# Tuning the Diels–Alder Reaction for Bioconjugation with Maleimide Drug-linkers.

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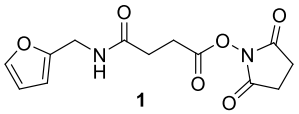
## Supporting Information – Table of Contents

**1. Materials and Methods**.

Unless stated otherwise, reactions were conducted under an atmosphere of $N_2$ using reagent grade solvents. DCM was stored over 3Å molecular sieves. THF was dried through an activated alumina column under $N_2$. All commercially obtained reagents were used as received. Compounds **1** and **vcMMAE** were purchased from SynChem, Inc. (Elk Grove Village, IL). $PPh_3AuNTf_2$ was a gift provided by Hongyi Chen and Liming Zhang. Thin-layer chromatography (TLC) was conducted with E. Merck silica gel 60 F254 pre-coated plates (0.25 mm) and visualized by exposure to UV light (254 nm) or stained with *p*-anisaldehyde or potassium permanganate. Flash column chromatography was performed using normal phase silica gel (60 Å, 0.040 – 0.063 mm, Geduran). $^1$H NMR spectra were recorded on Varian spectrometers (400, 500, or 600 MHz) and are reported relative to deuterated solvent signals. Data for $^1$H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz) and integration. $^{13}$C NMR spectra were recorded on Varian Spectrometers (100, 125, or 150 MHz). Data for $^{13}$C NMR spectra are reported in terms of chemical shift (δ ppm). Mass spectra were obtained from the UC Santa Barbara Mass Spectrometry Facility on a (Waters Corp.) GCT Premier high resolution Time-of-flight mass spectrometer with an electron ionization (EI) or chemical ionization (CI) source.

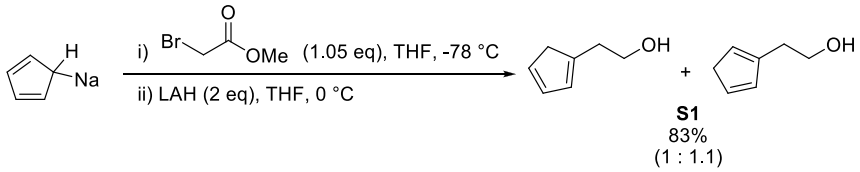


**1**

**2,5-Dioxopyrrolidin-1-yl 4-((furan-2-ylmethyl)amino)-4-oxobutanoate** (**1**):

$^1$H NMR (400 MHz, $CDCl_3$) δ 7.34 (dd, *J* = 0.8, 1.8 Hz, 1 H), 6.31 (dd, *J* = 1.9, 3.2 Hz, 1 H), 6.22 (dd, *J* = 0.8, 3.1 Hz, 1 H), 6.17 (br.s., 1 H), 4.43 (d, *J* = 5.7 Hz, 2 H), 2.98 (t, *J* = 7.3 Hz, 2 H), 2.81 (s, 4 H), 2.61 (t, *J* = 7.1 Hz, 2 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) δ 169.7, 169.0, 168.1, 151.0, 142.1, 110.4, 107.5, 36.6, 30.5, 26.7, 25.5 ppm.

**2. Synthesis of 2a, 3, 4, 5, and 2b.**



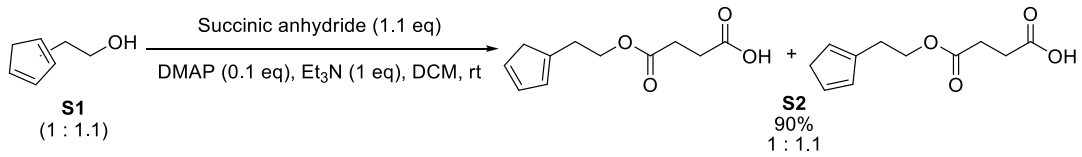

**2-(Cyclopenta-1,3-dienyl)ethanol & 2-(cyclopenta-1,4-dienyl)ethanol** (**S1**):

A solution of methyl bromoacetate (6.0 mL, 63 mmol, 1.05 equiv) in THF (60 mL) was cooled to -78 °C. A sodium cyclopentadienylide solution (2 M in THF, 30 mL, 60 mmol, 1 equiv) was added dropwise over 10 min and the reaction was stirred a further 2 h at -78 °C. The reaction was quenched with $H_2O$ (6 mL) and silica gel (6 g) then allowed to warm to rt. The reaction mixture was filtered through a plug of silica with DCM (100 mL) and the solvent removed to yield the methyl ester which was used directly in the next reaction.

A solution of lithium aluminum hydride (4.55 g, 120 mmol, 2 equiv) in THF (300 mL) was cooled to 0 °C. The crude methyl ester (~60 mmol) was dissolved in THF (10 mL) and added dropwise

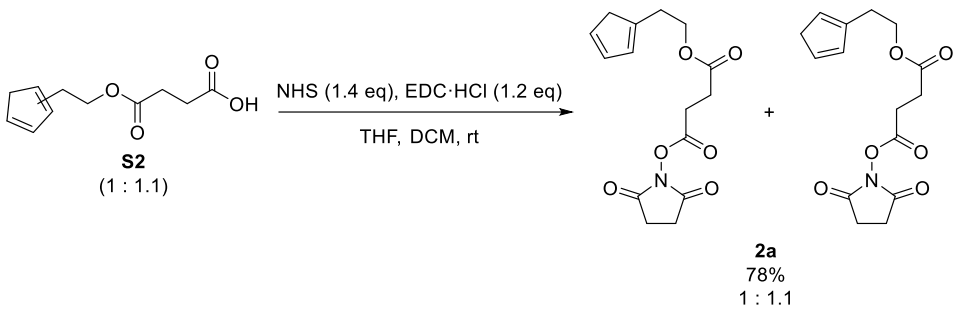
in 4 portions over 1 h at 0 °C then stirred for a further 2 h at rt. The reaction mixture was cooled to 0 °C and carefully quenched with $H_2O$ (20 mL), NaOH (4 M in $H_2O$, 5 mL), then $H_2O$ (20 mL). The reaction mixture was filtered, rinsed with $Et_2O$ (100 mL), then transferred to a separatory funnel. Brine (100 mL) was added then extracted with $Et_2O$ (3 x 100 mL). The organic layers were combined, washed with brine (100 mL), dried over $MgSO_4$, filtered, and the solvent removed. The residue was filtered through a silica plug (EtOAc:Hexane, 2:1, 200 mL) and the solvent removed to yield **S1** (5.45 g, 83% over two steps) as an amber oil. Dimerization of the cyclopentadiene occurs slowly when stored at -20 °C, for long term storage **S1** was frozen in a matrix of benzene. Spectral data matched that of literature reported data.[1]

**S1**: Rf (Hexane:EtOAc, 4:1): 0.14; $^1$H NMR (400 MHz, $CDCl_3$) δ 6.52 - 6.10 (m, 3 H), 3.88 - 3.71 (m, 2 H), 3.07 - 2.89 (m, 2 H), 2.77 - 2.57 (m, 2 H), 1.52 (br.s., 2 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) δ 145.3, 143.2, 134.5, 134.2, 132.4, 131.5, 128.7, 128.4, 62.2, 61.6, 43.4, 41.6, 34.0, 33.2 ppm.




**4-(2-(Cyclopenta-1,3-dienyl)ethoxy)-4-oxobutanoic acid & 4-(2-(cyclopenta-1,4-dienyl)ethoxy)-4-oxobutanoic acid** (**S2**):
To a solution of **S1** (0.33 g, 3.0 mmol, 1 equiv) in DCM (1.5 mL) in a vial was added $Et_3N$ (0.42 mL, 3.0 mmol, 1 equiv), 4-dimethylaminopyridine (37 mg, 0.3 mmol, 0.1 equiv), and succinic anhydride (0.33 g, 3.3 mmol, 1.1 equiv). The reaction was capped under an atmosphere of air and stirred at rt for 1 h, then transferred to a separatory funnel with DCM (50 mL). The organic layer was washed with HCl (1 M in $H_2O$, 50 mL) then $H_2O$ (50 mL). The organic layer was dried over $MgSO_4$, filtered, and the solvent removed to yield **S2** (0.57 g, 90%) as a tan powder.

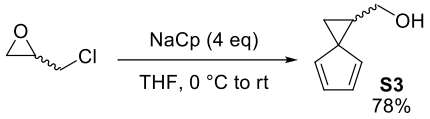
**S2**: Rf (EtOAc): 0.67; $^1$H NMR (400 MHz, $CDCl_3$) δ 11.49 (br.s., 1 H), 6.48 - 6.05 (m, 3 H), 4.33 - 4.21 (m, 2 H), 2.98 - 2.89 (m, 2 H), 2.83 - 2.52 (m, 6 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) δ 178.4, 172.0, 144.3, 142.3, 134.2, 134.0, 132.2, 131.4, 128.3, 127.9, 64.3, 63.9, 43.4, 41.4, 29.7, 29.0, 28.8, 28.8 ppm; IR (ATR) 2934, 2671, 2568, 1720, 1706, 1693, 1418, 1358, 1235, 1178 $cm^{-1}$; HRMS (CI) Exact mass cald. for $C_{11}H_{13}O_4$ $[M-H]^+$: 209.0814, found: 209.0815.




**2-(Cyclopenta-1,3-dienyl)ethyl 2,5-dioxopyrrolidin-1-yl succinate & 2-(cyclopenta-1,4-dienyl)ethyl 2,5-dioxopyrrolidin-1-yl succinate** (**2a**):

To a solution of **S2** (0.42 g, 2.0 mmol, 1 equiv) in THF (10 mL) in a vial was added *N*-hydroxysuccinimide (0.32 g, 2.8 mmol, 1.4 equiv), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.46 g, 2.4 mmol, 1.2 equiv) and DCM (5 mL). The reaction was capped under an atmosphere of air and stirred at rt overnight. The solvent was removed and the residue was subjected to flash column chromatography (Hexane:EtOAc, 1:1) to yield **2a** (0.48 g, 78%) as a clear, viscous oil. At room temperature **2a** will dimerize, but it is stable for several months when stored at -20 °C.

**2a**: Rf (Hexane:EtOAc, 1:1): 0.33; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.49 - 6.06 (m, 3 H), 4.37 - 4.25 (m, 2 H), 3.03 - 2.90 (m, 4 H), 2.85 (br.s., 4 H), 2.80 - 2.68 (m, 4 H) ppm; $^{13}$C NMR (125 MHz, $CDCl_3$) δ 170.8, 168.9, 167.6, 144.3, 142.3, 134.2, 134.1, 132.3, 131.4, 128.4, 128.0, 64.5, 64.2, 43.5, 41.4, 29.7, 29.0, 28.7, 26.2, 25.5 ppm; IR (ATR) 2953, 1814, 1783, 1731, 1362, 1201, 1068, 993 $cm^{-1}$; HRMS (CI) Exact mass cald. for $C_{15}H_{16}NO_6$ $[M-H]^+$: 306.0978, found: 306.0981.



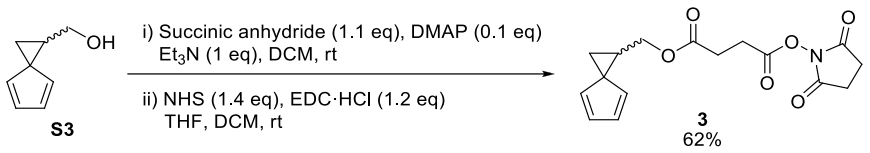

**Spiro[2.4]hepta-4,6-dien-1-ylmethanol** (**S3**):

Sodium cyclopentadienide (2 M solution in THF, 10 mL, 20 mmol, 4 equiv) was added to THF (40 mL) and cooled to 0 °C. (±)-Epichlorohydrin (0.39 mL, 5.0 mmol, 1 equiv) was added dropwise and the reaction was stirred at 0 °C for 1.5 h then a further 2 h at rt. The reaction was quenched with $H_2O$ (40 mL) then transferred to a separatory funnel. A saturated solution of $NaHCO_3$ in $H_2O$ (40 mL) was added then extracted with $Et_2O$ (40 mL). The organic layer was washed with brine (40 mL), dried over $MgSO_4$, filtered, and the solvent removed. The residue was subjected to flash column chromatography (Hexane:EtOAc, 2:1) to yield **S3** (0.48 g, 78%) as a brown oil.

Spectral data matched that of literature reported data.[2]

**S3**: Rf (Hexane:EtOAc, 2:1): 0.22; $^{1}$H NMR (500 MHz, $CDCl_3$) δ 6.64 (td, *J* = 1.6, 5.1 Hz, 1 H), 6.57 - 6.43 (m, 1 H), 6.34 - 6.21 (m, *J* = 1.0, 1.0, 5.2 Hz, 1 H), 6.12 (td, *J* = 1.7, 5.1 Hz, 1 H), 4.08 - 3.88 (m, 1 H), 3.59 (dd, *J* = 8.8, 11.7 Hz, 1 H), 2.51 - 2.37 (m, *J* = 6.0, 7.3, 8.6, 8.6 Hz, 1 H),

1.87 (dd, *J* = 4.3, 8.7 Hz, 1 H), 1.69 (dd, *J* = 4.4, 7.0 Hz, 1 H), 1.57 (br.s., 1 H) ppm; $^{13}$C NMR (125 MHz, $CDCl_3$) δ 139.4, 133.9, 131.7, 128.6, 64.9, 41.9, 30.0, 17.6 ppm.

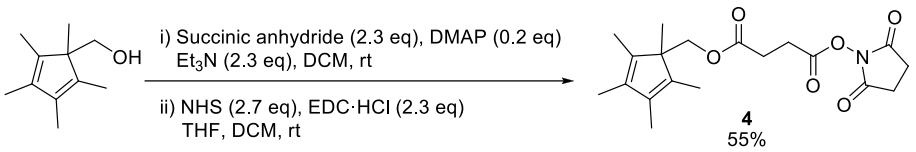



**2,5-Dioxopyrrolidin-1-yl spiro[2.4]hepta-4,6-dien-1-ylmethyl succinate** (**3**):
DCM (1.5 mL) was added to a vial containing **S3** (0.37 g, 3.0 mmol, 1 equiv). $Et_3N$ (0.42 mL, 3.0 mmol, 1 equiv), 4-dimethylaminopyridine (37 mg, 0.30 mmol, 0.1 equiv) and succinic anhydride (0.33 g, 3.3 mmol, 1.1 equiv) were added, the reaction capped under an atmosphere of air, and stirred at rt 1.75 h. The reaction mixture was poured into a separatory funnel with DCM (50 mL) and washed with aqueous HCl (1 M, 50 mL). The aqueous layer was extracted with DCM (50 mL), the organic layers combined, dried over $Na_2SO_4$, filtered, and the solvent removed to yield acid **S4** which was used directly in the next reaction.

**S4**: Rf (EtOAc): 0.56; $^1$H NMR (400 MHz, $CDCl_3$) δ 10.60 (br.s., 1 H), 6.57 (td, *J* = 1.9, 5.3 Hz, 1 H), 6.50 (td, *J* = 1.8, 5.1 Hz, 1 H), 6.21 (td, *J* = 1.7, 5.2 Hz, 1 H), 6.07 (td, *J* = 1.8, 5.1 Hz, 1 H), 4.37 (dd, *J* = 7.4, 11.7 Hz, 1 H), 4.20 (dd, *J* = 7.0, 11.7 Hz, 1 H), 2.74 - 2.57 (m, 4 H), 2.42 (quin, *J* = 7.8 Hz, 1 H), 1.85 (dd, *J* = 4.5, 8.4 Hz, 1 H), 1.69 (dd, *J* = 4.3, 7.0 Hz, 1 H) ppm.

THF (10 mL) was added to a vial containing **S4** (~3 mmol). *N*-hydroxysuccinimide (0.48 g, 4.2 mmol, 1.4 equiv), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.69 g, 3.6 mmol, 1.2 equiv) and DCM (5 mL) were added, the reaction capped under an atmosphere of air, and stirred at rt overnight. The solvent was removed and the residue was subjected to flash column chromatography (Hexane:EtOAc, 1:1) to yield **3** (0.59 g, 62% over two steps) as a colorless, viscous oil.

**3**: Rf (Hexane:EtOAc, 1:1): 0.34; $^1$H NMR (400 MHz, $CDCl_3$) δ 6.56 (td, J = 1.8, 5.1 Hz, 1 H), 6.48 (td, J = 1.8, 5.1 Hz, 1 H), 6.21 (td, J = 1.6, 3.4 Hz, 1 H), 6.06 (td, J = 1.6, 3.4 Hz, 1 H), 4.36 (dd, J = 7.4, 11.7 Hz, 1 H), 4.21 (dd, J = 7.4, 11.7 Hz, 1 H), 2.93 (t, J = 7.0 Hz, 2 H), 2.83 (s, 4 H), 2.73 (t, J = 7.4 Hz, 2 H), 2.42 (quin, J = 7.6 Hz, 1 H), 1.83 (dd, J = 4.3, 8.6 Hz, 1 H), 1.68 (dd, J = 4.5, 6.8 Hz, 1 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) δ 170.8, 168.9, 167.6, 138.8, 134.3, 131.2, 129.0, 66.6, 41.5, 28.6, 26.2, 25.5, 25.1, 17.3 ppm; IR (ATR) 2945, 1814, 1783, 1732, 1366, 1202, 1068 $cm^{-1}$; HRMS (EI) Exact mass cald. for $C_{16}H_{17}NO_6$ $[M]^+$: 319.1056, found: 319.1051.

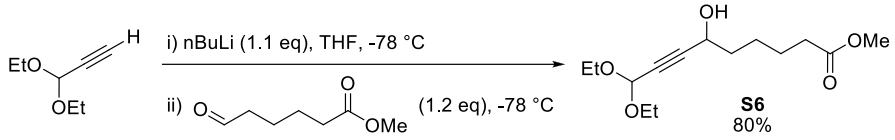



**2,5-Dioxopyrrolidin-1-yl (1,2,3,4,5-pentamethylcyclopenta-2,4-dienyl)methyl succinate** (**4**):
DCM (8 mL) was added to a vial containing (1,2,3,4,5-pentamethylcyclopenta-2,4-dienyl)methanol[3] (0.33 g, 2.0 mmol, 1 equiv). $Et_3N$ (0.64 mL, 4.6 mmol, 2.3 equiv), 4-dimethylaminopyridine (46 mg, 0.38 mmol, 0.2 equiv) and succinic anhydride (0.46 g, 4.6 mmol, 2.3 equiv) were added, the reaction capped under an atmosphere of air, and stirred at rt overnight. The reaction was quenched with $H_2O$ (1 mL) then poured into a separatory funnel. HCl (1 M, 50 mL) was added and extracted with DCM (2 x 50 mL). The organic layers were combined, washed with brine (50 mL), dried over $Na_2SO_4$, filtered, and the solvent removed to yield the acid **S5** which was used directly in the next reaction.

**S5**: Rf (EtOAc): 0.24; $^1$H NMR (400 MHz, $CDCl_3$) δ 3.98 (s, 2 H), 2.64 - 2.59 (m, 2 H), 2.59 - 2.54 (m, 2 H), 1.76 (s, 6 H), 1.74 (s, 6 H), 0.95 (s, 3 H) ppm.

THF (10 mL) was added to a vial containing **S5** (~2 mmol). *N*-hydroxysuccinimide (0.61 g, 5.3 mmol, 2.7 equiv), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (0.87 g, 4.6 mmol, 2.3 equiv) and DCM (6 mL) were added, the reaction capped under an atmosphere of air, and stirred at rt overnight. The solvent was removed and the residue was subjected to flash column chromatography (Hexane:EtOAc, 3:1 → 2:1) to yield **4** (0.39 g, 55% over two steps) as a white solid.

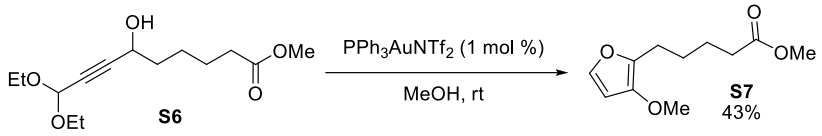
**4**: Rf (Hexane:EtOAc, 7:3): 0.27; $^1$H NMR (400 MHz, $CDCl_3$) δ 4.00 (s, 2 H), 2.89 (t, *J* = 6.7 Hz, 2 H), 2.85 (br.s., 4 H), 2.67 (t, *J* = 7.8 Hz, 2 H), 1.77 (s, 6 H), 1.74 (s, 6 H), 0.95 (s, 3 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) δ 170.7, 168.9, 167.6, 138.4, 135.0, 68.2, 55.3, 28.6, 26.2, 25.5, 16.8, 11.0, 10.1 ppm; IR (ATR) 2973, 2935, 1815, 1782, 1729, 1208, 1089, 1069, 967 cm$^{-1}$; HRMS (EI) Exact mass cald. for $C_{19}H_{25}NO_6$ $[M]^+$: 363.1682, found: 363.1676.




**Methyl 9,9-diethoxy-6-hydroxynon-7-ynoate** (**S6**):
3,3-Diethoxyprop-1-yne (0.72 mL, 5.0 mmol, 1 equiv) was added to THF (15 mL) then cooled to -78 °C. nBuLi (2.33 M in hexanes, 2.4 mL, 5.5 mmol, 1.1 equiv) was added dropwise then the reaction mixture stirred a further 30 min at -78 °C. Methyl 6-oxohexanoate (0.87 g, 6.0 mmol, 1.2 equiv) dissolved in THF (5 mL) was added dropwise, then the reaction mixture stirred at -78 °C for 1 h. The reaction mixture was poured into a separatory funnel containing a saturated aqueous solution of sodium bicarbonate (100 mL) then extracted with $Et_2O$ (2 x 50 mL). The combined organic layers were washed with brine (50 mL), dried over $MgSO_4$, filtered, the solvent removed,

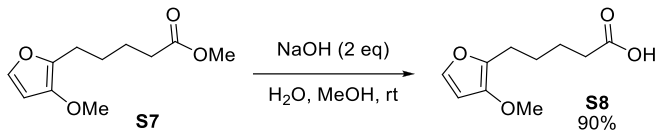
and the residue subjected to flash column chromatography (Hexane:EtOAc, 2:1) to yield **S6** (1.1 g, 80%) as a clear and colorless oil.

**S6**: Rf (Hexane:EtOAc, 6:4): 0.41; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.28 (d, *J* = 1.6 Hz, 1 H), 4.47 - 4.36 (m, *J* = 3.5 Hz, 1 H), 3.76 - 3.67 (m, 2 H), 3.67 - 3.63 (m, 3 H), 3.56 (qd, *J* = 7.0, 9.4 Hz, 2 H), 2.34 - 2.27 (m, 3 H), 1.76 - 1.59 (m, 4 H), 1.54 - 1.42 (m, 2 H), 1.21 (t, *J* = 7.0 Hz, 6 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.0, 91.2, 86.2, 80.0, 61.8, 60.8, 60.8, 51.5, 36.9, 33.8, 24.6, 24.4, 15.0 ppm; IR (ATR) 3451, 2932, 1736, 1437, 1328, 1135, 1051, 1012 cm$^{-1}$; HRMS (EI) Exact mass cald. for $C_{14}H_{23}O_5$ $[M-H]^+$: 271.1545, found: 271.1546.



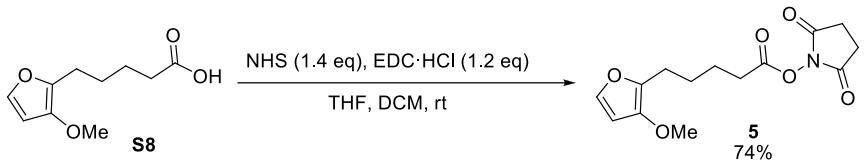

**Methyl 5-(3-methoxyfuran-2-yl)pentanoate** (**S7**):
MeOH (3.9 mL) was added to a vial containing **S6** (1.06 g, 3.89 mmol, 1 equiv). $PPh_3AuNTf_2$ (29 mg, 0.039 mmol, 0.01 equiv) was added, the reaction capped under an atmosphere of air, and stirred at rt overnight. The reaction mixture was poured into a separatory funnel containing brine (50 mL) then extracted with DCM (2 x 50 mL). The combined organic layers were washed with brine (50 mL), dried over $Na_2SO_4$, filtered, the solvent removed, and the residue subjected to flash column chromatography (Hexane:EtOAc, 15:1 → 9:1) to yield **S7** (0.35 g, 43%) as a clear and colorless oil.

**S7**: Rf (Hexane:EtOAc, 9:1): 0.35; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.11 (d, *J* = 2.0 Hz, 1 H), 6.27 (d, *J* = 2.0 Hz, 1 H), 3.72 (s, 3 H), 3.66 (s, 3 H), 2.61 (t, *J* = 6.8 Hz, 2 H), 2.33 (t, *J* = 7.2 Hz, 2 H), 1.69 - 1.60 (m, 4 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.1, 143.3, 139.2, 138.9, 102.9, 59.4, 51.4, 33.7, 27.5, 24.5, 24.3 ppm; IR (ATR) 2950, 1734, 1662, 1600, 1230, 1179, 1111 cm$^{-1}$; HRMS (EI) Exact mass cald. for $C_{11}H_{16}O_4$ $[M]^+$: 212.1049, found: 212.1045.



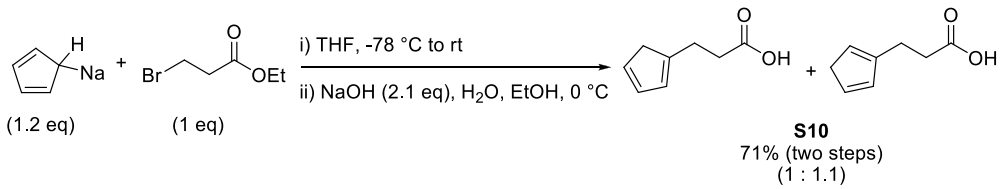

**5-(3-Methoxyfuran-2-yl)pentanoic acid** (**S8**):
To a vial containing **S7** (0.331 g, 1.56 mmol, 1 equiv) dissolved in MeOH (4 mL) was added a solution of NaOH (0.125 g, 3.12 mmol, 2 equiv) in $H_2O$ (4 mL). The reaction was capped under an atmosphere of air, and stirred at rt for 30 min. The reaction mixture was poured into a separatory funnel containing $H_2O$ (50 mL), and HCl (1 M in $H_2O$) was added to pH 2-3 (~4 mL). The aqueous layer was extracted with DCM (2 x 50 mL). The combined organic layers were washed with brine (50 mL), dried over $Na_2SO_4$, filtered, the solvent removed to yield **S8** (0.280 g, 90%) as a clear and colorless oil.

**S8**: Rf (Hexane:EtOAc, 1:1): 0.55; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 10.37 (br.s., 1 H), 7.12 (d, *J* = 2.0 Hz, 1 H), 6.28 (d, *J* = 2.0 Hz, 1 H), 3.73 (s, 3 H), 2.62 (t, *J* = 6.5 Hz, 2 H), 2.38 (t, *J* = 6.5 Hz, 2 H), 1.73 - 1.61 (m, 4 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) δ 179.8, 143.4, 139.1, 139.0, 102.9, 59.4, 33.7, 27.4, 24.4, 24.0 ppm; IR (ATR) 3133, 2940, 1706, 1662, 1454, 1411, 1279, 1236, 1109 $cm^{-1}$; HRMS (EI) Exact mass cald. for $C_{10}H_{14}O_4$ $[M]^+$: 198.0892, found: 198.0890.




**2,5-Dioxopyrrolidin-1-yl 5-(3-methoxyfuran-2-yl)pentanoate** (**5**):
THF (5 mL) was added to a vial containing **S8** (0.265 g, 1.34 mmol, 1 equiv). *N*-hydroxysuccinimide (0.216 g, 1.87 mmol, 1.4 equiv), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.308 g, 1.61 mmol, 1.2 equiv) and DCM (3 mL) were added, the reaction capped under an atmosphere of air, and stirred at rt overnight. The solvent was removed and the residue was subjected to flash column chromatography (Hexane:EtOAc, 2:1 → 1:1) to yield **5** (0.293 g, 74%) as a colourless, viscous oil.

**5**: Rf (Hexane:EtOAc, 2:1): 0.33; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.11 (d, *J* = 2.0 Hz, 1 H), 6.27 (d, *J* = 2.0 Hz, 1 H), 3.72 (s, 3 H), 2.82 (br.s., 4 H), 2.69 - 2.54 (m, 4 H), 1.81 - 1.60 (m, 4 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) δ 169.1, 168.5, 143.5, 139.1, 138.7, 102.8, 59.3, 30.5, 27.0, 25.5, 24.2, 23.8 ppm; IR (ATR) 2948, 1814, 1735, 1638, 1413, 1206, 1058, 1046 $cm^{-1}$; HRMS (EI) Exact mass cald. for $C_{14}H_{17}NO_6$ $[M]^+$: 295.1056, found: 295.1062.

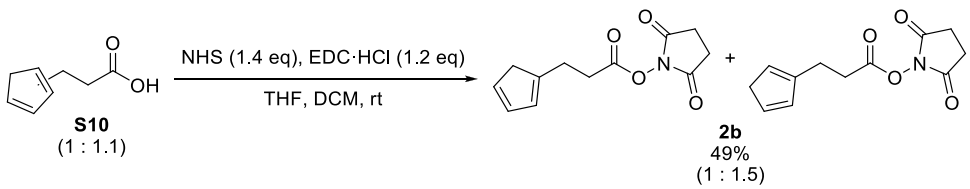



**3-(Cyclopenta-1,3-dienyl)propanoic acid & 3-(cyclopenta-1,4-dienyl)propanoic acid** (**S10**):
Sodium cyclopentadienide (2 M solution in THF, 30 mL, 60 mmol, 1.2 equiv) was added to THF (100 mL) and cooled to -78 °C. Ethyl 3-bromopropionate (6.41 mL, 50 mmol, 1 equiv) was added dropwise and the reaction was stirred at -78 °C for 3 h, removed from the cooling bath, and stirred a further 1 h. Water (6 mL) and silica gel (6 g) were added and the suspension stirred 5 min. The reaction mixture was filtered through silica gel with DCM (50 mL) and the solvent removed to yield **S9** as a yellow oil which was used directly in the next reaction. Spectral data matched that of literature reported data.[4]

**S9**: Rf (Hexane:EtOAc, 9:1): 0.45; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.47 - 6.02 (m, 3 H), 4.17 - 4.11 (m, 2 H), 2.96 (s, 0.31 H), 2.91 (d, *J* = 1.4 Hz, 1.69 H), 2.78 - 2.68 (m, *J* = 1.7 Hz, 2 H), 2.59 - 2.53 (m, 2 H), 1.26 (t, *J* = 7.1 Hz, 3 H).

A solution of **S9** (~50 mmol) dissolved in EtOH (36 mL) was cooled to 0 °C. A solution of NaOH (3.63 g, 90.72 mmol, 2.1 equiv) in $H_2O$ (36 mL) was added and the reaction stirred at 0 °C for 1.5 h. The reaction mixture was poured into a separatory funnel containing HCl (1 M in $H_2O$, 100 mL) and extracted with DCM (3 x 50 mL). The combined organic layers were washed with brine (50 mL), dried over $Na_2SO_4$, filtered, the solvent removed to yield **S10** (4.90 g, 71% two steps) as a yellow solid that decomposes at room temperature.

**S10**: Rf (Hexane:EtOAc, 1:2): 0.69; $^1$H NMR (400 MHz, $CDCl_3$) δ 10.57 (br.s., 1 H), 6.49 - 6.02 (m, 3 H), 2.97 (d, *J* = 1.6 Hz, 1.07 H), 2.92 (d, *J* = 1.2 Hz, 0.93 H), 2.82 - 2.68 (m, 2 H), 2.68 - 2.58 (m, 2 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) δ 179.7, 179.7, 147.1, 144.9, 134.2, 134.1, 132.3, 131.1, 127.0, 126.4, 43.3, 41.3, 33.9, 33.3, 25.5, 24.7 ppm; IR (ATR) 3070, 2926, 1705, 1412, 1283, 1205, 913 cm$^{-1}$; HRMS (EI) Exact mass cald. for $C_8H_{10}NO_2$ $[M]^+$: 138.0681, found: 138.0678.




**2,5-Dioxopyrrolidin-1-yl 3-(cyclopenta-1,3-dienyl)propanoate & 2,5-dioxopyrrolidin-1-yl 3-(cyclopenta-1,4-dienyl)propanoate** (**2b**):
**S10** (4.90 g, 35.5 mmol, 1 equiv) was dissolved in THF (50 mL). *N*-hydroxysuccinimide (5.71 g, 49.7 mmol, 1.4 equiv), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (8.17 g, 42.6 mmol, 1.2 equiv) and DCM (50 mL) were added, the reaction capped under an atmosphere of air, and stirred 2 h. The reaction mixture was filtered through a silica plug with DCM (100 mL) and the solvent removed. The residue was subjected to flash column chromatography (Hexane:EtOAc, 2:1 → 1:1) to yield **2b** (4.00 g, 49%) as an eggshell powder that must be stored in the freezer to prevent dimerization.

**2b**: Rf (Hexane:EtOAc, 2:1): 0.29; $^1$H NMR (400 MHz, $CDCl_3$) δ 6.47 - 6.08 (m, 3 H), 2.97 (d, *J* = 1.2 Hz, 1.2 H), 2.92 (d, *J* = 1.6 Hz, 0.8 H), 2.90 - 2.75 (m, 8 H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 169.1, 168.2, 168.1, 145.7, 143.9, 134.4, 133.8, 132.2, 131.4, 127.7, 127.1, 43.2, 41.4, 30.8, 30.2, 25.5, 25.3, 24.5; IR (ATR) 2947, 1810, 1779, 1735, 1420, 1366, 1204, 1062, 1046 cm$^{-1}$; HRMS (EI) Exact mass cald. for $C_{12}H_{13}NO_4$ $[M]^+$: 235.0845, found: 235.0848.

**3. Kinetic experiments.**

Dienes **1**, **2b**, **3**, **4**, or **5** (1 equiv) and **NEM** (*N*-ethylmaleimide, 1 equiv) in $CDCl_3$ were combined in an NMR tube (final concentration 0.05 M for **1**, 0.01 M for **2b**, **3**, **4**, and **5**) and monitored by $^1H$ NMR at room temperature. [A] = concentration of diene = [**NEM**] was calculated using the integration of **NEM**'s ethyl peaks (3.59 or 1.20 ppm) and those of the product (typically 4.40 or 1.05 ppm).

[A] = Initial Concentration * (integration of starting material) / (integration of starting material + product)

The inverse concentration (1 / [A]) was plotted against time (s). The second order reaction rate ($M^{-1}s^{-1}$) was obtained from the slope of the line of best fit. The average and standard deviation of three experiments was used.

