Electronic supplementary materials *Mendeleev Commun.*, 2026, **36**, 12–14

Substituted 3-aryl-4-nitroisoxazoles as potential blockers of the transport protein GLUT5: molecular design, synthesis and primary biotesting

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Contents

1. Molecular modeling

Molecular docking of compound **2a** and MSNBA (**1**) was performed using a spatial model of the crystal structure of the bovine fructose transporter GLUT5 in an open inward-facing conformation (PDB ID: 4YB9) into a region that includes amino acid residues Ser143, Gln288, Gln 289, Asn 294, Tyr 297, His 387. Atomic charges of protein amino acids were assigned by standard Kollman method using AutoDock Tools 1.5.6. Two-dimensional structures of ligands were converted to the 3D structures and were submitted to a conformational MMFF Amber ff14SB optimization using Gasteiger charges in USCF Chimera 1.15 software.[S1] The docking procedure was performed with the AutoDock Vina 1.1.2 software[S2] (grid box 18.0 Å × 20.25 Å × 24.00 Å, grid center size x = 25.58 Å, y = –12.454 Å, z = – 32.013 Å; energy range = 4, exhaustiveness = 20). The resulting complexes *ligand – protein* with the best scoring functions were selected and visualized using UCSF Chimera 1.15 software.[S1]

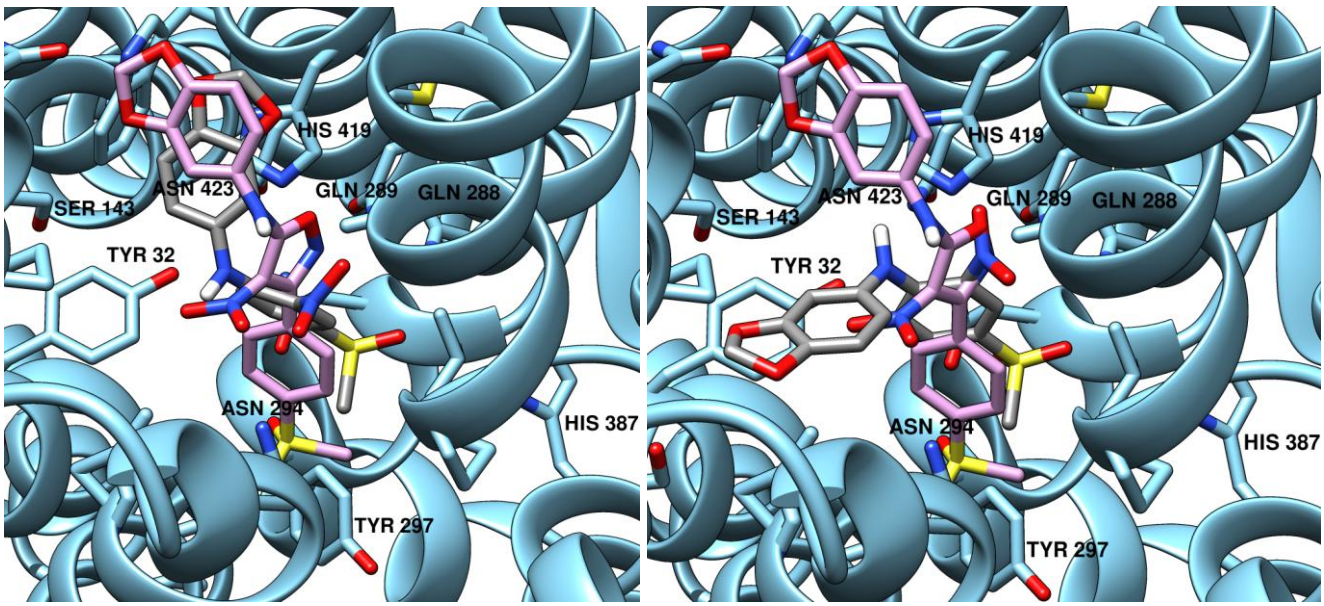


Figure S1. Binding mode of compound **2a** (represented by pink-colored stick model) in the presumed binding site of MSNBA (**1**)[S3] in bovine the bovine fructose transporter GLUT5 in an open inward-facing conformation (PDB ID: 4YB9) predicted by molecular docking. Two predicted positions of MSNBA molecule (**a** and **b**) are shown by a gray-colored stick model. Most of the hydrogen atoms are omitted for clarity.

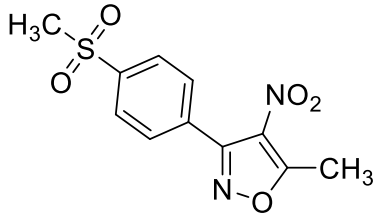
Two predicted positions of MSNBA (**1**) and one predicted position of the compound **2a** in GLUT5 are shown at Figures S1, *a*,*b*. It is seen that the methylsulfonyl groups of both molecules are located near the amino acid residue His 387 and can form a hydrogen bond with the imidazole of the side chain of this amino acid residue. Other key hydrogen bonds with residues Gln288, Gln 289[S3] are provided by the oxygen atoms of the nitro group of ring A in MSNBA, and by the nitrogen and oxygen atoms of the isoxazole ring in molecule **2a**. Nitro group at C^4 of isoxazole in **2a** is located near the residue Asn 294 and can be hydrogen-bonded with it. The modeling predicts two positions of the benzodioxole moiety of MSNBA in the protein, one of which is close to that in molecule **2a** (Figure S1, *a*) and the other is different from it (Figure S1, *b*). Oxygen atoms of the dioxolane ring of compound **2a** can form hydrogen bonds with the side chain of the Ser143 residue, which is also located in the GLUT5 ligand binding region. If MSNBA is located as shown in Figure S1 *b*, then an increase in the conformational flexibility of the chain between the amino group and the benzodioxole moiety of molecule **2a** could theoretically lead to a rotation of the latter in the binding site and bring it closer to a position similar to that in the parent molecule **1**.

2. Chemistry

NMR spectra were recorded on spectrometers Bruker Avance 400 and Agilent 400-MR (400.0 MHz for ^{1}H; 100.6 MHz for ^{13}C) at ambient temperature; the chemical shifts (δ scale) are referenced to the $CDCl_3$ (^{1}H: δ = 7.26 ppm, ^{13}C: δ = 77.16 ppm) and DMSO-d_6 (^{1}H: δ = 2.50 ppm, ^{13}C: δ = 39.52 ppm). Chemical shifts (δ) are given in ppm; *J* values are given in Hz. Accurate mass measurements (HRMS) were performed on a Bruker micrOTOF II instrument using electrospray ionization (ESI). The measurements were done in a positive ion mode (interface capillary voltage 4500 V). Melting points (mp) are uncorrected. Thin layer chromatographic method (TLC) was conducted on DC-Fertigfolien ALUGRAM pre-coated silica gel 60-F254 plates; the detection was done by UV lamp (254 and 365 nm) and chemical staining (5% aqueous solution of $KMnO_4$). Column chromatography was performed on silica gel (230–400 mesh, Merck). Isoxazole **3** was obtained according to the methods developed in our group[S4].

5-Methyl-3-(4-methylsulfonylphenyl)-4-nitroisoxazole (4)

75% *m*-Chloroperbenzoic acid (1.97 g, 8 mmol, 2 equiv) was added to a solution of isoxazole **3** (1.00 g, 4 mmol, 1 equiv) in CH_2Cl_2 (16 mL) in three portions. The resulting mixture was stirred for 3 h at room temperature. After the reaction was completed, the obtained precipitate was filtered off and washed with CH_2Cl_2 (2×20 mL). The filtrate was washed with aqueous $NaHCO_3$ (30 mL) and NaCl (30 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated under vacuum. The product was purified by recrystallization from chloroform.

