Total Syntheses of Cyclohelminthol I-IV Reveals a New Covalent Reactive Group

Thomas T. Paulsen[1,#], Anders E. Kiib[1,#], Gustav J. Wørmer[1], Stephan M. Hacker[2] and Thomas B. Poulsen[1]*

[1]Department of Chemistry, Aarhus University, Langelandsgade 140, 8000 Aarhus C, Denmark

[2]Leiden Institute of Chemistry, Leiden University, Einsteinweg 55, 2333CC Leiden, The Netherlands

[#] These authors contributed equally

Email: thpou@chem.au.dk

Supporting Information

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1. Supporting figures

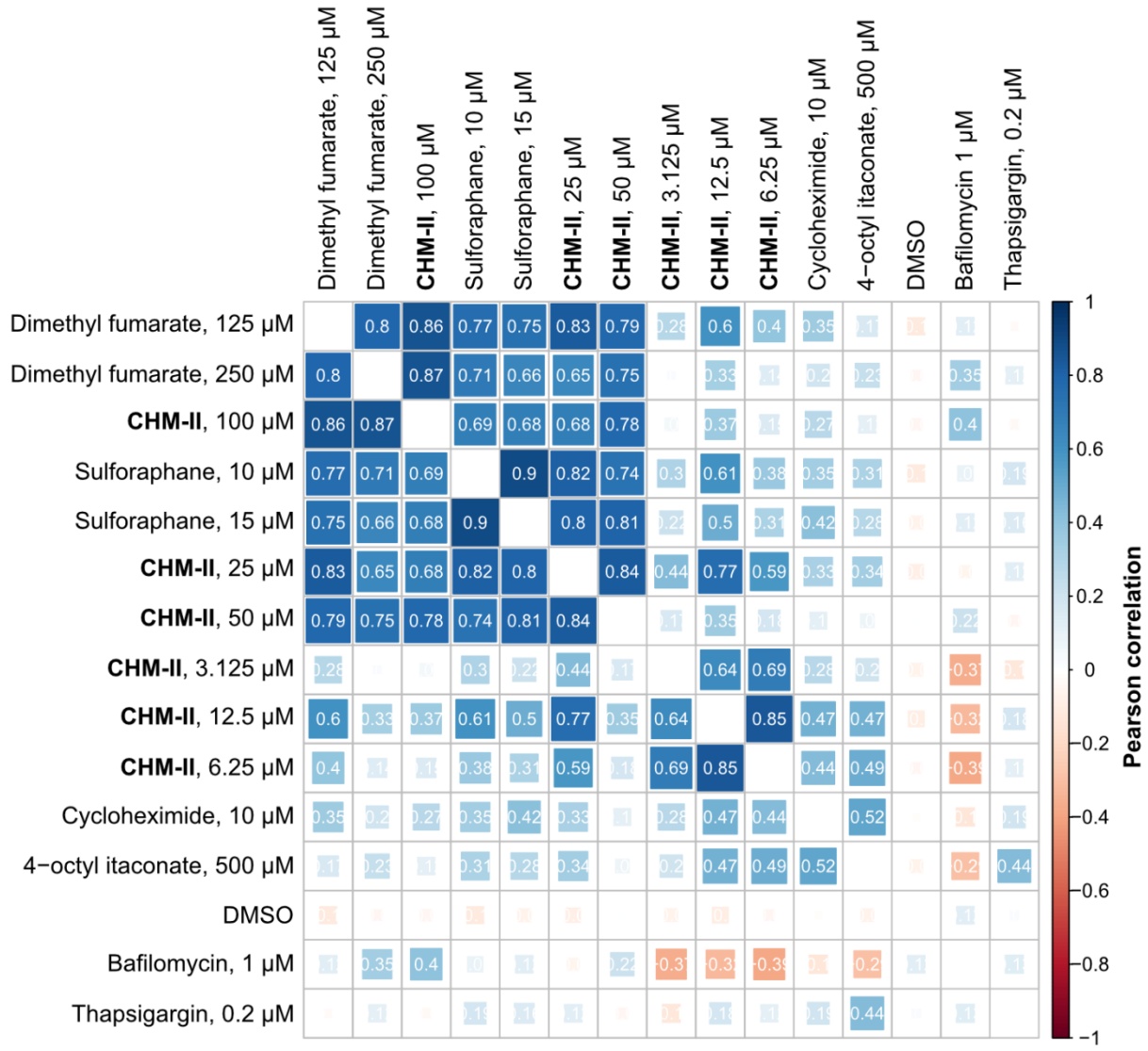


Figure S1. Correlation matrix containing **CHM-II** at various concentrations as well as select compounds from the in-house library of the Poulsen group. Pearson correlations > 0.7 are considered strong.

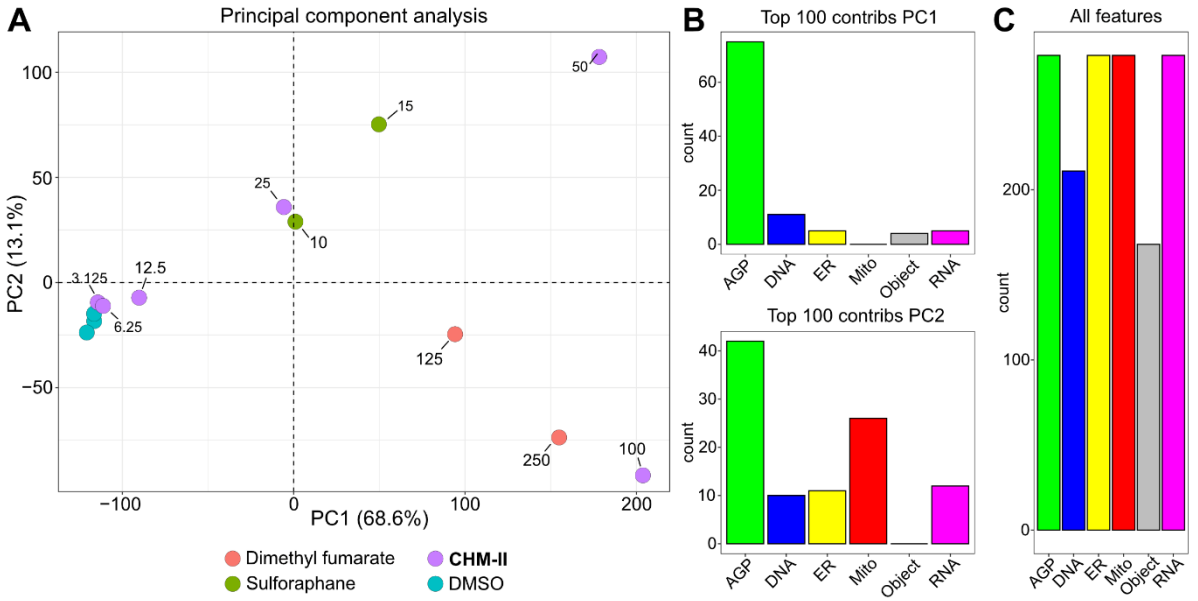


Figure S2. Results of PCA analysis with the morphological profiles of a number of selected compounds (**CHM-II**, DMF, Sulforaphane, and DMSO) at varying concentrations. **A)** A PCA plot of the first two principal components, which explain > 80 % of the variance in the data subset. The observation that the lower, active concentrations of **CHM-II** sub-cluster with sulforaphane, while the 100 µM profile sub-clusters with DMF, is observed once more in the notable shift in the contribution of PC2 going from 50 µM to 100 µM. While some of the shift might be explained by the onset of toxicity, it may also well be due to a concentration-dependent shift in the morphologically dominating mechanism of action of the compound. B) Fluorescence channels associated with the 100 highest contributing features of the first two principal components. A strong enrichment in features associated with the AGP (actin, Golgi, and plasma membrane) channel can be observed for both principal components, while PC2 also has a significant contribution from the mitochondrial channel. The differences in the main contributing features at different concentrations may aid future work to elucidate the mechanism of action of **CHM-II**. C) Distribution of features over all channels ('object' represents features not directly related to a specific channel).

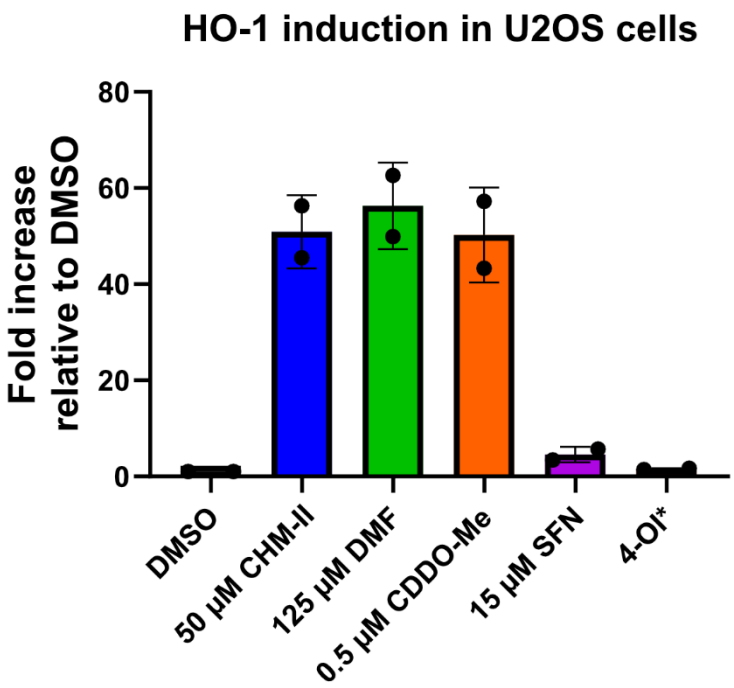


Figure S3. Quantification of HO-1 induction by **CHM-II** in U-2OS cells. Background-subtracted intensities were extracted in ImageQuant v8.2.0, the intensities were adjusted based on the β-actin signals (loading control), and the adjusted intensities were normalized to the DMSO-levels. The data presented is from two biological replicate experiments for all compounds except for 4-OI, where the concentration was increased from 80 µM in the first experiment to 125 µM in the second experiment. As the increase in HO-1 was similar between the two doses of 4-OI (1.5- and 1.7-fold of DMSO, respectively), the data has been included even as the experiments are no longer exact replicates for 4-OI. HO-1: Heme oxygenase 1, DMF: Dimethyl fumarate, CDDO-Me: Bardoxolone methyl ester, SFN: Sulforaphane, 4-OI: 4-octyl itaconate.

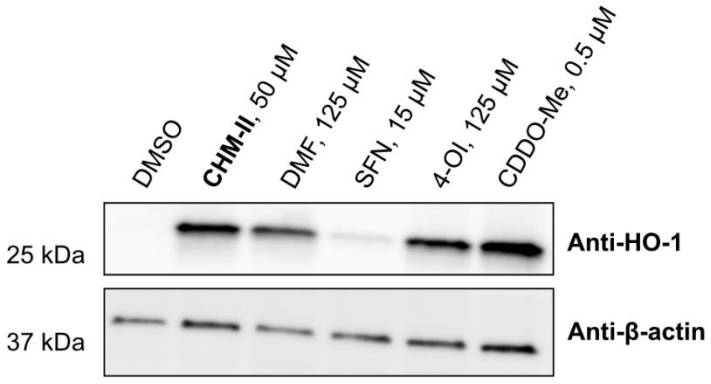


Figure S4. Induction of HO-1 by **CHM-II** after 24 hours of treatment in live 786-O cells. DMF: Dimethyl fumarate, SFN: Sulforaphane, 4-OI: 4-octyl itaconate, CDDO-Me: Bardoxolone methyl ester. HO-1: Heme oxygenase 1.

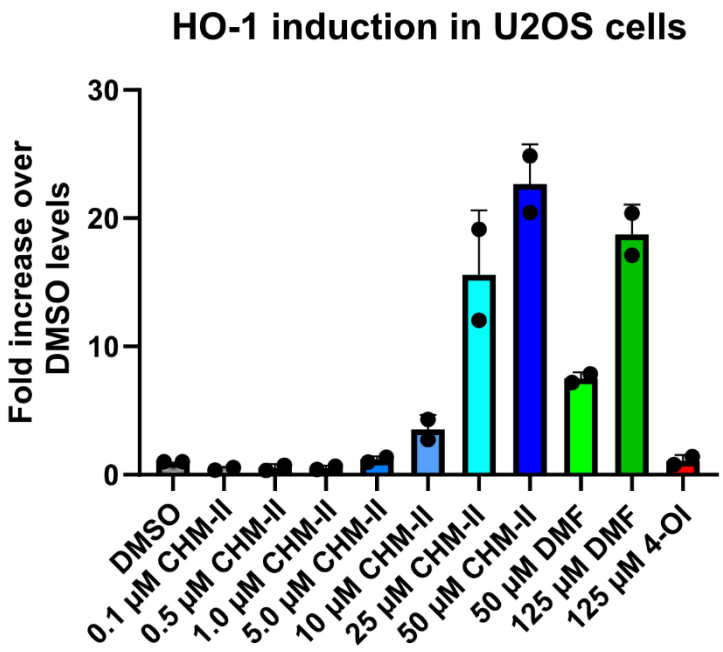


Figure S5. Quantification of HO-1 induction at varying concentrations of **CHM-II,** DMF, and 4-OI in U-2OS cells. Background-subtracted intensities were extracted in ImageQuant v8.2.0, the intensities were adjusted based on the β-actin signals (loading control), and the adjusted intensities were normalized to the DMSO-levels. DMF: Dimethyl fumarate, 4-OI: 4-octyl itaconate.

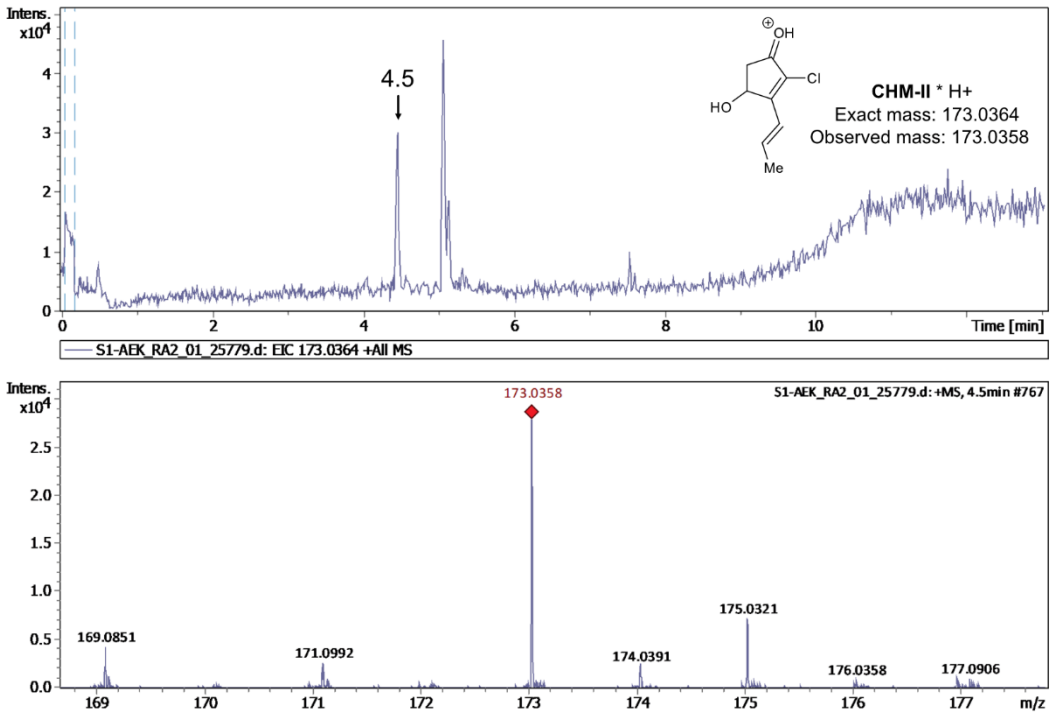


Figure S6. Extracted ion chromatogram (EIC) of remaining **CHM-II** after 1.5 hours of incubation of **CHM-II** with MTG in TEAB-buffer (pH 8.1, 10 % MeCN). The exact mass of the protonated species was determined in ChemDraw 22.2.0.3300, and the EIC was extracted

using Bruker Compass DataAnalysis 5.1. S1: The sample number in the LC-MS-run, for the reaction mixture. The spectrum view (bottom) has been zoomed to highlight the presence of the M+2 peak expected for the chlorine atom. The peaks with retention times between 5 and 6 minutes are likely results of fragmentation of the C-S bond in the monoconjugate species **19** (refer to figure S7). The DataAnalysis file and raw data have been uploaded to OSF (see main text).

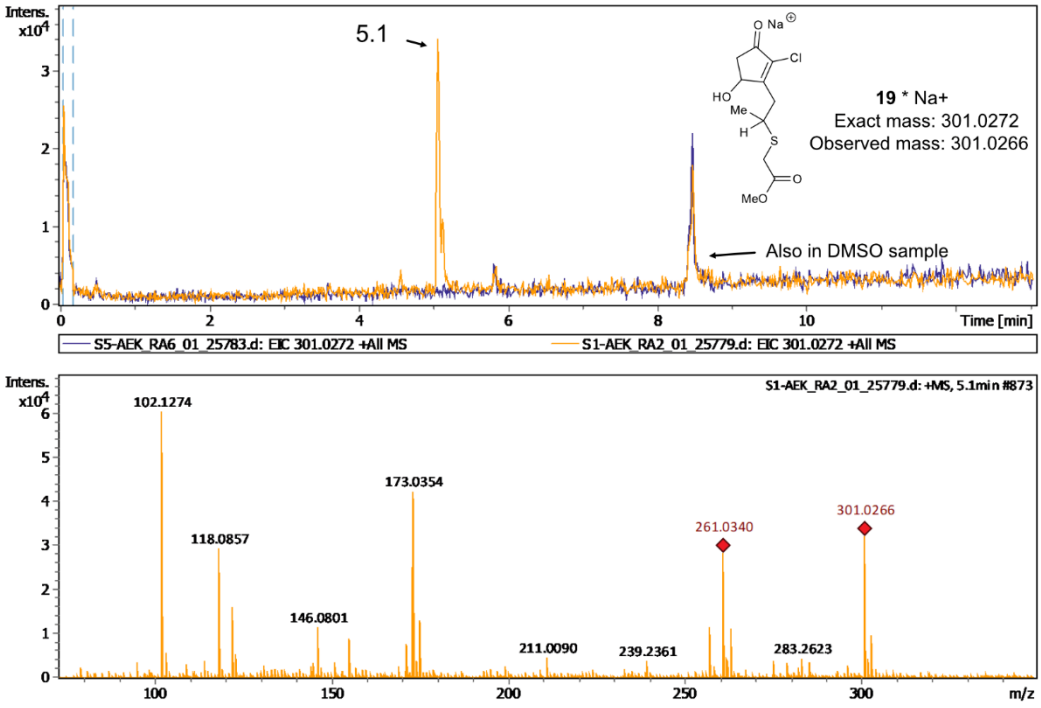


Figure S7. Extracted ion chromatogram (EIC) indicating the formation of monoconjugate species **19**, after 1.5 hours of incubation of **CHM-II** with MTG in TEAB-buffer (pH 8.1, 10 % MeCN). The exact mass of the sodium ion coordinated species was determined in ChemDraw 22.2.0.3300, and the EIC was extracted using Bruker Compass DataAnalysis 5.1. The peak with a retention time > 8 minutes was found in both the reaction mixture (S1, orange) and the DMSO control sample (S5, dark blue). The DataAnalysis file and raw data have been uploaded to OSF (see main text).

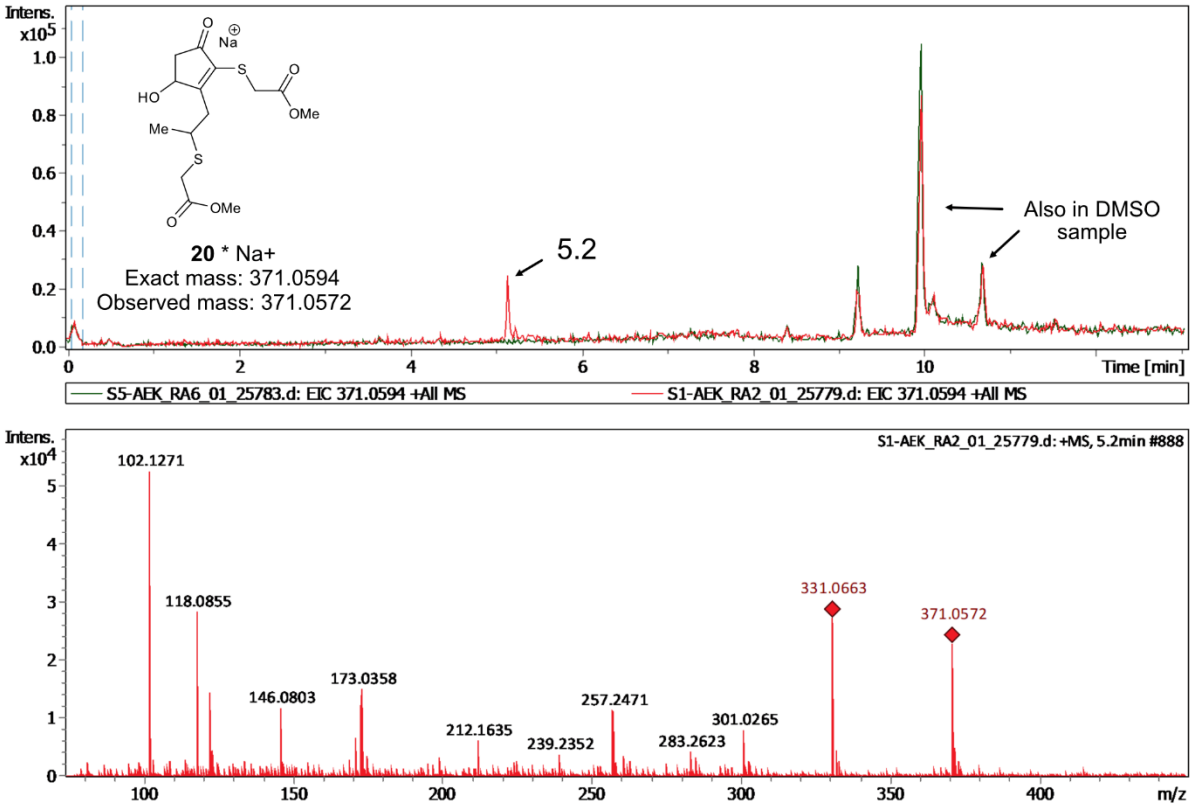


Figure S8. Extracted ion chromatogram (EIC) indicating the formation of diconjugate species **20**, after 1.5 hours of incubation of **CHM-II** with MTG in TEAB-buffer (pH 8.1, 10 % MeCN). The exact mass of the sodium ion coordinated species was determined in ChemDraw 22.2.0.3300, and the EIC was extracted using Bruker Compass DataAnalysis 5.1. The peak with a retention time > 8 minutes was found in both the reaction mixture (S1, red) and the DMSO control sample (S5, green). The DataAnalysis files and raw data have been uploaded to OSF (see main text).

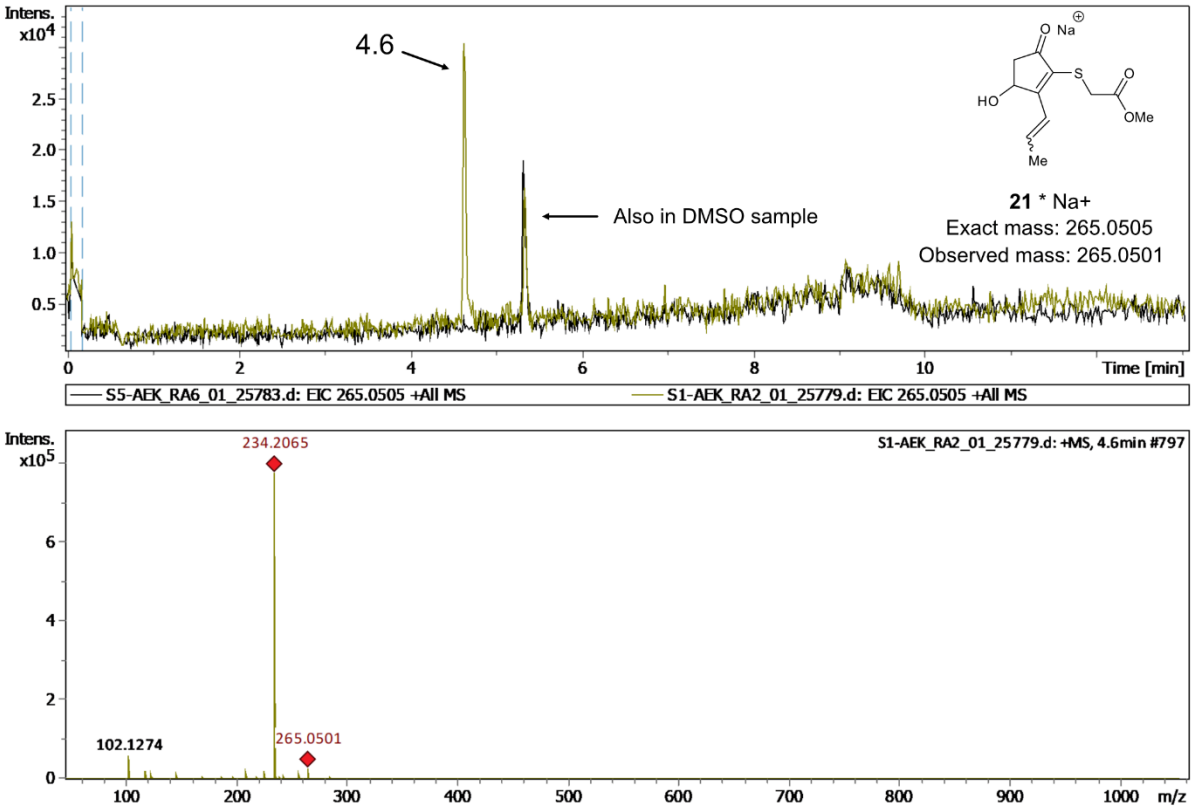


Figure S9. Extracted ion chromatogram (EIC) indicating the formation of compound **21**, after 1.5 hours of incubation of **CHM-II** with MTG in TEAB-buffer (pH 8.1, 10 % MeCN). The exact mass of the sodium ion coordinated species was determined in ChemDraw 22.2.0.3300, and the EIC was extracted using Bruker Compass DataAnalysis 5.1. The second peak was found in both the reaction mixture (S1, green) and the DMSO control sample (S5, black). The DataAnalysis file and raw data have been uploaded to OSF (see main text).

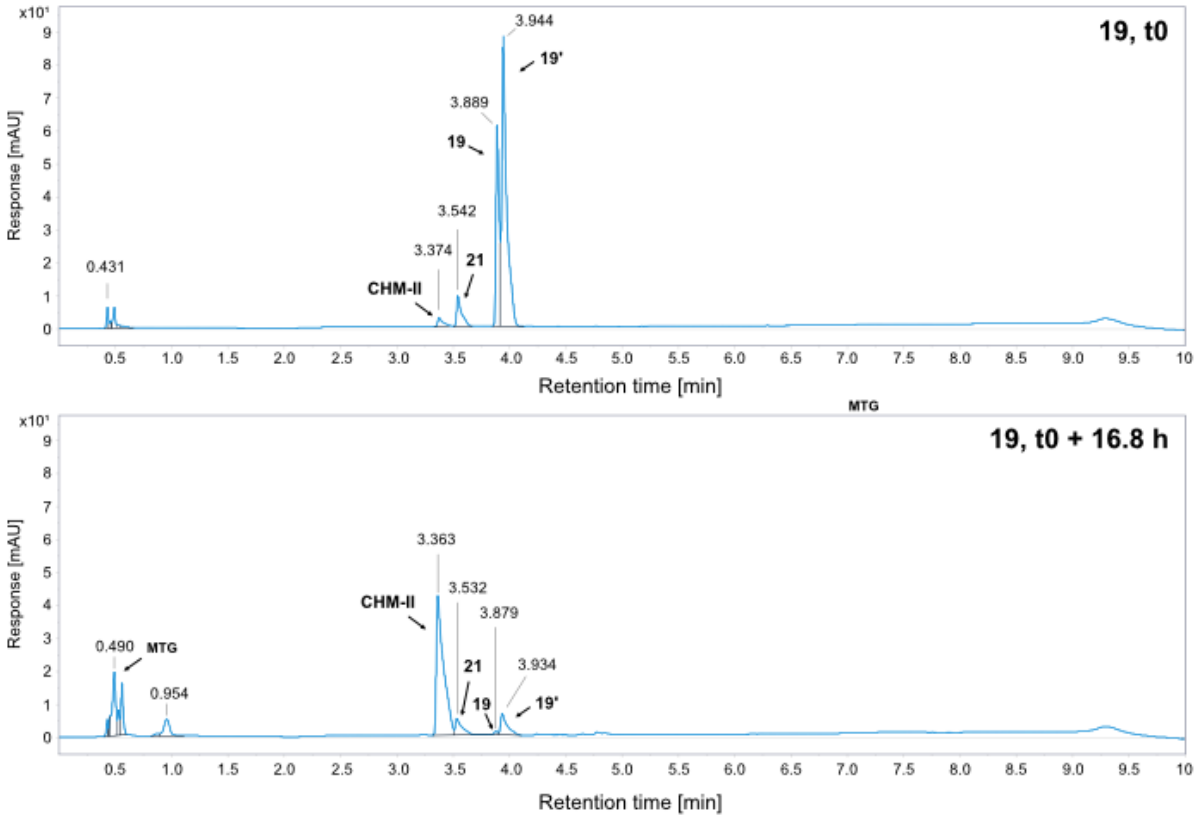


Figure S10. Stability of monoconjugate **19** (both diastereomers) in potassium phosphate buffer (pH 8.1, 10 % MeCN) at initial addition to the buffer (top) and after 16.8 hours (bottom). The displayed chromatogram was recorded at 260 nm. **19** and **19'** denote the two diastereomers, and the faint peak corresponding to 21 is attributed either to a small impurity in the **19** stock solution or to a minor amount of in-situ formation from liberated MTG and **CHM-II**. Only peaks for which a reference had been prepared for confirmation of the retention time have been annotated, but the un-annotated peaks with retention time < 1 min are expected to be various hydrolysis products generated upon hydrolysis of the MTG ester bond.

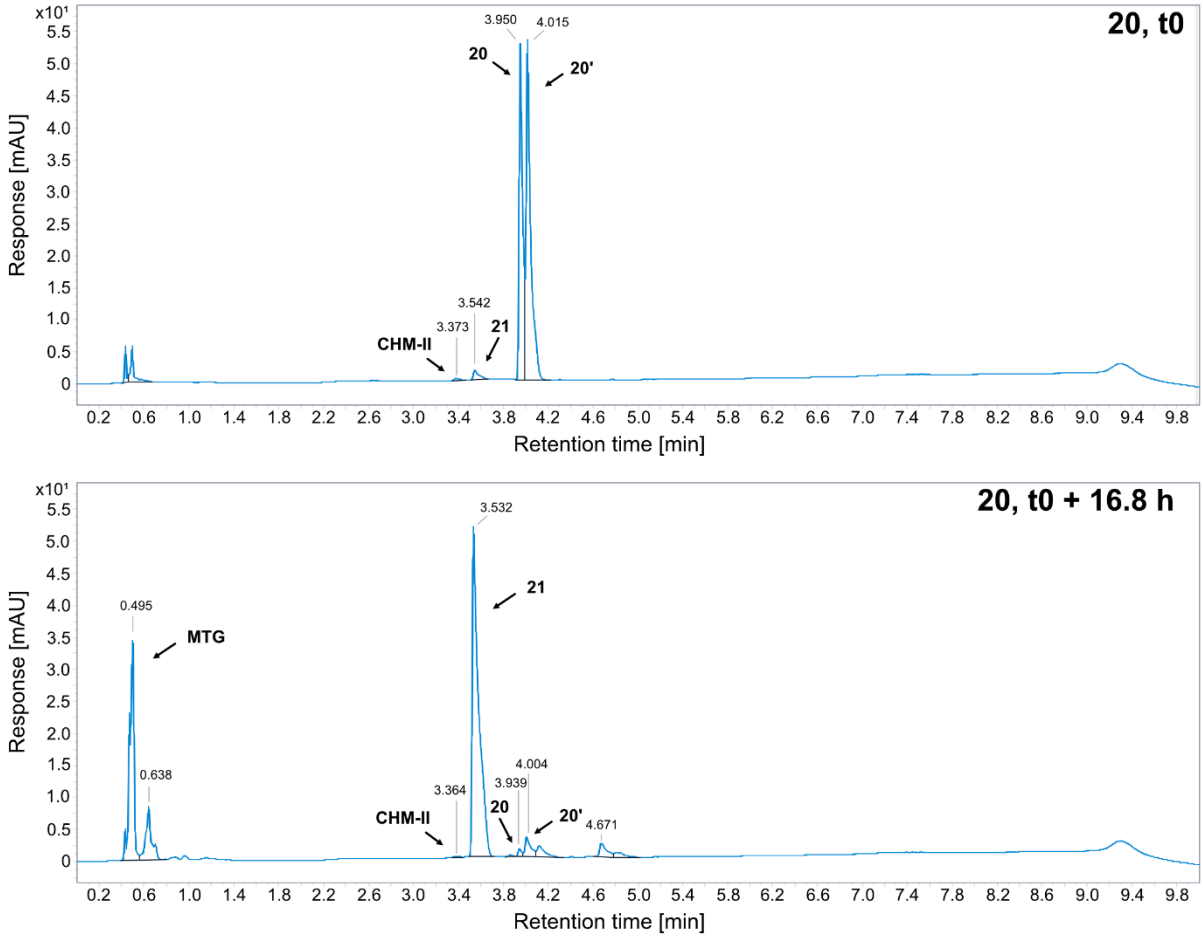


Figure S11. Stability of diconjugate **20** (both diastereomers) in potassium phosphate buffer (pH 8.1, 10 % MeCN) at initial addition (top) to the buffer and after 16.8 hours (bottom). The displayed chromatogram was recorded at 260 nm. **20** and **20'** denote the two diastereomeres of the diconjugation product. The faint peak corresponding to **CHM-II** is attributed to a minor remnant in the stock solution of **20**. Only peaks for which a reference had been prepared for confirmation of the retention time have been annotated, but the un-annotated peaks with retention time < 1 min are expected to be various hydrolysis products generated upon hydrolysis of the MTG ester bond.

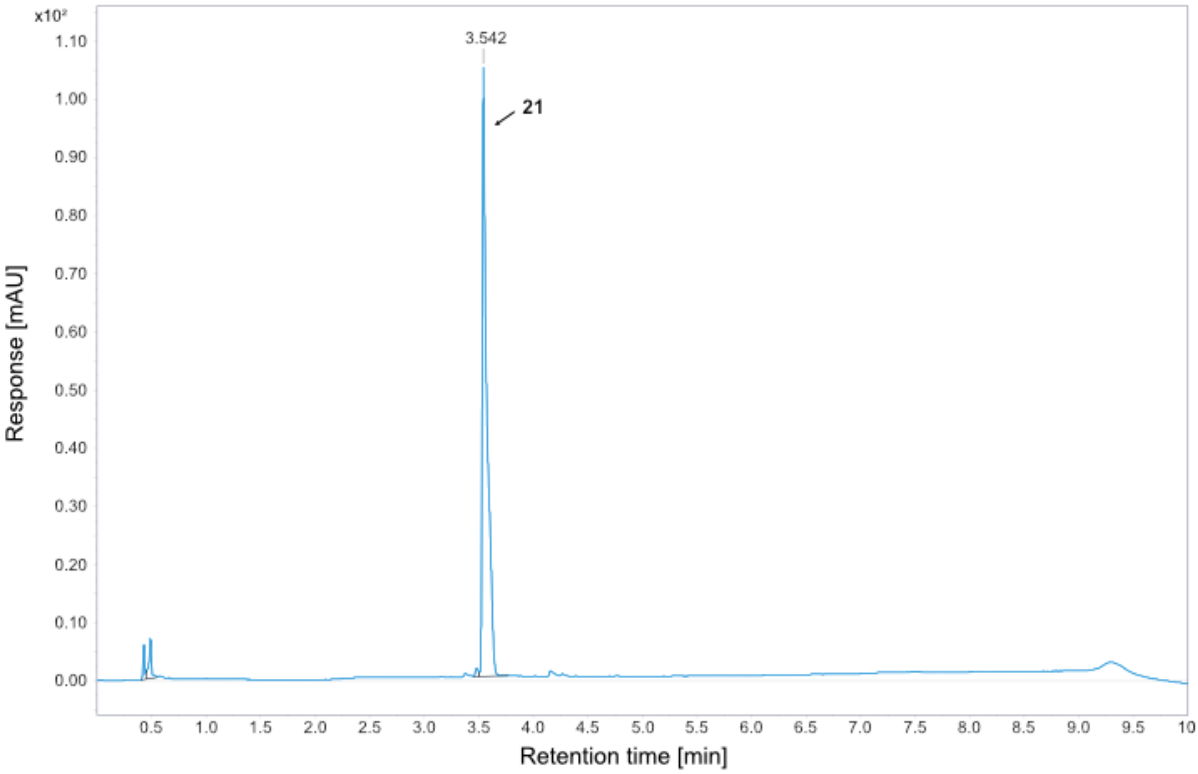


Figure S12. HPLC chromatogram of a stock solution of **21** using the standard elution method to confirm the identity of the previously observed peak with a retention time of 3.5 minutes.

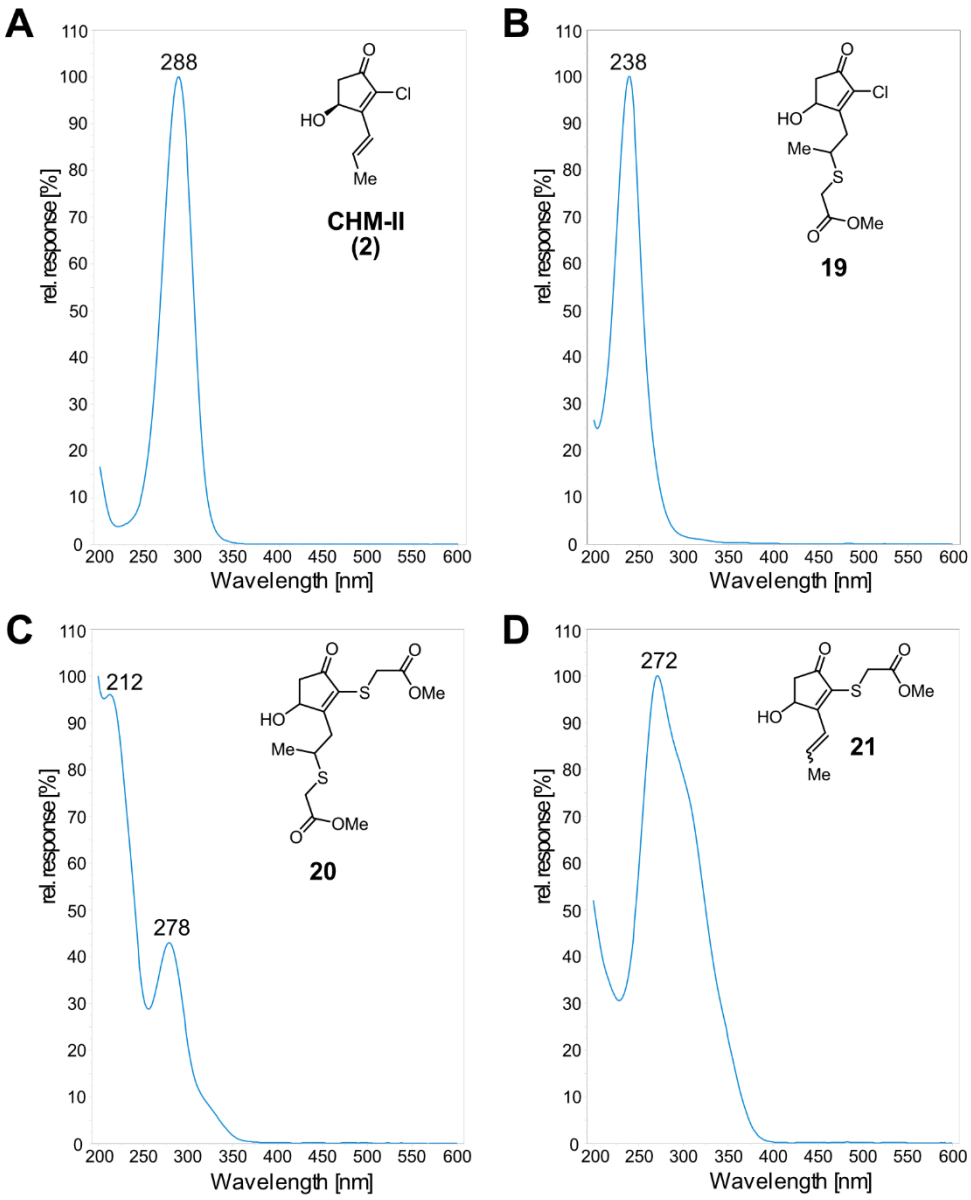


Figure S13. UV-absorbance spectra of **CHM-II**, **19**, **20**, and **21** as measured by the HPLC. A) Absorbance spectrum of **CHM-II**. B) Absorbance spectrum of **19**. C) Absorbance spectrum of **20**. B) Absorbance spectrum of **21**.

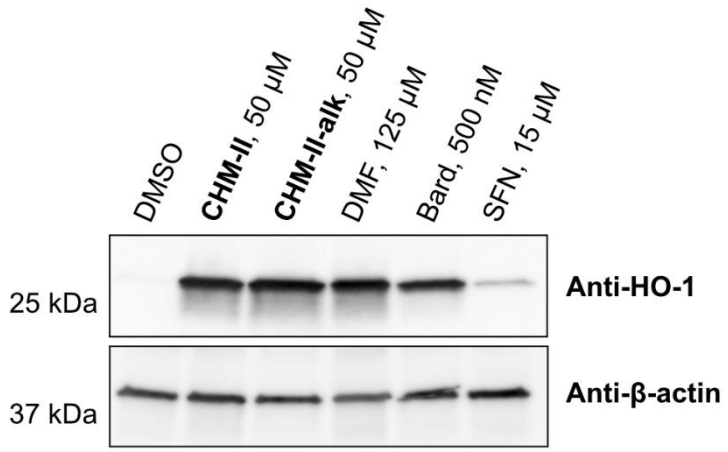


Figure S14. Induction of HO-1 by **CHM-II-alk** (**22**) after 21 hours of treatment in live U-2OS cells. DMF: Dimethyl fumarate, CDDO-Me: Bardoxolone methyl ester, SFN: Sulforaphane, HO-1: Heme oxygenase 1. The presented western blot is representative of two biological replicates.

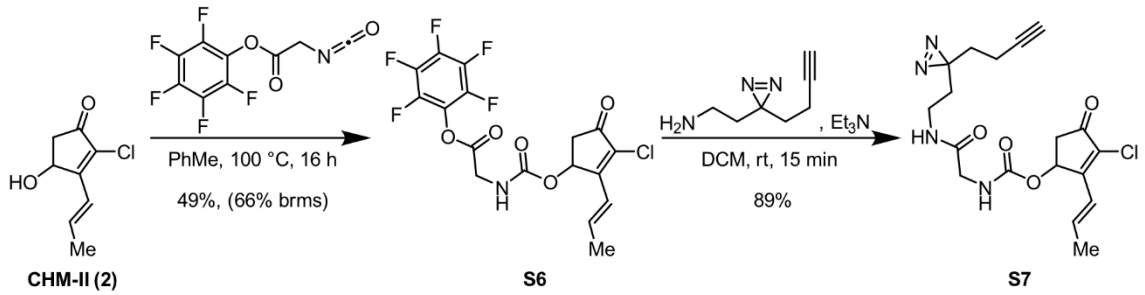


Figure S15. Oxy-functionalization of **CHM-II** to afford a versatile PFP-ester derivative for subsequent amide coupling.

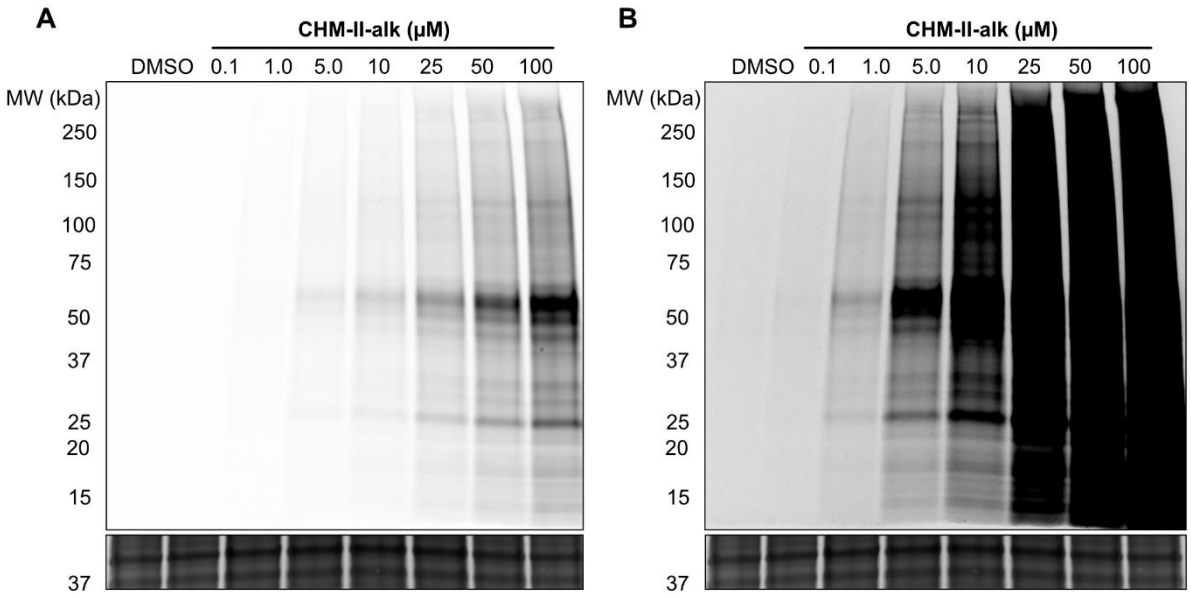


Figure S16. In-gel fluorescence after two hours of labelling with **CHM-II-alk** (**22**) at various concentrations in U-2OS cell lysate. **A)** Fluorescence with contrast adjusted based on the intensities observed for 100 μM **CHM-II-alk.** B) Same image as in A, this time with the contrast adjusted to enable visualization of bound targets at 1 μM **CHM-II-alk**

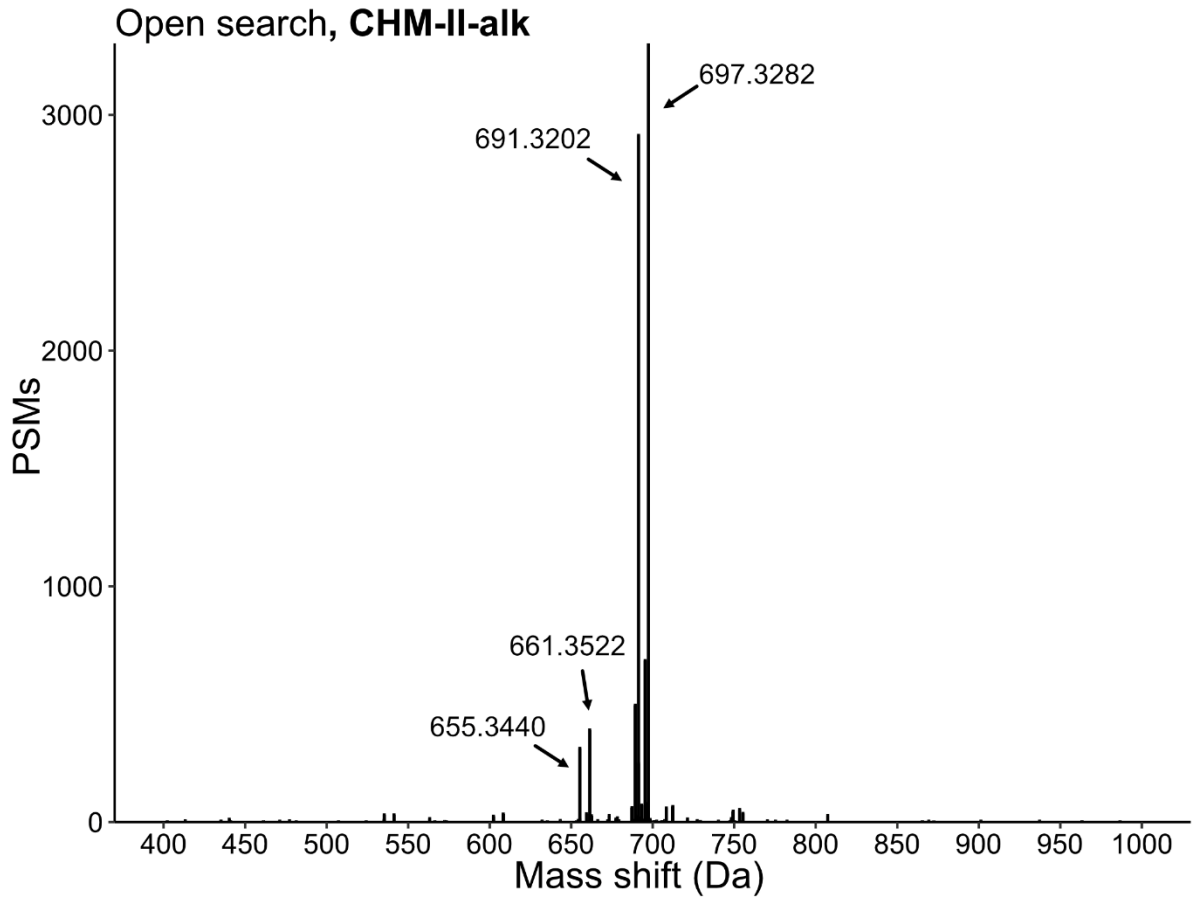


Figure S17. Plot of modification masses observed in the open search. The modification masses of 691.3209 Da and 697.3282 Da correspond to the masses expected for light- and heavy-tag

modified **CHM-II** mono-conjugation adducts still containing chloride (expected modification masses: 691.3209 Da and 697.3289), while the modification masses of 661.3522 Da and 655.3440 Da correspond to mono-conjugation with loss of chloride (expected modification masses: 655.3447 Da and 661.3522 Da). The data was plotted using an R script (available through OSF) and exact modification masses were determined using ChemDraw version 22.2.0.3300.

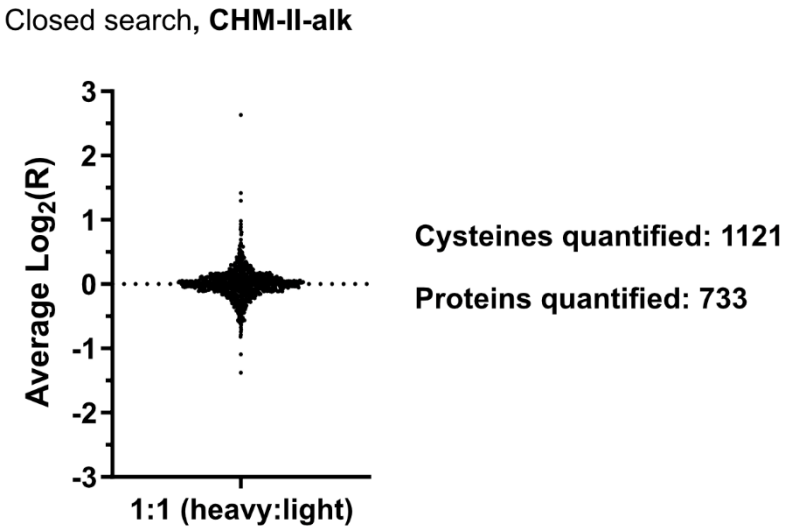


Figure S18. Violin plot of log2(R) ratios of the 1121 light- and heavy-tag modified peptides observed in the closed search analysis (filtered to contain only peptides present in three of four samples). While a few outliers are observed, the median log2(R) average of 0.004 is considered well within the acceptable range for the 1:1 mixture of light and heavy-tagged samples.

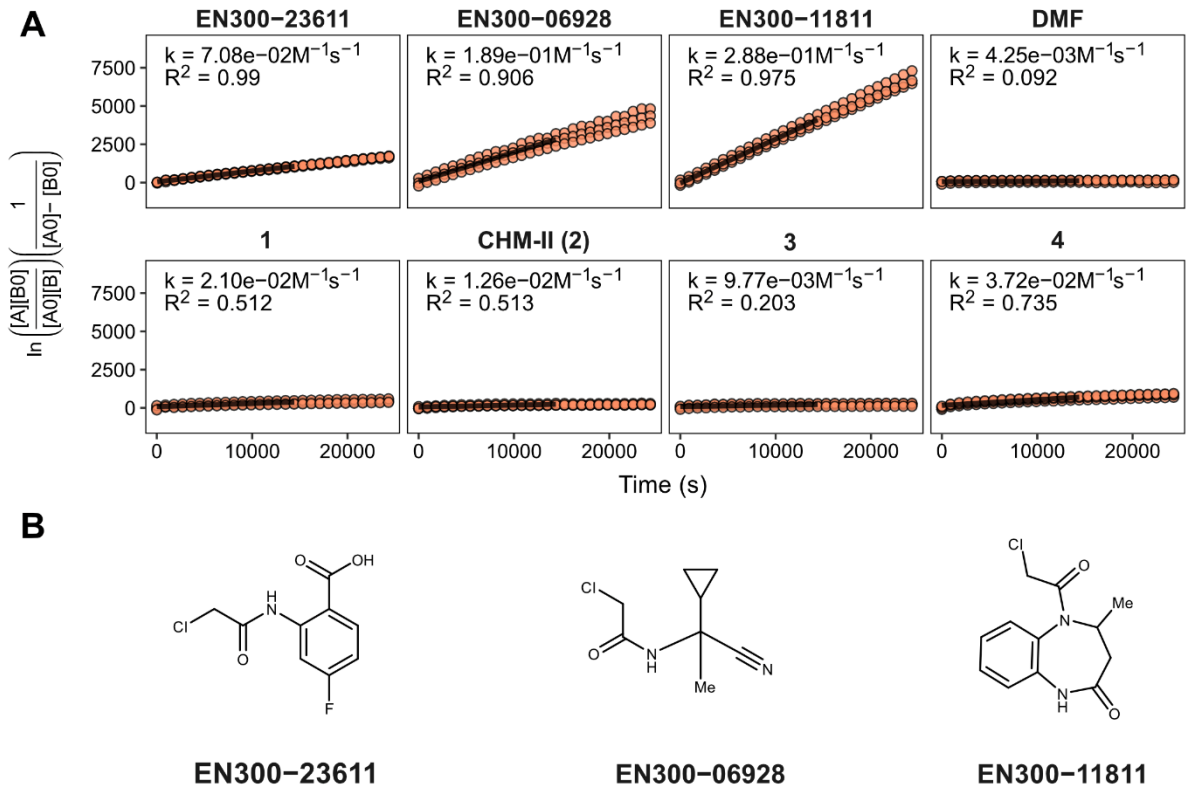


Figure S19. Results from the thiol-reactivity assay using reduced Ellman's reagent. **A)** Plots and linear regression lines obtained for the examined compounds. The flat curves and low R2-values observed for **1**, **CHM-II**, **3**, and **DMF** indicate that the cysteine reactivity of these compounds is below the detection limit of the assay. **B)** The structures of the utilized reference electrophiles. The names correspond to the catalogue IDs in the Enamine store. DMF: Dimethyl fumarate.

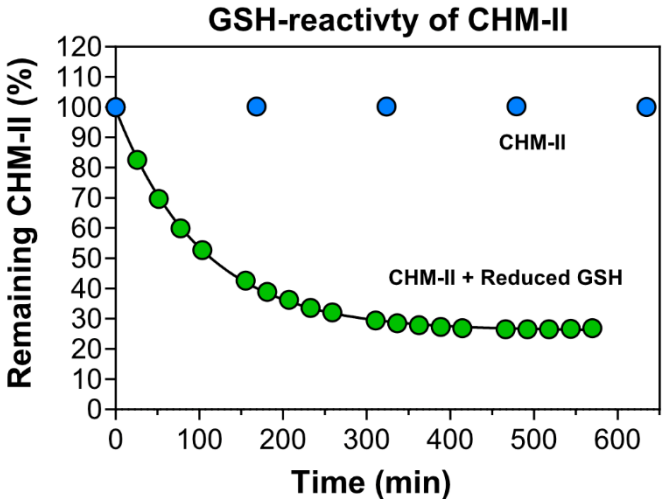


Figure S20. HPLC-based reactivity assay with **CHM-II** (200 µM) and GSH (1 mM) in potassium phosphate buffer (100 mM, pH 8.1) containing 10 % MeCN. The integrals of the **CHM-II** peak at different times were normalized to the integral of the first measurement, and the half-life was determined by fitting the **CHM-II** + GSH data to a one-phase decay equation in GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. GSH: glutathione.

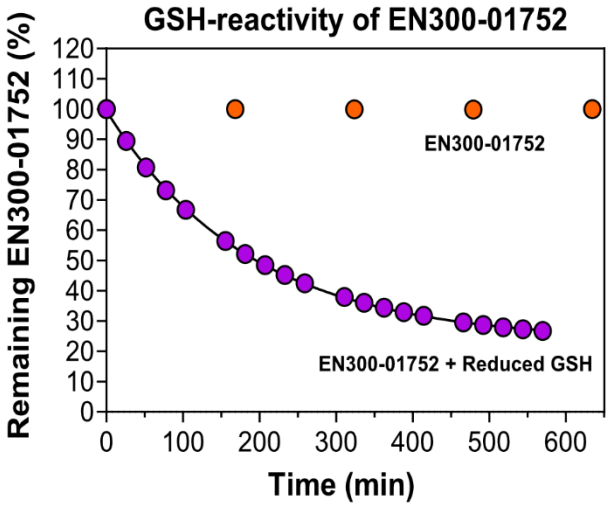


Figure S21. HPLC-based reactivity assay with **EN300-01752** (200 µM) and GSH (1 mM) in potassium phosphate buffer (100 mM, pH 8.1) containing 10 % MeCN. The integrals of the **EN300-01752** peak at different times were normalized to the integral of the first measurement, and the half-life was determined by fitting the **EN300-01752** + GSH data to a one-phase decay equation in GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. GSH: glutathione.

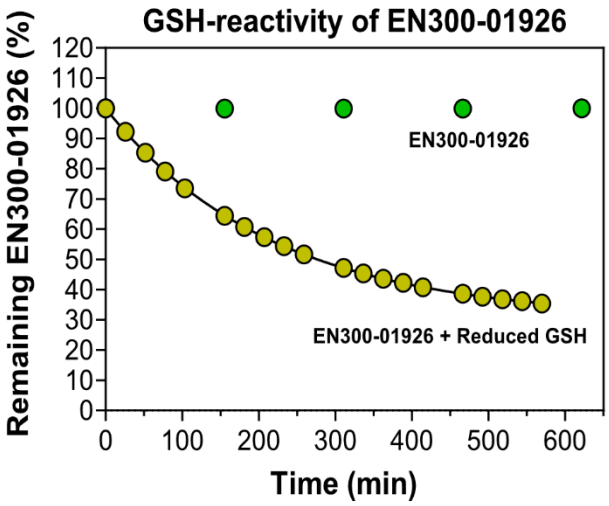


Figure S22. HPLC-based reactivity assay with **EN300-01926** (200 µM) and GSH (1 mM) in potassium phosphate buffer (100 mM, pH 8.1) containing 10 % MeCN. The integrals of the **EN300-01926** peak at different times were normalized to the integral of the first measurement, and the half-life was determined by fitting the **EN300-01926** + GSH data to a one-phase decay equation in GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. GSH: glutathione.

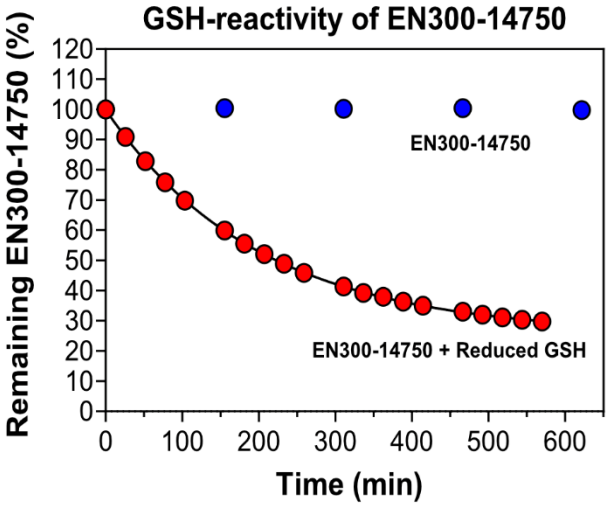


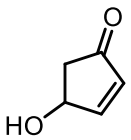
Figure S23. HPLC-based reactivity assay with **EN300-14750** (200 µM) and GSH (1 mM) in potassium phosphate buffer (100 mM, pH 8.1) containing 10 % MeCN. The integrals of the **EN300-14750** peak at different times were normalized to the integral of the first measurement, and the half-life was determined by fitting the **EN300-14750** + GSH data to a one-phase decay equation in GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. GSH: glutathione.

2. General methods – Organic synthesis

All reactions were conducted in flame-dried glassware under an atmosphere of argon unless otherwise stated. DCM, MeCN, THF and PhMe were dried over aluminium oxide via an MBraun SPS-800 solvent purification system and further stored over preactivated molecular sieves (3 Å or 4 Å). MeOH, 1,4-dioxane, DMSO and DMA were purchased as anhydrous. TBME and benzene were purchased as anhydrous and then transferred to a flask containing preactivated molecular sieves (3 Å or 4 Å). Pyridine was purchased anhydrous and distilled onto preactivated molecular sieves (4 Å). *n*-Hexane was distilled onto preactivated molecular sieves (3 Å or 4 Å). AcOH were purchased as absolute and used without further purification. Et_3N, DBU and HMPA were dried by stirring for at least 30 minutes over CaH_2 followed by distillation onto preactivated molecular sieves (3 Å or 4 Å). The dryness of solvents was controlled via Karl Fischer titration. Reagents were used as received from commercial suppliers unless otherwise stated (Sigma Aldrich, Merck, AK Scientific, Fluorochem, BLD Pharm and TCI). Concentration *in vacuo* was performed using a rotary evaporator with the water bath temperature at 35 °C, followed by further concentration using a high vacuum pump unless otherwise stated. TLC analysis was carried out on silica coated aluminum foil plates (Merck Kieselgel 60 F254). The TLC plates were visualized by UV irradiation and/or by staining with $KMnO_4$ stain ($KMnO_4$ (5.0 g), 5 % aq. NaOH (8.3 mL) and K_2CO_3 (33.3 g) in H_2O (500 mL)). Molecular sieves were activated by drying in the oven at 120 °C for at least 24 h, before they were heated in a microwave at maximum power for 2 minutes, followed by evaporation of the formed vapor under high vacuum. This was repeated 3-4 times and finished by gently flame-drying the flask containing the molecular sieves. Automated flash column chromatography (AFCC) was carried out with Interchim PuriFlash 420 or 5.050 using 30 µm prepacked columns unless otherwise stated. Infrared spectra (IR) were acquired on a PerkinElmer Spectrum TwoTM UATR. Mass spectra (HRMS) were recorded on a Bruker Daltonics MicrOTOF time-of-flight spectrometer with positive electrospray ionization, or negative ionization when stated. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury 400 MHz spectrometer or a Bruker BioSpin GmbH 400 MHz spectrometer, running at 400 and 101 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts (δ) are reported in ppm relative to the residual solvent signals (chloroform-d: 7.26 ppm ^{1}H NMR, 77.16 ppm ^{13}C NMR and methanol-d4: 3.31 ppm ^{1}H NMR, 49.00 ppm ^{13}C NMR). Multiplicities are indicated using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad.

