\textbf{19F NMR Monitoring of the Eukaryotic 20S Proteasome Chymotrypsin-like Activity: Investigative tool for studying Allosteric Regulation **}

Massaba Keita, Julia Kaffy,∗ Claire Troufflard, Estelle Morvan, Benoît Crousse, and Sandrine Ongeri∗

\begin{itemize}
  \item[a] Molécules Fluorées et Chimie Médicinale, BioCIS UMR-CNRS 8076, LabEx LERMIT, Université Paris-Sud, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry Cedex, France. E-mail: sandrine.ongeri@u-psud.fr and julia.kaffy@u-psud.fr; Tel : +33 146835737
  \item[b] NMR service, BioCIS UMR-CNRS 8076, Université Paris-Sud, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry Cedex, France.
\end{itemize}

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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 µm). TLC analyses were performed on silica gel 60 F254 (0.26 mm thickness) plates. The plates were visualized with UV light (λ = 254 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 Phamacy 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 (1H, 300 MHz, 13C, 75 MHz) or a Bruker 400 (1H, 400 MHz, 13C, 100 MHz, 19F 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 Boc$_2$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 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 (S$_2$O$_2$, EtOAc/Cyclohexane: 8:2); $^1$H NMR (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 (t, 3H, J = 6.7 Hz); 13C NMR (CDCl3, 75 MHz) δ 171.4, 156.2, 81.2, 61.0, 52.8, 28.2, 14.2; ESI+ MS m/z: 241 [M+Na]+; Anal. Calcd for C9H18N2O4: 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 MgSO4, 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%); 1H NMR (DMSO-d6, 300 MHz) δ 8.30 (bs, 1H), 7.52 (bs, 1H), 3.93 (s, 2H), 1.38 (s, 9H); 13C NMR (DMSO-d6, 75 MHz) δ 171.1, 156.2, 79.5, 52.8, 28.1; MS (ESI+) m/z: 213 [M+Na]+; Anal. Calcd for C7H14N2O4: C, 44.20; H, 7.42; N, 14.73; Found C, 44.22; H, 7.88; N, 14.48.

Methyl (2S)-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 K2CO3 (2 x 20 mL), brine (20 mL) and dried over Na2SO4, 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: Rf: 0.5 (S2O52-, EtOAc); 1H NMR (CDCl3, 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); 13C NMR (CDCl3, 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
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. \(^1\)H NMR (DMSO-\(d_6\), 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); \(^{13}\)C NMR (DMSO-\(d_6\), 75 MHz) \(\delta\) 171.6, 168.2, 136.9, 129.0, 128.3, 126.6, 53.7, 53.5, 50.2, 36.6; ESI\(^+\) MS \(m/z\): 252 [M+H]\(^+\).

Methyl (2S)-2-[[2-[(2S)-2-(benzyloxy carbonylamino)-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\(_2\)CO\(_3\) (2 x 20 mL), brine (20 mL) and dried over Na\(_2\)SO\(_4\), 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: \(R_f\): 0.5 (SiO\(_2\), EtOAc); \(^1\)H NMR (CDCl\(_3\), 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); \(^{13}\)C NMR (CDCl\(_3\), 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 (cm\(^{-1}\)): 3300 (N-H), 1652 –C=O), 1506 (C=C), 1234 (C-O); ESI\(^+\) MS \(m/z\): 594 [M+Na]\(^+\); Anal. Calcd for C\(_{31}\)H\(_{33}\)N\(_5\)O\(_6\)+H\(_2\)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.

\[ \delta 8.51 \text{ (bs, 2H), 8.32 \text{ (bs, 1H), 7.51 \text{ (m, 2H), 7.25 \text{ (m, 1H), 7.13-6.97 \text{ (m, 8H), 6.91 \text{ (s, 1H), 4.76 \text{ (m, 1H), 3.60 \text{ (s, 3H), 3.53 \text{ (m, 1H), 3.11-2.98 \text{ (m, 6H); 13C NMR}}}}}} \]

\[ \delta 174.5, 172.1, 170.0, 136.3, 136.1, 129.7, 129.1, 127.5, 127.1, 123.4, 122.1, 119.5, 118.7, 114.4, 110.8, 54.8, 54.7, 52.9, 52.4, 37.6, 30.7; ESI^+ MS m/z: 460 [M+Na^+]. \]


