

Supporting Information

Synthesis and Pharmacological Characterization of Novel Peripheral Cannabinoid-1 Receptor Blockers Based on a Tricyclic Scaffold

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Table of Content

Supplementary Experimental Methods	3
Brain membrane preparation for CB₁R binding.....	3
CB₁R and CB₂R binding.	3
Tissue preparation for [³⁵S]GTPγS binding.....	5
[³⁵S]GTPγS binding - CB₁R activity.....	6
Bidirectional permeability assessment in MDR1-MDCK cells.	6
Measurements of BNS808 using LC-MS/MS.....	7
Supplementary Table 1. The collision energy (CE), declustering potential (DP), and collision cell exit potential (CXP) for the monitored transitions.....	9
Mini Ames assay.....	9
Protein binding of test compound in mouse brain using equilibrium dialysis method.	10
hERG assay.	12
Cell viability assay.....	13
Supplementary Table 2. Summary of in vitro MDR-MDCKII permeability of BNS8 derivatives.....	14
Supplementary Table 3. Mini Ames Study.....	15
Supplementary Table 4 – Mini Ames Study.....	17
Supplementary Table 5. Cell viability assay in HepG2 cells at 72 h and mutagenic effect in a Mini Ames assay.....	19
Supplementary Table 6. The IC₅₀ of BNS8 derivative compounds on whole cell hERG currents.....	20
Supplementary Table 7. Stability of the test compounds in simulated gastric fluid (SGF).....	21
Supplementary Table 8. Stability of the test compounds in simulated intestine fluid (SIF).....	21
Supplementary Table 9. Protein binding of BNS808 in mouse brain, using equilibrium dialysis method.	22
Supplementary Figure 1. In vitro activity assessment of BNS compounds using the [³⁵S]GTPγS binding assay.....	23
Supplementary Figure 2. BNS808 biodistribution and pharmacokinetics after chronic PO administration.	24
Supplementary Figure 3. The NMR, HPLC, and LC-MS analysis of BNS808	25
Supplementary Figure 4. The NMR, HPLC, and LC-MS analysis of BNS807	28
Supplementary Figure 5. The NMR, HPLC, and LC-MS analysis of BNS809	31
Supplementary Figure 6. The NMR, HPLC, and LC-MS analysis of BNS813	34
Supplementary Figure 7. The NMR, HPLC, and LC-MS analysis of BNS816	37
Supplementary Figure 8. The NMR, HPLC, and LC-MS analysis of BNS825	40

Supplementary Experimental Methods

Brain membrane preparation for CB₁R binding. The mouse cortex, excluding the cerebellum, was dissected from 6-week-old C57Bl/6Jhds mice (Harlan, Israel). The tissue was homogenized in 4 mL Buffer 1 (320 mM sucrose, 2 mM EDTA, 5 mM MgCl₂) using a glass-Teflon homogenizer and Hei-TORQUE 200 (Heidolph; Cat# 17-012-002-25-1) homogenizer set to 132 rpm. After homogenization, the mixture was centrifuged at $1,600 \times g$ for 10 min at four °C, and the resulting supernatant was kept on ice. The pellet (P1) was discarded, and the remaining supernatant was subjected to additional rounds of resuspension in 4 mL Buffer 1 followed by centrifugations, repeating this process three times. The pellets obtained from these centrifugations were discarded, and the three supernatant fractions were combined and subjected to higher-speed centrifugation at $39,000 \times g$ for 15 min at four °C. The resulting supernatant was discarded, and the pellet (P2) was resuspended in 5 mL Buffer 2 containing 50 mM TRIS HCl pH 7.0, 0.2 mM EDTA, and five mM MgCl₂. This resuspended pellet (P2) was incubated at 37 °C for 10 min and then centrifuged at $23,000 \times g$ for 10 min at 4°C. After centrifugation, the pellet (P2) was resuspended in 5 mL Buffer 2 and further incubated at 30 °C for 40 min. Following this incubation, the suspension was centrifuged at $11,000 \times g$ for 15 min at 4 °C. The pellet (P2) was resuspended in 2 mL Buffer 3 containing 50 mM TRIS HCl pH 7.4, 1 mM EDTA, and 3 mM MgCl₂. The protein concentration of the final suspension was determined using the BCA assay. Subsequently, the suspension was diluted to a final concentration of 2 mg/mL in Buffer 3, aliquoted on ice, and stored at -80 °C for further use.

CB₁R and CB₂R binding. Binding to the CB₁R/CB₂R was assessed using competition displacement assays with [³H]CP-55,940 as the radioligand. Purified membranes from the mouse brain were utilized for CB₁R assessment (as described above). Purified

membranes from cells overexpressing human CB₂R (Charles River CAT# A618) were employed for CB₂R evaluation.

For mouse CB₁R binding assays, the novel compounds were dissolved in dimethyl sulfoxide (DMSO) to reach a final concentration of 10 mM. Each compound was then diluted in Buffer 3 (as described above) supplemented with 0.14% BSA, resulting in concentrations ranging from 10⁻⁴ M to 10⁻¹⁰ M. Triplicates of 100 µL of each concentration were dispensed into siliconized glass tubes. At the same time, 100 µL of Buffer 3 with 0.14% BSA was dispensed into three tubes as blanks. CB₁R brain membranes (20 µg) were added to each tube and diluted with 800 µL Buffer 3 with 0.14% BSA. Subsequently, 5 nM of [³H]CP-55,940 (100 µL) was added to each tube. The tubes were sealed with parafilm and incubated with shaking (120 rpm) in a water bath at 30 °C for 1 hour. Following incubation, 2 mL of cold Buffer 4 (50 mM TRIS HCl pH 7.4, 1 mg/mL BSA) was added to each tube.

To harvest the content of each tube, a Brandel M-24R Vacuum Harvester was employed, with a wet WHATMAN GF/B filter paper (Fired) (one membrane for 12 tubes) placed in the harvester after being washed twice with Buffer 4. The filter membrane in the harvester absorbed the full content of each tube. Then, the filter membrane was washed six times with Buffer 4. Each filter-glass membrane tube was excised and transferred to a 20 mL glass tube containing a 5 mL ULTIMA GOLD scintillation cocktail. The glass tubes were subjected to shaking for one hour at 25 °C and 200 rpm, following which they were read with a Tri-Carb 4910TR liquid scintillation counter.

Radioligand binding assays were performed to assess the binding affinity of the different compounds to hCB₁R (RBHCB1M400UA; PerkinElmer) or hCB₂R (RBXCB2M400UA; PerkinElmer). Competition displacement assays were employed,

utilizing [³H]CP-55,940 as the radioligand and crude membranes from humans for CB₁R or CB₂R. The compounds were tested at various concentrations (eight points with a five-fold serial dilution starting at two mM), and their ability to displace [³H]CP-55,940 was evaluated. Upon completion of the binding assays, the reaction mixture was filtered through GF/C plates, followed by four washes with cold wash buffer. After drying for one hour, 50 µL of Perkin Elmer Microscint 20 cocktail was added to the filter plate wells, and the bound [³H]CP-55,940 radioligand was measured using a β counter. All data points were duplicated, and *K_i* values were determined from two independent experiments using GraphPad Prism 7.02 analysis software, extracted through nonlinear regression analysis.

Tissue preparation for [³⁵S]GTPγS binding. The mouse cortex, excluding the cerebellum, from C57Bl/6Jhds mice (Harlan, Israel) was dissected in 10 mL of centrifugation buffer containing 50 mM TRIS HCl pH 7.4, 1 mM EGTA and three mM MgCl₂. The tissue was homogenized using a glass-Teflon and Hei-TORQUE 200 homogenizer (Heidolph; Cat#17-012-002-25-1) set to 132 rpm. The resulting suspension underwent centrifugation at 48,000 × g for 20 min at four °C, and the supernatant was discarded. The pellet obtained was homogenized again and subjected to a second centrifugation under the same conditions. Subsequently, the resulting pellet was resuspended in 1 mL of assay buffer composed of 50 mM TRIS HCl pH 7.4, 0.2 mM EGTA, 9 mM MgCl₂, and 150 mM NaCl, followed by homogenization. The protein concentration of the suspension was determined using the BCA assay, and the suspension was diluted to a final concentration of 2 mg/mL in assay buffer. The prepared suspension was aliquoted on ice and stored at -80 °C for further use.

[³⁵S]GTP γ S binding - CB₁R activity. The tested compounds were initially dissolved in DMSO to a final concentration of 10 mM and subsequently diluted in assay buffer containing 0.14% BSA to achieve concentrations ranging from 10⁻⁴ M to 10⁻¹⁰ M. Samples of each concentration (50 μ L) were then dispensed into siliconized glass tubes, with three tubes allocated for each concentration.

For non-specific binding assessment, 50 μ L of assay buffer was added to additional tubes; for basal binding assessment, 50 μ L of 100 μ M cold GTP was added to three glass tubes. Furthermore, 50 μ L of the solvent (assay buffer with 0.14% BSA) was dispensed in three extra tubes. Subsequently, 50 μ L of 1 mM GDP was added to each tube, followed by ten μ g CB₁R (mouse) membranes to each tube. The volume was then adjusted to 350 μ L with an assay buffer containing 0.14% BSA. Finally, 50 μ L of 0.5 nM [³⁵S]GTP γ S was added to each tube.

The glass tubes were covered with parafilm and incubated for one hour at 30 °C and 120 rpm. After one hour of incubation, the tubes were removed from the bath, and 2 mL of cold wash buffer composed of 50 mM TRIS HCl pH 7.4, 5 mM MgCl₂, and 1 mg/mL BSA was added to each tube. The content of each tube was harvested, allowing it to be absorbed into a wetted WHATMAN GF/B filter paper placed in the vacuum Brandel M-24R Harvester. The WHATMAN GF/B membrane was washed three times, cut, and transferred to a 20 mL glass tube with 5 mL of ULTIMA GOLD scintillation cocktail. The glass tubes were shaken at 25 °C for 1 hour at 200 rpm, and a Tri-Carb 4910TR liquid scintillation counter was used to read the radioactivity.

