Electronic Supplementary Material (ESI) for Analyst.


# Supplementary Information

## Construction of A Near-infrared Fluorescent Probe for Ratiometric Imaging of Peroxynitrite during tumor progression

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## Materials

All the reagents and materials in our experiments, including 2-(4-Diethylamino -2-hydroxybenzoyl) benzoicacid, cyclohexanone, 4-(Methylthio)benzaldehyde, 3-morpholinosydnoniminehydrochloride (SIN-1), 3,3-bis(aminoethyl)-1-hydroxy -2-oxo-1-triazene (NOC-18), menadione sodium bisulfite (MSB), apocynin, interferon-gamma(IFN-γ), aminoguanidine (AG), FeTMPyP, Lipopolysaccharides (LPS) and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma-Aldrich (USA). Concentrated $H_2SO_4$, perchloric acid were purchased from Energy Chemical Co., Ltd (Shanghai, China). Dimethyl sulfoxide (DMSO), Methanol, Ethanol were analytical grade without further purification. The stock solution of **CDMS** was prepared in dimethyl sulfoxide (DMSO) at the concentration of 1 mM and reserved in refrigerator. Analytical condition is the phosphate buffered saline (PBS) solution (20 mM, pH=7.4, with 0.1% DMSO).

## Apparatus

NMR spectra were measured on a Bruker AVANCEIIIHD 500 MHz ($^1$H NMR 500 MHz, $^{13}$C NMR 125 MHz) spectrometers at 37 ± 1 °C with TMS as the internal standard. Mass spectrometry data were obtained on Agilent 1290/6545 UHPLC-Q-TOF mass spectrometer. Absorption spectra were obtained on the UV-vis spectrophotometer (Shimadzu, UV-3600 plus). Fluorescence spectra were determined on a fluorescence spectrometer (Edinburgh, FLS 1000). Fluorescence imaging were acquired using a laser scanning confocal microscope (Olympus FV3000) with an objective lens (× 60). Intracellular fluorescence analysis was carried out on flow cytometry (FACSAria, BD). In vivo fluorescence images were collected by PerkinElmer IVIS® Lumina XR Series Ⅲ System. Mice pathological splices were imaged by Nikon Model Eclipse Ci-L microscope. Ultrapure water was prepared using a Milli-Q A10 system. All pH measurements were made with a JENCO 6230 M pH meter.

## Determination of the in vitro detection limit

The limit of detection was calculated based on the fluorescence titration curve of **CDMS**. The fluorescence intensity of **CDMS** was measured by twelve times and the standard deviation of blank measurement was obtained. The detection limit was calculated with the following equation: LOD= 3σ/k. Where σ is the standard deviation of blank measurement, k is the slop between the fluorescence intensity versus $ONOO^-$ concentrations.

## Determination of Absolute quantum yields

**CDMS** was diluted to 10 μM for absolute quantum yields (QY), and the determination was measured by fluorometer (Edinburgh，FLS 1000). For **CDMS**, $\lambda_{ex}$ = 500 nm.

## Generation of ROS/RNS

The sources of ROS/RNS are described as follows, $H_2O_2$ solution was purchased from Sigma-Aldrich and diluted before using. $KO_2$ was dissolved in DMSO to generate superoxide ($O_2^{\bullet-}$).[1] Potassium Nitroprusside Dihydrate was used to generate •NO. *tert*-butyl hydroperoxide (TBHP) was diluted from 70 % stock solution.[2] The source of NaOCl was from NaClO solution that contains 5% available chlorine. Hydroxyl radical (•OH) was generated by Fenton reaction. Briefly, ferrous chloride ($FeCl_2$) was added in the presence of 10 equiv. of $H_2O_2$, the concentration of •OH was equal to the Fe(II) concentration.[3] Peroxynitrite ($ONOO^-$) solution was synthesized according to the reported literature. Simply, hydrogen peroxide (0.7 M) was firstly acidified with hydrochloric acid (0.6 M), and then mixed with sodium nitrite (0.6 M). Finally, sodium hydroxide (1.5 M) was added within 1-2 seconds to make the solution alkaline. A short column of manganese dioxide was used to remove excess hydrogen peroxide. The concentration of $ONOO^-$ was determined by measuring the absorption of the solution at 302 nm. The $ONOO^-$ concentration was estimated by using an extinction coefficient of 1670 ± 50 $cm^{-1}M^{-1}$ at 302 nm.[4]

## Information of confocal imaging

Fluorescent images were acquired on an Olympus FV3000 confocal laser-scanning microscope. Fluorescence collection windows for red channel: 650−720 nm ($\lambda_{ex}$=594 nm), green channel: 520−590 nm ($\lambda_{ex}$ = 488 nm), and blue channel: 430−480 nm ($\lambda_{ex}$=405 nm). Cells were plated in the culture dish and allowed to adhere for 24 h before imaging. Cell nucleus dye 4, 6-diamidino-2-phenylindole (DAPI, 100 ng/mL, 1 μL) and **CDMS** (10 μL, 1.0 mM) were simultaneously added to the culture plates which were filled with 1 mL fresh complete medium. Cell imaging was carried out after washing cells with corresponding medium for three times after the different treatment.

