# Supporting Information

## Translation of Microbiota Short-Chain Fatty Acid Mechanisms Affords Anti-infective Acyl-Salicylic Acid Derivatives

Xinglin Yang[1], Emily R. Forster[2,3], Narek Darabedian[4], Alexander T. Kim[1], Matthew R. Pratt[4], Aimee Shen[2], Howard C. Hang[1]

[1]Laboratory of Chemical Biology and Microbial Pathogenesis, The Rockefeller University, New York, NY 10065, USA
[2]Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111, USA.
[3]Graduate School of Biomedical Sciences, Tufts University, Boston, MA 02111, USA.
[4]Department of Chemistry, University of Southern California, Los Angeles, CA 90089, USA.

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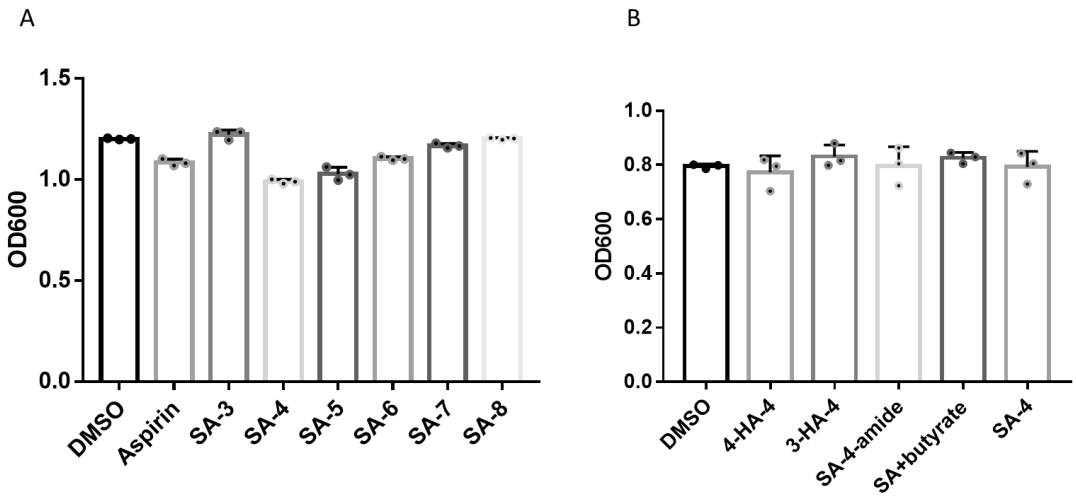



**Figure S1. Aspirin analogs at 1 mM have little effect on *Salmonella* growth.** An overnight culture of *S*. Typhimurium was diluted 1:50 into 4 mL SPI-1 inducing LB aliquots containing 1 mM aspirin analogs and incubated for 4 h at 37 °C with 220 rpm shaking. $OD_{600}$ is measured for 3 independent cultures per condition.

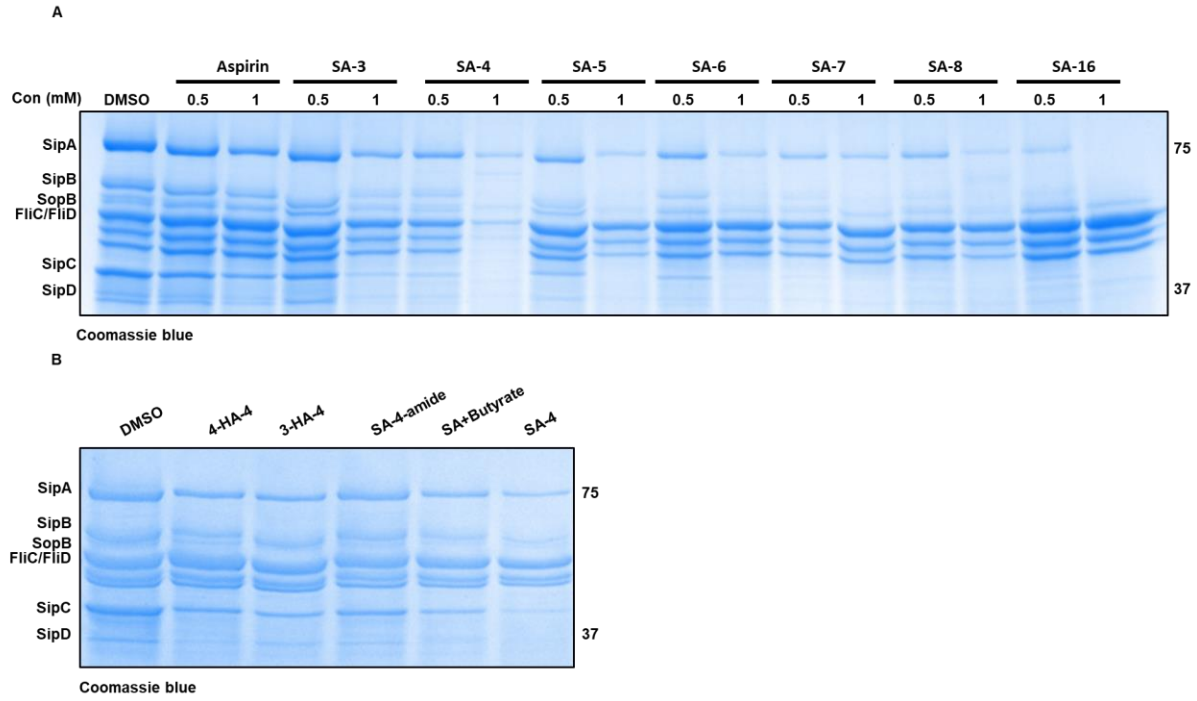



**Figure S2. Aspirin analogs inhibit *S*. Typhimurim protein secretion.** An overnight culture of *S*. Typhimurium was diluted 1:50 into 4 mL SPI-1-inducing LB aliquots containing 1 mM aspirin analogs and incubated for 4 h at 37 °C with 220 rpm shaking. SPI-1 effectors and flagellar components levels in *S*. Typhimurium growth media were monitored by SDS-PAGE followed by Coomassie blue staining. (A) Acylated salicylic acids (1 mM). (B) SA-4 analogs (0.8 mM).

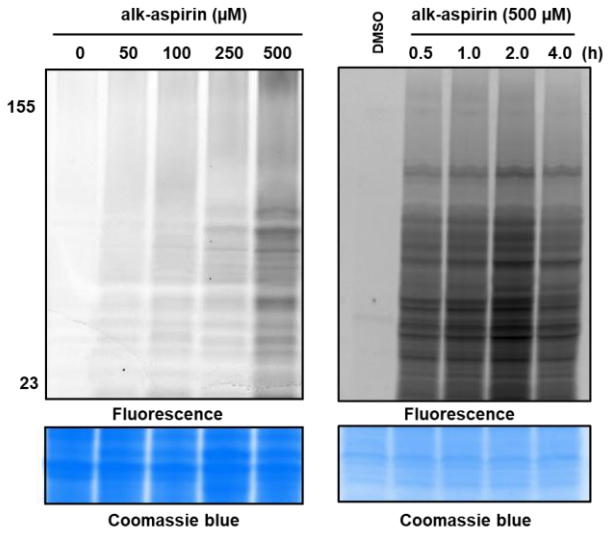



**Figure S3. Dose-dependent and time-course labeling of *S*. Typhimurium proteins by alk-aspirin.** 1:50 dilutions of Miller LB cultures of *S.* Typhimurium overnight culture were grown in 4 mL SPI-1 inducing LB 37 °C with 220 rpm shaking. Alk-aspirin in DMSO or DMSO were added as indicated time and concentration. Cell lysates were reacted with azide-Rho, and proteins were separated by SDS-PAGE and visualized by in-gel fluorescence gel. Coomassie blue staining demonstrates comparable loading.

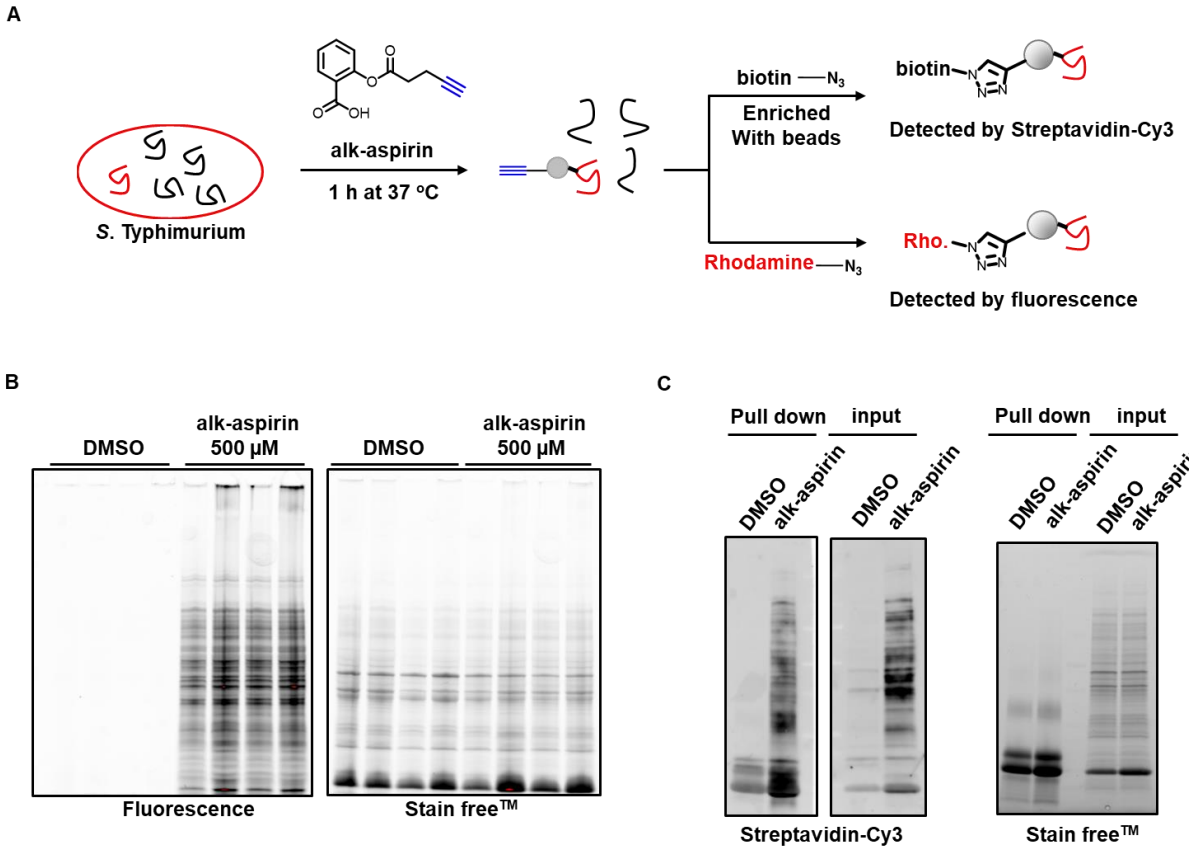



**Figure S4. Proteomics study of alk-aspirin-labeled *S.* Typhimurium proteins. (A)** Experimental procedures for profiling of alk-aspirin-labeled proteins. (B) Profiling of alk-aspirin-labeled proteins for proteomics samples preparation (n = 4). (C) The labeled proteins were reacted with azide-biotin and enriched with streptavidin beads. Input samples before enrichment ("Input") and enriched proteins ("pull down") are detected by Stain-Free ™ or Streptavidin-Cy3.

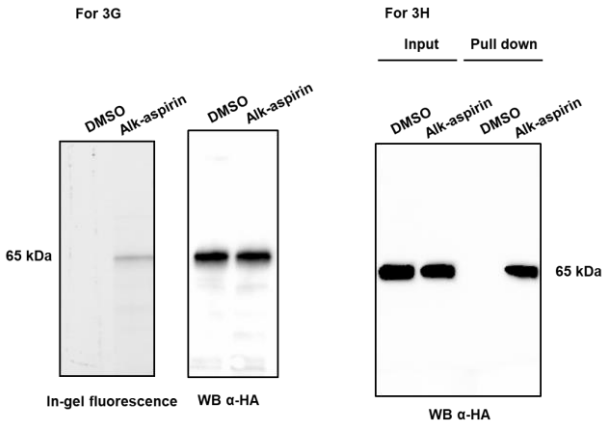



**Figure S5. Uncropped immunoblots for indicated Figure 3G, H.**

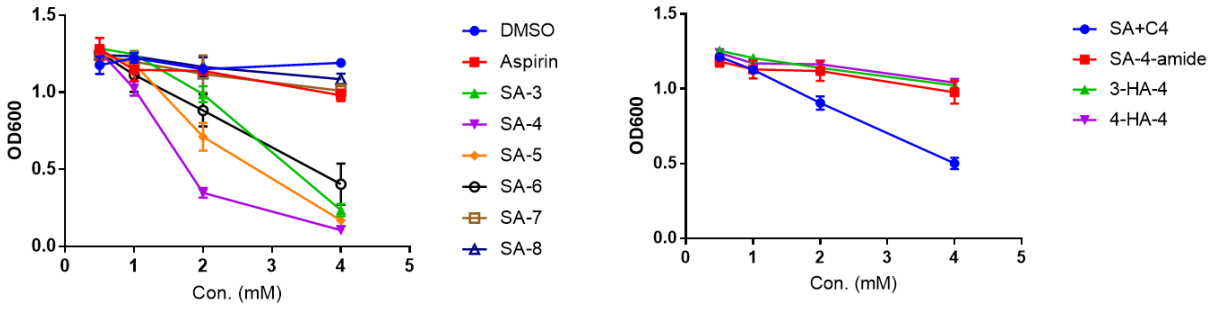



**Figure S6. SA-4 inhibits *S.* Typhimurium growth at higher concentrations.** Overnight culture of *S*. Typhimurium was diluted 1:50 in 4 mL aspirin analogs contained SPI-1-inducing LB for 4 h at 37 °C with 220 rpm shaking. $OD_{600}$ is measured for 3 independent cultures.

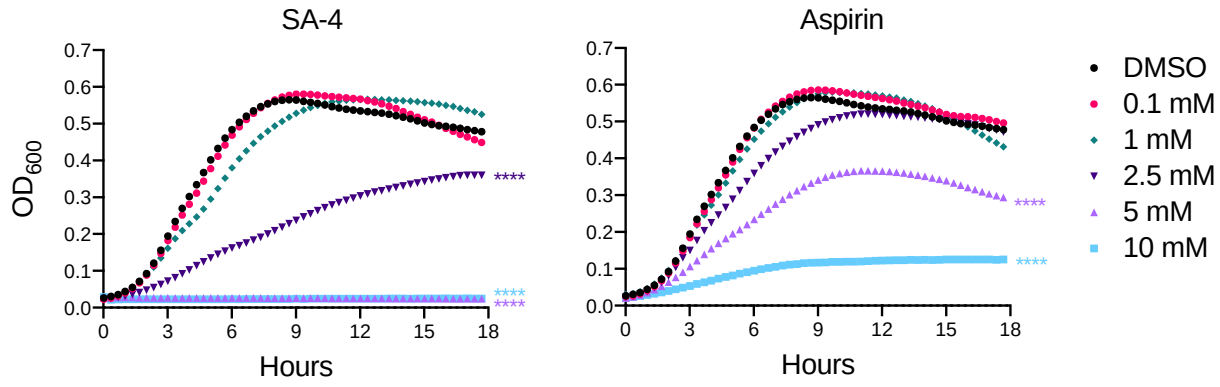
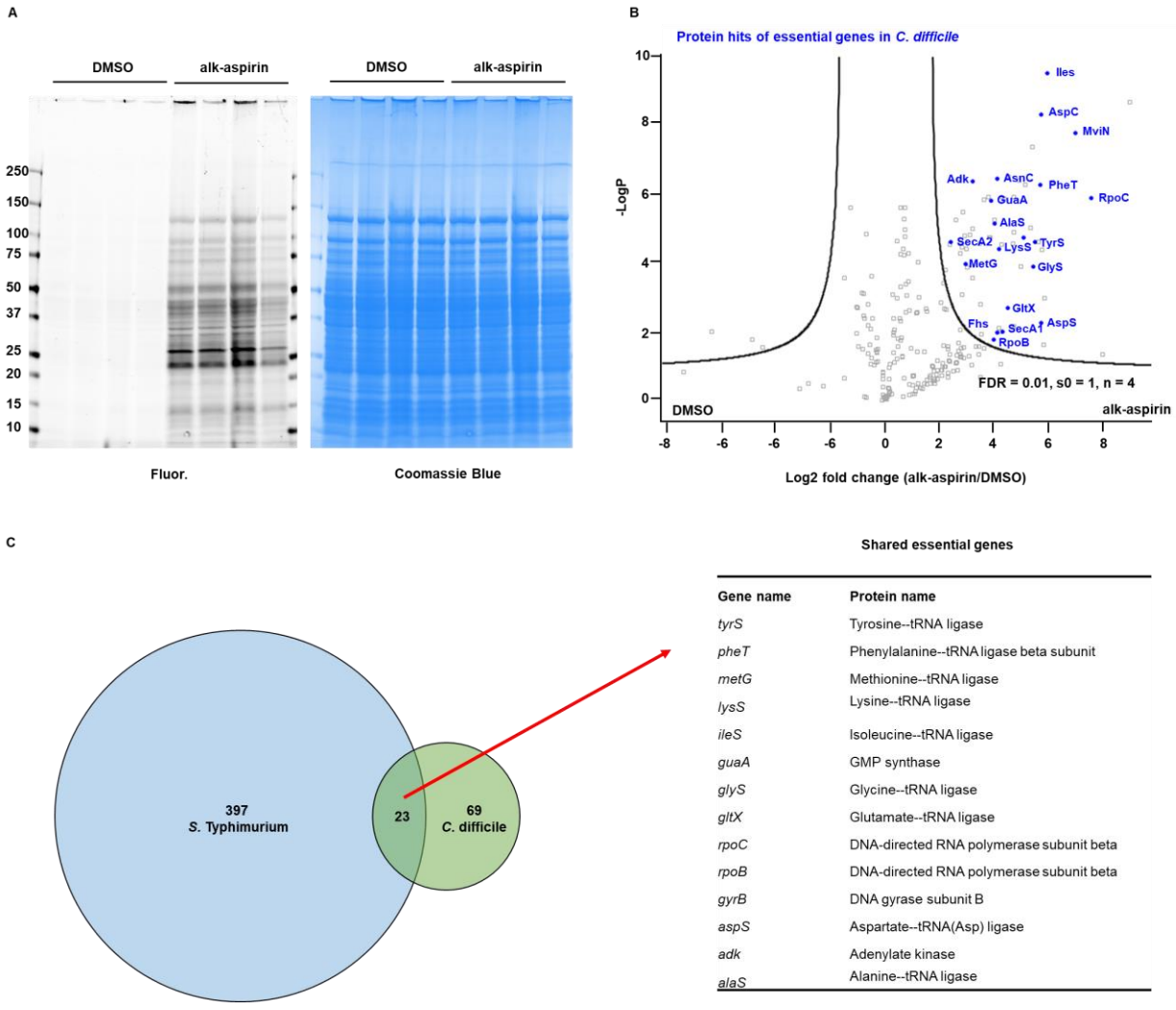



**Figure S7. SA-4 inhibits *C. difficile* growth at higher concentrations.** Wild-type *C. difficile* was grown in BHIS with the indicated concentrations of SA-4 or aspirin. Shading indicates SEM and statistical significance was determined by two-way ANOVA with Tukey correction. (****, $P$<0.0001 relative to DSMO).




