

Supplementary Information for:

A Four-Step Biocatalytic and Organocatalytic Cascade for Single-Flask Production of Dinitroalkanes from Alcohols in Aqueous Buffer

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General Materials

Unless otherwise noted, all chemicals and solvents were used as received from commercial sources. Water (dd-H₂O) used in biological procedures or as a reaction solvent was deionized using a Barnstead E-pure Series 1090 water purification system (Thermo Fisher Scientific, USA). Centrifugations were carried out in a Sorvall Biofuge Stratos Centrifuge (Thermo Electron Corp., USA). Optical density measurements were carried out with Evolution 260 Bio UV-visible spectrometer (Thermo Fisher Scientific, USA) by measuring the optical density at 600 nm. Liquid cultures were shaken in an I24 Incubator (New Brunswick Scientific, USA). Sterilization was carried out in a Sterilmatic Autoclave (Market Forge Industries, USA). Solvent evaporations were carried out with a USA Lab RE-200A rotary evaporator with a recirculating chiller (Glycol Power Pack H35G).

S.1 Gas Chromatography Mass Spectrometry

Gas chromatography mass spectrometry analysis was carried out with an Agilent Technologies 7890B GC system paired with a 7693 Autosampler and 5977B MSD. A 30 m Rxi®-5sil MS column with Integra-Guard (Restek #13623-127) was fitted. GC oven temp was initial held at 60 °C for 1 minute, followed by a 15 °C / min ramp to 120 °C and held for 1 minute. Helium was used as the carrier gas. The injector was maintained at 300 °C, the MSD transfer temperature was 280 °C, and the detector was maintained at 230 °C. 0.5 µL of sample was injected in split injection mode (1:100 split ratio) using 1-nitrohexane and 4-nitrobenzaldehyde as internal standards. Selected ion monitoring (SIM) was used. Calibration curves were generated by diluting 50 mg of the product to 5 mL in a volumetric flask with ethyl acetate. The 10 mg mL⁻¹ stock solution was diluted with ethyl acetate to generate calibration levels. 1-nitrohexane (1 mg mL⁻¹) and 4-nitrobenzaldehyde (1 mg mL⁻¹) were used as an internal standard. Samples were diluted (1:50) in isoctane (1 mL) before analysis. Spectra were analyzed with Agilent MassHunter Quantitative software.

S2. Nuclear Magnetic Resonance

Proton nuclear magnetic resonance (¹H spectra) were acquired with a JEOL ECA-500 spectrometer operating at 500 MHz. Chemical shifts are expressed as parts per million and referenced to residual solvent signal (CDCl₃, δ 7.26 ppm; DMSO-d, δ 2.50 ppm). Carbon-13 NMR (¹³C NMR) spectra were acquired with a JEOL-ECA 500 at 125 MHz. Chemical shifts are expressed in parts per million and referenced to residual solvent (CDCl₃, δ 77.16 ppm; DMSO-d₆, δ 39.50 ppm). All NMR solvents were acquired from Cambridge Isotope Laboratories.

S.3. Media, Strains, Culture Conditions

YPD media was prepared from 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose; the solution pH was adjusted to 5.6 with 10% v/v HCl. The solution was then autoclaved at 121 °C for 20 minutes.

Maintenance media was prepared with 2.6 g/L potassium phosphate monobasic, 0.3 g/L potassium phosphate dibasic, 1.5 g/L ammonium sulfate, 0.3 g/L magnesium sulfate heptahydrate, 1 mg/L iron sulfate heptahydrate, 5 µg/L copper sulfate pentahydrate, 10 µg/L boric acid, 10 µg/L manganese sulfate monohydrate, 70 µg/L zinc sulfate, 10 µg/L sodium molybdate, and 1 g/L yeast extract; pH of the solution was adjusted to 5.8 with 10% v/v HCl. The solution was autoclaved at 121 °C for 20 minutes. After the solution had cooled, 10 g/L methanol was added.

YPD agar media was prepared from 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose. The solution pH was adjusted to 5.6 with 10% v/v HCl. 20 g/L agar was added to the solution. The solution was then autoclaved at 121 °C for 20 minutes. The agar solution was allowed to cool slightly and poured into culture plates.

Freeze-dried *Komagataella pastoris* ATCC® 28485TM was cultured in ~125 mL YPD media at 30 °C to mid-exponential phase (~48 hours). The *K. pastoris* cultures were diluted 1:1 with 20% glucose then left to rest for 15 minutes. The diluted cell cultures were then divided in 1 mL aliquots in cryogenic tubes, placed in an insulated Styrofoam cooler and frozen at -80 °C. To induce alcohol oxidase expression, frozen cells were thawed in a water bath at room temperature and resuspended in ~125 mL maintenance media at 30 °C.

S.4. Buffer Screen

In a 250 mL Erlenmeyer with a screw cap water (10 mL), Tris-HCl (10 mL, 100 mM, pH 7.4), PB (10 mL, 100 mM, pH 7.4), or HEPES (10 mL, 100 mM, pH 7.4) were spiked with butyraldehyde (5 mg/mL) and nitromethane (2 equivalents). The flasks were capped and transferred to a shaker incubator (25 °C, 200 RPM). Each reaction was prepared in triplicate. After 24 h, 1-nitrohexane (30 mg) and 4-nitrobenzaldehyde (30 mg) were added as internal standards and the reaction was extracted with ethyl acetate (1 × 3 mL). The product yield was determined by GC-MS.

S.5. Initial Amino Acid Screen

In a 250 mL Erlenmeyer with a screw cap PB (10 mL, 100 mM, pH 7.4), PB/Phenylalanine (10 mL, 100 mM/50mM, pH 7.4), PB/Leucine (10 mL, 100 mM/50mM, pH 7.4), or PB/Lysine (10 mL, 100 mM/50mM, pH 7.4) were spiked with butyraldehyde (5 mg/mL) and nitromethane (2 equivalents). The flasks were capped and transferred to a shaker incubator (25 °C, 200 RPM). Each reaction was prepared in triplicate. After 24 h, 1-nitrohexane (30 mg) and 4-nitrobenzaldehyde (30 mg) were added as internal standards

and the reaction was extracted with ethyl acetate (1 × 3 mL). The product yield was determined by GC-MS.

S.5. Nitromethane Equivalence Henry/Michael Reaction

In a 250 mL Erlenmeyer with a screw cap PB/lysine buffer (10 mL, 100 mM PB with 50 mM lysine, pH 7.4) was spiked with butyraldehyde (5 mg/mL) and nitromethane (2, 4, 8, or 16 equivalents). The flasks were capped and transferred to a shaker incubator (25 °C, 200 RPM). Each reaction was prepared in triplicate. After 24 h, 1-nitrohexane (30 mg) and 4-nitrobenzaldehyde (30 mg) were added as internal standards and the reaction was extracted ethyl acetate (1 × 3 mL). The product yield was determined by GC-MS.

S.6. Amino Acid Screen

The amino acid (50 mM) was dissolved in PB (~9 mL, 100 mM, pH 7.4). The pH was adjusted back to 7.4 with hydrochloric acid (10% v/v) or sodium hydroxide (10% w/v) if necessary. PB was added to a final volume of 10 mL. Each reaction was prepared in a 250 mL Erlenmeyer flask with a screw cap, the amino acid buffer solution (10 mL) was spiked with butyraldehyde (5 mg/mL) and nitromethane (16 equivalents). The flasks were capped and transferred to a shaker incubator (25°C, 200 RPM). After 24 h, 1-nitrohexane (30 mg) and 4-nitrobenzaldehyde (30 mg) were added as internal standards, and the reaction was extracted ethyl acetate (1 × 3 mL). The product yield was determined by GC-MS.

S.7. *K. Pastoris* Tandem System

K. pastoris cells were grown from frozen stock in ~125 mL maintenance media at 30 °C for 72 hours to OD₆₀₀ ~2.2. 10 mL cell cultures were pelleted at 5000 x g for ~10 minutes and washed with PBS buffer (10 mL, 100 mM, pH 7.4). The cell pellets were resuspended in 10 mL 100 mM PBS buffer and amino acid organocatalyst (L-lysine or L-phenylalanine) and transferred to a 250 mL screw-cap Erlenmeyer flask. 1- butanol (5 mg/mL) and 5 or 10 equivalents nitromethane relative to 1-butanol were added to each flask. The flasks were capped and placed in a shaker incubator (30 °C, 200 RPM). After 48 hours, products were extracted with 2 mL ethyl acetate. Yields were determined by GC-MS.

S.8. *K. Pastoris* Nitromethane Toxicity

K. pastoris cells were grown in ~125 mL maintenance media for 72 hours, then pelleted (~10 minutes, 5000 x g) and resuspended in maintenance media (without methanol). 10 mL of resuspended cells were each transferred to 250 mL screw-cap Erlenmeyer flasks. Each cell culture was spiked with 0, 200, 500, or 1000 mM nitromethane. The flasks were capped and placed in a shaker incubator (30 °C, 200 RPM). After 48 hours the cells were plated on YPD agar media. The cells were placed in an incubator at 30 °C. After 48 hours, the cells were observed for growth.

S.9. Nitromethane Equivalence Tandem Reaction with Alcohol Oxidase

A stock solution of catalase (1 mg/mL) in PB/lysine buffer (100 mM PB with 50 mM lysine, pH 7.4) was prepared. The stock solution was aliquoted (2 mL) into 12 ~20mL glass vials with screw caps. Alcohol oxidase (6 units/mL) was added to each reaction. The reactions were spiked with butanol (5 mg/mL) and nitromethane (2, 4, 8, or 16 equivalents). Each reaction was prepared in triplicate. The vials were capped and transferred to a shaker incubator (25 °C, 120 RPM). After 24 h, 1-nitrohexane (6 mg) and 4-nitrobenzaldehyde (6 mg) were added as internal standards and the reaction was extracted with ethyl acetate (1 × 0.6 mL). The product yield was determined by GC- MS.

