Isothiocyanates as H_2S Donors Triggered by Cysteine: Reaction Mechanism and Structure and Activity Relationship

Yi Lin,[†] Xin Yang,[†] Yuyun Lu, Dong Liang,[‡] Dejian Huang*[, †, §]

[†] Food Science and Technology Programme, Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore

[‡] Food Chemistry and Molecular Sensory Science, Technische Universität München, Lise-Meitner-Straße 34, 85354 Freising, Germany

[§] National University of Singapore (Suzhou) Research Institute, 377 Lin Quan Street, Suzhou Industrial Park, Jiangsu 215123, China.

Supporting Information:

Tables of Contents

1. Experimental Section.

1.1. Chemicals Reagents.

L-cysteine, 4-methoxyphenylisothiocyanate (MeOPITC), phenylisothiocyanate (PITC), 4-chlorophenylisothiocyanate (ClPITC), 4-nitrophenylisothiocyanate (NO2PITC), benzylisothiocyanate (BITC), phenethylisothiocyanate (PEITC), and fluorescein isothiocyanate isomer I (FITC), sodium sulfide (Na_2S), N,N-dimethylformamide (DMF), formic acid, acetonitrile (ACN), sodium deuteroxide solution (NaOD), hydrochloric acid (HCl) and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich (Singapore, Singapore). Phosphate buffered saline (ultra-pure grade) was purchased from Vivantis Technologies (Selangor, Malaysia). Dulbecco's Modified Eagle Medium (DMEM) was purchased from HyClone Laboratories, Inc. (Utah, USA).

1.2. H_2S releasing capacity of isothiocyanates upon reaction with cysteine.

The evidence of H_2S releasing from the isothiocyanate was qualitatively confirmed by colorimetric assay using a lead (II) acetate paper, which was prepared by dipping commercial filter paper into 20 mM lead acetate aqueous solution for 30 seconds, and the pre-treated filter paper was dried at 40 ℃ under reduced pressure for 24 h. The dried filter paper was cut into 12 mm small circular shape before use. The papers were attached on top of the inner cover of each 24 well plate. ITCs with 6 different concentrations (from 0.2 mM to 1.2 mM) were prepared in DMF. L-cysteine with same concentration were prepared in PBS (10 mM, pH 7.4). The ITC solution (0.2 mL) was gently mixed with L-cysteine solution (0.4 mL). Then the pH of mixture was adjusted to 7.4 by using 0.1 M HCl or NaOH and appropriate amount of PBS (10 mM, pH 7.4) solution was added to ensure the final volume of mixture is 1.0 mL. The plates were incubated at 37 ℃ for 24 h. During incubation, the produced H_2S evaporated into the headspace of the microplate and reacted with the lead (II) acetate on the test paper, resulting in the black lead (II) sulfide (PbS). The paper was removed from the cover, and its colour was measured with a colorimeter (Konica Minolta, Inc., Japan) with an 8 mm probe. The standard curve was established by using Na_2S. The results were expressed in Hunter L* (0 = Blank to 100 diffuse white), a* (negative values indicate green, while positive values indicate red) and b* (negative values indicate blue and positive ones indicate yellow) values. The colour intensity (CI) of a sample was calculated by:

$$\mathrm{CI} = \sqrt{(100 - \mathrm{L}*)^2 + a*^2 + b*^2}$$

1.3. Determination of reaction rate constants for aryl isothiocyanates with cysteine.

The first order rate constants for the reaction of aryl isothiocyanates with L-cysteine (2 equivalents) were determined by measuring the concentration of H_2S. The amount of H_2S was analysed by lead acetate paper method mentioned above. All first-order rate constants were derived from at least three independent experiments each employing 4-5 different substrate concentrations with 5 kinetic traces averaged at each concentration.

1.4. General reaction procedure of isothiocyanates with L-cysteine for generation of 2-alkyl(aryl)amino-4,5-dihydro-4-thiazolecarboxylic acid, 5.

A solution of isothiocyanates (0.4 mmol) in 20 mL of DMF was added to a solution of L-cysteine (0.8 mmol) in 80 mL of PBS (10 mM, pH 7.4). Then the pH of the reaction mixture was adjusted to 7.4 by using 1 M HCl or 1 M NaOH. The reaction was conducted at 37 ℃ for 24 h. The reaction mixture was prepared as mentioned above. The mixture was sequentially extracted with dimethyl chloride (DCM) and ethyl acetate (EA). Then aqueous layer was concentrated and purified by semi-prep HPLC. Isolations were performed on a Waters HPLC system equipped with 717 plus auto-sampler, a Waters 515 HPLC pump, and a 2996 photodiode array (PDA) detector (Waters, Ireland). The column used was a 250 × 10 mm i.d., 5 μm, C18 (Luna, Phenomenex, USA). The mobile phase consisting of 0.1% formic acid in H_2O and 0.1% formic acid in ACN. The flow rate was 5.0 mL/min and the column temperature was 25 ℃. ^1H and ^{13}C NMR spectra were recorded with an AX 400 spectrometer at 400 MHz or an AV500 spectrometer (Bruker, Karlsruhe, Germany) at 500 MHz.

2-(Phenylamino)-4,5-dihydro-4-thiazolecarboxylic acid (5-Ph). Compound 4a was obtained as white solid. Yield: 9.0 mg (10%). ^1H NMR (500 MHz, D_2O, NaOD) δ 7.33 – 7.25 (m, 2H), 7.11 – 7.01 (m, 3H), 4.37 (dd, J = 8.1, 5.6 Hz, 1H), 3.51 (dd, J = 11.1, 8.0 Hz, 1H), 3.31 (dd, J = 11.2, 5.6 Hz, 1H). ^{13}C NMR (126 MHz, D_2O, NaOD) δ 178.68, 165.61, 147.93, 129.38, 129.38, 124.10, 122.05, 122.05, 64.04, 34.08. HR-MS (ESI positive): *m/z* calculated for $[C_{10}H_{11}N_2O_2S + H]^+$ 223.5036; found 223.0536.

2-(*p*-Chlorophenylamino)-4,5-dihydro- 4-thiazolecarboxylic acid (5-ClPh). Compound 4b was obtained as white solid. Yield: 19 mg (18%). ^1H NMR (500 MHz, D_2O, NaOD) δ 7.41 – 7.25 (m, 2H), 7.12 – 7.00 (m, 2H), 4.42 (dd, J = 8.0, 5.4 Hz, 1H), 3.58 (dd, J = 11.2, 8.0 Hz, 1H), 3.38 (dd, J = 11.1, 5.5 Hz, 1H). ^{13}C NMR (126 MHz, D_2O, NaOD) δ

178.54, 165.93, 146.98, 129.14, 129.14, 128.50, 123.58, 123.58, 63.65, 34.04. HR-MS (ESI positive): *m/z* calculated for $[C_{10}H_{10}ClN_2O_2S + H]^+$ 257.0146; found: 257.0150.

2-(*p*-Nitrophenylamino)-4,5-dihydro-4-thiazolecarboxylic acid (5-NO2Ph). Compound 4c was obtained as yellow solid. Yield: 38 mg (36%). ^{1}H NMR (400 MHz, D_2O, NaOD) δ 8.06 – 7.95 (m, 2H), 7.19 – 7.08 (m, 2H), 4.46 (dd, J = 8.2, 5.7 Hz, 1H), 3.55 (dd, *J* = 11.1, 8.3 Hz, 1H), 3.35 (dd, *J* = 11.1, 5.7 Hz, 1H). ^{13}C NMR (101 MHz, D_2O, NaOD) δ 178.55, 165.04, 154.15, 142.09, 125.30, 125.30, 121.36, 121.36, 65.85, 34.59. HR-MS (ESI positive): *m/z* calculated for $[C_{10}H_{10}N_3O_4S + H]^+$ 268.0387; found: 268.0386.

2-(*p*-Methoxyphenylamino)-4,5-dihydro-4-thiazolecarboxylic acid (5-MeOPh). Compound 4d was obtained as white solid. Yield: 11mg (11%). ^{1}H NMR (500 MHz, D_2O, NaOD) δ 7.08 – 7.02 (m, 2H), 6.99 – 6.91 (m, 2H), 4.42 (dd, *J* = 8.0, 5.5 Hz, 1H), 3.79 (s, 3H), 3.58 (dd, *J* = 11.0, 8.0 Hz, 1H), 3.37 (dd, *J* = 11.1, 5.4 Hz, 1H). ^{13}C NMR (126 MHz, D2O, NaOD) δ 178.48, 166.64, 155.78, 141.09, 123.70, 123.70, 114.74, 114.74, 63.82, 55.74, 34.08. HR-MS (ESI positive): *m/z* calculated for $[C_{11}H_{13}N_2O_3S + H]^+$ 253.0461; found: 253.0646.

2-(Benzylamino)-4,5-dihydro-4-thiazolecarboxylic acid (5-Bz). Compound 4e was obtained as white solid. Yield: 8.7 mg (9.2%).^{1}H NMR (400 MHz, D_2O, NaOD) δ 7.38 – 7.21 (m, 5H), 4.52 (dd, *J* = 8.4, 6.2 Hz, 1H), 4.42 (d, *J* = 15.2 Hz, 1H), 4.35 (d, *J* = 15.2 Hz, 1H), 3.54 (dd, *J* = 10.9, 8.4 Hz, 1H), 3.31 (dd, *J* = 10.9, 6.2 Hz, 1H). ^{13}C NMR (126 MHz, D_2O, NaOD) δ 180.33, 171.09, 165.51, 138.84, 128.80, 128.80, 127.41, 127.41, 73.82, 48.43, 37.32. HR-MS (ESI positive): *m/z* calculated for $[C_{11}H_{13}N_2O_2S + H]^+$ 237.0692; found: 237.0695.

2-(Phenethylamino)-4,5-dihydro- 4-thiazolecarboxylic acid (5-PE). Compound 4f was obtained as white solid. Yield: 5.2 mg (5.2%).^{1}H NMR (400 MHz, D_2O, NaOD) δ 7.39 – 7.14 (m, 5H), 4.52 (dd, *J* = 8.4, 6.1 Hz, 1H), 3.56 – 3.36 (m, 3H), 3.27 (dd, *J* = 10.9, 6.2 Hz, 1H), 2.82 (t, *J* = 6.9 Hz, 2H). ^{13}C NMR (101 MHz, D_2O, NaOD) δ 180.44, 165.35, 139.67, 129.01, 129.01, 128.5, 128.64, 126.45, 74.09, 45.77, 37.20, 34.75. HR-MS (ESI positive): *m/z* calculated for $[C_{12}H_{15}N_2O_2S + H]^+$ 251.0849; found: 251.0851.

