Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Novel α-ketoamide based diazeniumdiolates as hydrogen peroxide responsive nitric oxide donors with anti-lung cancer activity

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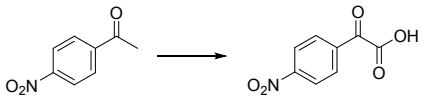
1. General Chemistry Experimental Information

All reagents and solvents were received from commercial suppliers and used without further purification. Sodium diazeniumdiolates and JS-K were received as a gift from Professor Zhangjian Huang at China Pharmaceutical University. When necessary, solvents were dried using standard protocols. TLC was performed on silica gel plates and visualized under UV light (254 nm). Flash chromatography was performed using chromatography grade silica gel (200–300 mesh) purchased from Qingdao Haiyang Chemical Co., Ltd. Melting points were determined on a Mel-TEMP II melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectrum were recorded with a JEOL 400 MHz spectrometer at 300 K. Mass spectrum were obtained on an Agilent Q-TOF 6540 spectrometer. High resolution mass spectra were obtained on Agilent LC/MSD TOF.

2. Synthesis and characterizations of target compounds

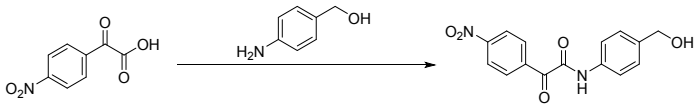
2-(4-Nitrophenyl)-2-oxoacetic acid (1)



1

1-(4-Nitrophenyl)ethan-1-one (1 g, 6.06 mmol) was dissolved in pyridine (10 mL) and SeO_2 (1008 mg, 9.08 mmol) was added. The reaction mixture was heated in an oil bath at 90 °C under nitrogen for 5 h. The reaction was monitored by TLC. Upon completion, the reaction mixture was filtered, and the residue was washed with ethyl acetate (EA) (3 × 20 mL). The combined filtrate was treated with 1 M HCl (40 mL), the organic layer was separated, and the aqueous layer was re-extracted with EA (3 × 20 mL). The organic layers were combined and treated with 1 M NaOH (50 mL), and the aqueous layer was separated. The aqueous layer was acidified with 1 M HCl to pH 1.5. The mixture was extracted with EA (3 × 50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated. The crude products were further purified by column chromatography using petroleum ether (PE)/EA (9:1 to 7:3) to afford **1** as a yellow solid in 35% yield.

***N*-(4-Hydroxymetheyl)phenyl)-2-(4-nitrophenyl)-2-oxoacetamide (2)**



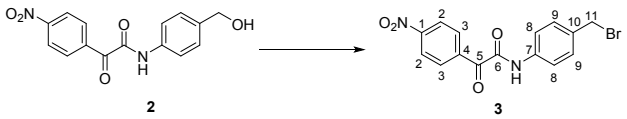
1 **2**

(4-Aminophenyl)methanol (633 mg, 5.13 mmol) was added to a solution of **1** (1 g, 5.13 mmol) in DMF (10 mL). EDCI (7.7 mmol, 1475 mg) and DIEA (7.7 mmol, 1272 μL) were added in turn. The reaction mixture was stirred at r.t. for 1.5 h and monitored by TLC. Upon completion, the reaction mixture was extracted with EA (3 × 50 mL), dried over Na_2SO_4, filtered, and concentrated to give **2** in 33% yield, which was used directly in the following reaction without purification.

ESI-MS: 299.3 $[M - H]^-$.

HRMS (m/z): $[M + H]^+$ calcd. $[C_{15}H_{13}ClN_2O_5]^+$ 301.0824, found 310.0817.

***N*-(4-(Bromomethyl)phenyl)-2-(4-nitrophenyl)-2-oxoacetamide (3)**



2 **3**

Compound **2** (300 mg, 1 mmol) was dissolved in DCM (10 mL) at 0 °C under nitrogen. PPh_3 (394 mg, 1.5 mmol) and CBr_4 (498 mg, 1.5 mmol) were added in turn, and the reaction mixture was allowed to warm

to r.t. and stirred for 2 h. The mixture was concentrated under reduced pressure, and the residue was further purified by column chromatography (PE/EA v/v, 9:1) to obtain **3** as a yellow solid in 50% yield.

m.p. 133–135 °C.

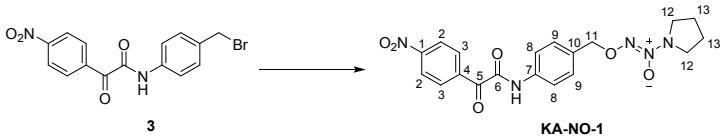
^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.98 (s, 1H, NH), 8.66–8.54 (m, 2H, H_2), 8.41–8.29 (m, 2H, H_3), 7.74–7.63 (m, 2H, H_8), 7.50–7.39 (m, 2H, H_9), 4.51 (s, 2H, H_{11}).

^{13}C NMR (100 MHz, Chloroform-*d*) δ 185.94 (C_5), 157.80 (C_6), 151.10 (C_1), 137.64 (C_4), 136.30 (C_7), 135.35 (C_{10}), 132.76 (C_3 × 2), 130.29 (C_9 × 2), 123.71 (C_2 × 2), 120.33 (C_8 × 2), 32.98 (C_{11}).

ESI-MS: 361.1 $[M - H]^-$.

HRMS (m/z): $[M - H]^-$ calcd. $[C_{15}H_{10}BrN_2O_4]^-$ 360.9829, found 360.9833.

KA-NO-1



Sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (64 mg, 0.42 mmol) and $NaHCHO_3$ (23 mg, 0.28 mmol) were dissolved in DMF (2 mL). The mixture was cooled to 0 °C and compound **3** (100 mg, 0.28 mmol) was added under nitrogen. The reaction mixture was allowed to stir and gradually warm to r.t. during 1–2 h. Upon completion, EA (50 mL) was added and the mixture was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography to afford KA-NO-1 (PE/EA v/v, 7:3) as a yellow solid in 38% yield.

m.p. 117–118 °C.

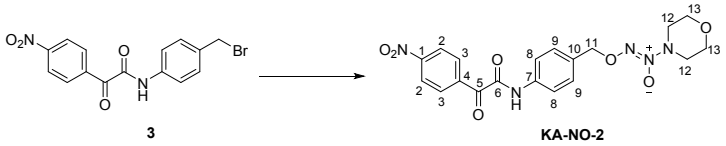
^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.99 (s, 1H, NH), 8.64–8.60 (m, 2H, H_2), 8.39–8.34 (m, 2H, H_3), 7.72–7.69 (m, 2H, H_8), 7.48–7.45 (m, 2H, H_9), 5.18 (s, 2H, H_{11}), 3.54–3.50 (m, 4H, H_{12}), 1.97–1.90 (m, 4H, H_{13}).

^{13}C NMR (100 MHz, Chloroform-*d*) δ 186.07 (C_5), 157.94 (C_6), 151.12 (C_1), 137.74 (C_4), 136.42 (C_7), 133.65 (C_{10}), 132.73 (C_3 × 2), 129.92 (C_9 × 2), 123.69 (C_2 × 2), 120.12 (C_8 × 2), 74.66 (C_{11}), 51.06 (C_{12} × 2), 22.95 (C_{13} × 2).

ESI-MS: 412.4 $[M - H]^-$.

HRMS (m/z): $[M - H]^-$ calcd. $[C_{19}H_{18}N_5O_6]^-$ 412.1257, found 412.1263.

KA-NO-2



Using a similar procedure as described above for the synthesis of KA-NO-1, KA-NO-2 was obtained from sodium 1-(morpholine-4-yl)diazen-1-ium-1,2-diolate and **3** as a yellow solid in 27% yield

m.p. 149–150 °C.

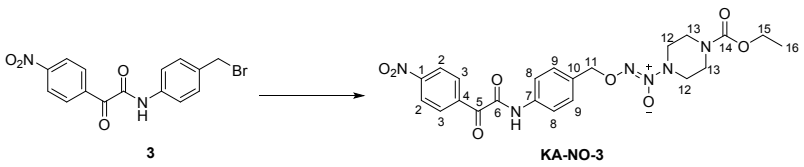
^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.01 (s, 1H, NH), 8.62–8.58 (m, 2H, H_2), 8.37–8.34 (m, 2H, H_3), 7.73–7.70 (m, 2H, H_8), 7.45–7.43 (m, 2H, H_9), 5.22 (s, 2H, H_{11}), 3.84–3.81 (m, 4H, H_{13}), 3.42–3.40 (m, 4H, H_{12}).

^{13}C NMR (100 MHz, Chloroform-*d*) δ 186.00 (C_5), 157.91 (C_6), 151.11 (C_1), 137.68 (C_4), 136.62 (C_7), 133.08 (C_{10}), 132.74 (C_3 × 2), 130.00 (C_9 × 2), 123.70 (C_2 × 2), 120.16 (C_8 × 2), 75.15 (C_{11}), 65.80 (C_{13} × 2), 51.73 (C_{13} × 2).

ESI-MS: 428.3 $[M - H]^-$.

HRMS (m/z): $[M - H]^-$ calcd. $[C_{19}H_{18}N_5O_7]^-$ 428.1206, found 428.1212.

KA-NO-3



3 KA-NO-3

Using a similar procedure as described above for the synthesis of KA-NO-1, KA-NO-3 was obtained from sodium 1-(4-ethyloxylcarbonylpiperazine-1-yl)diazen-1-ium-1,2-diolate and **3** as a yellow solid in 47% yield

m.p. 145–146 °C.

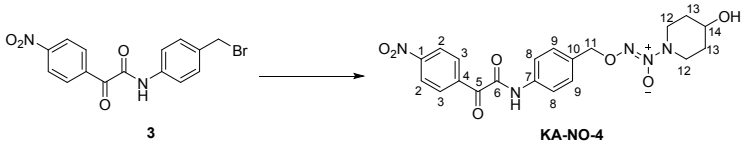
^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.01 (s, 1H, NH), 8.59–8.56 (m, 2H, H_2), 8.35–8.31 (m, 2H, H_3), 7.71–7.67 (m, 2H, H_8), 7.42–7.40 (m, 2H, H_9), 5.20 (s, 2H, H_{11}), 4.13 (q, *J* = 7.1 Hz, 2H, H_{15}), 3.62 (dd, *J* = 6.0, 4.4 Hz, 4H, H_{13}), 3.36–3.33 (m, 4H, H_{12}), 1.25 (t, *J* = 7.1 Hz, 3H, H_{16}).

^{13}C NMR (100 MHz, Chloroform-*d*) δ 185.95 (C_5), 157.90 (C_6), 155.18 (C_{14}), 151.05 (C_1), 137.63 (C_4), 136.64 (C_7), 132.90 (C_{10}), 132.68 (C_3 × 2), 129.95 (C_9 × 2), 123.65 (C_2 × 2), 120.14 (C_8 × 2), 75.19 (C_{11}), 61.94 (C_{15}), 51.17 (C_{12} × 2), 42.38 (C_{13} × 2), 14.70 (C_{16}).