3. Experimental procedures and compound characterization– Organic synthesis

Compound 6



A 1 L flask was charged with furfuryl alcohol (25.0 g, 0.255 mol, 1.0 equiv.) and 740 mL of MilliQ water. To the solution was added KH_2PO_4 (1.262 g, 9.27 mmol, 0.037 equiv.) and the pH was adjusted to 4.10 (pH meter) by addition of phosphoric acid (0.5 M, 4 drops). The solution was heated to reflux for 40 h before being cooled to rt and washed with DCM (2 x 100 mL). The combined organic layers were extracted with MilliQ water (2 x 100 mL) and the combined aqueous layers were concentrated *in vacuo* (rot. evap., 70 °C, 125 mbar) to give an orange oil. The oil was dissolved in DCM (200 mL) and the resulting solution was dried over Na_2SO_4, filtered and concentrated *in vacuo* to a dark brown oil (11.087 g).

R_f (3:1 EtOAc/Heptane) 0.24

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 7.57 (dd, *J*=5.7, 2.4 Hz, 1H), 6.24 (dd, *J*=5.6, 1.4 Hz, 1H), 5.07 (m, 1H), 2.79 (dd, *J*=18.4, 6.1 Hz, 1H), 2.29 (dd, *J*=18.4, 2.0 Hz, 1H)

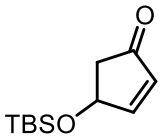
^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 206.7, 163.4, 135.3, 70.6, 44.4

$\tilde{\nu}_{max}$ (ATR) 3406, 2928, 1710, 1673, 1586, 1404, 1341, 1103, 1043, 947, 796, 659

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_5H_7O_2^+$ 99.0441; found 99.0434

The obtained data are in accordance with the literature.[1]

Compound 7



The resulting oil from above containing 4-hydroxycyclopent-2-en-1-one (**6**) (11.087 g, assumed 111.1 mmol, 1.0 equiv.) was dissolved in anhydrous THF (57 mL) and anhydrous triethylamine (24.5 mL, 175.8 mmol, 1.6 equiv.) was added followed by DMAP (272 mg, 2.23 mmol, 0.02 equiv.). The solution was cooled to 0 °C and TBSCl (15.045 g, 105.8 mmol, 0.95 equiv.) was added portion wise. After 10 min, the ice bath was removed, and the resulting mixture was stirred for 16 h. The solution was poured into aq. HCl (0.5 M, 57 mL), the phases were separated, and the aqueous phase was extracted with heptane (2 x 60 mL). The organic layers were combined and washed with HCl (0.5 M, 2 x 30 mL), 5% $NaHCO_3$ solution (30 mL) and brine (30 mL), dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo*. The crude material was azeotroped twice with PhMe (2 x 100 mL) to remove TBS-OH yielding a brown oil. The residual oil was distilled in a Kugelrohr apparatus (2 mbar, 115 °C) yielding TBS-protected alcohol **7** as a colorless oil (14.891 g, 70.12 mmol, 28% over 2 steps).

R_f (4:1 Heptane/EtOAc) 0.44

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 7.47 (dd, *J*=5.7, 2.3 Hz, 1H), 6.18 (d, *J*=5.7 Hz, 1H), 4.99 (m, 1H), 2.71 (dd, *J*=18.1, 6.0 Hz, 1H), 2.25 (dd, *J*=18.1, 2.1 Hz, 1H), 0.91 (s, 9H), 0.13 (s, 3H), 0.12 (s, 3H)

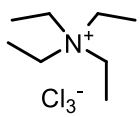
^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 206.6, 164.0, 134.6, 71.0, 45.1, 25.9, 18.2, -4.6, -4.6

$\tilde{\nu}_{max}$ (ATR) 2955, 2930, 2886, 2858, 1723, 1472, 1355, 1253, 1183, 1108, 1072, 900, 836, 778, 670

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{11}H_{21}O_2Si^+$ 213.1305; found 213.1316

The obtained data are in accordance with the literature.[1]

Mioskowski's reagent (Et_4NCl_3)



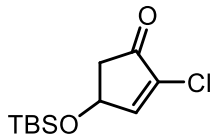
$NaOCl \cdot 5H_2O$ (98.996 g, 601 mmol, 3.0 equiv.) was dissolved in 400 mL water in a 1 L three-necked flask equipped with a stopper, a gas adaptor and a dropping funnel containing aqueous HCl (240 mL, 6 M, 1.44 mol, 7.2 equiv.). The gas adaptor was connected to a washing bottle containing Et_4NCl (33.206 g, 200 mmol, 1.0 equiv.) in 80 mL dichloromethane, which further lead into an empty washing bottle to protect the first, and then finally into a third washing bottle containing 20 wt% NaOH in water to neutralize any residual chlorine gas. The aqueous HCl was added dropwise to the NaOCl solution until almost complete addition. An argon stream was then added to the setup to flush through chlorine gas and to always ensure overpressure. The resulting yellow solution in the first washing bottle was concentrated *in vacuo* and further dried under high vacuum yielding the desired trichloride as yellow crystalline solid (41.141 g, 174 mmol, 87%)

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 3.49 (q, J = 7.2 Hz, 8H), 1.39 (t, J = 6.9 Hz, 12H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 53.0, 8.1

The obtained data are in accordance with the literature.[2]

Compound 8



To a solution of 4-((*tert*-butyldimethylsilyl)oxy)cyclopent-2-en-1-one (**7**) (1540 mg, 7.26 mmol, 1.0 equiv.) in anhydrous DCM (60 mL) was added a solution of Et_4NCl_3 (2580 mg, 10.9 mmol, 1.5 equiv.) in anhydrous DCM (10 mL) over 5 min at 0 °C. The mixture was stirred at this temperature for 30 min. Then anhydrous triethylamine (1.51 mL, 10.8 mmol, 1.5 equiv.) was added dropwise. The reaction mixture was stirred for another 30 min at 0 °C. The reaction was quenched by addition of sat. aq. $NaHCO_3$ (30 mL). The layers were separated, and the aqueous layer extracted with Et_2O (3 x 25 mL). The combined organic layers were washed with brine (30 mL), dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to a dark brown oil (1.781 g). The crude material was used directly.

R_f (4:1 Heptane/EtOAc) 0.62

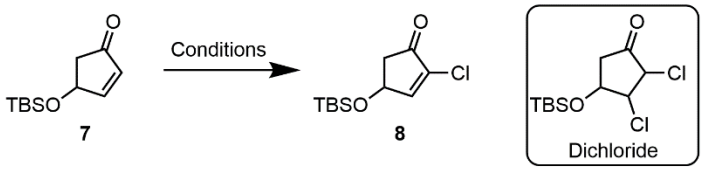
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 7.34 (d, J=2.7 Hz, 1H), 4.95 (m, 1H), 2.87 (dd, J=18.4, 6.1 Hz, 1H), 2.37 (d, J=18.4 Hz, 1H), 0.89 (s, 9H), 0.13 (s, 3H), 0.11 (s, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 197.8, 156.6, 137.4, 67.9, 44.3, 25.8, 18.2, -4.6, -4.6

$\tilde{\nu}_{max}$ (ATR) 2954, 2930, 2886, 2858, 1736, 1667, 1602, 1362, 1349, 1283, 1257, 1173, 1086, 1006, 968, 950, 900, 832, 779, 669, 556

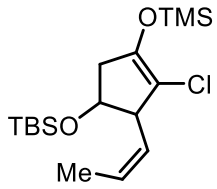
HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{11}H_{20}{}^{35}ClO_2Si^+$ 247.0916; found 247.0909, calc. for $C_{11}H_{20}{}^{37}ClO_2Si^+$ 249.0887; found 249.0891

Overview of chlorination attempts:



Entry	Chlorination attempts	Result
1	PhSeCl, pyr, DCM, 40 °C, 21 h	Complex mixture including **8**
2	PhSeCl, NCS, MeCN, rt, 24 h	Insignificant conversion
3	*m*-CPBA, HCl·dioxane, rt, 30 min	Complex mixture
4	Pyr·HCl, bisacetoxyiodobenzene, DCM, rt, 24 h	Decomposition of **7**
5	Et_4NCl_3, DCM, 0 °C, 30 min	**8** + dichloride
6	Et_4NCl_3, DCM, 0 °C, 30 min, then Et_3N quench	**8**

Compound 9



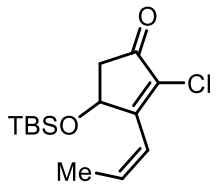
$CuBr \cdot SMe_2$ (1698 mg, 8.26 mmol, 2.0 equiv.) was charged to a flame-dried 100 mL round-bottomed flask. 1-propenylmagnesium bromide (0.5 M in THF, 33 mL, 16.5 mmol, 4.0 equiv) was added slowly at -78 °C. The mixture was stirred for 30 min at -78 °C. After 30 min TMEDA (2.49 mL, 16.5 mmol, 4.0 equiv.) and TMSCl (2.64 mL, 20.6 mmol, 5.0 equiv.) were added followed by dropwise addition of a solution of crude 4-((*tert*-butyldimethylsilyl)oxy)-2-chlorocyclopent-2-en-1-one (**8**) (1019 mg, 4.13 mmol, 1.0 equiv.) in anhydrous THF (5.0 mL). The mixture was stirred at -78 °C for 15 min then allowed to warm to rt. After 1 h at rt, the solution was diluted with pentane (100 mL) and the organic phase was washed with water (4 x 50 mL), dried over Na_2SO_4, filtered through sand and celite and concentrated in vacuo to a brown opaque oil, which was taken up in pentane (10 mL) and filtered through cotton yielding a brown oil (1.211 g). The crude material was used directly.

R_f (9:1 Heptane/EtOAc) 0.84

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm 5.68 (dqd, J = 10.7, 6.9, 1.1 Hz, 1H), 5.15 (tq, J = 10.5, 1.8 Hz, 1H), 4.03 (dt, J = 7.5, 4.7 Hz, 1H), 3.58 – 3.49 (m, 1H), 2.58 (ddd, J = 15.4, 7.5, 1.8 Hz, 1H), 2.29 (ddd, J = 15.4, 5.0, 1.7 Hz, 1H), 1.71 (dd, J = 6.8, 1.8 Hz, 3H), 0.87 (s, 9H), 0.24 (s, 9H), 0.03 (s, 6H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 144.9, 130.3, 127.5, 107.7, 74.8, 52.3, 42.2, 25.9, 18.1, 13.4, 0.8, -4.7, -4.7

Compound 10



To a solution of crude (*Z*)-*tert*-butyl((3-chloro-2-(prop-1-en-1-yl)-4-((trimethylsilyl)oxy)cyclopent-3-en-1-yl)oxy)dimethylsilane (**9**) (506 mg, assumed 1.40 mmol, 1.0 equiv.) in anhydrous DCM (14.0 mL) at rt was added DDQ (637 mg, 2.80 mmol, 2.0 equiv.). The solution was stirred at rt for 16 h before sat. aq. $NaHCO_3$ (20 mL) was added. The mixture was

extracted with Et_2O (3x20 mL). The combined organic layers were washed with sat. aq. $NaHCO_3$ (30 mL) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to a reddish-brown oil. The crude material was purified by AFCC (Heptane/DCM 100:0 → 50:50) yielding the (*Z*)-configured enone **10** as a yellow oil (104 mg, 0.362 mmol, 20% over 3 steps).

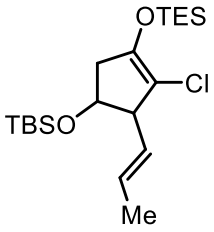
R_f (9:1 Heptane/EtOAc) 0.40

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.16 – 6.04 (m, 2H), 4.94 (dd, *J* = 6.1, 1.9 Hz, 1H), 2.89 (dd, *J* = 18.3, 6.1 Hz, 1H), 2.43 (dd, *J* = 18.3, 1.9 Hz, 1H), 1.80 (d, *J* = 5.4 Hz, 3H), 0.88 (s, 10H), 0.11 (s, 3H), 0.10 (s, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 197.5, 165.3, 135.9, 132.7, 121.3, 70.4, 44.1, 25.8, 18.2, 17.1, -4.2, -4.6

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{14}H_{24}{}^{35}ClO_2Si^+$ 287.1229; found 287.1226, calc. for $C_{14}H_{24}{}^{37}ClO_2Si^+$ 289.1200; found 289.1199

Compound 12



$Ni(acac)_2$ (18.5 mg, 0.072 mmol, 1 mol%), neocuproine (15.4 mg, 0.074 mmol, 1 mol%) and Mn powder (795 mg, 14.5 mmol, 2.0 equiv.) were charged to a flame-dried round bottomed flask. A solution of crude 4-((*tert*-butyldimethylsilyl)oxy)-2-chlorocyclopent-2-en-1-one (**8**) (1781 mg, presumed 7.22 mmol, 1.0 equiv.) in anhydrous DMA (21.5 mL) was added followed by 1-bromopropene (0.62 mL, 7.2 mmol, 1.0 equiv.) and TESCl (2.42 mL, 14.4 mmol, 2.0 equiv.). The mixture was heated to 40 °C. After 3 h, the mixture was diluted with pentane (100 mL) washed with water (3 x 30 mL). The organic layer was dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to an orange oil (3.132 g). The crude material was used directly.

Note: The silyl enol ether may be subjected to silica gel purification using 0.1% Et_3N buffered solvents (AFCC eluent: Heptane/EtOAc 100:0 → 98:2 + 0.1% Et_3N).

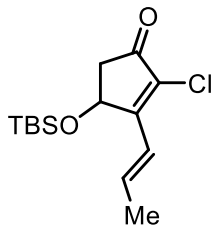
R_f (9:1 Heptane/EtOAc) 0.79

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 5.58 (dqd, *J* = 15.2, 6.5, 0.8 Hz, 1H), 5.23 (ddq, *J* = 15.1, 8.8, 1.6 Hz, 1H), 4.00 (dt, *J* = 7.2, 4.2 Hz, 1H), 3.03 (dd, *J* = 9.2, 3.7 Hz, 1H), 2.58 (ddd, *J* = 15.4, 7.2, 1.7 Hz, 1H), 2.23 (ddd, *J* = 15.3, 4.4, 1.6 Hz, 1H), 1.70 (dd, *J* = 6.4, 1.6 Hz, 4H), 1.00 (t, *J* = 7.9 Hz, 9H), 0.87 (s, 9H), 0.70 (q, *J* = 7.8 Hz, 6H), 0.03 (s, 3H), 0.03 (s, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 145.3, 130.5, 128.3, 107.4, 74.4, 58.2, 41.9, 25.9, 18.2, 18.1, 6.7, 5.5, -4.4, -4.7

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{20}H_{40}{}^{35}ClO_2Si_2^+$ 403.2250; found 403.2239, calc. for $C_{20}H_{40}{}^{37}ClO_2Si_2^+$ 405.2221; found 405.2214

Compound 11



To a solution of crude (*E*)-*tert*-butyl((3-chloro-2-(prop-1-en-1-yl)-4-((triethylsilyl)oxy)cyclopent-3-en-1-yl)oxy)dimethylsilane (**12**) (3.132 g, assumed 7.22 mmol, 1.0 equiv) in anhydrous DCM (230 mL) was added DDQ (3288 mg, 14.5 mmol, 2.0 equiv.) at rt. The solution was stirred for 18 h, before sat. aq. $NaHCO_3$ (50 mL), H_2O (50 mL) and Et_2O (100 mL) were added. The layers were separated and the aqueous layer was extracted with Et_2O (2 x 50 mL). The combined organic layers were washed with sat. aq. $NaHCO_3$ (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to a dark red oil. The crude material was purified by AFCC (Heptane/EtOAc 100:0 → 92:8) yielding the (*E*)-configured enone **11** as a yellow oil (683 mg, 2.38 mmol, 33% over 3 steps).

R_f (9:1 Heptane/EtOAc) 0.37

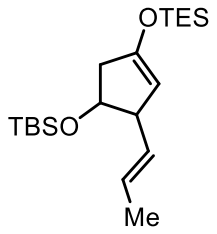
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.71 (dq, *J* = 15.9, 6.8 Hz, 1H), 6.52 (dq, *J* = 15.8, 0.6 Hz, 1H), 5.10 (dd, *J* = 6.0, 1.6 Hz, 1H), 2.86 (dd, *J* = 18.2, 6.2 Hz, 1H), 2.40 (dd, *J* = 18.2, 1.9 Hz, 1H), 1.98 (dd, *J* = 6.8, 1.6 Hz, 3H), 0.89 (s, 8H), 0.17 (s, 3H), 0.12 (s, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 197.3, 162.7, 140.2, 130.4, 123.4, 68.3, 44.1, 25.8, 19.8, 18.0, -3.7, -4.8

$\tilde{\nu}_{max}$ (ATR) 2954, 2930, 2858, 1720, 1640, 1581, 1471, 1253, 1084, 967, 925, 893, 832, 777

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{14}H_{24}{}^{35}ClO_2Si^+$ 287.1229; found 287.1248, calc. for $C_{14}H_{24}{}^{37}ClO_2Si^+$ 289.1200; found 289.1213

Compound S2



$Ni(acac)_2$ (1.3 mg, 5.1 µmol, 1.1 mol%), neocuproine (1.0 mg, 4.8 µmol, 1 mol%) and Mn powder (53.2 mg, 0.968 mmol, 2.0 equiv.) were charged to a flame-dried reaction tube. A solution of 4-((*tert*-butyldimethylsilyl)oxy)cyclopent-2-en-1-one (**7**) (101.5 mg, 0.478 mmol, 1.0 equiv.) in anhydrous DMA (1.5 mL) was added followed by 1-bromopropene (41.0 µL, 0.478 mmol, 1.0 equiv.) and TESCl (160 µL, 0.956 mmol, 2.0 equiv.). The mixture was heated to 40 °C. After 2.5 h, another portion of 1-bromopropene (41 µL, 0.478 mmol, 1.0 equiv.) was added. After a total of 5 h, the mixture was diluted with pentane (30 mL) washed with water (3 x 15 mL). The organic layer was dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to a clear colorless oil. The crude material was purified by AFCC (Heptane/EtOAc 100:0 → 98:2 + 0.1% Et_3N) yielding the desired silyl enol ether **S2** as a colorless oil (114.7 mg, 0.311 mmol, 65%)

R_f (9:1 Heptane/EtOAc) 0.72

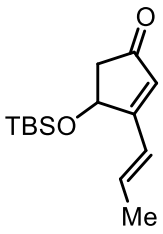
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 5.46 (dq, *J* = 15.5, 6.0 Hz, 1H), 5.34 (ddq, *J* = 15.1, 7.8, 1.4 Hz, 1H), 4.45 (q, *J* = 1.8 Hz, 1H), 3.98 (ddd, *J* = 7.4, 5.6, 4.7 Hz, 1H), 3.09 – 2.98 (m, 1H), 2.49 (ddt, *J* = 15.7, 7.4, 1.4 Hz, 1H), 2.26 (ddt, *J* = 15.7, 5.7, 1.8 Hz, 1H), 1.66 (dq,

J = 6.3, 0.8 Hz, 3H), 0.97 (t, *J* = 7.9 Hz, 9H), 0.88 (s, 9H), 0.68 (q, *J* = 7.5 Hz, 6H), 0.03 (s, 3H), 0.03 (s, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 152.0, 133.9, 125.0, 103.6, 77.3, 54.9, 43.2, 26.0, 18.3, 18.0, 6.8, 4.9, -4.5, -4.6.

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{20}H_{41}O_2Si_2^+$ 369.2640; found 369.2646

Compound 13



To a solution of (*E*)-*tert*-butyldimethyl((2-(prop-1-en-1-yl)-4-((triethylsilyl)oxy)cyclopent-3-en-1-yl)oxy)silane (**S2**) (114.7 mg, 0.311 mmol, 1.0 equiv.) in anhydrous DCM (3 mL) at rt was added DDQ (144.9 mg, 0.638 mmol, 2.1 equiv.) The solution was stirred at rt for 17 h. Then sat. aq. $NaHCO_3$ (15 mL) was added and the mixture was extracted with Et_2O (3 x 20 mL). The combined organic layers were washed with sat. aq. $NaHCO_3$ (25 mL) and brine (25 mL), dried over Na_2SO_4, filtered through sand and celite and concentrated in vacuo to a red oil. The crude material was purified by AFCC (Heptane/EtOAc 100:0 → 90:10) yielding TBS-protected cyclohelminthol I (**13**) as a colorless oil (42.6 mg, 0.169 mmol, 54%)

***R*$_f$** (4:1 Heptane/EtOAc) 0.39

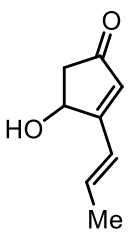
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.53 (dq, *J* = 15.8, 6.8 Hz, 1H), 6.32 (dq, *J* = 15.9, 1.1 Hz, 1H), 5.99 (s, 1H), 5.04 (dd, *J* = 6.2, 2.2 Hz, 1H), 2.74 (dd, *J* = 18.0, 6.1 Hz, 1H), 2.33 (dd, *J* = 18.0, 2.2 Hz, 1H), 1.92 (dd, *J* = 6.8, 1.6 Hz, 3H), 0.90 (s, 9H), 0.16 (s, 3H), 0.11 (s, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 205.4, 172.3, 138.5, 128.1, 125.4, 70.7, 46.1, 25.8, 19.3, 18.1, -3.9, -4.8.

$\tilde{\nu}_{max}$ (ATR) 2955, 2930, 2886, 2857, 1710, 1644, 1583, 1463, 1352, 1253, 1188, 1085, 965, 837, 776, 674

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{14}H_{25}O_2Si^+$ 253.1618; found 253.1615

Compound 1



To a solution of TBAF (1 M in THF, 220 µL, 0.220 mmol, 1.1 equiv.) was added glacial AcOH (12.5 µL, 0.218 mmol, 1.1 equiv.). The mixture was stirred for 5 min and then added to a solution of (*E*)-4-((*tert*-butyldimethylsilyl)oxy)-3-(prop-1-en-1-yl)cyclopent-2-en-1-one (**13**) (50 mg, 0.198 mmol, 1.0 equiv.) in THF (2 mL). The reaction mixture was stirred at rt for 3 h, before being diluted with water (10 mL) and extracted with EtOAc (3 x 15 mL). The combined organic layers were dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to a colorless oil. The crude material was purified by AFCC (Heptane/EtOAc 75:25 → 25:75) yielding *rac*-cyclohelminthol I (**1**) as a white solid (21.5 mg, 0.156 mmol, 70%)

***R*$_f$** (3:1 EtOAc/Heptane) 0.37

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.65 (dq, *J* = 16.0, 6.8 Hz, 1H), 6.44 (dq, *J* = 15.8, 1.7 Hz, 1H), 5.98 (s, 1H), 5.13 (t, *J* = 6.2 Hz, 1H), 2.84 (dd, *J* = 18.5, 6.4 Hz, 1H), 2.37 (dd, *J* = 18.5, 1.9 Hz, 1H), 1.96 (dd, *J* = 6.8, 1.6 Hz, 3H), 1.90 (d, *J* = 6.7 Hz, 1H)

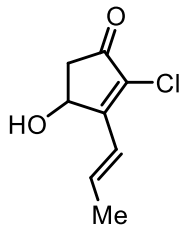
^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 205.4, 170.9, 139.2, 129.2, 125.6, 69.9, 45.6, 19.5

$\tilde{\nu}_{max}$ (ATR) 3386, 2923, 1679, 1638, 1578, 1443, 1288, 1192, 1057, 968

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_8H_{11}O_2^+$ 139.0754; found 130.0759

The obtained data are in accordance with the literature.[3]

Compound 2



To a solution of (*E*)-4-((*tert*-butyldimethylsilyl)oxy)-2-chloro-3-(prop-1-en-1-yl)cyclopent-2-en-1-one (**11**) (99.0 mg, 0.345 mmol, 1.0 equiv.) in MeOH (3.5 mL) was added acetyl chloride (100 µL, 1.40 mmol, 4.1 equiv.) at 0 °C. The mixture was stirred at 0 °C for 15 min, after which it was warmed to rt and stirred for further 1 h. The mixture was diluted with water (25 mL) and extracted with EtOAc (3 x 25 mL). The combined organic layers were dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to a yellow oil. The crude material was purified by AFCC (Heptane/EtOAc 75:25 → 60:40) yielding *rac*-cyclohelminthol II (**2**) as a white solid (48.2 mg, 0.279 mmol, 81%)

***R*$_f$** (2:1 Heptane/EtOAc) 0.21

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.82 (dq, *J* = 16.0, 6.8 Hz, 1H), 6.59 (dq, *J* = 15.9, 2.0 Hz, 1H), 5.15 (brt, *J* = 6.3 Hz, 1H), 2.93 (dd, *J* = 18.7, 6.4 Hz, 1H), 2.45 (dd, *J* = 18.8, 1.6 Hz, 1H), 2.33 (d, *J* = 7.0 Hz, 1H), 2.02 (dd, *J* = 6.8, 1.7 Hz, 3H)

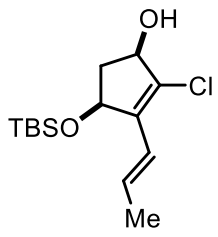
^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 197.2, 161.8, 141.3, 131.0, 123.3, 67.7, 43.6, 20.0.

$\tilde{\nu}_{max}$ (ATR) 3417, 2932, 1702, 1634, 1578, 1442, 1289, 1241, 1195, 1058, 1017, 969, 853

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_8H_{10}{}^{35}ClO_2^+$ 173.0364; found 173.0366, calc. for $C_8H_{10}{}^{37}ClO_2^+$ 175.0335; found 175.0334

The obtained data are in accordance with the literature.[3]

Compound 14



To a slurry of NaI (11.8 mg, 0.0787 mmol, 0.51 equiv.) in anhydrous PhMe (0.9 mL) at -30 °C was added Red-Al (70 wt% in PhMe, 29 µL, 0.104 mmol, 0.67 equiv.). The mixture was then treated with a solution of (*E*)-4-((*tert*-butyldimethylsilyl)oxy)-2-chloro-3-(prop-1-en-1-yl)cyclopent-2-en-1-one (44.5 mg, 0.155 mmol, 1.0 equiv.) (**11**) in TBME (0.5 mL) dropwise. The reaction mixture was stirred at -30 °C for 4 h, before being treated with sat. aq. NH_4Cl (10 mL) and extracted with DCM (3 x 15 mL). The combined organic layers were dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to a light-yellow

oil. The crude material was purified by AFCC (Heptane/EtOAc 100:0 → 90:10) yielding TBS-protected cyclohelminthol III (**14**) as a colorless oil (24.5 mg, 0.0848 mmol, 55%)

R_f (9:1 Heptane/EtOAc) 0.26

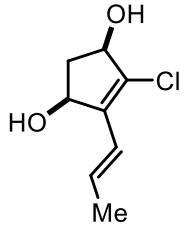
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.25 – 6.12 (m, 2H), 4.82 (dd, *J* = 6.8, 3.6 Hz, 1H), 4.45 (td, *J* = 7.6, 3.8 Hz, 1H), 2.67 (dt, *J* = 13.9, 7.0 Hz, 1H), 2.04 (d, *J* = 7.8 Hz, 1H), 1.87 – 1.81 (m, 3H), 1.73 (dt, *J* = 13.7, 3.7 Hz, 1H), 0.89 (s, 9H), 0.12 (s, 3H), 0.12 (s, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 139.1, 132.5, 132.4, 122.5, 76.1, 73.1, 42.8, 25.9, 19.1, 18.1, -3.8, -4.7

$\tilde{\nu}_{max}$ (ATR) 3390, 2955, 2929, 2885, 2856, 1253, 1074, 966, 892, 834, 774

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_{14}H_{25}{}^{35}ClNaO_2Si^+$ 311.1205; found 311.1211, calc. for $C_{14}H_{25}{}^{37}ClNaO_2Si^+$ 313.1176; found 313.1181

Compound 3



To a solution of *rac*-(1*R*,4*S*)-4-((*tert*-butyldimethylsilyl)oxy)-2-chloro-3-((*E*)-prop-1-en-1-yl)cyclopent-2-en-1-ol (**14**) (24.5 mg, 0.0848 mmol, 1.0 equiv.) in MeOH (1 mL) was added acetyl chloride (24.0 µL, 0.339 mmol, 4.0 equiv.) at 0 °C. The mixture was stirred at 0 °C for 30 min, after which the mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 15 mL). The combined organic layers were dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to a colorless oil. The crude material was purified by AFCC (Heptane/EtOAc 70:30 → 45:55) yielding *rac*-cyclohelminthol III (**3**) as a colorless oil (13.4 mg, 0.0767 mmol, 91%)

R_f (1:1 Heptane/EtOAc) 0.31

^{1}H NMR (400 MHz, CD_3OD) δ_H (ppm) 6.35 (dq, *J* = 15.9, 6.3 Hz, 1H), 6.24 (d, *J* = 16.8 Hz, 1H), 4.72 (dd, *J* = 7.6, 3.9 Hz, 1H), 4.40 (dd, *J* = 7.7, 4.2 Hz, 1H), 2.78 (dt, *J* = 14.1, 7.6 Hz, 1H), 1.84 (d, *J* = 6.4 Hz, 3H), 1.61 (dt, *J* = 14.0, 4.1 Hz, 1H)

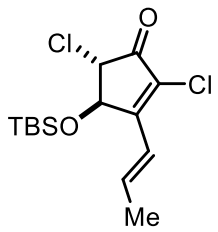
^{13}C NMR (101 MHz, CD_3OD) δ_C (ppm) 139.6, 134.0, 133.1, 123.6, 75.7, 72.7, 42.9, 19.1.

$\tilde{\nu}_{max}$ (ATR) 3315, 2912, 1436, 1326, 1122, 1054, 967, 900, 850, 694

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_8H_{11}{}^{35}ClNaO_2{}^+$ 197.0340; found 197.0346, calc. for $C_8H_{11}{}^{37}ClNaO_2{}^+$ 199.0311; found 199.0315

The obtained data are in accordance with the literature.[3]

Compound 15



To a solution of LiHMDS (1 M in THF, 765 µL, 0.765 mmol, 1.0 equiv.) in anhydrous THF (3.8 mL) at -78 °C was added a solution of (*E*)-4-((*tert*-butyldimethylsilyl)oxy)-2-chloro-3-(prop-1-en-1-yl)cyclopent-2-en-1-one (**11**) (218.9 mg, 0.763 mmol, 1.0 equiv.) in anhydrous THF (3.0 mL) dropwise. The mixture was stirred at -78 °C for 30 min. Trifluoromethanesulfonyl chloride (89 µL, 0.84 mmol, 1.1 equiv.) was then added at -78 °C in one portion. The mixture was stirred for 15 min at -78 °C, then allowed to warm to rt. After 1 h at rt, the reaction was quenched by addition of sat. aq. NH_4Cl (10 mL), diluted with water (5 mL) and extracted with DCM (3x15 mL). The combined organic layers were washed with brine (15 mL), dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo*. The crude material was purified by AFCC (Heptane/EtOAc 100:0 → 97:3) yielding α-chloroenone **15** as a light-yellow oil (180.0 mg, 0.560 mmol, 73%)

***R*$_f$** (9:1 Heptane/EtOAc) 0.58

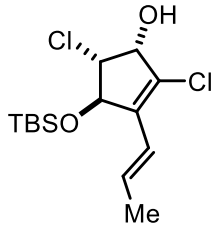
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.75 (dq, *J* = 15.9, 6.9 Hz, 1H), 6.51 (dq, *J* = 15.9, 0.8 Hz, 1H), 5.01 (d, *J* = 1.0 Hz, 1H), 4.15 (d, *J* = 1.8 Hz, 1H), 2.00 (dd, *J* = 6.9, 1.7 Hz, 3H), 0.90 (s, 9H), 0.24 (s, 3H), 0.24 (s, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 190.7, 160.9, 143.3, 128.7, 122.9, 78.2, 61.2, 25.8, 19.9, 18.0, -3.6, -5.2

$\tilde{\nu}_{max}$ (ATR) 2954, 2930, 2886, 2858, 1732, 1636, 1576, 1471, 1255, 1095, 964, 863, 830, 778, 737

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{14}H_{23}{}^{35}Cl_2O_2Si^+$ 321.0839; found 321.0845 calc. for $C_{14}H_{23}{}^{35}Cl^{37}ClO_2Si^+$ 323.0810; found 323.0816, Calc. for $C_{14}H_{23}{}^{35}Cl_2O_2Si^+$ 325.0781; found 325.0786

Compound 17



To a solution of *rac*-(4*R*,5*S*)-4-((*tert*-butyldimethylsilyl)oxy)-2,5-dichloro-3-((*E*)-prop-1-en-1-yl)cyclopent-2-en-1-one (**15**) (102.7 mg, 0.320 mmol, 1.0 equiv.) and $CeCl_3 \cdot 7H_2O$ (156 mg, 0.419 mmol, 1.3 equiv.) in MeOH (3.2 mL) at 0 °C was added $NaBH_4$ (42.2 mg, 1.12 mmol, 3.5 equiv.) portion wise. The mixture was stirred for 15 min. The reaction was then quenched with water (15 mL) and extracted with DCM (3 x 20 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo* to a colorless oil. The crude material was purified by AFCC (Heptane/EtOAc 100:0 → 90:10) yielding the alcohol **17** as a colorless oil (58.2 mg, 0.180 mmol, 56%)

***R*$_f$** (9:1 Heptane/EtOAc) 0.30

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.22 (dq, *J* = 15.9, 1.7 Hz, 1H), 6.06 (dq, *J* = 15.9, 6.6 Hz, 1H), 4.96 (d, *J* = 2.2 Hz, 1H), 4.84 (dd, *J* = 8.8, 5.3 Hz, 1H), 4.29 (dd, *J* = 5.4, 2.3 Hz,

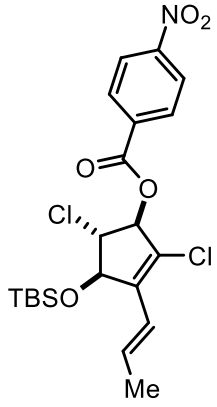
1H), 2.36 (d, J = 8.8 Hz, 1H), 1.86 (dq, J = 6.6, 0.9 Hz, 3H), 0.88 (s, 8H), 0.19 (s, 3H), 0.15 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 137.2, 132.9, 130.7, 122.2, 80.6, 75.4, 66.7, 25.8, 19.0, 18.1, -3.9, -4.7

$\tilde{\nu}_{max}$ (ATR) 3391, 2955, 2930, 2885, 2857, 1254, 1069, 964, 860, 834, 774

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_{14}H_{24}{}^{35}Cl_2NaO_2Si^+$ 345.0815; found 345.0819, Calc. for $C_{14}H_{24}{}^{35}Cl^{37}ClNaO_2Si^+$ 347.0786; found 347.0791, $C_{14}H_{24}{}^{37}Cl_2NaO_2Si^+$ 349.0757; found 349.0764

Compound 18



To a solution of *rac*-(1*S*,4*R*,5*R*)-4-((*tert*-butyldimethylsilyl)oxy)-2,5-dichloro-3-((*E*)-prop-1-en-1-yl)cyclopent-2-en-1-ol (**17**) (58.2 mg, 0.180 mmol, 1.0 equiv.) in anhydrous THF (1.4 mL) at rt was added triphenylphosphine (94.3 mg, 0.360 mmol, 2.0 equiv.) and 4-nitrobenzoic acid (60.5 mg, 0.362 mmol, 2.0 equiv.) followed by dropwise addition of a solution of DIAD (71 µL, 0.36 mmol, 2.0 equiv.) in anhydrous THF (0.4 mL). The mixture was stirred at rt for 2 h before the solvent was removed *in vacuo* yielding a brown sticky oil. The crude material was purified by AFCC (Heptane/EtOAc 100:0 → 96:4) yielding the ester **18** as an orange oil (73.9 mg, 0.156 mmol, 74%)

R_f (9:1 Heptane/EtOAc) 0.55

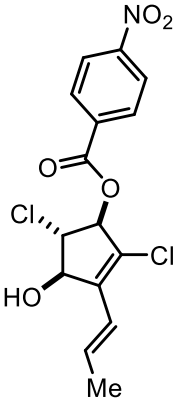
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 8.32 – 8.23 (m, 4H), 6.26 – 6.19 (m, 2H), 5.97 (d, J = 2.9 Hz, 1H), 4.92 (d, J = 2.5 Hz, 1H), 4.05 (t, J = 2.7 Hz, 1H), 1.91 – 1.85 (m, 3H), 0.87 (s, 9H), 0.19 (s, 3H), 0.18 (s, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 164.0, 150.9, 140.9, 134.9, 134.7, 131.2, 124.8, 123.7, 121.7, 84.4, 82.0, 65.0, 25.8, 19.1, 18.1, -3.7, -4.8

$\tilde{\nu}_{max}$ (ATR) 2955, 2930, 2886, 2857, 1734, 1608, 1529, 1346, 1256, 1093, 1014, 965, 861, 852, 836, 777, 717

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_{21}H_{28}{}^{35}Cl_2NNaO_5Si^+$ 494.0928; found 494.0934, Calc. for $C_{21}H_{28}{}^{35}Cl^{37}ClNNaO_5Si^+$ 496.0899; found 496.0907, Calc. for $C_{21}H_{28}{}^{37}Cl_2NNaO_5Si^+$ 498.0870; found 498.0883

Compound S3



To a suspension of *rac*-(1*R*,4*R*,5*R*)-4-((*tert*-butyldimethylsilyl)oxy)-2,5-dichloro-3-((*E*)-prop-1-en-1-yl)cyclopent-2-en-1-yl 4-nitrobenzoate (**18**) (61.6 mg, 0.130 mmol, 1.0 equiv.) in MeOH (1.3 mL) was added acetyl chloride (37 µL, 0.52 mmol, 4.0 equiv.) at 0 °C. The mixture was stirred at 0 °C for 15 min, after which it was heated to rt and stirred for 4 h. The mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 15 mL). The combined organic layers were washed with brine (10 mL), dried over Na_2SO_4, filtered through sand and celite and concentrated in vacuo to light brown crystals. The crude material was purified by AFCC (Heptane/EtOAc 100:0 → 80:20) yielding the alcohol **S3** as a white solid (32.8 mg, 0.0916 mmol, 70%)

***R**f* (2:1 Heptane/EtOAc) 0.44

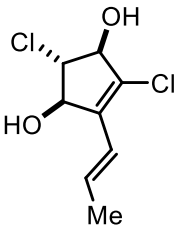
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 8.30 (d, *J* = 8.9 Hz, 2H), 8.24 (d, *J* = 8.9 Hz, 2H), 6.43 (dq, *J* = 16.0, 6.5 Hz, 1H), 6.31 (d, *J* = 16.3 Hz, 1H), 5.92 (d, *J* = 3.0 Hz, 1H), 4.96 (dd, *J* = 7.3, 2.8 Hz, 1H), 4.22 (t, *J* = 2.9 Hz, 1H), 2.62 (d, *J* = 7.4 Hz, 1H), 1.92 (d, *J* = 6.5 Hz, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 164.0, 151.0, 140.2, 135.6, 134.6, 131.2, 125.5, 123.8, 121.5, 84.5, 81.2, 64.7, 19.3

$\tilde{\nu}_{max}$ (ATR) 3453, 3112, 2939, 1732, 1654, 1527, 1346, 1260, 1097, 966, 718

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_{15}H_{13}{}^{35}Cl_2NNaO_5^+$ 380.0063; found 380.0065, calc. for $C_{15}H_{13}{}^{35}Cl^{37}ClNNaO_5^+$ 382.0034; found 382.0036, calc. for $C_{15}H_{13}{}^{37}Cl_2NNaO_5^+$ 384.0005; found 384.0006

Compound 4



To a solution of *rac*-(1*R*,4*R*,5*S*)-2,5-dichloro-4-hydroxy-3-((*E*)-prop-1-en-1-yl)cyclopent-2-en-1-yl 4-nitrobenzoate (**S3**) (28.4 mg, 0.0793 mmol, 1.0 equiv.) in MeOH (1 mL) at 0 °C was added K_2CO_3 (22.5 mg, 0.163 mmol, 2.1 equiv.) The mixture was stirred at 0 °C for 15 min, before water (10 mL) was added and the mixture was extracted with EtOAc (3 x 15 mL). The combined organic layers were dried over Na_2SO_4, filtered through sand and celite and concentrated in vacuo to a white solid. The crude material was purified by AFCC (Heptane/EtOAc 95:5 → 70:30) yielding *rac*-cyclohelminthol IV (**4**) as a white solid (11.5 mg, 0.0550 mmol, 69%).

***R**f* (2:1 Heptane/EtOAc) 0.30

^{1}H NMR (400 MHz, CD_3OD) δ_H (ppm) 6.41 (dq, *J* = 15.6, 6.5 Hz, 1H), 6.23 (d, *J* = 15.9 Hz, 1H), 4.66 (d, *J* = 4.6 Hz, 1H), 4.40 (d, *J* = 4.8 Hz, 1H), 3.80 (t, *J* = 4.7 Hz, 1H), 1.85 (d, *J* = 6.7 Hz, 3H)

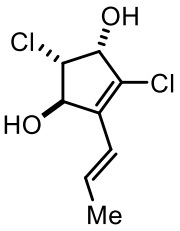
^{13}C NMR (101 MHz, CD_3OD) δ_C (ppm) 138.1, 134.1, 130.9, 123.0, 82.1, 80.6, 70.6, 19.2

$\tilde{\nu}_{max}$ (ATR) 3316, 2912, 1720, 1653, 1607, 1530, 1445, 1302, 1264, 1211, 1116, 1047, 967, 897, 849, 806, 751

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_8H_{10}{}^{35}Cl_2NaO_2^+$ 230.9950; found 230.9945, calc. for $C_8H_{10}{}^{35}Cl^{37}ClNaO_2^+$ 232.9921; found 232.9916, calc. for $C_8H_{10}{}^{37}Cl_2NaO_2^+$ 234.9897; found 234.9888

The obtained data are in accordance with the literature.[2]

Compound S4



To a solution of *rac*-(1*S*,4*R*,5*R*)-4-((*tert*-butyldimethylsilyl)oxy)-2,5-dichloro-3-((*E*)-prop-1-en-1-yl)cyclopent-2-en-1-ol (**17**) (18.1 mg, 0.056 mmol, 1.0 equiv.) in MeOH (1.0 mL) was added acetyl chloride (16 µL, 0.22 mmol, 4.0 equiv.) at 0 °C. The mixture was stirred at 0 °C for 15 min, after which it was heated to rt and stirred. After 2 h, the mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 15 mL). The combined organic layers were washed with brine (10 mL), dried over Na_2SO_4, filtered through sand and celite and concentrated in vacuo to a yellow oil. The crude material was purified by AFCC (Heptane/EtOAc 95:5 → 75:25) yielding the cyclohelminthol IV epimer **S4** as a white solid (9.1 mg, 0.0435 mmol, 78%).

***R**f* (2:1 Heptane/EtOAc) 0.21

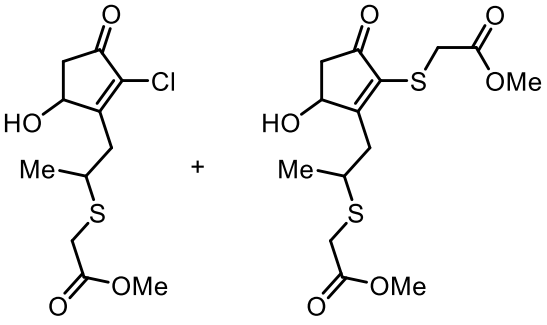
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.30 (br d, *J* = 16.1 Hz, 1H), 6.22 (dq, *J* = 16.0, 5.7 Hz, 1H), 5.03 (dd, *J* = 5.6, 3.0 Hz, 1H), 4.82 (dd, *J* = 8.0, 5.6 Hz, 1H), 4.38 (dd, *J* = 5.4, 3.0 Hz, 1H), 2.40 (d, *J* = 8.0 Hz, 1H), 2.05 (d, *J* = 5.7 Hz, 1H), 1.89 (br d, *J* = 6.3 Hz, 3H)

^{1}H NMR (400 MHz, CD_3OD) δ_H (ppm) 6.35 (dq, *J* = 16.1, 5.6 Hz, 1H), 6.26 (br d, *J* = 16.4 Hz, 1H), 4.60 (br d, *J* = 5.7 Hz, 1H), 4.20 (dd, *J* = 5.7, 4.3 Hz, 1H), 1.86 (br d, *J* = 6.1 Hz, 3H)

^{13}C NMR (101 MHz, CD_3OD) δ_C (ppm) 139.0, 134.3, 131.0, 123.1, 81.1, 76.0, 67.4, 19.2

$\tilde{\nu}_{max}$ (ATR) 3337, 2925, 2852, 1651, 1443, 1303, 1126, 1093, 1058, 1024, 967, 899, 833, 800, 705

Compound 19 and 20



To a solution of cyclohelminthol II (**4**, 33.7 mg, 0.195 mmol, 1.0 equiv.) and methylthioglycolate (52 uL, 0.59 mmol, 3.0 equiv.) in DCM (5 mL) was added DBU (58 uL, 0.39 mmol, 2.0 equiv.) at 0 °C. The mixture was stirred at this temperature for 20 min. The mixture was diluted with water (25 mL) and extracted with DCM (3 x 25 mL). The combined organic layers were dried

over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to a colorless oil. The crude material was purified by AFCC (Heptane/EtOAc 60:40 → 25:75) yielding **19** (22.0 mg, 41%) and **20** (27.0 mg, 40%) both as colorless oils. The compounds were repurified by AFCC to obtain analytically pure samples of each diastereoisomer.

Compound 19 data (Monoaddition):
Isomer 1:

R_f (1:1 Heptane/EtOAc) 0.26

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 5.00 (td, *J* = 6.3, 2.3 Hz, 1H), 3.79 – 3.73 (m, 1H), 3.77 (s, 3H), 3.39 (d, *J* = 14.6 Hz, 1H), 3.33 – 3.23 (m, 1H), 3.29 (d, *J* = 14.6 Hz, 1H), 3.01 – 2.93 (m, 2H), 2.76 (dd, *J* = 13.9, 8.3 Hz, 1H), 2.47 (dd, *J* = 18.5, 2.3 Hz, 1H), 1.34 (d, *J* = 6.8 Hz, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 197.1, 171.9, 168.7, 134.9, 68.7, 53.2, 43.1, 38.2, 34.5, 32.0, 21.1

$\tilde{\nu}_{max}$ (ATR) 3452, 2956, 2937, 1722, 1624, 1436, 1279, 1142, 1060, 1006, 972, 947

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_{11}H_{15}{}^{35}ClNaO_4S^+$ 301.0272; found 301.0279, Calc. for $C_{11}H_{15}{}^{37}ClNaO_4S^+$ 303.0243; found 301.0245

Isomer 2:

R_f (1:1 Heptane/EtOAc) 0.26

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 5.12 (td, *J* = 6.3, 2.1 Hz, 1H), 3.75 (s, 3H), 3.49 – 3.26 (m, 4H), 2.97 (dd, *J* = 18.5, 6.3 Hz, 1H), 2.89 (d, *J* = 6.5 Hz, 2H), 2.46 (dd, *J* = 18.6, 2.2 Hz, 1H), 1.38 (d, *J* = 6.9 Hz, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 197.2, 171.7, 168.3, 135.4, 69.6, 53.0, 43.4, 39.5, 34.9, 33.3, 21.6

$\tilde{\nu}_{max}$ (ATR) 3451, 2955, 2926, 1722, 1623, 1437, 1295, 1160, 1064, 1007, 973

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_{11}H_{15}{}^{35}ClNaO_4S^+$ 301.0272; found 301.0278, Calc. for $C_{11}H_{15}{}^{37}ClNaO_4S^+$ 303.0243; found 301.0245

Compound 20 data (Diaddition):
Isomer 1:

R_f (1:1 Heptane/EtOAc) 0.15

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 4.93 (td, *J* = 6.4, 2.6 Hz, 1H), 3.83 (d, *J* = 15.2 Hz, 1H), 3.76 (s, 3H), 3.70 (d, *J* = 15.3 Hz, 1H), 3.68 (s, 3H), 3.65 (d, *J* = 6.5 Hz, 1H), 3.38 (d, *J* = 14.5 Hz, 1H), 3.31 – 3.21 (m, 2H), 3.00 (dd, *J* = 13.5, 6.4 Hz, 1H), 2.91 (dd, *J* = 18.4, 6.4 Hz, 1H), 2.81 (ddd, *J* = 13.5, 8.0, 0.8 Hz, 1H), 2.43 (dd, *J* = 18.4, 2.6 Hz, 1H), 1.31 (d, *J* = 6.8 Hz, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 201.5, 174.3, 171.9, 170.1, 135.9, 69.6, 53.1, 52.6, 44.4, 38.9, 35.6, 32.1, 32.0, 21.2

$\tilde{\nu}_{max}$ (ATR) 3475, 2954, 1731, 1707, 1597, 1436, 1276, 1196, 1136, 1059, 1006

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_{14}H_{20}NaO_6{}^{32}S_2{}^+$ 371.0594; found 371.0603

Isomer 2:

***R*$_f$** (1:1 Heptane/EtOAc) 0.15

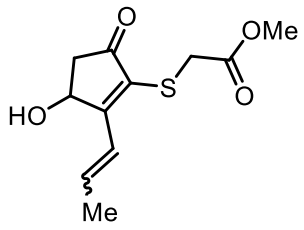
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 5.02 (td, *J* = 6.4, 2.4 Hz, 1H), 3.77 (d, *J* = 2.1 Hz, 2H), 3.74 (s, 3H), 3.68 (s, 3H), 3.45 – 3.36 (m, 1H), 3.35 (d, *J* = 4.4 Hz, 2H), 3.25 (d, *J* = 6.8 Hz, 1H), 2.99 – 2.86 (m, 3H), 2.41 (dd, *J* = 18.4, 2.5 Hz, 1H), 1.35 (d, *J* = 6.9 Hz, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 201.6, 173.9, 171.6, 170.2, 136.3, 70.2, 52.9, 52.7, 44.7, 39.9, 35.8, 33.0, 32.0, 21.7

$\tilde{\nu}_{max}$ (ATR) 3484, 2954, 1732, 1709, 1598, 1436, 1410, 1279, 1196, 1158, 1064, 1007

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_{14}H_{20}NaO_6{}^{32}S_2{}^+$ 371.0594; found 371.0595

Compound 21



To a 1:1 mixture of diastereomers of **20** (7.0 mg, 0.020 mmol, 1.0 equiv.) in anhydrous DCM (1 mL) was added DBU (10 µL, 0.066 mmol, 3.3 equiv.). The mixture was stirred for 30 min and then concentrated *in vacuo* to a brown oil. The crude material was purified by AFCC (Heptane/EtOAc 60:40 → 25:75) yielding **21** as a colorless oil (1.5 mg, 0.0062 mmol, 31%)

***R*$_f$** (1:1 Heptane/EtOAc) 0.22

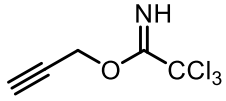
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.79 – 6.67 (m, 2H), 5.11 (td, *J* = 6.8, 1.7 Hz, 1H), 3.81 (d, *J* = 15.2 Hz, 1H), 3.75 (d, *J* = 15.2 Hz, 1H), 3.69 (s, 3H), 2.89 (dd, *J* = 18.6, 6.5 Hz, 1H), 2.44 (dd, *J* = 18.6, 1.7 Hz, 1H), 2.01 (d, *J* = 5.1 Hz, 3H), 1.98 (d, *J* = 7.1 Hz, 1H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 201.9, 170.2, 167.1, 140.1, 131.9, 124.8, 68.2, 52.7, 44.9, 32.2, 19.9

$\tilde{\nu}_{max}$ (ATR) 3450, 2954, 1735, 1700, 1631, 1551, 1437, 1279, 1189, 1017, 971

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_{11}H_{14}NaO_4{}^{32}S^+$ 265.0505; found 265.0507

Propargyl trichloroacetimidate



A solution of propargyl alcohol (590 µL, 10.0 mmol, 1.0 equiv) and trichloroacetonitrile (1.2 mL, 12 mmol, 1.2 equiv.) in anhydrous DCM (25 mL) was cooled to 0 °C in an ice bath and DBU (150 µL, 1.0 mmol, 0.1 equiv.) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 1 h, then warmed to room temperature and stirred for another 30 min. The mixture was passed through a short silica plug, which was washed with EtOAc. The solvent was removed *in vacuo* yielding a yellow oil. The crude material was purified by AFCC (Heptane/EtOAc 100:0 → 92:8) yielding propargyl trichloroacetimidate as a colorless oil (1343 mg, 6.70 mmol, 67%)

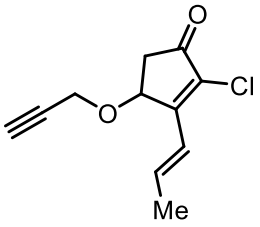
***R*$_f$** (4:1 Heptane/EtOAc) 0.56

^{1}H NMR (400 MHz, CDCl$_3$) δ_H (ppm) 8.50 (s, 1H), 4.92 (d, *J* = 2.4 Hz, 2H), 2.55 (t, *J* = 2.4 Hz, 1H).

^{13}C NMR (101 MHz, CDCl$_3$) δ_C (ppm) 162.0, 90.8, 77.1, 75.7, 56.7

The obtained data are in accordance with the literature.[4]

Compound 22



To a stirred solution of cyclohelminthol II (5.0 mg, 0.029 mmol, 1.0 equiv.) and propargyl trichloroacetimidate (12.1 mg, 0.0604 mmol, 2.1 equiv.) in 2:1 anhydrous *n*-hexane/anhydrous DCM (1 mL) was added a drop of triflic acid. After 22 h, the solvent was removed *in vacuo* yielding a brown solid. The crude material was repurified by AFCC (Heptane/EtOAc 100:0 → 75:25) yielding alkyne tagged cyclohelminthol II (**22**) as a colorless oil (1.2 mg, 0.0057 mmol, 20%)

***R*$_f$** (2:1 Heptane/EtOAc) 0.53

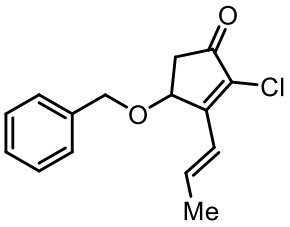
^{1}H NMR (400 MHz, CDCl$_3$) δ_H (ppm) 6.83 (dq, *J* = 15.9, 6.8 Hz, 1H), 6.59 (dq, *J* = 15.9, 2.1 Hz, 1H), 5.14 (d, *J* = 5.5 Hz, 1H), 4.33 (dd, *J* = 16.0, 2.4 Hz, 1H), 4.17 (dd, *J* = 16.1, 2.3 Hz, 1H), 2.82 (dd, *J* = 18.3, 6.0 Hz, 1H), 2.54 (dd, *J* = 18.3, 1.6 Hz, 1H), 2.54 (t, *J* = 2.4 Hz, 1H), 2.01 (dd, *J* = 6.9, 1.7 Hz, 3H)

^{13}C NMR (101 MHz, CDCl$_3$) δ_C (ppm) 196.8, 160.0, 141.7, 131.6, 123.3, 78.8, 76.0, 72.8, 56.4, 39.7, 20.1

$\tilde{\nu}_{max}$ (ATR) 3267, 2912, 2110, 1716, 1638, 1580, 1443, 1349, 1281, 1241, 1191, 1077, 970

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{11}H_{12}{}^{35}ClO_2^+$ 211.0521; found 211.0518, calc. for $C_{11}H_{12}{}^{37}ClO_2^+$ 213.0491; found 213.0487

Compound S5



To a stirred solution of cyclohelminthol II (5.0 mg, 0.029 mmol, 1.0 equiv.) and benzyl 2,2,2-trichloroacetimidate (11 µL, 0.059 mmol, 2.0 equiv.) in 2:1 anhydrous *n*-hexane/anhydrous DCM (1 mL) was added a drop of triflic acid at rt. After 17 h, the mixture was diluted with DCM (20 mL) and washed with sat. aq. $NaHCO_3$ (10 mL) and water (10 mL). The organic layer was dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo* to a yellow solid. The crude material was purified by AFCC (Heptane/EtOAc 100:0 → 80:20) yielding benzyl-protected cyclohelminthol II (**S5**) as a colorless oil (4.8 mg, 0.018 mmol, 63%)

***R*$_f$** (2:1 Heptane/EtOAc) 0.62

^{1}H NMR (400 MHz, CDCl$_3$) δ_H (ppm) 7.41 – 7.30 (m, 5H), 6.67 (dq, *J* = 15.8, 6.3 Hz, 1H), 6.59 (brd, *J* = 16.5 Hz, 1H), 4.95 (dd, *J* = 6.1, 1.7 Hz, 1H), 4.59 (d, *J* = 11.3 Hz, 1H), 4.50 (d,

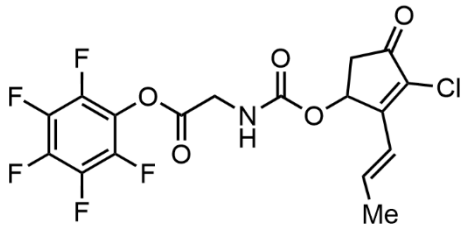
J = 11.3 Hz, 1H), 2.79 (dd, *J* = 18.4, 6.1 Hz, 1H), 2.57 (dd, *J* = 18.5, 1.7 Hz, 1H), 1.98 (dd, *J* = 6.5, 1.2 Hz, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 197.1, 160.5, 141.3, 137.0, 131.5, 128.8, 128.4, 128.3, 123.5, 73.6, 70.9, 39.9, 20.0

$\tilde{\nu}_{max}$ (ATR) 3032, 2867, 1714, 1636, 1581, 1497, 1454, 1443, 1339, 1277, 1239, 1190, 1070,. 962, 737, 698

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{15}H_{16}{}^{35}ClO_2^+$ 263.0833; found 263.0837, Calc. for $C_{15}H_{16}{}^{37}ClO_2^+$ 265.0804; found 265.0807

Compound S6



Cyclohelminthol II (28.1 mg, 0.163 mmol, 1.0 equiv.) was dissolved in PhMe (2 x 1.5 mL) and added to a flask charged with isocyanate-PFP-glycine (216.6 mg, 0.815 mmol, 5.0 equiv.). The resulting solution was then heated in a sealed tube to 100 °C. After 16 h at 100 °C, the reaction mixture was cooled to rt and concentrated by nitrogen flow. The resulting material was directly subjected to purification by AFCC (Heptane/EtOAc 100:0 → 55:45) to isolate **S6** (35.2 mg, 49%) as a white solid and remaining starting material (24.9 mg). Note, the reisolated starting material was contaminated by an isocyanate derivative which could not be removed by another purification. The amount of reisolated starting material (34%) was therefore determined using an internal standard (1,2,4,5-tetrachlorobenzene).

***R*$_f$** (2:1 Heptane/EtOAc) 0.24

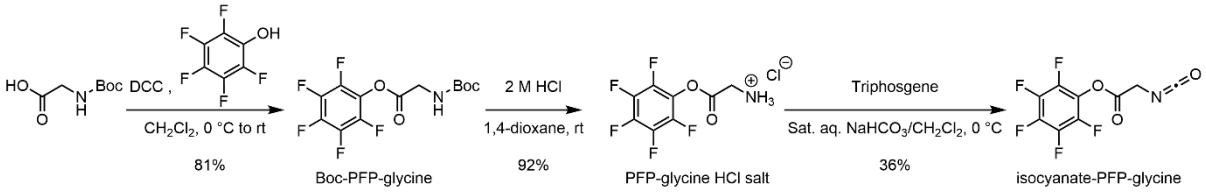
^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.63 – 6.49 (m, 2H), 6.11 (dd, *J* = 6.5, 1.0 Hz, 1H), 5.29 (t, *J* = 6.0 Hz, 1H), 4.43 (dd, *J* = 18.7, 6.2 Hz, 1H), 4.33 (dd, *J* = 18.7, 5.8 Hz, 1H), 3.03 (dd, *J* = 18.9, 6.5 Hz, 1H), 2.49 (dd, *J* = 18.9, 1.5 Hz, 1H), 1.98 (d, *J* = 5.6 Hz, 3H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 196.3, 166.3, 158.3, 155.5, 141.2, 132.4, 122.9, 69.7, 42.4, 41.1, 20.0
Note, the aromatic carbon signals of the PFP ester were of negligible intensity and are not reported

$\tilde{\nu}_{max}$ (ATR) 3335, 1807, 1722, 1638, 1520, 1282, 1142, 1114, 1055, 995

HRMS (ESI+) (m/z) $[M+H]^+$ Calc. for $C_{17}H_{12}{}^{35}ClF_5NO_5^+$ 440.0319; found 440.0317, calc. for $C_{17}H_{12}{}^{37}ClF_5NO_5^+$ 442.0290; found 442.0293

Isocyanate-PFP-glycine



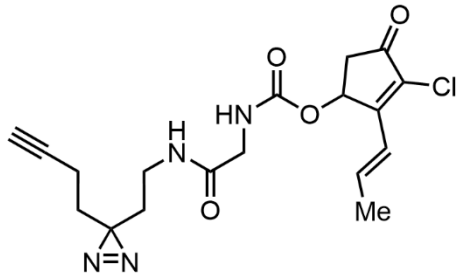
The Boc-PFP-glycine product was obtained following a literature protocol:[5]

A flask was charged with Boc-PFP-glycine (5510.5 mg, 16.149 mmol, 1.0 equiv.) and a stirring bar followed by addition of 1,4-dioxane (0.01 w/w water, 25 mL, transferred using a measuring cylinder) and lastly 4 M HCl in 1,4-dioxane (25 mL) at rt. During the first 10 min, the solution went from clear and colorless to a turbid suspension. After 18 h at rt, full conversion was obtained. The reaction mixture was diluted with heptane (50 mL) and the resulting solid was collected by filtration. The solid material was washed with pentane (50 mL) and the material was collected and dried under high vacuum to afford the PFP-glycine HCl salt as a white fluffy solid (4124.5 mg, 92%).

