Supporting Information

Fluorescence Imaging of Cellular Glutathione using a Latent Rhodamine

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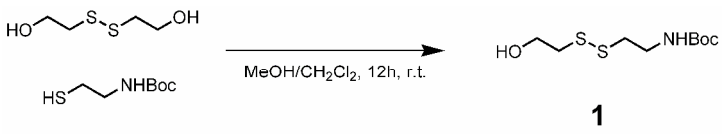
Contents:

Materials and Methods:

General Methods and Reagents. All chemicals were purchased from Sigma-Aldrich and used without purification. MALDI-TOF mass spectrometric measurements were performed on Applied Biosystems Voyager – DE MALDI-TOF. Analytical RP-HPLC was performed on a Vydac C18 column using the same solvent system.

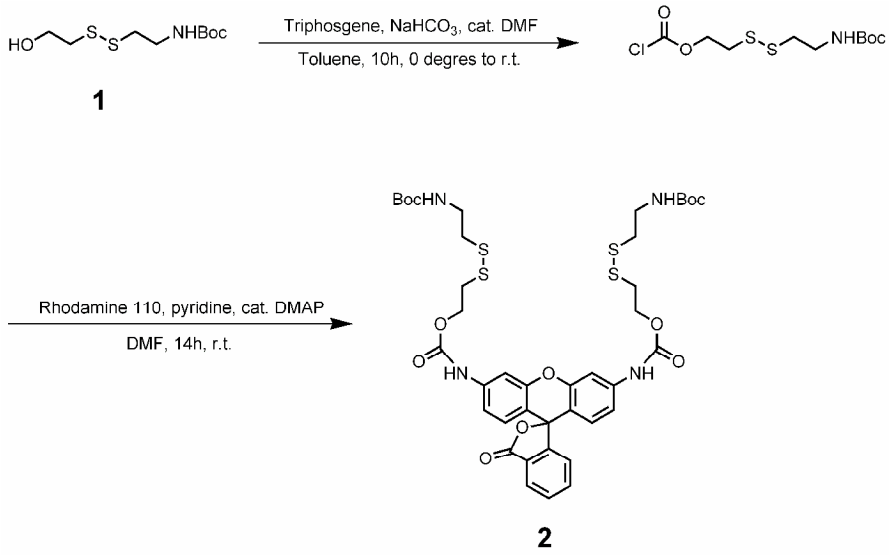
Synthesis of Compound 1.



1

2,2'-Dithiodiethanol (2 g, 11.28 mmol) was dissolved in 1:1 DCM:MeOH (50 mL) and the 2-(Boc-amino)ethanethiol (0.667 g, 3.76 mmol) was added neat in 5 portions over 3 hours with stirring. The solution was stirred at room temperature in the dark for 12 h followed by addition of a solution of iodine (0.5 g) dissolved in MeOH (10 mL) until the solution remained slightly yellow. The solvents were removed *in vacuo* and the residue was dissolved in ethyl acetate. The solution was washed 3X with a saturated sodium bicarbonate solution, and the organic layer was dried over $MgSO_4$. Purification by flash chromatographic (silica column) afforded the desired product **1** (0.82 g, 75% yield) as a light yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.93 (N, br s, 1H), 3.96 (t, J = 6 Hz, 2H), 3.44 (t, J = 6 Hz, 2H), 2.85 (t, J = 6 Hz, 2H), 2.77 (t, J = 6 Hz, 2H), 1.41 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.01, 79.82, 60.28, 41.81, 39.39, 38.16, 28.27. HRMS (ESI): $[M+Na]^+$: 276.0704 (calculated) 276.0702 (found).

Synthesis of Compound 2.

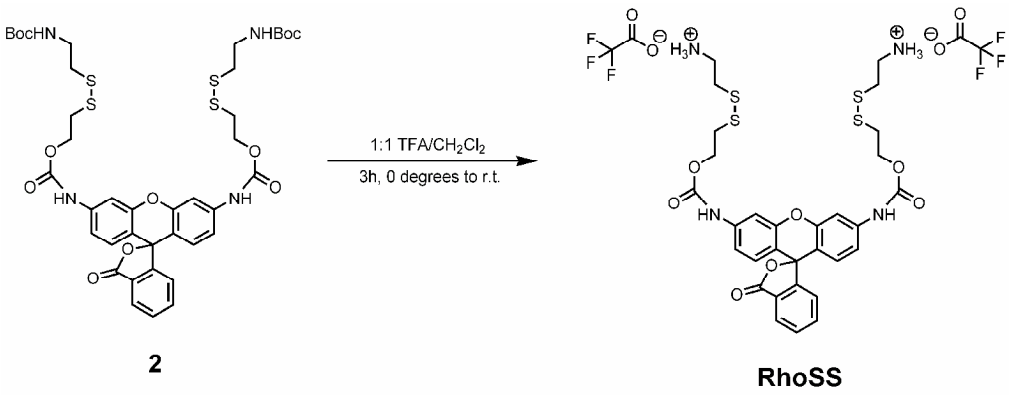


Sodium carbonate (845 mg, 7.98 mmol), triphosgene (585 mg, 1.973 mmol), and 60 mL of dry toluene were added to a flame dried flask under nitrogen. The heterogenous solution was stirred at 0 ºC (ice bath) for 30 min, and a solution of compound **1** (500 mg, 1.973 mmol) and DMF (20 μL, 0.25 mmol) dissolved in 1:1 diethyl ether/toluene (20 mL) was added slowly over 10 min. The reaction mixture was stirred vigorously for 10 hours, and the flask was flushed with nitrogen for 1 h to remove unreacted phosgene. The solution was filtered, the solvents were removed *in vacuo*, and the remaining (crude chloroformate) was used without further purification.

The crude chloroformate (300 mg, 0.950 mmol), pyridine (383 μL, 4.75 mmol), Rhodamine 110 (100 mg, 0.280 mmol), and a catalytic amount (15 mg, 0.122 mmol) of DMAP were dissolved in dry DMF (3 mL). The solution was stirred for 14 h at room temperature, and the solvent was removed *in vacuo*. The pink residue was dissolved in CH_2Cl_2 (100 mL) and washed with 2X with a solution of 1% HCl. The organic layer was

dried over $MgSO_4$, and the solvent was removed *in vacuo*. Purification by silica column afforded product **2** (110 mg, 44 % yield) as a pink powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.01 (d, *J* = 6 Hz, 1H), 7.63 (m, 2H), 7.32 (d, *J* = 2 Hz, 2H), 7.12 (d, *J* = 7 Hz, 1H), 7.04 (d, *J* = 6 Hz, 2H), 6.70 (d, *J* = 6 Hz, 2H), 4.42 (t, *J* = 6 Hz, 4H), 3.46 (q, *J* = 6 Hz, 4H), 2.98 (t, *J* = 6 Hz, 2H), 2.80 (t, *J* = 6 Hz, 2H), 1.23 (s, 18 H) . ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.51, 162.97, 155.78, 152.30, 151.97, 135.15, 129.82, 128.83, 126.56, 123.98, 123.57, 117.55, 115.05, 109.18, 79.62, 61.77, 41.83, 40.29, 39.78, 39.22, 38.63, 38.12 28.44. HRMS (ESI): $[M+H]^+$: 889.2281 (calculated) 889.2290 (found).

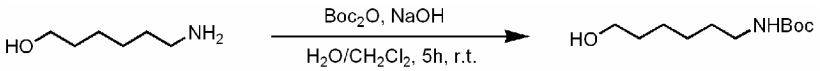
Synthesis of RhoSS.



Compound **2** (70 mg, 0.0788 mmol) was dissolved in a cold (0 ºC) solution of 1:1 TFA and CH_2Cl_2 (10 mL). The solution was kept in the dark and stirred for 1 h at 0 ºC before allowing it to come to room temperature and stirred for an additional 2 h. The solvents were removed *in vacuo,* and the residue was dissolved in DMSO and filtered. **RhoSS** was purified to homogeneity by reverse phase HPLC using a Vydac C8 column with an eluent consisting of solvent A (H_2O /0.1% TFA) and solvent B (CH_3CN /0.1% TFA) with a 60 minute gradient consisting of 10 to 70% A, a flow rate of 8 mL/min, and monitoring at 214 and 280 nm. After lyophilization, **RhoSS** was isolated as a pink powder (50 mg, 60% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.11 (d, *J* = 6 Hz, 1H), 7.88 (m, 2H), 7.70 (d,

J = 2 Hz, 2H), 7.29 (d, *J* = 6 Hz, 1H), 7.16 (d, *J* = 6 Hz, 2H), 6.77 (d, *J* = 6 Hz, 2H), 4.50 (t, *J* = 6 Hz, 4H), 3.39 (t, *J* = 6 Hz, 4H), 3.14 (t, *J* = 6 Hz, 4H), 3.07 (t, *J* = 6 Hz, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.85, 153.91, 153.00, 151.88, 135.60, 130.21, 128.40, 126.72, 124.87, 124.11, 114.64, 113.35, 106.08, 83.82, 63.40, 38.89, 37.70, 35.25. HRMS (ESI): $[M+H]^+$: 689.1232 (calculated) 689.1248 (found).

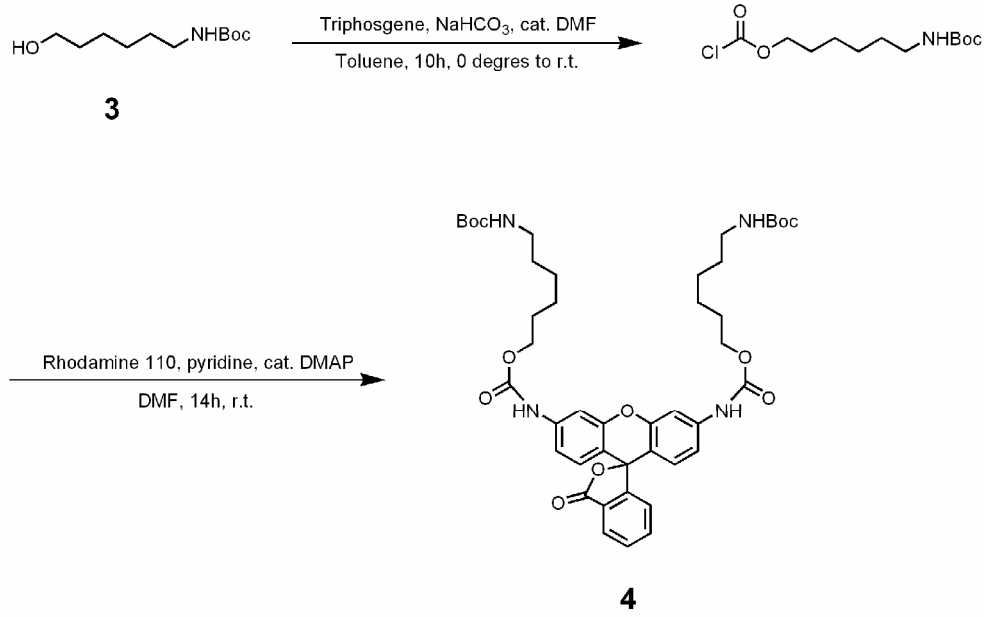
Synthesis of Compound 3.



