

## **Design and evaluation of poly-nitrogenous adjuvants capable of potentiating antibiotics in Gram-negative bacteria**

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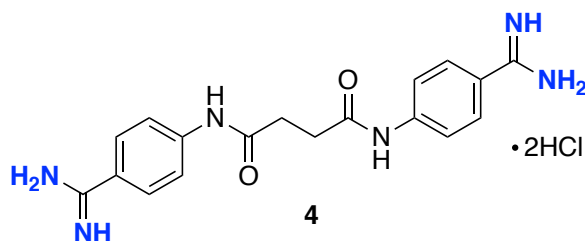
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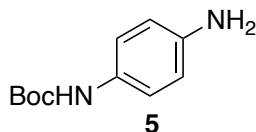
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## Synthesis and Spectroscopic Data.

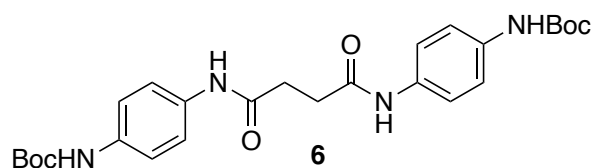
*General.* Reagents and solvents were purchased reagent-grade and used without further purification. All reactions were performed in flame-dried glassware under an Ar or N<sub>2</sub> atmosphere. Evaporation and concentration *in vacuo* was performed at 40 °C. TLC was conducted using precoated SiO<sub>2</sub> 60 F254 glass plates from EMD with visualization by UV light (254 or 366 nm). NMR (<sup>1</sup>H or <sup>13</sup>C) were recorded on an Oxford Varian-400 spectrophotometer at 298 K. Residual solvent peaks were used as an internal reference. Coupling constants (J) (H,H) are given in Hz. Coupling patterns are designated as singlet (s), doublet (d), triplet (t), quadruplet (q), quintuplet (qu), multiplet (m), or broad singlet (br). IR spectra were recorded on a Thermo Scientific Nicolet iS10 FT-IR spectrophotometer and measured neat. Low-resolution mass spectral data were acquired on a Shimadzu single quadrupole LCMS-2020. High-resolution mass spectral data were acquired on a Thermo Scientific Q Exactive Plus mass spectrometer coupled to a Waters Acquity UPLC, and the detected masses are given as *m/z* with *m* representing the molecular ion. The purity of each tested compound (>95%) was determined via <sup>1</sup>H NMR.



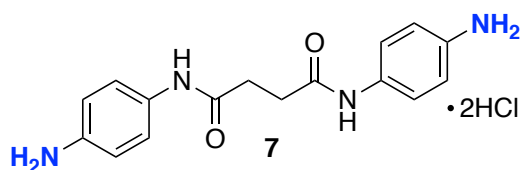
***N*<sup>1</sup>,*N*<sup>4</sup>-bis(4-Carbamimidoylphenyl)succinimide dihydrochloride (4):** 4-Aminobenzamidine dihydrochloride (2.00 g, 14.8 mmol, 2 equiv.) and pyridine (11.9 mL, 148 mmol, 20 equiv.) were dissolved in *N,N*-dimethyl formamide (0.06 M), and succinyl chloride (0.815 mL, 7.40 mmol, 1 equiv.) was added dropwise to the reaction, which was then warmed to reflux for 2 hours. The resultant precipitate was filtered and washed successively with ethanol and diethyl ether to yield **4** (1.85 g, 70%) as a brown solid. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz): δ 10.64 (s, 2H), 9.24 (br, 4H), 8.95 (br, 4H), 7.81 (s, 8H), 2.74 (s, 4H). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz): δ 171.30, 164.72, 144.32, 129.30, 121.49, 118.38, 31.05. IR (film)  $\nu_{\max}$  3308, 3092, 1669, 1605, 1495, 1352 cm<sup>-1</sup>. HRMS (ESI) *m/z* [*M*]<sup>2+</sup>: calcd for C<sub>18</sub>H<sub>20</sub>N<sub>6</sub>O<sub>2</sub> 177.08997; found: 177.08966.



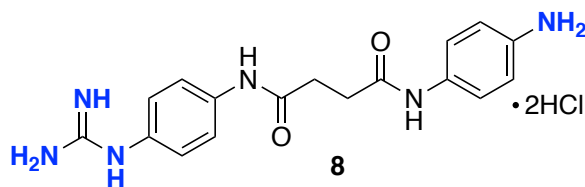
***tert*-Butyl-(4-aminophenyl)carbamate (5):** *p*-Phenylenediamine dihydrochloride (5.00 g, 28.0 mmol, 1 equiv.) and potassium carbonate (8.00 g, 58.0 mmol, 2.07 equiv.) were dissolved in a 2:1 mixture of dioxane/water (0.28 M). Boc anhydride (6.20 g, 28.0 mmol, 1 equiv.) was added and the reaction mixture was stirred at room temperature for 16 hours. The crude reaction mixture was concentrated *in vacuo*, 100 mL of water was added, the mixture was then filtered, and subsequently washed with water (100 mL x 3) to afford **5** (5.65 g, 98%) as a pink solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ 7.13 (d, *J* = 7.7 Hz, 2H), 6.63 (d, *J* = 8.7 Hz, 2H), 6.26 (s, 1H), 3.53 (s, 2H), 1.50 (s, 9H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): δ 153.31, 142.36, 129.71, 120.90, 115.61, 80.04, 28.38. IR (film)  $\nu_{\max}$  3394, 3361, 2985, 2934, 1691, 1513, 1234, 1158, 1028 cm<sup>-1</sup>. HRMS (ESI) *m/z* [*M*+H]<sup>+</sup>: calcd for C<sub>11</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub> 209.12845; found: 209.12824.



**di-tert-Butyl-((succinylbis(azanediyl))bis(4,1-phenylene))dicarbamate (6):** *p*-Phenylenediamine dihydrochloride (**5**) (2.02 g, 9.70 mmol, 2 equiv.) was dissolved in dioxane (0.33 M), and succinyl chloride (0.543 mL, 4.85 mmol, 1 equiv.) was added in dropwise, and the reaction mixture was stirred at room temperature for 16 hours. The crude reaction mixture was concentrated *in vacuo*, and 100 mL of saturated sodium bicarbonate was added, the mixture was then filtered, and subsequently washed with water (100 mL x 3) to afford **6** (1.69 g, 70%) as a grey solid. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz): δ 9.87 (s, 2H), 9.23 (s, 2H), 7.45 (d, *J* = 9.2 Hz, 4H), 7.34 (d, *J* = 9.5 Hz, 4H), 2.59 (s, 4H), 1.46 (s, 18H). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz): δ 169.95, 152.83, 134.69, 133.90, 119.37, 118.43, 66.37, 31.16, 28.18. IR (film)  $\nu_{\max}$  3327, 1692, 1655, 1543, 1405, 1161 cm<sup>-1</sup>. HRMS (ESI) *m/z* [M+H]<sup>+</sup>: calcd for C<sub>26</sub>H<sub>34</sub>N<sub>4</sub>O<sub>6</sub> 499.2557; found: 499.2559.

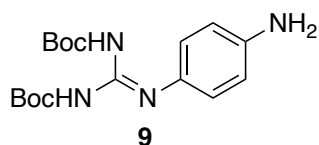


***N*<sup>1</sup>,*N*<sup>4</sup>-bis(4-Aminophenyl)succinimide dihydrochloride (7):** Compound **6** (364 mg, 0.730 mmol, 1 equiv.) was dissolved in 4M HCl in dioxane (0.2 M), and the reaction mixture was stirred at room temperature for 16 hours. The crude reaction mixture was concentrated *in vacuo* followed by trituration with DCM to afford **7** (298 mg, 98%) as a greyish-pink solid. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz): δ 10.45 (s, 2H), 10.42 (s, 6H), 7.71 (d, *J* = 9.0 Hz, 4H), 7.32 (d, *J* = 8.6 Hz, 4H), 2.67 (s, 4H). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz): δ 170.67, 138.95, 126.55, 123.52, 119.71, 31.21. IR (film)  $\nu_{\max}$  3243, 3192, 2964, 1668, 1550, 1422, 1333 cm<sup>-1</sup>. HRMS (ESI) *m/z* [M]<sup>2+</sup>: calcd for C<sub>16</sub>H<sub>20</sub>N<sub>4</sub>O<sub>2</sub> 150.0793; found: 150.0794.

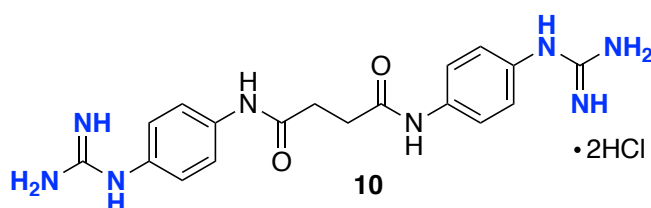


***N*<sup>1</sup>-(4-Aminophenyl)-*N*<sup>4</sup>-(4-guanidinophenyl)succinamide dihydrochloride (8):** Compound **7** (783 mg, 2.11 mmol, 1 equiv.) and triethylamine (2.35 mL, 16.9 mmol, 8 equiv.) were dissolved in dioxane (0.1 M). 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (2.48 g, 6.33 mmol, 3 equiv.) was added and the reaction mixture was stirred at room temperature for 16 hours. The crude reaction mixture was passed through a plug of silica and then eluted with DCM to remove unreacted guanidinylation reagent, followed by 5% MeOH/DCM which was then concentrated *in vacuo*. The resulting light brown solid was taken up in 1M HCl and let sonicate for half an hour. The crude reaction mixture was filtered, and the aqueous filtrate was concentrated *in vacuo* to afford **8** (47.0 mg, 5%). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz): δ 10.25 (s, 1H), 10.23 (s, 1H), 9.71 (s, 1H), 7.66 (m, 4H), 7.36 (s, 4H), 7.25 (d, *J* = 8.3 Hz, 2H), 7.15 (d, *J* = 6.9 Hz, 2H), 2.67

