Supplementary Information

Ratiometric analysis of reversible thia-Michael reactions using nitrile-tagged molecules by Raman microscopy

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1. Supplementary figures

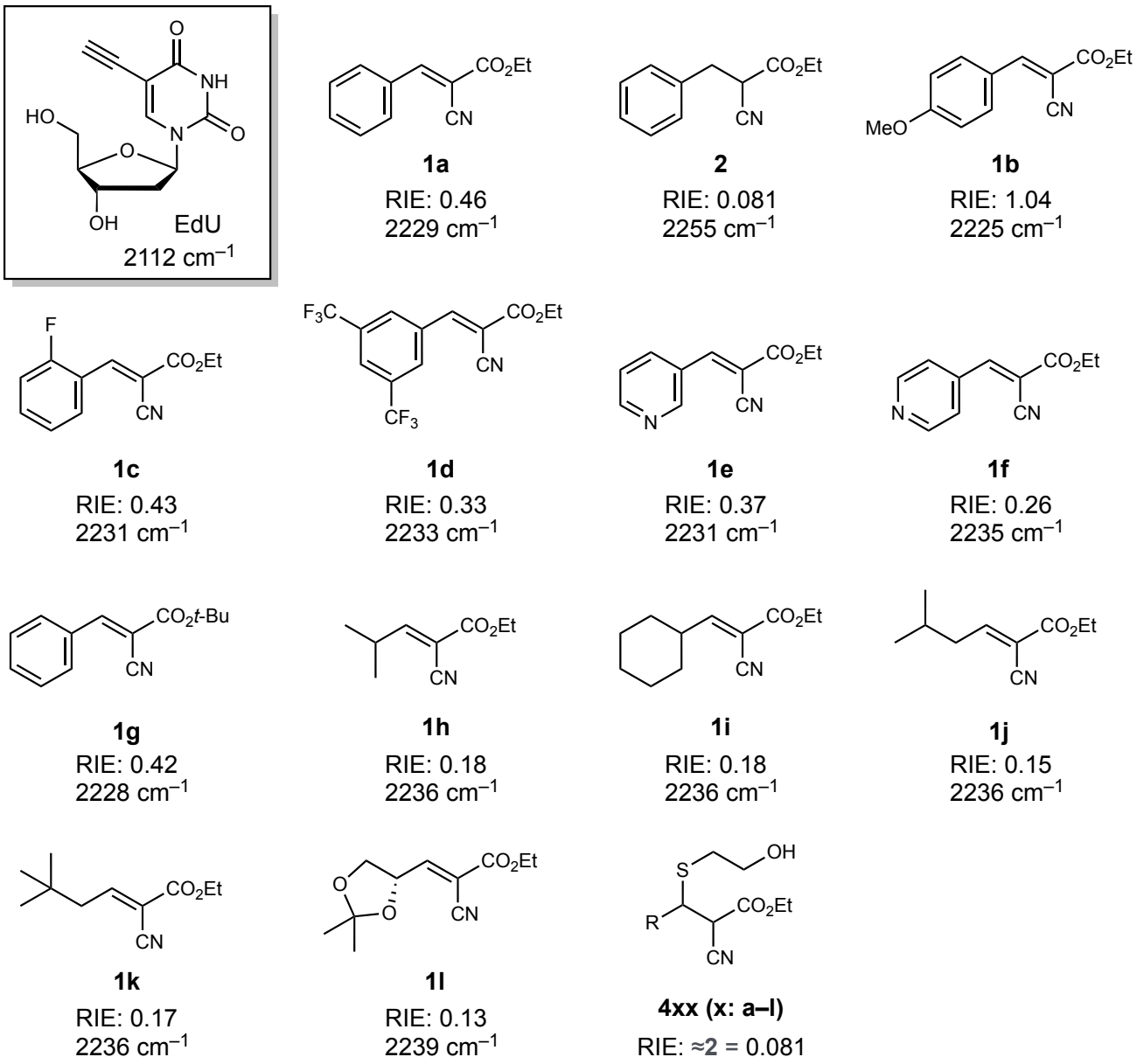


Figure S1. Relative Raman intensity vs. 5-ethynyl-2'-deoxyuridine (EdU) (RIE) values of nitriles. The laser wavelength was set to 532 nm. According to reference 3 (*J. Am. Chem. Soc.* **2012**, *134*, 20681), the RIE values were calculated from the ratios of the peak areas using mixtures of nitriles and EdU diluted in DMSO. The RIE values of thiol adducts **4xx** (**x: a–l**) approximated the value of alkyl nitrile **2** (0.081) because **4xx** was unisolable.

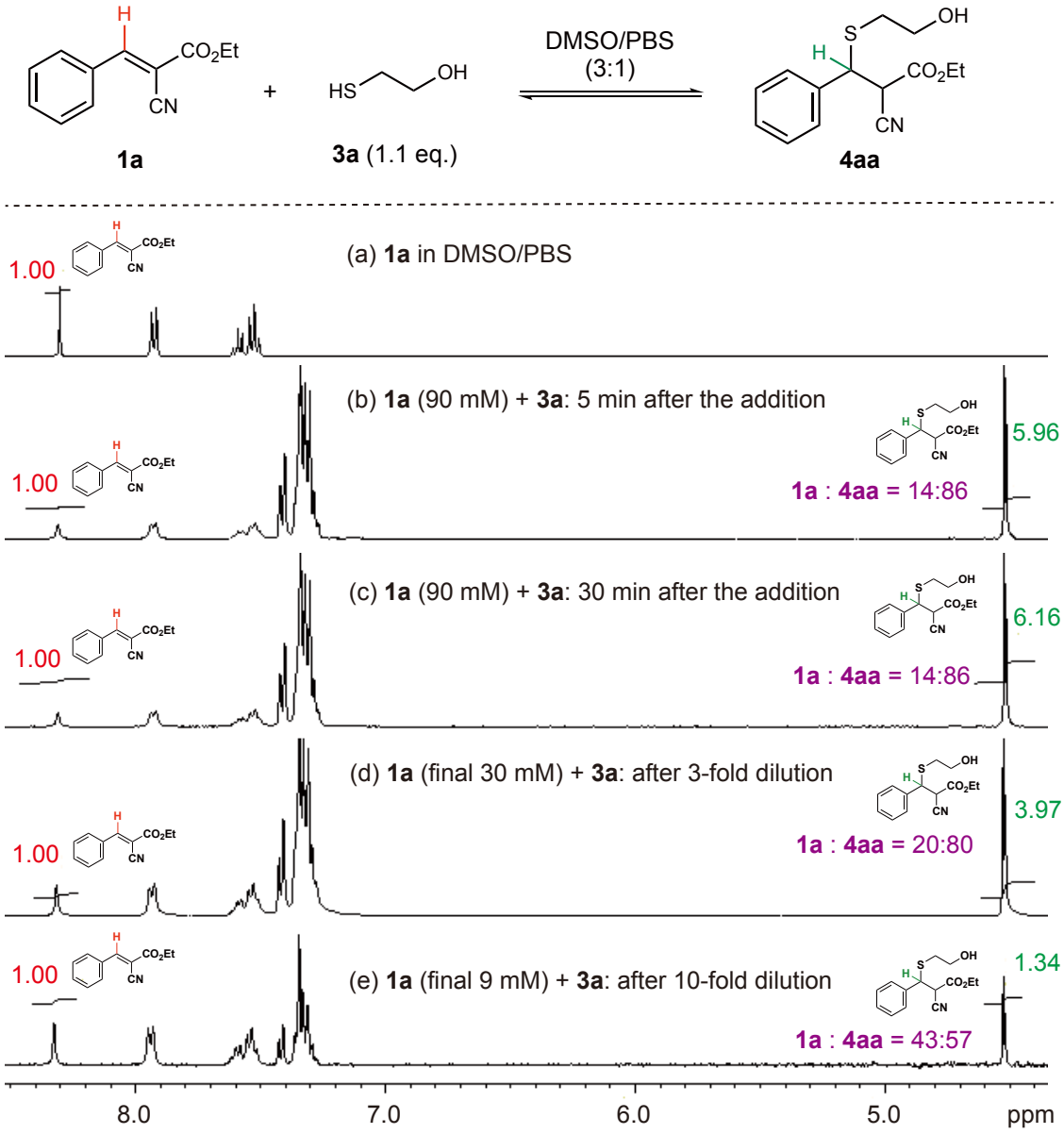


Figure S2. ^{1}H-NMR analysis of the thia-Michael reaction. The ratio between αCNA **1a** and the adduct **4aa** was calculated from the integration values of the proton signal in red for **1a** and in green for **4aa**. The values highlighted in the corresponding colors represent the respective integral values. The integral of the proton signal highlighted in red (**1a**) was normalized to 1. The integral of **4aa** accounts for the sum of the diastereomers. (a) ^{1}H-NMR spectrum of **1a** in DMSO-*d*6/PBS-*d*. (b) **1a** was treated with 1.1 equivalents of βME (**3a**). After 5 min, a 14:86 mixture of **1a** and **4aa** was obtained. (c) After 30 min, the **1a**:**4aa** ratio (13:87) was not changed, indicating that the thia-Michael reaction was completed within 5 min. (d, e) Upon a 3- or 10-fold dilution of the mixture without altering the DMSO percentage (c), a higher proportion of **1a** was detected, which confirmed the reversible nature of the thia-Michael reaction with **1a** and **3a**. NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; βME, β-mercaptoethanol; PBS, phosphate-buffered saline; αCNA, α-cyanoacrylic acid.

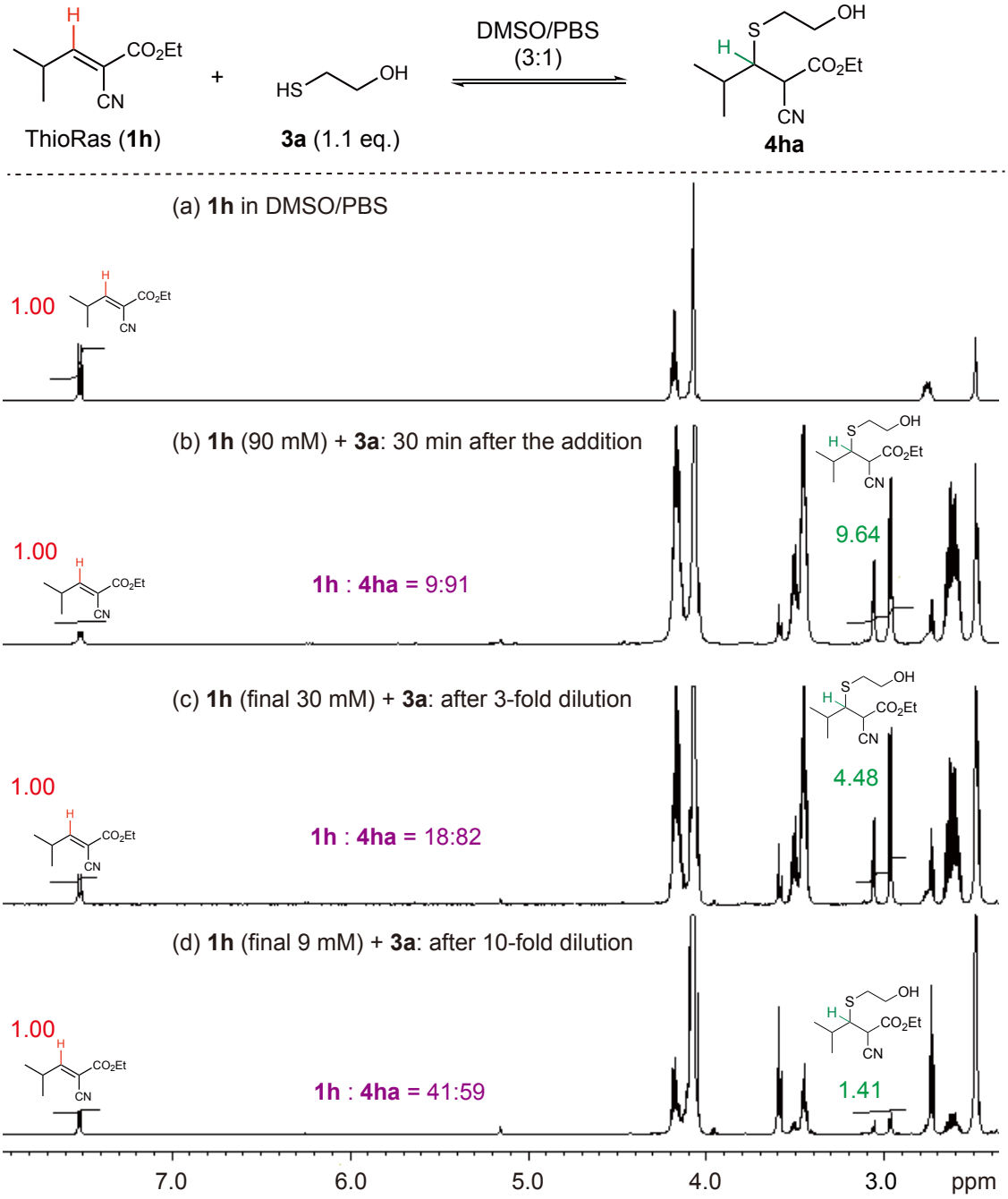


Figure S3. ^{1}H-NMR analysis of the thia-Michael reaction. The ratio between αCNA **1a** and the adduct **4ha** was calculated from the integration values of the proton signal in red for **1a** and in green for **4ha**. The values indicated in the corresponding colors represent the respective integral values. The integral of the proton highlighted in red (**1a**) was set to 1. The integral of **4ha** is the sum of the diastereomers. (a) ^{1}H-NMR spectrum of ThioRas (**1h**) in DMSO-*d*6/PBS-*d*. (b) **1h** was treated with 1.1 equivalents of βME (**3a**). After 30 min, the **1h**:**4ha** ratio was 9:91. (c, e) Upon a 3- or 10-fold dilution of the mixture without altering the DMSO percentage (b), a higher proportion of **1h** was detected, confirming the reversible nature of the thia-Michael reaction with **1h** and **3a**. NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; βME, β-mercaptoethanol; PBS, phosphate-buffered saline.

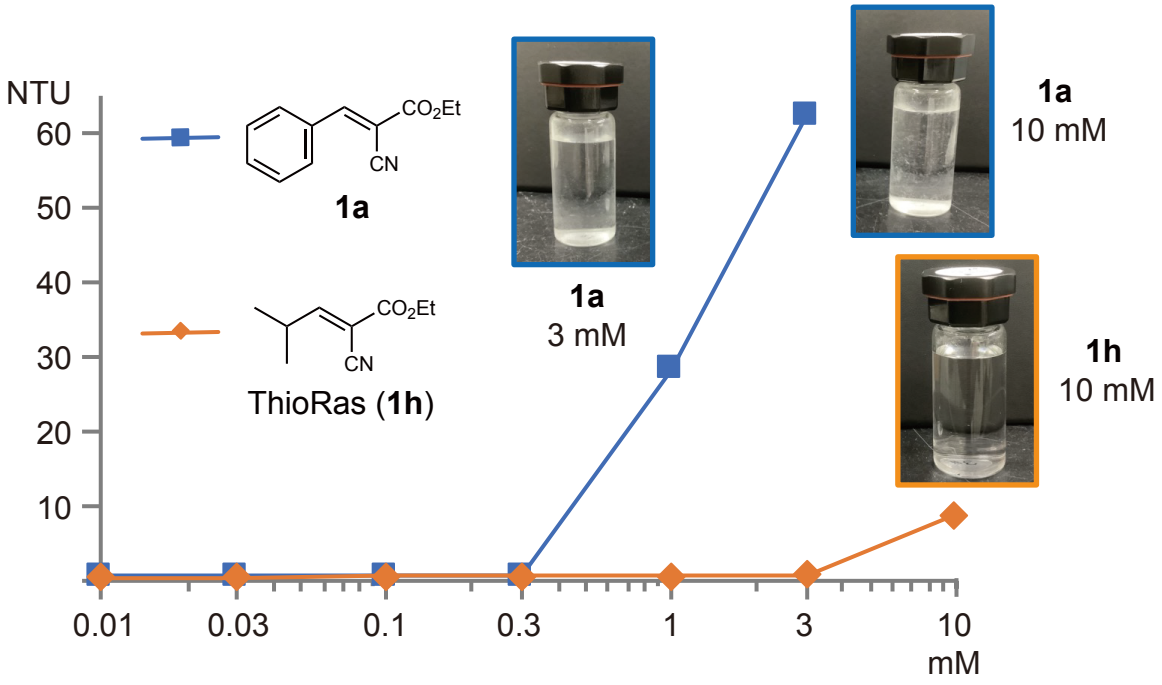


Figure S4. Turbidity assay of αCNA **1a** and ThioRas **1h** in PBS. The images within the blue box represent solutions with 3 mM or 10 mM **1a**. The image within the orange box represents a solution with 10 mM ThioRas (**1h**). Of note, 1,3-dioxolane **1l** was more water soluble; however, it was unstable under physiological conditions. Furthermore, ThioRas was completely dissolved in water at concentrations of >10 mM in the presence of thiols.

PBS, phosphate-buffered saline; αCNA, α-cyanoacrylic acid.

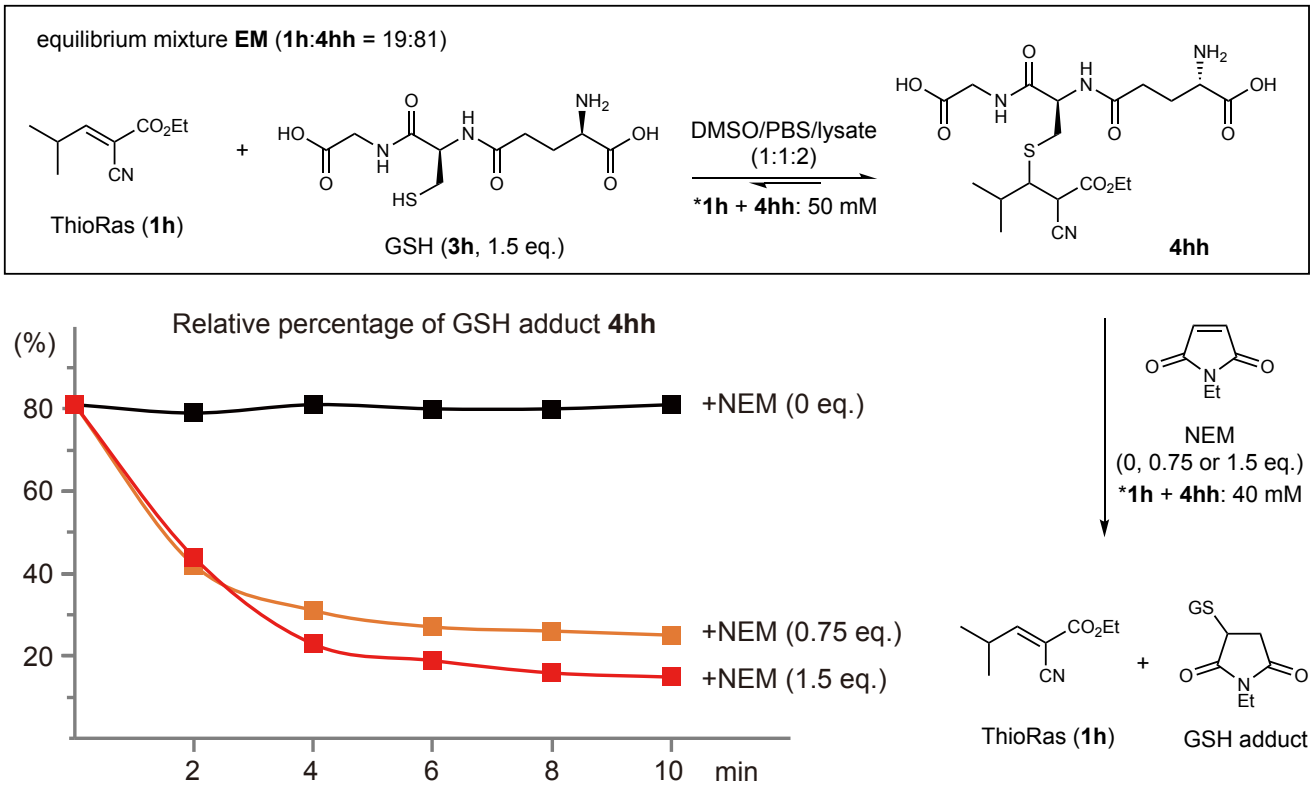


Figure S5. Raman analysis of the thia-Michael reaction between ThioRas (**1h**) and GSH (**3h**). ThioRas (**1h**) was treated with 1.5 equivalents of GSH (**3h**) to generate an equilibrium mixture, **EM** (**1h**: **4hh** = 19:81). After the addition of NEM without altering the DMSO percentage, the proportion of **4hh** was gradually decreased with the irreversible thia-Michael reaction of GSH (**3h**) and NEM.
GSH, glutathione; NEM, *N*-ethylmaleimide

Experimental procedures used for the experiments represented in Figure S5

Cell lysate was prepared by the homogenization of 3.3×10^5 HeLa cells in 1 mL of lysis buffer (20 mM HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mM NaCl, 1 mM EDTA, 0.5% Triton-X, pH 7.4) on ice. ThioRas (**1h**, 200 mM in DMSO), GSH (**3h**, 300 mM in pH 7.4 PBS), and cell lysate (3.3×10^5 HeLa cells/mL) were mixed in a 1:1:2 volumetric ratio. The Raman spectrum of the resulting equilibrium mixture (**EM**) was measured (**1h** + **4hh** = 50 mM; **1h**:**4hh** = 19:81). **EM** and *N*-ethylmaleimide (0, 150, or 300 mM in DMSO) were mixed at a 4:1 ratio. The **1h**:**4hh** ratio in the resulting mixture (**1h** + **4hh** = 40 mM) was monitored using Raman microscopy at different time points (2, 4, 6, 8, and 10 min). The **1h** and **4hh** molar ratios were calculated from peak heights corrected for intensity by the RIE values shown in Supplementary Fig. 1 (**1a**: 0.46; the RIE value for the thiol adduct **4hh** was approximated as 0.081 [the value of alkyl nitrile **2**] because **4hh** is unisolable).