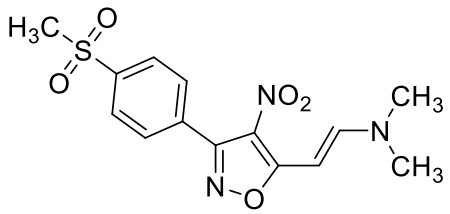


The yield was 0.96 g (85%), colorless solid, mp: 184-186°C. ^{1}H NMR ($CDCl_3$), δ, (*J*, Hz): 2.93 (s, 3H, CH_3), 3.12 (s, 3H, CH_3), 7.81–7.87 (m, 2H, 2CH), 8.06–8.12 (m, 2H, 2CH). ^{13}C NMR ($CDCl_3$), δ: 14.3 (CH_3), 44.6 (CH_3), 127.7 (2CH), 130.7 (2CH), 131.5 (C), 142.6 (C), 156.6 (C), 173.5 (C). HRMS (m/z); $(M+NH_4)^+$, $C_{11}H_{14}N_3O_5S^+$: found 300.0651, calculated: 300.0649.

(*E*)-*N*,*N*-Dimethyl-2-(3-(4-methylsulfonylphenyl)-4-nitroisoxazol-5-yl)ethen-1-amine (5)

N,*N*-Dimethylformamide dimethyl acetal (0.63 mL, 4.5 mmol, 1.5 equiv) was added to a solution of 4-nitroisoxazole **4** (0.85 g, 3 mmol, 1 equiv) in toluene (15 mL, 0.3 M). The reaction mixture was stirred at 80°C for 8 h. Then the dark reaction mixture was diluted with the excess of petroleum ether

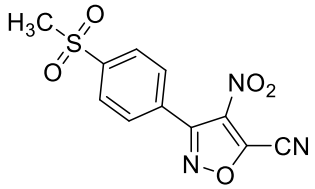
(60 mL) and stirred until the precipitate has completely formed. The solid was filtered and washed with petroleum ether to yield the desired product **5**, which was used in the next reaction without further purification.



The yield was 0.90 g (89%), brown solid, mp: 230-232°C. ^{1}H NMR (DMSO-d_6), δ, (*J*, Hz): 3.03 (s, 3H, CH_3), 3.29 (s, 3H, CH_3), 3.31 (s, 3H, CH_3), 5.86 (d, ^{3}J = 12.9 Hz, 1H, CH), 7.85–7.91 (m, 2H, 2CH), 8.03–8.05 (m, 2H, 2CH), 8.15 (d, ^{3}J = 12.9 Hz, 1H, CH). ^{13}C NMR (DMSO-d_6), δ: 37.3 (CH_3), 43.3 (CH_3), 45.2 (CH_3), 80.1 (CH=), 119.3 (C-NO_2), 126.7 (2CH), 130.4 (2CH), 132.6 (C), 142.0 (C), 154.0 (=CH), 156.6 (C), 170.4 (C). HRMS (m/z); $(M+H)^+$, $C_{14}H_{16}N_3O_5S^+$: found 338.0797, calculated: 338.0805.

3-(4-Methylsulfonylphenyl)-4-nitroisoxazole-5-carbonitrile (6)

tert-BuONO (0.62 mL, 4 mmol, 2 equiv) was added to a solution of enamine **5** (0.67 g, 2 mmol, 1 equiv) in dry CH_3CN (4 mL, 0.5 M) and the resulting mixture was stirred at room temperature for 2 h. Then $BF_3{\cdot}Et_2O$ (0.24 mL, 2 mmol, 1 equiv) was added dropwise, and the resulting mixture was stirred at room temperature for 12 h. The reaction was quenched with water (40 mL), and the mixture was extracted with CH_2Cl_2 (4×20 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated under vacuum. The residue was purified by column chromatography using petroleum ether/ethyl acetate (2:1) as the eluent to give product **6**.

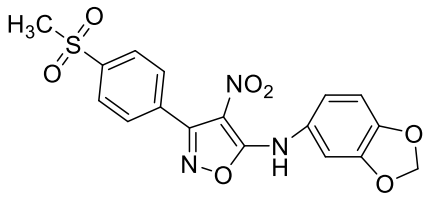


The yield was 370 mg (63%), colorless solid, mp: 156-158°C, R*f* 0.34 (petroleum either : EtOAc = 2:1). ^{1}H NMR ($CDCl_3$), δ, (*J*, Hz): 3.14 (s, 3H, CH_3), 7.87–7.93 (m, 2H, 2CH), 8.12–8.18 (m, 2H, 2CH). ^{13}C NMR (DMSO-d_6) , δ: 43.2 (CH_3), 106.6 (CN), 127.4 (2CH), 128.5 (C), 130.8 (2CH), 137.9 (C-NO_2), 143.1 (C), 143.5 (C), 156.7 (C). Found (%): C 45.17.; H 2.51; N 14.28. $C_{11}H_7N_3O_5S$. Calculated (%): C 45.05; H 2.41; N 14.33.

***N*-(Benzo[*d*][1,3]dioxol-5-yl)-3-(4-methylsulfonylphenyl)-4-nitroisoxazol-5-amine (2a)**

DIPEA (0.087 mL, 0.5 mmol, 1 equiv), 4-nitroisoxazole-5-carbonitrile **6** (147 mg, 0.5 mmol, 1 equiv) and benzo[*d*][1,3]dioxol-5-amine (69 mg, 0.5 mmol, 1 equiv) were mixed under an Ar atmosphere in

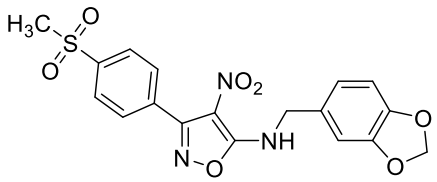
dry THF (2 mL, 0.25 M). The resulting mixture was stirred at 50°C for 6 h. After the reaction was finished, the mixture was poured into water and was extracted by CH_2Cl_2 (3×15 mL), the organic layer was dried over anhydrous $MgSO_4$ and the solvent was removed under reduced pressure. The residue was purified by column chromatography using petroleum ether/ethyl acetate (1:1) as the eluent to give product **2a**.



The yield was 119 mg (59%), yellow solid, mp: 243-245°C, R*f* 0.46 (petroleum either : EtOAc = 1:1). ^{1}H NMR (DMSO-d_6), δ, (*J*, Hz): 3.31 (s, 3H, CH_3), 6.09 (s, 2H, CH_2), 6.96–7.05 (m, 2H, 2CH), 7.12–7.17 (m, 1H, CH), 7.89–7.95 (m, 2H, 2CH), 8.05–8.11 (m, 2H, 2CH), 10.90 (br. s, 1H, NH). ^{13}C NMR (DMSO-d_6) , δ: 43.3 (CH_3), 101.7 (CH_2), 105.5 (CH), 108.2 (CH), 110.4 (C-NO_2), 117.3 (CH), 126.9 (2CH), 129.9 (C), 130.4 (2CH), 131.8 (C), 142.4 (C), 145.7 (C), 147.5 (C), 156.9 (C), 163.3 (C). HRMS (m/z); $(M+Na)^+$, $C_{17}H_{13}N_3O_7SNa^+$: found 426.0362, calculated: 426.0366.