**Shared essential genes**

| Gene name | Protein name |
|---|---|
| *tyrS* | Tyrosine--tRNA ligase |
| *pheT* | Phenylalanine--tRNA ligase beta subunit |
| *metG* | Methionine--tRNA ligase |
| *lysS* | Lysine--tRNA ligase |
| *ileS* | Isoleucine--tRNA ligase |
| *guaA* | GMP synthase |
| *glyS* | Glycine--tRNA ligase |
| *gltX* | Glutamate--tRNA ligase |
| *rpoC* | DNA-directed RNA polymerase subunit beta |
| *rpoB* | DNA-directed RNA polymerase subunit beta |
| *gyrB* | DNA gyrase subunit B |
| *aspS* | Aspartate--tRNA(Asp) ligase |
| *adk* | Adenylate kinase |
| *alaS* | Alanine--tRNA ligase |

**Figure S8. Summary for alk-aspirin labeled proteins in *C. difficile*. (**A) Profiling of alk-aspirin-labeled proteins in *C. difficile* for proteomics samples preparation (n = 4). (B) LFQ proteomic analysis of alk-aspirin-labeled proteins in *C. difficile* (n = 4). Cell lysates were reacted with az-biotin for the enrichment of alk-aspirin-labeled proteins with streptavidin beads and identification by LC-MS/MS. Reported essential genes[1] are in blue. (C) Venn diagram between the mass spectrometry dataset of *S*. Typhimurium and *C. difficile*.

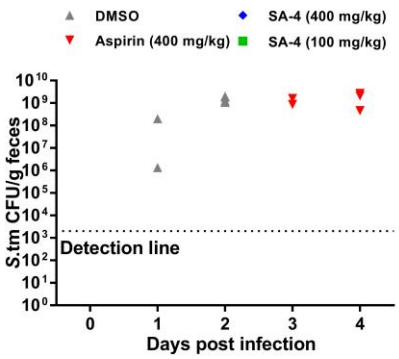



**Figure S9. *S.* Typhimurium fecal loads in mice as in Figure 4.** The DMSO-treated group was monitored on days 0-2. SA-4 and aspirin treated groups were monitored on days 0-4.

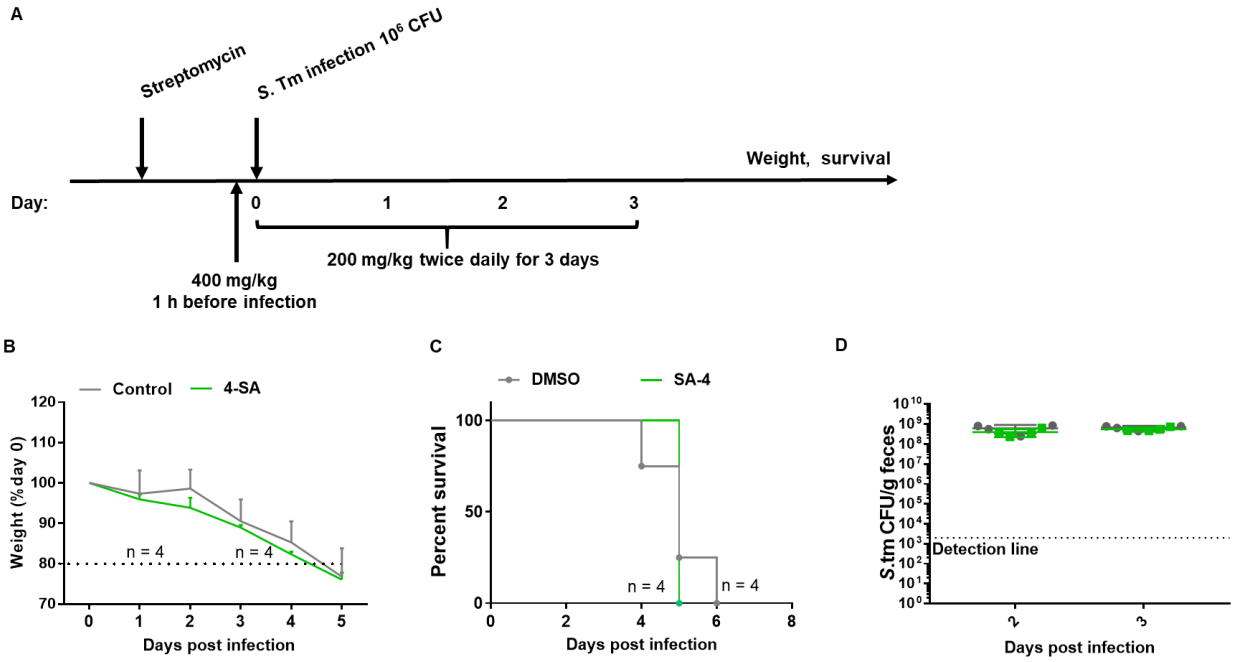



**Figure S10. Pre-treatment of SA-4 failed to inhibit *Salmonella* virulence *in vivo.*** (A) Protocol schematic for mice study. C57BL/6 mice were orally treated with 20 mg of streptomycin 24 hour before infection. SA-4 was suspended in 0.5% of sodium carboxymethyl cellulose and C57BL/6 mice were orally treated with 400 mg of SA-4 1 hour before infection. Then the mice were gavaged with 200 mg/kg twice daily. Mouse weight and the survival are monitored daily. (B) Weight of mice after challenged with *S*. Typhimurium on day 0. (C) Survival of infected mice treated with SA-4 and DMSO control. (D) *S.* Typhimurium fecal loads.

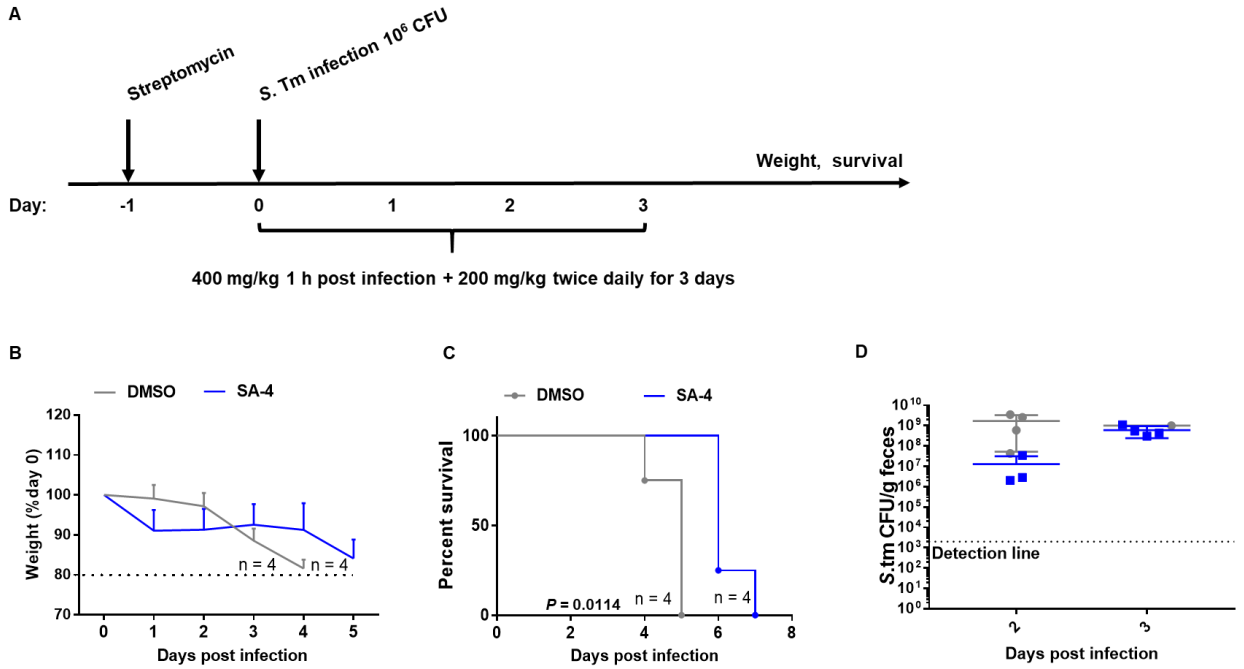



**Figure S11. SA-4 inhibits *S.* Typhimurium virulence *in vivo* less effectively when administered post-infection.** (A) Protocol schematic for mice study. Generally, C57BL/6 mice were orally treated with 20 mg of streptomycin 24 hour before infection. The mice were then orally infected with $10^6$ CFU of *S.* Typhimurium. SA-4 was suspended in 0.5% of sodium carboxymethyl cellulose and the mice were gavaged with SA-4 suspension at the indicated times and concentrations. Mouse weight and the survival are monitored daily. (B) Weight of mice after challenged with *S.* Typhimurium on day 0. (C) Survival of infected mice treated with SA-4 and DMSO control. Gehan-Breslow-Wilcoxon test. (D) *S.* Typhimurium fecal loads.

**Table S1. Bacterial strains used in this study.**

| Organism | Genotype | Antibiotic resistance |
|---|---|---|
| *Salmonella* Typhimurium 14028s[2] | WT | None |
| *Salmonella* Typhimurium 14028s | HilA-HA[3] | None |
| *C. difficile* 630Δ*erm* | WT | None |

**Table S2. Primers used for qRT-PCR.**

| Primer Name | Sequence (5' to 3') |
|---|---|
| sipA qPCR F | CGCCGGTAAGAAAGCGATAA |
| sipA qPCR R | CACCTTCATCAGTAGCGTCTTC |
| sipB qPCR F | ATTACTAGCGGTCTGGGTAATG |
| sipB qPCR R | GCCCAACGCCACTTTATTTAG |
| invF qPCR F | TCCTGAGTTTCGCGCTATTT |
| invF qPCR R | GTAACAGCGCCAGTACCTTAT |
| spaO qPCR F | CCGACCAATGCTGAACTTAAC |
| spaO qPCR R | TTCATGGATCTCAACGCCTAAG |
| prgH qPCR F | ACAGCAGGCGTTACCTTATTC |
| prgH qPCR R | AATTGACGGGCTCTGAGTATTT |
| orgB qPCR F | ATCAGACAATGGCCTGGAAG |
| orgB qPCR R | AAATCCCTTAGCCACTCATCC |
| ftsZ qPCR F | ATGTTTGAACCTATGGAACTAACCAAC |
| ftsZ qPCR R | TTAATCAGCTTGCTTACGCAGGA |

**Table S3. SPI-1 virulence factors and potential essential proteins in alk-aspirin-labeled *S.* Typhimurium proteomics.**

| Gene name | $Log_2$ fold change (alk-aspirin/DMSO) | -logP |
|---|---|---|
| | SPI-1 virulence factors | |
| *hilA* | 5.170 | 5.221 |
| *slyA* | 4.229 | 4.314 |
| *sptP* | 3.333 | 7.079 |
| *sopE2* | 2.731 | 2.637 |
| *arcA* | 2.577 | 3.563 |
| *sipA* | 2.296 | 6.859 |
| *sipB* | 2.010 | 3.939 |
| *invC* | 1.912 | 2.499 |
| *invA* | 1.883 | 1.481 |
| *invE* | 1.792 | 2.959 |
| *phoP* | 1.425 | 2.917 |
| *sopB* | 1.376 | 6.782 |
| *prgH* | 1.293 | 2.681 |
| *arcB* | 1.180 | 3.745 |
| | potential essential proteins | |
| *gyrB* | 5.298 | 7.381 |
| *thrS* | 5.020 | 5.967 |
| *glyS* | 4.922 | 3.370 |
| *glnS* | 4.339 | 6.244 |
| *metG* | 4.263 | 4.854 |
| *accA* | 4.208 | 4.714 |
| *hisS* | 4.207 | 7.544 |
| *rpoD* | 4.086 | 3.711 |
| *plsB* | 4.048 | 5.800 |
| *ileS* | 3.179 | 8.176 |
| *dfp* | 3.153 | 4.598 |
| *clpX* | 3.096 | 4.016 |
| *vacB* | 3.035 | 4.587 |
| *pyrG* | 2.945 | 6.904 |
| *msbA* | 2.784 | 7.257 |
| *gyrA* | 2.781 | 3.463 |
| *polA* | 2.741 | 3.170 |
| *clpB* | 2.687 | 6.126 |
| *nusA* | 2.644 | 6.563 |
| *pheT* | 2.603 | 3.333 |
| potential essential proteins | | |
| *infB* | 2.575 | 2.892 |
| *hflB* | 2.492 | 4.209 |
| *mrp* | 2.412 | 5.894 |
| *rnb* | 2.386 | 2.733 |
| *ftsA* | 2.361 | 3.882 |
| *rpoB* | 2.276 | 6.374 |
| *alaS* | 2.235 | 6.561 |
| *adk* | 2.219 | 5.845 |
| *yigC* | 2.217 | 3.656 |
| *secY* | 2.127 | 3.984 |
| *folD* | 2.083 | 3.649 |
| *sbcB* | 2.016 | 4.695 |
| *mutS* | 1.986 | 3.186 |
| *rpsG* | 1.926 | 4.457 |
| *grpE* | 1.924 | 3.531 |
| *fabD* | 1.897 | 2.957 |
| *rfbK* | 1.820 | 2.477 |
| *hflK* | 1.705 | 4.130 |
| *lysS* | 1.698 | 5.851 |
| *rpoC* | 1.393 | 4.646 |
| *rpoA* | 1.250 | 4.736 |
| *glyQ* | 1.215 | 1.742 |
| *aspS* | 1.169 | 1.725 |
| *pyrH* | 1.141 | 1.632 |
| *rplF* | 1.135 | 5.843 |
| *eno* | 1.122 | 6.338 |
| *prfB* | 1.122 | 1.785 |
| *pssA* | 1.122 | 1.710 |
| *yfiF* | 1.028 | 2.561 |
| *tsf* | 0.944 | 4.948 |
| *yidC* | 0.787 | 1.820 |
| *lpdA* | 0.762 | 6.315 |
| *lpxA* | 0.735 | 2.042 |
| *fusA* | 0.698 | 5.474 |
| *rplE* | 0.575 | 3.418 |
| *adhE* | 0.526 | 4.040 |

