Supplementary Information

for

Synthesis of rhodamine-labelled dieckol: Its unique intracellular localization and potent anti-inflammatory activity

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General methods

General procedures. NMR experiments were performed on a Bruker AVANCE III 700 spectrometers working at 700 MHz for proton and 176 MHz for carbon with the usual pulse sequences. ^{1}H and ^{13}C-NMR spectra were recorded in DMSO-d_6 for all compounds except for **3** ($CDCl_3$). NMR signals are reported in parts per million (δ), referenced to DMSO-d_6 and $CDCl_3$. ESI-MS and MALDI-TOF-MS data were obtained on an Agilent 1100 LC/MSD trap classic and a MALDI-TOF/TOF system (UltrafleXtreme TOF/TOF, Bruker, Germany). All reactions were carried out under argon atmosphere with dry, freshly distilled solvents under anhydrous conditions. The solutions of compounds were typically prepared from 1.0 mM stock solutions in DMSO.

Materials and reagents. All solvents and reagents used were reagent grade. Silica (230-400 mesh; Merck, Darmstadt, Germany) was used for flash column chromatography for purifications. Water used in all experiments was doubly purified by Milli-Q Systems equipment. Dulbecco's modified Eagle's minimum essential medium (DMEM) was purchased from WelGENE (Seoul, Korea). Fetal bovine serum (FBS) and antibiotics (streptomycin/penicillin) were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA). LPS (*Escherichia coli* O55:B5) and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma (St Louis, MO, USA). Antibodies to phospho-IκB (inhibitor of kappa B), IκB, phospho-p38, p38, phospho-ERK (extracellular signal-regulated kinase), ERK, phospho-JNK (c-Jun-terminal kinase), JNK were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin antibody was purchased from Sigma (St Louis, MO, USA). All chemical reagents (piperazine, HATU, DIPEA, Rhodamine B and some organic solvents) were purchased from Aldrich-Sigma, TCI, or Acros Company.

Procedure for the synthesis of compound 1

Synthesis of *N*-(6-(diethylamino)-9-(2-(piperazine-1-carbonyl)phenyl)-3H-xanthen-3-ylidene)-*N*-ethylethanaminium (3): To a stirring solution of piperazine (194 mg, 2.25 mmol) in DMF (6 mL) was added HATU (198 mg, 0.52 mmol), DIPEA (0.17 mL, 1.0 mmol) and rhodamine B (200 mg, 0.45 mmol). The reaction mixture was stirred at room temperature for 3 h and then quenched with dilute HCl. The organic layer was thoroughly washed with dilute HCl and brine, dried over Na_2SO_4, and concentrated under vacuum. The crude product was purified by silica gel column chromatography (CH_2Cl_2-MeOH, 30:1) to afford **3** (163 mg, 71%) as a dark purple solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.66 (ArH, 2H, m); 7.54 (ArH, 1H, m), 7.32 (ArH, 1H, m), 7.27 (ArH, 1H, s), 7.23 (ArH, 1H, s), 6.93 (ArH, 2H, m), 6.76 (ArH, 2H, d, J = 4 Hz), 3.61 (CH_2, 8H, m), 3.35 (CH_2, 4H, m), 2.69 (CH_2,

4H, t, J = 8 Hz), 1.32 (CH_3, 12H, t, J = 8 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 167.63, 157.94, 156.28, 155.82, 135.91, 132.34, 130.75, 130.35, 130.28, 129.89, 127.87, 114.25, 113.93, 96.41, 49.05, 46.25, 43.10, 12.77.

Synthesis of *N*-(9-(2-(4-(2-azidoacetyl)piperazine-1-carbonyl)phenyl)-6-(diethylamino)-3H-xanthen-3-ylidene)-*N*-ethylethanaminium (4): To a stirring solution of **3** (150 mg, 0.294 mmol) in DMF (5 mL) was added HATU (128 mg, 0.34 mmol), DIPEA (0.12 mL, 0.66 mmol) and 2-azidoacetic acid (33 mg, 0.32 mmol). The reaction mixture was stirred at room temperature for overnight and then the solvent was removed under vacuum. The crude product was purified by silica gel column chromatography (CH_2Cl_2-MeOH, 35:1) to afford **4** (131 mg, 75%) as a dark purple solid. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.70 (ArH, 3H, m), 7.50 (ArH, 1H, s), 7.10 (ArH, 4H, m), 6.91 (ArH, 2H, s), 4.09 (CH_2, 2H, s), 3.62 (CH_2, 8H, m), 3.13 (CH_2, 4H, m), 2.65 (CH_2, 4H, m), 1.17 (CH_3, 12H, m); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 167.27, 166.69, 157.73, 156.24, 155.77, 135.78, 132.40, 131.39, 131.03, 130.54, 128.24, 128.04, 114.91, 113.70, 96.56, 50.32, 47.31, 46.06, 38.94, 13.10; ESI-MS: *m/z* 594.50 (M^+) (594.32, calcd for $C_{34}H_{40}N_7O_3^+$).

Synthesis of 4-(4-(6-(3,5-dihydroxyphenoxy)-7,9-dihydroxy-4-(prop-2-ynyloxy)dibenzo[b,e][1,4]-dioxin-2-yloxy)-3,5-dihydroxyphenoxy)dibenzo[b,e][1,4]-dioxine-1,3,6,8-tetraol (6): To a mixture of **5** (50 mg, 0.067 mmol) and anhydrous potassium carbonate (14 mg, 0.1 mmol) in dry DMF (5 mL) was added propargyl bromide (0.04 ml, 0.08 mmol) slowly. The reaction mixture was stirred at room temperature for overnight, then the solvent was firstly evaporated under vacuum, then the residue was washed with water (5 mL); then the obtained mixture was separated by centrifuge to remove the aqueous solution. The remained solid compound was subsequently washed with acetone to remove the water; the collected solid was dried under vacuum. Then the solid was isolated by HPLC (acetonitrile-water binary gradient solvent system) to give the pure product **6** as a brown solid 20 mg, yield 38%. ^{1}H-NMR (700 MHz, DMSO-d_6): δ 9.14~9.68 (ArOH, 10H, m), 6.21(ArH, 1H, s), 6.19 (ArH, 1H, d, J = 2,7Hz), 6.16(ArH, 1H, s), 6.05 (ArH, 1H, d, J = 2.7Hz), 6.04 (ArH, 1H, d, J = 2.8Hz), 5.97 (ArH, 2H, s), 5.81 (ArH, 1H, t, J = 2.0Hz), 5.80 (ArH, 1H, d, J = 2.9Hz), 5.73 (ArH, 2H, d, J = 2.0Hz), 4.68 (CH_2, 2H, d, J = 2.2Hz), 3.57 (CH, 1H, t, J = 2.3Hz); ^{13}C-NMR (176 MHz, DMSO-d_6): δ 160.27, 158.79, 155.83, 154.17, 153.15, 151.19, 146.11, 146.06, 145.93, 145.81, 142.45, 142.37, 142.03, 141.86, 137.03, 136.81, 124.24, 124.02, 124.00, 123.09, 123.05, 122.25, 122.15, 98.49, 98.28, 98.18, 98.13, 96.32, 96.18, 94.42, 93.60, 93.39, 78.91, 78.83. 56.54; ESI-MS: *m/z* 781.07 $[M+H]^+$ (781.10, calcd for $C_{39}H_{25}O_{18}$).