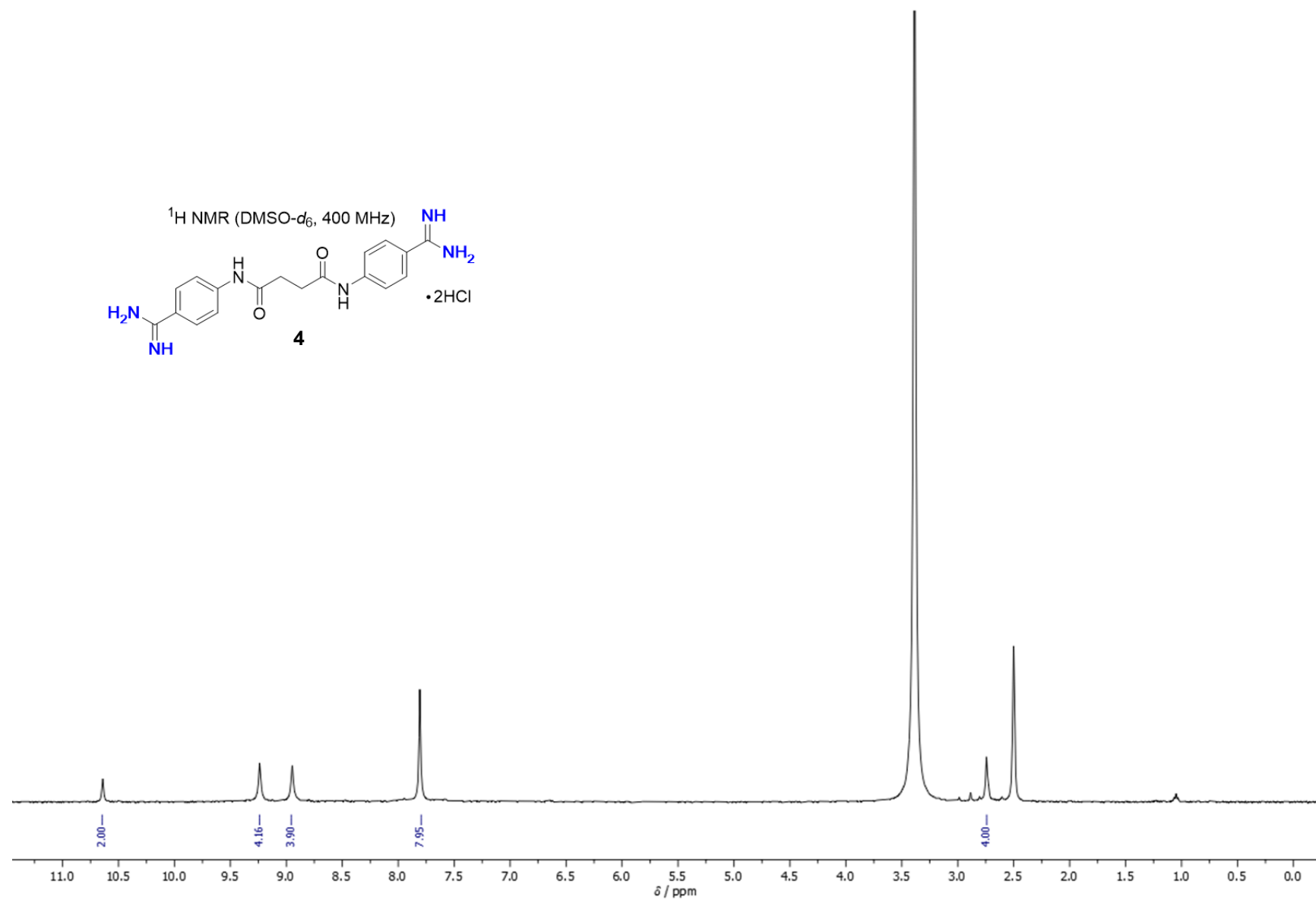
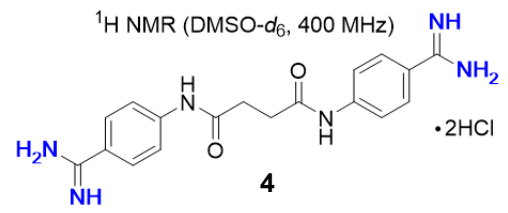
(br, 4H).  $^{13}\text{C}$  NMR (DMSO- $d_6$ , 100 MHz):  $\delta$  170.53 (2C), 156.21, 138.15, 129.52, 125.79 (2C), 122.90, 122.86, 119.84 (2C), 31.14 (2C). IR (film)  $\nu_{\text{max}}$  3245, 2980, 2624, 2500, 1668, 1549, 1332  $\text{cm}^{-1}$ . HRMS (ESI)  $m/z$   $[\text{M}]^{2+}$ : calcd for  $\text{C}_{17}\text{H}_{22}\text{N}_6\text{O}_2$  171.0897; found: 171.0898.

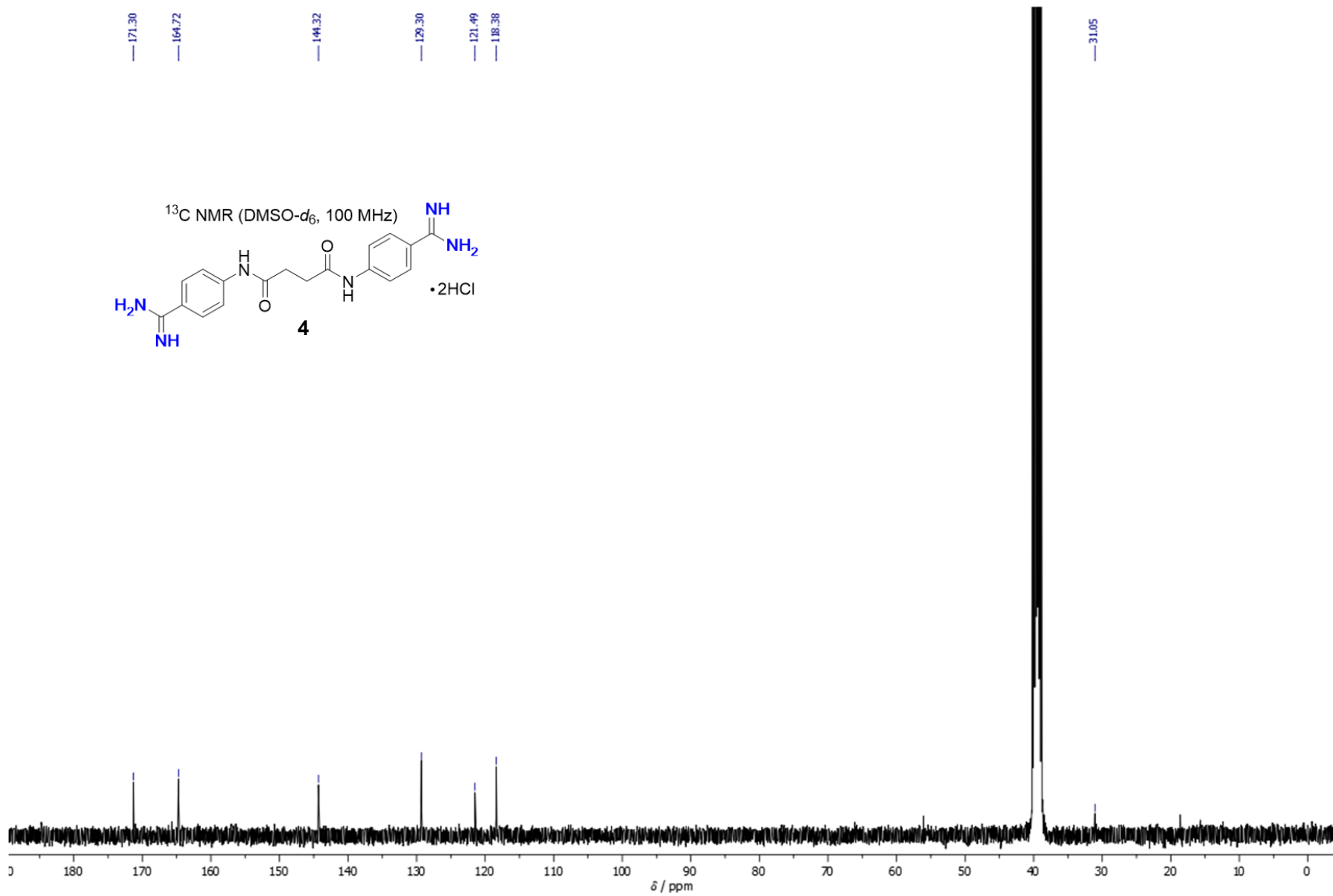


**4-[2,3-di(*tert*-Butoxycarbonyl)guanidino]aniline (9):** *p*-Phenylenediamine dihydrochloride (152 mg, 0.842 mmol, 1 equiv.) and triethylamine (0.470 mL, 3.37 mmol, 4 equiv.) were dissolved in DCM (0.5 M). 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (329 mg, 0.842 mmol, 1 equiv.) was added and the reaction mixture was stirred at room temperature for 16 hours. The crude reaction mixture was passed through a plug of silica and then eluted with DCM to remove unreacted guanidinylation reagent, followed by 5% MeOH/DCM which was then concentrated *in vacuo* to afford **9** (193 mg, 65%) as an off-white solid.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  11.65 (br, 1H), 10.07 (s, 1H), 7.30 (d,  $J = 8.8$  Hz, 2H), 6.63 (d,  $J = 8.8$  Hz, 2H), 3.62 (br, 2H), 1.52 (s, 9H), 1.48 (s, 9H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz):  $\delta$  163.78, 153.92, 153.47, 143.98, 127.95, 124.33, 115.41, 83.54, 79.52, 28.34, 28.21. IR (film)  $\nu_{\text{max}}$  3269, 2981, 2920, 1718, 1630, 1402, 1155, 1057  $\text{cm}^{-1}$ . HRMS (ESI)  $m/z$   $[\text{M}+\text{H}]^+$ : calcd for  $\text{C}_{17}\text{H}_{26}\text{N}_4\text{O}_4$  351.2026; found: 351.2027.