**Table S4. Data for alk-aspirin-labeled *C. difficile* proteomics.**

| Gene name | $Log_2$ fold change (alk-aspirin/DMSO) | -logP | Gene name | $Log_2$ fold change (alk-aspirin/DMSO) | -logP |
|---|---|---|---|---|---|
| potential essential proteins | | | *ptb* | 4.906 | 6.433 |
| *rpoC* | 7.553 | 5.909 | *CD630_19530* | 4.693 | 4.910 |
| *mviN* | 6.959 | 7.811 | *CD630_27170* | 4.686 | 5.947 |
| *ileS* | 5.931 | 9.555 | *tdcB* | 4.665 | 4.581 |
| *aspS* | 5.706 | 2.298 | *CD630_22610* | 4.515 | 6.530 |
| *adk* | 5.701 | 8.350 | *carB1;carB2* | 4.339 | 5.893 |
| *pheT* | 5.659 | 6.294 | *cat1* | 4.258 | 5.951 |
| *alaS* | 5.464 | 4.628 | *CD630_02400* | 4.202 | 2.039 |
| *secA1* | 5.418 | 3.925 | *4hbD* | 4.147 | 2.126 |
| *tyrS* | 5.047 | 4.768 | *CD630_17300* | 3.987 | 5.290 |
| *lysS* | 4.465 | 2.732 | *CD630_25610* | 3.785 | 4.760 |
| *rpoB* | 4.268 | 2.030 | *upp* | 3.620 | 5.854 |
| *gltX* | 4.152 | 4.425 | *CD630_26190* | 3.501 | 1.958 |
| *secA2* | 4.077 | 6.484 | *cat2* | 3.472 | 2.814 |
| *glyS* | 4.074 | 1.994 | *purA* | 3.186 | 5.019 |
| *metG* | 3.985 | 5.181 | *rpsA* | 3.170 | 3.593 |
| *fhs* | 3.963 | 1.811 | *CD630_16370* | 3.102 | 4.845 |
| *prfA* | 3.857 | 5.845 | *CD630_08410* | 2.987 | 3.904 |
| *asnC* | 3.179 | 6.389 | *crt2* | 2.880 | 4.811 |
| *gyrB* | 2.940 | 3.996 | *htpG* | 2.846 | 5.506 |
| *guaA* | 2.388 | 4.630 | *recA* | 2.808 | 3.093 |
| other proteins | | | *cbiK* | 2.803 | 4.482 |
| *CD630_21810* | 8.947 | 8.706 | *hisF* | 2.749 | 4.303 |
| *CD630_14190* | 7.945 | 1.374 | *sucD* | 2.745 | 4.587 |
| *CD630_16390* | 6.133 | 6.364 | *clpB* | 2.711 | 6.488 |
| *aspC* | 5.846 | 3.008 | *CD630_30180* | 2.567 | 5.284 |
| *proS1* | 5.802 | 1.657 | *atpD* | 2.334 | 4.556 |
| *CD630_08750* | 5.730 | 4.408 | *rnfC* | 2.194 | 3.814 |
| *hisC* | 5.603 | 4.639 | *clpP1* | 2.960 | 4.425 |
| *glnA* | 5.391 | 7.392 | *tetM* | 4.692 | 2.187 |
| *CD630_36680* | 5.315 | 5.052 | *CD630_02420* | 4.237 | 4.574 |
| *CD630_27160* | 5.133 | 6.302 | *rnj* | 3.820 | 4.768 |
| *CD630_30280* | 4.965 | 3.924 | *ychF* | 3.764 | 5.931 |
| *CD630_02440* | 3.434 | 4.583 | | | |

**Table S5. tRNA ligases in alk-aspirin-labeled *C. difficile* proteomics.**

| Gene name | protein name | $Log_2$ fold change (alk-aspirin/DMSO) | -logP |
|---|---|---|---|
| *tyrS* | Tyrosine--tRNA ligase | 5.047 | 4.768 |
| *pheT* | Phenylalanine--tRNA ligase beta subunit | 5.659 | 6.294 |
| *metG* | Methionine--tRNA ligase | 3.985 | 5.181 |
| *lysS* | Lysine--tRNA ligase | 4.465 | 2.732 |
| *ileS* | Isoleucine--tRNA ligase | 5.931 | 9.555 |
| *glyS* | Glycine--tRNA ligase | 4.074 | 1.994 |
| *gltX* | Glutamate--tRNA ligase | 4.152 | 4.425 |
| *aspS* | Aspartate--tRNA(Asp) ligase | 5.706 | 2.298 |
| *alaS* | Alanine--tRNA ligase | 5.464 | 4.628 |

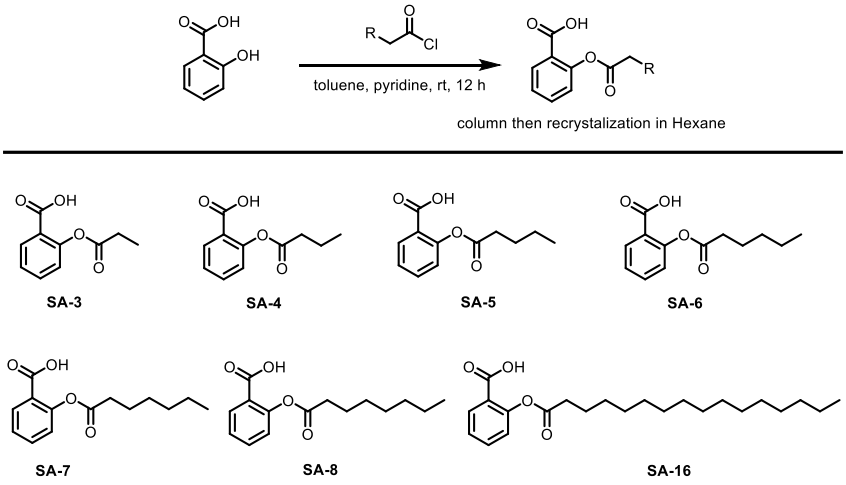
## Materials and Methods

### Chemical reagents

DMSO, Aspirin, salicylic acid and sodium butyrate was purchased from Sigma-Aldrich (303410). Az-Rho was synthesized in the lab as previously described[4]. Az-biotin was purchased from Sigma-Aldrich (762024). Streptavidin-Cy3 was purchased form Thermo Fisher (434315).

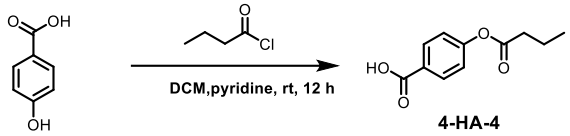
### Synthesis of aspirin analogs

All commercial materials were used without further purification. All solvents were analytical grade. The 1H NMR and 13C NMR spectra were recorded on a Bruker 600 MHz spectrometer in indicated deuterated solvents using TMS or solvent peak as a standard. All 13C NMR spectra were recorded with complete proton decoupling.



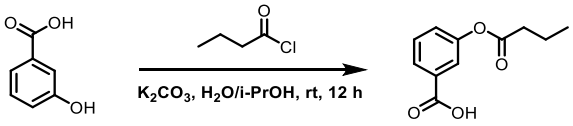

To 100 mL round bottom flask were added salicylic acid (1.0 g), acyl chloride (1.0 equiv.), pyridine (0.6 mL) and toluene (15 mL) at 0 ℃. The reaction was stirred at room temperature for 16 h and monitored by TLC. The reaction was then quenched with 50 mL 1M HCl and extracted by ethyl acetate for three times. The organic layer is dried over anhydrous $Na_2SO_4$ concentrated on rotavapor under reduced pressure. Finally, the residue was purified by silica gel column chromatography (dichloromethane). The desired product was obtained by recrystallization in

Hexane/ethyl acetate. The H and C NMR spectrum are shown in the NMR spectrum section and correlated well with reported data[5].




**4-HA-4**

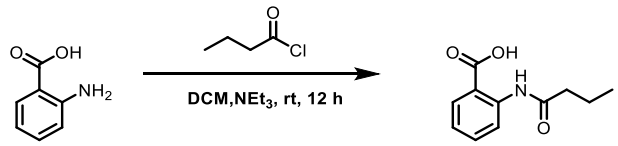
To 100 mL round bottom flask were added 4-hydroxybenzoic acid (1.0 g), acyl chloride (1.0 equiv.), pyridine (0.6 mL) and DCM (50 mL) at 0 ℃. The reaction was stirred at room temperature for 16 h and monitored by TLC. The reaction was then quenched with 50 mL 1M HCl and extracted by ethyl acetate for three times. The organic layer is dried over anhydrous $Na_2SO_4$ concentrated n rotavapor under reduced pressure. Finally, the residue was purified by silica gel column chromatography (Hexane/ethyl acetate 10:1). White solid, 85%. $^1$H-NMR (600 MHz, $CDCl_3$) δ (ppm) 8.17 (d, 1H, *J* = 8.4 Hz), 7.24 (d, 1H, *J* =8.4 Hz), 2.60 (t, 2H, *J* = 7.2 Hz), 1.86-1.80 (m, 2H), 1.08 (t, 3H, *J* = 7.8 Hz); $^{13}$C-NMR (150 MHz, $CDCl_3$) δ (ppm) 171.64, 171.32, 155.30, 132.0, 126.80, 121.93, 36.37, 18.51, 13.75; LRMS (ESI) calcd for $C_{11}H_{12}O_4$ $[M-H]^-$: 207.06, found 207.1.




**3-HA-4**

To 100 mL round bottom flask were added 3-hydroxybenzoic acid (1.0 g), acyl chloride (1.0 equiv.), $K_2CO_3$ (3 g) and H2O/i-PrOH (9 mL/3 mL) at 0 ℃. The reaction was stirred at room temperature for 16 h and monitored by TLC. The reaction was then quenched with 50 mL 1M HCl and extracted by ethyl acetate for three times. The organic layer is dried over anhydrous $Na_2SO_4$ concentrated on rotavapor under reduced pressure. Finally, the residue was purified by silica gel column chromatography (Hexane/ethyl acetate 10:1). White solid, 70%. $^1$H-NMR (600 MHz, $CDCl_3$) δ (ppm) 7.99 (d, 1H, *J* = 7.8 Hz), 7.827 (d, 1H, *J* =1.2 Hz), 7.50 (t, 1H, *J* = 7.8 Hz), 7.35(dd, 1H, *J* = 7.8 Hz, *J* =1.2 Hz), 2.58, (t, 2H, *J* = 7.2 Hz), 1.84-1.79 (m, 2H), 1.06 (t, 3H, *J* = 7.8 Hz); $^{13}$C-NMR

(150 MHz, $CDCl_3$) δ (ppm) 172.0, 171.2, 150.9, 130.9, 129.7, 127.7, 127.4, 123.6, 36.3, 18.5, 13.8; LRMS (ESI) calcd for $C_{11}H_{11}O_4$ $[M-H]^-$: 207.06, found 207.1.




**SA-4-amide**

To 100 mL round bottom flask were added 2-aminobenzoic acid (1.0 g), acyl chloride (1.0 equiv.), $NEt_3$ (1.2 equiv.) and DCM (50 mL) at 0 ℃. The reaction was stirred at room temperature for 16 h and monitored by TLC. The reaction was then quenched with 50 mL 1M HCl and extracted by ethyl acetate for three times. The organic layer is dried over anhydrous $Na_2SO_4$ concentrated on rotavapor under reduced pressure. Finally, the residue was purified by silica gel column chromatography (Hexane/ethyl acetate 10:1). White solid, 82%. $^1$H-NMR (600 MHz, $CDCl_3$) δ (ppm) 11.01 (s, 1H), 8.77 (d, 1H, *J* = 9.0 Hz), 8.14 (d, 1H, *J* =6.6 Hz), 7.60 (t, 1H), 7.35(d, 1H, *J* = 7.2 Hz), 2.47, (t, 2H, *J* = 7.8 Hz), 1.83-1.79 (m, 2H), 1.04 (t, 3H, *J* = 7.2 Hz); $^{13}$C-NMR (150 MHz, $CDCl_3$) δ (ppm) 172.9, 172.2, 142.1, 135.7, 131.9, 122.8, 120.8, 114.3, 40.7, 19.1, 13.9; LRMS (ESI) calcd for $C_{11}H_{14}NO_3$ $[M+H]^+$: 208.097, found 208.2.

**Microbial Strains and Growth Conditions**

All strains used are listed in **Table S1**. All *Salmonella* Typhimurium strains used were derivatives of S. Typhimurium 14028S[2]. *Salmonella* strains were cultured at 37°C in liquid Miller Luria-Bertani (LB) medium [10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl] (Becton Dickinson, Difco™), SPI-1 inducing LB medium [10 g/L tryptone, 5 g/L yeast extract, 300 mM NaCl], or on *Salmonella Shigella* agar (Becton Dickinson). Cultures were grown at 37°C in Multitron shaking incubator (INFORS HT) at 220 rpm. *C. difficile* was grown in BHIS (brain-heart infusion supplemented with 0.1% L-cysteine) in an anaerobic mixture of 5% $CO_2$, 10% $H_2$, and 85% $N_2$ with gentle swirling at 37˚C for all experiments.

**Preparation of *Salmonella* bacterial total cell lysates**

1:50 dilutions of overnight Miller LB cultures of *Salmonella* Typhimurium strain 14028 WT overnight culture were grown in 4 mL SPI-1 inducing LB for 4 h at 37°C with 220rpm shaking. For alk-aspirin labeling experiments, cultures were incubated with 0.5 mM alk-aspirin in DMSO or DMSO as the negative control. *S.* Typhimurium cells were pelleted at 15000 g for 1 min, and pellets washed with cold PBS once and were lysed with 200 µL lysis buffer (phosphate-buffered saline (PBS) containing 0.5% Nonidet P-40, 1X EDTA-free protease inhibitor cocktail (Roche), 0.5 mg/mL lysozyme (in $dH_2O$) (Sigma), and 1:1,000 dilution of Benzonase (Millipore)). After re-suspension, pellets were sonicated for 10 sec 3 times, then were incubated on ice for 30 min. Cell lysates were centrifuged at 8200 g for 5 min to remove cell debris and supernatants were collected. Protein concentration was estimated by BCA assay with BCA Protein Assay Kit (Thermo).

**In-gel fluorescence analysis of alk-aspirin labeling**

For in-gel fluorescence analysis of alk-aspirin labeled *Salmonella* proteome, from the alk-aspirin-treated or control total cell lysates prepared as described above, 45 µL of each total cell lysates (~50 µg) was added with 5 µL of click chemistry reagents as a 10X master mix (az-Rho: 0.1 mM, 10 mM stock solution in DMSO; tris(2-carboxyethyl)phosphine hydrochloride (TCEP): 1 mM, 50 mM freshly prepared stock solution in $dH_2O$; tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA): (0.1 mM, 2 mM stock in 4:1 t-butanol: DMSO); $CuSO_4$ (1 mM, 50 mM freshly prepared stock in $dH_2O$). Samples were mixed well and incubated at room temperature for 1 h. After incubation, samples were mixed with 200 µL cold methanol and incubate at -20 ℃ overnight. Sample proteins were precipitated at 18000 g for 1 min at 4 °C. After gently removing the aqueous layer, protein pellets were washed with 200 µL cold methanol, spinning down at 18000 g for 1 min at 4 °C, and liquid was gently decanted. After washing twice, pellets were allowed air-dried before boiling with 1X Laemmli buffer. Samples were boiled with 1X Laemmli buffer 95°C for 5 min before

being loaded onto a 4-20% Tris-HCl gel (Bio-Rad) for SDS-PAGE. In-gel fluorescence scanning was performed using a Typhoon 9400 imager (Amersham Biosciences).

For in-gel fluorescence analysis of alk-aspirin labeled HilA-HA, from the alk-aspirin-treated or control total cell lysates prepared as described above, 250 µg of each total cell lysates were immunoprecipitated with 20 µL PBS-T-washed Pierce™ Anti-HA Magnetic Beads (Thermo Scientific). After samples were washed with 500 µL PBS-T 3 times, then 44 µL of PBS-T was added to each sample. 5 µL of click chemistry reagents as a 10X master mix mentioned above were added to each sample. Samples were mixed well and incubated at 4 °C for 1 hour with end-to-end rotation. After incubation, samples were washed with 500 µL PBS-T 3 times. Samples were boiled with 1X Laemmli buffer 95°C for 5 min before being loaded onto a 4-20% Tris-HCl gel (Bio-Rad) for SDS-PAGE. In-gel fluorescence scanning was performed using a Typhoon 9400 imager (Amersham Biosciences).

Proteins were then transferred onto 0.45 µm nitrocellulose membrane (Bio-Rad) with Trans-Blot Turbo Transfer System (Bio-Rad) at 25 V for 30 min. The membrane was blocked with 5% non-fat milk in PBS with 0.1% Tween-20 (PBS-T) for 60 min, and 1:5,000 anti-HA rabbit antibody H6908 (Sigma) was added to solution before incubating membrane at 4°C overnight. The membrane was washed with PBS-T 3 times, and incubated with 1:10,000 goat polyclonal anti-rabbit HRP ab97051 (Abcam) in PBS-T with 5% non-fat milk at room temperature for 1 hour. The membrane was washed with PBS-T 3 times, and imaged with Clarity Western ECL substrate (Bio-Rad) and ChemiDoc XRS+ System (Bio-Rad).

**Label-free quantitative proteomics of alk-aspirin targets**

***S.*** **Typhimurium proteomics.** 1:50 dilutions of overnight Miller LB cultures of *Salmonella* Typhimurium strain 14028S WT were grown in 20 mL SPI-1 inducing LB for 3 h at 37°C with

220rpm shaking. Cultures were incubated with 0.5 mM alk-aspirin in DMSO or DMSO as the negative control for another 1 h at 37°C with 220rpm shaking. The culture medium was centrifuged at 5000 g for 10 min. Bacteria pellets was re-suspended in 3 mL cold PBS and centrifuged 5000xg 4℃ for 5 min. After re-suspension in 1 mL lysis buffer, bacteria were sonicated for 15 sec with a Sonic Dismembrator Model 500 (Fisher Scientific) with 5 sec on and 10 sec off per cycle. Cell lysates were centrifuged at 15000 g for 1 min to remove cell debris and supernatants were collected.

