Supplementary Material

Synthesis and characterization of sulphanilamide and benzimidazole pharmacophores containing γ-amino acid derivatives as dual antimicrobial and anticancer agents

Meta Mackeviciute,[1] Rita Vaickelioniene,[1] Kazimieras Anusevicius,[1] Jurate Siugzdaite,[2] Raimundas Lelesius,[2] Povilas Kavaliauskas,[1,3-6] and Vytautas Mickevicius[1]

[1]Kaunas University of Technology, Radvilenu Road 19, 50254, Kaunas, Lithuania. [2]Department of Patobiology, Lithuanian University of Health Sciences, Tilzes Street 18, 47181 Kaunas, Lithuania. [3]Transplantation-Oncology Infectious Diseases Program, Division of Infectious Diseases, Department of Medicine, Weill Cornell Medicine of Cornell University, 1300 York Avenue, New York, NY 10065, USA. [4]Institute for Genome Sciences, School of Medicine, University of Maryland, Baltimore School of Medicine, 655 W. Baltimore Street, Baltimore, MD 21201, USA. [5]Institute of Infectious Diseases and Pathogenic Microbiology, Birstono Street 38A, 59116 Prienai, Lithuania. [6]Biological Research Center, Lithuanian University of Health Sciences, Tilzes Street 18,
47181 Kaunas, Lithuania
Email: rita.vaickelioniene@ktu.lt

Table of Contents

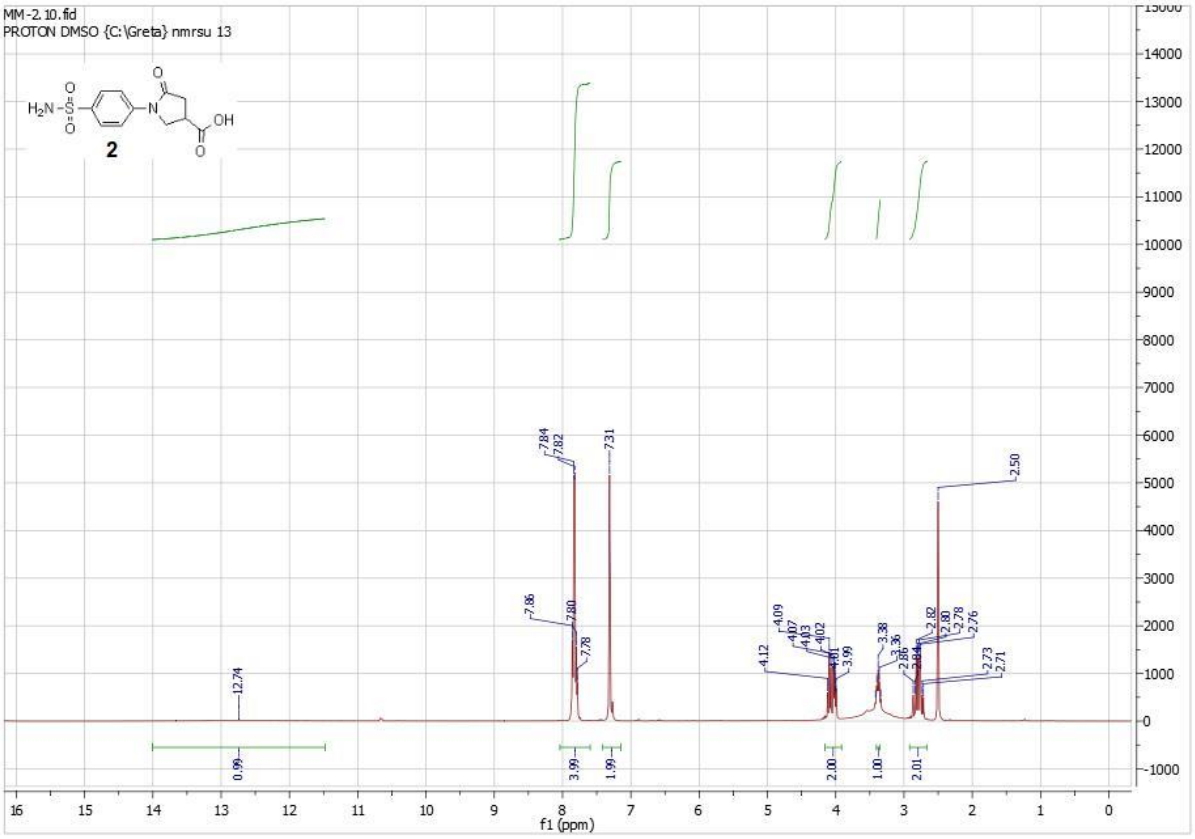

Figure S1. ^{1}H NMR of compound **2** at 400 MHz (DMSO-d_6).

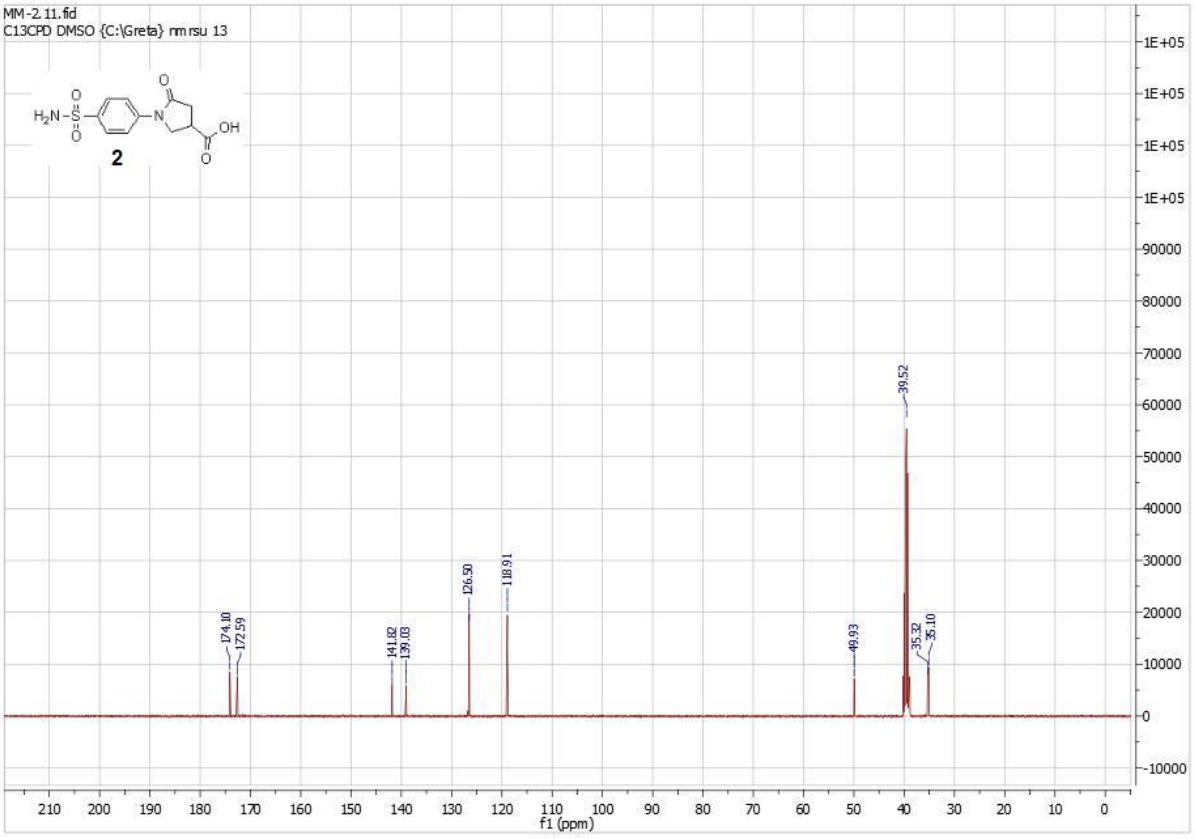

Figure S2. 13C NMR of compound **2** at 101 MHz (DMSO-d_6).

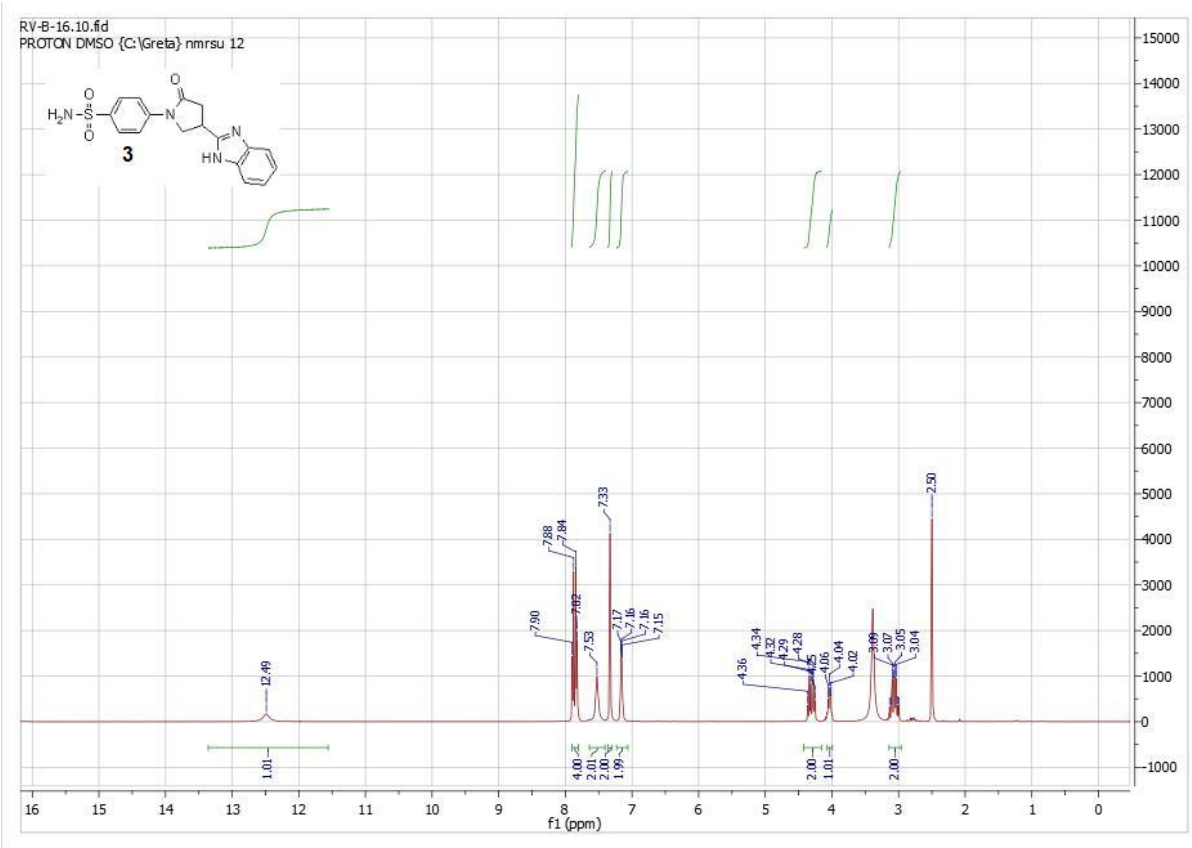

Figure S3. ^{1}H NMR of compound **3** at 400 MHz (DMSO-d_6).

