

## **<sup>19</sup>F NMR Monitoring of the Eukaryotic 20S Proteasome Chymotrypsin-like Activity : Investigative tool for studying Allosteric Regulation \*\***

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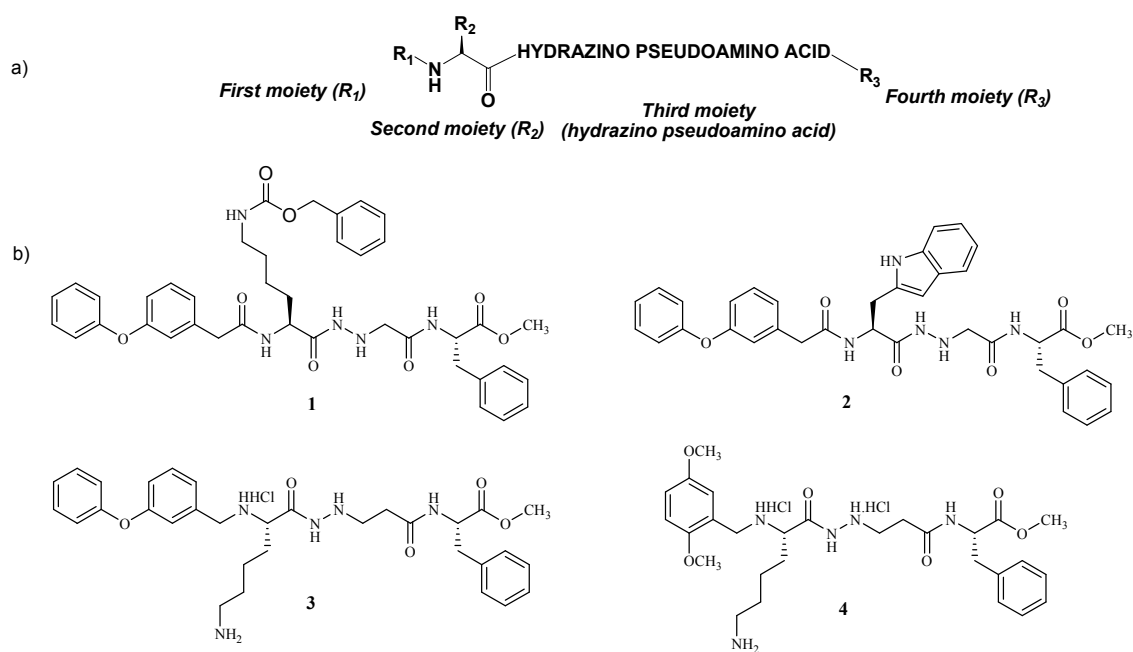
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## 1- Synthetic procedures and characterization of the hydrazino acid-based pseudopeptides 2-4.



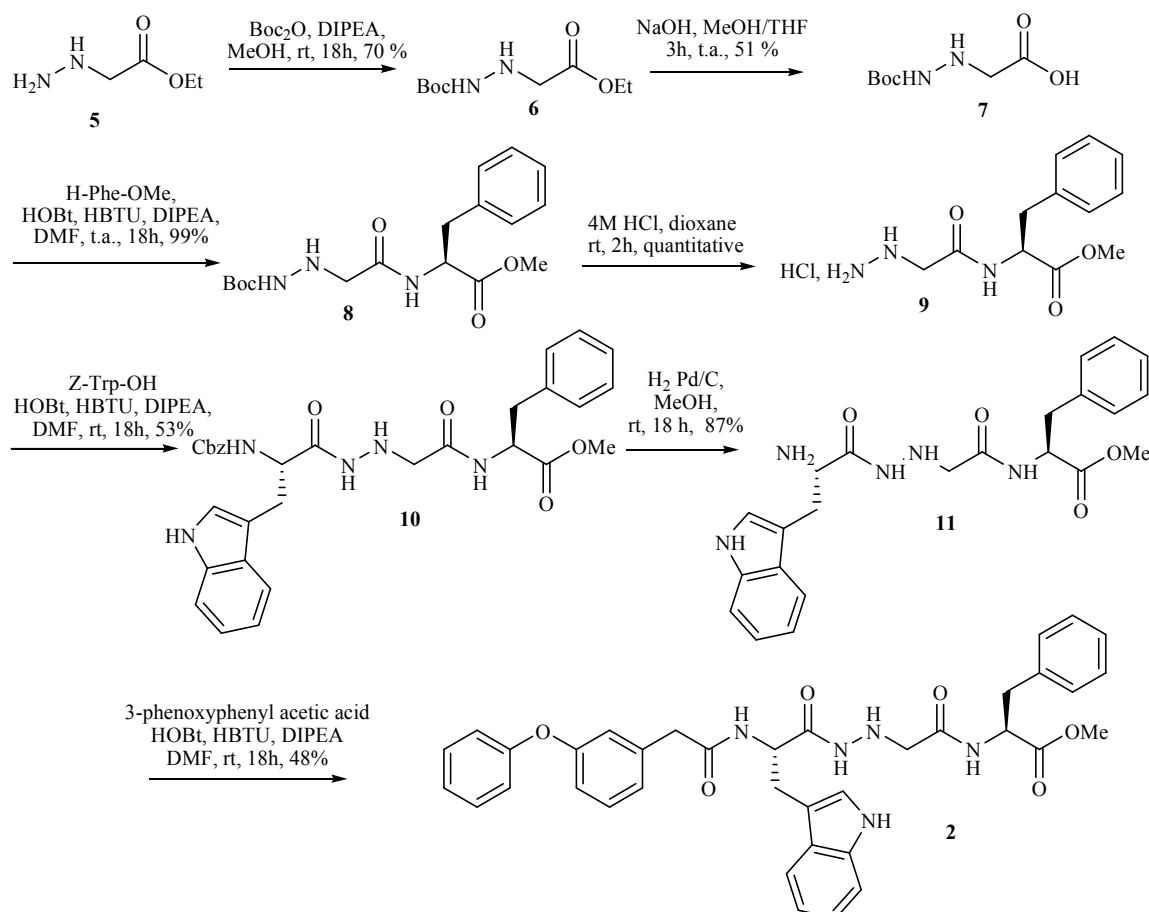
**Figure 1S.** a) Schematic representation of hydrazino acid based pseudopeptides. b) Structures of molecules 1-4.

### Chemistry

Usual solvents were purchased from commercial sources and dried and distilled by standard procedures. Pseudopeptides **1** and **12** were prepared according to published methods [15]. Pure products were obtained after liquid chromatography using Merck silica gel 60 (40-63  $\mu\text{m}$ ). TLC analyses were performed on silica gel 60 F<sub>250</sub> (0.26 mm thickness) plates. The plates were visualized with UV light ( $\lambda = 254 \text{ nm}$ ) or revealed with a 4 % solution of phosphomolybdic acid or ninhydrin in EtOH. Elemental analyses (C, H, N) were performed on a Perkin-Elmer CHN Analyser 2400 at the Microanalyses Service of the Faculty of Pharmacy in Châtenay-Malabry (France). Mass spectra were obtained using a Bruker Esquire electrospray ionization apparatus at the SAMM (Faculty of Pharmacy at Châtenay-Malabry, France). HRMS were obtained using a TOF LCT Premier apparatus (Waters), with an electrospray ionization source. NMR spectra were recorded on an ultrafield AVANCE 300 ( $^1\text{H}$ , 300 MHz,  $^{13}\text{C}$ , 75 MHz) or a Bruker 400 ( $^1\text{H}$ , 400 MHz,  $^{13}\text{C}$ , 100 MHz,  $^{19}\text{F}$  376 MHz).

Chemical shift  $\delta$  are in parts per million (ppm) and the following abbreviations are used: singlet (s), doublet (d), doublet of doublet (dd), triplet (t), multiplet (m), and broad singlet (bs). Melting points were determined on Kofler melting point apparatus and are uncorrected.

### Synthesis of compound 2.



**Scheme 1S.** Synthesis of compound 2.

### Ethyl 2-(2-tert-butoxycarbonylhydrazino)acetate 6

To a solution of ethyl hydrazinoacetate **5** (1.53 g, 13.00 mmol, 1.0 eq) in MeOH (20 mL), was added DIPEA (6.72 g, 52.00 mmol, 4.0 eq) and  $\text{Boc}_2\text{O}$  (3.00 g, 14.20 mmol, 1.1 eq). The mixture was stirred for 18 hours at room temperature. The solvent was removed and the residue obtained was dissolved in EtOAc (20 mL). The organic layer was washed successively with a 10% aqueous solution of citric acid (2 X 15 mL), brine (20 mL), dried over  $\text{MgSO}_4$  filtrated and evaporated. The crude product was purified by column chromatography (EtOAc/Cyclohexane: 8:2) to afford **6** (2.00 g, 9.20 mmol, 70%) as a colorless oil. Rf: 0.7 ( $\text{SiO}_2$ , EtOAc/Cyclohexane: 8:2);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  6.43 (bs,

1H), 4.52 (m, 1H), 4.17 (q, 2H, J = 6.7 Hz), 4.06 (m, 2H), 1.42 (s, 9H), 1.23 (t, 3H, J = 6.7 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ 171.4, 156.2, 81.2, 61.0, 52.8, 28.2, 14.2; ESI<sup>+</sup> MS *m/z*: 241 [M+Na]<sup>+</sup>; Anal. Calcd for C<sub>9</sub>H<sub>18</sub>N<sub>2</sub>O<sub>4</sub>: C, 49.53; H, 8.31; N, 12.84; Found C, 50.07; H, 8.84; N, 12.59.

### **2-(2-tert-butoxycarbonylhydrazino)acetic acid 7**

To a solution of **6** (1.86 g, 8.53 mmol, 1.0 eq) in 20 mL of a THF/MeOH mixture (1/1), 4.7 mL (9.4 mmol, 1.1 eq) of a 2N aqueous solution of NaOH were added. The reaction mixture was stirred at room temperature for 3 hours. The solvent was removed under reduced pressure (without distilling the water) and the remaining solution was acidified to pH = 5 using a 1N aqueous solution of HCl. The aqueous phase was extracted with EtOAc (3 x 20 mL). The combined organic layers were dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to obtain **7** as a white solid which was used in the next step without further purification (823 mg, 4.33 mmol, 51%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 300 MHz) δ 8.30 (bs, 1H), 7.52 (bs, 1H), 3.93 (s, 2H), 1.38 (s, 9H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 75 MHz) δ 171.1, 156.2, 79.5, 52.8, 28.1; MS (ESI<sup>+</sup>) *m/z*: 213 [M+Na]<sup>+</sup>; Anal. Calcd for C<sub>7</sub>H<sub>14</sub>N<sub>2</sub>O<sub>4</sub>: C, 44.20; H, 7.42; N, 14.73; Found C, 44.22; H, 7.88; N, 14.48.

### **Methyl (2*S*)-2-[[2-(2-tert-butoxycarbonylhydrazino)acetyl]amino]-3-phenyl-propanoate 8**

To a solution of **7** (800 mg, 4.21 mmol, 1.0 eq) in 10 mL of dry DMF, was added H-Phe-OMe (1.10 g, 5.04 mmol, 1.2 eq), DIPEA (2.14 g, 16.8 mmol, 4 eq), HBTU (1.9 g, 5.04 mmol, 1.2 eq), and HOBt (681 mg, 5.04 mmol, 1.2 eq) in this order. The reaction mixture was stirred for 18 h under argon atmosphere at room temperature. The solvent was evaporated under reduced pressure and the crude residue obtained was dissolved in EtOAc (20 mL). The organic layer was washed with a 10% aqueous solution of citric acid (2 x 20 mL), water (20 mL), a 10% aqueous solution of K<sub>2</sub>CO<sub>3</sub> (2 x 20 mL), brine (20 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated. The crude product was purified by column chromatography (EtOAc/cyclohexane: 8:2) to afford **8** (1.30 g, 3.70 mmol, 88%) as a white foam: R<sub>f</sub>: 0.5 (SiO<sub>2</sub>, EtOAc); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 7.26 (m, 5H), 7.08 (m, 1H), 6.42 (d, J = 5.1 Hz, 1H), 5.90 (m, 1H), 4.88 (m, 1H), 4.03 (s, 2H), 3.72 (s, 3H), 3.20-3.10 (m, 2H), 1.44 (s, 9H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ 171.8, 168.7, 156.9, 135.9, 129.3, 128.6, 127.2, 81.2, 54.9, 53.6, 53.0, 37.8, 28.2; IR

( $\text{cm}^{-1}$ ): 3300 (N-H), 2970 (C-H) (1652  $\text{-C=O}$ ), 1506 (C=C), 1234 (C-O); ESI<sup>+</sup> MS  $m/z$ : 374 [M+Na]<sup>+</sup>.

### **Methyl (2S)-2-[(2-hydrazinoacetyl)amino]-3-phenyl-propanoate 9**

A solution of **8** (662 mg, 1.89 mmol, 1.0 eq) in 15 mL of 4M HCl/dioxane was stirred for 2 h at room temperature. The solvent was evaporated under reduced pressure to afford the hydrochloride salt of **9** in quantitative yield as a yellow oil, which was used without further purification. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 300 MHz)  $\delta$  8.82 (d, 1H,  $J = 5.3$  Hz), 8.2 (bs, 3H), 7.31-7.21 (m, 6H), 4.52 (m, 1H), 4.03 (s, 2H), 3.62 (s, 3H), 3.03 (dd,  $J = 13.6, 6.3$  Hz, 1H), 2.92 (m, 1H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 75 MHz)  $\delta$  171.6, 168.2, 136.9, 129.0, 128.3, 126.6, 53.7, 53.5, 50.2, 36.6; ESI<sup>+</sup> MS  $m/z$ : 252 [M+H]<sup>+</sup>.

### **Methyl (2S)-2-[[2-[2-[(2S)-2-(benzyloxycarbonylamino)-3-(1H-indol-3-yl)propanoyl]hydrazino]acetyl]amino]-3-phenyl-propanoate 10**

To a solution of **9** (1.3 g, 3.70 mmol, 1.0 eq) in dry DMF (20mL) was added Z-Trp-OH (1.0 g, 2.96 mmol, 0.8 eq), DIPEA (3.3 g, 25.90 mmol, 7.0 eq), HBTU (1.7 g, 4.45 mmol, 1.2 eq), and HOBt (601 mg, 4.45 mmol, 1.2 eq) in this order. The mixture was stirred at room temperature under argon atmosphere for 18 hours. The solvent was evaporated under reduced pressure and the residue obtained was dissolved in EtOAc (20 ml). The organic layer was washed successively with a 10% aqueous solution of citric acid (2 x 20 mL), water (20 mL), a 10% aqueous solution of K<sub>2</sub>CO<sub>3</sub> (2 x 20 mL), brine (20 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated. The crude product was purified by column chromatography (EtOAc) to afford **10** (1.12 g, 1.96 mmol, 53%) as a white foam: Rf: 0.5 (S<sub>i</sub>O<sub>2</sub>, EtOAc); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  8.38 (bs, 1H), 7.60 (m, 2H), 7.32 (m, 2H), 7.30 (m, 2H), 7.24 (m, 1H), 7.22-7.15 (m, 8H), 7.08 (m, 2H), 6.89 (bs, 1H), 5.59 (d,  $J = 5.1$  Hz, 1H), 5.09 (m, 2H), 4.72 (m, 1H), 4.40 (m, 1H), 3.61 (s, 3H), 3.25 (m, 1H), 3.18 (m, 2H), 3.11 (m, 1H), 3.09 (m, 1H), 2.98 (dd,  $J = 13.9, 7.3$  Hz, 1H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz)  $\delta$  172.1, 171.2, 169.5, 156.9, 136.2, 136.1, 136.0, 135.9, 129.1, 128.6, 128.5, 128.2, 127.2, 123.6, 122.2, 118.6, 111.4, 109.6, 67.1, 54.3, 53.0, 52.4, 37.5, 28.7; IR ( $\text{cm}^{-1}$ ): 3300 (N-H), 1652  $\text{-C=O}$ ), 1506 (C=C), 1234 (C-O); ESI<sup>+</sup> MS  $m/z$ : 594 [M+Na]<sup>+</sup>; Anal. Calcd for C<sub>31</sub>H<sub>33</sub>N<sub>5</sub>O<sub>6</sub>+1H<sub>2</sub>O: C, 63.14; H, 6.00; N, 11.88; Found C, 63.17; H, 5.79; N, 11.81.