***C. difficile* proteomics.** Four overnight cultures of *C. difficile 630Δerm* were diluted 1:25 into 20 mL BHIS twice such that each overnight culture was used to start one DMSO and one alk-aspirin culture (n=4 per condition). These cultures were grown 6 hours anaerobically until the $OD_{600}$ was ~1.2, then 0.5 mM alk-aspirin or DMSO was added for 1 hour. Cells were pelleted and washed once with PBS, then resuspended in 500 µL cold lysis buffer (0.5 mg/mL lysozyme, 0.1% benzonase, 0.1% NP-40, 1X Halt protease inhibitor [Thermo Scientific] in PBS) and lysed by bead beating 4 x 30 seconds at speed 6 (MP Biomedicals). Beads were pelleted at 3,000g for 2 min and 1 mg lysate (supernatant) per sample was flash frozen in liquid nitrogen and stored at -80˚C until being further processed.

Each total cell lysates was added with 100 µL of click chemistry reagents as a 10X master mix (az-Biotin: 0.1 mM, 10 mM stock solution in DMSO; tris(2-carboxyethyl)phosphine hydrochloride (TCEP): 1 mM, 50 mM freshly prepared stock solution in $dH_2O$; tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA): (0.1 mM, 2 mM stock in 4:1 t-butanol: DMSO); $CuSO_4$ (1 mM, 50 mM freshly prepared stock in $dH_2O$). Samples were mixed well and incubated at room temperature for 1 h. After incubation, samples were mixed with 4 mL cold methanol and incubated at -20°C overnight.

Protein pellets were centrifuged at 5000 g for 30 min at 4°C, pellets were transferred to 2.0 mL centrifuge tube and were washed with 1 mL cold methanol 3 times. After last wash, pellets were let air dried (37 degree for 1 h) before being re-solubilized in 250 uL 4% SDS PBS with bath sonication. Solutions were diluted with 750 uL PBS, and incubated with 60 uL PBS-T-washed High Capacity NeutrAvidin agarose (Pierce) (500 uL PBS-T-washed twice, 2500gX60s) at room temperature for 1 h with end-to-end rotation. The agarose was washed with 500 uL 1% SDS PBS 3 times, 500 uL 1M Urea PBS 3 times, and 500 uL PBS 3 times and then reduced with 500 uL 10 mM DTT (Sigma) in PBS for 30 min at 37°C, and alkylated with 500 uL 50 mM iodoacetamide (Sigma) in PBS for 30 min in dark. 50 uL $NH_4HCO_3$ (10 mM) was added to the tube. Neutravidin-bound proteins were digested on bead with 400 ng Trypsin/Lys-C mix (Promega) at 37°C overnight with shaking. Digested peptides were collected (2500 g for 60s) and lyophilized before being desalted with custom-made stage-tip567 containing Empore SPE Extraction Disk (3M). Peptides were eluted with 2% acetonitrile, 2% formic acid in dH2O.

Peptide LC-MS analysis was performed with a Dionex 3000 nano-HPLC coupled to an Orbitrap XL mass spectrometer (Thermo Fisher). Peptide samples were pressure-loaded onto a home-made C18 reverse-phase column (75 µm diameter, 15 cm length). A 180-minute gradient increasing from 95% buffer A (HPLC grade water with 0.1% formic acid) and 5% buffer B (HPLC grade acetonitrile with 0.1% formic acid) to 75% buffer B in 133 minutes was used at 200 nL/min. The Orbitrap XL was operated in top-8-CID-mode with MS spectra measured at a resolution of 60,000@m/z 400. One full MS scan (300–2000 MW) was followed by three data-dependent scans of the nth most intense ions with dynamic exclusion enabled. Peptides fulfilling a Percolator calculated 1% false discovery rate (FDR) threshold were reported. Label-free quantification of alk-aspirin-labeled proteins was performed with the label-free MaxLFQ algorithm in MaxQuant software as described[6]. The search results from MaxQuant were analyzed by Perseus

(http://www.perseusframework.org/). Briefly, the DMSO replicates and alk-aspirin labeled sample replicates were grouped correspondingly. The results were cleaned to filter off reverse hits and contaminants. Only proteins that were identified in 3 out of 4 alk-aspirin labeled sample replicates and with more than two unique peptides were subjected to subsequent statistical analysis. LFQ intensities were used for measuring protein abundance and logarithmized. Signals that were originally zero were imputed with random numbers from a normal distribution, whose mean and standard deviation were chosen to best simulate low abundance values below the noise level (Replace missing values by normal distribution – Width = 0.3; Shift = 1.8). Significant proteins that were more enriched in alk-aspirin labeled sample group versus control group were determined by a threshold strategy, which combined t test p-values with ratio information. The summary of proteomis data is shown in the excel file of proteomics summary.

### *Salmonella* Quantitative Reverse-Transcription PCR

500 uL of *Salmonella* cultures were processed with RNeasy Mini Kit (Qiagen) per manufacturer's manual. Concentrations of purified RNA were normalized to 100 ng/uL with RNase-free water. Quantitative Reverse-Transcription PCR (qRT-PCR) were performed with Power SYBR Green RNA-to-$C_T$ 1-Step Kit (Applied Biosystems) per manufacturer's manual and primers listed in **Table S2**. *rpoS* and *rpoD* genes are used as $\Delta$CT calculating controls.

### Gentamicin protection assay of *S*. Typhimurium grown with aspirin analogs

HT-29 cells (human colon epithelium cell) were cultured in 12-well tissue culture plates about 90% of confluency. Wells were added with *Salmonella* cells at an MOI = 10:1 and centrifuged at 1000 g for 5 min at room temperature. Cells were incubated at 37°C with 5% $CO_2$ for 2 h to allow invasion. The media was removed and cells are washed with PBS (100 μg/mL gentamicin) three times. Then new medium containing 100 μg/mL gentamicin are added and cells are incubated for an additional hour to kill extracellular *Salmonella*. Wells were then washed 3 times with PBS, and

cells were lysed with 500 uL 1% Triton X-100 PBS. Lysates were serially diluted and drip-dropped on *Salmonella Shigella* agar plates (BD 211597) to determine the number of invaded bacteria.

**Animal Experiments**

C57BL/6J mice were purchased from the Jackson Laboratory and maintained at the Rockefeller University animal facilities under SPF conditions. Animal care and experimentation were consistent with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of the Rockefeller University.

***S.* Typhimurium infection of mice**

To ensure effective colonization and induce infection susceptibility, C57BL/6J mice (7-9 weeks) were gavaged with a single dose of 20 mg of streptomycin 24 hours before infection. Bacterial cultures of *S*. Typhimurium (WT) strain were washed and re-suspended in sterile phosphate-buffered saline (PBS) at $10^7$ CFU/mL. For the prevention model, the mice are gavaged with $10^6$ CFU bacteria and SA-4 (400 mg/kg, 100 mg/kg), aspirin (400 mg/kg) or 0.5% DMSO suspended in 0.5% of Sodium carboxymethyl cellulose at the same time. For the treatment model, mice were gavaged with $10^6$ CFU of bacterial. SA-4 was suspended in 0.5% of sodium carboxymethyl cellulose and the mice were gavaged with the SA-4 suspension at indicated time. (400 mg/kg 1h post infection, 200 mg/kg twice daily for 3 days). For pretreatment model, SA-4 was suspended in 0.5% of sodium carboxymethyl cellulose and C57BL/6 mice were orally treated with 400 mg of SA-4 1 hour before infection. Then the mice were orally infected with $10^6$ CFU of *S*. Typhimurium. SA-4 was gavaged with 200 mg/kg twice daily. For *S.* Typhimurium infection survival assay, mice weight loss was monitored just before infection, and mice were euthanized when they reached 80% baseline weight, appeared hunched or moribund, or exhibited a visibly distended abdomen (indicative of peritoneal effusion), whichever occurred first. Death was not used as an end point.

Colony-forming units (CFU) in the feces were determined by plating five serial dilutions of feces suspended in sterile PBS on *Salmonella Shigella agar plates (BD 211597).*

### *C. difficile* growth curves

*C. difficile* was grown to log phase (~3 hours) and diluted to an $OD_{600}$ of 0.05 in BHIS. 1 mL per well of diluted culture was added to a 24-well non tissue culture-treated flat bottom plate (CellStar) in technical duplicate containing 10, 5, 2.5, 1, or 0.1 mM 4-SA or aspirin, or an equivalent volume of DMSO (1% v/v final) as a vehicle control. The plate was then sealed with a clear plate seal (R&D systems) to maintain an anaerobic environment in the wells when read in a Synergy H1 plate reader (Biotek) outside of the anaerobic chamber. $OD_{600}$ was measured every 10 minutes for 18 hours at 37˚C with 1 minute shaking before each measurement, and BHIS + 1% v/v DMSO served as a blank to be subtracted from each well. Technical duplicate measurements were averaged to give one biological replicate measurement. The assay was performed with at least three biological replicates.