Synthesis of *N*-(6-(diethylamino)-9-(2-(4-(2-(4-((3-(2,6-dihydroxy-4-(2,4,7,9-tetrahydroxydibenzo[b,e][1,4]-dioxin-1-yloxy)phenoxy)-9-(3,5-dihydroxyphenoxy)-6,8-dihydroxydibenzo[b,e][1,4]dioxin-1-yloxy)methyl)-1H-1,2,3-triazol-1-yl)acetyl)piperazine-1-carbonyl)phenyl)-3H-xanthen-3-ylidene)-*N*-ethylethanaminium (**1**): Compounds **6** (52 mg, 0.067 mmol) and **4** (40 mg, 0.067 mmol) were dissolved in DMF (5 mL), and the solution was degassed by bubbling argon for 1 h;

copper perchlorate ($Cu(ClO_4)_2$, 5.3 mg, 0.02 mmol) and soduim L-ascorbate (7.9 mg, 0.04 mmol) were dissolved in 3 mL DMF, then the solution was also degassed by argon bubbling for 1 h, the solution color changed into light yellow from brown; And then the copper (I) solution was transferred into the reaction solution through a syringe. The obtained mixture was stirred at room temperature for 48 h, then poured the reaction solution into large amount of diethyl ether, the mixture suspended and the solid was collected by centrifuge. The solid was washed by water (5 mL X 3), methanol (15 mL X 3), acetone (10 mL X 3) and DCM (15 mL X 3), then obtained solid was dried under vacuum to provide product **1** (32 mg, 35 %) as a purple solid. ^{1}H-NMR (700 MHz, DMSO-d_6): δ 9.12~9.7 (dieckol), 6.54~7.75 (rhodamine), 5.41~6.2 (dieckol), 1.1~4.36 (rhodamine); ^{13}C-NMR (176 MHz, DMSO-d_6): δ 206.57, 204.58, 172.77, 166.66, 158.86, 158.74, 157.03, 155.46, 155.08, 146.02, 135.06, 131.75, 130.40, 129.83, 127.51, 126.40, 124.60, 124.17, 123.83, 123.11, 122.10, 121.84, 121.28, 119.76, 115.59, 114.23, 112.99, 111.65, 105.39, 98.25, 95.87, 94.00, 93.55, 91.28, 87.86, 74.77, 73.22, 62.74, 45.36, 35.77, 31.26, 30.67, 18.53, 18.21, 12.41; MALDI-TOF-MS: *m/z* 1374.507 (M^+).

HPLC analysis of compound 1. To determine the purity of compound **1**, HPLC analysis was performed by reverse phase C-18 column using an isocratic elution [0.3% trifluoroacetic acid in MeOH/H_2O (3:2)]. The HPLC system consisted of a Knauer Smartline Manager 5000, two Knauer Smartline Pump 1000 and a Knauer Smartline UV Detector 2500 equipped with a phenomenex Gemini 5μ C18 110A column (150 × 4.60 mm). The flow rate was 1 ml/min, and column temperature was maintained at 30°C. UV absorption was measured at wavelength 560 nm. Compound **1** was determined to be above 90% pure by HPLC analysis with 560 nm UV detection.

Methods for biological evaluation

Two-photon fluorescence microscopy. Two-photon fluorescence microscopy images of labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with ×10, × 40 dry and ×100 oil objectives, numerical aperture (NA) = 0.30, 0.75 and 1.30. The two-photon fluorescence microscopy images were obtained with a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 740 nm and output power 1260 mW, which corresponded to approximately 5 mW average power in the focal plane.

Cell culture. RAW 264.7, a mouse macrophage cell line, was obtained from the Korean Cell Line Bank (Seoul, Korea). RAW 264.7 macrophages were cultured in DMEM medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. One day before imaging, the cells were passed and plated on glass bottomed dishes (MatTek). All the cells were maintained in a humidified atmosphere of 5/95 (v/v) of CO_2/air at 37 °C. The cells were treated and incubated with compounds **1**

and **4** at 37 °C under 5% CO_2 for 20 min. The cells were washed three times with phosphate buffered saline (PBS; Gibco) and then imaged after further incubation in colorless serum-free media for 15 min.

Colocalization experiments for rhodamine labeled dieckol (1). Raw 264.7 cells were cultured in DMEM medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). One day before imaging, cells were seeded into 24-well flat-bottomed plates. The next day, the cells colabeled with compounds **1** and **4** (2 μM) and ER tracker (1.0 μM), Mito tracker (0.2 μM) and Lyso tracker (2.0 μM) for 20 min at 37 ^{o}C under 5% CO_2 and washed with phosphate-buffered saline (PBS) three times. Confocal microscopy images of Raw 264.7 cells were obtained using a Leica TCS-SP2 confocal fluorescence microscope, 100 × objective lens. The excitation wavelength was 488 nm and the emission was collected at 560-620 nm (compounds **1** and **4**). The excitation and emission were 488 nm and 500-540 nm (ER Tracker Green), 488 nm and 660-750 nm (Mito Tracker Deep Red), 740 nm and 360-520 nm (Lyso tracker blue DND-22), Image size 75×75 μm^2.

Measurement of cell viability. Cell viability was determined colorimetrically using MTT reagent (Duchefa, Haarlem, Netherlands). RAW 264.7 cells at the exponential phase were seeded (5 × 104 cells/mL) in a 96-well plate. The cells were pre-treated with the indicated doses of dieckol or rhodamine labeled dieckol (1) for 15 hr before LPS (100 ng/ml) stimulation. 24 hr after LPS treatment, 10 μl of 5 mg/mL MTT solution was added to each well (0.1 mg/ml) and incubated for 4 hr. The supernatants were aspirated, and the formazan crystals in each well were dissolved in 200 μL of dimethyl sulfoxide for 30 min at 37°C, and the 24-well plates were read at 570 nm in a microplate reader (Bio-Rad, Hercules, CA, USA).