S.10. Time Course – Tandem Reaction

A stock solution of catalase (1 mg/mL) in PB/lysine buffer (100 mM PB with 50 mM lysine, pH 7.4) was prepared. The stock solution was aliquoted (2 mL) into 8 ~20mL glass vials with screw caps. Alcohol oxidase (6 units/mL) was added to each reaction. The reactions were spiked with butanol (5mg/mL) and nitromethane (8 mol eq.). Each reaction was prepared in duplicate. The vials were capped and transferred to a shaker incubator (25 °C, 120 RPM). At 4, 10, 24, or 36 hours 1-nitrohexane (6 mg) and 4-nitrobenzaldehyde (6 mg) were added as internal standards, and the reaction was extracted with ethyl acetate (1 × 0.6 mL). The product yield was determined by GC-MS.

S.11. Time Course – Henry/Michael Reaction

In a 20 mL glass vial with a screw cap, PB/lysine buffer (2 mL, 100 mM PB, 50 mM lysine, pH 7.4) was spiked with butyraldehyde (5 mg/mL) and nitromethane (8 equivalents). Each reaction was prepared in duplicate. At 4, 10, 24, or 36 hours 1-nitrohexane (6 mg) and 4-nitrobenzaldehyde (6 mg) were added as internal standards, and the reaction was extracted with ethyl acetate (1 × 0.6 mL). The product yield was determined by GC- MS.

S.12. Substrate Scope

A stock solution of catalase (1 mg/mL) in PB/lysine buffer (100 mM PB with 50 mM lysine, pH 7.4) was prepared. The stock solution was aliquoted (2 mL) into 6 ~20mL glass vials with screw caps. Alcohol oxidase (6 units/mL) was added to each reaction. The reactions were spiked with propanol or pentanol (5mg/mL) and nitromethane (8 mol equivalents). Each reaction was prepared in triplicate. The vials were capped and transferred to a shaker incubator (25 °C, 120 RPM). At 24 hours, the reaction was extracted with ethyl acetate (1 × 0.6 mL). The product yield was determined by GC-MS.

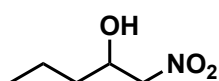
S.13. One-Pot Sequential Reaction

A stock solution of catalase (1 mg/mL) in PB (100 mM PB, pH 7.4) was prepared. The stock solution was aliquoted (10 mL) into 9 × 250 mL glass Erlenmeyer flasks with screw caps. Alcohol oxidase (6 units/mL) was added to each reaction. The reactions were spiked with butanol (2, 5, or 10 mg/mL). Each reaction was prepared in triplicate. The

flasks were capped and transferred to a shaker incubator (25°C, 120 RPM). At 24 hours, lysine (50 mM) and nitromethane (16 mol equivalents) were added, and the reaction was capped and returned to the shaker/incubator for an additional 10 hours. At that point, 1-nitrohexane (30 mg) and 4-nitrobenzaldehyde (30 mg) were added as internal standards, and the reaction was extracted with ethyl acetate (1 × 3 mL). The product yield was determined by GC-MS.

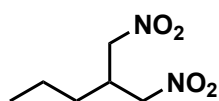
S.14. Synthetic Procedures

1-nitropentan-2-ol:

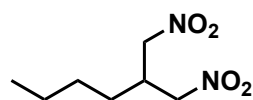


The synthesis of 1-nitropentan-2-ol was adapted from Lucet *et al.*¹ In a 100 mL round bottom flask submerged in an ice bath, 2 mL absolute ethanol, butyraldehyde (900 μ L), and nitromethane (552 μ L) was stirred. While stirring, NaOH (1 mL, 10 M) was added dropwise to the solution. When the solution had turned a yellow color glacial acetic acid (572 μ L) was added to the solution. The solution was washed with diethyl ether (2 × 10 mL) and the diethyl ether layers were combined. The combined diethyl ether layers were washed with DI water until the pH of the solution reached 5.5. The diethyl ether layer was dried with Na₂SO₄, and the solvent was removed using rotary evaporation. The product was purified using column chromatography with 5:1 hexanes:ethyl acetate. The fractions were combined and concentrated under reduced pressure to give 1-nitropentan-2-ol (182.5 mg, 5.4%). ¹H NMR (500 MHz, CDCl₃) δ 4.47 – 4.28 (m, 3H), 2.60 (s, 1H), 1.59 – 1.35 (m, 4H), 0.95 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 80.75, 68.49, 35.81, 18.53, 13.86.

1-nitro-2-(nitromethyl)pentane:



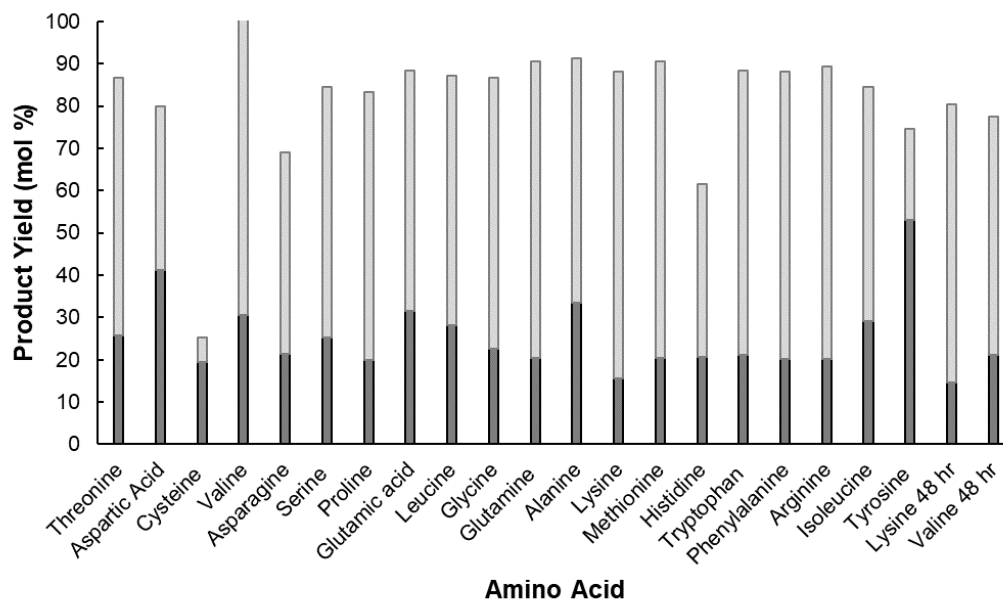
The synthesis of 1-nitro-2-(nitromethyl)pentane was adapted from Ballini *et al.*² In a 25 mL round bottom flask, nitromethane (8.02 mL) and butyraldehyde (721 μ L) was stirred under reflux at 100 °C. ~4 g basic alumina (Brockmann Activity I) was added to the solution and the mixture was allowed to stir. After 4 hours, the mixture was cooled to room temperature. The mixture was washed with ethyl acetate (3 × 50 mL), and the ethyl acetate layers were combined. The combined ethyl acetate layers were gravity filtered to remove the alumina catalyst. The combined ethyl acetate layers were then washed with DI water (3 × 30 mL). The solvent was removed using rotary evaporation to give the dinitroalkane (178.0 mg, 9.2% yield). ¹H NMR (500 MHz, CDCl₃-*d*) δ 4.64 – 4.55 (m, 2H), 4.55 – 4.48 (m, 2H), 2.92 (ddd, *J* = 11.9, 6.6, 5.2 Hz, 1H), 1.50 – 1.39 (m, 4H), 0.98 – 0.92 (m, 3H). ¹³C NMR (126 MHz, CDCl₃-*d*) δ 76.07, 35.88, 31.15, 19.67, 13.79.



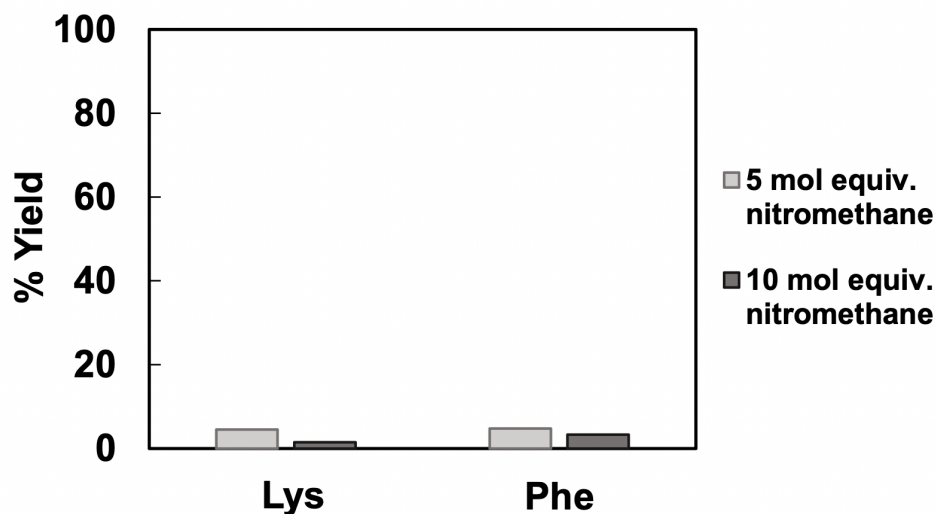
1-nitro-2-(nitromethyl)hexane: Nitromethane (8 mL), pentanal (850 μ L), and 4 g basic alumina were combined in a round bottom flask and refluxed at 100°C for 4 hours. After allowing the reaction mixture to cool, ethyl acetate was added (20 mL), and the reaction was filtered to remove the excess alumina. If necessary, the filtration was repeated to remove any residual alumina. The combined organic layers were washed with water (3 × 30 mL). The

washed organic layer was dried over sodium sulfate and concentrated with rotary evaporation and dried under vacuum (215 mg, 14%). ^1H NMR (500 MHz, CDCl_3) δ 4.58 (dd, $J = 13.5, 6.6$ Hz, 2H), 4.50 (dd, $J = 13.4, 5.3$ Hz, 2H), 2.89 (ddd, $J = 12.2, 6.8, 5.4$ Hz, 1H), 1.47 (dt, $J = 9.5, 6.4$ Hz, 2H), 1.34 (ttt, $J = 14.3, 6.5, 2.7$ Hz, 5H), 0.90 (t, $J = 7.1$ Hz, 4H). ^{13}C NMR (126 MHz, CDCl_3) δ 76.11, 36.07, 28.80, 28.44, 22.41, 13.81.