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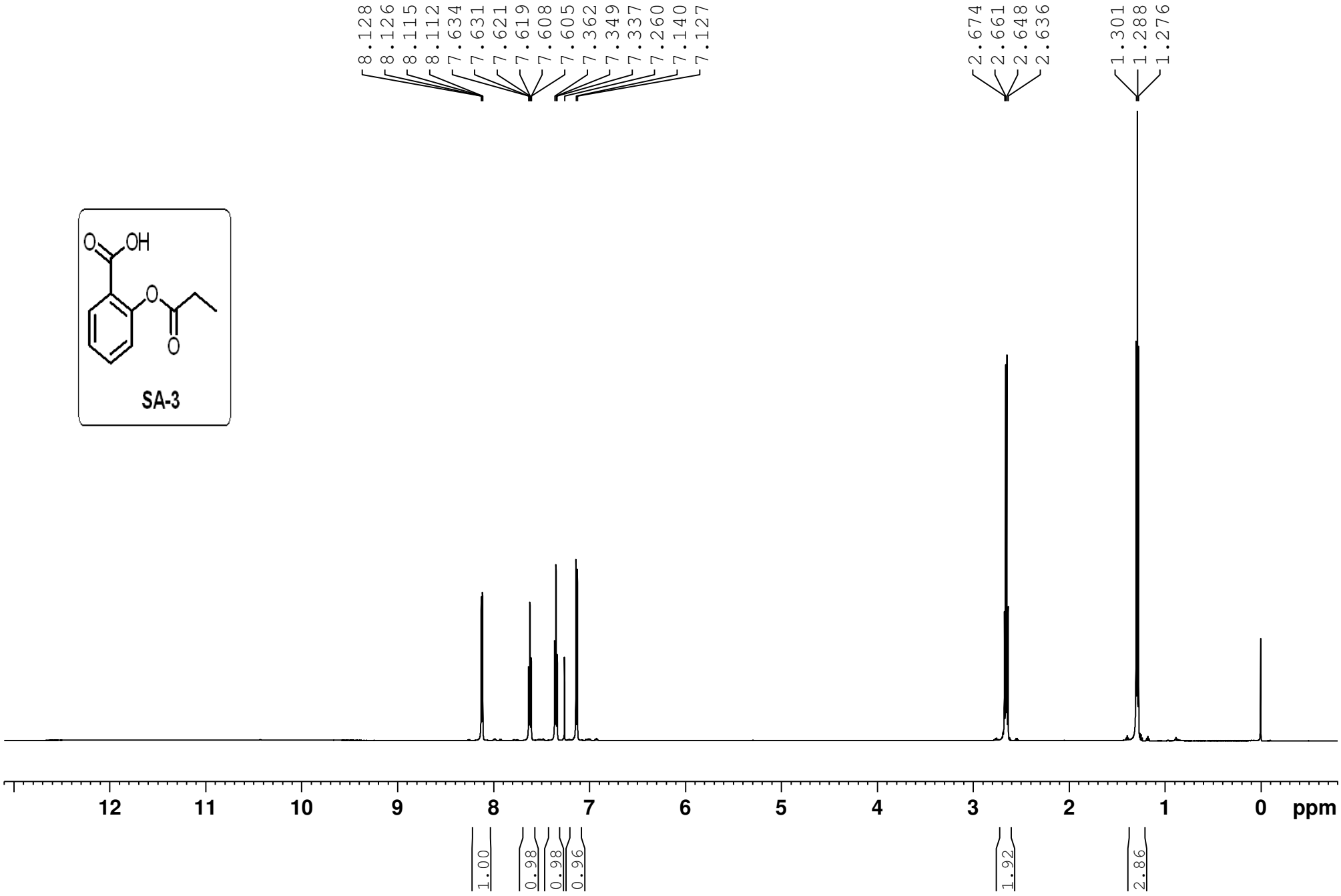
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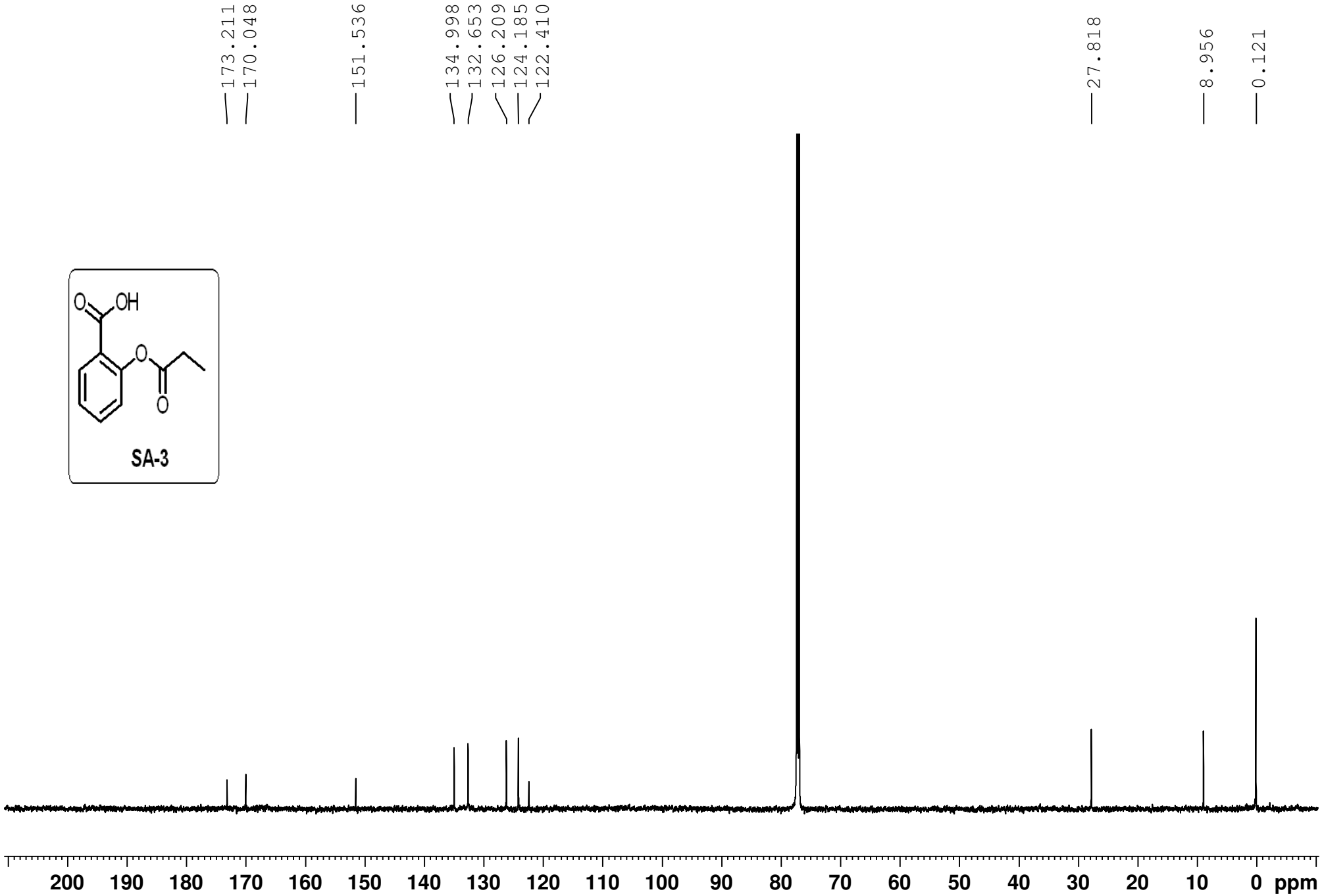
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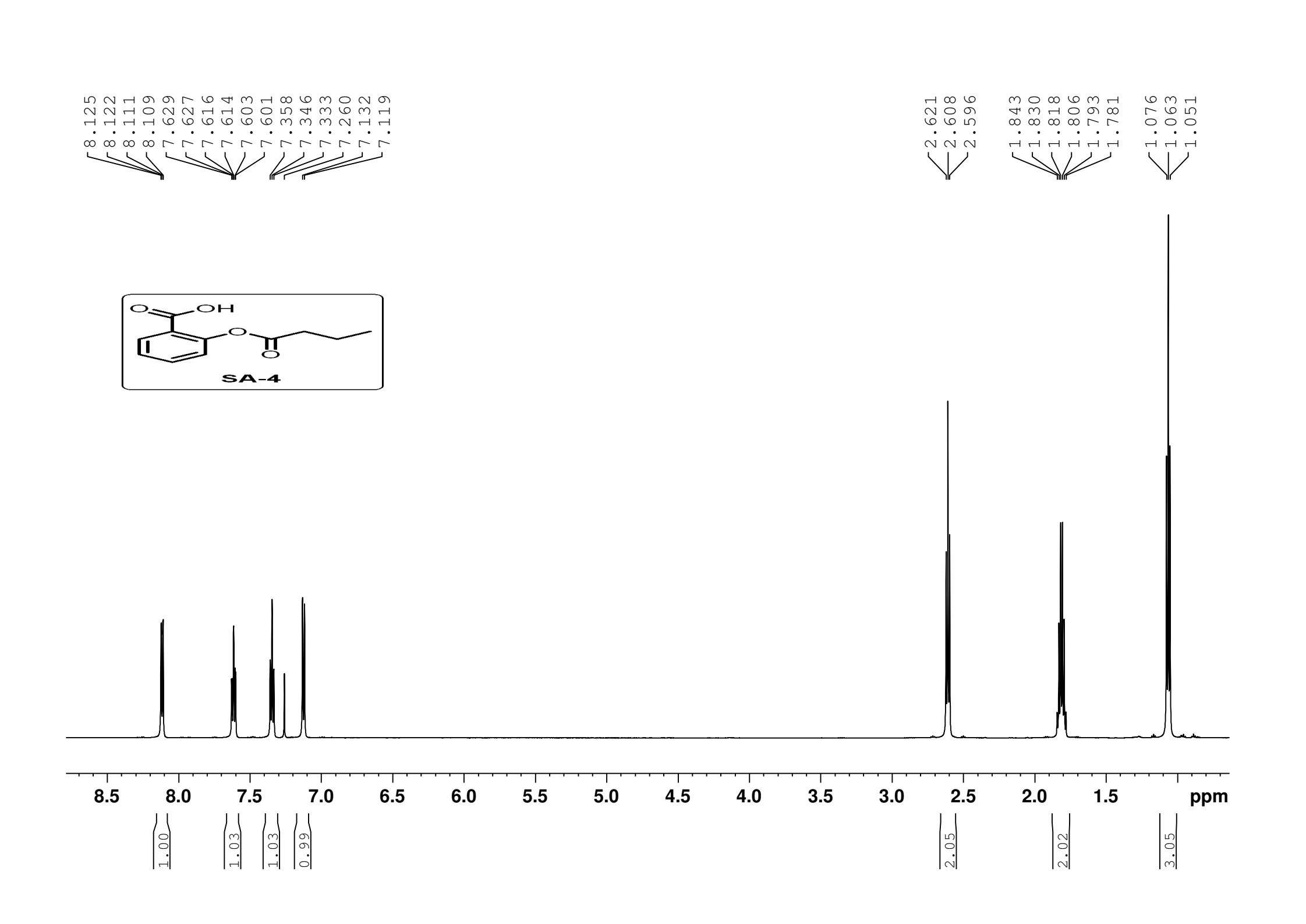
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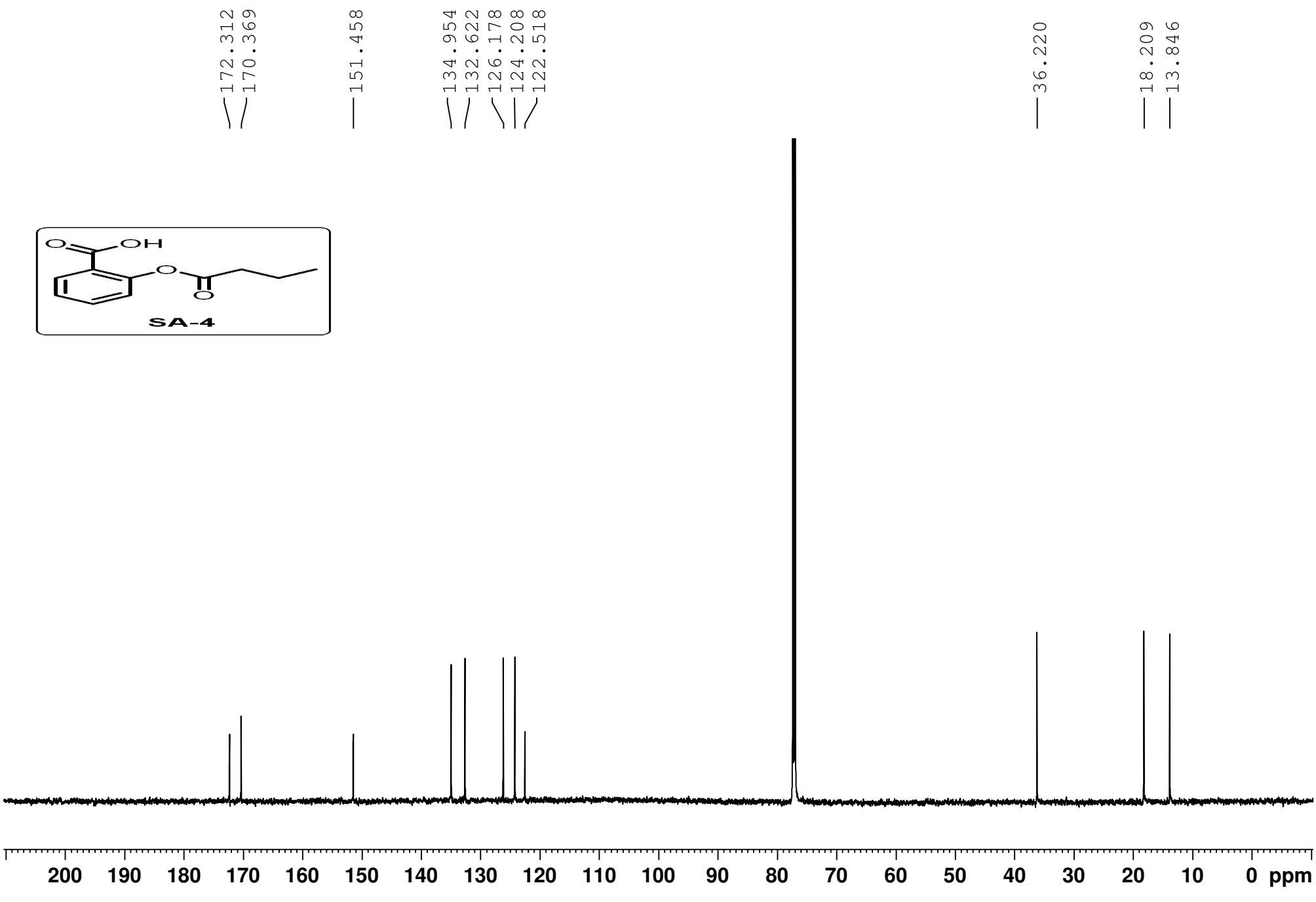
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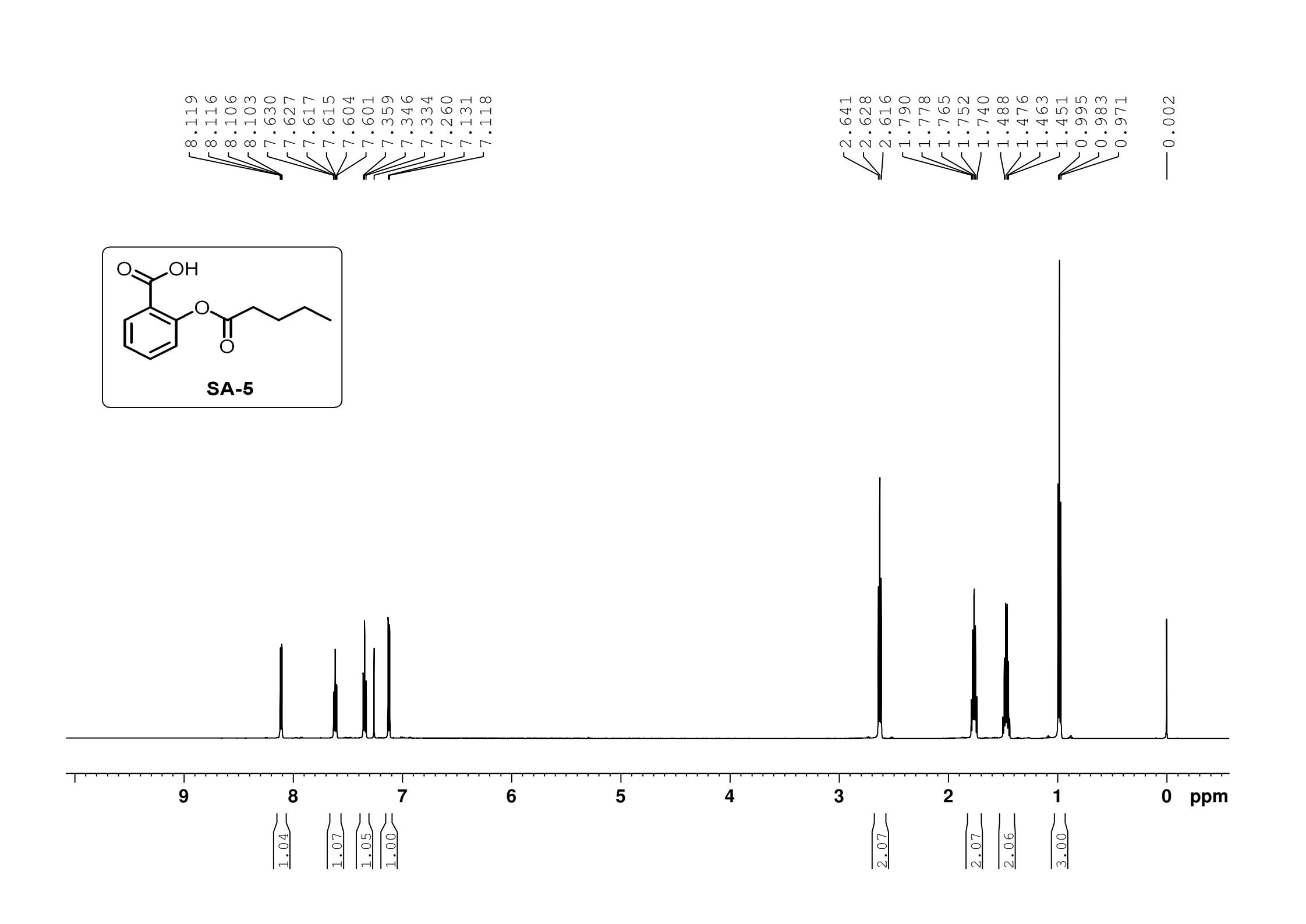
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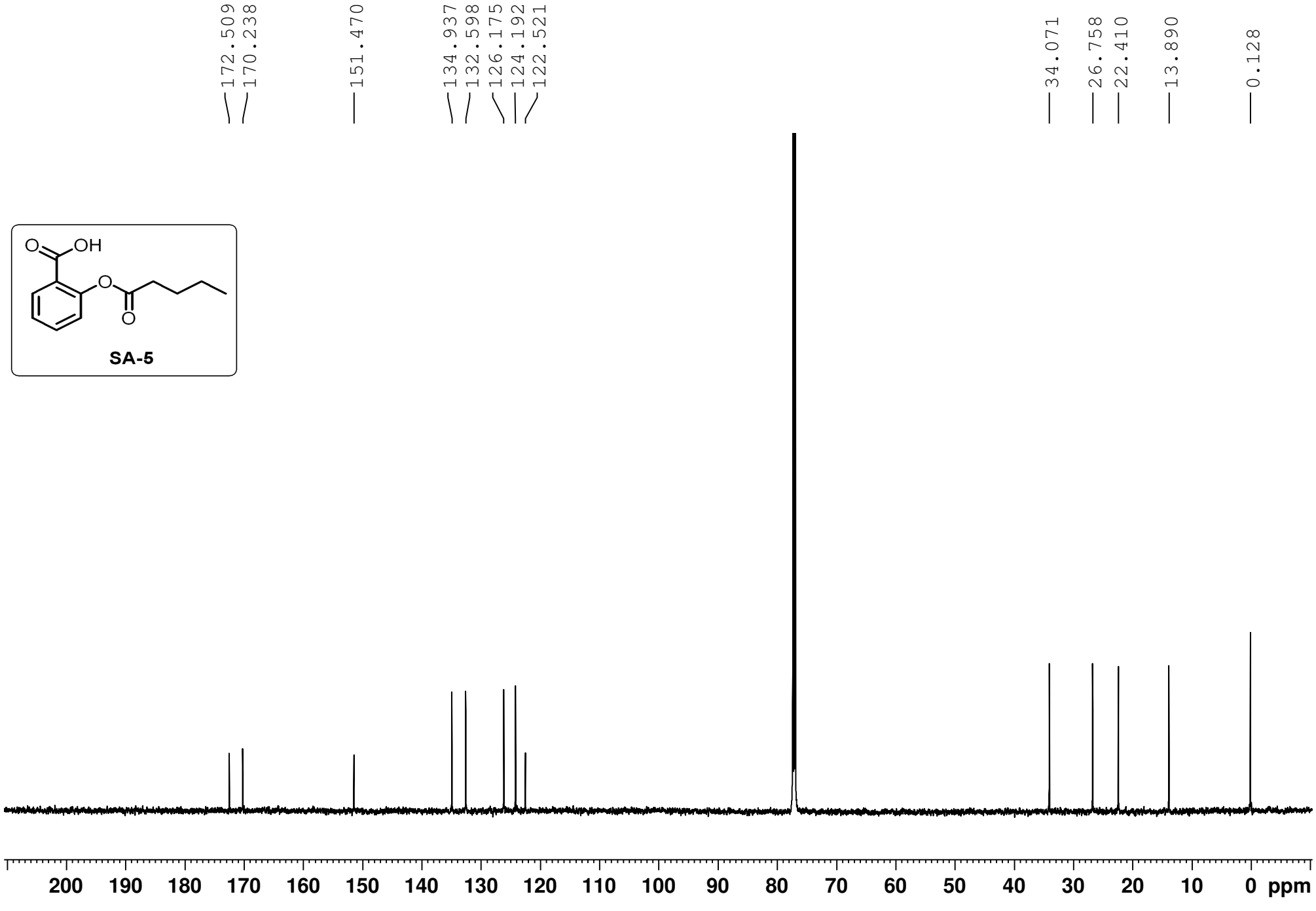
