Supporting Information

Synthesis and Evaluation of Conjugates of Novel TLR7 Inert Ligands as Self-Adjuvanting Immunopotentiators

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EXPERIMENTAL PROCEDURES

All of the reagents and solvents were reagent grade and were obtained from commercial companies, and used without further purification. Moisture- or air-sensitive reactions were conducted under nitrogen atmosphere in oven-dried glass apparatus. The solvents were removed under reduced pressure using standard rotary evaporators. Agilent 6530 ESI-QTOF mass spectrometer was used operating in the positive ion acquisition mode. All the compounds synthesized were obtained as solids. 5-6 weeks BALB/c and C57BL/6 mice were purchased from Guangdong Medical Laboratory Animal Center and were bred at 22 ± 0.5 °C on a 12/12 h light-dark cycle from 7 a.m. to 7 p.m. All procedures and protocols were approved by the Institutional Animal Care and Use Committee.

Synthesis of Compound 2: 2-chloroadenine (**1**) (256 mg), methyl 4-bromobutyrate (546 mg), K_2CO_3 (500 mg) and KI (50 mg) were combined in 2 mL of DMF, and the mixture was stirred at room temperature for 12 h. After filtration to remove insoluble inorganic salts, the dried filtrate was subjected to flash silica gel chromatography using 1:19 MeOH/DCM to obtain 9-methylbutyrate-2-chloroadenine (**2**), ^{1}H NMR (500 MHz, *DMSO-d*$_6$) δ 8.111 (s, 1H), 7.726 (s, 2H), 4.109 (s, 2H), 3.512 (s, 3H), 2.288 (s, 2H), 2.018 (s, 2H). ^{13}C NMR (126 MHz, DMSO-*d*$_6$) δ 173.124, 157.314, 153.501, 151.247, 142.039, 118.360, 51.929, 42.993, 30.882, 25.269. MS (ESI) calculated for $C_{10}H_{12}ClN_5O_2$, *m/z* [M + 1] 270.0680; found 270.0747 $(M + H)^+$.

Synthesis of Compound 3: The sodium salt of 2-methoxyethanol was prepared by dissolving 200 mg of sodium metal in 10 mL of 2-methoxyethanol, and then 540 mg of compound **2** was added. The reaction mixture was heated for 6 h at 90°C and cooled in ice, pH was adjusted to 3, the separated crystals were filtered. The residue was chromatographed on silica gel using 1:24 MeOH/DCM to yield 9-(3-carboxypropyl)-2-(2-methoxyethoxy)-adenine (**3**), ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 12.153 (brs, 1H), 7.909 (s, 1H), 7.184 (s, 2H), 4.313 (t, *J* = 4.80 Hz, 2H), 4.054 (t, *J* = 6.80 Hz, 2H), 3.611 (t, *J* = 4.80 Hz, 2H), 3.281 (s, 3H), 2.194 (t, *J* = 7.20 Hz, 2H), 1.993 (t, *J* = 6.80 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 174.15, 161.63, 157.16, 151.72, 140.00, 115.65, 70.81, 65.72, 58.57, 42.52, 31.13, 25.30. MS (ESI) calculated for $C_{12}H_{17}N_5O_4$, *m/z* [M + 1] 296.1281; found 296.1352 $(M + H)^+$.

Synthesis of Compound 4: 100 mg of compound **3** was dissolved in DCM (60 mL) and bromine (1 mL) was added dropwise. The mixture was stirred for 12 h at 30°C and extracted first with 250 mL of a 0.1 M aqueous sodium thiosulfate solution, then with 60 mL of aqueous sodium bicarbonate (saturated). The residue from the organic layer was chromatographed on silica gel using 1:19 MeOH/DCM to yield 9-(3-carboxypropyl)-8-bromo-2-(2-methoxyethoxy)-adenine (**4**), ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 12.207 (brs, 1H), 7.398 (brs, 2H), 4.314 (t, *J* = 4.80 Hz, 2H), 4.059 (t, *J* = 6.80 Hz, 2H), 3.612 (t, *J* = 4.80 Hz, 2H), 3.454 (s, 3H), 2.234 (t, *J* = 7.20 Hz, 2H), 1.957 (t, *J* = 10.40 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 173.98, 161.49, 156.01, 152.74, 124.20, 115.85, 70.71, 65.94, 58.57, 43.32, 30.93, 24.59. MS (ESI) calculated for $C_{12}H_{16}BrN_5O_4$, *m/z* [M + 1] 374.0386; found 374.0460 $(M + H)^+$.

Synthesis of Compound 5: Compound **4** (100 mg) was added to 1:1 MeOH /10M NaOH (20 mL) and the mixture refluxed for 36 h. The reaction mixture was allowed to cool and acidified pH to 3 with conc HCl. The aqueous solution was extracted with DCM (3 × 20 mL) and the organic layer concentrated *in vacuo* to yield a mixture of compound **5** and 9-(3-carboxypropyl)-8-methoxy-2-(2-methoxyethoxy)-adenine. The residue was chromatographed on silica gel using 1:19 MeOH/DCM to yield 9-(3-carboxypropyl)-8-hydroxy-2-(2-methoxyethoxy)-adenine (**5**), ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 12.084 (brs, 1H), 9.954 (s, 1H), 6.486 (s, 2H), 4.249 (t, *J* = 3.60 Hz, 2H), 3.693 (t, *J* = 5.20 Hz, 2H), 3.584 (t, *J* = 3.60 Hz, 2H), 3.266 (s, 3H), 2.205 (t, *J* = 6.00 Hz, 2H), 1.844 (t, *J* = 5.60 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 174.15, 160.09, 152.67, 149.75, 147.88, 98.61, 70.62, 65.65, 58.45, 38.86, 31.14, 23.80. MS (ESI) calculated for $C_{12}H_{17}N_5O_5$, *m/z* [M + 1] 312.1230; found 334.1120 $(M + Na)^+$.

Conjugation. The 9-(3-carboxypropyl) group on GD2 enabled facile coupling to a variety of antigens with free amino groups by using well established conjugation chemistry. We chose EDCI as crosslinking agent and OVA, BSA, MSA, MG7 (Lys-Pro-His-Val-His-Thr-Lys) and Thymosin α1 bovine (Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn) (Sigma, St. Louis, MO) as the antigens. Initially, to a solution of GD2 (5.879 mg) in DMSO (100 μL) were added EDCI (2.979 mg) and NHS (2.332 mg), and the reaction mixture was shaken for 24 h to link the 3-carboxypropyl groups on GD2

with EDCI. Then the modified GD2 was added to the antigens at 10-, 20-, or 40-fold molar excess in PBS (for OVA, BSA and MSA) or DMSO (for MG7 and Thymosin) and incubated for 24 h at 4°C.[23] The excess EDCI and unreacted GD2 were removed by size-exclusion gel filtration (millipore UFC901024 15M, 10KD), eluted with PBS from GD2-OVA, GD2-BSA, GD2-MSA. GD2-MG7, GD2-Thymosin purified by affinity chromatography.

