Supplementary Information I

Synthesis, characterization, and impact of carbon spacer on geometry and antibacterial activity of a new series of 1,n-bis(5-cycloalkylsulfenyl-1,3,4-thiadiazole-2-sulfenyl) alkanes (n = 4 and 6)

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1. Experimental

1.1. Materials and methods

All chemicals were purchased from either Sigma Aldrich or Merck Chemical Companies. All yields refer to the isolated products and GC-MS analysis on an Agilent GC-MS 6890 instrument under 70eV conditions. The purity determination of the substrates and reaction monitoring were conducted by TLC using silica gel 60 F254 on an aluminum sheet. The FTIR spectra were recorded by a Perkin Elmer 781 spectrophotometer with UATR in the 4000-400 cm^{-1} range. The ^{1}H and ^{13}C NMR spectra were recorded on a Brüker Avance III 600 MHz instrument (δ in ppm). All chemical shifts are quoted in parts per million (ppm) relative to TMS in a deuterated solvent. Positive and negative ion ESI-MS analyses were performed using an Agilent 6550 iFunnel Q-TOF LC/MS system equipped with a Dual AJS electrospray ionization (ESI) source operating in positive ion mode and AutoMS² acquisition mode. Chromatographic separation was conducted by a ZORBAX Eclipse Plus C18 column (4.6 mm × 100 mm, 3.5 µm) maintained at 37 °C. Melting points were recorded on an IA9100 digital melting point apparatus at a temperature rise of 0.5-2.0 °C min^{-1} in open capillary tubes. For sample purification, 0.5 m column chromatography was used with half of it packed with Merck silica gel 60 (0.040- 0.063 mm) completely saturated with *n*-hexane. Glass wool was used to prevent the stationary phase from being washed out of the column. For the mobile phase, a volume ratio of 80:20 of *n*-hexane: ethyl acetate was used as eluent. Differential scanning calorimetry (DSC) curves were obtained using a DSC-Mettler Toledo DSC 822e calorimeter. The heat flow calibration was conducted with 10.000 mg of indium and silver as the reference materials by the method IndCal at 5.0 K min^{-1}. The measurement was taken in the aluminum pans with a pierced lid under a dry nitrogen gas atmosphere (10 mL min^{-1}). Dynamic scans were performed at a heating rate of 10 K min^{-1} in three temperature cycles. TG-DTG curves were recorded using a Mettler Toledo TGA/SDTA 851e. Baseline optimization was conducted with a scan rate of 20.0 °C at 303.15 to 1273.15 K for starting and ending temperatures, respectively. All measurements were taken in the Al_2O_3 crucible under a nitrogen atmosphere (10 mL min^{-1}). Dynamic scans were performed with a temperature range from 298.15 to 1073.15 K at a heating rate of 10 K min^{-1}. The reproducibility was checked by repeating each reaction three times, and it was found to be within acceptable limits (±5%).

1.2. Synthesis of CmCnTD derivatives

In a round-bottom flask, 5-cycloalkylsulfenyl-1,3,4-thiadiazole-2-thione (10 mmol) was dissolved in ethanol (5 mL) at 50 °C. After 5 min stirring at room temperature, a solution of potassium carbonate (0.69 ± 0.01g, 5 mmol) in deionized water (1 mL) was added to the solution, and the stirring was continued at the same temperature. After 3h, 1,4-dichloromobutane or 1,6-dichlorohexane (5 mmol) was added to the solution, and the stirring was continued for 4 h under reflux conditions (monitored by TLC). After completion of the reaction, the solvent was removed by a rotary evaporator, and the residue was dissolved in ethyl acetate. The organic phase was washed with deionized water several

times to remove unreacted reactants and residual salts. Then, the crude product was purified by column chromatography after drying with $MgSO_4$ anhydride. The corresponding pure products were isolated in 69±2 % yield (see Supplementary Information I, Table S1).

1.3. Cell viability assay

The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT) assay was performed to evaluate the cell viability and the cytotoxic effect of the biguanidine derivatives on cell cultures using the RAW 264.7 mouse macrophage cell line. The RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC), Sigma-Aldrich, and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-100 µg/mL streptomycin (Gibco: catalogue no 15140122). The cells were seeded (20,000 cells/well) in 96-well plates and incubated at 37 °C in a humidified atmosphere overnight with 5% CO_2 for optimal growth. The cells were cultured in sterile cell-culture flasks until they reached 80-90 % confluency. After the incubation, the cells were treated with 10 µM of biguanidine derivatives 1-4. Next, the MTT solution (0.5 mg/mL) was added and incubated at 37 ℃ for 3 h. The viable and metabolically active live cells reduced the MTT to form purple water-insoluble formazan crystals. Then, 100 µL of dimethyl sulfoxide (DMSO) was added to the media to dissolve the purple formazan crystals. The absorbance of purple formazan crystals was recorded at 570 nm using a microplate reader (Flex Station 3 Instrument, Molecular Devices).