A flask was charged with a stirring bar (the size should allow for vigorous stirring), sat. aq. $NaHCO_3$ (50 mL) and DCM (30 mL). The suspension was cooled to 0 °C followed by addition of PFP-glycine HCl salt (3113.1 mg, 11.215 mmol, 1.0 equiv., the flask was rinsed with 2 x 5 mL DCM) and lastly triphosgene (892.6 mg, 3.372 mmol, 0.3 equiv., rinse vial with 2 x 5 mL DCM). The resulting suspension was stirred at 0 °C for 30 min after which the reaction mixture was diluted with DCM (150 mL) and water (100 mL). The organic phase was collected and the aqueous phase was extracted twice more with DCM (2 x 50 mL). The combined organic layers were dried over Na_2SO_4, filtered through sand and celite and concentrated *in vacuo*. The resulting material was dissolved in refluxing heptane/EtOAc (approx. 8:1, 75 mL) and the solution was cooled to -24 °C overnight. The resulting solid was removed by filtration and the liquid phase was concentrated *in vacuo* (the liquid phase contains the product). The material was collected and suspended in a minimum pentane/Et_2O (approx. 4 mL, 2:1) and filtered through a PTFE filter. The resulting liquid was concentrated by nitrogen flow which induced precipitation. The resulting oily substance was dried under high vacuum to yield the product (1275.7 mg, 36%). The obtained material was used without further purification.

Spectroscopic data were in agreement with the literature.[6]

Compound S7



PFP-ester **S6** (7.2 mg, 0.016 mmol, 1.0 equiv.) was charged to a flask and dissolved in DCM (0.6 mL) followed by addition of 2-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl)ethan-1-amine (4.7 mg, 0.034 mmol, 2.1 equiv.) and Et_3N (5 µL, 0.036 mmol, 2.3 equiv.) at rt and the flask was covered in aluminum foil. After 15 min at rt, full conversion was obtained. The reaction mixture was cooled to -24 °C for 1 h (only for practical reasons) and then directly subjected to purification by AFCC (Heptane/EtOAc 90:10 → 0:100) to isolate **S7** (5.7 mg, 89%) as a thick oil.

***R**_f* (3:1 EtOAc/heptane) 0.32.

^{1}H NMR (400 MHz, $CDCl_3$) δ_H (ppm) 6.64 – 6.49 (m, 2H), 6.04 (d, J = 6.4 Hz, 1H), 5.90 (t, J = 4.3 Hz, 1H), 5.43 (t, J = 4.8 Hz, 1H), 3.88 (d, J = 5.5 Hz, 2H), 3.16 (q, J = 6.3 Hz, 2H),

3.02 (dd, *J* = 18.9, 6.4 Hz, 1H), 2.50 (dd, *J* = 18.8, 1.5 Hz, 1H), 2.07 – 1.96 (m, 6H), 1.74 (t, *J* = 6.5 Hz, 2H), 1.65 (t, *J* = 7.1 Hz, 2H)

^{13}C NMR (101 MHz, $CDCl_3$) δ_C (ppm) 196.4, 168.4, 158.4, 155.7, 141.1, 132.3, 123.0, 82.9, 69.7, 69.6, 44.6, 41.3, 34.6, 32.5, 32.2, 26.9, 20.1, 13.3

$\tilde{\nu}_{max}$ (ATR) 3303, 2933, 2852, 1719, 1638, 1522, 1443, 1280, 1168, 1003, 973

HRMS (ESI+) (m/z) $[M+Na]^+$ Calc. for $C_{18}H_{21}{}^{35}ClN_4O_4Na^+$ 415.1144; found 415.1145, calc. for $C_{18}H_{21}{}^{37}ClN_4O_4Na^+$ 417.1115; found 417.1122.

4. Biological and HPLC-based methods

Cell culturing

Cells were cultured in vented T75 and T175 flasks (Thermo Scientific, cat. no. 130190 and 130191, respectively) under a humidified atmosphere at 37 °C containing 5 % CO2, with passaging to new flask approximately every third day depending on the cell line. During passaging, the cells were washed twice with warm DPBS (Sigma-Aldrich, cat. no. D8537)) and dissociated from the flask through incubation with a commercial trypsin/EDTA mixture (Sigma-Aldrich, cat. No. T4049). Subsequently, the trypsin mixture was diluted with medium, and one fifth to one twelfth of the resulting solution was transferred to a new flask containing additional fresh culture medium.

U-2OS cells (ATCC HTB-96) were cultured in McCoy's 5A Medium (Sigma-Aldrich, cat. no. M9309) supplemented with FBS (10 %, Gibco, cat. no. A3160801) and penicillin/streptomycin (1 %, Gibco, cat. no. 15150-022). 786-O cells were cultured in RPMI-1640 medium supplemented with FBS (10 %) and penicillin/streptomycin (1 %). Finally, Both HepG2 (Sigma Aldrich, 85011430) and BJ cells (ATCC CRL-2522) were cultured in MEM + GlutaMAX™ (Gibco, cat. no. 41090-028) supplemented with FBS (10 %), penicillin/streptomycin (1 %), MEM non-essential amino acids (1 %, Gibco, cat. no. 11140-050, and sodium pyruvate (1 %, Gibco, cat. no. 11360-070).

Cell Viability (cytotoxicity) Assay

Cells were seeded in 75 µL full growth medium into the inner 60 wells of black-bottomed 96-well plates (Thermo Scientific, cat. no. 137101) at cell densities of 2000 cells/well (U2OS, HepG2, and 786-O cells) or 1500 cells/well (BJ cells) and allowed to adhere through overnight incubation (37 °C, 5 % CO_2, humid). The following day, the examined compounds were prepared as 4X solutions in a four-fold dilution series, through 50-fold dilution of 200X stock solutions in DMSO into full growth medium. The cells were then treated, in technical triplicates, through addition of 25 µL 4X solution to the wells, yielding the desired compound concentrations at a final DMSO concentration of 0.5 %, followed by 46.5 hours of incubation (37 °C, 5 % CO2, humid). Next, 20 µL CellTiter-Blue reagent (Promega, cat. no. G8081) was added to each well, and the plates were returned to the incubator for an additional 1.5 hours of incubation. Finally, the resorufin fluorescence (excitation at 552 ±10 nm and emission at 598 ±10 nm) was measured on a Tecan Spark 10M multimode plate reader. Data treatment consisted of subtraction of background fluorescence (the average fluorescence measured in wells containing medium and CellTiter-Blue reagent but no cells), followed by normalization to the average fluorescence measured from DMSO-treated cells. Finally, the data was plotted and fitted to a 4-parameter nonlinear regression in GraphPad Prism 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. All IC50-values presented represent the mean of three biological replicates.

Cell Painting Assay

The cell painting assay was conducted using the previously described protocol,[7,8] which has been adapted to 96-well format from the protocol published by Bray *et al*.[9]

Cells (U-2OS: 4000 cells/well) were seeded into the inner 60 wells of a 96-well plate with optical bottom (Corning cat. no. 3603) in complete medium (75 µL) and incubated (37 °C, 5% CO_2, humid) for 24 h. Compounds or DMSO were dosed in the designated culture plates in quadruplicates, distributed over 4 plates, as 4X solutions in 25 µL medium with a normalized DMSO concentration (0.5%). A total of 12 DMSO control wells were included on each plate for normalization. After 24 h, 75 µL medium was removed and replaced with 75 µL complete medium containing 500 nM MitoTracker Deep Red (final C = 325 nM) and plates were incubated in the dark for 30 min. Wells were then aspirated and 75 µL medium were added, before adding 25 µL 16% paraformaldehyde (Electron Microscopy Sciences, cat. no. 15710-S) (final PFA = 4%) and incubating in the dark for 20 min. Plates were washed once with 1X HBSS (Invitrogen, cat. no. 14065056) and 75 µL 0.1% (vol/vol) Triton X-100 (BDH, cat. no. 306324N) in 1X HBSS was added and incubated for 15 min in the dark. Plates were washed twice with 1X HBSS before addition of 75 µL multiplex staining solution (Hoechst 33342: 5 µg/mL; Concanavalin-Alexa Fluor 488 conjugate: 35 µg/mL; SYTO 14 Green Fluorescence Nuclei Acid Stain: 3 µM; Phalloidin-Alexa Fluor 568 conjugate: 5 µL/mL; Wheat-Germ agglutinin-Alexa Fluor 555 conjugate: 1.5 µg/mL) in HBSS containing 1% BSA (Sigma-Aldrich cat. no. A9647) and incubation for 30 min in the dark. Plates were then washed three times with 1X HBSS with no final aspiration and imaged immediately in a Zeiss Celldiscoverer 7 automated microscope. 9 images were acquired in each well with 2x2 binning using the AxioCam 702 CMOS 12-bit camera with 4x analog gain in Zen 3.0 software for Celldiscoverer 7 using the following imaging settings:

Table S1. CellDiscoverer 7 imaging settings.

Channel	Dyes	Excitation LED (nm)	Beamsplitter	Emission filter
DNA	Hoechst 33342	385	RTBS 405 + 493 + 610	TBP 425/30 + 524/50 + 688/145
ER	Concanavalin-AF488	470	RTBS 405 + 493 + 610	TBP 425/30 + 524/50 + 688/145
RNA	SYTO 14 green fluorescent nucleic acid stain	511	RTBS 450 + 538 + 610	TBP 467/24 + 555/25 + 687/145
AGP	Phalloidin-AF568 + Wheat-germ agglutinin-AF555	567	RQBS 405 + 493 + 575 + 653	QBP 425/30 + 514/30 + 592/25 + 709/100
Mito	MitoTracker Deep Red	625	RQBS 405 + 493 + 575 + 653	QBP 425/30 + 514/30 + 592/25 + 709/100

To generate the bioactivity profiles the workflow outlined in Svenningsen & Poulsen[7] was followed.

In short, CellProfiler 2.1.1 was used to correct images for uneven illumination followed by image segmentation and extraction of 1476 features across nuclei, cytoplasm and the whole cell on a per-cell basis. Features were then averaged to per-well profiles after which the data was normalized on a per-plate basis followed by per-treatment aggregation which affords the final profiles using the cytominer 0.1.0 package in R 3.6.0.

The heatmap of morphological profiles is visualized with heatmap.2 in the gplots 3.0.3 package. The Pearson correlation matrix is calculated using the stats package in R 3.6.0 and visualized using the corrplot 0.84 package. Hierarchical clustering of the correlation matrix is performed using the stats package using Pearson correlation coefficients as distance metric and average linkage method.

Pearson correlation coefficients > 0.7 were considered strong for the purpose of morphological correlation, and profiles for which the mp-value < 0.1 were considered significantly active.

Finally, PCA analysis was performed for selected profiles (those included in the hierarchical clustering in the main text, as well as a few additional DMSO-profiles) using the FactoMineR 2.11 package. The R script used for the PCA analysis has been uploaded to OSF (DOI: 10.17605/OSF.IO/FEHN5)

HPLC-based Experiments with CHM-II and MTG conjugates

*Stability and reactivity studies with **CHM-II** in aqueous buffer*

200 µM solutions of **CHM-II** were prepared through 100-fold dilution of a 20 mM stock solution (in DMSO) with a 9:1 mixture of potassium phosphate buffer (100 mM, pH 8.1) and acetonitrile (MeCN) with or without the presence of 1 mM methyl thioglycolate (MTG, from a 100 mM stock solution in MeCN). The samples, as well as the relevant DMSO and MTG controls, were prepared just prior to injection on the HPLC by addition of **CHM-II,** thorough mixing, and rapid filtration through a 0.2 µM PTFE syringe filter (Frisenette, Q-Max®, 13PT022-100) into a sample vial (Mikrolab, ML 33003SA, 1-3 minutes from addition of **CHM-II** to sampling). Each sample was measured several times over the course of 5 hours, as well as one measurement on the following day (after ~ 21.7 hours). The HPLC-analysis was conducted using an Agilent 1260 Infinity II instrument equipped with an InfinityLab Poroshell 120 EC-C18 column (3.0 mm x 100 mm, 2.7 µm, Agilent) with a corresponding guard column (Agilent, cat. no. 823750-911), and the column oven was held at a temperature of 40 °C. The mobile phase consisted of Mili-Q water (A) and MeCN (B), the flowrate was 1 mL/min, and the injection volume was 10 µL. The elution method was: 10 % B during 0-1 min, gradient from 10 – 95 % B during 1 - 6 min, gradient from 95-100% B during 6.5 - 7 min, 100% B during 7-8 min, 100-10 % B during 8 – 9 min, and finally 10 % B during 9 – 10 min (the method description has been uploaded to OSF).

UV-traces were collected at several wavelengths with 260 nm, 280 nm, and 230 nm being the main ones of interest. Data analysis was conducted using the OpenLab CDS software (Agilent, peak integral extraction and chromatogram comparisons) and GraphPad Prism 10.2.3 (plotting and half-life determination through fitting to a one-phase decay equation).

*HPLC-based investigation of the stability of **19** and **20** in aqueous buffer*

Samples containing diastereomeric mixtures of either 200 µM monoconjugate (**19**) or 100 µM diconjugate (**20**) were prepared through 100-fold dilutions of DMSO stock solutions in potassium phosphate buffer (100 mM, pH 8.1) containing 10 % (v/v) MeCN. Addition of the

compound, thorough mixing, and filtration through a 0.2 µM PTFE syringe filter (Frisenette, Q-Max®, 13PT022-100) into a sample vial was conducted just prior to the first sampling (1-3 minutes). HPLC- and data analysis was conducted as previously described.

LC-MS investigation of cyclohelminthol II (CHM-II, 2) thiol conjugation

LC-MS-based experiments were conducted on a Dionex UltiMate 3000 UHPLC system equipped with a Ascentis® Express C18 column (100 mm x 2.1 mm, 2 µm, Supelco) coupled to a Bruker MaXis Impact time-of-flight spectrometer using positive electrospray ionization. The column oven was held at 40 °C, the injection volume was 5 µL, and the flowrate was 0.4 mL/min. The mobile phase consisted of Mili-Q water (A) and MeCN (B), both containing 0.1 % (v/v) formic acid, and the elution method used was: 5% B during 0-1 min, gradient from 5-95 % B during 1-9 min, 95 % B during 9-12 min, and finally 95–5% B during 12-13 min. Data analysis was conducted in Bruker Compass DataAnalysis 5.1 with internal calibration to a calibrant solution (sodium formate clusters) by inspection of the UV-traces, as well as searching the data for peaks with masses corresponding to hypothesized conjugation products (protonated or sodium-ion coordinated) by construction of extracted ion chromatograms (EICs). Sample preparation was conducted in varying ways, depending on the experiment, as described below.

*Conjugation of **CHM-II** and methyl thioglycolate in TEAB buffer*

Samples were prepared as described for the HPLC-based experiments with the exception that a triethylammonium bicarbonate buffer (TEAB, 100 mM, pH 8.1) was used in place of a phosphate buffer. After 1.5 hours, the samples were diluted 50-fold with 9:1 MQ-H_2O/MeCN, filtered through 0.2 µM PTFE syringe filter, and submitted for LC-MS analysis, which was conducted within 30 minutes for the reaction mixture sample, and after approximately 1.5 h for the DMSO control sample.

LC-MS analysis of mono- and diconjugate samples after stability measurements

Samples of **19** and **20** were prepared as described in the 'HPLC-based investigation of the stability **19** and **20**' section during a preliminary stability experiment and submitted for LC-MS-analysis approximately 36 hours after compound addition.

Gel-based ABPP in live cells

Compound treatment and cell harvest

U-2OS cells were seeded in a 6-well plate (Thermo Scientific, cat. no. 140675) and allowed to adhere overnight (400,000 cells/well in 2.5 mL medium). Next day, the growth medium was exchanged for 2 mL fresh medium containing the alkyne-tagged probe, **22**, at the desired concentration (diluted 200-fold from stock solutions in DMSO) or DMSO (0.5 % (v/v)) as vehicle control, followed by overnight (approximately 18 hours) incubation (37 °C, 5 % CO_2, humid). At the end of the incubation the cells were washed twice with 1 mL ice-cold DPBS (Sigma-Aldrich, cat. no. D8537) and harvested into 1.5 mL tubes by way of cell scrapers

(Sarstedt, cat. no. 83.3950) in 1 mL ice-cold DPBS (2x 0.5 mL). Next, the cells were pelleted through centrifugation (400 g, 4 °C, 5 min), the supernatant was aspirated, and the cell-pellets were briefly stored on ice prior to cell lysis.

Cell lysis and sample preparation

The cells were lysed via resuspension in 40 µL PBS containing 10 % (v/v) of a broad-spectrum protease inhibitor cocktail (Thermo Scientific, cat. no. A32955), followed by tip-sonication (3x10 seconds with cooling on ice between each round) using a Branson Sonifier 250 equipped with a microtip. The lysates were then cleared through centrifugation (16,000 g, 4 °C, 5 min) and transferred to fresh 1.5 mL tubes, where the protein concentration was determined by way of a commercial BCA assay (Thermo Scientific, cat. no. 23225). Next, samples containing 80 µg protein in 50 µL total volume were prepared by dilution of suitable volumes of lysate with PBS.

Click chemistry (CuAAC)

Next, copper-catalyzed alkyne-azide cycloaddition (CuAAC) was conducted with 5-tetramethylrhodamine (TAMRA) alkyne through addition of 5 µL of a 3:7 CuSO4:THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine, TCI chemicals, cat. no. T3171) mixture (3 µL 50 mM CuSO4 in H2O and 7 µL 100 mM THPTA in H2O, premixed and stored at – 20 °C), 1.5 µL 5-TAMRA-alkyne (10 mM in DMSO, Lumiprobe, cat. no. D7130, and 2.5 µL 100 mM sodium ascorbate. After the addition of the sodium ascorbate, the samples were vortexed and incubated at rt. for 1 h (covered by aluminum foil). At the end of the incubation, the proteins were precipitated and the reaction quenched through addition of 900 µL (9 volumes) of ice-cold MeOH, and the samples were stored overnight at – 20 °C.

SDS-PAGE and fluorescence visualization

The samples were centrifuged (7000 g, 4 °C, 5 min) and the resulting protein pellets were washed via resuspension in 500 µL ice-cold 9:1 MeOH/MQ-H_2O and re-pelleting through centrifugation (7000 g, 4 °C, 5 min). The supernatant was decanted, and the pellets were air-dried for 5-10 minutes while any visible droplets were carefully removed with rolled-up pieces of paper towel. Next, the proteins were redissolved in 36 µL 4 % SDS solution in PBS followed by addition of 12 µL reducing 4X SDS-sample buffer (Bio Rad, cat. no. 1610747) containing 10 % (v/v) β-mercaptoethanol (protein concentration: 1.667 µg/µL). The samples were then heated to 95 °C for 5 minutes and centrifuged briefly in a minicentrifuge before 14 µL (23.3 µg protein/well) was loaded onto a 4-15 % Mini-PROTEAN TGX gel (15 wells, Bio Rad, cat. no. 4561086). 3 µL Precision Plus Protein™ All Blue Standards (Bio Rad, cat. no. 1610373) solution was used as molecular mass marker, and electrophoresis was carried out at 200 V for 26-30 minutes using a Bio Rad running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, diluted from a 10X formulation (Bio Rad, cat. no. 1610732) using MQ-H2O).

After electrophoresis, the gel was washed briefly in MQ-H_2O, and the in-gel fluorescence was detected on a ImageQuant LAS 4000 system in green fluorescence mode (Green (Epi-RGB), 520 nm, 575DF20 filter). Finally, the gel was stained with SimplyBlue SafeStain (ThermoFisher Scientific, cat. no. LC6060) according to the manufacturer's microwave

protocol and digitalized using the ImageQuant LAS 4000 in trans-illumination mode. The contrast of the resulting images was adjusted in Fiji (ImageJ) 2.3.0 to improve visualization, and the final figures were prepared using Affinity Designer 2.5.3.

Gel-based ABPP in cell lysate

U-2OS cells were seeded in a T175 flask (Thermo Scientific, cat. no. 130191) and allowed to grow to 80-90 % confluence before being washed twice with 10 mL ice-cold DPBS, harvested in 10 mL ice-cold DPBS by way of cell-scrapers (Sarstedt, cat. no. 83.3952), and pelleted through centrifugation (400 g, 4 °, 5 min). Subsequently, the supernatant was replaced with 300 µL PBS containing 10 % (v/v) of a broad-spectrum protease inhibitor cocktail (Thermo Scientific, cat. no. A32955), and cell lysis through tip-sonication, lysate clearing, and determination of the protein concentration was conducted as previously described. Once the protein concentration of the lysate was known, samples containing 100 µg protein in 98 µL PBS-diluted lysate were prepared in 1.5 mL tubes, and 2 µL 50X DMSO solution of **22**, or DMSO as vehicle control, was added to each sample, followed by 2 hours of incubation with rotation at rt.

At the end of the treatment, CuAAC conjugation with 5-TAMRA-alkyne was conducted by addition of 10 µL $CuSO_4$:THPTA mixture (as described for the live cell experiment), 3 µL 10 mM 5-TAMRA-alkyne (in DMSO), and 5 µL 100 mM sodium ascorbate (in MQ-H_2O) prior to 1 hour of incubation (dark, no rotation) at rt. After the incubation, the proteins were precipitated, and the reaction quenched, through the addition of 900 µL ice-cold MeOH and overnight storage of the samples at –20 °C.

The remaining parts of the experiment were conducted as described for the live cell-based experiment with the exceptions that 45 µL 4 % SDS solution (in PBS) and 15 µL reducing 4X sample buffer was used to redissolve the pellets after washing, and that a 12 well gel (Bio Rad, cat. no. 4561085) was used (15 µL sample/well: 25 µg protein/well).

Competitive gel-based ABPP with iodoacetamide and 22 in lysate

U-2OS cell lysate was generated as previously described, and after determination of the protein concentration, samples containing 200 µg protein in 96 µL were prepared through suitable dilution of the lysate with PBS. Pre-treatment of the samples with iodoacetamide (IA) was conducted through addition of 2 µL 49X IA solutions (in MQ-H2O), or MQ-H2O as vehicle control, followed by 1 hour of incubation at rt. (dark, with shaking at 300 rpm in an Eppendorf Thermomixer® C. Subsequently, the samples were treated with 22 via addition of 2 µL 50X solution (in DMSO) and another two hours of incubation (dark, shaking). As in the other gel-based experiments, the reaction was quenched by addition of 900 µL ice-cold MeOH, and the samples were stored overnight at –20 °C.

The remaining parts of the experiment were conducted as previously described, with 90 µL 4 % SDS solution and 30 µL reducing 4X sample buffer being used to redissolve the pellets, and with 15 µL sample being loaded onto each well on a 12-well gel.

Western blotting

Western blotting experiments were conducted in 6- or 12-well formats depending on the number of conditions examined in each experiment. As the harvesting and cell lysis steps differed slightly in each format, both are described in the following section.

6-well format

U-2OS (400,000/well) or 786-O (350,000 cells/well) cells were seeded in a 6-well plate (Thermo Scientific, cat. no. 140675) in 2.5 mL medium and allowed to adhere overnight. Next day, the medium was replaced with fresh medium containing the investigated compounds at the desired concentrations (diluted in medium from 400X DMSO solutions) or DMSO as a vehicle control (0.25 %), and the plate was returned to the incubator for 21-24 hours of incubation (always similar for replicate experiments).

At the end of the incubation the cells were washed twice with 1 mL ice-cold DPBS (Sigma-Aldrich, cat. no. D8537) and harvested into 1.5 mL tubes by way of cell scrapers (Sarstedt, cat. no. 83.3950) in 1.3 mL ice-cold DPBS (2x 0.5 mL + 0.3 mL). Next, the cells were pelleted through centrifugation (400 g, 4 °C, 5 min), the supernatant was aspirated, and the cell-pellets were briefly stored on ice prior to cell lysis.

Cell lysis was conducted by resuspension of the cell pellet in 35-50 µL RIPA lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris HCl, 0.5 % (m/v) sodium deoxycholate, 1 % (v/v) NP-40, 0.1 % (m/v) SDS) containing 10 % (v/v) broad-spectrum protease inhibitor cocktail (Thermo Scientific, cat. no. A32955), thorough vortexing, and 10 minutes of incubation on ice. After another round of vortexing, the samples were subjected to tip-sonication (2x10 pulses with cooling on ice in between) before the lysates were cleared by centrifugation (16,000 g, 4 °C, 5 min) and transferred to fresh tubes.

12-well format

U-2OS (125,000 cells/well) cells were seeded in a 12-well plate (Thermo Scientific, Nunc™, cat. no. 150628) in 1.5 mL medium and allowed to adhere overnight. On the following day, the medium was exchanged medium containing the compounds at the desired concentrations (diluted to 1X from 400X solutions in DMSO), or DMSO as a vehicle control (0.25 % (v/v)), and the plate was returned to the incubator for 21- 24 hours. After the incubation, the cells were washed twice with 0.5 mL ice-cold DPBS followed by the addition of 50 µL protease inhibitor containing RIPA buffer (*vide supra*) to each well. Once the lysis buffer had been added, the plate was placed on ice for 10 minutes with occasional swirling of the buffer every couple of minutes by gentle shaking of the plate. Next, the resulting lysate was collected in 1.5 mL tubes, subjected to tip-sonication, cleared by centrifugation (16,000 g, 4 °C, 5 min), and transferred to fresh tubes.

Sample preparation, SDS-PAGE

Once lysates had been generated through one of the two workflows described above, the protein concentration was determined by way of a commercial BCA assay (Thermo Scientific, cat. no. 23225). Subsequently, 7.5 µL reducing 4X sample buffer (Bio Rad, cat. no. 1610747, with 10 % (v/v) added β-mercaptoethanol) was added to samples containing 30 µg protein in 22.5 µL

PBS-diluted lysate to give a final protein concentration of 1 µg/µL (when 15-well gels were used, the final protein concentration was 1.15 µg/µL). Next, the samples were heated to 95 °C for 5 minutes, and 15 µL (14 µL for 15-well gels) was loaded onto 12- or 15-well 4-15 % Mini-PROTEAN TGX gels (Bio Rad, cat. nos. 4561086 and 4561086, respectively) to ensure a protein concentration of 15 µg/well. Electrophoresis was then conducted at 200 V for 26-30 minutes, using 3 µL Precision Plus Protein™ All Blue Standards (Bio Rad, cat. no. 1610373) solution as molecular mass marker, and a 1X Tris/SDS/Glycine solution as running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, diluted from a 10X formulation (Bio Rad, cat. no. 1610732) using MQ-H_2O).

Protein transfer and visualization

Protein transfer was achieved using a Bio-Rad Trans-Blot Turbo Transfer System with Trans-Bot Turbo Mini 0.2 µM PVDF Transfer Packs (Bio-Rad, cat. no. 1704156), and blocking of the membrane was conducted via incubation in a blocking buffer consisting of 5 % (wt/vol) skim milk powder in TBST (tris-buffered saline: 20 mM Tris-base, 150 mM NaCl, 0.1 (vol/vol) % Tween-20, pH = 7.6) for 1 hour at rt. Next, the membrane was incubated overnight with a 1:1000 solution of heme oxygenase 1 (HO-1) specific antibody (monoclonal from rabbit, Cell Signaling Technology, D60G11, cat. no. 5853) in blocking buffer with gentle agitation at 4 °C. Next day, the membrane was washed thrice with TBST (10 minutes each) and incubated with a 1:10,000 solution of HRP-conjugated antibody (Goat Anti-Rabbit IgG H&L (HRP), abcam, cat. no. ab6721) in TBST for 1-2 hours at room temperature. The membrane was then washed thrice with TBST (5 minutes each), developed using SuperSignal™ West Femto detection reagent (Thermo Fisher, cat. no. 34094), and imaged on a ImageQuant LAS 4000 system in chemiluminescence mode.

After the initial visualization, the membrane was stripped through incubation (2x 10 min) with stripping buffer (0.1 % SDS, 1 % Tween-20, 200 mM glycine, pH = 2.2), washed thrice with TBST and reblocked through one hour of incubation with blocking buffer at rt. Finally, the membrane was re-probed for β-actin through the same workflow utilizing the following primary and secondary antibodies: Anti-β-actin (monoclonal from mouse, 1:5000 in blocking buffer, Sigma-Aldrich, A5441) and sheep anti-mouse (1:10,000 in TBST, Cytiva, NA931).

Signal quantification was conducted in ImageQuant v8.2.0 using the β-actin signals as loading controls, and images used in figures were contrast adjusted in Fiji (ImageJ) 2.3.0 to improve visualization, with the final figures being prepared in Affinity Designer 2.5.3.

Thiol-reactivity assay with Ellman's reagent

This experiment was conducted based on the work of Resnick *et al.*[10]

45 µL 444.4 µM TCEP (final C: 200 µM, TCI, cat. No. T1656) solution in PBS was added to the wells of a clear, non-coated 96-well plate (Thermo Scientific, cat. no. 260895), followed by 45 µL PBS or 45 µL 111.1 µM Ellman's reagent (DTNB, 5,5-dithio-bis-(2-nitrobenzoic acid), final C: 50 µM, which yields 100 µM 2-nitro-5-thiobenzoate (TNB^-) upon reduction, Sigma-Aldrich, cat. no. D218200) in PBS. The plate was then incubated 10 minutes at rt to allow for reduction of the disulfide, while 2 mM stock solutions of the compound of interest

(**1-4** as well as the three reference electrophiles EN300-11811, EN300-06928, and EN300-23611) were prepared through 5-fold dilution of 10 mM DMSO stock solutions with PBS. Finally, 10 µL of the 2 mM stock solutions were added to the wells of the 96-well plate, which was swiftly placed in a humidity cassete and taken to a pre-heated (37 °C) Tecan Spark 10M Multimode plate-reader, where the absorbance at 412 nm was measured every 15 minutes over the course of 7 hours. Each compound was examined in three technical replicates in the presence of DTNB and three control replicates without DTNB.

The data analysis, which was likewise based on the work of Resnick *et al.*[10], was conducted using a slightly modified version of the .R scripts prepared by Esben Svenningsen for a previous project[11].

Briefly, the background absorbance, defined as the mean absorbance measured in the triplicate wells with no DTNB for a given compound, was subtracted from each timepoint, and the data was fit a general integrated second-order rate equation for a situation where the initial concentrations of the reactants are not equal:

$$\ln\left(\frac{[A][B]_0}{[B][A]_0}\right) = k \cdot ([B]_0 - [A]_0 \cdot t)$$

Which rearranges to:

$$\ln\left(\frac{[A][B]_0}{[B][A]_0}\right) \cdot \frac{1}{[A]_0 - [B]_0} = k \cdot t$$

Where A is the examined electrophile and B is reduced Ellman's reagent (TNB^{2-}), meaning that $[A]_0$ = 200 µM and $[B]_0$ = 100 µM (assuming complete reduction of DNTB). Using the absorbance to determine the concentration of TNB^{2-} leads to the following expression:

$$[B] = \frac{abs}{abs_0} \cdot [B]_0$$

Furthermore, by assuming a 1:1 consumption ratio between TNB^{2-} and the electrophiles, and knowing that $[A]_0 = [B]_0 + 100$ µM in our experiment, an expression for [A] becomes:

$$[A] = [B] + 100\ \mu M = \frac{abs}{abs_0} \cdot [B]_0 + 100\ \mu M$$

Finally, by utilizing the above expressions for [B] and [A], the second equation can be written as:

$$\ln\left(\frac{\left(\frac{abs}{abs_0} \cdot [B]_0 + 100\ \mu M\right) \cdot [B]_0}{\left(\frac{abs}{abs_0} \cdot [B]_0\right) \cdot [A]_0}\right) \cdot \frac{1}{[A]_0 - [B]_0} = y(t) = k \cdot t$$

The left-hand side of the equation can be calculated for each data point using the absorbance measurement followed by linear regression to find k. Only the first 4 hours of data was used for the fitting and the aforementioned .R scripts used for data processing and plotting is available along with the raw and processed data on OSF.

The Reference electrophiles were purchased from Enamine and used as racemic mixtures. The IDs refer to the catalogue IDs in the Enamine store, and the structures are shown in figure S15.

Reactivity studies with L-glutathione – CHM-II and chloroacetamides

Samples containing 200 µM of either **CHM-II** or one of the investigated chloroacetamide containing compounds (EN300-01752, EN300-01926, and EN300-14750) were prepared through 100-fold dilution of 20 mM DMSO stock solutions in a 9:1 mixture of potassium phosphate buffer (100 µM, pH 8.1) and MeCN, with and without the presence of 1 mM reduced L-glutathione (GSH, freshly dissolved to 100 mM in MQ-H2O, Sigma-Aldrich, cat. no. G4251). Each sample, as well as the relevant controls, were prepared just prior to injection on the HPLC (1-3 minutes) through addition of both glutathione and electrophile and rapid filtration through a Chromafil® Xtra 0.2 µM PET syringe filter (Macherey-Nagel, cat. no. 729222) into HPLC sample vials (Screening Devices, cat. No. KG 09 0188). Each sample was subsequently measured several times over a period of approximately 10 hours. These experiments were conducted using an Agilent 1260 Infinity II instrument equipped with a NUCLEODUR C18 Gravity column (Macherey-Nagel, cat. no. 760080.46) and a corresponding guard column (Macherey-Nagel, cat. no. 761902.30). The column oven temperature was held constant at 40 °C, and the mobile phase consisted of Mili-Q H2O (A), MeCN (B), and 1 % TFA in Mili-Q H2O (C) of which the latter was held constant at 10 %. The flowrate was 1 mL/min, the injection volume was 10 µL, and the elution method was: 80 % A, 10 % B and 10 % C during 0 – 0.5 min, gradient from 10 – 90 % B with 10 % C during 0.5 - 8.5 min, 90 % B and 10 % C during 8.5 - 10.5 min. After each run, the mobile phase was returned to 80 % A, 10 % B, and 10 % C over the course of 2.5 minutes. The method file has been uploaded to OSF.

UV-traces were collected at 260 nm, 280 nm, and 230 nm, and the resulting chromatograms were analyzed in Agilent MassHunter Qualitative Analysis 10.0, by manual inspection and automatic integration of the peaks corresponding to the electrophilic compounds. These integrals were subsequently normalized to the ones observed for the first measurement for each compound to yield a measure of remaining electrophile as a function of time. Finally, half-lives of each compound in the presence of GSH were determined through fitting to a one-phase decay equation in GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

Unbiased amino acid selectivity chemoproteomics workflow

Cell lysis, sample preparation, compound treatment, and CuAAC.

Pellets of U-2OS cells, harvested from T175-flasks as previously described and stored at – 20 °C until lysis, were thawed on ice, resuspended in 300 µL PBS, and lysed by way of ultra-sonication (3x10 seconds at 50 % amplitude with 10 s off in between sets, in ice-containing water). The resulting lysates were cleared by centrifugation (20,000 g, 4 °C, 20 min) and combined in a 1.5 mL tube, while the pellets were discarded. The protein concentration of the lysate was determined by way of a commercial BCA assay (Thermo Scientific, cat. no. 23225) and eight samples containing 200 µg protein in a volume of 100 µL were prepared in protein

LoBind Eppendorf tubes (Eppendorf, cat. no. 0030108442) through dilution of the lysate with PBS. Compound treatment was then conducted through the addition of 2 µL 15.3 mM stock solution of **CHM-II-alk** in DMSO to each sample (final concentration of CHM-II-alk: 300 µM), followed by 4 hours of incubation at rt with shaking at 1000 rpm (in a thermomixer).

Next, copper-catalyzed alkyne-azide cycloaddition (CuAAC) was conducted with heavy- and light isoDTB-azide tags[12] (four samples for each tag type) by addition of 6 µL TBTA (0.9 mg/mL, 1.7 mM) in 4:1 tBuOH:DMSO, 2 µL CuSO4 (8 mg/mL, anhydrous, 50 mM) in MQ-H2O, 2 µL isoDTB-azide tag (50 mM) in DMSO (heavy or light), 2 µL freshly prepared TCEP (13 mg/mL, 50 mM) in MQ-H2O, and 2 µL 10 % SDS in MQ-H2O to each sample prior to one hour of incubation with shaking (1000 rpm) at rt. At the end of the incubation, 0.1 µL benzonase solution (GENIUS™ Nuclease, Santa Cruz, cat. no. SC-202391, 10 U/µL with 20 mM Tris, pH 8, 2 mM MgCl2, 20 mM NaCl, 50 % glycerol) was added, followed by 30 minutes of incubation at 37 °C with shaking (1000 rpm). Finally, the samples were flash-frozen and stored overnight at – 20 °C.

SP3 cleanup and on-bead tryptic digestion

For each pair of samples (one heavy and one light sample. 4 pairs in total), 20 µL hydrophobic and 20 µL hydrophilic SpeedBeads magnetic carboxylate modified particle solution (Cytiva, cat. nos. 45152105050250 and 65152105050250) was added to a single 1.5 mL tube, washed thrice with MQ-H2O, and resuspended in the initial volume of MQ-H2O. Next, the samples were thawed, vortexed, and mixed 1:1 heavy:light in protein LoBind tubes to yield a total of four final samples, to which 40 µL bead mixture was subsequently added, followed by 5 minutes of incubation at rt with shaking (1000 rpm). After the incubation, 920 µL absolute EtOH was added prior to another 5 minutes of incubation at rt. (shaking at 1000 rpm), addition of an additional 300 µL absolute EtOH, and a final 5 minutes of incubation. The samples were then placed on a magnetic rack and washed three times with 80 % EtOH in MQ-H2O. After the final wash the supernatant was removed, and the beads were resuspended in 200 µL freshly prepared 2 M urea (proteomics grade) in PBS containing 0.5 % SDS. Next, 10 µL 0.2 M DTT solution (31 mg/mL) in MQ-H2O was added, followed by 15 minutes of incubation at 65 °C (with shaking at 1000 rmp), after which alkylation was conducted through the addition of 10 µL 0.4 M iodoacetamide (71 mg/mL) in MQ-H2O and incubation at 37 °C for another 30 minutes (with shaking).

880 µL absolute EtOH was subsequently added to each sample, followed by 5 minutes of incubation (with shaking) at rt, three washing steps with 80 % EtOH, and resuspension of the beads in 200 µL 2 M urea in PBS. Finally, 2 µL trypsin (0.5 µg/µL, Promega, cat. no. V5111A) solution was added, and on-bead digestion was conducted overnight at 37 °C (shaking at 800 rpm).

Enrichment of bound peptides

The supernatant was transferred to fresh 2 mL protein low-binding tubes (Sarstedt, cat. no. 72.695.600), and the remaining peptides were through addition of 50 µL 2 % DMSO in MQ-H2O and 30 minutes of incubation at 37 °C (shaking at 1000 rpm). The supernatant was transferred to the aforementioned 2 mL tubes, and the process was repeated once more. During the incubation 50 µL high-capacity streptavidin resin (Thermo Scientific, cat. no. 20361) was added to a falcon tube for each sample and washed thrice with PBS (by centrifugation at 1000

g for 2 min and removal of the supernatant). After the final wash, the beads were resuspended in 1200 µL per sample, and 1200 µL of the suspension was distributed into each of the supernatant-containing 2 mL tubes, followed by 2 hours of incubation (with rotation) at rt.

At the end of the incubation, the samples were centrifuged (1000 g, rt, 3 min), the supernatant aspirated, and the beads/resin transferred to centrifuge columns (Thermo Scientific, cat. no 89868) after the addition of 600 µL PBS (MS-grade). The beads were then washed twice more with 600 µL PBS, thrice with 600 µL MQ-H2O, and thrice with 600 µL 50 % MeCN in MQ-H2O. The columns were subsequently transferred to fresh 1.5 mL protein LoBind tubes, into which the bound peptides were eluted by addition of 340 µL (200 µL + 2x70 µL) 50 % MeCN in MQ-H2O containing 0.1 % TFA (MS-grade), followed by centrifugation (3000 g, rt, 3 min) and evaporation of the eluent on a SpeedVac system. The dry samples were stored at – 20 °C until just prior to measurement.

Resuspension and filtration

The peptides were dissolved in 30 µL 0.1 % TFA in MQ-H2O by pipetting and 3 minutes of sonication (10 %) before being centrifuged (20,000 g, rt, 1.5 min) to collect all the solution in the bottom of the tube. Simultaneously, one PTFE micro-spin filter (BGB, cat. no. SPFMICPT02) was washed with 300 µL 0.1 % TFA in MQ-H2O for each sample by centrifugation (20000 g, rt, 2 min). Next, the washed filters were transferred to fresh protein LoBind tubes, the 30 µL sample solutions were loaded onto the middle of the filters, and the solution was forced through the filters by centrifugation (20,000, rt, 2 min). Finally, the sample solutions were transferred into MS sample vials and taken for measurement.

Measurement of the samples

Proteomics samples were analyzed by liquid chromatography tandem mass-spectrometry (LC-MS/MS) using an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Scientific) coupled to a Vanquish Neo UHPLC system and autosampler (Thermo Scientific) in a trap-elute configuration. For trapping peptides, a PepMap Neo 5 µm C18, 300 µm x 5 mm trap (Thermo Scientific, 174500) was used which was eluted into an Easy-Spray PepMap Neo 2 µm C18, 75 µm x 500 mm analytical column (Thermo Scientific, ES75500PN) kept at 50 °C with a constant flowrate of 0.3 µL/min. The mobile phase consisted of a mixture of mobile phase A (0.1% FA in ULC-MS grade water (Biosolve) and mobile phase B (0.1% FA in 80% ULC-MS grade acetonitrile (Biosolve), and the separation was performed with a gradient from 6 – 44 % B over 40 minutes, followed by an increase to 90 % B over five minutes (min 40-45), washing of the column with 90 % B for 14 minutes (min 45-59) before column re-equilibration.

Data was acquired in data-dependent acquisition (DDA) mode with the following scan sequence: MS1 master scan (Orbitrap analysis, resolution 240,000, scan range 375-1500 m/z, RF lens 30%, AGC target standard, maximum injection time 80 ms, polarity positive, data type profile) with dynamic exclusion enabled (repeat count 1, exclusion duration 60 s, mass tolerance 10 ppm, dependent scan on single charge state per precursor only). The top precursors were then selected for MS2 analysis with an intensity threshold of 1.0·104 within a 1.7 second duty cycle, through quadrupole isolation (isolation window 1.2 m/z) followed by higher-energy collisional dissociation in the ion routing multipole (collision energy 30%) and analysis of the resulting fragments in the orbitrap (resolution 15,000, mass range normal, maximum injection

time 100 ms, data type centroid). A file containing the full list of MS/MS parameters has been uploaded to OSF.

Proteomics data analysis

General data analysis software setup

Raw data of the LC-MS/MS analysis was converted to mzML format with the MSconvert (version 3.0.19172-57d620127) included in the ProteoWizard software package[13] using the standard settings with vendor's peak picking. Data analysis was conducted using FragPipe (version 22) with MSFragger (version 4.1)[14,15], IonQuant (version 1.10.27)[16], Philosopher (version 5.1.1)[17], and Python (version 3.9.13) installed. The utilized FASTA database (Homo Sapiens, Uniprot ID: UP000005640, reviewed sequences only) was downloaded from UniProt (UP000005640), with decoys and common contaminants added, through the FragPipe interface on September 30th, 2024. All workflows described below are based on the work of Zanon *et al*.[18] **The workflows for the FragPipe based analyses described below have been uploaded to OSF.**

Open search

To investigate the modification masses observed for the enriched peptides in samples prepared as described above, an Open Search was conducted with the following settings:

MSFragger - Peak matching: Precursor mass tolerance (Da): -150 – 1000, Fragment mass tolerance (PPM): 20, Calibration and Optimization: "Mass calibration, parameter optimization". **Protein digestion:** Cleavage: Enzymatic, clip N-term M: enabled, Enzyme name 1: Trypsin, Load rules: Trypsin, Cuts 1: KR, No cuts: P, Missed cleavages: 2, Sense 1: C, Peptide length: 6 – 50, Peptide mass range: 500 – 5,000, Split database: 1. **Modifications:** No variable modifications and fixed modifications of 0.0 enabled for all residues and termini. **Mass offsets:** 0, Restrict delta mass to: "all", Use detailed mass offsets: disabled. **Glyco/Labile mode**: Off. **Spectral Processing:** Activation type filter: all, Precursor mass mode: selected, Check spectral files: enabled, Require precursor: disabled, Analyzer filter: all, Min peaks: 15, Use top N peaks: 150, Min ratio: 0.01, Clear m/z range: 0 – 0, Intensity transform: square root, Reuse DIA fragment peaks: disabled, Remove precursor peak: Peaks with all charge states, Removal m/z range: -1.5 - 1.5. **Open search options:** Report mass shifts as variable mod: No, Track zero top N: 0, Zero bin accept except: 0, Zero bin multiply expect: 1, Delta mass exclude range: (-1.5,3.5). Localize mass shift (LOS): enabled. **Advanced output options:** Report top N for DDA: 1, Report alternative proteins: enabled, Output format: PEPXML_PIN (TSV_PEPXML_PIN can be used for manual inspection of data), Report top N for DDA+: 5, Write calibrated mzML: disabled, Group variable: None, Report top N for DIA: 5, Write uncalibrated MGF: disabled, Output max expect: 50. **Advanced peak matching options:** Min frags modeling: 2, Min matched frags: 4, Max fragment charge: 2, Deisotope: Yes, Fragment ion series: "b, y", Add custom ion series: empty, Deneutralloss: Yes, Precursor true tolerance (PPM): 20, Override charge with precursor charge: disabled.

Validation – Run validation tools: Enabled. Run Crystal C: Enabled. Run MSBooster: disabled. **PSM validation:** Enabled, Run PeptideProphet: Enabled, Defaults for: Open search,

Single combined pepxml file per experiment/group: enabled, Cmd line opts: "--nonparam --expectscore --decoyprobs --masswidth 1000.0 --clevel –2", Run Percolator: disabled. **PTM Site Localization:** disabled. **Protein Inference:** Run ProteinProphet: enabled, Cmd line opts: "--maxppmdiff 2000000". **FDR Filter and Report:** Generate report: enabled, Filter: "--sequential --prot 0.01 --mapmods", Do not use ProteinProphet file: disabled, Remove contaminants: disabled, Print decoys: disabled, Generate peptide-level summary: disabled, Generate protein-level summary: enabled.

PTMs: Run PTM-Shepherd: enabled. Defaults for Open Search loaded, Extended output: disabled. **PTM profiling:** Smoothing factor: 2, Precursor tolerance 0.01 Da, Prominence ratio: 0.3, Peak picking width: 0.002, Peak minimum PSMs: 1, Max fragment charge: 2, Fragment mass tolerance (PPM): 20. **Annotation:** Annotation tolerance (Da): 0.01, Annotation source: A custom annotation file shift list was used including only UniMod modifications with less than 400 Da molecular weight (available on OSF). **Amino acid propensity analysis:** "b" and "y" enabled with the rest disabled. **Diagnostic Feature Discovery:** Mine for diagnostic ions and fragments: enabled, Min peptide ions per MS1 delta mass peak: 25, Min % of spectra with ion (diagnostic ions, peptide ions, and fragment ions): 25, Min intensity fold change: 3, Min fragment ions per spec: 2, Min fragment propensity 12.5. Extract known diagnostic ions from spectra: disabled, Iterative localization of PSMs (experimental feature): disabled.

Tabs in FragPipe not mentioned above (e.g. Glyco and Quant (MS1)) were disabled.

For downstream analysis, the "global.modsummary.tsv" file found in the ptm-shepherd-output folder was utilized. By virtue of the mass of the isoDTB-tags, only modification masses above 400 Da were considered.

Offset search and amino acid selectivity

MSFragger: Enabled. P**eak matching:** Precursor mass tolerance (PPM): -20 – 20, Fragment mass tolerance (PPM): 20, Calibration and optimization: "Mass calibration, parameter optimization", Isotope error: "0/1/2". **Protein digestion:** Cleavage: Enzymatic, clip N-term M: enabled, Enzyme name 1: Trypsin, Load rules: Trypsin, Cuts 1: KR, No cuts: P, Missed cleavages: 2, Sense 1: C, Peptide length: 6 – 50, Peptide mass range: 500 – 5,000, Split database: 1. **Modifications:** Variable modification of 57.02146 Da on Cys with max. 3 occurrences and no fixed modifications. **Mass offsets:** 691.3209/697.3282 (main modification observed in the open search, expected modification masses: 691.3209 Da and 697.3289), Restrict delta mass to: all, Use detailed mass offsets: disabled. **Glyco/Labile mods:** disabled. **Spectral Processing:** Activation Type Filter: all, Precursor mass mode: selected, Check spectral files: enabled, Require precursor: disabled, Analyzer filter: all, Min peaks: 15, Use top N peaks: 150, Min ratio: 0.01, Clear m/z range: 0 – 0, Intensity transform: square root, Reuse DIA fragment peaks: disabled, Remove precursor peak: Peaks with all charge states, Removal m/z range: -1.5 - 1.5. **Open search options:** Report mass shifts as variable mod: "Yes, remove delta mass", Track zero top N: 0, Zero bin accept except: 0, Zero bin multiply expect: 1, Delta mass exclude range: (-1.5,3.5). Localize mass shift (LOS): enabled. **Advanced output options:** Report top N for DDA: 1, Report alternative proteins: disabled, Output format: TSV_PEPXML_PIN, Report top N for DDA+: 5, Write calibrated mzML: disabled, Group

variable: None, Report top N for DIA: 5, Write uncalibrated MGF: disabled, Output max expect: 50. **Advanced peak matching options:** Min frags modeling: 2, Min matched frags: 4, Max fragment charge: 2, Deisotope: Yes, Fragment ion series: ”b, y”, Add custom ion series: empty, Deneutralloss: Yes, Precursor true tolerance (PPM): 20, Override charge with precursor charge: disabled.

Validation – Run validation tools: enabled. Run Crystal C: disabled. Run MSBooster: disabled. **PSM validation:** Enabled, Run PeptideProphet: Enabled, Defaults for: Offset search, Single combined pepxml file per experiment/group: enabled, Cmd line opts: “- --nonparam --expectscore --decoyprobs --masswidth 1000.0 --clevel -2", Run Percolator: disabled. **PTM Site Localization:** disabled. PTM Prophet: disabled. **Protein Inference:** Run ProteinProphet: enabled, Cmd line opts: “--maxppmdiff 2000000”. **FDR Filter and Report:** Generate report: enabled, Filter: “--sequential --mapmods --prot 0.01”, “Do not use ProteinProphet file”: disabled, Remove contaminants: disabled, Print decoys: disabled, Generate peptide-level summary: disabled, Generate protein-level summary: enabled.

PTMs: Run PTM-Shepherd: enabled. Extended output: disabled. **PTM profiling:** Smoothing factor: 2, Precursor tolerance 20 PPM, Prominence ratio: 0.3, Peak picking width: 20 PPM, Peak minimum PSMs: 10, Max fragment charge: 2, Fragment mass tolerance (PPM): 20. **Annotation:** Annotation tolerance (Da): 0.01, Annotation source: A custom annotation file shift list was used including only UniMod modifications with less than 400 Da molecular weight (available on OSF). **Amino acid propensity analysis:** “b” and “y” enabled with the rest disabled. **Diagnostic Feature Discovery:** Mine for diagnostic ions and fragments: enabled, Min peptide ions per MS1 delta mass peak: 25, Min % of spectra with ion (diagnostic ions, peptide ions, and fragment ions): 25, Min intensity fold change: 3, Min fragment ions per spec: 2, Min fragment propensity 12.5. Extract known diagnostic ions from spectra: disabled, Iterative localization of PSMs (experimental feature): disabled.

Tabs in FragPipe not mentioned above (e.g. Glyco and Quant (MS1)) were disabled.

Downstream analysis was conducted using the psm.tsv file output by FragPipe. Initially, the following analyses were conducted separately for each sample. For each PSM a “Delta Score” was calculated as the difference between “Score Best Position” and “Score Second Best Position” and after that point only the columns “Peptide”, “MSFragger Localization”, “Protein ID”, “Protein Start”, “Protein End”, and “Delta Score” were retained. The FASTA database file was converted into a .csv file containing only the protein ID and full sequence of each protein (with reverse sequences and contaminants removed), and the full sequences were matched to each entry based on “Protein ID”. The length of each full sequence was calculated, and the data was filtered to contain only PSMs for which the modification was localized to exactly one amino acid, as indicated by exactly one lower case letter in the “MSFragger Localization” column, and for which the “Delta Score” was ≥1. The position of the modification on the peptide, and which amino acid this corresponded to, was then identified, and a column named “Modified Amino Acid” was created. The data was subsequently filtered to contain only peptides which occurred exactly once in the full sequence, and modifications were assigned as N-terminal (and not as the modified amino acid) if the modified amino acid was in position 1 in the full sequence, or position 2 of the full sequence if the peptide started at position 2 of the full sequence. Modifications were assigned as C-terminal (and not as the modified amino acid)

if the modified amino acid was in the last position of the full sequence. Within each sample, the number of PSMs with modification on each amino acid (and terminals) were counted to obtain the absolute number and percentage. Next, each PSM was given an "Identifier" from "Modified Amino Acid" and "Modified Residue Number", and filtering to retain each "Identifier" only once allowed for counting and calculating the number and percentage of modifications observed for each unique site. Finally, the data was concatenated, using the "Identifier", for all samples and the number of samples a specific modification was observed in was counted. For the final data analysis, the number of modifications and corresponding percentage were calculated for each amino acid (and terminals) based on unique modifications (sites), which were observed in at least three out of four samples. The resulting percentages were plotted in GraphPad Prism 10.2.3. for visualization purposes.

Closed search

Note that for the closed search, each sample was numbered in the "Experiment" column under the "Workflow" tab in FragPipe for downstream data analysis purposes.

MSFragger: Enabled. P**eak matching:** Precursor mass tolerance (PPM): -20 – 20, Fragment mass tolerance (PPM): 20, Calibration and optimization: "Mass calibration, parameter optimization", Isotope error: "0/1/2". **Protein digestion:** Cleavage: Enzymatic, clip N-term M: enabled, Enzyme name 1: Trypsin, Load rules: Trypsin, Cuts 1: KR, No cuts: P, Missed cleavages: 2, Sense 1: C, Peptide length: 6 – 50, Peptide mass range: 500 – 5,000, Split database: 1. **Modifications: Variable modifications:** 15.9949 Da on M (max 2 occurences); 42.0106 Da on N-termini (site [^, max 1 occurrence); 634.2987 Da on C (light modification mass with the fixed/expected mass of carbamidomethylation on C subtracted); 640.3067 Da (heavy modification mass with the fixed carbamidomethylation mass on C subtracted). Fixed modifications: 57.02146 on C. **Mass offsets:** 0, Restrict delta mass to: all, Use detailed mass offsets: disabled. **Glyco/Labile mods:** disabled. **Spectral Processing:** Activation Type Filter: all, Precursor mass mode: selected, Check spectral files: enabled, Require precursor: disabled, Analyzer filter: all, Min peaks: 15, Use top N peaks: 150, Min ratio: 0.01, Clear m/z range: 0 – 0, Intensity transform: square root, Reuse DIA fragment peaks: disabled, Remove precursor peak: Peaks with all charge states, Removal m/z range: -1.5 - 1.5. **Open search options:** Report mass shifts as variable mod: No, Track zero top N: 0, Zero bin accept except: 0, Zero bin multiply expect: 1, Delta mass exclude range: (-1.5,3.5). Localize mass shift (LOS): disabled. **Advanced output options:** Report top N for DDA: 1, Report alternative proteins: disabled, Output format: PEPXML_PIN, Report top N for DDA+: 5, Write calibrated mzML: disabled, Group variable: None, Report top N for DIA: 5, Write uncalibrated MGF: disabled, Output max expect: 50. **Advanced peak matching options:** Min frags modeling: 2, Min matched frags: 4, Max fragment charge: 2, Deisotope: Yes, Fragment ion series: "b, y", Add custom ion series: empty, Deneutralloss: Yes, Precursor true tolerance (PPM): 20, Override charge with precursor charge: disabled.

Validation – Run validation tools: enabled. Run Crystal C: disabled. **Rescoring using Deep Learning Prediction:** Run MSBooster: enabled, Predict RT: enabled, Model: DIA-NN, Find best RT mode: disabled, Predict spectra: enabled, Model: DIA-NN, **PSM validation:** Enabled, Run PeptideProphet: disabled, Run Percolator: enabled, Keep intermediate files: disabled, Min probability: 0.5, Cmd line opts: "--only-psms --no-terminate --post-processing-tdc". **PTM Site**

Localization: disabled. PTM Prophet: disabled. **Protein Inference:** Run ProteinProphet: enabled, Cmd line opts: "--maxppmdiff 2000000". **FDR Filter and Report:** Generate report: enabled, Filter: "--sequential --prot 0.01", "Do not use ProteinProphet file": disabled, Remove contaminants: disabled, Print decoys: disabled, Generate peptide-level summary: disabled, Generate protein-level summary: disabled.

PTMs: Run PTM-Shepherd: disabled.

Quant (MS1): Run MS1 quant: enabled. IonQuant: Enabled, LFQ: disabled, Add MaxLFQ: enabled, MaxLFQ min ions: 1, Labelling: enabled, Requantify: enable, Light: C634.2987 , Heavy: C640.3067, Match between runs (MBR): disabled, Normalize intensity between runs: disabled, Peptide-protein uniqueness: "unique + razor". **Advanced options:** Min scans: 1, Min isotopes: 2, m/z tolerance (PPM): 10, RT tolerance (minutes): 0.4, IM tolerance (1/KD): 0.05, Top N ions: 0, Min freq: 0, Min site localization probability: 0.75, Keep index on disk: disabled. FreeQuant (deprecated): disabled, RT window (minutes): 0.4, m/z window (PPM): 10.

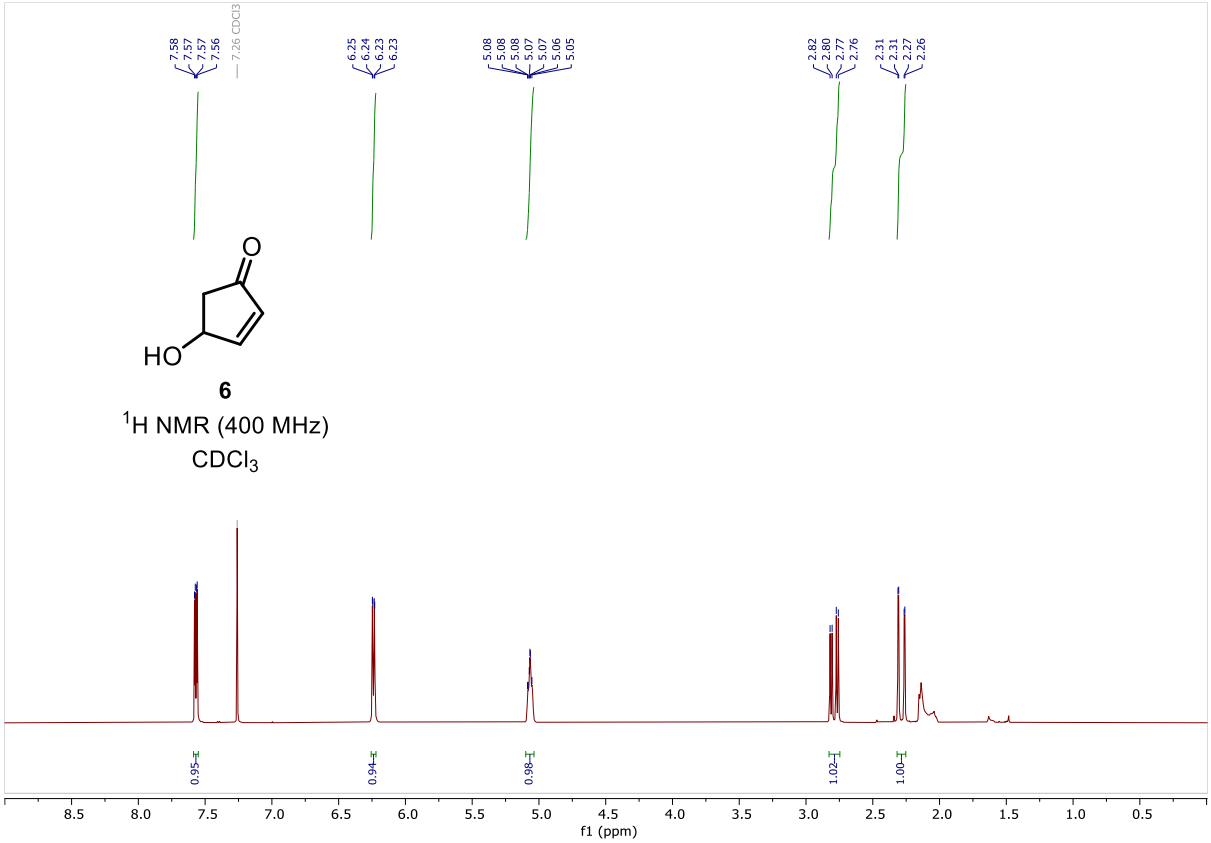
Tabs in FragPipe not mentioned above (e.g. Glyco) were disabled.