Quantification of GD2 molecules covalently bound to antigens. Different concentrations of GD2 or antigens were subjected to spectrophotometric analysis and the readout was used to derive standard curve (GD2, y=39.695x-0.0087, R^2=0.998; BSA, y=0.6546x+0.0771, R^2=0.9997; OVA, y=0.5537x+0.075, R^2=0.9995; MSA, y=0.6645x+0.0698, R^2=0.9999; MG7, y=11.408x+0.0283, R^2=0.9999; Thymosin α1 bovine, y=7.4612x+0.0162, R^2=0.9997). The concentrations of conjugates were determined with Coomassie Brilliant Blue method and the absorbance were subjected to spectrophotometric analysis. The concentration of GD2 in conjugates can be calculated by standard curves above. Thus, the GD2 numbers bound to antigens were calculated as [GD2 : antigen = (Conc. of GD2/mass of GD2)/(Conc. of antigen/mass of antigen). The coupling ratio using different material input ratios was done and shown in the Table S1. The conjugates of 40:1 set was chosen for further study. The whole experiment procedure can be found in the previously published paper (Gao et al., 2014, http://dx.doi.org/10.1016/j.bmcl.2014.10.034).

Table S1

Material input ratio (antigen:GD2)	Approximate GD2 molecules linked to antigen				
	GD2-BSA	GD2-OVA	GD2-MSA	GD2-MG7	GD2-Thymosin
40:1	6.1500	5.1436	4.9125	1.0542	3.9453
20:1	3.6053	2.9845	2.0024	1.1954	3.1342
10:1	2.1235	1.9887	1.4524	0.6079	1.7899

***In Vitro* Stimulation of Splenocyte.** Splenocytes were isolated from BALB/c mice and were seeded in 96-well plates at a density of 1×10^6/mL cells. Compound GD2 and UC-1 V150 were added to wells at a final concentration ranging from 0.01 to 10 μM. We used the conjugation ratio to determine the added concentration of conjugates and antigens. The concentrations of BSA, OVA, MSA, MG7, thymosin and their conjugates were used with one-sixth, -fifth, -fifth, equal, or a quarter of the number as indicated at the x-axis, respectively. After 24 h of incubation, culture supernatants were collected and the cytokines were determined by ELISA (eBioscience) according to the manufacturer's instructions. To confirm that the GD2 conjugates stimulated via TLR7 activation, we analyzed IL-12 cytokine inductions with BMDM derived from TLR7 deficient mice (Model Animal Research Center of Nanjing University). The wild type or $TLR7^{-/-}$ cells (1×10^6/mL) were incubated with the conjugates for 24 h. Culture supernatants were collected and assayed for cytokine inductions by ELISA (eBioscience).

***In Vivo* Experimental Procedures.** For *in vivo* pharmacokinetic experiments, 6 to 8 week old C57BL/6 mice were intravenously injected with 0.18 μmol of free drugs or conjugates per mouse. Blood samples were collected 2, 4, 6, 24, or 48 h after the injections. The levels of cytokines in the sera were measured by Luminex bead microassay (eBioscience). For the adaptive immune responses experiment, we based on the conjugation ratio to determine immune dosage. Mice were immunized subcutaneously at the base of the tail on days 0 and 7 with 5 nmol of GD2, 0.83 nmol of BSA, 0.83 nmol of GD2-BSA, (0.83 nmol of BSA + 5 nmol of GD2), 1 nmol of OVA, 1 nmol of GD2-OVA, or (1 nmol of OVA + 5 nmol of GD2), respectively. Sera samples were collected at 7 days intervals from days 0 to 30. Anti-BSA and anti-OVA IgG (IgG1 and IgG2a) levels were measured in mouse sera as previously described.[16] Each ELISA plate contained a titration of a previously quantitated serum to generate a standard curve. The titer of this standard was calculated as the highest dilution of serum that gave an absorbance reading that was double the background. The various sera samples were tested at a 1:100 dilution. The results are expressed in units per mL, calculated based on the units/mL of the standard serum.

Endotoxin Contamination Exclusion. Endotoxin levels in the materials for immunological activities' assessment *in vitro* and *in vivo* were measured using the Limulus Amoebocyte Lysate (LAL) assay (Pyrosate kit, Associates of Cape Cod, Inc.). BMDM from $TLR4^{-/-}$ mice (Model Animal Research Center of Nanjing University) were used to test contamination by endotoxin. One $\times10^6$/mL cells were incubated with 10 μM GD2 or 2 μM conjugates for 24 hours. The levels of IL-12 in the culture supernatants were measured by ELISA.

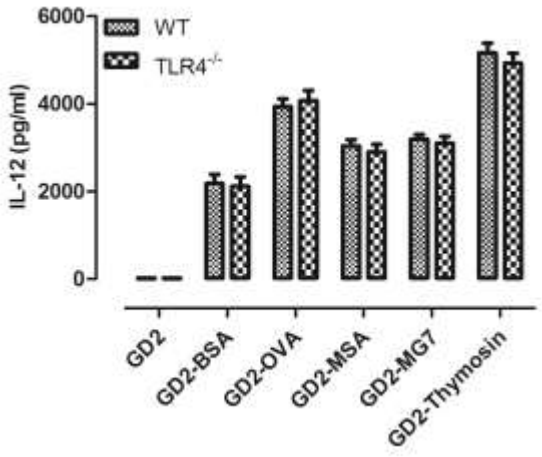
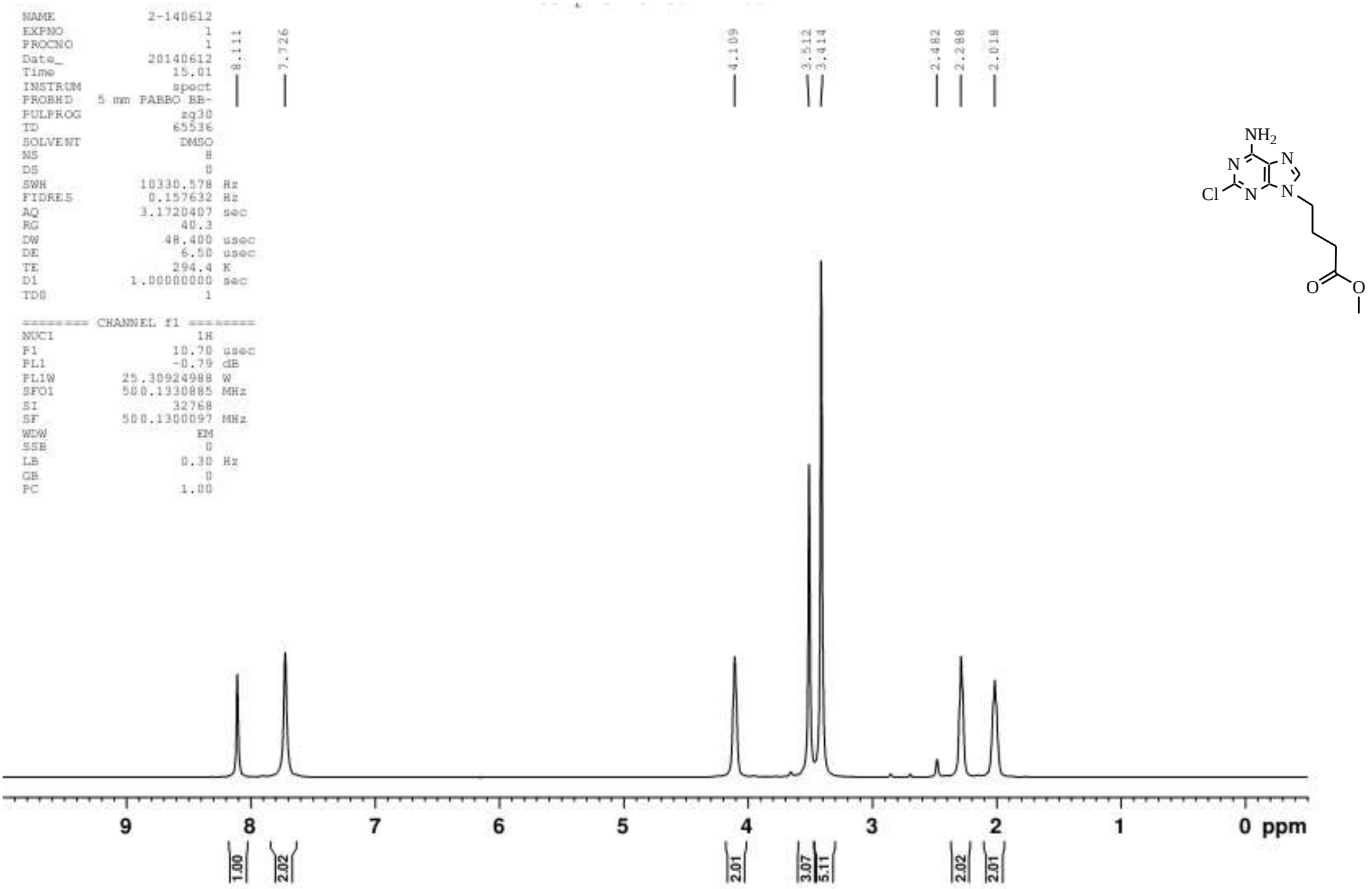