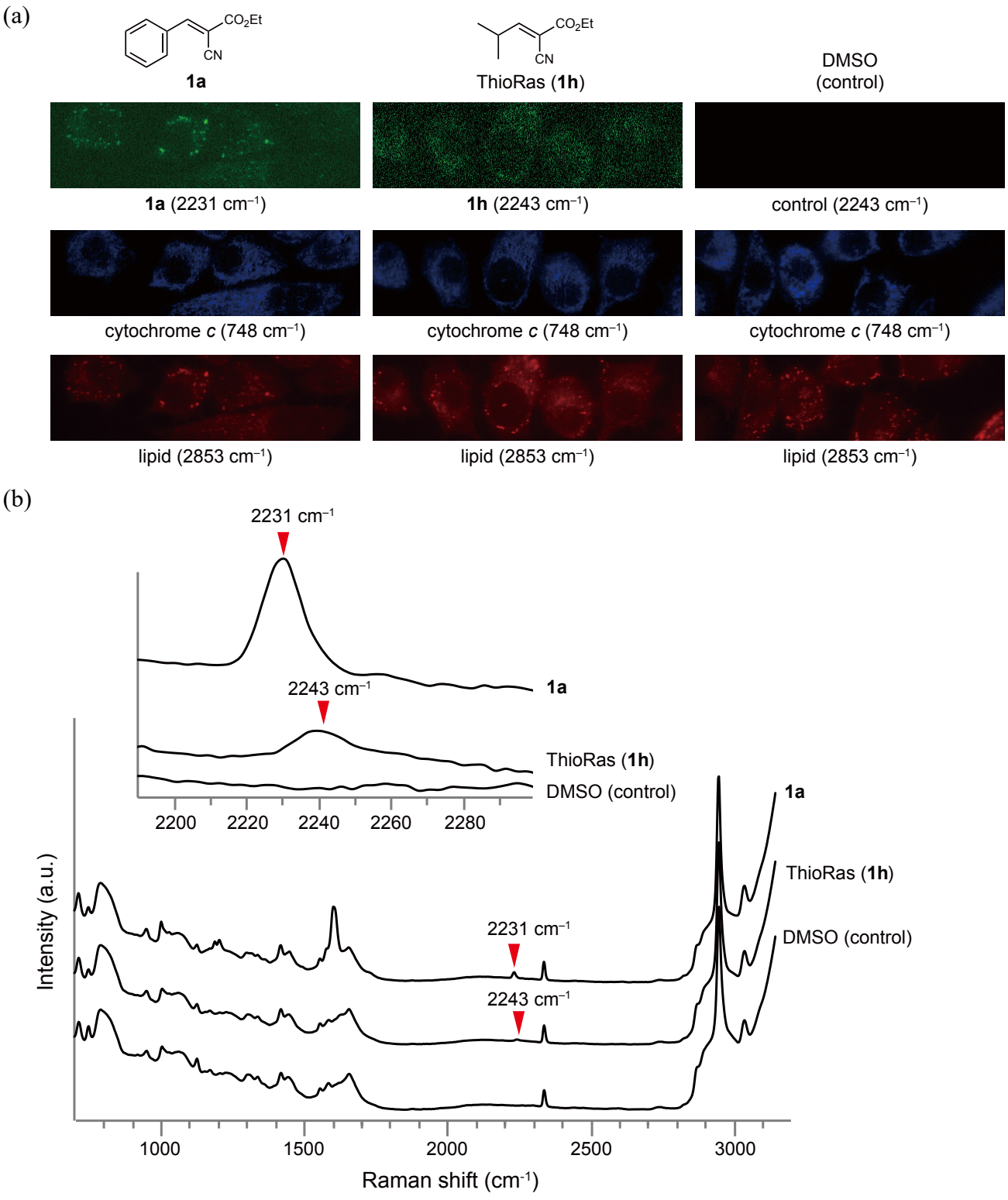


Figure S6. Raman imaging of HeLa cells with 600 μM **1a** or 8 mM ThioRas (**1h**). Laser wavelength: 532 nm; objective lens: 25×; laser-power density: 3 mW/μm^2; exposure time: 5 s/line. (a) Raman images showing the distributions of **1a** at 2231 cm^{-1} (Green), **1h** at 2243 cm^{-1} (Green), cytochrome *c* at 748 cm^{-1} (Blue), and lipid at 2853 cm^{-1} (Red). (b) Average Raman spectra of HeLa cells (3000 pixels). DMSO, dimethyl sulfoxide; a.u., arbitrary units

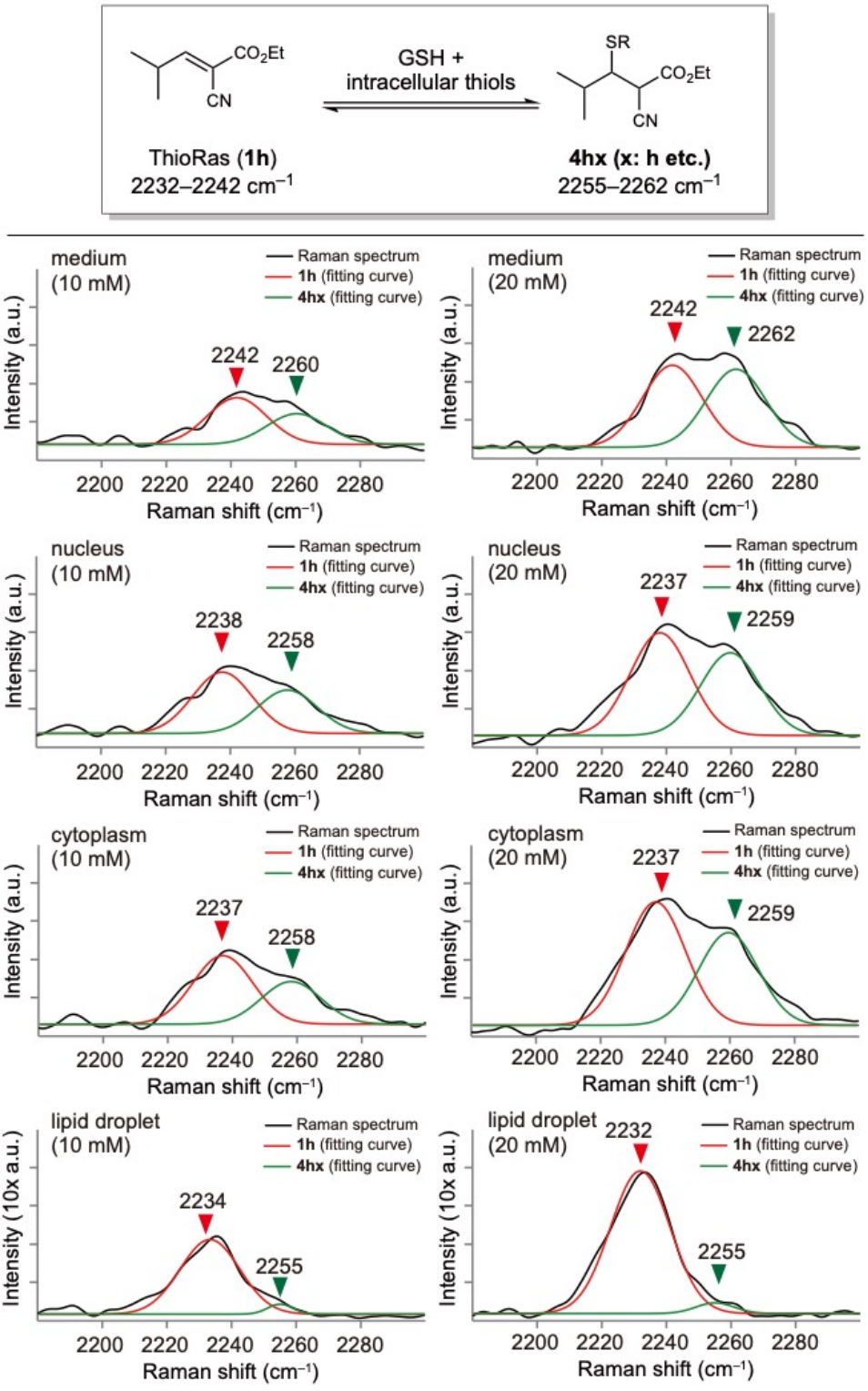
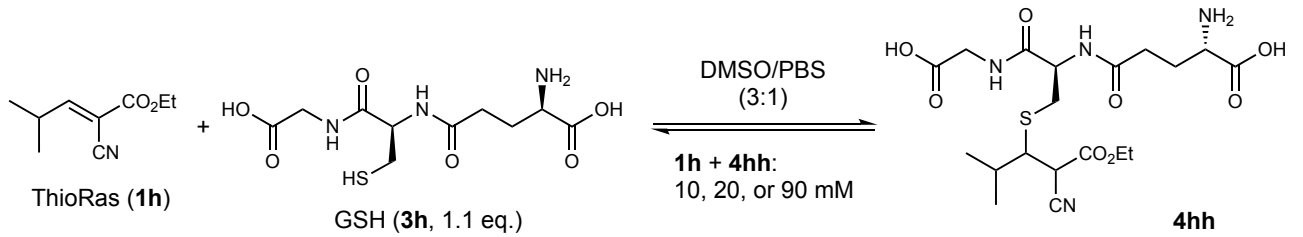


Figure S7. Live-cell analysis of thiols after exposure to 10 or 20 mM ThioRas in the presence of glutathione (GSH) (1.2 equivalents).



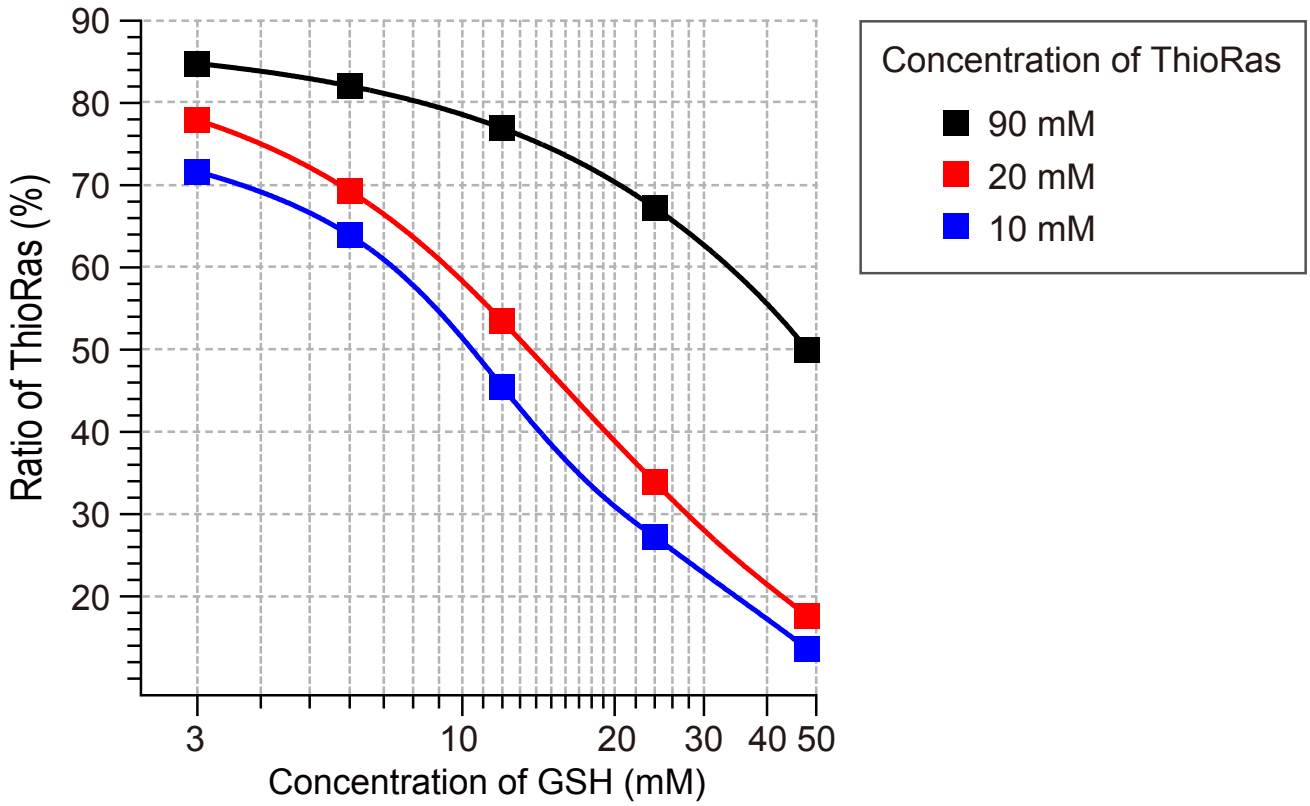


Figure S8. Thiol (GSH) concentration calibration curve. ThioRas (**1h** in DMSO, final concentration: 10, 20, or 90 mM) and GSH (**3h** in PBS, pH 7.4, final concentration: 3, 6, 12, 24, or 48 mM) were mixed in a 3:1 ratio. After 5 min, the Raman spectra of the reaction mixture were recorded. The **1h**:**4hh** molar ratio was calculated from the relative peak heights of the fitted spectra after correcting for intensity using the RIE value shown in Figure S1, where the RIE value of thiol adduct **4hh** was approximated as 0.081 (the value of alkyl nitrile **2**), since **4hh** is unisolable. The curve was obtained (interpolated) by spline function.

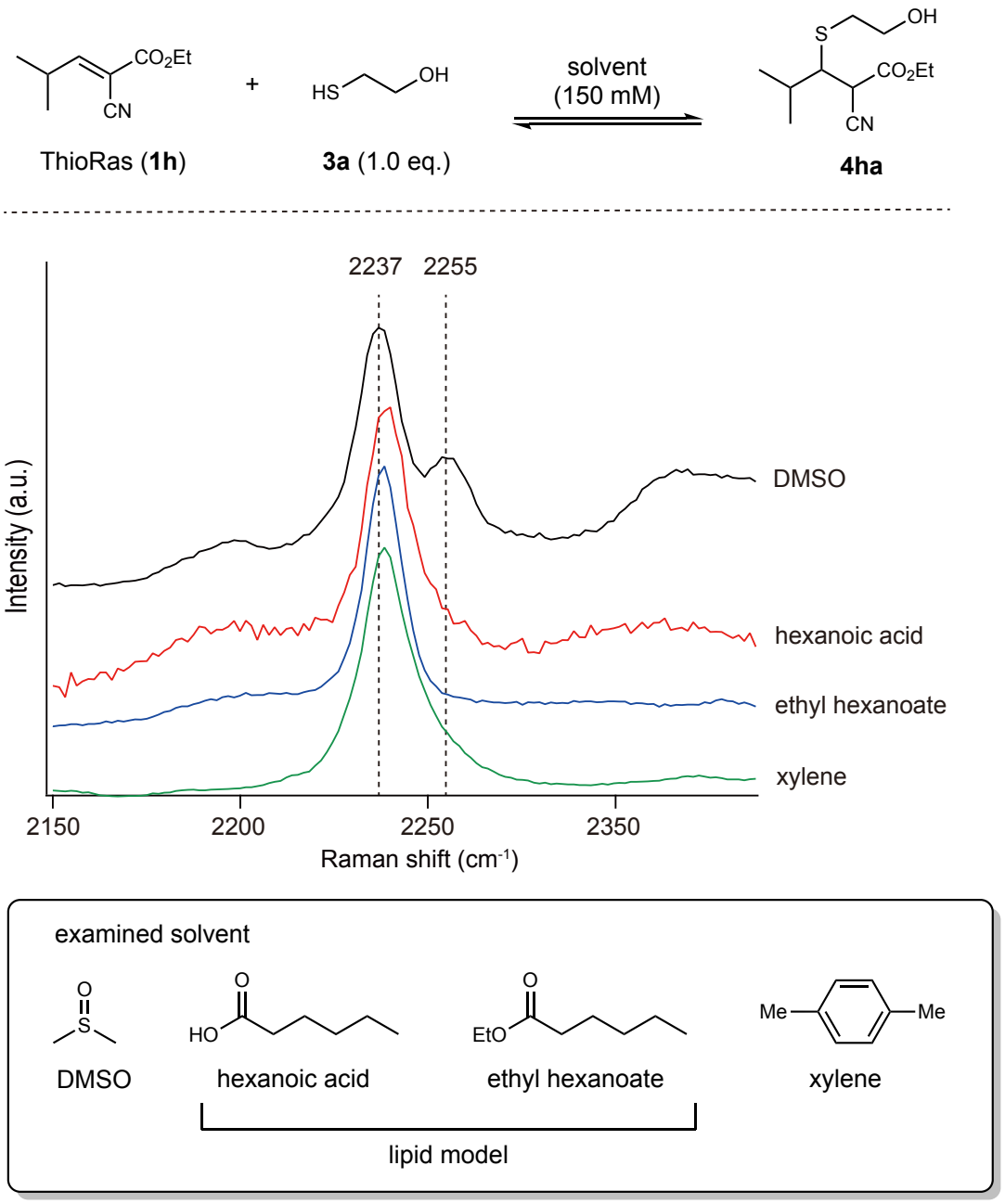


Figure S9. Raman analysis of thia-Michael reactions between 150 mM ThioRas (**1h**) and 150 mM βME (**3a**) in various solvents.

DMSO, dimethyl sulfoxide; βME, β-mercaptoethanol.

	ThioRas (**1h**) +adducts **4hx** (**1h** + **4hx**: 10 mM)			
	1h (mM)	**4hx** (mM)	**1h**:**4hx**	estimated thiol concentration
medium	3.9	6.1	42:58	13 mM using the 10 mM curve
nucleus	5.1	8.4	41:59	14 mM using the 10 mM curve
cytoplasm	5.7	8.3	42:58	13 mM using the 10 mM curve
lipid droplet	61.7	19.1	80:20	8 mM using the 90 mM curve

	ThioRas (**1h**) +adducts **4hx** (**1h** + **4hx**: 20 mM)			
	1h (mM)	**4hx** (mM)	**1h**:**4hx**	estimated thiol concentration
medium	6.1	13.9	28:72	30 mM using the 20 mM curve
nucleus	7.8	14.8	35:65	23 mM using the 20 mM curve
cytoplasm	9.3	16.5	38:62	21 mM using the 20 mM curve
lipid droplet	107	18.6	84:16	4 mM using the 90 mM curve

Figure S10. Live-cell analysis of thiols after exposure to 10 or 20 mM ThioRas in the presence of glutathione (1.2 equivalents). Concentrations of **1h** and **4hx** were calculated from the peak heights of the fitted spectra (Figure S7) after correcting for the intensities of the RIE values shown in Figure S1. Thiol concentrations were estimated from the calibration curve (Fig. S8). Each thiol concentration was estimated using the closest calibration curve (10, 20, or 90 mM) to the concentration of ThioRas (**1h**+**4h**).

2. Experimental procedures

Cell culture

HeLa human cervical cancer cells were cultured in Dulbecco's modified Eagle's medium (043-30085, Wako) supplemented with 10% fetal bovine serum, 5×10^4 U/L penicillin G, and 50 mg/L streptomycin sulfate (15070-063, Gibco) at 37 °C and 5% CO_2.