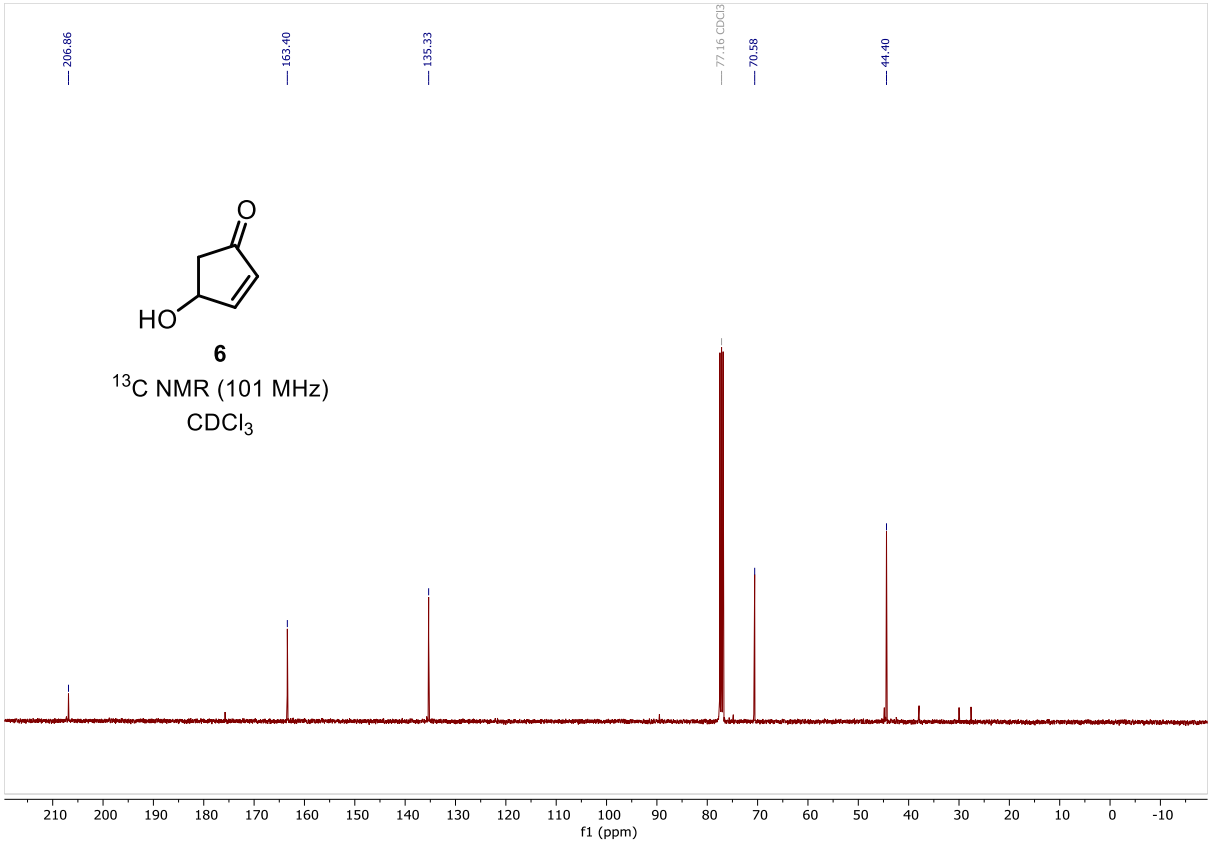
For downstream analysis each sample was initially processed individually using the ion_label_quant.tsv" file generated by the FragPipe workflow. For ions where both light- and heavy modifications were observed (in the "Heavy Modified Peptide" and "Light Modified Peptide" columns) the one belonging to the higher intensity value was used as the "Modified peptide" for downstream analysis (for finding the position of the modification etc.). For ions where only one of the modifications was observed, this modification was used without regard for the intensity. Next, the modification masses corresponding to carbamidomethylation, N-terminal acetylation, and oxidation were removed from the "Modified peptide", while the masses of the light- or heavy modifications were converted to "*" for subsequent identification of the modification sites. Filtering was then conducted to only retain ions with exactly one modification (as indicated by "*"), and the FASTA database was used to match each peptide ion to the corresponding full sequence (with additional filtering to remove any ions not found in the FASTA). The modified amino acid, as well as its position in the peptide, was then identified, along with the start position of the peptide in the full sequence. At this stage, ions were only retained if the peptide occurred in the full sequence, taking into account that "I" (Ile) and "L" (Leu) cannot be differentiated by MS, and for peptides occurring more than once a label was added to indicate this. The position of the modified residue was subsequently identified in the full sequence ("Modified Residue Number"), with the first occurrence being reported for peptides occurring multiple times in the full sequence. Next, an "Identifier" was generated as "Protein ID"_"Modified Amino Acid"_"Modified Residue Number", and ions only appearing with either heavy or light modifications were labelled as "Light Singletons" (only observed with the light modification) or "Heavy Singletons" (only observed with the heavy modification). In this experiment, "pseudo-tag-switching" was conducted (i.e. half the samples were treated as heavy/light, while the other half was treated as light/heavy). For the H/L samples the "Heavy Singletons" were renamed to "Positive singletons and "Light Singletons" to "Negative Singletons" and vice versa for the "L/H" samples for which the "Log2 Ratio HL"-value also was multiplied by –1. Finally, data was combined across samples for the same identifier by keeping the shortest peptide, modified peptide, and peptide length, while

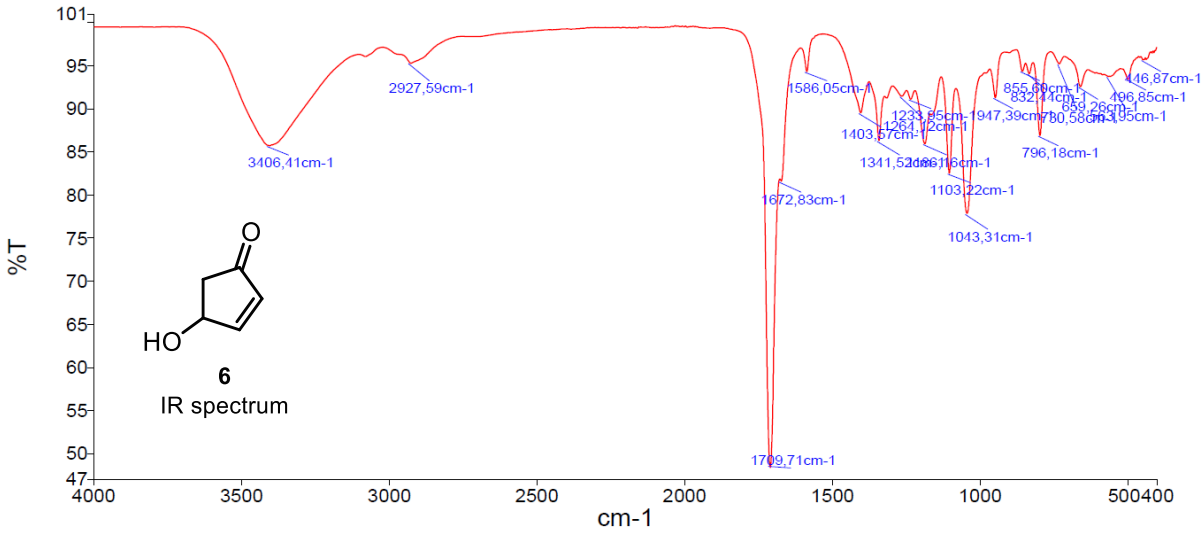
taking the median of the Log2(R)-values for the ions. Finally, quantification was conducted, using only residues (cysteines) observed modified in at least three out of four samples.

5. ^{1}H, ^{13}C and IR spectra of products and intermediates

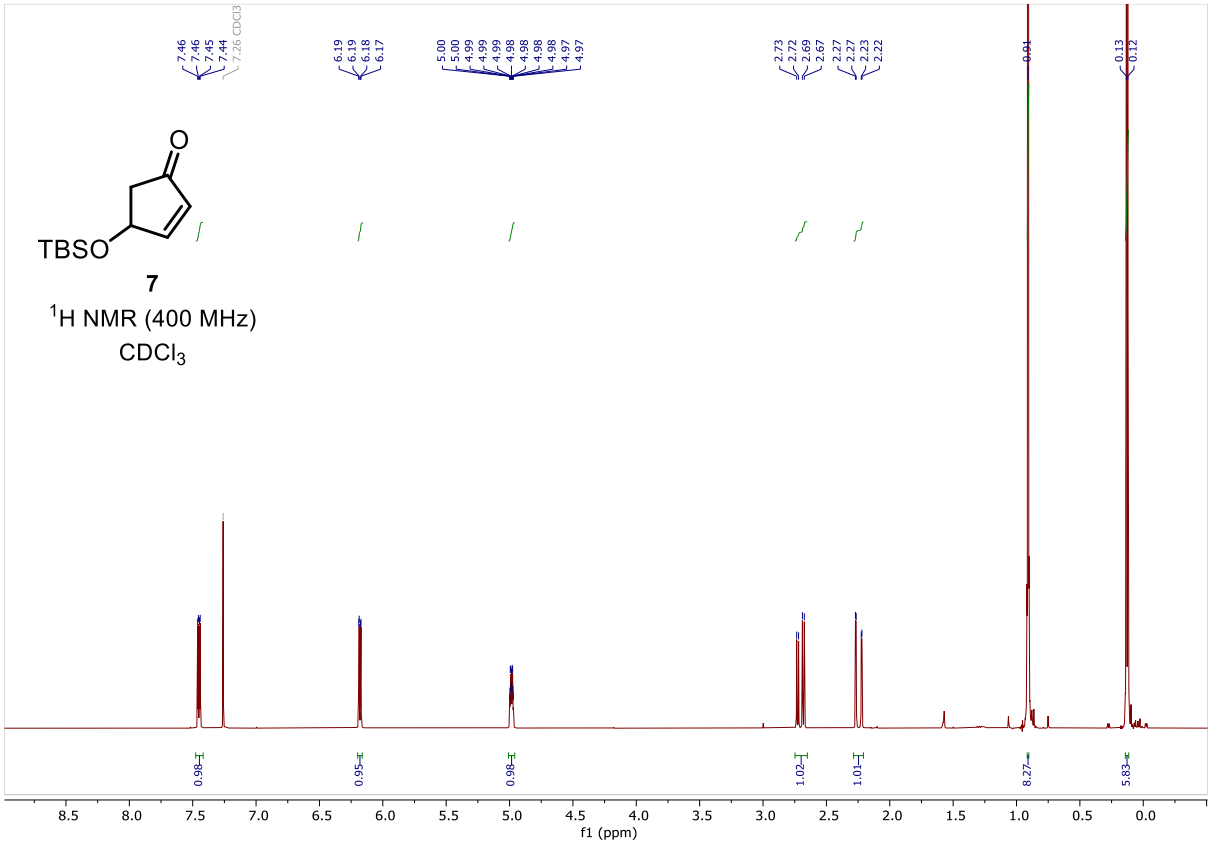
Compound 6

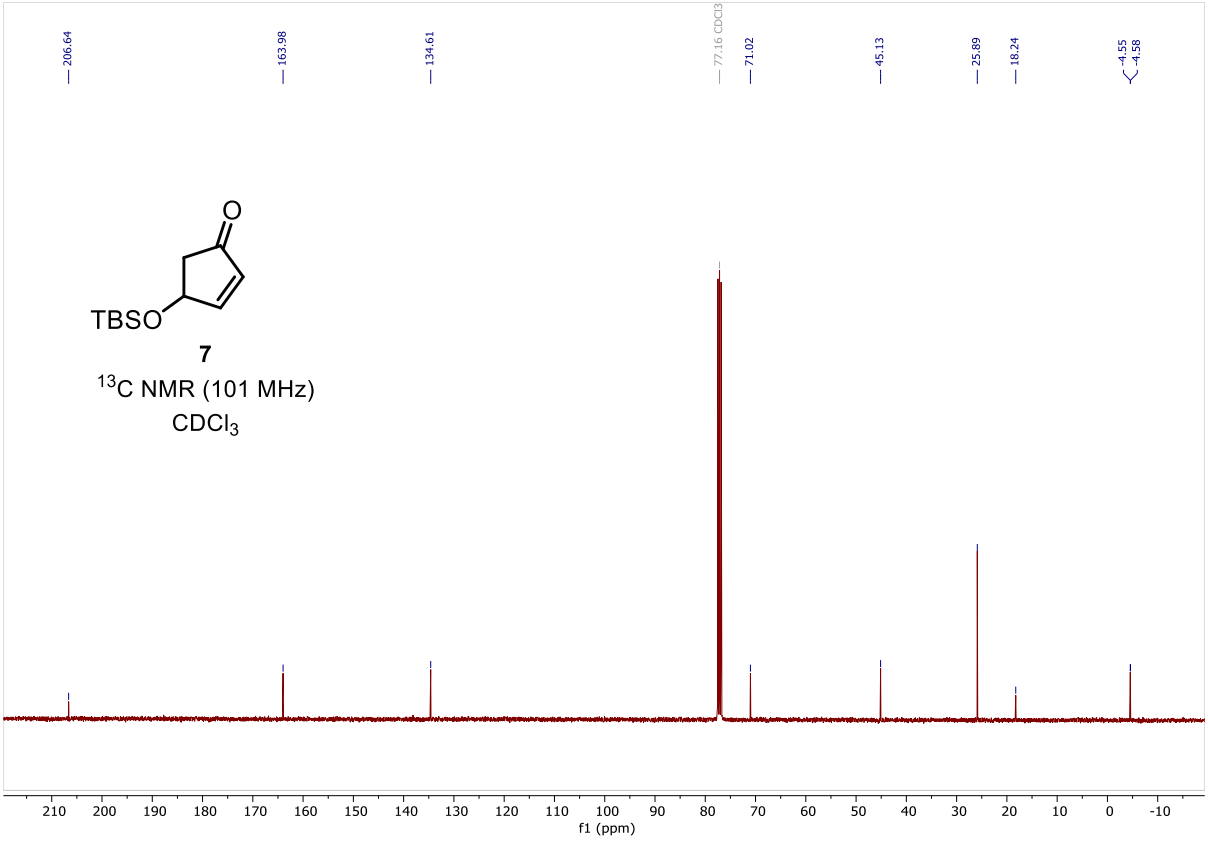


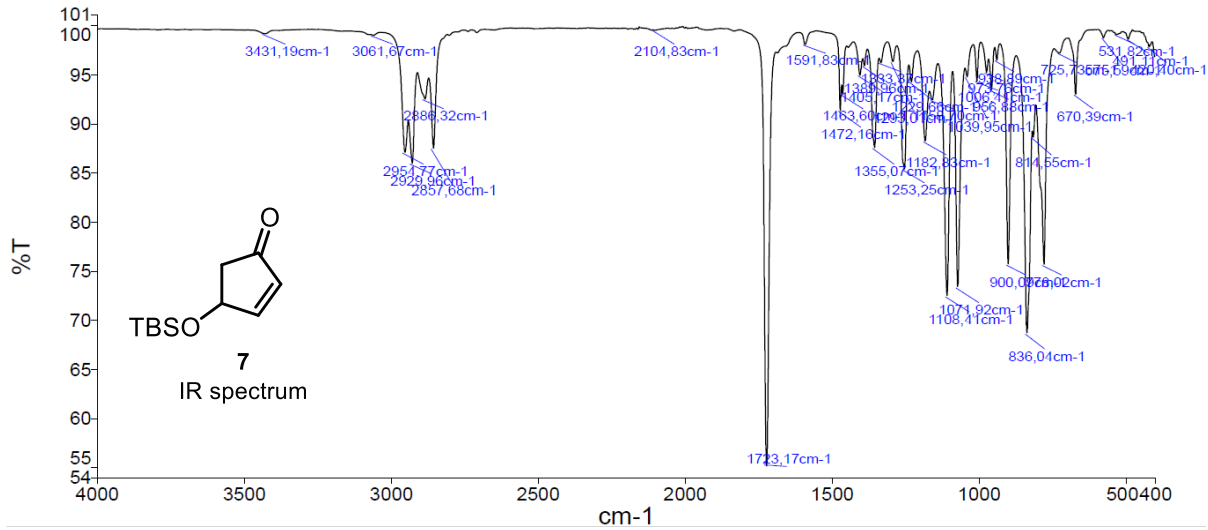



%T
cm-1
3406,41cm-1
2927,59cm-1
1709,71cm-1
1672,83cm-1
1586,05cm-1
1403,67cm-1
1264,72cm-1
1233,85cm-1
1103,22cm-1
1043,31cm-1
796,18cm-1
HO
O
6
IR spectrum

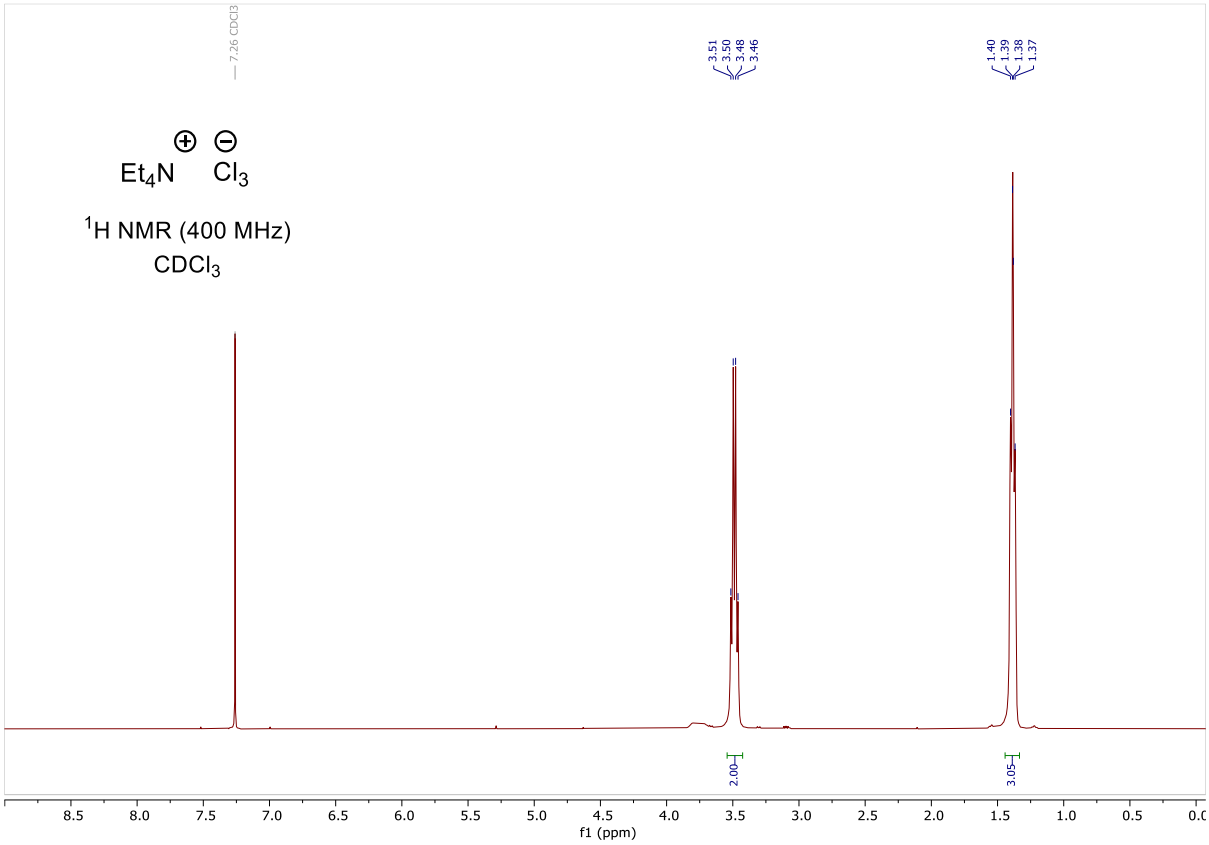
Compound 7

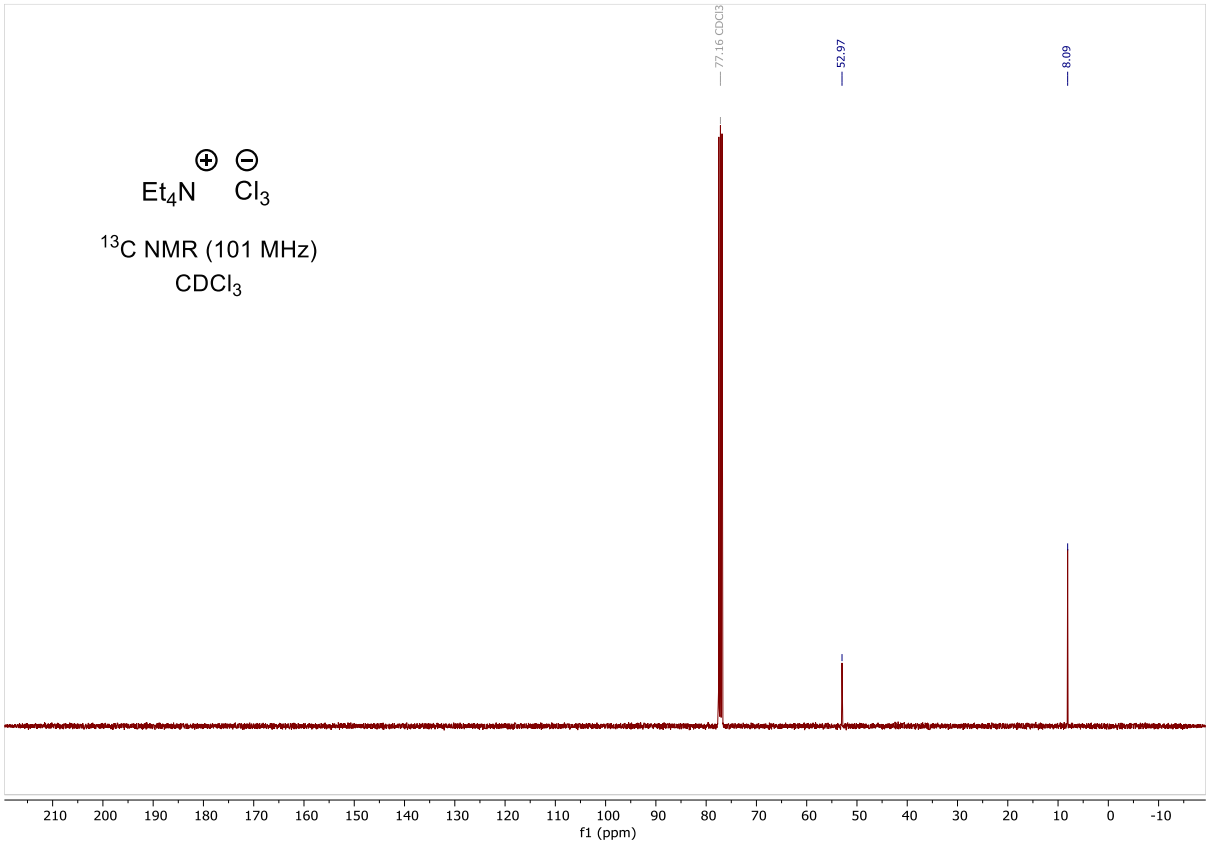

TBSO
O
7
1H NMR (400 MHz)
CDCl3
f1 (ppm)


206.64
163.98
134.61
77.16 CDCl3
71.02
45.13
25.89
18.24
4.55
4.58
O
TBSO
7
13C NMR (101 MHz)
CDCl3
f1 (ppm)

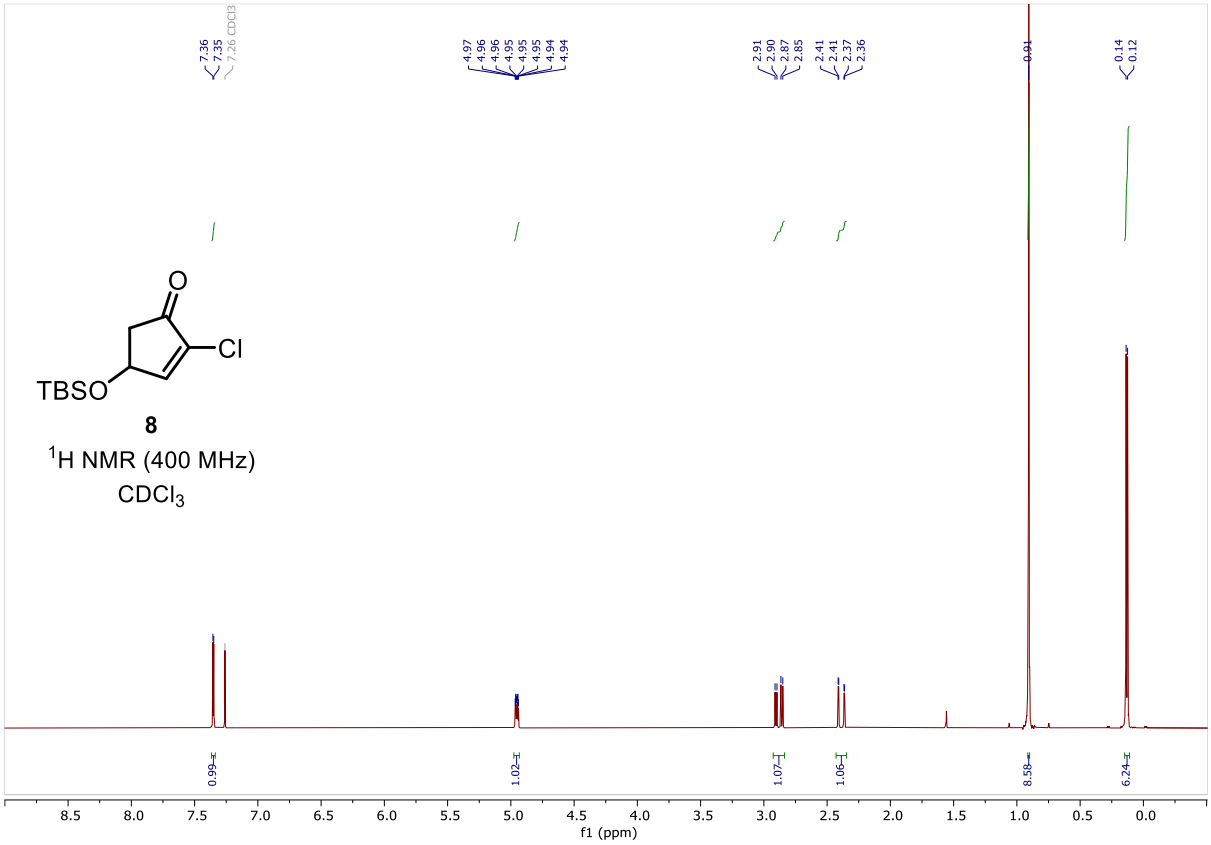

%T
cm-1
3431,19cm-1
3061,67cm-1
2886,32cm-1
2957,77cm-1
2926,96cm-1
2857,68cm-1
2104,83cm-1
1591,83cm-1
1472,16cm-1
1355,07cm-1
1253,25cm-1
1182,83cm-1
1071,92cm-1
1108,41cm-1
836,04cm-1
814,55cm-1
670,39cm-1
1723,17cm-1
O
TBSO
7
IR spectrum

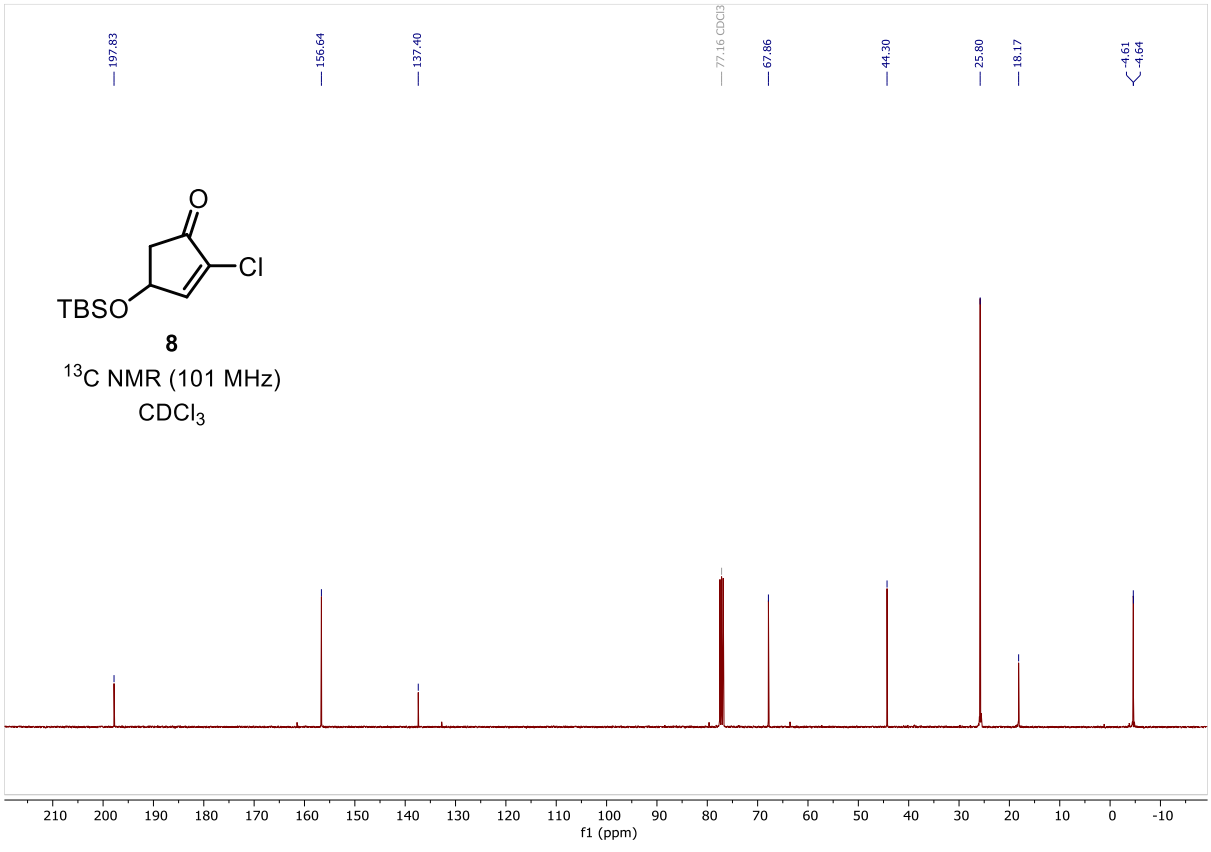
Mioskowski's reagent (Et_4NCl_3)

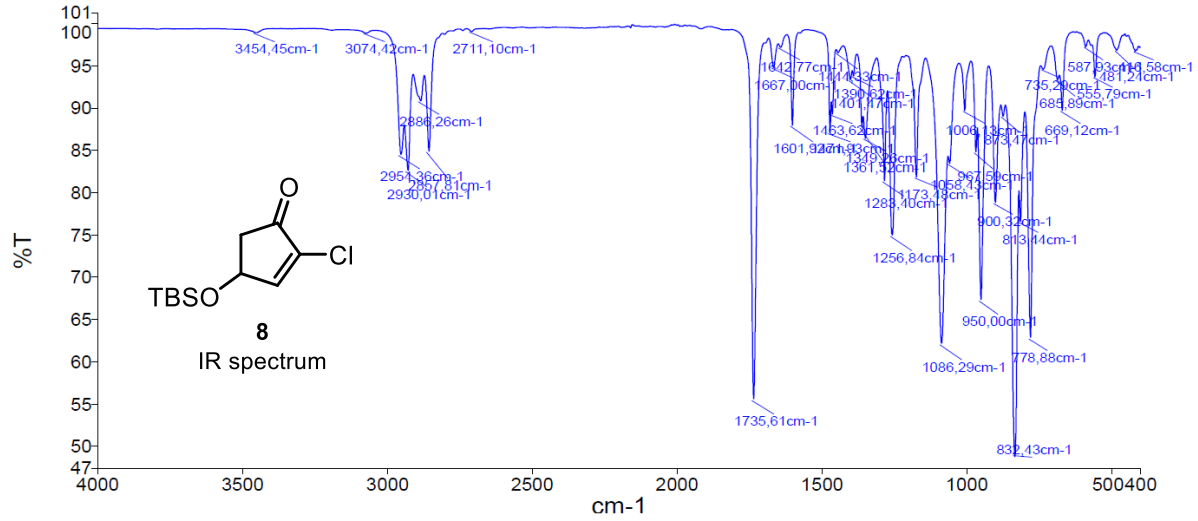




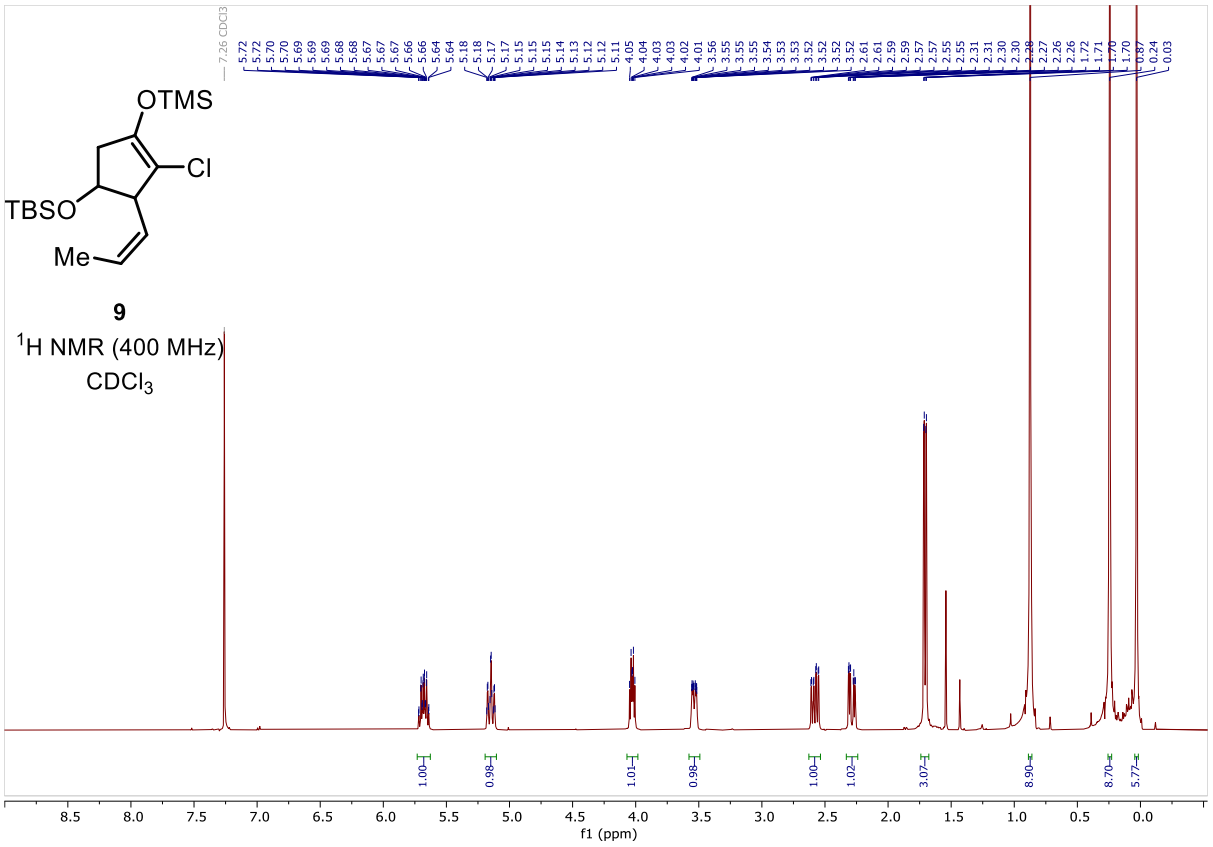
Compound 8

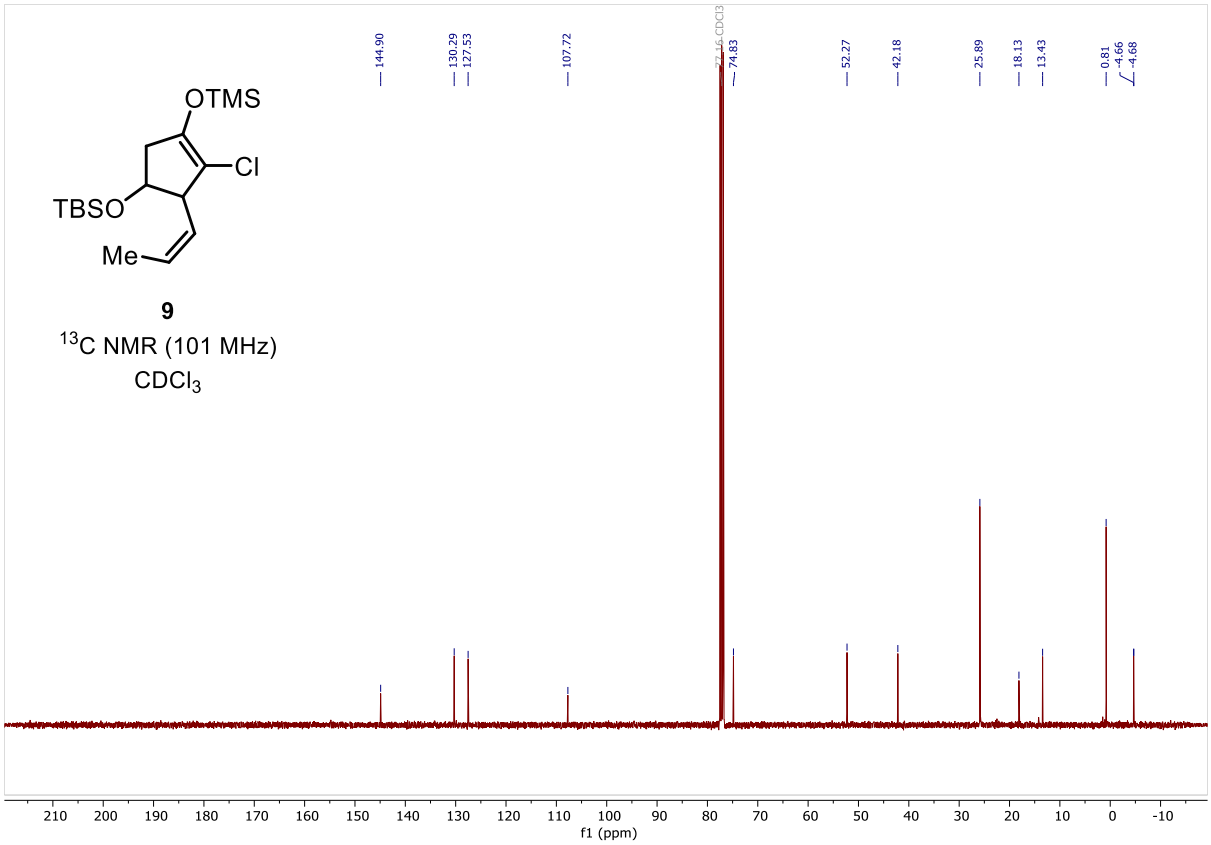

7.36
7.35
7.26 CDCl3
4.97
4.96
4.96
4.95
4.95
4.95
4.94
4.94
2.91
2.90
2.87
2.85
2.41
2.41
2.37
2.36
0.01
0.14
0.12
O
Cl
TBSO
8
1H NMR (400 MHz)
CDCl3
0.99
1.02
1.07
1.06
8.58
6.24
f1 (ppm)


197.83
156.64
137.40
77.16 CDCl3
67.86
44.30
25.80
18.17
-4.61
-4.64
O
Cl
TBSO
8
13C NMR (101 MHz)
CDCl3
f1 (ppm)

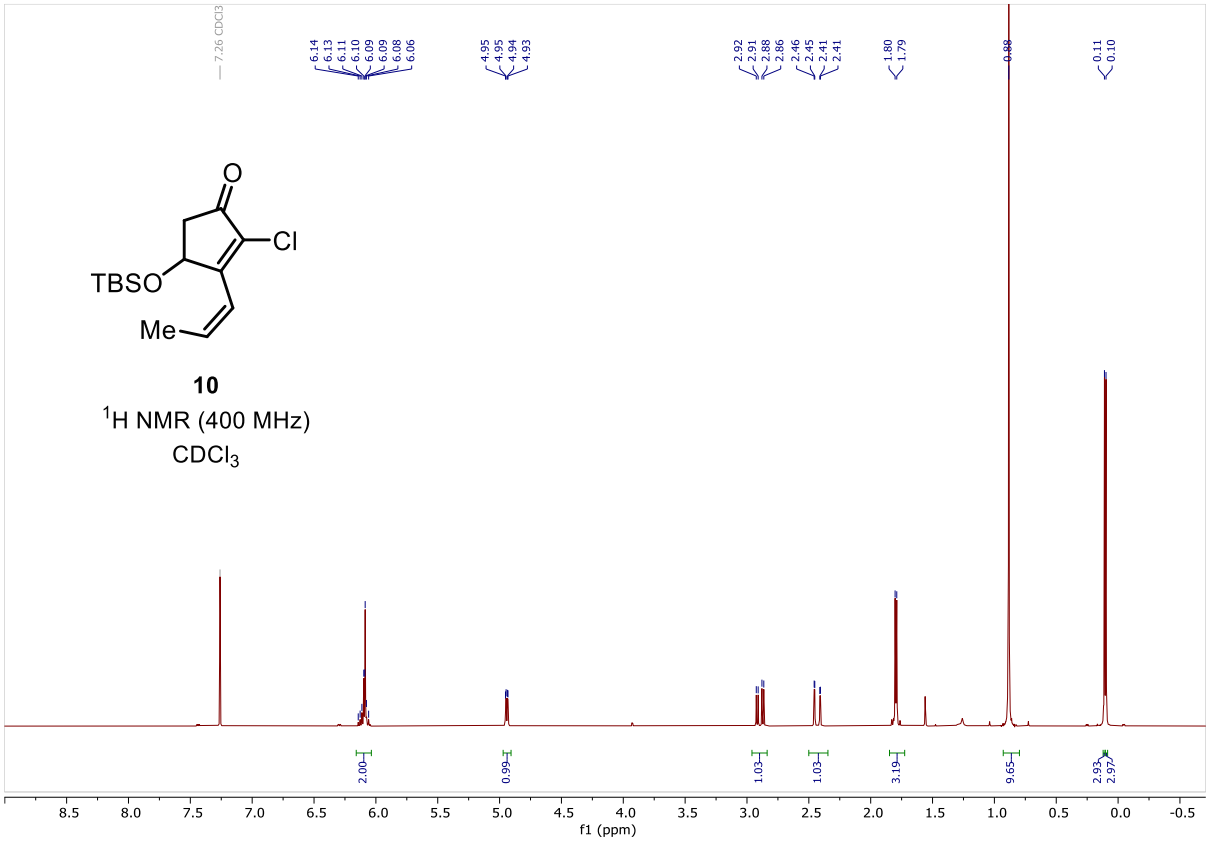

3454,45cm-1
3074,42cm-1
2711,10cm-1
2886,26cm-1
2951,36cm-1
2867,81cm-1
2930,01cm-1
1735,61cm-1
1086,29cm-1
832,43cm-1
950,00cm-1
778,88cm-1
1256,84cm-1
900,32cm-1
813,14cm-1
669,12cm-1
%T
cm-1
TBSO
Cl
O
8
IR spectrum

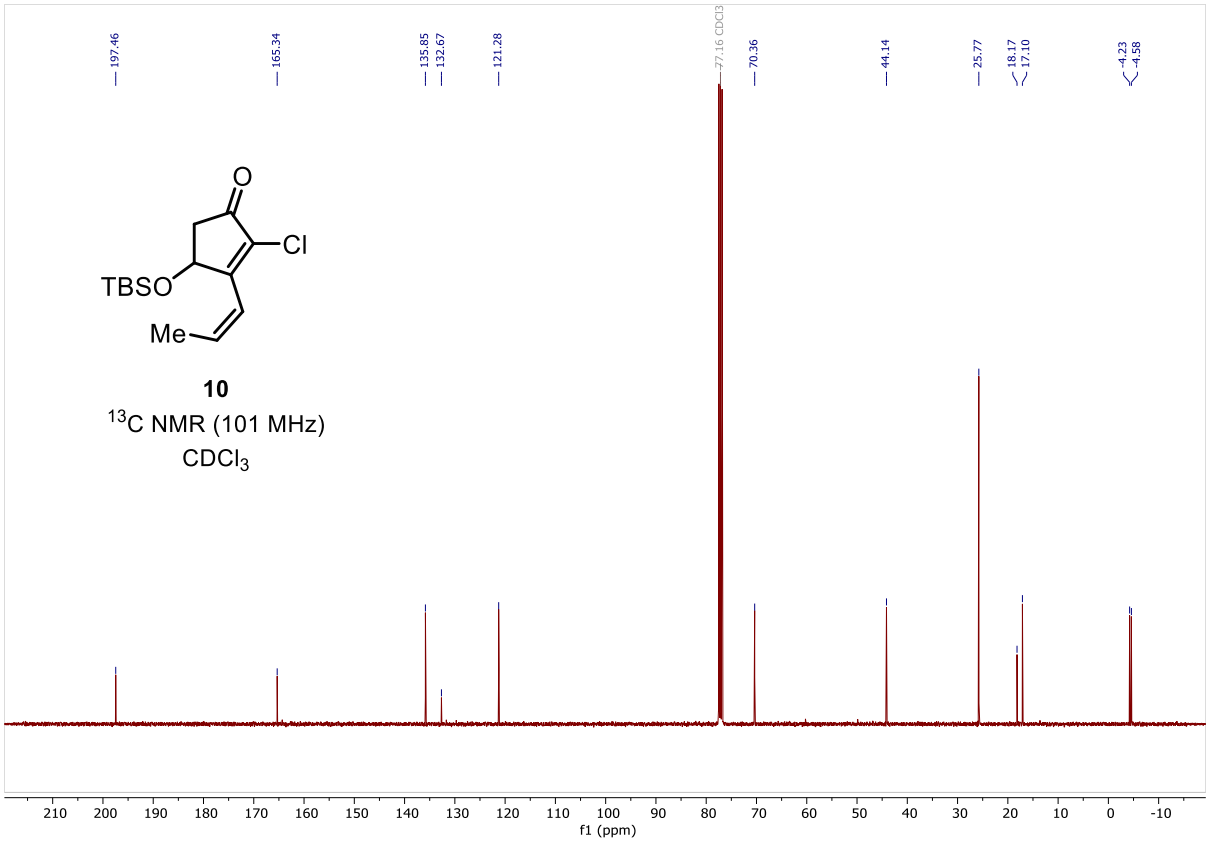
Compound 9


OTMS
Cl
TBSO
Me
9
1H NMR (400 MHz)
CDCl3
f1 (ppm)

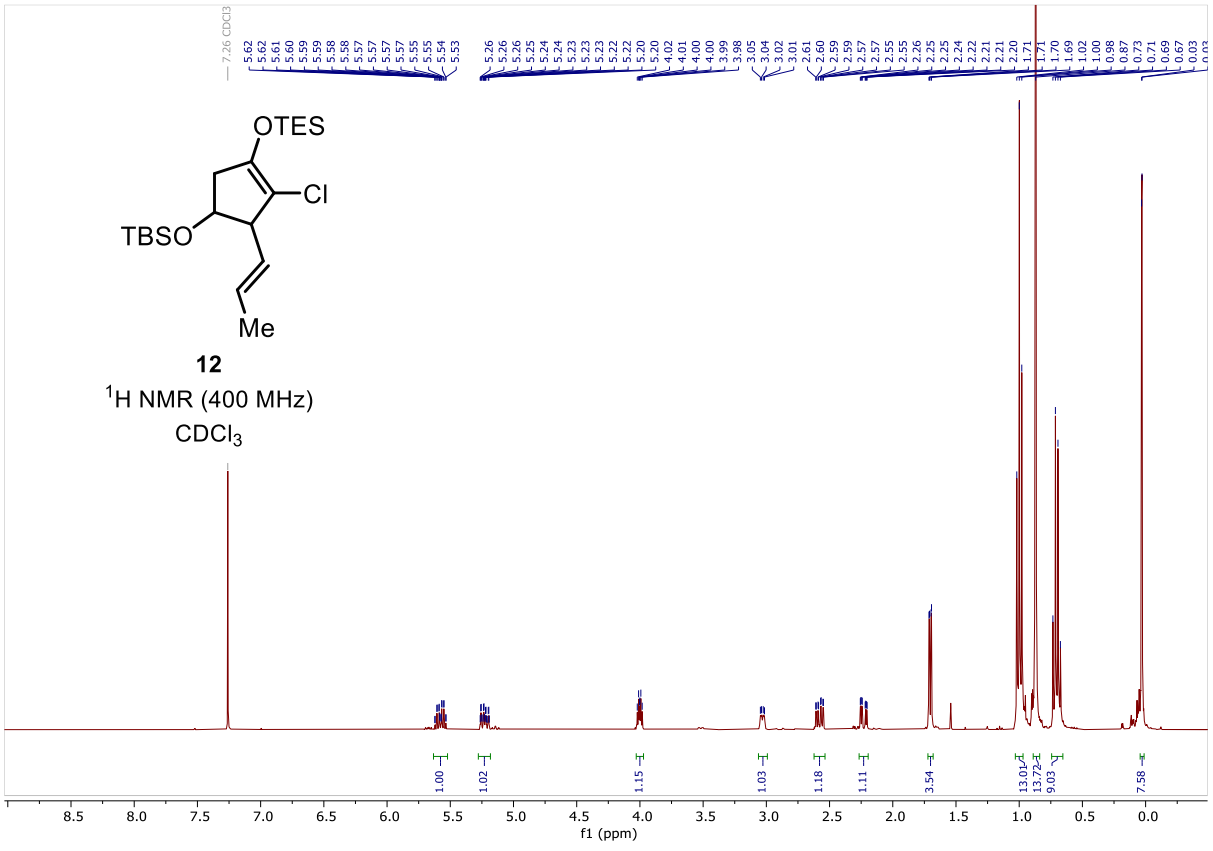

OTMS
Cl
TBSO
Me
9
13C NMR (101 MHz)
CDCl3
144.90
130.29
127.53
107.72
77.16 CDCl3
74.83
52.27
42.18
25.89
18.13
13.43
0.81
-4.66
-4.68
f1 (ppm)

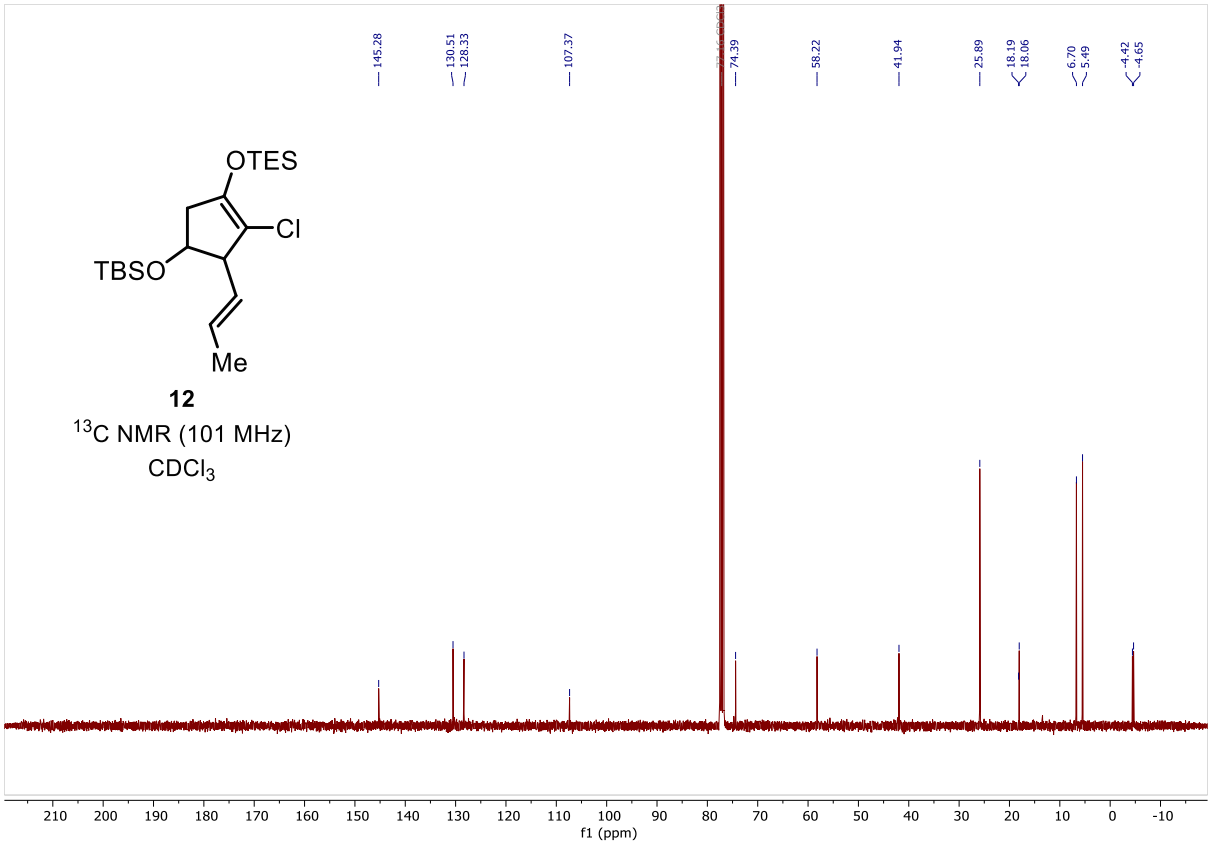
Compound 10


7.26 CDCl3
6.14
6.13
6.11
6.10
6.09
6.09
6.08
6.06
4.95
4.95
4.94
4.93
2.92
2.91
2.88
2.86
2.46
2.45
2.41
2.41
1.80
1.79
0.88
0.11
0.10
O
Cl
TBSO
Me
10
1H NMR (400 MHz)
CDCl3
2.00
0.99
1.03
1.03
3.19
9.65
2.93
2.97
f1 (ppm)

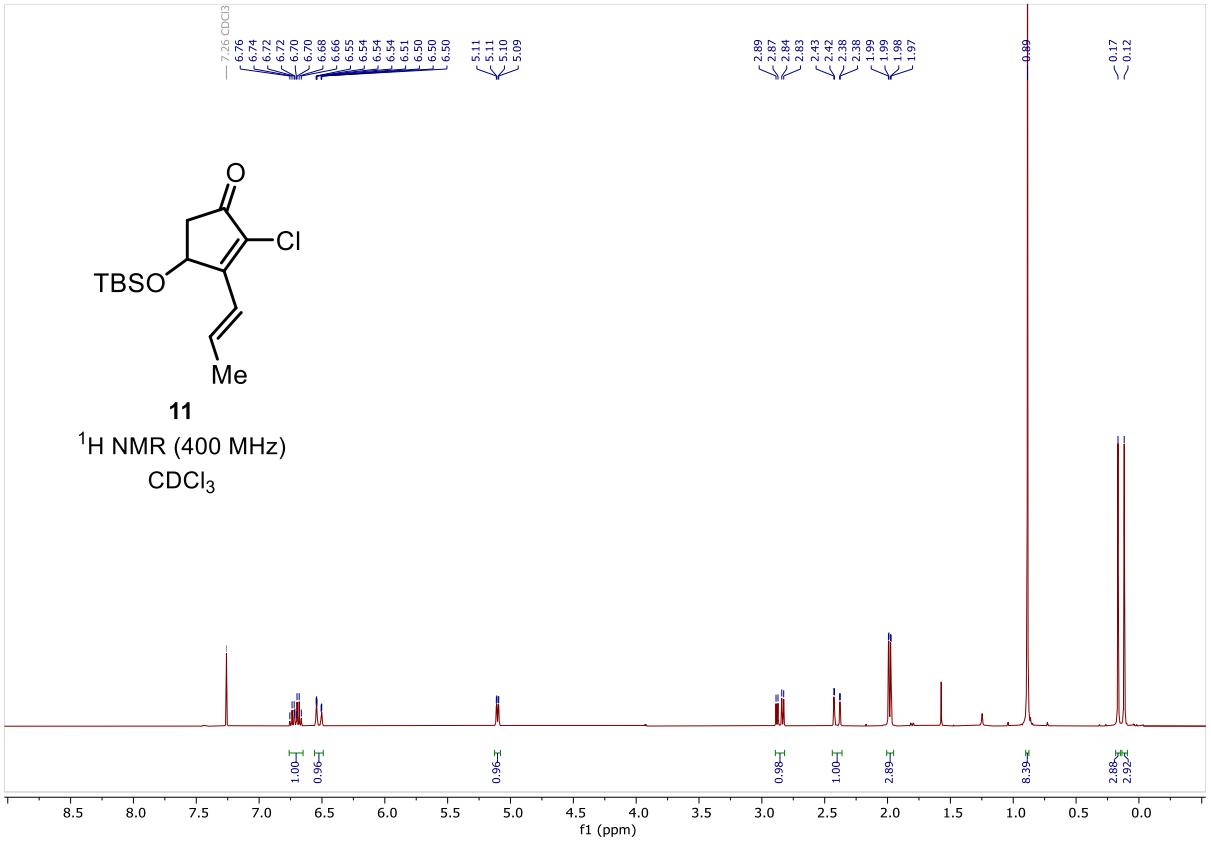

197.46
165.34
135.85
132.67
121.28
77.16 CDCl3
70.36
44.14
25.77
18.17
17.10
-4.23
-4.58
O
Cl
TBSO
Me
10
13C NMR (101 MHz)
CDCl3
f1 (ppm)

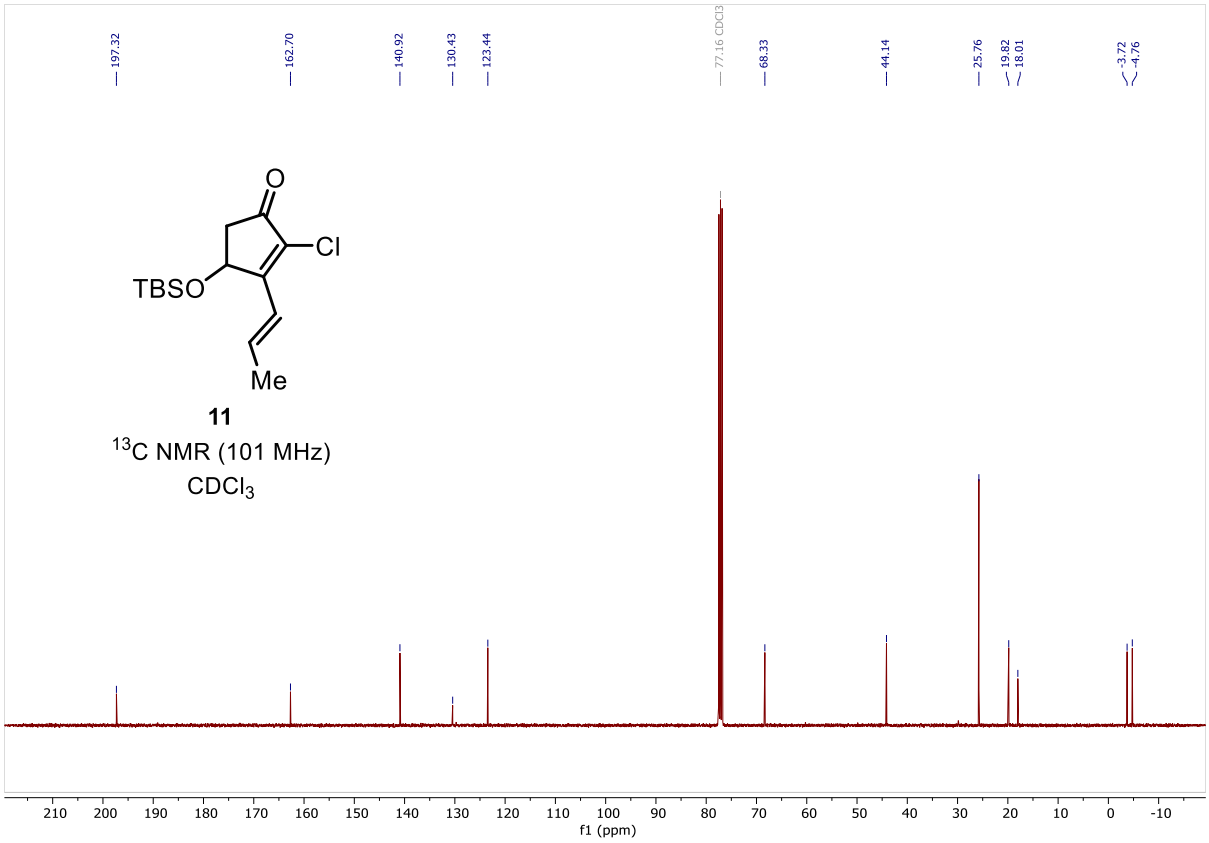
Compound 12

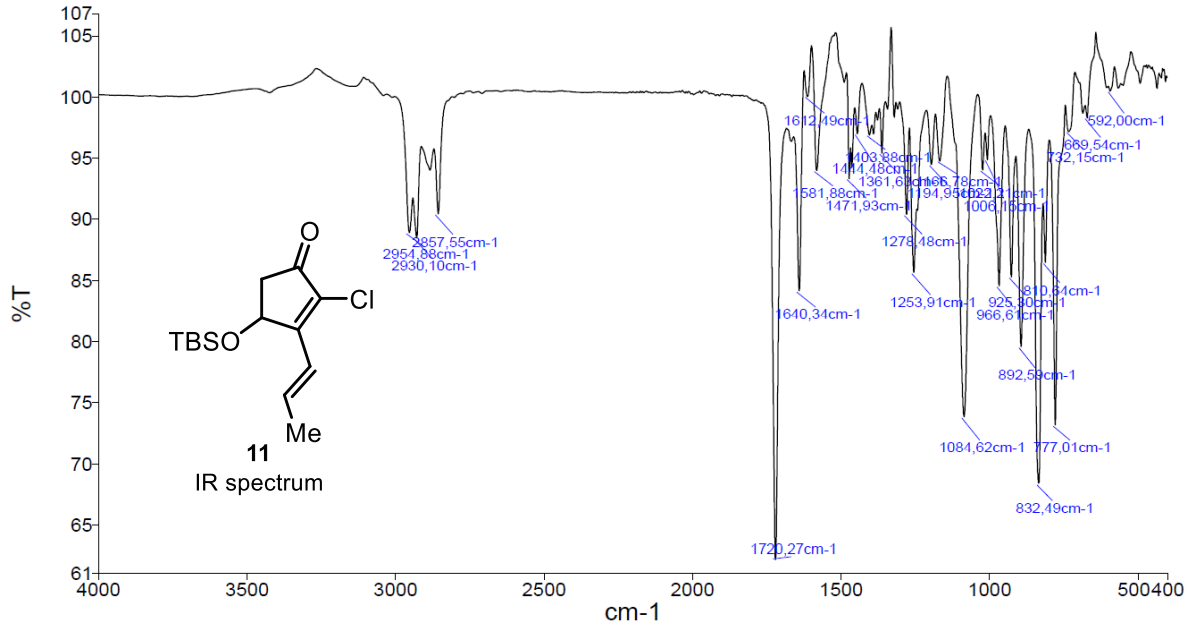

7.26 CDCl3
OTES
Cl
TBSO
Me
12
1H NMR (400 MHz)
CDCl3
1.00
1.02
1.15
1.03
1.18
1.11
3.54
13.01
13.72
9.03
7.58
f1 (ppm)


145.28
130.51
128.33
107.37
77.16 CDCl3
74.39
58.22
41.94
25.89
18.19
18.06
6.70
5.49
-4.42
-4.65
OTES
Cl
TBSO
Me
12
13C NMR (101 MHz)
CDCl3
f1 (ppm)

