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Anti-proliferative, -migratory and -clonogenic effects of long-lasting nitric oxide release in HepG2 cells

Rakhi Bormon[a], Ekta Srivastava[b], Rafat Ali[a], Prerna Singh[b], Ashok Kumar*[bcdef], and Sandeep Verma*[acdef]

[a]Department of Chemistry, Indian Institute of Technology Kanpur, Kanpur 208016, UP, India.

[b]Department of Biological Sciences & Bioengineering, Indian Institute of Technology Kanpur, Kanpur 208016, UP, India.

[c]Centre for Environmental Science and Engineering, Indian Institute of Technology Kanpur, Kanpur-208016, UP, India

[d]Centre for Nanosciences, Indian Institute of Technology Kanpur, Kanpur-208016, UP, India

[e]Mehta Family Centre for Engineering in Medicine, Indian Institute of Technology Kanpur, Kanpur-208016, UP, India

[f]Gangwal School of Medical Sciences and Technology, Indian Institute of Technology Kanpur, Kanpur-208016, UP, India

E-mail: sverma@iitk.ac.in

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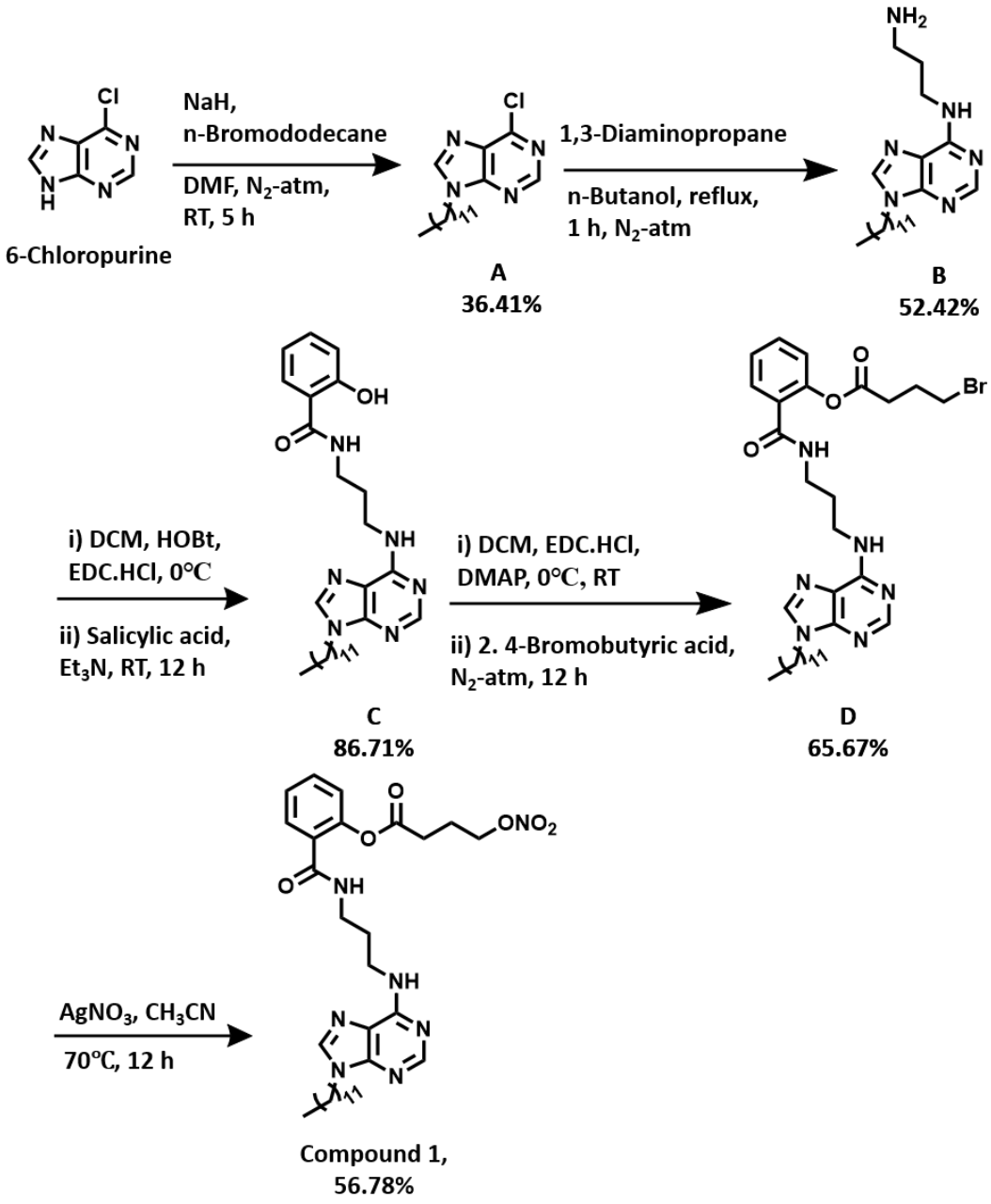
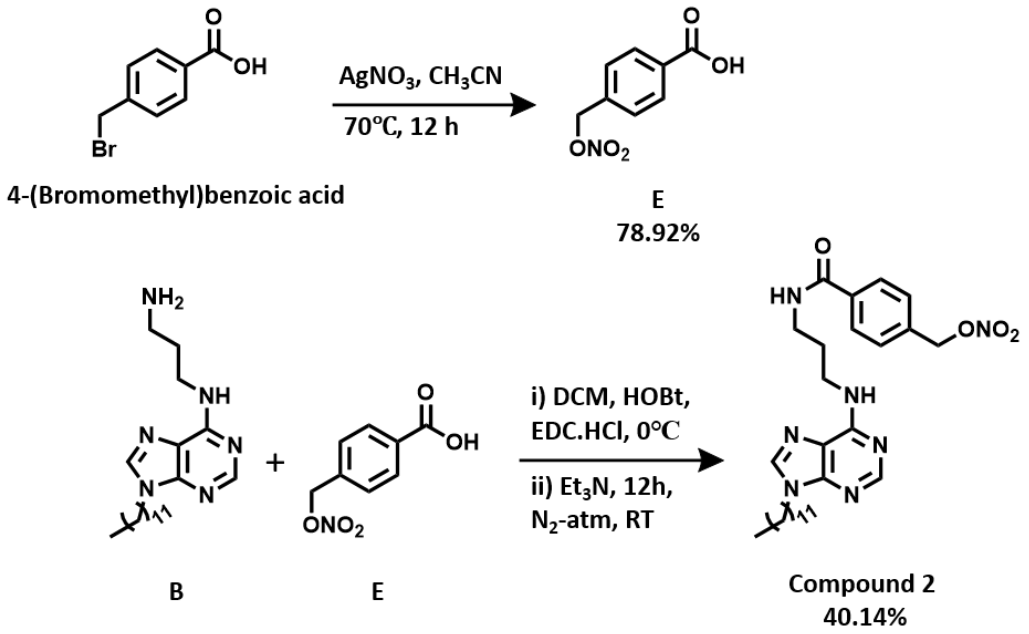
1. General information: Acetonitrile (CH_3CN), dichloromethane (DCM), methanol (CH_3OH), and trimethylamine (Et_3N) were distilled following standard procedures of distillation. 6-

Chloropurine, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl), Triethyl amine (Et_3N), Glutathione reduced (GSH), n-Butanol, Sodium phosphate monobasic dihydrate (NaH_2PO_4. $2H_2O$), Sodium phosphate dibasic dihydrate (Na_2HPO_4. $2H_2O$), Sodium hydroxide (NaOH), and n-Bromododecane were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai India). Sodium hydride (NaH) and acetonitrile were purchased from S D Fine Chem Ltd. (Mumbai India). Dimethylformamide (DMF), dichloromethane (DCM), methanol, ethyl acetate, hexane, hydrochloric acid (HCl), and Sodium chloride were obtained from Finar Ltd. (Gujarat India). Salicylic acid was purchased from Sigma-Aldrich Chemicals Pvt. Ltd. (Bangalore India). 1-Hydroxybenzotriazole (HOBt) and 1,3-Diaminopropane were obtained from Avra Synthesis Pvt. Ltd. (Hyderabad India). 4-dimethylaminopyridine (DMAP), Tetrahydrofuran (THF) and 4-bromobutyric acid were obtained from Spectrochem Pvt. Ltd. (Mumbai India). Silver nitrate ($AgNO_3$) was purchased from RFCL Ltd. (New Delhi, India). Sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) were purchased from Alfa Aesar (England). Celite 545 and Orthophosphoric acid were purchased from Merck Specialities Pvt. Ltd. (Darmstadt, Germany). 4-(Bromomethyl)benzoic acid was purchased from Tokyo Chemical Industry Co. Ltd. (TCI, Tokyo, Japan). Anhydrous sodium sulphate was purchased from Avantor Performance Materials India Ltd. (Maharastra, India). The completion of the reactions was monitored by thin layer chromatography (TLC) using readymade TLC silica gel $60F_{254}$ plates purchased from Merck, Darmstadt, Germany. For the purification of the reaction mixture, column chromatography was done using 100−200 mesh silica gel (S. D. Fine-Chem Pvt. Ltd.). ^{1}H NMR (400 MHz & 500 MHz) and ^{13}C NMR (100 MHz & 125 MHz) spectra were recorded on JEOL-JNM spectrometer at 25 °C using appropriate solvent and TMS as internal standard. HRMS spectra were recorded on Waters, Q-Tof Premier Micromass HAB 213 mass spectrometer using capillary voltage 2.6-3.2 Kv. UV-Vis absorption spectra were recorded on Varian CARY 100 Bio UV-Vis spectrophotometer with 10 mm quartz cell at 25 °C. The cell culture studies were performed on mouse neuroblast (Neuro 2a), mouse embryonic fibroblast (NIH3T3) and human hepatocellular carcinoma (HepG2) cell lines, purchased from National Centre for Cell Science (NCCS), Pune. The cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, UK) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic incubated at 37 °C and 5% CO_2.

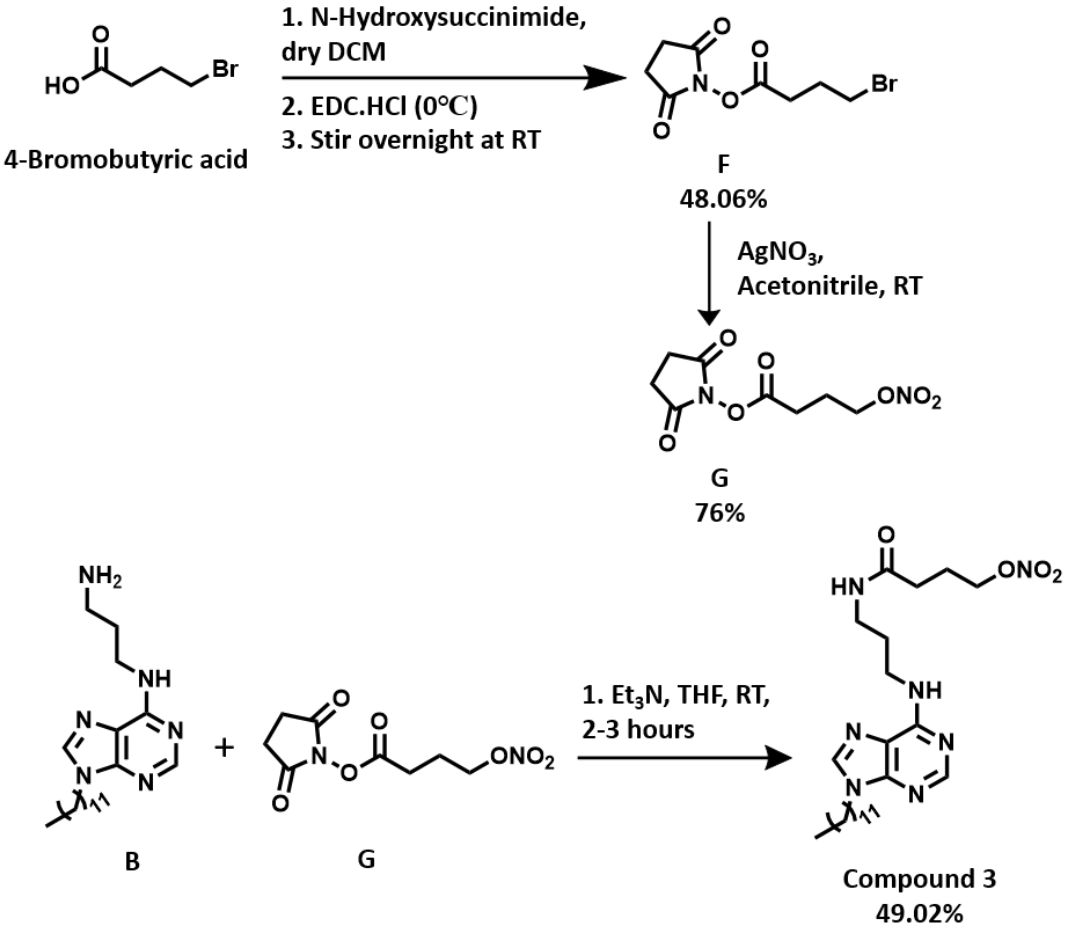
2. Synthetic schemes from compounds 1, 2 and 3:



Scheme S1: Synthesis of compound **1**



Scheme S2: Synthesis of compound **2**



Scheme S3: Synthesis of compound **3**

3. General experimental procedure and spectral data of compounds:

Synthesis of 6-chloro-9-dodecyl-9*H*-purine (A): To a solution of Sodium hydride (372 mg, 15.527 mmol) in DMF (50 mL), 6-chloropurine (2 gm, 12.939 mmol) was added under nitrogen atmosphere, and the reaction was left on stirring for 15 minutes. Then n-bromododecane was added to the solution and left the solution on stirring for 6-7 h at room temperature (RT). After completion of the reaction, the solvent was evaporated under reduced pressure. The reaction mixture was then dissolved in ethyl acetate and washed with water, followed by brine.[1] Then the organic layer was separated and dried over anhydrous sodium sulphate. The organic layer was evaporated under reduced pressure and the crude was purified by silica gel column chromatography using 10% ethyl acetate/hexane. Yield (1.521 gm, 36.41%); white solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm= 8.76 (s, 1H), 8.12 (s, 1H), 4.30 (t, J = 7.3 Hz, 2H), 1.94 (p, J = 7.2 Hz, 2H), 1.33 (d, J = 6.1 Hz 4H), 1.26 (d, J = 11.9 Hz, 14H), 0.88 (t, J = 6.9 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ/ppm= 152.01 (s), 151.14 (s), 145.26 (s), 131.71 (s), 44.69 (s), 32.00 (s), 29.98 (s), 29.53 (dd, J = 22.8, 9.4 Hz), 29.07 (s), 26.72 (s), 22.78 (s), 14.21 (s); ESI-HRMS: $[M+H]^+$, calculated for 323.1997, found= 323.1997.