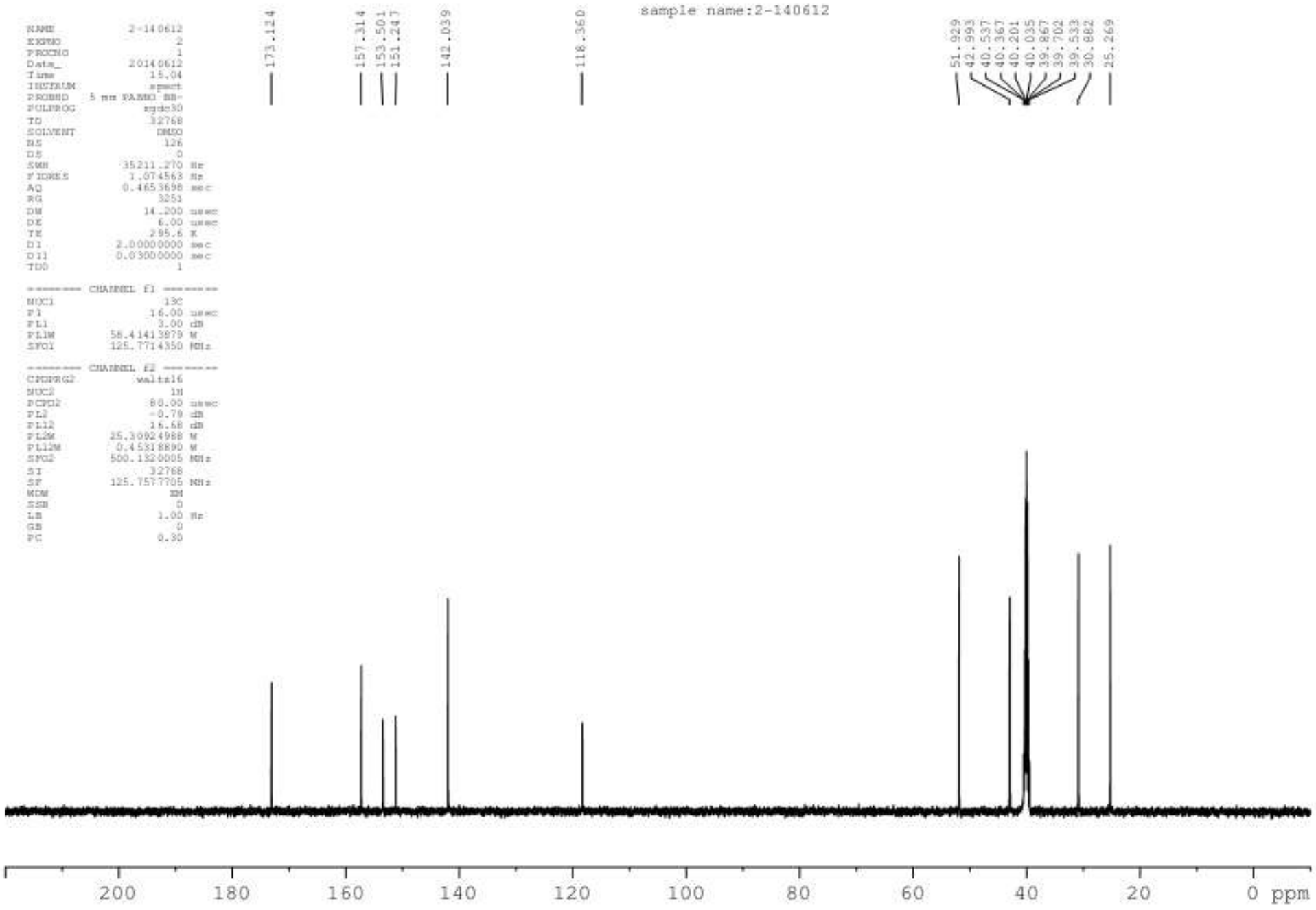
Figure S1. Evaluation of endotoxin contamination using $TLR4^{-/-}$ and wild-type macrophages. Each conjugates and GD2 induced similar levels of IL-12 both in $TLR4^{-/-}$ and wild-type mice, indicating endotoxin contamination is minimum.

Graph. The graph were performed by using Prism software version 4.0c (GraphPad, San Diego, CA) to compare differences in IL-12 and IFN-γ.

