Supporting information

GNE-064: a potent, selective, and orally bioavailable chemical probe for the bromodomains of SMARCA2 and SMARCA4 and the fifth bromodomain of PBRM1

Alexander M. Taylor*,[†] Chris Bailey,[†] Lisa D. Belmont,[‡] Robert Campbell,[†] Nico Cantone,[†] Alexandre Côté,[†] Terry D. Crawford,[‡] Richard Cummings,[†] Kevin DeMent,[‡] Martin Duplessis,[†] Megan Flynn,[‡] Andrew C. Good,[†] Hon-Ren Huang,[†] Shivangi Joshi,[†]Yves Leblanc,[†] Jeremy Murray,[‡] Christopher G. Nasveschuk,[†] Adrianne Neiss,[†] Florence Poy,[†] F. Anthony Romero,[‡] Peter Sandy,[†] Yong Tang,[†] Vickie Tsui,[‡] Laura Zawadzke,[†] Robert J. Sims, III,[†] James E. Audia,[†] Steven F. Bellon,[†] Steven R. Magnuson,[‡] Brian K. Albrecht,[†] Andrea G. Cochran*,[‡]

[†]Constellation, a Morphosys Company, 215 First St., Suite 200, Cambridge, Massachusetts 02142, United States

[‡]Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, United States

Corresponding Authors
* Email: ataylor@relaytx.com, cochran.andrea@gene.com

Supporting Experimental Procedures

Time-resolved fluorescence resonance energy transfer (TR-FRET) screening assay. Initial screening was conducted by displacement of a biotinylated peptide (ARTKQTARKSTGG-K(Ac)-APR-K(Ac)-QLAT-K(Ac)-AAR-K(Ac)-SAPGG-K(Biotin), Anaspec) from a his-tagged SMARCA4 bromodomain. The bromodomain construct was as indicated, where the underlined protion represents the epitope tag added to the bromodomain and the following "GS" is a residue of the cloning site:

MHHHHHHGSLVPR\GS A_{1448}EKLSPNPPNLTKKMKKIVDAVIKYKDSSSGRQLSEVFIQLPSRKELPEYYE LIRKPVDFKKIKERIRNHKYRSLNDLEKDVMLLCQNAQTFNLEGSLIYEDSIVLQSVFTSVRQKIEKEDD SEGEES$_{1575}$

DMSO stock solutions of compounds were dispensed to the wells of white, 384-well ProxiPlates (PerkinElmer) to a final volume of 150 nL (1% final DMSO concentration). A working stock (10 µL, 1.5x in assay buffer) of his-SMARCA4 (9 nM) and peptide (37.5 nM), or 10 µL assay buffer [50 mM HEPES (Sigma), pH 7.5, 50 mM NaCl, 1 mM TCEP, 0.069 mM Brij-35 (Calbiochem), 0.1 mg/mL BSA (New England Biolabs)] for MIN controls, was added to wells. After incubation for 10 min at room temperature, 5 µL Eu/APC mixture [3x in assay buffer, 150 nM Streptavidin Surelight (SA-APC, PerkinElmer), 0.9 nM Eu-W1024 anti-6xhis antibody (PerkinElmer)] was added and the plate incubated an additional 20 min in the dark. Plates were read on an Envision reader (PerkinElmer).

TR-FRET IC_{50} determinations. Assays for SMARCA2, SMARCA4 and PBRM1(5) bromodomains were conducted in a similar manner as the screens described above, except using a biotinylated small-molecule ligand instead of the peptide. The detailed protocols were as previously described[1].

Isothermal titration calorimetry (ITC). Binding constants were determined by injection of aliquots of his6-Flag-SMARCA4 bromodomain (same construct as above, except with the following tag: MHHHHHHGSLVPR\GSMDYKDDDDKENLYFQ\) into the cell of the calorimeter (MicroCal iTC200; GE Healthcare) filled with compound at an appropriate concentration.

Protein was diluted in ITC buffer (50 mM HEPES pH 7.2, 100 mM NaCl), with the aim of a final concentration of ~200 µM (or higher, as required). The protein was then dialyzed in a D-Tube Dialyzer Midi (6-8 kDa cut off; Novagen) against ITC buffer at 4 °C overnight. After dialysis, protein was centrifuged to pellet any precipitate and then filtered using a Spin-X tube (0.22 µM filter; Corning) to remove any additional insoluble protein. Concentration was then determined by measuring A_{280} on a NanoDrop spectrophotometer (Thermo). Dialysate from buffer exchange was degassed and filtered for use in experiments as buffer control or for dilution of reagents. DMSO was carefully matched between injectant and sample cell and was typically 0.2%. Injections (16; 2.43 µL) were performed at 25 °C, and data were analyzed using the MicroCal software.

For the SMARCA2 bromodomain experiments, N-terminally tagged wildtype (MHHHHHHGSLVPRGS\S1377–Q1486; Uniprot P51531-2) or the L1412P, P1413V, S1414N variant ("PVN" mutant) binding to **4** or to a peptide ligand was evaluated in replicate runs performed on a MicroCal Auto-iTC200 (Malvern Panalytical, Inc. Westborough, MA). Binding of **4** was characterized through a "reverse injection" procedure at matched DMSO concentration similar to that described above. Protein was dialyzed into 50 mM HEPES, pH 7.5, 150 mM NaCl and then centrifuged to remove any insoluble protein. Injections (35; 1 at 0.2 µl, 34 at 1.1 µL) were performed at 15 °C. Peptide (histone $H3_{9–19}$ K14ac with an added C-terminal tyrosine for spectrophotometric quantitation) was instead injected into bromodomain (in 20 mM HEPES, pH

7.5, 150 mM NaCl, 1 mM TCEP) in the calorimeter cell (40 injections total: 1 at 0.1 µl, 39 at 1 µl; 10 °C). For all experiments, protein and peptide conentrations were determined from calculated extinction coefficients by measuring A_{280} in a quartz cuvette using a SpectraMax M5 spectrophotometer (Molecular Devices). Raw data were processed in NITPIC[2], and control runs (injection of syringe component into buffer) were subtracted. Replicate data were fitted globally in SEDPHAT[3] to a 1:1 binding model, allowing the concentration of the component in the cell to vary locally to adjust to small stoichiometry differences among runs. After fits had converged, confidence intervals for fitted parameters were simulated in SEDPHAT as described[4, 5]. Final figures showing the global fit overlaid on representative runs were prepared using the ITC module of GUSSI[5].

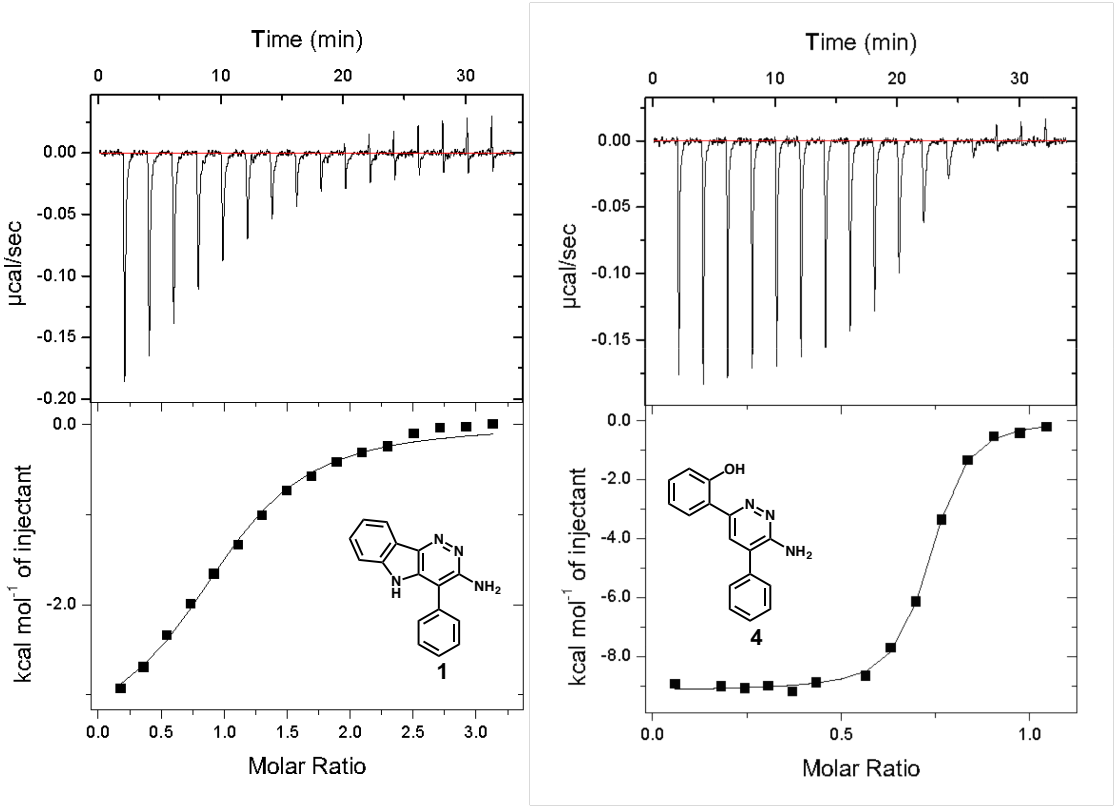
Crystallography. The SMARCA4 bromodomain used for crystallography was the same construct listed in the TR-FRET assay section above, except that the N-terminal tag was removed by treatment with thrombin. The protein was concentrated in 20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM TCEP, to a final concentration of 15.55 mg/mL (1.03 mM). Compounds were dissoved in DMSO at 100 mM. SMARCA4 bromodomain (30 µL) was mixed throughly with 1 µL compound stock and incubated on ice for 60 min. The sample tube was then centrifuged briefly at 8 °C to remove any precipitated material. Using a Mosquito® Crystal crystallization robot (TTP Labtech), 200 nL protein mixture was mixed with 200 nL reservoir solution (30% Jeffamine® ED-2001, pH 7.0 in 0.1 M Tris-HCl, pH 8.5) and 20 nL of a suitable additive (determined by screening Hampton Research kit HR2-138; see Table S1). The mixtures were equilibrated against 75 µL of reservoir solution at 4 °C in a low profile Intelli-Plate 96-2 (Hampton Research) via the sitting-drop vapor-diffusion method. Plate-like crystals were obtained, usually within 1-3 days. A suitable piece was cracked and transferred into oil (Paratone:Paraffin=1:1), washed to remove excess aqueous solution on the crystal surface, and flash-frozen in liquid nitrogen. Data were collected by Shamrock Structures, LLC (Woodbridge, IL) at either the Canadian Light Source (CLS; beamline CMCF-08ID; Rayonix 300 detector) for **1** or SER-CAT (APS, Argonne National Laboratory; beamline 22-ID; MAR 300 detector). Structures were solved by molecular replacement and refined using the CCP4 suite of programs[6] and REFMAC5[7]. Structural statistics are reported in Table S1.

Cellular target engagement ("dot") assay. A U2OS-derived stable cell line was engineered to express in a Tet-inducible fashion the bromodomain (BD) and surrounding sequences of SMARCA2 (isoform B) fused to a nuclear localization signal (NLS) and the ZsGreen fluorescence tag. Upon addition of bromodomain inhibitors, the homogeneous nuclear localization of NLS-ZsGreen-SMARCA2 fusion protein changes to a more pronounced dot-like pattern. These foci can be counted using appropriate imaging tools and software. The number of foci is dose-dependent and shows a typical sigmoidal curve from which the EC_{50} can be computed.

Cells were seeded in 384-well imaging plates (5000 cells per well) in DMEM containing 10% FBS, 1% Pen/Strep, 1% HEPES and 15 µg/mL blasticidin supplemented with 200 ng/mL doxycycline just before plating. Cells were incubated overnight at 37 °C, 5% CO_2. After removal of medium, compound-containing medium (30 µL) was added to each well, followed by incubation at 37°C for 1h. Medium was removed and cells were fixed for 10 min at room temperature by addition of 30 µL 4% PFA [16% paraformaldehyde stock diluted with 10x PBS (to 1x final concentration) and dH_2O]. Fixative was removed, wells were washed once with PBS, and fresh PBS was added (30 µL). Plates were stored in the dark at room temperture (or at 4 °C longer term) until read. Images were acquired on an ImageXpress Micro high-content screening system (Molecular Devices) and analyzed with the MetaXpress 3.0 software supplied.

Pharmacokinetics study. Exposure and oral availability of **5** were evaluated in female CD-1 mice (Charles River Laboratory; N=3 per timepoint) in a cassette format[8] (6 different compounds dosed together). For the IV arm, compounds were dissolved at a final concentration of 0.1 mg mL^{-1} in a solution of 35% PEG400 in H_2O and dosed at 0.5 mg (5 mL) kg^{-1}. The formulation for oral dosing was a suspension of 0.2 mg mL^{-1} **5** in medium-chain triglyceride (MCT) oil; the administered dose was 1 mg kg^{-1}. All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and were approved by the internal Animal Care and Use Committee.

Figure S1. Isothermal titration calorimetry (ITC) analysis of binding of **1** and **4** to SMARCA4 bromodomain.



parameter[a]	1 (run 1)	1 (run 2)	4
N	1.02 ± 0.03	0.96 ± 0.04	1.410 ± 0.004
K_D, µM	3.5 ± 0.5	3.2 ± 0.7	0.063 ± 0.005
ΔH, cal mol^{-1}	–3430 ± 80	–3300 ± 100	–6480 ± 30
ΔS, cal mol^{-1} K^{-1}	13.4	13.9	11.2

[a]Fitted parameters (N, K_D, and ΔH) and curve-fitting errors were generated by the MicroCal software.

Figure S2. Comparison of NMP (PDB:3MB4[9]; yellow protein, orange ligand) and salicylic acid (PDB:4Y03[10]; purple protein, pink ligand) binding to PBRM1(5). Yellow non-bonded spheres indicate conserved water molecules 1–4 that are displaced in the salicylic acid complex.

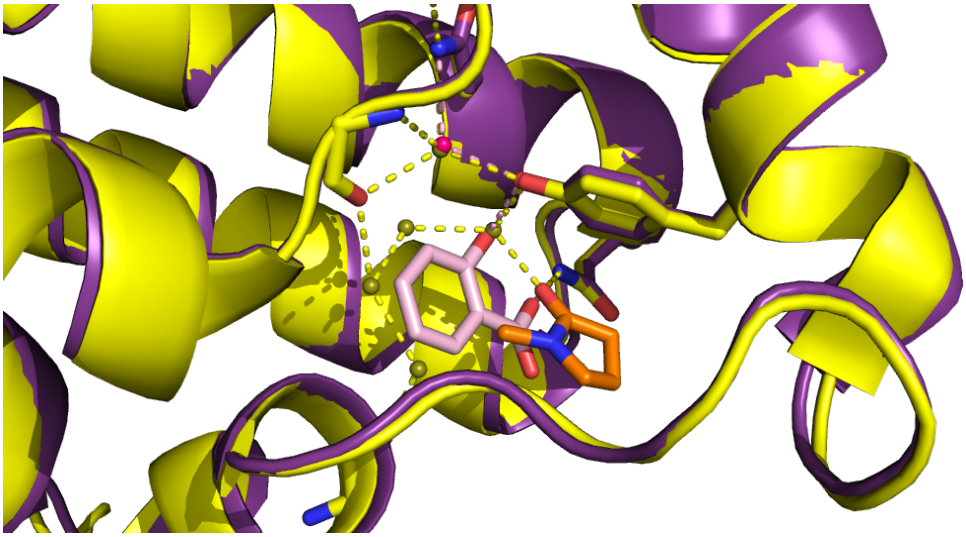


Figure S3. Comparison of the dynamic "open" and "closed" states of SMARCA2 bromodomain. Published models of each state[11] were overlaid using the αB and αC helices. A. Open state (gray) and closed state (cyan) showing the shift of ZA loop residues into the ZA channel upon opening. One hydrogen bond characteristic of the closed state is shown (dotted lines); this is the equivalent of the $L653^{O}$–$E675^{N}$ H-bond of PBRM1(5) mentioned in the text. In the open state, the O to N distance increases from 3.1 Å to 8.4 Å. The "LPS" motif is shown in stick representation. B. The overlaid models described in A (which include no structural waters) are rotated and overlaid with the water network from the PBRM1(5) NMP complex (PDB:3MB4[9]; water 0 shown as a yellow sphere; waters 1–4 rendered as red spheres). The leucine sidechain from the "LPS" motif results in unusual positions for waters 3 and 4 in the PBRM1(5) crystal structure (closed state) as described in the text. In the open state model, this leucine residue shifts well into the water pocket and would cause a severe steric clash with waters 1–4. This clash is expected to result in either ejection of the waters or a substantial rearrangement.