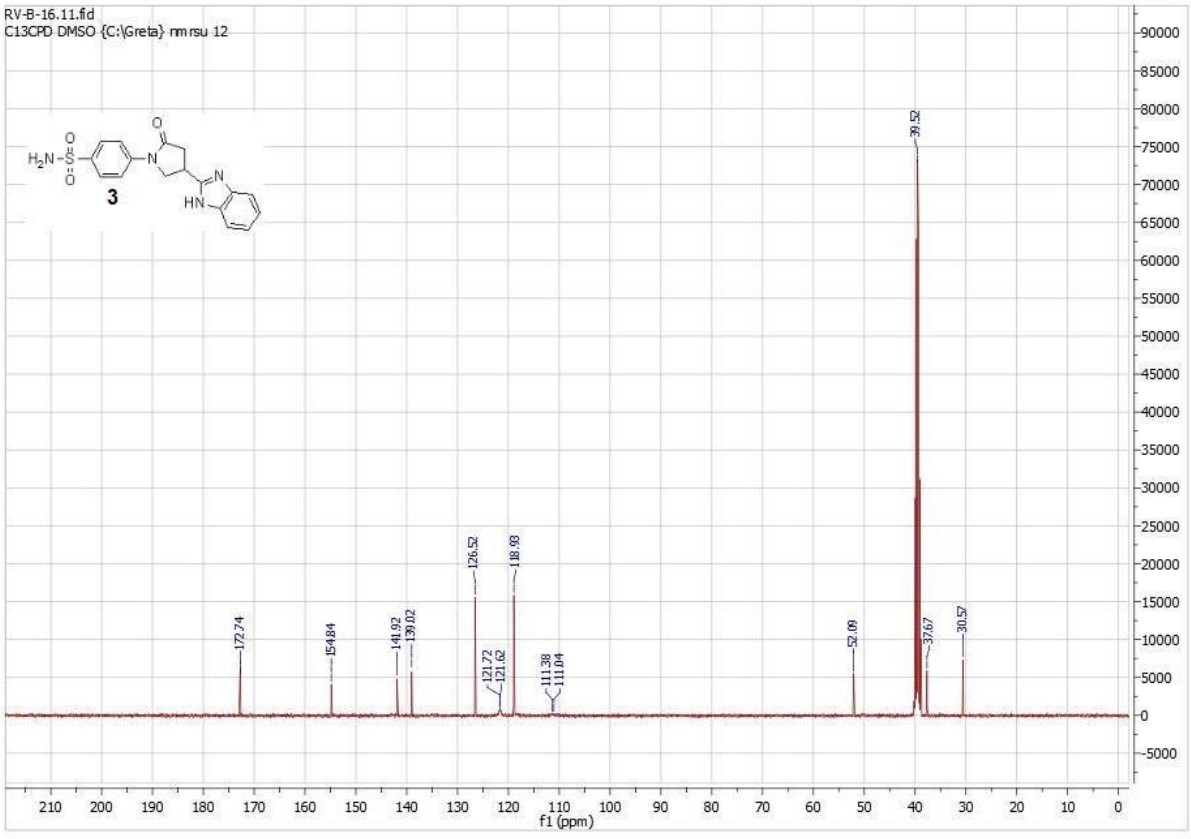

Figure S4. ^{13}C NMR of compound **3** at 101 MHz (DMSO-d_6).

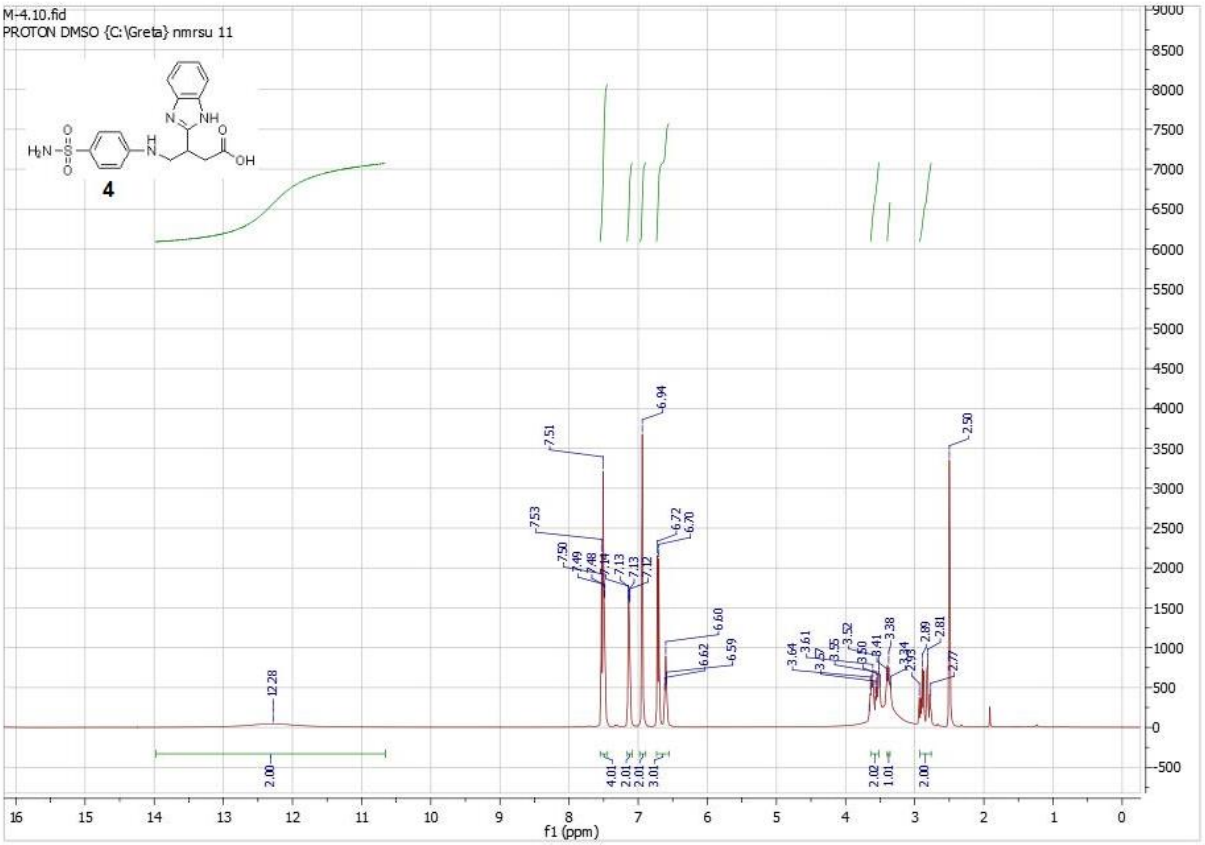

Figure S5. ^{1}H NMR of compound **4** at 400 MHz (DMSO-d_6).

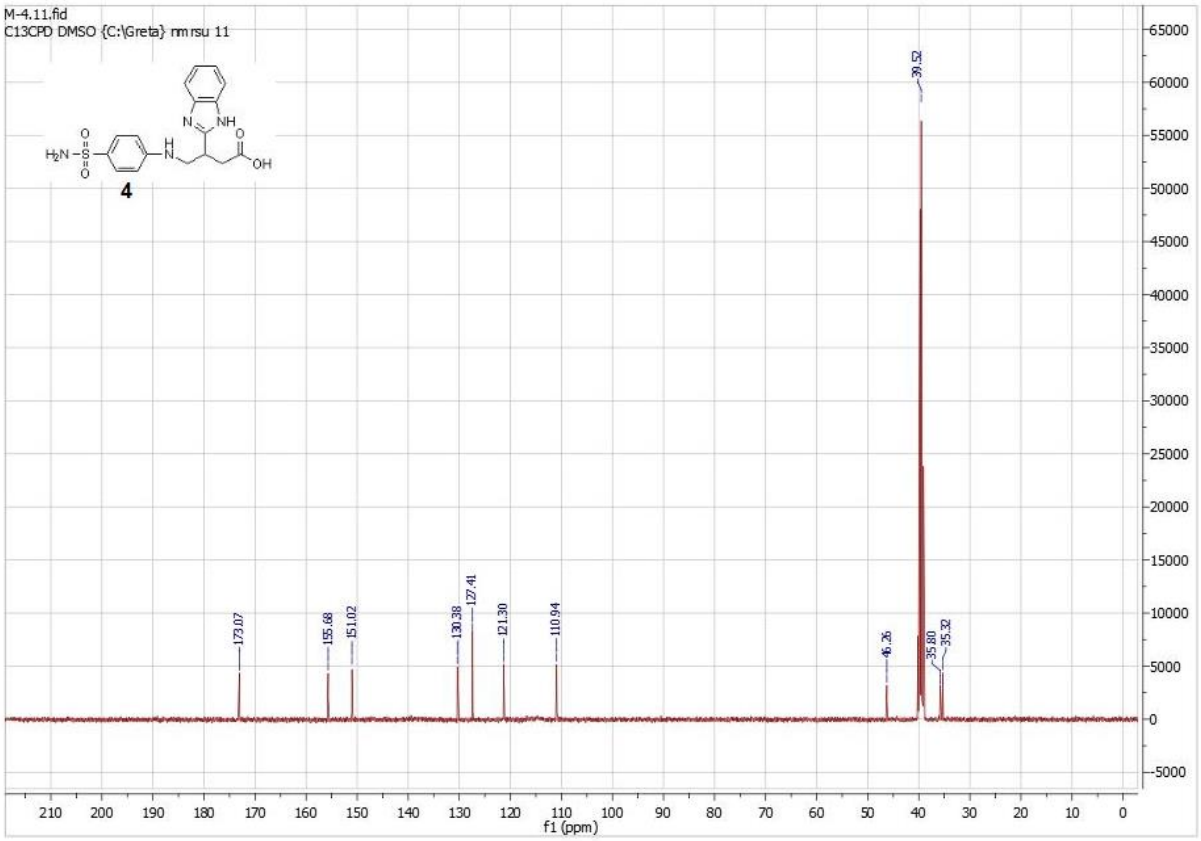

Figure S6. ^{13}C NMR of compound **4** at 101 MHz (DMSO-d_6).

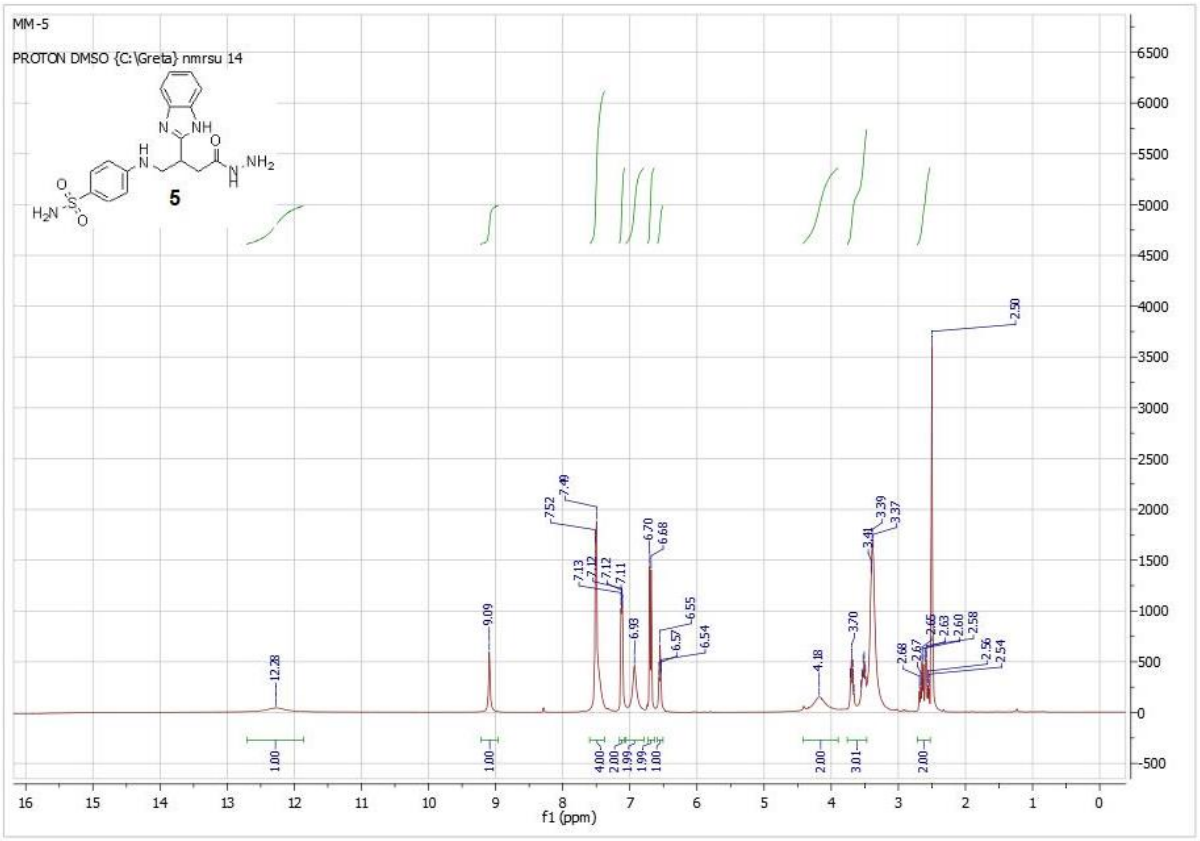

Figure S7. ^{1}H NMR of compound **5** at 400 MHz (DMSO-d_6).