Raman microscopy

Raman spectra and images were obtained using a multiconfocal Raman microscope (Phalanx-R, Tokyo Instruments) equipped with an inverted microscope (Nikon). The details of the Raman microscopy experiments have been described previously[1]. Briefly, the excitation beam from a 532 nm continuous wave laser was split into 10 × 10 beamlets, and all 100 beamlets were focused on a sample using an objective lens (60×, 1.49 NA oil immersion, plan Apo 60X, Nikon). The Raman signal from each spot was collected and transferred to an optical fiber bundle to rearrange the 10 × 10 matrix into a single line. A single-line Raman signal was introduced into the spectrograph having a holographic transmission grating (2400 line/mm), and 100 Raman spectra were simultaneously detected using a thermoelectrically cooled charge-coupled device (CCD) camera (Andor Technology). The culture medium was replaced with Hank's balanced salt solution (HBSS) (H8264, Sigma) immediately before measurement, and all measurements were carried out at 20 °C. A single Raman image was constructed using 30 × 30 points at intervals of 1.1 μm. The laser-irradiation intensity and exposure time per point were 2–3 mW and 60 s, respectively.

The Raman spectra shown in Figures 5a and S6a were obtained using a slit-scanning Raman microscope[2] (built in our laboratory by modifying a Nikon Ti2 microscope) equipped with a 532 nm excitation laser (Millennia eV 15HA-W, Spectra Physics). The excitation-laser output was shaped into a line using cylindrical lenses and focused onto each sample using a water-immersion objective lens (25×/1.1 NA; Nikon). The Raman-scattering light emanating from each position on the illuminated line passed through a slit mounted on a spectrophotometer (MK300, Bunkoukeiki), was dispersed by grating (groove density: 600 line/mm), and was detected using a cooled CCD camera (Pixis 400 B, Princeton Instruments,

Teledyne) to obtain the Raman spectra. The excitation-laser intensity along the sample plane was 3 mW/mm^2. The exposure time for each line was 5 s. The laser beam was scanned using a galvanometer mirror in the longitudinal and perpendicular directions of each line on the sample surface. A total of 110 lines were used.

The Raman hyperspectral dataset was further processed using the singular-value decomposition (SVD) technique for noise reduction[3]. The wavenumber regions for the SVD calculations were 2120 cm^{-1} to 2321 cm^{-1} (nitrile images) and 697 cm^{-1} to 3200 cm^{-1} (other images). Owing to the differing autofluorescence-background signals present at each point in the Raman spectrum, we used a modified polynomial-fitting technique[4] to determine the autofluorescence-baseline signal, which was subtracted from the original Raman spectrum. Finally, a Raman image was constructed by displaying the intensity of each vibrational band of interest at each spatial position. All nitrile images were obtained by subtracting the intensities of the lower nitrile peaks from those at the top of the nitrile peaks.

Reversible thia-Michael reactions monitored by Raman microscopy (for Figure 3)

Nitrile **1a** (200 mM in DMSO), thiol **3x** (200 mM in DMSO), and PBS (pH 7.4) were mixed in a 3:3:2 ratio. For GSH experiments involving **3h**, **1a** (100 mM in DMSO) and **3h** (300 mM in PBS) were mixed in a 3:1 ratio. After 5 min, Raman spectra of the reaction mixture were recorded. The **1a**:**4ax** molar ratio was calculated based on the relative area of each peak corrected for intensity by the RIE value shown in Figure S1 (**1a**: 0.46, the RIE value of the thiol adduct **4ax** was approximated as 0.081 [the value of alkyl nitrile **2**], since **4ax** is unisolable). The Raman shifts of the peaks were as follows:

1a	shift (1/cm)	**4ax**	shift (1/cm)
1a	2228	**4aa**	2253
1a	2228	**4ab**	2253
1a	2228	**4ac**	2253
1a	2228	**4ad**	2253
1a	2228	**4ae**	2254

1a	shift (1/cm)	**4ax**	shift (1/cm)
1a	2230	**4af**	2255
1a	2228	**4ag**	2254
1a	2228	**4ah**	2254
1a	2227	**4ai**	2251

Reversible thia-Michael reactions monitored by Raman microscopy (for Figure 4)

Nitrile **1x** (100 mM in DMSO) and thiol **3a** (400 mM in PBS, pH 7.4) were mixed in a 3:1 ratio. After 5 min, the Raman spectra of the reaction mixture were recorded. The **1x**:**4xa** molar ratio was calculated from the relative area of each peak corrected for intensity by the RIE value shown in Figure S1, where the RIE value of thiol adduct **4xa** was approximated as 0.081 (the value of alkyl nitrile **2**), since **4xa** is unisolable. The Raman shifts of the peaks were as follows:

1x	shift (1/cm)	**4xa**	shift (1/cm)
1a	2230	**4aa**	2256
1b	2227	**4ba**	2256
1c	2230	**4ca**	2257
1d	2234	**4da**	2257
1e	2233	**4ea**	2257
1f	2234	**4fa**	2257

1x	shift (1/cm)	**4xa**	shift (1/cm)
1g	2229	**4ga**	2255
1h	2238	**4ha**	2256
1i	2237	**4ia**	2256
1j	2237	**4ja**	2257
1k	2237	**4ka**	2256
1l	2239	**4la**	2258

Live-cell analysis of thiols (for Figures 6 and S7)

ThioRas (**1h**, 1 M or 2 M in DMSO), GSH (**3h**, 600 mM in saturated aqueous $NaHCO_3$), and HBSS were mixed in a 1:2:100 ratio to prepare mixture "A." Because GSH is an acidic compound, mixture A had a neutral pH. HeLa cells were cultured on a glass-bottom dish, and the culture medium was washed twice with HBSS and then replaced with mixture "A" immediately before measurements were taken. Raman imaging was performed as described above in the section titled "Raman microscopy". The inhomogeneity of the laser intensity was corrected using a Raman image of the surrounding homogeneous medium. The classifications of medium, nucleus, cytoplasm, and lipid droplet were based on the peak intensity ratio of cytochrome *c* (pyrrole breathing: 750 cm^{-1}), CH_2 and CH_3 stretching (2840–3030 cm^{-1}), and water (O–H stretching: 3100–3700 cm^{-1}).[5] The SVD technique was applied for the spectra in the 2100–2400 cm^{-1}. Because the total cell volume was negligible compared to that of the medium and the cellular ThioRas concentration was only approximately 10 times the maximum in the medium, the total **1h** and **4hx** concentrations in the medium were approximated as the treatment concentration (10 or 20 mM). Using the nitril peaks of the medium as internal standards, the cellular **1h** and **4hx** concentrations were calculated from the peak heights of fitting spectra corrected for the intensities of the RIE values shown in Figure S1 (**1h**: 0.18), where the RIE value of the thiol adduct **4hx** was approximated as 0.081 (the value of alkyl nitrile **2**), since **4hx** is unisolable. SVD and the fitting analyses were performed by Igor

Pro 8 (Wavemetrics).

Turbidity assay

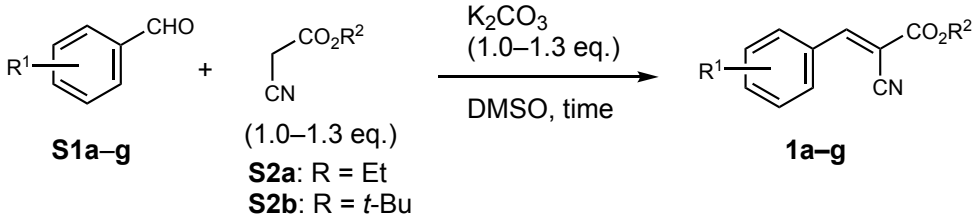
Nephelometric turbidity units (NTUs) were measured using a digital turbidimeter TBD700 (AS ONE) with an infrared radiation diode (wavelength: 850 nm). DMSO solutions containing each compound were diluted 1:100 in PBS (pH 7.4). After mixing, the measurements were repeated four times, and the average NTU values were calculated.

General information for chemical synthesis

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded with tetramethylsilane (δ_H 0.00) or $CHCl_3$ (δ_H 7.26) as an internal standard. Coupling constants (*J*) are reported in hertz (Hz). Abbreviations of multiplicity are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. The data are presented as follows: chemical shift, multiplicity, coupling constants, and integration. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded with $CDCl_3$ (δ_C 77.0) as an internal standard. Infrared (IR) spectra were recorded on an FT-IR spectrophotometer, and absorbance bands are reported in wavenumbers (cm^{-1}).

Column chromatography was carried out on silica gel 60 N (63–210 μm or 40–50 μm). Analytical thin layer chromatography (TLC) was carried out with 0.25-mm silica gel plates. Visualization was accomplished with ultraviolet light and anisaldehyde or phosphomolybdic acid stain, followed by heating. Reagents and solvents were purified by standard means or used as received, unless otherwise noted. Dehydrated dichloromethane (CH_2Cl_2) and tetrahydrofuran (THF, stabilizer-free) were purchased. All reactions were conducted in an argon atmosphere, unless otherwise noted.

Table S1. Synthesis of 3-aryl-2-cyanoacrylates 1a, 1j–o



S1a–g

(1.0–1.3 eq.)

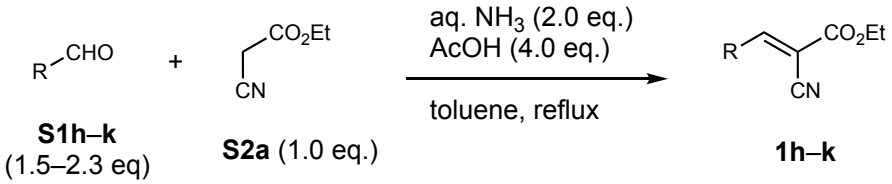
S2a: R = Et

S2b: R = *t*-Bu

1a–g

entry	aldehyde S1a–g	R^1	R^2	product 1aj–g	time
1	**S1a**, 2.00 g 18.8 mmol	H	Et	**1a**, 1.31 g 6.51 mmol, 35%	12 h
2	**S1b**, 2.00 g 14.6 mmol	4-OMe	Et	**1b**, 437 mg 1.90 mmol, 13%	9 h
3	**S1c**, 1.50 g 12.1 mmol	2-F	Et	**1c**, 748 mg 3.41 mmol, 28%	1 h
4	**S1d**, 1.50 g 6.20 mmol	3,5-CF_3	Et	**1d**, 1.06 g 3.14 mmol, 51%	1 h
5	**S1e**, 1.50 g 14.0 mmol	3-*N*-pyrid	Et	**1e**, 953 mg 4.71 mmol, 34%	8 h
6	**S1f**, 1.50 g 14.0 mmol	4-*N*-pyrid	Et	**1f**, 901 mg 4.46 mmol, 32%	1 h
7	**S1g**, 2.04 g 18.8 mmol	H	*t*-Bu	**1g**, 2.85 g 12.4 mmol, 66%	12 h

Table S2. Synthesis of 3-alkyl-2-cyanoacrylates 1p–s



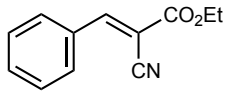
S1h–k (1.5–2.3 eq) **S2a** (1.0 eq.) **1h–k**

entry	aldehyde S1h–k	R	product 1h–k	time
1	**S1h**, 2.06 g 41.6 mmol	*i*-Pr	**1h**, 791 mg 4.73 mmol, 26%	4 h
2	**S1i**, 1.05 g 9.36 mmol	cyclohexyl	**1i**, 1.44 6.95 mmol, 74%	2 h
3	**S1j**, 1.20 g 13.9 mmol	CH_2*i*-Pr	**1j**, 21.5 mg 119 μmol, 1%	18 h
4	**S1k**, 1.88 g 18.8 mmol	CH_2*t*-Bu	**1k**, 1.84 g 8.97 mmol, 95%	8 h

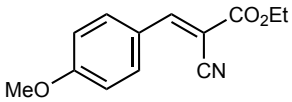
Method for the synthesis of 3-aryl-2-cyanoacrylates 1a–g (Table S1)

K_2CO_3 (1.0–1.3 eq) was added to a solution of arylaldehyde (**S1a–g**, 1.0 eq) and ethyl cyanoacetate* (**S2a**, 1.3 eq) in DMSO (0.7–1.4 M) at room temperature. After 1–12 h of stirring, the reaction mixture was diluted with H_2O and extracted with EtOAc. The organic layer was washed with H_2O and brine and dried over anhydrous Na_2SO_4. Filtration and evaporation in vacuo furnished the crude product, which was purified by silica gel column chromatography or recrystallization to afford 3-aryl-2-cyanoacrylate **1a**–**g**.

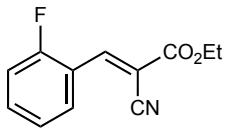
tert*-butyl cyanoacetate (S2b**, 1.2 eq) was used to synthesize **1g**.



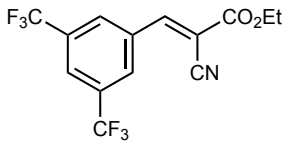
Ethyl (*E*)-2-cyano-3-phenylacrylate (1a): Colorless solid; R_f = 0.40 (4:1 *n*-hexane/AcOEt); IR (ATR) ν_{max}/cm^{-1} 2224, 1726, 1607, 1573, 1261; δ_H (597 MHz, $CDCl_3$) 8.26 (1 H, s), 8.02 – 7.97 (2 H, m), 7.59 – 7.52 (1 H, m), 7.54 – 7.48 (2 H, m), 4.39 (2 H, q, J = 7.1 Hz), 1.40 (3 H, t, J = 7.1 Hz); δ_C (150 MHz, $CDCl_3$) 162.6 (C), 155.2 (CH), 133.4 (CH), 131.6 (C), 131.2 (CH), 129.4 (CH), 115.6 (C), 103.2 (C), 62.9 (CH_2), 14.3 (CH_3); HRMS (EI) *m*/*z* $[M]^+$ calcd for $C_{12}H_{11}NO_2$ 201.0790; found 201.0780.



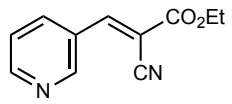
Ethyl (*E*)-2-cyano-3-(4-methoxyphenyl)acrylate (1b): Pale yellow solid; R_f = 0.66 (2:1 *n*-hexane/AcOEt); IR (ATR) ν_{max}/cm^{-1} 2216, 1714, 1585, 1513, 1185; δ_H (597 MHz, $CDCl_3$) 8.18 (1 H, s), 8.04–7.97 (2 H, m), 7.03–6.97 (2 H, m), 4.37 (2 H, q, J = 7.1 Hz), 3.90 (3 H, s), 1.39 (3 H, t, J = 7.1 Hz); δ_C (150 MHz, $CDCl_3$) 163.9 (C), 163.3 (C), 154.6 (CH), 133.8 (CH), 124.5 (C), 116.4 (C), 114.9 (CH), 99.5 (C), 62.6 (CH_2), 55.8 (CH_3), 14.4 (CH_3); HRMS (EI) *m*/*z* $[M]^+$ calcd for $C_{13}H_{13}NO_3$ 231.0895; found 231.0890.



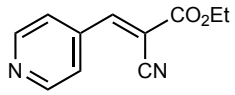
Ethyl (*E*)- 2-cyano-3-(2-fluorophenyl)acrylate (1c): Colorless solid; R_f = 0.52 (3:1 *n*-hexane/AcOEt); IR (ATR) ν_{max}/cm^{-1} 2226, 1718, 1612, 1481, 1260; δ_H (597 MHz, $CDCl_3$) 8.56 (1 H, s), 8.38 (1 H, ddd, J = 7.9, 1.65 Hz, $^4J_{HF}$ = 7.3 Hz), 7.56 (1 H, dddd, J = 8.6, 7.3, 1.7 Hz, $^4J_{HF}$ = 5.4 Hz), 7.30 (1 H, ddd, J = 7.3, 1.2 Hz, $^4J_{HF}$ = 7.7 Hz), 7.19 (1 H, ddd, J = 8.6, 1.1 Hz, $^4J_{HF}$ = 10.1 Hz), 4.40 (2 H, q, J = 7.1 Hz), 1.41 (3 H, t, J = 7.1 Hz); δ_C (150 MHz, $CDCl_3$) 162.1 (C), 161.9 (C, d, $^1J_{CF}$ = 257.4 Hz), 146.4 (CH, d, $^3J_{CF}$ = 7.8 Hz), 135.3 (CH, d, $^3J_{CF}$ = 9.2 Hz), 129.3 (CH), 125.1 (CH, d, $^3J_{CF}$ = 3.9 Hz), 120.1 (C, d, $^2J_{CF}$ = 10.8 Hz), 116.3 (CH, d, $^2J_{CF}$ = 21.6 Hz), 115.3 (C), 105.1 (C), 63.0 (CH_2), 14.3 (CH_3); HRMS (EI) *m*/*z* $[M]^+$ calcd for $C_{12}H_{10}FNO_2$ 219.0696; found 219.0688.



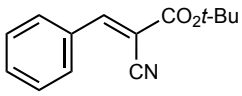
Ethyl (*E*)- 3-(3,5-bis(trifluoromethyl)phenyl)-2-cyanoacrylate (1d): Colorless solid; R_f = 0.50 (4:1 *n*-hexane/AcOEt); IR (ATR) ν_{max}/cm^{-1} 2232, 1723, 1585, 1512, 1282, 1134; δ_H (597 MHz, $CDCl_3$) 8.40 (2 H, d, J = 1.6 Hz), 8.30 (1 H, s), 8.06 – 8.03 (1 H, m), 4.43 (2 H, q, J = 7.1 Hz), 1.43 (3 H, t, J = 7.1 Hz); δ_C (150 MHz, $CDCl_3$) 161.3, 151.0 (CH, d, $^3J_{CF}$ = 4.1 Hz), 133.4, 133.2 (C, q, $^2J_{CF}$ = 257 Hz), 130.3, 126.1, 122.8 (CF_3, q, $^1J_{CF}$ = 272.9 Hz), 114.3, 107.8, 63.6 (CH_2), 14.2 (CH_3); HRMS (EI) *m*/*z* $[M]^+$ calcd for $C_{14}H_9F_6NO_2$ 337.0538; found 337.0547.



Ethyl (*E*)-2-cyano-3-(pyridin-3-yl)acrylate (1e): Colorless solid; R_f = 0.52 (AcOEt); IR (ATR) ν_{max}/cm^{-1} 2221, 1717, 1610, 1584, 1220; δ_H (396 MHz, $CDCl_3$) 8.93 (1 H, d, J = 2.2 Hz), 8.77 (1 H, dd, J = 4.8, 1.9 Hz), 8.58 (1 H, ddd, J = 8.2, 2.2, 1.9 Hz), 8.27 (1 H, s), 7.48 (1 H, dd, J = 8.2, 4.8 Hz), 4.42 (2 H, q, J = 7.2 Hz), 1.42 (3 H, t, J = 7.2 Hz); δ_C (150 MHz, $CDCl_3$) 161.9 (C), 153.6 (CH), 153.1 (CH), 151.4 (CH), 136.1, 127.7, 124.2, 115.0 (C), 105.8 (C), 63.2 (CH_2), 14.3 (CH_3); HRMS (EI) *m*/*z* $[M]^+$ calcd for $C_{11}H_{10}N_2O_2$ 202.0742; found 202.0770.



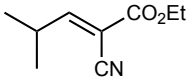
Ethyl (*E*)-2-cyano-3-(pyridin-4-yl)acrylate (1f): Pale pink solid; R_f = 0.57 (AcOEt); IR (ATR) ν_{max}/cm^{-1} 2223, 1721, 1618, 1500, 1223; δ_H (396 MHz, $CDCl_3$) 8.83 (2 H, d, J = 6.2 Hz), 8.20 (1 H, s), 7.76 (2 H, d, J = 6.2 Hz), 4.43 (2 H, q, J = 7.1 Hz), 1.42 (3 H, t, J = 7.1 Hz); δ_C (99 MHz, $CDCl_3$) 161.5 (C), 152.3 (CH), 151.3 (CH), 138.2 (C), 123.5 (CH), 114.4 (C), 108.5 (C), 63.5 (CH_2), 14.2 (CH_3); HRMS(EI) m/z $[M]^+$ calcd for $C_{11}H_{10}N_2O_2$ 202.0742; found. 202.0734.



***tert*-Butyl (*E*)-2-cyano-3-phenylacrylate (1g)**: Colorless solid; R_f = 0.60 (4:1 *n*-hexane/AcOEt); IR (ATR) ν_{max}/cm^{-1} 2221, 1717, 1604, 1448, 1207; δ_H (396 MHz, $CDCl_3$) 8.17 (1 H, s), 7.99 – 7.95 (2 H, m), 7.57 – 7.45 (3 H, m), 1.59 (9 H, s); δ_C (99 MHz, $CDCl_3$) 161.5 (C), 154.3 (CH), 133.2, 131.8, 131.1, 129.4, 115.9 (C), 104.8 (C), 83.9 (C), 28.0 (CH_3); HRMS (EI) m/z $[M]^+$ calcd for $C_{14}H_{15}NO_2$ 229.1103; found 229.1091.