SI Figures



SI Figure 1. Yields of dinitroalkane (light grey) and nitroalcohol (dark grey) as a function of amino acid. Reaction conditions: 50 mM amino acid, 100 mM buffer, 5 mg/mL butyraldehyde, 16 equivalents nitromethane. Reactions were run for 24 h at room temp. Nitroalcohol and dinitroalkane yields were determined by GC-MS.



SI Figure 2. Yields of nitroalcohol and dinitroalkane produced from butanol in system utilizing *K. pastoris* as the bio-oxidative catalyst. Reaction conditions: 100 mM PBS buffer, 50 mM amino acid, 5 mg/mL butanol, 5 or 10 mol equivalents nitromethane per mol butanol, 48 hours at 30 °C.

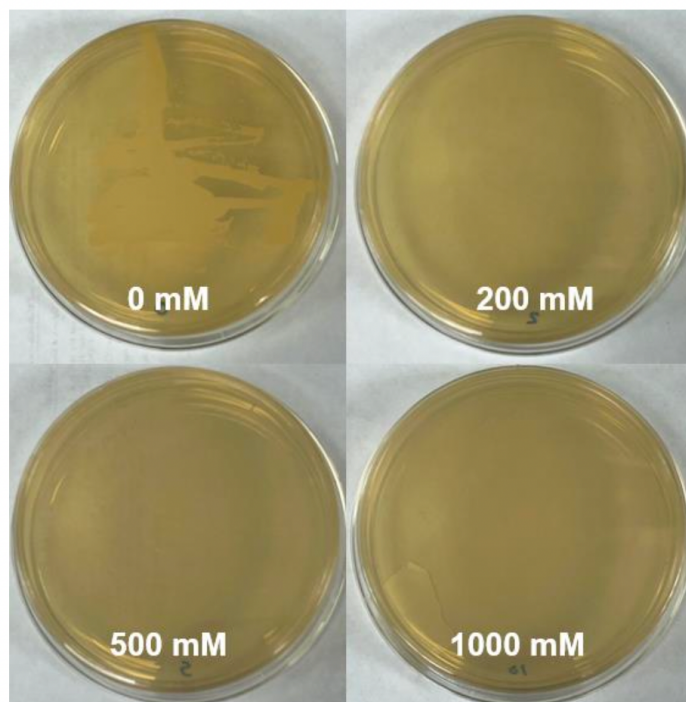


Figure S3. Nitromethane toxicity assay. *K. pastoris* cultured for 48 hours at 30 °C after being suspended in media containing 0, 200, 500, and mM nitromethane for 48 hours at 30 °C.

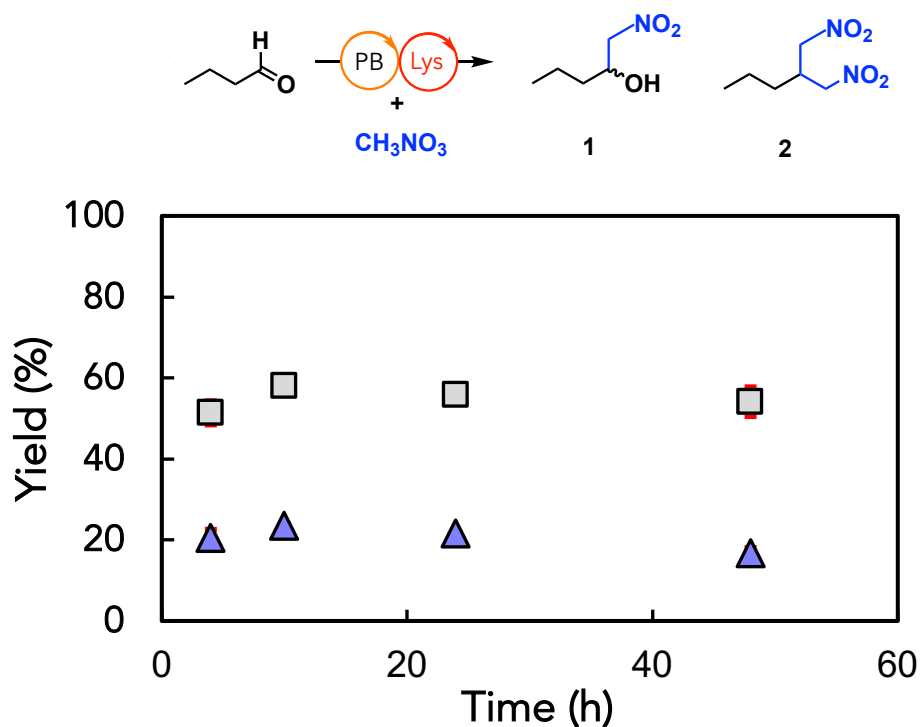


Figure S4. Time-course yield of nitroalcohol (1, blue triangles) and dinitroalkane (2, grey squares) from n-butylaldehyde starting material. Reaction conditions: Phosphate buffer (100 mM, pH 7.4), lysine (50 mM), butylaldehyde (5 mg/mL), nitromethane (8 equiv.). Reactions were stopped at the indicated time point and nitroalcohol and dinitroalkane yields were determined by GC-MS. Each timepoint represents a separate reaction. Each data point represents the average \pm SD of duplicate reactions.

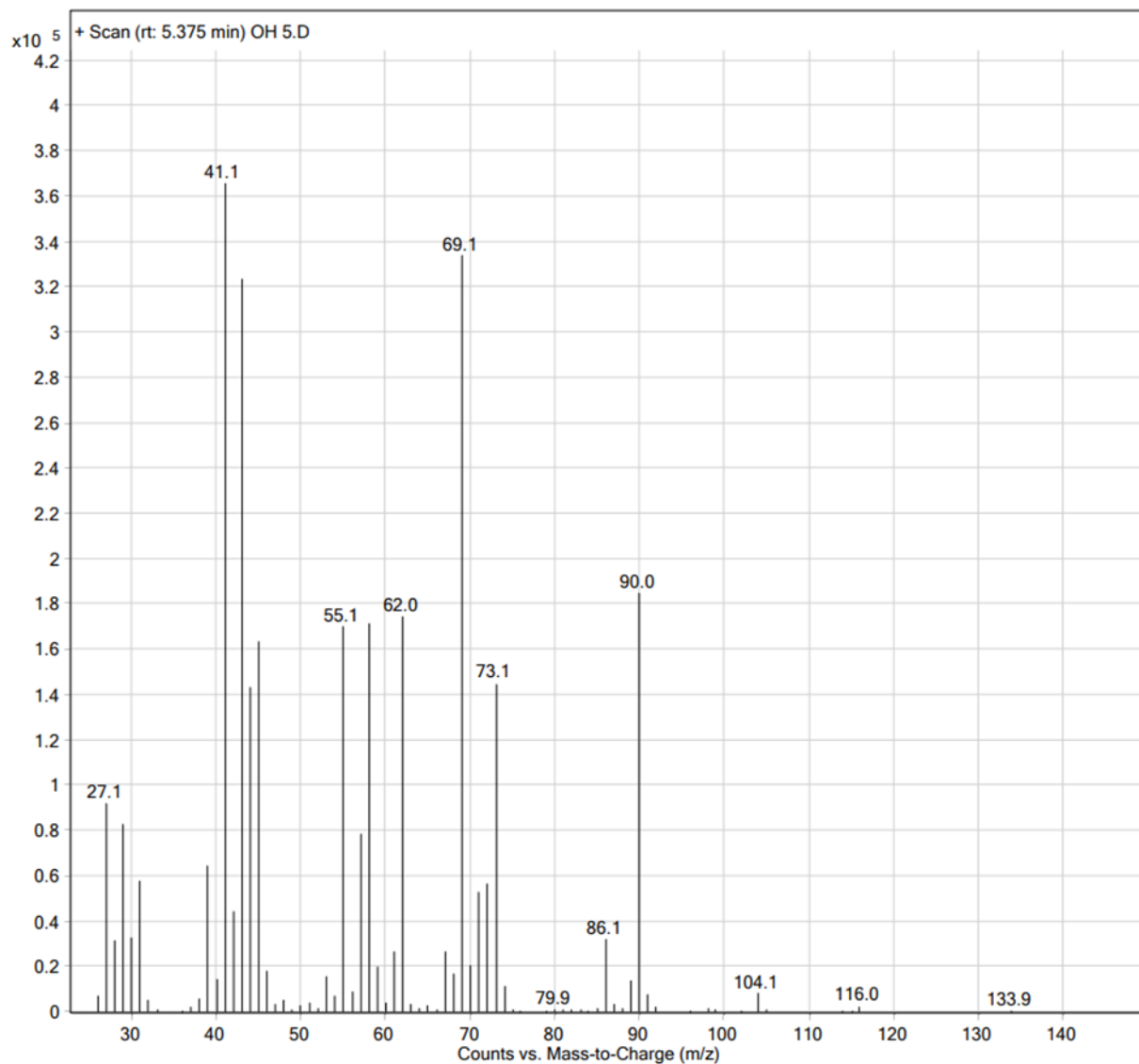


Figure S5. Mass spectrum of 1-nitro-2-pentanol.

