ELECTRONIC SUPPORTING INFORMATION

– New Journal of Chemistry

Herbicidal ionic liquids based on esterquats

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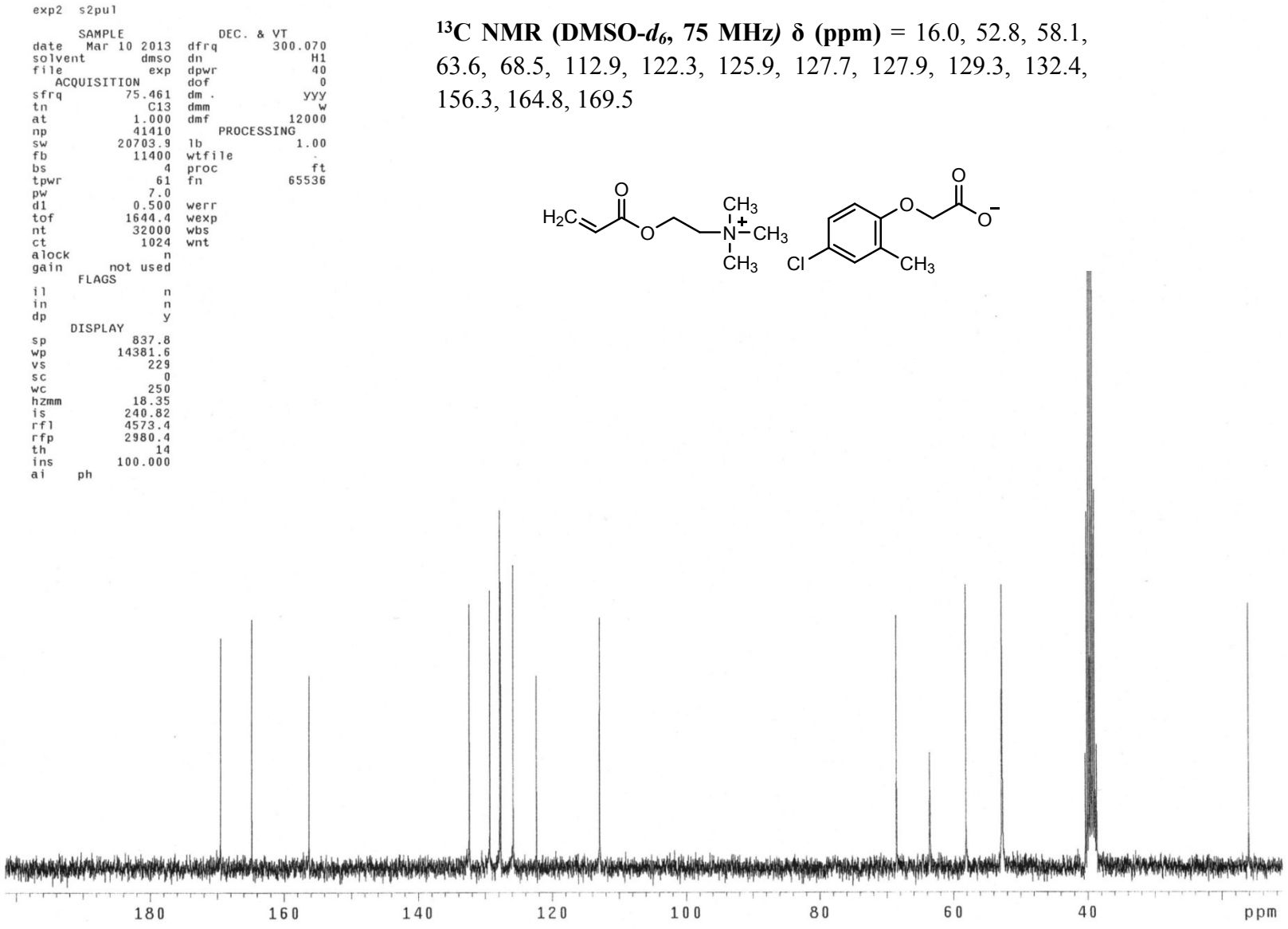
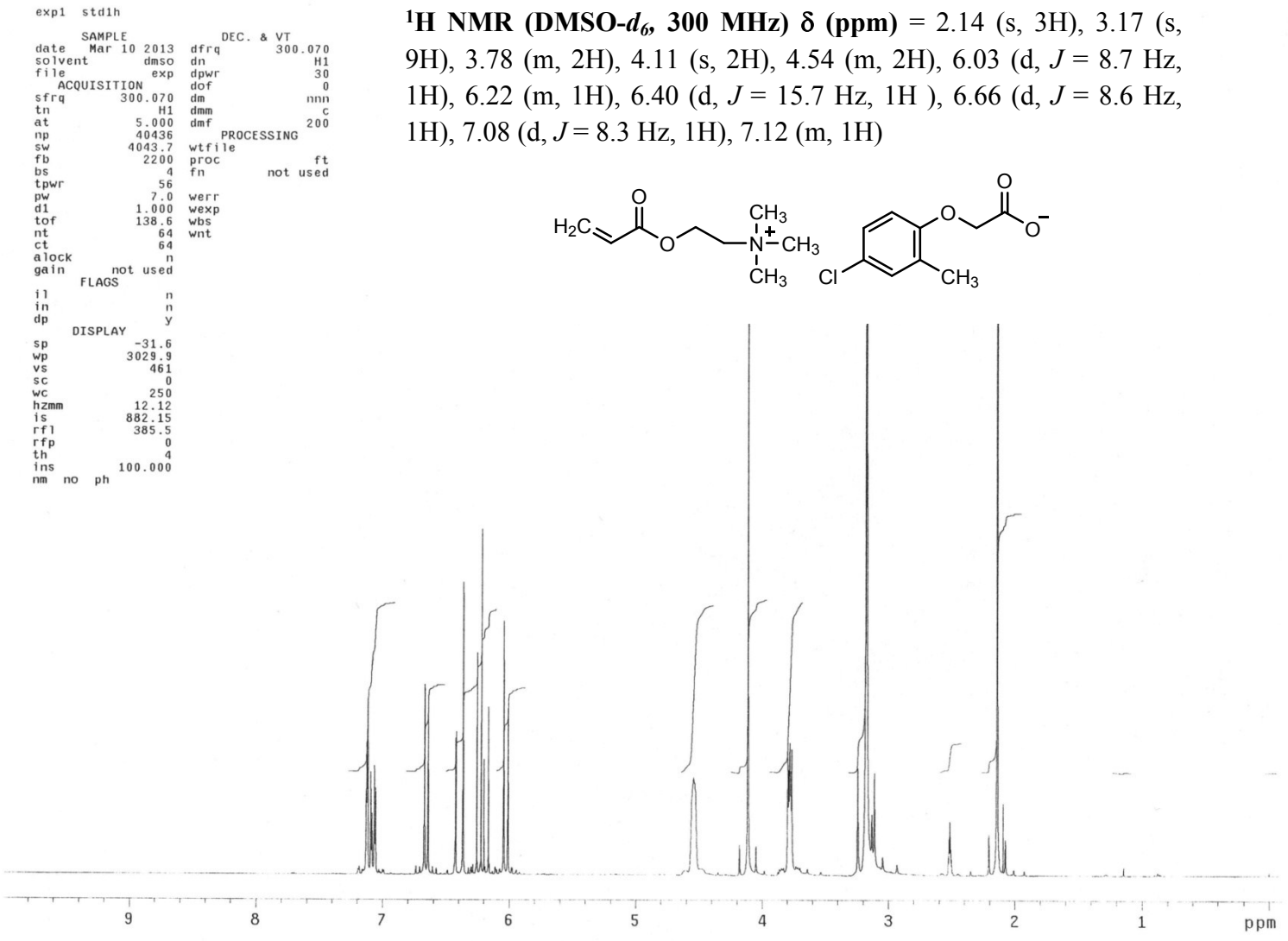
1. NMR spectra

Figure S1. ^{13}C NMR spectrum of **1** ([2-(acryloyloxy)ethyl]trimethylammonium (4-chloro-2-methylphenoxy)acetate).



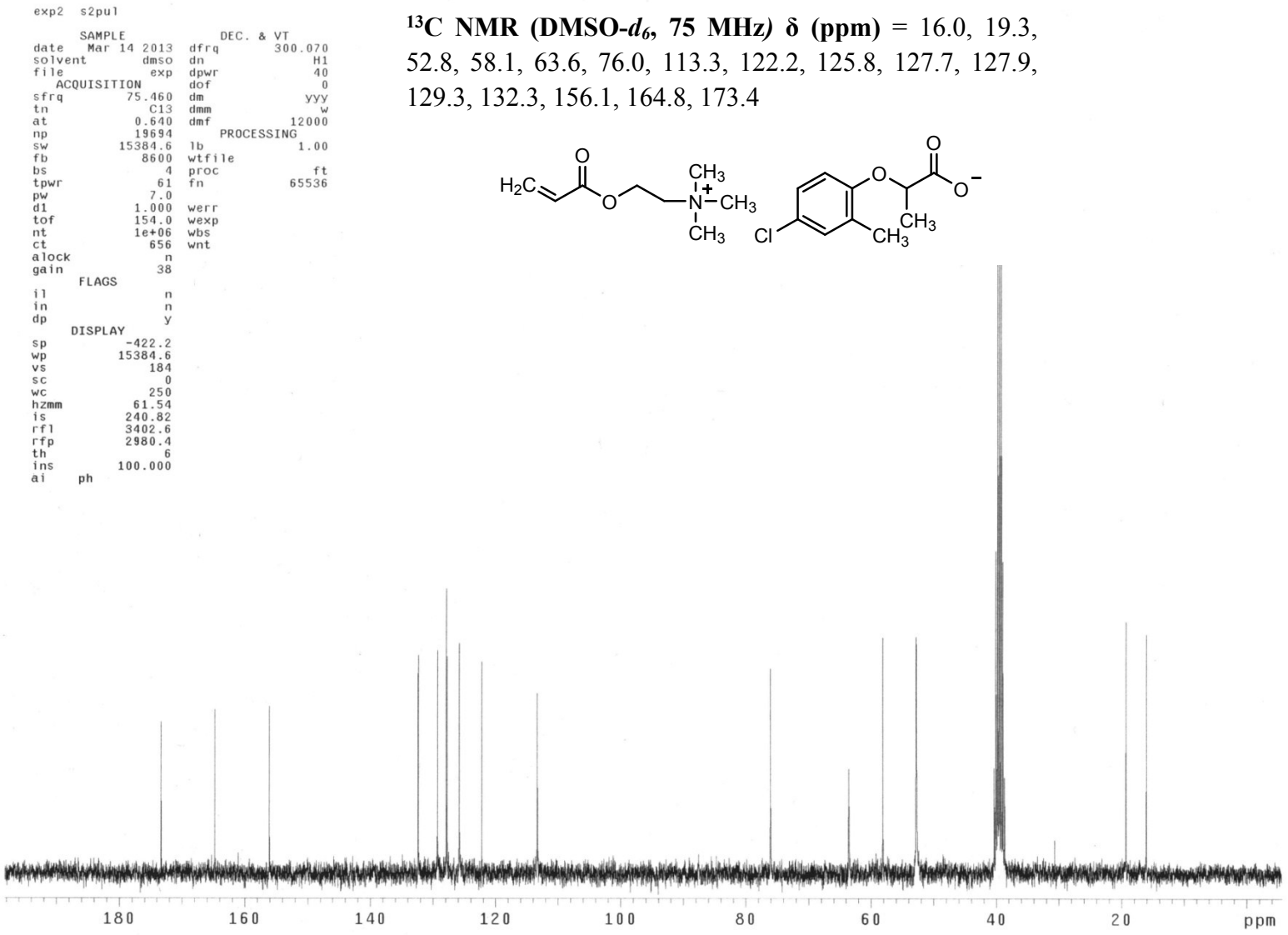
^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) = 16.0, 52.8, 58.1, 63.6, 68.5, 112.9, 122.3, 125.9, 127.7, 127.9, 129.3, 132.4, 156.3, 164.8, 169.5

Figure S2. ^{1}H NMR spectrum of **1** ([2-(acryloyloxy)ethyl]trimethylammonium (4-chloro-2-methylphenoxy)acetate).



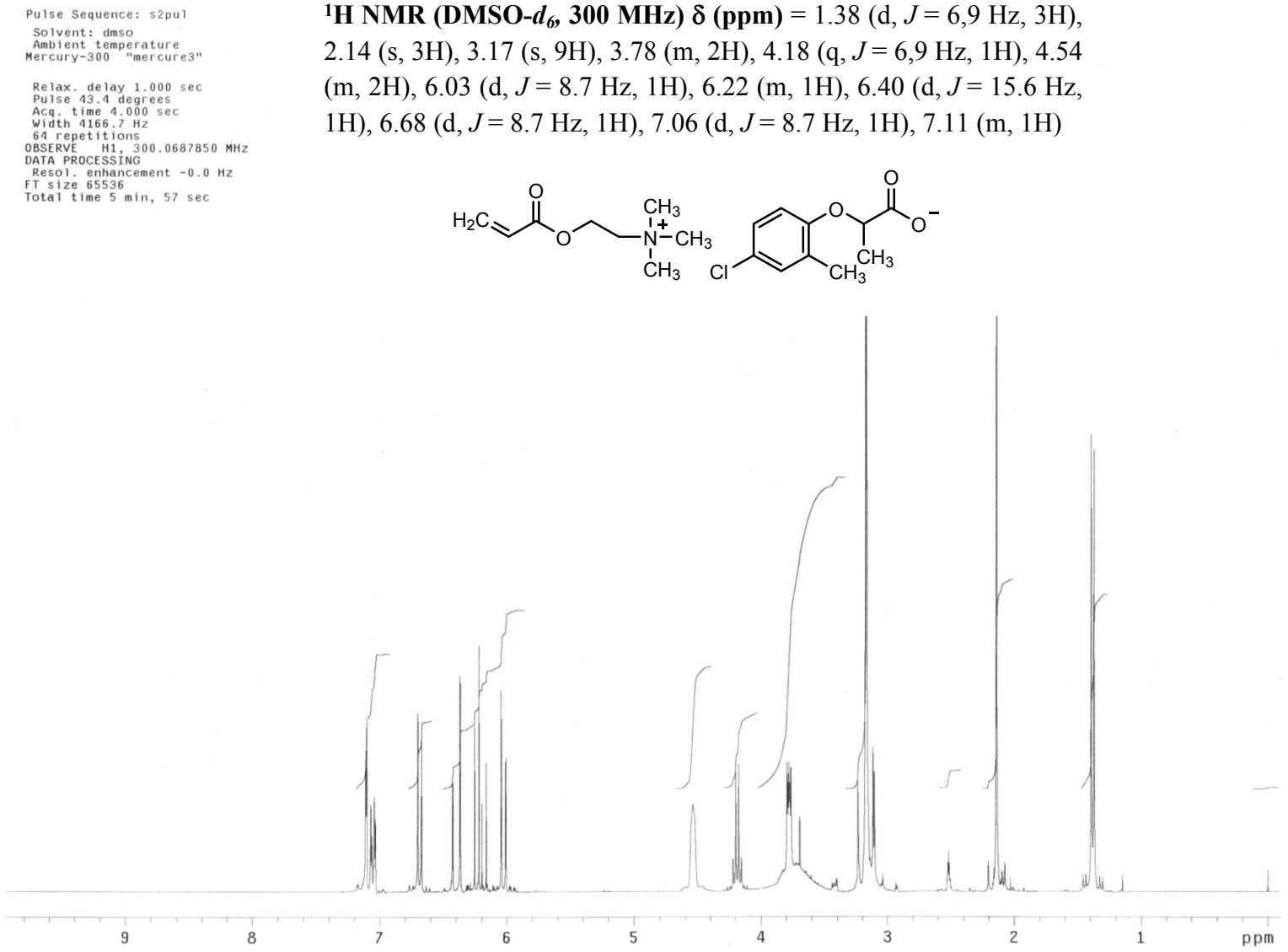
^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm) = 2.14 (s, 3H), 3.17 (s, 9H), 3.78 (m, 2H), 4.11 (s, 2H), 4.54 (m, 2H), 6.03 (d, J = 8.7 Hz, 1H), 6.22 (m, 1H), 6.40 (d, J = 15.7 Hz, 1H), 6.66 (d, J = 8.6 Hz, 1H), 7.08 (d, J = 8.3 Hz, 1H), 7.12 (m, 1H)

Figure S3. ^{13}C NMR spectrum of **2** ([2-(acryloyloxy)ethyl]trimethylammonium 2-(4-chloro-2-methylphenoxy)propioniate).