**Figure S1.** Kinetic analysis of the reaction of **1** and **NEM**:

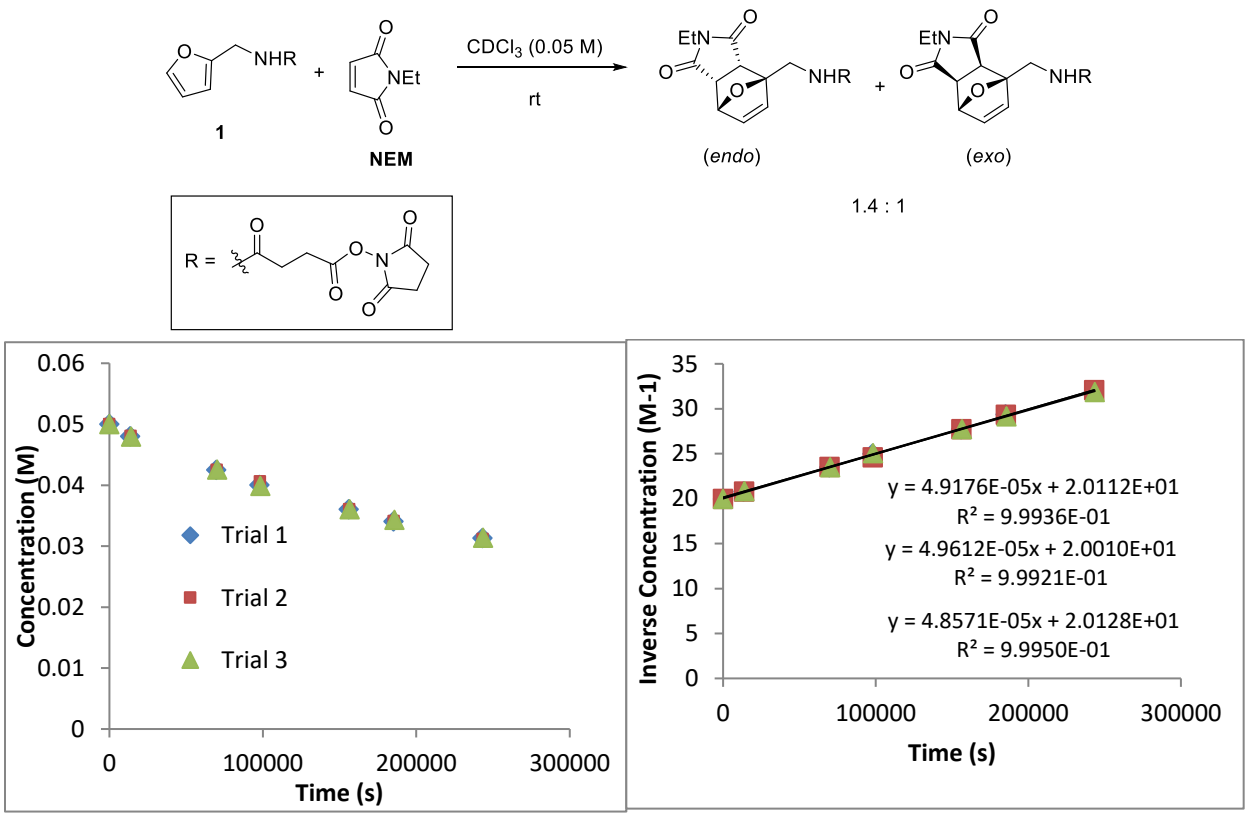
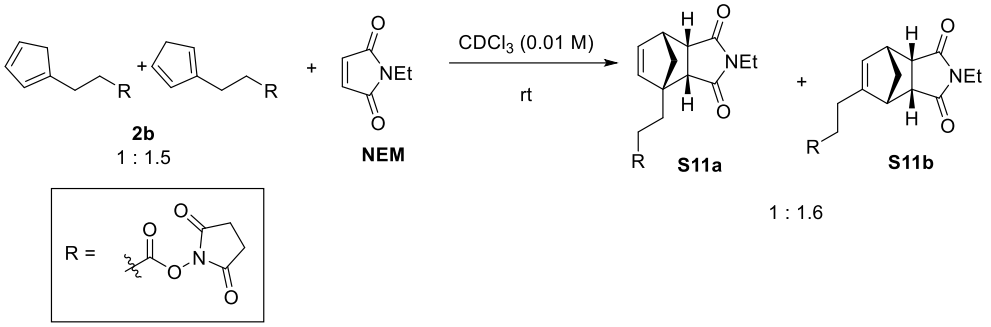


**Figure S2.** Kinetic analysis of the reaction of **2b** and **NEM**:



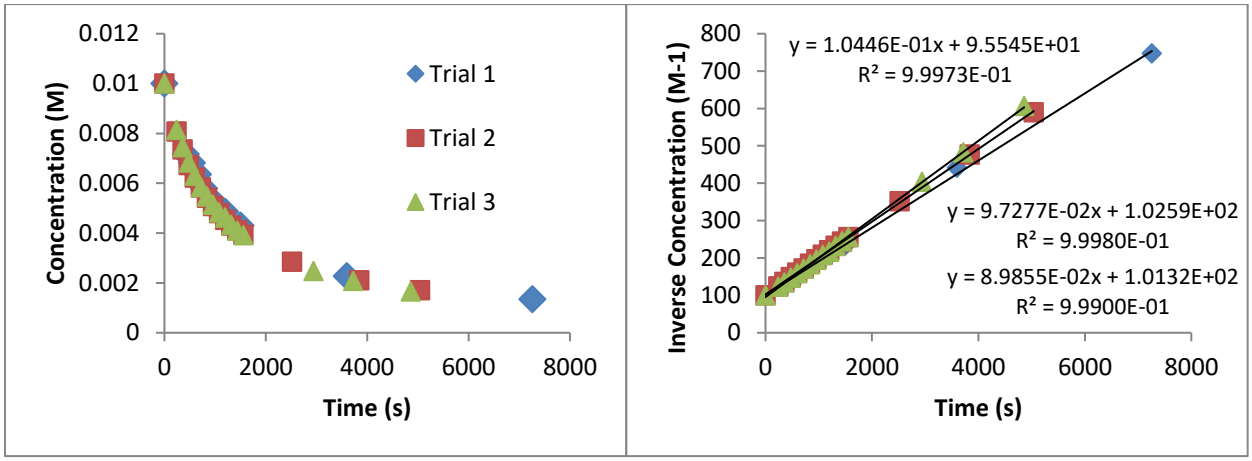
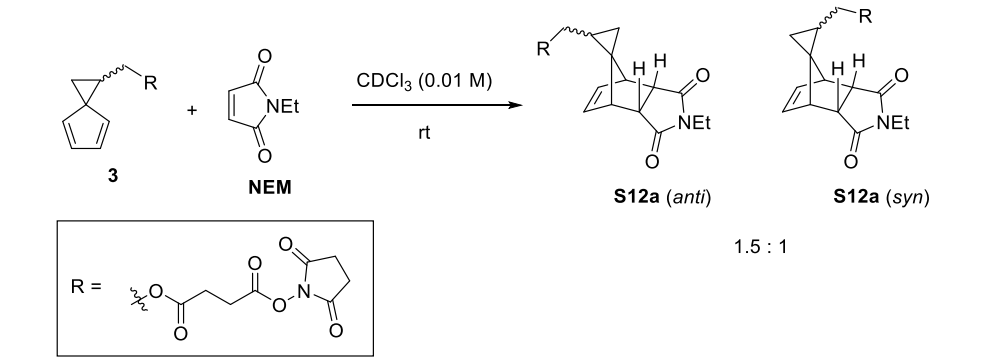




**Figure S3.** Kinetic analysis of the reaction of **3** and **NEM**:



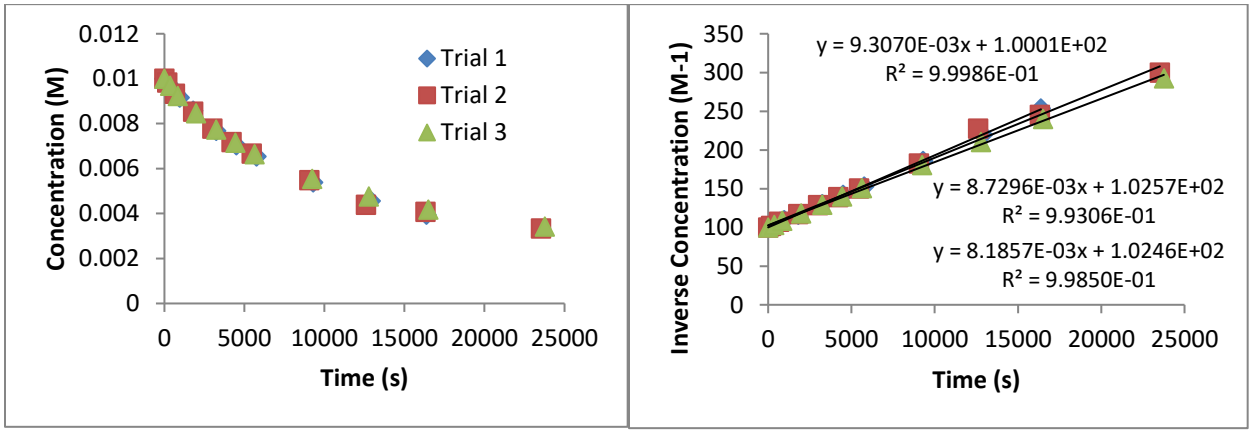



**Figure S4.** Kinetic analysis of the reaction of **4** and **NEM**:

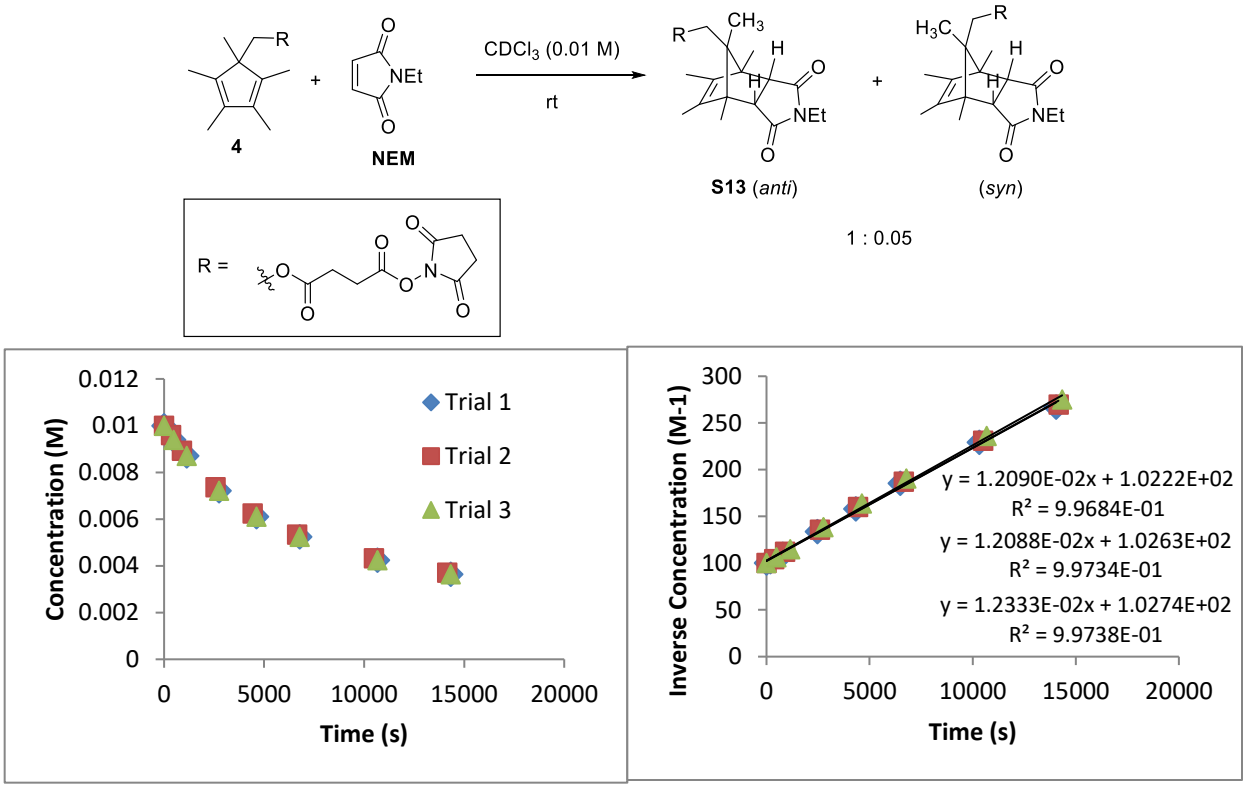
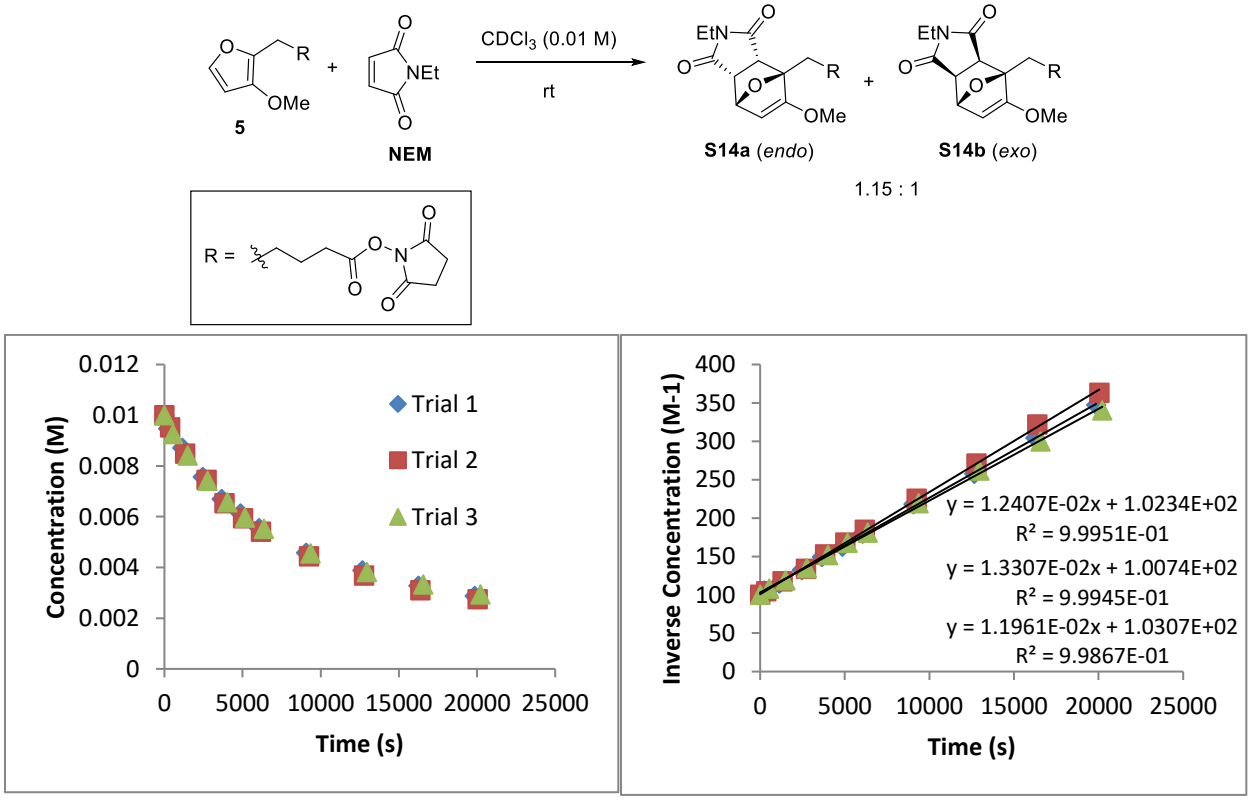
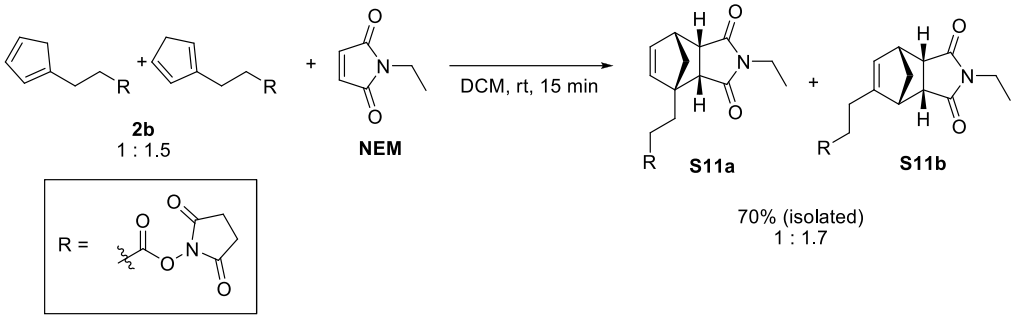



**Figure S5.** Kinetic analysis of the reaction of **5** and **NEM**



**4. Characterization of Diels-Alder Adducts.**

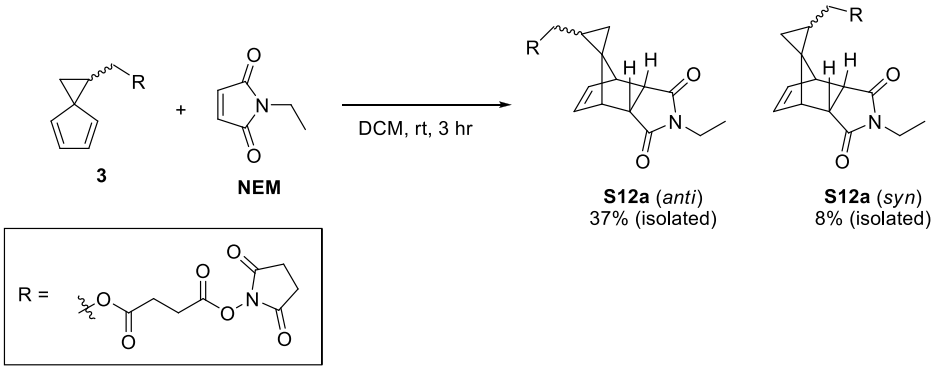
Note: The conversion for the DA reaction is high, but significant amount of material is not recovered due to hydrolysis of the NHS-ester on silica gel. For a more accurate representation of the *endo*/*exo* and *syn*/*anti* ratios see **Figures S1** to **S5**.




***Endo* Diels–Alder adducts of 2,5-Dioxopyrrolidin-1-yl 3-(cyclopenta-1,3-dienyl)propanoate (S11a) & 2,5-dioxopyrrolidin-1-yl 3-(cyclopenta-1,4-dienyl)propanoate (S11b)**:

To a solution of **2b** (105 mg, 0.446 mmol, 1 equiv) dissolved in DCM (5 mL) was added **NEM** (75 mg, 0.60 mmol, 1.3 equiv) and the solution was stirred for 15 min. The solvent was removed and the residue was subjected to flash column chromatography (Hexane:EtOAc, 1:2) to yield **S11a** and **S11b** (1:1.7, 112 mg, 70%) as a white foam.

**S11a** & **S11b**: Rf (Hexane:EtOAc, 1:2): 0.33; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.11 (dd, *J* = 2.2, 5.7 Hz, 0.38 H, **S11a**), 5.92 (d, *J* = 5.9 Hz, 0.38 H, **S11a**), 5.73 (d, *J* = 1.2 Hz, 0.62 H, **S11b**), 3.41 - 2.54 (m, 12 H, **S11a** & **S11b**), 2.53 - 2.36 (m, 1.24 H, **S11b**), 2.28 (ddd, *J* = 6.5, 8.9, 14.8 Hz, 0.38 H, **S11a**), 1.79 (d, *J* = 8.6 Hz, 0.62 H, **S11b**), 1.67 (d, *J* = 8.6 Hz, 0.38 H, **S11a**), 1.52 (d, *J* = 9.0 Hz, 0.62 H, **S11b**), 1.44 (d, *J* = 8.6 Hz, 0.38 H, **S11a**), 1.01 (t, *J* = 7.2 Hz, 3 H, **S11a** & **S11b**); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 177.5, 177.2, 177.1, 176.8, 169.1, 169.0, 168.3, 167.6, 146.7, 136.1, 135.4, 126.9, 57.5, 55.0, 52.4, 48.8, 47.8, 47.4, 46.8, 45.7, 45.3, 44.7, 33.2, 33.1, 28.7, 27.9, 26.2, 25.5, 25.5, 25.3, 13.0, 13.0; IR (ATR) 2984, 2941, 1812, 1780, 1727, 1684, 1443, 1399, 1339, 1208, 1139, 1064 $cm^{-1}$; HRMS (CI) Exact mass cald. for $C_{18}H_{21}N_2O_6$ $[M+H]^+$: 361.1400, found: 361.1398.



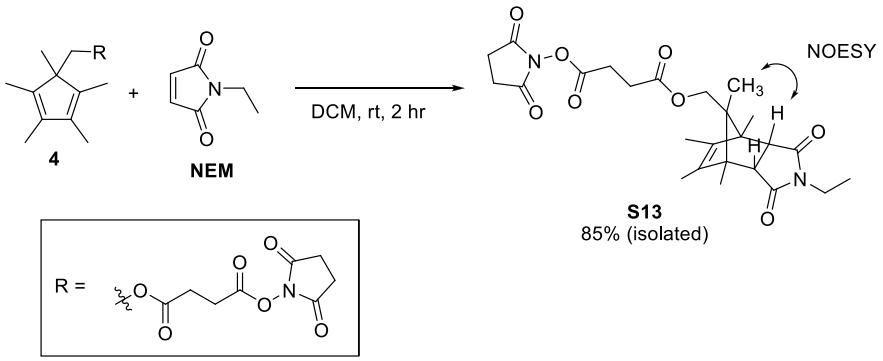

***Endo* Diels–Alder adducts of 2,5-Dioxopyrrolidin-1-yl spiro[2.4]hepta-4,6-dien-1-ylmethyl succinate** (**S12a**, *anti*) **&** (**S12b**, *syn*):

To a solution of **3** (116 mg, 0.364 mmol, 1 equiv) dissolved in DCM (5 mL) was added **NEM** (64 mg, 0.51 mmol, 1.4 equiv) and the solution was stirred for 3 h. The solvent was removed and the residue was subjected to flash column chromatography (Hexane:EtOAc, 1:2 → 1:3) to yield **S12a** (59.9 mg, 37%) and **S12b** (13.3 mg, 8%) as a white foams.

The isomers were assigned through the $^{13}$C NMR shift of the cyclopropane methylene (*syn* = 10.2 ppm, *anti* = 12.5 ppm).[5]

**S12a**: Rf (Hexane:EtOAc, 1:2): 0.26; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.15 (t, *J* = 2.0 Hz, 2 H), 4.11 (dd, *J* = 7.2, 11.5 Hz, 1 H), 3.87 (dd, *J* = 8.2, 11.3 Hz, 1 H), 3.39 (q, *J* = 7.0 Hz, 2 H), 3.36 - 3.33 (m, 2 H), 3.04 - 2.98 (m, 1 H), 2.97 - 2.88 (m, 2 H), 2.84 (s, 4 H), 2.80 - 2.75 (m, 1 H), 2.70 (t, *J* = 6.7 Hz, 2 H), 1.38 (dq, *J* = 5.3, 8.0 Hz, 1 H), 1.04 (t, *J* = 7.2 Hz, 3 H), 0.76 (dd, *J* = 5.9, 9.0 Hz, 1 H), 0.47 (t, *J* = 5.5 Hz, 1 H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 177.0, 176.9, 170.7, 168.9, 167.6, 134.3, 133.9, 66.3, 52.3, 50.3, 46.2, 45.7, 45.3, 33.3, 28.7, 26.3, 25.5, 17.8, 13.0, 12.5; IR (ATR) 2991, 2954, 1820, 1783, 1731, 1686, 1399, 1375, 1346, 1203, 1137, 1088, 1066 cm$^{-1}$; HRMS (CI) Exact mass cald. for $C_{22}H_{25}N_2O_8$ $[M+H]^+$: 445.1611, found: 445.1630.

**S12b**: Rf (Hexane:EtOAc, 2:1): 0.39; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.21 - 6.13 (m, 2 H), 4.42 (dd, *J* = 5.5, 11.7 Hz, 1 H), 3.67 (dd, *J* = 9.8, 11.7 Hz, 1 H), 3.48 - 3.30 (m, 4 H), 3.06 - 3.02 (m, 1 H), 2.96 (dt, *J* = 3.7, 6.6 Hz, 2 H), 2.86 (br.s., 4 H), 2.79 - 2.72 (m, 3 H), 1.35 - 1.29 (m, 1 H), 1.05 (t, *J* = 7.0 Hz, 3 H), 0.82 (dd, *J* = 6.1, 9.2 Hz, 1 H), 0.47 (t, *J* = 5.5 Hz, 1 H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 177.2, 170.8, 169.0, 167.6, 134.0, 133.9, 77.2, 66.2, 52.6, 50.4, 46.2, 45.8, 45.6, 33.3, 28.9, 26.5, 25.6, 19.0, 13.0, 10.2; IR (ATR) 2986, 2941, 1815, 1783, 1732, 1687, 1399, 1375, 1351, 1203, 1137, 1068 cm$^{-1}$; HRMS (CI) Exact mass cald. for $C_{22}H_{25}N_2O_8$ $[M+H]^+$: 445.1611, found: 445.1631.

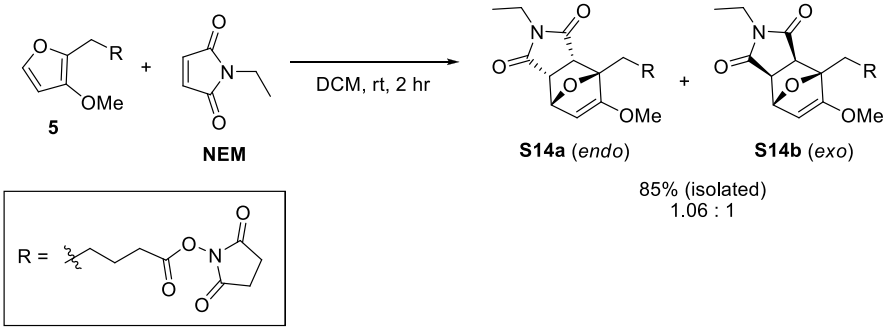



***Endo* Diels–Alder adduct of 2,5-Dioxopyrrolidin-1-yl (1,2,3,4,5-pentamethylcyclopenta-2,4-dienyl)methyl succinate** (**S13**, *anti*):

To a solution of **4** (108 mg, 0.297 mmol, 1 equiv) dissolved in DCM (5 mL) was added **NEM** (52 mg, 0.42 mmol, 1.4 equiv) and the solution was stirred for 2 h. The solvent was removed and the residue was subjected to flash column chromatography (Hexane:EtOAc, 2:3) to yield **S13** (72.7 mg, 50%) as a white foam.

The *anti* configuration of the Diels-Alder adduct was assigned through the NOESY signal of the methyl group indicated.[6]

**S13**: Rf (Hexane:EtOAc, 1:1): 0.21; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 3.97 (s, 2 H), 3.35 (q, *J* = 7.2 Hz, 2 H), 2.94 (t, *J* = 7.0 Hz, 2 H), 2.91 (s, 2 H), 2.84 (s, 4 H), 2.72 (t, *J* = 7.0 Hz, 2 H), 1.52 (s, 6 H), 1.30 (s, 6 H), 0.98 (t, *J* = 7.0 Hz, 3 H), 0.88 (s, 3 H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 177.2, 170.9, 168.8, 167.6, 135.1, 77.2, 67.4, 66.4, 59.1, 51.1, 32.8, 28.7, 26.2, 25.5, 13.1, 12.6, 12.4, 11.3; IR (ATR) 2965, 2938, 1815, 1785, 1733, 1686, 1441, 1401, 1380, 1347, 1224, 1202, 1142, 1088, 1068 cm$^{-1}$; HRMS (CI) Exact mass cald. for $C_{25}H_{33}N_2O_8$ $[M+H]^+$: 489.2237, found: 489.2239.




**Diels–Alder adducts of 2,5-dioxopyrrolidin-1-yl 5-(3-methoxyfuran-2-yl)pentanoate** (**S14a**, *endo*) **&** (**S14b**, *exo*):

To a solution of **5** (100. mg, 0.339 mmol, 1 equiv) dissolved in DCM (5 mL) was added **NEM** (59 mg, 0.47 mmol, 1.4 equiv) and the solution was stirred for 2 h. The solvent was removed and the residue was subjected to flash column chromatography (Hexane:EtOAc, 1:2) to yield **S14a** and **S14b** (1.06:1, 121 mg, 85%) as a white foam.

The *endo* and *exo* isomers were assigned through analogy with Sheppard's work.[7]

**S14a** & **S14b**: Rf (Hexane:EtOAc, 1:1): 0.33; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.15 - 5.09 (m, 3 H), 4.99 (d, *J* = 2.0 Hz, 1 H, **S14a**), 3.66 - 3.60 (m, 4 H), 3.54 - 3.46 (m, 5 H), 3.39 (q, *J* = 7.0 Hz, 2 H, **S14a**), 3.17 (d, *J* = 7.8 Hz, 1 H, **S14a**), 3.07 (d, *J* = 6.7 Hz, 1 H, **S14b**), 2.86 - 2.78 (m, 8 H), 2.62 (q, *J* = 7.4 Hz, 4 H), 2.31 - 2.20 (m, 1 H), 2.11 - 1.53 (m, 12 H), 1.13 (t, *J* = 7.2 Hz, 3 H, **S14b**), 1.06 (t, *J* = 7.0 Hz, 3 H, **S14a**); $^{13}$C NMR (100 MHz, $CDCl_3$) δ; IR (ATR) 2942, 1813, 1783, 1733, 1689, 1627, 1442, 1401, 1347, 1203, 1135, 1065 $cm^{-1}$; HRMS (CI) Exact mass cald. for $C_{12}H_{13}NO_4$ $[M+H]^+$: 421.1611, found: 421.1607.

