# Supporting Information

## In silico-guided identification of new potent inhibitors of carbonic anhydrases expressed in Vibrio cholerae

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## *1. Chemistry*

All reagents were used without further purification and bought from common commercial suppliers. Microwave-assisted reactions were carried out in a Focused Microwawe TM Synthesis System, Model Discover (CEM Technology Ltd Buckingham, UK). Melting points were determined on a Buchi B-545 apparatus (BUCHI Labortechnik AG Flawil, Switzerland) and are uncorrected. By combustion analysis (C, H, N) carried out on a Carlo Erba Model 1106-Elemental Analyzer we determined the purity of synthesized compounds; the results confirmed a ≥95% purity. Merck Silica Gel 60 F254 plates were used for analytical TLC (Merck KGaA, Darmstadt, Germany,). $^{1}$H-NMR and $^{13}$C-NMR spectra were measured in dimethylsulfoxide-$d_6$ (DMSO-$d_6$) with a Varian Gemini 500 spectrometer (Varian Inc. Palo Alto, California USA); chemical shifts are expressed in δ (ppm) and coupling constants (*J*) in hertz. All exchangeable protons were confirmed by addition of $D_2O$. $R_f$ values were determined on TLC plates ($SiO_2$) using a mixture of DCM/MeOH (9:1, v/v) as eluent. For compounds **20a-i** retrieved in SciFinder platform the registered CAS numbers have been already assigned. However, their synthetic procedures, chemical properties and structural characterization are not available in literature. Therefore, the experimental details and spectral data are reported below.

**Synthetic procedures for N-(4-sulfamoylbenzyl) amides 20a-i**

**Pathway i (A):** To a solution of 4-aminomethylbenzenesulfonamide hydrochloride (1 molar equivalent) in a mixture of DCM/DMF (6 mL, 2:1, v/v) placed in a cylindrical quartz tube (Ø 2 cm), N,N-Diisopropylethylamine (DIPEA) (2.5 molar equivalents) and the appropriate acyl chloride (0.8 molar equivalent) were added. The mixture was subjected to microwave irradiation at 250W for 10 minutes at 25 °C. The reaction progresses were monitored by thin layer chromatography (TLC) ($SiO_2$), by using a solution of DCM/MeOH (9:1, v/v) as eluent. Then, the solvent was removed under vacuum, the resulted residue diluted with of $H_2O$ (10 mL) and extracted with EtOAc (3 x 10 mL). The combined organic extracts were washed with brine solution, dried ($Na_2SO_4$) and concentrated until dryness under reduced pressure. The crude product was purified by crystallization with $Et_2O$ and EtOH to afford compounds **20a-e** and **20h** as white powders.

**Pathway i (B):** The suitable carboxylic acid derivative (1 molar equivalent) and N,N,N,N-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU) (1 molar equivalent) in DMF (2 mL) were placed in a cylindrical quartz tube (Ø 2 cm), stirred and irradiated in a microwave oven at 250 W for 5 minutes at 25 °C. Then, *N,N*-Diisopropylethylamine (DIPEA) (2.5 molar equivalents) and 4-aminomethylbenzenesulfonamide hydrochloride (1 molar equivalent) were added and the resulted solution was stirred and subjected to microwave irradiation for 25 minutes in the

same condition reported above. The reaction progresses were monitored by thin layer chromatography (TLC) ($SiO_2$), by using a solution of DCM/MeOH (9:1, v/v) as eluent. Then, the solvent was removed under vacuum, the resulted residue diluted with of $H_2O$ (10 mL) and extracted with EtOAc (3 x 10 mL). The combined organic extracts were washed with brine solution, dried ($Na_2SO_4$) and concentrated until dryness under reduced pressure. The crude product was purified by crystallization with $Et_2O$ and EtOH to afford compounds **20f-g** and **20i** as white powders.

*3-Methyl-N-(4-sulfamoylbenzyl)benzamide*, (CAS Number: 349136-13-6)**, 20a**

Yield: 68%; M.p.: 197-199 °C; $^1$H-NMR (500 MHz, DMSO-$d_6$): (δ) 9.07 (t, *J*= 5.9, 1H, NH), 7.78 (d, *J*= 8.3, 2H, ArH), 7.68-7.72 (m, 2H, ArH), 7.48 (d, *J*= 8.3, 2H, ArH), 7.36-7.37 (m, 2H, ArH), 7.30 (*bs*, 2H, $NH_2$), 4.52 (d, *J*= 5.9, 2H, $CH_2$), 2.36 (s, 3H, $CH_3$); $^{13}$C-NMR (126 MHz, DMSO-$d_6$): (δ) 166.6, 143.9, 142.7, 137.8, 134.3, 132.1, 128.4, 128.0, 127.7, 125.9, 124.6, 42.5, 21.1. Calcd. for $C_{15}H_{16}N_2O_3S$: C 59.19%, H 5.30%, N 9.20%. Found: C 59.09%, H 5.44%, N 9.15%.

*3-Chloro-N-(4-sulfamoylbenzyl)benzamide,* (CAS Number: 349136-17-0)**, 20b**

Yield: 59%; M.p.: 157-159 °C; $^1$H-NMR (500 MHz, DMSO-$d_6$): (δ) 9.25 (t, *J*= 5.9, 1H, NH), 7.94 (m, 1H, ArH), 7.85-7.87 (m, 1H, ArH), 7.78 (d, *J*= 8.3, 2H, ArH), 7.62-7.64 (m, 1H, ArH), 7.51-7.54 (m, 1H, ArH), 7.49 (d, *J*= 8.3, 2H, ArH), 7.31 (*bs*, 2H, $NH_2$), 4.52 (d, *J*= 5.9, 2H, $CH_2$), 2.36 (s, 3H, $CH_3$); $^{13}$C-NMR (126 MHz, DMSO-$d_6$ ): (δ) 164.9, 143.4, 142.7, 136.1, 133.3, 131.2, 130.4, 127.6, 127.1, 126.1, 125.7, 42.5, 39.5. Calcd. for $C_{14}H_{13}ClN_2O_3S$: C 51.77%, H 4.03%, N 8.63%. Found: C 51.97%, H 4.33%, N 8.41%.

*2,5-Dichloro-N-(4-sulfamoylbenzyl)benzamide,* (CAS Number: 349136-29-4) )**, 20c**

Yield: 57%; M.p.: 213-215 °C; $^1$H-NMR (500 MHz, DMSO-$d_6$): (δ) 9.16 (*t*, *J*= 5.9, 1H, NH), 7.80 (m, 2H, ArH), 7.53-7.61 (m, 5H, ArH), 7.33 (*bs*, 2H, $NH_2$), 4.52 (*d*, *J*= 5.9, 2H, $CH_2$). $^{13}$C-NMR (126 MHz, DMSO-$d_6$): 42.2, 125.8, 127.6, 128.6, 128.8, 130.7, 131.4, 131.8, 138.1, 142.8, 142.9, 165.2. Calcd. for $C_{14}H_{12}Cl_2N_2O_3S$: C 46.81%, H 3.37%, N 7.80%. Found: C 46.96%, H 3.44%, N 7.56%.

*4-Bromo-N-(4-sulfamoylbenzyl)benzamide*, (CAS Number: 349136-03-4)**, 20d**

Yield: 97% M.p.: 233-235 °C; $^1$H-NMR (500 MHz, DMSO-$d_6$): (δ) 9.20 (*t*, *J*= 5.9, 1H, NH), 7.83 (*d*, *J*= 8.3, 2H, ArH), 7.77 (*d*, *J*= 8.1, 2H, ArH), 7.69 (*d*, *J*= 8.3, 2H, ArH), 7.47 (*d*, *J*= 8.1, 2H, ArH), 7.30 (*bs*, 2H, $NH_2$), 4.52 (*d*, *J*= 5.9, 2H, $CH_2$). $^{13}$C-NMR (126 MHz, DMSO-$d_6$): 42.4, 125.1, 125.8, 127.6, 129.4, 131.4, 133.2, 142.7, 143.5, 165.4. Calcd. for $C_{14}H_{13}BrN_2O_3S$: C 45.54%, H 3.55%, N 7.59%. Found: C 45.33%, H 3.41%, N 7.68%;

*N-(4-Sulfamoylbenzyl)-[1,1'-biphenyl]-4-carboxamide,* (CAS Number: 349084-68-0)**, 20e**

Yield: 88%; M.p.: 367-369 °C; $^1$H-NMR (500 MHz, DMSO-$d_6$): (δ) 9.27 (t, *J*= 5.9, 1H, NH), 8.01 (d, *J*= 8.3, 2H, ArH), 7.78 (d, *J*= 8.3, 2H, ArH), 7.61-7.74 (m, 4H, ArH), 7.32-7.51 (m, 7H, ArH),

4.55 (d, *J*= 5.9, 2H, $CH_2$); $^{13}$C-NMR (126 MHz, DMSO-$d_6$): 165.4, 143.5, 142.6, 133.2, 131.4, 131.4, 129.4, 127.6, 125.7, 125.7, 125.1, 42.4, 39.5. Calcd. for $C_{20}H_{18}N_2O_3S$: C 65.55%, H 4.95%, N 7.64%. Found: C 65.46%, H 4.81%, N 7.50%.