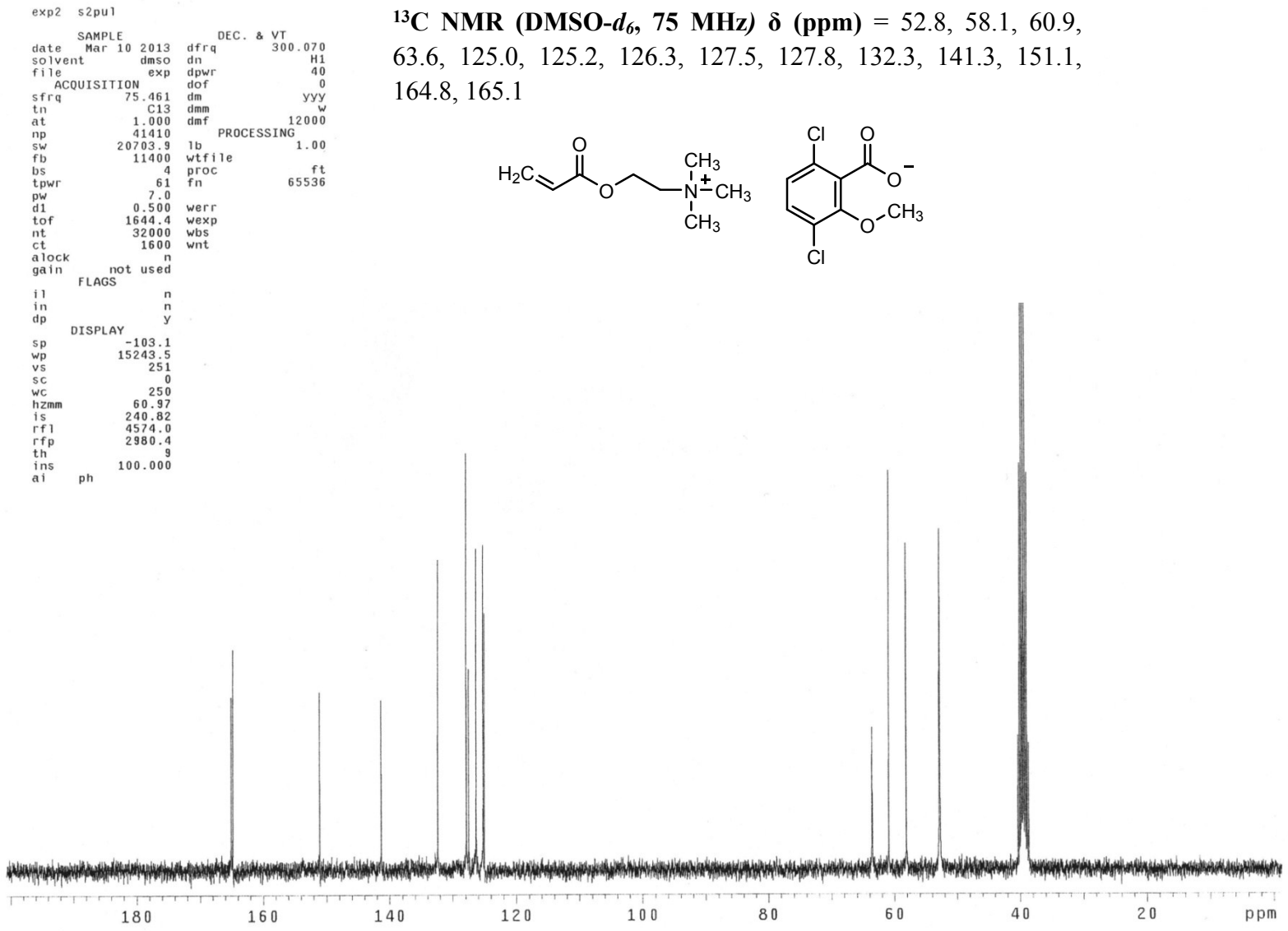
^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) = 16.0, 19.3, 52.8, 58.1, 63.6, 76.0, 113.3, 122.2, 125.8, 127.7, 127.9, 129.3, 132.3, 156.1, 164.8, 173.4

Figure S4. [1]H NMR spectrum of **2** ([2-(acryloyloxy)ethyl]trimethylammonium 2-(4-chloro-2-methylphenoxy)propioniate).



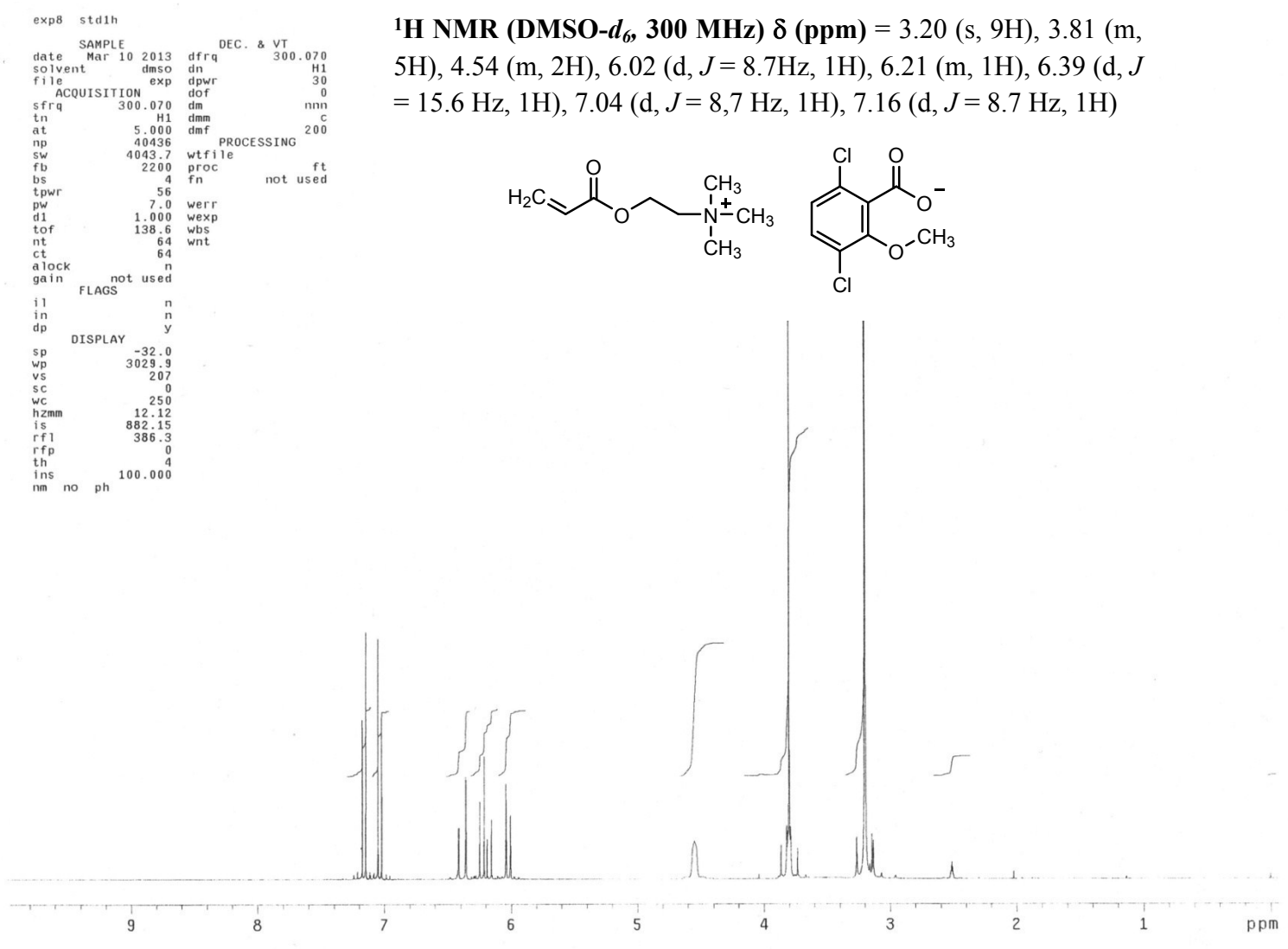
[1]H NMR (DMSO-*d*$_6$, 300 MHz) δ (ppm) = 1.38 (d, *J* = 6,9 Hz, 3H), 2.14 (s, 3H), 3.17 (s, 9H), 3.78 (m, 2H), 4.18 (q, *J* = 6,9 Hz, 1H), 4.54 (m, 2H), 6.03 (d, *J* = 8.7 Hz, 1H), 6.22 (m, 1H), 6.40 (d, *J* = 15.6 Hz, 1H), 6.68 (d, *J* = 8.7 Hz, 1H), 7.06 (d, *J* = 8.7 Hz, 1H), 7.11 (m, 1H)

Figure S5. ^{13}C NMR spectrum of **3** ([2-(acryloyloxy)ethyl]trimethylammonium 3,6-dichloro-2-methoxybenzoate).



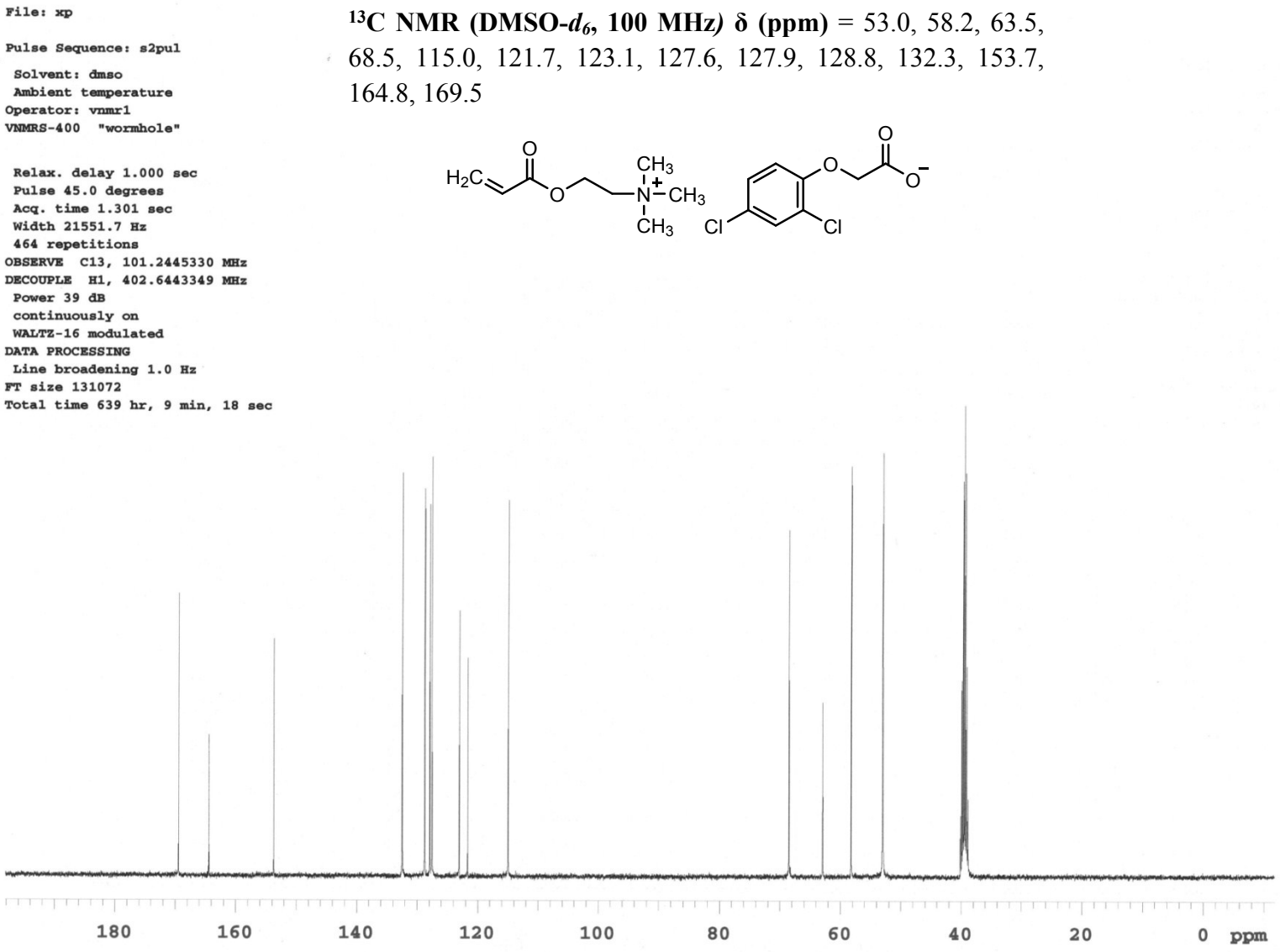
^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) = 52.8, 58.1, 60.9, 63.6, 125.0, 125.2, 126.3, 127.5, 127.8, 132.3, 141.3, 151.1, 164.8, 165.1

Figure S6. [1]H NMR spectrum of **3** ([2-(acryloyloxy)ethyl]trimethylammonium 3,6-dichloro-2-methoxybenzoate).



