Chemical Site-Selective Prenylation of Proteins

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General procedures

Proton nuclear magnetic resonance (δ_H) spectra were recorded on a Bruker AV400 (400 MHz) or Bruker AV200 (200 MHz). Carbon nuclear magnetic resonance (δ_C) spectra were recorded on a Bruker AV400 (100.7 MHz) spectrometer. Spectra were fully assigned using COSY and HMQC; multiplicities were assigned using DEPT 135. All chemical shifts are quoted on the δ scale in ppm using residual solvent as the internal standard (1H NMR: $CDCl_3$ = 7.26; ^{13}C NMR: $CDCl_3$ = 77.0). The following splitting abbreviations were used: s = singlet, d = doublet, t = triplet, q = quartet, a = apparent.

Infrared spectra were recorded on a Bruker Tensor 27 Fourier Transform spectrophotometer using thin films on NaCl plates for oils. Absorption maxima (υ_{max}) are reported in wavenumbers (cm^{-1}) and classified as strong (s) or broad (br).

Low resolution mass spectra were recorded on a Micromass Platform 1 spectrometer using electrospray ionization (ESI) or by Mr. Robin Proctor using a Walters 2790-Micromass LCT electrospray ionization mass spectrometer. High resolution mass spectra were recorded by Mr. Robin Proctor on a Walters 2790-Micromass LCT electrospray ionization mass spectrometer. m/z values are reported in Daltons.

Thin layer chromatography (TLC) was carried out using Merck aluminium backed sheets coated with $60F_{254}$ silica gel. Visualization of the silica plates was achieved using a UV lamp (λ_{max} = 254 nm) and potassium permanganate (5% in 1M NaOH). Flash column chromatography was carried out using BDH PROLAB® 40-63 mm silica gel (VWR).

Anhydrous solvents were purchased from Fluka or Acros. All other solvents were used as supplied (Analytical or HPLC grade), without prior purification. Milli-Q water for protein modifications. Reagents were purchased from Aldrich and used as supplied. 'Petrol' refers to the fraction of light petroleum ether boiling in the range 40-60 ºC. All reactions using anhydrous conditions were performed using flame-dried apparatus under an atmosphere of argon.

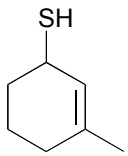
Protein Mass Spectrometry: Liquid chromatography-mass spectrometry (LC-MS) was performed on a Micromass LCT (ESI-TOF-MS) coupled to a Waters Alliance 2790

HPLC using a Phenomenex Jupiter C4 column (250 x 4.6 mm x 5µm). Water:acetonitrile, 95:5 (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, were used as the mobile phase at a flow rate of 1.0 mL min^{-1}. The gradient was programmed as follows: 5% B, 3 min isocratic, then gradient to 100% B over 16 min, then isocratic at 100% B for 2 min. The electrospray source of LCT was operated with a capillary voltage of 3.2 kV and a cone voltage of 25 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 l hr^{-1}. Spectra were calibrated using a calibration curve constructed from a minimum of 17 matched peaks from the multiply charged ion series of equine myoglobin, which was also obtained at a cone voltage of 25V. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.0 from Waters) according to manufacturer's instructions.

General Procedure for the Preparation of Allylic Thiols

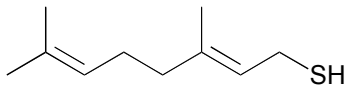
The substrate was dissolved in anhydrous toluene (2 mL for a 200 mg scale reaction). Lawesson's reagent (0.6 equivalents) was added, and the reaction mixture heated to 80 °C under an atmosphere of argon for the indicated reaction time. After this time the reaction mixture was cooled to room temperature and purified directly by flash column chromatography (petrol→petrol:ethyl acetate; 95:5).

3-Methyl-1-thio-cyclohex-2-ene 2



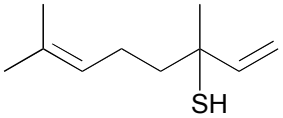
Using the general procedure, **2** was prepared from 3-methyl-cyclohex-2-ene-1-ol **1** (16 hour reaction time) and isolated as a yellow oil on a 3.80 mmol scale; Yield: 71%; R_f 0.8 (petrol:ethyl acetate, 95:5); υ_{max} (thin film) 2305 (br, SH) 1440 (s, C=C) cm^{-1}; δ_H (400 MHz, $CDCl_3$) 1.64-1.69 (6H, m), 1.88-1.97 (4H, m), 3.53-3.56 (1H, m, C<u>H</u>SH), 5.46-5.47 (1H, m, =C<u>H</u>); δ_C (100.7 MHz, $CDCl_3$) 19.7, 23.7, 29.6, 33.1, 35.6, 124.8, 135.9; *m/z* (FI) 254 [2M]; HRMS (FI) Calcd. for $C_{14}H_{22}S_2$ 254.1163. Found: 254.1174.