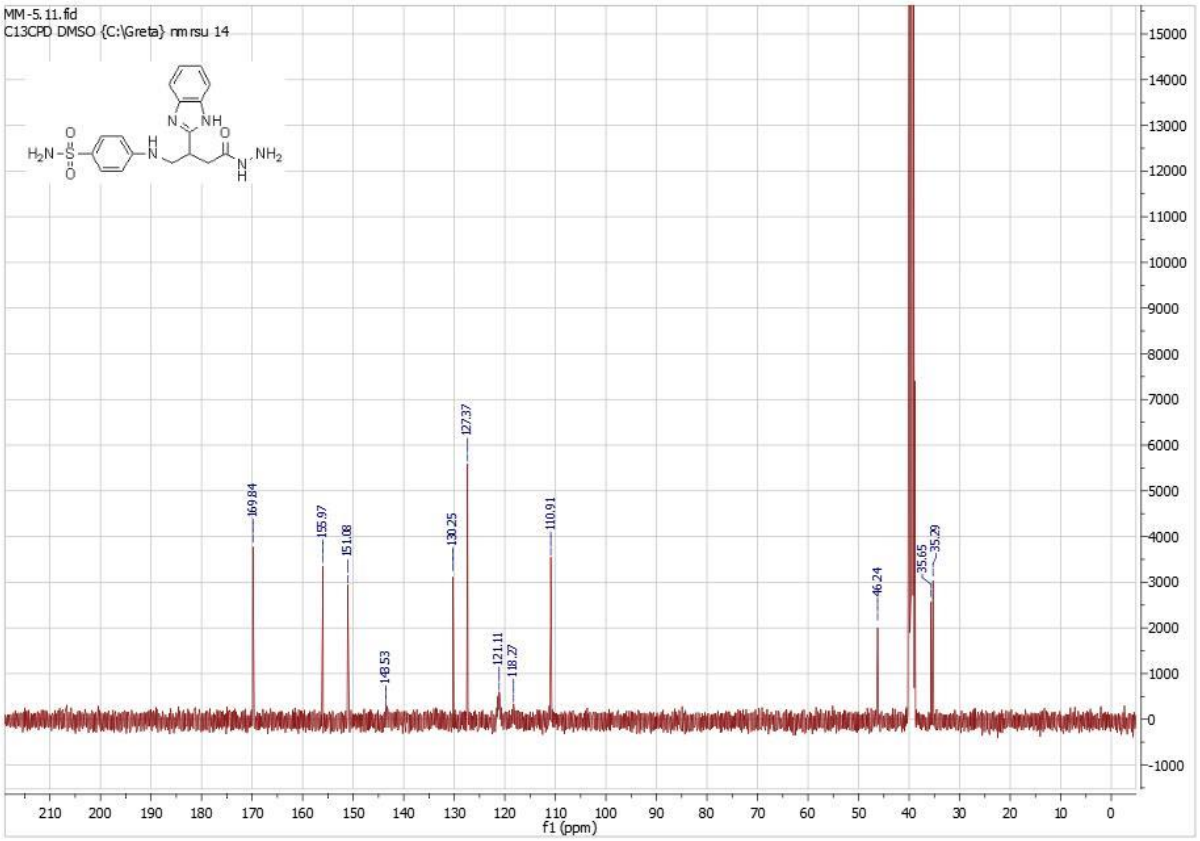

Figure S8. ^{13}C NMR of compound **5** at 101 MHz (DMSO-d_6).

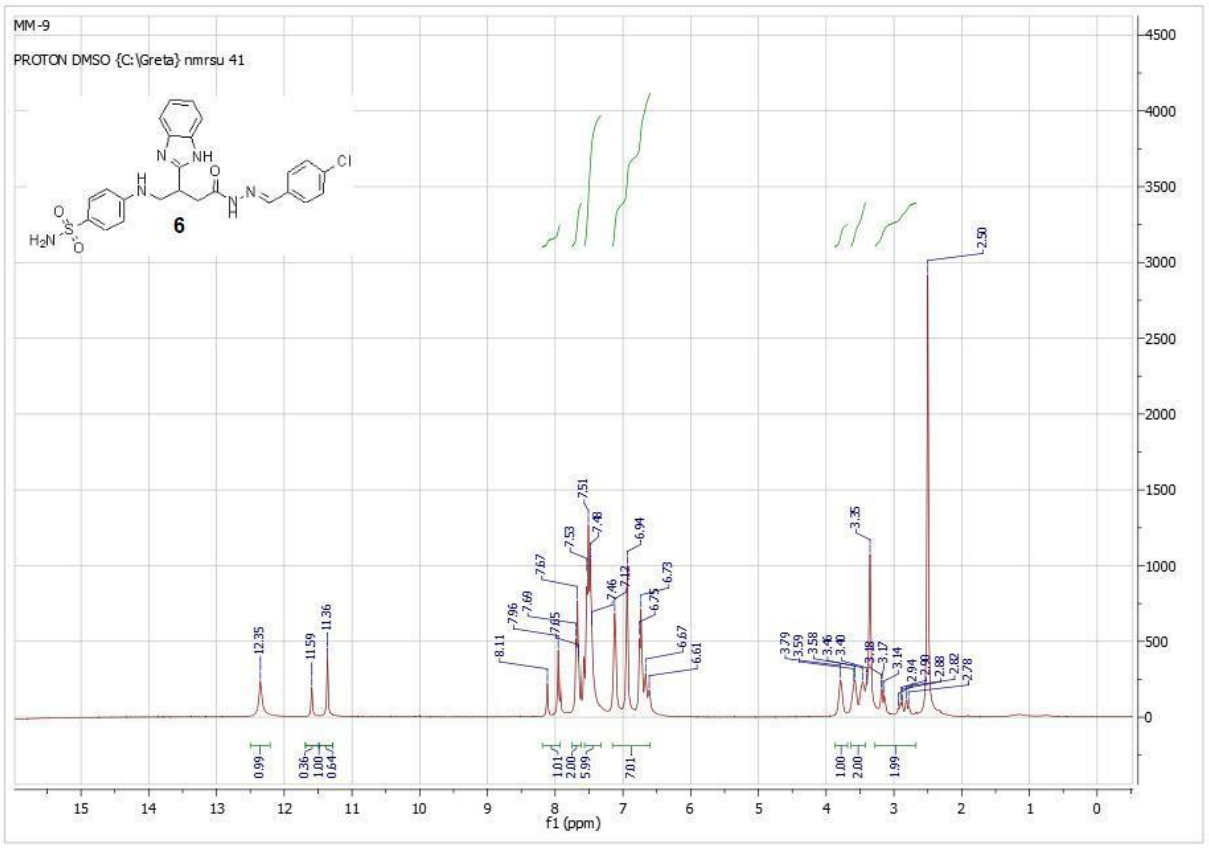

Figure S9. ^{1}H NMR of compound **6** at 400 MHz (DMSO-d_6).

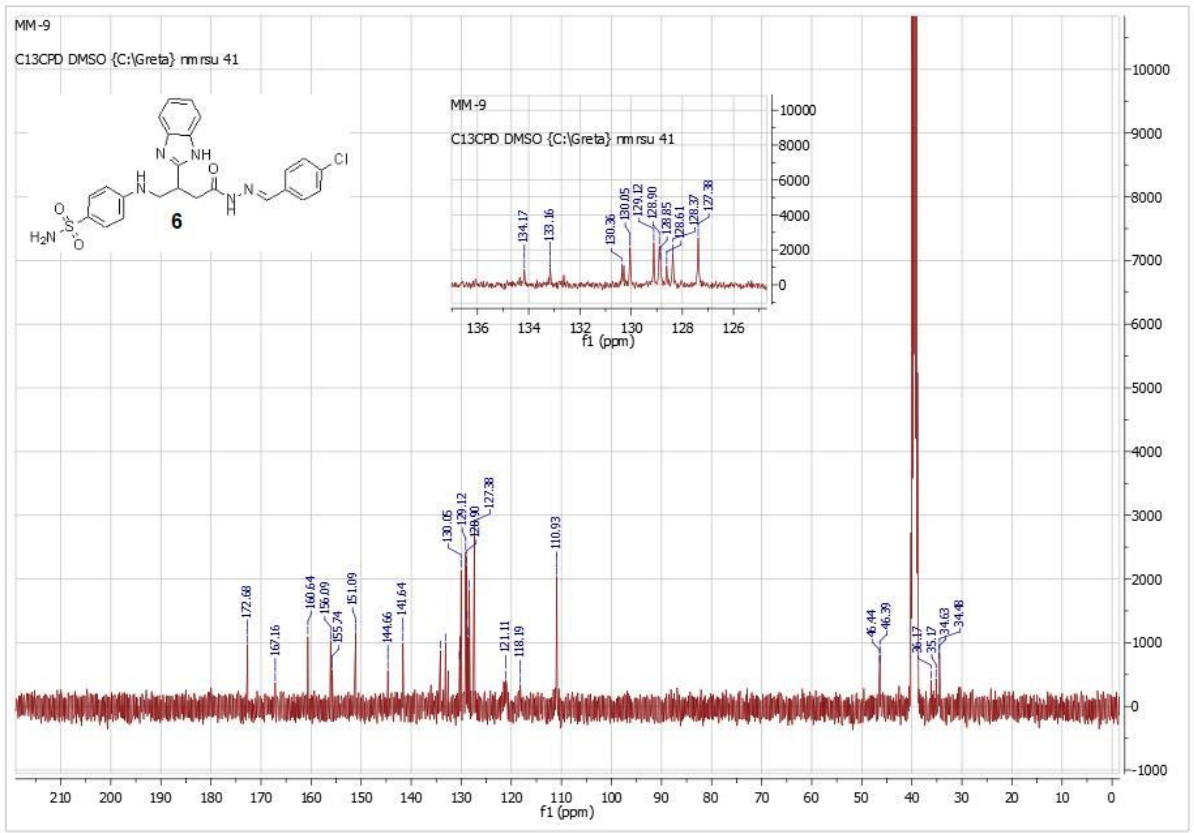

Figure S10. ^{13}C NMR of compound **6** at 101 MHz (DMSO-d_6).