1.5. LC-MS^n characterization of reaction products of *p*-chlorophenyl-NCS and cysteine.

The samples (30 μL) were filtered through 0.2 μm membrane (Merck Millipore, USA) before being injected into the LC-MS/MS system. LC- MS^2 was acquired using a Bruker Amazon ion trap mass spectrometer (Billerica, MA, USA) equipped with a Dionex Ultimate 3000 rapid separation LC system (Bannockburn, IL). The heated capillary and spray voltage were maintained at 250 °C and 4.5 kV, respectively. Nitrogen was operated at 80 psi for sheath gas flow rate and 20 psi for auxiliary gas flow rate. The MS^2 collision gas was helium with collision energy of 30% of the 5 V end-cap maximum tickling voltage. The full scan mass spectra from *m/z* 100-1500 were acquired in both positive and negative ion mode with a scan speed at one scan per second. The column used was a 250 × 4.6 mm i.d., 5 μm, C18 (Luna, Phenomenex, USA). A binary mobile phase consisting of 0.1% formic acid in H_2O and 0.1% formic acid in acetonitrile was employed. The column was equilibrated with 100% solvent A (0.1% formic acid in water) for 10 min before gradient elution of solvent A ratio from 100% to 0% in 48 min in linear increase at a flow rate of 1.0 mL/min, and then remain 100% solvent B (0.1% formic acid in acetonitrile) for the following 12 min.

1.6. Quantitation of 2-alkyl(aryl)amino-4,5-dihydro-4-thiazolecarboxylic acid and raphanusamic acid in reaction mixture.

HPLC analysis was carried out on a Waters HPLC system (Milford, MA, USA) with an Alliance 2659 separation module and a 2996 photodiode array (PDA) detector. The method are the same as LC-MS^2 section. Detection and quantification were performed at 235 and 254 nm. Cyclic compounds were quantified based on external standards using six-point analytical curves ($R^2 > 0.99$) for **5-Ph** (0.27-1.79 mM), **5-ClPh** (0.13-1.33 mM), **5-NO2Ph** (0.28 -1.84 mM), **5-MeOPh** (0.15-0.97 mM), **5-Bz** (0.24-1.59 mM), **5-PE** (0.22-1.48 mM) and raphanusamic acid (0.61-6.12 mM).

1.7. Cell culture.

RAW 264.7 cell line was purchased from the American Type Culture Collection (Rock-vile, MD), and were grown in DMEM medium at 37 ℃ with 5% CO_2. RAW 264.7 cells were seeded into cell culture flasks at a density of 10^5. After 24 h, to allow cell attachment, the medium was replaced and the cells were treated for 24 h with FITC at the concentration of 100 μM. At the end of treatment, the medium sample was collected and passed through a C18

SPE cartridge (1 g, Strata, Phenomenex Inc, USA) pre-conditioned with methanol and water. After washing with water and 50% methanol, metabolites were eluted with 12 mL methanol. After evaporation, the sample was suspended in 200 μL methanol. LC-MS^2 analysis was performed under the same condition as LC-MS^2 section.

2. Data

2.1. Dose response curve of H_2S and the concentration of ITCs

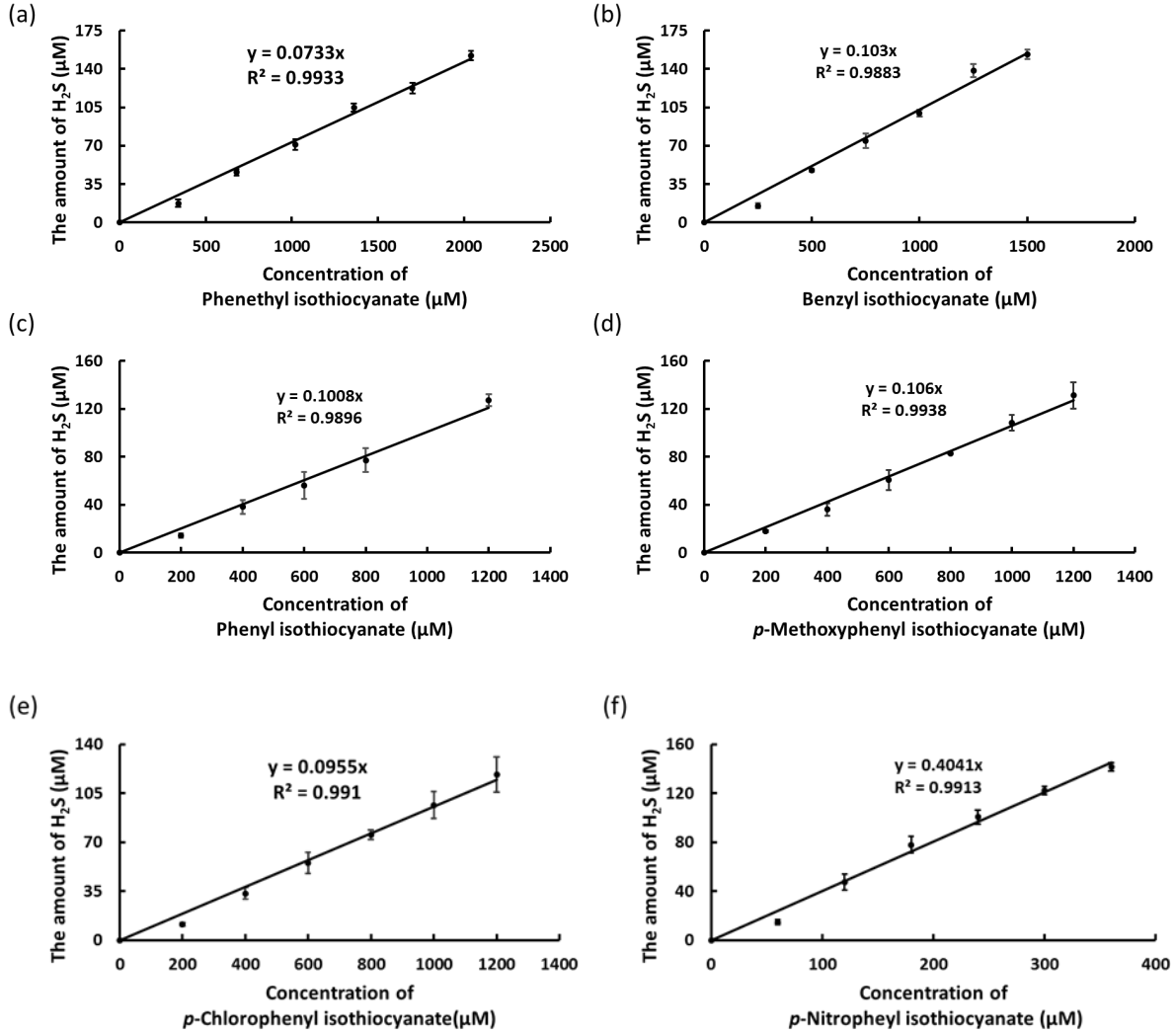


Figure S1. The dose response curve of the amount of H_2S produced in 24h and the concentration of ITCs. (a) Phenethyl isothiocyanate; (b) Benzyl isothiocyanate; (c) phenyl isothiocyanate; (d) *p*-Methoxyphenyl isothiocyanate; (e) *p*-Chlorophenyl isothiocyanate; (f) *p*-Nitrophenyl isothiocyanate; (c) and (d) *p*-Chlorophenyl isothiocyanate; (e) and (f) phenyl isothiocyanate; (g) and (h) *p*-methoxyphenyl isothiocyanate. Cysteine concentration is two times of the ITCs.

2.2. Kinetics of H_2S releasing by aryl isothiocyanates in the presence of L-cysteine.

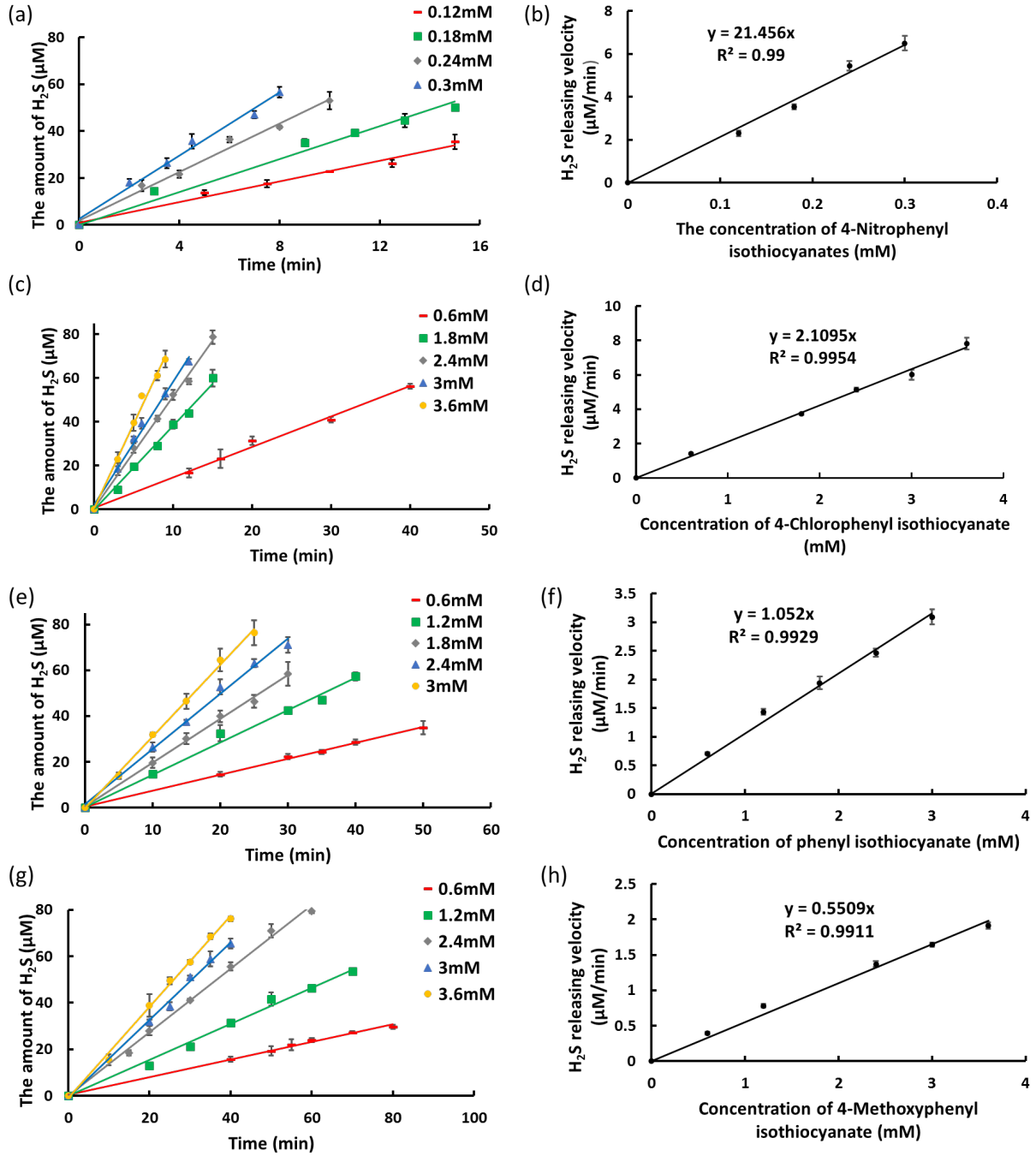


Figure S2. Relationship of initial formation rates of H_2S and the concentration of ITCs (a) and (b) *p*-nitrophenyl isothiocyanate; (c) and (d) *p*-Chlorophenyl isothiocyanate; (e) and (f) phenyl isothiocyanate; (g) and (h) *p*-methoxyphenyl isothiocyanate. Cysteine concentration is two times of the ITCs. The linear relationship of rates and the concentration of ITCs give apparent rate constant from the slopes.