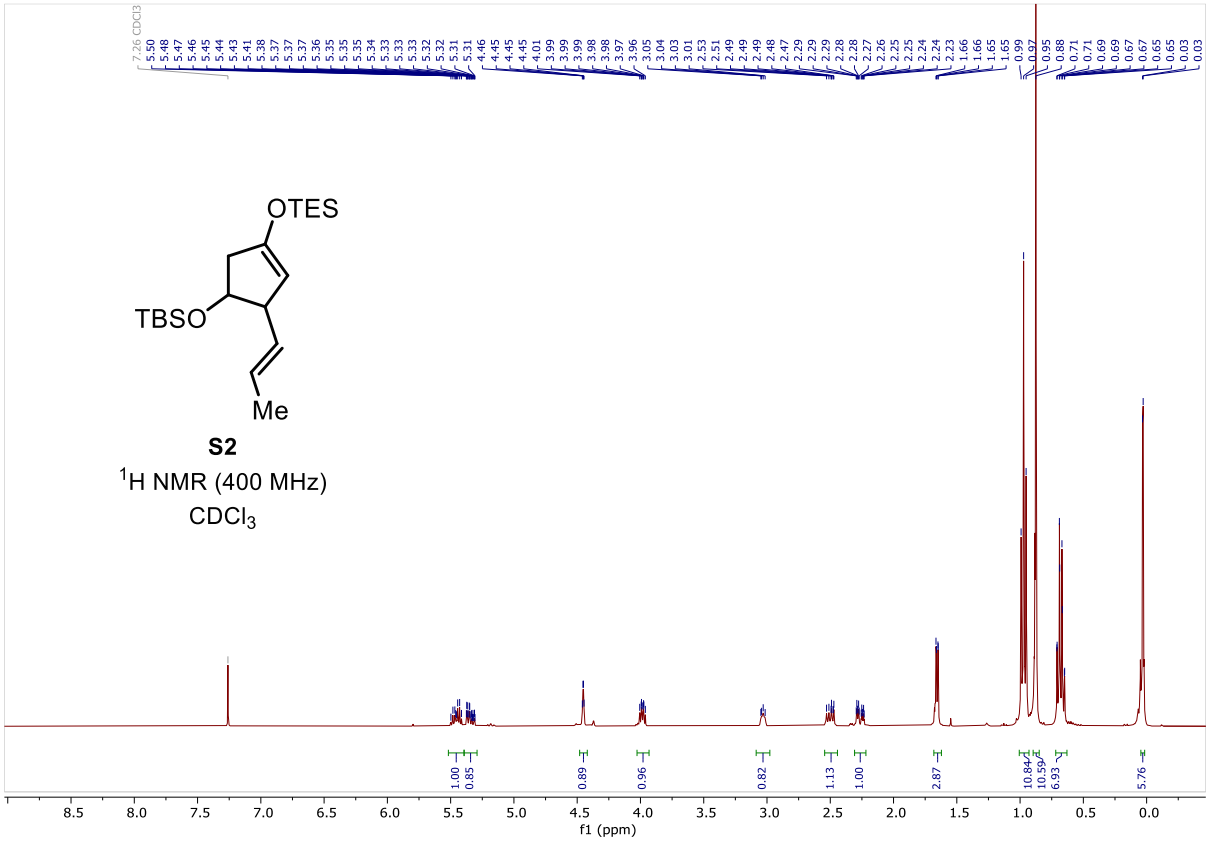
Compound 11

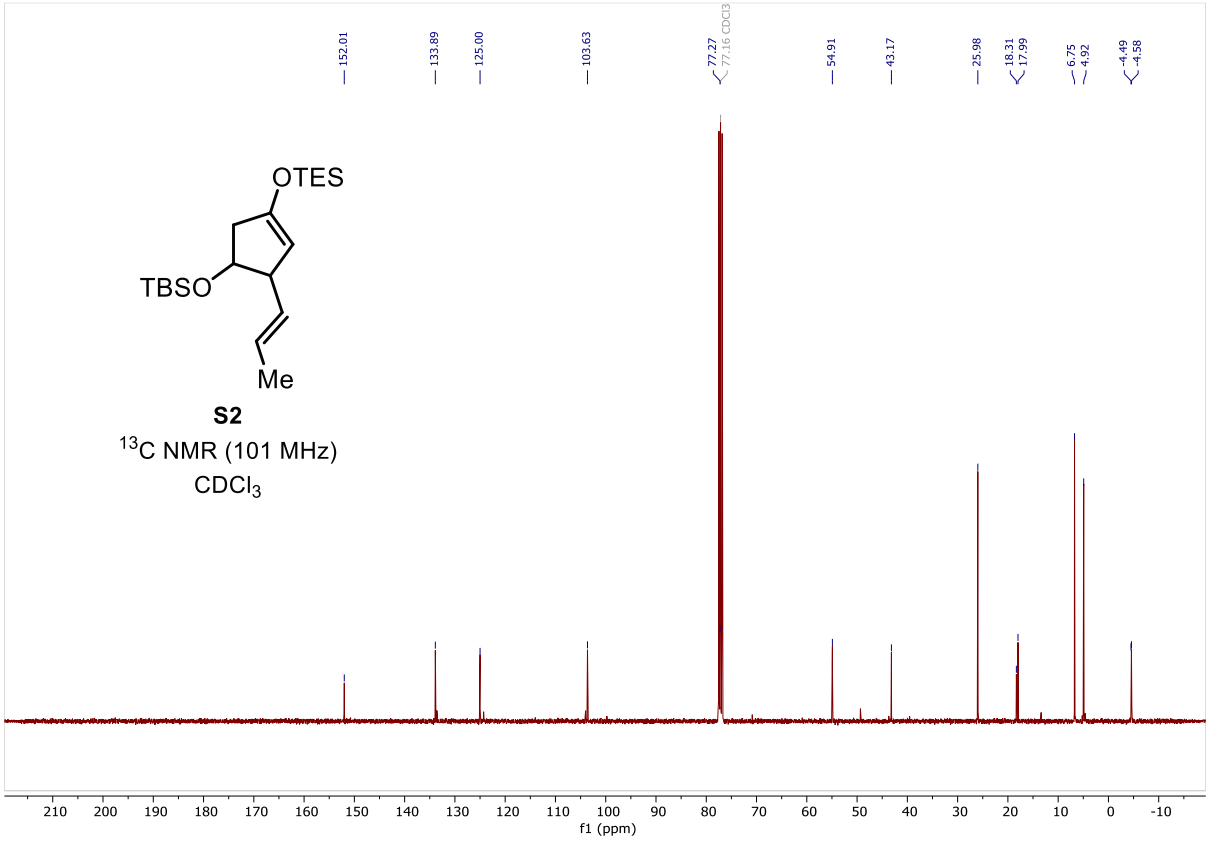

7.26 CDCl3
6.76
6.74
6.72
6.72
6.70
6.70
6.68
6.66
6.55
6.54
6.54
6.54
6.51
6.50
6.50
6.50
5.11
5.11
5.10
5.09
2.89
2.87
2.84
2.83
2.43
2.42
2.38
2.38
1.99
1.99
1.98
1.97
0.89
0.17
0.12
O
Cl
TBSO
Me
11
1H NMR (400 MHz)
CDCl3
1.00
0.96
0.96
0.98
1.00
2.89
8.39
2.88
2.92
f1 (ppm)


197.32
162.70
140.92
130.43
123.44
77.16 CDCl3
68.33
44.14
25.76
19.82
18.01
-3.72
-4.76
O
Cl
TBSO
Me
11
13C NMR (101 MHz)
CDCl3
f1 (ppm)

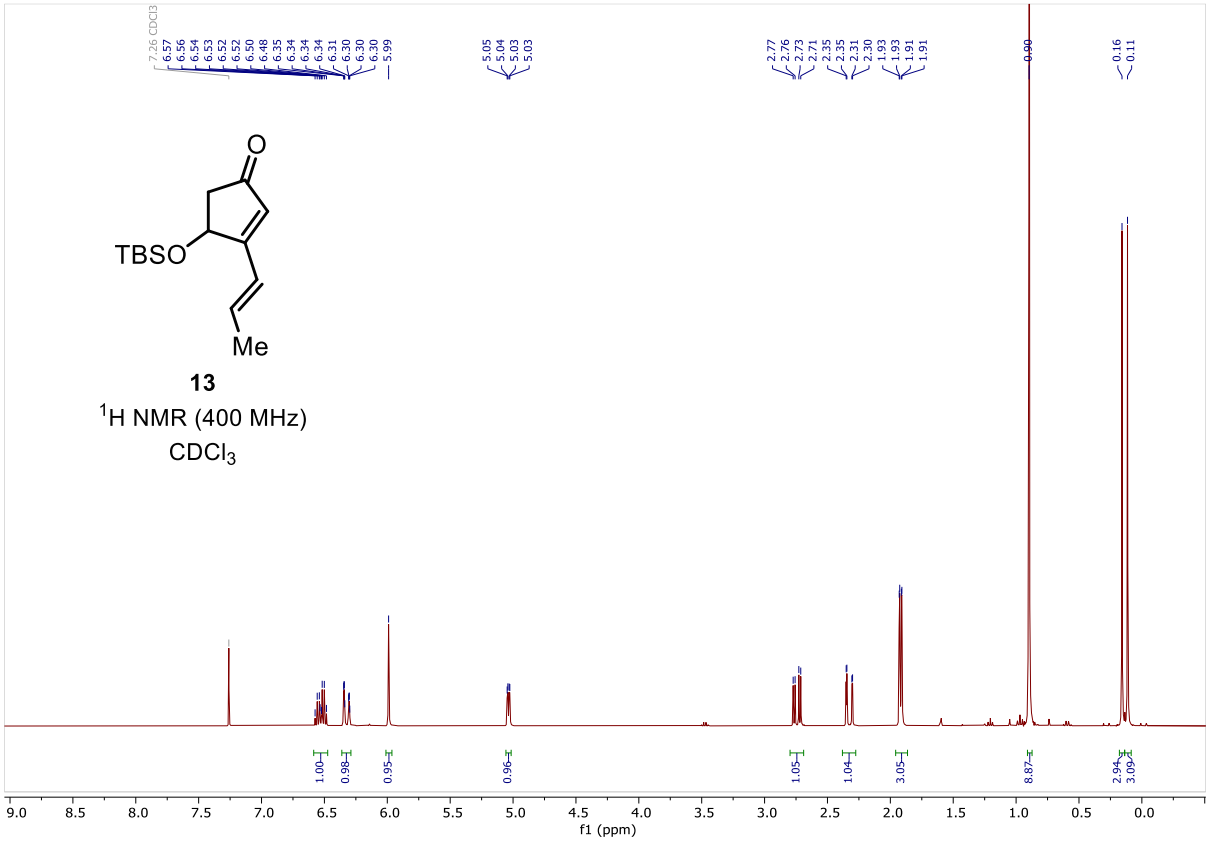

%T
cm-1
O
Cl
TBSO
Me
11
IR spectrum
2857,55cm-1
2954,88cm-1
2930,10cm-1
1640,34cm-1
1720,27cm-1
1084,62cm-1
832,49cm-1
892,59cm-1
592,00cm-1
689,54cm-1
732,15cm-1

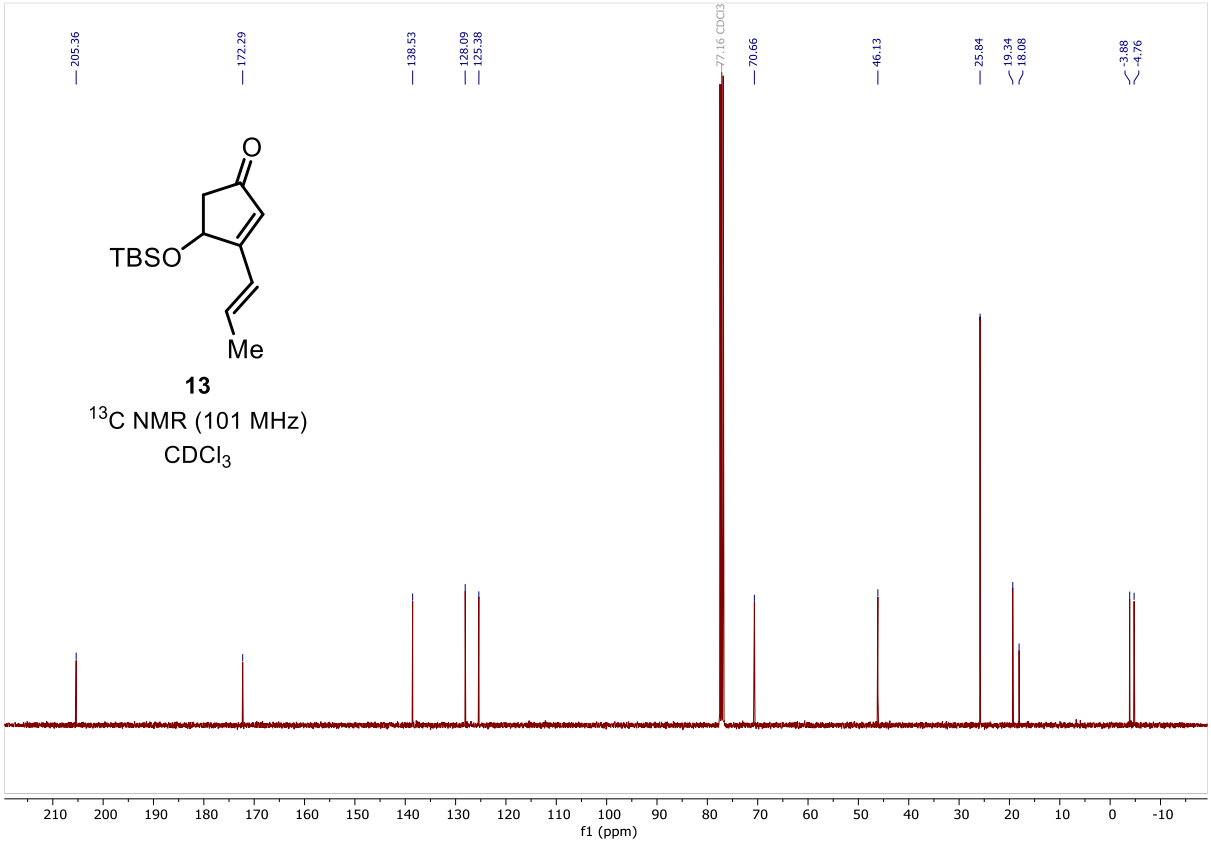
Compound S2

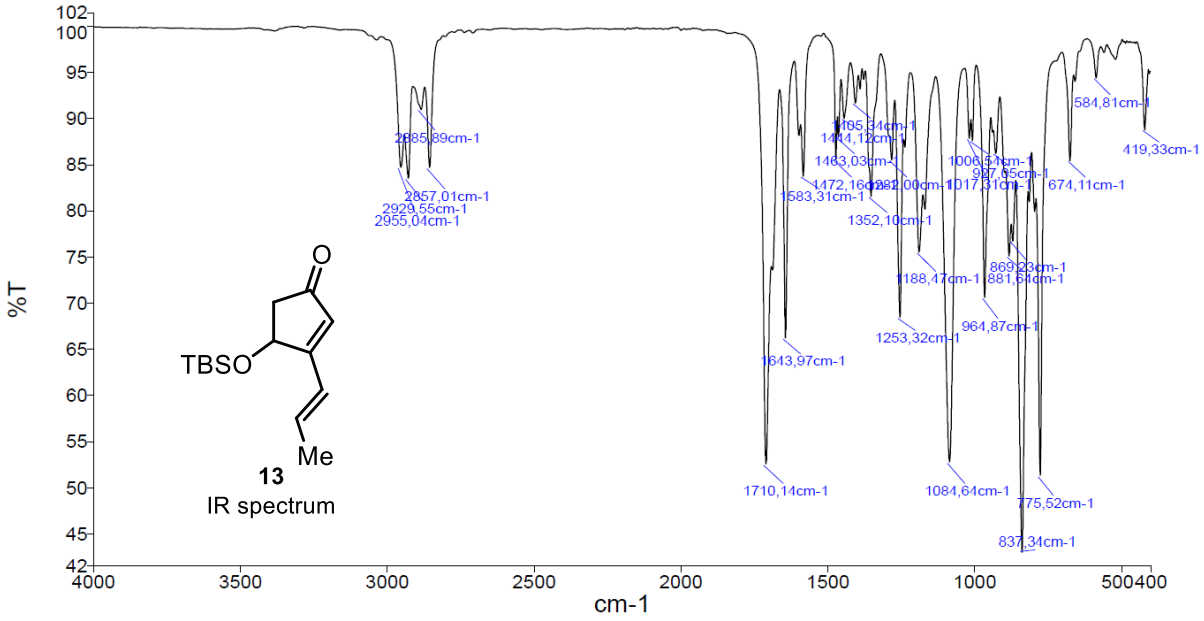

OTES
TBSO
Me
S2
1H NMR (400 MHz)
CDCl3
f1 (ppm)


OTES
TBSO
Me
S2
13C NMR (101 MHz)
CDCl3
f1 (ppm)

Compound 13


TBSO
O
Me
13
1H NMR (400 MHz)
CDCl3
f1 (ppm)

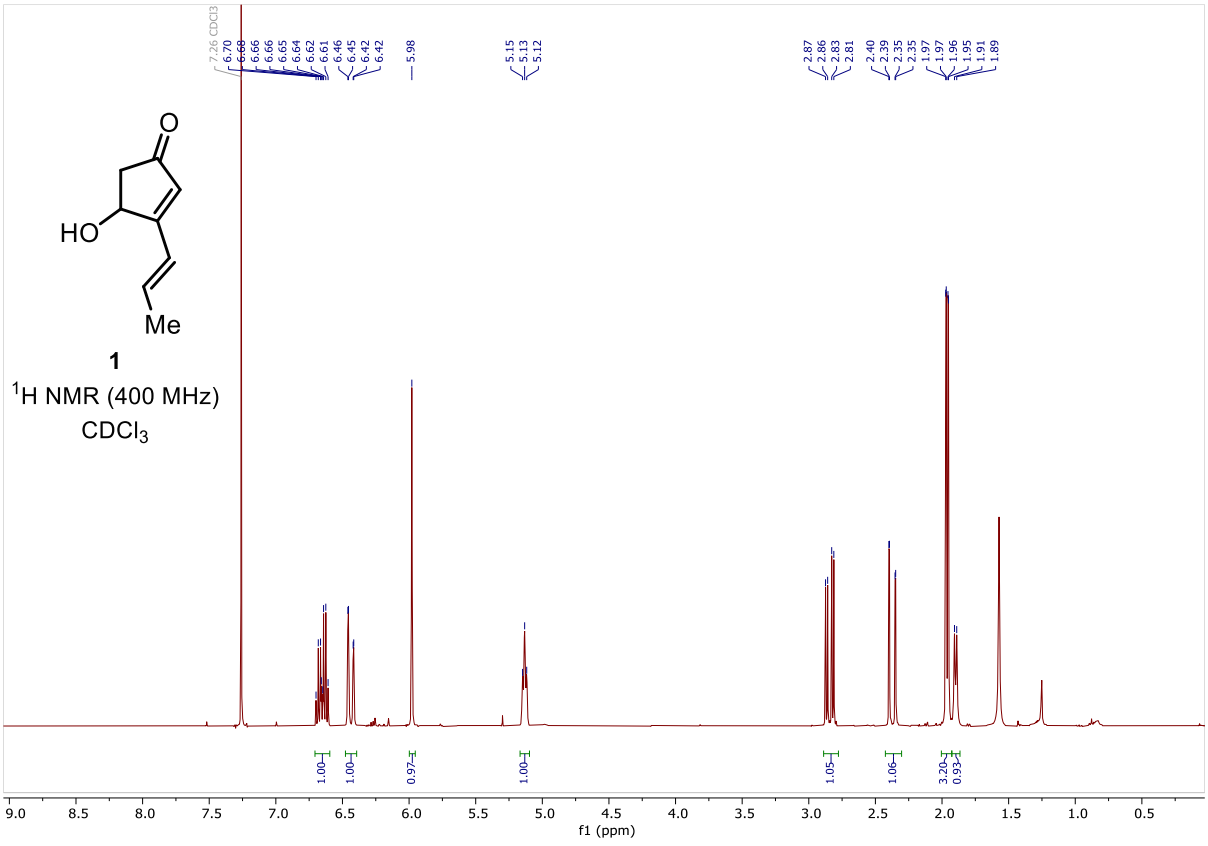

TBSO
O
Me
13
13C NMR (101 MHz)
CDCl3
f1 (ppm)



13

IR spectrum

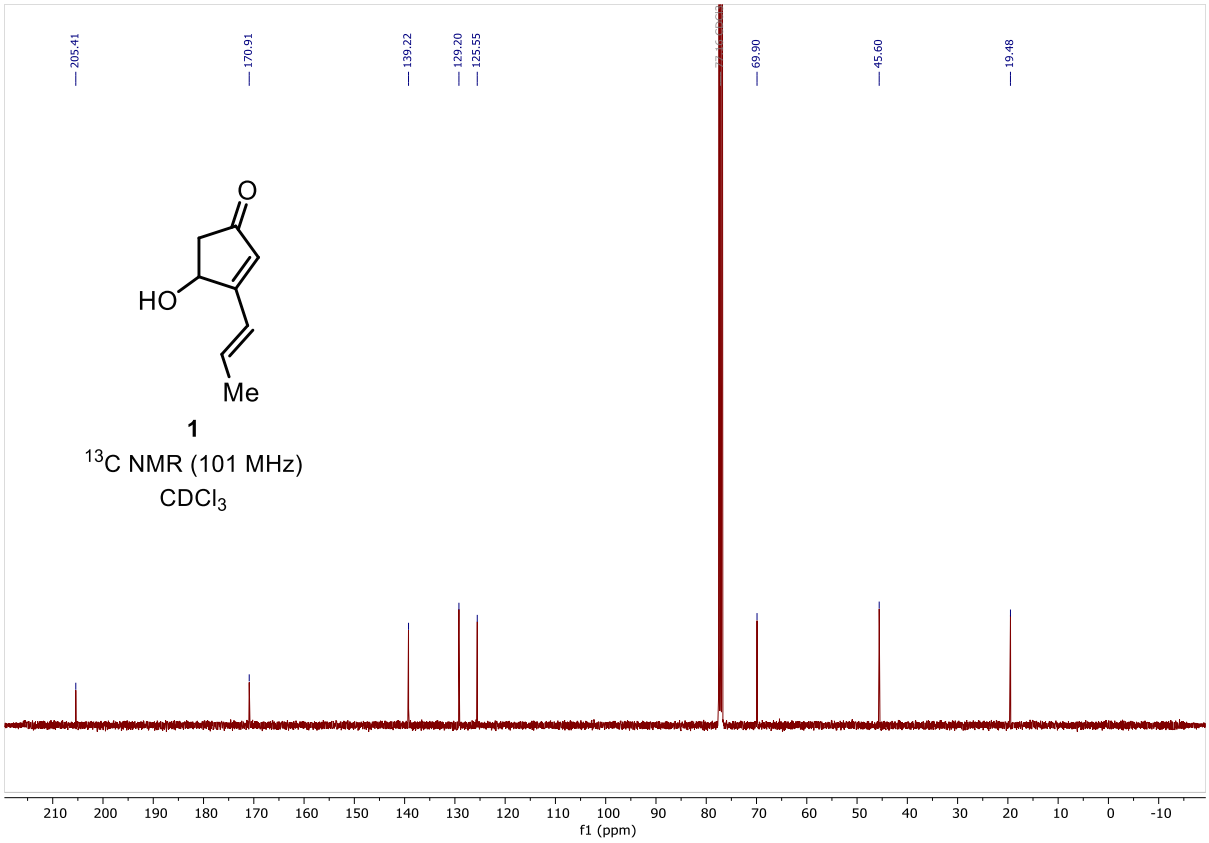
Compound 1

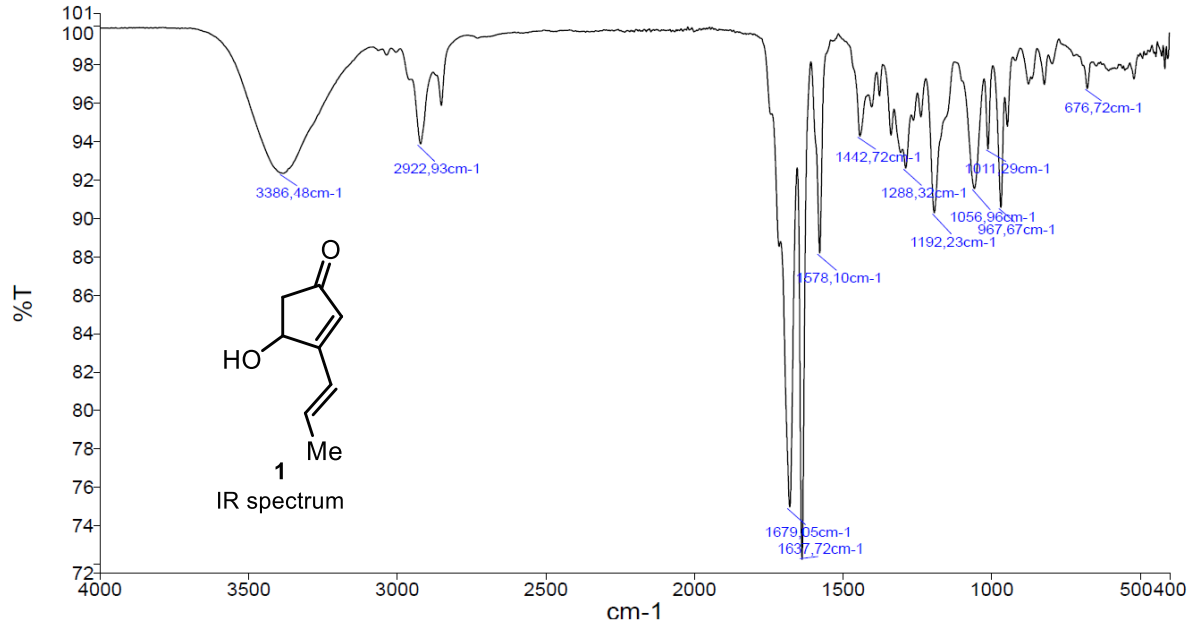


1

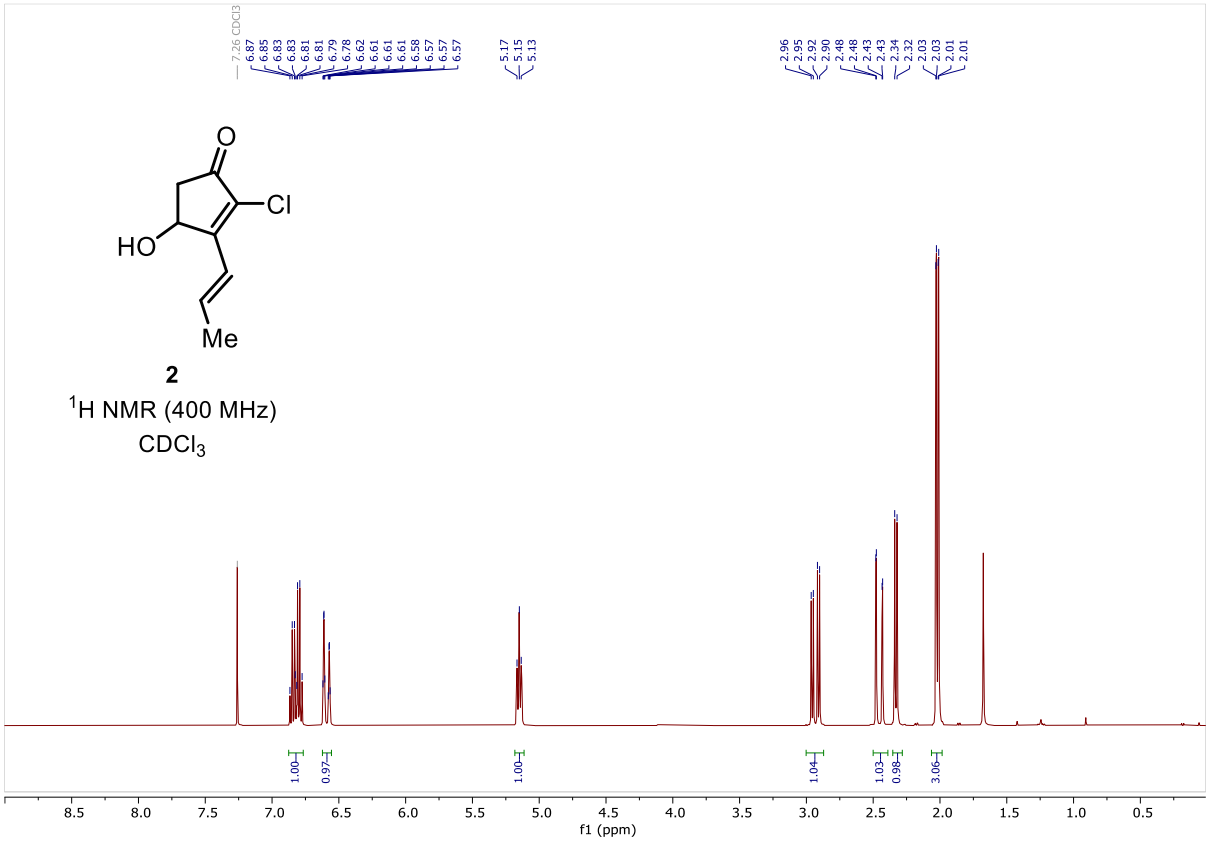
^{1}H NMR (400 MHz)

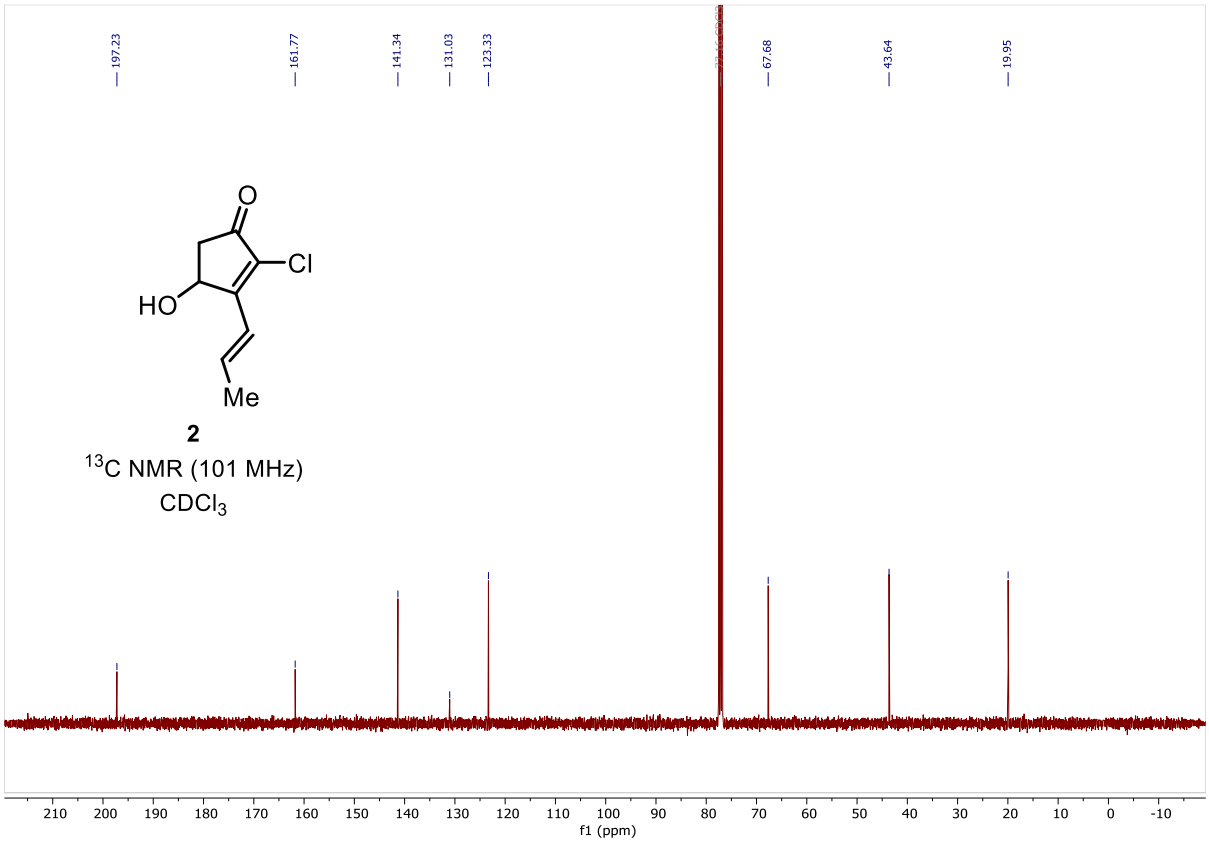
$CDCl_3$

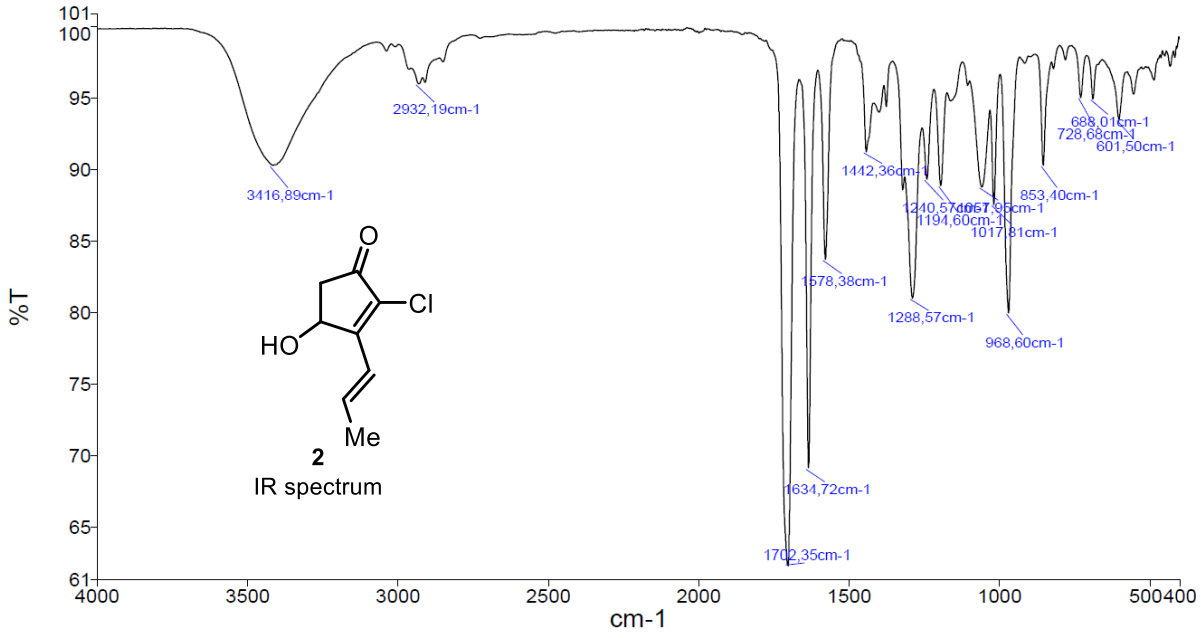

205.41
170.91
139.22
129.20
125.55
69.90
45.60
19.48
O
HO
Me
1
13C NMR (101 MHz)
CDCl3
f1 (ppm)


%T
cm-1
3386,48cm-1
2922,93cm-1
1679,05cm-1
1637,72cm-1
1578,10cm-1
1442,72cm-1
1288,32cm-1
1192,23cm-1
1056,96cm-1
1011,29cm-1
967,67cm-1
676,72cm-1
O
HO
Me
1
IR spectrum

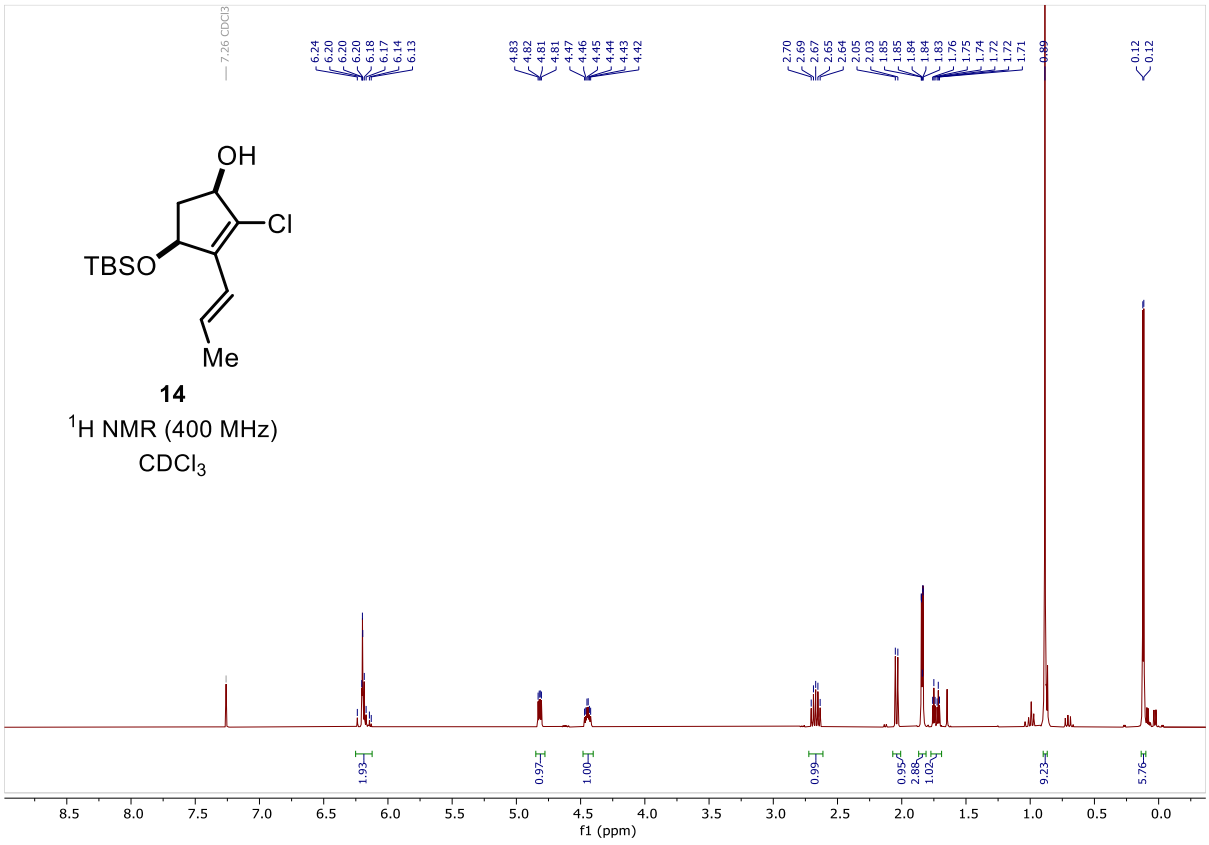
Compound 2

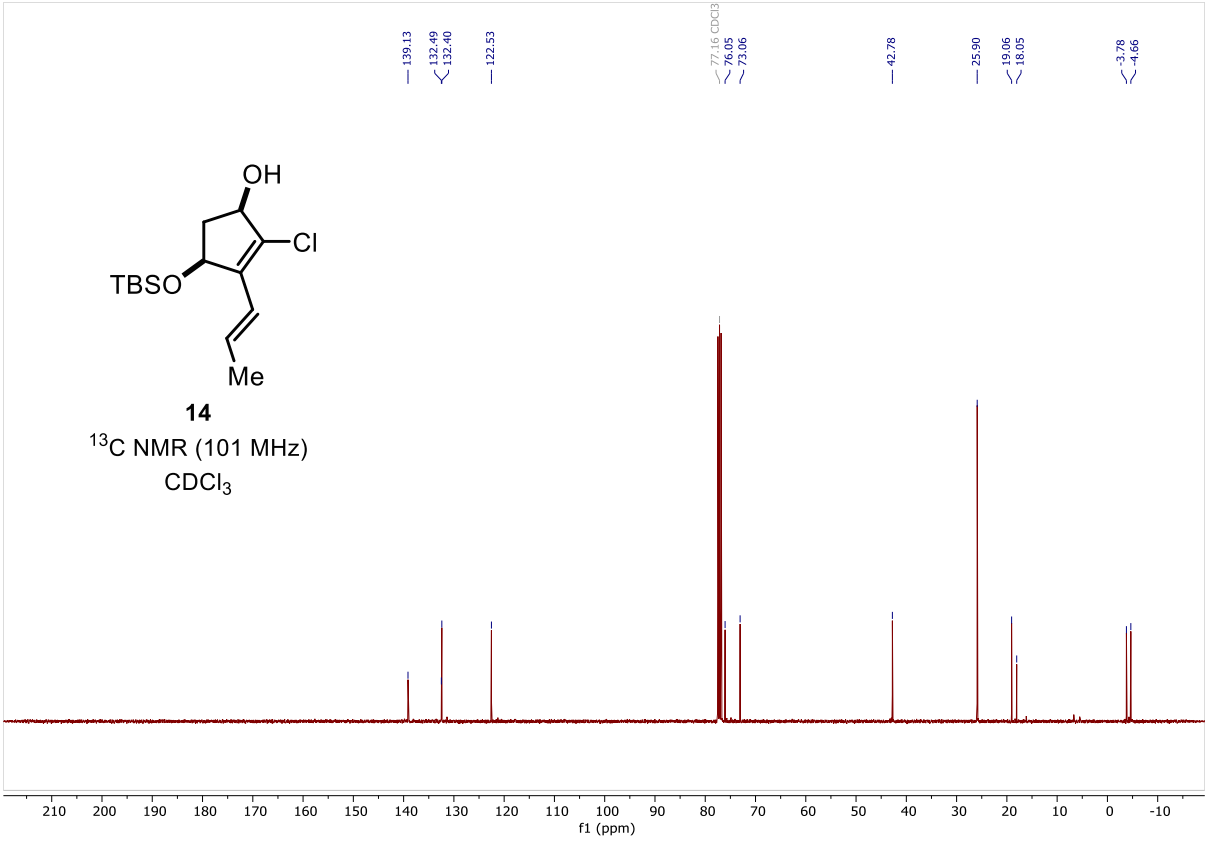

7.26 CDCl3
6.87
6.85
6.83
6.83
6.81
6.81
6.79
6.78
6.62
6.61
6.61
6.61
6.58
6.57
6.57
6.57
5.17
5.15
5.13
2.96
2.95
2.92
2.90
2.48
2.48
2.43
2.43
2.34
2.32
2.03
2.03
2.01
2.01
O
Cl
HO
Me
2
1H NMR (400 MHz)
CDCl3
1.00
0.97
1.00
1.04
1.03
0.98
3.06
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)

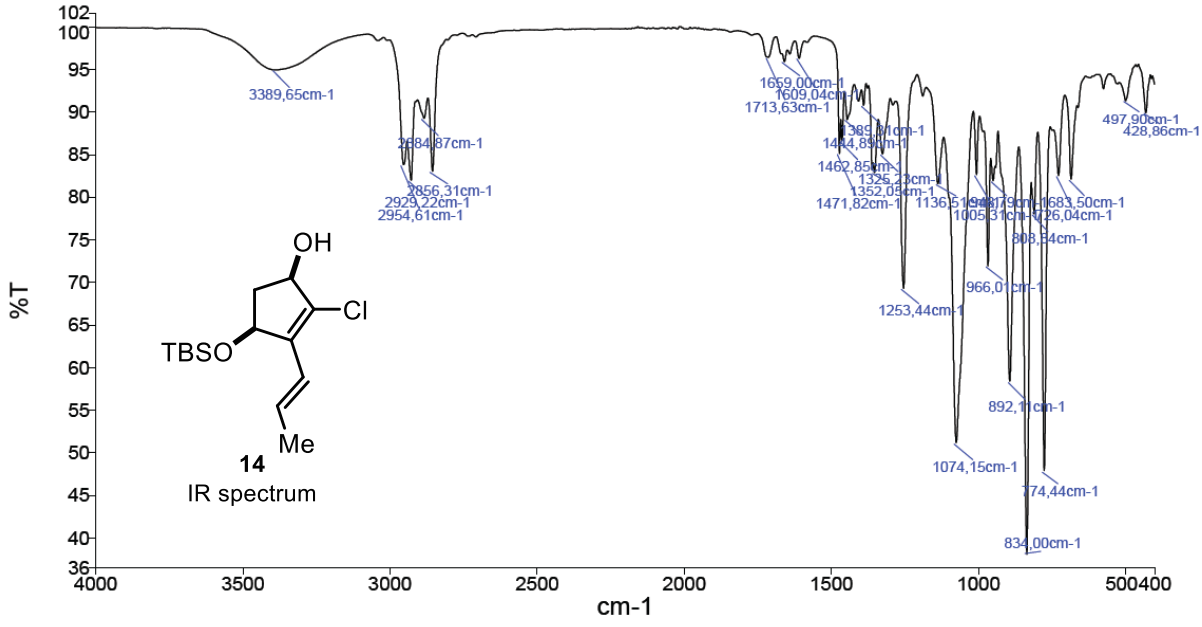

197.23
161.77
141.34
131.03
123.33
77.16 CDCl3
67.68
43.64
19.95
O
Cl
HO
Me
2
13C NMR (101 MHz)
CDCl3
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)


3416,89cm-1
2932,19cm-1
1634,72cm-1
1702,35cm-1
1578,38cm-1
1442,36cm-1
1288,57cm-1
968,60cm-1
853,40cm-1
688,01cm-1
728,68cm-1
601,50cm-1
%T
cm-1
HO
Cl
Me
2
IR spectrum

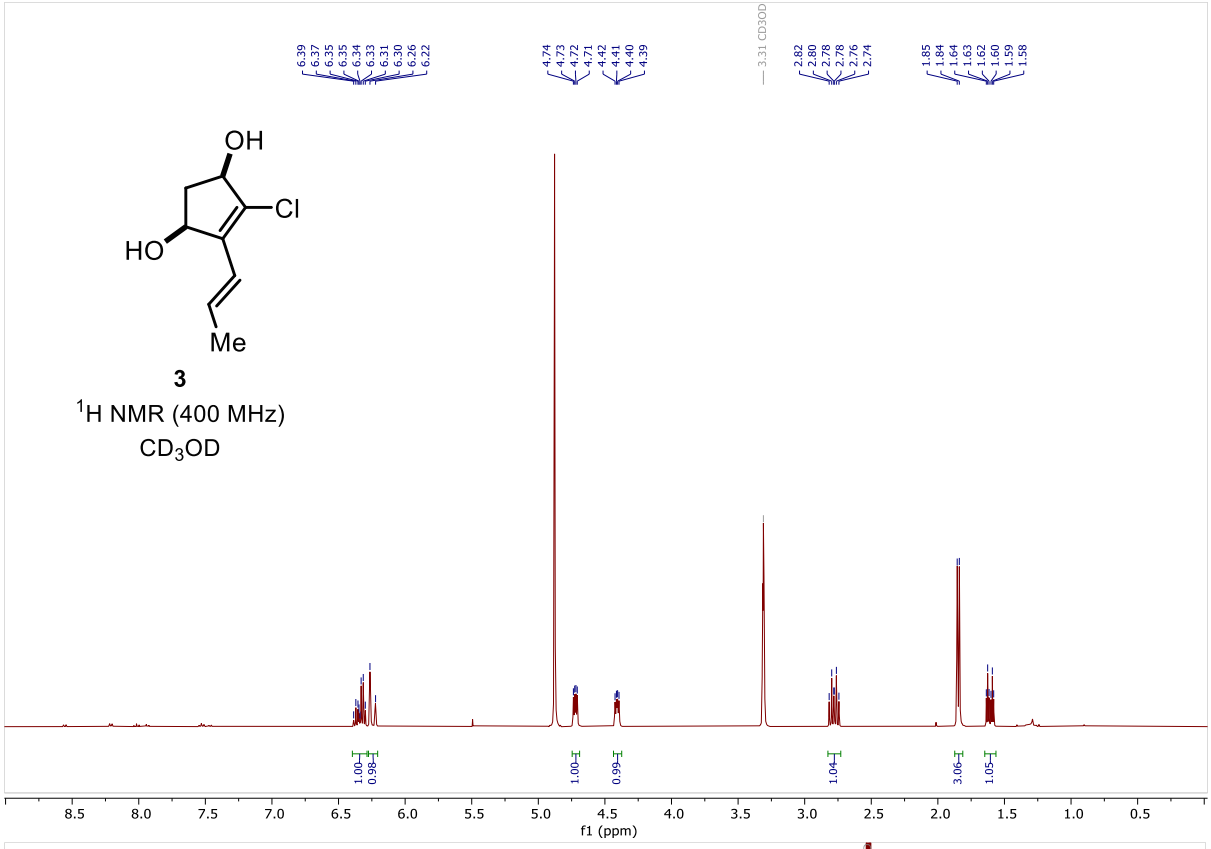
Compound 14

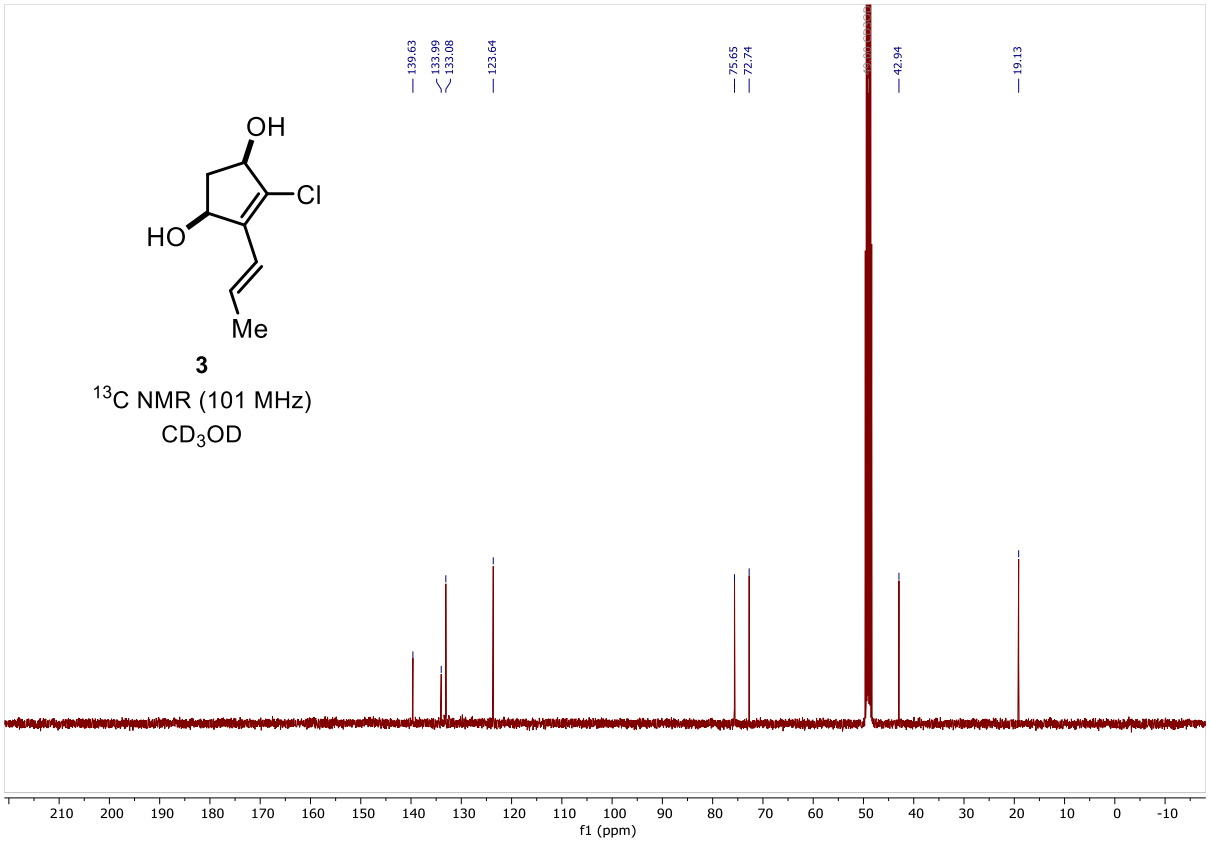

OH
Cl
TBSO
Me
14
1H NMR (400 MHz)
CDCl3
f1 (ppm)

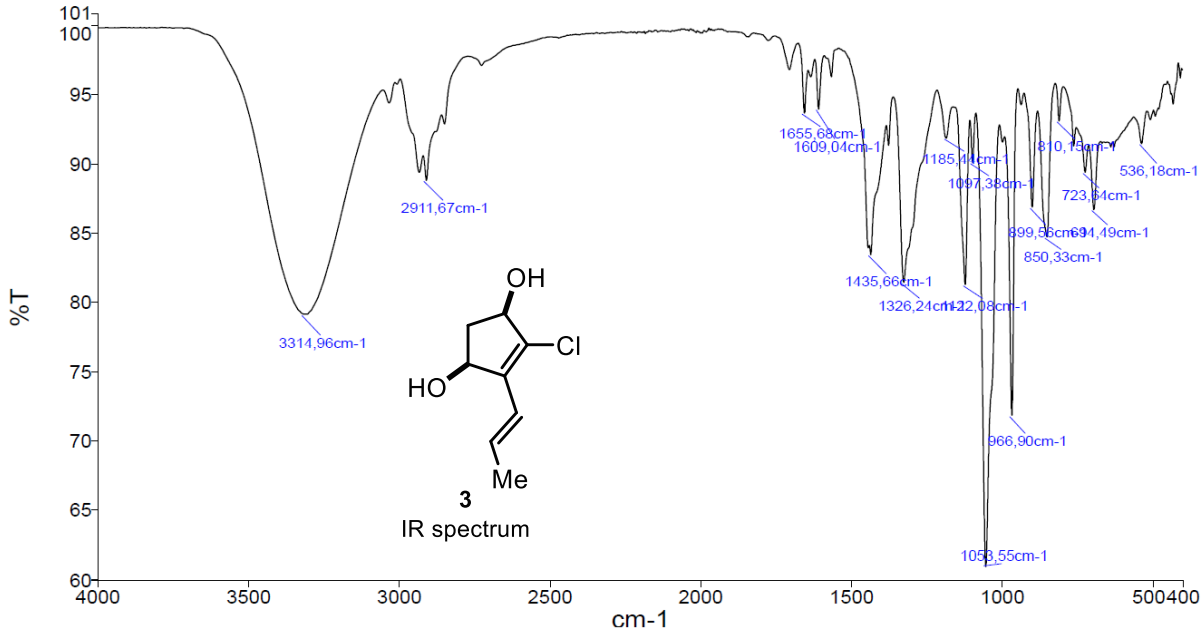

139.13
132.49
132.40
122.53
77.16 CDCl3
76.05
73.06
42.78
25.90
19.06
18.05
-3.78
-4.66
OH
Cl
TBSO
Me
14
13C NMR (101 MHz)
CDCl3
f1 (ppm)


%T
cm-1
3389,65cm-1
2884,87cm-1
2856,31cm-1
2929,22cm-1
2954,61cm-1
1659,00cm-1
1609,04cm-1
1713,63cm-1
1471,82cm-1
1253,44cm-1
1074,15cm-1
834,00cm-1
OH
Cl
TBSO
Me
14
IR spectrum

Compound 3


OH
Cl
HO
Me
3
1H NMR (400 MHz)
CD3OD
f1 (ppm)

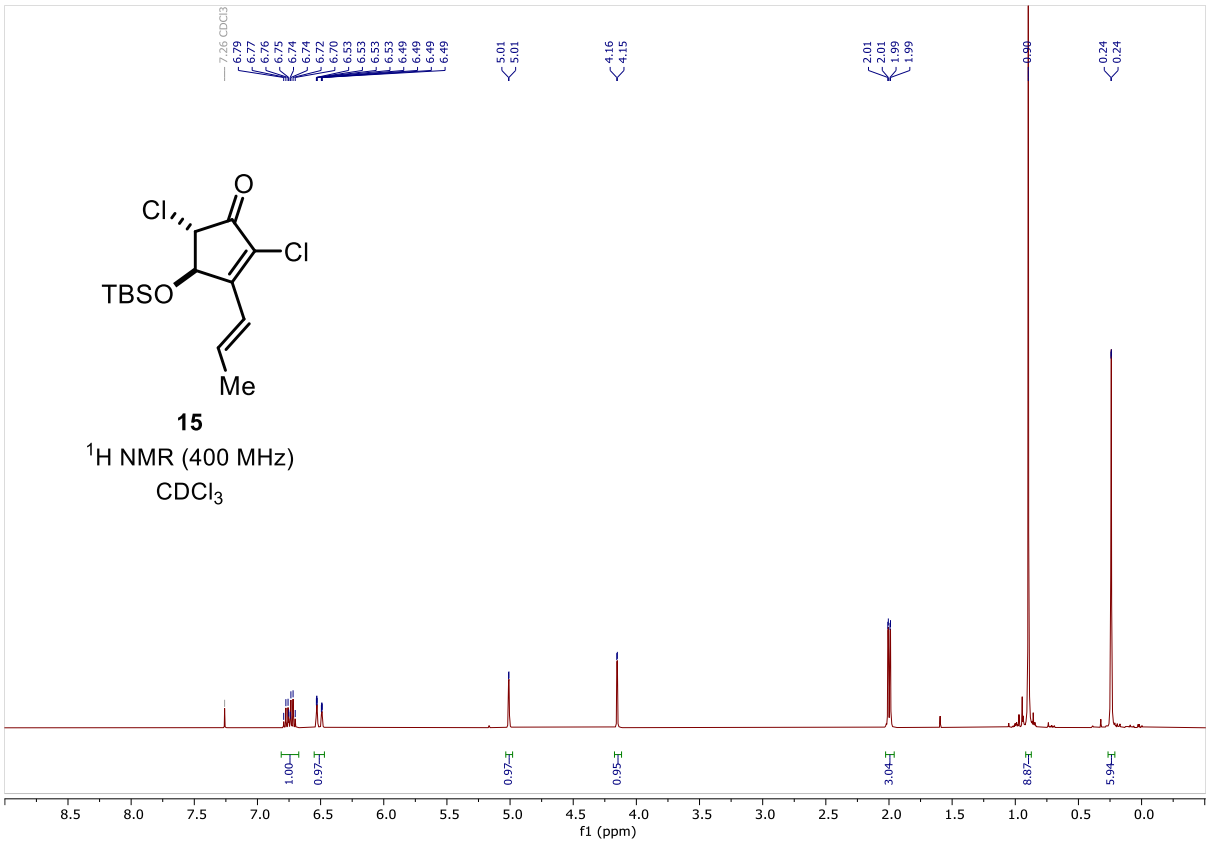

OH
Cl
HO
Me
3
13C NMR (101 MHz)
CD3OD
f1 (ppm)



3

IR spectrum

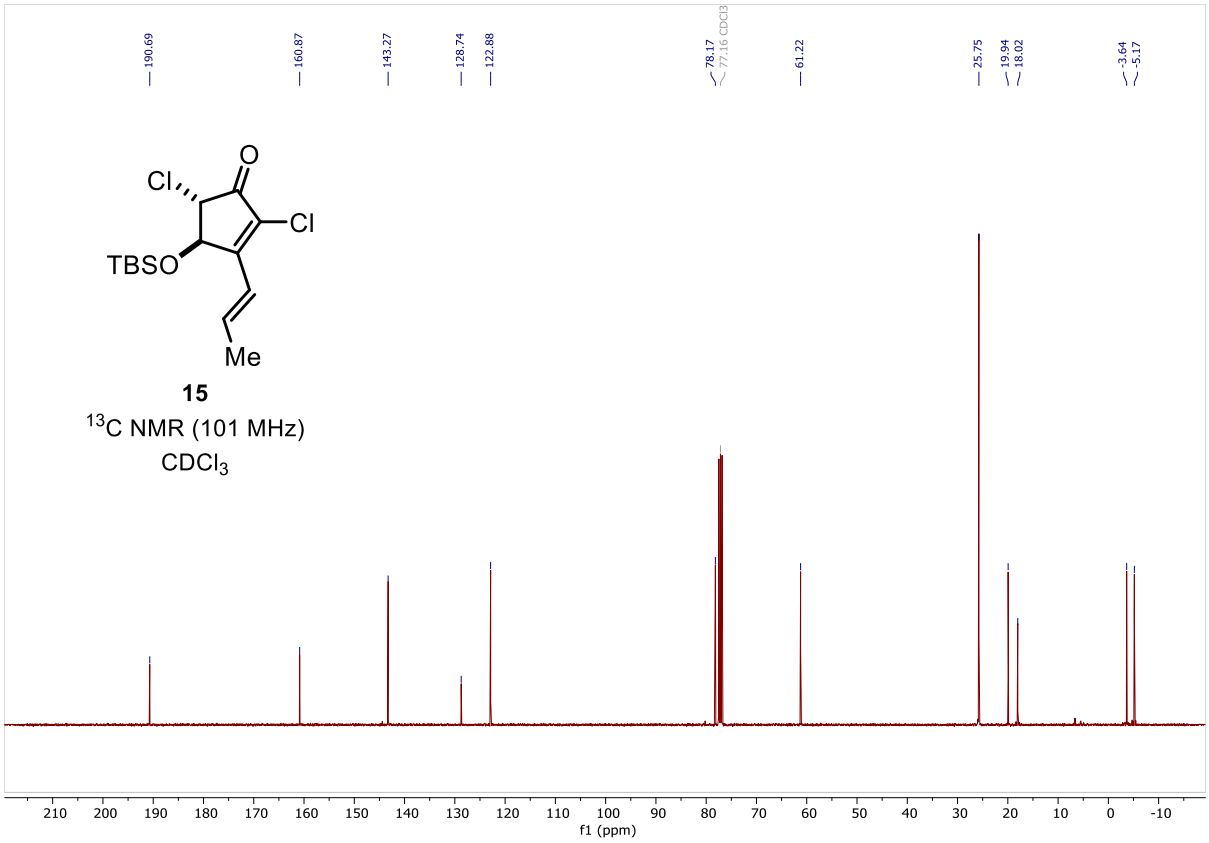
Compound 15

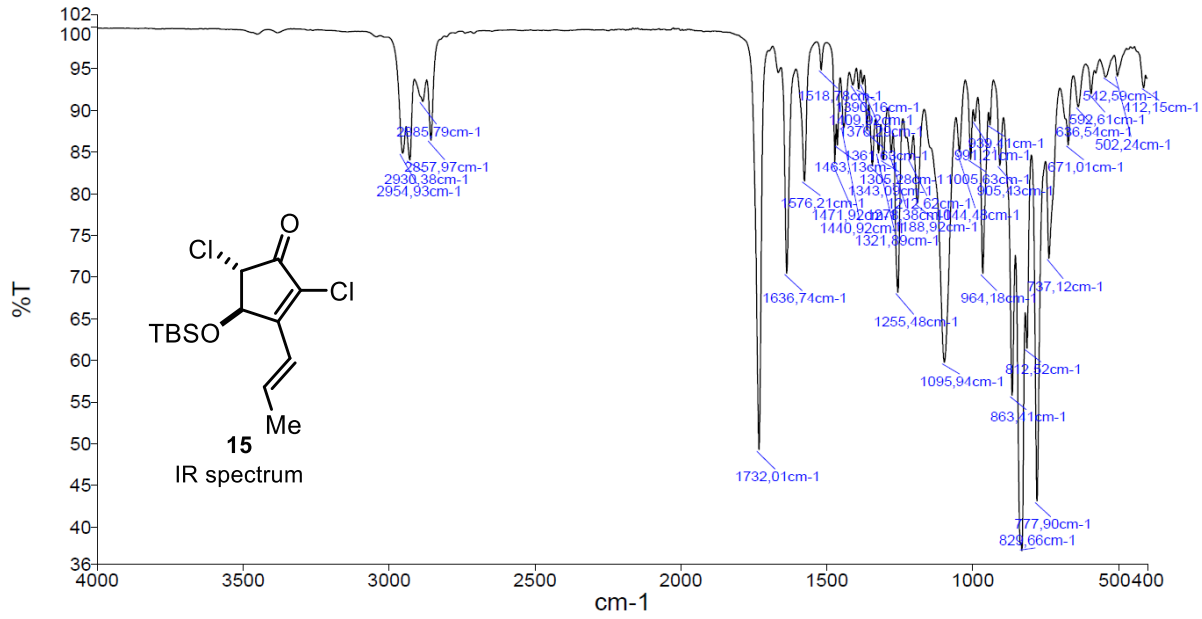


15

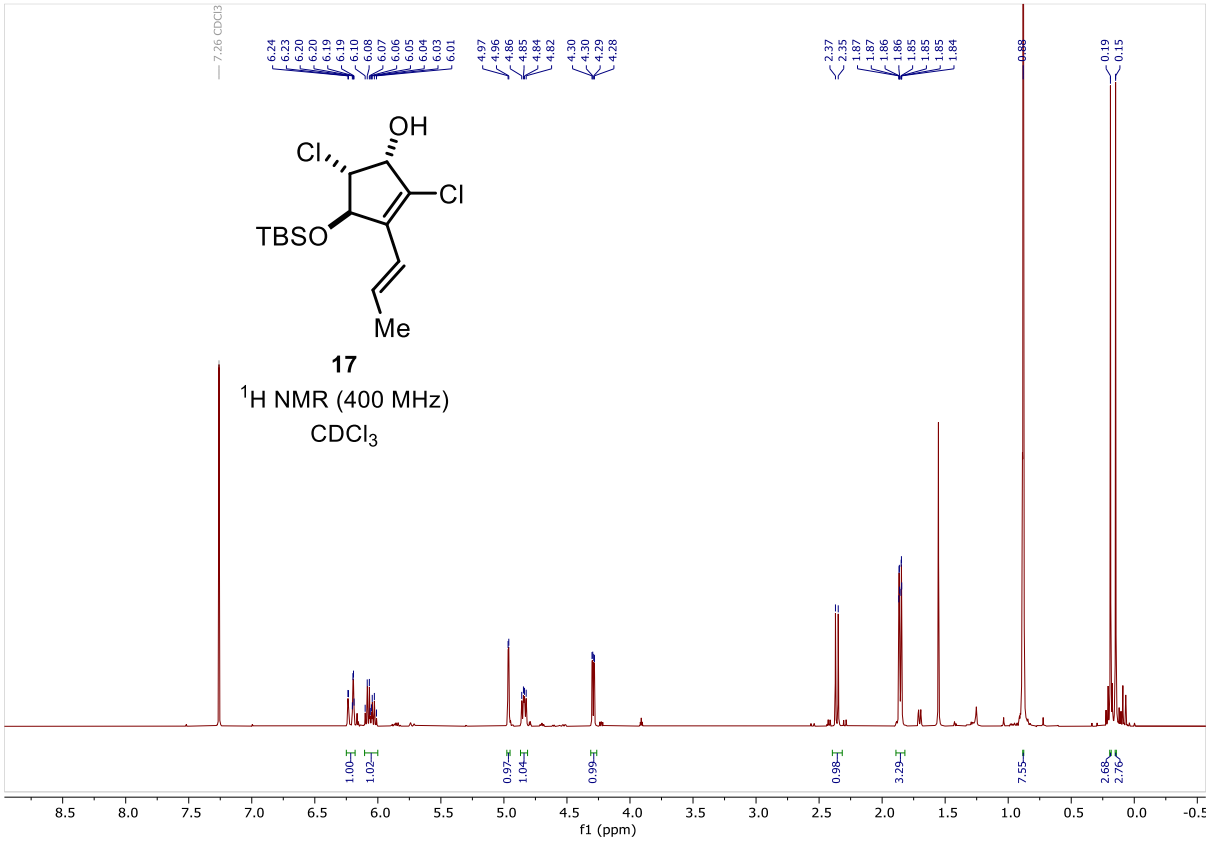
^{1}H NMR (400 MHz)