3

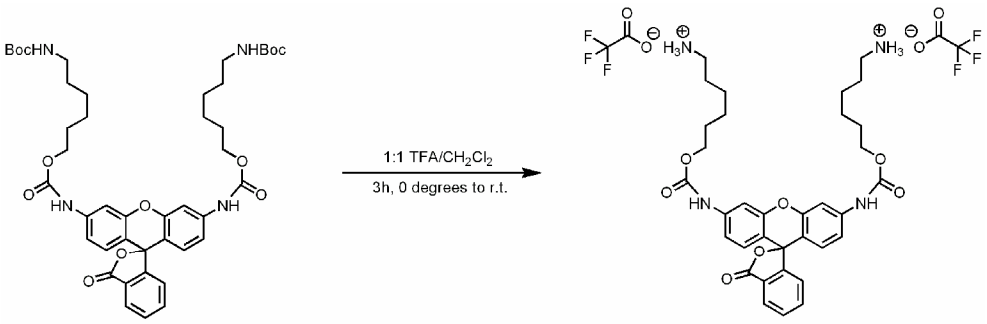
6-Amino-1-hexanol (2 g, 17.07 mmol) was added to a stirring solution of 1:1 CH_2Cl_2/H_2O (80 mL). The pH of the solution was adjusted to pH 9 and kept at this pH throughout the reaction with 6 M NaOH. Di-*tert*-butyl dicarbonate (3.35 g, 15.36 mmol) dissolved in CH_2Cl_2 (20 mL) was added slowly over 1 h. After 5 h, additional CH_2Cl_2 (200 mL) was added and the pH was adjusted to 2. The organic layer was washed 3X with a solution of 5% HCl, and dried over $MgSO_4$. After removal of the solvents *in vacuo*, compound **3** (3.04 g, 91% yield) was isolated as a clear oil. This compound was pure enough to be used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.57 (N, br s, 1H), 3.58 (t, *J* = 6 Hz, 2H), 3.07 (q, *J* = 3 Hz, 2H), 3.07 (q, *J* = 3 Hz, 2H), 1.56-1.49 (m, *J* = 6 Hz, 2H), 1.39 (s, 9H), 1.32-1.25 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.18, 79.14, 62.64, 40.40, 32.63, 30.10, 28.47, 26.45, 25.32. HRMS (ESI): $[M+Na]^+$: 240.1576 (calculated) 240.1578 (found).

Synthesis of Compound 4.



Compound **3** (434 mg, 2.0 mmol) was activated as the chloroformate as described above. The crude chloroformate (280 mg, 1.0 mmol) was reacted with Rhodamine 110 using the same procedure as described above to yield compound **2**. Flash Purification by silica column afforded product **4** (120 mg, 52 % yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.98 (d, J = 7 Hz, 1H), 7.63 (m, *J* = 6 Hz, 2H), 7.49 (d, *J* = 2 Hz, 2H), 7.27 (d, *J* = 6 Hz, 1H), 7.11 (d, *J* = 6 Hz, 2H), 6.7 (d, *J* = 6 Hz, 2H), 4.14 (t, J = 6 Hz, 4H), 3.11 (q, J = 6 Hz, 4H), 1.53 (b, 8H), 1.38 (b, 8H), 1.23 (s, 18H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.49, 156.13, 153.53, 153.00, 151.95, 135.31, 130.02, 128.91, 126.76, 125.37, 124.28, 114.64, 113.73, 106.73, 83.49, 66.37, 41.52, 30.92, 29.57, 27.42, 26.73. HRMS (ESI): $[M+H]^+$: 817.4024 (calculated) 817.4033 (found).

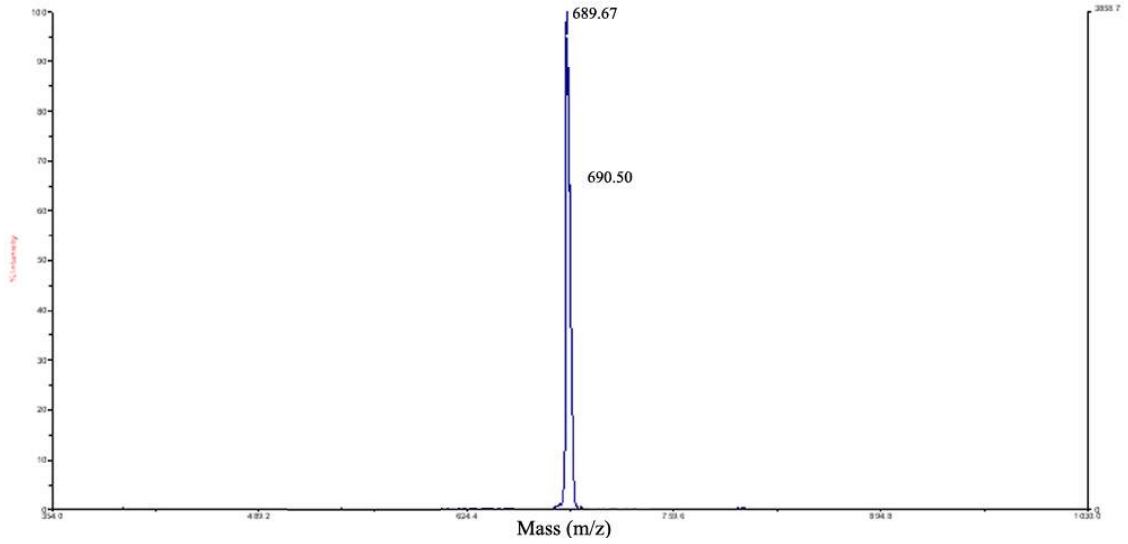
Synthesis of RhoCC.



Compound **4** (71 mg, 0.0870 mmol) was dissolved in a cold (0 ºC) solution of 1:1 TFA and CH_2Cl_2 (10 mL). The solution was kept in the dark and stirred for 1 h at 0 ºC before allowing it to come to room temperature and stirred for an additional 2 h. The solvents were removed *in vacuo,* and ,the residue was dissolved in DMSO and filtered. **RhoCC** was purified to homogeneity by reverse phase HPLC using a Vydac C8 column with an eluent consisting of solvent A (H_2O /0.1% TFA) and solvent B (CH_3CN /0.1% TFA) with a 60 minute gradient consisting of 10 to 70% A, a flow rate of 8 mL/min, and monitoring at 214 and 280 nm. After lyophilization, **RhoCC** was isolated as a pink powder (55 mg, 75 % yield). ^{1}H NMR (300 MHz, CD_3OD): δ 8.11 (d, 1H), 7.83 (m, *J* = 1, 6 Hz, 2H), 7.70 (d, *J* = 2 Hz, 2H), 7.28 (d, *J* = 6 Hz, 1H), 7.13 (d, 6 Hz, 2H), 6.75 (d, *J* = 6 Hz, 2H), 4.25 (t, *J* = 6 Hz, 4H), 3.00 (t, *J* = 6 Hz, 4H), 2.40 (s, 2H), 1.75 (b, 8H), 1.55 (b, 8H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.88, 154.42, 153.00, 151.90, 135.58, 130.19, 128.84, 128.34, 128.13, 126.75, 125.25, 124.85, 124.12, 114.57, 113.16, 105.98, 84.03, 65.42, 40.35, 29.63, 28.33, 26.89, 26.30. HRMS (ESI): $[M+H]^+$: 617.2975 (calculated) 617.2974 (found).

Figure S1.

MALDI-TOF of RhoSS



MALDI-TOF of RhoCC

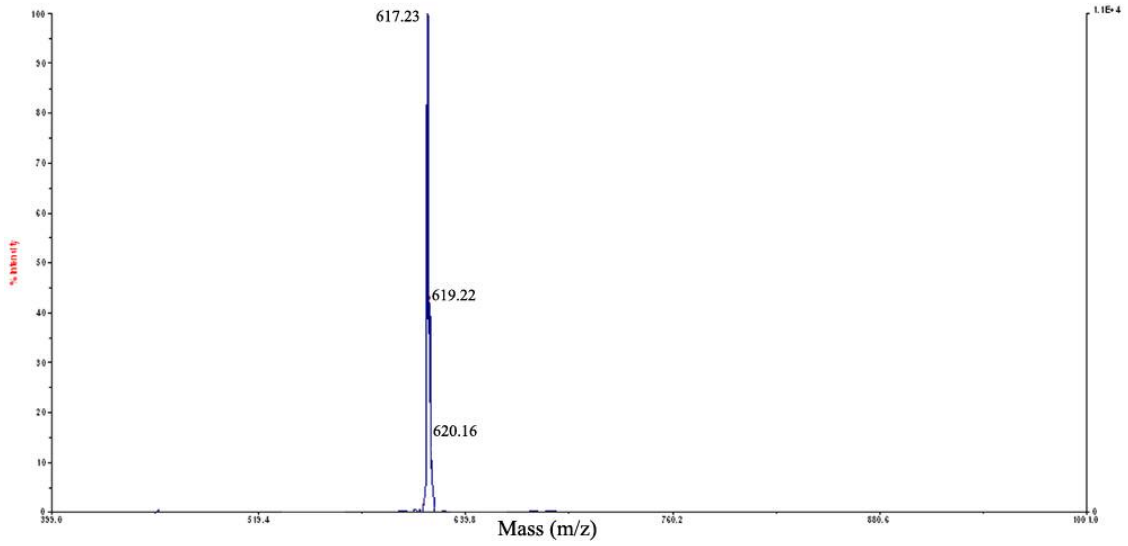
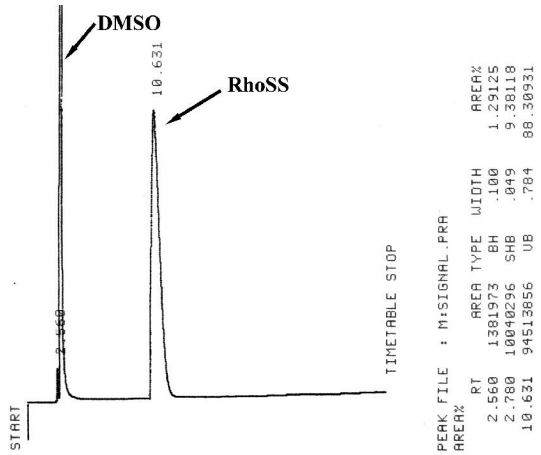


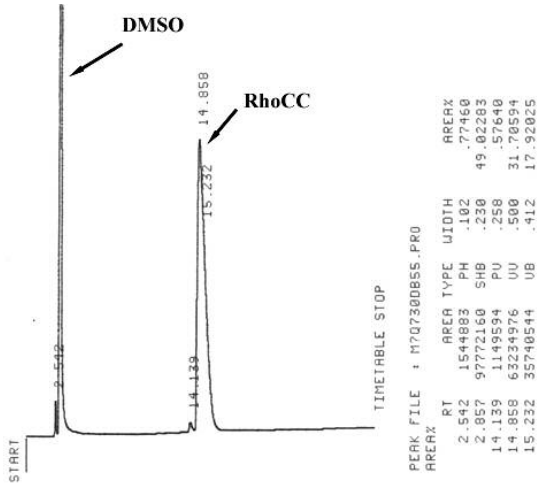
Figure 2S.

