Aminopropargyl Derivative of Terpyridine-bis(Methyl-enamine) Tetraacetic Acid Chelate of Europium (Eu (TMT)-AP_3): A New Reagent for Fluorescent Labelling of Proteins and Peptides

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Supporting Information

S1 Experimental section : synthesis of Eu(III) chelate dimer **18** and FRET substrate **21**

S2 RP-HPLC elution profile of purified **21**

S3 Fluorescence emission time course of **21** incubated with recombinant human caspase-3

-S1- Experimental section : synthesis of Eu(III) chelate dimer 18 and FRET substrate 21

High-performance liquid chromatography separations

Several chromatographic systems were used for the analytical experiments and the purification steps. System J: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5μm, 4.6 x 150 mm) with CH_3CN and TEAA buffer (100 mM, pH 7.0) as the eluents [100% TEAA (5 min), linear gradient from 0 to 45% (30 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. Dual UV detection was achieved at 260 and 285 nm. System K: RP-HPLC (Waters Xterra MS C_{18} column, 5μm, 7.8 x 100 mm) with CH_3CN and deionised water as the eluents [100% H_2O (5 min), linear gradient from 0 to 60% (40 min) of CH_3CN] at a flow rate of 2.5 mL min^{-1}. Dual UV detection was achieved at 260 and 285 nm. System L: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5μm, 4.6 x 150 mm) with CH_3CN and aq. TFA (0.1%, pH 2.0) as the eluents [100% TFA (5 min), linear gradient from 0 to 60% (40 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. Dual UV/Visible detection was achieved at 260 and 650 nm. System M: RP-HPLC (Waters Xterra MS C_{18} column, 5μm, 7.8 x 100 mm) with CH_3CN and 0.1% aq. formic acid (aq. FA, 0.1%, v/v) as the eluents [100% FA (5 min), linear gradient from 0 to 20% (20 min) and 20 to 80% (80 min) of CH_3CN] at a flow rate of 2.5 mL min^{-1}. UV detection was achieved at 260 nm. System N: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5μm, 4.6 x 150 mm) with CH_3CN and TEAA buffer (100 mM, pH 7.0) as the eluents [100% TEAA (5 min), linear gradient from 0 to 10% (10 min) and 10 to 70 (120 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. Dual UV/Visible detection was achieved at 260 and 650 nm. System O: RP-HPLC (Waters Xterra MS C_{18} column, 5μm, 7.8 x 100 mm) with CH_3CN and TEAB buffer (50 mM, pH 7.5) as the eluents [100% TEAB (5 min), linear gradient from 0 to 10% (10 min) and 10 to 60% (120 min) of CH_3CN] at a flow rate of 3.0 mL min^{-1}. UV detection was achieved at 260 nm. System P: System N with the following gradient [100% TEAA (5 min), then linear gradient from 0 to 60% (40 min) of CH_3CN].

Eu(III) chelate dimer (18): 2.18 mg of Eu (TMT)-AP_3 chelate **3** (2.64 μmol, weighed in a 1.0 mL Eppendorf tube) was dissolved in deionised water (75 μL). After complete solubilisation by vortexing, the resulting solution was slowly added to a $CHCl_3$ solution containing thiophosgene (2.0 μL, 26.4 μmol) and solid $NaHCO_3$ (2.83 mg, 33.7 μmol). The reaction mixture was protected from light and vigorously stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system J). Finally, the aq. layer was taken, diluted with deionised water (~ 1 mL) and purified by RP-HPLC (system K). The product-containing fractions were lyophilised to give the thiourea **18** as a white amorphous powder. Quantification was achieved by UV/Visible measurements at λ_{max} = 298 nm of the Eu(III) chelate by using the ε value 42000 L mol^{-1} cm^{-1} value (yield after RP-HPLC purification : quantitative). HPLC (system J): t_R = 29.4 min; MS (ESI-): *m/z* 821.7 $[M-2H]^{2-}$, 843.3 $[M-2H+2Na]^{2-}$, calcd for $C_{69}H_{54}N_{12}O_{16}SEu_2$ 1643.23.

Synthesis and characterisation of fluorogenic caspase-3 substrate Ac-Lys(Cy 5.0)-Asp-Glu-Val-Asp-Lys(Eu (TMT)-AP_3)-NH_2 (21):

Synthesis of Ac-Lys(Cy 5.0)-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 (19): 5.6 mg of peptide Ac-Lys-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 **17** (4.6 μmol, weighed in a 1.0 mL Eppendorf tube) was dissolved in 100 μL of dry NMP and 5.6 μL of DIEA (32.3 μmol) was added. After complete solubilisation by vortexing, the resulting solution was added to the crude reaction mixture containing the succinimidyl ester of Cy 5.0 dye (4.9 μmol). The resulting reaction mixture was protected from light and stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system L). Finally, the reaction mixture was quenched by dilution with 2 mL of aq. FA 0.1% and purified by RP-HPLC (system M, 2 injections). The product-containing fractions were lyophilised to give the peptide-Cy 5.0 conjugate **19** as a blue amorphous powder. Quantification was achieved by UV/Visible measurements at λ_{max} = 650 nm of the Cy 5.0 dye by using the ε value 250000 L mol^{-1} cm^{-1} (yield after RP-HPLC purification : 85%). HPLC (system L): t_R = 27.9 min; MS (MALDI-TOF, positive mode, CHCA matrix): *m/z* 1625.91 $[M+H]^+$, calcd exact mass for $C_{73}H_{100}N_{12}O_{22}S_4$ 1625.93.