***N*-(Benzo[*d*][1,3]dioxol-5-ylmethyl)-3-(4-(methylsulfonyl)phenyl)-4-nitroisoxazol-5-amine (2b)**

DIPEA (0.087 mL, 0.5 mmol, 1 equiv) was added to the solution of 4-nitroisoxazole-5-carbonitrile **6** (147 mg, 0.5 mmol, 1 equiv) and benzo[*d*][1,3]dioxol-5-ylmethnamine (76 mg, 0.5 mmol, 1 equiv) in CH_3CN (2 mL, 0.25 M). The resulting mixture was stirred at r.t. for 24 h. After the reaction was finished, the mixture was poured into water and extracted with CH_2Cl_2 (3×15 mL), the organic layer was dried over anhydrous $MgSO_4$ and the solvent was removed under reduced pressure. The residue was purified by column chromatography using petroleum ether/ethyl acetate (1:1) as the eluent to give product **2b**.



The yield was 127 mg (61%), yellow solid, mp: 213-214°C, R*f* 0.53 (petroleum either : EtOAc = 1:1). ^{1}H NMR (DMSO-d_6), δ, (*J*, Hz): 3.12 (s, 3H, CH_3), 4.57 (d, ^3J = 6.4 Hz, 2H, CH_2), 6.01 (s, 2H, CH_2), 6.82–6.89 (m, 3H, 3CH), 7.86–7.90 (m, 2H, 2CH), 7.93 (br. s, ^3J = 6.4 Hz, 1H, NH), 8.05–8.09 (m, 2H, 2CH). ^{13}C NMR (DMSO-d_6) , δ: 43.3 (CH_3), 45.5 (CH_2), 101.1 (CH_2), 108.3 (CH), 109.6 (C-NO_2), 121.2 (CH), 126.8 (2CH), 127.9 (C), 130.4 (2CH), 131.0 (CH), 131.7 (C), 142.4 (C), 146.8 (C), 147.4 (C), 157.0 (C), 165.1 (C). HRMS (m/z); $(M+Na)^+$, $C_{18}H_{15}N_3O_7SNa^+$: found 440.0521, calculated: 440.0523.

3. Biological Assays

Cell culture. The studies were performed on cultured human chronic myeloid K562 leukemia cell line (obtained from Russian Cell Culture Collection, Institute of Cytology, Russian Academy of Sciences). Cells that were cultured under standard conditions – RPMI-1640 medium containing glucose (Biolot, Russia), 10% fetal bovine serum (Biolot, Russia) and 40 μg/ml antibiotic gentamicin in humidified incubator at 37ºC, 5% CO_2 were transferred to a culture medium containing 6 mM fructose (PanEco, Russia) instead of glucose. Then the cells were cultured in a fructose-containing medium for 2 weeks to adapt the cell culture to new conditions. Cells cultured under standard conditions were used in parallel for control experiments. The number of live/dead cells were measured using a "ThermoFisher Countess 2" automatic cell counter (ThermoFisher Scientific, USA).

Cell proliferation and viability assay. Standard colorimetric MTT test[S5] was performed according to the manufacturer's protocol (Servicebio, China) after 24, 48 and 72 h from the addition of the compounds to the cells. As the "zero" (starting) point 30000 cells per 0.1 ml of medium in each well of 96-well plates (NEST, China) were used). The stock solutions of all compounds were dissolved in dimethyl sulfoxide (DMSO). For each timepoint, 6 experimental replicates (wells) were used with each of the compounds. 20 μl MTT were added to the required number of wells of the plate with cells and incubated for 4 h to carry out the MTT reaction. To test the non-specific effect of the DMSO on proliferation and viability, the amount of solvent that corresponded to its presence at the maximum concentration of the studied reagent was added to the cells. After incubation with MTT the medium with the cells was collected from each well to a separate tube (0.5 ml), centrifuged for 5 min at 3500g and then the supernatant was carefully collected and discarded. The pellet in each tube was resuspended in 100 μl DMSO, mixed thoroughly and transferred to the wells of a new 96-well plate. To evaluate the intensity of purple formazan, the optical density (OD600) value was measured on a microplate reader (BK-EL10C, Biobase, China) at an absorbance wavelength of 600 nm. 100 μl DMSO was used as a background that was subtracted from the experimental values.

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, USA). Data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to assess the reliability of the effect of the tested compounds on K562 cell proliferation. The mean OD600 values obtained for cells in the presence of each compound were compared with the value obtained in the cells with 0.3% DMSO; a level of $p<0.05$ was considered a reliable difference. The differences in statistical significance at the Figure 2 are indicated by asterisks: *$p<0.05$, **$p<0.005$, ***$p<0.0005$. The absence of statistically significant differences between the control and tested compounds is not indicated.

4. References

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S3. N. Nomura, G. Verdon, H. J. Kang, T. Shimamura, Y. Nomura, Y. Sonoda, S. A. Hussien, A. A. Qureshi, M. Coincon, Y. Sato, H. Abe, Y. Nakada-Nakura, T. Hino, T. Arakawa, O. Kusano-Arai, H. Iwanari, T. Murata, T. Kobayashi, T. Hamakubo, M. Kasahara, S. Iwata and D. Drew, *Nature*, 2015, **526**, 397; https://doi.org/10.1038/nature14909.

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S5. T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55; https://doi.org/10.1016/0022-1759(83)90303-4.