Synthesis of N^1-(9-dodecyl-9*H*-purin-6-yl)propane-1,3-diamine (B): 1,3-Diaminopropane (3.44 gm, 46.45 mmol) was added to the solution of 6-chloro-9-dodecyl-9*H*-purine, **A** (1.5 gm, 4.645 mmol) in n-butanol (50 mL). After 1 h of reflux, the completion of the reaction was monitored by TLC. The solvent was evaporated under reduced pressure and the crude was dissolved in DCM. Then this reaction mixture was washed with water and the organic layer was collected and dried over anhydrous sodium sulphate. The organic solvent was then removed under reduced pressure and the crude was purified by crystallization method using diethyl ether solvent.[2] Yield (878 mg, 52.42%); white solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm= 8.37 (s, 1H), 7.73 (s, 1H), 6.63 (s, 1H), 4.16 (t, J = 7.3 Hz, 2H), 3.78 (s, 2H), 3.58 (s, 2H), 2.91 (t, J = 6.4 Hz, 2H), 1.92 – 1.83 (m, 4H), 1.32 (s, 4H), 1.29 – 1.19 (m, 14H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ/ppm= 155.14 (s), 153.24 (s), 139.68 (s), 44.00 (s), 40.21 (s), 32.95 (s), 32.03 (s), 30.26 (s), 29.58 (dd, J = 21.2, 11.8 Hz), 29.19 (s), 26.80 (s), 22.80 (s), 14.23 (s); ESI-HRMS: $[M+H]^+$, calculated for 361.3074, found= 361.3063.

Synthesis of *N*-(3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)-2-hydroxybenzamide (C): Salicylic acid (268.09 mg, 1.941 mmol) and 1-hydroxybenzotriazole (HOBt) (314.69 mg, 2.329 mmol) were dissolved in DCM (25 mL) under nitrogen atmosphere and cooled at 0°C in ice bath. Then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC. HCl) (446.47 mg, 2.329 mmol) was added at 0°C and the reaction mixture was stirred for half an hour. Then N^1-(9-dodecyl-9*H*-purin-6-yl)propane-1,3-diamine, **B** (700 mg, 1.941 mmol) was added to it, followed by the addition of triethylamine (Et_3N) (0.541 mL, 3.882 mmol). Then the reaction mixture was stirred at RT for overnight. The completion of the reaction was monitored by TLC. After completion, the reaction mixture was washed with 1N HCl, followed by 10% aqueous $NaHCO_3$ solution, and then with brine. The organic layer was collected and dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude was purified by silica gel column chromatography (3% methanol/DCM). Yield (809 mg, 86.71%); white solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm= 12.57 (s, 1H), 8.67 (s, 1H), 8.44 (s, 1H), 7.77 (s, 1H), 7.74 (d, J = 7.0 Hz, 1H), 7.41 (ddd, J = 8.6, 7.2, 1.6 Hz, 1H), 7.00 (dd, J = 8.4, 1.1 Hz, 1H), 6.94 – 6.88 (m, 1H), 6.09 (s, 1H), 4.19 (t, J = 7.3 Hz, 2H), 3.86 (s, 2H), 3.53 (dd, J = 11.7, 6.0 Hz, 2H), 1.90 (dt, J = 14.6, 7.2 Hz, 4H), 1.41 – 1.17 (m, 18H), 0.87 (dd, J = 8.3, 5.7 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ/ppm= 170.21 (s), 161.77 (s), 155.73 (s), 152.85 (s), 140.24 (s), 134.04 (s), 125.83 (s), 118.66 (s), 115.09 (s), 44.20 (s), 35.20 (s), 32.03 (s), 30.30 (d, J = 11.9 Hz), 29.59 (dd, J = 22.0, 11.5 Hz), 29.19 (s), 26.81 (s), 22.81 (s), 14.25 (s); ESI-HRMS: $[M+H]^+$, calculated for 481.3286, found= 481.3276.

Synthesis of 2-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)phenyl 4-bromobutanoate (D): EDC. HCl (193.746 mg, 1.248 mmol) was added to a solution of 4-bromobutyric acid (173.68 mg, 1.040 mmol) in DCM (15 mL) at 0°C under a nitrogen atmosphere. Then 4-dimethylaminopyridine (12.71 mg, 0.1040 mmol) was added to it and left on stirring at RT for half an hour. After that, *N*-(3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)-2-hydroxybenzamide, **C** (500 mg, 1.040 mmol) was added and left on stirring at RT for overnight. After completion of the reaction, the reaction mixture was washed with 1N HCl solution, followed by 10% aqueous $NaHCO_3$ solution, and then with brine. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. Then the crude was purified by silica gel column chromatography (3% methanol/DCM). Yield (430 mg, 65.67%); white solid; ^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm= 8.30 (s, 1H), 7.74 (dt, J = 22.9, 11.5 Hz, 3H), 7.48 (td, J = 7.8, 1.4 Hz, 1H), 7.32

(t, J = 7.7 Hz, 1H), 7.13 (d, J = 8.0 Hz, 1H), 6.19 (s, 1H), 4.16 (t, J = 7.2 Hz, 2H), 3.81 (s, 2H), 3.49 (q, J = 6.4 Hz, 4H), 2.78 (t, J = 7.1 Hz, 2H), 2.26 (p, J = 6.7 Hz, 2H), 1.94 – 1.83 (m, 4H), 1.32 (s, 4H), 1.26 (d, J = 16.3 Hz, 14H), 0.88 (t, J = 6.9 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.15 (s), 166.39 (s), 155.40 (s), 152.93 (s), 148.32 (s), 140.02 (s), 131.73 (s), 129.06 (s), 126.27 (s), 123.36 (s), 44.09 (s), 36.35 (s), 32.65 (d, J = 13.8 Hz), 32.02 (s), 29.97 (d, J = 66.1 Hz), 29.71 – 29.33 (m), 29.19 (s), 27.63 (s), 26.80 (s), 22.80 (s), 14.24 (s); ESI-HRMS: $[M+H]^+$, calculated for 629.2809, found= 629.2811.

Synthesis of 2-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)phenyl 4-(nitrooxy)butanoate (Compound 1): 2-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)phenyl 4-bromobutanoate, **D** (300 mg, 0.317 mmol) was dissolved in acetonitrile and then $AgNO_3$ (134.54 mg, 0.792 mmol)was added to it under dark condition. Then the reaction mixture was left on stirring at 70℃ for overnight. After the completion of the reaction, the reaction mixture was filtered using celite 545 and the filtrate was evaporated under reduced pressure. Then the residue was dissolved in ethyl acetate and washed with water twice. The organic layer was collected and dried over anhydrous sodium sulphate and evaporated under reduced pressure. The crude was then purified by silica gel column chromatography (3% methanol/DCM). Yield (110 mg, 56.78%); yellowish liquid. ^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm= 8.29 (s, 1H), 7.74 (dd, J = 7.6, 1.4 Hz, 3H), 7.50 (dd, J = 8.3, 7.1 Hz, 1H), 7.33 (t, J = 7.6 Hz, 1H), 7.14 (d, J = 7.7 Hz, 1H), 6.22 (s, 1H), 4.57 (t, J = 6.2 Hz, 2H), 4.16 (t, J = 7.3 Hz, 2H), 3.82 (s, 2H), 3.47 (dd, J = 11.9, 6.0 Hz, 2H), 2.74 (t, J = 7.0 Hz, 2H), 2.15 (dd, J = 13.3, 6.8 Hz, 2H), 1.93 – 1.83 (m, 4H), 1.32 (s, 4H), 1.26 (d, J = 16.3 Hz, 14H), 0.87 (t, J = 6.9 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ/ppm= 171.05 (s), 166.39 (s), 155.40 (s), 152.91 (s), 148.52 (s), 140.03 (s), 131.83 (s), 128.75 (s), 126.31 (s), 123.48 (s), 72.03 (s), 44.12 (s), 36.23 (s), 32.03 (s), 30.53 – 29.83 (m), 29.60 (dd, J = 21.9, 11.5 Hz), 29.19 (s), 26.81 (s), 22.81 (s), 22.20 (s), 14.25 (s); ESI-HRMS: $[M+H]^+$, calculated for 612.3504, found= 612.3515.

Synthesis of 4-((nitrooxy)methyl) benzoic acid (E): The compound was synthesized following the above-mentioned process (synthesis of compound **1**) using 4-(bromomethyl)benzoic acid (500 mg, 2.325 mmol). Yield (360 mg, 78.92%); White solid; ^{1}H NMR (500 MHz, DMSO-D_6): δ/ppm= 13.08 (s, 1H), 8.01 – 7.96 (m, 2H), 7.61-7.56 (m, 2H), 5.65 (s, 2H); ^{13}C NMR (100 MHz, DMSO-

D_6): δ/ppm= 166.87 (s), 137.28 (s), 131.37 (s), 129.63 (s), 128.99 (s), 74.22 (s); ESI-HRMS: $[M-H]^+$, calculated for 196.0251, found= 196.0254.

Synthesis of 4-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)benzyl nitrate (Compound 2): The compound was synthesized using *N*[1]-(9-dodecyl-9*H*-purin-6-yl)propane-1,3-diamine, **B** (320 mg, 0.887 mmol), 4-((nitrooxy)methyl) benzoic acid, **E** (208.69 mg, 1.064 mmol), HOBt (143.77 mg, 1.064 mmol), EDC.HCl (203.97 mg, 1.064 mmol), and Et_3N (247.3 µL, 1.774 mmol) as reactants, following the same procedure of synthesis of *N*-(3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)-2-hydroxybenzamide. Yield (192 mg, 40.14%); White solid, ^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm= 8.37 (d, J = 6.0 Hz, 1H), 8.25 – 8.08 (m, 1H), 7.98 (d, J = 8.2 Hz, 1H), 7.93 (d, J = 8.2 Hz, 1H), 7.73 (s, 1H), 7.50 (dd, J = 8.0, 4.8 Hz, 2H), 6.16 (s, 1H), 5.49 (s, 1H), 4.64 (s, 1H), 4.18 (t, J = 7.3 Hz, 2H), 3.85 (s, 2H), 3.54 (dd, J = 11.3, 5.7 Hz, 2H), 1.96 – 1.84 (m, 4H), 1.33 (s, 4H), 1.24 (s, 14H), 0.87 (t, J = 6.9 Hz, 3H); ^{13}C NMR (125 MHz,) δ 155.58 (s), 152.83 (s), 143.46 (s), 140.15 (s), 136.30 (s), 130.01 (s), 128.20 (s), 127.91 (s), 127.73 (s), 124.74 (s), 120.28 (s), 108.73 (s), 81.83 (s), 44.16 (s), 36.05 (s), 32.02 (s), 30.26 (s), 29.59 (dd, J = 21.8, 12.1 Hz), 29.20 (s), 26.81 (s), 22.80 (s), 14.23 (s); ESI-HRMS: $[M+H]^+$, calculated for 540.3293, found= 540.3293.

Synthesis of 2,5-dioxopyrrolidin-1-yl 4-bromobutanoate (F): 4-Bromobutyric acid (500 mg, 2.994 mmol) was dissolved in dry DCM and EDC. HCl (688.7 mg, 3.5928 mmol) was added to it at 0°C under an inert atmosphere. After 10 min, N-hydroxysuccinimide (413.5 mg, 3.5928 mmol) was added to it and left the reaction mixture on stirring at RT for overnight. After completion of the reaction, the reaction mixture was washed with 1N HCl, followed by 10% aqueous $NaHCO_3$ solution, and then with brine. The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure. Yield (380 mg, 48.06%); white solid. ^{1}H NMR (400 MHz, DMSO-D_6): δ/ppm= 3.60 (t, J = 6.6 Hz, 2H), 2.88 – 2.78 (m, 6H), 2.20 – 2.11 (m, 2H); ^{13}C NMR (100 MHz, DMSO-D_6): δ/ppm= 170.25 (s), 168.29 (s), 32.94 (s), 29.03 (s), 27.53 (s), 25.48 (s).

Synthesis of 2,5-dioxopyrrolidin-1-yl 4-(nitrooxy)butanoate (G): The compound was synthesized using 2,5-dioxopyrrolidin-1-yl 4-bromobutanoate, **F** (360 mg, 1.363 mmol) as a reactant and following the same procedure of synthesis of 2-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)phenyl 4-(nitrooxy)butanoate (Compound **1)** but at RT. Yield (255 mg, 76%); white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm= 4.57 (t, J = 6.3 Hz, 2H), 2.80 (dd, J

= 20.9, 13.6 Hz, 6H), 2.25 – 2.13 (m, 2H); ^{13}C NMR (100 MHz, DMSO-D_6): δ/ppm= 170.20 (s), 168.46 (s), 72.12 (s), 26.71 (s), 25.46 (s), 21.53 (s).