1.4. Antibacterial studies and determining the minimum inhibitory concentration (MIC)

Antibacterial activities of the new novel 5-cyclopentylsulfenyl-[1,3,4]thiadiazole-2-thione were assayed in vitro against *Escherichia coli* ATCC 25922 (*E. Coli*) and *Staphylococcus aureus* ATCC 25923 (*S. Aureus*). *E. coli* ATCC 25922 is a well-characterized control strain in antibiotic susceptibility laboratory experiments. It is of serotype O6 and biotype 1 and is designated as a Gram-negative control bacterium. *S. aureus* strain ATCC 25923, also known as Seattle 1945, is a well-characterized, methicillin-susceptible strain commonly used as a control in antibiotic susceptibility experiments and quality control for commercial products, providing a reliable and well-understood strain for a wide range of biological testing. The concentrations from 62.5 to 1000 µg mL^{-1} of TDS derivatives were prepared using DMSO as solvent. Then, 1 mL of the as-prepared solutions was added to 3 mL of inoculated broth containing specific bacteria. Moreover, the untreated Luria-Bertani (LB) broth was kept as a negative control. A single colony of the organism, which was picked from an LB streak plate, was dissolved in 3 mL LB broth and incubated overnight at 310.15 K at 220 rpm. The optical density in 600 nm (OD_{600}) (1 $OD_{600} = 1 \times 10^9$ colony-forming unit(CFU) mL^{-1}) was checked with a spectrophotometer. The bacterial solution was diluted with LB broth to get 0.1 OD600 suspensions and then

incubated at 310.15 K at 220 rpm till mid-log phase (~2 h). 1 mL mid-log phase bacterial solution was put in a 1.5 mL Eppendorf tube, centrifuged at 6000 rpm for 5 min, and washed two times with 1 mL phosphate-buffered saline solution. The bacterial pellet was dissolved with 1 mL of LB broth, and 20 µl of the bacterial solution was mixed with 180 µL of LB broth, and then OD600 was checked with a spectrophotometer. The bacterial concentration was adjusted to 1×10^7 CFU mL^{-1} with LB broth (1 OD_{600} ~10^9 CFU mL^{-1}).

The bacterial cultures were inoculated uniformly into each tube containing different concentrations of the test sample. An uninoculated broth was kept as a blank. The tubes were incubated at 310.15 K for 24 h. The OD_{600} with the spectrophotometer was checked. The MIC endpoint is the lowest concentration of antibiotic at which there is no visible growth of bacteria, viz, no solution turbidity on the naked eye, and the absorbance measured keeping uninoculated broth (blank) should be less than 0.010. The inhibition was assessed based on turbidity. Each antibacterial test was performed in triplicate.

1.5. Crystallography

The Crystallographic Information Files (CIFs) were deposited in the Cambridge Crystallographic Data Centre. The CCDC number for 1,6-bis(5-cyclopentylsulfenyl-1,3,4-thiadiazole-2-sulfenyl) hexane (C5C6TD) is 2470371.

1.6. Statistical analysis

For the statistical analysis, a completely randomized design was utilized, and all experiments were performed in triplicate. Analysis of variance (ANOVA) was done, and the mean comparison was performed by the Duncan test. The significance level was set at ($P<0.05$). The SPSS software (version 16.0) was used for statistical analysis.

Figure S1. FT-IR spectrum of C5C4TD.

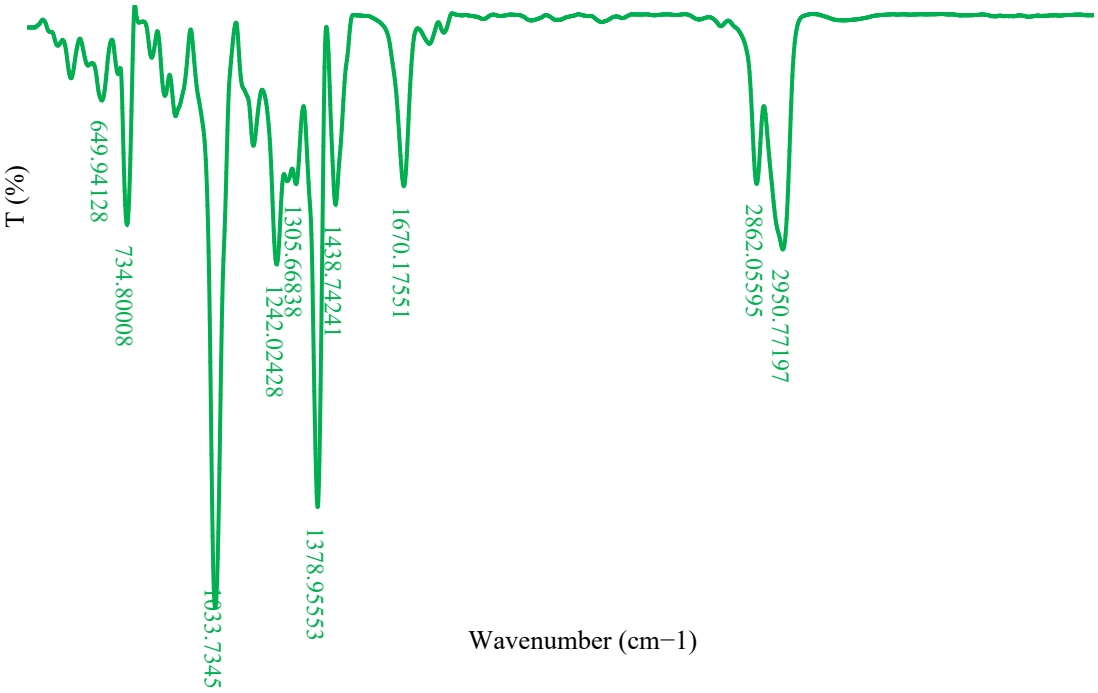


Figure S2. FT-IR spectrum of C5C6TD.