***trans*-Geranylmercaptan[1] 4**



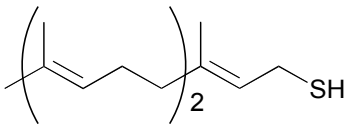
Using the general procedure, **4** was prepared from *trans*-geraniol **3** (22 hour reaction time) and isolated as as an oil on a 0.94 mmol scale; Yield: 72%; R_f 0.8 (petrol:ethyl acetate, 9:1); υ_{max} (thin film) 2564 (br, SH) 1462 (s, C=C) cm^{-1}; δ_H (400 MHz, $CDCl_3$) 1.41 (1H, t, *J* 7.0 Hz, SH), 1.60, 1.66, 1.69 (9H, 3 x s, 3 x CH_3), 1.96-2.11 (4H, m, 2 x CH_2), 3.17 (2H, at, *J* 7.3 Hz, C<u>H</u>$_2$SH), 5.10 (1H, m, CH=C), 5.35 (1H, dt, J_{vic} 7.8 Hz, *J* 1.2 Hz, C<u>H</u>CH_2SH); δ_C (100.7 MHz, $CDCl_3$) 15.8, 15.9, 16.0, 22.1, 39.2, 39.7, 123.7, 124.3; *m/z* (ESI) 363 $[2M+Na]^+$.

NOTE: If the reaction was worked up after 3 hours of reaction time, **4** was isolated along with the allylic isomer shown below in a 87:13 ratio. With prolonged heating (20+ hours), this isomer was not observed.



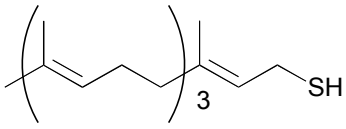
[1] J. A. Maddry, N. Bansal, L. E. Bermudez, R. N. Comber, I. M. Orme, W. J. Suling, L. N. Wilson and R. C. Reynolds, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 237-242.

***trans,trans*-Farnesylmercaptan[2] 6**



Using the general procedure, **6** was prepared from *trans,trans*-farnesol **5** (6 hours reaction time) and isolated as a yellow oil along with 7% inseparable allylic isomer; 0.91 mmol scale; Yield: 84%; R_f 0.9 (petrol:ethyl acetate, 9:1); υ_{max} (thin film) 2453 (br, SH) 1476 (s, C=C) cm^{-1}; δ_H (400 MHz, $CDCl_3$) 1.40 (1H, t, *J* 7.0 Hz, SH), 1.61, 1.66, 1.68 (12H, 3 x s, 4 x CH_3) 1.99-2.08 (8H, m, 4 x CH_2), 3.17 (2H, at, *J* 7.4 Hz, C$\underline{H}_2$SH), 5.06-5.14 (2H, m, 2 x CH=C), 5.35 (1H, dt, J_{vic} 7.8 Hz, *J* 1.2 Hz, C$\underline{H}$CH_2SH); *m/z* (ESI) 237 $[M-H]^-$.

***trans,trans,trans*-Geranylgeranylmercaptan[2] 8**



Using the general procedure, **8** was prepared from *trans,trans,trans*-geranyl geraniol **7** (6 hours reaction time) and isolated as a yellow oil along with 4% inseparable allylic isomer; 0.91 mmol scale; Yield: 91%; R_f 0.9 (petrol:ethyl acetate, 9:1); υ_{max} (thin film) 2397 (br, SH) 1444 (s, C=C) cm^{-1}; δ_H (200 MHz, $CDCl_3$) 1.40 (1H, t, *J* 7.0 Hz, SH), 1.61, 1.66, 1.69 (15H, 3 x s, 5 x CH_3) 2.02-2.08 (12H, m, 6 x CH_2), 3.17 (2H, at, *J* 7.4 Hz, C$\underline{H}_2$SH), 5.08-5.15 (3H, m, 3 x CH=C), 5.35 (1H, dt, J_{vic} 7.5 Hz, *J* 1.1 Hz, C$\underline{H}$CH_2SH); *m/z* (ESI) 305 $[M-H]^-$.