Synthesis of 4-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)amino)-4-oxobutyl nitrate (Compound 3): The compound was synthesized by dissolving *N*^{1}-(9-dodecyl-9*H*-purin-6-yl)propane-1,3-diamine, **B** (350 mg, 0.971 mmol) and 2,5-dioxopyrrolidin-1-yl 4-(nitrooxy)butanoate, **G** (238.97 mg, 0.9707 mmol) in THF, followed by addition of Et_3N (135.3 µL, 0.9707 mmol). The reaction mixture was left on stirring for 2-3 h at RT. The completion of the reaction was checked by TLC chromatography. Purification was done by column chromatography (4% methanol/DCM) Yield (234 mg, 49.02%); Colourless liquid; ^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm= 10.30 (s, 1H), 8.16 (s, 1H), 8.05 (s, 2H), 4.80 (s, 6H), 4.16 (d, J = 27.8 Hz, 4H), 3.08 (s, 2H), 2.12 (s, 2H), 1.84 (s, 2H), 1.29 (s, 4H), 1.21 (s, 14H), 0.84 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ/ppm= 175.02 (s), 172.09 (s), 166.75 (s), 118.37 (s), 73.50 (s), 72.68 (s), 68.70 (s), 66.44 (s), 63.81 (s), 63.11 – 62.82 (m), 62.16 (d, J = 118.2 Hz), 46.27 (s), 44.54 (s), 36.69 (s), 32.65 – 32.41 (m), 32.15 (d, J = 25.8 Hz), 30.13 (s), 29.94 – 29.26 (m), 29.13 (s), 26.72 (s), 23.16 – 23.04 (m), 22.91 (d, J = 22.4 Hz), 14.23 (s), 8.74 (s); ESI-HRMS: $[M+H]^+$, calculated for 492.3293, found= 492.3298.

4. Critical micelle concentration (CMC) determination: CMC of the compounds was determined by fluorescence assay using Nile red dye as a lipophilic stain. This dye shows

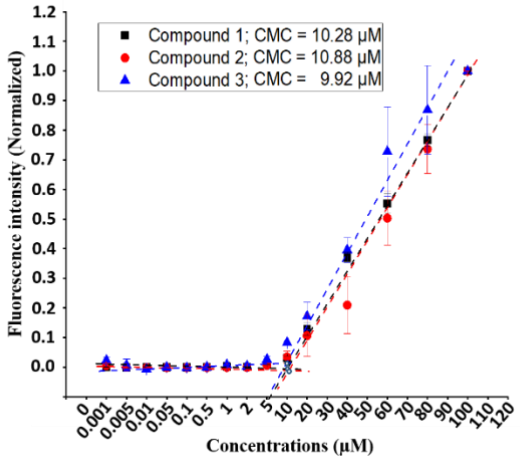


Figure S1: CMC determination by the graph plotted as fluoresce intensity (normalized) vs concentration (µM) for compound **1**. The experiment was done in triplicate. Each bas represents the mean ± SD of three independent experiments.

significant fluorescence enhancement when it goes into a hydrophobic environment from an aqueous one.[4] Different concentrations of compounds **1, 2**, and **3** ranging from 100 μM to 0.0001 μM were prepared from the 1 mg/mL stock solution in water. 2mL of each of the concentrations was treated with 2 μL of Nile red solution (1 mg/mL in acetone) and keep it in rest for overnight in a 96-well plate to evaporate the acetone. After evaporation, samples were excited (λ_{ex}=530 nm) using a plate reader and the respective fluorescence emission intensities were measured at 630 nm. Emission intensity values were normalized and plotted against the log of respective concentrations of the compounds. CMC value was considered as the X-axis value (concentration) of the intersection point of the two lines of best fits of the points of higher concentrations and lower concentrations. CMC values for compounds **1**, **2**, and **3** were found to be 10.28 μM, 10.88 μM, and 9.92 μM respectively (Figure S1).

5. Dynamic light scattering (DLS) experiment: The formation of micelle was confirmed by DLS method. For the experiment, a concentration of 10 mM of each of the compounds was prepared in ethanol and diluted to 30 μM using distilled water. The diameters of the micelles were assessed by the dynamic light scattering (DLS) measurements. For the experiment, a concentration of 30 μM

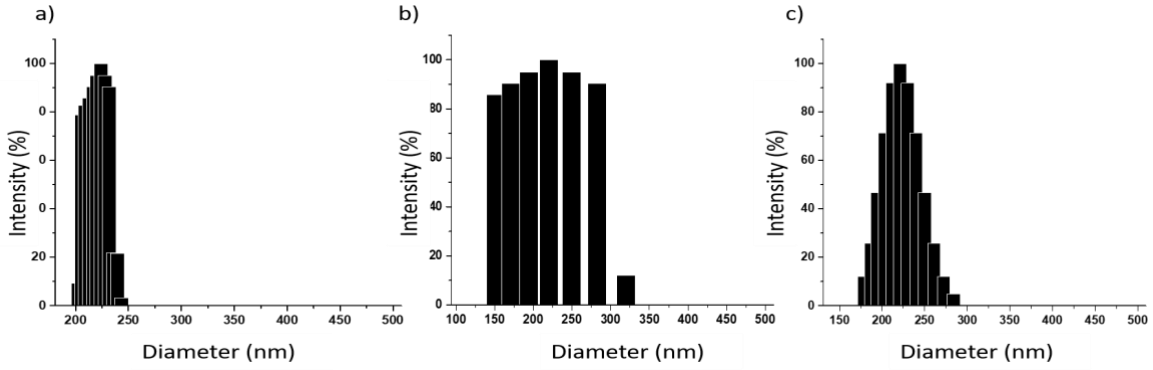


Figure S2: Size distribution of compounds a) **1**, b) **2**, and c) **3** by dynamic light scattering measurements.

of each of the compounds was taken and the diameters were measured and found to be 223 nm, 220 nm, and 220 nm for compounds **1**, **2**, and **3** respectively (Figure S2).

6. cLogP values: cLogP values of the compounds are calculated using licensed PerkinElmer ChemDraw software and it came out to be 5.25, 4.56, and 4.24 for compound **1**, **2**, and **3**, respectively.

7. *In vitro* nitrite ion release experiment: *In vitro* nitrite ion release experiment was done indirectly by using Griess reagent in a UV-Vis spectrophotometer. Firstly, a stock solution of 10 mM of each compound was prepared and diluted to 30 µM concentration using phosphate buffer (20 mM, pH=7.4) at 37°C containing 5mM GSH. Phosphate buffer (20 mM) was prepared by dissolving $NaH_2PO_4.2H_2O$ (0.004919 mM) and $Na_2HPO_4.2H_2O$ (0.01508 mM) in distilled water and titrated the buffer solution to physiological pH (7.4) by adding 0.01N NaOH. Griess reagent preparation: Sulfanilamide (0.232 M) and N-(1-Naphthyl)ethylenediamine dihydrochloride (0.0077 M) were dissolved in a mixture of 85% H_3PO_4 (10%, 5 mL) and distilled water (90%, 45 mL). The formation of azo dye by these reagents in the presence of nitrite ions gives the absorbance at 540 nm.

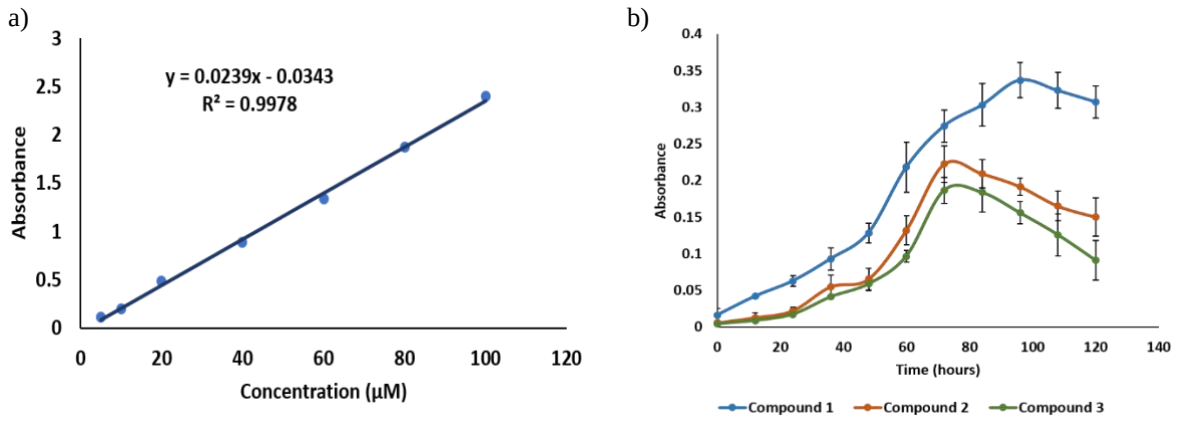


Figure S3: a) Standard calibration curve of nitrite ion (NO_2^-) plotted as absorbance vs concentration, b) Time-dependent UV-vis spectrum of compounds **1**, **2**, and **3** incubated with GSH at pH 7.4 and 37°C.

For the standard curve of concentration vs absorbance, different concentrations (1-100 µM) of sodium nitrite solutions were prepared by diluting it from a stock solution of 2 mM using phosphate buffer and incubated at 37°C. An aliquot of 900 µL from each of the concentrations was taken and mixed with 300 µL of Griess reagent followed by incubation at 37°C for 15 minutes. After that, absorbance was checked at 540 nm wavelength for each of the solutions (Figure S3 a).[3] These data were used to plot the standard concentration vs absorbance curve that was used to calculate the nitrite ion release from compounds **1**, **2**, and **3**. 30 µM of the compounds (15 mL) were prepared from the stock solution of 10 mM in phosphate buffer and GSH was added to it so that the concentration of GSH would be 5 mM. Then the solution was incubated at 37°C. An aliquot of 900 µL of the solution was treated with 300µL of Griess reagent and incubated at 37°C for 15

minutes. Then UV absorbance was measured for the same using a blank solution (phosphate buffer+GSH+Griess reagent) (Figure S3 b). The concentration of nitrite ion release was calculated using the standard curve where the absorbance was converted to the concentration of nitrite release (Fig 4a). All the experiments were done in triplicate.

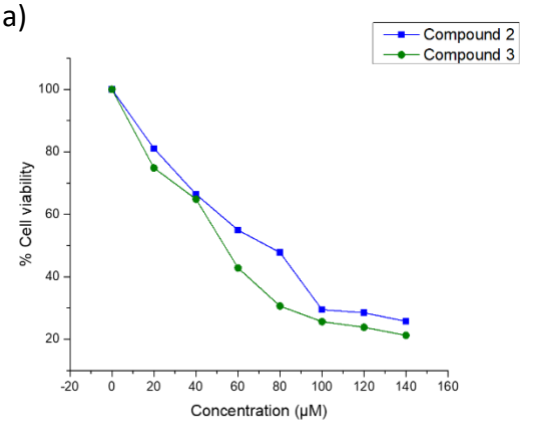
8. Nitric-oxide release kinetics: Nitrite ion release kinetic was checked from the data obtained from the *in vitro* nitrite ion release experiment. The graph for the first-order model was plotted as natural logarithm 'lnA' vs time (h) where A is the absorbance at 540 nm at 72 h. This model was found to be the best-fitted one with the R^2 values of 0.9662, 0.9875, and 0.9899 for compounds **1**, **2**, and **3** respectively (Fig 4b). Therefore, it can be demonstrated that nitrite ion release from our compounds follows first-order kinetics which also implies that the change in concentration of the nitrite ion with respect to time depends only on the concentration of the compounds undergoing reaction.

9. IC_{50} and SI determination with NIH3T3: For calculating the % cellular viability, NIH3T3 and HepG2 cells (3*10^4 cell number) were seeded in a 96 well treated plate and incubated for 24 h. Further, the cells were treated with different concentrations of all three compounds varying from 0 to 120 µM and incubated for 24 h. The viability of cells was calculated with MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay, where the cells were incubated with MTT (0.5 mg/mL, Sigma-Aldrich, UK) for 4h in a humidified incubator and absorbance was recorded at 570 nm using microplate reader (EonBioteK, USA) after dissolving the formazan crystals with dimethyl sulfoxide (DMSO). The 50% inhibitory concentration was calculated by utilizing % cell viability against concentration plot of compounds and performing curve fitting analysis using origin software. Further IC_{50} value was utilized to calculate the selectivity index as per the following formula:

$$\%\boldsymbol{cell\ viability} = \frac{[absorbance\ (treated) - absorbance\ (blank)]}{absorbance\ (untreated) - absorbance\ (blank)} * 100$$

$$\boldsymbol{Selectivity\ index\ (SI)} = \frac{[IC\ 50\ value\ for\ normal\ cell\ line\ (NIH3T3)]}{IC\ 50\ value\ for\ cancer\ cell\ line\ (HepG2)} * 100$$

a)

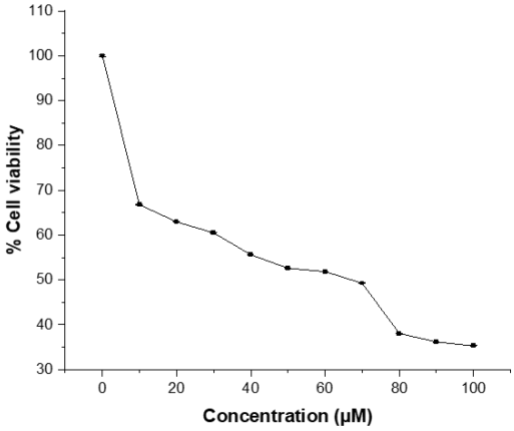


b)

Cell line	Compounds	IC_{50}±sd (µM)
NIH3T3	2	60.99±35
	3	34.54±15

Figure S4: a) The graph represents % cell viability of NIH3T3 cell line after treatment with different concentrations of compounds **2** and **3**; b) The chart represents IC_{50} values for compounds **2** and **3** in NIH3T3 cell line.

a)



b)

Cell lines	IC_{50} ± sd (µM)
HepG2	49.13±0.09

Figure S5: a) The graph represents % cell viability of HepG2 cell line after treatment with different concentrations of 6,9-disubstituted purine synthetic intermediate (**C**) without NO-donor group (*N*-(3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)-2-hydroxybenzamide), b) The chart represents IC_{50} value of the intermediate (**C**) for HepG2 cells. The experiment was done in triplicate.

10. Antiproliferative assay: The effect of compound **1** over proliferation of cancer cells (HepG2) was determined with MTT assay for a period of 72 h. 3*10^4 cells were seeded in a 96 well treated plate and allowed to adhere and proliferate for 24 h. The cells were further treated with varying concentration of compound **1** (0-300) and MTT assay was performed after 24 h, 48 h, and 72 h. The experiments were performed in triplicates and mean ± sem was plotted (Fig 5b).