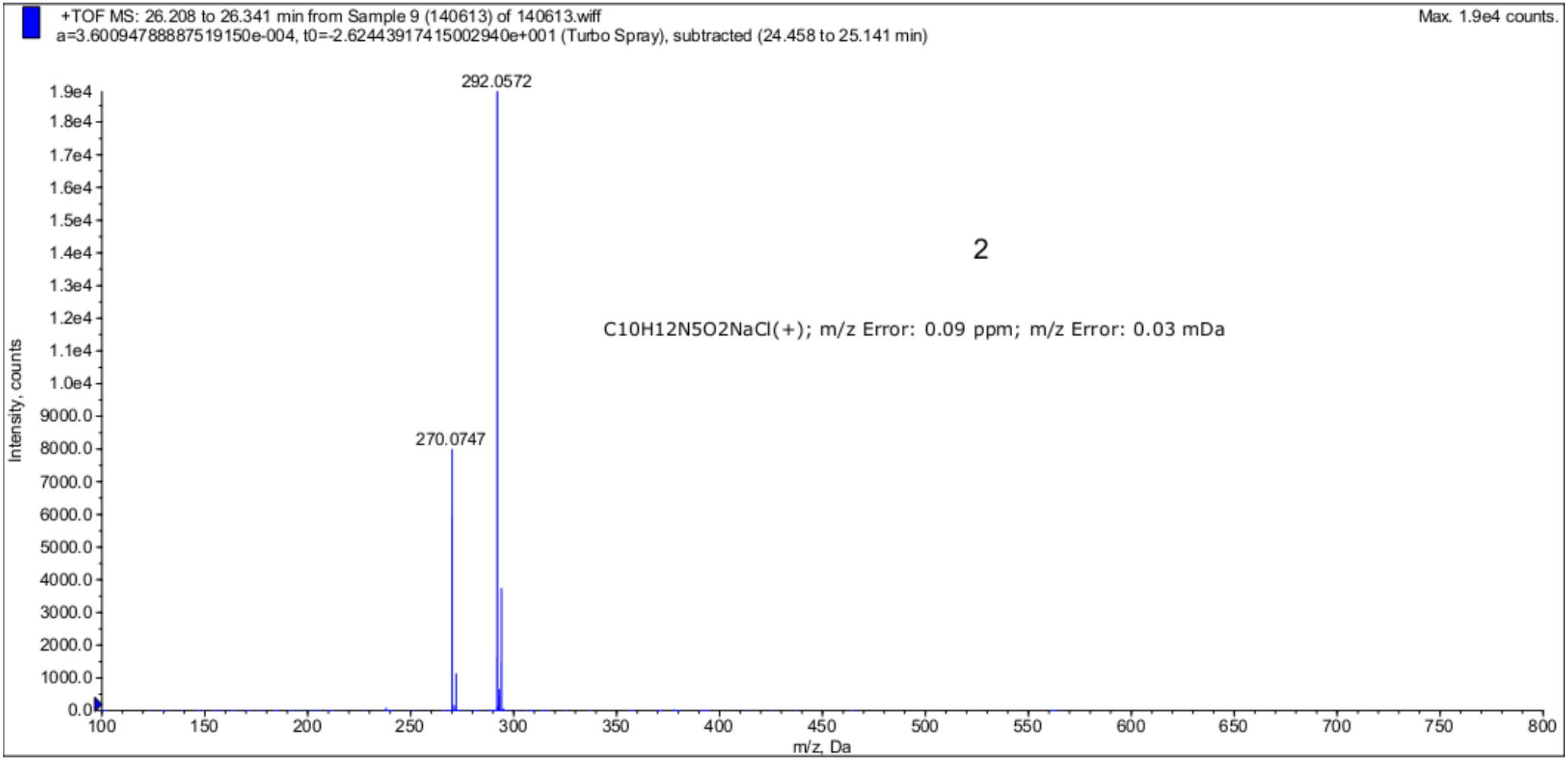
^{1}H specturm of compound 2 ($C_{10}H_{12}ClN_5O_2$; 500 MHz, DMSO-d_6)



^{1}C specturm of compound 2 ($C_{10}H_{12}ClN_5O_2$; 126 MHz, DMSO-d_6)



MS of compound 2 ($C_{10}H_{12}ClN_5O_2$)



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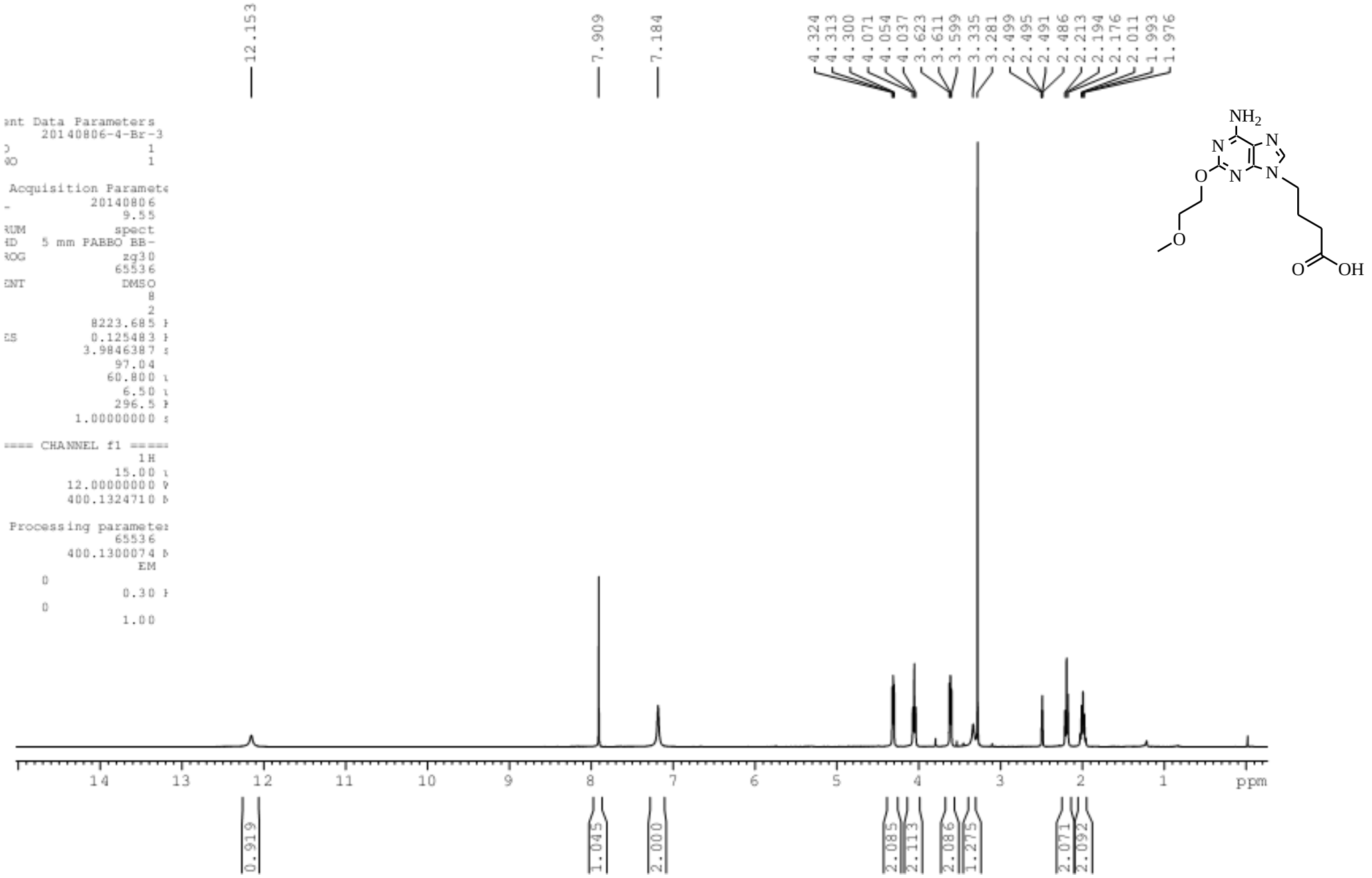