Pre-activation of Tagged Mutant Protein

SBL-S156C mutant (2 mg) was dissolved in degassed aqueous buffer solution (1 mL, 70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5). A solution of phenyl selenenyl bromide (5 mg, 0.02 mmol) in acetonitrile (200 µL) was added, along with irreversible inhibitor phenyl methylsulfonyl fluoride (PMSF) (650 µL of a 15 mg/mL solution in acetonitrile), to the protein solution and placed on an end-over-end rotator. After 30 min, the reaction mixture was loaded onto a PD10 Sephadex® G25 column pre-equilibrated with the above buffer. The protein fraction was analyzed by LC-mass spectrometry revealing formation of the pre-activated tagged mutant protein (calculated mass, 27026; observed mass, 27024).

Procedure for Protein Prenylation

Prenyl thiol (100 equivalents) was dissolved in DMSO (250 µL) and added to a solution of the pre-activated protein (1 mg) in aqueous buffer solution (1 mL, 70 mM

[2] W. W. Epstein, Z. Wang, *Chem. Commun.*, 1997, 863-864.

CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5). The mixture was sonicated for 2 h in 10 minute pulses (10 minutes on, 10 minutes off) at room temperature. The mixture was centrifuged and analyzed by LC-mass spectrometry, revealing formation of the disulfide-linked prenylated protein.

Thiol	Product	ESI-MS Theor.	Mass Found	Yield
Geranyl	**10**	27037	27042	90%
Farnesyl	**11**	27105	27110	50%

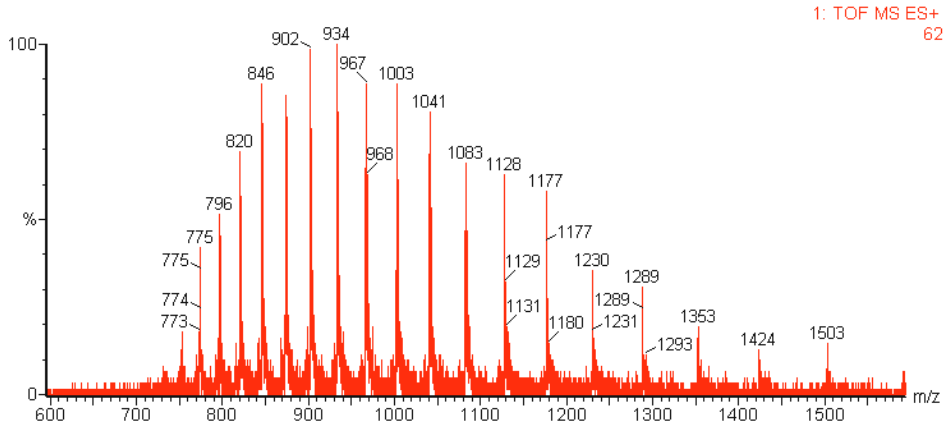


Figure 1. ESI-MS spectrum of SBL-geranyl **10**.

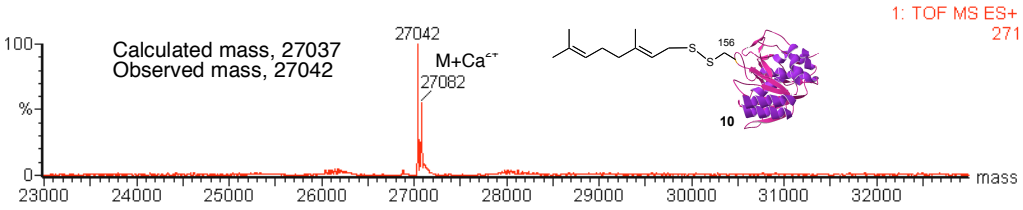


Figure 2. Deconvoluted ESI-MS spectrum of SBL-geranyl **10**.

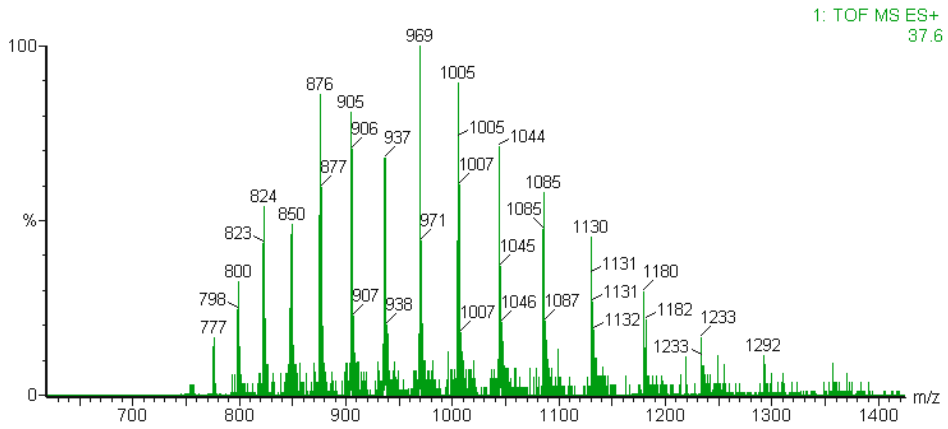


Figure 3. ESI-MS spectrum of SBL-farnesyl **11**.

