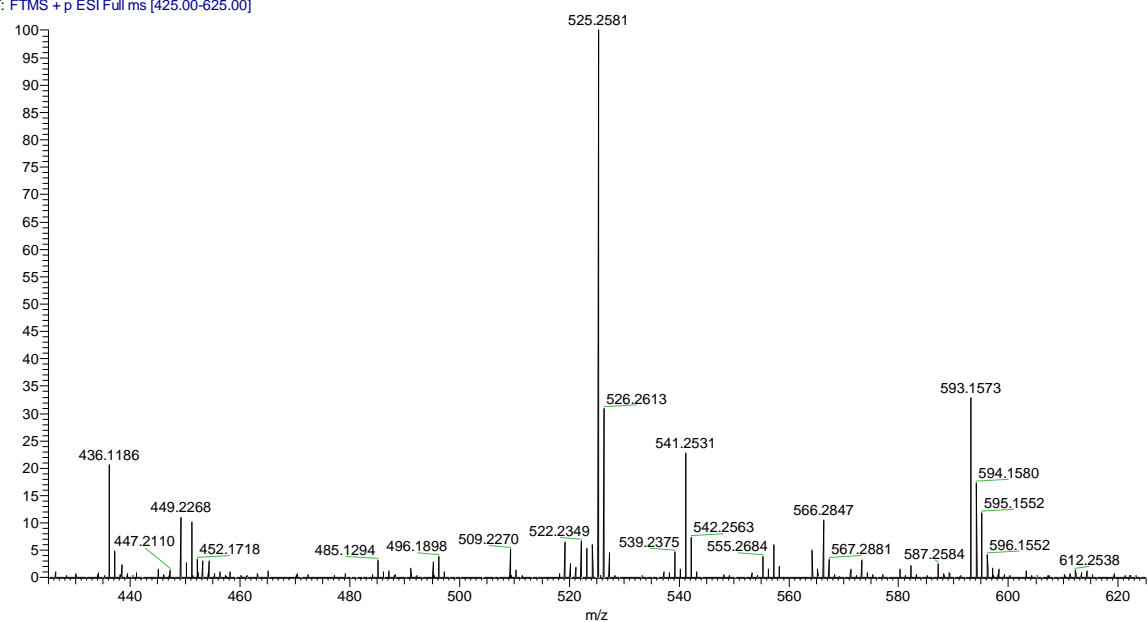
Mass spectrum of compound 4j

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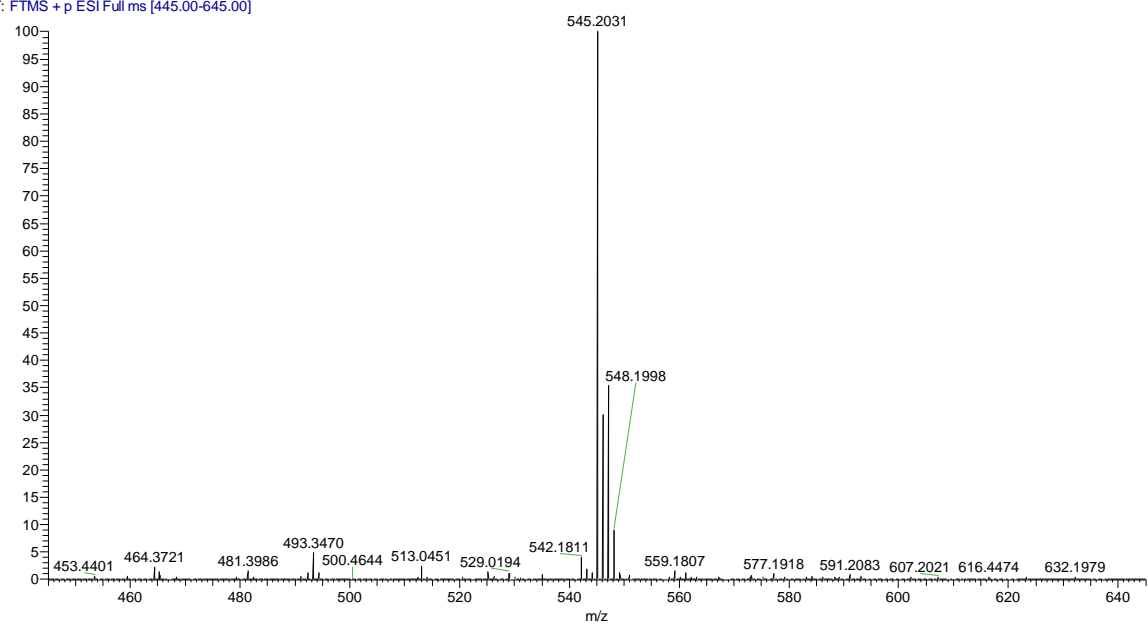
Mass spectrum of compound **4k**