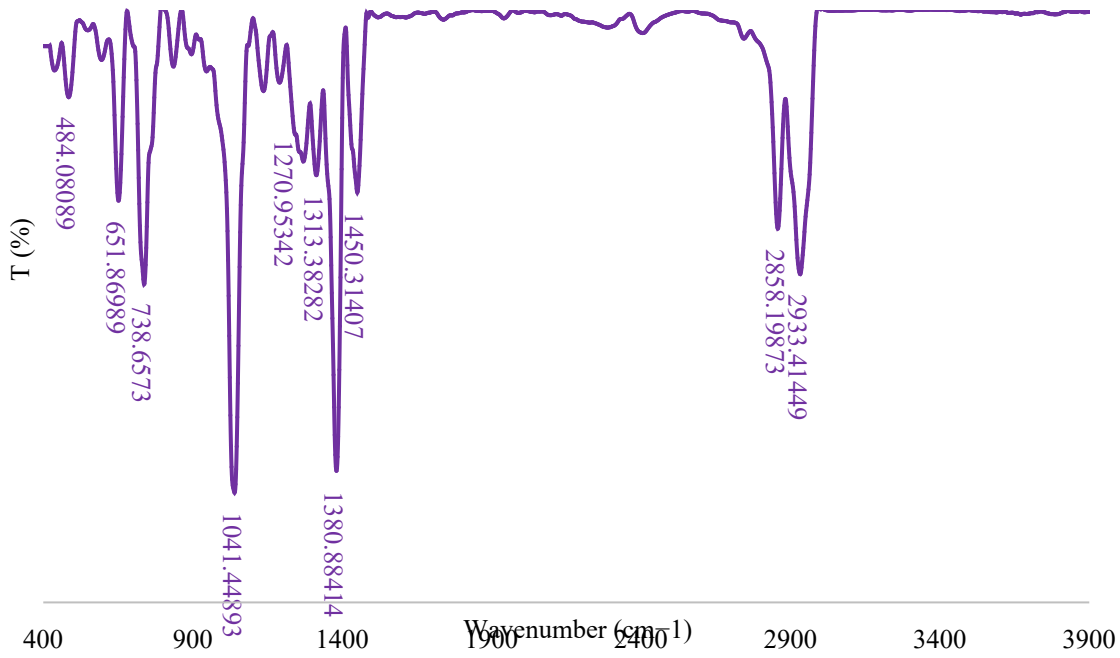


Figure S3. FTIR spectrum of C6C4TD.

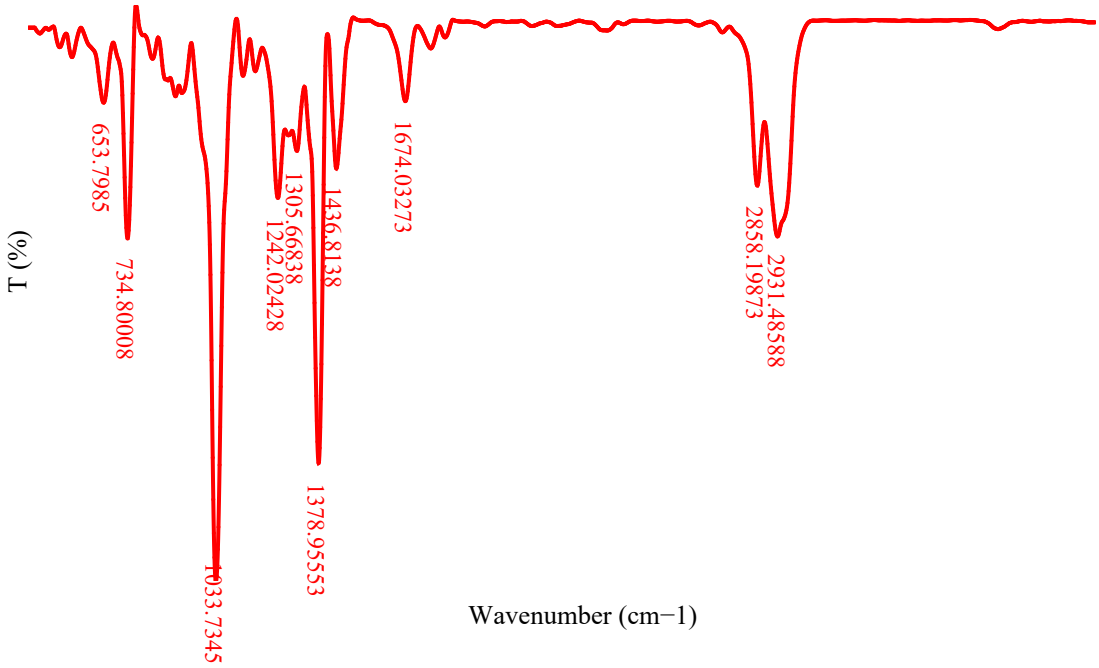


Figure S4. FTIR spectrum of C6C6TD.