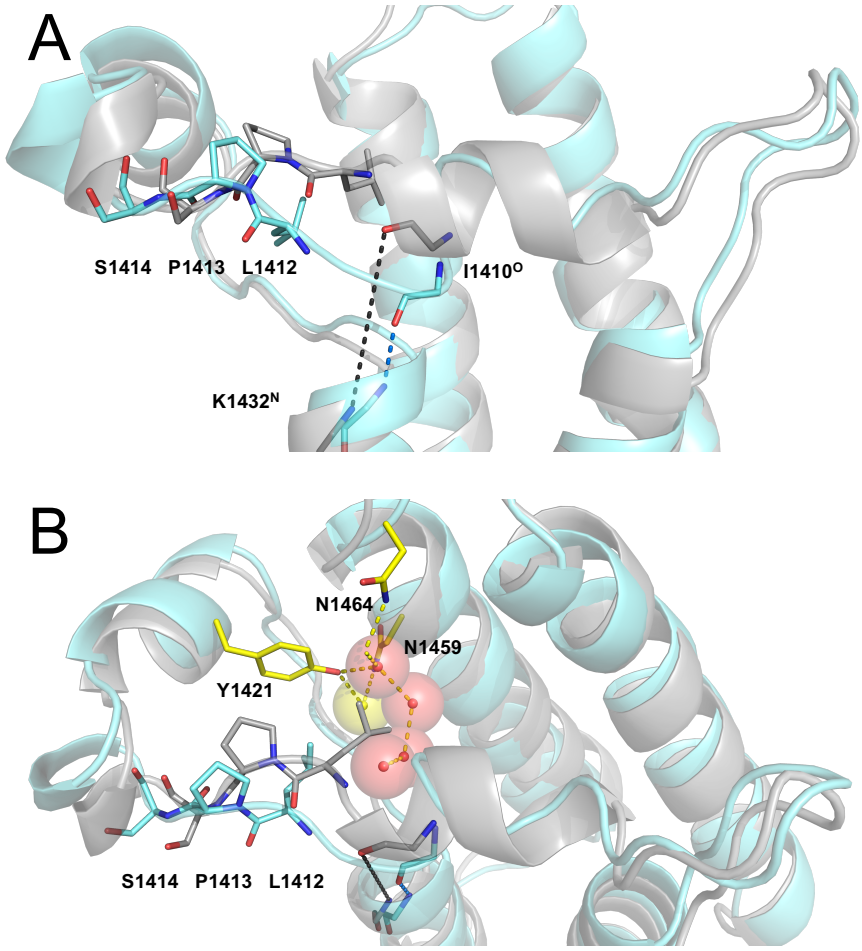


Figure S4. Comparison of the dynamic open and closed states of the first bromodomain of BRD4. Published models[11] were aligned and colored as in Fig. S3. A. Comparison of the two states. The closed state hydrogen bond Q84O to G108N is indicated by the dotted lines. The O to N distance increases from 3.4 Å to 7.0 Å in the open state. B. The water network from the BRD4(1) crystal structure (PDB:3UVW9) is overlaid onto the models shown in A. In contrast to the case of SMARCA2 (Fig. S3B), the open state model does not indicate any steric conflict with the structural waters.

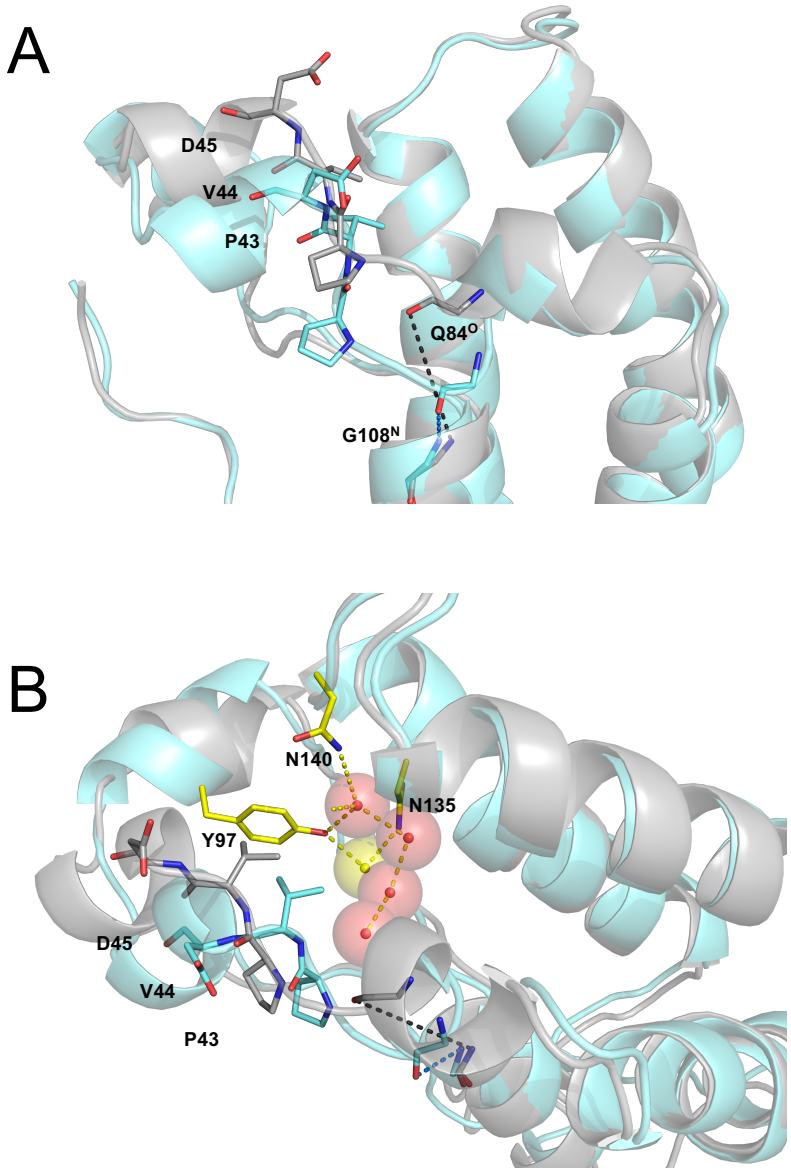


Table S1. X-ray data collection and refinement statistics

	SMARCA4 BD in complex with **1**	SMARCA4 BD in complex with **4**
PDB ID	7TD9	7TAB
Data collection		
Space group	P65	P21
Cell constants a, b, c, α, β, γ	90.26 Å, 90.26 Å, 96.79 Å 90.00°, 90.00°, 120.00°	30.23 Å, 28.60 Å, 65.74 Å 90.00°, 91.31°, 90.00°
Resolution (Å)	41.15 – 1.61	32.88 – 1.16
% Data completeness (in resolution range)	99.9 (41.15-1.61)	98.1 (32.88-1.16)
R_{merge} (%)	7.0	8.0
<I> / <σI>	6.03 (at 1.61 Å)	6.43 (at 1.16 Å)
Refinement		
R_{work} / R_{free} (%)	19.6/23.7	18.0/20.1
R_{free} test set	2933 reflections (5.08%)	1943 reflections (5.03%)
Wilson B-factor (Å^2)	24.4	9.8
Anisotropy	0.000	0.032
Bulk solvent k_{sol}(e/Å^3), B_{sol}(Å^2)	0.34 , 40.8	0.38 , 47.5
L-test for twinning	< |L| > = 0.49, < L^2 > = 0.32	< |L| > = 0.49, < L^2 > = 0.32
Estimated twinning fraction	0.033 for h,-h-k,-l	0.027 for h,-k,-l
F_O,F_C correlation	0.96	0.96
Total number of atoms	3163	1170
Average B, all atoms (Å^2)	29.0	13.0
Crystallization		
Protein solution	20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM TCEP, 1.03 mM (15.55 mg/mL) protein	20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM TCEP, 1.03 mM (15.55 mg/mL) protein
Reservoir solution	30% Jeffamine ED-2001 (pH 7.0), 0.1 M Tris-HCl (pH 8.5), 5% v/v ethyl acetate	30% Jeffamine ED-2001 (pH 7.0), 0.1 M Tris-HCl (pH 8.5), 4% PEG 400

Table S2. Thermodynamic parameters for binding of acetylated peptide and compound **4** to wild-type (WT) and "PVN" mutant (MUT) SMARCA2 bromodomain

	parameter[a]	Value (WT)	CI 95%[b]	Value (MUT)	CI 95%
Peptide[c] (283 K)	log10 K_A	3.7	[3.57, 3.83]	3.3	[3.10, 3.53]
	K_D, μM	190	[147, 263]	480	[300, 800]
	ΔH, kcal mol^{-1}	–4.0	[–6.27, –3.13]	–2.5	[–3.45, –1.95]
	ΔS, cal mol^{-1} K^{-1}	2.7		6.3	
	TΔS, kcal mol^{-1}	0.76		1.8	
4 (288 K)	log10 K_A	7.2	[6.82, 7.75]	6.0	[4.81, 7.27]
	K_D, nM	61	[18, 150]	1100	[54, 16000]
	ΔH, kcal mol^{-1}	–9.2	[-10.5, -7.86]	–2.7	[–4.67, –1.77]
	ΔS, cal mol^{-1} K^{-1}	1.1		18	
	TΔS, kcal mol^{-1}	0.32		5.2	

[a]Thermodynamic parameters resulting from global fit of replicate datasets to a 1:1 model as described in Supporting Experimental Procedures. [b]Simulated 95% confidence intervals for the fitted parameters. [c]Peptide derived from histone H3 with acetylation at lysine 14.

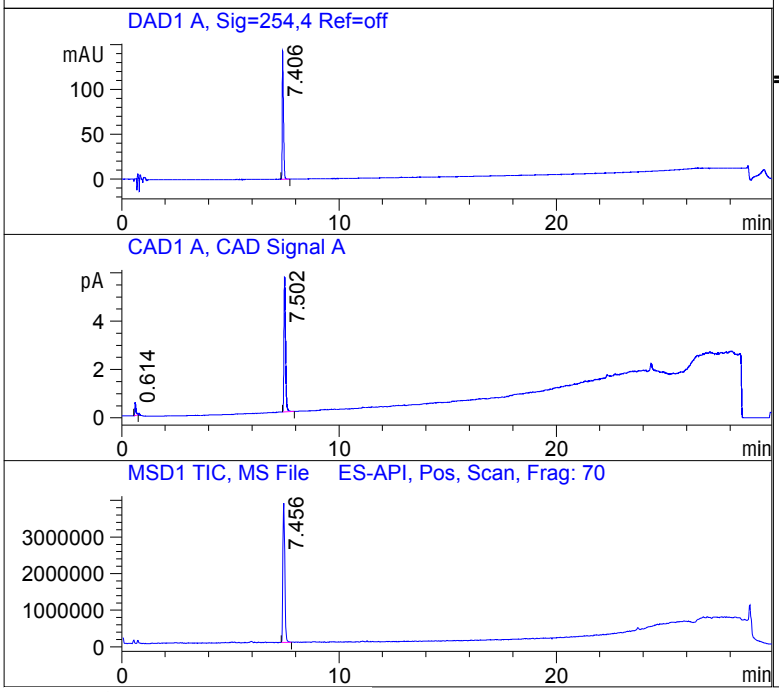
Table S3. BROMO*scan* profiling of compounds **4** and **5** (GNE-064)

BROMOscan assay	op	**4**, K_D, nM	op	**5**, K_D, nM
ATAD2A	>	10000	=	9100
ATAD2B	>	10000	>	10000
BAZ2A	>	10000	>	10000
BAZ2B	>	10000	>	10000
BRD1	>	10000	>	10000
BRD2(1)	>	10000	>	10000
BRD2(1,2)	>	10000	>	10000
BRD2(2)	>	10000	>	10000
BRD3(1)	>	10000	>	10000
BRD3(1,2)	>	10000	>	10000
BRD3(2)	>	10000	>	10000
BRD4(1)	>	10000	>	10000
BRD4(1,2)	>	10000	>	10000
BRD4(2)	>	10000	>	10000
BRD4(full-length,short-iso.)	>	10000	>	10000
BRD7	=	6500	=	6000
BRD8(1)	>	10000	>	10000
BRD8(2)	>	10000	>	10000
BRD9	=	7900	=	5000
BRDT(1)	>	10000	>	10000
BRDT(1,2)	>	10000	>	10000
BRDT(2)	=	8200	>	10000
BRPF1	>	10000	>	10000
BRPF3	>	10000	>	10000
CECR2	>	10000	=	4500
CREBBP	>	10000	>	10000
EP300	>	10000	>	10000
FALZ	>	10000	>	10000
GCN5L2	>	10000	>	10000
PBRM1(2)	=	550	=	490
PBRM1(5)	=	69	=	18
PCAF	>	10000	>	10000
SMARCA2	=	71	=	10
SMARCA4	=	86	=	16
TAF1(2)	>	10000	>	10000
TAF1L(2)	>	10000	>	10000
TRIM24(Bromo.)	>	10000	>	10000
TRIM24(PHD,Bromo.)	>	10000	>	10000
TRIM33(PHD,Bromo.)	>	10000	>	10000
WDR9(2)	>	10000	>	10000

Supporting References

1. Albrecht, B. K.; Cote, A.; Crawford, T.; Duplessis, M.; Good, A. C.; LeBlanc, Y.; Magnuson, S. R.; Nasveschuk, C. G.; Romero, F. A.; Tang, Y.; Taylor, A. M. Therapeutic Compunds and Uses Thereof. US 10,308,614 B2, June 4, 2019.
2. Keller, S.; Vargas, C.; Zhao, H.; Piszczek, G.; Brautigam, C. A.; Schuck, P., High-precision isothermal titration calorimetry with automated peak-shape analysis. *Anal Chem* **2012,** *84* (11), 5066-73.
3. Zhao, H.; Piszczek, G.; Schuck, P., SEDPHAT--a platform for global ITC analysis and global multi-method analysis of molecular interactions. *Methods* **2015,** *76*, 137-148.
4. Paketuryte, V.; Petrauskas, V.; Zubriene, A.; Abian, O.; Bastos, M.; Chen, W. Y.; Moreno, M. J.; Krainer, G.; Linkuviene, V.; Sedivy, A.; Velazquez-Campoy, A.; Williams, M. A.; Matulis, D., Uncertainty in protein-ligand binding constants: asymmetric confidence intervals versus standard errors. *Eur Biophys J* **2021,** *50* (3-4), 661-670.
5. Brautigam, C. A.; Zhao, H.; Vargas, C.; Keller, S.; Schuck, P., Integration and global analysis of isothermal titration calorimetry data for studying macromolecular interactions. *Nat Protoc* **2016,** *11* (5), 882-94.
6. Collaborative Computational Project, N., The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* **1994,** *50* (Pt 5), 760-3.
7. Murshudov, G. N.; Skubak, P.; Lebedev, A. A.; Pannu, N. S.; Steiner, R. A.; Nicholls, R. A.; Winn, M. D.; Long, F.; Vagin, A. A., REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr D Biol Crystallogr* **2011,** *67* (Pt 4), 355-67.
8. Smith, N. F.; Raynaud, F. I.; Workman, P., The application of cassette dosing for pharmacokinetic screening in small-molecule cancer drug discovery. *Mol Cancer Ther* **2007,** *6* (2), 428-40.
9. Filippakopoulos, P.; Picaud, S.; Mangos, M.; Keates, T.; Lambert, J. P.; Barsyte-Lovejoy, D.; Felletar, I.; Volkmer, R.; Muller, S.; Pawson, T.; Gingras, A. C.; Arrowsmith, C. H.; Knapp, S., Histone recognition and large-scale structural analysis of the human bromodomain family. *Cell* **2012,** *149* (1), 214-31.
10. Fedorov, O.; Castex, J.; Tallant, C.; Owen, D. R.; Martin, S.; Aldeghi, M.; Monteiro, O.; Filippakopoulos, P.; Picaud, S.; Trzupek, J. D.; Gerstenberger, B. S.; Bountra, C.; Willmann, D.; Wells, C.; Philpott, M.; Rogers, C.; Biggin, P. C.; Brennan, P. E.; Bunnage, M. E.; Schule, R.; Gunther, T.; Knapp, S.; Muller, S., Selective targeting of the BRG/PB1 bromodomains impairs embryonic and trophoblast stem cell maintenance. *Sci Adv* **2015,** *1* (10), e1500723.
11. Raich, L.; Meier, K.; Gunther, J.; Christ, C. D.; Noe, F.; Olsson, S., Discovery of a hidden transient state in all bromodomain families. *Proc Natl Acad Sci U S A* **2021,** *118* (4), e2017427118.