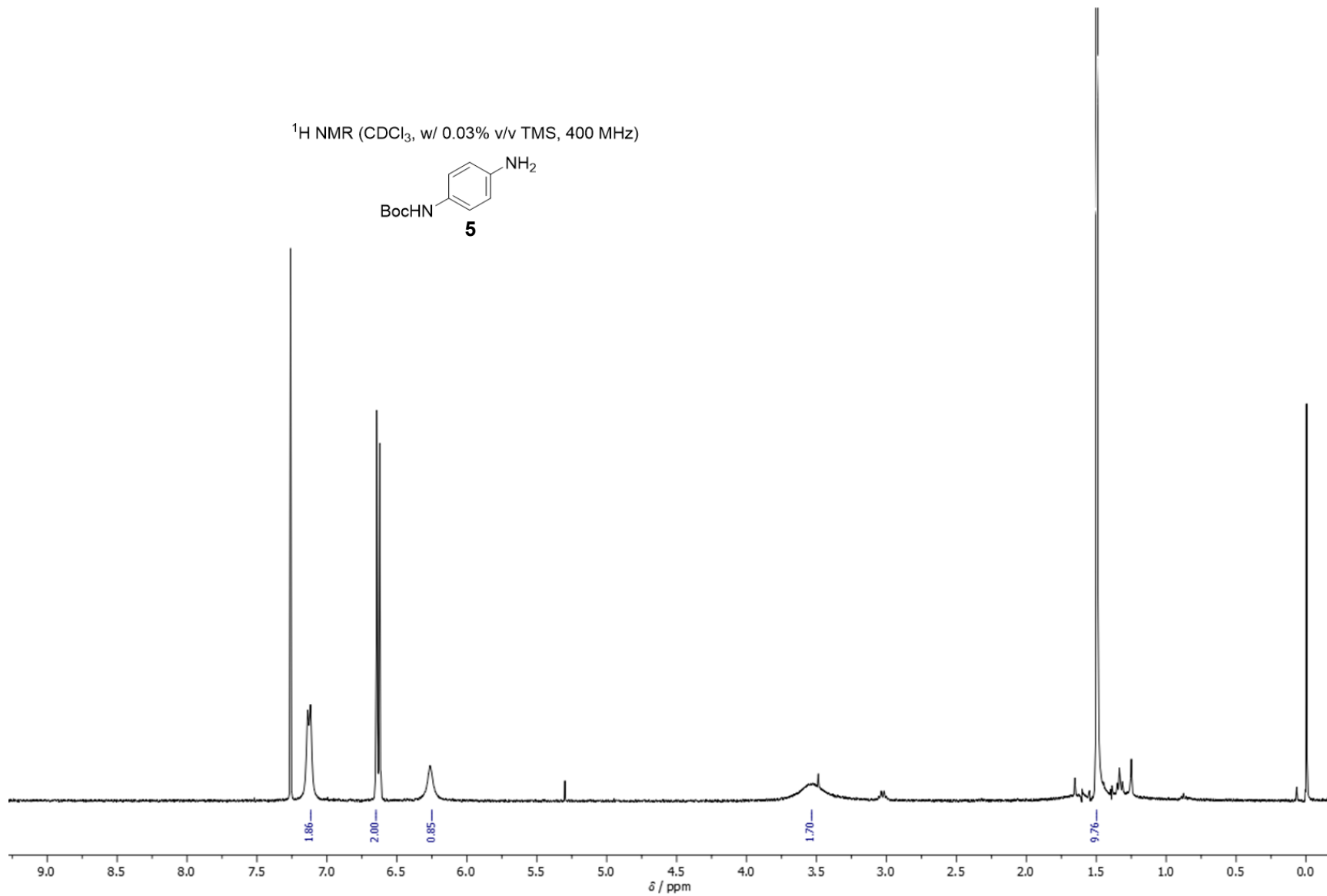
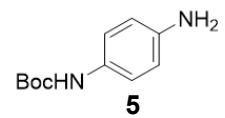


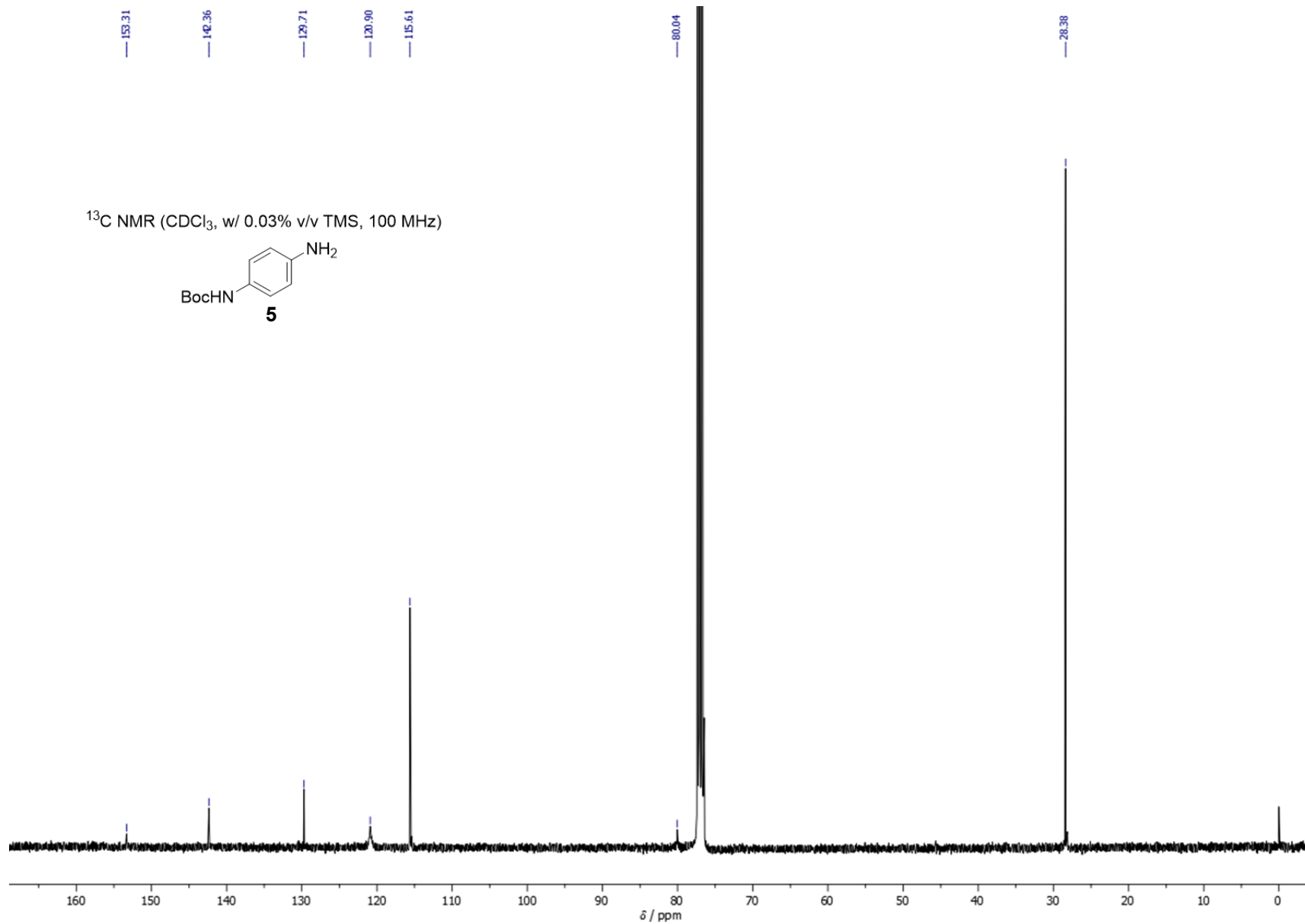
***N*<sup>1</sup>,*N*<sup>4</sup>-bis(4-Guanidinophenyl)succinimide dihydrochloride (10):** Compound **9** (81.1 mg, 0.231 mmol, 2 equiv.) was dissolved in dioxane (0.05 M), and succinyl chloride (12.7  $\mu\text{L}$ , 0.116 mmol, 1 equiv.) was added in dropwise, and the reaction mixture was stirred at room temperature for 16 hours. The crude reaction mixture was concentrated *in vacuo*, and 100 mL of saturated sodium bicarbonate was added, the mixture was then filtered, and subsequently washed with water (100 mL x 3). The resulting brown solid was dissolved in 4M HCl in dioxane (0.1 M) and stirred at room temperature for 16 hours. The crude reaction mixture was concentrated *in vacuo* followed by trituration with DCM to afford **10** (42.7 mg, 41%) as a dark yellow foam.  $^1\text{H}$  NMR (DMSO- $d_6$ , 400 MHz):  $\delta$  10.30 (s, 2H), 9.87 (s, 2H), 7.68 (d,  $J = 8.9$  Hz, 4H), 7.43 (s, 8H), 7.15 (d,  $J = 8.9$  Hz, 4H), 2.67 (s, 4H).  $^{13}\text{C}$  NMR (DMSO- $d_6$ , 100 MHz):  $\delta$  170.54, 156.34, 138.14, 129.51, 125.70, 119.82, 31.28. IR (film)  $\nu_{\text{max}}$  3382, 1670, 1628, 1515, 1405, 1184  $\text{cm}^{-1}$ . HRMS (ESI)  $m/z$   $[\text{M}]^{2+}$ : calcd for  $\text{C}_{18}\text{H}_{24}\text{N}_8\text{O}_2$  192.1006; found: 192.1007.



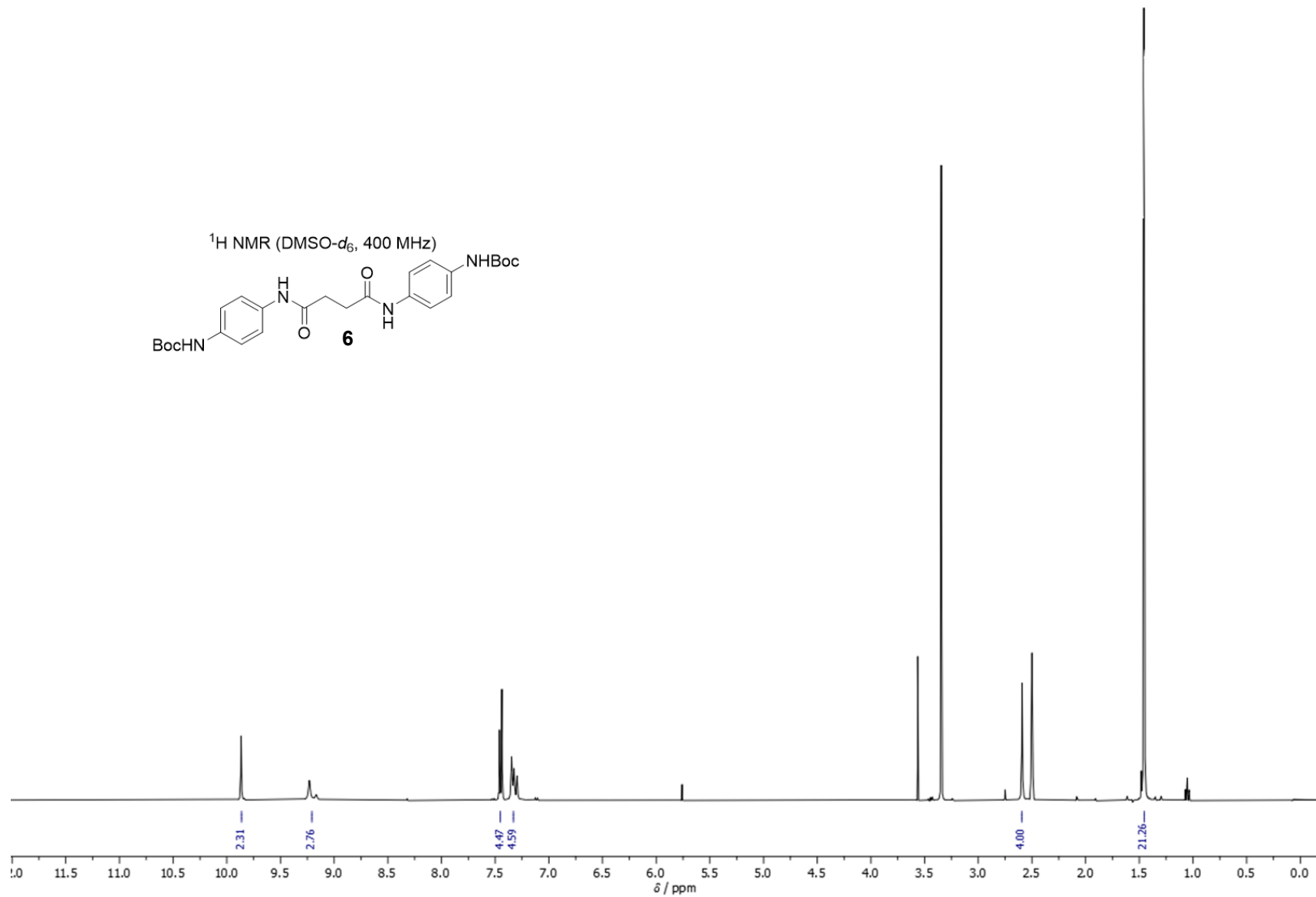
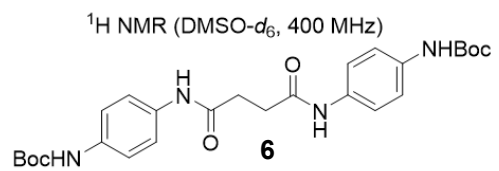