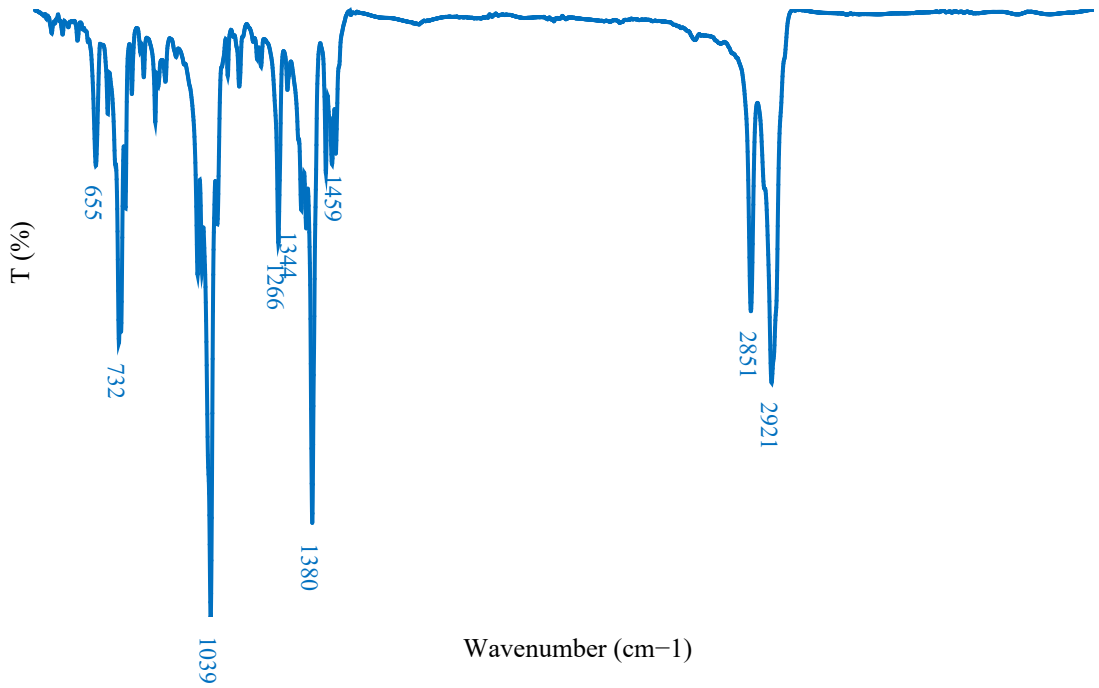
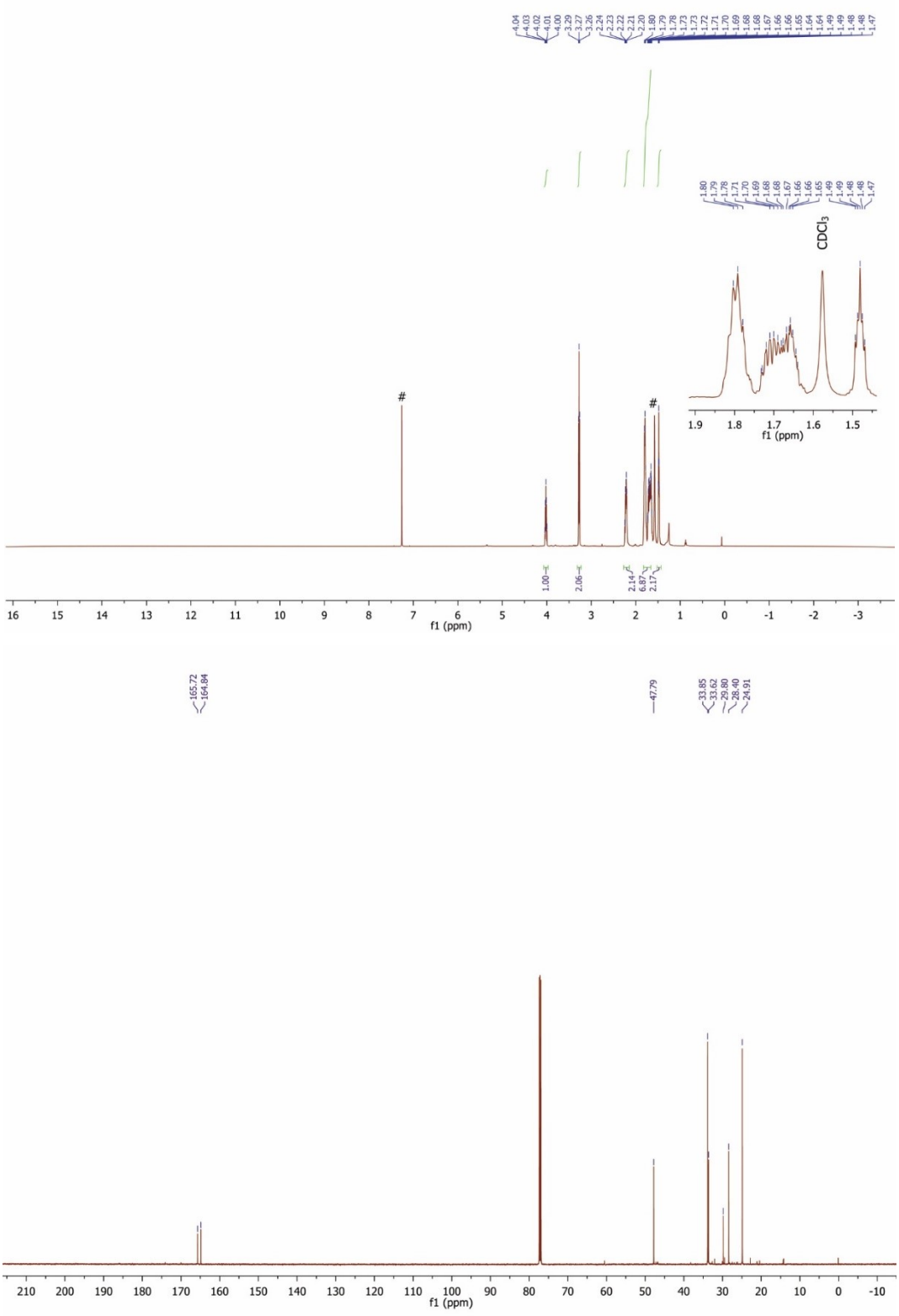
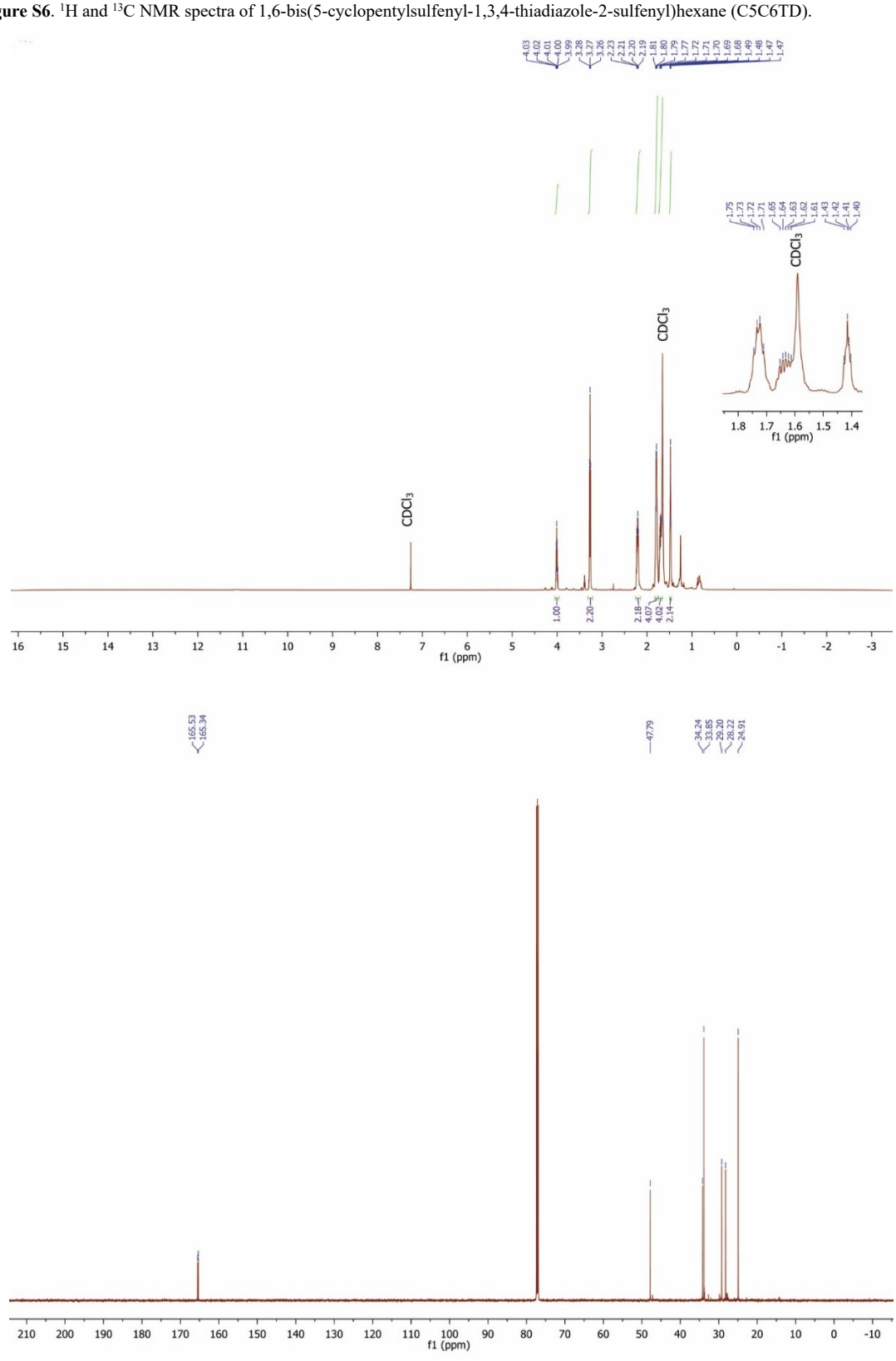