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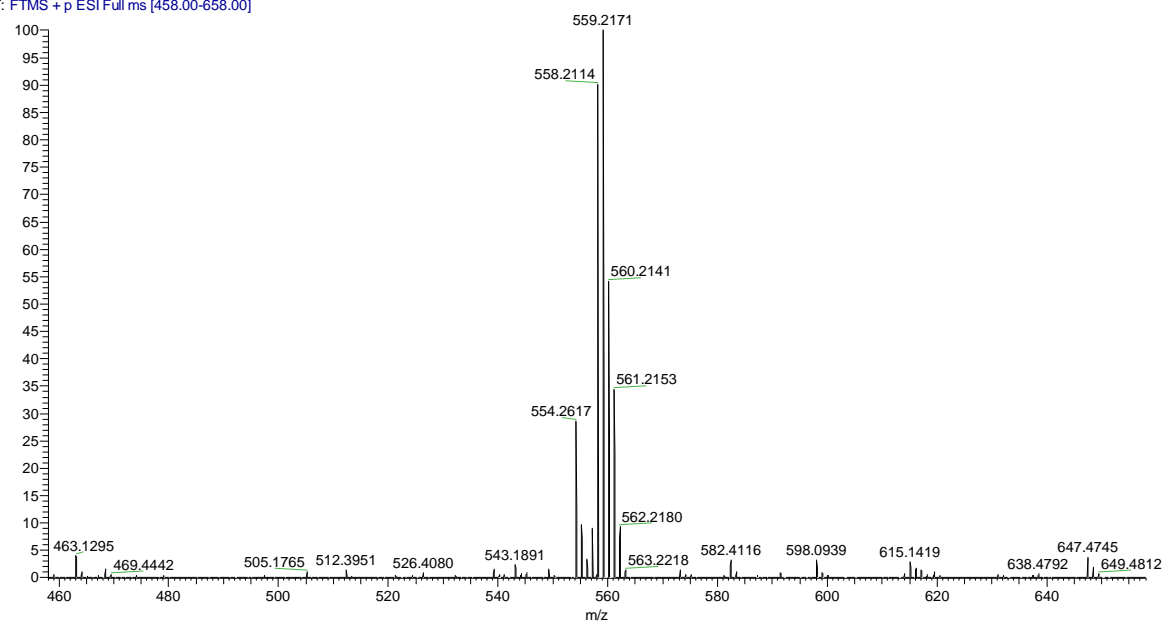
Mass spectrum of compound **4l**

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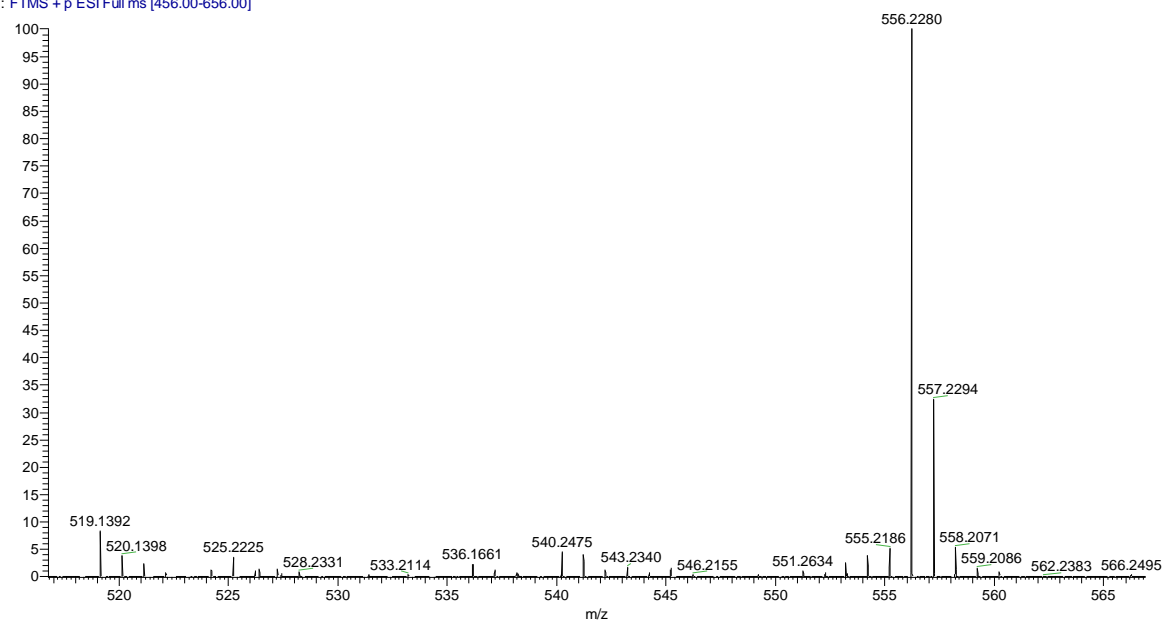
Mass spectrum of compound 4m