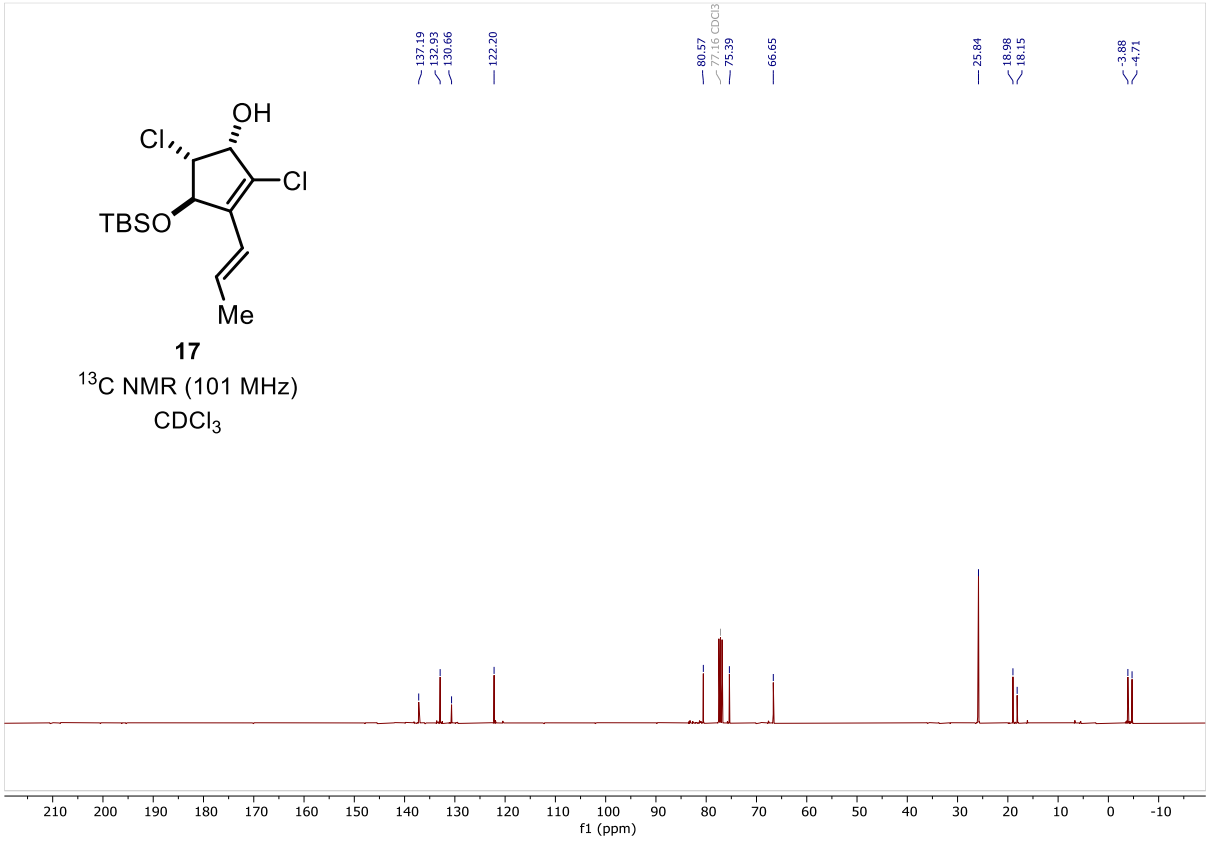
$CDCl_3$

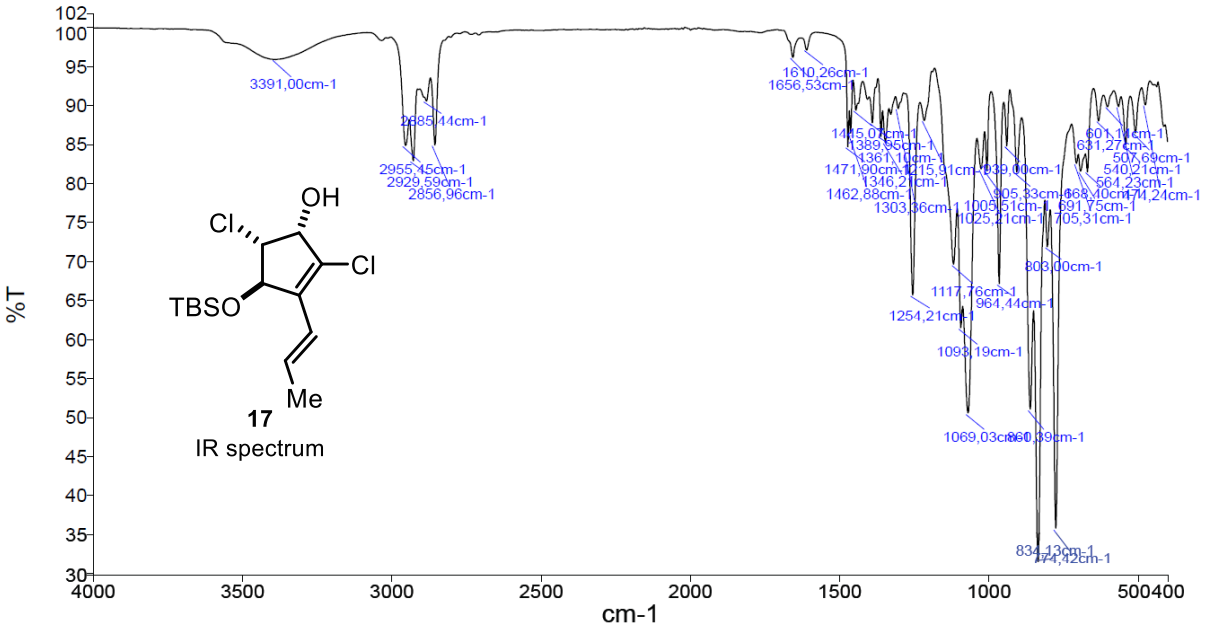

190.69
160.87
143.27
128.74
122.88
78.17
77.16 CDCl3
61.22
25.75
19.94
18.02
-3.64
-5.17
O
Cl
Cl
TBSO
Me
15
13C NMR (101 MHz)
CDCl3
f1 (ppm)


%T
cm-1
2885,79cm-1
2857,97cm-1
2930,38cm-1
2951,93cm-1
1636,74cm-1
1732,01cm-1
1255,48cm-1
1095,94cm-1
964,18cm-1
737,12cm-1
671,01cm-1
502,24cm-1
O
Cl
Cl
TBSO
Me
15
IR spectrum

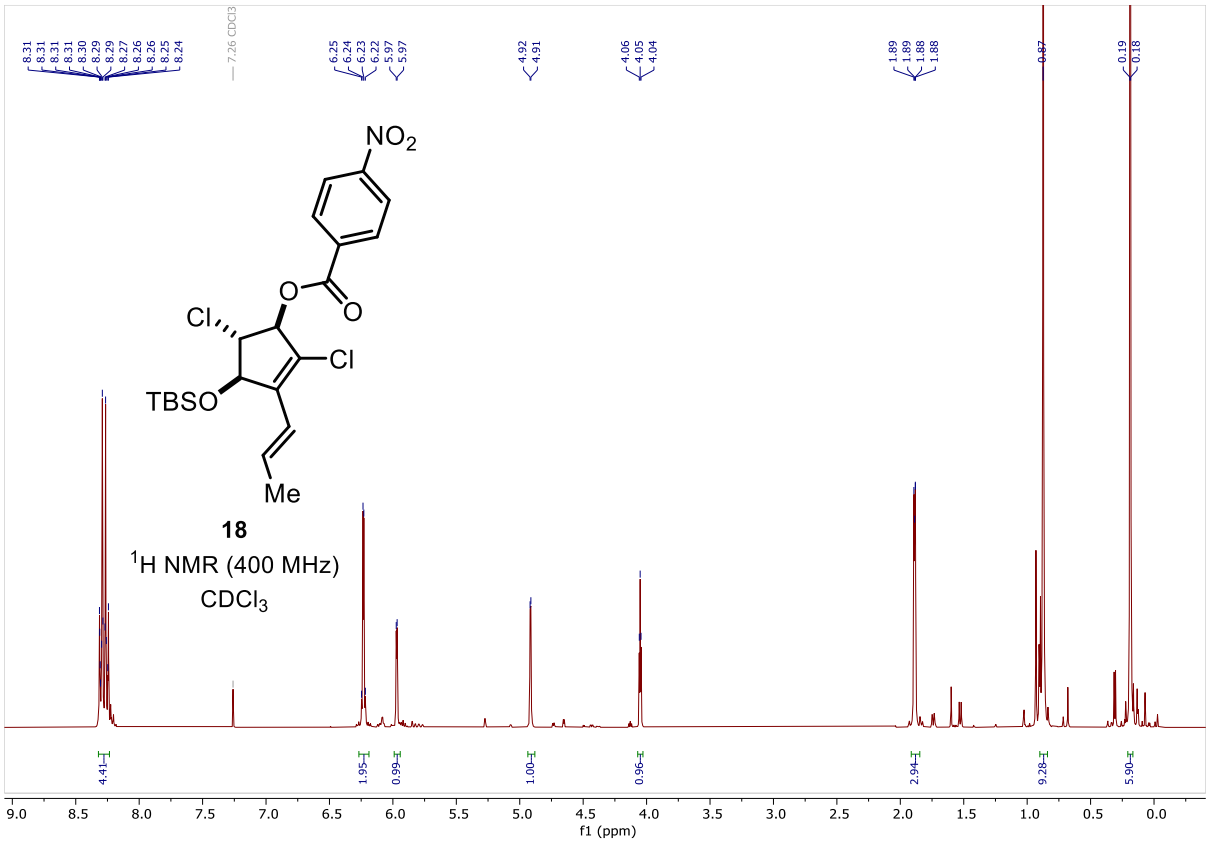
Compound 17

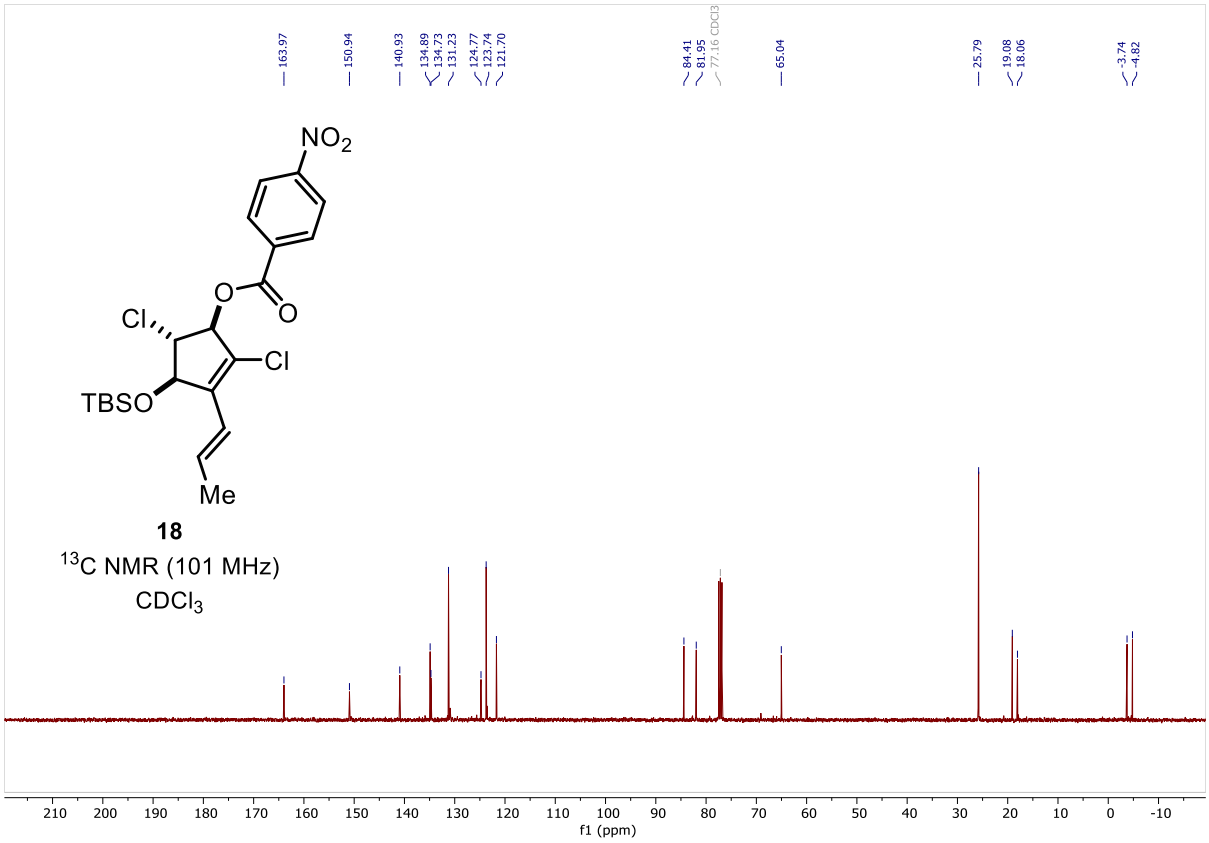

OH
Cl
Cl
TBSO
Me
17
1H NMR (400 MHz)
CDCl3
f1 (ppm)

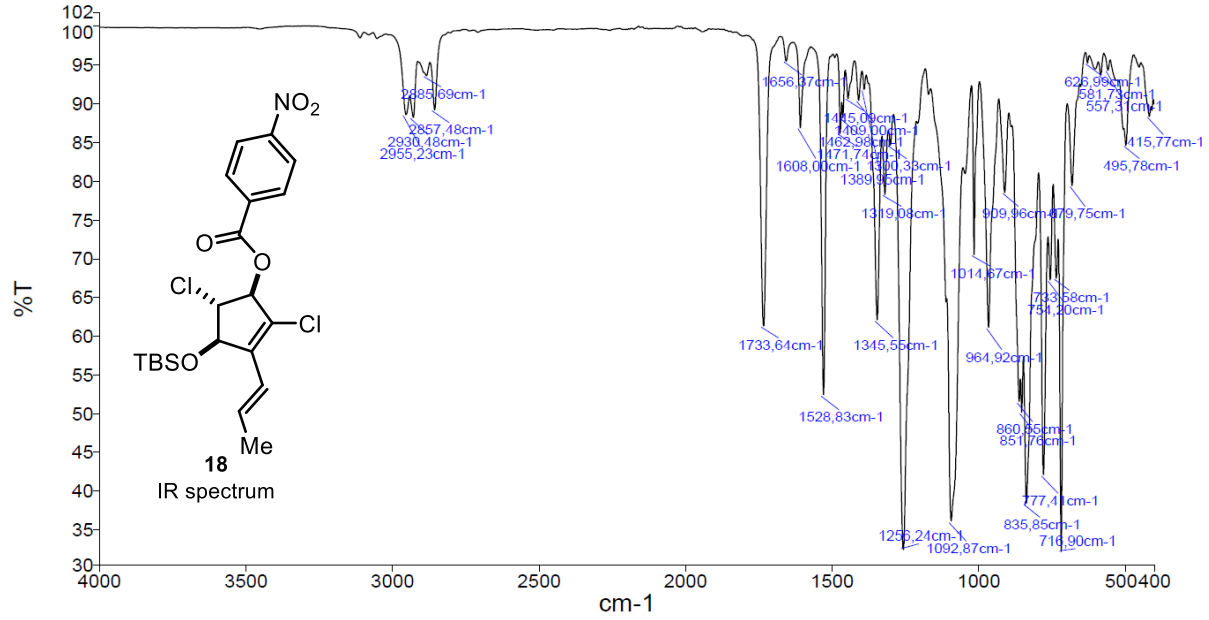

OH
Cl
Cl
TBSO
Me
17
13C NMR (101 MHz)
CDCl3
f1 (ppm)


%T
cm-1
3391,00cm-1
2955,45cm-1
2929,59cm-1
2856,96cm-1
1610,26cm-1
1656,53cm-1
1462,88cm-1
1303,36cm-1
1254,21cm-1
1117,76cm-1
1093,19cm-1
1069,03cm-1
964,44cm-1
834,13cm-1
OH
Cl
Cl
TBSO
Me
17
IR spectrum

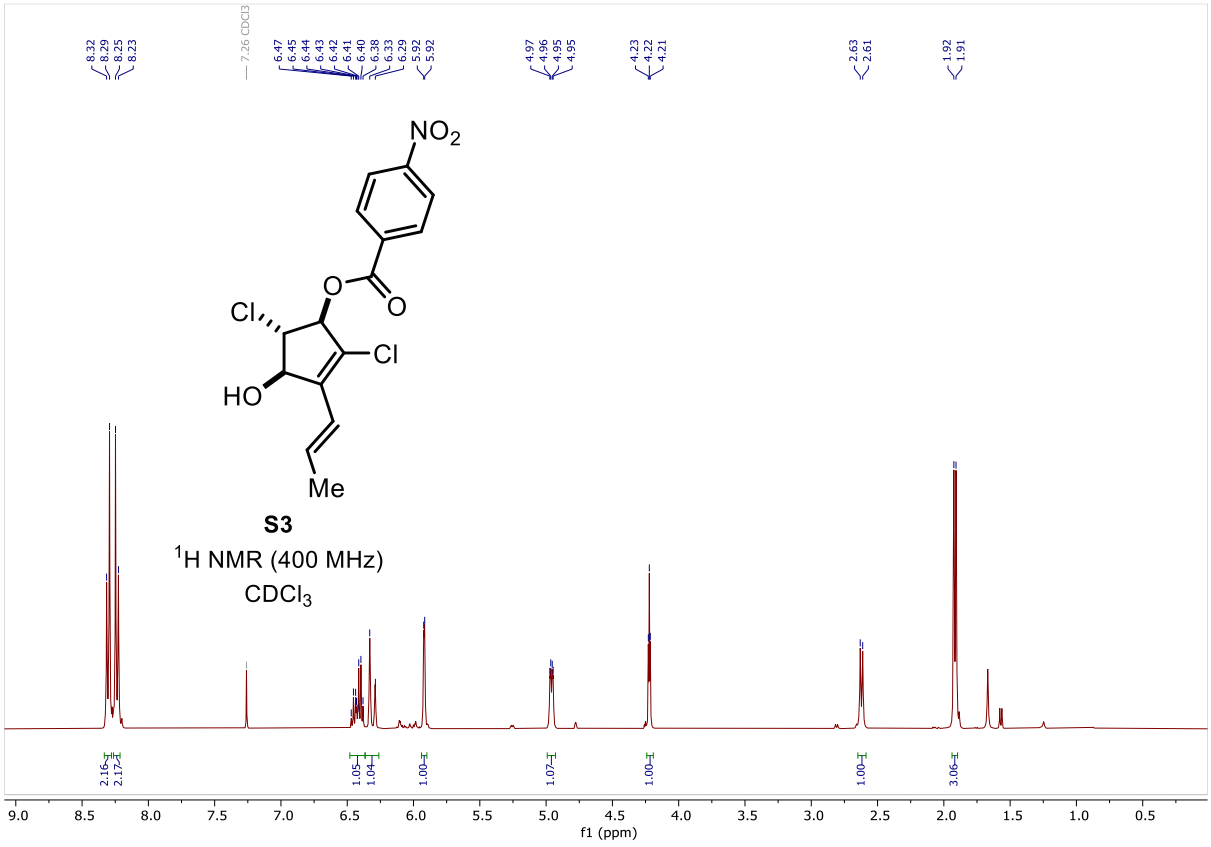
Compound 18

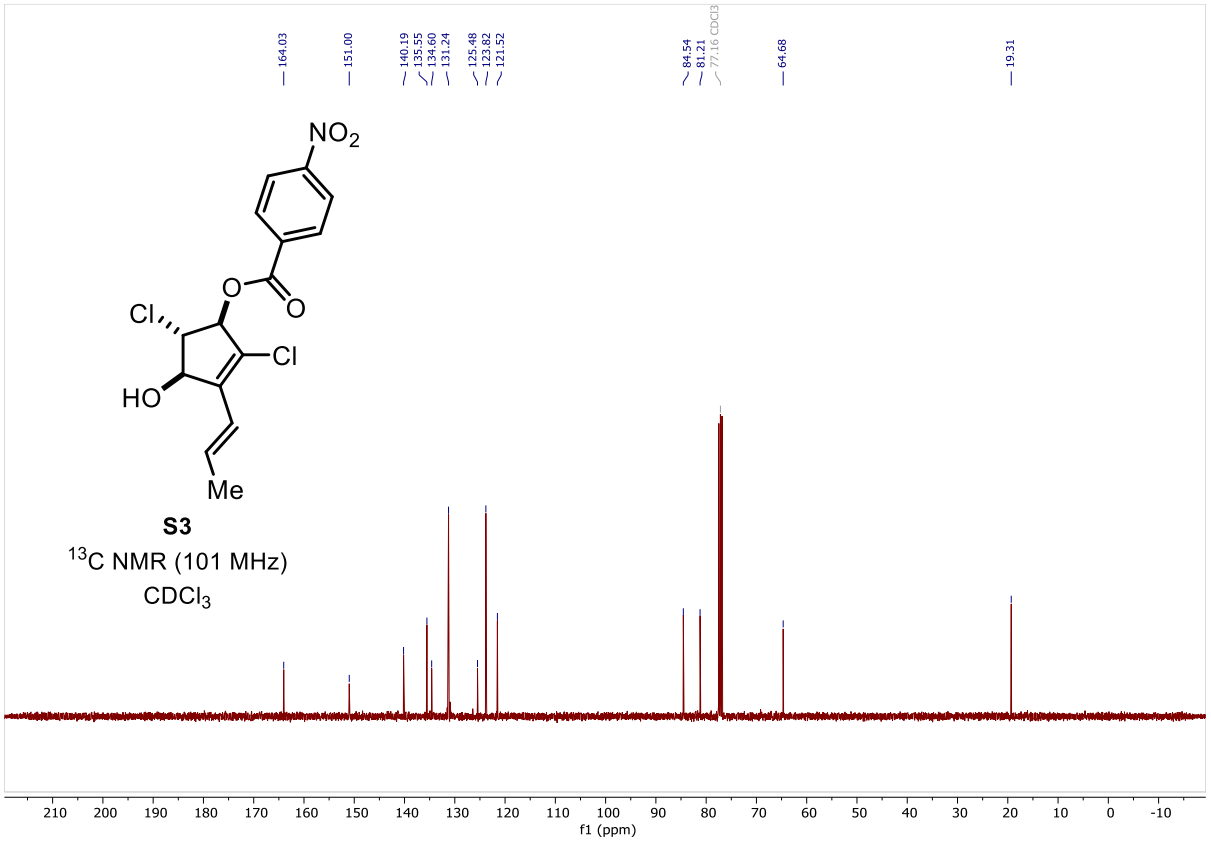

NO2
O
O
Cl
Cl
TBSO
Me
18
1H NMR (400 MHz)
CDCl3
f1 (ppm)

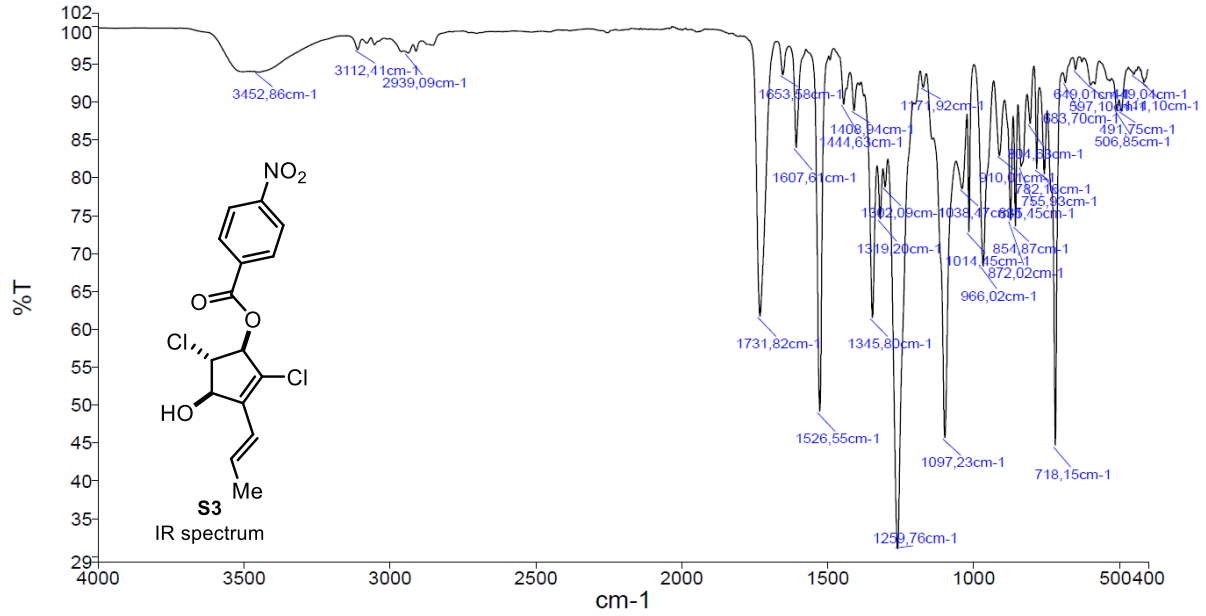

163.97
150.94
140.93
134.89
134.73
131.23
124.77
123.74
121.70
84.41
81.95
77.16 CDCl3
65.04
25.79
19.08
18.06
-3.74
-4.82
NO2
O
O
Cl
Cl
TBSO
Me
18
13C NMR (101 MHz)
CDCl3
f1 (ppm)


%T
cm-1
NO2
O
O
Cl
Cl
TBSO
Me
18
IR spectrum
2885,69cm-1
2857,48cm-1
2930,48cm-1
2955,23cm-1
1656,37cm-1
1608,00cm-1
1733,64cm-1
1528,83cm-1
1345,55cm-1
1319,08cm-1
1256,24cm-1
1092,87cm-1
1014,67cm-1
964,92cm-1
909,96cm-1
860,85cm-1
851,76cm-1
835,85cm-1
716,90cm-1
733,58cm-1
754,30cm-1
779,75cm-1
626,99cm-1
581,73cm-1
557,31cm-1
495,78cm-1

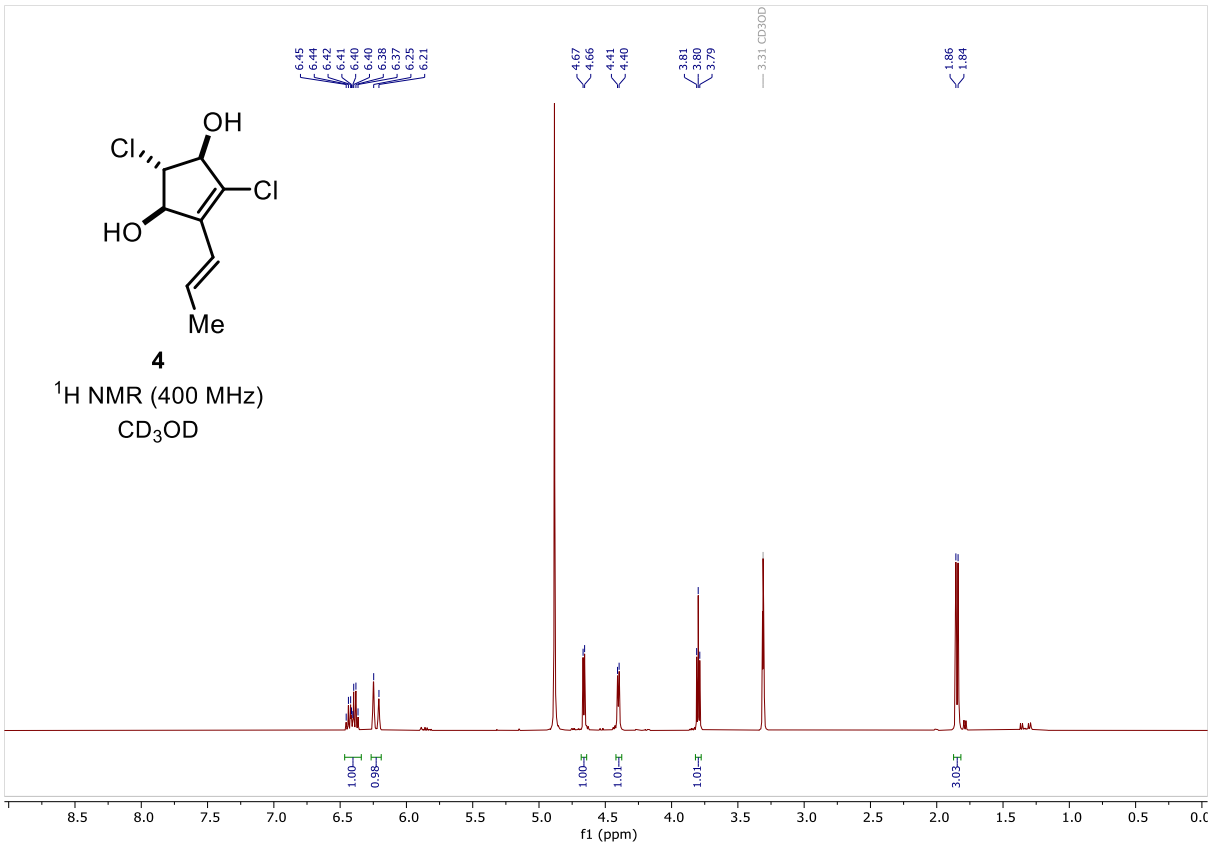
Compound S3

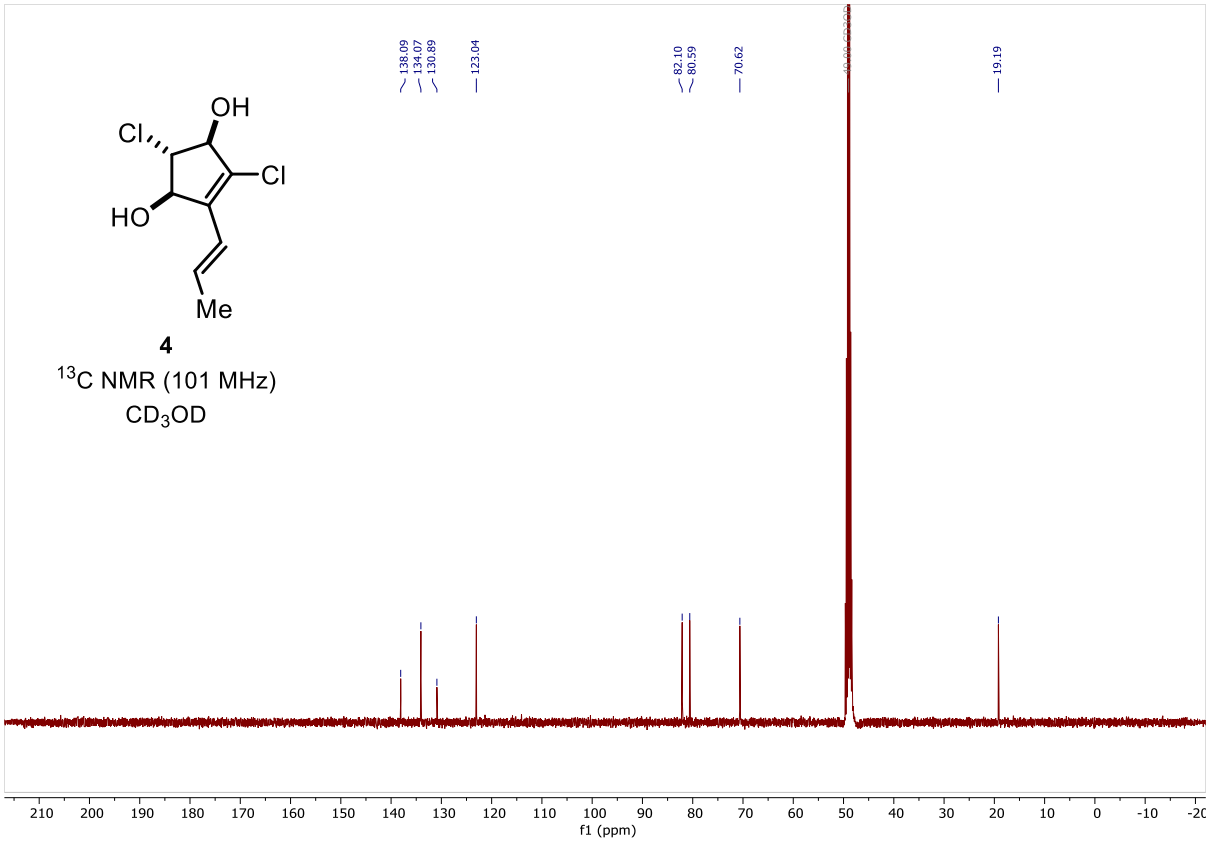

8.32
8.29
8.25
8.23
7.26 CDCl3
6.47
6.45
6.44
6.43
6.42
6.41
6.40
6.38
6.33
6.29
5.92
5.92
4.97
4.96
4.95
4.95
4.23
4.22
4.21
2.63
2.61
1.92
1.91
NO2
O
O
Cl
Cl
HO
Me
S3
1H NMR (400 MHz)
CDCl3
2.16
2.17
1.05
1.04
1.00
1.07
1.00
1.00
3.06
f1 (ppm)


164.03
151.00
140.19
135.55
134.60
131.24
125.48
123.82
121.52
84.54
81.21
77.16 CDCl3
64.68
19.31
NO2
O
O
Cl
Cl
HO
Me
S3
13C NMR (101 MHz)
CDCl3
f1 (ppm)


%T
cm-1
3452,86cm-1
3112,41cm-1
2939,09cm-1
1731,82cm-1
1607,61cm-1
1526,55cm-1
1444,63cm-1
1408,94cm-1
1345,80cm-1
1319,20cm-1
1259,76cm-1
1097,23cm-1
718,15cm-1
NO2
O
O
Cl
Cl
HO
Me
S3
IR spectrum

Compound 4

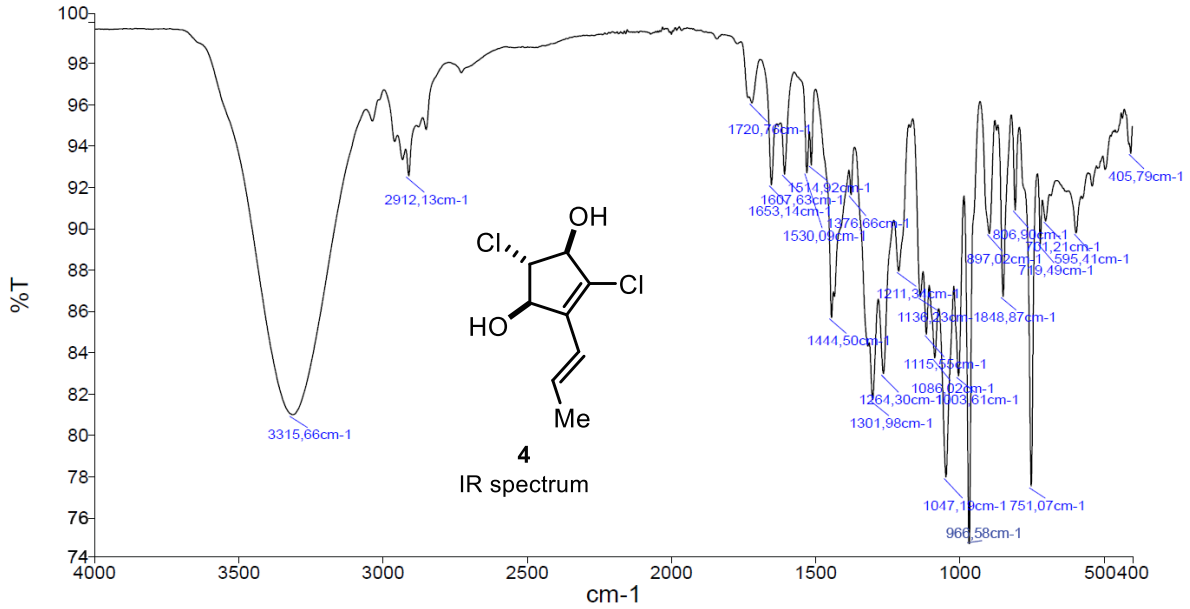

OH
Cl
Cl
HO
Me
4
1H NMR (400 MHz)
CD3OD
f1 (ppm)



4

^{13}C NMR (101 MHz)

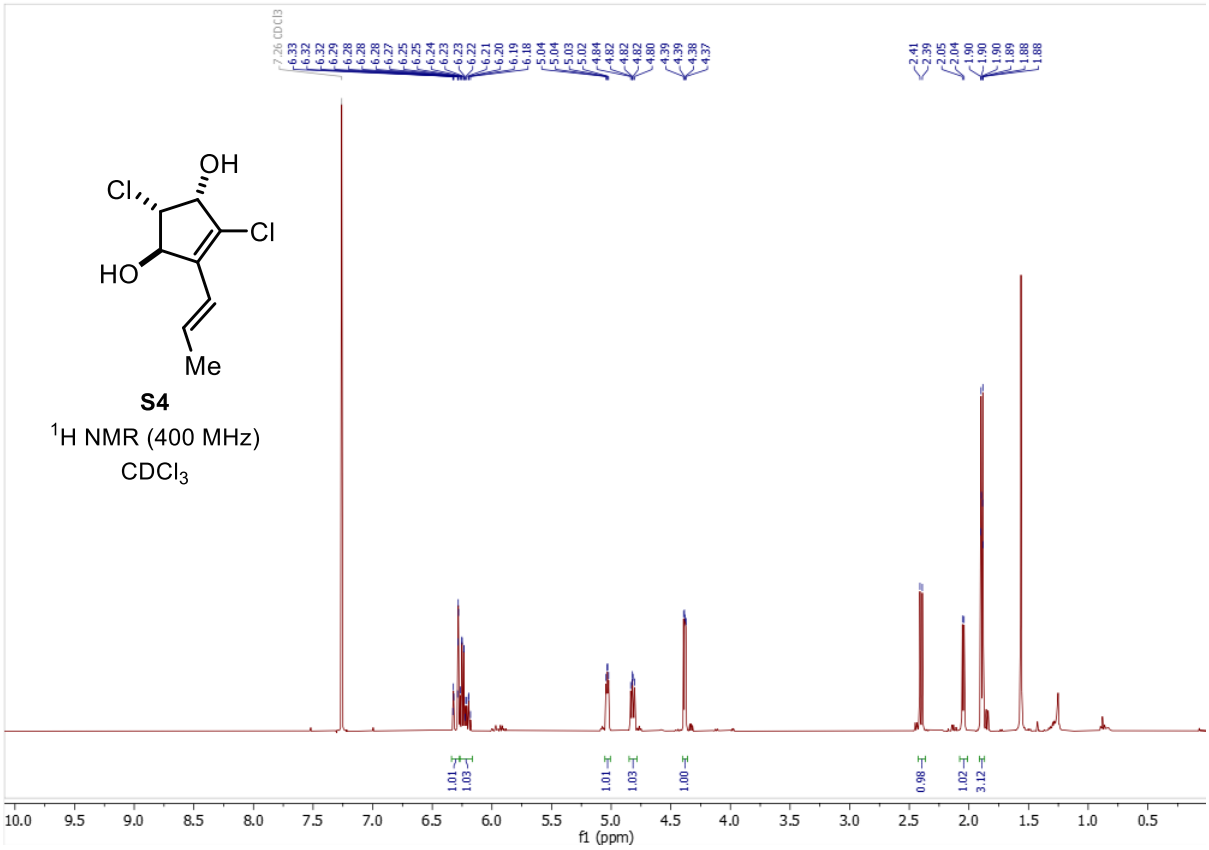
CD_3OD

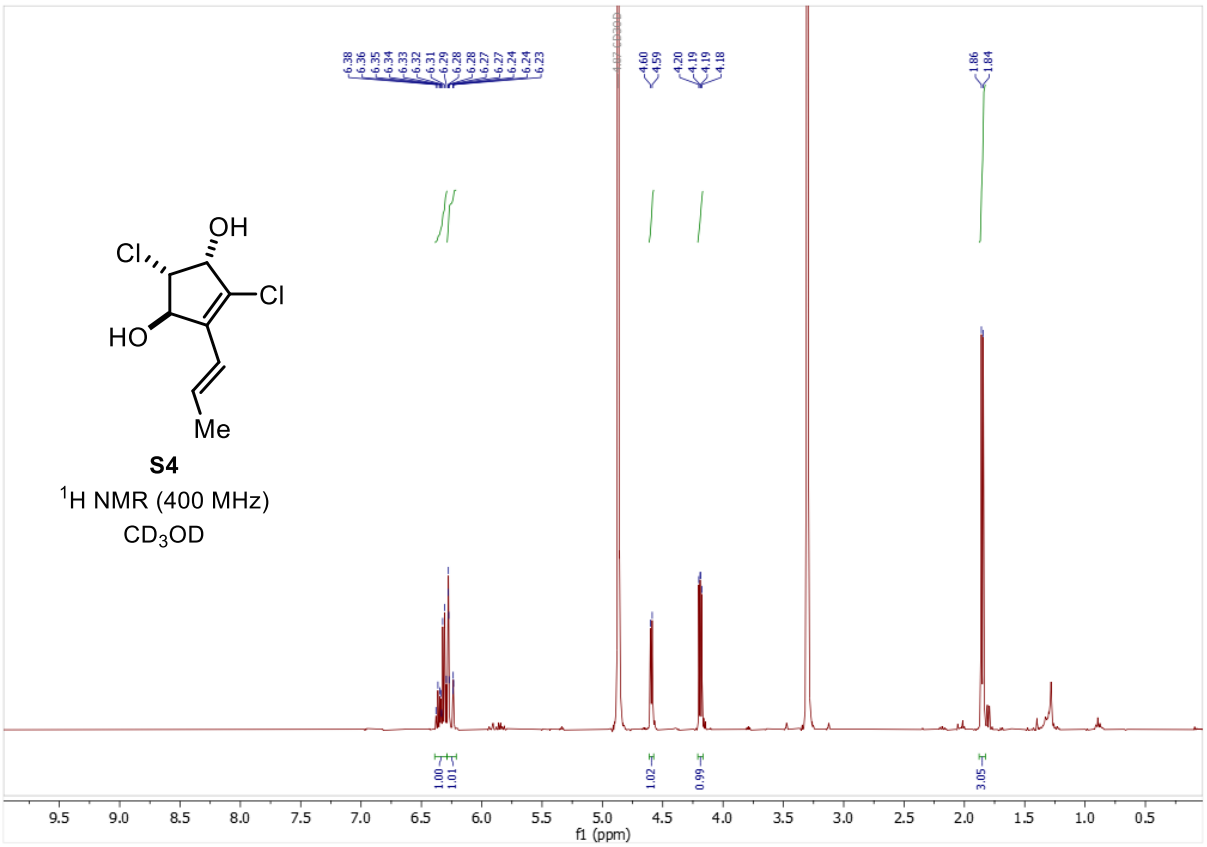


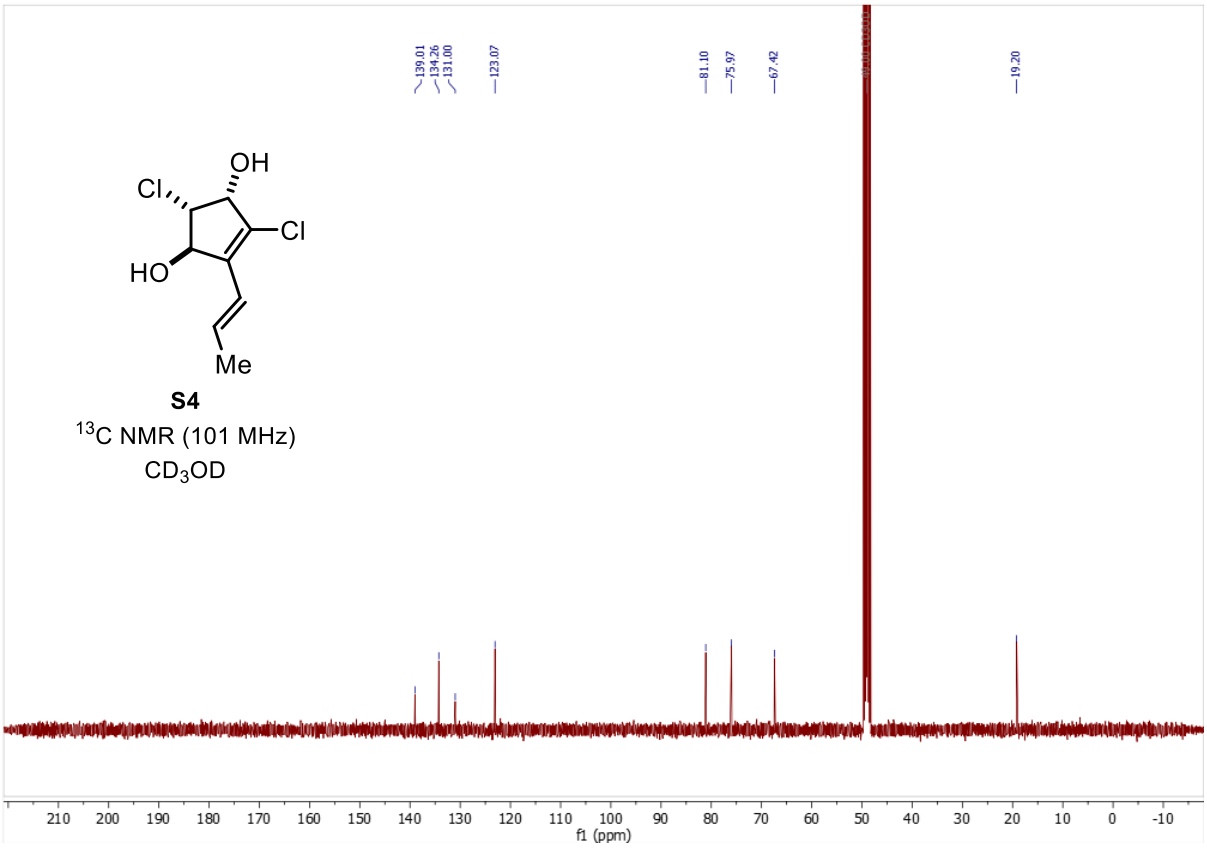
4

IR spectrum

Compound S4


OH
Cl
Cl
HO
Me
S4
1H NMR (400 MHz)
CDCl3
f1 (ppm)


OH
Cl
Cl
HO
Me
S4
1H NMR (400 MHz)
CD3OD
f1 (ppm)

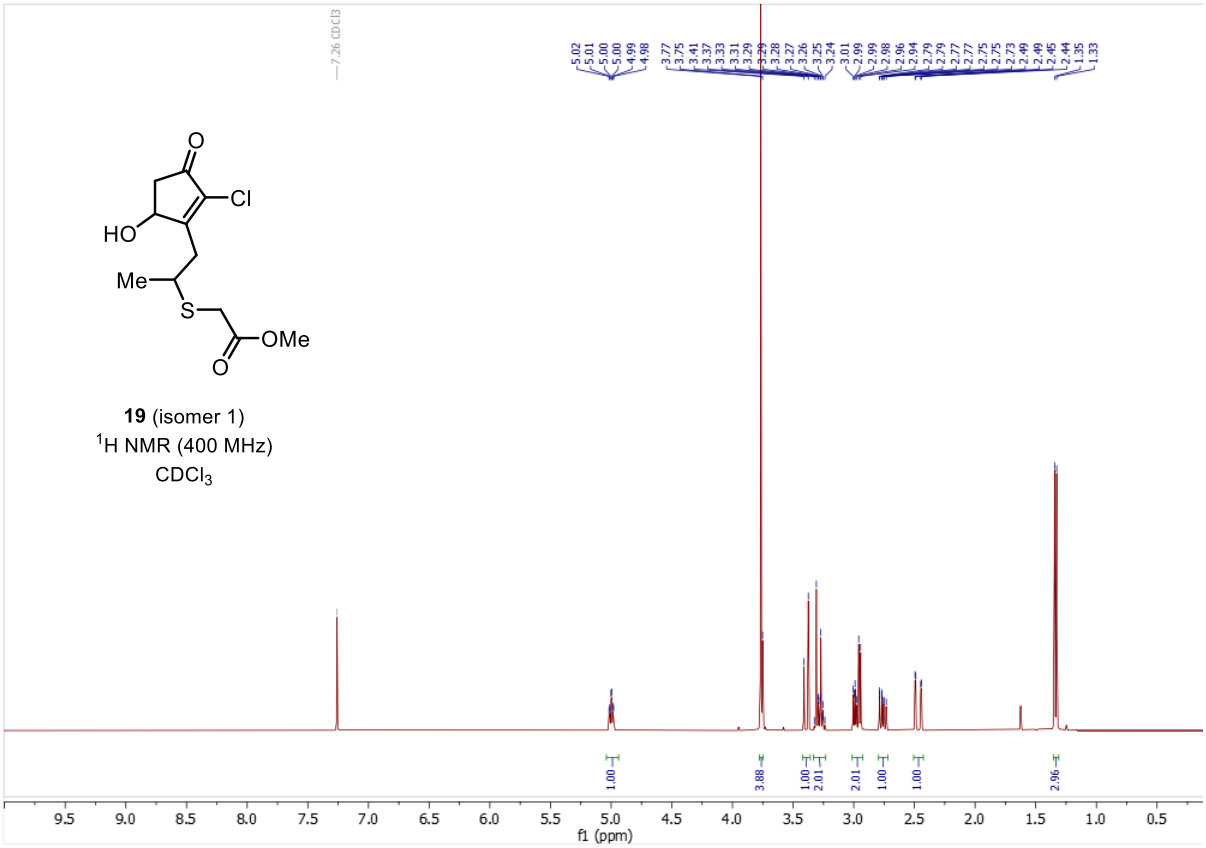


S4

^{13}C NMR (101 MHz)

CD_3OD

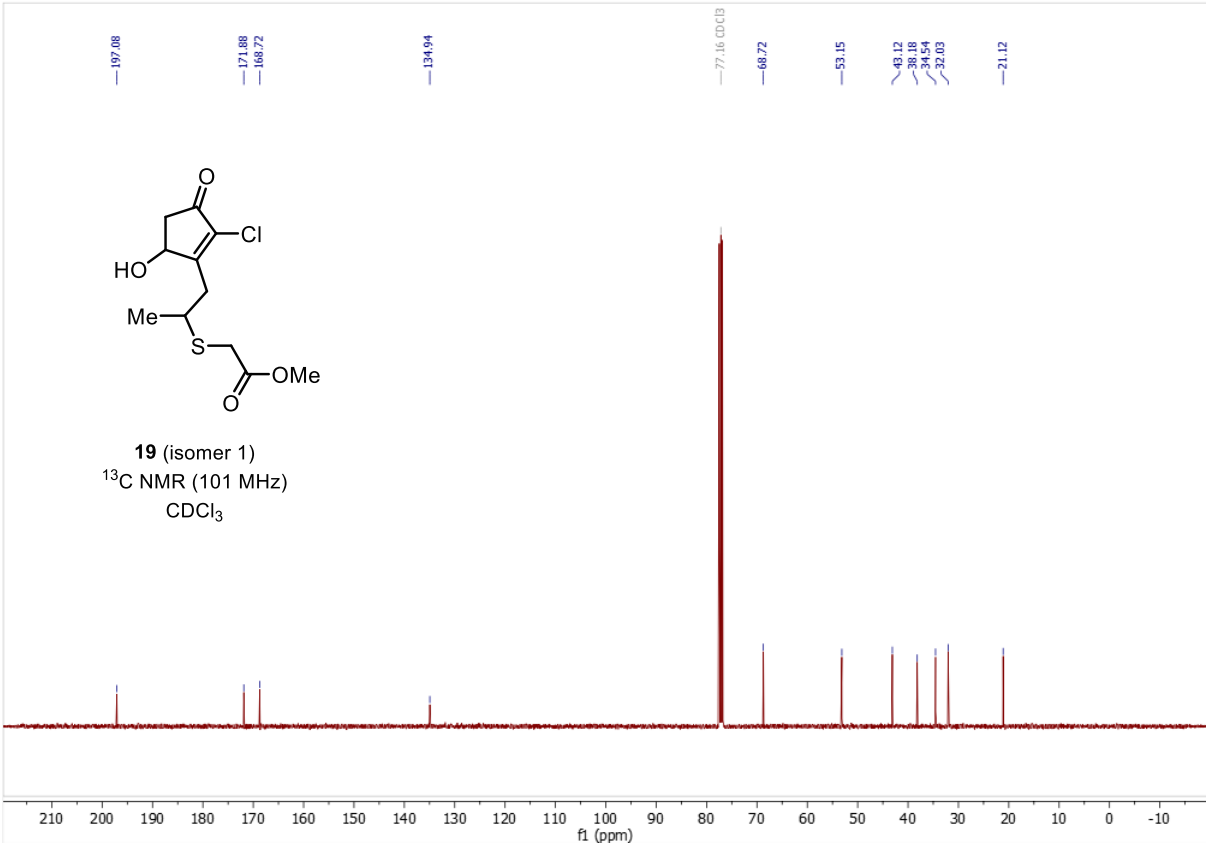
Compound 19 (Isomer 1)



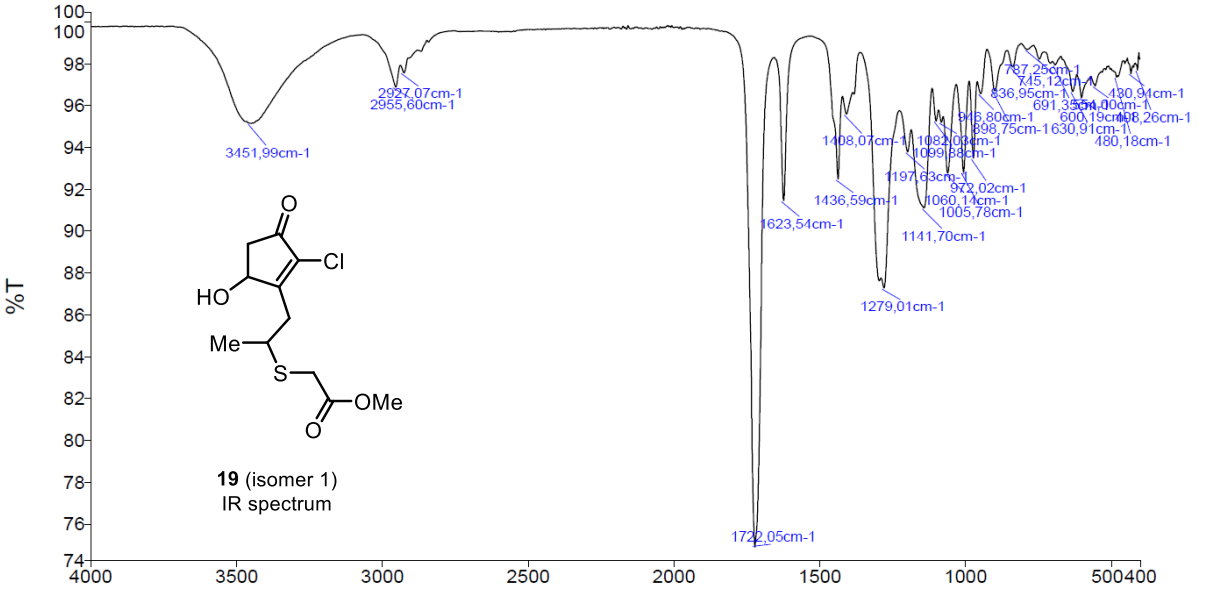
19 (isomer 1)

^{1}H NMR (400 MHz)

$CDCl_3$

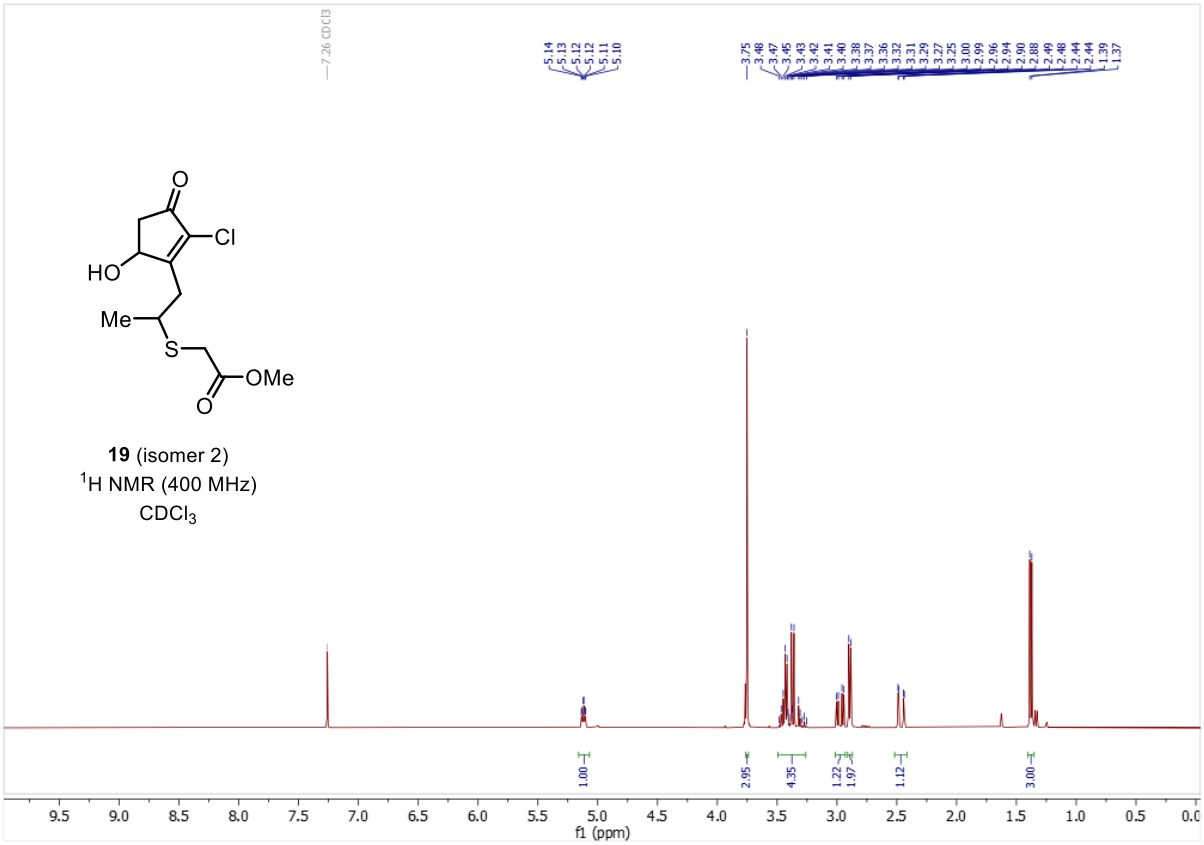


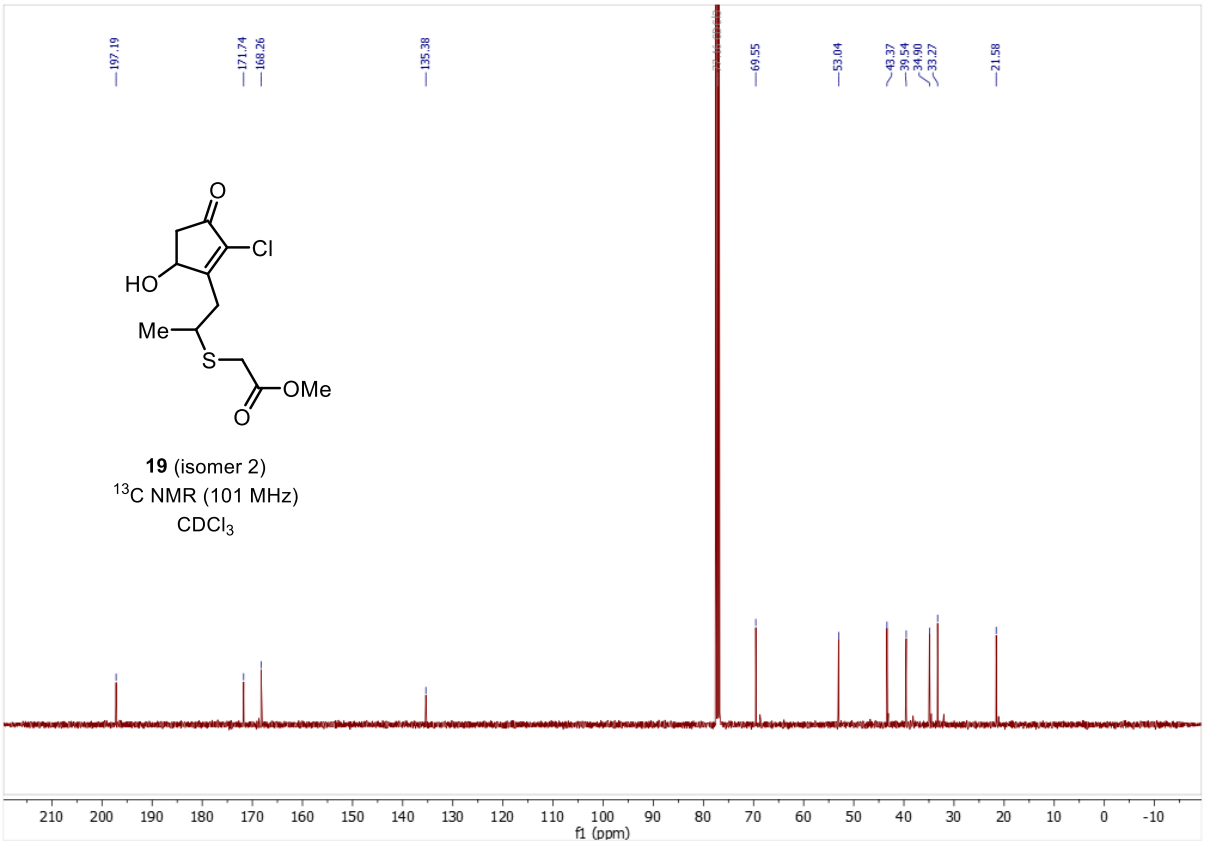
19 (isomer 1)
^{13}C NMR (101 MHz)
$CDCl_3$