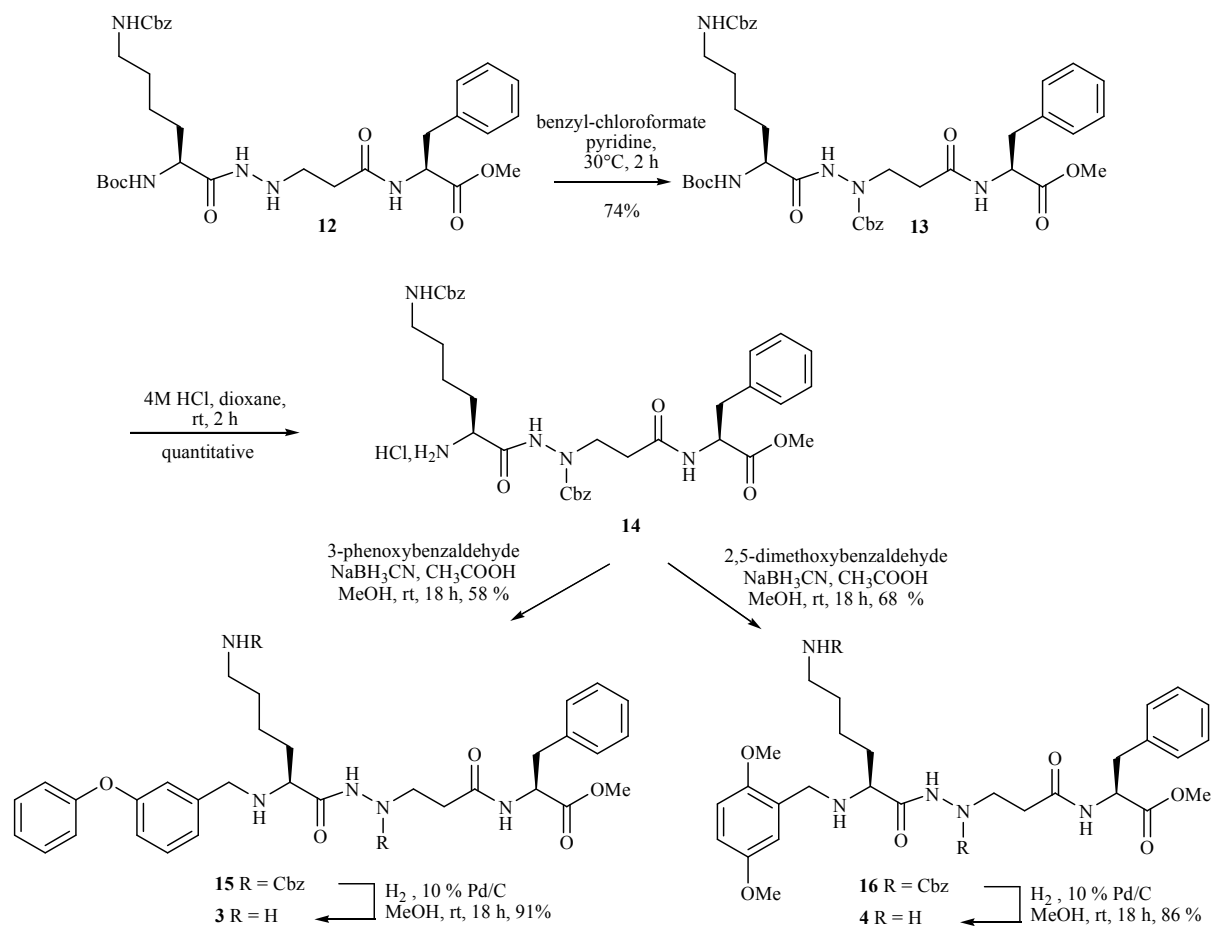
**Methyl (2S)-2-[[2-[2-[(2S)-2-amino-3-(1H-indol-3-yl)propanoyl]hydrazino]acetyl]amino]-3-phenyl-propanoate 11**

To a solution of **10** (866 mg, 1.50 mmol, 1 eq) in dry MeOH (15 mL) was added Pd/C 10% (85 mg). The mixture was stirred overnight under hydrogen atmosphere at room temperature and filtered on a celite pad. After concentration under reduced pressure, **11** was obtained as a yellowish solid (576 mg, 1.32 mmol, 87%) and was used without further purification. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 8.51 (bs, 2H), 8.32 (bs, 1H), 7.51 (m, 2H), 7.25 (m, 1H), 7.13-6.97 (m, 8H), 6.91 (s, 1H), 4.76 (m, 1H), 3.60 (s, 3H), 3.53 (m, 1H), 3.11-2.98 (m, 6H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ 174.5, 172.1, 170.0, 136.3, 136.1, 129.7, 129.1, 127.5, 127.1, 123.4, 122.1, 119.5, 118.7, 114.4, 110.8, 54.8, 54.7, 52.9, 52.4, 37.6, 30.7; ESI<sup>+</sup> MS *m/z*: 460 [M+Na<sup>+</sup>].

**Methyl (2S)-2-[[2-[2-[(2S)-3-(1H-indol-3-yl)-2-[[2-(3-phenoxyphenyl)acetyl]amino]propanoyl]hydrazino]acetyl]amino]-3-phenyl-propanoate 2**

To a solution of **11** (616 mg, 1.41 mmol, 1.0 eq) in dry DMF (15 mL) was added 3-phenoxyphenylacetic acid (356 mg, 1.56 mmol, 1.1 eq), DIPEA (672 mg, 5.22 mmol, 3.7 eq), HBTU (595 mg, 1.56 mmol, 1.1 eq), and HOBt (211 mg, 1.56 mmol, 1.1 eq) in this order. The reaction mixture was stirred overnight at room temperature under argon atmosphere. The solvent was evaporated under reduced pressure and the obtained residue was dissolved in EtOAc (20 mL). The organic layer was successively washed with a 10% aqueous solution of citric acid (2 x 15 mL), water (15 mL), a 10% aqueous solution of K<sub>2</sub>CO<sub>3</sub> (2 x 15 mL), brine (15 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated. The crude product was purified by column chromatography (EtOAc) to afford **2** (407 mg, 0.63 mmol, 45%) as a white foam: Rf: 0.5 (SiO<sub>2</sub>, EtOAc/MeOH: 9/1); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 8.34 (bs, 1H), 7.69 (bs, 1H), 7.50 (d, J = 7.5 Hz, 1H), 7.43 (d, J = 7.9 Hz, 1H), 7.49 (m, 2H), 7.43 (m, 2H), 7.22 (m, 2H), 7.19 (m, 1H), 7.17 (m, 1H), 7.15 (m, 1H), 7.13 (m, 1H), 7.03 (m, 2H), 6.96 (d, J = 8.1 Hz, 2H), 6.87 (m, 3H), 6.76 (m, 1H), 6.71 (s, 1H), 6.39 (d, J = 7.4 Hz, 1H), 4.65 (m, 1H), 4.50 (m, 1H), 3.53 (s, 3H), 3.38 (s, 2H), 3.08 (m, 2H), 3.06 (m, 1H), 3.03 (m, 1H), 2.94 (m, 1H), 2.91 (dd, J = 13.9, 7.3 Hz, 1H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ 172.3, 171.1, 170.7, 170.0, 157.6, 156.9, 136.4, 136.1, 136.0, 130.1, 129.8, 129.0, 128.6, 127.1, 124.1, 123.4, 119.7, 119.0, 118.7, 118.0, 117.4, 114.4, 109.8, 54.4, 53.1, 52.6, 52.4, 43.0, 37.5, 28.2; IR (cm<sup>-1</sup>): 3300 (N-H), 1643 (C=O), 1506 (C=C), 1234 (C-O); ESI<sup>+</sup> MS *m/z*: 670 [M+Na<sup>+</sup>]; Anal. Calcd for C<sub>37</sub>H<sub>37</sub>N<sub>5</sub>O<sub>6</sub>: C, 68.61; H, 5.76; N, 10.81; Found C, 68.29; H, 6.03; N, 10.73.

## 1.1 Synthesis of compounds **3** and **4**.



**Scheme 2S.** Synthesis of compounds **3** and **4**.

### Methyl (2*S*)-2-[3-[benzyloxycarbonyl-[(2*S*)-6-(benzyloxycarbonylamino)-2-(tert-butoxycarbonylamino)hexanoyl]amino]amino]propanoylamino]-3-phenyl-propanoate **13**

To a solution of **12** (650 mg, 1.04 mmol) in dry pyridine (20 mL) was added benzyl chloroformate (355 mg, 2.08 mmol, 2 eq) and the mixture was stirred during 3 hours at 30°C. After removal of pyridine under reduced pressure, the residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and successively washed with a 10% aqueous solution of K<sub>2</sub>CO<sub>3</sub> (2 x 15 mL) and brine (20 mL). The organic layer was dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford a crude product which was purified by column chromatography (EtOAc/Cyclohexane:

6/4) to afford **13** as a colorless oil (583 mg, 0.77 mmol, 74%). Rf: 0.6 (SiO<sub>2</sub>, EtOAc); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 9.05 (bs, 1H), 7.76 (bs, 1H), 7.47 (bs, 1H), 7.37-7.22 (m, 15H), 5.13 (m, 1H), 5.07 (s, 4H), 4.80 (m, 1H), 4.04 (m, 1H), 3.77 (m, 2H), 3.72 (s, 3H), 3.19 (m, 1H), 2.95 (m, 1H), 2.94 (m, 2H), 2.33 (m, 2H), 1.82 (m, 1H), 1.68 (m, 1H), 1.60 (m, 2H), 1.44 (s, 9H), 1.33 (m, 2H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ 174.7, 171.7, 156.2, 156.0, 136.6, 136.3, 128.9, 128.5, 128.4, 128.0, 127.9, 126.9, 80.6, 66.6, 66.5, 53.8, 52.8, 52.7, 45.4, 40.4, 37.0, 32.7, 31.2, 28.9, 28.3, 22.2; IR (cm<sup>-1</sup>): 3322 (N-H), 2952 (C-H), 1689 (C=O), 1525 (N-H), 1454 (C-H), 1225 (C-N); ESI<sup>+</sup> MS *m/z*: 784 [M+Na<sup>+</sup>]; Anal. calcd for C<sub>40</sub>H<sub>51</sub>N<sub>5</sub>O<sub>10</sub> + 0.25 H<sub>2</sub>O: C. 63.23, H. 7.30, N. 8.92 Found C. 62.90, H. 7.34, N. 8.88.

**Methyl (2S)-2-[3-[[[(2S)-2-amino-6-(benzyloxycarbonylamino)hexanoyl]amino]-benzyloxycarbonyl-amino]propanoylamino]-3-phenyl-propanoate hydrochloride **14****

A solution of **13** (523 mg, 0.69 mmol, 1.0 eq) in 15 mL of 4M HCl in dioxane was stirred for 2 h at room temperature. The solvent was evaporated under reduced pressure to afford **14** as a white solid which was used without further purification. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 300 MHz) δ 10.80 (s, 1H), 8.55 (d, J = 7.8 Hz, 1H), 8.35 (bs, 3H), 7.30-7.25 (m, 15H), 5.01 (s, 4H), 4.80 (m, 1H), 4.45 (d, 1H), 4.04 (m, 1H), 3.55 (s, 3H), 3.02 (m, 6H), 2.41 (m, 2H), 1.8-1.2 (m, 6H). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 75 MHz) δ 177.2, 175.1, 173.4, 161.3, 159.9, 142.4, 141.4, 134.3; 133.6; 133.4; 133.2; 133.0; 132.8; 131.8, 72.3; 71.6, 58.8, 57.0, 56.1, 44.8, 44.7, 43.9, 41.9, 35.7, 34.0, 26.5; ESI<sup>+</sup> MS *m/z*: 662 [M+H<sup>+</sup>].

**Methyl (2S)-2-[3-[benzyloxycarbonyl-[(2S)-6-(benzyloxycarbonylamino)-2-[(3-phenoxyphenyl)methylamino]hexanoyl]amino]amino]propanoylamino]-3-phenyl-propanoate **15****

To a solution of **14** (272 mg, 0.39 mmol, 1.0 eq) in 7 mL of methanol was added DIPEA (100 mg, 0.78 mmol, 2.0 eq) and a solution of 3-phenoxybenzaldehyde (78 mg, 0.39 mmol, 1.0 eq) in methanol (3 mL). After stirring for 5 min at room temperature, NaBH<sub>3</sub>CN (25 mg, 0.39 mmol, 1.0 eq) was added and the solution was acidified to pH = 5 by addition of acetic acid. The reaction mixture was stirred overnight at room temperature and then, quenched by addition of a 2N HCl solution. After stirring for 10 min at room temperature, the mixture was basified to pH = 10 by addition of a saturated solution of Na<sub>2</sub>CO<sub>3</sub>. The methanol was then evaporated under reduced pressure (without distilling water) and the aqueous mixture was extracted with EtOAc (2 x 15 mL). The organic layer was dried over MgSO<sub>4</sub>, filtered and



concentrated to give a crude product which was purified by column chromatography (EtOAc) to afford **15** (190 mg, 0.23 mmol, 58 %) as a colorless oil.  $R_f = 0.6$  ( $S_iO_2$ , EtOAc);  $^1H$  NMR ( $CDCl_3$ , 400 MHz)  $\delta$  8.90 (bs, 1H), 7.32 (m, 2H), 7.26 (m, 10H), 7.23 (m, 1H), 7.11 (m, 1H), 7.09 (m, 5H), 7.08 (m, 2H), 6.98 (m, 2H), 6.87 (m, 1H), 6.42 (bs, 1H), 5.08 (s, 4H), 4.80 (m, 1H), 3.86 (m, 2H), 3.63 (s, 3H), 3.42 (s, 2H), 3.14 (m, 1H), 3.13 (m, 2H), 3.07 (m, 1H), 2.97 (m, 1H), 2.39 (m, 2H), 1.66 (m, 1H), 1.57 (m, 1H), 1.37 (m, 2H), 1.30 (m, 2H);  $^{13}C$  NMR ( $CDCl_3$ , 100 MHz)  $\delta$  172.3, 172.0, 171.8, 157.4, 157.1, 156.3, 141.6, 136.7, 135.8, 129.7; 129.1; 128.6; 128.5, 128.2; 128.0; 127.1, 123.3, 118.8, 117.5, 68.2, 66.5, 60.9, 53.3, 52.3, 51.9, 47.7, 40.6, 37.7, 35.2, 32.9, 29.5, 22.7; IR ( $cm^{-1}$ ): 3295 (N-H), 2949 (C-H), 1707 (C=O), 1498 (C=C), 1453 (C-H); ESI<sup>+</sup> MS  $m/z$ : 867 [ $M+Na$ ]<sup>+</sup>; Anal. Calcd for  $C_{48}H_{53}N_5O_9 + 1.5 H_2O$ : C, 66.19; H, 6.49; N, 8.04; Found C, 66.20; H, 6.44; N, 7.54.