*2-Benzyl-N-(4-sulfamoylbenzyl)benzamide* (CAS Number: 848052-23-3), **20f**

Yield: 85%; M.p.: 197-198 °C; $^1$H-NMR (500 MHz, DMSO-$d_6$): (δ) 8.94 (*t*, *J*= 5.9, 1H, NH), 7.75 (*m,* 2H, ArH), 7.36-7.43 (*m*, 4H, ArH), 7.33 (*bs*, 2H, $NH_2$), 7.13-7.30 (*m*, 7H, ArH), 4.47 (*d*, *J*= 5.9, 2H, $CH_2$), 4.12 (*s*, 2H, $CH_2$); $^{13}$C NMR (126 MHz, DMSO-$d_6$): (δ) 169.1, 143.4, 142.4, 140.9, 138.9, 136.3, 130.4, 129.6, 128.6, 128.1, 127.4, 127.4, 125.9, 125.8, 125.6, 42.0, 39.5, 37.6. Calcd. for $C_{21}H_{20}N_2O_3S$: C 66.29%, H 5.30%, N 7.36%. Found: C 66.10%, H 5.22%, N 7.00%.

*2-(2-Fluorophenyl)-N-(4-sulfamoylbenzyl)acetamide*, (CAS Number: 794539-67-6), **20 g**

Yield: 58%; M.p.: 196-198 °C; $^1$H-NMR (500 MHz, DMSO-$d_6$): (δ) 8.66 (*t*, *J*= 5.9, 1H, NH), 7.76 (*d*, *J*= 8.3, 2H, ArH), 7.42 (*d*, *J*= 8.3, 2H, ArH), 7.30-7.35 (m, 4H, ArH and $NH_2$), 7.15-7.16 (m, 2H, ArH), 4.34 (*d*, *J*= 5.9, 2H, $CH_2$); $^{13}$C NMR (126 MHz, DMSO-$d_6$): (δ) 169.4, 143.7, 142.8, 132.1, 132.0, 128.8, 128.8, 127.6, 125.8, 124.4, 115.3, 115.1, 42.1, 39.5, 35.5. Calcd. for $C_{15}H_{15}FN_2O_3S$: C 55.89%, H 4.69%, N 8.69%. Found: C 55.91%, H 4.38%, N 8.55%.

*N-(4-Sulfamoylbenzyl)thiophene-3-carboxamide,* (CAS Number: 1090372-43-2), **20h**

Yield: 76%; M.p.: 222-224 °C; $^1$H-NMR (500 MHz, DMSO-$d_6$): (δ) 9.00 (*t*, *J*= 5.9, 1H, NH), 8.18 (*dd*, *J*= 2.98, *J*= 1.28), 7.78 (*d*, *J*= 8.3, 2H, ArH), 7.60 (*dd*, *J*= 7.82, *J*= 2.98, 1H, ArH), 7.55 (*dd*, *J*= 7.82, *J*= 1.28, 1H, ArH), 7.48 (*d*, *J*= 8.3, 2H, ArH), 7.31 (*bs*, 2H, $NH_2$), 4.50 (*d*, *J*= 5.9, 2H, $CH_2$); $^{13}$C NMR (126 MHz, DMSO-$d_6$): (δ) 162.1, 143.8, 142.6, 137.5, 129.1, 127.6, 126.9, 126.7, 125.7, 41.9, 39.5. Calcd. for $C_{12}H_{12}N_2O_3S_2$: C 48.63%, H 4.08%, N 9.45. Found: C 48.75%, H 4.01%, N 9.66%.

*3-(Methylthio)-N-(4-sulfamoylbenzyl)propanamide*, (CAS Number: 851629-70-4), **20i**

Yield: 84%; M.p.: 128-130 °C; $^1$H-NMR (500 MHz, DMSO-$d_6$): (δ) 8.50 (*t*, *J*= 5.9, 1H, NH), 7.76 (*d*, *J*= 8.3, 2H, ArH), 7.43 (*d*, *J*= 8.3, 2H, ArH), 7.30 (*bs*, 2H, $NH_2$), 4.33 (*d*, *J*= 5.9, 2H, $CH_2$), 2.69 (*d*, *J*= 6.9 2H, $CH_2$), 2.46 (*d*, *J*= 6.9 2H, $CH_2$), 2.06 (*s*, 3H, $CH_3$). Calcd. for $C_{11}H_{16}N_2O_3S_2$: C 45.81%, H 5.59%, N 9.71%. Found: C 45.75%, H 5.50%, N 9.58%.

### *2. Carbonic anhydrase inhibition assay*

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed $CO_2$ hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 – 20 mM Hepes (pH 7.5) or Tris (pH 8.3) as buffers, and 20 mM $Na_2SO_4$ or 20 mM $NaClO_4$ (for maintaining constant the ionic strength), following the

initial rates of the CA-catalyzed $CO_2$ hydration reaction for a period of 10-100s. The $CO_2$ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier, and represent the mean from at least three different determinations. CA isoforms were recombinant ones obtained in-house.

### 3. *Modelled structure of the opening of the active site of β–carbonic anhydrase from Vibrio cholerae*

The protein structure used to perform docking studies was built by following the procedure reported in literature from our group.[1, 2]

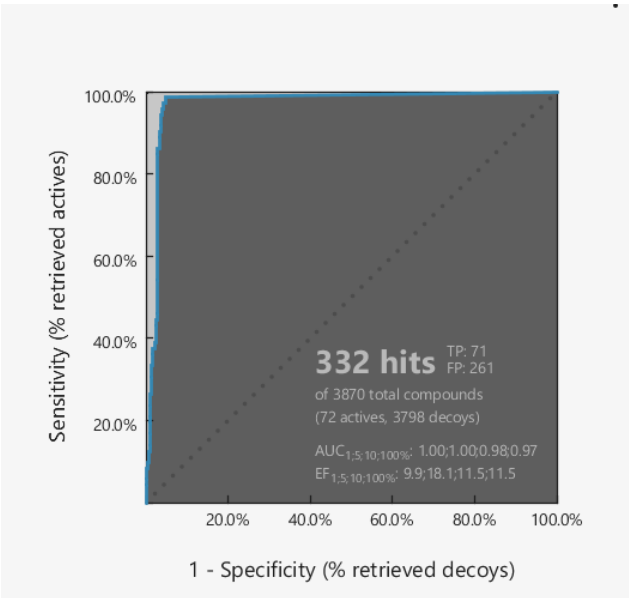
### 4. *Docking studies*

The complex obtained was used to perform docking studies by GOLD software[3] and the AAZ was discarded. The ligand structures were constructed by Vega 3.1.1 and the energy was minimized by using the Conjugate Gradient method (1000 steps). The minimized ligands were docked in their corresponding proteins using Gold Suite 5.0.1. The region of interest, which was used by the Gold program, was defined as containing the residues within 10 Å of the original position of the acetazolamide in the model structure. A scaffold constraint (penalty = 5.0) was used to restrict the solutions in which the sulfonamide moiety was able to coordinate the metal within the catalytic binding site. ChemPLP was chosen as the fitness function. The standard default settings were used in all the calculations and the ligands were submitted to 100 genetic algorithm runs. The "allow early termination" command was deactivated. Results differing by less than 0.75 Å in the ligand−all atom RMSD were clustered together. The conformations with the highest fitness values were chosen for both analysis and representation. All obtained complexes were minimized keeping the Zn ion fixed and using the conjugated gradients algorithm by NAMD.[4] The results were displayed using the PyMOL software (https://pymol.org ).