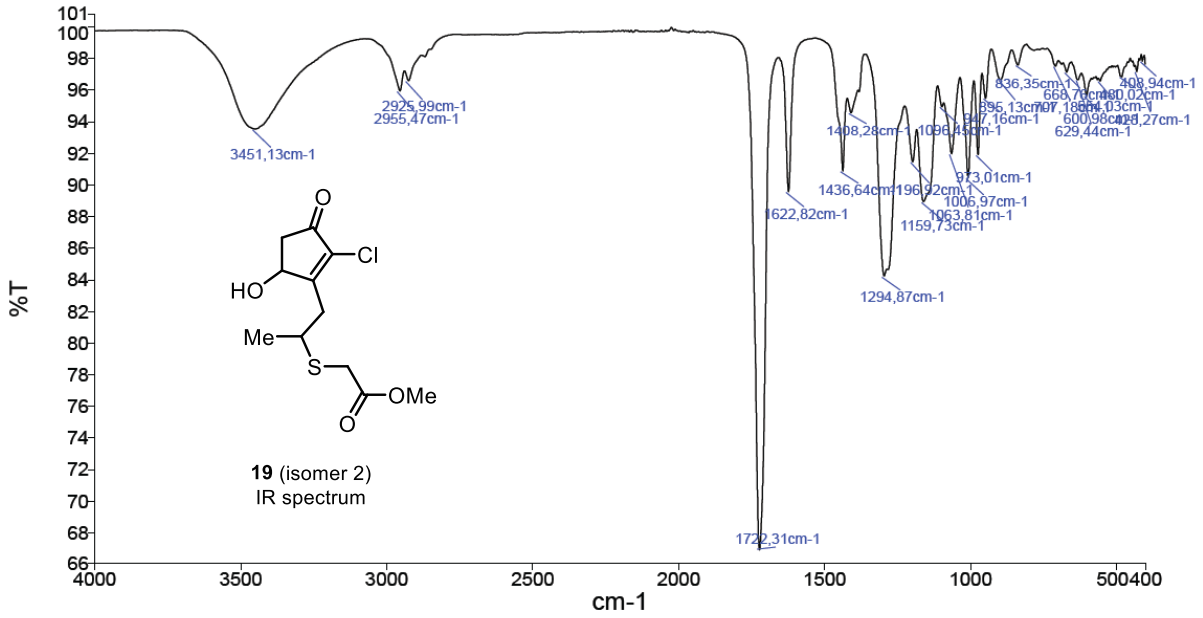


19 (isomer 1)
IR spectrum

Compound 19 (Isomer 2)

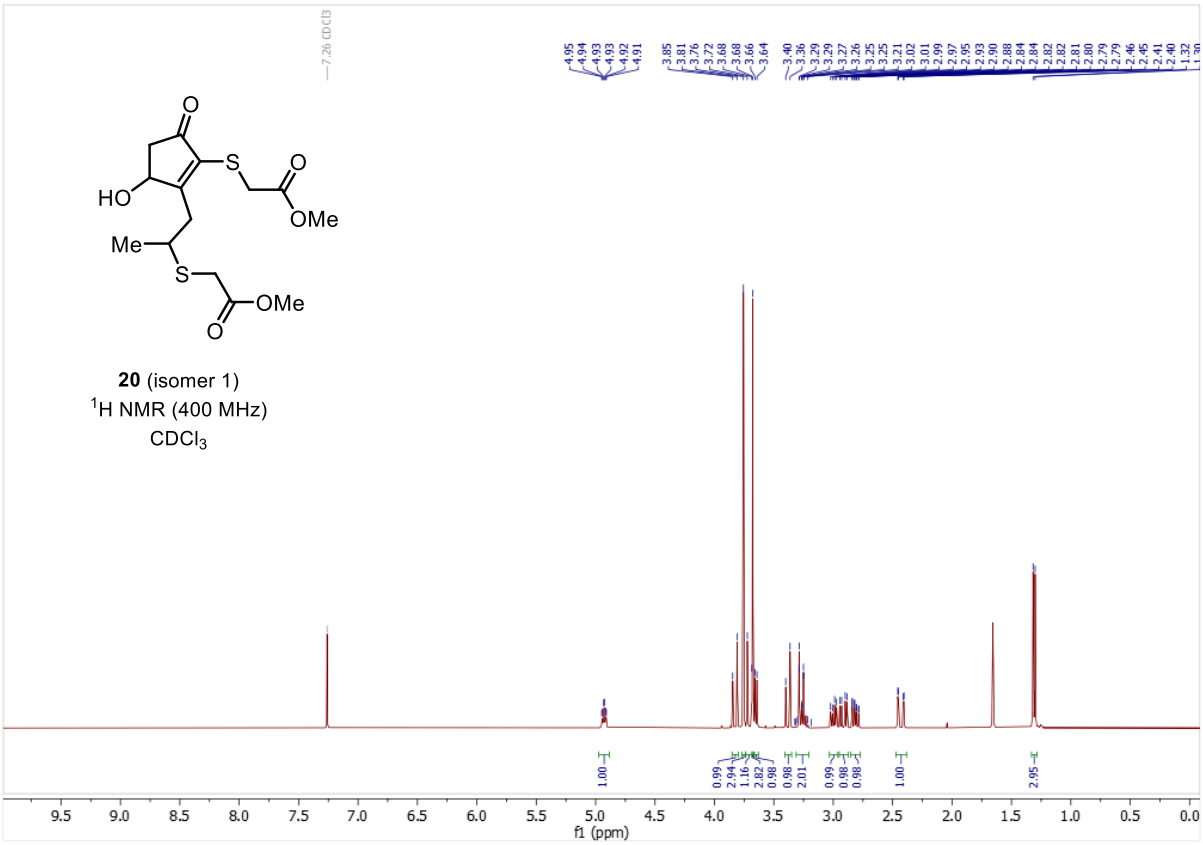




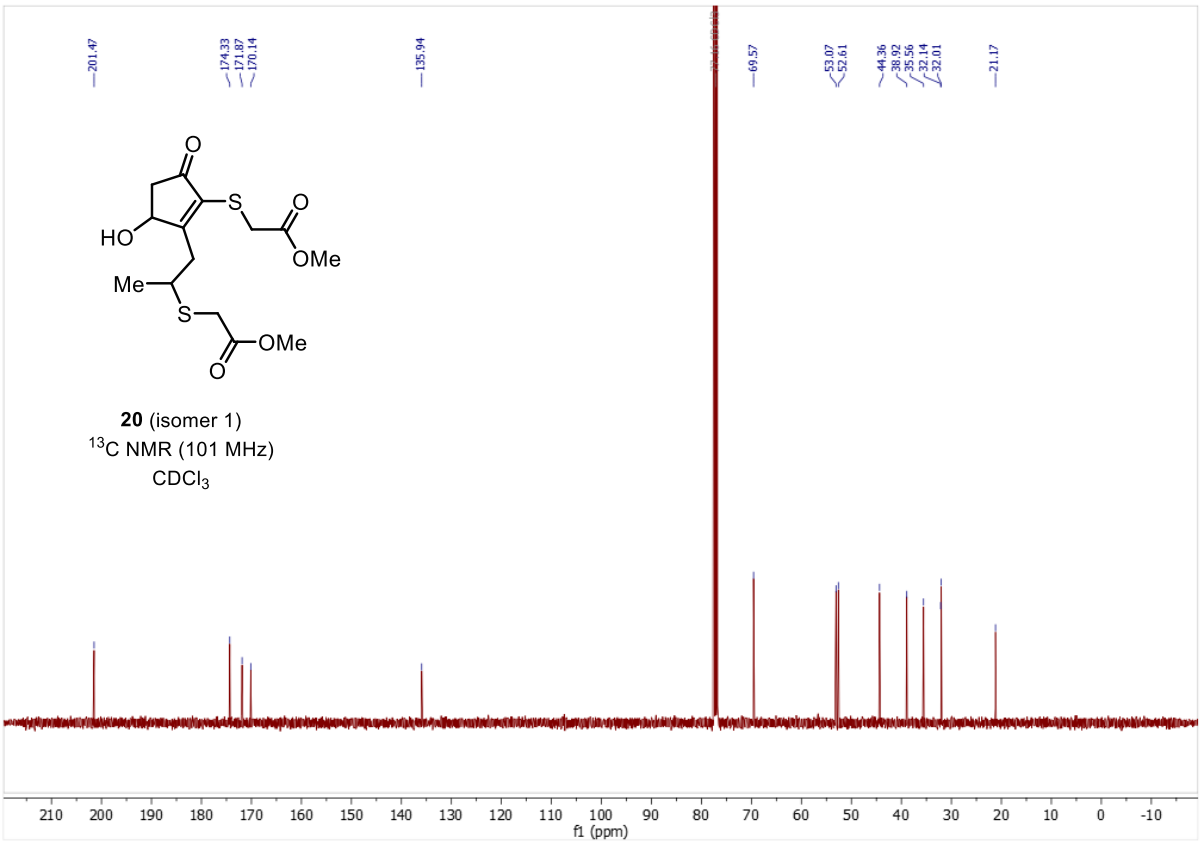


19 (isomer 2)
IR spectrum

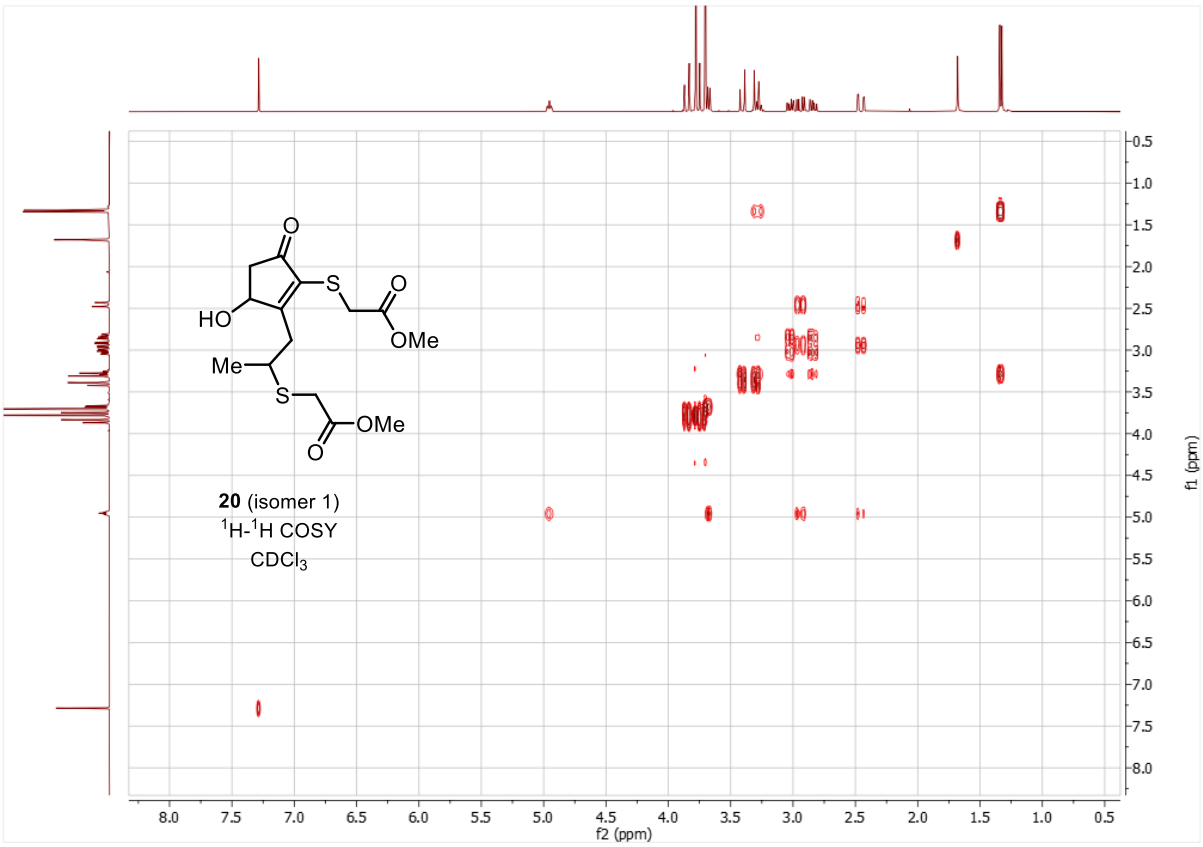
Compound 20 (Isomer 1)



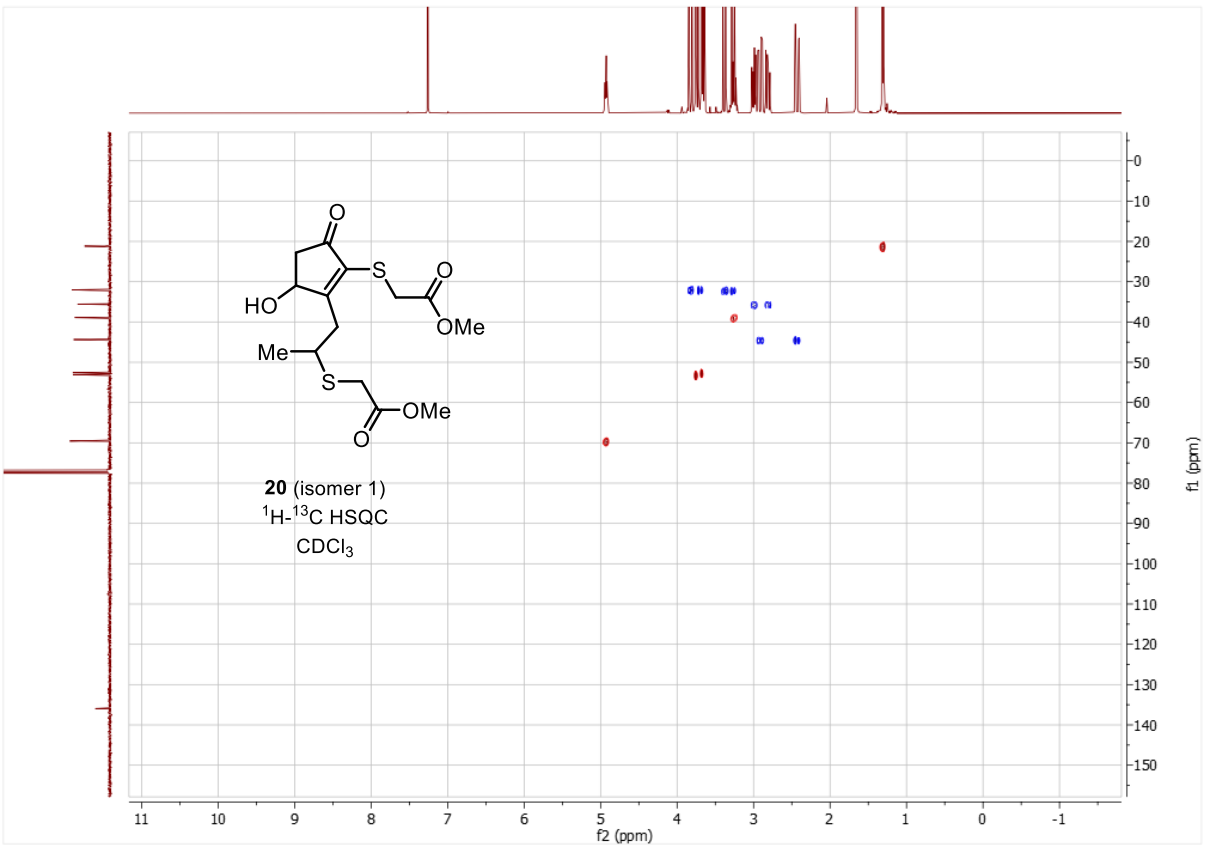
20 (isomer 1)
^{1}H NMR (400 MHz)
$CDCl_3$

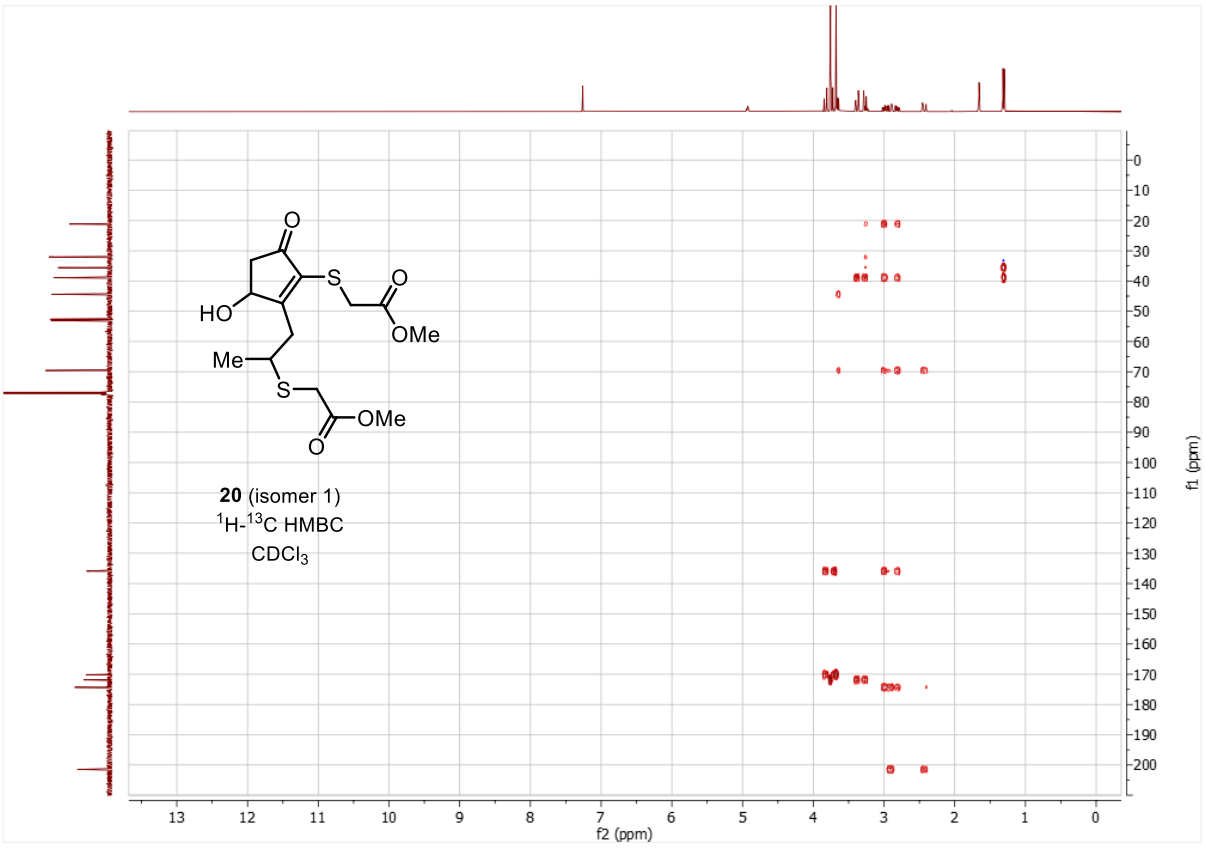


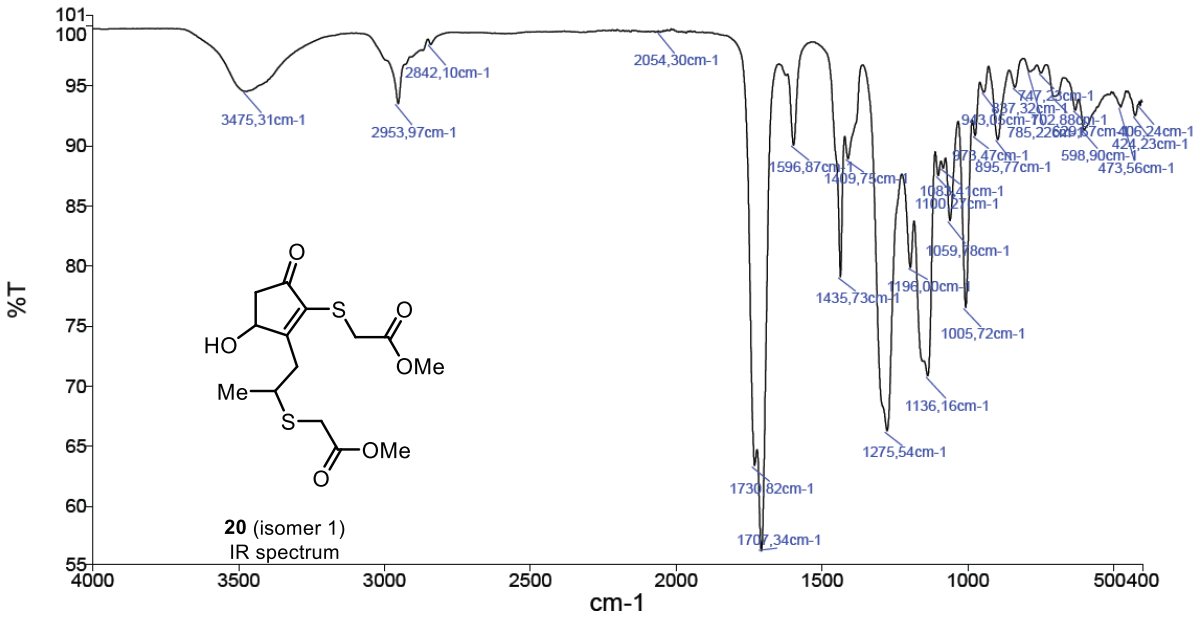
20 (isomer 1)
^{13}C NMR (101 MHz)
$CDCl_3$

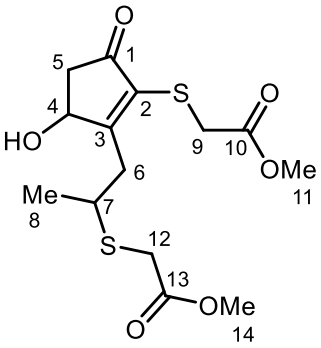


20 (isomer 1)
^{1}H-^{1}H COSY
$CDCl_3$


HO
O
S
O
OMe
Me
S
OMe
O
20 (isomer 1)
1H-13C HSQC
CDCl3
f2 (ppm)
f1 (ppm)


HO
O
S
O
OMe
Me
S
OMe
O
20 (isomer 1)
1H-13C HMBC
CDCl3
f2 (ppm)
f1 (ppm)



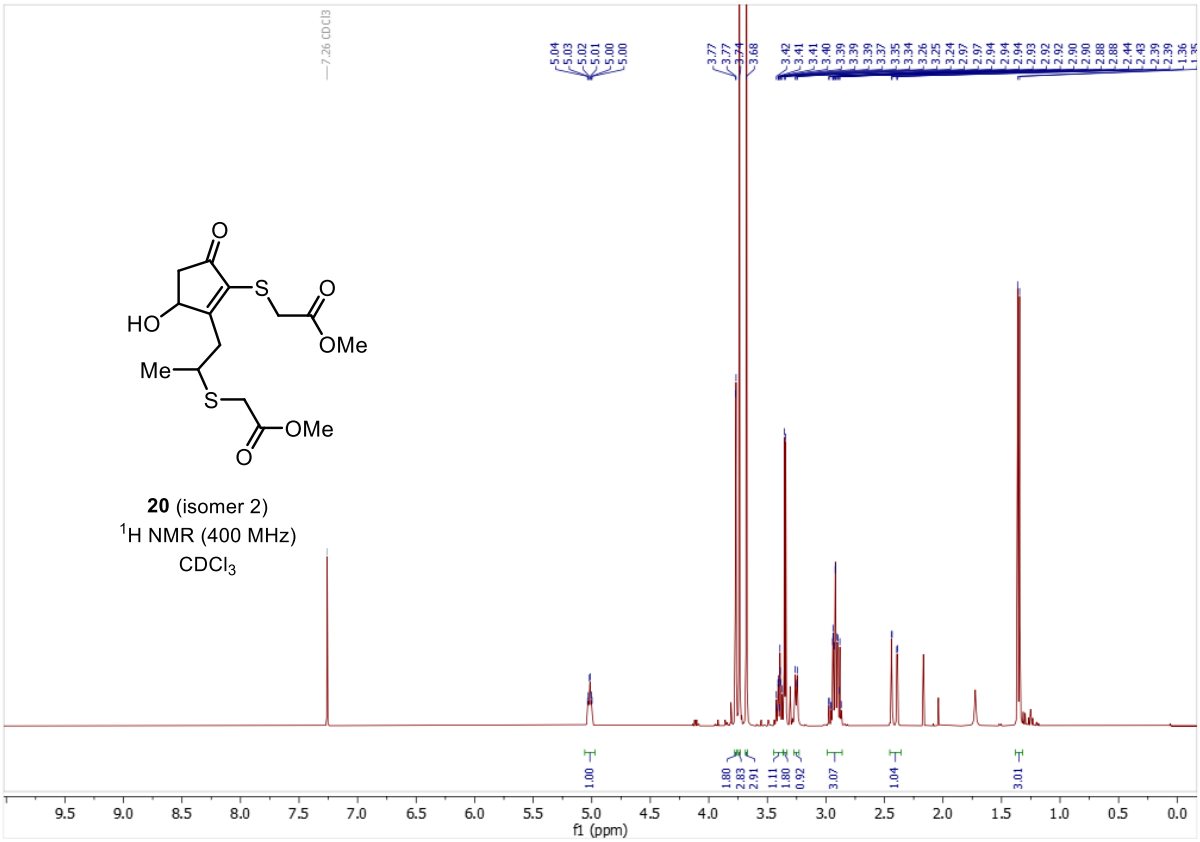


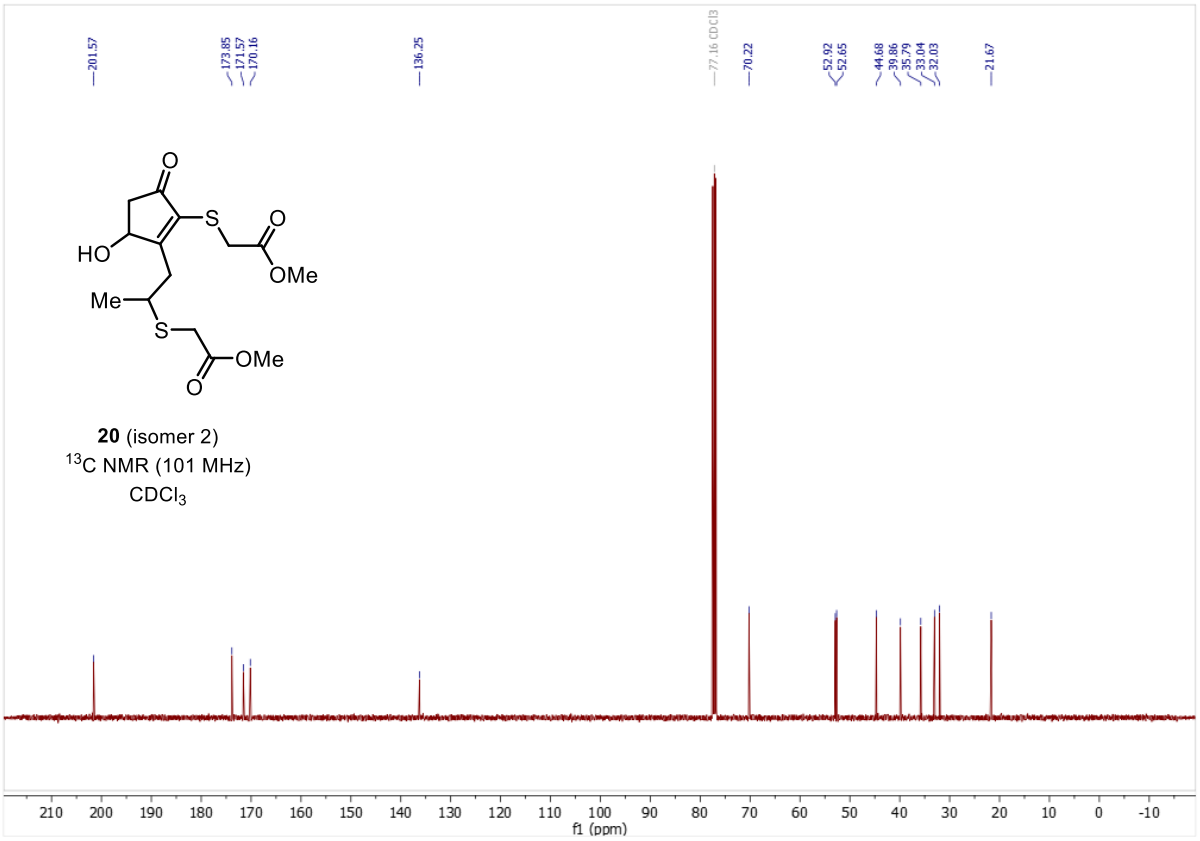
20 (isomer 1)

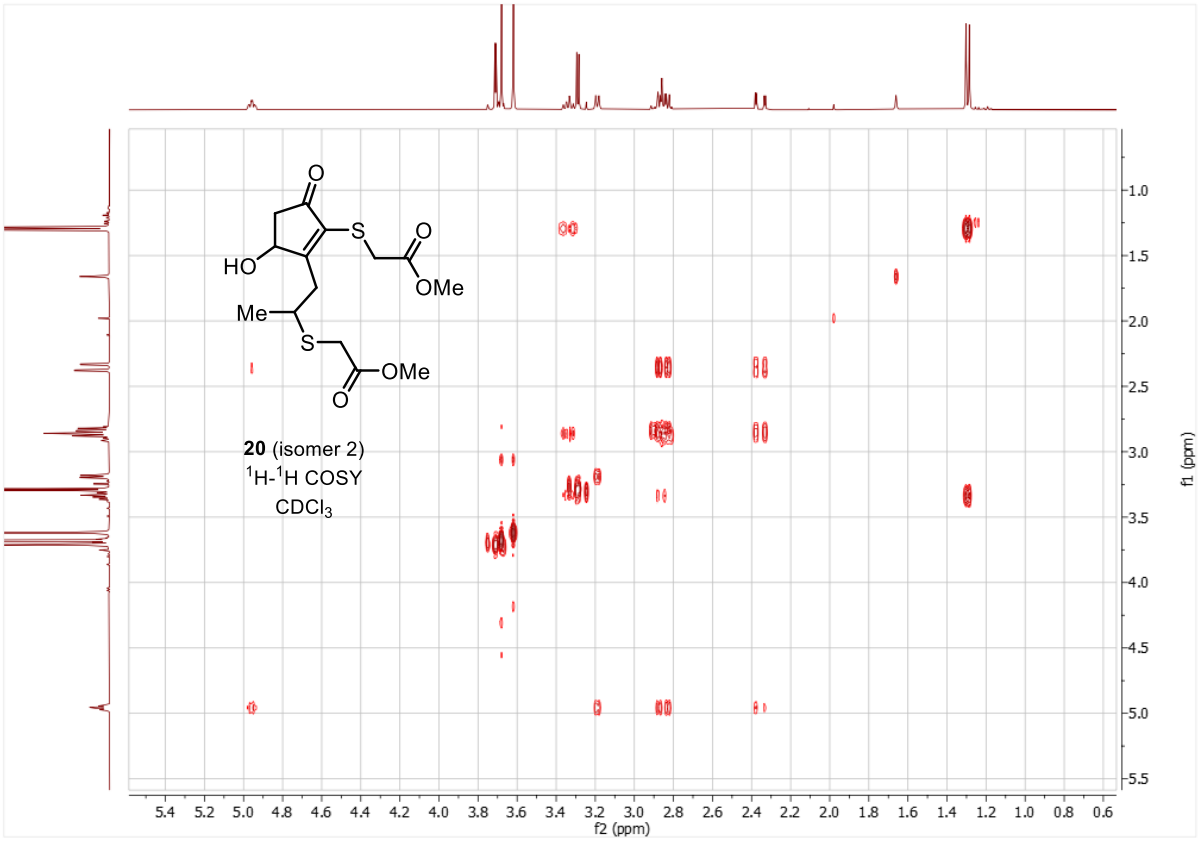
#	δ_H (ppm)	δ_C (ppm)
1	-	201.5
2	-	135.9
3	-	174.3
4	4.94	69.6
5	2.91 + 2.43	44.4
6	3.00 + 2.81	35.6
7	3.26	38.9
8	1.31	21.2
9	3.83 + 3.70	32.0
10	-	170.1
11	3.68	52.6
12	3.38 + 3.27	32.1
13	-	171.9
14	3.76	53.1

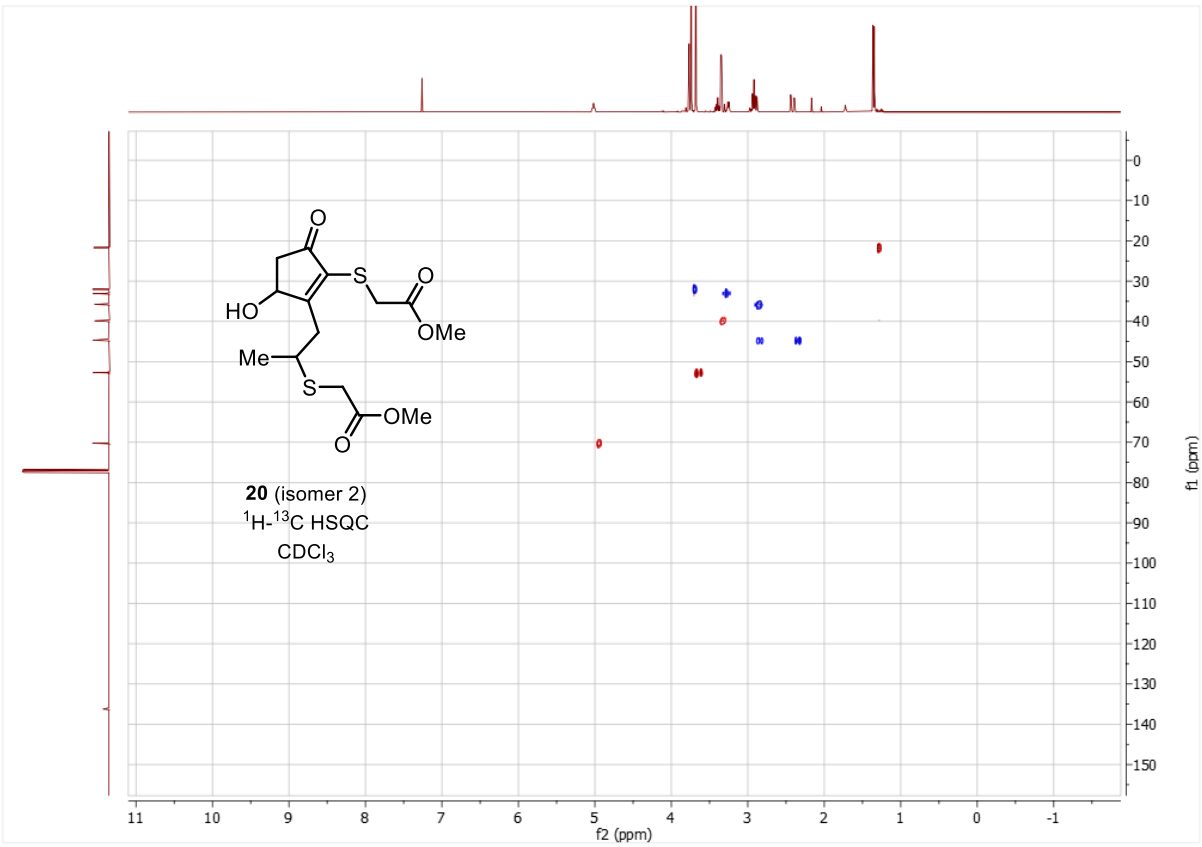
OH: 3.65 ppm

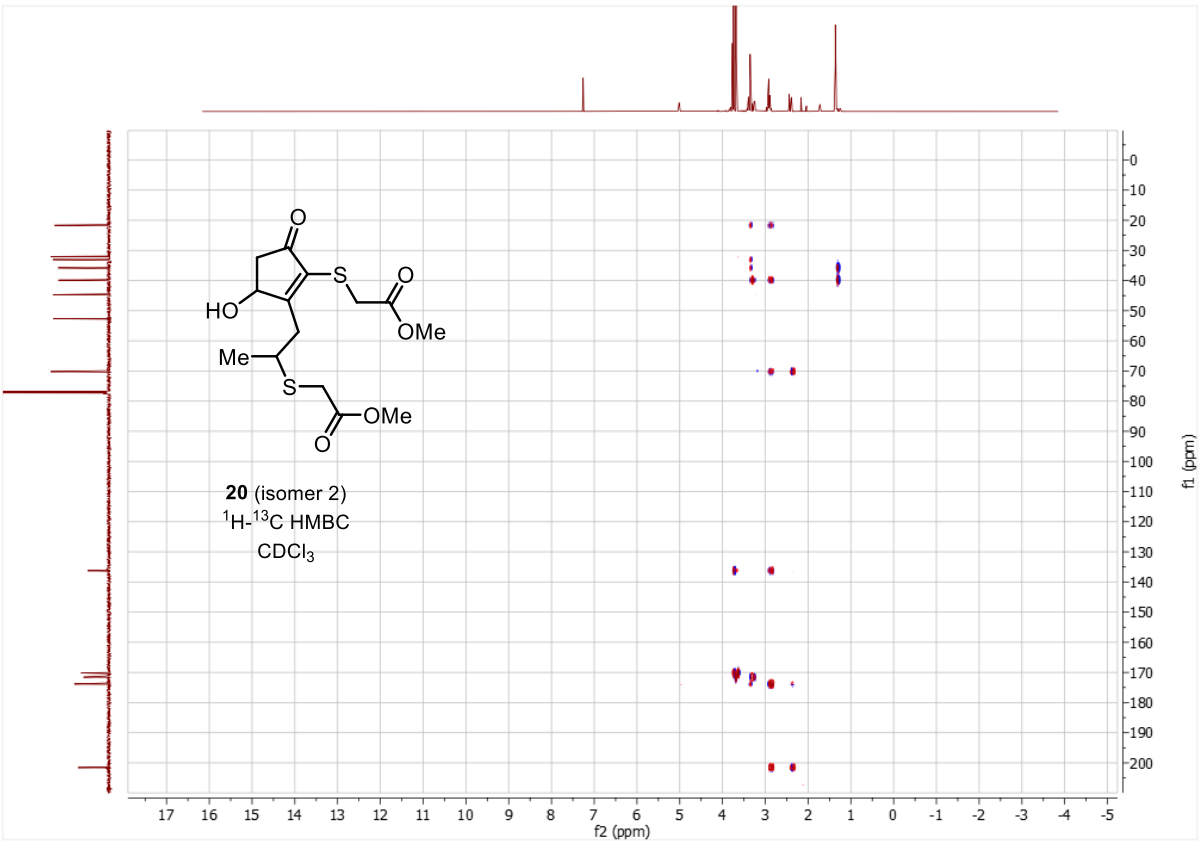
Compound 20 (Isomer 2)

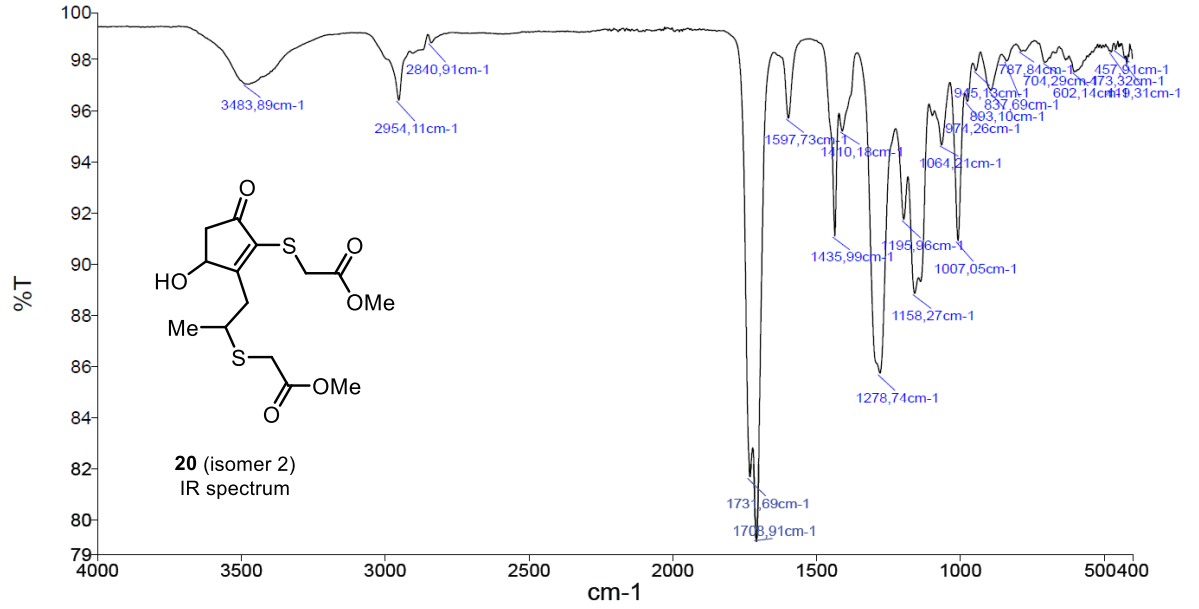




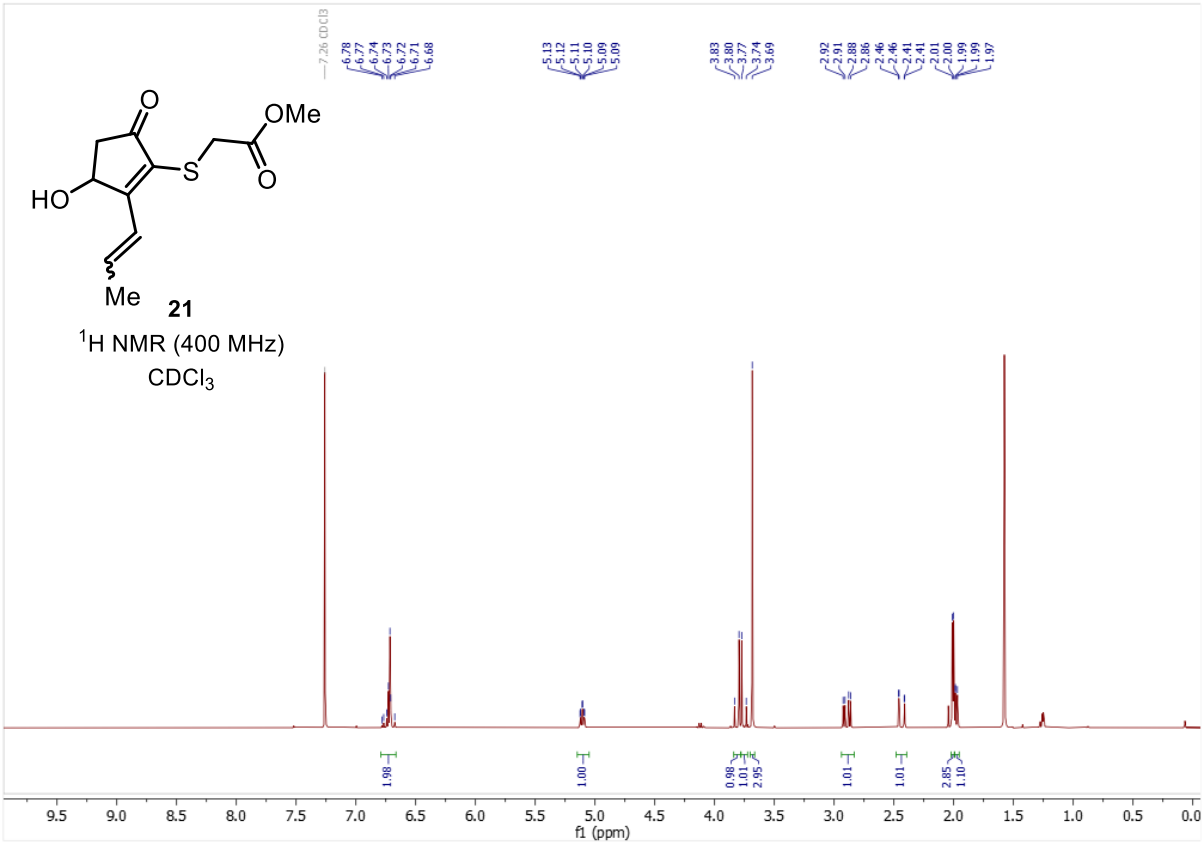

HO
O
S
O
OMe
Me
S
OMe
O
20 (isomer 2)
1H-1H COSY
CDCl3
f2 (ppm)
f1 (ppm)

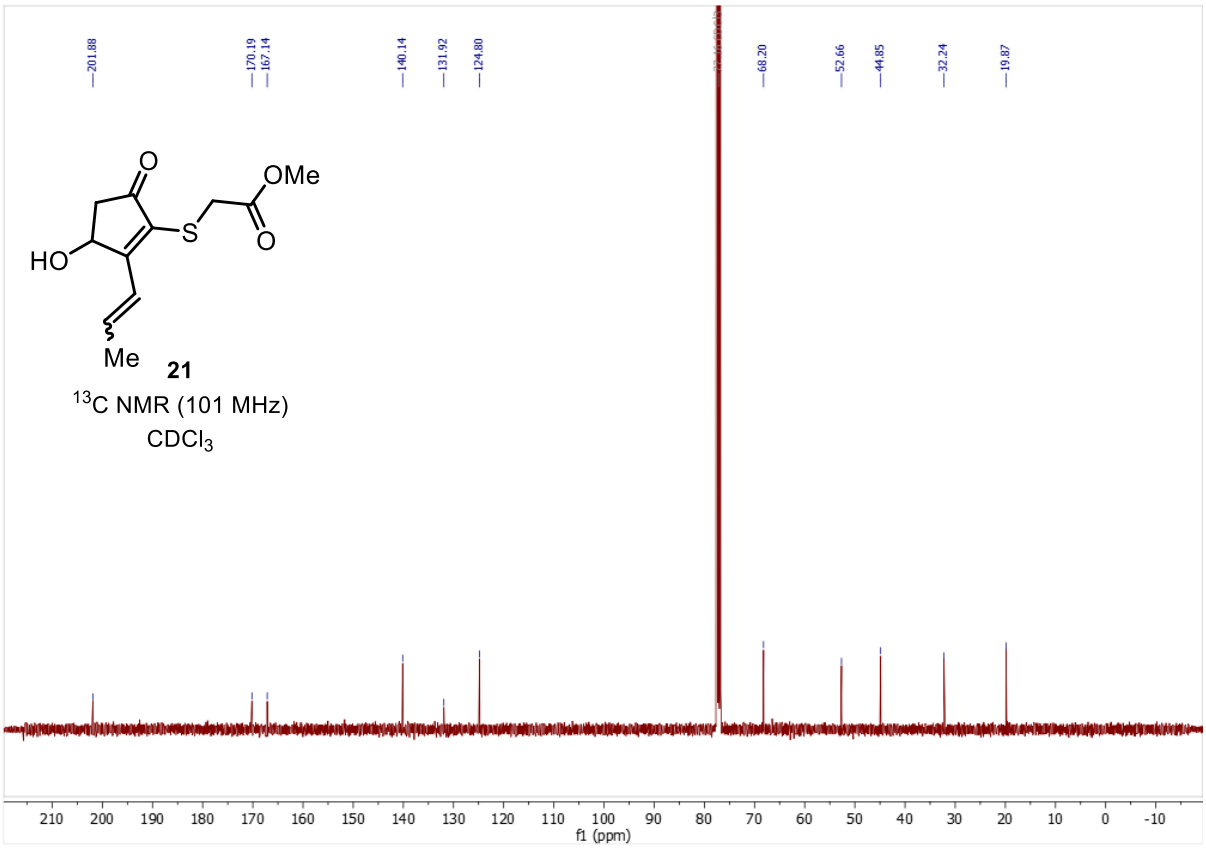

HO
O
S
O
OMe
Me
S
OMe
O
20 (isomer 2)
1H-13C HSQC
CDCl3
f2 (ppm)
f1 (ppm)

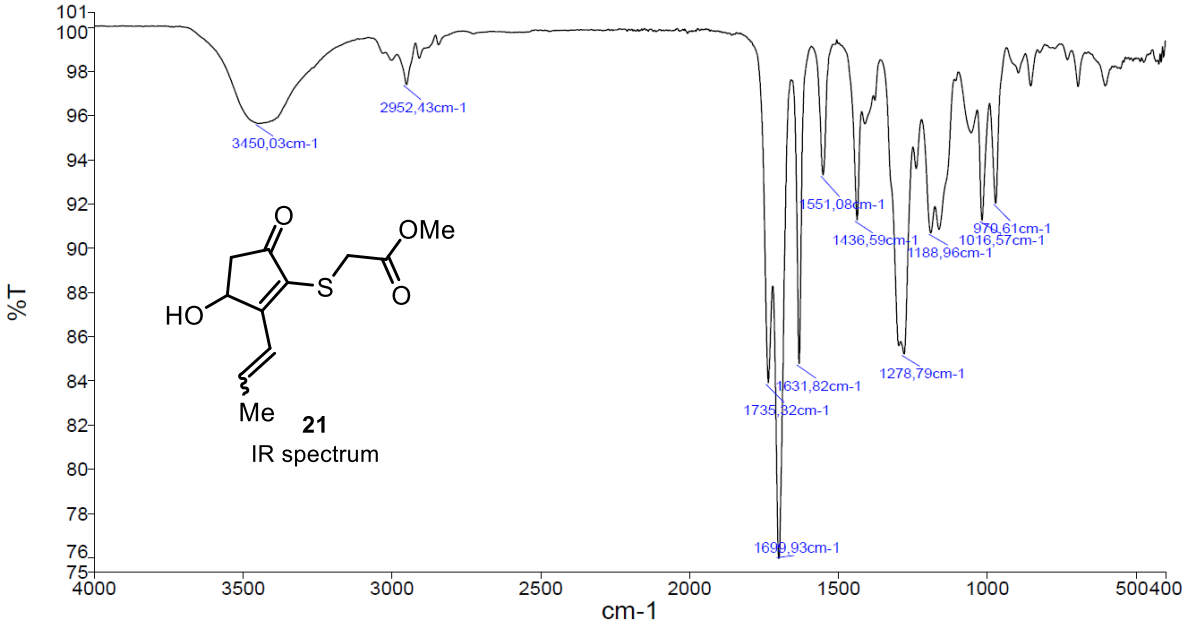

HO
O
S
O
OMe
Me
S
OMe
O
20 (isomer 2)
1H-13C HMBC
CDCl3
f2 (ppm)
f1 (ppm)


3483,89cm-1
2840,91cm-1
2954,11cm-1
1597,73cm-1
1435,99cm-1
1195,96cm-1
1158,27cm-1
1278,74cm-1
1064,21cm-1
1007,05cm-1
974,26cm-1
893,10cm-1
837,69cm-1
787,84cm-1
704,29cm-1
602,14cm-1
HO
O
S
O
OMe
Me
S
OMe
O
20 (isomer 2)
IR spectrum
%T
cm-1

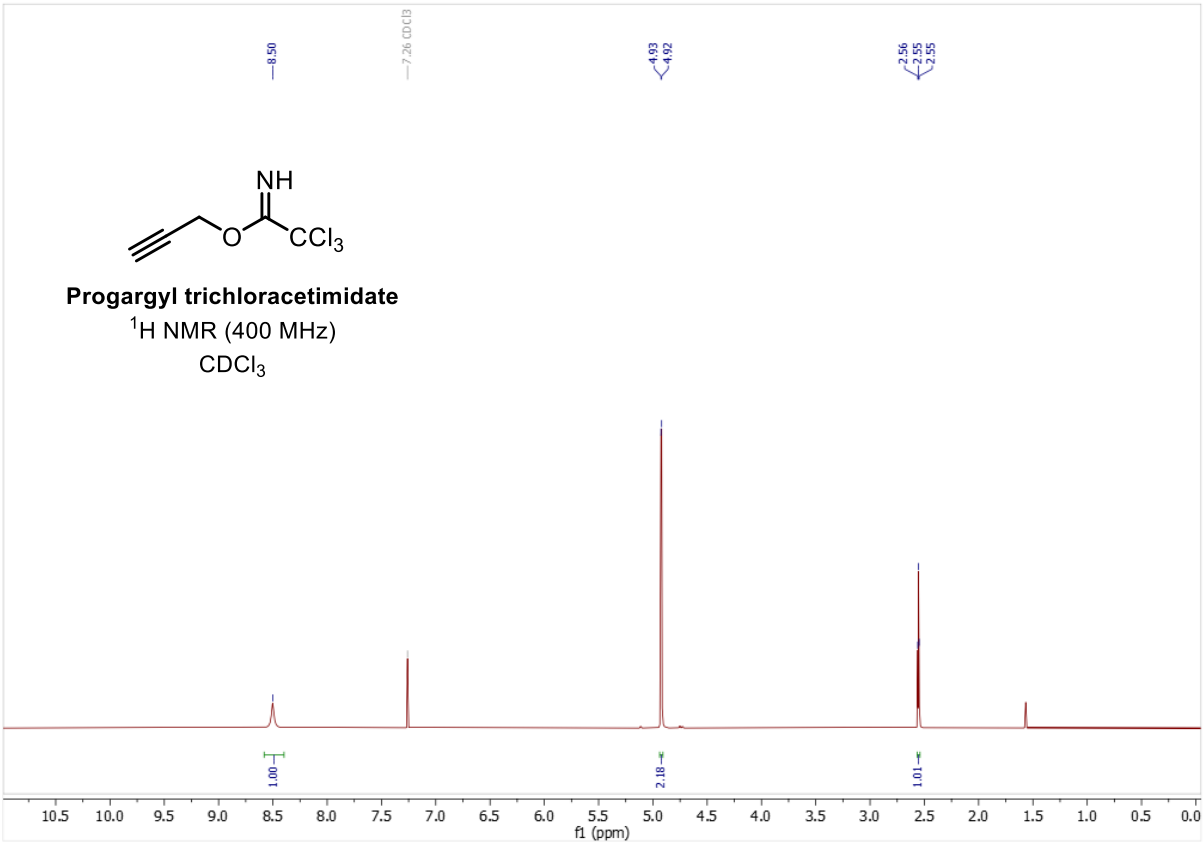
Compound 21

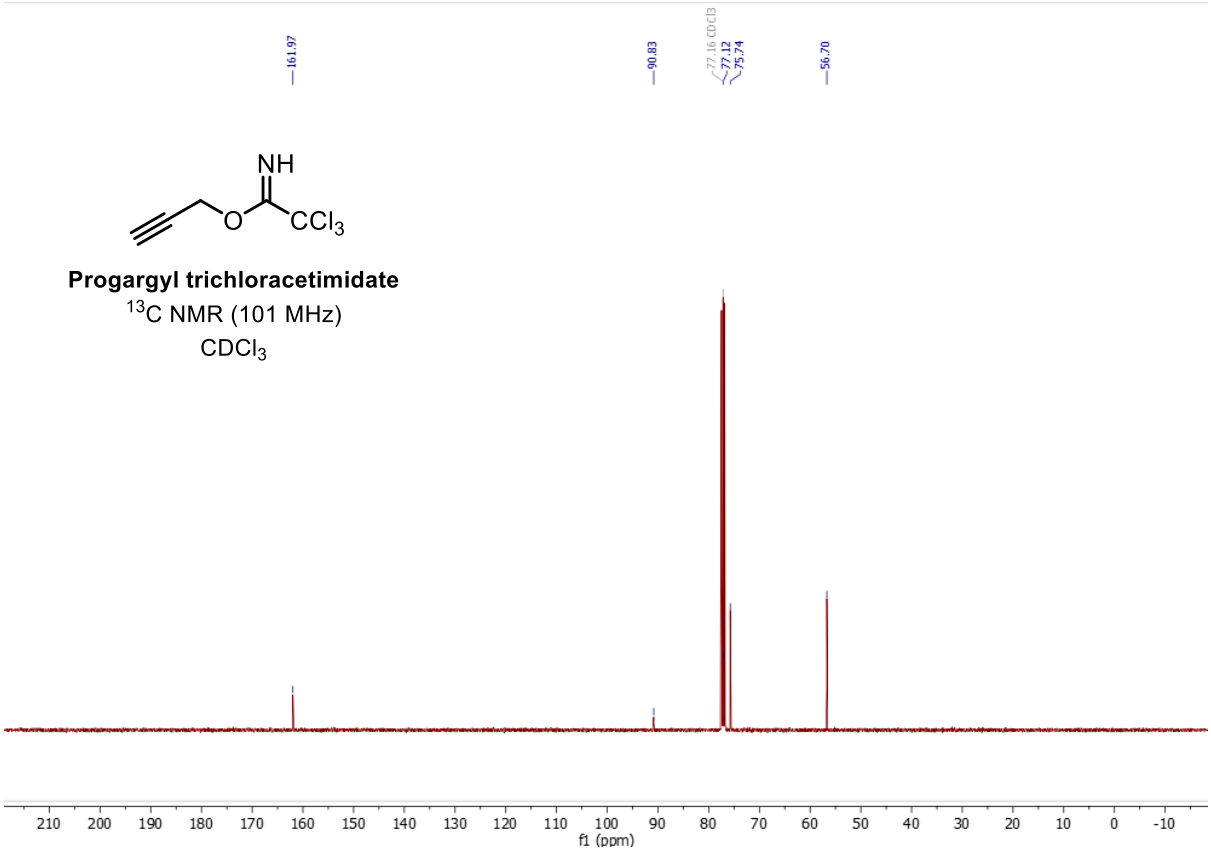






Propargyl trichloroacetimidate



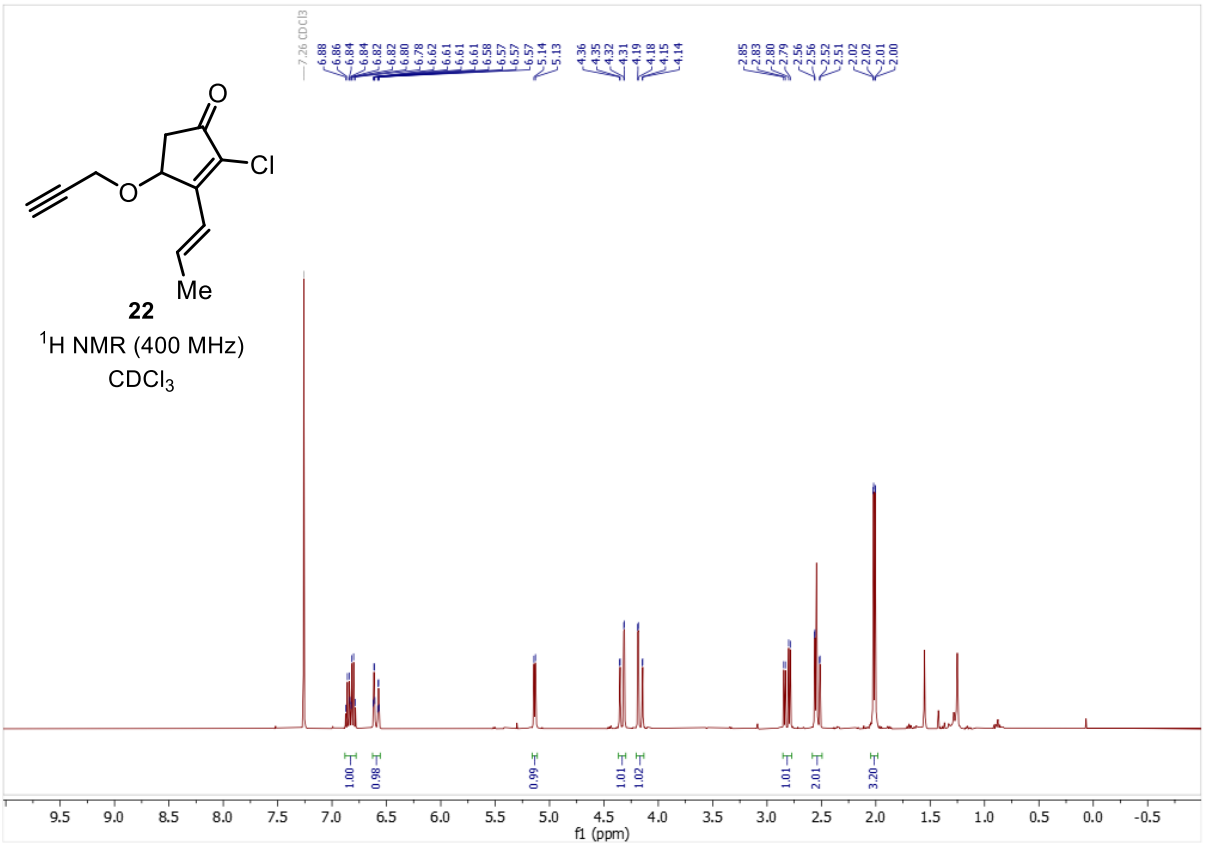


Progargyl trichloracetimidate

^{13}C NMR (101 MHz)