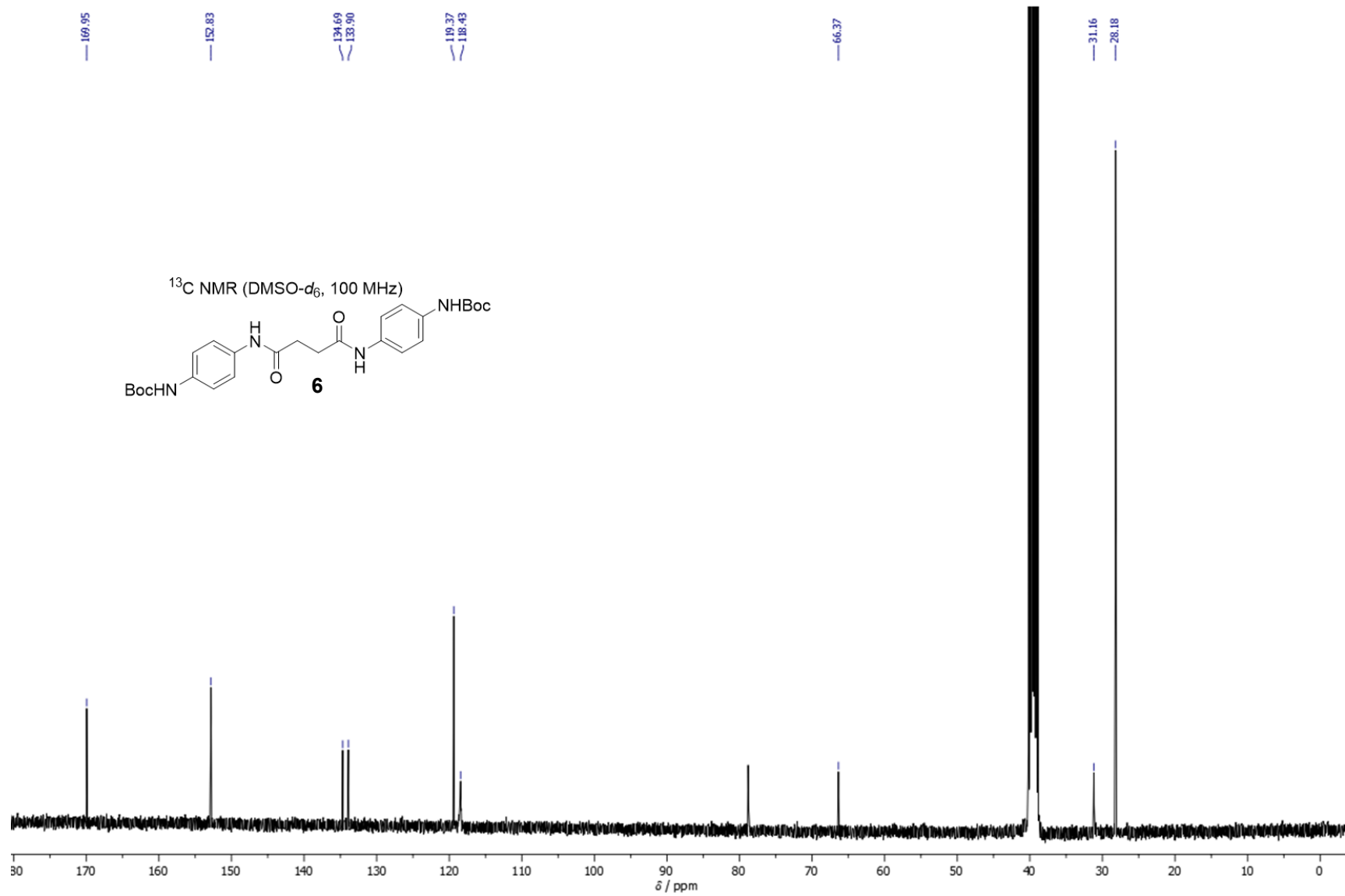
$^1\text{H}$  NMR ( $\text{CDCl}_3$ , w/ 0.03% v/v TMS, 400 MHz)



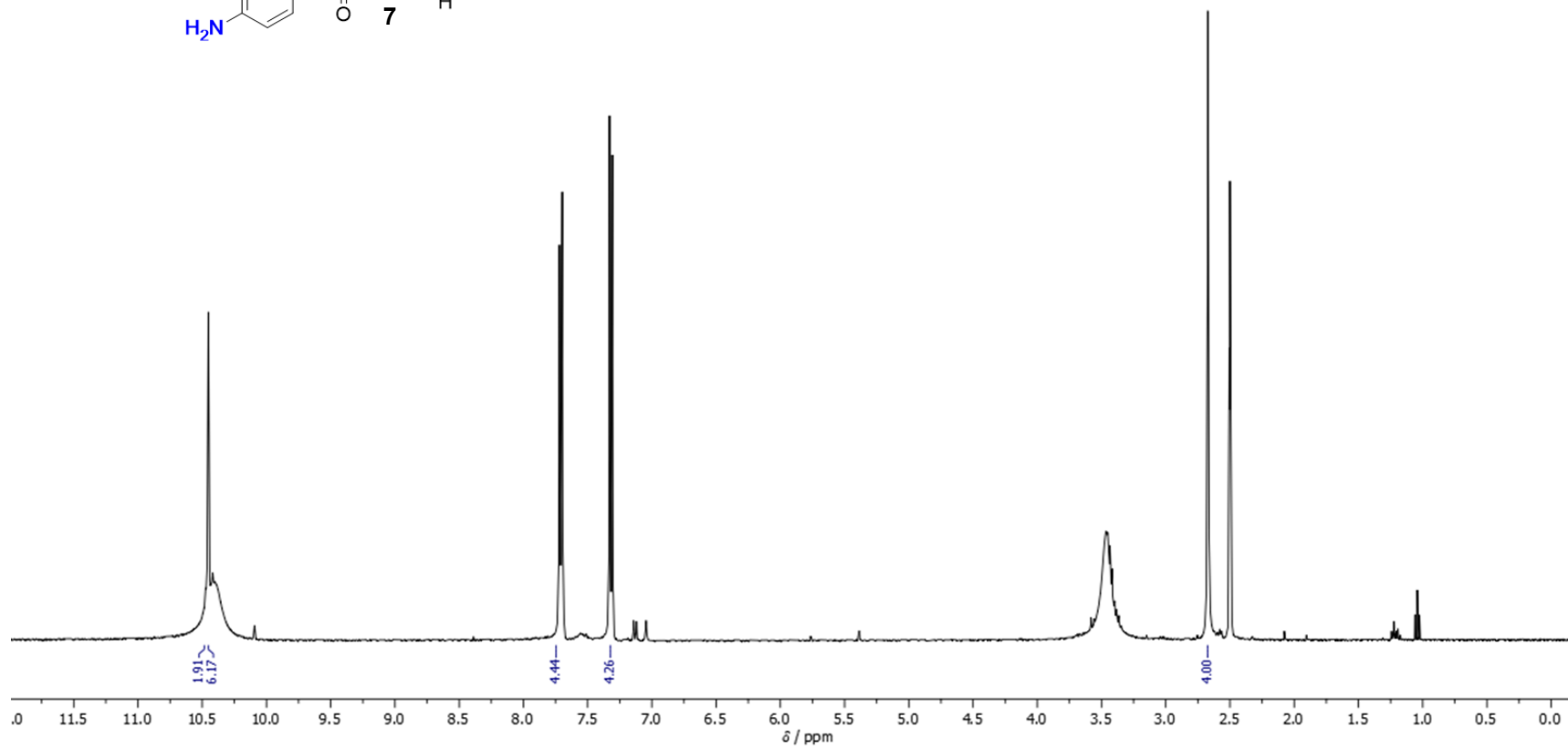
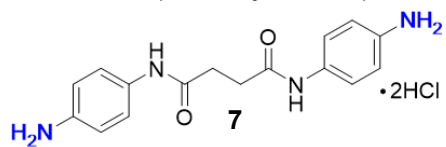