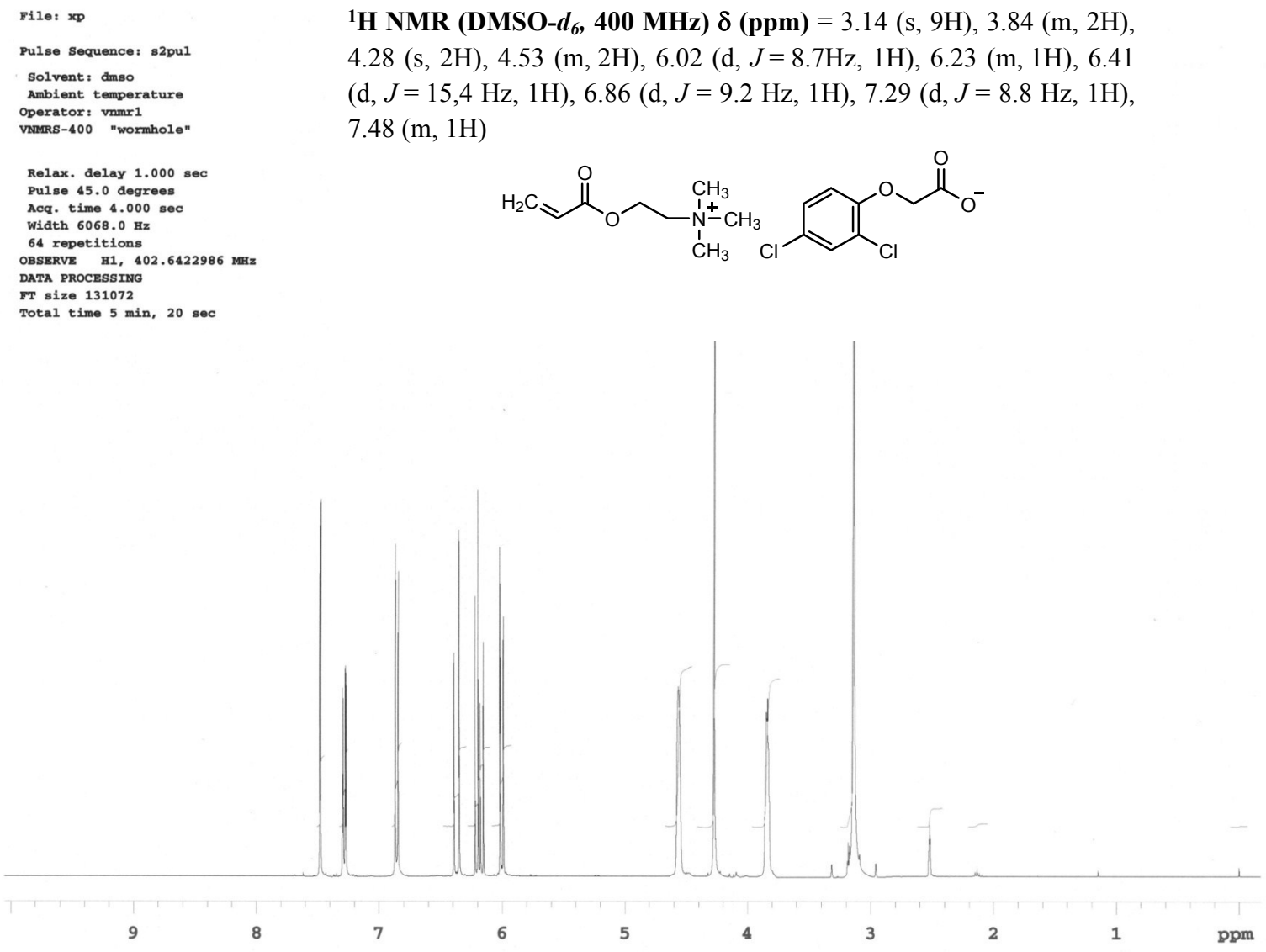
[1]H NMR (DMSO-d_6, 300 MHz) δ (ppm) = 3.20 (s, 9H), 3.81 (m, 5H), 4.54 (m, 2H), 6.02 (d, J = 8.7Hz, 1H), 6.21 (m, 1H), 6.39 (d, J = 15.6 Hz, 1H), 7.04 (d, J = 8,7 Hz, 1H), 7.16 (d, J = 8.7 Hz, 1H)

Figure S7. ^{13}C NMR spectrum of **4** ([2-(acryloyloxy)ethyl]trimethylammonium (2,4-dichlorophenoxy)acetate).



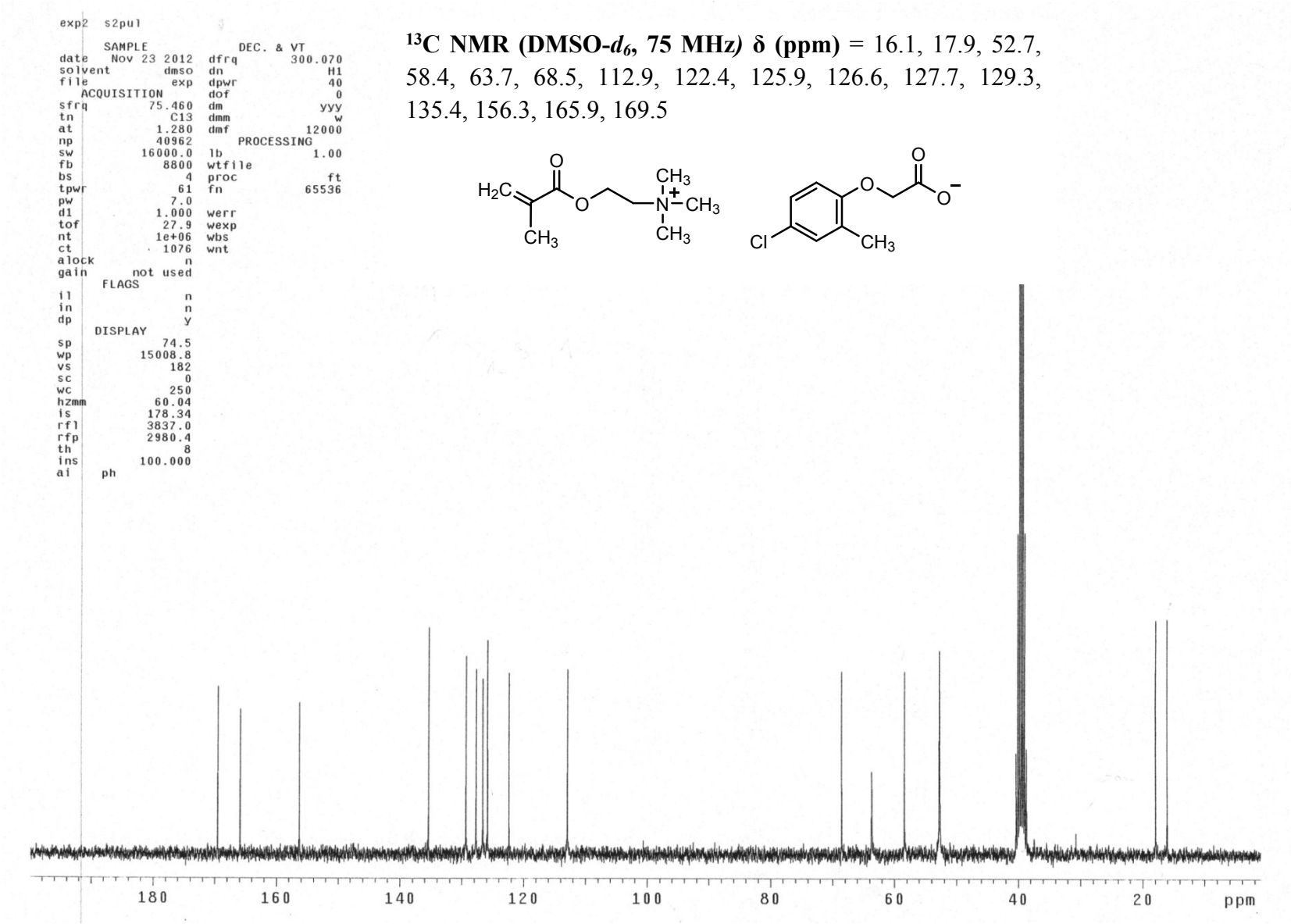
^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm) = 53.0, 58.2, 63.5, 68.5, 115.0, 121.7, 123.1, 127.6, 127.9, 128.8, 132.3, 153.7, 164.8, 169.5

Figure S8. ^{1}H NMR spectrum of **4** ([2-(acryloyloxy)ethyl]trimethylammonium (2,4-dichlorophenoxy)acetate).



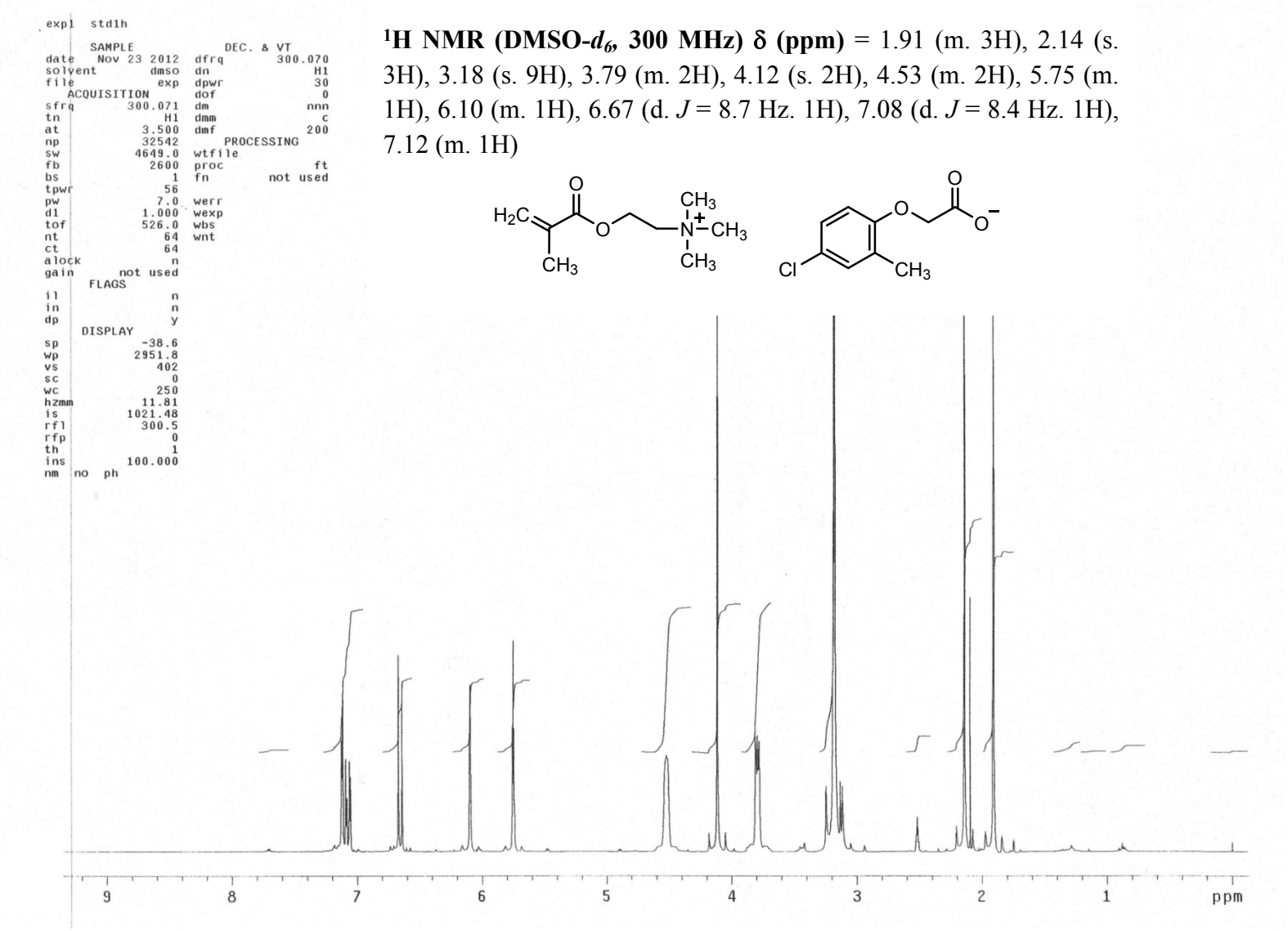
^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) = 3.14 (s, 9H), 3.84 (m, 2H), 4.28 (s, 2H), 4.53 (m, 2H), 6.02 (d, J = 8.7Hz, 1H), 6.23 (m, 1H), 6.41 (d, J = 15,4 Hz, 1H), 6.86 (d, J = 9.2 Hz, 1H), 7.29 (d, J = 8.8 Hz, 1H), 7.48 (m, 1H)

Figure S9. ^{13}C NMR spectrum of **5** ([2-(methacryloyloxy)ethyl]trimethylammonium (4-chloro-2-methylphenoxy)acetate).



^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) = 16.1, 17.9, 52.7, 58.4, 63.7, 68.5, 112.9, 122.4, 125.9, 126.6, 127.7, 129.3, 135.4, 156.3, 165.9, 169.5

Figure S10. [1]H NMR spectrum of **5** ([2-(methacryloyloxy)ethyl]trimethylammonium (4-chloro-2-methylphenoxy)acetate).



[1]H NMR (DMSO-d_6, 300 MHz) δ (ppm) = 1.91 (m. 3H), 2.14 (s. 3H), 3.18 (s. 9H), 3.79 (m. 2H), 4.12 (s. 2H), 4.53 (m. 2H), 5.75 (m. 1H), 6.10 (m. 1H), 6.67 (d. J = 8.7 Hz. 1H), 7.08 (d. J = 8.4 Hz. 1H), 7.12 (m. 1H)

Figure S11. ^{13}C NMR spectrum of **6** ([2-(methacryloyloxy)ethyl]trimethylammonium 2-(4-chloro-2-methylphenoxy)propioniate).