2.3. The yield of 2-alkyl(or aryl)amino-4,5-dihydro-4-thiazolecarboxylic acid (5) and raphanusamic acid (6).

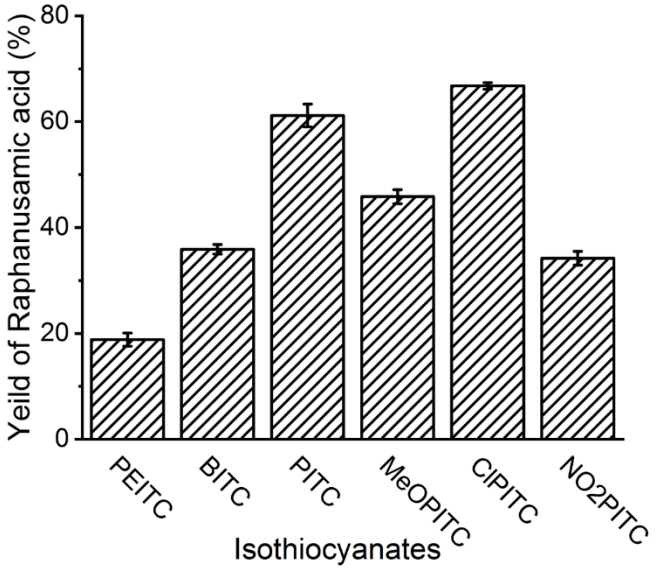


Figure S3. The yield of raphanusamic acid (in the reaction mixture of isothiocyanate and L-cysteine (1:2) at 37 ºC after 24 h. (PEITC, $PhCH_2CH_2NCS$; BITC, $PhCH_2NCS$, PITC, PhNCS; MeOPITC, *p*-$CH_3OC_6H_4NCS$, ClPITC, *p*-Cl-C_6H_4NCS; NO2PITC, *p*-O_2N- C_6H_4NCS)

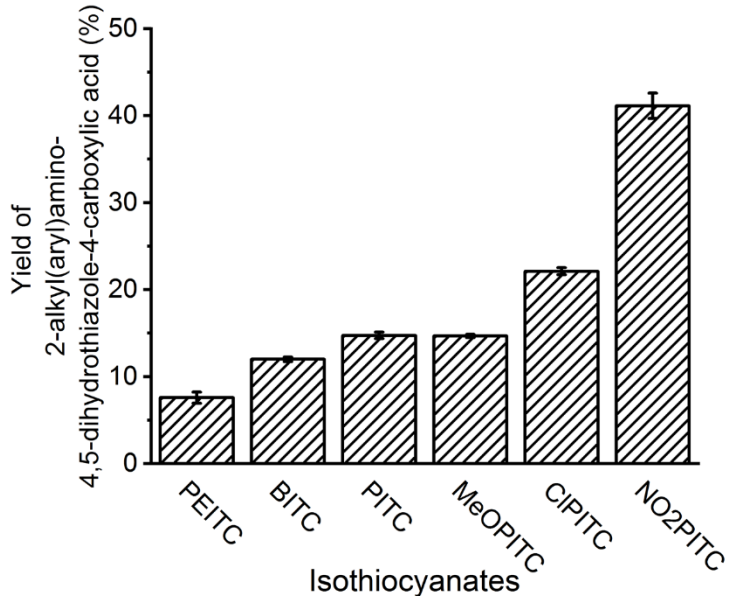


Figure S4. The yield of 2-N-alkyl(or aryl)amino-4,5-dihydro-4-thiazolecarboxylic acid (**5**) in the reaction mixture of isothiocyanate and L-cysteine (1:2) at 37 ºC after 24 h. (PEITC, $PhCH_2CH_2NCS$; BITC, $PhCH_2NCS$, PITC, PhNCS; MeOPITC, *p*-$CH_3OC_6H_4NCS$, ClPITC, *p*-Cl-C_6H_4NCS; NO2PITC, *p*-O_2N- C_6H_4NCS)

2.4. LC-MS/ MS analysis of the products from ITC (4 mM) + L-cysteine (8 mM).

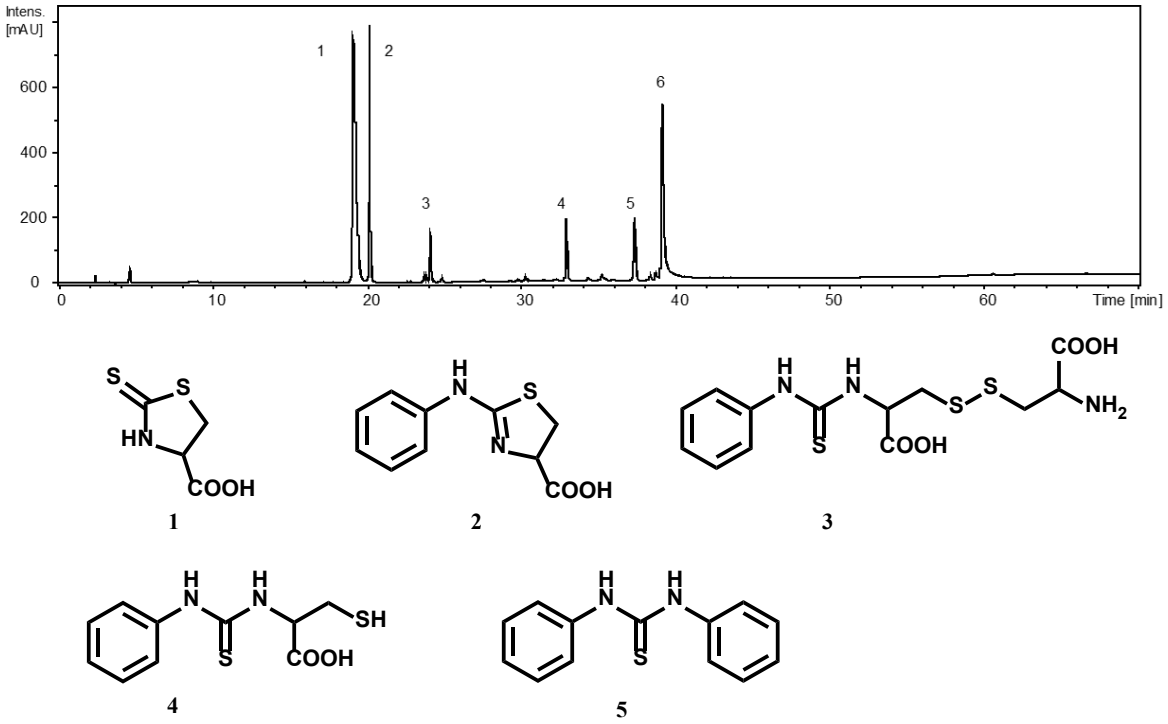


Figure S5. HPLC chromatogram of products from the reaction of PITC (4 mM) and L-cysteine (8 mM) at 254 nm.

Table S1. Molecular ions for the peaks in Figure S5 and their proposed structures.

Peak No.	Rete-ntion time (min)	Molecular weight	m/z (ms^2) Positive Mode	m/z (ms^2) Negative mode	Formula
1	19.3	163	164	162, 325 (118)	$C_4H_5NO_2S_2$
2	20.3	222	223 (177)	221, 443 (187)	$C_{10}H_{10}O_2N_2S$
3	24.2	375	376 (255)	374, 745 (253, 239，221, 187)	$C_{13}H_{17}N_3O4S_3$
4	33.1	256	257 (122)	255, 511 (221)	$C_{10}H_{12}N_2O_2S_2$
5	37.5	228	229 (94)	227	$C_{13}H_{12}N_2S$
6	39.2	-	-	-	-

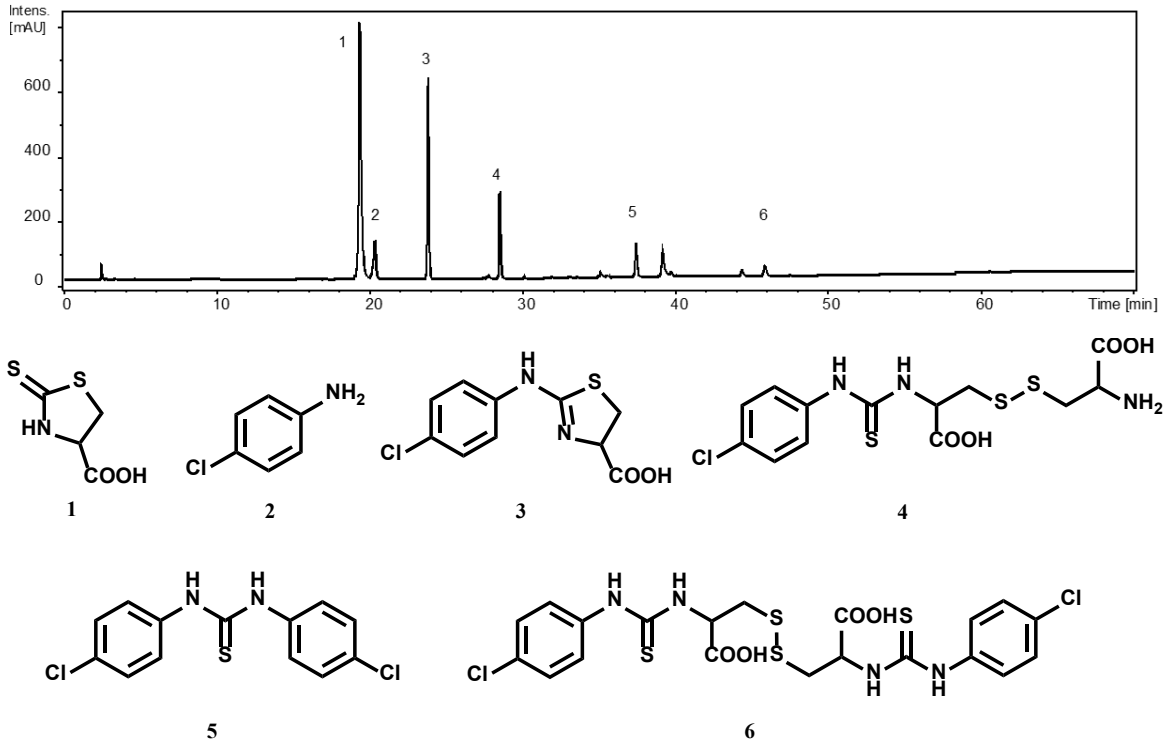


Figure S6. HPLC chromatogram of products from the reaction of p-chlorophenylisothiocyanates (ClPITC) (4 mM) and L-cysteine (8 mM) at 254 nm.

Table S2. Molecular ions for the peaks in Figure **S6** and their proposed structures.