5. NMR Spectra

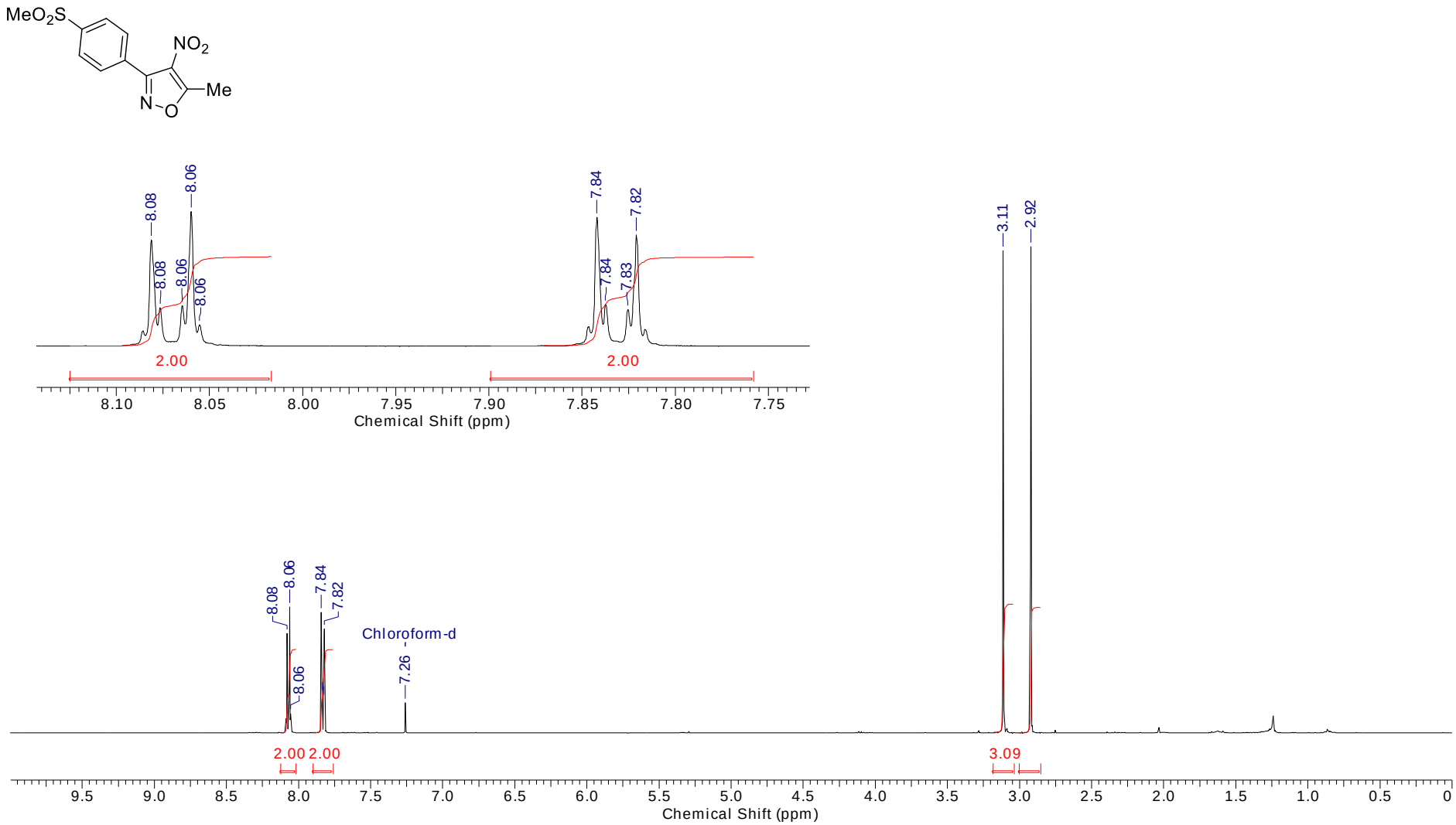

Figure S2. ^{1}H NMR spectrum of methyl-3-(4-methylsulfonylphenyl)-4-nitroisoxazole **4** in $CDCl_3$

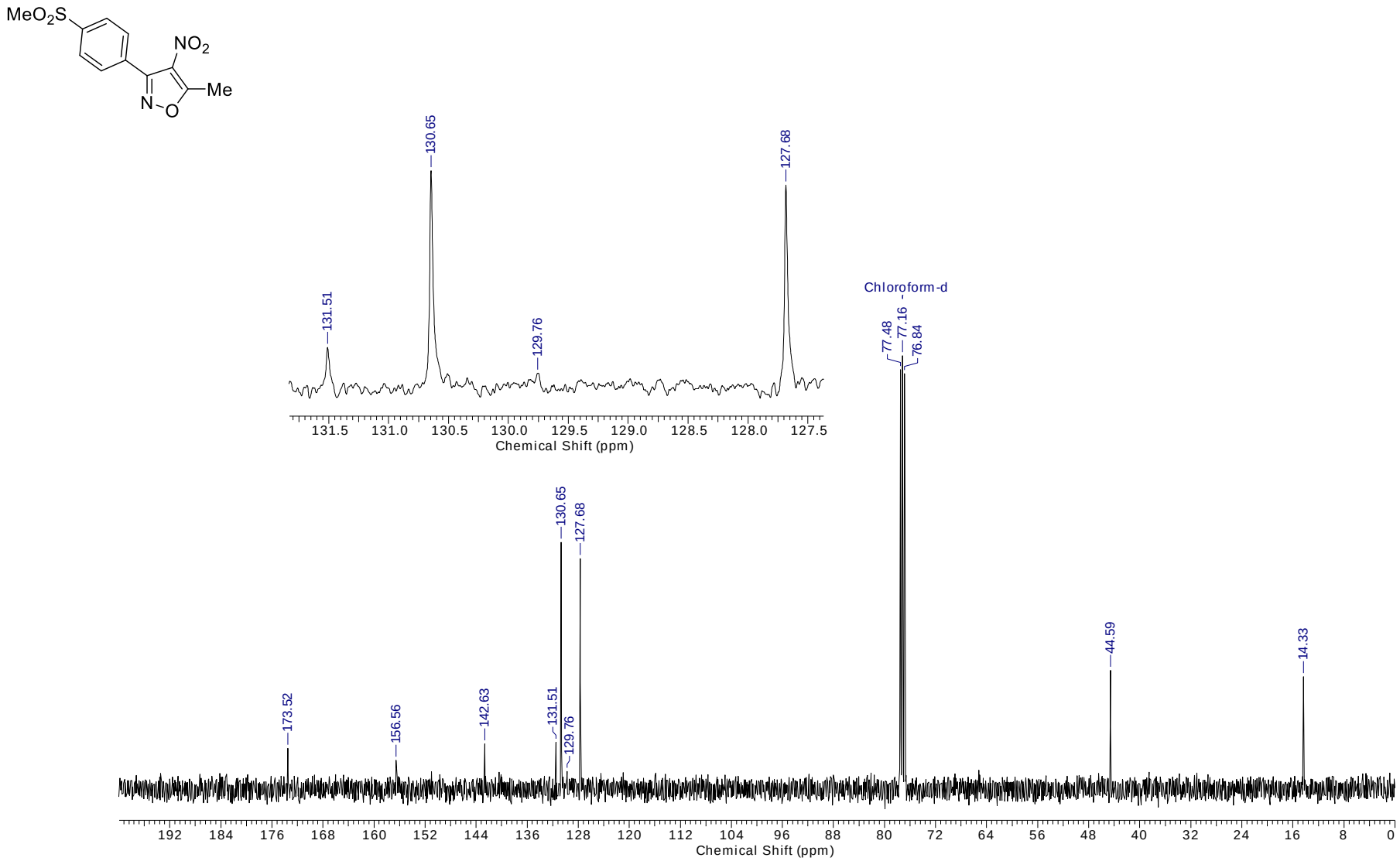

Figure S3. $^{13}C\{^{1}H\}$ NMR spectrum of methyl-3-(4-methylsulfonylphenyl)-4-nitroisoxazole **4** in $CDCl_3$

