Electronic Supplementary Material (ESI) for MedChemComm.

Supplementary material

4-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1,2,5-thiadiazole-3-carboxamide: a novel inhibitor of the canonical NF-kB cascade.

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Table of contents

Additional biochemical data.

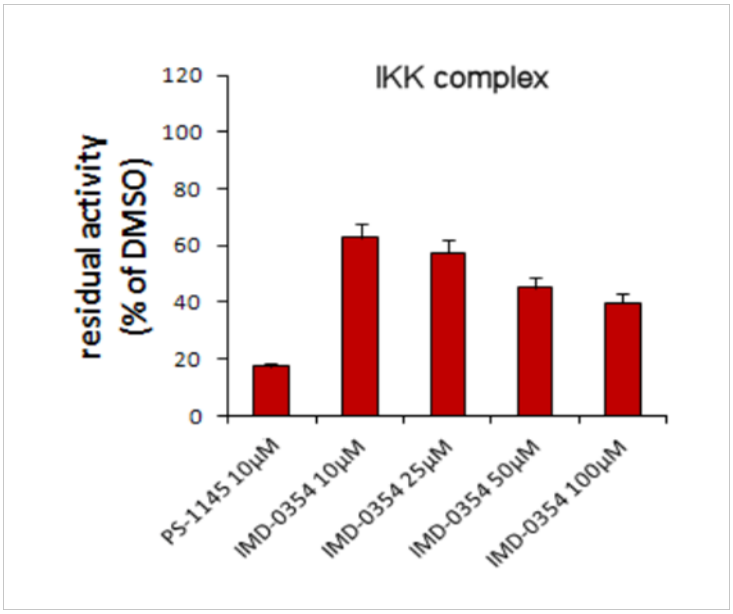


Figure S1. Effects of IMD-0354 on IKK complex activity. IKK complex was immunoprecipitated from Jurkat cells treated with 15ng/ml *h*TNFα using an antibody against IKKγ. The activity of the complex in the presence of IMD-0354 at increasing concentrations was measured using kinase assay. Values are means ± SD of three independent experiments.

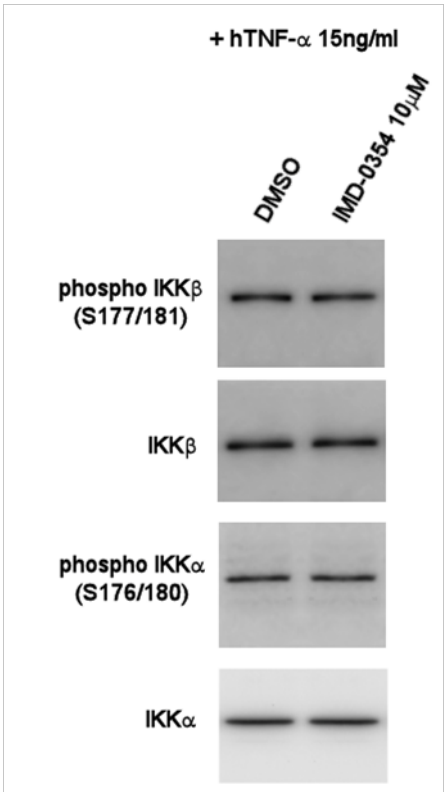


Figure S2. Effects of IMD-0354 on phosphorylation of IKKs.
Jurkat cells were exposed to 10 μM IMD-0354 for 60 min and then treated for 20 minutes with TNFα (15ng/mL). Protein expression and phosphorylation of IKKα and IKKβ was evaluated by immunoblot analysis as described in biochemical protocols section (see IκBα degradation assay). Values are means ± SD of three independent experiments.

Chemistry.

General methods. Final compounds **1** – **11** were assayed in biological experiments. Their purity was measured by HPLC analyses, showing a chromatogram where the main peak (area at least 95% of all detected peaks) was attributable to the final compound. HPLC analyses was performed on a Waters HPLC system composed by: Waters 1525EF binary pump, Waters 717 plus autosampler and Waters 2996 PDA detector. The analytical column was Waters XTerra Phenyl (4.6 x 150 mm, 5µm particle size) column, flow 1 ml/min; compounds were dissolved in CH_3CN or MeOH. The mobile phase consisted of MeOH (or CH_3CN)/water with 0.1% trifluoroacetic acid; two gradient profiles of mobile phase were used to assay the purity of each compound. Melting points (m.p.) were measured on a capillary apparatus (Büchi 540). The final m.p. determination was achieved by placing the sample at a temperature 10° C below the m.p. and applying a heating rate of 2 °C min^{-1}. All compounds were routinely checked by ^{1}H- and ^{13}C-NMR and mass spectrometry. ^{1}H- and ^{13}C-NMR spectra were performed on a Bruker Avance 300 instrument or Jeol Resonance ECZ600R. For coupling patterns, the following abbreviations are used: br = broad, s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet. HRMS spectra were recorded on an LTQ Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an atmospheric pressure interface and an ESI ion source instrument.

4-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1,2,5-oxadiazole-3-carboxamide (**1**). White solid (m.p. 153.7 – 155.2 °C from diisopropyl ether/hexane). ^{1}H-NMR (300 MHz, $(CD_3)_2CO$): δ 7.87 (1H, s, *aromatic protons),* 8.54 (2H, s, *aromatic protons*), 10.47 (1H, s,-CON*H*-), 11.25 (1H, very br s, -O*H*); ^{13}C-NMR (75 MHz $(CD_3)_2CO$): δ 118.2 (q, $^3J_{CF}$ = 3.8 Hz), 120.8 (q, $^3J_{CF}$ = 4.1 Hz), 123.7 (q, $^1J_{CF}$ = 272.0 Hz), 132.4 (q, $^2J_{CF}$ = 33.8 Hz), 139.9, 141.5, 156.4, 163.04. ESI-HRMS (m/z) $[M - H]^-$ calcd. for $C_{11}H_4F_6N_3O_3$ 340.0151, obsd. 340.0153.

4-Hydroxy-N-[3-(trifluoromethyl)phenyl]-1,2,5-oxadiazole-3-carboxamide (**2**). White solid (m.p. 165.3 – 167.5 °C from diisopropyl ether). ^{1}H-NMR (300 MHz, $(CD_3)_2CO$): δ 7.56 (1H, d, J = 7.8 Hz, *aromatic protons),* 7.69 (1H, t, J = 8.0 Hz, *aromatic protons*), 8.08 (1H, d, J = 8.1 Hz, *aromatic protons),* 8.30 (1H, s, *aromatic protons*), 10.24 (1H, s,-CON*H*-), 11.21 (1H, very br s, -O*H);* ^{13}C-NMR (75 MHz $(CD_3)_2CO$): δ 117.2 (q, $^3J_{CF}$ = 3.9 Hz), 121.7 (q, $^3J_{CF}$ = 3.8 Hz), 124.3, 124.6 (q, $^1J_{CF}$ = 272.1Hz), 130.4, 131.0 (q, $^2J_{CF}$ = 32.1 Hz), 138.7, 141.7, 156.2, 163.1. ESI-HRMS (m/z) $[M - H]^-$ calcd. for $C_{10}H_5F_3N_3O_3$ 272.0278, obsd. 272.0274.

4-Amino-N-[3,5-bis(trifluoromethyl)phenyl]-1,2,5-oxadiazole-3-carboxamide (**3**). White solid (m.p. 166.9 – 168.1 °C). ^{1}H-NMR (300 MHz, $(CD_3)_2CO$): δ 6.16 (2H, br s, NH_2), 7.88 (1H, s, *aromatic proton),* 8.59 (2H, *s*, *aromatic protons*), 10.52 (1H, s,-CON*H*-)*;* ^{13}C-NMR (75 MHz $(CD_3)_2CO$): δ 118.6 (q, $^3J_{CF}$ = 3.8 Hz), 121.4 (q, $^3J_{CF}$ = 4.1 Hz), 124.3 (q, $^1J_{CF}$ = 272.0 Hz), 132.6 (q, $^2J_{CF}$ = 34.0 Hz), 140.6, 141.0, 157.4, 158.8. ESI-HRMS (m/z) $[M - H]^-$ calcd. for $C_{11}H_5F_6N_4O_2$ 339.0311, obsd. 339.0312.

4-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1,2,5-thiadiazole-3-carboxamide (**4**). White solid (m.p. 208.0 – 209.6 °C from hexane). ^{1}H-NMR (300 MHz, CD_3OD): δ 7.73 (1H, s, *aromatic proton),* 8.45 (2H, s, *aromatic protons*)*;* ^{13}C-NMR (75 MHz CD_3OD): δ 118.5 (q, $^3J_{CF}$ = 3.8 Hz), 121.4 (q, $^3J_{CF}$ = 4.1 Hz), 124.7 (q, $^1J_{CF}$ = 271.6 Hz), 133.3 (q, $^2J_{CF}$ = 33.4 Hz), 140.5, 141.1, 160.7, 166.0. ESI-HRMS (m/z) $[M - H]^-$ calcd. for $C_{11}H_4F_6N_3O_2S$ 355.9923, obsd. 355.9921.

4-Hydroxy-N-[3-(trifluoromethyl)phenyl]-1,2,5-thiadiazole-3-carboxamide (**5**). White solid (m.p. 177.6 – 178.9 °C). ^{1}H-NMR (300 MHz, CD_3OD): δ 7.45 (1H, d, J = 7.9 Hz *aromatic protons),* 7.55 (1H, t, J = 8.0 Hz, *aromatic protons),* 7.97 (1H, d, J = 8.2 Hz, *aromatic protons),* 8.20 (1H, s, *aromatic protons);* ^{13}C-NMR (75 MHz CD_3OD) δ 118.3 (q, $^{3}J_{CF}$ = 4.0 Hz), 122.3 (q, $^{3}J_{CF}$ = 4.0 Hz), 125.1, 125.5 (q, $^{1}J_{CF}$ = 272.5 Hz), 130.8, 132.2 (q, $^{2}J_{CF}$ = 32.3 Hz), 139.7, 140.8, 160.7, 166.0. ESI-HRMS (m/z) [M - H]$^{-}$ calcd. for $C_{10}H_5F_3N_3O_2S$ 288.0049, obsd. 288.0047.

4-Methoxy-N-[3,5-bis(trifluoromethyl)phenyl]-1,2,5-thiadiazole-3-carboxamide (**6**). Pale yellow solid (m.p. 113.0 – 114.4 °C from diisopropyl ether). ^{1}H-NMR (300 MHz, CD_3OD): δ 4.18 (3H, s, -OC*H*$_3$), 4.90 (1H, s, -CON*H*-), 7.71 (1H, s, *aromatic proton*), 8.42 (2H, s, *aromatic protons*); ^{13}C-NMR (75 MHz CD_3OD) δ 58.8, 118.4 (q, $^{3}J_{CF}$ = 3.8 Hz), 121.28 (q, $^{3}J_{CF}$ = 3.0 Hz), 124.69 (q, $^{1}J_{CF}$ = 271.8 Hz), 133.29 (q, $^{2}J_{CF}$ = 33.4 Hz), 141.4, 142.2, 159.5, 166.5. ESI-HRMS (m/z) [M - H]$^{-}$ calcd. for $C_{12}H_6F_6N_3O_2S$ 370.0079, obsd. 370.0083.