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 K₂CO₃ (2 x 15 mL), brine (15 mL) and dried over Na₂SO₄, 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 (SiO₂, EtOAc/MeOH: 9/1); \[ 1^H NMR (CDCl₃, 300 MHz) \delta 8.34 \text{ (bs, 1H), 7.69 \text{ (bs, 1H), 7.50 \text{ (d, J = 7.5 Hz, 1H), 7.43 \text{ (d, J = 7.9 Hz, 1H), 7.49 \text{ (m, 2H), 7.43 \text{ (m, 2H), 7.22 \text{ (m, 2H), 7.19 \text{ (m, 1H), 7.17 \text{ (m, 1H), 7.15 \text{ (m, 1H), 7.13 \text{ (m, 1H), 7.03 \text{ (m, 2H), 6.96 \text{ (d, J = 8.1 Hz, 2H), 6.87 \text{ (m, 3H), 6.76 \text{ (m, 1H), 6.71 \text{ (s, 1H), 6.39 \text{ (d, J = 7.4 Hz, 1H), 4.65 \text{ (m, 1H), 4.50 \text{ (m, 1H), 3.53 \text{ (s, 3H), 3.38 \text{ (s, 2H), 3.08 \text{ (m, 2H), 3.06 \text{ (m, 1H), 3.03 \text{ (m, 1H), 2.94 \text{ (m, 1H), 2.91 \text{(dd, J = 13.9, 7.3 Hz, 1H); 13C NMR}}}}}})} \]

\[ \delta 172.3, 171.1, 170.7, 170.0, 157.6, 156.9, 136.4, 136.1, 136.0, 130.1, 129.8, 129.0, 128.6, 127.1, 124.1, 123.4, 119.7, 119.0, 118.7, 118.0, 114.4, 109.8, 54.4, 53.1, 52.6, 52.4, 43.0, 37.5, 28.2; IR (cm⁻¹): 3300 (N-H), 1643 (C=O), 1506 (C=C), 1234 (C-O); ESI^+ MS m/z: 670 [M+Na^+]; Anal. Caled for C₃₇H₃₇N₂O₆: C, 68.61; H, 5.76; N, 10.81; Found C, 68.29; H, 6.03; N, 10.73. \]
### 1.1 Synthesis of compounds 3 and 4.

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

**Methyl (2S)-2-[3-[benzyloxy carbonyl-][[(2S)-6-(benzyloxy carbonylamino)-2-(tert-butoxy carbonylamino)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$_2$Cl$_2$ and successively washed with a 10% aqueous solution of K$_2$CO$_3$ (2 x 15 mL) and brine (20 mL). The organic layer was dried over MgSO$_4$, 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 (S_{2}O_{2}, EtOAc); \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 300 MHz) \(\delta\) 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); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75 MHz) \(\delta\) 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\textsuperscript{-1}): 3322 (N-H), 2952 (C-H), 1689 (C=O), 1525 (N-H), 1454 (C-H), 1225 (C-N); ESI\textsuperscript{+} MS \(m/z\): 784 [M+Na\textsuperscript{+}]. Anal. calcd for C\textsubscript{40}H\textsubscript{51}N\textsubscript{5}O\textsubscript{10}+0.25 H\textsubscript{2}O: C. 63.23, H. 7.30, N. 8.92 Found C. 62.90, H. 7.34, N. 8.88.

**Methyl (2S)-2-[[2S)-2-amino-6-(benzyloxycarbonylamino)hexanoylamino]-benzyloxycarbonylamino]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. \textsuperscript{1}H NMR (DMSO-\textsubscript{d}6, 300 MHz) \(\delta\) 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). \textsuperscript{13}C NMR (DMSO-\textsubscript{d}6, 75 MHz) \(\delta\) 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\textsuperscript{+} MS \(m/z\): 662 [M+H\textsuperscript{+}].


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\textsubscript{3}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\textsubscript{2}CO\textsubscript{3}. 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\textsubscript{4}, 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. Rf = 0.6 (S$_2$O$_2$, EtOAc); $^1$H NMR (CDCl$_3$, 400 MHz) δ 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) δ 172.3, 172.0, 171.8, 157.4, 157.1, 156.3, 141.6, 136.7, 136.3, 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$^+$ MS m/z: 867 [M+Na]$^+$; Anal. Calcd for C$_{48}$H$_{53}$N$_5$O$_9$ + 1.5 H$_2$O: 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]hydrazinolpropanoylamino]-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$_2$O (92 mg, 0.16 mmol, 91%); $^1$H NMR (CD$_3$OD, 300 MHz) δ 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); $^1$H NMR (DMSO-d$_6$, 400 MHz) δ 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$_3$OD, 100 MHz) δ 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$^+$ MS m/z: 598 [M+Na]$^+$; Anal. Calcd for C$_{32}$H$_{44}$Cl$_3$N$_5$O$_9$ + 1 H$_2$O: C, 54.66; H, 6.61; N, 9.96; Found C, 54.34; H, 6.81; N, 10.09.