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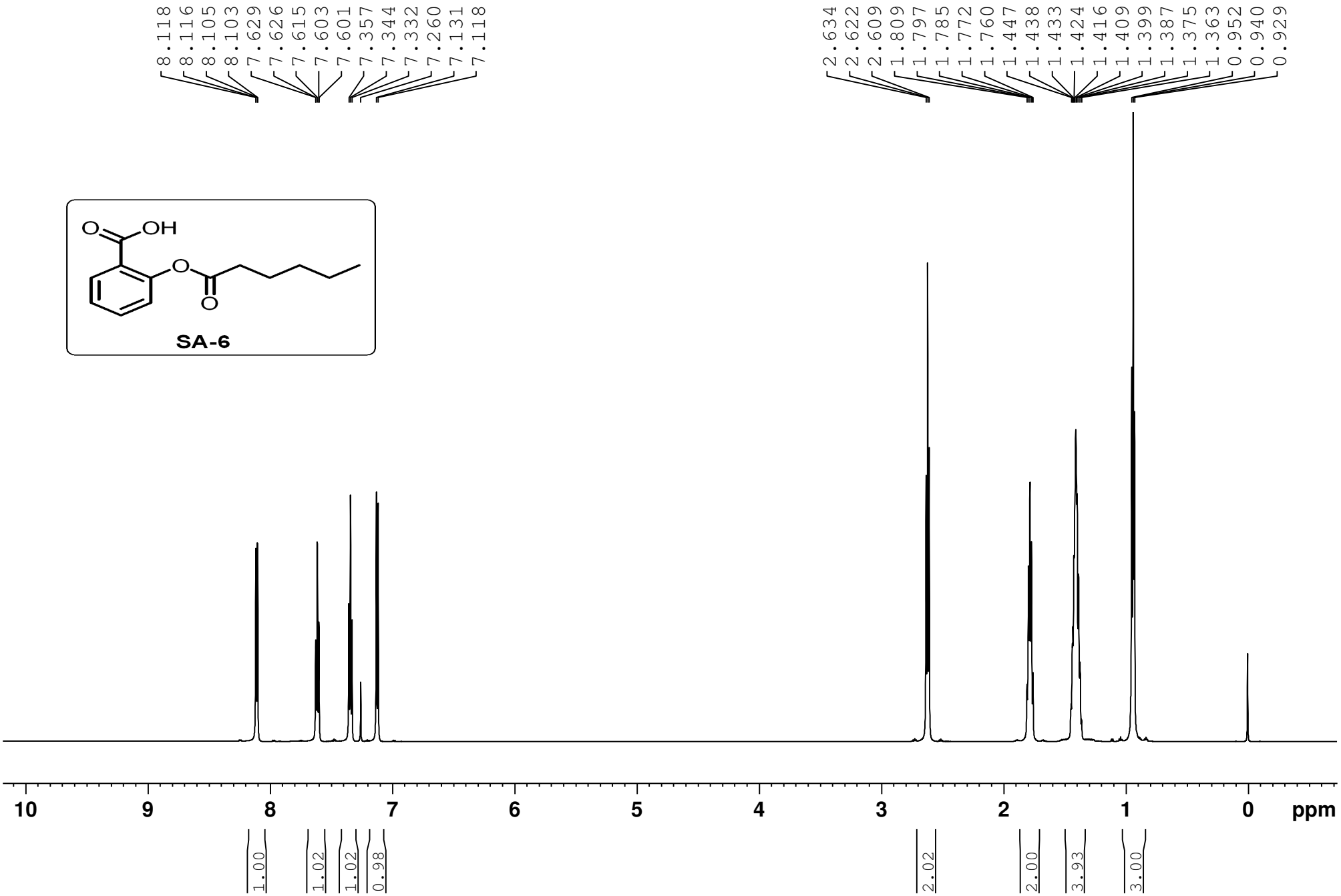
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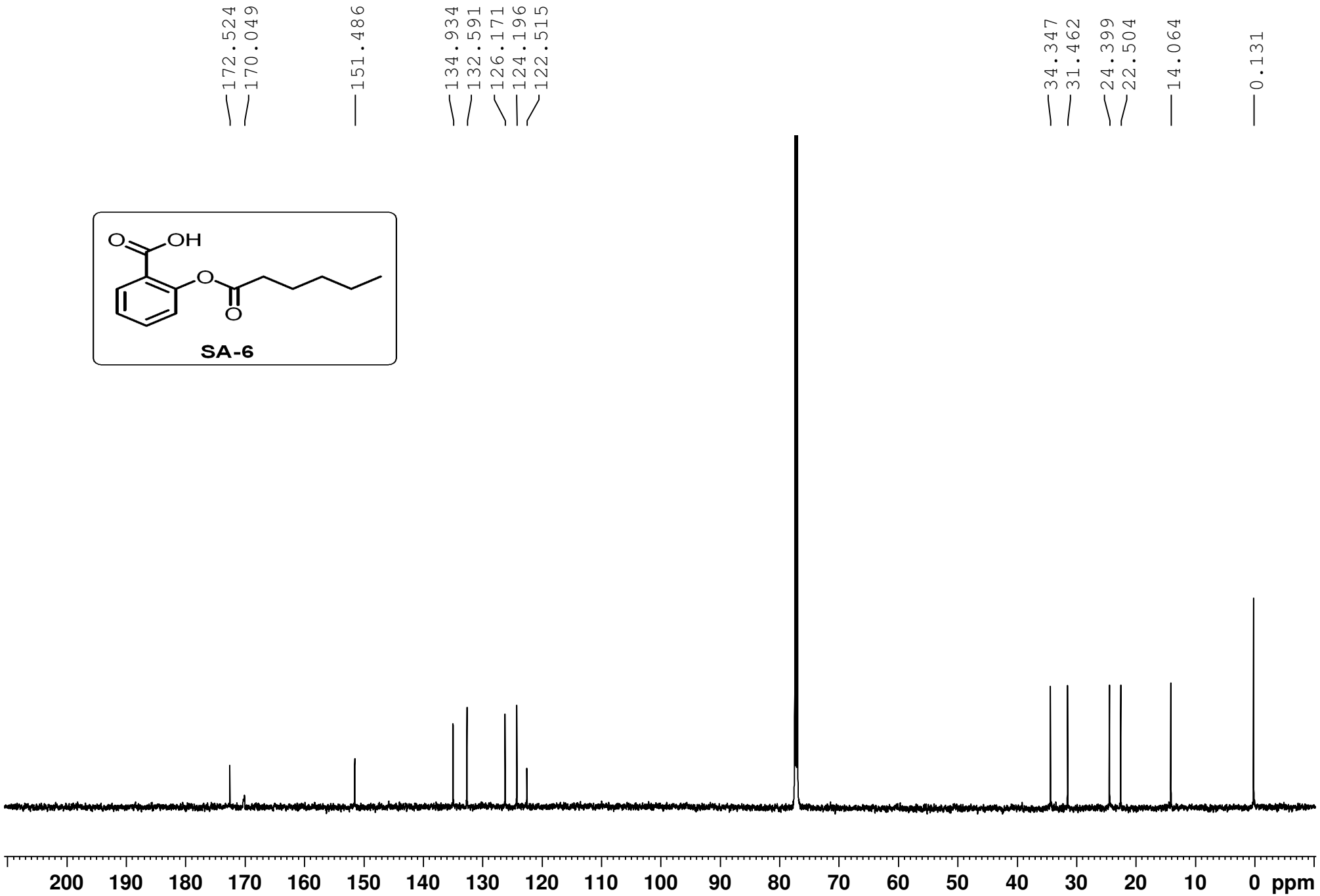
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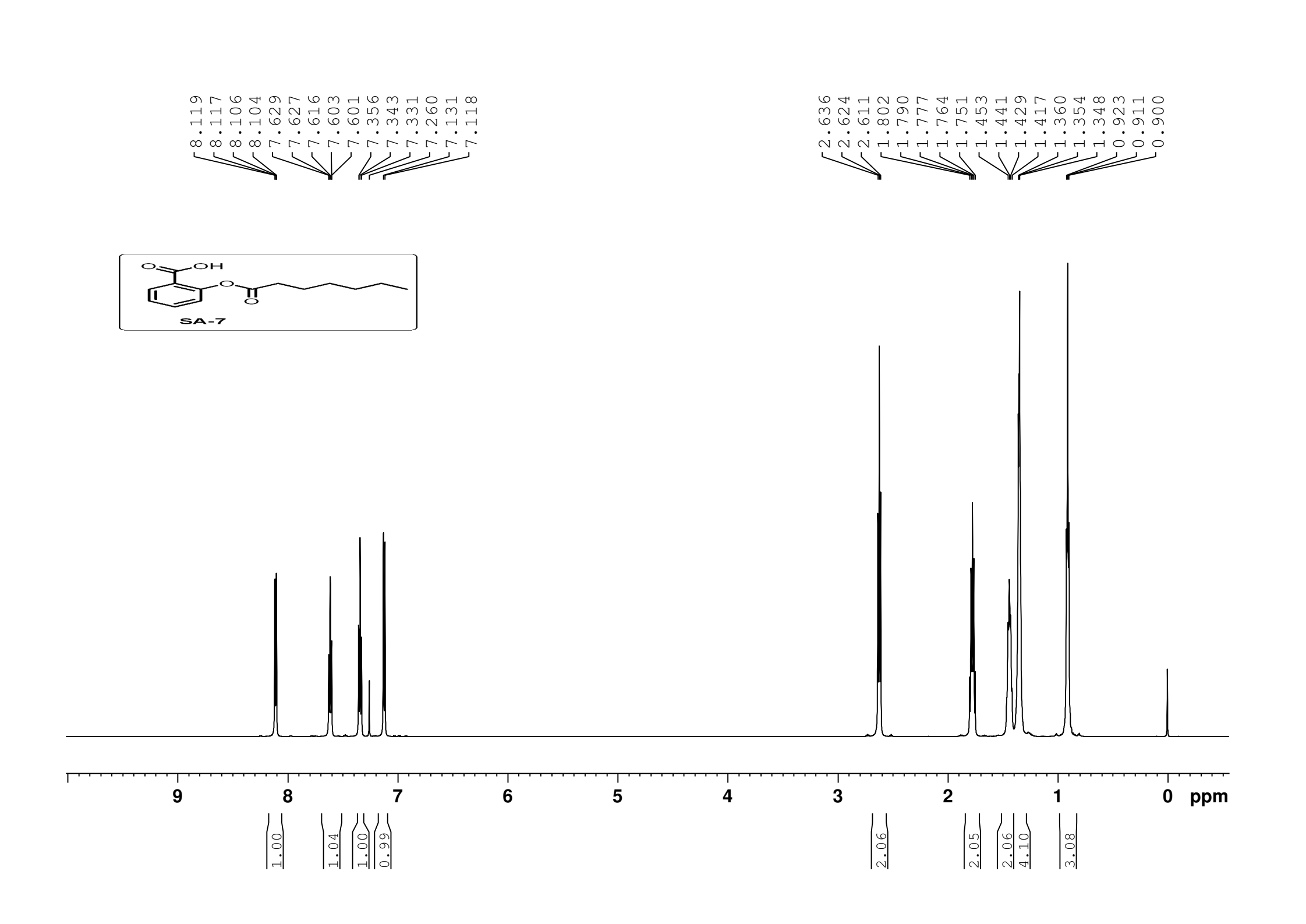
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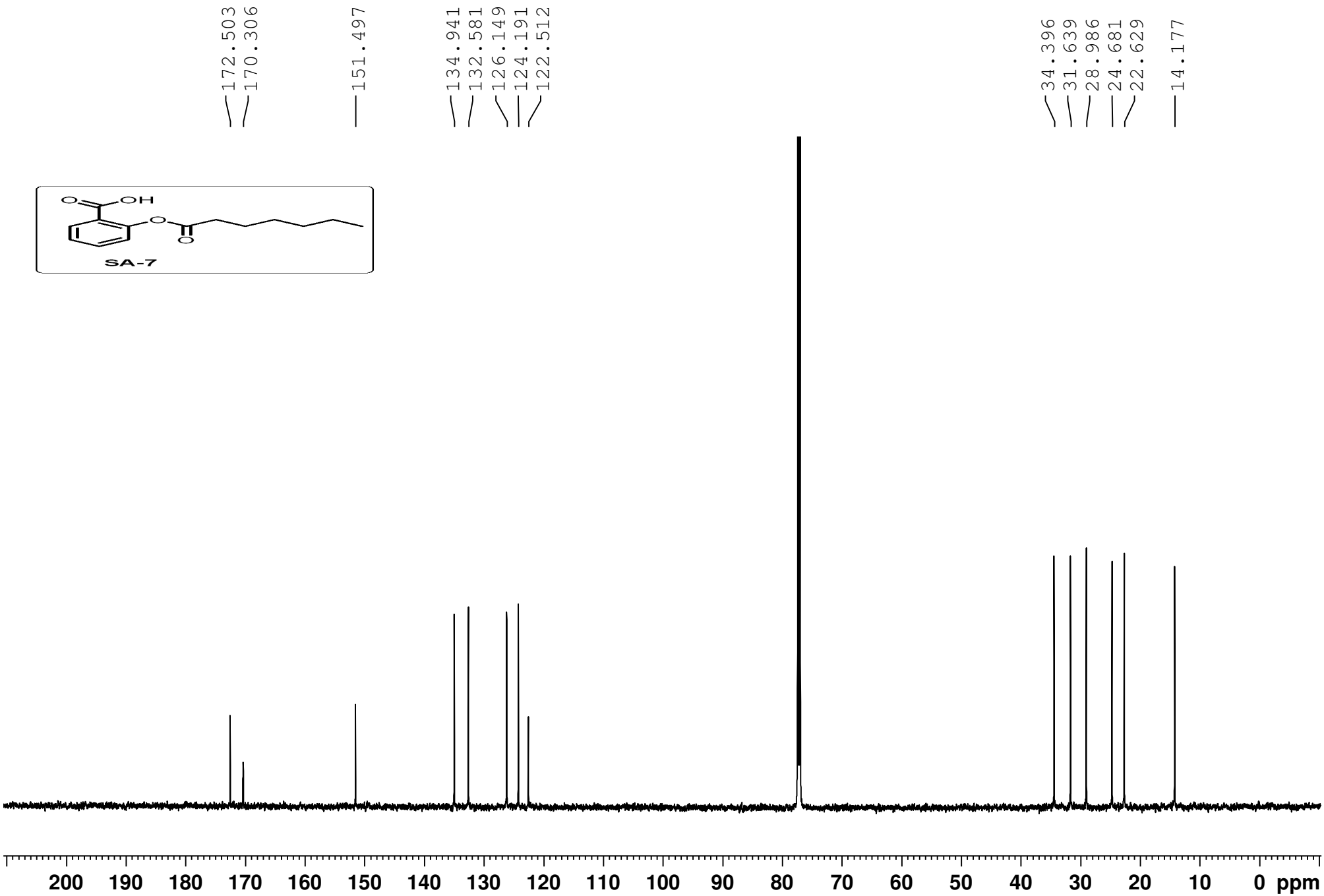
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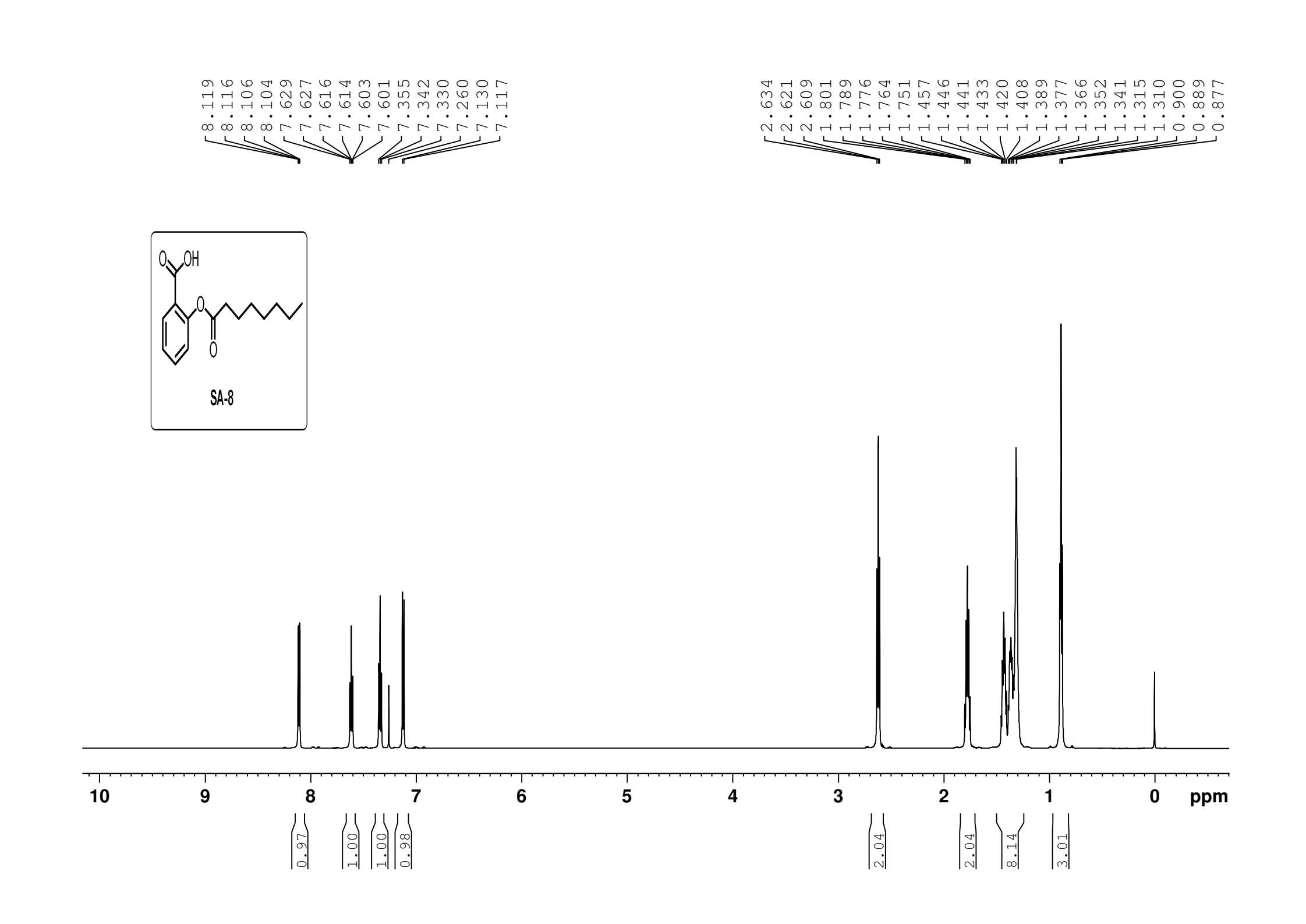