### 5. Pharmacophore Modelling and Virtual Screening

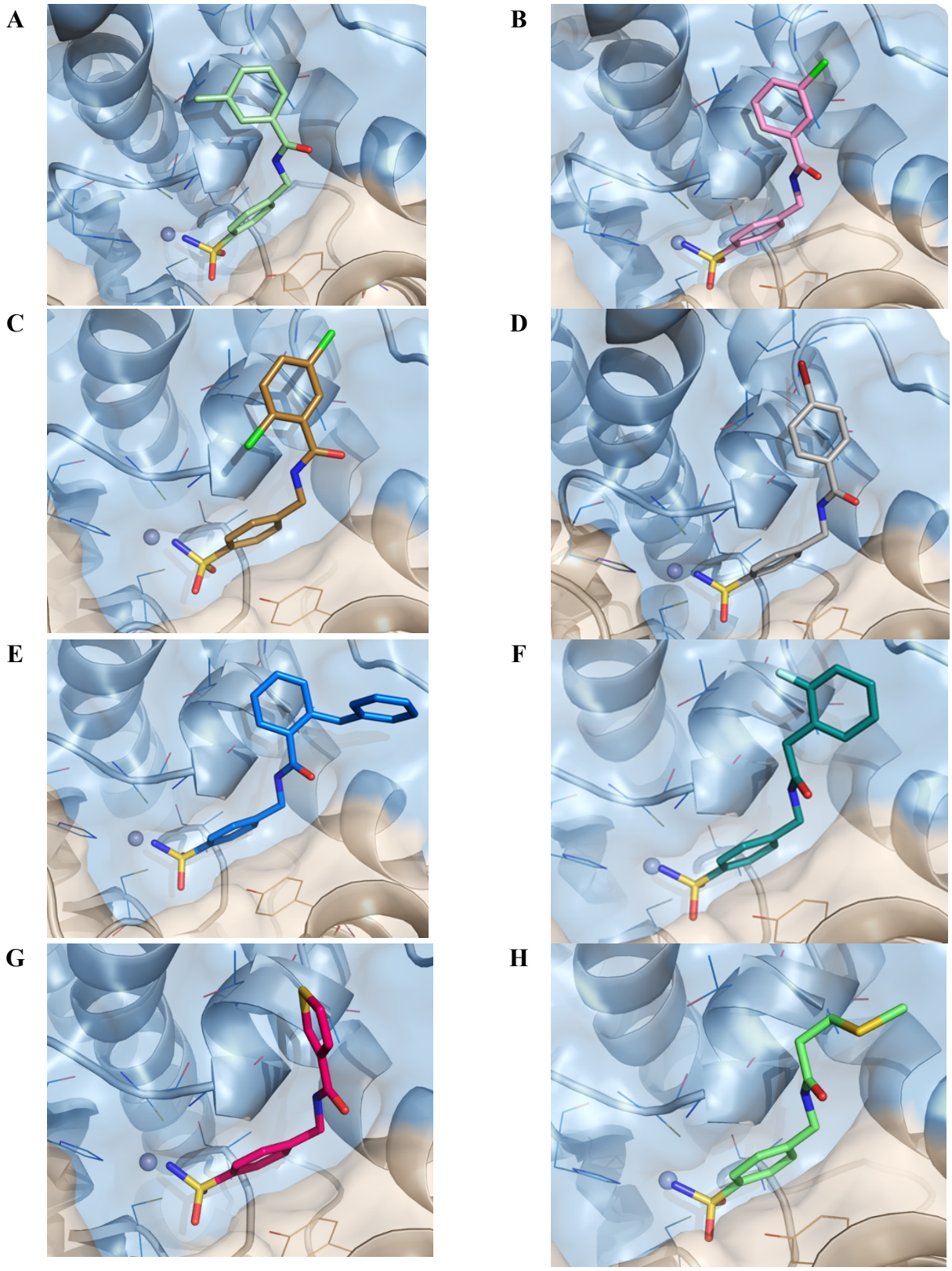
LigandScout V4.4.2[5] was used for the pharmacophore generation and the virtual screening process. All selected molecules (Training Set and Test Set) were constructed by Vega 3.1.1.[6] The ligand-based pharmacophore model was generated using "merged features" option and maintaining the default parameters. Our model was validated by using two datasets a) the first one containing 72 inhibitors of the β-class of VchCA presenting a sulfonamide moiety and with a $K_i$ value < 10000 nM; b) the second one was generated throughout a web tool online DUD-E (http://dude.docking.org/) obtaining 3899 decoys. The ligand-based pharmacophore model was used for a virtual screening against a SciFinder database containing 8.208 para-benzenesulfonamide derivatives. All virtual screening runs were conducted by setting the option "Get best matching conformation" as retrieval mode and the obtained hits from screening were ranked based on their pharmacophore fit scores.

## 6. Figure 1: ROC curve of the best performing pharmacophore model




The active set was composed of 72 compounds collected from the literature and the decoys were 3798 compounds with unknown activity toward β CA isozyme. The Figure 1S displays the ROC plot, the AUC and the EF factor values at 1%, 5%, 10% and 100% of the best pharmacophore hypothesis that has been used for the following studies. As shown in Figure 3, the early enrichment (EF 1%) is equal to 9.9 with an AUC value of 1.00 demonstrating that our pharmacophore model was able to distinguish active compounds.

7. **Figure 2. Predicted binding mode for compounds 20 a-d and 20f-i into "modelled" VchCAβ.**