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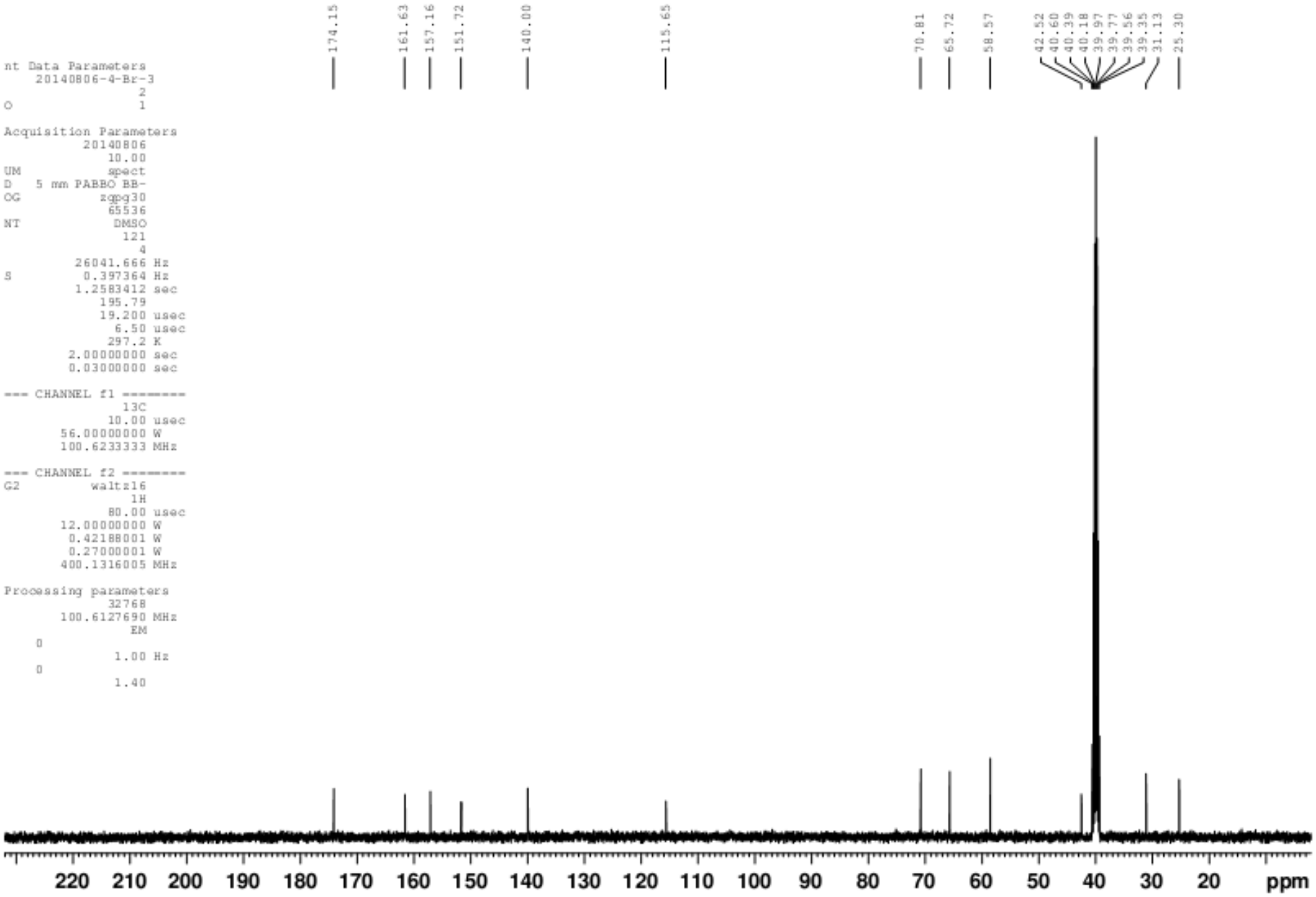