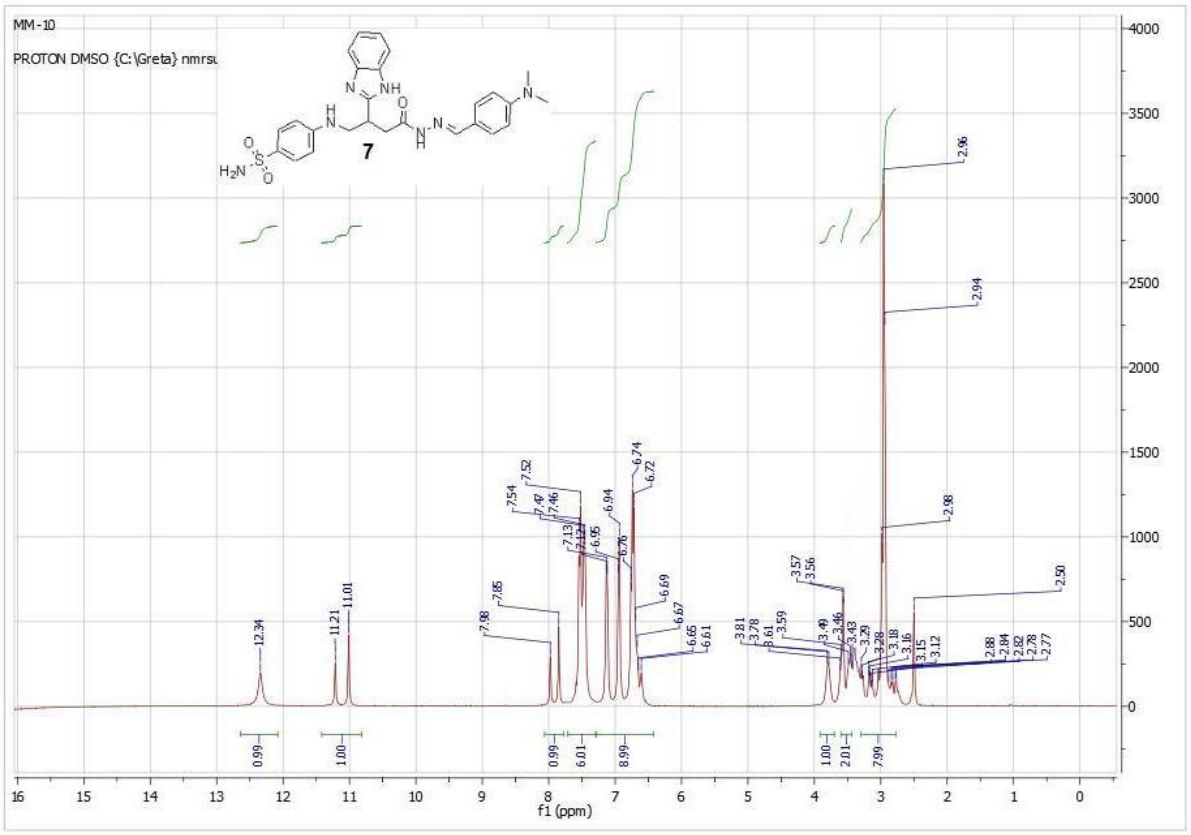

Figure S11. ^{1}H NMR of compound **7** at 400 MHz (DMSO-d_6).

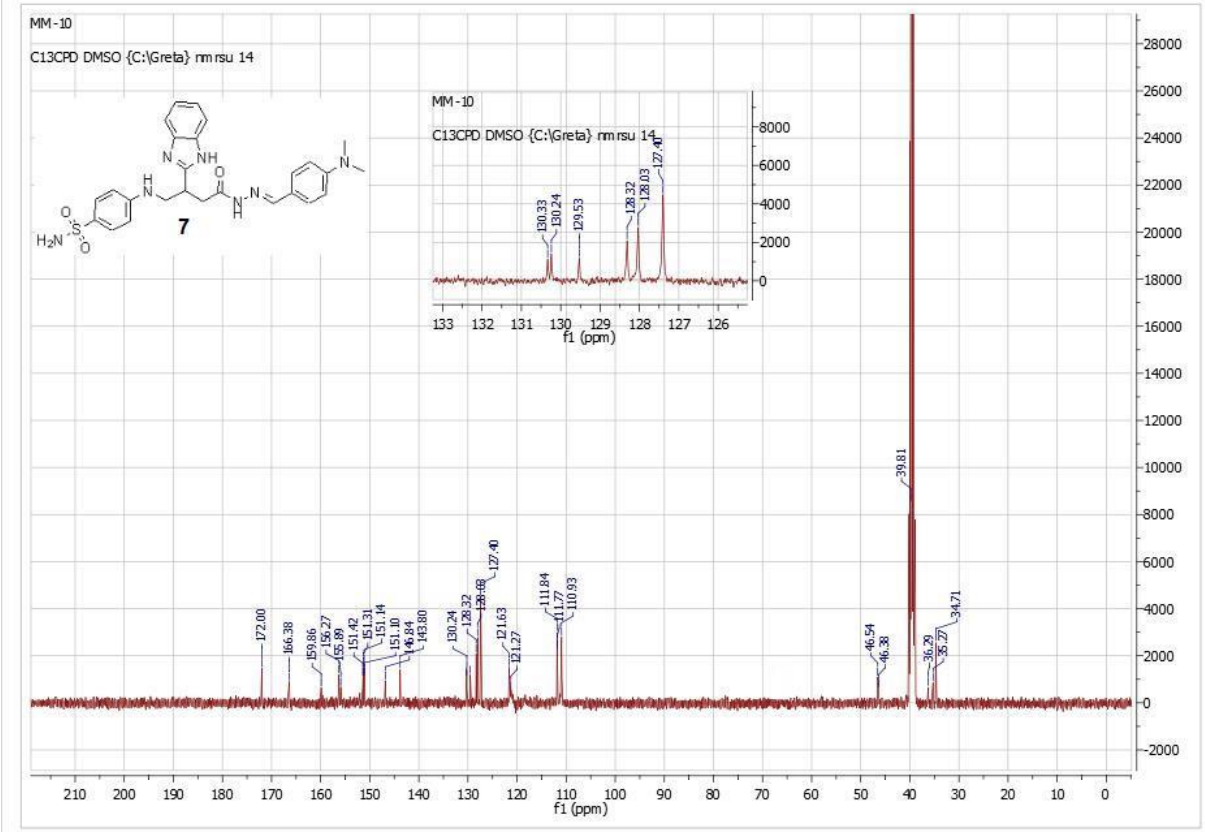

Figure S12. ^{13}C NMR of compound **7** at 101 MHz (DMSO-d_6).

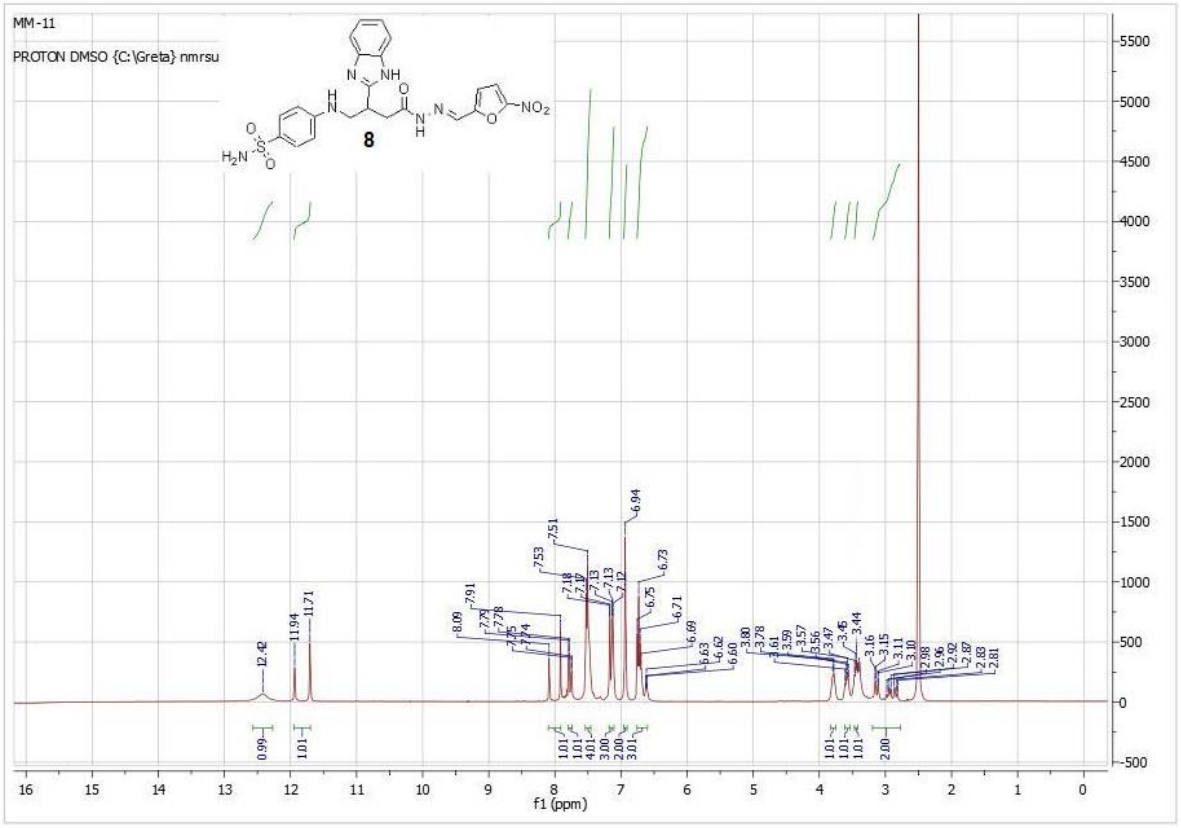

Figure S13. ^{1}H NMR of compound **8** at 400 MHz (DMSO-d_6).

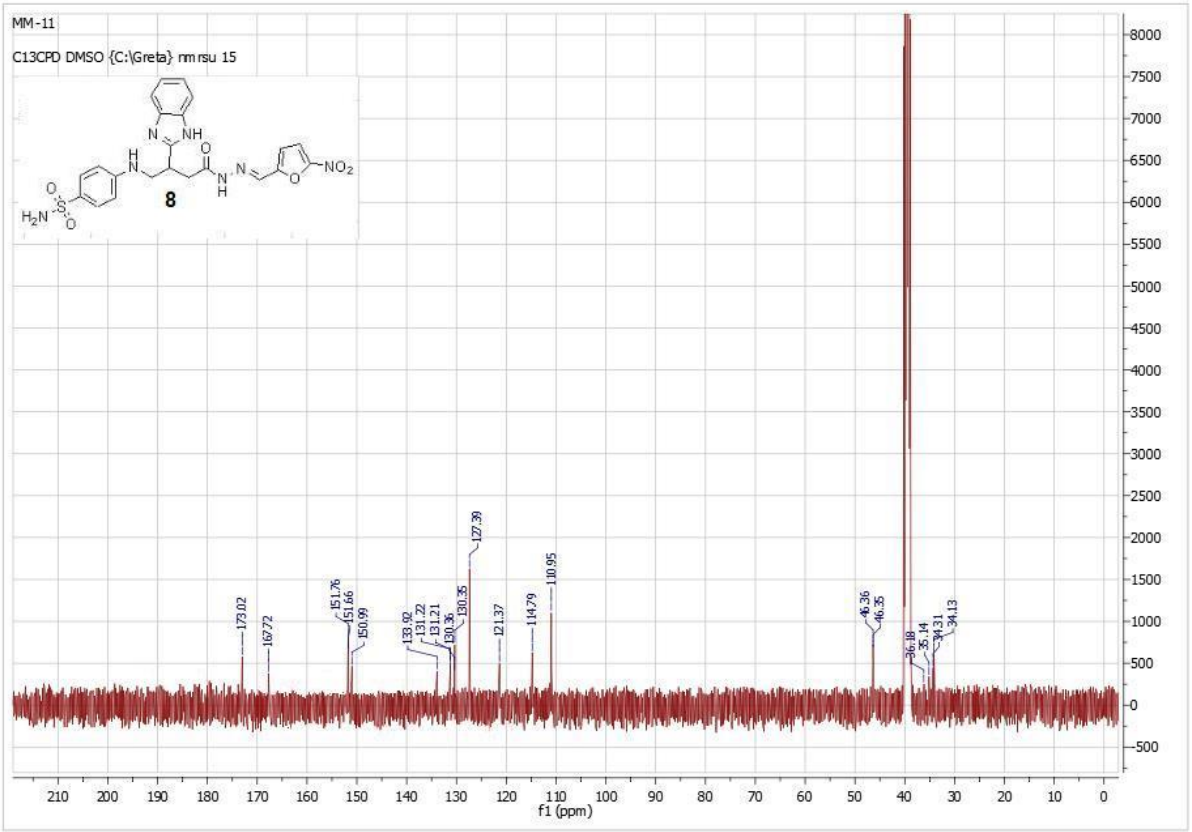

Figure S14. ^{13}C NMR of compound **8** at 101 MHz (DMSO-d_6).