Bidirectional permeability assessment in MDR1-MDCK cells. Test and reference compounds were quantified using LC-MS/MS analysis based on the peak area ratio of analyte to internal standard (IS). The apparent permeability coefficient (P_{app}) in cm/s was computed using the equation: $P_{app} = (dC_r/dt) \times V_r / (A \times C_0)$, where dC_r/dt

represents the cumulative concentration of the compound in the receiver chamber over time ($\mu\text{M/s}$), V_r is the solution volume in the receiver chamber (0.075 mL on the apical side, 0.25 mL on the basolateral side), A is the surface area for transport (0.0804 cm^2 for the monolayer area), and C_0 is the initial concentration in the donor chamber (μM).

The efflux ratio was determined using the equation **Efflux Ratio** = $P_{\text{app}}(\text{B:A}) / P_{\text{app}}(\text{A:B})$. The percent recovery was calculated with the formula: **% Recovery** = $100 \times [(V_r \times C_r) + (V_d \times C_d)] / (V_d \times C_0)$, where V_d represents the volume in the donor chambers (0.075 mL on the apical side, 0.25 mL on the basolateral side), and C_d and C_r are the final concentrations of transport compound in donor and receiver chambers, respectively.

Additionally, the percent of lucifer yellow in the basolateral well was calculated using the equation:

$$\text{Lucifer yellow} = \frac{V_{\text{basolatera}} \times \text{RFU}_{\text{basolatera}}}{V_{\text{apical}} \times \text{RFU}_{\text{apical}} + V_{\text{basolatera}} \times \text{RFU}_{\text{basolatera}}} \times 100$$

where $\text{RFU}_{\text{Apical}}$ and $\text{RFU}_{\text{Basolateral}}$ demote the relative fluorescence unit values of lucifer yellow in the apical and basolateral wells, respectively, and V_{Apical} and $V_{\text{Basolateral}}$ represent the volumes of apical and basolateral wells (0.075 mL and 0.25 mL, respectively). The %Lucifer Yellow should be less than 2, indicating minimal leakage.

Measurements of BNS808 using LC-MS/MS. MS/MS analyses were conducted using a Sciex QTRAP[®] 6500+ mass spectrometer (Framingham, MA, USA). Air was generated using an SF4FF compressor (Atlas Copco, Belgium) and purified using an Infinity 1031 nitrogen generator (Peak Scientific, Inchinnan, Scotland). Purified air served as the source and exhaust gases, while purified nitrogen functioned as curtain and collision gases. A receiver was positioned between the compressor and the nitrogen generator to ensure a continuous and stable air supply.

Chromatographic separation was performed under reverse phase conditions using a Shimadzu UHPLC System (Kyoto, Japan), comprising a Nexera X2 LC-30AD pump, Shimadzu DGU-20A5R degasser, a Shimadzu SIL-30AC autosampler, and a Shimadzu CTO-20AC column oven. A CORTECS[®] column (C18, 2.7 μ m particle size, 50 $\times$ 2.1 mm, Waters Corp., Milford, MA, USA) protected by a VanGuard[®] precolumn (Waters Corp., Milford, MA, USA) was employed for chromatographic separation. The autosampler was set at 15 $^{\circ}$ C, while the column was maintained at 40 $^{\circ}$ C throughout the analysis. Data acquisition was performed using Analyst 1.7.1 on a Dell Optiplex XE2 computer, and data analysis was conducted using Sciex OS Software. Gradient elution was achieved using mobile phases consisting of 0.1% formic acid (FA) in water (phase A) and 0.1% FA in acetonitrile (phase B). The gradient elution rate was set to 300 μ L/min with an initial 5% B for the first 3 min, followed by a linear increase towards 65% B over 3 min, maintained at 65% B for 2 min, further increased linearly to 95% B in 0.5 min, and held at 95% B for 3 min.

BNS808 was analyzed in positive ion mode using electron spray ionization (ESI) and multiple reaction monitoring (MRM) acquisition modes, with BNS807 as the IS. The Turbo Spray IonDrive[™] Turbo V source temperature was set at 400 $^{\circ}$ C, and the ion spray voltage was maintained at 3500 V. The curtain gas pressure was set at 35 psi. The nebulizer gas (Gas 1) was adjusted to 40 psi, while the turbo heater gas (Gas 2) was set to 50 psi. A dwell time of 30 msec was configured for the analysis. The collision energy (CE), declustering potential (DP), and collision cell exit potential (CXP) for the monitored transitions were determined and documented in **Supplementary Table 1**.

Supplementary Table 1. The collision energy (CE), declustering potential (DP), and collision cell exit potential (CXP) for the monitored transitions.

<i>Compound</i>	<i>Molecular ion [M+H]⁺ (m/z)</i>	<i>Fragment (m/z)</i>	<i>CE (volts)</i>	<i>DP (volts)</i>	<i>CXP (volts)</i>	<i>Retention time (min)</i>
<i>BNS808</i>	550	350 (quantifier)	30	1	18	11.1
		250 (qualifier)	35	1	18	
		357(quantifier)	25	1	26	
<i>BNS807</i>	528	111 (qualifier)	79	1	12	10.8

Mini Ames assay. To conduct the assay, top agar was melted with a microwave and maintained at a temperature ≥ 47 °C in a water bath. The assay was performed using a heat block set to $45 \text{ °C} \pm 2 \text{ °C}$. 12×75 mm test tubes were prepared in duplicates. For each concentration, the following components were added sequentially to a test tube: 1600 μL of top agar, 80 μL of the test compound or control substances, 400 μL of S9 mix (a liver enzyme mixture) or PBS buffer, and 80 μL of overnight culture. The mixture was vortexed and then dispensed into wells using a disposable pipette. The plates were then incubated at 37 ± 2 °C for approximately 48 to 72 hours.

$$\text{BoldBoldf} = \frac{\text{Average number of revertants foe each dose group}}{\text{Average number of revertants for DMSO negative group}}$$

The mutagenicity of the compound was determined by evaluating the number of revertant colonies in strains TA98 and TA100. A compound was classified as mutagenic if the mean number of revertant colonies showed a dose-related and if the fold increase in revertants (F) was equal to or greater than 2 for both strains.

In addition, the cytotoxicity assessment included an evaluation of the background lawn growth on the agar plates. A scoring system ranging from 0 to 4 was employed for this assessment: 0 indicated no background lawn growth or complete cytotoxicity, while scores 1, 2, and 3 represented 25%, 50%, and 75% growth, respectively. A score of 4 signified full '100%' growth. The background lawn was evaluated using a stereoscope, with all observations compared to the vehicle control. The colonies appeared very small in plates with an entire background lawn and required magnification for visualization. In plates exhibiting toxicity, lawn colonies manifested as microcolonies (with a small diameter) or colonies (with a diameter similar to the revertant colony), which were visible without magnification.

Protein binding of test compound in mouse brain using equilibrium dialysis method. Solution A (0.5 mM) was prepared by mixing 10 μ L of a 10 mM stock solution with 190 μ L of DMSO. Subsequently, Solution B (0.02 mM) was prepared by adding 8 μ L of solution A to 192 μ L of 0.05 M sodium phosphate buffer. The final concentration of DMSO in Solution B was 4%.

A 96-well plate was initially prepared by dispensing 380 μ L aliquots of brain tissue into wells designated for the brain while the buffer was added to the remaining wells to serve as a control. Subsequently, 20 μ L of Solution B (containing 0.02 mM of test and reference compounds) was spiked into the pre-loaded brain samples in the plate. This step yielded a final test concentration of 1 μ M, containing 0.2% DMSO. A duplicate system was established for the dialysis samples wherein brain tissue was prepared against buffer. Initially, 100 μ L aliquots of blank dialysis buffer were applied to the receiver side of the dialysis chambers. Subsequently, 100 μ L aliquots of the brain

tissue spiked with test and reference compounds were applied to the donor side of the dialysis chambers.

T0 brain samples for initial concentrations were prepared in duplicate. A 25 µL aliquot of the brain spiked with test and reference compounds was transferred into a 96-well sample preparation plate. An equivalent volume of blank buffer was then added to each well. After the 5-hour incubation period, aliquots of 25 µL were taken from the dialysis apparatus's donor and receiver sides and transferred into new sample preparation plates. An equal volume of the opposite matrix (blank buffer to brain and vice versa) was added to each well, resulting in a final volume of 50 µL in each well. The samples (both T0 and five h) were then quenched with 200 µL of acetonitrile containing internal standard (IS) and vortexed at 600 rpm for 10 minutes. After vortexing, the samples were centrifuged at $5594 \times g$ for 15 minutes. Subsequently, 50 µL of the supernatants were transferred to a new 96-well plate and mixed with 50 µL of Milli-Q water. The sample plates were covered and stored in a freezer (-20 °C) until LC/MS/MS analysis.

Warfarin and quinidine were used as moderate and high brain-protein binding, respectively. For data analysis, the following equations were used:

$$\% \text{ Bound} = 100 \times ([\text{Donor}]5\text{h} - [\text{Receiver}]5\text{h}) / [\text{Donor}] 5\text{h}$$

$$\% \text{ Recovery} = 100 \times ([\text{Donor}]5\text{h} + [\text{Receiver}] 5\text{h}) / [\text{Initial}] 0\text{h}$$

$$\text{Fu} (\%) = 100 - \% \text{Bound}$$

$$\text{Undiluted Fu} (\%) = (1/D) / [(1/\text{Fu}^2 - 1) + 1/D]$$

Whereas; D: Dilution factor = Dilution factor of brain: 4

hERG assay. The electrophysiological assay was conducted with a holding potential of -80 mV. A brief voltage step to -50 mV was applied for 80 ms to eliminate any leak current. Subsequently, the voltage was stepped to +20 mV for 4,800 ms to induce the opening of hERG channels. After this activation step, the voltage was back down to -50 mV for 5,000 ms, resulting in a "rebound" or tail current, which was recorded for data analysis. Finally, the voltage was returned to the holding potential (-80 mV) for 1,000 ms.

For each experiment, a 40 μ L volume of the vehicle (control) was added, and a baseline period of 300 sec was recorded. Then, doses of the test compounds were administered in 40 μ L aliquots. The exposure of each concentration of the test compound lasted for no less than 300 sec. Five concentrations (0.30 μ M, 1.00 μ M, 3.00 μ M, 10.00 μ M, and 30.00 μ M) were tested for each compound, with a minimum of 2 replicates per concentration. Data analysis was done using DataControl, Excel 2013 (Microsoft), and GraphPad Prism 5.0.

