Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Electronic Supporting Information

for

Structure-Based Traceless Specific Fluorescence Labeling of Smoothened Receptor

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SUPPLEMENTARY TABLES AND FIGURES

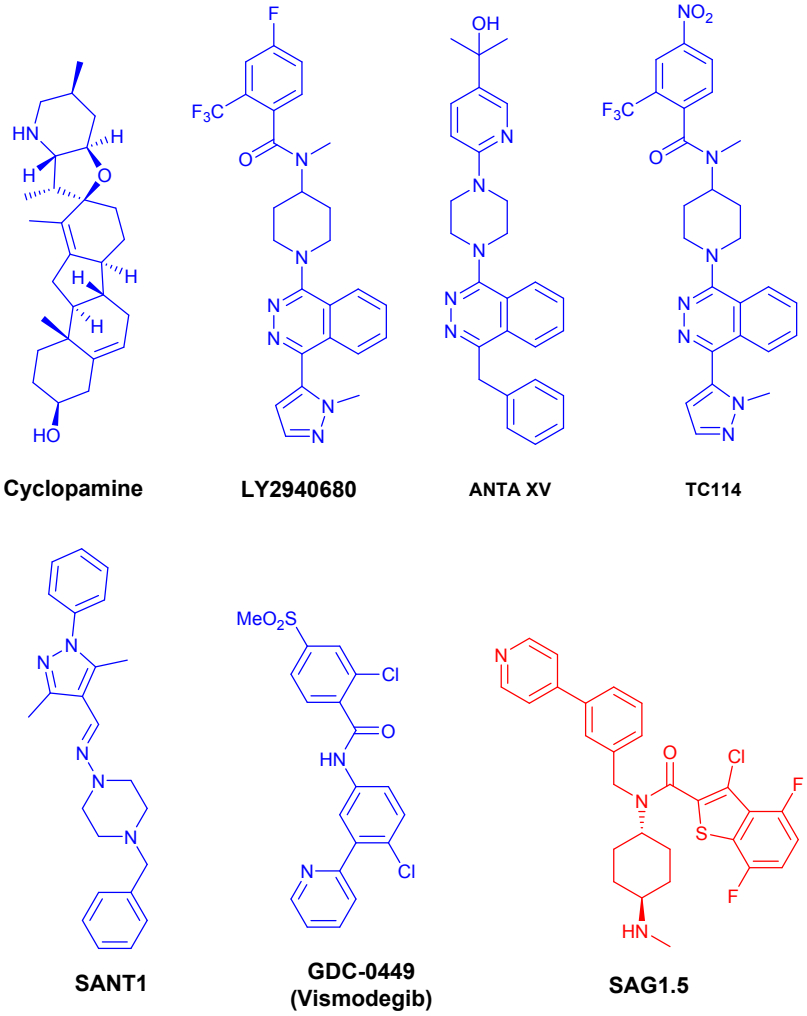


Figure S1. Chemical structures of co-crystallized SMO-TMD ligands.

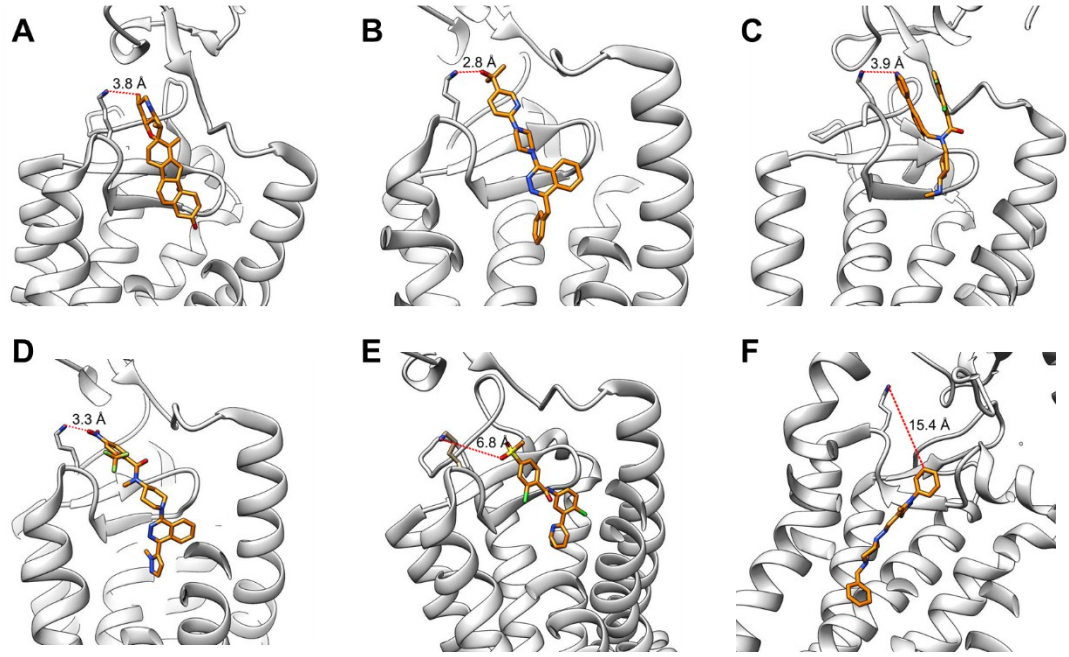


Figure S2. Distances between K395 and ligands in crystal structures of SMO receptor. The distances between the ε-amine of K395 and the nearest non-hydrogen atoms of the ligands are 3.8 Å (A, 4O9R), 2.8 Å (B, 4QIM), 3.9 Å (C, 4QIN), 3.3 Å (D, 5V57) 6.8 Å (E, 5L7I) and 15.4 Å (F, 4N4W).

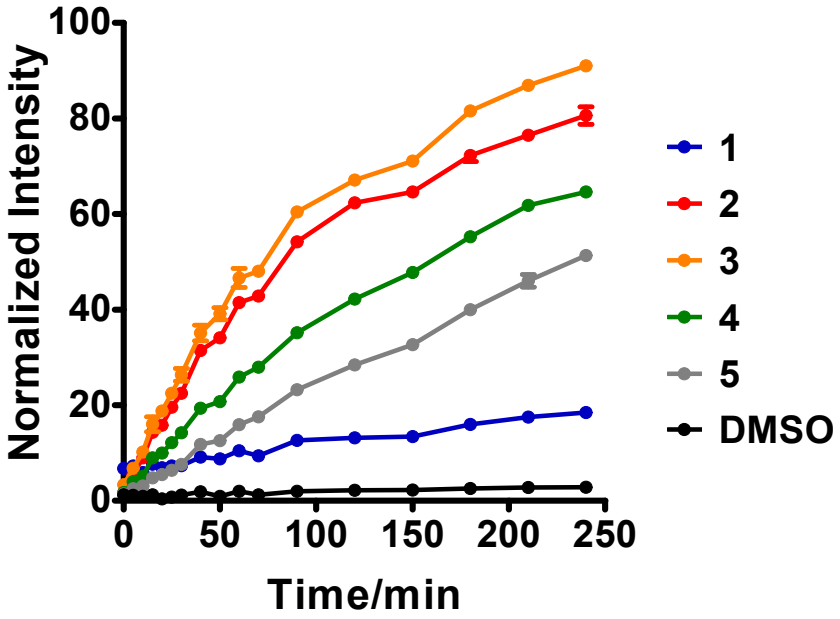


Figure S3. Time course of the fluorescence intensity of SMO construct treated with probe 1~5. Fluorescence was recorded by a microplate fluorescence reader.

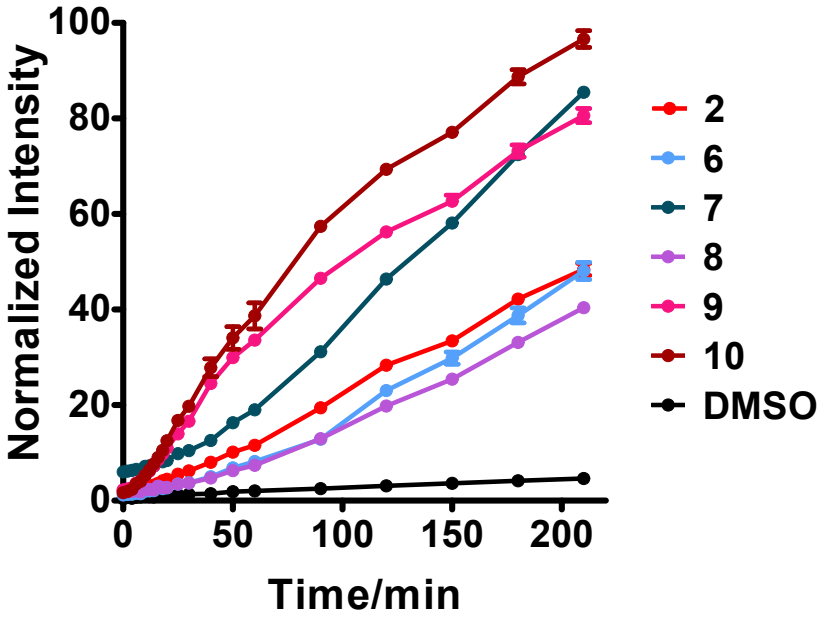
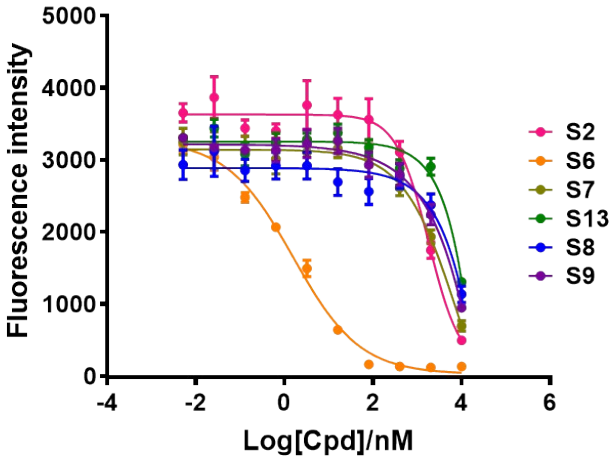


Figure S4. Time course of the fluorescence intensity of SMO construct treated with probe 2 and probe 6~10. Fluorescence was recorded by a microplate fluorescence reader.

A



B

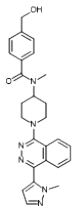
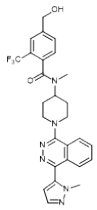
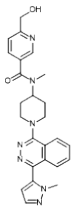
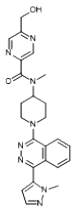
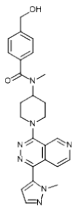
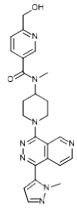
No.	S2	S6	S7	S13	S8	S9
Chemical structure						
IC_{50} (nM)	1795	1.56	8373	>10000	>10000	>10000

Figure S5. Gli-luciferase reporter activity of precursors of probes as antagonists. (A) Dose-response curves of precursors as antagonists. The signaling pathway was stimulated by 100 nM SAG. (B) Structures and IC_{50} values of precursors of probe **2** (**S2**), **6** (**S6**), **7** (**S7**), **8** (**S13**), **9** (**S8**), **10** (**S9**).

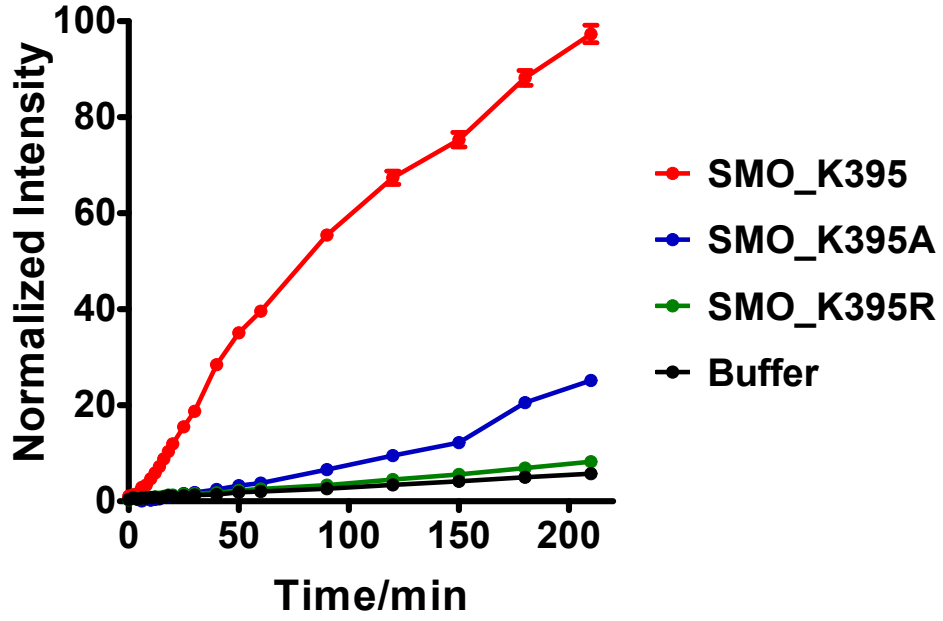


Figure S6. Time course of the fluorescence intensity of SMO constructs treated with probe 9. Fluorescence was recorded for the SMO construct (SMO_K395), the K395A mutant (SMO_K395A) and the K395R mutant (SMO_K395R) treated with probe **9** within 210 minutes by a microplate fluorescence reader.

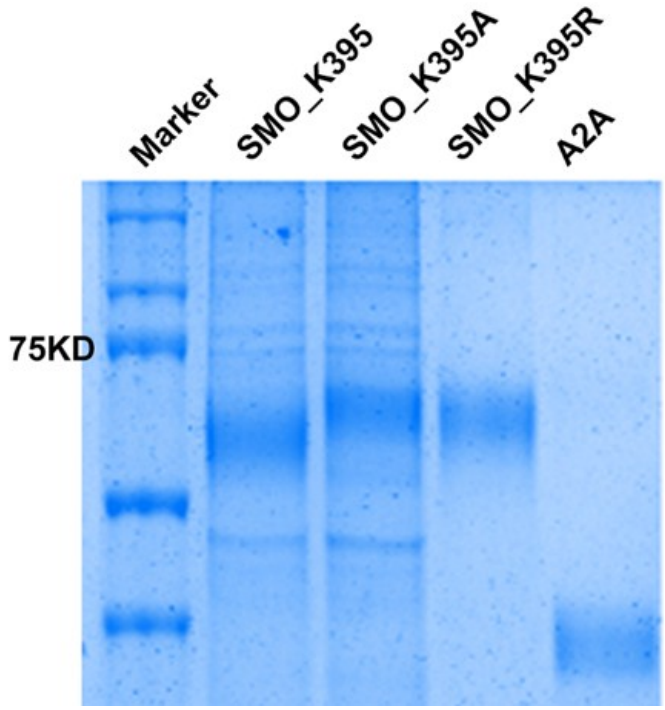


Figure S7. CBB staining gel of probe 9 treated proteins. The SMO construct (SMO_K395), the K395A mutant (SMO_K395A), the K395R mutant (SMO_K395R), and A2A were incubated with probe **9** for 3.5 hours followed by SDS-PAGE analysis.

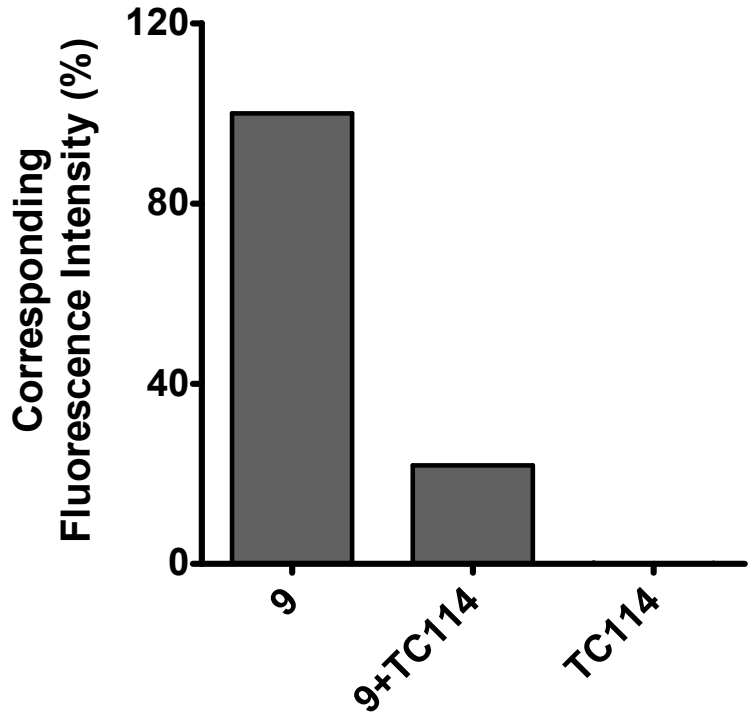


Figure S8. Normalized fluoresce (488/530 nm) intensity of SDS page of SMO_K395 treated with probe 9 and/or TC114. A 10 times final concentration of TC114 was pre-incubated with SMO for 30 min before probe **9** was added.

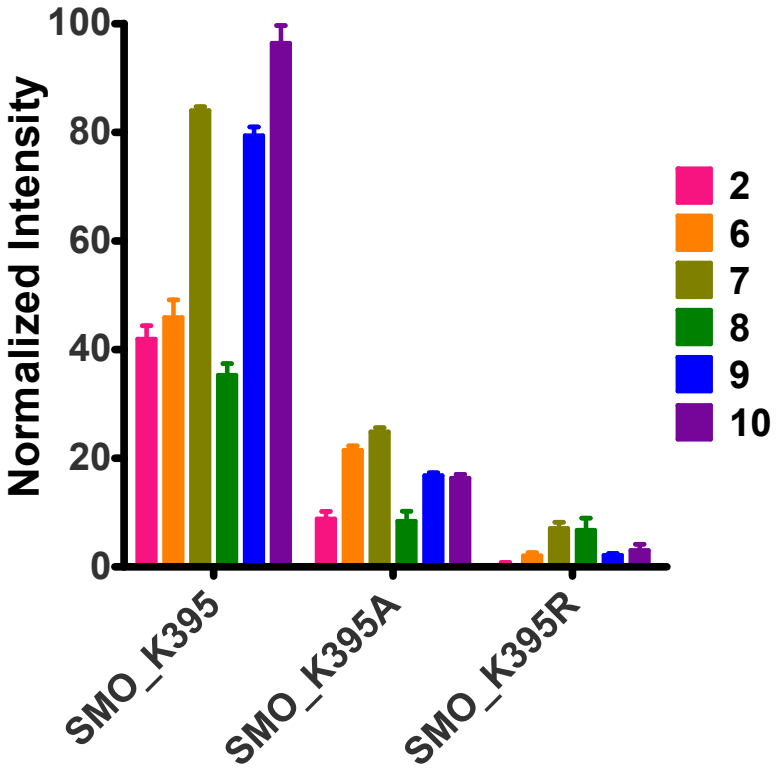


Figure S9. Labeling selectivity of probe 2, 6 ~10 on different SMO constructs. Fluorescence was recorded for The FLA-SMO construct (SMO_K395), the K395A mutant (SMO_K395A), and the K395R mutant (SMO_K395R) treated with probe **2** and probe **6~10** at room temperature within 210 min by a microplate fluorescence reader.

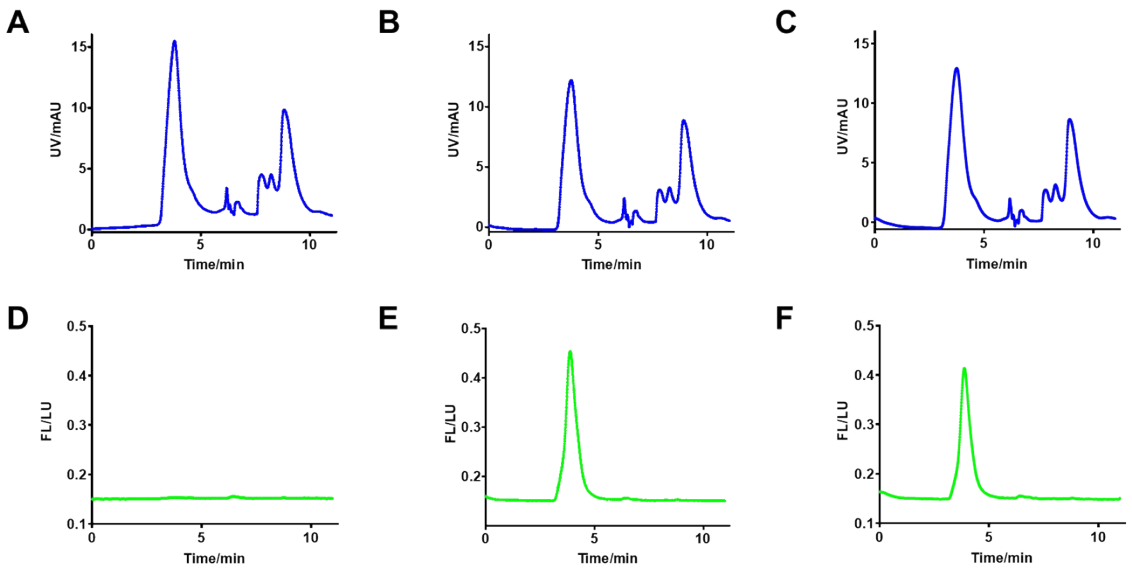


Figure S10. Probe 9 selectively labeled SMO on membrane. Size exclusive chromatography analysis results of SMO membranes treated without labeling probe (A, D) or with probe **9** (B, E) and probe **10** (C, F) at 4 °C. Results were recorded by a 280 nm UV detector or a fluorescence detector with excitation and emission wavelengths at 488 nm and 530 nm respectively.

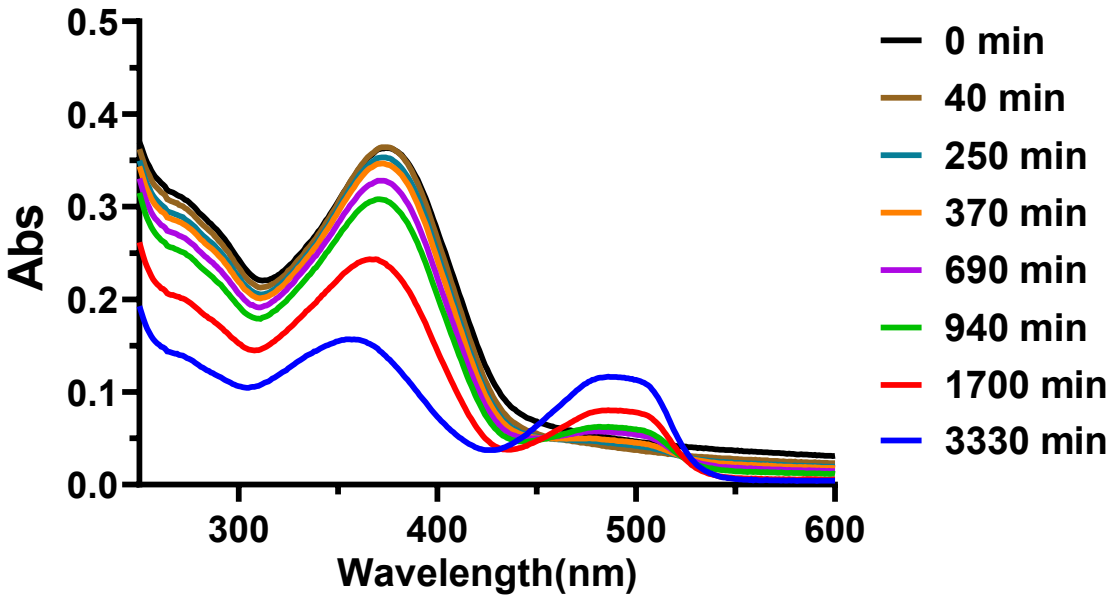


Figure S11. The stability of probe 9 in aqueous buffer. Time-dependent spectral change of probe 9 (25 μM) in 25 mM HEPES buffer (pH 7.5) after 0 min, 40 min, 250 min, 370 min, and 690 min, 940 min, 1700 min and 3330 min.

EXPERIMENTAL SECTION

Expression and purification of GPCRs

The expression and purification of SMO-FLA fusion proteins (wild-type, K395A, K395R) was carried out following the literature.[1] Typically, the engineered SMO constructs were expressed in HEK293F cells in the presence of 5 μM GDC-0449. HEK293F cells at a cell density of 1.0-1.3×10^6 cells/mL were transiently transfected with PEI:DNA at a ratio of 2:1 and cultured at 37 °C. Cells were collected by centrifugation at 48 hours after transfection and stored at −80 °C until use. Cell membranes were lysed by thawing frozen cell pellets in a hypotonic buffer (10 mM HEPES, 10 mM $MgCl_2$, 20 mM KCl, pH 7.5) and EDTA-free complete protease inhibitor cocktail tablets (Roche). Extensive washing of the raw membranes was performed by repeated centrifugation (three times) in a high osmotic buffer comprising 1.0 M NaCl. The washed membranes were re-suspended into a buffer containing 2 mg/mL iodoacetamide (Sigma) and EDTA-free complete protease inhibitor cocktail tablets, and incubated at 4 °C for 1 h before solubilization or tested in membrane-involved assays.

The membranes were then solubilized in a buffer (50 mM HEPES, 200 mM NaCl, 1% (w/v) n-dodecyl-β-D-maltopyranoside (DDM; Anatrace), 0.2% (w/v) cholesteryl hemisuccinate (CHS, Sigma), pH 7.5) for 2.5 hours at 4 °C. The supernatant containing solubilized SMO protein was isolated from the cell debris by a high-speed centrifugation, and subsequently incubated with TALON IMAC resin (Clontech) overnight at 4 °C in the presence of 20 mM imidazole and 1 M NaCl. After binding, the resin was washed with 10-column volumes of wash I buffer (50 mM HEPES, 800 mM NaCl, 10% glycerol, 0.5% LMNG (Anatrace)/0.1% CHS, 20 mM imidazole, 10 mM $MgCl_2$, 6 mM ATP, pH 7.5). The beads with 2 mL wash I buffer were transferred to a 5 mL tube in order to change the detergent and incubated on a rocker at 4 °C for 2 hours, followed by

washing with 6-column volumes of wash II buffer (25 mM HEPES, 500 mM NaCl, 10% glycerol, 0.03% LMNG/0.006% CHS, 40 mM imidazole, pH 7.5). The protein was then eluted by 3-column volumes of elution buffer (25 mM HEPES, 300 mM NaCl, 10% glycerol, 0.01% LMNG/0.002% CHS, 220 mM imidazole, pH 7.5). The protein was then concentrated to >0.9 mg/mL (>11.5 μM) with a 100 kDa cutoff Vivaspin concentrator. Protein monodispersity was tested by analytical size-exclusion chromatography (aSEC). Typically, the aSEC profile showed a monodispersed peak. Sequences of FLA-SMO WT and mutant constructs are listed below.

HA-Flag-His-TEV-SMO (Fla)

SMO (SMO_K395)

MKTIIALSYIFCLVFADYKDDDDAKLQTMHHHHHHHHHHHENLYFQGAVTGPPPPLSHC
GRAAPCEPLRYNVCLGSVLPYGATSTLLAGDSDSQEEAHGKLVLWSGLRNAPRCWAVI
QPLLCAVYMPKCENDRVELPSRTLCQATRGPCAIVERERGWPDFLRCTPDRFPEGCTNE
VQNIKFNSSGQCEVPLVRTDNPKSWYEDVEGCGIQCQNPLFTEAEHQDMHSYIAAFGAV
TGLCTLFTLATFVADWRNSNRYPAVILFYVNACFFVGSIGWLAQFMDGARREIVCRAD
GTMRLGEPTSNETLSCVIIFVIVYYALMAGVVWFVVLTYAWHTSFKALGTTYQPLSGKT
SYFHLLTWSLPFVLTVAILAVAQVDGDSVSGICFVGY**K**NYRYRAGFVLAPIGLVLIVGG
YFLIRGVMTLFSIKSNHAKALIVYGSTTGNTEYTAETIARELADAGYEVDSRDAASVEAG
GLFEGFDLVLLGCSTWGDDSIELQDDFIPLFDSLEETGAQGRKVACFGCGDSSWEYFCG
AVDAIEEKLKNLGAEIVQDGLRIDGDPRAARDDIVGWAHDVRGAIKINETMLRLGIFGFL
AFGFVLITFSCHFYDFFNQAEWERSFRDYVLCQANVTIGLPTKQPIPDCEIKNRPSLLVEK
INLFAMFGTGIAMSTWVWTKATLLIWRRTWCRLTGQSDDHHHHHHHHHH

SMO_K395A

MKTIIALSYIFCLVFADYKDDDDAKLQTMHHHHHHHHHHENLYFQGGTRGAASSGNAT
GPGPRSAGGSARRSAAVTGPPPPLSHCGRAAPCEPLRYNVCLGSVLPYGATSTLLAGDS
DSQEEAHGKLVLWSGLRNAPRCWAVIQPLLCAVYMPKCENDRVELPSRTLCQATRGPC
AIVERERGWPDFLRCTPDRFPEGCTNEVQNIKFNSSGQCEVPLVRTDNPKSWYEDVEGC
GIQCQNPLFTEAEHQDMHSYIAAFGAVTGLCTLFTLATFVADWRNSNRYPAVILFYVNA
CFFVGSIGWLAQFMDGARREIVCRADGTMRLGEPTSNETLSCVIIFVIVYYYALMAGVVW
FVVLTYAWHTSFKALGTTYQPLSGKTSYFHLLTWSLPFVLTVAILAVAQVDGDSVSGIC
FVGY**A**NYRYRAGFVLAPIGLVLIVGGYFLIRGVMTLFSIKSNHAKALIVYGSTTGNTEYT
AETIARELADAGYEVDSRDAASVEAGGLFEGFDLVLLGCSTWGDDSIELQDDFIPLFDSL
EETGAQGRKVACFGCGDSSWEYFCGAVDAIEEKLKNLGAEIVQDGLRIDGDPRAARDDI
VGWAHDVRGAIKINETMLRLGIFGFLAFGFVLITFSCHFYDFFNQAEWERSFRDYVLCQ
ANVTIGLPTKQPIPDCEIKNRPSLLVEKINLFAMFGTGIAMSTWVWTKATLLIWRRTWCR
LTGQSDDHHHHHHHHHH

SMO K395R

MKTIIALSYIFCLVFADYKDDDDAKLQTMHHHHHHHHHHENLYFQGGTRGAASSGNAT
GPGPRSAGGSARRSAAVTGPPPPLSHCGRAAPCEPLRYNVCLGSVLPYGATSTLLAGDS
DSQEEAHGKLVLWSGLRNAPRCWAVIQPLLCAVYMPKCENDRVELPSRTLCQATRGPC
AIVERERGWPDFLRCTPDRFPEGCTNEVQNIKFNSSGQCEVPLVRTDNPKSWYEDVEGC
GIQCQNPLFTEAEHQDMHSYIAAFGAVTGLCTLFTLATFVADWRNSNRYPAVILFYVNA
CFFVGSIGWLAQFMDGARREIVCRADGTMRLGEPTSNETLSCVIIFVIVYYYALMAGVVW

FVVLTYAWHTSFKALGTTYQPLSGKTSYFHLLTWSLPFVLTVAILAVAQVDGDSVSGIC

FVGY**R**NYRYRAGFVLAPIGLVLIVGGYFLIRGVMTLFSIKSNHAKALIVYGSTTGNTEYT

AETIARELADAGYEVDSRDAASVEAGGLFEGFDLVLLGCSTWGDDSIELQDDFIPLFDSL

EETGAQGRKVACFGCGDSSWEYFCGAVDAIEEKLKNLGAEIVQDGLRIDGDPRAARDDI

VGWAHDVRGAIKINETMLRLGIFGFLAFGFVLITFSCHFYDFFNQAEWERSFRDYVLCQ

ANVTIGLPTKQPIPDCEIKNRPSLLVEKINLFAMFGTGIAMSTWVWTKATLLIWRRTWCR

LTGQSDDHHHHHHHHHH

The expression and purification of A2A-BRIL-ΔC[2] fusion protein was carried out in a similar way.

