Supporting Information

An N-nitrosation Reactivity-based Two-photon Fluorescent Probe for Specific *in Situ* Detection of Nitric Oxide

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## Contents

1. Materials and Apparatus ................................................................. S3
2. Experimental detail ................................................................. S3
3. Synthesis and characterization .............................................. S7
4. Optical properties of NCNO ................................................. S11
5. Cytotoxicity assay .............................................................. S15
6. TP imaging study of NCNO stained tissues ................. S16
7. NMR and MS data ............................................................... S19
8. Reference ................................................................. S26
1. Materials and Apparatus Lipopolysaccharide (LPS), Interferon-γ (IFN-γ), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (Carboxyl-PTIO) and 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine (NOC-9, NO release: $t_{1/2} = 3 \text{ min (phosphate buffer at 22 °C)}$) were purchased from Sigma-Aldrich. 3-(4, 5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Aladdin Reagent, Co., Ltd (Shanghai, China). Other reagents were from commercial sources and used without further purification. All reactions were performed under argon atmosphere unless otherwise stated. Anhydrous solvents for organic synthesis were prepared by standard methods. All aqueous solutions were prepared in ultrapure water with a resistivity of 18.25 M$\Omega$•cm (purified by the Milli-Q system supplied by Millipore). Two-photon excited fluorescence data were measured by exciting with a mode-locked Ti: sapphire femtosecond pulsed laser (Chameleon Ultra I, Coherent Inc.) with a pulse width of 140 fs and repetition rate of 80 MHz. The two-photon excited fluorescence intensity was recorded on a DCS200PC Photon Counting (Beijing Zolix Instruments Co., Ltd.) with single-photon sensitivity through an Omni-λ5008 monochromator (Beijing Zolix Instruments Co., Ltd.). One-photon excited fluorescence was measured on a RF-5301 fluorescence spectrophotometer (Shimadzu Scientific Instruments Inc.). Absorption measurements were conducted on a UV2550 UV-vis spectrophotometer (Shimadzu Scientific Instruments Inc.). Two-photon microscopy was performed on a Zeiss Axio Examiner LSM 780 multiphoton laser scanning confocal microscope (Carl Zeiss, Germany).

2. Experimental detail

Theoretical Calculation Owing to the diglycol group barely contribute to $\pi$-electron delocalization system, the structures of NCNO were simplified in which contains mono-methyl substituted $p$-phenylenediamine moiety. Geometries of NCNO and its N-nitrosation product were optimized at B3LYP/6-31G (d) level with Grimme’s D3 empirical dispersion correction. The orbital graphs and levels were obtained at same level of theory.

Spectroscopic Measurements The fluorescence quantum yield was determined with fluorescein in 0.1 M NaOH solution ($\Phi=0.92$) as the reference with a literature method. Nitric oxide (NO) stock
solution (1.9 mM) was prepared by purging PBS (0.01 M, pH 7.4) with N₂ gas for 30 min and then with NO gas (99.9 %) for 30 min at 25 °C. Other ROS and RNS were prepared according to previous literature.²

(1) ClO⁻: It was prepared by dilution of commercial NaClO solution in deionized water and the concentration of the ClO⁻ stock solution was determined by measuring the absorbance at 209 nm with a molar extinction coefficient of 350 M⁻¹cm⁻¹.

(2) H₂O₂: Commercial H₂O₂ was used as H₂O₂ source. The concentration of the H₂O₂ stock solution was determined by measuring the absorbance at 240 nm with a molar extinction coefficient of 43.6 M⁻¹cm⁻¹.

(3) •OH: It was generated in the Fenton system from ferrous ammonium sulfate and hydrogen peroxide. To a solution of H₂O₂ (1.0 mM, 1.0 mL) in PBS (10 mM, pH 7.4) was added FeSO₄ solution (1.0 mM, 100 μL) at ambient temperature (stock solution 0.1 mM).

(4) O₂⁻: Solid potassium superoxide was dissolved in dry DMSO to make a superoxide radical anion solution (0.1 mM).

(5) NO₂⁻: NaNO₂ was dissolved in deionized water to make a NO₂⁻ stock solution (1 mM).