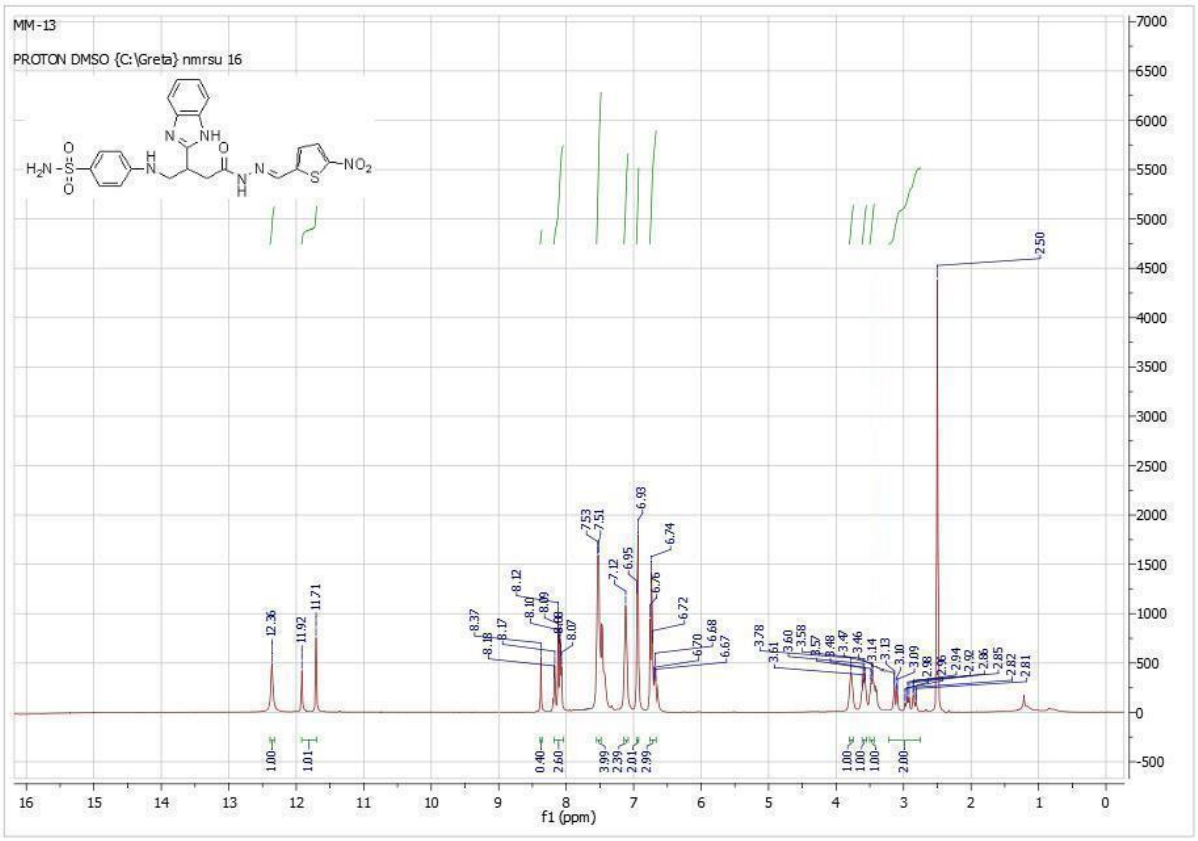

Figure S15. ^{1}H NMR of compound **9** at 400 MHz (DMSO-d_6).

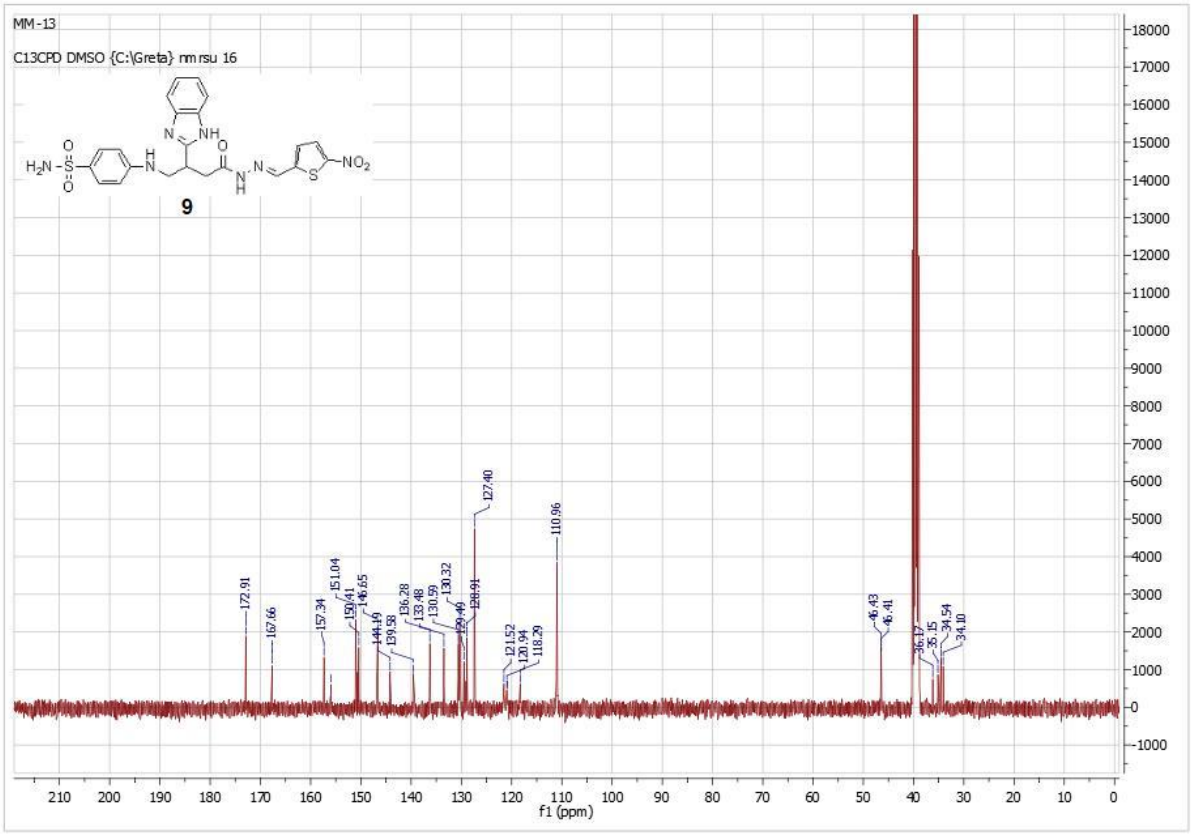

Figure S16. ^{13}C NMR of compound **9** at 101 MHz (DMSO-d_6).

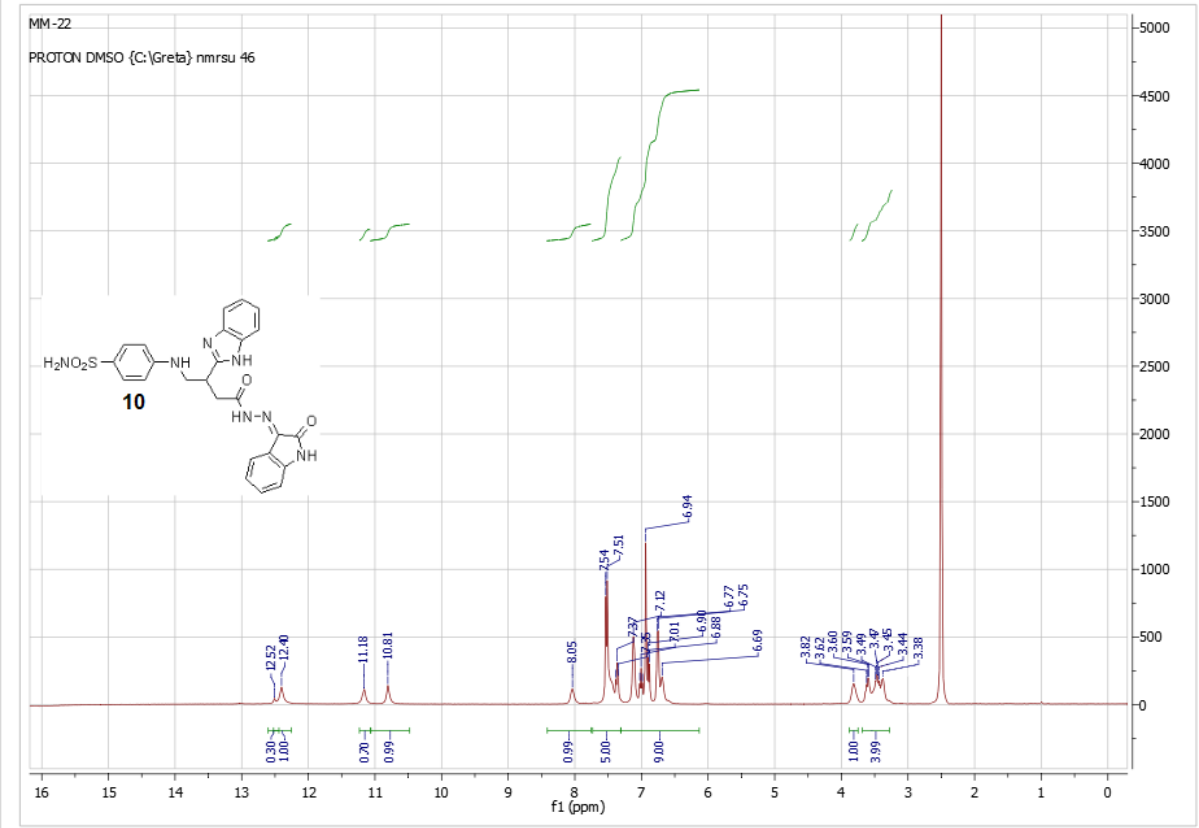

Figure S17. ^{1}H NMR of compound **10** at 400 MHz (DMSO-d_6).