ESI-MS: 499.1 $[M - H]^-$.

HRMS (m/z): $[M - H]^-$ calcd. $[C_{22}H_{23}N_6O_8]^-$ 499.1577, found 499.1583.

KA-NO-4



3 KA-NO-4

Using a similar procedure as described above for the synthesis of KA-NO-1, KA-NO-4 was obtained from sodium 1-(4-hydroxypiperidine-1-yl)diazen-1-ium-1,2-diolate and **3** as a pale yellow solid in 47% yield

m.p. 142–143 °C.

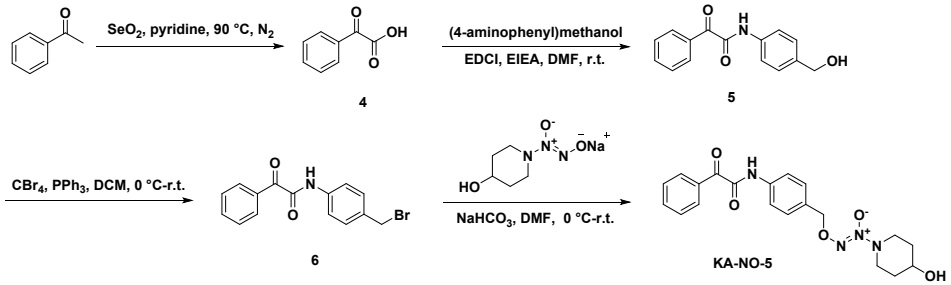
^{1}H NMR (400 MHz, DMSO-d_6) δ 11.02 (s, 1H, NH), 8.39 (d, *J* = 8.8 Hz, 2H, H_2), 8.29 (d, *J* = 8.8 Hz, 2H, H_3), 7.71 (d, *J* = 8.3 Hz, 2H, H_8), 7.30 (d, *J* = 8.2 Hz, 2H, H_9), 4.58 (s, 1H, OH), 3.45 (brs, 3H, H_{11} and H_{14}), 2.67 (d, *J* = 10.5 Hz, 2H, H_{12}), 2.06 (brs, 2H, H_{12}), 1.75–1.62 (m, 2H, H_{13}), 1.45–1.33 (m, 2H, H_{13}).

^{13}C NMR (100 MHz, DMSO-d_6) δ 187.71 (C_5), 161.60 (C_6), 150.52 (C_1), 137.59 (C_4), 136.25 (C_7), 131.55 (C_3 × 2 and C_{10}), 129.36 (C_9 × 2), 123.94 (C_2 × 2), 120.10 (C_8 × 2), 61.54 (C_{11}), 50.72 (C_{14}), 34.34 (C_{12} × 2), 30.33 (C_{13} × 2).

ESI-MS: 442.2 $[M - H]^-$.

HRMS (m/z): $[M - H]^-$ calcd. $[C_{20}H_{20}N_5O_7]^-$ 442.1363, found 442.1368.

Synthetic route of KA-NO-5



4 5 6 KA-NO-5

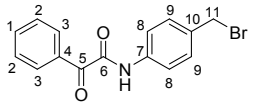
2-Oxo-2-phenylacetic acid (4)

Using a similar procedure as described above for the synthesis of **1**, compound **4** was obtained from acetophenone as a pale yellow oil in 87% yield.

***N*-(4-(Hydroxymethyl)phenyl)-2-oxo-2-phenylacetamide (5)**

Using a similar procedure as described above for the synthesis of **2**, compound **5** was obtained from **4** and (4-aminophenyl)methanol as a pale yellow oil in 64% yield and was used directly in the following reaction without purification.

***N*-(4-(Bromomethyl)phenyl)-2-oxo-2-phenylacetamide (6)**



6

Using a similar procedure as described above for the synthesis of **3**, compound **6** was obtained from **5** as a yellow solid in 85% yield.

m.p. 124–125 °C.

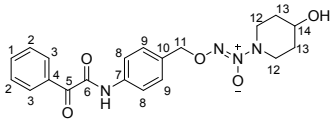
^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.01 (s, 1H, NH), 8.43–8.40 (m, 2H, H_3), 7.71–7.69 (m, 2H, H_2), 7.68–7.65 (m, 1H, H_1), 7.54–7.50 (m, 2H, H_8), 7.45–7.41 (m, 2H, H_9), 4.51 (s, 2H, H_{11}).

^{13}C NMR (100 MHz, Chloroform-*d*) δ 187.25 (C_5), 158.91 (C_6), 136.80 (C_7), 134.90 (C_1), 134.82 (C_4), 133.07 (C_{10}), 131.63 ($C_3 \times 2$), 130.19 ($C_9 \times 2$), 128.75 ($C_2 \times 2$), 120.22 ($C_8 \times 2$), 33.19 (C_{11}).

ESI-MS: 318.1 $[M + H]^+$.

HRMS (m/z): $[M + H]^+$ calcd. $[C_{15}H_{13}BrNO_2]^+$ 318.0130, found 318.0124.

KA-NO-5



KA-NO-5

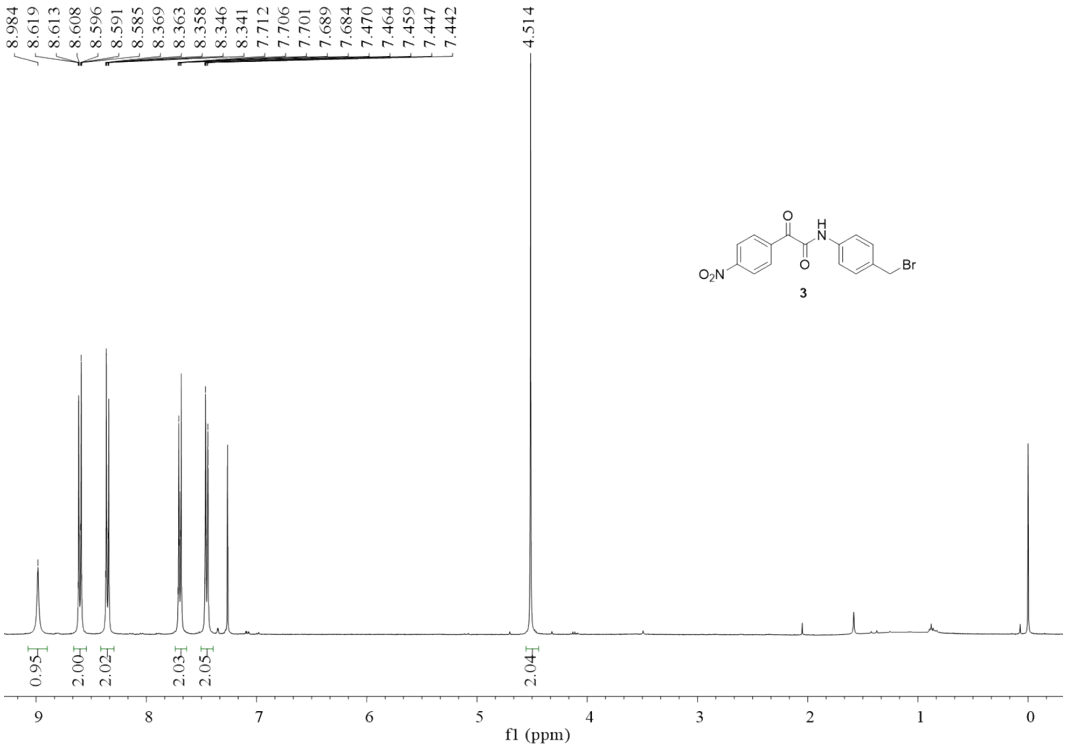
Using a similar procedure as described above for the synthesis of KA-NO-4, KA-NO-5 was obtained from sodium 1-(4-hydroxypiperidine-1-yl)diazen-1-ium-1,2-diolate and **6** as a pale yellow solid in 42% yield

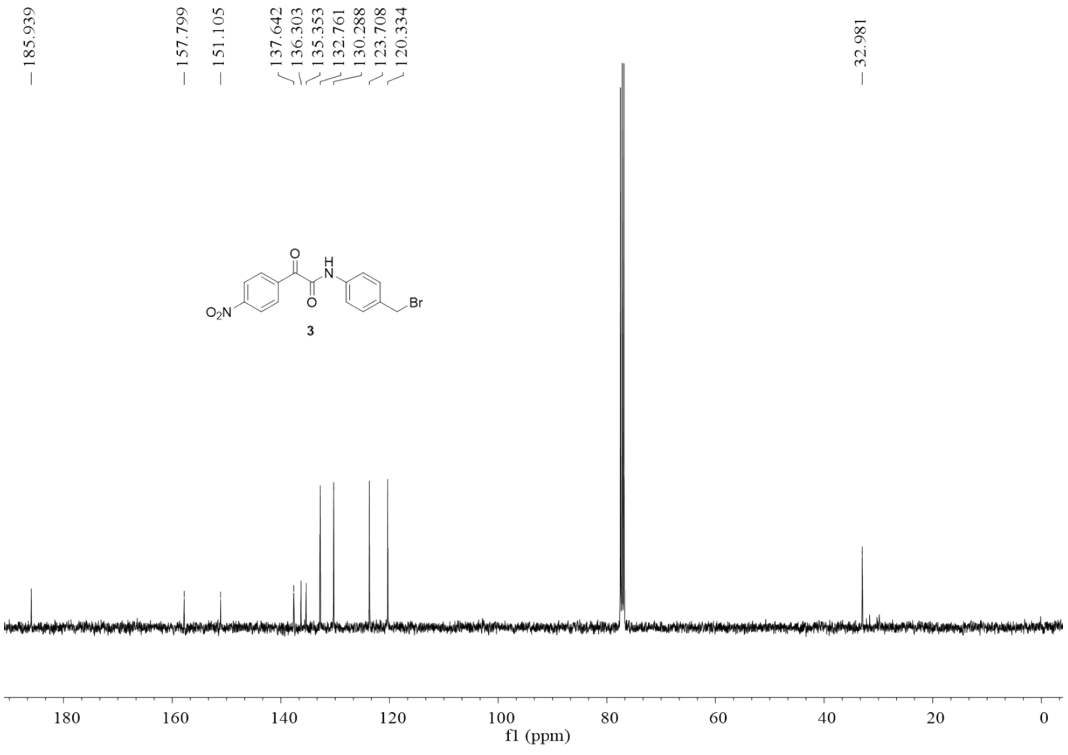
m.p. 130–131 °C.