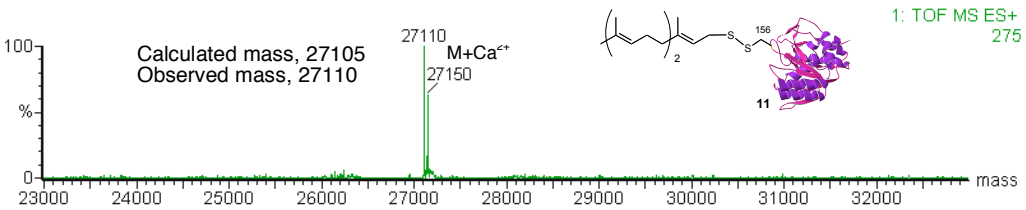


Figure 4. Deconvoluted ESI-MS spectrum of SBL-farnesyl **11**.

Protein Activity Assay

Disulfide-linked geranyl SBL was prepared as described above (phenyl selenyl bromide activation then sonication with geranyl thiol, 20% DMSO) with the exception that the inhibitor PMSF was not used to deactivate the enzyme. A 200 μL aliquot of the prenylated protein was diluted with the same buffer to a final concentration of 0.2 mg/mL and 475 μL was transferred to a plastic micro-centrifuge tube. A solution of suc-AAPF-pNA (25 μL of a 0.20 M solution in DMSO) was added to the protein solution. A bright yellow product was formed immediately, indicating enzymatic activity (Figure 5).

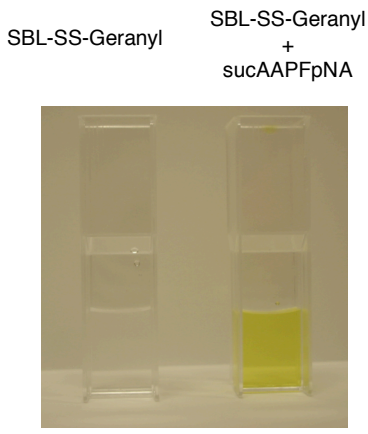
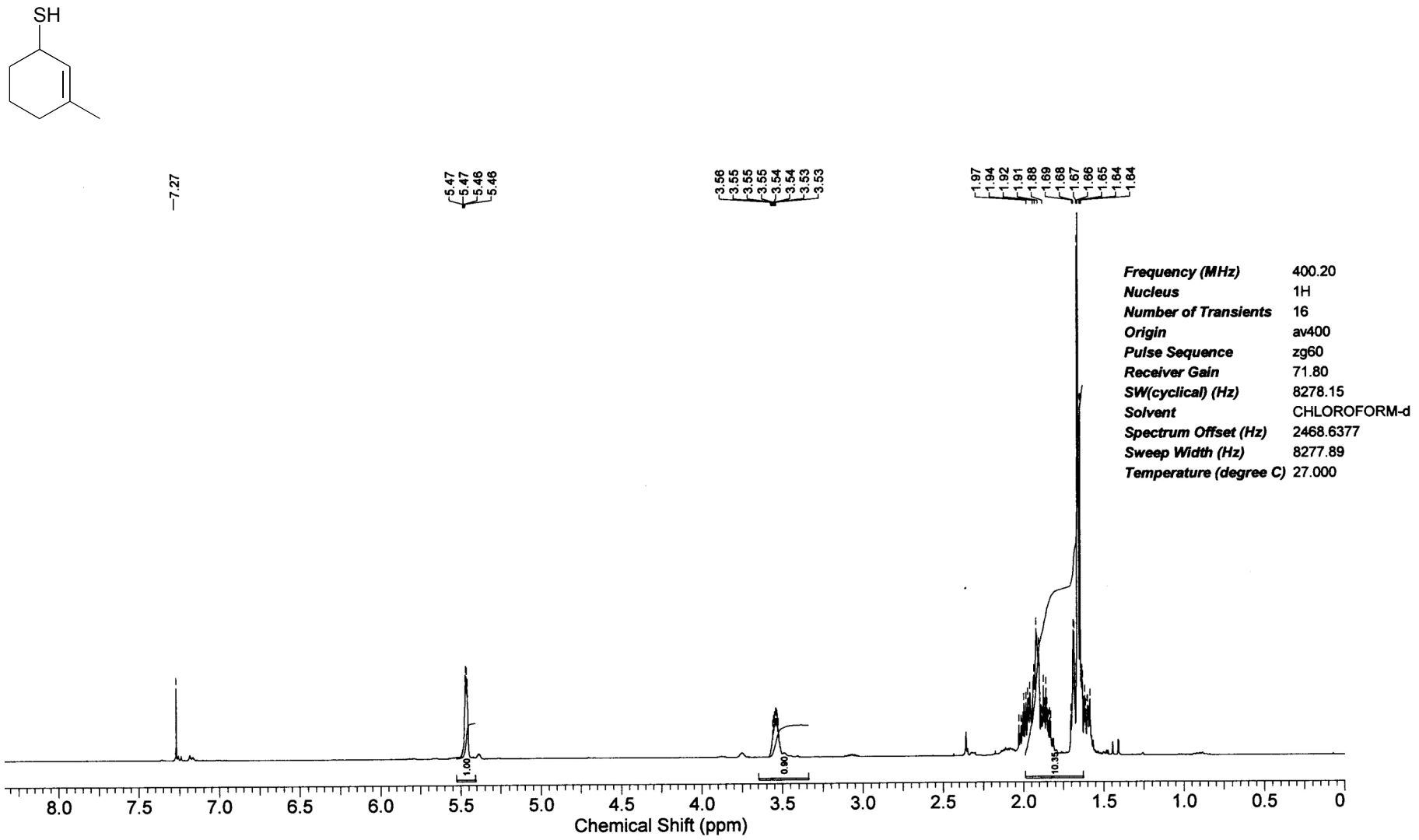