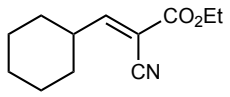
Method for the Synthesis of 3-Alkyl-2-cyanoacrylates 1h–k (Table S2)

A mixture of aldehyde **S1h–k**, ethyl 2-cyanoacrylate, 30% aqueous NH_3, and AcOH in toluene (0.4 M) was refluxed. The water produced by the reaction was removed by azeotropic distillation using a Dean−Stark apparatus. The mixture was successively washed with saturated aqueous NH_4Cl (2 × 50 mL), saturated aqueous $NaHCO_3$ (3 × 50 mL), and brine (3 × 50 mL), and dried over anhydrous Na_2SO_4. Filtration and evaporation in vacuo furnished the crude product, which was purified to give **1h**–**k**.

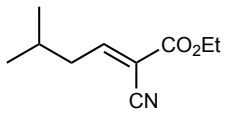


ThioRas: Ethyl (*E*)-2-cyano-4-methylpent-2-enoate (1h): Colorless oil; R_f = 0.50 (4:1 *n*-hexane/AcOEt); IR (neat) ν_{max}/cm^{-1} 2970, 2231, 1733, 1625, 1467, 1258; δ_H (600 MHz, $CDCl_3$) 7.46 (1 H, d, J = 10.6 Hz), 4.31 (2 H, q, J = 7.1 Hz), 3.00 (1 H, dsept, J = 10.6, 6.6 Hz), 1.36 (3 H, t, J = 7.1 Hz), 1.16 (6 H, d, J = 6.6 Hz); δ_C (151 MHz, $CDCl_3$) 169.3 (CH), 161.6 (C), 113.7 (C), 107.7 (C), 62.6 (CH_2), 31.7

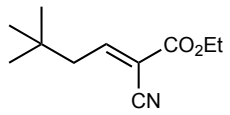
(CH), 21.4 (CH_3), 14.2 (CH_3); HRMS (ESI) *m*/*z* $[M + Na]^+$ calcd for $C_9H_{13}NO_2Na$ 190.0838; found 190.0838.



Ethyl (*E*)-2-cyano-3-cyclohexylacrylate (1i): Colorless oil; R_f = 0.28 (10:1 *n*-hexane/AcOEt); IR (neat) ν_{max}/cm^{-1} 2931, 2231, 1732, 1623, 1449, 1261; δ_H (600 MHz, $CDCl_3$) 7.48 (1 H, d, *J* = 10.7 Hz), 4.31 (2 H, q, *J* = 7.1 Hz), 2.71 (1 H, ddddd, *J* = 10.7, 10.5, 10.5, 3.6, 3.6 Hz), 1.83 – 1.69 (3 H, m), 1.42 – 1.36 (1 H, m), 1.35 (5 H, t, *J* = 7.1 Hz), 1.31 – 1.18 (4 H, m); δ_C (151 MHz, $CDCl_3$) 168.0 (CH or C), 161.8 (CH or C), 113.9 (C), 107.7 (C), 62.5 (CH_2), 41.3 (CH), 31.3 (CH_2), 25.5 (CH_2), 25.0 (CH_2), 14.2 (CH_3); HRMS (EI) *m*/*z* $[M]^+$ calcd for $C_{12}H_{17}NO_2$ 207.1259; found 207.1259.

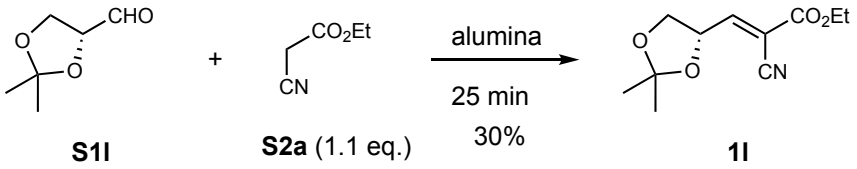


Ethyl (*E*)-2-cyano-5-methylhex-2-enoate (1j): Colorless oil; R_f = 0.50 (4:1 *n*-hexane/AcOEt); IR (neat) ν_{max}/cm^{-1} 2961, 2231, 1732, 1625, 1282, 1258; δ_H (594 MHz, $CDCl_3$) 7.67 (1 H, t, *J* = 8.0 Hz), 4.32 (2 H, q, *J* = 7.1 Hz), 2.47 (2 H, dd, *J* = 8.0, 6.8 Hz), 1.92 (1 H, dsep, *J* = 6.8, 6.7 Hz), 1.36 (3 H, t, *J* = 7.1 Hz), 1.00 (6 H, d, *J* = 6.7 Hz); δ_C (149 MHz, $CDCl_3$) 162.9 (CH), 161.4 (C), 113.9 (C), 110.6 (C), 62.6 (CH_2), 40.9 (CH_2), 28.3 (CH), 22.5 (CH_3), 14.2 (CH_3); HRMS (EI) *m*/*z* $[M]^+$ calcd for $C_{10}H_{15}NO_2$ 181.1103; found 181.1105.



Ethyl (*E*)-2-cyano-5,5-dimethylhex-2-enoate (1k): Colorless oil; R_f = 0.60 (4:1 *n*-hexane/AcOEt); IR (neat) ν_{max}/cm^{-1} 2961, 2231, 1733, 1624, 1262, 1234; δ_H (594 MHz, $CDCl_3$) 7.73 (1 H, t, *J* = 8.2 Hz), 4.33 (2 H, q, *J* = 7.1 Hz), 2.47 (2 H, d, *J* = 8.2 Hz), 1.37 (3 H, t, *J* = 7.1 Hz), 1.02 (9 H, s); δ_C (149 MHz, $CDCl_3$) 161.8 (CH), 161.3 (C), 113.9 (C), 111.1 (C), 62.5 (CH_2), 45.9 (CH_2), 32.8 (C), 29.5 (CH_3), 14.2

(CH_3); HRMS (FAB) *m*/*z* $[M+H]^+$ calcd for $C_{11}H_{18}NO_2$ 196.1332; found 196.1346.

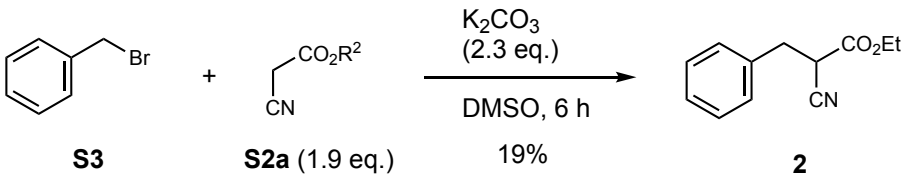


S1l **S2a** (1.1 eq.) **1l**

Ethyl (*S*,*E*)-2-cyano-3-(2,2-dimethyl-1,3-dioxolan-4-yl)acrylate (1l): Alumina (5.95 g) was added to a solution of (*R*)-2,2-dimethyl-1,3-dioxolane-4-carbaldehyde (**S1l**, 2.52 g, 19.4 mmol) and ethyl 2-cyanoacetate (**S2a**, 2.17 mL, 20.4 mmol) at 0 °C. After 25 min of stirring at room temperature, the mixture was diluted with CH_2Cl_2 (100 mL) and passed through a Celite pad. Evaporation of the filtrate in vacuo furnished the crude product, which was purified by silica gel column chromatography (100:1 → 10:1 *n*-hexane/acetone) to give acrylate **1l** (172.9 mg) and diethyl 2,4-dicyano-3-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)pentanedioate (5.11 g).

SiO_2 (40–50 μm, 1.6 g) was added to a solution of diethyl 2,4-dicyano-3-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)pentanedioate (5.11 g) in *n*-hexane/AcOEt (1:1, 150 mL). After 23 h of stirring, the mixture was passed through a Celite pad. Evaporation of the filtrate in vacuo furnished the crude product, which was purified by flash column chromatography (10:1 *n*-hexane/acetone) to give acrylate **1l** (1.17 g) as a colorless oil. The combined yield of acrylate **1l** was 30% (1.33 g, *E*/*Z* = 16:1).

Colorless oil; R_f = 0.38 (4:1 *n*-hexane/AcOEt); IR (neat) ν_{max}/cm^{-1} 2989, 2234, 1735, 1635, 1456, 1373; δ_H (600 MHz, $CDCl_3$) 7.54 (1 H, d, *J* = 7.9 Hz), 5.04 (1 H, ddd, *J* = 7.9, 6.8, 6.2 Hz), 4.36–4.25 (3 H, m), 3.78 (1 H, dd, *J* = 8.6, 6.2 Hz), 1.47 (3 H, s), 1.41 (3 H, s), 1.35 (3 H, t, *J* = 7.1 Hz); δ_C (151 MHz, $CDCl_3$) 160.6 (C), 159.4 (CH), 112.9 (C), 111.6 (C), 110.5 (C), 73.9 (CH), 68.4 (CH_2), 63.1 (CH_2), 26.5 (CH_3), 25.5 (CH_3), 14.2.; HRMS (EI) *m*/*z* $[M]^+$ calcd for $C_{11}H_{15}NO_4$ 225.1001; found 225.1001.



S3 **S2a** (1.9 eq.) 19% **2**

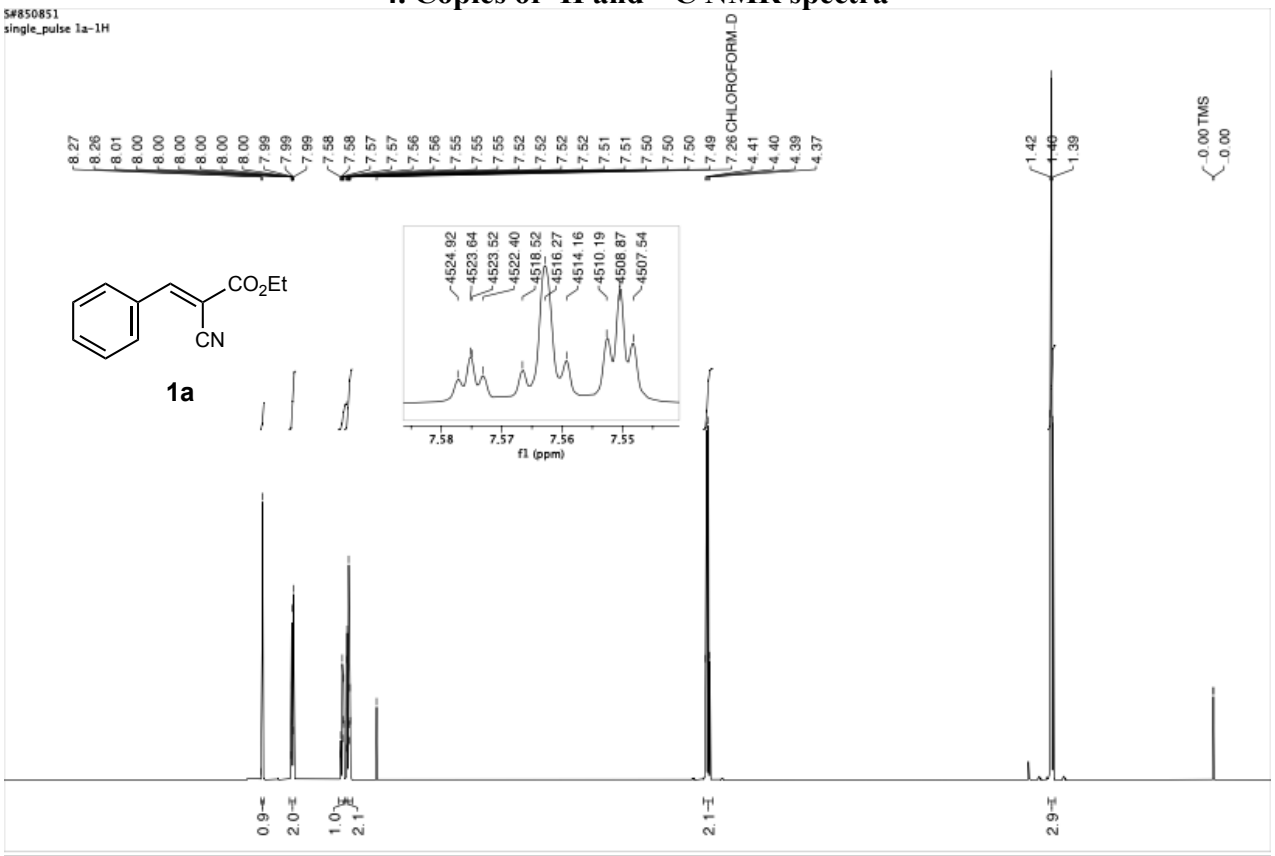
Ethyl 2-cyano-3-phenylpropanoate (2): K_2CO_3 (1.77 g, 19.5 mmol) was added to a solution of

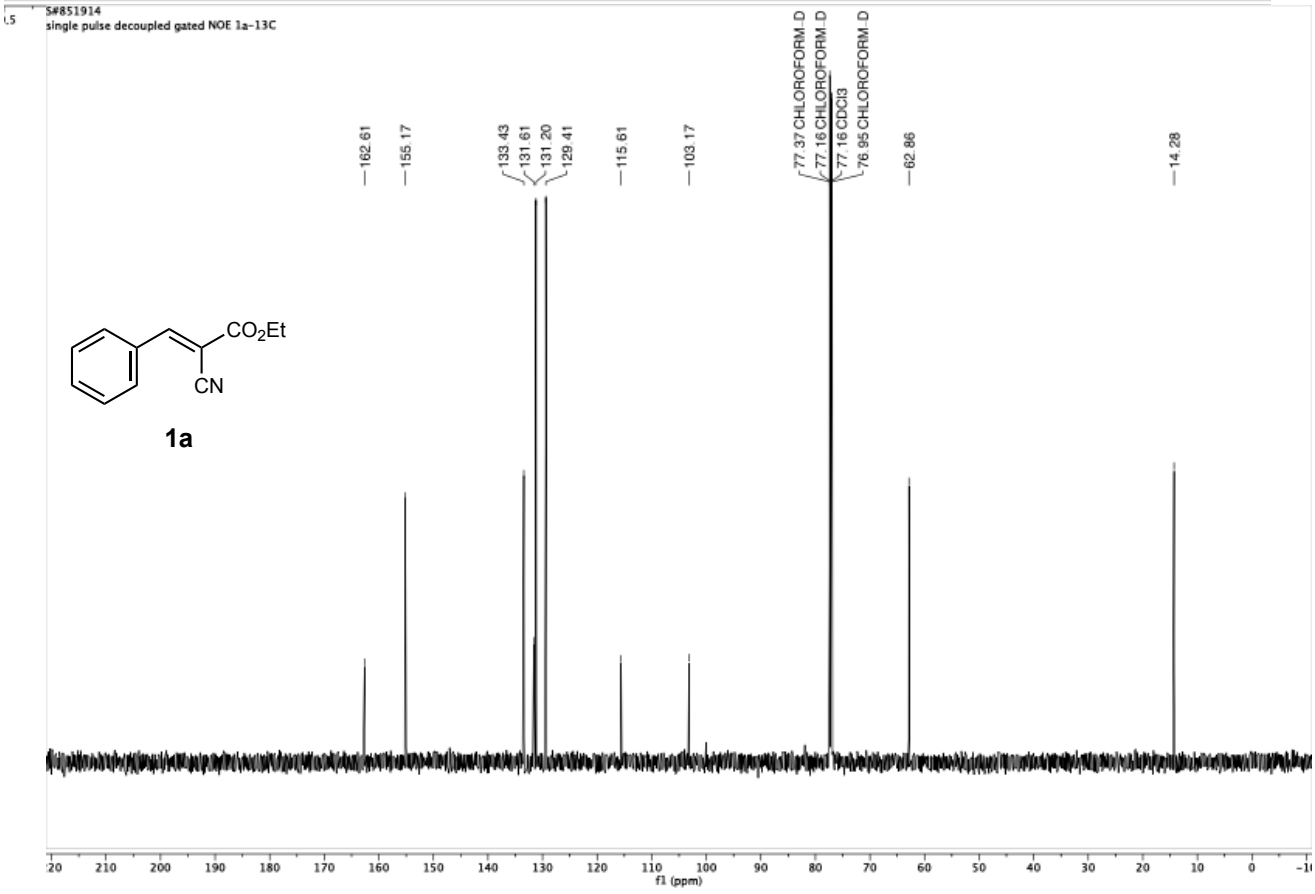
benzylbromide (**S3**, 1.0 mL, 8.42 mmol) and ethyl 2-cyanoacrylate (2.32 g, 16.4 mmol) in DMSO (20 mL) at 20 °C. After 6 h of stirring, H_2O (50 mL) was added to the reaction mixture, and the aqueous layer was extracted with AcOEt (50 mL). The organic extract was successively washed with H_2O (50 mL) and brine (50 mL), and dried over anhydrous Na_2SO_4. Filtration and evaporation in vacuo furnished the crude product (3.72 g), which was purified by flash column chromatography (silica gel 85 g, 30:1 → 20:1 *n*-hexane/AcOEt) to give alkylated product **2** (362 mg, 19%) as a colorless oil. The ^{1}H NMR spectrum of **2** was identical with that reported by Deng and co-workers[6].

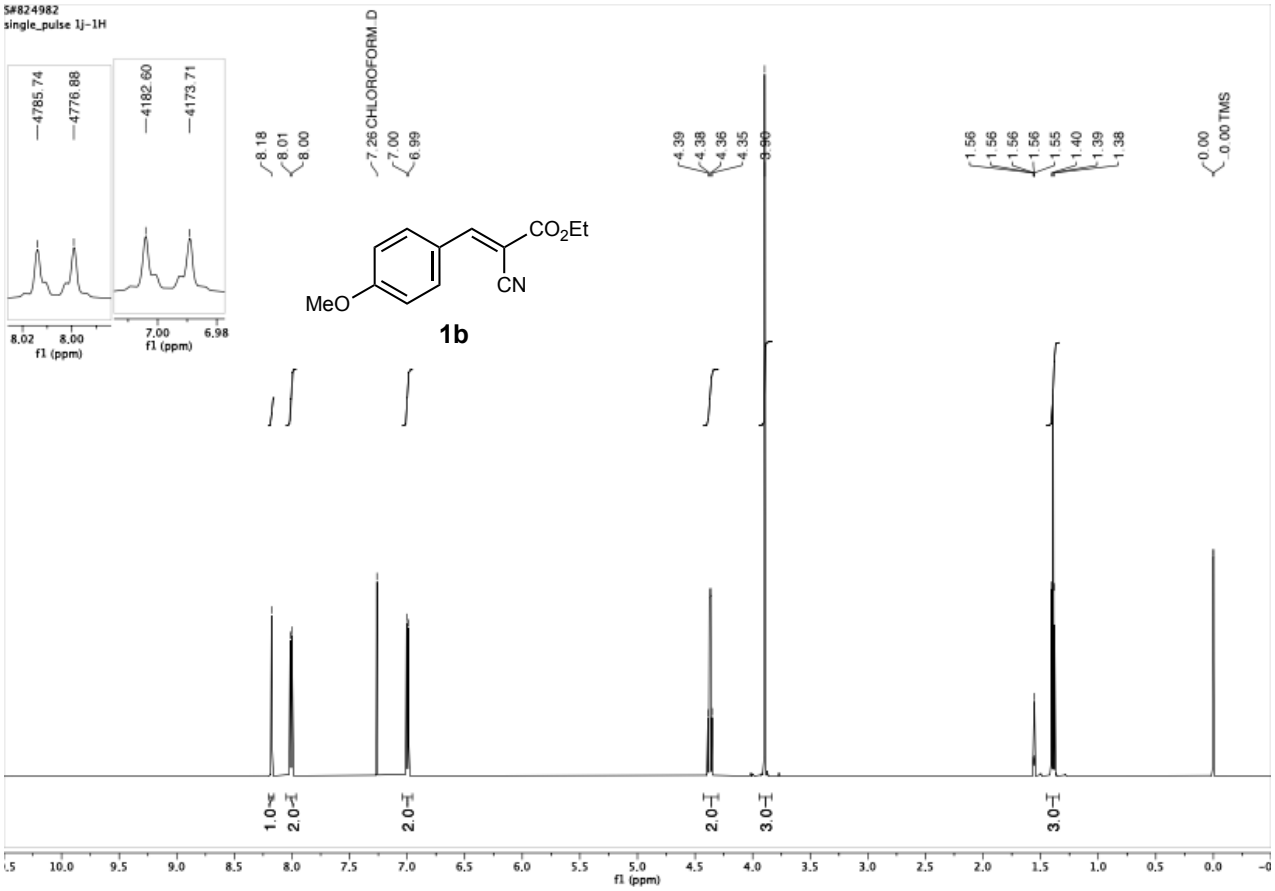
3. Additional references

1. Takeuchi, M., Kajimoto, S. & Nakabayashi, T. Experimental Evaluation of the Density of Water in a Cell by Raman Microscopy. *J. Phys. Chem. Lett.* **8**, 5241–5245 (2017).
2. Palonpon, A. F. *et al.* Raman and SERS microscopy for molecular imaging of live cells. *Nat. Protoc.* **8**, 677–692 (2013).
3. van Manen, H.-J., Kraan, Y. M., Roos, D. & Otto, C. Intracellular Chemical Imaging of Heme-Containing Enzymes Involved in Innate Immunity Using Resonance Raman Microscopy. *J. Phys. Chem. B* **108**, 18762–18771 (2004).
4. Lieber, C. A. & Mahadevan-Jansen, A. Automated method for subtraction of fluorescence from biological Raman spectra. *Appl. Spectrosc.* **57**, 1363–1367 (2003).
5. Sugimura, T., Kajimoto, S. & Nakabayashi, T. Label-Free Imaging of Intracellular Temperature by Using the O-H Stretching Raman Band of Water. *Angew. Chem. Int. Ed Engl.* **59**, 7755–7760 (2020).
6. Xue, D. *et al.* Transfer hydrogenation of activated C=C bonds catalyzed by ruthenium amido complexes: reaction scope, limitation, and enantioselectivity. *J. Org. Chem.* **70**, 3584–3591 (2005).