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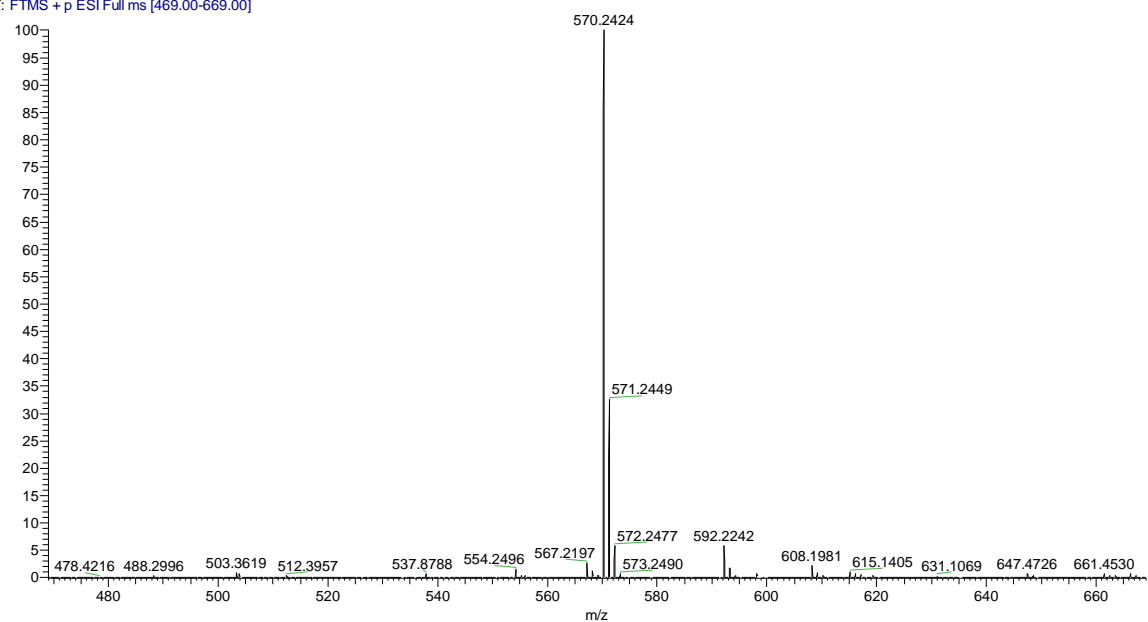
Mass spectrum of compound 4n

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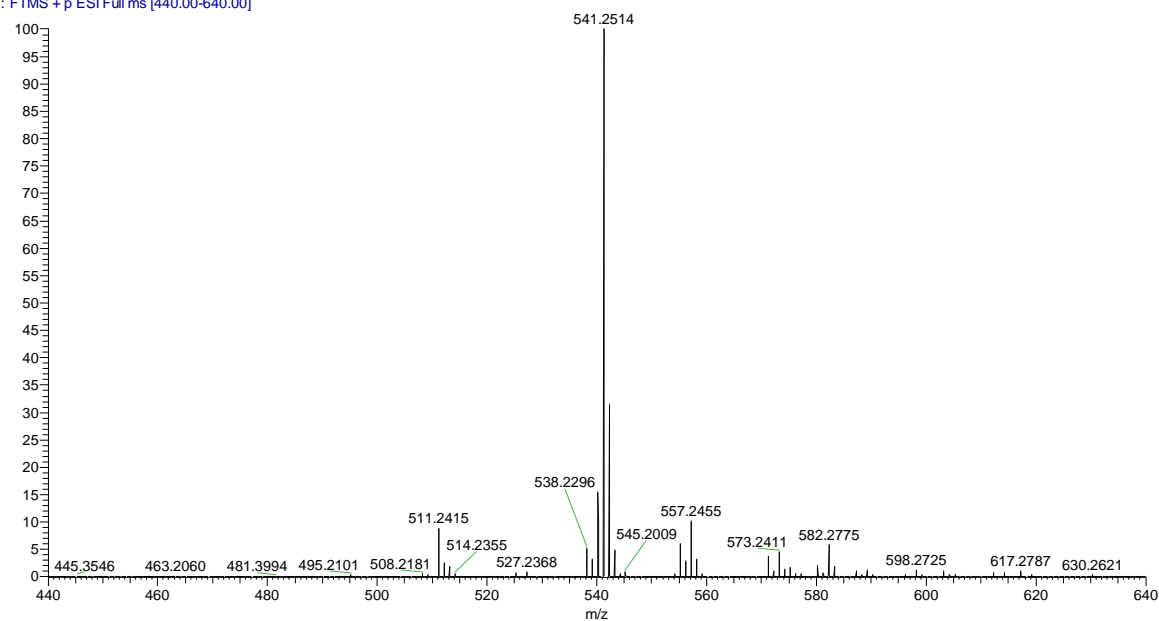
Mass spectrum of compound **4o**

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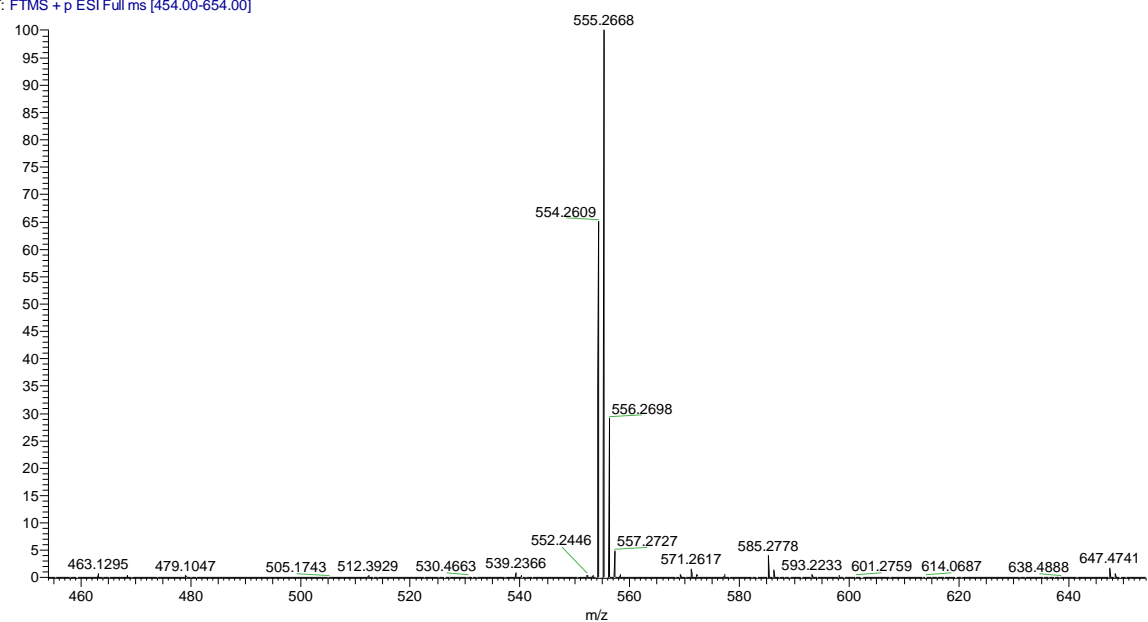
Mass spectrum of compound **4p**