Within each recording well, the percent of control values was calculated for each test compound concentration based on peak current amplitude in the presence of reference control, using the formula: (current response/peak current) $\times$ 100%. Subsequently, dose-response curves were fitted to the standard Hill equation to determine the IC₅₀ values using the equation:

$$I_{\text{post cpd}} / I_{\text{pre cpd}} = \text{Bottom} + (\text{Top}-\text{Bottom}) / [1+10^{(\text{LogIC}_{50}\text{X}) \times \text{HillSlope}}]$$

where X is the logarithm of the concentration, $I_{\text{post cpd}} / I_{\text{pre cpd}}$ is the normalized peak current amplitude, the Top is 1, and the Bottom is equal to 0. Curve fitting and IC₅₀ calculations were performed using GraphPad Prism 5.0. If the inhibition obtained at the lowest concentration tested was over 50%, or at the highest

concentration tested was less than 50%, the IC₅₀ was reported as less than the lowest concentration or higher than the highest concentration, respectively.

Cell viability assay. To assess the cell viability, which indicates the toxicity of the novel compounds, a total of 3,000 HepG2 cells were seeded into a 384-well plate. The compounds were then dispensed onto the cell plate using a Tecan D300e digital dispenser. Following compound addition, 35 µL CellTiter Glo reagent was added to all wells of the assay plate, and the plate was mixed for 30 sec on an orbital shaker to lyse cells. Subsequently, the plate was incubated at room temperature for 10 minutes. The luminescence signal was measured using a 384-well plate reader (EnVision). The obtained data were analyzed using XL-fit software (ID Business Solutions Ltd., Software version: XL fit 5.5.0.5). The percentage of inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = (1 - \text{Sample} / \text{ZPE}) \times 100$$

Sample represents the test compound's luminescence signal, and ZPE represents the luminescence signal from the zero-percent enzyme control.

Supplementary Table 2. Summary of in vitro MDR-MDCKII permeability of BNS8 derivatives.

ID#	GF120918 (+/-)	Mean Papp (10 ⁻⁶ cm/s)		Efflux Ratio	Mean recovery %		Rank	Note	P-gp substrate
		A to B	B to A		A to B	B to A			
Nadolol	-	0.42	ND	ND	101.89	ND	Low	Low permeability marker	-
Metoprolol	-	28.73	ND	ND	107.2	ND	High	High permeability marker	-
Digoxin	-	0.19	10.88	58.34	84.22	80.51	Low	P-gp substrate	Yes
	+	1.55	3.06	1.98	85.13	85.64			
BNS808	-	0.26	1.01	3.95	7.90	15.26	Low	*	Yes
	+	0.26	0.48	1.88	7.87	23.44			
BNS815	-	10.09	18.87	1.87	75.25	76.08	High	*	Yes
	+	15.82	9.96	0.63	63.83	77.57			
BNS816	-	6.99	5.37	0.77	41.56	48.84	High	*	No
	+	10.65	23.07	2.6	75.04	61.35			
10q (BNS8)	-	8.86	23.07	2.6	75.04	87.59	High	--	Yes
	+	20.08	13.91	0.69	63.83	86.10			

*The insufficient recovery (<50) might be caused by non-specific binding, cellular metabolism, cellular retention, or other issues

Supplementary Table 3. Mini Ames Study

TA98												
Conditions	Compound ID	Concentration (µg/well)	Number of revertants						Growth of lawn			solubility
			Replicate 1	Replicate 2	Replicate 3	MEAN	SD	F value	Replicate 1	Replicate 2	Replicate 3	
+S9	DMSO	0	9	13	11	11.00	2.00	1.00	4	4	4	P0
	BNS808	31.25	13	9	10	10.67	2.08	0.97	4	4	4	P0
		62.5	10	9	15	11.33	3.21	1.03	4	4	4	P0
		125	9	12	13	11.33	2.08	1.03	4	4	4	P0
		250	13	12	14	13.00	1.00	1.18	4	4	4	P1
		500	14	10	6	10.00	4.00	0.91	4	4	4	P2
		1000	8	10	11	9.67	1.53	0.88	4	4	4	P3
	2- aminoanthra cene (2-AA)	2	362	320	364	34 8.67	24.8 5	31.70	4	4	4	P0
-S9	DMSO	0	7	6	9	7.33	1.53	1.00	4	4	4	P0
	BNS808	31.25	2	7	11	6.67	4.51	0.91	4	4	4	P0
		62.5	10	9	7	8.67	1.53	1.18	4	4	4	P0
		125	6	5	11	7.33	3.21	1.00	4	4	4	P0
		250	1	6	5	4.00	2.65	0.55	4	4	4	P1

		500	3	8	5	5.33	2.52	0.73	4	4	4	P2
		1000	3	2	5	3.33	1.53	0.45	4	4	4	P3
	2-nitrofluorene (2-NF)	4	112	92	104	102.67	10.0 7	14.00	4	4	4	P0

P0: no precipitations in solutions or growth of crystals on plates;

P1: 0~20% growth of crystals;

P2: 20%~60% growth of crystals;

P3: 60%~100% growth of crystals.

Supplementary Table 4 – Mini Ames Study TA100												
Conditions	Compound ID	Concentration (µg/well)	Number of revertants						Growth of lawn			solubility
			Replicate 1	Replicate 2	Replicate 3	MEAN	SD	F value	Replicate 1	Replicate 2	Replicate 3	
+S9	DMSO	0	40	34	45	39.67	5.51	1.00	4	4	4	P0
	BNS808	31.25	39	37	46	40.67	4.73	1.03	4	4	4	P0
		62.5	32	32	40	34.67	4.62	0.87	4	4	4	P0
		125	27	26	37	30.00	6.08	0.76	4	4	4	P0
		250	30	33	25	29.33	4.04	0.74	4	4	4	P1
		500	16	17	25	19.33	4.93	0.49	4	4	4	P2
		1000	16	13	21	16.67	4.04	0.42	4	4	4	P3
	2-aminoanthracene (2-AA)	2	316	324	312	317.33	6.11	8.00	4	4	4	P0
-S9	DMSO	0	28	35	27	30.00	4.36	1.00	4	4	4	P0
	BNS808	31.25	33	26	28	29.00	3.61	0.97	4	4	4	P0
		62.5	30	25	16	23.67	7.09	0.79	4	4	4	P0
		125	23	22	23	22.67	0.58	0.76	4	4	4	P0
		250	25	21	23	23.00	2.00	0.77	4	4	4	P1
		500	22	18	16	18.67	3.06	0.62	4	4	4	P2

		1000	19	11	16	15.33	4.04	0.51	4	4	4	P3
	Methylnitronitrosoguanidine (MNNG)	1	182	162	154	166.00	14.42	5.53	4	4	4	P0

P0: no precipitations in solutions or growth of crystals on plates;

P1: 0~20% growth of crystals;

P2: 20%~60% growth of crystals;

P3: 60%~100% growth of crystals.

Supplementary Table 5. Cell viability assay in HepG2 cells at 72 h and mutagenic effect in a Mini Ames assay.

ID#	Absolute IC₅₀ (μM)	Relative IC₅₀ (μM)	Top (%)	Bottom (%)	Slope	Ames assay
BNS807	>100	17.95	21.39	-12.17	1.52	ND
BNS808	26.2	16.84	62.98	-8.11	3.39	*Non mutagenic
BNS815	55.88	46.69	80.41	-9.25	3.71	ND
BNS825	>100	>100	6.27	-11.62	-0.21	ND
Staurosporine	0.08	0.06	71.33	1.13	2.53	ND

ND = not determined. *Detailed Results in the **Supplementary Tables 3-4**

Supplementary Table 6. The IC₅₀ of BNS8 derivative compounds on whole cell hERG currents.

ID#	IC₅₀ (μM)
Amitriptyline	3.49
Amitriptyline	2.58
BNS807	18.04
BNS808	5.39
BNS815	4.86

Supplementary Table 7. Stability of the test compounds in simulated gastric fluid (SGF).

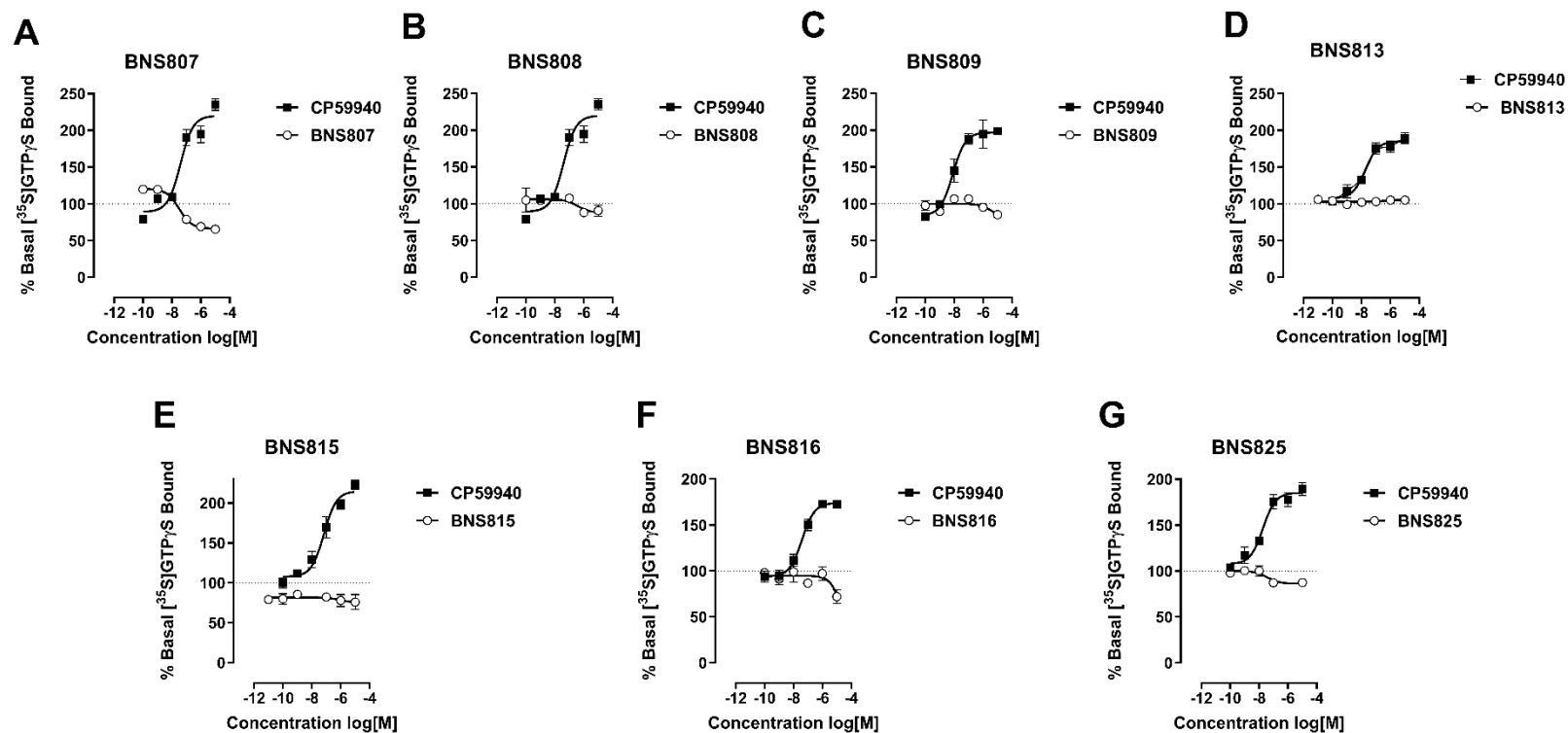
ID#	Stability in SGF					
	Time (h)	With pepsin (mg)	Initial amount (%)	No pepsin (mg)	Initial amount (%)	Stability
10q	0	2.882	100	2.945	100	Stable
	1	2.879	100	2.968	101	
BNS808	0	2.753	100	2.826	100	Stable
	1	2.69	98	2874	102	