## Histopathological Analyses

In this work, all animal experiments were performed according to the guidelines issued by The Ethical Committee of Qufu Normal University. H&E staining was carried out according to a protocol provided by the vendor (BBC Biochemical). The tumors of different sizes in mice model were excised, subsequently the 8 μm of tumor cryogenic slides and normal tissue were prepared and into fixed in 10% formaldehyde. After washing with running water, the slides were treated with gradient concentrations of alcohol (100 %, 95 % and 70 %), each for 20 s. The hematoxylin staining was performed for 3 min and washed with water for 1 min. The eosin staining was performed for 1 min. The slides were washed, treated with xylene, and mounted with Canada balsam. The images were captured using a Nikon Eclipse 90i.

## *In Vivo* Imaging

The *in vivo* imaging experiments were performed in accordance with the guidelines issued by The Ethical Committee of Qufu Normal University. Mice were anesthetized prior to injection and during imaging via inhalation of isoflurane. Mice were imaged after injection of probes for 30 min. PerkinElmer IVIS® Lumina XR Series III System was used for the bio-imaging of animal models. The excitation and emission wavelength were chosen as described in the manuscript.

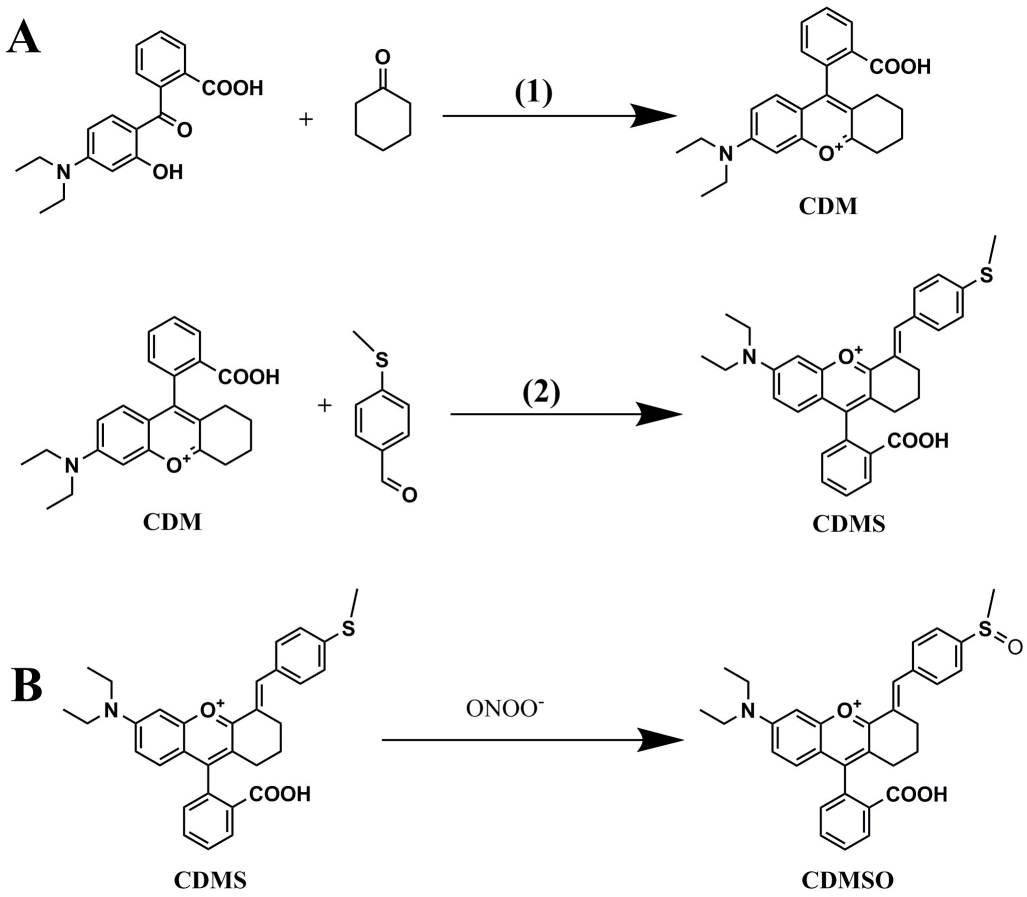


**Figure S1.** (A) General synthetic routes for the probe **CDMS**. (1). Condensed $H_2SO_4$, 95 °C, 3 h. (2). Ethanol, 80 °C, 8 h. (B) Proposed responding mechanism toward $ONOO^-$.

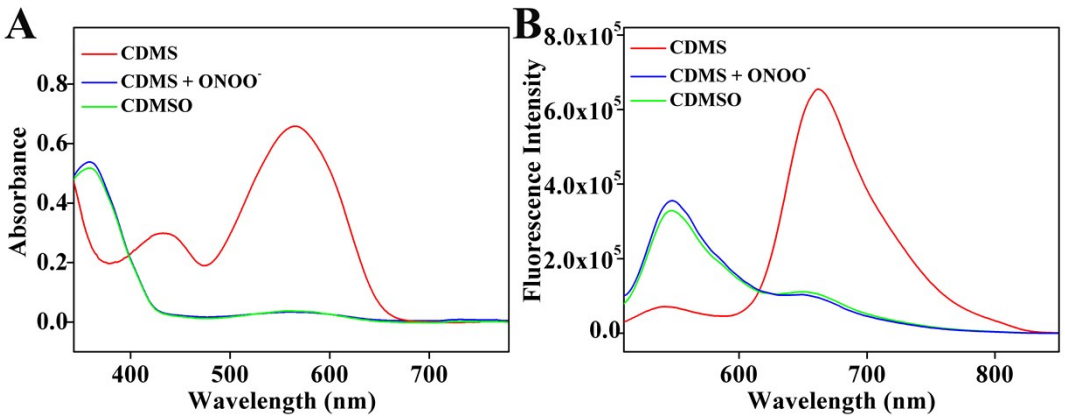


**Figure S2. (**A) UV-vis absorption spectra and (B) fluorescence spectra of **CDMS** (10 μM), **CDMS** (10 μM) + (100 μM) $ONOO^-$ and **CDMSO** (10 μM) in PBS solution (pH=7.4, 20 mM, with 0.1% DMSO).