SDS-PAGE analysis

Purified proteins were diluted in a 5 μM concentration with the HPLC buffer (25 mM HEPES, 300 mM NaCl, 0.01% LMNG/0.002% CHS, pH 7.5). Probes were dissolved as a 50 μM HPLC buffer stock containing <0.2% v/v DMSO. For each sample, 10 μL of the protein stock/HPLC buffer was mixed with 10 μL of the probe stock/HPLC buffer and incubated for 3.5 hours at room temperature. The sample was adjusted to 30 μL, followed by mixing with 10 μL 4 × SDS page loading buffer and subjecting to SDS page. The gel was then visualized with an in-gel fluorescence imager (ChemiDoc MP Imaging System, Bio-Rad Laboratories, Inc., 488/530 nm) and stained with Coomassie Brilliant Blue (CBB). Quantitative analysis was performed by ImageLab charted with GraphPad Prism.

In the case of competing experiments, a 500 μM final concentration of TC114 was subjected to the purified protein solution. The sample was incubated at 4 °C for 30 minutes before mixing up with fluorescence labeling probes.

Time course of the fluorescence intensity

Purified proteins were diluted in a 5 μM concentration with the HPLC buffer. Probes were dissolved as a 5 μM HPLC buffer stock containing <0.2% v/v DMSO. For each sample, 9 μL of the protein stock/HPLC buffer was mixed with 9 μL of the probe stock/HPLC buffer in a 384 well plate at room temperature. The fluorescence intensity was measured by a microplate reader (FlexStation 3, Molecular Devices, LLC., 488/530 nm).

Cell-based luciferase reporter assay

The activities of precursors of labeling probes were measured with cell based luciferase reporter assay. NIH3T3 cells expressed firefly luciferase gene under the control of Gli responsive. The cells were cultured to confluency in 96-well plates using DMEM (Gibco) containing 10% (v/v) newborn calf serum (NCS, Gibco) and 175 μg/mL hygromycin (Gibco), and then treated with various concentrations of compounds in DMEM containing 0.5% NCS. After 2 hours' incubation at 37 °C, SAG (commercial source) was added to the final concentration of 100 nM. After another 24 hours' incubation at 37 °C, the intensity of the firefly luciferase was tested with Bright-Glo® Luciferase Assay System (Promega) on the Envision (PerkinElmer) under the guidance of description. The inhibition curve and IC50 of these antagonists were obtained with GraphPad Prism. Each data point was the mean of the duplicated results.

Digestion and HPLC-MS/MS analysis

The SDS-PAGE gel of SMO protein labeled by probe 9 was stained with CBB to visualize the SMO band. The gel was fixed with buffer containing 50% methanol and 7% acetic acid solution and then washed with deionized water three times. The gel was then stained with gelcode reagent solution (Invitrogen) according to the manufacturer's instruction. The band was cut into 2 regions and diced to small cubes. After washed with Milli Q water, the sample was destained with 25 mM NH_4HCO_3/50% MeCN. Then the gel was dehydrated with 100% MeCN. After removing MeCN, the sample was dissolved in 25 mM NH_4HCO_3 appended with 10 mM dithiothreitol (DTT). After shaking for 1 hour at 56 °C, the sample was cooled to room temperature and added 55 mM iodoacetamide (IAA). After incubation for 45 min in the dark. The gel was then washed with Milli Q water for three times, dehydrated by 25 mM NH_4HCO_3, 25 mM NH_4HCO_3/50% MeCN and 100% MeCN sequentially. After dried by speed Vac, the sample was added trypsin and Glu-C in Tris buffer (pH 8.8), followed by incubation at 37 °C overnight. The digested peptides were collected and cleaned with ZipTips (Millipore; ZTC18S096). The combined extracts were concentrated and subjected to HPLC-MS/MS spectrometer.

UV/Fluorescence HPLC analysis

The washed membrane was incubated with 10 μM probe 9 at 4 °C for 24 hours. After that, labeled SMO was purified following the routing process described above. SMO receptor was analyzed by SEC with a 280 nm UV detector and a 488/530 nm fluorescence detector.

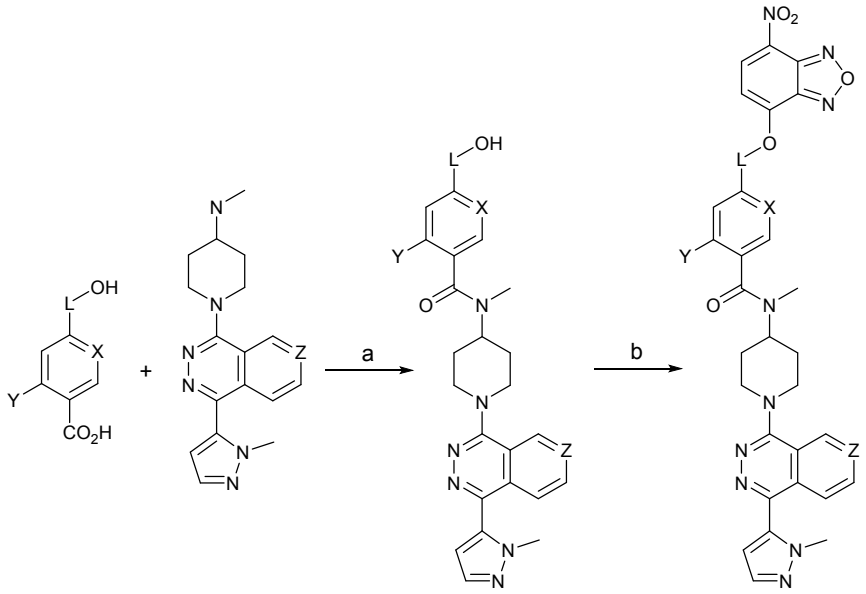
General methods for synthetic chemistry

All commercial reagents and solvents were used without further purification. Benzoic acid and second amine intermediates were synthesized according to the literatures.[3-6] High-resolution mass

spectra (HRMS) were recorded on an Agilent 6230 mass spectrometer using ESI (electrospray ionization). Chromatography was performed on silica gel 200-300 mesh. NMR spectra were recorded on a Bruker AVANCE III 500, 600 or 800 spectrometer (FT, 500/600/800 MHz for ^{1}H NMR; 125/150/200 MHz for ^{13}C NMR) at 298K or 273K with $CDCl_3$ and CD_3OD as the solvent and tetramethylsilane (TMS) as the internal standard. Chemical shifts were reported in units (ppm) by assigning TMS resonance as 0.00 ppm, in the ^{1}H spectrum, $CDCl_3$ resonance as 77.00 ppm in the ^{13}C spectrum. All coupling constants (*J* values) were reported in Hertz (Hz).

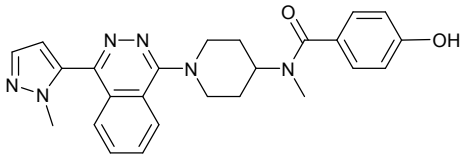
Synthesis of SMO labeling probes

Scheme S1. Synthesis of LY2040680 analogues as precursors and labeling probes.



Reagents and conditions. a. HATU, DIPEA, DCM, r.t., 2 h; b. F-NBD, Et_3N, DMAP, DCM, r.t., overnight.

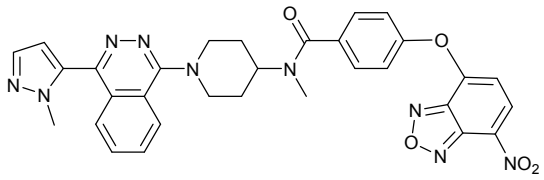
General method for synthesis of labeling probes. To a solution of the carboxylic acid (1.2 mmol) in 5 mL CH_2Cl_2 was added 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU, 570 mg, 1.5 mmol). The mixture was stirred for 15 min at room temperature before added to a mixture of the second amine (1.0 mmol) and *N*,*N*-diisopropylethylamine (DIPEA, 322 mg, 2.5 mmol) in 5 mL CH_2Cl_2. The reaction mixture was stirred at room temperature for 2 hours before being quenched by the addition of brine. The reaction mixture was extracted 3 times with CH_2Cl_2. The combined organic layer was washed with saturated $NaHCO_3$ solution and brine sequentially, dried over Na_2SO_4. After filtration, the solution was concentrated in vacuum and the crude product was purified by flash column chromatography on silica gel to yield the LY2940680 analogue as the precursor of the probe.



*4-hydroxy-N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)benzamide (**S1**)* Colorless solid, isolated yield 45% (21 mg). ^{1}H NMR (500 MHz, 298K, $CDCl_3$), δ (ppm) 1.95 (br, 2H, CH_2), 2.22 (br, 2H, CH_2), 2.83 and 3.03 (s, 3H, CH_3), 3.05-3.36 (m, 2H, CH_2), 3.56-3.61 (m) and 4.81 (br) (1H, CH), 4.01 (s, 3H, CH_3), 4.15 (m, 2H, CH_2), 6.61 (d, J = 1.0 Hz, 1H, CH), 6.89 (d, J = 8.5 Hz, 2H, CH), 7.29 (d, J = 8.0 Hz, 2H, CH), 7.67 (d, J = 1.0 Hz, 1H, CH), 7.84-7.93 (m, 2H, CH), 8.03 (d, J = 8.0 Hz, 1H, CH), 8.13 (d, J = 8.0 Hz, 1H, CH); HRMS calcd for $C_{25}H_{26}N_6O_2$ $[M+H]^+$: 443.2190; found: 443.2164.

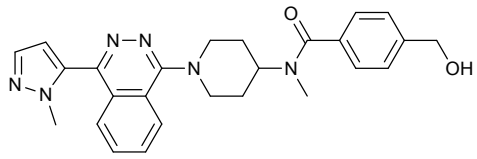
To a solution of the precursor (0.05 mmol) in 0.5 mL CH_2Cl_2 was added 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (F-NBD, 0.15 mmol), trimethylamine (0.2 mmol) and 4-dimethylaminopyridine

(DMAP, 0.01 mmol). The reaction mixture was stirred at room temperature overnight before being quenched by the addition of brine. The reaction mixture was extracted 3 times with CH_2Cl_2. The combined organic layer was washed with saturated $NaHCO_3$ solution and brine sequentially, dried over Na_2SO_4. After filtration, the solution was concentrated in vacuum and the crude product was purified by flash column chromatography on silica gel followed by purification by HPLC to yield the corresponding fluorescence labeling probe.

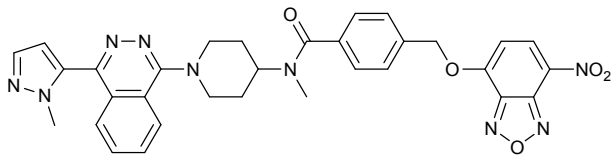


*N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)-4-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)benzamide (**1**)* Yellow solid, isolated yield 50 % (15 mg). ^{1}H NMR (500 MHz, 298K, $CDCl_3$) δ (ppm) 1.92-2.03 (m, 2H, CH_2), 2.22-2.35 (m, 2H, CH_2), 3.03 (s, 3H, CH_3), 3.14 (br, 1H, CH_2), 3.43 (br, 1H, CH_2), 3.88 and 4.91 (br, 1H, CH_2), 4.06 (s, 3H, CH_3), 4.11-4.25 (m, 2H, CH_2), 6.60 (d, J = 2.0 Hz, 1H, CH), 6.64 (d, J = 8.0 Hz, 1H, CH), 7.37 (d, J = 7.5 Hz, 2H, CH), 7.64-7.66 (m, 3H, CH), 7.84-7.93 (m, 2H, CH), 8.07-8.13 (m, 2H, CH), 8.47 (d, J = 8.5 Hz, 1H, CH); ^{13}C NMR (125 MHz, 298K, $CDCl_3$) δ (ppm) 28.5, 29.1, 29.2, 29.8, 32.4, 38.2, 50.4, 50.6, 51.8, 53.4, 108.1, 109.0, 111.5, 121.0, 121.3, 124.5, 126.2, 127.8, 128.1, 128.9, 129.7, 130.8, 131.4, 132.0, 133.0, 133.2, 135.78, 135.81, 136.6, 138.0, 144.0, 145.0, 147.4, 150.2, 153.4, 153.5, 157.0, 159.3; HRMS calcd for $C_{31}H_{27}N_9O_5$ $[M+H]^+$: 606.2231; found: 606.2241.

Probe **2-7**, **9**, **10** were synthesized followed the same procedure of probe **1**.

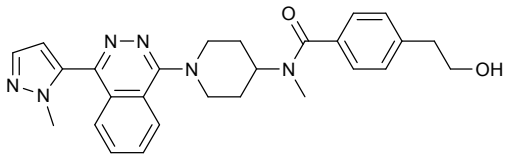


*4-(hydroxymethyl)-N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)benzamide (**S2**)* Colorless solid, isolated yield 98% (42 mg). ^{1}H NMR (800 MHz, 273K, $CDCl_3$ and CD_3OD) δ (ppm) 1.95-2.10 (m, 2H, CH_2), 2.24-2.28 (m, 2H, CH_2), 2.93 and 3.07 (s, 3H, CH_3) 3.31 and 3.70 (t, J = 12.0 Hz, 2H, CH_2), 3.95 and 4.83 (br, 1H, CH), 3.98 and 4.00 (s, 3H, CH_3), 4.24 and 4.45 (d, J = 12.0 Hz, 2H, CH_2) 4.70 (s, 2H, CH_2), 6.66 and 6.68 (s, 1H, CH), 7.36-7.44 (m, 4H, CH), 7.69 (br, 1H, CH), 8.07-8.09 (m, 2H, CH), 8.17-8.31 (m, 2H, CH); HRMS calcd for $C_{26}H_{28}N_6O_2$ $[M+H]^+$: 457.2352; found: 457.2381.

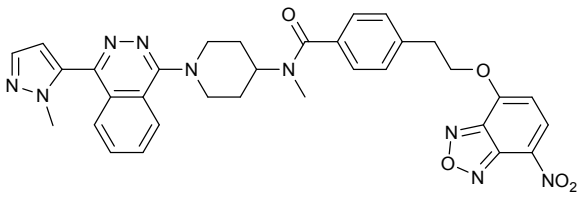


*N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)-4-(((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)methyl)benzamide (**2**)* Yellow solid, isolated yield 37% (21 mg). ^{1}H NMR (800 MHz, 273K, $CDCl_3$ and CD_3OD) δ (ppm) 1.99-2.13 (m, 2H, CH_2), 2.28-2.33 (m, 2H, CH_2), 2.96 and 3.11 (s, 3H, CH_3) 3.34 and 3.74 (t, J = 12.0 Hz, 2H, CH_2), 3.96 and 4.87 (br, 1H, CH), 4.00 and 4.02 (s, 3H, CH_3), 4.48 (d, J = 12.0 Hz, 2H, CH_2) 5.54 and 5.55 (s, 2H, CH_2), 6.66 and 6.68 (s, 1H, CH), 6.83 and 6.87 (d, J = 8.0 Hz, 1H, CH), 7.50-7.53 (m, 2H, CH), 7.59-7.64 (m, 2H, CH), 7.69 (br, 1H, CH), 8.07-8.09 (m, 2H, CH), 8.18-8.32 (m, 2H, CH), 8.57-8.60 (m, 1H, CH); ^{13}C NMR (200 MHz, 298K, $CDCl_3$ and CD_3OD) δ (ppm) 28.4, 29.4, 29.6,

31.6, 32.3, 37.58, 37.64, 46.14, 46.17, 50.2, 50.5, 51.7, 56.7, 71.8, 105.5, 108.9, 121.2, 124.4, 124.59, 124.60, 125.9, 126.5, 127.2, 127.3, 127.7, 127.8, 129.6, 131.8, 132.2, 134.09, 134.12, 135.2, 136.6, 137.3, 138.0, 143.7, 145.0, 145.2, 147.0, 147.3, 153.8, 159.5; HRMS calcd for $C_{32}H_{29}N_9O_5$ $[M+H]^+$: 620.2370; found: 620.2363.

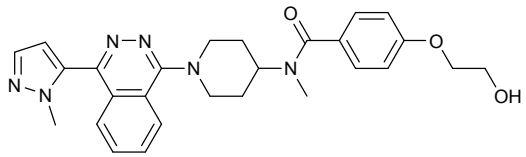


*4-(2-hydroxyethyl)-N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)benzamide (**S3**)* Colorless solid, isolated yield 86% (50 mg). ^{1}H NMR (500 MHz, 298K, $CDCl_3$), δ (ppm) 1.91-2.04 (m, 2H, CH_2), 2.21-2.24 (m, 2H, CH_2), 2.88 (t, J = 7.5 Hz, 2H, CH_2), 2.93 and 3.05 (s, 3H, CH_3), 3.19 (br, 1H, CH_2), 3.56 (br, 1H, CH_2), 3.84 (t, J = 7.0 Hz, 2H, CH_2), 3.98 (s, 3H, CH_3), 4.18 and 4.35 (br, 2H, CH_2), 4.57 and 4.83 (br, 1H, CH), 6.63 (s, 1H, CH), 7.27-7.35 (m, 2H, CH), 7.66 (d, J = 2.0 Hz, 1H, CH), 7.99-8.01 (m, 2H, CH), 8.23 (br, 1H, CH); HRMS calcd for $C_{27}H_{30}N_6O_2$ $[M+H]^+$: 471.2503; found: 471.2532.

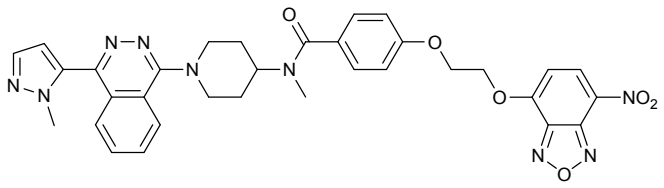


*N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)-4-(2-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)ethyl)benzamide (**3**)* Yellow solid, isolated yield 26% (16 mg). ^{1}H NMR (500 MHz, 298K, $CDCl_3$) δ (ppm) 1.95-2.07 (m, 2H, CH_2), 2.25-2.27 (m, 2H, CH_2),

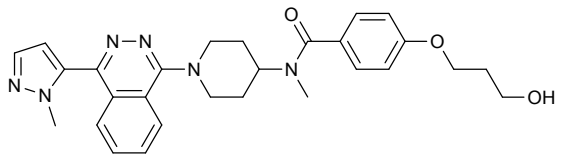
2.93 and 3.21, (s, 3H, CH_3), 3.07 (br, 1H, CH_2), 3.34 (t, J = 6.0 Hz, 2H, CH_2), 3.65 (br, 1H, CH), 3.94 and 3.99 (s, 3H, CH_3), 4.19-4.42 (m, 2H, CH_2), 4.60 (t, J = 6.5 Hz, 2H, CH_2), 4.85 (br, 1H, CH), 6.64 (d, J = 1.5 Hz, 1H, CH), 6.70 (d, J = 7.0 Hz, 1H, CH), 7.41 (br, 4H, CH), 7.68 (d, J = 1.5 Hz, 1H, CH), 8.03-8.06 (m, 2H, CH), 8.12-8.27 (m, 2H, CH), 8.51 (d, J = 8.5 Hz, 1H, CH); ^{13}C NMR (125 MHz, 298K, $CDCl_3$) δ (ppm) 28.6, 29.3, 29.7, 35.0, 38.3, 50.71, 50.76, 71.3, 104.6, 109.1, 114.7, 121.4, 124.6, 126.3, 126.9, 127.5, 128.0, 128.1, 129.3, 130.0, 131.5, 132.0, 133.8, 135.7, 136.6, 138.2, 144.0, 145.2, 146.0,147.8,152.4, 154.4, 159.5; HRMS calcd for $C_{33}H_{31}N_9O_5$ $[M+H]^+$: 634.2526; found: 634.2515.



*4-(2-hydroxyethoxy)-N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)benzamide (**S4**)* Colorless solid, isolated yield 82% (40 mg). ^{1}H NMR (500 MHz, 298K, $CDCl_3$) δ (ppm) 1.96 (br, 2H, CH_2), 2.22 (br, 2H, CH_2), 3.01 and 3.35 (s, 3H, CH_3), 3.12-3.17 (m, 1H, CH_2), 3.63-3.68 (m, 1H, CH_2), 3.99 (t, J = 4.0 Hz, 2H, CH_2), 4.04 (s, 3H, CH_3), 4.11 (t, J = 4.0 Hz, 2H, CH_2), 4.14-4.17 (m, 2H, CH_2), 4.82 (br, 1H, CH), 6.60 (s, 1H, CH), 6.96 (d, J = 8.0 Hz, 2H, CH), 7.40 (d, J = 8.0 Hz, 2H, CH), 7.66 (br, 1H, CH), 7.83-7.92 (m, 2H, CH), 8.03-8.13 (m, 2H, CH); HRMS calcd for $C_{27}H_{30}N_6O_3$ $[M+H]^+$: 487.2458; found: 487.2462.

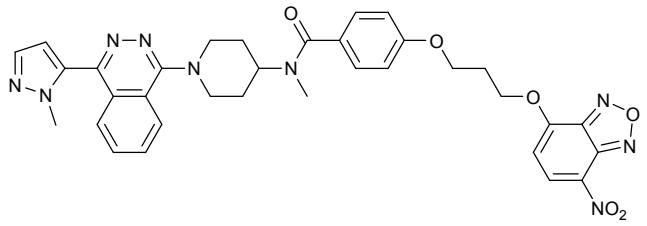


*N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)-4-(2-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)ethoxy)benzamide (**4**)* Yellow solid, isolated yield 17% (9 mg). ^{1}H NMR (800 MHz, 273K, $CDCl_3$ and CD_3OD) δ (ppm) 1.99-2.15 (m, 2H, CH_2), 2.30-2.32 (m, 2H, CH_2), 3.04 and 3.09 (s, 3H, CH_3), 3.28 and 3.68 (br, 2H, CH_2), 4.01 and 4.03 (s, 3H, CH_3), 4.22 and 4.28 (br, 2H, CH_2), 4.59 (br, 2H, CH_2), 4.84 (br, 3H, CH_2 and CH), 6.71 (s, 1H, CH), 6.96 and 6.97 (s, 1H, CH), 7.04-7.06 (m, 2H, CH), 7.42-7.47 (m, 2H, CH), 7.71 (br, 1H, CH), 8.06-8.37 (m, 4H, CH), 8.67 (d, J = 8.0 Hz, 1H, CH); ^{13}C NMR (200 MHz, 298K, $CDCl_3$ and CD_3OD) δ (ppm) 29.8, 38.2, 49.4, 51.3, 63.5, 66.0, 69.7, 105.6, 110.06, 110.09, 114.6, 114.7, 116.0, 120.2, 121.0, 121.6, 122.5, 127.4, 129.1, 129.2, 129.3, 130.1, 133.05, 133.08, 133.27, 133.32, 133.4, 134.6, 138.7, 144.2, 144.6, 145.4, 154.6, 159.7; HRMS calcd for $C_{33}H_{31}N_9O_6$ $[M+H]^+$: 650.2476; found: 650.2484.

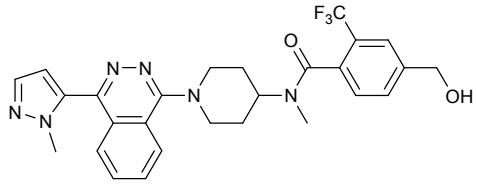


*4-(3-hydroxypropoxy)-N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)benzamide (**S5**)* Colorless solid, isolated yield 87% (42 mg). ^{1}H NMR (500 MHz, 298K, $CDCl_3$) δ (ppm) 1.95 (br, 2H, CH_2), 2.05 (pent, J = 6.0 Hz, 2H,CH_2), 2.22 (br, 2H, CH_2), 3.01 (s, 3H, CH_3), 3.11-3.16 (m, 2H, CH_2), 3.35 (br, 1H, CH_2), 3.62-3.67 (m, 1H, CH_2), 3.84 (t, J = 6.0 Hz, 2H, CH_2), 4.03 (s, 3H, CH_3), 4.15 (t, J = 6.5 Hz, 2H, CH_2), 4.80 (br, 1H, CH), 6.59 (d, J = 1.5 Hz,

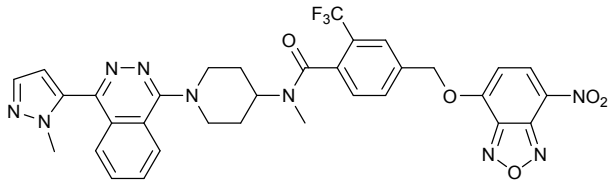
1H, CH), 6.95 (d, J = 6.5 Hz, 2H, CH), 7.38 (d, J = 8.0 Hz, 2H, CH), 7.65 (d, J = 2.0 Hz, 1H, CH), 7.83-7.92 (m, 2H, CH), 8.03 (d, J = 8.0 Hz, 1H, CH), 8.13 (d, J = 8.5 Hz, 1H, CH); HRMS calcd for $C_{28}H_{32}N_6O_2$ $[M+H]^+$: 501.2614; found: 501.2636.



*N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)-4-(3-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)propoxy)benzamide (**5**)* Yellow solid, isolated yield 40% (21 mg). ^{1}H NMR (500 MHz, 298K, $CDCl_3$) δ (ppm) 1.94-2.05 (m, 2H, CH_2), 2.20-2-23 (m, 2H, CH_2), 2.51 (pent, 2H, CH_2), 3.00 (br, 3H, CH_3), 3.32-3.46 (m, 2H, CH_2), 4.04 (s, 3H, CH_3), 4.10-4.16 (m, 2H, CH_2), 4.29 (t, J = 6.0 Hz, 2H, CH_2), 4.63 (t, J = 6.0 Hz, 2H, CH_2), 4.83 (br, 1H, CH), 6.59 (d, J = 2.0 Hz, 1H, CH), 6.77 (d, J = 8.0 Hz, 1H, CH), 6.95 (d, J = 9.0 Hz, 2H, CH), 7.40 (d, J = 8.5 Hz, 2H, CH), 7.65 (d, J = 2.0 Hz, 1H, CH), 7.82-7.91 (m, 2H, CH), 8.05-8.12 (m, 2H, CH), 8.54 (d, J = 8.5 Hz, 1H, CH); ^{13}C NMR (125 MHz, 298K, $CDCl_3$) δ (ppm) 28.5, 29.1, 29.2, 29.6, 38.2, 50.59, 50.63, 50.66, 53.4, 60.3, 63.5, 67.5, 104.6, 109.2, 114.1, 121.3, 124.50, 124.53, 126.1, 127.8, 128.3, 128.7, 129.3, 129.6, 131.5, 131.96, 131.99, 134.1, 136.6, 138.0, 143.8, 145.0, 154.5, 159.3, 159.4; HRMS calcd for $C_{34}H_{33}N_9O_6$ $[M+H]^+$: 664.2632; found: 664.2612.

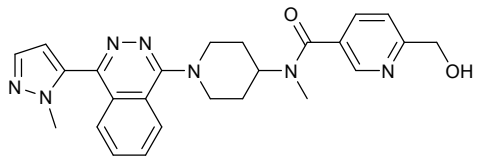


*4-(hydroxymethyl)-N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)-2-(trifluoromethyl)benzamide (**S6**)* Colorless solid in 95% yield (45 mg). ^{1}H NMR (800 MHz, 273K, $CDCl_3$), δ (ppm) 1.88-2.09 (m, 2H, CH_2), 2.16-2.34 (m, 2H, CH_2), 2.74, 2.77, 3.11 and 3.13 (s, 3H, CH_3), 3.23-3.28 and 3.56-3.76 (m, 4H, CH_2 and CH), 3.99, 4.02 and 4.03 (s, 3H, CH_3), 4.22-4.24 (m, 1H, CH_2),4.44-4.52 (m, 1H, CH_2), 4.78, 4.80, 5.44 and 5.47 (s, 2H, CH_2), 4.91-4.94 (m, 1H, CH), 6.65 and 6.67 (s, 1H, CH), 7.32-7.33 and 7.43-7.46 (m, 1H, CH), 7.59 and 7.61 (d, J = 8.0 Hz, 1H, CH), 7.69-7.76 (m, 2H, CH), 8.05-8.08 (m, 2H, CH),8.16- 8.29 (m, 2H, CH); ^{13}C NMR (125 MHz, 298K, $CDCl_3$) δ (ppm) 27.2, 27.6, 28.0, 28.6, 29.1, 29.2, 29.29, 29.31, 29.45, 29.49, 29.52, 29.58, 29.67, 29.74, 31.85, 31.88, 35.9, 38.2, 38.3, 42.0, 49.9, 50.2, 50.5, 51.0, 51.2, 53.7, 56.6, 63.6, 63.7, 109.24, 109.27, 121.6, 122.6, 124.7 (q, J = 4.6 Hz), 125.0, 126.2, 126.41, 126.45, 126.6, 127.0 (q, J = 173.5 Hz), 128.1, 128.2, 129.85, 129.88, 130.1, 130.2, 131.7, 131.8, 132.4, 132.5, 133.8, 134.4 (q, J = 1.5 Hz), 136.3, 136.4, 138.20, 138.23, 142.7, 143.1, 147.2, 147.6, 159.0, 168.9, 169.1; HRMS calcd for $C_{27}H_{27}F_3N_6O_2$ $[M+H]^+$: 525.2226; found: 525.2212.



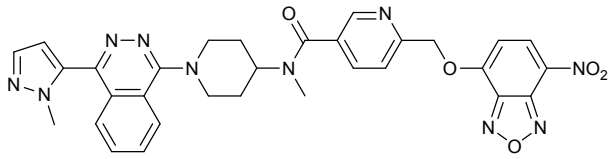
*N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)-4-(((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)methyl)-2-(trifluoromethyl)benzamide (**6**)* Yellow solid,

isolated yield 57% (18 mg). ^{1}H NMR (800 MHz, 298K, $CDCl_3$), δ ppm 1.94-2.13 (m, 2H, CH), 2.23-2.42 (m, 4H, CH_2), 2.74, 2.78, 3.11 and 3.13 (s, 3H, CH_3), 3.38-3.42 and 3.75-3.81 (m, 2H, CH_2), 3.62-3.68 (m, 1H, CH), 4.00 and 4.03 (s, 3H, CH_3), 4.32-4.35 and 4.52-4.63 (m, 2H ,CH_2), 5.58 (s, 2H, CH_2), 4.90-4.93 and 5.34-5.35 (m, 1H, CH), 5.57 and 5.60 (s, 2H, CH_2), 6.65-6.69 (m, 1H, CH), 6.81 and 6.85 (d, J = 8.0 Hz, 1H, CH), 7.36 and 7.48 (d, J = 8.0 Hz, 1H, CH), 7.69 and 7.70 (s, 1H, CH), 7.81 and 7.83 (d, J = 8.0 Hz, 1H, CH), 7.86 and 7.91 (m, 1H, CH), 8.06-8.10 (m, 2H, CH), 8.18 (d, J = 8.0 Hz, 1H, CH), 8.21 and 8.30 (d, J = 8.0 Hz, 1H, CH), 8.52-8.58 (m, 1H, CH); ^{13}C NMR (125 MHz, 298K, $CDCl_3$) δ (ppm) 27.2, 27.6, 28.0, 28.6, 29.1, 29.3, 29.56, 29.63, 29.7, 31.9, 38.16, 38.20, 42.3, 50.1, 50.4, 50.9, 51.1, 51.3, 54.1, 56.8, 71.2, 71.4, 105.72, 105.78, 109.0, 109.23, 109.26, 121.5, 122.2, 124.4, 124.6, 124.8, 125.97, 126.07, 126.35, 126.40, 127.1, 127.4, 127.6, 128.0 (q, J = 5.5 Hz), 128.4, 128.8, 130.4, 131.6 (q, J = 25 Hz), 132.2, 132.5, 133.66, 133.70, 135.1, 135.4, 135.9, 136.46, 136.51, 138.2, 144.0, 145.0, 145.1, 146.4, 147.3, 147.7, 153.55, 153.58, 159.34, 159.36, 168.0, 168.2; HRMS calcd for $C_{33}H_{28}F_3N_9O_5$ $[M+H]^+$: 688.2244; found: 688.2218.