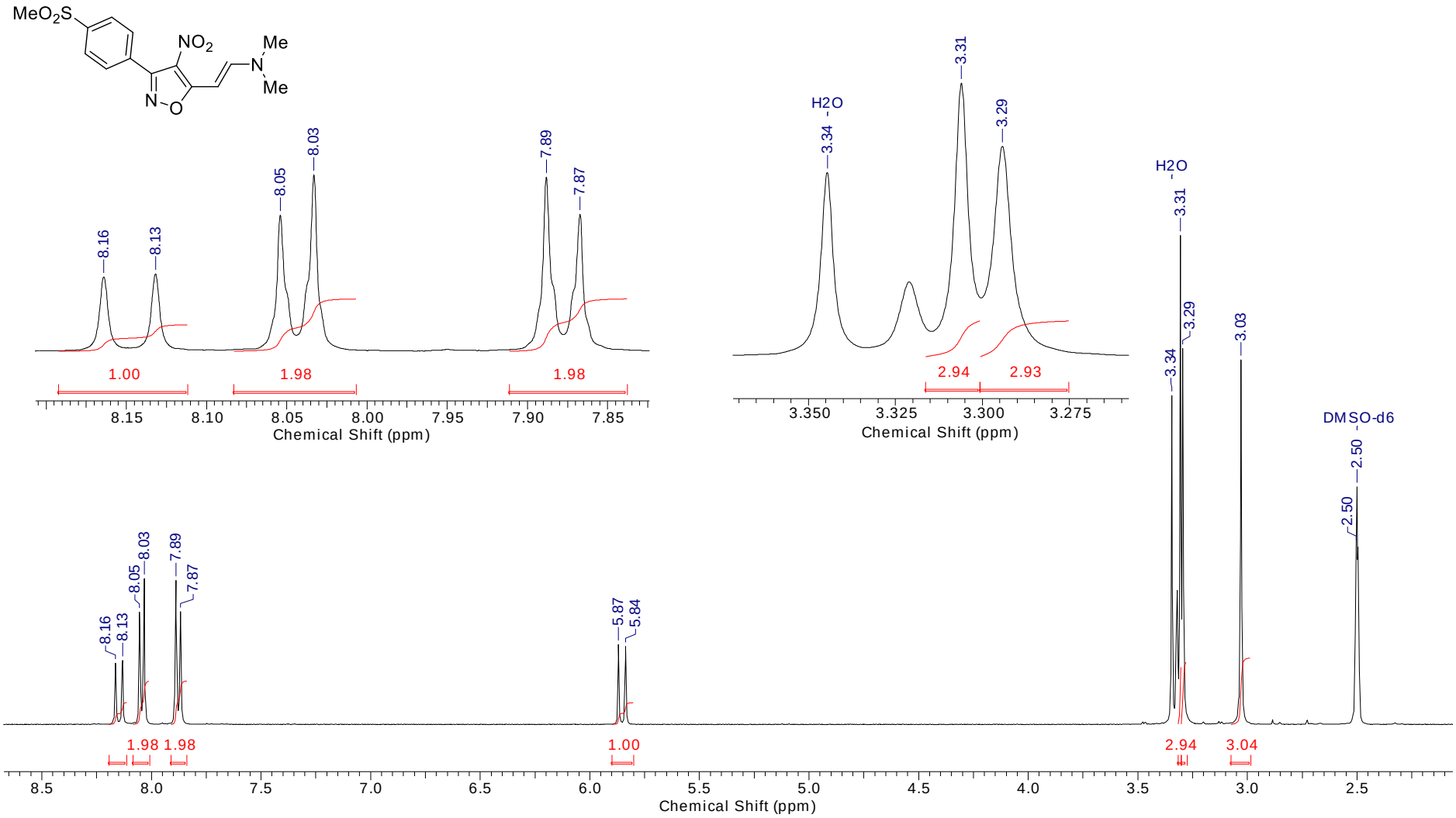

Figure S4. ^{1}H NMR spectrum of (*E*)-*N*,*N*-Dimethyl-2-[3-(4-methylsulfonylphenyl)-4-nitroisoxazol-5-yl]ethen-1-amine **5** in DMSO-d_6

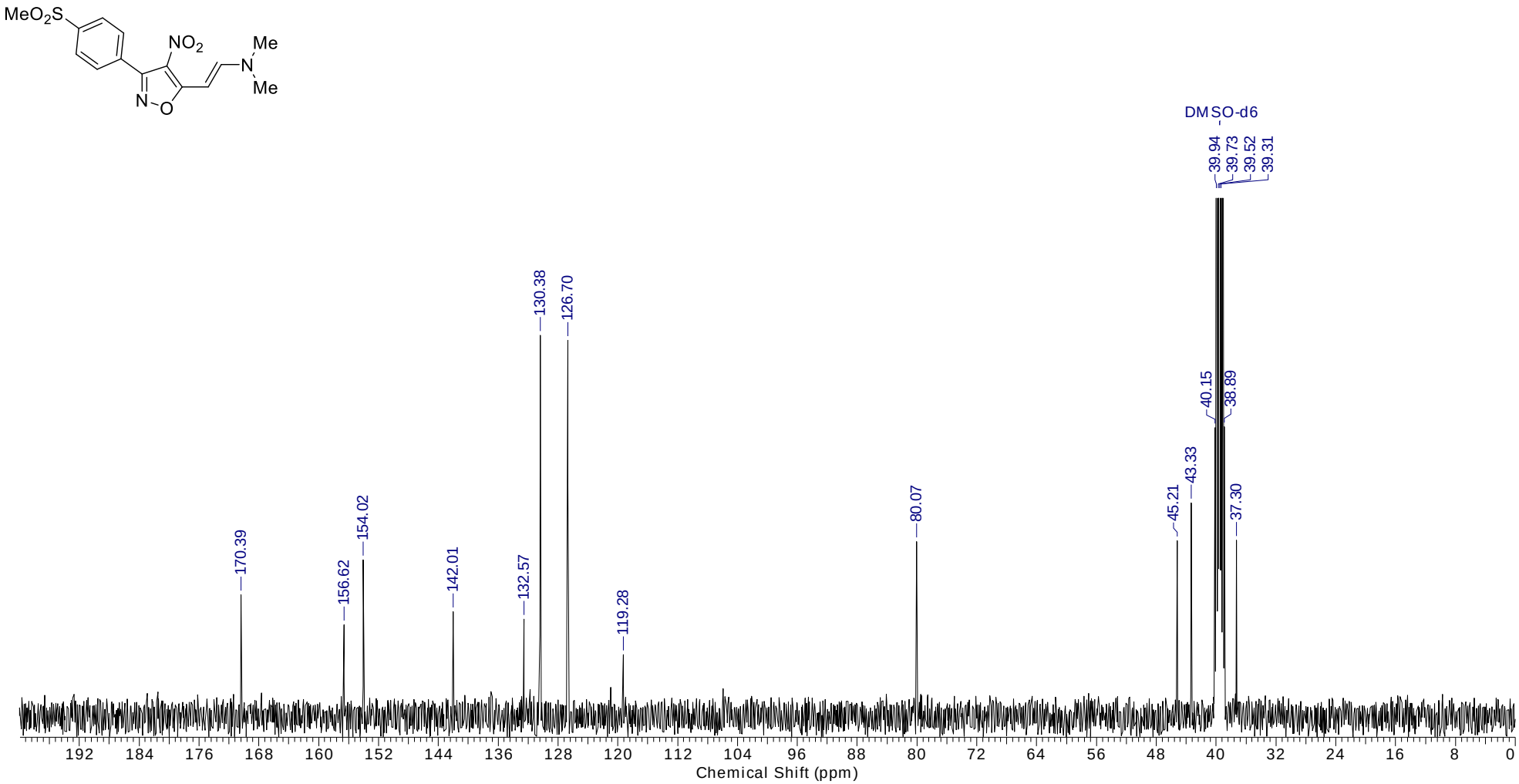

Figure S5. $^{13}C\{^{1}H\}$ NMR spectrum of (*E*)-*N*,*N*-Dimethyl-2-[3-(4-methylsulfonylphenyl)-4-nitroisoxazol-5-yl]ethen-1-amine **5** in DMSO-d_6