DataFile: ..nts\ChemStation\1\Data\AGILENT-070522-01 2022-07-05 15-26-56\80823396.D
Inj.Date: 05-Jul-22, 16:00:08
Sample Name : 80823396 Location: D1F-A1
AcqOper: SYSTEM Inj: 1
SpecRptd: UV Peaks InjVol: 2
Tgt.Mass: (CHM) SeqLine: 2
AcqMethod: ..Station\1\Data\AGILENT-070522-01 2022-07-05 15-26-56\CAD_30MIN_07ML.M
AnalyMeth: ..Station\1\Data\AGILENT-070522-01 2022-07-05 15-26-56\CAD_30MIN_07ML.M
SampInfo:
MethInfo: 30 min LCMS with 0.7mL/min



Integration for DAD1 A, Sig=254,4 Ref=off

R.T.	Width	Area	Height	Area%	MS+
7.41	0.06	572.70	143.47	100.00	248.1

Integration for CAD1 A, CAD Signal A

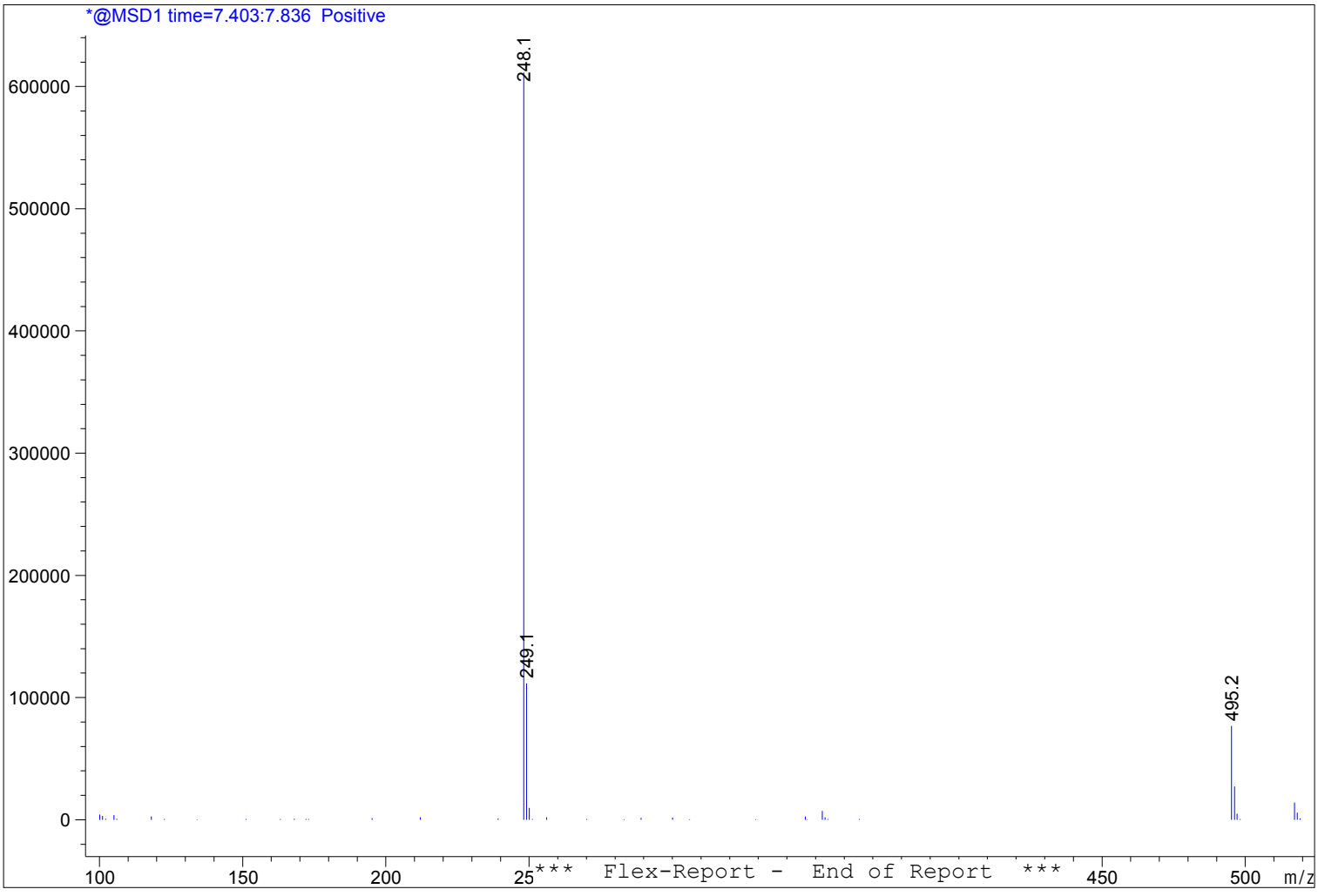
R.T.	Width	Area	Height	Area%	MS+
0.61	0.05	2.02	0.55	6.44	346.3
7.50	0.08	29.29	5.59	93.56	248.1

Integration for MSD1 TIC, MS File

R.T.	Width	Area	Height	Area%	MS+
7.46	0.10	23504336.00	3825900.50	100.00	248.1

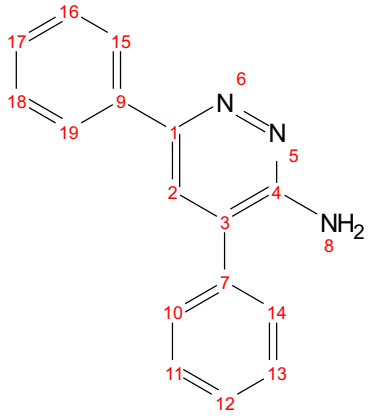
Compound **2**

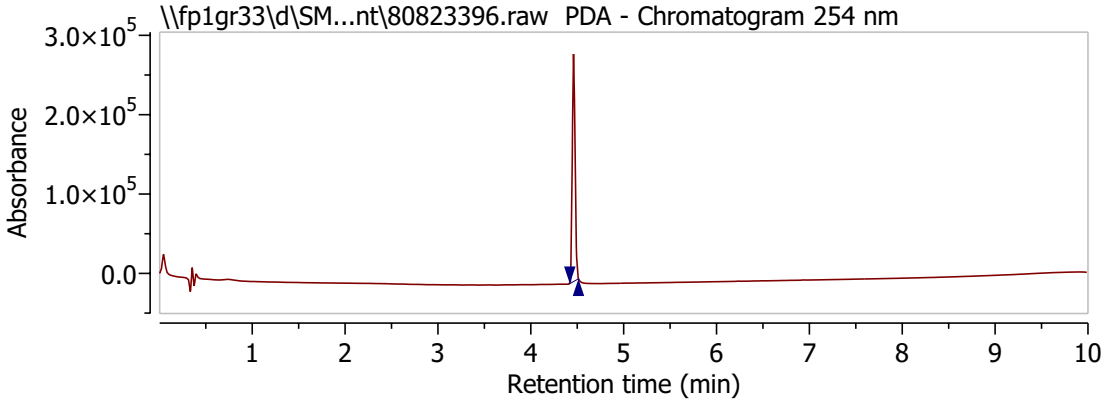
Ret. Time: 7.406 DAD1 A



Molecule matched at RT 4.80 min. Adduct/Loss = H+/. MF=C16H13N3.
Matched m/z=248.1178. Predicted m/z=248.1182. Error = -1.72ppm.

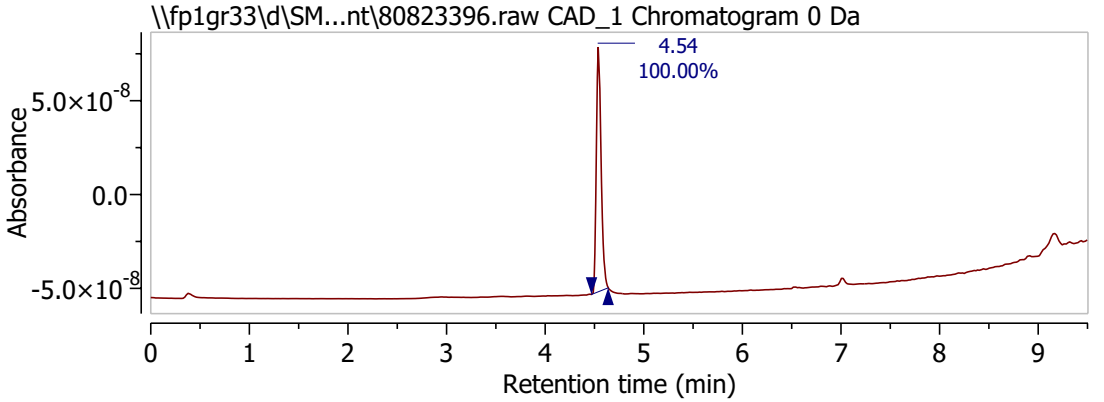
File : D:\SMDICurrent\80823396.raw
Acquired Date : 2022-07-05
Acquired Time : 16:11:16
Sample Name : 80823396
Operator : Kewei
Instrument : Thermo
Sample Position : GC3
Method : D:\Kewei\methods\Test_CE.meth





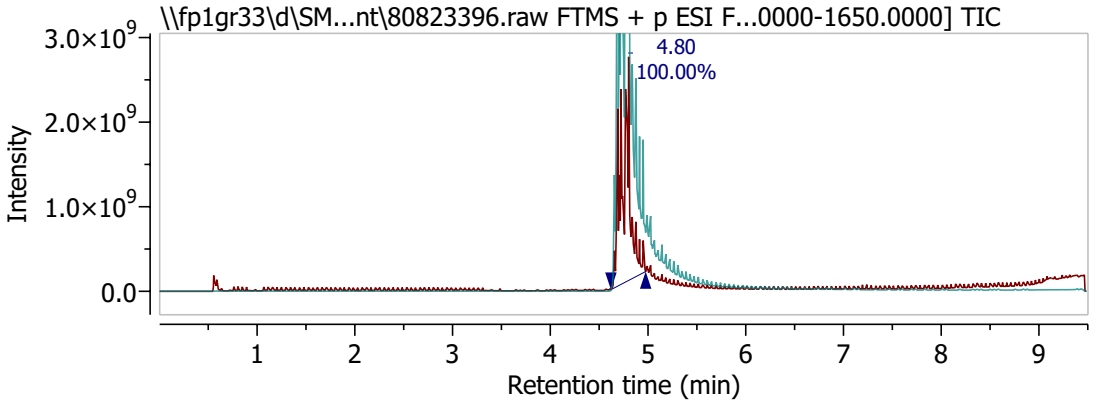
DAD, Sig=254 (>=1.5%)

RT	Area	Area %	MS+
4.46	2920290.00	100.00	248.12



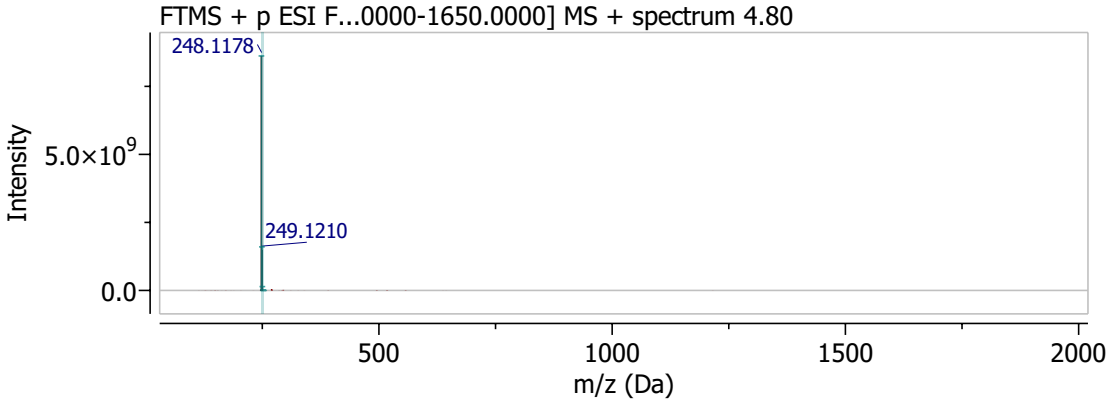
CAD_1 (>=5%)

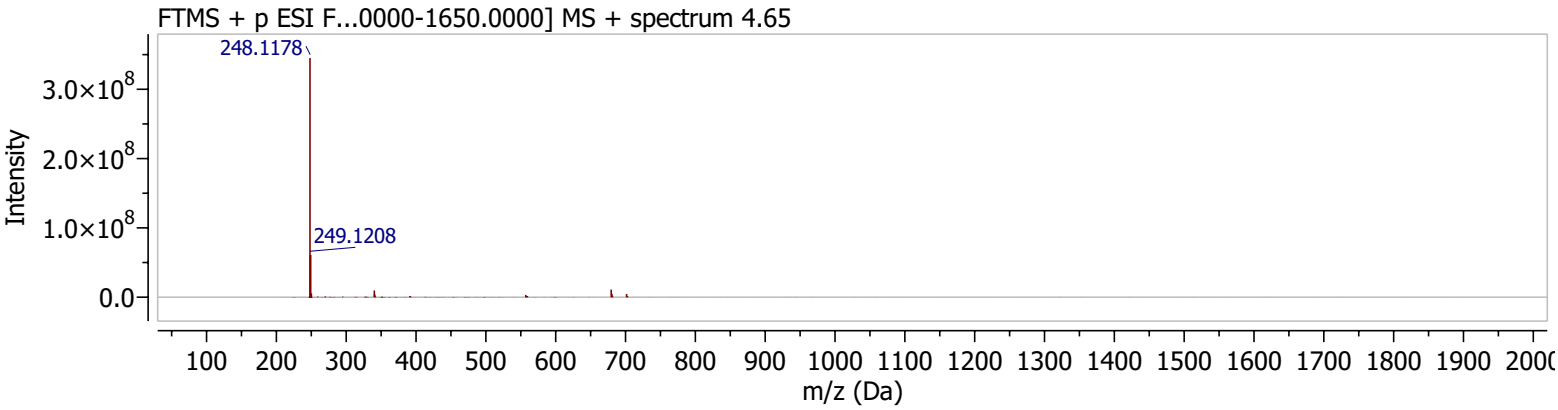
RT	Area	Area %	MS+
4.54	8772.27	100.00	248.12



TIC (>=2%)

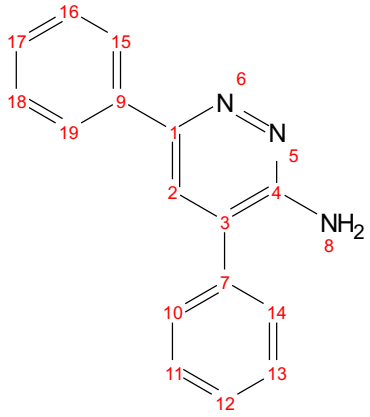
RT	Area	Area %	MS+
4.80	38796273480.00	100.00	248.12

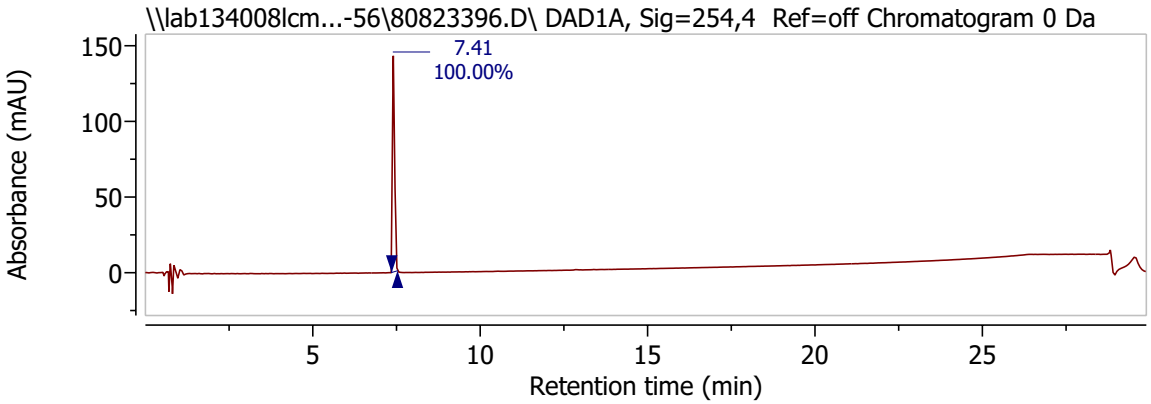



FTMS + p ESI F...0000-1650.0000] MS + spectrum 4.65
248.1178
249.1208
Intensity
3.0×10^8
2.0×10^8
1.0×10^8
0.0
100 200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900 2000
m/z (Da)

Molecule matched at RT 7.46 min. Adduct/Loss = H+/. MF=C16H13N3.
Matched m/z=248.1000. Predicted m/z=248.1182. Error = -73.45ppm.