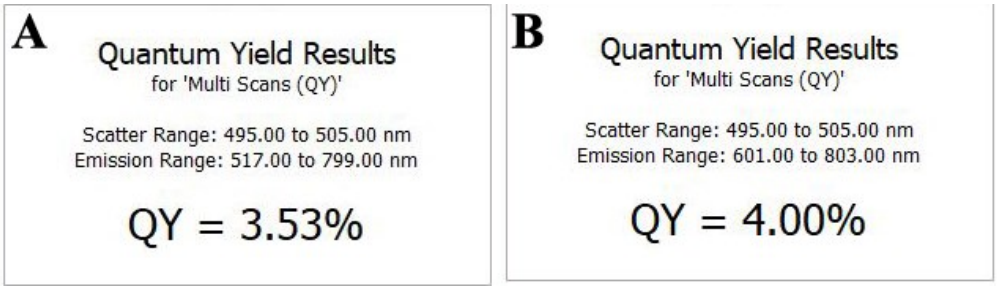


**Figure S3.** Fluorescence quantum yields of (A) **CDMS** (10 μM) and (B) **CDMSO** (10 μM) in buffer solution.

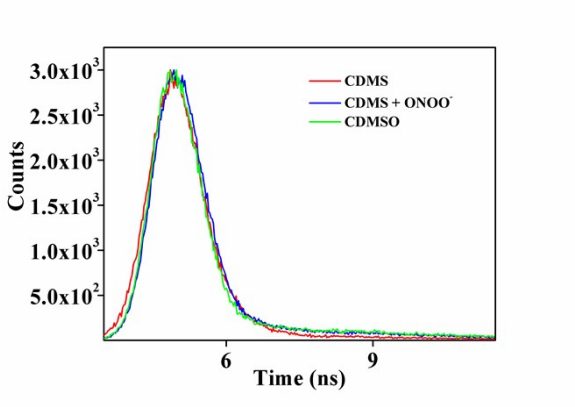


**Figure S4.**Fluorescence life time spectra of (a) **CDMS** (10 μM), (b) **CDMS** (10 μM) + $ONOO^-$ (100μM) and (c) **CDMSO**.

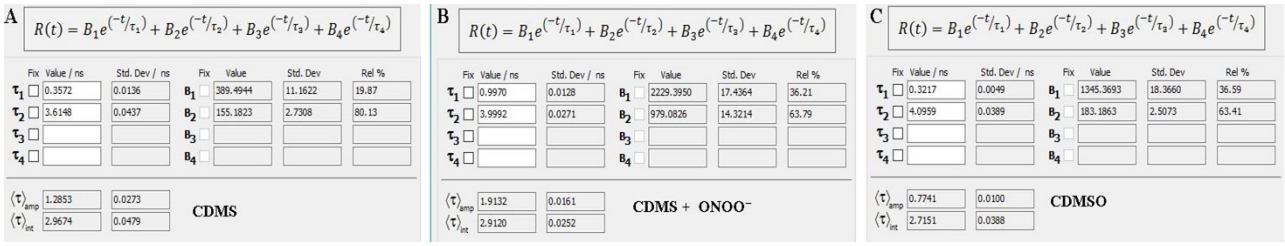


**Figure S5.** The fluorescence lifetimes of (A) **CDMS**, (B) **CDMS** + $ONOO^-$ and (C) **CDMSO.**

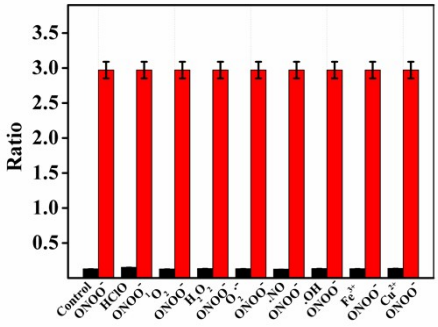


**Figure S6.** Competitive fluorescence responses of 10 μM **CDMS** in the presence of

various substrates (50 equivalents).