5-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-2-methyl-2H-1,2,3-triazole-4-carboxamide (**7**). White solid (m.p. 244.5 – 245.2 °C). ^{1}H NMR (300 MHz, CD_3OD): δ 4.09 (3H, s, -NC*H*$_3$), 7.67 (1H, s, *aromatic proton*), 8.41 (2H, s, *aromatic protons*); ^{13}C NMR (75 MHz CD_3OD): δ 42.7, 117.7 (q, $^{3}J_{CF}$ = 3.8 Hz), 121.0 (q, $^{3}J_{CF}$ = 3.8 Hz), 124.7 (q, $^{1}J_{CF}$ = 271.4 Hz), 125.6, 133.1 (q, $^{2}J_{CF}$ = 33.2Hz), 141.7, 160.9, 161.5. ESI-HRMS (m/z) [M - H]$^{-}$ calcd. for $C_{12}H_7F_6N_4O_2$ 353.0468, obsd. 353.0469.

4-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1-methyl-1H-1,2,3-triazole-5-carboxamide (**8**). White solid (m.p. 240.0 – 242.6 °C). ^{1}H NMR (300 MHz, $(CD_3)_2CO$): δ 4.28 (3H, s, -NC*H*$_3$), 7.75 (1H, s, *aromatic proton*), 8.44 (2H, s, *aromatic protons*) 10.05 (1H, s, -CON*H*-); ^{13}C NMR (75 MHz, $(CD_3)_2CO$): δ 39.48, 112.1, 117.3 (q, $^{3}J_{CF}$ = 3.8 Hz), 120.5 (q, $^{3}J_{CF}$ = 3.8 Hz), 124.3 (q, $^{1}J_{CF}$ = 273.4 Hz), 132.4 (q, $^{2}J_{CF}$ = 33.1 Hz), 141.3, 157.9, 159.8. ESI-HRMS (m/z) [M - H]$^{-}$ calcd. for $C_{12}H_7F_6N_4O_2$ 353.0468, obsd. 353.0470.

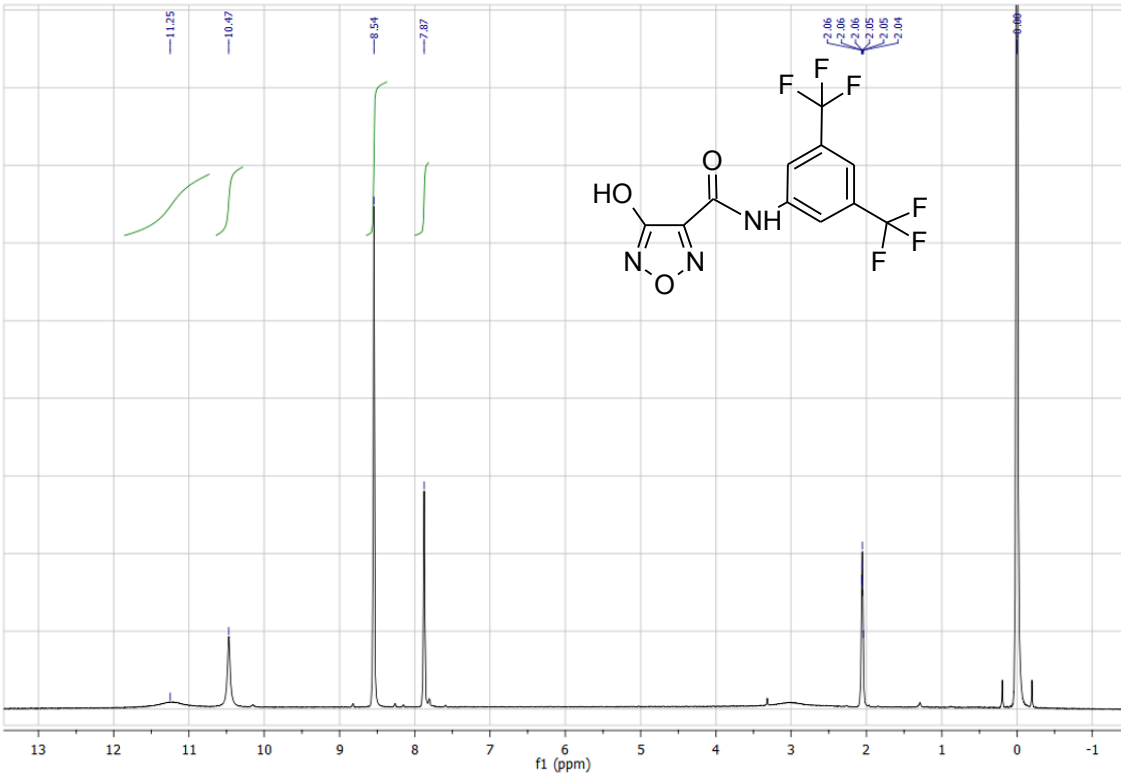
5-hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-2H-1,2,3-triazole-4-carboxamide (**9**). White solid (m.p. 163.7 – 164.9 °C). ^{1}H NMR (600 MHz, $(CD_3)_2SO$)): δ 7.77 (1H, s, *aromatic proton*), 8.57 (2H, s, *aromatic proton*), 10.71 (1H, s, -CON*H*-/-O*H*), 10.89 (0.22H, br s, -CON*H*-/-O*H* minor tautomer) and 11.08 (0.77H, s, -CON*H*-/-O*H* major tautomer), 14.67 (0.76H, s, triazole N*H* major tautomer) and 15.03 (0.23H, br s, triazole N*H* minor tautomer). ^{13}C NMR (75 MHz $(CD_3)_2CO$)): 110.6, 117.3, 120.5, 124.08 (q, $^{1}J_{CF}$ = 272.0 Hz), 132.24 (q, $^{2}J_{CF}$ = 33.2 Hz), 141.0, 160.1, 161.0. ESI-HRMS (m/z) [M - H]$^{-}$ calcd. for $C_{11}H_5F_6N_4O_2$ 339.0311, obsd. 339.0313.

3-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1-methyl-1H-pyrazole-4-carboxamide (**10**). White solid (m.p. 308.2 – 310.6 °C, from diisopropyl ether/hexane). ^{1}H NMR (300 MHz, $(CD_3)_2SO$): δ 3.70 (3H, s, -NC*H*$_3$), 7.72 (1H, s, *aromatic proton*), 8.07 (1H, s, *aromatic proton*), 8.39 (2H, s, *aromatic protons*), 9.81 (1H, s, -CON*H*-), 11.24 (1H, br s, -O*H*); ^{13}C NMR (75 MHz $(CD_3)_2SO$)): δ 38.8, 100.4,115.8 (q, $^{3}J_{CF}$ = 3.7 Hz), 119.5 (q, $^{3}J_{CF}$ = 3.8 Hz), 123.3 (q, $^{1}J_{CF}$ = 273.5 Hz), 130.6 (q,$^{2}J_{CF}$ = 32.7 Hz), 133.9, 140.8, 159.8, 161.5. ESI-HRMS (m/z) [M - H]$^{-}$ calcd. for $C_{13}H_8F_6N_3O_2$ 352.0515, obsd. 352.0523.

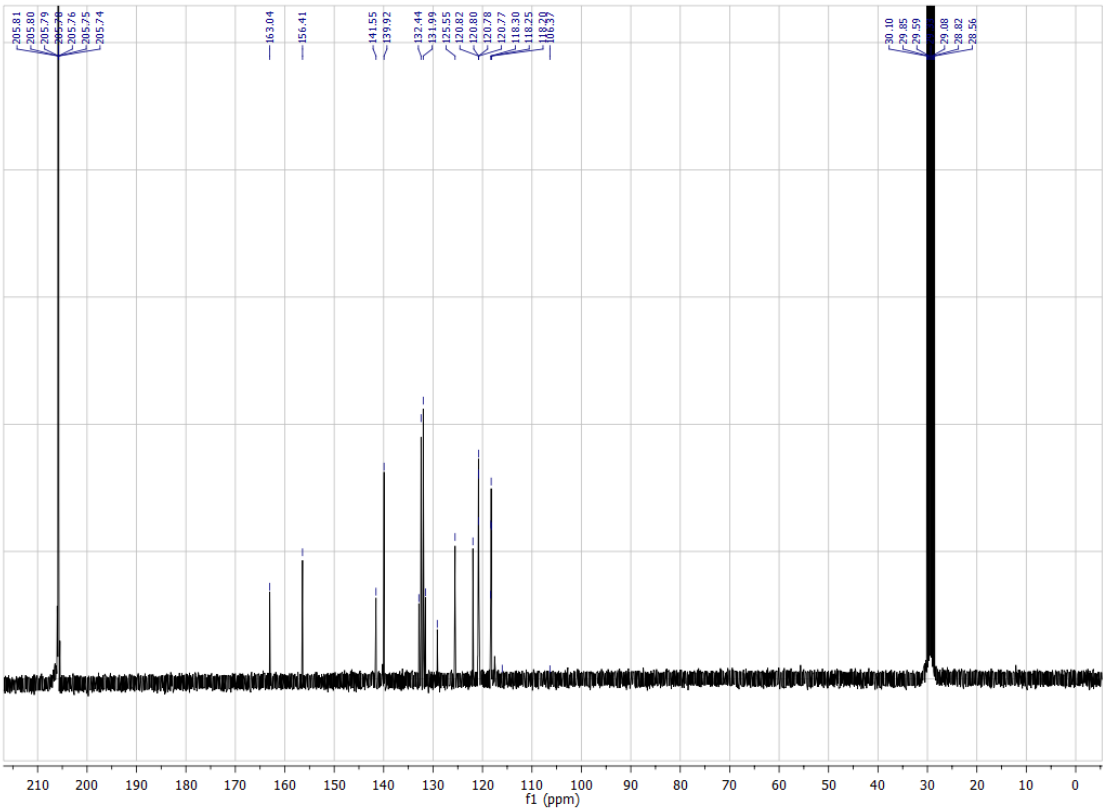
3-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1,5-dimethyl-1H-pyrazole-4-carboxamide (**11**). White solid (m.p. 302.2 – 303.3 °C, from diisopropyl ether/hexane). ^{1}H NMR (300

MHz, $(CD_3)_2SO$): δ 2.47 (3H, s, pyrazol ring -C*H*$_3$), 3.57 (3H, s, -NC*H*$_3$), 7.70 (1H, s, *aromatic protons*), 8.36 (2H, s, *aromatic protons*), 9.88 (1H, s, -CON*H*-), 11.94 (1H, br s, -O*H*). ^{13}C NMR (75 MHz $(CD_3)_2SO$)): δ 10.6, 35.0, 97.4, 115.5 (q, $^3J_{CF}$ = 3.8 Hz), 119.3 (q, $^3J_{CF}$ = 3.8 Hz), 123.9 (q, $^1J_{CF}$ = 270.5 Hz), 131.2 (q, $^2J_{CF}$ = 33.6 Hz), 140.9, 144.6, 159.0, 162.2. ESI-HRMS (m/z) $[M - H]^-$ calcd. for $C_{14}H_{10}F_6N_3O_2$ 366.0675, obsd. 366.0678.

4-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1,2,5-oxadiazole-3-carboxamide (**1**).