Figure 5. Activity assay post-prenylation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5.47
5.46
5.46
3.56
3.55
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3.55
3.54
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3.53
1.97
1.94
1.92
1.91
1.88
1.69
1.68
1.67
1.66
1.65
1.64
1.64
Frequency (MHz) 400.20
Nucleus 1H
Number of Transients 16
Origin av400
Pulse Sequence zg60
Receiver Gain 71.80
SW(cyclical) (Hz) 8278.15
Solvent CHLOROFORM-d
Spectrum Offset (Hz) 2468.6377
Sweep Width (Hz) 8277.89
Temperature (degree C) 27.000
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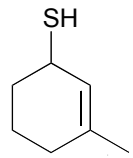
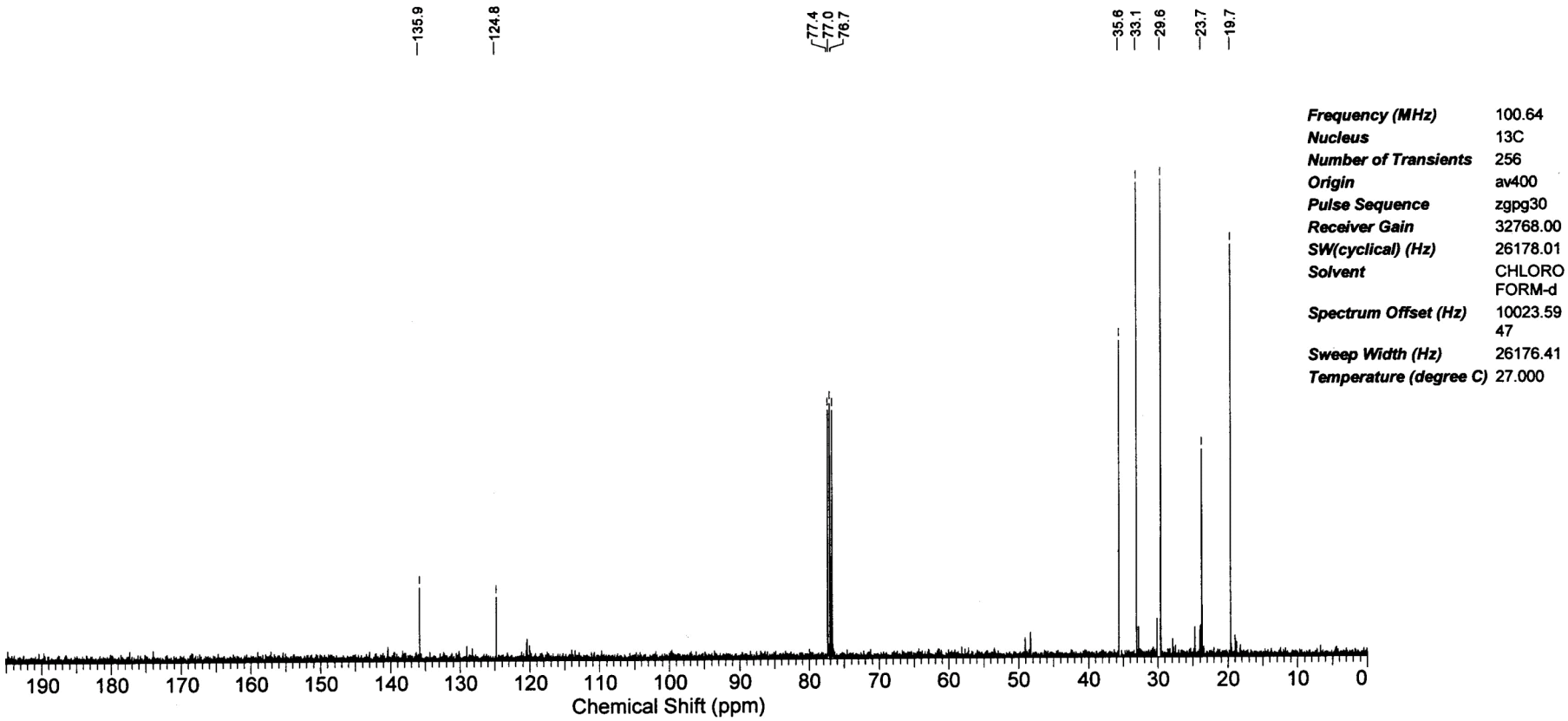
0.90
10.35
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Chemical Shift (ppm)