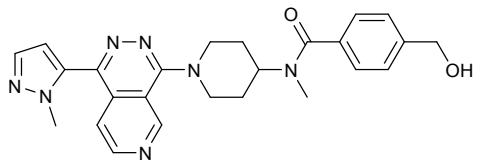


*6-(hydroxymethyl)-N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)nicotinamide (**S7**)* Colorless solid, isolated yield 83% (33 mg). ^{1}H NMR (800 MHz, 273K, $CDCl_3$ and CD_3OD) δ (ppm) 2.05-2.12 (m, 2H, CH_2), 2.26-2.33 (m, 2H, CH_2), 3.02 and 3.12 (s, 3H, CH_3), 3.45 and 3.66 (t, J = 12.8 Hz, 2H, CH_2), 3.96 and 4.81 (br, 1H, CH), 3.99 and 4.01 (s, 3H, CH_3) 4.25 and 4.42 (d, J = 12.8 Hz, 2H, CH_2), 4.98 and 5.00 (s, 2H, CH_2), 6.68 and 6.69 (s,

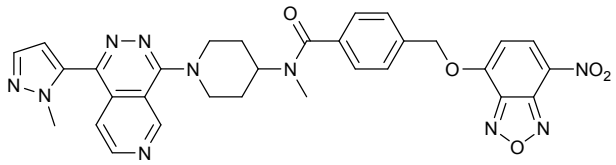
1H, CH), 7.70 (br, 1H, CH), 7.91 and 7.94 (d, J = 8.0 Hz, 1H, CH), 8.08-8.11 (m, 2H, CH), 8.16 and 8.17 (s, 1H, CH), 8.27-8.34 (m, 2H, CH), 8.84 and 8.87 (s, 1H, CH); ^{13}C NMR (200 MHz, 298K, $CDCl_3$ and CD_3OD) δ (ppm) 27.9, 29.2, 34.9, 37.1, 39.0, 42.4, 50.5, 54.3, 56.7, 63.5, 109.0, 120.3, 120.5, 125.9, 127.9, 128.24, 128.26, 132.4, 133.1, 134.5, 136.0, 136.2, 137.9, 139.2, 146.0, 150.1, 158.7, 161.5; HRMS calcd for $C_{25}H_{27}N_7O_2$ $[M+H]^+$: 458.2304; found: 458.2299.



N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)-6-(((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)methyl)nicotinamide (7) Yellow solid, isolated yield 41% (13 mg). ^{1}H NMR (500 MHz, 298K, $CDCl_3$) δ (ppm) 1.89-2.05 (m, 2H, CH_2), 2.20-2.34 (m, 2H, CH_2), 2.99 and 3.13 (s, 3H, CH_3) 3.04 and 3.41 (br, 2H, CH_2), 3.66 and 4.89 (br, 1H, CH), 3.78 (br) and 4.23 (d, J = 11.0 Hz) (2H, CH_2), 4.07 (s, 3H, CH_3), 5.64 and 5.67 (s, 2H, CH_2), 6.60 (s, 1H, CH), 6.88 (d, J = 8.0 Hz, 1H, CH), 7.66 (s, 1H, CH), 7.73 (br, 1H, CH), 7.83-7.91 (m, 3H, CH), 8.07-8.12 (m, 2H, CH), 8.55 (d, J = 8.0 Hz, 1H, CH), 8.74 (s, 1H, CH); ^{13}C NMR (200 MHz, 298K, $CDCl_3$) δ (ppm) 28.7, 29.3, 29.6, 29.7, 31.9, 32.1, 32.9, 38.5, 51.5, 51.8, 70.5, 72.5, 72.6, 105.9, 109.7, 121.1, 121.4, 121.7, 122.1, 125.0, 126.8, 127.2, 130.4, 132.5, 133.7, 133.98, 134.05, 136.2, 138.3, 144.0, 145.2, 150.9, 153.6, 155.1, 158.4; HRMS calcd for $C_{31}H_{28}N_{10}O_5$ $[M+H]^+$: 621.2322; found: 621.2309.

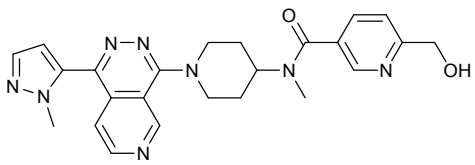


*4-(hydroxymethyl)-N-methyl-N-(1-(1-(1-methyl-1H-pyrazol-5-yl)pyrido[3,4-d]pyridazin-4-yl)piperidin-4-yl)benzamide (**S8**)* Colorless solid, isolated yield 98% (70 mg). ^{1}H NMR (800 MHz, 273K, $CDCl_3$) δ (ppm) 1.92-2.09 (m, 2H, CH_2), 2.17-2.31 (m, 2H, CH_2), 2.84, 2.94, 3.07 and 3.08 (s, 3H, CH_3), 3.11, 3.22 and 3.62 (d, J = 12.0 Hz, 2H, CH_2), 3.51 and 4.89 (d, J = 12.0 Hz, 1H, CH), 3.92-3.99, 4.17-4.24, 4.30-4.35 and 4.46-4.47 (m, 2H, CH_2), 4.06, 4.08, 4.09 and 4.11 (s, 3H, CH_3), 4.69, 4.70 and 4.72 (s, 2H, CH_2), 6.67, 6.69, 6.72 and 6.74 (s, 1H, CH), 7.37-7.45 (m, 4H, CH), 7.71, 7.72 and 7.74 (s, 1H, CH), 7.92, 7.97, 7.99 and 8.01 (d, J = 5.6 Hz, 1H, CH), 9.00, 9.03, 9.05 and 9.07 (d, J = 5.6 Hz, 1H, CH), 9.53, 9.54, 9.56 and 9.64 (s, 1H, CH); ^{13}C NMR (200 MHz, 298K, $CDCl_3$) δ (ppm) 28.5, 29.6, 32.3, 38.28, 38.35, 41.9, 50.3, 50.7, 53.6, 64.0, 109.0, 109.8, 114.9, 117.3, 121.8, 124.5, 126.2, 126.5, 126.8, 129.8, 130.8, 135.2, 135.4, 138.2, 138.3, 143.1, 145.6, 149.3, 149.8, 150.9, 157.5, 171.6; HRMS calcd for $C_{25}H_{27}N_7O_2$ $[M+H]^+$: 458.2304; found: 458.2318.



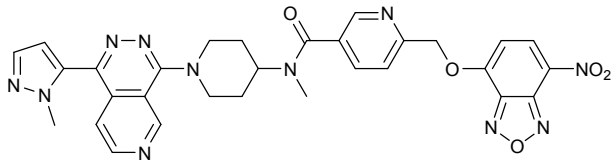
*N-methyl-N-(1-(1-(1-methyl-1H-pyrazol-5-yl)pyrido[3,4-d]pyridazin-4-yl)piperidin-4-yl)-4-(((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)methyl)benzamide (**9**)* Yellow solid, isolated yield 54% (22 mg). ^{1}H NMR (800 MHz, 273K, $CDCl_3$) δ (ppm) 1.96-2.13 (m, 2H, CH_2), 2.21-2.35 (m, 2H, CH_2), 2.97, 3.10 and 3.11 (s, 3H, CH_3), 3.21, 3.35, 3.56 and 3.67 (t, J = 12.0 Hz, 2H, CH_2), 3.94-

4.00 and 4.90-4.96 (m, 1H, CH), 4.09, 4.11, 4.12 and 4.15 (s, 3H, CH_3), 4.23-4.25 (m) and 4.41 (br) (1H, CH_2), 4.55 (d, J = 12.8 Hz, 1H, CH_2), 5.54 (s, 2H, CH_2), 6.69, 6.70, 6.73 and 6.75 (s, 1H, CH), 6.82 and 6.86 (d, J = 8.0 Hz, 1H, CH), 7.52-7.54 (m, 2H, CH), 7.60-7.64 (m, 2H, CH), 7.74-7.76 (m, 1H, CH), 7.97-8.06 (m, 1H, CH), 8.55-8.57 (m, 1H, CH), 9.02-9.11 (m, 1H, CH), 9.57, 9.58, 9.64 and 9.70 (s, 1H, CH); ^{13}C NMR (200 MHz, 298K, $CDCl_3$) δ (ppm) 28.5, 28.8, 29.7, 30.4, 32.3, 38.36, 38.43, 38.8, 50.7, 51.3, 53.4, 56.5, 60.3, 67.6, 72.0, 105.5, 109.0, 109.8, 114.9, 116.4, 117.3, 124.5, 126.8, 127.6, 127.7, 127.9, 129.3, 129.9, 130.8, 133.8, 135.1, 135.4, 137.5, 138.2, 138.3, 143.8, 145.2, 149.4, 149.9, 151.0, 153.9, 157.5, 157.8, 171.0; HRMS calcd for $C_{31}H_{28}N_{10}O_5$ $[M+H]^+$: 621.2322; found: 621.2341.



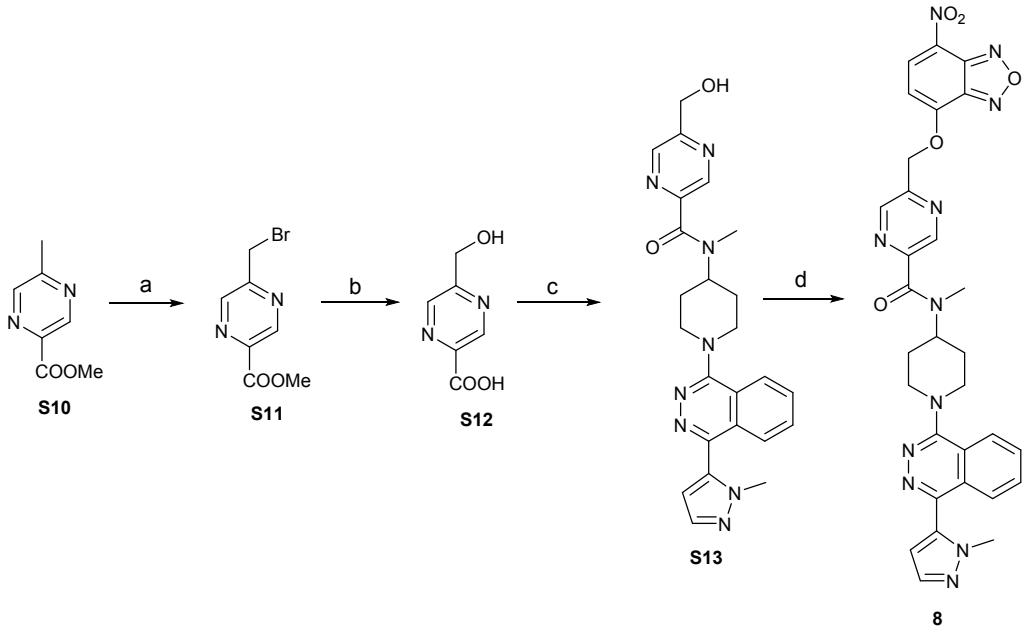
*6-(hydroxymethyl)-N-methyl-N-(1-(1-(1-methyl-1H-pyrazol-5-yl)pyrido[3,4-d]pyridazin-4-yl)piperidin-4-yl)nicotinamide (**S9**)* Colorless solid, isolated yield 87% (35 mg). ^{1}H NMR (500 MHz, 298K, $CDCl_3$) δ (ppm) 1.89-2.04 (m, 2H, CH_2), 2.21-2.27 (m, 2H, CH_2), 2.97 and 3.09 (s, 3H, CH_3), 3.16-3.19 and 3.44-3.50 (m, 2H, CH_2), 3.69-3.74 and 4.90-4.91 (m, 1H, CH), 4.11 and 4.14 (s, 3H, CH_3), 4.28-4.39 (m, 2H, CH_2), 4.83 (s, 2H, CH_2), 6.62 and 6.67 (d, J = 1.5 Hz, 1H, CH), 7.40 (br, 1H, CH), 7.68 and 7.69 (d, J = 1.5 Hz, 1H, CH), 7.80 (d, J = 8.0 Hz, 1H, CH), 7.87 (d, J = 6.0 Hz, 1H, CH), 8.65 (br, 1H, CH), 8.96 and 9.02 (d, J = 5.5 Hz, 1H, CH), 9.55 (s, 1H, CH); ^{13}C NMR (200 MHz, 298K, $CDCl_3$) δ (ppm) 28.4, 29.7, 32.4, 38.3, 38.4, 41.9, 50.7, 51.6, 53.4, 53.6, 64.1, 64.3, 109.0, 109.8, 114.9, 117.3, 120.1, 120.3, 121.8, 124.5, 128.8, 130.86,

130.92, 135.4, 135.81, 135.89, 138.0, 138.2, 138.3, 145.8, 146.7, 149.3, 149.8, 150.3, 151.0, 161.2, 168.9; HRMS calcd for $C_{24}H_{26}N_8O_2$ $[M+H]^+$: 459.2257; found: 459.2284.

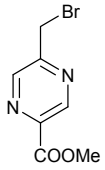


*N-methyl-N-(1-(1-(1-methyl-1H-pyrazol-5-yl)pyrido[3,4-d]pyridazin-4-yl)piperidin-4-yl)-6-(((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)methyl)nicotinamide (**10**)* Yellow solid, isolated yield 63% (16 mg). ^{1}H NMR (800 MHz, 273K, $CDCl_3$) δ (ppm) 1.96-2.13 (m, 2H, CH_2), 2.20-2.39 (m, 2H, CH_2), 3.01, 3.02 and 3.13 (s, 3H, CH_3), 3.19, 3.30, 3.54 and 3.63 (t, J = 12.0 Hz, 2H, CH_2), 3.85-3.91 and 4.91-4.96 (m, 1H, CH), 4.11, 4.14 and 4.17 (s, 3H, CH_3), 4.22-4.24 (m) and 4.37 (d, J = 12.8 Hz) (1H, CH_2), 4.50 (d, J = 12.8 Hz, 1H, CH_2), 5.69, 5.70 and 5.71 (s, 2H, CH_2), 6.68, 6.69, 6.73 and 6.74 (s, 1H, CH), 6.91 (d, J = 8.0 Hz, 1H, CH), 7.77-7.79 (m, 1H, CH), 7.83-7.88 (m, 1H, CH), 8.00-8.05 (m, 2H, CH), 8.58 (d, J = 8.8 Hz, 1H, CH), 8.79 (br, 1H, CH), 9.01, 9.03 and 9.09 (d, J = 5.6 Hz, 1H, CH), 9.59, 9.60, 9.64 and 9.69 (s, 1H, CH); ^{13}C NMR (200 MHz, 298K, $CDCl_3$ and CD_3OD) δ (ppm) 27.0, 28.3, 29.1, 29.4, 29.5, 31.7, 32.6, 38.1, 50.8, 51.8, 72.2, 105.8, 109.2, 121.7, 130.1, 132.2, 133.9, 135.1, 136.4, 138.3, 138.4, 143.8, 145.0, 149.3, 150.0, 153.4, 153.5, 155.0; HRMS calcd for $C_{30}H_{27}N_{11}O_5$ $[M+H]^+$: 622.2275; found: 622.2294.

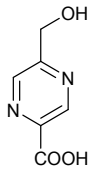
Scheme S2. Synthesis of probe 8.



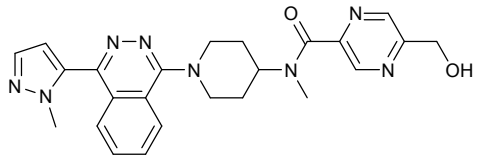
Reagents and condition. a. NBS, AIBN, CCl_4, 95 °C, overnight; b. $CaCO_3$, 1,4-dioxane, H_2O, 100 °C, overnight; c. second amine, HATU, DIPEA, DCM, r.t., 2 h; d. F-NBD, DMAP, DCM, r.t., overnight.



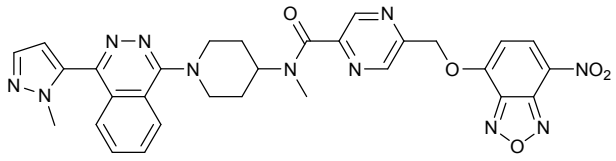
*methyl 5-(bromomethyl)pyrazine-2-carboxylate (**S11**)* To a solution of **S10** (6.58 mmol, 1 g) in 15 mL CCl_4 was added *N*-bromosuccinimide (NBS, 7.24 mmol, 1.3 g) and azodiisobutyronitrile (AIBN, 0.66 mmol, 108 mg). The reaction mixture was heated at 95 °C overnight before being quenched by the addition of water. The reaction mixture was extracted 3 times with CH_2Cl_2. The combined organic layer was washed with brine sequentially, dried over Na_2SO_4. After filtration, the solution was concentrated in vacuum and the crude product was purified by flash column chromatography on silica gel to give **S11** as colorless solid in 41% yield. (620 mg).



*5-(hydroxymethyl)pyrazine-2-carboxylic acid (**S12**)* To a solution of **S11** (1.3 mmol, 300 mg) in 5 mL H_2O and 5 mL MeOH was added $CaCO_3$ (3.9 mmol, 390 mg). The reaction mixture was heated at 100 °C overnight. After the reaction was complete, the solution was concentrated in vacuum and the crude product was directly used in the next step without further purification.



*5-(hydroxymethyl)-N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)pyrazine-2-carboxamide (**S13**)* Synthesis of **S13** followed the same method of synthesis of LY2940680 analogues. Colorless solid, isolated yield (24 mg, 75 %). ^{1}H NMR (500 MHz, 298K, $CDCl_3$) δ (ppm) 2.02-2.08 (m, 2H, CH_2), 2.17-2.31 (m, 2H, CH_2), 3.06 and 3.16 (s, 3H, CH_3), 3.06 and 3.40 (t, J = 7.5 Hz, 2H, CH_2), 3.65-3.66 and 4.97-4.98 (m, 1H, CH), 4.03 and 4.06 (s, 3H, CH_3), 4.08 and 4.22 (d, J = 12.5 Hz, 2H, CH_2), 4.92 and 4.93 (s, 2H, CH_2), 6.59 and 6.60 (br, 1H, CH), 7.66 (d, J = 1.0 Hz, 1H, CH), 7.83-7.91 (m, 2H, CH), 8.05-8.14 (m, 2H, CH), 8.67 (s, 1H, CH), 8.87 and 8.91 (br, 1H, CH); ^{13}C NMR (125 MHz, 298K, $CDCl_3$) δ (ppm) 28.3, 28.4, 29.6, 29.9, 31.9, 38.2, 50.6, 52.2, 63.0, 109.1, 121.4, 124.5, 124.6, 126.2, 127.9, 131.5, 131.6, 132.0, 132.1, 136.6, 136.7, 138.1, 140.4, 143.8, 144.0, 147.4, 147.6, 148.3, 148.5, 155.9, 159.5, 166.8; HRMS calcd for $C_{24}H_{26}N_8O_2$ $[M+H]^+$: 459.2257; found: 459.2236.



N-methyl-N-(1-(4-(1-methyl-1H-pyrazol-5-yl)phthalazin-1-yl)piperidin-4-yl)-5-(((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)methyl)pyrazine-2-carboxamide ***(8)*** Synthesis of **8** followed the same method of labeling probe preparation. Yellow solid, isolated yield 40% (10 mg). ^{1}H NMR (500 MHz, 298K, $CDCl_3$) δ (ppm) 2.02-2.10 (m, 2H, CH_2), 2.19-2.34 (m, 2H, CH_2), 3.09 and 3.17 (s, 3H, CH_3), 3.07-3.12 (m, 1H, CH_2), 3.41 (t, J = 12.5 Hz, 1H, CH_2), 4.04 and 4.07 (s, 3H, CH_3), 4.11-4.24 (m, 2H, CH_2), 4.90-4.94 (m, 1H, CH), 5.69 and 5.71 (s, 2H, CH_2), 6.60 (d, J = 5.5 Hz, 1H, CH), 6.94 (d, J = 8.0 Hz, 1H, CH), 7.65 and 7.66 (s, 1H, CH), 7.82-7.92 (m, 2H, CH), 8.07-8.14 (m, 2H, CH), 8.57 (d, J = 8.0 Hz, 1H, CH), 8.91 (d, J = 4.0 Hz, 1H, CH), 8.99 and 9.03 (s, 1H, CH); ^{13}C NMR (150 MHz, 298K, $CDCl_3$ and CD_3OD) δ (ppm) 28.29, 28.33, 29.1, 29.5, 29.8, 31.7, 31.9, 37.79, 37.82, 50.5, 50.6, 52.3, 56.6, 70.67, 70.70, 105.9, 109.10, 109.14, 121.4, 121.5, 124.6, 124.8, 126.16, 126.21, 128.0, 129.72, 129.75, 130.4, 131.8, 132.0, 132.4, 132.5, 133.71, 133.75, 136.5, 136.6, 138.1, 141.3, 141.4, 143.8, 144.4, 144.6, 145.0, 147.2, 147.5, 149.5, 149.8, 150.0, 153.2, 159.3, 166.1, 166.3; HRMS calcd for $C_{30}H_{27}N_{11}O_5$ $[M+H]^+$: 622.2275; found: 622.2280.

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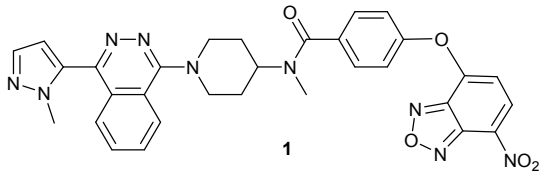
NMR and HRMS Spectra of new compounds



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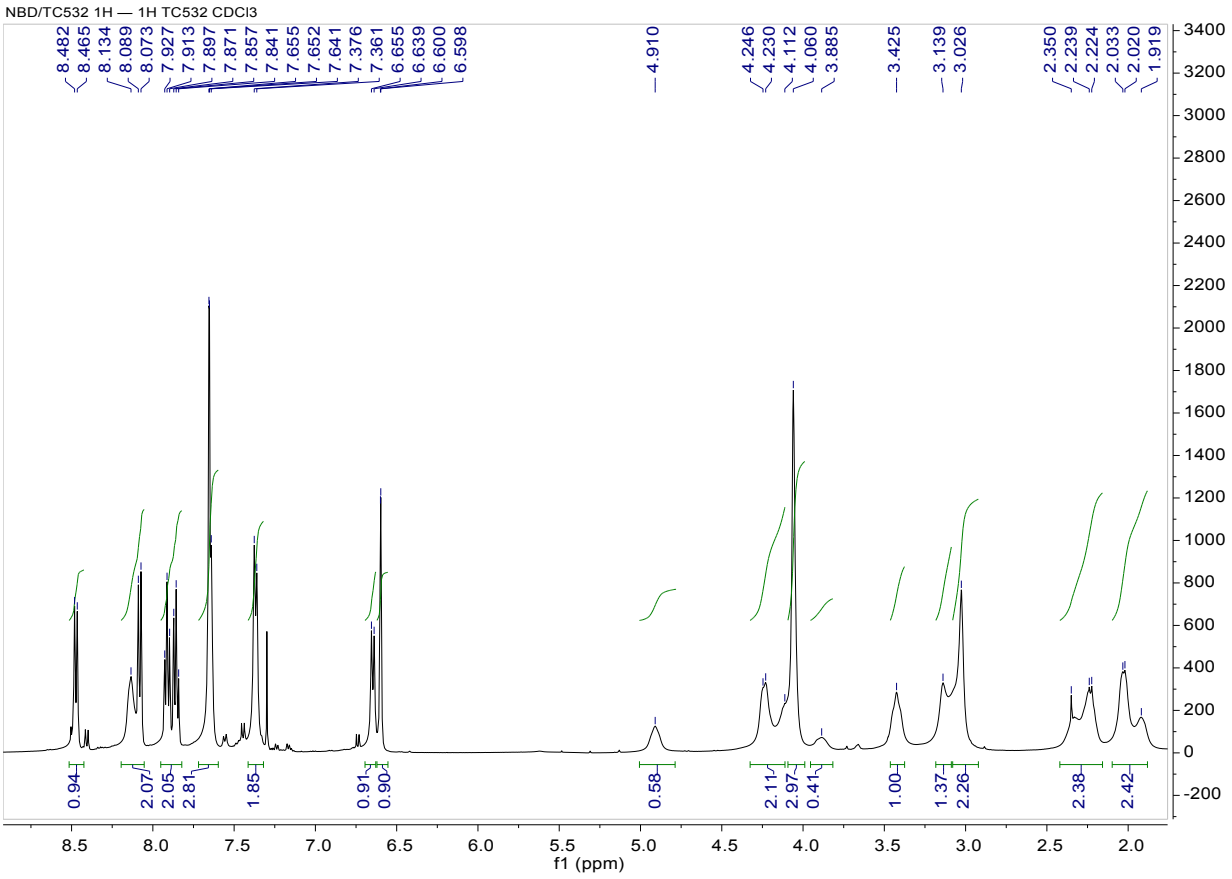