11. Apoptosis assessment with C-AM/PI staining: HepG2 cells were seeded in a 48 well treated plate and allowed to adhere for 24 h. At this point, cells are treated with compound **1** at IC_{50} concentration and incubated for 24 h. They are further stained with Calcein acetoxymethyl ester (C-AM, Sigma Aldrich, 4 mM) and Propidium iodide (PI, Sigma Aldrich, 1.5 mM) followed by incubation for 30 minutes at 37°C. Fluorescence images were further captured with green and red filters at 10X magnification using fluorescence microscope (Leica DM 2500) (Fig 6).

12. Cellular morphology assay: To determine the effect of compound **1** on cellular morphology, HepG2 cells were seeded in 48 well treated plate and after 24 h, treatment was performed at IC_{50} value. A Bright field microscope (NIKON) was used to capture cellular morphology at 40X magnification keeping intensity constant for all the groups (Fig 7).

13. Cellular migration assay: HepG2 cells were seeded in a 96 well treated plate and cultured until they reach 90-95% confluency. The developed monolayer of the cells was scraped with a 10µL sterile pipette longitudinally to develop microinjury, followed by PBS wash. The cells were further treated with complete media (as positive control) and with compound **1** at IC_{50} concentration. No migration of cells after treatment with compound **1** was monitored for a period of 24 h. The bright field images (NIKON) were captured at 20X magnification (Fig 7).

14. Colony formation assay: HepG2 cells are seeded in a treated 6 well-plate with approximately 1000 cells per well. They are treated with compound **1** at IC_{50} concentration and cultured for a period of 15 days until cellular colonies develop. The colonies are fixed with 4% formaldehyde and stained with crystal violet. Images of microscopic colonies were captured with bright field microscope (NIKON). The experiment is performed in triplicates (Fig 7).

15. Copies of ^{1}H, ^{13}C NMR, and Mass spectra:

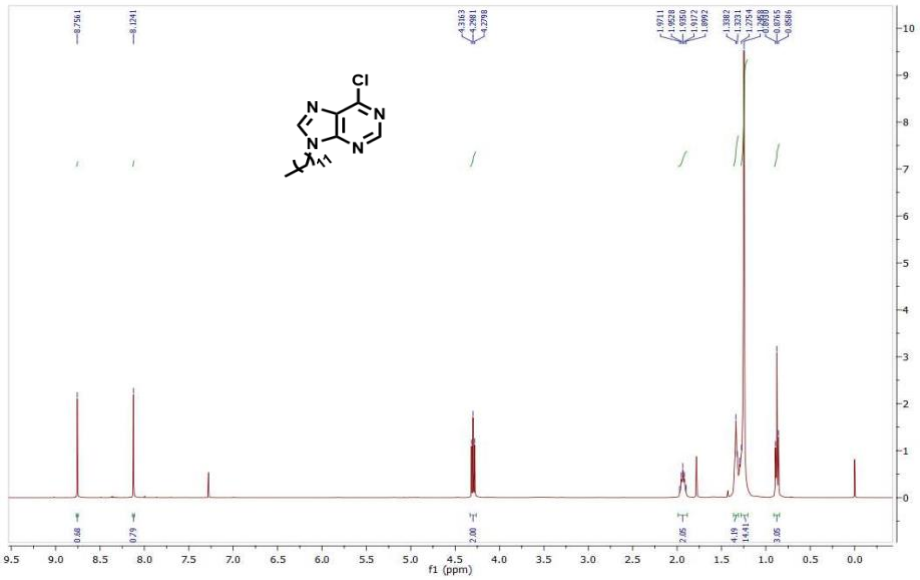


Figure S6: ^{1}H NMR spectrum of **6-Chloro-9-dodecyl-9*H*-purine** (400 MHz, $CDCl_3$)

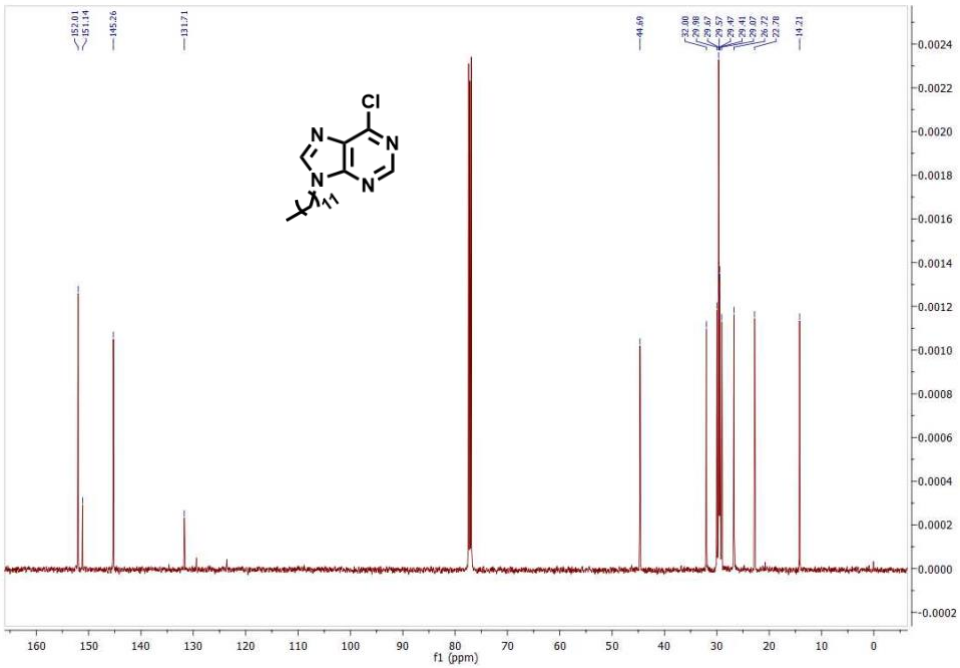


Figure S7: ^{13}C NMR spectrum of **6-Chloro-9-dodecyl-9*H*-purine** (125 MHz, $CDCl_3$)

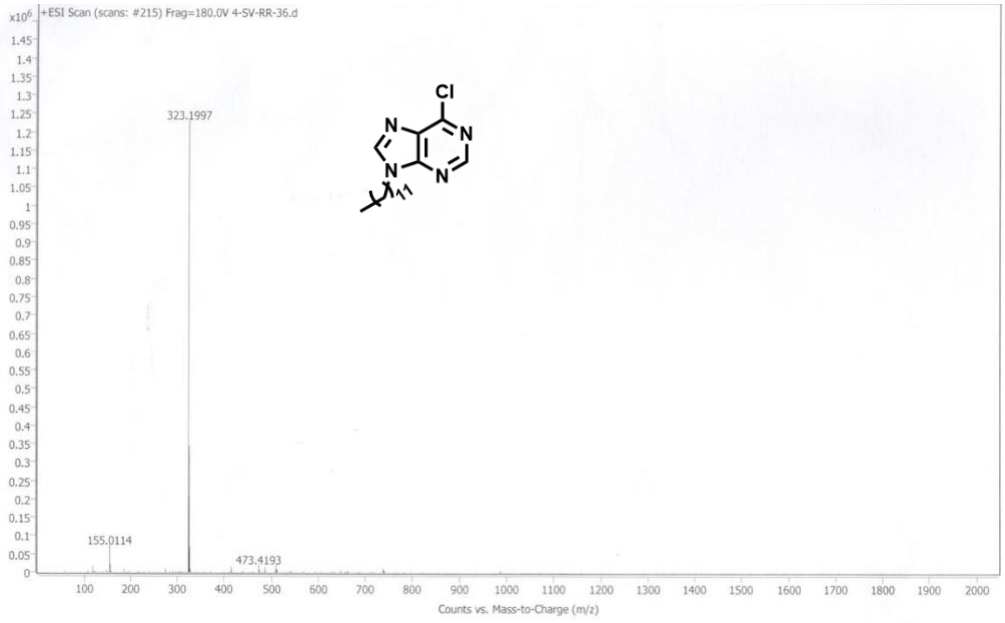


Figure S8: ESI-HRMS spectrum of **6-Chloro-9-dodecyl-9*H*-purine**

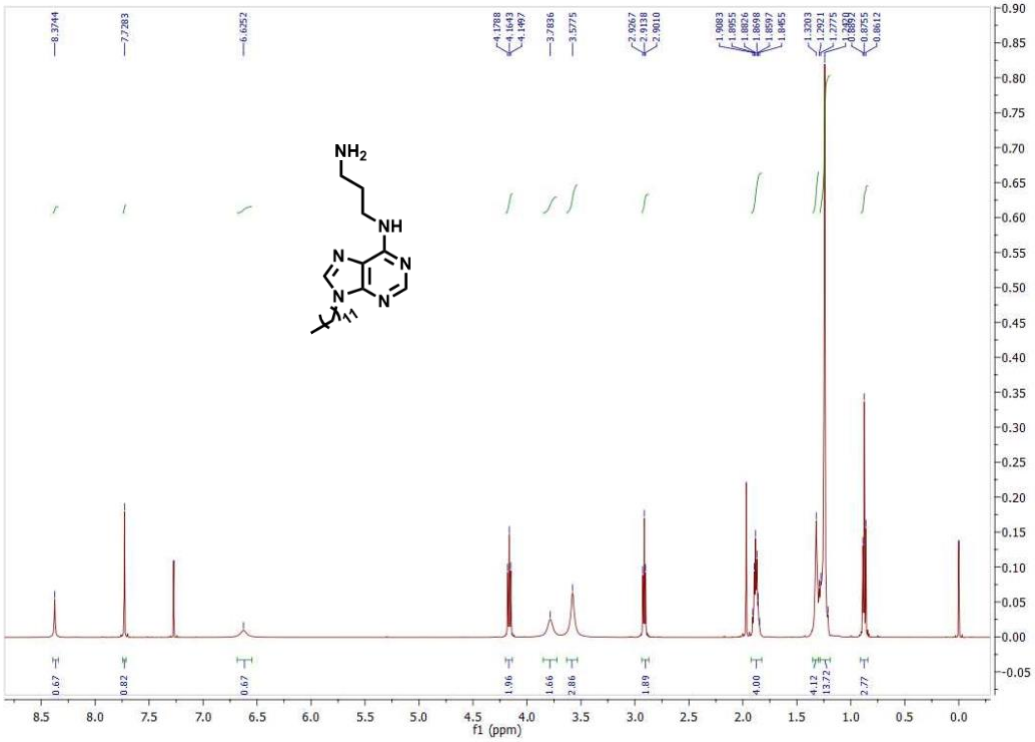


Figure S9: ^{1}H NMR spectrum of ***N*^{1}-(9-dodecyl-9*H*-purin-6-yl)propane-1,3-diamine** (500 MHz, $CDCl_3$)

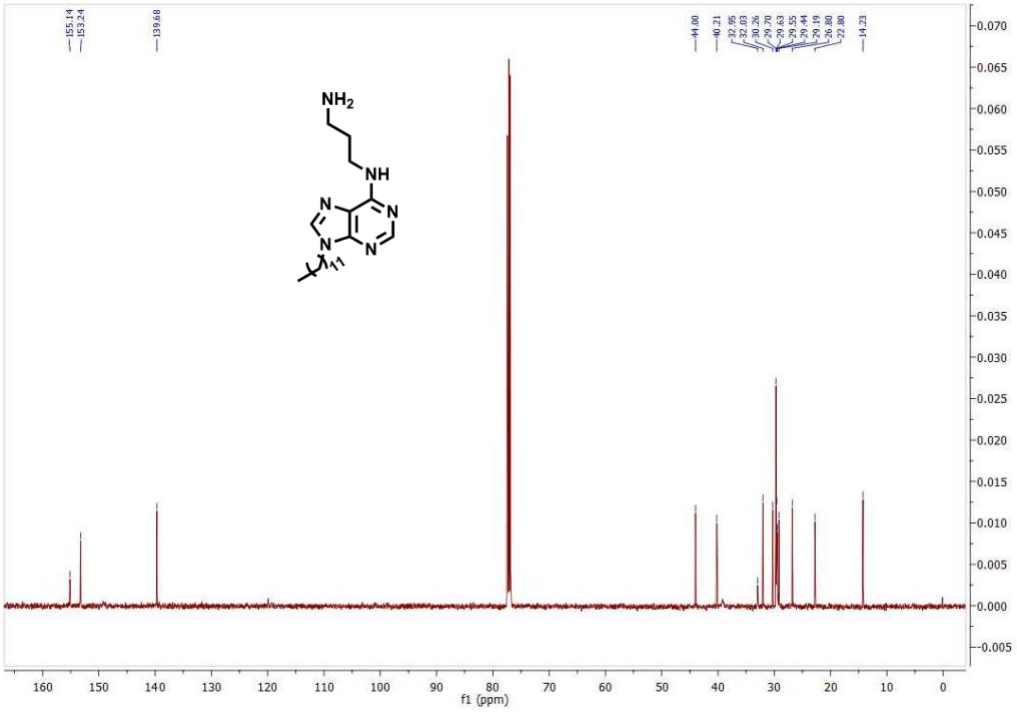


Figure S10: ^{13}C NMR spectrum of ***N*^{1}-(9-dodecyl-9*H*-purin-6-yl)propane-1,3-diamine** (125 MHz, $CDCl_3$)