Analytical RP-HPLC Analysis

RhoSS – 15% acetonitrile/85% water to 50% acetonitrile/water (containing 0.1 % TFA) over 30 min, flow rate of 1 mL/min, monitored at 214 nm



RhoCC – 15% acetonitrile/85% water to 50% acetonitrile/water (containing 0.1 % TFA) over 30 min, flow rate of 1 mL/min, monitored at 214 nm



Cell Culture. All cells were cultured at 37°C with 5% CO_2. MCF-7 cells (breast adenocarcinoma) were grown in RPMI 1640 medium supplemented with 10% fetal

bovine serum (Cambrex Bio Science Walkersville, Inc.), 2 mM L-glutamine (Cellgro, Mediatech), 50 units/mL penicillin and 50 μg/mL streptomycin (Cellgro, Mediatech). HeLa cells (cervical cancer) were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 50 units/mL penicillin and 50 μg/mL streptomycin.

Fluorescence Reader. Fluorescence assays were performed on a Tecan SpectrafluorPlus 96-well plate reader. Total volume for each run was 200 μL. Samples were excited at 495 nm and fluorescence emission was read at 525 nm.

Flow Cytometry Assay. For assays performed from a 24 well plate, the plates were evenly seeded with cells and allowed to grow to 70-80% confluence. Cells were then incubated with the desired amount of **RhoCC** or **RhoSS** for 60 min or 6 h at 37 °C. After the removal of the media, the cells were washed with cold PBS, trypsized, and resuspended in cold culture media (cells were kept on ice until analysis). For experiments in which cellular thiols were blocked, cells were treated with the desired concentration of NEM in PBS at 37 °C for 30 min, prior to the addition of **RhoCC** or **RhoSS**. The harvested cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488-nm argon laser and a 530 band pass filter (FL1). A minimum of 10,000 events were counted for each data point. The fluorescence data were expressed as mean arbitrary fluorescence units.

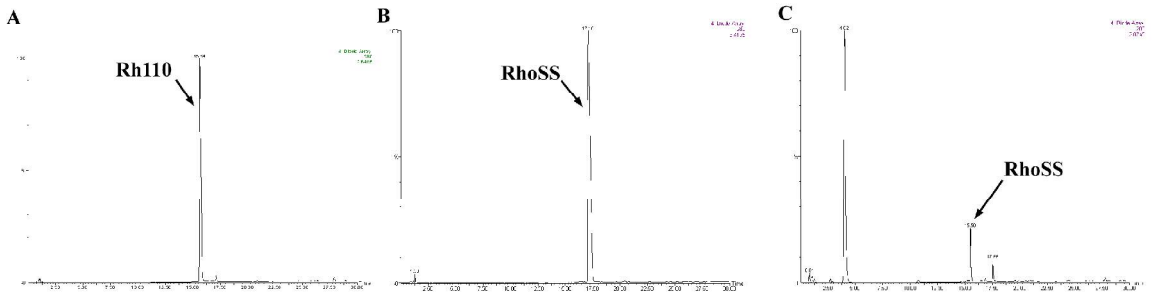
Confocal Microscopy. HeLa cells were plated onto 4-chambered Lab-Tek slides at a density of 50,000 cells per well, and were allowed to grow to 60-70% confluence. The cells were incubated with **RhoSS**, **RhoCC**, or NEM followed by **RhoSS** in DMEM media for 1h. Following this incubation, the cells were either washed with cold PBS and

fresh media was added to each well, or the cells were not washed (Figure 4e). For the co-staining experiments cells were incubated with 40 μM of **RhoSS** and 500 nM of Hoescht 33342 (Invitrogen) or 40 μM of **RhoSS** and 100 nM of MitoFluoro 589 Red (Invitrogen) in DMEM media for 4 h. Following this incubation, cells that were co-stained were washed with cold PBS and fresh media was added to each well. Images were acquired using a Radiance 2100 MP Rainbow (Bio-Rad, Hemel Hempstead, England) on a TE2000 (Nikon, Tokoyo, Japan) inverted microscope using a 60x oil 1.4 NA lens. Mitofluor Red 589, Rhodamine 110 and Hoechst 33342 fluorescence images were collected sequentially to avoid any possible bleed through. The Mitofluor Red 589 was excited at 543 nm using the green HeNe laser and the fluorescence emission greater than 560 nm in wavelength was collected. Then Rhodamine 110 was excited with the 488 nm line of the 4-line argon and the emission was collected with a 500LP, 550SP filter combination. Multi-photon excitation for the Hoechst 33342 was provided by the Mai Tai laser (Spectra-Physics, Mountain View, CA) at 750 nm and the emission between 420 and 480 nm was collected. A transmission image was also collected to show cell morphology.

In vitro **Fluorescence Unmaking.** The desired rhodamine derivative was dissolved in buffered solution (Tris-HCl 80 mM) at the specified pH. The thiol-containing solution (DTT or glutathione) was prepared fresh in the same buffer as the rhodamine solution. 100 μL of the rhodamine solution was added to a Costar 96-well plate, followed by adding 100 μL of the thiol solution. Temperature was maintained at 37 ºC. Fluorescence data was obtained by exciting at 485 nm and the emission data was collected at 535 nm every 2 min.

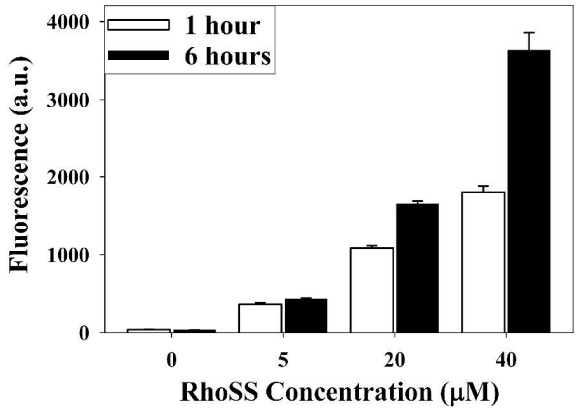
Cellular Viability (MTT Assay). HeLa cells were seeded into wells of a 96-well plate in 200 μL of cell culture media at a density of 12,000 cells per well. The cells were incubated for 18 h at 37 ºC. After removing the cell culture media, cells were incubated for 4 h at 37 ºC with fresh culture media alone or fresh culture media with varying concentrations of **RhoSS** (20 μM, 40 μM, 80 μM). Following this incubation period, the media was replaced with fresh culture media containing 0.5 mg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and cell were incubated for 4 h at 37 ºC. The media was gently removed and 200 μL of DMSO was added to each well. Absorbance was measured at 580 nm and % cell viability was calculated based on the viability of cells incubated with no **RhoSS**.

Figure 3S.



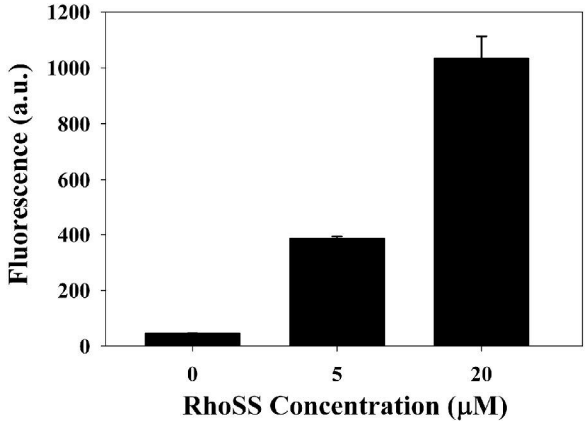
HPLC trace of Rhodamine 110 and **RhoSS**. Conditions: 0.6 mL/min flow rate, C18 analytical column, 5% A to 75% A over 30 min, detected at 280 nm. (a) rhodamine 110 (b) **RhoSS** (c) **RhoSS** after incubation for 2 h with 10 mM DTT at 37 ^{o}C pH 7.4. Assigned peaks were confirmed with ESI^{+}. Solvent A is acetonitrile with 0.1% formic acid and solvent B is water with 0.1% formic acid.

Figure 4S.



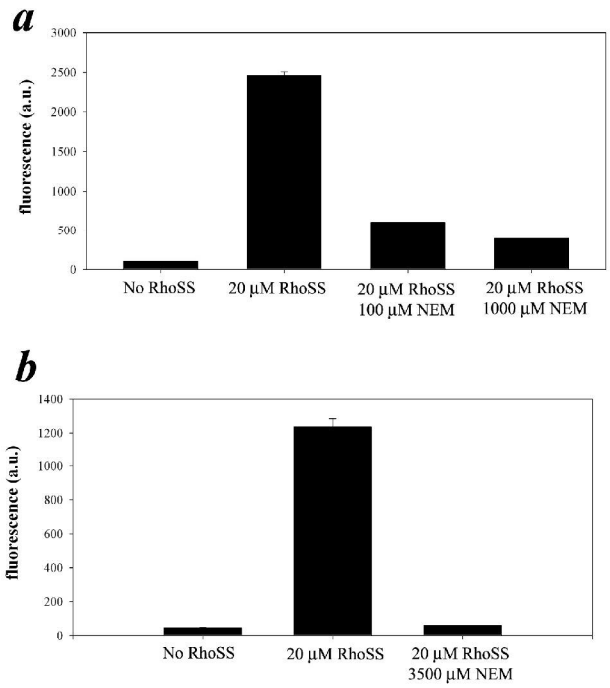
Time dependence of cellular fluorescence by flow cytometry. HeLa cells were incubated with varying concentrations of **RhoSS** for 1 and 6 h at 37 ^{o}C.

Figure 5S.