## 8. *Figures 3-18: Selected $^{1}$H- and $^{13}$C-NMR spectra for representative compounds*

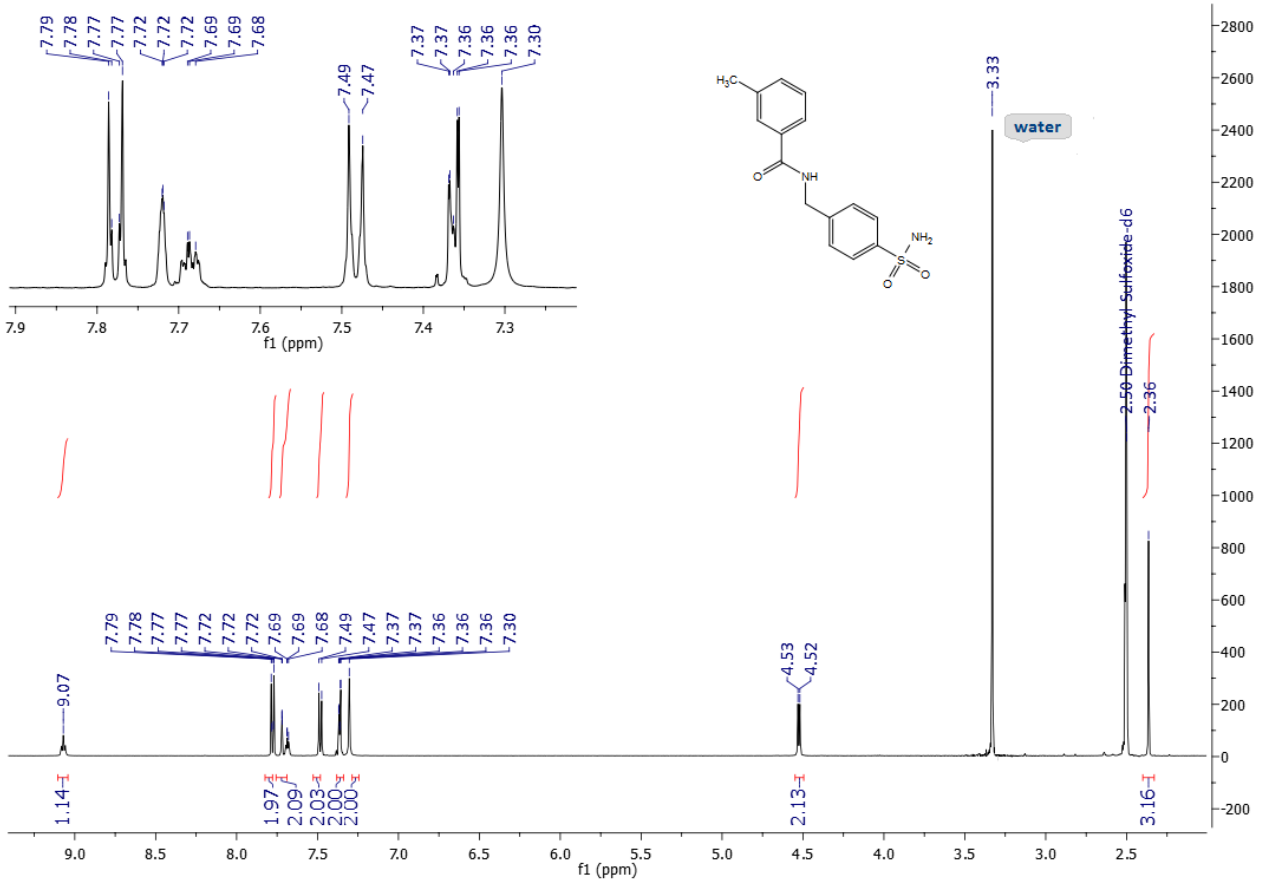



**Figure 3.** $^{1}$H-NMR (DMSO-$d_6$) spectrum of for 3-methyl-N-(4-sulfamoylbenzyl)benzamide (**20a**)

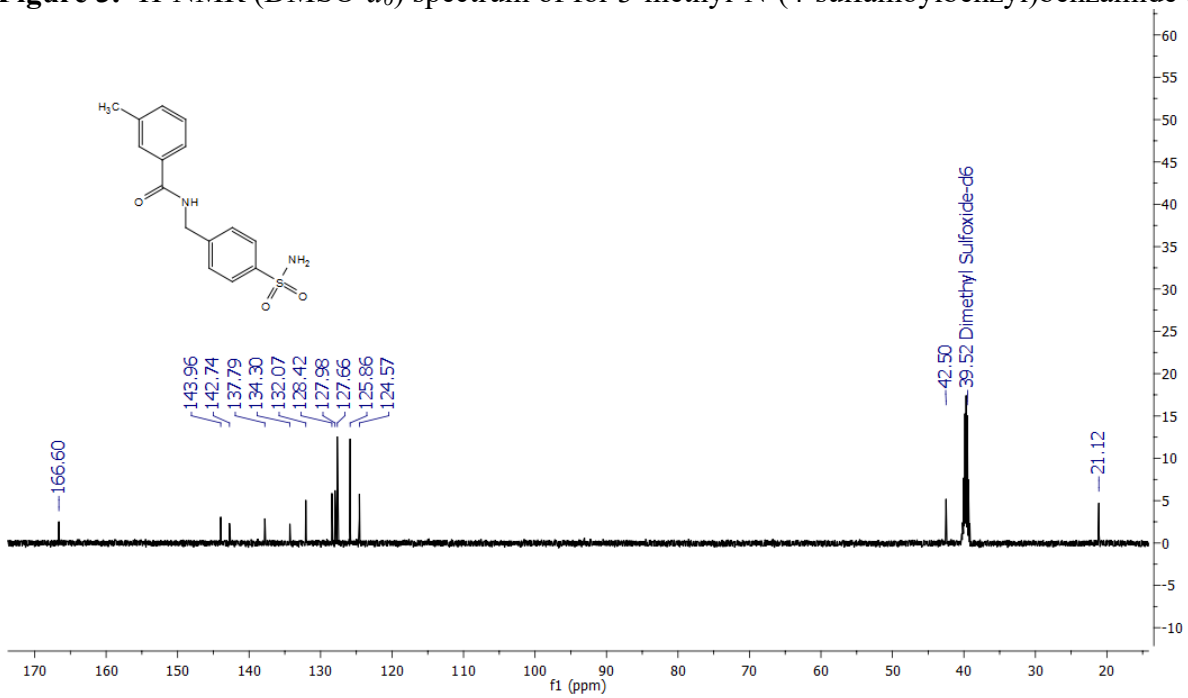



**Figure 4.** $^{13}$C-NMR (DMSO-$d_6$) spectrum for 3-methyl-N-(4-sulfamoylbenzyl)benzamide (**20a**)

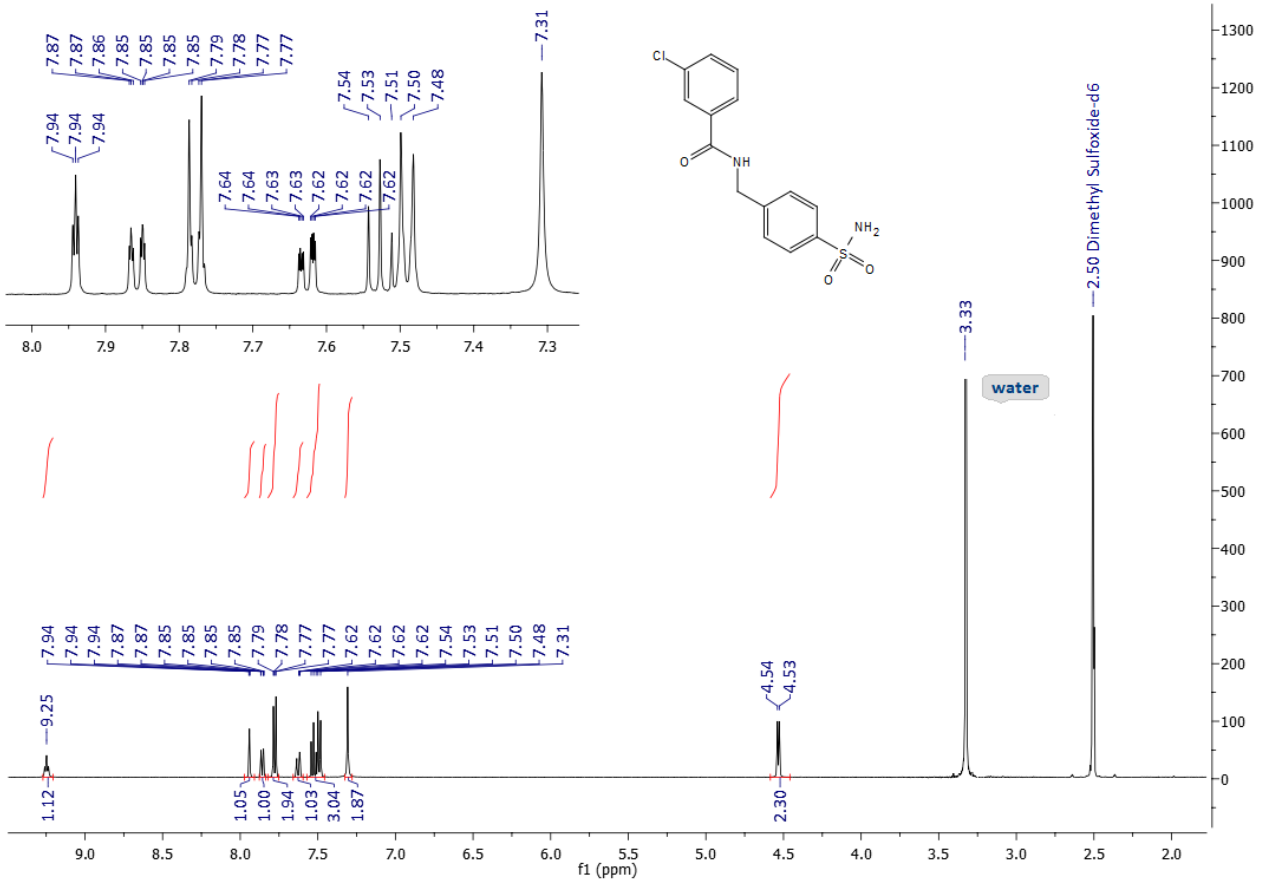



**Figure 5**. $^{1}$H-NMR (DMSO-$d_6$) spectrum for 3-chloro-N-(4-sulfamoylbenzyl)benzamide (**20b**)

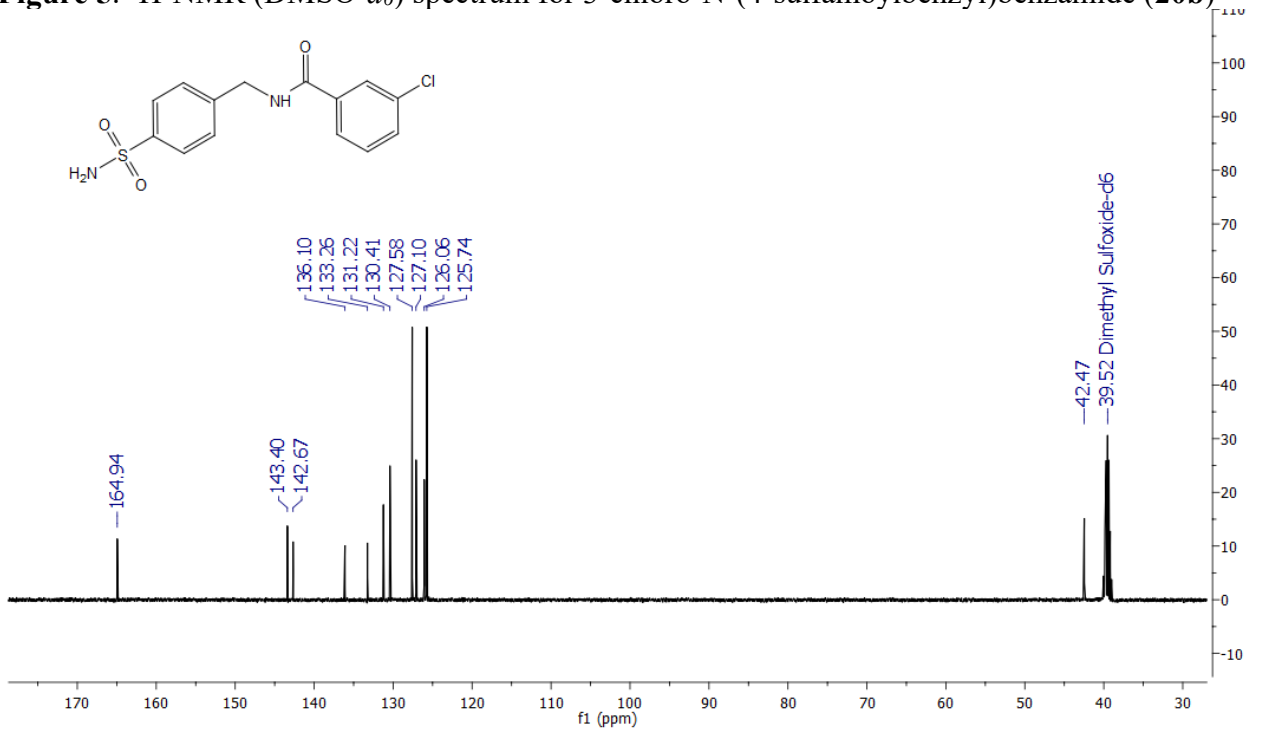