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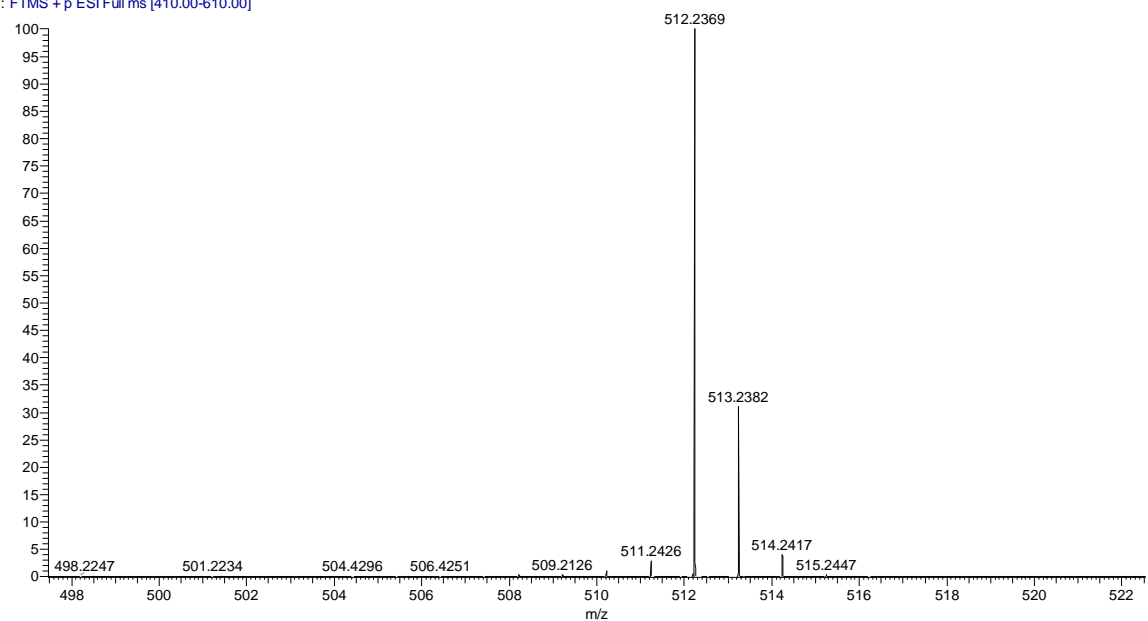
Mass spectrum of compound 4q

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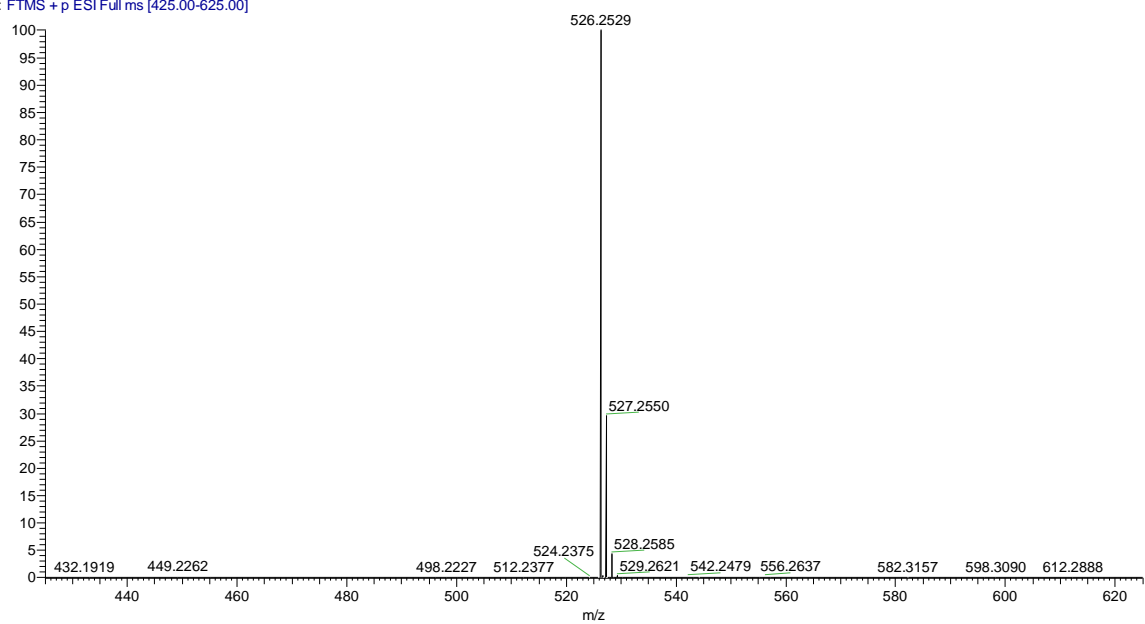
Mass spectrum of compound 4r