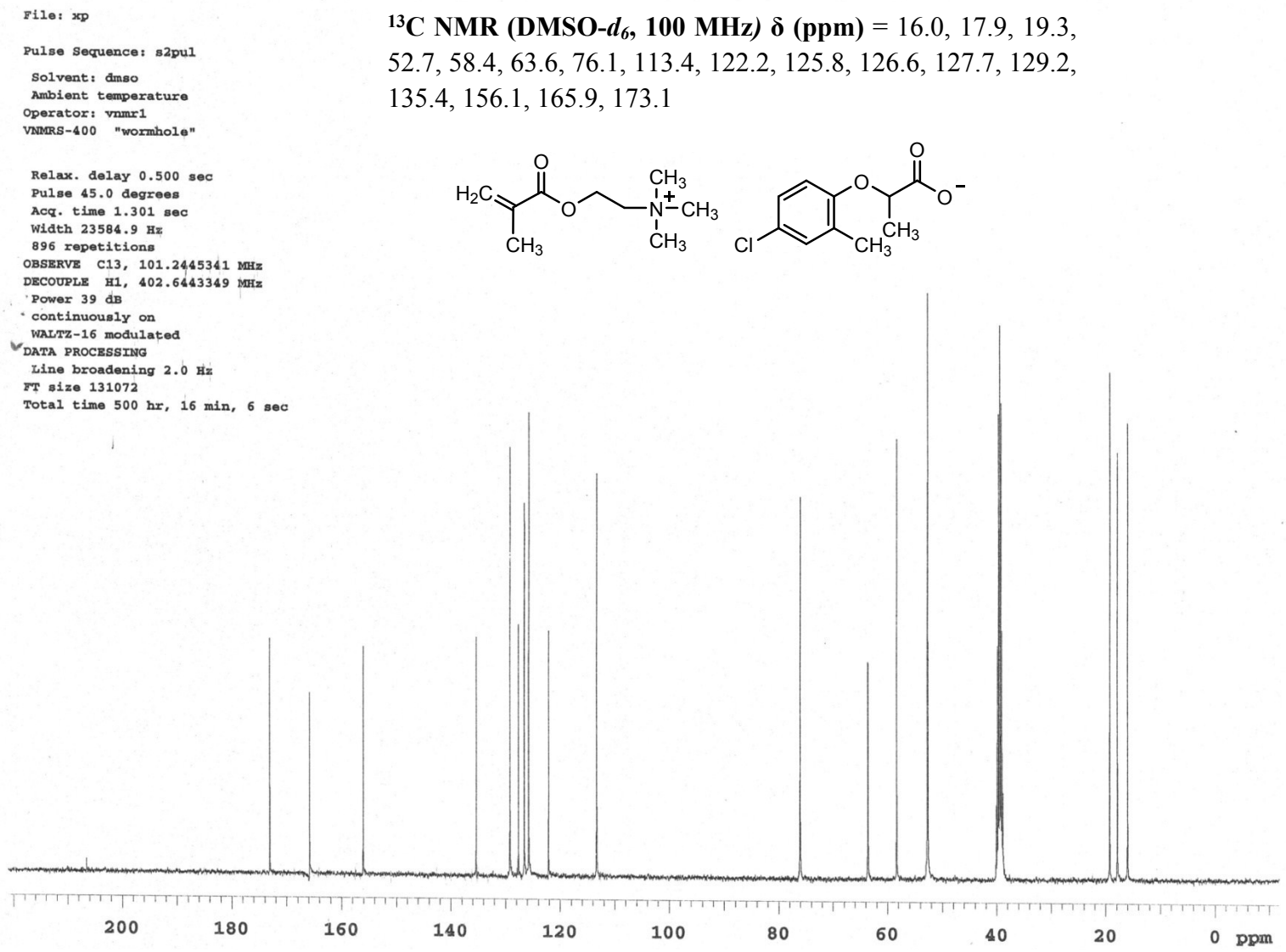
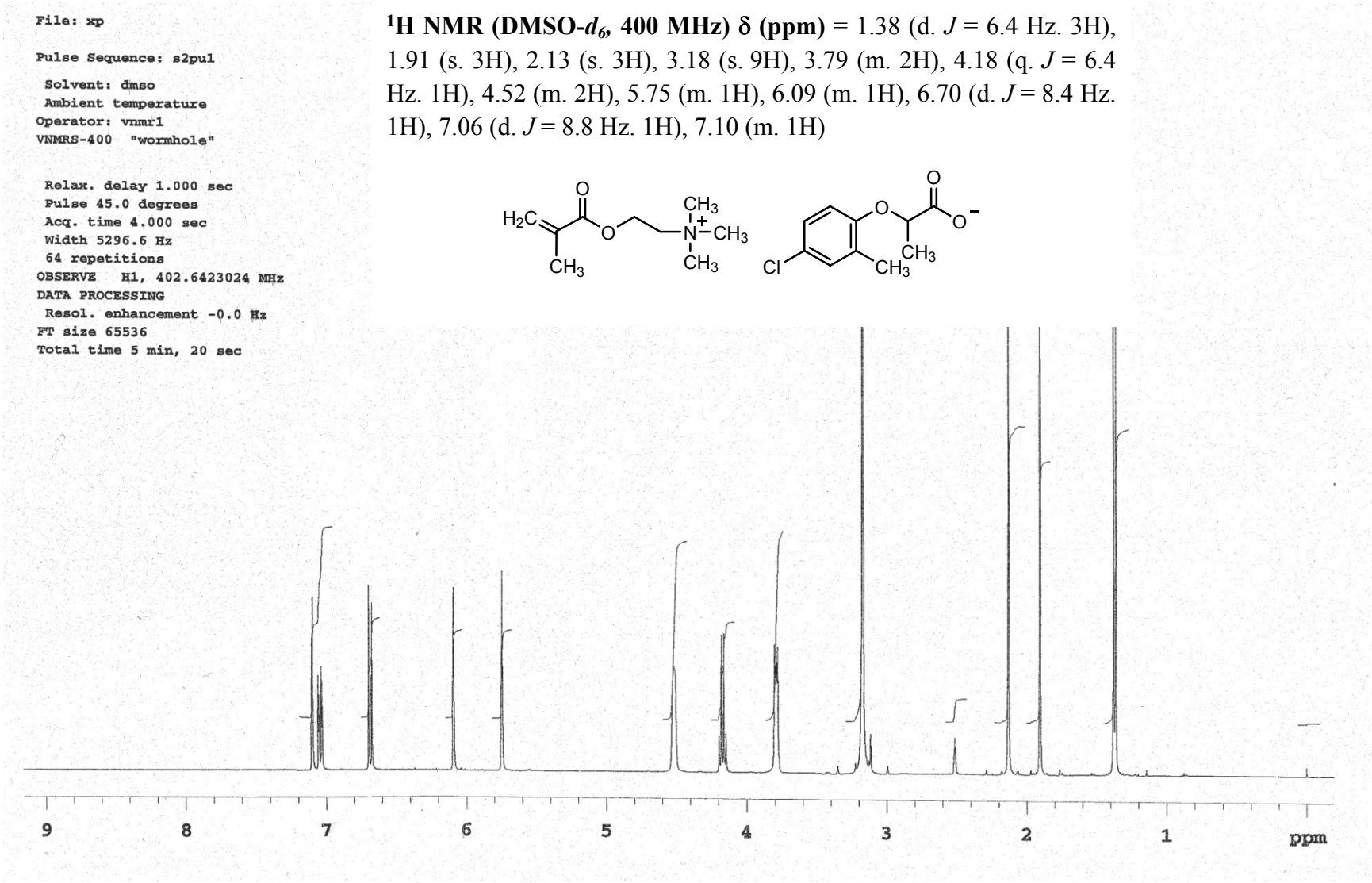
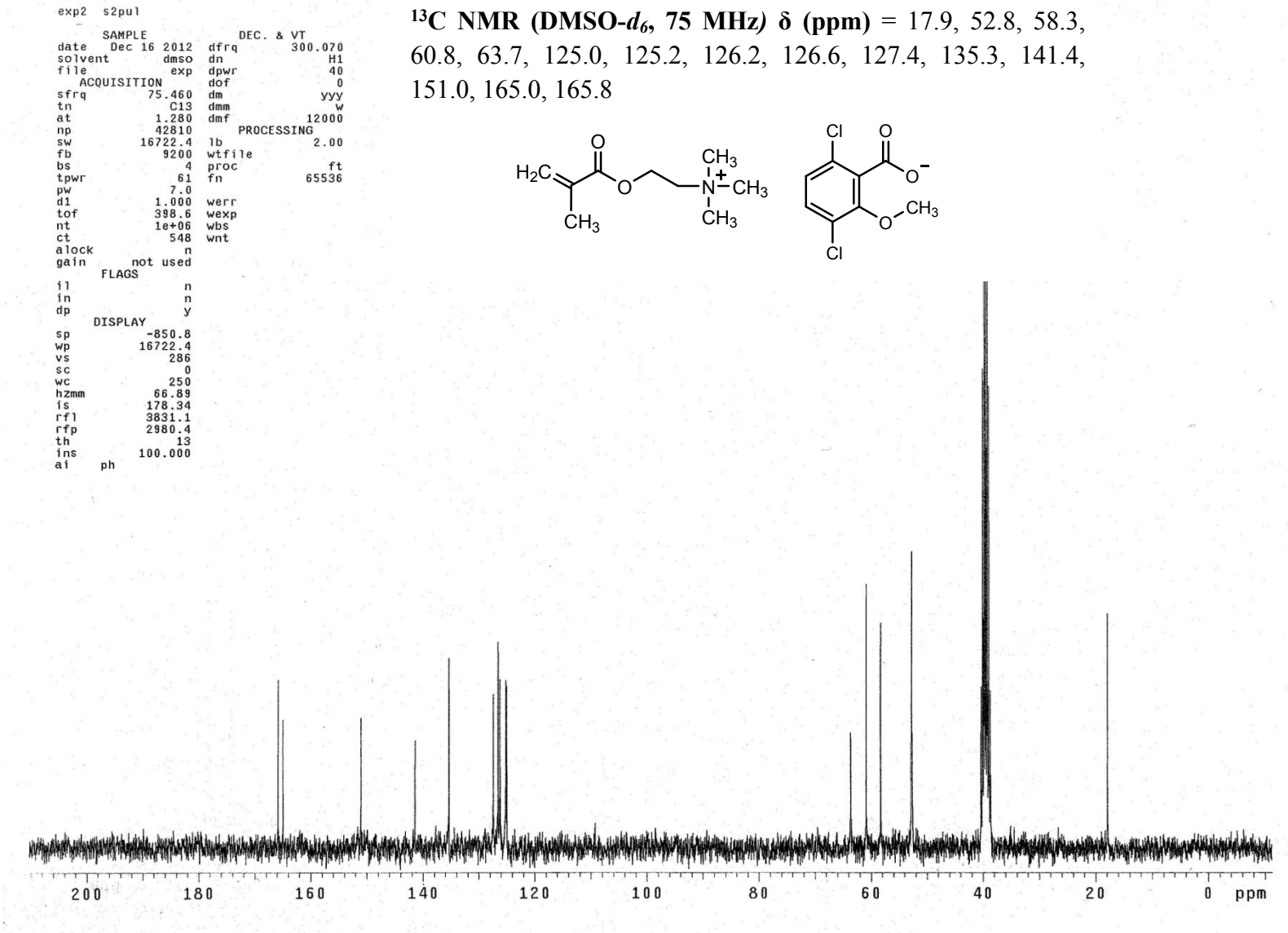


Figure S12. [1]H NMR spectrum of **6** ([2-(methacryloyloxy)ethyl]trimethylammonium 2-(4-chloro-2-methylphenoxy)propioniate).



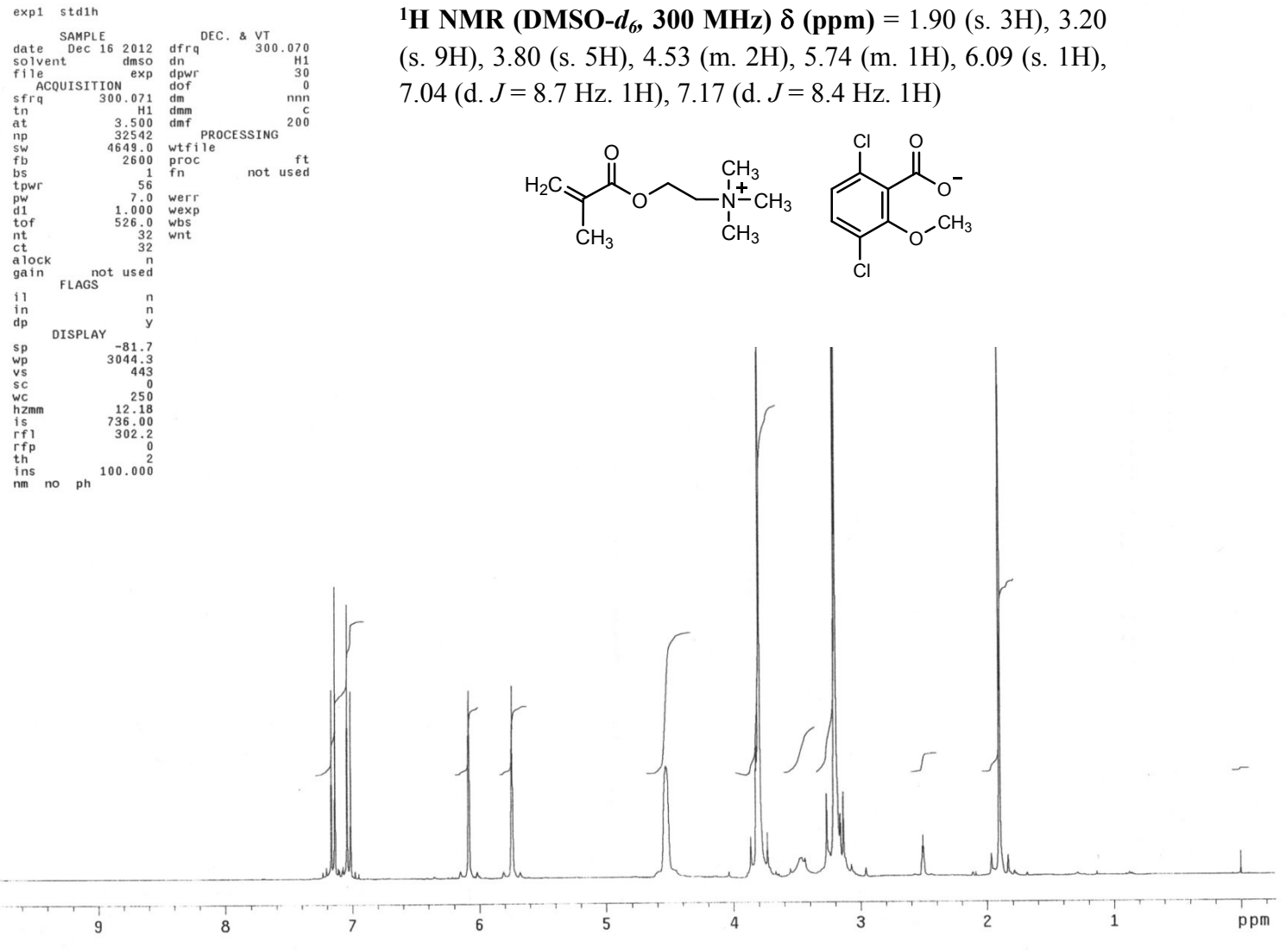
[1]H NMR (DMSO-d_6, 400 MHz) δ (ppm) = 1.38 (d. *J* = 6.4 Hz. 3H), 1.91 (s. 3H), 2.13 (s. 3H), 3.18 (s. 9H), 3.79 (m. 2H), 4.18 (q. *J* = 6.4 Hz. 1H), 4.52 (m. 2H), 5.75 (m. 1H), 6.09 (m. 1H), 6.70 (d. *J* = 8.4 Hz. 1H), 7.06 (d. *J* = 8.8 Hz. 1H), 7.10 (m. 1H)

Figure S13. ^{13}C NMR spectrum of **7** ([2-(methacryloyloxy)ethyl]trimethylammonium 3.6-dichloro-2-methoxybenzoate).