## 5. $^{1}$H and $^{13}$C NMR Spectra.

**Figure S6.** $^{1}$H NMR (400 MHz, $CDCl_3$) **1**

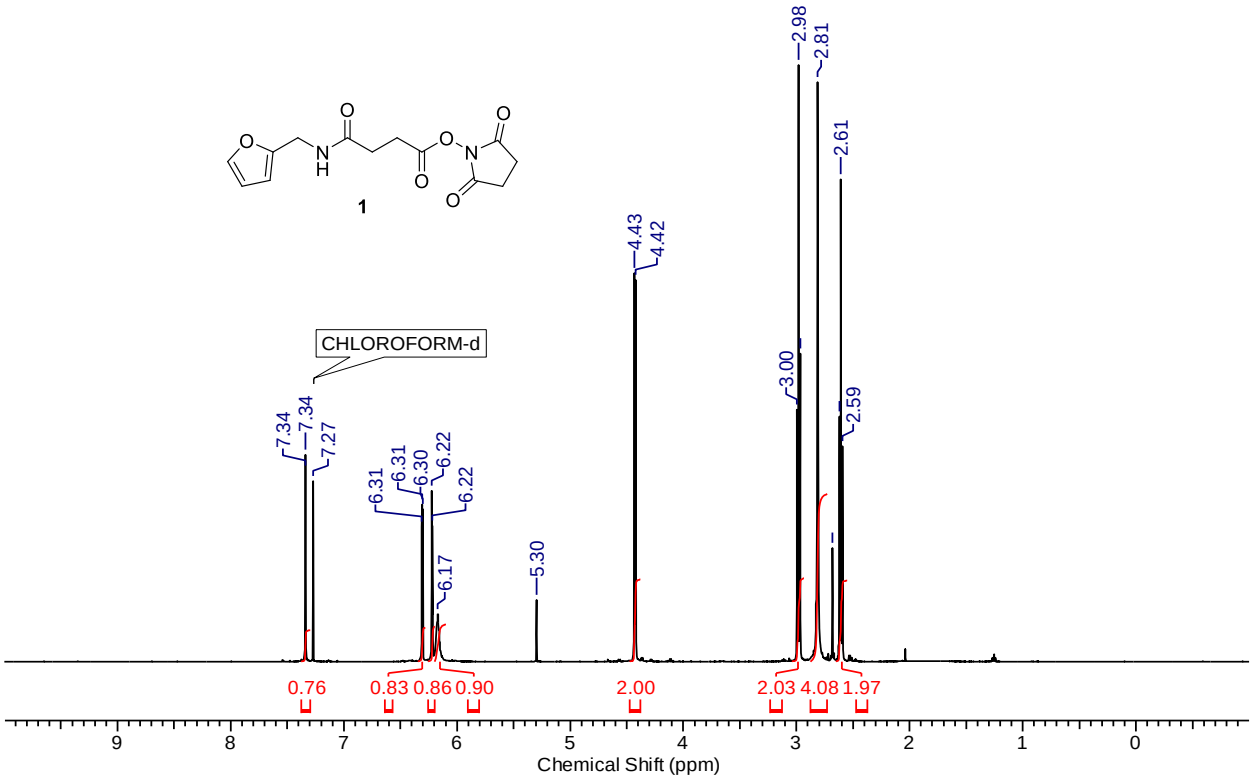



**Figure S7.** $^{13}$C NMR (100 MHz, $CDCl_3$) **1**

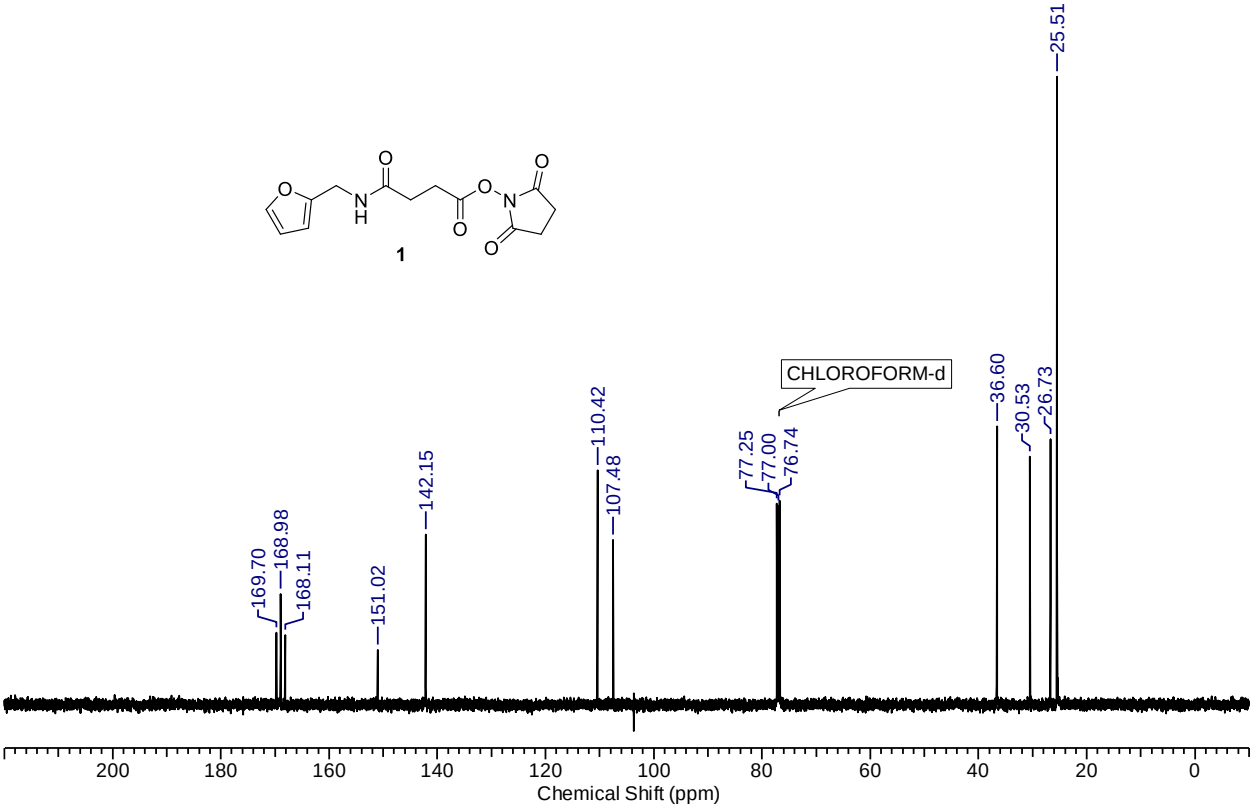


**Figure S8.** $^{1}$H NMR (400 MHz, $CDCl_3$) **S1**

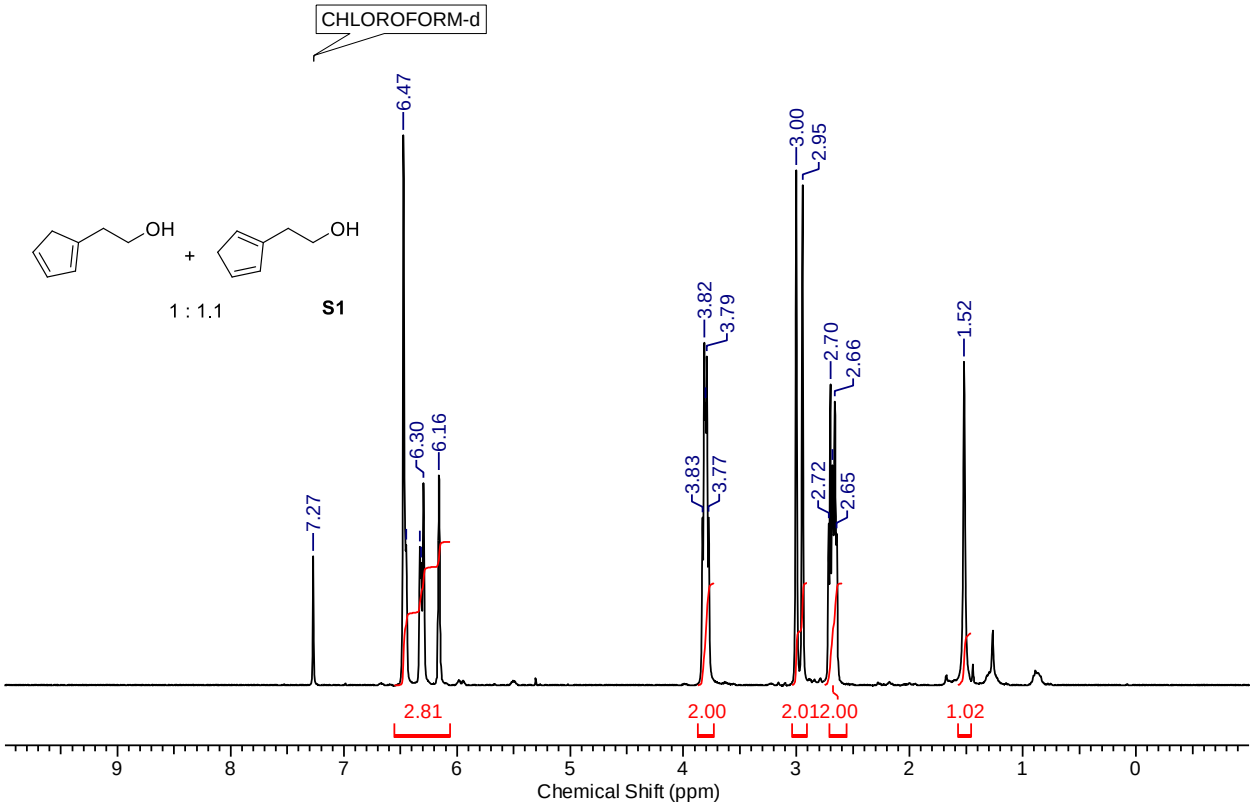



**Figure S9.** $^{13}$C NMR (100 MHz, $CDCl_3$) **S1**

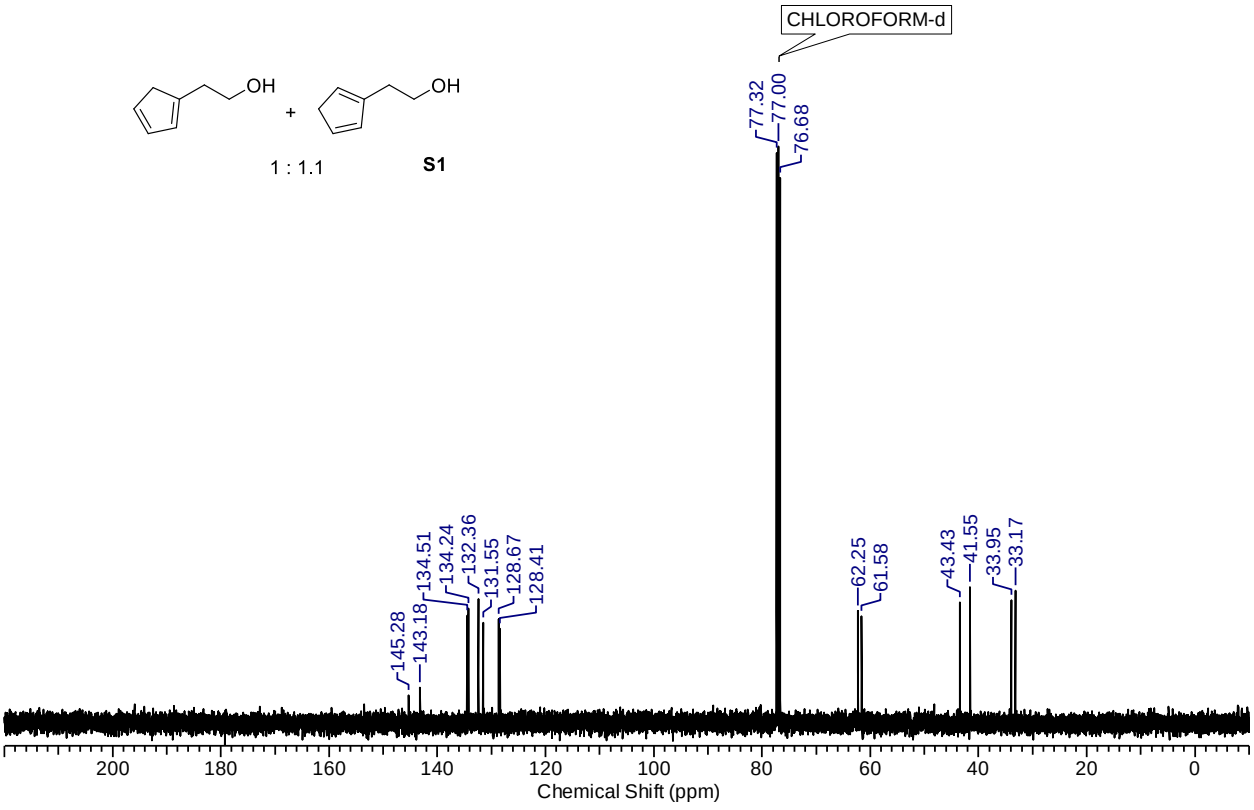


**Figure S10.** $^{1}$H NMR (400 MHz, $CDCl_3$) **S2**

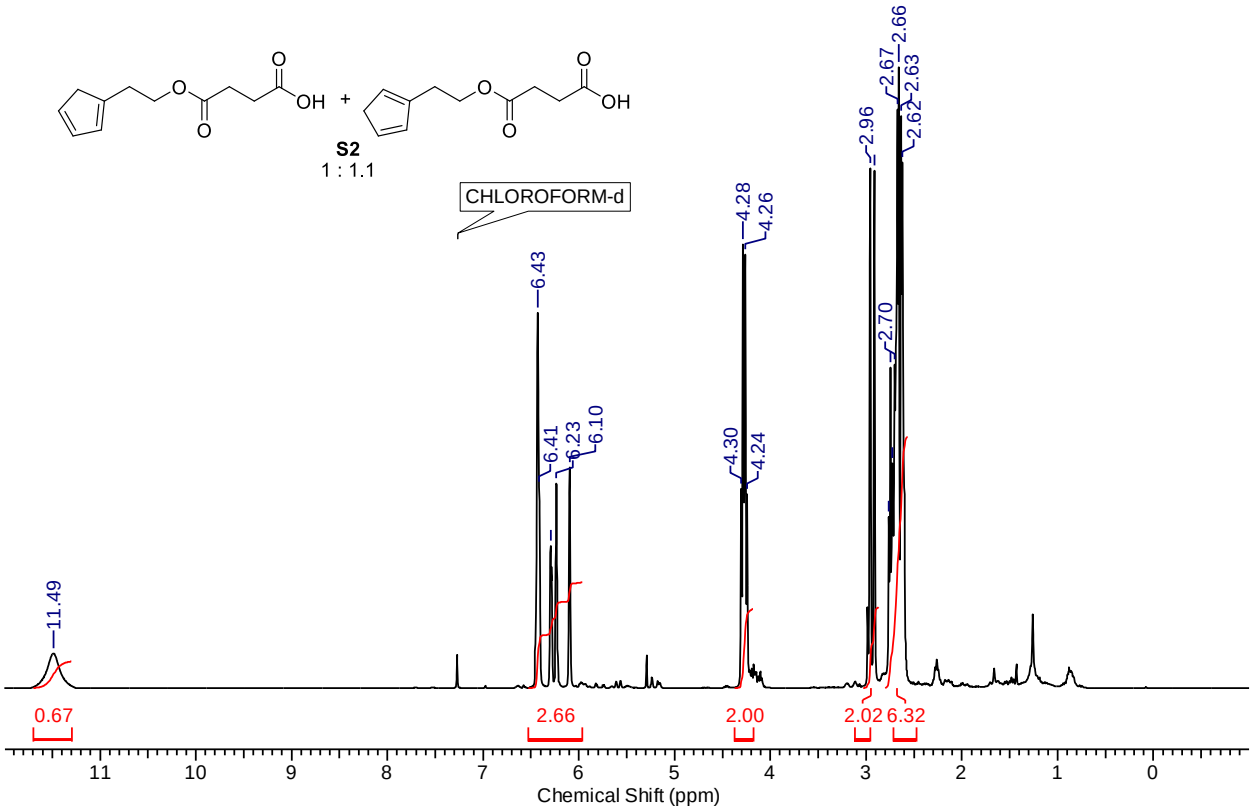



**Figure S11.** $^{13}$C NMR (100 MHz, $CDCl_3$) **S2**

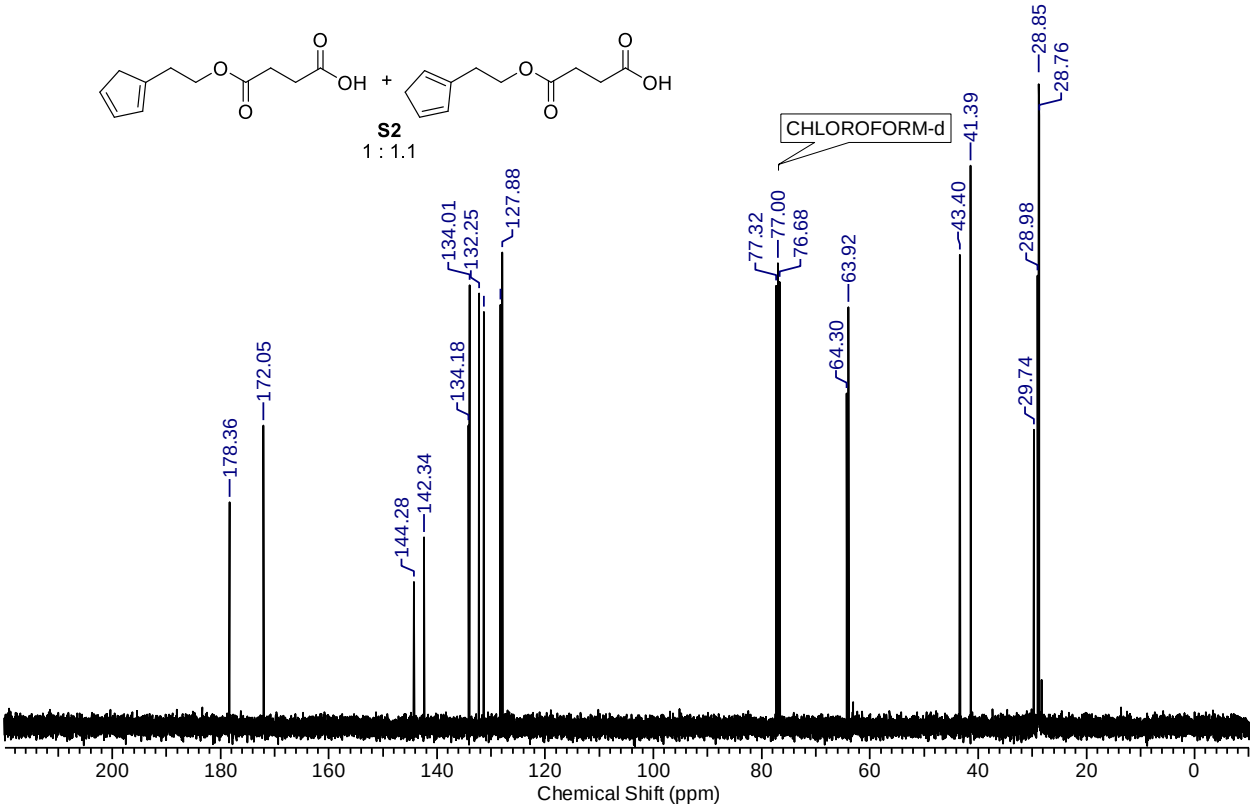


**Figure S12.** $^{1}$H NMR (400 MHz, $CDCl_3$) **2a**

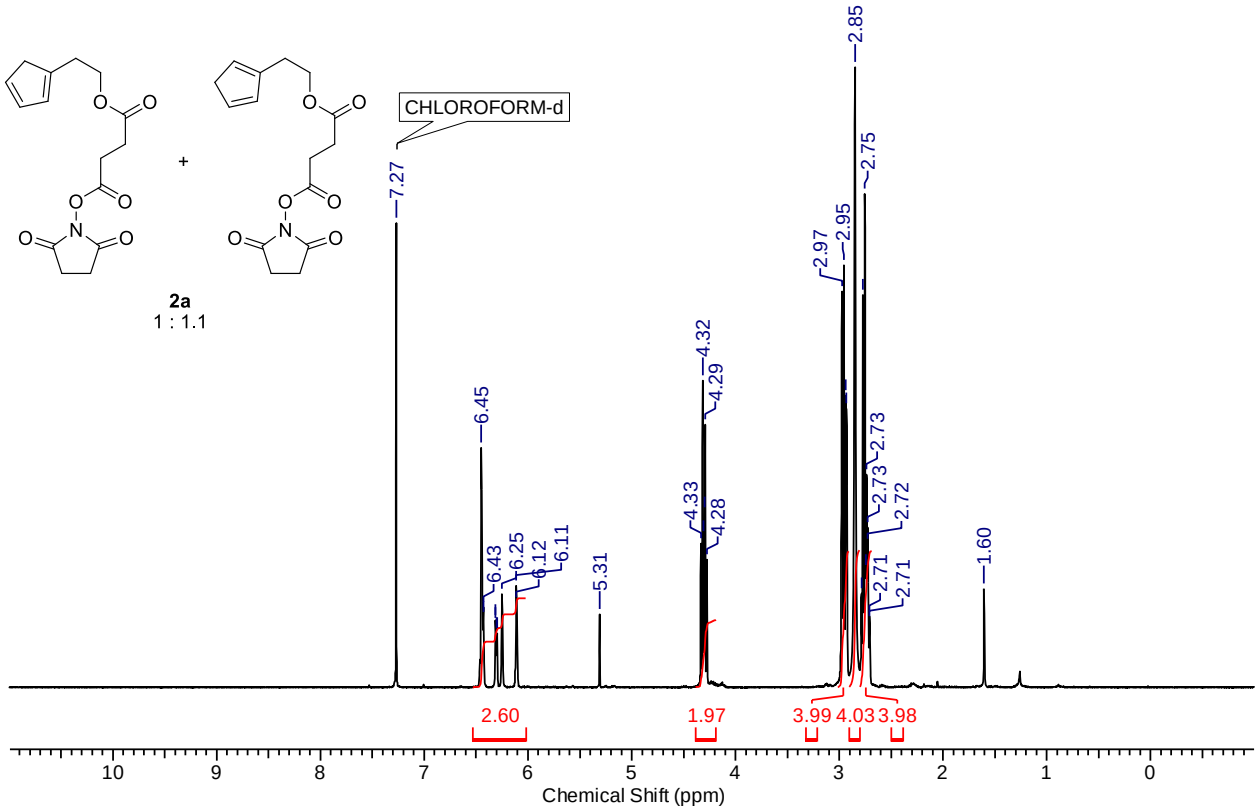



**Figure S13.** $^{13}$C NMR (125 MHz, $CDCl_3$) **2a**

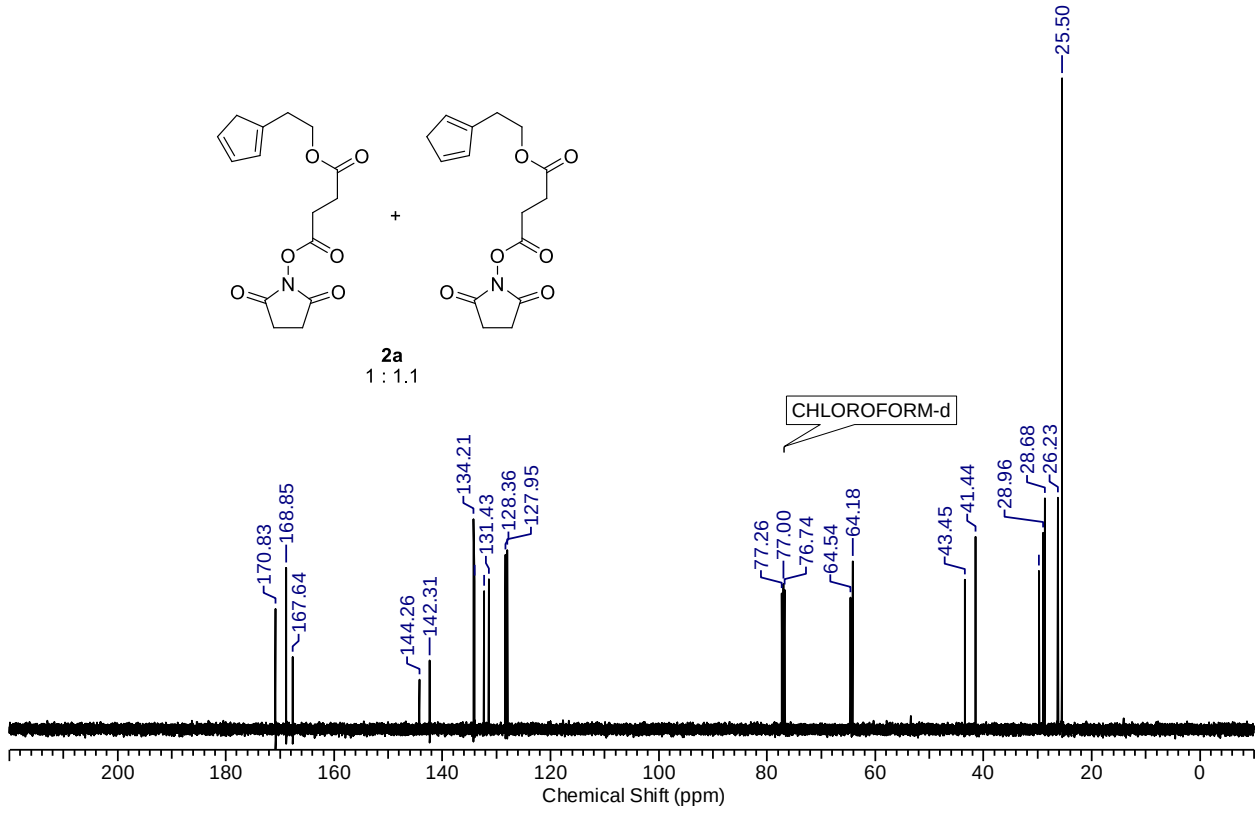


**Figure S14.** $^{1}$H NMR (500 MHz, $CDCl_3$) **S3**

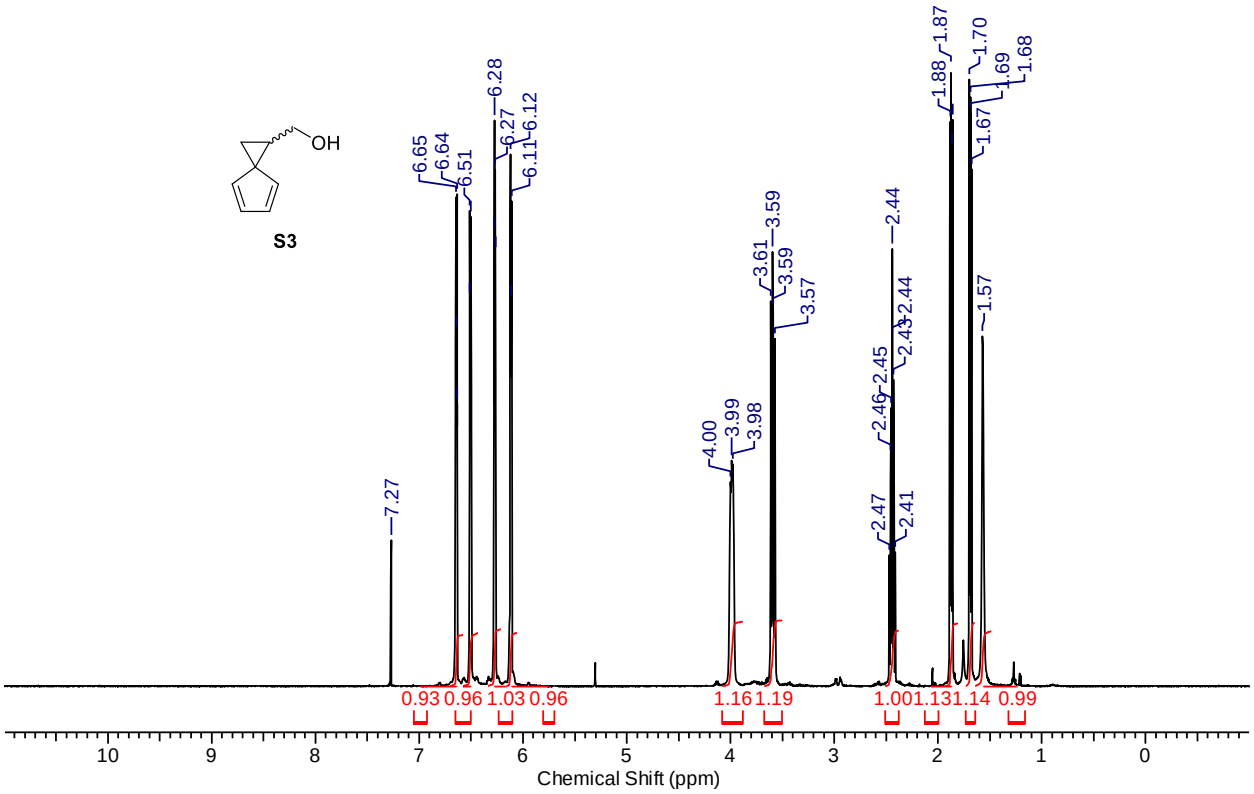



**Figure S15.** $^{13}$C NMR (125 MHz, $CDCl_3$) **S3**

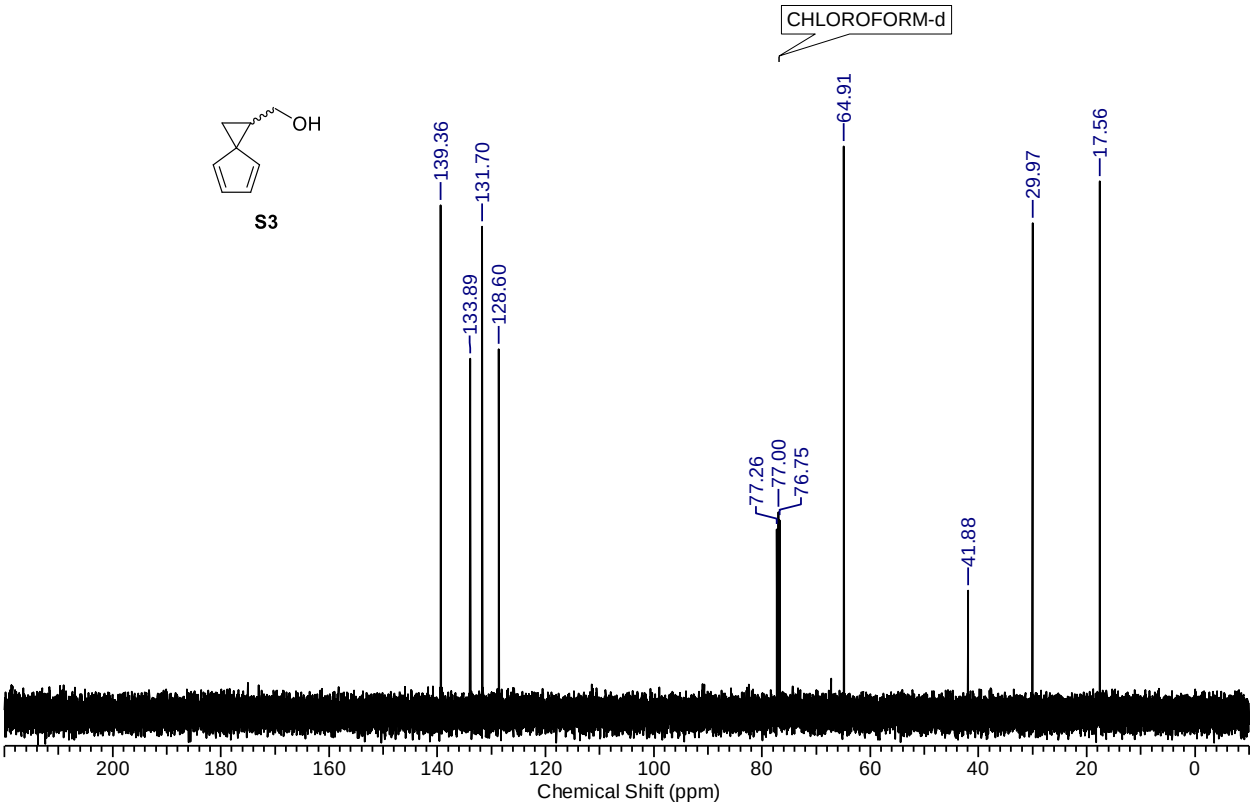


**Figure S16.** $^{1}H$ NMR (400 MHz, $CDCl_3$) **S4**

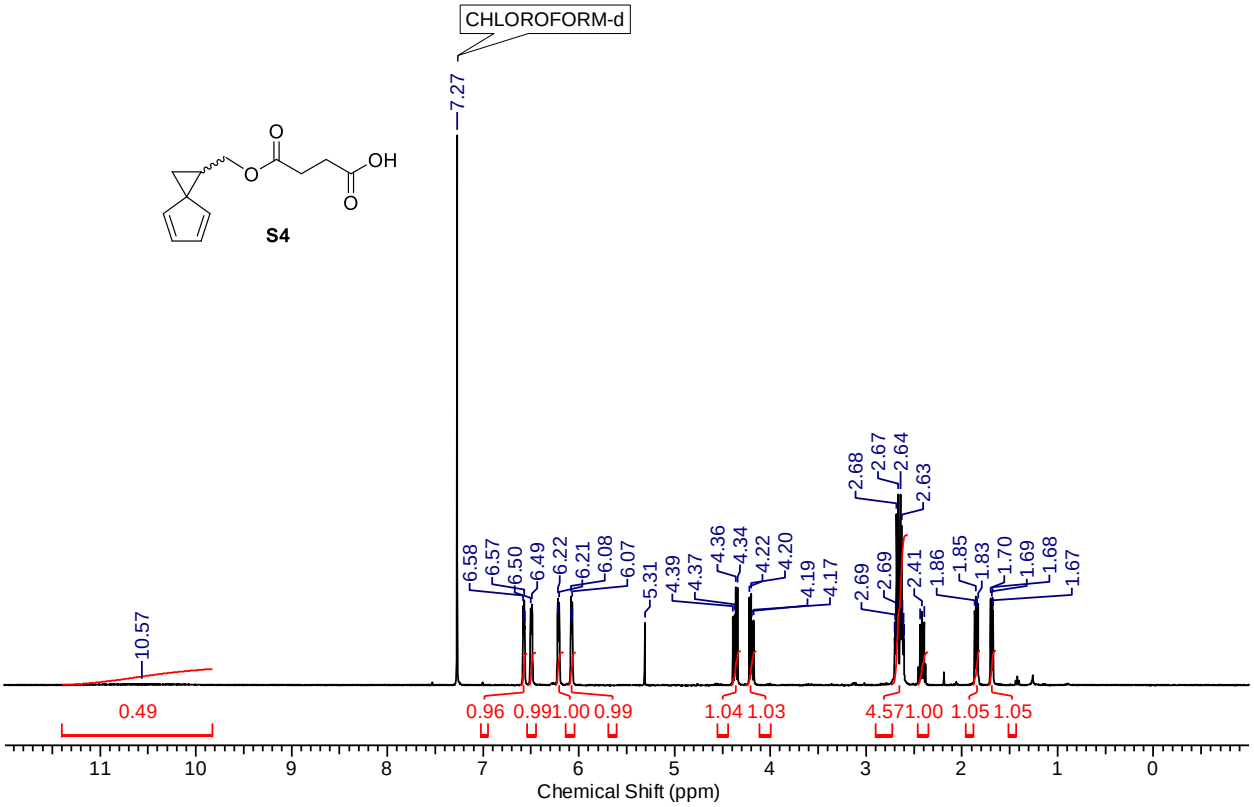


**Figure S17.** $^{1}H$ NMR (400 MHz, $CDCl_3$) **3**

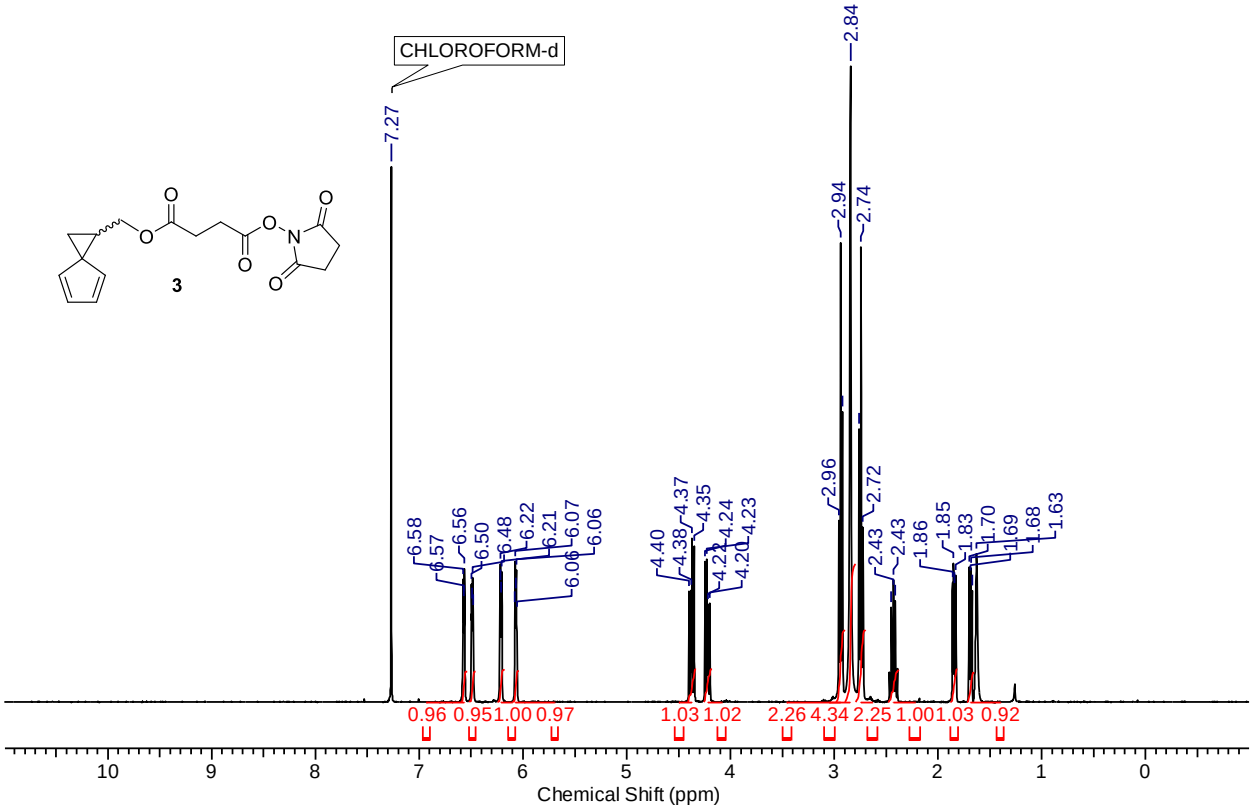



**Figure S18.** $^{13}C$ NMR (100 MHz, $CDCl_3$) **3**

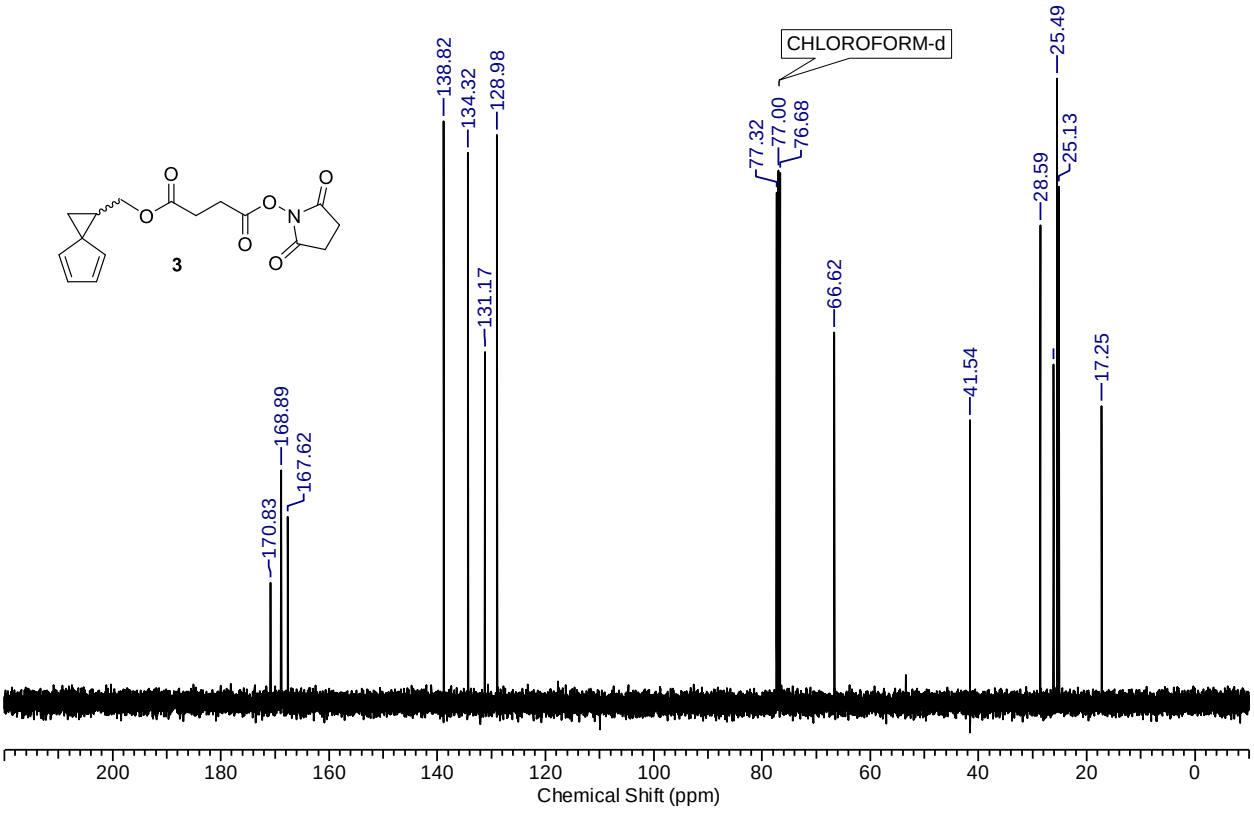


**Figure S19.** $^{1}$H NMR (400 MHz, $CDCl_3$) **4**

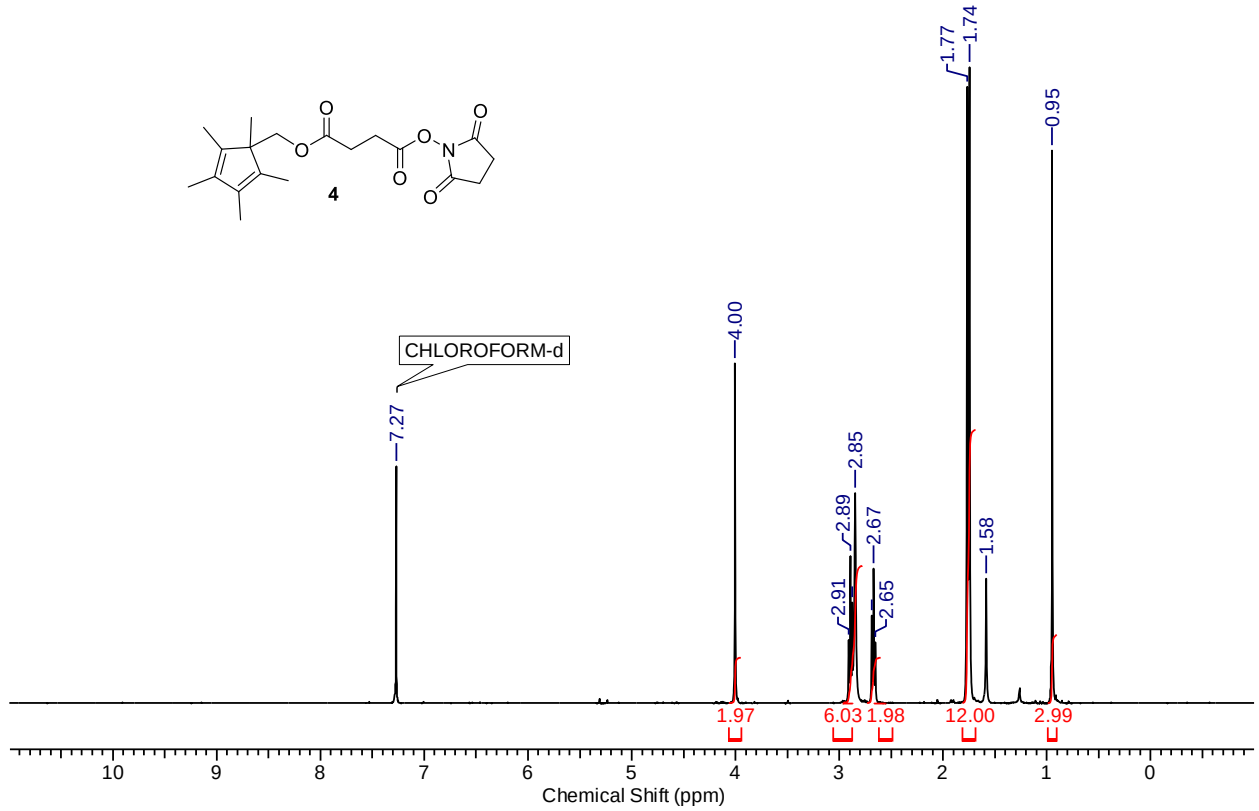



**Figure S20.** $^{13}$C NMR (100 MHz, $CDCl_3$) **4**

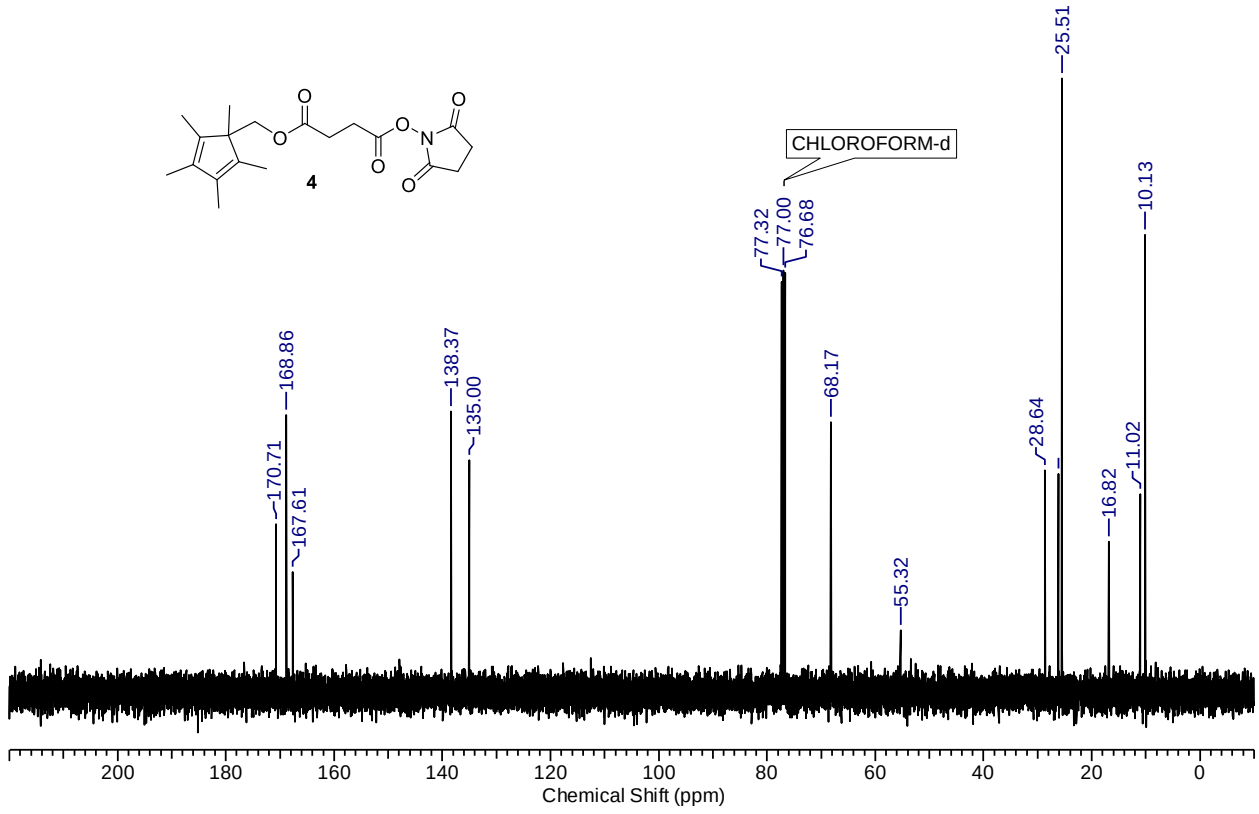


**Figure S21.** $^{1}H$ NMR (400 MHz, $CDCl_3$) **S6**

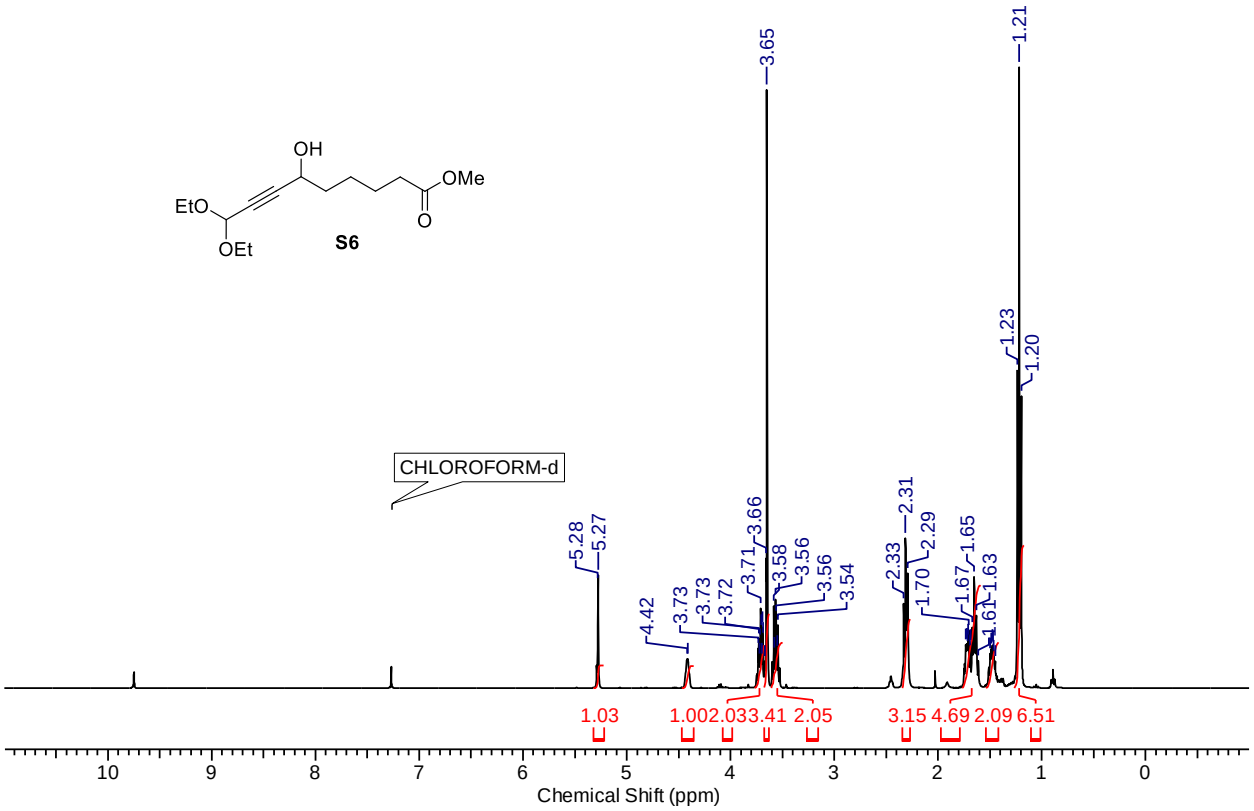



**Figure S22.** $^{13}C$ NMR (100 MHz, $CDCl_3$) **S6**

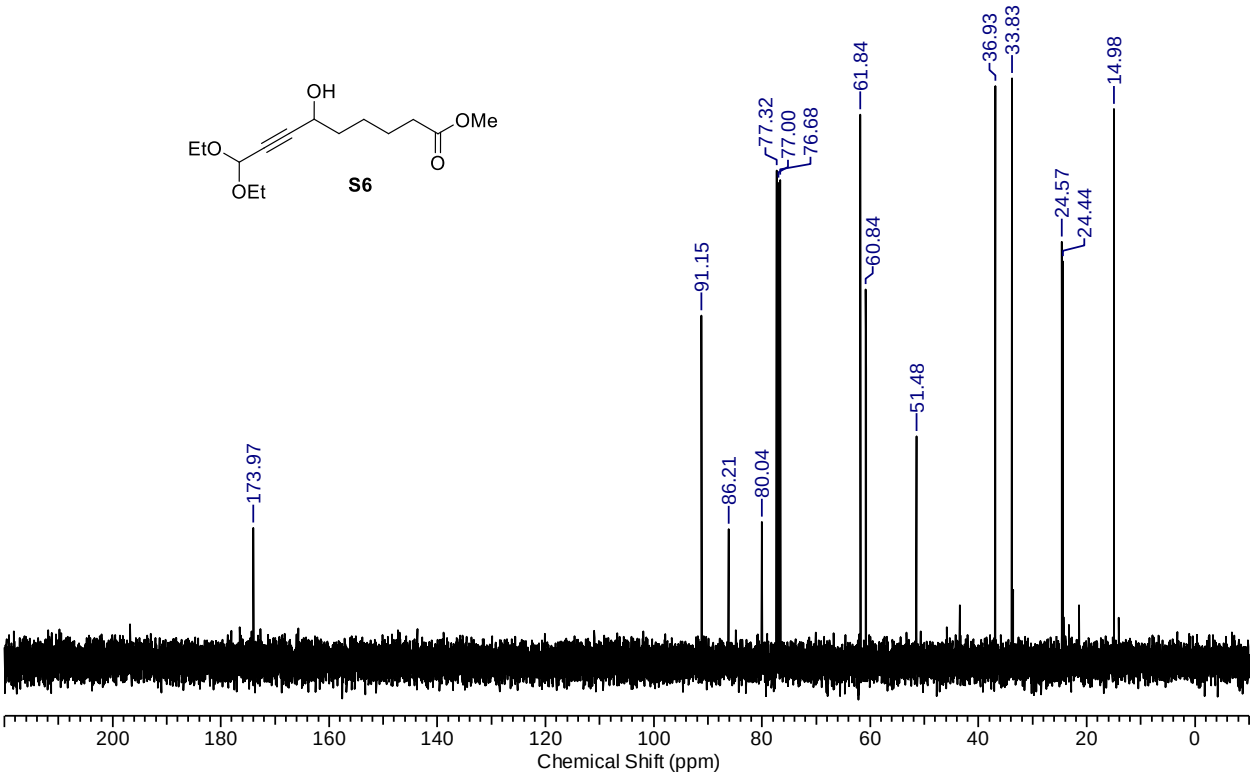


**Figure S23.** $^{1}$H NMR (400 MHz, $CDCl_3$) **S7**

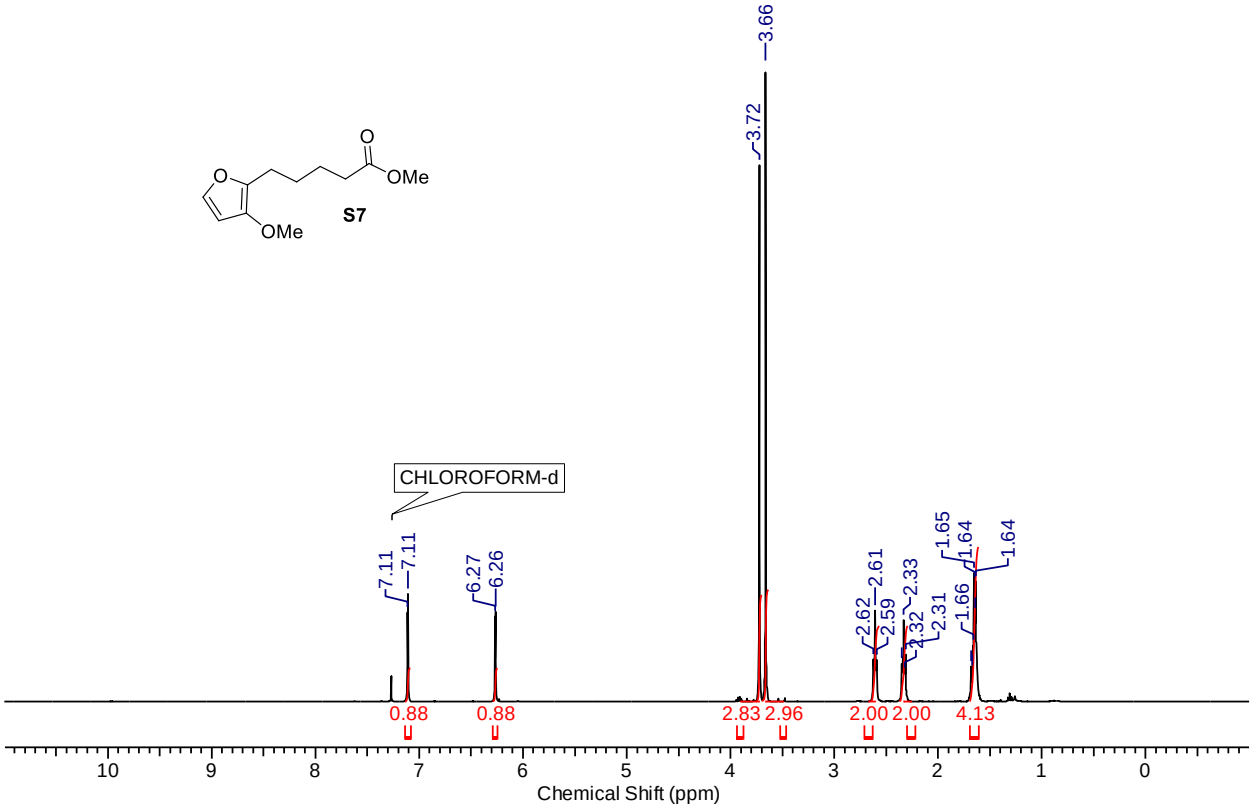



**Figure S24.** $^{13}$C NMR (100 MHz, $CDCl_3$) **S7**

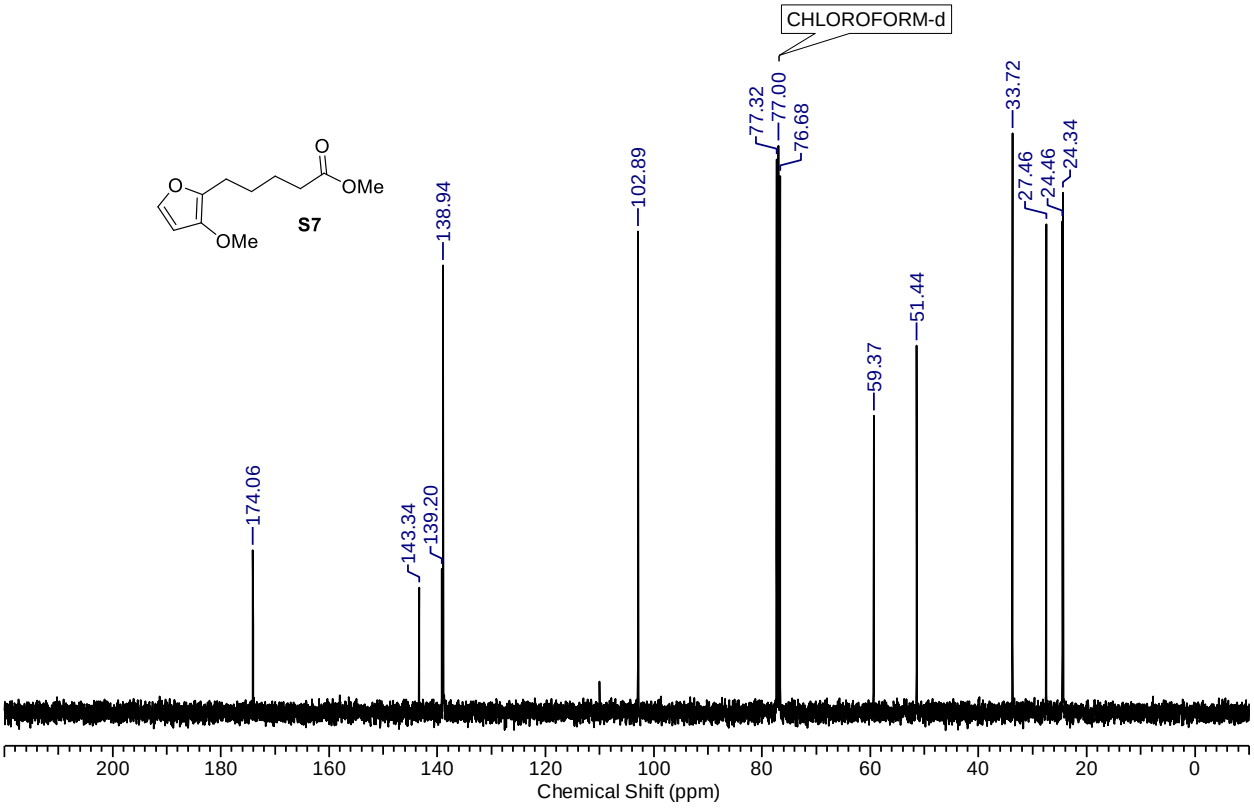


**Figure S25.** $^{1}$H NMR (400 MHz, $CDCl_3$) **S8**

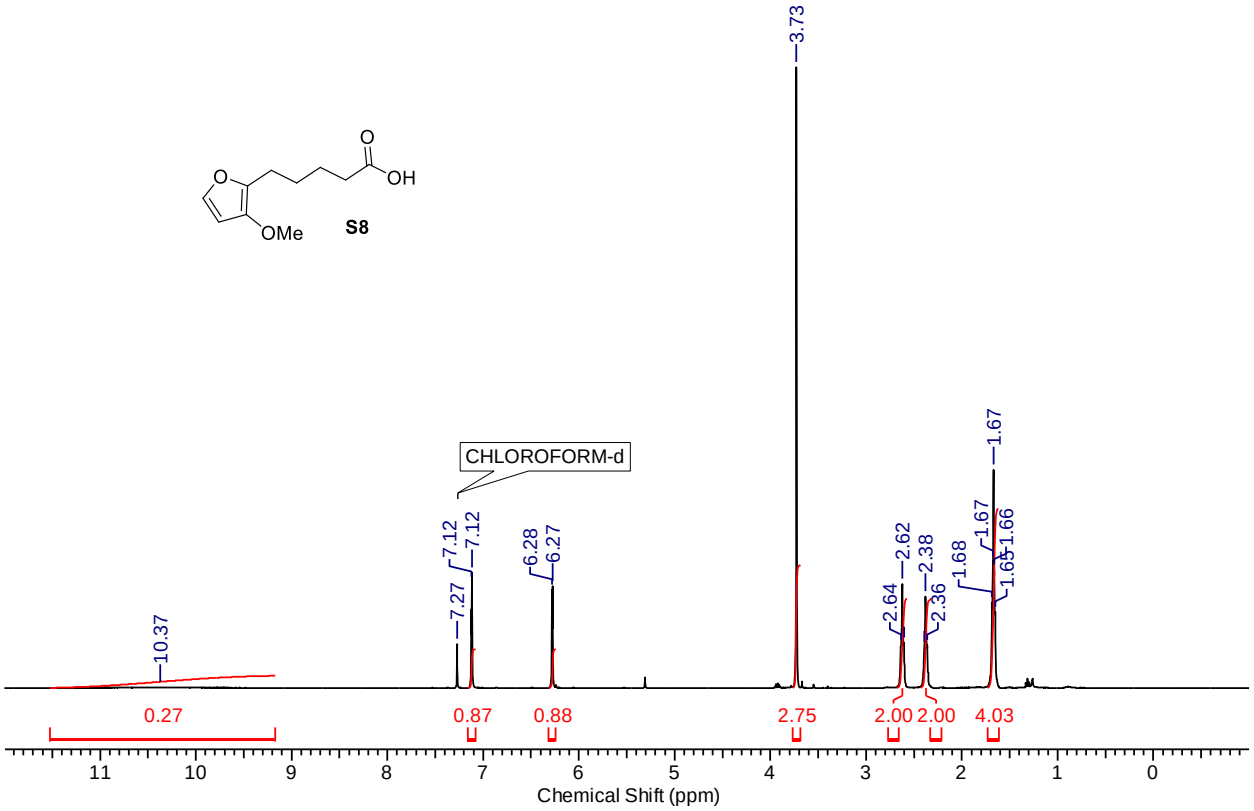



**Figure S26.** $^{13}$C NMR (100 MHz, $CDCl_3$) **S8**

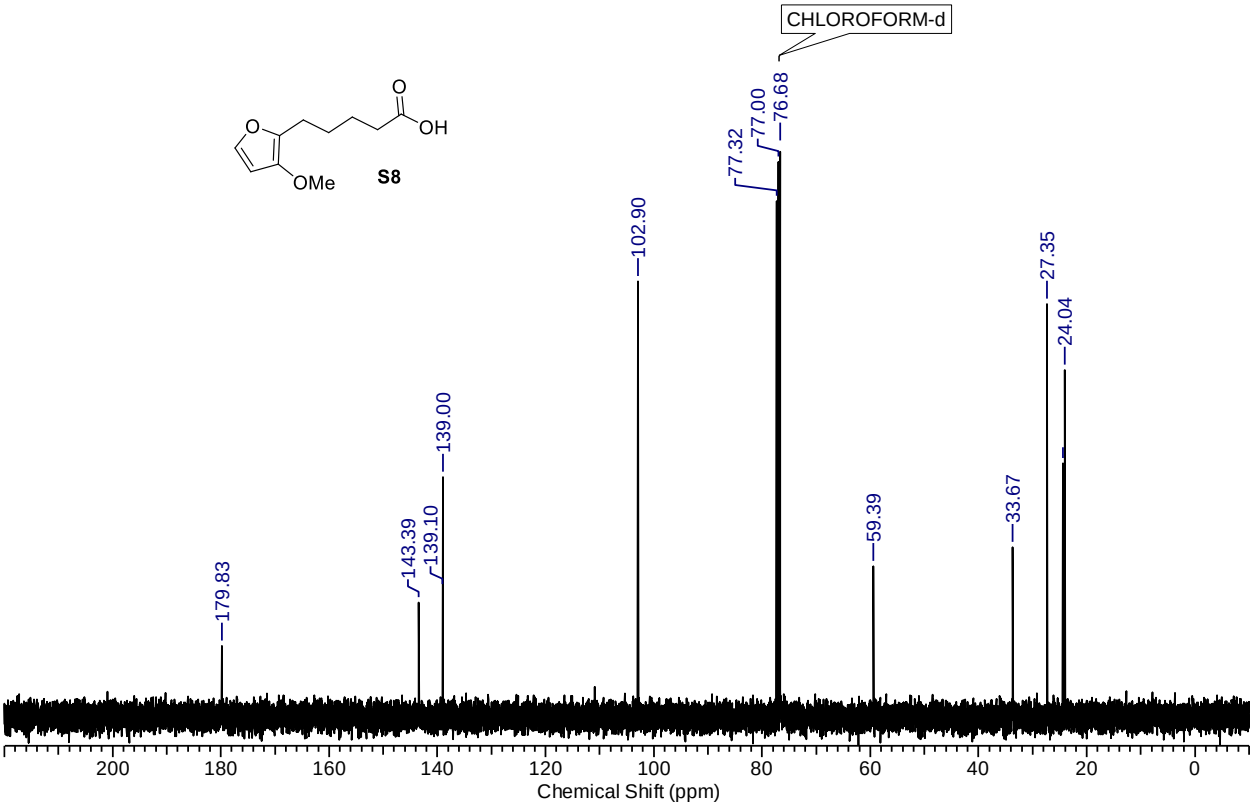


**Figure S27.** $^{1}$H NMR (400 MHz, $CDCl_3$) **5**

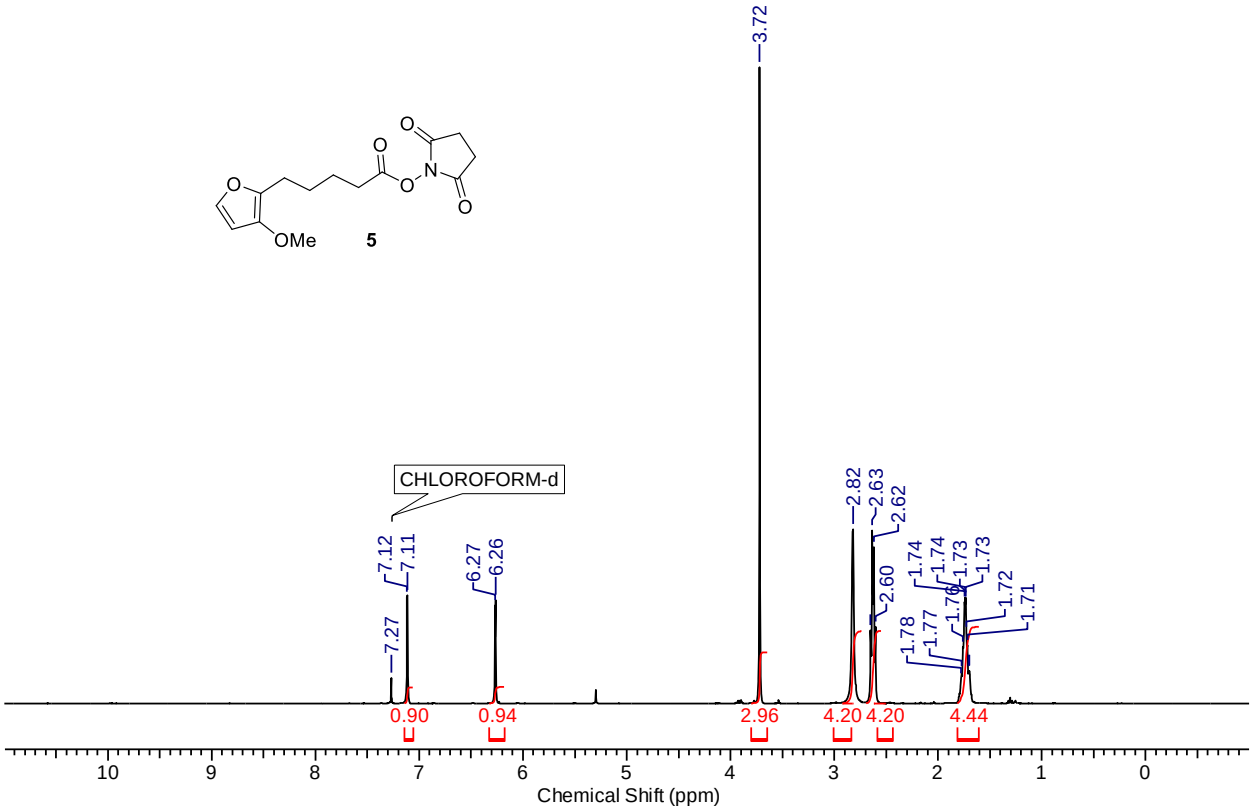



**Figure S28.** $^{13}$C NMR (100 MHz, $CDCl_3$) **5**

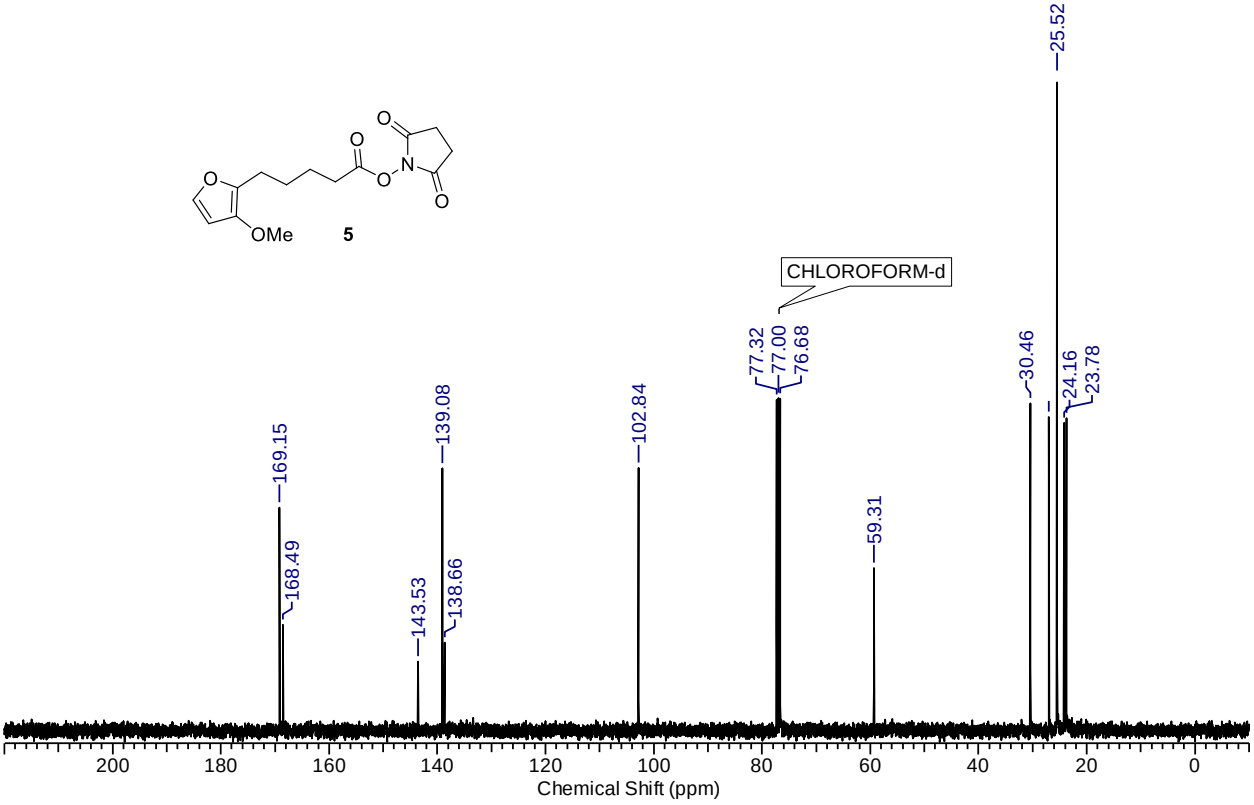


**Figure S29.** $^{1}H$ NMR (400 MHz, $CDCl_3$) **S9**

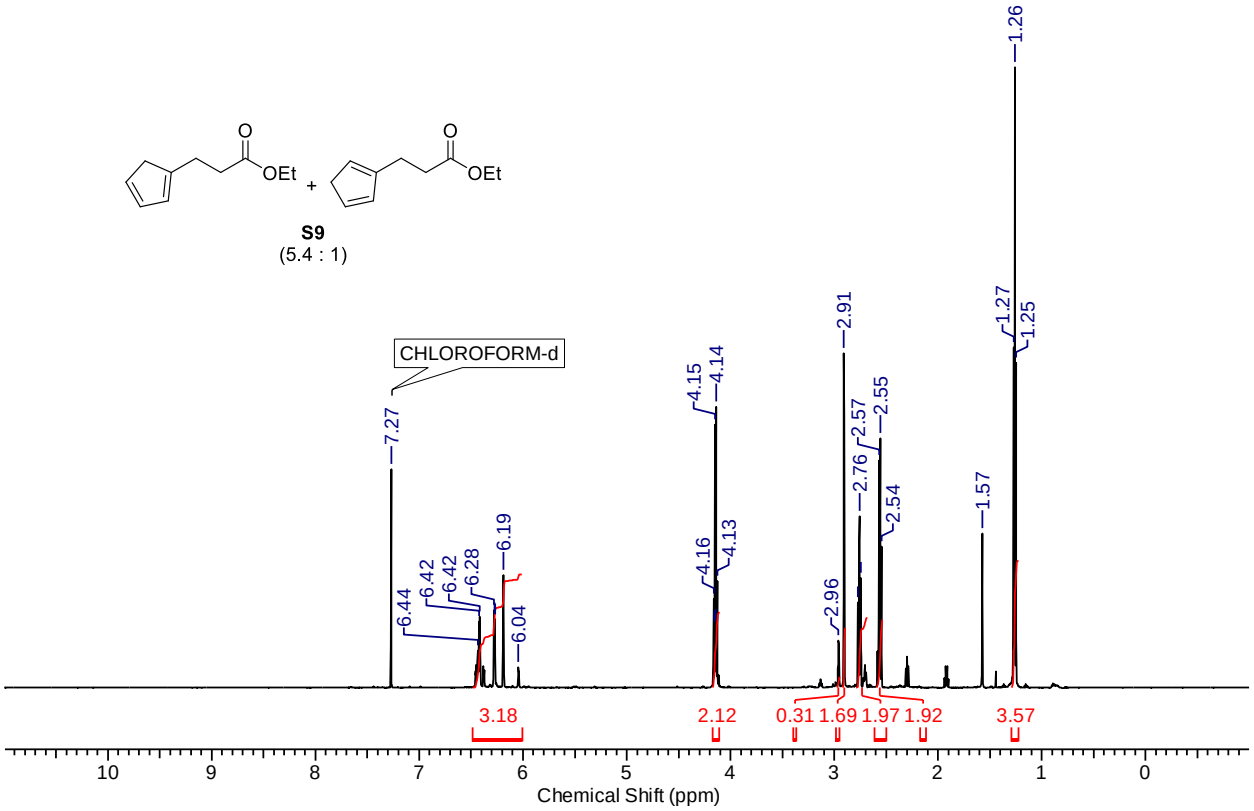


**Figure S30.** $^{1}$H NMR (400 MHz, $CDCl_3$) **S10**

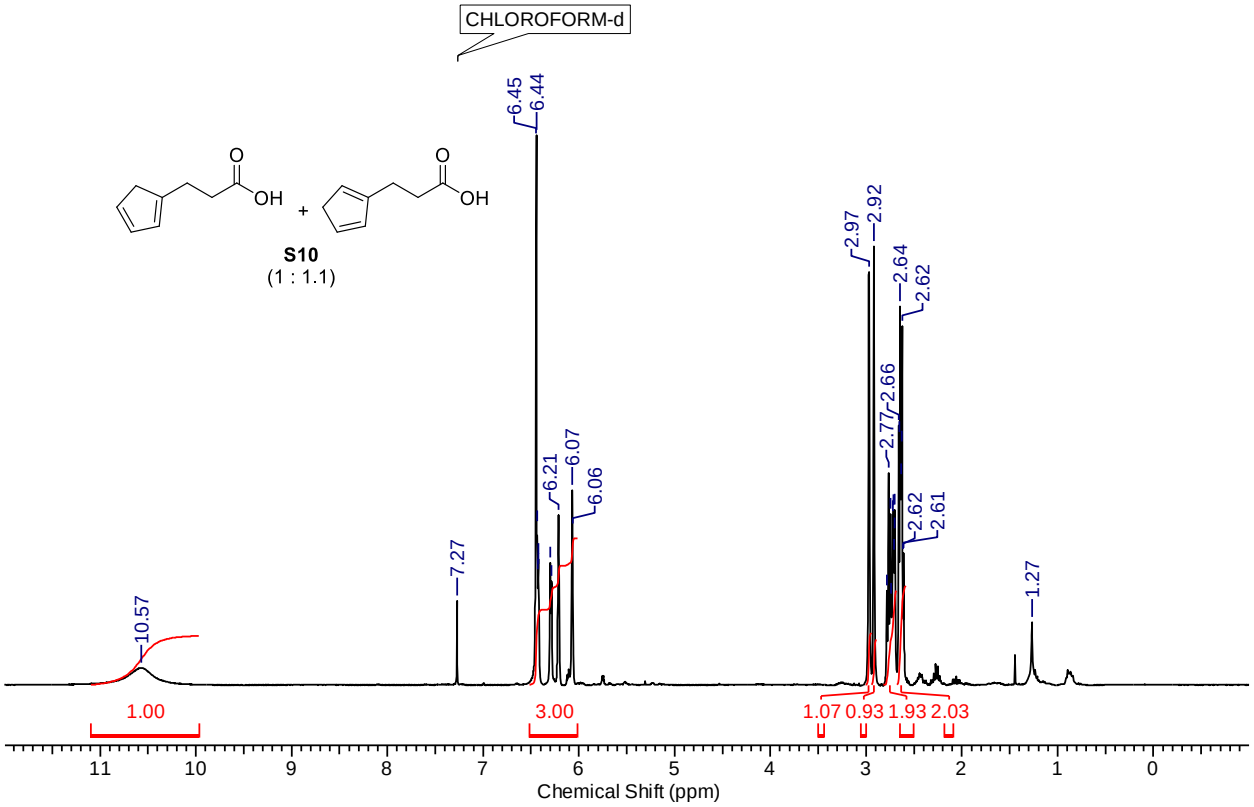



**Figure S31.** $^{13}$C NMR (100 MHz, $CDCl_3$) **S10**

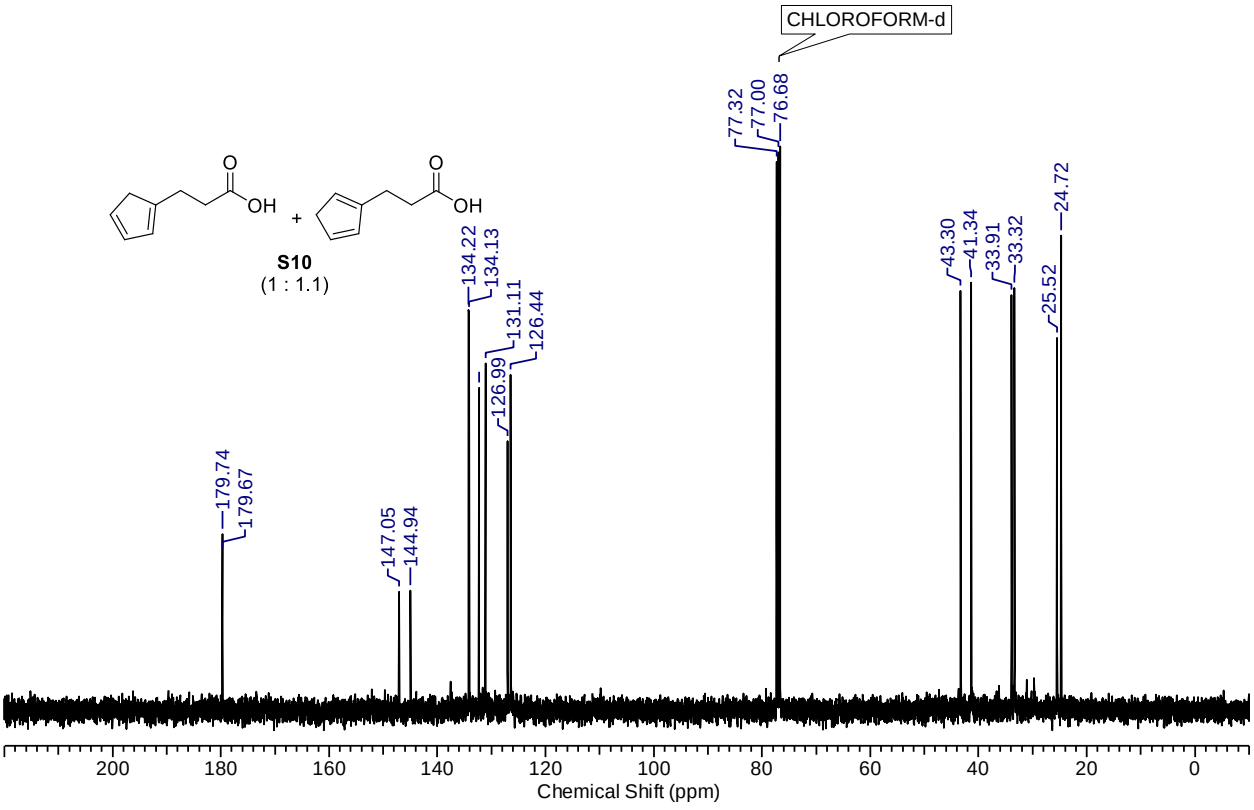