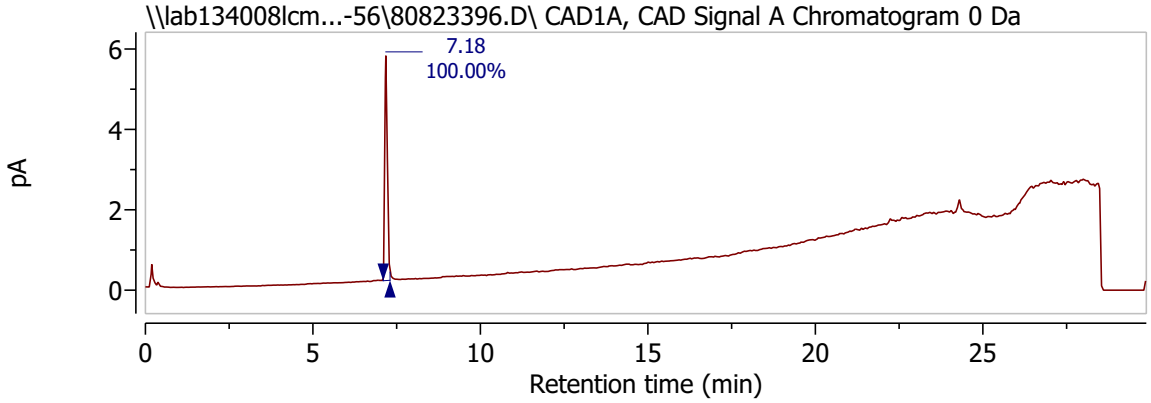
File : C:\Users\Public\Documents\ChemStation\1\Data\AGILENT-070522-01 2022-07-05 15-26-56\80823396.D
Acquired Date : 2022-07-05
Acquired Time : 16:30:12
Sample Name : 80823396
Operator : Kewei
Instrument : Agilent(New)
Sample Position : undefined
Acq. Method: CAD_30MIN_07ML.M





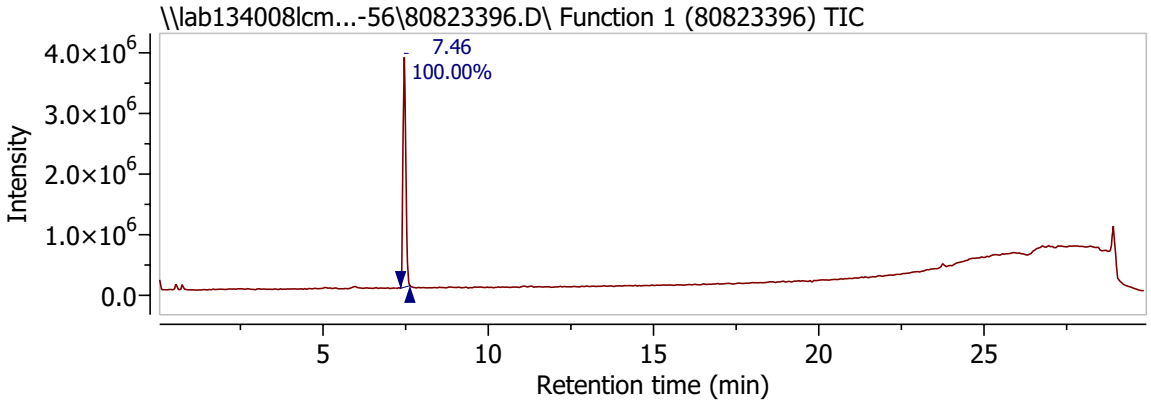
DAD1A, Sig=254,4 Ref=off (>=1.5%)

RT	Area	Area %	MS+
7.41	5611.12	100.00	248.10



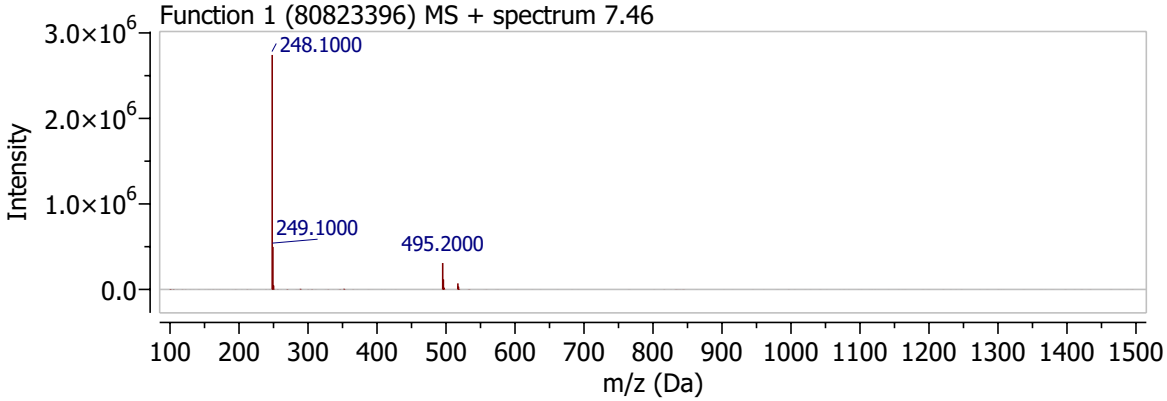
CAD CHANNEL A (>=5%)

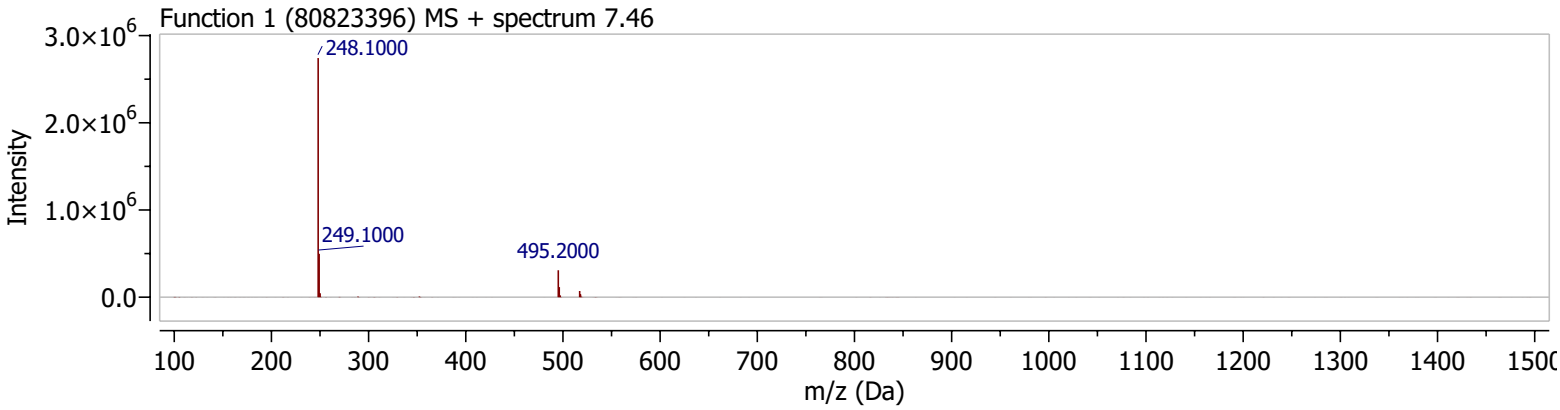
RT	Area	Area %	MS+
7.18	287.51	100.00	248.10



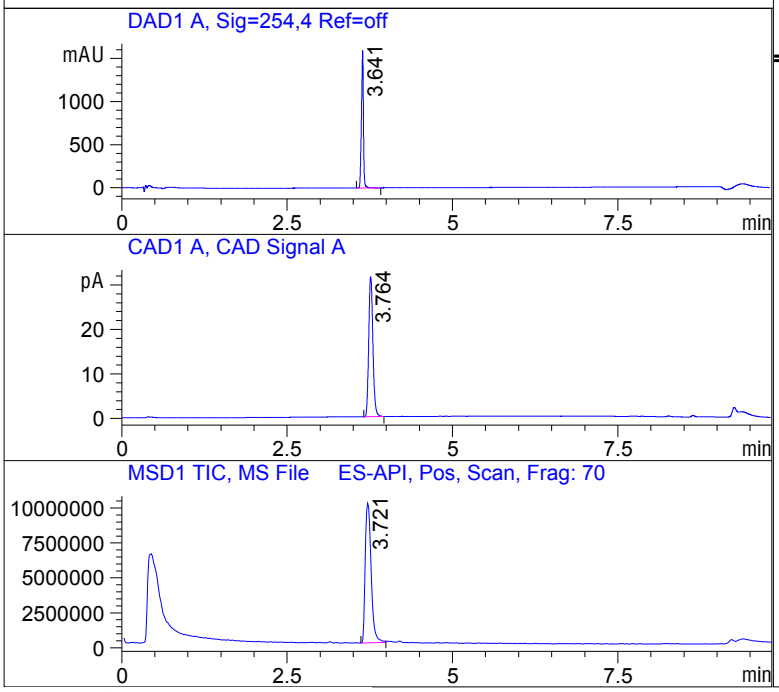
TIC (>=2%)

RT	Area	Area %	MS+
7.46	20352013.00	100.00	248.10




Function 1 (80823396) MS + spectrum 7.46
3.0×10^6
2.0×10^6
1.0×10^6
0.0
Intensity
248.1000
249.1000
495.2000
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z (Da)

DataFile:	..nts\ChemStation\1\Data\AGILENT-013122-01 2022-01-31 14-57-44\80962855.D
Inj.Date:	31-Jan-22, 17:38:58
Sample Name :	80962855
Location:	D1F-B1
AcqOper:	SYSTEM
Inj:	1
SpecRptd:	UV Peaks
InjVol:	2
Tgt.Mass:	(CHM)
SeqLine:	12
AcqMethod:	..Station\1\Data\AGILENT-013122-01 2022-01-31 14-57-44\CAD_10MIN_04ML.M
AnalyMeth:	..Station\1\Data\AGILENT-013122-01 2022-01-31 14-57-44\CAD_10MIN_04ML.M
SampInfo:	
MethInfo:	10 min LCMS with 0.4mL/min



Integration for DAD1 A, Sig=254,4 Ref=off

R.T.	Width	Area	Height	Area%	MS+
3.64	0.03	3339.61	1588.85	100.00	264.1

Integration for CAD1 A, CAD Signal A

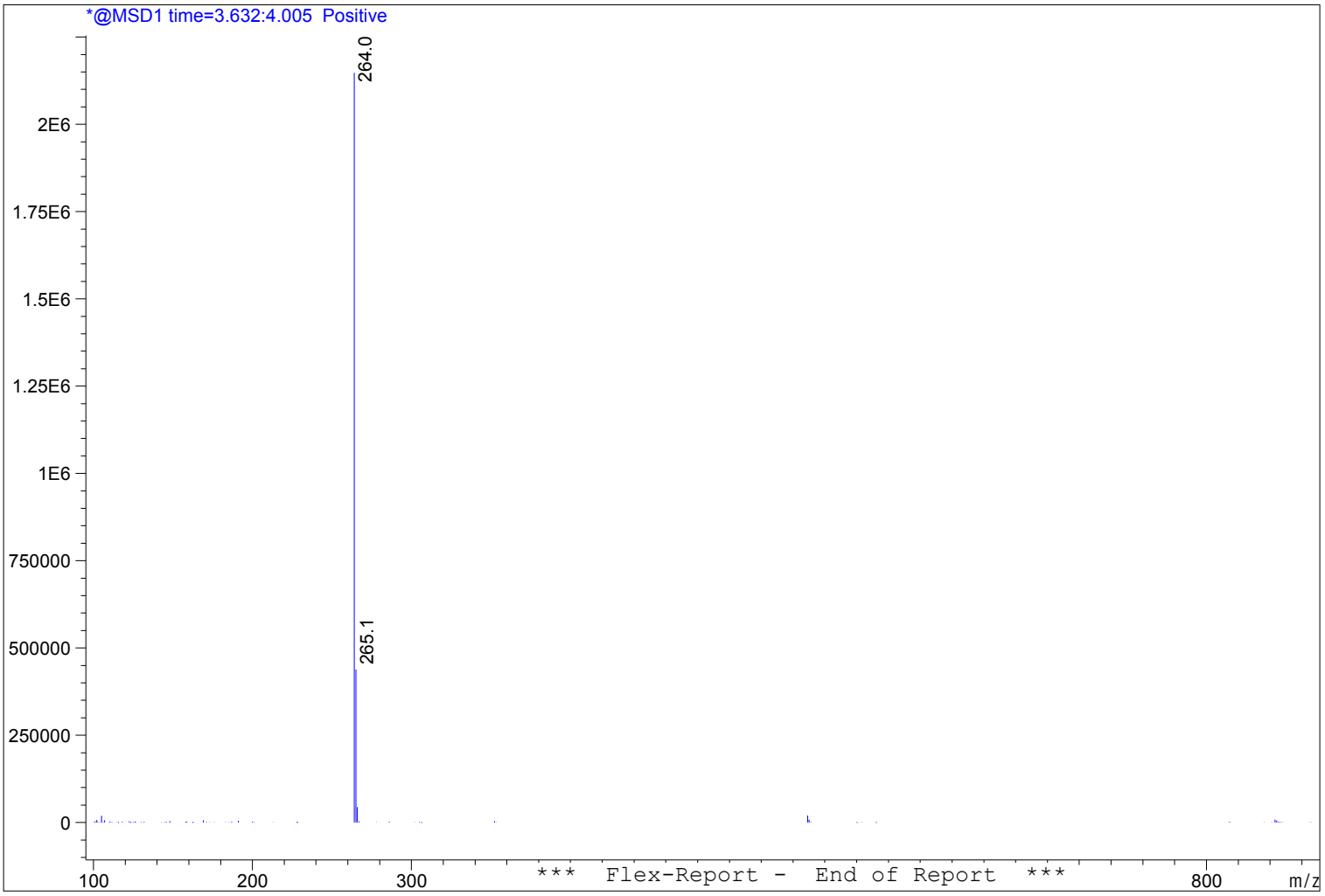
R.T.	Width	Area	Height	Area%	MS+
3.76	0.07	137.19	31.40	100.00	264.1

Integration for MSD1 TIC, MS File

R.T.	Width	Area	Height	Area%	MS+
3.72	0.10	61455148.00	10024956.00	100.00	264.1

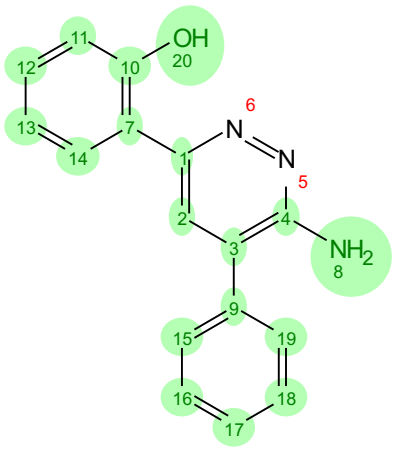
Compound **4**

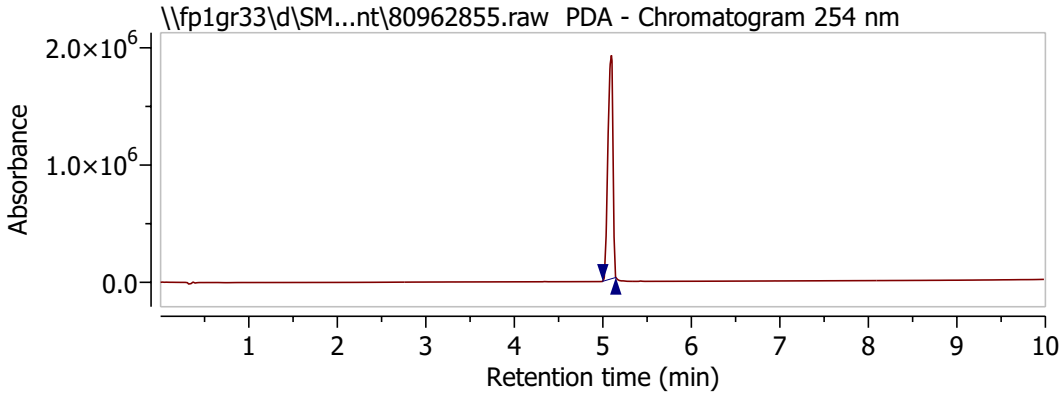
Ret. Time: 3.641 DAD1 A



Molecule matched at RT 5.32 min. Adduct/Loss = H+/. MF=C16H13N3O.
Matched m/z=264.1127. Predicted m/z=264.1131. Error = -1.76ppm.