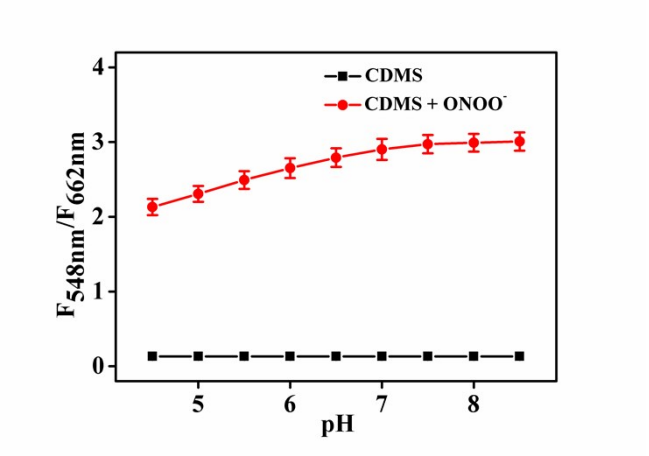


**Figure S7.** Investigation on the stability of **CDMS** (10 μM) for detecting $ONOO^-$ at different pH.

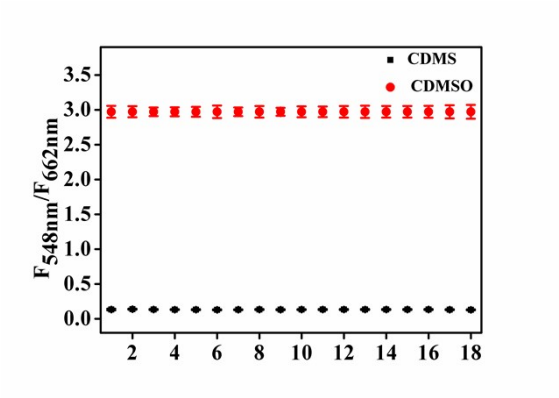


**Figure S8.** The ratio values of **CDMS** and **CDMSO** (10 μM) in different solvents: 1. Blank; 2. Ethanol; 3. Ethylene Glycol; 4. Methanol; 5. Dichloromethane; 6. Isobutanol; 7. Water; 8. Dimethyl Sulfoxide; 9. N,N-Dimethyl formamide; 10. Acetonitrile; 11. Chloroform; 12. Acetone; 13. Toluene; 14. Ethylacetate; 15. Dioxane; 16. Diethyl Ether; 17. Ethylether; 18. Acetic acid.

**HPLC assay**

To further verify the response mechanism of **CDMS** towards $ONOO^-$. High Performance Liquid Chromatography (HPLC) experiments were performed. Eluent A was $H_2O$ solution containing 5% methanol, and eluent B was pure methanol. The linear gradient condition was as follows: 0 min = 20% B, 10 min = 100% B, 15 min = 100% B using Hypersil BDS C18, 5μm, 4.6×250 mm column in Agilent 1260 infinity II. The flow rate was set at 0.1 mL · $min^{-1}$ and the monitor wavelength was constant at

254 nm. As shown in Figure S8, These results verify the response mechanism during detection process of **CDMS** towards $ONOO^-$.

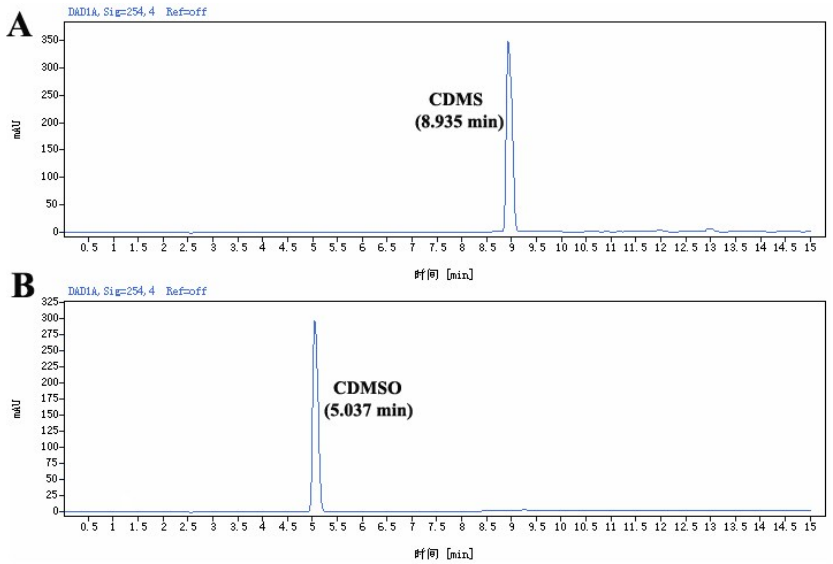


**Figure S9.** HPLC analysis of **CDMS** and **CDMSO** + $ONOO^-$ (100 μM). Data were recorded ten min after the addition of $ONOO^-$.

The cytotoxicity of probe **CDMS** against RAW 264.7 cells was assessed by the CCK-8 assay. The cells were seeded into a 96-well cell culture plate at a final density of $5\times10^3$ cells/well. After the cells were incubated for 24 h at 37 °C under 5% $CO_2$, different concentrations of **CDMS** (0 − 100 μM) were added to the wells. Subsequently, the cells were incubated for 24 h at 37 °C under 5% $CO_2$. Then, 10 μL CCK-8 solution was added to each well of the plate and incubated for 4 h at 37 °C under 5% $CO_2$. The optical density (OD) was measured at 450 nm using a microplate reader (Tecan, Austria). When the concentration of **CDMS** was 0 μM, the cell viability value was set to 100 %. Accordingly, the $IC_{50}$ value of RAW264.7 cells was calculated to be 376.58 μM. As shown in Figure S9, the cells viability indicated that our probe displayed low cytotoxicity in living cells.