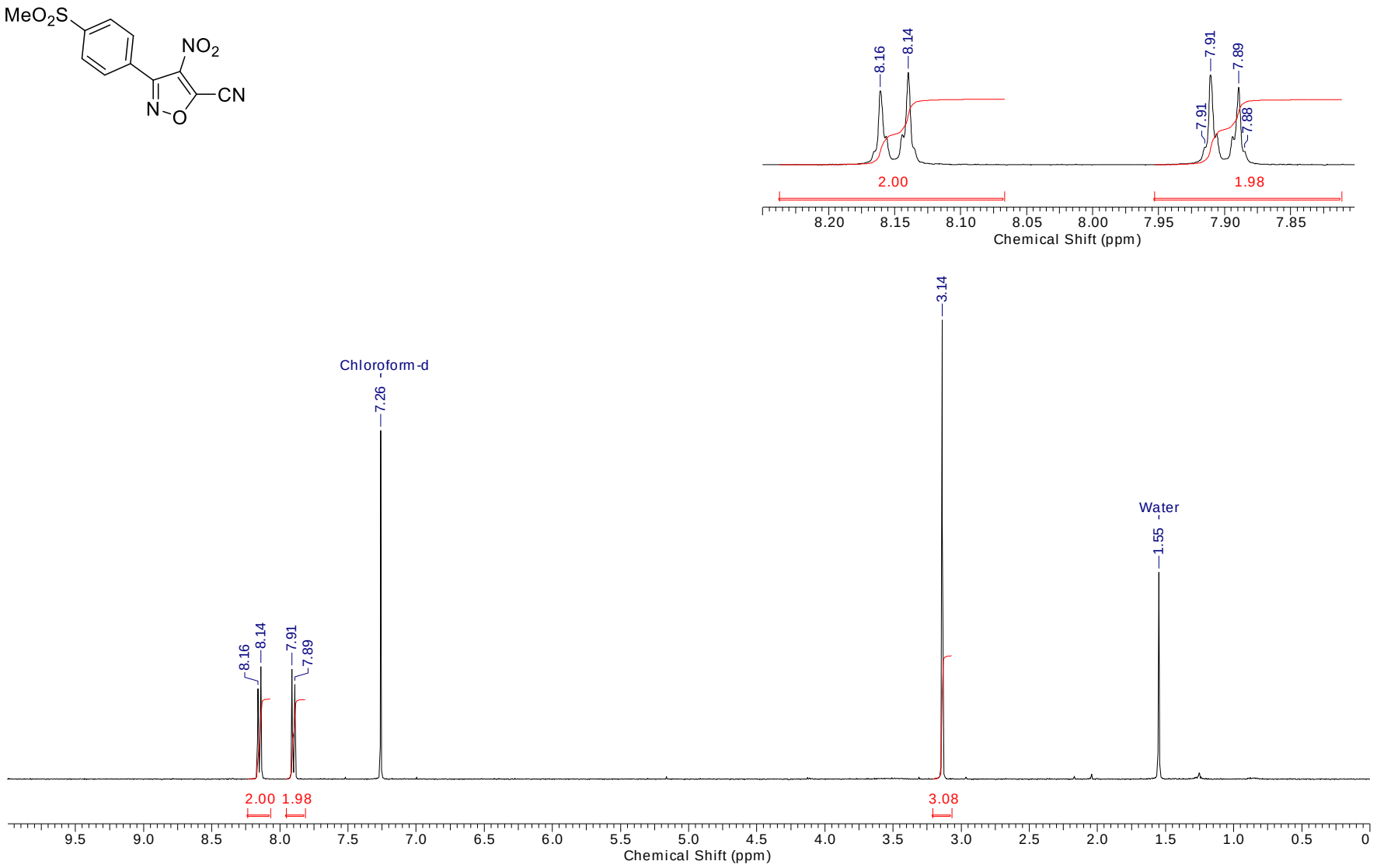

Figure S6. ^{1}H NMR spectrum of 3-(4-methylsulfonylphenyl)-4-nitroisoxazole-5-carbonitrile **6** in $CDCl_3$

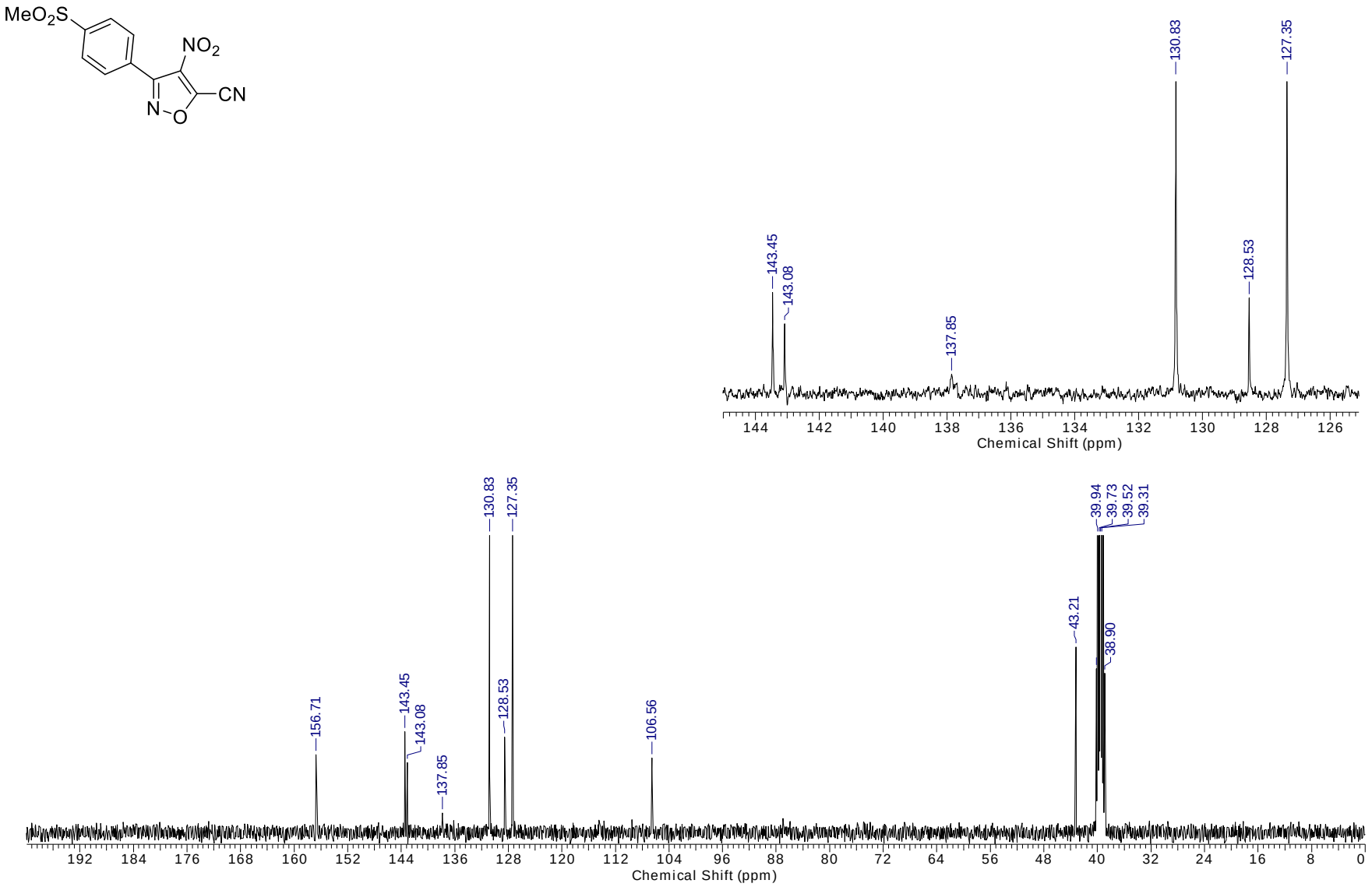

Figure S7. $^{13}C\{^{1}H\}$ NMR spectrum of 3-(4-methylsulfonylphenyl)-4-nitroisoxazole-5-carbonitrile **6** in DMSO-d_6