Measurement of NO, IL-6, and TNF-α. The nitrite concentration in the supernatants of RAW 264.7 cell cultures was determined using a NO Detection Kit (iNtRON Biotechnology, Korea), according to the manufacturer's instructions. The levels of cytokines (TNF-α and IL-6) in the supernatants of RAW 264.7 cell cultures were determined by enzyme-linked immunosorbent assay (ELISA) kits (BD Science, San Diego, CA, USA) according to the manufacturer's instructions.

Western blot. RAW 264.7 cells (1×10^6) were collected by centrifugation and washed once with cold PBS. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, protease and phosphatase inhibitor cocktail) and centrifuged. Protein concentrations in the supernatant fractions were determined using a BCA protein assay (Sigma, St. Louis, MO, USA). Then, 30 μg aliquots of protein were subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membranes (Bio-Rad). The membranes were incubated in 5% skim milk solution and then with antibodies against phosphorylated IκB, phosphorylated JNK, phosphorylated p38, or phosphorylated ERK. The membranes were washed eight times with PBS containing 0.1% Tween-20, incubated with

horseradish peroxidase-conjugated secondary antibody for 1 hr, and washed eight times with PBS containing 0.1% Tween-20. The protein bands were visualized by enhanced chemiluminescence.

Statistical analysis. Results are expressed as mean ± standard error, and compared using Student's *t*-test. $P<0.05$ was considered statistically significant.

Cell viability, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR and mass spectra

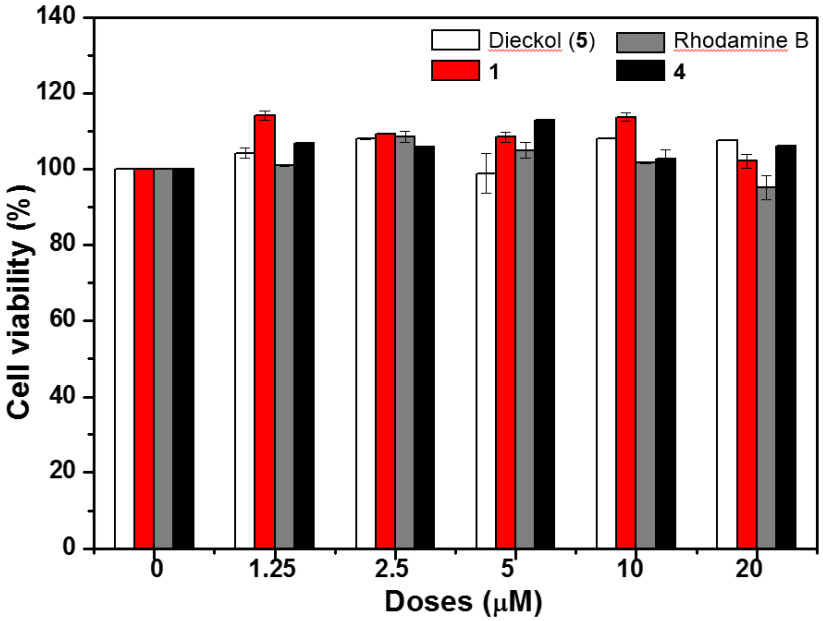


Fig. S1. Cytotoxicity of various compounds. RAW 264.7 cells were treated with the indicated doses of various compounds in the presence of LPS (100 ng/ml). Cell viability was determined by the MTT assay.

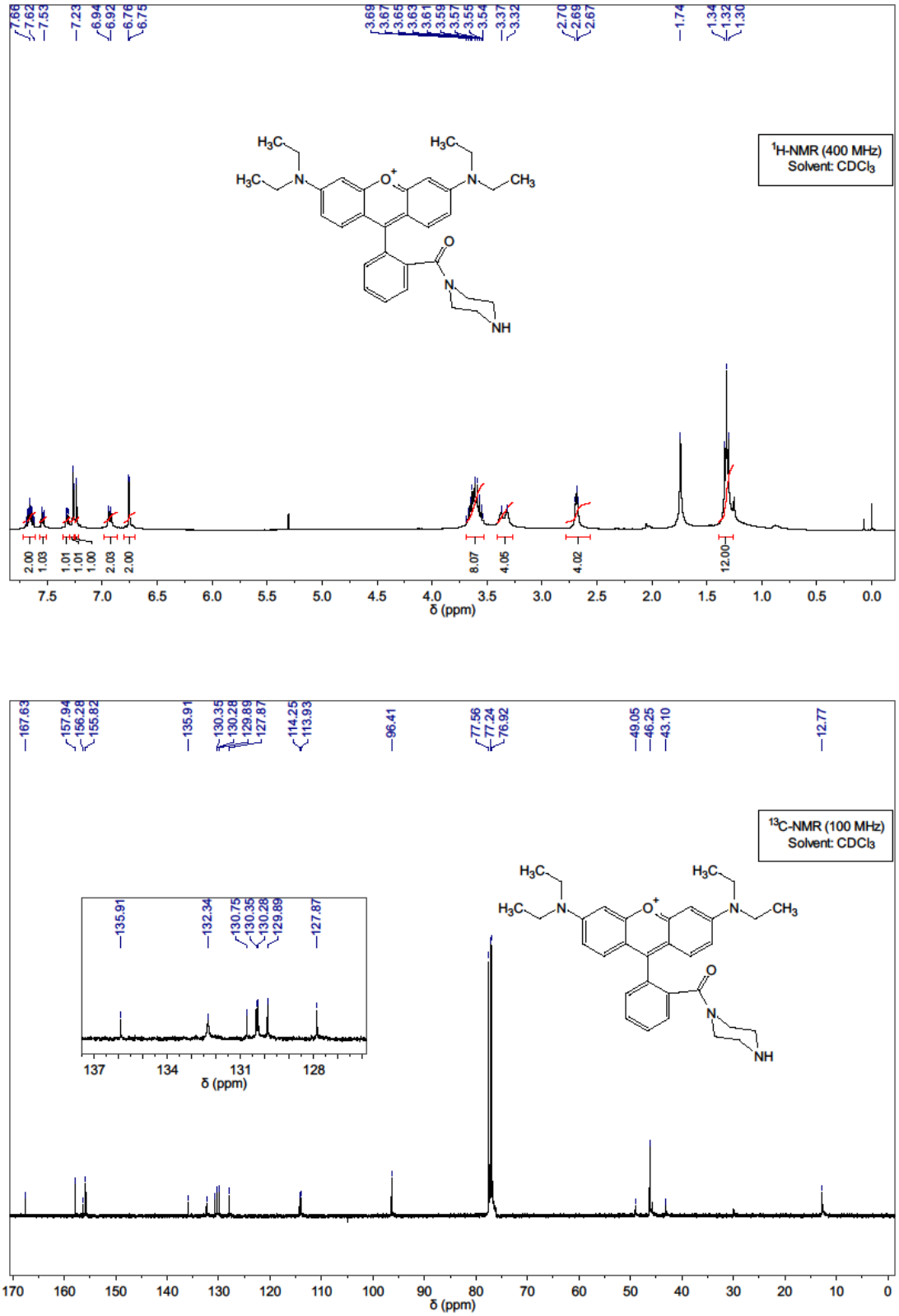


Fig. S2. ^{1}H- and ^{13}C-NMR spectra of compound **3 (**400 and 100 MHz, $CDCl_3$)