Peak No.	Retention time (min)	Molecular weight	m/z (ms^2) Positive Mode	m/z (ms^2) Negative mode	Formula
1	19.5	163	164	162, 325 (118)	$C_4H_5NO_2S_2$
2	20.4	127	128	-	C_6H_6ClN
3	23.9	256	257, 513 (211)	255, 512 (212)	$C_{10}H_9ClN_2O_2S$
4	28.6	409	410 (289)	408, 819 (392, 289,257, 239,223)	$C_{13}H_{16}ClN_3O_4S_3$
5	37.5	296	297	295	$C_{13}H_{10}Cl_2N_2S$
6	46.0	579	-	578 (410, 321, 257)	$C_{20}H_{20}Cl_2N_4O_4S_4$

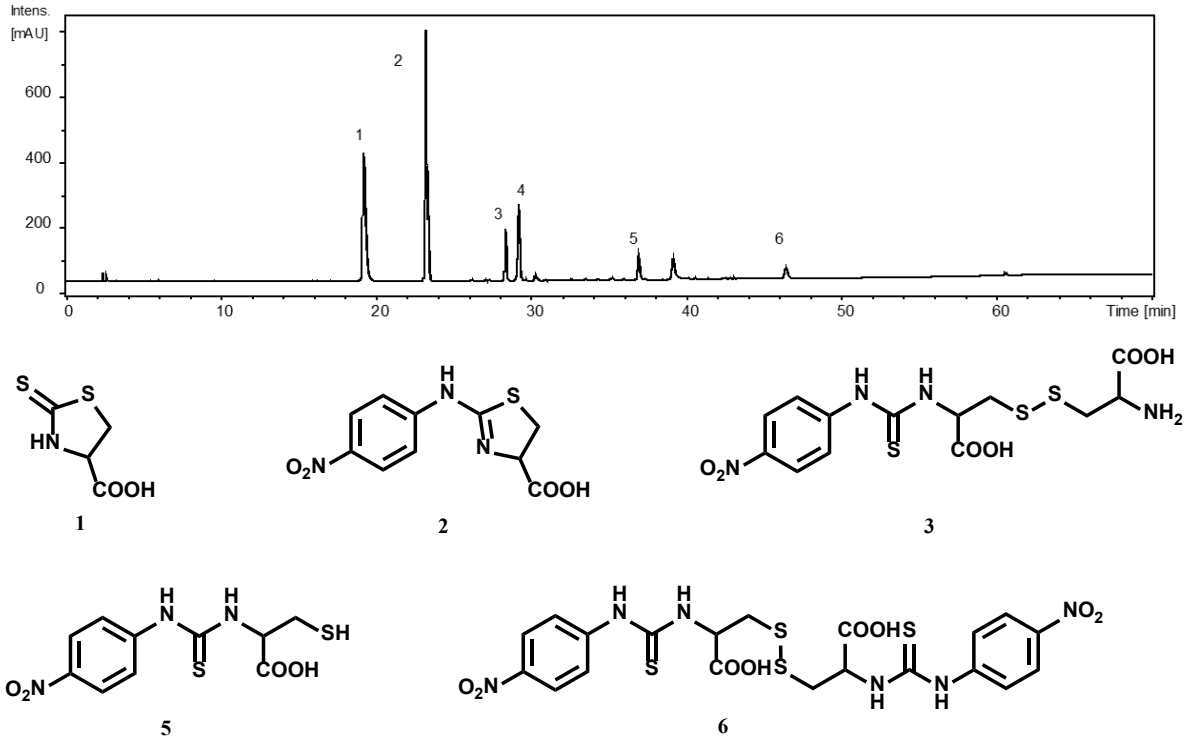


Figure S7. HPLC chromatogram of products from the reaction of NO_2PITC (4 mM) + L-cysteine (8 mM) at 254 nm.

Table S3. Molecular ions for the peaks in Figure **S7** and their proposed structures.

Peak No.	Rete-ntion time (min)	Molecular weight	m/z (ms^2) Positive Mode	m/z (ms^2) Negative mode	Formula
1	19.3	163	164	162,325 (11 8)	$C_4H_5NO_2S_2$
2	23.5	267	268 (222)	266, 533 (223)	$C_{10}H_9N_3O_4S$
3	28.5	420	421 (300)	419, 839 (298, 266, 232)	$C_{13}H_{16}N_4O_6S_3$
4	29.2	-	-	-	
5	37.1	301	302 (122)	300, 601 (266)	$C_{10}H_{11}N_3O_4S_2$
6	46.5	600	-	599	$C_{20}H_{20}N_6O_8S_4$

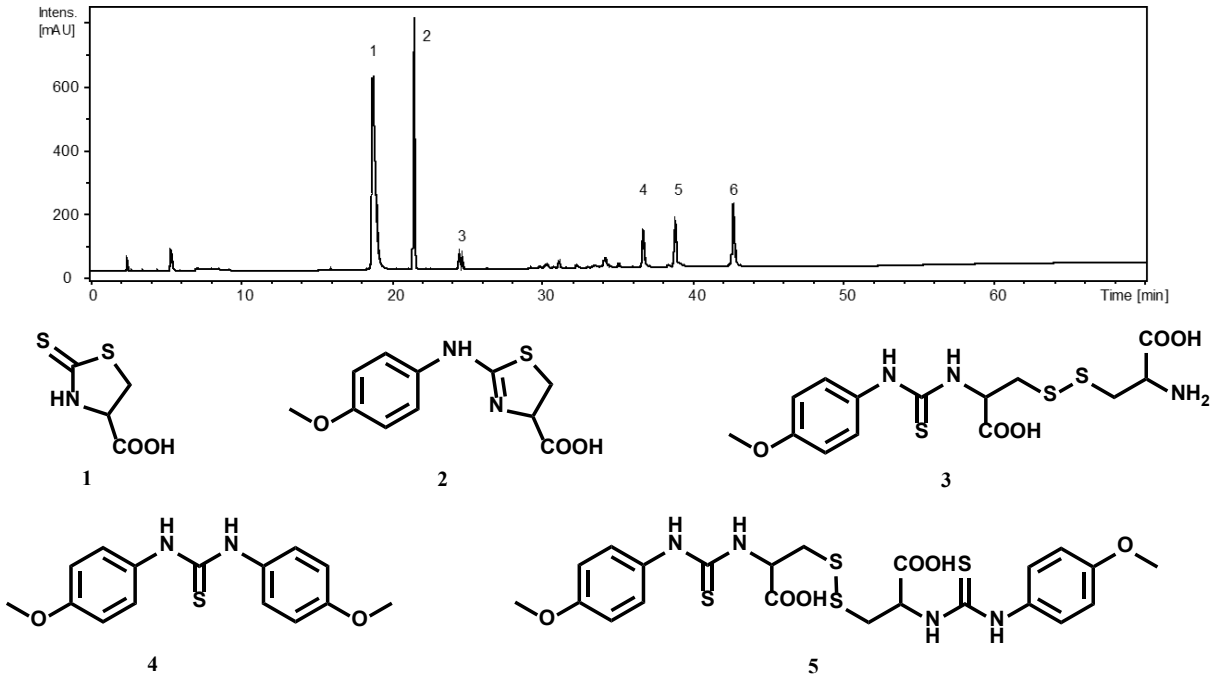


Figure S8. HPLC chromatogram of products from the reaction of MeOPITC (4 mM) + L-cysteine (8 mM) at 254 nm.

Table S4. Molecular ions for the peaks in Figure S8 and their proposed structures.

Peak No.	Retention time (min)	Molecular weight	m/z (ms^2) Positive Mode	m/z (ms^2) Negative mode	Formula
1	19.5	163	164	162,325 (118)	$C_4H_5NO_2S_2$
2	21.6	252	253,505 (207)	251,503 (208)	$C_{11}H_{12}N_2O_3S$
3	24.4	405	406 (120,196, 239,251)	404,809 (152,239, 283)	$C_{14}H_{19}N_3O_5S_3$
4	36.8	288	289,577 (166,124)	287	$C_{15}H_{16}N_2O_2S$
5	37.5	570	571 (285)	569 (404, 251)	$C_{22}H_{26}N_4O_6S_4$
6	42.6	-	-	-	

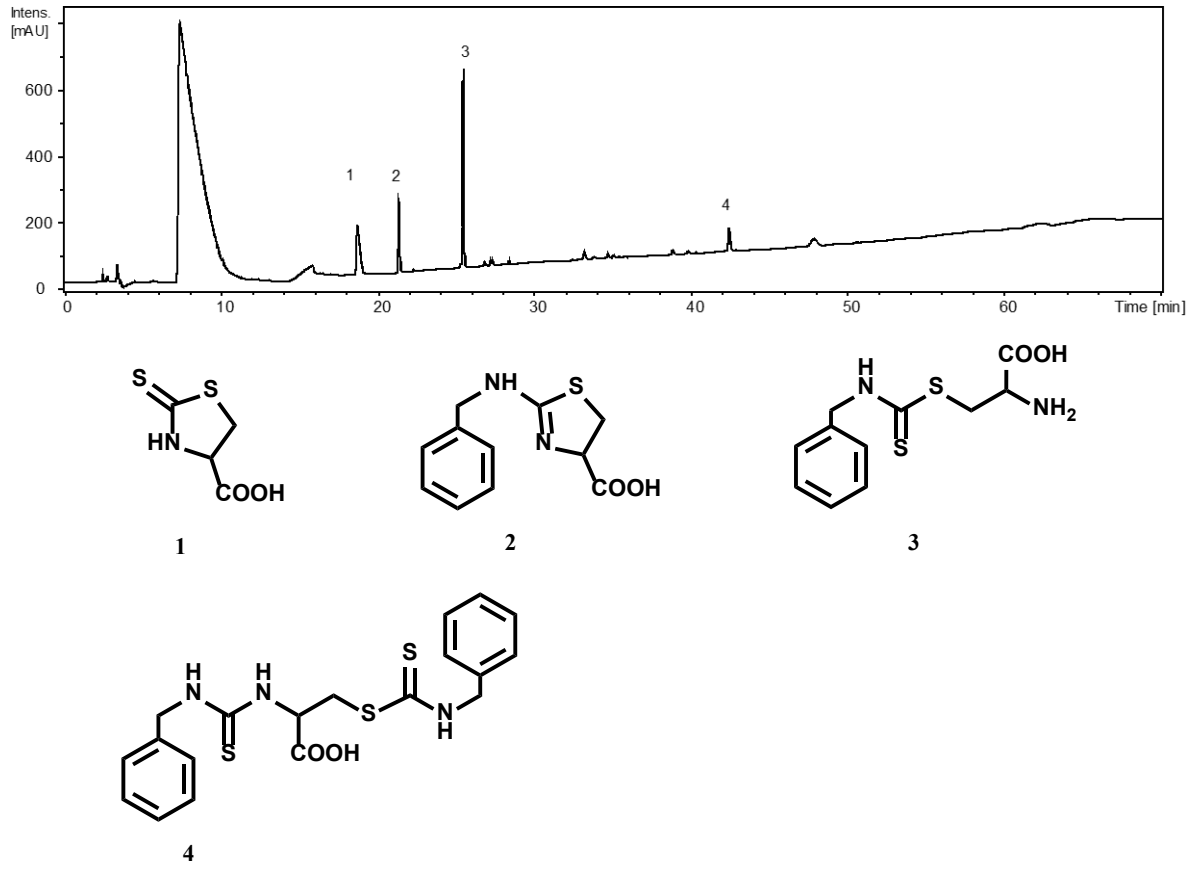


Figure S9. HPLC chromatogram of products from the reaction of benzylisothiocyanate (BITC) (4 mM) and L-cysteine (8 mM) at 235 nm.