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-dimethoxy benzaldehyde (47 mg, 0.30 mmol, 1.0 eq) in methanol (3 mL). After stirring for 5 min at room temperature, NaBH$_3$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$_2$CO$_3$. 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$_4$, 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. Rf = 0.3 (SiO$_2$, EtOAc); $^1$H NMR (CDCl$_3$, 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); $^{13}$C NMR (CDCl$_3$, 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$^{-1}$): 3295 (N-H), 2949 (C-H), 1707 (C=O), 1498 (C=C); ESI$^+$ MS m/z: 834 [M+Na]$^+$; Anal. Calcd for C$_{44}$H$_{53}$N$_5$O$_{10}$: 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-phenyl-propanoate 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$_2$O (101 mg, 0.19 mmol, 86%). $^1$H NMR (CDCl$_3$, 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); $^{13}$C NMR (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 (cm$^{-1}$): 3295 (N-H), 2949 (C-H), 1707 (C=O), 1498 (C=C), 1453 (C-H); ESI$^+$ MS m/z: 566 [M+Na]$^+$; Anal. Calcd for C$_{28}$H$_{44}$Cl$_3$N$_5$O$_6$ + 1.5 H$_2$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-LVVY-AFC

![Scheme 3S. Synthesis of Suc-LVVY-AFC.](image-url)
**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 µl, 8.73 mmol, 1.0 eq) at -15°C. After 15 min. of stirring at -15°C, the solution was poured into water and extracted with EtOAc. The organic layer was then washed with a saturated aqueous solution of NaHCO$_3$, a 10% aqueous solution of citric acid, brine, and dried over Na$_2$SO$_4$. After filtration and evaporation under reduced pressure, the crude material was purified by silica gel flash column chromatography (DCM/Et$_2$O: 8/2) to give 18 (2.00 g, 4.06 mmol, 46%) as a white solid. m.p.: 224-226°C; Rf: 0.4 (SiO$_2$, DCM/Et$_2$O: 8/2); $^1$H NMR (DMSO-d$_6$, 400 MHz) δ 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); $^{13}$C NMR (DMSO-d$_6$, 100 MHz) δ 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; $^{19}$F NMR (DMSO-d$_6$, 188 MHz) δ -63.99 (s); IR (cm$^{-1}$): 3400 (N-H), 2959 (C-H), 1678 (C=O), 1644 (C=C), 1528 (N-H); ESI$^+$ MS m/z: 515 [M+Na]$^+$; Anal. Calcd for C$_{24}$H$_{23}$F$_3$N$_2$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. $^1$H NMR (DMSO-d$_6$, 300 MHz) δ 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); $^{13}$C NMR (DMSO-d$_6$, 75 MHz) δ 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; $^{19}$F NMR (DMSO-d$_6$, 188 MHz) δ -64.02 (s); ESI$^+$ MS m/z: 393 [M+H]$^+$. 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$_2$CO$_3$ (2 x 20 mL) and brine (20 mL), dried over Na$_2$SO$_4$, 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$_2$DCM/Et$_2$O: 7/3); $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 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); $^{13}$C NMR (DMSO-$d_6$, 100 MHz) $\delta$ 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; $^{19}$F NMR (DMSO-$d_6$, 188 MHz) $\delta$ -64.02 (s); IR (cm$^{-1}$): 3308 (N-H), 2959 (C-H), 1678 (C=O), 1644 (C=C), 1528 (N-H); ESI-MS m/z: 590 [M-H]$^-$; HRMS (TOF, ESI, ion polarity positive): calcd for Anal. Calcd for C$_{29}$H$_{32}$F$_3$N$_3$O$_7$Na: 614.2090; found 614.2092.

tert-butyl N-[(1S)-1-][(1S)-1-][(1S)-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. $^1$H NMR (DMSO-$d_6$, 300 MHz) $\delta$ 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); $^{13}$C NMR (DMSO-$d_6$, 75 MHz) $\delta$ 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; $^{19}$F (DMSO-$d_6$, 188 MHz) $\delta$ -64.02 (s); MS (ESI-) m/z: 492 [M+H]$^+$. 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$_2$CO$_3$ (2 x 20 mL) and brine (20 mL), dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (DCM/Et$_2$O: 6/4) to give 20 (526 g, 0.75 mmol, 46%) as a white foam. Rf: 0.4 S$_1$O$_2$ DCM/ether: 6/4); $^1$H NMR (DMSO-d$_6$, 400 MHz) $\delta$ 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), 7.30 (m, 1H), 7.05 (d, J = 8.2 Hz, 2H), 4.58 (m, 1H), 4.23 (m, 1H), 3.97 (m, 1H), 3.94 (m, 1H), 3.94 (m, 1H), 1.70 (m, 1H), 1.48 (m, 2H), 0.83 (m, 6H), 0.77 (m, 3H), 0.75 (m, 3H); $^{13}$C NMR (DMSO-d$_6$, 100 MHz) $\delta$ 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; $^{19}$F NMR (DMSO-d$_6$, 188 MHz) $\delta$ -64.01 (s); IR (cm$^{-1}$): 3320 (N-H), 2972 (C-H), 1678 (C=O), 1645 (C=C), 1516 (N-H), 1407 (OH); MS (ESI+) m/z: 727 [M+Na]$^+$; Anal. Calcd for C$_{35}$H$_{43}$F$_3$N$_4$O$_8$: C, 59.42; H, 6.18; N, 7.92; Found C, 59.03; H, 6.19; N, 7.70.