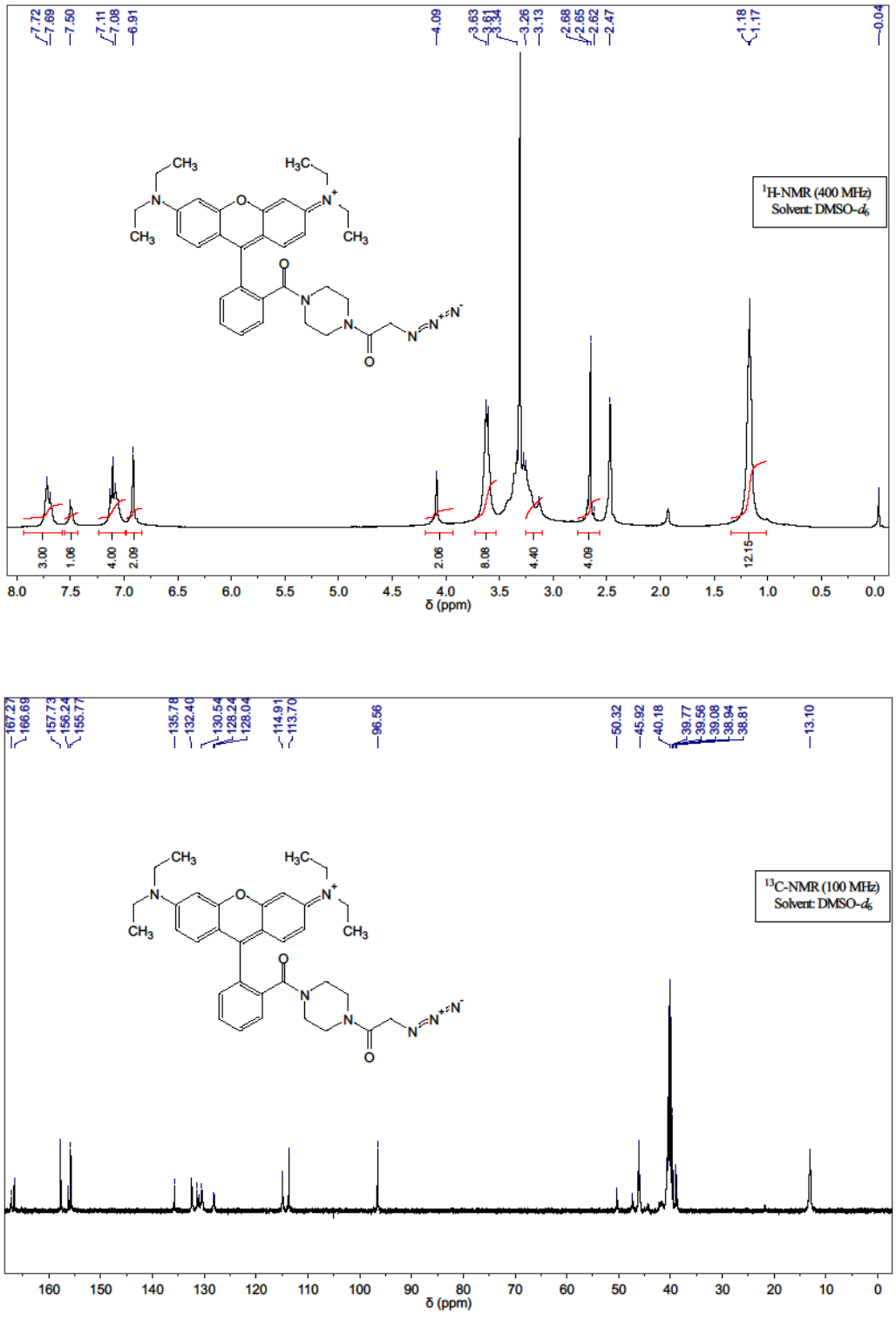


Fig. S3. ^{1}H- and ^{13}C-NMR spectra of compound **4** (400 and 100 MHz, DMSO-d_6)