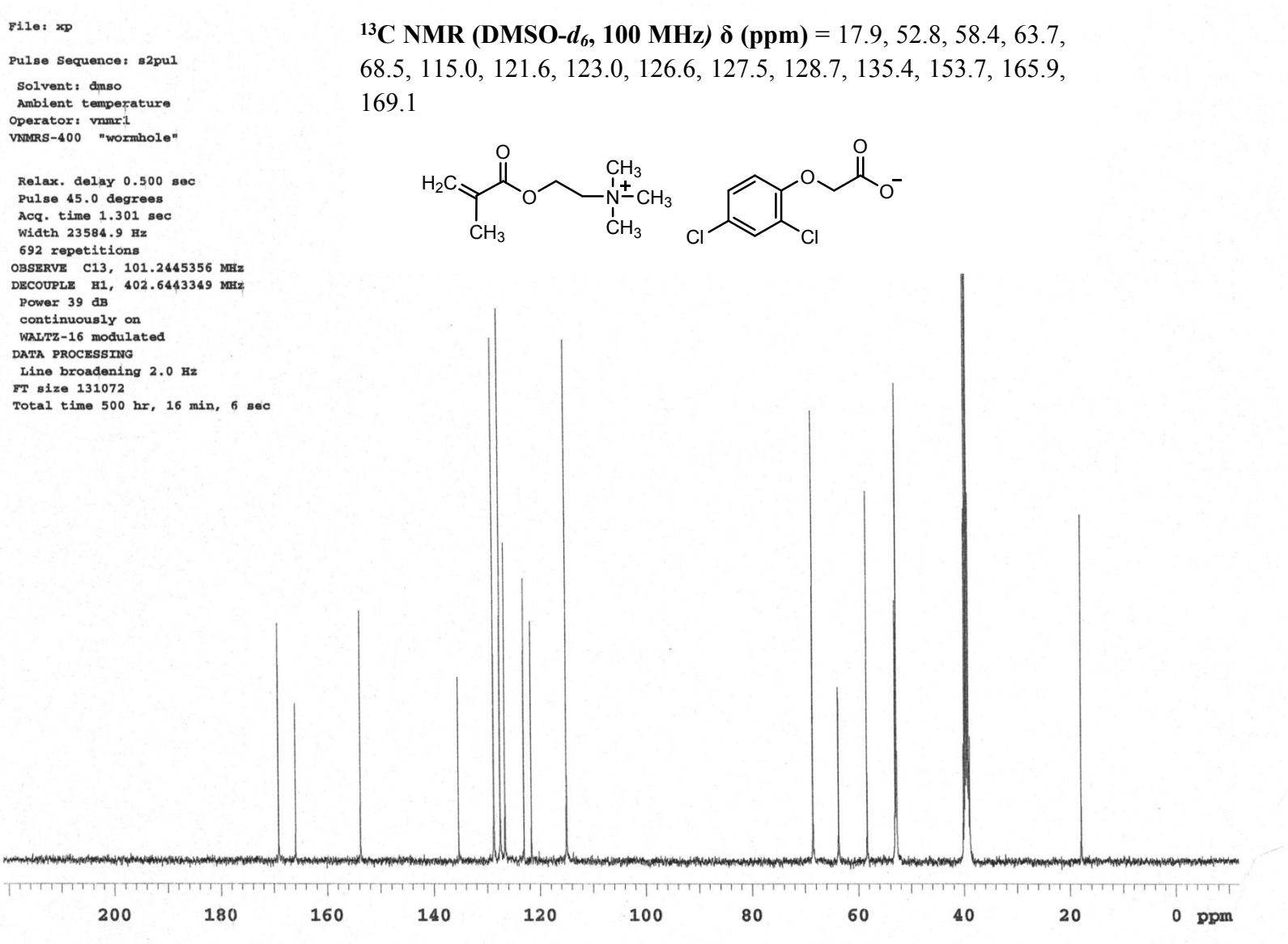
^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) = 17.9, 52.8, 58.3, 60.8, 63.7, 125.0, 125.2, 126.2, 126.6, 127.4, 135.3, 141.4, 151.0, 165.0, 165.8

Figure S14. [1]H NMR spectrum of **7** ([2-(methacryloyloxy)ethyl]trimethylammonium 3.6-dichloro-2-methoxybenzoate).



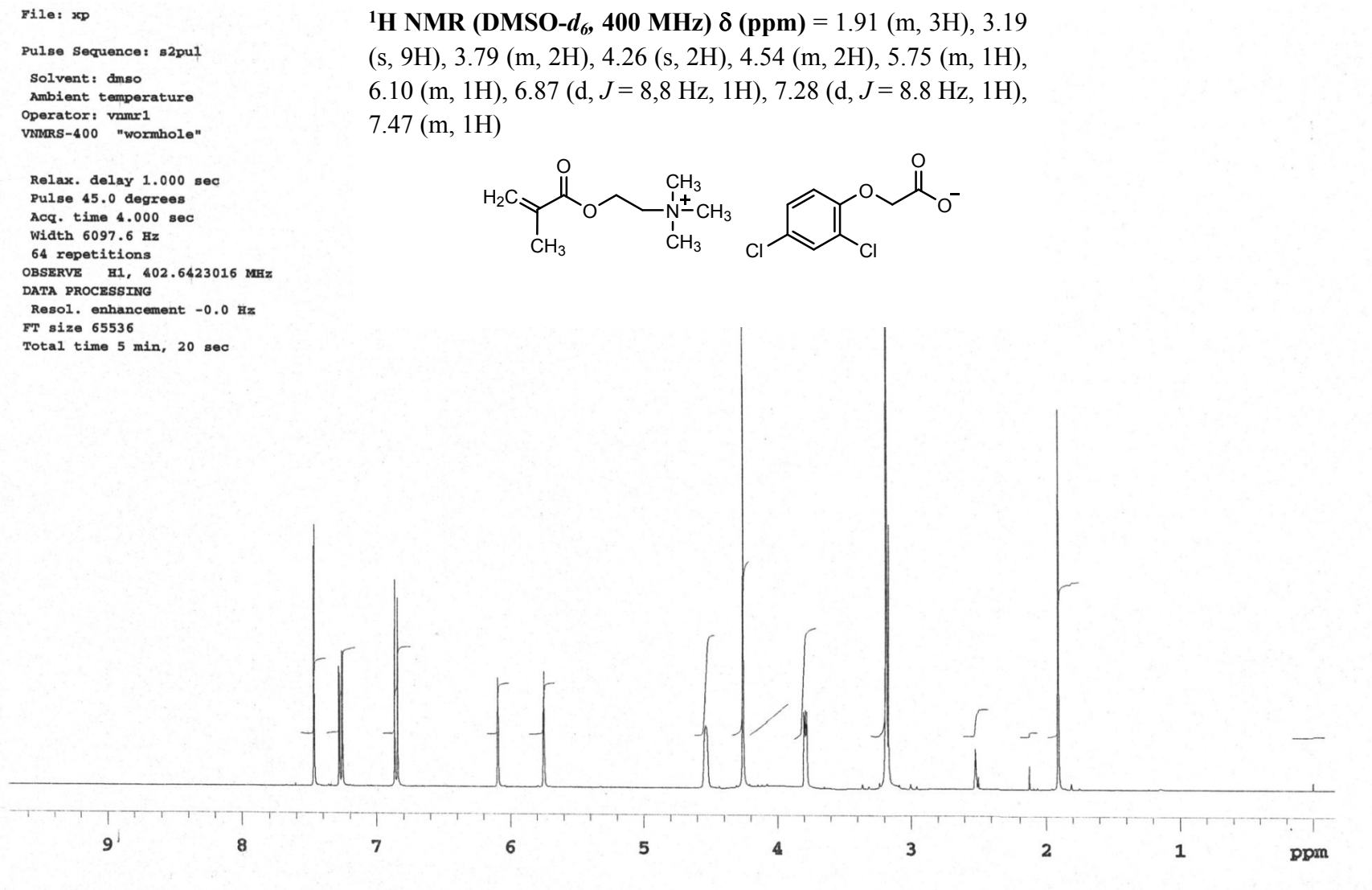
[1]H NMR (DMSO-d_6, 300 MHz) δ (ppm) = 1.90 (s. 3H), 3.20 (s. 9H), 3.80 (s. 5H), 4.53 (m. 2H), 5.74 (m. 1H), 6.09 (s. 1H), 7.04 (d. J = 8.7 Hz. 1H), 7.17 (d. J = 8.4 Hz. 1H)

Figure S15. ^{13}C NMR spectrum of **8** ([2-(methacryloyloxy)ethyl]trimethylammonium (2,4-dichlorophenoxy)acetate).



^{13}C NMR (DMSO-*d*$_6$, 100 MHz) δ (ppm) = 17.9, 52.8, 58.4, 63.7, 68.5, 115.0, 121.6, 123.0, 126.6, 127.5, 128.7, 135.4, 153.7, 165.9, 169.1

Figure S16. [1]H NMR spectrum of **8** ([2-(methacryloyloxy)ethyl]trimethylammonium (2,4-dichlorophenoxy)acetate).



[1]H NMR (DMSO-d_6, 400 MHz) δ (ppm) = 1.91 (m, 3H), 3.19 (s, 9H), 3.79 (m, 2H), 4.26 (s, 2H), 4.54 (m, 2H), 5.75 (m, 1H), 6.10 (m, 1H), 6.87 (d, J = 8,8 Hz, 1H), 7.28 (d, J = 8.8 Hz, 1H), 7.47 (m, 1H)

Figure S17. ^{13}C NMR spectrum of **9** (di(tallowoyloxyethyl)dimethylammonium (4-chloro-2-methylphenoxy)acetate).

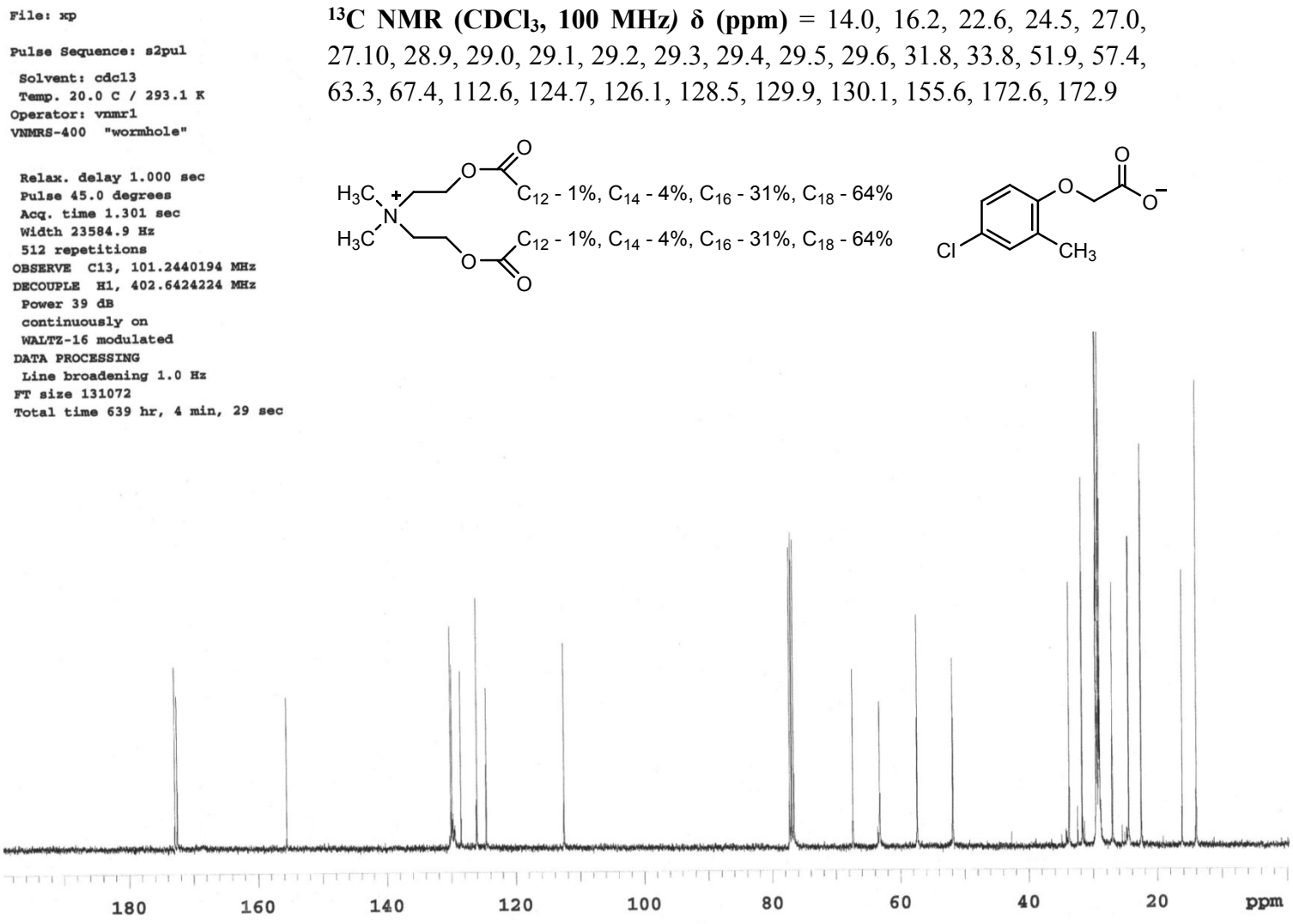
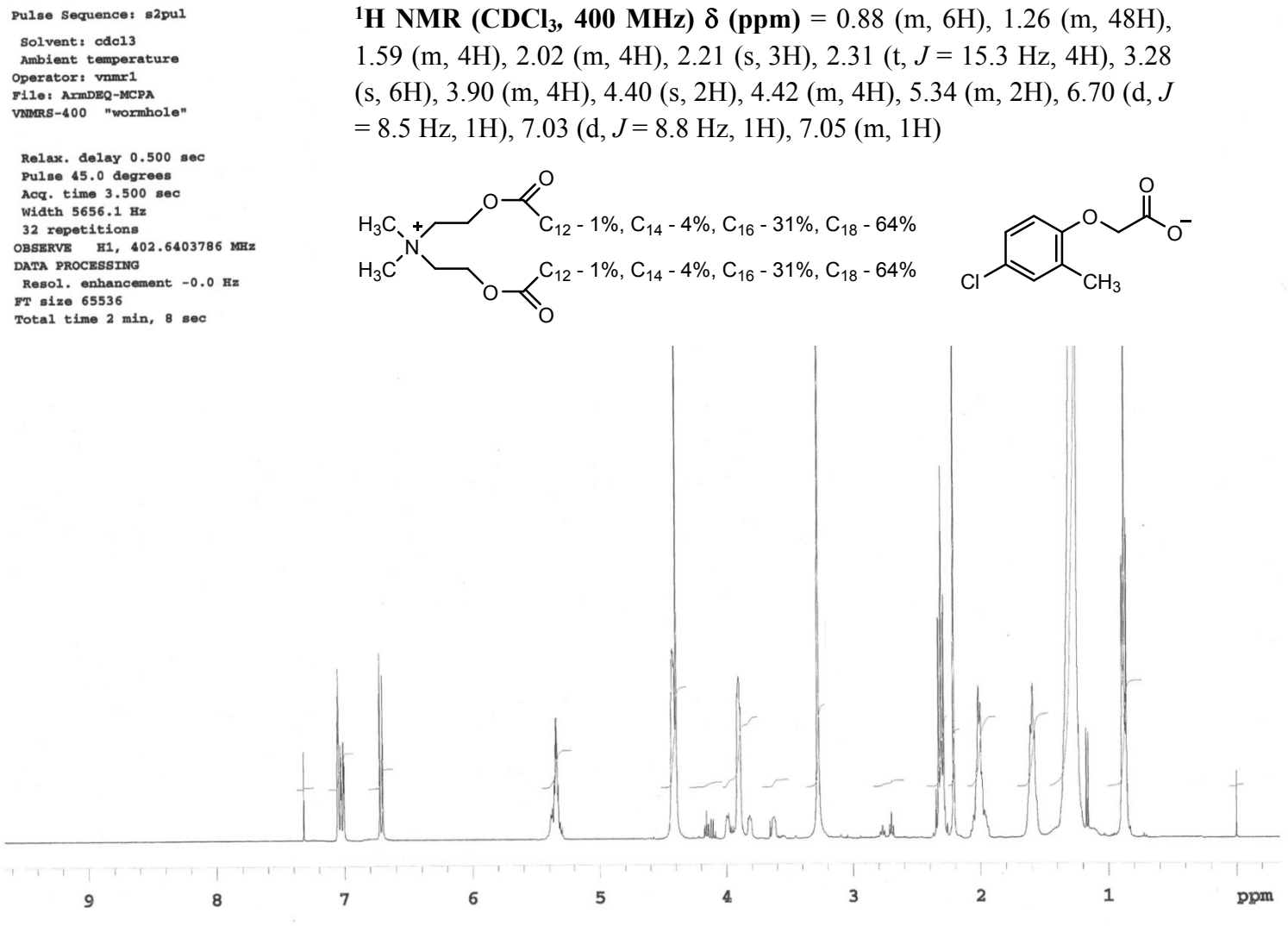
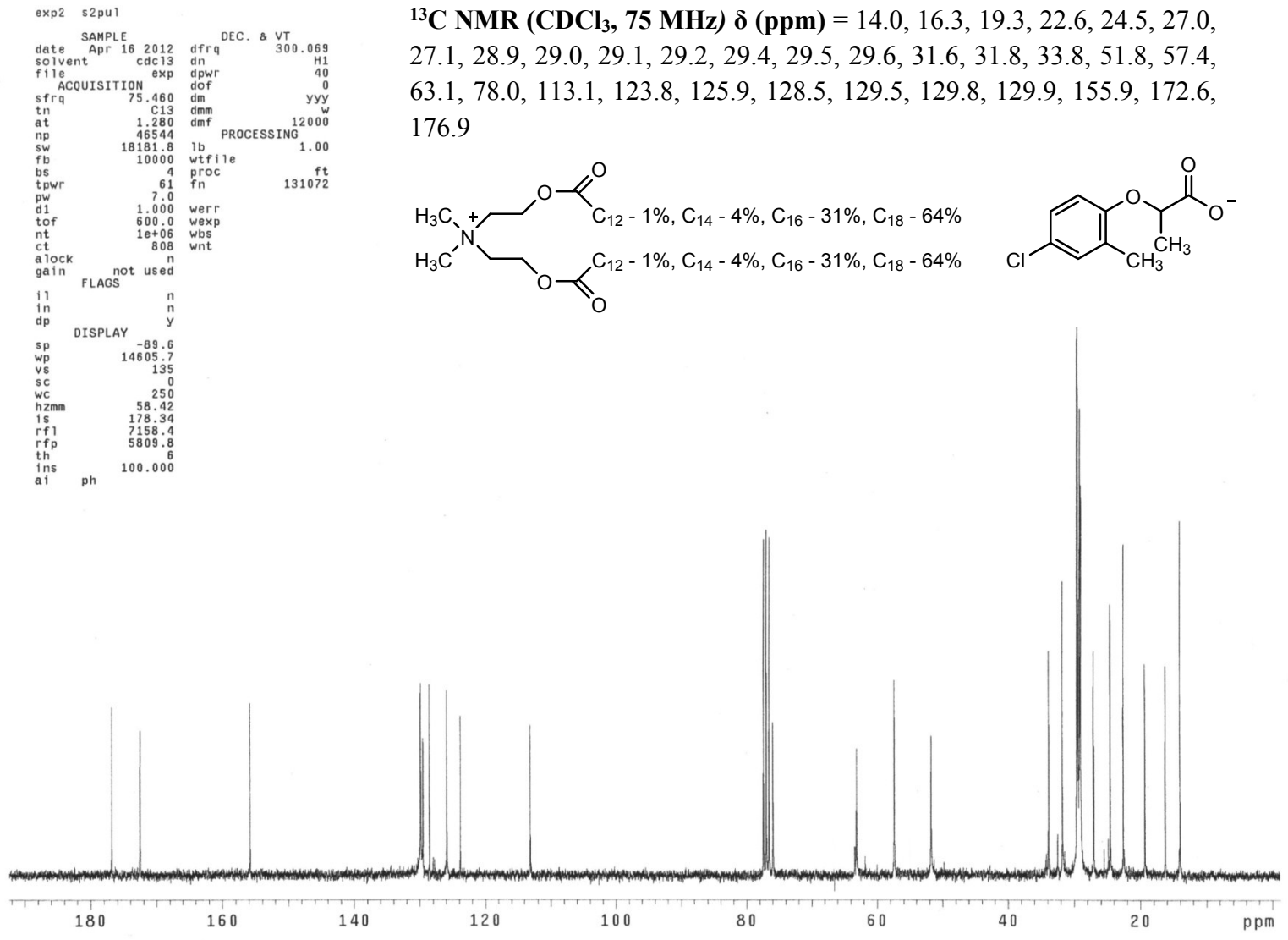


