

## **Electronic Supporting Information**

# **Analytical determination of heroin, fentanyl and fentalogues using High-Performance Liquid Chromatography with Diode Array and Amperometric Detection**

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## Instrumentation

Voltammetric measurements were conducted using a ‘μAutolab type III’ (MetrohmAutolab, The Netherlands) potentiostat /galvanostat interfaced to a PC loaded with NOVA 2.1 software. All measurements were performed using a 10 mL voltammetric cell and a conventional three-electrode system. A platinum wire and Ag/AgCl were used as counter and reference electrodes, respectively. Screen-printed graphite macroelectrodes (SPEs), with a 3.1 mm diameter, were used as working electrodes, they were fabricated in-house as previously reported <sup>9, 25</sup>. Note that due to their scales of economy and reproducibility, a new SPE was used for each experiment performed.

### *Preparation of Standard Stock Solutions and Working Solutions for Cyclic Voltammetry*

12.5 mg of each of HRN, cocaine (COC), fentanyl hydrochloride (**2c**) and its derivatives (**2a**, **2b**, **2d – 2k**) were weighted separately into thirteen 25.0 mL glass volumetric flasks and diluted with ultrapure deionized water to obtain stock solutions of 0.5 mg mL<sup>-1</sup> of each. Working solutions were prepared as follows: 0.5 mL of each drug stock solution were transferred separately into 5.0 mL volumetric flasks, the volume was made to the mark using 0.04 M Britton-Robinson buffer (B-R buffer, pH 2.0) to obtain solutions of 50 μg mL<sup>-1</sup> of each drug. Each solution was transferred quantitatively to an electrochemical cell, degassed with pure nitrogen for 10 minutes, and cyclic voltammograms were recorded between +0.6 to +1.3 V (vs. Ag/AgCl) using the following scan rates: 5, 15, 25, 50, 100, 250 and 500 mV s<sup>-1</sup>. Similarly, working solutions in 0.04 M B-R buffer pHs 7.0 and 10.0 were prepared for each drug at a concentration level of 50 μg mL<sup>-1</sup>. Cyclic voltammograms were recorded for each drug at three different pHs: 2.0, 7.0 and 10.0, using scan rates t: 5, 15, 25, 50, 100, 250 and 500 mV s<sup>-1</sup>. Three new SPEs were used for each measurement and the average of the three anodic peak currents ( $I_{Pa}$ ) and the three anodic peak potentials ( $E_{Pa}$ ) were recorded. All solutions were protected from light by aluminum foil and the stock solutions were refrigerated at 4 °C for two weeks.

### *System suitability parameters*

System suitability parameters are listed within **Tables 2** and **3** (within the main paper) which include: (i) retention time in minutes ( $t_R$ ) for each drug eluting from the chromatographic column (**Method I**); (ii) Relative retention time ( $RRT$ ), which is determined with respect to fentanyl retention time obtained from **Method I**; (iii) Retention time (in minutes) for drugs eluted from the flow-cell system (**Method II**); (iv) Relative retention time ( $RRT$ ) determined with respect to fentanyl retention time obtained from **Method II**; (v) Capacity factor ( $k'$ ), ideally  $k'$  value is > 2; (vi) Number of theoretical plates expressed in plates per m ( $N$ ), generally  $N$  is > 2000; (vii) Height equivalent to theoretical plate expressed in m (HETP); (viii) Resolution between two successive eluted peaks ( $R_s$ ), ideally  $R_s$  is > 2; (ix) Asymmetry factor ( $A_s$ ) which indicates how symmetrical is the shape of the eluted peak and it is important factor

in quantification of peak areas.  $A_s$  has to be between 0.8 – 1.2; (x) Relative retention factor ( $\alpha$ ) which should be > 1.

**Table S1.** Comparison of the peak potential ( $E_p$  vs. Ag/AgCl) of the investigated drugs in 0.04 M B-R Buffer at three different pH's over a range of scan rates.

Analyte Scan rate (mV s <sup>-1</sup> ) \	HRN <sup>a</sup>	COC <sup>a</sup>	(2c) <sup>a</sup>	(2a) <sup>a</sup>	(2b) <sup>a</sup>	(2d) <sup>a</sup>	(2e) <sup>a</sup>	(2f) <sup>a</sup>	(2g) <sup>a</sup>	(2h) <sup>a</sup>	(2i) <sup>a</sup>	(2j) <sup>a</sup>	(2k) <sup>a</sup>
<b>pH 2.0</b>													
No peak for all the target analytes in all the studied scan rates at this pH.													
<b>pH 7.0</b>													
5	0.87 ± 0	1.00 ± 0.01	0.89 ± 0.01	0.87 ± 0	0.88 ± 0.01	0.85 ± 0.02	0.87 ± 0.01	0.86 ± 0.01	0.85 ± 0	0.89 ± 0.01	0.87 ± 0.01	0.87 ± 0.02	0.85 ± 0.01
15	0.89 ± 0	1.03 ± 0.01	0.90 ± 0	0.87 ± 0	0.88 ± 0	0.86 ± 0.02	0.88 ± 0.01	0.87 ± 0.01	0.88 ± 0.01	0.89 ± 0.01	0.90 ± 0	0.90 ± 0	0.88 ± 0.01
25	0.89 ± 0.01	1.05 ± 0	0.91 ± 0.01	0.87 ± 0.01	0.89 ± 0	0.87 ± 0.01	0.88 ± 0.01	0.87 ± 0.02	0.86 ± 0.02	0.89 ± 0.01	0.91 ± 0.01	0.90 ± 0.01	0.89 ± 0.01
50	<b>0.91 ± 0</b>	<b>1.06 ± 0.01</b>	<b>0.91 ± 0.01</b>	<b>0.88 ± 0.01</b>	<b>0.89 ± 0.01</b>	<b>0.88 ± 0.01</b>	<b>0.90 ± 0.01</b>	<b>0.89 ± 0</b>	<b>0.89 ± 0.01</b>	<b>0.92 ± 0.01</b>	<b>0.91 ± 0.01</b>	<b>0.92 ± 0.01</b>	<b>0.90 ± 0</b>
100	0.92 ± 0.01	1.08 ± 0	0.92 ± 0	0.90 ± 0	0.89 ± 0.01	0.90 ± 0	0.90 ± 0.01	0.91 ± 0.01	0.90 ± 0.01	0.92 ± 0	0.93 ± 0.01	0.92 ± 0	0.92 ± 0.01
250	0.94 ± 0	1.11 ± 0	0.96 ± 0.01	0.92 ± 0.01	0.91 ± 0	0.92 ± 0.01	0.93 ± 0.01	0.93 ± 0.01	0.93 ± 0.01	0.95 ± 0.01	0.95 ± 0	0.94 ± 0	0.94 ± 0
500	0.95 ± 0	1.13 ± 0.01	0.99 ± 0.02	0.94 ± 0.01	0.95 ± 0	0.95 ± 0.02	0.95 ± 0.01	0.96 ± 0.01	0.95 ± 0.01	0.97 ± 0	0.98 ± 0.01	0.96 ± 0.01	0.97 ± 0.01
<b>pH 10.0</b>													
5	No peak	0.87 ± 0.01	0.75 ± 0.01	0.76 ± 0	0.74 ± 0.02	0.78 ± 0.01	No peak	No peak	No peak	0.80 ± 0.03	0.79 ± 0.02	0.77 ± 0	No peak
15	No peak	0.90 ± 0.01	0.77 ± 0.01	0.77 ± 0	0.79 ± 0.01	0.79 ± 0	No peak	No peak	No peak	0.84 ± 0.01	0.85 ± 0.04	0.84 ± 0.01	0.78 ± 0
25	0.78 ± 0.01	0.91 ± 0	0.78 ± 0.02	0.79 ± 0	0.82 ± 0.01	0.81 ± 0.01	No peak	No peak	No peak	0.86 ± 0.01	0.86 ± 0.02	0.87 ± 0.01	0.81 ± 0.02
50	0.80 ± 0.01	0.94 ± 0	0.79 ± 0.02	0.81 ± 0	0.85 ± 0	0.84 ± 0.01	No peak	0.83 ± 0.01	0.83 ± 0.01	0.88 ± 0.02	0.88 ± 0.02	0.90 ± 0.02	0.85 ± 0.01
100	0.82 ± 0.01	0.95 ± 0	0.82 ± 0	0.83 ± 0	0.87 ± 0	0.84 ± 0.02	0.84 ± 0	0.83 ± 0	0.82 ± 0	0.91 ± 0.01	0.90 ± 0.01	0.93 ± 0	0.86 ± 0
250	0.85 ± 0	0.97 ± 0	0.86 ± 0	0.86 ± 0	0.89 ± 0	0.85 ± 0.01	0.85 ± 0	0.85 ± 0.01	0.82 ± 0	0.93 ± 0.01	0.94 ± 0.02	0.92 ± 0.03	0.87 ± 0.01
500	0.87 ± 0	1.00 ± 0	0.89 ± 0	0.90 ± 0	0.93 ± 0	0.87 ± 0	0.86 ± 0	0.87 ± 0	0.85 ± 0	0.97 ± 0	0.97 ± 0.02	0.97 ± 0.03	0.87 ± 0.01

<sup>a</sup> Mean ± SD of peak potential ( $E_p$ ) of each drug at the studied scan rate. (n=3)

**Table S2.** Evaluation of the robustness of the proposed HPLC-DAD Method (**Method I**) for the determination of HRN, fentanyl and its 10 fentalogues.

<b>Analyte Parameters</b>	<b>HRN</b>	<b>(2a)</b>	<b>(2b)</b>	<b>(2c)</b>	<b>(2d)</b>	<b>(2e)</b>	<b>(2f)</b>	<b>(2g)</b>	<b>(2h)</b>	<b>(2i)</b>	<b>(2j)</b>	<b>(2k)</b>
<b>Temperature (25 ± 2 °C)</b>	100.58 ± 0.33	100.61 ± 0.31	100.03 ± 0.33	100.63 ± 0.42	100.81 ± 0.20	100.10 ± 0.64	99.83 ± 0.58	100.60 ± 0.84	100.27 ± 0.36	100.97 ± 0.49	99.67 ± 0.48	99.99 ± 0.62
<b>Molarity of buffer (20 ± 2.0 mM)</b>	100.82 ± 1.16	100.56 ± 1.00	100.51 ± 0.99	100.15 ± 1.04	100.98 ± 0.89	100.19 ± 1.24	100.43 ± 0.86	100.44 ± 0.45	99.36 ± 0.17	100.64 ± 0.79	100.27 ± 0.69	100.42 ± 1.07
<b>pH of buffer (7.0 ± 0.2 pH units)</b>	100.47 ± 0.53	100.66 ± 0.67	100.14 ± 0.98	100.08 ± 0.84	100.76 ± 0.54	100.24 ± 0.65	100.02 ± 1.26	100.40 ± 0.75	100.68 ± 0.98	100.23 ± 0.91	100.91 ± 0.66	100.19 ± 0.77
<b>RSD%<sup>b</sup></b>												
<b>Temperature (25 ± 2 °C)</b>	0.33	0.31	0.33	0.42	0.20	0.64	0.58	0.83	0.36	0.49	0.48	0.62
<b>Molarity of buffer (20 ± 2.0 mM)</b>	1.15	0.99	0.98	1.04	0.88	1.24	0.86	0.45	0.17	0.78	0.69	1.07
<b>pH of buffer (7.0 ± 0.2 pH units)</b>	0.53	0.67	0.98	0.84	0.54	0.65	1.26	0.75	0.97	0.91	0.65	0.77
<b>t<sub>R</sub> ± SD<sup>c</sup></b>												
<b>Temperature (25 ± 2 °C)</b>	2.79 ± 0.02	4.09 ± 0.01	4.79 ± 0.02	8.10 ± 0.04	10.79 ± 0.08	13.20 ± 0.05	14.26 ± 0.07	18.08 ± 0.13	20.65 ± 0.29	25.50 ± 0.15	27.64 ± 0.20	29.42 ± 0.22
<b>Molarity of buffer (20 ± 2.0 mM)</b>	2.79 ± 0.05	4.09 ± 0.04	4.79 ± 0.04	8.10 ± 0.09	10.79 ± 0.14	13.20 ± 0.17	14.26 ± 0.18	18.08 ± 0.24	20.65 ± 0.31	25.50 ± 0.36	27.64 ± 0.38	29.42 ± 0.41
<b>pH of buffer (7.0 ± 0.2 pH units)</b>	2.79 ± 0.20	4.09 ± 0.39	4.79 ± 0.48	8.10 ± 0.94	10.79 ± 1.34	13.20 ± 1.69	14.26 ± 1.84	18.08 ± 2.43	20.65 ± 2.99	25.50 ± 3.55	27.64 ± 3.83	29.42 ± 4.17

<sup>a</sup> Mean ± SD of percentage recoveries of peak areas of each drug at the three studied parameters. (n=3)<sup>b</sup> Percentage relative standard deviation of peak areas of each drug at the three studied parameters.<sup>c</sup> Mean ± SD of retention time of each drug at the three studied parameters. (n=3)