**Figure 6**. $^{13}$C-NMR (DMSO-$d_6$) spectrum for 3-chloro-N-(4-sulfamoylbenzyl)benzamide (**20b**)

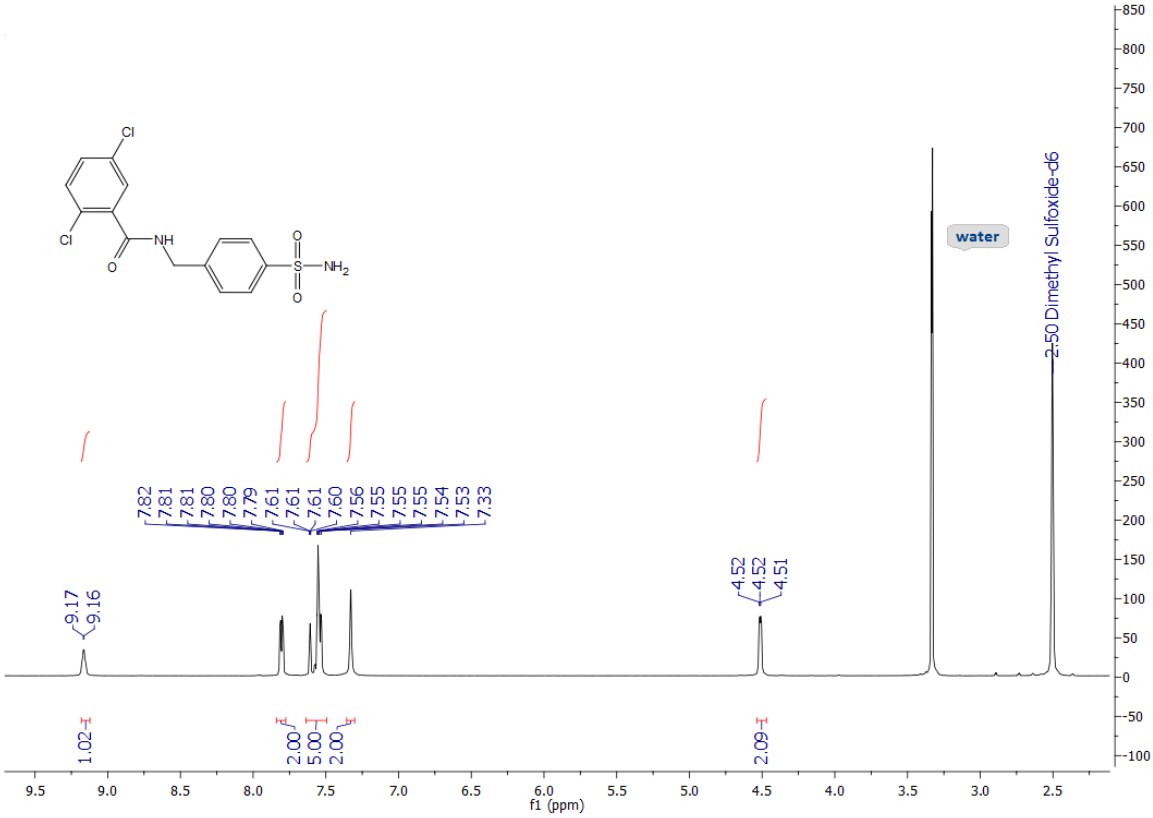



**Figure 7**. $^{1}$H-NMR (DMSO-$d_6$) spectrum for 2,5-dichloro-N-(4-sulfamoylbenzyl)benzamide (**20c**)

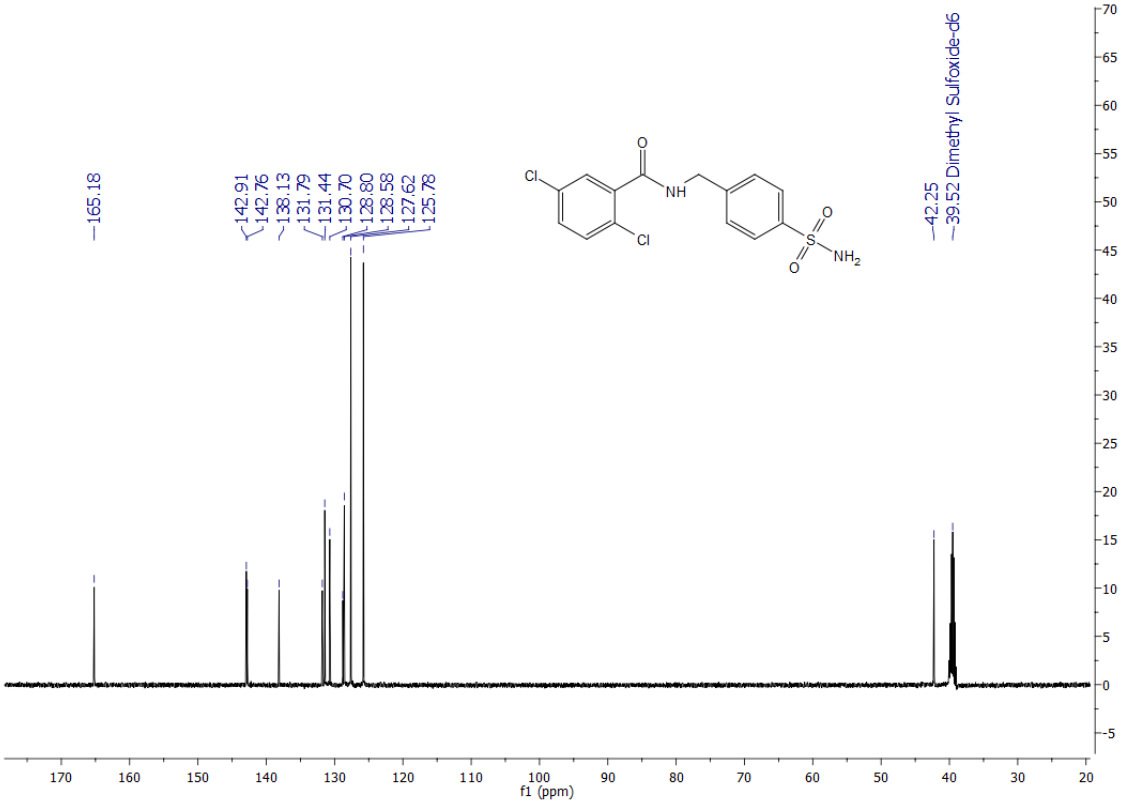



**Figure 8**. $^{13}$C-NMR (DMSO-$d_6$) spectrum for 2,5-dichloro-N-(4-sulfamoylbenzyl)benzamide (**20c**)

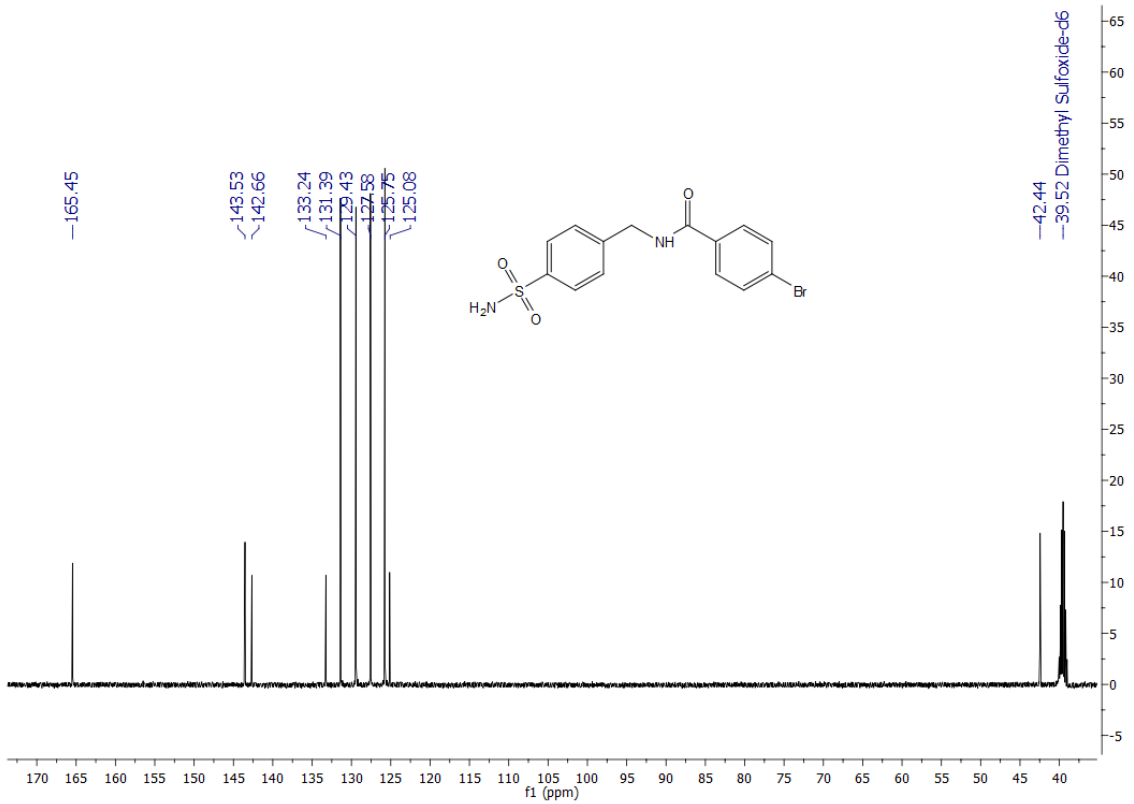