Table S5. Molecular ions for the peaks in **Figure S9** and their proposed structures.

Peak No.	Retention time (min)	Molecular weight	m/z (ms^2) Positive Mode	m/z (ms^2) Negative mode	Formula
1	19.5	163	164	162, 325 (118)	$C_4H_5NO_2S_2$
2	21.6	236	237 (191, 91)	235	$C_{11}H_{12}N_2O_2S$
3	25.6	270	271, 541 (254, 184,122, 91)	269 (235, 182, 120)	$C_{11}H_{14}N_2O_2S_2$
4	42.6	419	420 (271, 237, 191)	418, 837 (269, 235, 182)	$C_{19}H_{21}N_3O_2S_3$

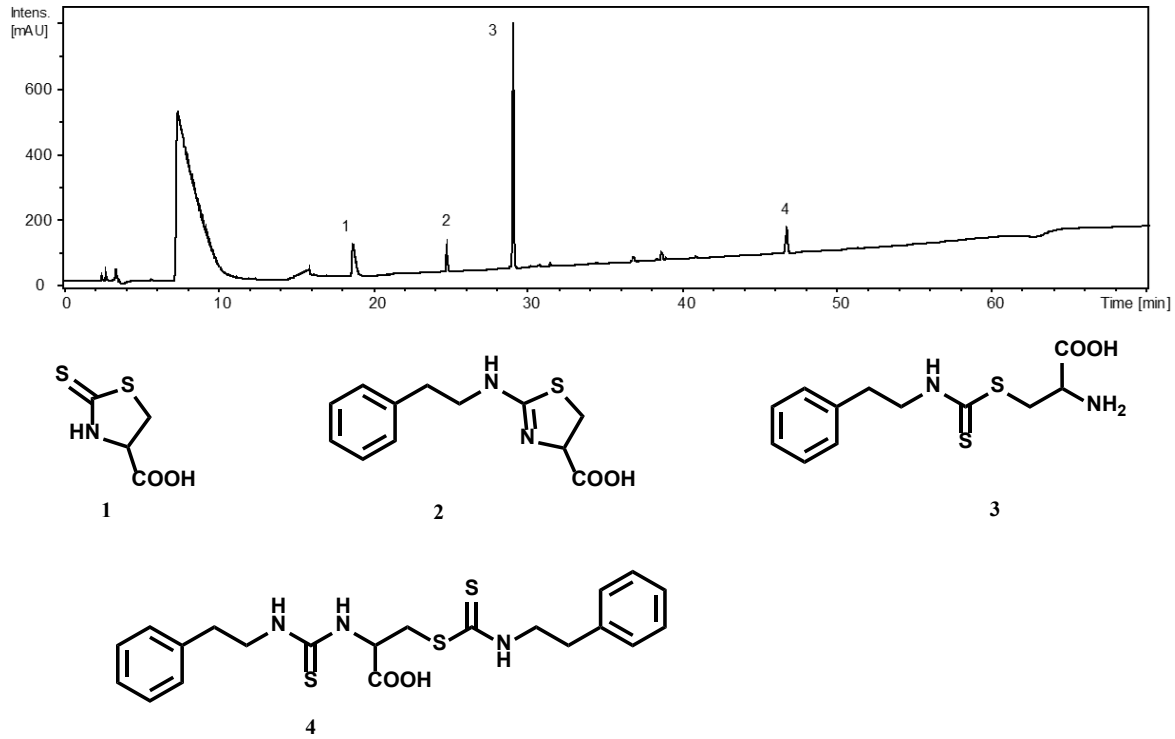


Figure S10. HPLC chromatogram of products from the reaction of phenethylisothiocyanate (PEITC) (4 mM) and L-cysteine (8 mM) at 235 nm.

Table S6. Molecular ions for the peaks in **Figure S10** and their proposed structures.

Peak No.	Retention time (min)	Molecular weight	m/z (ms^2) Positive Mode	m/z (ms^2) Negative mode	Formula
1	19.5	163	164	162, 325 (118)	$C_4H_5NO_2S_2$
2	24.4	250	251, 501 (205, 105)	249, 499	$C_{12}H_{14}N_2O_2S$
3	29.1	284	285, 569 (268, 105)	283, 567	$C_{12}H_{16}N_2O_2S_2$
4	46.6	447	448 (251,205,105)	446, 893	$C_{21}H_{25}N_3O_2S_3$

2.5. LC-MS/ MS analysis of metabolites of fluorescein isothiocyanate in RAW 264.7 cells.

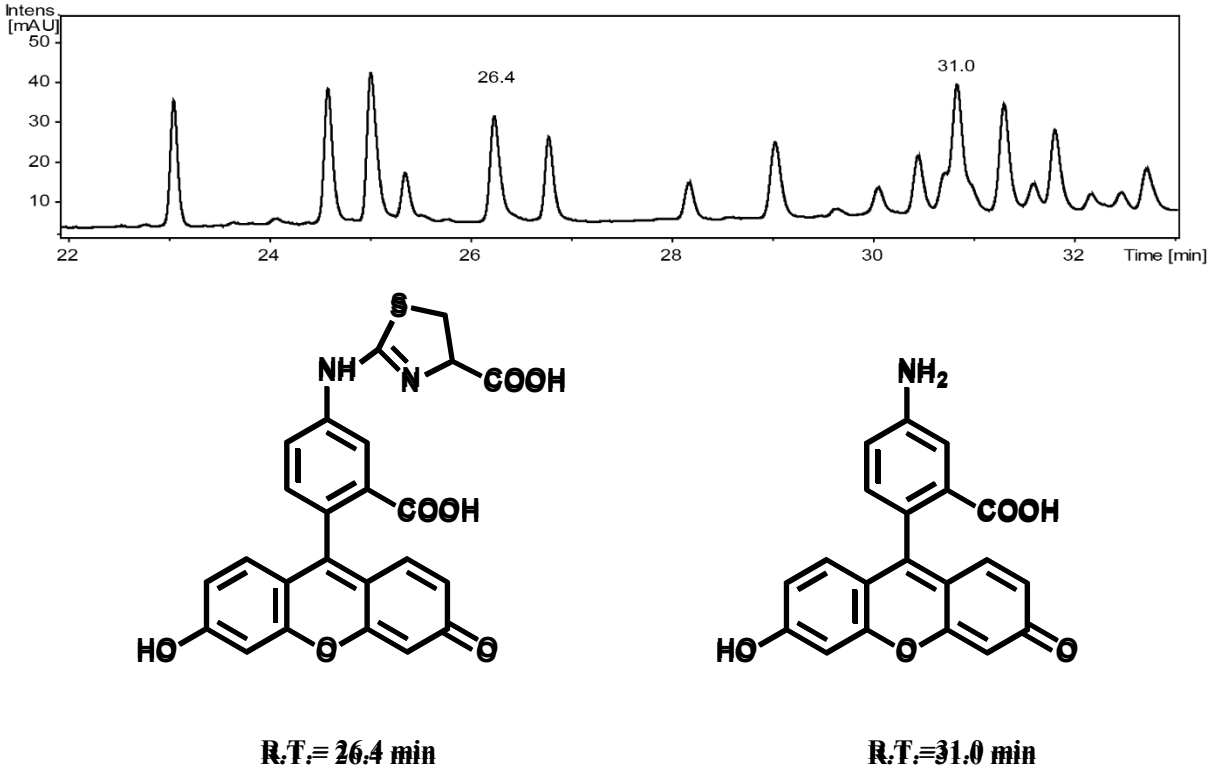


Figure S11. HPLC chromatograms (254 nm) of metabolites of RAW 264.7 cells after incubated with 100 μM fluorescein isothiocyanate (FITC) at 37 ℃ for 24 h.

Table S7. Molecular ions for the peaks in **Figure S11** and their proposed structures.

Peak No.	Retention time (min)	Molecular weight	m/z (ms^2) Positive Mode	m/z (ms^2) Negative mode	Formula
1	26.4	476	477	475	$C_{24}H_{16}N_2O_7S$
2	31.0	347	348	346	$C_{20}H_{13}NO_5$

2.6. NMR spectra of isolate compounds

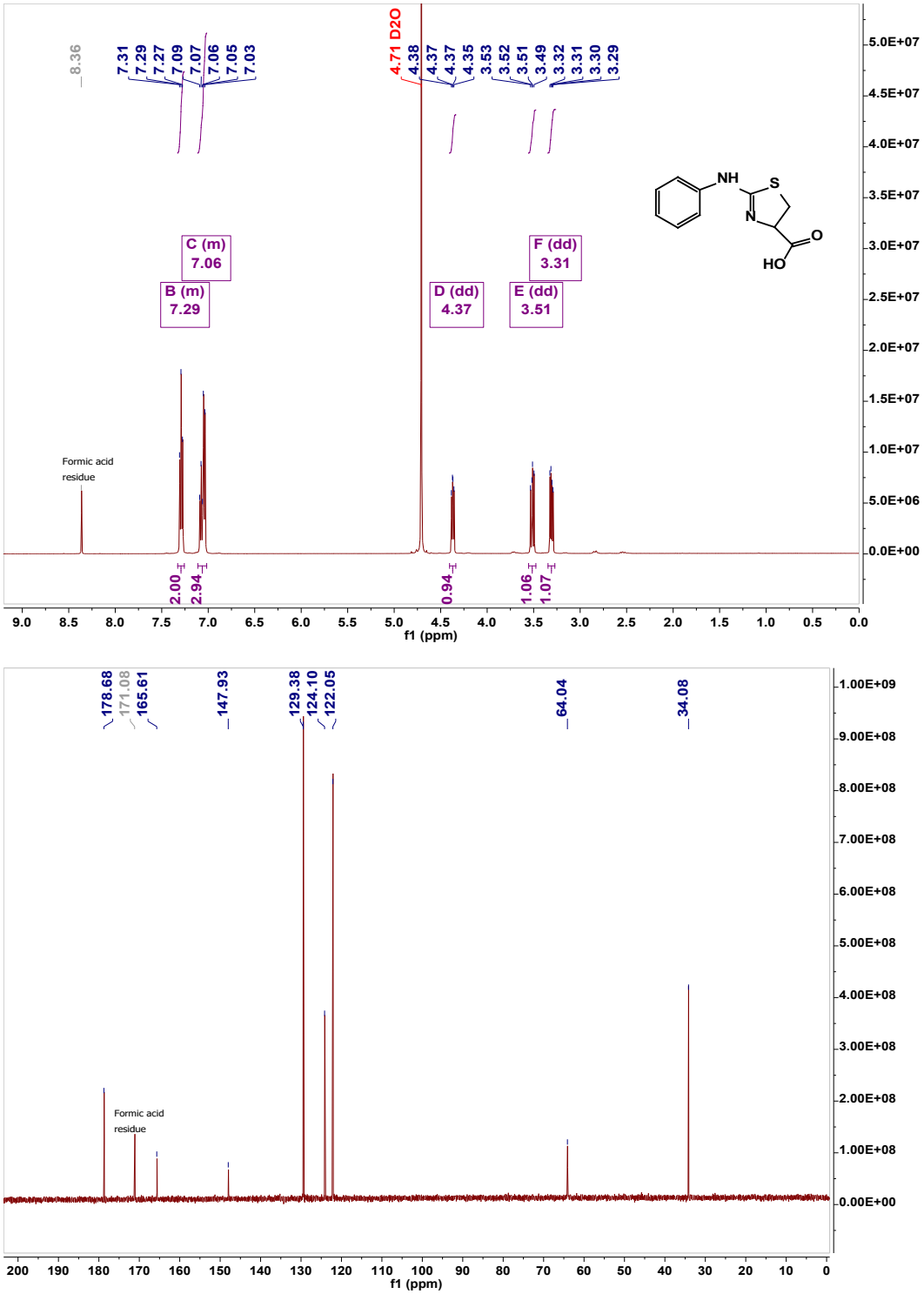


Figure S12.Top: ^{1}H (500 MHz) and (bottom) ^{13}C NMR (126 MHz) spectra of **5-Ph**. Solvent: D_2O with NaOD.