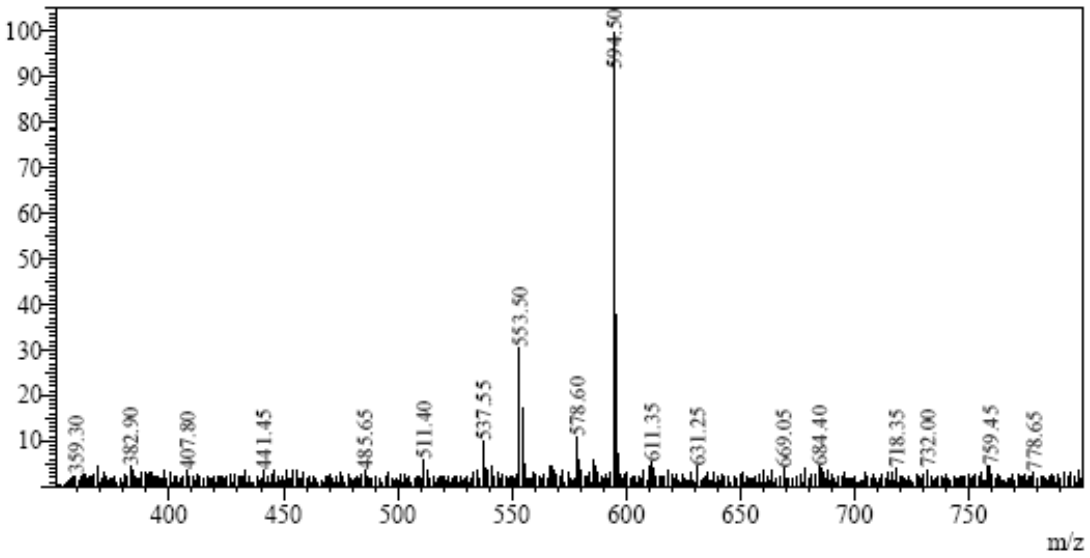


Fig. S4. ESI-MS spectra of compound **4**

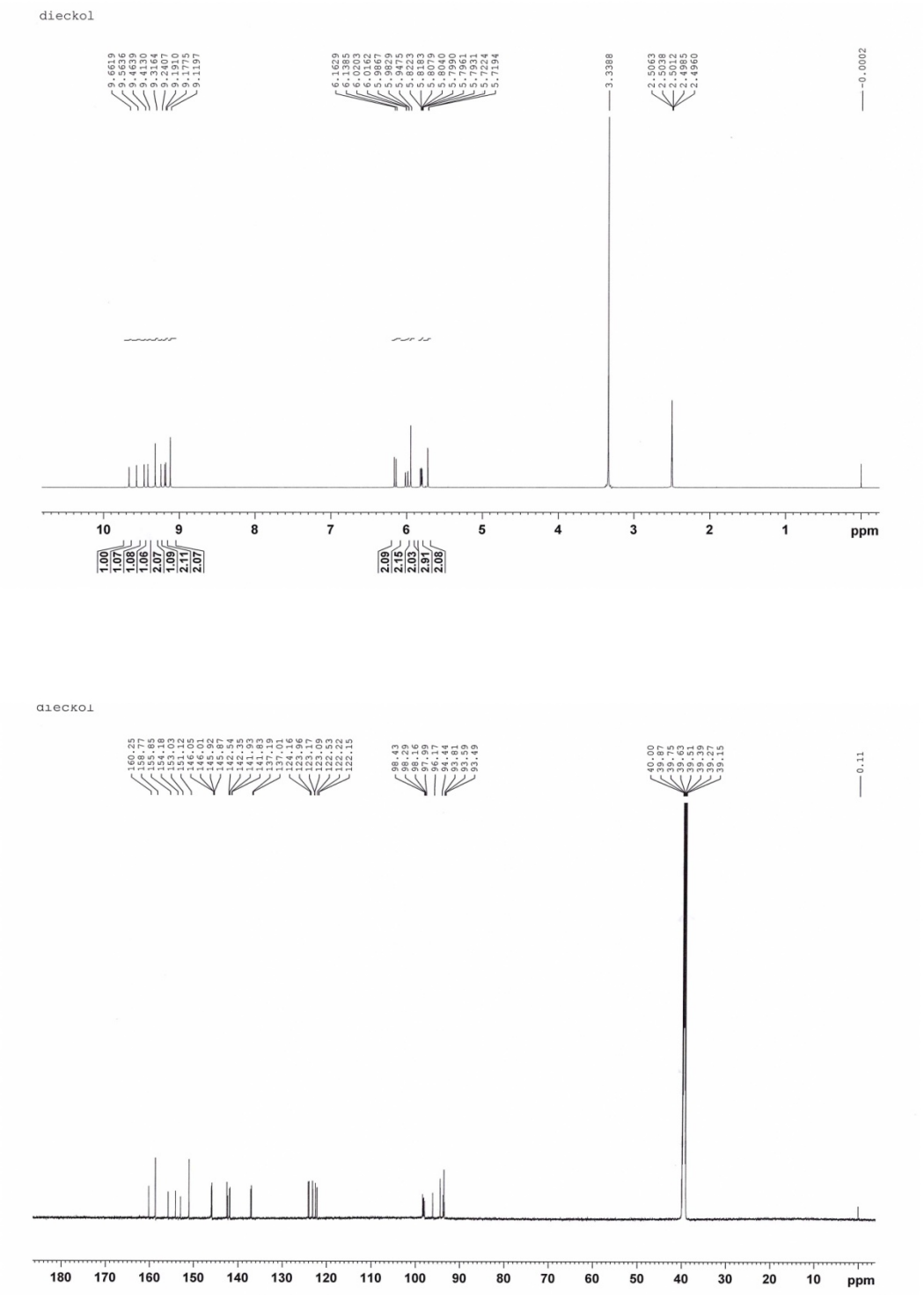


Fig. S5. ^{1}H- and ^{13}C-NMR spectra of compound **5** (700 and 176 MHz, DMSO-d_6)