**Figure 9**. $^{13}$C-NMR (DMSO-d$_6$) spectrum for 4-Bromo-N-(4-sulfamoylbenzyl)benzamide (**20d**)

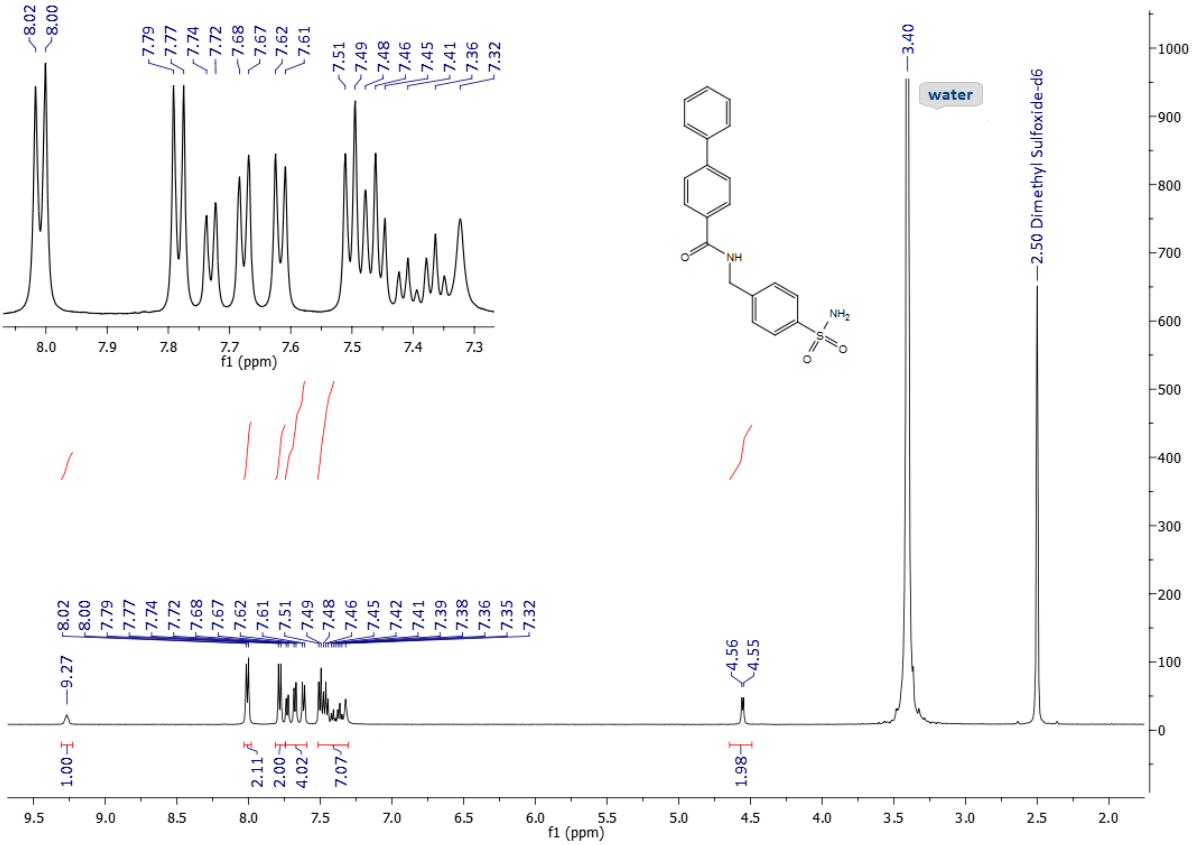



**Figure 10.** $^{1}$H-NMR (DMSO-d$_6$) spectrum for N-(4-sulfamoylbenzyl)-[1,1'-biphenyl]-4-carboxamide (**20e**)

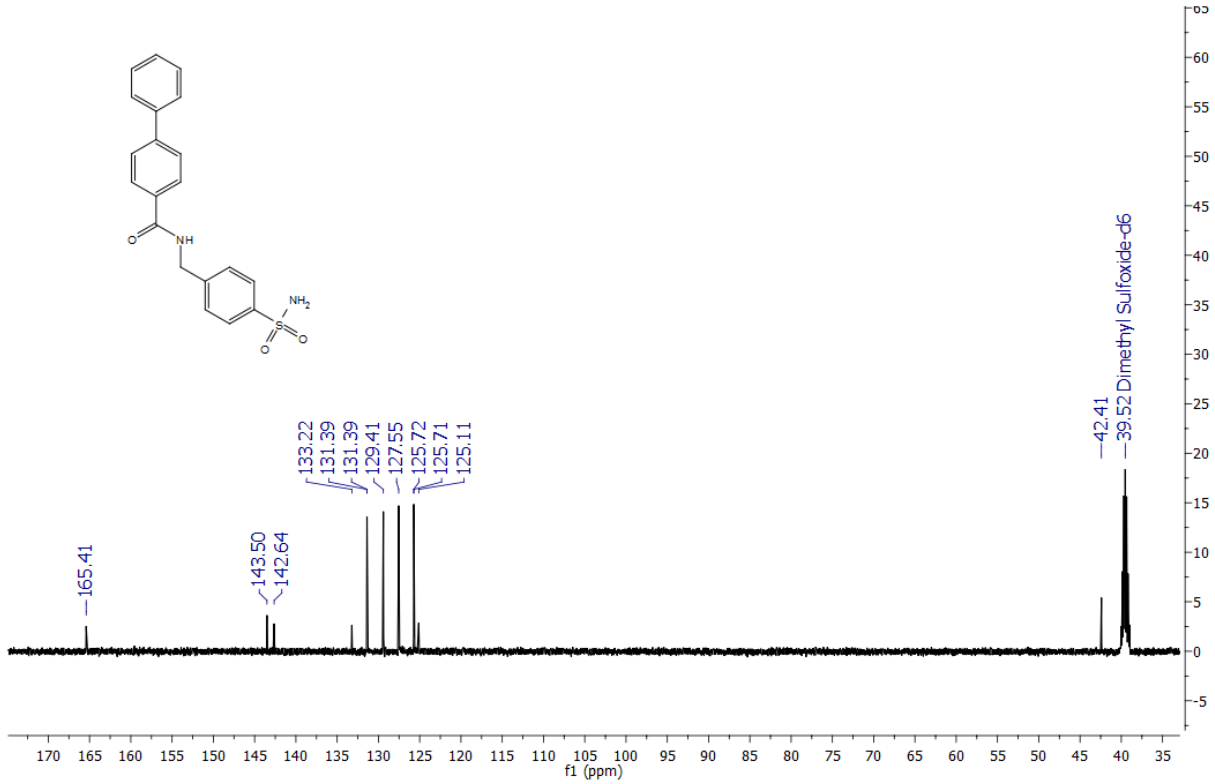



**Figure 11**. $^{13}$C-NMR (DMSO-d$_6$) spectrum for N-(4-sulfamoylbenzyl)-[1,1'-biphenyl]-4-carboxamide (**20e**)

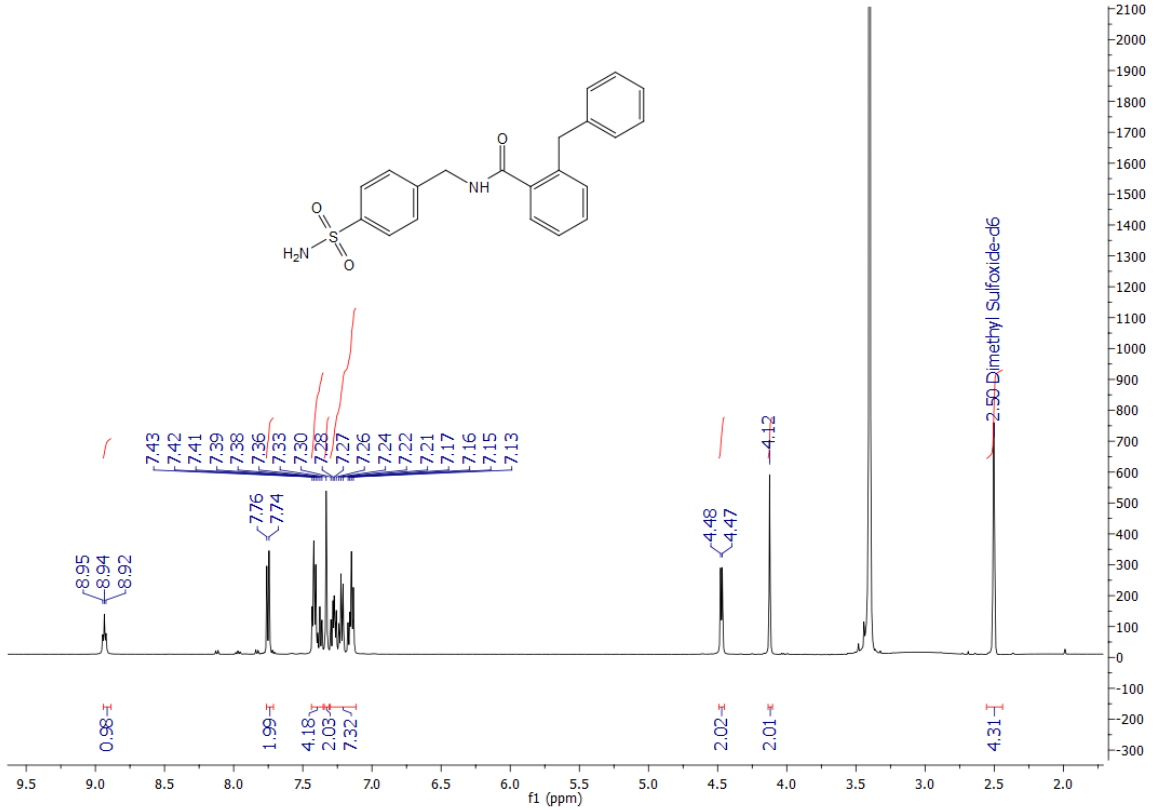