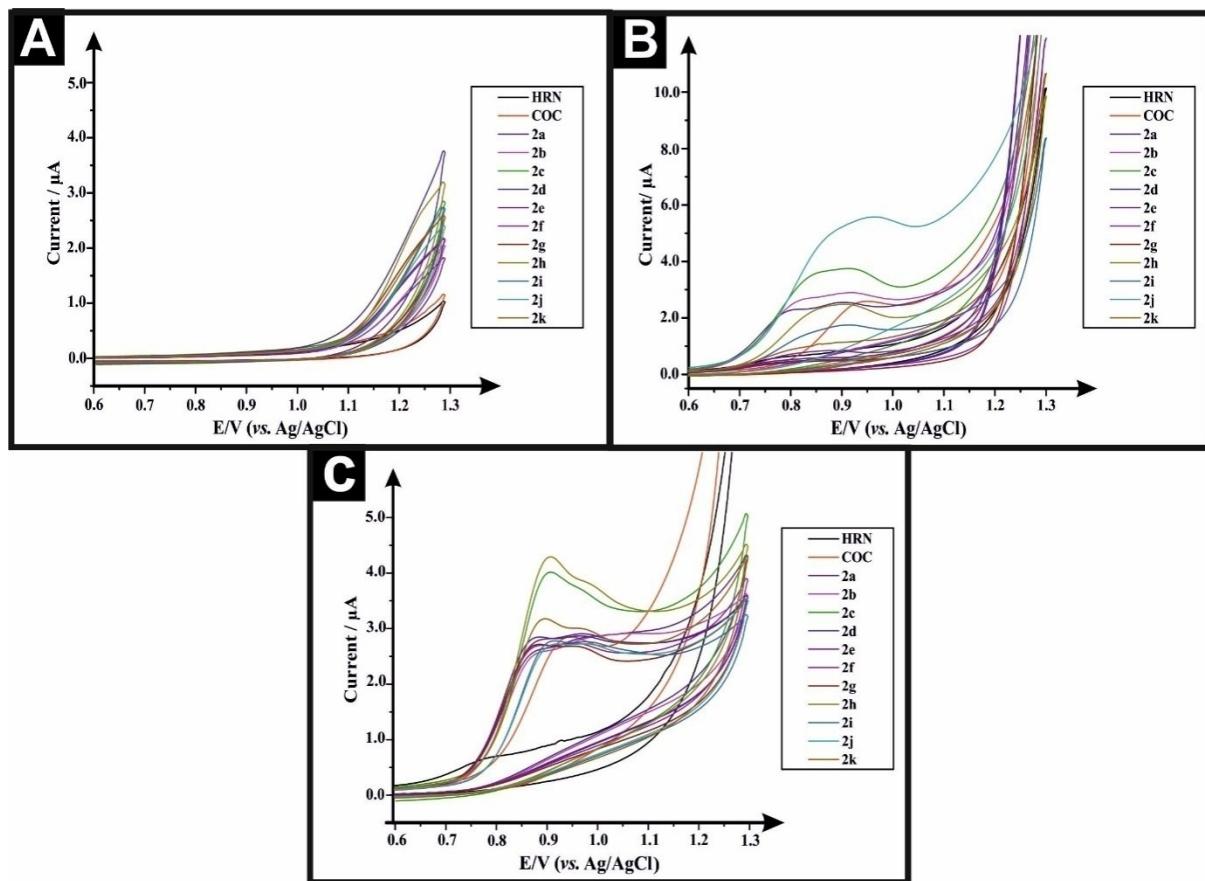
**Table S3.** Evaluation of the robustness of the proposed HPLC-AD Method (**Method II**) for the determination of HRN, fentanyl and its 10 fentalogues.

Analyte Parameters	Mean % recovery ± SD <sup>a</sup>											
	HRN	(2a)	(2b)	(2c)	(2d)	(2e)	(2f)	(2g)	(2h)	(2i)	(2j)	(2k)
Temperature (25 ± 2 °C)	99.48 ± 0.82	99.73 ± 0.66	100.38 ± 0.66	100.52 ± 0.73	100.30 ± 0.98	100.47 ± 0.63	100.07 ± 0.75	99.76 ± 1.34	99.73 ± 0.53	100.39 ± 0.76	100.56 ± 0.75	100.10 ± 0.64
Molarity of buffer (20 ± 2.0 mM)	100.39 ± 0.73	100.50 ± 0.72	100.37 ± 0.65	100.50 ± 0.58	100.51 ± 0.80	100.43 ± 0.59	101.12 ± 1.18	100.30 ± 0.60	99.65 ± 0.48	100.09 ± 0.81	100.53 ± 0.63	100.39 ± 0.75
pH of buffer (7.0 ± 0.2 pH units)	99.75 ± 0.51	100.56 ± 0.63	100.42 ± 0.65	100.62 ± 1.06	100.36 ± 0.60	100.54 ± 0.58	100.30 ± 0.92	100.28 ± 0.55	99.66 ± 0.40	100.51 ± 0.58	100.20 ± 0.93	99.94 ± 0.97
RSD% <sup>b</sup>												
Temperature (25 ± 2 °C)	0.82	0.66	0.66	0.73	0.98	0.63	0.75	1.34	0.53	0.76	0.75	0.64
Molarity of buffer (20 ± 2.0 mM)	0.73	0.72	0.65	0.58	0.80	0.59	1.17	0.60	0.48	0.81	0.63	0.75
pH of buffer (7.0 ± 0.2 pH units)	0.51	0.63	0.65	1.05	0.60	0.58	0.92	0.55	0.40	0.58	0.93	0.97

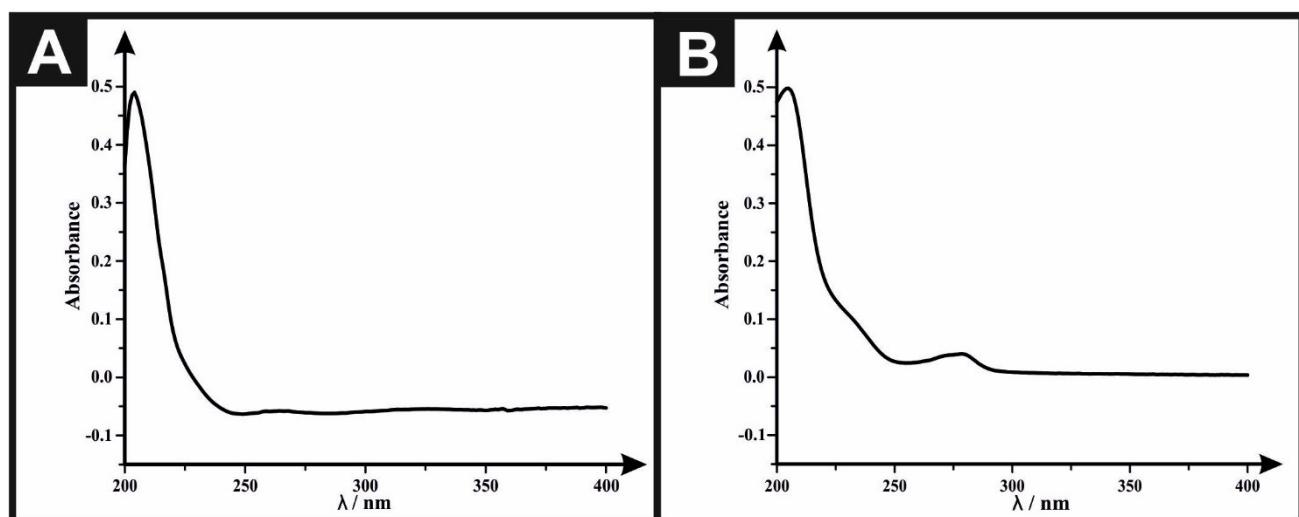
<sup>a</sup> Mean ± SD of percentage recoveries of peak heights (current, µA) of each drug at the three studied parameters. (n=3)

<sup>b</sup>Percentage relative standard deviation of peak heights (current, µA) of each drug at the three studied parameters.

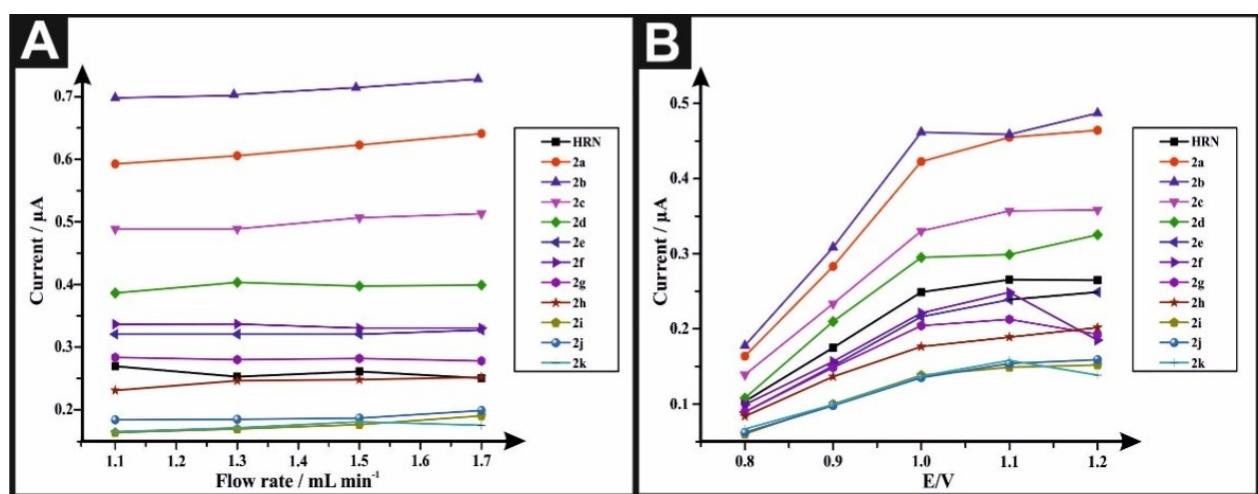
**Figure S1.** Overlay of cyclic voltammograms (CV) for 50  $\mu\text{g mL}^{-1}$  of HRN, COC, fentanyl and its 10 fentalogues in 0.04 MB-R buffer in 3 different pHs: **(A)** pH 2.0, **(B)** pH 10.0, and **(C)** pH 7.0; Scan rate: 50 mV s $^{-1}$ .



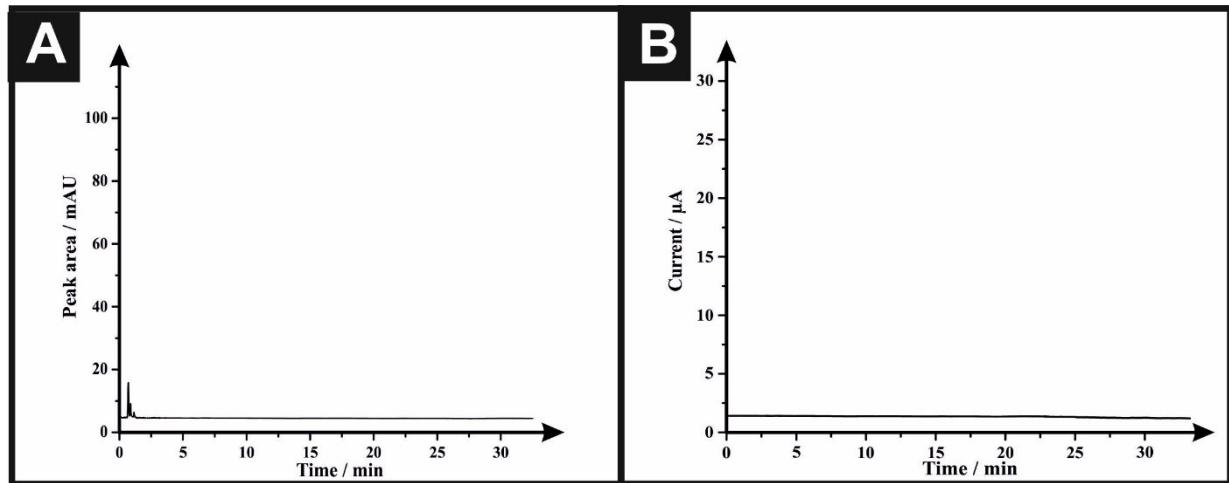
**Figure S2.** UV absorption spectrum of (A)  $10 \mu\text{g mL}^{-1}$  fentanyl and (B)  $20 \mu\text{g mL}^{-1}$  HRN in solution of the mobile phase showing their  $\lambda_{\max}$  at 205 nm.



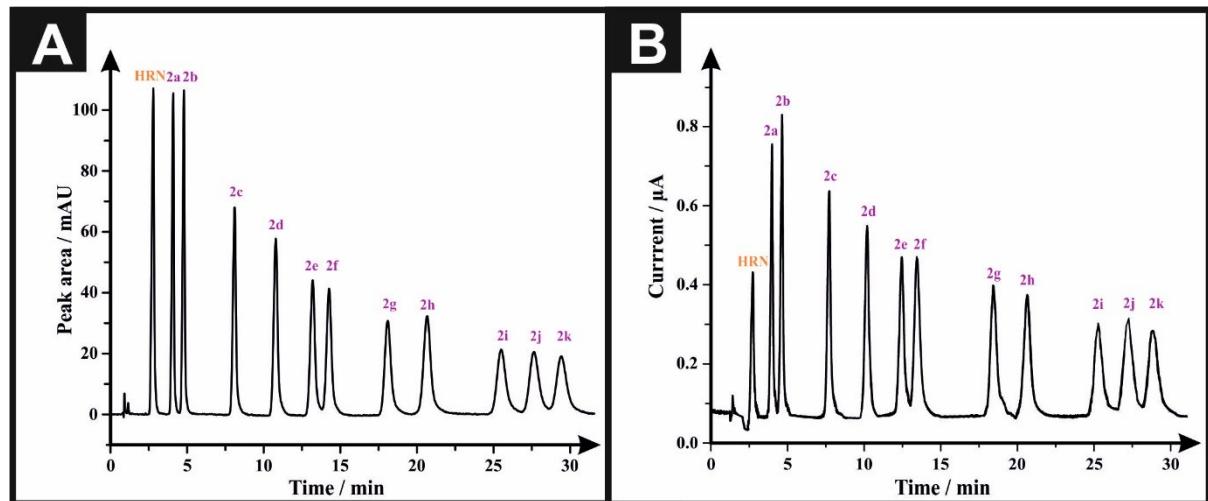
**Figure S3.** Effect of (A) flow rate and (B) potential ( $E\text{ V}^{-1}$ ) on current intensity in HPLC-AD flow cell system.