4. Copies of ^{1}H and ^{13}C NMR spectra



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f1 (ppm)
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8.01
8.00
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7.00
6.99
4.39
4.38
4.36
4.35
3.90
1.56
1.56
1.56
1.56
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1.38
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3.0
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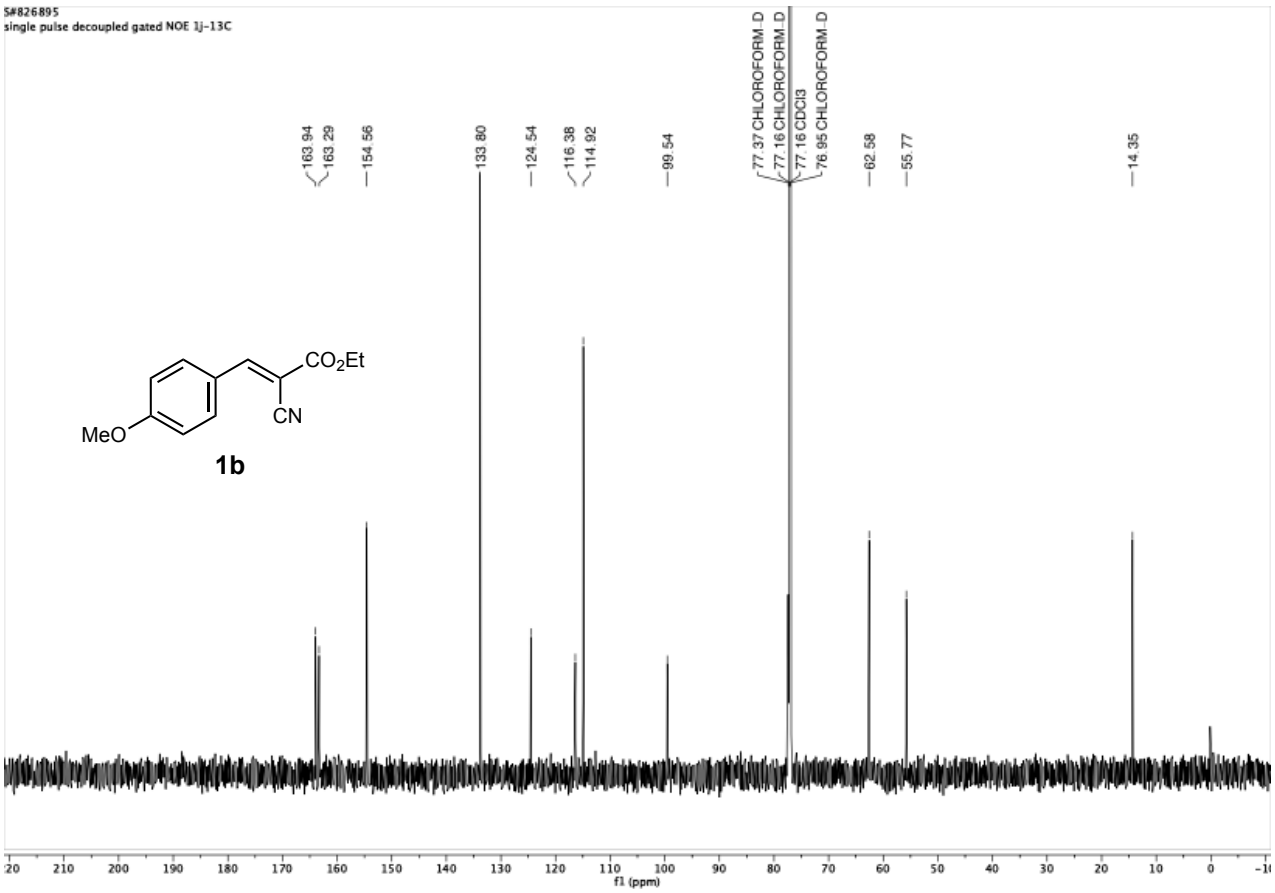
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1b
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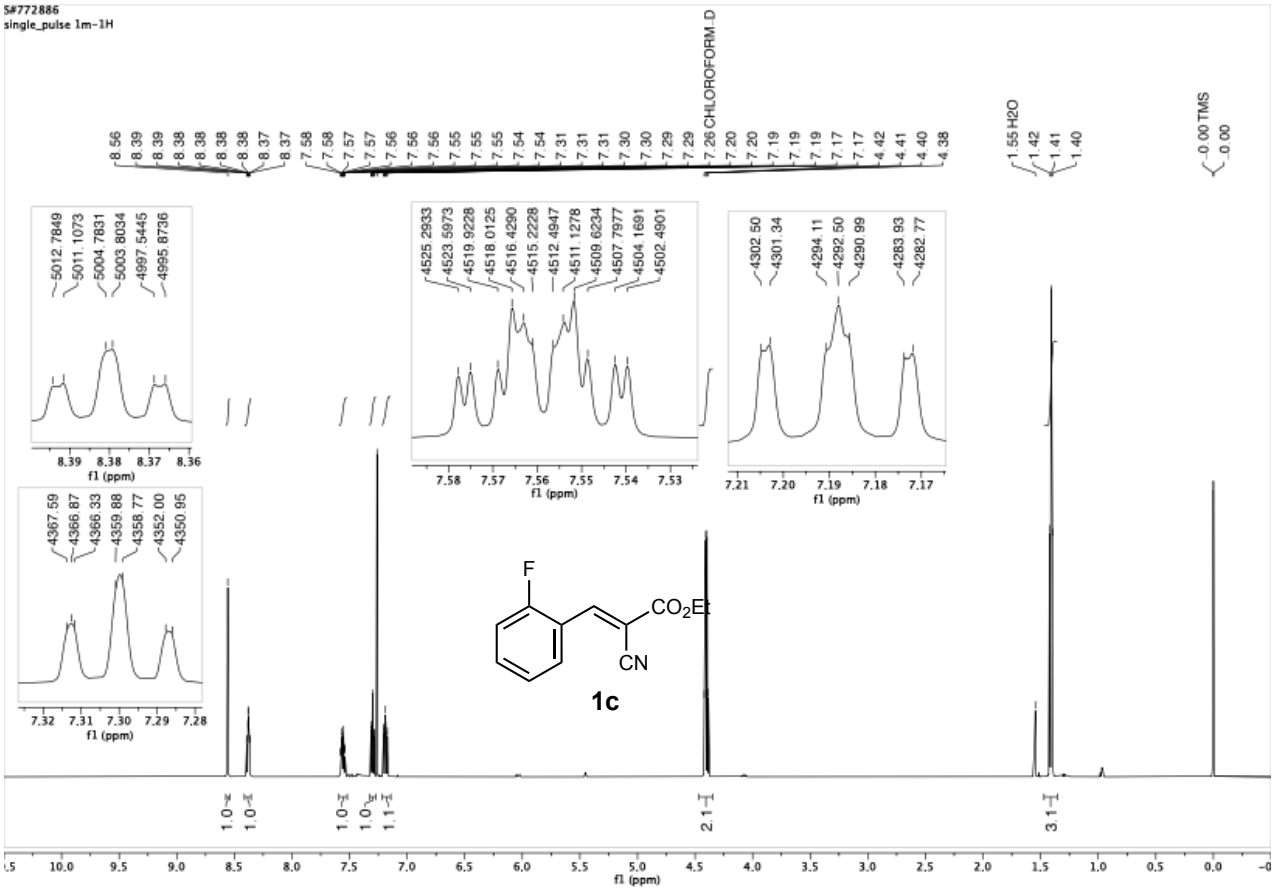
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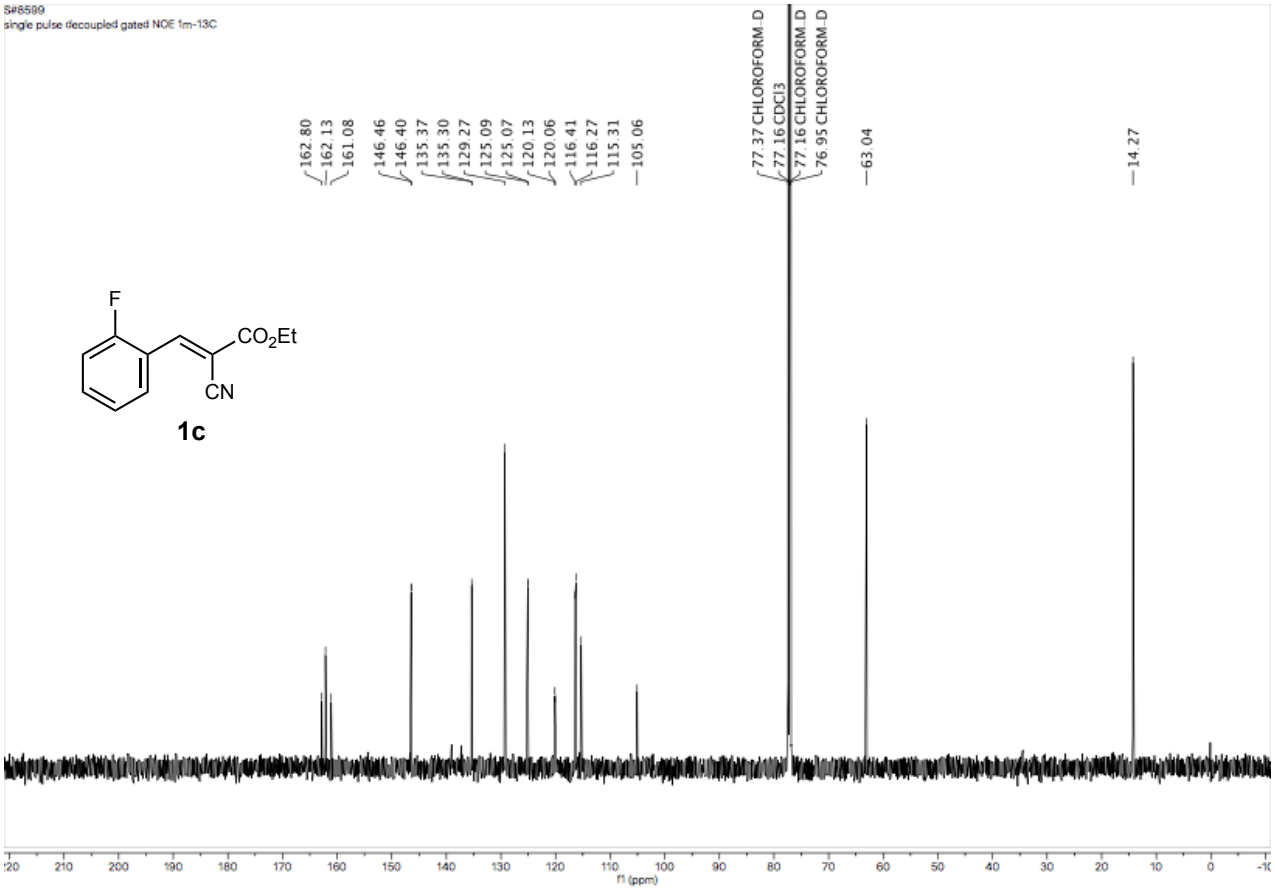
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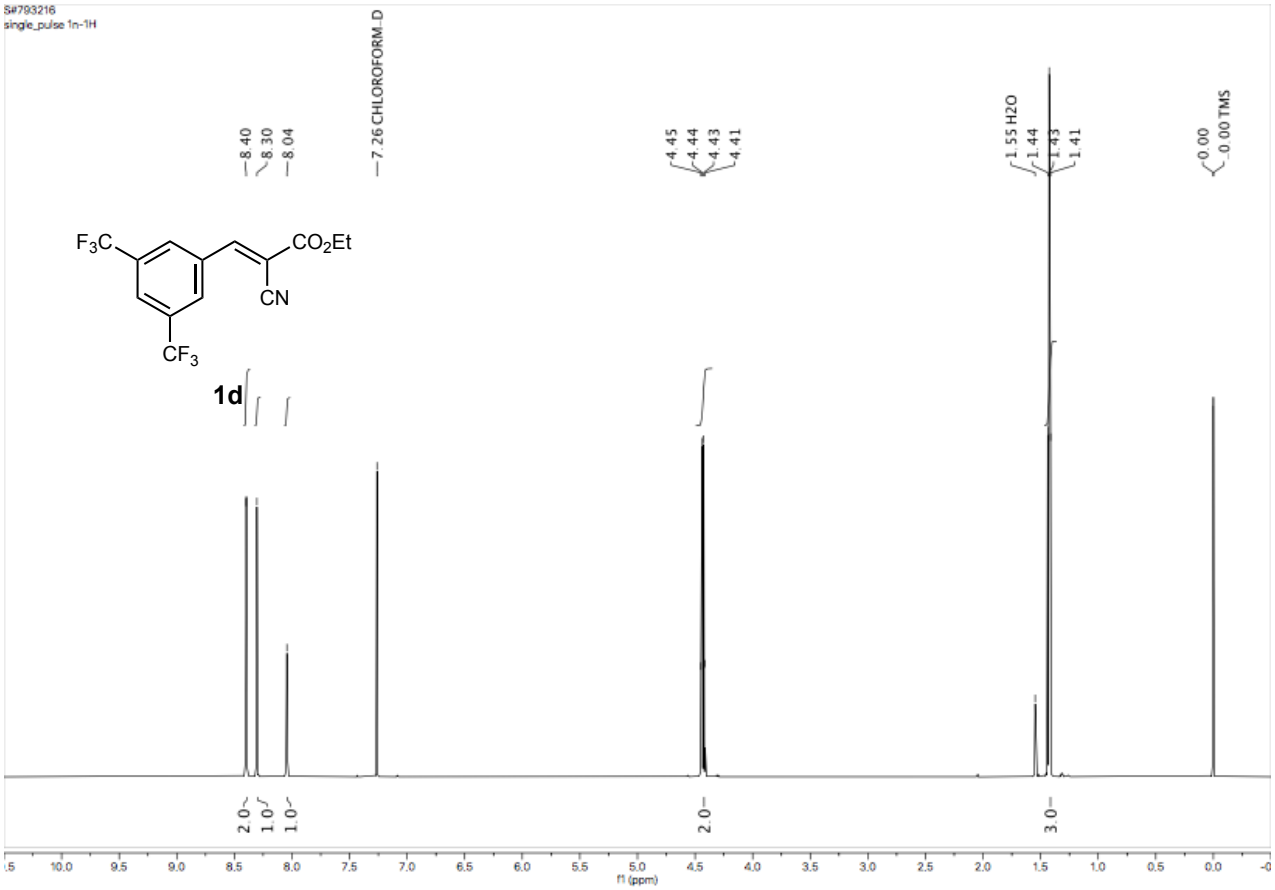
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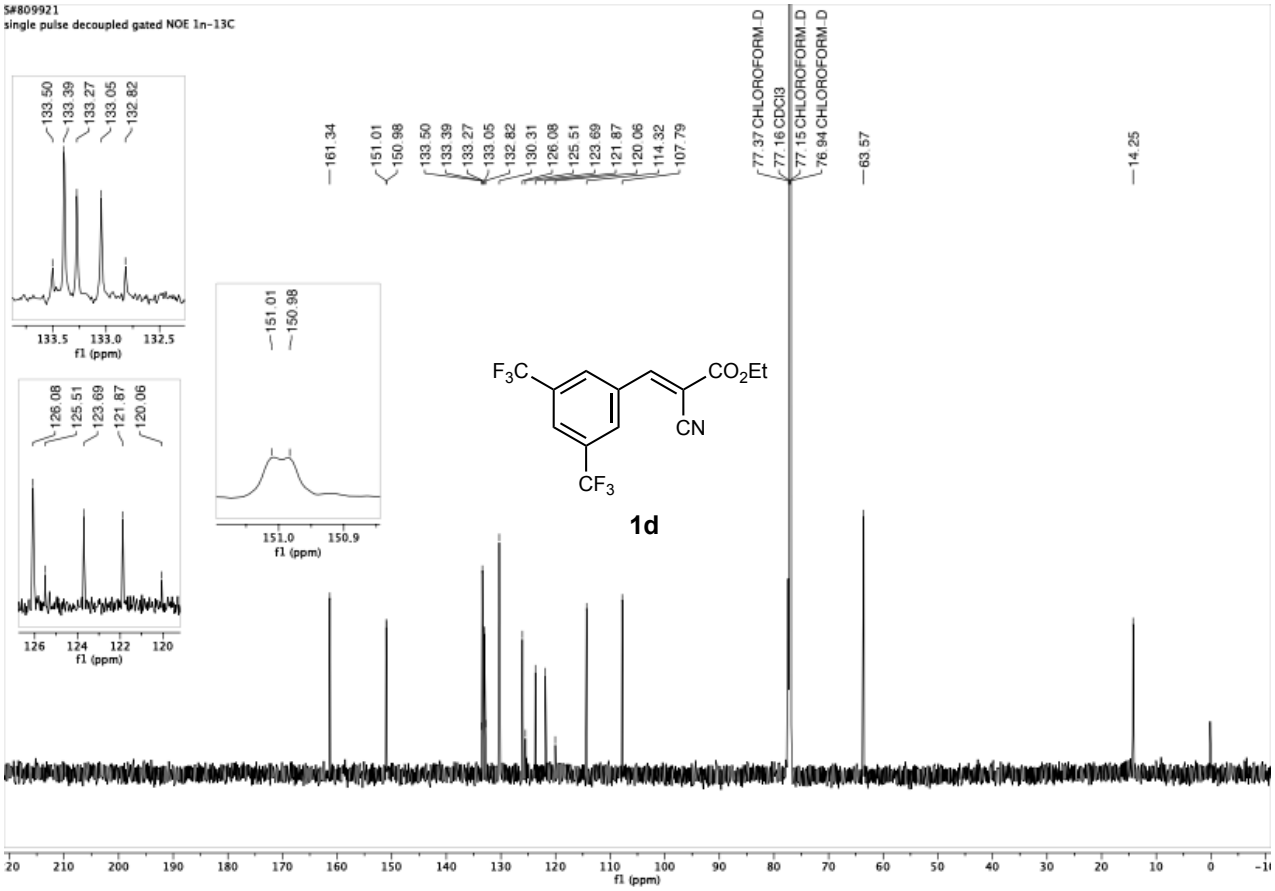
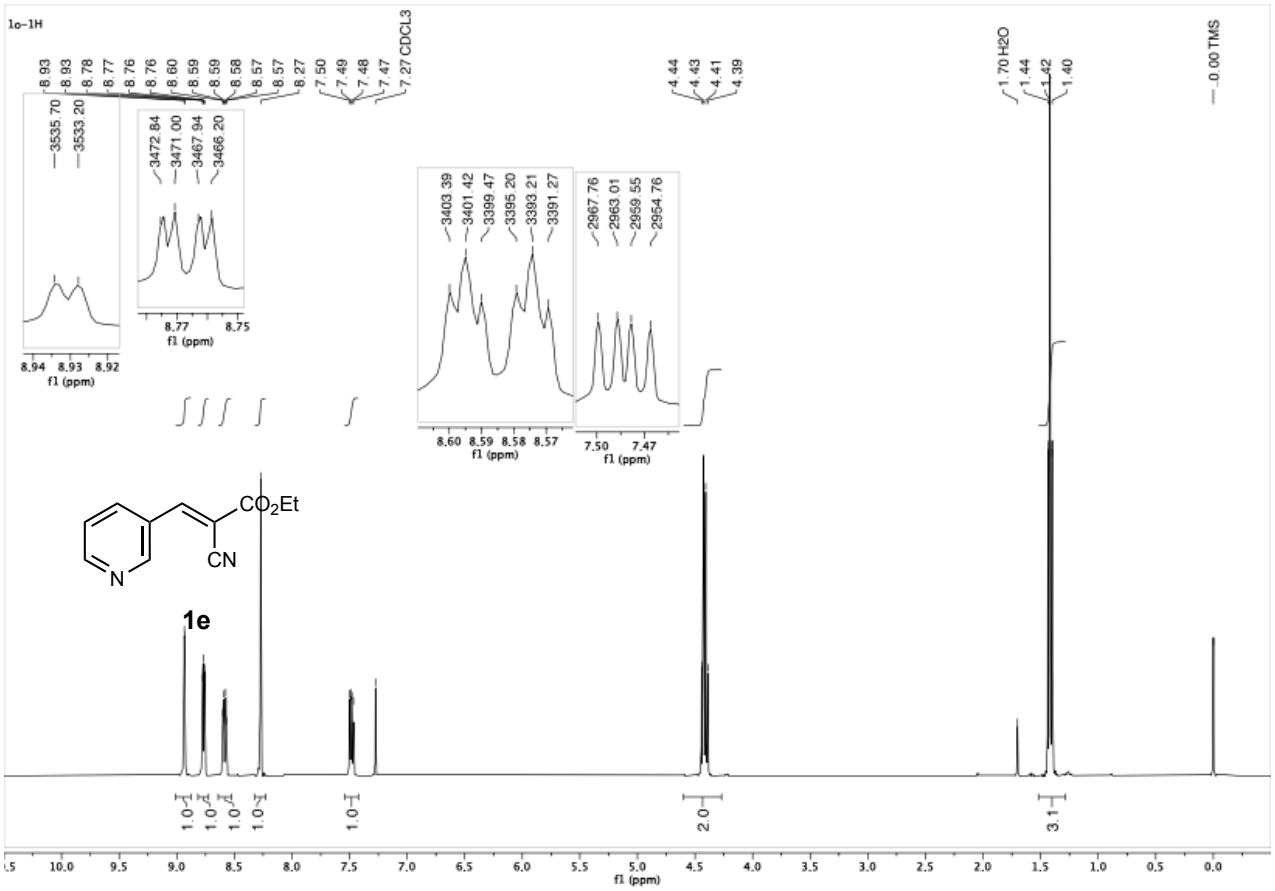
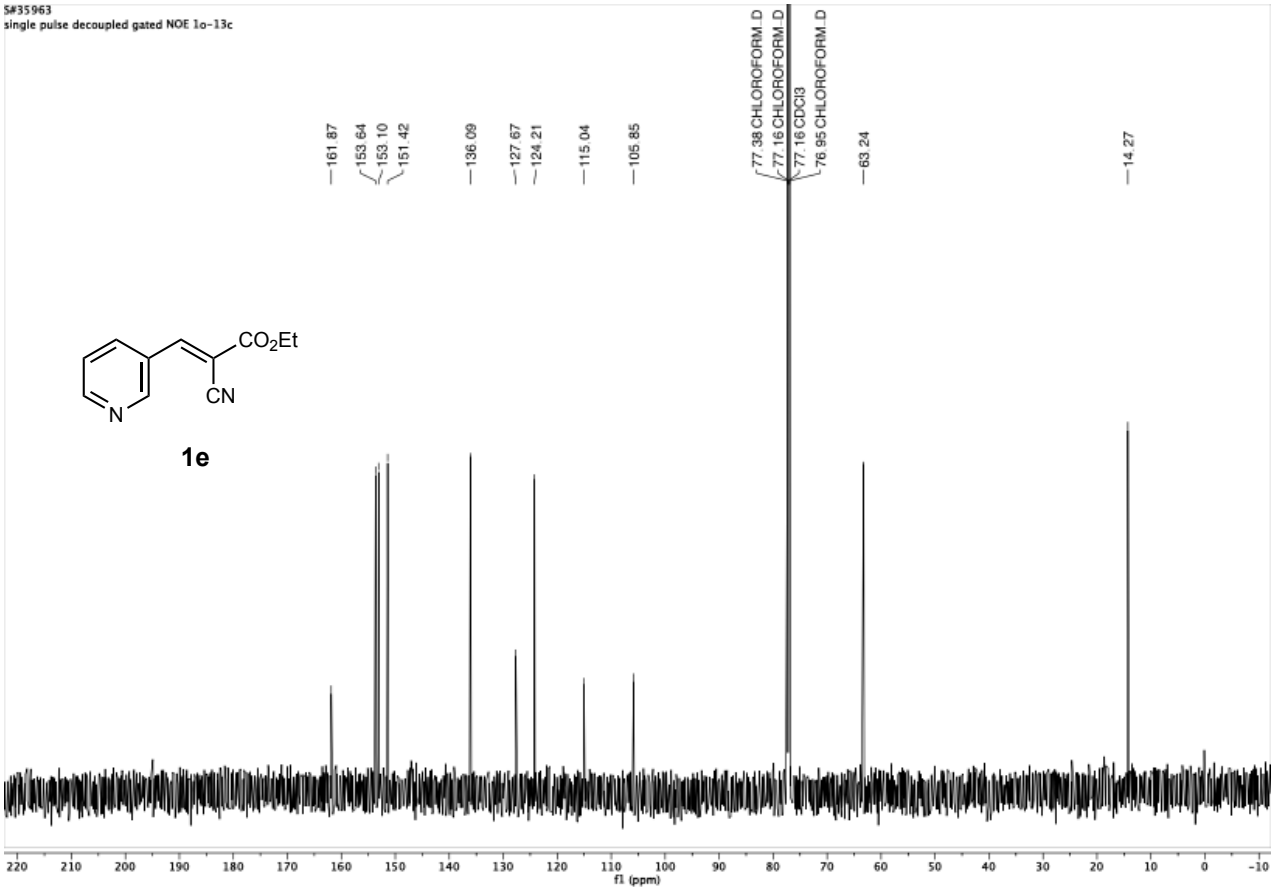
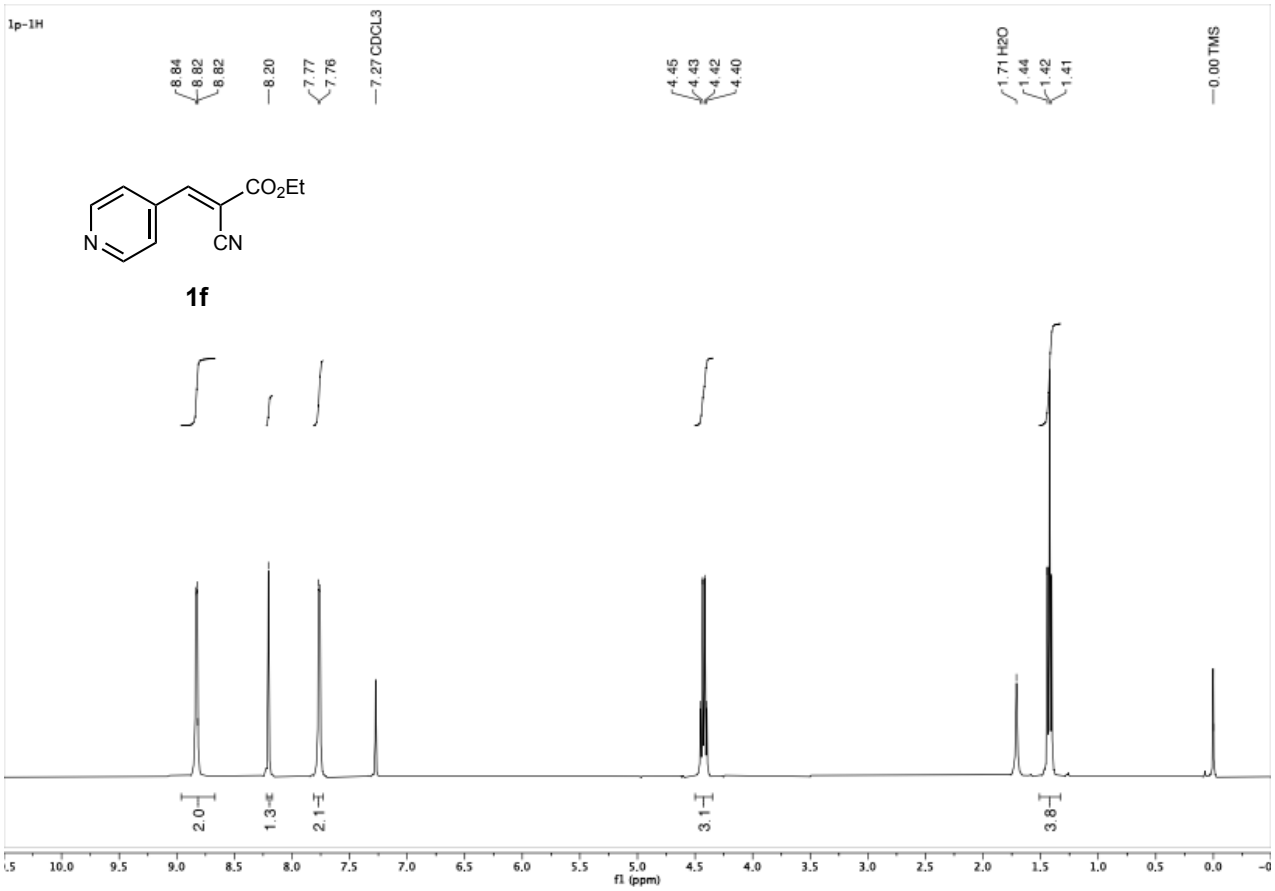
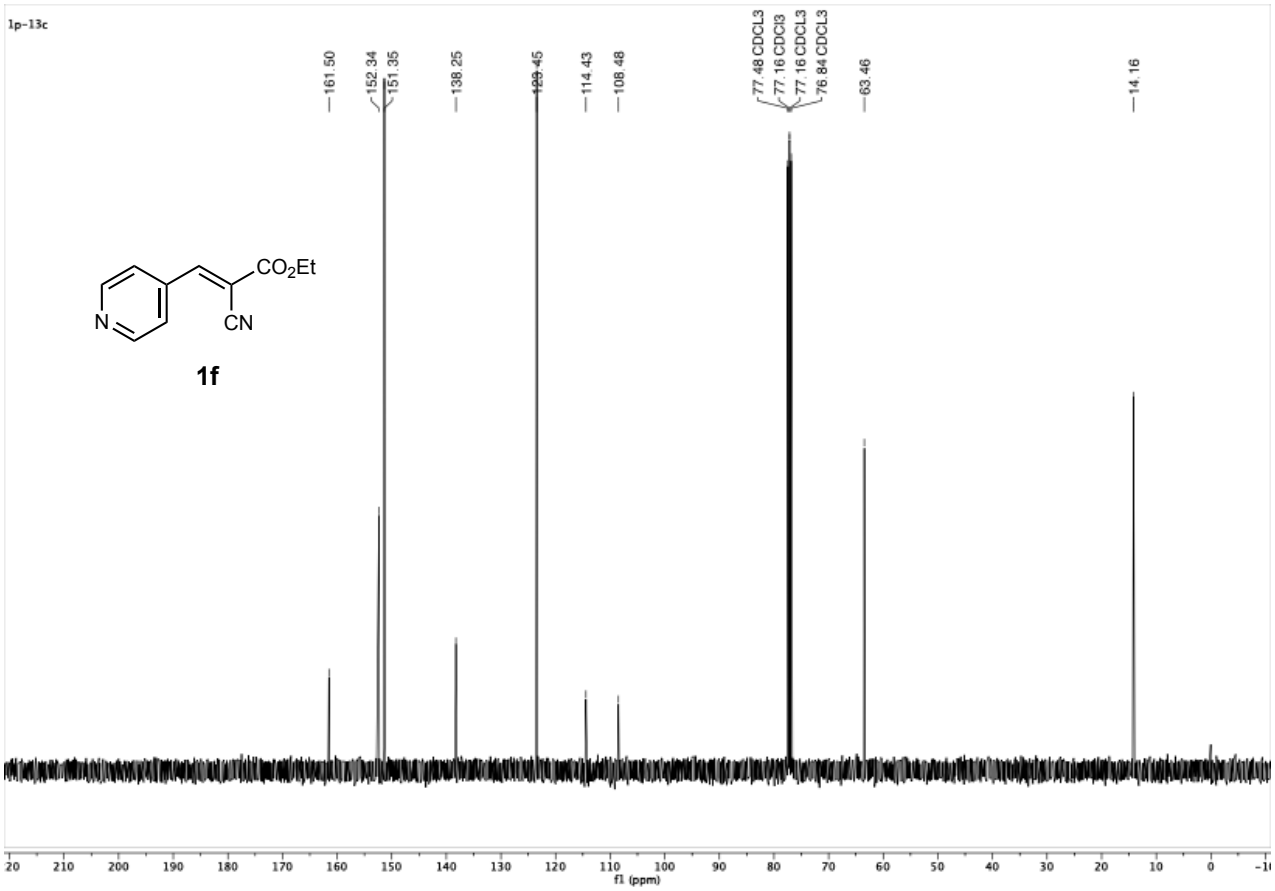
1d
2.0
1.0
1.0
2.0
3.0
f1 (ppm)