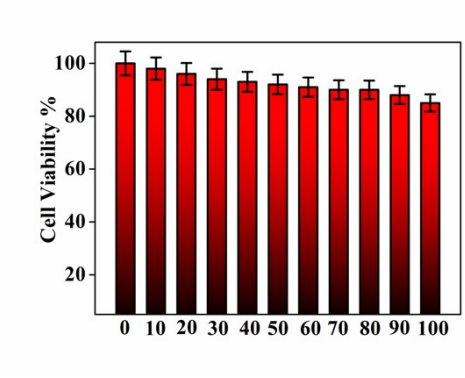


**Figure S10**. The cell viability of RAW264.7 cells with **CDMS**.

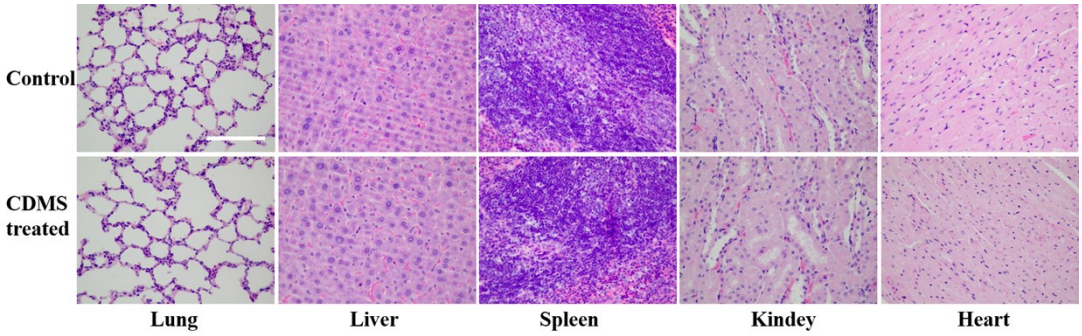


**Figure S11.** H&E staining of main organs from control mouse (above) and treated mouse after 24 hours' administration. **CDMS** (1 mM in saline, 20 μL) was intravenously injected in the tail. Scale bar: 200 μm.