**Methyl (2S)-2-[3-[2-[(2S)-6-amino-2-[(3-phenoxyphenyl)methylamino]hexanoyl]hydrazino]propanoylamino]-3-phenyl-propanoate trihydrochloride **3****

To a solution of **15** (150 mg, 0.18 mmol, 1.0 eq) in dry MeOH (25 mL) were added 15 mg of Pd/C 10 %. After 18 h of stirring under hydrogen atmosphere at room temperature, the reaction mixture was filtrated on a celite pad. The solvent was then removed under reduced pressure, HCl/MeOH was added and **3** was obtained as a white solid after a precipitation with  $Et_2O$  (92 mg, 0.16 mmol, 91%);  $^1H$  NMR ( $CD_3OD$ , 300 MHz)  $\delta$  7.31-7.03 (m, 8H), 6.98 (m, 2H), 6.93-6.81 (m, 3H), 6.75 (dd,  $J = 8.1, 1.6$  Hz, 1H), 4.57 (m, 1H), 3.65 (s, 3H), 3.38 (m, 2H), 3.12 (m, 1H), 3.00-2.79 (m, 4H), 2.56 (t,  $J = 7.1$  Hz, 2H), 2.22 (t,  $J = 7.1$  Hz, 2H), 1.56 (m, 1H), 1.49 (m, 1H), 1.33 (m, 2H), 1.26 (m, 2H);  $^1H$  NMR ( $DMSO-d_6$ , 400 MHz)  $\delta$  8.59 (bs, 1H), 8.52 (bs, 1H), 8.18 (bs, 1H), 8.0-7.94 (bs, 5H), 7.40 (m, 1H), 7.26-7.16 (m, 10H), 7.03 (m, 1H), 6.87-6.75 (m, 3H), 4.46 (m, 1H), 4.22 (m, 1H), 3.59 (s, 3H), 3.45 (m, 2H), 3.0-2.92 (m, 2H), 2.91 (m, 2H), 2.74 (m, 2H), 2.30 (m, 2H), 1.64-1.59 (m, 2H), 1.56 (m, 2H), 1.33 (m, 2H);  $^{13}C$  NMR ( $CD_3OD$ , 100 MHz)  $\delta$  172.1, 171.8, 171.5, 155.0, 153.1, 140.9, 138.0, 131.8; 131.0; 129.5; 127.9, 128.2, 124.9, 120.7, 120.3, 61.2, 56.6, 56.2, 52.8, 48.5, 40.6, 38.3, 36.2, 33.7, 28.3, 22.3; IR ( $cm^{-1}$ ): 3295 (N-H), 2949 (C-H), 1707 (C=O), 1498 (C=C), 1453 (C-H); ESI<sup>+</sup> MS  $m/z$ : 598 [ $M+Na$ ]<sup>+</sup>; Anal. Calcd for  $C_{32}H_{44}Cl_3N_5O_5 + 1 H_2O$ : C, 54.66; H, 6.61; N, 9.96; Found C, 54.34; H, 6.81; N, 10.09.

**Methyl (2S)-2-[3-[benzyloxycarbonyl-[(2S)-6-(benzyloxycarbonylamino)-2-[(2,5-dimethoxyphenyl)methylamino]hexanoyl]amino]amino]propanoylamino]-3-phenylpropanoate 16**

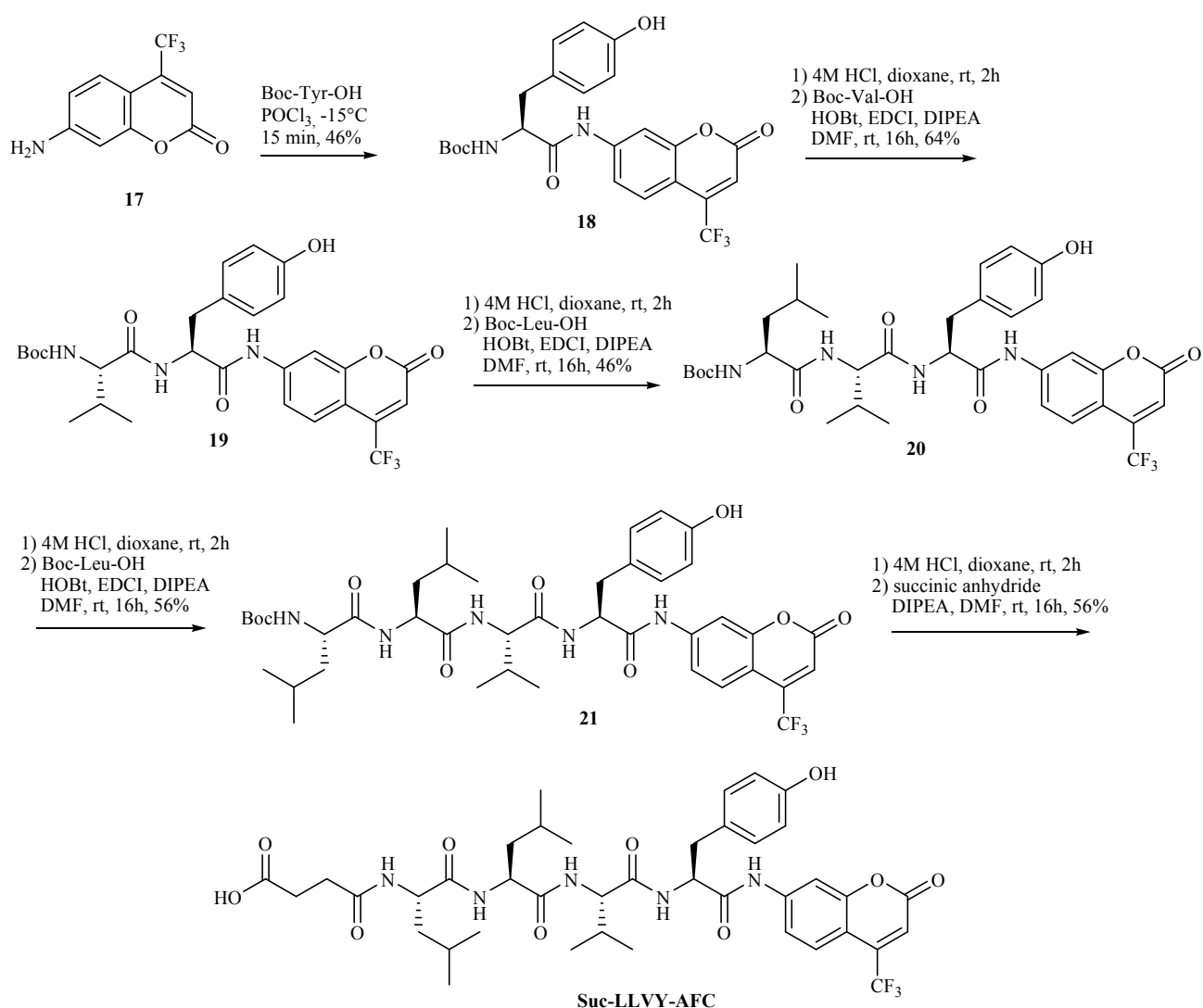
To a solution of the **14** (209 mg, 0.30 mmol, 1.0 eq) in methanol (7 mL) was added DIPEA (0.19 mL, 0.60 mmol, 2.0 eq) and a solution of 2,5-dimethoxybenzaldehyde (47 mg, 0.30 mmol, 1.0 eq) in methanol (3 mL). After stirring for 5 min at room temperature, NaBH<sub>3</sub>CN (19 mg, 0.30 mmol, 1.0 eq) was added and the solution was acidified to pH = 5 by addition of acetic acid. The reaction mixture was stirred overnight at room temperature and then, was quenched by addition of a 2N solution of HCl. After stirring for 10 min at room temperature, the mixture was basified to pH = 10 by addition of a saturated solution of Na<sub>2</sub>CO<sub>3</sub>. The methanol was then evaporated under reduced pressure (without distilling water) and the aqueous mixture was extracted with EtOAc (2 x 20 mL). The organic layer was dried over MgSO<sub>4</sub>, filtered and concentrated to give a crude product which was purified by column chromatography (EtOAc) to afford **16** (165 mg, 0.20 mmol, 68%) as a colorless oil. R<sub>f</sub> = 0.3 (SiO<sub>2</sub>, EtOAc); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 9.26 (s, 1H), 7.32-7.21 (m, 15H), 7.12 (d, J = 7.3 Hz, 1H), 6.80 (m, 1H), 6.76 (m, 1H), 6.68 (m, 1H), 5.13 (m, 1H), 5.05 (s, 4H), 4.80 (m, 1H), 4.66 (s, 2H), 3.82 (m, 2H), 3.76 (s, 3H), 3.74 (s, 3H), 3.64 (s, 3H), 3.17 (m, 1H), 3.08 (m, 2H), 3.06 (m, 2H), 2.46 (m, 2H), 1.68 (m, 1H), 1.54 (m, 1H), 1.45 (m, 2H), 1.26 (m, 2H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) δ 172.2, 170.6, 156.5, 155.3, 153.5, 151.8, 136.7, 136.1, 135.8, 129.1; 128.5; 128.4; 128.2; 128.1; 127.0, 116.9, 113.0, 111.3, 68.2, 66.5, 62.1, 60.9, 55.8, 55.7, 53.5, 52.2, 47.3, 37.9, 34.8, 32.8, 29.4, 22.6; IR (cm<sup>-1</sup>): 3295 (N-H), 2949 (C-H), 1707 (C=O), 1498 (C=C), 1453 (C-H); ESI<sup>+</sup> MS *m/z*: 834 [M+Na]<sup>+</sup>; Anal. Calcd for C<sub>44</sub>H<sub>53</sub>N<sub>5</sub>O<sub>10</sub>: C, 65.09; H, 6.59; N, 3.45; Found C, 64.66; H, 6.90; N, 3.82.

**Methyl (2S)-2-[3-[2-[(2S)-6-amino-2-[(2,5-dimethoxyphenyl)methylamino]hexanoyl]hydrazino]propanoylamino]-3-phenylpropanoate trihydrochloride 4**

To a solution of **16** (181 mg, 0.22 mmol, 1.0 eq) in dry MeOH (25 mL) were added 118 mg of Pd/C 10%. The reaction mixture was stirred at room temperature overnight under hydrogen atmosphere and then, filtered on a celite pad. After removal of the solvent under reduced pressure, HCl/MeOH was added and **4** was obtained as a white solid after a precipitation with Et<sub>2</sub>O (101 mg, 0.19 mmol, 86%). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 11.48 (bs, 1H), 9.92 (bs, 1H), 9.37 (bs, 1H), 8.68 (d, J = 7.7 Hz, 1H), 8.11 (bs, 5H), 7.27 (m, 2H), 7.24 (m, 1H), 7.23 (m, 2H), 7.21 (m, 1H), 6.98 (m, 1H), 6.95 (m, 1H), 4.46 (m, 1H), 4.04 (s, 2H), 3.80 (m, 1H),

3.75 (s, 3H), 3.72 (s, 3H), 3.59 (s, 3H), 3.07 (m, 2H), 3.02 (m, 1H), 2.91 (m, 1H), 2.74 (m, 2H), 2.49 (m, 2H), 1.96 (m, 1H), 1.86 (m, 1H), 1.59 (m, 2H), 1.36 (m, 2H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)  $\delta$  172.0, 169.8, 165.9, 152.8, 151.6, 137.2, 129.1, 128.2, 126.5, 119.7, 117.5, 115.5, 112.1, 57.2, 56.0, 55.6, 53.7, 51.8, 46.4, 43.3, 38.2, 36.7, 31.5, 28.8, 26.3, 21.1; IR ( $\text{cm}^{-1}$ ): 3295 (N-H), 2949 (C-H), 1707 (C=O), 1498 (C=C), 1453 (C-H); ESI $^+$  MS  $m/z$ : 566  $[\text{M}+\text{Na}]^+$ ; Anal. Calcd for  $\text{C}_{28}\text{H}_{44}\text{Cl}_3\text{N}_5\text{O}_6 + 1.5 \text{H}_2\text{O}$ : C, 49.45; H, 6.98; N, 10.30; Found C, 49.69; H, 6.78; N, 10.16.

## 2- Synthetic procedures and characterization of the substrate Suc-LLVY-AFC



**Scheme 3S.** Synthesis of Suc-LLVY-AFC.

***tert*-butyl N-[(1S)-1-[(4-hydroxyphenyl)methyl]-2-oxo-2-[[2-oxo-4-(trifluoromethyl)chromen-7-yl]amino]ethyl]carbamate **18****

To a solution of 7-amino-4-(trifluoromethyl)coumarin **17** (2.00 g, 8.73 mmol, 1.0 eq) and Boc-Tyr-OH (2.45 g, 8.73 mmol, 1.0 eq) in dry pyridine (60 mL) was added phosphoryl chloride (814  $\mu$ l, 8.73 mmol, 1.0 eq) at  $-15^{\circ}\text{C}$ . After 15 min. of stirring at  $-15^{\circ}\text{C}$ , the solution was poured into water and extracted with EtOAc. The organic layer was then washed with a saturated aqueous solution of  $\text{NaHCO}_3$ , a 10% aqueous solution of citric acid, brine, and dried over  $\text{Na}_2\text{SO}_4$ . After filtration and evaporation under reduced pressure, the crude material was purified by silica gel flash column chromatography (DCM/ $\text{Et}_2\text{O}$ : 8/2) to give **18** (2.00 g, 4.06 mmol, 46%) as a white solid. m.p.:  $224\text{--}226^{\circ}\text{C}$ ; Rf: 0.4 ( $\text{SiO}_2$ , DCM/ $\text{Et}_2\text{O}$ : 8/2);  $^1\text{H}$  NMR (DMSO-*d*<sub>6</sub>, 400 MHz)  $\delta$  10.58 (s, 1H), 9.17 (s, 1H), 7.90 (d,  $J = 2.0$  Hz, 1H), 7.68 (d,  $J = 8.9$  Hz, 1H), 7.53 (dd,  $J = 8.9$  and  $2.0$  Hz, 1H), 7.15 (d,  $J = 7.8$  Hz, 1H), 7.09 (d,  $J = 8.6$  Hz, 2H), 6.90 (s, 1H), 6.65 (d,  $J = 8.6$  Hz, 2H), 4.26 (m, 1H), 2.88 (m, 1H), 2.76 (m, 1H), 1.34 (s, 9H);  $^{13}\text{C}$  NMR (DMSO-*d*<sub>6</sub>, 100 MHz)  $\delta$  172.1, 158.6, 155.8, 154.7, 155.4, 143.1, 139.2 (q,  $J = 31.7$  Hz), 130.1, 127.5, 125.4, 121.8 (q,  $J = 274.5$  Hz), 116.1, 114.9, 114.4, 108.3, 106.3, 78.2, 57.1, 36.4, 28.1;  $^{19}\text{F}$  NMR (DMSO-*d*<sub>6</sub>, 188 MHz)  $\delta$  -63.99 (s); IR ( $\text{cm}^{-1}$ ): 3400 (N-H), 2959 (C-H), 1678 (C=O), 1644 (C=C), 1528 (N-H); ESI<sup>+</sup> MS  $m/z$ : 515 [ $\text{M}+\text{Na}$ ]<sup>+</sup>; Anal. Calcd for  $\text{C}_{24}\text{H}_{23}\text{F}_3\text{N}_2\text{O}_6$ : C, 58.54; H, 4.71; N, 5.69; Found C, 58.20; H, 5.14; N, 6.07.