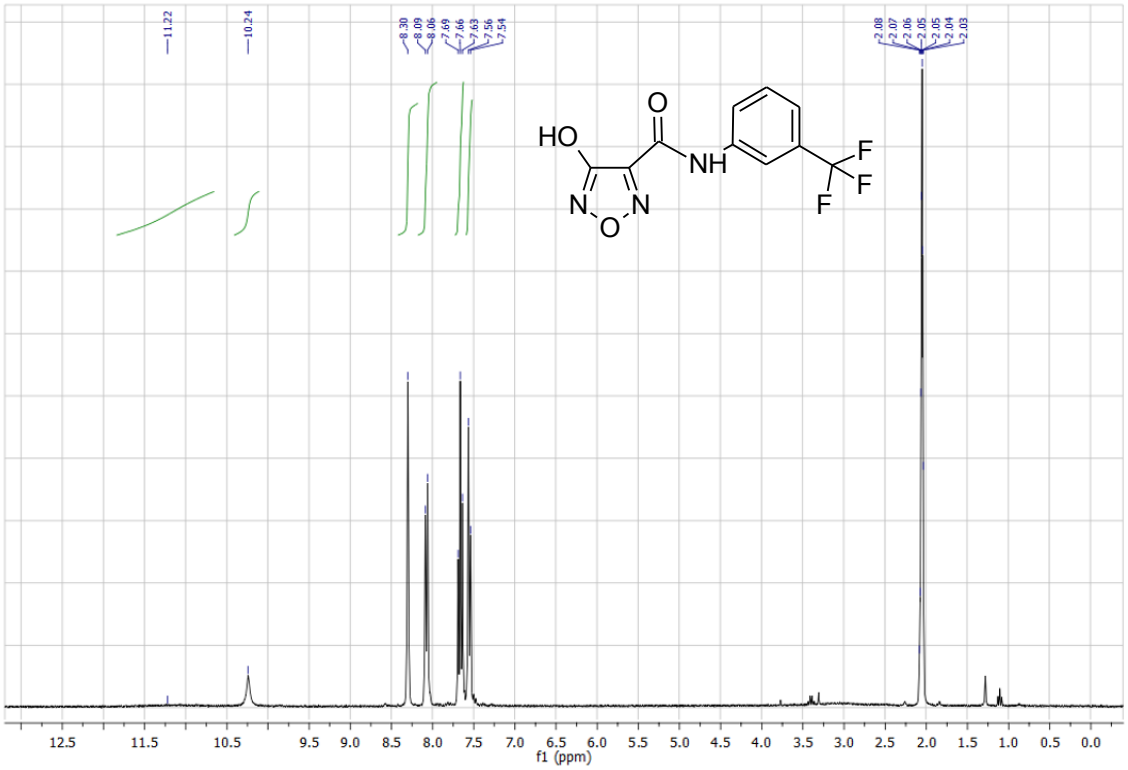


^{1}H NMR spectrum $(CD_3)_2CO$

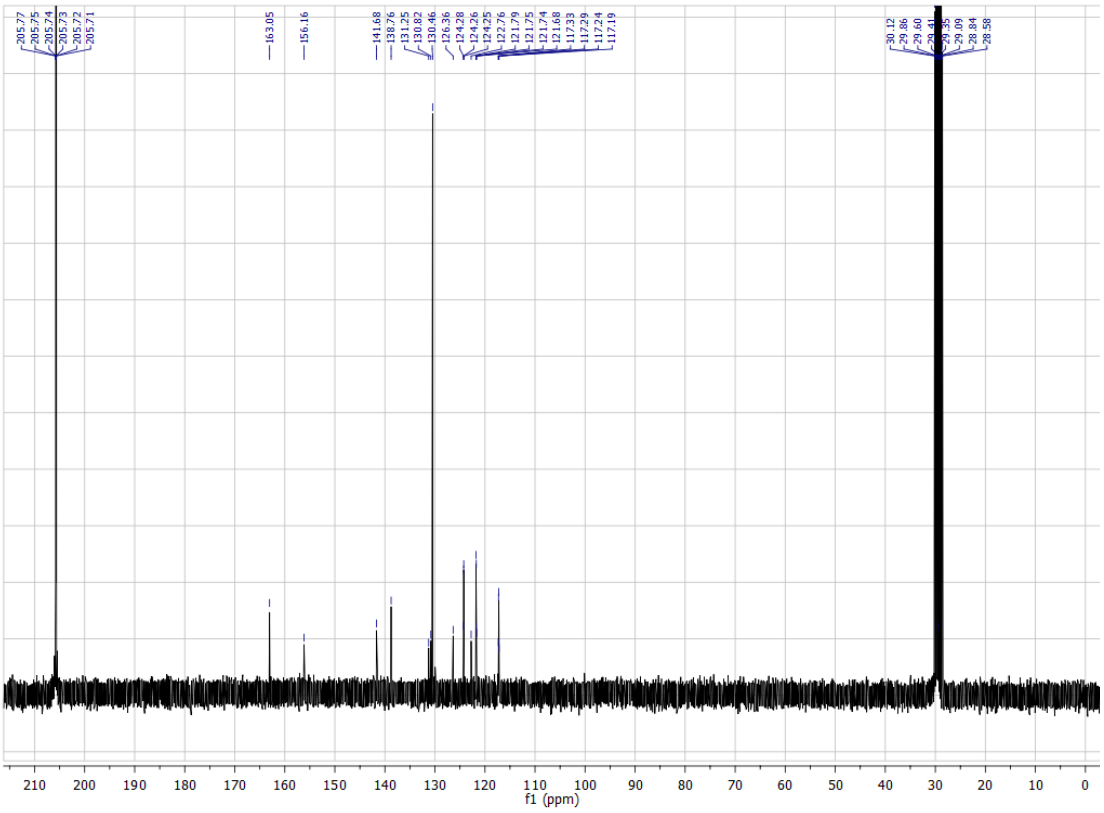


^{13}C NMR spectrum $(CD_3)_2CO$

4-Hydroxy-N-[3-(trifluoromethyl)phenyl]-1,2,5-oxadiazole-3-carboxamide (**2**).

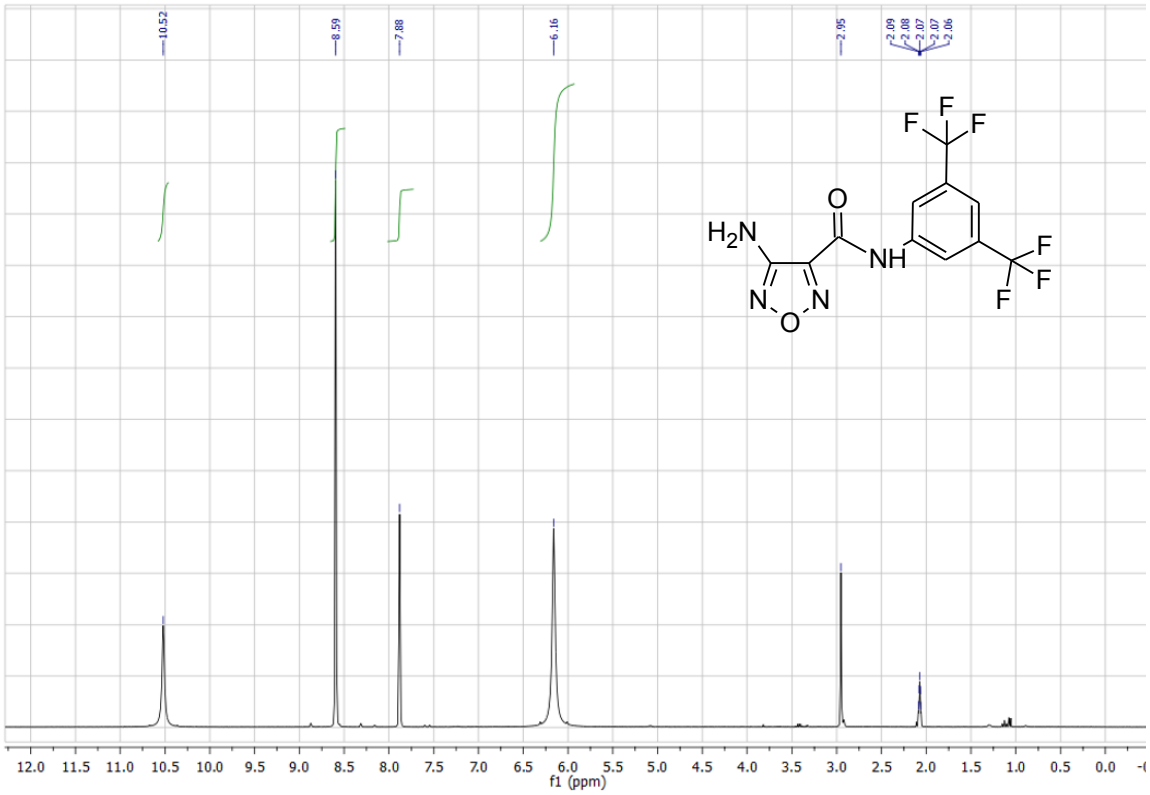


^{1}H NMR spectrum $(CD_3)_2CO$



^{13}C NMR spectrum $(CD_3)_2CO$

4-Amino-N-[3,5-bis(trifluoromethyl)phenyl]-1,2,5-oxadiazole-3-carboxamide (**3**).