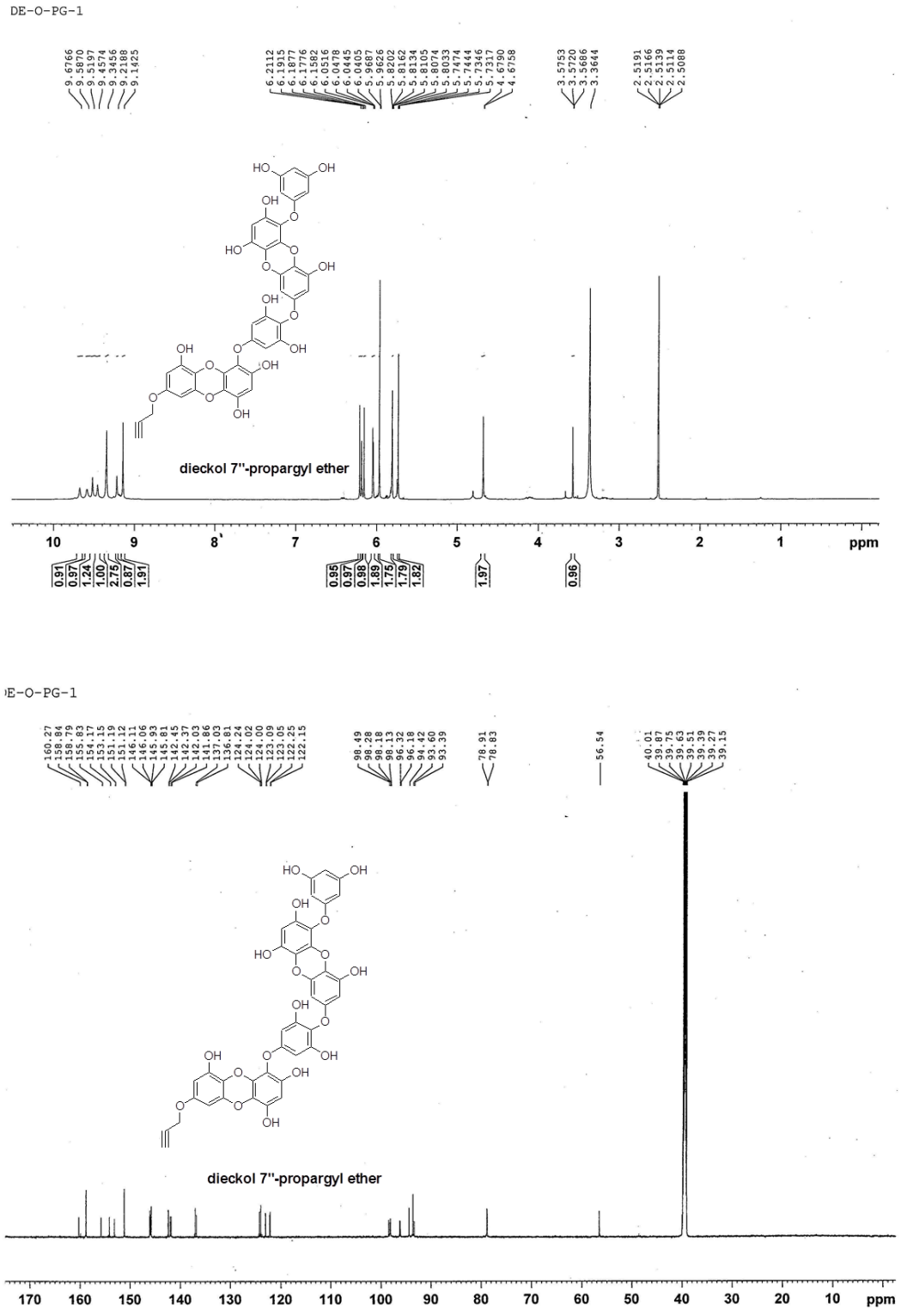


Fig. S6. ^{1}H- and ^{13}C-NMR spectra of compound **6** (700 and 176 MHz, DMSO-d_6)

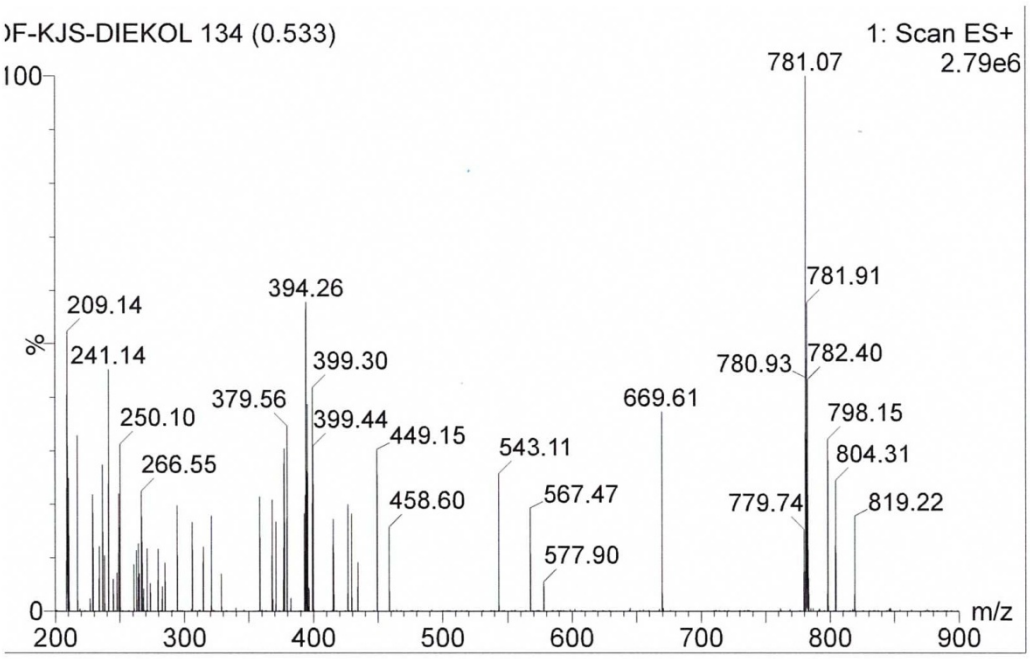


Fig. S7. ESI-MS spectrum of compound **6**

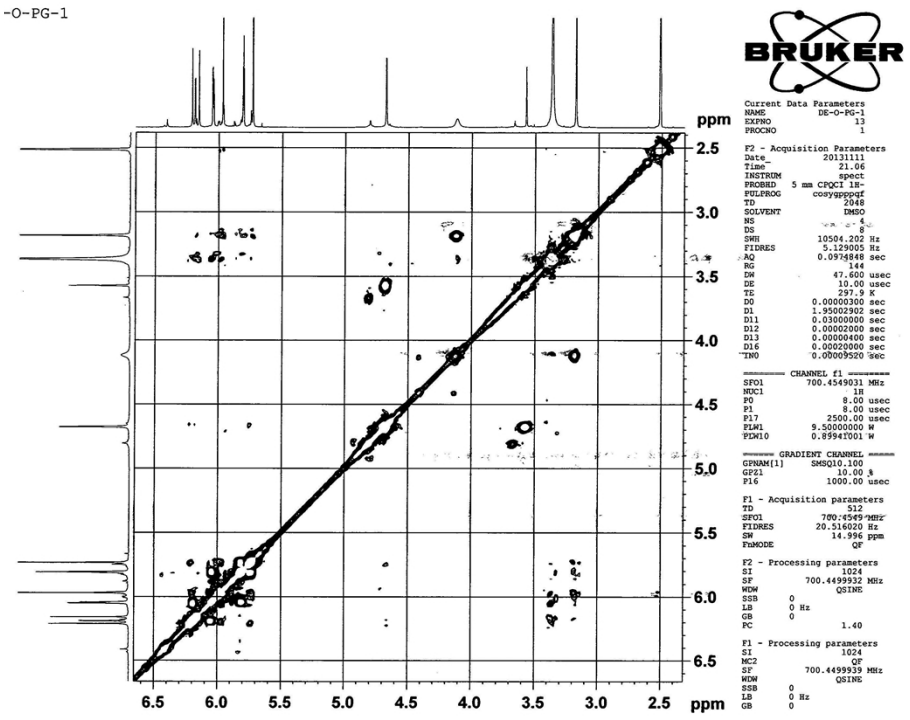
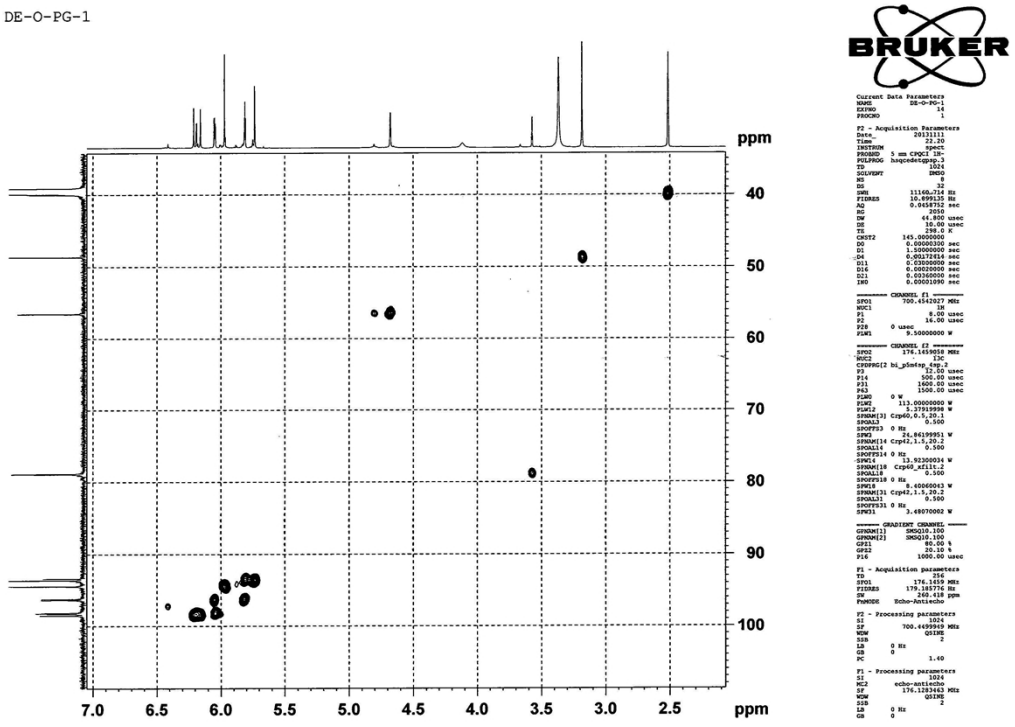