***tert*-butyl N-[(1S)-1-[[[(1S)-1-[(4-hydroxyphenyl)methyl]-2-oxo-2-[[2-oxo-4-(trifluoromethyl)chromen-7-yl]amino]ethyl]carbamoyl]-2-methyl-propyl]carbamate **19****

A solution of compound **18** (1.30 g, 2.64 mmol, 1.0 eq) in 15 mL of 4M HCl/dioxane was stirred for 2 h at room temperature. The reaction mixture was concentrated *in vacuo* to afford the hydrochloride salt of the free amine as a yellow oil (quantitative yield), which was used without further purification.  $^1\text{H}$  NMR (DMSO-*d*<sub>6</sub>, 300 MHz)  $\delta$  11.58 (s, 1H), 8.86 (s, 1H), 8.46 (bs, 3H), 7.91 (d,  $J = 2.1$  Hz, 1H), 7.70 (d,  $J = 9.0$  Hz, 1H), 7.60 (dd,  $J = 9.0, 2.1$  Hz, 1H), 7.10 (d,  $J = 8.6$  Hz, 2H), 6.93 (s, 1H), 6.68 (d,  $J = 8.6$  Hz, 2H), 4.30 (m, 1H), 3.12 (dd,  $J = 13.8, 6.2$  Hz, 1H), 3.07 (dd,  $J = 13.8, 7.3$  Hz, 1H);  $^{13}\text{C}$  NMR (DMSO-*d*<sub>6</sub>, 75 MHz)  $\delta$  168.0, 158.5, 156.6, 154.5, 143.2, 139.2 (q,  $J = 31.7$  Hz), 130.5, 126.6, 124.4, 120.2 (q,  $J = 274.5$  Hz), 116.2, 115.3, 114.9, 108.9, 106.6, 54.6, 36.0;  $^{19}\text{F}$  NMR (DMSO-*d*<sub>6</sub>, 188 MHz)  $\delta$  -64.02 (s); ESI<sup>+</sup> MS  $m/z$ : 393 [ $\text{M}+\text{H}$ ]<sup>+</sup>. To a solution of the hydrochloride salt of the free amine (2.64 mmol, 1 eq) in dry DMF (20 mL) was added Boc-Val-OH (642 mg, 2.95 mmol, 1.2 eq), DIPEA (1.36 g, 10.56 mmol, 4.0 eq), EDCI (566 mg, 2.95 mmol, 1.2 eq) and HOBT (397 mg, 2.95 mmol, 1.2 eq) in this order. The reaction mixture was stirred overnight at room

temperature under argon atmosphere. The residue was concentrated under reduced pressure and dissolved in EtOAc (20 mL). The organic layer was washed successively with a 10% aqueous solution of citric acid (2 x 20 mL), water (20 mL), a 10% aqueous solution of K<sub>2</sub>CO<sub>3</sub> (2 x 20 mL) and brine (20 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (DCM/ether: 7/3) to give **19** (1.00 g, 1.70 mmol, 64%) as a white foam. Rf: 0.3 (SiO<sub>2</sub>, DCM/Et<sub>2</sub>O: 7/3); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz) δ 10.51 (s, 1H), 9.16 (s, 1H), 8.12 (d, J = 7.8 Hz, 1H), 7.87 (d, J = 2.0 Hz, 1H), 7.67 (d, J = 8.7 Hz, 1H), 7.49 (dd, J = 8.7, 2.0 Hz, 1H), 7.05 (d, J = 8.6 Hz, 2H), 6.90 (s, 1H), 6.68 (d, J = 7.8 Hz, 1H), 6.65 (d, J = 8.6 Hz, 2H), 4.65 (m, 1H), 3.79 (m, 1H), 2.94 (m, 1H), 2.88 (m, 1H), 1.88 (m, 1H), 1.27 (s, 9H), 0.77 (m, 3H), 0.75 (m, 3H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz) δ 171.4, 171.2, 158.6, 155.9, 155.4, 154.6, 142.9, 139.2 (q, J = 32.3 Hz), 130.3, 127.0, 125.4, 121.6 (q, J = 274.5 Hz), 116.1, 114.6, 114.5, 108.3, 106.3, 78.1, 59.8, 57.1, 36.8, 30.5, 28.1, 19.1, 18.2; <sup>19</sup>F NMR (DMSO-*d*<sub>6</sub>, 188 MHz) δ -64.02 (s); IR (cm<sup>-1</sup>): 3308 (N-H), 2959 (C-H), 1678 (C=O), 1644 (C=C), 1528 (N-H); ESI<sup>-</sup> MS *m/z*: 590 [M-H]<sup>-</sup>; HRMS (TOF, ESI, ion polarity positive): calcd for Anal. Calcd for C<sub>29</sub>H<sub>32</sub>F<sub>3</sub>N<sub>3</sub>O<sub>7</sub>Na: 614.2090; found 614.2092.

**tert-butyl N-[(1*S*)-1-[[[(1*S*)-1-[[[(1*S*)-1-(4-hydroxyphenyl)methyl]-2-oxo-2-[[2-oxo-4-(trifluoromethyl)chromen-7-yl]amino]ethyl]carbamoyl]-2-methyl-propyl]carbamoyl]-3-methyl-butyl]carbamate **20****

A solution of **19** (965 mg, 1.63 mmol, 1.0 eq) in 15 mL of 4M HCl/dioxane was stirred for 2 h at room temperature. The reaction mixture was concentrated *in vacuo* to afford the hydrochloride salt of the free amine as a yellow oil (quantitative yield), which was used without further purification. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 300 MHz) δ 11.01 (s, 1H), 8.96 (s, 1H, OH), 8.20 (bs, 3H), 8.09 (d, J = 7.8 Hz, 1H), 7.90 (d, J = 2.0 Hz, 1H), 7.69 (d, J = 8.8 Hz, 1H), 7.42 (dd, J = 8.8, 2.0 Hz, 1H), 7.15 (d, J = 8.4 Hz, 1H), 6.89 (s, 1H), 6.65 (d, J = 8.4 Hz, 2H), 4.70 (m, 1H), 3.69 (m, 1H), 3.01 (dd, J = 13.8, 6.2 Hz, 1H), 2.89 (m, 1H), 2.10 (m, 1H), 0.93 (d, J = 6.9 Hz, 3H), 0.91 (d, J = 6.9 Hz, 3H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 75 MHz) δ 170.8, 168.0, 158.4, 156.0, 154.6, 155.4, 143.0, 139.0 (q, J = 32.3 Hz), 130.1, 127.1, 125.4, 123.4 (q, J = 274.5 Hz), 116.1, 115.0, 114.5, 108.3, 106.3, 57.0, 55.8, 36.7, 29.8, 18.3, 17.4; <sup>19</sup>F (DMSO-*d*<sub>6</sub>, 188 MHz) δ -64.01 (s); MS (ESI<sup>-</sup>) *m/z*: 492 [M+H]<sup>+</sup>. To a solution of the hydrochloride salt of the free amine (1.63 mmol, 1.0 eq) in dry DMF (20 mL) was added Boc-Leu-OH (453 mg, 1.96 mmol, 1.2 eq), DIPEA (843 mg, 6.52 mmol, 4.0 eq), EDCI (376 mg, 1.96 mmol, 1.2 eq) and HOBt (265 mg, 1.96 mmol, 1.2 eq) in this order. The reaction mixture

was stirred overnight at room temperature under argon atmosphere. The residue was concentrated under reduced pressure and dissolved in EtOAc (20 mL). The organic layer was washed with a 10% aqueous solution of citric acid (2 x 20 mL), water (20 mL), a 10% aqueous solution of K<sub>2</sub>CO<sub>3</sub> (2 x 20 mL) and brine (20 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (DCM/Et<sub>2</sub>O: 6/4) to give **20** (526 g, 0.75 mmol, 46%) as a white foam. Rf: 0.4 S<sub>i</sub>O<sub>2</sub> DCM/ether: 6/4); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz) δ 10.55 (s, 1H), 9.16 (s, 1H), 8.31 (d, J = 7.2 Hz, 1H), 7.86 (d, J = 2.0 Hz, 1H), 7.67 (d, J = 8.7 Hz, 1H), 7.51 (dd, J = 8.7, 2.0 Hz, 1H), 7.50 (m, 1H), 7.05 (d, J = 8.2 Hz, 2H), 7.03 (m, 1H), 6.89 (s, 1H), 6.62 (d, J = 8.2 Hz, 2H), 4.58 (m, 1H), 4.23 (m, 1H), 3.97 (m, 1H), 2.94 (dd, J = 14.0, 6.5 Hz, 1H), 2.83 (dd, J = 14.0, 8.2 Hz, 1H), 1.96 (m, 1H), 1.57 (m, 1H), 1.40 (m, 2H), 1.37 (s, 9H), 0.86 (m, 6H), 0.77 (m, 3H), 0.75 (m, 3H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz) δ 172.7, 171.5, 171.4, 159.1, 156.4, 155.9, 155.1, 143.5, 139.6 (q, J = 32.3 Hz), 130.4, 127.4, 125.9, 122.2 (q, J = 274.5 Hz), 116.6, 115.4, 114.9, 108.8, 106.8, 78.6, 57.4, 55.8, 53.4, 40.9, 37.0, 31.5, 28.6, 24.7, 23.4, 22.0, 19.5, 18.3; <sup>19</sup>F NMR (DMSO-*d*<sub>6</sub>, 188 MHz) δ -64.01 (s); IR (cm<sup>-1</sup>): 3320 (N-H), 2972 (C-H), 1678 (C=O), 1645 (C=C), 1516 (N-H), 1407 (OH); MS (ESI+) *m/z*: 727 [M+Na]<sup>+</sup>; Anal. Calcd for C<sub>35</sub>H<sub>43</sub>F<sub>3</sub>N<sub>4</sub>O<sub>8</sub> + 0.15 H<sub>2</sub>O: C, 59.42; H, 6.18; N, 7.92; Found C, 59.03; H, 6.19; N, 7.70.

**tert-butyl N-[(1*S*)-1-[[[(1*S*)-1-[[[(1*S*)-1-[[[(1*S*)-1-[(4-hydroxyphenyl)methyl]-2-oxo-2-[[2-oxo-4-(trifluoromethyl)chromen-7-yl]amino]ethyl]carbamoyl]-2-methyl-propyl]carbamoyl]-3-methyl-butyl]carbamoyl]-3-methyl-butyl]carbamate **21****

A solution of **20** (500 mg, 0.71 mmol, 1.0 eq) in 10 mL of 4M HCl/dioxane was stirred for 2 h at room temperature. The reaction mixture was concentrated *in vacuo* to afford the hydrochloride salt of the free amine as a yellow oil (quantitative yield), which was used without further purification. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz) δ 10.80 (s, 1H), 9.20 (s, 1H), 8.47 (d, J = 7.2 Hz, 1H), 8.41 (m, 1H), 8.29 (bs, 3H), 7.90 (d, J = 1.9 Hz, 1H), 7.67 (d, J = 8.7 Hz, 1H), 7.53 (dd, J = 8.7, 1.9 Hz, 1H), 7.06 (d, J = 8.2 Hz, 2H), 6.89 (s, 1H), 6.63 (d, J = 8.2 Hz, 2H), 4.61 (m, 1H), 4.24 (m, 1H), 3.86 (m, 1H), 2.97 (dd, J = 14.0, 6.5 Hz, 1H), 2.83 (dd, J = 14.0, 8.2 Hz, 1H), 1.98 (m, 1H), 1.57 (m, 1H), 1.48 (m, 2H), 0.83 (m, 6H), 0.82 (m, 6H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz) δ 171.1, 170.5, 168.5, 158.6, 156.0, 154.6, 143.1, 139.0 (q, J = 32.3 Hz), 129.9, 127.0, 125.4, 123.5 (q, J = 274.5 Hz), 116.1, 114.9, 114.4, 108.2, 106.2, 57.8, 55.4, 50.7, 40.8, 36.5, 30.8, 23.6, 22.6, 22.0, 19.0, 18.3; <sup>19</sup>F NMR (DMSO-*d*<sub>6</sub>, 188 MHz) δ -64.00 (s); ESI<sup>-</sup> MS *m/z*: 603 [M-H]<sup>-</sup>. To a solution the hydrochloride salt of the free

amine (0.62 mmol, 1.0 eq) in dry DMF (10 mL) was added Boc-Leu-OH (173 mg, 0.75 mmol, 1.2 eq), DIPEA (321 mg, 2.48 mmol, 4.0 eq), EDCI (143 mg, 0.75 mmol, 1.2 eq) and HOBt (101 mg, 1.96 mmol, 1.2 eq) in this order. The reaction mixture was stirred overnight at room temperature under argon atmosphere. The residue was concentrated under reduced pressure and dissolved in 10 mL of EtOAc. The organic layer was washed successively with a 10% aqueous solution of citric acid (2 x 10 mL), water (10 mL), a 10% aqueous solution of K<sub>2</sub>CO<sub>3</sub> (2 x 10 mL) and brine (10 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (DCM/Et<sub>2</sub>O: 6/4) to give the compound **21** (300 mg, 0.37 mmol, 56%) as a white foam. Rf: 0.5 (SiO<sub>2</sub> DCM/ether: 6/4); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz) δ 10.53 (s, 1H), 9.15 (s, 1H), 8.20 (d, J = 7.2 Hz, 1H), 7.86 (s, 1H), 7.84 (d, J = 7.4 Hz, 1H), 7.67 (d, J = 8.7 Hz, 1H), 7.66 (m, 1H), 7.49 (d, J = 8.7 Hz, 1H), 7.03 (d, J = 8.2 Hz, 2H), 6.91 (m, 1H), 6.89 (s, 1H), 6.62 (d, J = 8.2 Hz, 2H), 4.59 (m, 1H), 4.37 (m, 1H), 4.17 (m, 1H), 3.95 (m, 1H), 2.93 (dd, J = 14.0, 6.5 Hz, 1H), 2.82 (dd, J = 13.9, 8.2 Hz, 1H), 1.94 (m, 1H), 1.58 (m, 2H), 1.44 (m, 2H), 1.38 (m, 2H), 1.36 (s, 9H), 0.86 (m, 6H), 0.81 (m, 6H), 0.78 (m, 3H), 0.76 (m, 3H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz) δ 172.4, 171.7, 171.1, 170.8, 158.6, 155.9, 155.2, 154.6, 143.0, 139.1 (q, J = 33.3 Hz), 129.9, 126.9, 125.4, 121.7 (q, J = 276.0 Hz), 116.1, 114.9, 114.5, 108.3, 106.3, 78.0, 57.3, 55.3, 52.8, 50.8, 40.6, 36.5, 30.7, 28.1, 24.2, 23.9, 23.0, 21.6, 19.0, 17.9; <sup>19</sup>F NMR (DMSO-*d*<sub>6</sub>, 188 MHz) δ -64.01 (s); IR (cm<sup>-1</sup>): 3340 (N-H), 2971 (C-H), 1678 (C=O), 1637 (C=C), 1515 (N-H), 1407 (OH); MS (ESI<sup>+</sup>) m/z: 841 [M+Na]<sup>+</sup>; Anal. Calcd for C<sub>41</sub>H<sub>54</sub>F<sub>3</sub>N<sub>5</sub>O<sub>9</sub> + 0.15 H<sub>2</sub>O: C, 60.04; H, 6.69; N, 8.54; Found C, 59.72; H, 6.82; N, 8.25.