Figure S18. [1]H NMR spectrum of **9** (di(tallowoyloxyethyl)dimethylammonium (4-chloro-2-methylphenoxy)acetate).



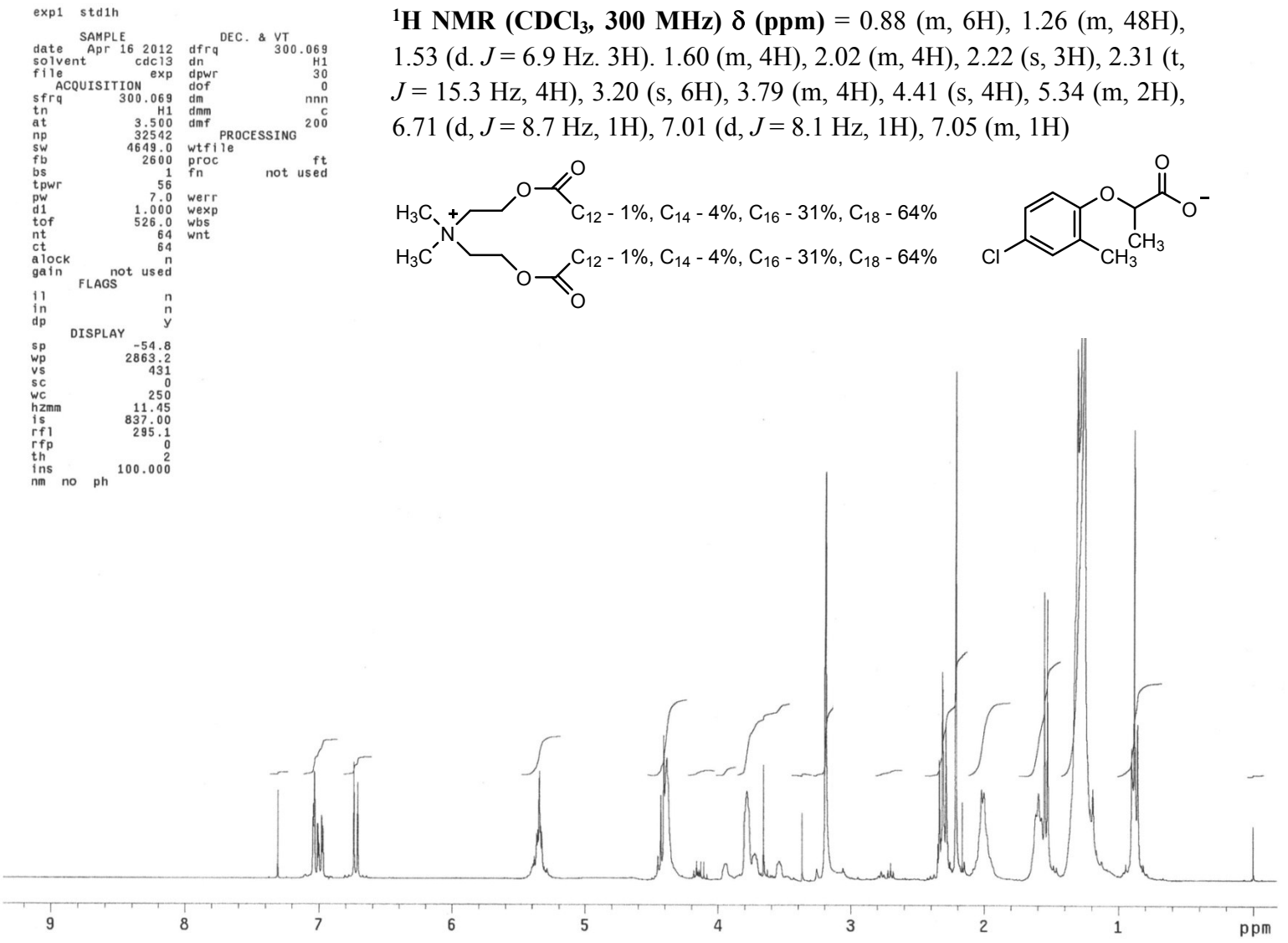
[1]H NMR ($CDCl_3$, 400 MHz) δ (ppm) = 0.88 (m, 6H), 1.26 (m, 48H), 1.59 (m, 4H), 2.02 (m, 4H), 2.21 (s, 3H), 2.31 (t, J = 15.3 Hz, 4H), 3.28 (s, 6H), 3.90 (m, 4H), 4.40 (s, 2H), 4.42 (m, 4H), 5.34 (m, 2H), 6.70 (d, J = 8.5 Hz, 1H), 7.03 (d, J = 8.8 Hz, 1H), 7.05 (m, 1H)

Figure S19. ^{13}C NMR spectrum of **10** (di(tallowoyloxyethyl)dimethylammonium 2-(4-chloro-2-methylphenoxy)propioniate).



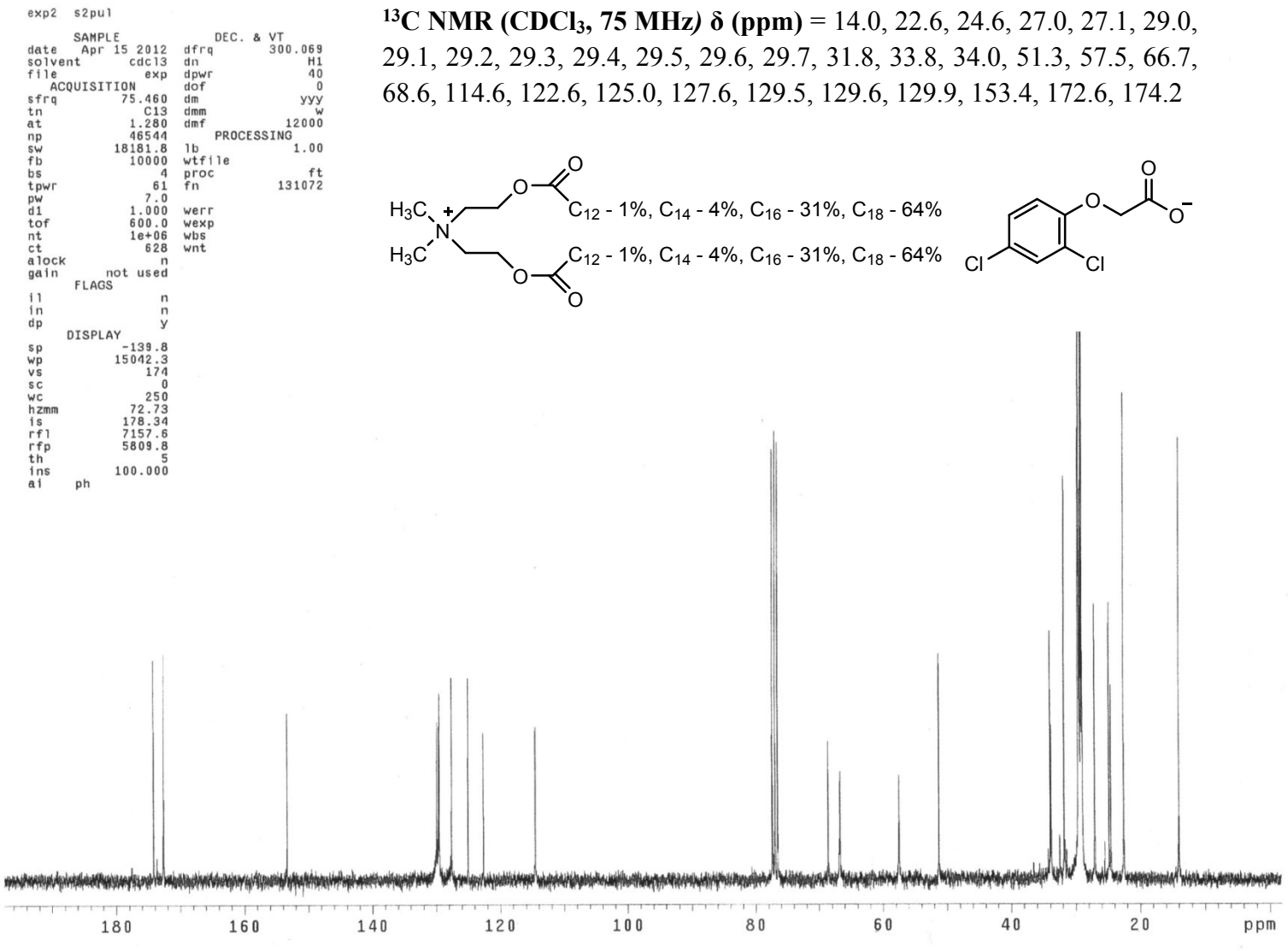
^{13}C NMR ($CDCl_3$, 75 MHz*)* δ (ppm) = 14.0, 16.3, 19.3, 22.6, 24.5, 27.0, 27.1, 28.9, 29.0, 29.1, 29.2, 29.4, 29.5, 29.6, 31.6, 31.8, 33.8, 51.8, 57.4, 63.1, 78.0, 113.1, 123.8, 125.9, 128.5, 129.5, 129.8, 129.9, 155.9, 172.6, 176.9

Figure S20. [1]H NMR spectrum of **10** (di(tallowoyloxyethyl)dimethylammonium 2-(4-chloro-2-methylphenoxy)propioniate).