tert-butyl N-[(1S)-1-][(1S)-1-][(1S)-1-][(1S)-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 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. $^1$H NMR (DMSO-d$_6$, 400 MHz) $\delta$ 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); $^{13}$C NMR (DMSO-d$_6$, 100 MHz) $\delta$ 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; $^{19}$F NMR (DMSO-d$_6$, 188 MHz) $\delta$ -64.00 (s); ESI MS m/z: 603 [M-H]$^-$; 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$_2$CO$_3$ (2 x 10 mL) and brine (10 mL), dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (DCM/Et$_2$O: 6/4) to give the compound 21 (300 mg, 0.37 mmol, 56%) as a white foam. Rf: 0.5 (S$_2$O$_2$ DCM/ether: 6/4); $^1$H NMR (DMSO-$d_6$, 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) ; $^{13}$C NMR (DMSO-$d_6$, 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; $^{19}$F NMR (DMSO-$d_6$, 188 MHz) δ -64.01 (s); IR (cm$^{-1}$): 3340 (N-H), 2971 (C-H), 1678 (C=O), 1637 (C=C), 1515 (N-H), 1407 (OH); MS (ESI$^+$) m/z: 841 [M+Na]$^+$; Anal. Calcd for C$_{41}$H$_{54}$F$_3$N$_5$O$_9$ + 0.15 H$_2$O: C, 60.04; H, 6.69; N, 8.54; Found C, 59.72; H, 6.82; N, 8.25.

Suc-LLVY-AFC

4-[[1(S)-1-][(1S)-1-][(1S)-1-][(1S)-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][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. $^1$H NMR (DMSO-$d_6$, 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),

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0.81 (m, 6H), 0.79 (m, 3H), 0.60 (m, 3H); $^{13}$C NMR (DMSO-$d_6$, 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; $^{19}$F NMR (DMSO-$d_6$, 188 MHz) $\delta$ -64.0 (s); ESI$^+$ MS $m/z$: 718 [M+H]$^+$. 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$_2$O to give Suc-LLVY-AFC (138 mg, 0.17 mmol, 56%) as a white solid. m.p.:230°-232°C; $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 12.10 (brs, 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); $^{13}$C NMR (DMSO-$d_6$, 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; $^{19}$F NMR (DMSO-$d_6$, 188 MHz) $\delta$ -64.01 (s); IR (cm$^{-1}$): 2959 (C-H), 1678 (C=O), 1644 (C=C), 1528 (N-H); MS (ESI$^+$) $m/z$: 816 [M-H]$^-$; Anal. Calcd for C$_{40}$H$_{50}$F$_3$N$_5$O$_{10}$ + 3 H$_2$O: C, 55.10; H, 6.49; N, 8.03; found C, 55.35; H, 6.38; N, 7.79.
3- NMR spectra of pseudopeptides 2-4 and of Suc-LLVY-AFC substrate
Suc-LLVY-AFC
Suc-LLVY-AFC
Suc-LLVY-AFC
Suc-LLVY-AFC
Suc-LLVY-AFC
4- **K\textsubscript{M} determination of the Suc-LLVY-AFC substrate by fluorescence**

\(K\textsubscript{M}\) 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 \(K\textsubscript{M}\) value, equal to 20 \(\mu\)M, was calculated from \(1/V_0\) against \(1/S\) plot (Linweaver-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 (\(\lambda_{\text{ex}} = 360 \text{ nm}, \lambda_{\text{em}} = 480 \text{ nm}\)) and by 3-FABS.**

\begin{align*}
2 \text{ (fluorescence)} &\quad \text{IC}_{50} = 3.9 \pm 0.6 \\
2 \text{ (3-FABS)} &\quad \text{IC}_{50} = 8.5 \pm 1.1
\end{align*}
3 (fluorescence) IC₅₀ = 1.7 ± 0.3

3 (3-FABS) IC₅₀ = 3.1 ± 0.3

4 (fluorescence) IC₅₀ = 9.4 ± 1.4

4 (3-FABS) IC₅₀ = 10.5 ± 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.