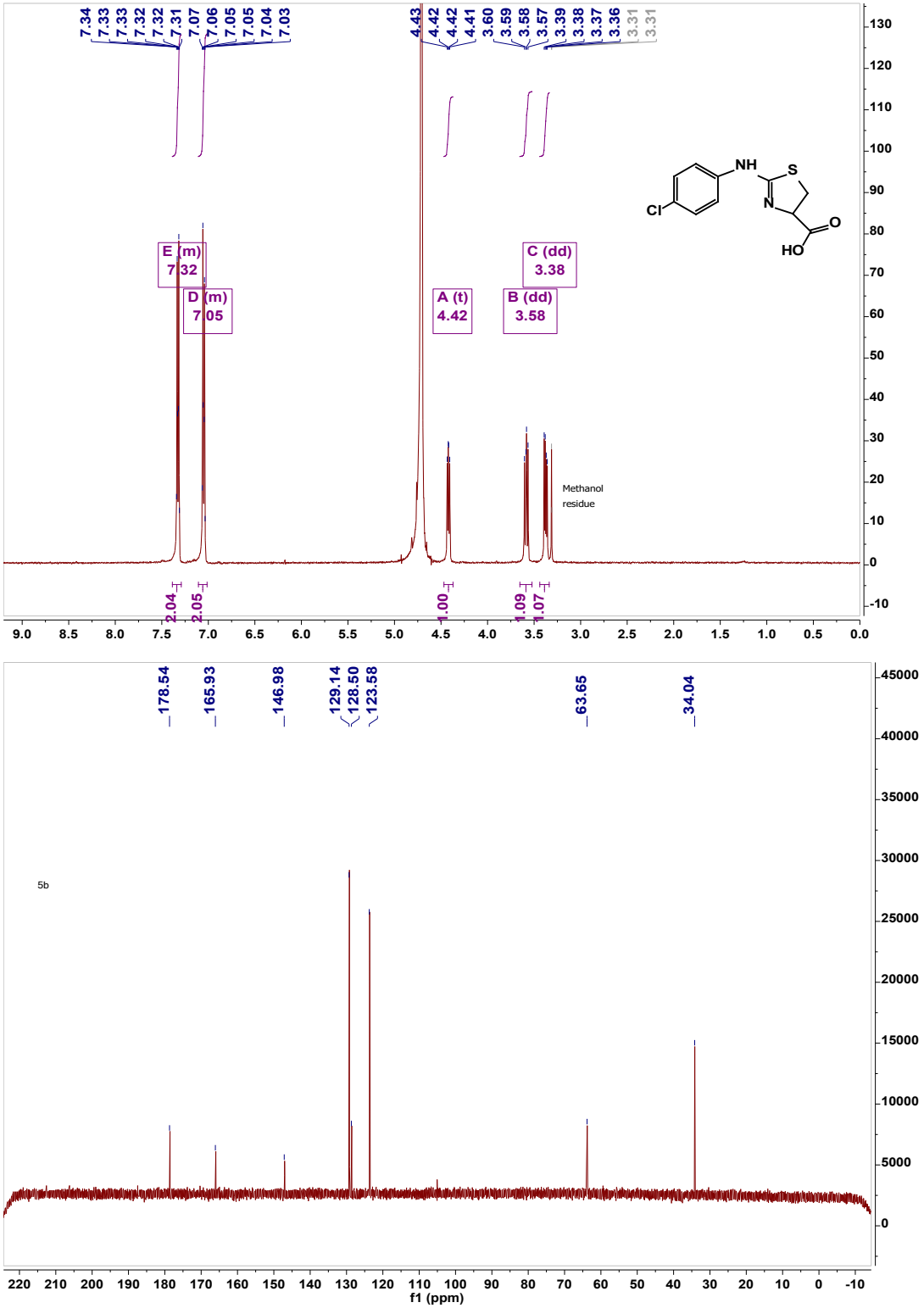


Figure 13. ^{1}H (500 MHz) spectrum (top) and ^{13}C NMR (126 MHz) spectrum (bottom) of **5-ClPh**. Solvent: basic D_2O.

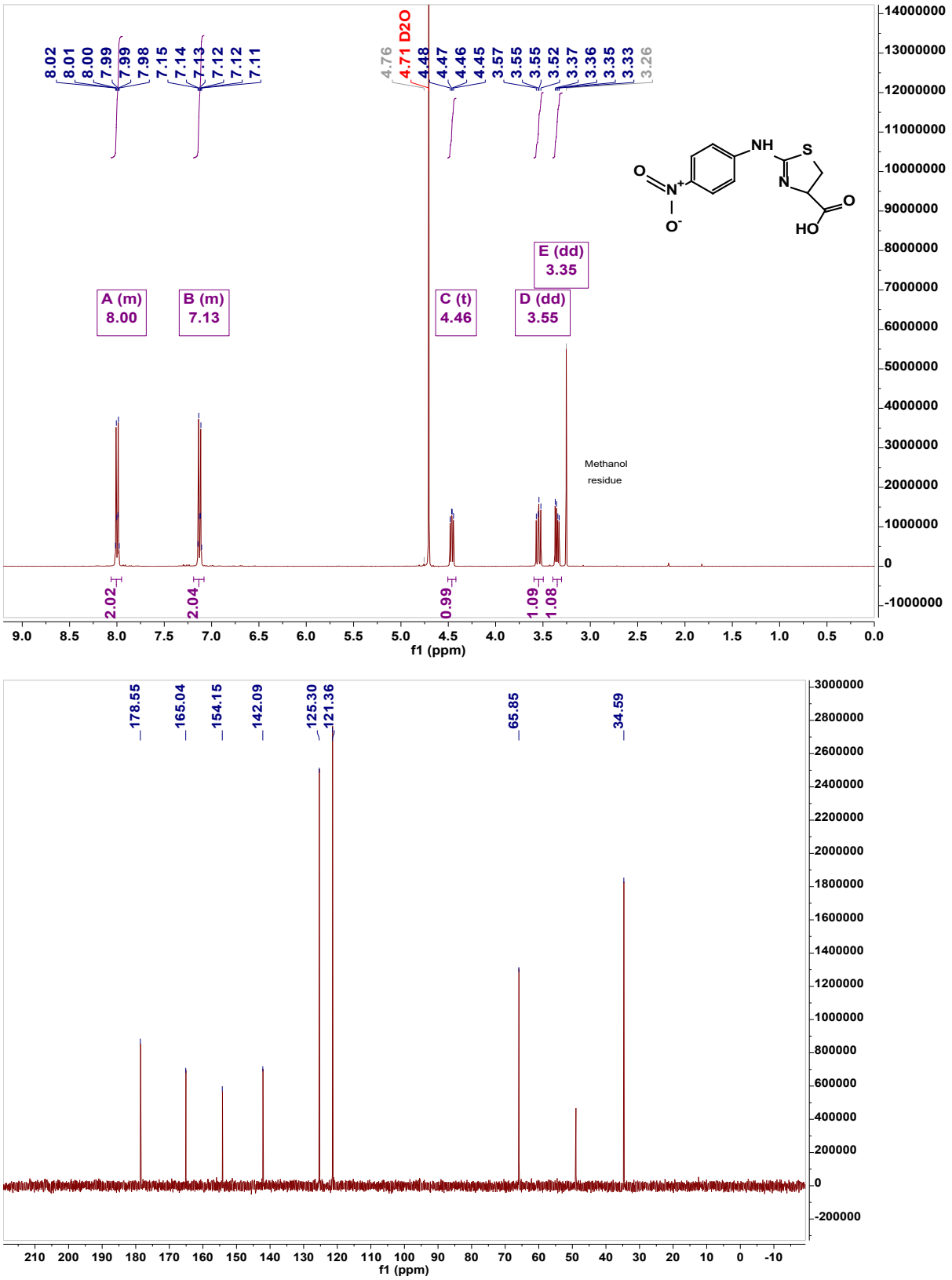


Figure 14. ^{1}H (400 MHz) spectrum (top) and ^{13}C NMR (101 MHz) spectrum (bottom) of 5-NO_2Ph. Solvent: Basic D_2O (with NaOD).