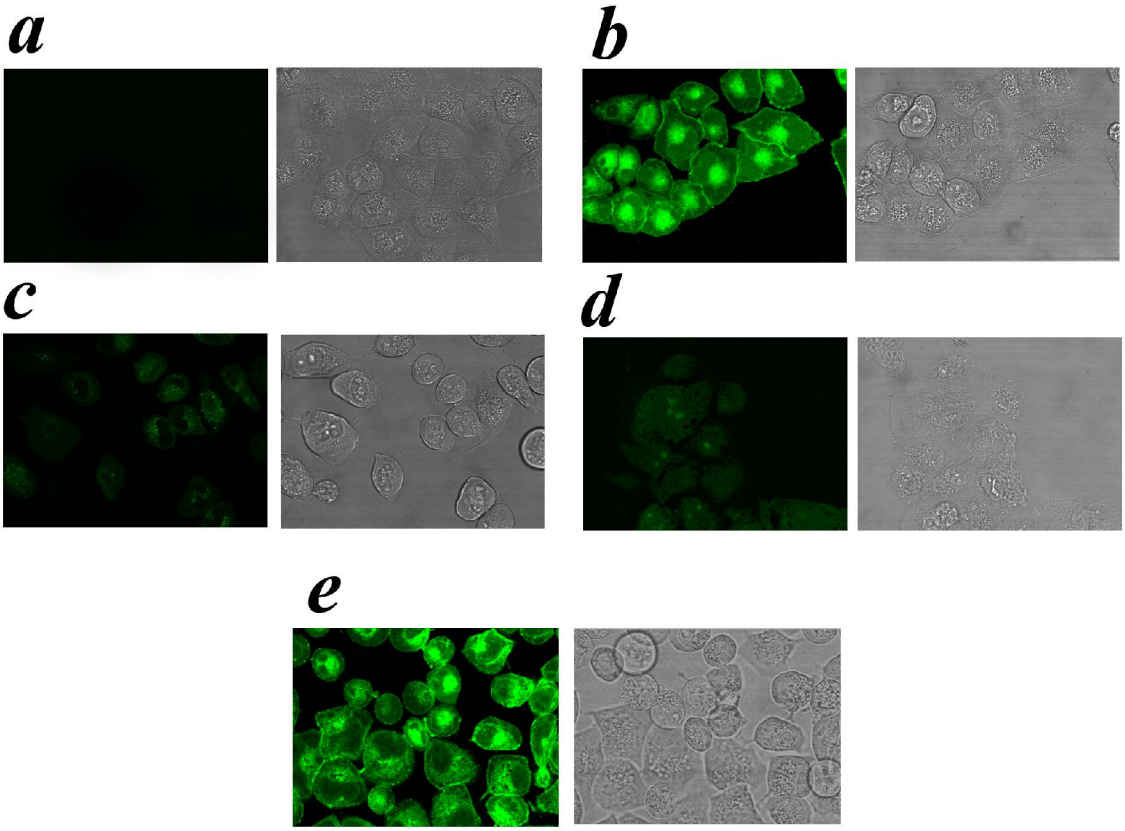
Cellular fluorescence of MCF-7 cells. MCF-7 cells were incubated with varying concentrations of **RhoSS** for 1 h at 37 ^{o}C. Uptake was quantified using FACS.

Figure 6S.



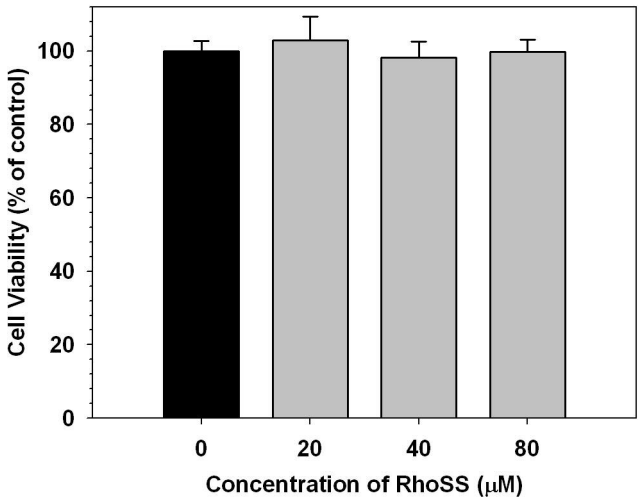
Cellular fluorescence after blocking of thiols. Thiol blocking agent (NEM) was pre-incubated with cells at the specified concentration for 30 min at 37 ^{o}C. (a) HeLa cells with and without pre-incubation with NEM followed by addition of **RhoSS**. The pre-incubation of cells with 100 μM and 1000 μM of NEM caused a major decrease in cellular fluorescence. (b) MCF-7 cells without pre-incubation of NEM showed similar fluorescence as previously shown with and without 20 μM of **RhoSS**. The pre-incubation of cells with 3500 μM with NEM followed by addition of **RhoSS** also caused a major decrease in cellular fluorescence.

Figure 7S.



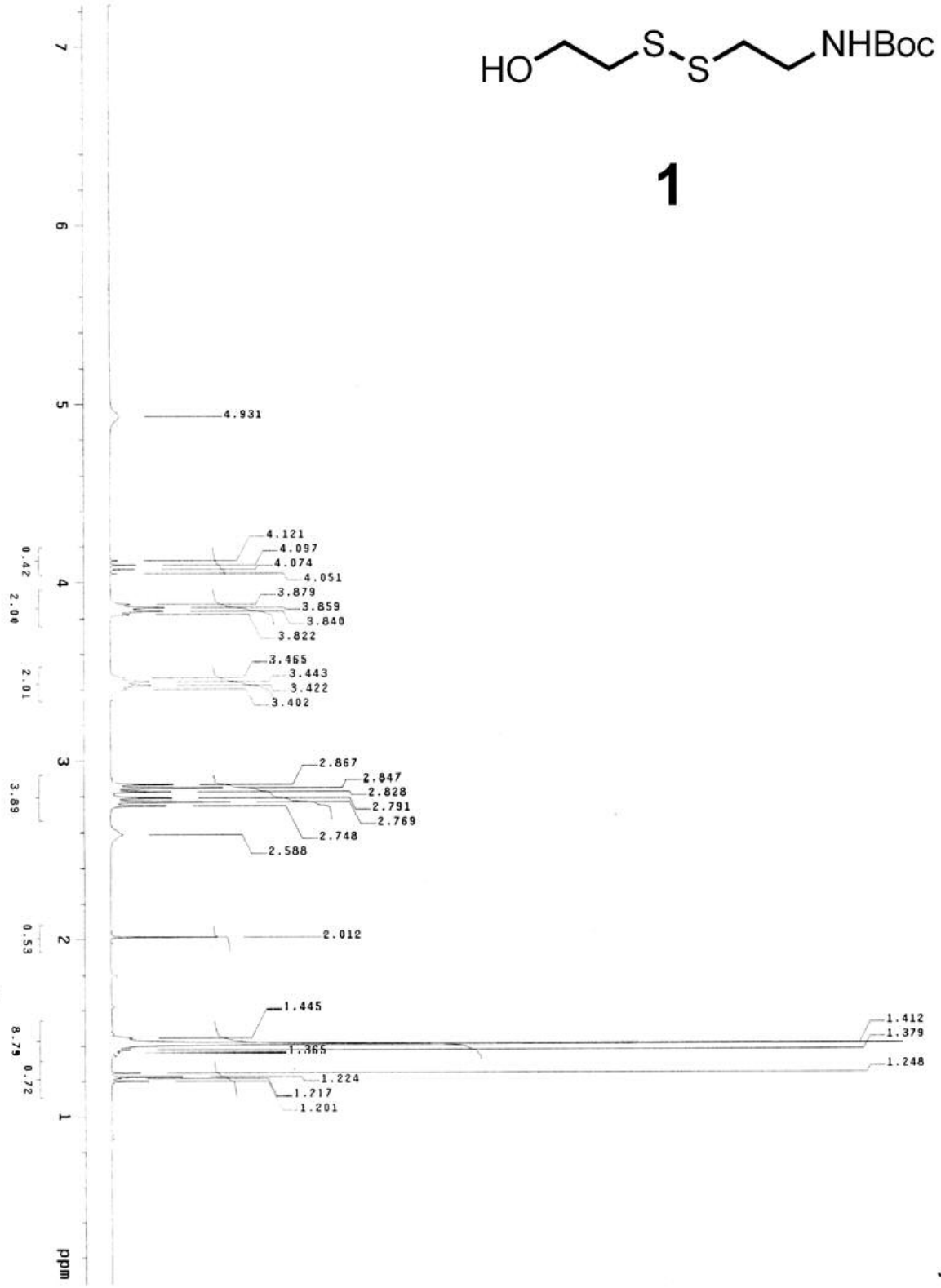
Confocal images of live HeLa cells. HeLa cells were incubated with or without specified molecule(s) for 1 h. (a) No probe (b) Treated with 20 μM of **RhoSS** (c) Treated with 20 μM of **RhoCC** (d) Pre-incubated with 100 mM of NEM (30 min) and treated with 20 μM of **RhoSS** (e) Treated with 40 μM of **RhoSS** with no washing prior to microscopy.

Figure 8S.



Cellular viability of HeLa cells incubated with **RhoSS**. HeLa cells were incubated with varying concentrations of **RhoSS** for 4 h. Viability of the cells was assessed using the MTT assay. Percent viability was determined relative to control survival.