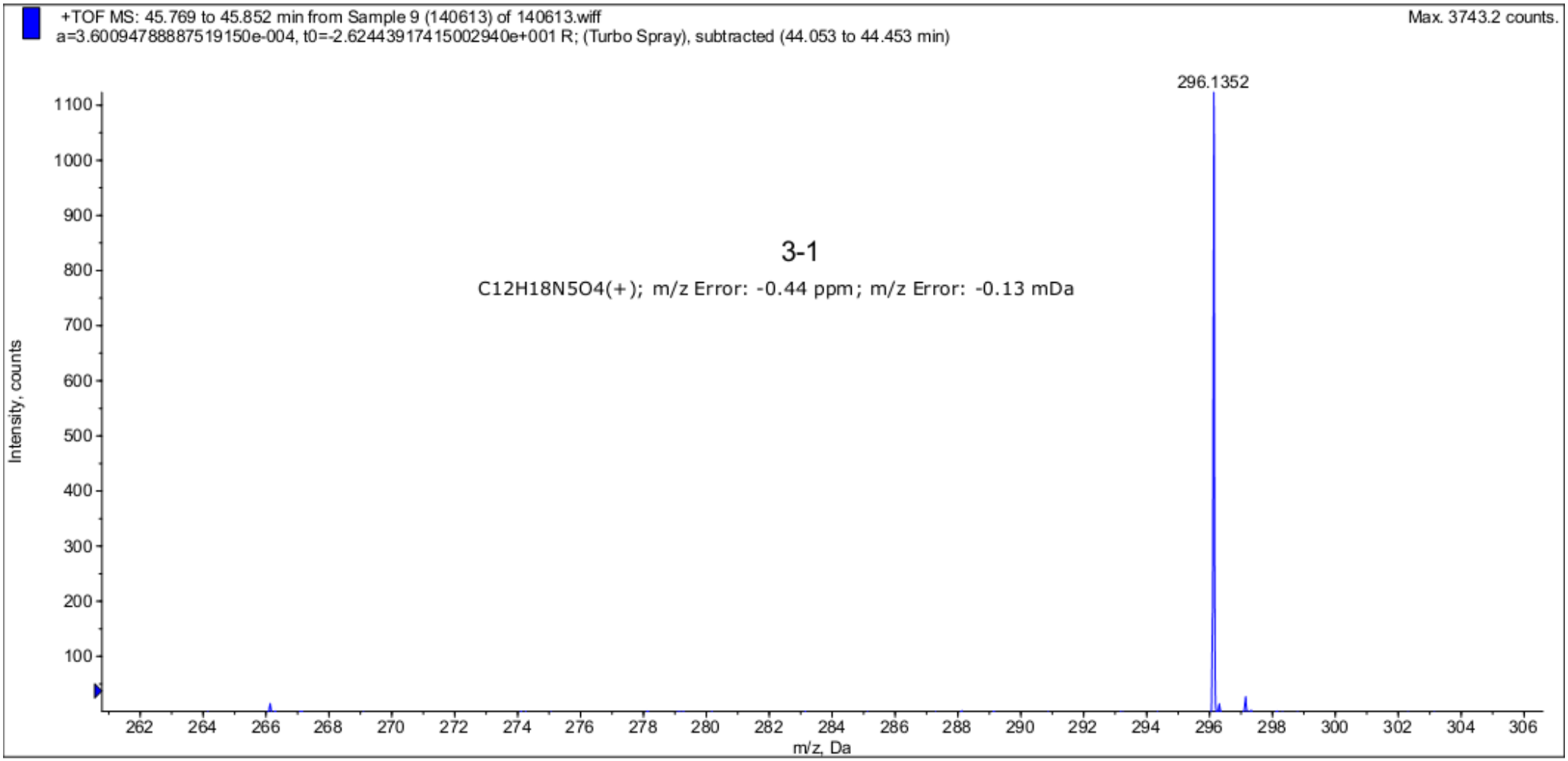
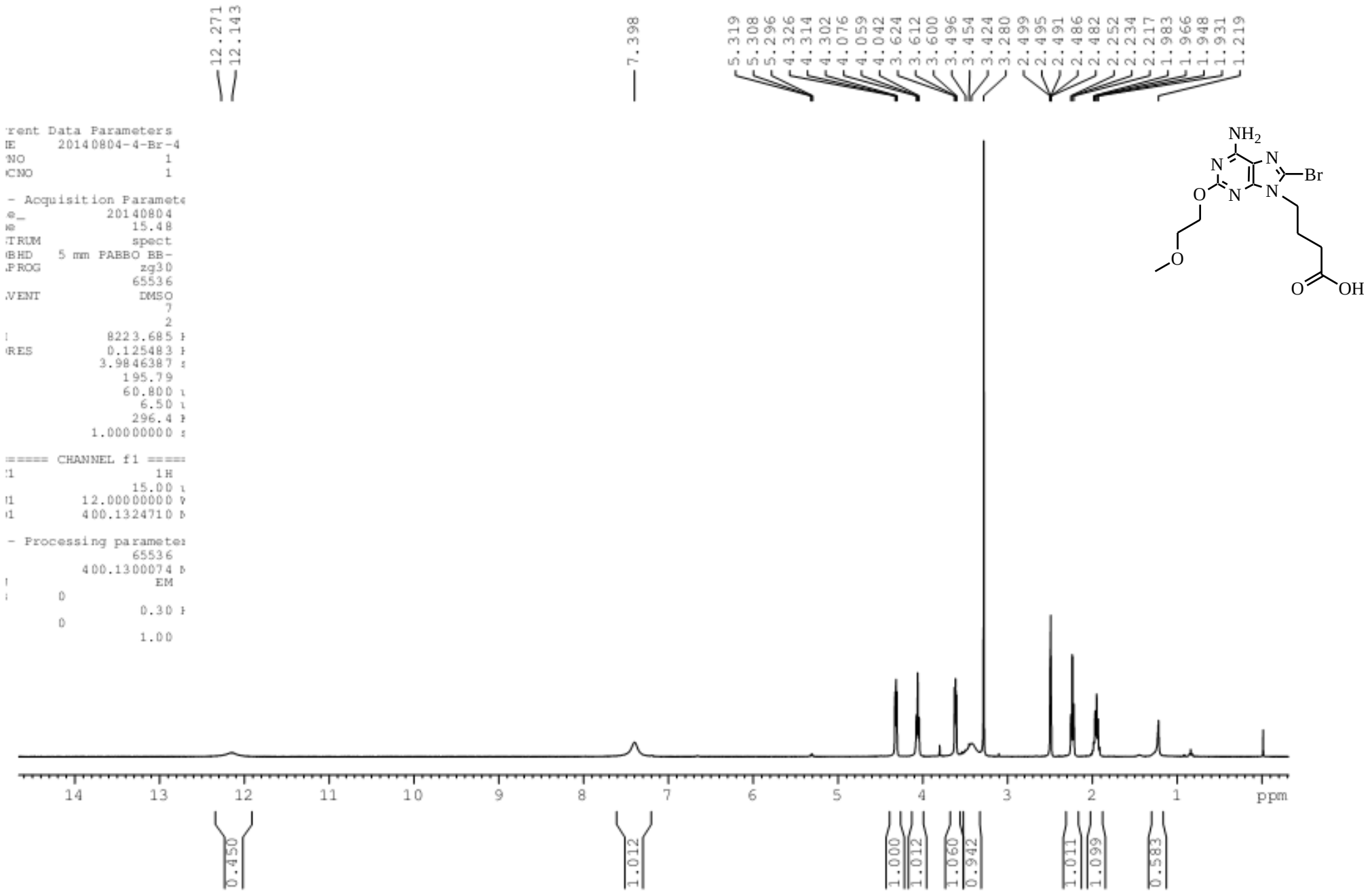