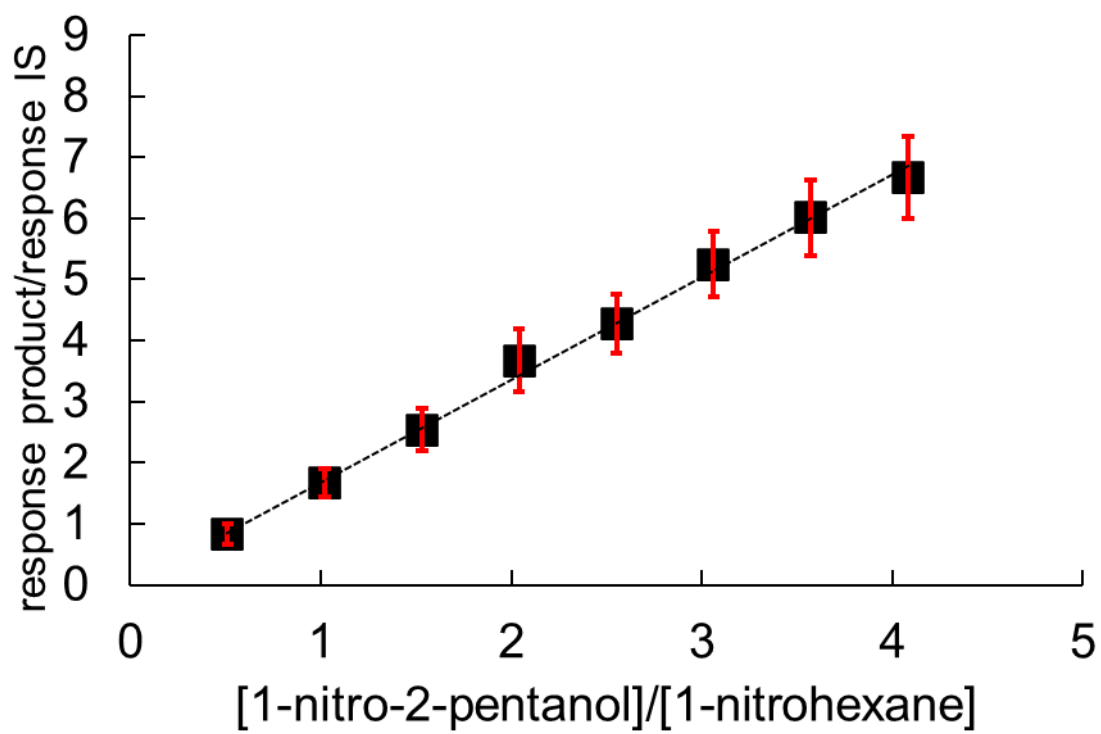


Figure S6. Calibration curve for 1-nitro-2-pentanol with 1-nitrohexane as an internal standard. Data points are averages \pm SD of triplicate samples with duplicate runs.

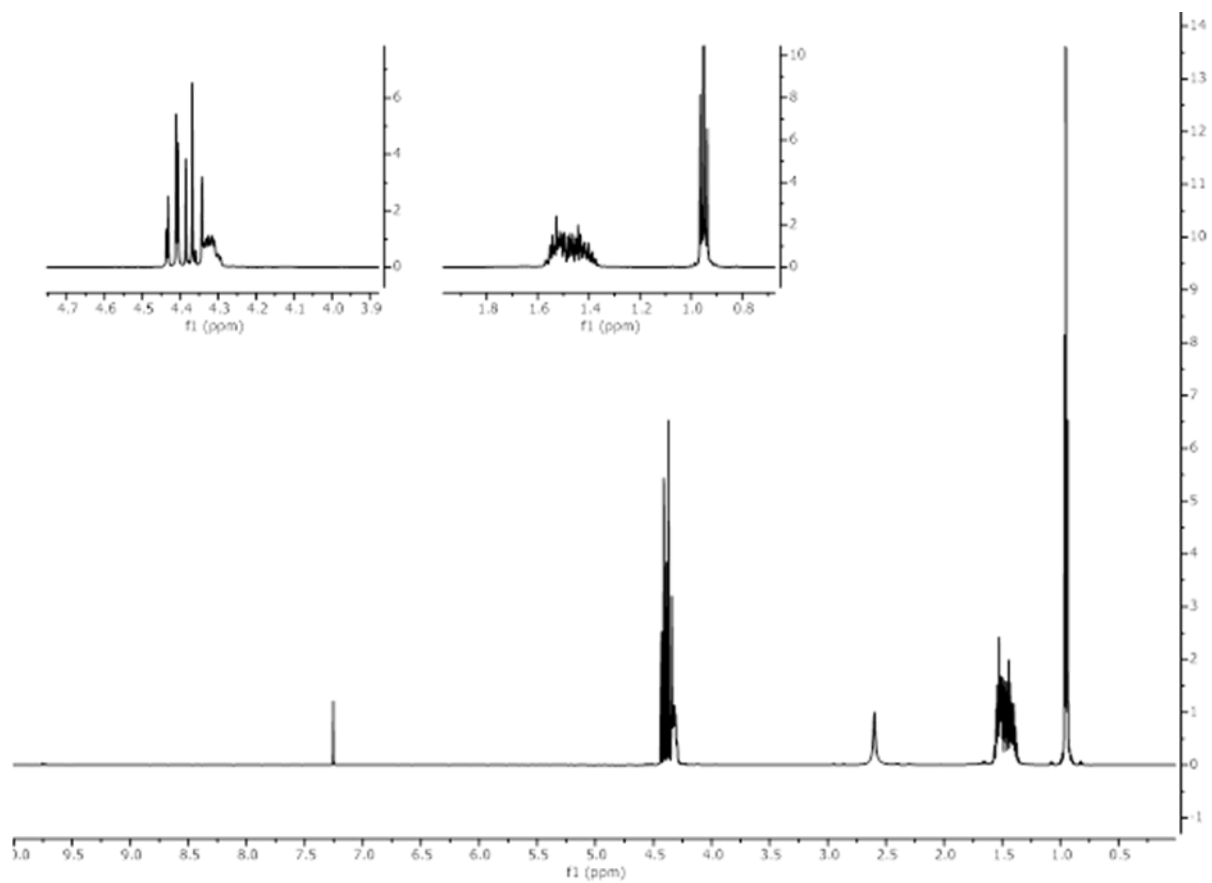


Figure S7. ^1H NMR spectrum of 1-nitro-2-pentanol.

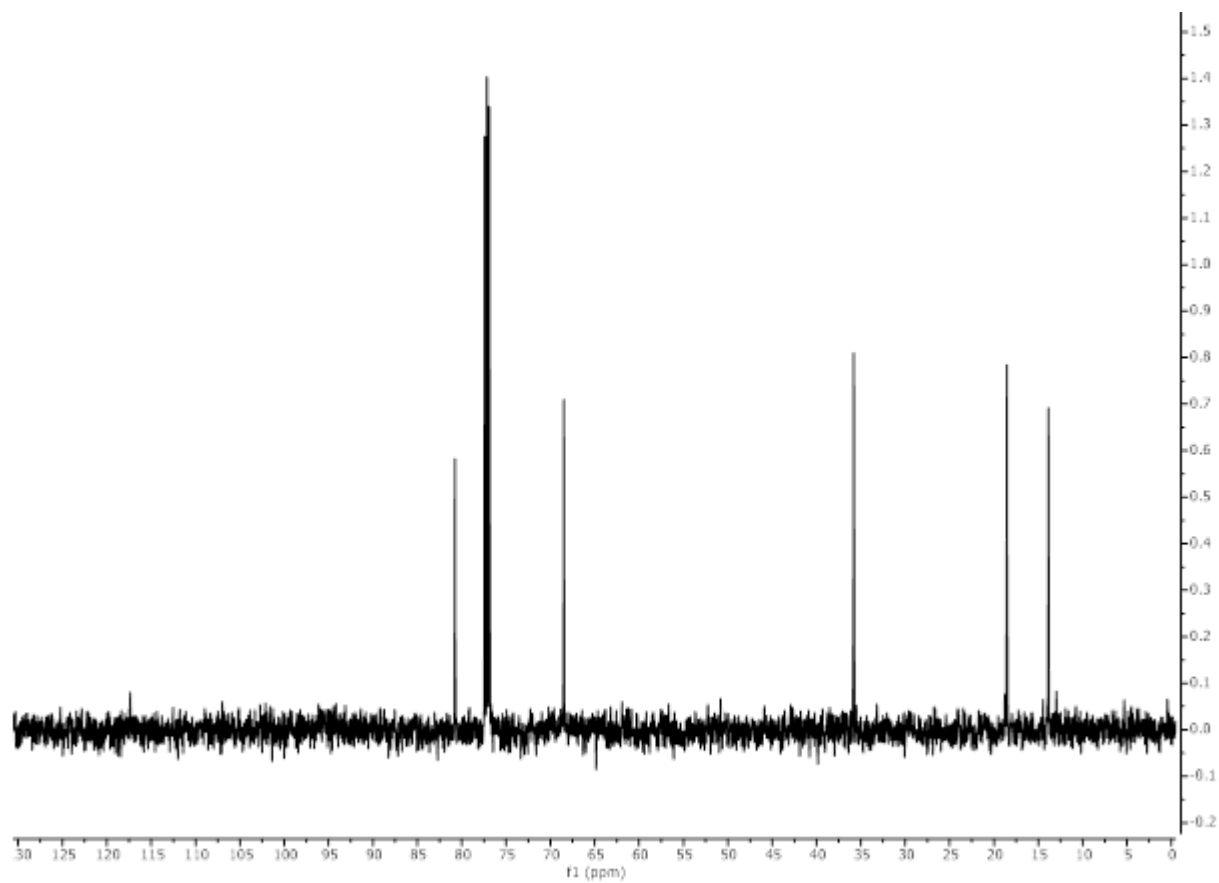


Figure S8. ^{13}C NMR spectrum of 1-nitro-2-pentanol.