**Figure S4.** (A) Representative HPLC-DAD chromatogram (B) Representative amperogram for a solution containing  $300 \mu\text{g mL}^{-1}$  of each of d-glucose, d-fructose, sucrose, lactose, starch, aerosil 200, sodium lauryl sulfate, stearic acid and sodium carboxymethyl cellulose using Eclipse XDB-C8 column (150 x 4.6 mm, i.d. 5  $\mu\text{m}$ ); mobile phase: acetonitrile : 20 mM ammonium formate –100 mM potassium chloride buffer (pH 7.0) (30 : 70% v/v); flow rate 1.5  $\text{mL min}^{-1}$ , detector wavelength (UV): 205 nm and column temperature 25 °C.

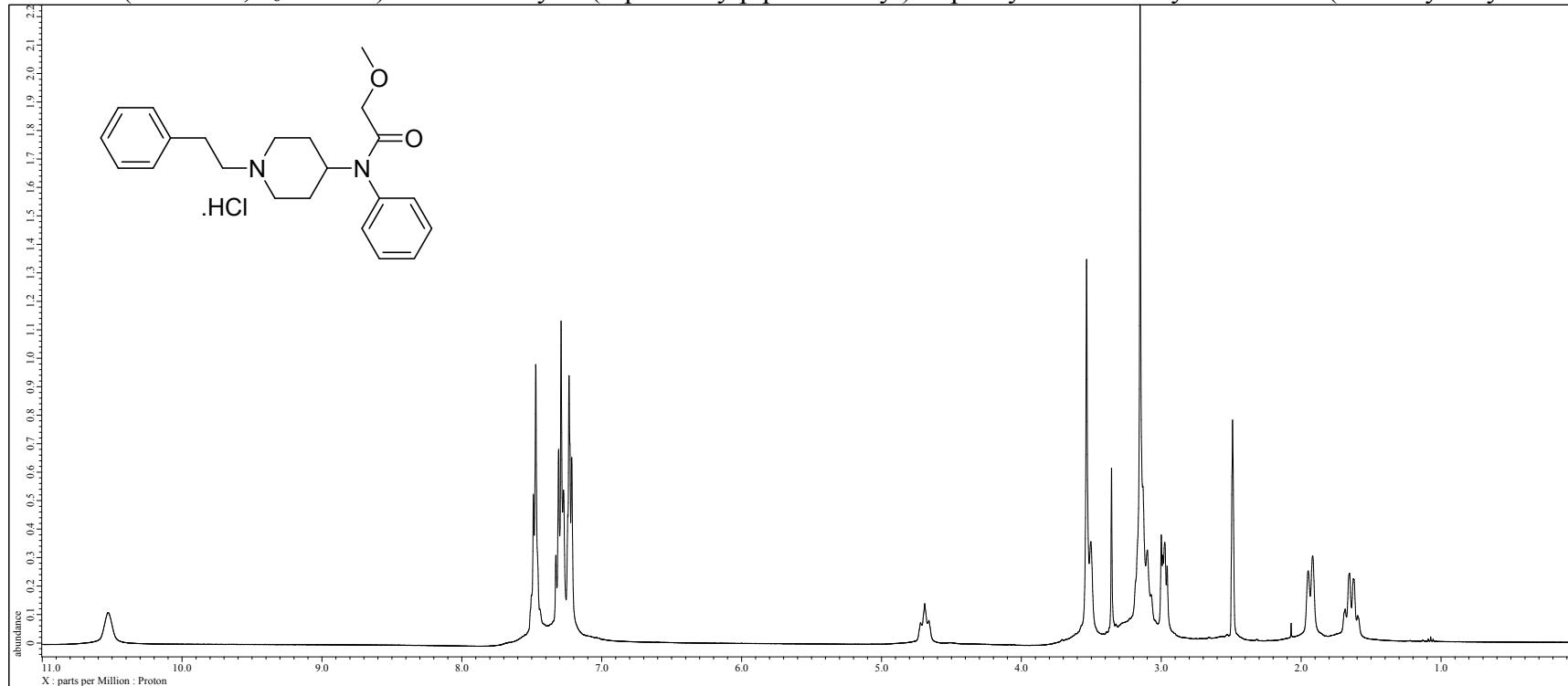


**Figure S5.** (A) Representative HPLC-DAD chromatogram (B) Representative amperogram for a solution containing  $50 \mu\text{g mL}^{-1}$  of each of HRN, fentanyl (2c), fentalogues (2a, 2b, 2d – 2k) and  $300 \mu\text{g mL}^{-1}$  of each of d-glucose, d-fructose, sucrose, lactose, starch, aerosil 200, sodium lauryl sulfate, stearic acid and sodium carboxymethyl cellulose using Eclipse XDB-C8 column (150 x 4.6 mm, i.d. 5  $\mu\text{m}$ ); mobile phase: acetonitrile : 20 mM ammonium formate – 100 mM potassium chloride buffer (pH 7.0) (30 : 70% v/v); flow rate  $1.5 \text{ mL min}^{-1}$ , detector wavelength (UV): 205 nm and column temperature  $25^\circ\text{C}$ .

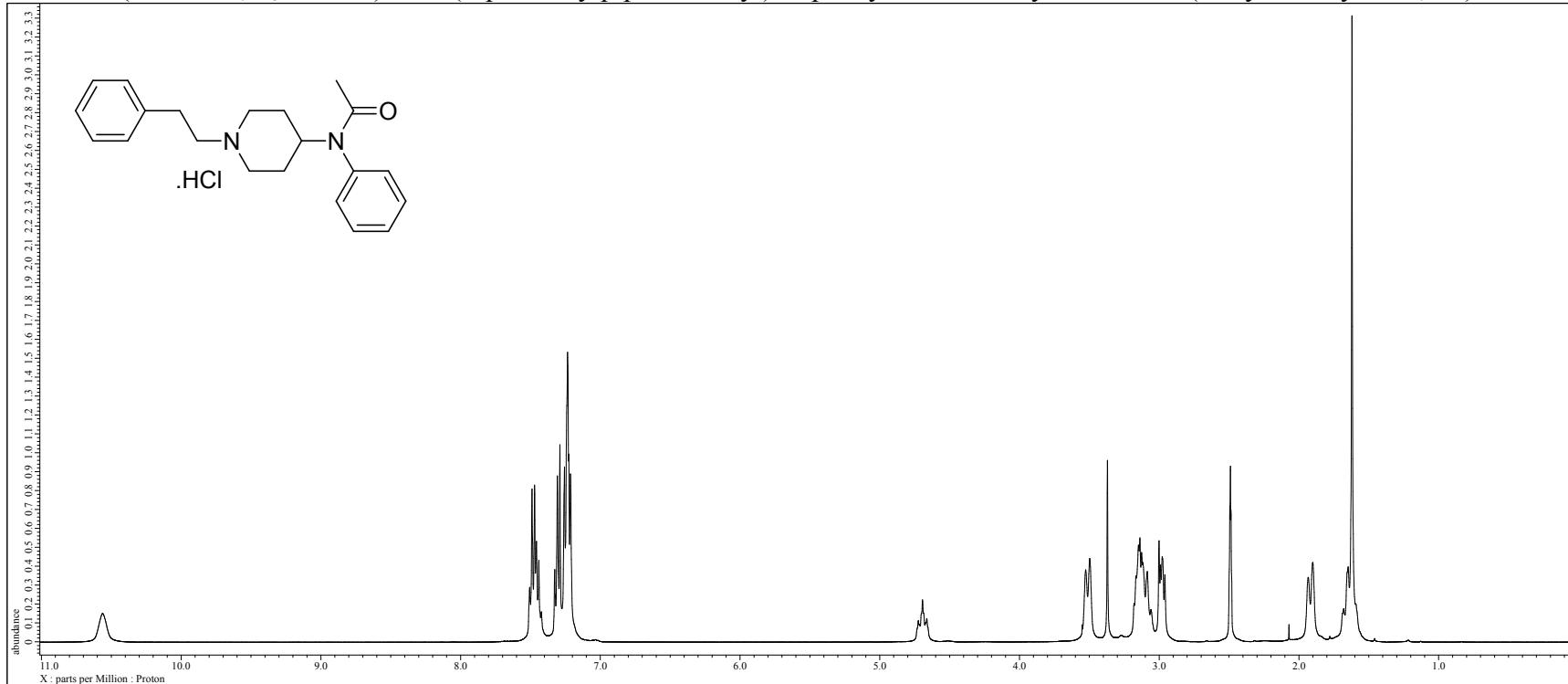


*H-NMR and C-NMR spectra of the fentalogues (2a-2k) utilised within the study are presented below.*

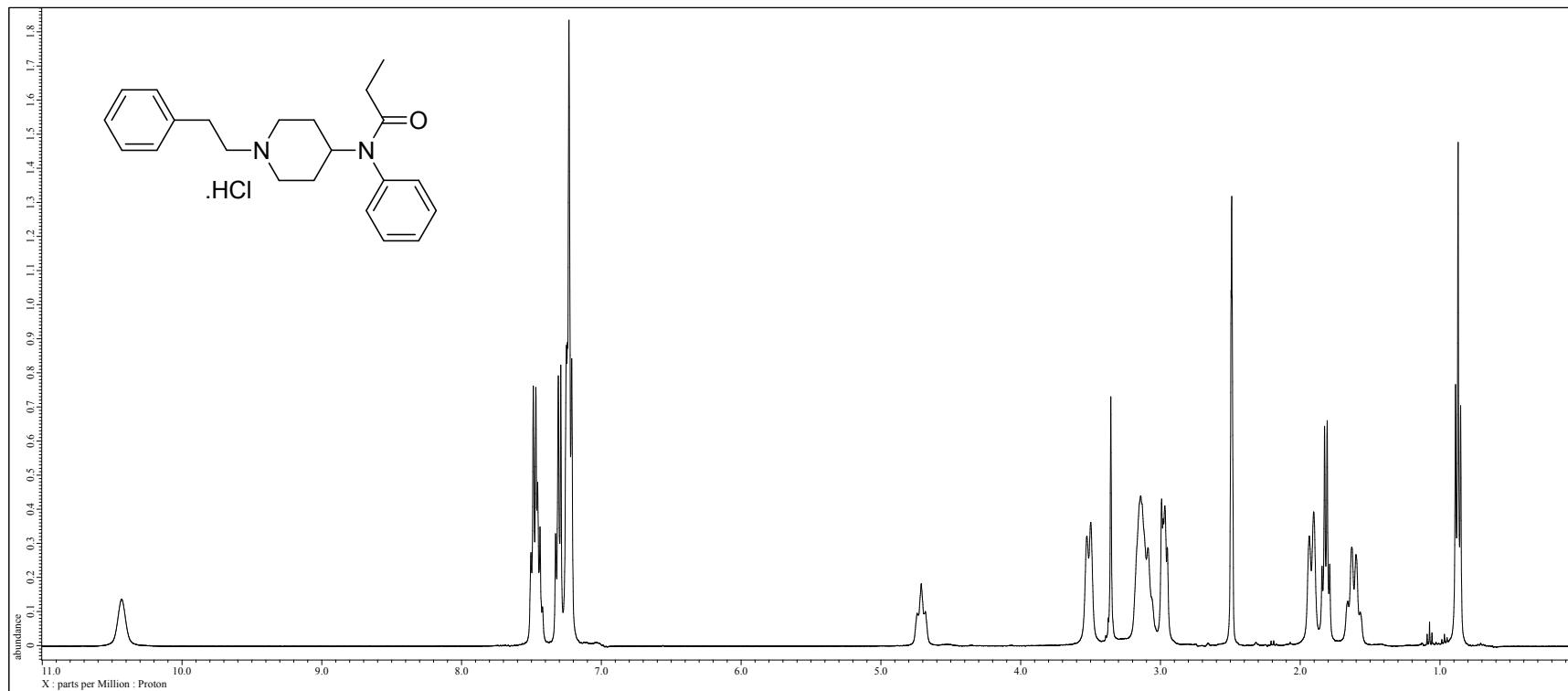
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of 2-methoxy-N-(1-phenethylpiperidin-4-yl)-N-phenylacetamide hydrochloride (methoxyacetylfentanyl.HCl, 2a)



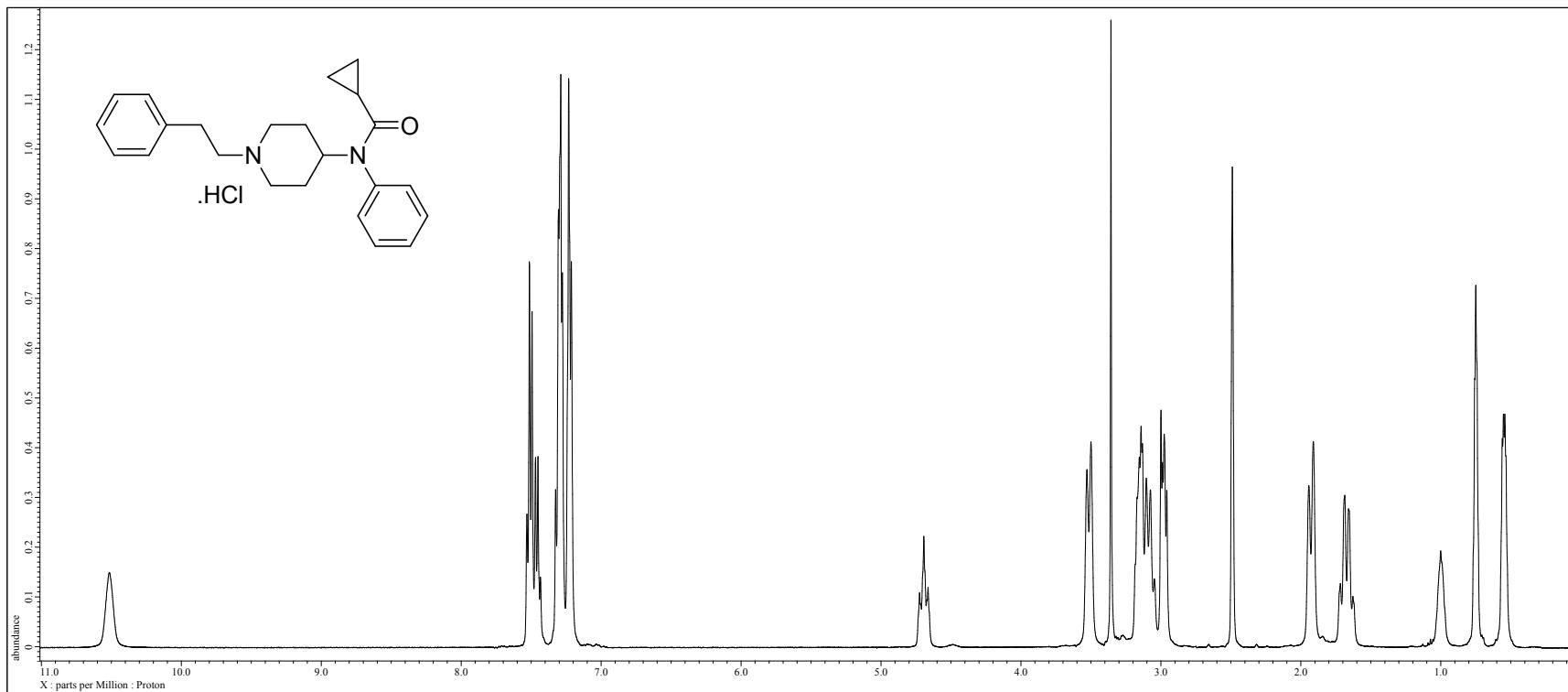
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of N-(1-phenethylpiperidin-4-yl)-N-phenylacetamide hydrochloride (acetylfentanyl.HCl, **2b**)



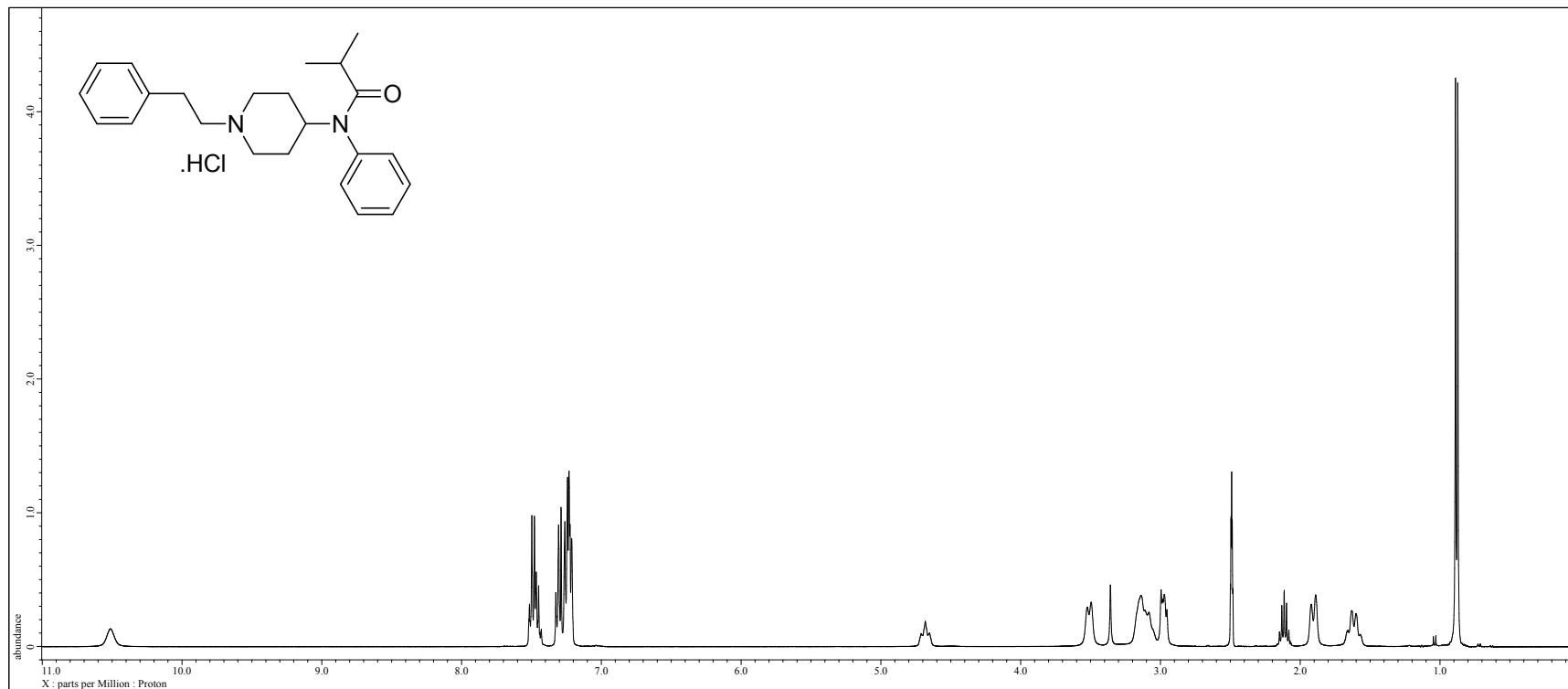
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of N-(1-phenethylpiperidin-4-yl)-N-phenylpropionamide hydrochloride (fentanyl.HCl, **2c**)



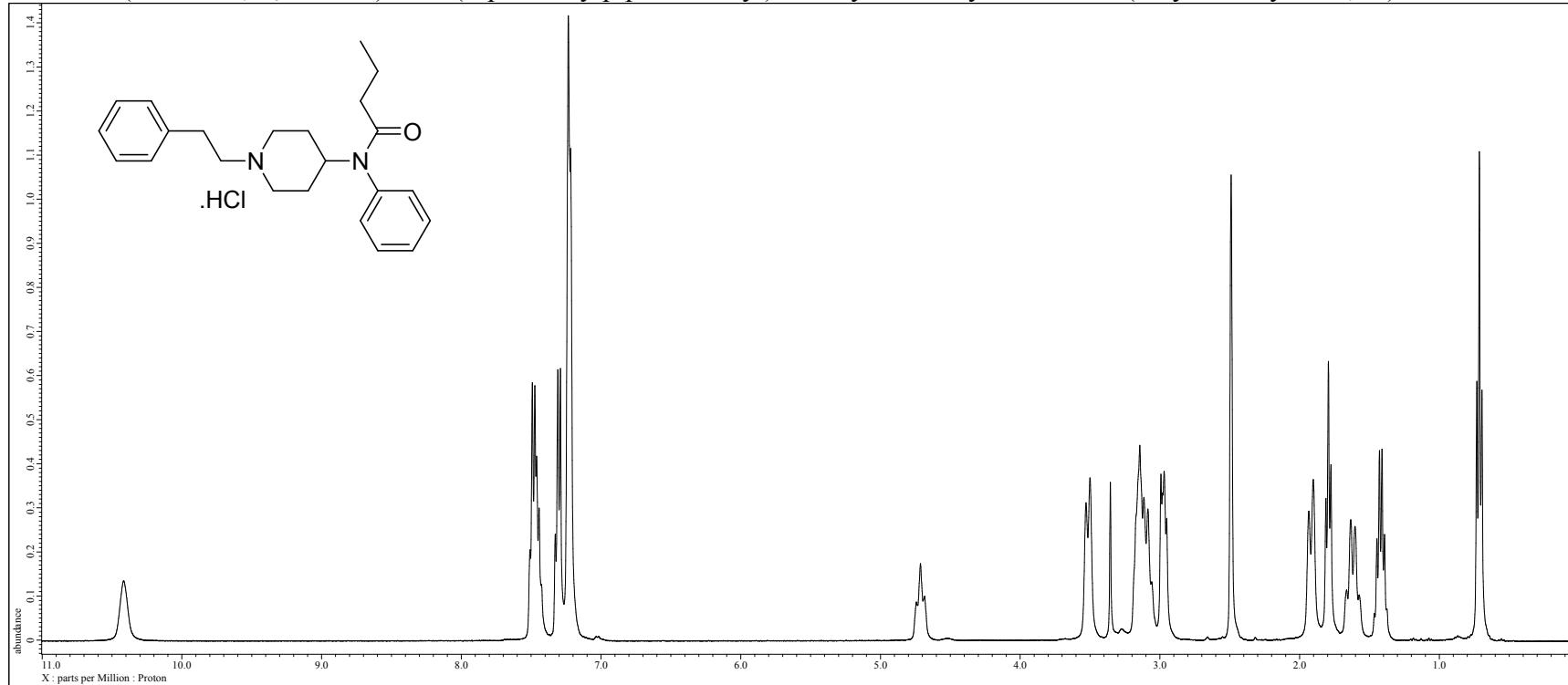
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylcyclopropanecarboxamide hydrochloride (cyclopropylfentanyl.HCl, **2d**)



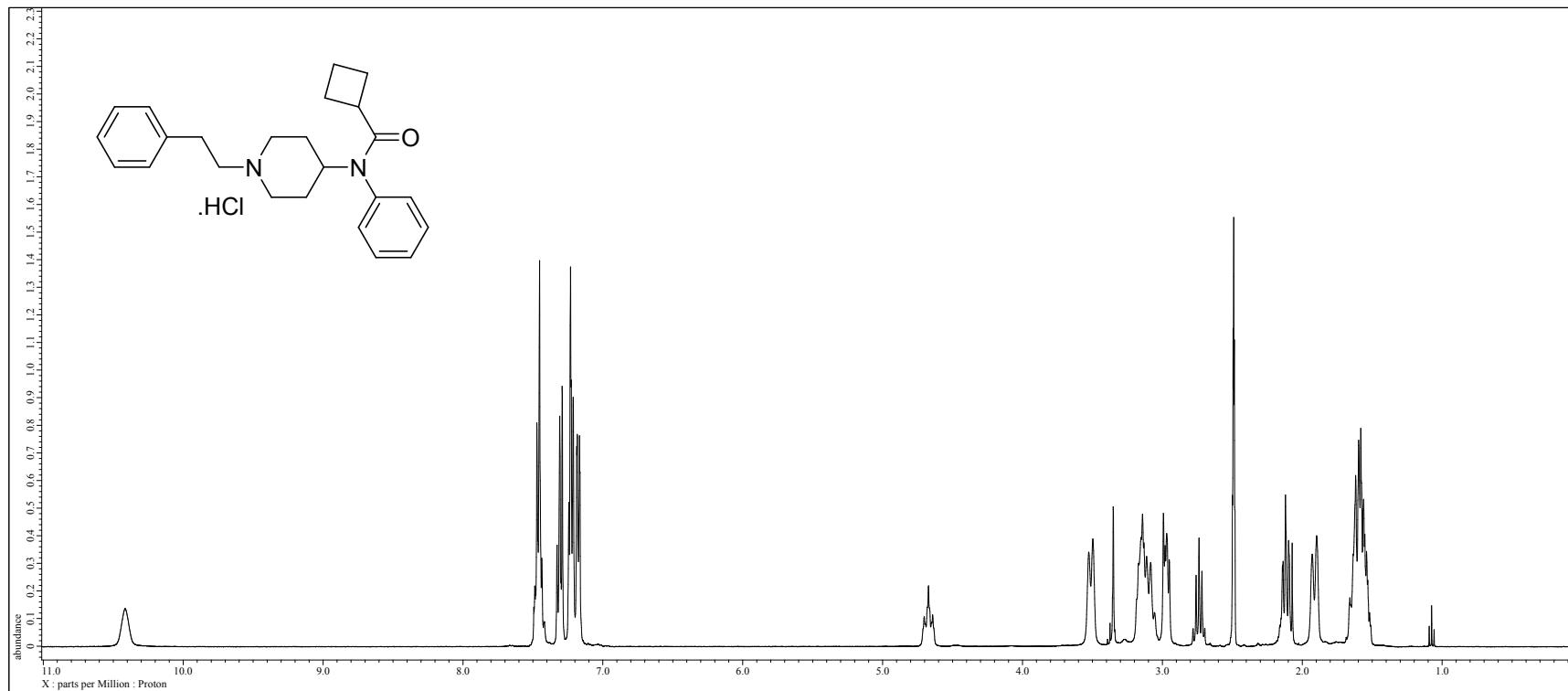
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of N-(1-phenethylpiperidin-4-yl)-N-phenylisobutyramide hydrochloride (isobutyrfentanyl.HCl, 2e)



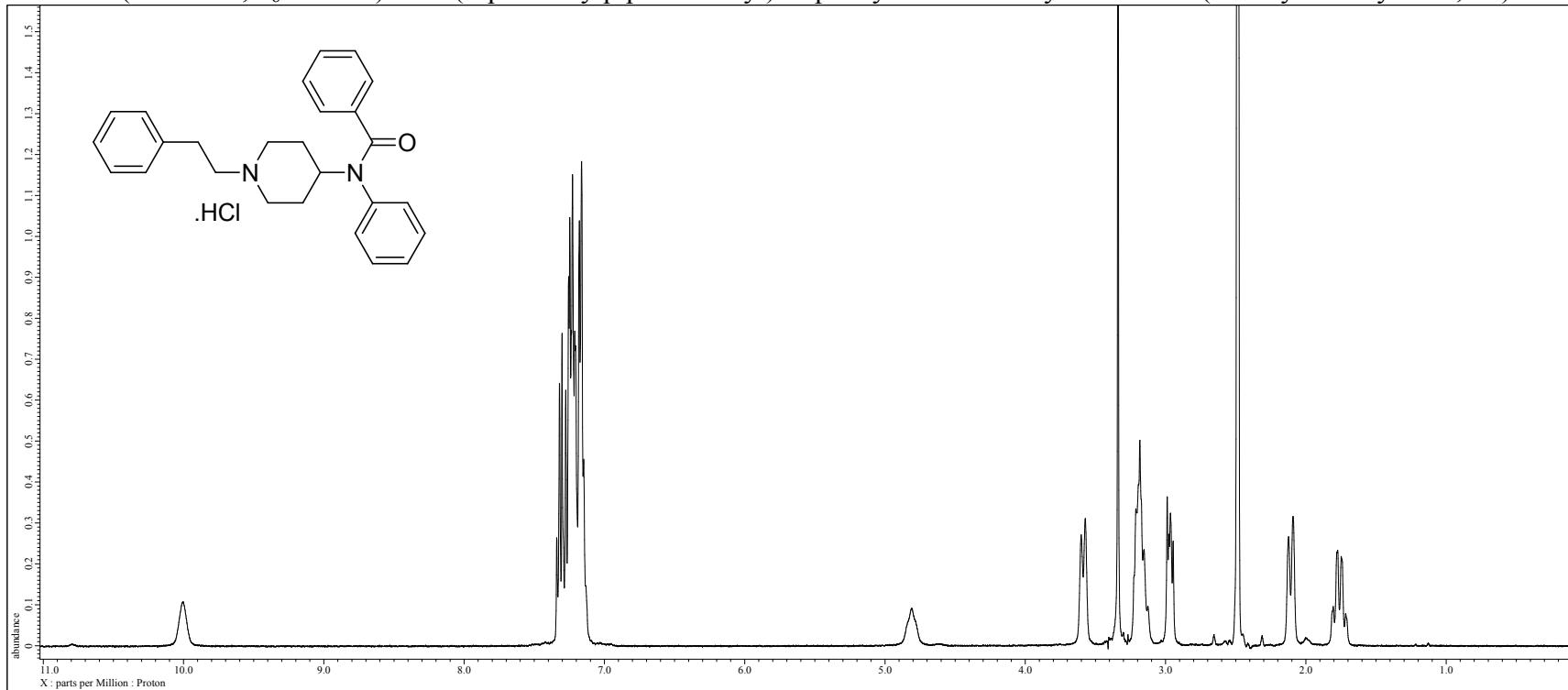
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of *N*-(1-phenethylpiperidin-4-yl)-*N*-butyramide hydrochloride (butyrfentanyl.HCl, **2f**)



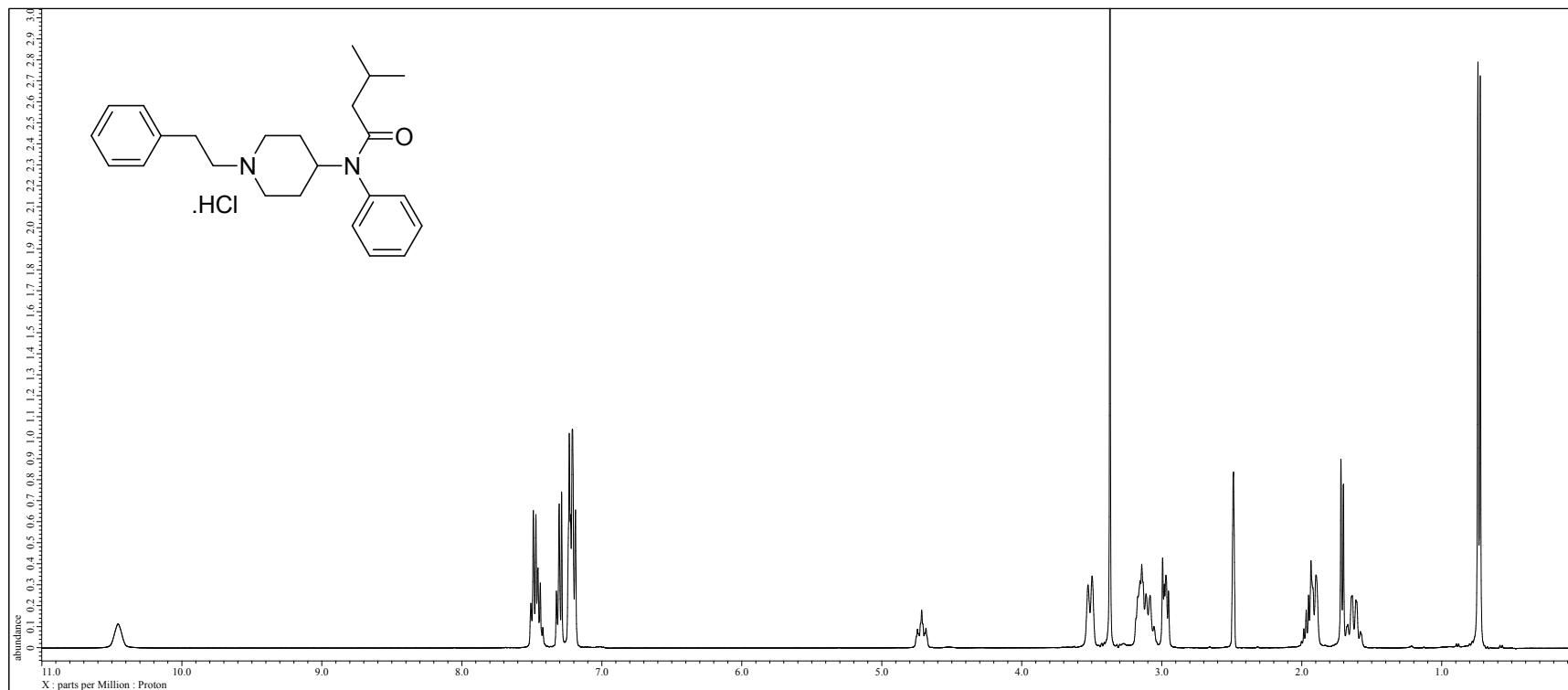
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of N-(1-phenethylpiperidin-4-yl)-N-phenylcyclobutanecarboxamide hydrochloride (cyclobutylfentanyl.HCl, **2g**)



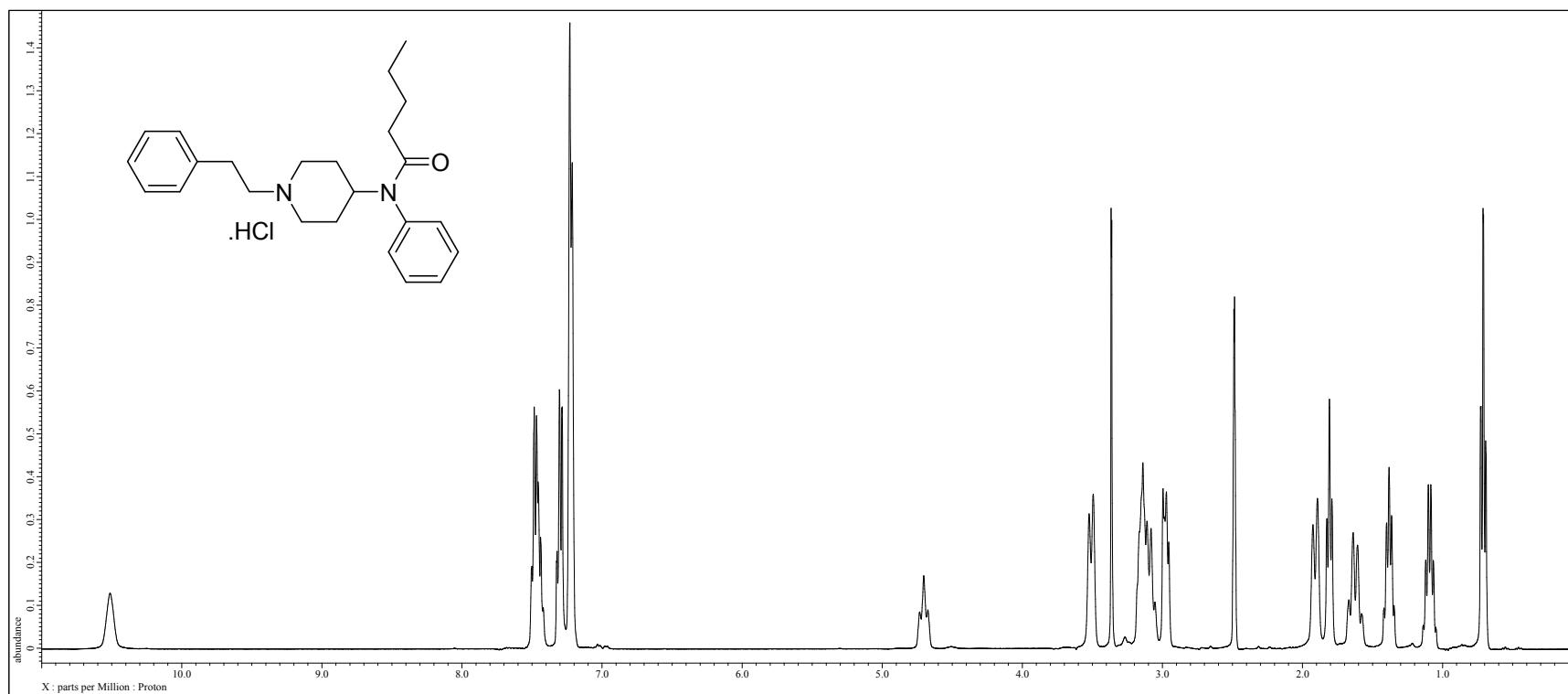
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of N-(1-phenethylpiperidin-4-yl)-N-phenylbenzamide hydrochloride (benzoylfentanyl.HCl, 2h)



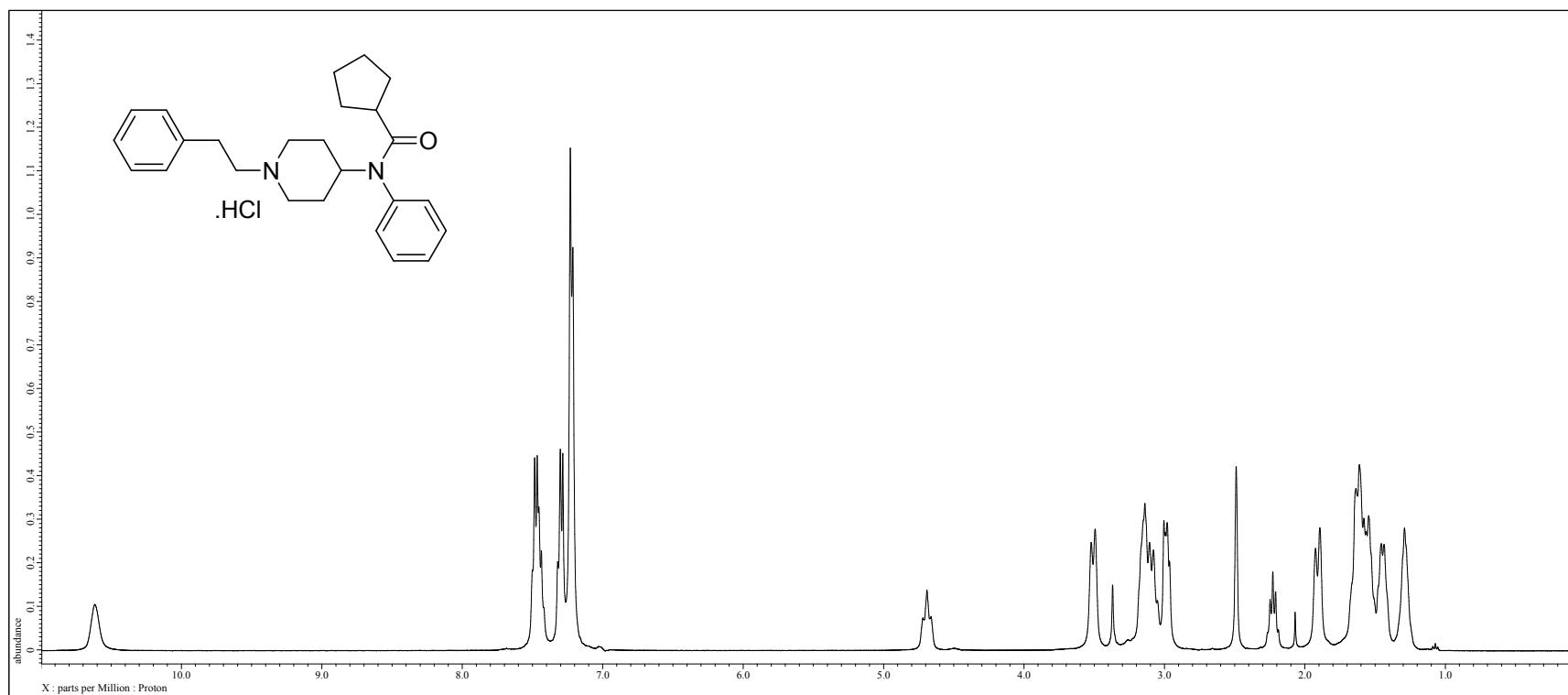
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of 3-methyl-N-(1-phenethylpiperidin-4-yl)-N-phenylbutanamide hydrochloride (isovalerylfentanyl.HCl, **2i**)



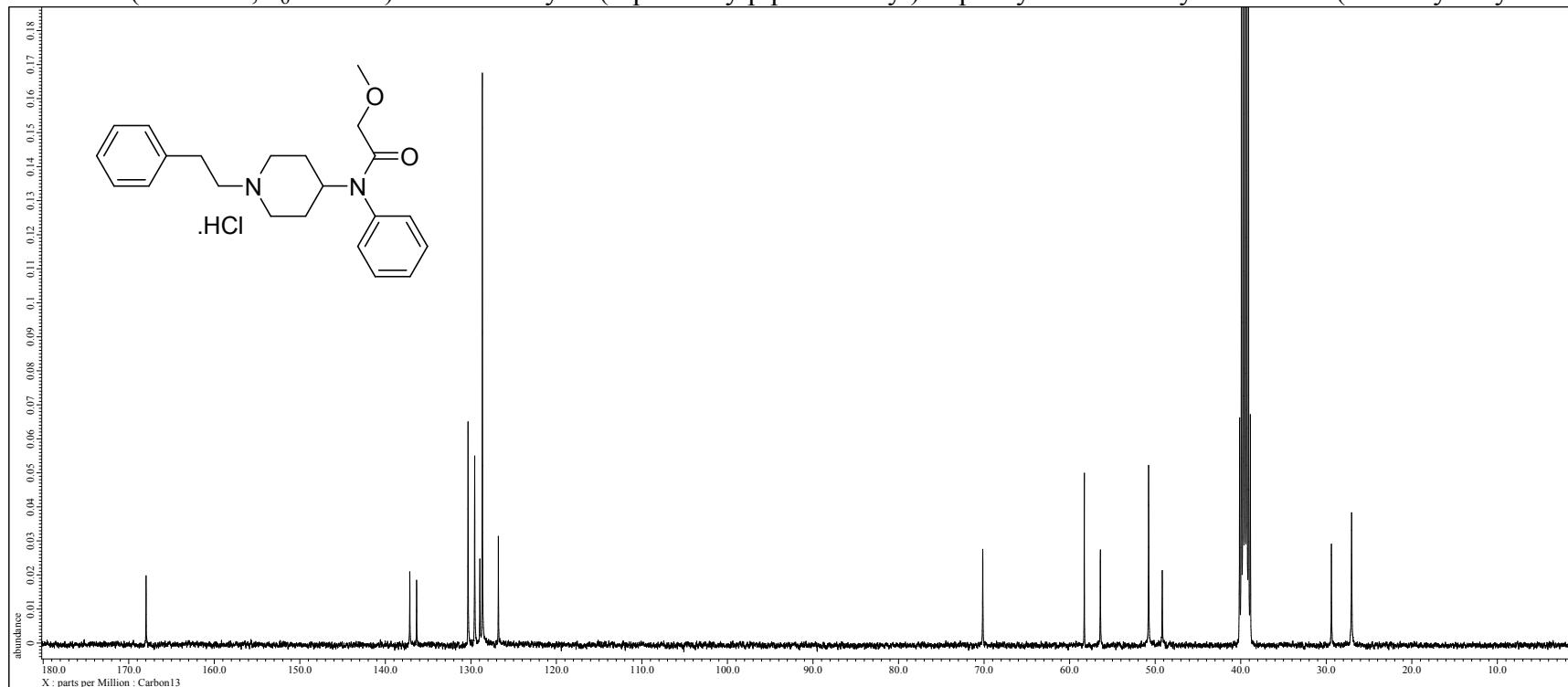
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of N-(1-phenethylpiperidin-4-yl)-N-phenylpentanamide hydrochloride (valerylfentanyl.HCl, **2j**)



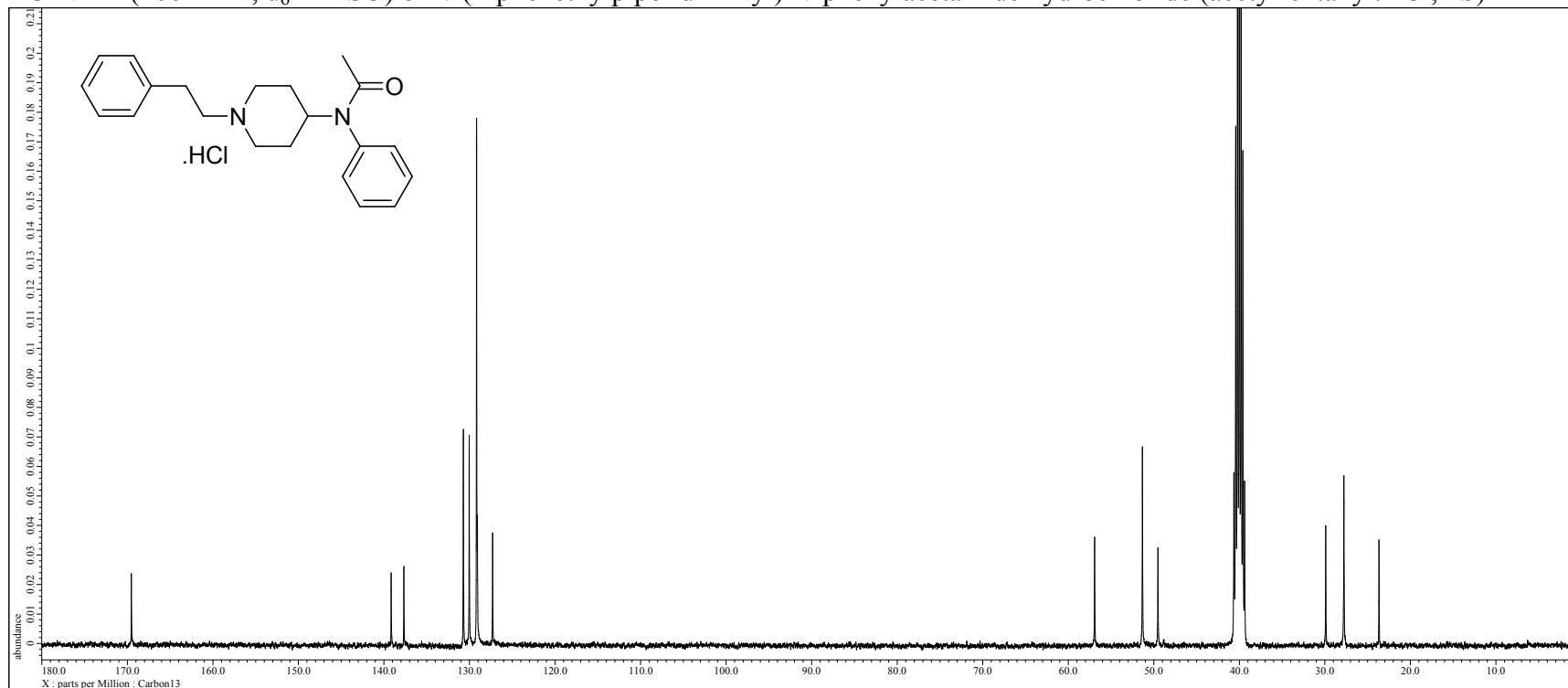
<sup>1</sup>H-NMR (400 MHz, d<sub>6</sub>-DMSO) of N-(1-phenethylpiperidin-4-yl)-N-phenylcyclopentanecarboxamide hydrochloride (cyclopentylfentanyl.HCl, **2k**)



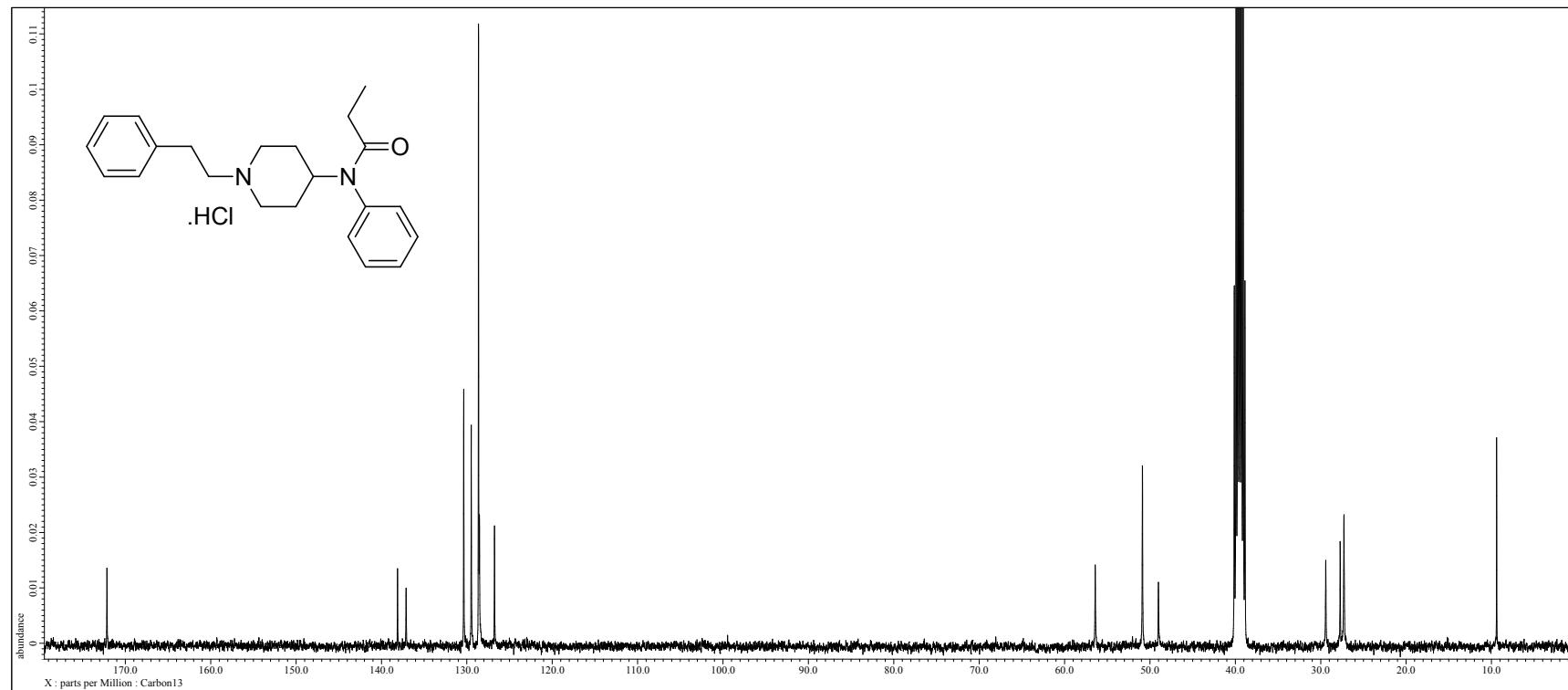
<sup>13</sup>C-NMR (100 MHz, d<sub>6</sub>-DMSO) of 2-methoxy-N-(1-phenethylpiperidin-4-yl)-N-phenylacetamide hydrochloride (methoxyacetylentanyl.HCl, **2a**)



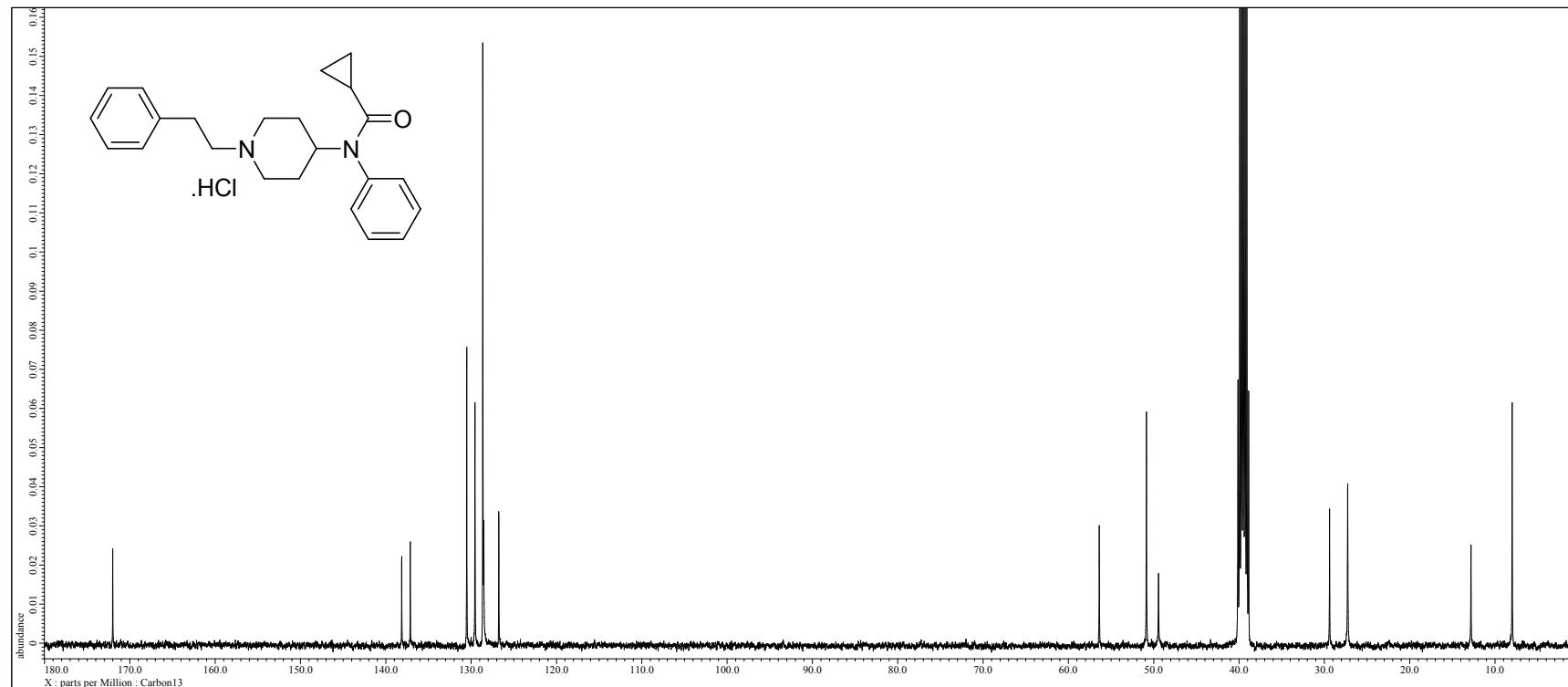
<sup>13</sup>C-NMR (100 MHz, d<sub>6</sub>-DMSO) of *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylacetamide hydrochloride (acetylfentanyl.HCl, **2b**)



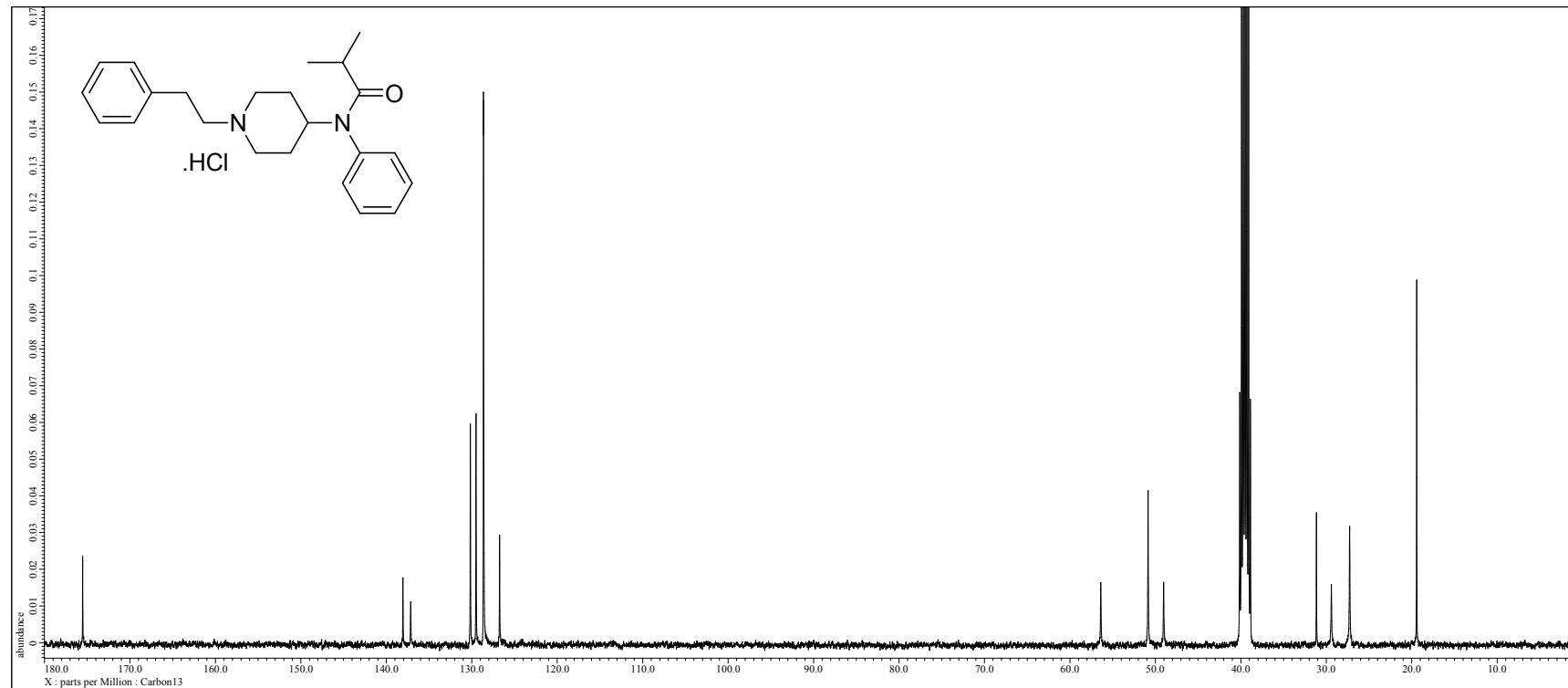
$^{13}\text{C}$ -NMR (100 MHz,  $\text{d}_6\text{-DMSO}$ ) of *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylpropionamide hydrochloride (fentanyl.HCl, **2c**)



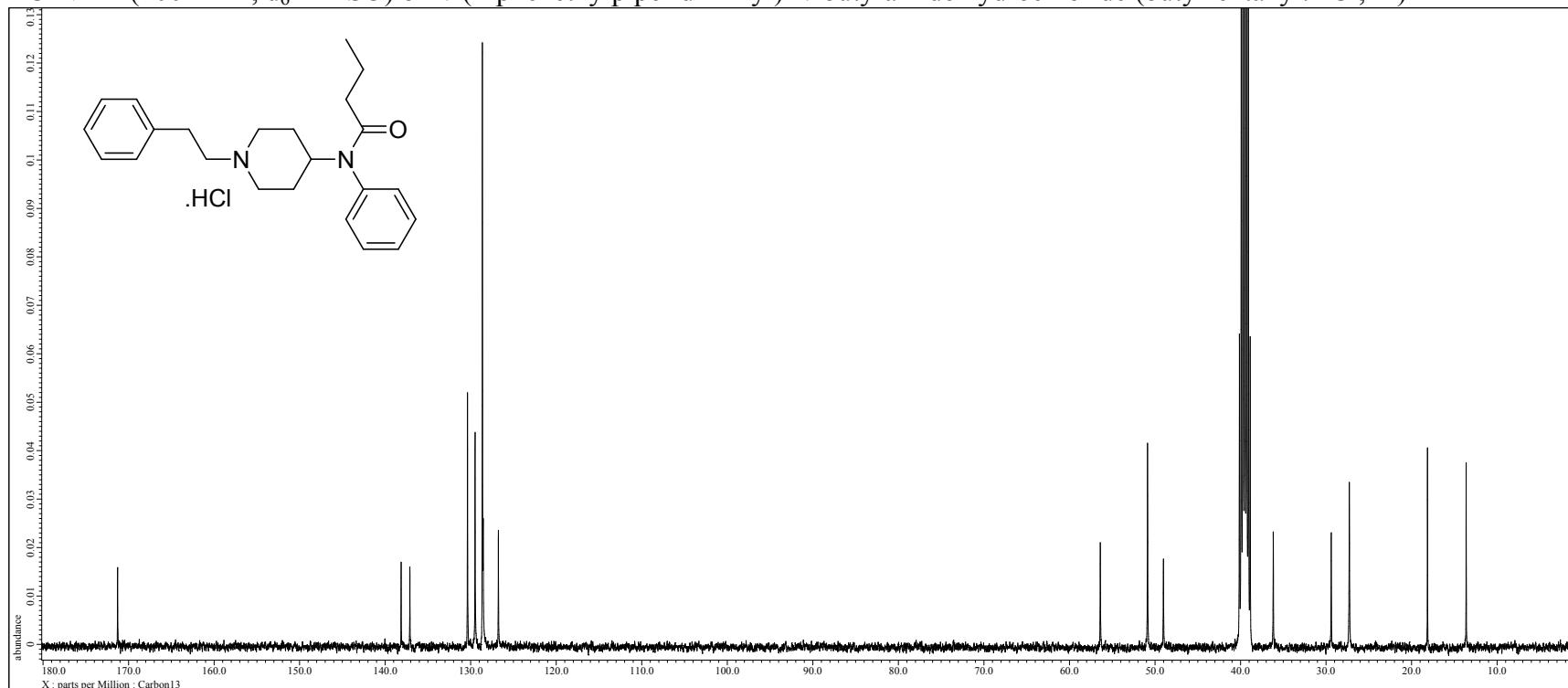
<sup>13</sup>C-NMR (100 MHz, d<sub>6</sub>-DMSO) of *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylcyclopropanecarboxamide hydrochloride (cyclopropylfentanyl.HCl, **2d**)



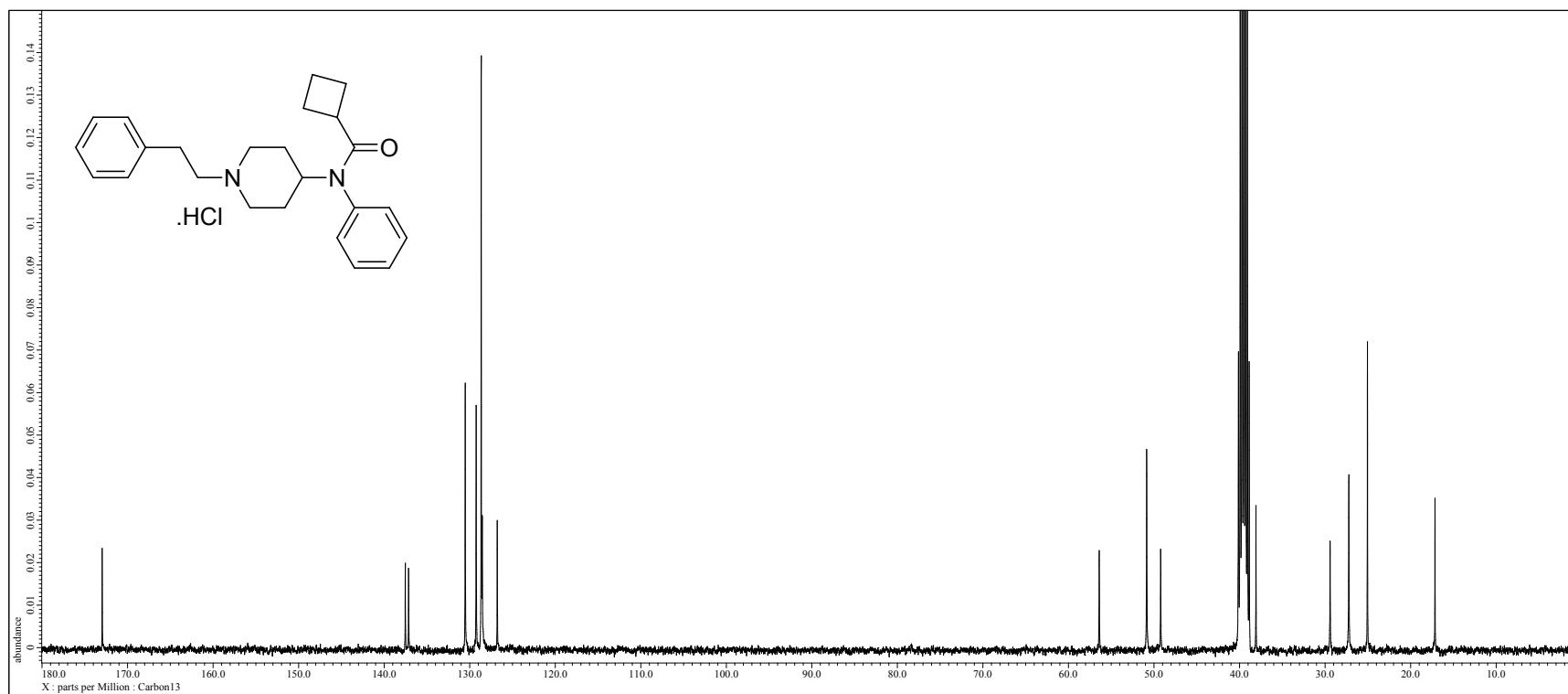
<sup>13</sup>C-NMR (100 MHz, d<sub>6</sub>-DMSO) of *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylisobutyramide hydrochloride (isobutyrfentanyl, HCl, **2e**)



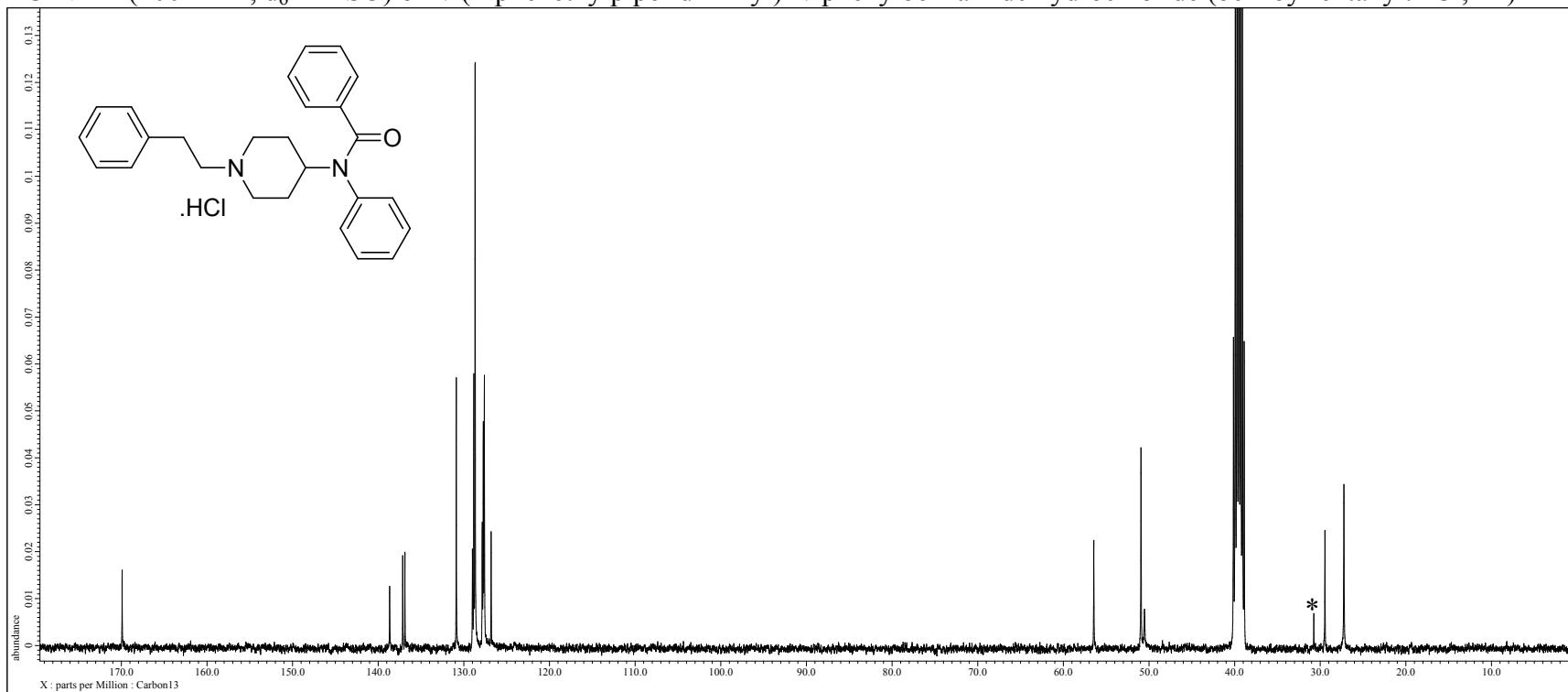
<sup>13</sup>C-NMR (100 MHz, d<sub>6</sub>-DMSO) of *N*-(1-phenethylpiperidin-4-yl)-*N*-butyramide hydrochloride (butyrfentanyl.HCl, 2f)



<sup>13</sup>C-NMR (100 MHz, d<sub>6</sub>-DMSO) of *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylcyclobutanecarboxamide hydrochloride (cyclobutylfentanyl.HCl, **2g**)

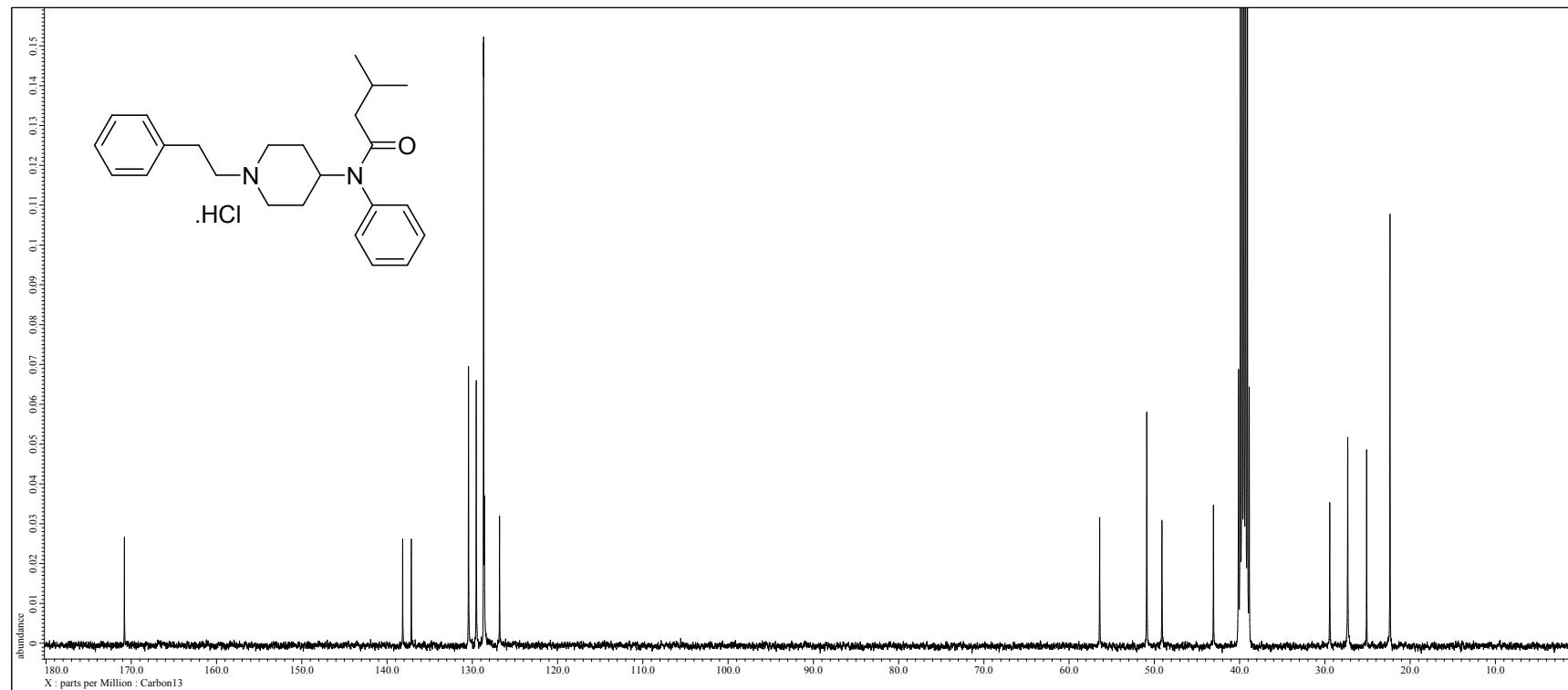


<sup>13</sup>C-NMR (100 MHz, d<sub>6</sub>-DMSO) of *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylbenzamide hydrochloride (benzoylfentanyl.HCl, **2h**)

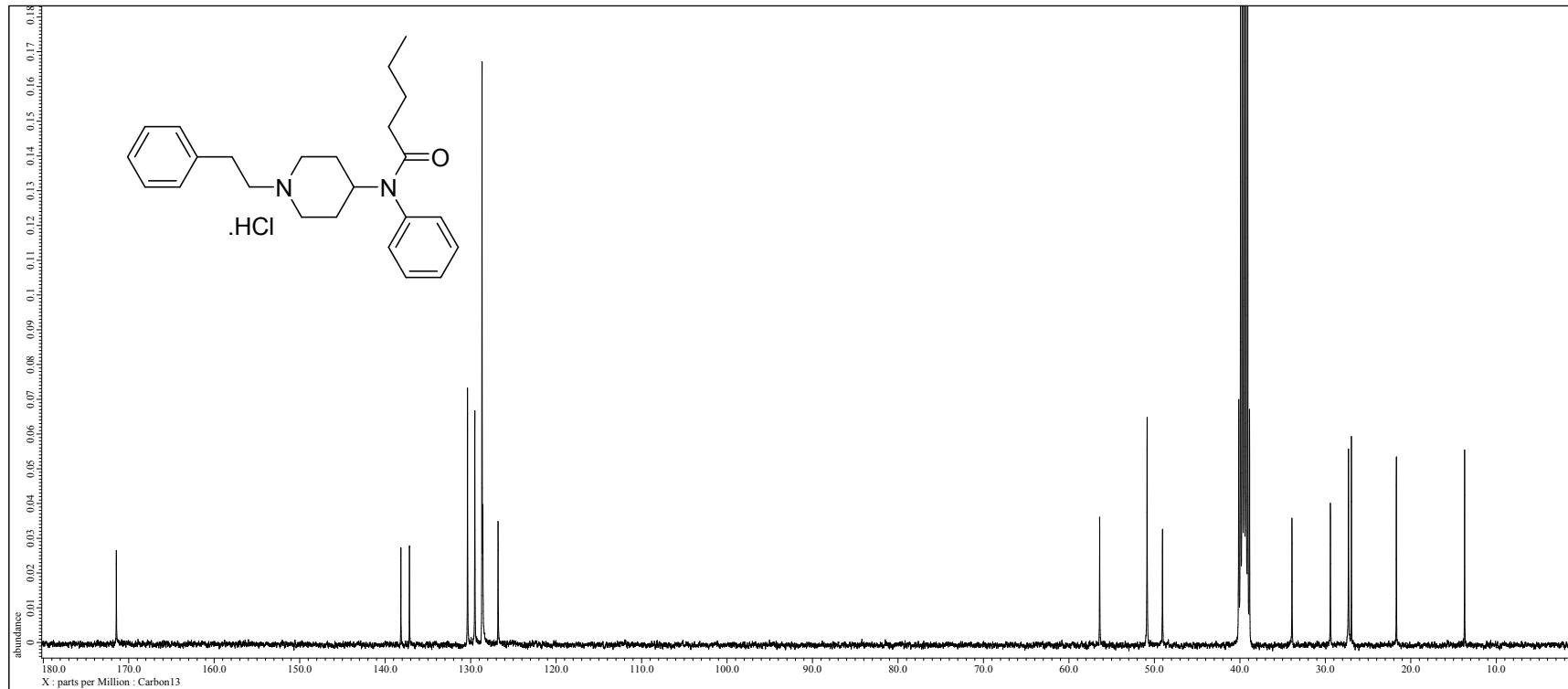


Peak denoted by (\*) is residual acetone.

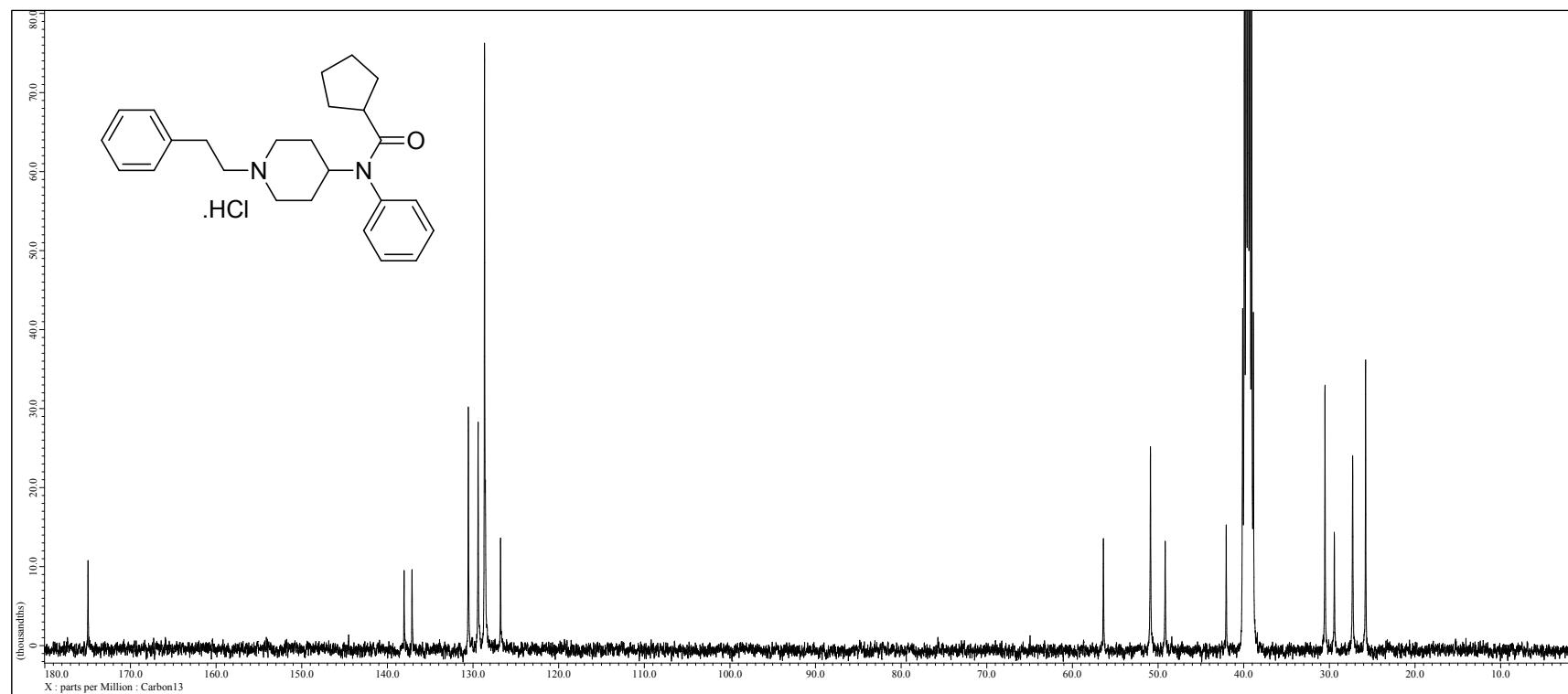
<sup>13</sup>C-NMR (100 MHz, d<sub>6</sub>-DMSO) of 3-methyl-N-(1-phenethylpiperidin-4-yl)-N-phenylbutanamide hydrochloride (isovalerylfentanyl.HCl, **2i**)



<sup>13</sup>C-NMR (100 MHz, d<sub>6</sub>-DMSO) of *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylpentanamide hydrochloride (valerylfentanyl.HCl, **2j**)



$^{13}\text{C}$ -NMR (100 MHz,  $\text{d}_6\text{-DMSO}$ ) of *N*-(1-phenethylpiperidin-4-yl)-*N*-phenylcyclopentanecarboxamide hydrochloride (cyclopentylfentanyl.HCl, **2k**)



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