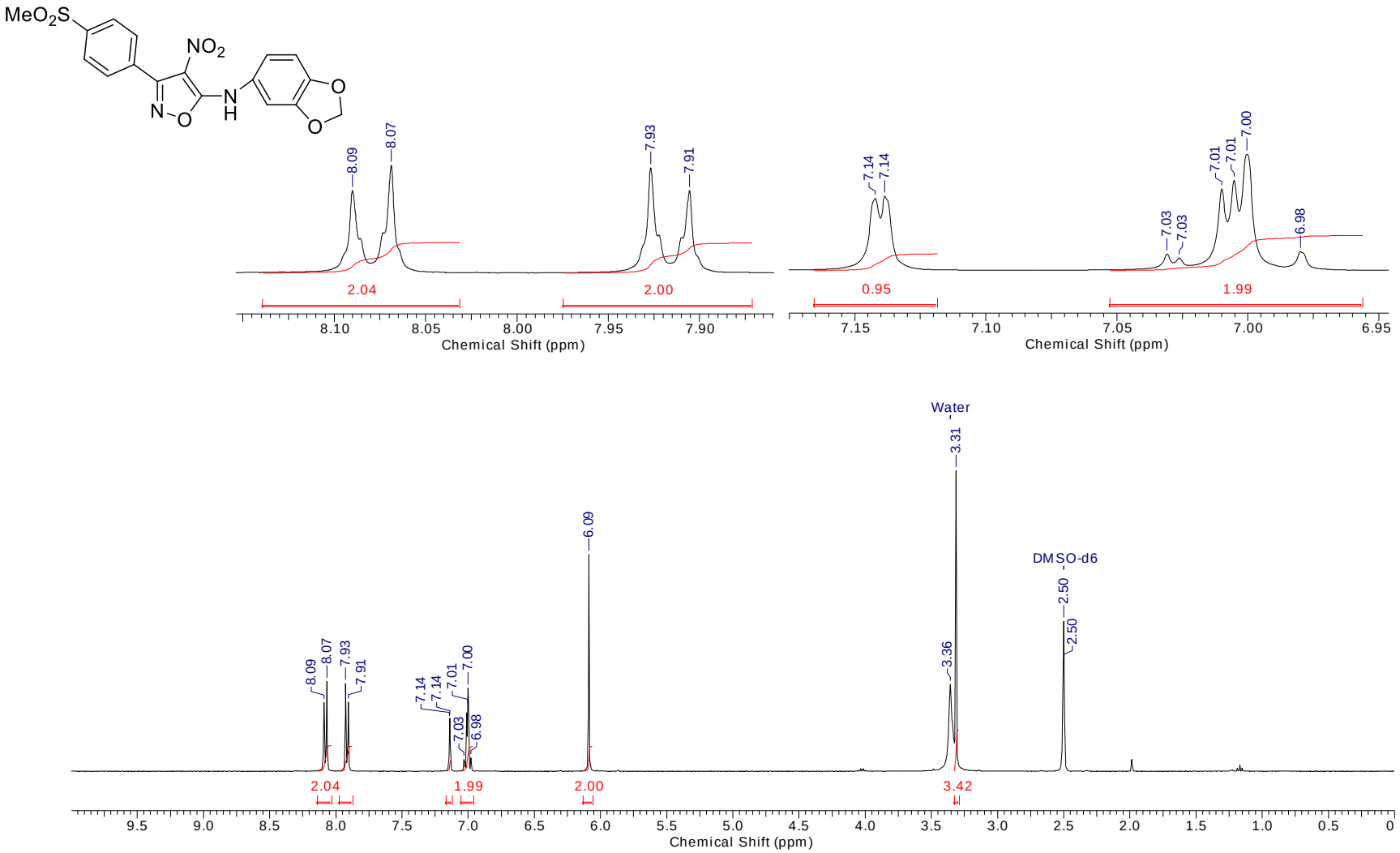

Figure S8. ^{1}H NMR spectrum of *N*-(benzo[*d*][1,3]dioxol-5-yl)-3-(4-methylsulfonylphenyl)-4-nitroisoxazol-5-amine **2a** in DMSO-d$_6$

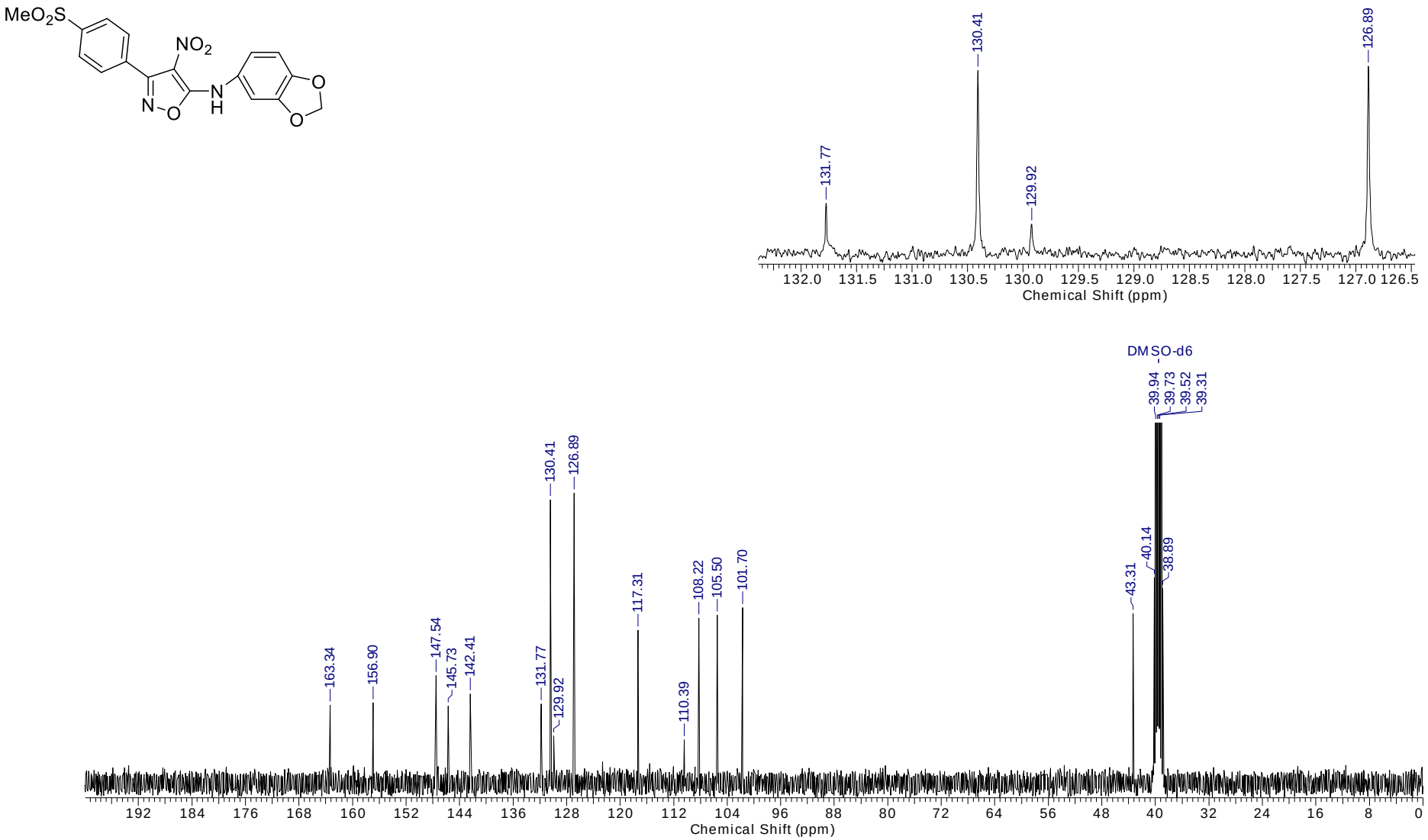

Figure S9. $^{13}C\{^{1}H\}$ NMR spectrum of *N*-(benzo[*d*][1,3]dioxol-5-yl)-3-(4-methylsulfonylphenyl)-4-nitroisoxazol-5-amine **2a** in DMSO-d_6