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Mass spectrum of compound 4s

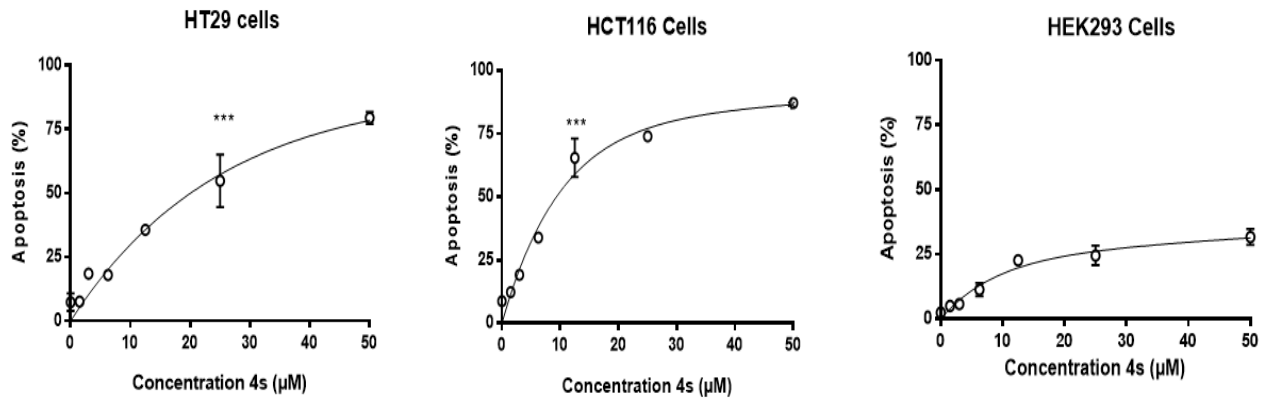
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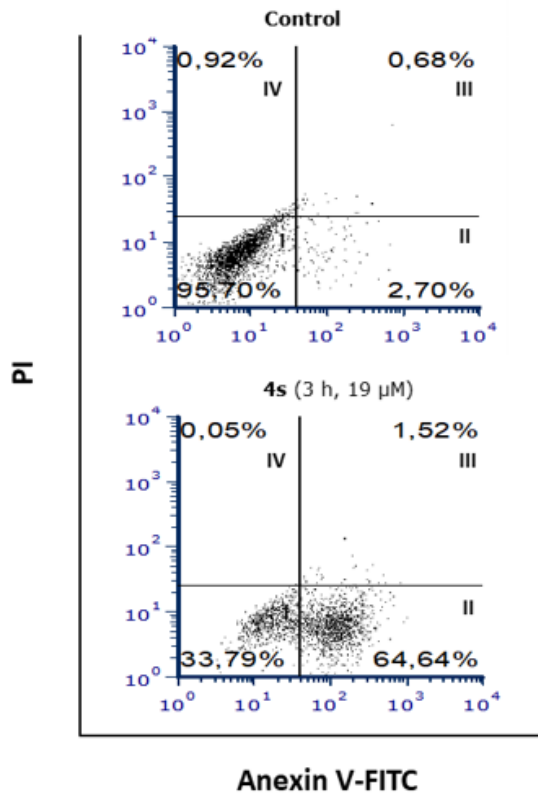
Supplemental Table S1: In vitro cytotoxicity of purine derivatives 4a-s at 50 μ M on four cancer cell lines and control cells (HEK293).

Compound	% Cytotoxicity (50 μ M)				
	HCT116	HT29	H1975	DAOY	HEK293
4a	68 \pm 0.3	<50	66 \pm 0.9	82 \pm 6.2	<50
4b	53 \pm 0.9	<50	30 \pm 1.0	94 \pm 0.4	<50
4c	<50	<50	19 \pm 0.8	69 \pm 0.9	<50
4d	<50	<50	<50	<50	<50
4e	<50	<50	<50	<50	<50
4f	<50	<50	<50	<50	<50
4g	<50	<50	<50	<50	<50
4h	<50	<50	61 \pm 1.2	81 \pm 1.1	<50
4i	<50	<50	<50	<50	<50
4j	<50	<50	<50	<50	<50
4k	<50	<50	<50	<50	<50
4l	<50	<50	<50	<50	<50
4m	<50	<50	<50	<50	<50
4n	<50	<50	<50	<50	<50
4o	<50	<50	<50	<50	<50
4p	<50	<50	<50	<50	<50
4q	<50	<50	<50	<50	<50
4r	81 \pm 0.8	<50	<50	92 \pm 0.4	<50
4s	99 \pm 0.4	25 \pm 2.7	99 \pm 0.2	98 \pm 0.3	<50
Vismodegib	<50	<50	53 \pm 1.2	<50	<50
Etoposide	90 \pm 0.5	-	60 \pm 1.1	-	65 \pm 0.7
Cisplatin	-	-	-	88 \pm 0.7	88 \pm 0.7
5-FU	62 \pm 1.3	66 \pm 4.9-	-	-	<50