SH


135.9
124.8
77.4
77.0
76.7
35.6
33.1
29.6
23.7
19.7
Frequency (MHz) 100.64
Nucleus 13C
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Receiver Gain 32768.00
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Solvent CHLOROFORM-d
Spectrum Offset (Hz) 10023.5947
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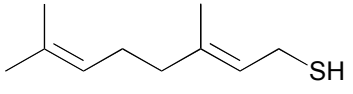
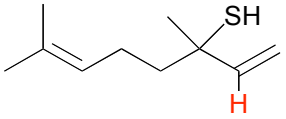
Chemical Shift (ppm)





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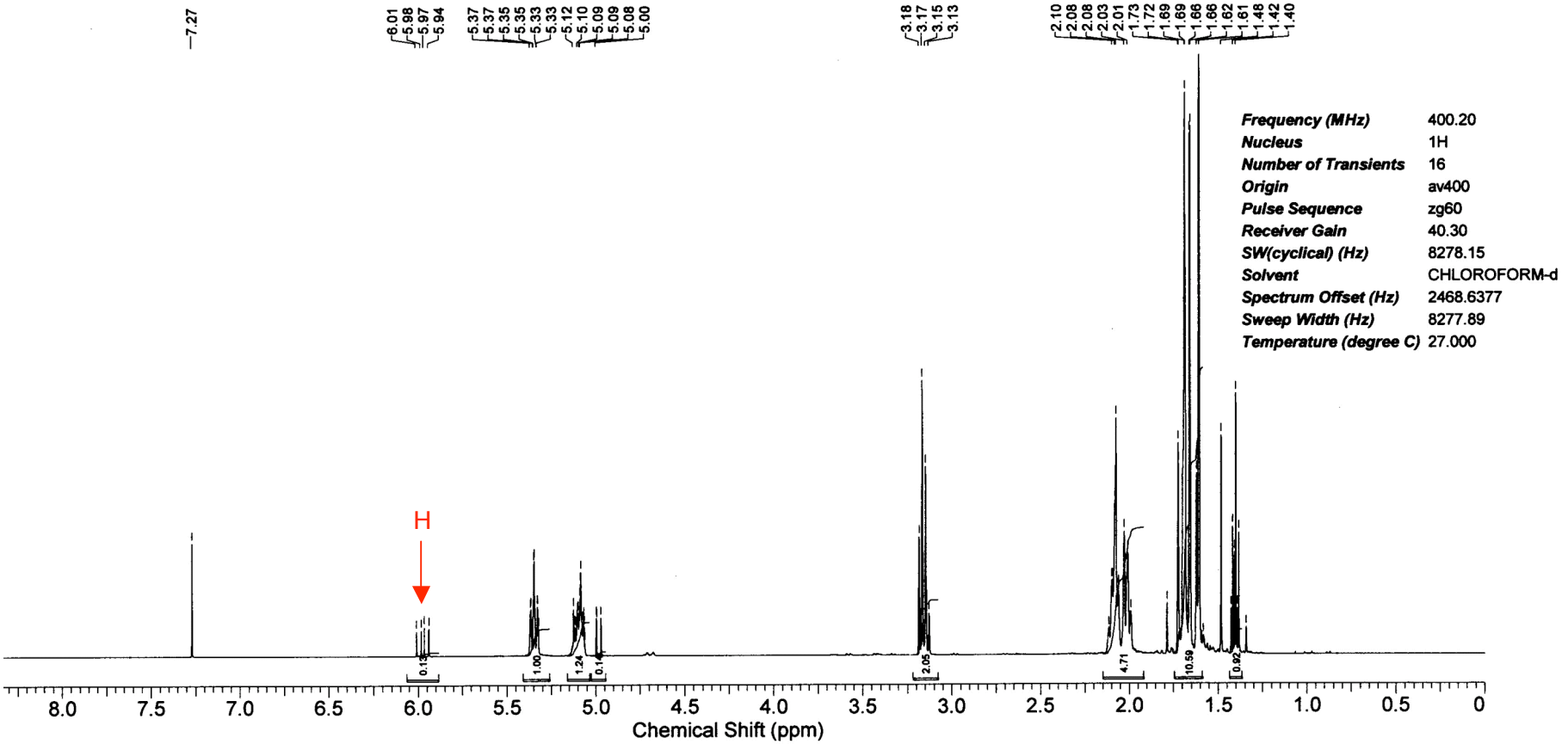