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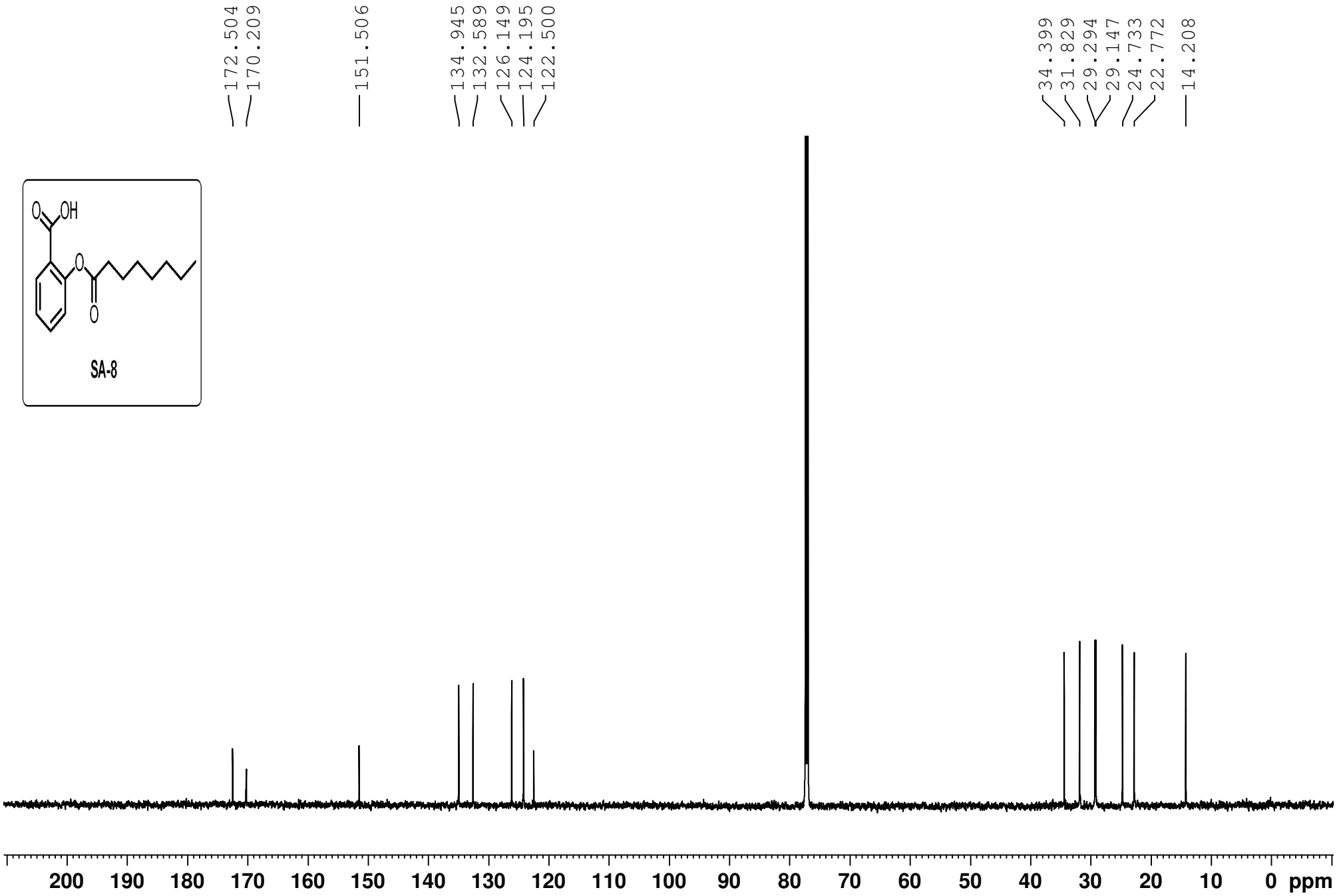
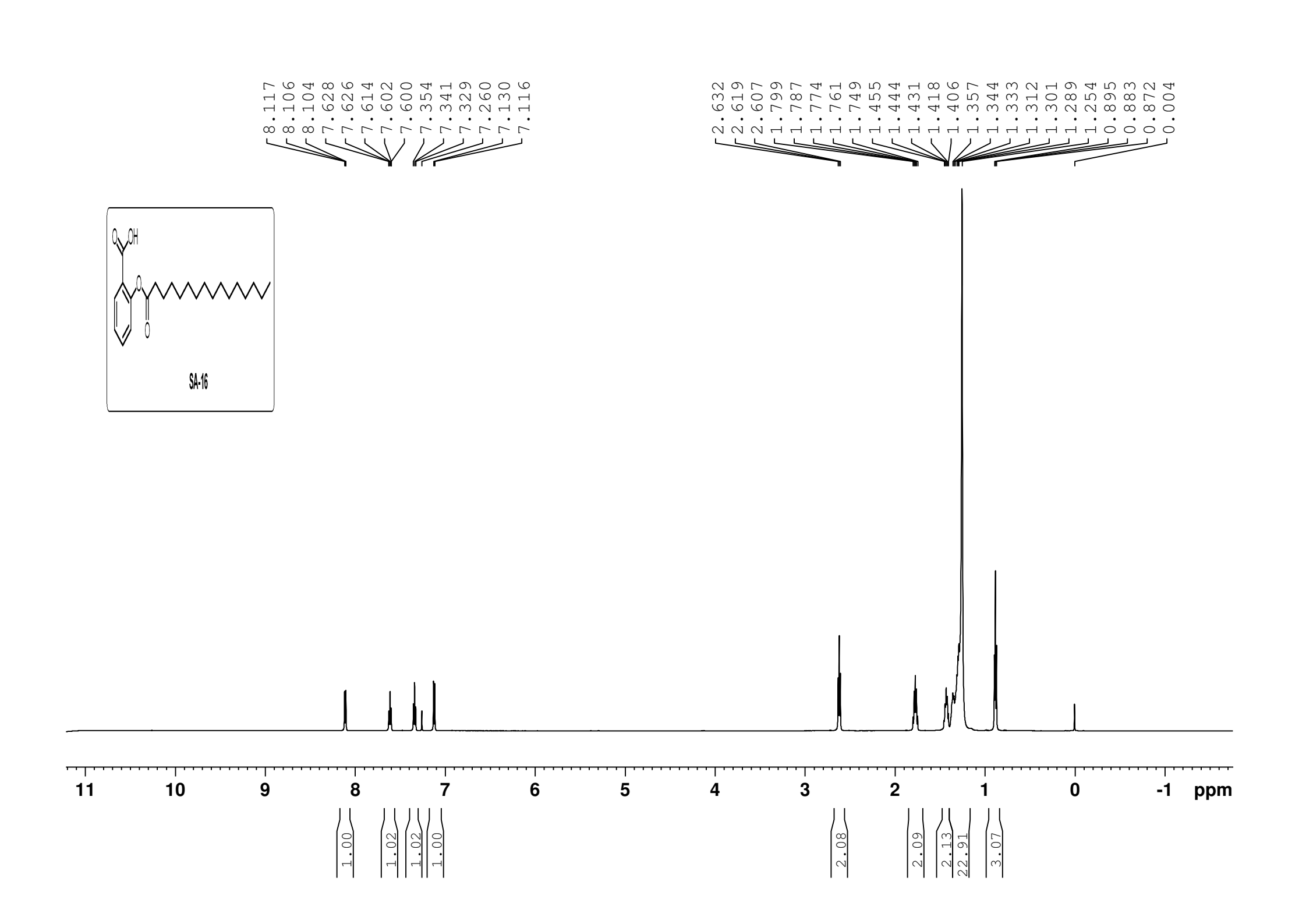
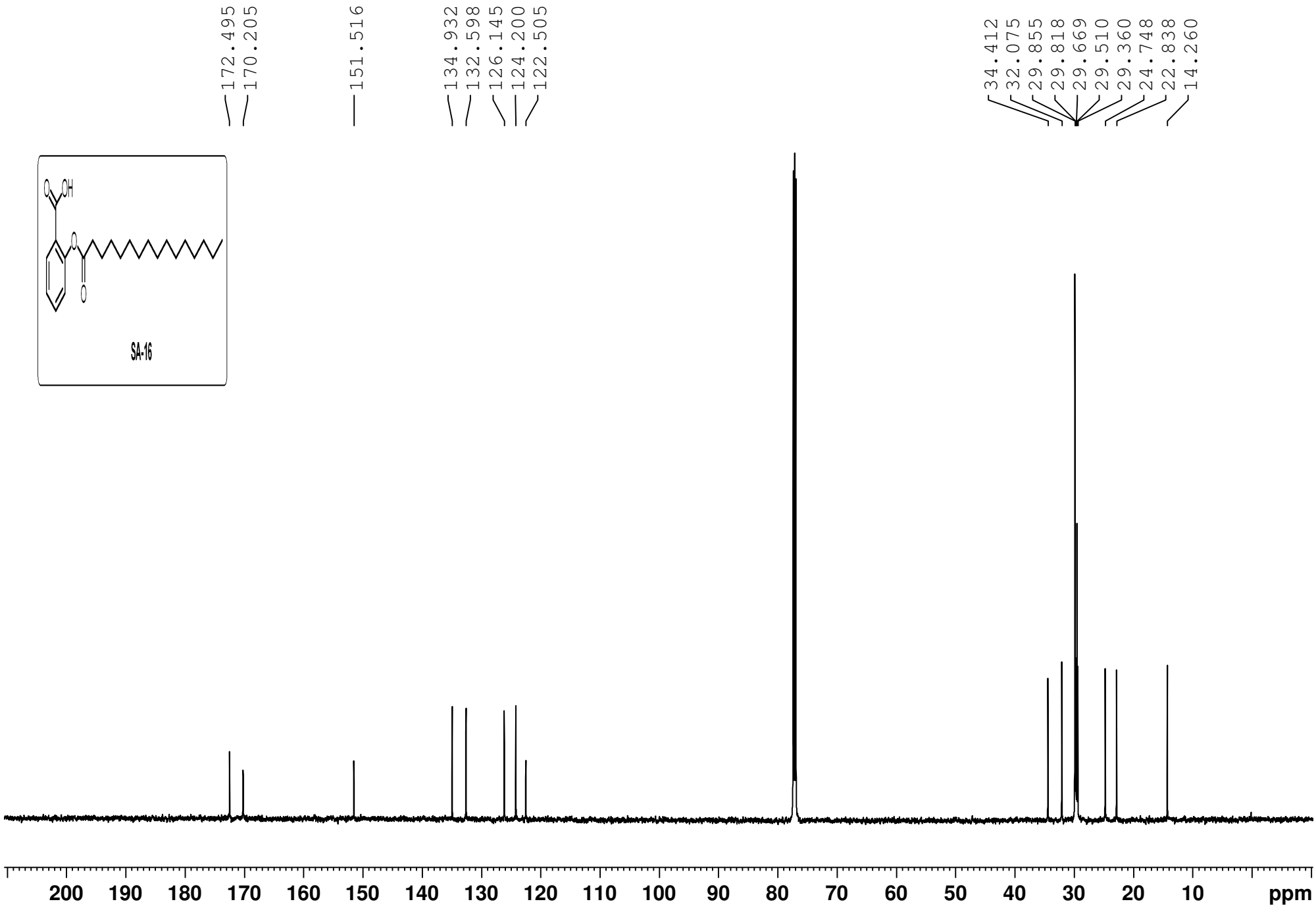
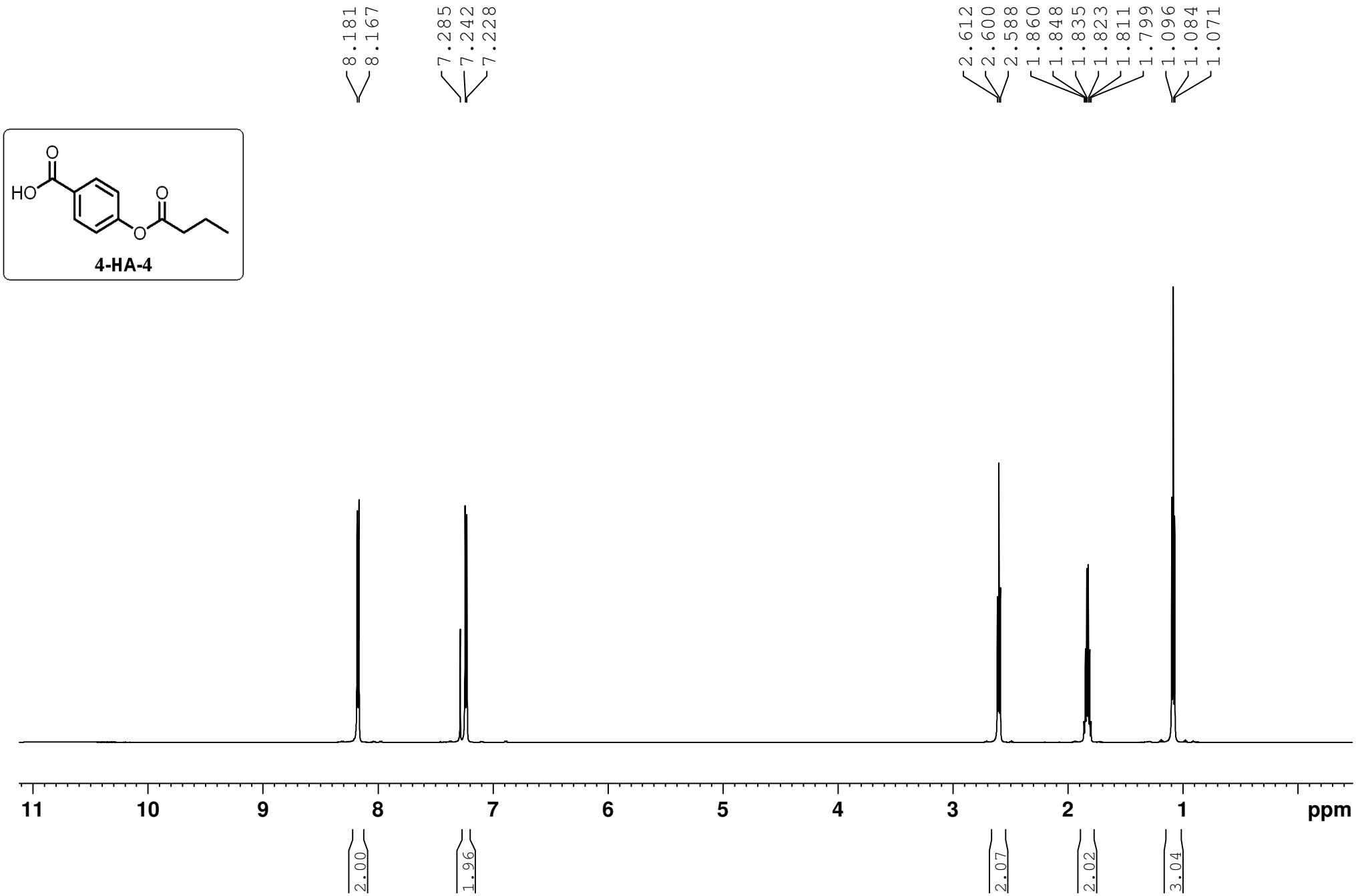
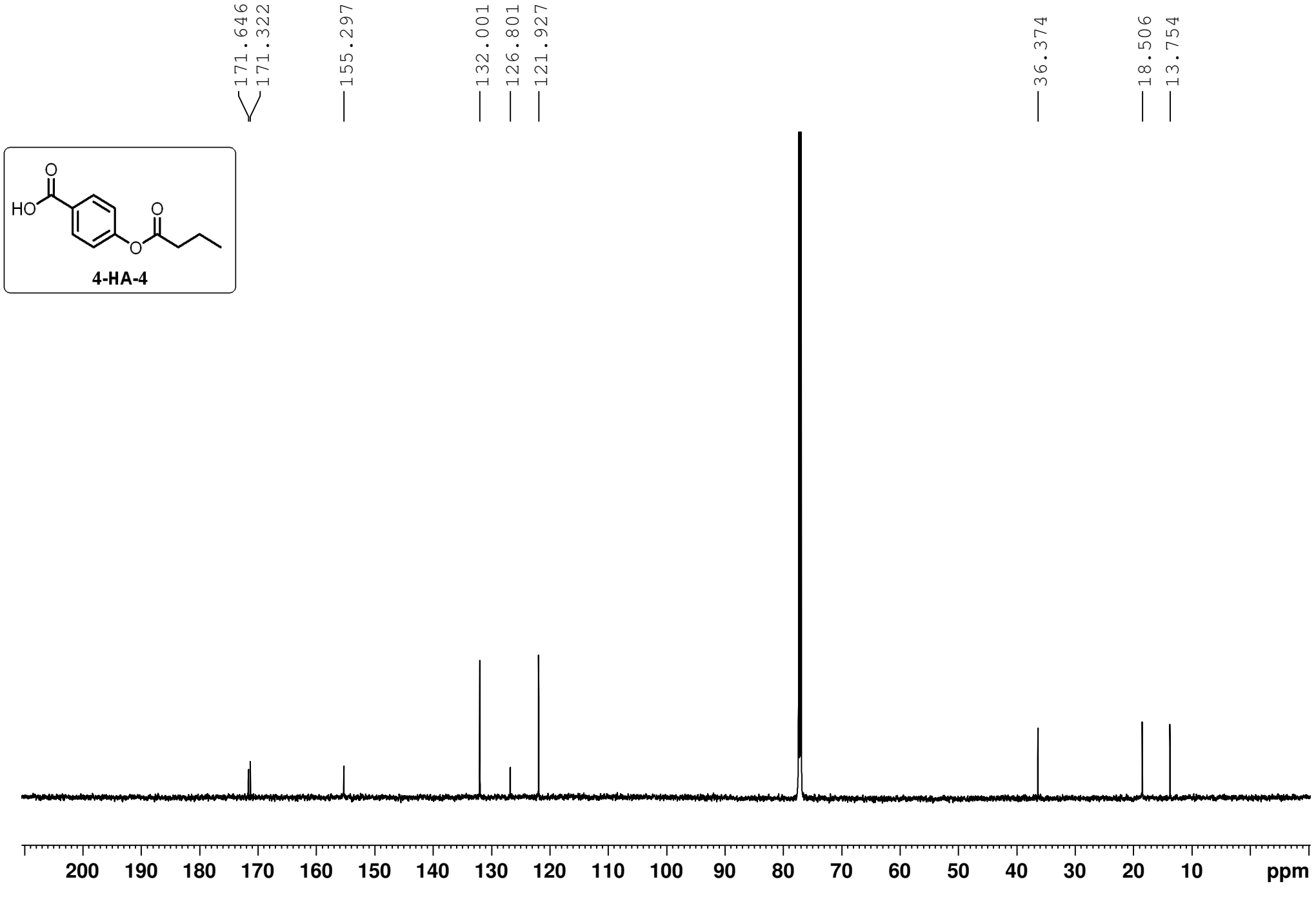
7.629
7.627
7.616
7.614
7.603
7.601
7.355
7.342
7.330
7.260
7.130
7.117
2.634
2.621
2.609
1.801
1.789
1.776
1.764
1.751
1.457
1.446
1.441
1.433
1.420
1.408
1.389
1.377
1.366
1.352
1.341
1.315
1.310
0.900
0.889
0.877
OH
O
O
O
SA-8
10
9
8
7
6
5
4
3
2
1
0
ppm
0.97
1.00
1.00
0.98
2.04
2.04
8.14
3.01