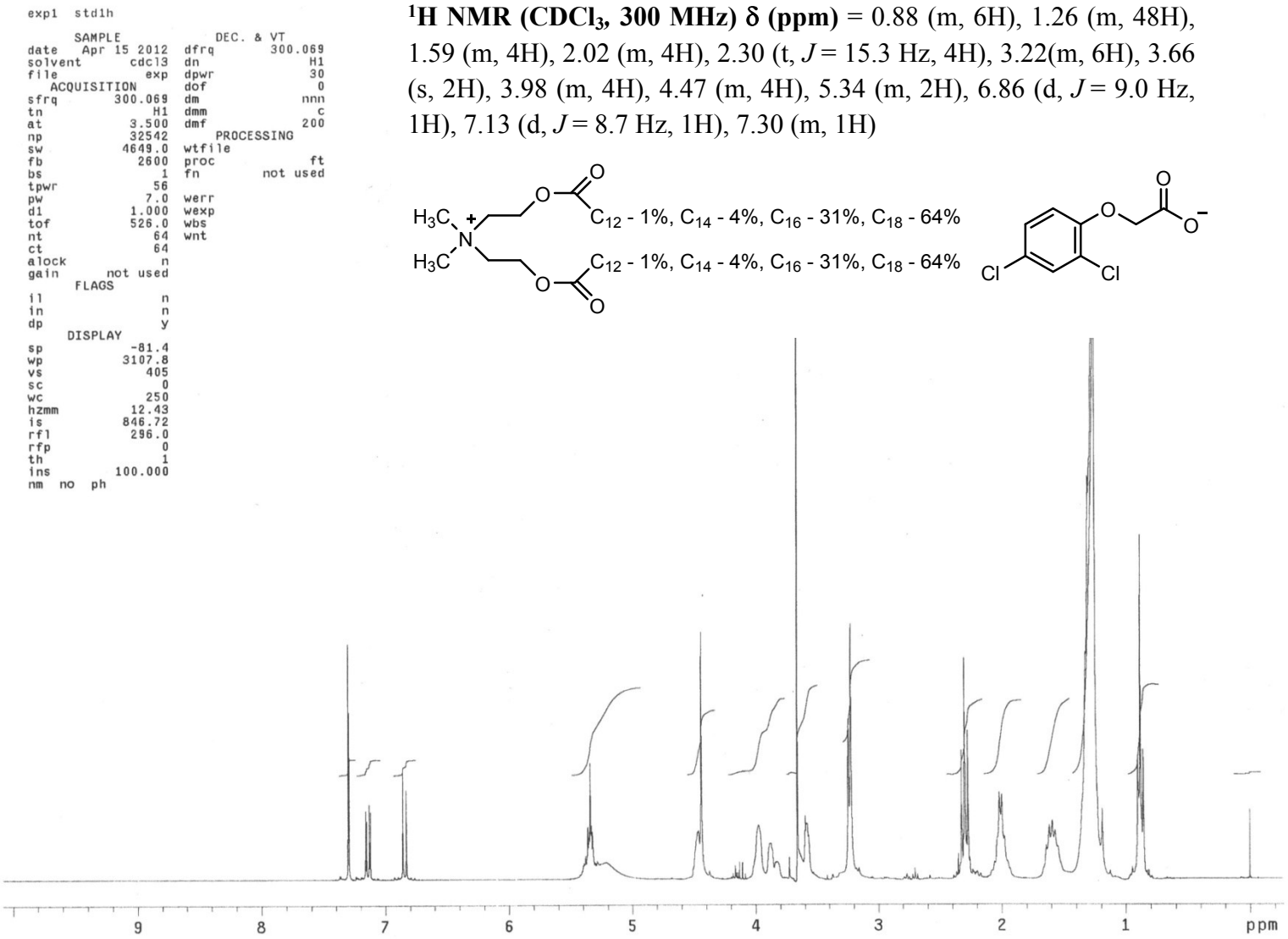
[1]H NMR ($CDCl_3$, 300 MHz) δ (ppm) = 0.88 (m, 6H), 1.26 (m, 48H), 1.53 (d. J = 6.9 Hz. 3H). 1.60 (m, 4H), 2.02 (m, 4H), 2.22 (s, 3H), 2.31 (t, J = 15.3 Hz, 4H), 3.20 (s, 6H), 3.79 (m, 4H), 4.41 (s, 4H), 5.34 (m, 2H), 6.71 (d, J = 8.7 Hz, 1H), 7.01 (d, J = 8.1 Hz, 1H), 7.05 (m, 1H)

Figure S21. ^{13}C NMR spectrum of **11** (di(tallowoyloxyethyl)dimethylammonium (2,4-dichlorophenoxy)acetate).



^{13}C NMR ($CDCl_3$, 75 MHz*)* δ (ppm) = 14.0, 22.6, 24.6, 27.0, 27.1, 29.0, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 31.8, 33.8, 34.0, 51.3, 57.5, 66.7, 68.6, 114.6, 122.6, 125.0, 127.6, 129.5, 129.6, 129.9, 153.4, 172.6, 174.2

Figure S22. [1]H NMR spectrum of **11** (di(tallowoyloxyethyl)dimethylammonium (2,4-dichlorophenoxy)acetate).



[1]H NMR ($CDCl_3$, 300 MHz) δ (ppm) = 0.88 (m, 6H), 1.26 (m, 48H), 1.59 (m, 4H), 2.02 (m, 4H), 2.30 (t, J = 15.3 Hz, 4H), 3.22(m, 6H), 3.66 (s, 2H), 3.98 (m, 4H), 4.47 (m, 4H), 5.34 (m, 2H), 6.86 (d, J = 9.0 Hz, 1H), 7.13 (d, J = 8.7 Hz, 1H), 7.30 (m, 1H)

2. Elemental analysis

[2-(Acryloyloxy)ethyl]trimethylammonium (4-chloro-2-methylphenoxy)acetate (**1**):
calcd (%) for $C_{17}H_{24}ClNO_5$ (M = 357.83): C 57.06, H 6.76, N 3.91; found: C 57.28, H 6.59, N 4.13.

[2-(Acryloyloxy)ethyl]trimethylammonium 2-(4-chloro-2-methylphenoxy)propioniate (**2**):
calcd (%) for $C_{18}H_{26}ClNO_5$ (M = 371.86): C 58.14, H 7.05, N 3.77; found: C 58.37, H 6.81, N 3.92.

[2-(Acryloyloxy)ethyl]trimethylammonium 3,6-dichloro-2-methoxybenzoate (**3**):
calcd (%) for $C_{16}H_{21}Cl_2NO_5$ (M = 378.25): C 50.81, H 5.60, N 3.70, found; C 50.64, H 5.82, N 3.89.

[2-(Acryloyloxy)ethyl]trimethylammonium (2,4-dichlorophenoxy)acetate (**4**):
calcd (%) for $C_{16}H_{21}Cl_2NO_5$ (M = 378.25): C 50.81, H 5.60, N 3.70, found; C 51.05, H 5.57, N 3.43.

[2-(Methacryloyloxy)ethyl]trimethylammonium (4-chloro-2-methylphenoxy)acetate (**5**):
calcd (%) for $C_{18}H_{26}ClNO_5$ (M = 371.86): C 58.14. H 7.05. N 3.77, found: C 58.41, H 6.88, N 3.90.

[2-(Methacryloyloxy)ethyl]trimethylammonium 2-(4-chloro-2-methylphenoxy)propioniate (**6**)
calcd (%) for $C_{19}H_{28}ClNO_5$ (M = 385.88): C 59.14, H 7.31, N 3.63, found: C 58.89, H 7.45, N 3.81.

[2-(Methacryloyloxy)ethyl]trimethylammonium 3.6-dichloro-2-methoxybenzoate (**7**):
calcd (%) for $C_{17}H_{23}Cl_2NO_5$ (M = 392.27): C 52.05, H 5.91, N 3.57, found: C 52.33, H 5.62, N 3.78.

[2-(Methacryloyloxy)ethyl]trimethylammonium (2,4-dichlorophenoxy)acetate (**8**):
calcd (%) for $C_{17}H_{23}Cl_2NO_5$ (M = 392.27): C 52.05, H 5.91, N 3.57, found: C 52.38, H 5.72, N 3.74.

3. Acute toxicity test towards rats

3.1. Animals and conditions of the sighting and the main studies

3.1.1. Test animals (certificate no. 482324)

The experiment was conducted on the Wistar female rats (Crl: WI(Han); outbred) obtained from the husbandry of laboratory animals of the Experimental Medicine Centre at the Medical University in Białystok kept behind the breeding barrier (number in the register of units entitled to the husbandry of laboratory animals: 0043). The Health Certificate issued by the Experimental Medicine Centre at the Medical University in Białystok confirmed the animals' good health. animals were quarantined and observed daily for 5 days. A general medical-veterinary examination was performed on the day of the introduction of the animals to the quarantine, whereas a detailed medical-veterinary examination was performed before the beginning of the experiment. Animals without any clinical signs were randomly chosen to be used in the experiment. Their sex and body weights were taken into account. All animals were marked for individual identification. One 10-week-old animal weighing 227 g (dose of 300 mg/kg b.w.) and one 11-week-old animal weighing 201 g (dose of 2000 mg/kg b.w.) were used in the sighting study. Four 11-week-old animals whose average body weight was 205 g (dose of 2000 mg/kg b.w.) were used in the main study.

3.1.2. Housing conditions

During the quarantine, the sighting study, and the main study, the animals were kept in air-conditioned rooms under the following conditions: air temperature: 20 – 25 °C; relative air humidity: 42 – 65%; artificial fluorescent lighting; lighting cycle: 12 hours light/12 hours dark; facility air exchange: about 16 times/hour. The animals were kept in plastic cages covered with wire bar lids. The dimensions of the cages were 58 x 37 x 21 cm (length x width x height). In the sighting study, the animals were caged individually. In the main study, there were four animals in one cage. UV-sterilized wood shavings were used as bedding. Each cage was equipped with a label containing the study code, the dose, the dates of the commencement and the planned end of the experiment, and the animals' sex and numbers.

3.1.3. Food and water

The animals were given *ad libitum* access to the "Murigran" standard granulated laboratory fodder produced by AGROPOL, Motycz Poland and drinking tap water.

3.2. Course of the sighting and the main studies

3.2.1. Preparation of the animals

On the days before the start of the sighting and the main studies (about 19 hours before the administration of the test item), food was withheld, but water was still available. Food was given back 3 hours after the administration of the test item.

3.2.2. Administration of the test item

3.2.2.1. Sighting study

In the view of the fact that no information on toxicity of the test item was available, a single dose of 300 mg/kg b.w. was administered to one animal used in the sighting study. The test item in the form of an oil solution in a volume of 0.5 mL/100 g b.w. was administered with a metal stomach tube. One milliliter of the administered solution contained 60 mg of the test item (dose of 300 mg/kg b.w.). The oil solution of the test item was prepared on the administration day (shortly before the administration). Considering the fact that no evident toxicity was produced, the test item at a single dose of 2000 mg/kg b.w. was administered to the next animal. The test item in the form of an oil solution in a volume of 0.5 mL/100 g b.w. was administered with a metal stomach tube. One milliliter of the administered solution contained 400 mg of the test item (dose of 2000 mg/kg b.w.). The oil solution of the test item was prepared on the administration day (shortly before the administration). On the grounds of the sighting study results, the dose of 2000 mg/kg b.w. was selected to be used in the main study.

3.2.2.2. Main study

The test item at a single dose of 2000 mg/kg b.w. was administered to four animals. The animal from the sighting study which had received the dose of 2000 mg/kg b.w. was included in the main study. The test item in the form of an oil solution in a volume of 0.5 mL/100 g b.w. was administered with a metal stomach tube. One milliliter of the administered solution contained 400 mg of the test item. The oil solutions of the test item were prepared on the administration day (shortly before the administration).

3.3. Parameters, the scope of studies, and dates in the sighting and the main studies

3.3.1. Clinical observations

After the administration of the test item, the animals were observed for 14 days. These observations comprised the evaluation of general condition of the animals and detailed clinical observations. The evaluation of general condition of the animals, i.e. the observation of all animals for morbidity and mortality was conducted twice a day or once a day (on days off) during the 14-day experiment. The detailed clinical observations were performed on the

day of the test item administration (day 0), i.e. 10, 30, and 60 minutes after the administration and then at hourly intervals up to the 5th hour after the administration. From the 1st to the 14th day of the experiment, the detailed clinical observations were performed once a day.

3.3.2. Body weights of the animals

Body weights of the animals were determined on days 0 (directly before the administration of the test item), 7, and 14 (before euthanasia).

3.3.3. Gross examinations

After the 14-day observation period, all animals were euthanized by intraperitoneal administration of morbital at a dose of 200 mg/kg b.w. and subjected to gross examinations. The detailed gross examinations comprised the observation of the external body surface, all natural apertures, and the cranial, thoracic, and abdominal cavities with their contents. After the gross examinations, the animals were transferred to utilization.

4. Acute oral toxicity test towards *Oncorhynchus mykiss* (rainbow trout)

4.1. Test organism

The test was conducted on the rainbow trout, *Oncorhynchus mykiss*. The fish were obtained from 'The Culture of Salmonidae Fish in Zawoja', Poland. They were approximately 2 months old. To confirm the sensitivity of the fish, a test with a reference substance, i.e. 3,4 – dichloroaniline was conducted. After euthanasia, the length and weight of the fish used in the definitive test were measured. The average body length was 4.7 cm ± 0.3 cm (minimum: 4.0 cm, maximum: 5.2 cm), whereas the average weight was 1.23 g ± 0.02 g.

4.2. Adaptation - definitive test

After delivery, the fish were quarantined for 7 days. The aims of the quarantine were to confirm good health of the fish and to allow them to acclimatize to the test conditions. The adaptation was performed in an air-conditioned room (TADIRAN air conditioner, Israel). The fish were kept in a tank with a capacity of 2.5 m^3. Water circulation in the closed system was continuous. Water was continuously filtered (through the layers of a sponge, active carbon, and leca stones), aerated, and disinfected using a UV lamp. During the adaptation, the fish were fed with standard granulated food in the amount of 2% of their average body weight per day (Aller Aqua standard granulated food for fish, Denmark). Feeding was terminated 24 hours before the exposure initiation. The fish were healthy and free from external parasites and visible deformations (a veterinary control). Fish mortality during the quarantine period was lower than 5% (2 dead fish/1000 fish in the batch).

4.3. Acute toxicity test

The study was conducted in compliance with the OECD Guideline for the Testing of Chemicals No. 203 (1992) and the principles of Good Laboratory Practice.

4.3.1. Preliminary test (non-GLP)

The preliminary test (non-GLP) was conducted before the beginning of the study in order to determine the design of the definitive test and the range of concentrations to be used.

4.3.1.1. Preliminary test conditions

The preliminary test (non-GLP) on the rainbow trout was performed as a static test. The fish were exposed for 96 hours. Conditioned water (the same as during the adaptation period) was used. The pH value measured at the exposure initiation was 7.53. The hardness was 47 mg $CaCO_3$/L. The pH values and the dissolved oxygen concentrations were measured at the exposure initiation and after 24, 48, 72, and 96 hours of the exposure. The dissolved oxygen concentration was higher than 60% of the air saturation value. Water was constantly aerated. During the exposure, the water temperature was constantly recorded (HI 141 temperature logger, Hanna Instruments, USA; GEA air conditioner, Küba Kältetechnik GmbH, Germany). The test was performed under natural lighting, with additional fluorescent lighting of a daily cycle: 16 hours light: 8 hours dark (KANLUX electronic time programmer, Poland). The fish were not fed during exposure. The fish were observed for intoxication symptoms (loss of equilibrium and changes in swimming behavior, respiration, or pigmentation) and mortality after 3, 6, 24, 48, 72, and 96 hours of the exposure. Fish are considered dead if they do not react to external stimuli. In the preliminary test, three fish were introduced into each test item concentration and the control. The preliminary test was conducted in glass aquaria with a capacity of 10 L.

4.3.1.2. Preparing of the test item concentrations in the preliminary test

In the preliminary test, the concentrations of the test item of 0.1, 1.0, 10, 100 mg/L and a control were used. First, 1000.5 mg of the test item was weighed into a glass flask. Next, approximately 150 mL of conditioned water were added. The content of flask was sonicated for 1 minute. Next, the content of flask was transferred into glass flask with a capacity of 5 L by multiple washing with conditioned water. The flask was filled up into 4 L of conditioned water. The content of flask was sonicated for 25 minutes. The content of flask was visually homogeneous, nontransparent with foam. Next the content of flask was transferred into glass aquarium and filled up to total volume of 10 L with conditioned water. The lower test item concentrations were prepared by sequential dilutions with the conditioned water in ratio 1:9 (i.e. 1 L of the higher test item concentration was mixed with 9 L of conditioned water). The

test item, concentrations of 10 and 100 mg/L were visually homogeneous with foam. The control was 9 L of conditioned water.