MS of compound 3 ($C_{12}H_{17}N_5O_4$)



^{1}H specturm of compound 4 ($C_{12}H_{16}BrN_5O_4$; 400 MHz, DMSO-d_6)



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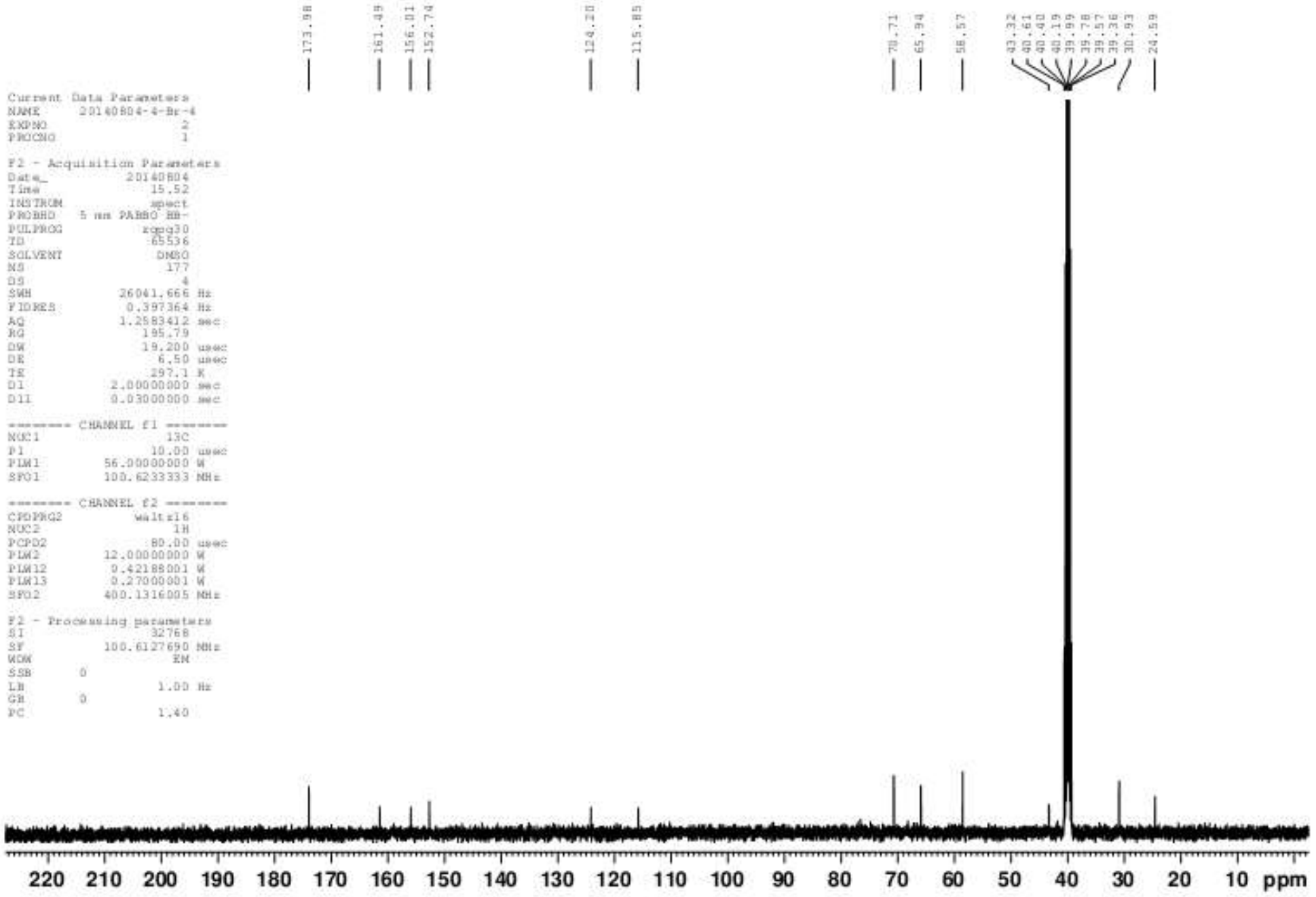
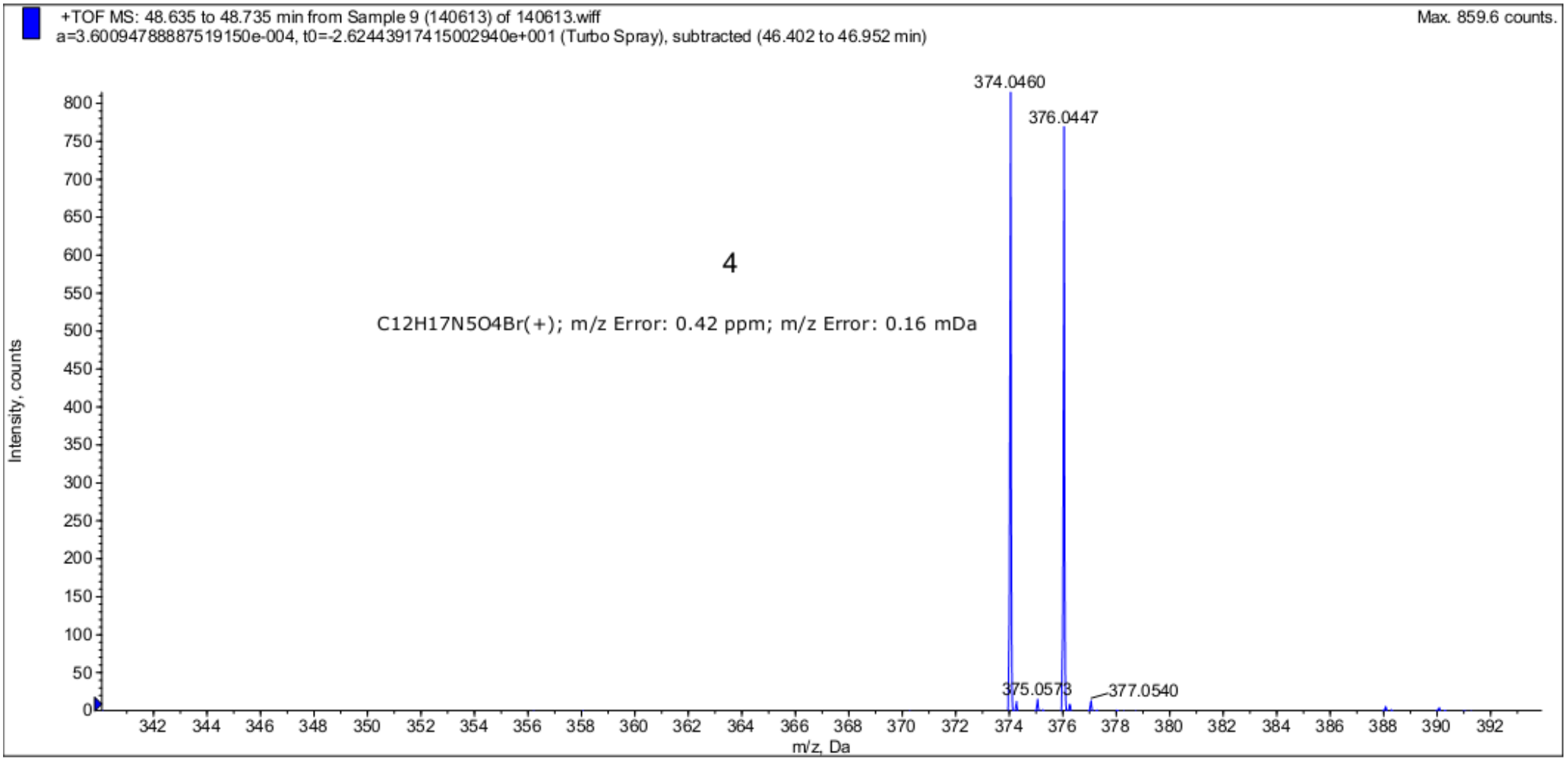


MS of compound 4 ($C_{12}H_{16}BrN_5O_4$)