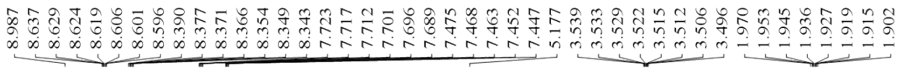
^{1}H NMR (400 MHz, Acetone-d_6) δ 9.92 (s, 1H, NH), 8.25–8.22 (m, 2H, H_3), 7.84–7.80 (m, 2H, H_2), 7.75–7.71 (m, 1H, H_1), 7.61–7.57 (m, 2H, H_8), 7.40–7.35 (m, 2H, H_9), 3.63–3.56 (m, 1H, H_{14}), 3.47 (s, 2H, H_{11}), 2.77–2.72 (m, 2H, H_{12}), 2.14–2.08 (m, 2H, H_{12}), 1.84–1.78 (m, 2H, H_{13}), 1.56–1.48 (m, 2H, H_{13}).

^{13}C NMR (100 MHz, Acetone-d_6) δ 189.46 (C_5), 162.49 (C_6), 137.62 (C_7), 136.56 (C_4), 135.33 (C_1), 134.31 (C_{10}), 131.49 ($C_3 \times 2$), 130.24 ($C_9 \times 2$), 129.61 ($C_2 \times 2$), 120.86 ($C_8 \times 2$), 63.02 (C_{11}), 52.04 (C_{14}), 35.65 ($C_{12} \times 2$), 30.45 ($C_{13} \times 2$, embedded in the solvent peak of acetone).