Figure S5. ^{1}H NMR and ^{13}C NMR of 1,4-bis(5-cyclopentylsulfenyl-1,3,4-thiadiazole-2-sulfenyl)butane (C5C4TD).



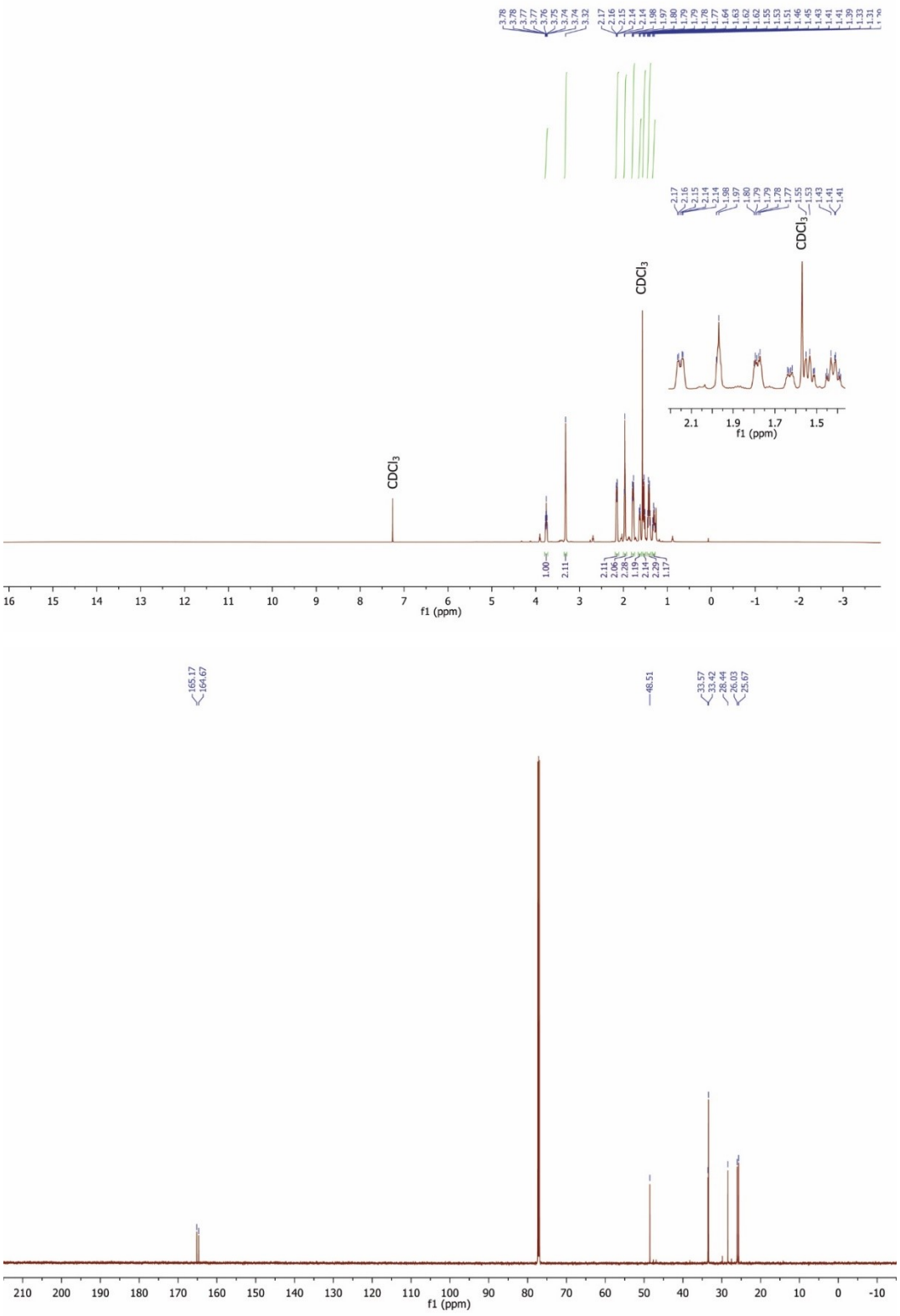
^{1}H NMR ($CDCl_3$, 600.17 MHz) δ 4.04-4.00 (m, 1H), 3.27 (t, J = 7.3 Hz, 2H), 2.24-2.20 (m, 2H), 1.81-1.65 (m, 6H), 1.46-1.47 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$, 150.91 MHz) δ 165.72, 164.84, 47.79, 33.85, 33.62, 29.80, 28.40, 24.91 ppm.