A)

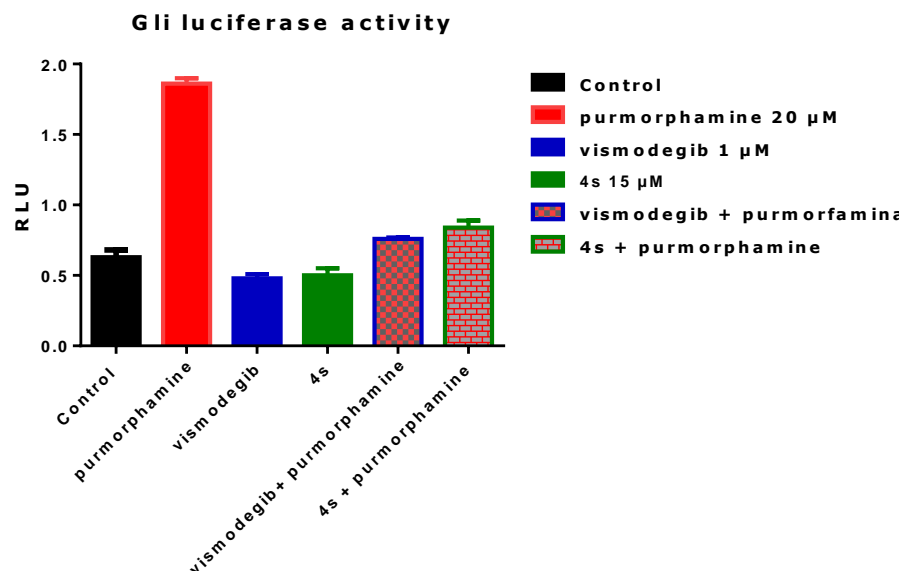


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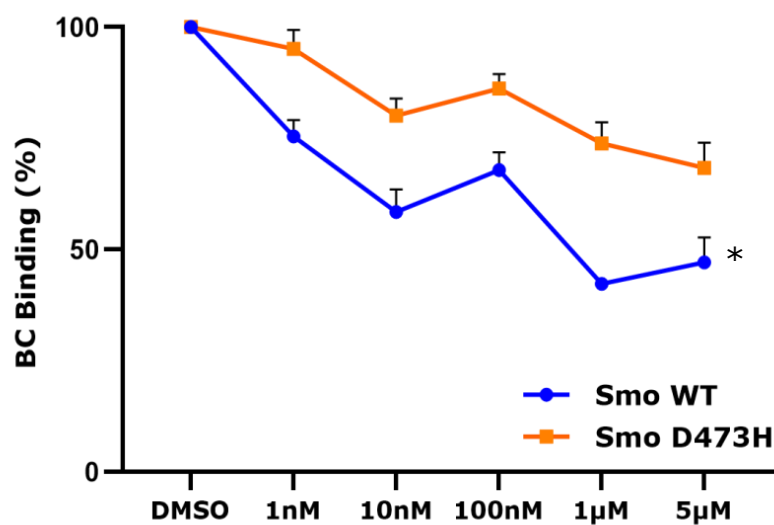
Supplemental Figure S1: 4s triggers apoptosis in various cancer cell lines.

A) Dose-response curves are shown for four neoplastic cancer cell lines and HEK293 cells as a control. Cells were treated with 4s at 1.5, 3.0, 6.25, 19, 25 and 50 μM for 72 h, stained with annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Results are presented as the mean ± SD of three independent experiments. (***) $p < 0.0001$ vs. control. Data were analyzed with a non-parametric t -test. **B)** Representative dot plot of HT29 cells untreated (control) or treated for 3 h with 4s (19 μM).

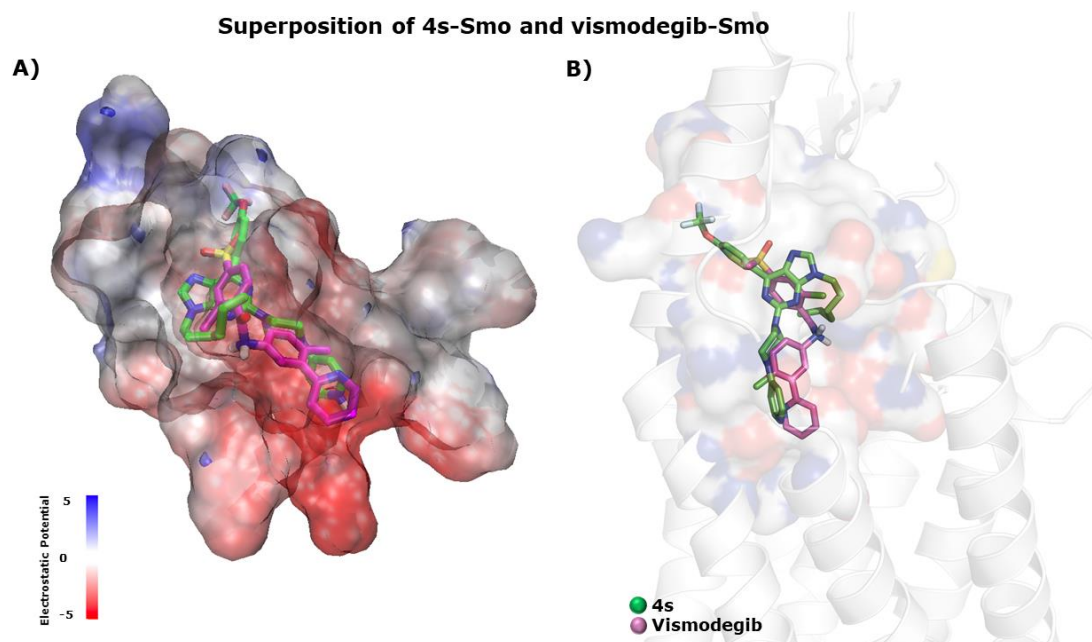


Supplemental Figure S2: Luciferase reporter activity dependent on the Hh pathway.