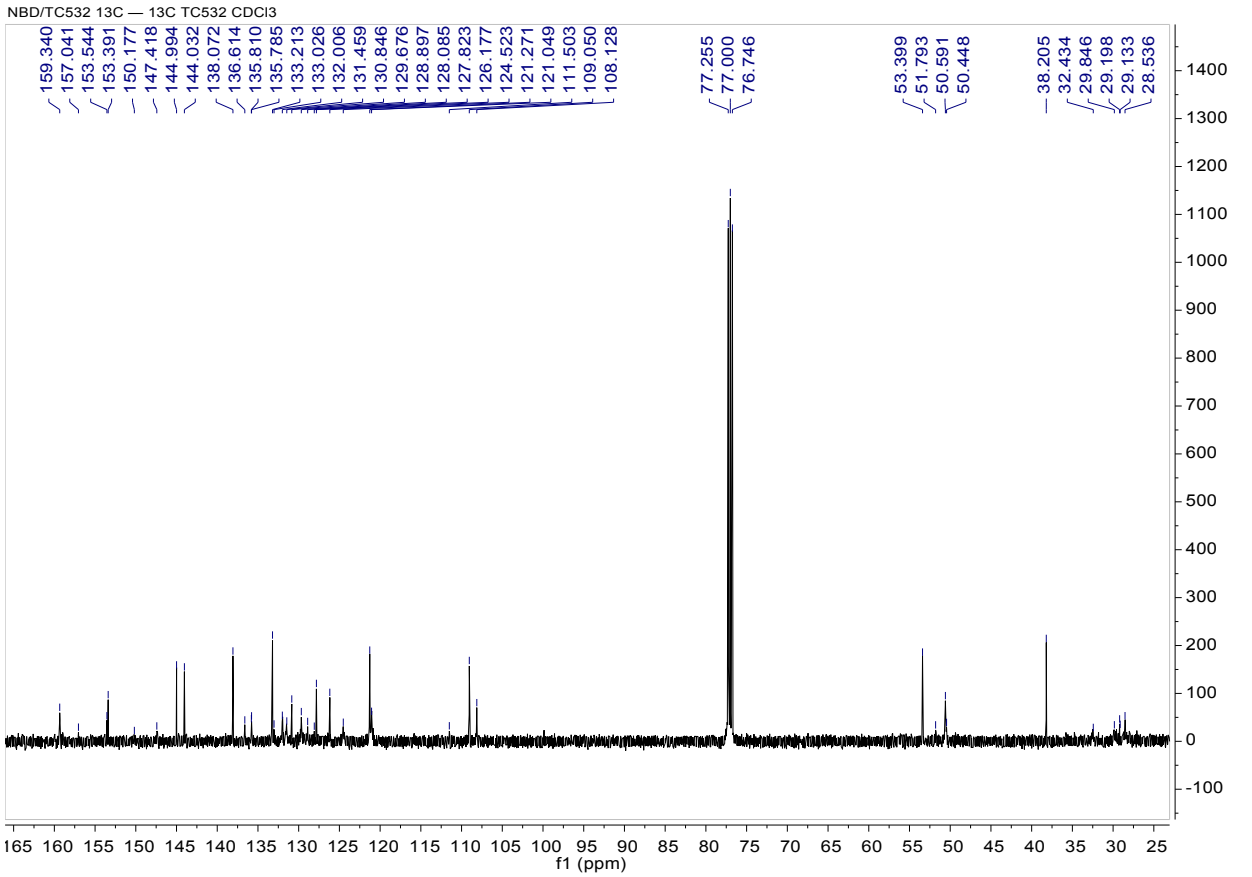
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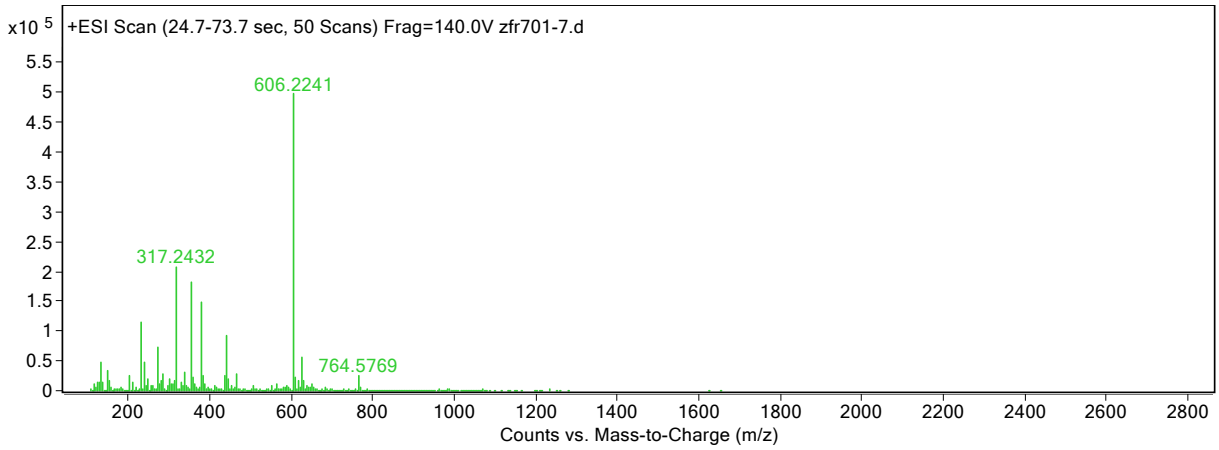
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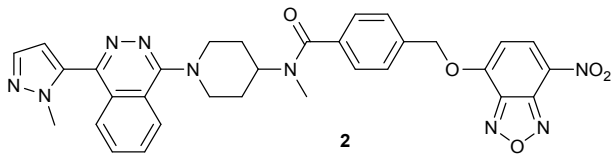
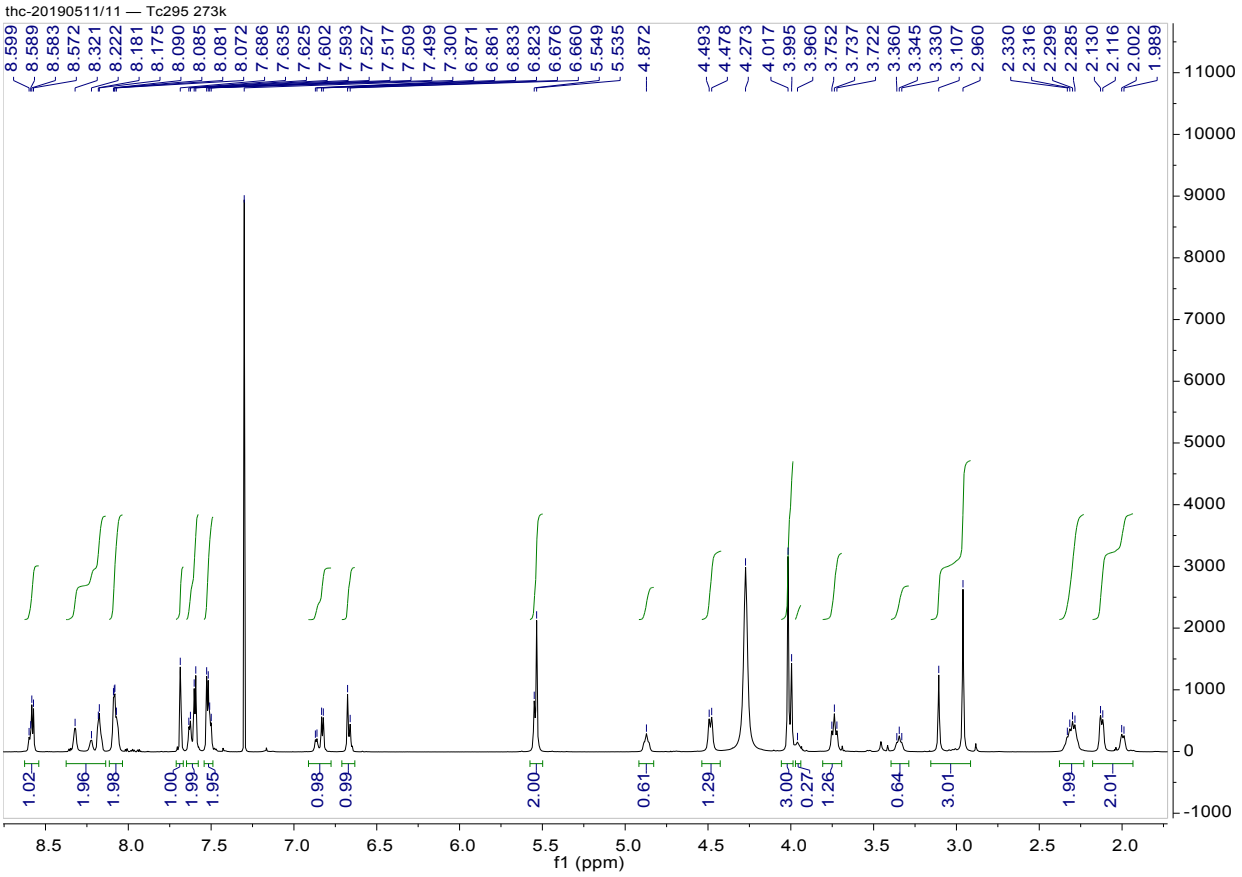
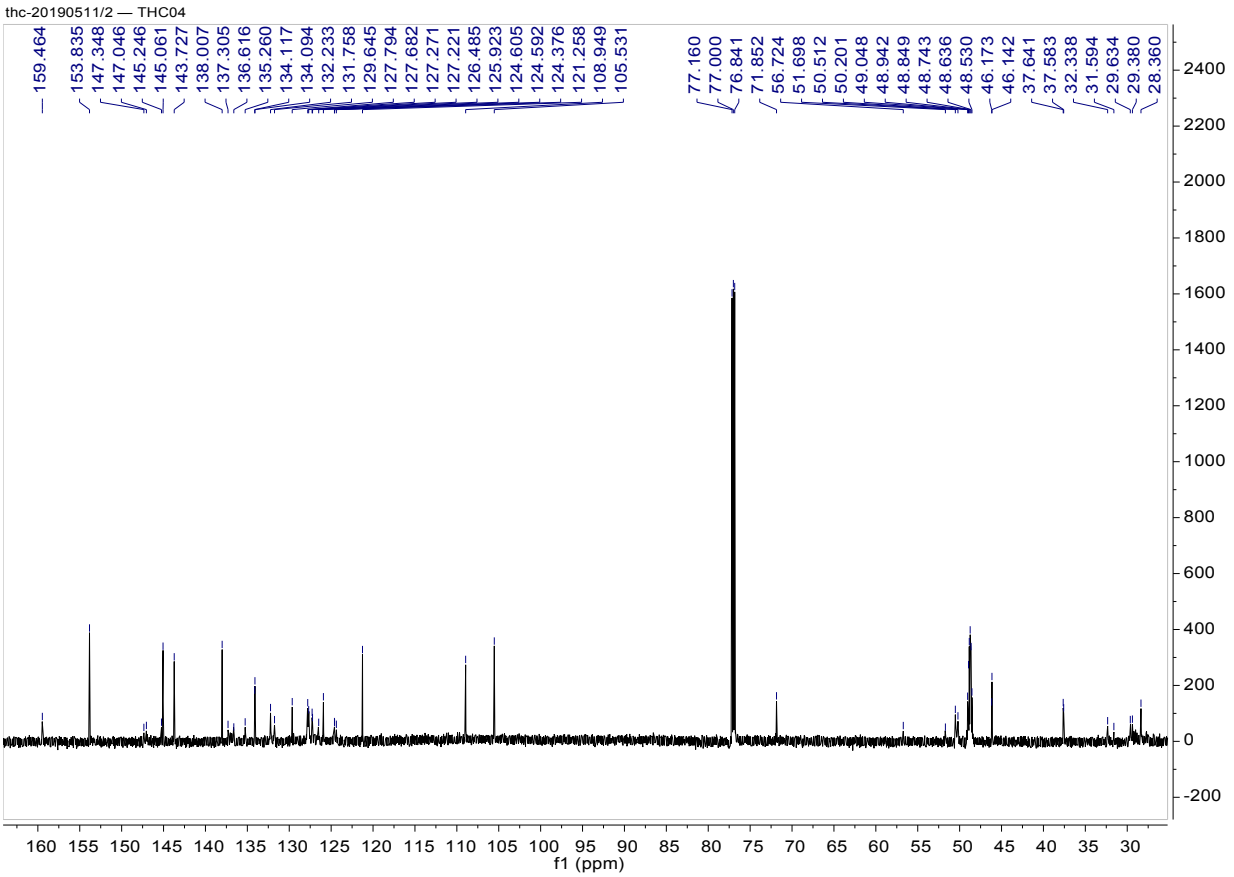
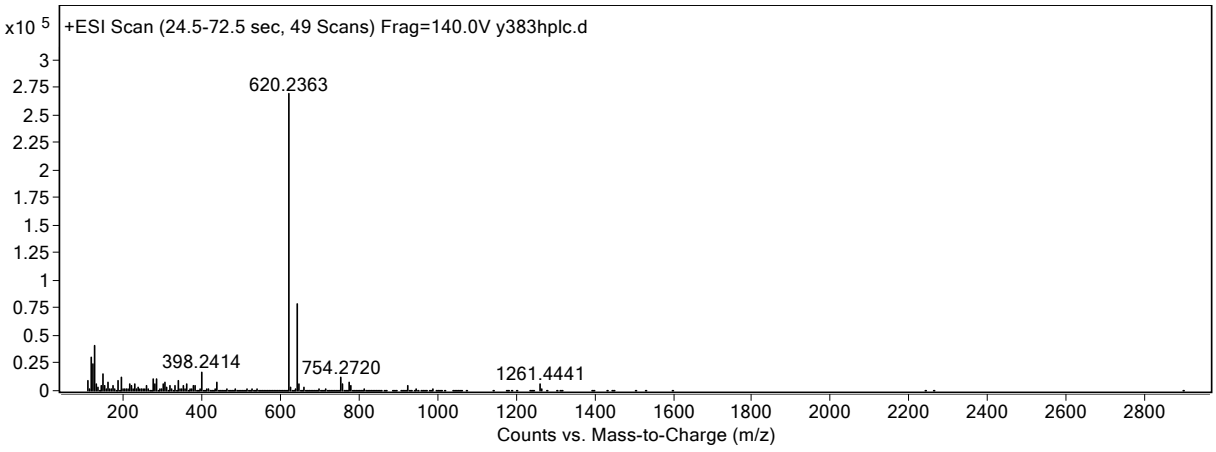
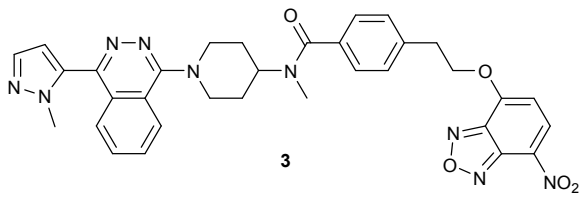


2

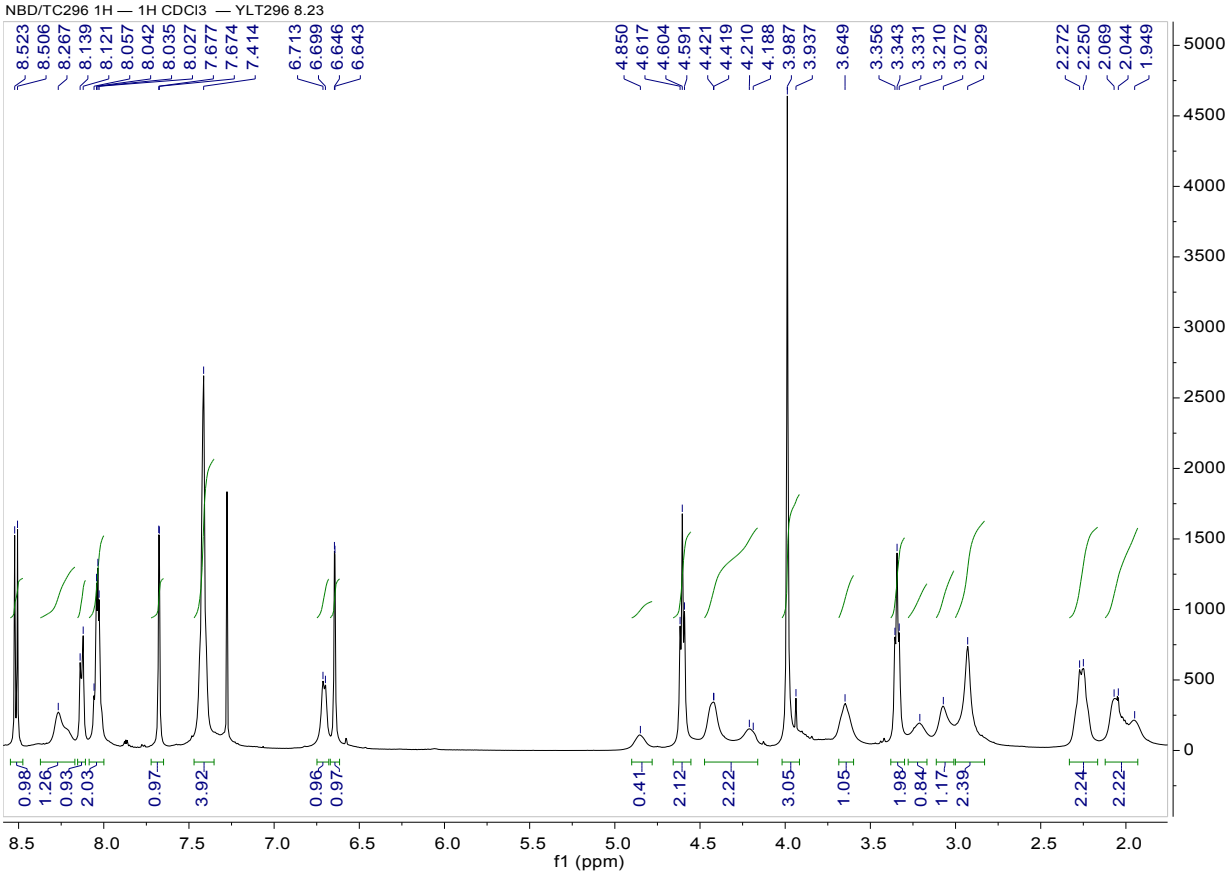

thc-20190511/11 — Tc295 273k
8.599
8.589
8.583
8.572
8.321
8.222
8.181
8.175
8.090
8.085
8.081
8.072
7.686
7.635
7.625
7.602
7.593
7.527
7.517
7.509
7.499
7.300
6.871
6.861
6.833
6.823
6.676
6.660
5.549
5.535
4.872
4.493
4.478
4.273
4.017
3.995
3.960
3.752
3.737
3.722
3.360
3.345
3.330
3.107
2.960
2.330
2.316
2.299
2.285
2.130
2.116
2.002
1.989
1.02
1.96
1.98
1.00
1.99
1.95
0.98
0.99
2.00
0.61
1.29
3.00
0.27
1.26
0.64
3.01
1.99
2.01
f1 (ppm)

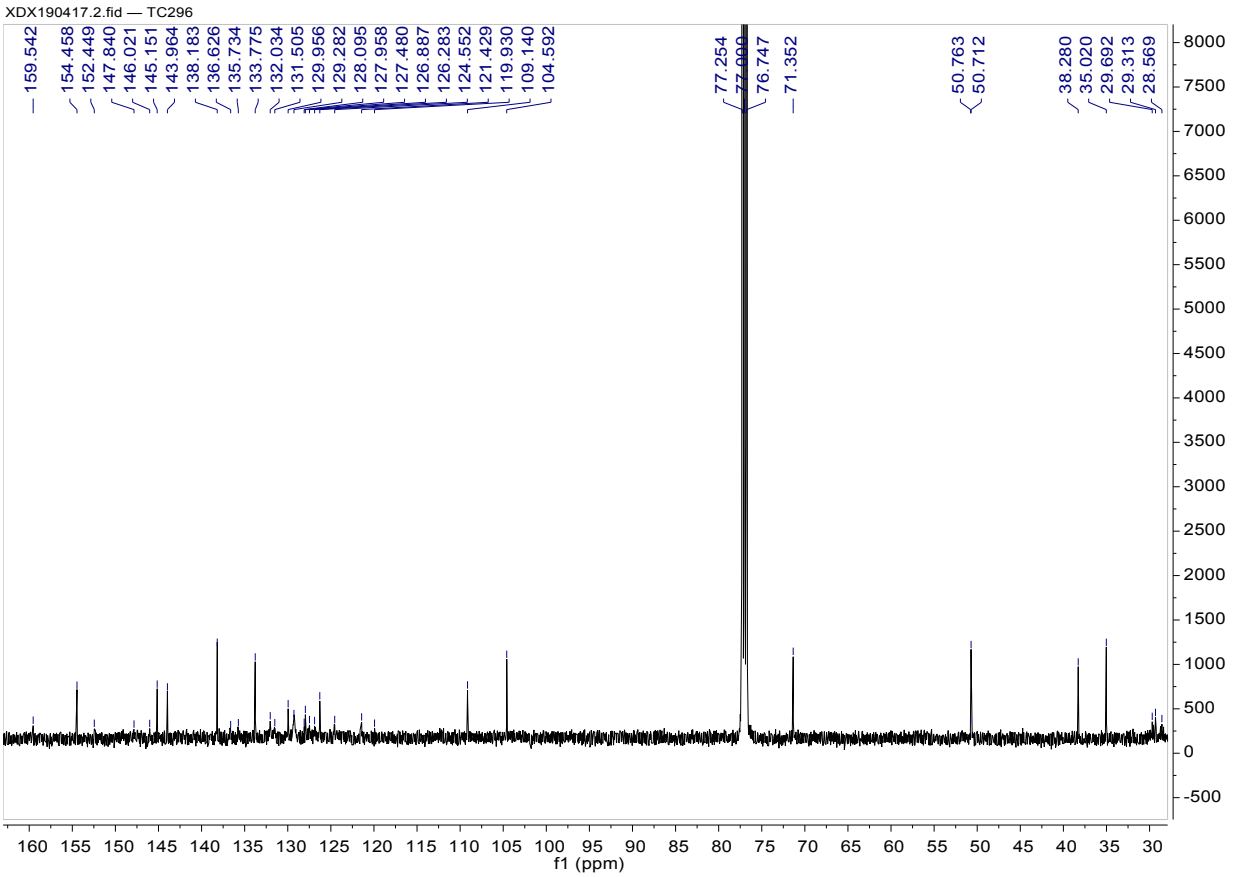

thc-20190511/2 — THC04
159.464
153.835
147.348
147.046
145.246
145.061
143.727
138.007
137.305
136.616
135.260
134.117
134.094
132.233
131.758
129.645
127.794
127.682
127.271
127.221
126.485
125.923
124.605
124.592
124.376
121.258
108.949
105.531
77.160
77.000
76.841
71.852
56.724
51.698
50.512
50.201
49.048
48.942
48.849
48.743
48.636
48.530
46.173
46.142
37.641
37.583
32.338
31.594
29.634
29.380
28.360
f1 (ppm)

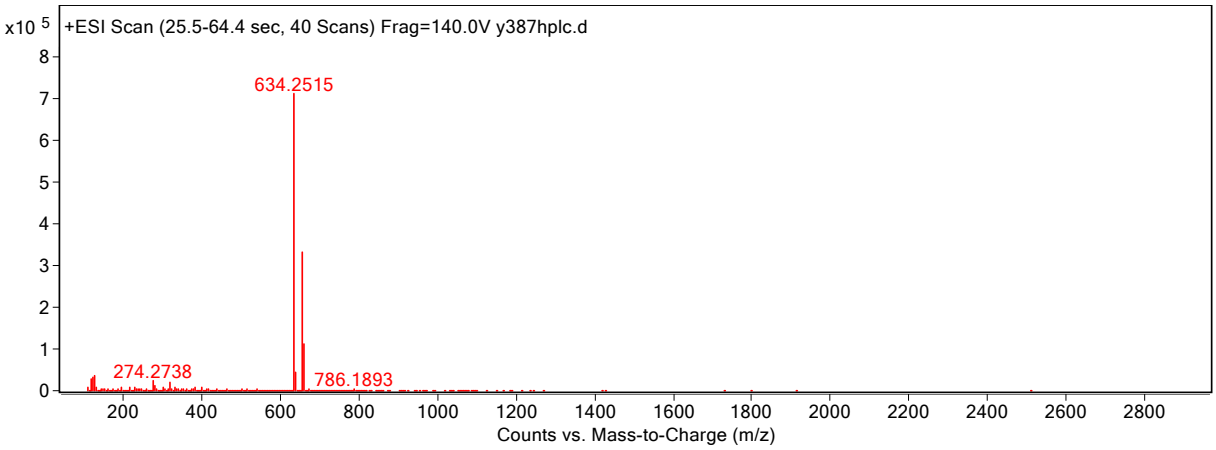

x10 5
+ESI Scan (24.5-72.5 sec, 49 Scans) Frag=140.0V y383hplc.d
620.2363
398.2414
754.2720
1261.4441
Counts vs. Mass-to-Charge (m/z)

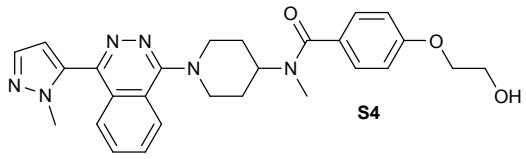


3

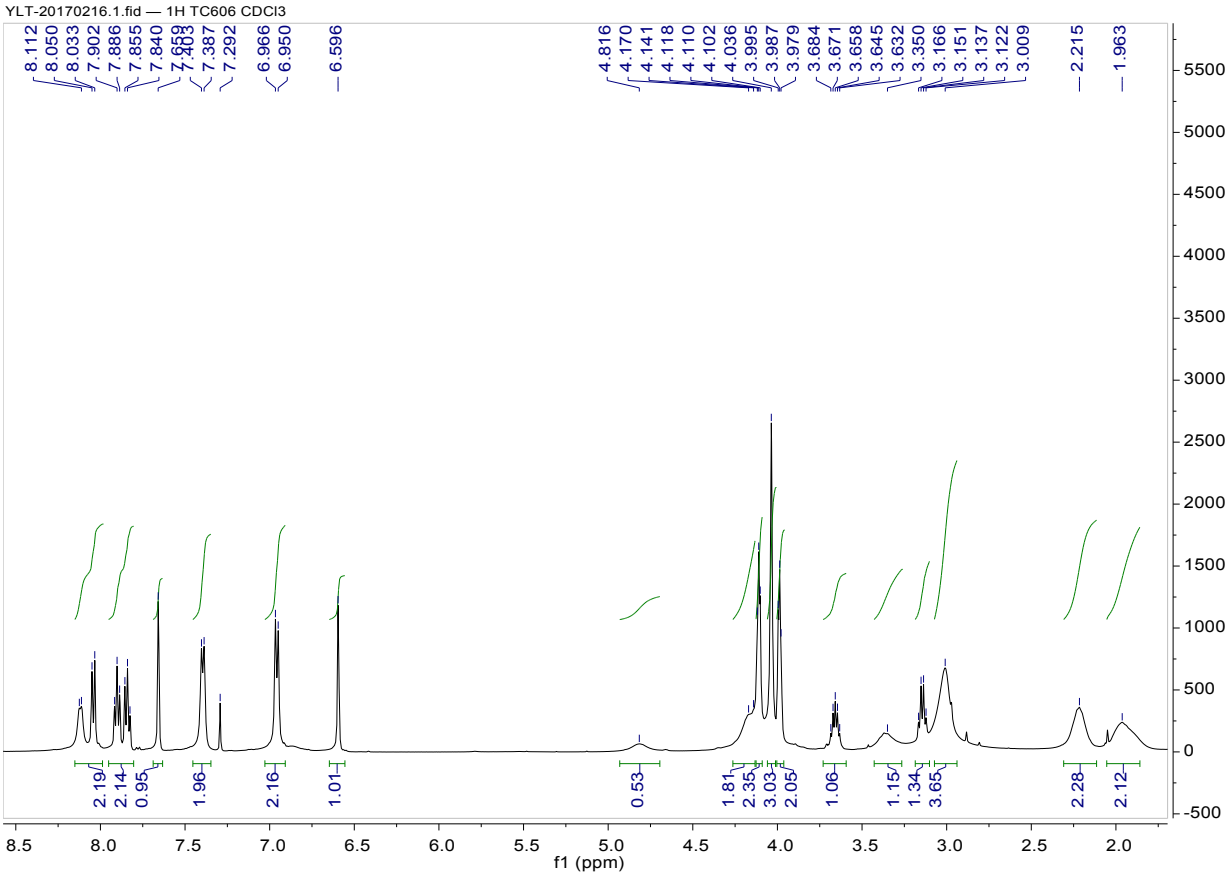

NBD/TC296 1H — 1H CDCl3 — YLT296 8.23
8.523
8.506
8.267
8.139
8.121
8.057
8.042
8.035
8.027
7.677
7.674
7.414
6.713
6.699
6.646
6.643
4.850
4.617
4.604
4.591
4.421
4.419
4.210
4.188
3.987
3.937
3.649
3.356
3.343
3.331
3.210
3.072
2.929
2.272
2.250
2.069
2.044
1.949
0.98
1.26
0.93
2.03
0.97
3.92
0.96
0.97
0.41
2.12
2.22
3.05
1.05
1.98
0.84
1.17
2.39
2.24
2.22
f1 (ppm)

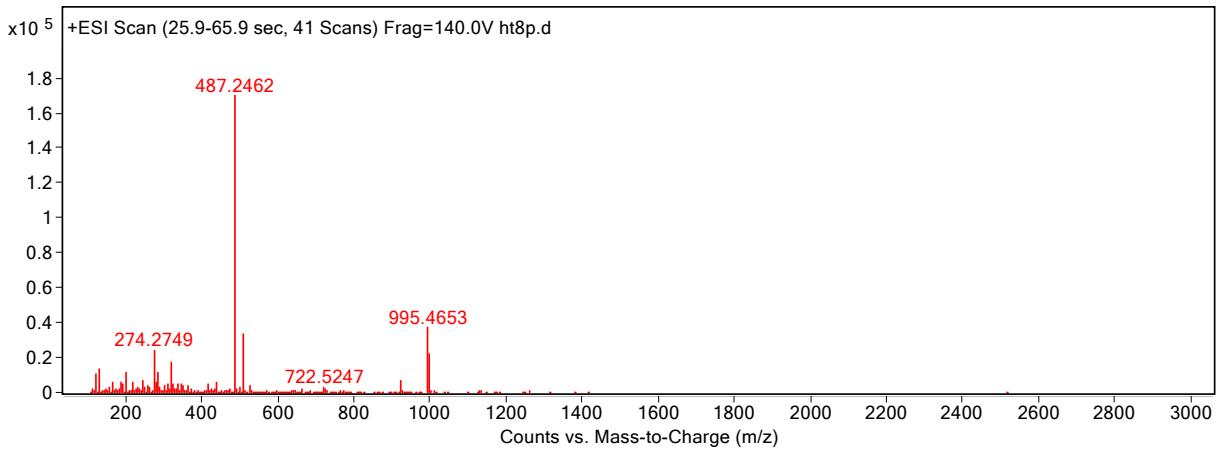

XDX190417.2.fid — TC296
159.542
154.458
152.449
147.840
146.021
145.151
143.964
138.183
136.626
135.734
133.775
132.034
131.505
129.956
129.282
128.095
127.958
127.480
126.887
126.283
124.552
121.429
119.930
109.140
104.592
77.254
77.000
76.747
71.352
50.763
50.712
38.280
35.020
29.692
29.313
28.569
f1 (ppm)

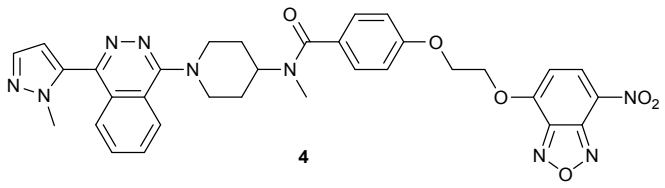

x10 5
+ESI Scan (25.5-64.4 sec, 40 Scans) Frag=140.0V y387hplc.d
634.2515
274.2738
786.1893
Counts vs. Mass-to-Charge (m/z)


O
N
N
N
N
N
O
OH

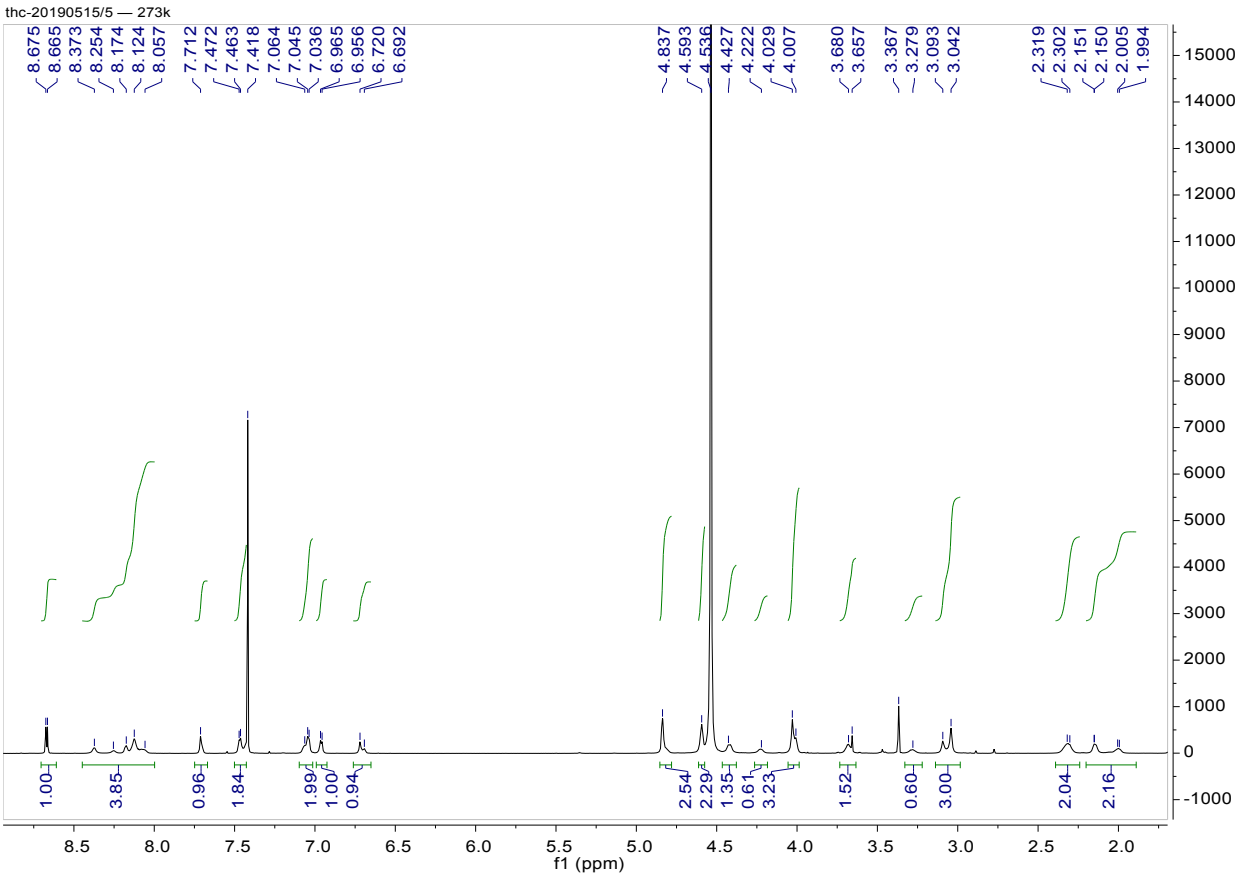
S4

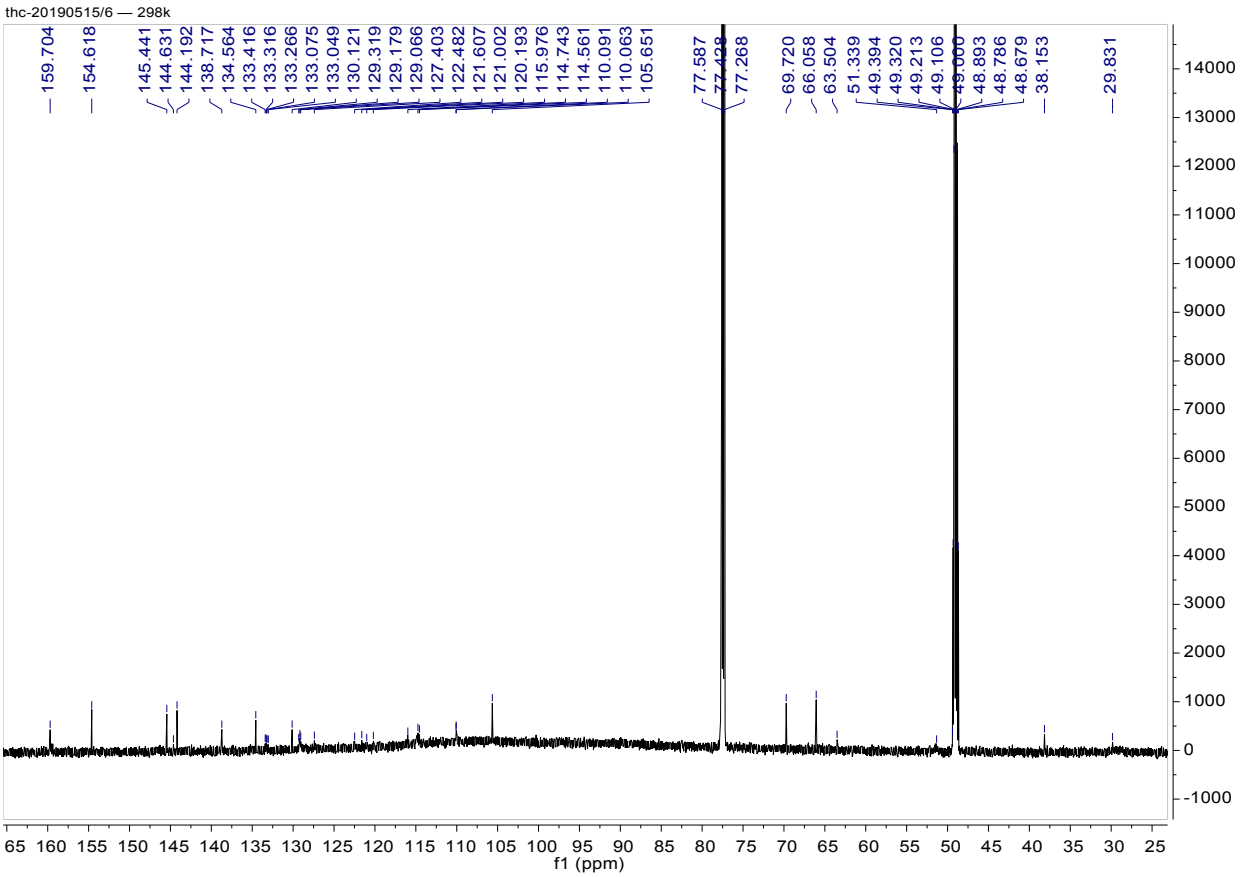

YLT-20170216.1.fid — 1H TC606 CDCl3
8.112
8.050
8.033
7.902
7.886
7.855
7.840
7.659
7.653
7.387
7.292
6.966
6.950
6.596
4.816
4.170
4.141
4.118
4.110
4.102
4.036
3.995
3.987
3.979
3.684
3.671
3.658
3.645
3.632
3.350
3.166
3.151
3.137
3.122
3.009
2.215
1.963
2.19
2.14
0.95
1.96
2.16
1.01
0.53
1.81
2.35
3.03
2.05
1.06
1.15
1.34
3.65
2.28
2.12
f1 (ppm)