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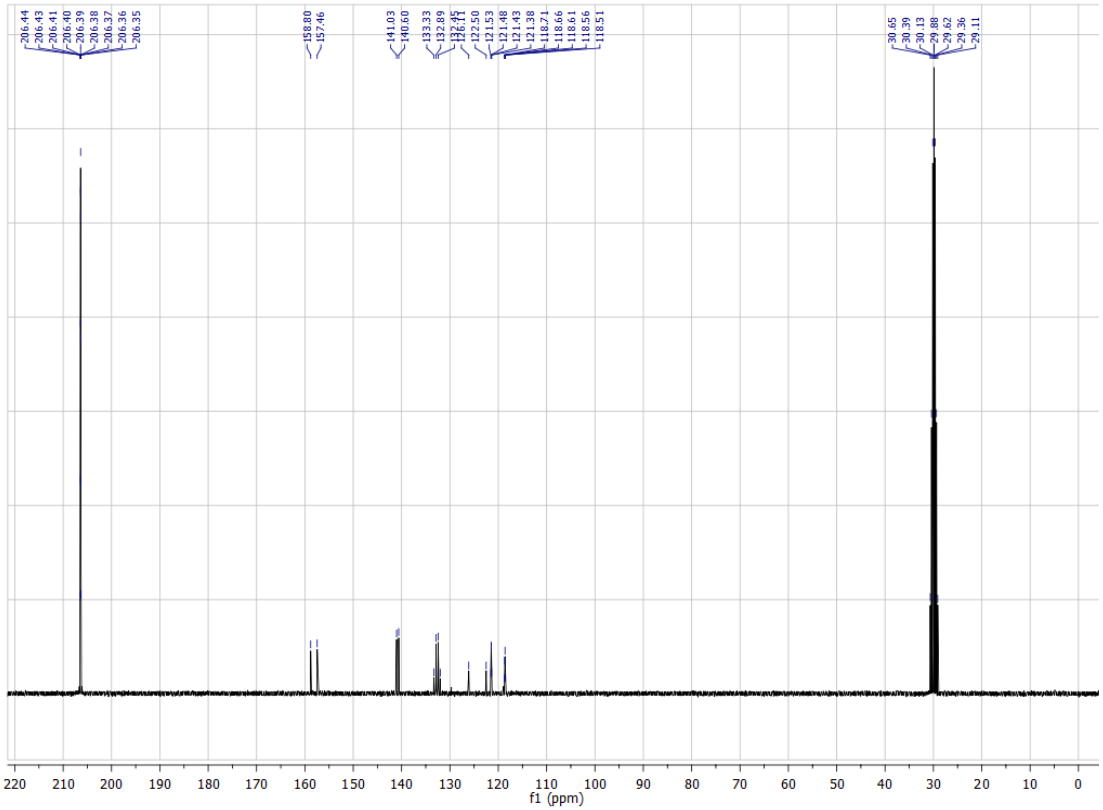
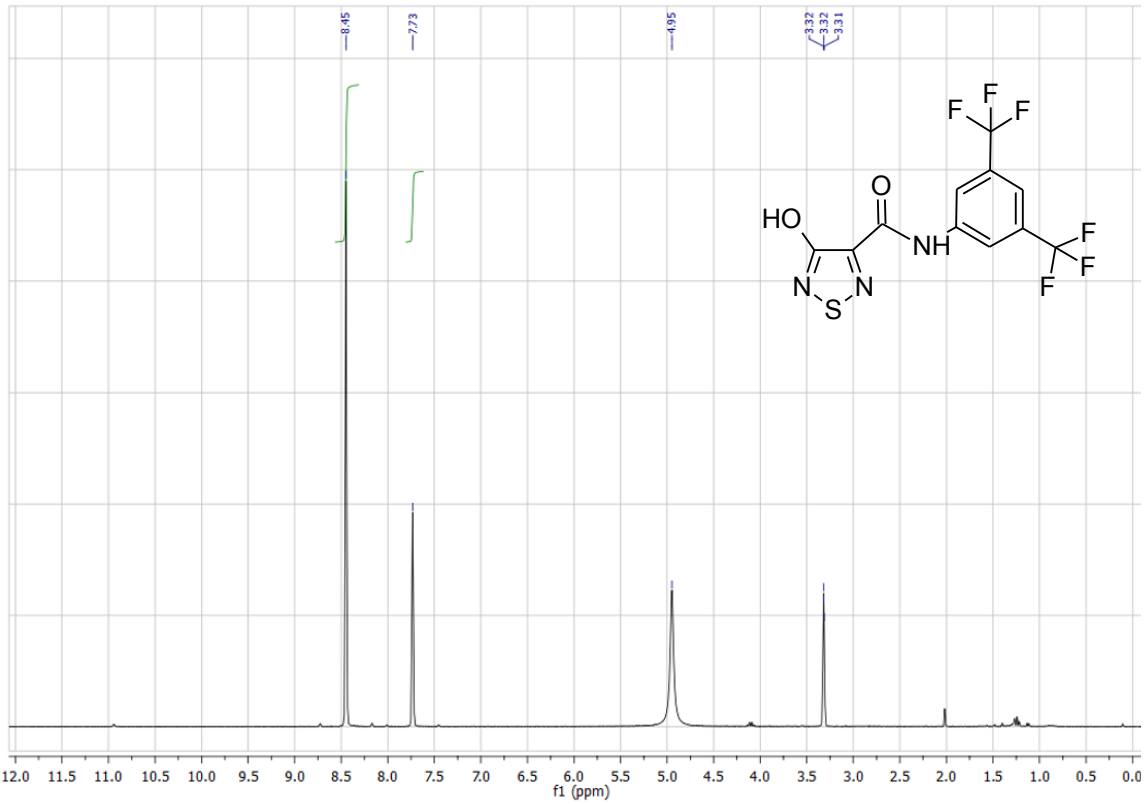
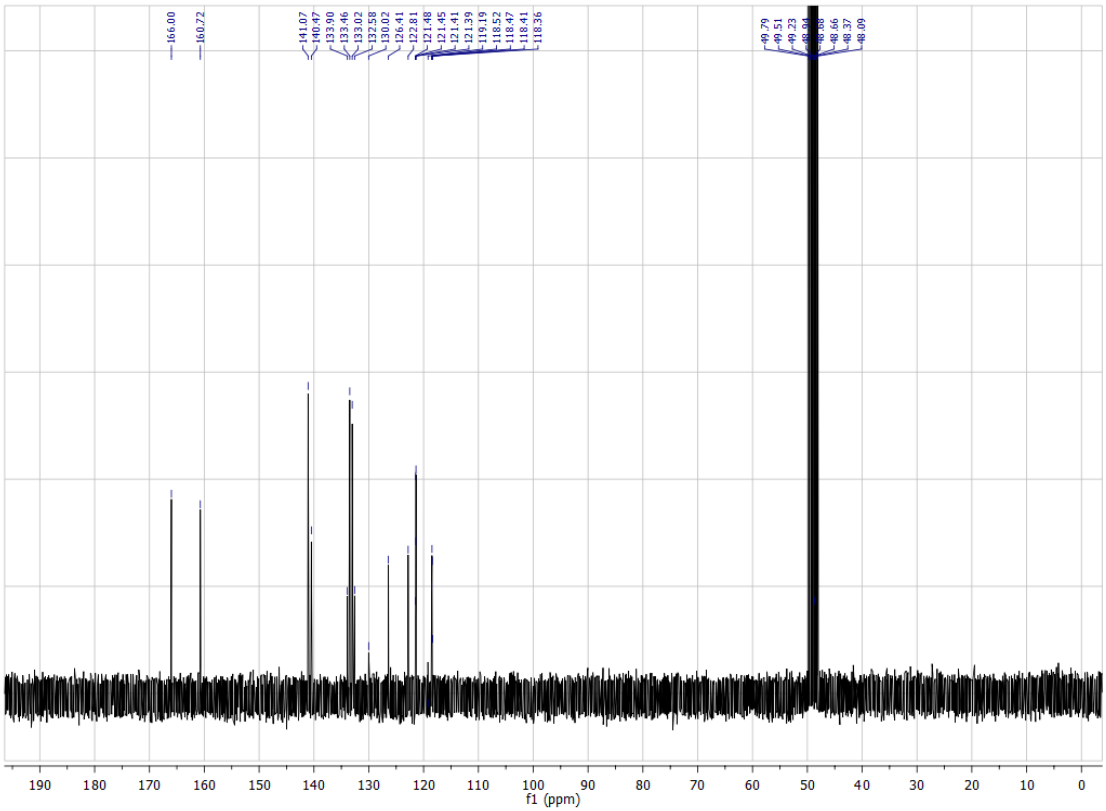


^{13}C NMR spectrum $(CD_3)_2CO$

4-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1,2,5-thiadiazole-3-carboxamide (**4**).

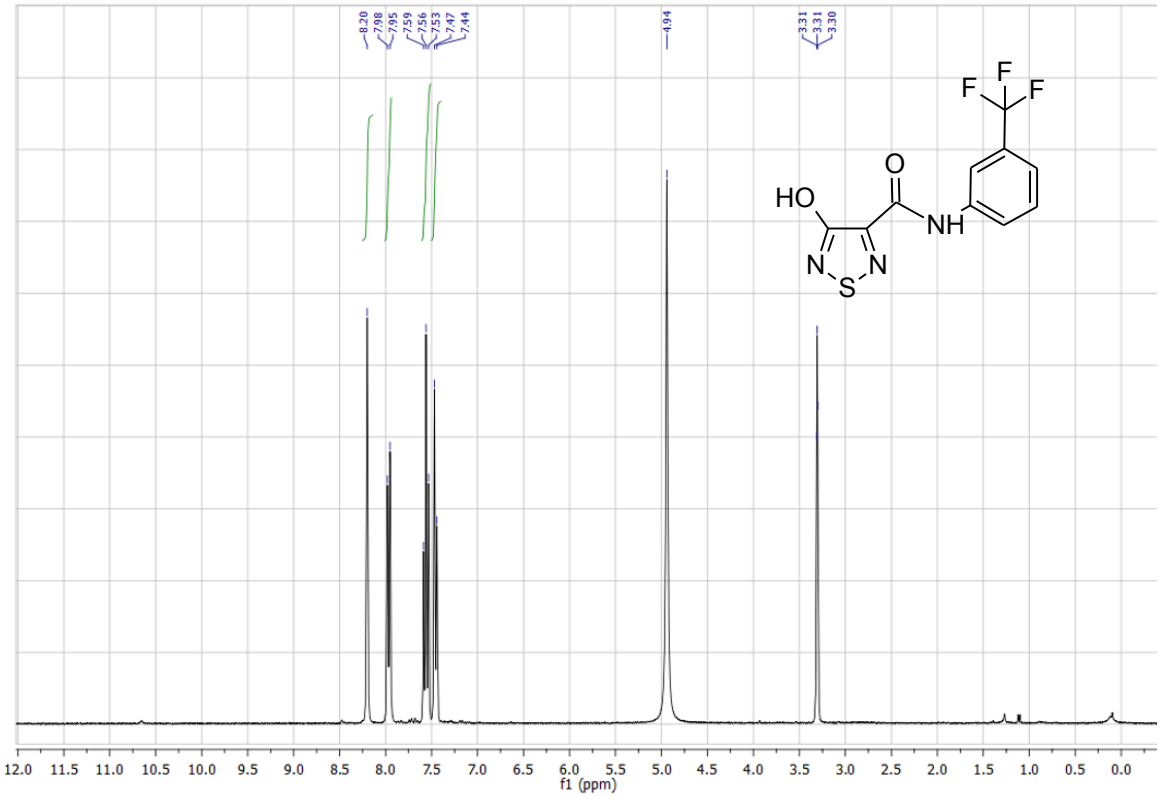


^{1}H NMR spectrum CD_3OD

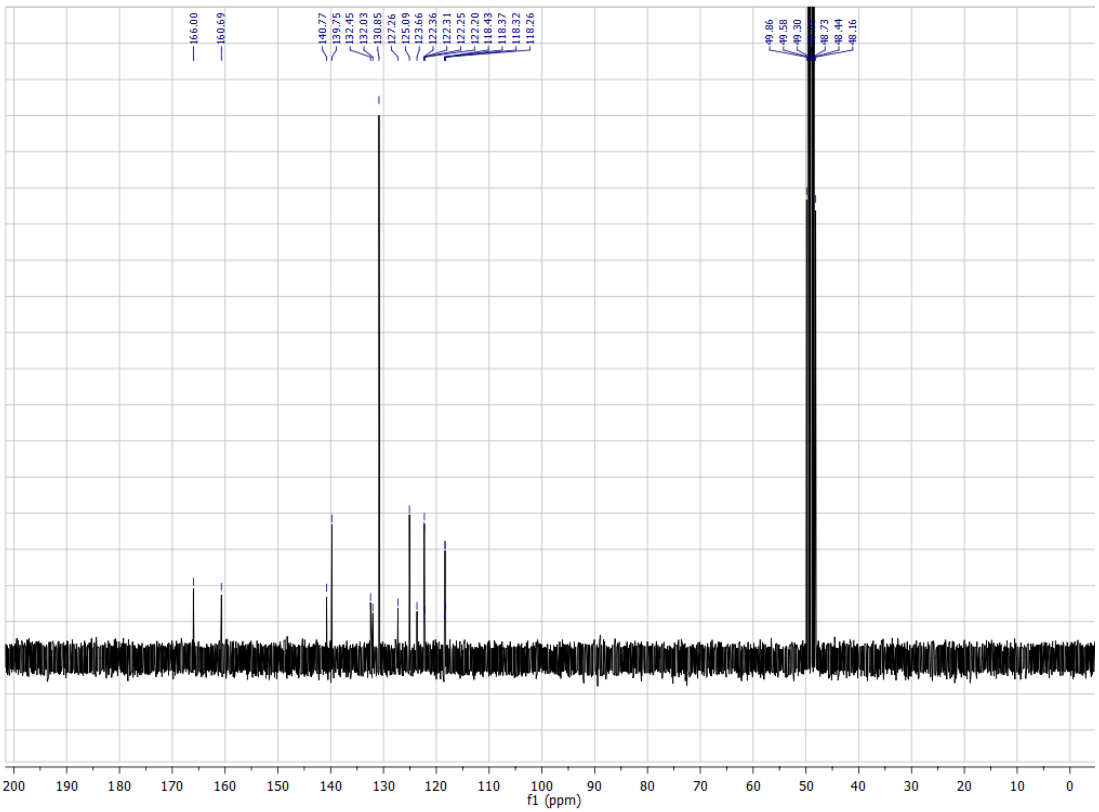


^{13}C NMR spectrum CD_3OD

4-Hydroxy-N-[3-(trifluoromethyl)phenyl]-1,2,5-thiadiazole-3-carboxamide (**5**).