Supplementary Table 8. Stability of the test compounds in simulated intestine fluid (SIF).

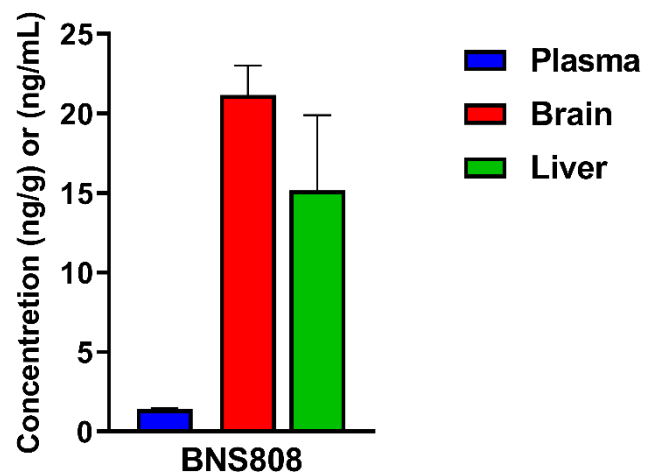
ID#	Stability in SIF						
	Time (h)	With pancreatin (mg)	Initial amount (%)	Time (h)	No pancreatin (mg)	Initial amount (%)	Stability
10 q	0	3.035	100	0	2.77	100	Stable
	1	3.113	103	1	2.79	100	
	3	3121	103	3	3.572	101	
BNS808	0	2.972	100	0	3.257	100	Stable
	1	3.023	102	1	3.277	101	
	3	3.0009	101	3	3.266	100	

Supplementary Table 9. Protein binding of BNS808 in mouse brain, using equilibrium dialysis method.

ID#	Species	Area ratio			Fraction of bound (%)	Fu (%)	Undiluted Bound (%)	Undiluted Fu (%)	Category	Recovery (%)
		Donor 0h	Donor 5h	Receiver 5h						
Warfarin	Mouse brain	-	0.146	0.088	39.5	60.5	72.3	27.7	Moderate	-
Quinidine	Mouse brain	-	0.070	0.013	82.0	18	94.8	5.2	High	-
BNS808	Mouse brain	1.135	0.928	LOD	100	0.0	100.0	0.0	High	81.8



Supplementary Figure 1. In vitro activity assessment of BNS compounds using the [^{35}S]GTP γ S binding assay. The assay was performed using mouse brain membrane preparations to evaluate CB $_1$ R activity. CP-55,940, a synthetic cannabinoid receptor agonist, was used as a reference ligand to stimulate CB $_1$ R, leading to G-protein activation. The tested compounds were evaluated for their ability to inhibit CB $_1$ R-mediated activation (A-G). The results indicate that all compounds, except BNS807 (an inverse agonist), act as neutral antagonists, as demonstrated by their lack of basal activation.

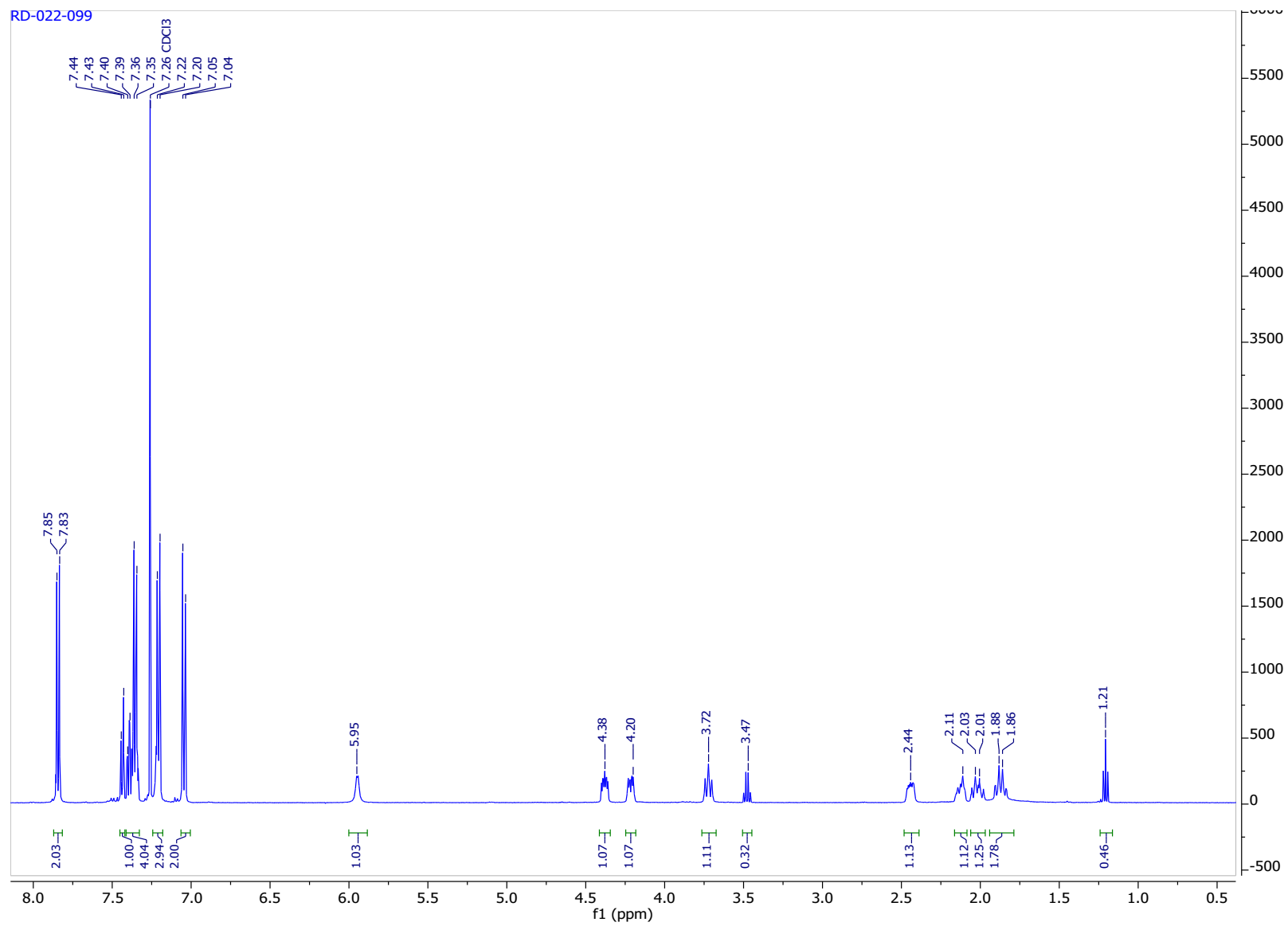


Supplementary Figure 2. BNS808 biodistribution and pharmacokinetics after chronic PO administration. The accumulation of BNS808 in organs/plasma was evaluated by analyzing the BNS808 levels in the plasma, brain, and liver post-chronic administration of 1 mg/kg PO for 24 days in diet-induced obese mice. Data represent the mean $\pm$ SEM of 4 mice per group.