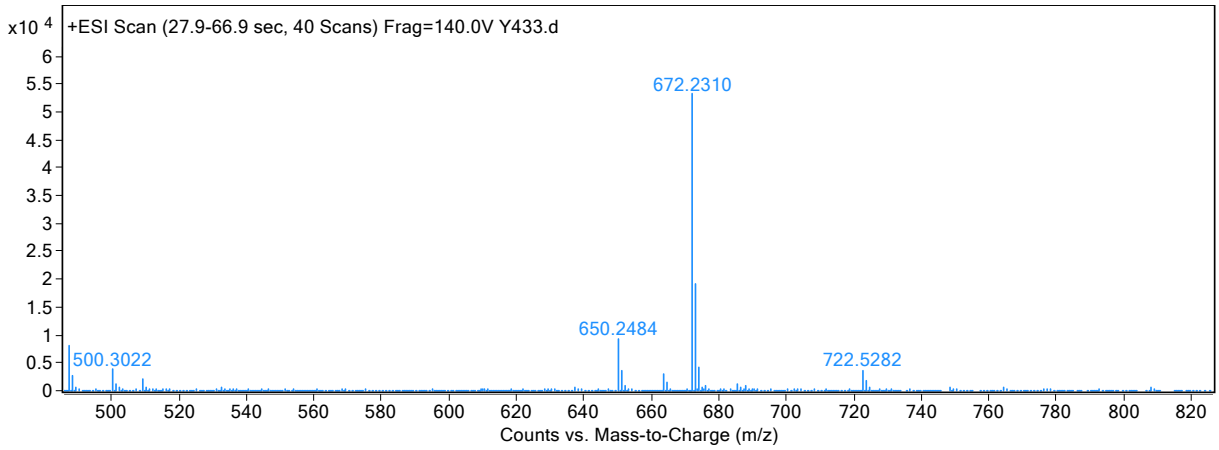

x10 5
+ESI Scan (25.9-65.9 sec, 41 Scans) Frag=140.0V ht8p.d
487.2462
995.4653
274.2749
722.5247
Counts vs. Mass-to-Charge (m/z)

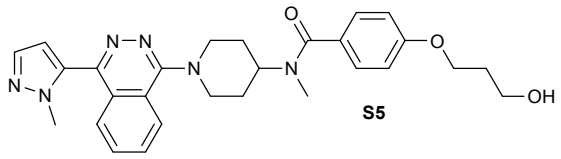


4

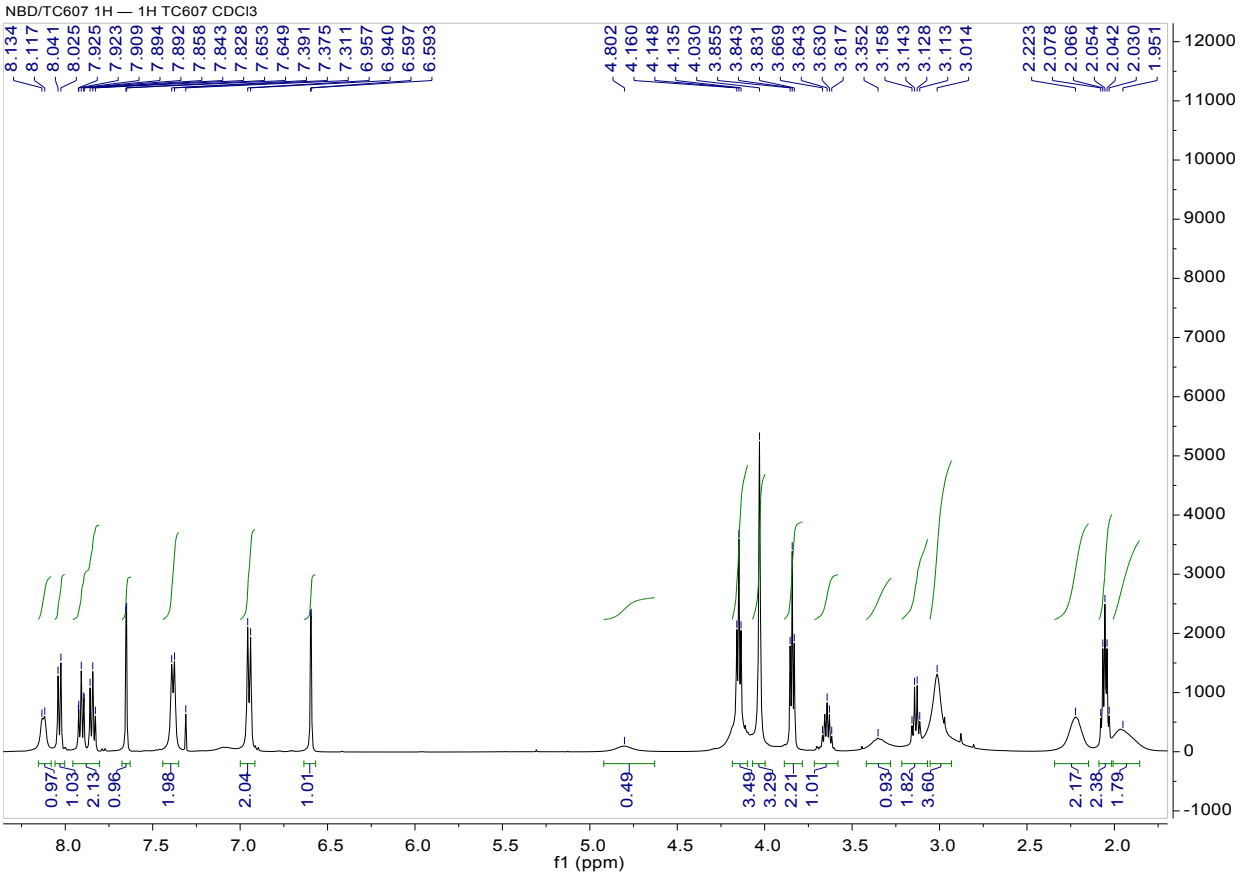

thc-20190515/5 — 273k
8.675
8.665
8.373
8.254
8.174
8.124
8.057
7.712
7.472
7.463
7.418
7.064
7.045
7.036
6.965
6.956
6.720
6.692
4.837
4.593
4.536
4.427
4.222
4.029
4.007
3.680
3.657
3.367
3.279
3.093
3.042
2.319
2.302
2.151
2.150
2.005
1.994
1.00
3.85
0.96
1.84
1.99
1.00
0.94
2.54
2.29
1.35
0.61
3.23
1.52
0.60
3.00
2.04
2.16
f1 (ppm)

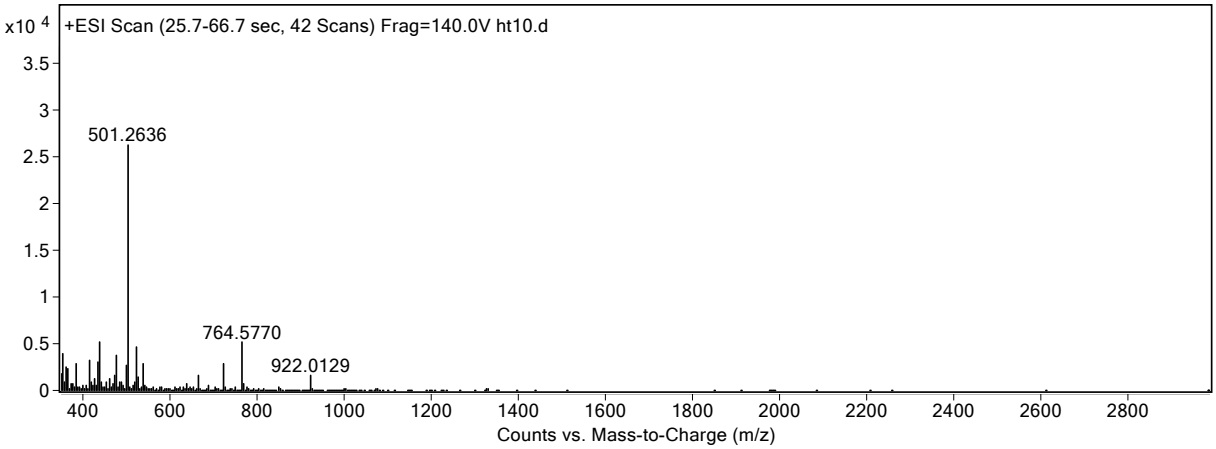

thc-20190515/6 — 298k
159.704
154.618
145.441
144.631
144.192
138.717
134.564
133.416
133.316
133.266
133.075
133.049
130.121
129.319
129.179
129.066
127.403
122.482
121.607
121.002
120.193
115.976
114.743
114.561
110.091
110.063
105.651
77.587
77.428
77.268
69.720
66.058
63.504
51.339
49.394
49.320
49.213
49.106
49.000
48.893
48.786
48.679
38.153
29.831
f1 (ppm)

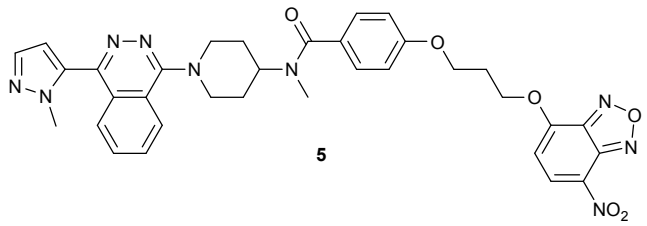

x10 4
+ESI Scan (27.9-66.9 sec, 40 Scans) Frag=140.0V Y433.d
672.2310
650.2484
500.3022
722.5282
Counts vs. Mass-to-Charge (m/z)


O
N
N
N
N
N
O
OH

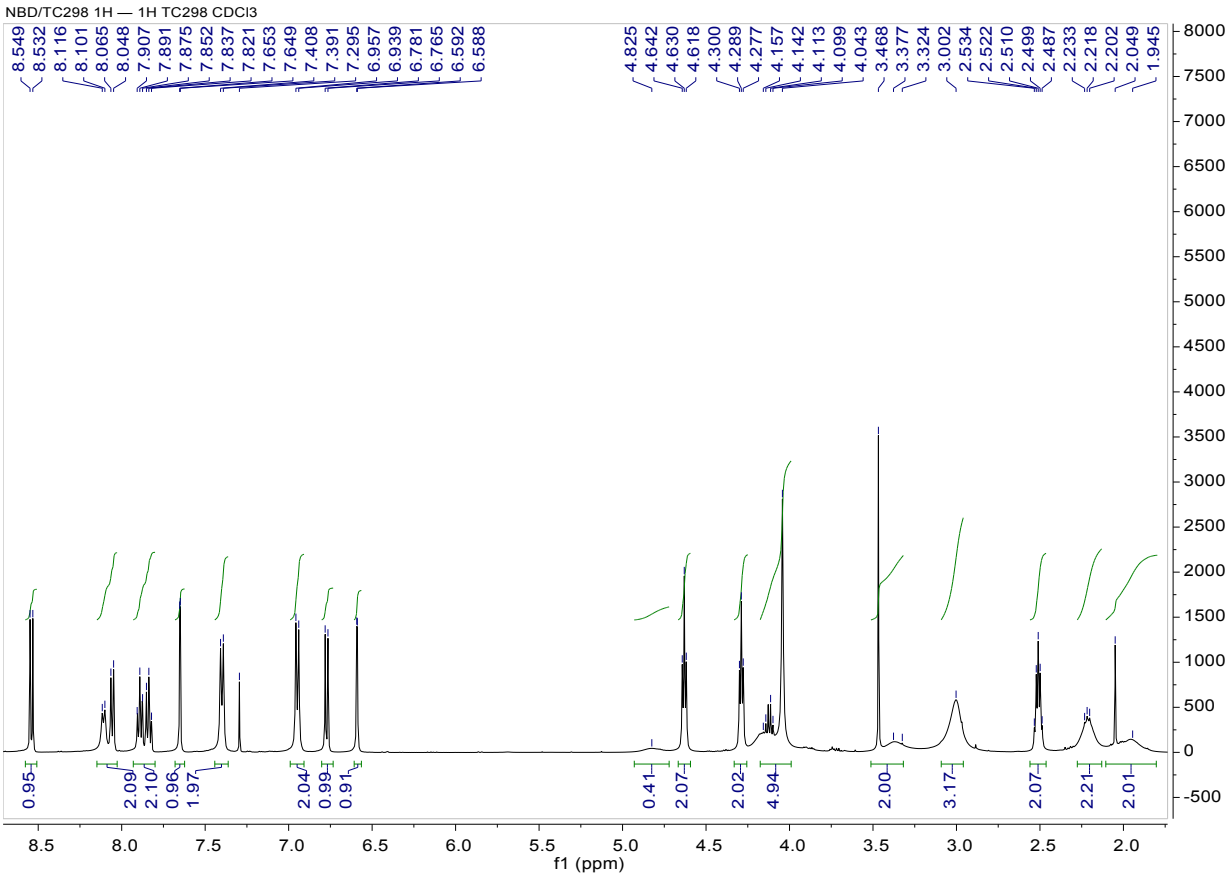
S5

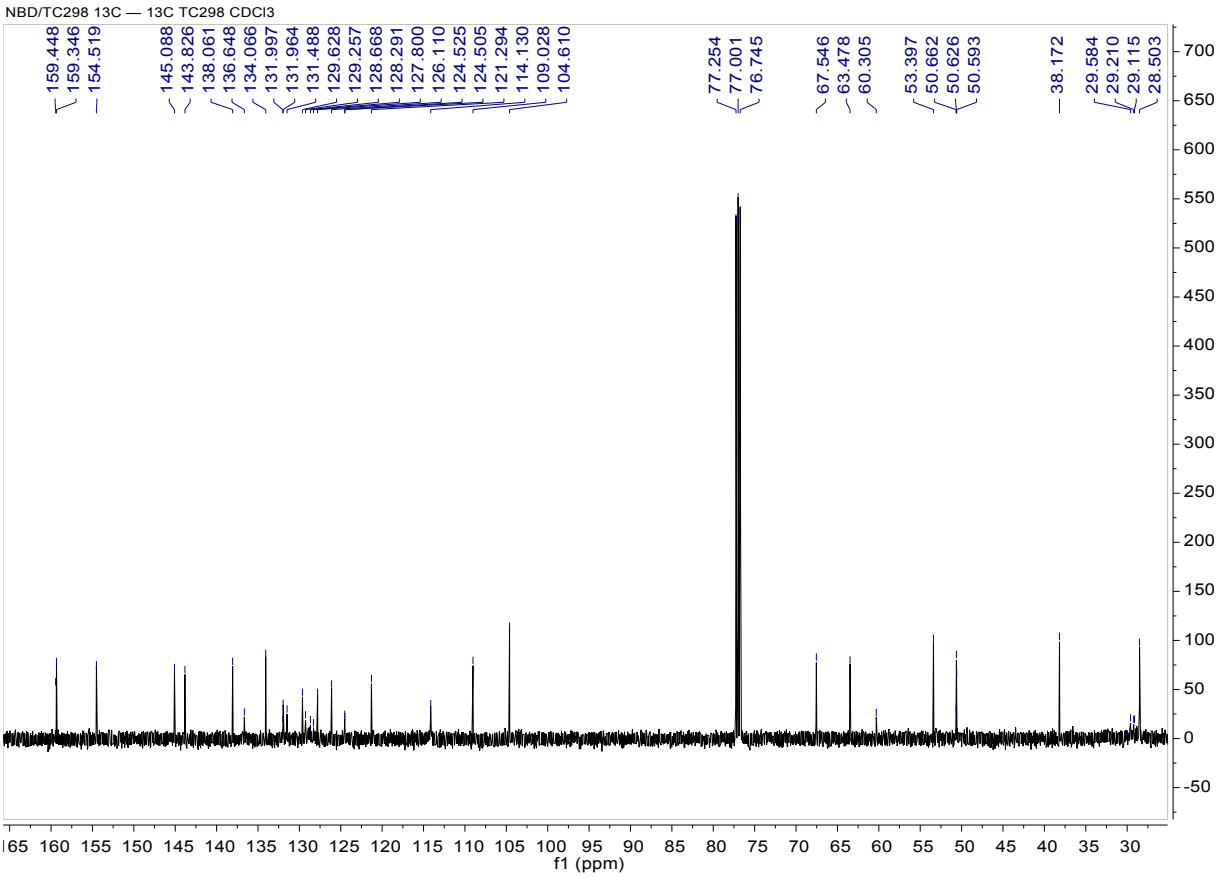

NBD/TC607 1H — 1H TC607 CDCl3
8.134
8.117
8.041
8.025
7.925
7.923
7.909
7.894
7.892
7.858
7.843
7.828
7.653
7.649
7.391
7.375
7.311
6.957
6.940
6.597
6.593
4.802
4.160
4.148
4.135
4.030
3.855
3.843
3.831
3.669
3.643
3.630
3.617
3.352
3.158
3.143
3.128
3.113
3.014
2.223
2.078
2.066
2.054
2.042
2.030
1.951
0.97
1.03
2.13
0.96
1.98
2.04
1.01
0.49
3.49
3.29
2.21
1.01
0.93
1.82
3.60
2.17
2.38
1.79
f1 (ppm)

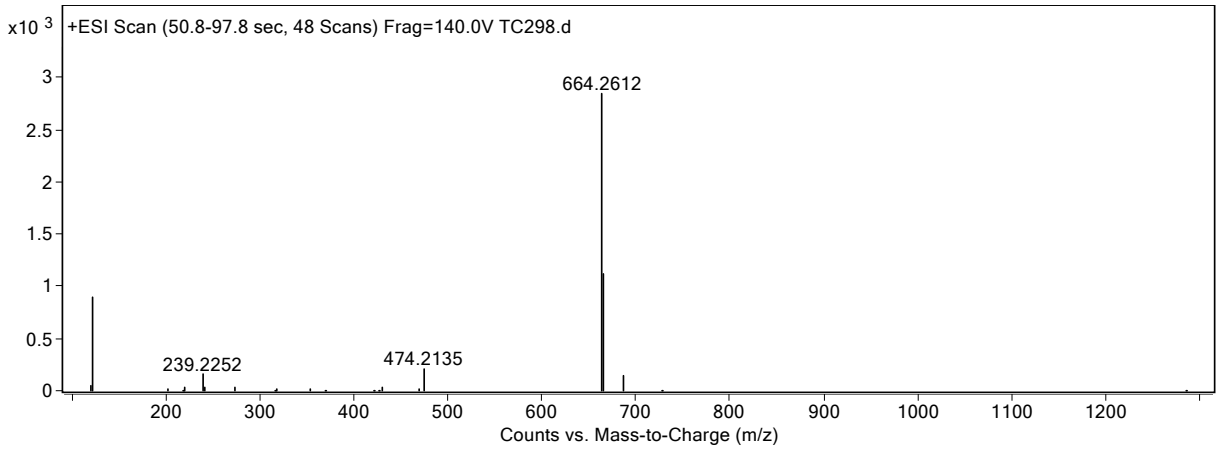

x10 4
+ESI Scan (25.7-66.7 sec, 42 Scans) Frag=140.0V ht10.d
501.2636
764.5770
922.0129
Counts vs. Mass-to-Charge (m/z)

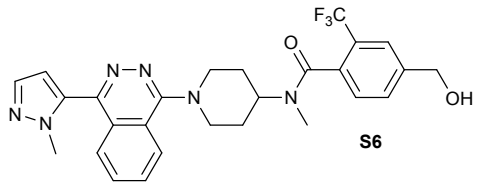


5

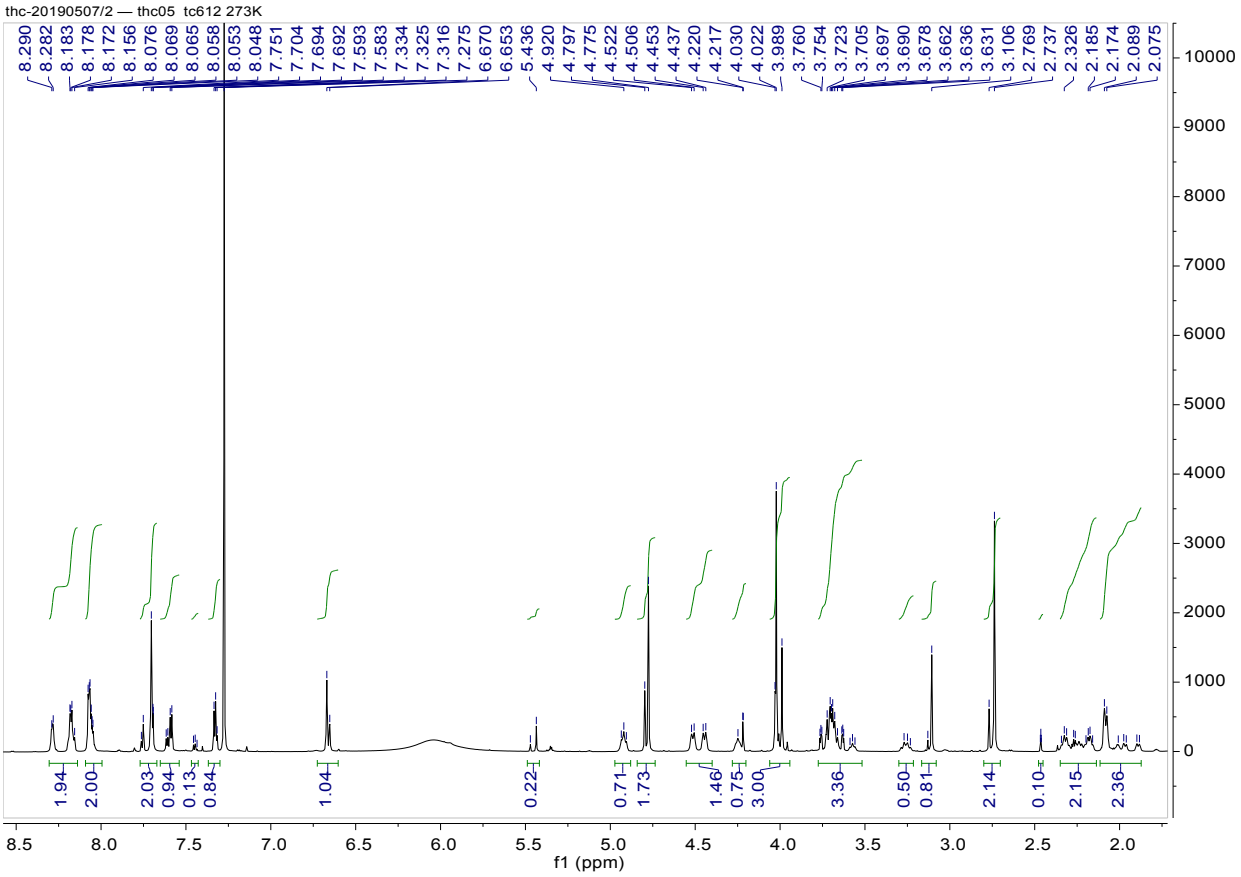

NBD/TC298 1H — 1H TC298 CDCl3
8.549
8.532
8.116
8.101
8.065
8.048
7.907
7.891
7.875
7.852
7.837
7.821
7.653
7.649
7.408
7.391
7.295
6.957
6.939
6.781
6.765
6.592
6.588
4.825
4.642
4.630
4.618
4.300
4.289
4.277
4.157
4.142
4.113
4.099
4.043
3.468
3.377
3.324
3.002
2.534
2.522
2.510
2.499
2.487
2.233
2.218
2.202
2.049
1.945
0.95
2.09
2.10
0.96
1.97
2.04
0.99
0.91
0.41
2.07
2.02
4.94
2.00
3.17
2.07
2.21
2.01
f1 (ppm)

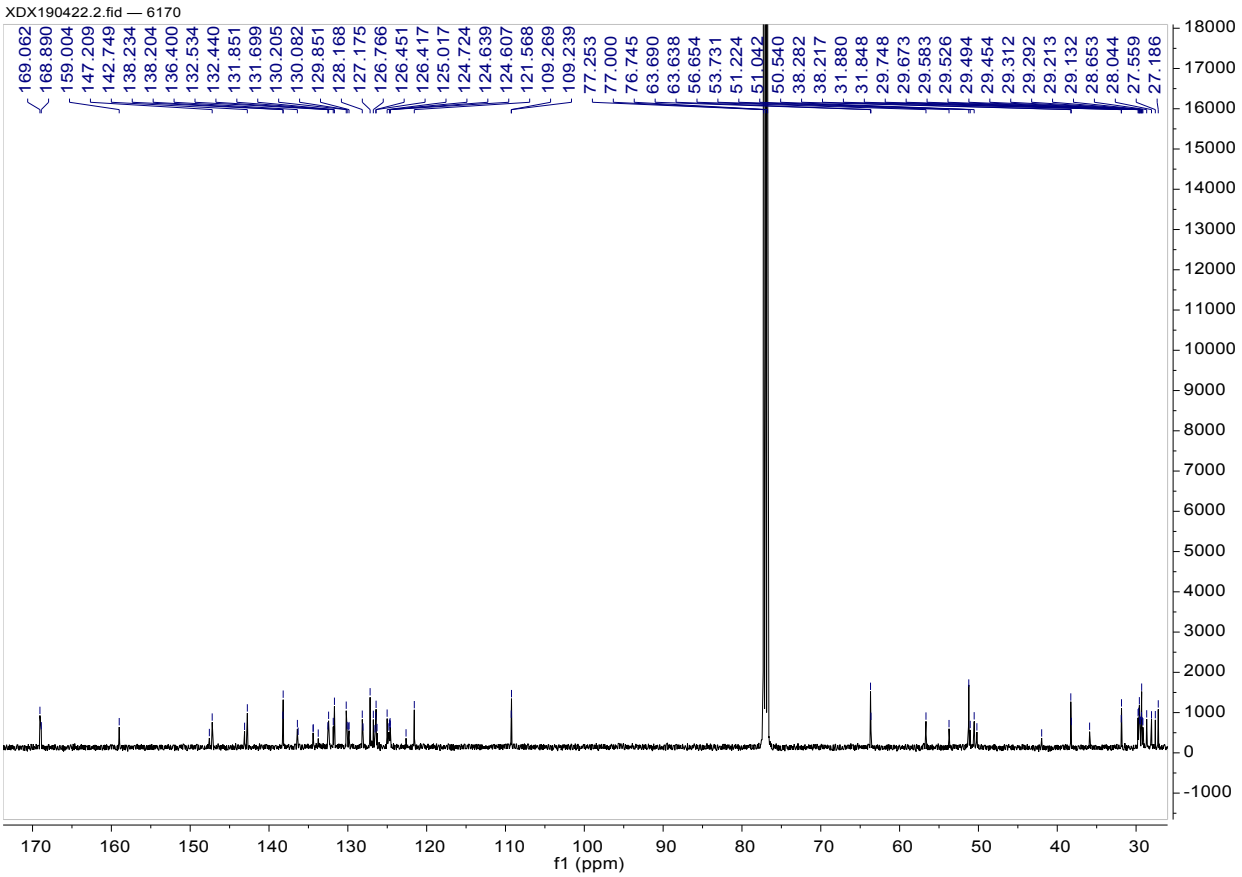

NBD/TC298 13C — 13C TC298 CDCl3
159.448
159.346
154.519
145.088
143.826
138.061
136.648
134.066
131.997
131.964
131.488
129.628
129.257
128.668
128.291
127.800
126.110
124.525
124.505
121.294
114.130
109.028
104.610
77.254
77.001
76.745
67.546
63.478
60.305
53.397
50.662
50.626
50.593
38.172
29.584
29.210
29.115
28.503
f1 (ppm)

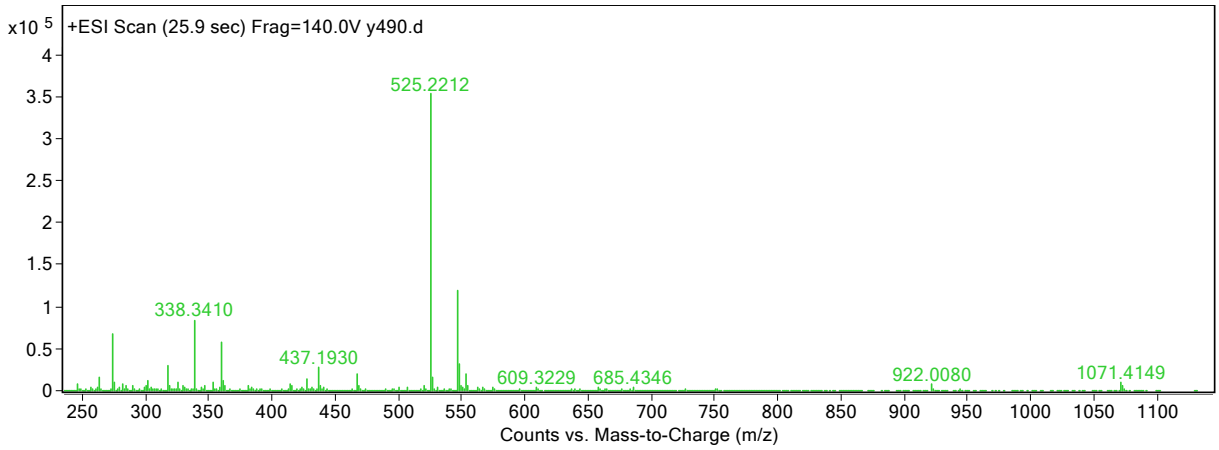

x10 3
+ESI Scan (50.8-97.8 sec, 48 Scans) Frag=140.0V TC298.d
664.2612
239.2252
474.2135
Counts vs. Mass-to-Charge (m/z)