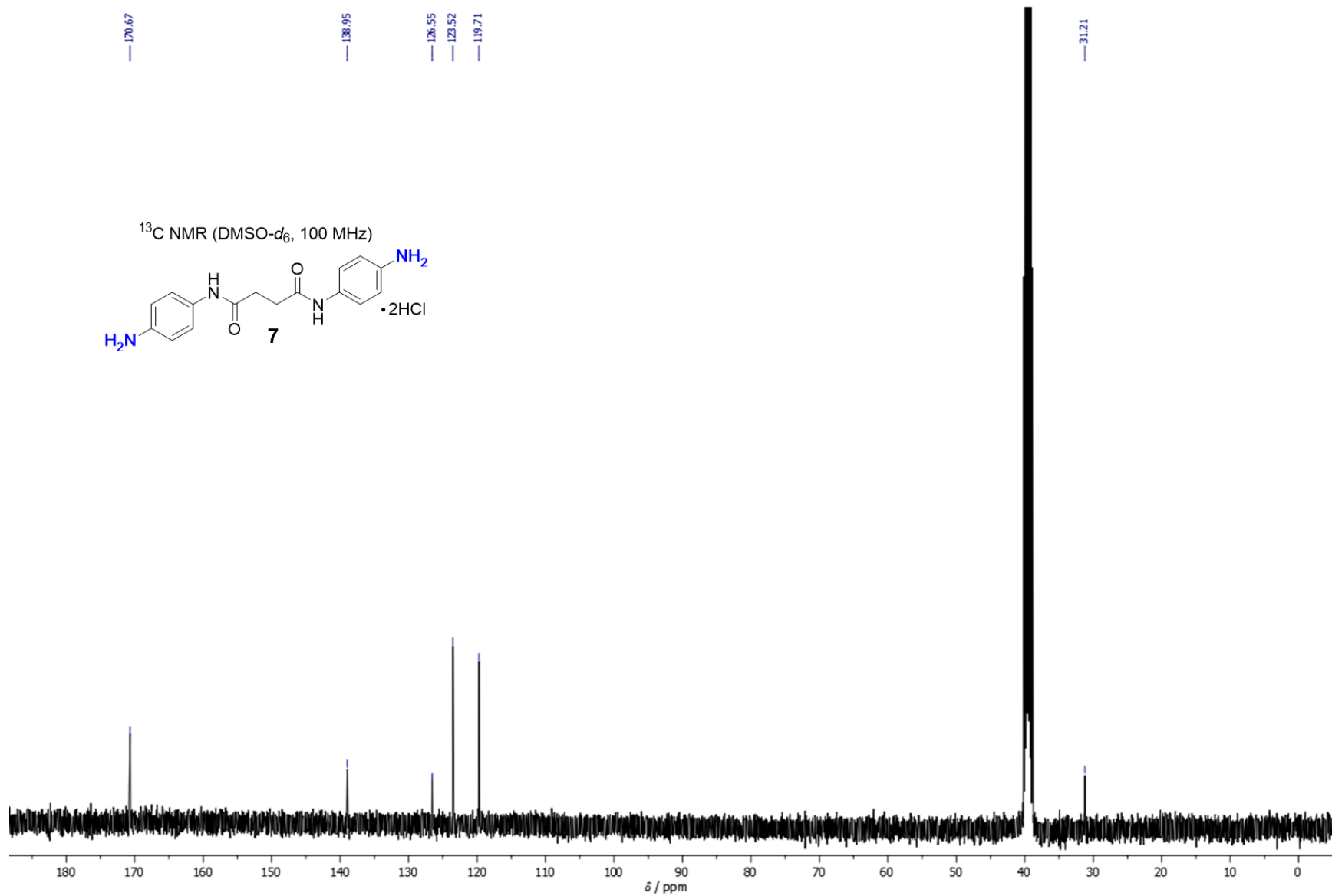


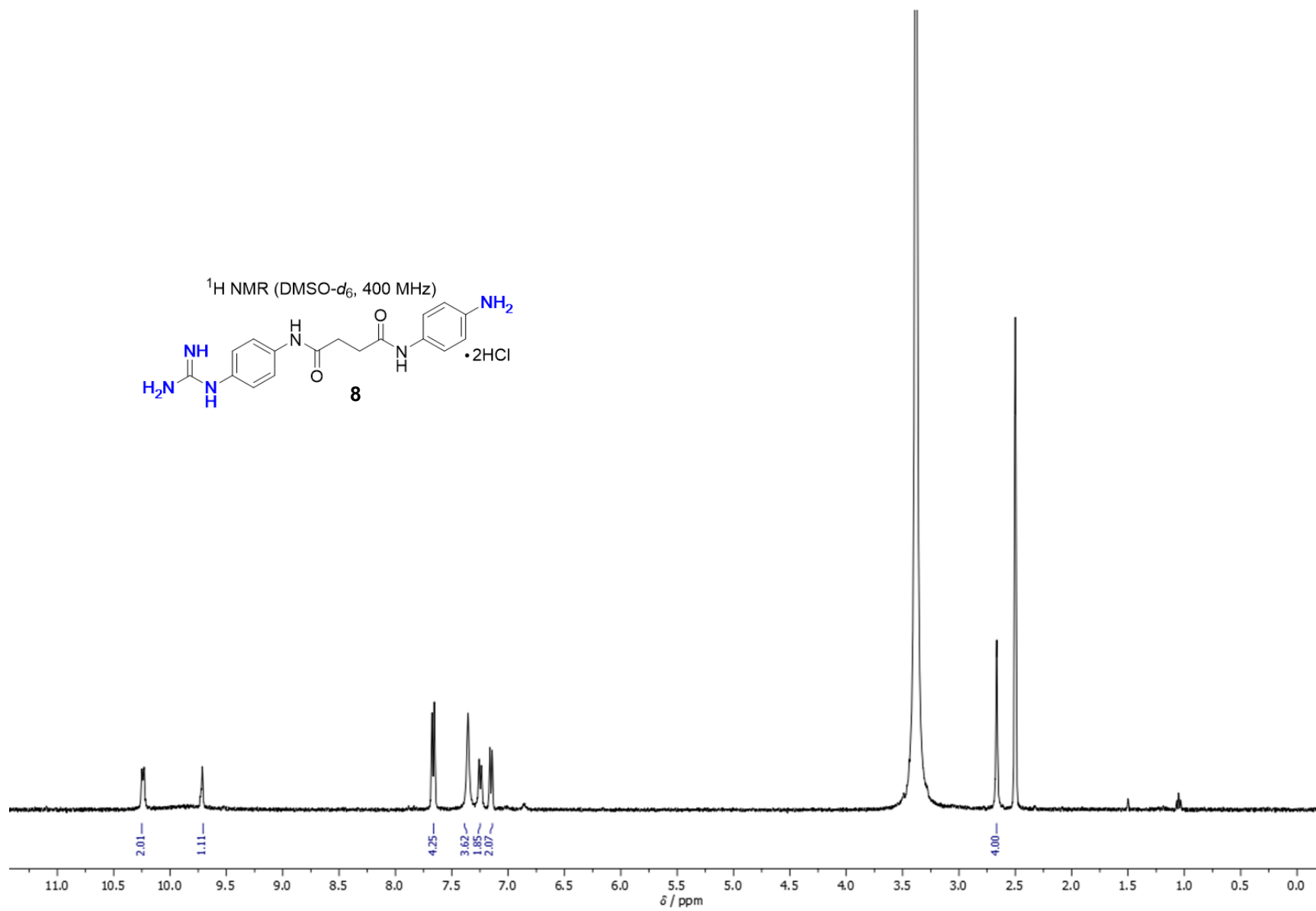


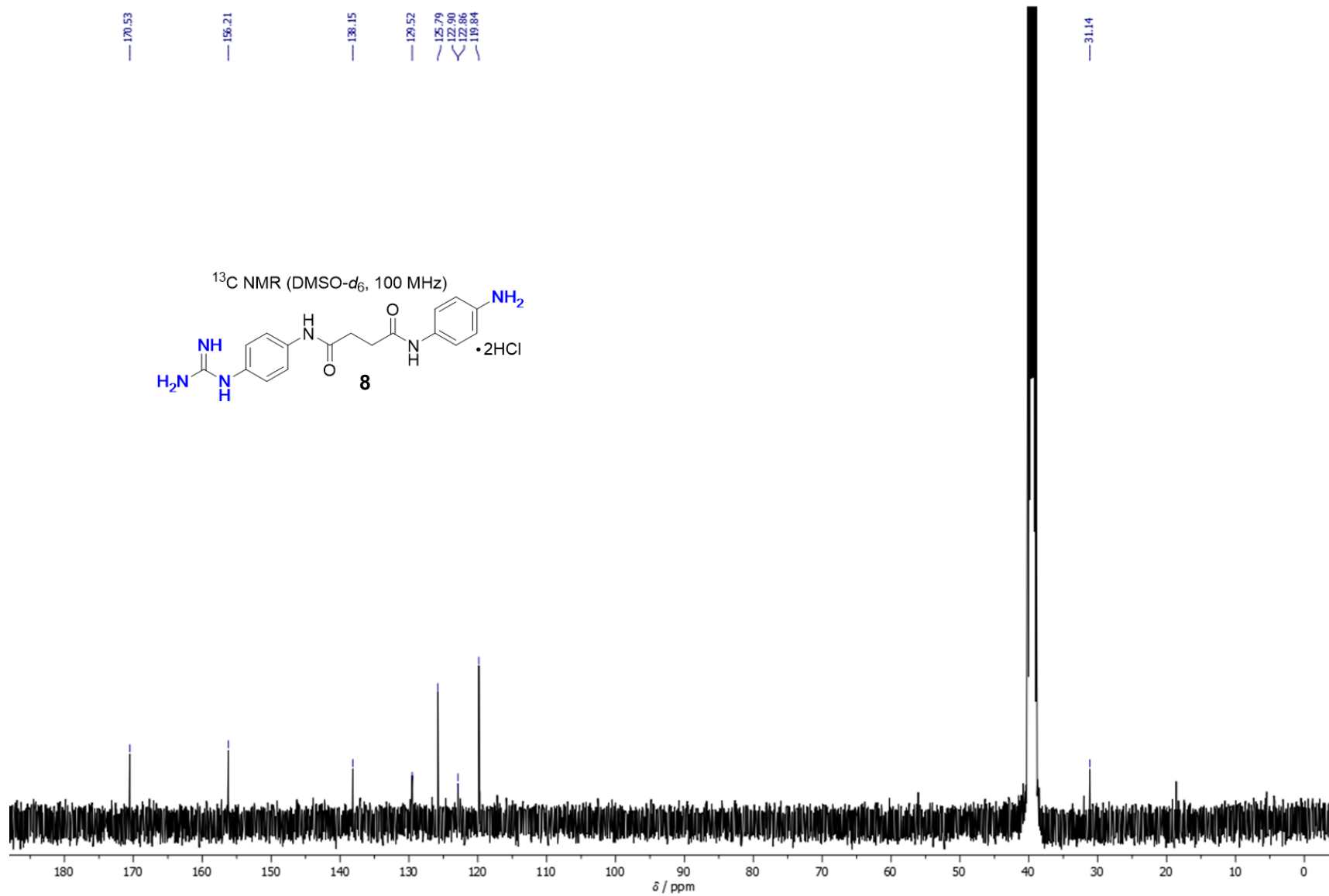


<sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz)