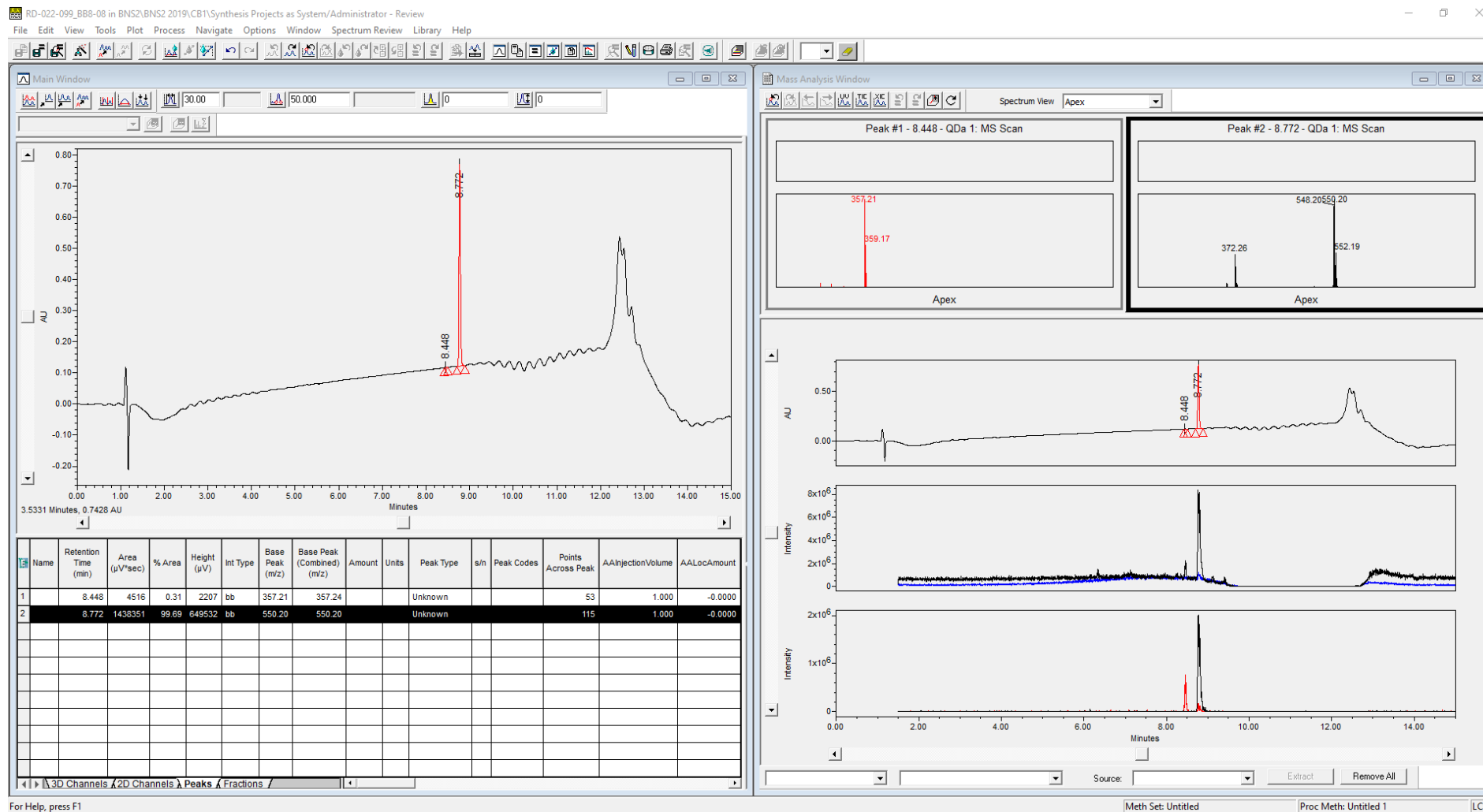
Supplementary Figure 3. The NMR, HPLC, and LC-MS analysis of BNS808

Compound Analysis				
HPLC	Column	Waters-XSelect, CSH-C18 2.5μm, 150mm×3mm		
	Method	Gradient ACN: (H ₂ O + 0.1% FA)		
	Detection	210nm		
	Purity	99%		
	Notes	LC peak at 8.772min corresponds to column and eluent.		
LC-MS	Main mass peak	550	Expected exact mass	549
	Source	ESI		
¹ H NMR	Frequency	500 MHz	Solvent	CDCl ₃
	Notes	Presence of traces of H ₂ O		

¹H NMR



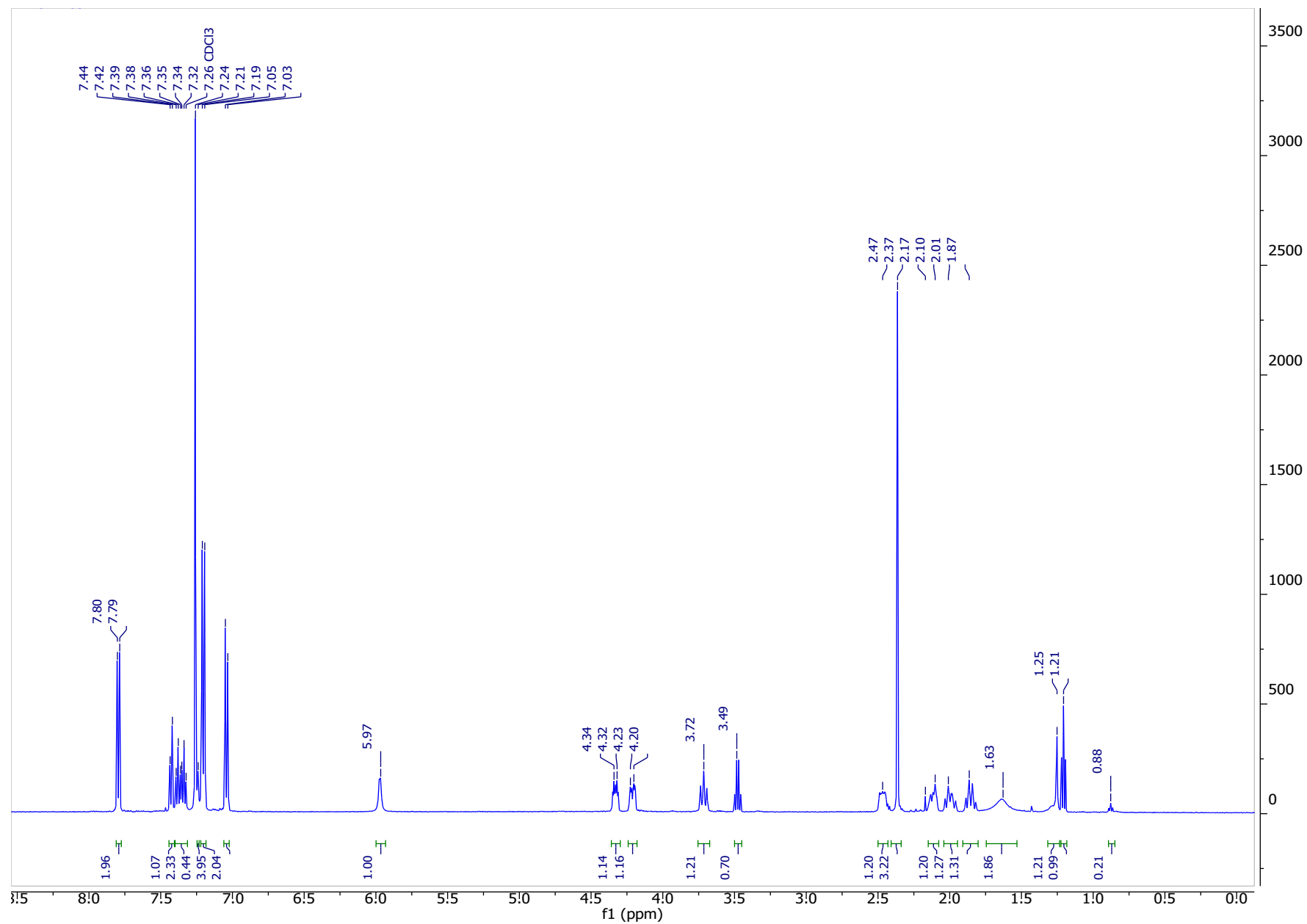
LC-MS



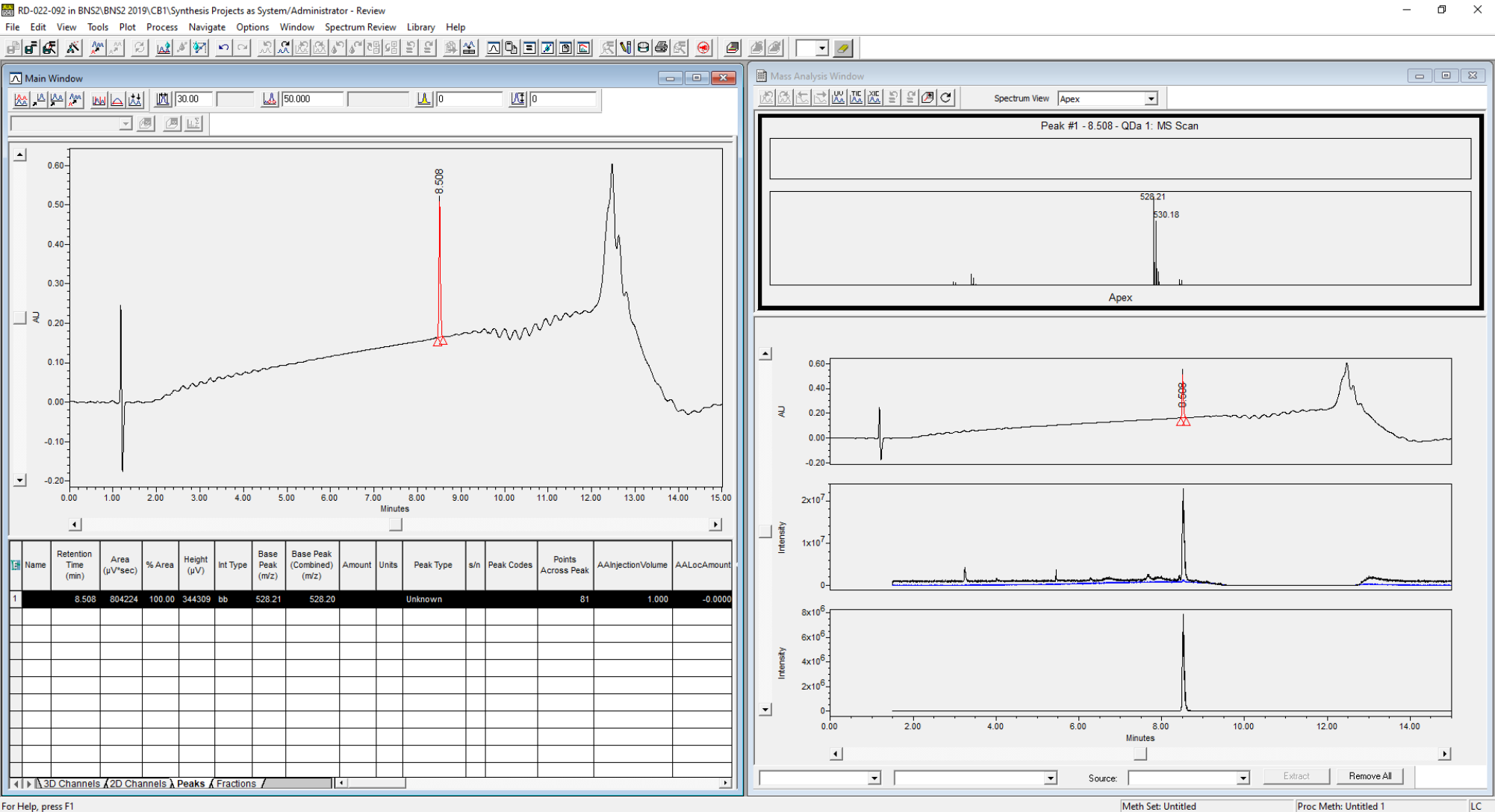
Supplementary Figure 4. The NMR, HPLC, and LC-MS analysis of BNS807