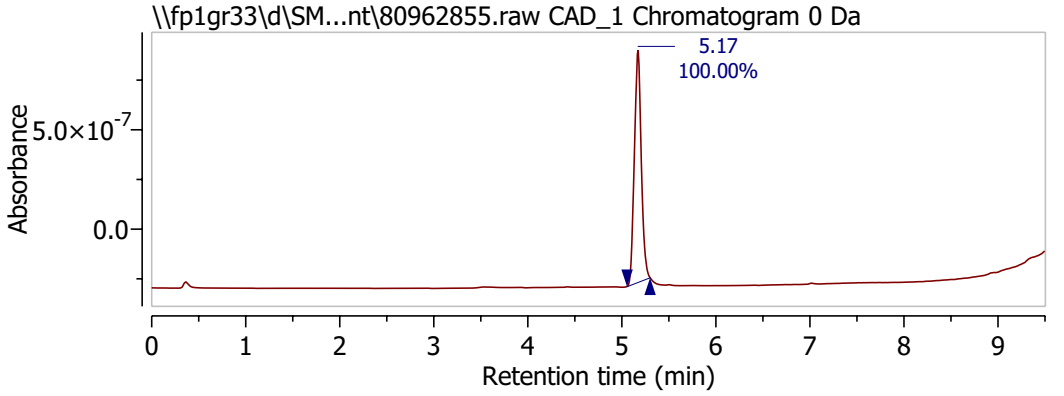
File : D:\SMDICurrent\80962855.raw
Acquired Date : 2022-01-31
Acquired Time : 15:16:12
Sample Name : 80962855
Operator : Kewei
Instrument : Thermo
Sample Position : GA4
Method : D:\Kewei\methods\Kewei_LCMS_10Min_Positive_Jan25.meth





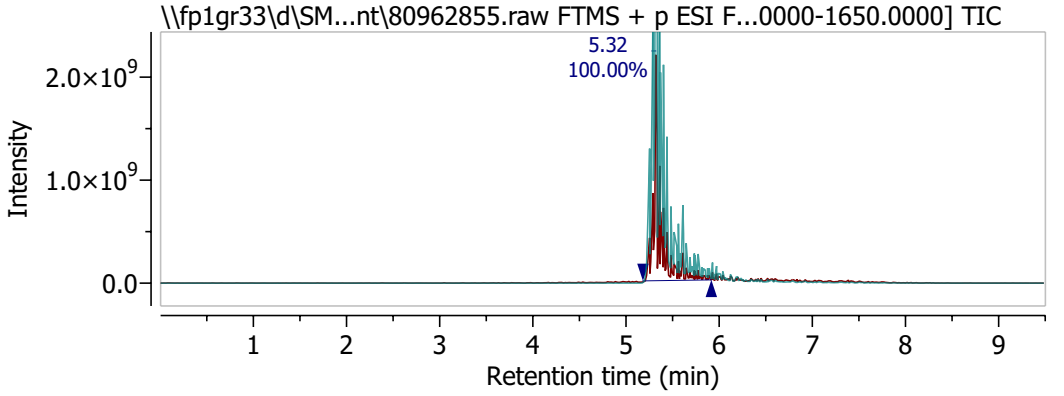
DAD, Sig=254 (>=1.5%)

RT	Area	Area %	MS+
5.10	37929345.50	100.00	264.11



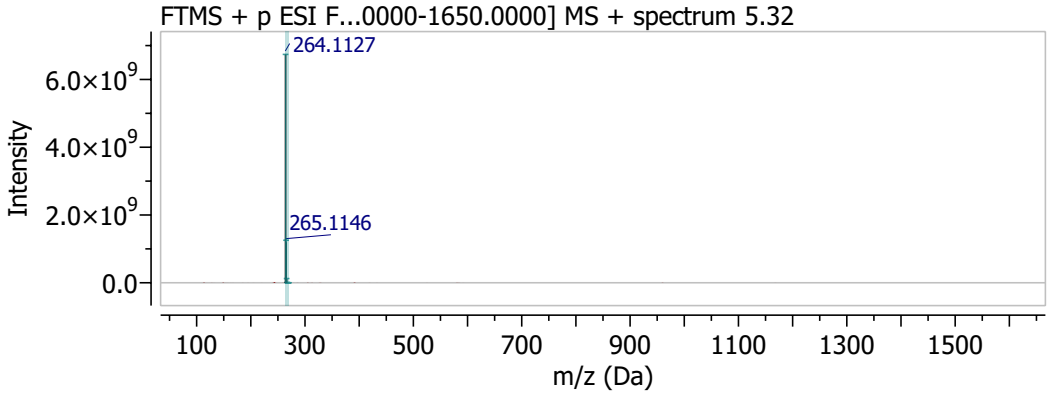
CAD_1 (>=5%)

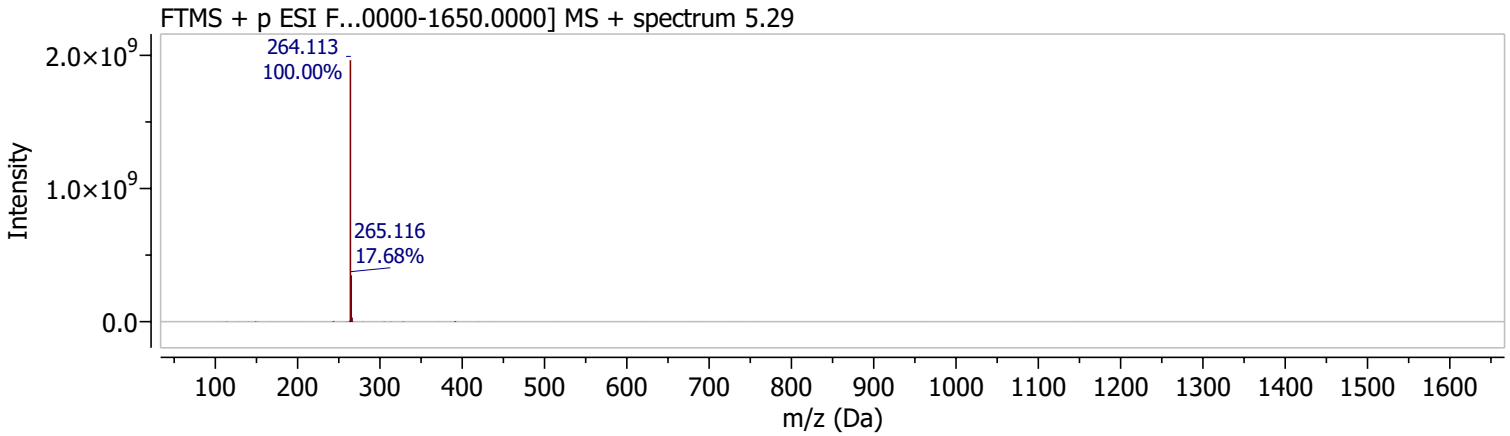
RT	Area	Area %	MS+
5.17	118046.17	100.00	264.11



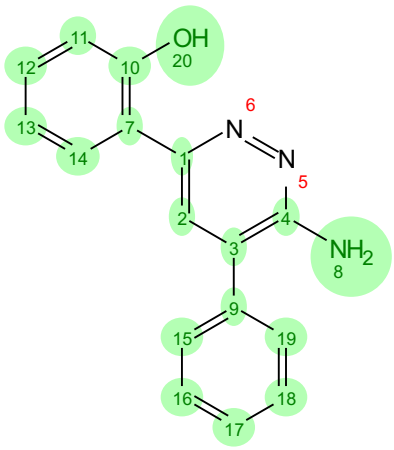
TIC (>=2%)

RT	Area	Area %	MS+
5.32	31484395507.07	100.00	264.11

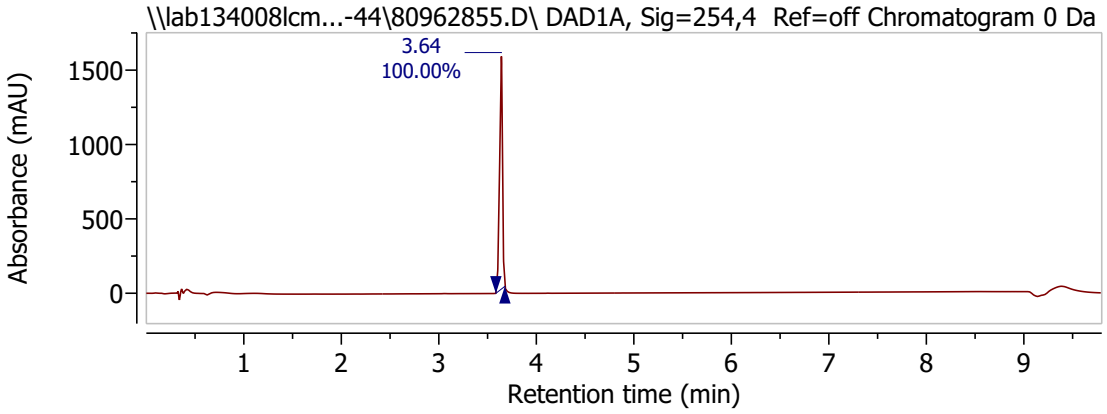



FTMS + p ESI F...0000-1650.0000] MS + spectrum 5.29
264.113
100.00%
265.116
17.68%
Intensity
2.0×10^9
1.0×10^9
0.0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
m/z (Da)

Molecule matched at RT 3.72 min. Adduct/Loss = H+/. MF=C16H13N3O.
Matched m/z=264.1000. Predicted m/z=264.1131. Error = -49.75ppm.

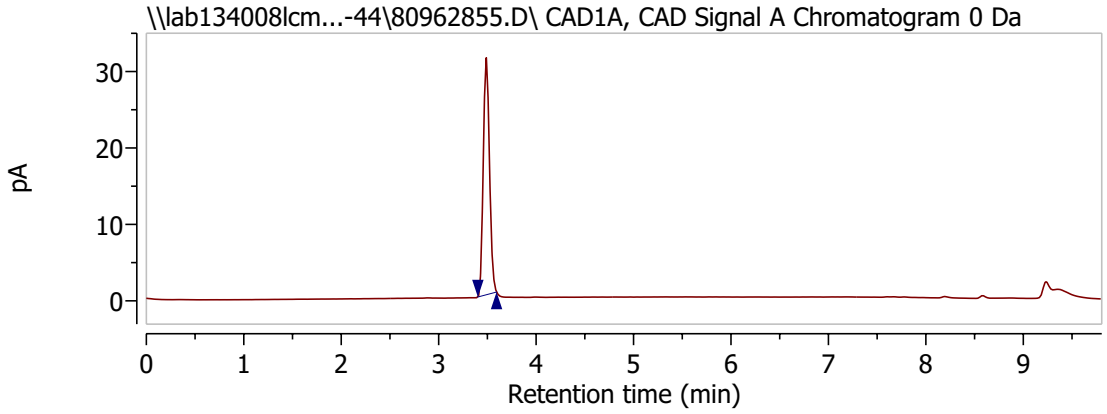


File : C:\Users\Public\Documents\ChemStation\1\Data\AGILENT-013122-01 2022-01-31 14-57-44\80962855.D
Acquired Date : 2022-01-31
Acquired Time : 17:48:55
Sample Name : 80962855
Operator : Kewei
Instrument : Agilent(New)
Sample Position : undefined
Acq. Method: CAD_10MIN_04ML.M



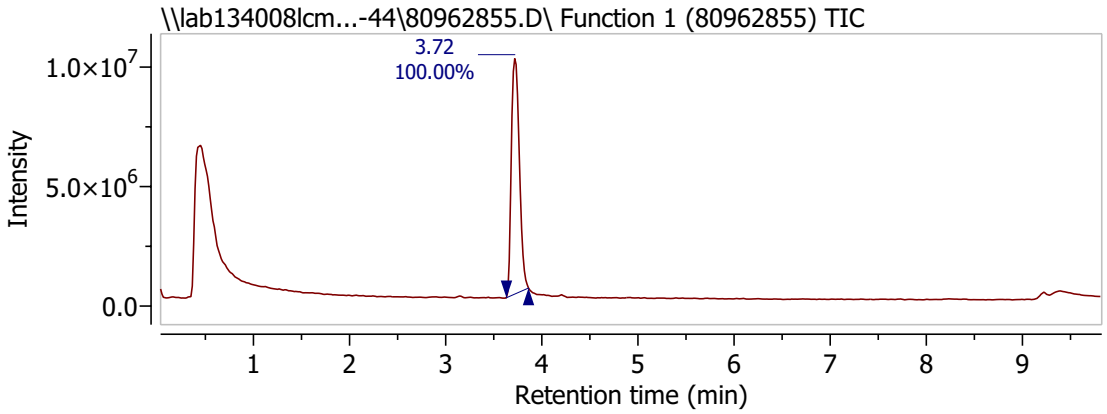
DAD1A, Sig=254,4 Ref=off (>=1.5%)

RT	Area	Area %	MS+
3.64	62804.08	100.00	264.10



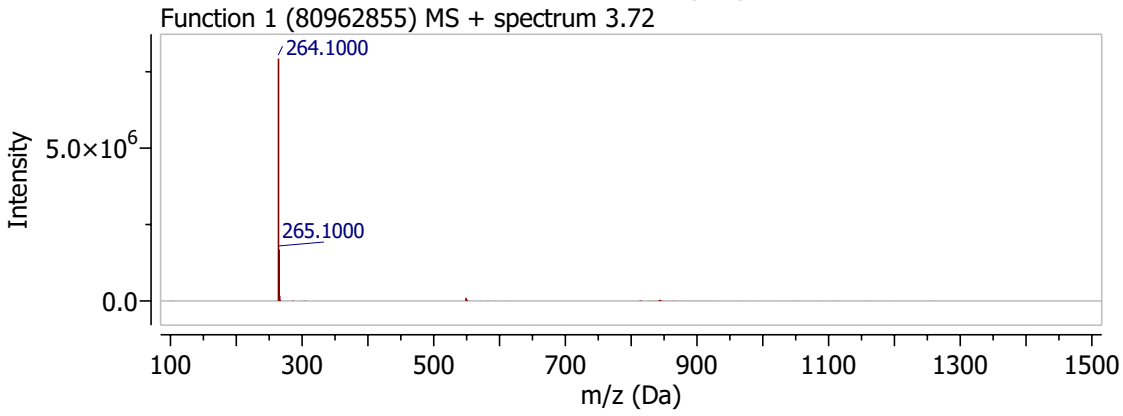
CAD CHANNEL A (>=5%)