Figure S6. ^{1}H and ^{13}C NMR spectra of 1,6-bis(5-cyclopentylsulfenyl-1,3,4-thiadiazole-2-sulfenyl)hexane (C5C6TD).



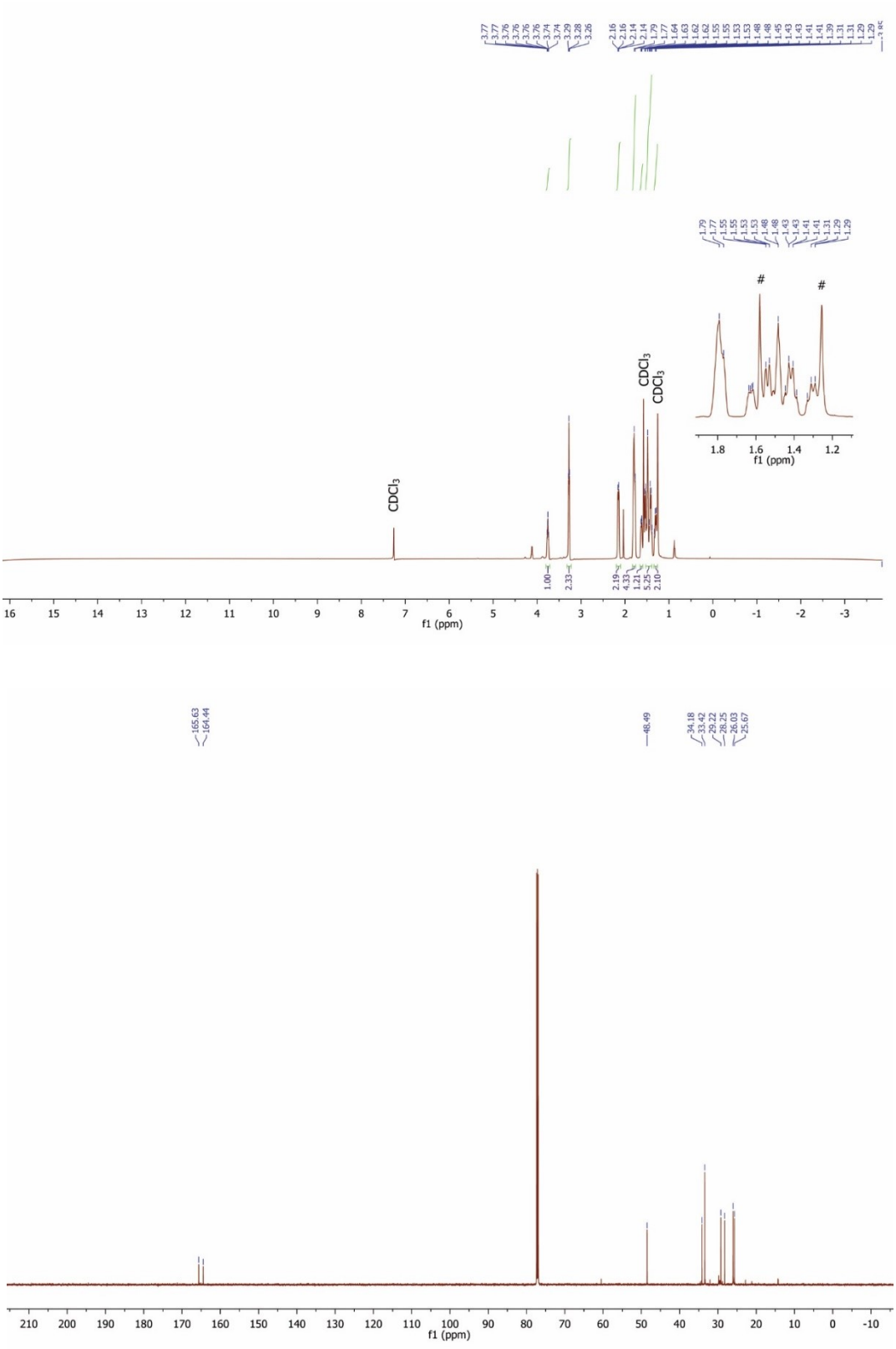
1H NMR (CDCl3, 600.17 MHz) δ 4.03-3.99 (m, 1H), 3.27 (t, *J* =7.3 Hz, 2H), 2.23-2.19 (m, 2H), 1.81-1.77 (m, 4H), 1.72–1.68 (m, 4H), 1.49-147 (m, 2H) ppm; 13C NMR (CDCl3, 150.93 MHz) δ 165.53, 165.34, 47.79, 34.24, 33.85, 29.20, 28.22, 24.91 ppm.