**Figure S32.** $^{1}$H NMR (400 MHz, $CDCl_3$) **2b**

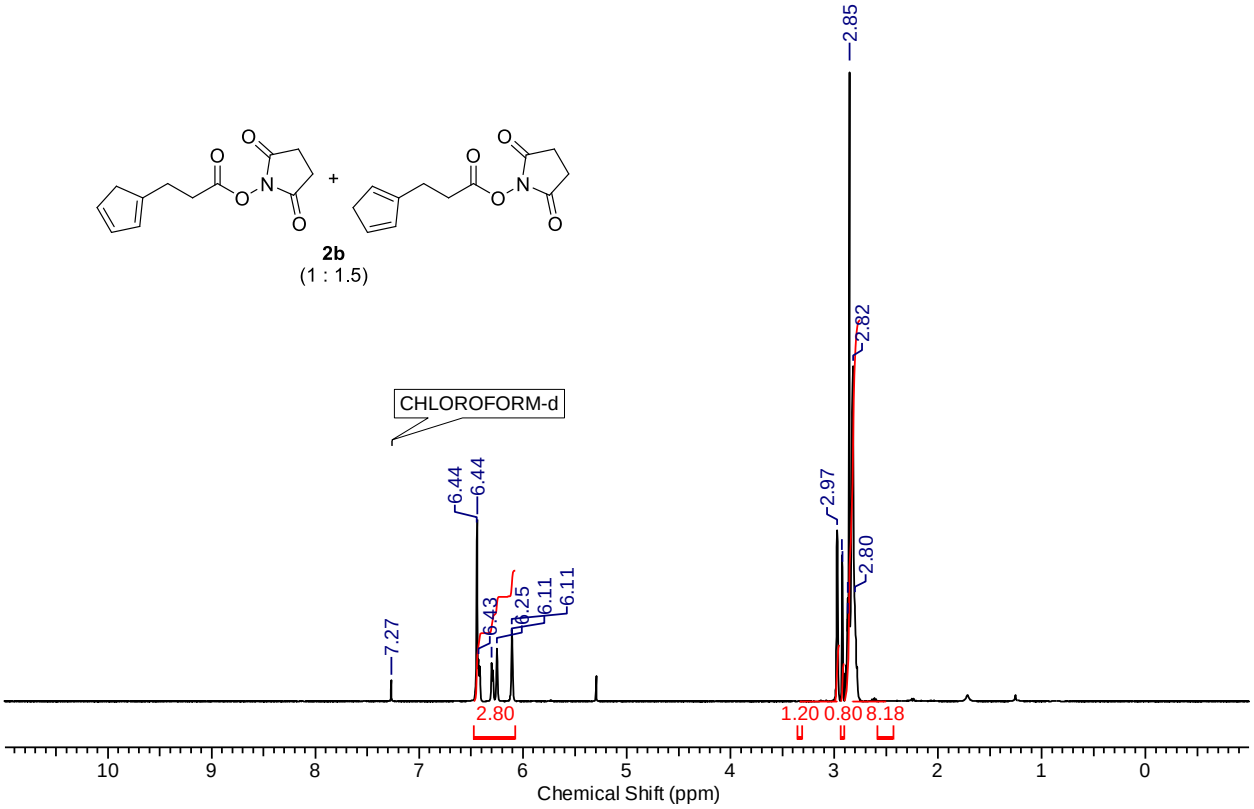



**Figure S33.** $^{13}$C NMR (100 MHz, $CDCl_3$) **2b**

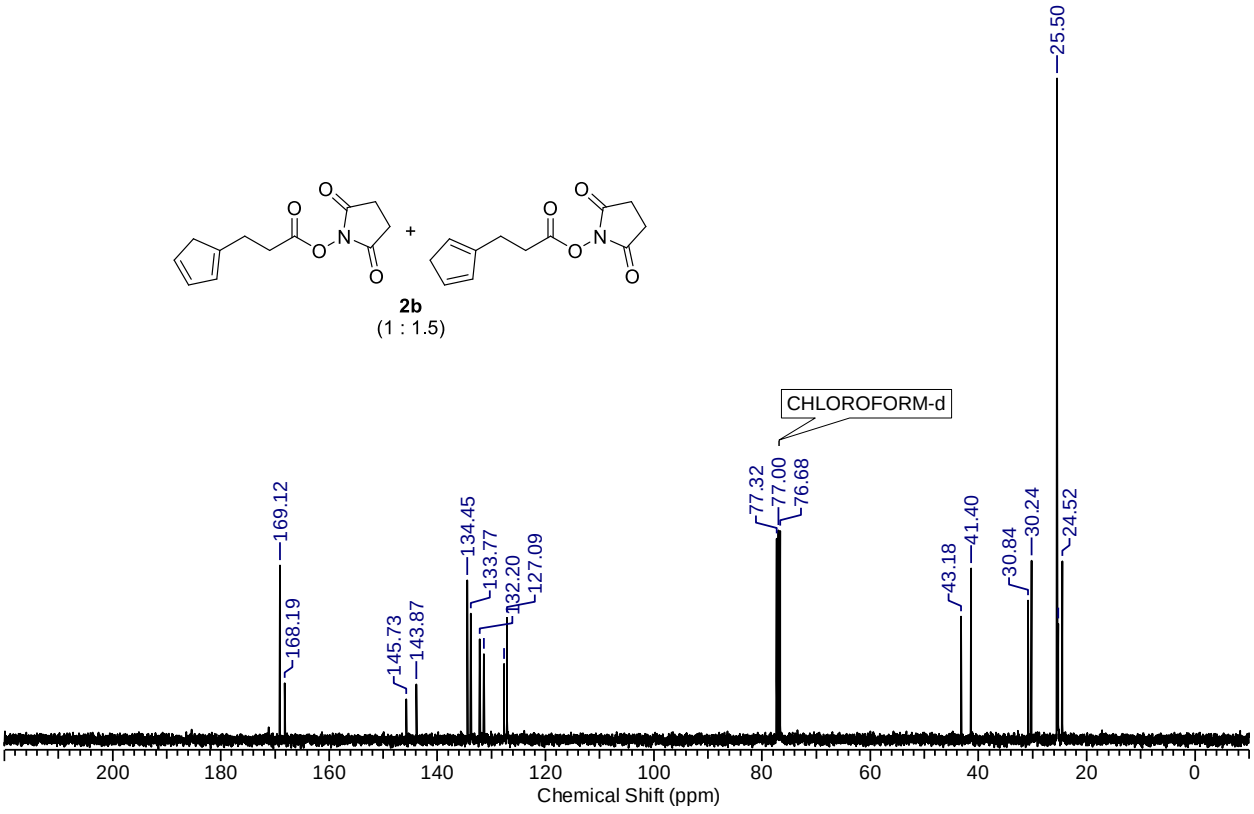


**Figure S34.** $^{1}$H NMR (400 MHz, $CDCl_3$) **S11a** & **S11b**

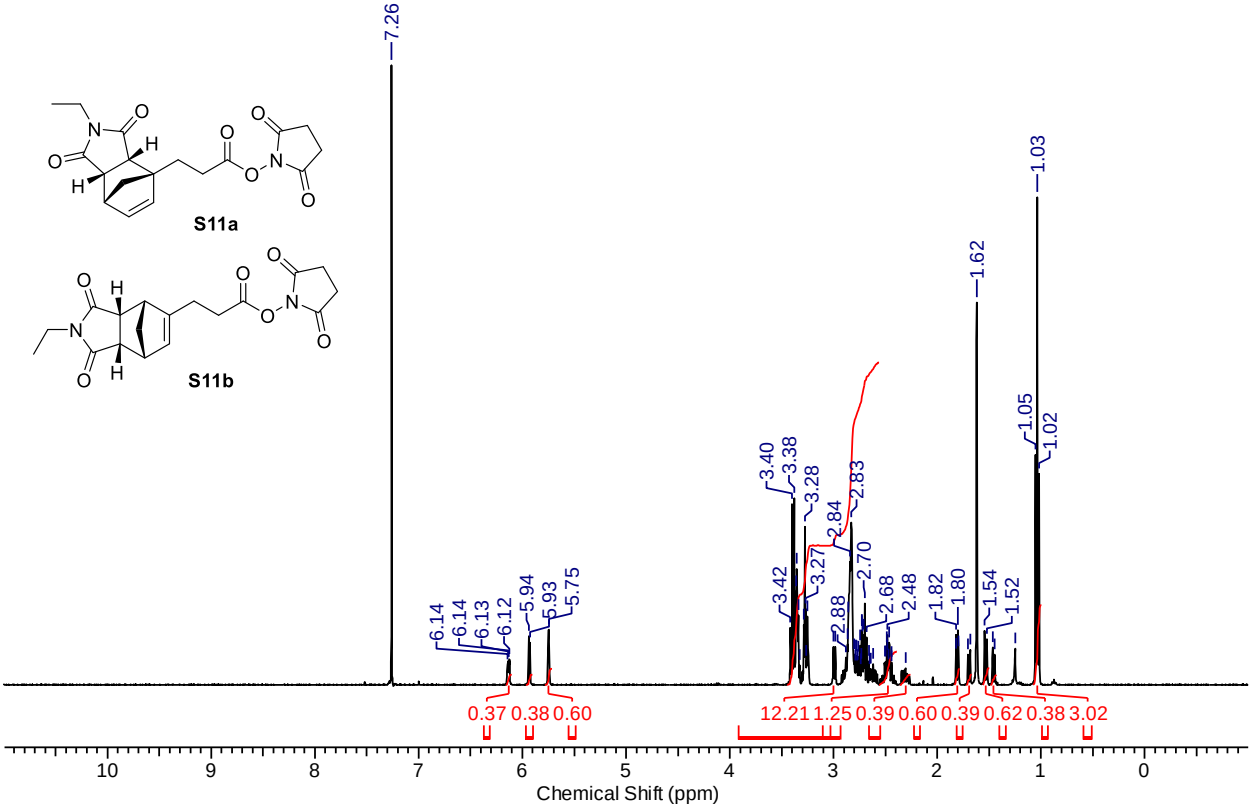



**Figure S35.** $^{13}$C NMR (100 MHz, $CDCl_3$) **S11a** & **S11b**

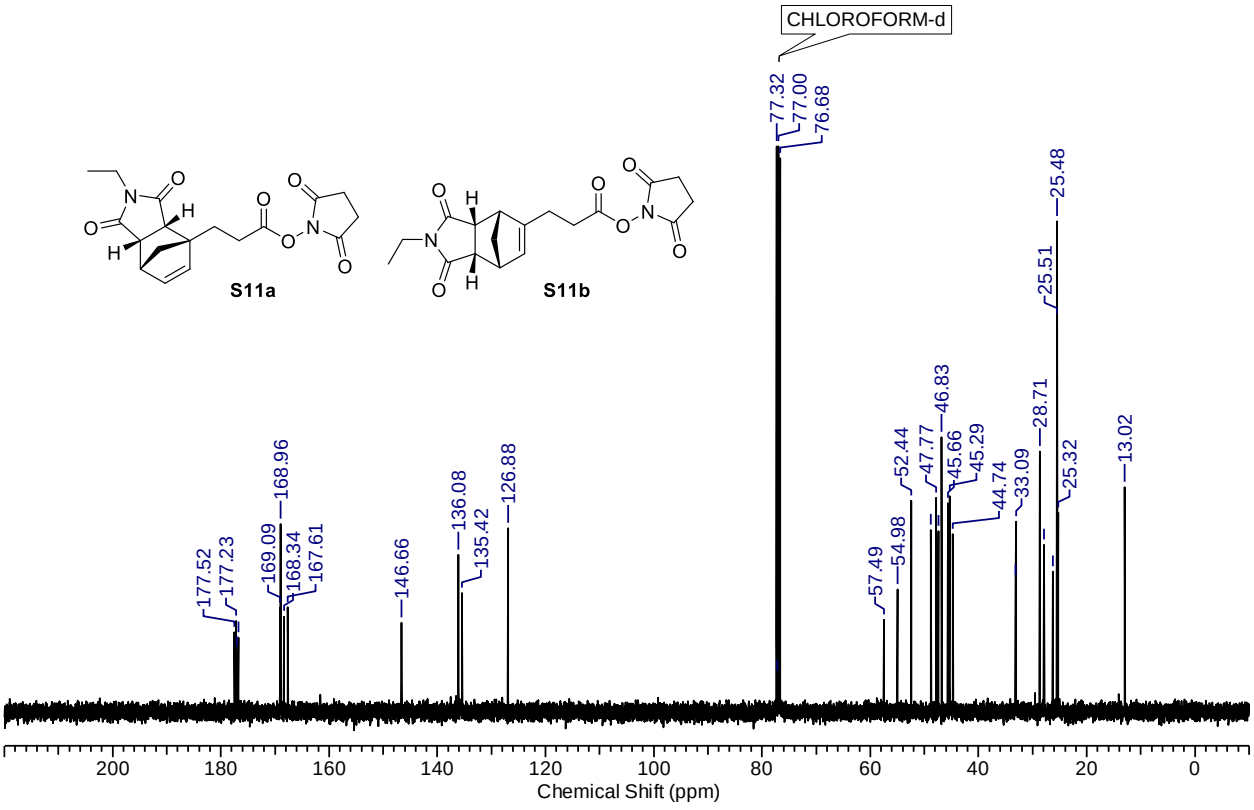


**Figure S36.** $^{1}$H NMR (400 MHz, $CDCl_3$) **S12a** (*anti*)

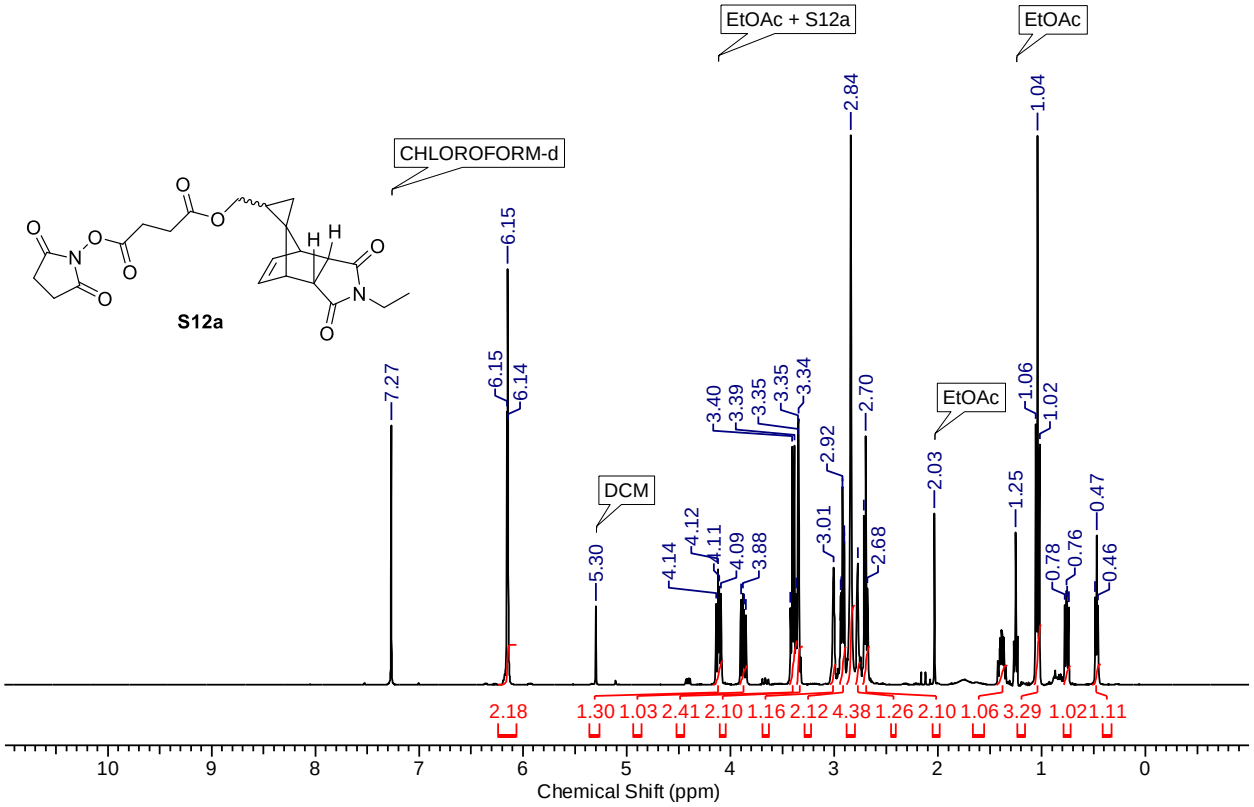



**Figure S37.** $^{13}$C NMR (100 MHz, $CDCl_3$) **S12a** (*anti*)

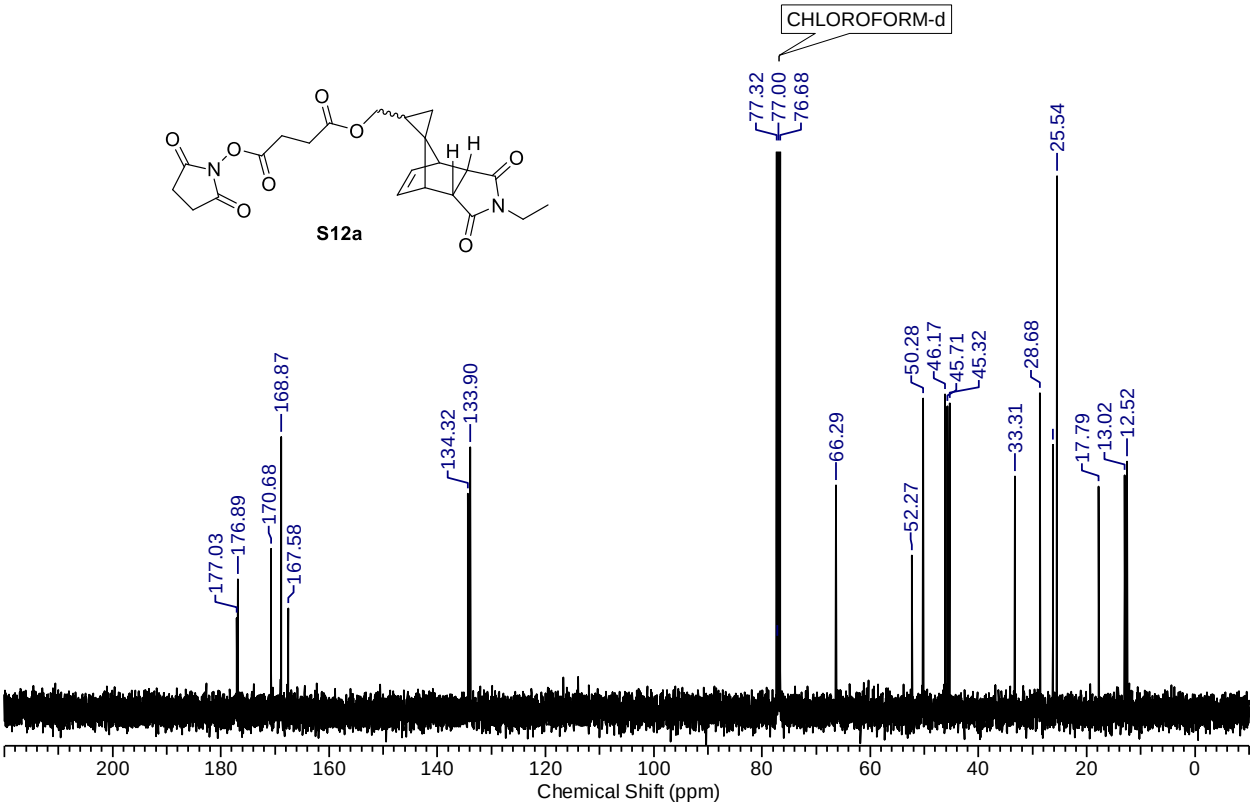


**Figure S38.** $^{1}$H NMR (400 MHz, $CDCl_3$) **S12b** (*syn*)

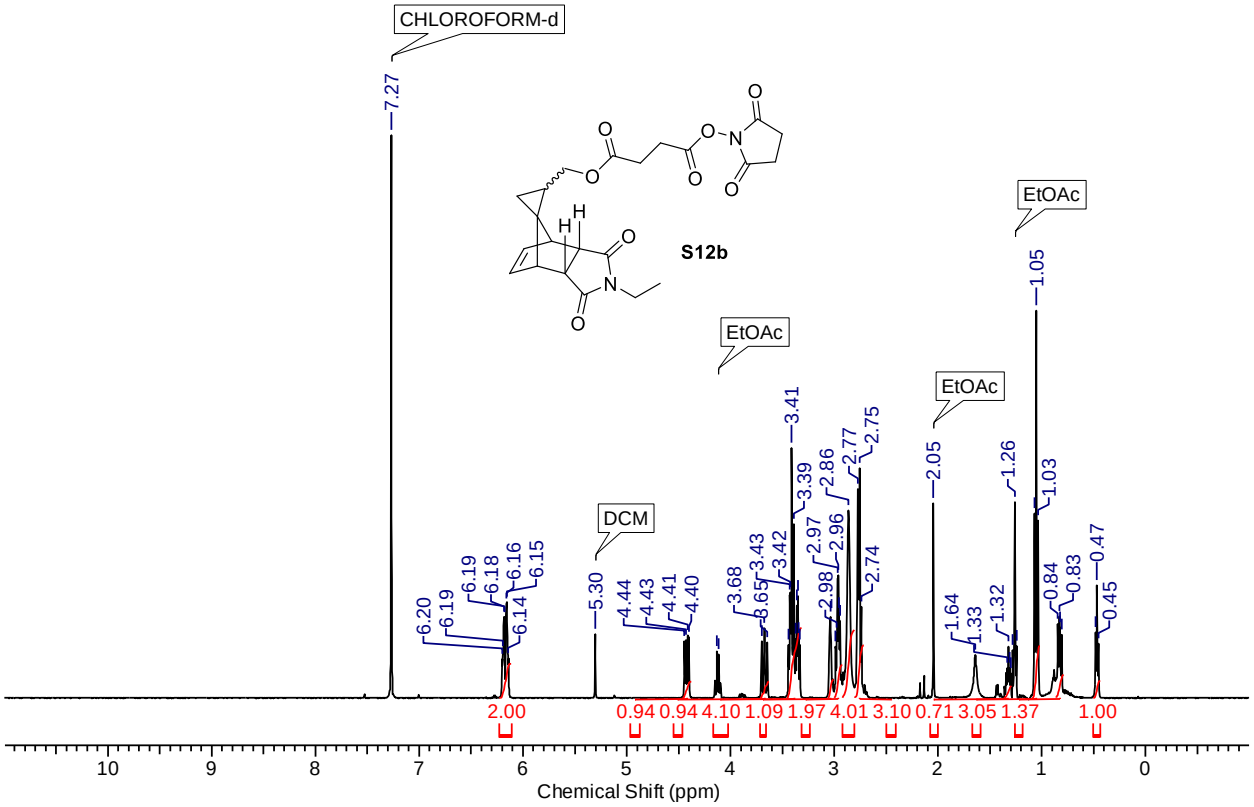



**Figure S39.** $^{13}$C NMR (100 MHz, $CDCl_3$) **S12b** (*syn*)

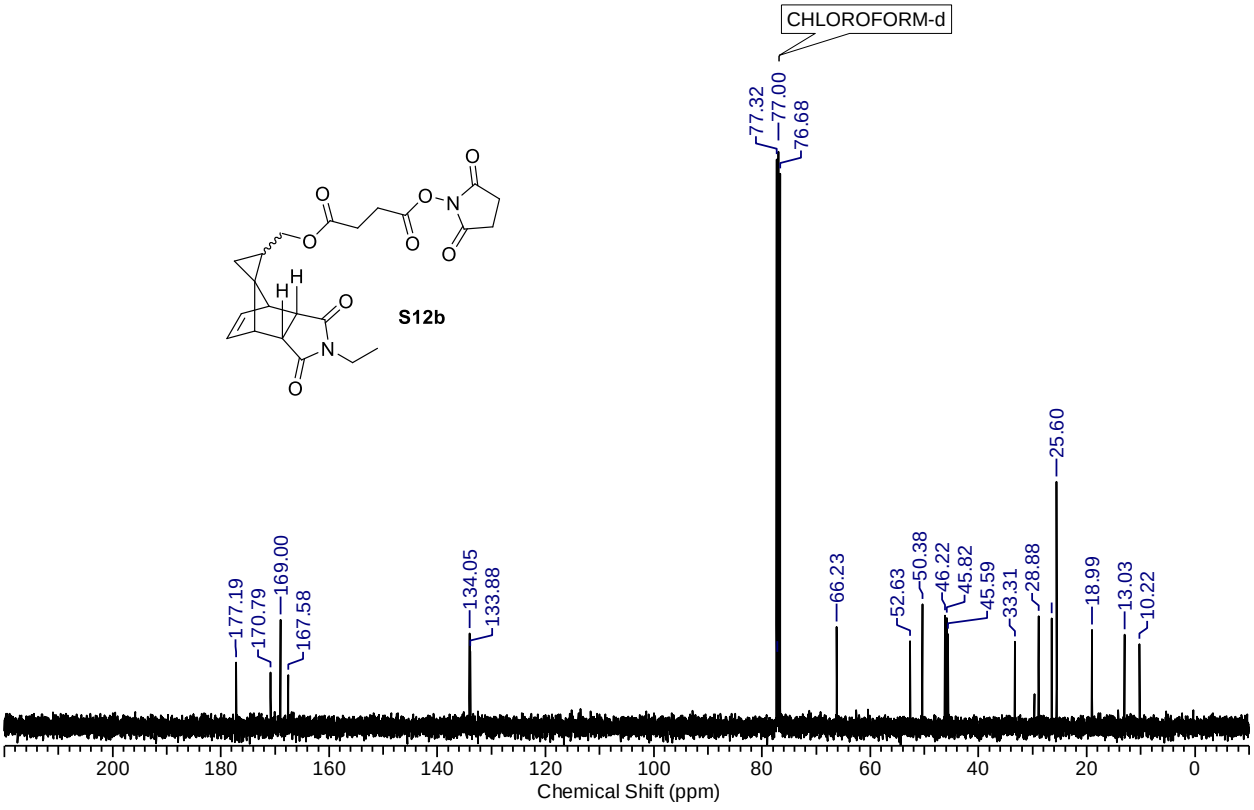


**Figure S40.** $^{1}$H NMR (400 MHz, $CDCl_3$) **S13**

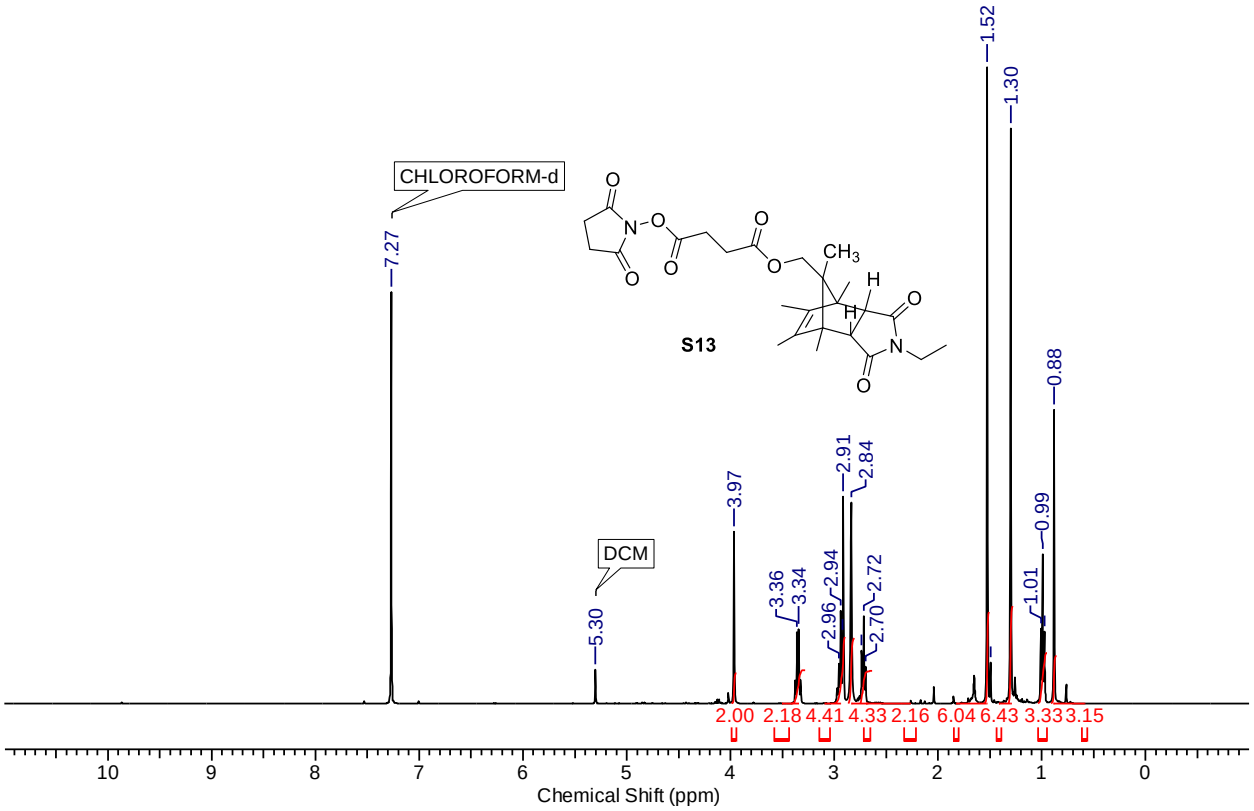



**Figure S41.** $^{13}$C NMR (100 MHz, $CDCl_3$) **S13**

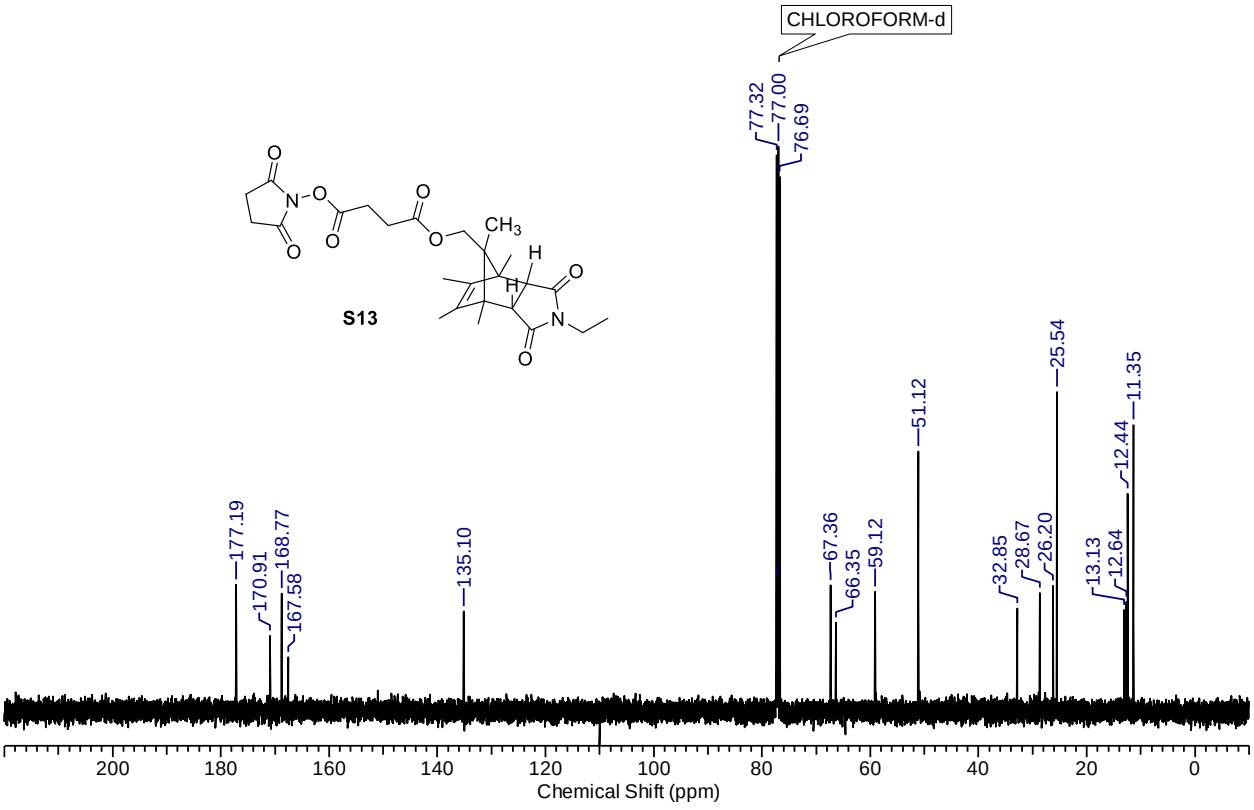


**Figure S42.** $^{1}$H NMR (400 MHz, $CDCl_3$) **S14a** (*endo*) & **S14b** (*exo*)

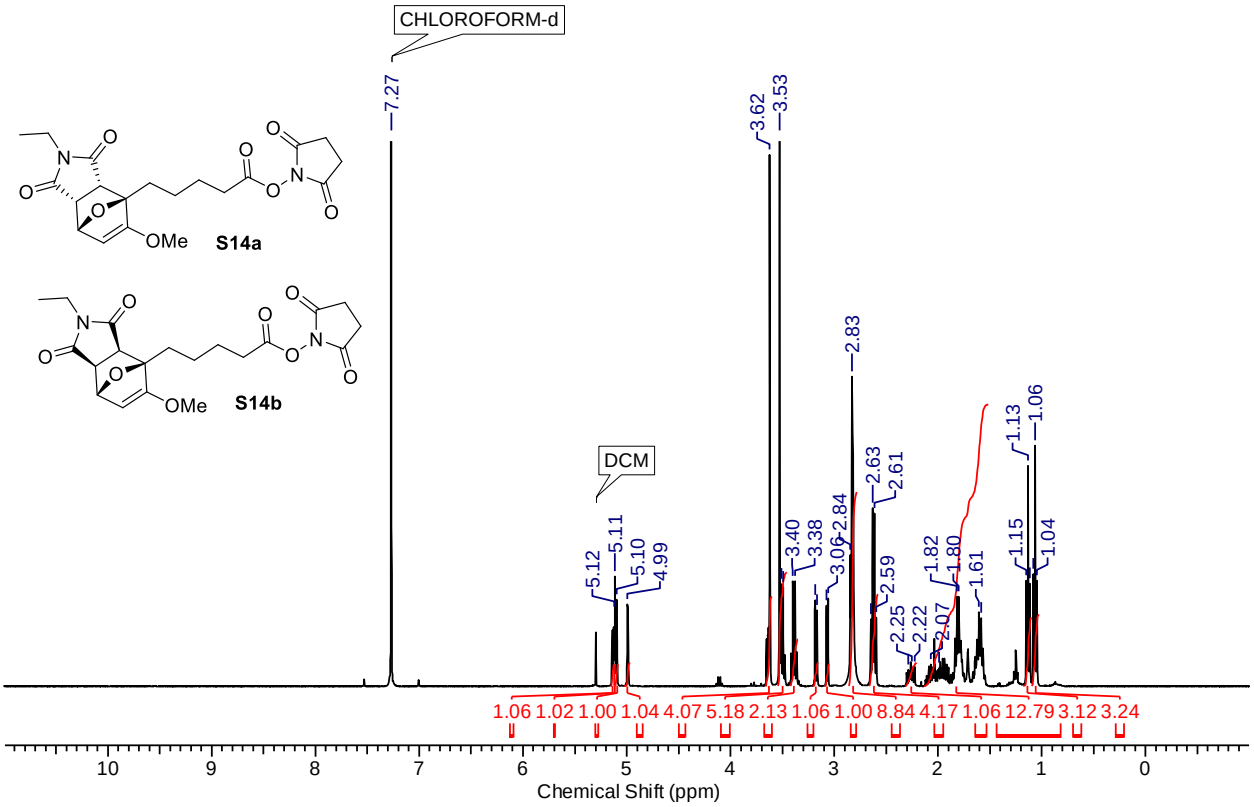
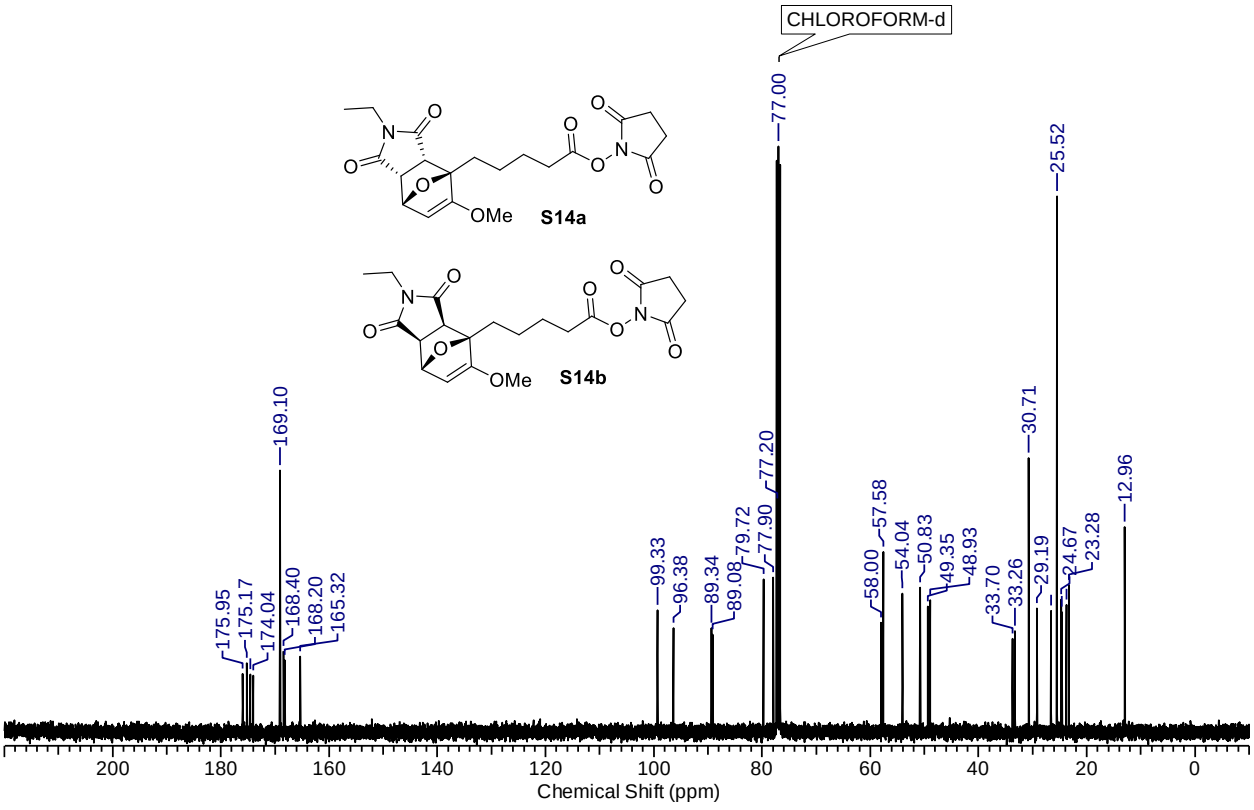



**Figure S43.** $^{13}$C NMR (100 MHz, $CDCl_3$) **S14a** (*endo*) & **S14b** (*exo*)



## 6. Preparation of mAb-Diene Conjugates.

**Materials.** All antibodies (IgG-1 format) were expressed and purified using standard molecular biology methods. All reagents were purchased from commercial vendors unless noted otherwise.

**Method.** Diene functionality was randomly incorporated into antibodies by reaction of the NHS ester-containing dienes described above with lysine amines. Degree of mAb modification was controlled by the amount of NHS-diene used in the reaction and different linker densities were targeted depending on the experiment. Two different mAbs were used in this experiment; R347 (**2a**, **3**, **4** and **5**) and 5T4 (**1**). A general procedure for modification of **mAb** with **2a** is described as follows: First, **mAb** solution was adjusted to 5 mg/mL (3 mL, 15 mg mAb, 100 nmol, 1 equiv) with PBS pH 7.2 followed by addition of 10% v/v 1M $NaHCO_3$. This solution was chilled on ice and 30 µL **2a** (10 mM stock in DMAc, 300 nmol, 3 equiv) was added. The reaction proceeded on ice for 5 minutes followed by reaction at room temperature for 1 h with continuous mixing. Reacted **mAb-2a** was purified by dialysis (Slide-A-Lyzer, 10 kDa MWCO) against PBS, 1 mM EDTA, pH 7.4, 4 $^{o}$C for 24 h. **2a** introduction was quantified by intact deglycosylated mass spectrometry as described below.