Compound Analysis				
HPLC	Column	Waters-XSelect, CSH-C18 2.5μm, 150mm×3mm		
	Method	Gradient ACN: (H ₂ O + 0.1% FA)		
	Detection	210nm		
	Purity	98%		
	Notes	LC peak at 8.508min corresponds to column and eluent.		
LC-MS	Main mass peak	528	Expected exact mass	528
	Source	ESI		
¹ H NMR	Frequency	500 MHz	Solvent	CDCl ₃
	Notes	Presence of traces of H ₂ O		

¹H NMR



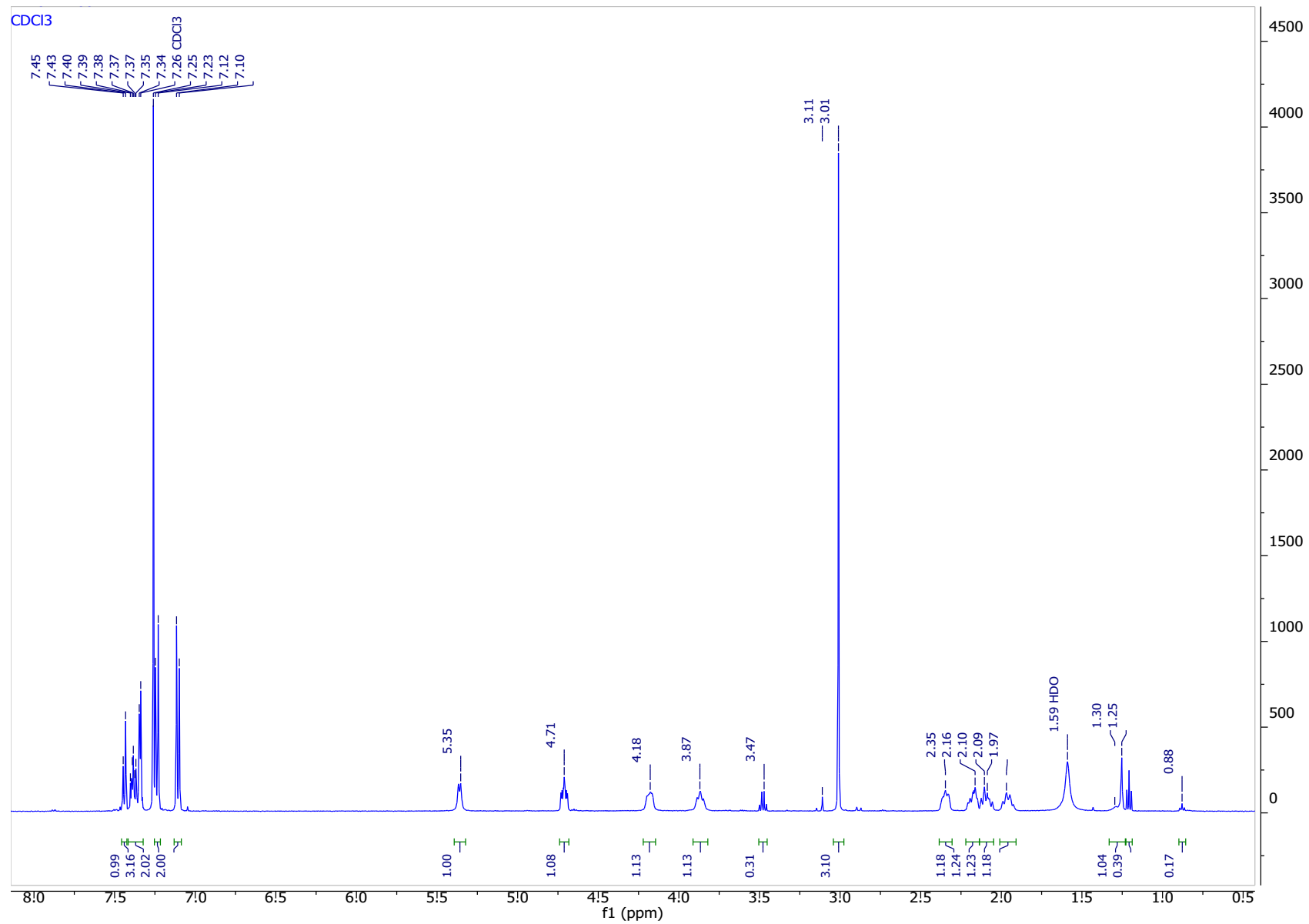
LC-MS



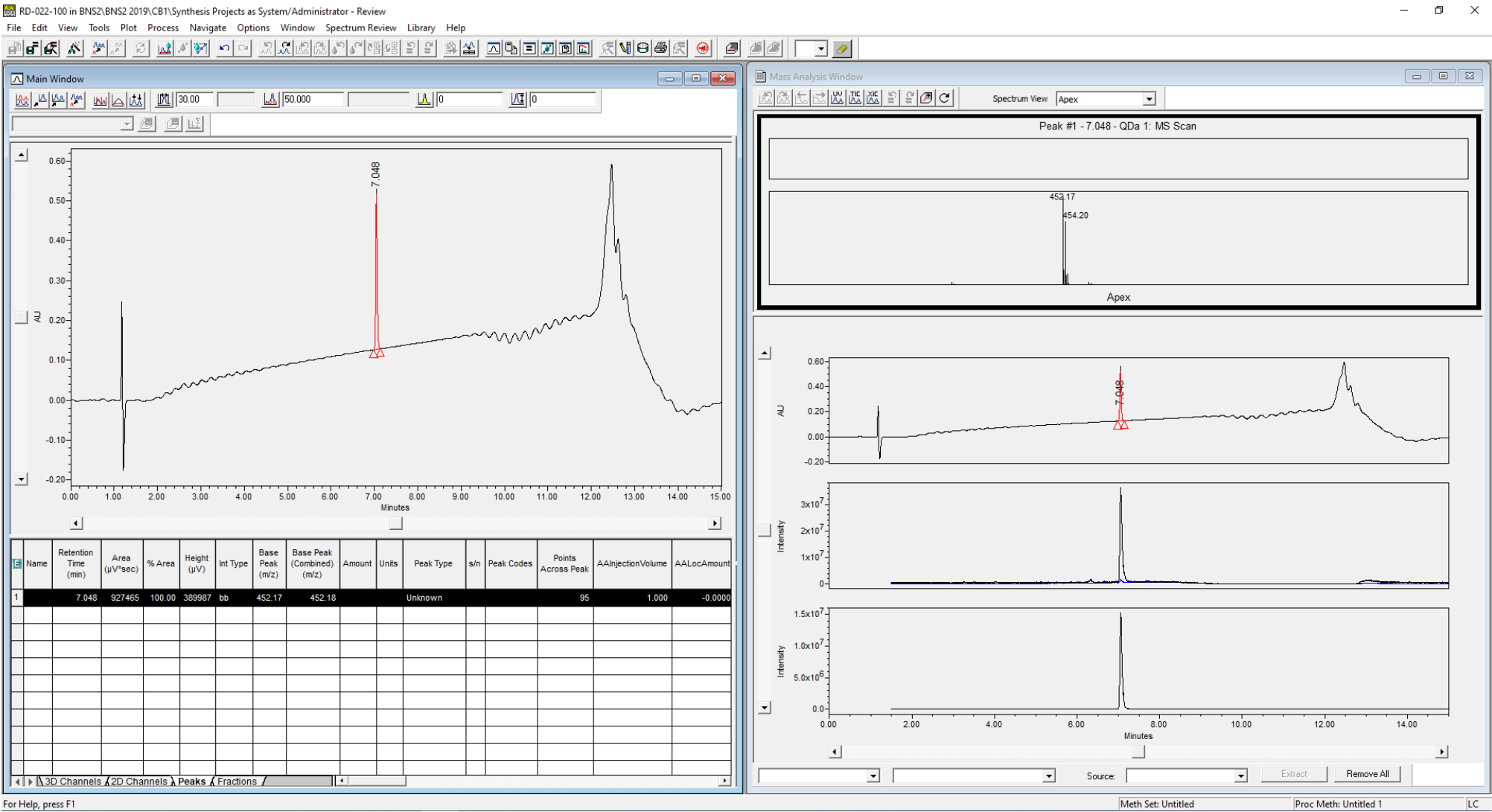
Supplementary Figure 5. The NMR, HPLC, and LC-MS analysis of BNS809

Compound Analysis				
HPLC	Column	Waters-XSelect, CSH-C18 2.5μm, 150mm×3mm		
	Method	Gradient ACN: (H ₂ O + 0.1% FA)		
	Detection	210nm		
	Purity	97%		
	Notes	LC peak at 7.048min correspond to column and eluent.		
LC-MS	Main mass peak	452	Expected exact mass	452
	Source	ESI		
¹ H NMR	Frequency	500 MHz	Solvent	CDCl ₃
	Notes	Presence of traces H ₂ O		