Figure S7. ^{1}H NMR and ^{13}C NMR of 1,4-bis(5-cyclohexylsulfenyl-1,3,4-thiadiazole-2-sulfenyl)butane (C6C4TD).



^{1}H NMR ($CDCl_3$, 600.17 MHz) δ 3.78-3.74 (m, 1H), 3.32 (t, *J* =7.3 Hz, 2H) 2.17-2.14 (m, 2H), 1.98-1.96 (m, 2H), 1.80-1.77 (m, 2H), 1.64-1.62 (m, 1H), 1.55-1.51 (m, 2H), 1.45-1.39 (m, 2H), 1.31-1.27 (m, 1H) ppm; ^{13}C NMR ($CDCl_3$, 150.93 MHz) δ 165.17, 164.67, 48.51, 33.57, 33.42, 28.44, 26.03, 25.67 ppm.

Figure S8. ^{1}H and ^{13}C NMR spectra of 1,6-bis(5-cyclohexylsulfenyl-1,3,4-thiadiazole-2-sulfenyl)hexane (C6C6TD).



^{1}H NMR ($CDCl_3$, 600.17 MHz) δ 3.77-3.74 (m, 1H), 3.28 (t, J = 7.3 Hz, 2H), 2.16-2.14 (m, 2H), 1.79-1.77 (m, 4H), 1.64-1.28 (m, 8H) ppm; ^{13}C NMR ($CDCl_3$, 150.93 MHz) δ 165.63, 164.44, 48.49, 34.18, 33.42, 29.22, 28.25, 26.03, 25.67 ppm.

Figure S9. ^{1}H NMR spectrum of the used deuterated chloroform ($CDCl_3$).

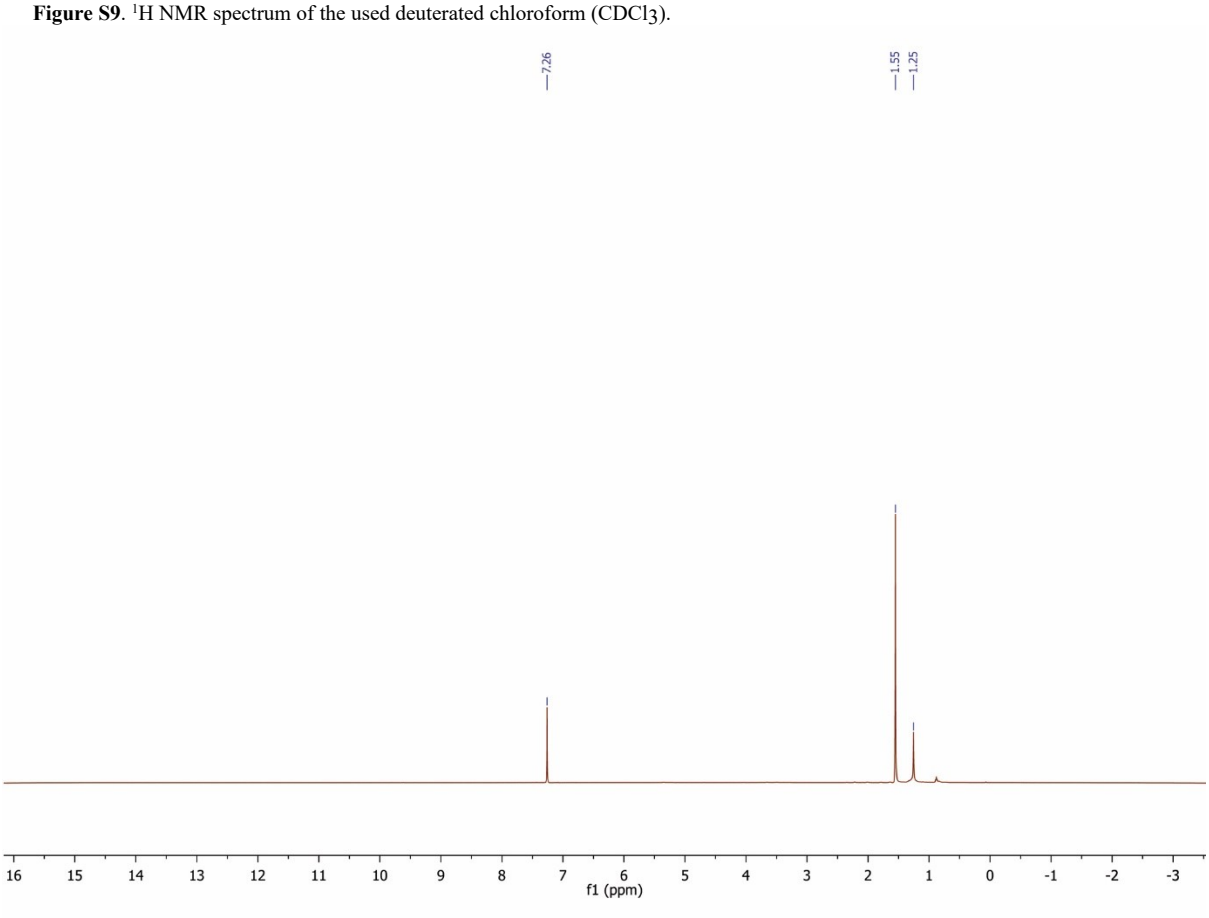


Figure S10. A copy of the mass spectrum of the CmCnTD derivatives from their LC-MS data.