**Mass Spectrometry Analysis.** For deglycosylated mAb analysis, EndoS (5 µL Remove-iT EndoS (1:10 dilution in PBS, 20,000 units/mL, New England BioLabs) was combined with 50 µL sample (1 mg/mL mAb) and 5 µL glyco buffer 1 (New England BioLabs) and followed by incubation for 1 h at 37 °C. Reduced samples were prepared by addition of 5 µL Bond-Breaker TCEP solution (0.5 M, Thermo Fisher Scientific) and incubation for 10 min at 37 °C. Mass spectrometry analysis was performed using an Agilent 6520B Q-TOF mass spectrometer equipped with a RP-HPLC column (ZORBAX 300 Diphenyl RRHD, 1.8 micron, 2.1 mm x 50 mm). High-performance liquid chromatography (HPLC) parameters were as follows: flow rate, 0.5 ml/min; mobile phase A was 0.1% (v/v) formic acid in HPLC-grade $H_2O$, and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The column was equilibrated in 90%A/10%B, which was also used to desalt the mAb samples, followed by elution in 20%A/80%B. Mass spec data were collected for 100-3000 *m/z*, positive polarity, a gas temperature of 350 $^{\circ}$C, a nebulizer pressure of 48 lb/in$^2$, and a capillary voltage of 5,000 V. Data were analyzed using vendor-supplied (Agilent v.B.04.00) MassHunter Qualitative Analysis software and peak intensities from deconvoluted spectra were used to derive the relative proportion of species in each sample as previously described.

**Mass Spectrum Analysis.** Antibodies were deglycosylated and analyzed intact. Peak masses are indicated as well as the number of linkers conjugated for each peak.

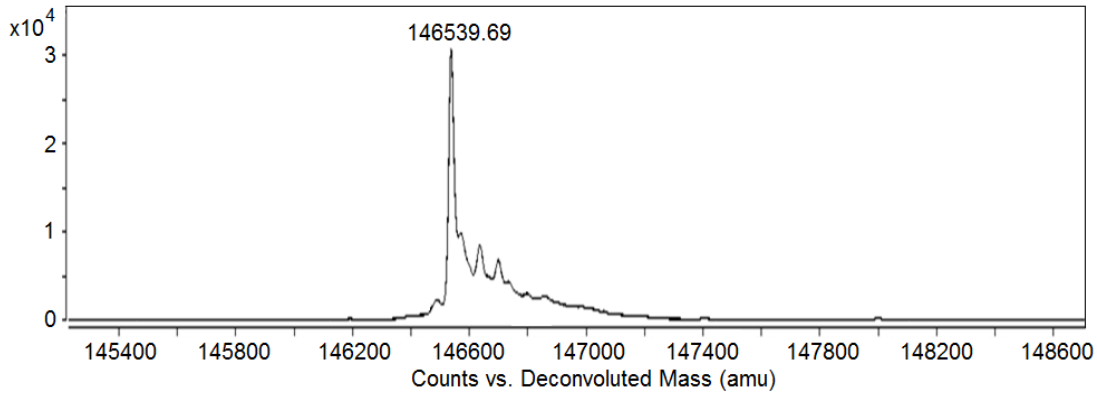



**Figure S44.** Mass spectrum of intact, deglycosylated **mAb** (5T4).

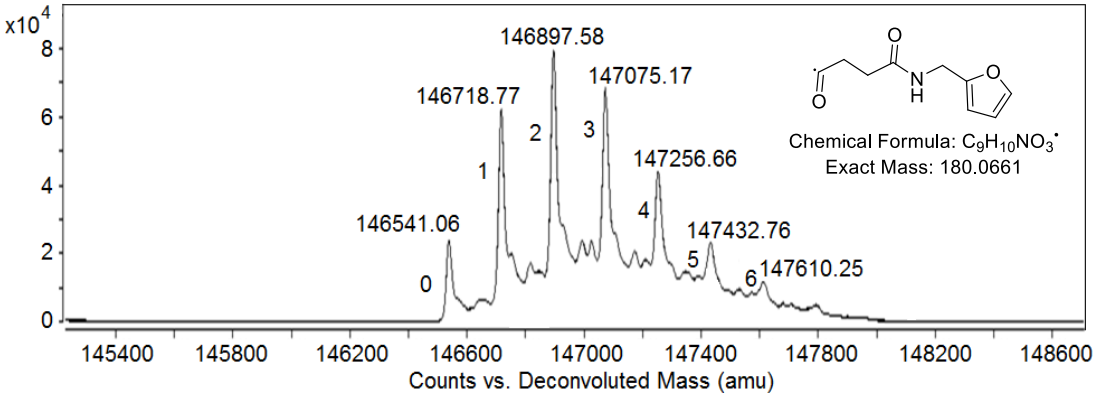



**Figure S45.** Mass spectrum of intact, deglycosylated antibody-diene conjugate **mAb-1** (LAR = 2.5).

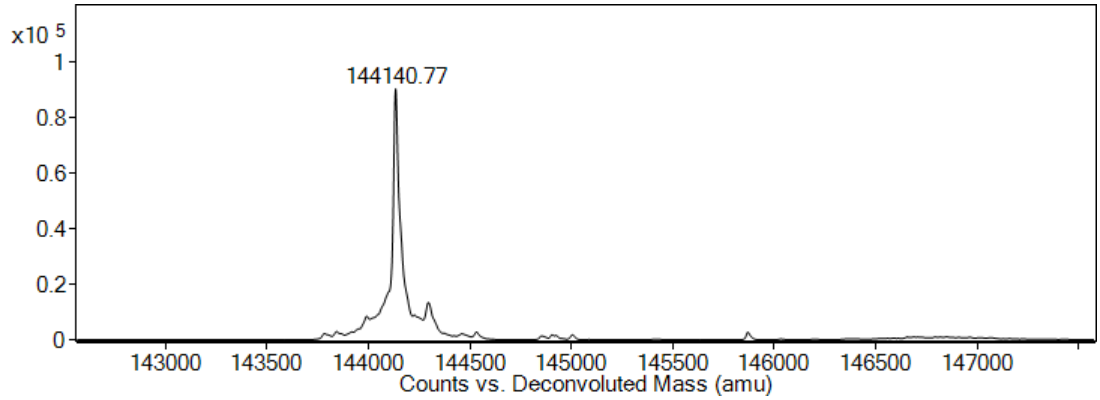



**Figure S46.** Mass spectrum of intact, deglycosylated **mAb** (R347).

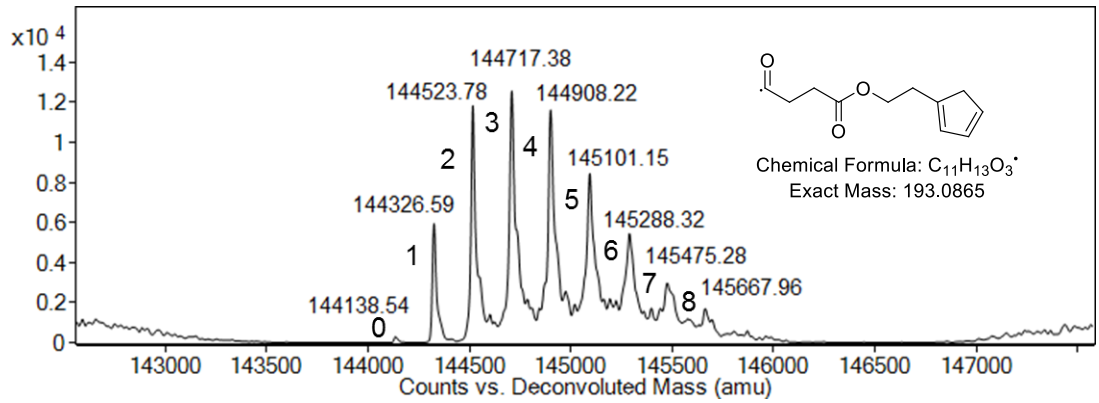



**Figure S47.** Mass spectrum of intact, deglycosylated antibody-diene conjugate **mAb-2a** (LAR = 2.3).

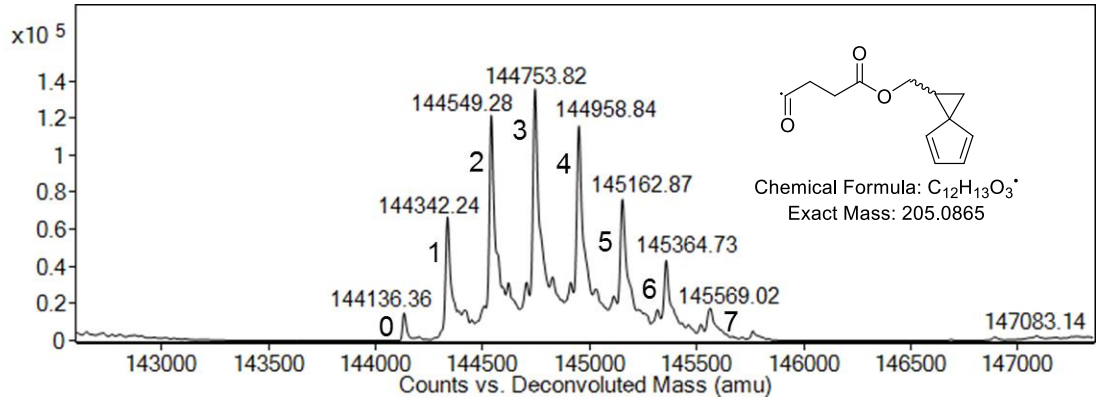



**Figure S48.** Mass spectrum of intact, deglycosylated antibody-diene conjugate **mAb-3** (LAR = 3.3).

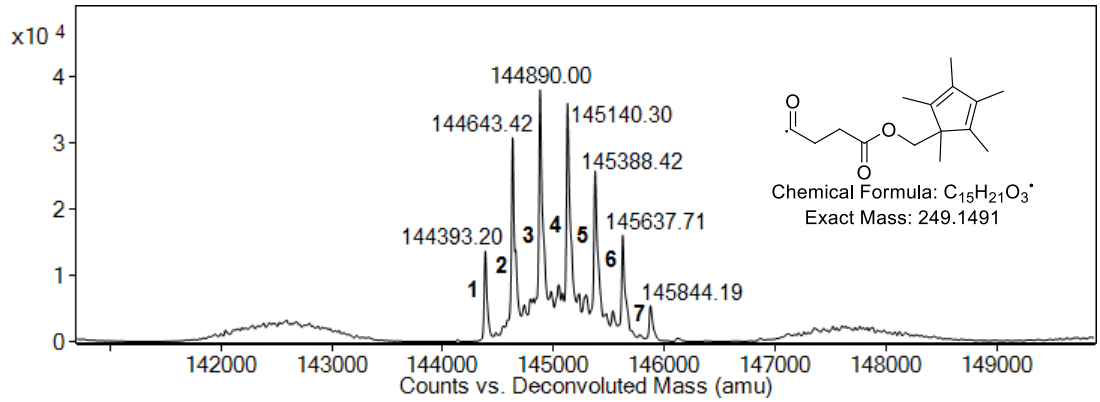



**Figure S49.** Mass spectrum of intact, deglycosylated antibody-diene conjugate **mAb-4** (LAR = 3.04).

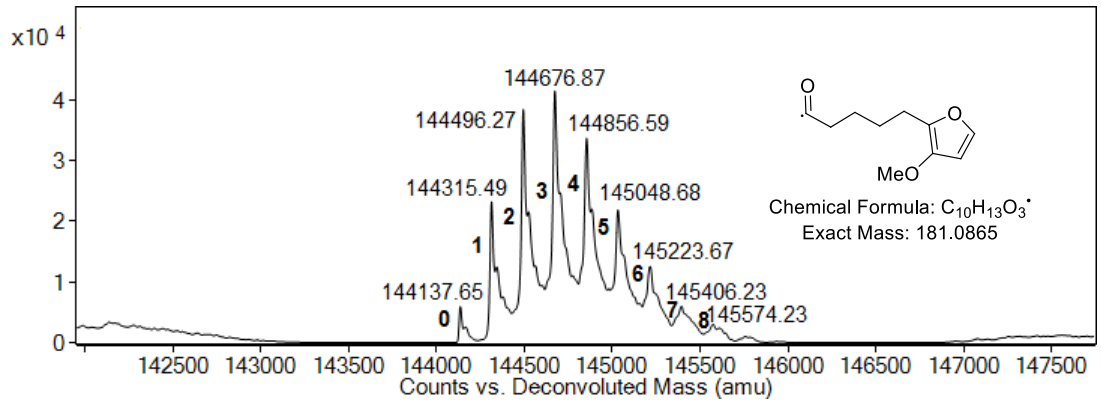