4.3.2. Definitive test

On the basis of the results of the preliminary test (non-GLP), the definitive test was performed according to the procedure described below.

4.3.2.1. Definitive test conditions

The definitive test on the rainbow trout was performed as a static test. The fish were exposed for 96 hours. Conditioned water (the same as during the adaptation period) was used. The pH value measured at the exposure initiation in the control was 7.25. The hardness was 60.5 mg $CaCO_3$/L. The pH values and the dissolved oxygen concentrations were measured at the exposure initiation and after 24, 48, 72, and 96 hours of the exposure. The dissolved oxygen concentration was higher than 60% of the air saturation value. Water was constantly aerated. During the exposure, the water temperature was constantly recorded (HI 141 temperature logger, Hanna Instruments, USA; GEA air conditioner, Küba Kältetechnik GmbH, Germany). The test was performed under natural lighting, with additional fluorescent lighting of a daily cycle: 16 hours light: 8 hours dark (KANLUX electronic time programmer, Poland). The fish were not fed during exposure. The fish were observed for intoxication symptoms (loss of equilibrium and changes in swimming behavior, respiration, or pigmentation) and mortality after 3, 6, 24, 48, 72, and 96 hours of the exposure. Fish are considered dead if they do not react to external stimuli. In the definitive test, seven fish were introduced into each aquarium. The ratio of fish weight per volume 10 L in the definitive test was 0.86 g/L.

4.3.2.2. Preparing of the test item concentrations in the definitive test

In the definitive test, the concentrations of the test item of 0.9, 1.9, 4.3, 9.4, 20.7, 45.5, 100 mg/L and a control were used. The appropriate amounts of the test item were separately weighted into glass beakers. Into each beaker 150 mL of conditioned water was added. The content of each beaker was sonicated for 1 minute and then quantitatively transferred into the flasks by multiple washing with conditioned water. The content of flasks was sonicated for a maximum of 30 minutes. Next, the content of each flask was introduced into the appropriate aquarium and filled up with conditioned water. All test item concentrations were visually homogeneous. The test item concentration of 100 mg/L was nontransparent with foam. The test item concentrations of 0.9, 1.9, 4.3, 9.4, 20.7, 45.5 mg/L were transparent. The aquaria contained 10 L of each test item concentration and the control (conditioned water). Two samples, of each test item concentration and the control were collected. One of them was transferred for chemical analyses at exposure initiation, whereas the other was located next to

the aquaria under test conditions and transferred for chemical analyses at exposure termination. Into each aquarium seven fish were introduced. The aquaria were covered with glass lids in order to minimize evaporation and to prevent accidental loss of fish (due to jumping out of aquarium).

4.4. Test with the reference substance

The test with a reference substance, i.e. 3,4 – dichloroaniline was performed. The conditions of the test were as follows: temperature: 14.8-16.5 ^{o}C; pH of the control: 7.43-7.56; oxygen concentration: 80.8- 99.8%; lighting: 16 hours light: 8 hours dark, feeding: no. The test was performed between 23.10.2014 - 27.10.2014. One concentration of the reference substance of 8.0 mg/L and a control (conditioned water) were used. Glass aquaria with a capacity of 35 L were used as test vessels. Ten fish were introduced into each aquarium. The fish were observed for intoxication symptoms and mortality after 3, 6, 24, 48, 72, and 96 hours of the exposure.

5. Growth inhibition test of *Pseudokirchneriella subcapitata* (green algae)

5.1. Test organism

The test was conducted on the unicellular green algae, *Pseudokirchneriella subcapitata* (Reinsch) Korshikov (syn. *Selenastrum capricornutum* Prinz) from a standard laboratory culture cultivated at the Institute of Industrial Organic Chemistry, Branch Pszczyna, Department of Ecotoxicology, Laboratory of Aquatic Toxicology Poland according to the recommendations specified in the OECD Guideline for the Testing of Chemicals No. 201. The algae, *Pseudokirchneriella subcapitata* SAG 61.81 on agar bevels were obtained from the Culture Collection of Algae at Göttingen University, Germany.

5.1.1. Culturing of algae

The algae were transferred from agar bevels to a fresh AAP medium) contained in Erlenmeyer flasks with a capacity of 250 mL, and incubated at temperature between 21- 24 °C under constant illumination (a pre-culture). The algal culture was renewed (transferred to a fresh medium) twice a week under sterile conditions. The pre-culture whose density was 1 x 10^4 cells/mL was renewed three days before the definitive test. The pre-culture was used for inoculation of the test item concentrations and the control. The sensitivity of the algal culture is monitored every six months using a reference substance - 3,5-dichlorophenol.

5.1.2. Culturing medium

The AAP medium (U.S. EPA) recommended by the OECD Guideline No. 201 was used as a culture medium for the test organism and a diluent/solvent of the test item. The AAP medium

was a source of nutrients necessary for algal growth. The AAP medium was prepared on the basis of deionized water (SolPure7 filtering system) by adding stock solutions of reagents of recognized analytical grade. The stock solutions were renewed, sterilised by autoclaving (with exception of sodium hydrogen carbonate – the buffering ingredient), and stored in a refrigerator.

5.2. Algal growth inhibition test

The *Pseudokirchneriella subcapitata* growth inhibition test was performed according to the OECD Guideline for the Testing of Chemicals No. 201 (2006).

5.2.1. Test conditions

To maintain constant conditions throughout the preliminary test (non-GLP) and the definitive test, a thermostatic chamber was used (ILW400STD incubator with a shaker, Pol-Eko-Aparatura, Poland). In the definitive test, the temperature was continuously measured using a sensor submerged in an additional test vessel containing 100 mL of the AAP medium (HI 141 thermologger, Hanna Instruments, USA). The tests were performed under constant illumination; a fluorescent lighting (six 24-W light bulbs) was used. In the preliminary test, the light intensity was measured with a lux meter with a 2π receptor (Sonopan L-50 planar lux meter, Poland) at exposure initiation and at exposure termination. In the definitive test, the light intensity was measured with a lux meter with a 2π receptor (Sonopan L-50 planar lux meter, Poland) at exposure initiation and every 24 hours. Each test item concentration was divided into three replicates, whereas the control into six replicates. An additional replicate without the algae was used as a background for spectrophotometric measurements. The glass Erlenmeyer flasks with a capacity of 250 mL containing 100 mL of the test item concentration and the control and closed with air permeable stoppers (replicates) were arranged at random and continuously mechanically shaken at 90 rounds per minute to maintain stable conditions during the tests. The pH values were measured at exposure initiation, before the division into replicates and at exposure termination in pooled replicates (inoLAB Level 3pH/Oxi-meter, WTW, Germany). In the preliminary test, and the definitive test the number of algal cells was determined with an indirect method which involves a spectrophotometric measurement of the absorbance of an algal suspension at 670 nm. The absorbance value is then converted into the number of cells using a standard curve. The transmittance was measured in an additional replicate without the algae at exposure initiation and at exposure termination. In the definitive test, morphology observations of the algae were performed at exposure termination.

5.2.2. Preparing test item concentrations

In the preliminary test (non-GLP), the following test item concentrations were used for exposure 0.01, 0.1, 1.0, 10, and 100 mg/L plus the control. The test item in the amount of 50.0 mg was weighed into a glass flask and 100 mL of the AAP medium were added. The content of the flask was sonicated for approximately 2 minutes and transferred to a volumetric flask by multiple washing with the AAP medium and filled up to 500 mL. The test item concentration of 100 mg/L was visually homogeneous, nontransparent and with foamy. The lower test item concentrations were prepared by sequential dilutions with the AAP medium in a ratio 1 : 9, volume per volume. The control was 1000 mL of the AAP medium. From each test item concentration and the control 100 mL was collected for spectrophotometric measurements of the background. In order to obtain the initial algal density of 1 x 10^4 cells/mL, 350 mL of each test item concentration and 900 mL of the control were inoculated with the algae. In the definitive test, the following test item concentrations were used for 0.01, 0.03, 0.1, 0.31, 1.0, 3.1, and 10 mg/L plus the control. The test item in the amount of 10.03 mg was weighed into a glass flask and approximately 150 mL of the AAP medium were added. The content of the flask was sonicated for 5 minutes and transferred to a volumetric flask by multiple washing with the AAP medium and filled up to 1000 mL. The test item concentration of 10 mg/L was visually homogeneous and transparent. The lower test item concentrations were prepared by sequential dilutions with the AAP medium. The control was 1000 mL of the AAP medium. Two samples (100 mL), of each test item concentration and the control were collected. One of them was transferred for chemical analyses at exposure initiation, whereas the other was located next to the test vessels under test conditions and transferred for chemical analyses at exposure termination. The cell density of the three-day-old algae pre-culture was determined by counting the number of algal cells in the Bűrker chamber under a microscope. The algae cell density in the pre-culture was 2.625x10^6 cells/mL. In order to obtain the initial algal density of 1 x 10^4 cells/mL, 300 mL of each test item concentration and 700 mL of the control were inoculated with the algae.

5.2.3. Determination of the density of the algal cells

The number of algal cells was determined with an indirect method. The indirect method involves

a spectrophotometric measurement of the absorbance of algal suspensions at 670 nm and converting its value into the number of cells using a standard curve. The standard curve represents the relationship between absorbance values and the number of cells in the Bürker chamber. In the definitive test, the number of algal cells was determined on the basis of the

following equation: ΔA670 = 0.3371 x [10^6 cells/mL] (R^2=0.9936). In the preliminary test the absorbance for each test item concentration and the control was determined at exposure termination. In the definitive test, the absorbance for each test item concentration and the control was determined after 24, 48, and 72 hours of exposure.

5.3. Test with reference substance

The test with reference substance (3,5-dichlorophenol, Sigma Aldrich) was performed between 24.05.2014 – 27.05.2014. The test conditions were as follows: temperature: 22.5-23.0 °C; pH of the control: 7.20-9.03; average light intensity: 6890-7075 lx. Six concentrations of the reference substance ranging from 0.56 to 10 mg/L were used. There were three replicates of each concentration and six replicates of the control.

6. Acute immobilization test using *Daphnia magna* (water flea)

6.1. Test organism

The test organism, i.e. *Daphnia magna* Straus originated from a standard laboratory culture cultivated at the Institute of Industrial Organic Chemistry, Branch Pszczyna, Department of Ecotoxicology, Laboratory of Aquatic Toxicology Poland. Only organisms aged less than 24 hours (not first brood progeny) were used in the tests. The sensitivity of the culture was monitored on a regular basis using a reference substance, potassium dichromate.

6.1.1. Culturing of *Daphnia magna*

Daphnia magna was cultured in glass beakers with a capacity of 150 mL (one parent per vessel) at room temperature ranging from 18 to 22 °C with daily cycle 16 hours light: 8 hours dark (KANLUX electronic time programmer, Poland). The Elendt M7 medium was used. The daphnids were fed with a suspension of algae, mixture of two species, *Pseudokirchneriella subcapitata* : *Desmodesmus subspicatus* (2:1) originating from cultures cultivated in the Laboratory of Aquatic Toxicology. Group B vitamins and micronutrients necessary for proper growth were supplied with a lyophilized suspension of *Spirulina* sp..

6.1.2. Culture medium

The Elendt M7 medium recommended by the OECD Guideline No. 202 (2004) was used for culturing and as a diluent/solvent of the test item. The medium was prepared on the basis of deionized water (SolPure7 filtering system) by adding stock solutions of reagents of recognized analytical grade. The stock solutions for the culture of *Daphnia magna* were renewed on a regular basis.

6.2. Acute immobilization test

The acute immobilization test on *Daphnia magna* was conducted according to the OECD Guideline No. 202 (2004).

6.2.1. Test conditions

The preliminary test (non-GLP) and the definitive test were conducted as static tests. The exposure lasted 48 hours. The Elendt M7 medium was used as a diluent necessary to prepare the treatments. The medium was aerated prior to the beginning of the test. The daphnids were exposed in glass beakers with a capacity of 150 mL. Each beaker contained 100 mL of a given test item concentration or the control. Five individuals of *Daphnia magna* were introduced into each replicate. In the preliminary test and the definitive test, four replicates of each test item concentration and the control were used. The beakers were covered with transparent lids in order to minimize evaporation and to prevent accidental contamination. During the exposure, the daphnids were not fed. The tests were conducted with daily cycle 16 hours light: 8 hours dark. In the preliminary test, the air temperature was continuously recorded with a thermograph. In the definitive test, the temperature was continuously measured using a sensor submerged in an additional test vessel containing the test medium (HI 141 thermologger, Hanna Instruments, USA). The pH values and the dissolved oxygen concentrations were measured at the exposure initiation, i.e. before the division into replicates and at the exposure termination (pooled replicates).

6.2.2. Preparing of the test item concentrations

In the preliminary test, four test item concentrations were used: 100, 10, 1.0, 0.1 mg/L plus the control. First, 100.2 mg of the test item was weighed into a glass flask. Next, approximately 150 mL of the Elendt M7 medium was added. The content of flask was sonicated for 5 minutes and transferred into a flask with a capacity of 1 L by multiplewashing with the Elendt M7 medium. Flask was filled up into 1 L. The flask was sonicated for 5 minutes. The test item concentration of 100 mg/L was visually homogeneous, nontransparent, and foaming. The lower test item concentrations were prepared by sequential dilutions with the Elend M7 medium in ratio 1:9 (i.e. 100 mL of the higher test item concentration was mixed with 900 mL of the Elendt M7 medium). In the definitive test, six test item concentrations of 0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 mg/L and a control were used. First, 10.04 mg of the test item was weighed into a glass flask. Next, approximately 200 mL of the Elendt M7 medium was added. The content of the flask was sonicated for 1 minute and transferred into a flask with a capacity of 1 L by multiple washing with the Elendt M7 medium. The volume was made up to 1 L. The stock concentration of 10 mg/L was visually

homogeneous and transparent. To prepare the test item concentration of 2 mg/L, 400 mL of the stock concentration of 10 mg/L were collected and added to 1600 mL of the Elendt M7 medium. The highest test item concentration of 2 mg/L was visually homogeneous and transparent. The lower test item concentrations were prepared by making sequential dilutions with the Elendt M7 medium. Two samples of the test item concentrations and the control (2 x 100 mL) were collected. One of them was transferred for chemical analyses at the exposure initiation, whereas the other was placed near the test vessels under the test conditions. It was transferred for chemical analyses at the exposure termination. The pH values and the dissolved oxygen concentrations were measured. The test item concentrations and the control were divided into four replicates (4 x 100 mL). Five young *Daphnia magna* were introduced into each replicate.

6.3. Test with the reference substance

The test with a reference substance, potassium dichromate was performed between October 23, 2014 – October 25, 2014. The results confirmed the sensitivity of *Daphnia magna* used in the definitive test. During the test, the temperature was in the range of 19.9- 20.7 °C, and the dissolved oxygen concentrations were higher than 3 mg/L. The Elendt M7 medium was used. Five concentrations of the reference substance, i.e. 0.32, 0.56, 1.00, 1.80, and 3.20 mg/L and a control were used. Each reference substance concentration and the control were divided into four replicates. The results are within the range given in the references. To make calculations and to conduct statistical analyses, the ToxRat Professional 2.10 commercial software was used.