3. ^{1}H-NMR and ^{13}C-NMR spectra



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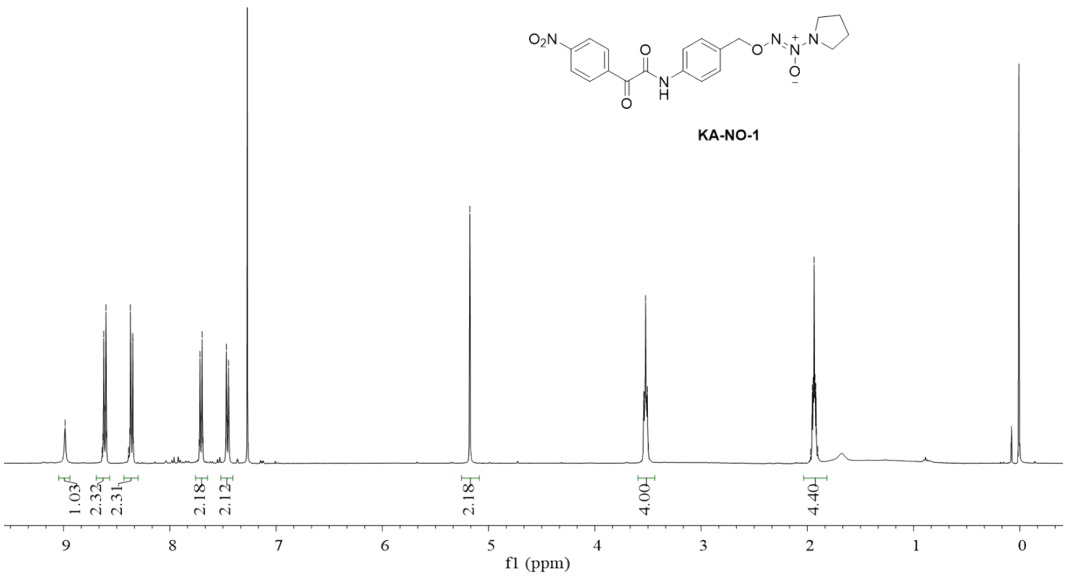
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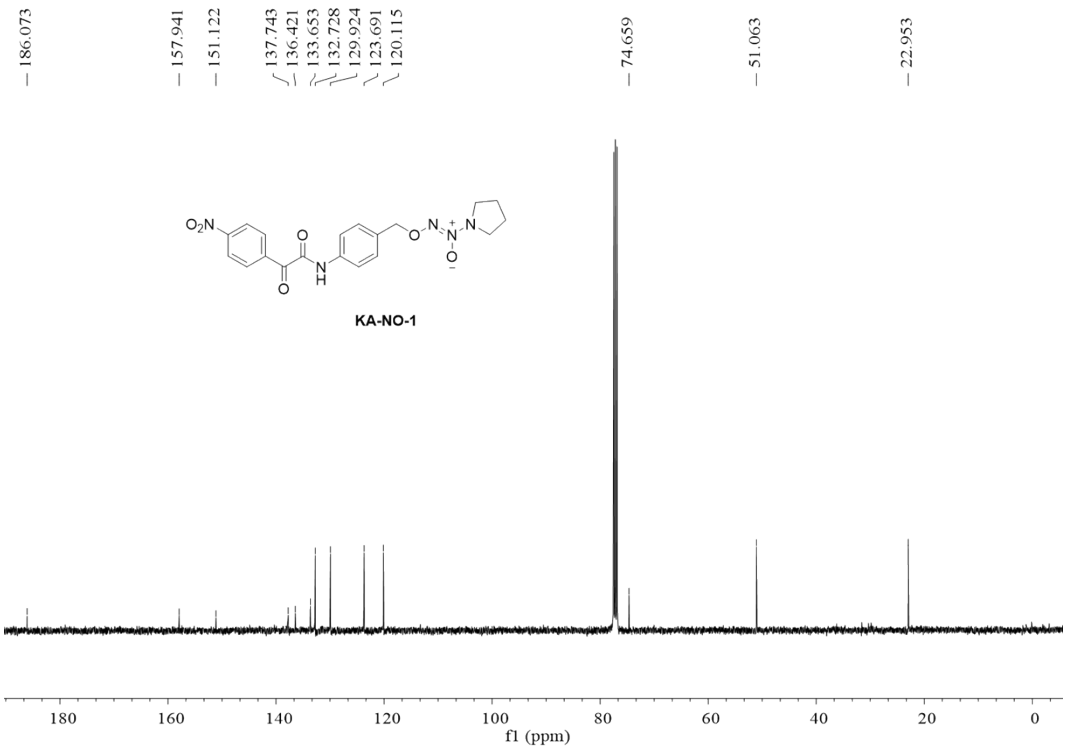
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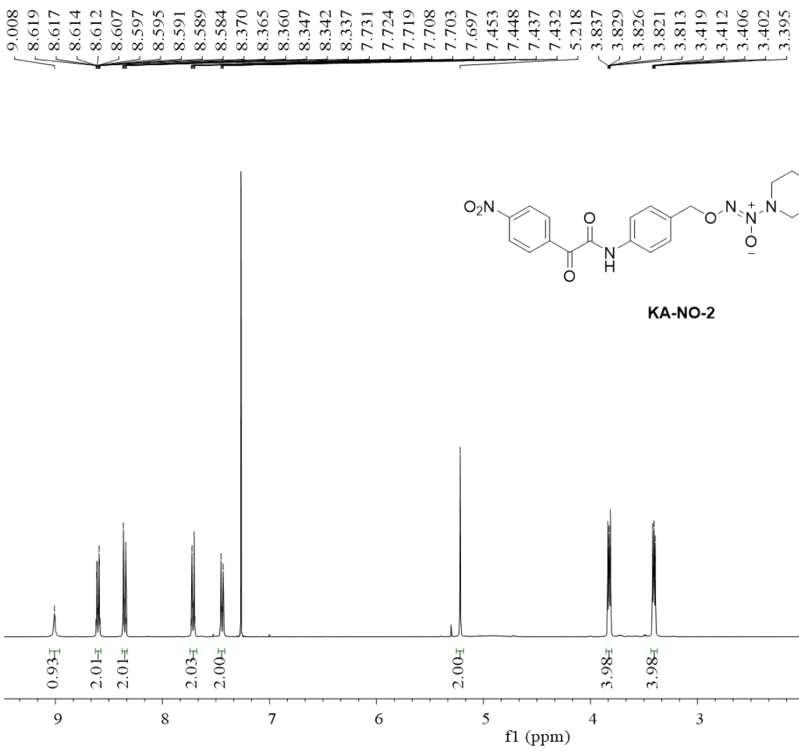
KA-NO-1
f1 (ppm)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3.829
3.826
3.821
3.813
3.419
3.412
3.406
3.402
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2.00
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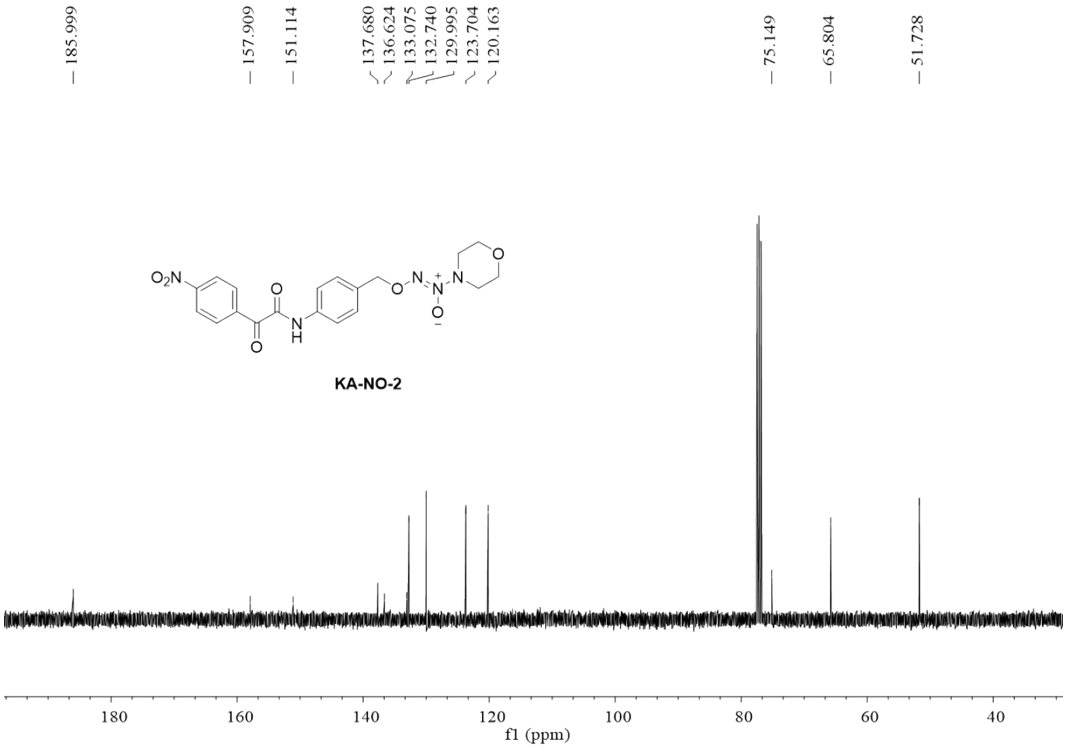
3.98
f1 (ppm)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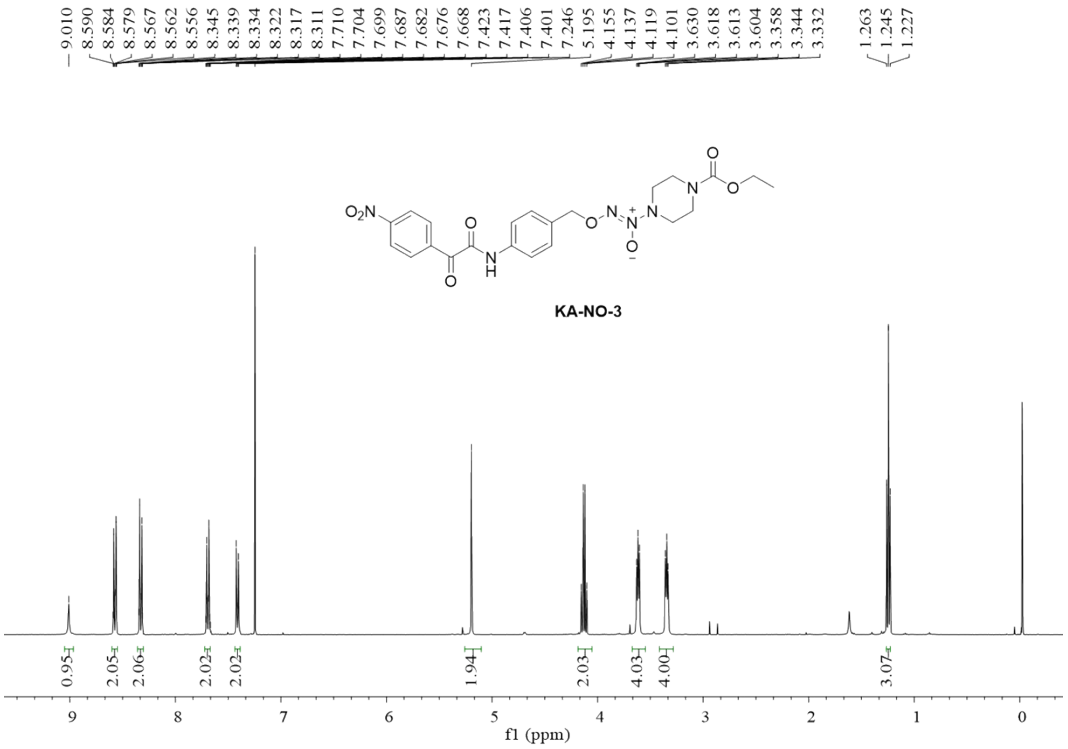
KA-NO-2
f1 (ppm)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KA-NO-3
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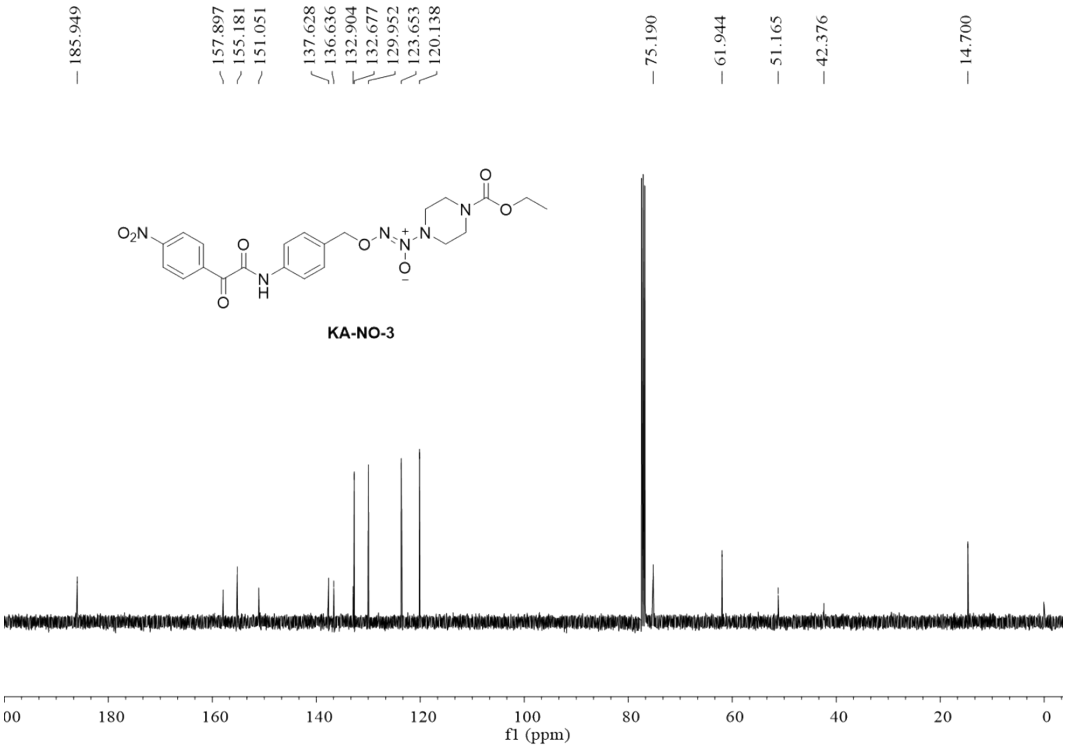
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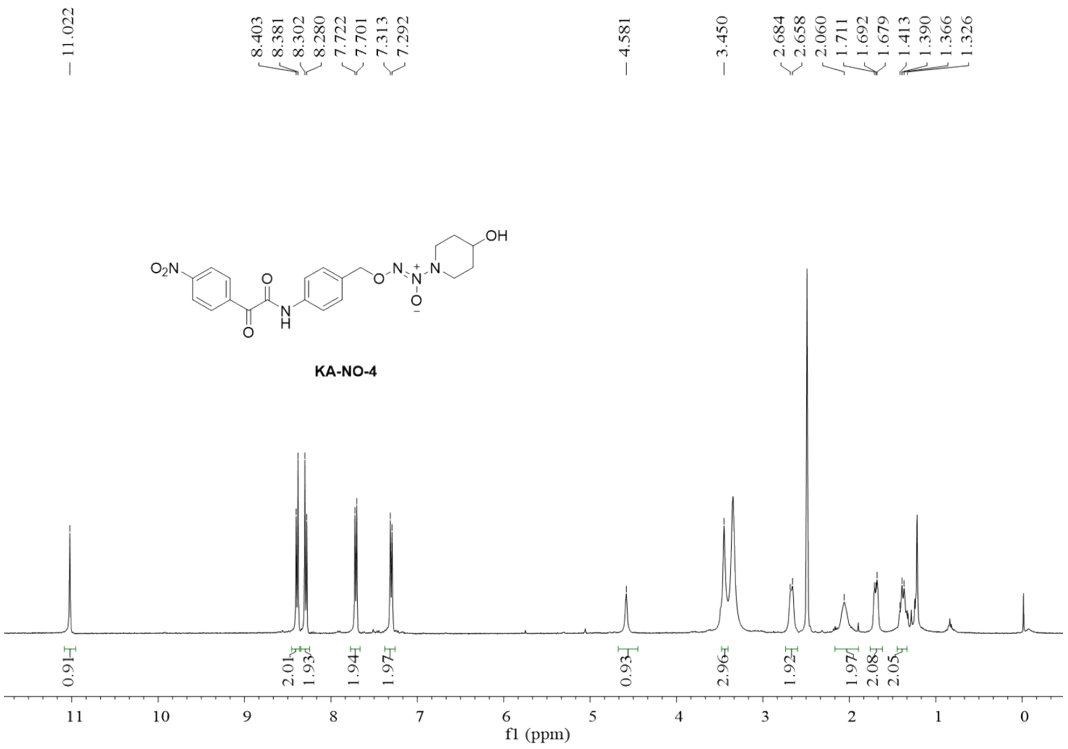