(6) OONO⁻: To a vigorously stirred solution of NaNO₂ (0.6 M, 10 mL) and H₂O₂ (0.7 M, 10 mL) in deionized H₂O at 0 °C was added HCl (0.6 M, 10 mL), followed by the rapid addition of NaOH solution (1.5 M, 20 mL). Excess hydrogen peroxide was removed by passing the solution through a short column of MnO₂. The concentration of ONOO⁻ was determined by UV analysis with the extinction coefficient at 302 nm (ε = 1670 M⁻¹cm⁻¹) according to c=A/(bε). Aliquots of the solution were stored at -20 °C for use.

**Measurement of Two-photon Cross Section** The two-photon cross section (δ) was determined by using femtosecond (fs) fluorescence measurement technique as described.³ Probe (5.0×10⁻⁶ M) was dissolved in 10 mM PBS buffer (pH 7.4, containing 10% CH₃CN), and the two-photon induced fluorescence intensity was measured at 760-900 nm by using rhodamine B as the reference, whose two-photon property has been well characterized in the literature. The intensities of the two-photon induced fluorescence spectra of the reference and sample at the same excitation wavelength were determined. The 2P absorption cross section was calculated by using δ=δ[SΦϕc]/(SΦϕc),
where the subscripts \( s \) and \( r \) stand for the sample and reference molecules. The intensity of the two-photon excited fluorescence was denoted as \( S \). \( \Phi \) is the fluorescence quantum yield, and \( \phi \) is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as \( c \). \( \delta_r \) is the 2P absorption cross section of the reference molecule.

**Experimental Calculation of Limit of Detection (LOD) Value** The detection limit was calculated based on the method reported in the previous literature. The fluorescence emission spectrum of \text{NCNO} \ was measured by eleven times and the standard deviation of blank measurement was achieved. The fluorescence intensity at 650 nm was plotted as a concentration of NO. The detection limit was calculated by using detection limit \( 3\sigma/m \): Where \( \sigma \) is the standard deviation of blank measurement, \( m \) is the slope between the fluorescence intensity versus NO concentration.

**Cytotoxicity Assay** The cytotoxicity was evaluated by MTT assay. HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) in 96-well microplates at 37 °C under 5% CO\(_2\) for 12 h. The medium was next replaced by fresh medium containing various concentrations of \text{NCNO} (0-50 µM). Each concentration was tested in three replicates. Cells were rinsed twice with phosphate buffer saline 24 h later and incubated with 0.5 mg/mL MTT reagent for 4h at 37 °C. 100 µL of DMSO was then added to dissolve formazan. The absorbance at 570 nm was measured in a microplate reader. Cell viability (%) was calculated according to following equation: Viability = (mean Abs. of treated wells/mean Abs. of control wells) ×100%.

**Cell Culture and Imaging** HepG2 cells were cultured with DMEM supplemented with 10% (v/v) newborn calf serum (Gibco), 100 U•mL\(^{-1}\) penicillin, and 100 µg•mL\(^{-1}\) streptomycin in a humidified atmosphere with 5/95 (v/v) of CO\(_2\)/air at 37 °C. One day before imaging, cells were detached with a treatment of 0.2% (w/v) trypsin-EDTA solution (Gibco) and suspended in culture media. The cell suspension was then transferred to confocal dishes to grow with adherence. For probe loading, the growth medium was replaced with 5.0 µM \text{NCNO} in culture media and incubated at 37 °C under 5% CO\(_2\) for 30 min. Next, the cells were washed with serum-free DMEM for three times. Various
concentration of NOC-9 solution was added to the dishes and incubated at 37 °C under 5% CO₂ for 30 min. Raw 264.7 cells were maintained with DMEM supplemented with 10% (v/v) newborn calf serum (Gibco), 100 U•mL⁻¹ penicillin, and 100 μg•mL⁻¹ streptomycin in a humidified atmosphere with 5/95 (v/v) of CO₂/air at 37 °C. For confocal imaging, RAW 264.7 cells at 80% confluence were harvested by scraping and transferred to confocal dishes to grow with adherence. For endogenous NO production, RAW 264.7 cells were pretreated with 20 µg/mL LPS, 200 U/mL IFN-γ and 0.5 mg/mL L-arginine for 14 hours. Then, Raw 264.7 cells were incubated with 5.0 µM NCNO at 37 °C for 30 min and washed with serum-free DMEM three times for imaging. One-photon and two-photon excited fluorescence images were obtained by Zeiss Axio Examiner LSM 780 multiphoton laser scanning confocal microscope.

**Tissue Imaging** Mouse was anesthetized and the body temperature was maintained at 37 °C for the duration of the operation. After performing a midline incision, the mouse was subjected to bilateral renal ischemia for 60 min, during which the renal arteries and veins were occluded by microaneurysm clamps. Then, the clamps were removed and the kidney restored reperfusion for various time (0-2 h). The mouse was injected 200 µL 1.0 mM NCNO via tail vein. Subsequently, the mouse kidney was isolated, the renal tissues were harvested and slices were cut to 300 µm by a vibrating-blade microtome. Then, the slices were washed with PBS three times and imaged by two-photon microscope immediately. Animal care and handing procedures were reviewed and approved by Animal Care and Use Committee of Wuhan University.

### 3. SYNTHESIS AND CHARACTERIZATION

Compound 1 was prepared by literature method. Synthesis of other compounds was described as
below.

Scheme S1. Synthesis of NCNO. Regests and conditions: (a) Piperidine, HOAc, EtOH, reflux. (b) DMSO, reflux. (c) 5%Pd/C, N$_2$H$_4$·H$_2$O, EtOH, reflux. (d) EDC·HCl, 4-dimethylaminopyridine (DMAP), CH$_2$Cl$_2$.

Synthesis of compound 2

A mixture of 0.28g (1.30 mmol) 6-(dimethylamino)-3-hydroxy-2-naphthaldehyde and 0.23g (1.60 mmol) 2, 2-dimethyl-1, 3-dioxane-4, 6-dione were dissolved in 20 mL dry absolute ethanol. Under argon atmosphere, 0.2 mL piperidine and 0.4 mL acetic acid were added dropwise to the mixture. Then the reaction mixture was refluxed at 90 ºC overnight. After cooling to room temperature, the precipitate was filtrated and washed by ethanol for twice. Compound 2 was obtained as red solid (300mg, 82%); m. p. 236.2-236.5 ºC. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.75 (s, 1H), 8.30 (s, 1H), 7.88 (d, $J = 9.3$ Hz, 1H), 7.51 (s, 1H), 7.29 (dd, $J = 9.3$, 2.4 Hz, 1H), 6.96 (d, $J = 2.2$ Hz, 1H), 3.12 (s, 6H).
Synthesis of compound 3

A mixture of 2.82 g (20 mmol) 4-fluoronitrobenzene and 10.5 g (100 mmol) 2-(2-Aminoethoxy) ethanol were dissolved in 20 mL dry DMSO. Under the argon atmosphere, the mixture was refluxed at 80 °C for 3 h. After the mixture cooling to room temperature, the mixture was poured into 100 mL water, extracted with 300 mL ethyl acetate for three times, dried with anhydrous Na$_2$SO$_4$. The solvent was evaporated under reduced pressure and the crude mixture was purified with silica gel column chromatography (ethyl acetate: petroleum ether = 1:4) to obtained yellow powder (4.30 g, 95%); m. p. 75.5-75.8 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.00 (d, J = 9.3 Hz, 2H), 7.35 (t, J = 5.4 Hz, 2H), 6.69 (d, J = 9.4 Hz, 2H), 4.64 (s, 1H), 5.36-3.49 (m, 2H), 3.46 (m, 2H), 3.41-3.30 (m, 3H).

$^{13}$C NMR (400 MHz, DMSO-$d_6$) δ 155.03, 136.05, 126.89, 111.34, 72.72, 88.98, 80.65, 42.83.

Synthesis of compound 4

A suspension of 1.20 g (5.3 mmol) compound 3, 200 mg Pd/C (5%) and 3.11 mL N$_2$H$_4$·H$_2$O in 30 mL ethanol was heat to reflux for 1 h. the mixture was filtrated to remove Pd/C and the filtrate was evaporated in vacuum. The residue was purified with silica gel column chromatography (ethyl acetate: triethylamine = 100:1) to give colorless oil (0.98 g, 94%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 6.52-6.31 (m, 4H), 4.63 (s, 1H), 4.38 (s, 3H), 3.51 (m, 4H), 3.46-3.41 (m, 2H), 3.05 (t, J = 5.8 Hz, 2H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) 140.54, 139.67, 115.93, 114.30, 72.67, 69.69, 60.70, 44.48.

Synthesis of compound NCNO

A mixture of (50 mg, 0.18 mmol) compound 2, 1.42 mg (0.22 mmol) EDC·HCl and 4 mg (0.036
mmol) DMAP in 20 mL dichloromethane was stirred under argon atmosphere for 60 min. Subsequently, 50 mg (0.22 mmol) compound 3 was added. The mixture was stirred at room temperature for 18 h. The solution was washed with saturated brine for 3 times and evaporated under vacuum. Then the residue was purified by silica gel column chromatography (methanol: dichloromethane=1:100 to 1:70) to give the crude product. The crude product was crystallized from methanol to give NCNO as orange red powder (40 mg, 67%); m. p. 226.9-227.3 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 10.43 (s, 1H), 8.90 (s, 1H), 8.39 (s, 1H), 7.91 (d, $J = 9.4$ Hz, 1H), 7.61 (s, 1H), 7.47 (d, $J = 8.8$ Hz, 2H), 7.31 (dd, $J = 9.3$, 2.4 Hz, 1H), 6.99 (s, 1H), 6.63 (d, $J = 8.8$ Hz, 2H), 5.56 (t, $J = 4.0$ Hz, 1H), 4.66 (t, $J = 5.5$ Hz, 1H), 3.59 (t, $J = 5.7$ Hz, 2H), 3.53 (dd, $J = 10.3$, 5.2 Hz, 2H), 3.50-3.45 (m, 2H), 3.20 (dd, $J = 11.4$, 5.7 Hz, 2H), 3.13 (s, 6H). $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 161.45, 158.95, 150.65, 150.38, 147.63, 145.69, 137.82, 137.28, 130.25, 127.15, 123.10, 121.22, 116.38, 115.80, 114.26, 111.98, 108.57, 103.39, 72.12, 68.84, 60.11, 42.81. DART-MS: calcd for [M+H]$^+$ C$_{26}$H$_{28}$N$_3$O$_4$ 462.2023, found 462.2024.

Scheme S2. (a) i) MeONa, (CH$_2$O)$_n$, MeOH, r.t. 16h; ii) NaBH$_4$, reflux for 1.0 h. (b) NO bubbling, CH$_3$CN-PBS ($v$: $v$, 2:1), 5 min.

**Synthesis of compound 5**

A mixture of 1.0 g (6.7 mmol) N-(4-aminophenyl)acetamide, 2.17g (40.2 mmol) MeONa and 0.402g (13.4 mmol) paraformaldehyde in 30 mL MeOH was stirred at room temperature for 16 h. Then, the mixture was slowly added 253 mg (6.7 mmol) NaBH$_4$ and refluxed at 70 °C for 1 h. After cool down to room temperature, the result mixture was treated with an aqueous solution of 1.0 M
KOH (10 mL). The mixture was extracted with CH₂Cl₂ (70×3 mL), dried with anhydrous Na₂SO₄ and concentrated in vacuo. Then the residue was purified by silica gel column chromatography (ethyl acetate: petroleum ether = 2:1 to 3:1) to give the product as white powder (692 mg, 63%); m. p. 92.4-92.7 °C. ¹H NMR (400 MHz, DMSO-dma) δ 9.52 (s, 1H), 7.37-7.17 (m, 2H), 6.54-6.36 (m, 2H), 5.41 (q, J = 4.9 Hz, 1H), 2.63 (d, J = 5.1 Hz, 3H), 1.96 (s, 3H). ¹³C NMR (101 MHz, DMSO-dma) δ 167.67, 146.65, 128.92, 121.30, 111.90, 30.51, 24.16. MS (EI): calcd for [C₉H₁₂N₂O]⁺ 164.21, found: 164.04.