**Figure S50.** Mass spectrum of intact, deglycosylated antibody-diene conjugate **mAb-5** (LAR = 2.95).

**7. Reaction of mAb-Dienes with vcMMAE.**

Feasibility of the diene-maleimide reaction for bioconjugation was determined by reaction of diene-modified mAbs with excess **vcMMAE** at both room temperature and 37 °C. Two different mAbs were used in this experiment; R347 (**2a**, **3**, **4** and **5**) and 5T4 (**1**). First, mAb-diene solution in PBS (286 µL, 3.5 mg/mL, 6.7 nmol mAb, 1 equiv) was combined with 57 µL DMSO and 29 µL 0.1M sodium phosphate, monobasic to yield a ~20% and ~10% v/v solution of each, respectively. Addition of all solution components yielded a mixture comprising 2.7 mg/mL mAb, 2.16 mM DMSO, 78 mM sodium phosphate, 115 mM NaCl, pH 5.5. **vcMMAE** (10 µL of a 10 mM stock solution in DMAc, 100 nmol, 15 equiv) was added to the antibody solution, the mixture was vortexed briefly, and the reaction was allowed to proceed at 22 °C or 37 °C with mixing. After 4 h reaction, *N*-acetylcysteine (8 µL of a 100 mM solution, 120 equiv) was added to quench unreacted maleimide groups. Samples were purified using PD Spintrap G-25 devices (GE Healthcare Life Sciences) to remove small molecule components from the reaction mixture. Samples were then analyzed by reduced deglycosylated mass spectrometry as described below.

**Mass spectrometry analysis. mAb-Diene** before and after reaction with **vcMMAE** (15 molar equivalents relative to mAb, 22 °C, 4 h*). Samples were deglycosylated and reduced prior to analysis, spectra are zoomed to show the heavy chain only.

*__mAb-1__ was reacted at 37 °C for 20 h.

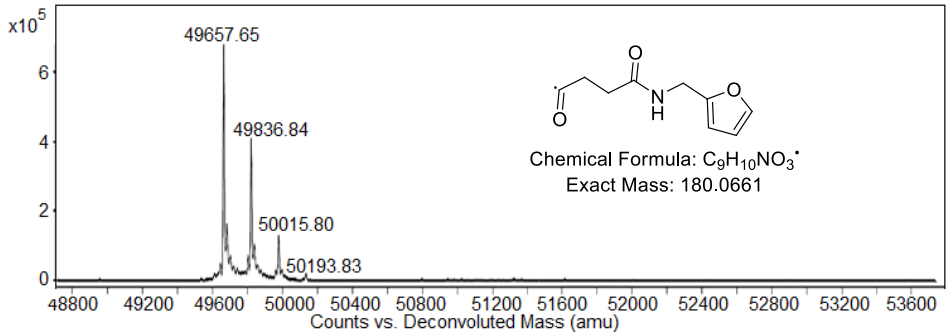



**Figure S51.** Mass spectrum of reduced, deglycosylated antibody-diene conjugate **mAb-1** (LAR = 2.5) showing the antibody heavy chain.

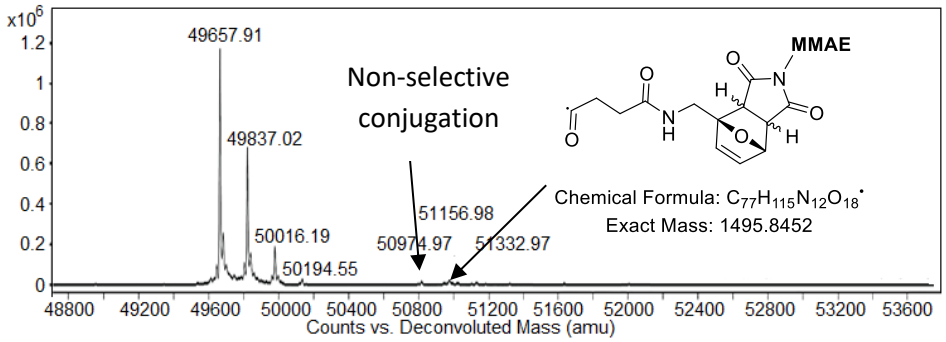



**Figure S52.** Mass spectrum of reduced, deglycosylated **mAb-1-MMAE** DAADC (DAR = 0.03) showing the antibody heavy chain. Non-selective conjugation is indicated; the chemical structure of this linkage is not known. Conjugation is considered non-selective because the drug-linker mass tracked from antibody heavy chain peak lacking furan (49,658 amu).

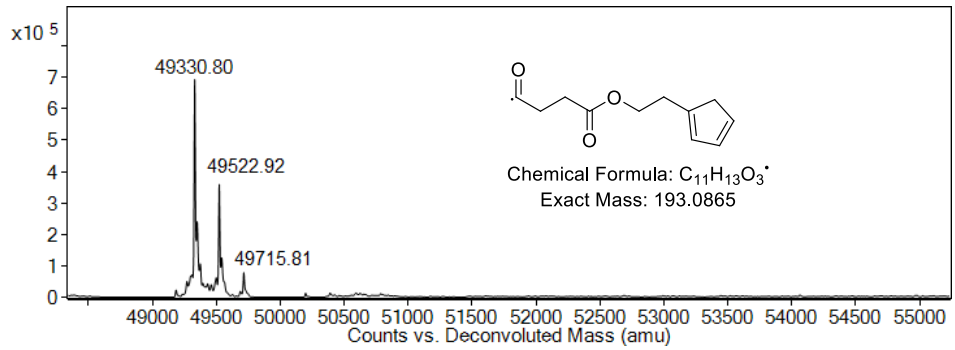



**Figure S53.** Mass spectrum of reduced, deglycosylated antibody-diene conjugate **mAb-2a** (LAR = 2.3) showing the antibody heavy chain.

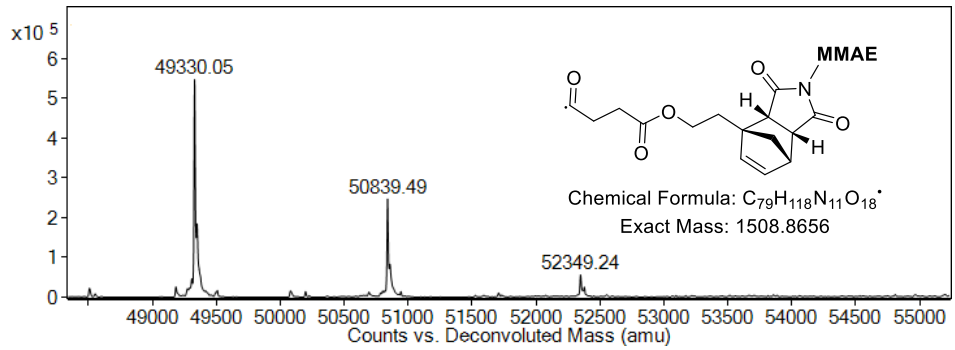



**Figure S54.** Mass spectrum of reduced, deglycosylated **mAb-2a-MMAE** (DAR = 2.3) showing the antibody heavy chain.

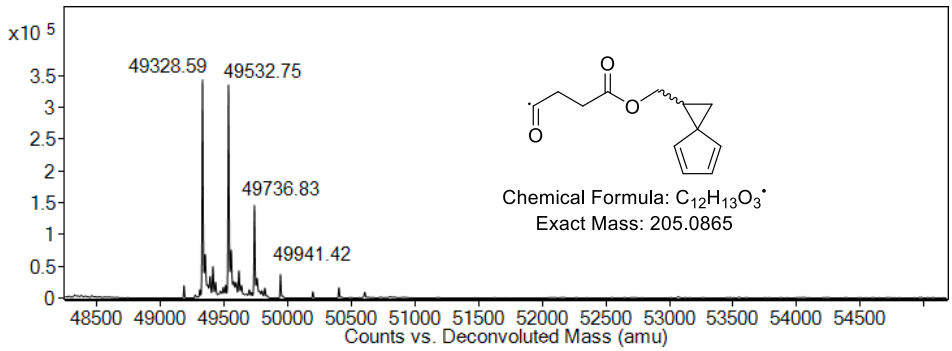



**Figure S55.** Mass spectrum of reduced, deglycosylated antibody-diene conjugate **mAb-3** (LAR = 3.3).

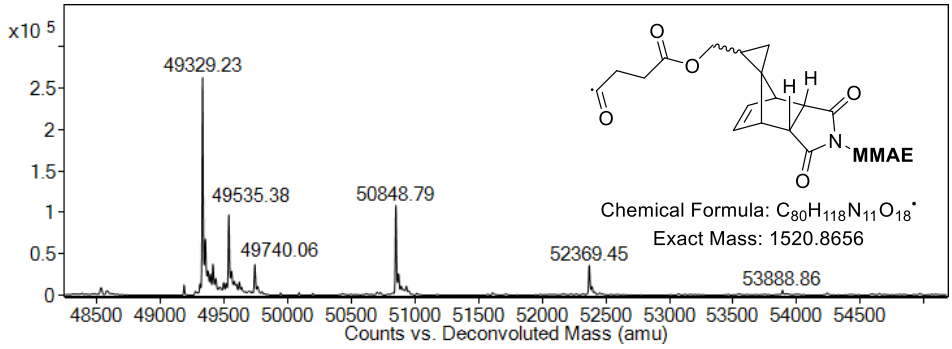



**Figure S56.** Mass spectrum of reduced, deglycosylated **mAb-3-MMAE** DAADC (DAR = 2.4) showing the antibody heavy chain.