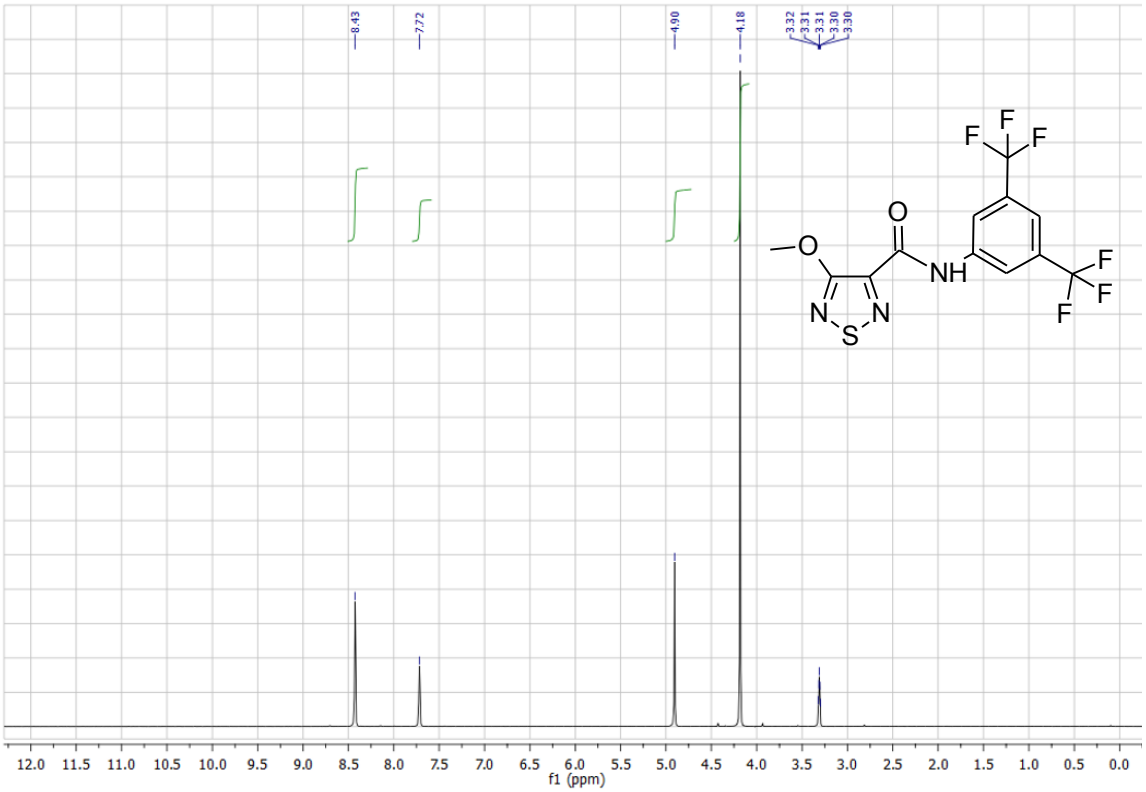


^{1}H NMR spectrum CD_3OD

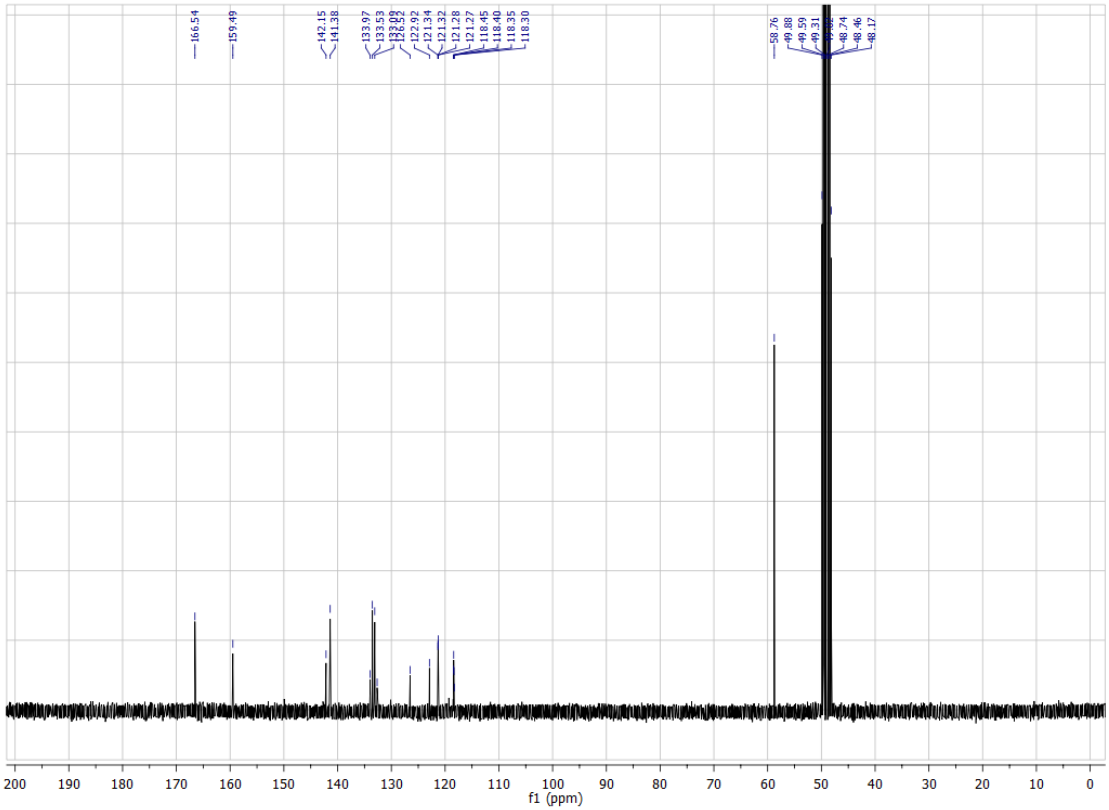


^{13}C NMR spectrum CD_3OD

4-Methoxy-N-[3,5-bis(trifluoromethyl)phenyl]-1,2,5-thiadiazole-3-carboxamide (**6**).