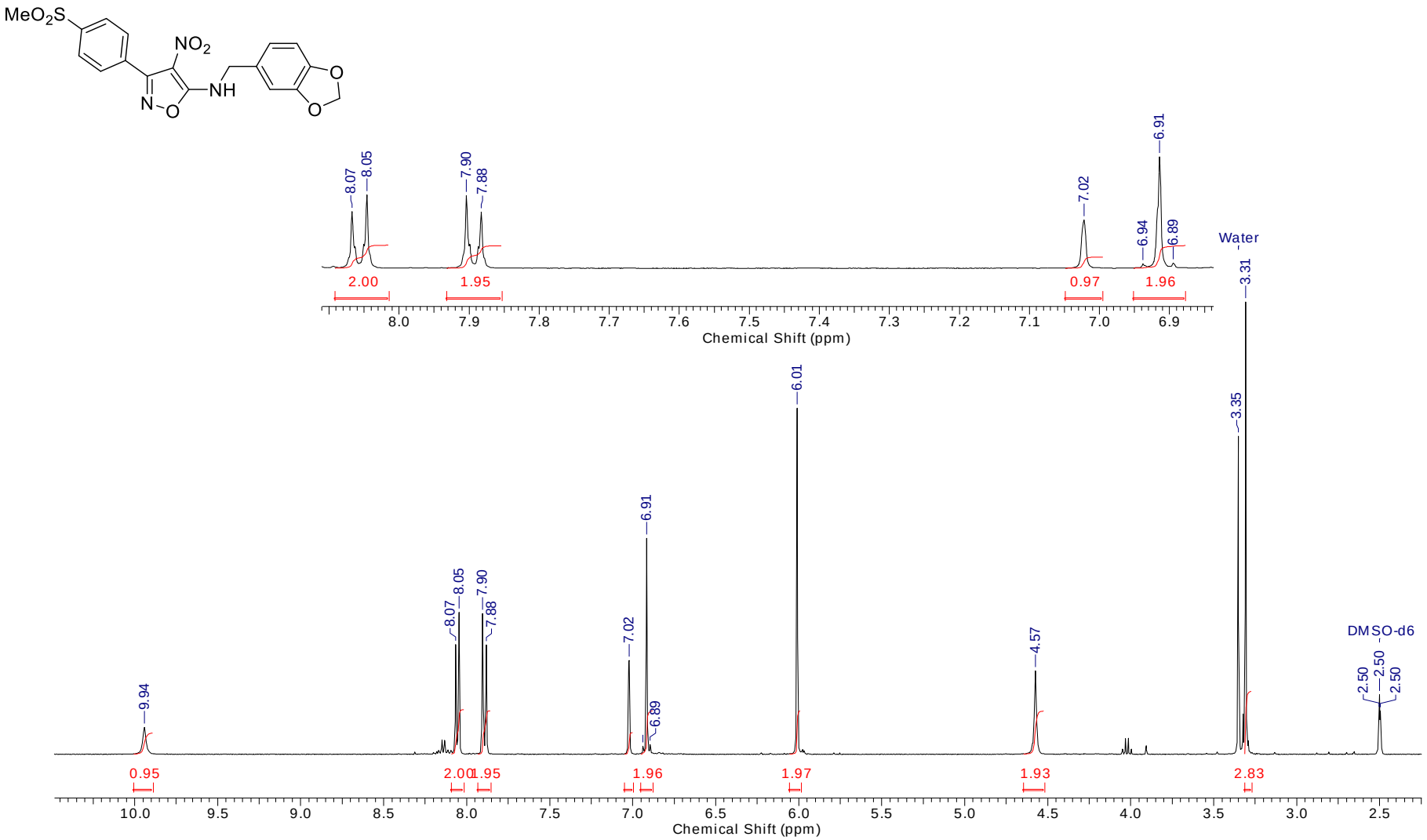

Figure S10. ^{1}H NMR spectrum of *N*-(benzo[*d*][1,3]dioxol-5-ylmethyl)-3-(4-methylsulfonylphenyl)-4-nitroisoxazol-5-amine **2b** in DMSO-d$_6$

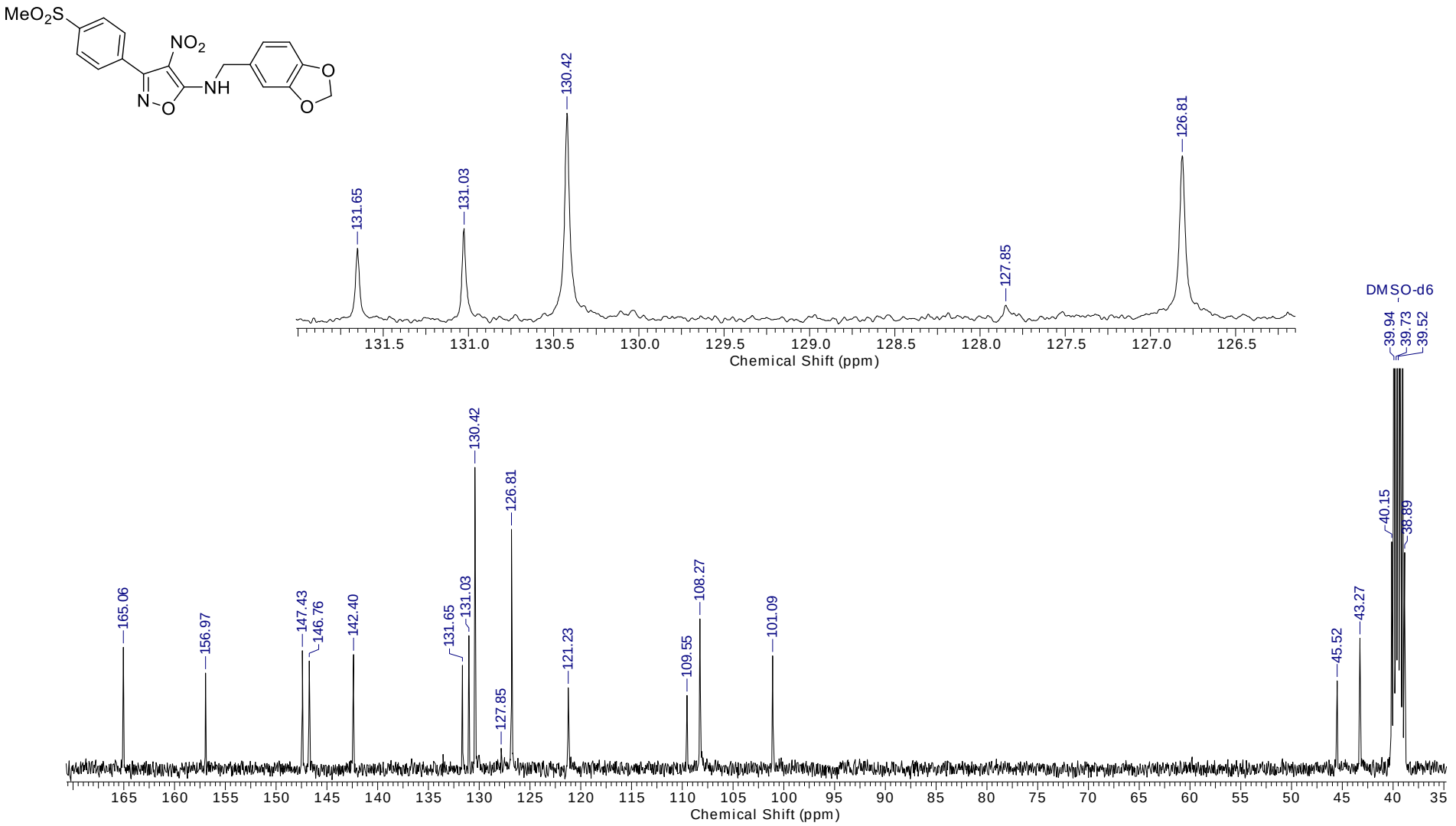

Figure S11. $^{13}C\{^{1}H\}$ NMR spectrum of *N*-(benzo[*d*][1,3]dioxol-5-ylmethyl)-3-(4-methylsulfonylphenyl)-4-nitroisoxazol-5-amine **2b** in DMSO-d_6