**Figure 12**. $^{1}$H-NMR (DMSO-d$_6$) spectrum for 2-benzyl-N-(4-sulfamoylbenzyl)benzamide (**20f**)

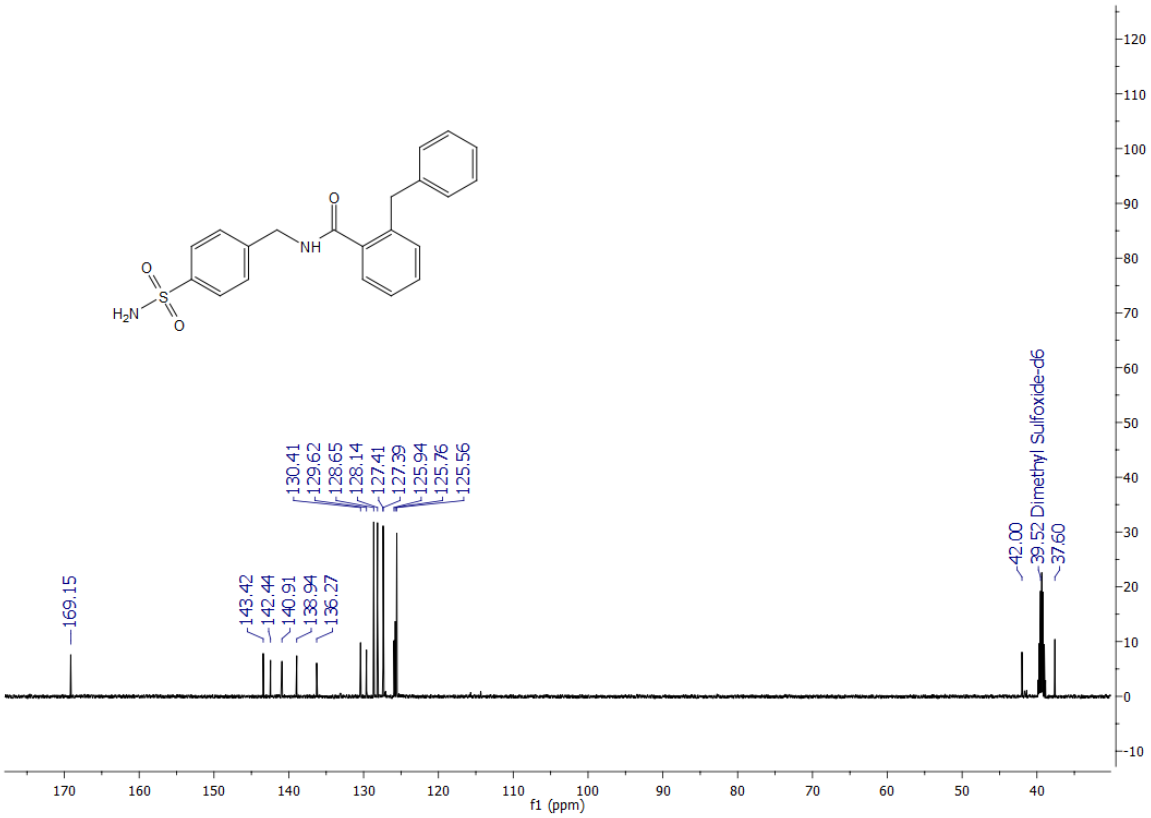



**Figure 13**. $^{13}$C-NMR (DMSO-$d_6$) spectrum for 2-benzyl-N-(4-sulfamoylbenzyl)benzamide (**20f**)

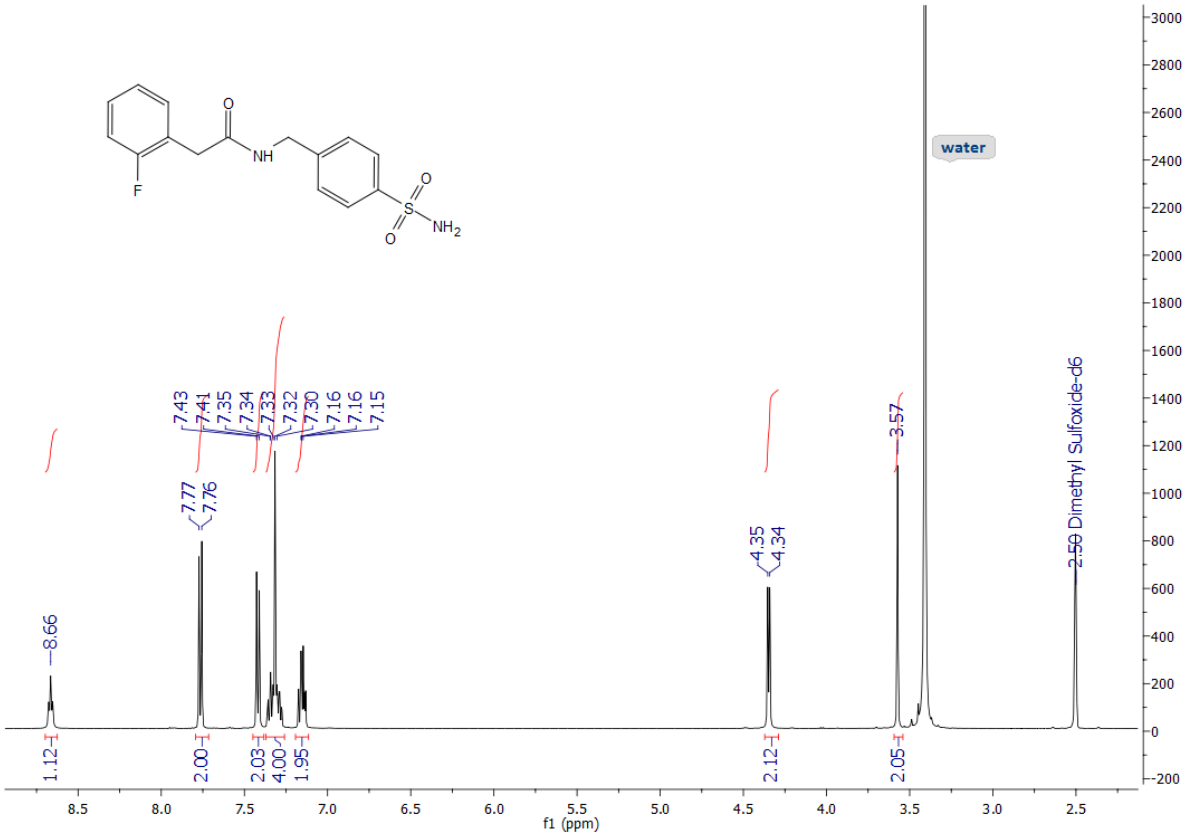



**Figure 14**. $^{1}$H-NMR (DMSO-$d_6$) spectrum for 2-(2-fluorophenyl)-N-(4-sulfamoylbenzyl)acetamide (**20g**)

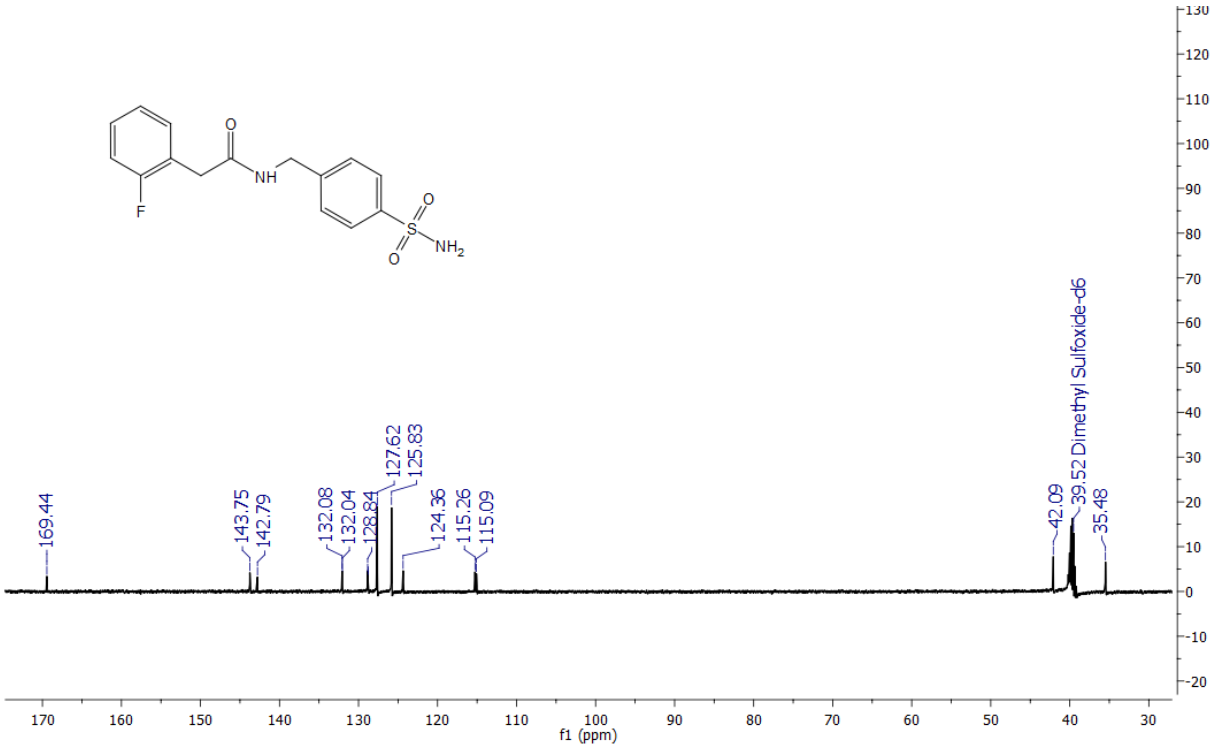



**Figure 15**. $^{13}$C-NMR (DMSO-$d_6$) spectrum for 2-(2-fluorophenyl)-N-(4-sulfamoylbenzyl)acetamide (**20g**)

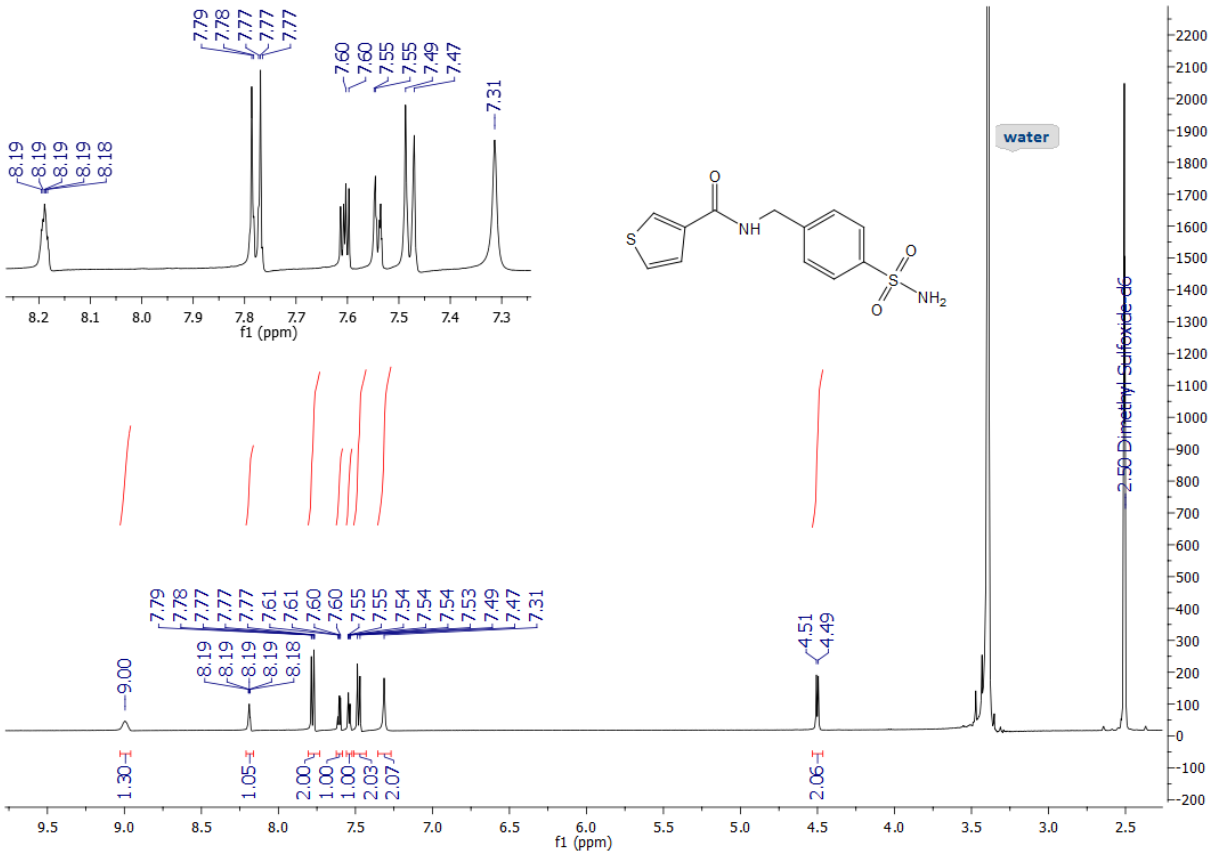