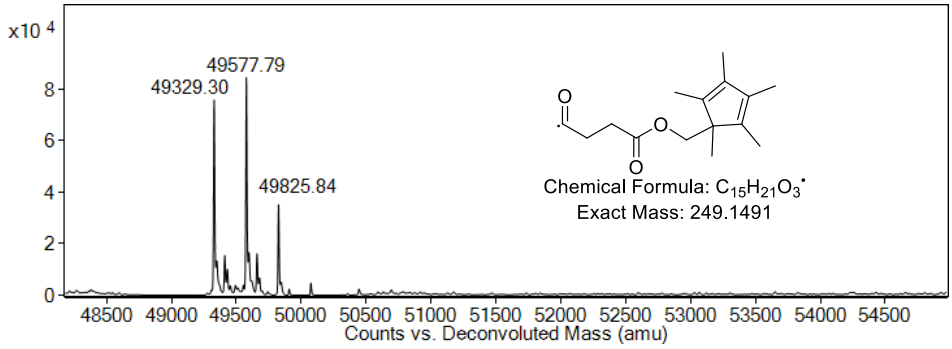



**Figure S57.** Mass spectrum of reduced, deglycosylated antibody-diene conjugate **mAb-4** (LAR = 3.04) showing the antibody heavy chain.

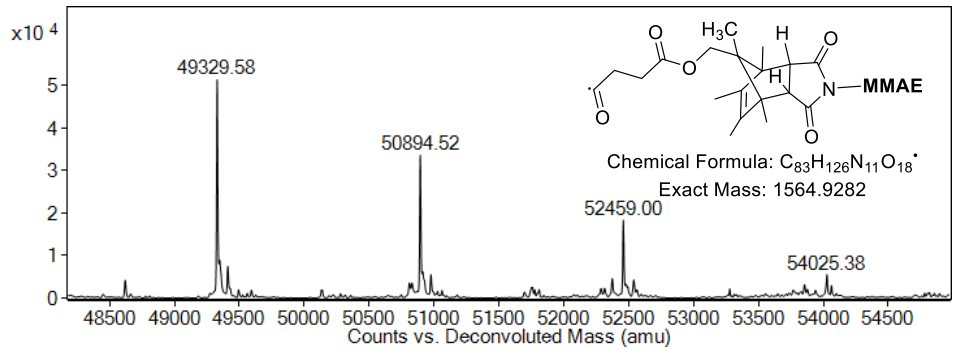



**Figure S58.** Mass spectrum of reduced, deglycosylated **mAb-4-MMAE** (DAR = 3.04) DAADC showing the antibody heavy chain.

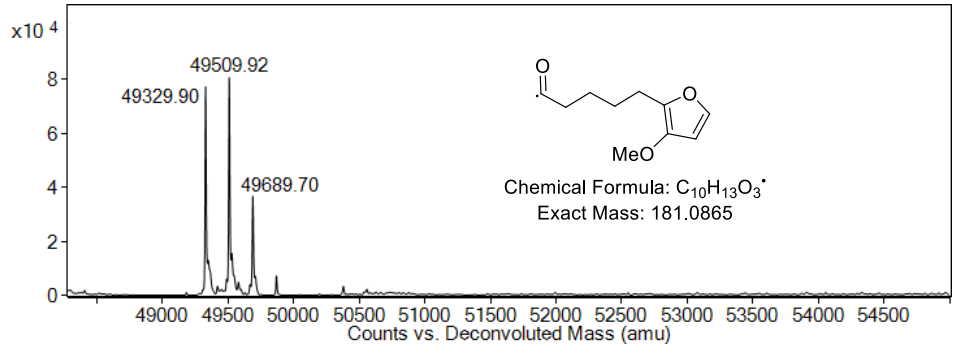



**Figure S59.** Mass spectrum of reduced, deglycosylated antibody-diene conjugate **mAb-5** (LAR = 2.95) showing the antibody heavy chain.

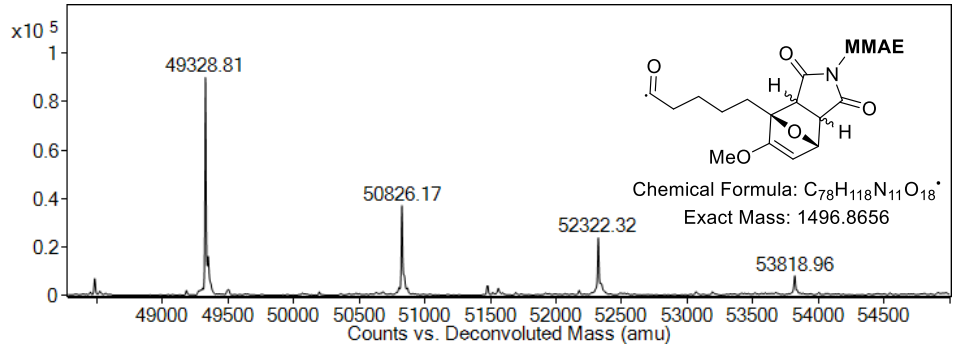



**Figure S60.** Mass spectrum of reduced, deglycosylated **mAb-5-MMAE** DAADC (DAR = 2.95) showing the antibody heavy chain.

### 8. Kinetic Study of vcMMAE with mAb-Dienes.

Reaction progress of **mAb-Diene** with **vcMMAE** was monitored by mass spectrometry. The same general protocol was followed for all samples, with **vcMMAE** feed adjusted for each sample to maintain a 1:1 molar ratio based on diene content. The procedure for analysis of **mAb-2a** is as follows: **mAb-2a** (3.7 **2a**/mAb, 3 mg, 74 nmol **2a**, 1 equiv) was diluted with PBS (pH 7.4) to a final concentration of 1.7 mg/mL. Next, DMSO was added to yield a 20% v/v solution followed by addition of 1 M sodium phosphate monobasic to yield a 10% v/v solution. Addition of all

solution components yielded a mixture comprising 1.3 mg/mL **mAb-2a**, 32.3 μM **2a**, 2.16 mM DMSO, 78 mM sodium phosphate, 115 mM NaCl, pH 5.5. Next, **vcMMAE** (7.4 μL of a 10 mM stock solution in DMSO, 74 nmol, 1 equiv) was added to the antibody solution. The reaction solution was mixed and allowed to proceed at 22 °C with continuous mixing. Aliquots (180 μL) were removed at various time points and *N*-acetylcysteine (3 μL of a 100 mM solution, 51 equivalents) was added to quench unreacted maleimide groups. Samples were then purified using PD Spintrap G-25 devices (GE Healthcare Life Sciences) to remove small molecule components from the mixture. Samples were then analyzed by reduced deglycosylated mass spectrometry as described below.

Relative abundance of unreacted diene was determined by peak intensities in reduced deglycosylated mass spectra using the following equation:

$$\text{diene per mAb} = \left[\left(\frac{b}{a+b+c+d} \times 1\right) + \left(\frac{c}{a+b+c+d} \times 2\right) + \left(\frac{d}{a+b+c+d} \times 3\right) + \left(\frac{f}{e+f+g} \times 1\right) + \left(\frac{g}{e+f+g} \times 2\right)\right] \times 2$$

a = peak intensity of unmodified heavy chain
b = sum of peak intensities of mAb heavy chains with one diene group
c = sum of peak intensities of mAb heavy chains with two diene groups
d = sum of peak intensity of mAb heavy chain with three diene groups
e = peak intensity of unmodified light chain
f = sum of peak intensities of mAb light chains with one diene group
g = sum of peak intensities of mAb light chains with two diene groups

Note that multiple peaks could contribute to each diene containing species. For example, mAb heavy chain with one diene and mAb heavy chain with two dienes, where one is reacted with **vcMMAE** would both be considered as a one-diene species in line (b) above.

Conjugation data were further analyzed in units of molar concentration to determine kinetic constants. Second order rate constants were determined from the slopes of curves generated from plotting 1/[diene] versus time and linear regression analysis. Reaction half-lives were calculated from second-order reaction rate constants using the equation shown below:

$$T_{1/2} = \frac{1}{k_2[\text{diene}]_0}$$

$k_2$ = second order rate constant
$[\text{diene}]_0$= diene concentration at time=0

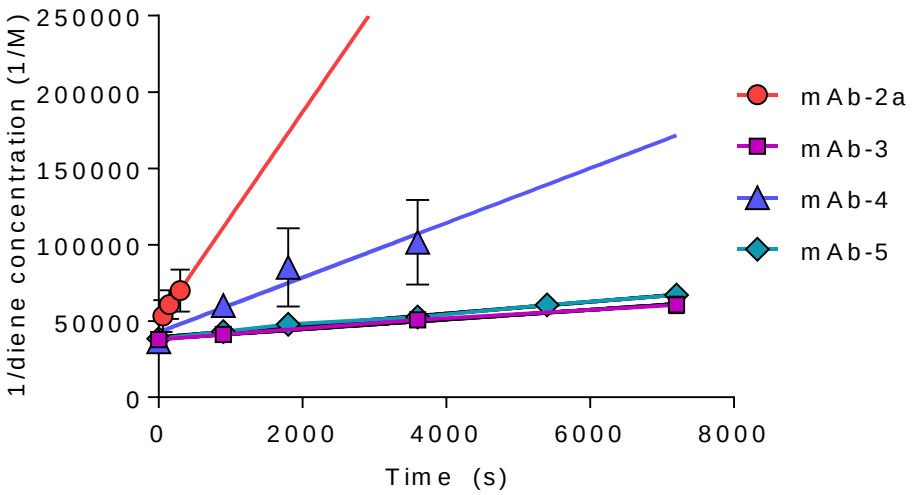



**Figure S61.** Kinetic analysis of the reaction of **mAb-Diene** (1 equiv of diene) with **vcMMAE** (1 equiv). All reactions were performed at 22 °C, unreacted diene concentration was determined by mass spectrometry. Data are plotted as the average value ± standard deviation, n=3, for **mAb-2a** and **mAb-3** samples. Data are plotted as the average value ± absolute error, n=2, for **mAb-4** and **mAb-5** samples. Kinetic experiments were repeated with independent samples. Best fit lines were extrapolated beyond experimental values for **mAb-2a** and **mAb-4** for illustrative purposes.

**9. Preparation of Functional DAADCs.**

Antibody drug-conjugates were prepared from Trastuzumab (**T**, on-target) or R347 (**mAb**, off-target) mAbs using both Diels-Alder conjugation via linkers and direct conjugation to antibody cysteine thiols. Diels-Alder ADCs were prepared from **2b** and **5** linker-modified antibodies using the same general procedure described for **mAb-2a** as follows: **mAb-2b** (10 mg, 67 nmol, 1 equiv) was diluted to 4.27 mg/mL with PBS, pH 7.4, followed by addition of DMSO (493 µL) and 1 M sodium phosphate monobasic (247 µL) to yield ~20% and 10% v/v solutions respectively. **vcMMAE** (53.3 µL of 10 mM stock in DMSO, 530 nmol, 8 equiv) was added to the antibody solution and the reaction continued at room temperature with mixing for 4 h. *N*-Acetylcysteine (43 µL of a 100 mM solution in water, 4.3 µmol, 64 equiv) was added to quench unreacted maleimides. ADC was purified from the reaction mixture using CHT chromatography. ADC solution was diluted 3-fold with distilled water and loaded onto a Bio-Scale Mini Cartridge CHT Type II 40 µm media column. ADC was eluted with a gradient from buffer A (10 mM phosphate, pH 7.0) to buffer B (10 mM phosphate pH 7.0 containing 2M NaCl) over 25 minutes at a flow rate of 5 mL/min. After CHT chromatography ADC sample was buffer exchanged to PBS using a slide-a-lyzer cassette at 4 °C. The same procedure was followed for ADCs prepared with **mAb-5** constructs, with the exception that the **vcMMAE** conjugation reaction continued for 24 h at room temperature. 8 equivalents of **vcMMAE** relative to mAb used for the conjugation reaction corresponds to approximately 2 molar equivalents relative to diene (linker-antibody ratio (LAR) or drug-antibody ratio (DAR) content for each mAb is provided on the mass spectra).

ADCs were also prepared by conjugation of **vcMMAE** to cysteine thiols contained in the antibody hinge region. First, antibody (10 mg, 67 nmol, 1 equiv) solution was adjusted to 2.5 mg/mL with PBS containing 1 mM EDTA. Next, TCEP (10 μL of 50 mM solution in water, 500 nmol, 7.5 equiv) was added to reduce hinge disulfides, and the mixture was incubated at 37 °C with mixing for 1 h. Next, DMSO (410 μL, 10% v/v final concentration in reaction) was added followed by addition of **vcMMAE** (30 μL of 10 mM solution in DMSO, 300 nmol, 4.5 equiv) and the reaction continued at room temperature with mixing for 1 h. *N*-Acetylcysteine was added to quench unreacted maleimide groups and ADC was purified by CHT chromatography and dialysis as described above.

**Mass Spectrum Analysis.** Antibodies were deglycosylated and analyzed intact. Peak masses are indicated as well as the number of linkers conjugated for each peak.

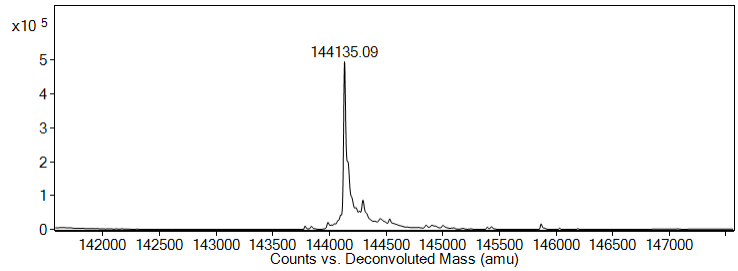
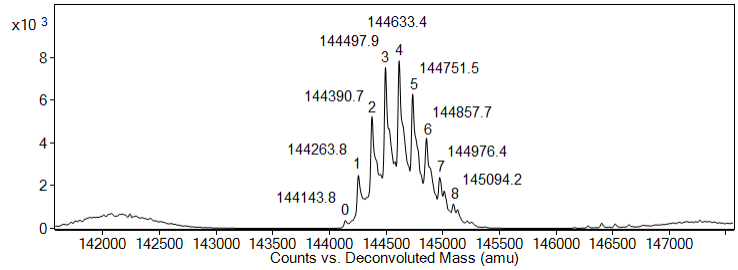
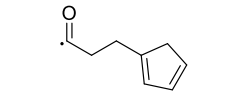


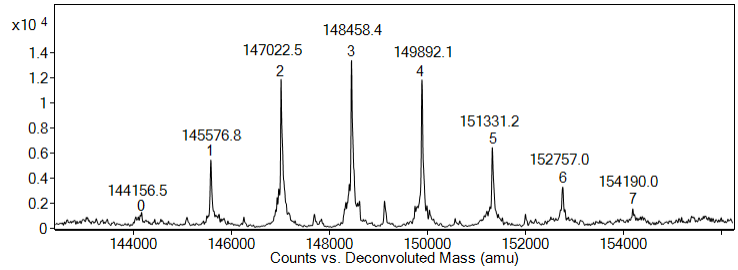

**Figure S62.** Mass spectrum of intact, deglycosylated non-targeting **mAb**.

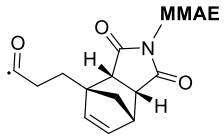


Chemical Formula: $C_8H_9O^{\bullet}$
Exact Mass: 121.07

**Figure S63.** Mass spectrum of intact, deglycosylated non-targeting antibody-diene conjugate **mAb-2b** (LAR = 3.7).




Chemical Formula: $C_{76}H_{114}N_{11}O_{16}^{\bullet}$
Exact Mass: 1436.84

**Figure S64.** Mass spectrum of intact, deglycosylated non-targeting **mAb-2b-MMAE** DAADC (DAR = 3.4).

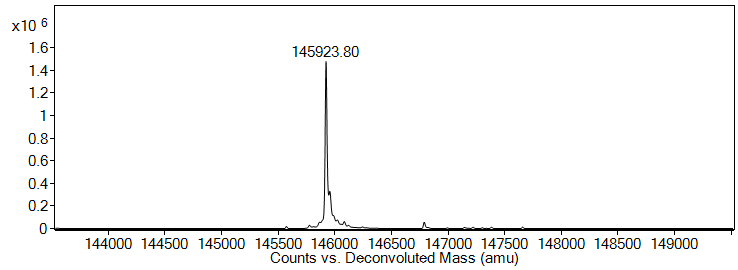
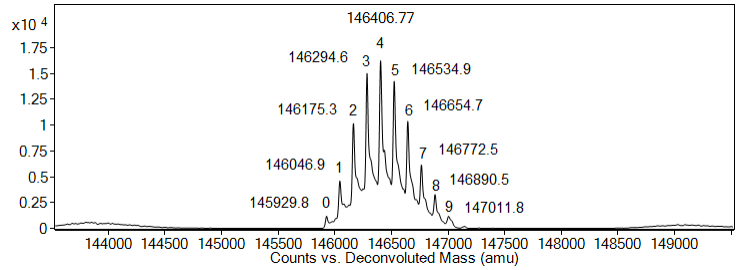



**Figure S65.** Mass spectrum of intact, deglycosylated antibody **T**.



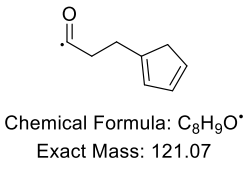
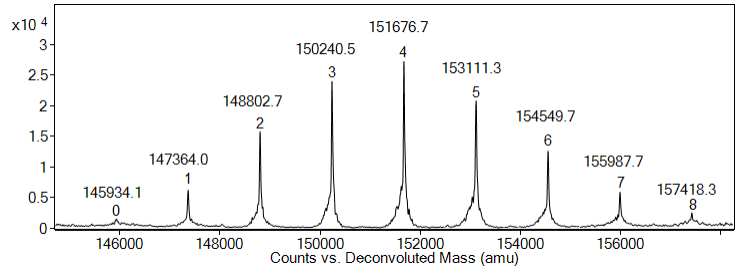




**Figure S66.** Mass spectrum of intact, deglycosylated antibody-linker conjugate **T-2b** (LAR = 4.1).



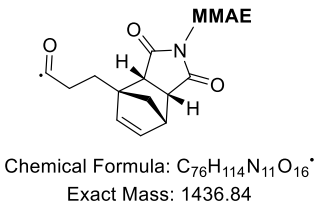
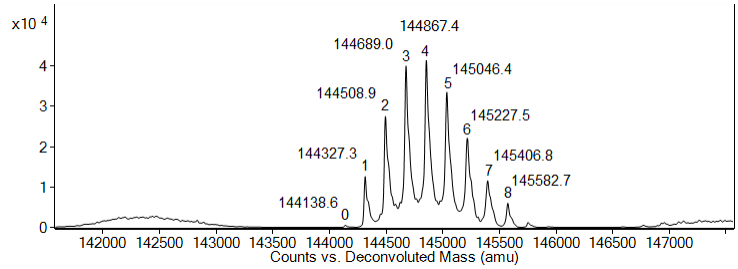




**Figure S67.** Mass spectrum of intact, deglycosylated **T-2b-MMAE** DAADC (DAR = 3.9).



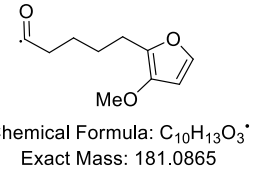
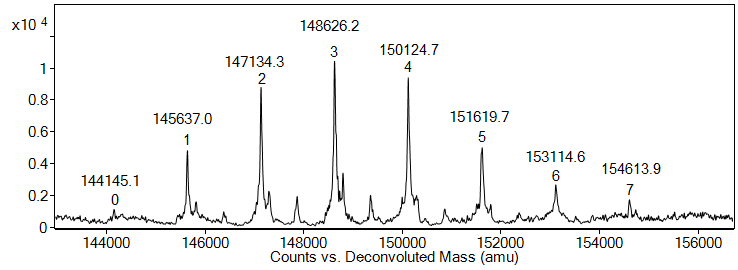




**Figure S68.** Mass spectrum of intact, deglycosylated non-targeting antibody-diene conjugate **mAb-5** (LAR = 3.8).



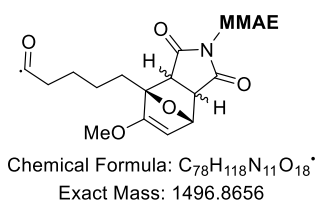




**Figure S69.** Mass spectrum of intact, deglycosylated non-targeting **mAb-5-MMAE** DAADC (DAR = 3.2).

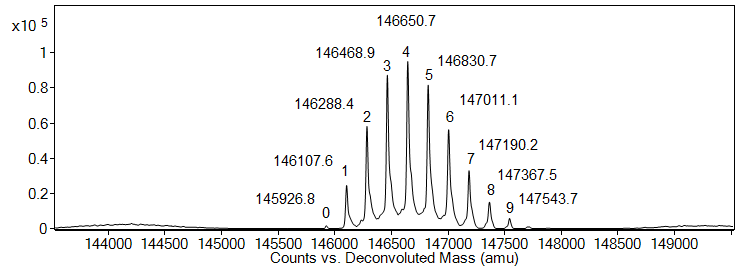



**Figure S70.** Mass spectrum of intact, deglycosylated antibody-diene conjugate **T-5** (LAR = 4.0).

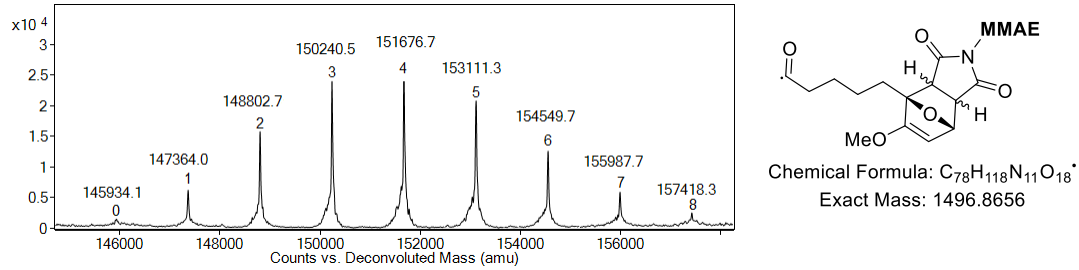



**Figure S71.** Mass spectrum of intact, deglycosylated **T-5-MMAE** DAADC (DAR = 3.5).

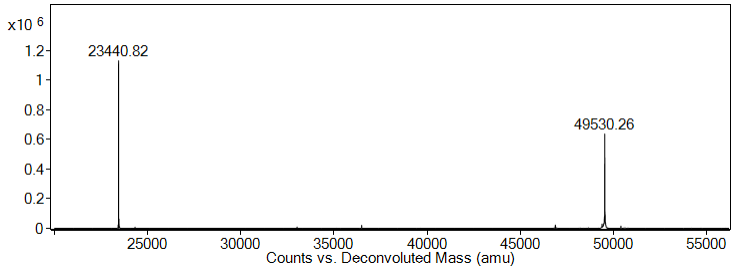



**Figure S72.** Mass spectrum of reduced, deglycosylated antibody **T**.

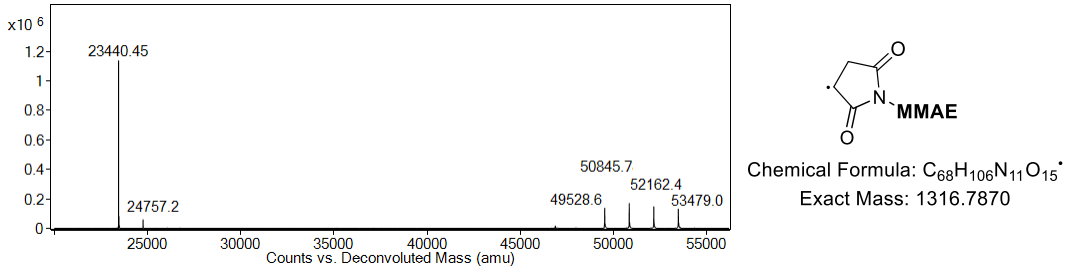



**Figure S73.** Mass spectrum of reduced, deglycosylated **T-Cys** ADC (DAR = 3.2).

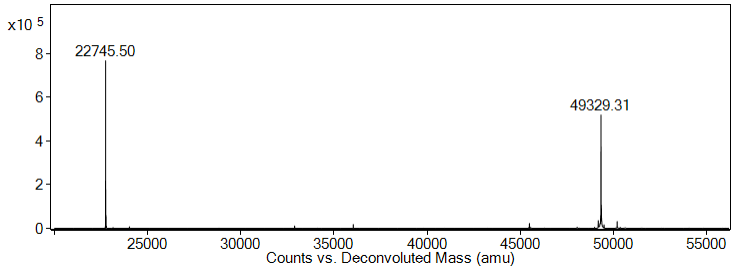



**Figure S74.** Mass spectrum of reduced, deglycosylated non-targeting **mAb**.

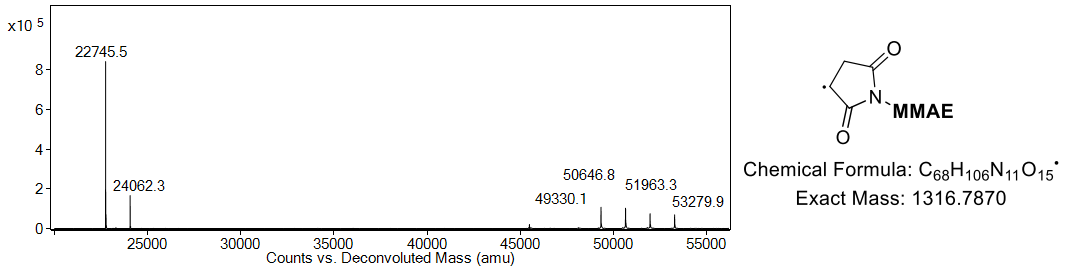



**Figure S75.** Mass spectrum of reduced, deglycosylated **mAb-Cys** ADC (DAR=2.9).

**rRP-HPLC analysis.** For each analysis, the antibodies and ADCs were reduced at 37 °C for 20 minutes using 42 mM dithiothreitol (DTT) in PBS pH 7.2. 10 µg of reduced antibodies and ADCs was loaded onto a PLRP-S, 1000Å column (2.1 x 50 mm, Agilent) and eluted at 40°C at a flow rate of 0.5 mL/min with a gradient of 5% B to 100% B over 60 minutes (mobile phase A: 0.1% trifluoroacetic acid in water, and mobile phase B: 0.1% trifluoroacetic acid in acetonitrile). Percent conjugation was determined using integrated peak areas from the chromatogram.

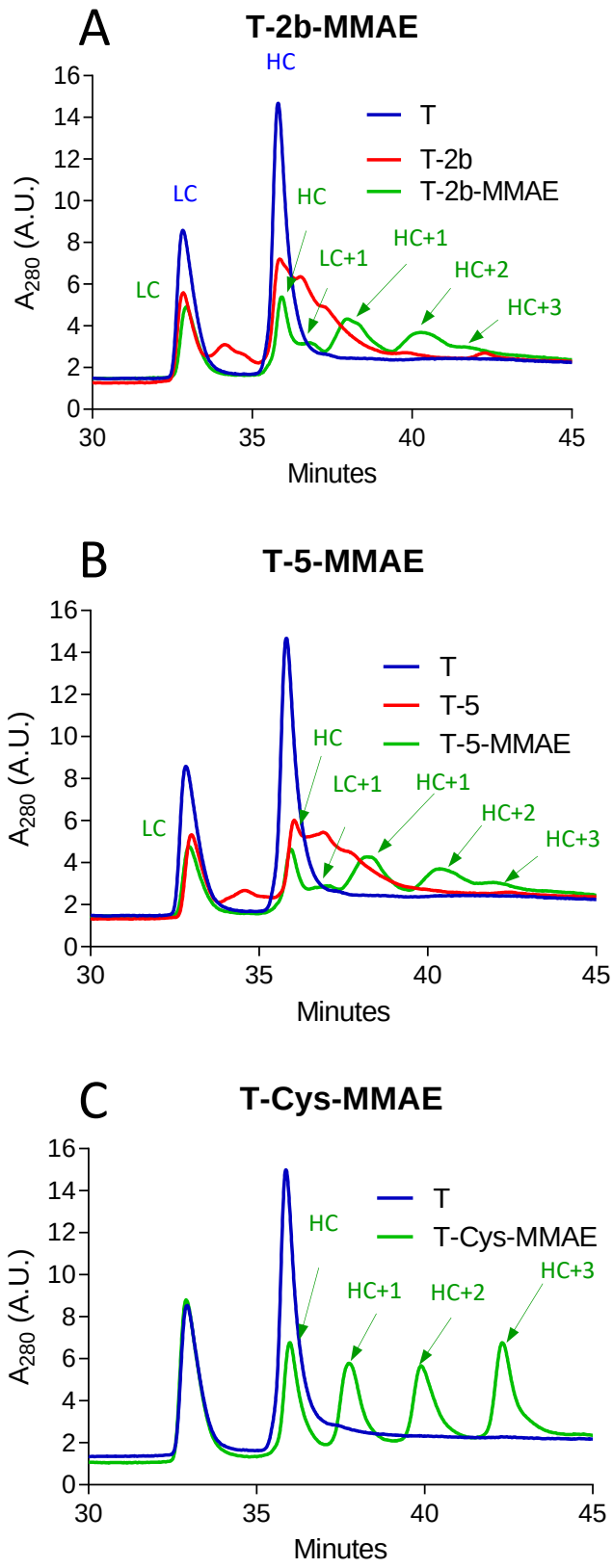



**Figure S76.** rRP-HPLC traces of **T** ADCs made using **2b** (A), **5** (B) or native thiols (C).

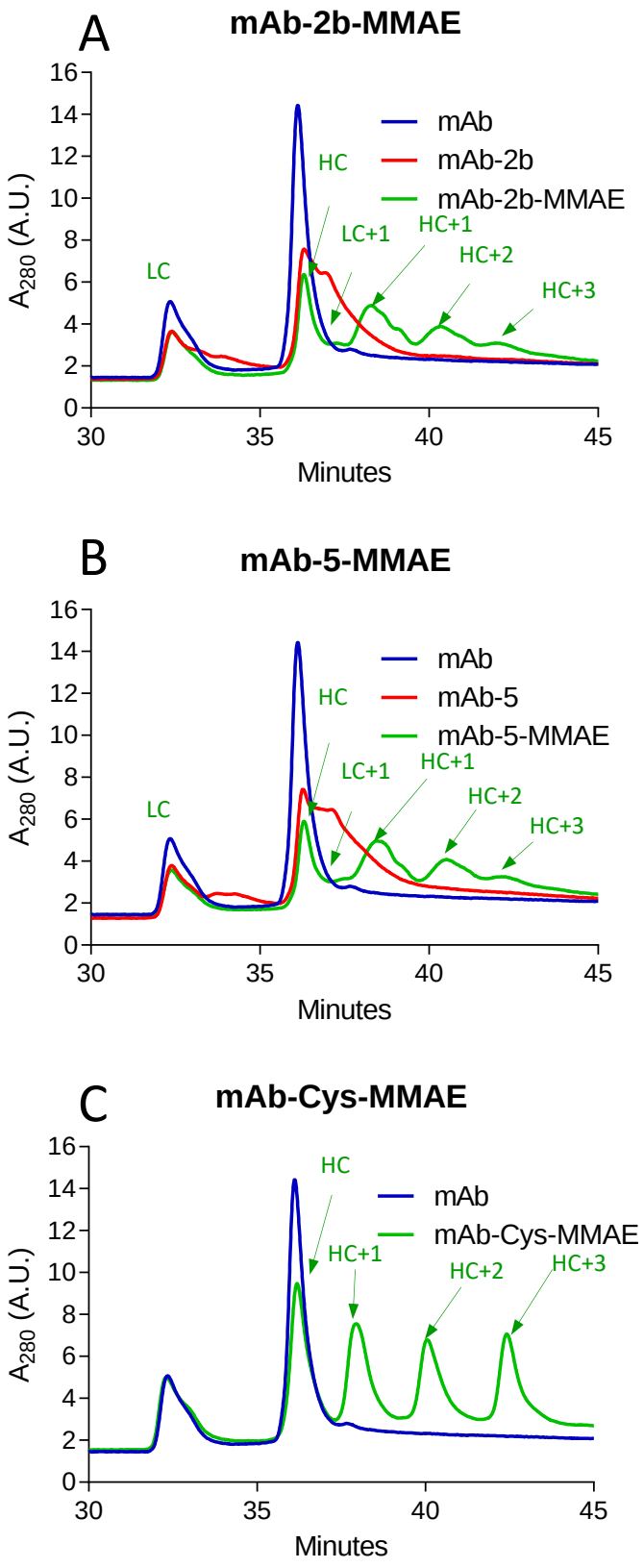



**Figure S77.** rRP-HPLC traces of control ADCs made using **2b** (A), **5** (B) or native thiols (C).

**Size exclusion chromatography.** SEC analysis was performed using an Agilent 1100 Capillary LC system equipped with a triple detector array (Viscotek 301, Viscotek, Houson, TX); the wavelength was set to 280 nm, and samples (50 μg) were run on a TSK-GEL G3000SWXL column (Toso Bioscience LLC, Montgomeryville, PA) using 100 mM sodium phosphate buffer, 10% isopropyl alcohol, pH 6.8 at a flow rate of 1 mL/min. All ADC samples showed >90% monomer content.

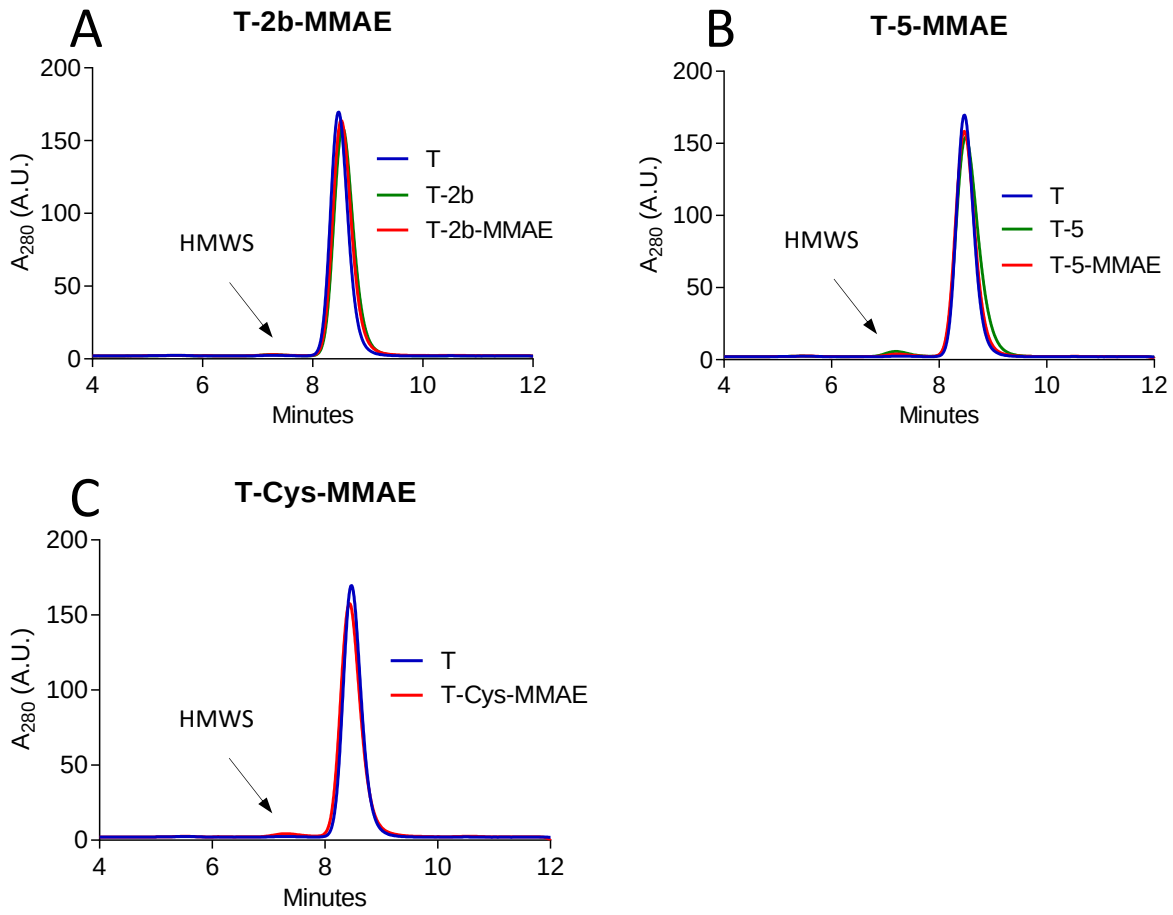



**Figure S78.** SEC traces of **T** ADCs made using **2b** (A), **5** (B) or native thiols (C). High molecular weight species (HMWS) are indicated.