Spectra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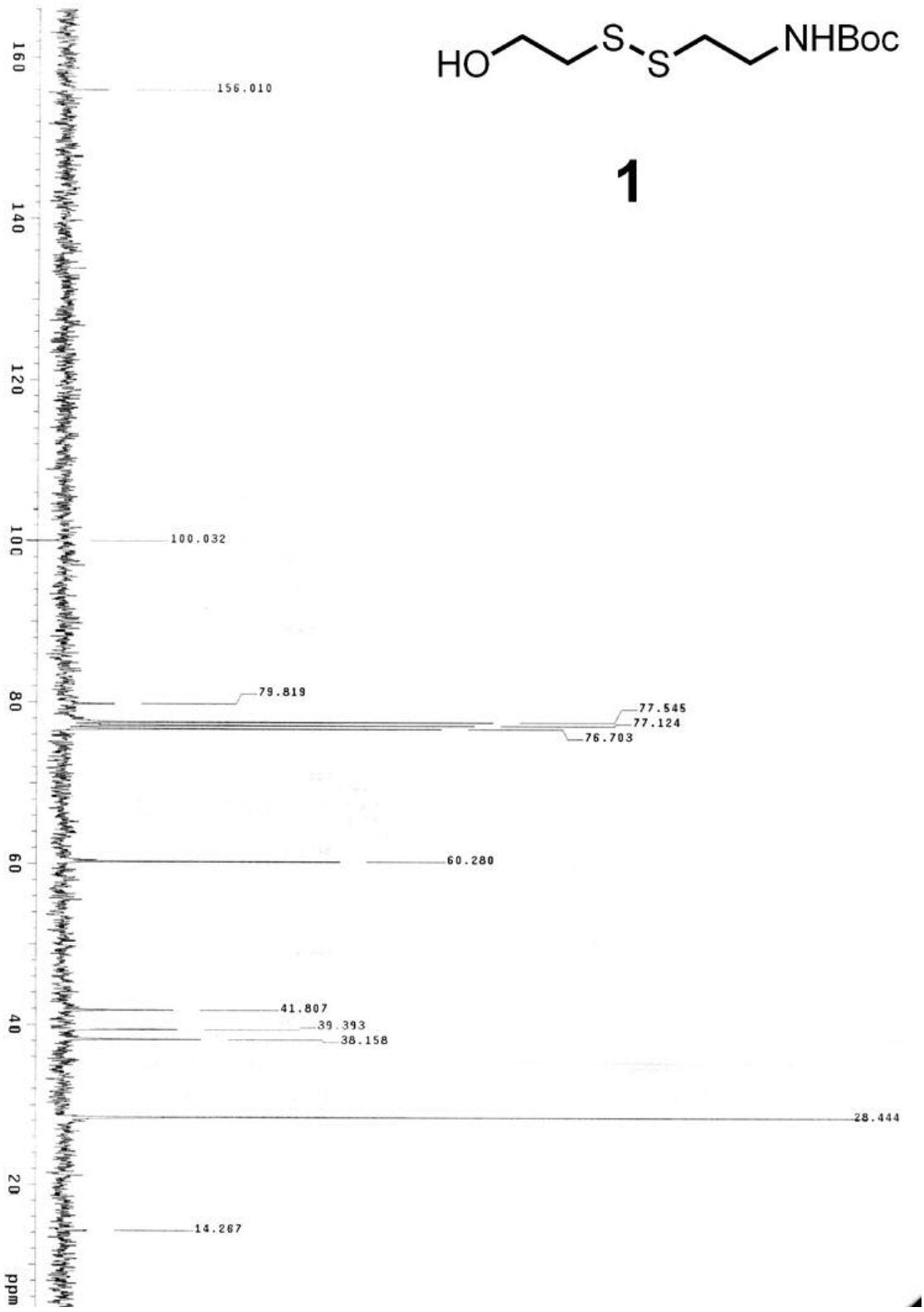
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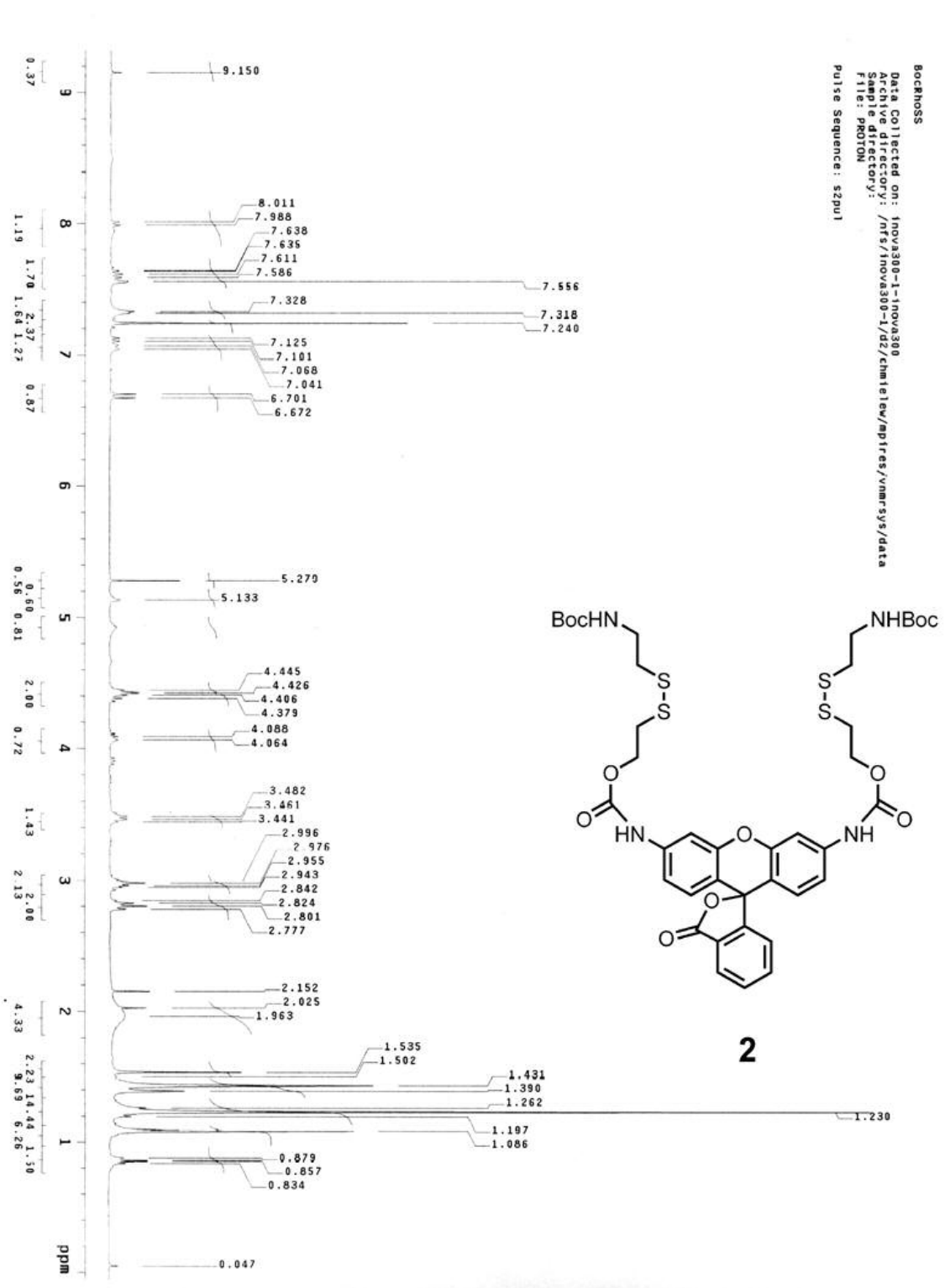
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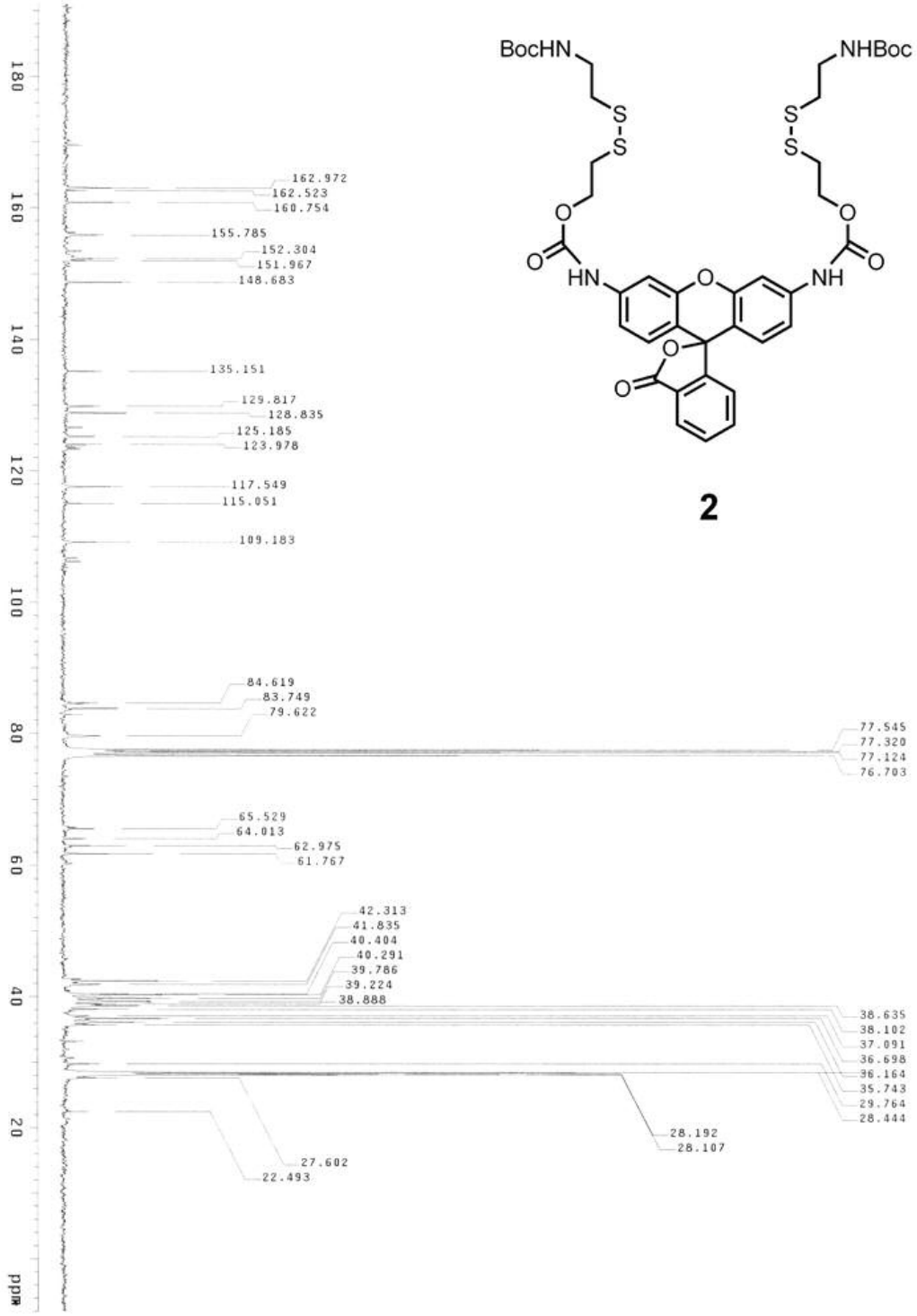
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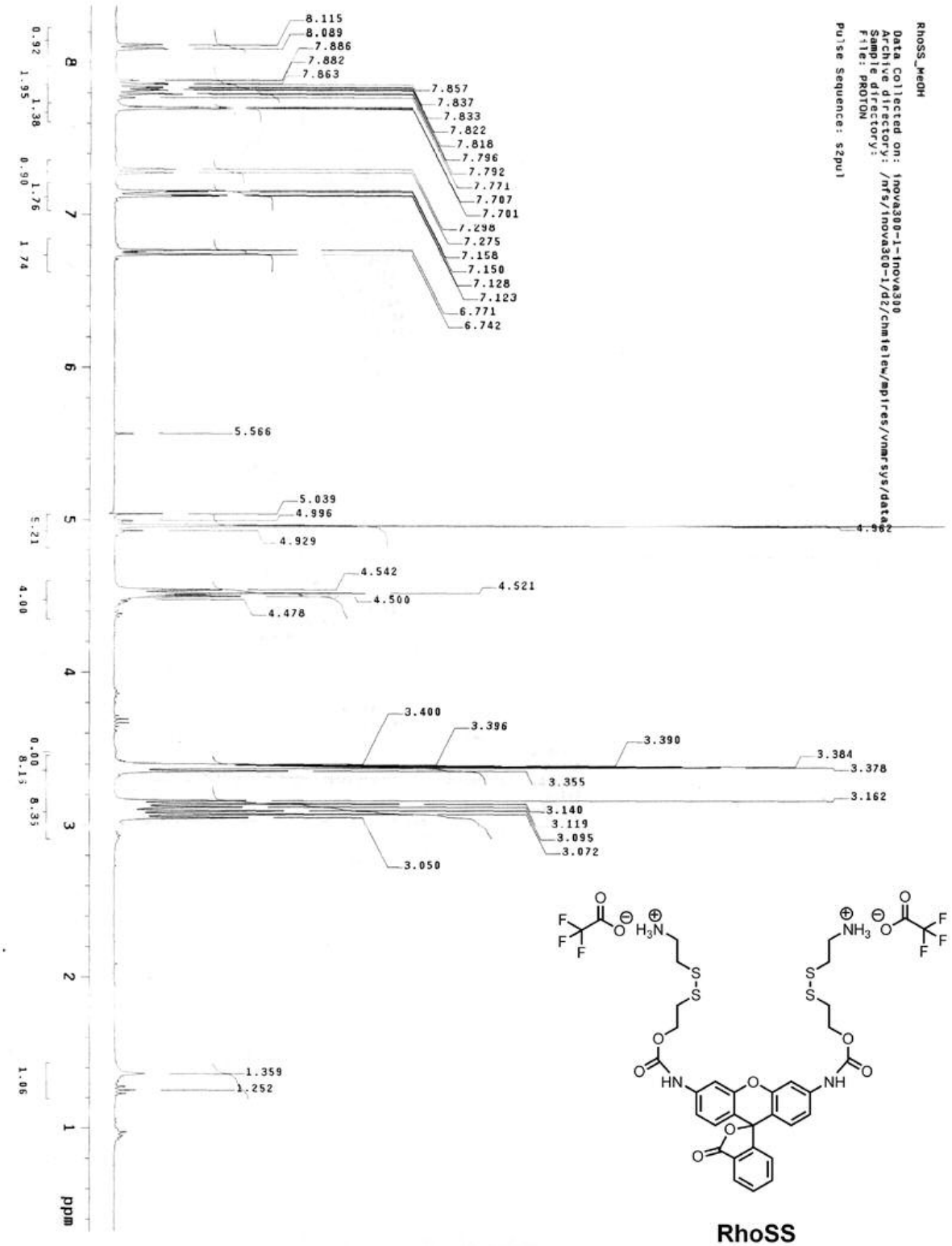
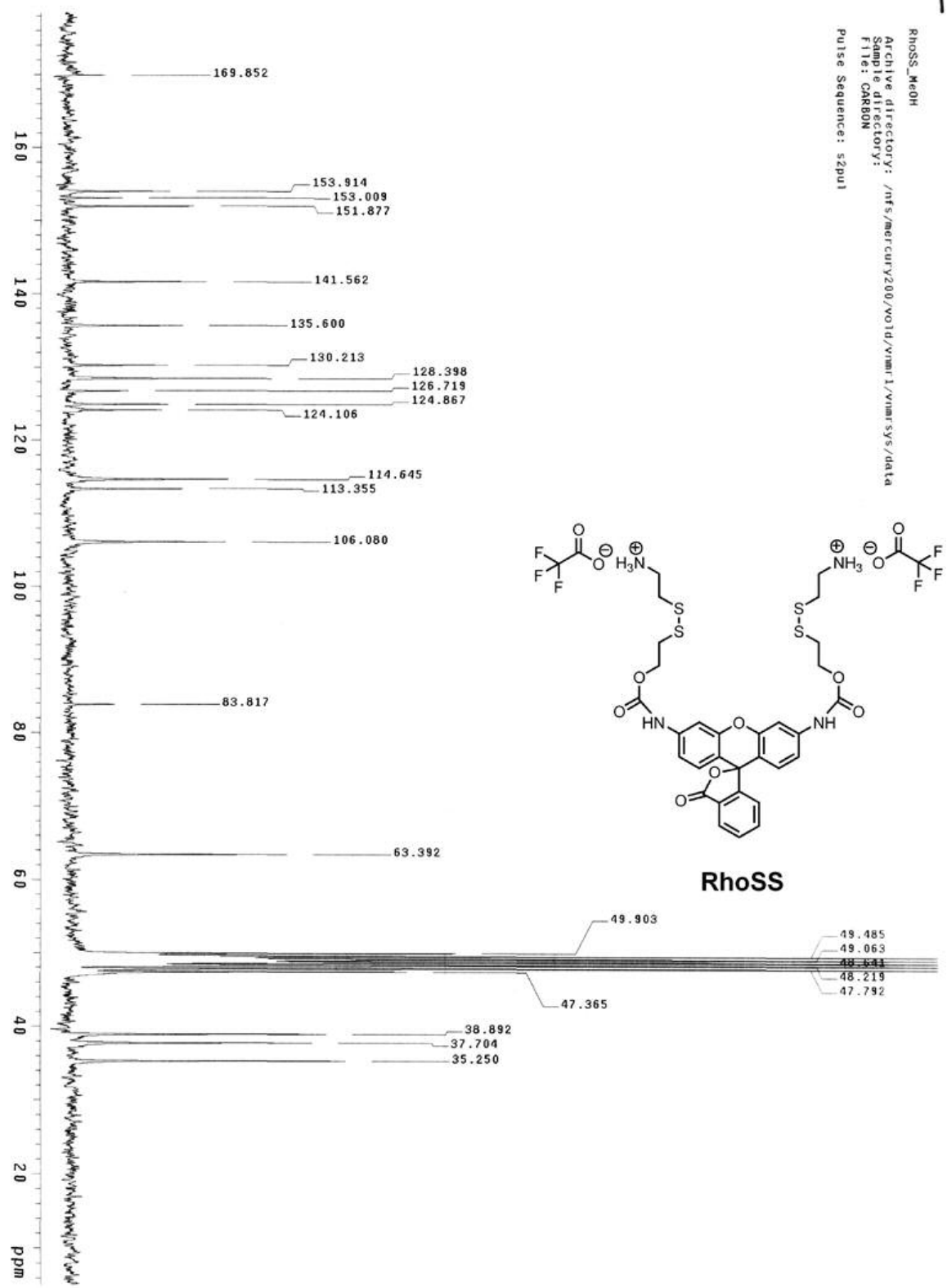
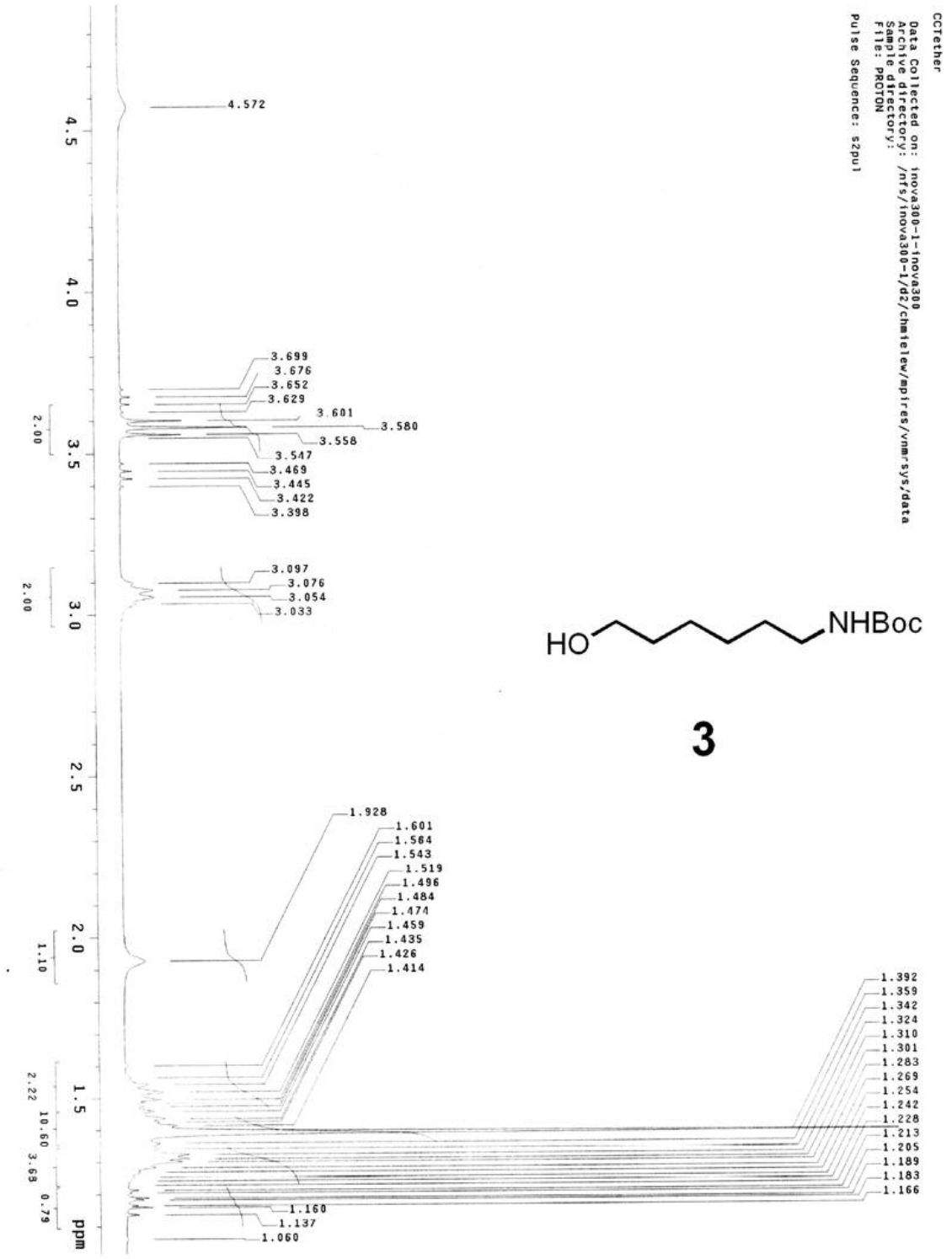