$CDCl_3$

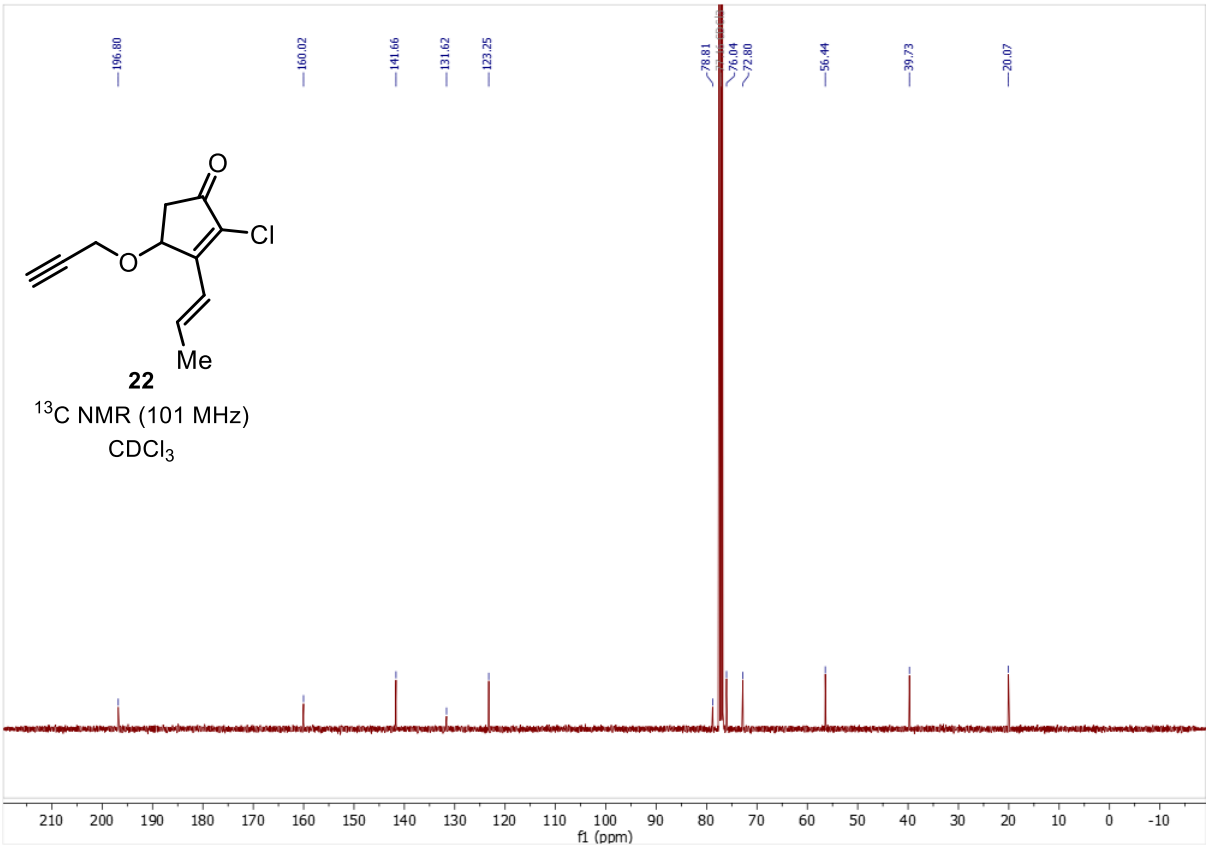
Compound 22

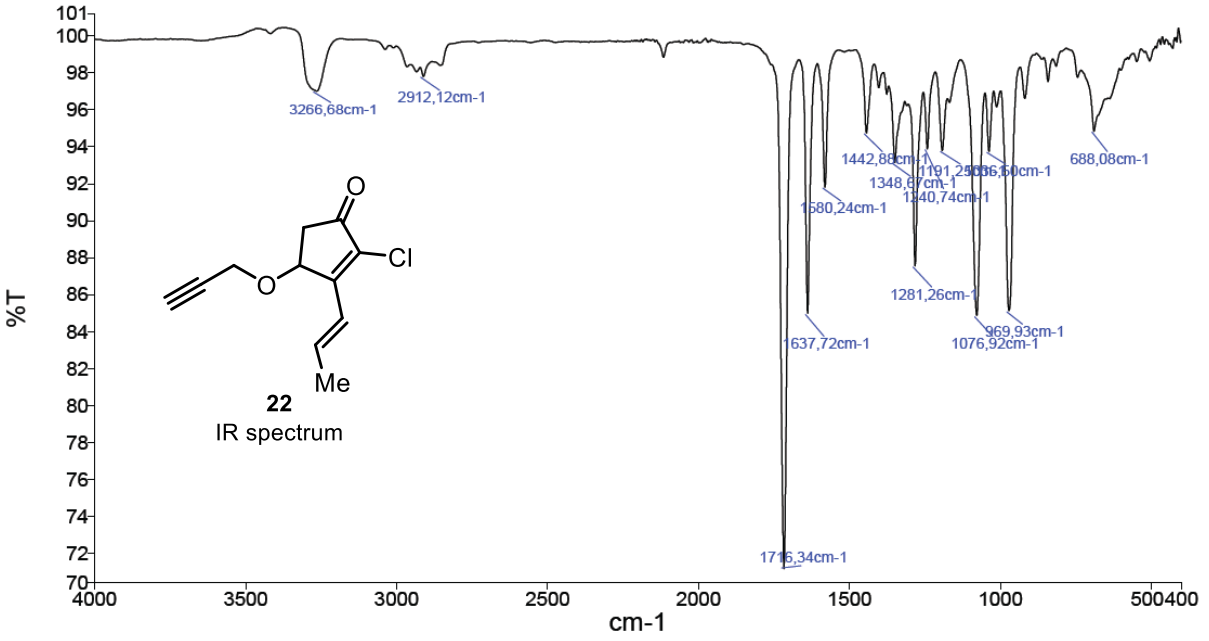


22

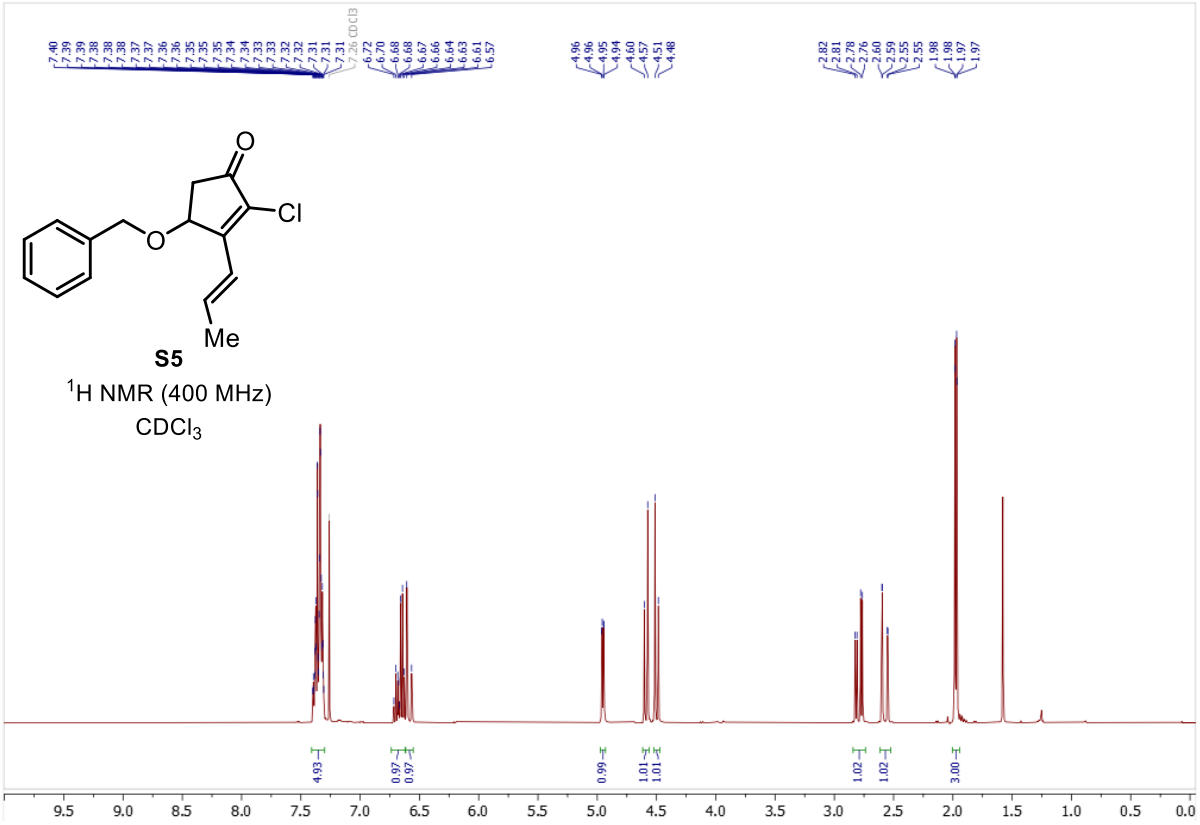
^{1}H NMR (400 MHz)

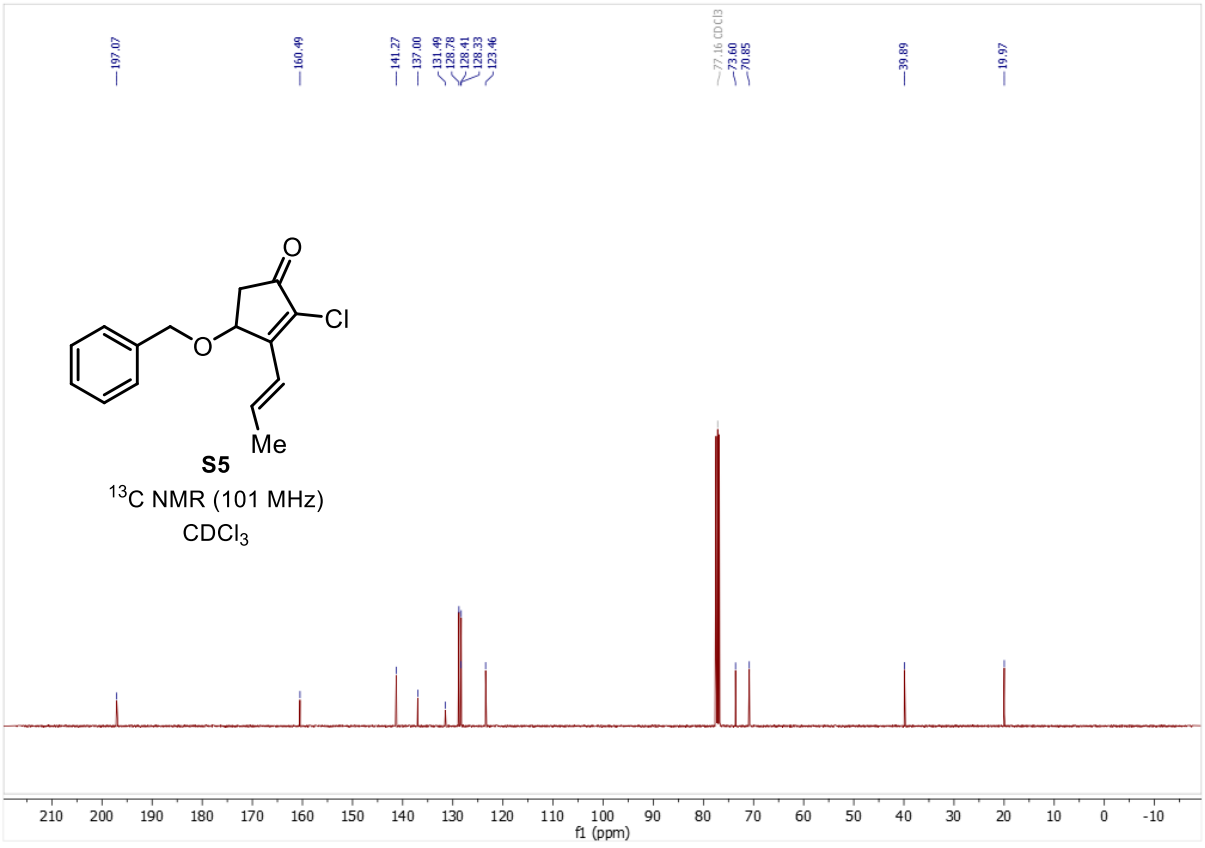
$CDCl_3$

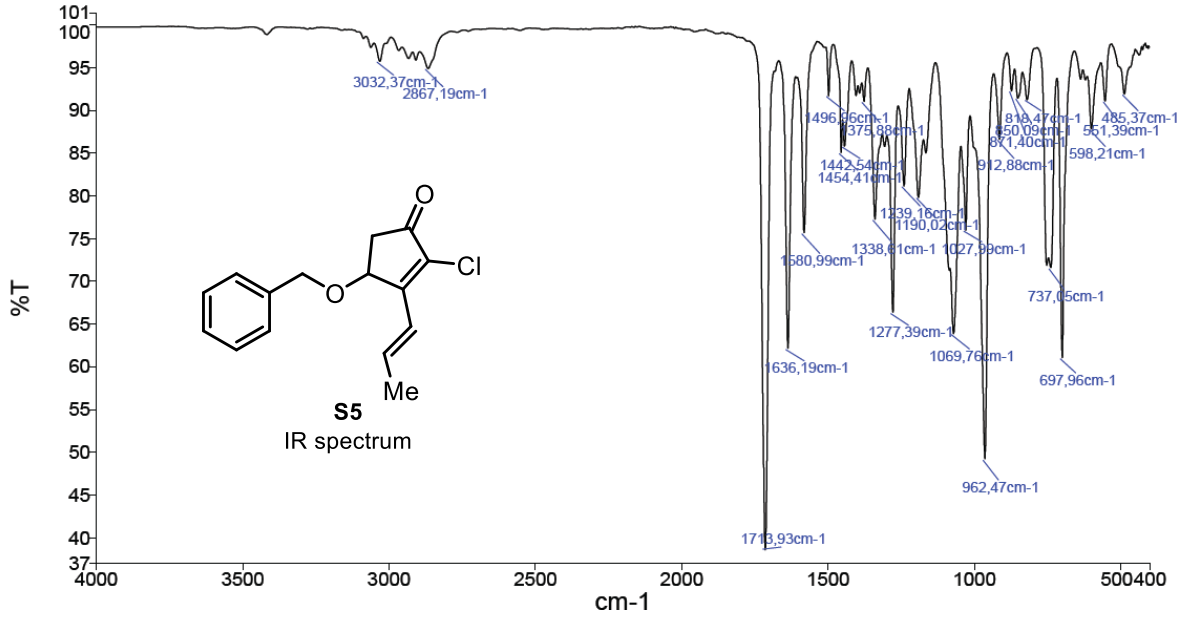

196.80
160.02
141.66
131.62
123.25
79.81
76.04
72.80
56.44
39.73
20.07
O
Cl
O
Me
22
13C NMR (101 MHz)
CDCl3
f1 (ppm)


%T
cm-1
3266,68cm-1
2912,12cm-1
1716,34cm-1
1637,72cm-1
1580,24cm-1
1442,88cm-1
1348,67cm-1
1340,74cm-1
1281,26cm-1
1191,25cm-1
1076,92cm-1
969,93cm-1
688,08cm-1
O
Cl
O
Me
22
IR spectrum

Compound S5


O
Cl
O
Me
S5
1H NMR (400 MHz)
CDCl3

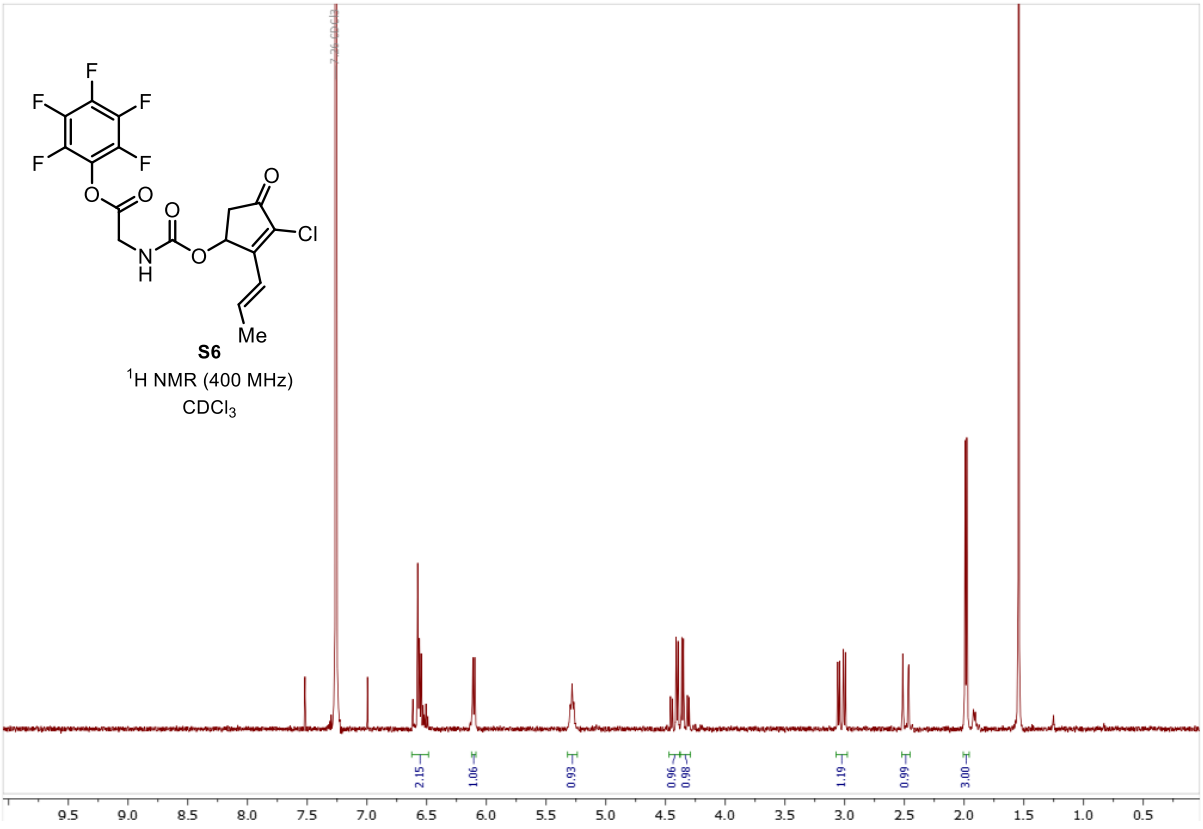

O
Cl
O
Me
S5
13C NMR (101 MHz)
CDCl3
f1 (ppm)



S5

IR spectrum

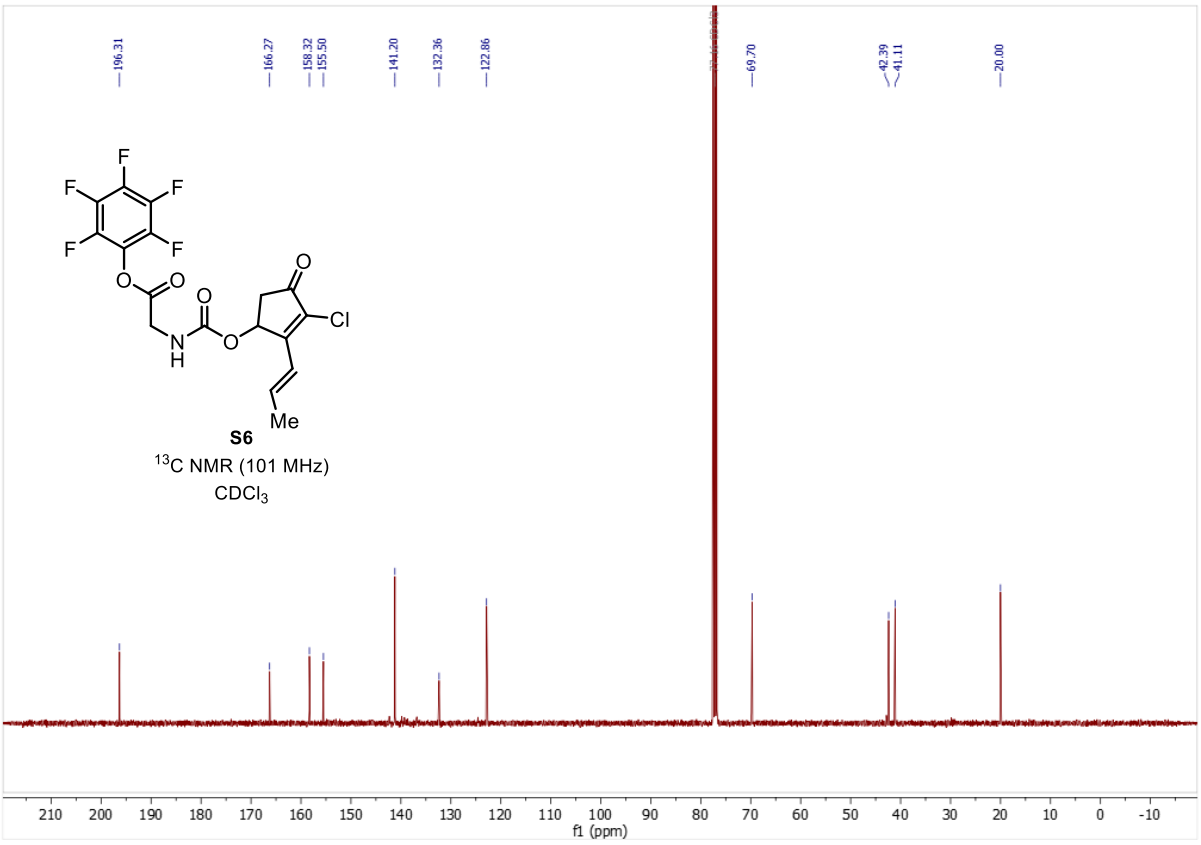
Compound S6



S6

^{1}H NMR (400 MHz)

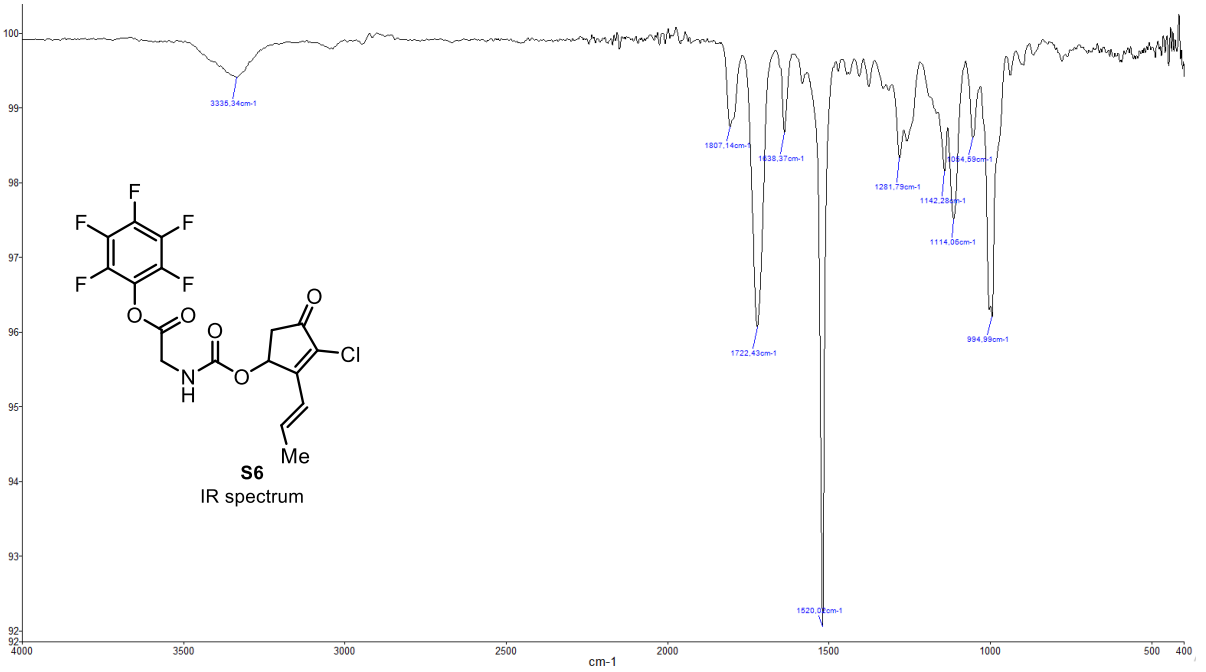
$CDCl_3$



S6

^{13}C NMR (101 MHz)

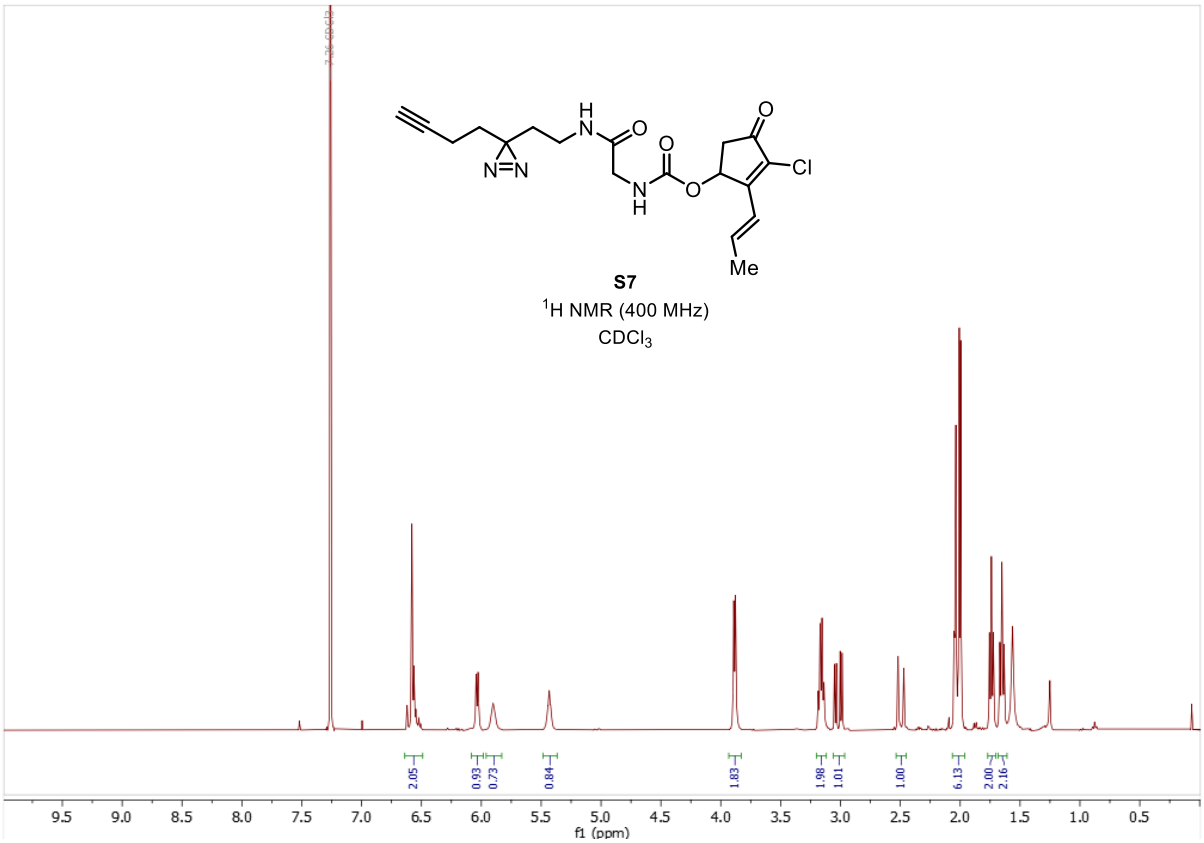
$CDCl_3$

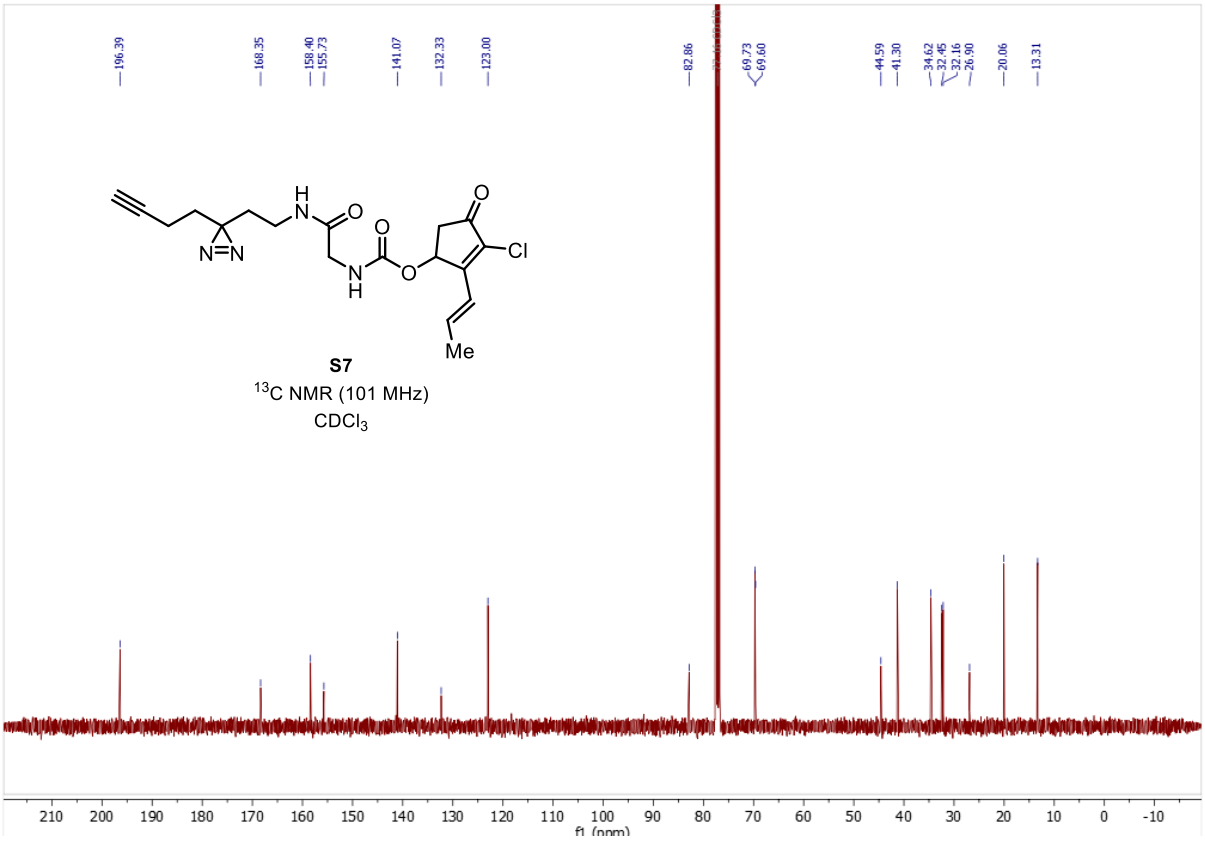


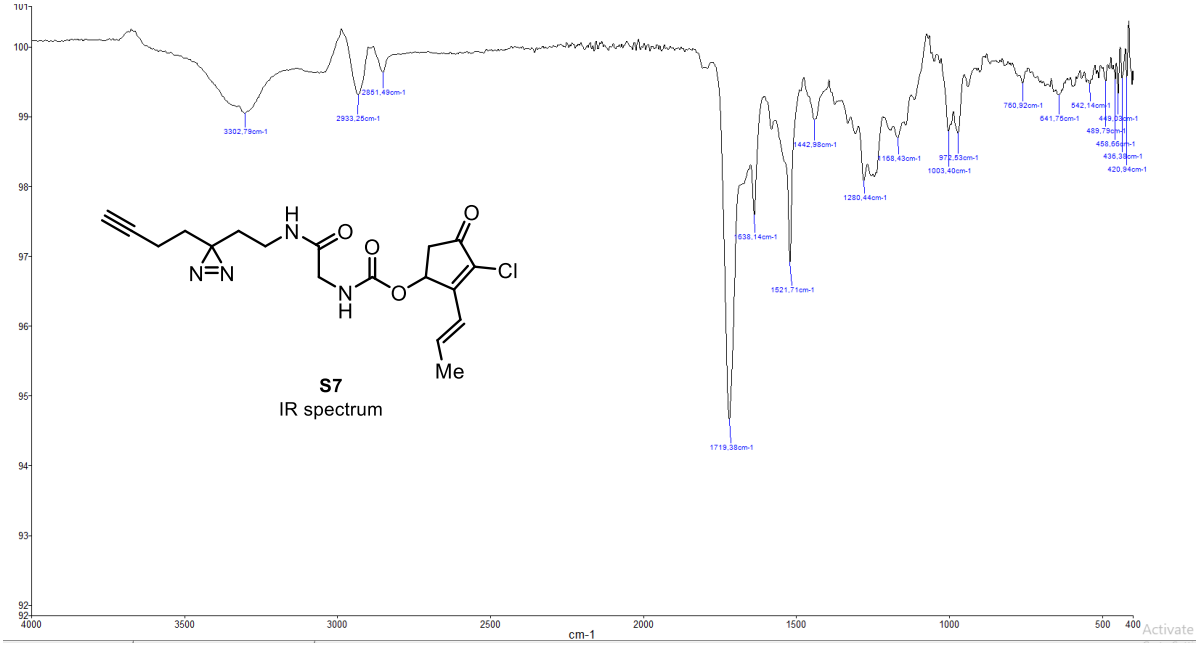
S6

IR spectrum

Compound S7

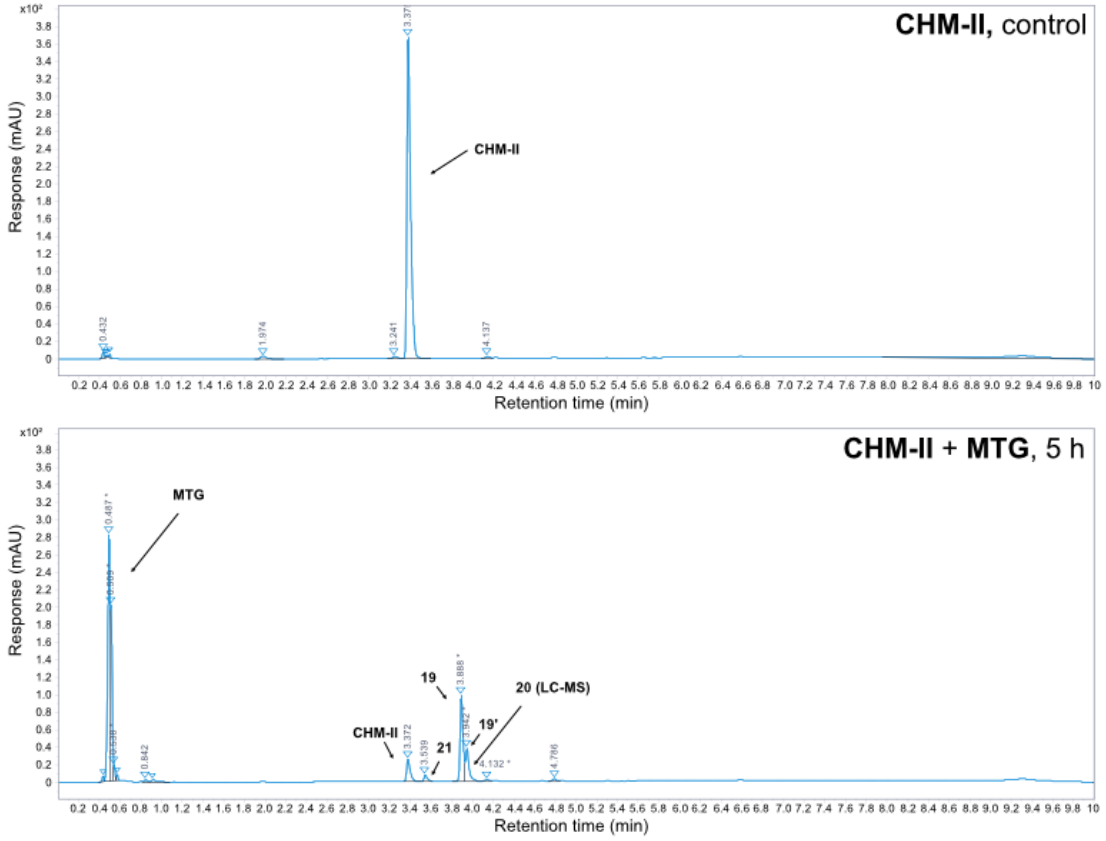






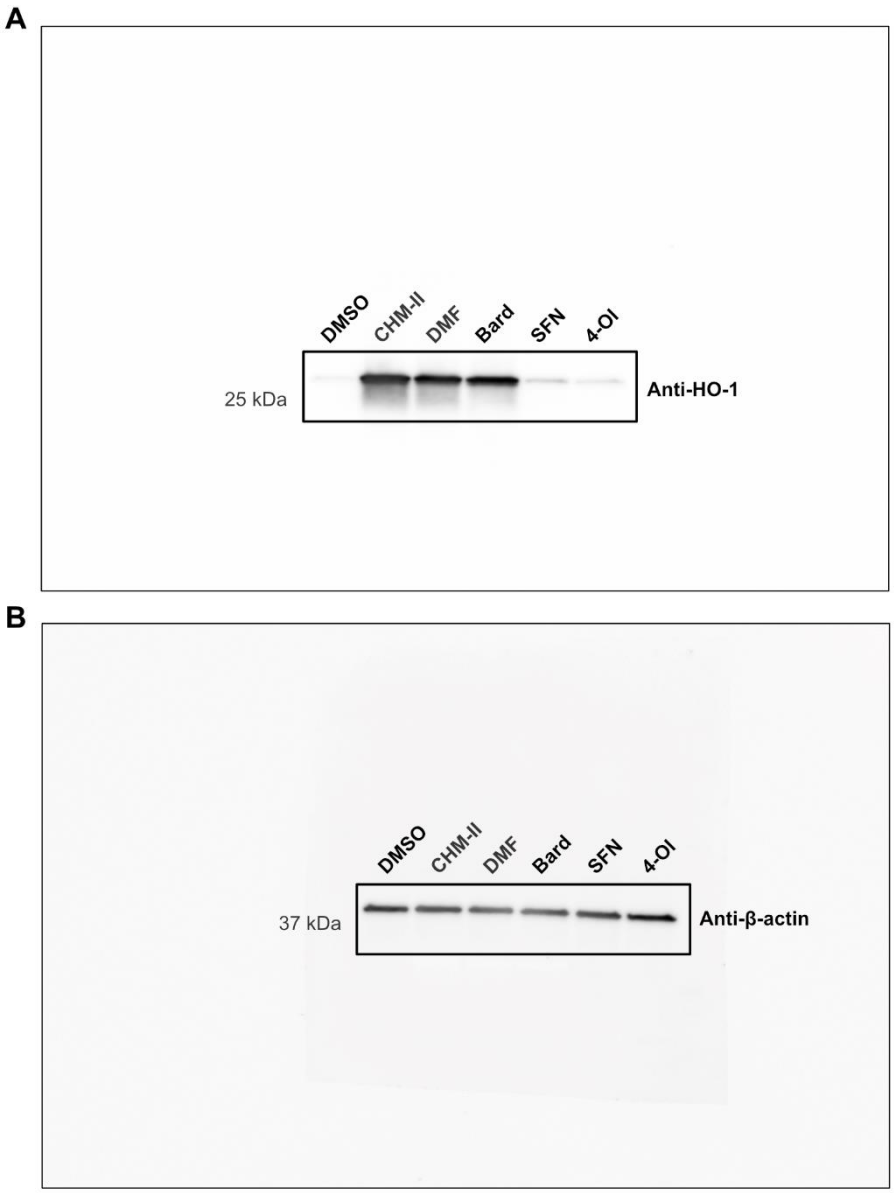
S7
IR spectrum

6. Full chromatograms



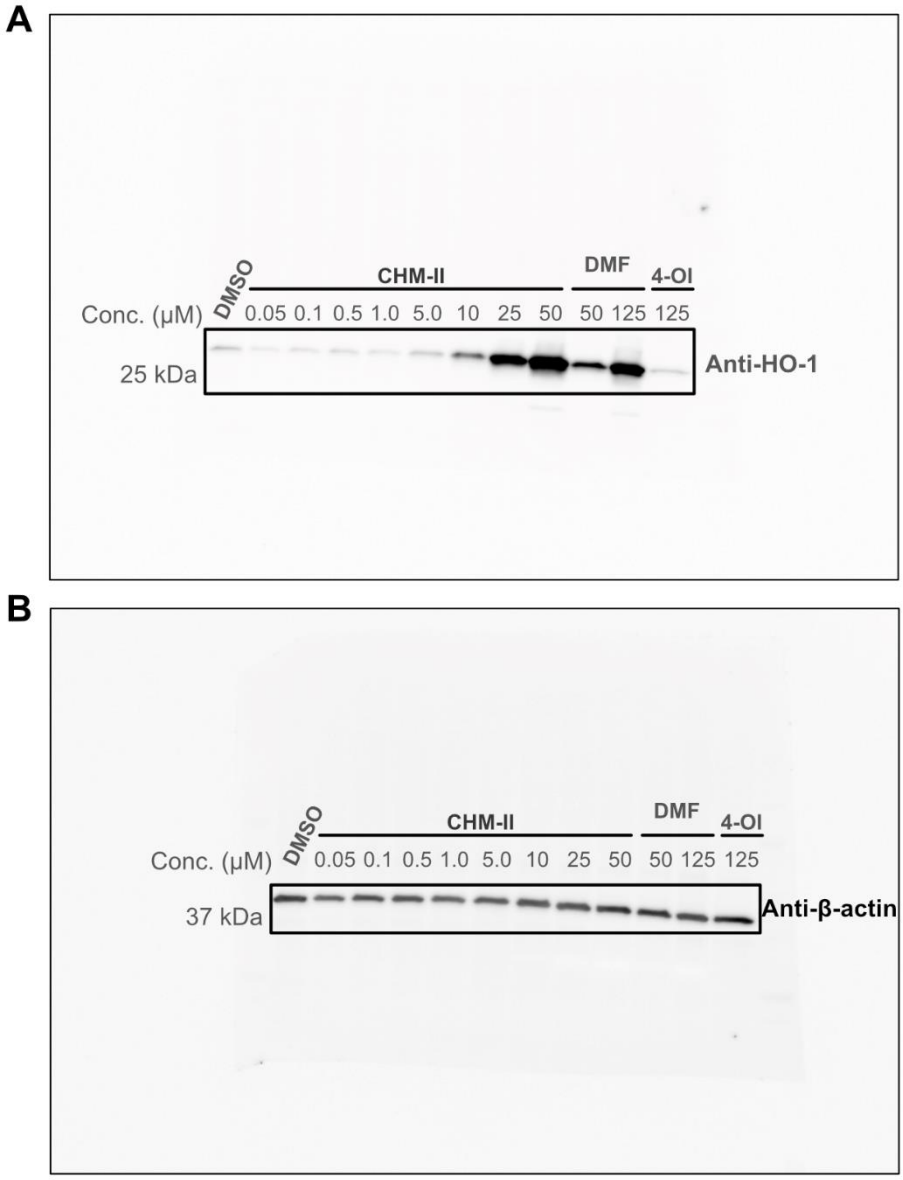
7. Uncropped gels and membranes

Uncropped membrane from figure 2E



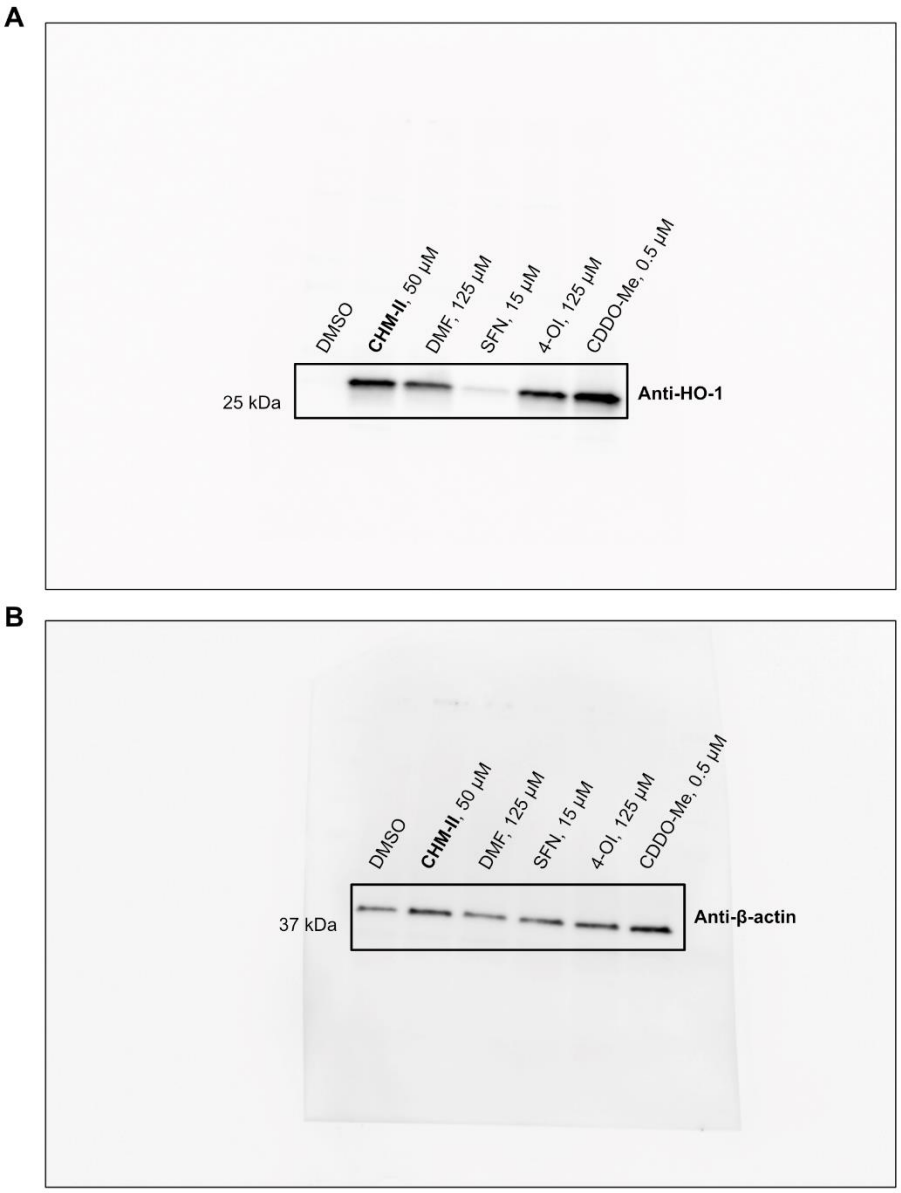
UC1. Uncropped versions of the western blotting membrane presented in figure 2E. A) Anti-HO-1. B) Anti-β-actin.

Uncropped membrane from figure 2F



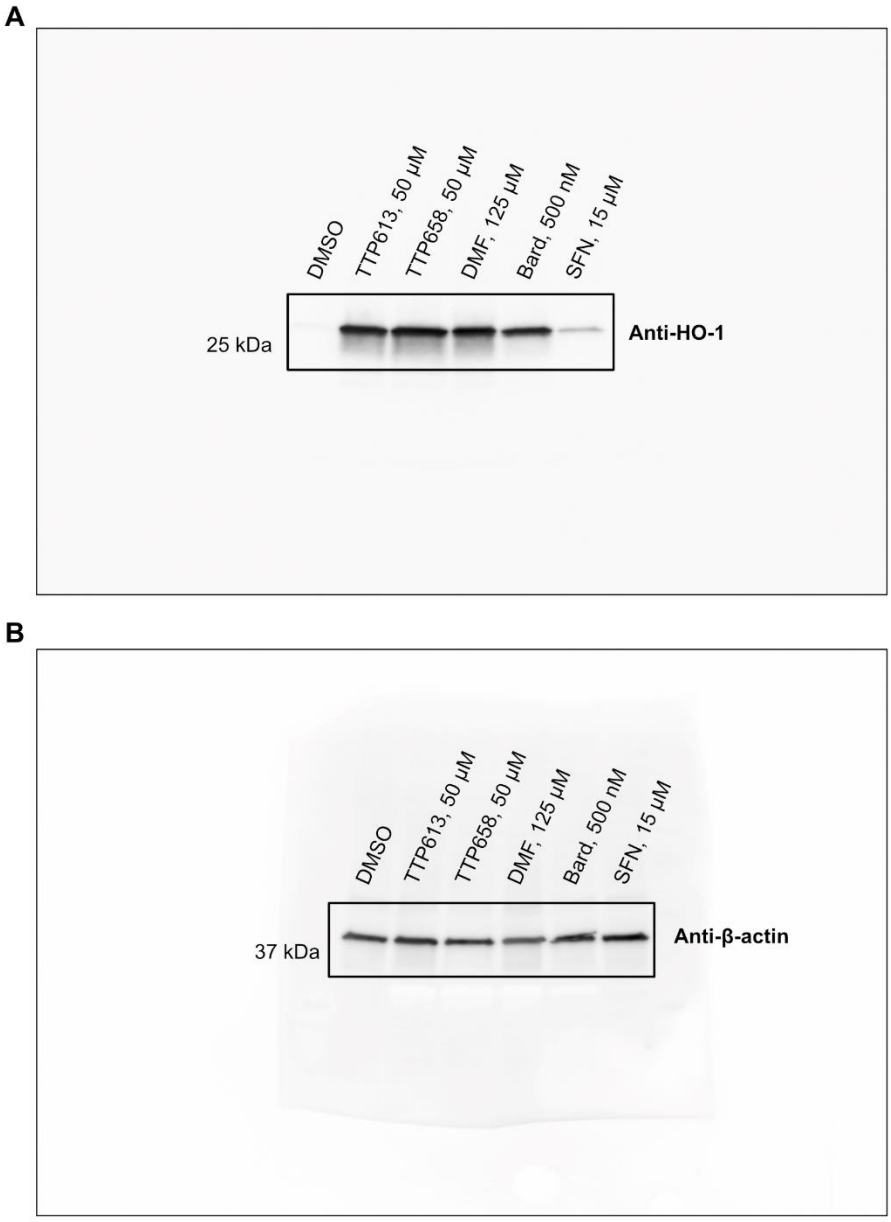
UC2. Uncropped versions of the western blotting membrane presented in figure 2F. A) Anti-HO-1. B) Anti-β-actin.

Uncropped membrane from figure S4



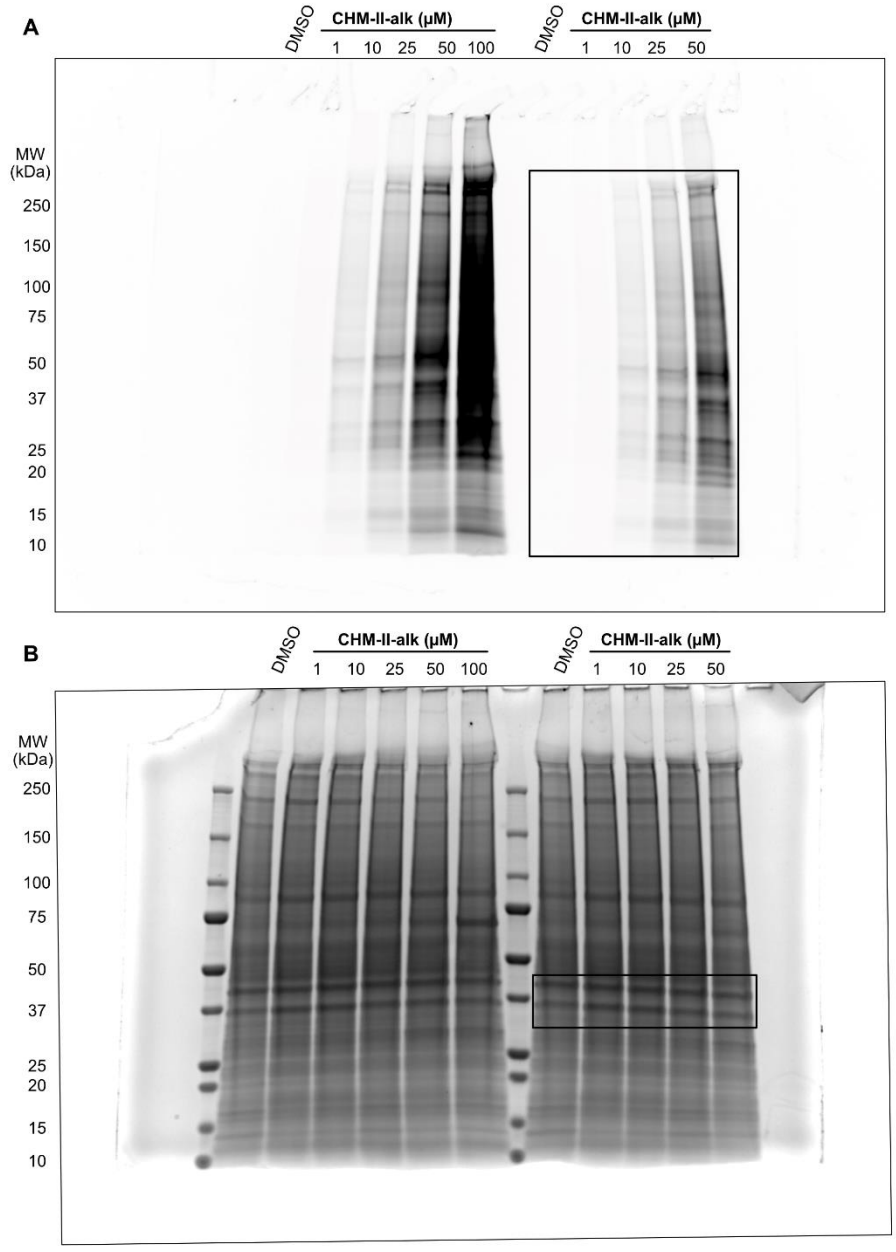
UC3. Uncropped versions of the western blotting membrane are presented in figure S4. A) Anti-HO-1. B) Anti-β-actin.

Uncropped membrane from figure S14



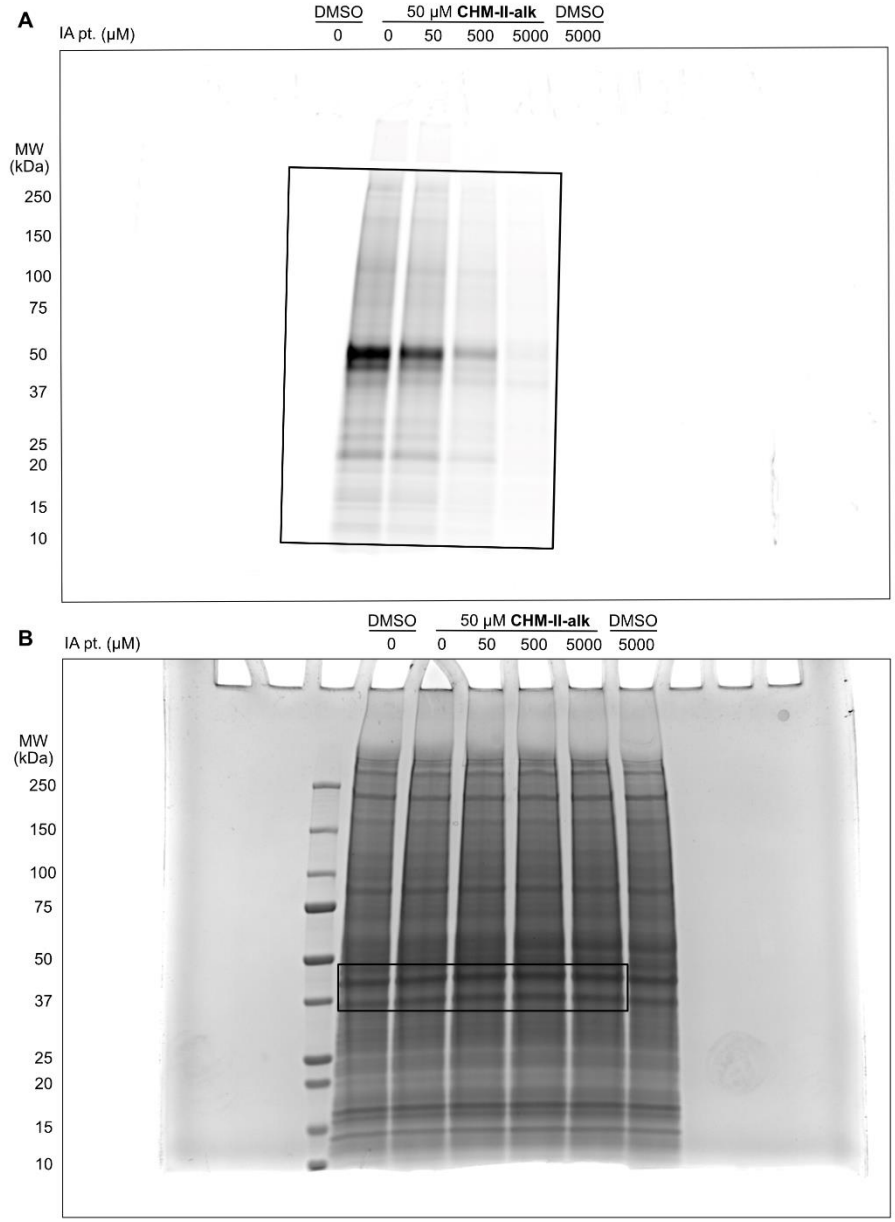
UC4. Uncropped versions of the western blotting membrane are presented in figure S14. A) Anti-HO-1. B) Anti-β-actin.

Uncropped gel from Figure 4C



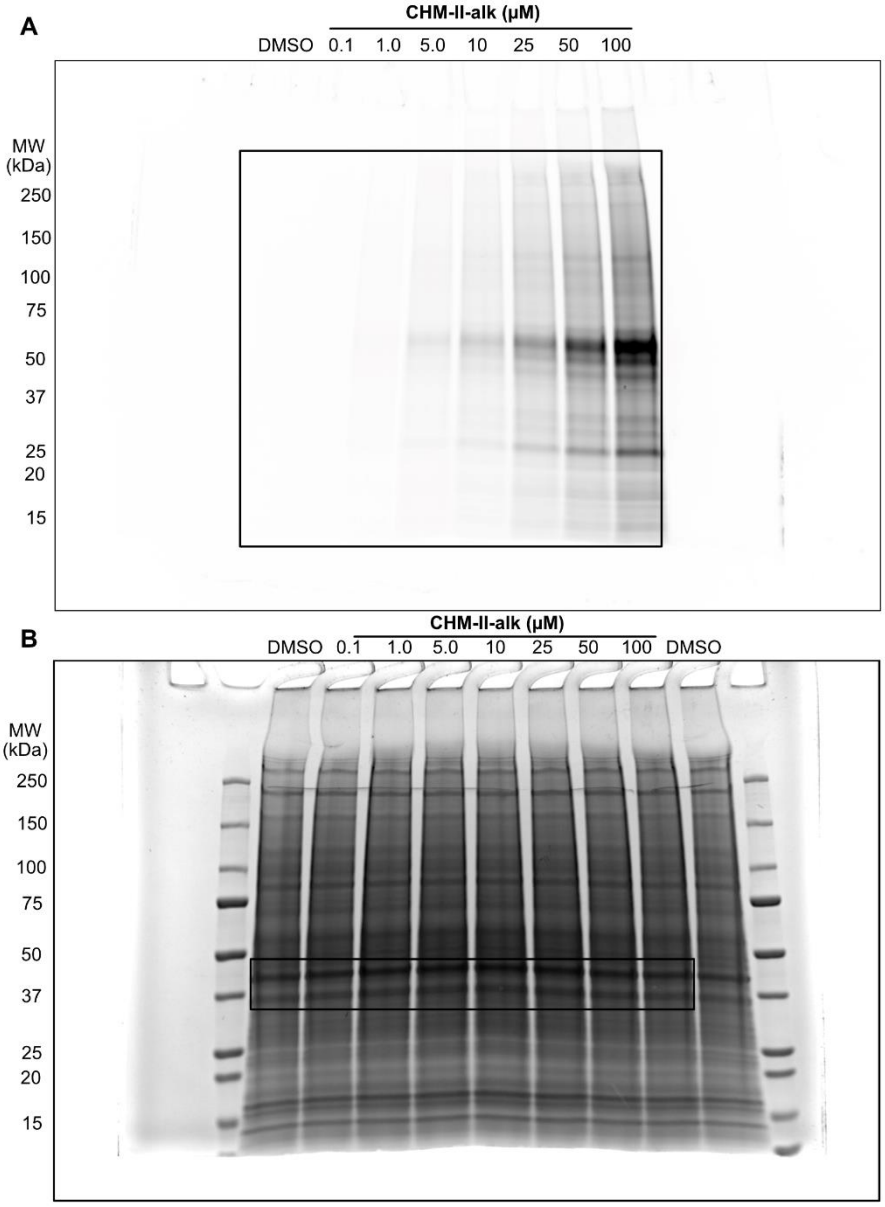
UC5. Uncropped versions of the gel presented in figure 4C. A) Fluorescence gel. B) Protein-stained gel image. The left side of the gel was not presented in the main text, due to the high fluorescence observed for the 100 µM **CHM-II**-treatment, which 'drowned out' the binding at lower concentrations and thus complicated the contrast adjustment.

Uncropped gel from Figure 4D



UC6. Uncropped versions of the gel presented in figure 4D. A) Fluorescence gel. B) Protein-stained gel image. The iodoacetamide (IA) pre-treated and DMSO-treated sample was left included in the experiment for good measures, but was left out of the final figure as it did not provide additional, relevant information.

Uncropped gel from Figure S16



UC7. Uncropped versions of the gel presented in figure S16. A) Fluorescence gel. An uncropped version of the high-contrast figure (Figure S16) gel has not been added, as the .gel file is available through OSF. B) Protein-stained gel image.

8. Supporting information references

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