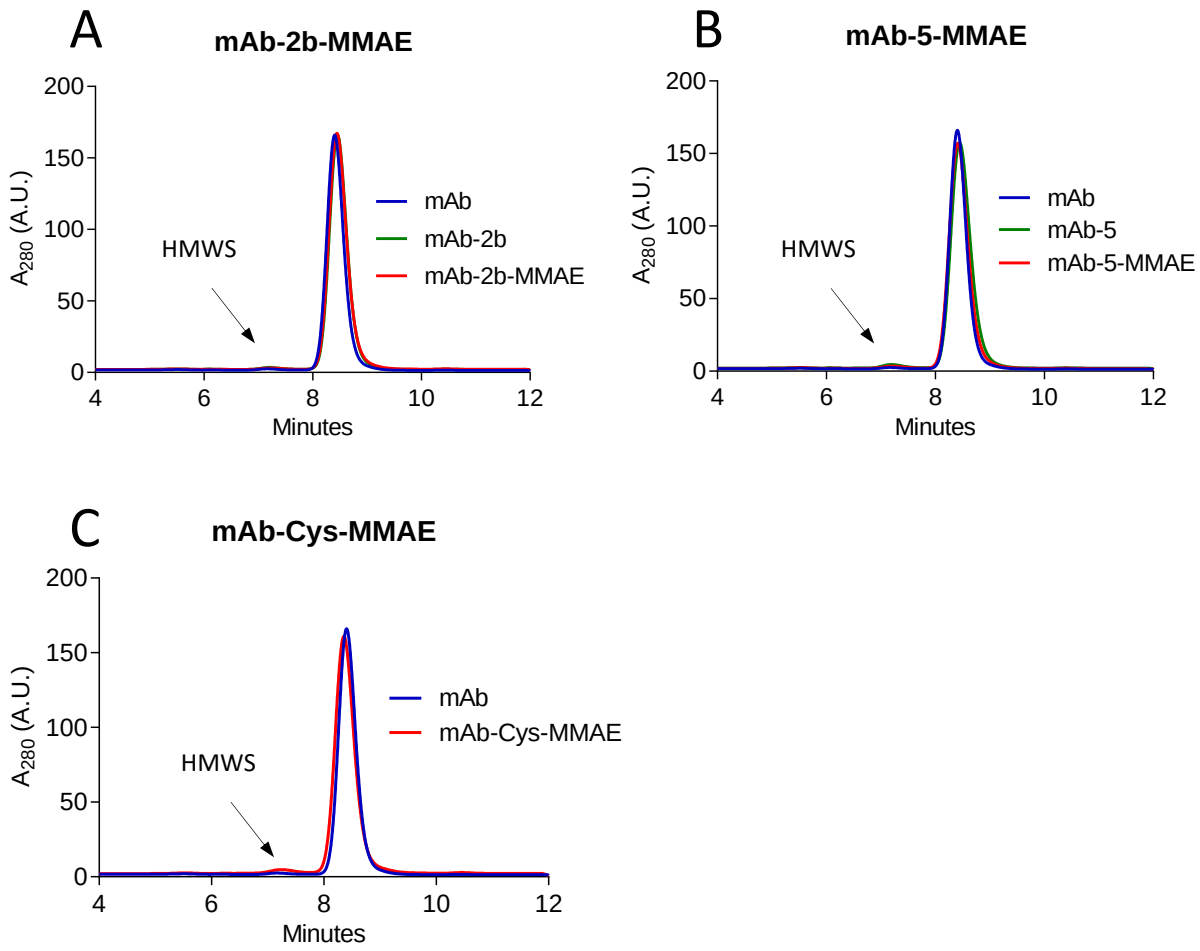



**Figure S79.** SEC traces of control antibody **mAb** ADCs made using **2b** (A), **5** (B) or native thiols (C). High molecular weight species (HMWS) are indicated.

**10. Serum Stability Study.**

**Method.** ADCs were incubated in rat and mouse serum to challenge the stability of the antibody-payload linkage. ADCs were added to normal rat or normal mouse serum (Jackson Immunoresearch) to achieve a final concentration of 0.2 mg/mL (1.33 µM antibody), with the total volume of ADC solution added to serum less than 10%. The ADC-serum mixture was sterile filtered and an aliquot was removed from this mixture and frozen as a t=0 control. Remaining sample was then further incubated at 37 ˚C in a sealed container for 7 d. Conjugated and unconjugated human antibody was recovered from serum by immunoprecipitation using Fc-specific anti-human IgG-agarose resin (Sigma-Aldrich). Resin was rinsed twice with PBS, once with IgG elution buffer, and then twice more with PBS. ADC-serum samples were then combined with anti-human IgG resin (100 µL of ADC-serum mixture, 50 µL resin slurry) and mixed for 15 minutes at room temperature. Resin was recovered by centrifugation and then washed twice with PBS. Washed resin was resuspended in 100 µL IgG elution buffer (Thermo Scientific) and further incubated for 5 minutes at room temperature. Resin was removed by centrifugation and then 20 µL of 10X glycobuffer 1 (New England Biolabs) was added to the supernatant. Recovered human

antibody solution was sterile filtered, and incubated with EndoS for 1 h at 37 °C. Deglycosylated mAbs were then reduced with TCEP and analyzed by LC/MS as described above. Percent conjugated antibody was determined from peak heights of mass spectra.

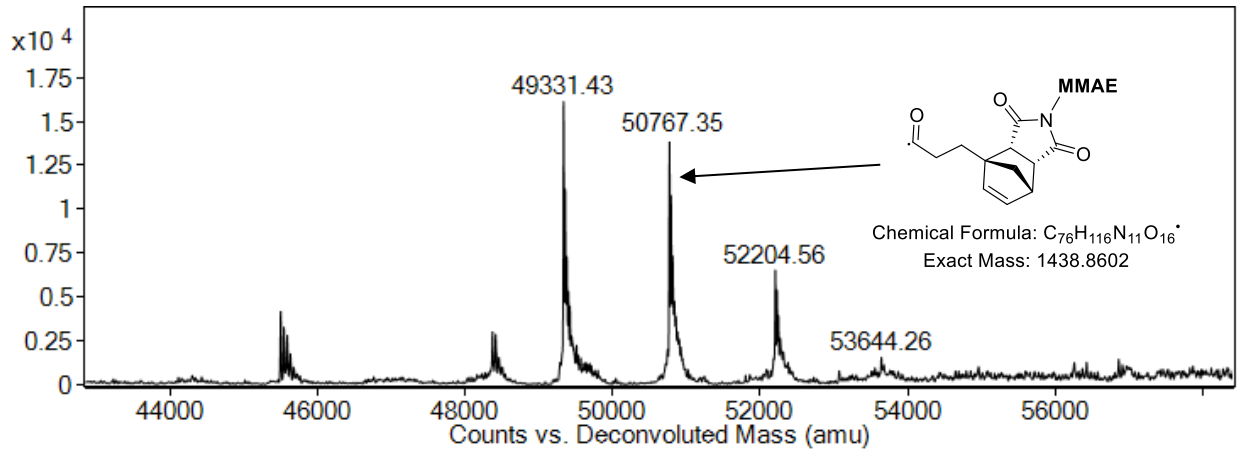



**Figure S80.** Rat serum stability study mass spectrum of reduced, deglycosylated **mAb-2b-MMAE** showing the antibody heavy chain, T = 0 days.

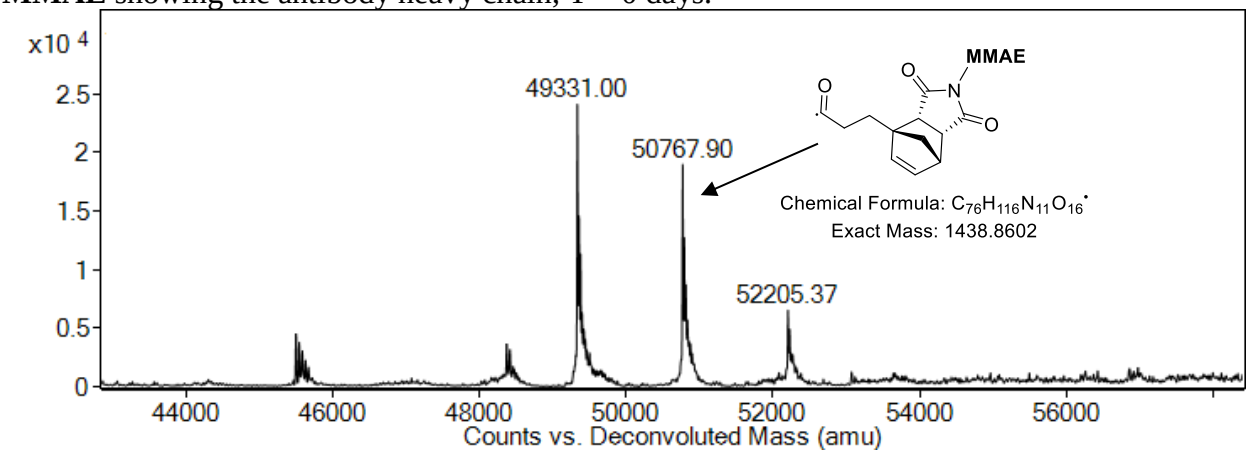



**Figure S81.** Rat serum stability study mass spectrum of reduced, deglycosylated **mAb-2b-MMAE** showing the antibody heavy chain, T = 7 days.

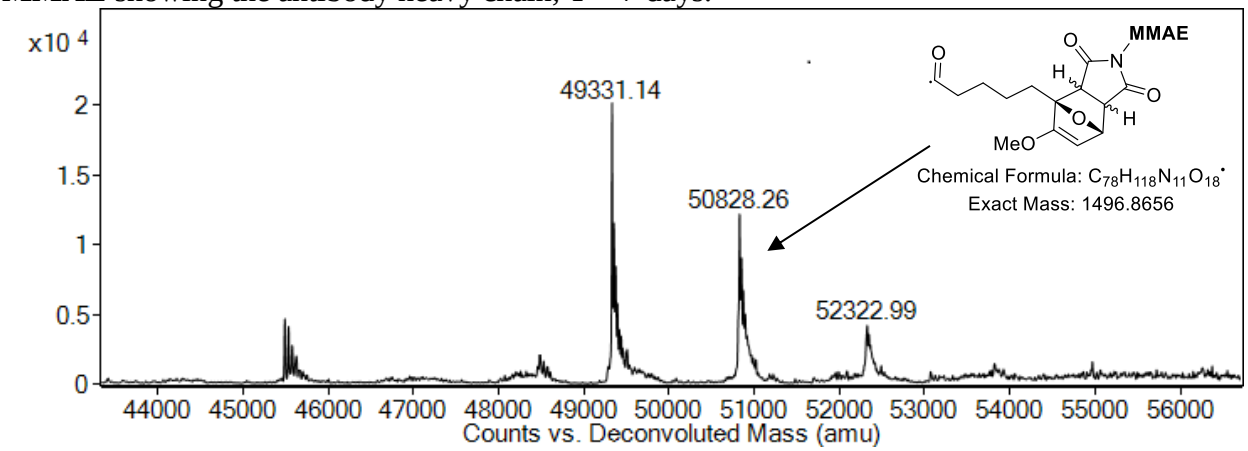



**Figure S82.** Rat serum stability study mass spectrum of reduced, deglycosylated **mAb-5-MMAE** showing the antibody heavy chain, T = 0 days.

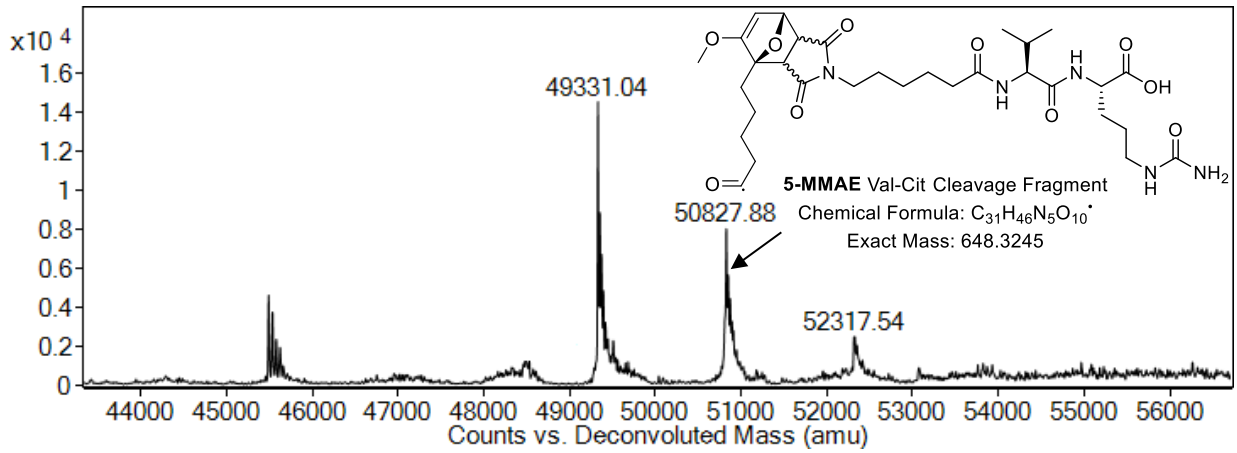



**Figure S83.** Rat serum stability study mass spectrum of reduced, deglycosylated **mAb-5-MMAE** showing the antibody heavy chain, T = 7 days.

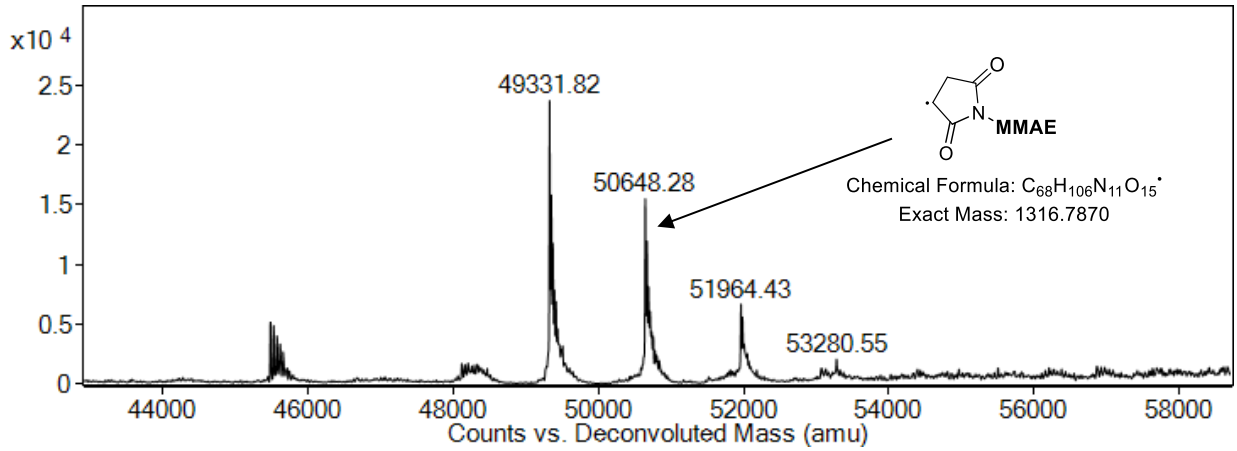



**Figure S84.** Rat serum stability study mass spectrum of reduced, deglycosylated **mAb-Cys-MMAE** showing the antibody heavy chain, T = 0 days.

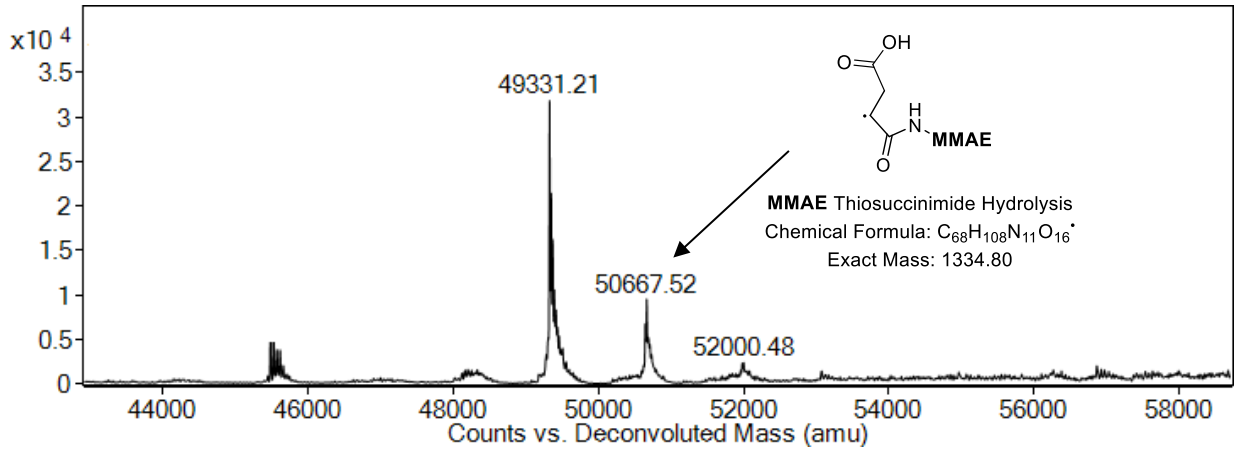



**Figure S85.** Rat serum stability study mass spectrum of reduced, deglycosylated **mAb-Cys-MMAE** showing the antibody heavy chain, T = 7 days. Remaining drug is attached through a hydrolyzed thiosuccinimide linkage.

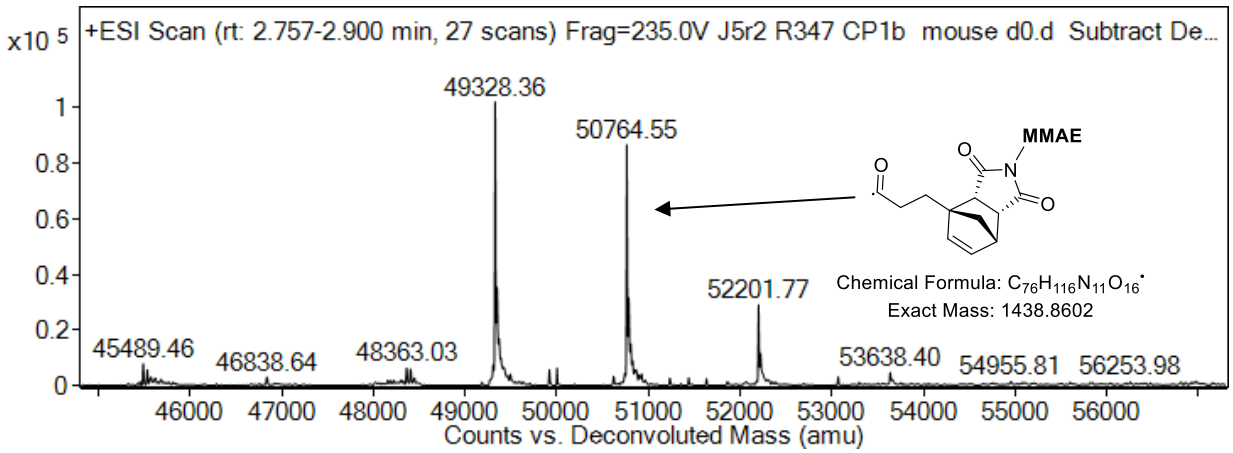



**Figure S86.** Mouse serum stability study mass spectrum of reduced, deglycosylated **mAb-2b-MMAE** showing the antibody heavy chain, T = 0 days.

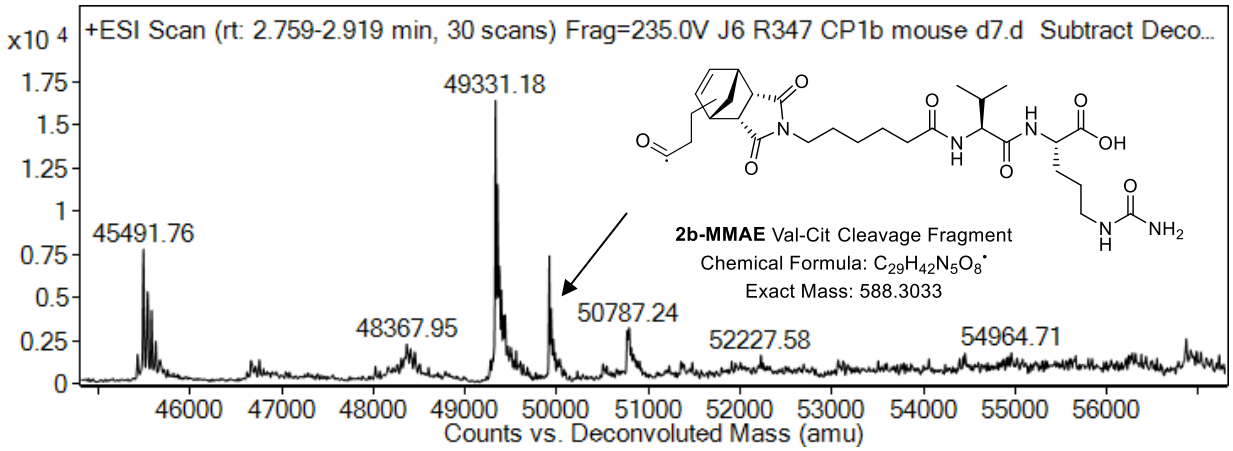



**Figure S87.** Mouse serum stability study mass spectrum of reduced, deglycosylated **mAb-2b-MMAE** showing the antibody heavy chain, T = 7 days. Drug loss through cleavage of the Val-Cit linker is indicated.

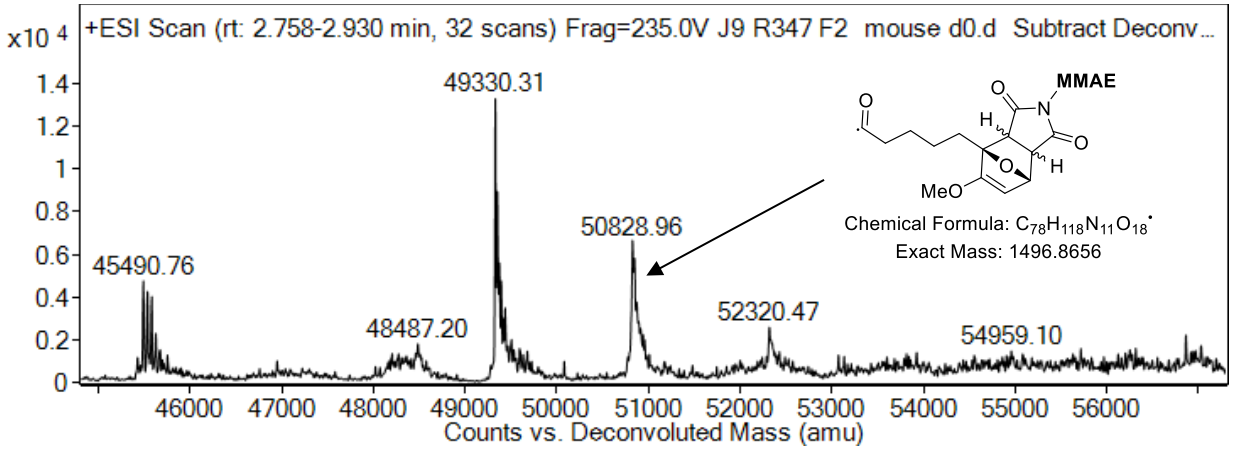



**Figure S88.** Mouse serum stability study mass spectrum of reduced, deglycosylated **mAb-5-MMAE** showing the antibody heavy chain, T = 0 days.

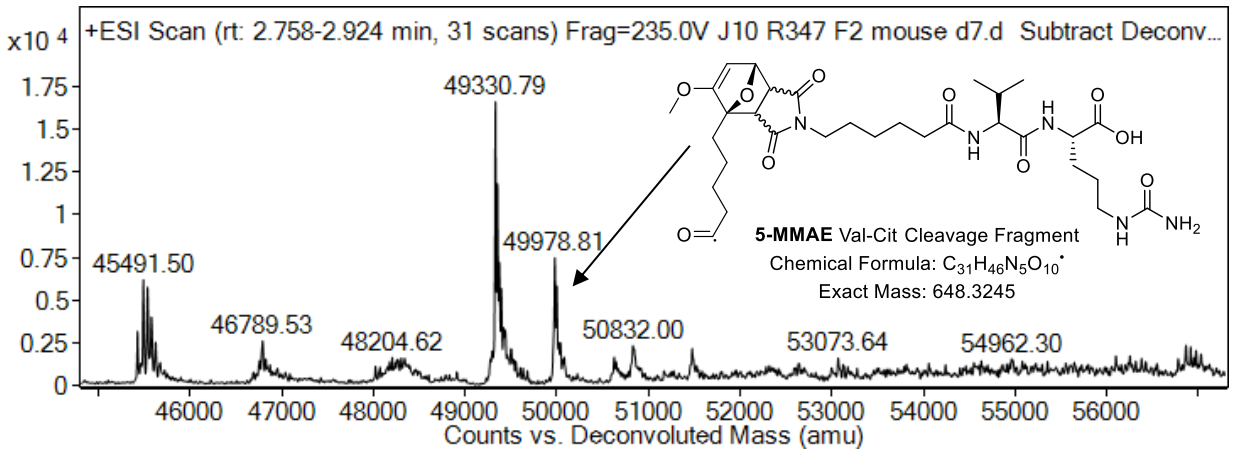



**Figure S89.** Mouse serum stability study mass spectrum of reduced, deglycosylated **mAb-5-MMAE** showing the antibody heavy chain, T = 7 days. Drug loss through cleavage of the Val-Cit linker is indicated.

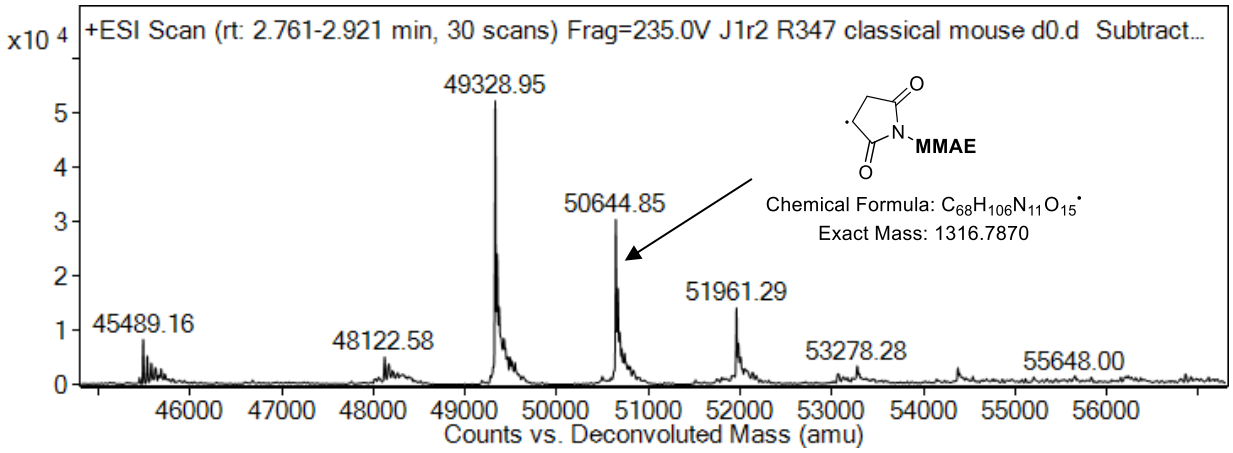



**Figure S90.** Mouse serum stability study mass spectrum of reduced, deglycosylated **mAb-Cys-MMAE** showing the antibody heavy chain, T = 0 days.

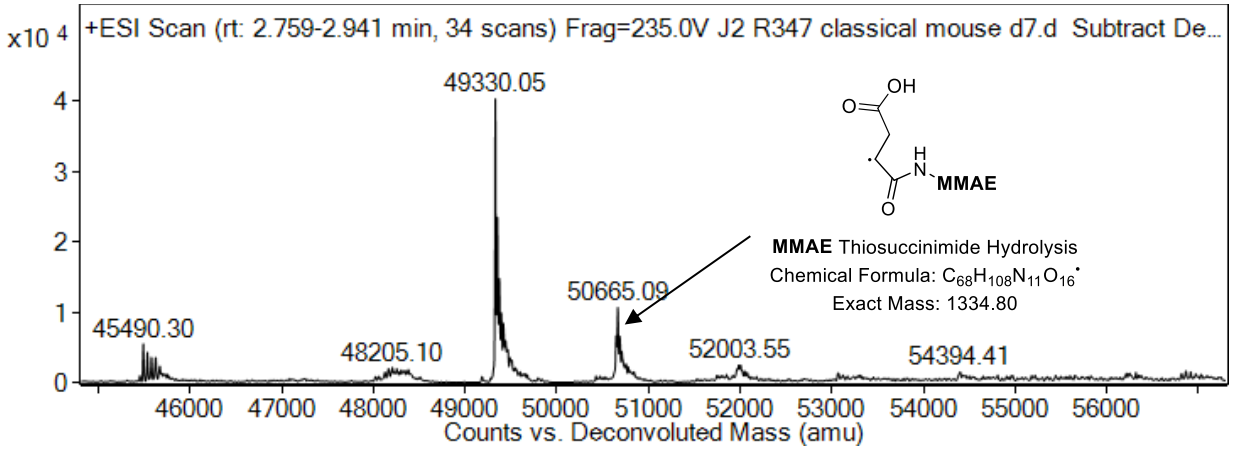



**Figure S91.** Mouse serum stability study mass spectrum of reduced, deglycosylated **mAb-Cys-MMAE** showing the antibody heavy chain, T = 7 days. Remaining drug is attached through a hydrolyzed thiosuccinimide linkage.

**11. *In Vitro* Cytotoxicity Study.**

Human gastric cancer cell line NCI-N87 and human breast cancer cell line SKBR3 were obtained from American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 media (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (Life Technologies) at 37°C in 5% $CO_2$. SKBR3 and NCI-N87 cells harvested in exponential growth phase were seeded in 96-well culture plates at 2500 and 2000 cells/well and allowed to adhere overnight. Cells were then treated on the following day with ADCs at 4-fold serial dilutions starting from 4000 or 64,000 ng/mL (9 concentrations) in duplicate. The treated cells were cultured for 6 days and cell viability was determined using the CellTiter-Glo Luminescent Viability Assay (Promega) following the manufacturer's protocol. Cell viability was calculated as a percentage of untreated control cells. $IC_{50}$ values were determined using logistic non-linear regression analysis with Prism software (GraphPad). $IC_{50}$s can be found in the manuscript text.

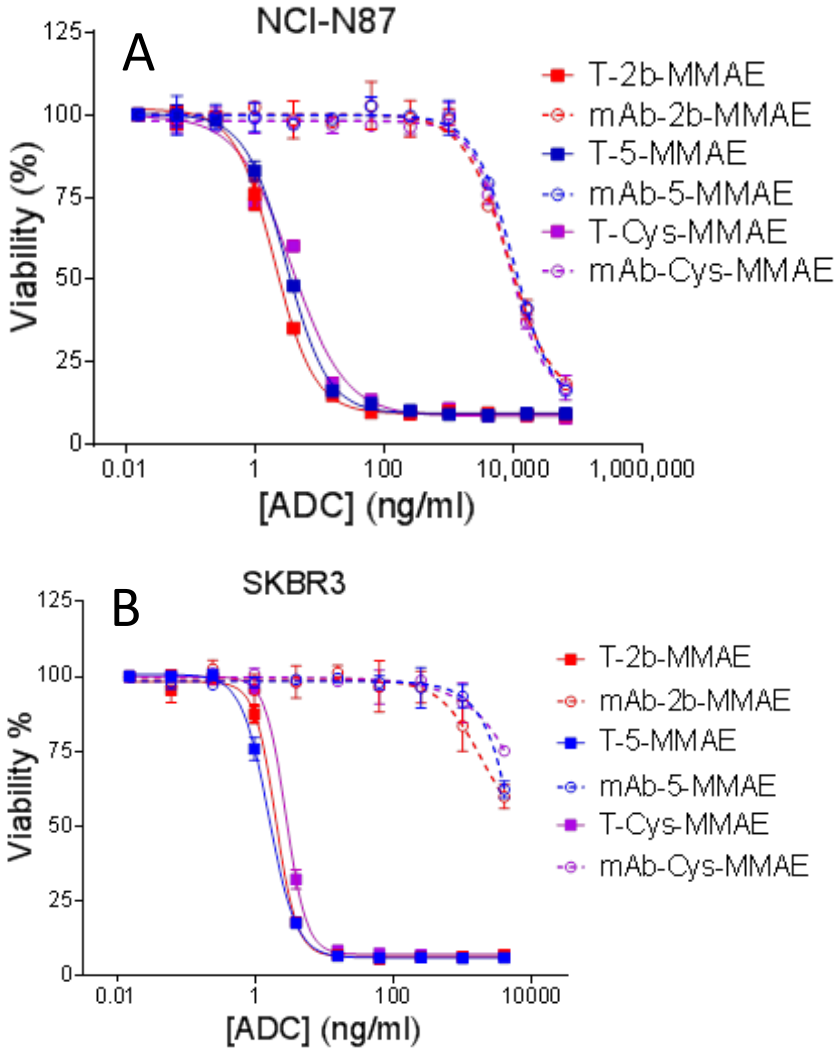



**Figure S92.** Cytotoxicity study of ADCs on human cancer cell lines NCI-N87 (**A**), and SKBR3 (**B**).

## 12. *In Vivo* Tumor Growth Inhibition Study.

Tumor growth inhibition studies were performed at MedImmune and all animal procedures were performed in accordance with appropriate regulatory standards under protocols approved by the MedImmune Institutional Animal Care and Use Committee. Trastuzumab ADCs **T-2b-MMAE**, **T-5-MMAE**, and **T-Cys-MMAE** were evaluated for antitumor activity in vivo in a subcutaneous N87 xenograft model in mice. Tumors were prepared by inoculation of N87 cells (5 million N87 cells in 50% Matrigel) subcutaneously into 4-6 week old female athymic nude mice. When tumors reached approximately 200 $mm^3$, mice were randomly assigned into groups, 5 mice per group. ADCs were administered IV at the indicated doses and dosed at day 5 post cell inoculation. Tumor dimensions (long axis and short axis) were measured twice weekly with calipers. Tumor volume was calculated using the equation:

$$V = \frac{1}{2} a \times b^2$$

Where,
a = tumor long axis in mm
b = tumor short axis in mm

## 13. References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