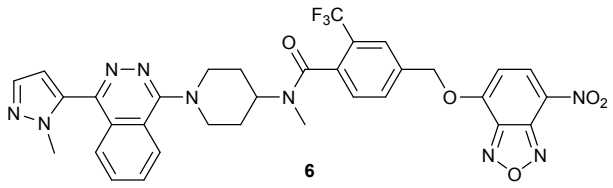

F3C
O
N–N
N
N
N
N
OH

S6

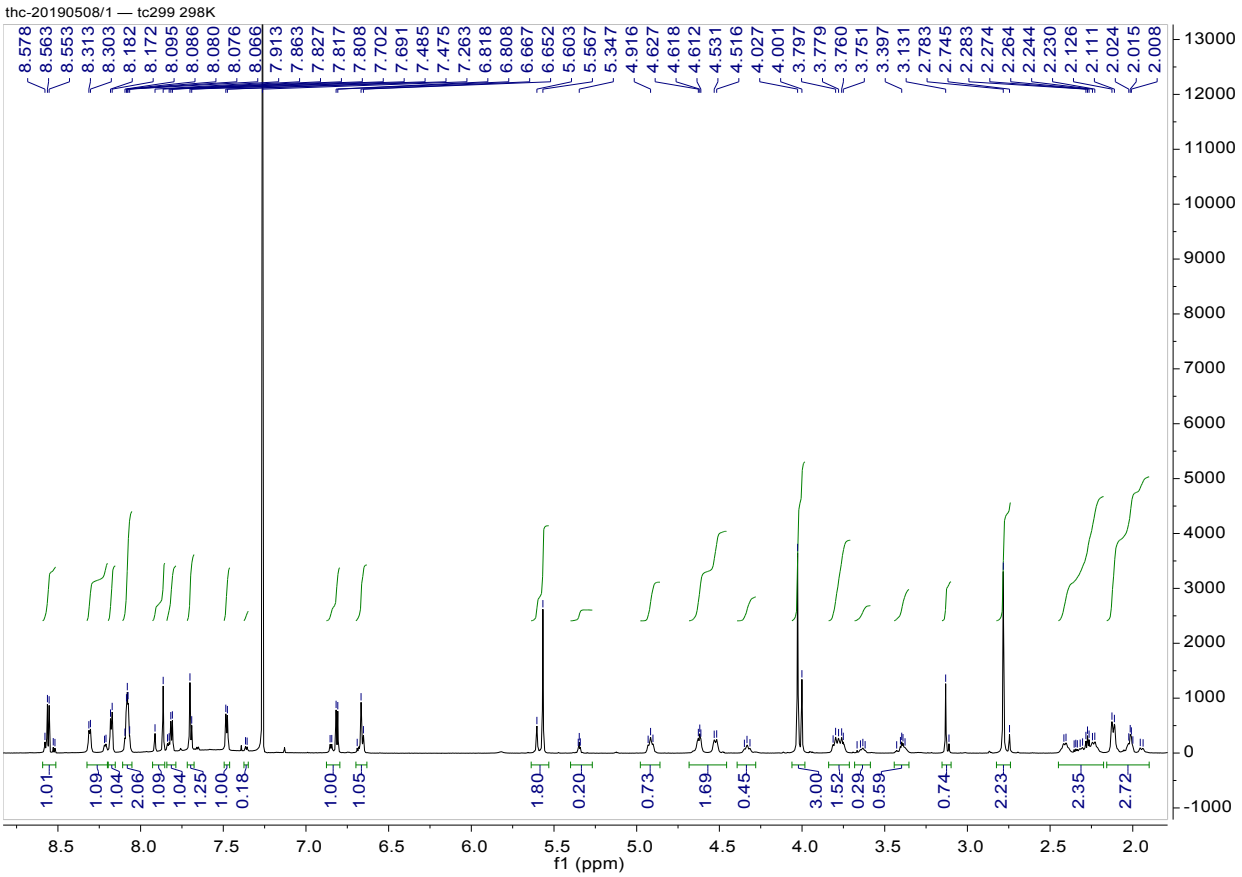

thc-20190507/2 — thc05 tc612 273K
8.290
8.282
8.183
8.178
8.172
8.156
8.076
8.069
8.065
8.058
8.053
8.048
7.751
7.704
7.694
7.692
7.593
7.583
7.334
7.325
7.316
7.275
6.670
6.653
5.436
4.920
4.797
4.775
4.522
4.506
4.453
4.437
4.220
4.217
4.030
4.022
3.989
3.760
3.754
3.723
3.705
3.697
3.690
3.678
3.662
3.636
3.631
3.106
2.769
2.737
2.326
2.185
2.174
2.089
2.075
1.94
2.00
2.03
0.94
0.13
0.84
1.04
0.22
0.71
1.73
1.46
0.75
3.00
3.36
0.50
0.81
2.14
0.10
2.15
2.36
f1 (ppm)

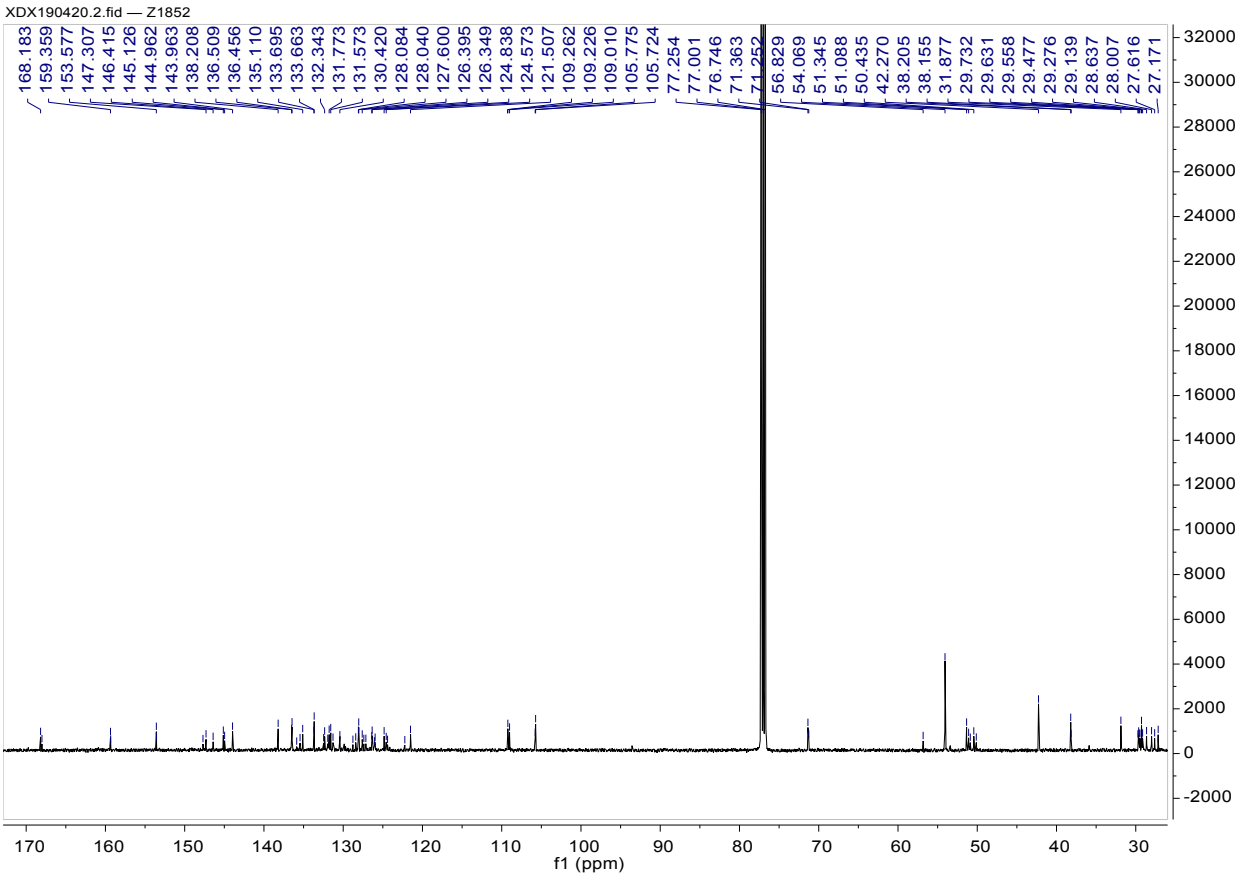

XDX190422.2.fid — 6170
169.062
168.890
159.004
147.209
142.749
138.234
138.204
136.400
132.534
132.440
131.851
131.699
130.205
130.082
129.851
128.168
127.175
126.766
126.451
126.417
125.017
124.724
124.639
124.607
121.568
109.269
109.239
77.253
77.000
76.745
63.690
63.638
56.654
53.731
51.224
51.042
50.540
38.282
38.217
31.880
31.848
29.748
29.673
29.583
29.526
29.494
29.454
29.312
29.292
29.213
29.132
28.653
28.044
27.559
27.186
18000
17000
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
f1 (ppm)

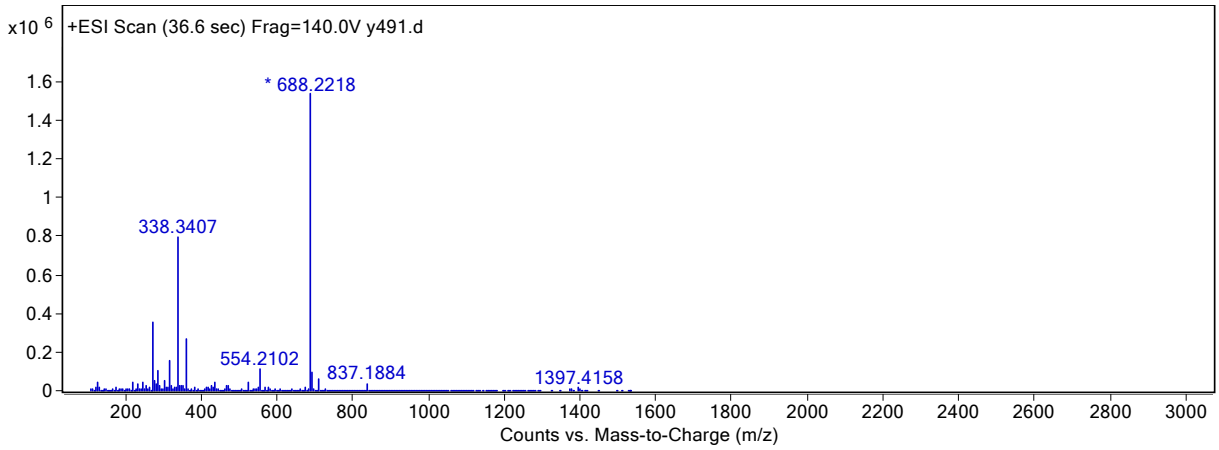

x10 5
+ESI Scan (25.9 sec) Frag=140.0V y490.d
4
3.5
3
2.5
2
1.5
1
0.5
0
525.2212
338.3410
437.1930
609.3229
685.4346
922.0080
1071.4149
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
1050
1100
Counts vs. Mass-to-Charge (m/z)

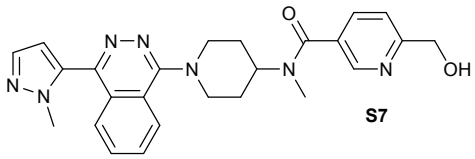

F3C
O
N
N
N
N
N
O
NO2
N
O
N

6

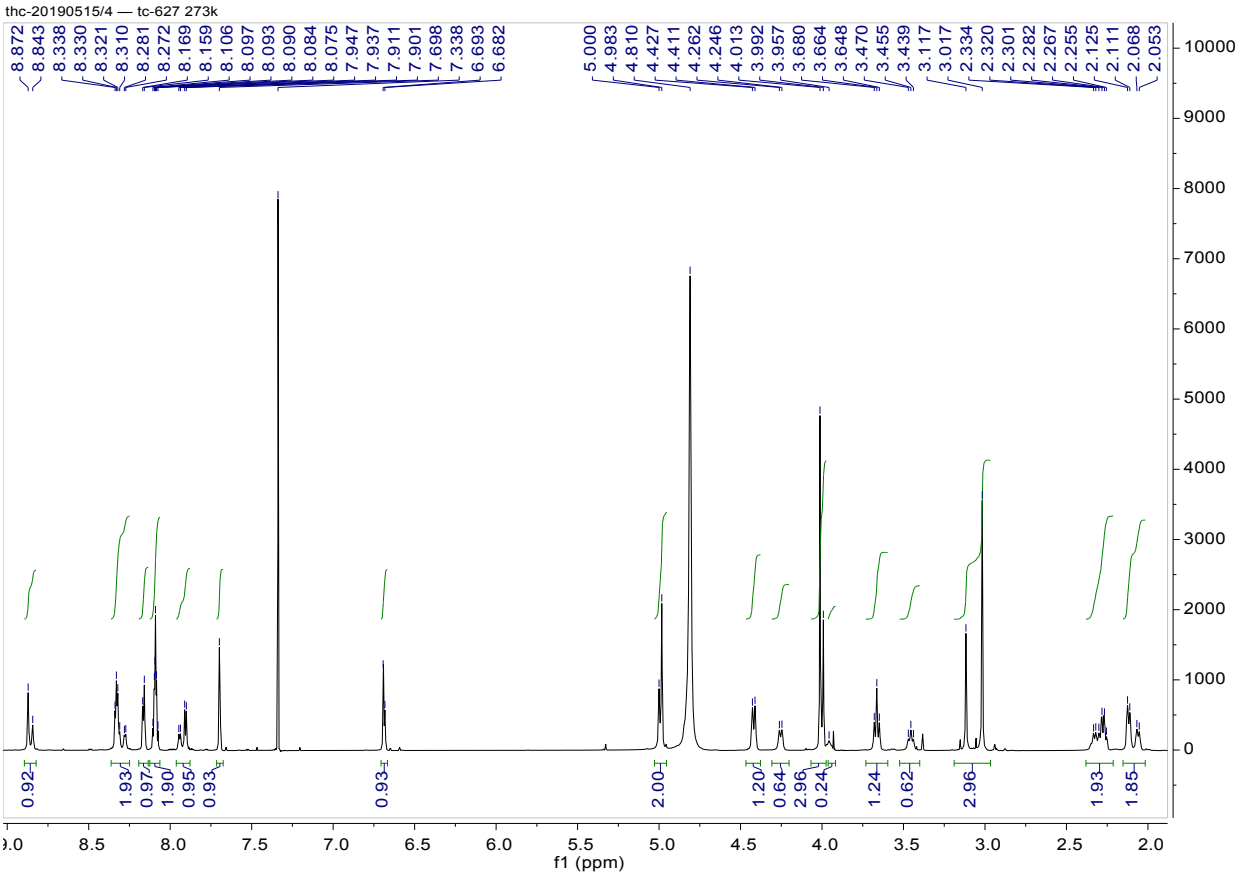

thc-20190508/1 — tc299 298K
8.578
8.563
8.553
8.313
8.303
8.182
8.172
8.095
8.086
8.080
8.076
8.066
7.913
7.863
7.827
7.817
7.808
7.702
7.691
7.485
7.475
7.263
6.818
6.808
6.667
6.652
5.603
5.567
5.347
4.916
4.627
4.618
4.612
4.531
4.516
4.027
4.001
3.797
3.779
3.760
3.751
3.397
3.131
2.783
2.745
2.283
2.274
2.264
2.244
2.230
2.126
2.111
2.024
2.015
2.008
1.01
1.09
1.04
2.06
1.09
1.04
1.25
1.00
0.18
1.00
1.05
1.80
0.20
0.73
1.69
0.45
3.00
1.52
0.29
0.59
0.74
2.23
2.35
2.72
f1 (ppm)

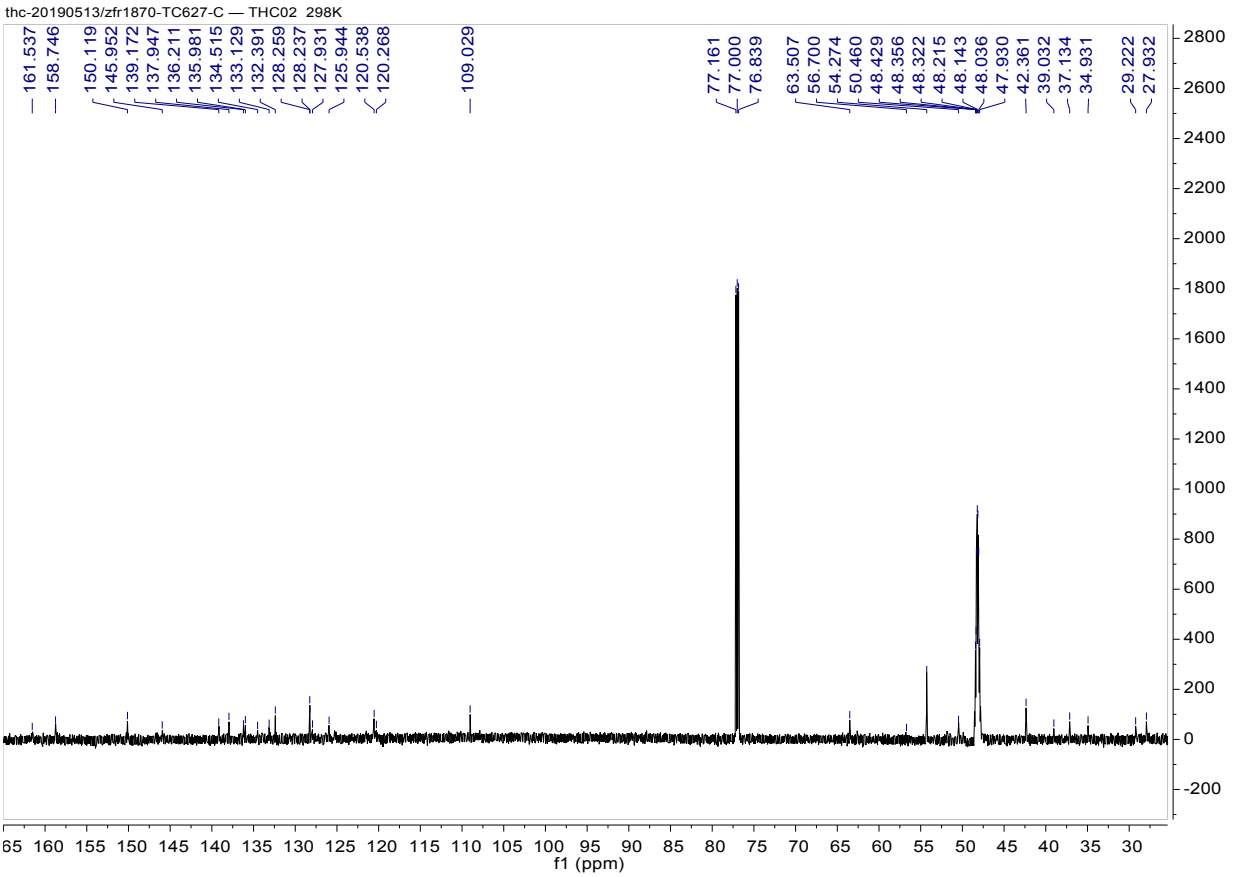

XDX190420.2.fid — Z1852
168.183
159.359
153.577
147.307
146.415
145.126
144.962
143.963
138.208
136.509
136.456
135.110
133.695
133.663
132.343
131.773
131.573
130.420
128.084
128.040
127.600
126.395
126.349
124.838
124.573
121.507
109.262
109.226
109.010
105.775
105.724
77.254
77.001
76.746
71.363
71.252
56.829
54.069
51.345
51.088
50.435
42.270
38.205
38.155
31.877
29.732
29.631
29.558
29.477
29.276
29.139
28.637
28.007
27.616
27.171
32000
30000
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
-2000
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
f1 (ppm)

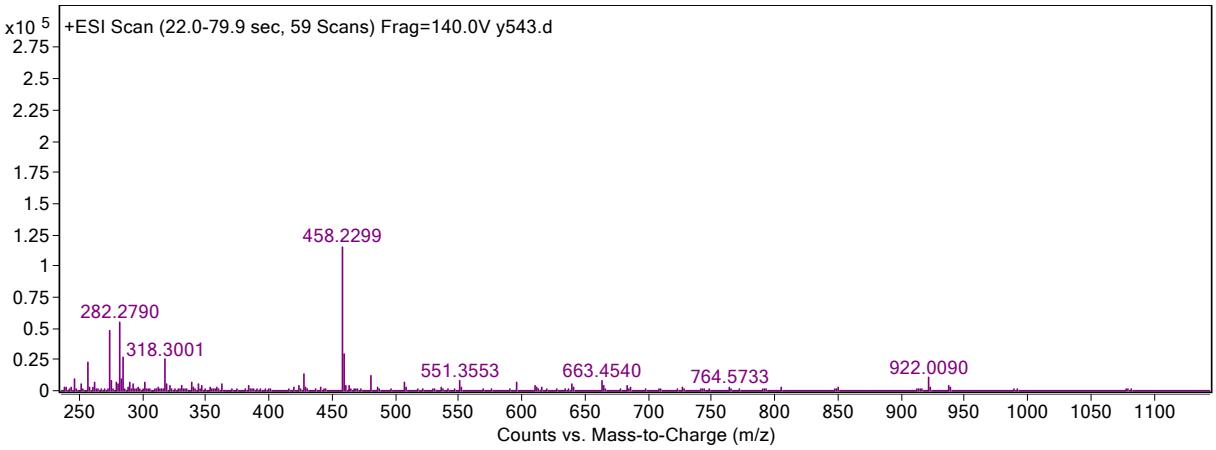

x10 6
+ESI Scan (36.6 sec) Frag=140.0V y491.d
* 688.2218
338.3407
554.2102
837.1884
1397.4158
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
3000
Counts vs. Mass-to-Charge (m/z)

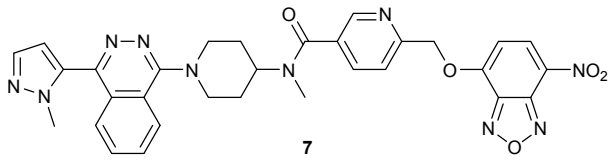

O
N
N
N
N
N
N
OH

S7


thc-20190515/4 — tc-627 273k
8.872
8.843
8.338
8.330
8.321
8.310
8.281
8.272
8.169
8.159
8.106
8.097
8.093
8.090
8.084
8.075
7.947
7.937
7.911
7.901
7.698
7.338
6.693
6.682
5.000
4.983
4.810
4.427
4.411
4.262
4.246
4.013
3.992
3.957
3.680
3.664
3.648
3.470
3.455
3.439
3.117
3.017
2.334
2.320
2.301
2.282
2.267
2.255
2.125
2.111
2.068
2.053
0.92
1.93
0.97
1.90
0.95
0.93
0.93
2.00
1.20
0.64
2.96
0.24
1.24
0.62
2.96
1.93
1.85
f1 (ppm)

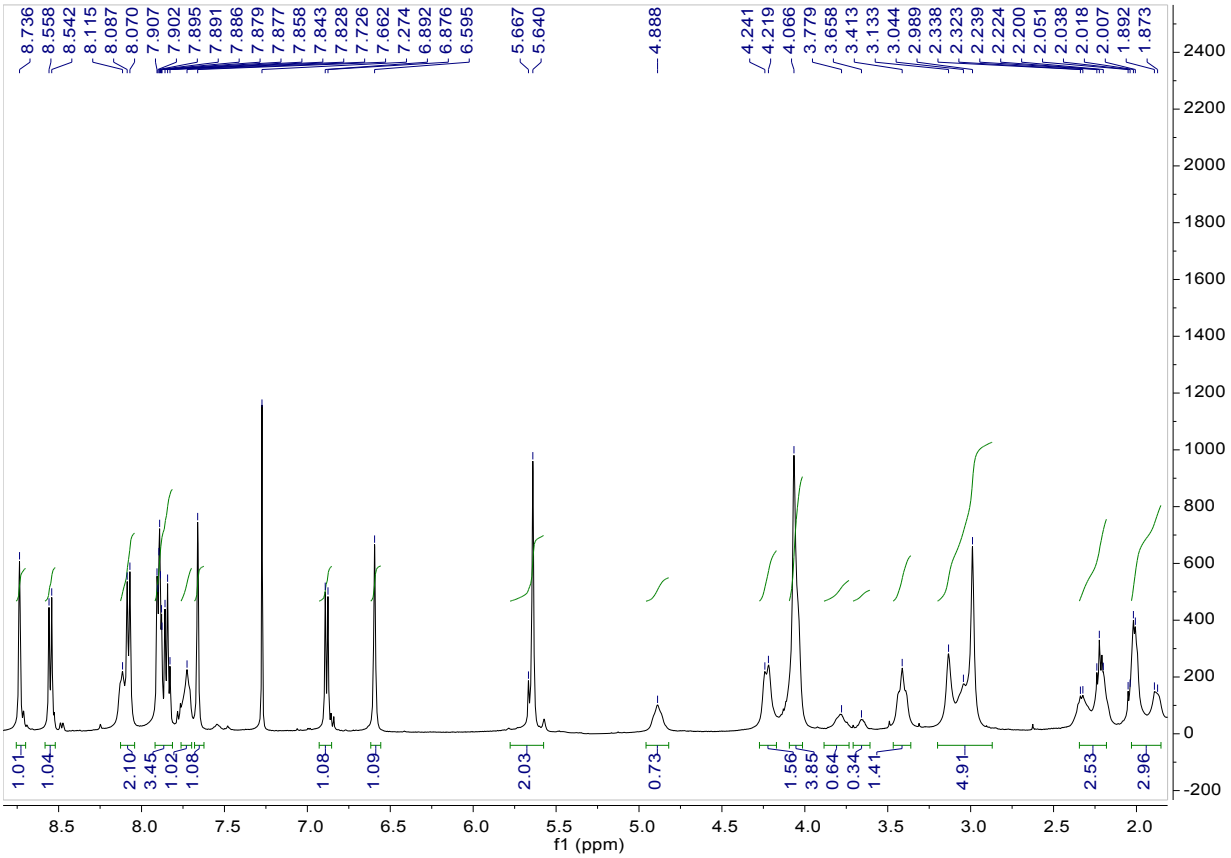

thc-20190513/zfr1870-TC627-C — THC02 298K
161.537
158.746
150.119
145.952
139.172
137.947
136.211
135.981
134.515
133.129
132.391
128.259
128.237
127.931
125.944
120.538
120.268
109.029
77.161
77.000
76.839
63.507
56.700
54.274
50.460
48.429
48.356
48.322
48.215
48.143
48.036
47.930
42.361
39.032
37.134
34.931
29.222
27.932
f1 (ppm)

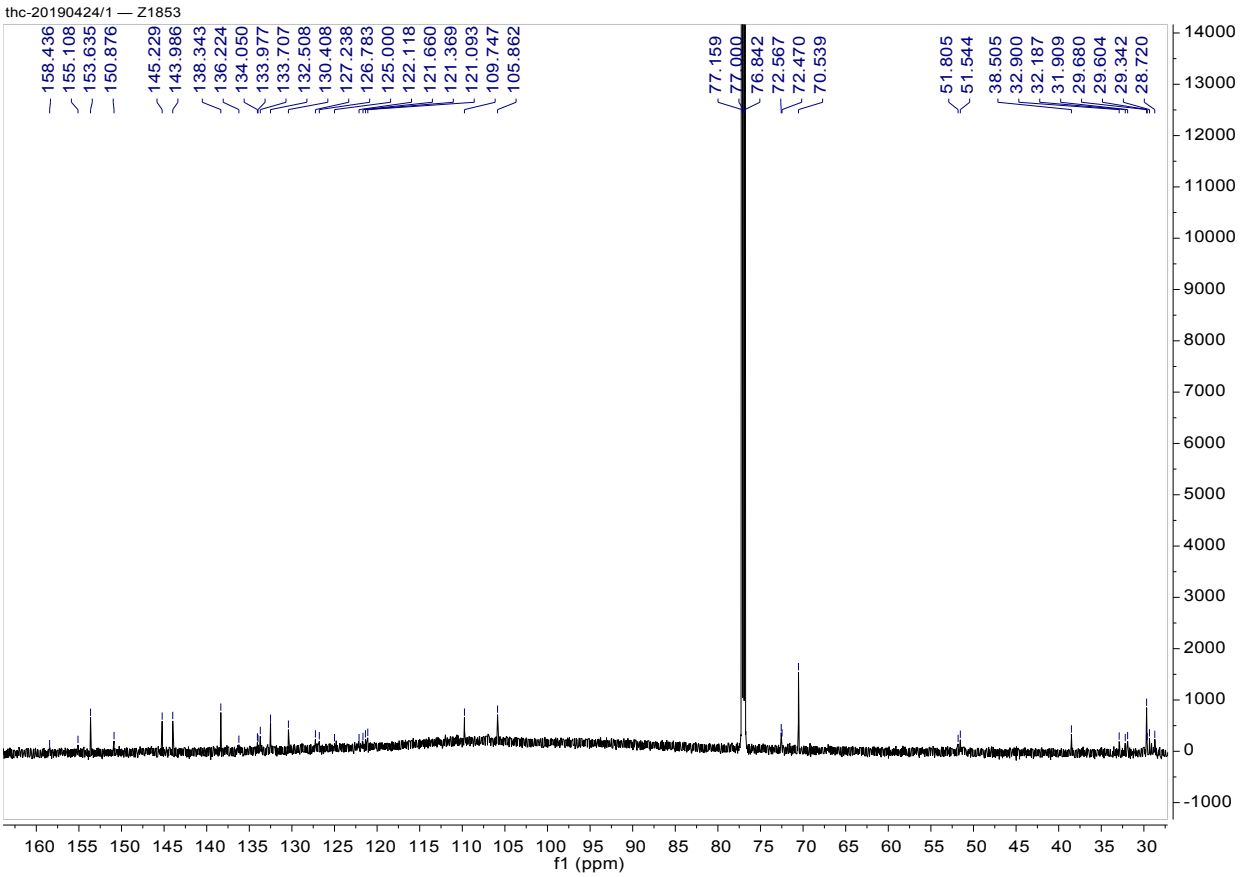

x10 5
+ESI Scan (22.0-79.9 sec, 59 Scans) Frag=140.0V y543.d
458.2299
282.2790
318.3001
551.3553
663.4540
764.5733
922.0090
Counts vs. Mass-to-Charge (m/z)