### Suc-LLVY-AFC

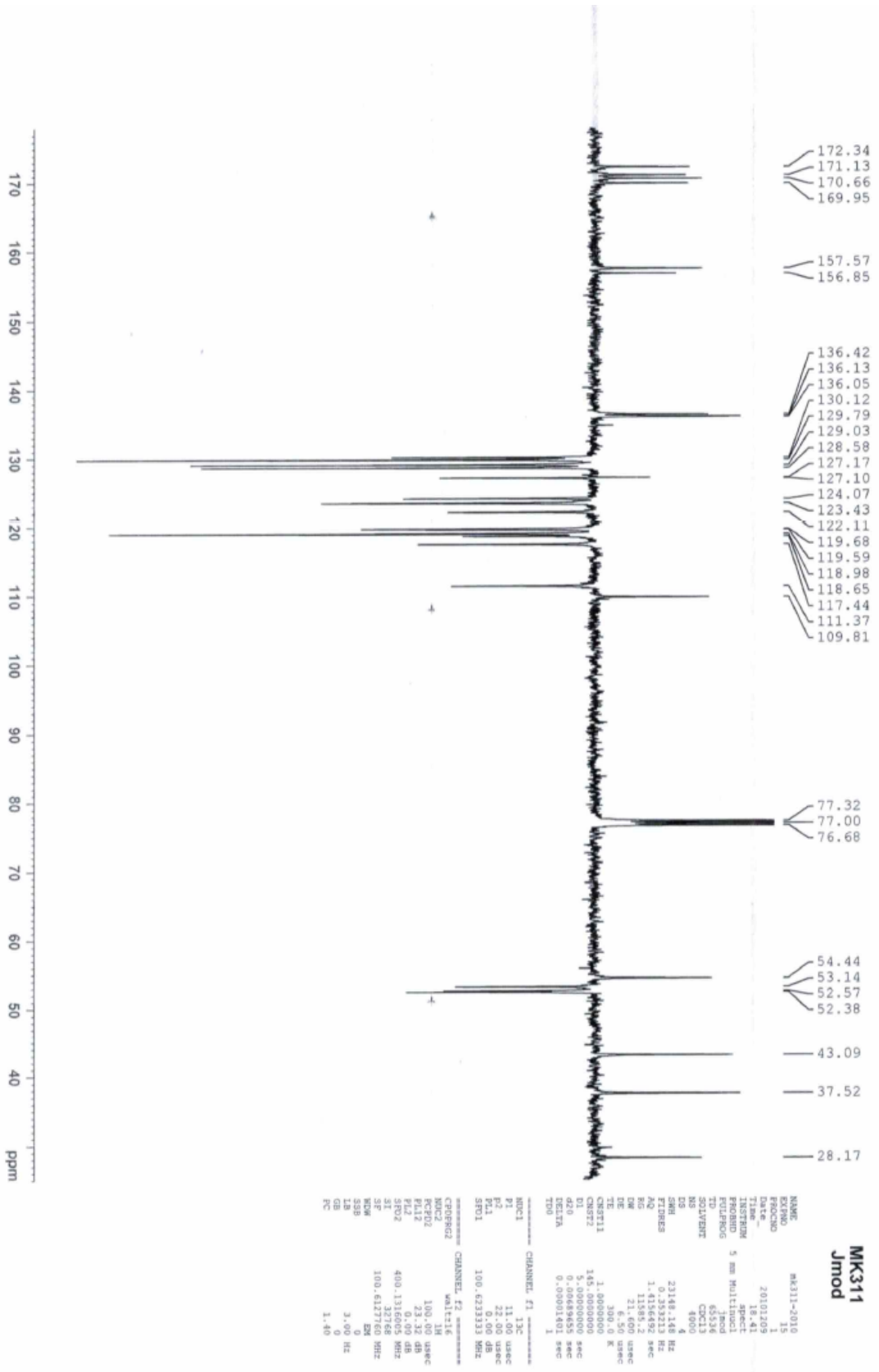
#### **4-[[[(1S)-1-[[[(1S)-1-[[[(1S)-1-[[4-hydroxyphenyl)methyl]-2-oxo-2-[[2-oxo-4-(trifluoromethyl)chromen-7-yl]amino]ethyl]carbonyl]-2-methyl-propyl]carbonyl]-3-methyl-butyl]carbonyl]-3-methyl-butyl]amino]-4-oxo-butanoic acid**

A solution of **21** (270 mg, 0.33 mmol, 1.0 eq) in 5 mL of HCl 4M /dioxane (5 mL) was stirred at room temperature for 2 h. The reaction mixture was concentrated *in vacuo* to afford the hydrochloride salt of the free amine as a yellow oil (quantitative yield), which was used without further purification. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 300 MHz) δ 10.77 (s, 1H), 9.22 (s, 1H), 8.62 (d, J = 7.2 Hz, 1H), 8.27 (d, J = 7.4 Hz, 1H), 8.20 (bs, 3H), 8.01 (m, 1H), 7.89 (d, J = 1.9 Hz, 1H), 7.67 (d, J = 8.8 Hz, 1H), 7.51 (dd, J = 8.8, 1.9 Hz, 1H), 7.08 (d, J = 8.2 Hz, 2H), 6.90 (s, 1H), 6.63 (d, J = 8.2 Hz, 2H), 4.60 (m, 1H), 4.47 (m, 1H), 4.17 (m, 1H), 3.79 (m, 1H), 2.93 (m, 1H), 2.87 (m, 1H), 1.96 (m, 1H), 1.59 (m, 2H), 1.47 (m, 2H), 1.40 (m, 2H), 0.86 (m, 6H),

0.81 (m, 6H), 0.79 (m, 3H), 0.60 (m, 3H) ;  $^{13}\text{C}$  NMR (DMSO-*d*<sub>6</sub>, 75 MHz)  $\delta$  172.0, 171.2, 170.8, 168.6, 158.6, 155.9, 154.6, 143.0, 139.2 (q,  $J = 33.3$  Hz), 129.9, 127.0, 125.4, 121.8 (q,  $J = 276.0$  Hz), 116.0, 114.9, 114.6, 108.3, 106.2, 57.6, 55.3, 51.1, 50.6, 40.6, 36.5, 30.6, 23.9, 23.4, 23.0, 22.1, 21.8, 19.0, 17.9;  $^{19}\text{F}$  NMR (DMSO-*d*<sub>6</sub>, 188 MHz)  $\delta$  -64.0 (s); ESI<sup>+</sup> MS  $m/z$ : 718 [M+H]<sup>+</sup>. To a solution of the hydrochloride salt of the free amine (228 mg, 0.30 mmol, 1.0 eq) in dry DMF (10 mL) was added succinic anhydride (33 mg, 0.33 mmol, 1.1 eq) and DIPEA (77 mg, 0.60 mmol, 2.0 eq). The mixture was stirred at room temperature overnight. The solution was acidified to pH = 5 using a 10% aqueous solution of citric acid. The white precipitate formed was filtered and washed with 20 mL of Et<sub>2</sub>O to give **Suc-LLVY-AFC** (138 mg, 0.17 mmol, 56%) as a white solid. m.p.:230°-232°C;  $^1\text{H}$  NMR (DMSO-*d*<sub>6</sub>, 400 MHz)  $\delta$  12.10 (bs, 1H), 10.50 (s, 1H), 9.15 (s, 1H), 8.16 (d,  $J = 7.2$  Hz, 1H), 8.02 (d,  $J = 7.4$  Hz, 1H), 7.93 (m, 1H), 7.86 (d,  $J = 1.9$  Hz, 1H), 7.67 (d,  $J = 8.9$  Hz, 1H), 7.56 (m, 1H), 7.49 (dd,  $J = 8.9, 1.9$  Hz, 1H), 7.03 (d,  $J = 8.2$  Hz, 2H), 6.90 (s, 1H), 6.62 (d,  $J = 8.2$  Hz, 2H), 4.59 (m, 1H), 4.28 (m, 2H), 4.15 (m, 1H), 2.94 (dd,  $J = 14.0, 6.5$  Hz, 1H), 2.83 (dd,  $J = 13.9, 8.2$  Hz, 1H), 2.70 (m, 2H), 2.61 (m, 2H), 1.93 (m, 1H), 1.58 (m, 2H), 1.48 (m, 2H), 1.43 (m, 2H), 0.86 (m, 6H), 0.81 (m, 6H), 0.78 (m, 3H), 0.77 (m, 3H) ;  $^{13}\text{C}$  NMR (DMSO-*d*<sub>6</sub>, 100 MHz)  $\delta$  173.8, 172.2, 172.1, 171.1, 171.0, 170.8, 158.6, 155.9, 154.6, 143.0, 139.1 (q,  $J = 33.3$  Hz), 130.0, 127.0, 125.4, 121.7 (q,  $J = 274.5$  Hz), 116.1, 114.9, 114.5, 108.4, 106.3, 57.5, 55.3, 51.1, 40.8, 40.1, 30.0, 29.1, 36.6, 30.6, 24.1, 23.0, 21.6, 19.0, 17.9;  $^{19}\text{F}$  NMR (DMSO-*d*<sub>6</sub>, 188 MHz)  $\delta$  -64.01 (s); IR (cm<sup>-1</sup>): 2959 (C-H), 1678 (C=O), 1644 (C=C), 1528 (N-H); MS (ESI<sup>-</sup>)  $m/z$ : 816 [M-H]<sup>-</sup>; Anal. Calcd for C<sub>40</sub>H<sub>50</sub>F<sub>3</sub>N<sub>5</sub>O<sub>10</sub> + 3 H<sub>2</sub>O: C, 55.10; H, 6.49; N, 8.03; found C, 55.35; H, 6.38; N, 7.79.