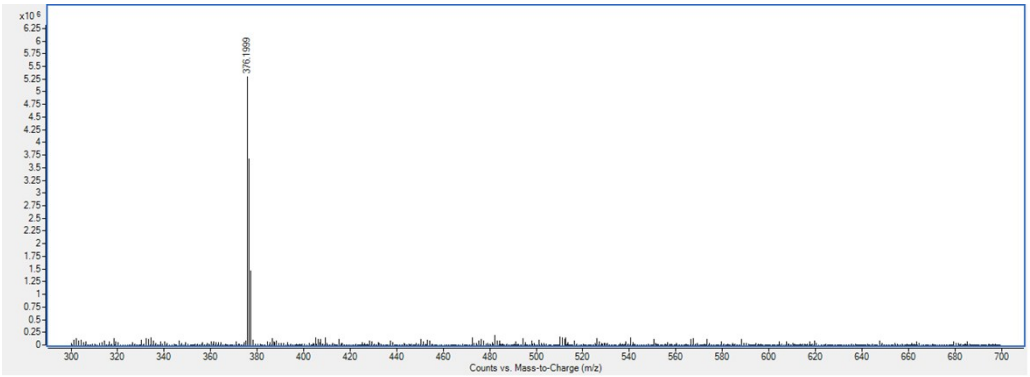


**Figure S12.** ESI-MS of **CDM**

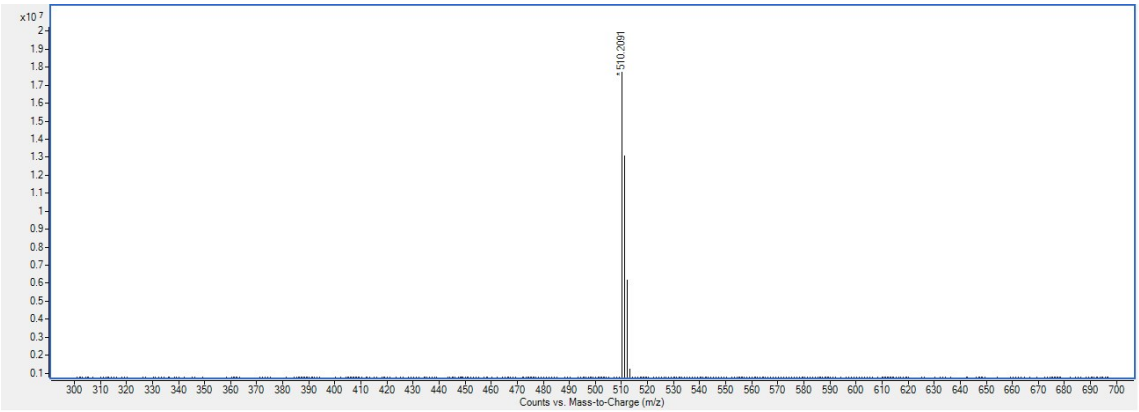


**Figure S13.** ESI-MS of **CDMS**

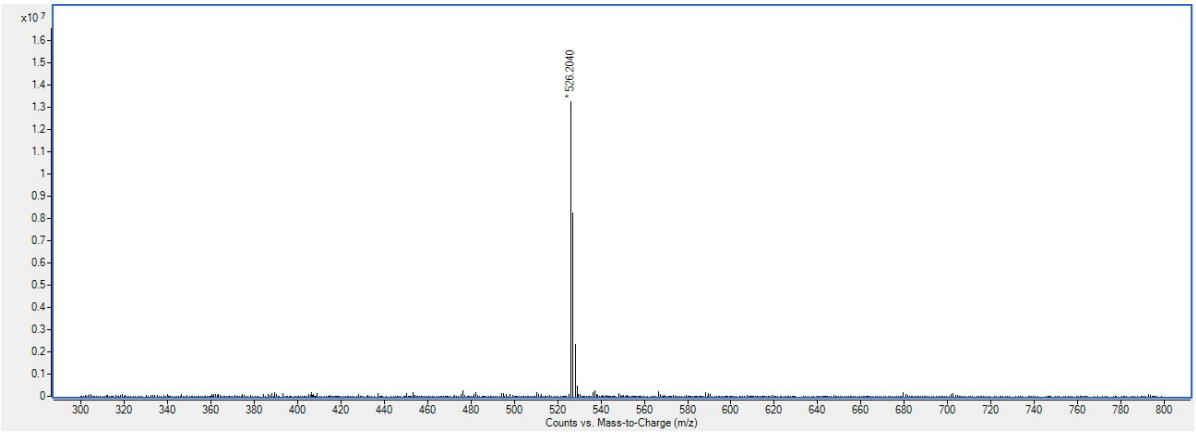


**Figure S14.** ESI-MS of **CDMSO**

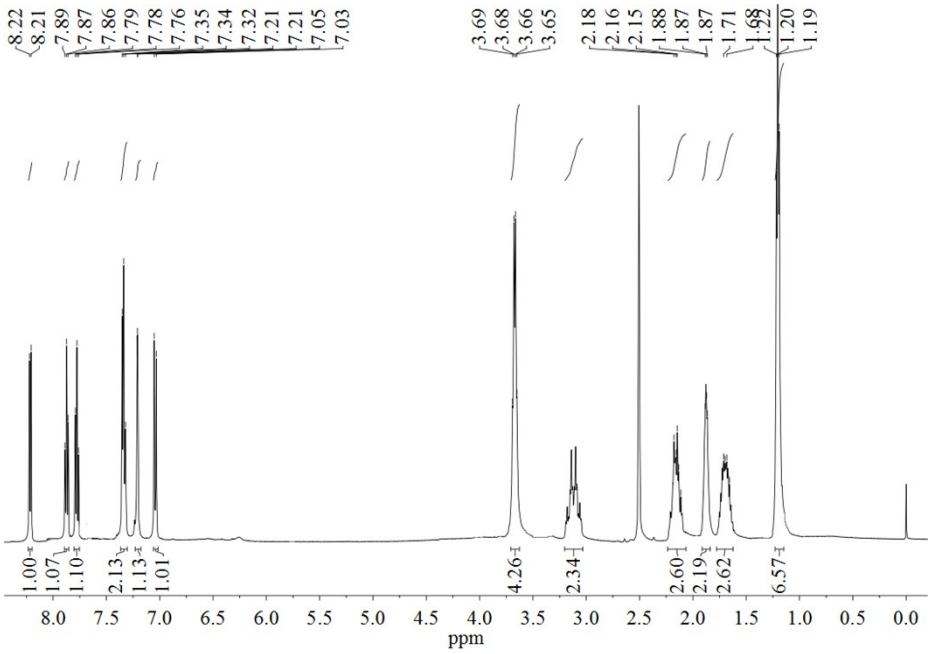