KA-NO-3
f1 (ppm)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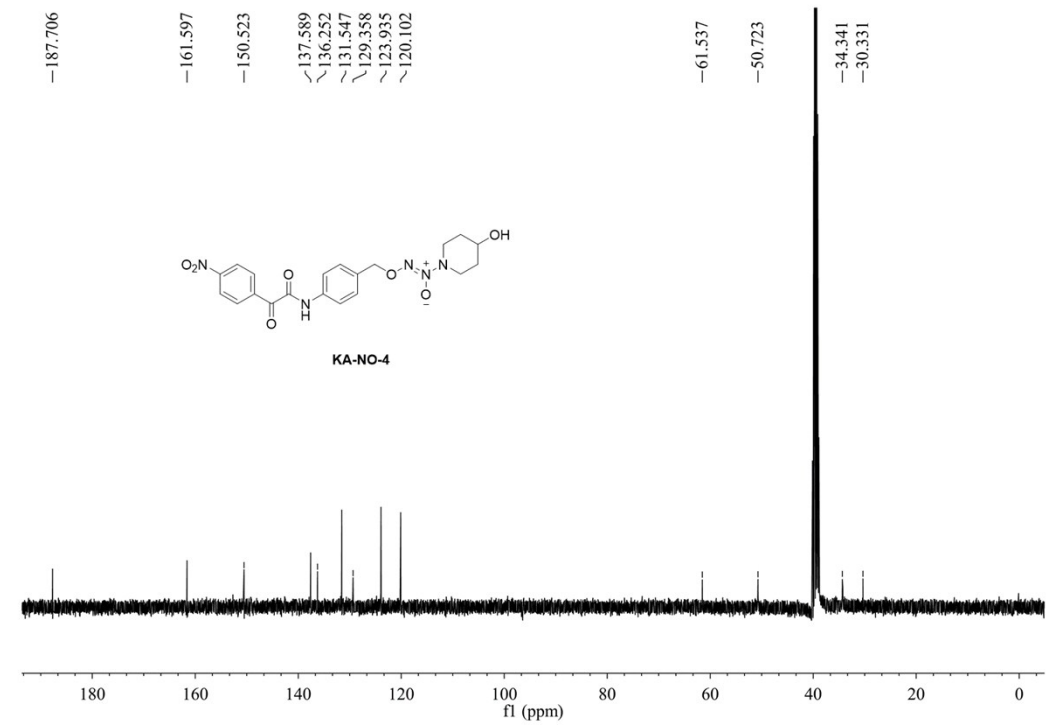
2.060
1.711
1.692
1.679
1.413
1.390
1.366
1.326
KA-NO-4
0.91
2.01
1.93
1.94
1.97
0.93
2.96
1.92
1.97
2.08
2.05
f1 (ppm)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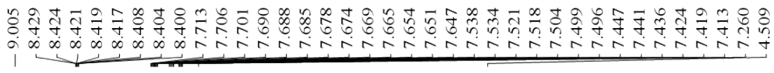
f1 (ppm)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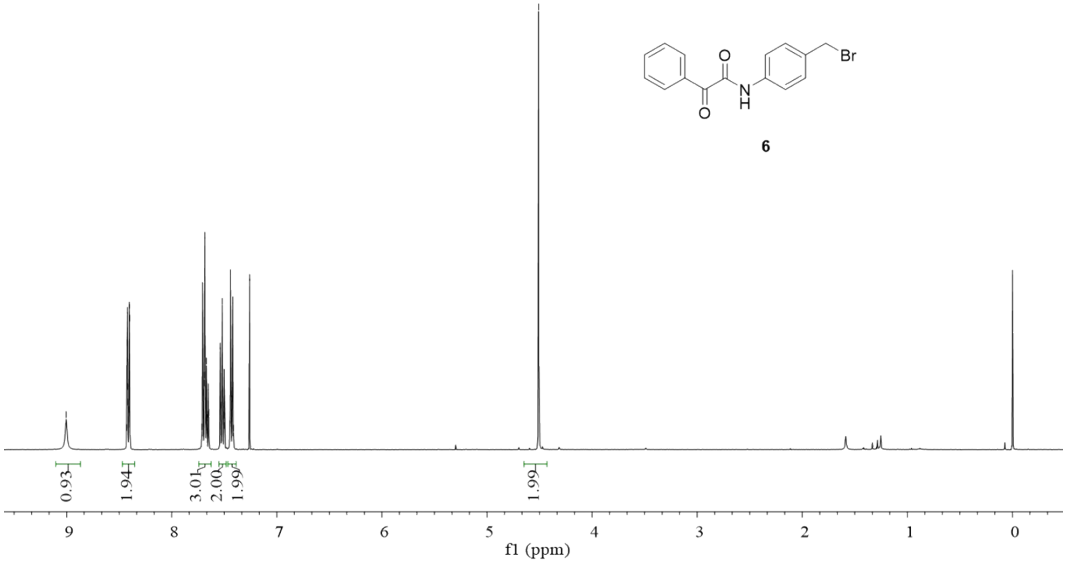
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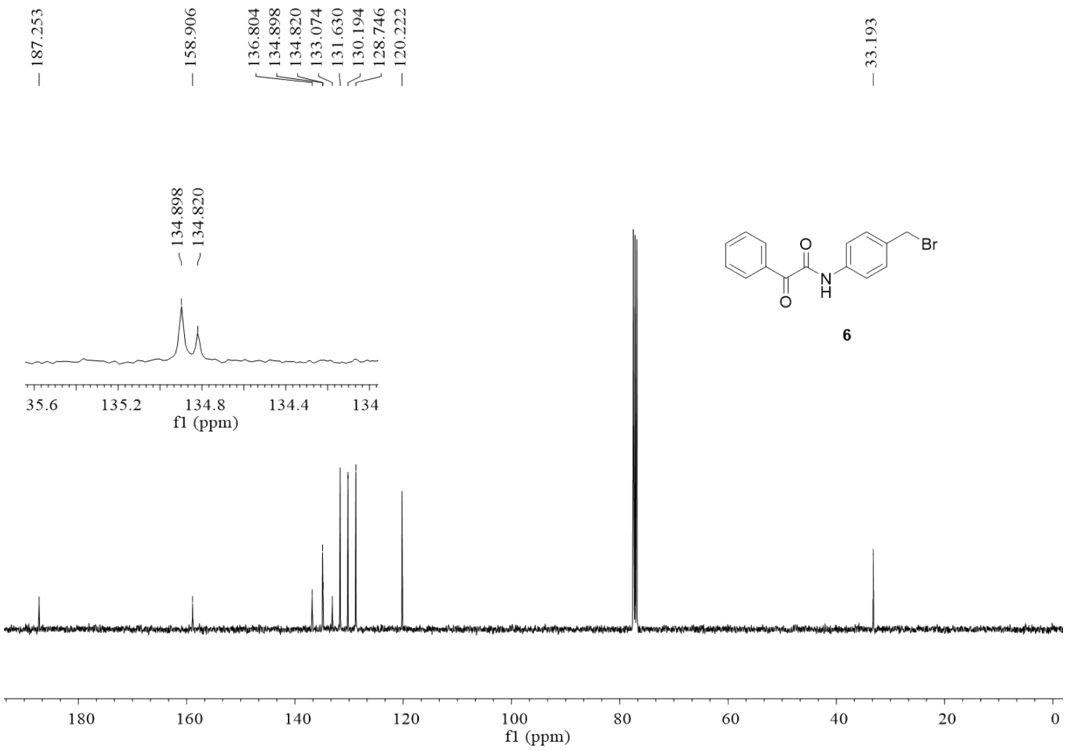
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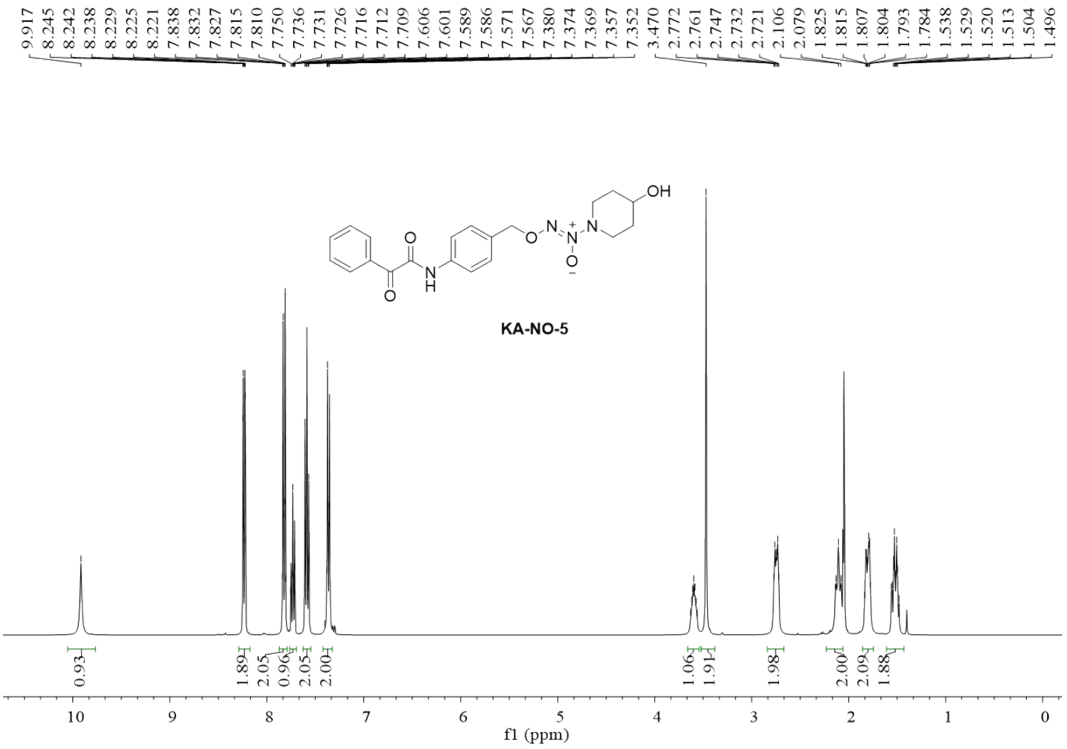
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NH
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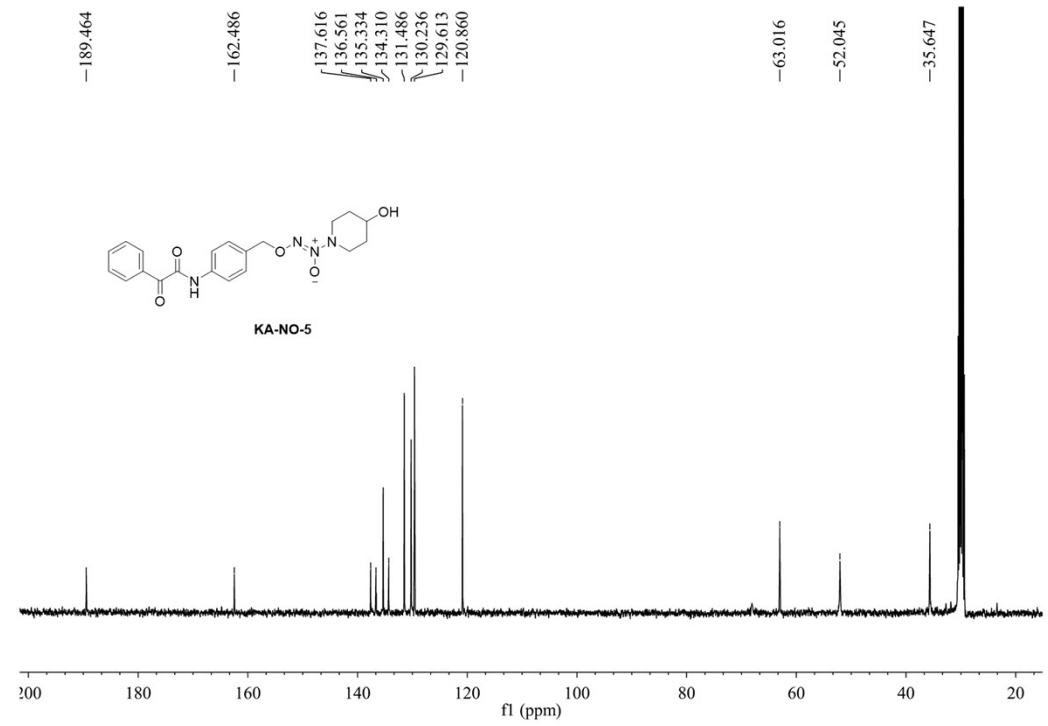
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KA-NO-5
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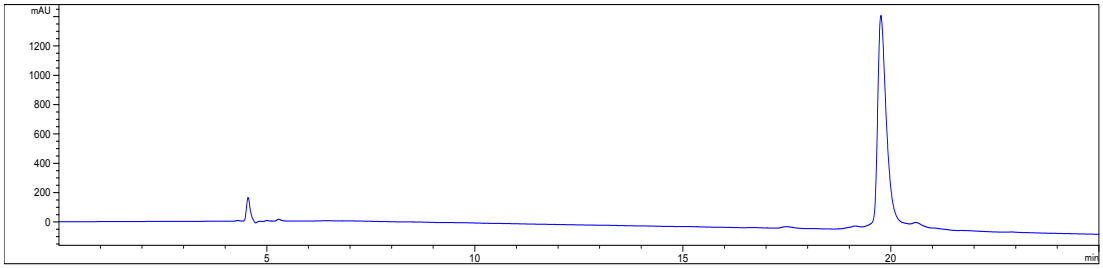
20
f1 (ppm)

4. HPLC purity of targeted compounds

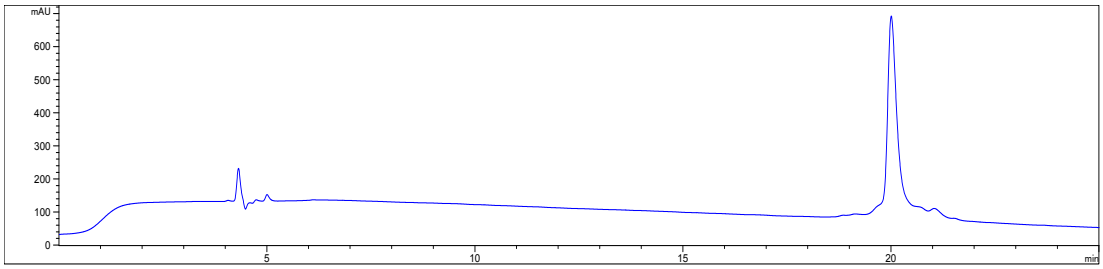
KA-NO-1

96.3%



KA-NO-2

94.0%



KA-NO-3

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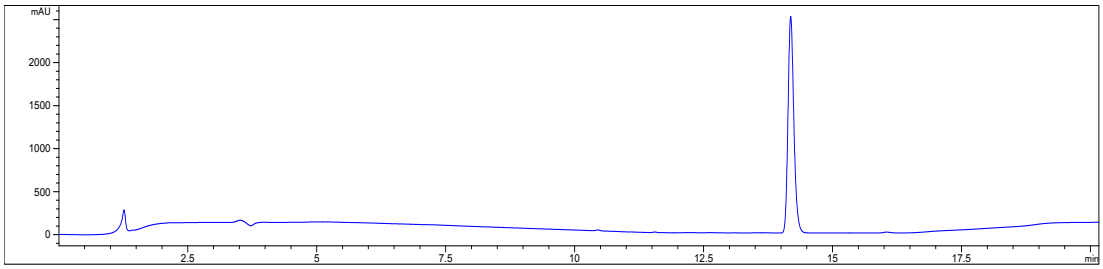
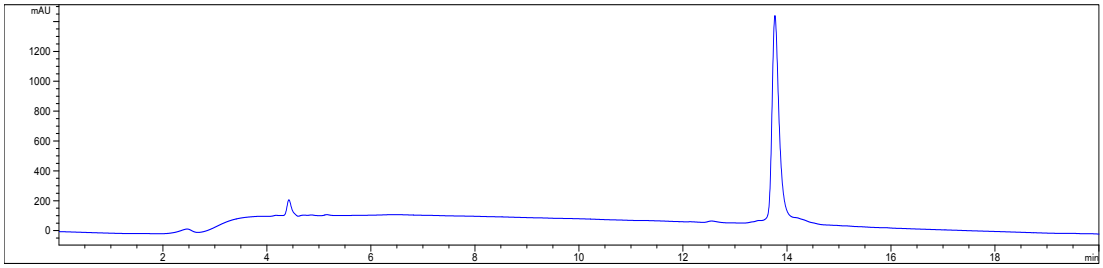


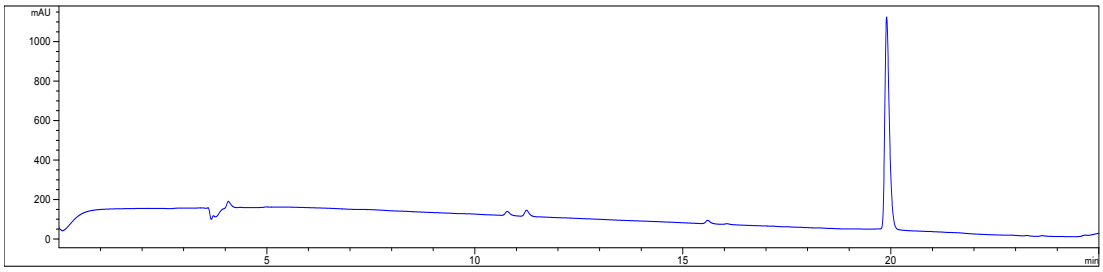
KA-NO-4

96.2%



KA-NO-5

97.2%



5. Analytical and biological experiments

HPLC analysis

HPLC analysis was performed on LC-10A HPLC system (C18 column, 4.6 mm × 200 mm, LC-10ATvp pumps, and SPD-10Avp UV detector). The gradients of solvent A (CH_3CN, 0.1% TFA) and B (H_2O, 0.1% TFA) are listed below:

0–5 min: 20% A

5–20 min: 20–95% A

20–25 min: 95% A

Detection wavelength is 254 nm.

Cytotoxicity assays

The cytotoxicity of adhesive cells was determined using MTT assay. All the cell lines were purchased from American Tissue Culture Collection (ATCC, Rockville, MD, USA). Cells were planked in a 96 well plate with a concentration of 6×10^4 cells per well and cultured at 37 °C with 5% CO_2 for 24 h. Then cells were treated with tested compounds and incubated for an additional 72 h. MTT (5 mg/mL, 20 μL) was added and the plate was kept at 37 °C for another 4 h. Then the medium was carefully removed. Dimethyl sulfoxide (150 μL per well) was added and oscillated gently to make crystal dissolved. The absorbance at 490 nm was measured using a microplate reader.

The cytotoxicity of suspension cell HL-60 was determined using cell counting kit-8 (CCK-8) (MCE, NJ, USA) according to the manufacturer's protocol. HL-60 cells (1×10^4 per well) were seeded in 96-well plates and the plates were placed at 37 °C with 5% CO_2 for 12 h. Then cells were treated with tested compounds and incubated for an additional 72 h. CCK-8 (10 μL) was added and the plates were incubated at 37 °C with 5% CO_2. for another 4 h. The fluorescent signal was recorded at the wavelength of 450 nm on a microplate reader (Thermo Labsystems, Helsinki, Finland). In case of *N*-acetylcysteine (NAC) pretreatment, cells were treated with 20 mM NAC for 1 h at 37 °C, and the following procedures were the same as described above.

The percentage of cell death was calculated using the formula:

$$\text{Cell death\%} = 1 - \frac{\text{mean OD of drug treated cells} - \text{mean OD of medium}}{\text{mean OD of drug untreated cells} - \text{mean OD of medium}} \times 100\%$$

Stability of KA-NO-4 in bovine plasma

A solution of KA-NO-4 (1 mM) in bovine plasma (10 mL) containing 5% DMSO was stirred at 37 °C. Aliquots (500 μL) of the plasma solution were added to methanol (500 μL), followed by centrifugation at 12000 rpm for 5 min. The obtained clear solutions were analyzed by HPLC to determine the remaining concentrations of KA-NO-4.

Selectivity of KA-NO-4 toward H_2O_2

The stock solutions of reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS) were prepared as follow:

H_2O_2, *tert*-butylhydroperoxide (TBHP), and hypochlorite (NaOCl) were purchased from 30%, 70% and 13% commercial aqueous solutions, respectively.

H_2O_2 stock solution (100 mM) was prepared by adding 11 μL of 30% (w/v) H_2O_2 to 989 μL of H_2O.

TBHP stock solution (100 mM) was prepared by adding 12 μL of 70% (w/v) TBHP to 998 μL H_2O.

NaOCl stock solution (100 mM) was prepared by adding 22 μL of 13% (w/v) NaOCl to 978 μL H_2O.

Hydroxyl radical (OH•) and *tert*-butoxy radical (^{t}BuO•) stock solutions (200 μM) were generated by reaction of 1 mM Fe^{2+} with 200 μM H_2O_2 or 200 μM TBHP respectively.

Peroxynitrite solution ($ONOO^-$) was freshly prepared before use as reported. A mixture of H_2O_2 (0.7 M, 1.5 mL), $NaNO_2$ solution (0.6 M, 3 mL) in an ice bath was acidified with HCl (0.6 M, 1.5 mL), and NaOH solution (1.5 M, 3 mL) was added within 1−2 s to make the solution alkaline. The final concentration of $ONOO^-$ was determined by measuring the absorbance at 302 nm (ε = 1670 M^{-1} cm^{-1}). [$ONOO^-$] (mM) = $Abs_{302\ nm}$ /1.67.

NO stock solution (100 mM) was freshly prepared before use by dissolving 29.8 mg sodium nitroprusside dihydrate in 1000 μL H_2O.

GSH (200 mM), Cys (200 mM) and Hcy (200 mM) stock solutions were prepared by adding 62.3 mg GSH, 12.1 mg Cys and 13.52 mg Hcy to 500 μL H_2O, respectively.

KA-NO-4 (100 μM) was incubated with ROS (1 mM), RNS (1 mM) or RSS (5 mM) in PBS (pH 7.4, 10 mM)/MeCN (v/v, 80/20) solution at 37 °C for 1 h, and the percentage of decomposed KA-NO-4 was determined by RP-HPLC.

Griess assay

For extracellular NO release study, KA-NO prodrugs (50 μM) in PBS (pH 7.4, 10 mM) /MeCN (v/v, 80/20) solution were treated with 10 eq. of H_2O_2 at 37 °C for 8 h. The nitrite content in each group was detected by the Griess assay using the nitrite colorimetric assay kit (Beyotime, Nanjing, China). The absorbance was recorded at 540 nm on a spectrophotometer (Smart spec, Bio-Rad) using $NaNO_2$ as a standard to establish the calibration curve.

For intracellular NO release study, A549 cells were treated with 6 μM of each compound for 8 h, or pretreated with NAC (20 mM) for 1 h, followed by treatment with KA-NO-4 for 8 h. The nitrite contents of cell lysates were detected by Griess assay using the same method as described above.

Intracellular NO release assay by DAF-FM DA

The NO released from tested compounds in A549 cells were measured using an NO-sensitive reagent fluorophore DAF-FM DA. When cells grown in a 96- well plate reached 80% confluence, they were washed with PBS. After being loaded with 5 μM DAF-FM DA at 37 °C for 20 min, the cells were rinsed three times with PBS and incubated with test compounds for 8 h. In case of NAC or C-PTIO pretreatment, cells were treated with NAC (20 mM) or C-PTIO (50 μM) for 1h, and then incubated with 6 μM KA-NO-4 for 24 h. NO production was measured with the flow cytometer with excitation and emission wavelengths of 495 and 515 nm, respectively.

Nitrated mitochondrial cytochrome c (3-NT Cyt-c) determination

A549 cells were treated with different concentrations of KA-NO-4 or JS-K for 24 h, In case of NAC or C-PTIO pretreatment, cells were treated with NAC (20 mM) or C-PTIO (50 μM) for 1h, and then incubated with 6 μM KA-NO-4 for 24 h. Mitochondrial proteins were extracted by centrifugation. After being lysed, the mitochondrial protein lysates (30 μg/lane, ~500 ng Cyt-c) were separated by SDS-PAGE, transferred on PVDF membranes, and probed with anti-3NT antibody (Proteintech Group Inc., IL, USA).

Annexin V-FITC/PI assay

A549 cells were planted at 5 × 10^5 per well in 6-well plate and incubated for 24 h. Cells were treated with different concentrations of KA-NO-4 or JS-K for 24 h, In case of NAC or C-PTIO pretreatment, cells were treated with NAC (20 mM) or C-PTIO (50 μM) for 1h, and then incubated with 6 μM KA-NO-4 for 24 h. Cells were collected and washed twice with ice-cold PBS, resuspended in 300 μL 1× binding buffer. Annexin V-FITC (KGA105, Keygentec, China) (5 μL) was added into each sample and incubated for 15 min at r.t. in darkness. PI (KGA105, Keygentec, China) (5 μL) was added into cells and stained for 5 min at r.t. in darkness. At last, 200 μL 1× binding buffer was added, and cellular apoptosis was analysed by using flow cytometry with a BD FACScalibur (BD Bioscience, Heidelberg, Germany).

Cell cycle analysis

A549 cells were planted at 5×10^5 per well in 6-well plate and incubated for 24 h. Cells were treated with different concentrations of KA-NO-4 or JS-K for 24 h. In case of NAC or C-PTIO pretreatment, cells were treated with NAC (20 mM) or C-PTIO (50 μM) for 1h, and then incubated with 6 μM KA-NO-4 for 24 h. Cells were washed in ice-cold PBS, and fixed in ice-cold 70% alcohol for at least 24 h. After that, cells were washed twice in ice-cold PBS, resuspended in 0.4 mL PBS and treated with 30 μL RNase A (1 mg/mL) (Thermo Scientific, MA, USA). Then 50 μL PI (500 ug/mL) (KGA105, Keygentec, Nanjing, China) was added into cells to stain the cellular DNA, and the staining process lasted 30 min at 4 °C in darkness. The DNA content of the stained cells was analyzed by flow cytometry with a BD FACScalibur (BD Bioscience, Heidelberg, Germany), and the cycle distribution was quantified.

Cell mitochondrial membrane potential assay

A549 cells were planted at 5×10^5 per well in 6-well plate and incubated for 24 h. Cells were treated with different concentrations of KA-NO-4 or JS-K for 24 h. In case of NAC or C-PTIO pretreatment, cells were treated with NAC (20 mM) or C-PTIO (50 μM) for 1h, and then incubated with 6 μM KA-NO-4 for 24 h. The cells were stained with the lipophilic cationic dye JC-1 (KGA602, Keygentec, China) according to the manufacturer's instruction. The percentage of cells with healthy or collapsed mitochondrial membrane potentials was monitored by flow cytometry with a BD FACScalibur (BD Bioscience, Heidelberg, Germany).

Western blot assay

A549 cells were planted at 5×10^5 per well in 6-well plate and incubated at 37 °C for 24 h. Then cells were treated with different concentration of KA-NO-4 or JS-K for indicated time. In case of NAC or C-PTIO pretreatment, cells were treated with NAC (20 mM) or C-PTIO (50 μM) for 1h, and then incubated with 6 μM KA-NO-4 for 24 h.

A549 cells were placed on ice for 10 min, followed by two rounds of centrifugation at 10000 rpm for 15 min and protein concentration was measured using a BCA (KGA902, Keygentec, China) protocol. Protein was stored at -70 °C until further use. SDS-PAGE (KGP113, Keygentec, China) was used to separate the proteins and then transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in 5% milk in Tris-buffered saline. Membranes were incubated in antibodies, including anti-Cleaved Caspase3 antibody (ab2302, Abcam, UK), anti-Cleaved Caspase9 antibody (ab2324, Abcam, UK), anti-Bax antibody (ab32503, Abcam, UK), anti-Bcl-2 antibody (ab182858, Abcam, UK), anti-MMP-2 antibody (ab37150, Abcam, UK), and anti-NM23-H1 antibody (Novus Biologicals LLC, CO, USA), overnight at 4 °C. Following three washes in TBST (1×) (NBP1-47398, KGP103X, Keygentec, China), and then incubated with the secondary antibodies conjugated to horseradish peroxidase at r.t for 2 h. Membranes were washed three times in TBST and then incubated in an enhanced chemiluminescence (ECL) (KGP116, Keygentec, China) detection substrate for 5 min. Densitometry of the appropriate bands was performed using G: Box ChemiXR5 (Syngene, MD, USA).

Transwell assay

A549 cells were seeded on Matrigel-coated chambers and treated with KA-NO-4 for 48 h. In case of NAC or C-PTIO pretreatment, cells were treated with NAC (20 mM) or C-PTIO (50 μM) for 1h, and then incubated with 200 nM KA-NO-4 for 48 h. Cells that migrated through the Matrigel-coated chambers were stained with crystal violet. Representative images were captured, and the cells were counted from three independent experiments.

6. Supplementary Figures

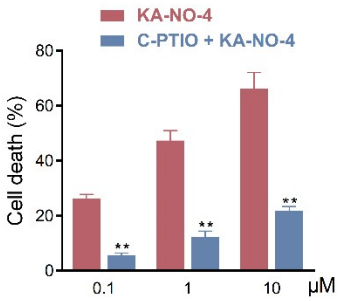


Fig. S1 The effects of C-PTIO pretreatment on the cytotoxicity of KA-NO-4 in A549 cells. A549 cells were treated with or without C-PTIO (50 μM) for 1 h, followed by incubated with different concentrations of KA-NO-4 for 48 h. The percentage of cell death was evaluated by MTT. Mean ± SD, n = 3, $^{**}p < 0.01$ vs. KA-NO-4.

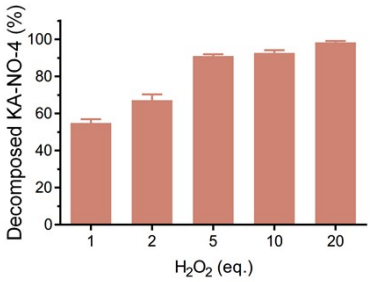


Fig. S2 H_2O_2 equivalent-dependent decomposition of KA-NO-4. KA-NO-4 (100 μM) in PBS (pH 7.4, 10 mM) /MeCN (v/v, 80/20) solution was treated with 1–20 eq. of H_2O_2 at 37 °C for 1 h, and the percentage of decomposed KA-NO-4 was determined by HPLC. Mean ± SD (n = 3).

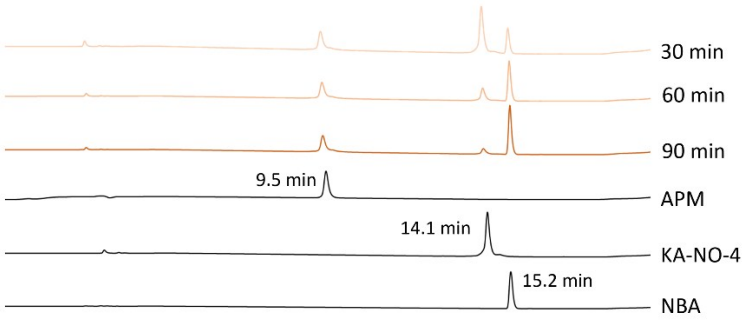


Fig. S3 Decomposition of 100 μM KA-NO-4 in presence of H_2O_2 (10 eq.) as monitored by RP-HPLC (254 nm).

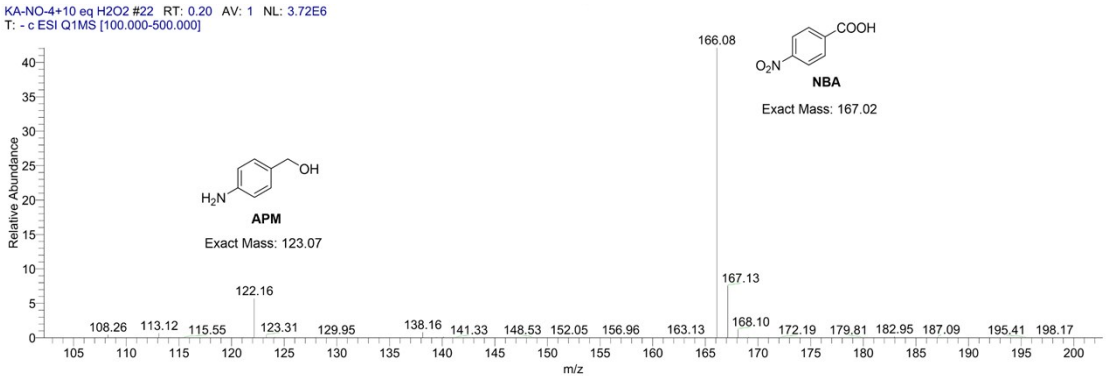


Fig. S4 ESI-MS spectrum of KA-NO-4 (100 µM) after 1 h H_2O_2 (10 eq.) treatment in PBS 7.4 (10 mM)/MeCN (v/v, 80/20).

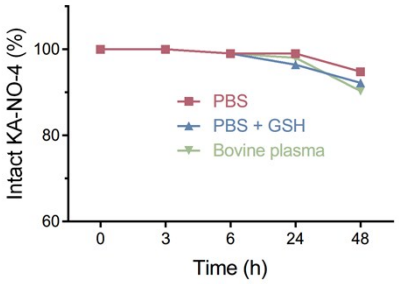


Fig. S5 Stability of KA-NO-4 (100 µM) in PBS (pH = 7.4,10 mM)/MeCN (v/v, 80/20) with or without 5 mM GSH and in bovine plasma (5% DMSO). The percentage of intact KA-NO-4 at indicated time point was determined by HPLC.

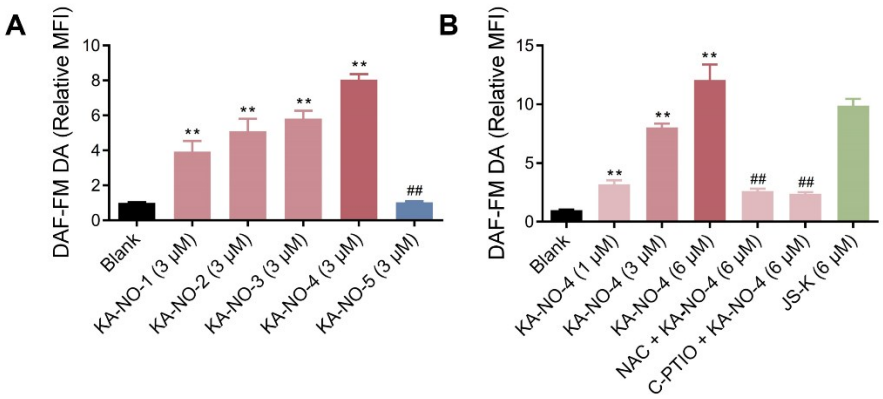


Fig. S6 Intracellular NO release from KA-NO prodrugs detected by DAF-FM DA. A549 cells were treated with indicated compound for 8 h, or pretreated with NAC (20 mM) or C-PTIO (50 µM) for 1 h, followed by treatment with KA-NO-4 for 8 h. Cells were stained with DAF-FM DA and analyzed by flow cytometry. Mean ± SD, n = 3, $^{**}p < 0.01$ vs. blank, $^{\#\#}p < 0.01$ vs KA-NO-4 (6 µM).

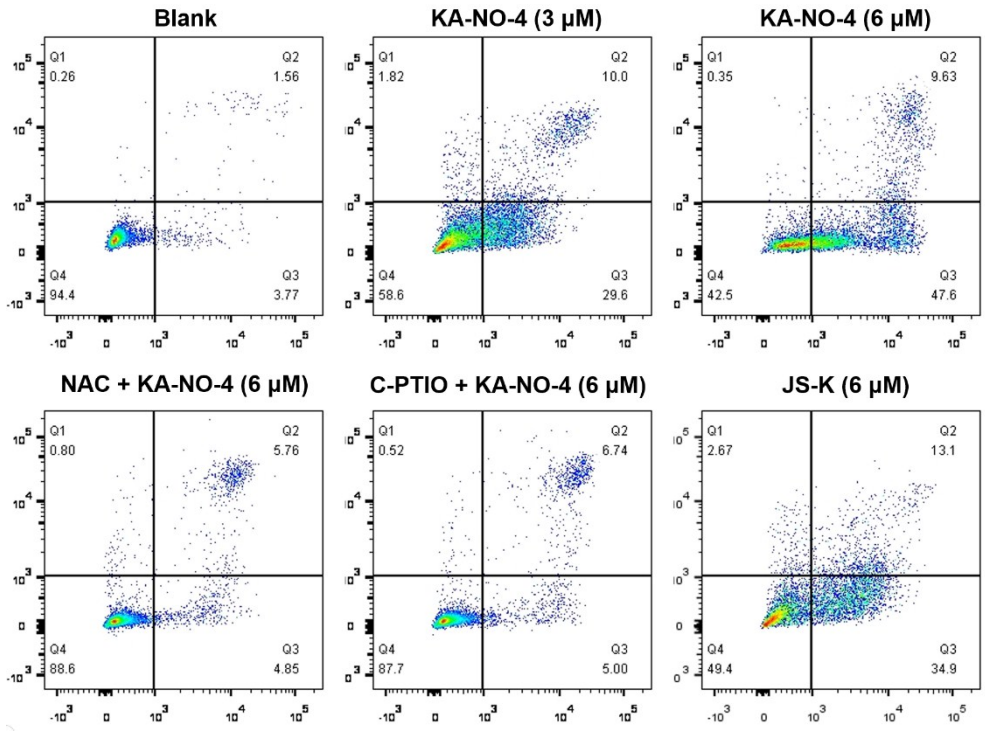


Fig. S7 Apoptotic effects of KA-NO-4 on A549 cells. A549 cells were treated with compounds at indicated concentrations for 24 h, or pre-incubated with ROS scavenger NAC (20 mM) for 1 h. In case of NAC or C-PTIO pretreatment, cells were treated with NAC (20 mM) or C-PTIO (50 μM) for 1h, and then incubated with 6 μM KA-NO-4 for 24 h. Apoptotic effects were measured by flow cytometry using annexin V-FITC/PI staining protocol. Percentage of cells in each quadrant (%) is labelled.

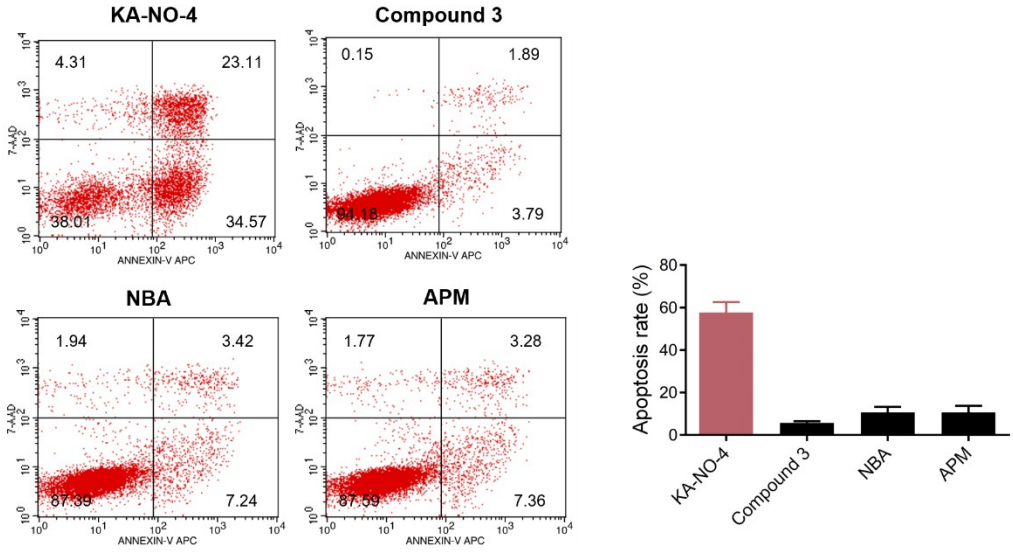


Fig. S8 Apoptotic effects of KA-NO-4, compound **3**, NBA, and APM on A549 cells. A549 cells were treated with 6 μM of each compound for 24 h. Apoptotic effects were measured by flow cytometry using annexin V-FITC/PI staining protocol. Percentage of cells in each quadrant (%) is labelled.

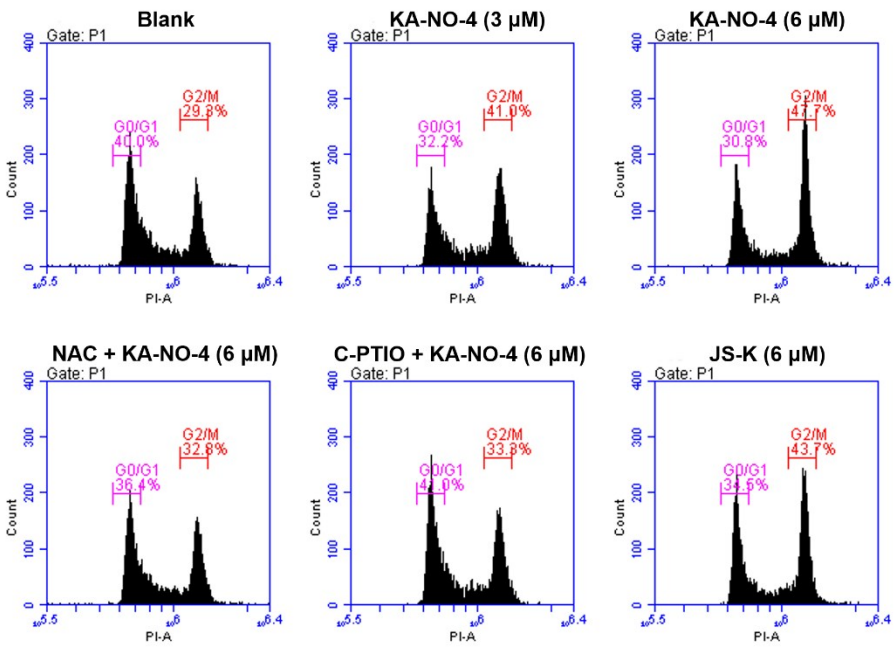


Fig. S9 Effects of KA-NO-4 on cell cycle arrest. A549 cells were treated with compounds at indicated concentrations for 24 h. In case of NAC or C-PTIO pretreatment, cells were treated with NAC (20 mM) or C-PTIO (50 μM) for 1h, and then incubated with 6 μM KA-NO-4 for 24 h. The percentage of cells in each cell cycle phase was analyzed by flow cytometry.

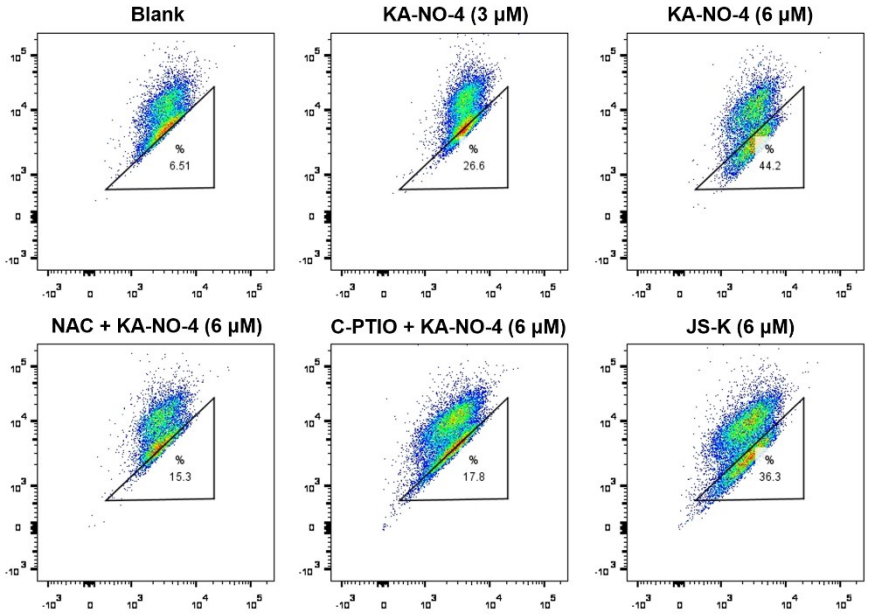


Fig. S10 Effect of KA-NO-4 on mitochondrial membrane potentials ($\Delta\Psi_m$) of A549 cells. A549 cells were treated with compounds at indicated concentrations for 24 h. In case of NAC or C-PTIO pretreatment, cells were treated with NAC (20 mM) or C-PTIO (50 μM) for 1h, and then incubated with 6 μM KA-NO-4 for 24 h. $\Delta\Psi_m$ was measured by flow cytometry using JC-1 staining protocol.

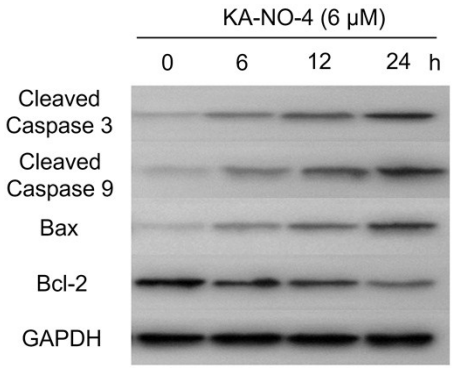


Fig. S11 Effect of KA-NO-4 on the expression of cleaved Capspase 3, cleaved Caspase 9, Bax, and Bcl-2. A549 cells were treatd with 6 μM KA-NO-4 for indicated time.