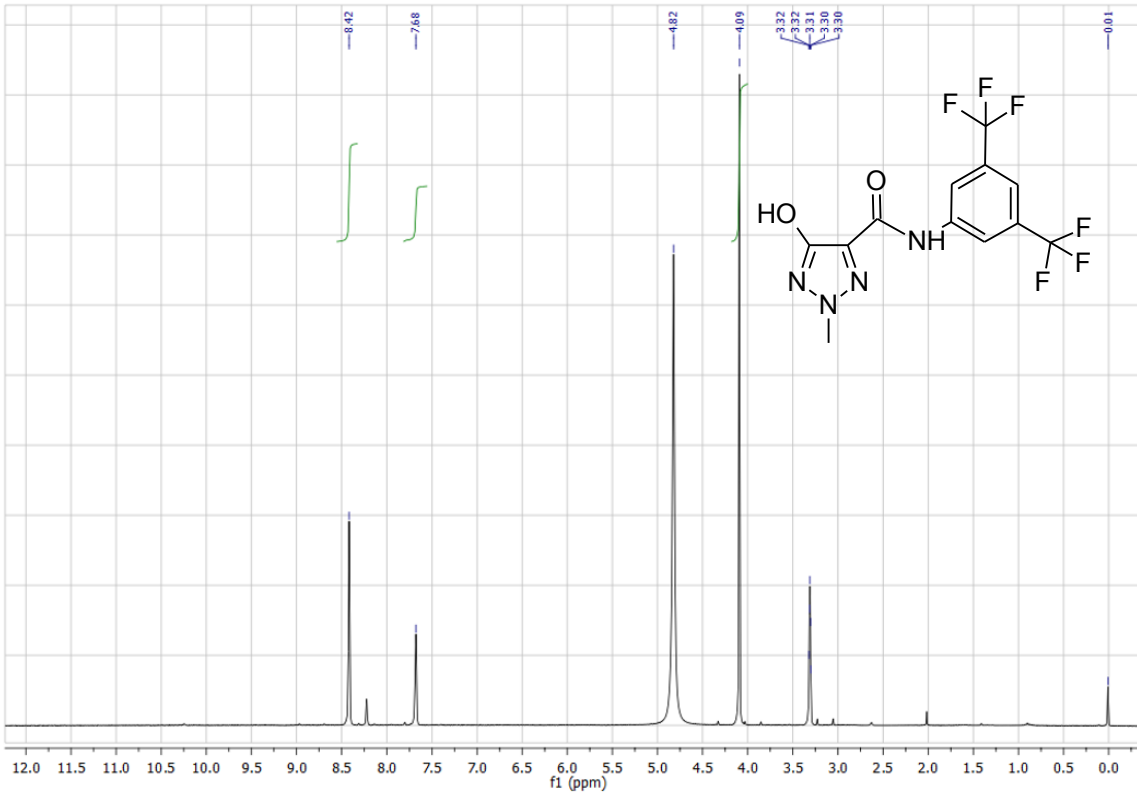


^{1}H NMR spectrum CD_3OD

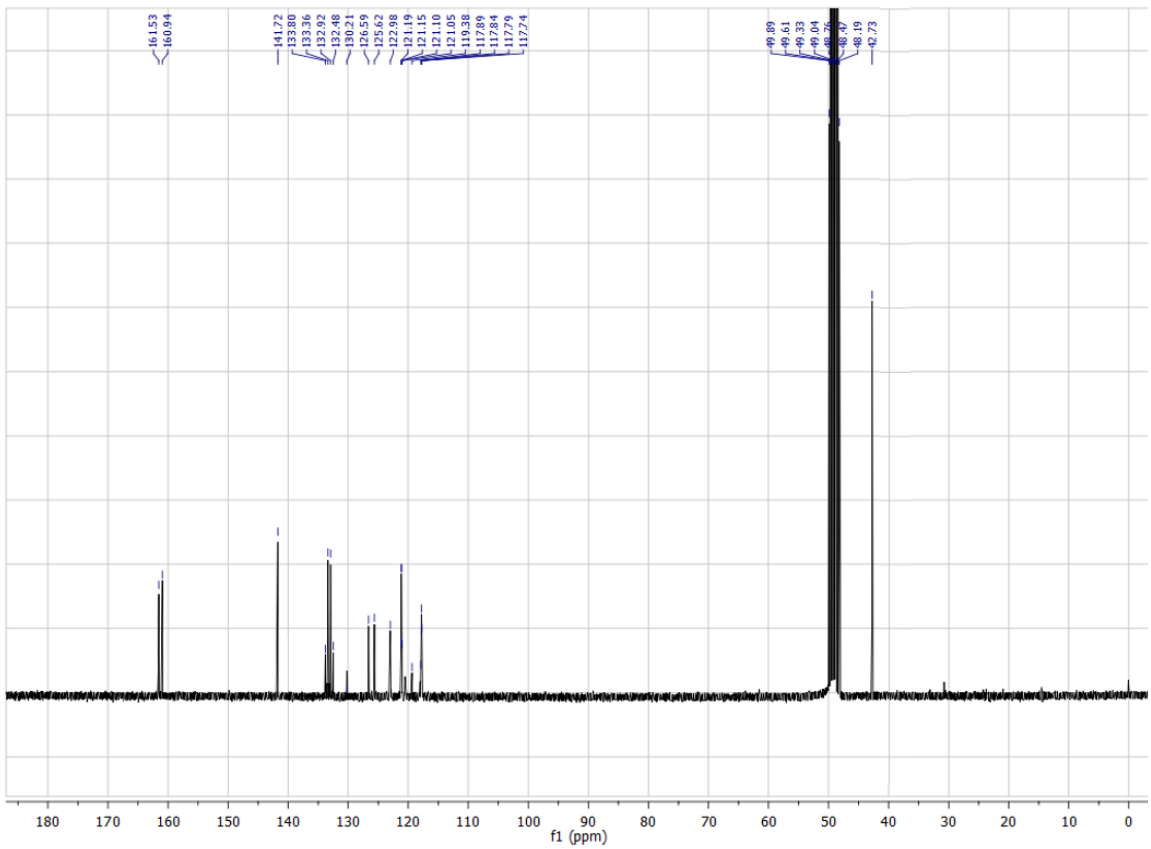


^{13}C NMR spectrum CD_3OD

5-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-2-methyl-2H-1,2,3-triazole-4-carboxamide (**7**).

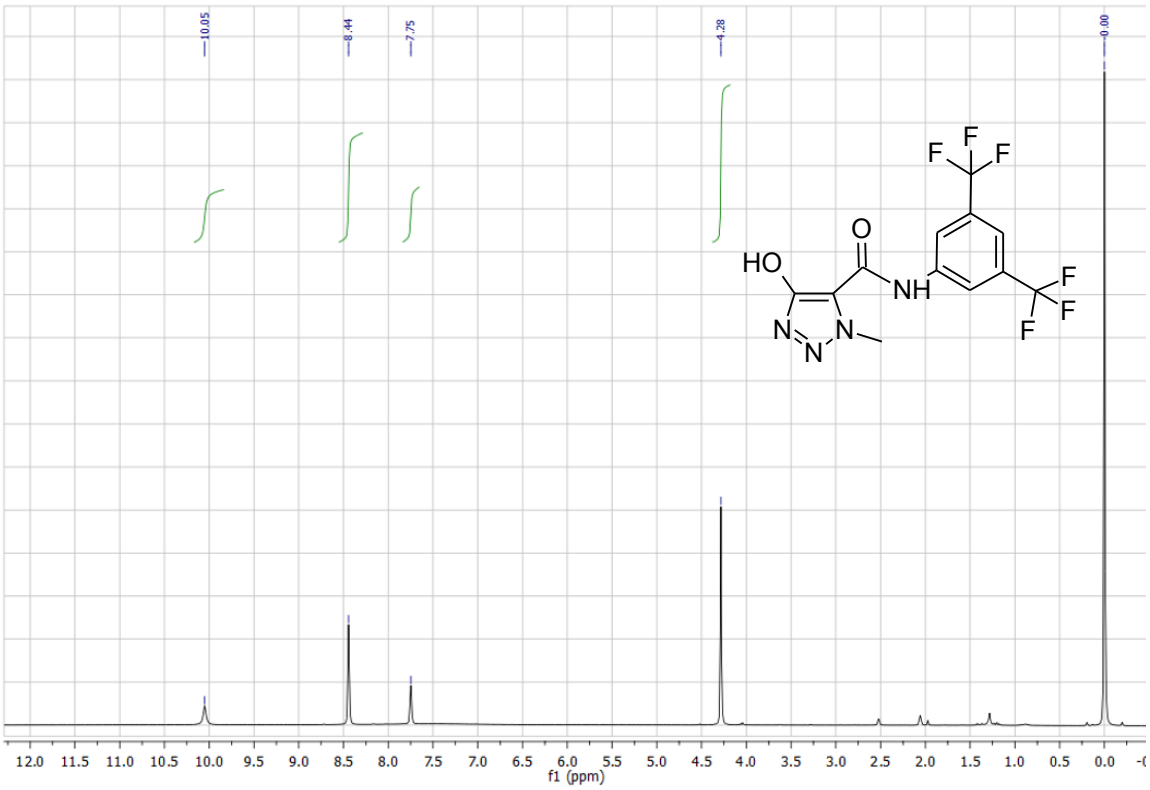


^{1}H NMR spectrum CD_3OD

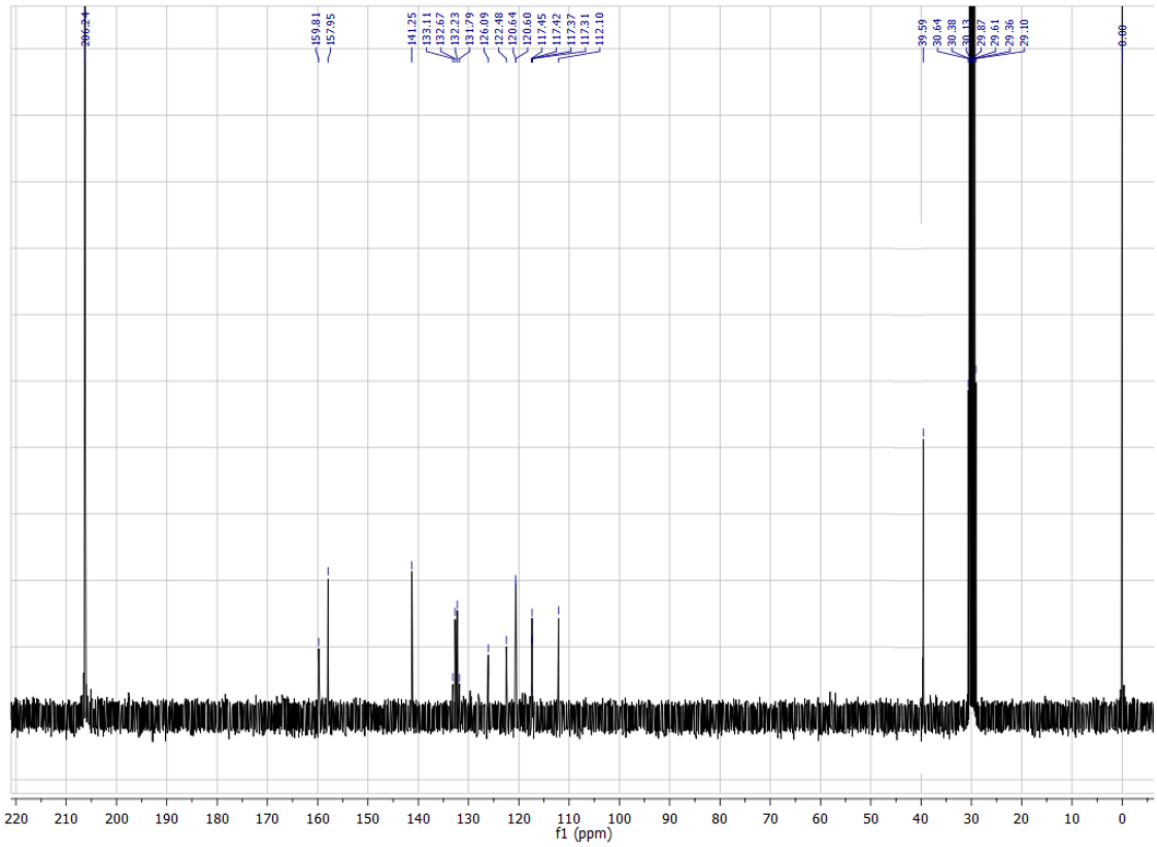


^{13}C NMR spectrum CD_3OD

4-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1-methyl-1H-1,2,3-triazole-5-carboxamide (**8**).