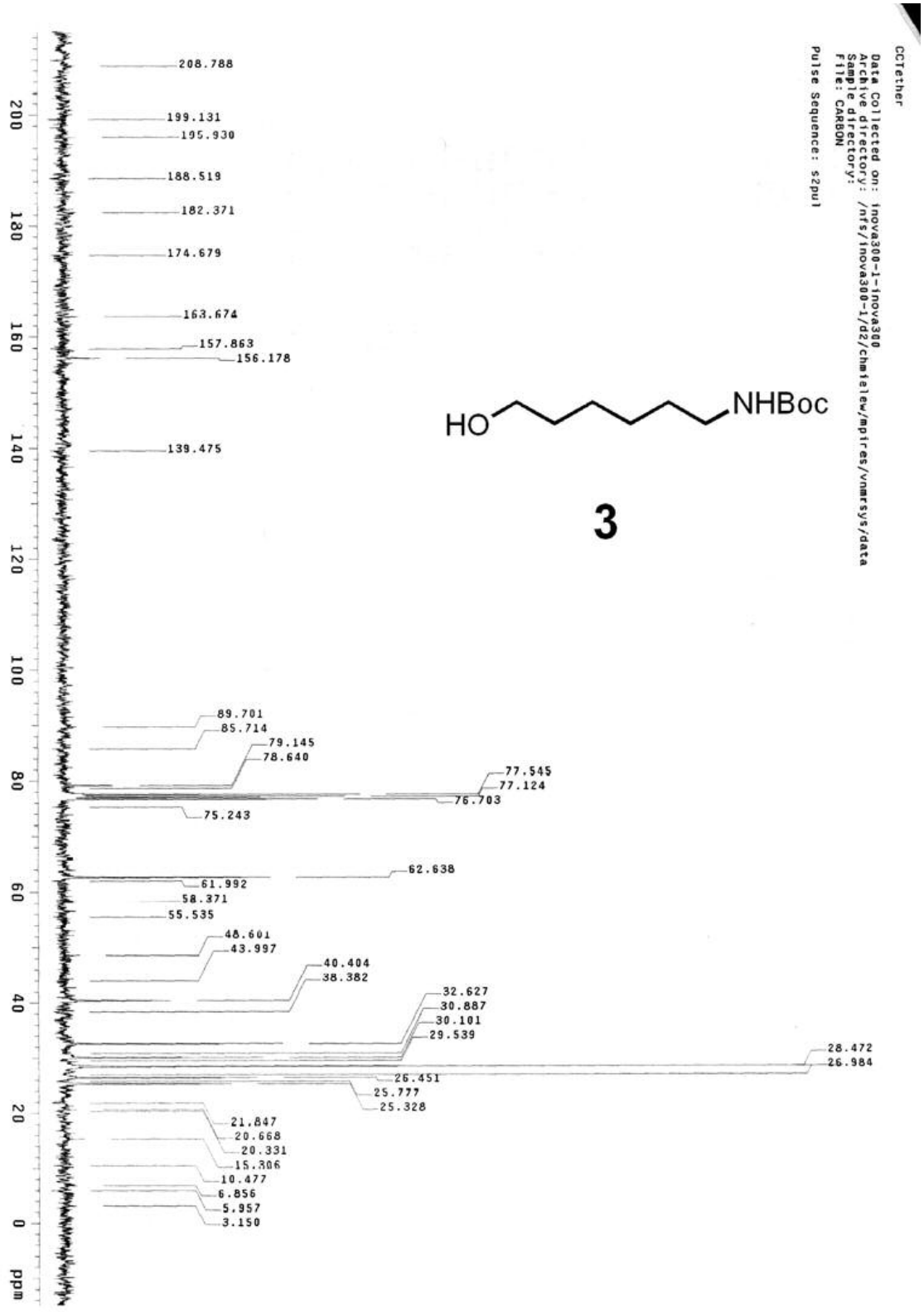
O
O
HN
NH
O
O
O
2
180
160
140
120
100
80
60
40
20
ppm
162.972
162.523
160.754
155.785
152.304
151.967
148.683
135.151
129.817
128.835
125.185
123.978
117.549
115.051
109.183
84.619
83.749
79.622
77.545
77.320
77.124
76.703
65.529
64.013
62.975
61.767
42.313
41.835
40.404
40.291
39.786
39.224
38.888
38.635
38.102
37.091
36.698
36.164
35.743
29.764
28.444
28.192
28.107
27.602
22.493