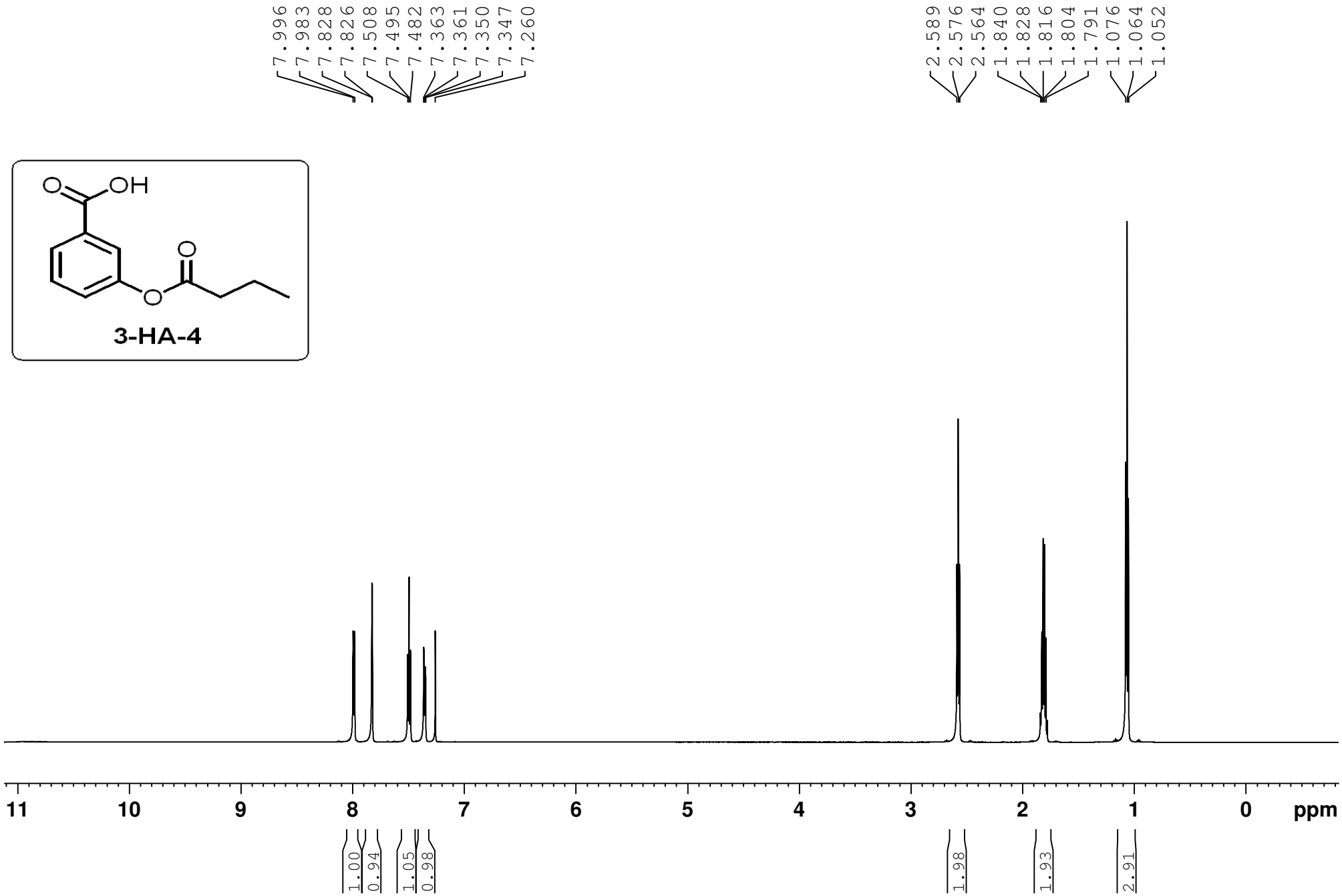

172.504
170.209
151.506
134.945
132.589
126.149
124.195
122.500
34.399
31.829
29.294
29.147
24.733
22.772
14.208
OH
O
O
O
SA-8
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

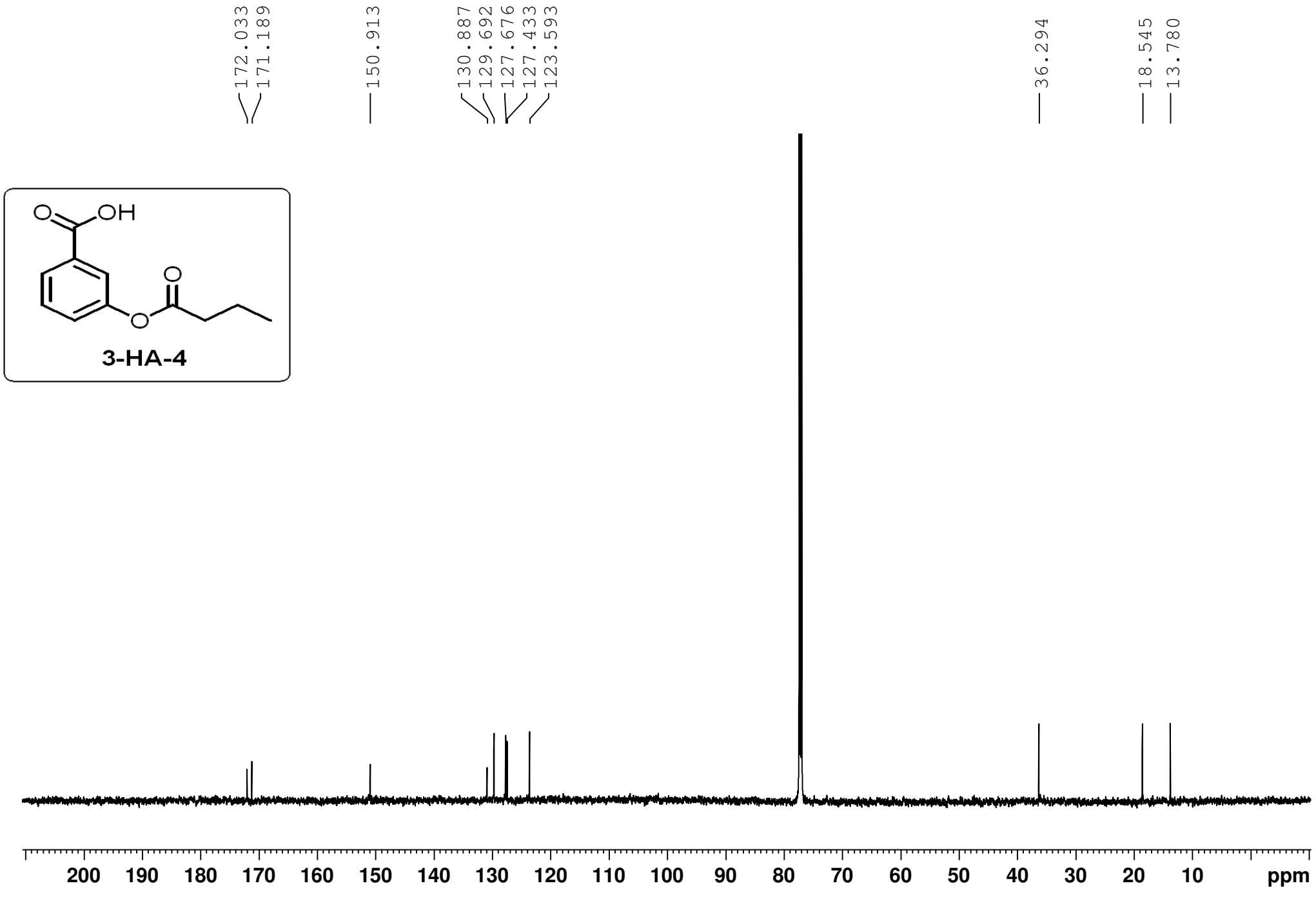

8.117
8.106
8.104
7.628
7.626
7.614
7.602
7.600
7.354
7.341
7.329
7.260
7.130
7.116
2.632
2.619
2.607
1.799
1.787
1.774
1.761
1.749
1.455
1.444
1.431
1.418
1.406
1.357
1.344
1.333
1.312
1.301
1.289
1.254
0.895
0.883
0.872
0.004
O
OH
O
O
SA-16
11
10
9
8
7
6
5
4
3
2
1
0
-1
ppm
1.00
1.02
1.02
1.00
2.08
2.09
2.13
22.91
3.07


172.495
170.205
151.516
134.932
132.598
126.145
124.200
122.505
34.412
32.075
29.855
29.818
29.669
29.510
29.360
24.748
22.838
14.260
OH
O
O
SA-16
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm


8.181
8.167
7.285
7.242
7.228
2.612
2.600
2.588
1.860
1.848
1.835
1.823
1.811
1.799
1.096
1.084
1.071
HO
O
O
O
4-HA-4
11
10
9
8
7
6
5
4
3
2
1
ppm
2.00
1.96
2.07
2.02
3.04


171.646
171.322
155.297
132.001
126.801
121.927
36.374
18.506
13.754
HO
O
O
O
4-HA-4
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm

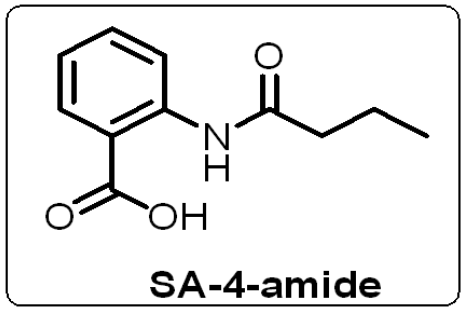

7.996
7.983
7.828
7.826
7.508
7.495
7.482
7.363
7.361
7.350
7.347
7.260
2.589
2.576
2.564
1.840
1.828
1.816
1.804
1.791
1.076
1.064
1.052
OH
O
O
O
3-HA-4
11
10
9
8
7
6
5
4
3
2
1
0
ppm
1.00
0.94
1.05
0.98
1.98
1.93
2.91


172.033
171.189
150.913
130.887
129.692
127.676
127.433
123.593
36.294
18.545
13.780
OH
O
O
O
3-HA-4
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm

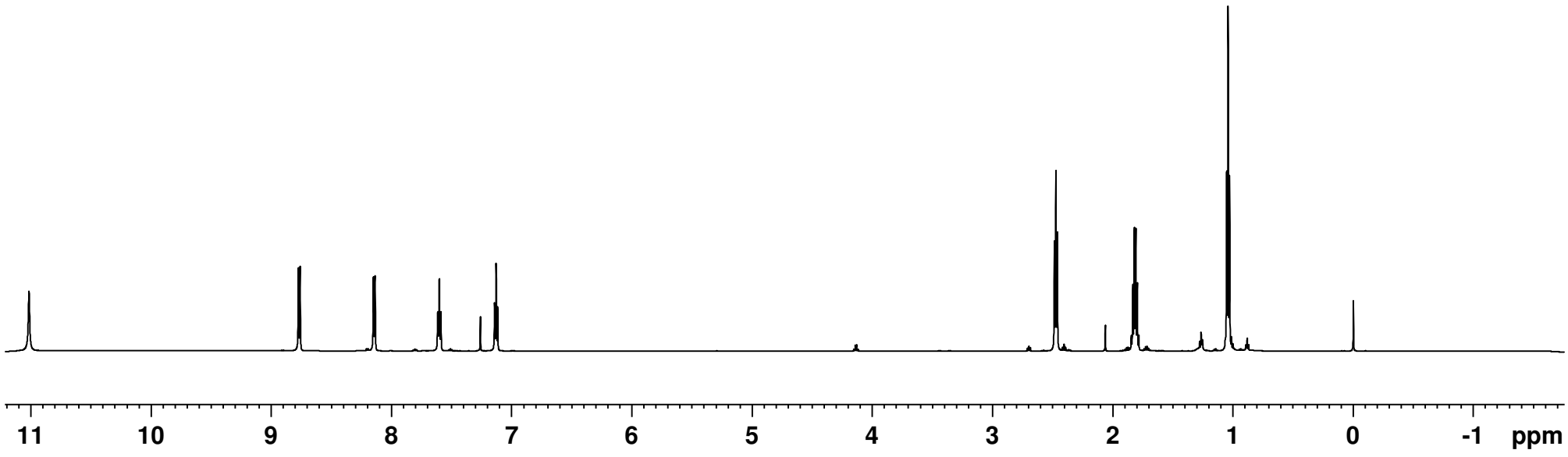
11.015

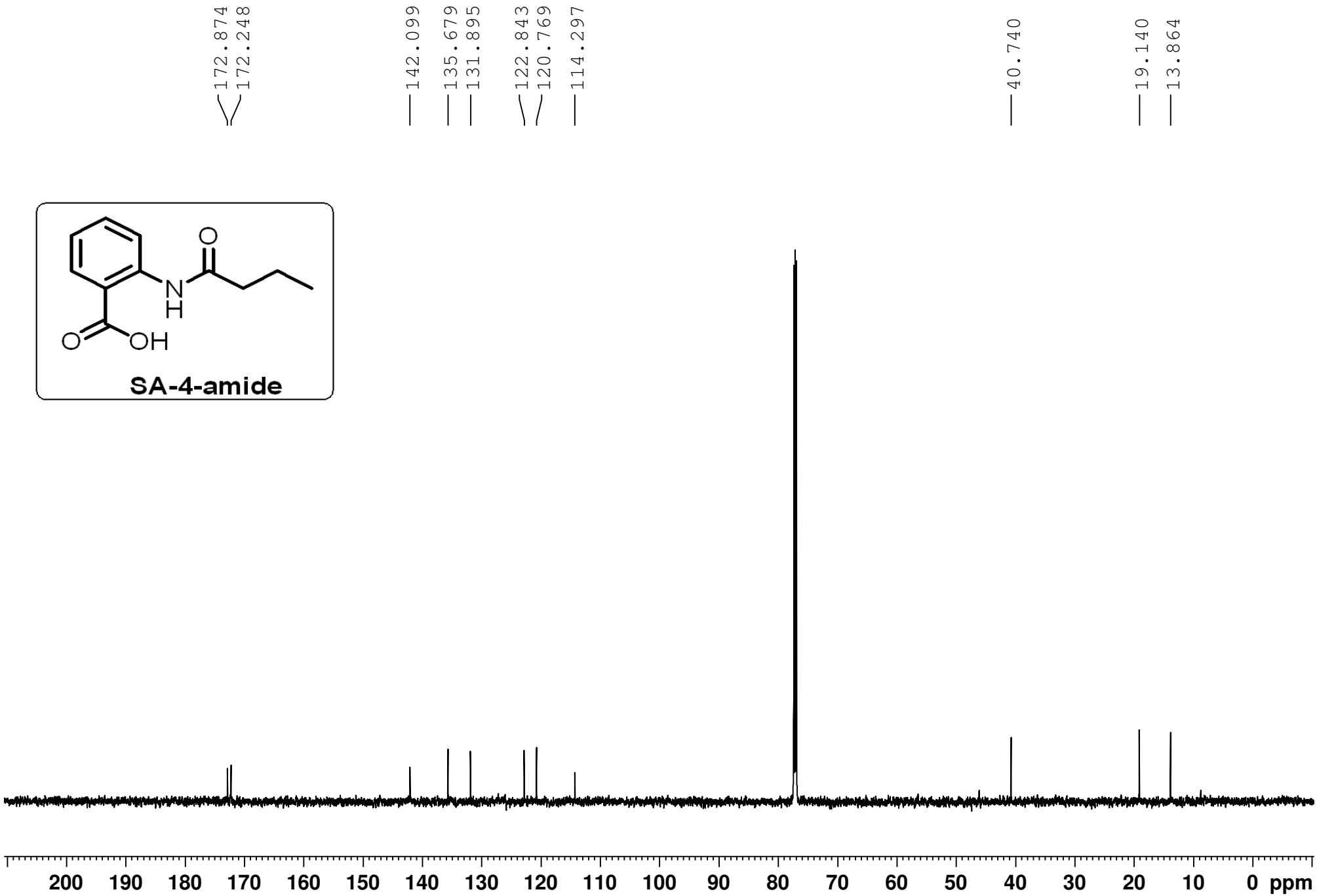
8.774
8.759
8.148
8.137
7.601
7.141
7.129

2.484
2.471
2.459
1.832
1.819
1.807
1.794
1.051
1.039
1.027




**SA-4-amide**




172.874
172.248
142.099
135.679
131.895
122.843
120.769
114.297
40.740
19.140
13.864
O
N
H
O
OH
SA-4-amide
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm