

## Supporting Information

### Identification of a Dual Autophagy and REV-ERB Inhibitor with in Vivo Anticancer Efficacy

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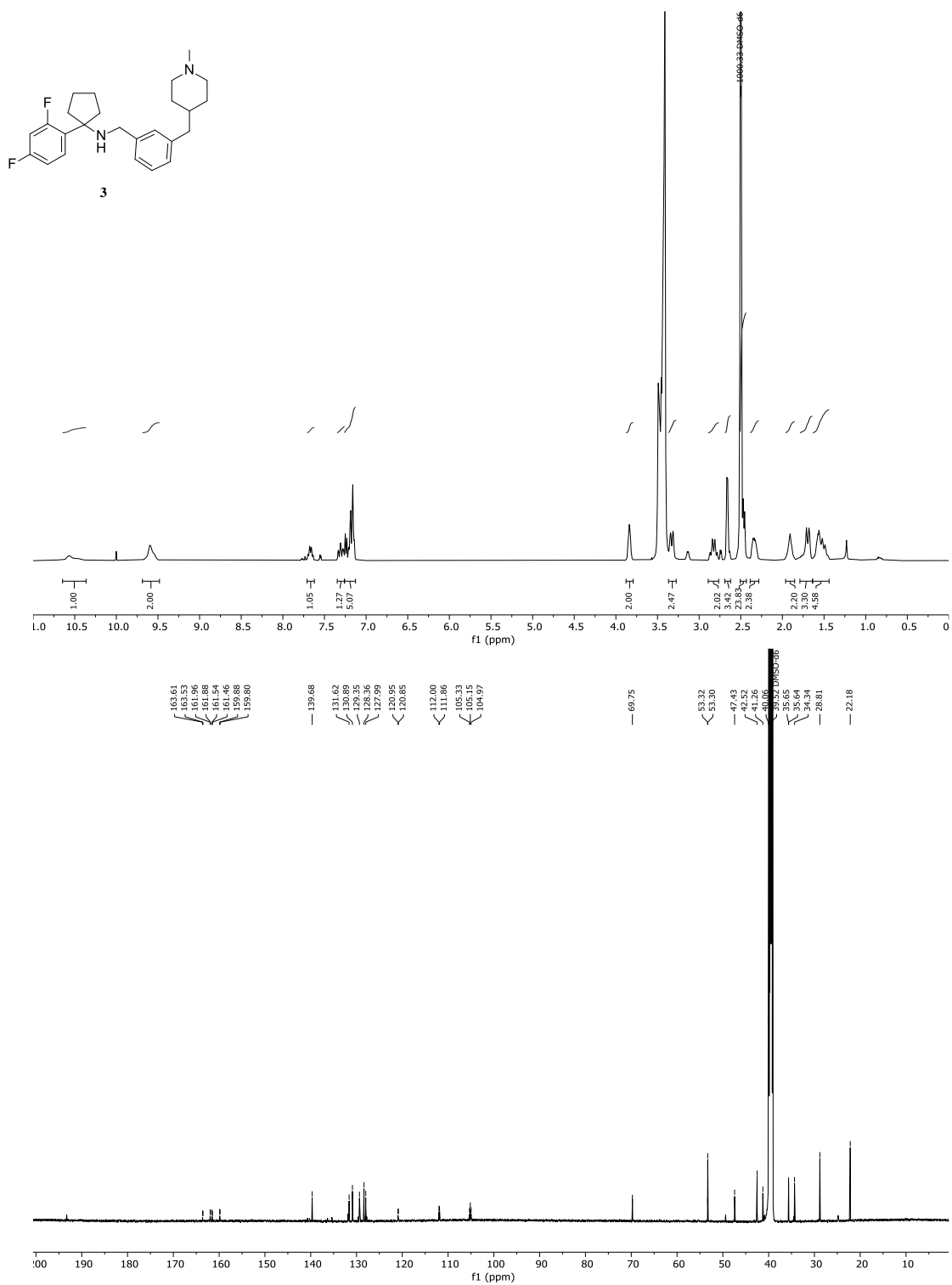
benedetto.grimaldi@iit.it

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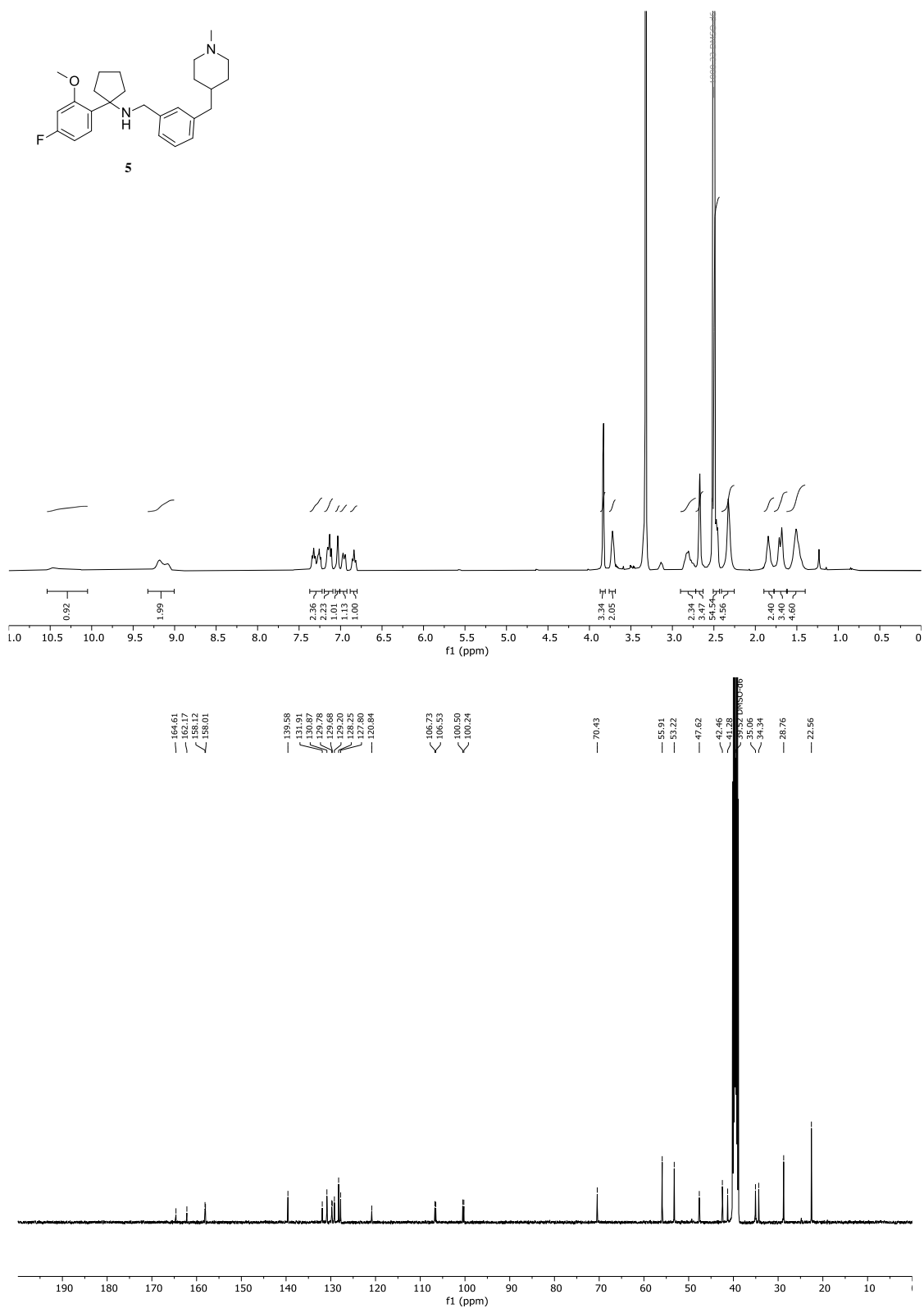
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Compound 3:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )

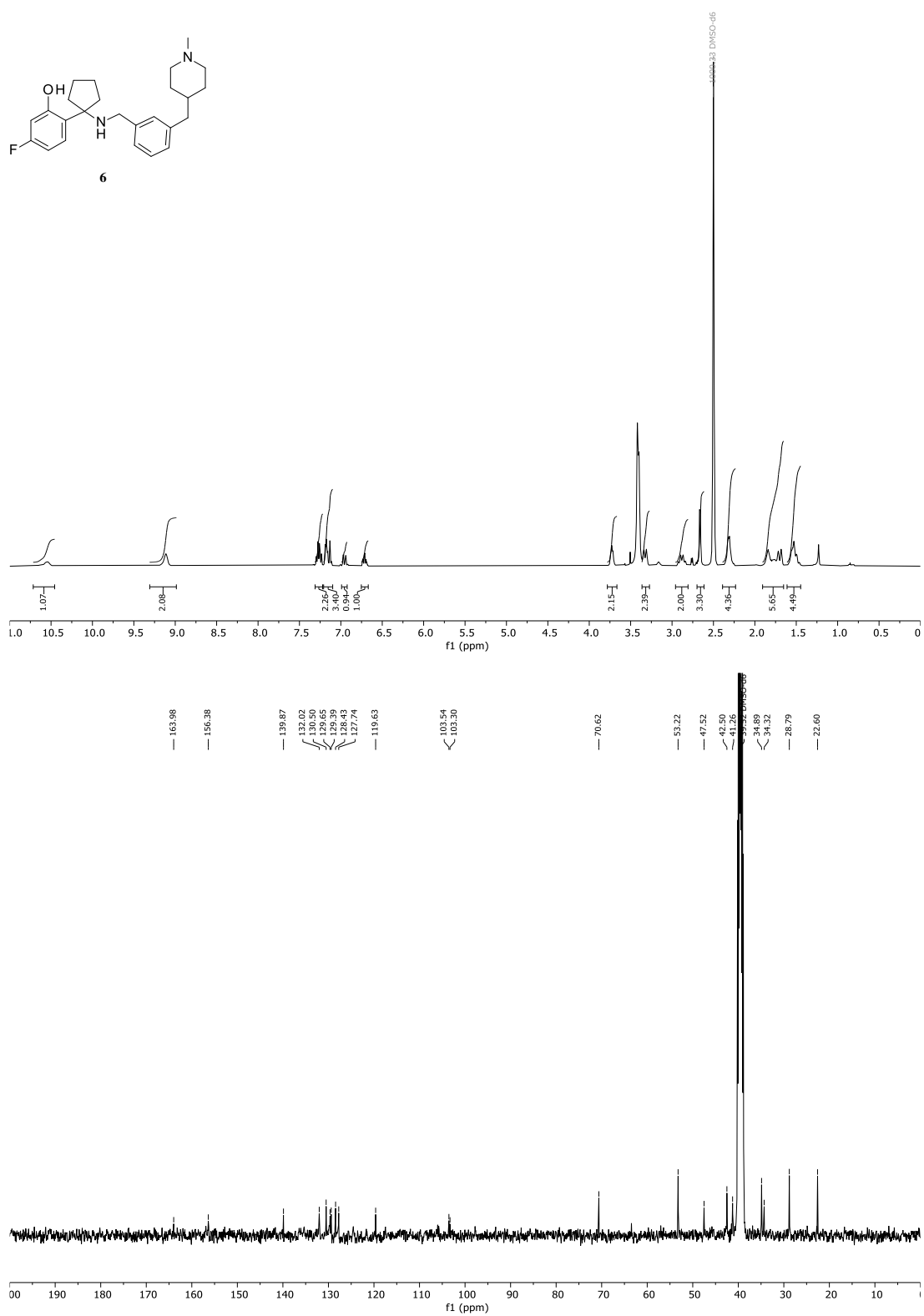




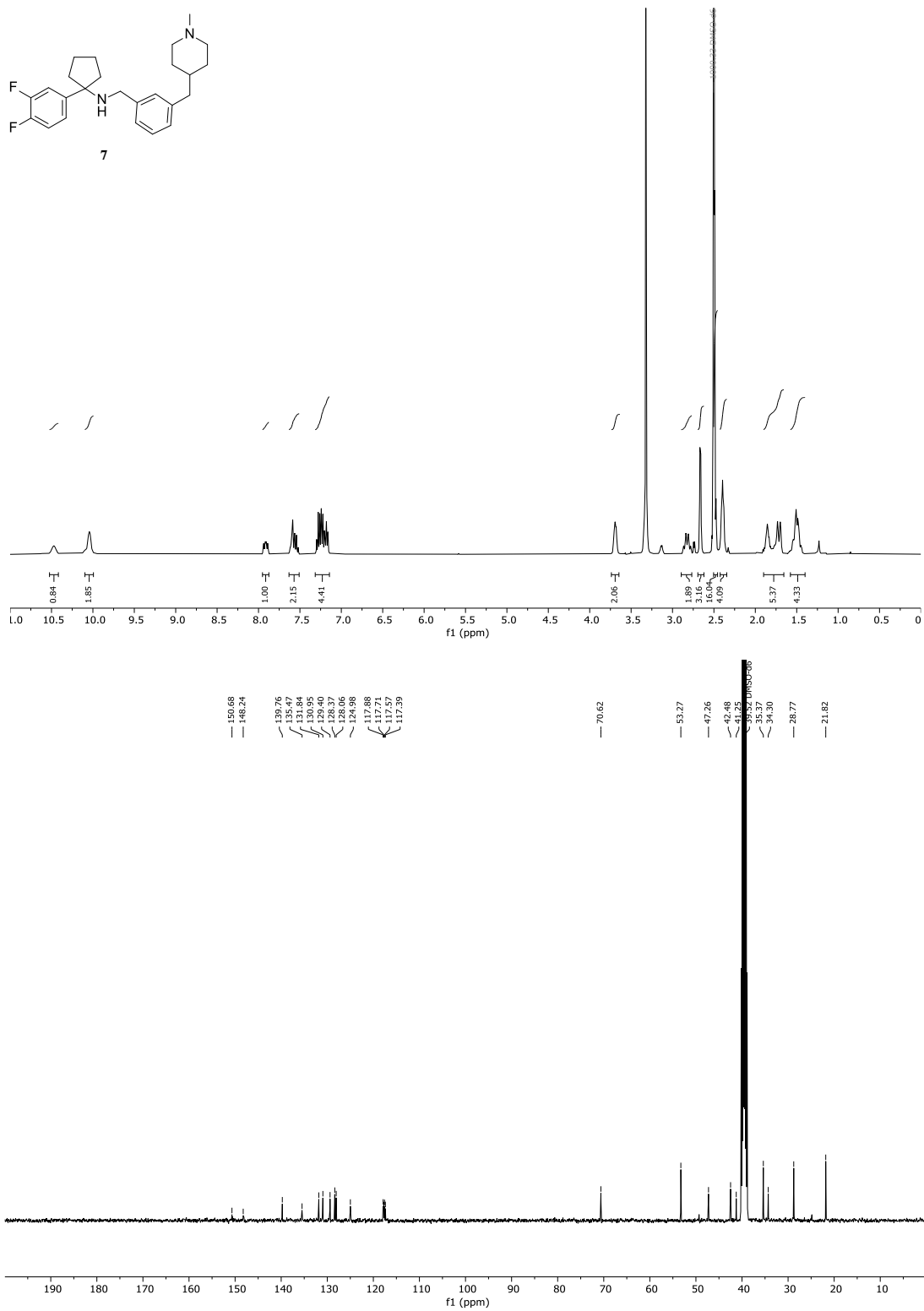
Compound **5**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )



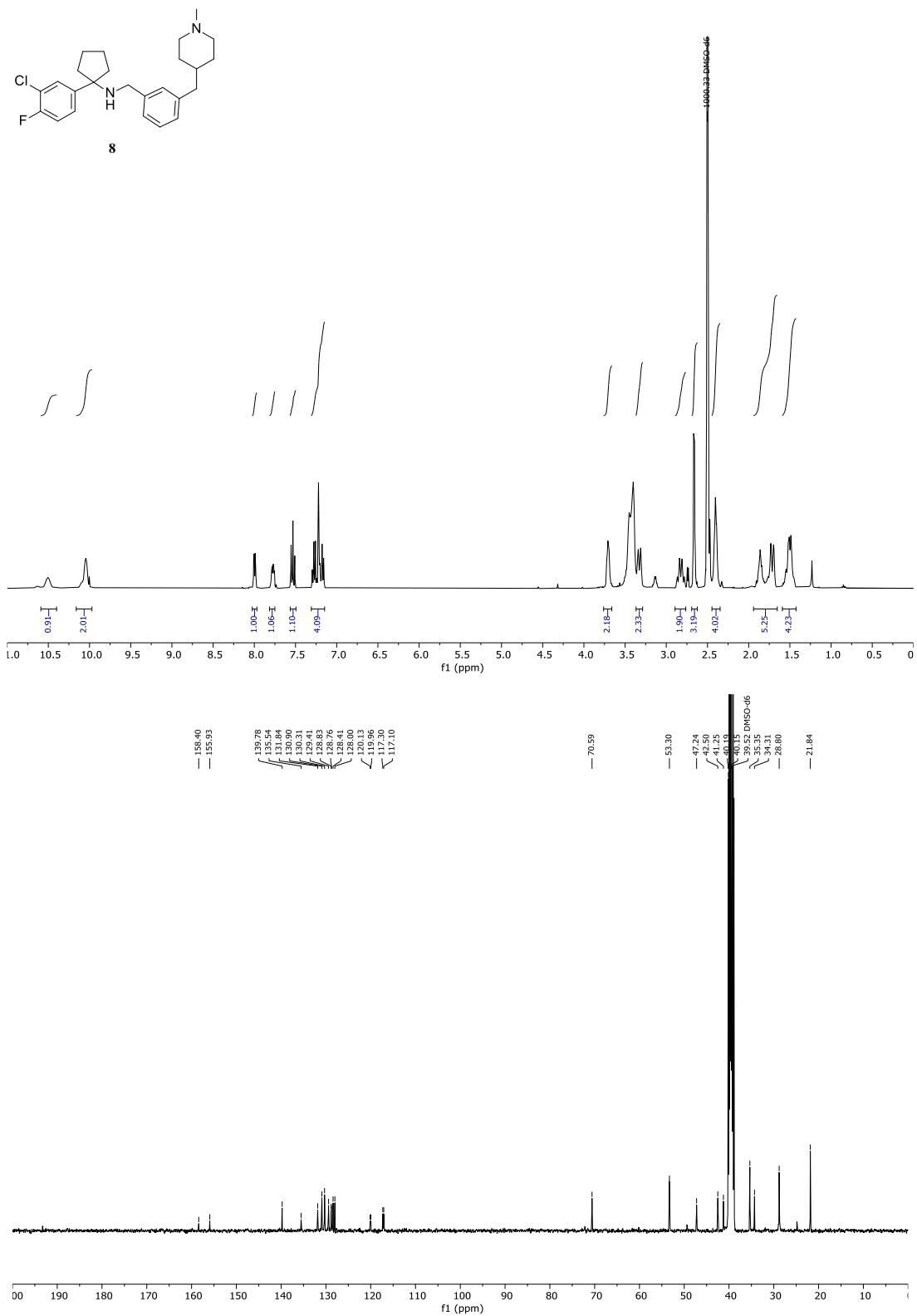
Compound **6**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )



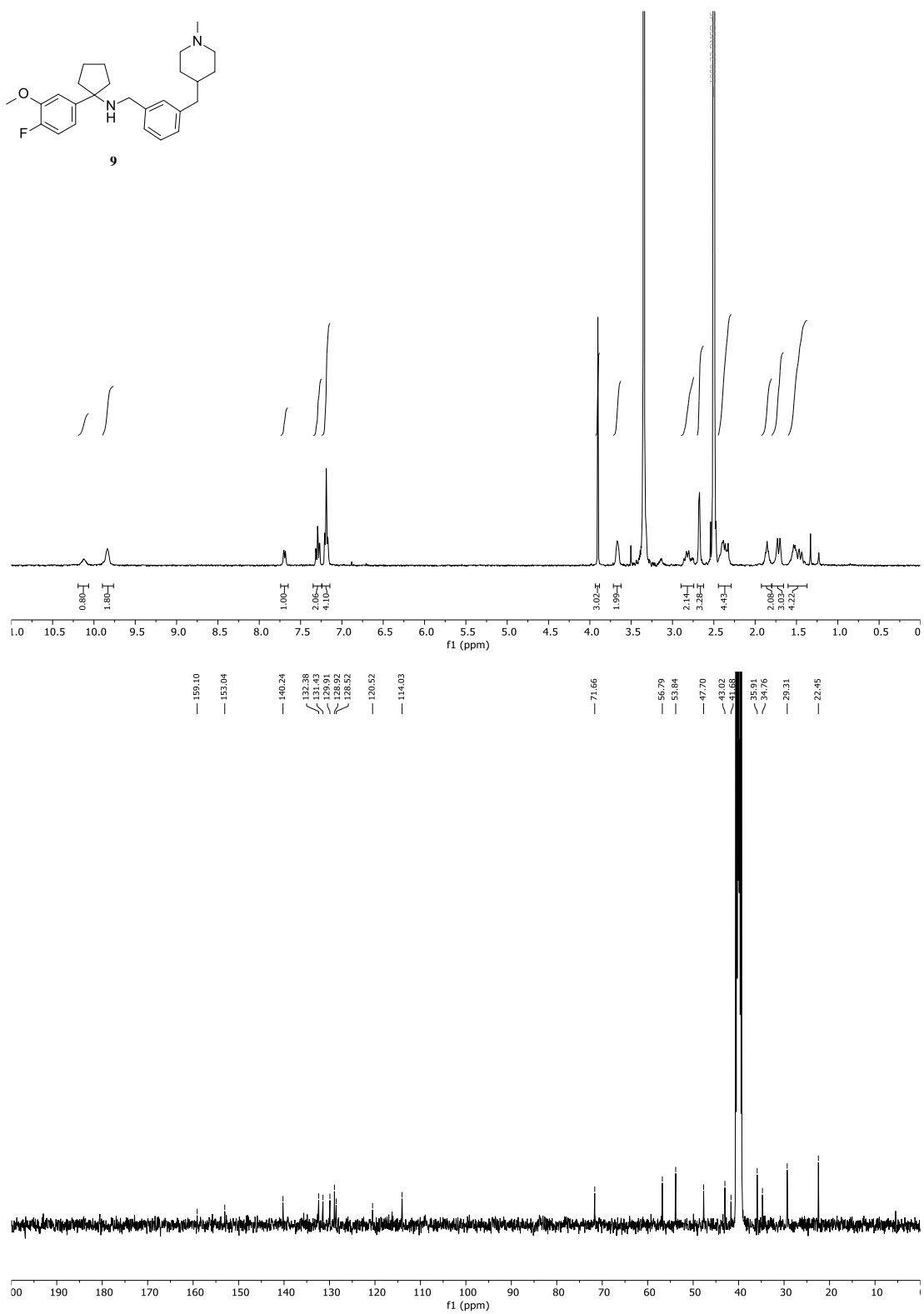
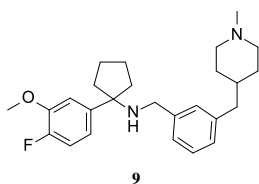
Compound 7:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )



Compound **8**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )

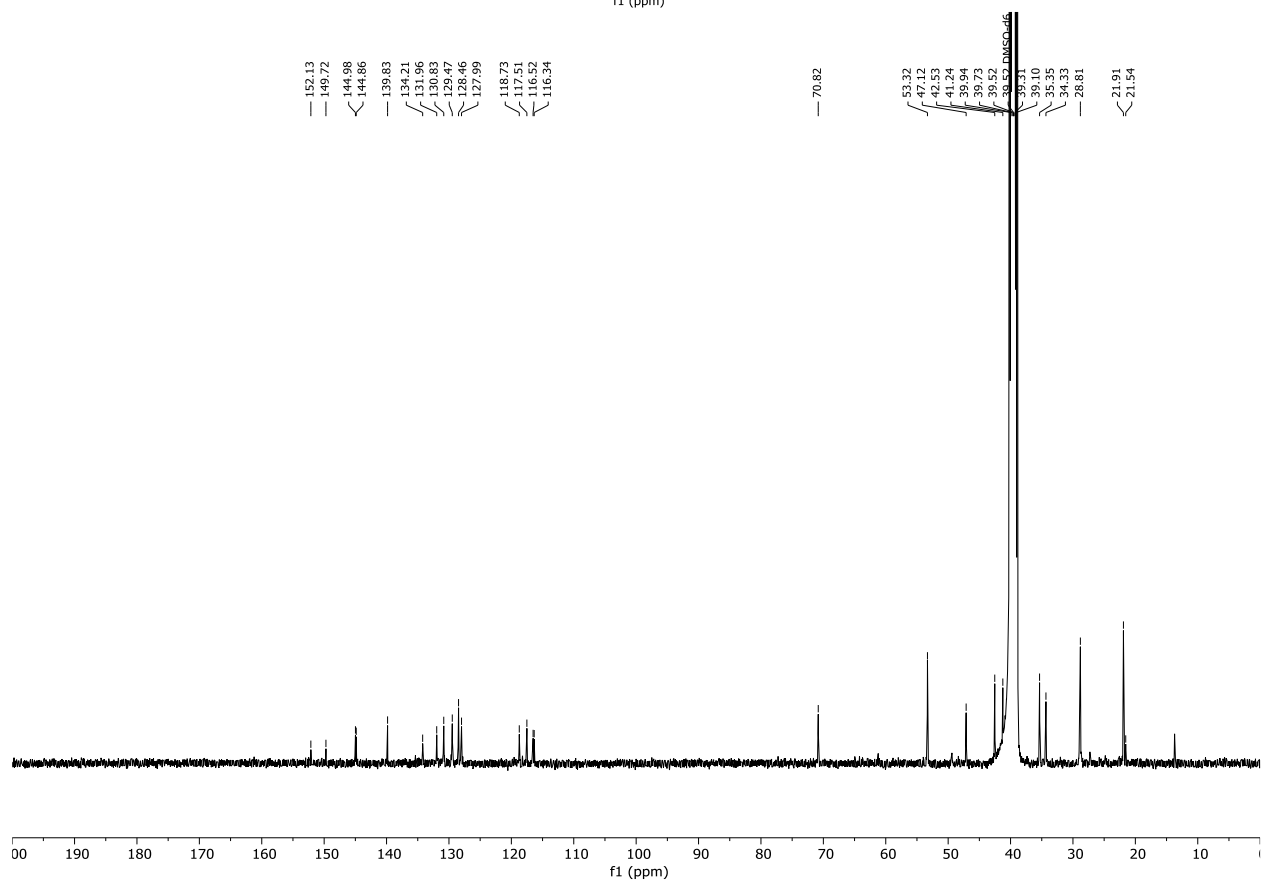
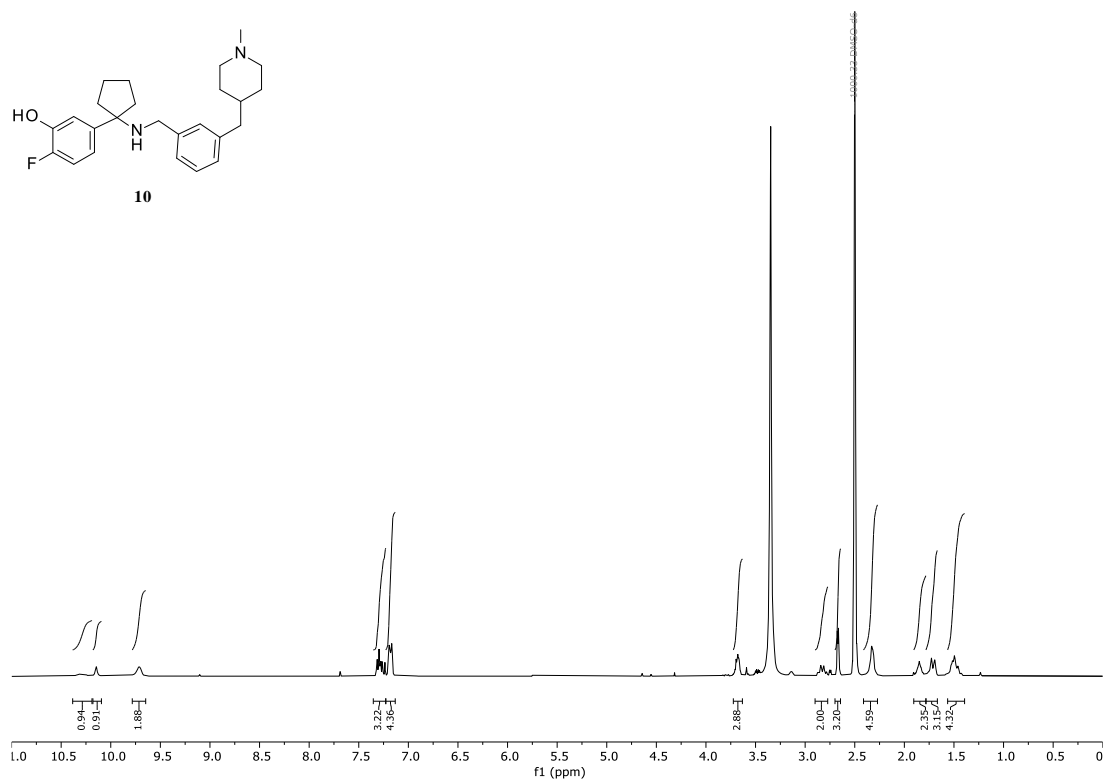
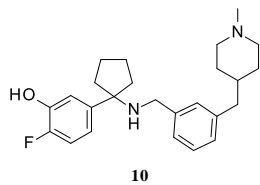


Compound **9**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )

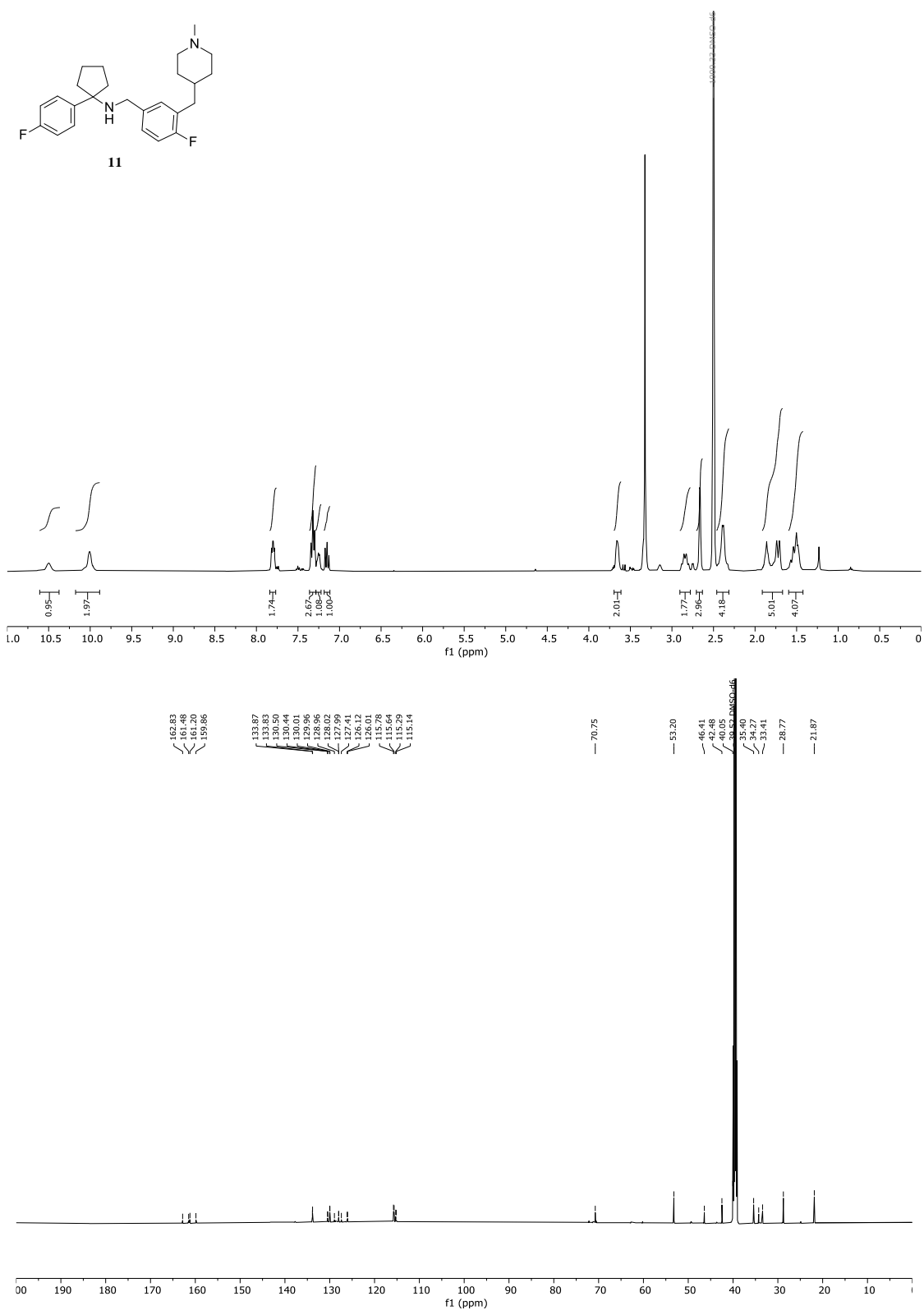




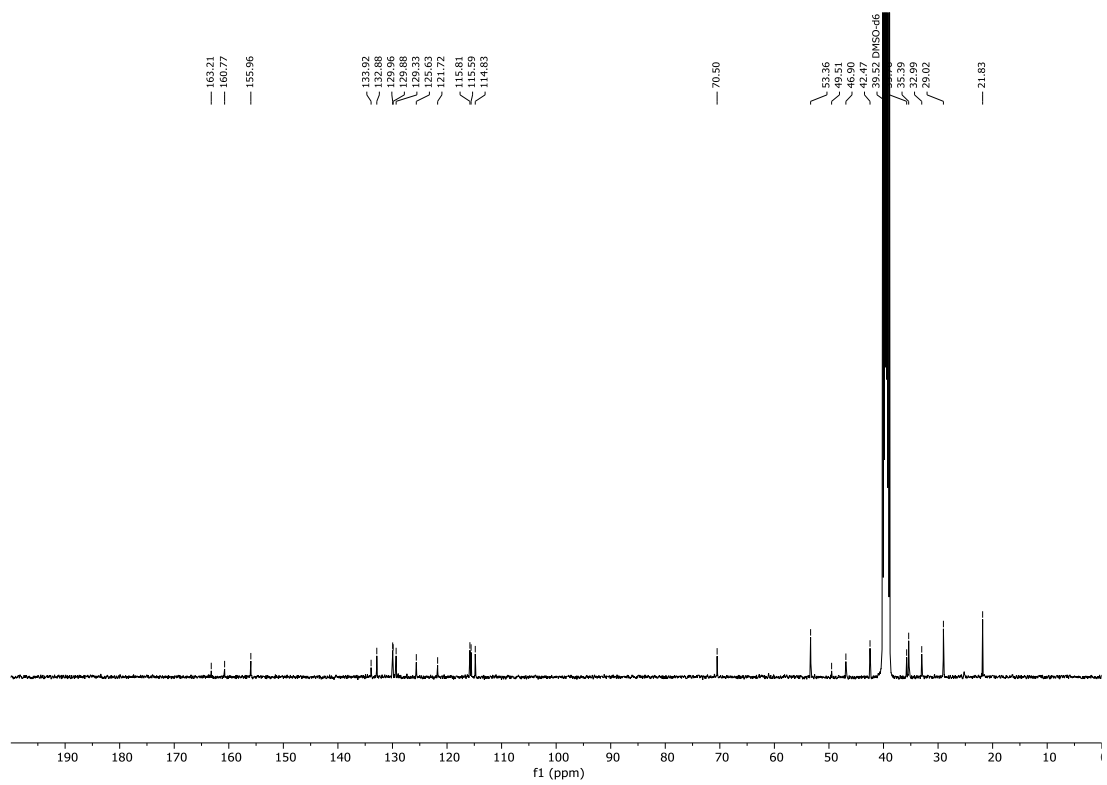
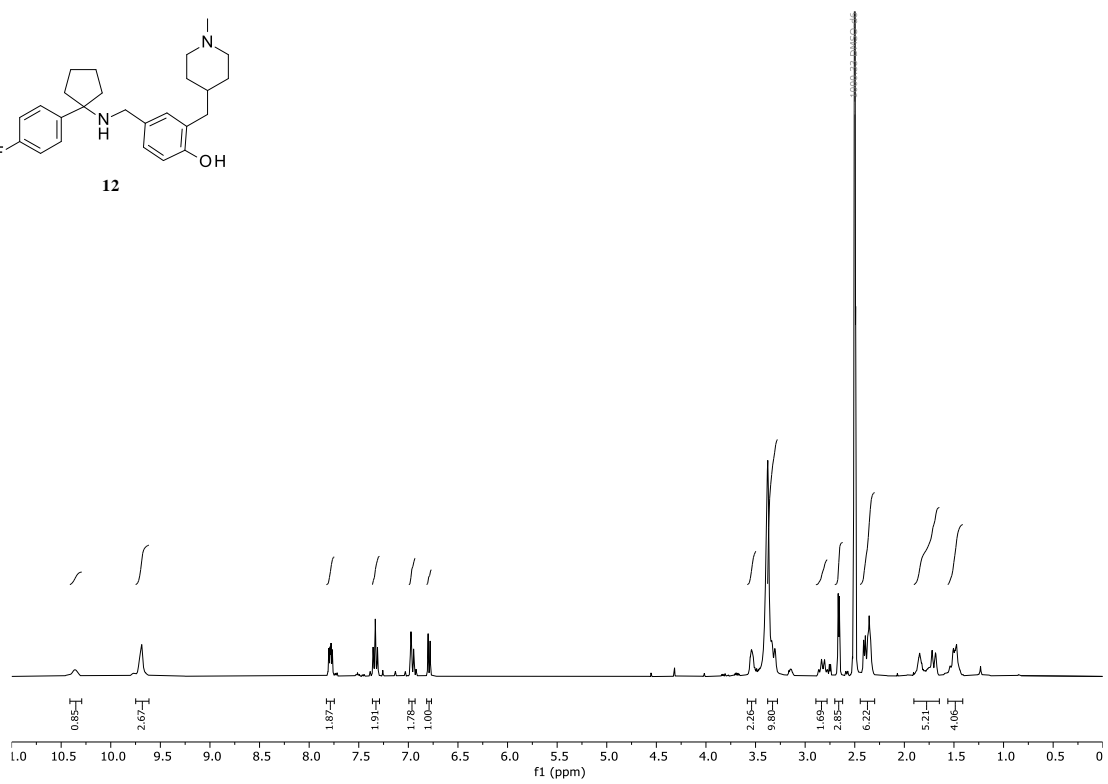
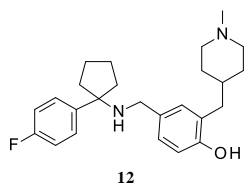
Compound **10**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )



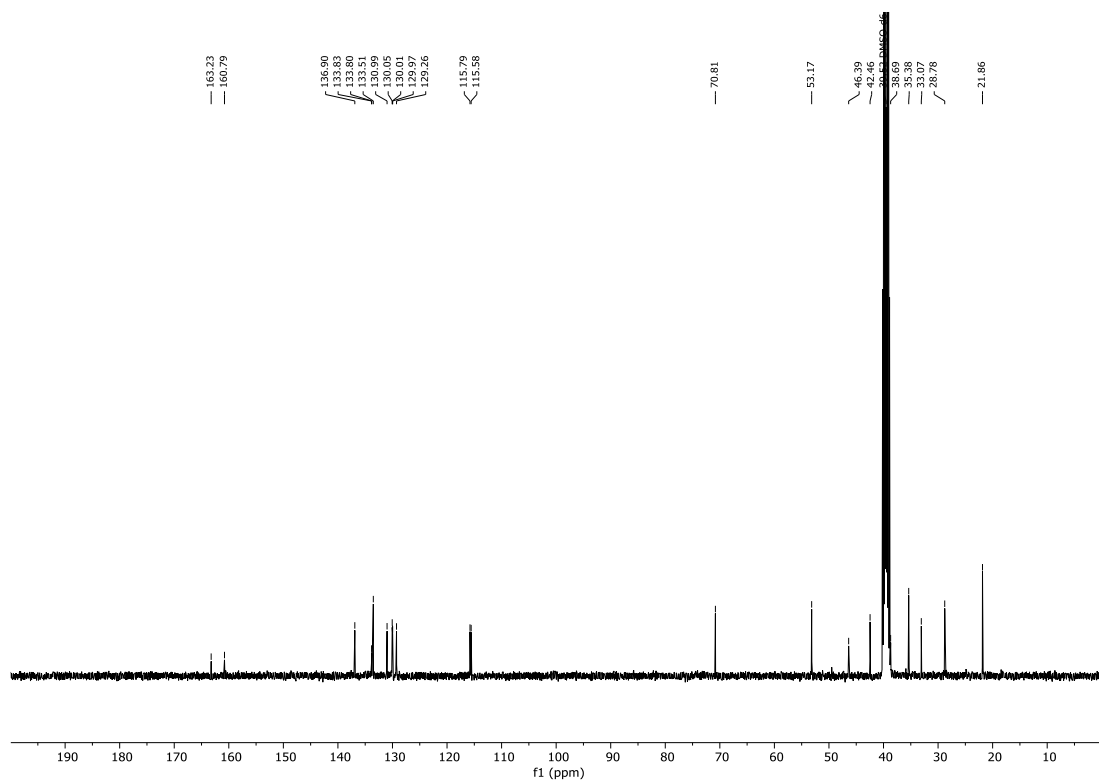
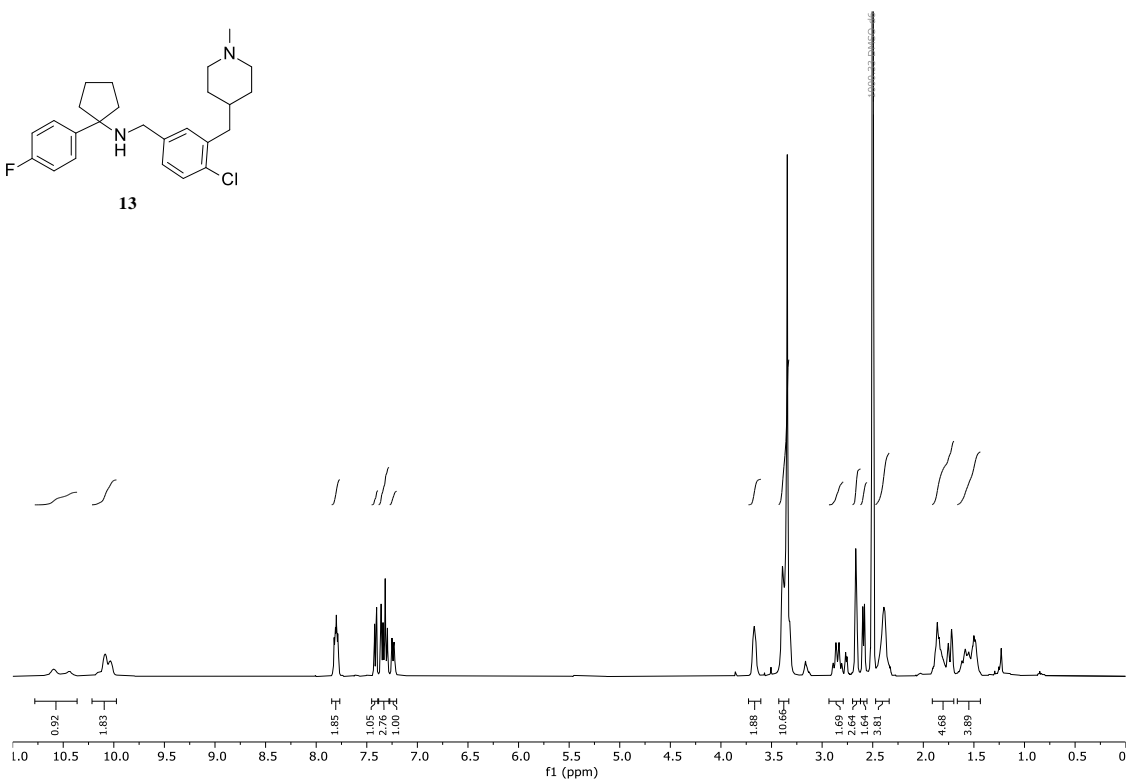
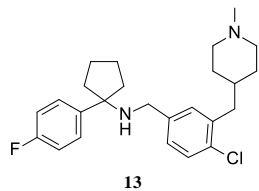
Compound **11**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )



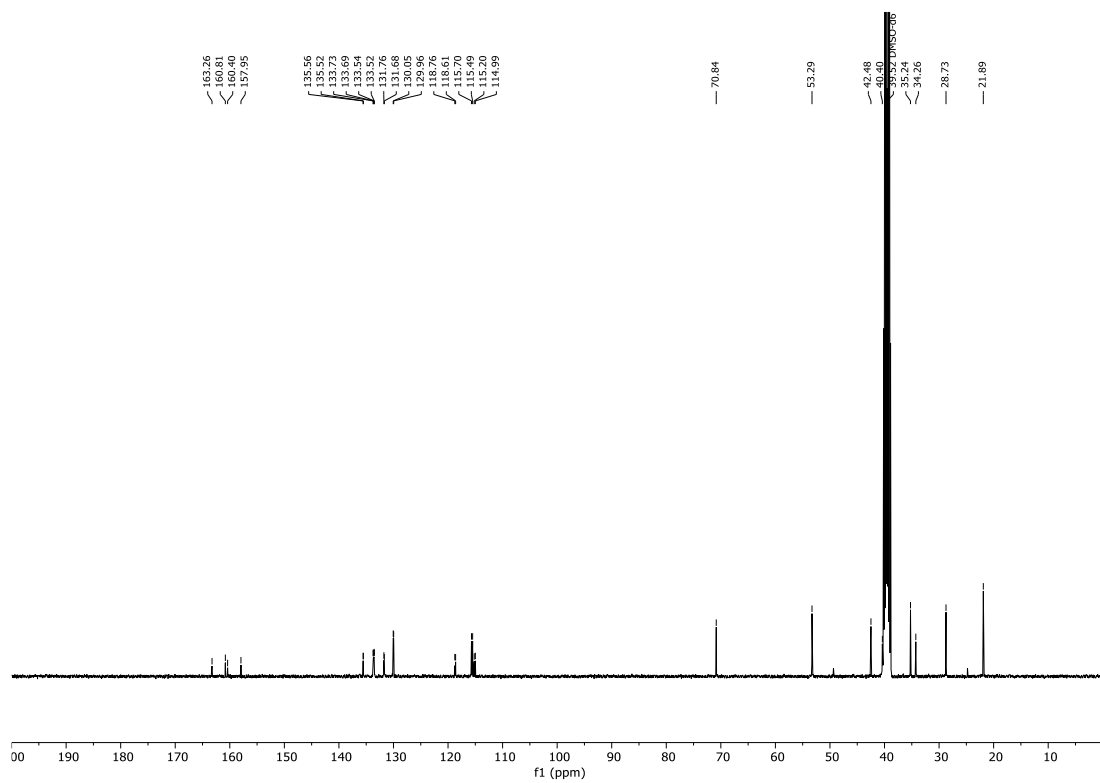
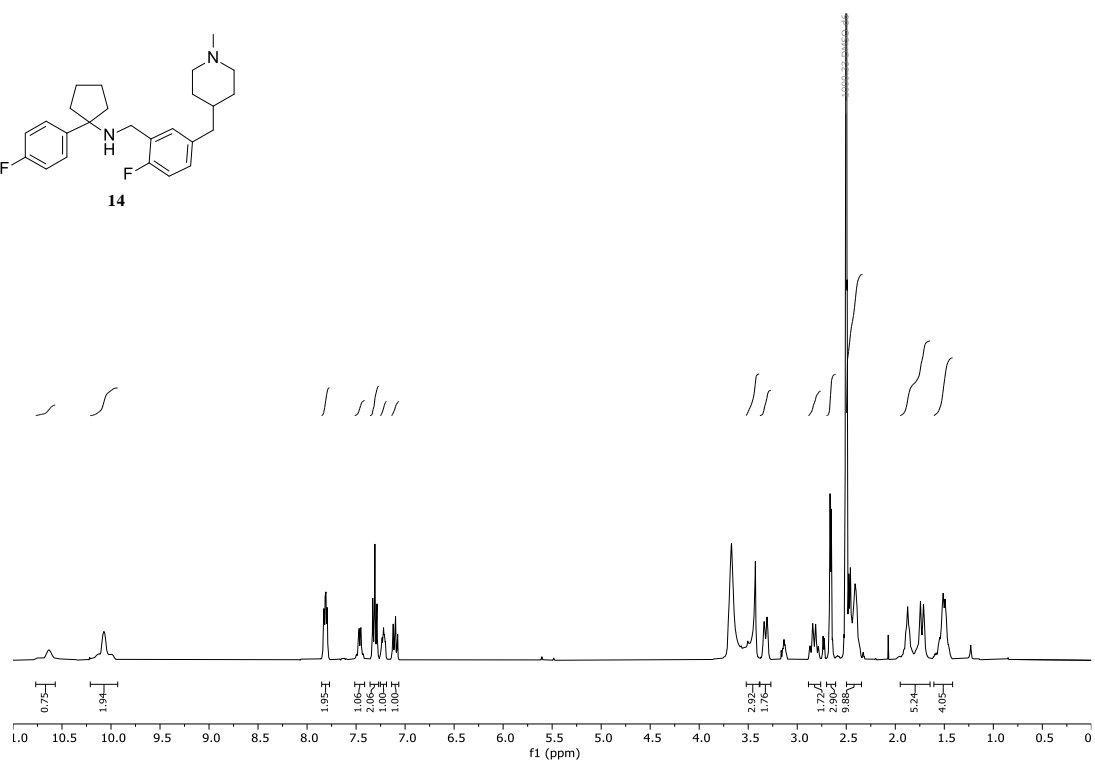
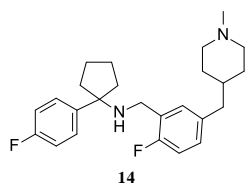
Compound **12**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )



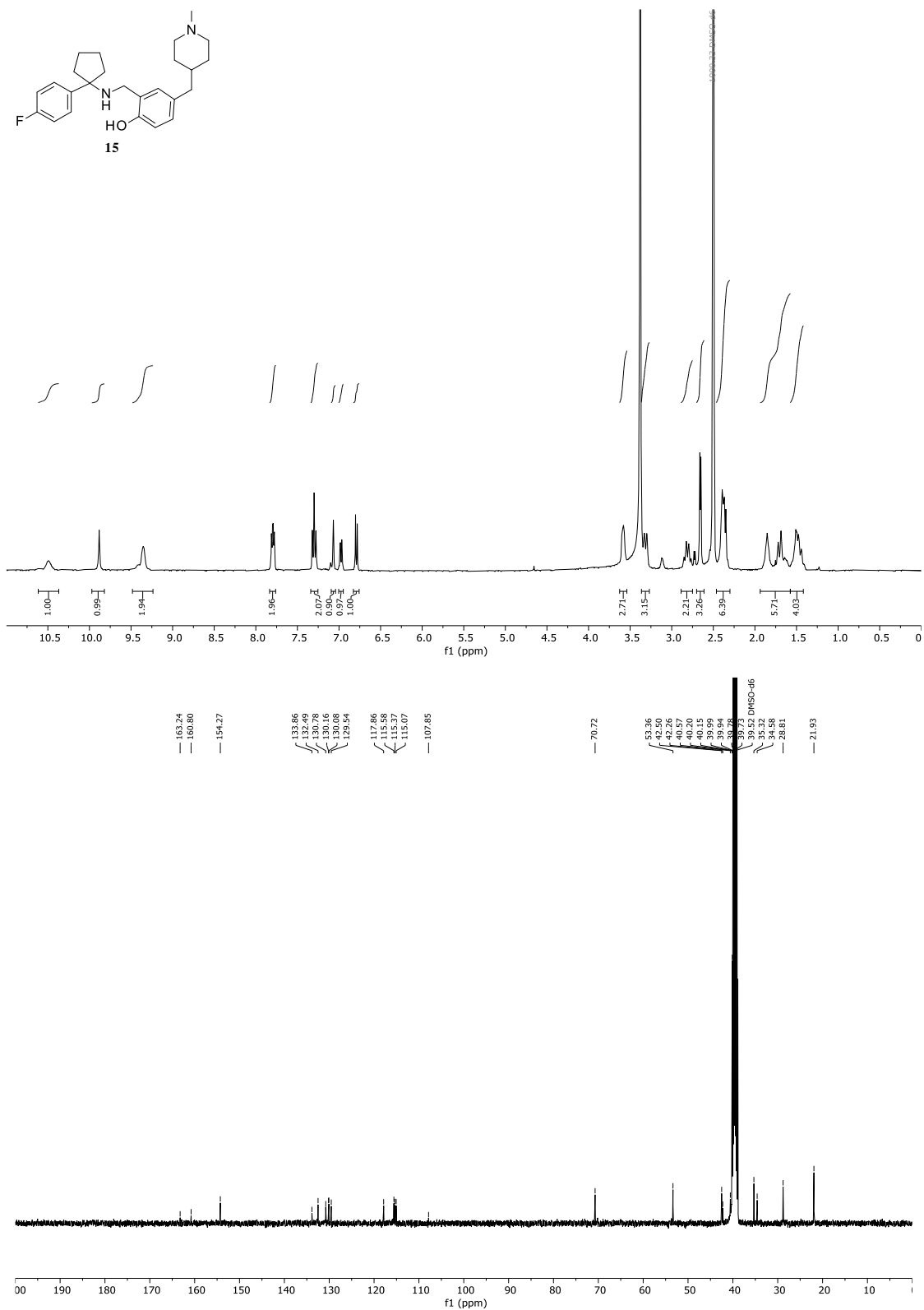
Compound **13**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )



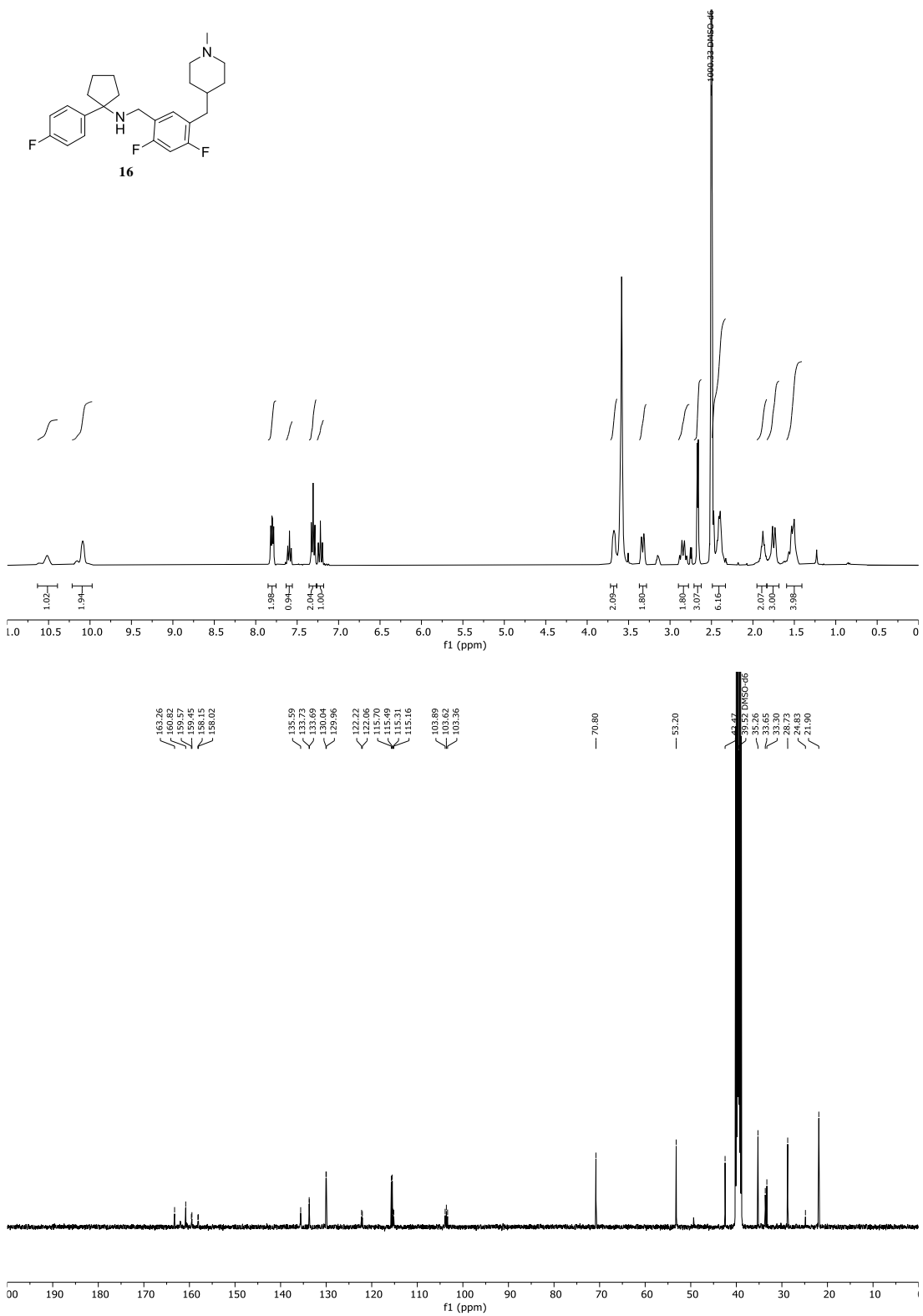
Compound **14**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )



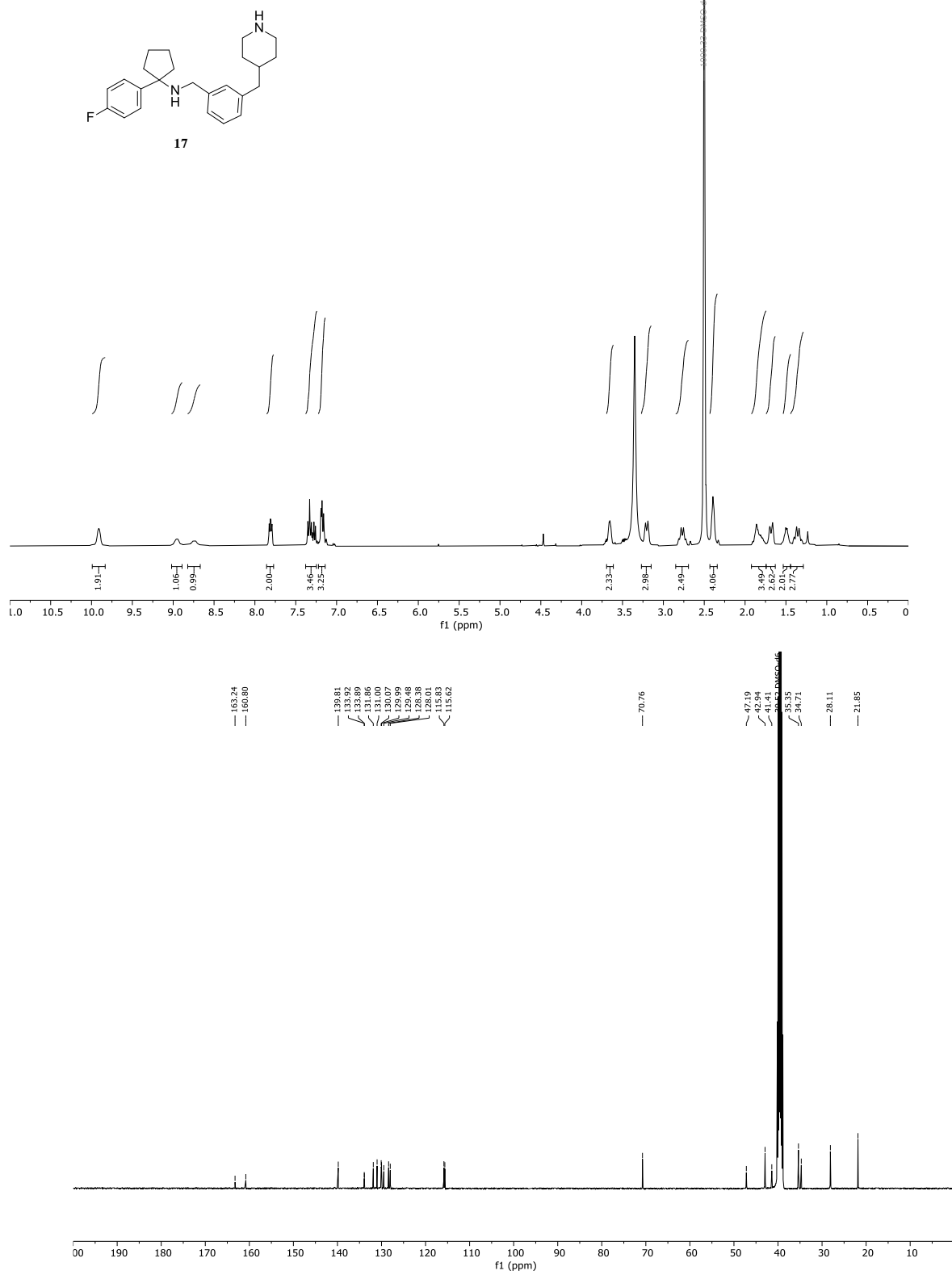
Compound **15**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )



Compound **16**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )

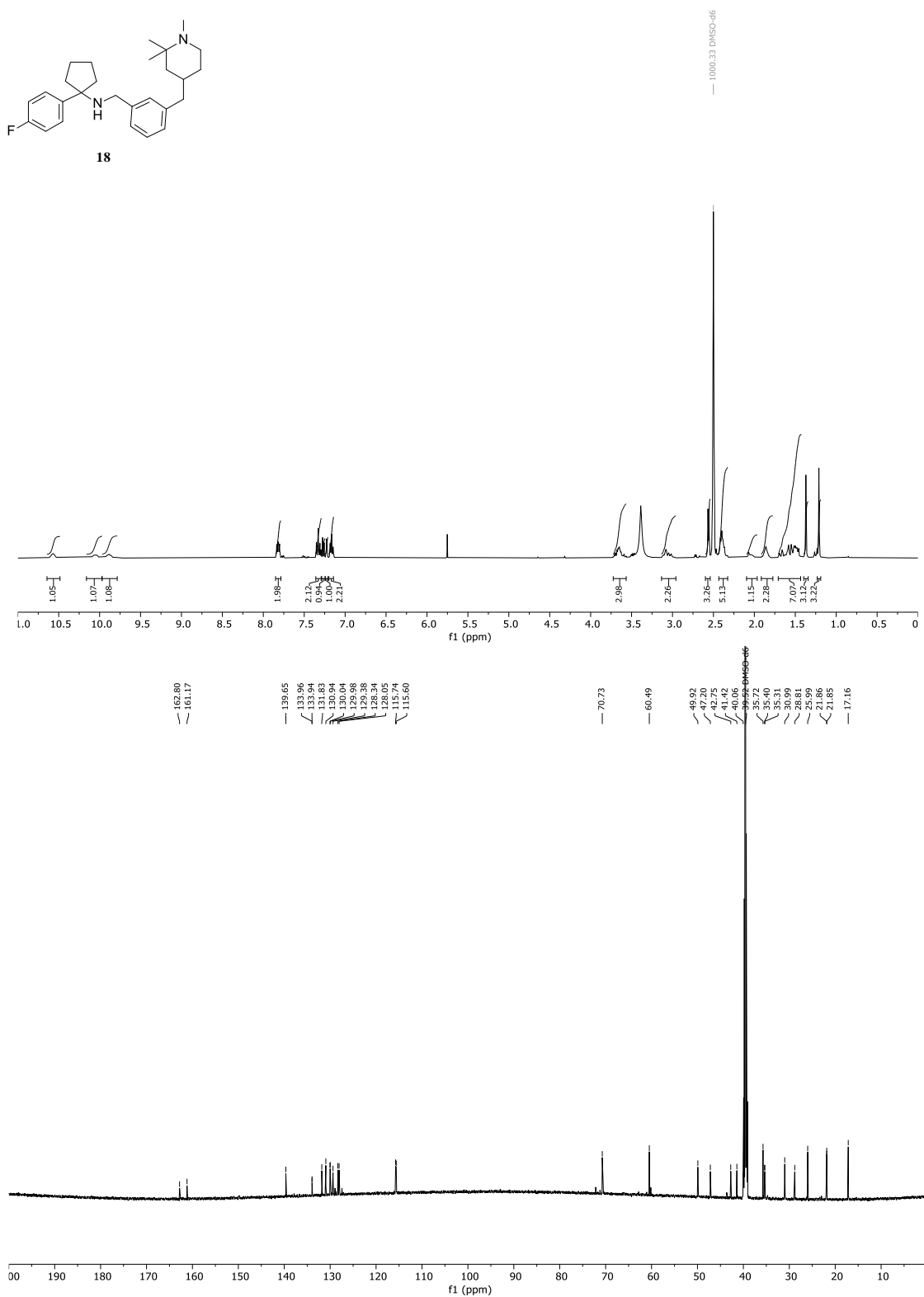
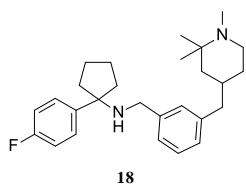


Compound **17**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )

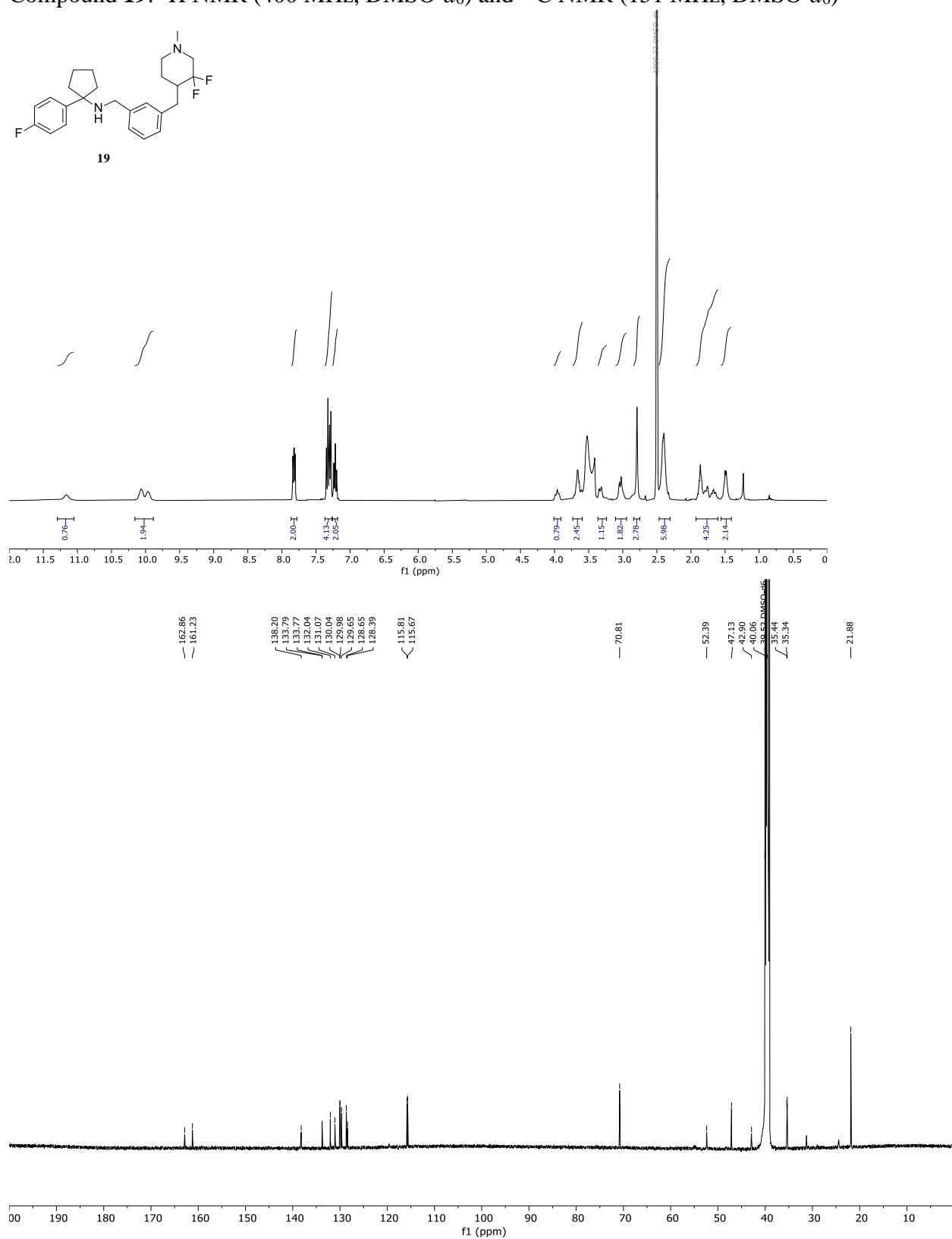
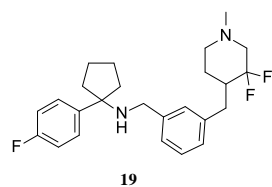




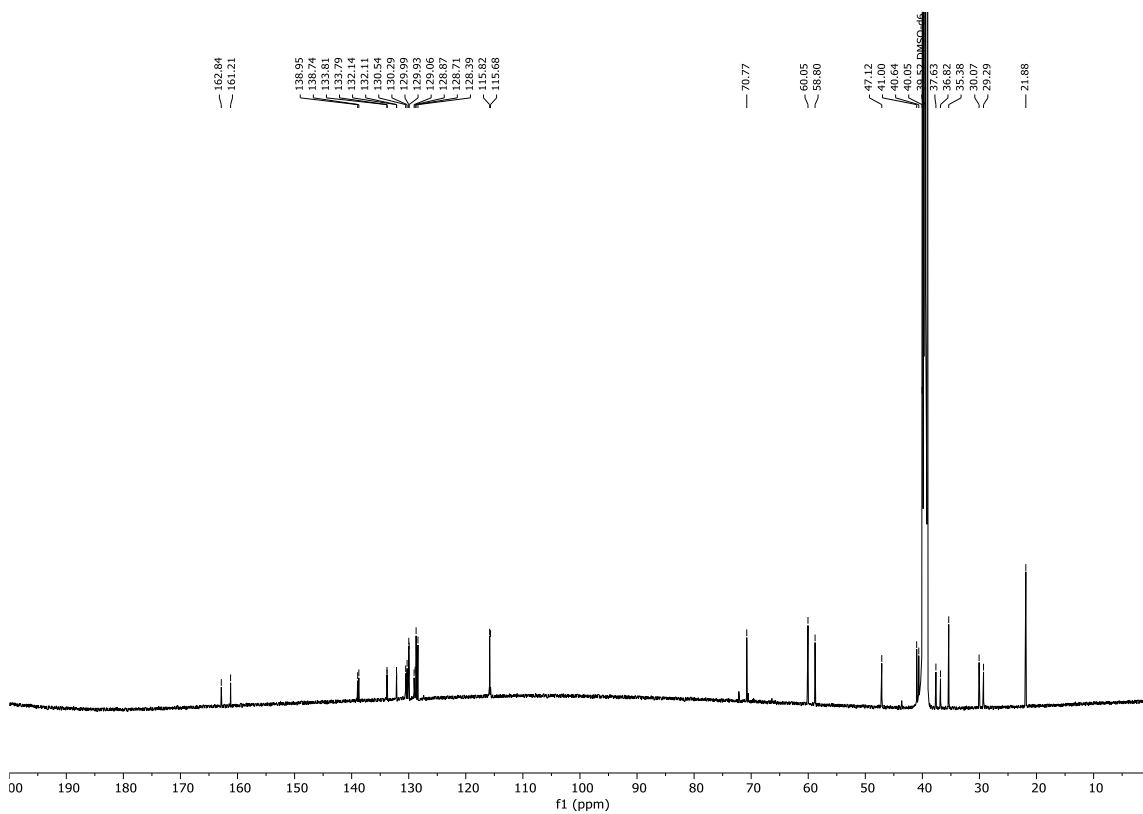
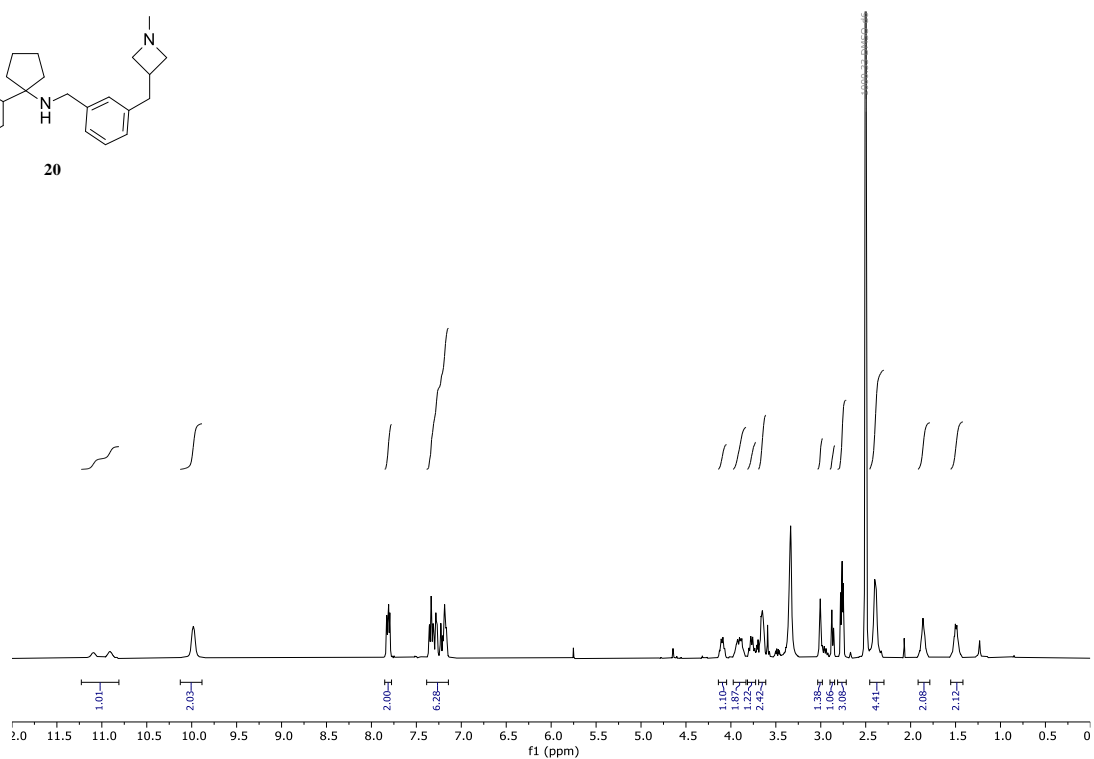
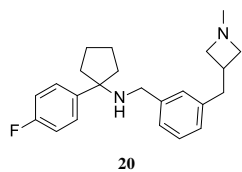
Compound **18**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )



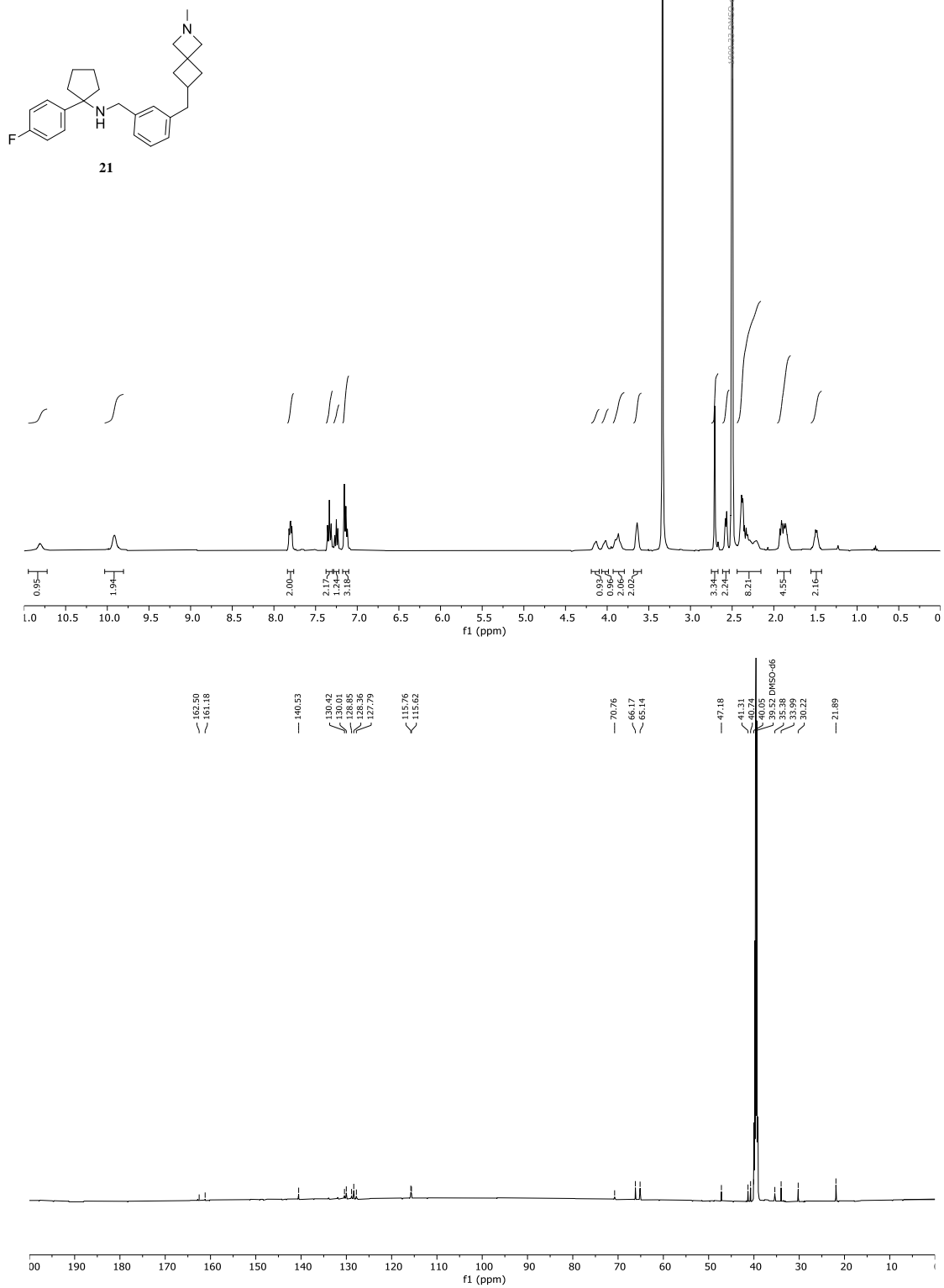
Compound **19**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )



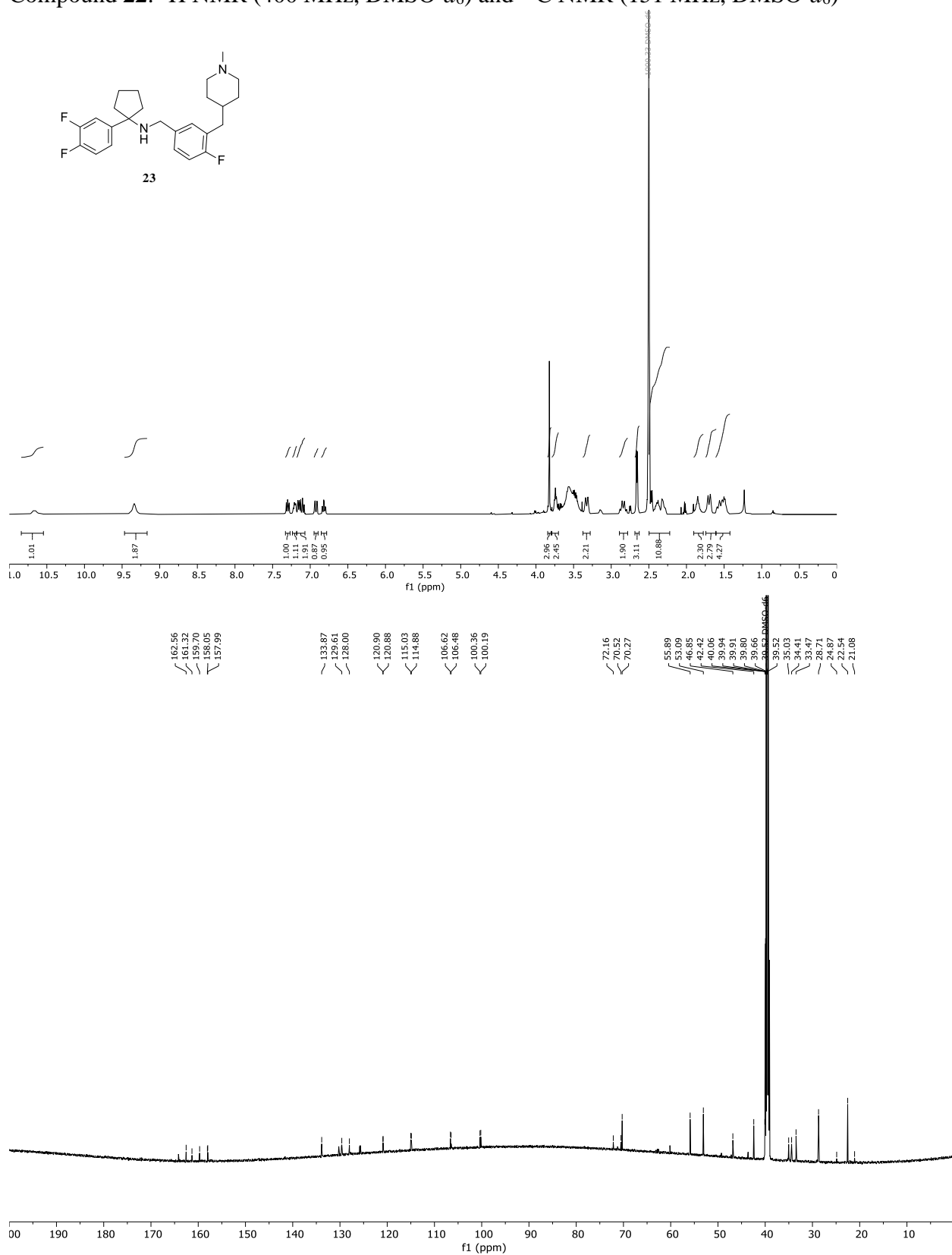
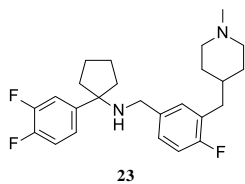
Compound **20**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )



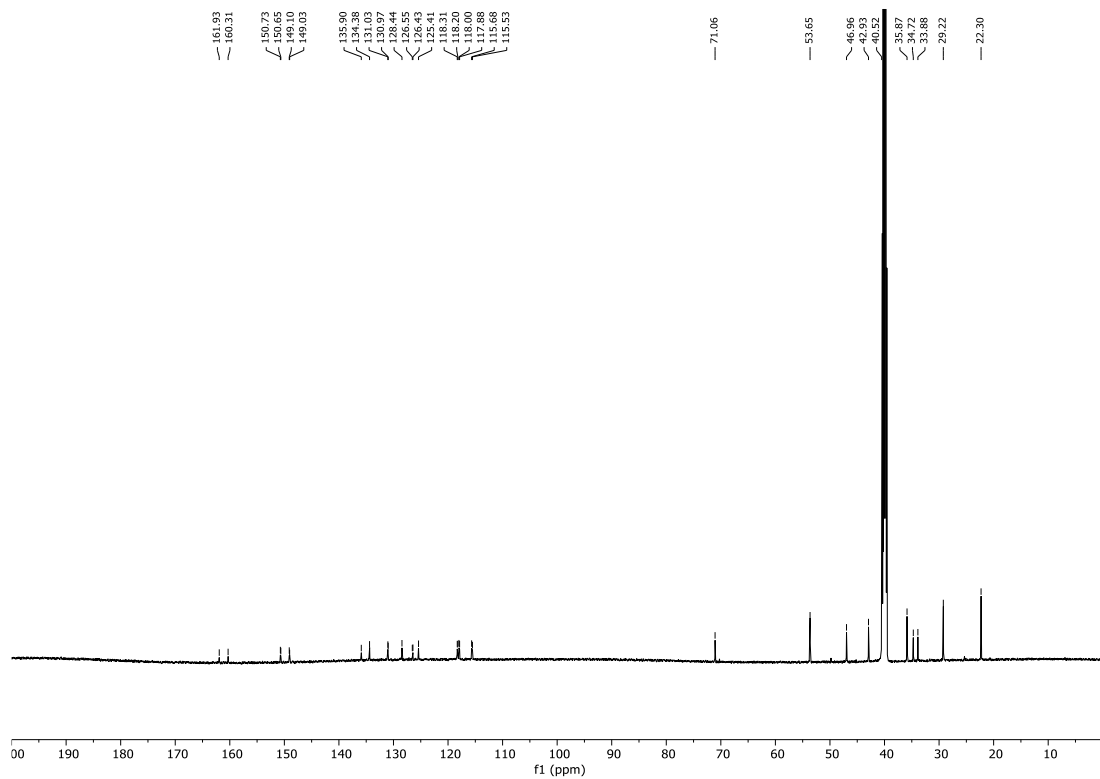
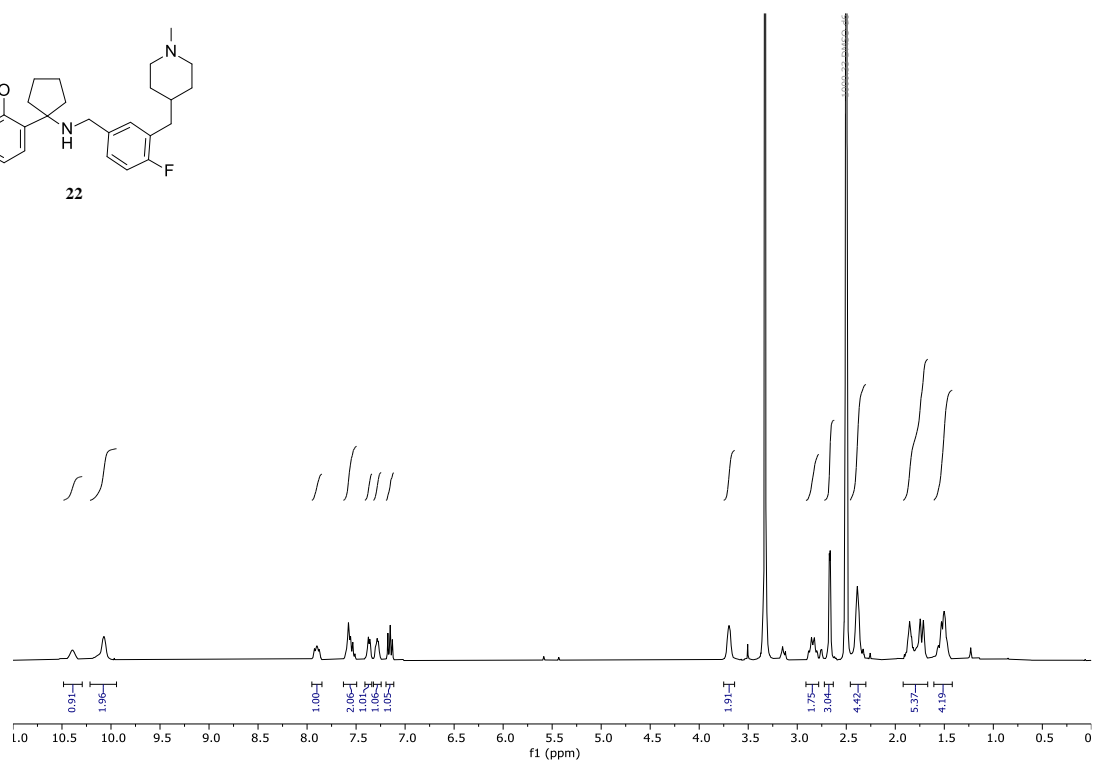
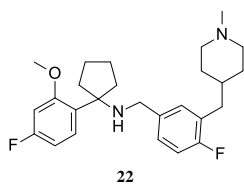
Compound **21**:  $^1\text{H}$ NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )



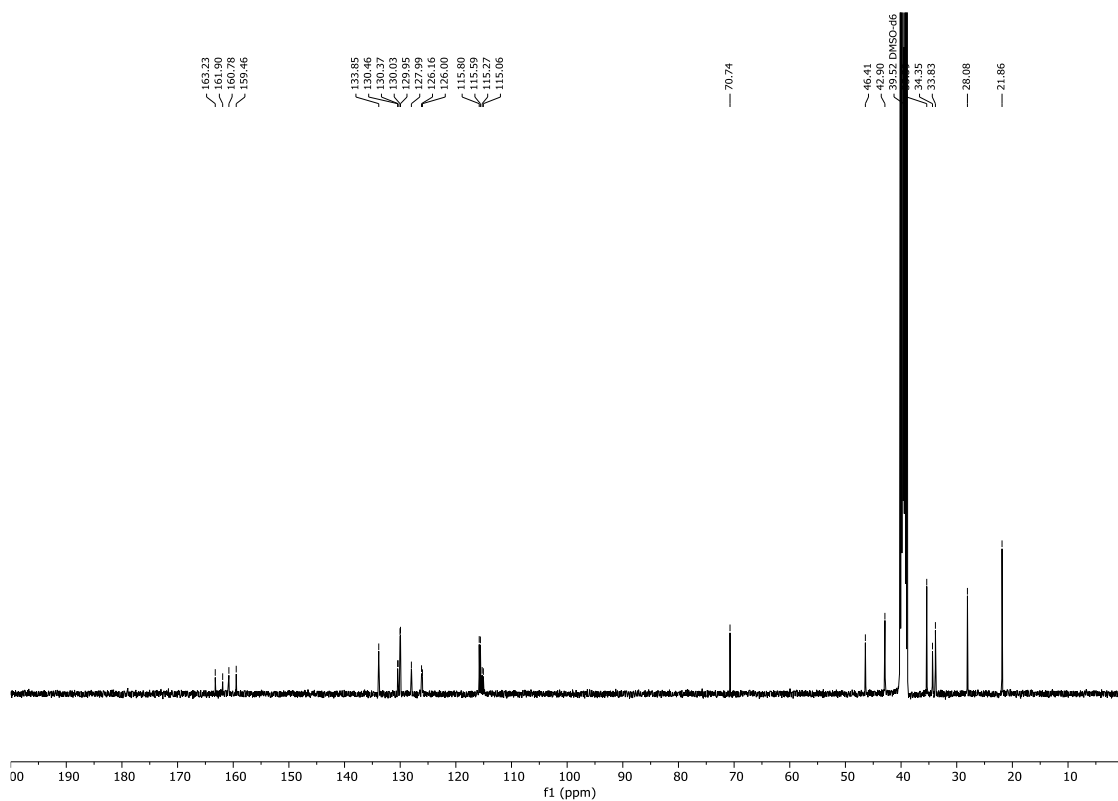
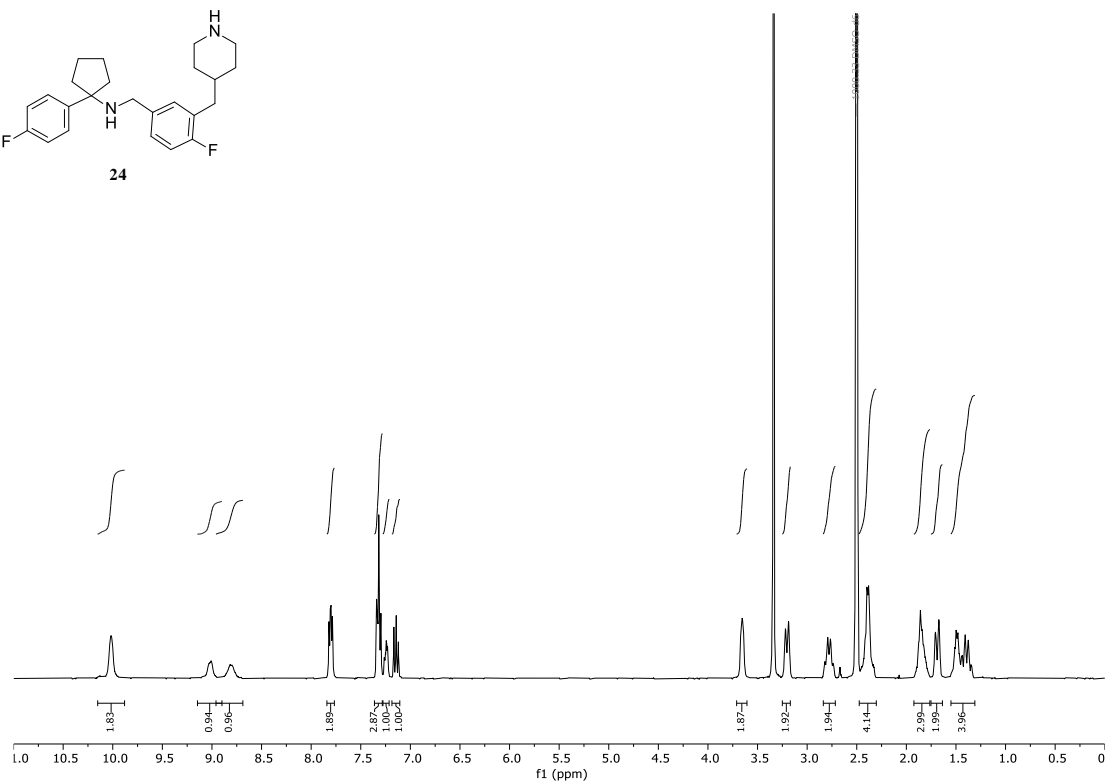
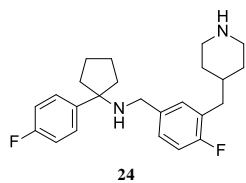
Compound **22**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )



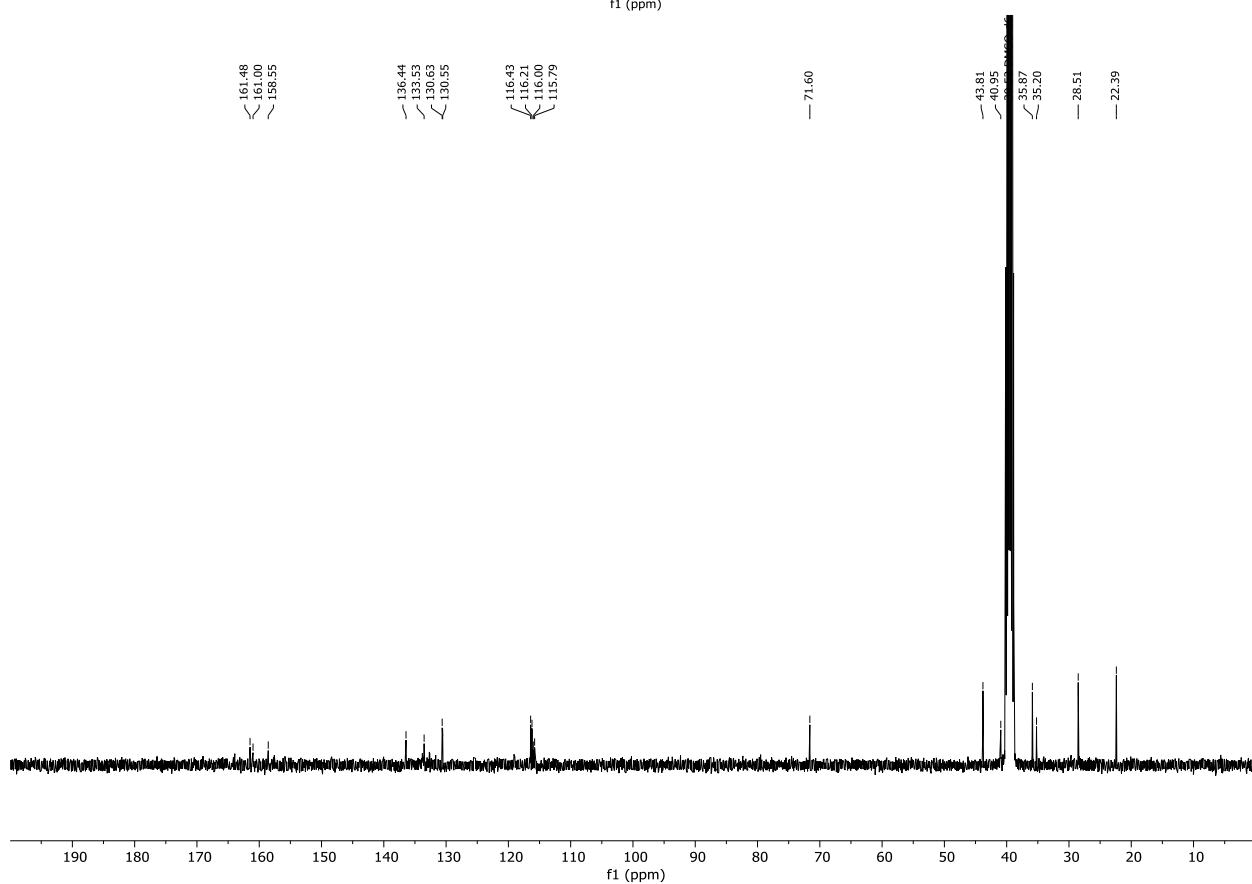
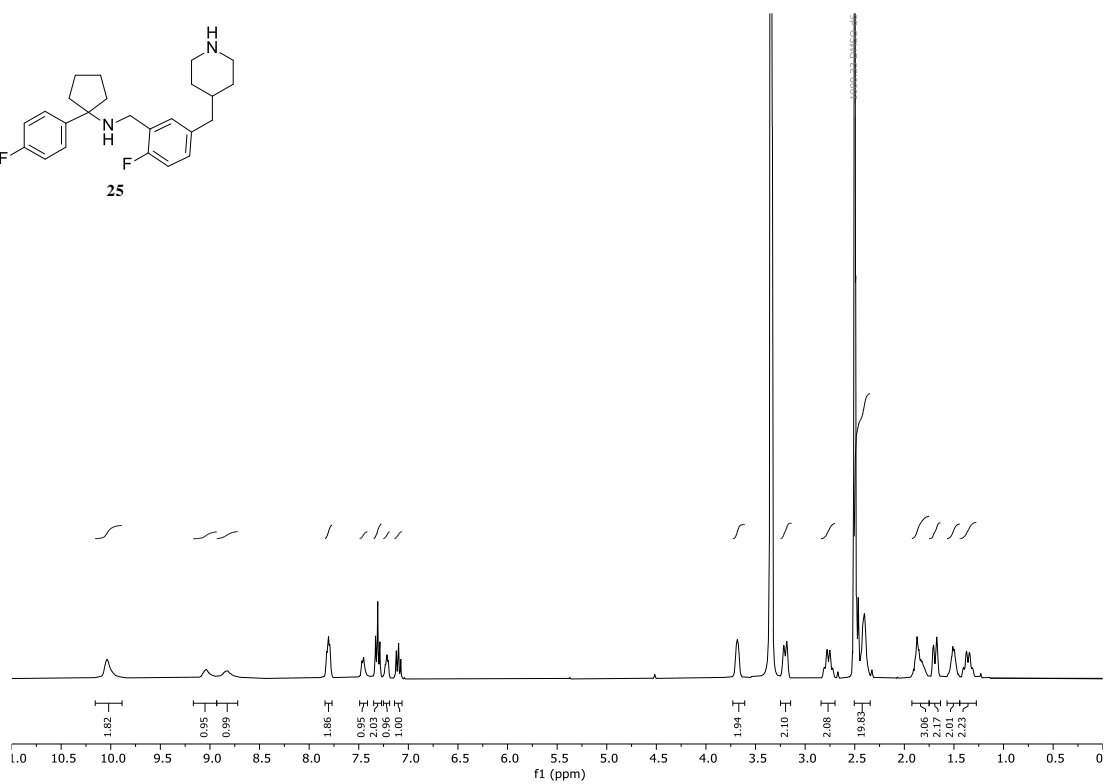
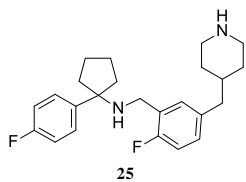
Compound **23**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )



Compound **24**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )

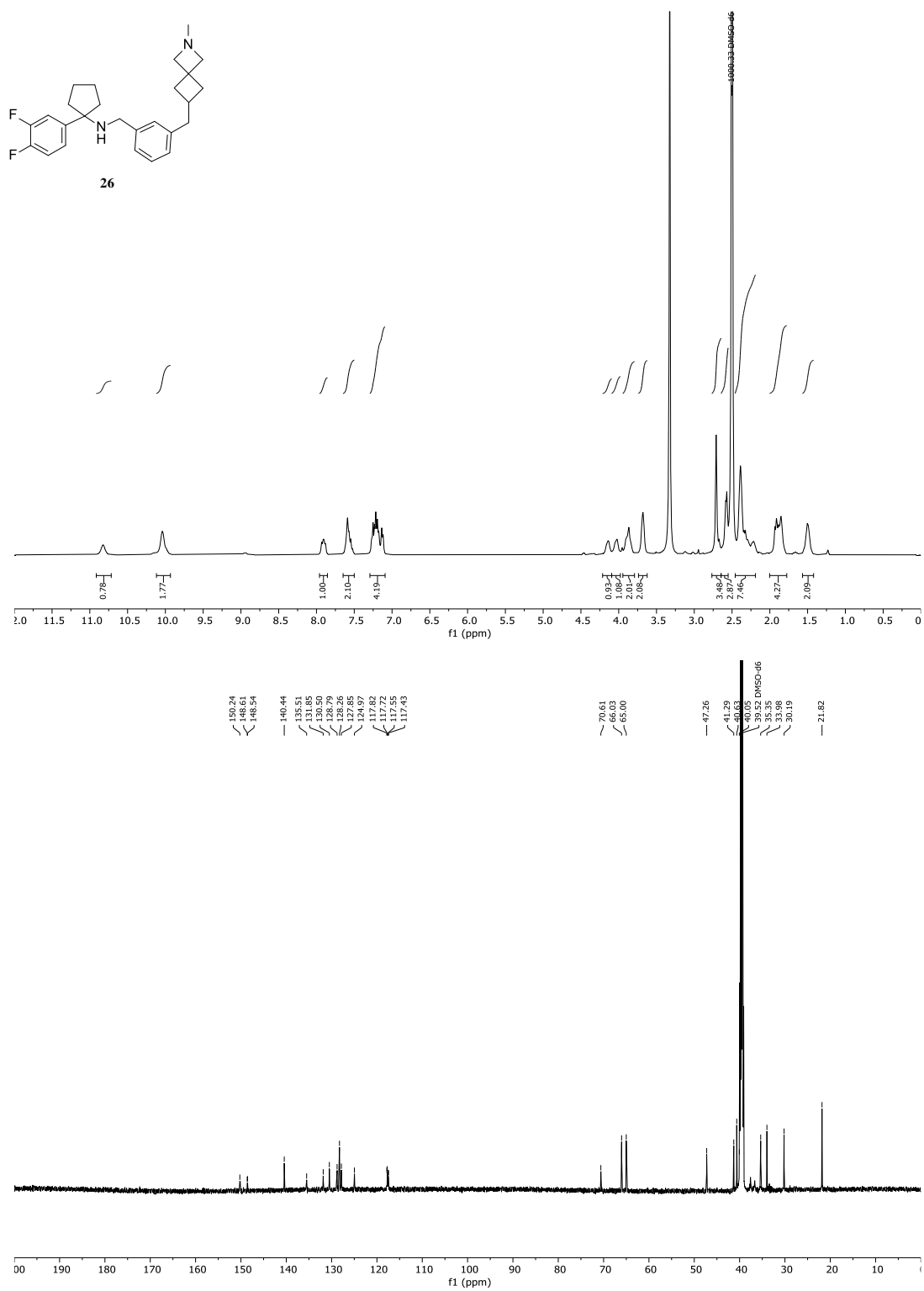


Compound **25**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )

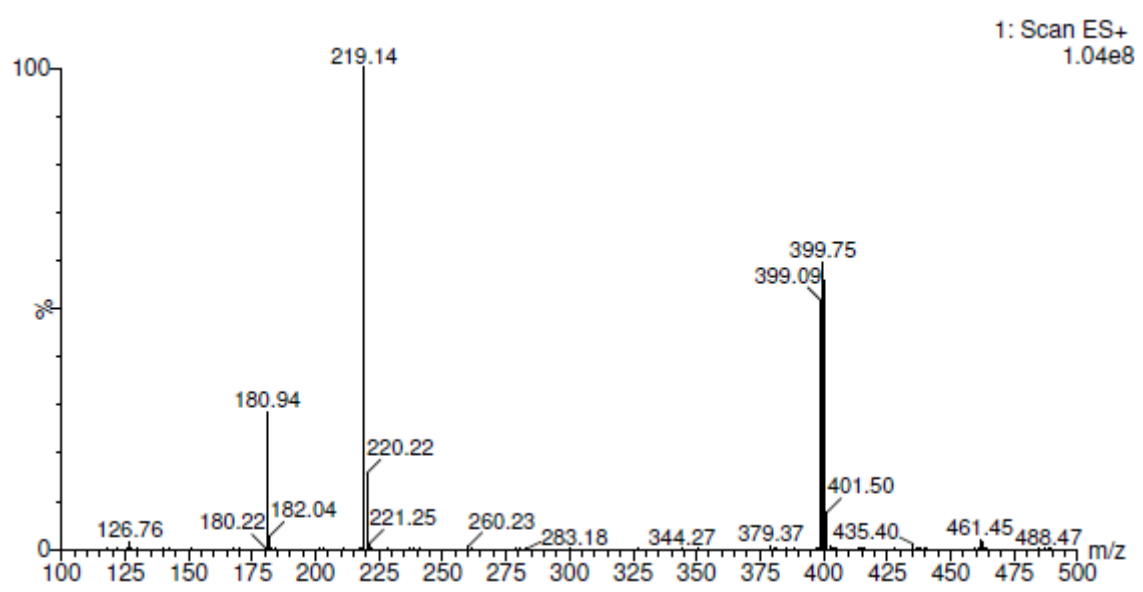
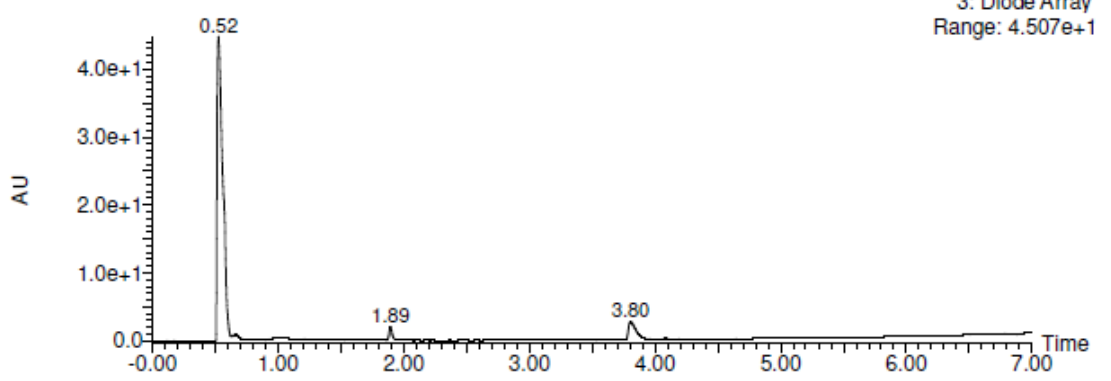
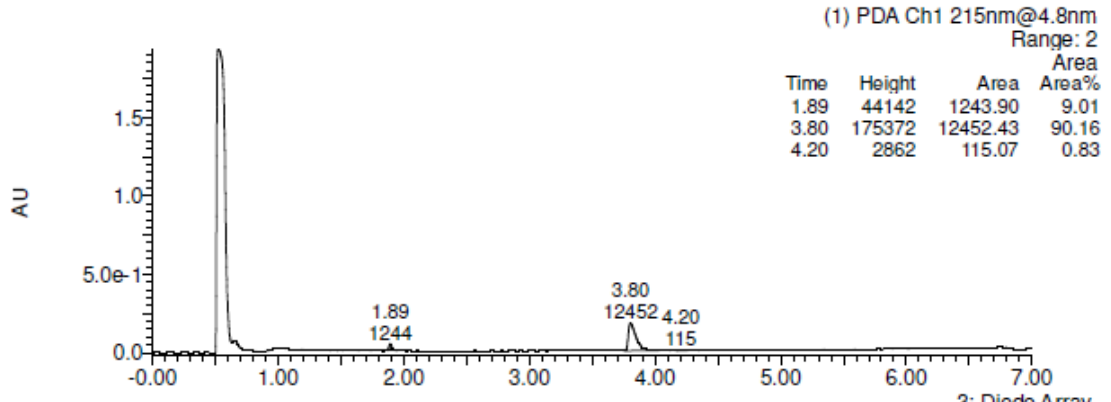




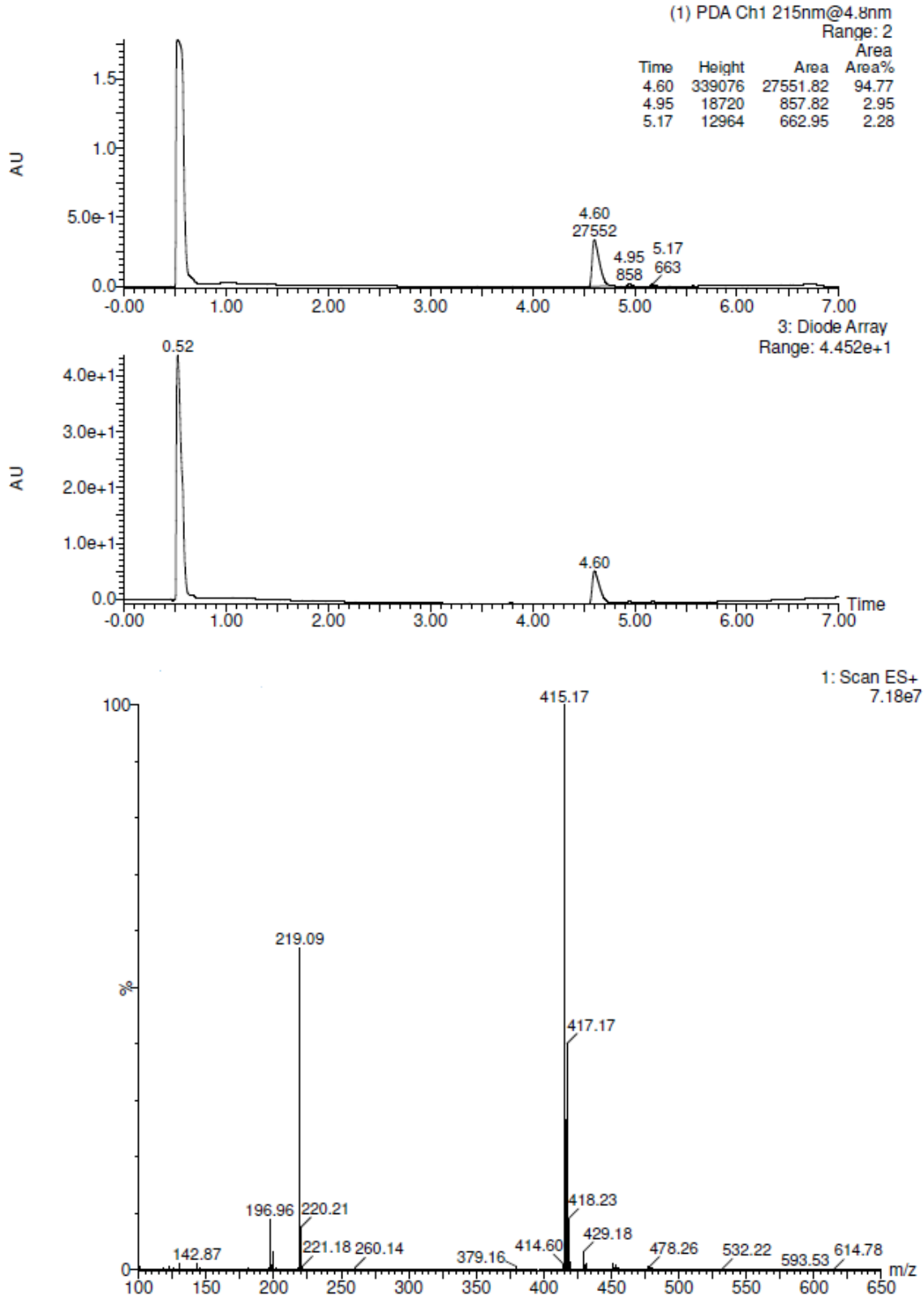
Compound **26**:  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ) and  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )



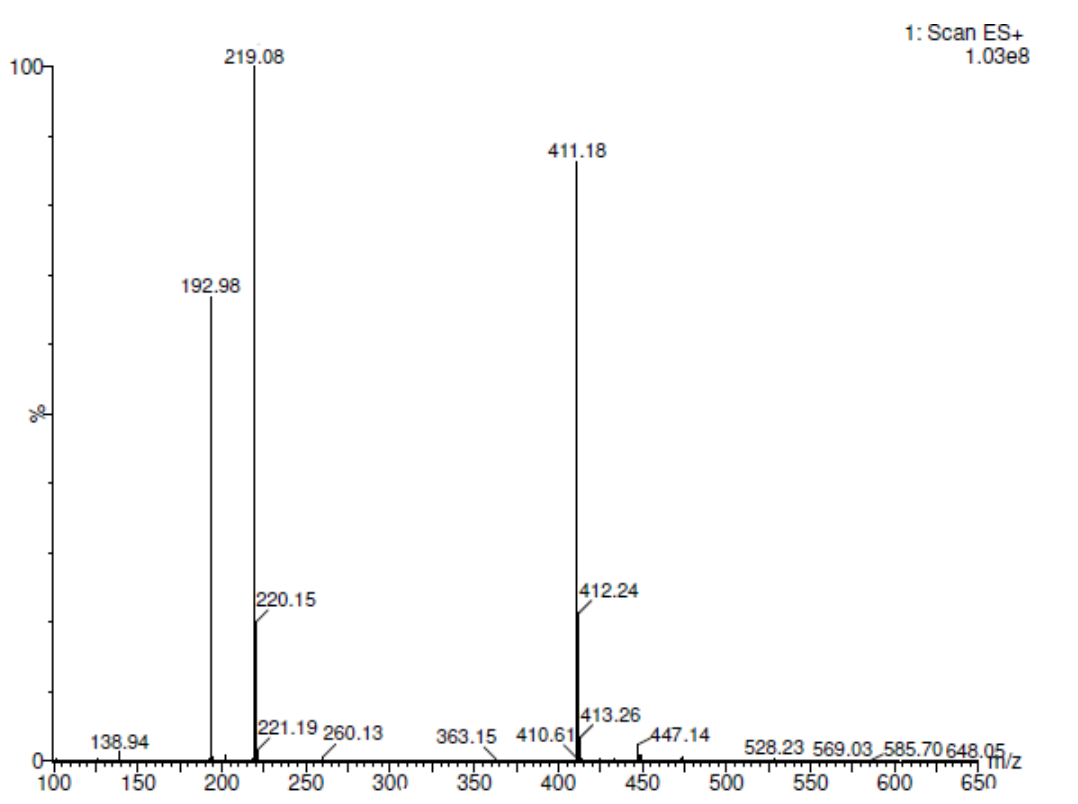
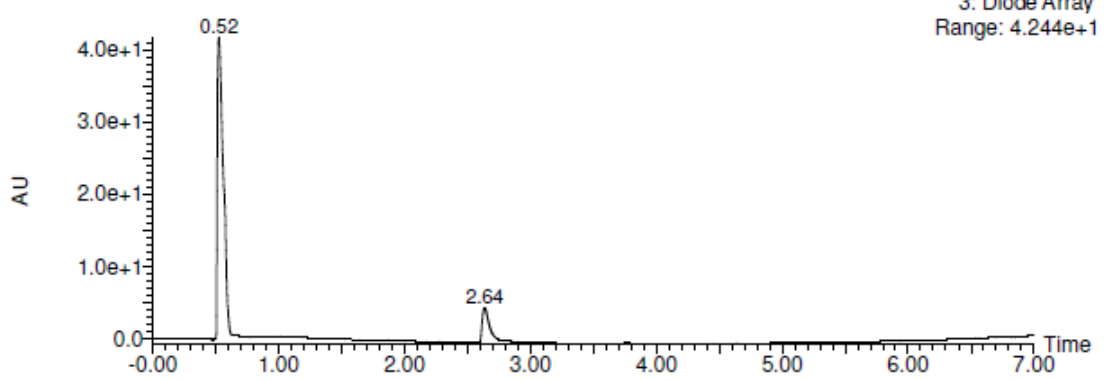
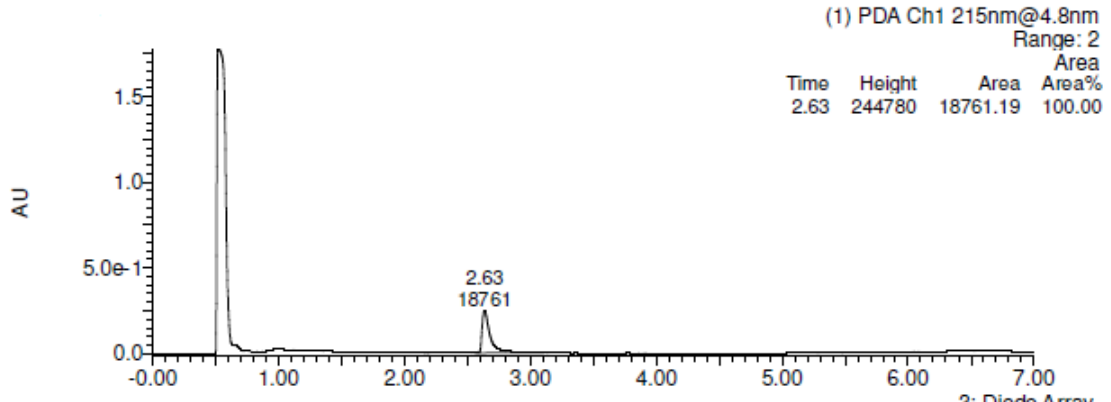
Compound 3: UPLC/MS analysis



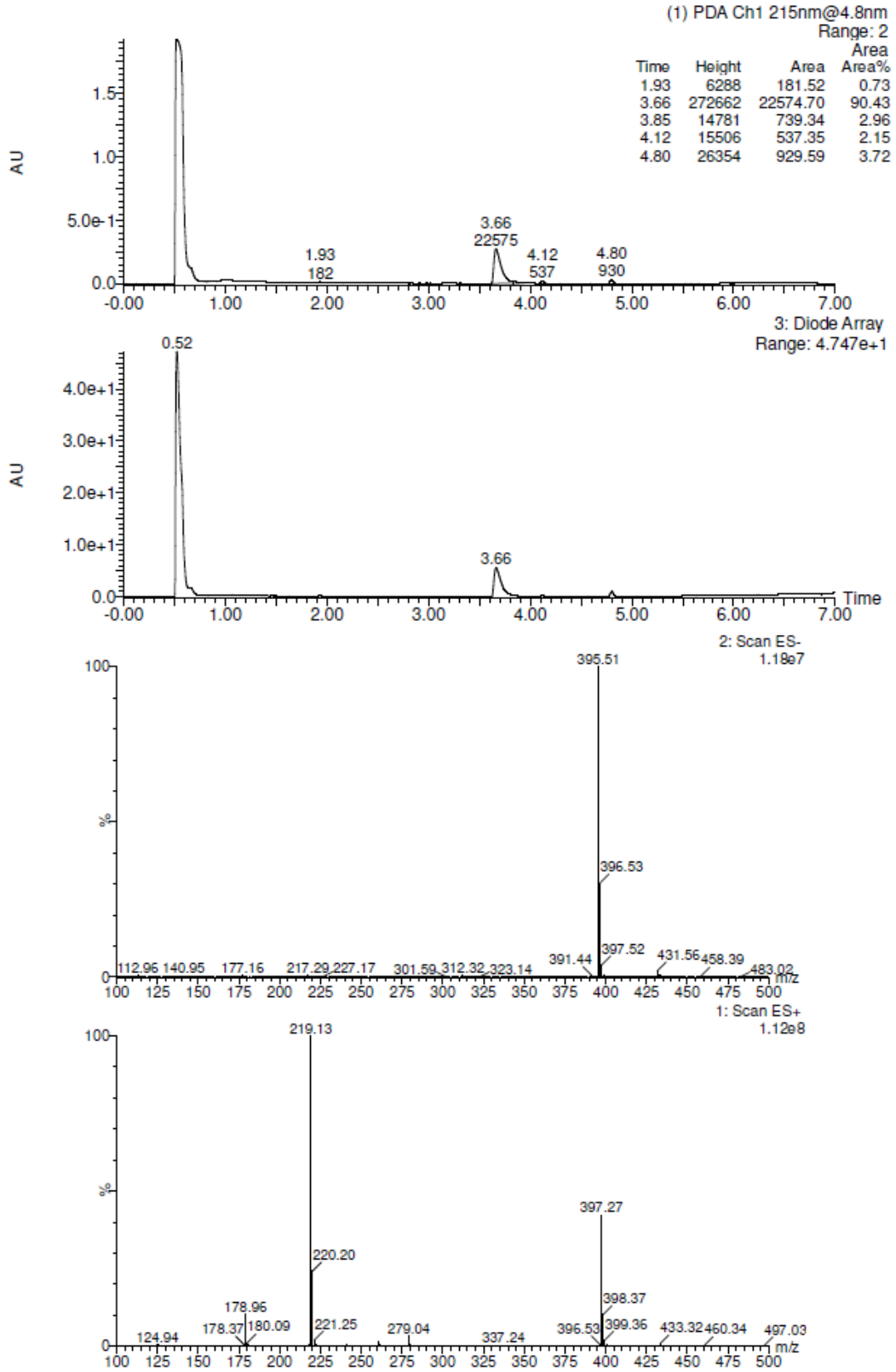
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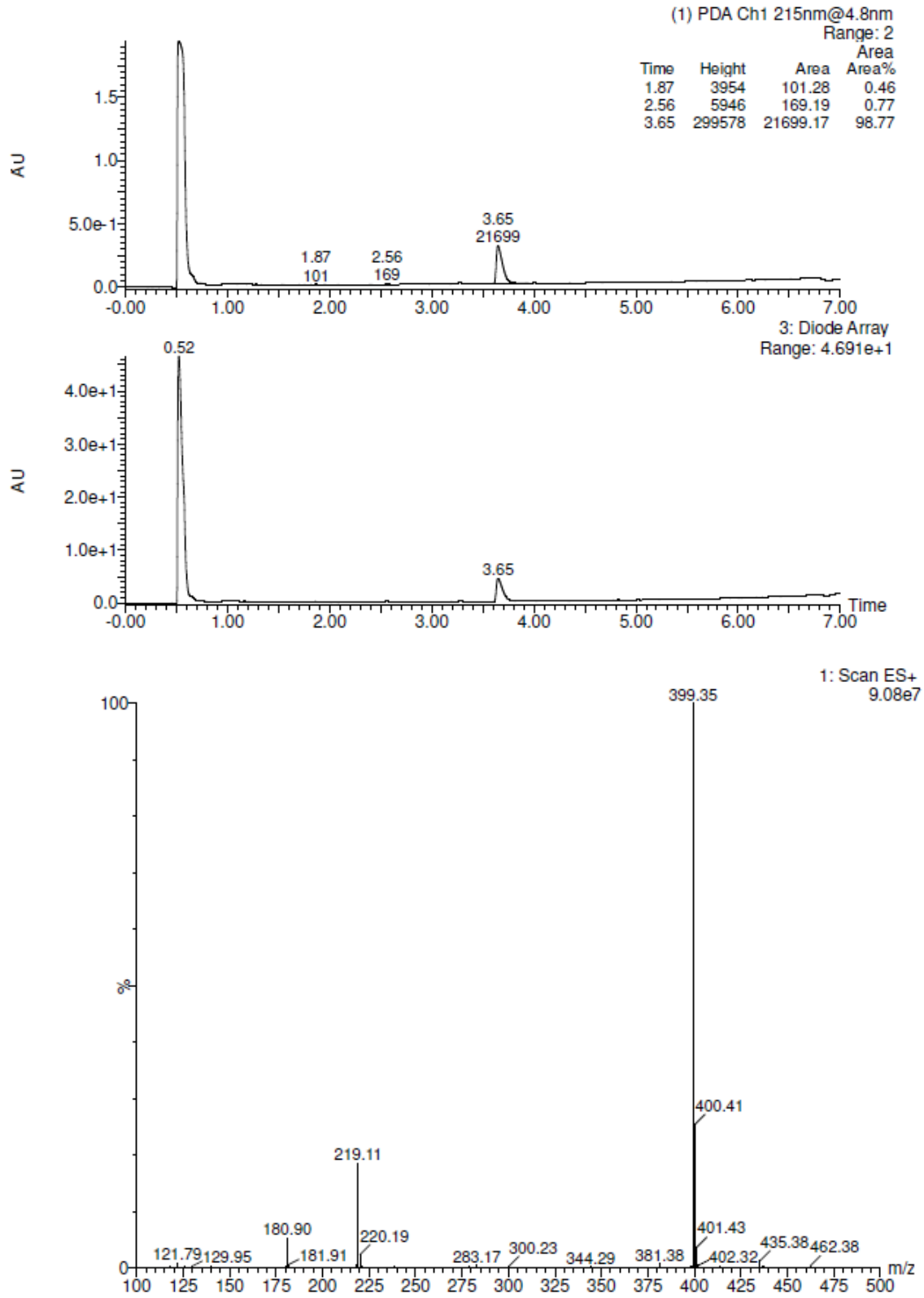
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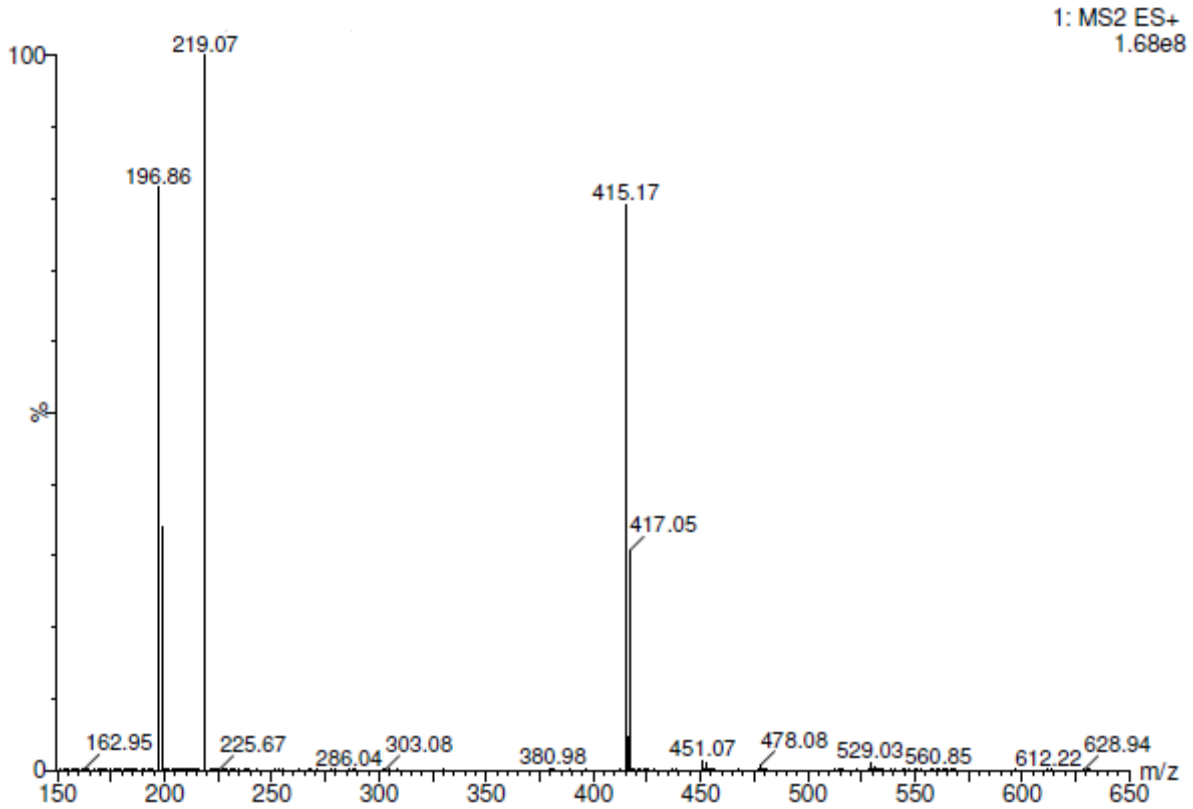
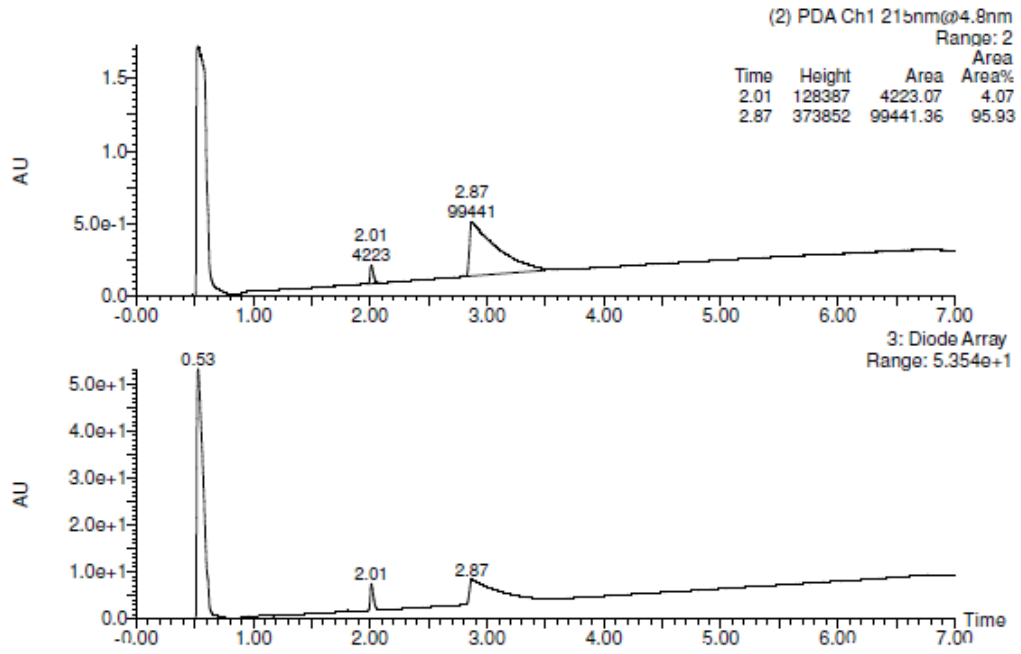
Compound 6: UPLC/MS analysis



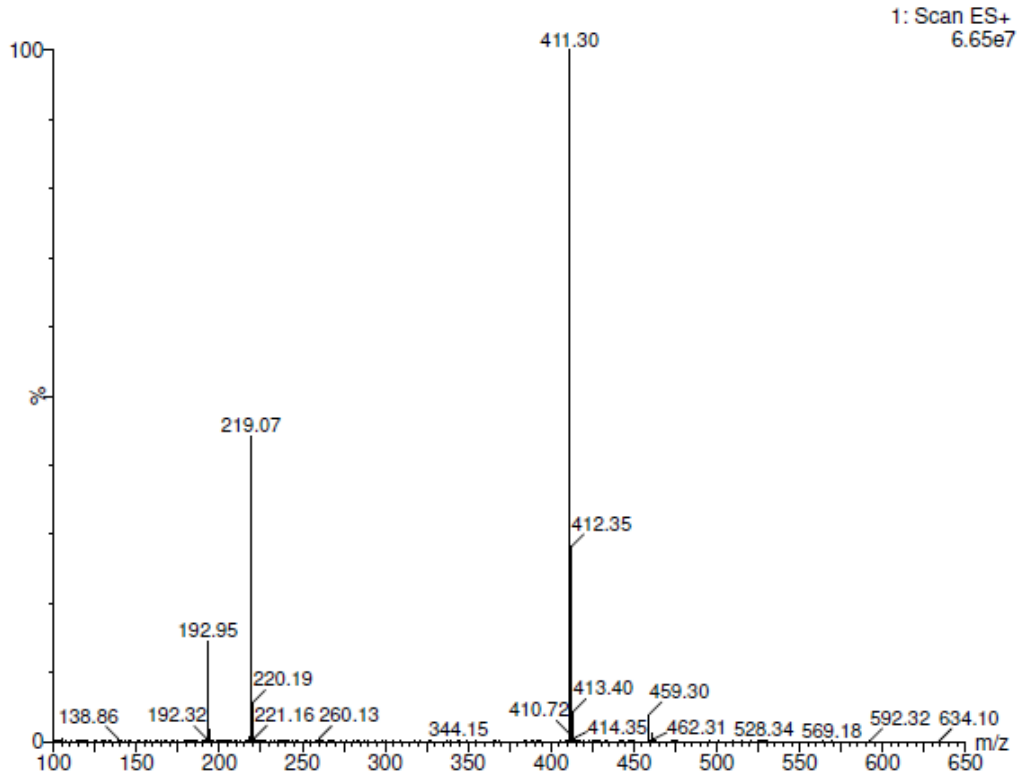
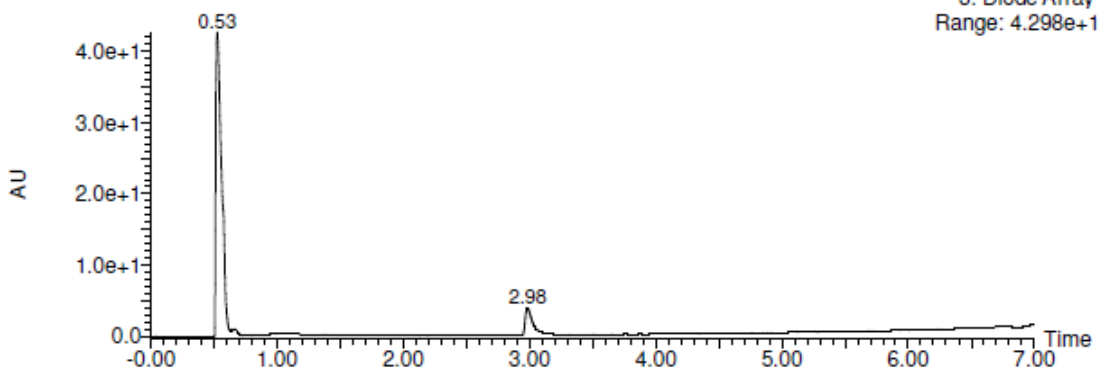
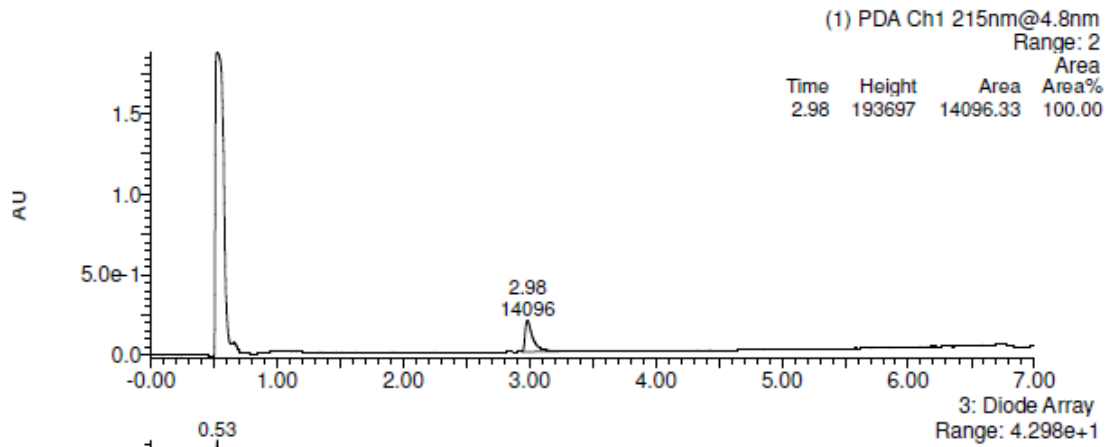
Compound 7: UPLC/MS analysis



Compound 8: UPLC/MS analysis

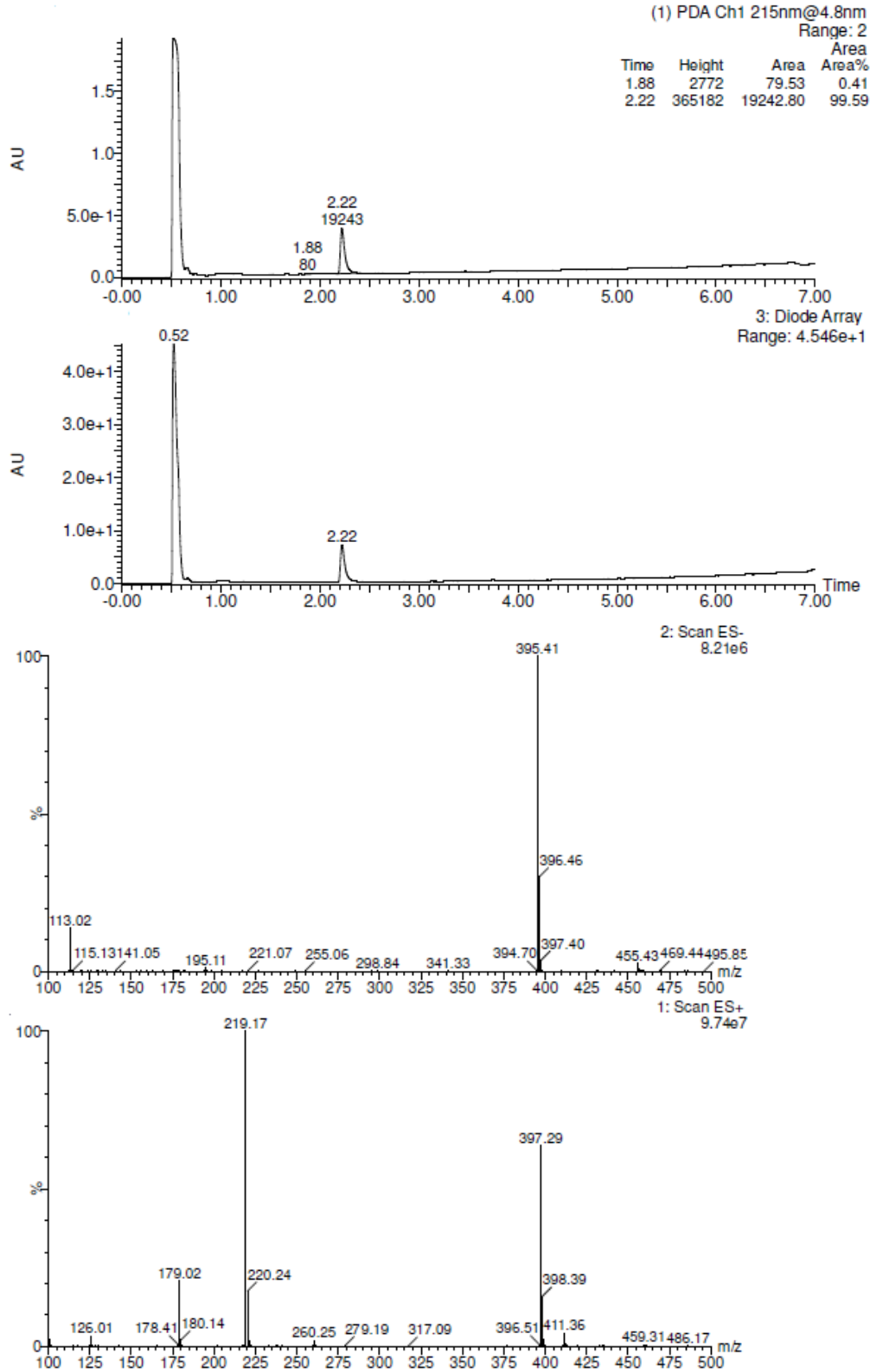


Compound 9: UPLC/MS analysis

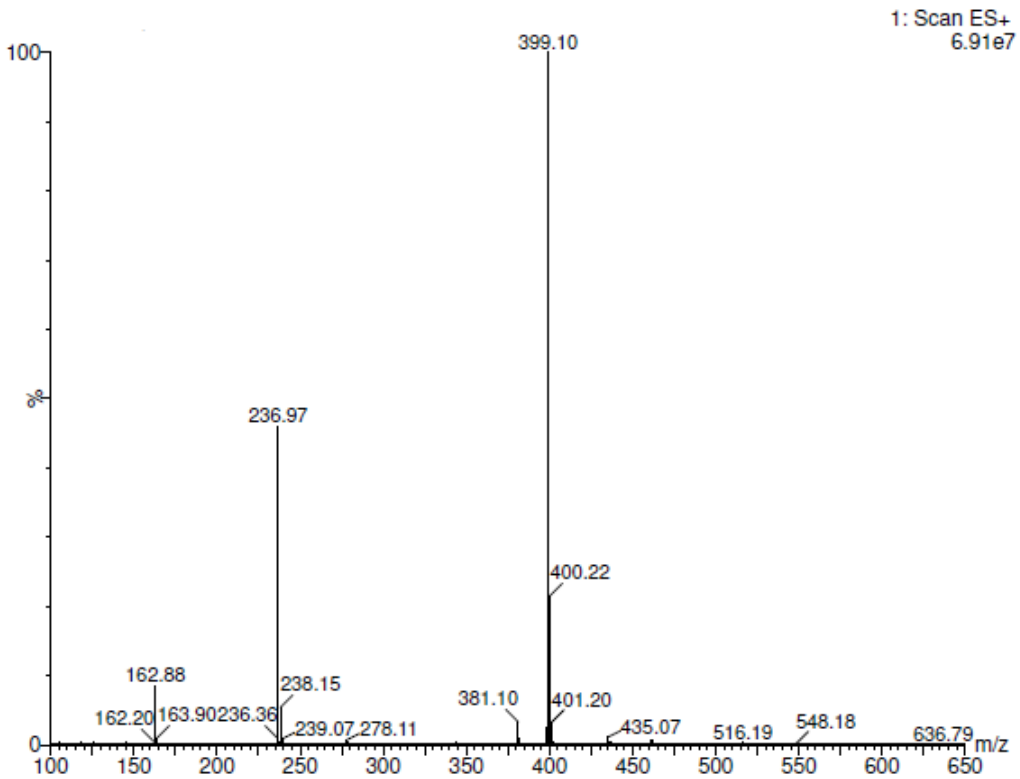
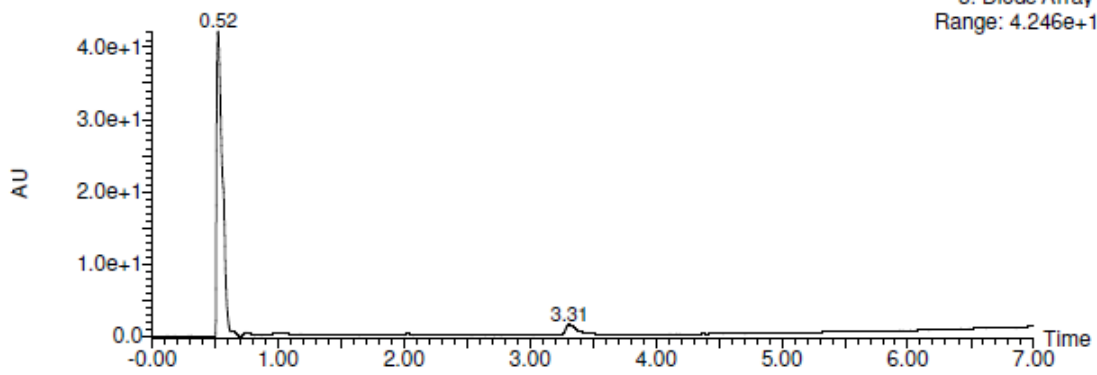
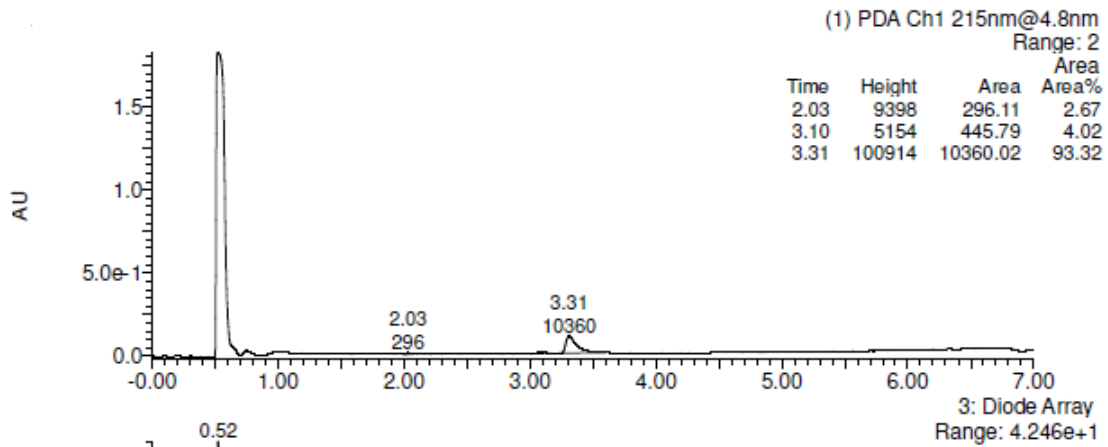




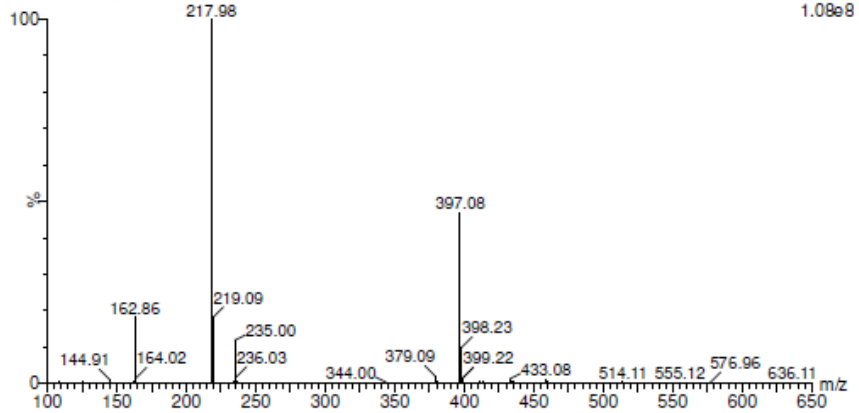
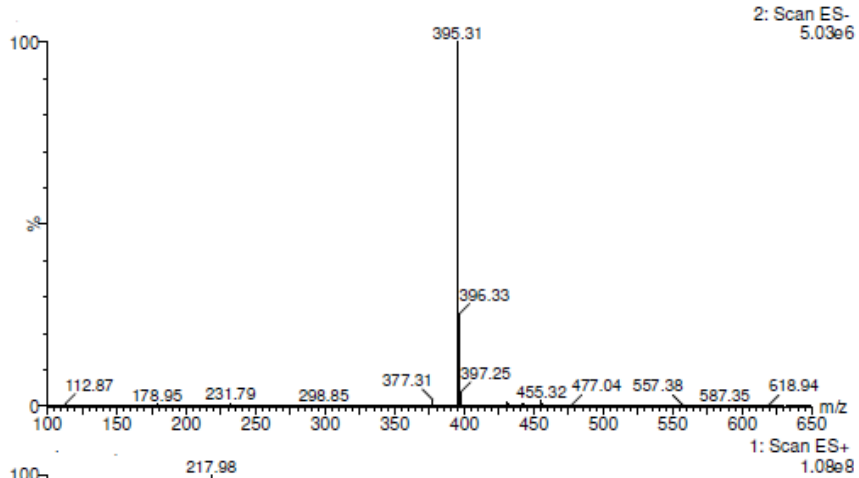
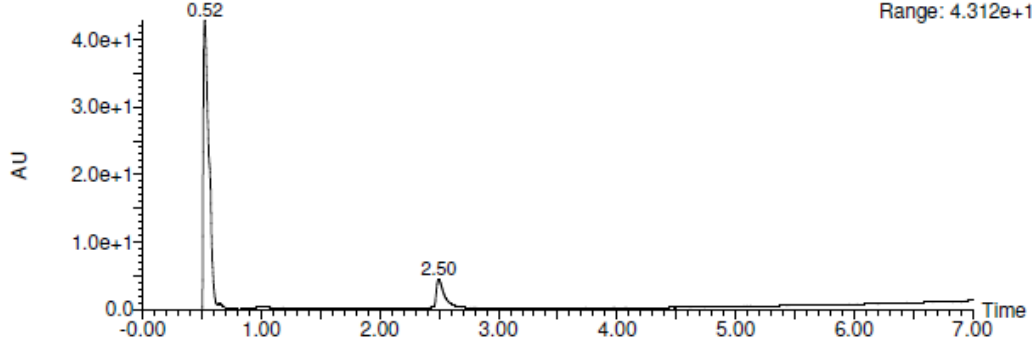
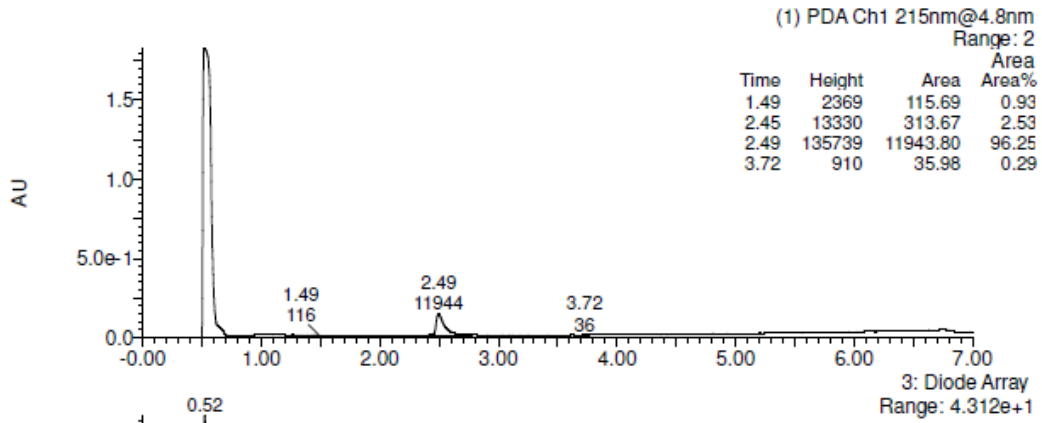
Compound **10**: UPLC/MS analysis



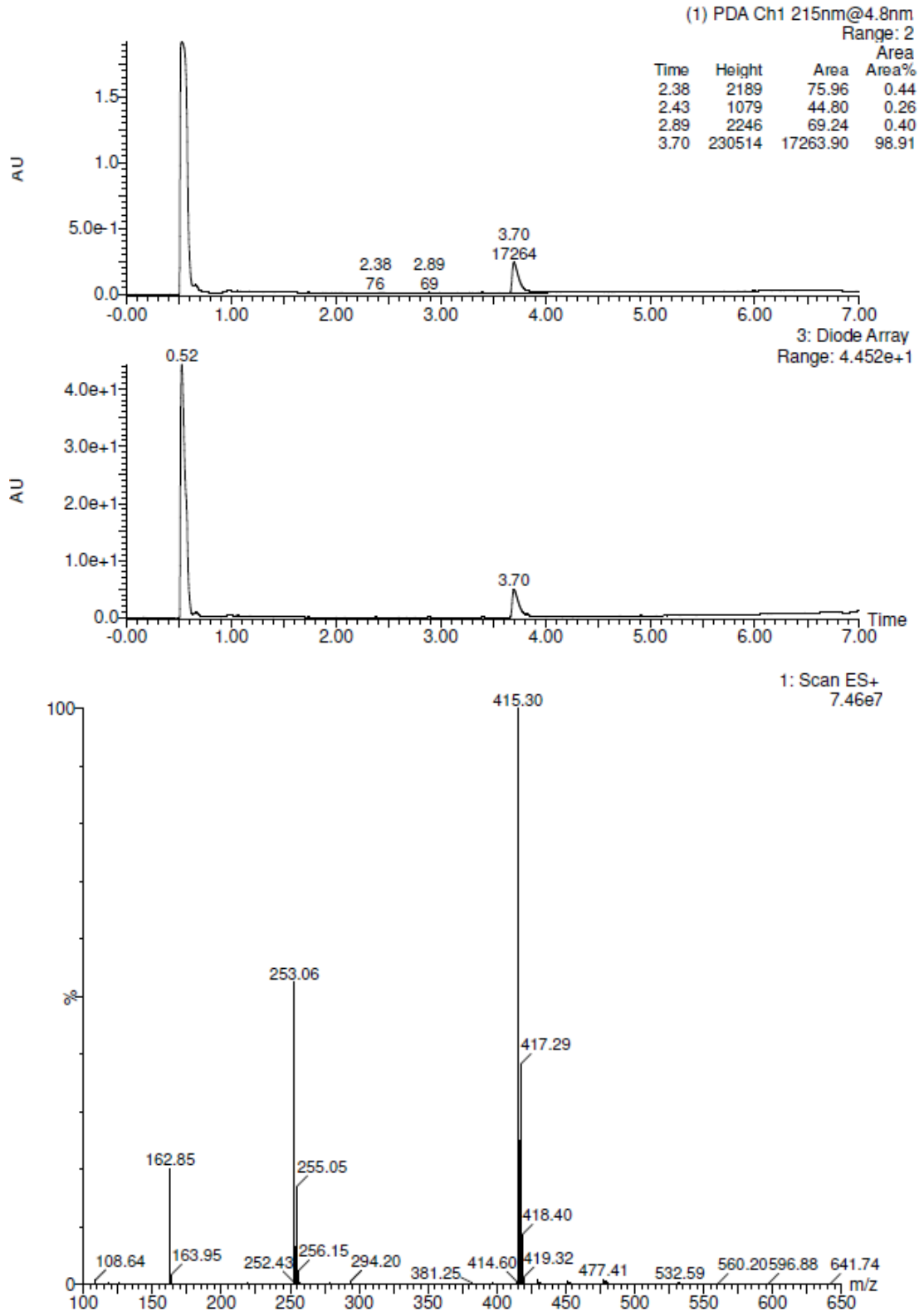
Compound 11: UPLC/MS analysis



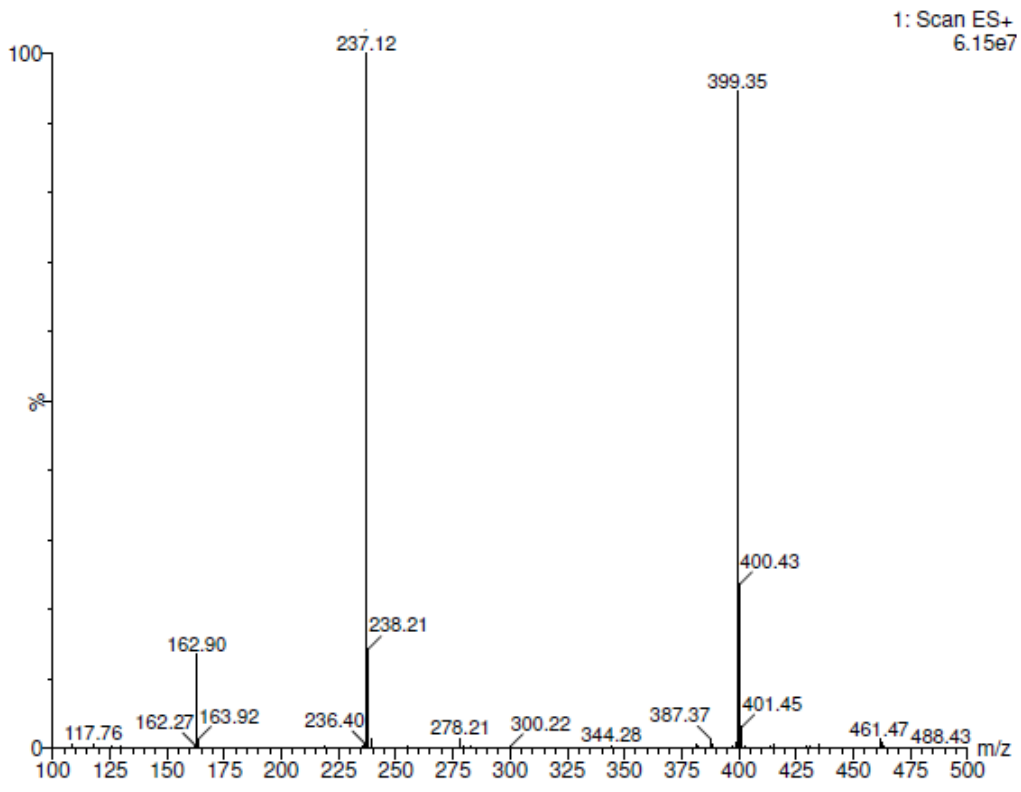
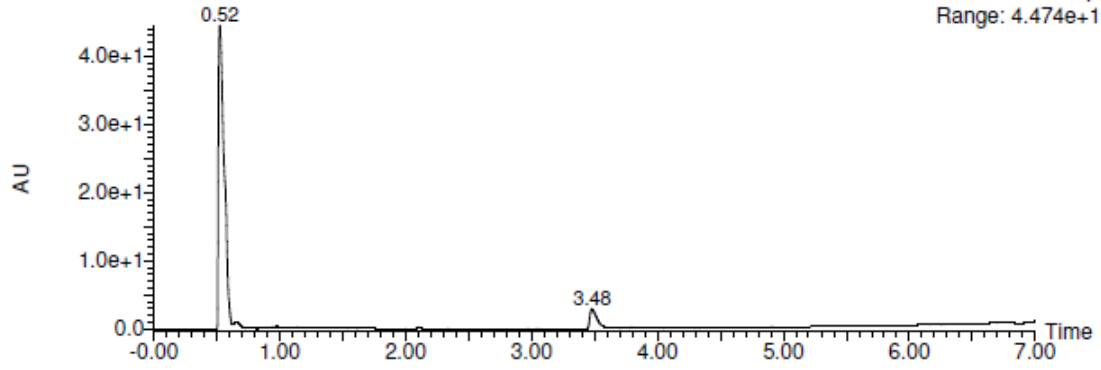
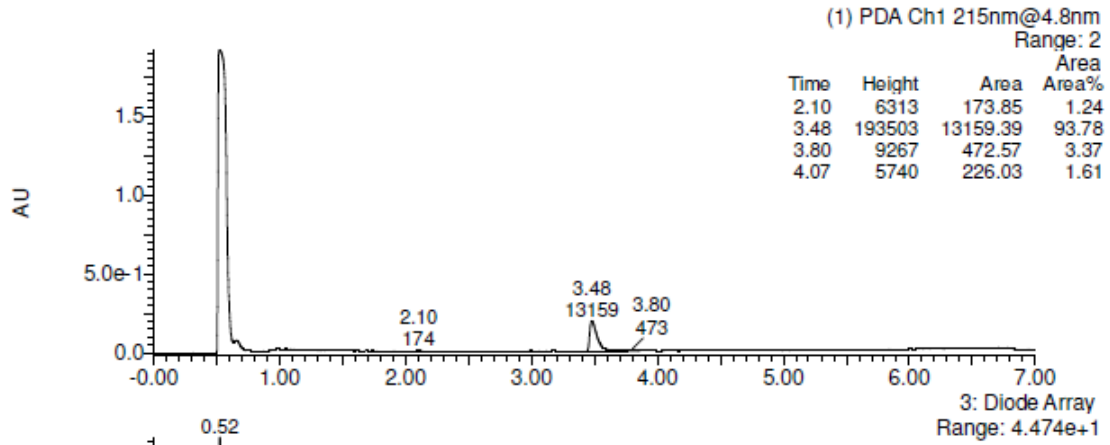
Compound 12: UPLC/MS analysis



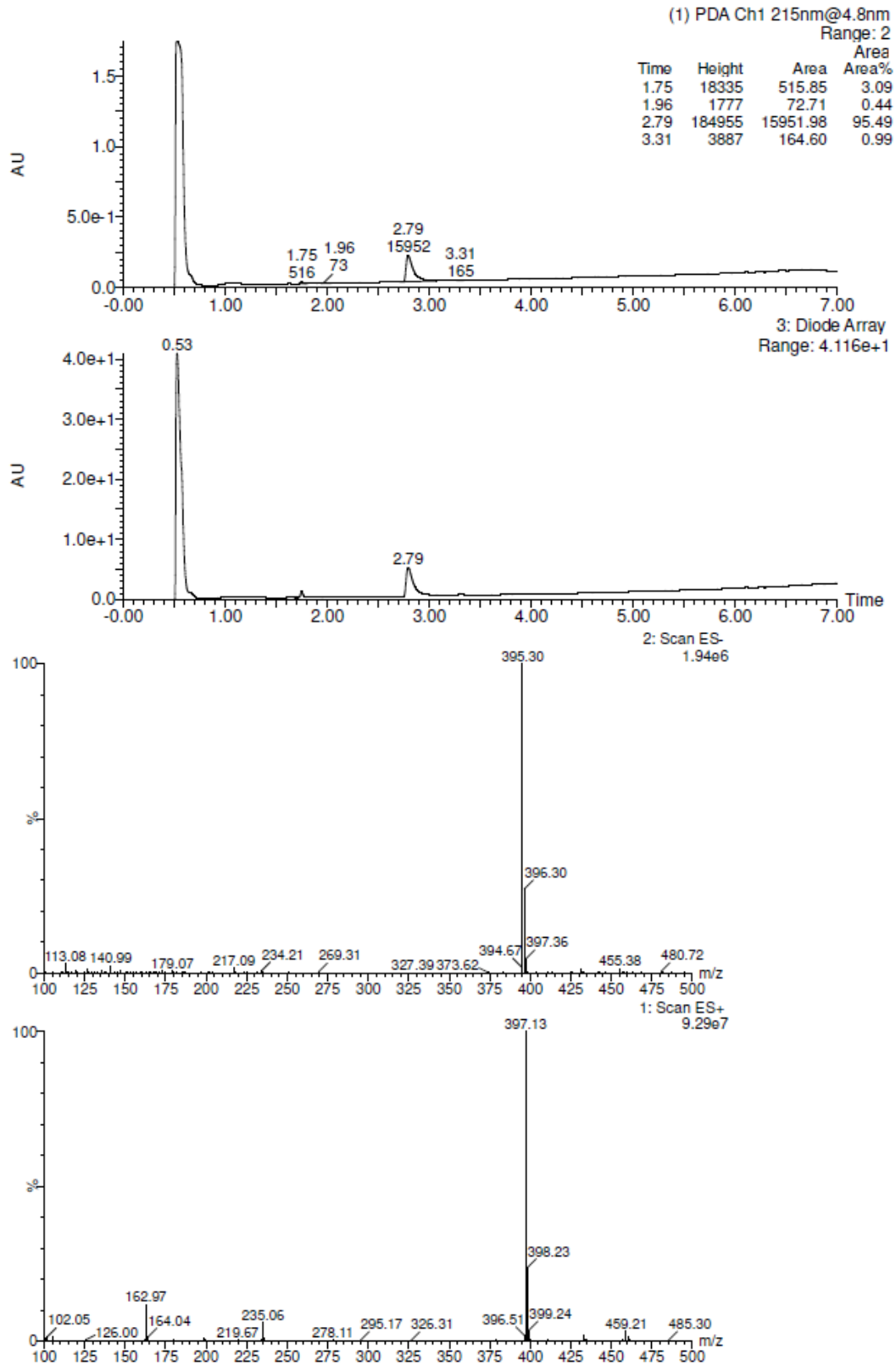
Compound 13: UPLC/MS analysis



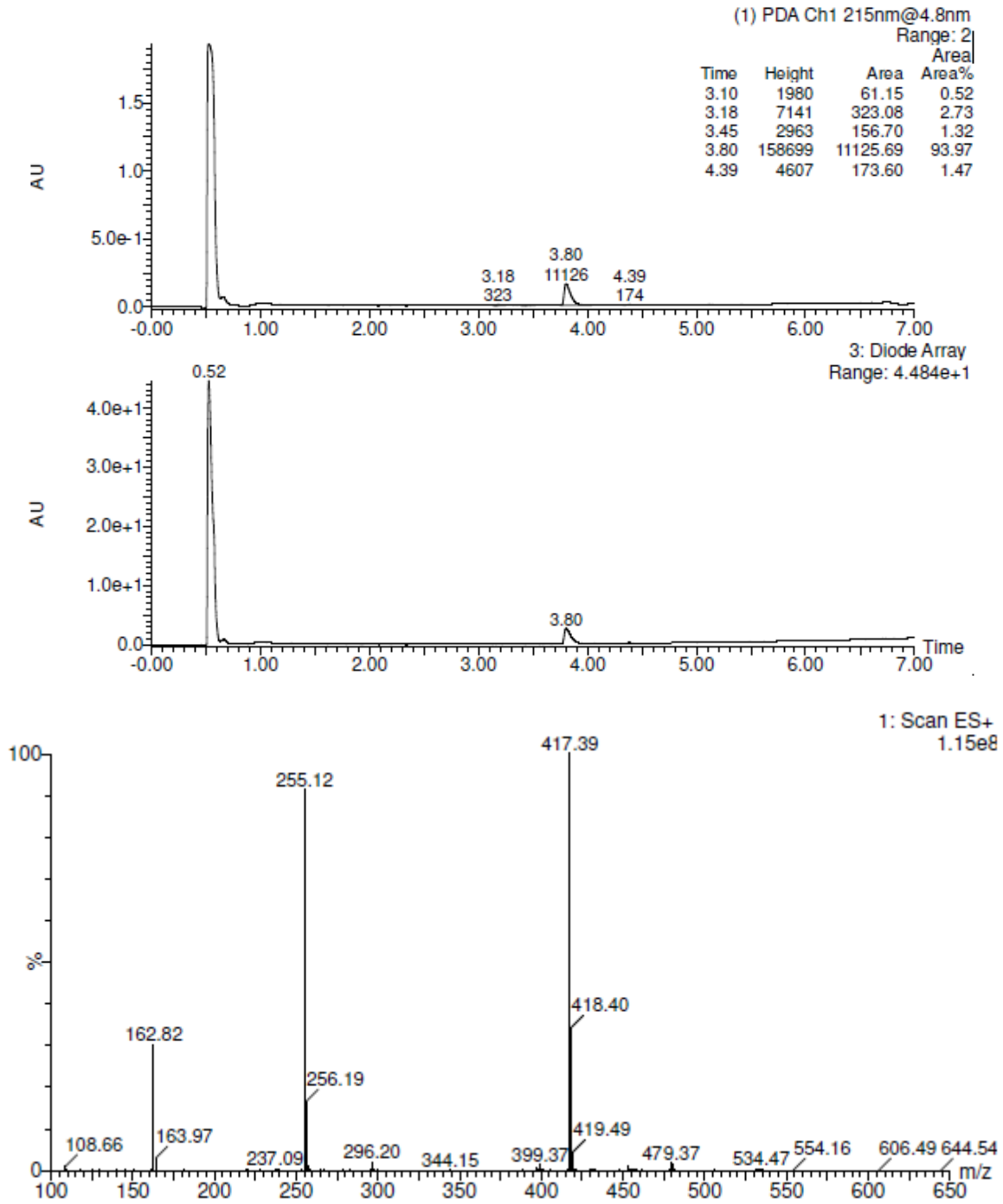
Compound 14: UPLC/MS analysis



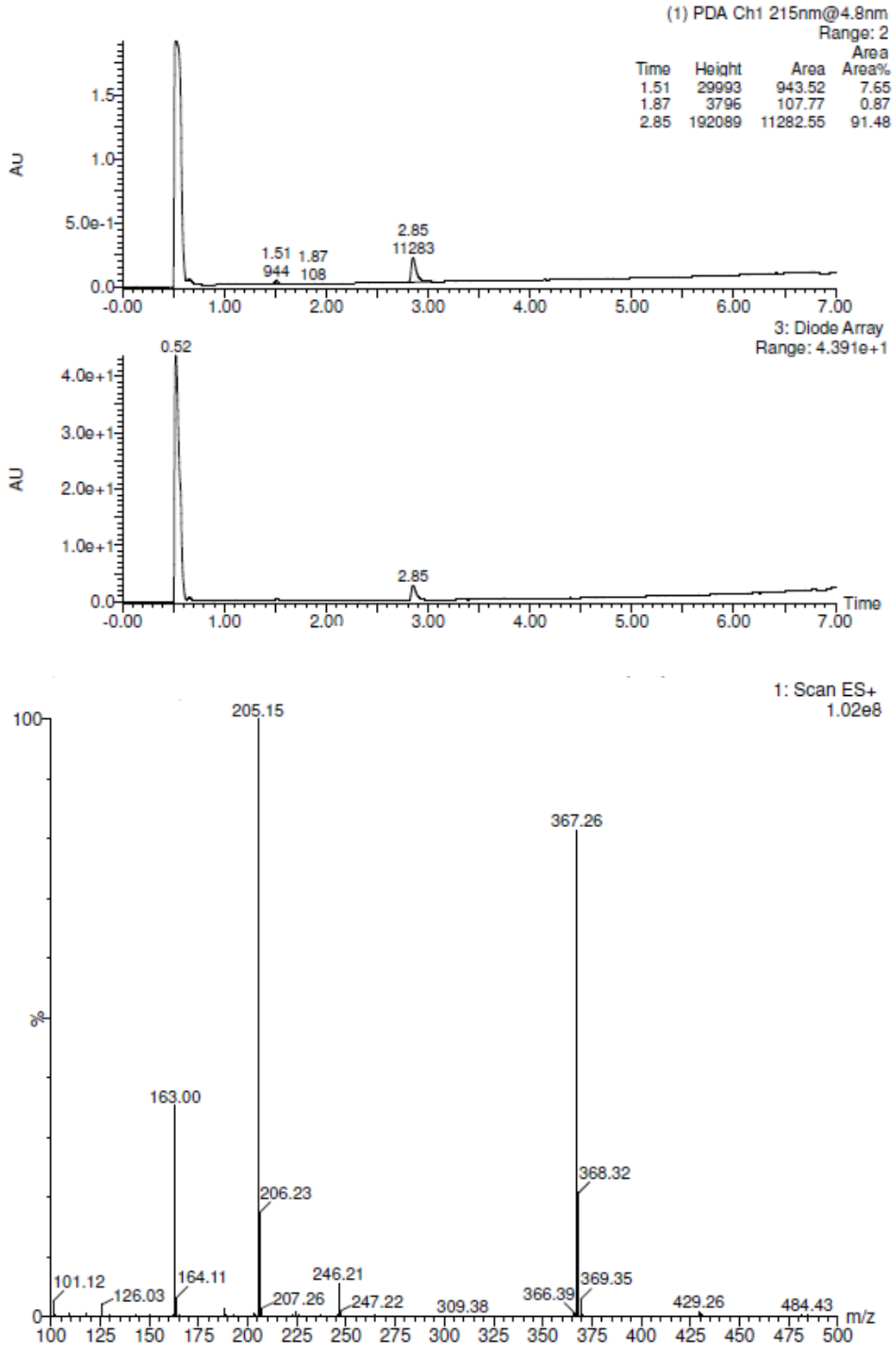
Compound **15**: UPLC/MS analysis



Compound **16**: UPLC/MS analysis

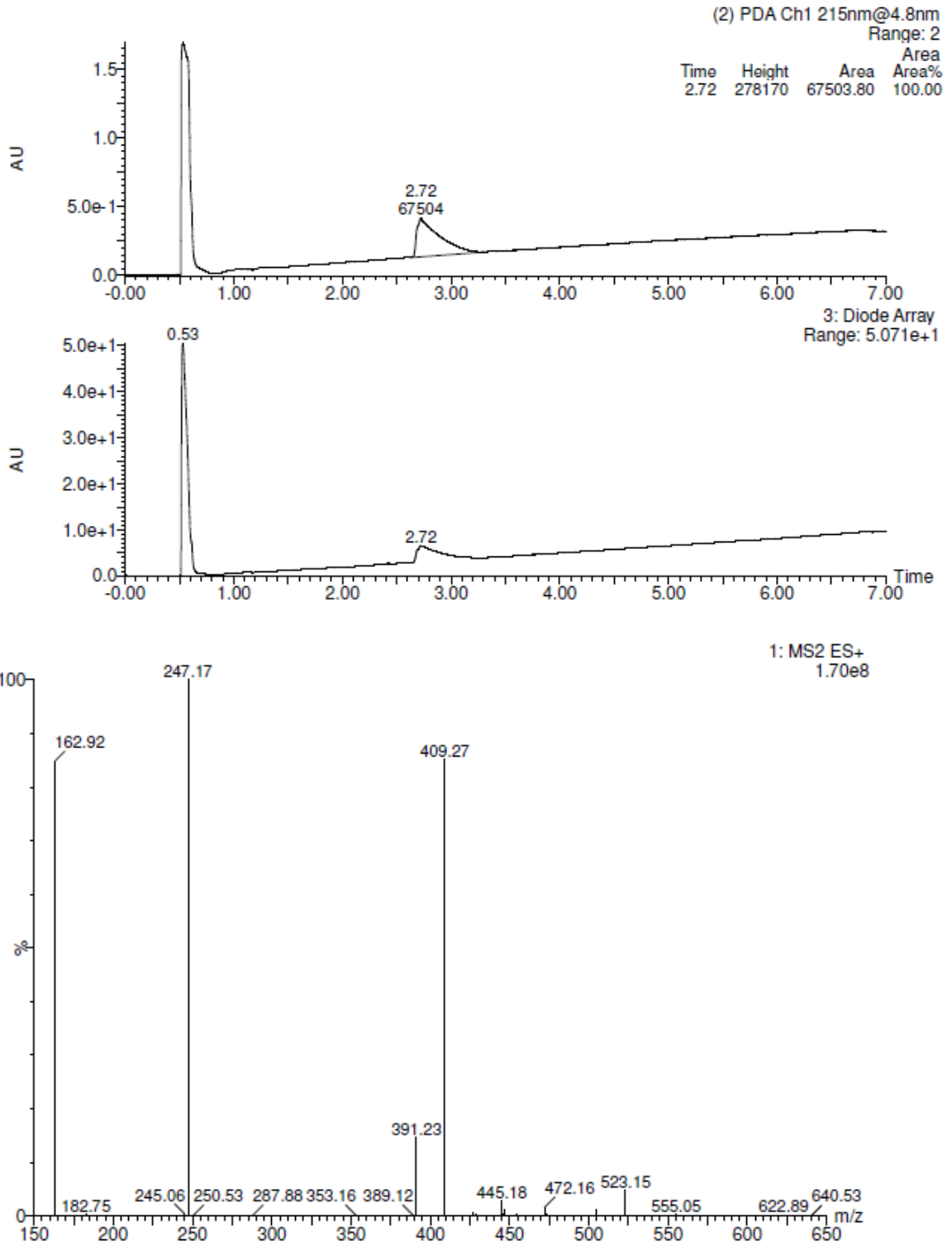


Compound 17: UPLC/MS analysis

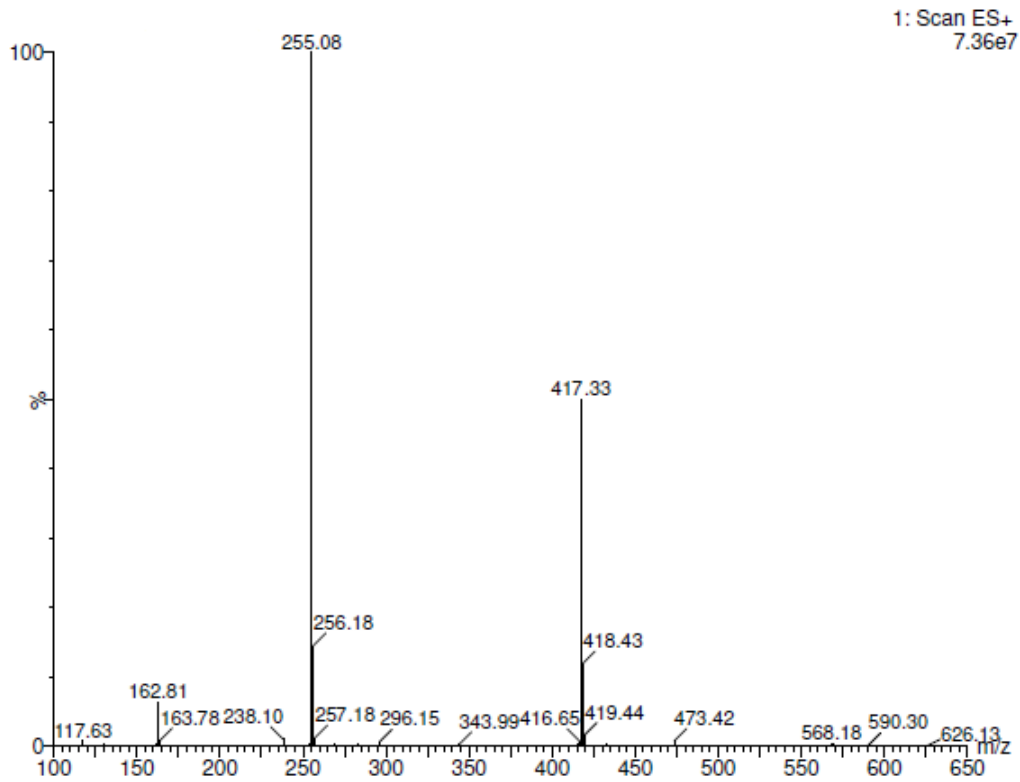
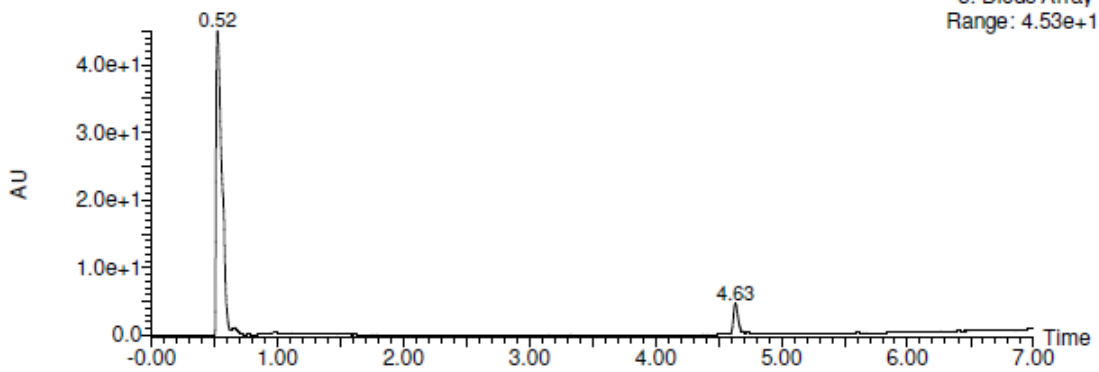
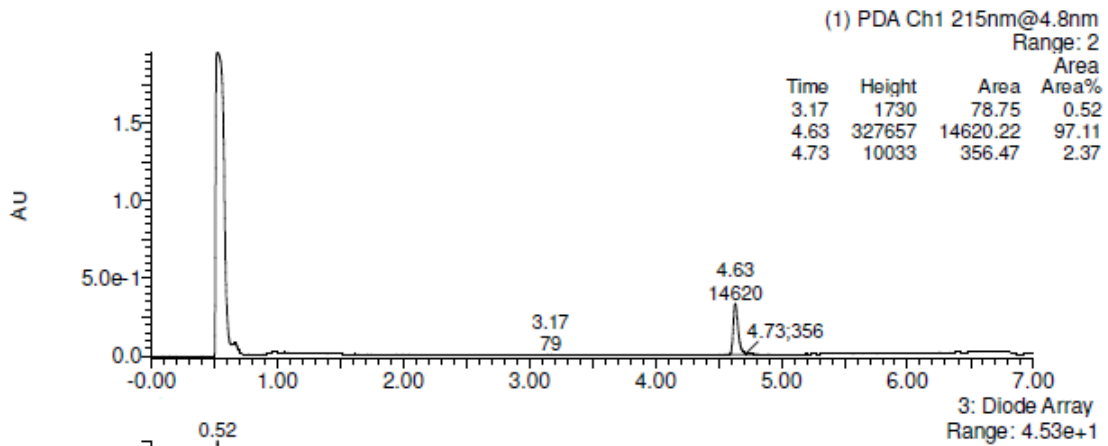




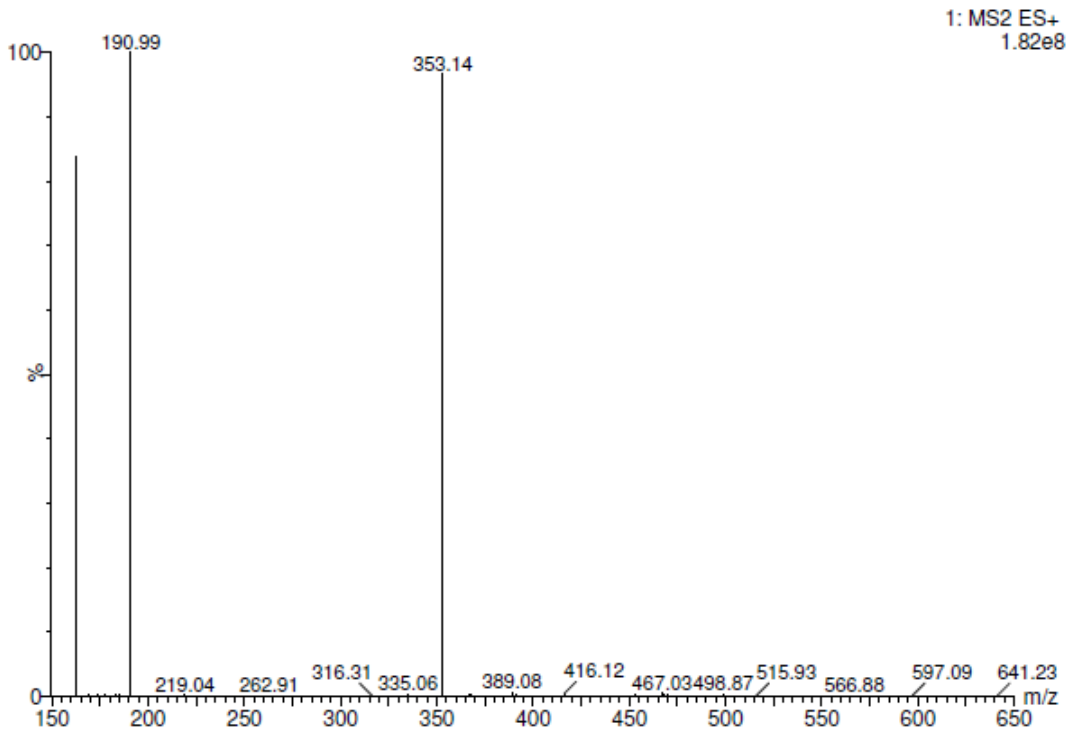
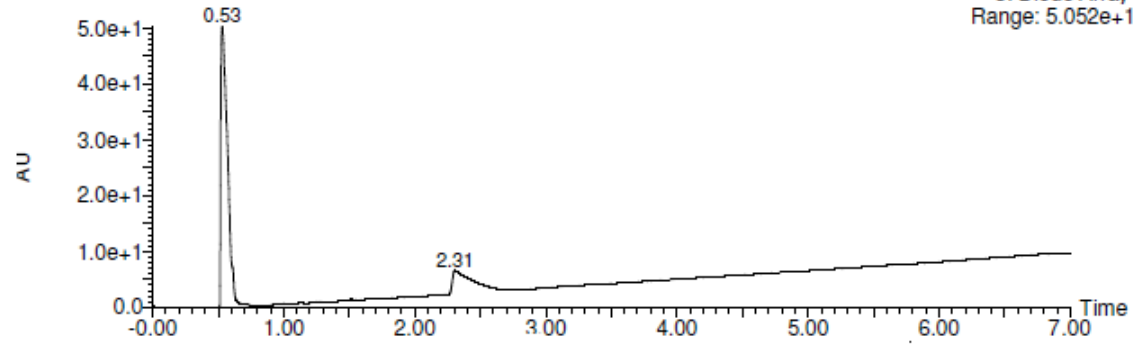
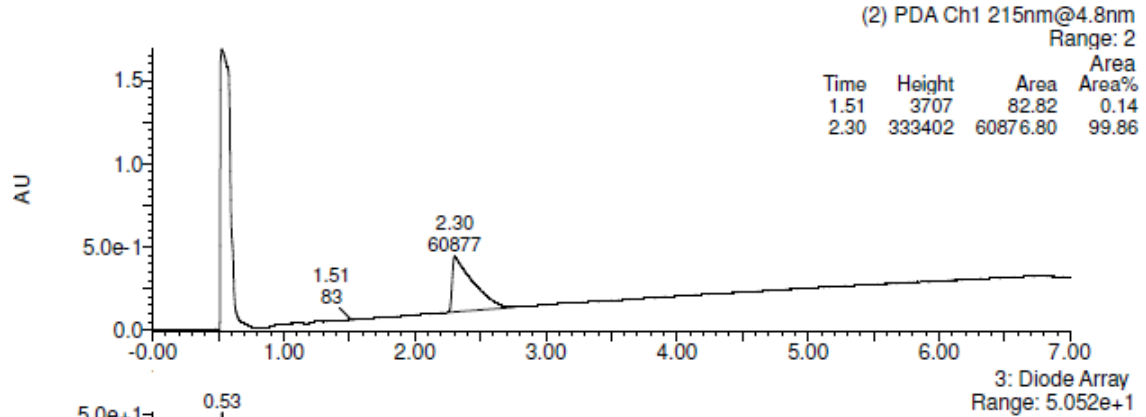
Compound 18: UPLC/MS analysis



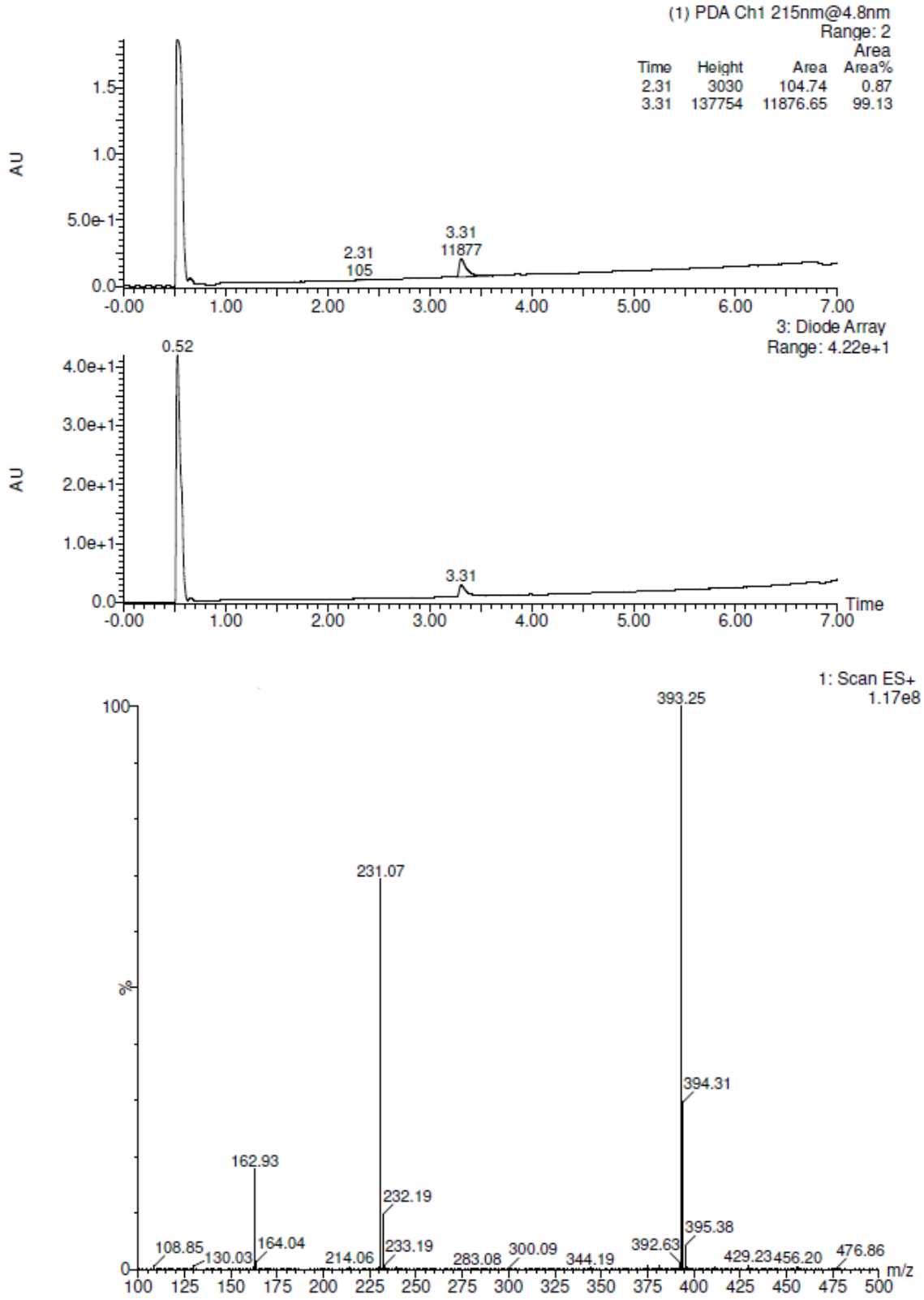
Compound 19: UPLC/MS analysis



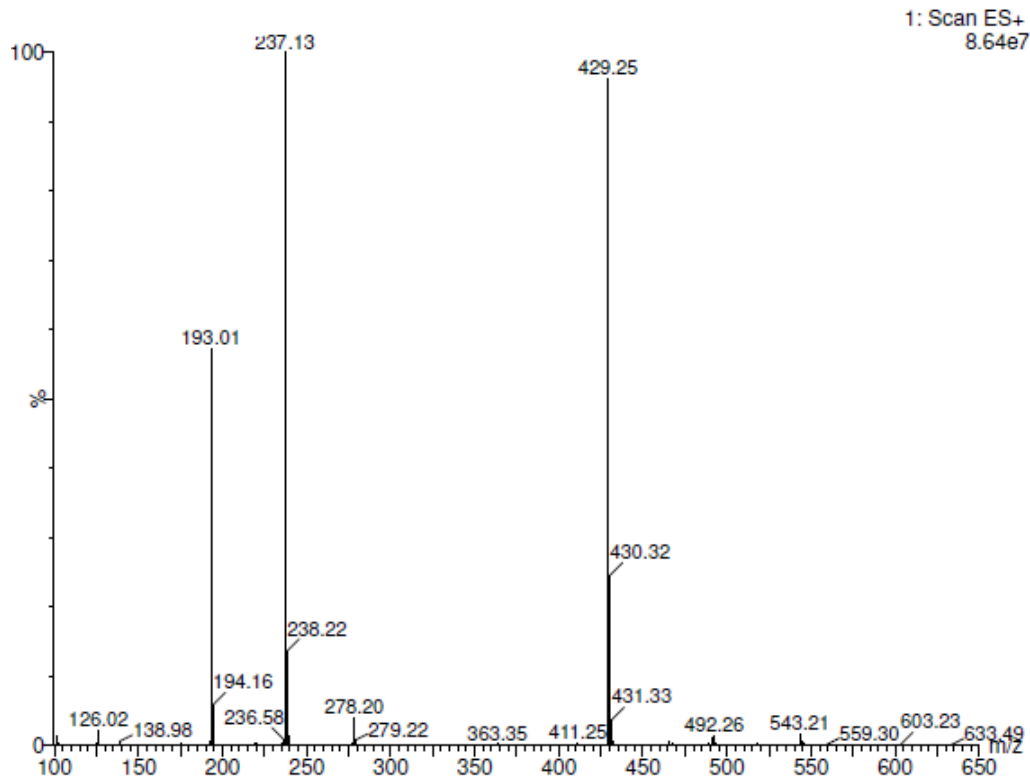
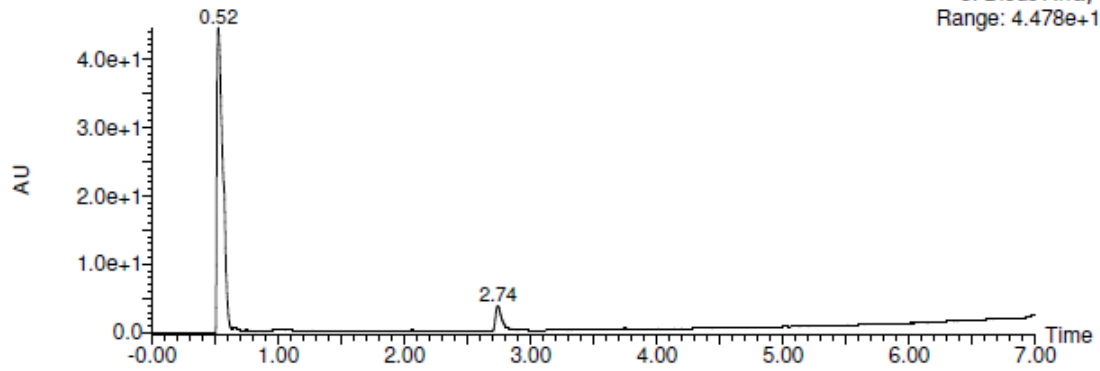
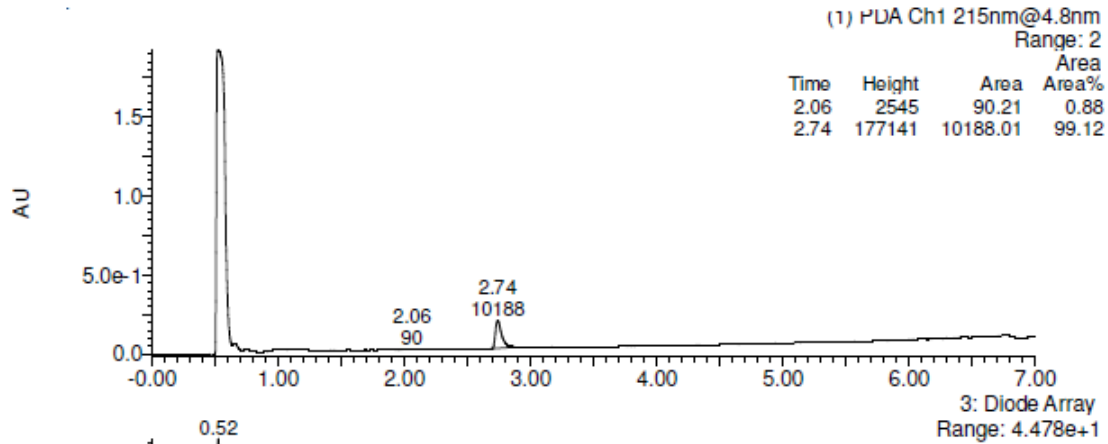
Compound 20: UPLC/MS analysis



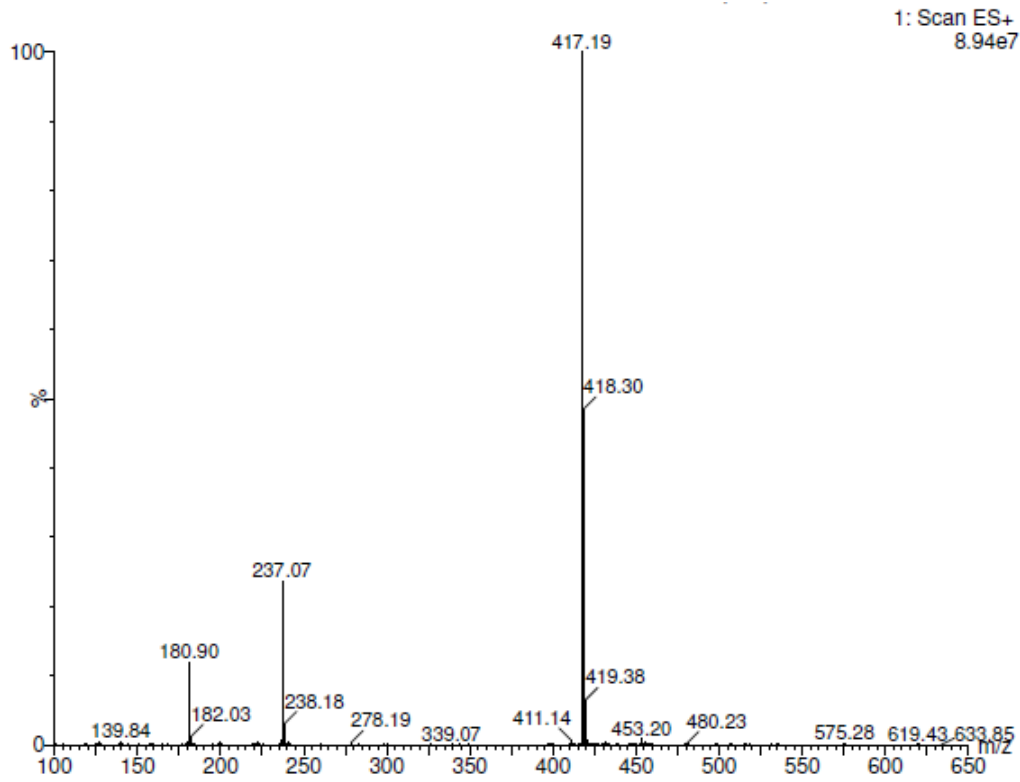
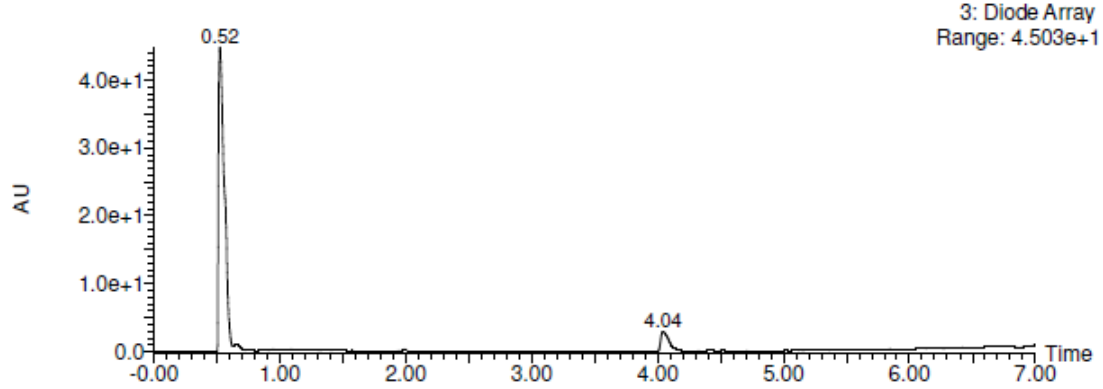
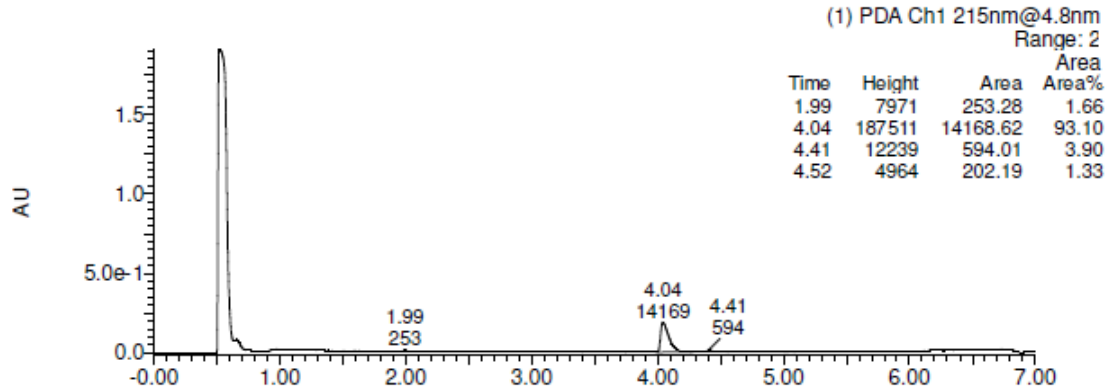
Compound **21**: UPLC/MS analysis



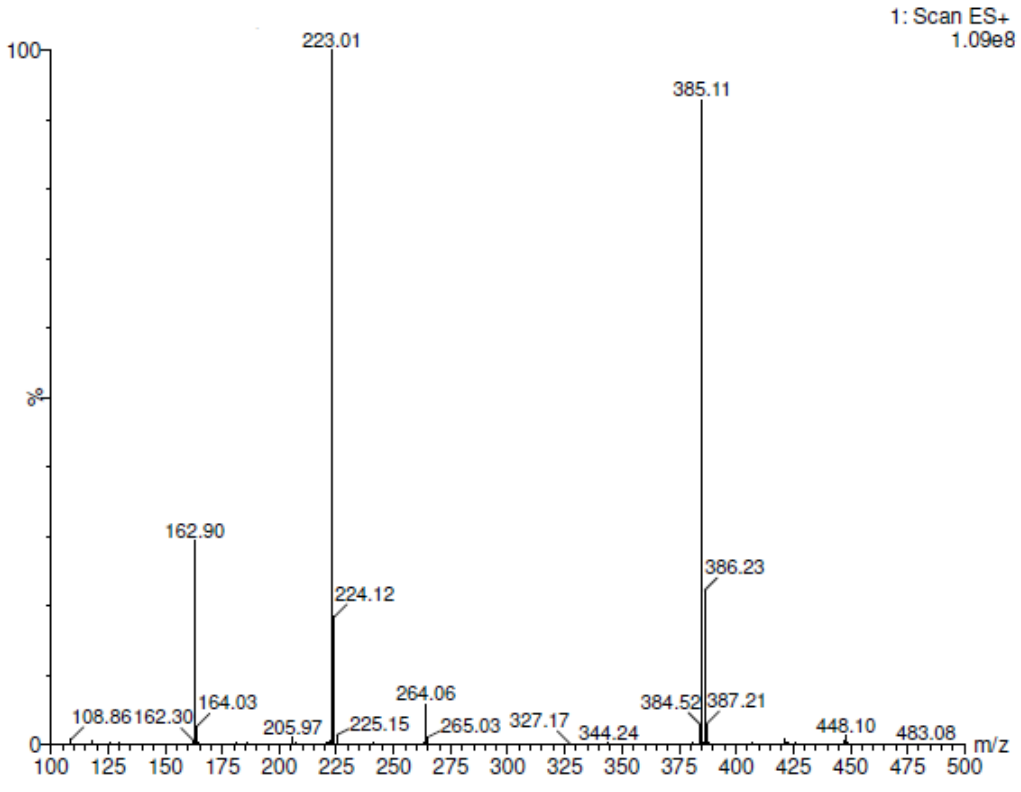
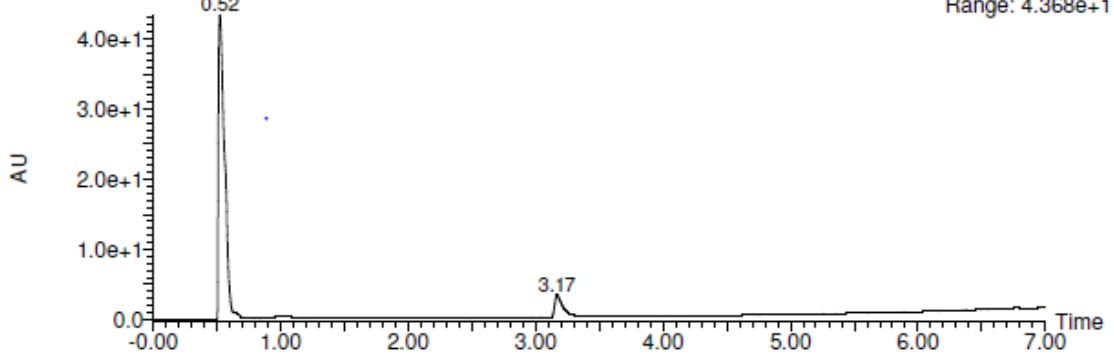
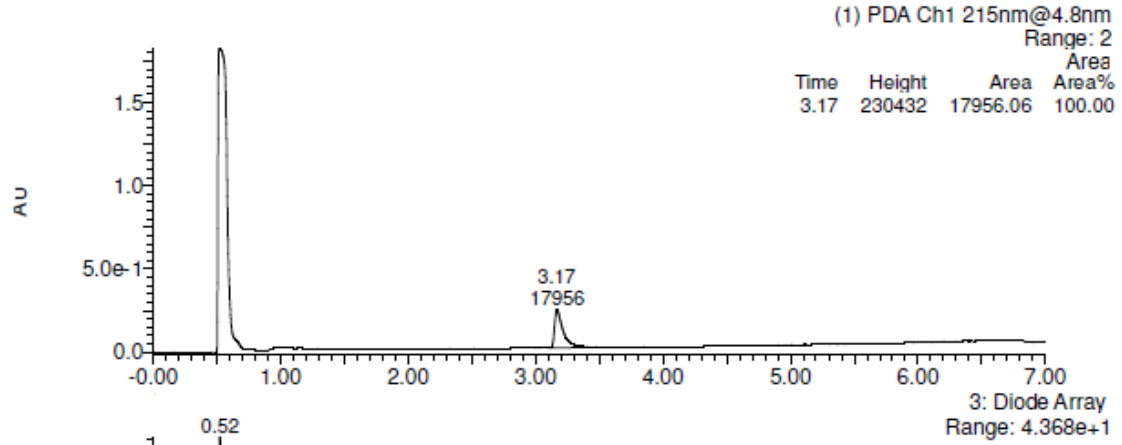
Compound 22: UPLC/MS analysis



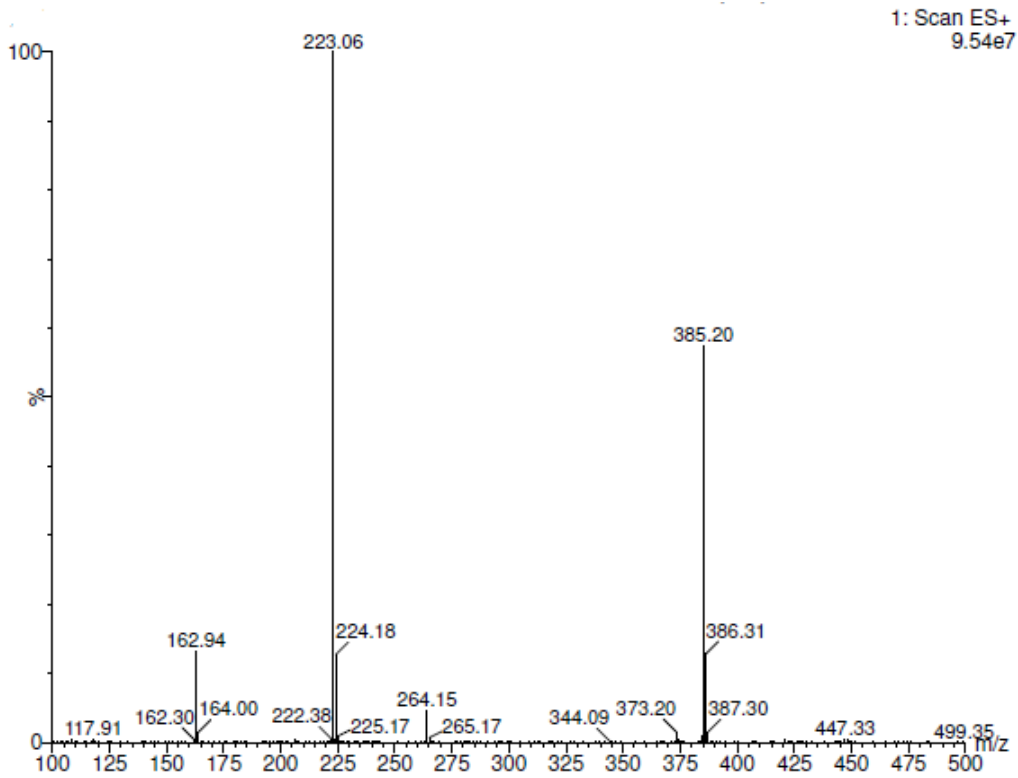
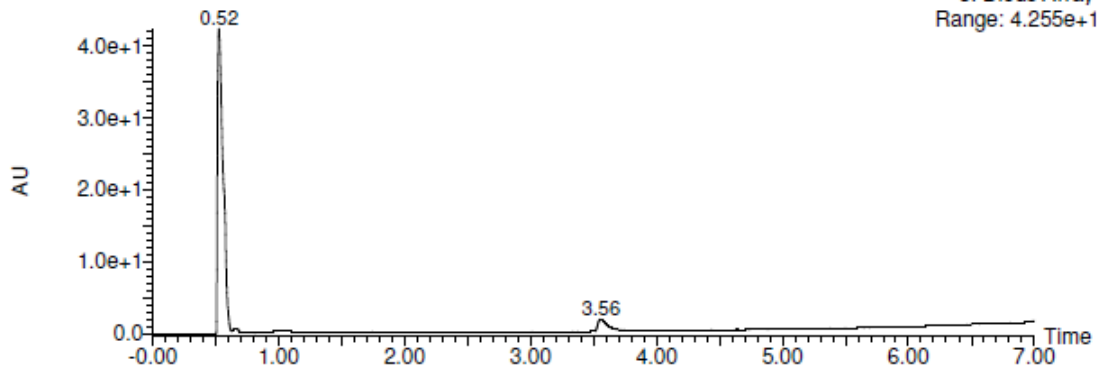
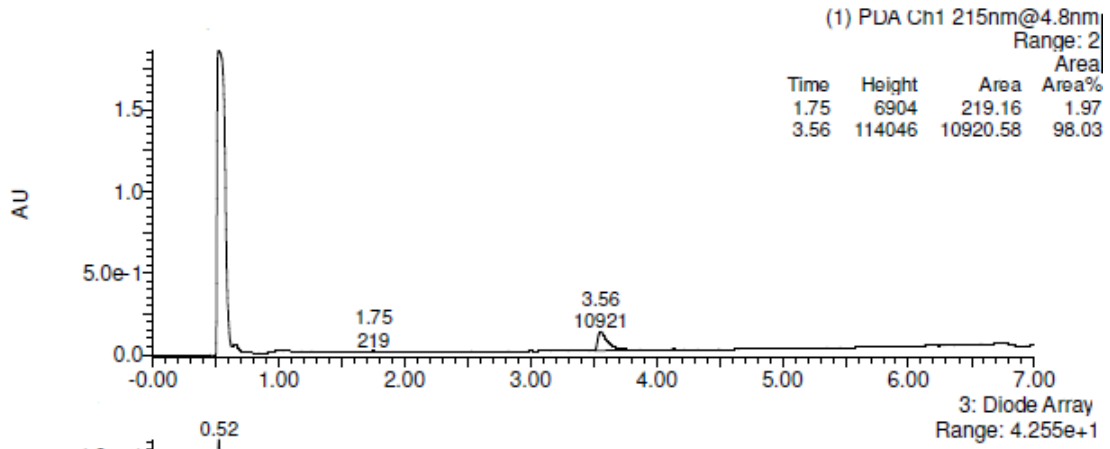
Compound 23: UPLC/MS analysis



Compound 24: UPLC/MS analysis

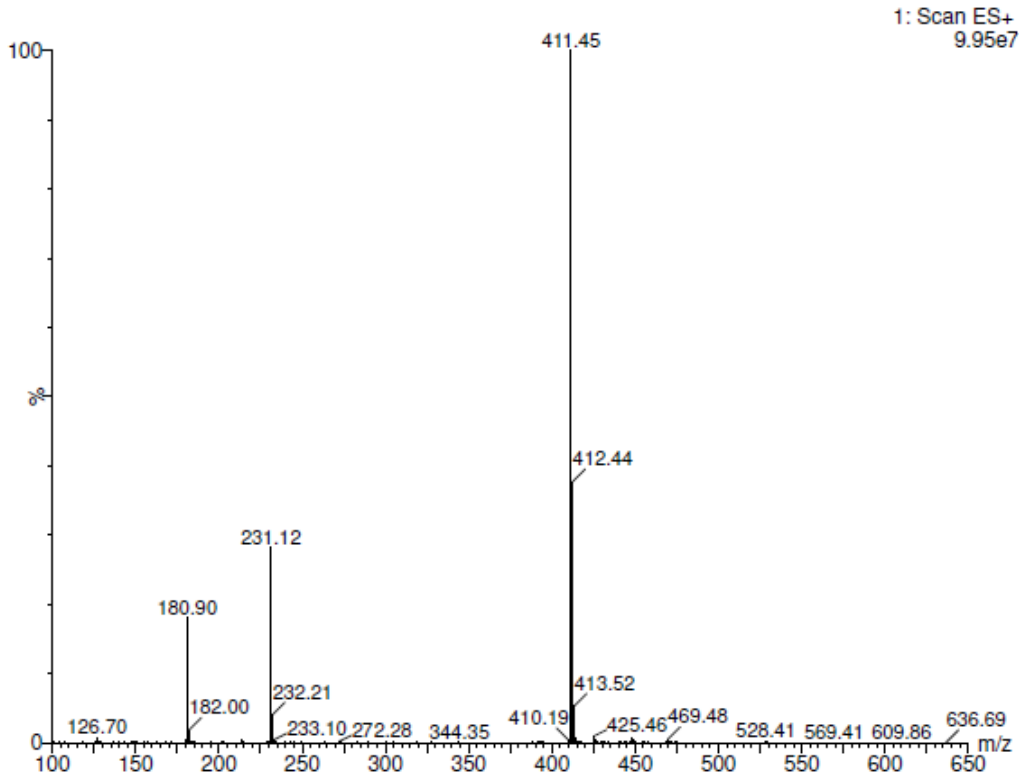
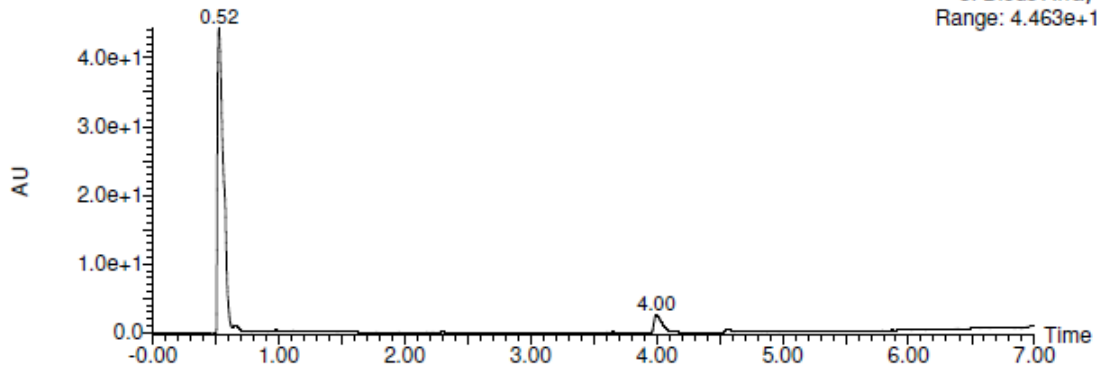
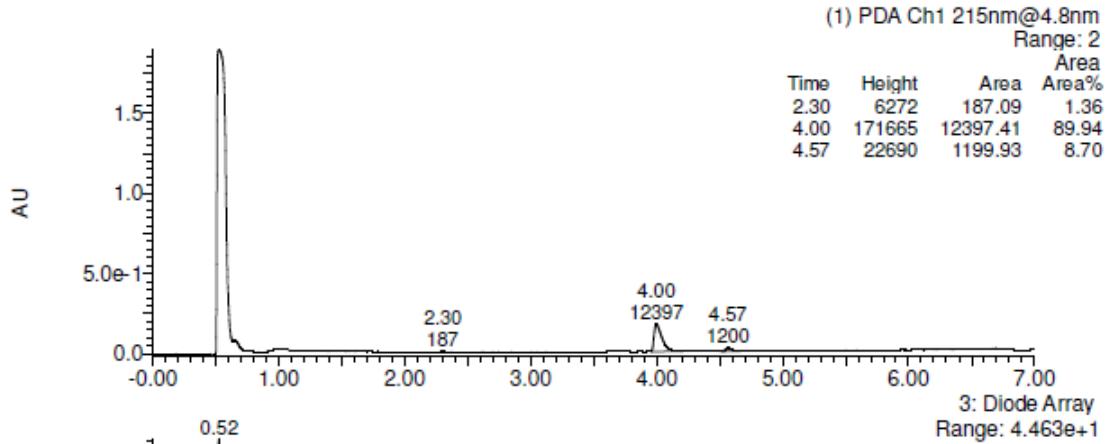


Compound 25: UPLC/MS analysis





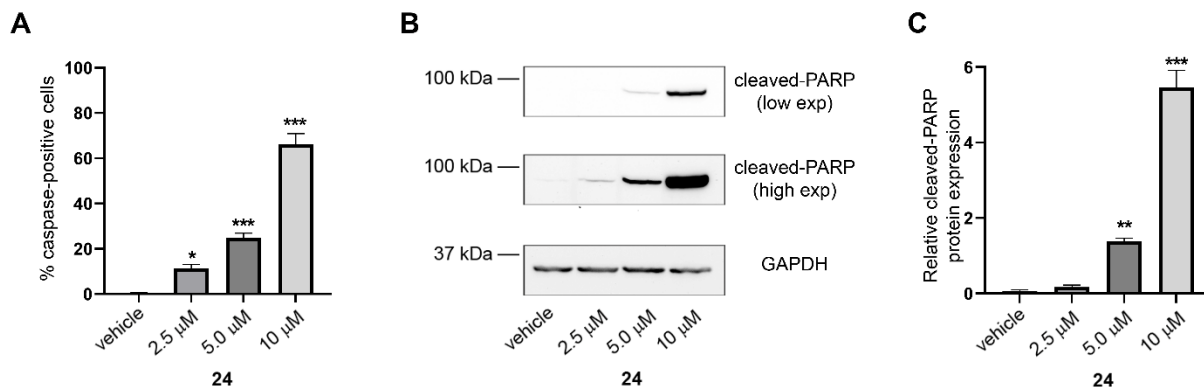
Compound **26**: UPLC/MS analysis



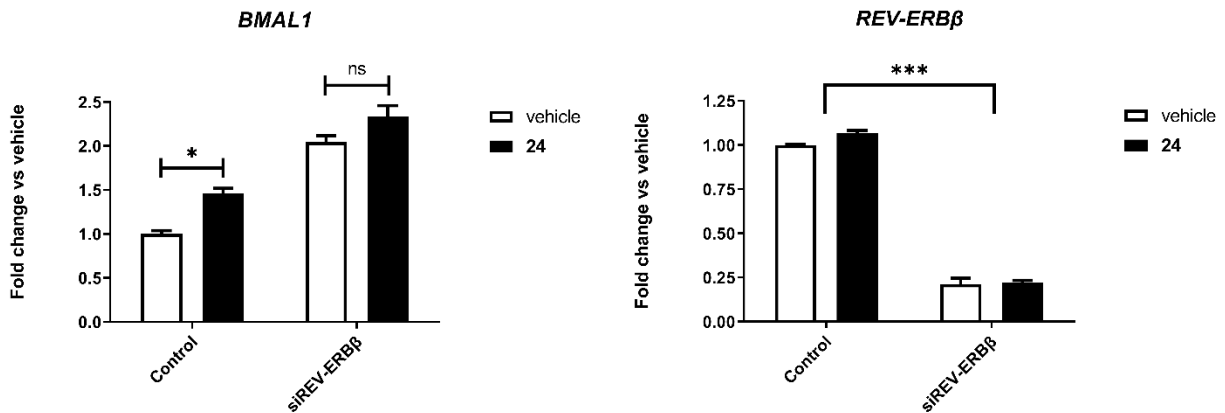
**Table S1.** Retention times and UPLC analytical method of the final compounds.<sup>a</sup>

<b>Compound</b>	<b>Rt (min)</b>	<b>Method</b>	<b>Compound</b>	<b>Rt (min)</b>	<b>Method</b>
<b>3</b>	3.80	<i>E</i>	<b>15</b>	2.79	<i>E</i>
<b>4</b>	4.60	<i>E</i>	<b>16</b>	3.80	<i>E</i>
<b>5</b>	2.63	<i>E</i>	<b>17</b>	2.85	<i>E</i>
<b>6</b>	3.66	<i>E</i>	<b>18</b>	2.72	<i>E</i>
<b>7</b>	3.65	<i>E</i>	<b>19</b>	4.63	<i>E</i>
<b>8</b>	2.87	<i>E</i>	<b>20</b>	2.30	<i>E</i>
<b>9</b>	2.96	<i>E</i>	<b>21</b>	3.31	<i>E</i>
<b>10</b>	2.22	<i>E</i>	<b>22</b>	2.74	<i>E</i>
<b>11</b>	3.31	<i>E</i>	<b>23</b>	4.04	<i>E</i>
<b>12</b>	2.49	<i>E</i>	<b>24</b>	3.17	<i>E</i>
<b>13</b>	3.70	<i>E</i>	<b>25</b>	3.56	<i>E</i>
<b>14</b>	3.48	<i>E</i>	<b>26</b>	4.00	<i>E</i>

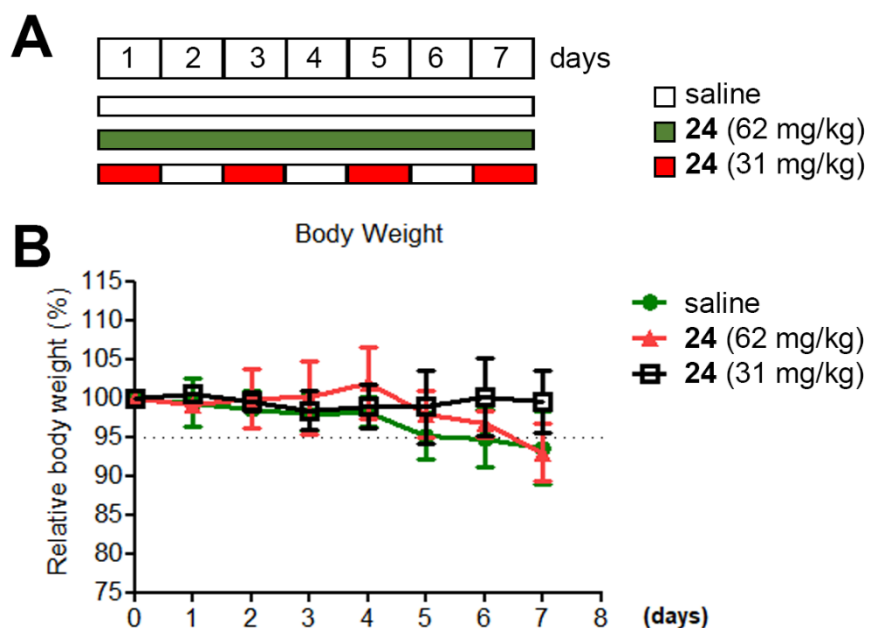
<sup>a</sup>Freshly prepared 10 mM DMSO-*d*<sub>6</sub> stock solutions (used for biological screenings), diluted 20-fold or 100 fold in CH<sub>3</sub>CN/H<sub>2</sub>O (1:1), and directly analyzed. The analysis was performed on an ACQUITY UPLC BEH C18 column (100x2.1mmID, particle size: 1.7μm) with a VanGuard BEH C18 pre-column (5x2.1mmID, particle size: 1.7μm) at 40 °C using 10mM NH<sub>4</sub>OAc in H<sub>2</sub>O at pH 5 adjusted with AcOH (A) and 10mM NH<sub>4</sub>OAc in CH<sub>3</sub>CN-H<sub>2</sub>O (95:5) at pH 5 (B) as mobile phase at 0.5mL/min. *Method E*: gradient 10 to 90% B over 6.0 min. Flow rate 0.5 mL min<sup>-1</sup>. Temperature 40 °C. The detection wavelength (λ) was set at 215 nm for relative purity determination.



**Figure S1.** Compound **24** induces apoptosis in BT-474 cells. (A) BT-474 cells were treated with the indicated concentrations of **24** or DMSO (vehicle). After 24 h, caspase activity was evaluated with a fluorescent inhibitor of caspases covalently bound to the poly-caspase-specific amino acid sequence valine-alanine-aspartic acid (VAD) (SR-VAD-FMK). Count of the percentage of caspase-positive cells is given as mean  $\pm$  SEM,  $n=3$ . \* $P<0.05$  and \*\*\* $P<0.001$ , (one-way ANOVA with Dunnett's multiple comparison test). (B) Protein samples from BT-474 cells treated as in A were probed with specific antibodies against cleaved-PARP (cleaved-PARP) and GAPDH proteins. (C) Quantification of immunoblot analysis from protein samples treated as in B. Relative cleaved PARP expression was calculated normalizing the optical density of cleaved-PARP signals with that of GAPDH. Shown as mean  $\pm$  SEM,  $n=3$ . \*\* $P<0.01$  and \*\*\* $P<0.01$  (one-way ANOVA with Dunnett's multiple comparison test).



**Figure S2.** REV-ERB $\beta$  silencing abolishes compound **24**-mediated transcriptional response. BT-474 cells were transfected with pooled siRNA sequences against *REV-ERB $\beta$*  (siREV-ERB $\beta$ ) or a non-targeting pool as a control (Control). One day post transfection, cells were treated with DMSO (vehicle) or 5  $\mu$ M of compound **24** for 24 h and the expression of the REV-ERB target gene, *BMAL1*, and *REV-ERB $\beta$*  was determined by quantitative reverse transcriptase-PCR (qRT-PCR) using *GAPDH* for normalization. Shown as mean  $\pm$  SEM, n = 3. \*P<0.05, **24**-treated versus vehicle treated control cells; \*\*\*P<0.001, **24**- and vehicle-treated siREV-ERB $\beta$  versus **24**- and vehicle-treated control cells (two-way ANOVA with Bonferroni's posttest analysis).



**Figure S3.** Tolerability of **24** in CD1 mice. (A) Female CD1 mice were treated with i.p. injections of **24** administered according to the schematized schedule treatments. (B) Animal body weight was monitored daily over a 7-day period. Weight of mice the day before starting the treatment was set as 100% and used for calculating the relative body weight (%) and is shown as mean  $\pm$  SEM,  $n = 3$  mice per each treatment group.