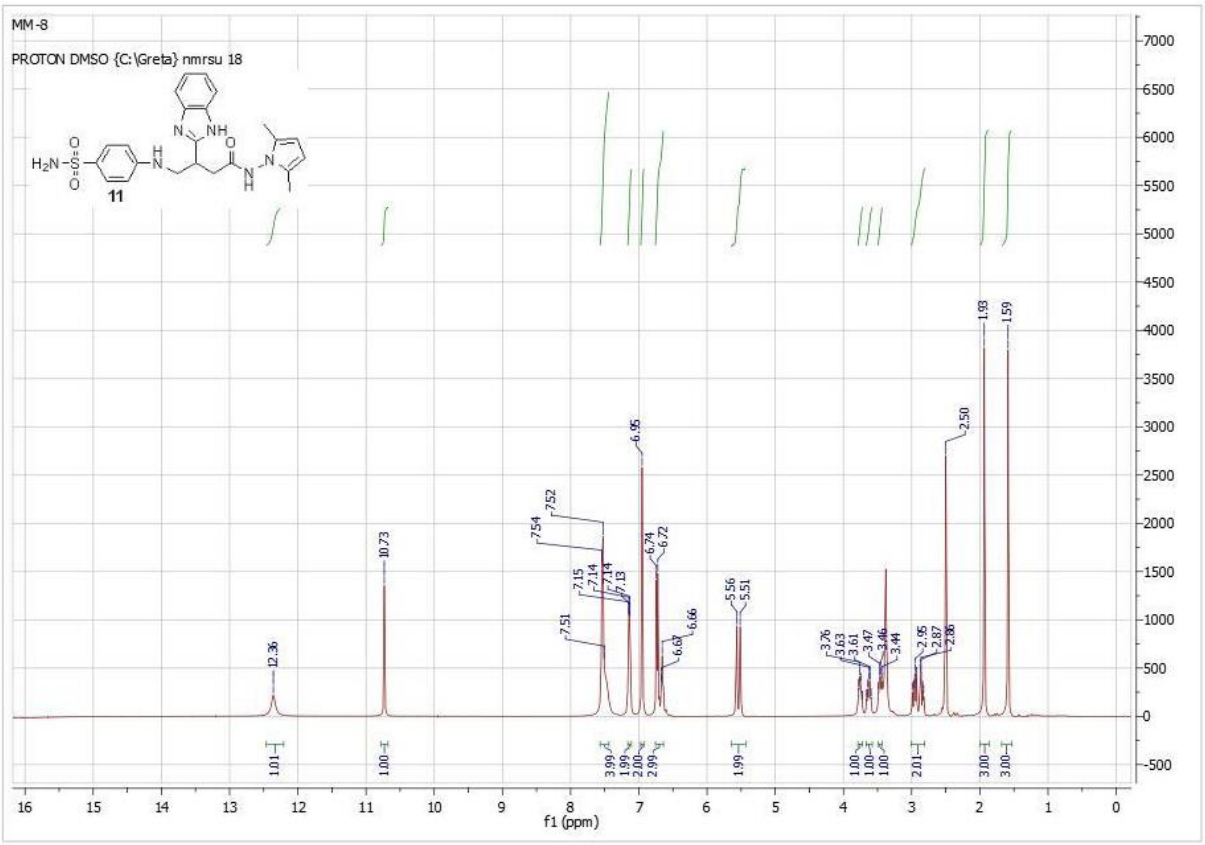

Figure S18. ^{1}H NMR of compound **11** at 101 MHz (DMSO-d_6).

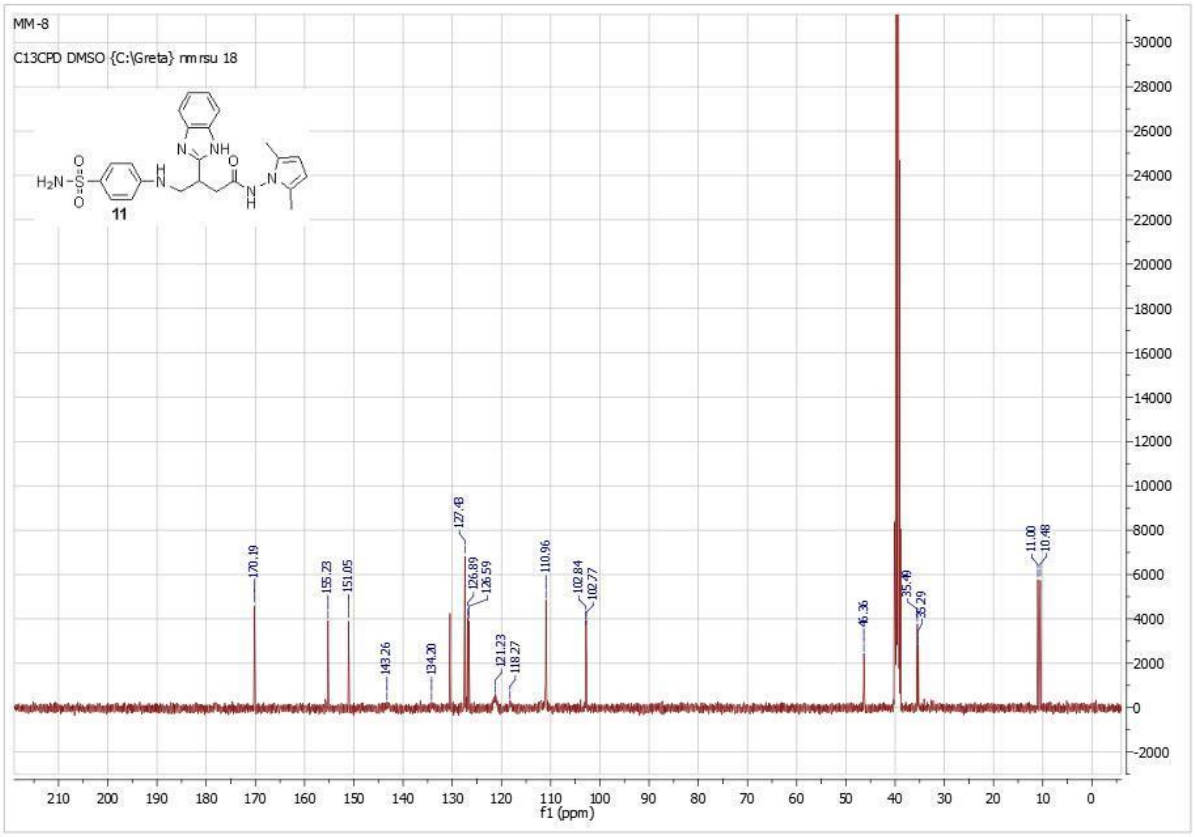

Figure S19. ^{13}C NMR of compound **11** at 400 MHz (DMSO-d_6).

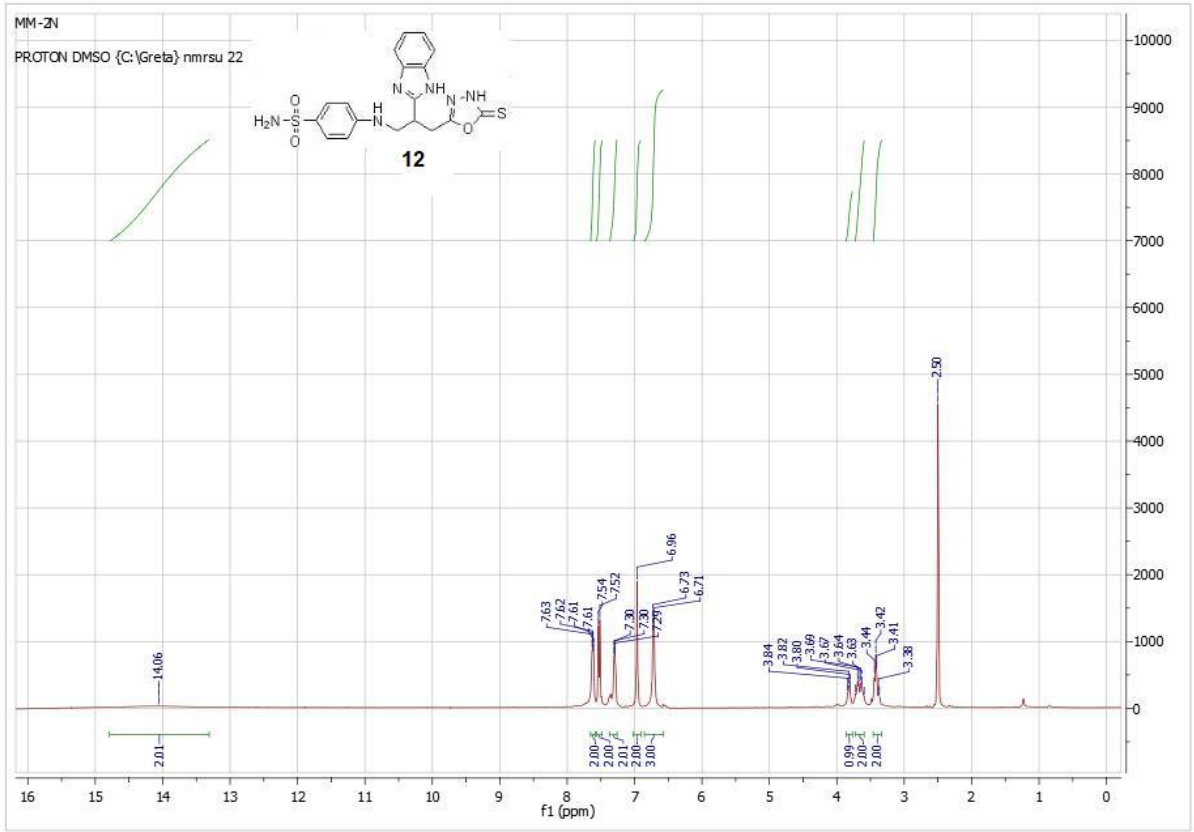

Figure S20. ^{1}H NMR of compound **12** at 101 MHz (DMSO-d_6).

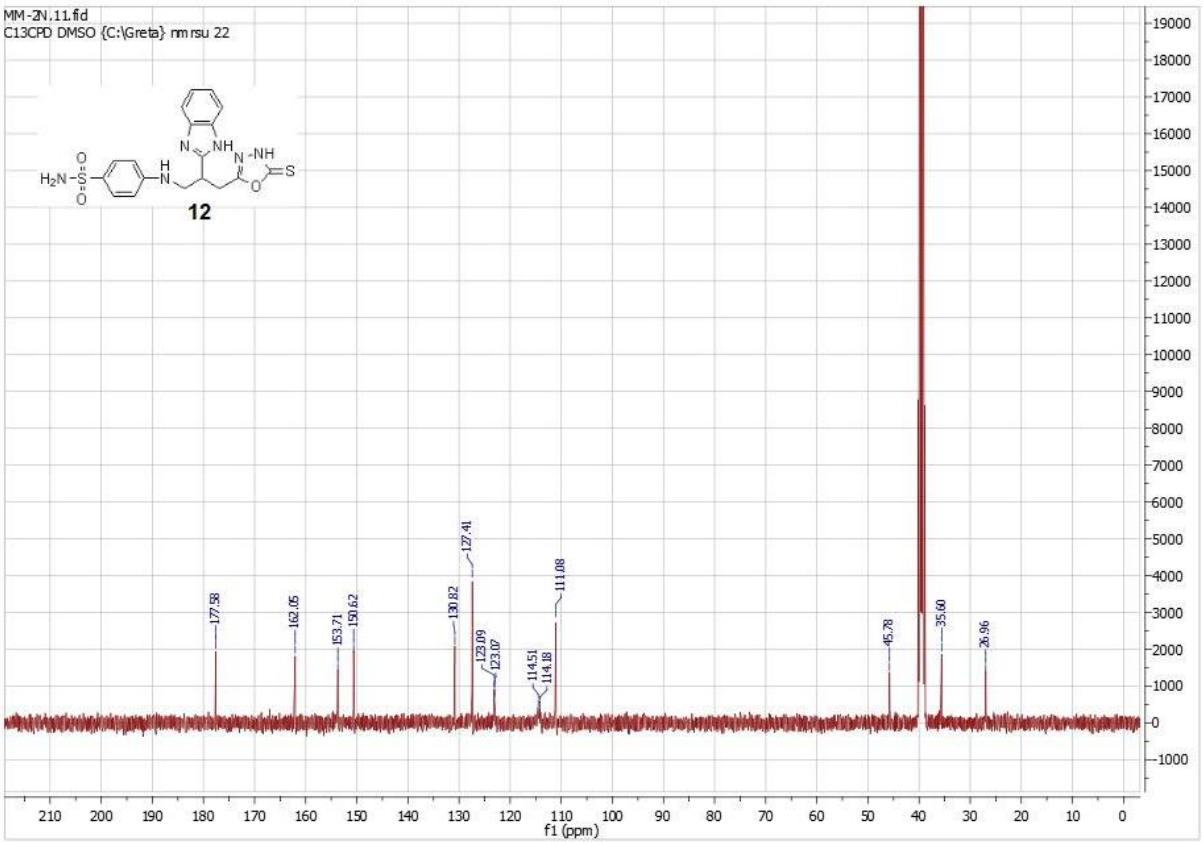

Figure S21. ^{13}C NMR of compound **12** at 400 MHz (DMSO-d_6).