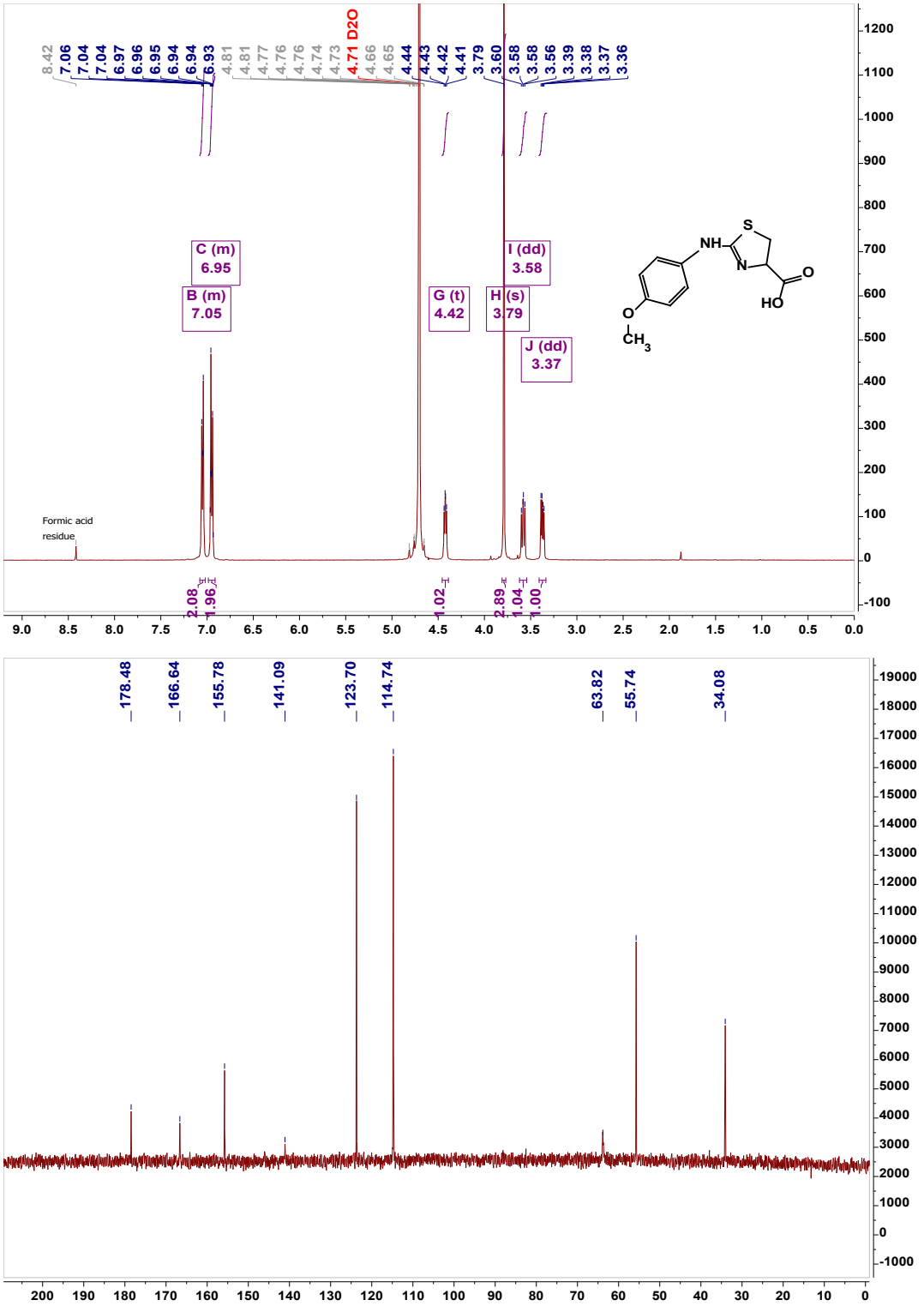


Figure 15. ^{1}H (500 MHz) spectrum (top) and ^{13}C NMR (126 MHz) spectrum (bottom) of 5-MeOPh. Solvent basic D_2O (with NaOD).

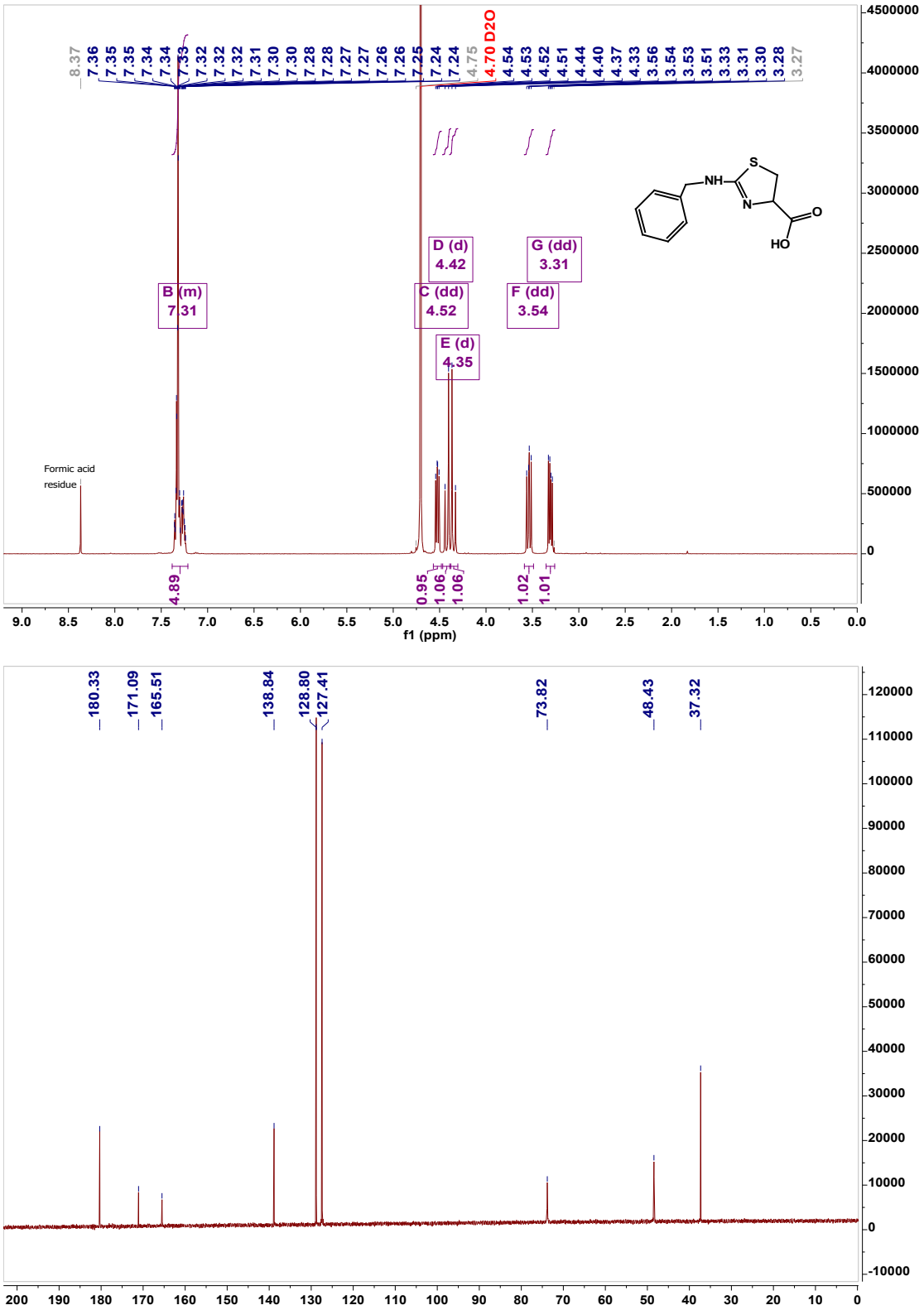


Figure 16. ^{1}H (400 MHz) spectrum (top) and ^{13}C NMR (101 MHz) spectrum (bottom) of **5-Bz**. Solvent: Basic D_2O (with NaOD).

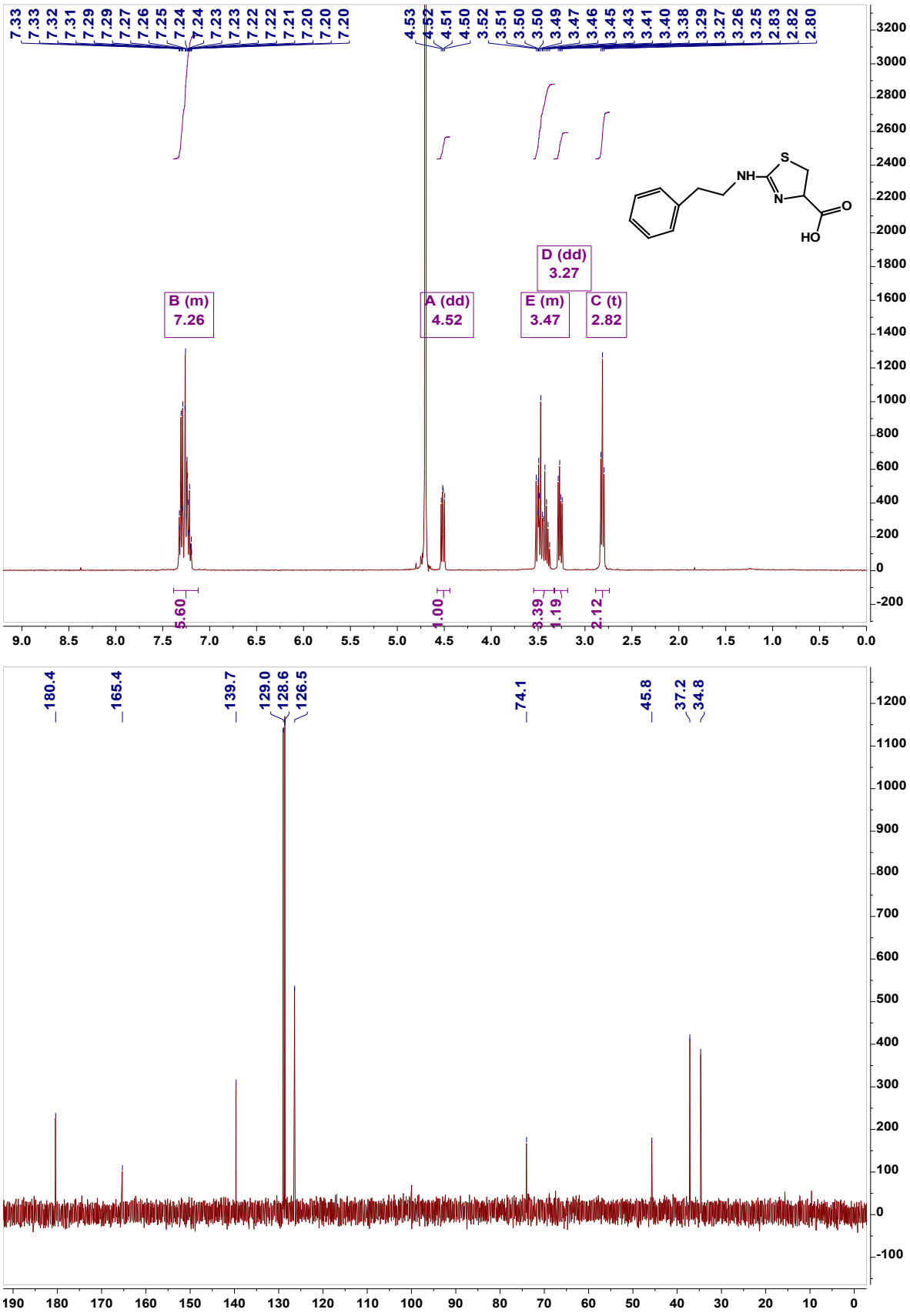
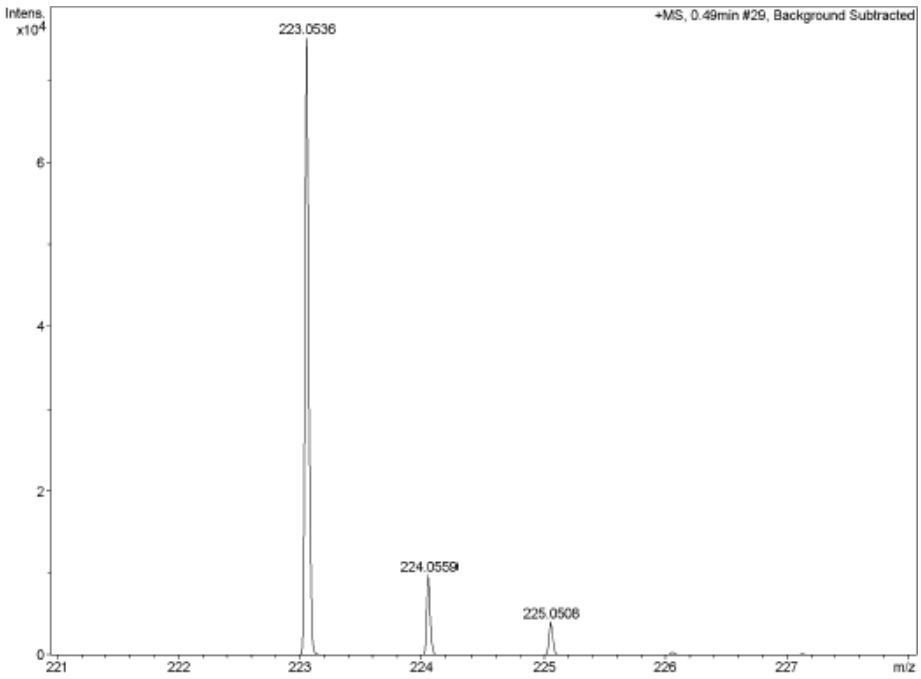