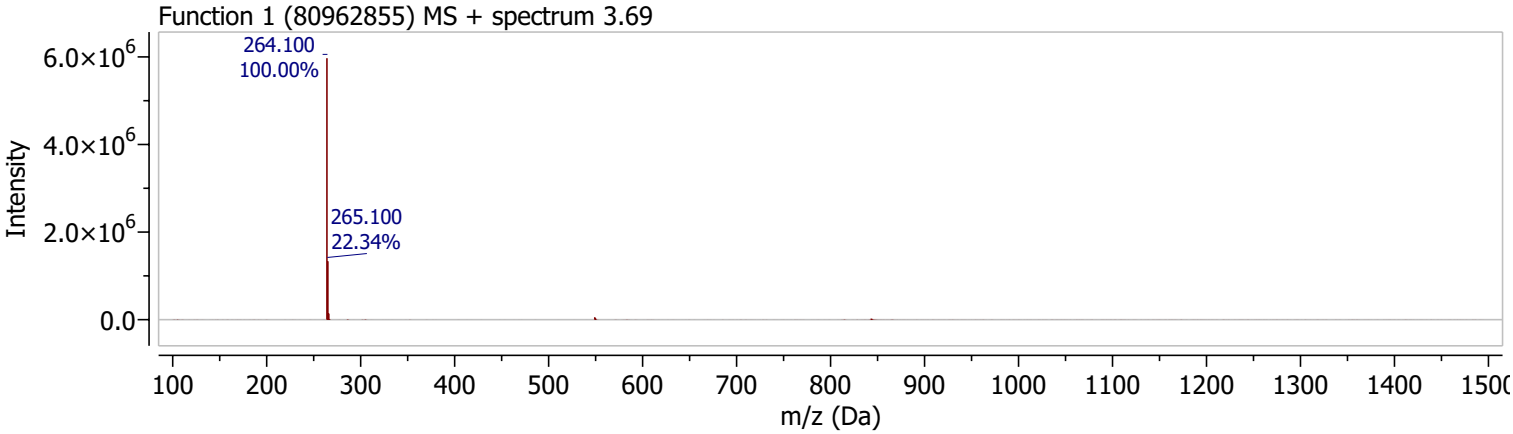
RT	Area	Area %	MS+
3.49	1318.87	100.00	264.10



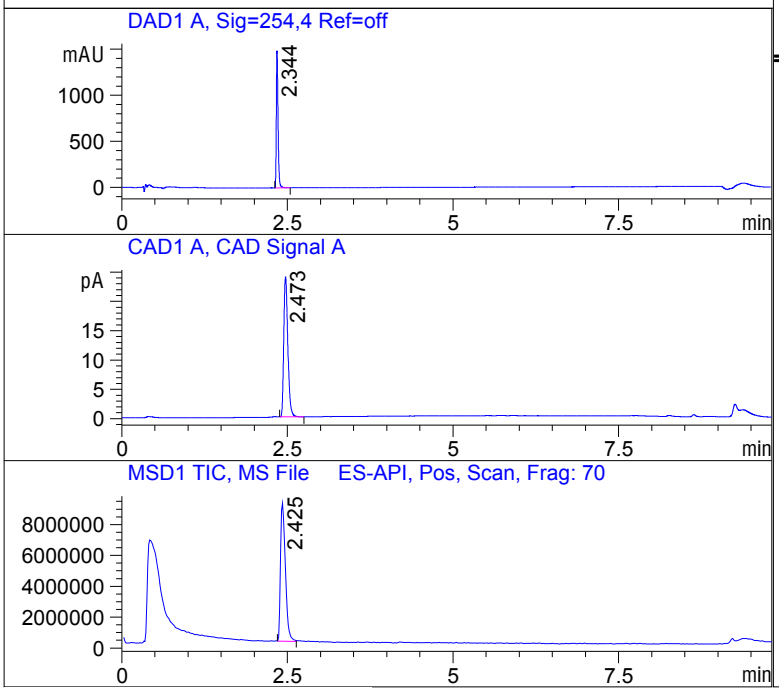
TIC (>=2%)

RT	Area	Area %	MS+
3.72	66699819.50	100.00	264.10




Function 1 (80962855) MS + spectrum 3.69
264.100
100.00%
265.100
22.34%
Intensity
6.0×10^6
4.0×10^6
2.0×10^6
0.0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z (Da)

DataFile: ..nts\ChemStation\1\Data\AGILENT-013122-01 2022-01-31 14-57-44\81098304.D
Inj.Date: 31-Jan-22, 17:49:42
Sample Name : 81098304 Location: D1F-B2
AcqOper: SYSTEM Inj: 1
SpecRptd: UV Peaks InjVol: 2
Tgt.Mass: (CHM) SeqLine: 13
AcqMethod: ..Station\1\Data\AGILENT-013122-01 2022-01-31 14-57-44\CAD_10MIN_04ML.M
AnalyMeth: ..Station\1\Data\AGILENT-013122-01 2022-01-31 14-57-44\CAD_10MIN_04ML.M
SampInfo:
MethInfo: 10 min LCMS with 0.4mL/min



Integration for DAD1 A, Sig=254,4 Ref=off

R.T.	Width	Area	Height	Area%	MS+
2.34	0.03	2681.74	1480.34	100.00	328.1

Integration for CAD1 A, CAD Signal A

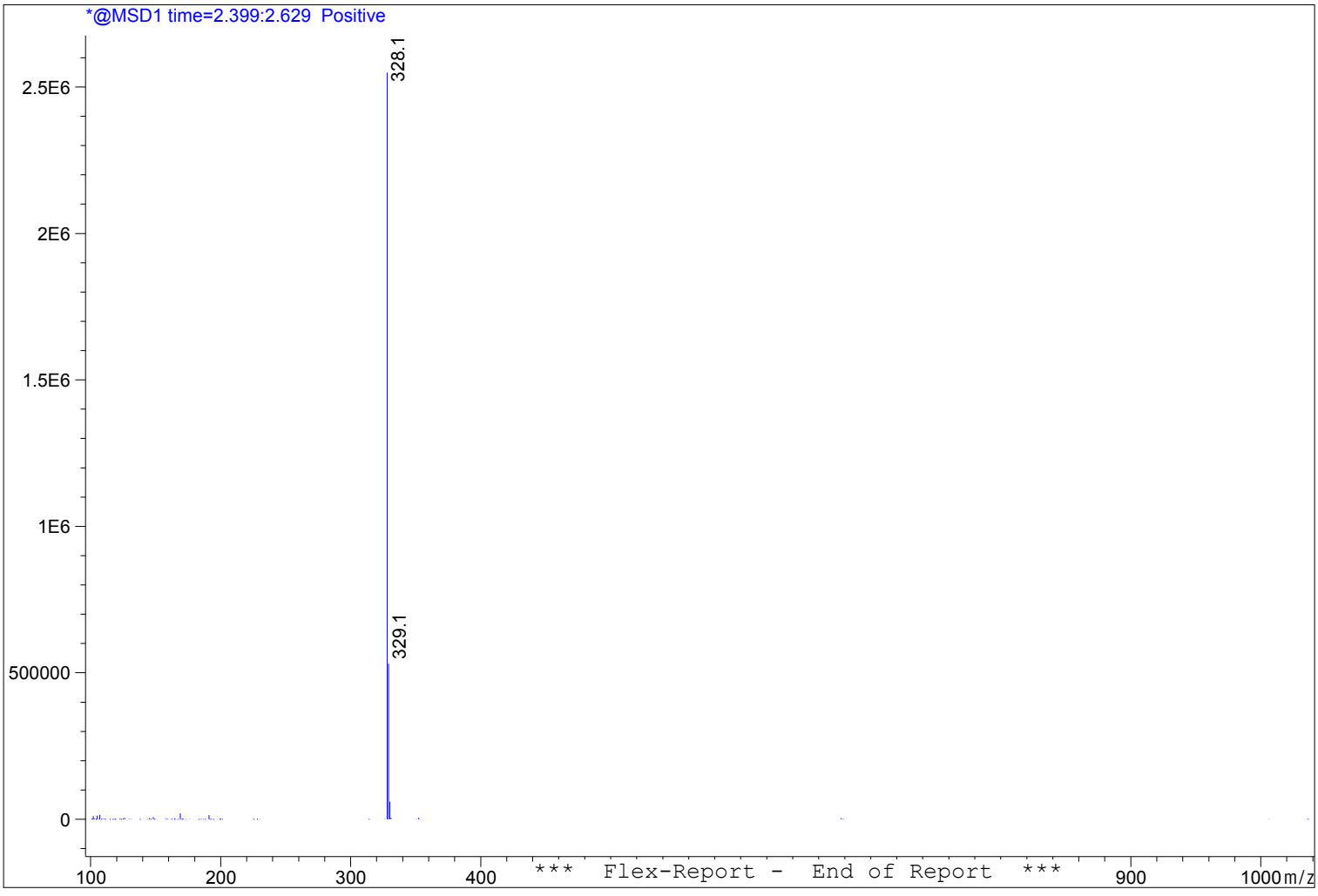
R.T.	Width	Area	Height	Area%	MS+
2.47	0.07	102.13	23.89	100.00	328.1

Integration for MSD1 TIC, MS File

R.T.	Width	Area	Height	Area%	MS+
2.43	0.08	45560748.00	8978201.00	100.00	328.1

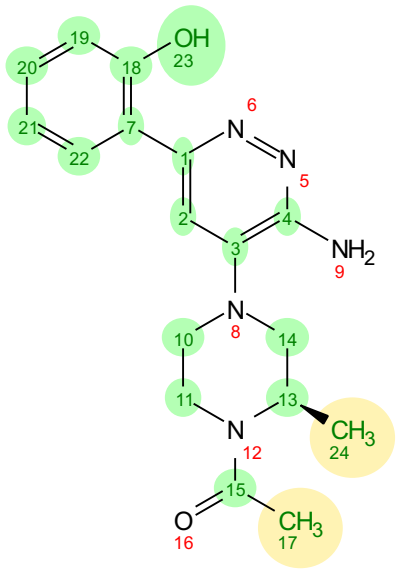
Compound **5** (GNE-064)

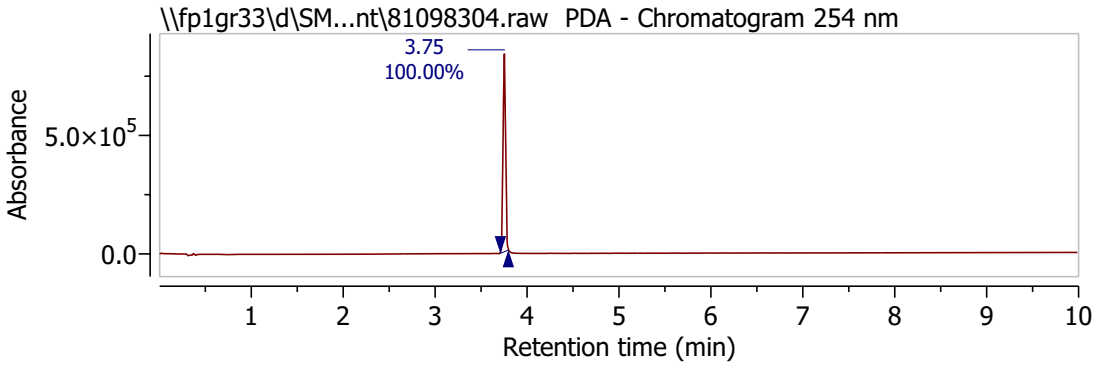
Ret. Time: 2.344 DAD1 A



Molecule matched at RT 3.94 min. Adduct/Loss = H+/. MF=C17H21N5O2.
Matched m/z=328.1760. Predicted m/z=328.1768. Error = -2.30ppm.

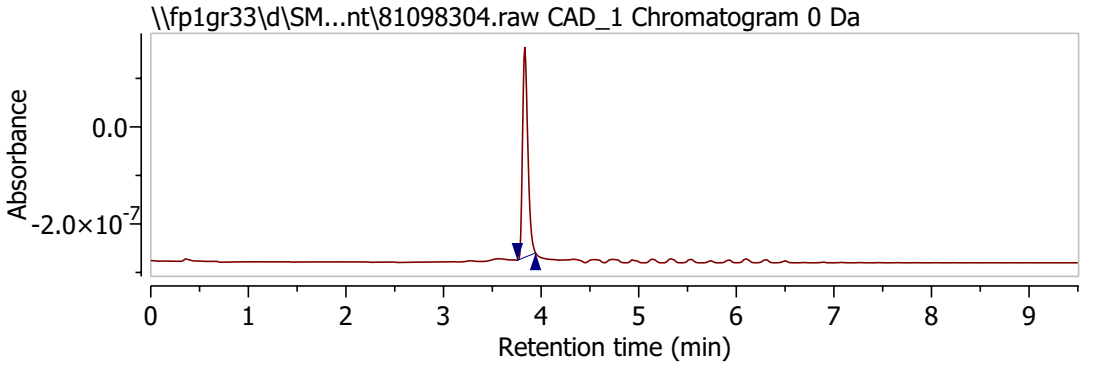
File : D:\SMDICurrent\81098304.raw
Acquired Date : 2022-01-31
Acquired Time : 15:27:29
Sample Name : 81098304
Operator : Kewei
Instrument : Thermo
Sample Position : GA5
Method : D:\Kewei\methods\Kewei_LCMS_10Min_Positive_Jan25.meth





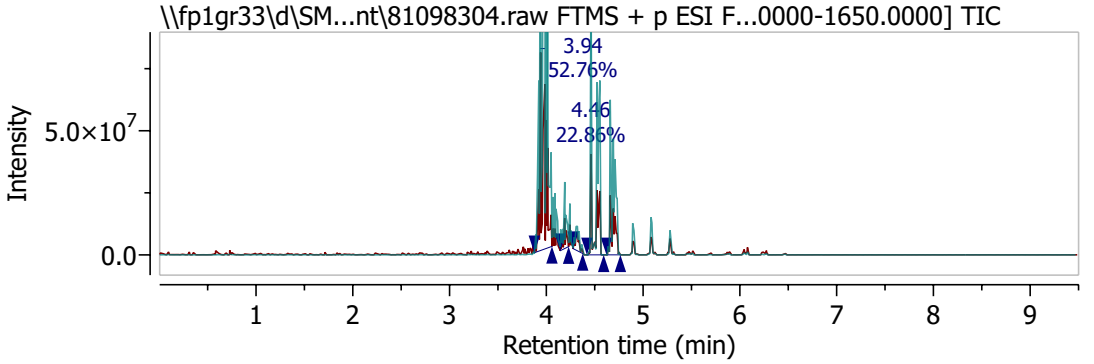
DAD, Sig=254 (>=1.5%)

RT	Area	Area %	MS+
3.75	7721150.50	100.00	328.18



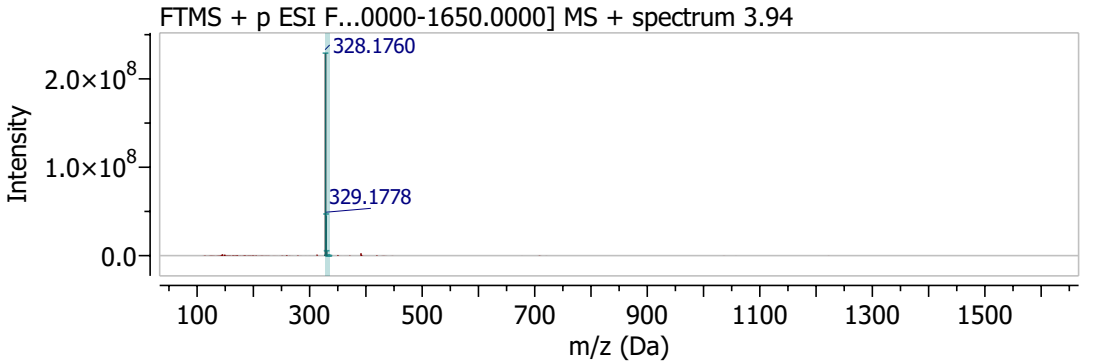
CAD_1 (>=5%)