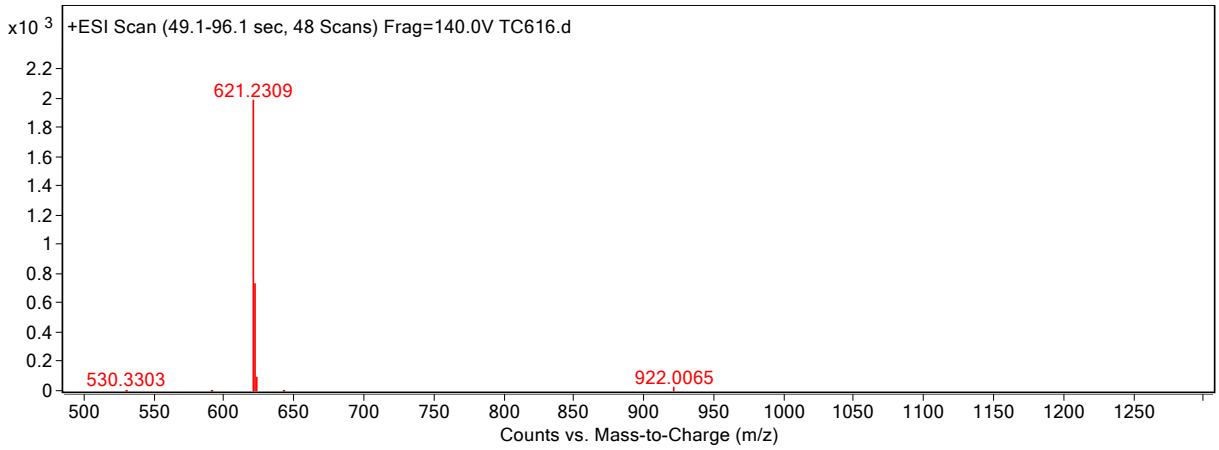


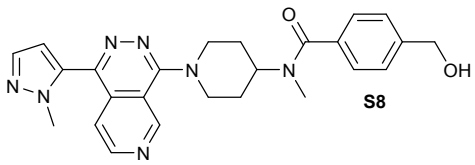
7

NBD/TC616 1H — 1H TC616 CDCl3

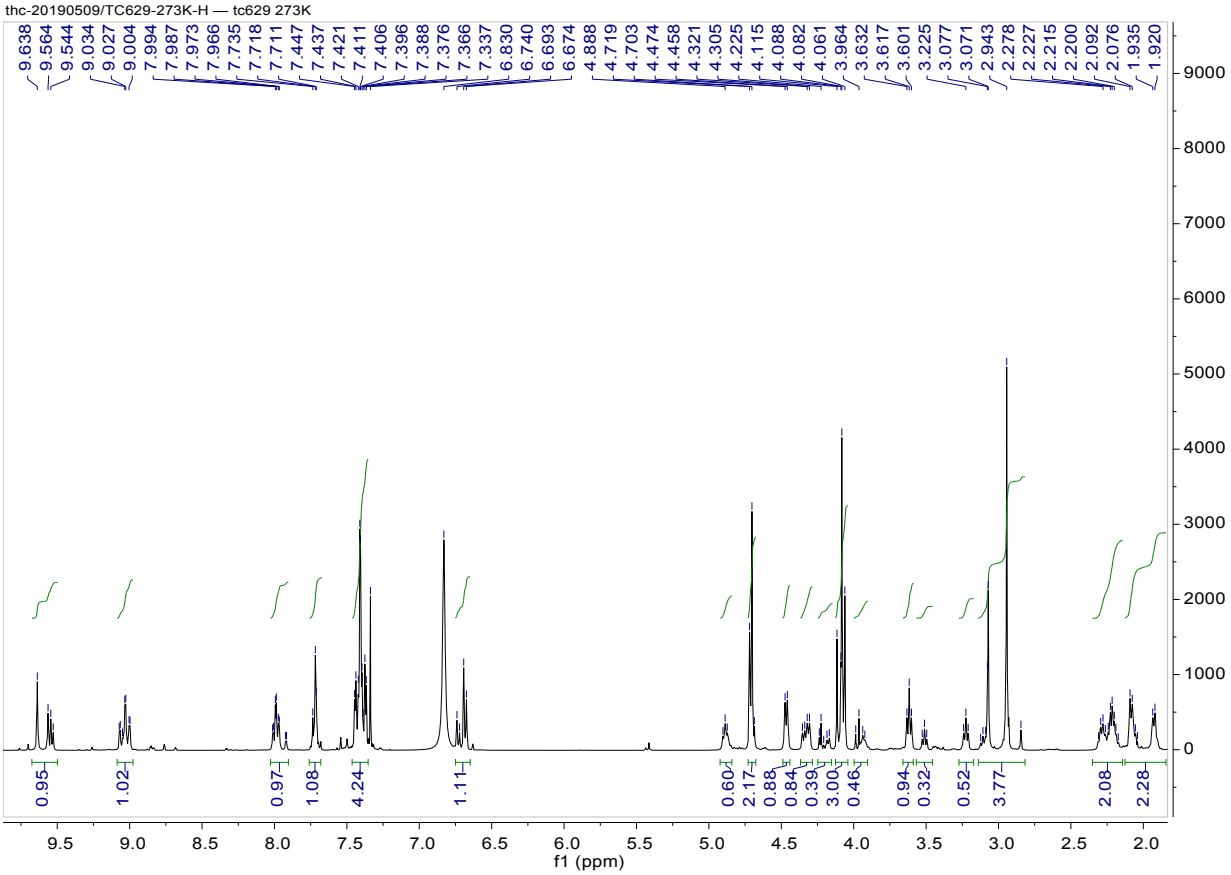


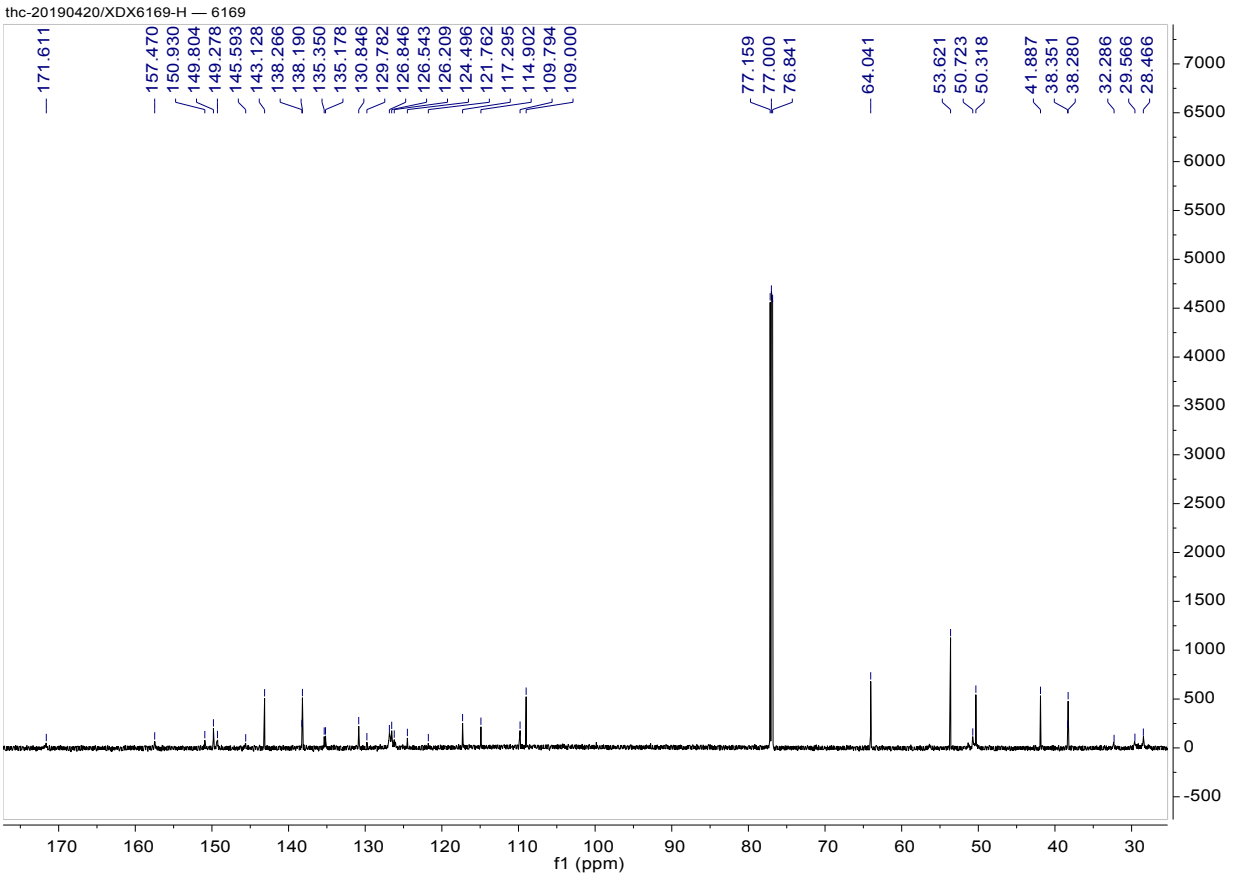

thc-20190424/1 — Z1853
158.436
155.108
153.635
150.876
145.229
143.986
138.343
136.224
134.050
133.977
133.707
132.508
130.408
127.238
126.783
125.000
122.118
121.660
121.369
121.093
109.747
105.862
77.159
77.000
76.842
72.567
72.470
70.539
51.805
51.544
38.505
32.900
32.187
31.909
29.680
29.604
29.342
28.720
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30
f1 (ppm)

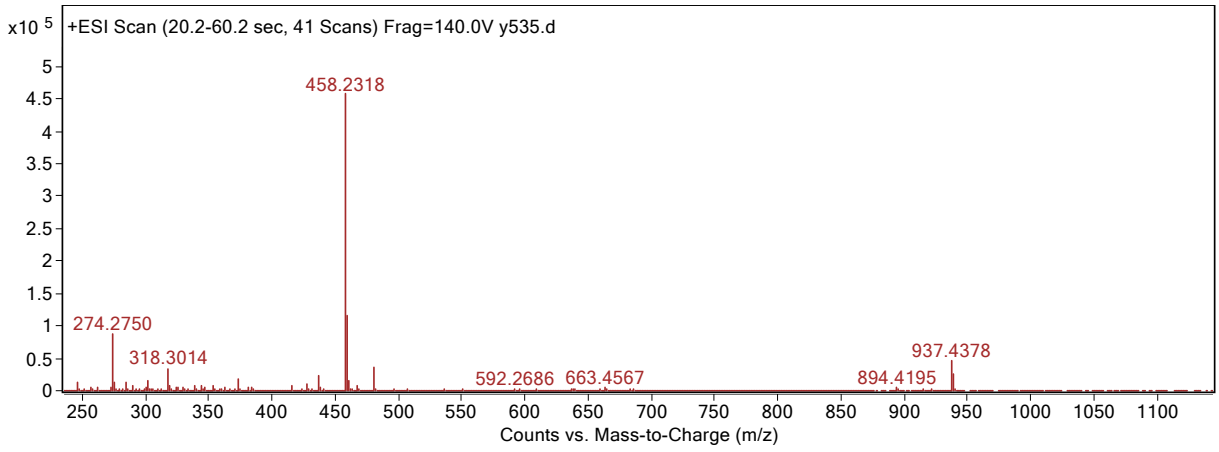

x10 3
+ESI Scan (49.1-96.1 sec, 48 Scans) Frag=140.0V TC616.d
621.2309
530.3303
922.0065
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
500 550 600 650 700 750 800 850 900 950 1000 1050 1100 1150 1200 1250
Counts vs. Mass-to-Charge (m/z)

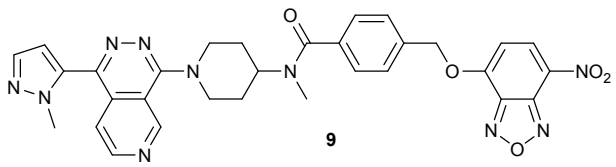

O
N
N
N
N
N
N
N
OH

S8

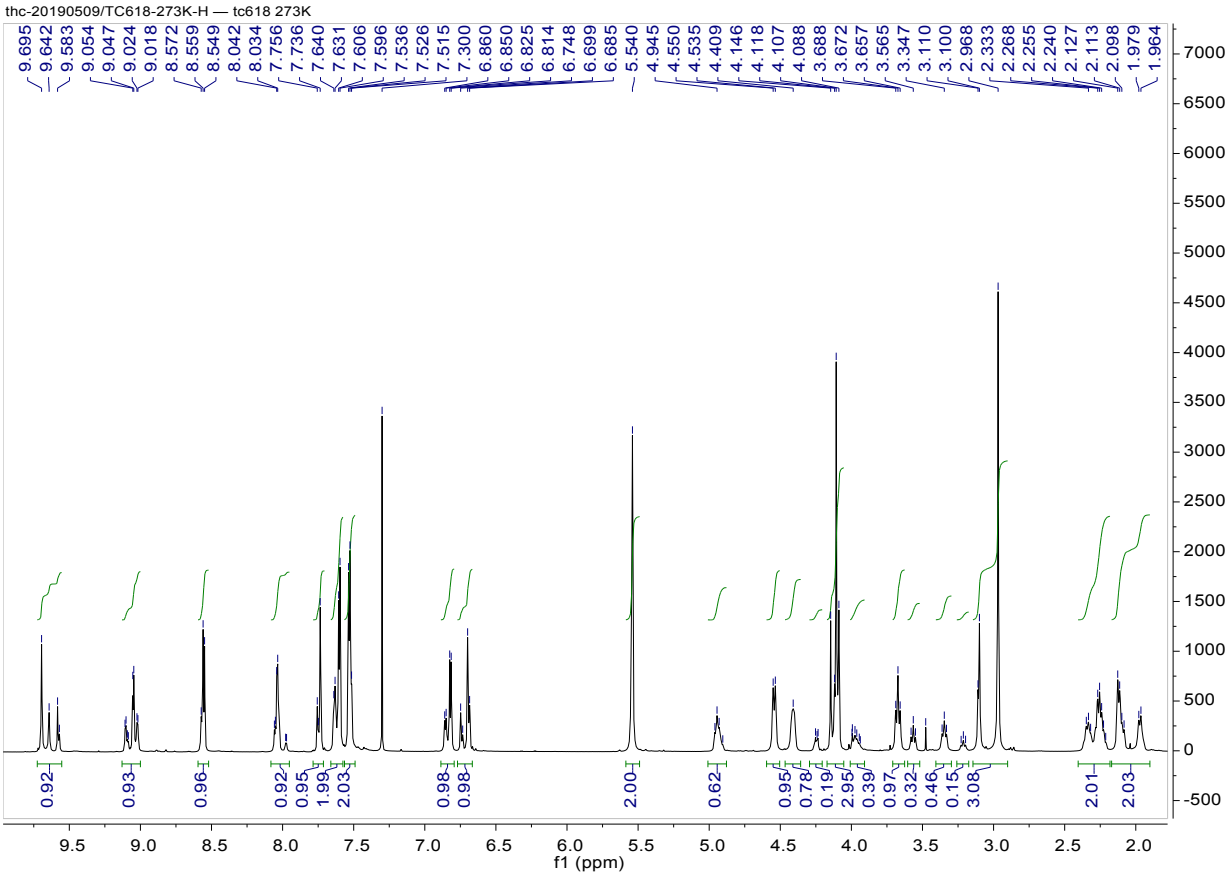

thc-20190509/TC629-273K-H — tc629 273K
9.638
9.564
9.544
9.034
9.027
9.004
7.994
7.987
7.973
7.966
7.735
7.718
7.711
7.447
7.437
7.421
7.411
7.406
7.396
7.388
7.376
7.366
7.337
6.830
6.740
6.693
6.674
4.888
4.719
4.703
4.474
4.458
4.321
4.305
4.225
4.115
4.088
4.082
4.061
3.964
3.632
3.617
3.601
3.225
3.077
3.071
2.943
2.278
2.227
2.215
2.200
2.092
2.076
1.935
1.920
0.95
1.02
0.97
1.08
4.24
1.11
0.60
2.17
0.88
0.84
0.39
3.00
0.46
0.94
0.32
0.52
3.77
2.08
2.28
f1 (ppm)

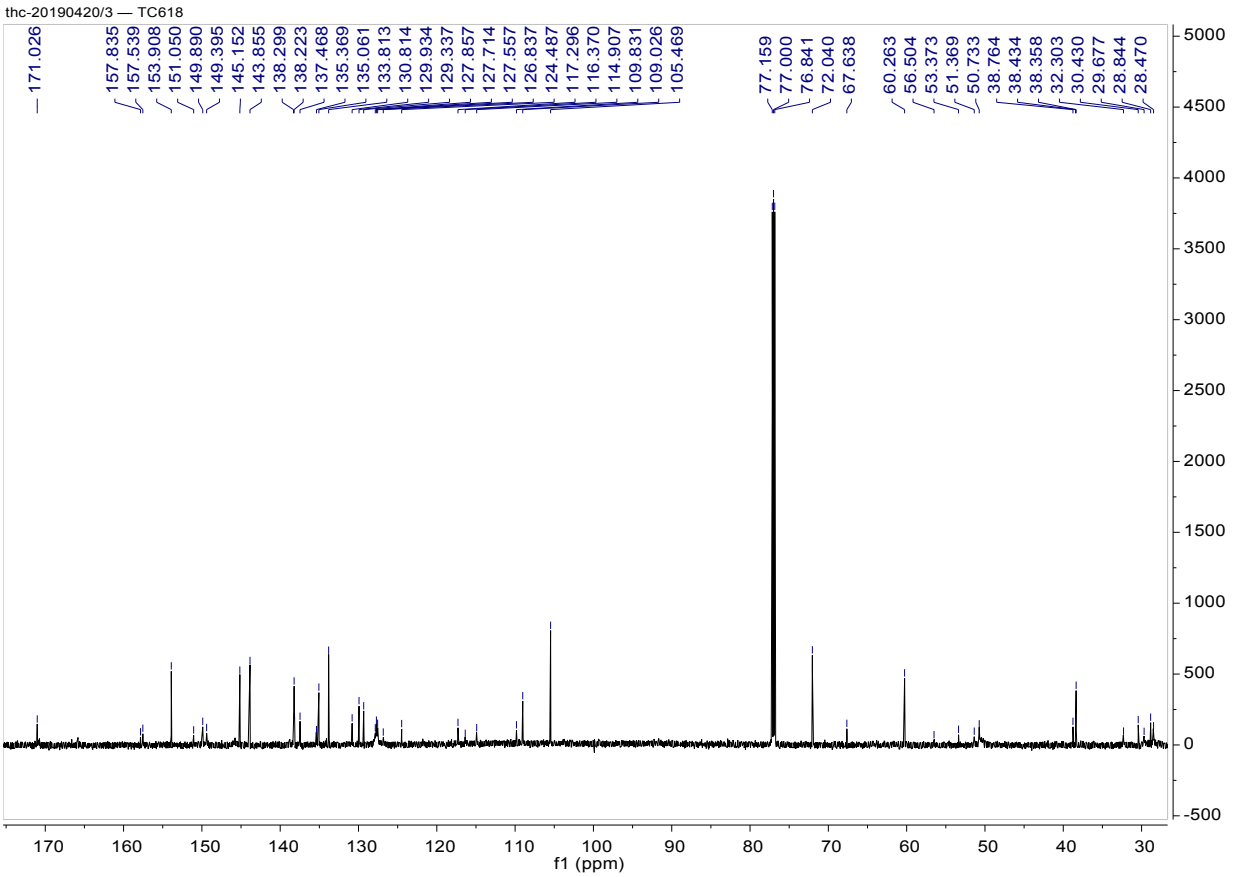

thc-20190420/XDX6169-H — 6169
171.611
157.470
150.930
149.804
149.278
145.593
143.128
138.266
138.190
135.350
135.178
130.846
129.782
126.846
126.543
126.209
124.496
121.762
117.295
114.902
109.794
109.000
77.159
77.000
76.841
64.041
53.621
50.723
50.318
41.887
38.351
38.280
32.286
29.566
28.466
f1 (ppm)

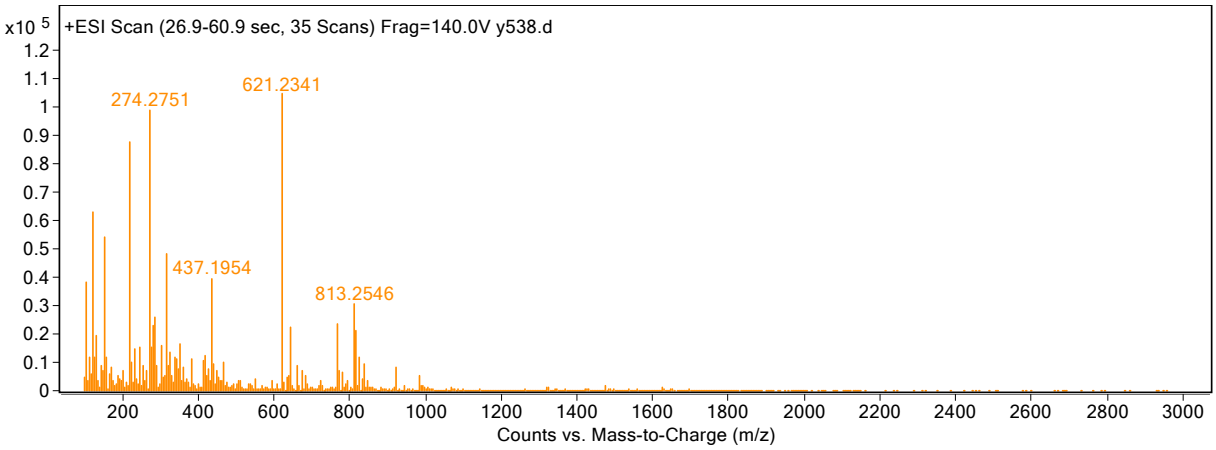

x10 5
+ESI Scan (20.2-60.2 sec, 41 Scans) Frag=140.0V y535.d
458.2318
274.2750
318.3014
592.2686
663.4567
894.4195
937.4378
Counts vs. Mass-to-Charge (m/z)

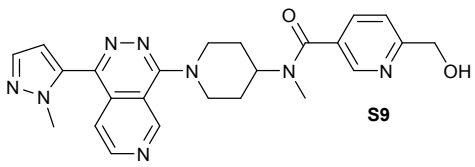


9


thc-20190509/TC618-273K-H — tc618 273K
9.695 9.642 9.583 9.054 9.047 9.024 9.018 8.572 8.559 8.549 8.042 8.034 7.756 7.736 7.640 7.631 7.606 7.596 7.536 7.526 7.515 7.300 6.860 6.850 6.825 6.814 6.748 6.699 6.685 5.540 4.945 4.550 4.535 4.409 4.146 4.118 4.107 4.088 3.688 3.672 3.657 3.565 3.347 3.110 3.100 2.968 2.333 2.268 2.255 2.240 2.127 2.113 2.098 1.979 1.964
0.92 0.93 0.96 0.92 0.95 1.99 2.03 0.98 0.98 2.00 0.62 0.95 0.78 0.19 2.95 0.39 0.97 0.32 0.46 0.15 3.08 2.01 2.03
f1 (ppm)

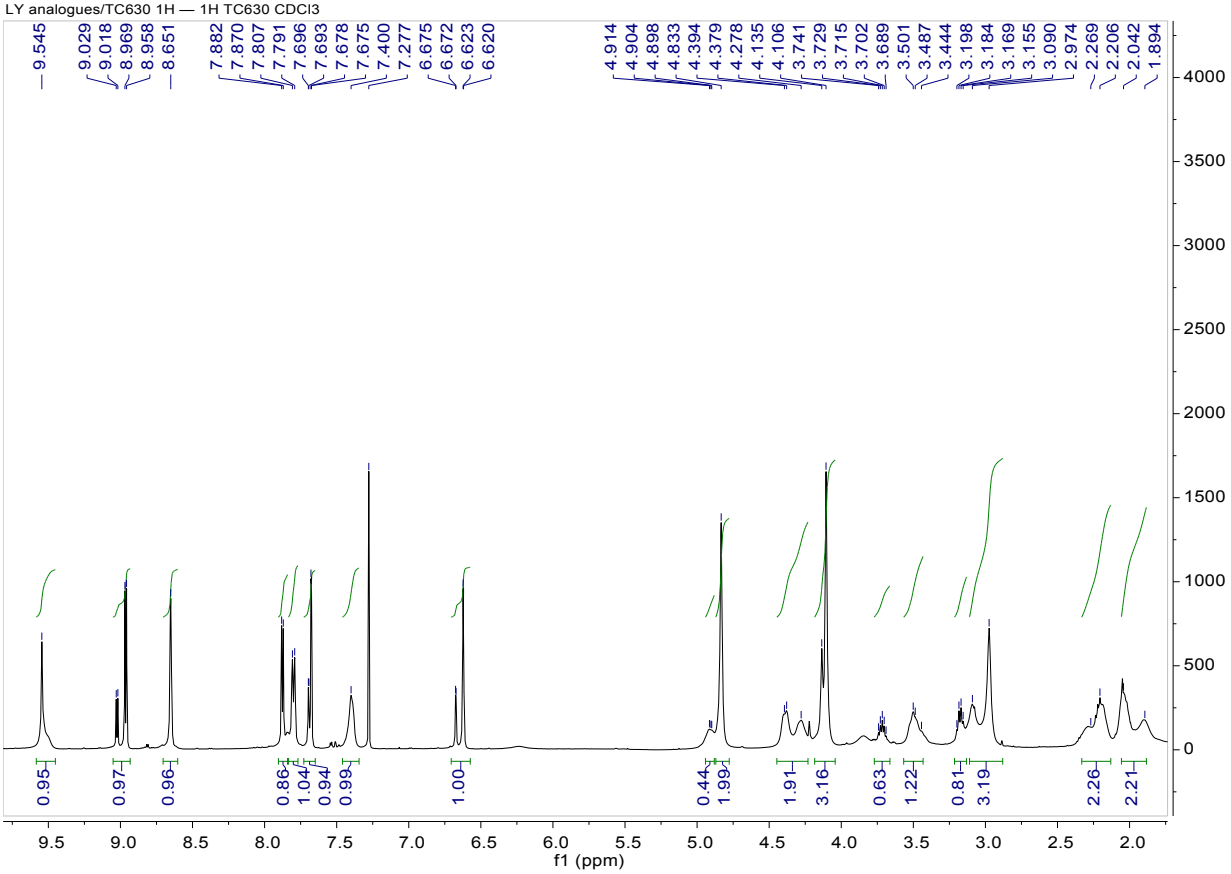

thc-20190420/3 — TC618
171.026
157.835
157.539
153.908
151.050
149.890
149.395
145.152
143.855
138.299
138.223
137.468
135.369
135.061
133.813
130.814
129.934
129.337
127.857
127.714
127.557
126.837
124.487
117.296
116.370
114.907
109.831
109.026
105.469
77.159
77.000
76.841
72.040
67.638
60.263
56.504
53.373
51.369
50.733
38.764
38.434
38.358
32.303
30.430
29.677
28.844
28.470
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
f1 (ppm)

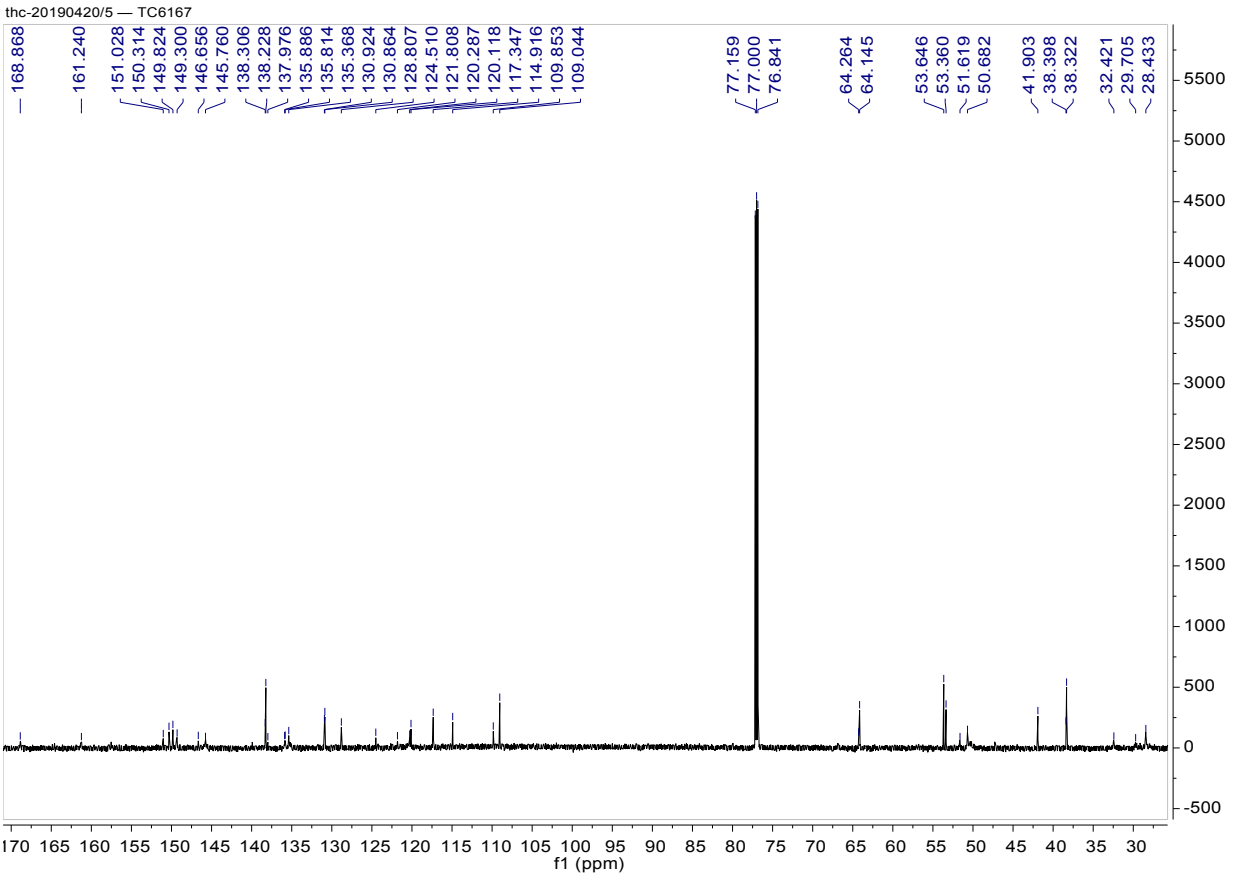

x10 5
+ESI Scan (26.9-60.9 sec, 35 Scans) Frag=140.0V y538.d
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
274.2751
621.2341
437.1954
813.2546
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
3000
Counts vs. Mass-to-Charge (m/z)