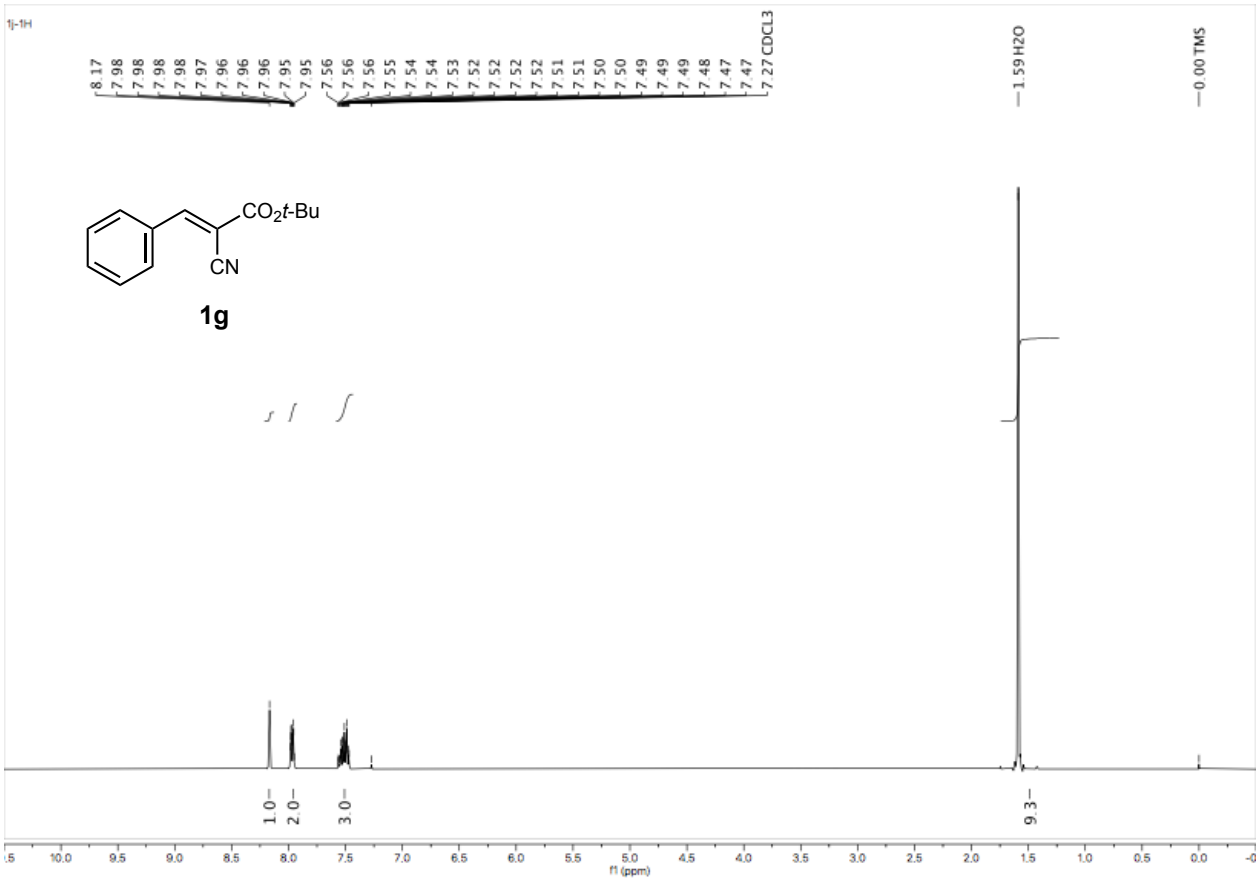

S#809921
single pulse decoupled gated NOE 1n-13C
161.34
151.01
150.98
133.50
133.39
133.27
133.05
132.82
130.31
126.08
125.51
123.69
121.87
120.06
114.32
107.79
77.37 CHLOROFORM-D
77.16 CDCl3
77.15 CHLOROFORM-D
76.94 CHLOROFORM-D
63.57
14.25
F3C
CO2Et
CN
CF3
1d
f1 (ppm)