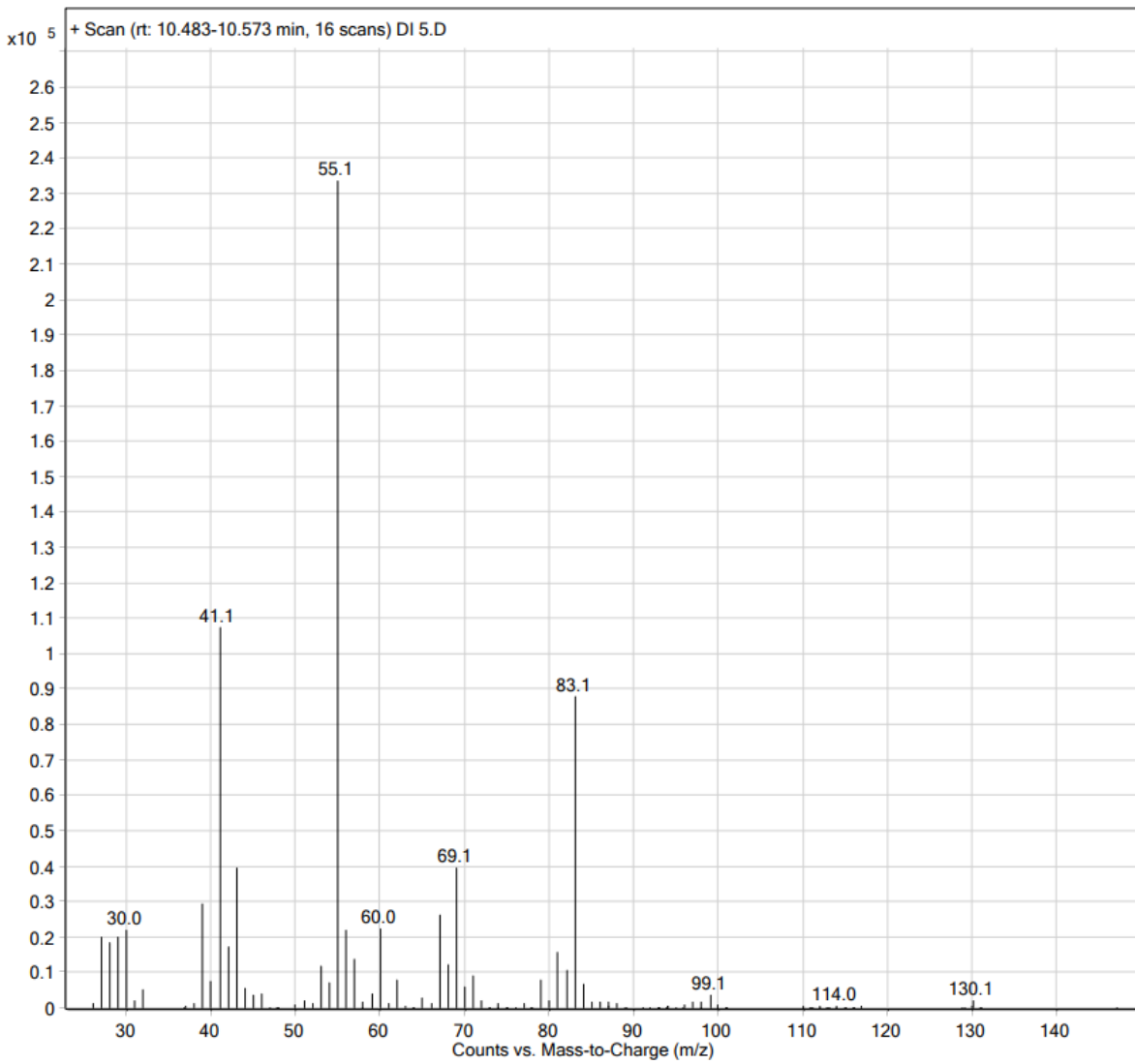


Figure S9. Mass spectrum of 1-nitro-2-(nitromethyl)pentane.

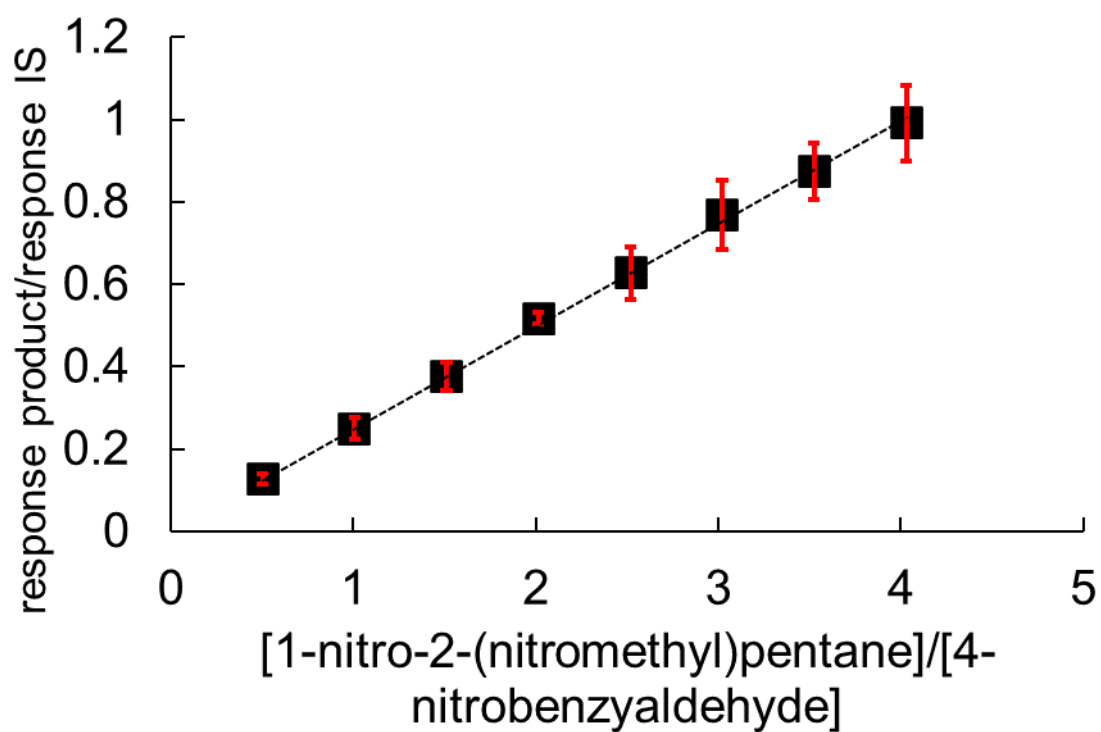


Figure S10. Calibration curve for 1-nitro-2-(nitromethyl)-pentane with 4-nitrobenzaldehyde as an internal standard. Data points are averages \pm SD of triplicate samples with duplicate runs.

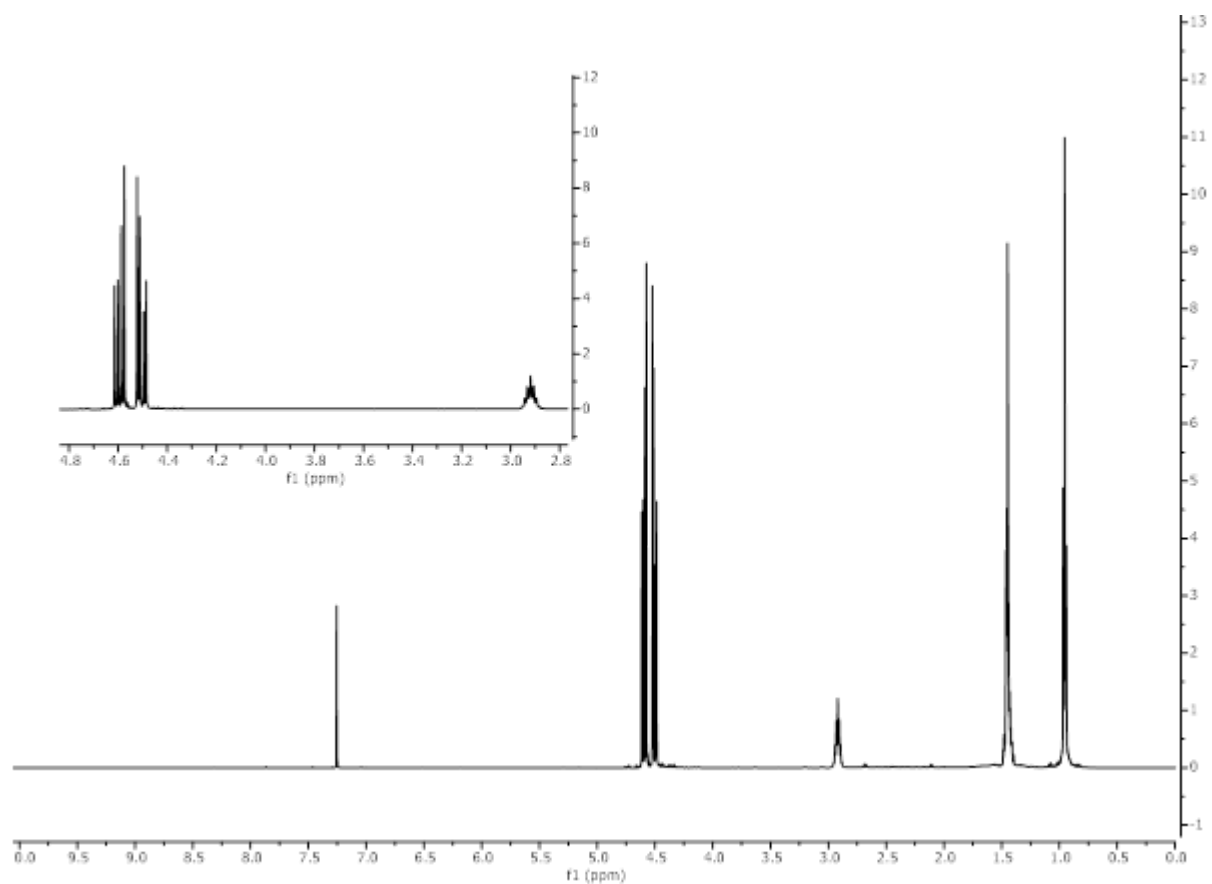


Figure S11. ^1H NMR spectra of 1-nitro-2-(nitromethyl)pentane.