Biology

The in vitro antibacterial evaluation

The antimicrobial properties of eleven synthesized compounds were investigated. Gram-positive and Gram-negative bacteria were used in the study. *Staphylococcus aureus* subs. *aureus* (ATCC 9144) and zoonotic agent *Listeria monocytogenes* (ATCC 35152) represented Gram-positive bacteria, while Gram-negative ones were represented by *Escherichia coli* (ATCC 13076), and zoonotic agent *Salmonella enterica* subs. *enterica* serovar Enteritidis (ATCC 8739). The minimal inhibitory concentration (MIC) was evaluated by the broth dilution method, while the minimal bactericidal concentration (MBC) was determined by plating. Antibiotic – Cefazolin was used as a control (C) for antibacterial activity screening.

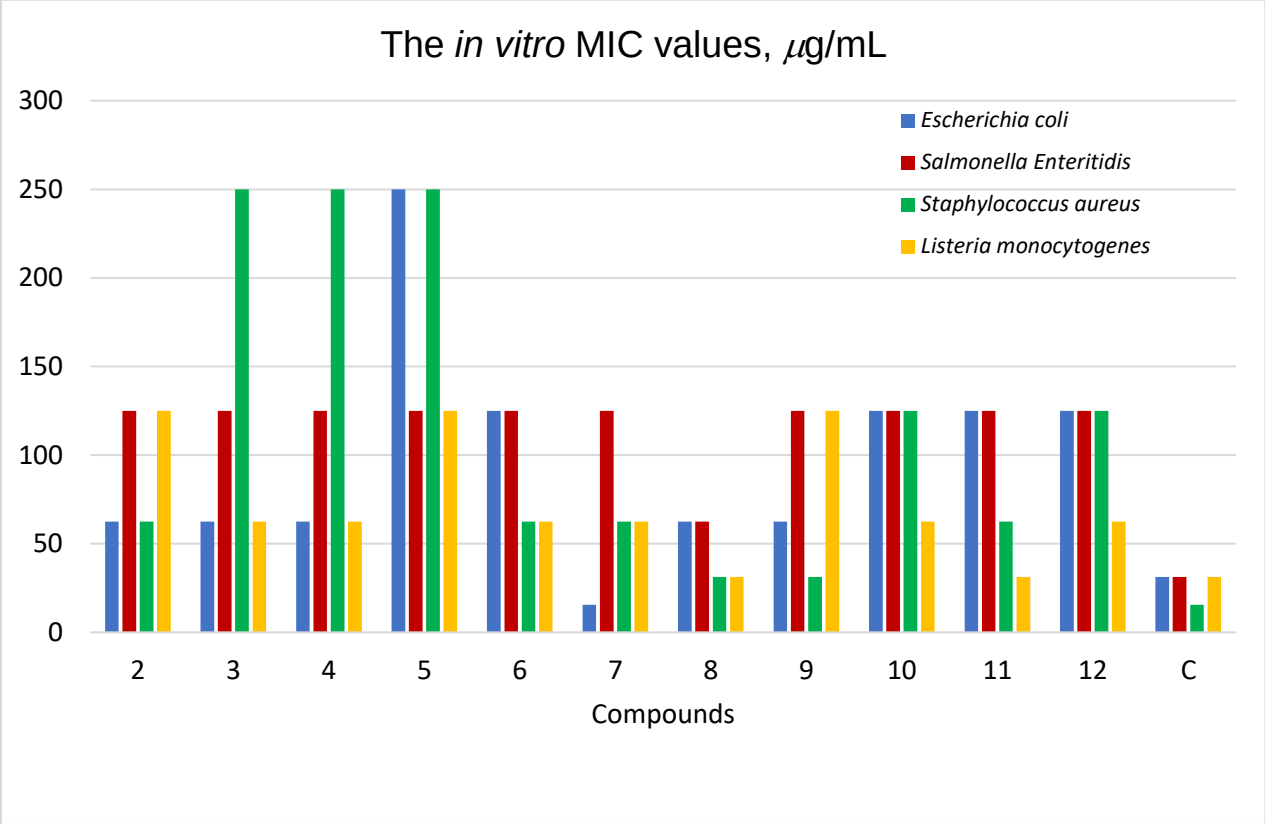

Figure 1. The *in vitro* minimum inhibition concentration (MIC) of the tested compounds, μg/mL.

The investigations *in vitro* demonstrated that all eleven compounds had the antibacterial activity of the broad spectrum for Gram-positive and Gram-negative bacteria. The studies showed that all synthesized compounds inhibited the growth of Gram-negative bacteria *E. coli* and *S.* Enteritidis. MIC values for *E. coli* from 15.6 to 250 μg/mL, and for *S.* Enteritidis from 62.5 μg/mL to 125 μg/mL were obtained (Figure 1). MBC values for *E. coli* and *S.* Enteritidis ranged from 62.5 to 250 μg/mL and from 125 to 500 μg/mL (Figure 2), respectively. Evaluation of MBC and MIC ratios showed that all compounds were bactericidal for all tested Gram-negative bacteria (Figure 3).

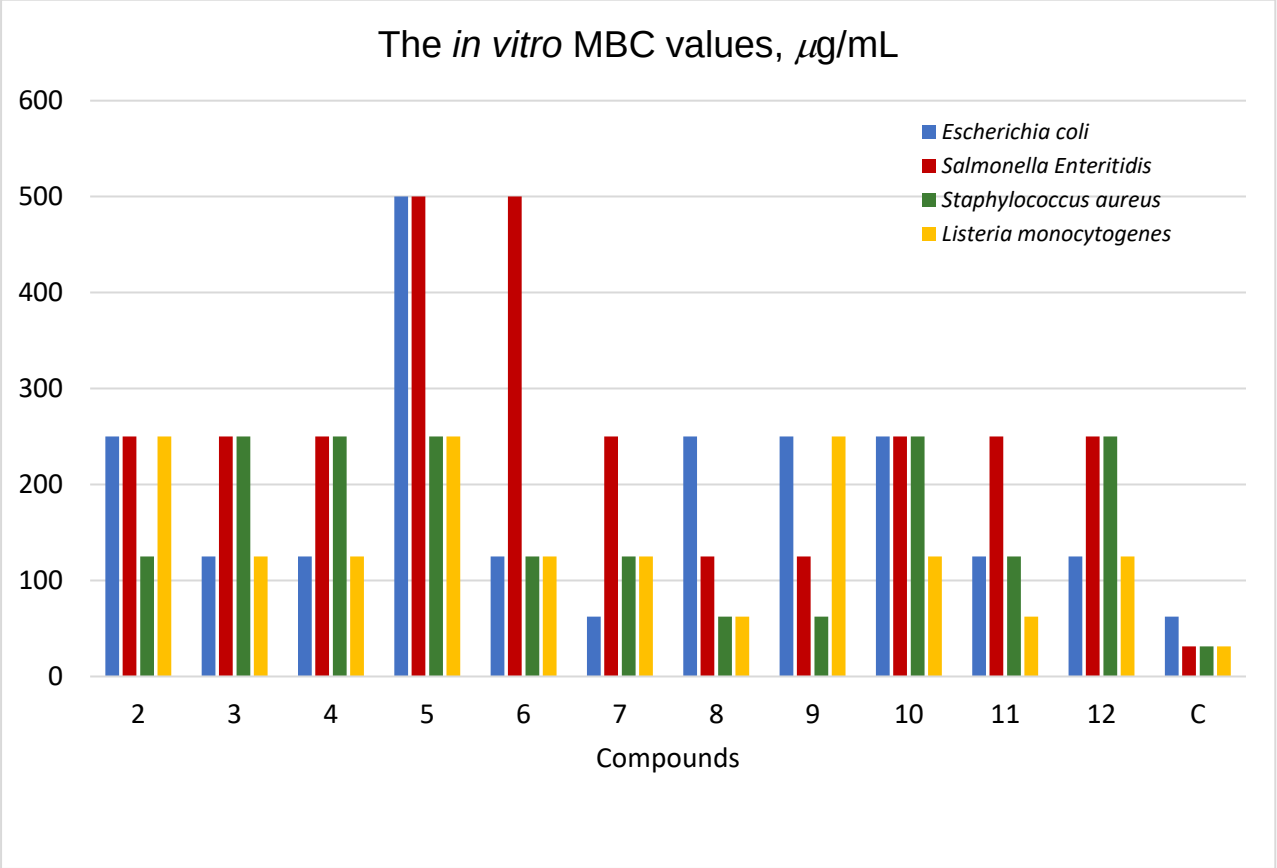

Figure 2. The *in vitro* minimum bactericidal concentration (MBC) of the tested compounds, μg/mL.

The effect was regarded as a bactericidal when a ratio of MBC/MIC was ≤4 and bacteriostatic – when the ratio of MBC/MIC>4. The MBC/MIC ratios of all tested compounds ranged from 1 to 4 for both *E. coli* and. *S.* Enteritidis.

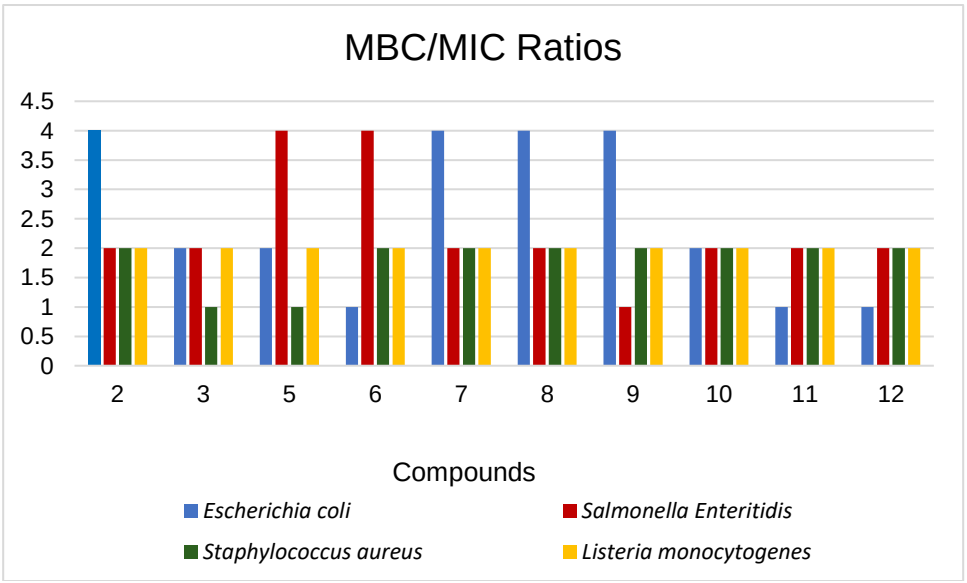

Figure 3. The Ratios of the MBC/MIC.

All compounds had an inhibitory effect on the growth of the Gram-positive bacterium *Listeria monocytogenes.* MIC values for the *L. monocytogenes* varied at the interval from 31.3 to 125 μg/mL, and MBC from 62.5 to 250 μg/mL. MIC and MBC values of *S. aureus* ranged from 31.3 to 250 μg/mL and 62.5 to 250 μg/mL, respectively. Evaluation of MBC and MIC ratios showed that all compounds were bactericidal for all tested Gram-positive bacteria. The MBC/MIC ratios of the tested compounds ranged from 1 to 2 for *S. aureus,* and 2 for *L. monocytogenes* strain.