**Figure S15.** $^{1}$H NMR of **CDM** in d$^{6}$-DMSO

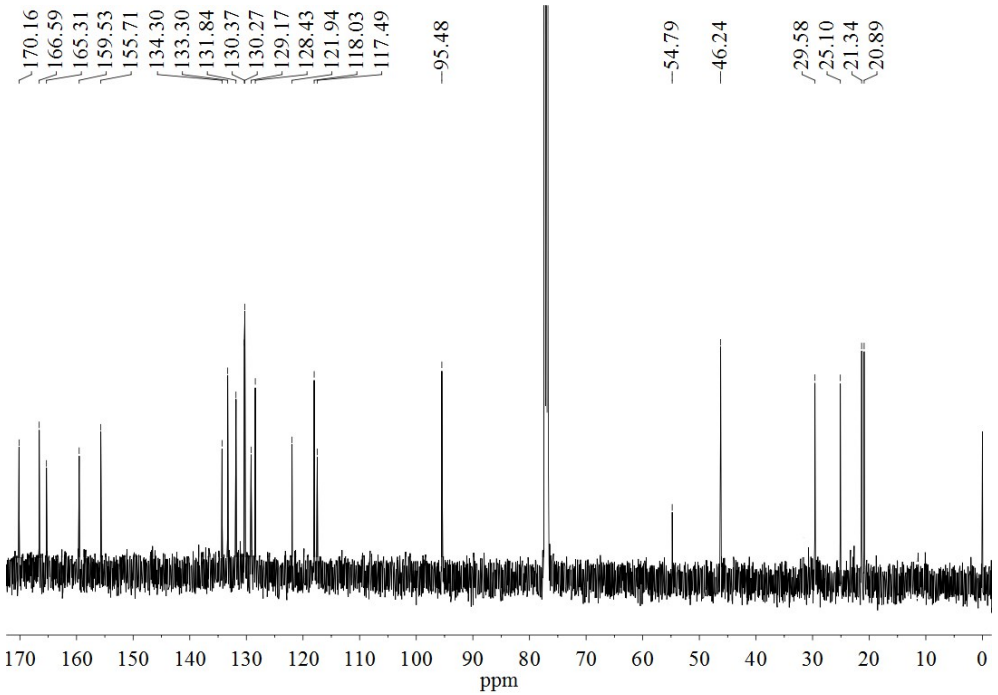


**Figure S16.** $^{13}C$ NMR of **CDM** in $CDCl_3$

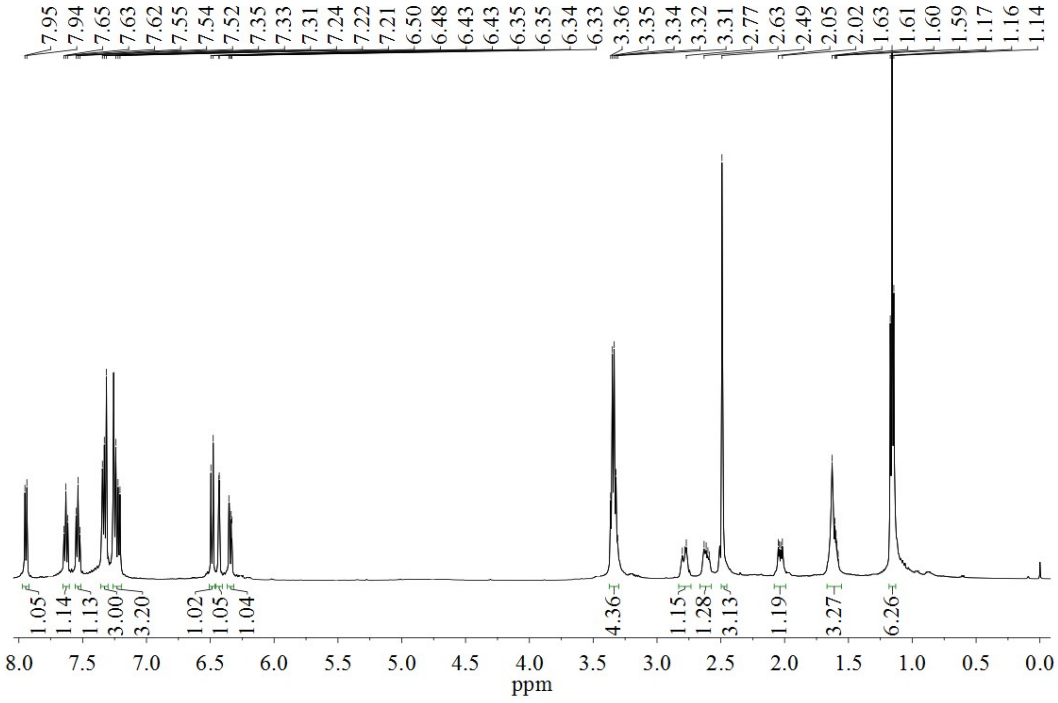


**Figure S17.** $^{1}H$ NMR of **CDMS** in $CDCl_3$

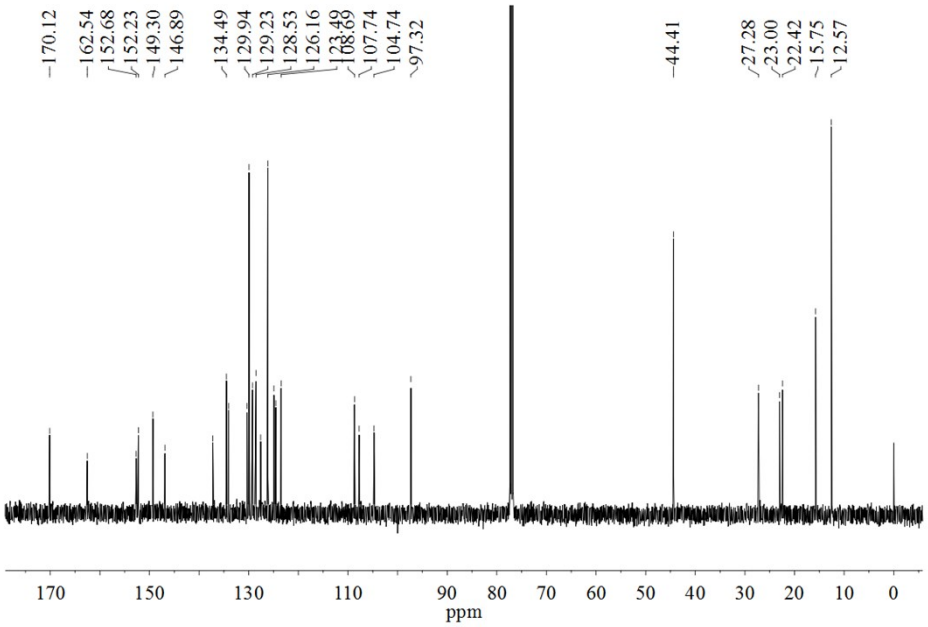


**Figure S18.** $^{13}C$ NMR of **CDMS** in $CDCl_3$

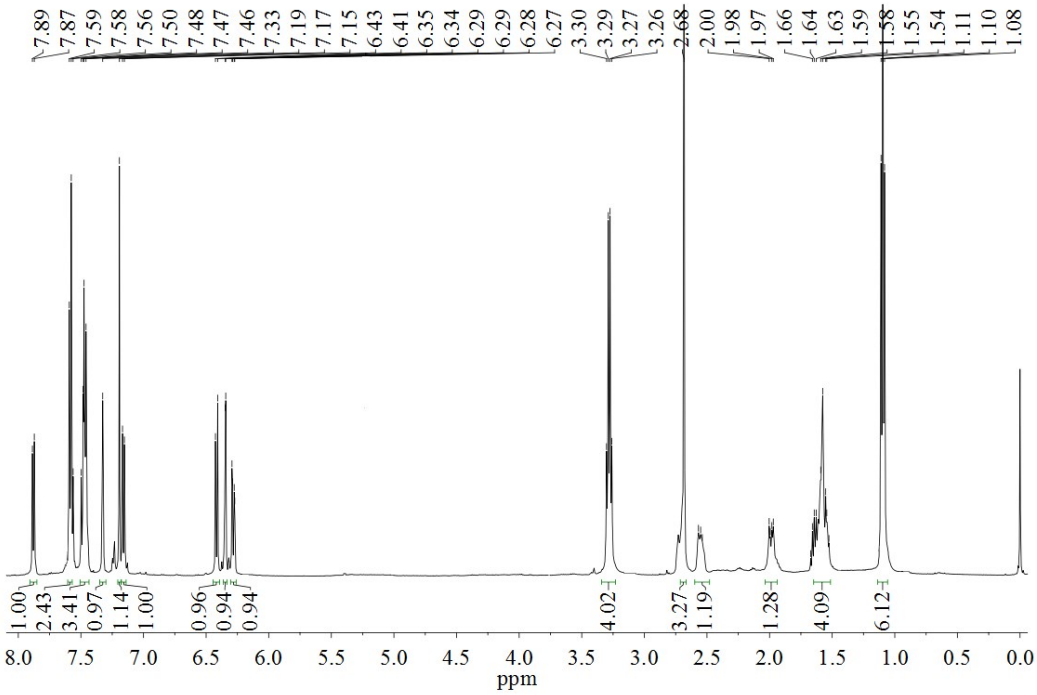