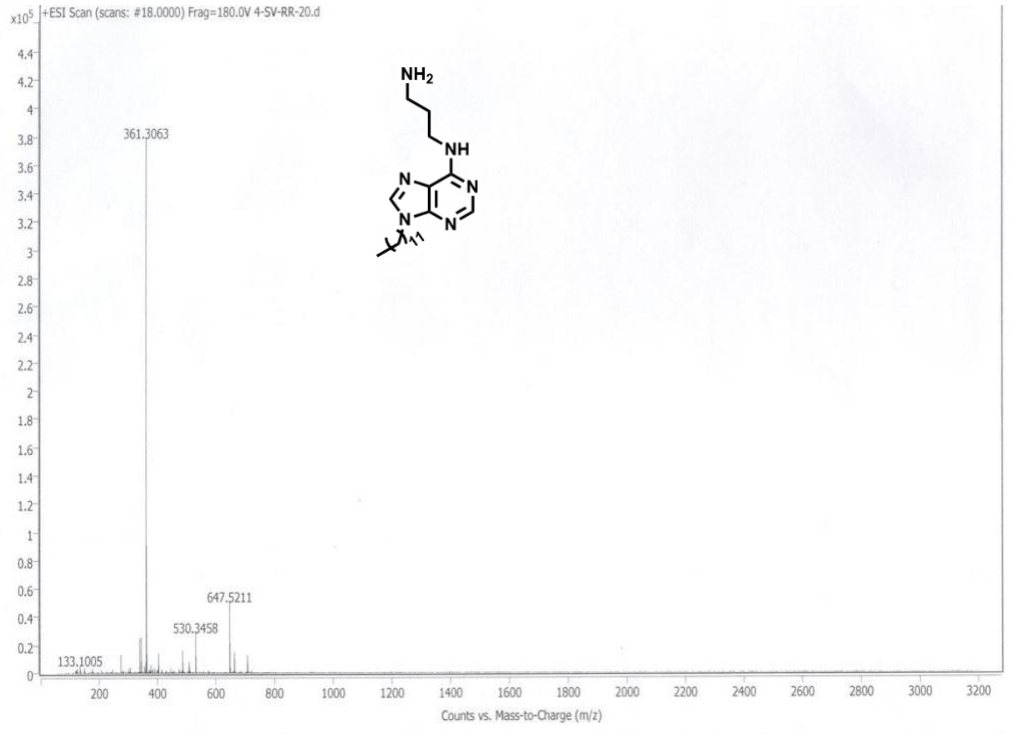


Figure S11: ESI-HRMS spectrum of ***N*^{1}-(9-dodecyl-9*H*-purin-6-yl)propane-1,3-diamine**

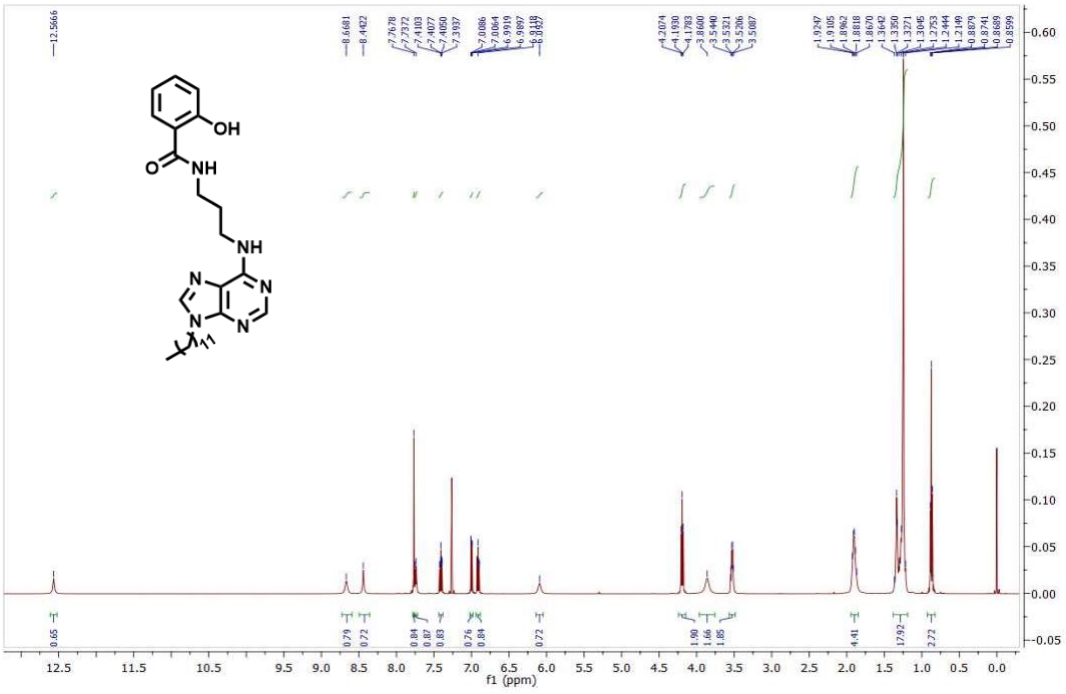


Figure S12: ^{1}H NMR spectrum of ***N*-(3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)-2-hydroxybenzamide** (500 MHz, $CDCl_3$)

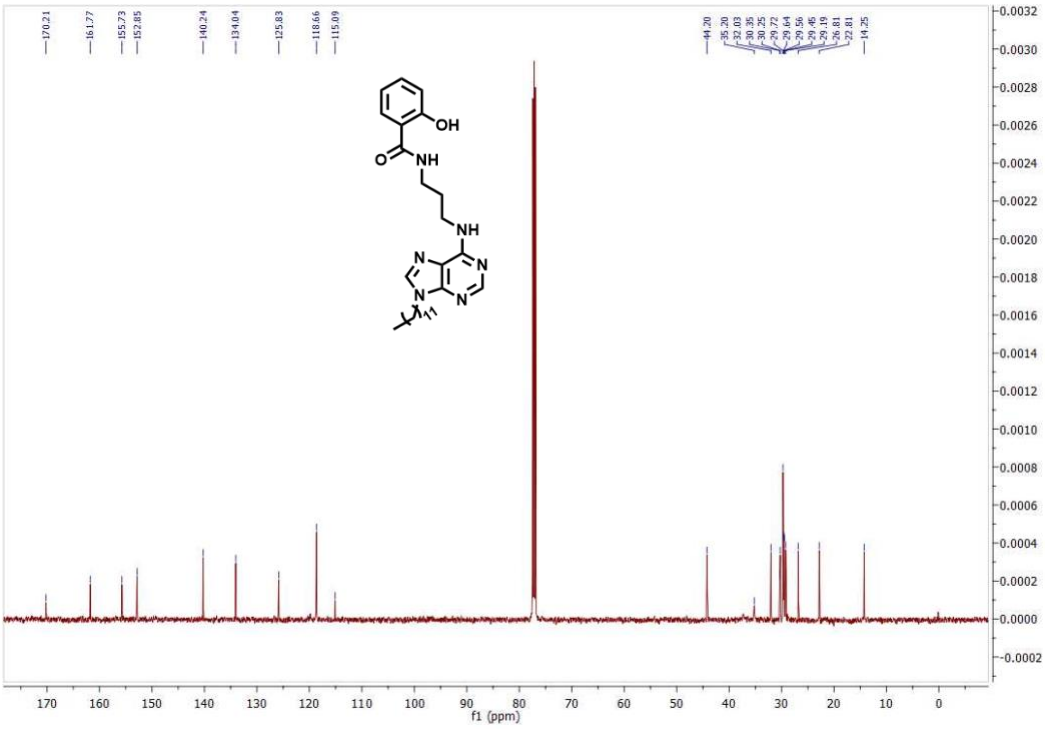


Figure S13: ^{13}C NMR spectrum of ***N*-(3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)-2-hydroxybenzamide** (125 MHz, $CDCl_3$)

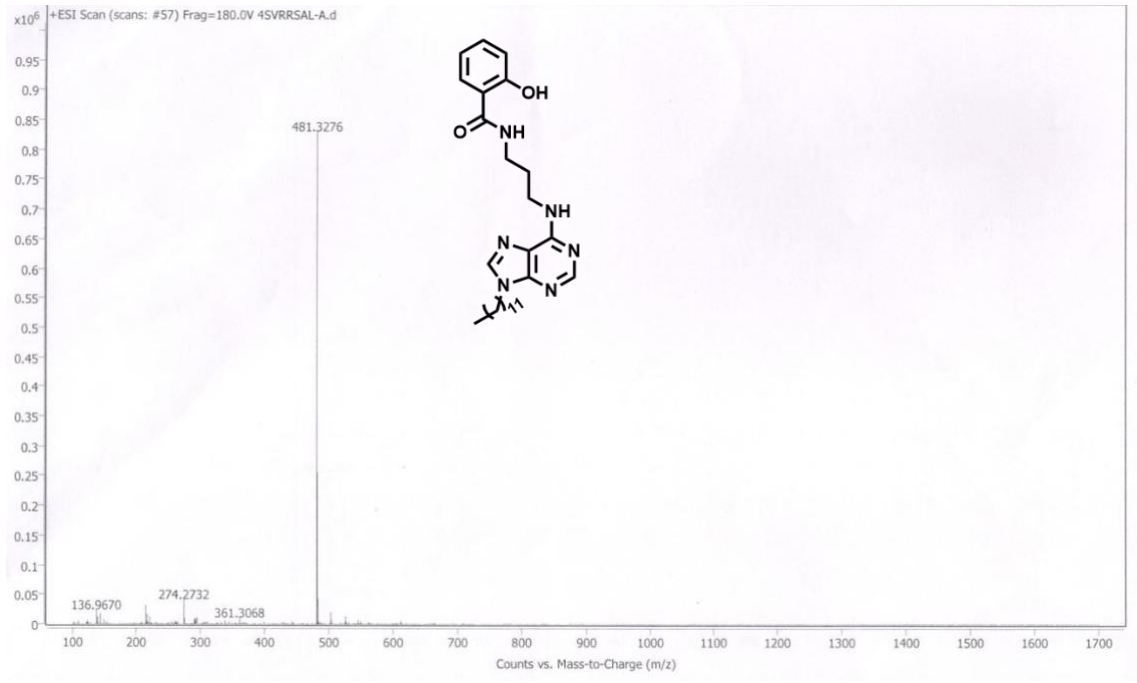


Figure S14: ESI-HRMS spectrum of ***N*-(3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)-2-hydroxybenzamide**

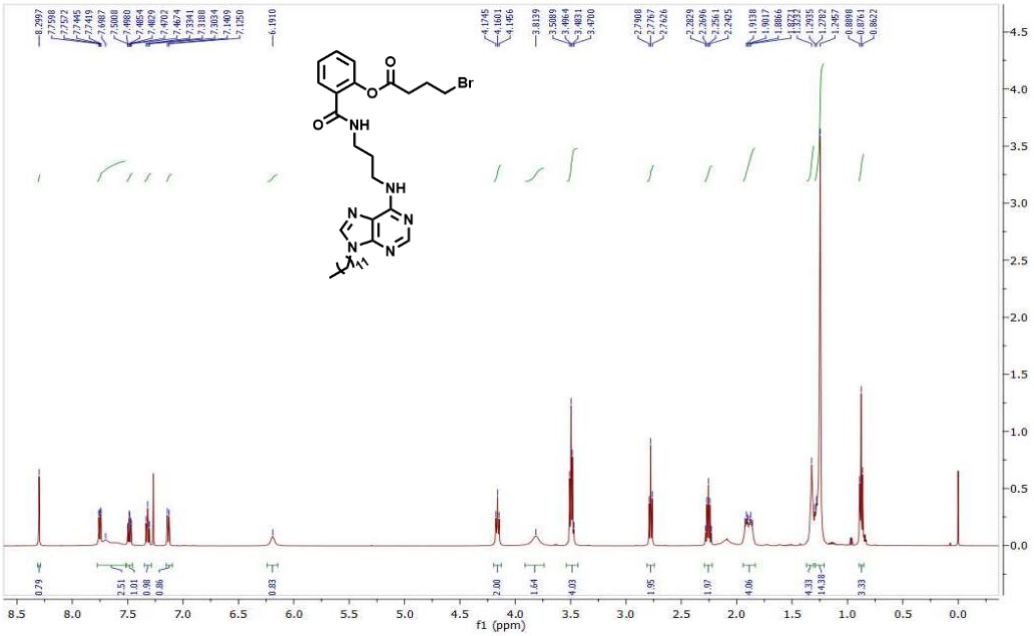


Figure S15: ^{1}H NMR spectrum of **2-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)phenyl 4-bromobutanoate** (500 MHz, $CDCl_3$)

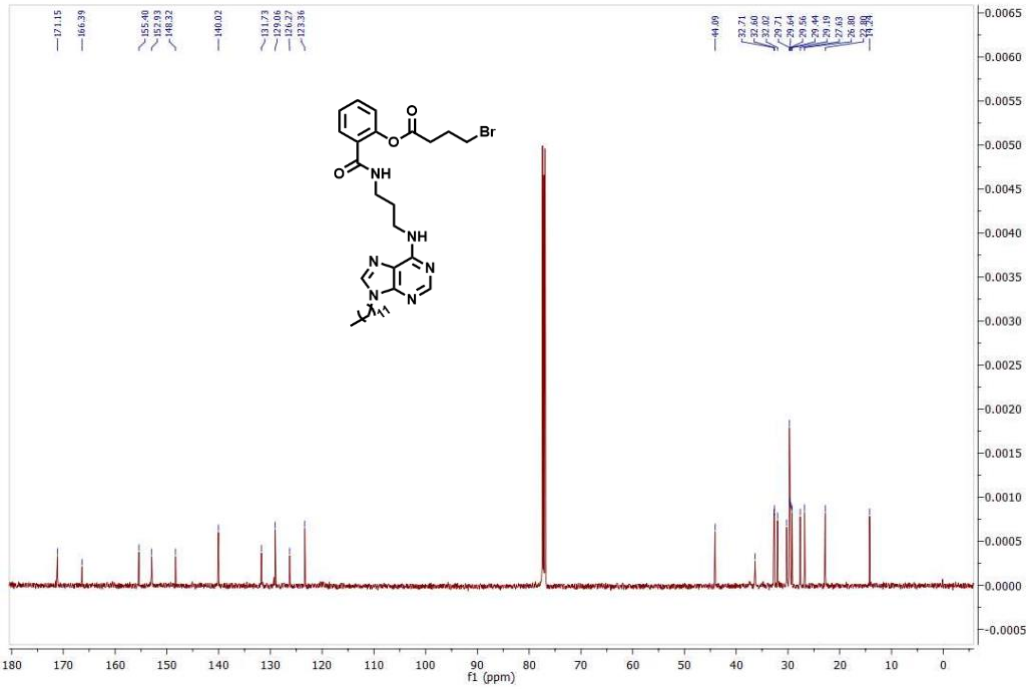


Figure S16: ^{13}C NMR spectrum of **2-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)phenyl 4-bromobutanoate** (125 MHz, $CDCl_3$)