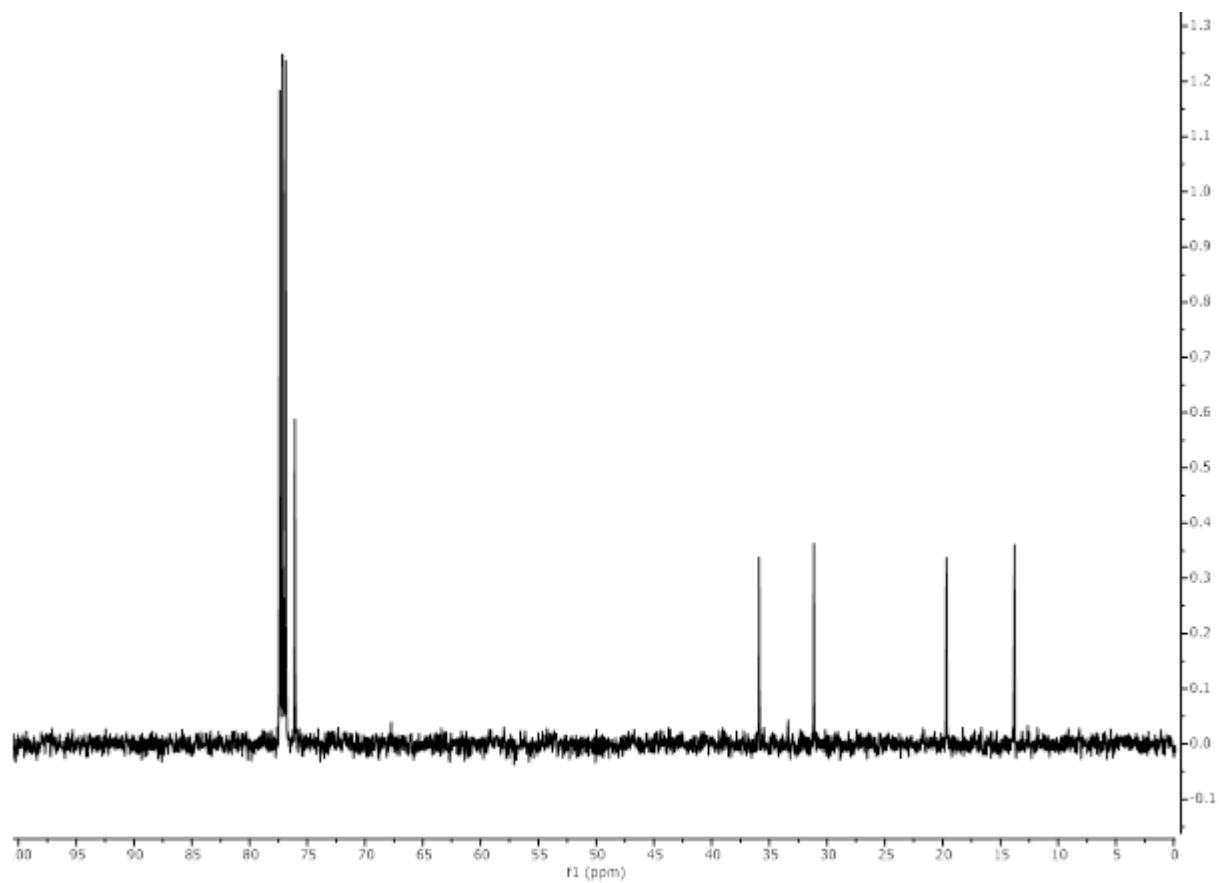


Figure S12. ^{13}C NMR spectra of 1-nitro-2-(nitromethyl)pentane.

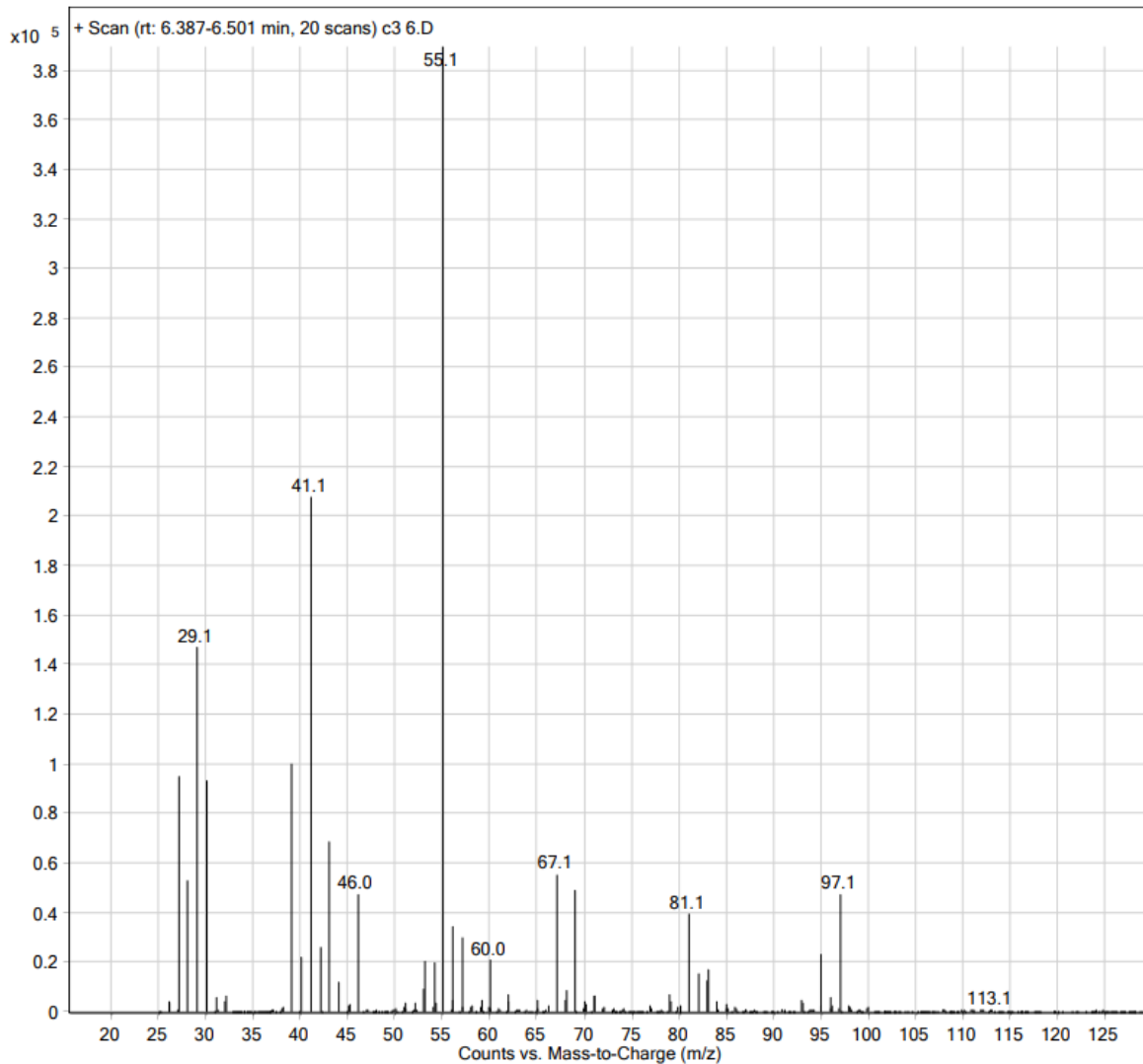


Figure S13. Mass spectrum of 1-nitro-2-(nitromethyl)butane.