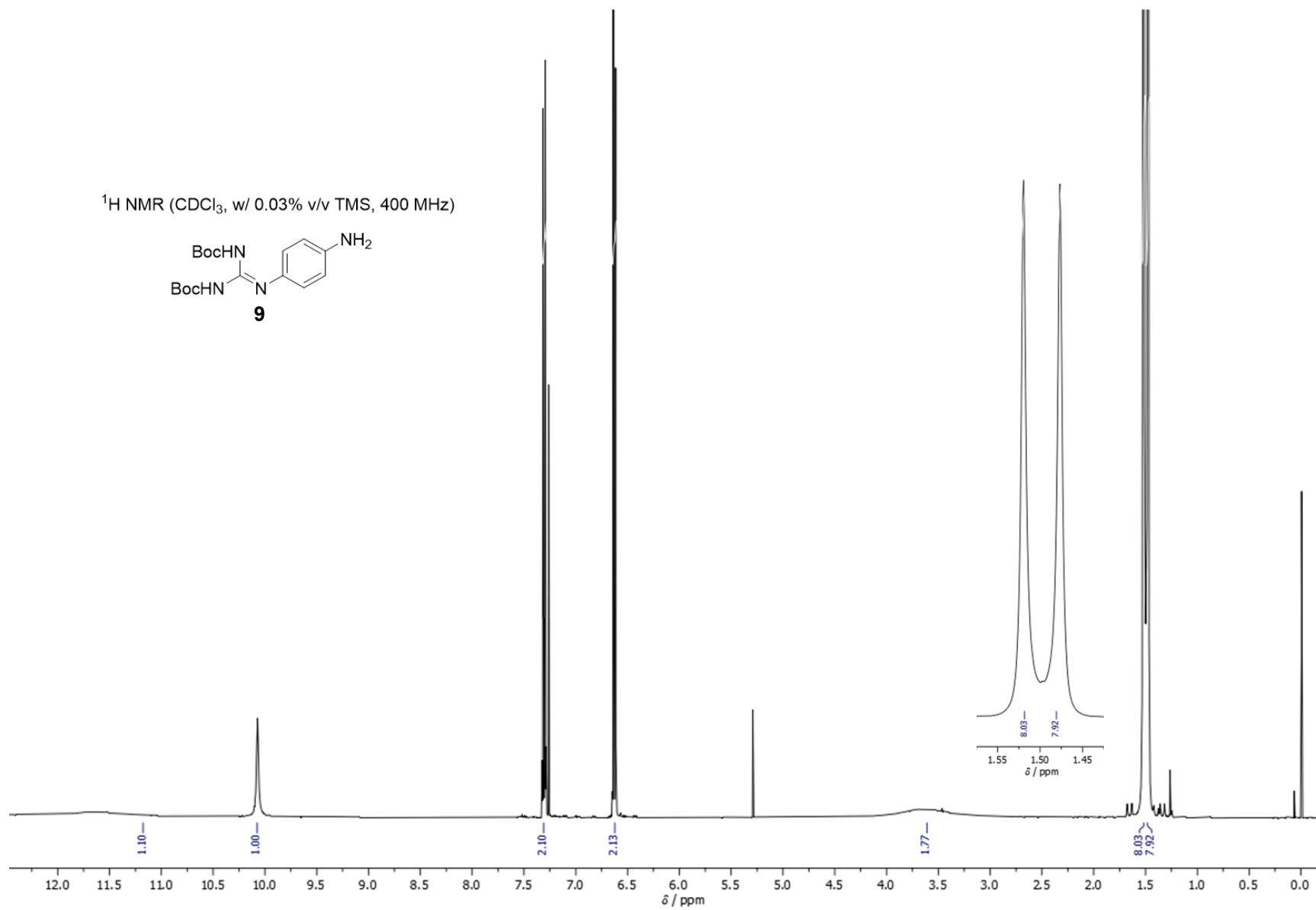
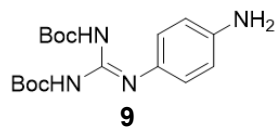


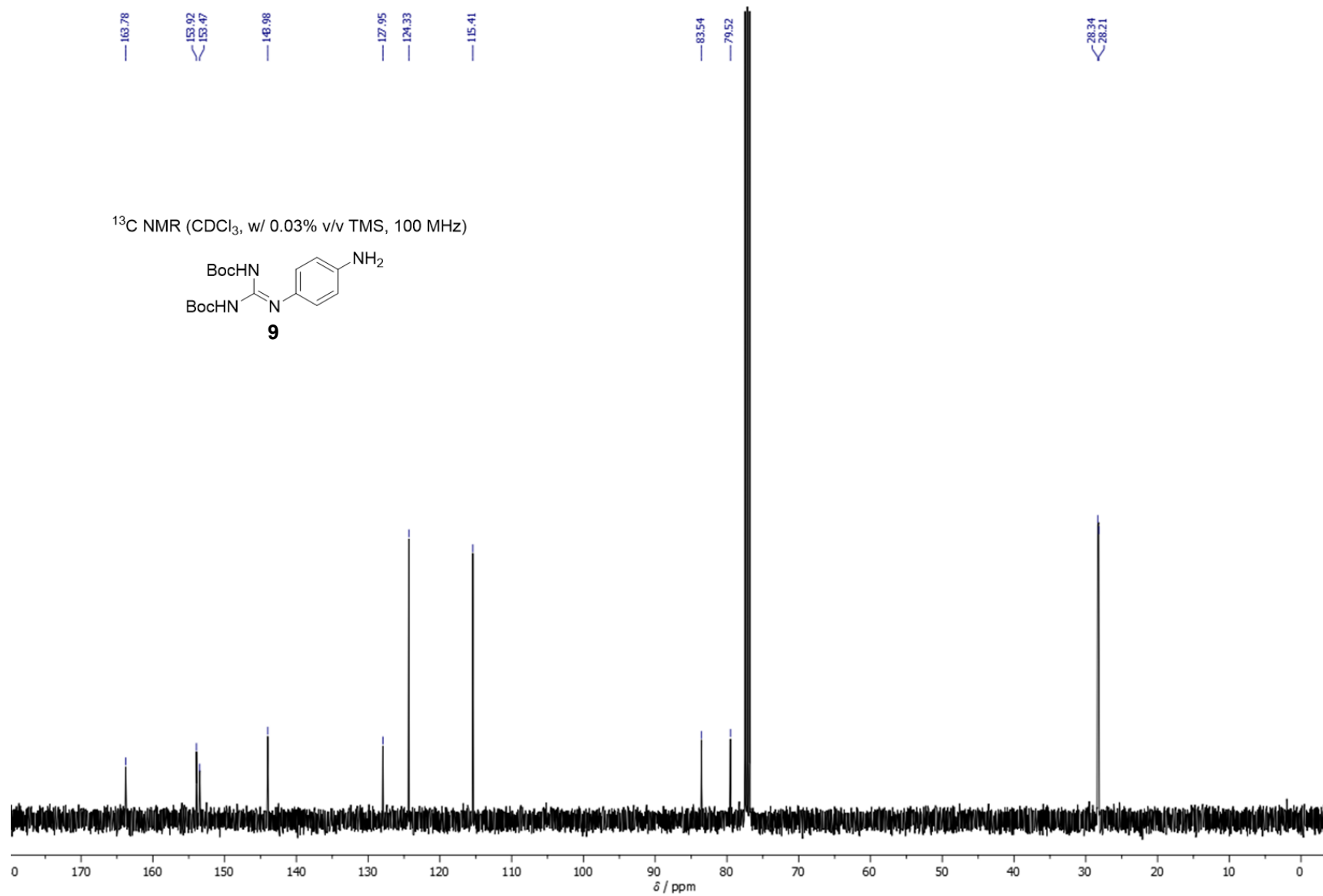




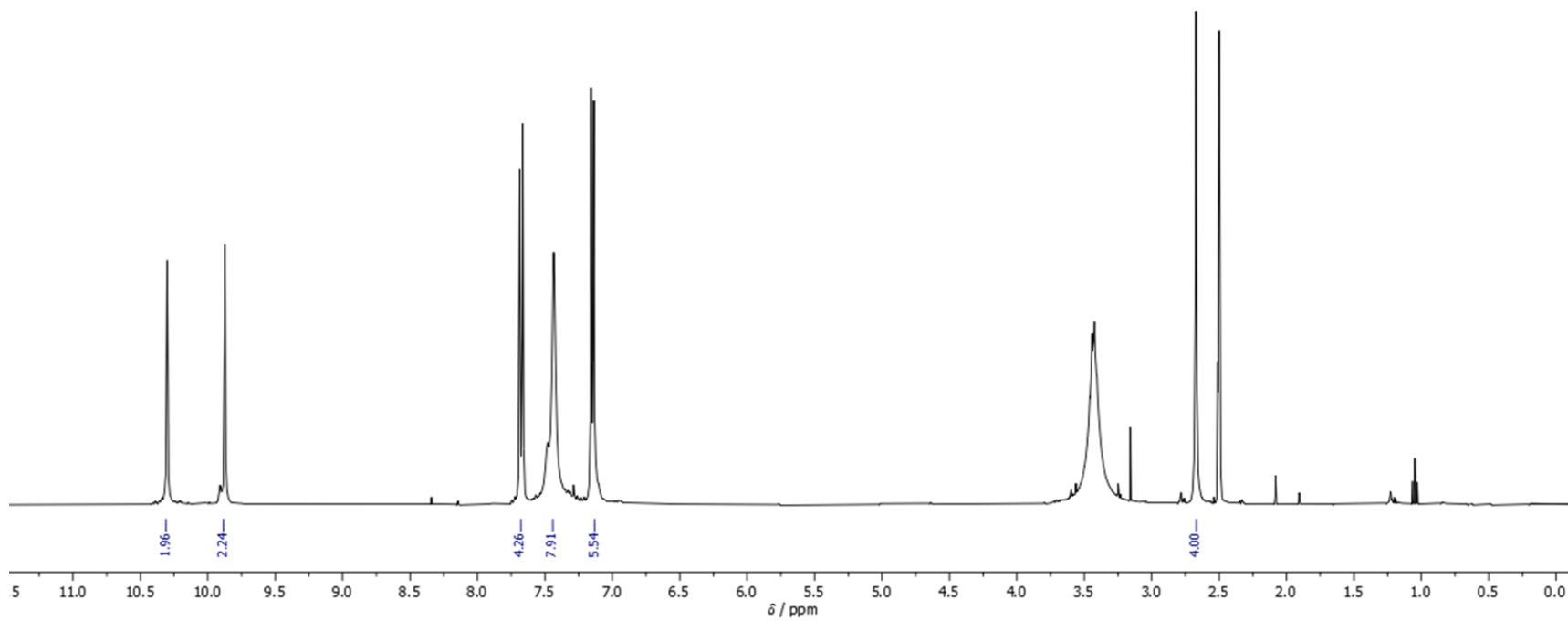
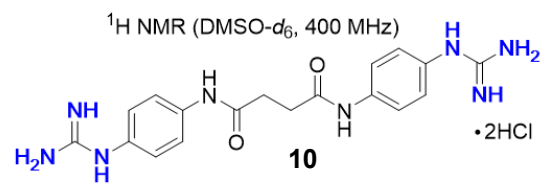