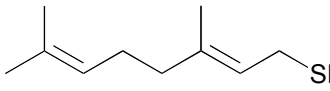

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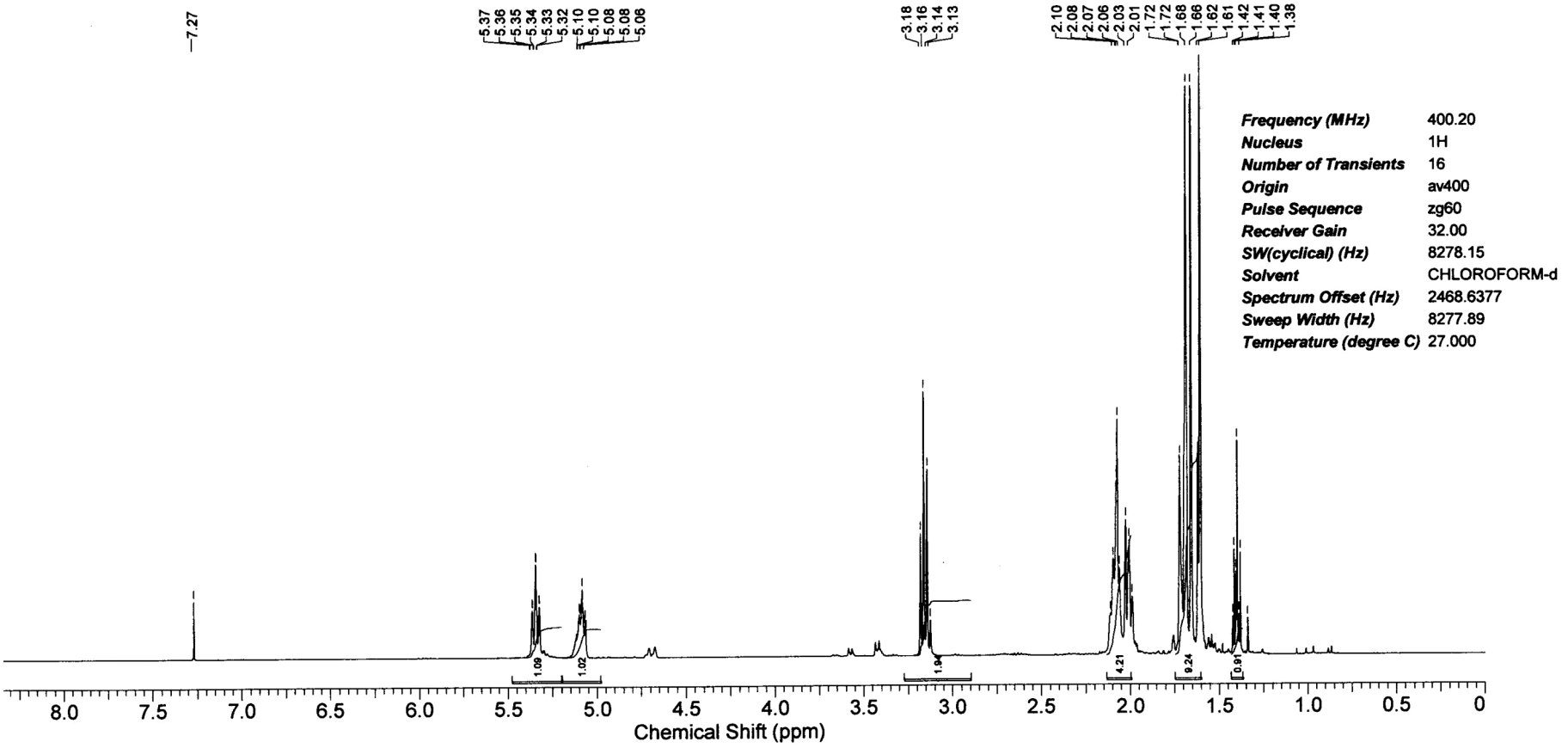