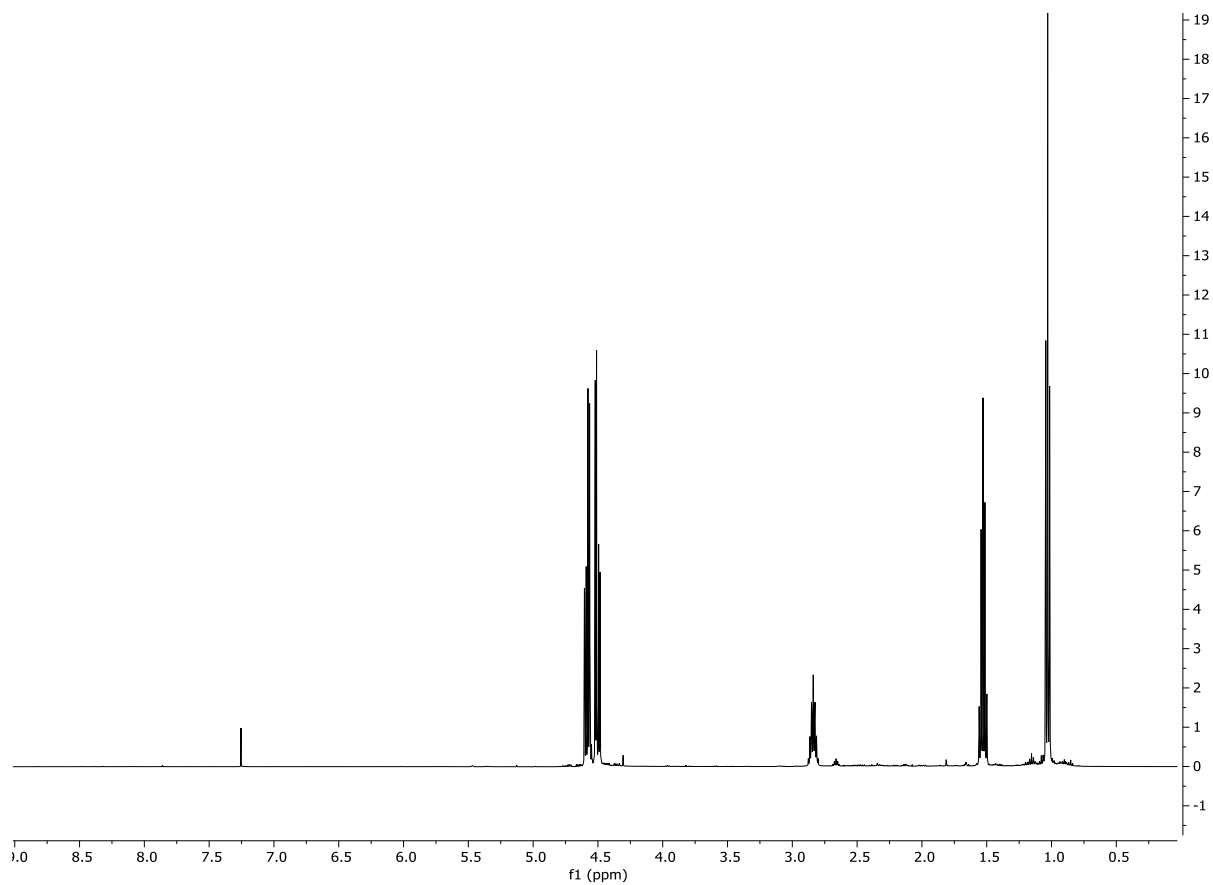


Figure S14. ^1H NMR spectra of 1-nitro-2-(nitromethyl)butane.

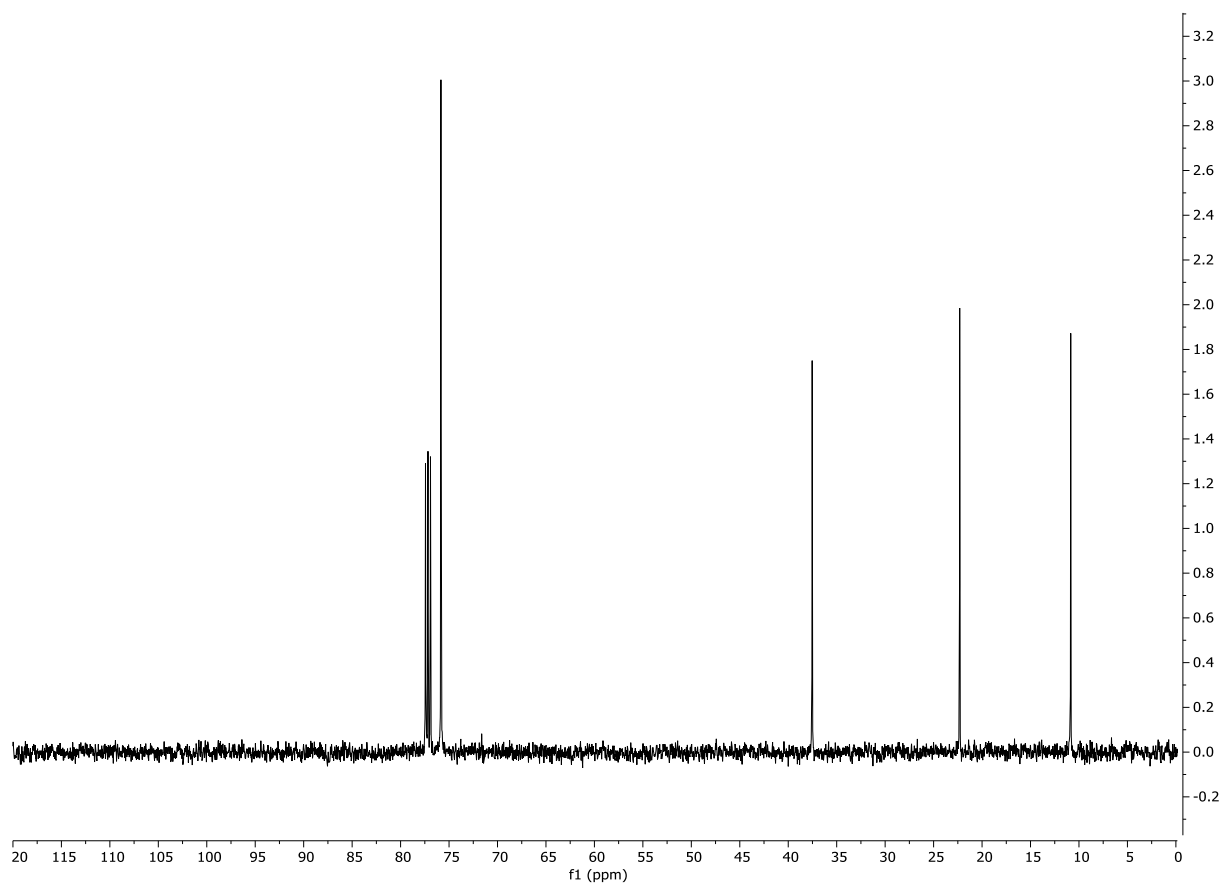


Figure S15. ^{13}C NMR spectra of 1-nitro-2-(nitromethyl)butane.

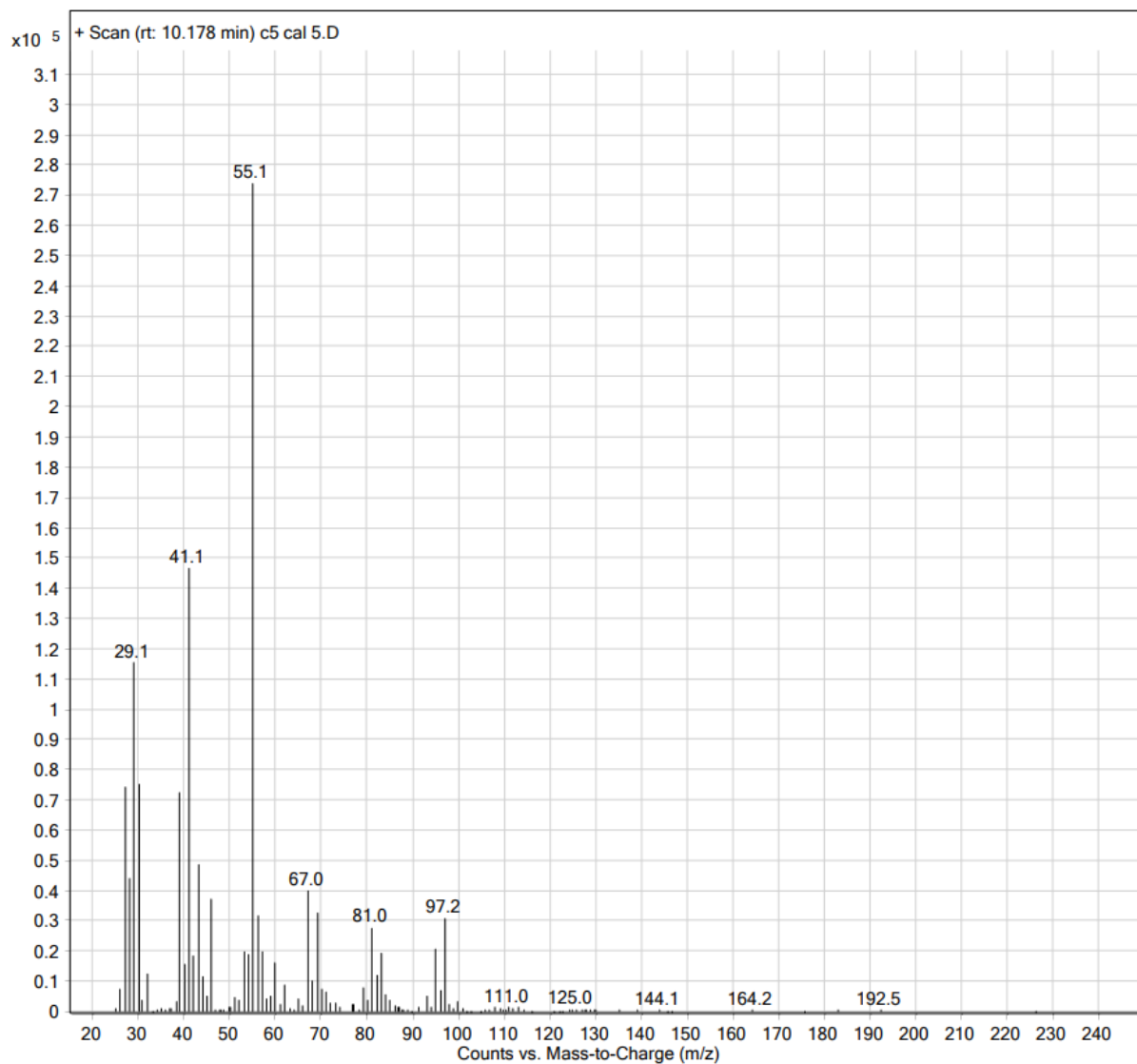


Figure S16. Mass spectrum of 1-nitro-2-(nitromethyl)hexane.