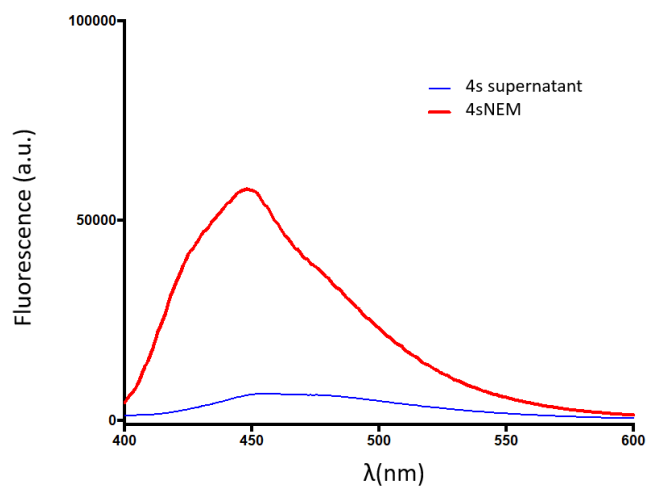
This assay was performed in HCT116 cells stably transformed with a Gli responsive luciferase reporter gene and a renilla luciferase gene (normalization control), treated for 24 h with purmorphamine (20 μ M), vismodegib (1 μ M) or **4s** (1.5 μ M). These concentrations were 100 times higher than the IC₅₀ values reported for each compound. Luciferase and renilla activity were assayed with the dual-luciferase assay system Signal Reporter Gli-luciferase (ADN, Qiagen). Results are expressed as luciferase/renilla activity ratios and represent the mean of triplicate values from one assay.



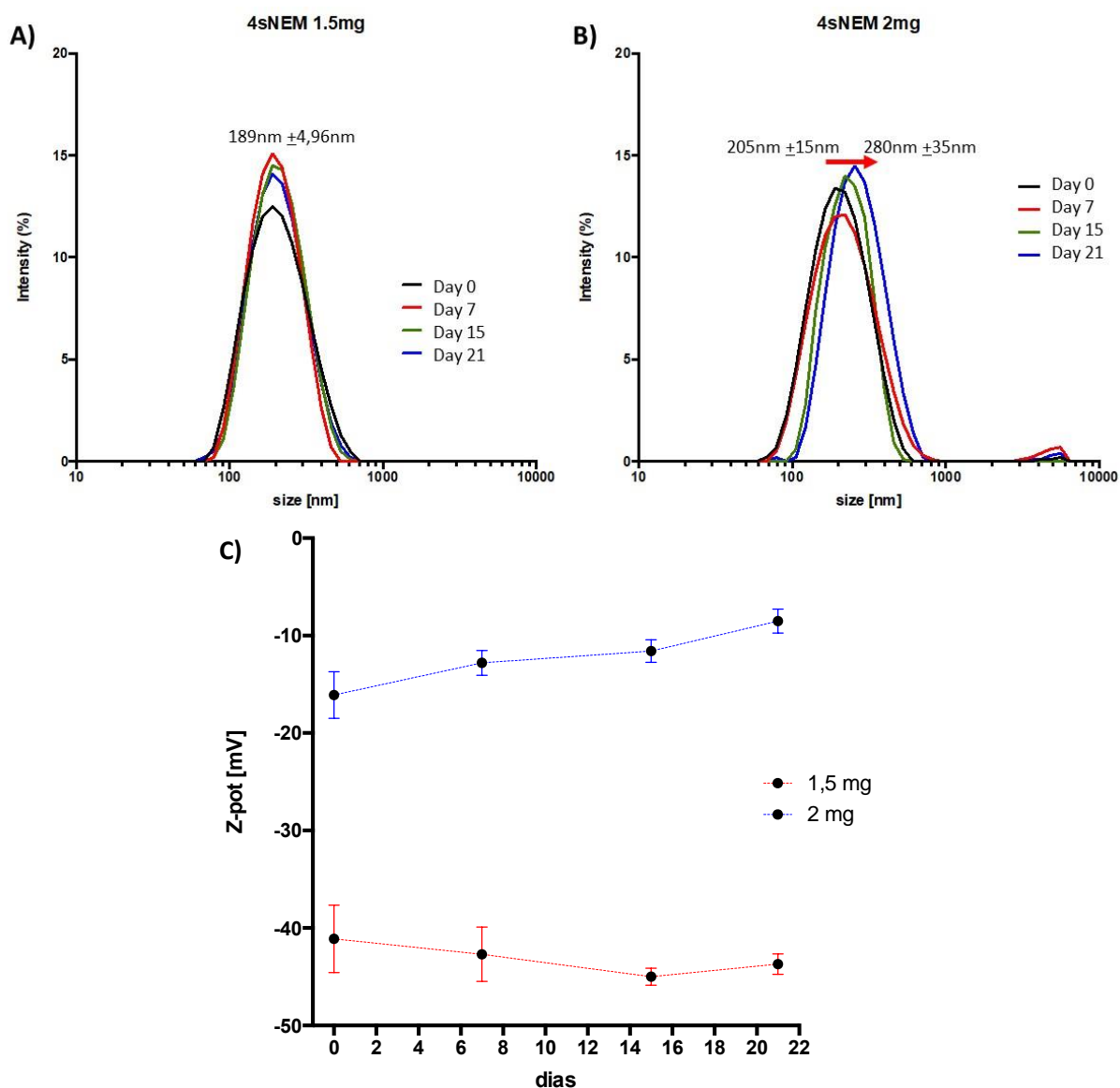
Supplemental Figure S3: Binding of 4s to wild-type and D473H mutant SMO cells. The concentration-response curves represent the percentage of BODIPY-cyclopamine (BC) incorporation into control HEK293T cells carrying either the wild type SMO gene (Smo WT, in blue) or the D473H mutant SMO gene (SMO-D473H, in orange) after treatment with **4s**. IC_{50} Smo WT = 0.95 μ M. At concentrations greater than 5 μ M, **4s** was toxic to SMO D473H. Data represent the average \pm SD of three independent experiments. Data are the average BC intensity of five fluorescence microscopy images.