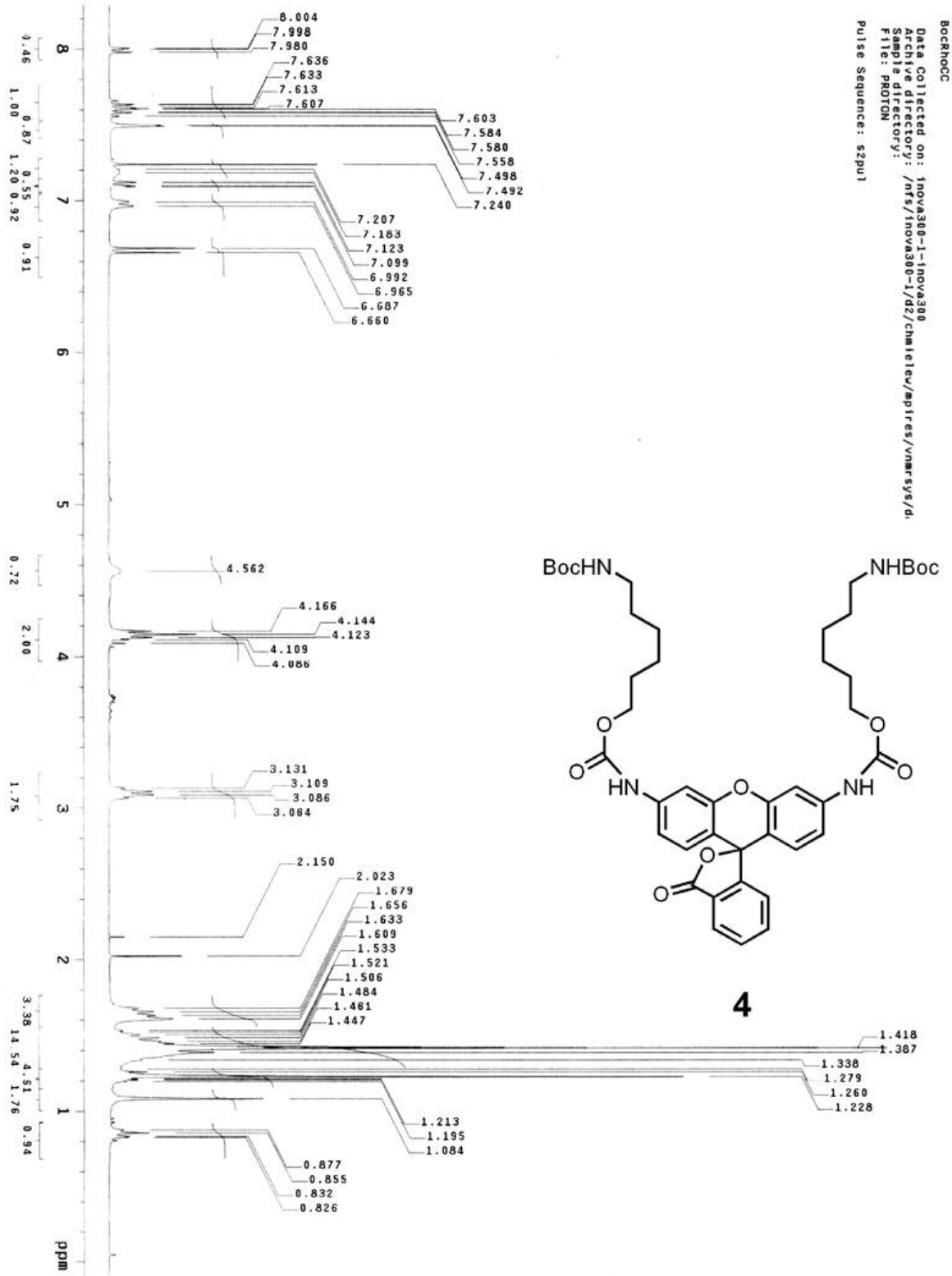

RhoSS_MeOH
Data Collected on: inova300-1-inova300
Archive directory: /nfs/inova300-1/d2/chmlelew/mpires/vnmrsys/data
Sample directory:
File: PROTON
Pulse Sequence: s2pul
8.115
8.089
7.886
7.882
7.863
7.857
7.837
7.833
7.822
7.818
7.796
7.792
7.771
7.707
7.701
7.298
7.275
7.158
7.150
7.128
7.123
6.771
6.742
5.566
5.039
4.996
4.962
4.929
4.542
4.521
4.500
4.478
3.400
3.396
3.390
3.384
3.378
3.355
3.162
3.140
3.119
3.095
3.072
3.050
1.359
1.252
ppm