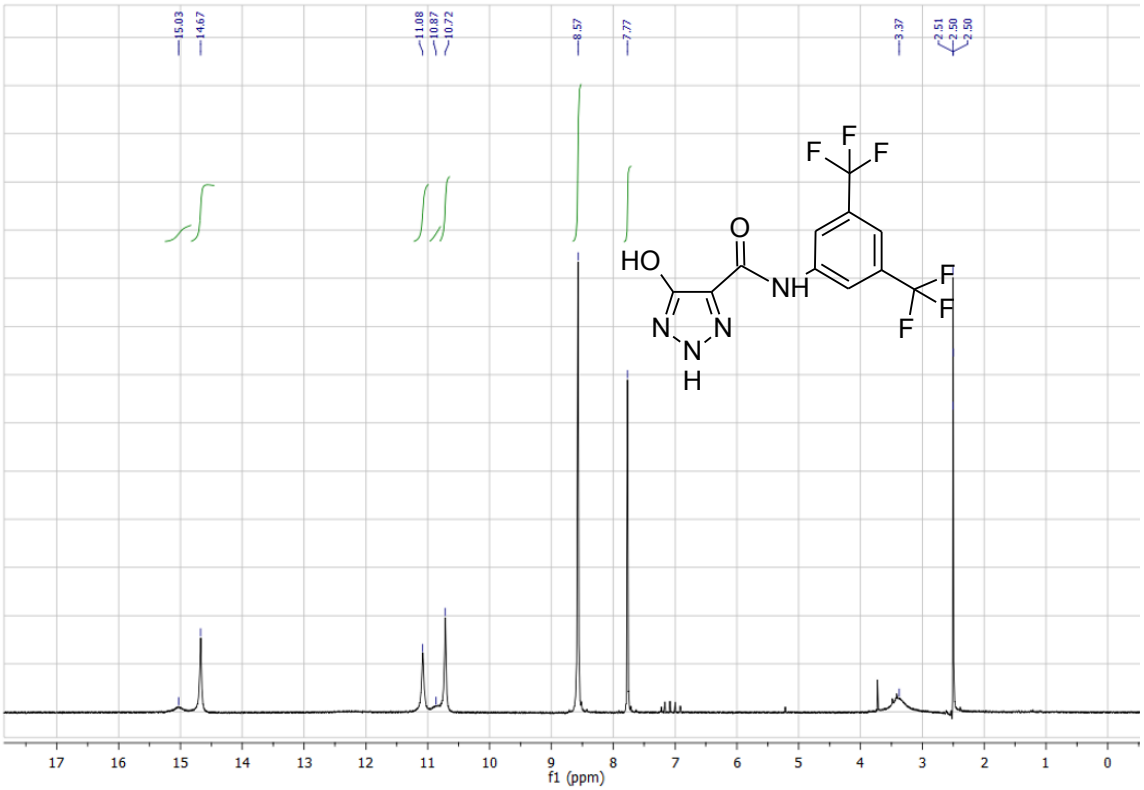


^{1}H NMR spectrum CD_3OD

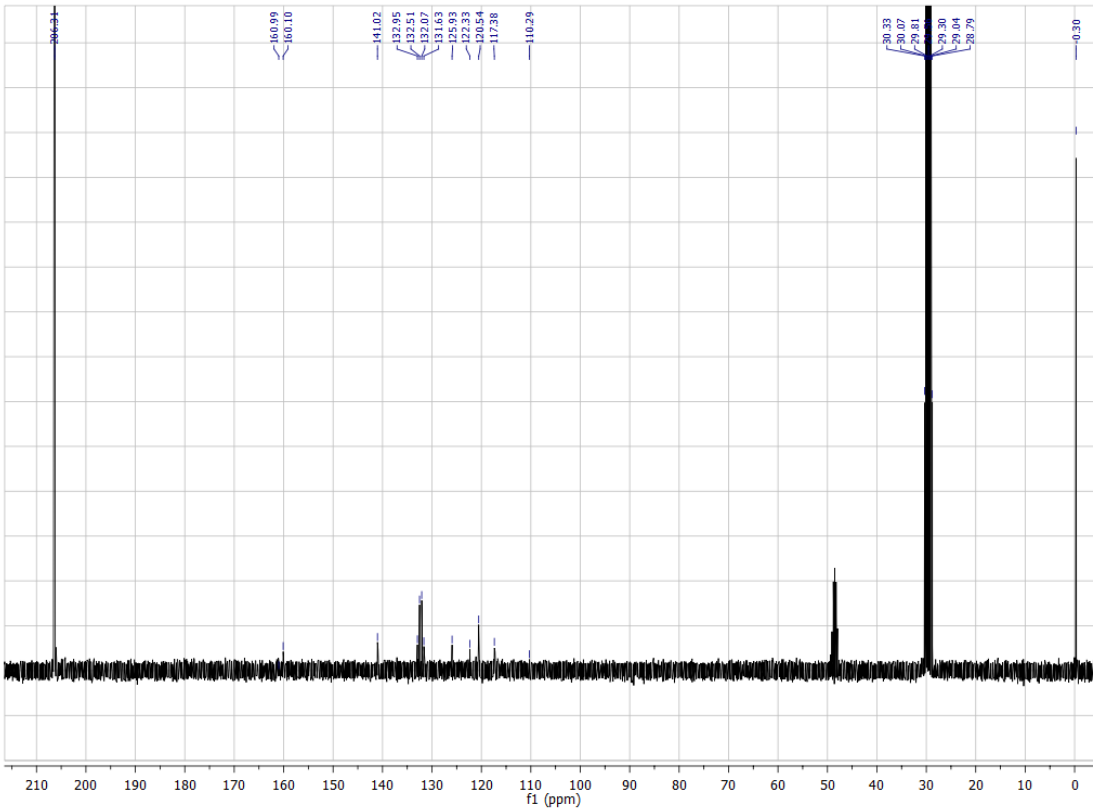


^{13}C NMR spectrum CD_3OD

5-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-2H-1,2,3-triazole-4-carboxamide (**9**).

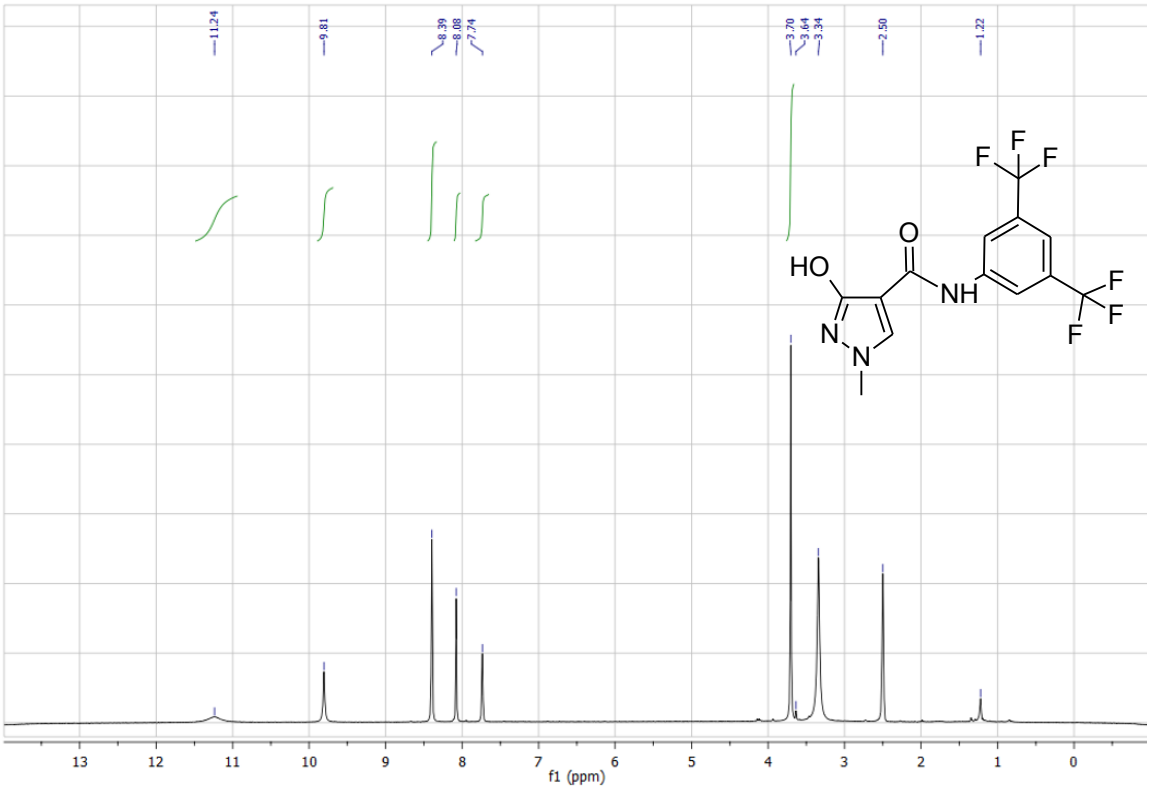


^{1}H NMR spectrum $(CD_3)_2SO$

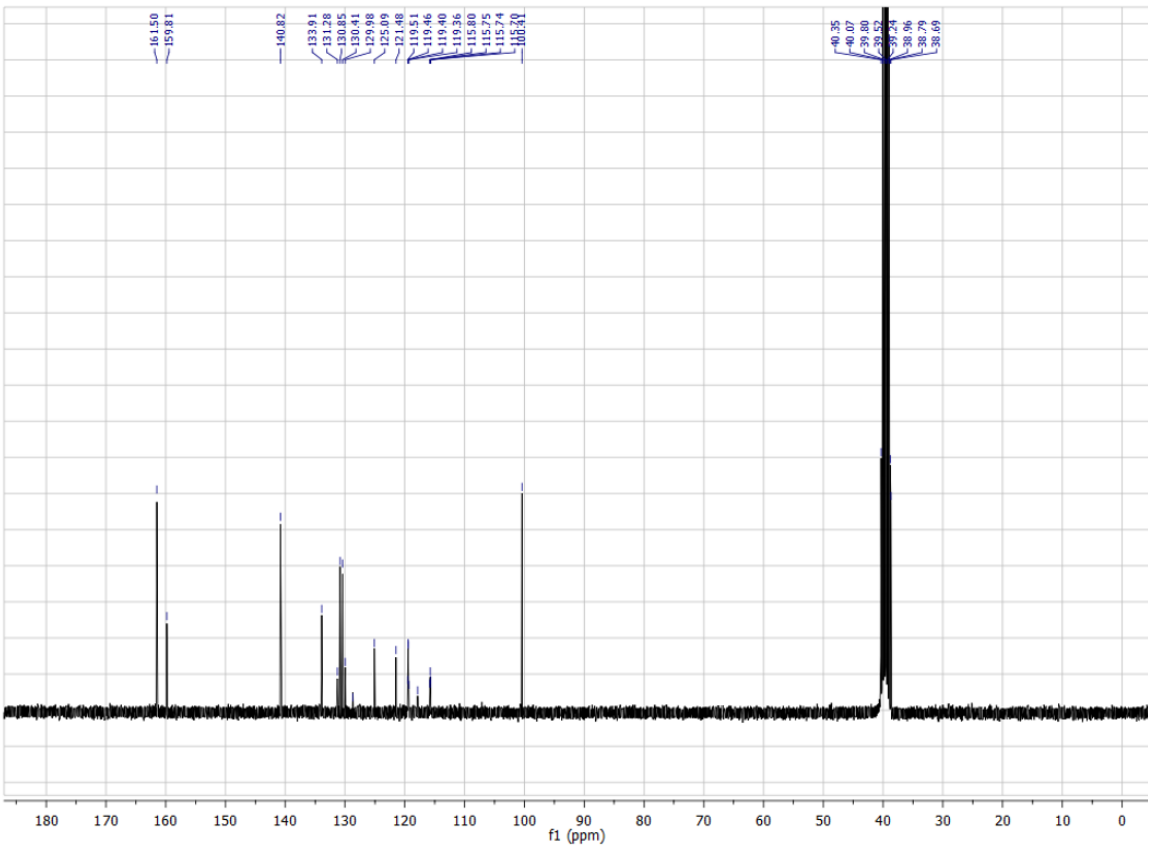


^{13}C NMR spectrum $(CD_3)_2CO$

3-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1-methyl-1H-pyrazole-4-carboxamide (**10**).