Supplemental Figure S4: Graphical superposition of 4s-SMO (green) and vismodegib-SMO (fuchsia). **A)** Electrostatic potential. The binding cavity (in red) provides a highly electron-rich environment. **B)** 3D representation of **4s** and vismodegib binding to the inner part of the heptahelical bundle of SMO.



Supplemental Figure S5: Fluorescence spectra of 4s and 4sNEM. The fluorescence was detected with the excitation wavelength 365 nm. Red indicates **4s** incorporate in NEM (**4s-NEM**) and blue indicates **4s** detected in supernatant.

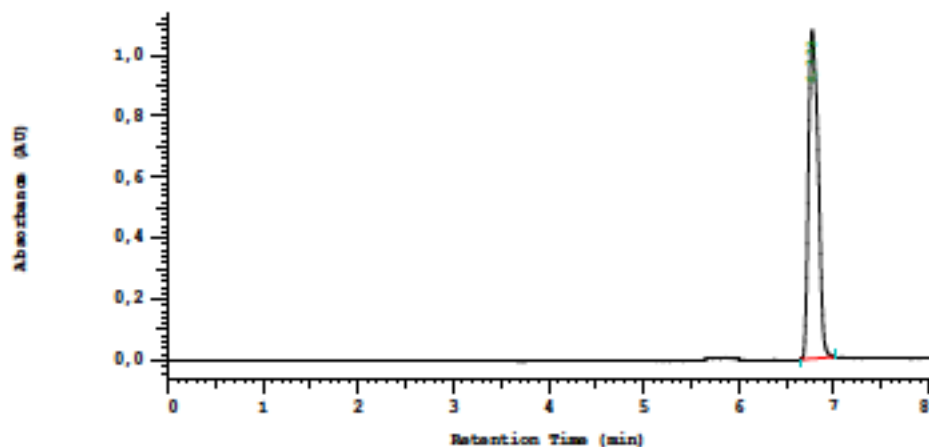


Supplemental Figure S6: Development of a formulation to nanoemulsify the SMO antagonist 4s. The stability of two systems using different amounts of 4s considering the hydrodynamic diameter: **A)** 1.5 mg and **B)** 2 mg, both evaluated for 21 d. **C)** The Z potential were measured in triplicate.

Chromaster System Manager Report

Analyzed Date and Time: 04-12-2020 17:18 Reported Date and Time: 04-12-2020 17:45:49
 Processed Date and Time: 04-12-2020 17:32
 Data Path: C:\WIN32APP\CHROMASTER\polen\DATA\1957\
 Processing Method: Christian
 System (acquisition): Chromaster DAD Series: 1957
 Application(data): polen Vial Number: 2
 Sample Name: 4sA Vial Type: UNK
 Injection from this vial: 1 of 1 Volume: 5,0 ul
 Sample Description:

Chrom Type: Fixed WL Chromatogram, 260 nm



Processing Method: Christian
 Method Developer: Paula
 Pump 1: 5160
 Pump 1 Solvent A: Pump 1 Solvent B:
 Pump 1 Solvent C: MeCN/formico Pump 1 Solvent D: aqua/formico
 Method Description:

Chrom Type: Fixed WL Chromatogram, 260 nm

Peak Quantitation: AREA
 Calculation Method: AREA%

No.	RT	Area	Conc 1	BC
1	6,773	3782079	100,000	BB
		3782079	100,000	

Peak rejection level: 0

Supplemental Figure S7: HPLC spectrum for 4s.

Supplemental Table S2: PAIN's report for purine derivatives **4a-s**. This analysis was done by Swiss Target Prediction platform (<http://www.swisstargetprediction.ch/>).

Compounds	PAIN
4a	0
4b	0
4c	0
4d	0
4e	0
4f	0
4g	0
4h	0
4i	0
4j	0
4k	0
4l	0
4m	0
4n	0
4o	0
4p	0
4q	0
4r	0
4s	0