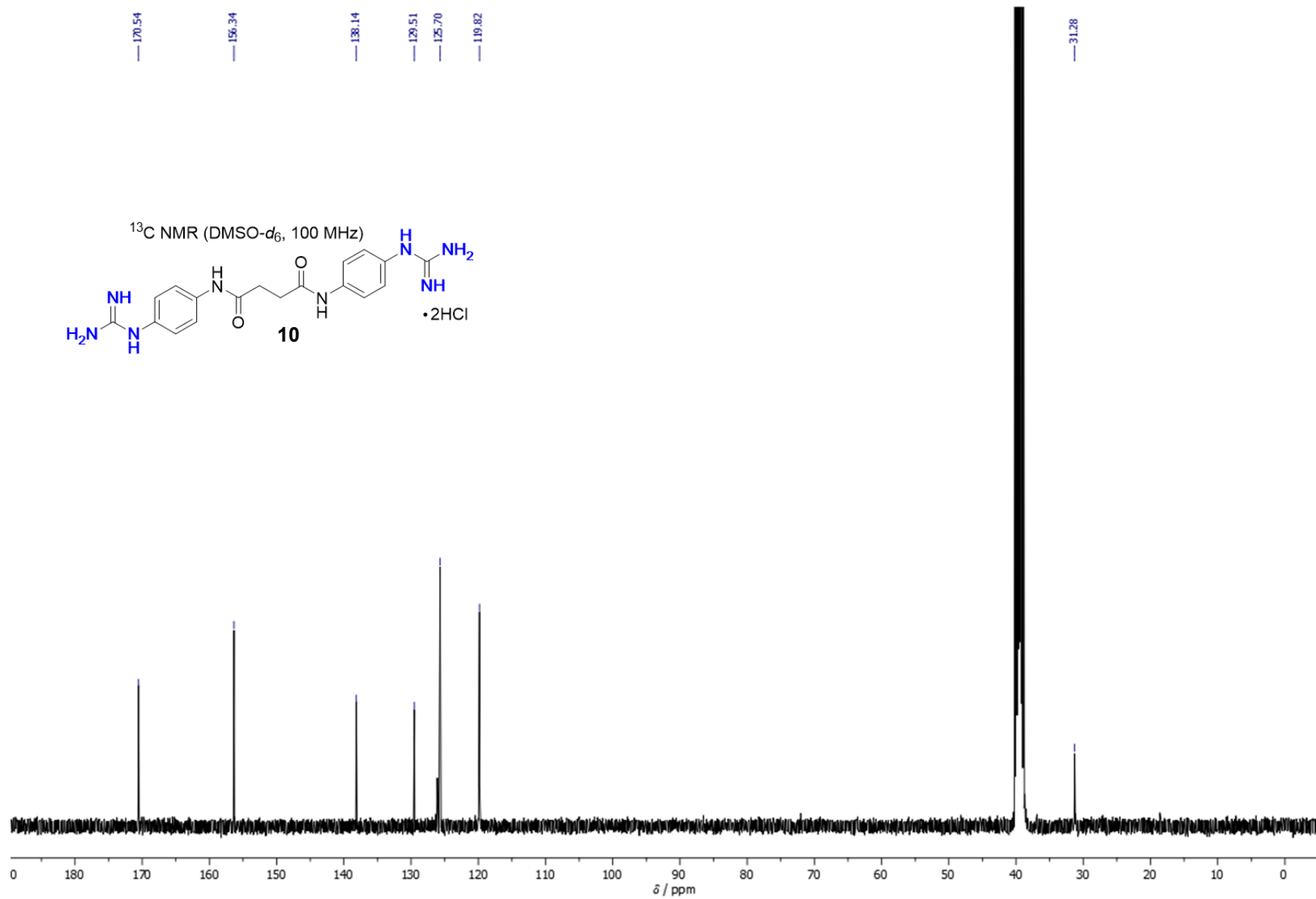
<sup>1</sup>H NMR (CDCl<sub>3</sub>, w/ 0.03% v/v TMS, 400 MHz)









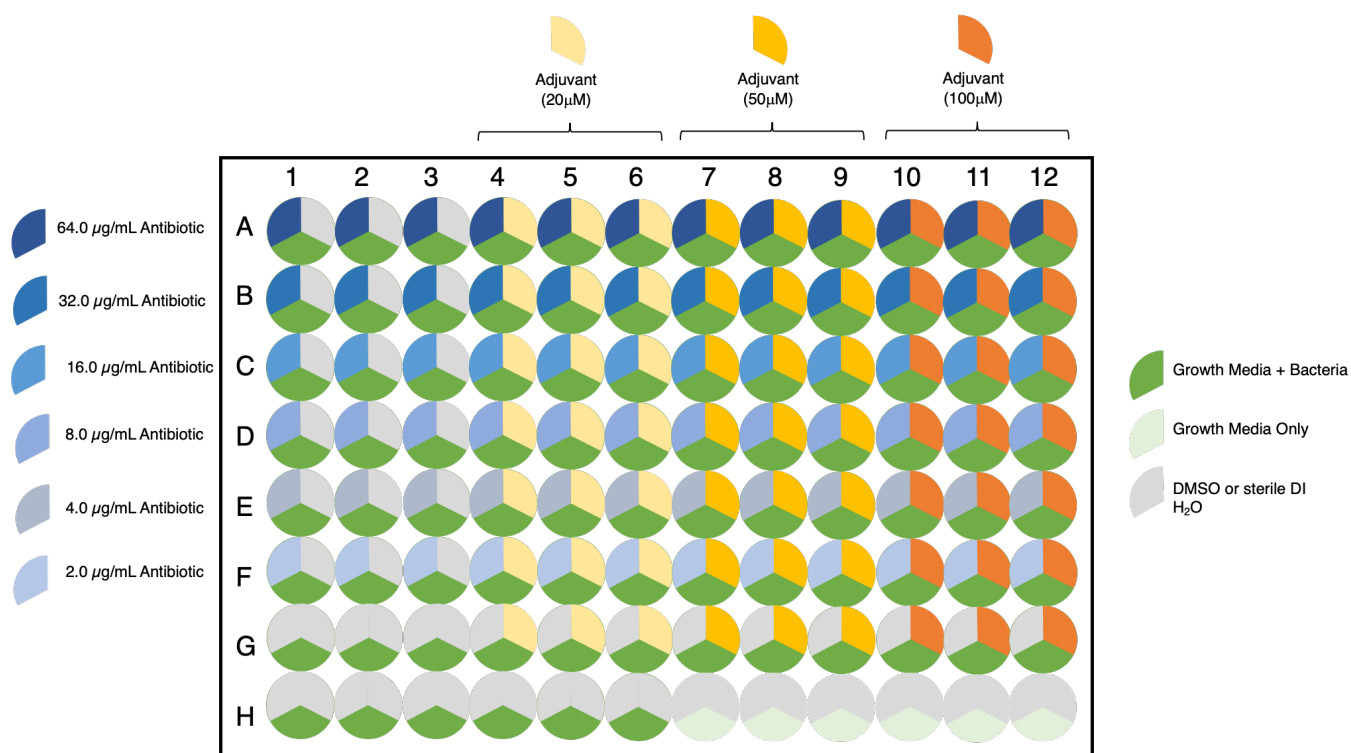


**Biological Evaluation.** All compounds evaluated in biological assays were >95% pure.

*General Sterilization Procedure.* The following are general steps, unless otherwise noted. All steps were completed with aseptic techniques. All media and glassware were sterilized via autoclave at 121 °C for 60 minutes. All agitation occurred at 160 rpm in a temperature-controlled console shaker (Excella E25) at 25 °C. 10% tryptic soy broth (TSB) was made by dissolving 3 g BD Bacto TSB powder in 1L deionized water. Full strength tryptic soy broth (FSTSB) was made by dissolving 30 g BD Bacto TSB powder in 1 L deionized water. 10% tryptic soy agar (TSA) was made by dissolving 3 g BD Bacto TSB powder and 20 g Bacto agar in 1 L deionized water. Purchased bacteria strains used were *Escherichia coli* (EC, ATCC 25922, CLSI control strain for antimicrobial susceptibility testing) and *Pseudomonas aeruginosa* (PA, ATCC 9027, standard efficacy testing strain).

*Antimicrobial Susceptibility Assay Procedure.* Susceptibility testing was performed in biological triplicate, using the micro-dilution broth method as outlined by the Clinical and Laboratory Standards Institute. Briefly, IC<sub>50</sub> determinations (**Table S1**) were carried out in 96-well microtiter plates with 10-fold serial dilutions of the compounds in duplicate. Briefly, to each well 1 µL of compound in DMSO, 89 µL of tryptic soy broth (BD Difco), and 10 µL of bacterium inoculum in tryptic soy broth were added. After incubation for 12–15 h at 37 °C, absorbance at 590 nm was read on a Biotek Synergy HTX Multi-mode plate reader. IC<sub>50</sub> values were then determined by plotting the concentration versus absorbance on Excel and using the trend function to determine the concentration at 50% inhibition. Bacteria strains used were *Escherichia coli* (ATCC 15022) and *Pseudomonas aeruginosa* (ATCC 9027).

*Adjuvant MIC Assay Procedure.* Growth inhibition was determined by broth microdilution according to the CLSI M100-S23 guidelines. Master plates were prepared for both adjuvants and antibiotics. Antibiotics spanned from concentrations of 0 µg/mL to 256 µg/mL in DMSO or sterile DI H<sub>2</sub>O. Adjuvants were tested at 20, 50, and 100 µM in DMSO. Liquid cultures of bacteria were grown for 4-6 hours by inoculating a single bacterial culture into 10 mL FSTSB. In each well of rows A-G, 178 µL full-strength TSB, 20 µL bacteria culture, and 1 µL from each master plate was added (**Figure S1**). Vehicle (DMSO or sterile DI H<sub>2</sub>O) controls (H1-H6), media blanks (H7-H12), antibiotic controls (A1-3 through F1-3) and adjuvant controls (G4-G12) were included in each assay, and each treatment was tested in triplicate. Plates were covered and incubated at 37 °C for 12-18 h. Optical density (OD<sub>590</sub>) was measured on a Biotek Synergy HTX-Multimode plate readers, and data was processed by background subtracting the media absorbance (H7-H12) and then normalizing the data to full bacteria growth with only vehicle (H1-H6). MIC is defined as the lowest concentration of antibiotic or antibiotic/adjuvant combination that achieves ≥ 90% growth inhibition.



**SI Figure 1.** Example adjuvant assay plate layout. Some antibiotics were tested up to 256 µg/mL.

#### *Accumulation Assay Procedure.*

The accumulation assay was performed as described in Perlmutter et al.<sup>8</sup> with minor modifications. Briefly, 16-hour overnight cultures of *P. aeruginosa* and *E. coli* were diluted 1:100 into 250 mL of fresh Tryptic Soy Broth (TSB) for *P. aeruginosa* or 250 mL Luria Bertani (LB) for *E. coli*. Subcultures were grown at 37 °C with shaking to an OD<sub>600</sub> of 0.55. Cultures were pelleted by centrifugation at 3,200 r.c.f. for 10 min at 4 °C and resuspended in 40 mL phosphate buffered saline (PBS). Pelleting was repeated, and bacteria were resuspended into a final volume of 8.8 mL PBS, which was used to create ten 875 µL aliquots in microfuge tubes. Colony forming units (CFUs) were determined at this point by serial dilution and plating for viable counts. The aliquots were incubated with shaking at 37 °C for 10 minutes. Following this, each compound was added at a final concentration of 50 µM, followed by shaking and incubation for 10 minutes at 37 °C. Serial dilution and viable count plating were repeated following incubation. No reduction in CFU was observed for any of the test compounds or ampicillin. The ciprofloxacin control resulted in a 1-log reduction in CFU for *E. coli* and a 4-log reduction for *P. aeruginosa*; however, OD<sub>600</sub> was unchanged for both, suggesting that cells remained intact. Following incubation, 800 µL of the samples were layered onto 700 µL of cold silicone oil (AR20/Sigma High Temperature, cooled to -80 °C) and centrifuged at room temperature at 13,000 r.c.f. for 2 minutes. The supernatant and oil were discarded carefully. Pellets were washed by dissolving in 500 µL PBS, which was layered onto another 700 µL of cold silicon oil and pelleted as before. After removing the oil and PBS, the pellet was dissolved in 200 µL of water and lysed via three cycles of 3 minutes in liquid nitrogen followed by 3 minutes in a 65 °C water bath. The lysates were then pelleted at 21,000 r.c.f. for 5 minutes at room temperature and supernatants collected (~180 µL for *E. coli*, and ~50 µL for *P. aeruginosa*, which forms a loose mucilaginous pellet). Pellets were resuspended in 100 µL methanol via vortexing, and the centrifugation step was repeated as before. Supernatants were combined with the original supernatants, then centrifuged at room temperature for 10 minutes at 21,000 r.c.f. Final supernatants were collected for LCMS.