Fig. S8. ^{1}H-^{1}H COSY spectrum of compound **6** (700 MHz, DMSO-d_6)



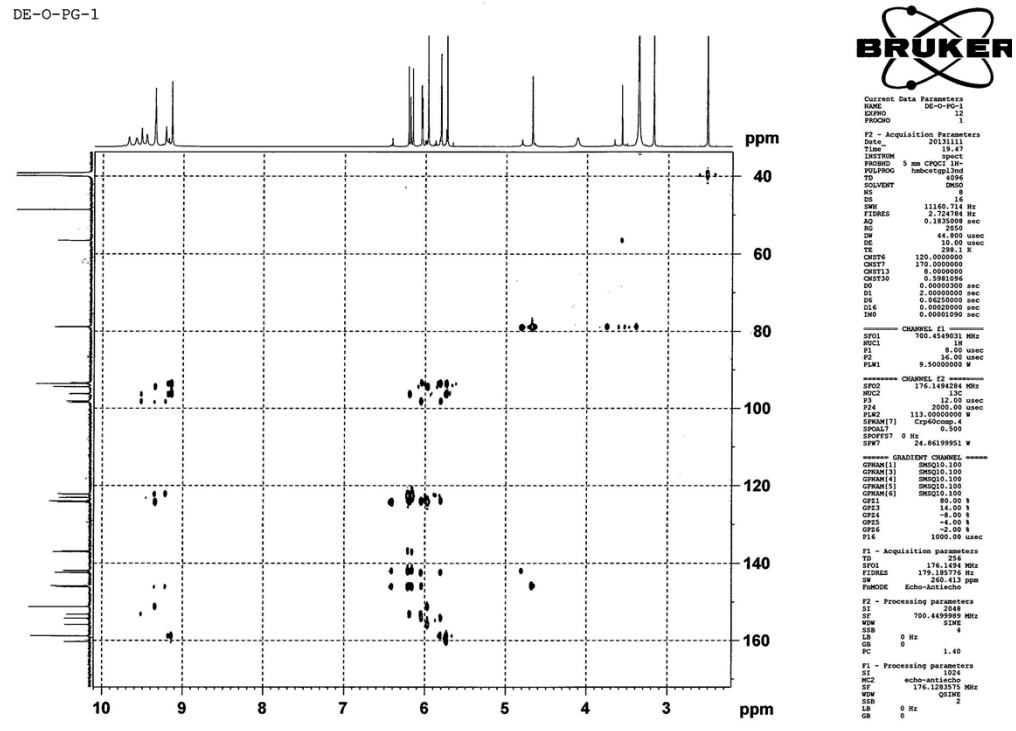


Fig. S9. HSQC and HMBC spectra of compound **6** (700 MHz, DMSO-d_6)