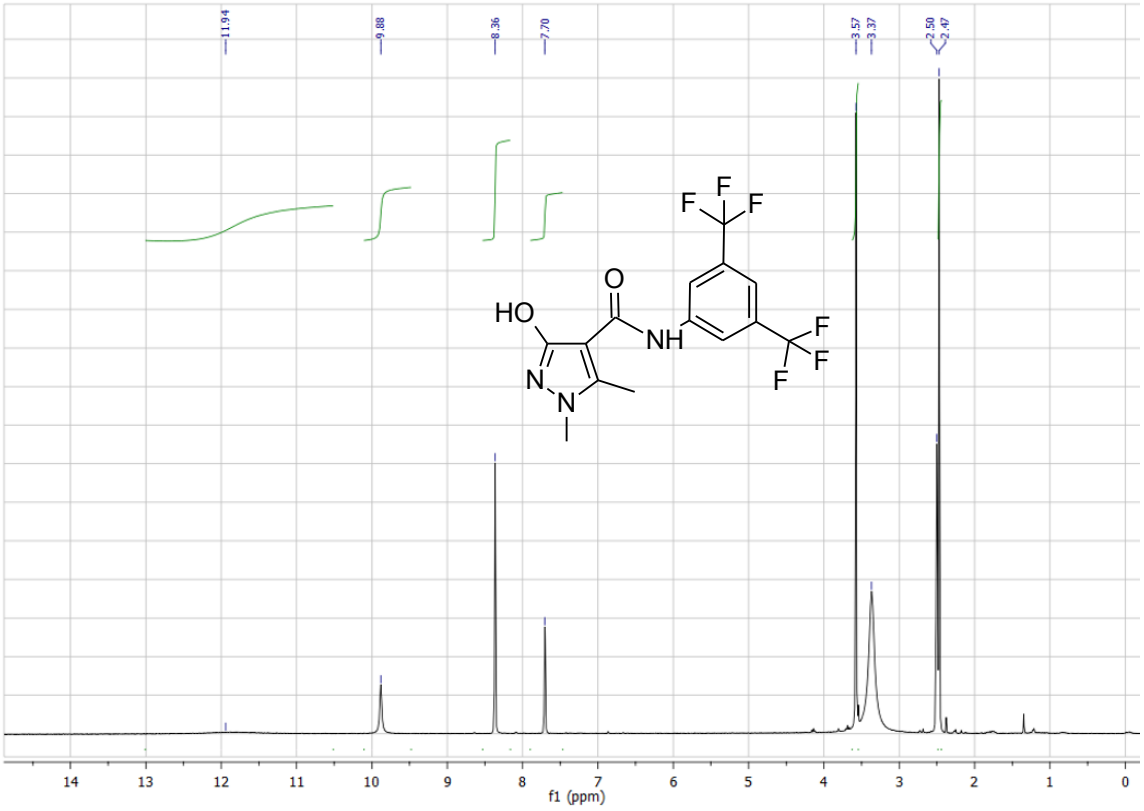


'H NMR spectrum $(CD_3)_2SO$

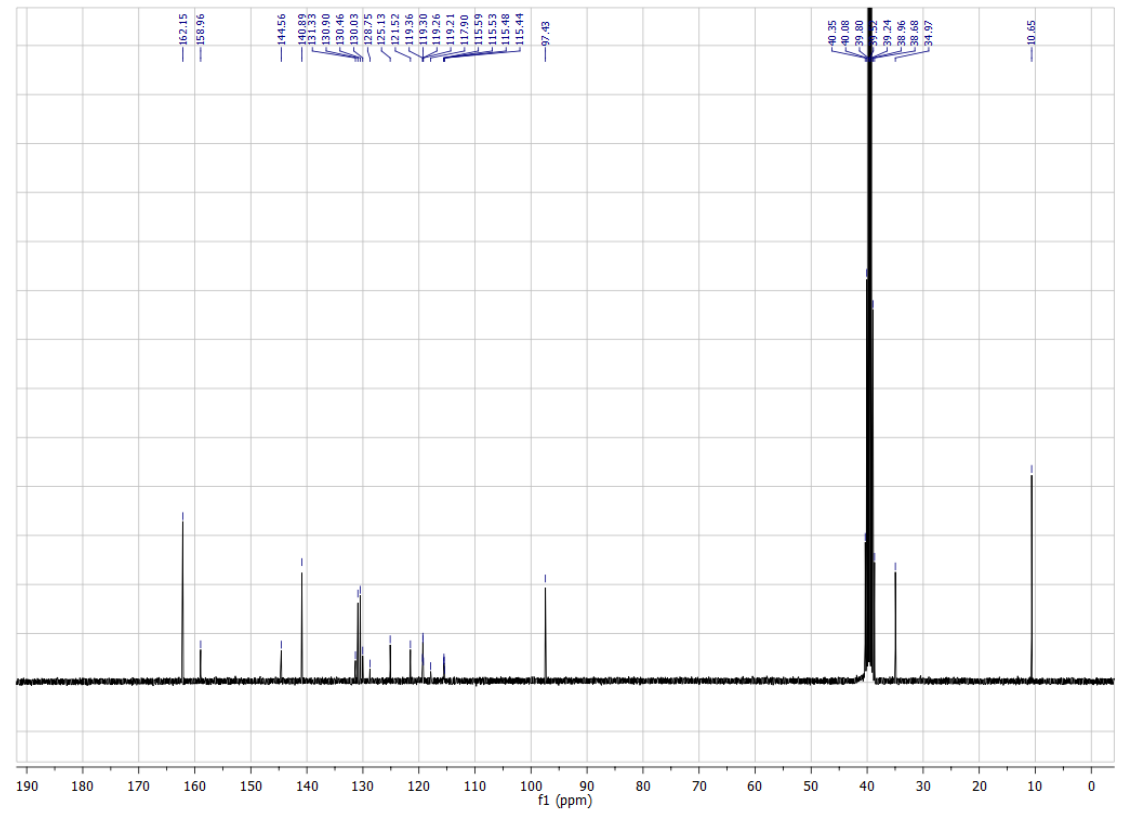


^{13}C NMR spectrum $(CD_3)_2SO$

3-Hydroxy-N-[3,5-bis(trifluoromethyl)phenyl]-1,5-dimethyl-1H-pyrazole-4-carboxamide (**11**).



^{1}H NMR spectrum $(CD_3)_2SO$



^{13}C NMR spectrum $(CD_3)_2SO$

Biochemical protocols.

Cell culture and drug treatments. Jurkat E6.1, THP-1 and MDA-MB-231 cells were cultured in X-VIVO 15 (BE02-060F, Lonza), RPMI-1640 (R8758, Sigma) and DMEM (D-5796, Sigma) media, respectively, supplemented with 10% v/v fetal bovine serum (F-7524, Sigma Aldrich) and 1% v/v antibiotic-antimycotic solution (A-5955, Sigma Aldrich) (complete medium). Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere. Cells were passaged every 2 – 3 days and discarded after 15 passages. Cells were routinely tested to confirm the absence of Mycoplasma using MycoAlert Plus detection kit (Lonza) and were used between passages 5 and 10 for all experiments. Each compound tested was solubilized in DMSO (drug vehicle, 41639, Fluka) at a final concentration of 10 mM, which was used as the stock solution for all experiments. Final dilutions were made in culture medium.

Kinase assay. Reactions were carried out in 96 well microtiter plates in the presence of : 1 μM ATP, enzyme and peptide substrate at optimal concentration (see Table S1), reaction buffer (40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 2 mM DTT, 100 μM Na_3VO_4, 0.1 mg/mL BSA), inhibitory compound or vehicle (DMSO), in a final volume of 25 μL. Temperature 25°C. Incubation length 60 min. Enzyme activity was evaluated using ADP-Glo kinase assay (Promega). Peptide substrates were synthesized by Caslo. Recombinant human IKKβ, IKKε and NIK were provided by ProQinase. Recombinant human IKKα was from Life Technologies.

	Peptide sequence	Substrate concentration	Enzyme concentration
IKKα assay	ERLLDDRHDSGLDSMKDEE	100 μM	8 ng/μL
IKKβ assay	ERLLDDRHDSGLDSMKDEE	200 μM	3.2 ng/μL
IKKε assay	ERLLDDRHDSGLDSMKDEE	200 μM	4 ng/μL
NIK assay	AKDVDQGSLCTSFVGTLQY	200 μM	5.2 ng/μL

Table S1. Sequence of peptide substrate used for each kinase assay. Final concentration of peptide substrate and enzyme in each kinase reaction.

IκBα degradation assay. Jurkat cells were exposed for 60 min to the designed compounds, IMD-0354 or PS-1145 at increasing concentrations (0.025 to 20 μM) and then treated for 20 minutes with TNFα (15ng/mL). THP-1 cells were exposed for 60 min to Cpd 4, IMD-0354, PS-1145, aspirin, indomethacin at the indicated concentrations and then treated for 30 minutes with LPS (1μg/mL). At the end of incubation, cells were collected using a cell scraper, washed with phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4), counted manually, lysed with cell extraction buffer (FNN0011, Life Technologies: 30 μL/1 × 10^6 cells) supplemented with 1% v/v protease inhibitor mixture (P8340, Sigma, Milan, Italy) and 4% v/v phosphatase inhibitor mixture (P-0044, Sigma, Milan, Italy) and centrifuged at 15000 × *g* for 20 min at 4 °C. Proteins were quantified using Protein Assay Kit II (500-0002, Bio-Rad). Proteins (20 μg/lane) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% gels and electro-transferred (16 v, 12 h) at 4 °C to polyvinylidene fluoride membranes (PVDF) (IPVH00010, Millipore) equilibrated in Towbin buffer. Membranes were blocked in 5% w/v BSA (sc-2323, Santa Cruz Biotechnology) in 20 mM Tris pH 7.6, 140 mM NaCl, 0.02% v/v Tween-20 (blocking buffer) and probed with the indicated primary antibodies diluted in blocking buffer. Monoclonal antibodies to IκBα (ALX-804-209, Alexis Biochemicals) and β-actin (A-

5441, Sigma) were used at dilution of 1:1000 and 1:20,000, respectively. After washing, membranes were incubated with HRP-conjugated secondary antibody goat anti-mouse IgG (81-6520, Life Technologies) diluted in blocking buffer. Immunoreactive bands were revealed by enhanced chemiluminscence (ECL) (Millipore) and visualized using G:Box Chemi-XT CCD gel-imaging system and GeneSnap image acquisition software (SynGene, Cambridge, UK). Immunoreactive bands were quantitated using GeneTool software (SynGene, Cambridge, UK). Normalized signals expressed as percentage of control (IκBα band intensity from cells not exposed to TNFα) were analyzed by non-linear regression to calculate the apparent IC_{50} using GraphPad Prism. Values are means ± SD of three independent experiments.

NF-κB gene reporter assay. Effects of IMD-0354, PS-1145 and compound **4** on NF-κB gene reporter assay in Jurkat and MDA-MB-231 cells. Cells were co-transfected with 3 µg of plasmids pGL4.32[*luc2P*/NF-κB-RE/Hygro] and pGL4.74[*hRluc*/TK] in the ratio 10:1 using Amaxa nucleofector II and cultured for 24 h at 37 °C in humidified CO_2 incubator. IMD-0354 PS-1145 or compound **4** were added at the indicated concentrations and cells incubated for a further 6 h for Jurkat cells and for further 6 h and 24 h for MDA-MB-231 cells. In Jurkat cells NF-κB pathway was activated through the treatment with TNFα in the last 20 min whilst in MDA-MB-231 cells the NF-κB signalling pathway is reported to be constitutively activated and driven by both IKKβ and IKKα. At the end of incubation the activities of firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*) luciferases were measured sequentially from a single sample. Luminescence of Photinus was measured and normalized to the luminescence of Renilla. Normalized signals were expressed as percentage of control (cells exposed to DMSO for MDA-MB-231 assay and DMSO+TNFα for Jurkat assay). Values are means ± SD of three independent experiments.

Cell proliferation. Growth of MDA-MB-231 cells was evaluated by quantitation of DNA content using the fluorescent dye Hoechst 33258. Cells (5×10^3 in 100 µL medium) were seeded in a white 96-well plate and exposed to increasing concentrations (0.001 - 200 µM) of each compound or vehicle (DMSO) for 72 h. At the end of incubation, medium was aspirated and wells washed twice with 100 µL PBS. Cells were exposed to 100 µL 0.02 % SDS solution in SSC buffer (154 mM NaCl, 15 mM sodium citrate, pH 7) for 1 h at 37 °C with occasional swirling. At the end, an equal volume of 1 µg/mL Hoechst 33258 solution in SSC buffer was added to each well and fluorescence measured at 355 nm (excitation) and 460 nm (emission) using a Fluoroskan Ascent-Thermo microplate fluorometer (Thermo Fisher Scientific, MA). IC_{50} values were determined using nonlinear regression plots with GraphPad Prism6. Values are means ± SD of three independent experiments.

Cytotoxicity assay. The cytotoxic effects of compounds on MDA-MB-231 cells were evaluated using CellTox green assay (Promega), a fluorimetric assay that measures changes in membrane integrity as a result of cell death. Cells (5×10^3 / well) were seeded in a white - opaque 96-well plate and exposed to increasing concentrations (0.001 - 100 µM) of each compound or vehicle (DMSO) for 72 h. IC_{50} values were determined using nonlinear regression plots with GraphPad Prism6. Values are means ± SD of three independent experiments.