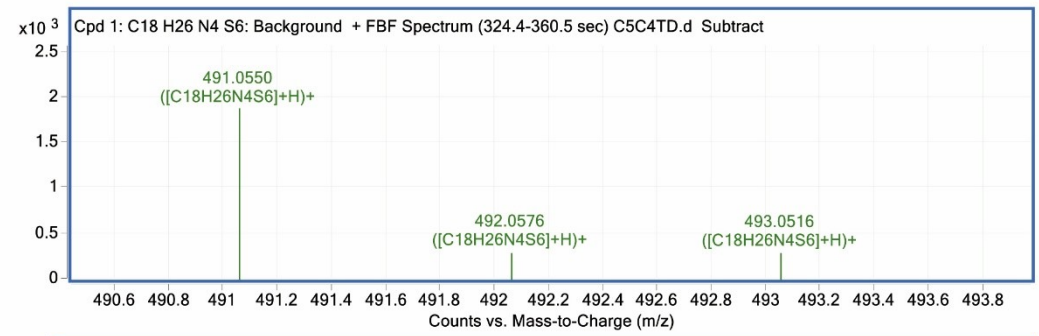
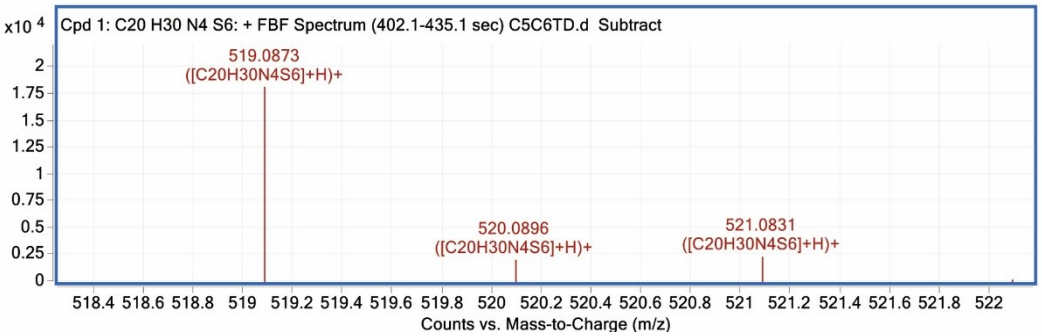
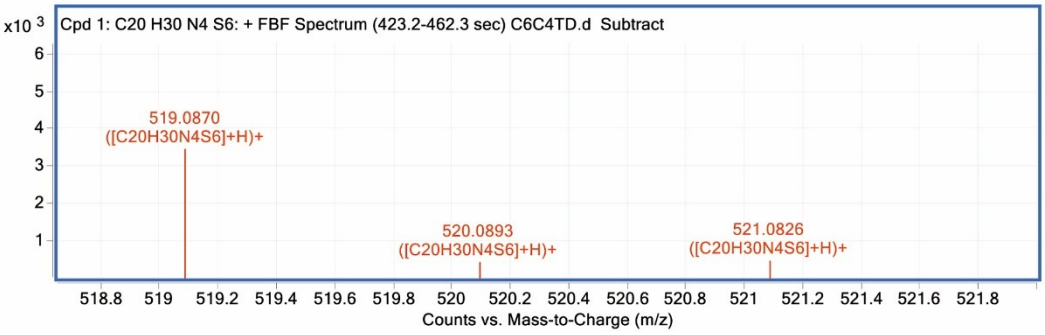
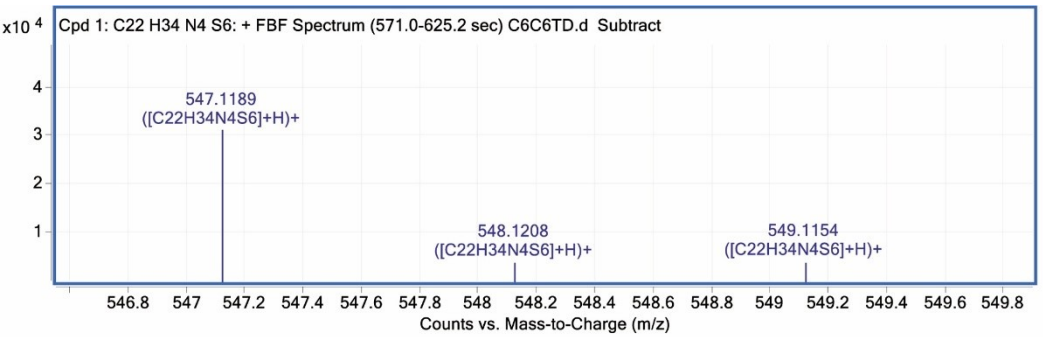
Derivative	Mass Spectrum
C5C4TD	
C5C6TD	
C6C4TD	
C6C6TD	

Table S1. Yield, appearance, and melting point of bis(5-cycloalkylsulfenyl-1,3,4-thiadiazole-2-sulfenyl) derivatives.

Derivative	Yield (g, %)	Apperance	Melting point (°C)
C5C4TD	1.693, 69	Clear crystals	59-60
C5C6TD	1.738, 67	Clear crystals	89-90
C6C4TD	1.842, 71	Clear crystals	77-78
C6C6TD	1.859, 68	White powder	59-60