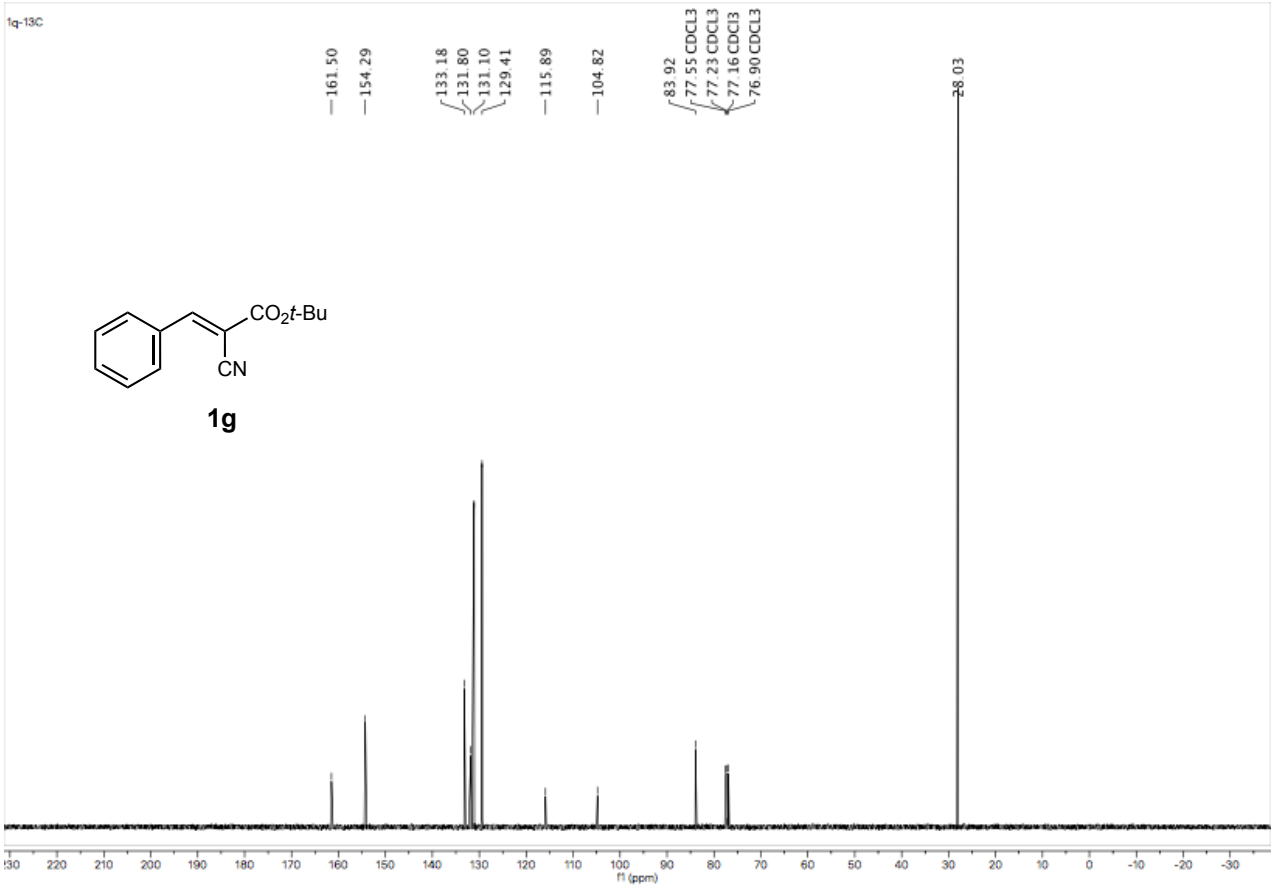

1o-1H
CO2Et
CN
N
1e

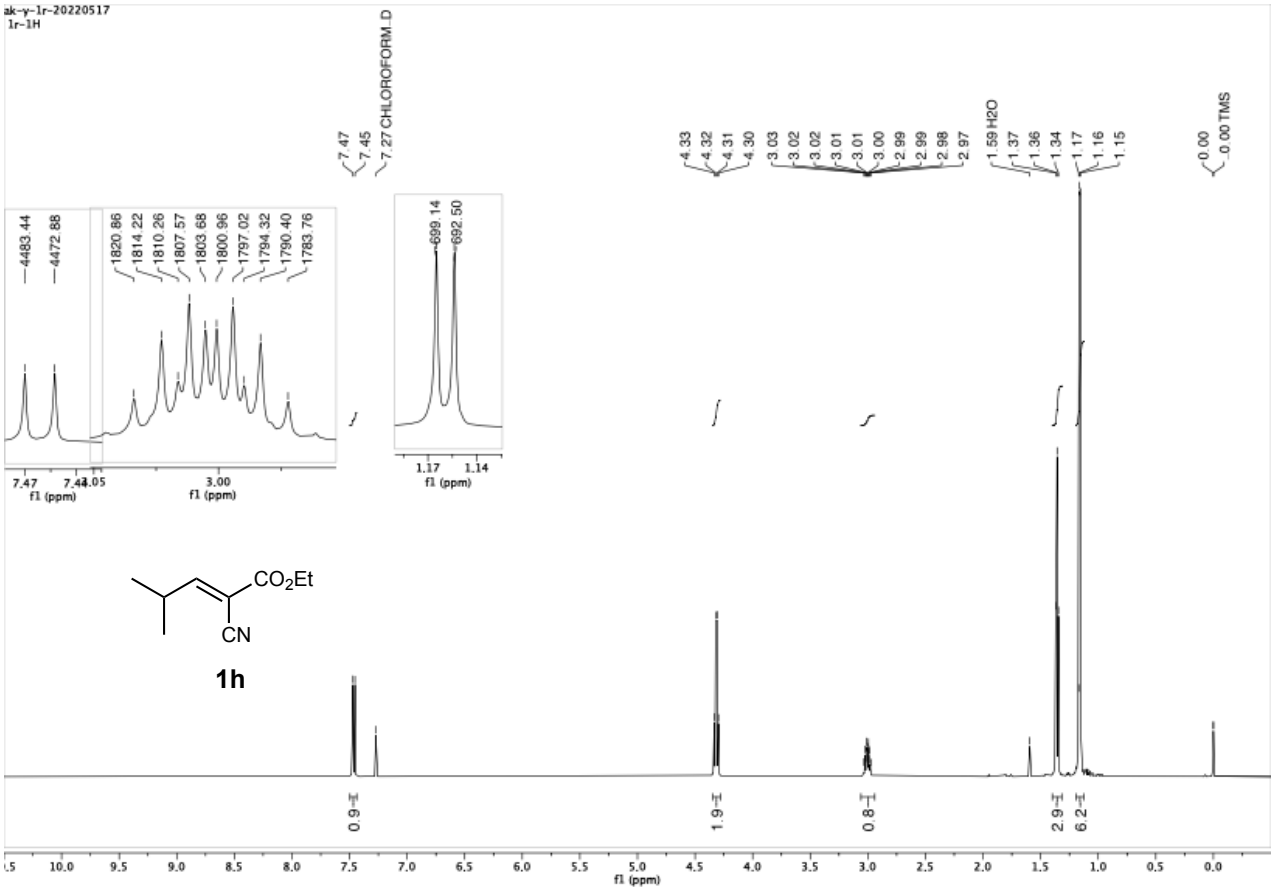

S#35963
single pulse decoupled gated NOE 1o-13c
CO2Et
CN
N
1e

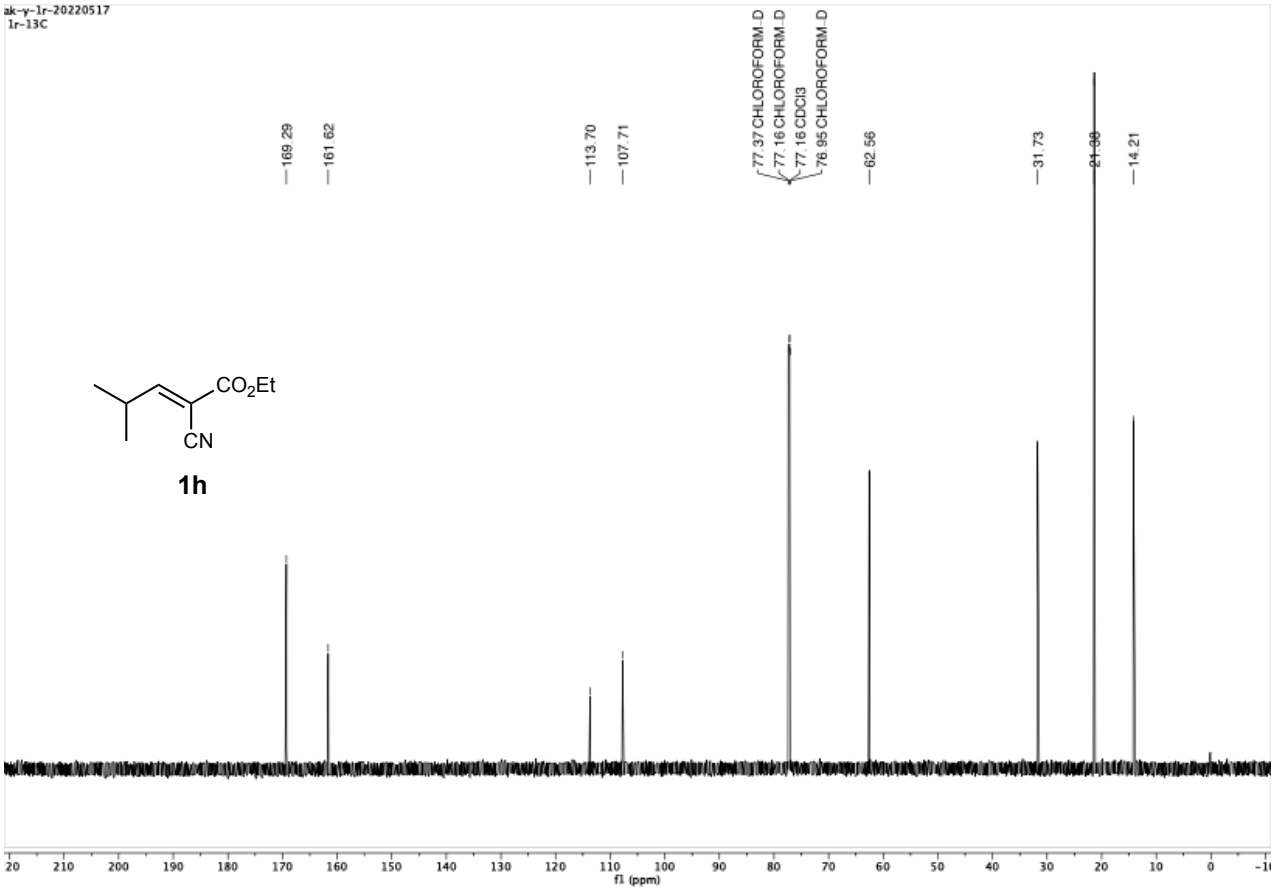

1p-1H
8.84
8.82
8.82
8.20
7.77
7.76
7.27 CDCL3
4.45
4.43
4.42
4.40
1.71 H2O
1.44
1.42
1.41
0.00 TMS
CO2Et
N
CN
1f
2.0
1.3
2.1
3.1
3.8
f1 (ppm)

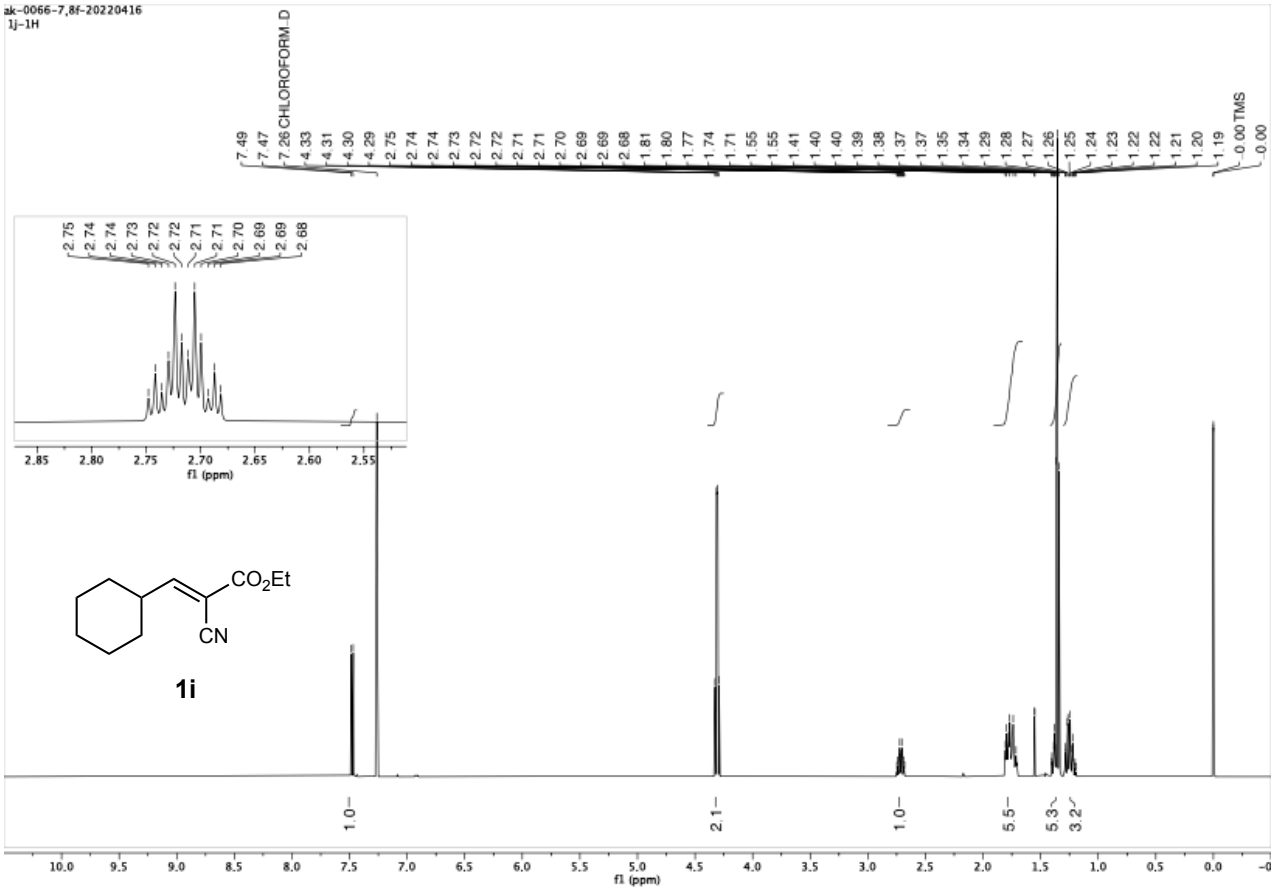

1p-13c
161.50
152.34
151.35
138.25
123.45
114.43
108.48
77.48 CDCL3
77.16 CDCl3
77.16 CDCL3
76.84 CDCL3
63.46
14.16
CO2Et
N
CN
1f
f1 (ppm)

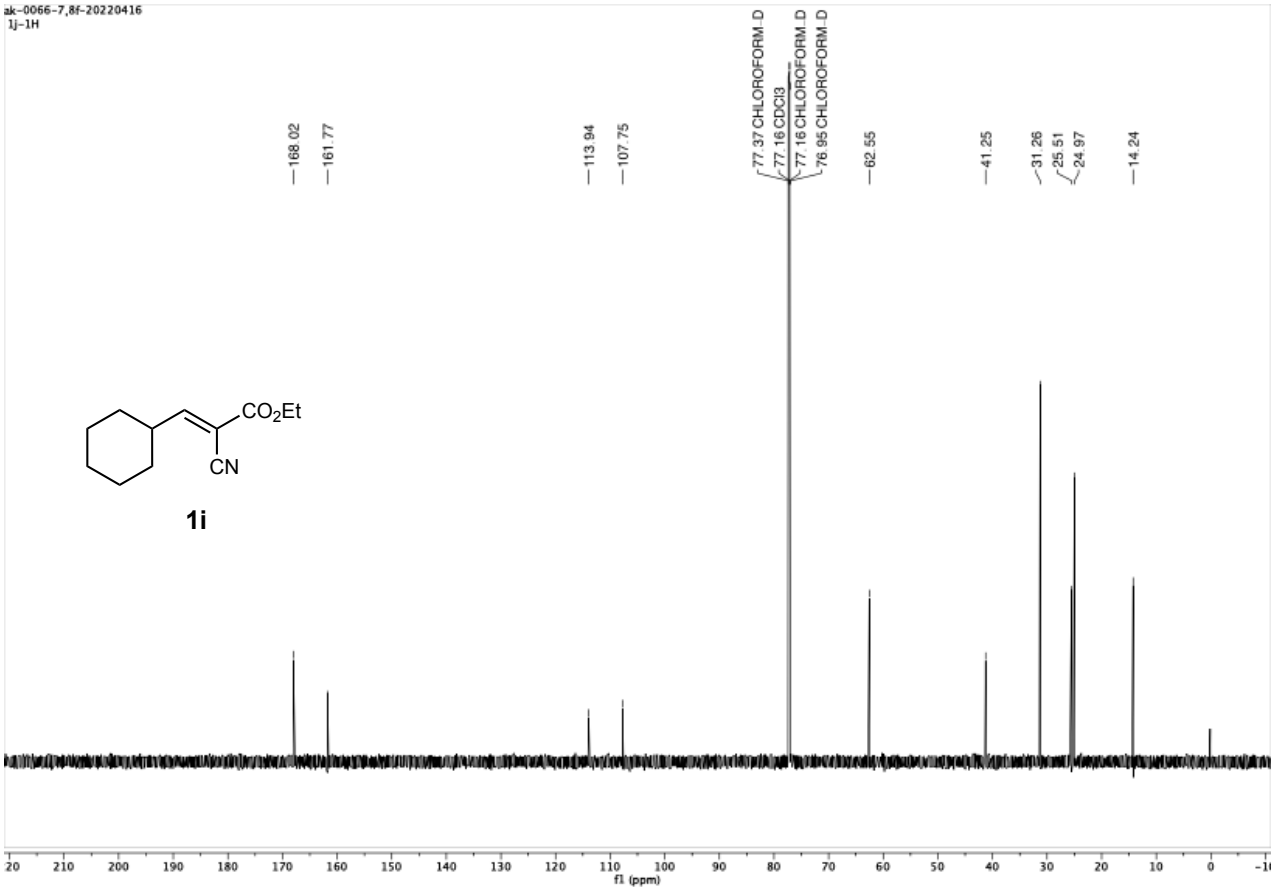

1j-1H
8.17
7.98
7.98
7.98
7.98
7.97
7.96
7.96
7.96
7.95
7.95
7.56
7.56
7.56
7.55
7.54
7.54
7.53
7.52
7.52
7.52
7.52
7.51
7.51
7.50
7.50
7.49
7.49
7.49
7.48
7.47
7.47
7.27 CDCL3
1.59 H2O
0.00 TMS
CO2t-Bu
CN
1g
1.0
2.0
3.0
9.3
f1 (ppm)

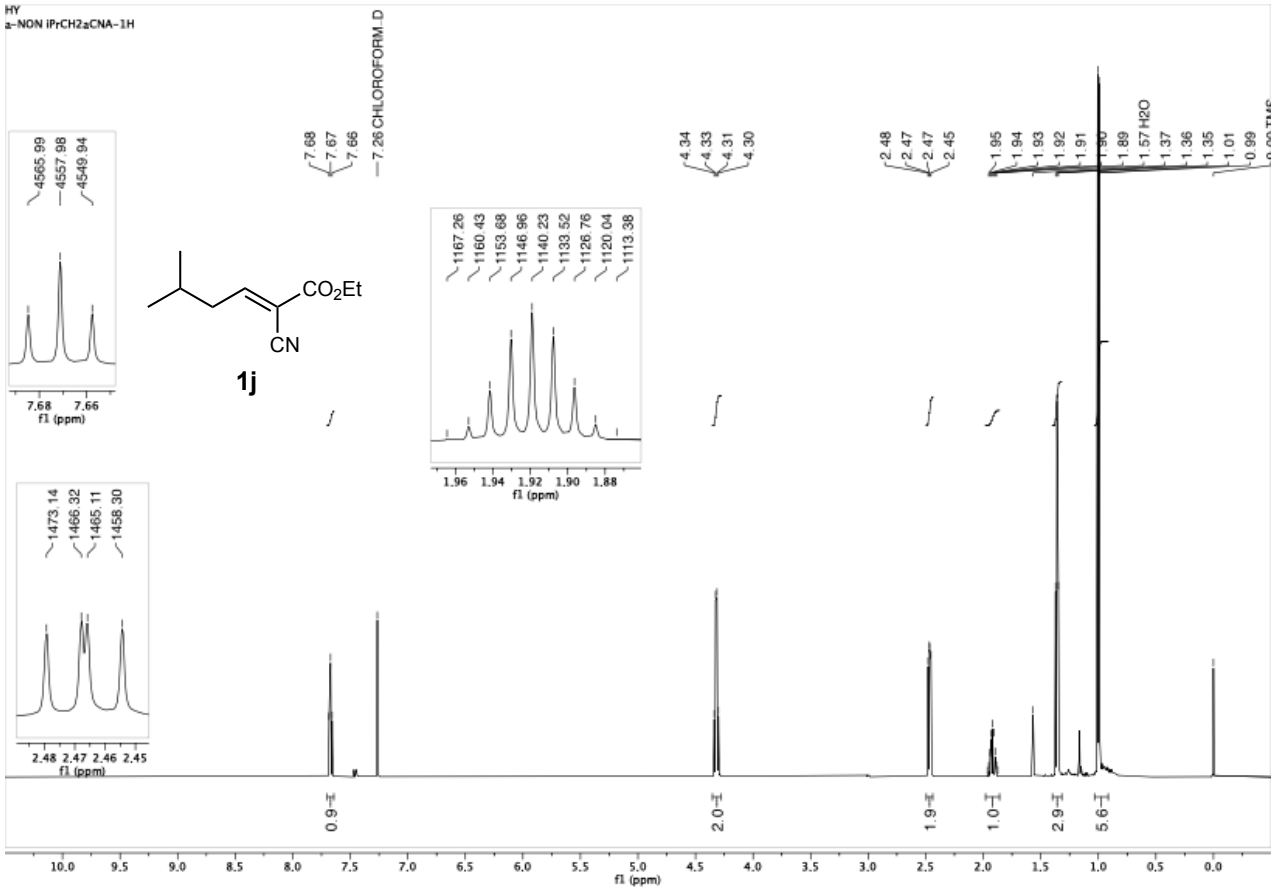

1q-13C
161.50
154.29
133.18
131.80
131.10
129.41
115.89
104.82
83.92
77.55 CDCL3
77.23 CDCL3
77.16 CDCl3
76.90 CDCL3
28.03
CO2t-Bu
CN
1g
f1 (ppm)