**Figure 16**. $^{1}$H-NMR (DMSO-$d_6$) spectrum for N-(4-sulfamoylbenzyl)thiophene-3-carboxamide (**20h**)

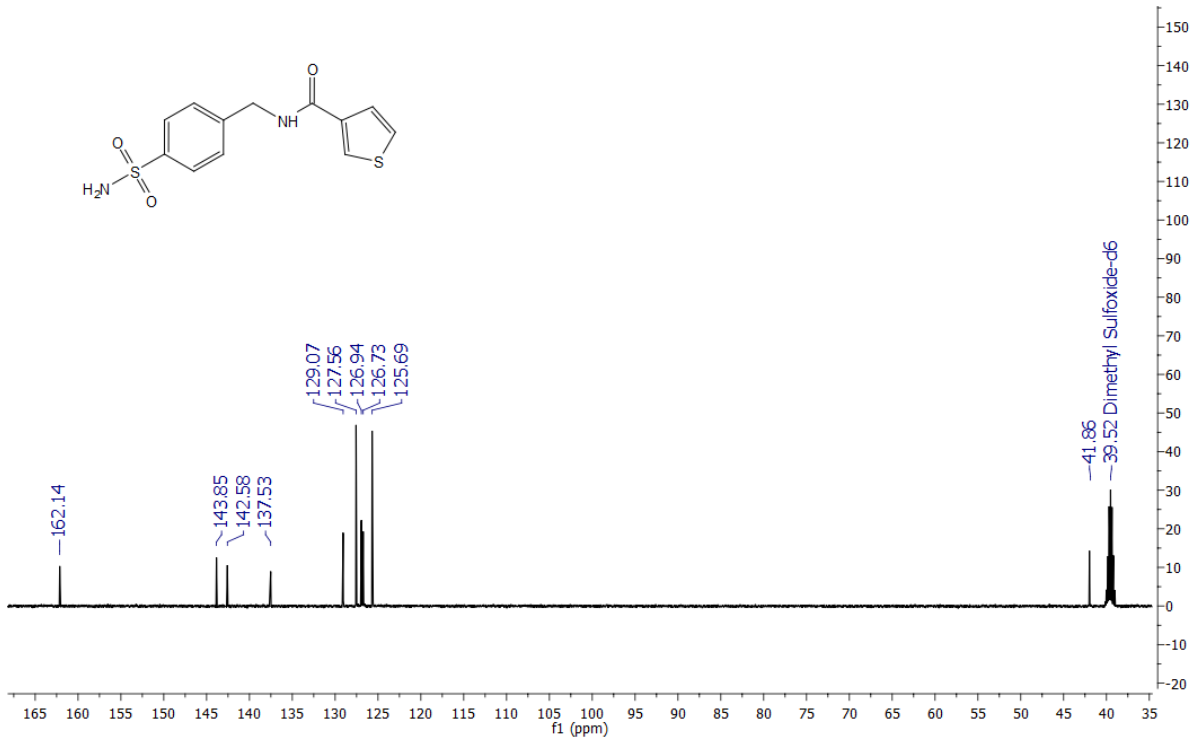



**Figure 17**. $^{13}$C-NMR (DMSO-$d_6$) spectrum for N-(4-sulfamoylbenzyl)thiophene-3-carboxamide (**20h**)

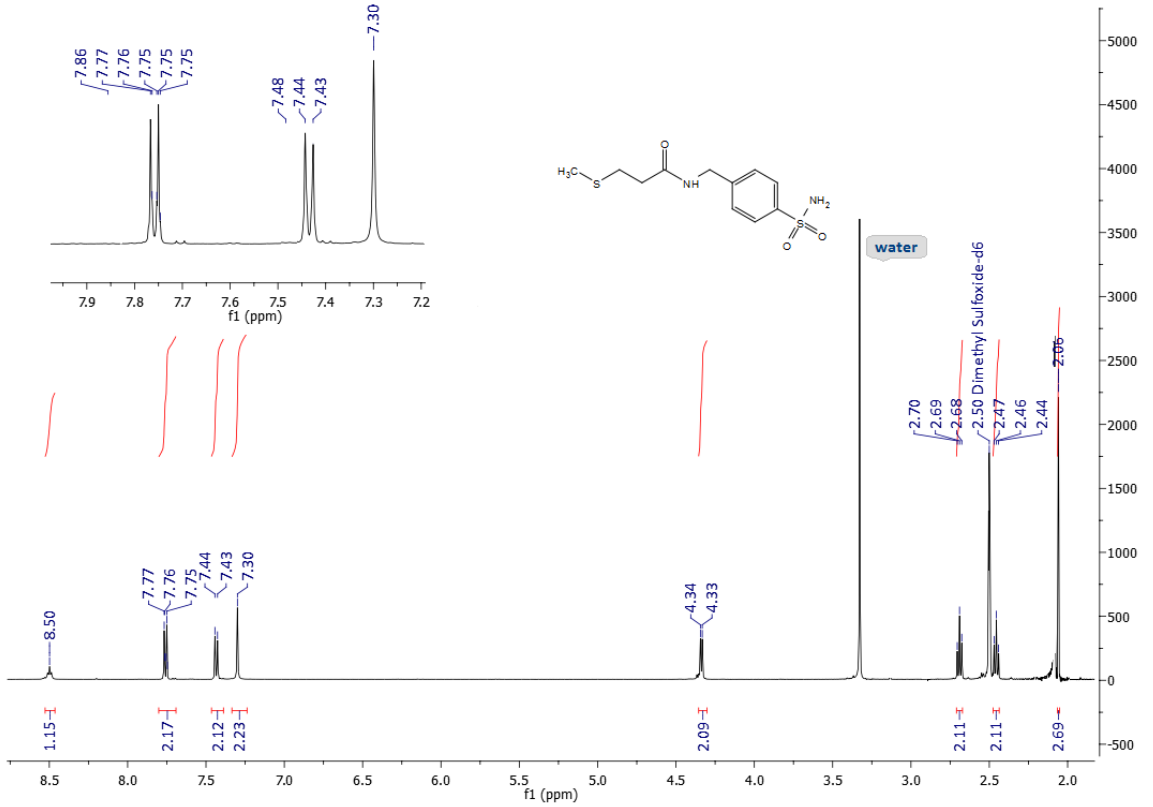



**Figure 18**. $^{1}$H-NMR (DMSO-$d_6$) spectrum for 3-(methylthio)-N-(4-sulfamoylbenzyl)propanamide (**20i**)

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