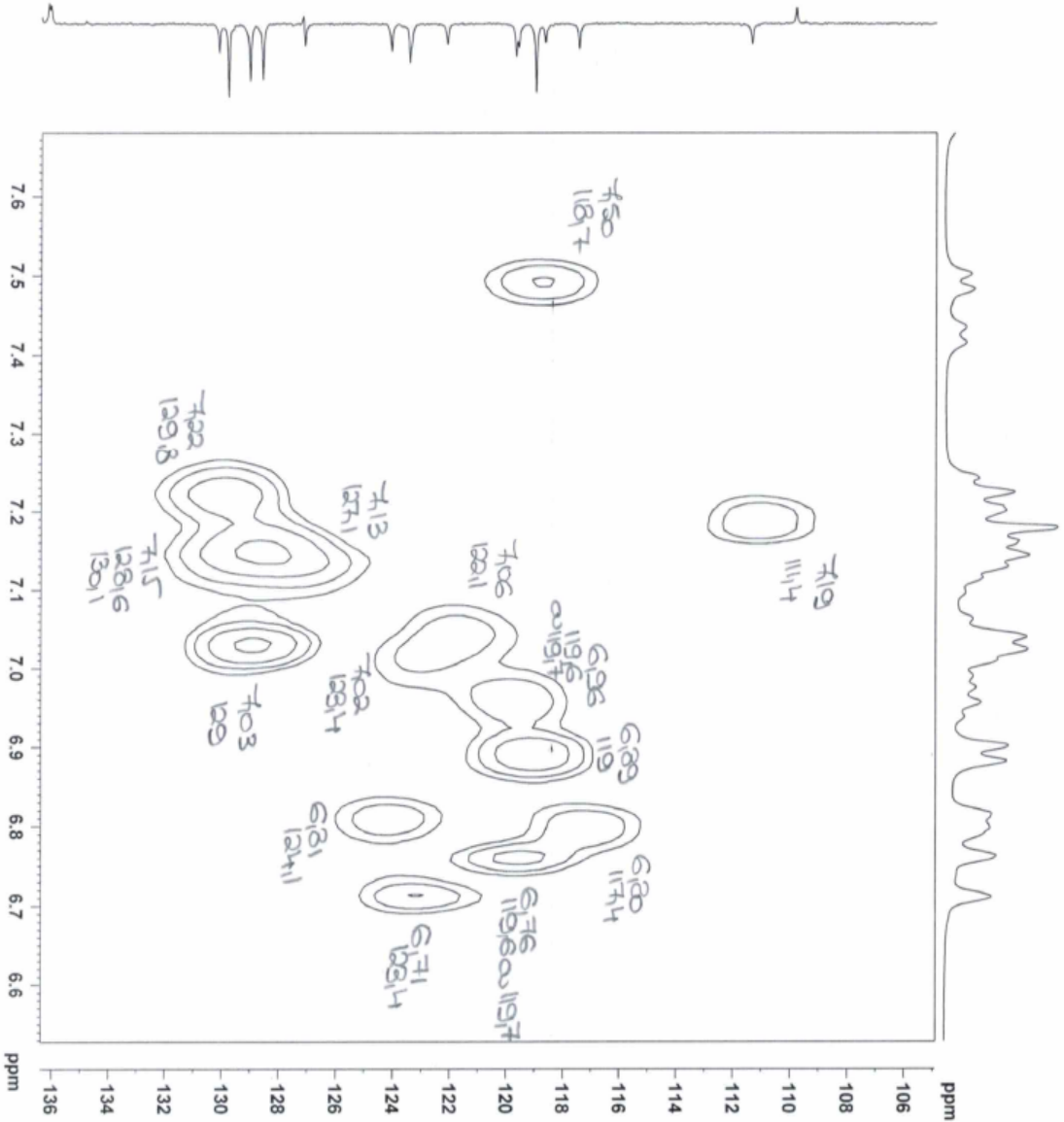








MK311  
HSQC

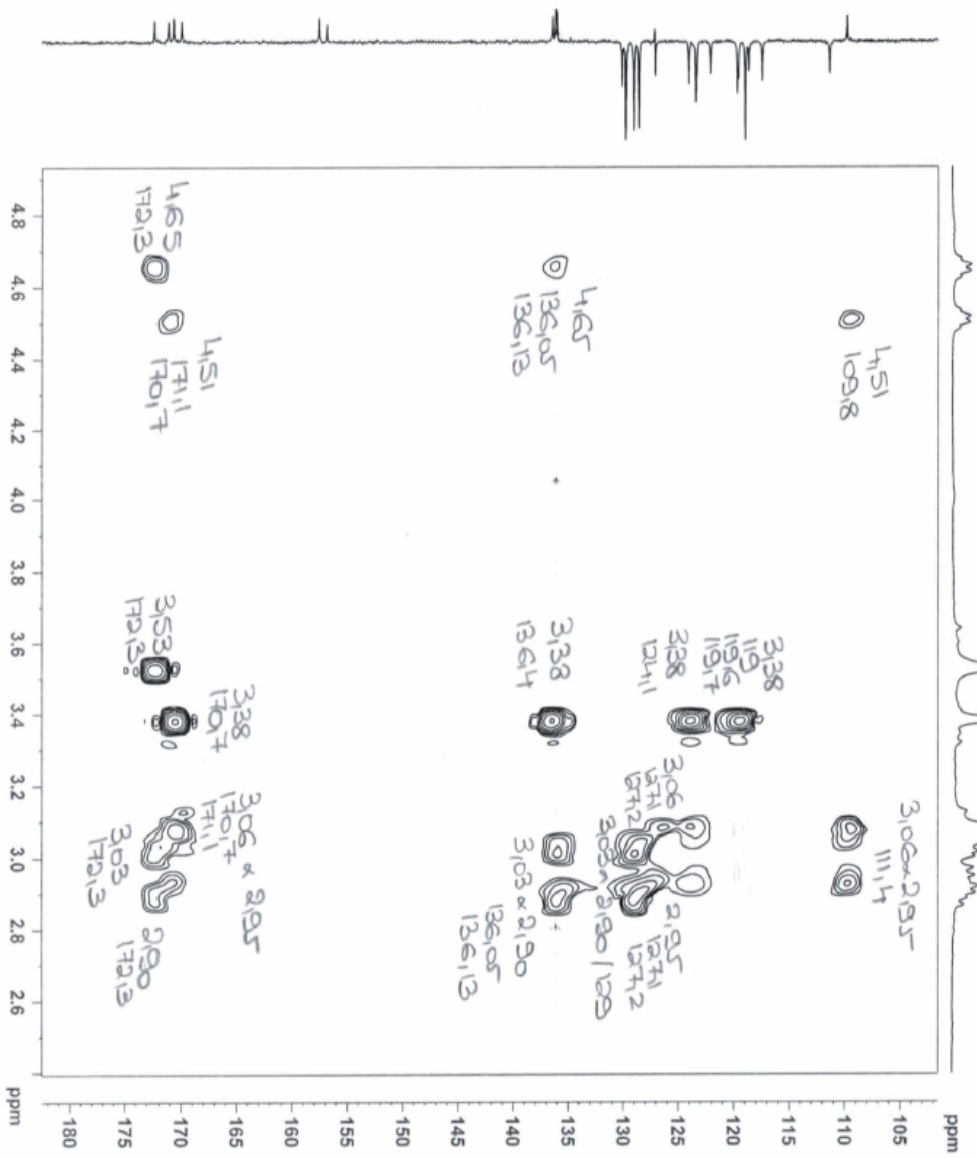


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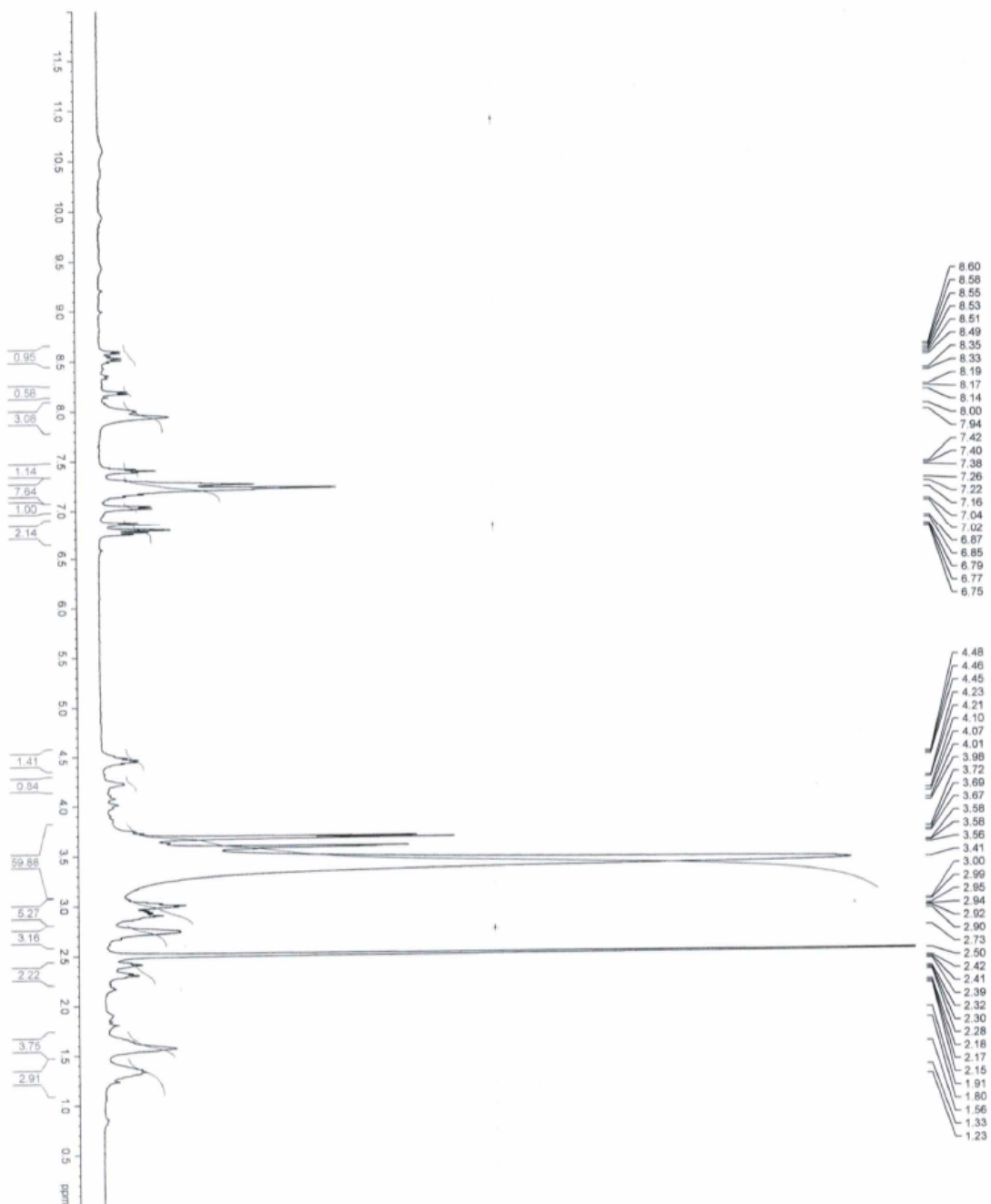
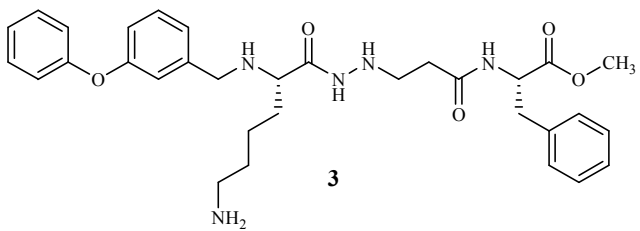




MK311  
HMBC

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P2	13.45 usec
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**MK159 in DMSO  
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PROCNO 1  
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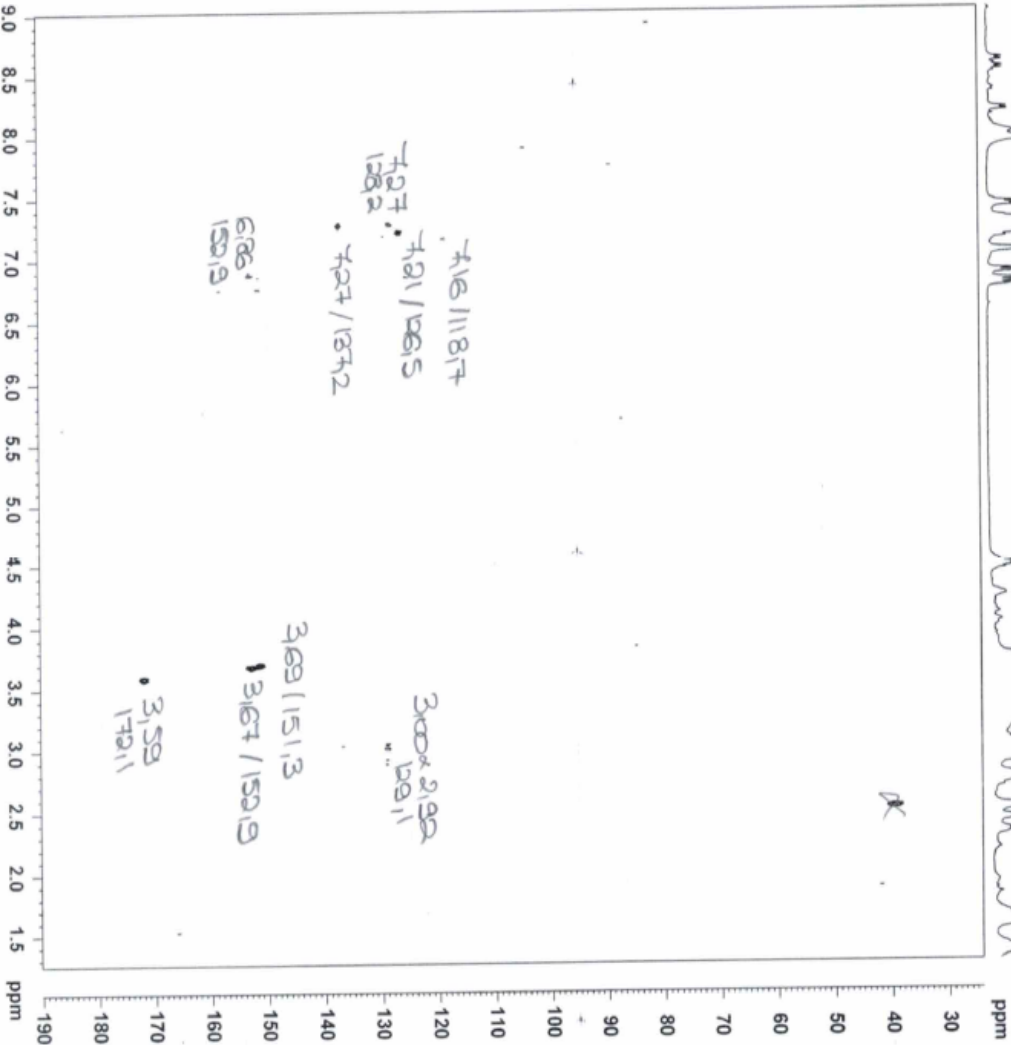
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MK159 in DMSO  
HMBC



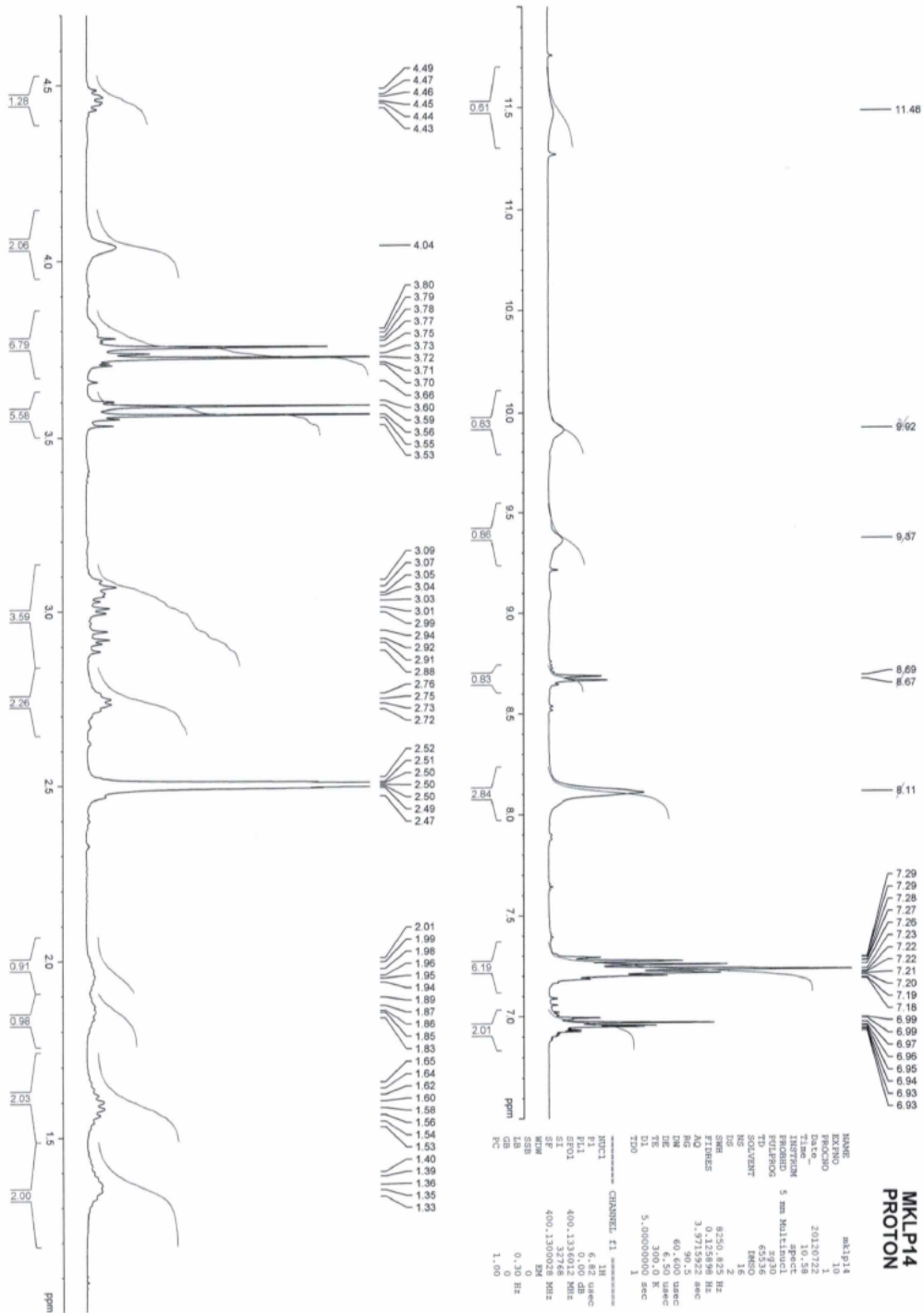
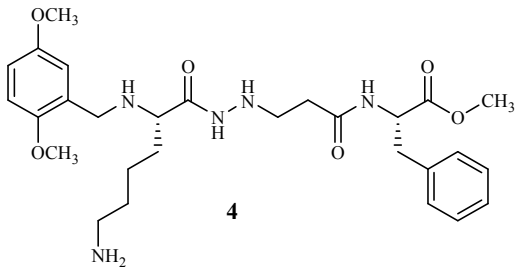
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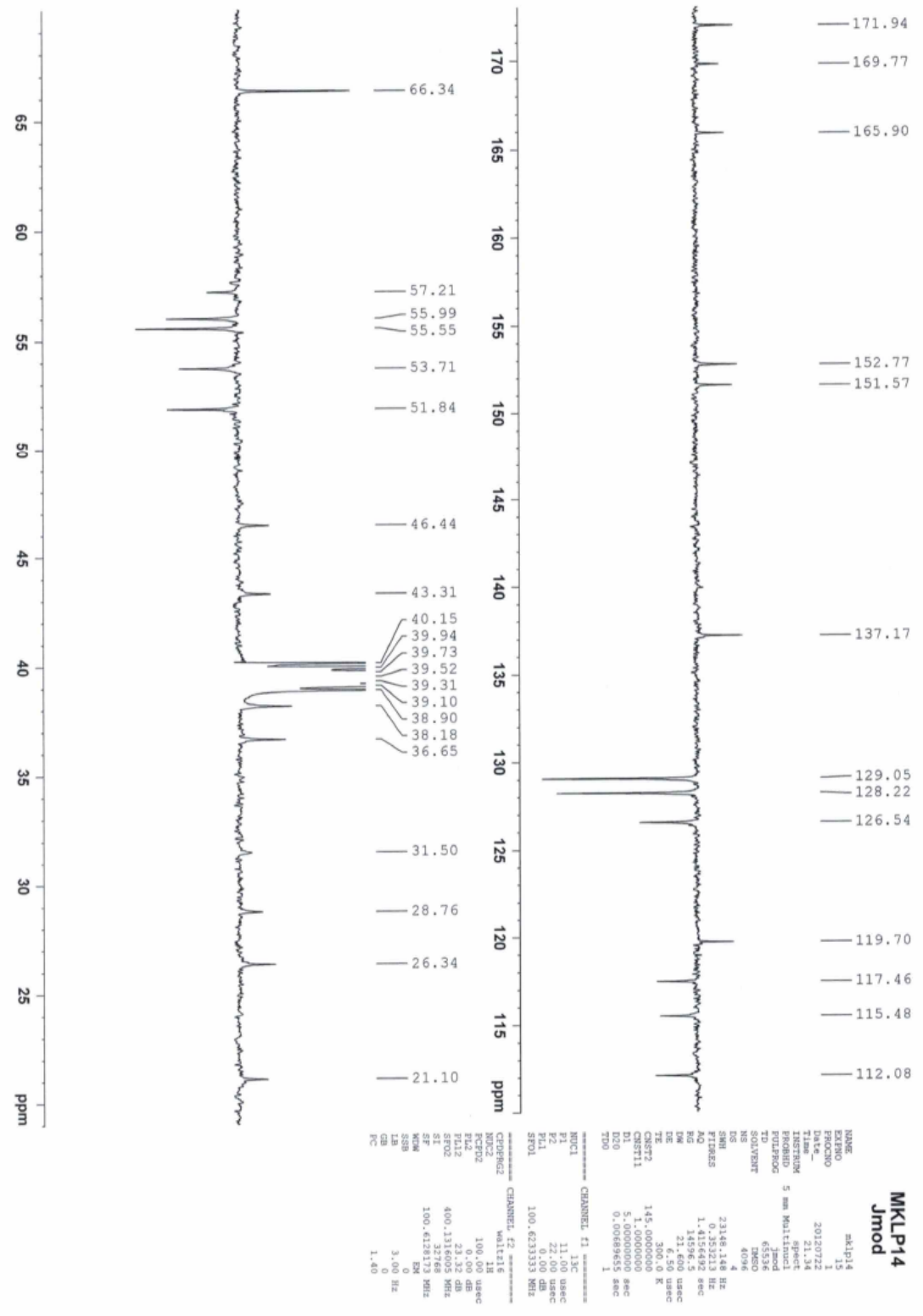
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GRADN4        SINE.100
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GR23          40.00 %
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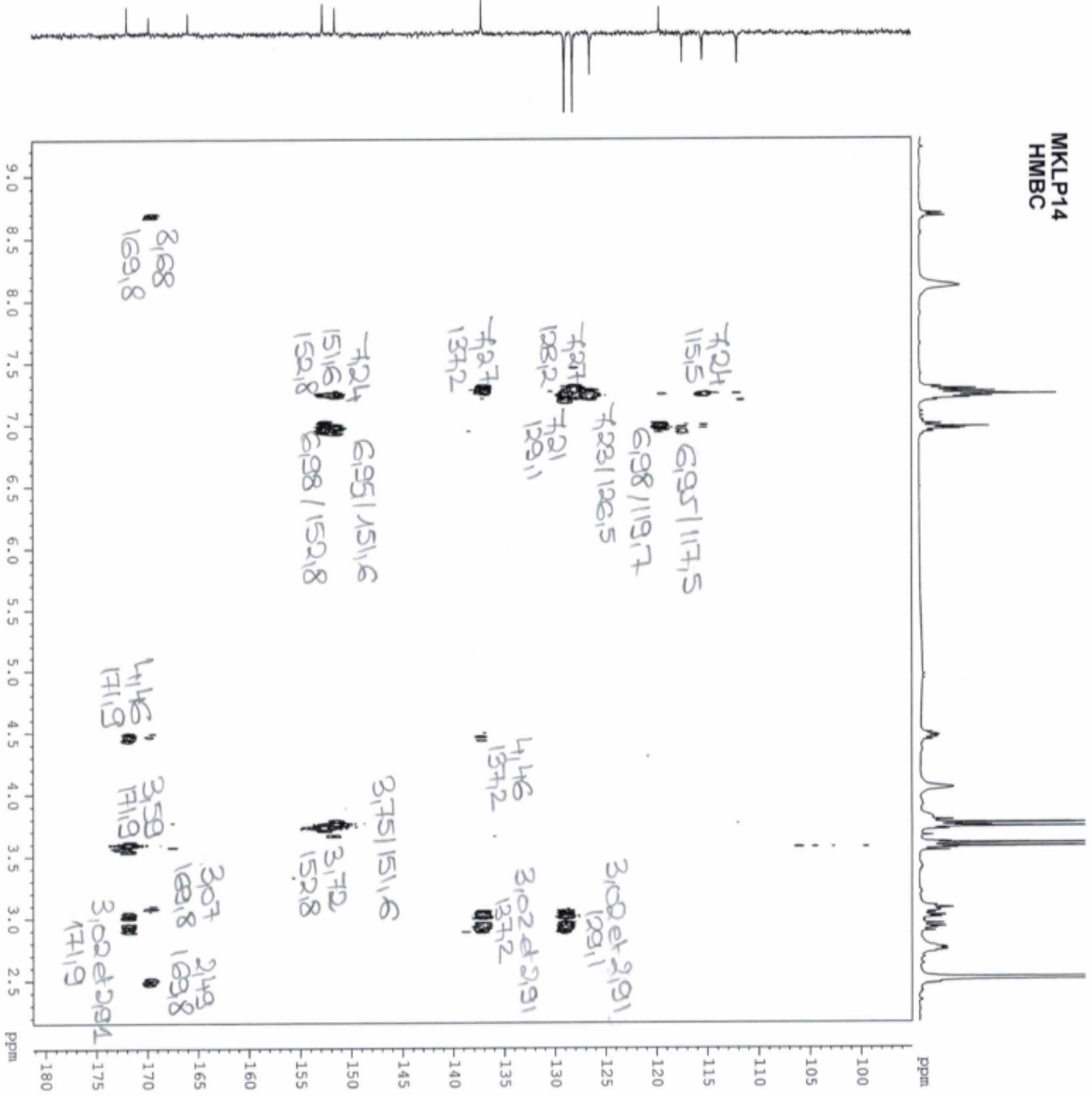