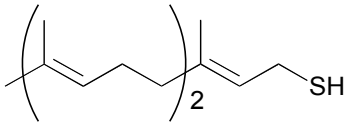

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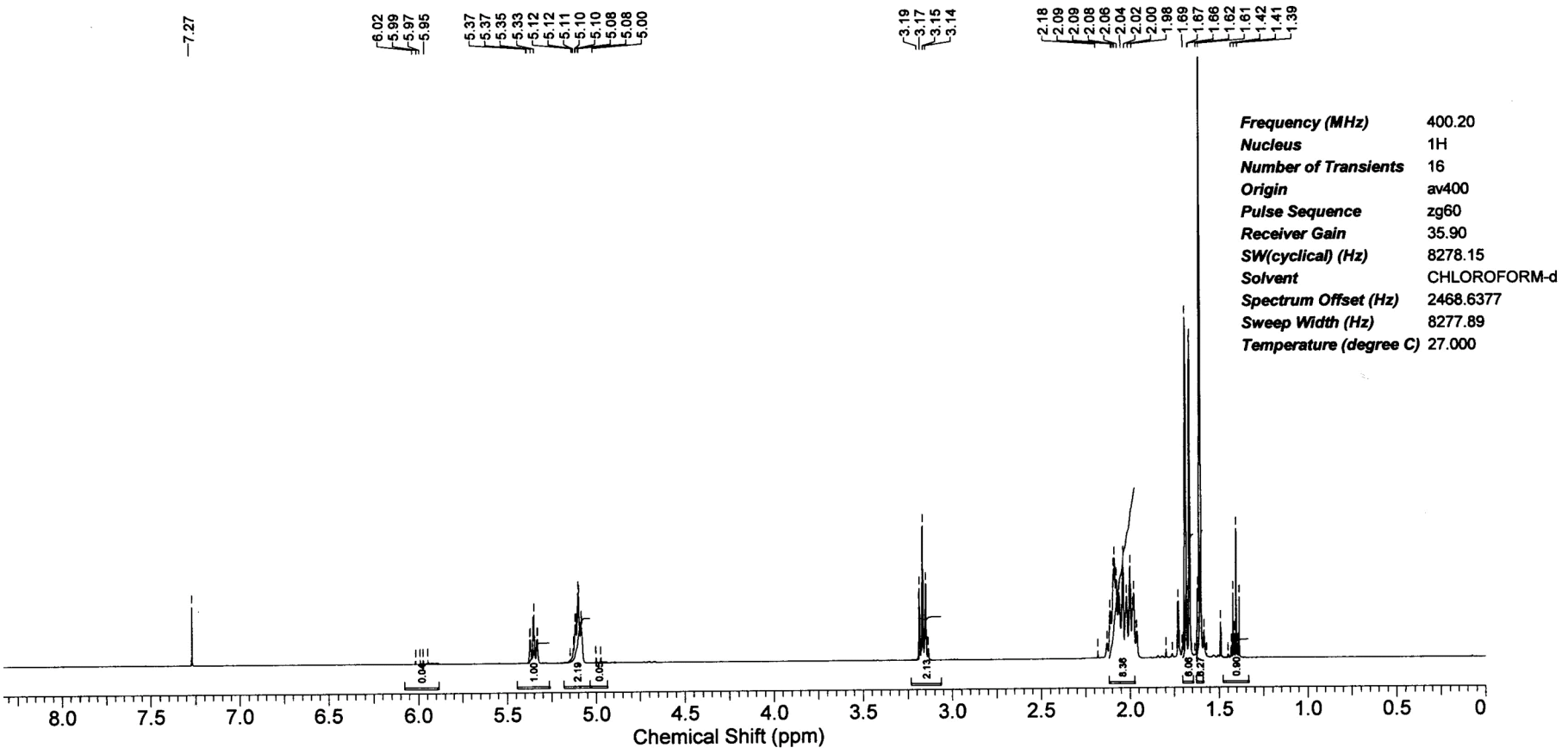

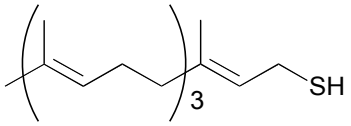

(6 hour reaction time)