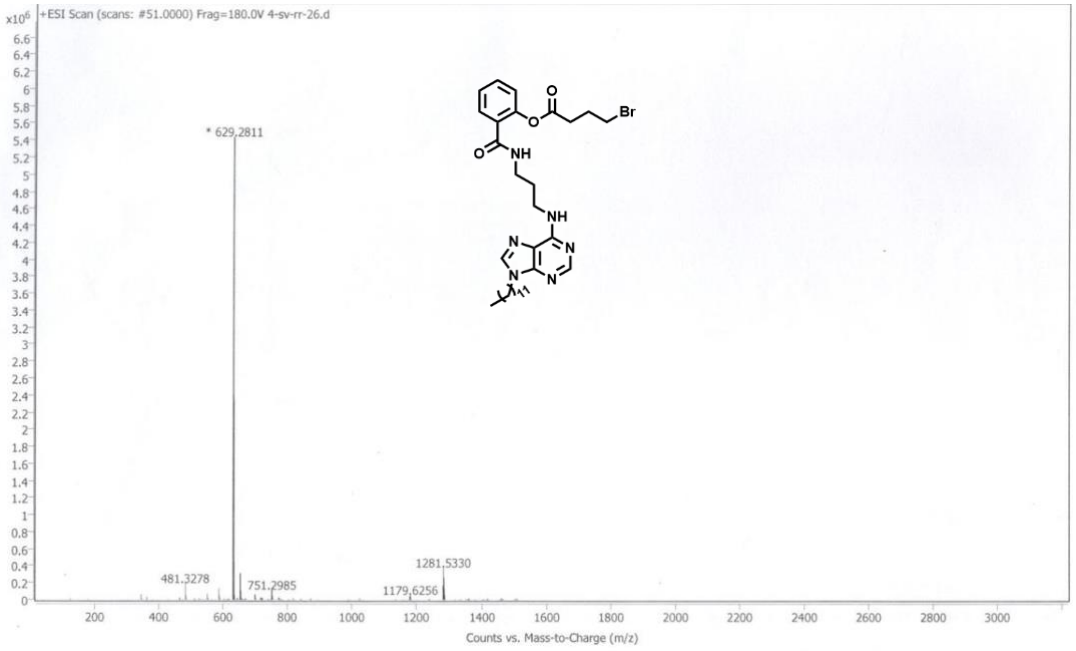


Figure S17: ESI-HRMS spectrum of **2-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)phenyl 4-bromobutanoate**

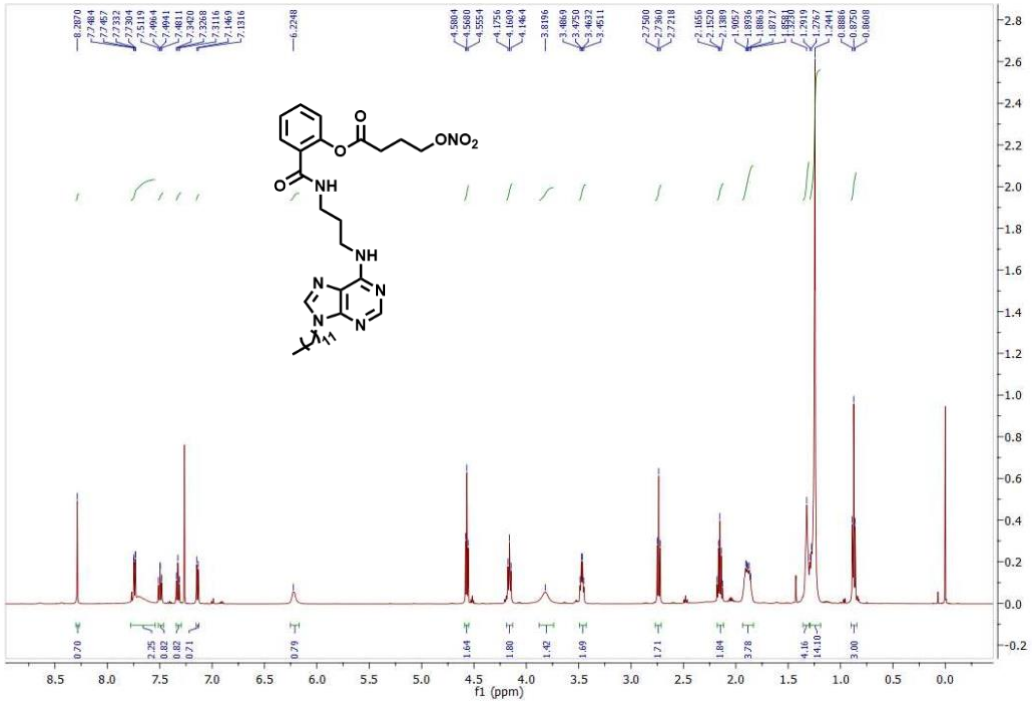


Figure S18: ^{1}H NMR spectrum of **2-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)phenyl 4-(nitrooxy)butanoate** (Compound **1**) (500 MHz,

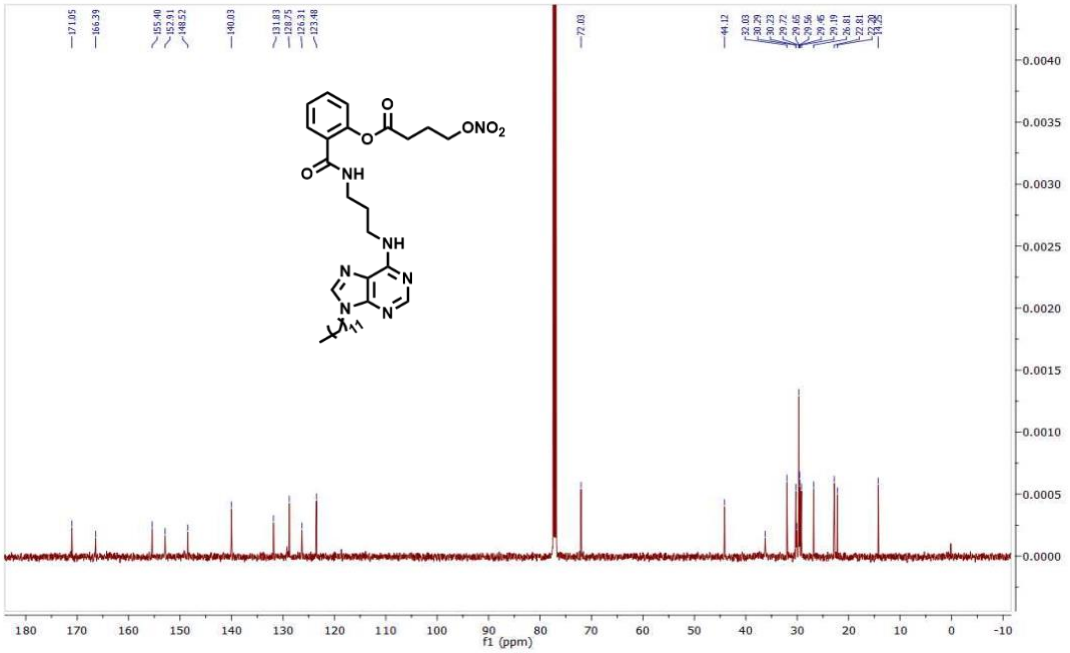


Figure S19: ^{13}C NMR spectrum of **2-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)phenyl 4-(nitrooxy)butanoate** (Compound **1**) (125 MHz, $CDCl_3$)

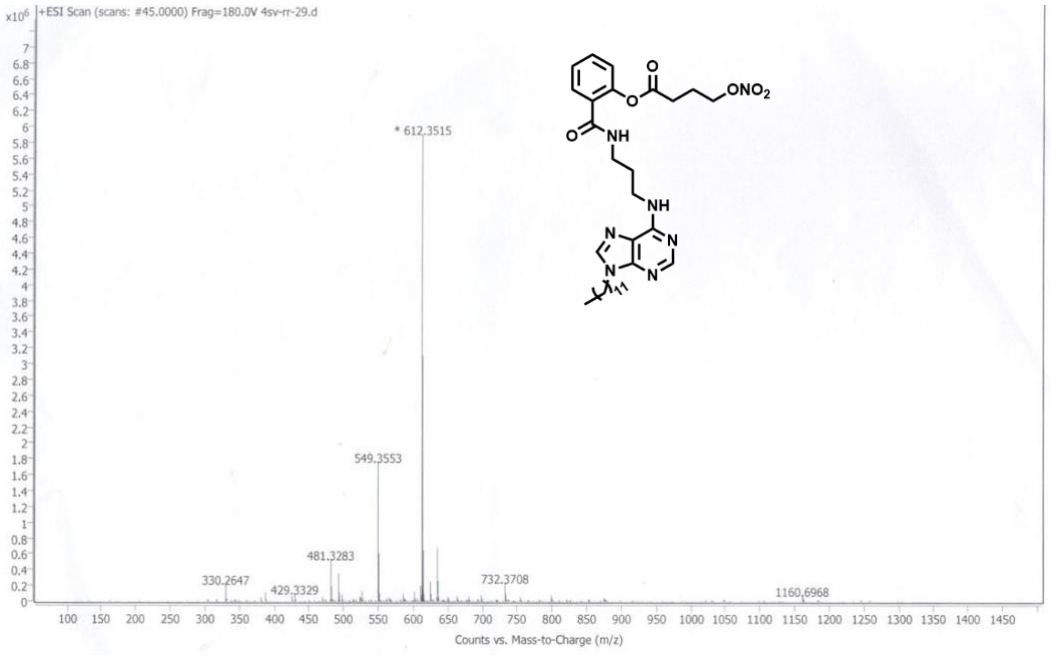


Figure S20: ESI-HRMS spectrum of **2-((3-((9-dodecyl-9*H*-purin-6-yl)amino)propyl)carbamoyl)phenyl 4-(nitrooxy)butanoate** (Compound **1**)

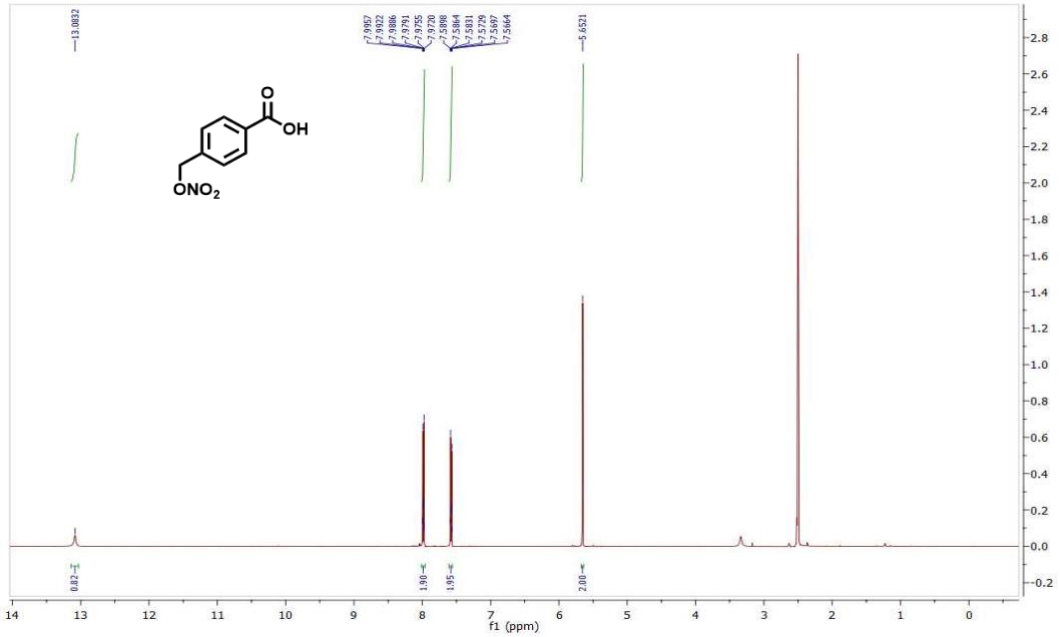


Figure S21: ^{1}H NMR spectrum of **4-((nitrooxy)methyl)benzoic acid** (500 MHz, DMSO-D_6)

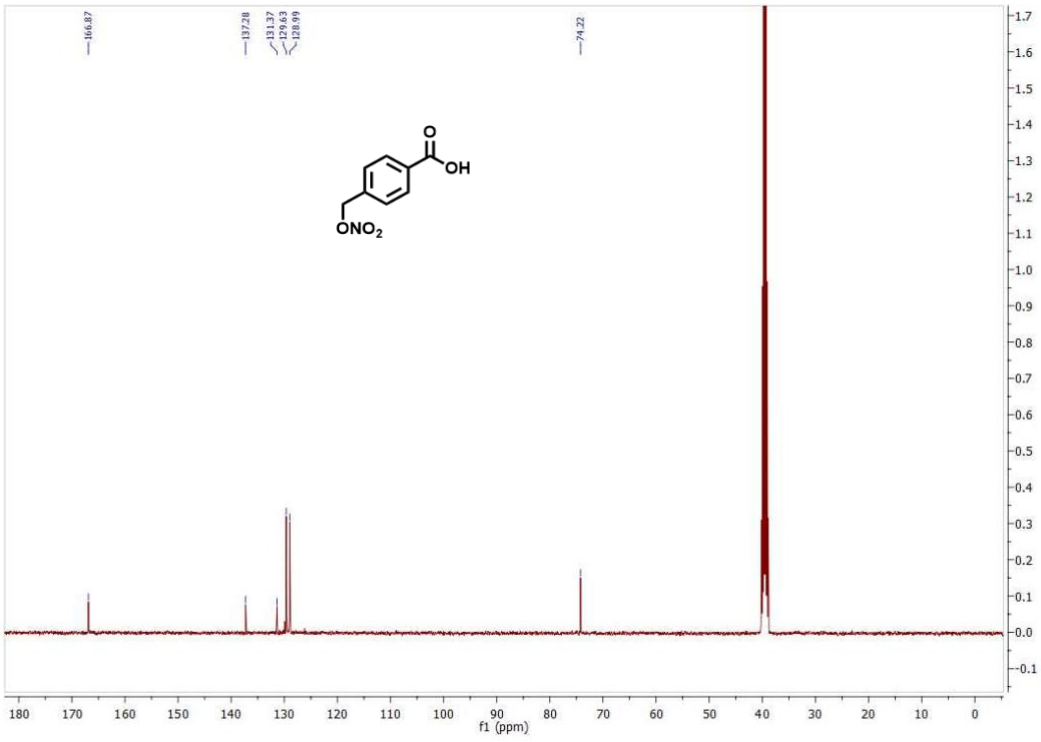


Figure S22: ^{13}C NMR spectrum of **4-((nitrooxy)methyl)benzoic acid** (100 MHz, DMSO-D_6)