Samples were analyzed with a single quadrupole Shimadzu LCMS-2020 system using LabSolutions version 5.99 SP2. Samples of **4**, **7**, **8**, **10**, and ciprofloxacin were subject to direct injection with a 1:1 ratio of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in methanol) at a flow rate of 0.2 mL/min for 4 minutes. The injection volume was 10  $\mu$ L. Samples for ampicillin were subject to injection through a Restek Ultra C18 3 $\mu$ m, 30 x 2.1mm column at a flow rate of 0.1 mL/min using a gradient of 0% mobile phase B to 100% mobile phase B at 9 minutes, followed by 0% mobile phase A until minute 15. Ampicillin peak monitored eluted at 8.6 min. The injection volume for ampicillin was 2  $\mu$ L. Mass spectra for **4**, **7**, **8**, **10**, and ciprofloxacin were acquired with the following parameters: positive electrospray ionization (ESI) with a voltage of 4.5 kV; detector voltage of 1.15 kV; nebulizing gas flow 1.5 mL/min; heat block temperature 200  $^{\circ}$ C; DL temperature 250  $^{\circ}$ C. Ampicillin mass spectra were acquired with similar parameters, using negative ESI with a voltage of -4.5kV. Single ion monitoring (SIM) of  $[M+H]^+$ ,  $[M-H]^-$ , or  $[M+2H]^{2+}$  (**SI Table 1**) were used to quantify metabolites based on the calibration curves generated for compounds **4**, **7**, **8**, **10**, and ciprofloxacin as a positive control (see **SI Figure 2**) and ampicillin as a negative control (see **SI Figure 3**).

<b>Compound</b>	<b>SIM m/z Monitored</b>	<b>Ionization Mode</b>
<b>7</b>	299	$[M+H]^+$
<b>4</b>	177	$[M+2H]^{2+}$
<b>8</b>	171	$[M+2H]^{2+}$
<b>10</b>	192	$[M+2H]^{2+}$
<b>Ciprofloxacin</b>	332	$[M+H]^+$
<b>Ampicillin</b>	348	$[M-H]^-$

**SI Table 1.** SIM mass table for **4**, **7**, **8**, **10**, ciprofloxacin, and ampicillin.

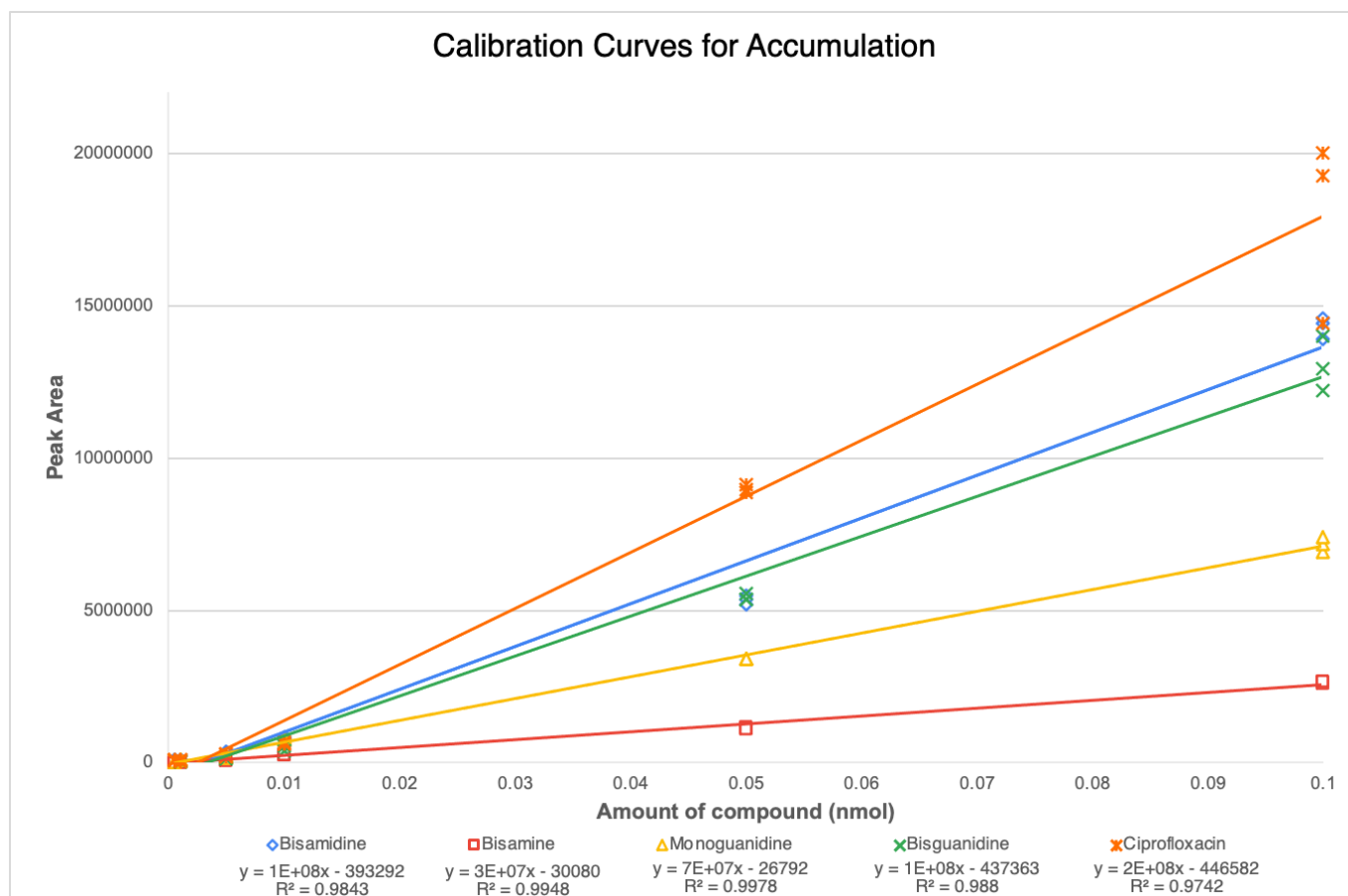
**Biological Data.***Antimicrobial Susceptibility Data*

<b>Compound</b>	<b>IC<sub>50</sub> (µg/mL)</b>	
	<b><i>E. coli</i></b>	<b><i>P. aeruginosa</i></b>
Bisamine (7)	>1000	>1000
Bisamidine (4)	>1000	>1000
Aminoguanidine (8)	>1000	>1000
Bisguanidine <sup>a</sup> (10)	>1000	>1000
Chloramphenicol	0.18 ± 0.1	4 ± 1.5

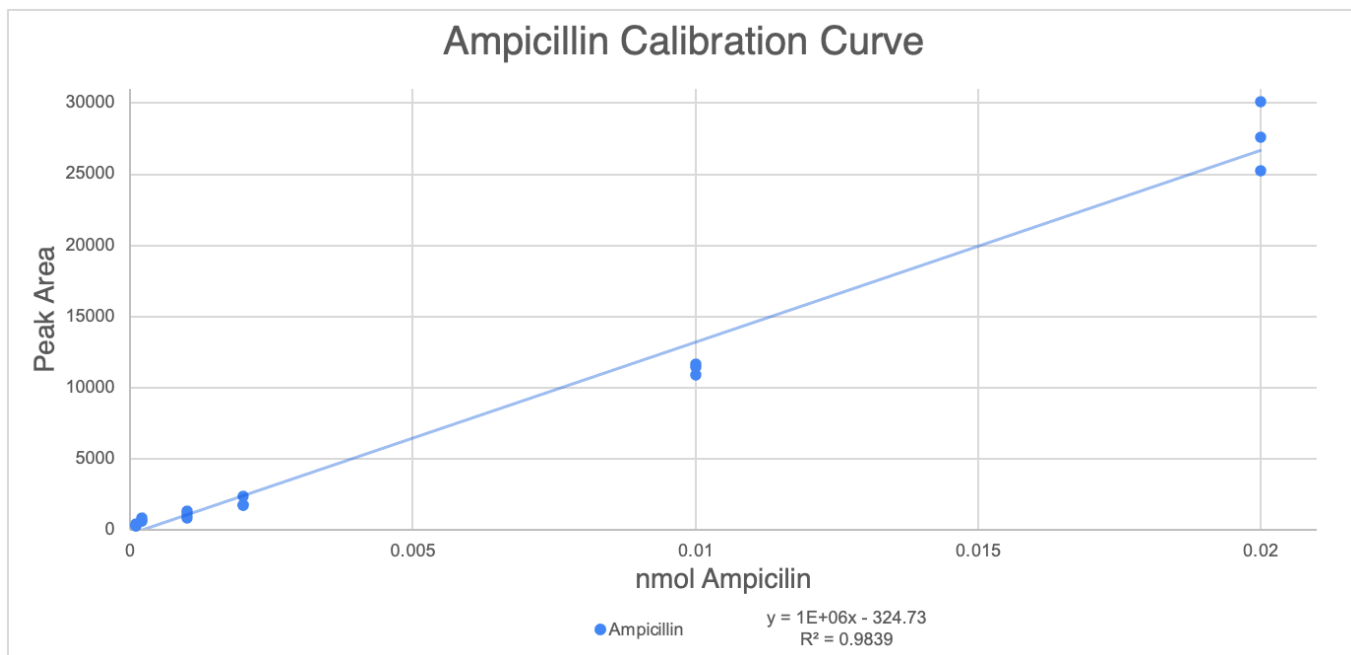
<sup>a</sup>Bisguanidine showed ~18% growth inhibition of EC at 100 µg/mL and ~33% growth inhibition of PA at 1000 µg/mL

**SI Table 2.** IC<sub>50</sub> data for adjuvants **4, 7, 8, 10**, and chloramphenicol, (n = 3).

Accumulation Assay Calibration Curves



SI Figure 2. Calibration curves for ciprofloxacin, 4, 7, 8, and 10, n > 3.



**SI Figure 3.** Calibration curve for ampicillin (n = 3).