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Electronic Supporting Information

Identifying urotropine derivatives as co-donors of formaldehyde and nitric oxide for improving antitumor therapy

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1. Experimental section and supporting figures

Materials and instrument

N_2O_4, N_2O_5 and 100%HNO_3 were prepared by reported procedure.[1,2] Other chemicals (AR grade) were obtained from commercial sources and used without further purification. B16F10 cell lines were obtained from Cell Bank of Chinese Academy of Sciences. B16F10 cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS (Gibco) and penicillin/streptomycin (1%, w/v), respectively. C57BL/6 mice and Sprague Dawley rats were purchased from Nanjing Qinglongshan Experimental Animal Center. C57BL/6 mice used in experiments were 6-8 weeks old and SD rats used in experiments were 3-4 weeks. All animal experiments were performed in accordance with the National Institute of Health Guidelines under the protocols, approved by the Ethics Committee at the Affiliated Drum Tower Hospital of Nanjing University Medical School.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance-III DRX spectrometer operatingat 500 MHz and 126 MHz respectively, using CDCl3 or DMSO-d6 as solvent. Electrospray ionization (ESI) mass spectra were recorded on a Finnigan TSQ Quantum ultra-AM mass spectrometer (Thermo Fisher Scientific, Waltham, MA USA). IR spectra were recorded with a Nicolet FTIR IS10 Spectrometer. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Merck Life Science, Shanghai, China). MTT assays were performed on a microplate reader (Tecan Group). TEM images were taken with an H-800 transmission electron microscope (Hitachi). DLS was performed on Lightsizer 500 (Anton Paar). Fluorescent images were taken by an inverted fluorescence microscope (Nikon Instruments) and a confocal laser scanning microscope (Zeiss, L710, Germany). The pH values of medium were measured by a digital pH-meter (pH 301, HANNA Instruments). In vivo images were obtained on PerkinElmer IVIS Spectrum (PerkinElmer).

Synthesis

*Synthetic procedures and identification of 3,7-dinitro-1,3,5,7-tetraazabicyclo[3.3.1]nonane (FANO**1**)*

To a stirred acetic acid (0.6 mL, 10.0 mmol) was added continuously and equivalently a solution of urotropine (HA) (1.4 g, 10.0 mmol) in acetic acid (2.3 mL, 40 mmol) and a solution of fuming nitric acid (0.9 mL, 21 mmol) in acetic anhydride (2.8 mL, 30.0 mmol) at 25℃ for 60 minutes. After that, the reaction mixture was stirred another 30 minutes at the same temperature. Then the reaction was quenched by the addition of hot water (20 mL,65℃). The precipitated product was filtered, washed with pure water and dried, FANO**1** was obtained as white powder, 79.2% yield. ^{1}H-NMR (DMSO-d_6, 500 MHz) δ = 5.50 (d, 4 H, J = 13.0 Hz), 4.94 (d, 4 H, J = 13.0 Hz), 4.12 (s, 2 H) ppm; ^{13}C-NMR (DMSO-d_6, 126 MHz) δ =

69.17, 65.57 ppm. IR (υ/cm^{-1}): 3031, 2974, 2937, 2880, 1602, 1524, 1456, 1289, 1207, 1077, 935, 816 and 780 cm^{-1}. ESI-MS: m/z [(M+H)$^{+}$]: 219.21. C5H10N6O4 calcd. C 27.53; N 38.52; H 4.62; found: C 27.58; N 38.47; H 4.65.

*Synthetic procedures and identification of 3,7-dinitroso-1,3,5,7-tetraazabicyclo[3.3.1]nonane (FANO**2**)*

To a stirred solution of urotropine (1.4 g, 10.0 mmol) and sodium nitrite (1.5 g, 22.0 mmol) in 15 mL pure water was added dropwise hydrochloric acid (3.5 mL, 40.0 mmol) at 0℃. After that, the reaction mixture was warmed to 25℃ and stirred for 2 hours. Then the reaction was quenched by the addition of ice water (30 mL) and neutralized by sodium bicarbonate. The precipitated product was filtered, washed with pure water and dried, FANO**2** was achieved as white powder, 82.0%, yield. ^{1}H-NMR (DMSO-d_6, 500 MHz,) δ = 5.80-5.74 (m, 2H), 5.51-5.46 (m, 2H), 5.36 (d, 1H), 5.23 (d, 1H), 4.46 (d, 2H), 4.26 (dd, 2H) ppm; ^{13}C-NMR (DMSO-d_6, 126 MHz) δ = 70.15, 69.45, 68.46, 68.23, 60.85, 59.97 ppm. IR (υ/cm^{-1}): 3010, 2953, 2880, 1440, 1348, 1331, 1257, 1174, 1106, 1000, 961, 944, 833, 809, 727, 611 cm^{-1}. ESI-MS: m/z [(M+H)$^{+}$]: 187.21. C10H18N6O6 calcd. C 32.26; N 45.14; H 5.41; found: C 32.29; N 45.11; H 5.44.

*Synthetic procedures and identification of compounds 3,7-Diacetyl-1,3,5,7-tetraazabicyclo[3.3.1]nonane (FANO**3**) and 3,7-Dipropionyl-1,3,5,7-tetraazabicyclo[3.3.1]nonane (FANO**4**)*

To a stirred solution of urotropine (1.4 g, 10 mmol) and anhydrous sodium acetate (2.5 g, 30 mmol) in 50 mL water was added dropwise acetic anhydride (3.1 g, 30 mmol) or propionic anhydride (3.9 g, 30 mmol) at 5℃. After stirring 1 hour, the solution was evaporated to dryness in vacuo. The wet, acid solid was then dissolved in 10 mL pure water and the solution made weakly alkaline with sodium carbonate. Next, evaporation to dryness gave a white solid, and the solid was extracted with acetone (10 x 3 mL). The combined extracts were evaporated to gain crude product.

FANO**3** was obtained as colorless cubic crystals through the recrystallization with acetone, 89.5% yield. ^{1}H-NMR (DMSO-d_6, 500 MHz,) δ = 5.48-5.45 (d, 2H), 4.90-4.87 (d, 2H), 4.71-4.68 (d, 2H), 4.20 (s, 2H), 4.17 (s, 2H), 1.89 (s, 6H) ppm; ^{13}C-NMR (DMSO-d_6, 126 MHz) δ = 170.21, 71.02, 67.39, 61.87, 21.44 ppm. IR (υ/cm^{-1}): 3062, 2926, 2885, 1633, 1354, 1213, 1206, 1083 cm^{-1}. ESI-MS: m/z [(M+H)$^{+}$]: 213.21. C9H16N4O2 calcd. C 50.93; N 26.40; H 7.60; found: C 50.98; N 26.03; H 7.71.

FANO**4** was obtained as colorless rhombic crystals through the recrystallization with acetone, 83.2% yield. ^{1}H-NMR ($CDCl_3$, 500 MHz,) δ = 5.74 (d, 2H, J=15 Hz), 5.00 (d, 2H, J=10 Hz), 4.71 (d, 2H, J=10 Hz), 4.23 (s, 2H), 4.22(d, 2H, J=15 Hz), 2.48-2.41 (m, 2H), 2.19-2.11 (m, 2H), 1.09-1.06 (m, 6H) ppm; ^{13}C-NMR ($CDCl_3$, 126 MHz) δ = 172.37, 70.61, 66.19, 62.47, 25.87, 8.83 ppm. IR (υ/cm^{-1}): 3140, 3000, 2920, 1620, 1322, 1216, 1188, 1090, 1025 cm^{-1}.

ESI-MS: m/z [(M+H)$^+$]: 241.44. C11H20N4O2 calcd. C 54.98; N 23.32; H 8.39; found: C 55.07; N 22.96; H 8.75.

*Synthesis procedures and identification of 1-Nitroso-3,5,7-trinitro-1,3,5,7-tetraazacyclooctane (FANO**5**)*

To a stirred solution of NH_4NO_3 (1.0 g, 12.5 mmol) in fuming HNO_3 (8.0 mL, 200.0 mmol) was added slowly FANO**1** (5.0 mmol) at -15℃. The reaction mixture was stirred for another 5 minutes. A solution of sodium nitrite (345.0 mg, 5.0 mmol) in water (5 mL) was added dropwise below 0℃, additional water (15 mL) was dropped at the same temperature. The precipitated product was filtered, washed with pure water and dried, FANO**5** was achieved as white powder, 82.2% yield. ^{1}H-NMR (DMSO-d_6, 500 MHz) δ = 6.36 (s, 2 H), 6.13 (s, 2 H), 6.03 (s, 2 H), 5.71 (s, 2 H) ppm; ^{13}C-NMR (DMSO-d_6, 126 MHz) δ = 65.84, 64.38, 64.05, 56.08 ppm. IR (υ/cm^{-1}): 3054, 1558, 1446, 1313, 1212, 1021, 757and 631 cm^{-1}. ESI-MS: m/z [(M+H)$^+$]: 281.06. C4H8N8O7 calcd. C 17.15; N 40.00; H 2.88; found: C 17.18; N 39.96; H 2.94.

*Synthesis procedures and identification of 1,5-Diacetyl-3-nitroso-7-nitro-1,3,5,7-tetraazacyclooctane (FANO**6**) and 1,5-dipropionyl-3-nitroso-7-nitro-1,3,5,7-tetraazacyclooctane (FANO**7**)*

To fuming HNO_3 (8.0 mL, 200.0 mmol), N_2O_4 (460.0 mg, 5.0 mmol) and NH_4NO_3 (1.0 g, 12.5 mmol) were added in succession. Then FANO**3** or **4** (5.0 mmol) was added slowly under vigorous stir at -15℃ and let reacting for 10 minutes. The reaction was quenched by ice water (30.0 mL) and neutralized by sodium bicarbonate. The precipitated product was filtered, washed with pure water and dried, FANO**6** and **7** were obtained respectively.

FANO**6** was obtained from FANO**3** as white solid, 92.5% yield. ^{1}H-NMR (DMSO-d_6, 500 MHz,) δ = 6.99-5.12 (m, 8 H) 2.27-2.20 (m, 6H) ppm; ^{13}C-NMR (DMSO-d_6, 126 MHz) δ = 171.12, 170.02, 65.17, 64.66, 64.16, 63.96, 61.76, 60.77, 60.48, 58.99, 58.59, 58.14, 55.05, 54.76, 22.40, 21.90 ppm. IR (υ/cm^{-1}): 3050, 1685, 1505, 1445, 1370, 1265, 1141, 965 cm^{-1}. ESI-MS: m/z [(M+H)$^+$]: 275.15. C8H14N6O5 calcd. C 35.04; N 30.65; H 5.15; found: C 35.09; N 30.58; H 5.18.

FANO**7** was obtained from FANO**4** as white solid, 94.4% yield. ^{1}H-NMR ($CDCl_3$): 5.93-5.09 (m, 8H), 2.61-2.46 (m, 4H), 1.21-1.16 (m, 6H) ppm. ^{13}C-NMR (DMSO-d_6): 174.11, 173.47, 63.84, 63.37, 62.72, 61.73, 61.11, 60.33, 58.95, 58.38, 57.07, 56.58, 54.79, 54.59, 26.01, 25.46, 8.70, 8.57. IR (υ/cm^{-1}): 2995, 2944, 1683, 1525, 1435, 1261, 1049, 810, 605 cm^{-1}. ESI-MS: m/z [(M+H)$^+$]: 303.12. C10H18N6O5 calcd. C 39.73; N 27.80; H 6.00; found: C 39.75; N 27.76; H 6.02.

*Synthetic procedures and identification of 1,5-Diacetyl-3,7-dinitro-1,3,5,7-tetraazacyclooctane (FANO**8**) and 1,5-Dipropionyl-3,7-dinitro-1,3,5,7-tetraazacyclooctane (FANO**9**)*

To solution of N_2O_5 (540.0 mg, 5 mmol) in anhydrous HNO_3 (10.5 mL, 250.0 mmol) was added slowly FANO**6** or **7** (5 mmol) at 0℃. After that, the reaction mixture was warmed to 25℃ and stirred for another 30 minutes. Then the reaction was quenched by the addition of ice water (30 mL). The precipitated product was filtered, washed with water and dried, FANO**8** and **9** were obtained respectively.

FANO**9** was obtained from FANO**6** as white solid, 89.5% yield. **1**H-NMR (DMSO-d_6, 500 MHz,) δ = 5.58-5.45 (m, 8 H) 2.27 (s, 6H) ppm; ^{13}C-NMR (DMSO-d_6, 126 MHz) δ = 170.24, 64.26, 64.02, 59.90, 59.75, 21.57 ppm. IR (υ/cm^{-1}): 3120, 1678, 1511, 1421, 1389, 1352, 1280, 1181, 996, 973, 944, 830 cm^{-1}. ESI-MS: m/z [(M+H)$^+$]: 291.43. C8H14N6O6 calcd. C 33.11; N 28.96; H 4.86; found: C 33.16; N 28.90; H 4.89.

FANO**10** was obtained from FANO**7** as white solid, 86.4% yield. ^{1}H-NMR (DMSO-d_6): 5.52 (s, 8H), 2.63 (q, J=7.2 Hz, 4H), 1.01 (t, J=5.0 Hz) ppm. ^{13}C-NMR (DMSO-d_6): 173.05, 62.50, 59.69, 25.15, 8.95 ppm. IR (υ/cm^{-1}): 2983, 2943, 1669, 1531, 1271, 762 cm^{-1}. ESI-MS: m/z [(M+H)$^+$]: 319.23. C10H18N6O6 calcd. C 37.74; N 26.40; H 5.70; found: C 37.78; N 26.34; H 5.73.

Preparation and characterization of lipo@FANO1 and lipo@FANO2

Lipo@FANO**1** and Lipo@FANO**2** were prepared by thin lipid film hydration and sequential extrusion method. HSPC, cholesterol, DSPE-mPEG2000, and FANO**1** or FANO**2** (5:3:1.5:1, w/w) were dissolved in the mixture of chloroform and methanol (1:2, v/v). The solution was evaporated by rotary evaporation to form a homogeneous film. The film was hydrated with 1 mL deionized water at 60 °C and sonicated for 5 min in an ice bath. Subsequently, the liposome suspension was extruded sequentially 21 times through polycarbonate membrane filters with pore sizes of 800, 400, 200, and 100 nm. Products were purified by Sephadex G50 column. The sizes and morphology were determined by TEM. The size distribution and zeta potential were measured using the Anton Paar Lightsizer 500.

In vitro cytotoxicity

For MTT assay, cancer cells including B16F10 and 4T1, as well as normal cells NCTC 1469 (5 x 10^3 cells per well) were seeded in 96-well plates in complete medium overnight, followed by incubating with different concentrations of SNAP, urotropine, FANO**1**-**9**, lipo@FANO**1**, and lipo@FANO**2** for 24 h. MTT solution (20 μL) was added to the culture medium, and the medium was removed after 4 h incubation, followed by adding dimethyl sulfoxide (200μL). The absorbance of individual well was measured at 570 nm using the microplate reader.

NO release evaluation

Total NO production was estimated by measurement of the accumulation of nitrite and nitrate using the Griess reagent in the Total Nitric Oxide Assay Kit (Beyotime, China). The B16F10 cells (5 x 10^3 cells per well) were seeded in 96-well plates in complete medium overnight, followed by incubating with different concentrations of FANO**1**-**9**, lipo@FANO**1**, and lipo@FANO**2** for 2 h or 8 h. Nitrate was measured after enzymatic conversion to nitrite

by nitrate reductase. Briefly, the lysis buffer of every sample was added in duplicate wells in a 96-well plate at room temperature. The mixture was incubated with 5 μL of nicotinamide adenine dinucleotide phosphate (NADPH), 10 μL of flavin adenine dinucleotide (FAD) and 5 μL of nitrate reductase for 30 min at 37°C. Then, 10 μL of lactate dehydrogenase (LDH) buffer and 10 μL of LDH were added in the above mixture for another 30 min at 37 °C. Finally, 50 μL of Griess reagent I and 50 μL of Griess reagent II were added into the wells before incubation for 10 min. The absorbance of individual well was measured at 540 nm using the microplate reader. Concentrations were calculated using a standard curve (80, 60, 40, 20, 10, 5 and 2 μM sodium nitrite).

Stock solutions of FANO**1** and FANO**2** (1 mM) were prepared in DMSO. A typical mixture consisted of FANO**1** or FANO**2** (1 mM) prepared by mixing FANO**1** or FANO**2** (12.5 mL of stock) with phosphate buffer (47.5 mL, 10 mM, pH=4.5, 5.5, 6.5 or 7.4) at 37°C. An aliquot of the mixture (10 mL) was injected into a Sievers NOA 280i nitric oxide analyzer (GE Instruments) in argon as the carrier gas.

Formaldehyde release evaluation

Formaldehyde Assay Kit (Sigma-aldrich, the USA) was used to evaluate the formaldehyde release. The B16F10 cells (5 x 10^3 cells per well) were seeded in 96-well plates in complete medium overnight, followed by incubating with different concentrations of FANO**1-9**, lipo@FANO**1**, and lipo@FANO**2** for 2 h or 8 h. Formaldehyde was measured after deproteinated and neutralized the protein in samples. Add 50 μL of 10% TCA per 100 μL of sample. Vortex and centrifuge for 5 minutes at 14,000 rpm. Transfer 100 μL of clear supernatant to a clean plate and neutralize with 25 μL of Neutralizer. Briefly, 50 μL of Reaction Mix was transferred to each of the sample and tapped plate briefly to mix. Incubate the samples at room temperature for 30 minutes protected from light. The fluorescence intensity of individual well was measured at E_x/E_m = 370/470 nm. Concentrations were calculated using a standard curve (0, 30, 60 and 100 μM of formaldehyde).

Stock solutions of FANO**1** and FANO**2** (1 mM) were prepared in DMSO. A typical mixture consisted of FANO**1** or FANO**2** (1 mM) prepared by mixing FANO**1** or FANO**2** (12.5 mL of stock) with phosphate buffer (47.5 mL, 10 mM, pH=4.5, 5.5, 6.5 or 7.4) at 37°C. Then we evaluated the formaldehyde release using formaldehyde Assay Kit, and the experiment method was the same described above.

Evaluation of FA and NO synergistic effect

The B16F10 cells (5 x 10^3 cells per well) were seeded in 96-well plates in complete medium overnight, then pretreated with hemoglobin at the indicated concentrations (0, 1, 5, 10, or 20 μM) for 1 h and then treated with FANO**1** or FANO**2** (0.25 μM) for 24 h. The cell viability was determined by the MTT assay.

The B16F10 cells (5 x 10^3 cells per well) were seeded in 96-well plates in complete medium overnight, then pretreated with or without 10 μM hemoglobin (H) for 1 h, followed by incubation with FANO**1** or FANO**2** (0.25 μM) for 2 h, and the content of FA and nitrite was determined by the Formaldehyde Assay Kit and Total Nitric Oxide Assay Kit, respectively.

Measurement of intracellular ROS levels.

B16F10 cells in 24-well plates (2.5 x 10^5 cells per well) were grown overnight to reach 70-90% confluence, followed by incubating with SNAP (0.25 mM), urotropine (0.25 mM), FANO**1** (0.25 mM), FANO**2** (0.25 mM), lipo@FANO**1** (0.05 mM of FANO**1**), lipo@FANO**2** (0.25 mM of FANO**2**) for 10 h. After washing three times with PBS, the cells were incubated with carboxy-H2DCFDA (1 μL, 10 mM) for 10 min in fresh culture medium (0.5 mL), and imaged by inverted fluorescence microscope (Ex/Em = 495/529 nm).

Measurement of mitochondrial membrane potential.

The mitochondrial membrane potential changes were measured using a mitochondrial detection kit (Beyotime C2006). 5 × 10^5 cells per well were seeded into confocal dishes (Costar, Washington, DC) and cultured at 37 °C for 24 h, followed by replacing with saline, CCCP (0.01 mM), urotropine (0.25 mM), SNAP (0.25 mM), FANO**1** (0.25 mM), FANO**2** (0.25 mM), lipo@FANO**1** (0.05 mM of FANO**1**) and lipo@FANO**2** (0.25 mM of FANO**2**) for 10 h. Depolarization of the mitochondrial membrane is characterized by a shift from red fluorescence to green fluorescence. The simultaneous measurement of fluorescence was performed by inverted fluorescence microscope. The green fluorescent emission (E_x/E_m = 514/529 nm) of J-monomerand and red fluorescent emission (E_x/E_m = 585/590 nm) of J-aggregate were measured and analyzed using ImageJ programs.

Western blot assay

B16F10 cells were incubated with 300 μL RIPA buffer supplemented with protease inhibitor cocktail and phenylmethylsulfonyl fluoride on the ice for 15 min, and whole proteins were extracted and quantified using bicinchoninic acid assay. After boiling at 95 °C for 10 min, equivalent amounts of proteins (50 μg) were analyzed using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes for 60 min, and were blocked with 5% skim milk for 2 h. the membranes were incubated with anti-p53, anti-caspase-3 at 4°C overnight. After washing with TBST, the membranes were incubated with secondary antibody at 1:5000 dilutions for additional 2 h at room temperature, followed by detecting with the Tanon-5200 chemiluminescent imaging system.

Assessment of EE and DL

Lipo@FANO**1** or lipo@FANO**2** was separately broken by adding the mixture of chloroform and methanol (1:2, v/v), and the amounts of compound were measured using the high-performance liquid chromatography (HPLC) method (Shimadzu LC-20A) with reverse phase

micro C18 column. The eluent used was the mixture of acetonitrile, methanol and water (5:35:60) with the flow rate 1 mL/min, and the detection was conducted at wavelength of 224 nm. The DL and EE were calculated as follows:

DL (%) = $M_d/M_s \times 100\%$

EE (%) = $M_d/M_o \times 100\%$

where M_d represents the total amount of drug in liposome, M_s represents the total amount of drug and liposomal formula, and M_o represents the initial amount of drug used to prepare liposome.

In vitro release behaviour

The in vitro release of FANO**1** or FANO**2** from liposome was carried out by dialysis membrane method. 1 mL sample (lipo@FANO**1** or lipo@FANO**2**, containing equivalent to 1 mg/mL of FANO**1** or FANO**2**, respectively) was transferred in dialysis bags with a molecular cut off of 14 kDa. The bag was suspended in 50 mL release medium of pH 7.4 PBS with/without 0.5% Tween 80) at 37°C in shaking water bath at 50 rpm. At specific time intervals, 1 mL of released medium was withdrawn and replaced with fresh medium. To calculate the total cumulative amount of FANO**1** or FANO**2** released from liposome, FANO**1** or FANO**2** was measured at sampling time by HPLC method. The cumulative release (%) of FANO**1** or FANO**2** was calculated using the equation below:

Cumulative release (%) = $\sum_{t=0}^{t}(C_t/C_0) \times 100$

where C_0 is the initial concentration of the samples in the release medium and C_t is the cumulative amount of FANO**1** or FANO**2** released at each sampling time point.

Cellular uptake

For flow cytometry analysis, B16F10 cells in 12-well plates (1×10^4 cells per well) were grown overnight to reach 70–80% confluence, followed by incubating with C6 labelled lipo@FANO**1** and lipo@FANO**2**. Cells were harvested at different time points (1, 2, 4, and 6 h), and analyzed by flow cytometry. The fluorescence intensity of C6 was measured using FL1 channel (Ex/Em = 488/525 nm).

Intracellular NO measurement

A NO-specific fluorescent probe, DAF-FM DA, purchased from Beyotime Institute of Biotechnology (China), was diluted to a final concentration of 5 μM with kit diluents. To incubate the probe and cells sufficiently, B16F10 cells (1×10^7) were pretreated with 5 μM DAF-FM DA at 37 °C for 20 min. The cells were washed 3 times with PBS to remove the remaining DAF-FM DA that did not enter into the cells. The cells then incubated with lipo@FANO**1** or lipo@FANO**2** at 37°C for 2 h, followed determining NO by using a laser confocal scanning microscope with excitation at 495 nm and emission at 515 nm.

Cell apoptosis

The cell apoptosis was determined using the Calcein/PI dual staining kit purchased from KeyGEN Biotech (Nanjing, China). B16F10 cells in six-well plates (1×10^5 cells per well) were grown overnight to reach 70-90% confluence, followed by incubating with saline, lipo@FANO**1** (0.05 mM FANO**1**), or lipo@FANO**2** (0.25 mM FANO**2**) for 24 h. After incubation, cells were washed with PBS, and stained with Calcein AM (2 μM) and propidium iodide (PI, 8 μM) for 30 min at 37°C, followed by imaging with a Zeiss fluorescence microscope. Green fluorescence emission of Calcein AM (Ex/Em = 495/520 nm) and red fluorescence (Ex/Em = 530/620 nm) of PI were detected.

In vivo biodistribution

For in vivo experiments, 1 x 10^7 B16F10 cells were subcutaneously injected into female C57BL/6 mice. When tumor volume reached ≈ 100 mm^3, mice were randomly assigned to three different groups (n = 5), and were used to evaluate the biodistribution of lipo@FANO**1** and lipo@FANO**2**. DiR-labeled lipo@FANO**1** (2.2 $mg \cdot kg^{-1}$ of FANO**1**, 1.0 $mg \cdot kg^{-1}$ of DiR) or lipo@FANO**2** (9.3 $mg \cdot kg^{-1}$ of FANO**2**, 1.0 $mg \cdot kg^{-1}$ of DiR) was injected into mice via the tail vein. The tumor region was imaged at the indicated time (2, 4, 8, 12, and 24 h). Moreover, after 24 h injection of DiR-labeled liposome, the tumor-bearing mice were sacrificed, and major organs were obtained and imaged.

Pharmacokinetic studies in rats

Sprague Dawley rats were used to evaluate the pharmacokinetics of lipo@FANO**1** and lipo@FANO**2**. The concentrations of FANO**1**, FANO**2**, lipo@FANO**1** and lipo@FANO**2** in plasma were measured using HPLC. FANO**1** (2.2 $mg \cdot kg^{-1}$), lipo@FANO**1** (2.2 $mg \cdot kg^{-1}$ of FANO**1**), FANO**2** (9.3 $mg \cdot kg^{-1}$) and lipo@FANO**2** (9.3 $mg \cdot kg^{-1}$ of FANO**2**) were separately injected through the tail vein (n = 6), and blood samples were collected at predetermined intervals. Plasma was obtained by centrifugation at 10 000 rpm for 10 min, followed by adding 200 μL of acetonitrile. The mixture was vortexed for 2 min, centrifuged at 12 000 rpm for 10 min, and measured by HPLC. Pharmacokinetic parameters were calculated by PKSolver V2.0.

In vivo antitumor effects

Female C57BL/6 mice bearing B16F10 tumors were intravenously injected with saline (control), lipo@FANO**1** (2.2 $mg \cdot kg^{-1}$ of FANO**1**), or lipo@FANO**2** (9.3 $mg \cdot kg^{-1}$ of FANO**2**) every 2 days for 10 days. Tumor volumes and body weights were measured every 2 days. At the end of the experiments, mice were sacrificed, and tumors were collected, weighted, imaged, and analyzed by H&E, and caspase-3 stains. The major organs, including heart, liver, spleen, lung, and kidneys were also harvested and analyzed by H&E stains.

Statistics

All experiments were repeated at least three times with 6-12 biological replicates. Data are represented as mean ± SD. Error bars represent standard error of the mean from

independent samples assayed within the experiments. Statistical analysis was done with GraphPad Prism 6 software. Statistical significance was calculated using unpaired Student's t-test: * represents $P < 0.05$, ** represent $P < 0.01$, and *** represent $P < 0.001$.

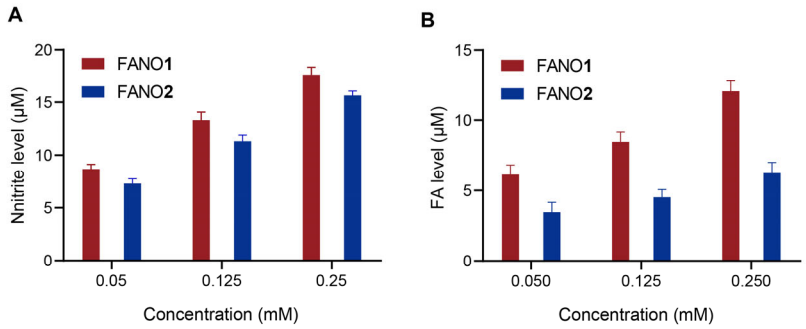

Fig. S1 Dose-dependent release of NO and formaldehyde in B16F10 cells after 2 h of treatment with FANO**1** and FANO**2**. The data are presented as mean ± SD (n = 6).

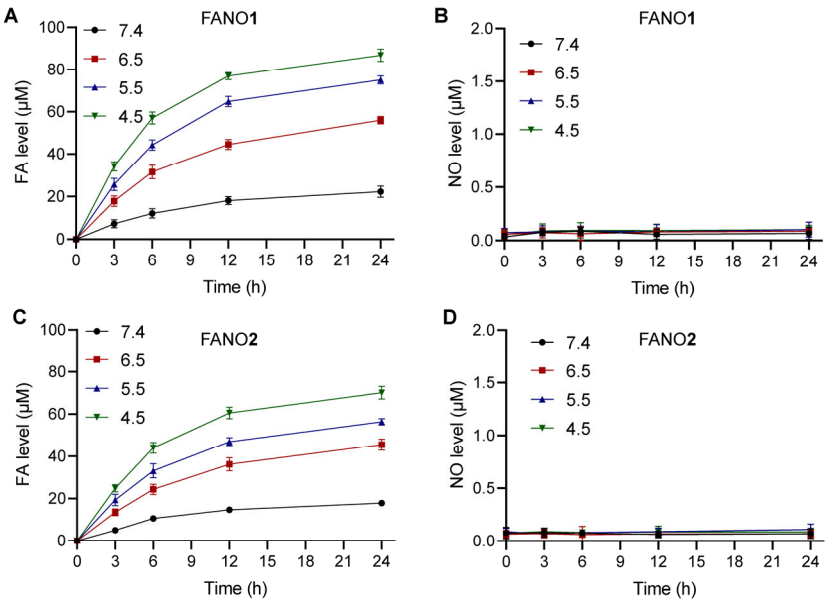

Fig. S2 The FA and NO release of FANO**1** and FANO**2** in PBS with varied pH at 37℃ for 24 h. The data are presented as mean ± SD (n = 6).

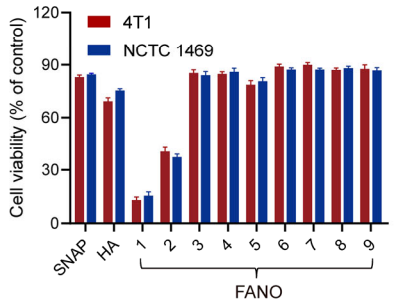

Fig. S3 Cytotoxicity of SNAP, HA and FANO**1**-**9** in 4T1 and NCTC 1469 cells. 4T1 and NCTC 1469 cells were incubated with 0.25 mM of SNAP, HA or FANO**1**-**9** for 24 h, followed by using MTT assay to measure cell viability. The data are presented as mean ± SD (n = 6).

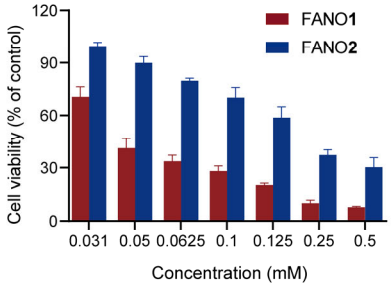

Fig. S4 Does-dependent cytotoxicity of FANO**1** and FANO**2** in B16F10 cells. Cancer cells were incubated with different concentrations of FANO**1** or FANO**2** for 24 h, followed by measuring cell viability with MTT assay. The data are presented as mean ± SD (n = 6).

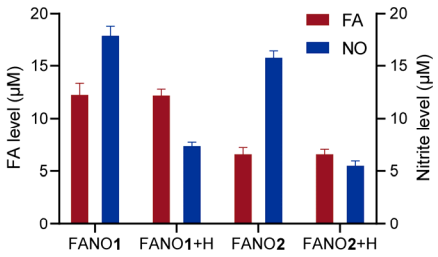

Fig. S5 Measurement of intracellular levels of FA and NO in B16F10 cells. Cancer cells were pretreated with 10 μM hemoglobin (H) for 1 h, and were then incubated with 0.25 mM FANO**1** or FANO**2** for 2 h, followed by measuring the release of FA and NO with the Formaldehyde Assay Kit and Total Nitric Oxide Assay Kit, respectively. The data are presented as mean ± SD (n = 6).

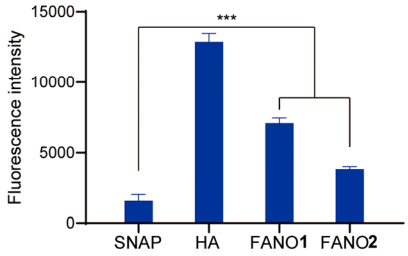

Fig. S6 Quantification of intracellular ROS intensity in B16F10 cells. Cancer cells were treated with 0.25 mM SNAP, HA, FANO**1** or FANO**2** for 10 h, and then were stained by ROS dye carboxy-H2DCFDA, followed by measuring the fluorescence intensity. The data are presented as mean ± SD (n = 6). *** P < 0.001 (two-tailed Student's t-test).

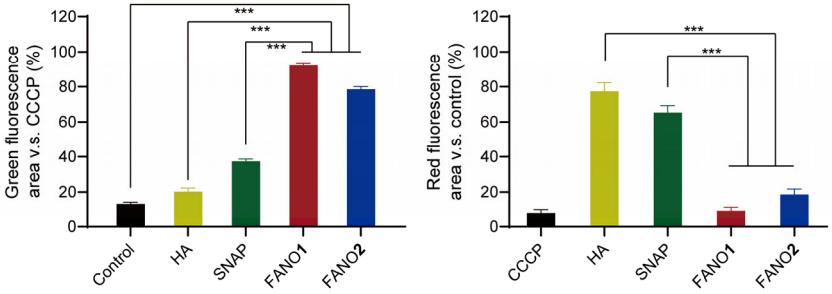

Fig. S7 Mitochondrial membrane potentials of B16F10 cells. Cancer cells were incubated with 0.25 mM HA, SNAP, FANO**1** or FANO**2** for 10 h, and the mitochondrial membrane potentials were determined by JC-1 assay. The data are presented as mean ± SD (n = 6). *** P < 0.001 (two-tailed Student's t-test).

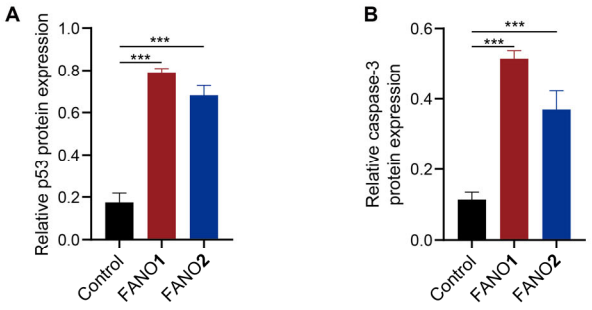

Fig. S8 Western Blot analysis of the changes of p53 and caspase-3 expression in B16F10 cells after incubating with 0.25 mM FANO**1** and FANO**2** for 24 h. The data are presented as mean ± SD (n = 6). *** P < 0.001 (two-tailed Student's t-test).

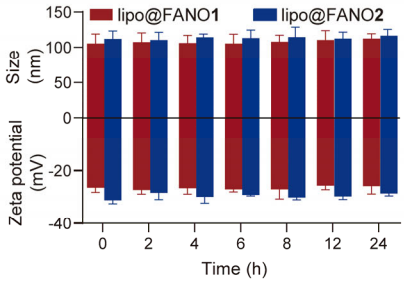

Fig. S9 Serum stability of lipo@FANO1 and lipo@FANO2 in 50% FBS at 37°C for 24h. The data are presented as mean ± SD (n = 6).

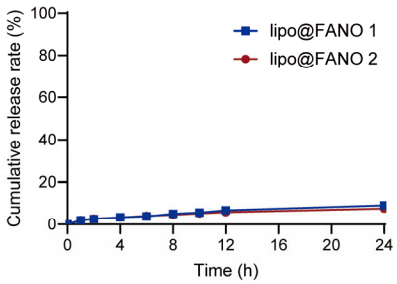

Fig. S10 *In vitro* drug release profiles of lipo@FANO**1** and lipo@FANO**2** in PBS at 37 °C for 24 h. The data are presented as mean ± SD (n = 6).

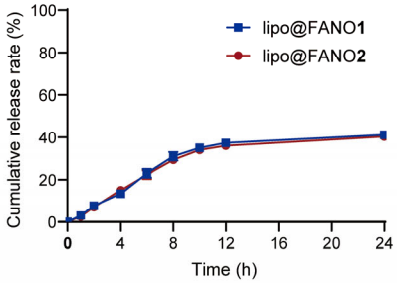

Fig. S11 *In vitro* drug release profiles of lipo@FANO**1** and lipo@FANO**2** in PBS with 0.5% Tween 80 at 37 °C for 24 h. The data are presented as mean ± SD (n = 6).

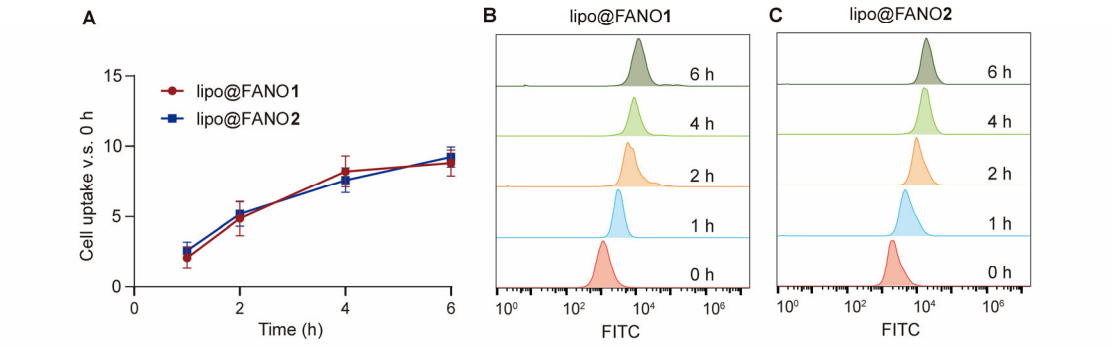

Fig. S12 Cellular uptake profiles of lipo@FANO**1** and lipo@FANO**2** in B16F10 cells. Cancer cells were incubated with C6-labelled liposomes, followed by monitoring their uptake with flow cytometry for 6 h.

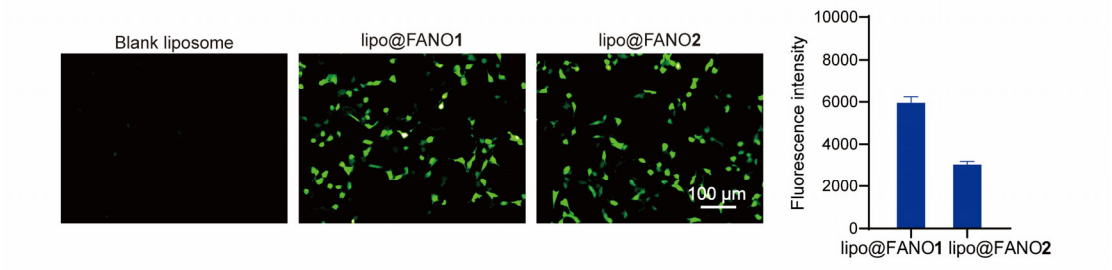

Fig. S13 Fluorescent images and Quantification of intracellular ROS in B16F10 cells stained by ROS dye carboxy-H2DCFDA, treated with lipo@FANO**1** (0.05 mM of FANO**1**) or lipo@FANO**2** (0.25 mM of FANO**2**) for 10 h. The data are presented as mean ± SD (n = 6). *** P < 0.001 (two-tailed Student's t-test).

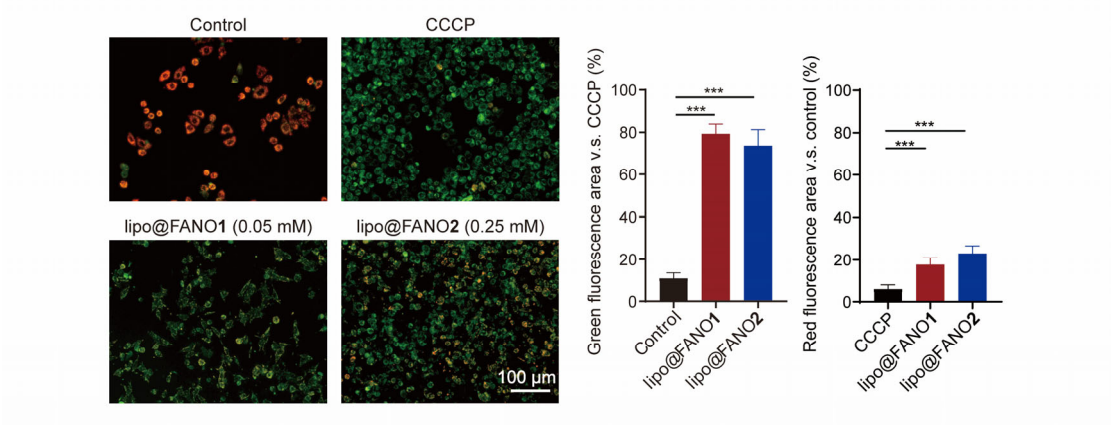

Fig. S14 Mitochondrial membrane potentials of in B16F10 cells determined by JC-1 assay after 10 h of incubation of lipo@FANO**1** (0.05 mM FANO **1**) and lipo@FANO**2** (0.25 mM FANO**2**). The data are presented as mean ± SD (n = 6). *** P < 0.001 (two-tailed Student's t-test).

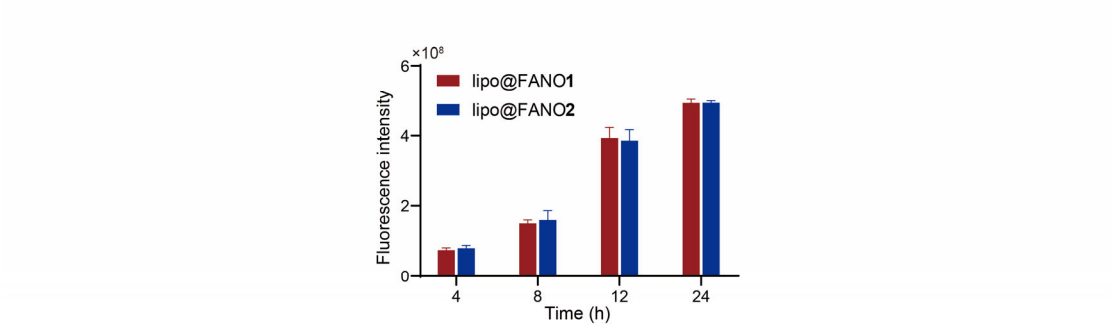

Fig. S15 Time-dependent fluorescent intensity of the tumors. Tumor bearing mice were intravenously injected DiR-labelled lipo@FANO**1** (2.2 mg·kg^{-1} of FANO**1**, 1.0 mg·kg^{-1} of DiR) and lipo@FANO**2** (9.3 mg·kg^{-1} of FANO**2**, 1.0 mg·kg^{-1} of DiR), and the fluorescent intensity of tumor areas was monitored for 24 h. The data are presented as mean ± SD (n = 6).

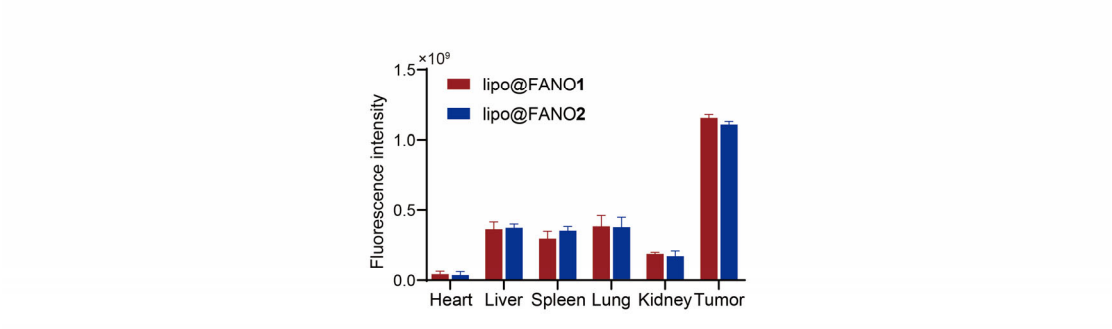

Fig. S16 Quantification of fluorescence intensity of major organs and tumors after intravenous injection of DiR-labelled lipo@FANO**1** (2.2 mg·kg^{-1} of FANO**1**, 1.0 mg·kg^{-1} of DiR) and lipo@FANO**2** (9.3 mg·kg^{-1} of FANO**2**, 1.0 mg·kg^{-1} of DiR) for 24 h. The data are presented as mean ± SD (n = 6)

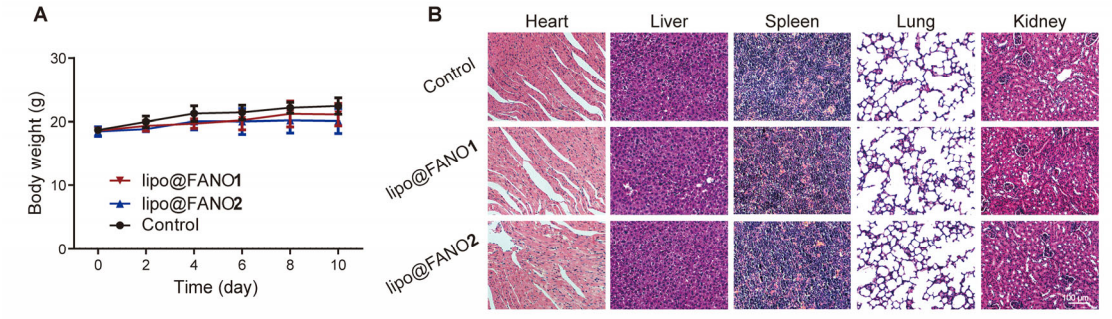

Fig. S17 (A) Body weights of mice during 10-day treatments. (B) H&E histopathological analysis of major organ tissues. The data are presented as mean ± SD (n = 6).

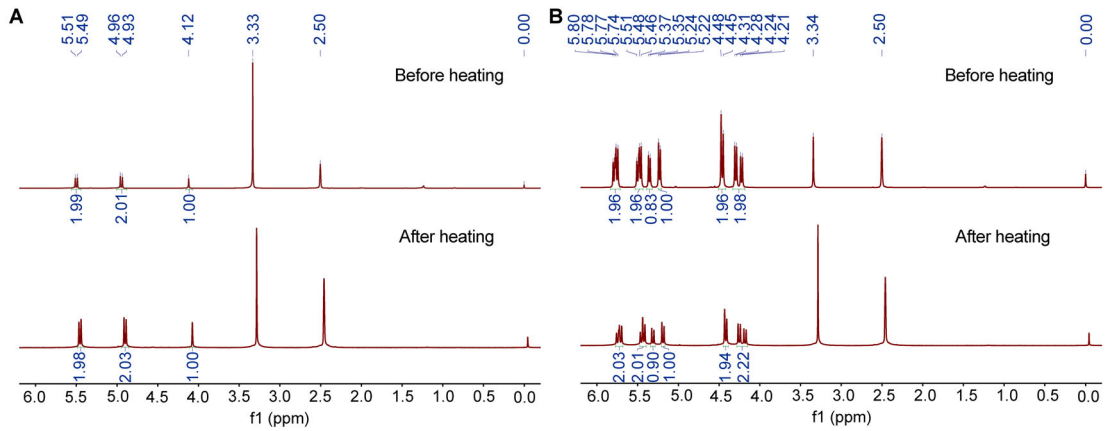

Fig. S18 ^{1}H-NMR spectra of (A) FANO**1** and (B) FANO**2** before and after heating. FANO**1** and FANO**2** were dissolved in DMSO-d_6, and their ^{1}H-NMR spectra were taken before and after heating at 60°C for 1 h.

Table S1 Encapsulation efficiency (EE) and drug loading rate (DL) of lipo@FANO**1** and lipo@FANO**2**. The data are presented as mean ± SD (n = 6).

	EE (%)	DL (%)
lipo@FANO**1**	96.26 ± 2.42	12.24 ± 2.84
lipo@FANO**2**	95.78 ± 1.78	11.36 ± 2.51

Table S2 Pharmacokinetic parameters of FANO**1**, lipo@FANO**1**, FANO**2** and lipo@FANO**2**. Parameters were calculated by PKSolver V2.0. The data are presented as mean ± SD (n = 6)

PK parameters	FANO**1**	lipo@FANO**1**	FANO**2**	lipo@FANO**2**
$T_{1/2}$ (h)	2.02 ± 0.29	7.22 ± 2.01	2.55 ± 0.55	7.34 ± 0.56
$AUC_{0\text{-inf}}$ (μg·h·mL^{-1})	55.08 ± 11.24	194.28 ± 14.29	117.21 ± 12.71	772.45 ± 29.79
$MRT_{0\text{-inf}}$ (h)	2.66 ± 0.36	9.47 ± 1.57	2.17 ± 0.24	9.32 ± 0.94

References

1 H. Zhi, J. Luo, G. Feng, C. Lv, *Chin. Chem. Lett.,* 2009, ***20***, 379-382.

2 Y. Zhang, Z. Xu, L. Zhang, Y. He, J. Luo, *React. Chem. Eng.*, 2019, ***4***, 610-617.

2. Copies of identification spectra

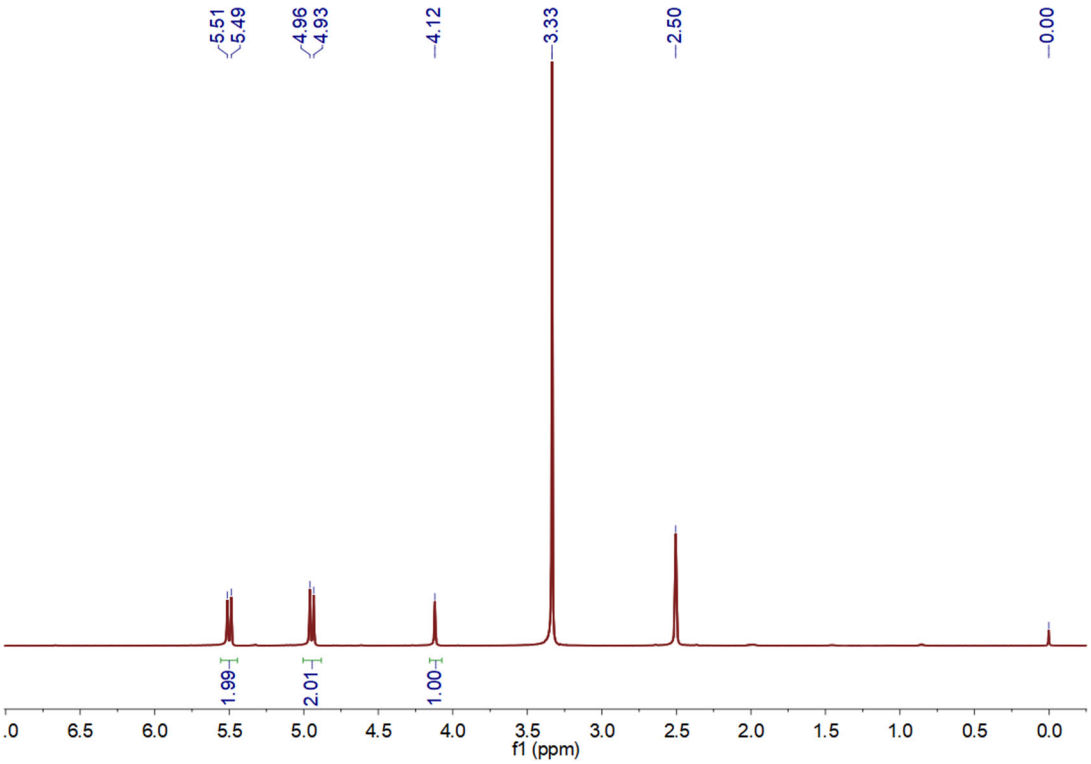

Figure S19. ^{1}H-NMR spectrum of FANO**1** (DMSO-d_6, 500 MHz)

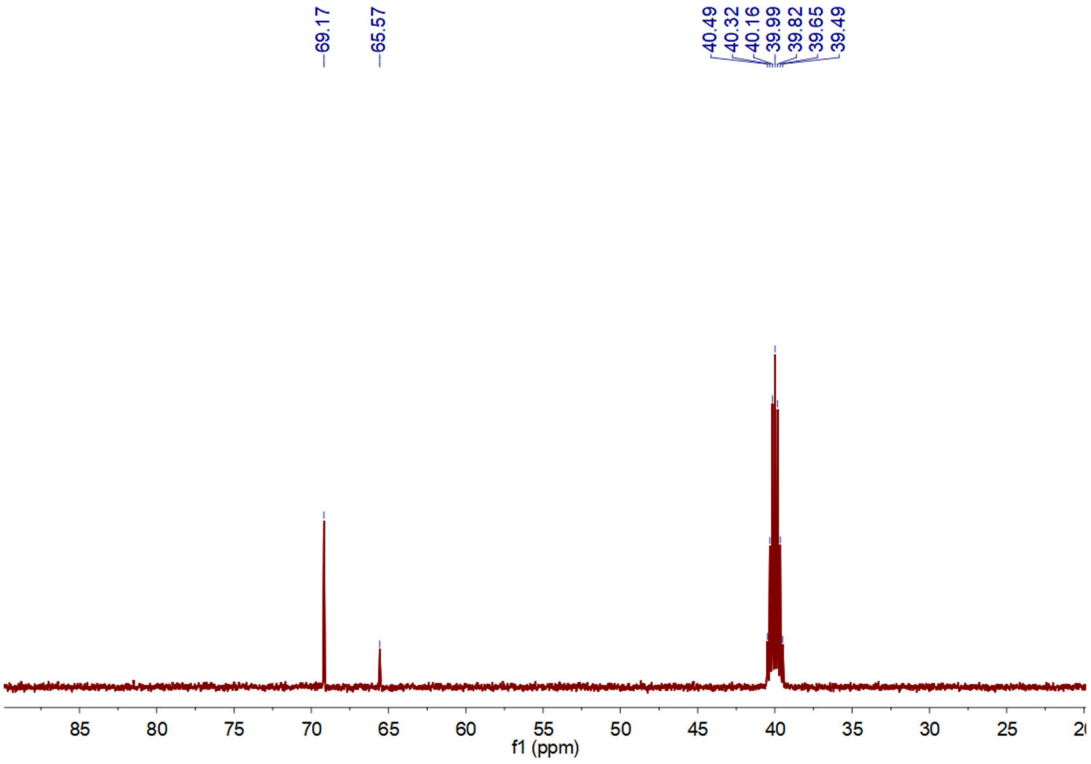

Figure S20. ^{13}C-NMR spectrum of FANO**1** (DMSO-d_6, 126 MHz)

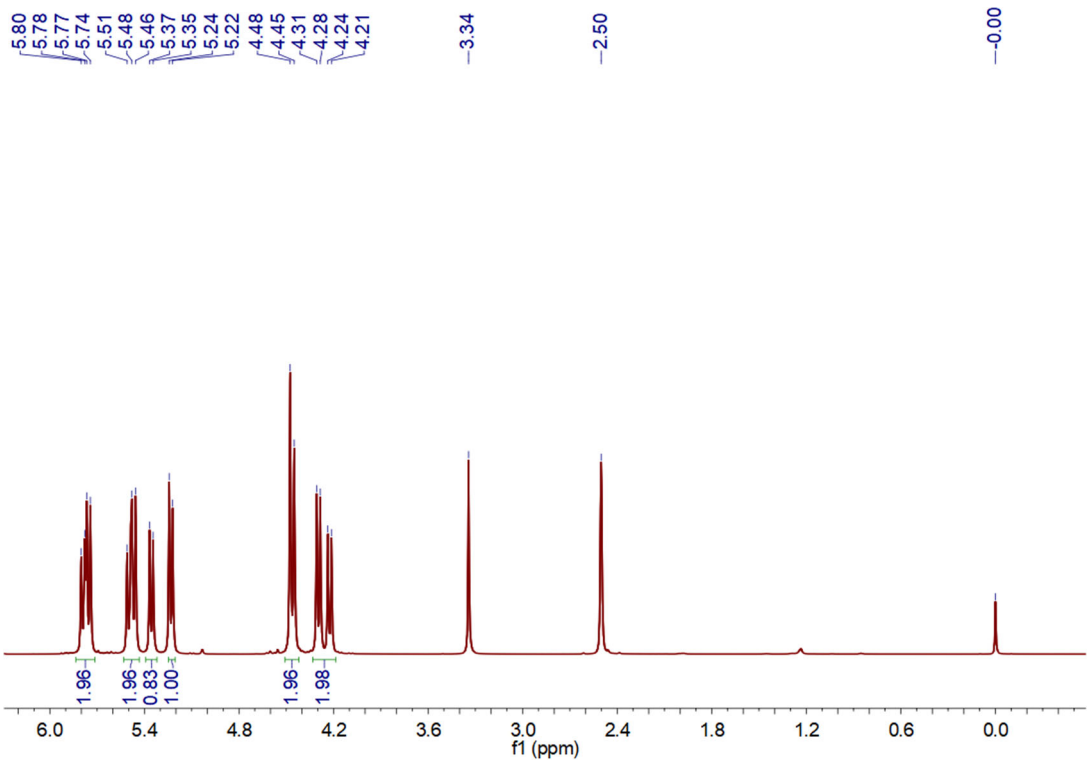

Figure S21. ^{1}H-NMR spectrum of FANO**2** (DMSO-d_6, 500 MHz)

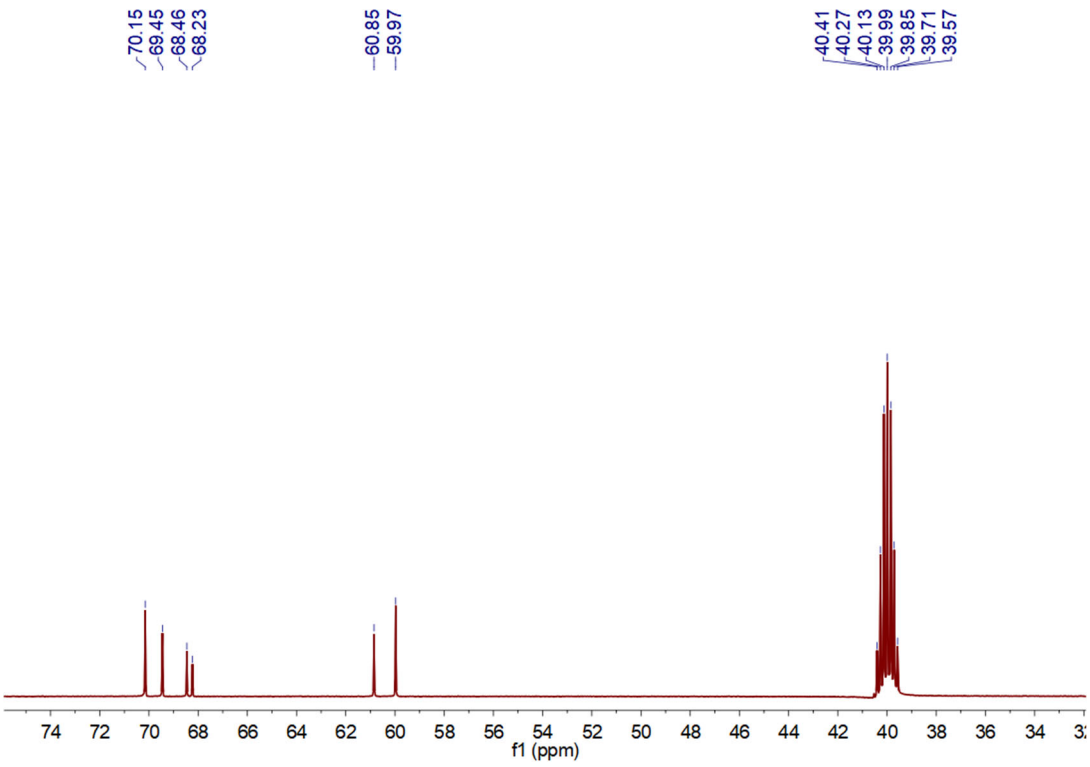

Figure S22. ^{13}C-NMR spectrum of FANO**2** (DMSO-d_6, 126 MHz)

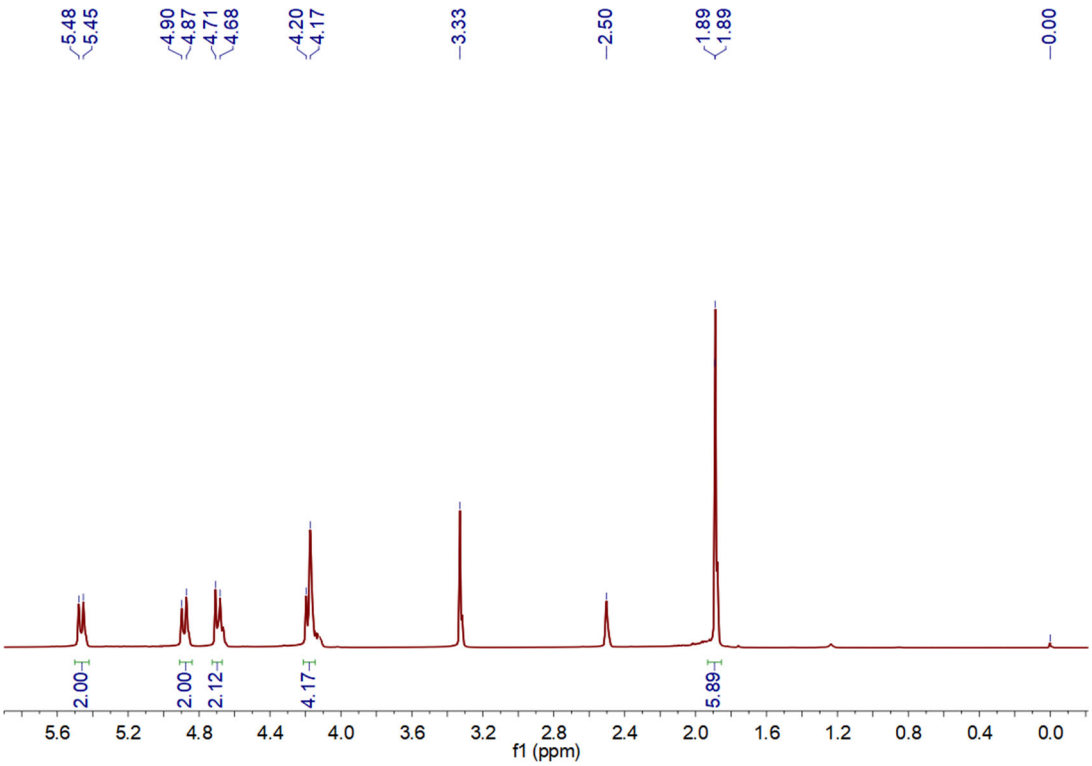

Figure S23. ^{1}H-NMR spectrum of FANO**3** (DMSO-d_6, 500 MHz)

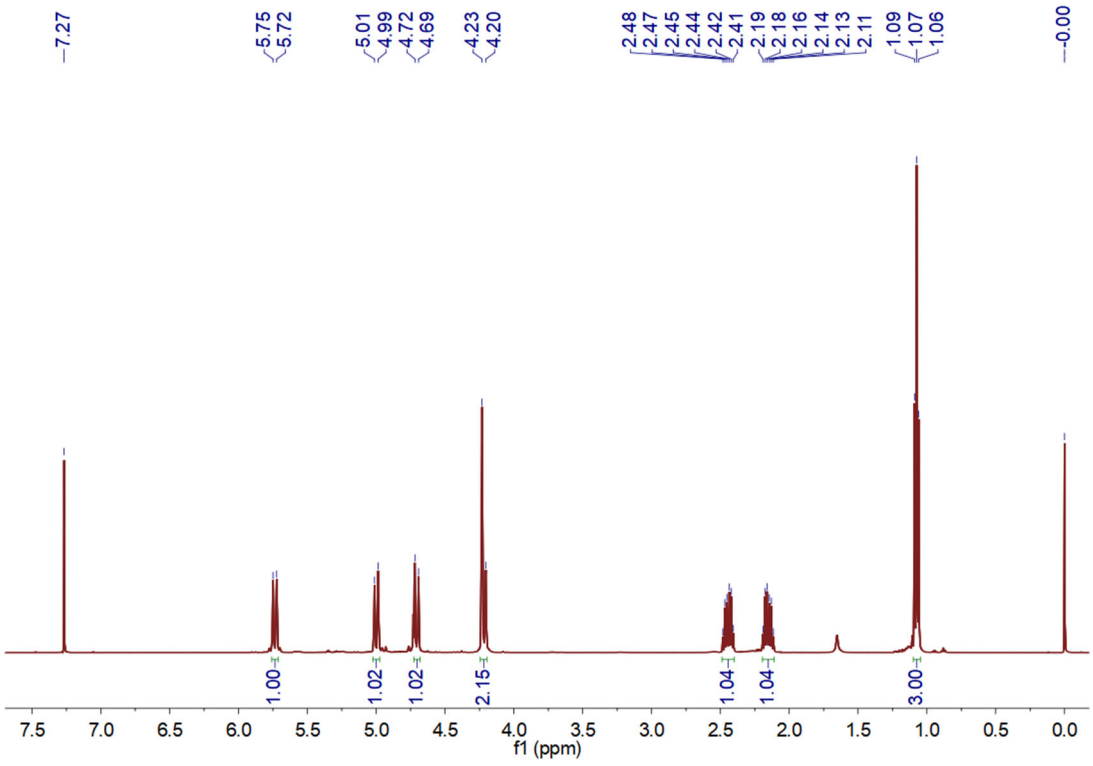

Figure S24. ^{1}H-NMR spectrum of FANO**4** ($CDCl_3$, 500 MHz)

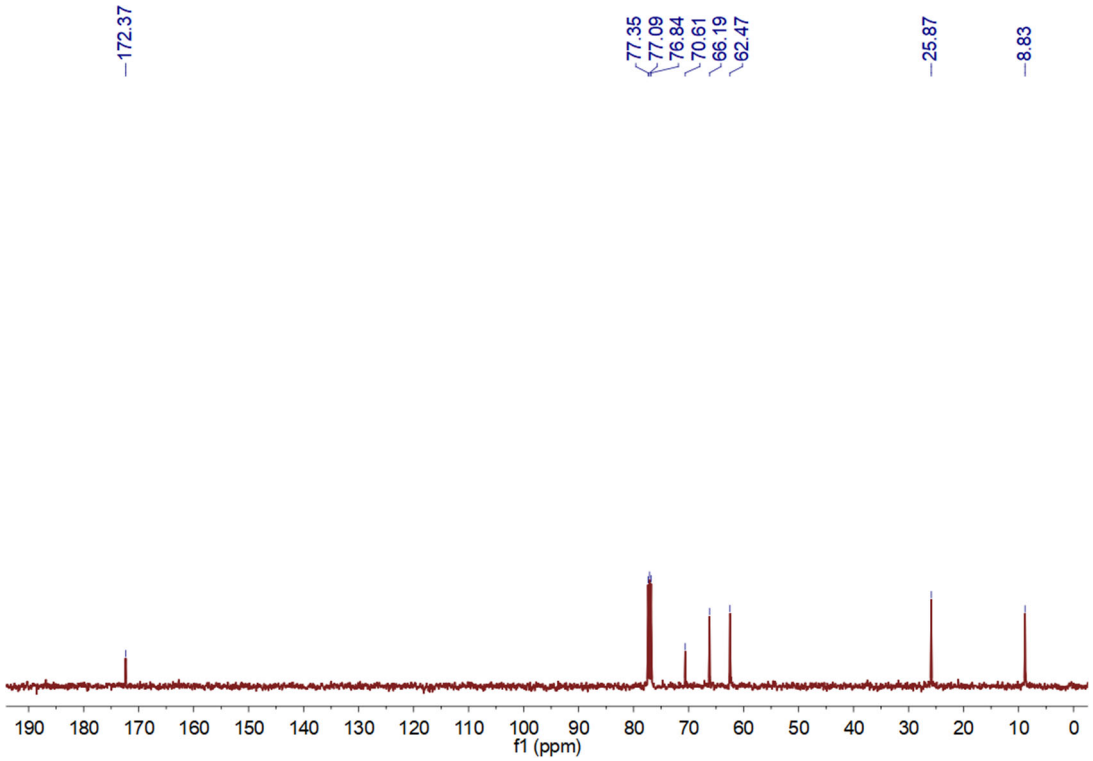

Figure S25. ^{13}C-NMR spectrum of FANO**4** ($CDCl_3$, 500 MHz)

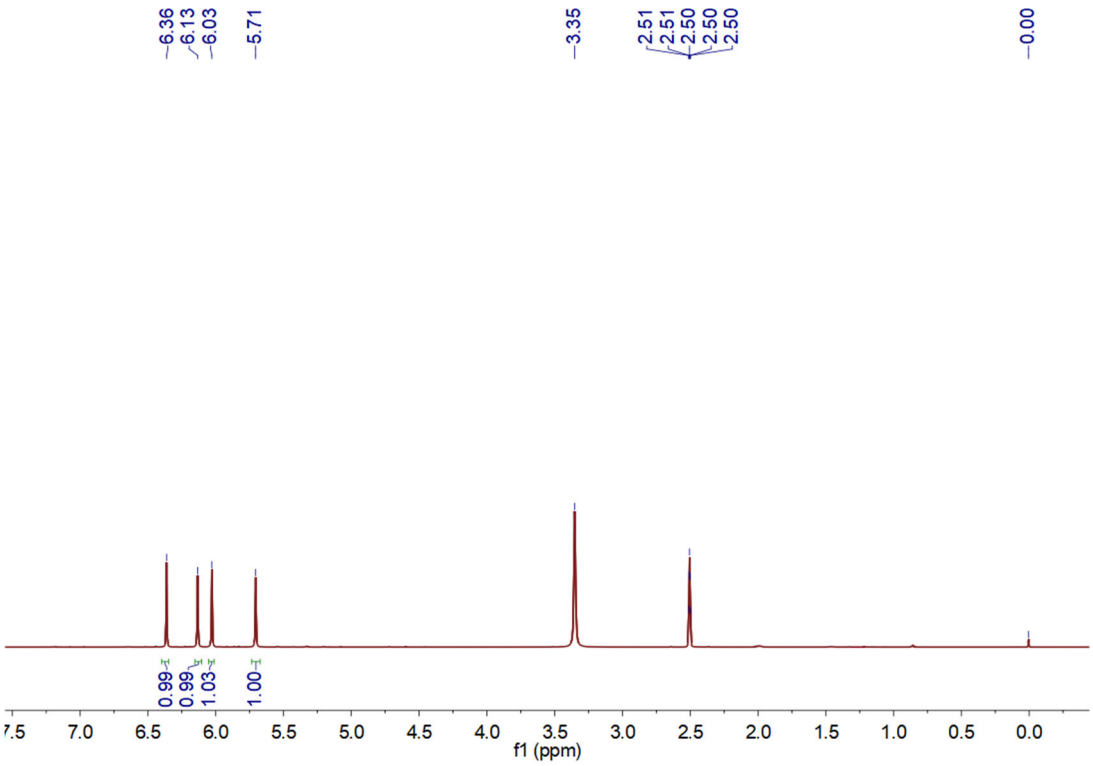

Figure S26. ^{1}H-NMR spectrum of FANO**5** (DMSO-d_6, 500 MHz)

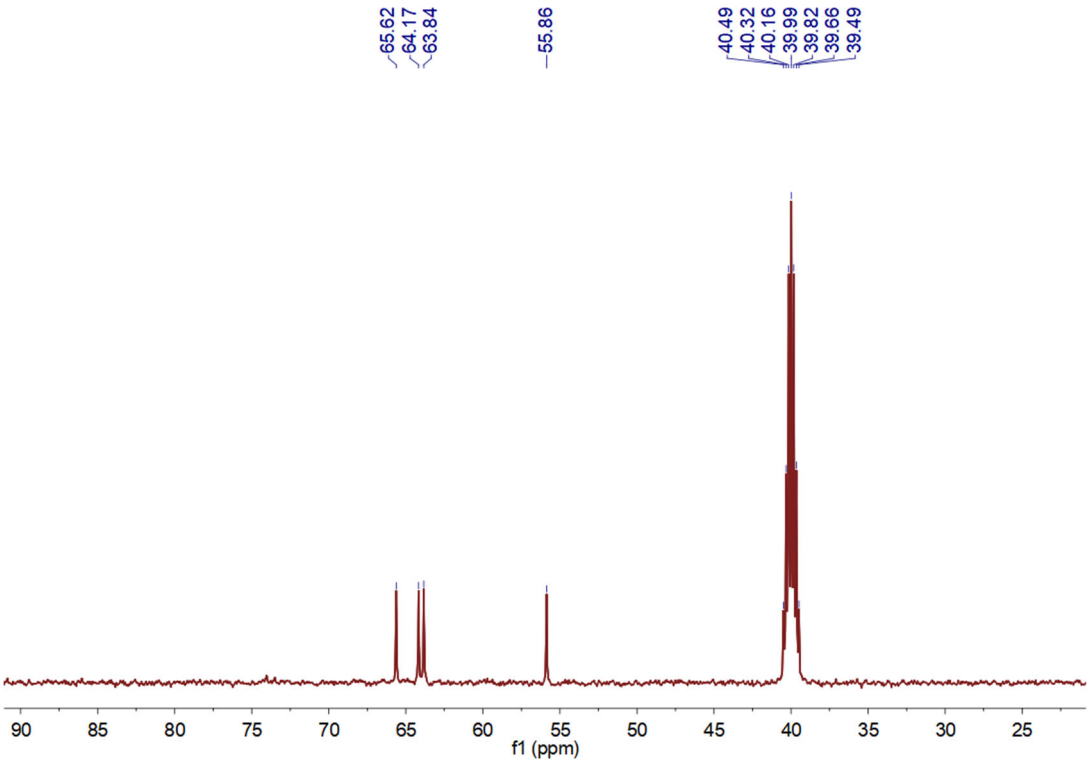

Figure S27. ^{13}C-NMR spectrum of FANO**5** (DMSO-d_6, 126 MHz)

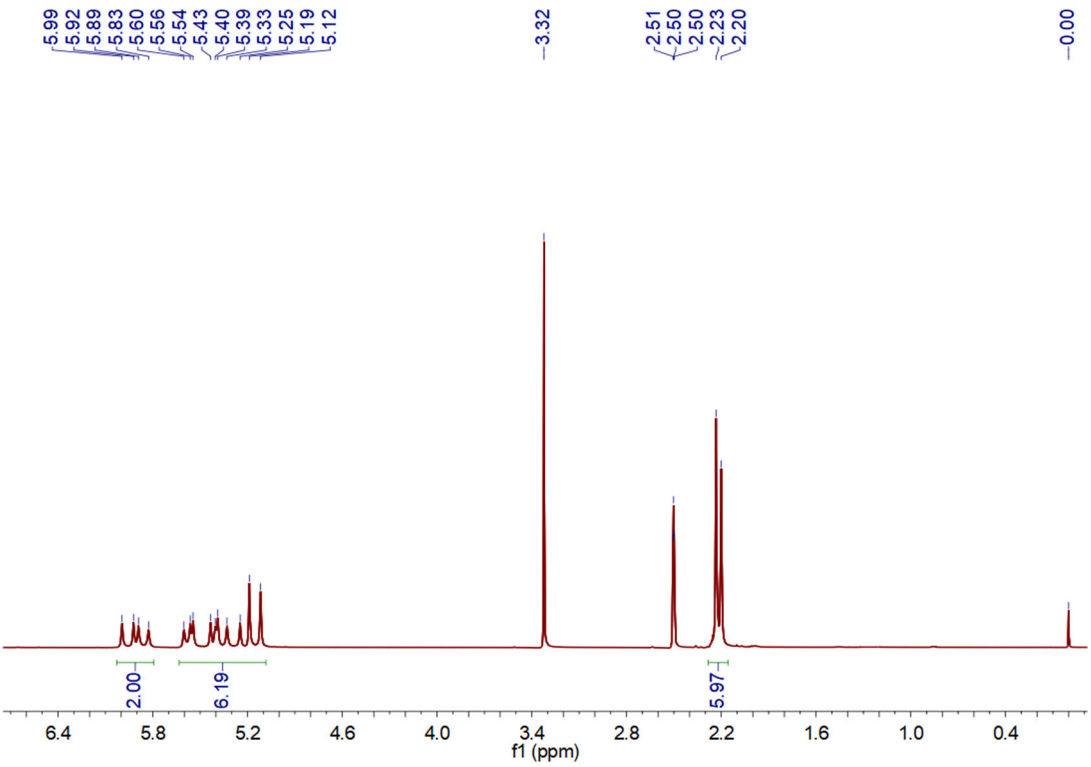

Figure S28. ^{1}H-NMR spectrum of FANO**6** (DMSO-d_6, 500 MHz)

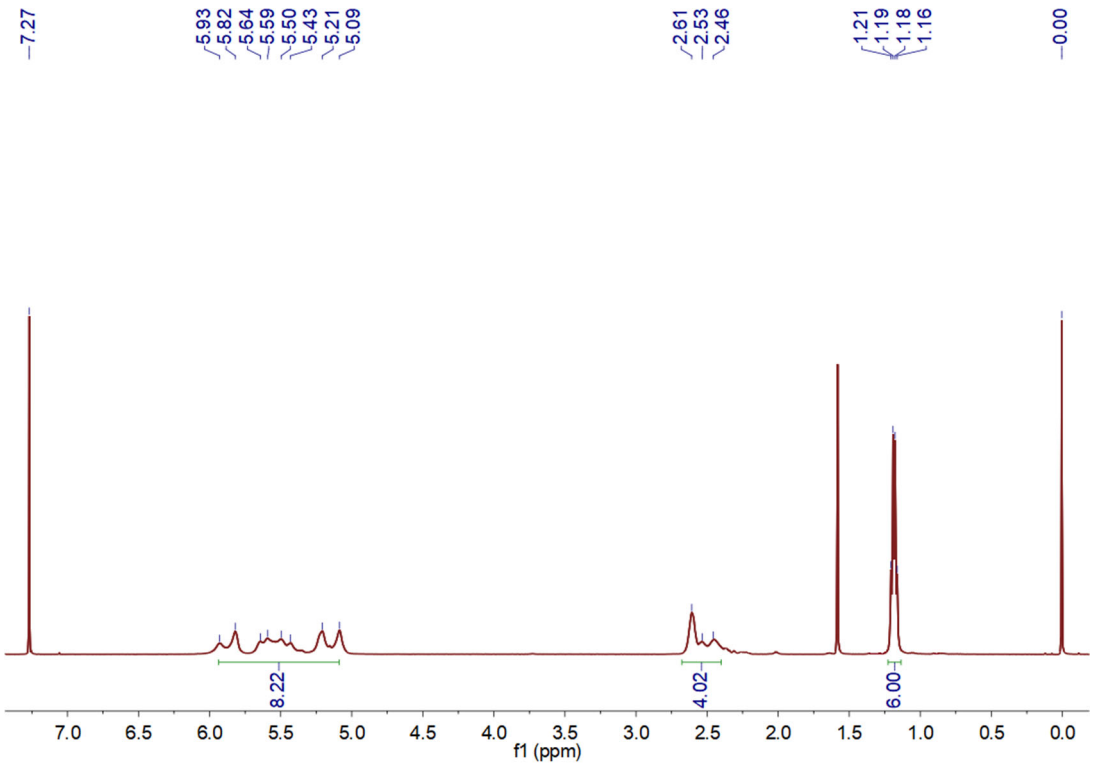

Figure S29. ^{1}H-NMR spectrum of FANO**7** ($CDCl_3$, 500 MHz)