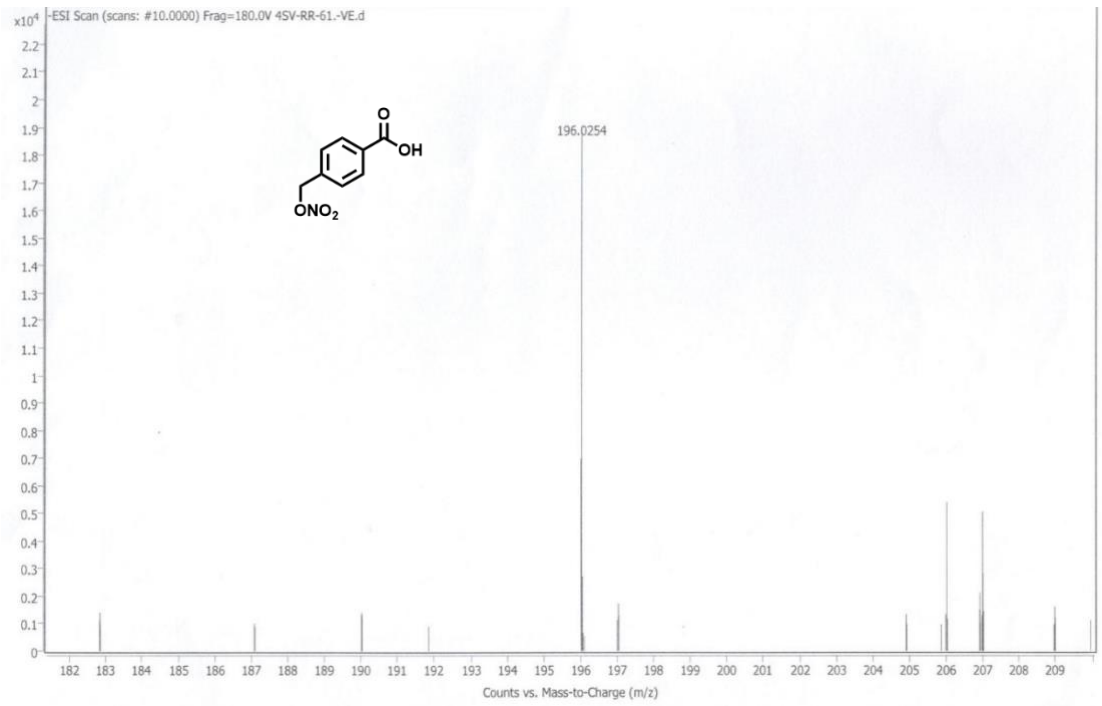


Figure S23: ESI-HRMS spectrum of **4-((nitrooxy)methyl)benzoic acid**

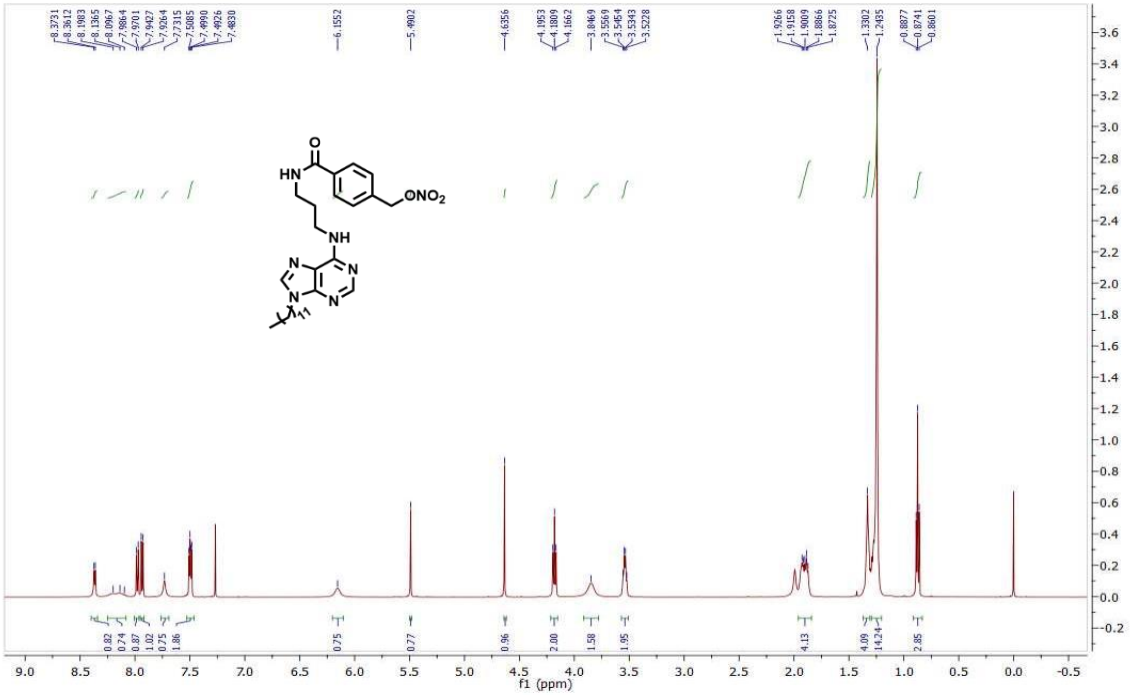


Figure S24: ^{1}H NMR spectrum of **4-((3-((9-dodecyl-9H-purin-6-yl)amino)propyl)carbamoyl)benzyl nitrate** (Compound **2**) (500 MHz, $CDCl_3$)

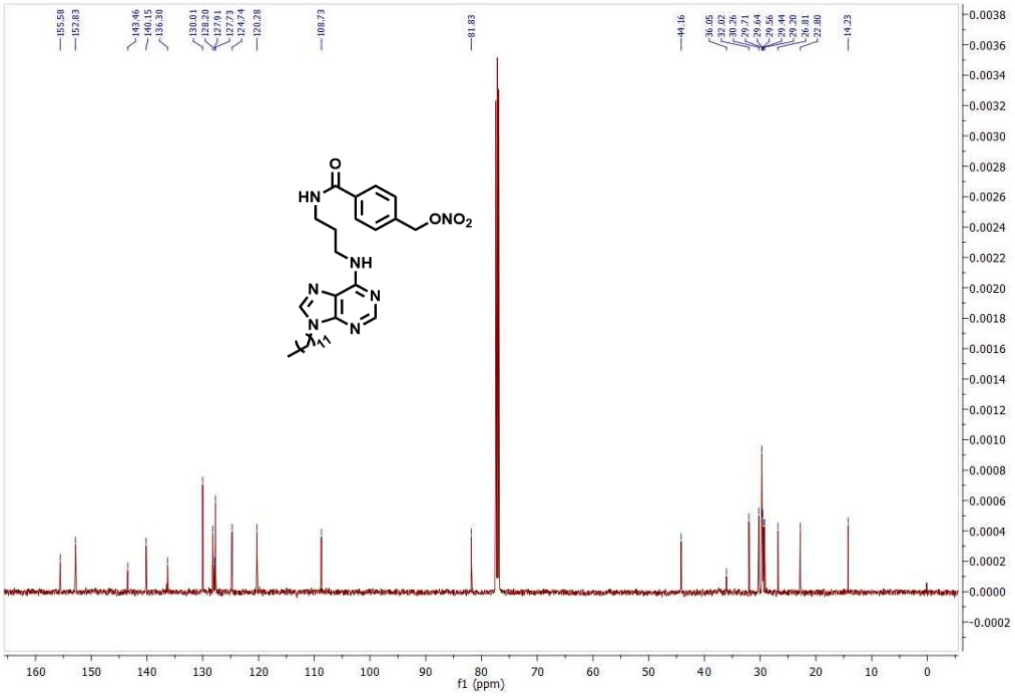


Figure S25: ^{13}C NMR spectrum of **4-((3-((9-dodecyl-9H-purin-6-yl)amino)propyl)carbamoyl)benzyl nitrate** (Compound **2**) (125 MHz, $CDCl_3$)

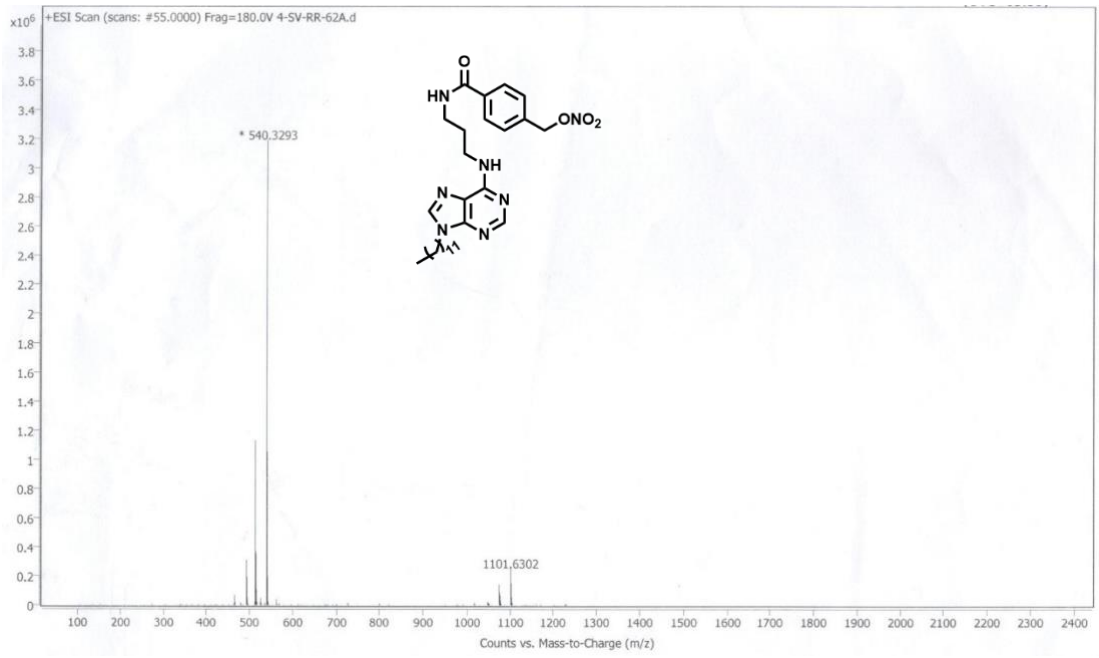


Figure S26: ESI-HRMS spectrum of **4-((3-((9-dodecyl-9H-purin-6-yl)amino)propyl)carbamoyl)benzyl nitrate** (Compound **2**)

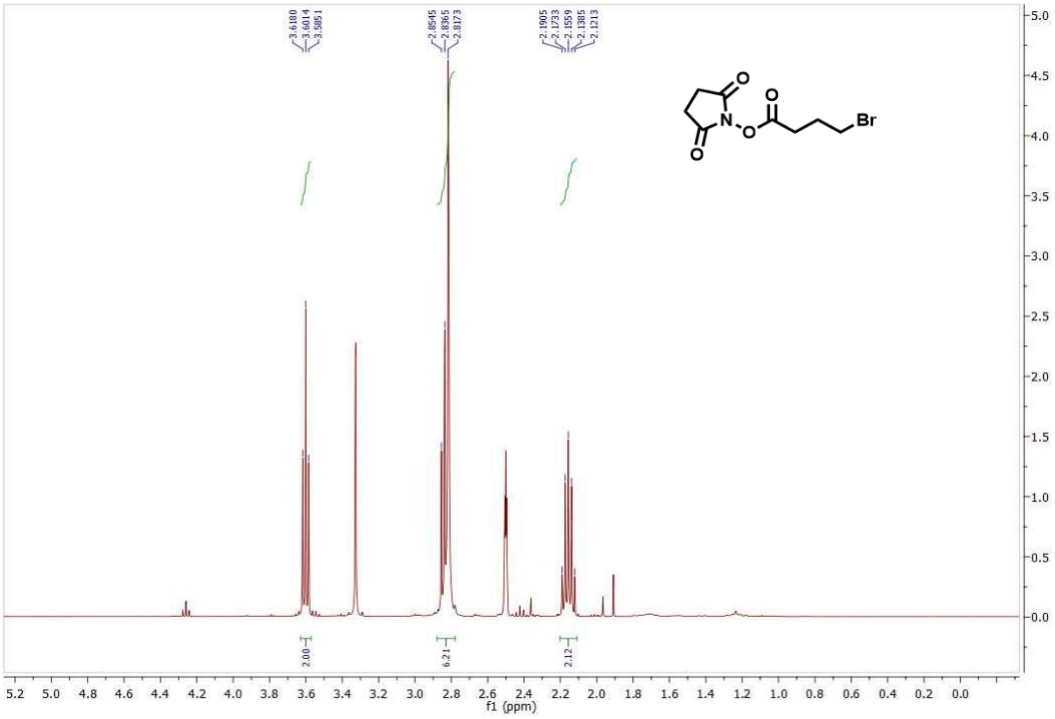


Figure S27: ^{1}H NMR spectrum of **2,5-dioxopyrrolidin-1-yl 4-bromobutanoate** (400 MHz, DMSO-D_6)