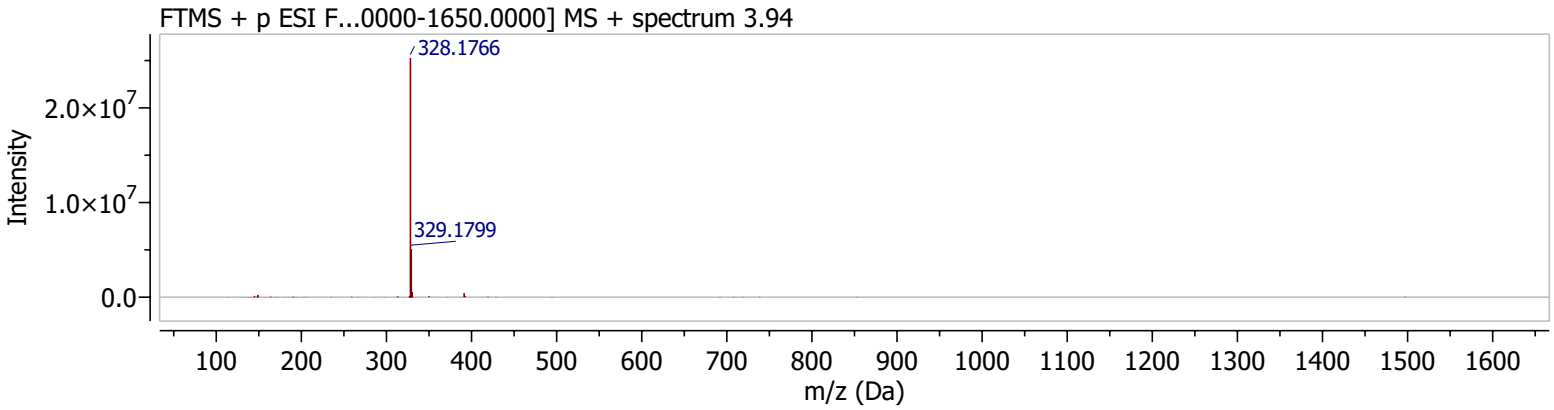
RT	Area	Area %	MS+
3.83	32115.40	100.00	328.18



TIC (>=2%)

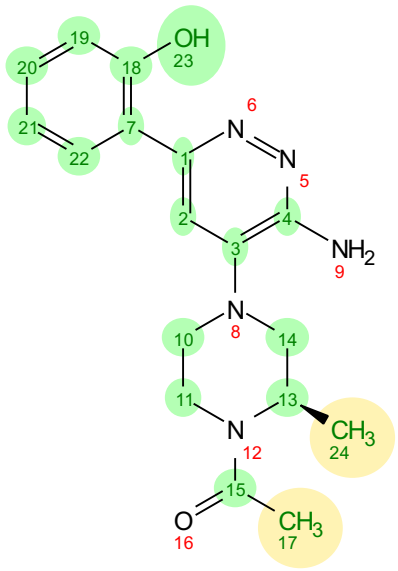
RT	Area	Area %	MS+
3.94	769604838.20	52.76	328.18
4.19	66278587.30	4.54	328.18
4.32	66018894.03	4.53	328.18
4.46	333448256.30	22.86	328.18
4.66	223439748.95	15.32	328.18

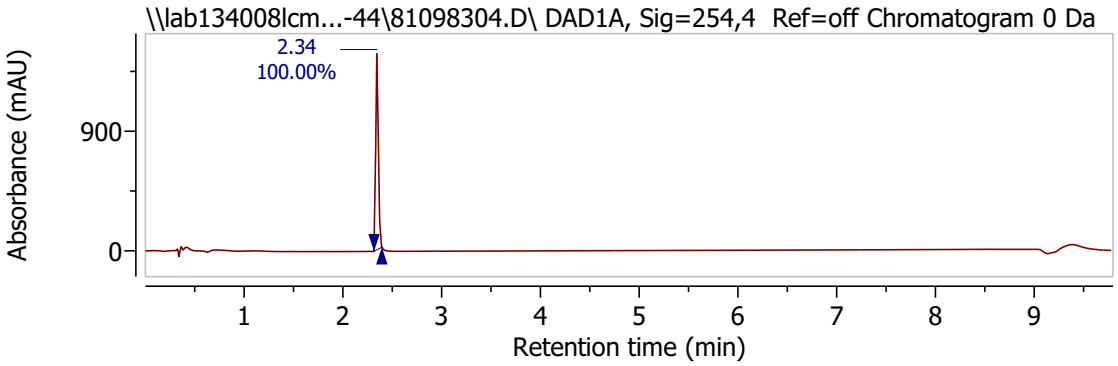



FTMS + p ESI F...0000-1650.0000] MS + spectrum 3.94
328.1766
329.1799
Intensity
2.0×10^7
1.0×10^7
0.0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
m/z (Da)

Molecule matched at RT 2.43 min. Adduct/Loss = H+/. MF=C17H21N5O2.
Matched m/z=328.1000. Predicted m/z=328.1768. Error = -234.02ppm.

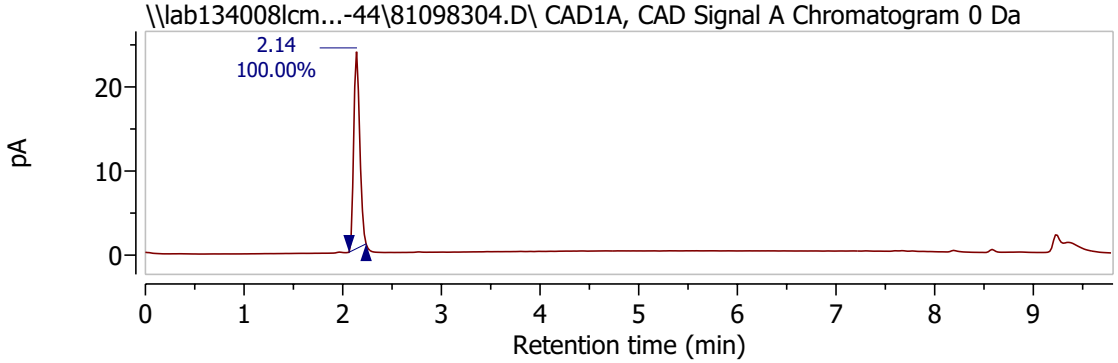
File : C:\Users\Public\Documents\ChemStation\1\Data\AGILENT-013122-01 2022-01-31 14-57-44\81098304.D
Acquired Date : 2022-01-31
Acquired Time : 17:59:41
Sample Name : 81098304
Operator : Kewei
Instrument : Agilent(New)
Sample Position : undefined
Acq. Method: CAD_10MIN_04ML.M





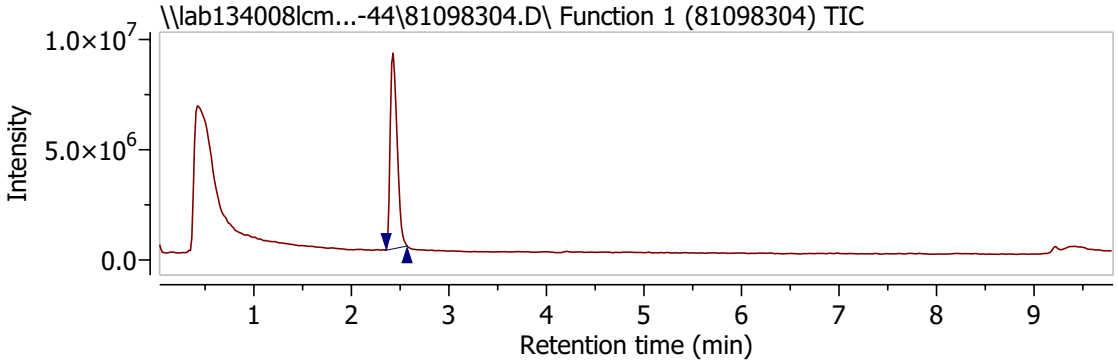
DAD1A, Sig=254,4 Ref=off (>=1.5%)

RT	Area	Area %	MS+
2.34	51213.56	100.00	328.10



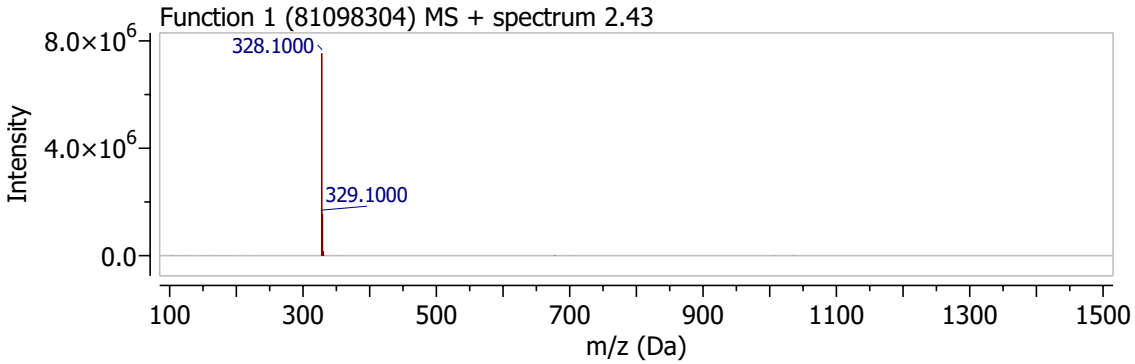
CAD CHANNEL A (>=5%)

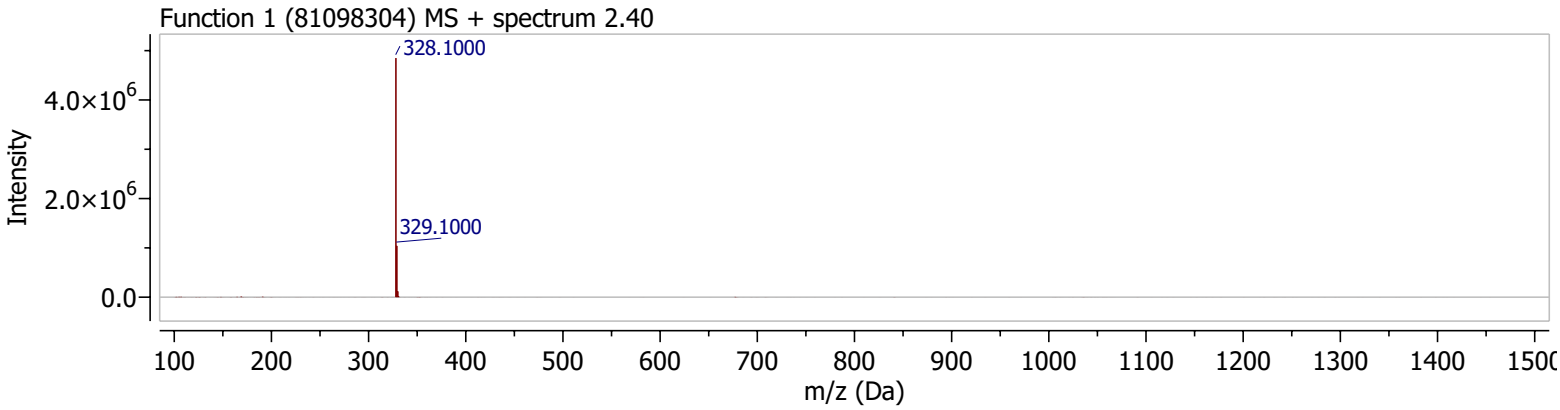
RT	Area	Area %	MS+
2.14	950.75	100.00	328.10



TIC (>=2%)

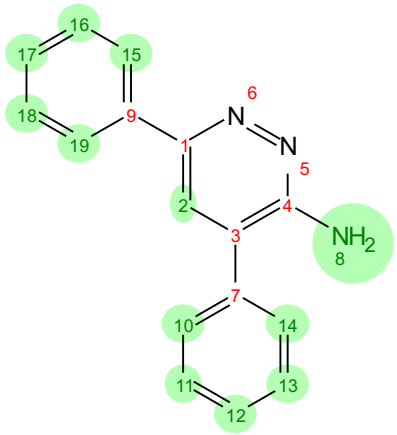
RT	Area	Area %	MS+
2.43	51034894.00	100.00	328.10




Function 1 (81098304) MS + spectrum 2.40
328.1000
329.1000
Intensity
4.0×10^6
2.0×10^6
0.0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z (Da)

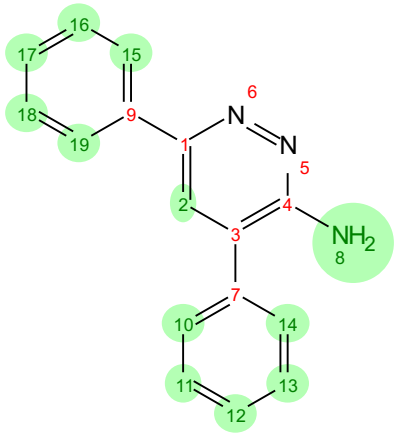
	Parameter	Value
1	Data File Name:	//pinot400/data/smapches/nmr/80823396/10/fid
2	Sample ID	80823396
3	Origin:	Bruker BioSpin GmbH
4	Ow ner:	smapches
5	Solvent:	DMSO
6	Pulse Sequence:	zg30
7	Acquisition Date:	2022-07-05T17:37:47
8	Temperature:	300.0002
9	Number of Scans:	32
10	Spectrometer Frequency	400.332471
11	Nucleus	1H

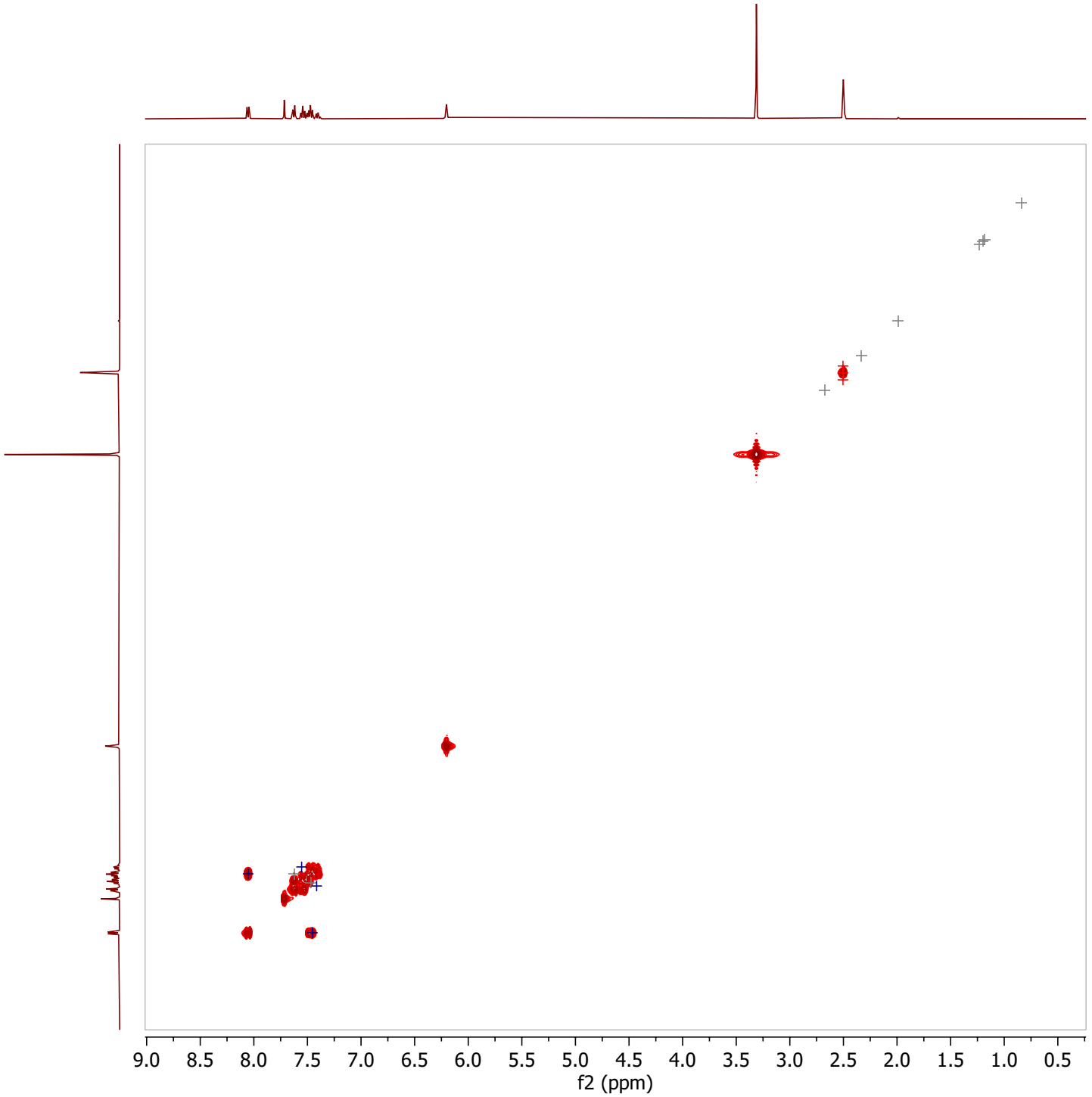
^{1}H NMR (400 MHz, DMSO) δ 8.08 – 8.03 (m, 2H), 7.72 (s, 1H), 7.65 – 7.60 (m, 2H), 7.58 – 7.44 (m, 5H), 7.44 – 7.36 (m, 1H), 6.20 (s, 2H).