**Synthesis of compound 6**

85 mg (0.52mmol) compound 5 was dissolved in 30 mL CH₃CN-PBS (pH 7.4, 10 mM) (v: v, 2:1) solution. NO gas was bubbling into the solution and the reaction was monitored by TLC analysis. After bubbling NO for 5 min, the mixture was extracted with dichloromethane, dried with anhydrous Na₂SO₄ and concentrated in vacuo. Then the residue was purified by silica gel column chromatography (ethyl acetate: petroleum ether = 2:1 to 3:1) to give the product as white powder (92 mg, 92%); m. p. 147.5-147.7 °C. ¹H NMR (400 MHz, DMSO-dma) δ 10.15 (s, 1H), 7.81-7.67 (m, 2H), 7.63-7.50 (m, 2H), 3.41 (s, 3H), 2.07 (s, 3H). ¹³C NMR (101 MHz, DMSO-dma) δ 168.95, 139.09, 137.39, 120.59, 119.97, 32.31, 24.50. MS (EI) for [C₉H₁₁N₃O₂]⁺ 193.00; [C₉H₁₁N₃O₂-NO]⁺ 163.04.

4. Optical properties of NCNO

**Table S1.** Photophysical properties of NCNO and the reaction product.

<table>
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<th>Compd.[a]</th>
<th>λ_{abs}[b]</th>
<th>φ[b]</th>
<th>λ_{em}[c]</th>
<th>Φ[c]</th>
<th>λ_{max}[d]</th>
<th>Φ[d]</th>
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510
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<th>NCNO+NO</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;</th>
<th>ε</th>
<th>Φ</th>
<th>( \sigma_{2\text{ph}} )</th>
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<td>613</td>
<td>0.008</td>
<td>830</td>
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[a] All data were measured in 10 mM PBS (pH=7.4, containing 10% CH<sub>3</sub>CN) in the absence and presence of 15 µM NO. [b, e] \( \lambda_{\text{max}} \) of the absorption and one-photon emission spectra in nm. [c] Molar extinction coefficient in cm<sup>-1</sup>-M<sup>-1</sup>. [d] Fluorescence quantum yield. [e] \( \lambda_{\text{max}} \) of the two-photon excitation spectra in nm. [f] The peak two-photon action cross section in GM, 1 GM= 10<sup>-50</sup>cm<sup>4</sup>-s-photon<sup>-1</sup>-molecule<sup>-1</sup>.

**Fig. S1** Geometries and frontier orbitals of (a) NCNO and (b) the N-nitrosation product of NCNO.

In the ball-and-stick model, carbon, nitrogen, oxygen and hydrogen are colored in grey, blue, red and white, respectively.
Fig. S2 (a) Fluorescence spectra for the reaction of 5.0 µM NCNO with excessive NO (15 µM) as a function of time. (b) Plot of fluorescence intensity versus time for the reaction of 5.0 µM NCNO and excessive NO (15 µM). Inset: photographs of NCNO before and after reacting with excess NO under the UV light at 365 nm.

Fig. S3 Fluorescence intensity of 5.0 µM NCNO reacting with 15 µM NO under anaerobic and aerobic conditions, respectively.
**Fig. S4** Relative fluorescence intensity of NCNO incubated with 50 μM various heavy metal ions (Ag⁺, Hg²⁺, Cu²⁺, Fe³⁺ and Cd²⁺) and 15 μM NO in 10 mM PBS solution (pH=7.4, containing 10% CH₃CN) for 0.5 h.

**Fig. S5** The fluorescence intensity of 5.0 μM NCNO reacting with 50 μM various ROS/RNS for 0.5h at 37 °C (grey bars), followed by reacting with 15 μM NO for another 0.5 h at 37 °C (black bars).
Fig. S6 Effect of pH on the fluorescence intensity of 5.0 µM NCNO before and after the reaction with 15 µM NO. The excitation wavelength was 475 nm, and the fluorescence intensity was measured at 613 nm in 10 mM PBS (pH7.4, containing 10% CH$_3$CN).