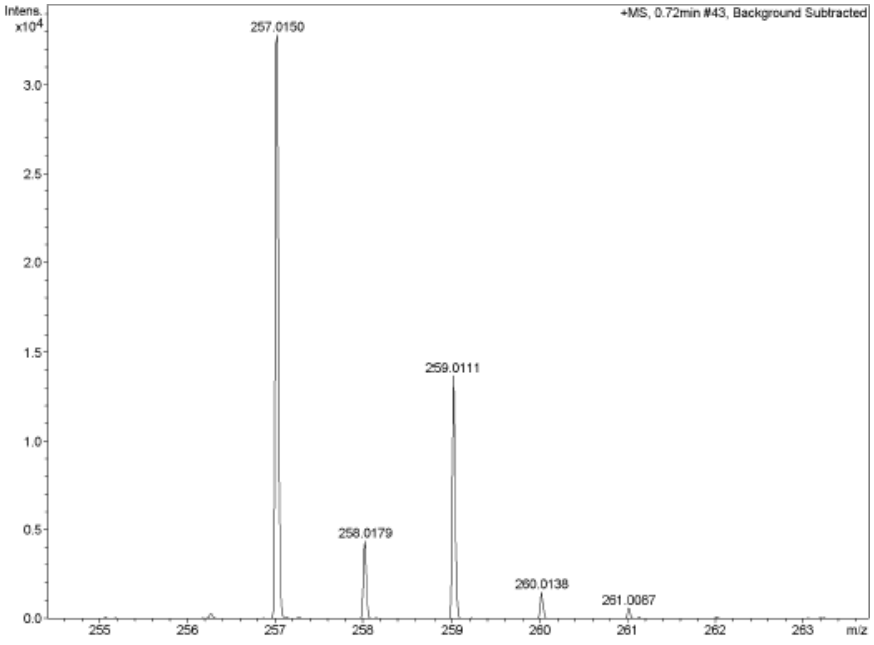
Figure 17. ^{1}H (400 MHz) spectrum (top) and ^{13}C NMR (101 MHz) spectrum (bottom) of **5-PE**. Solvent: basic D_2O (with NaOD).

2.7. High resolution mass spectra.

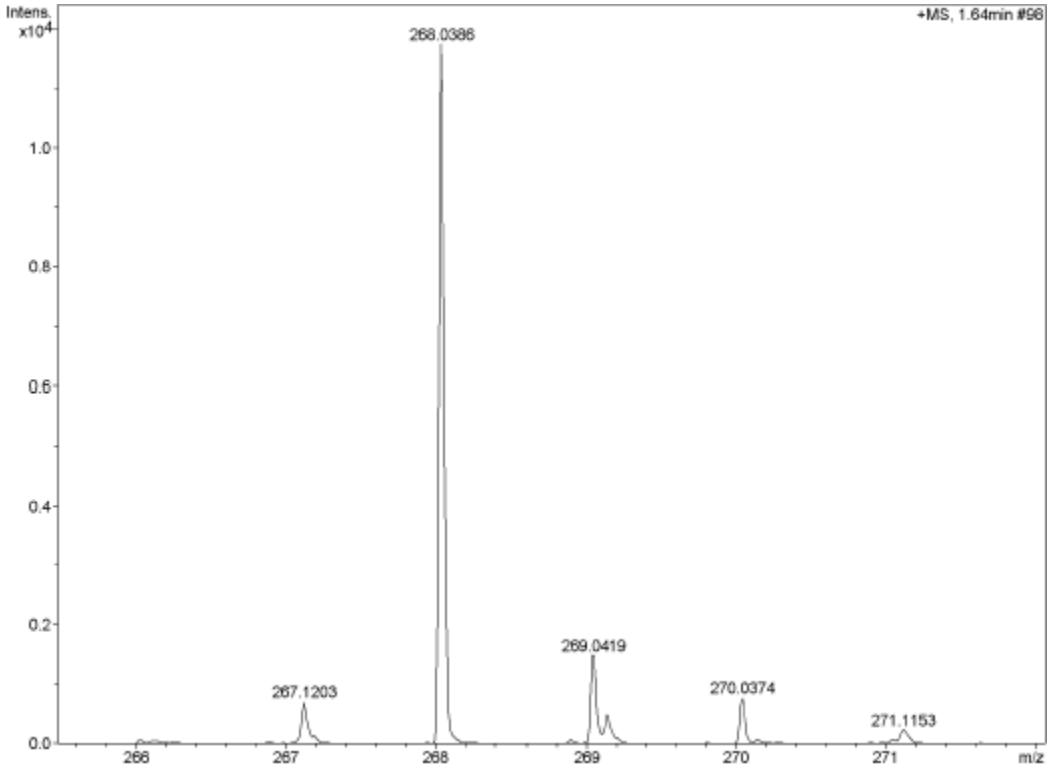
I. Compound 5-Ph



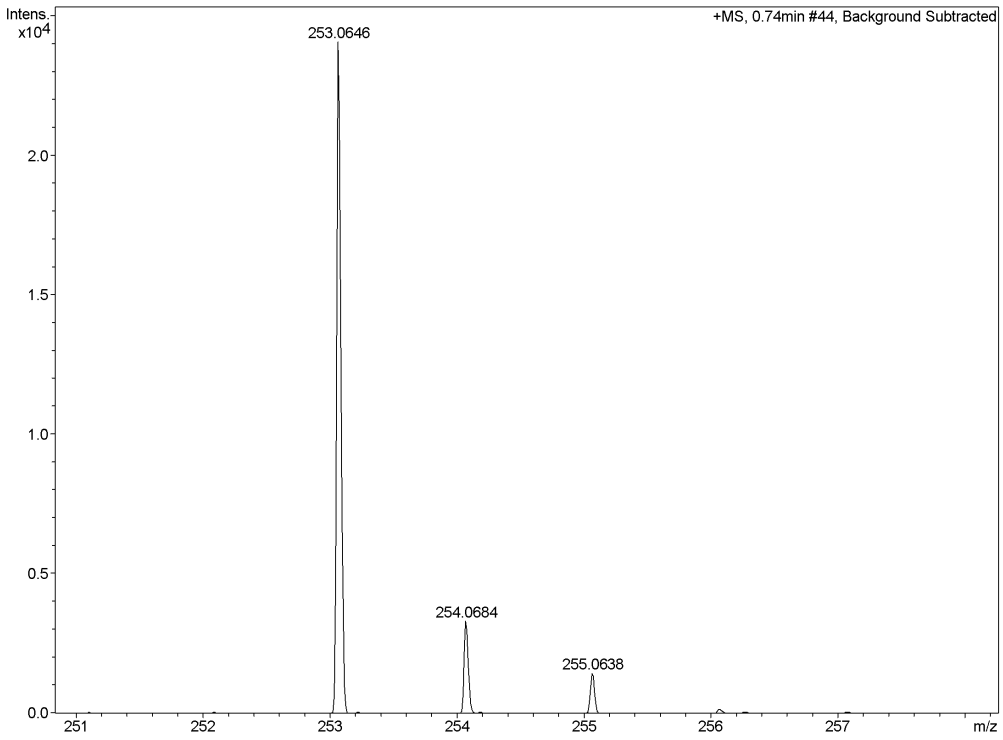
II. Compound 5-ClPh



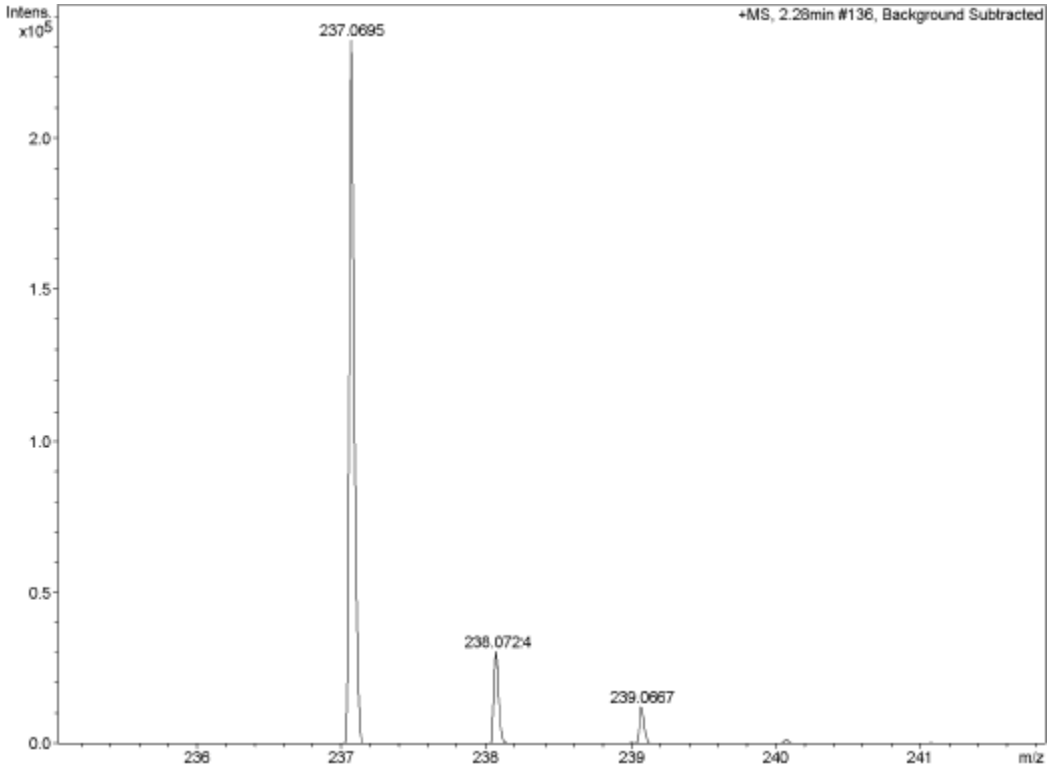
III. Compound 5-NO2Ph



IV. Compound 5-MeOPh



V. Compound 5-Bz



VI. Compound 5-PE