MKLP14  
HMBC



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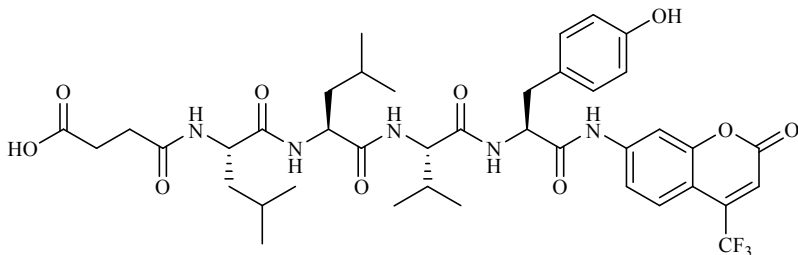
NAME          MKLP14
EXPNO         1
PROCNO        1
Date_         20120722
Time          11.54
INSTRUM       spect
PROBHD        5 mm Multispec1
PULPROG       zgpg30
TD            65536
SOLVENT       DMSO
NS            16
DS            4
AQ            5000.000 Hz
F2            120.003 MHz
RG            0.4097500 sec
RG            18390.4
DW            100.0000 usec
DE            6.50 usec
TE            300.2 K
CNSRT2        145.0000000
CNSRT3        8.00000000
D0            0.00000300 sec
D1            1.18902504 sec
D2            0.00344828 sec
DIF           0.00020000 sec
IND           0.00002160 sec

----- CHANNEL f1 -----
NUC1          1H
P1            4.72 usec
PL1           13.40 dB
PC1           0.00 dB
SFO1          400.1324750 MHz

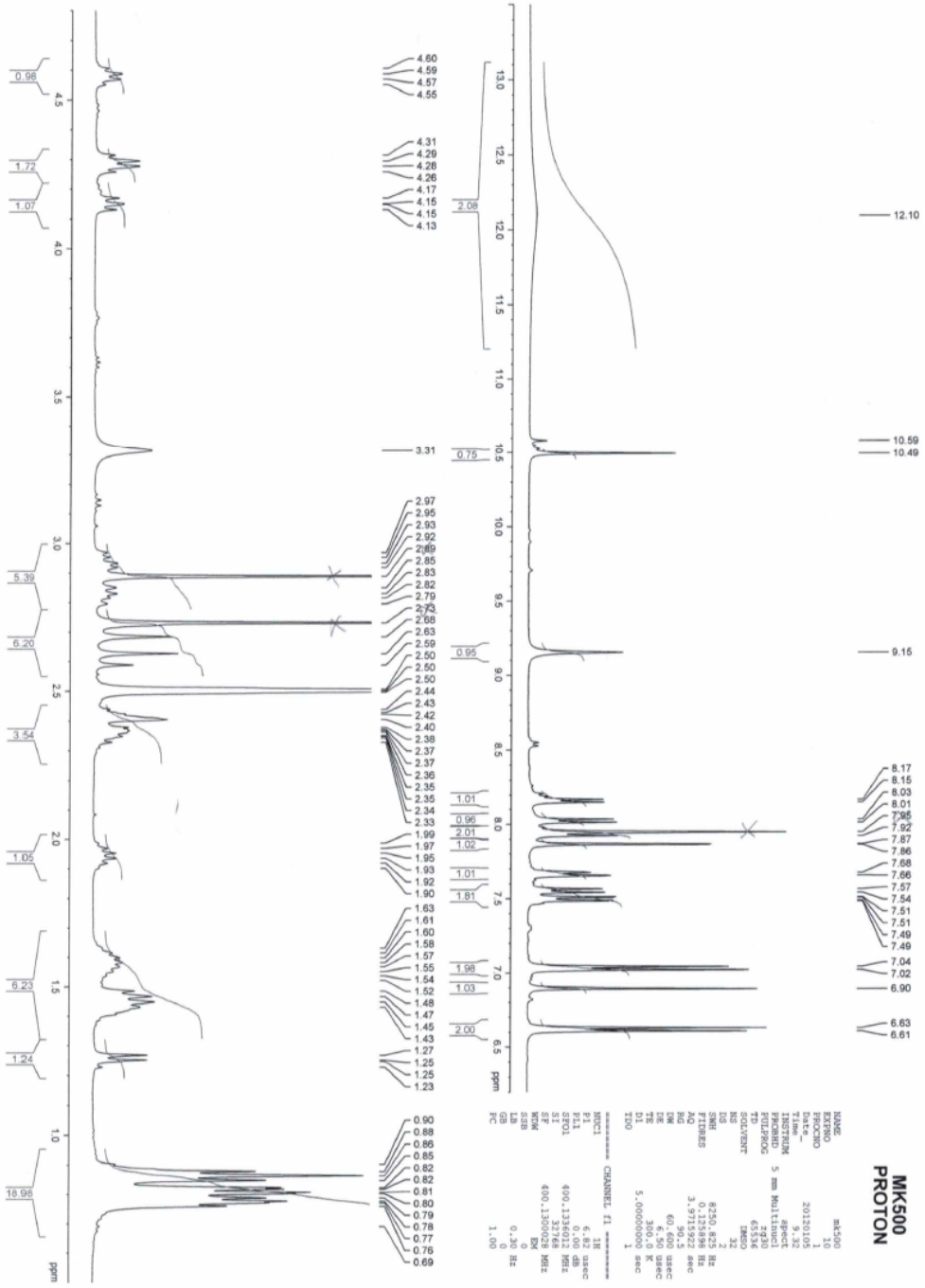
----- CHANNEL f2 -----
NUC2          13C
P2            11.00 usec
PL2           0.00 dB
SFO2          100.628364 MHz