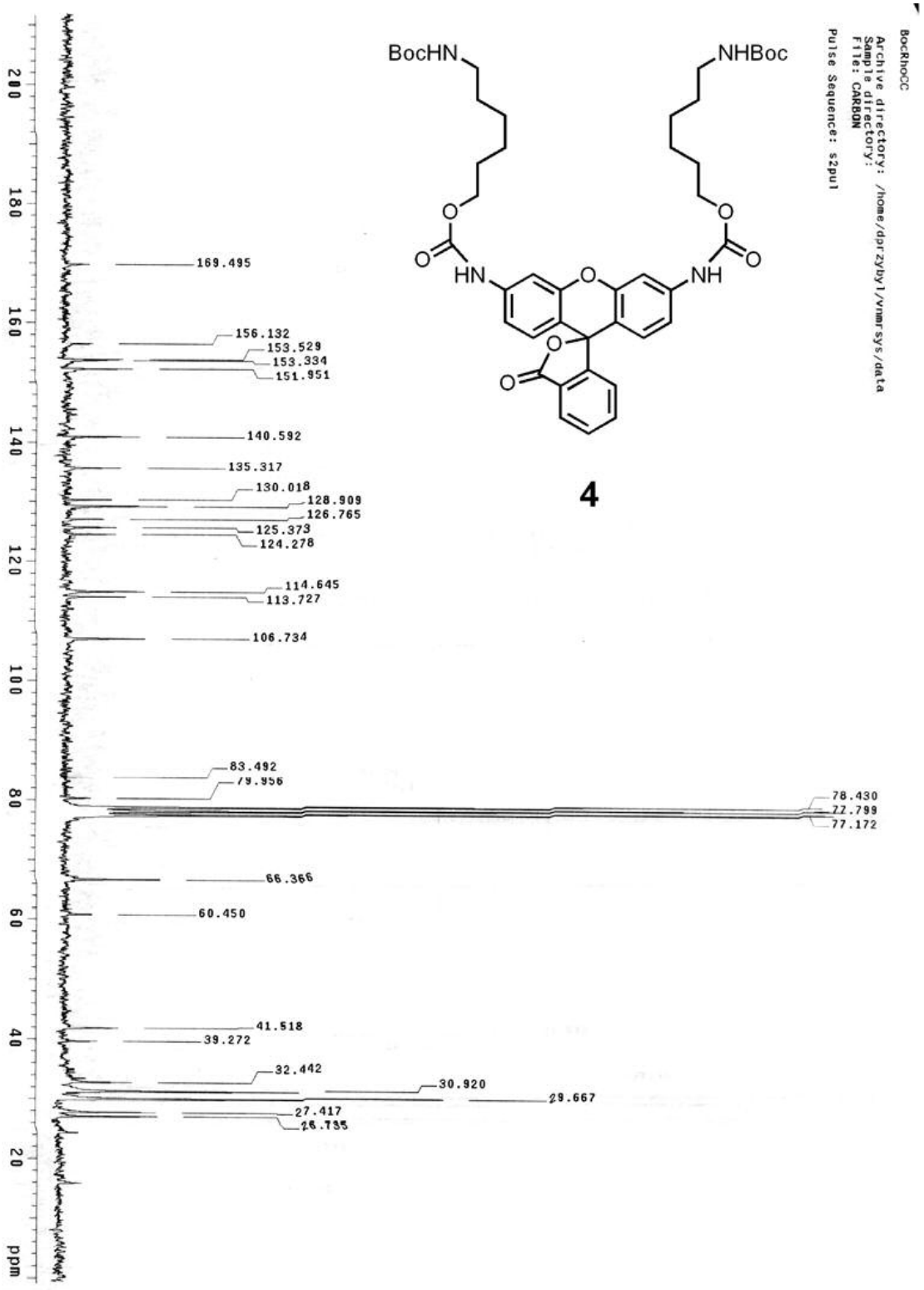
RhoSS

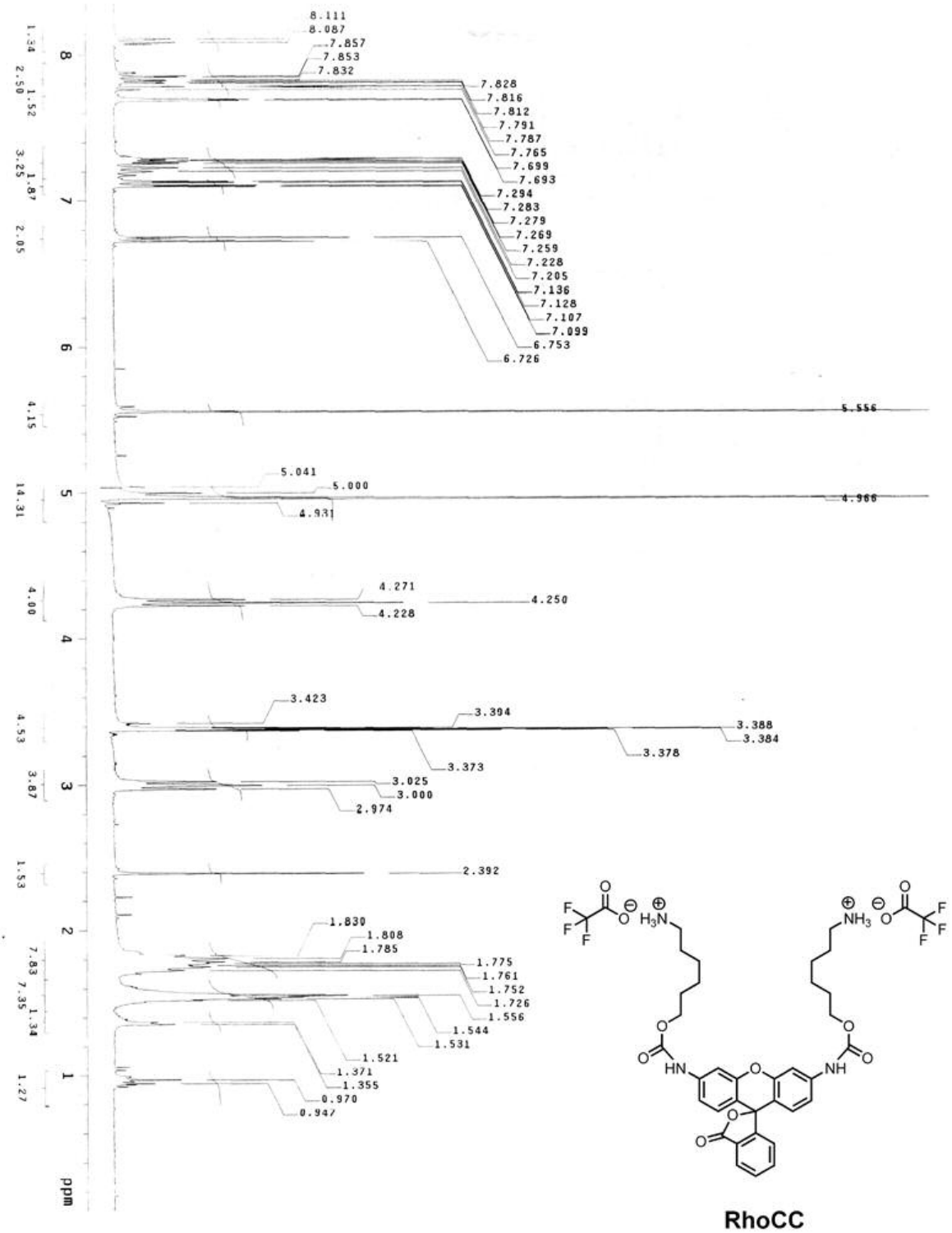

RhoSS_MeOH
Archive directory: /nfs/mercury200/vold/vnmr1/vnmrsys/data
Sample directory:
File: CARBON
Pulse Sequence: s2pul
169.852
153.914
153.009
151.877
141.562
135.600
130.213
128.398
126.719
124.867
124.106
114.645
113.355
106.080
83.817
63.392
49.903
49.485
49.063
48.641
48.219
47.792
47.365
38.892
37.704
35.250
160
140
120
100
80
60
40
20
ppm
RhoSS


CCTether
Data Collected on: inova300-1-inova300
Archive directory: /nfs/inova300-1/d2/chmielew/mpires/vnmrsys/data
Sample directory:
File: PROTON
Pulse Sequence: s2pul
4.572
3.699
3.676
3.652
3.629
3.601
3.580
3.558
3.547
3.469
3.445
3.422
3.398
3.097
3.076
3.054
3.033
1.928
1.601
1.564
1.543
1.519
1.496
1.484
1.474
1.459
1.435
1.426
1.414
1.392
1.359
1.342
1.324
1.310
1.301
1.283
1.269
1.254
1.242
1.228
1.213
1.205
1.189
1.183
1.166
1.160
1.137
1.060
HO
NHBoc
3
ppm

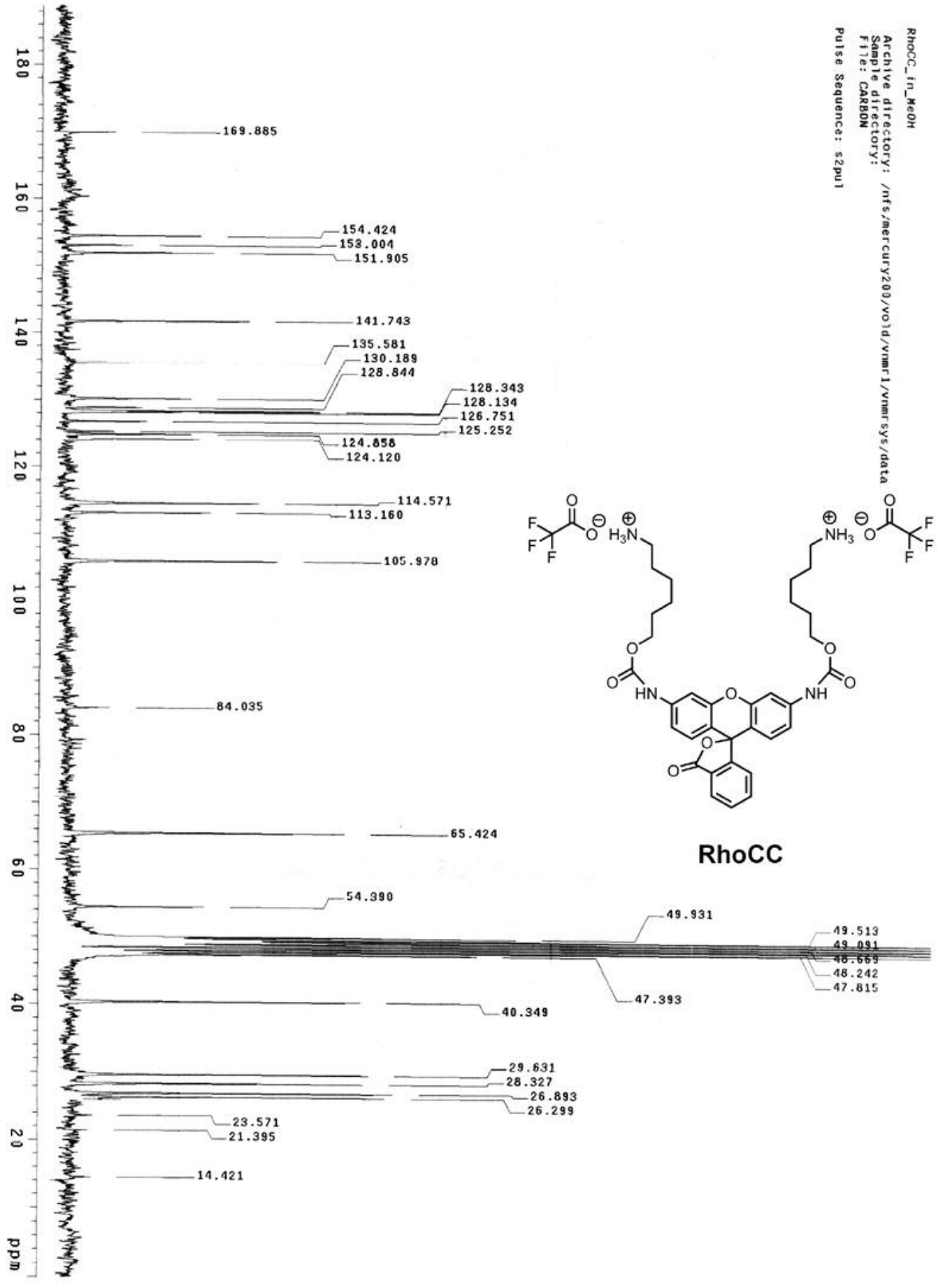

CCTether
Data Collected on: inova300-1-inova300
Archive directory: /nfs/inova300-1/d2/chmielew/mpires/vnmrsys/data
Sample directory:
File: CARBON
Pulse Sequence: s2pul
HO
NHBoc
3
208.788
199.131
195.930
188.519
182.371
174.679
163.674
157.863
156.178
139.475
89.701
85.714
79.145
78.640
77.545
77.124
76.703
75.243
62.638
61.992
58.371
55.535
48.601
43.997
40.404
38.382
32.627
30.887
30.101
29.539
28.472
26.984
26.451
25.777
25.328
21.847
20.668
20.331
15.306
10.477
6.856
5.957
3.150
200
180
160
140
120
100
80
60
40
20
0
ppm


BocRhoCC
Data Collected on: inova300-1-inova300
Archive directory: /nfs/inova300-1/d2/chamlew/mpires/vnmrsys/d.
Sample directory:
File: PROTON
Pulse Sequence: s2pul
8.004
7.998
7.980
7.636
7.633
7.613
7.607
7.603
7.584
7.580
7.558
7.498
7.492
7.240
7.207
7.183
7.123
7.099
6.992
6.965
6.687
6.660
4.562
4.166
4.144
4.123
4.109
4.086
3.131
3.109
3.086
3.084
2.150
2.023
1.679
1.656
1.633
1.609
1.533
1.521
1.506
1.484
1.461
1.447
1.418
1.387
1.338
1.279
1.260
1.228
1.213
1.195
1.084
0.877
0.855
0.832
0.826
ppm
BocHN
NHBoc
HN
NH
O
4


BocRhoCC
Archive directory: /home/dprzyby1/vnmrsys/data
Sample directory:
File: CARBON
Pulse Sequence: s2pul
BocHN
NHBoc
HN
NH
O
O
O
O
O
O
4
169.495
156.132
153.529
153.334
151.951
140.592
135.317
130.018
128.909
126.765
125.373
124.278
114.645
113.727
106.734
83.492
79.956
78.430
77.799
77.172
66.366
60.450
41.518
39.272
32.442
30.920
29.667
27.417
26.735
200
180
160
140
120
100
80
60
40
20
ppm


8.111
8.087
7.857
7.853
7.832
7.828
7.816
7.812
7.791
7.787
7.765
7.699
7.693
7.294
7.283
7.279
7.269
7.259
7.228
7.205
7.136
7.128
7.107
7.099
6.753
6.726
5.556
5.041
5.000
4.966
4.931
4.271
4.250
4.228
3.423
3.394
3.388
3.384
3.378
3.373
3.025
3.000
2.974
2.392
1.830
1.808
1.785
1.775
1.761
1.752
1.726
1.556
1.544
1.531
1.521
1.371
1.355
0.970
0.947
ppm

RhoCC


RhoCC_in_MeOH
Archive directory: /nfs/mercury200/void/vnmr1/vnmrsys/data
Sample directory:
File: CARBON
Pulse Sequence: s2pul
169.885
154.424
153.004
151.905
141.743
135.581
130.189
128.844
128.343
128.134
126.751
125.252
124.858
124.120
114.571
113.160
105.978
84.035
65.424
54.390
49.931
49.513
49.091
48.669
48.242
47.815
47.393
40.349
29.631
28.327
26.893
26.299
23.571
21.395
14.421
ppm
RhoCC