**Figure S19.** $^{1}H$ NMR of **CDMSO** in $CDCl_3$

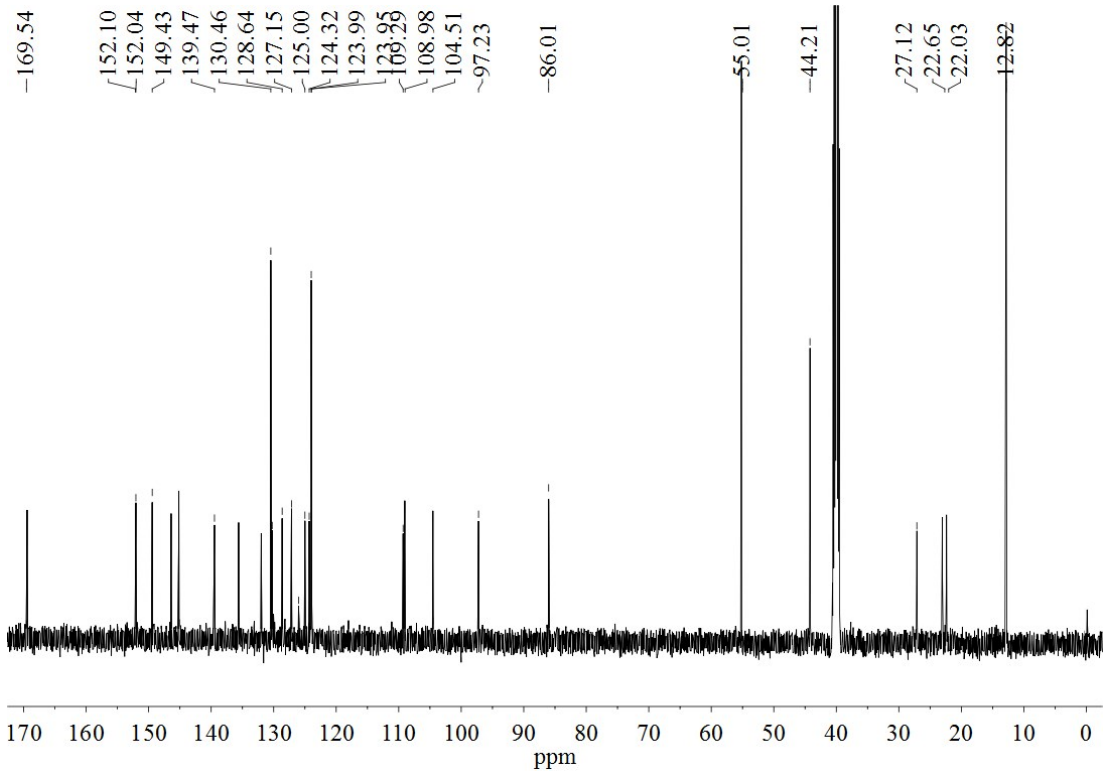


**Figure S20.** $^{13}$C NMR of **CDMSO** in d$^{6}$-DMSO

## References

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(4) Cheng, D.; Xu, W.; Yuan, L.; Zhang, X. *Anal. Chem.,* **2017**, *89*, 7693−7700.