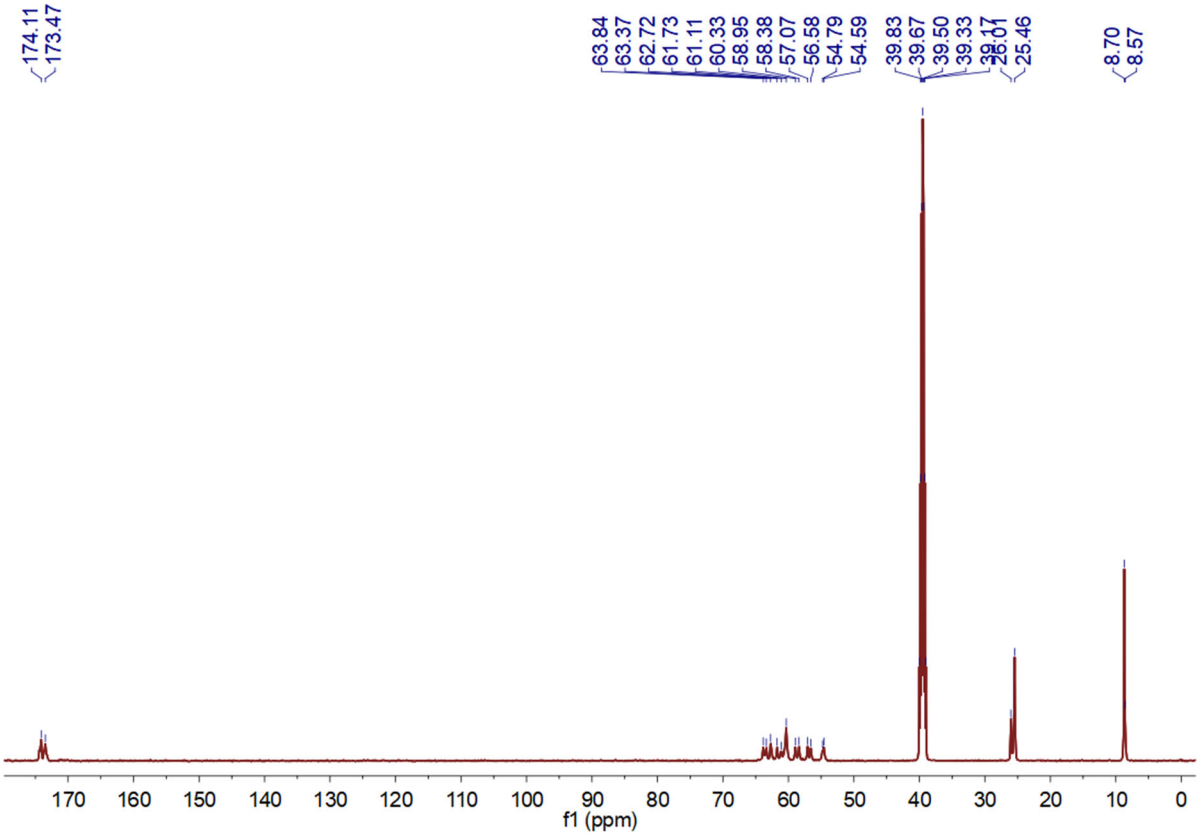

Figure S30. ^{13}C-NMR spectrum of FANO**7** ($CDCl_3$, 126 MHz)

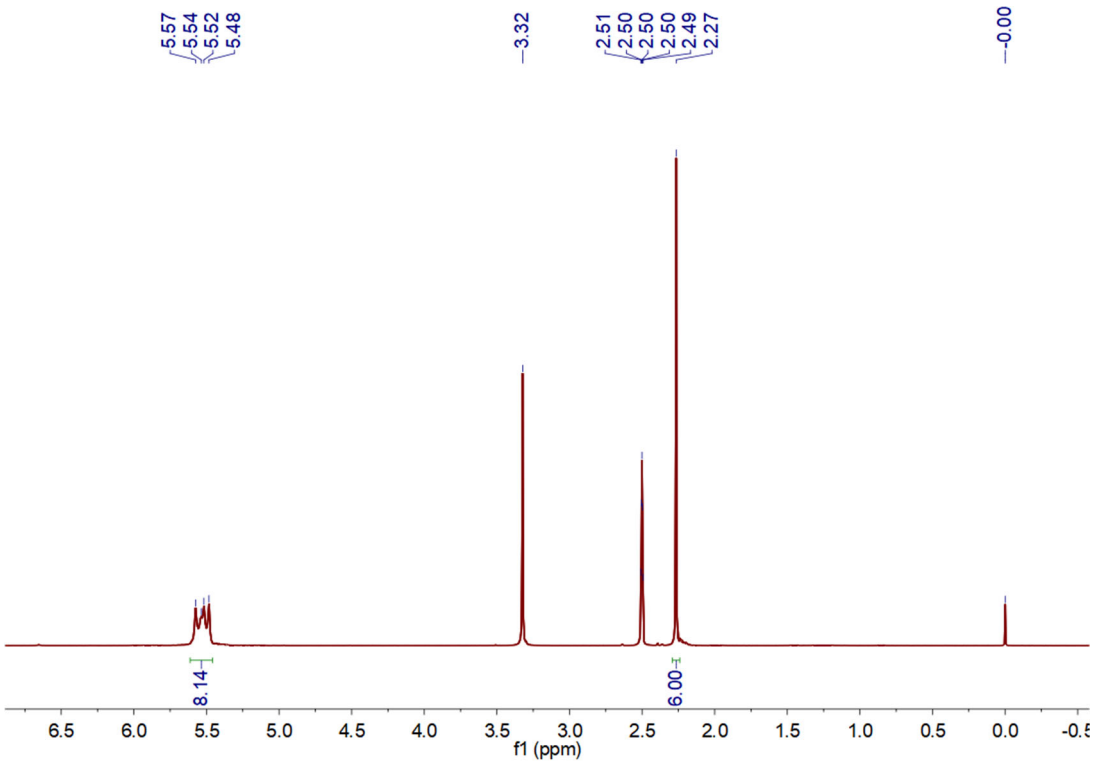

Figure S31. ^{1}H-NMR spectrum of FANO**8** (DMSO-d_6, 500 MHz)

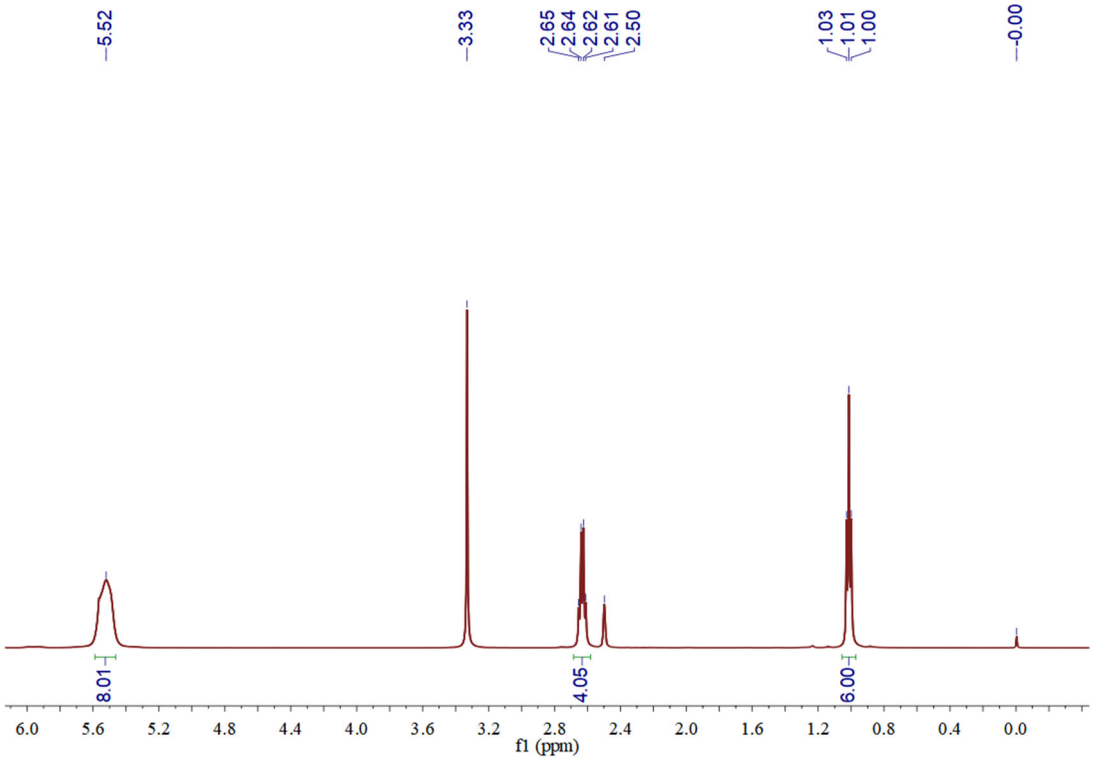

Figure S32. ^{1}H-NMR spectrum of FANO**9** (DMSO-d_6, 500 MHz)

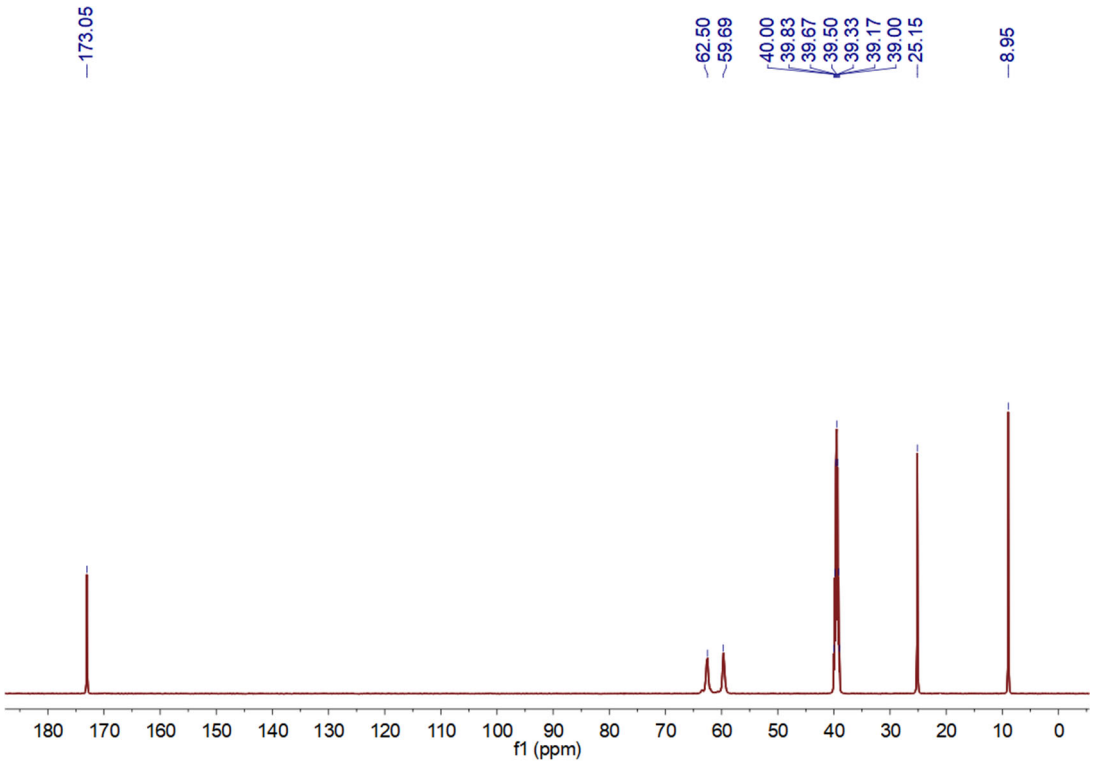

Figure S33. ^{13}C-NMR spectrum of FANO**9** (DMSO-d_6, 126 MHz)