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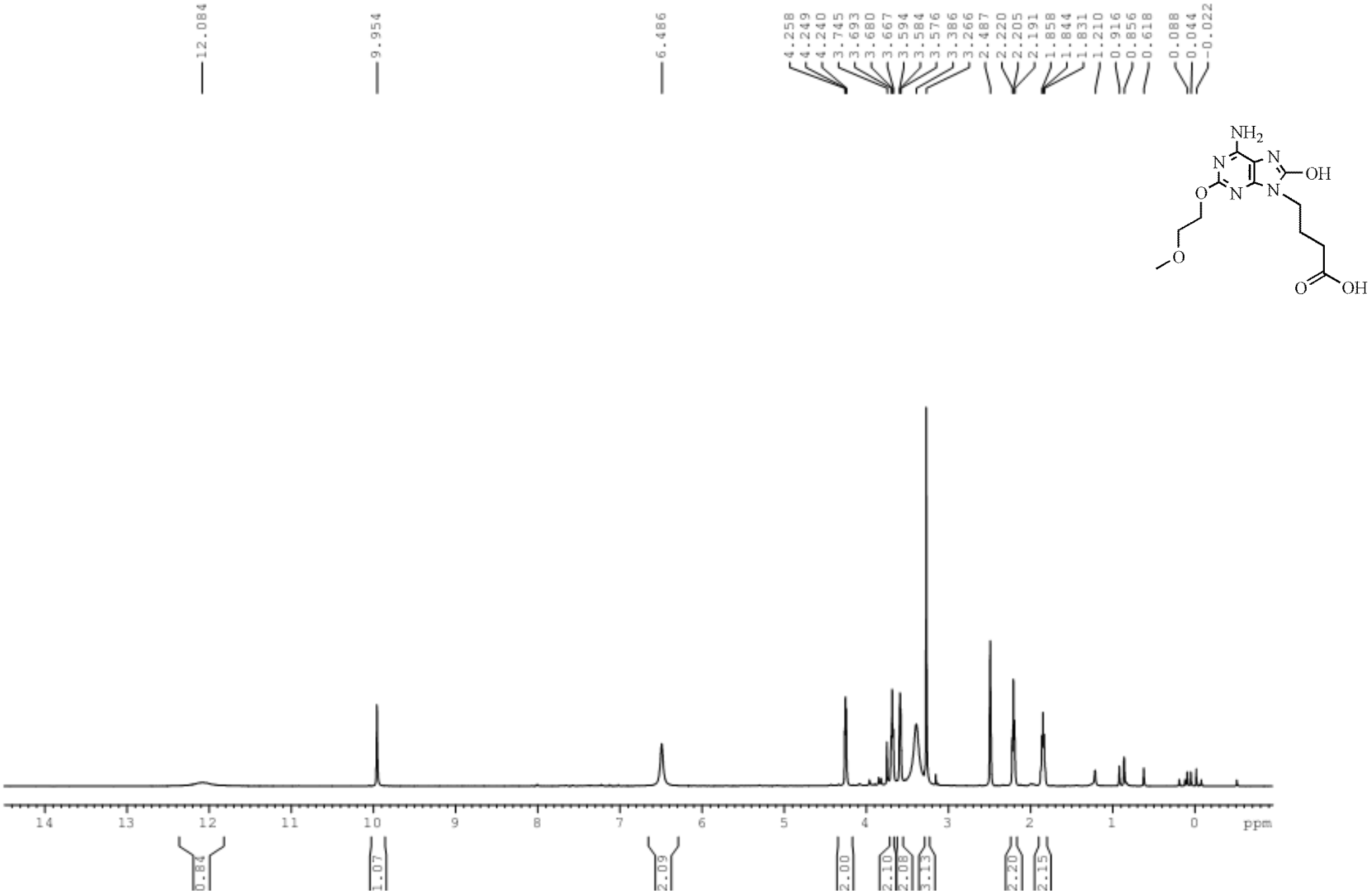

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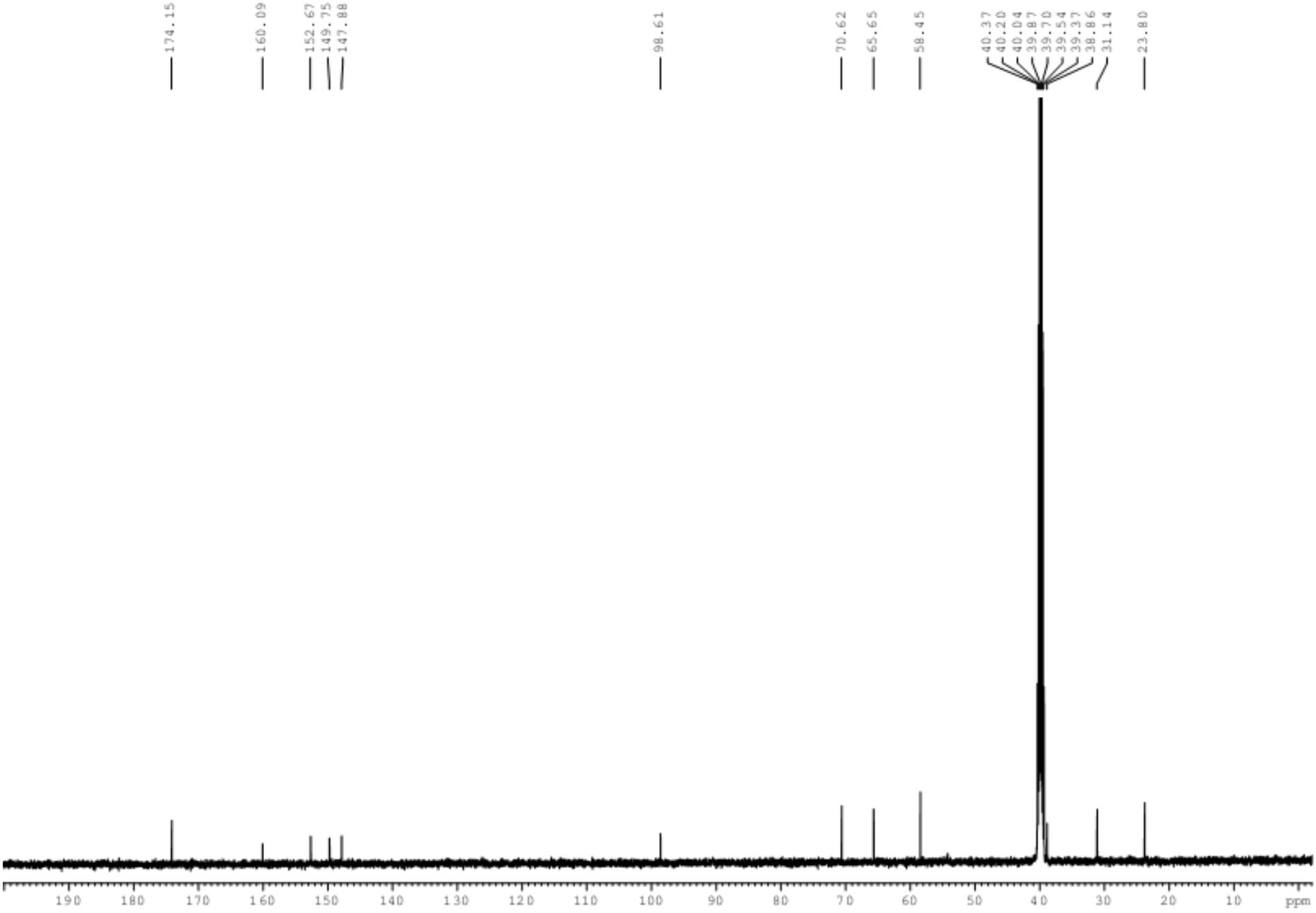

MS of compound 5 ($C_{12}H_{17}N_5O_5$)