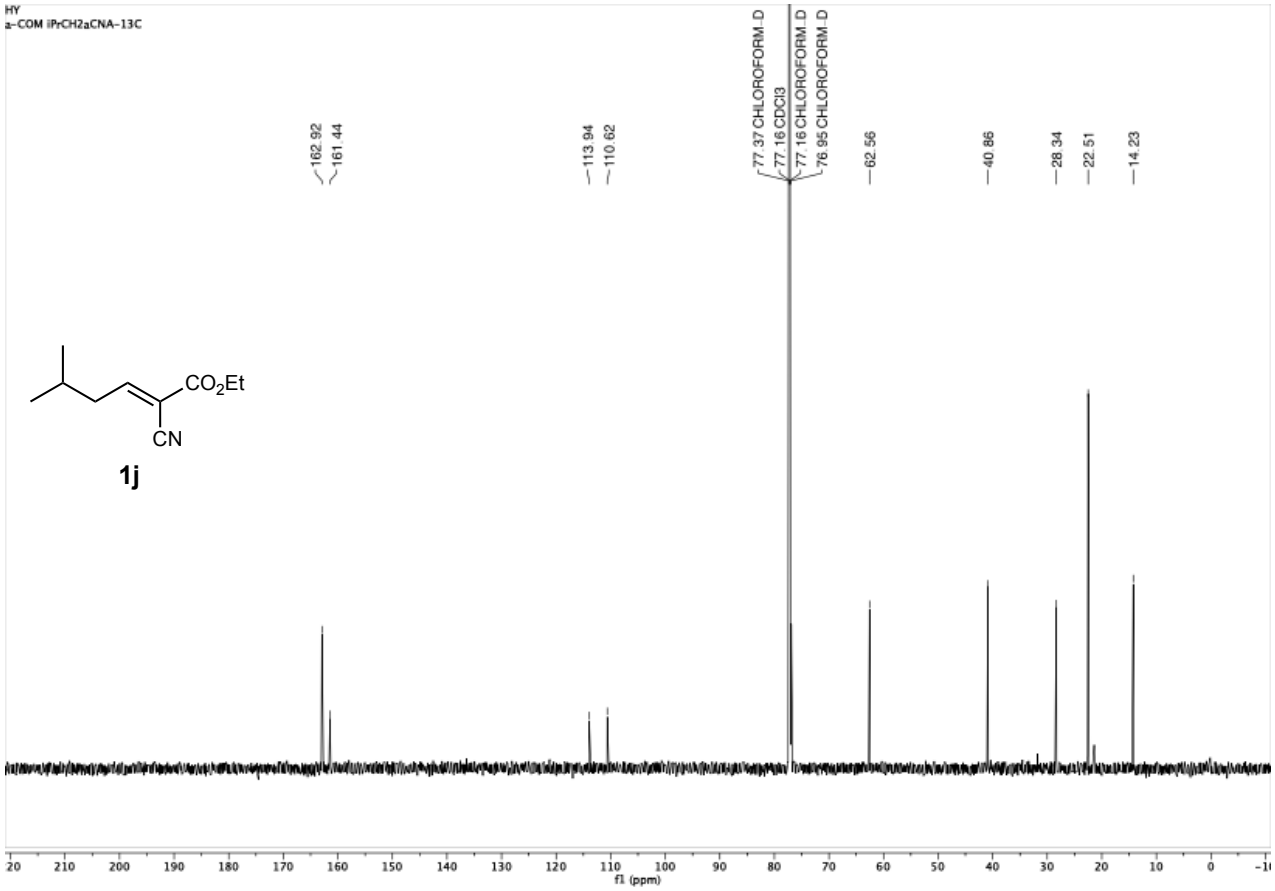

ak-y-1r-20220517
1r-1H
7.47
7.45
7.27 CHLOROFORM-D
4.33
4.32
4.31
4.30
3.03
3.02
3.02
3.01
3.01
3.00
2.99
2.99
2.98
2.97
1.59 H2O
1.37
1.36
1.34
1.17
1.16
1.15
0.00
0.00 TMS
4483.44
4472.88
1820.86
1814.22
1810.26
1807.57
1803.68
1800.96
1797.02
1794.32
1790.40
1783.76
699.14
692.50
7.47
7.44.05
f1 (ppm)
3.00
f1 (ppm)
1.17
1.14
f1 (ppm)
CO2Et
CN
1h
0.9
1.9
0.8
2.9
6.2
f1 (ppm)

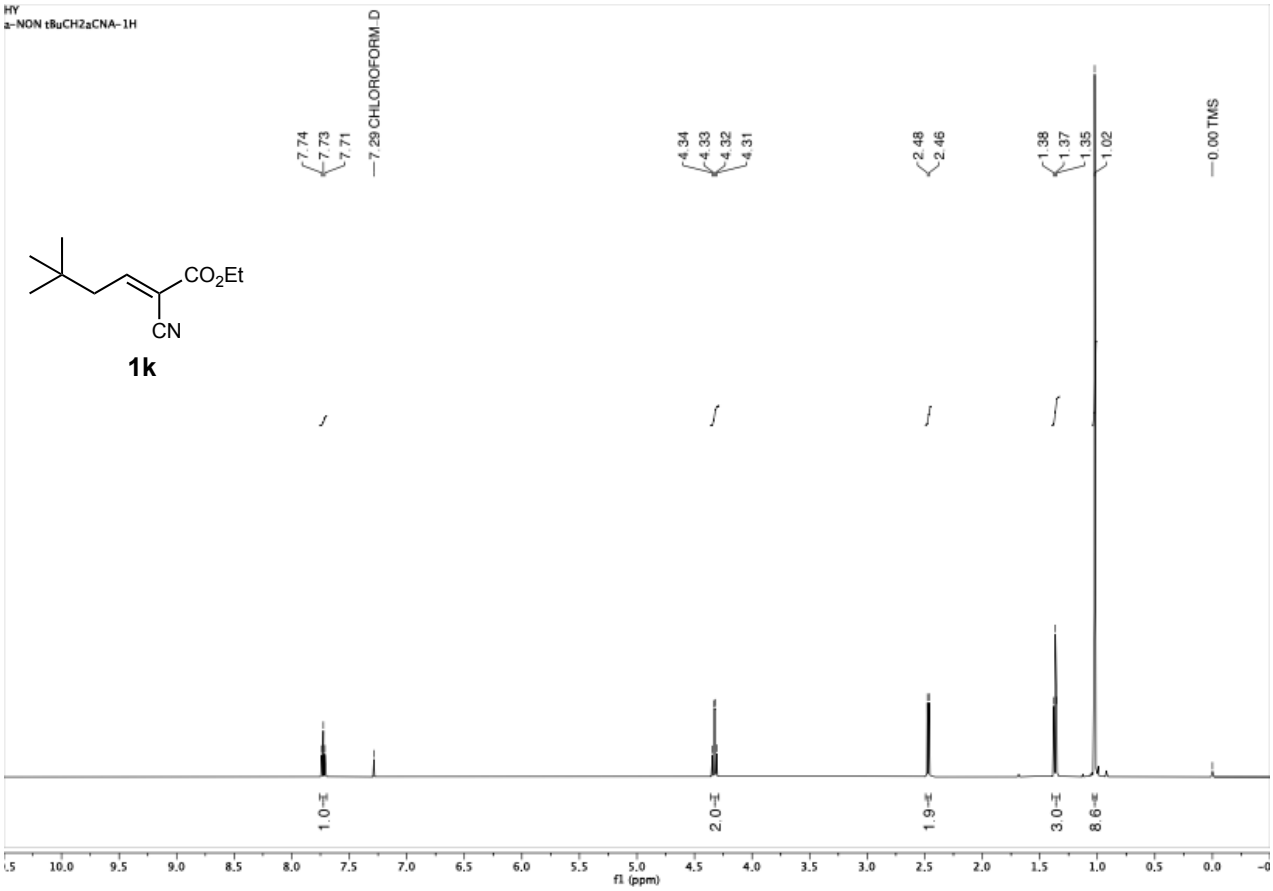

ak-y-1r-20220517
1r-13C
169.29
161.62
113.70
107.71
77.37 CHLOROFORM-D
77.16 CHLOROFORM-D
77.16 CDCl3
76.95 CHLOROFORM-D
62.56
31.73
21.38
14.21
CO2Et
CN
1h
f1 (ppm)

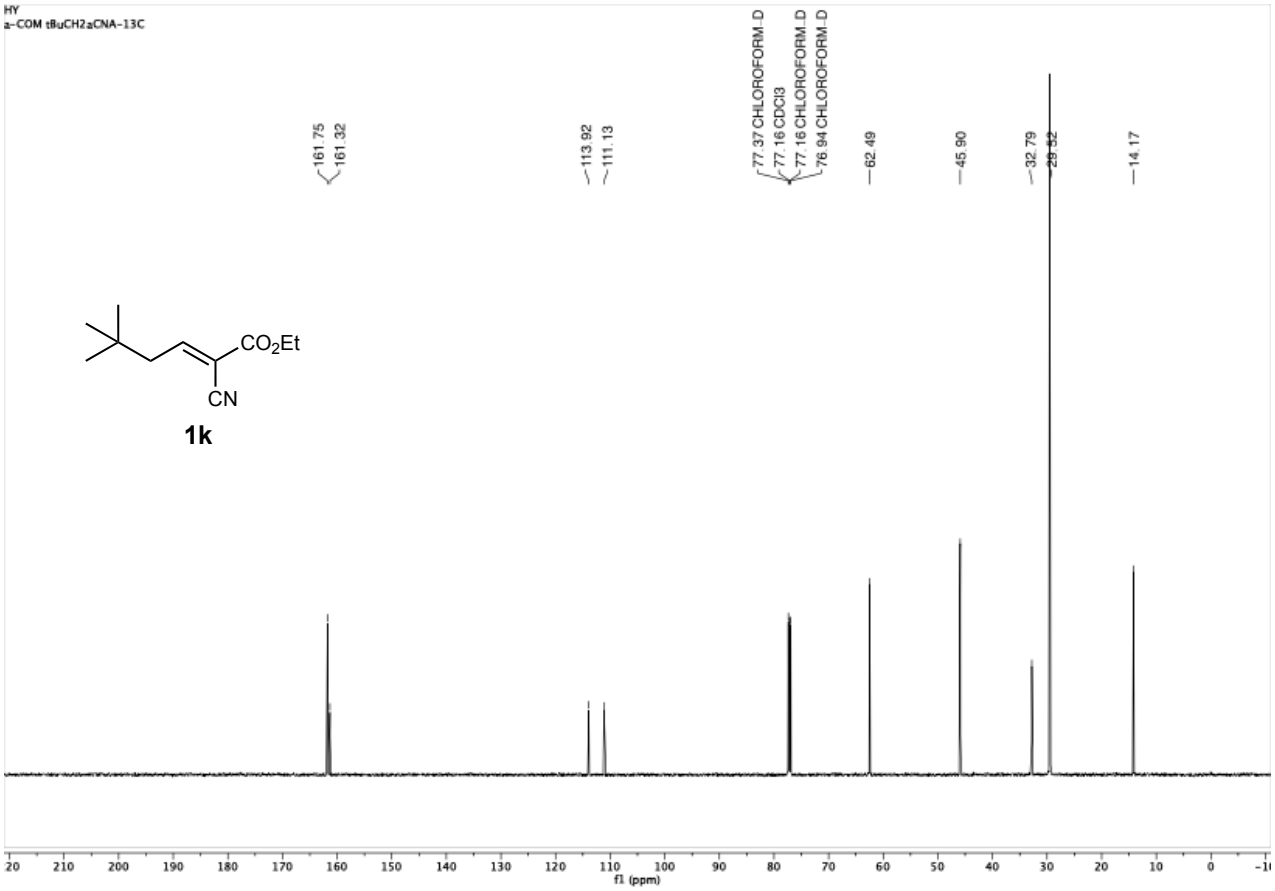

ak-0066-7,8f-20220416
1j-1H
CHLOROFORM-D
TMS
f1 (ppm)
CO_2Et
CN
1i

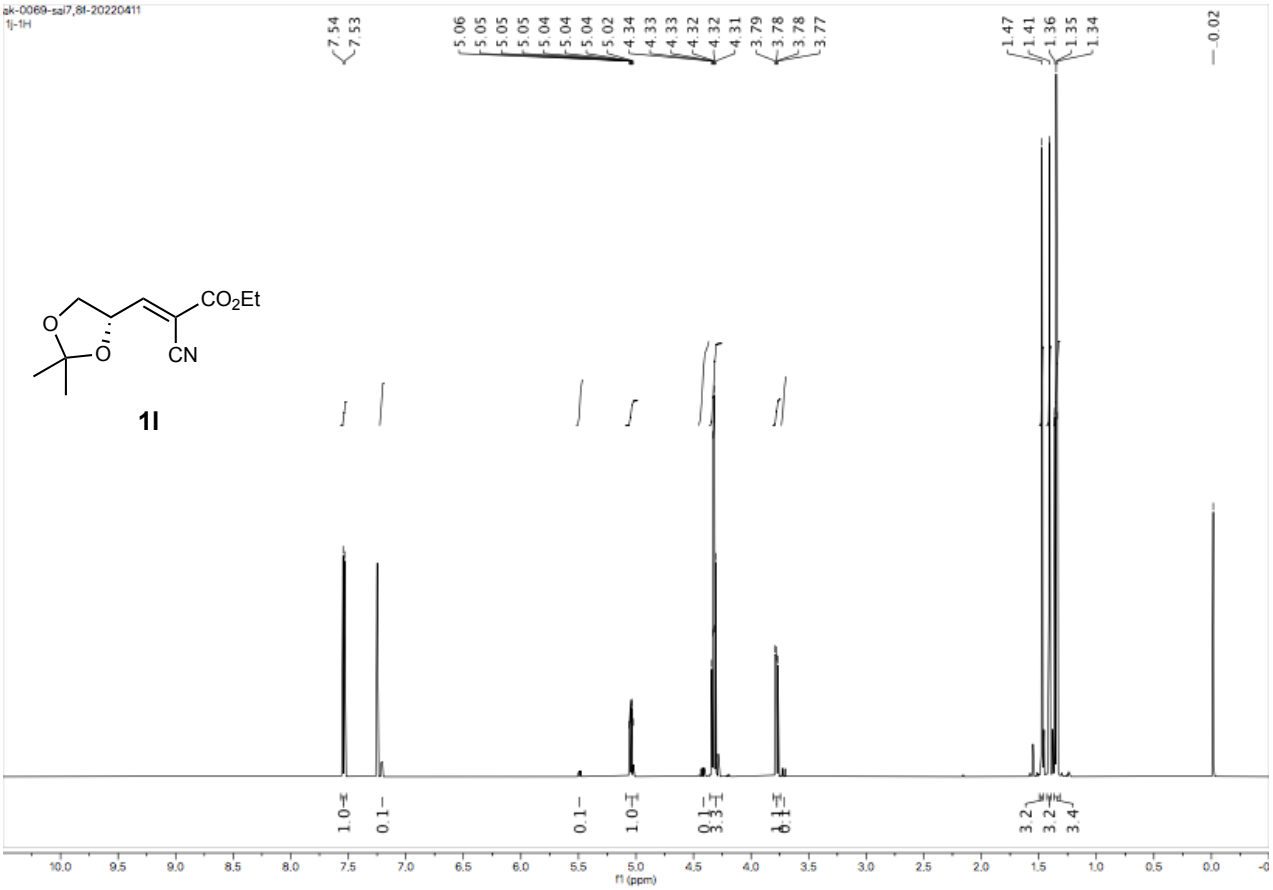

ak-0066-7,8f-20220416
1j-1H
168.02
161.77
113.94
107.75
77.37 CHLOROFORM-D
77.16 CDCl3
77.16 CHLOROFORM-D
76.95 CHLOROFORM-D
62.55
41.25
31.26
25.51
24.97
14.24
f1 (ppm)
CO_2Et
CN
1i


HY
a-NON iPrCH2aCNA-1H
7.68
7.67
7.66
7.26 CHLOROFORM-D
4.34
4.33
4.31
4.30
2.48
2.47
2.47
2.45
1.95
1.94
1.93
1.92
1.91
1.89
1.57 H2O
1.37
1.36
1.35
1.01
0.99
4565.99
4557.98
4549.94
7.68 7.66
f1 (ppm)
CO2Et
CN
1j
1167.26
1160.43
1153.68
1146.96
1140.23
1133.52
1126.76
1120.04
1113.38
1.96 1.94 1.92 1.90 1.88
f1 (ppm)
1473.14
1466.32
1465.11
1458.30
2.48 2.47 2.46 2.45
f1 (ppm)
0.9
2.0
1.9
1.0
2.9
5.6
f1 (ppm)

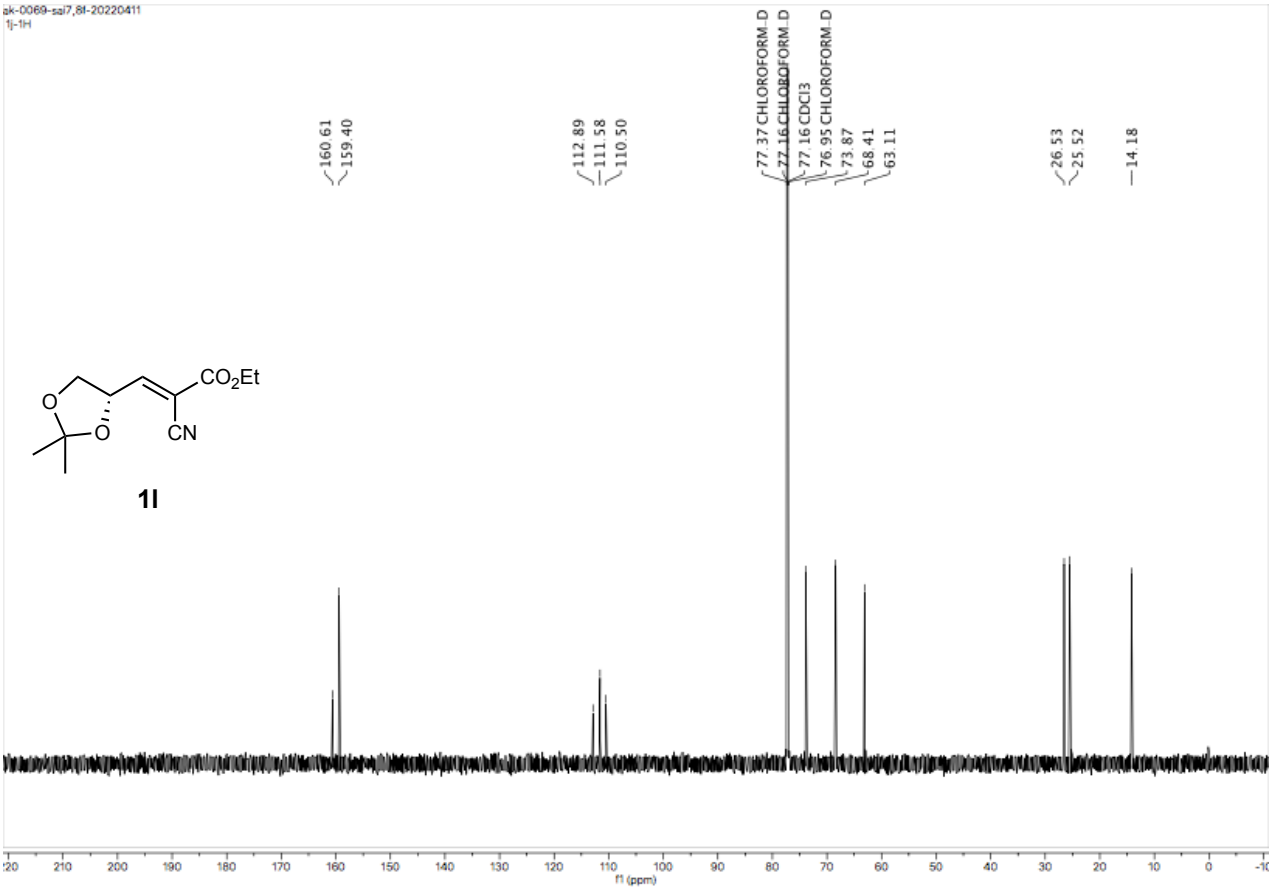

HY
a-COM iPrCH2aCNA-13C
162.92
161.44
113.94
110.62
77.37 CHLOROFORM-D
77.16 CDCl3
76.95 CHLOROFORM-D
62.56
40.86
28.34
22.51
14.23
CO2Et
CN
1j
f1 (ppm)


HY
a-NON tBuCH2aCNA-1H
7.74
7.73
7.71
7.29 CHLOROFORM-D
4.34
4.33
4.32
4.31
2.48
2.46
1.38
1.37
1.35
1.02
0.00 TMS
CO2Et
CN
1k
1.0
2.0
1.9
3.0
8.6
f1 (ppm)


HY
a-COM tBuCH2aCNA-13C
161.75
161.32
113.92
111.13
77.37 CHLOROFORM-D
77.16 CDCl3
77.16 CHLOROFORM-D
76.94 CHLOROFORM-D
62.49
45.90
32.79
29.32
14.17
CO2Et
CN
1k
f1 (ppm)


ak-0069-sai7,8f-20220411
1j-1H
7.54
7.53
5.06
5.05
5.05
5.05
5.04
5.04
5.04
5.02
4.34
4.33
4.33
4.32
4.32
4.31
3.79
3.78
3.78
3.77
1.47
1.41
1.36
1.35
1.34
0.02
CO2Et
CN
1l
1.0
0.1
0.1
1.0
0.1
3.3
0.1
3.2
3.2
3.4
f1 (ppm)

p


ak-0069-sai7,8f-20220411
1j-1H
160.61
159.40
112.89
111.58
110.50
77.37 CHLOROFORM-D
77.16 CHLOROFORM-D
77.16 CDCl3
76.95 CHLOROFORM-D
73.87
68.41
63.11
26.53
25.52
14.18
CO2Et
CN
1l
f1 (ppm)