Fig. S7 HPLC-MS analysis of the reaction of 5.0 µM NCNO and 15 µM NO in PBS buffer for 30 min. (a) HPLC chromatogram of the reaction between NCNO and NO. (b) MS spectrum of the reaction between NCNO and NO. The peak at m/z=491 is assigned to the product of the N-nitrosation of NCNO ([M+H]$^+$=491).
Fig. S8 Two-photon excitation spectra of 5.0 µM NCNO in the absence or presence of 15.0 µM NO in 10 mM PBS solution (pH=7.4, containing 10% CH₃CN).

5. Cytotoxicity assay

Fig. S9 Viability of HepG2 cells at different NCNO concentrations for 24 hours by MTT assays.
6. TP imaging study of NCNO stained tissues

![TPM images of a fresh mouse brain slice.](image)

**Fig. S10** TPM images of a fresh mouse brain slice. The fluorescence was collected at different windows (Blue 400-500 nm, a, d, g, j; Green 500-600 nm, b, e, h, k; Red 600-700 nm, c, f, i, l) under excitation at different wavelengths (700 nm (a-c), 750 nm (d-f), 800 nm (g-i), 850 nm (j-l)) with a femtosecond laser.
**Fig. S11** Fluorescence intensities of TP images collected at different windows (Blue: 400-500 nm; Green: 500-600 nm; Red: 600-700 nm) under excitation at different wavelengths (700 nm, 750 nm, 800 nm, 850 nm).

**Fig. S12** Z-direction TP images for 20 µM NCNO stained mouse liver tissue and treated with 50 µM NO. The TP excitation wavelength was 830 nm and emission was collected at 600-700 nm. Scale bar, 50 µm.
**Fig. S13** TPM images of a fresh mouse liver slice stained with 20 µM NCNO at ~110 µm for 60 min (a), followed by treatment with 50 µM NO solution for 30 min (b). The images were collected at 600-700 nm upon the excitation at 830 nm with femtosecond laser. Scale bar, 50 µm.

**Fig. S14** TP images of 20 µM NCNO stained tissues (brain, liver, spleen, lung and kidney) at a depth of 110 µm. The tissues were incubated with 20 µM NCNO for 1 h, then treated with 50 µM NO for 0.5 h and imaged. The fluorescence intensities were collected at 600-700 nm upon the excitation at 830 nm with a femtosecond laser. Scale bar, 500 µm.
7. NMR and MS spectra

Fig. S15 $^1$H NMR spectrum of compound 2 (DMSO-$d_6$, 298K, 400 MHz).

Fig. S16 $^1$H NMR spectrum of compound 3 (DMSO-$d_6$, 298K, 400 MHz).
Fig. S17 $^{13}$C NMR spectrum of compound 3 (DMSO-$d_6$, 298K, 101 MHz).

Fig. S18 $^1$H NMR spectrum of compound 4 (DMSO-$d_6$, 298K, 400 MHz).
Fig. S19 $^{13}$C NMR spectrum of compound 4 (DMSO-$d_6$, 298K, 101 MHz).

Fig. S20 $^1$H NMR spectrum of compound NCNO (DMSO-$d_6$, 298K, 400 MHz).
Fig. S21 $^{13}$C NMR spectrum of compound NCNO (DMSO-$d_6$, 298K, 101 MHz).

Fig. S22 HR-MS spectrum of NCNO.
Fig. S23 $^1$H NMR spectrum of compound 5 (DMSO-$d_6$, 298K, 400 MHz).

Fig. S24 $^{13}$C NMR spectrum of 5 (DMSO-$d_6$, 298K, 101 MHz).
Fig. S25 EI-MS spectrum of compound 5.

Fig. S26 $^1$H NMR spectrum of compound 6 (DMSO-$d_6$, 298K, 400 MHz).
Fig. S27 $^{13}$C NMR spectrum of compound 6 (DMSO-$d_6$, 298K, 101 MHz).

Fig. S28 EI-MS spectrum of compound 6.
8. Reference