Compound **2**

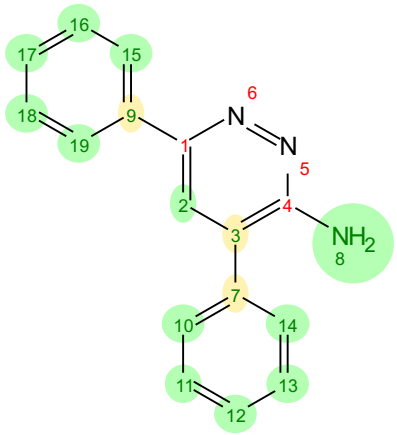
	Parameter	Value
1	Data File Name:	//pinot400/data/smapches/nmr/80823396/11/ser
2	Sample ID	80823396
3	Origin:	Bruker BioSpin GmbH
4	Ow ner:	smapches
5	Solvent:	DMSO
6	Pulse Sequence:	cosygpppqf
7	Acquisition Date:	2022-07-05T17:43:38
8	Temperature:	299.9995
9	Number of Scans:	2
10	Spectrometer Frequency	[400.33185133155, 400.33185133155]
11	Nucleus	[1H, 1H]



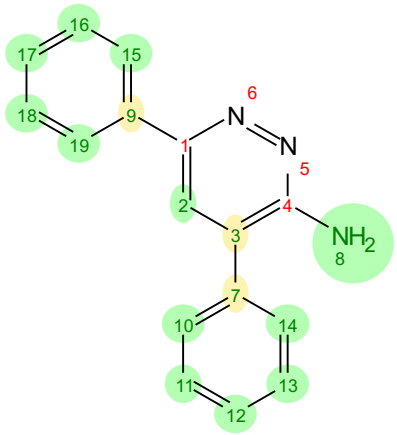


	Parameter	Value
1	Data File Name:	//pinot400/data/smapches/nmr/80823396/100013/fid
2	Sample ID	80823396
3	Origin:	Bruker BioSpin GmbH
4	Ow ner:	smapches
5	Solvent:	DMSO
6	Pulse Sequence:	zgpg30
7	Acquisition Date:	2022-07-08T02:20:18
8	Temperature:	299.9988
9	Number of Scans:	8192
10	Spectrometer Frequency	100.6731198
11	Nucleus	13C

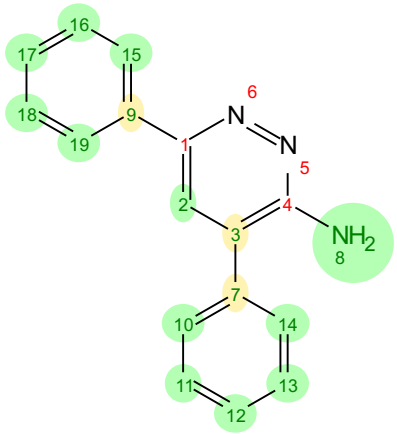
^{13}C NMR (101 MHz, DMSO) δ 157.91, 151.49, 137.42, 135.73, 129.56, 129.25, 129.15, 128.88, 128.80, 128.58, 126.29, 126.10, 125.00, 42.32, 41.47, 41.28, 40.65, 40.43, 40.22, 40.01, 39.79, 39.58, 39.37.

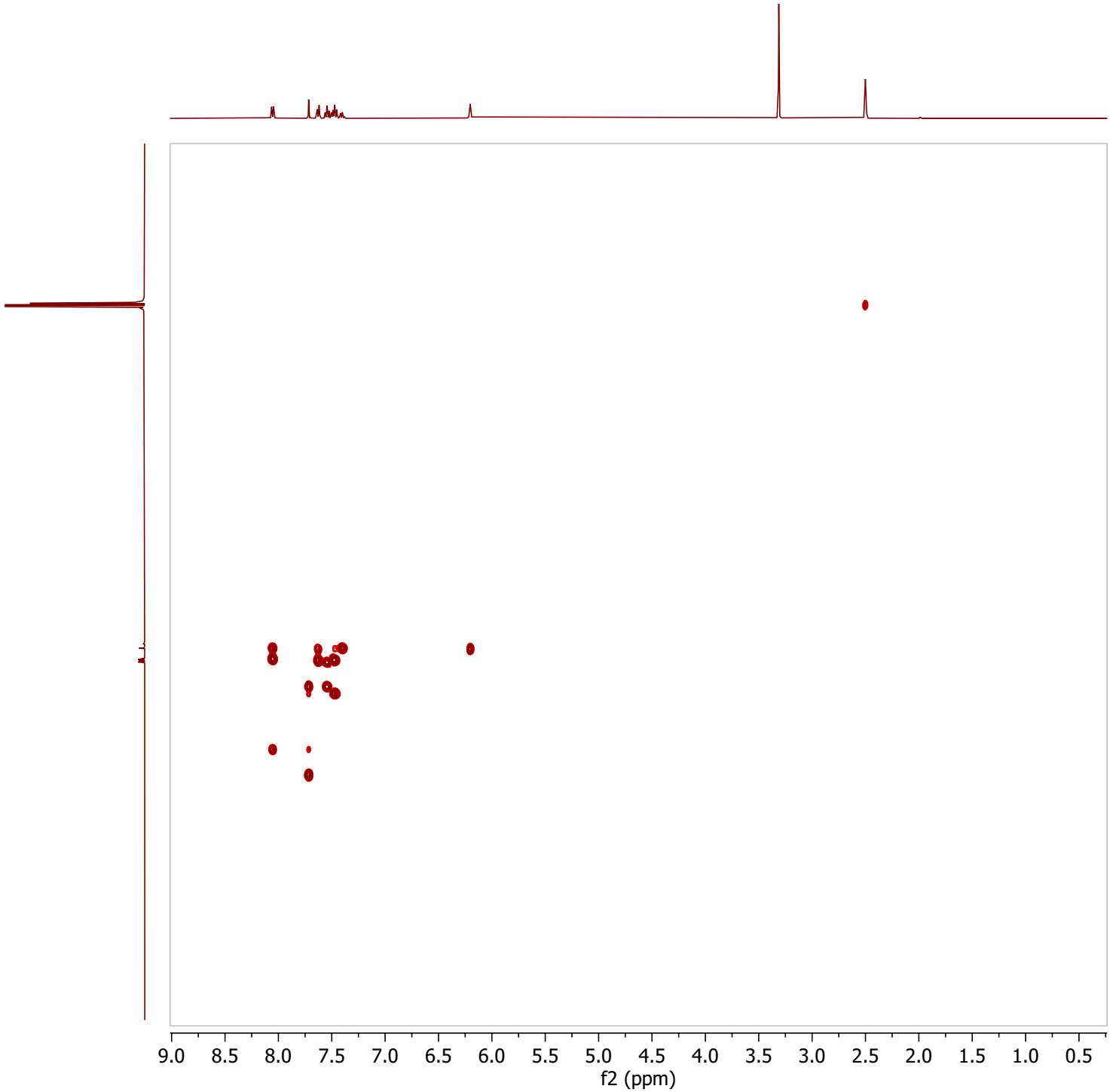


	Parameter	Value
1	Data File Name:	//pinot400/data/smapches/nmr/80823396/13/fid
2	Sample ID	80823396
3	Origin:	Bruker BioSpin GmbH
4	Owner:	smapches
5	Solvent:	DMSO
6	Pulse Sequence:	zgpg30
7	Acquisition Date:	2022-07-08T02:20:18
8	Temperature:	299.9988
9	Number of Scans:	8192
10	Spectrometer Frequency	100.6731198
11	Nucleus	13C



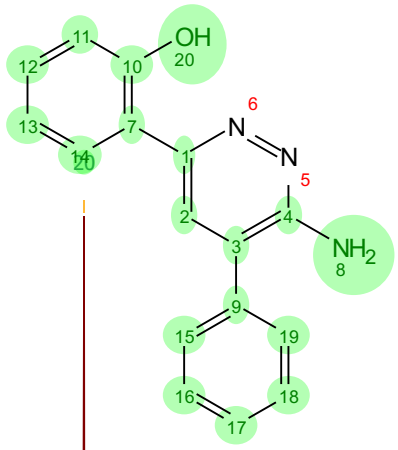
	Parameter	Value
1	Data File Name:	//pinot400/data/smapches/nmr/80823396/14/ser
2	Sample ID	80823396
3	Origin:	Bruker BioSpin GmbH
4	Ow ner:	smapches
5	Solvent:	DMSO
6	Pulse Sequence:	hmbcgplpndqf
7	Acquisition Date:	2022-07-08T03:45:01
8	Temperature:	299.9992
9	Number of Scans:	16
10	Spectrometer Frequency	[100.6741264, 400.33185133155]
11	Nucleus	[13C, 1H]





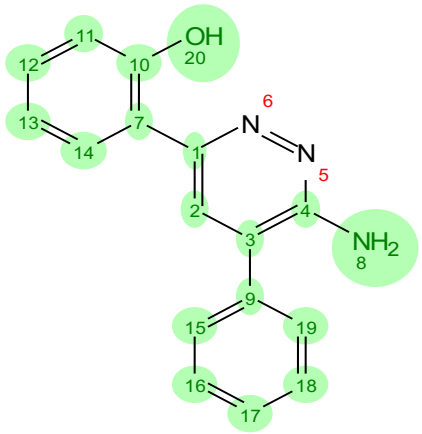
Parameter	Value
1 Data File Name:	//pinot400/data/smapches/nmr/80962855/10/fid
2 Sample ID	80962855
3 Origin:	Bruker BioSpin GmbH
4 Ow ner:	smapches
5 Solvent:	DMSO
6 Pulse Sequence:	zg30
7 Acquisition Date:	2022-01-31T15:58:07
8 Temperature:	300.0009
9 Number of Scans:	32
10 Spectrometer Frequency	400.332471
11 Nucleus	1H

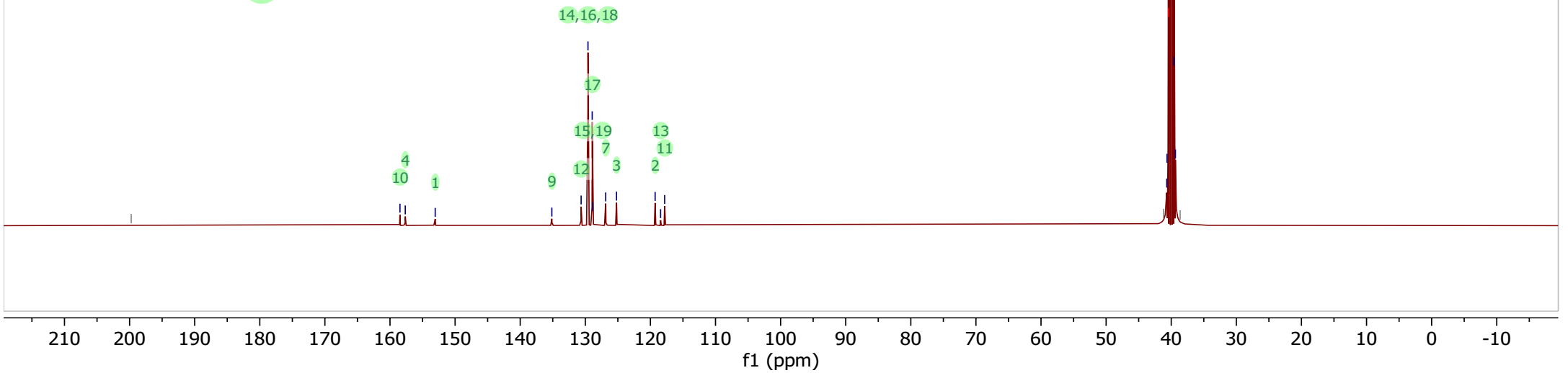
^{1}H NMR (400 MHz, DMSO) δ 13.59 (s, 1H), 8.01 (s, 1H), 7.95 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.66 – 7.61 (m, 2H), 7.61 – 7.48 (m, 3H), 7.25 (ddd, *J* = 8.5, 7.2, 1.6 Hz, 1H), 6.96 – 6.85 (m, 2H), 6.45 (s, 2H).



Compound **4**

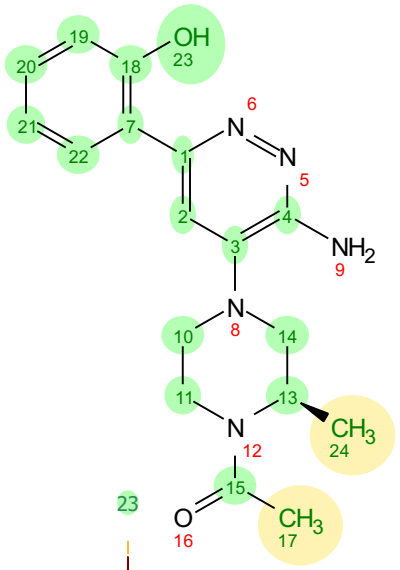
Parameter	Value
1 Data File Name:	//pinot400/data/smapches/nmr/80962855/100011/fid
2 Sample ID	80962855
3 Origin:	Bruker BioSpin GmbH
4 Ow ner:	smapches
5 Solvent:	DMSO
6 Pulse Sequence:	zgpg30
7 Acquisition Date:	2022-02-09T21:28:19
8 Temperature:	299.9996
9 Number of Scans:	8192
10 Spectrometer Frequency	100.6731198
11 Nucleus	13C





Parameter	Value
1 Data File Name:	//pinot400/data/smapches/nmr/81098304/10/fid
2 Sample ID	81098304
3 Origin:	Bruker BioSpin GmbH
4 Ow ner:	smapches
5 Solvent:	DMSO
6 Pulse Sequence:	zg30
7 Acquisition Date:	2022-01-31T16:03:06
8 Temperature:	299.9999
9 Number of Scans:	32
10 Spectrometer Frequency	400.332471
11 Nucleus	1H

^{1}H NMR (400 MHz, DMSO) δ 14.12 (s, 1H), 7.90 (d, *J* = 7.9 Hz, 1H), 7.51 (s, 1H), 7.28 – 7.21 (m, 1H), 6.93 – 6.85 (m, 2H), 6.33 (s, 2H), 4.74 (s, 0H), 4.25 (d, *J* = 13.7 Hz, 1H), 3.71 (s, 1H), 3.58 (d, *J* = 12.0 Hz, 1H), 3.22 (s, 1H), 3.09 (d, *J* = 11.8 Hz, 0H), 3.00 (d, *J* = 12.2 Hz, 1H), 2.48 – 2.36 (m, 1H), 2.07 (s, 1H), 2.02 (s, 1H), 1.37 (d, *J* = 6.1 Hz, 1H), 1.25 (d, *J* = 6.7 Hz, 1H).



Compound **5** (GNE-064)

Parameter	Value
1 Data File Name:	//pinot400/data/smapches/nmr/81098304/100011/fid
2 Sample ID	81098304
3 Origin:	Bruker BioSpin GmbH
4 Ow ner:	smapches
5 Solvent:	DMSO
6 Pulse Sequence:	zgpg30
7 Acquisition Date:	2022-02-09T23:26:56
8 Temperature:	299.9998
9 Number of Scans:	8192
10 Spectrometer Frequency	100.6731198
11 Nucleus	13C

^{13}C NMR (101 MHz, DMSO) δ 168.66, 158.95, 155.41, 153.74, 141.04, 130.64, 130.56, 126.64, 118.98, 118.92, 118.26, 117.86, 111.45, 44.02, 40.64, 40.48, 40.43, 40.21, 40.00, 39.79, 39.58, 39.36.