The results showed, that cefazolin was effective at 15.6 μg/mL concentration of MIC and at 31.3 μg/mL of MBC for *S. aureus*. The antibiotic was effective at 31.3 μg/mL concentration of MIC against *L. monocytogenes, E. coli, S.* Enteritidis and MBC at the same concentration against the test strains *L. monocytogenes* and *S.* Enteritidis. The MBC of *E. coli* was detected at 62.5 μg/mL.

The comparison of the efficacy of 5-oxopyrrolidine-3-carboxylic acid **2** and its benzimidazole derivative **3** did not show the differences in the spectrum of antibacterial activity, however, the values of MIC and MBC were identical only to the tested *Salmonella* strain. Compounds **2** and **3** were equally potent to inhibit the tested Gram-negative bacteria, while the differences were found when observing the inhibition of *S. aureus* and *L. monocytogenes*. Compound **2** caused the death of ≥99.9% of *E. coli* and *L. monocytogenes* at twice lower concentrations than compound **3**, while on the contrary, the effective concentration for *S. aureus* was twice higher.

The further comparison of the efficacy of compounds **3** and **4** did not show any differences in the spectrum of antibacterial activity, MIC, and MBC for all the tested bacteria. Decyclization of the pyrrolidinone ring neither positively nor negatively affected the antibacterial potency of the compound. The transformation of γ-amino acid **4** into its hydrazide **5** did not increase the antibacterial efficacy also. Furthermore, higher concentrations were required to inhibit or kill *E. coli*, *S.* Enteritidis or *L. monocytogenes.*

The study of the antibacterial activity of derivatives of hydrazide **5** showed the various success of different steps of synthesis to all or different bacteria. Hydrazones **6** and **7** with the 4-ClPh and 4-Me_2NPh moieties, respectively, showed increased antibacterial activity against Gram-positive bacteria. The hydrazones **7**, **8**, and **9** showed more promising results compared to other ones. Hydrazone **7** bearing 4-Me_2NPh moiety was the most effective against *E. coli* – the values of MIC and MBC, respectively, 15.6 μg/mL and 62.5 μg/mL, were the strongest ones. The introduction of the 5-nitrofur-2-yl moiety in the molecule (compound **8**) influenced the highest efficacy in inhibiting the growth of Gram-positive bacteria at the MIC of 31.2 μg/mL, while hydrazone **9** possessing S-analogue i. e. 5-nitrothien-2-yl moiety at the same concentration was effective only against *S. aureus*.

The incorporation of 2-oxindole fragment (compound **10**) or 1,3,4-oxadiazole thione moiety (compound **12**) into the hydrazide structure did not meet expectations – the compounds were not potent to show increased antibacterial activity, while 2,5-dimethylpyrrole derivative **11**, as derivative **8**, showed the highest efficacy against *L. monocytogenes*. Noteworthy, that compound **11** demonstrated reasonably good properties against the other Gram-positive strain, namely *S. aureus.*

The anticancer activity of sulphanilamide and benzimidazole-based γ-amino acid derivatives **2–12**

To compare the anticancer activity of compounds **2–12** we employed human lung and colonic adenocarcinoma models (A549 and Caco-2) *in vitro* and compared the anticancer activity of compounds **2–12** with doxorubicin (DOX) and cisplatin (CP) an approved anticancer drug used for treatment lung and colon cancers.

Compounds **2–12** exhibited the variable and structure-depended cytotoxic activity on A549 and Caco-2 cells by decreasing the viability by 33.7–89.0% respectively (Figure 3 AB). 5-Oxopyrrolidine-3-carboxylic acid **2** showed similar cytotoxicity on both A549 and Caco-2 cell lines by reducing the viability to 65.0% and 67.6% respectively. Generation of benzimidazole derivative **3** decreased the anticancer activity against both cell lines (75.8% and 84.1% respectively). The decyclization of pyrrolidinone cycle (**4**) enhanced the anticancer activity against A549 (60.1%) although had little activity against Caco-2 cells (79.2%). Subsequent transformations resulting in hydrazide **5**, and hydrazones **6, 7** showed weak anticancer activity against A549 and Caco-2 (approximately 70–80%), while hydrazone **8** bearing 5-nitrofurane substitution resulted in strong anticancer activity against Caco-2 cells (33.7%) in comparison to untreated control (p<0.05). On the other hand, moderate anticancer activity was observed against A549 (69.1%). Similar results were observed when 5-nitrofurane substitution was replaced by 5-nitro-2-thienyl group (**9**). Compound **9** significantly reduced Caco-2 viability to 45.5% in comparison to untreated control (p<0.05), suggesting that the 5-nitrofuryl and 5-nitrothienyl substituents in hydrazones are crucial for selective anticancer activity targeting Caco-2 colorectal adenocarcinoma cells (Figure 4).

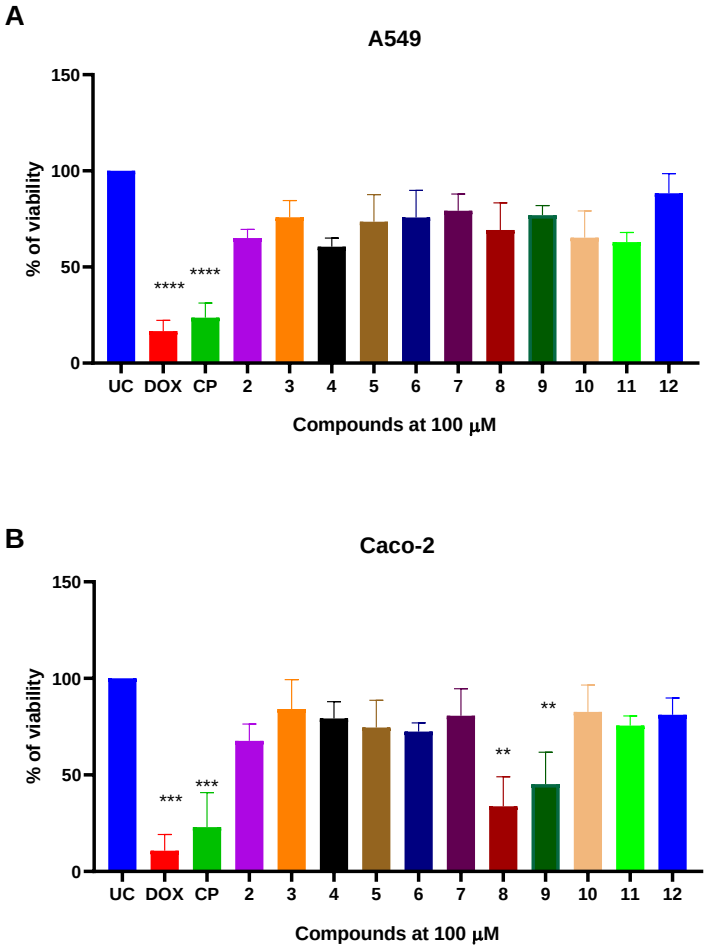

Figure 4. The *in vitro* anticancer activity of compounds **2–12** against A549 (Panel A) human lung adenocarcinoma and Caco-2 (Panel B) human colon adenocarcinoma cells. The cells were exposed with 100µM of each compound or doxorubicin (DOX) and cisplatin (CP) that were used as cytotoxicity controls. The compound mediated cytotoxicity was compared to untreated control (UC). Each bar represents mean±SD from tree experimental replicas.

The incorporation of 2-oxindole substitution in hydrazide nucleus resulted derivative **10**. Interestingly, 2-oxindole (isatin) containing derivative **10** demonstrated weak anticancer activity against Caco-2 (82.6%) while moderate anticancer activity was observed in A549 cells (62.7%) demonstrating that the 2-oxindole moiety is important for the A549 human lung adenocarcinoma-targeting activity (Figure 4 AB).

Compound **11** containing pyrrole ring exhibited moderate anticancer activity against A549 cells (62.9%), while weak activity was observed in Caco-2 cell culture model (75.5%). On the other hand, replacement of pyrrole with 1,3,4-oxadiazolethione substitution (compound **12**) diminished anticancer activity against both cell lines.

Biology

Strains and Culturing Conditions

Gram-positive bacteria *Staphylococcus aureus* subs. *aureus* (ATCC 9144) and *Listeria monocytogenes* (ATCC 35152) as well as Gram-negative ones, *Escherichia coli* Crooks (ATCC 8739) and *Salmonella enterica* subs. *enterica* serovar Enteritidis (ATCC 13076) were used.

Bacteria were cultured on Tryptone soya agar (OXOID, England) for 24-hours before the test. A turbidity concentration of 0.5 Mac Farland (10^8 CFU/ml) was prepared from the grown bacterial cultures.

Determination of minimum inhibitory concentration (MIC)

The broth microdilution method was used to study the antibacterial activity of symmetrical *N*-substituted 2,2‘-dithioaniline derivatives.[60,61] Bacterial growth was assessed in 96-well microplates (OXOID, England) in Muller Hinton (MH) broth. Serial two-fold dilutions of the compounds were used to determine the MIC, ranging from 500 μg/mL to 0.244 μg/mL concentrations. The concentration of bacteria used for the study was 5 x 10^5 CFU/mL. Each well contained 100 µL of the mixture (different concentrations of compounds and bacteria 5 x 10^4 CFU). The sterility of the medium, the growth of the tested bacteria and the sensitivity to the antibiotic cefazolin were also controlled. Microtitration plates with the test mixture were incubated at +37 °C for 24 hours. MIC was determined as the lowest concentration of the compound at which no bacterial growth (turbidity) can be seen in the plate wells.

Cefazolin used as a control (C) for antibacterial activity screening.

Determination of minimum bactericidal concentration (MBC)

MBC was considered as the lowest concentration of the compound causing ≥3 $\log_{10}$ reduction (≥ 99.9% kill) in number (5 x 10^4 CFU/100 µL) of bacteria.[62] Ten microliters of mixture were taken from the well in which the MIC value was determined and from up to three wells in which the concentration of the compound was 2, 4 and 8 times higher. The tested mixtures were spread on Mueller Hinton agar. The growth of bacteria and number of colonies were evaluated after 24 hours of incubation at +37 °C. MBC was considered as the lowest concentration of a compound when bacteria did not grow or formed up to 5 colonies.

Interpretation of MBC/MIC ratios

If the ratio MBC/MIC ≤4, the effect was considered as bactericidal, but if the ratio MBC/MIC > 4, the effect was defined as bacteriostatic.

The antibiotic cefazolin was effective for *S. aureus* at concentrations – 15.6 μg/mL (MIC) and 31.3 μg/mL (MBC). The MIC and MBC of cefazolin for *L. monocytogenes* and *S.* Enteritidis were 31.3 μg/mL. The MIC and MBC of cefazolin for *E. coli* were respectively 31.3 μg/mL and 62.5 μg/mL.

Determination of anticancer activity

Human A549 lung adenocarcinoma and human Caco-2 colon adenocarcinoma cell lines were obtained from American Type Culture Collection (ATCC) and were maintained at DMEM: F12 media supplemented with 10% of fetal bovine serum (FBS), 1X Penicilin-Streptomycin. Cells were trypsinized and counted using haematocytometer. The cell density was adjusted to reach 2.5 x 10^5 cells/mL and cells were plated (100 µl) to flat bottom, 96-well microplates (2.5 x 10^4 cells/well). Plates were incubated overnight at 37 °C, 5% CO_2 overnight to facilitate the cell attachment.

The symmetrical *N*-substituted 2,2'-dithioaniline derivatives (**2**–**12**) were dissolved in sterile DMSO and further diluted using cell culture media to achieve a 200 μM (2X concentration) concentration of each compound. Doxorubicin (DOX) and cisplatin (CP) were used as a control drug and were diluted in the same manner. The diluted compounds or control drugs were added to the wells resulting in reaction mixture of 200 μL containing 100 μM of each compound or control drug.

The plates were then incubated at 37 °C, 5% CO_2 for 24 hours and the viability was measured by using Piece MTT assay as described by the manufacturer.