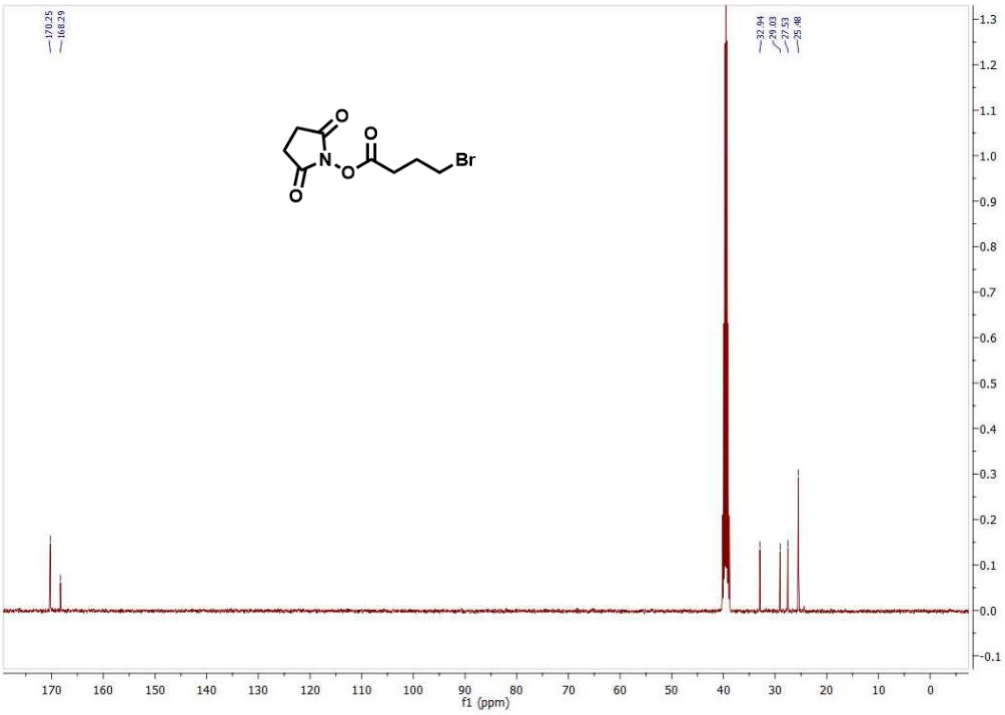


Figure S28: ^{13}C NMR spectrum of **2,5-dioxopyrrolidin-1-yl 4-bromobutanoate** (100 MHz, DMSO-D_6)

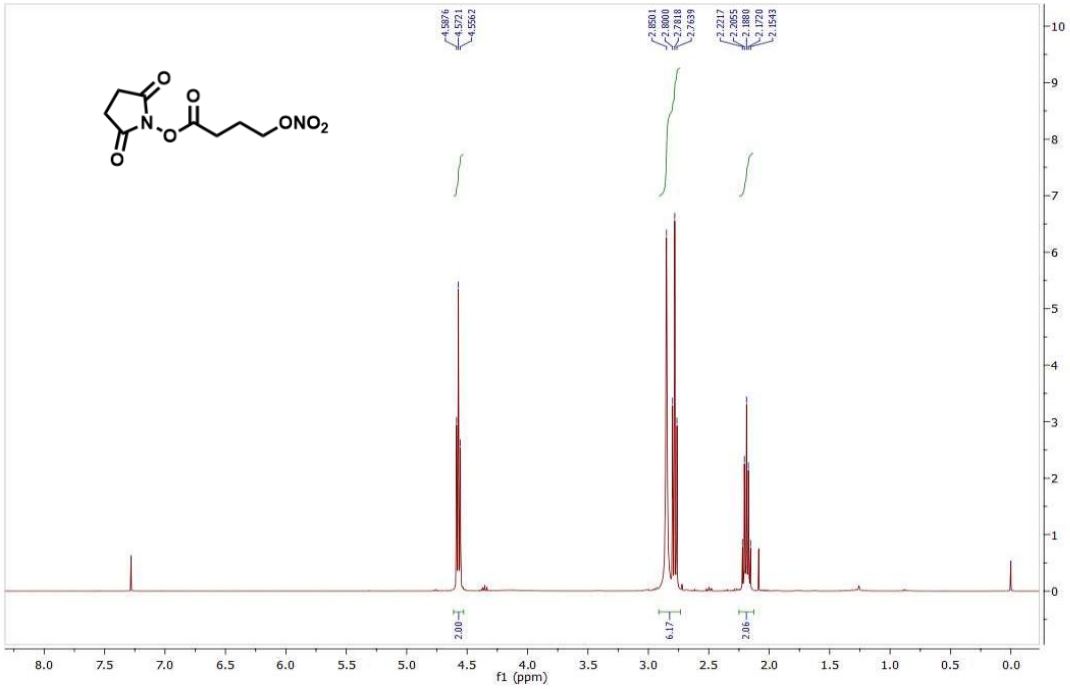


Figure S29: ^{1}H NMR spectrum of **2,5-dioxopyrrolidin-1-yl 4-(nitrooxy)butanoate** (400 MHz, $CDCl_3$)

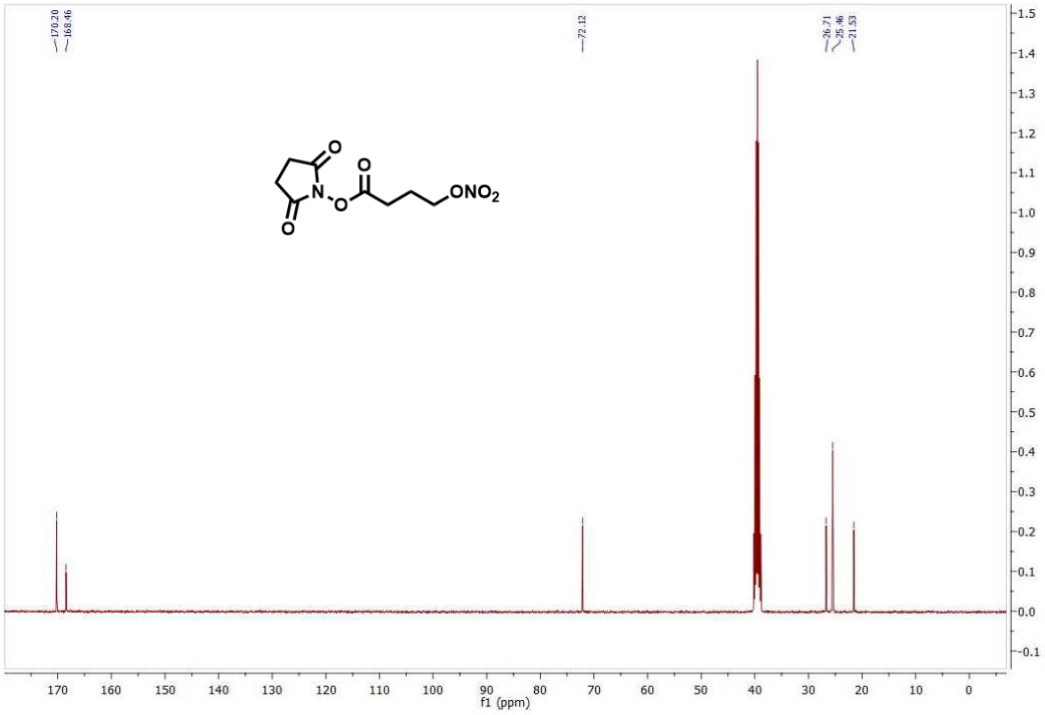


Figure S30: ^{13}C NMR spectrum of **2,5-dioxopyrrolidin-1-yl 4-(nitrooxy)butanoate** (100 MHz, DMSO-D_6)

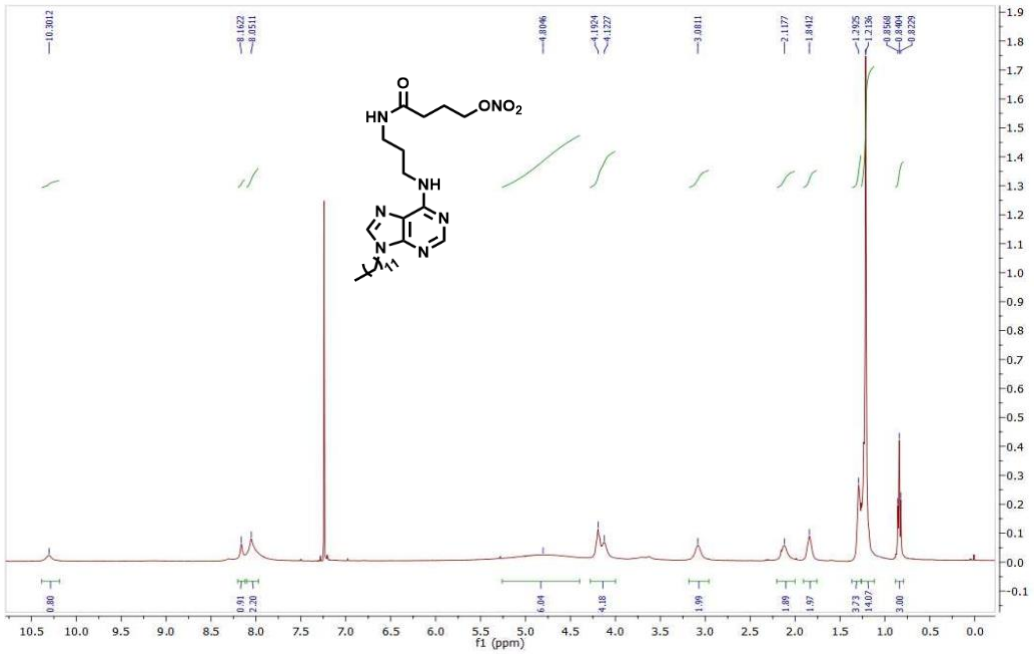


Figure S31: ^{1}H NMR spectrum of **4-((3-((9-dodecyl-9H-purin-6-yl)amino)propyl)amino)-4-oxobutyl nitrate** (Compound **3**) (400 MHz, $CDCl_3$)

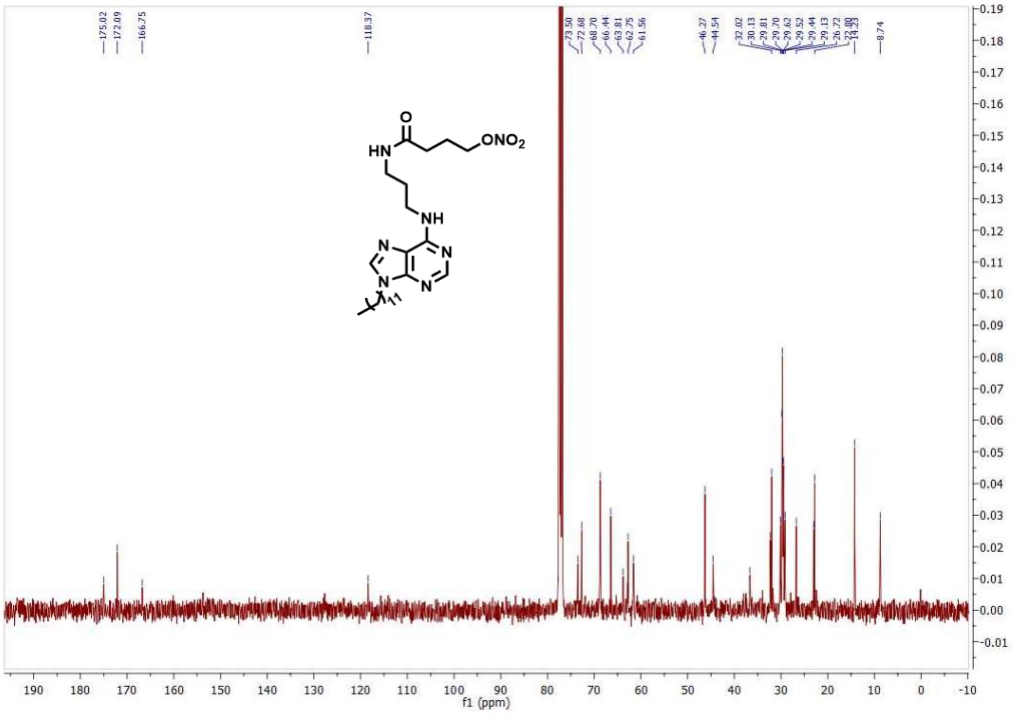


Figure S32: ^{13}C NMR spectrum of **4-((3-((9-dodecyl-9H-purin-6-yl)amino)propyl)amino)-4-oxobutyl nitrate** (Compound **3**) (100 MHz, $CDCl_3$)

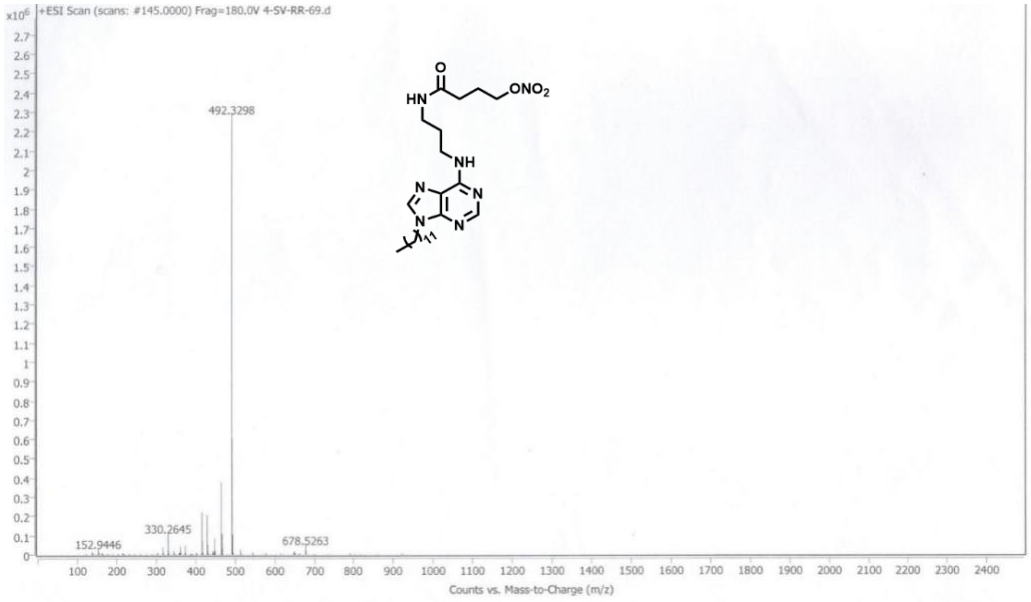


Figure S33: ESI-HRMS spectrum of **4-((3-((9-dodecyl-9H-purin-6-yl)amino)propyl)amino)-4-oxobutyl nitrate** (Compound **3**)

16. References:

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