¹H NMR



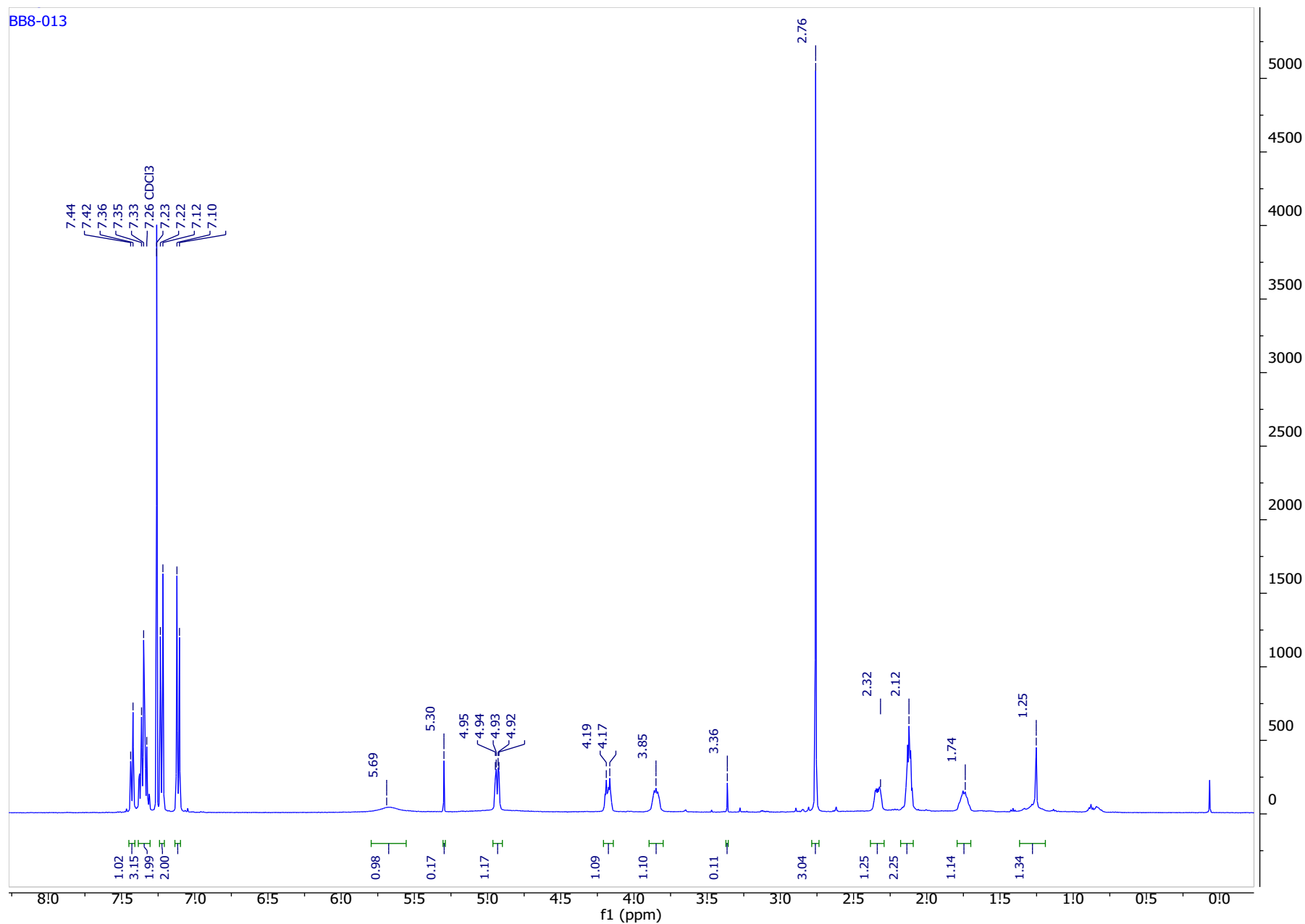
LC-MS



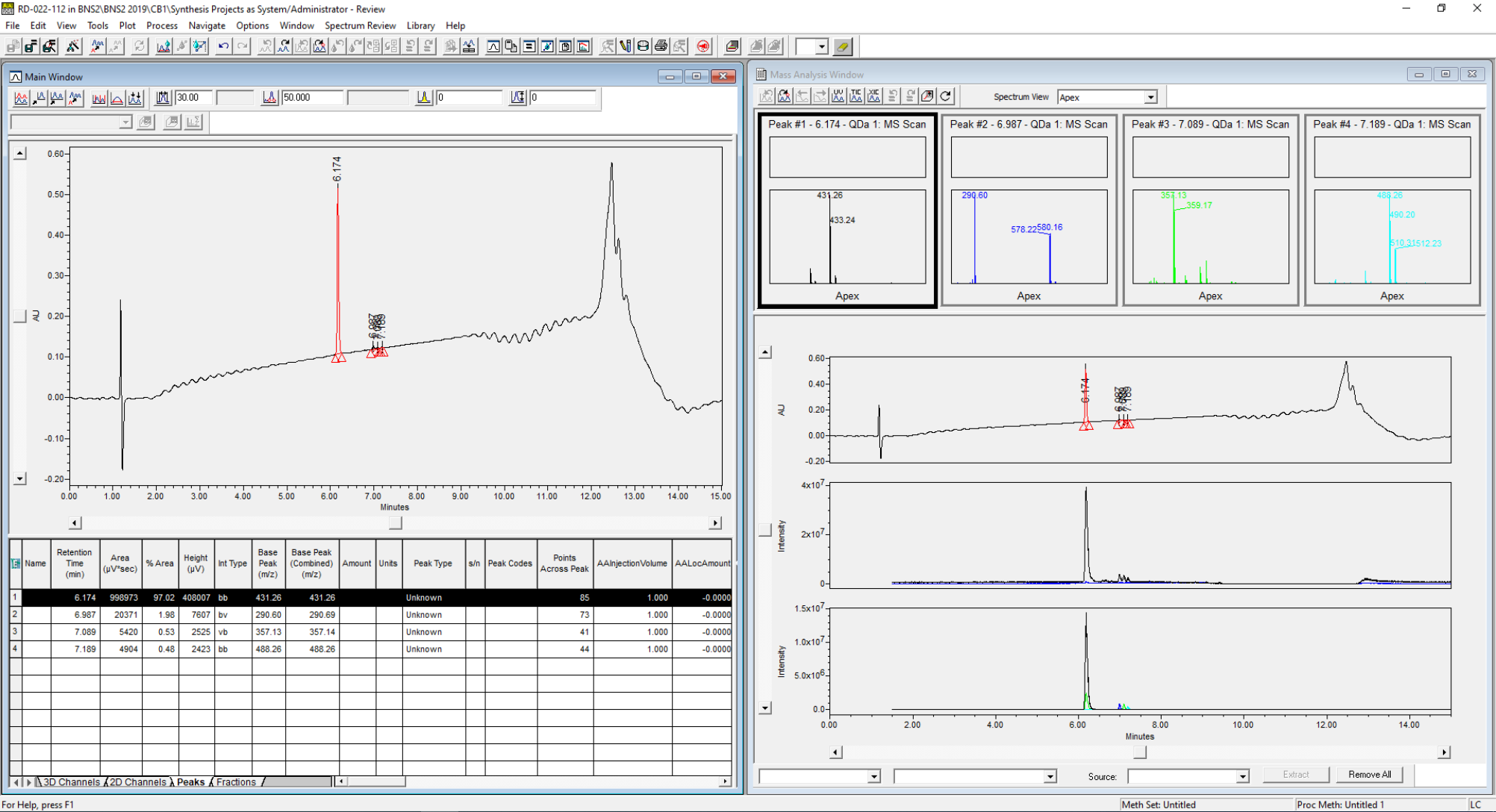
Supplementary Figure 6. The NMR, HPLC, and LC-MS analysis of BNS813

Compound Analysis				
HPLC	Column	Waters-XSelect, CSH-C18 2.5μm, 150mm×3mm		
	Method	Gradient ACN: (H ₂ O + 0.1% FA)		
	Detection	210nm		
	Purity	97%		
	Notes	LC peak at 6.174min correspond to column and eluent.		
LC-MS	Main mass peak	431	Expected exact mass	431
	Source	ESI		
¹ H NMR	Frequency	500 MHz	Solvent	CDCl ₃
	Notes	Presence of traces H ₂ O		