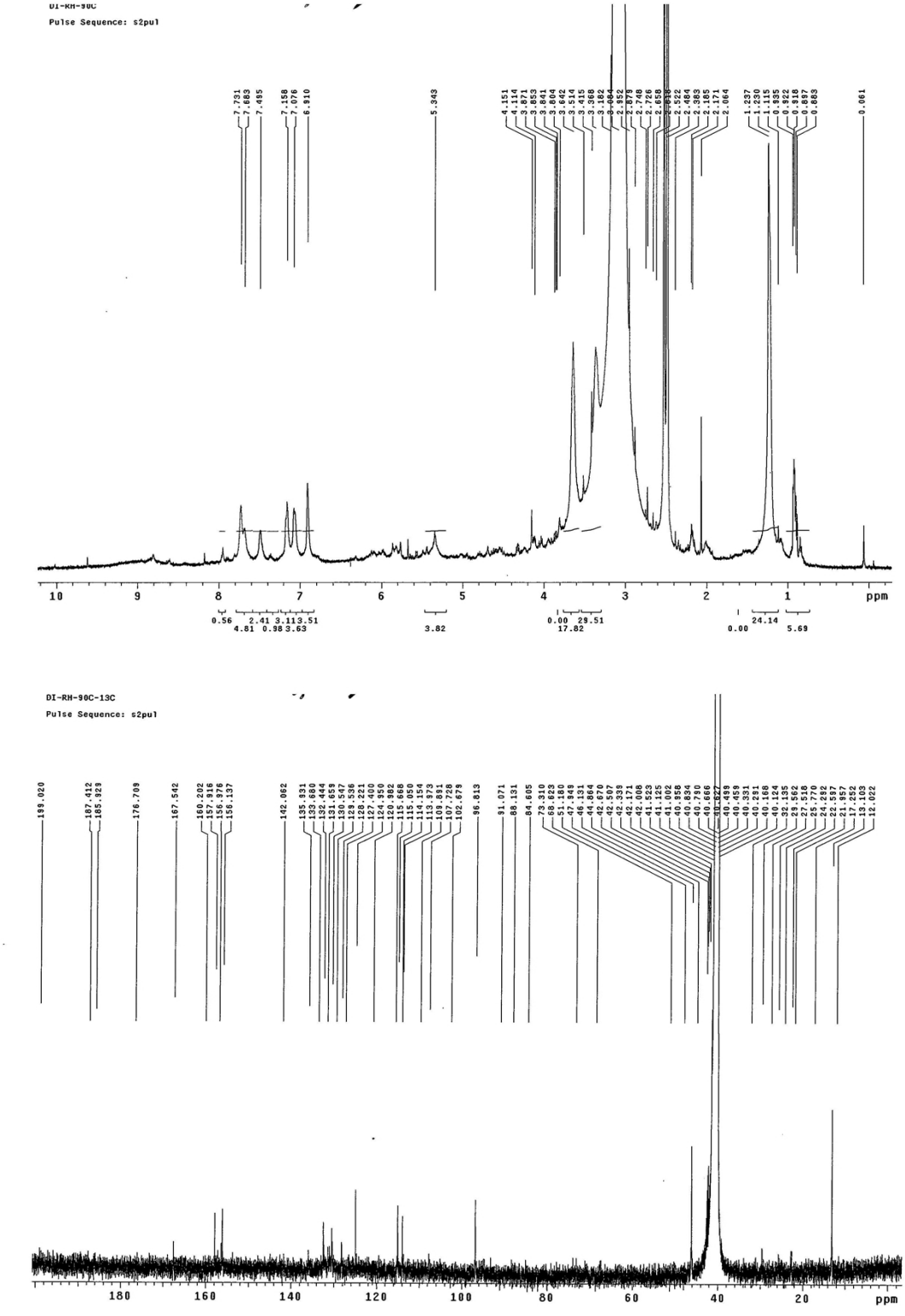


Fig. S10. ^{1}H- and ^{13}C-NMR spectra of compound **1** (500 and 125 MHz, DMSO-d_6)

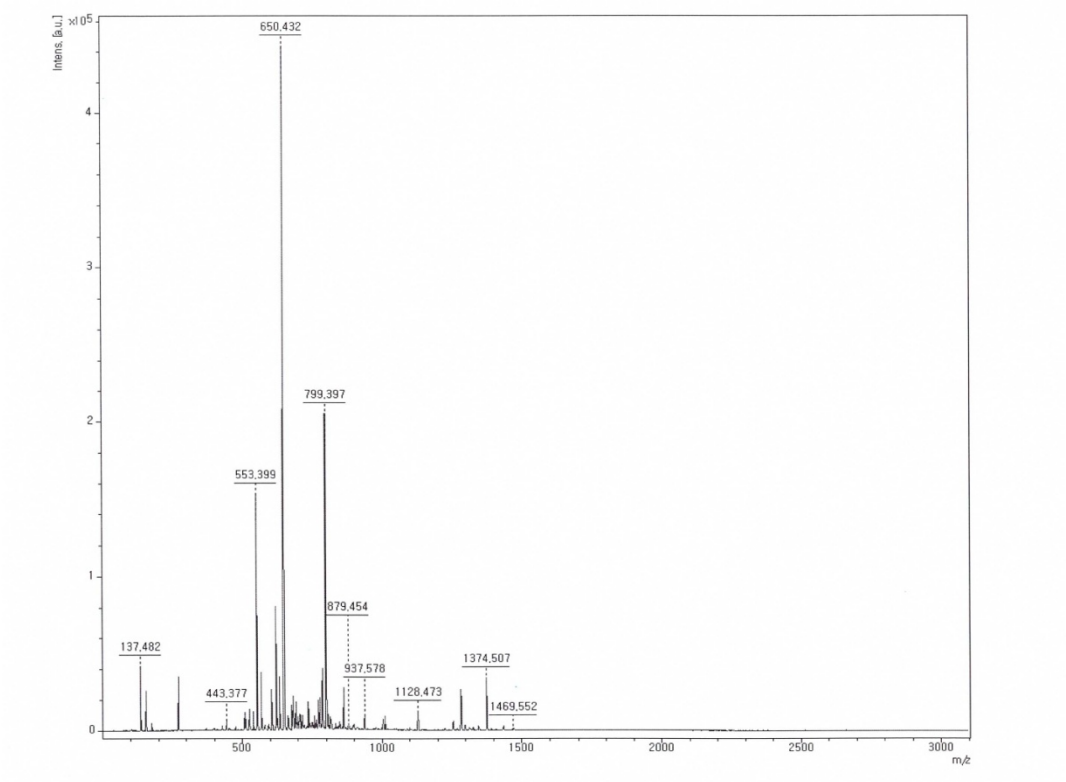


Fig. S11. MALDI-TOF-MS spectrum of compound **1**

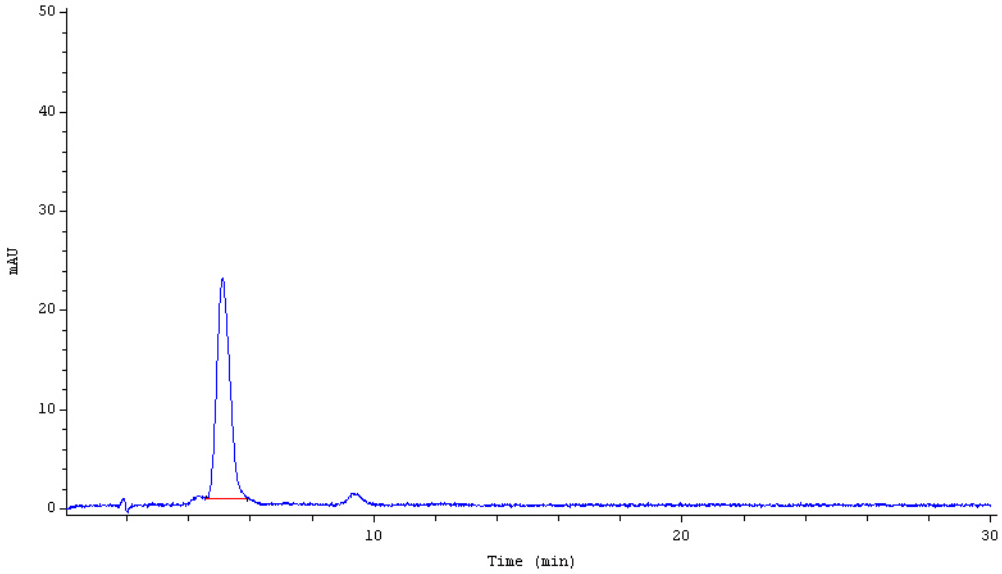


Fig. S12. HPLC chromatogram of compound **1**. HPLC analysis was carried out using an isocratic elution on a phenomenex Gemini 5µ C18 110A column (150 × 4.60 mm) at flow rate of 1.0 ml/min, with detection of wavelength 560 nm.