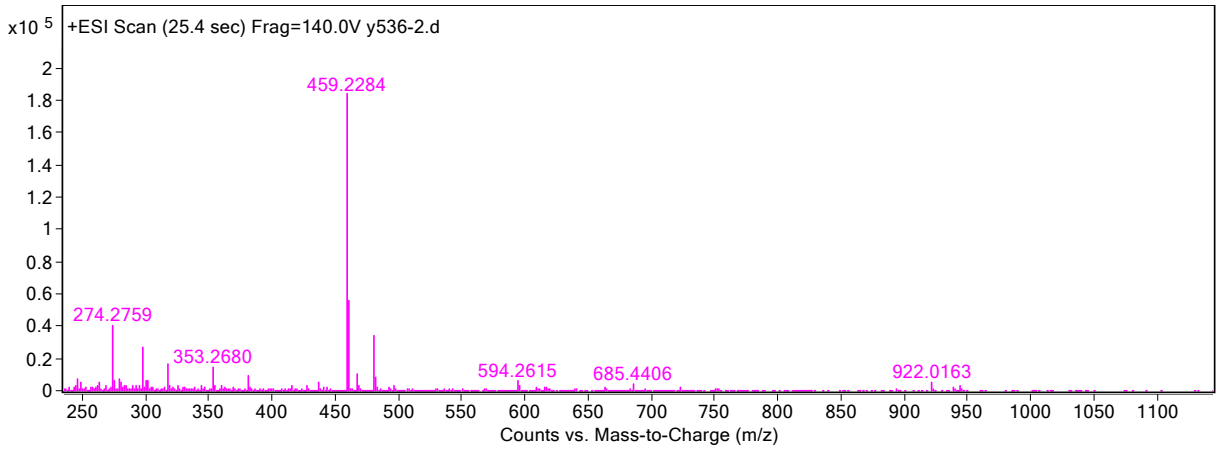


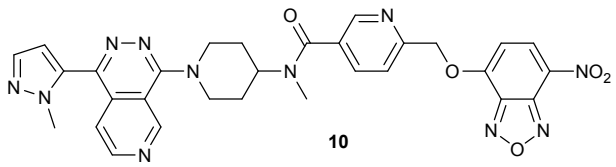
S9

LY analogues/TC630 1H — 1H TC630 CDCl3

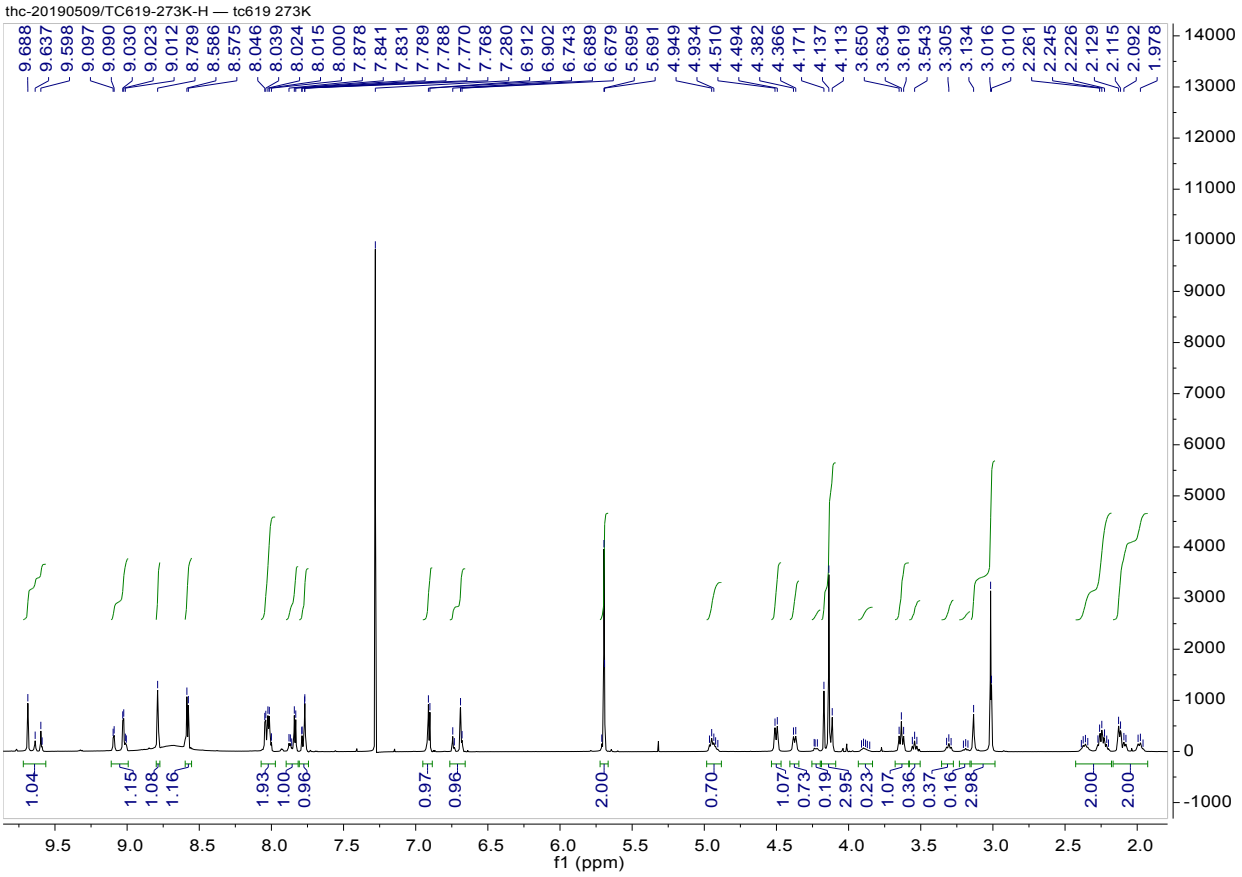


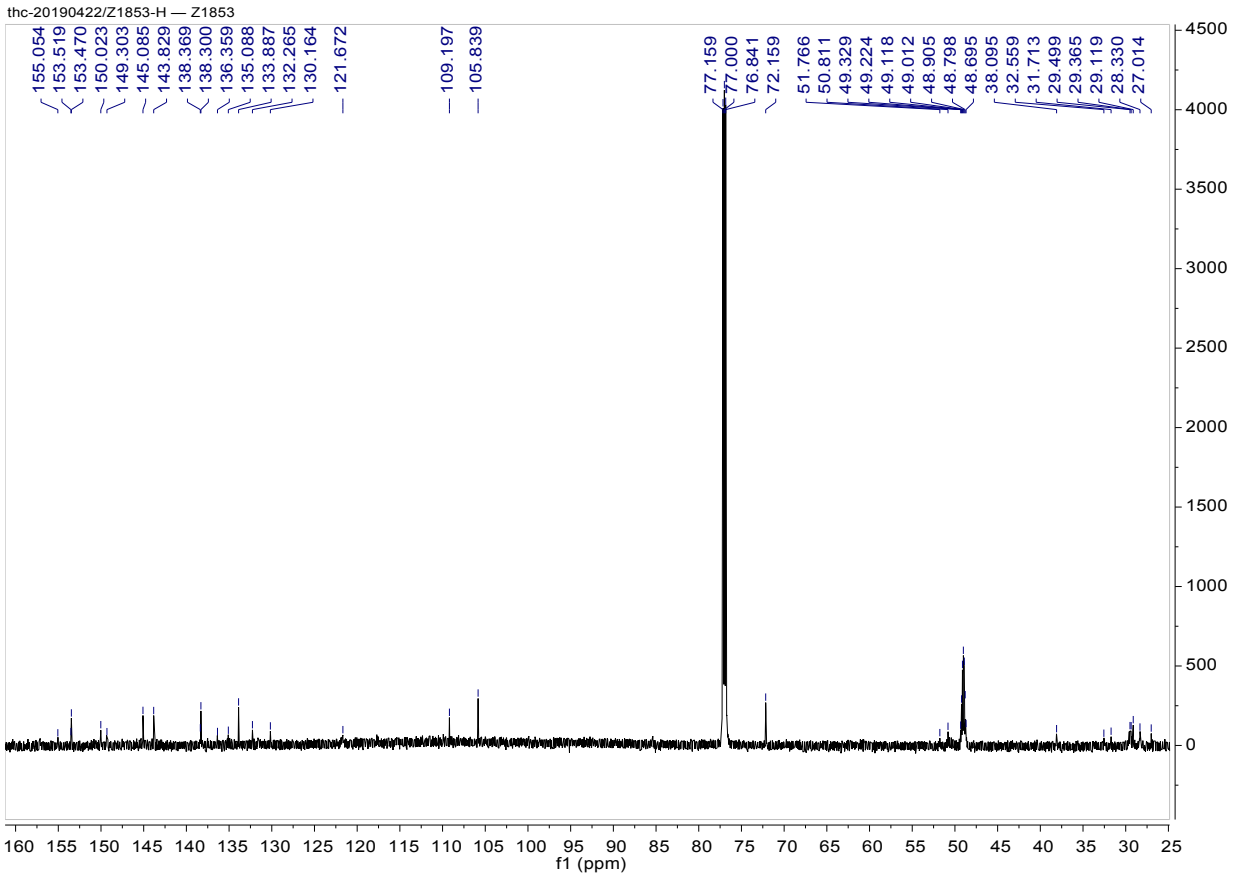

thc-20190420/5 — TC6167
168.868
161.240
151.028
150.314
149.824
149.300
146.656
145.760
138.306
138.228
137.976
135.886
135.814
135.368
130.924
130.864
128.807
124.510
121.808
120.287
120.118
117.347
114.916
109.853
109.044
77.159
77.000
76.841
64.264
64.145
53.646
53.360
51.619
50.682
41.903
38.398
38.322
32.421
29.705
28.433
f1 (ppm)

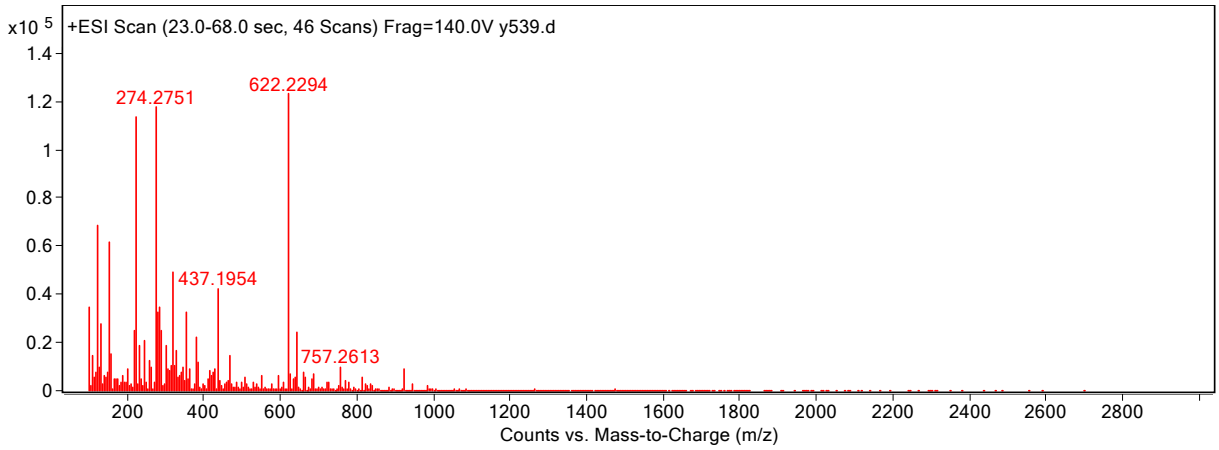

x10 5
+ESI Scan (25.4 sec) Frag=140.0V y536-2.d
459.2284
274.2759
353.2680
594.2615
685.4406
922.0163
Counts vs. Mass-to-Charge (m/z)

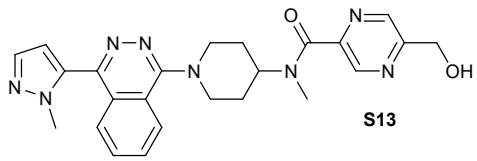


10

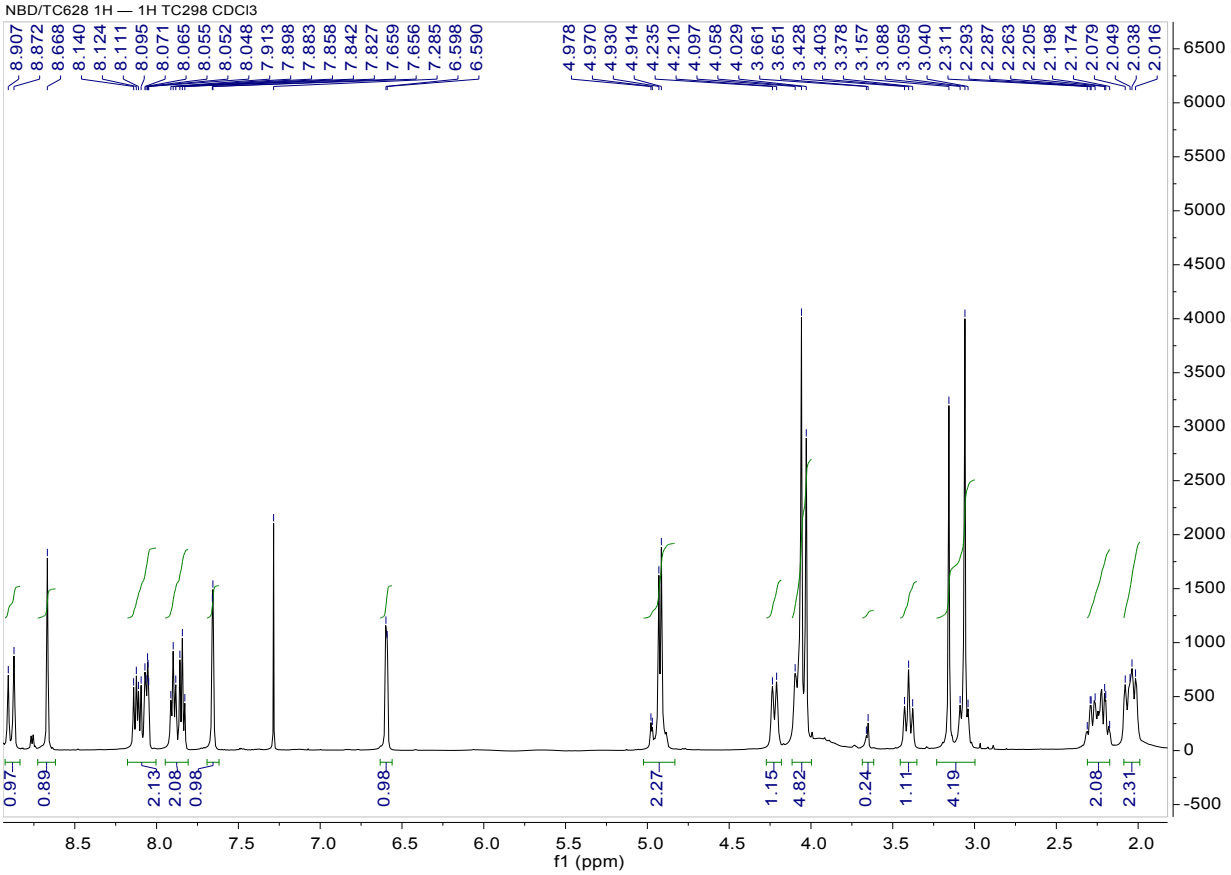

thc-20190509/TC619-273K-H — tc619 273K
9.688 9.637 9.598 9.097 9.090 9.030 9.023 9.012 8.789 8.586 8.575 8.046 8.039 8.024 8.015 8.000 7.878 7.841 7.831 7.789 7.788 7.770 7.768 7.280 6.912 6.902 6.743 6.689 6.679 5.695 5.691 4.949 4.934 4.510 4.494 4.382 4.366 4.171 4.137 4.113 3.650 3.634 3.619 3.543 3.305 3.134 3.016 3.010 2.261 2.245 2.226 2.129 2.115 2.092 1.978
1.04 1.15 1.08 1.16 1.93 1.00 0.96 0.97 0.96 2.00 0.70 1.07 0.73 0.19 2.95 0.23 1.07 0.36 0.37 0.16 2.98 2.00 2.00
f1 (ppm)

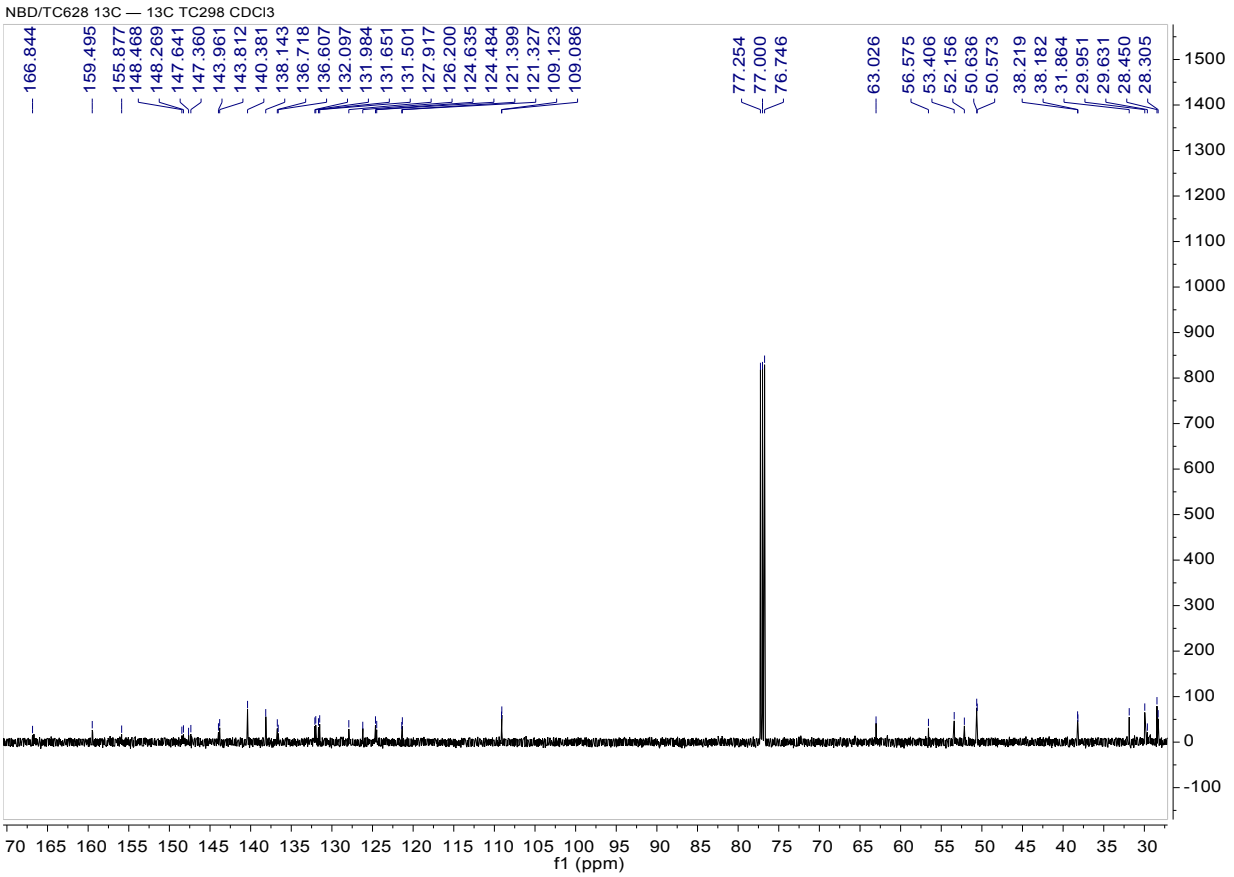

thc-20190422/Z1853-H — Z1853
155.054
153.519
153.470
150.023
149.303
145.085
143.829
138.369
138.300
136.359
135.088
133.887
132.265
130.164
121.672
109.197
105.839
77.159
77.000
76.841
72.159
51.766
50.811
49.329
49.224
49.118
49.012
48.905
48.798
48.695
38.095
32.559
31.713
29.499
29.365
29.119
28.330
27.014
4500
4000
3500
3000
2500
2000
1500
1000
500
0
160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25
f1 (ppm)

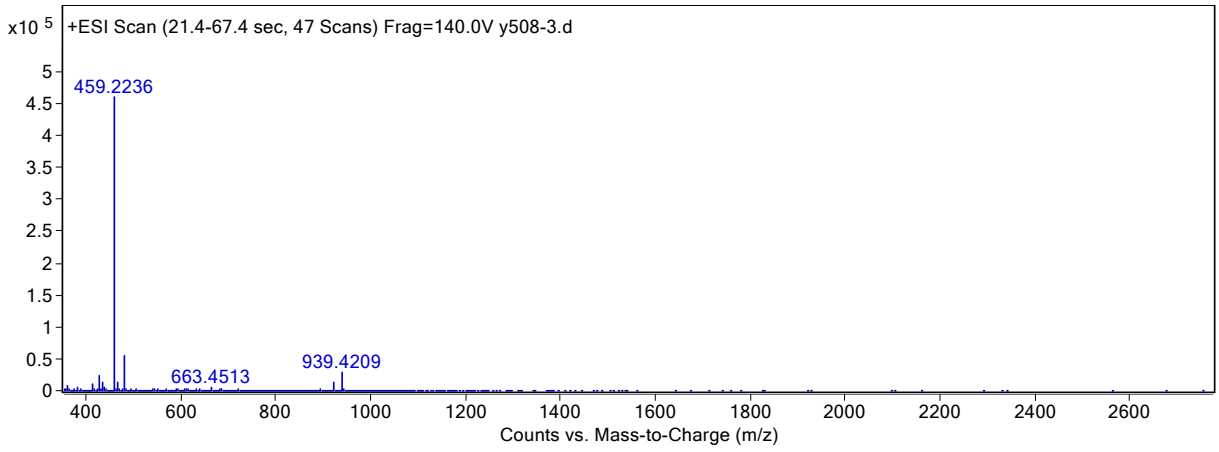

x10 5
+ESI Scan (23.0-68.0 sec, 46 Scans) Frag=140.0V y539.d
1.4
1.2
1
0.8
0.6
0.4
0.2
0
274.2751
622.2294
437.1954
757.2613
200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400 2600 2800
Counts vs. Mass-to-Charge (m/z)

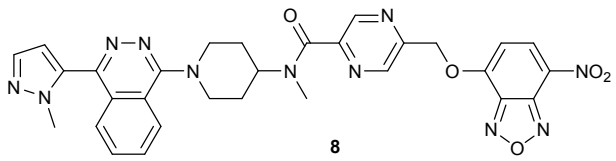

O
N
N–N
N
N
N
N
N
OH

S13

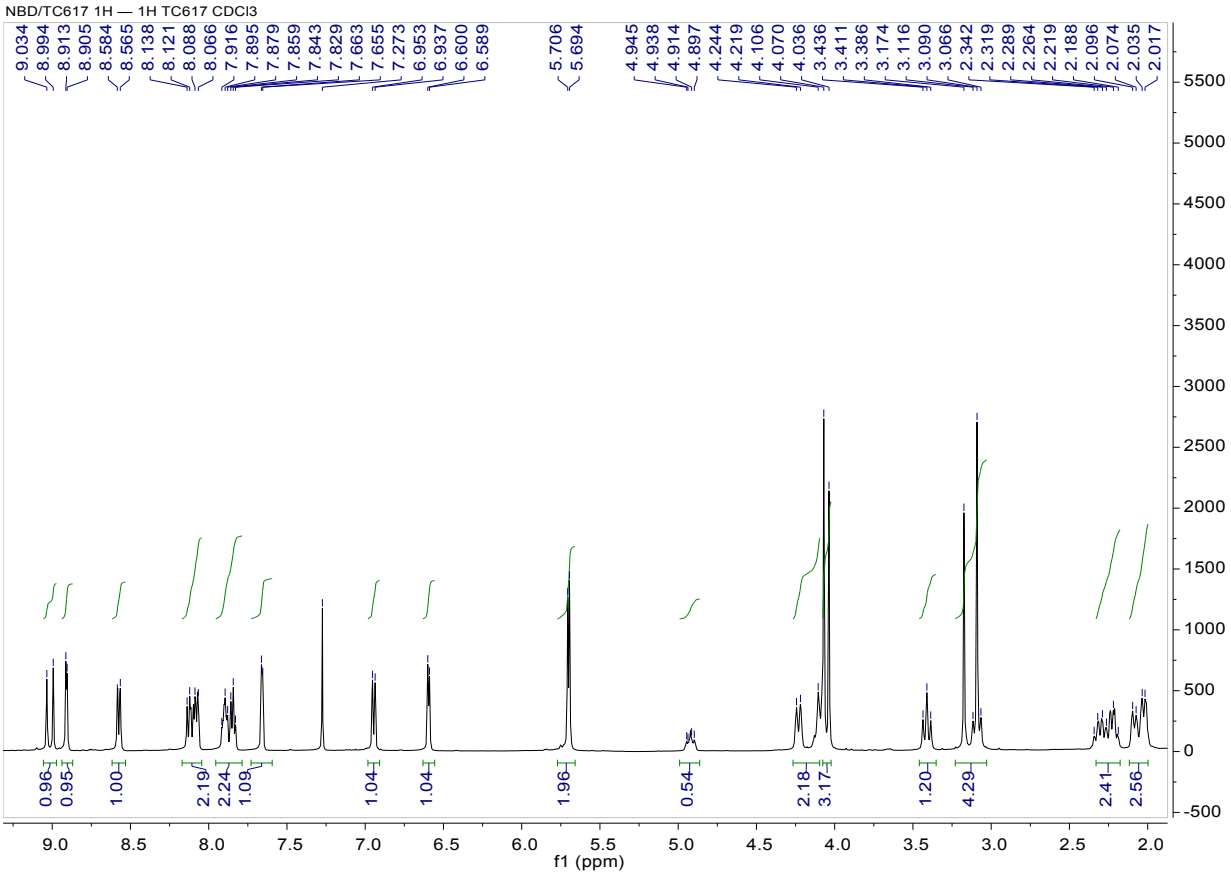

NBD/TC628 1H — 1H TC298 CDCl3
8.907
8.872
8.668
8.140
8.124
8.111
8.095
8.071
8.065
8.055
8.052
8.048
7.913
7.898
7.883
7.858
7.842
7.827
7.659
7.656
7.285
6.598
6.590
4.978
4.970
4.930
4.914
4.235
4.210
4.097
4.058
4.029
3.661
3.651
3.428
3.403
3.378
3.157
3.088
3.059
3.040
2.311
2.293
2.287
2.263
2.205
2.198
2.174
2.079
2.049
2.038
2.016
0.97
0.89
2.13
2.08
0.98
0.98
2.27
1.15
4.82
0.24
1.11
4.19
2.08
2.31
f1 (ppm)

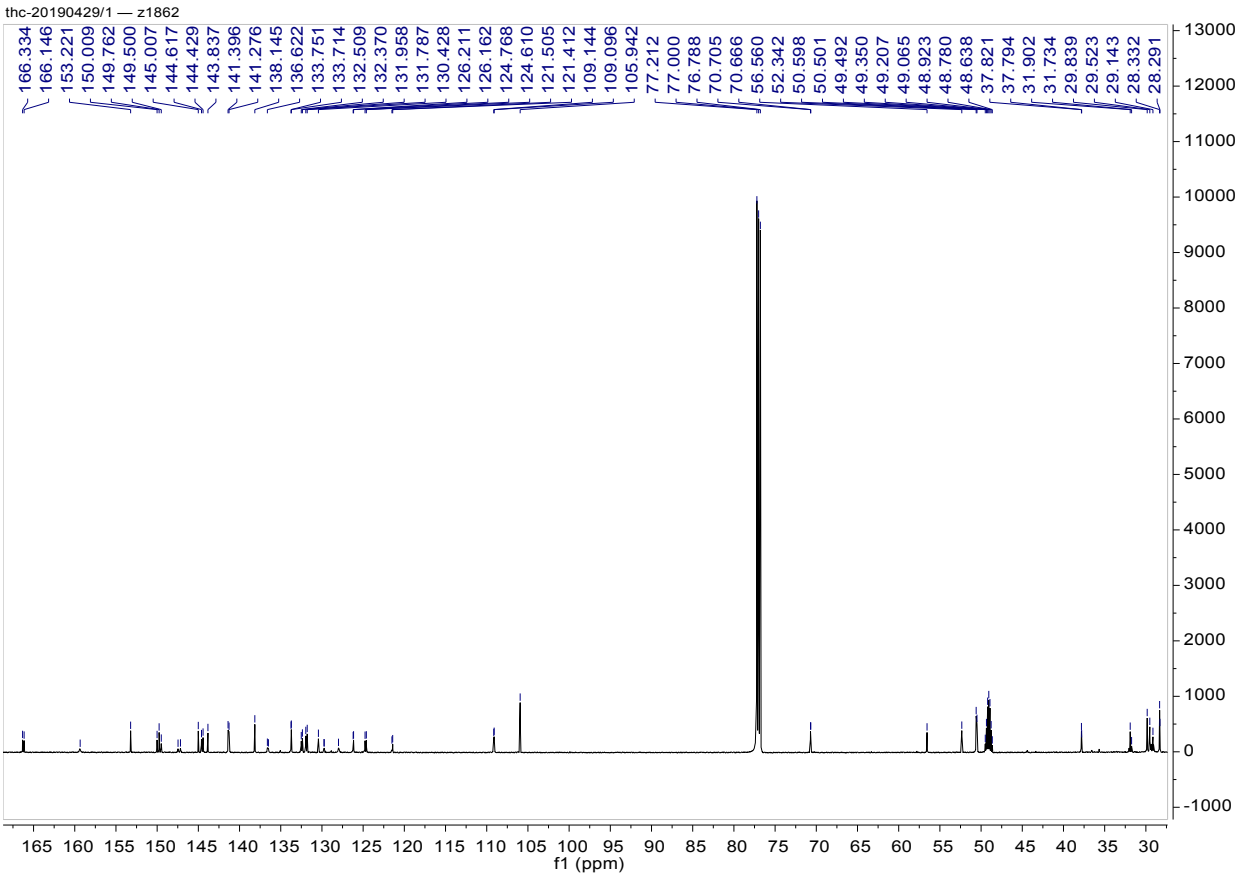

NBD/TC628 13C — 13C TC298 CDCl3
166.844
159.495
155.877
148.468
148.269
147.641
147.360
143.961
143.812
140.381
138.143
136.718
136.607
132.097
131.984
131.651
131.501
127.917
126.200
124.635
124.484
121.399
121.327
109.123
109.086
77.254
77.000
76.746
63.026
56.575
53.406
52.156
50.636
50.573
38.219
38.182
31.864
29.951
29.631
28.450
28.305
f1 (ppm)

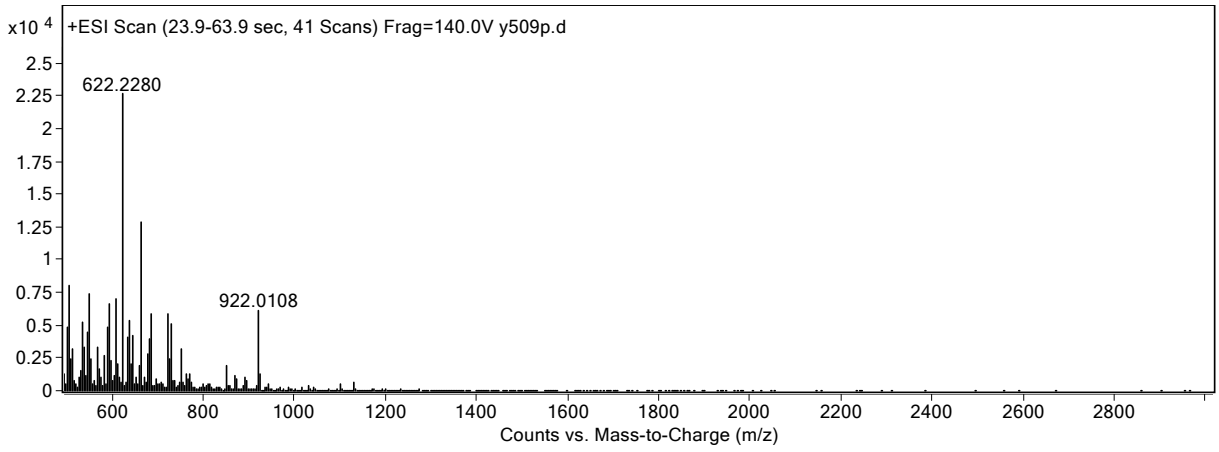

x10 5
+ESI Scan (21.4-67.4 sec, 47 Scans) Frag=140.0V y508-3.d
459.2236
663.4513
939.4209
Counts vs. Mass-to-Charge (m/z)



8


NBD/TC617 1H — 1H TC617 CDCl3
9.034
8.994
8.913
8.905
8.584
8.565
8.138
8.121
8.088
8.066
7.916
7.895
7.879
7.859
7.843
7.829
7.663
7.655
7.273
6.953
6.937
6.600
6.589
5.706
5.694
4.945
4.938
4.914
4.897
4.244
4.219
4.106
4.070
4.036
3.436
3.411
3.386
3.174
3.116
3.090
3.066
2.342
2.319
2.289
2.264
2.219
2.188
2.096
2.074
2.035
2.017
0.96
0.95
1.00
2.19
2.24
1.09
1.04
1.04
1.96
0.54
2.18
3.17
1.20
4.29
2.41
2.56
f1 (ppm)


thc-20190429/1 — z1862
166.334
166.146
153.221
150.009
149.762
149.500
145.007
144.617
144.429
143.837
141.396
141.276
138.145
136.622
133.751
133.714
132.509
132.370
131.958
131.787
130.428
126.211
126.162
124.768
124.610
121.505
121.412
109.144
109.096
105.942
77.212
77.000
76.788
70.705
70.666
56.560
52.342
50.598
50.501
49.492
49.350
49.207
49.065
48.923
48.780
48.638
37.821
37.794
31.902
31.734
29.839
29.523
29.143
28.332
28.291
f1 (ppm)


x10 4
+ESI Scan (23.9-63.9 sec, 41 Scans) Frag=140.0V y509p.d
622.2280
922.0108
Counts vs. Mass-to-Charge (m/z)