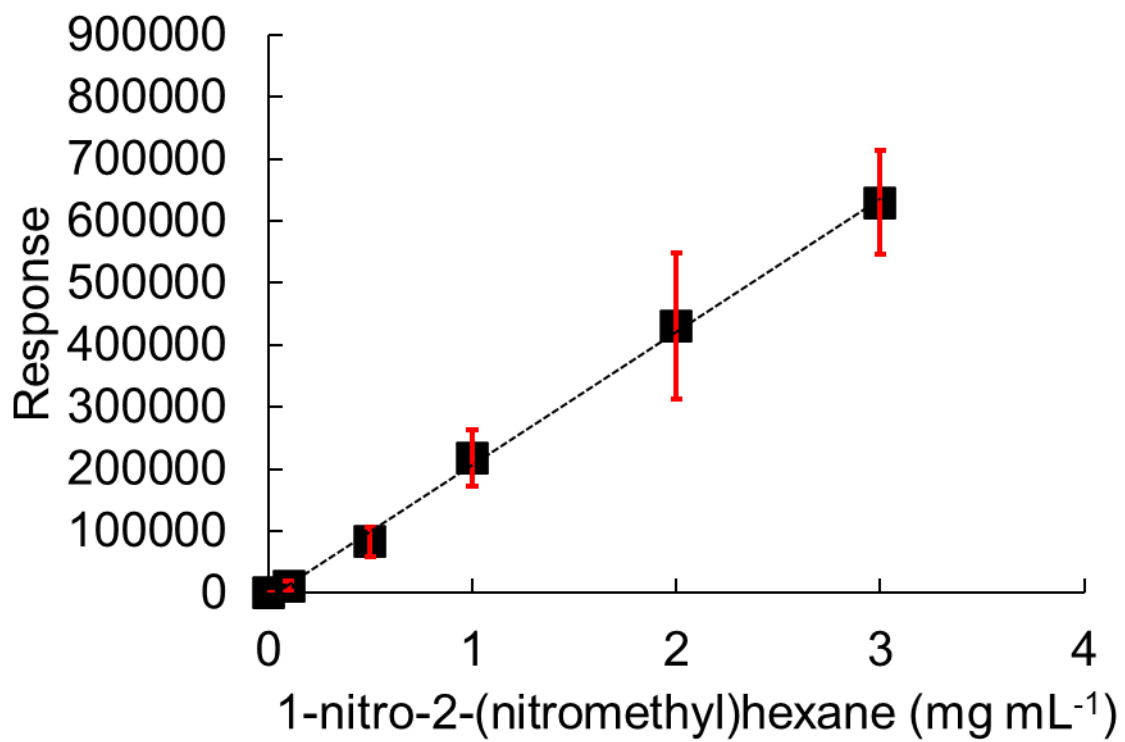


Figure S17. Calibration curve for 1-nitro-2-(nitromethyl)hexane. Each data point represents the average \pm SD of triplicate determinations.

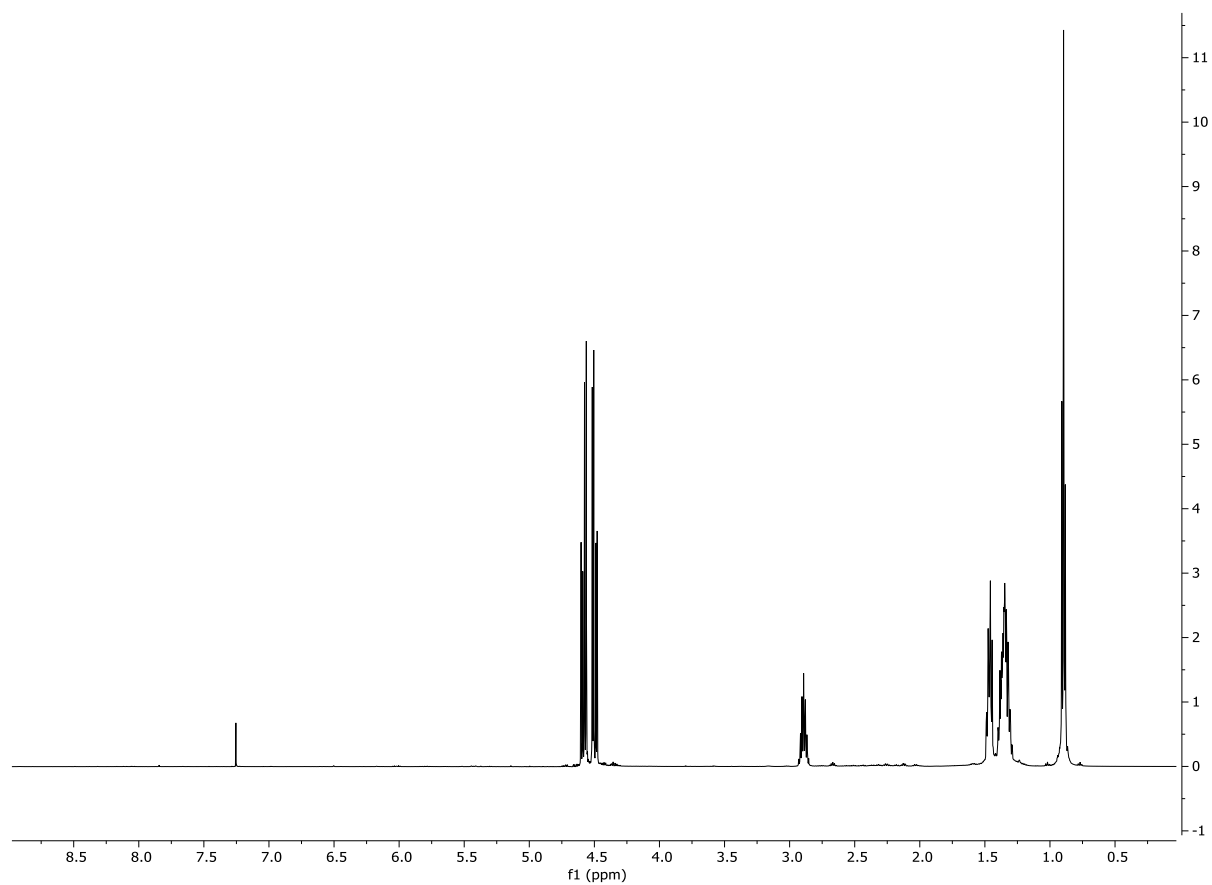


Figure S18. ^1H NMR spectra of 1-nitro-2-(nitromethyl)hexane.

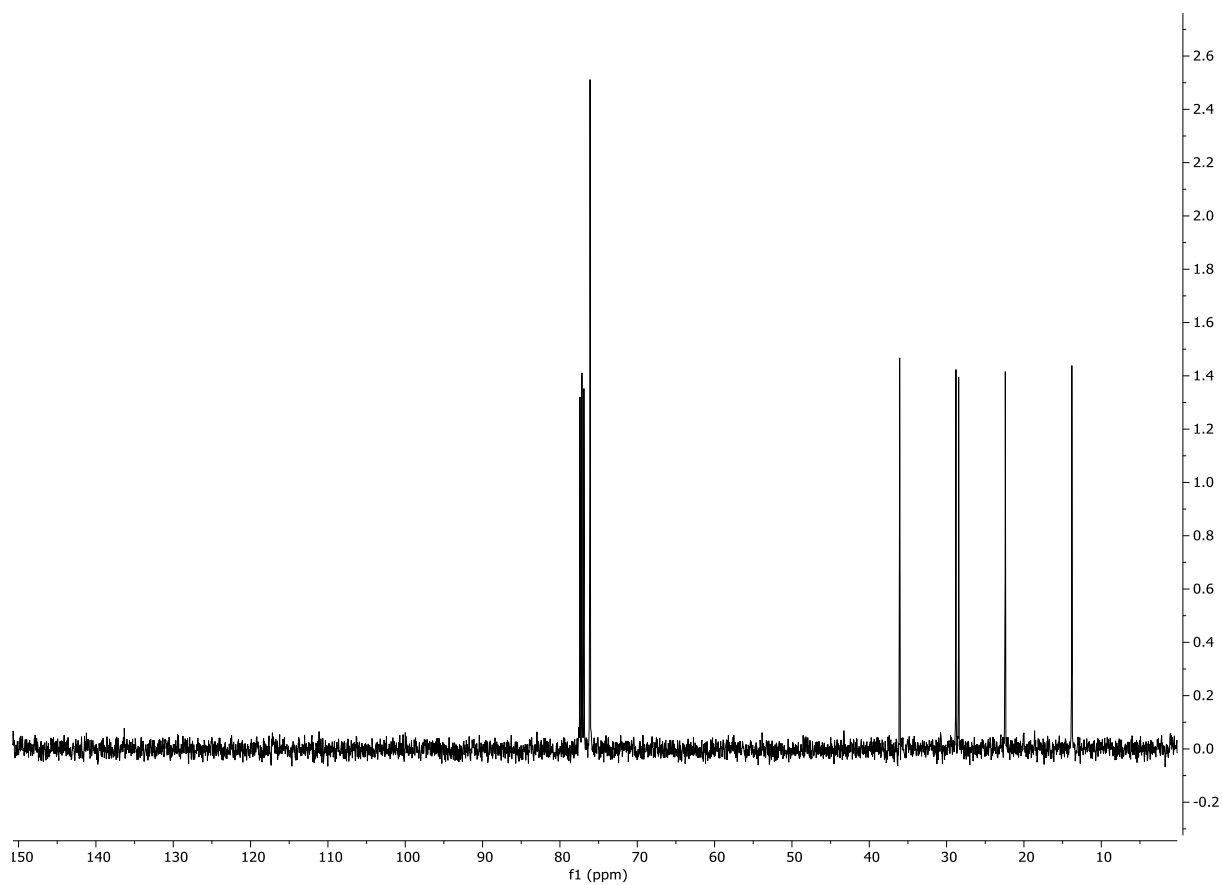


Figure S19. ^{13}C NMR spectra of 1-nitro-2-(nitromethyl)hexane.

References:

1. D. Lucet, S. Sabelle, O. Kostelitz, T. Le Gall and C. Mioskowski, *Eur. J. Org. Chem.*, 1999, **1999**, 2583–2591.
2. R. Ballini, G. Bosica, D. Fiorini and A. Palmieri, *Synthesis*, 2004, **2004**, 1938–1940.