¹H NMR



LC-MS

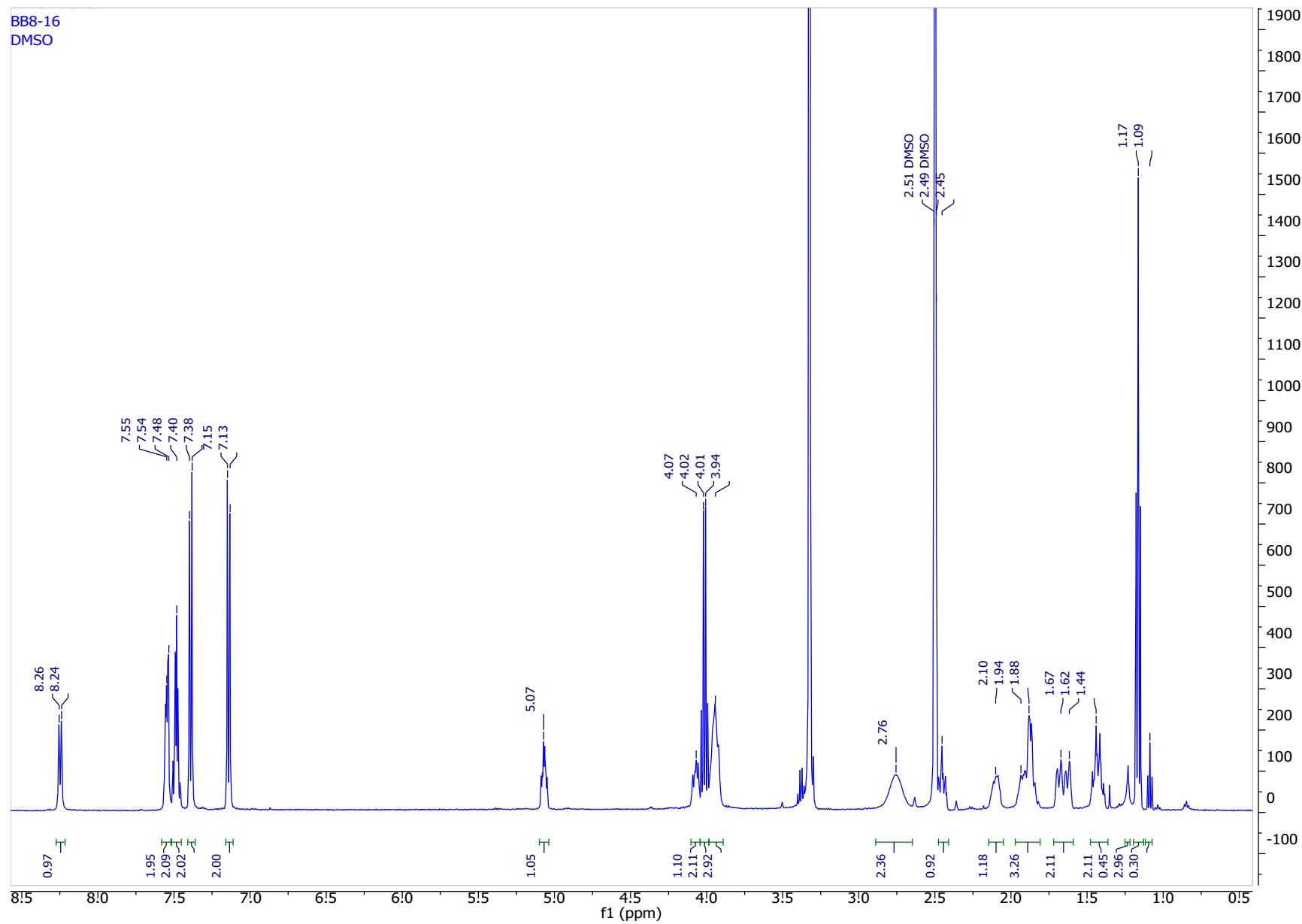


Supplementary Figure 7. The NMR, HPLC, and LC-MS analysis of BNS816

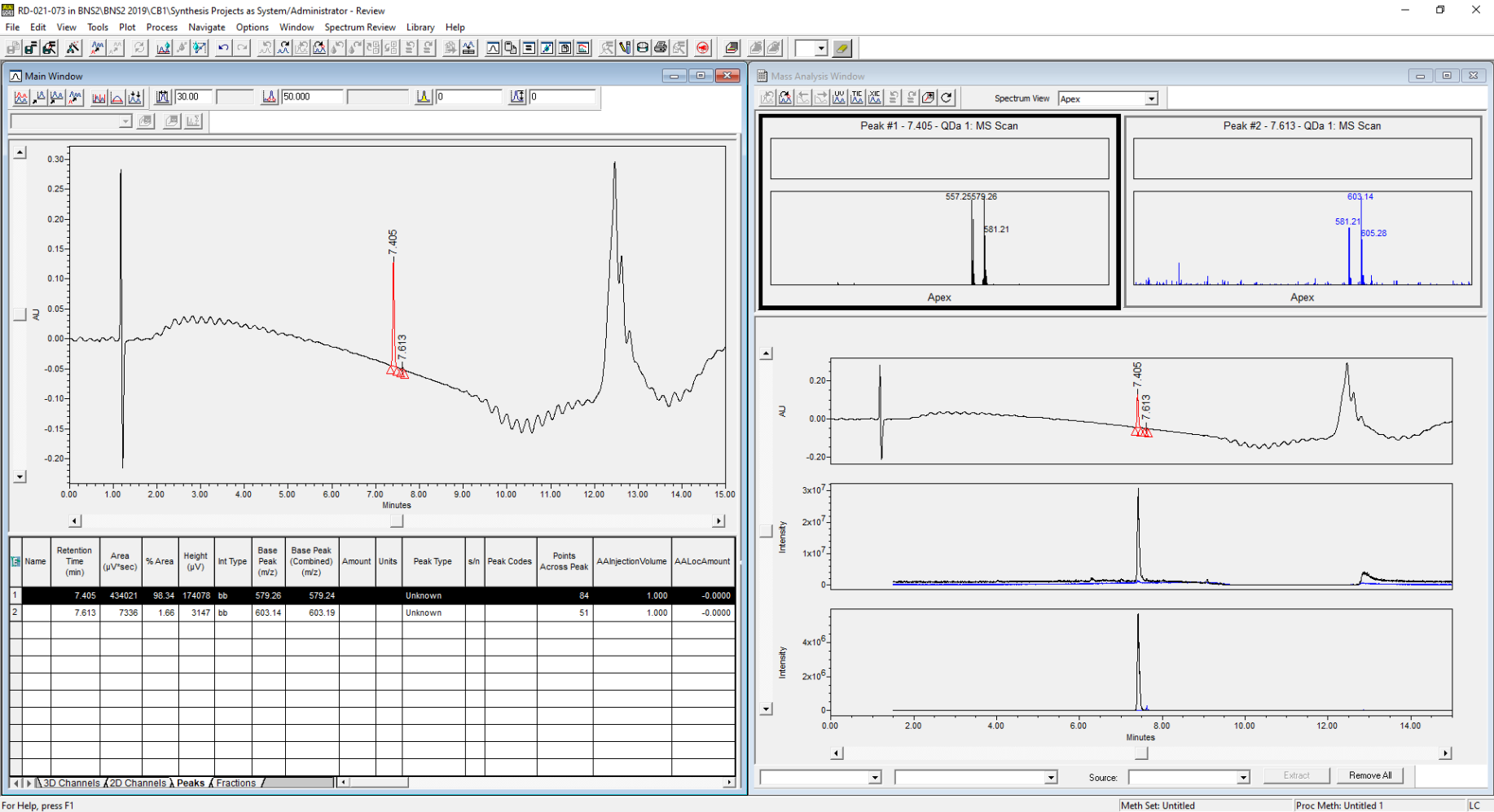
Compound Analysis				
HPLC	Column	Waters-XSelect, CSH-C18 2.5μm, 150mm×3mm		
	Method	Gradient ACN: (H ₂ O + 0.1% FA)		
	Detection	210nm		
	Purity	98%		
	Notes	LC peak at 7.405min correspond to column and eluent.		
LC-MS	Main mass peak	557	Expected exact mass	556
	Source	ESI		
¹ H NMR	Frequency	500 MHz	Solvent	DMSO
	Notes	Presence of traces H ₂ O		

¹H NMR

BB8-16
DMSO



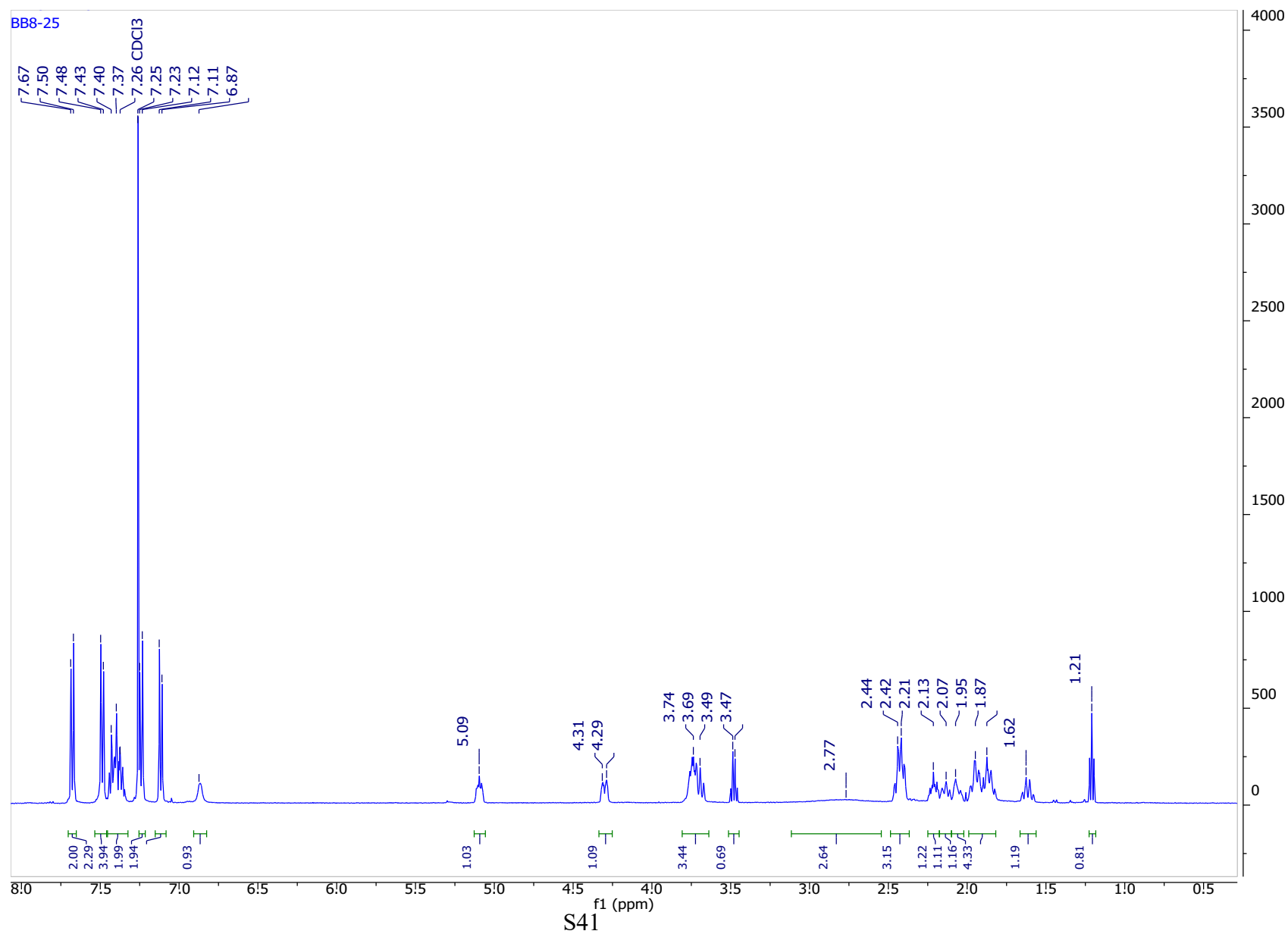
LC-MS



Supplementary Figure 8. The NMR, HPLC, and LC-MS analysis of BNS825

Compound Analysis				
HPLC	Column	Waters-XSelect, CSH-C18 2.5μm, 150mm×3mm		
	Method	Gradient ACN: (H ₂ O + 0.1% FA), 15min		
	Detection	210nm		
	Purity	100%		
	Notes	LC peak at 7.849min correspond to column and eluent.		
LC-MS	Main mass peak	659	Expected exact mass	660
	Source	ESI		
¹ H NMR	Frequency	500 MHz	Solvent	CDCl ₃
	Notes			

¹H NMR



LC-MS