----- GRADIENT CHANNEL -----
GENGRAD      SINE:100
GENSFO        100
GPRM03       SINE:100
GPRM04       56.00 %
GPRM05       30.00 %
GPRM06       40.10 %
GPRM07       1000.00 usec
MDO           2
TD            256
SFO1          100.62318 MHz
FIDRES        90.403236 Hz
AQ            229.99 usec
SOLVENT       DMSO-D6
SI            4036
SF            400.1300078 MHz
WDW           SINE
SSB           0
GB            0.00 Hz
PC            1.40
SI            1024
MC2           QF
SF            100.6128173 MHz
WDW           SINE
SI            0
GB            0.00 Hz
  
```

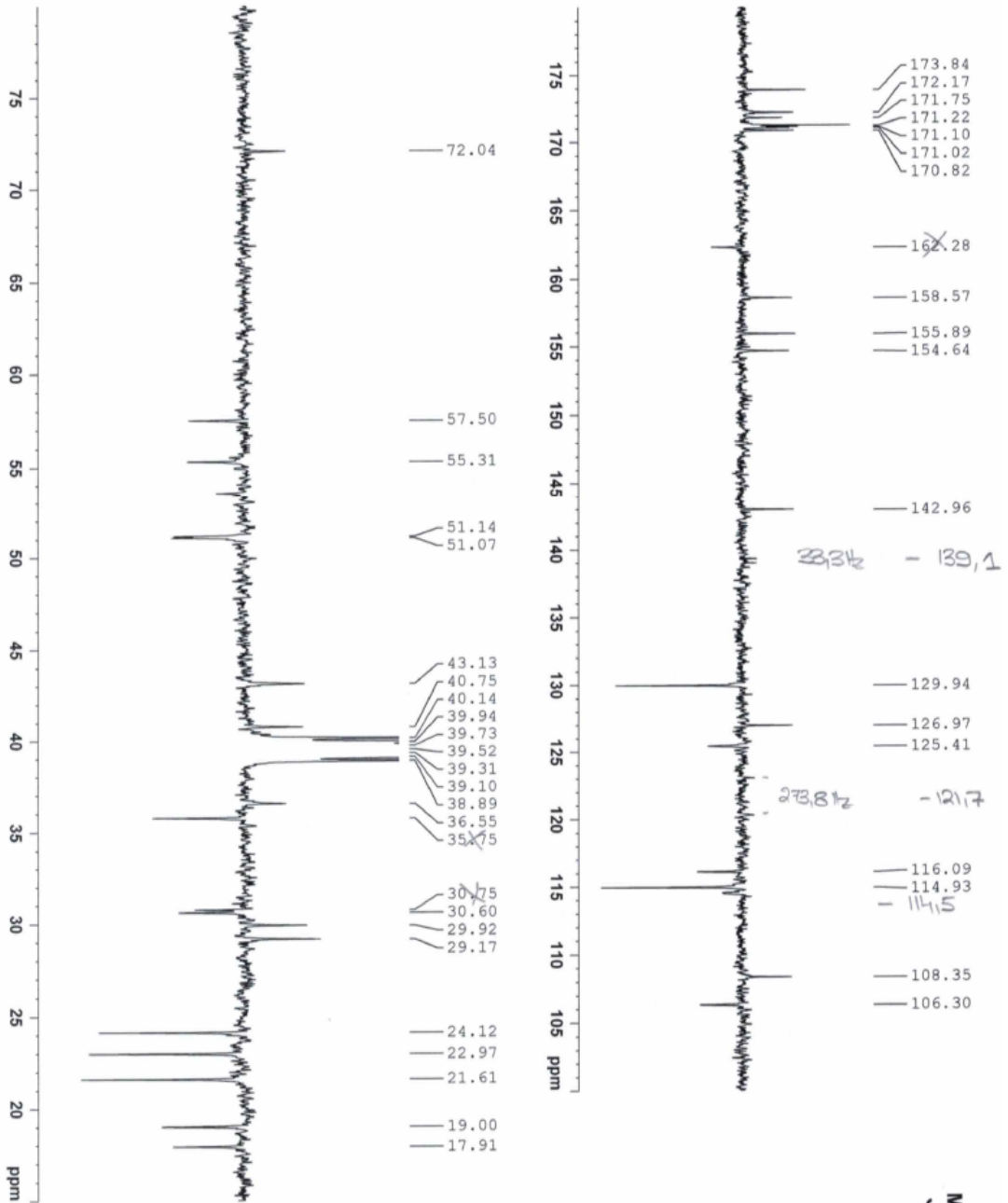




Suc-LLVY-AFC



Suc-LLVY-AFC



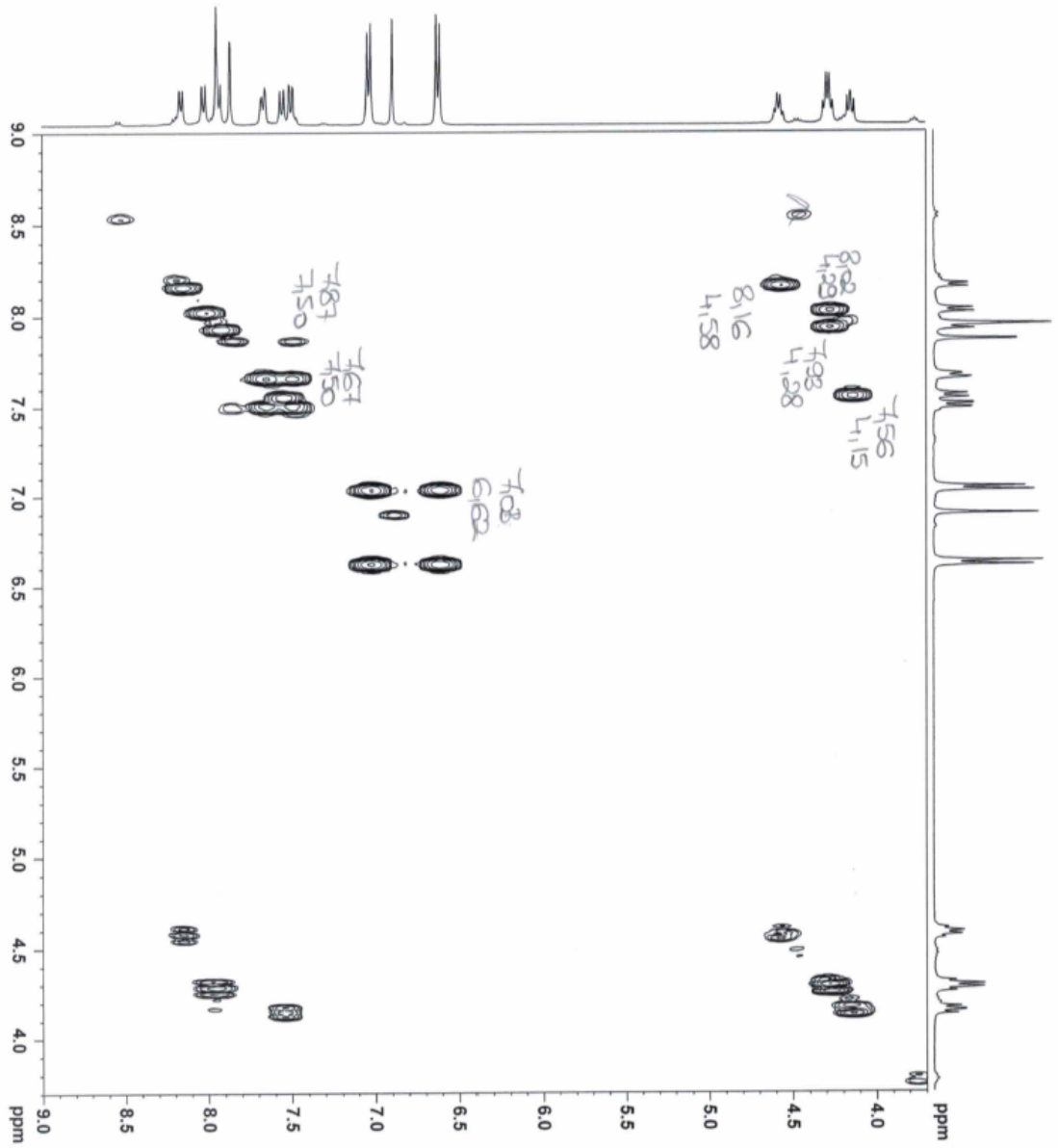
**MK500**  
**Jmod**  
 NAME mk500  
 EXPNO 15  
 PROCNO 20120105  
 Date\_ 19.06  
 Time 19.06  
 INSTRUM spect  
 F2PROB 5 mm Multinuc1  
 PULPROG zgpg30  
 TD 65386  
 SOLVENT DMSO  
 NS 4096  
 DS 4  
 SFO 23148.148 Hz  
 FIDRES 0.353213 Hz  
 AQ 1.4156492 sec  
 RG 327.6  
 IQ 21.76 usec  
 ISF 6.50 usec  
 IE 6.50 usec  
 TE 300.0 K  
 CHST2 145.000000  
 C1P2F1 4.120000  
 D1 5.0000000 sec  
 D2 0.00689655 sec  
 T20 1

----- CHANNEL f1 -----  
 NUC1 13C  
 P1 1.00 usec  
 PL 0.00 dB  
 F1 100.6283533 MHz  
 SFO1 100.6283533 MHz

----- CHANNEL f2 -----  
 CHPROG2 wahtz16  
 NUC2 1H  
 P2 100.00 usec  
 PL 0.00 dB  
 F2 400.1316005 MHz  
 SFO2 400.1316005 MHz  
 SI 32768  
 SF 100.6128180 MHz  
 NS 0  
 DS 0  
 SFO 3.00 Hz  
 LB 0  
 GB 1.40



Suc-LLVY-AFC



MK500  
COSY

```

NAME          mk500
EXPNO         11
PROCNO        1
Date_         20120105
Time          23:23
INSTRUM       spect
PROBHD        5 mm Multinuc1
PULPROG       zgpg30
F2 - F1       cosymprg2
TD            2134
SOLVENT       DMSO
NS            8
DS            4
SWH           5787.037 Hz
FIDRES        2.711826 Hz
AQ            0.1845140 sec
RG            655
DE            86.400 usec
TE            300.0 K
DO            0.00000000 sec
D1            0.00000000 sec
D16           0.00020000 sec
IN0           0.00017280 sec

----- CHANNEL f1 -----
NUC1          1H
P1            5.82 usec
PL1           0.00 dB
SFO1          400.1325552 MHz

----- CHANNEL CHANNEL -----
GRND1         SINE,100
GRND2         SINE,100
GRND3         SINE,100
GR21          16.00 Hz
GR22          16.00 Hz
GR23          40.00 Hz
P16           1000.00 usec
NUC0           1
TD0            256
FID0           400.1325552 MHz
FIDRES0        22.605614 Hz
SW0            14.463 GHz
PRMO0         OF
SI            2048
SF            400.1300028 MHz
WDW           EM
SSB           1
LB            0.00 Hz
GB            0
PC            1.40
SC            OF
MC2           OF
SF            400.1300028 MHz
WDW           GE
SSB           1
LB            0.00 Hz
GB            0
  
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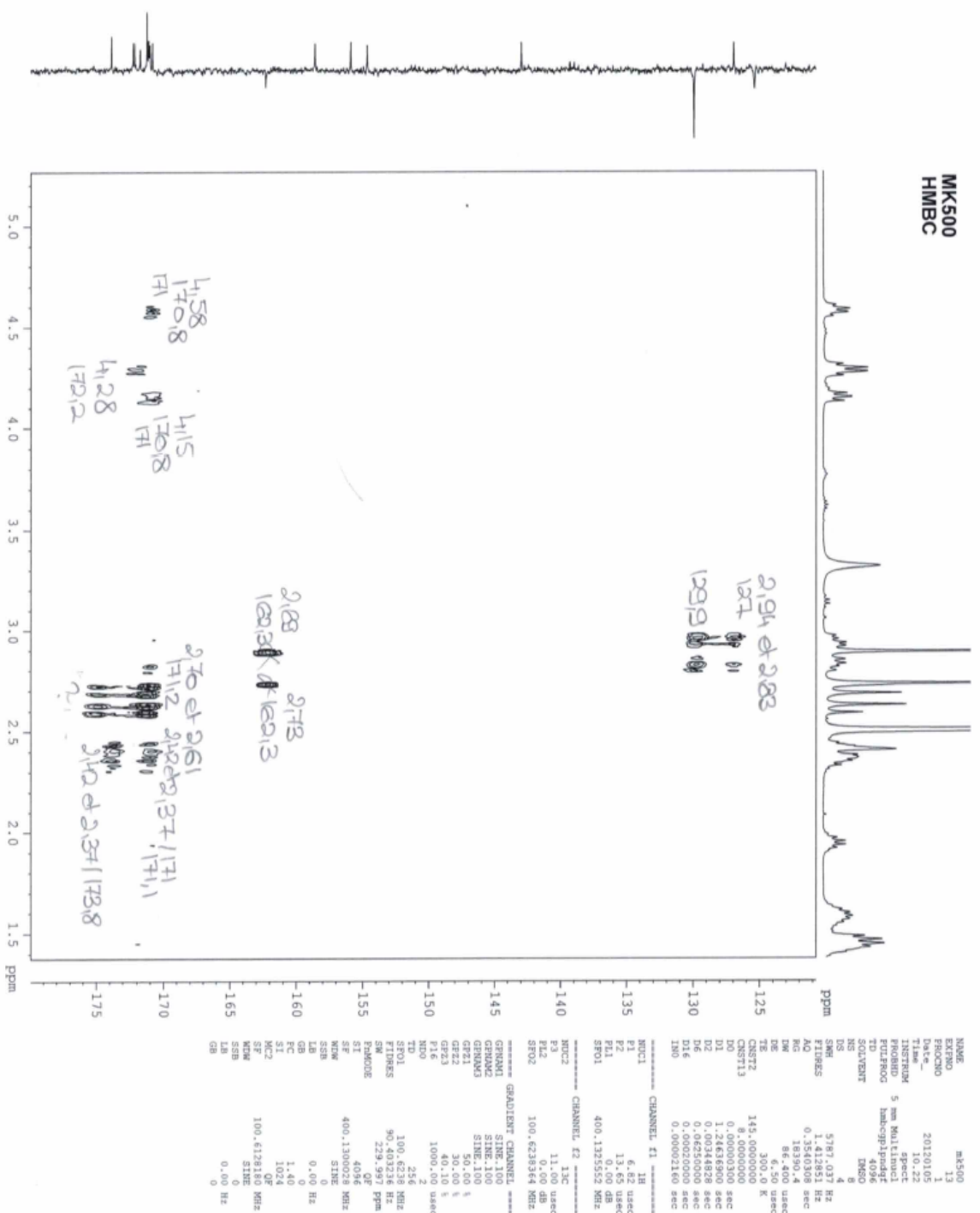








Suc-LLVY-AFC

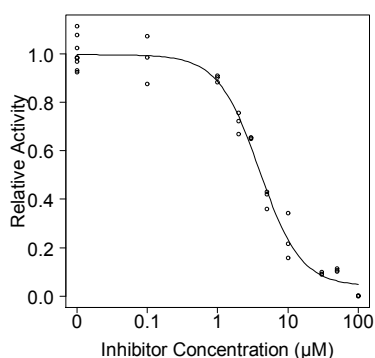


#### 4- $K_M$ determination of the Suc-LLVY-AFC substrate by fluorescence

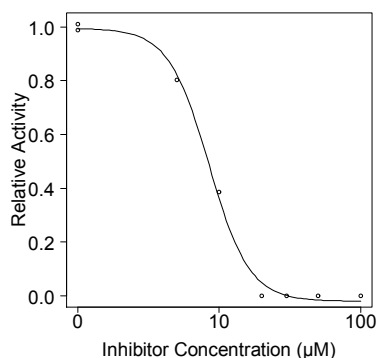
$K_M$  of Suc-LLVY-AFC was determined by monitoring its hydrolysis in the presence of purified 20S rabbit proteasome (BostonBioChem) (10 nM final concentration) which was incubated in 96-wells plates (200  $\mu$ L final volume) in the following buffer: 20 mM TrisHCl, 1mM DTT, 0.02% (w/v) SDS, 10 % glycerol, PH 7.4, in the presence of different concentrations of Suc-LLVY-AFC (from 10 to 90  $\mu$ M, in duplicate). Suc-LLVY-AFC was previously dissolved in DMSO, with the final concentration kept constant at 2% (v/v). The rate of hydrolysis of the substrate was monitored with a Fluostar Optima (BMG Labtech) microtiter plate reader by recording the fluorescence of the hydrolyzed AFC group (excitation filter : 360 nm, emission filter : 480 nm). The initial linear portion of the curves (20-100 min) gave access to  $V_0$  and the experimental  $K_M$  value, equal to 20  $\mu$ M, was calculated from  $1/V_0$  against  $1/S$  plot (Lineweaver-Burk plot).

#### 5- Inhibition of the ChT-L activity of the rabbit 20S proteasome by pseudopeptides 2-4, at pH 7.5 and 37 °C, using the substrate Suc-LLVY-AFC, followed by fluorescence spectroscopy ( $\lambda_{ex} = 360$ nm, $\lambda_{em} = 480$ nm) and by 3-FABS.

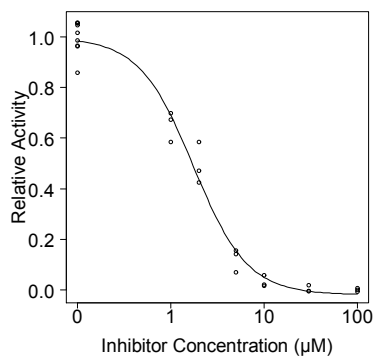
2 (fluorescence)  $IC_{50} = 3.9 \pm 0.6$



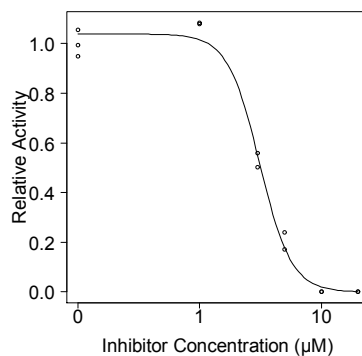
2 (3-FABS)  $IC_{50} = 8.5 \pm 1.1$



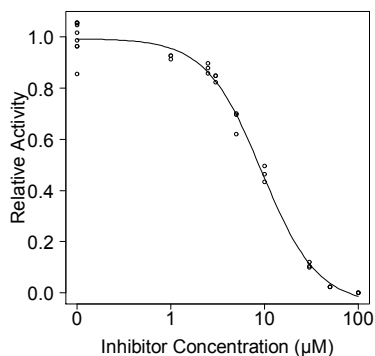
**3** (fluorescence)  $IC_{50} = 1.7 \pm 0.3$



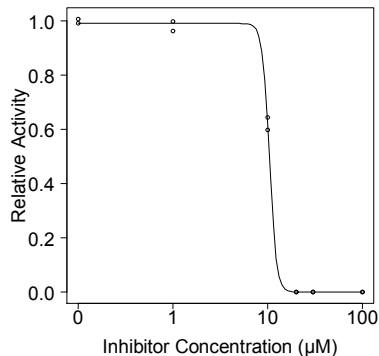
**3** (3-FABS)  $IC_{50} = 3.1 \pm 0.3$



**4** (fluorescence)  $IC_{50} = 9.4 \pm 1.4$



**4** (3-FABS)  $IC_{50} = 10.5 \pm 2.7$



## **6- Monitoring of the ChT-L activity as assessed with Suc-LLVY-AFC alone and in the presence of inhibitor 3 and PA or T-L substrates**

Initial hydrolysis rates were determined by linear regression with the initial linear part of progress curves. Inhibition percentage was calculated as the ratio of initial hydrolysis rate of Suc-LLVY-AFC in the presence of the inhibitor and/or PA and T-L substrates to initial hydrolysis rate of Suc-LLVY-AFC alone.