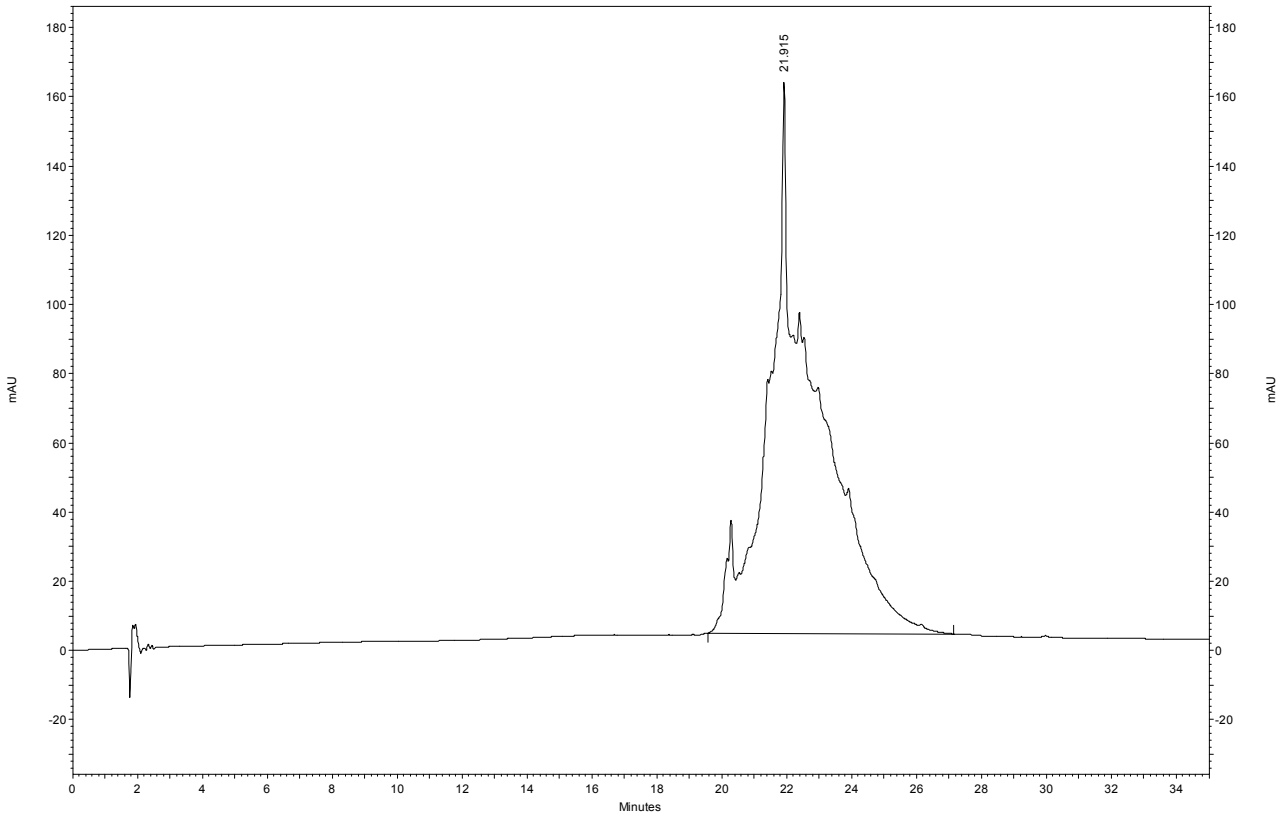
Removal of Pydec group: Peptide Ac-Lys(Cy 5.0)-Asp-Glu-Val-Asp-Lys(Pydec)-NH_2 **19** (2.4 mg, 1.3 μmol, weighed in a 10.0 mL Falcon tube) was dissolved in 2.5 mL of Tris.HCl buffer (0.1 M, pH 9.0) and a solution of DTT (10.8 mg in 0.43 mL, 70 μmol) in Tris.HCl buffer (0.1 M, pH 9.0) was added. The reaction mixture was protected from light and stirred at room temperature overnight. The deprotection was checked for completion by RP-HPLC (system L) and the reaction mixture was quenched by dilution with aq. FA (2 mL). Purification was achieved by RP-HPLC (system M, 2 injections). The product-containing fractions were lyophilised to give the peptide Ac-Lys(Cy 5.0)-Asp-Glu-Val-Asp-Lys-NH_2 **20** as a blue amorphous powder. Quantification was achieved by UV/Vis measurements at λ_{max} = 650 nm of the Cy 5.0 dye by using the ε value 250000 L mol^{-1} cm^{-1} (yield after RP-HPLC purification : 95%). HPLC (system M): t_R = 25.2 min; MS (MALDI-TOF, positive mode, CHCA matrix): *m/z* 1413.05 $[M+H]^+$, calcd exact mass for $C_{65}H_{93}N_{11}O_{20}S_2$ 1412.66.

Activation of Eu (TMT)-AP_3 chelate with glutaraldehyde: 1.37 mg of Eu (TMT)-AP_3 chelate **3** (1.66 μmol, weighed in a 1.0 mL Eppendorf tube) was dissolved in deionised water (50 μL). After complete solubilisation by vortexing, the resulting solution was slowly added to an aq. solution of glutaraldehyde (0.5 μL of 50% glutaraldehyde in 50 μL of deionised water). The resulting reaction mixture was protected from light and stirred at 4°C for 1 h.

Synthesis of Ac-Cys(Cy 5.0)-Asp-Glu-Val-Asp-Lys(Eu (TMT)-AP_3)-NH_2 (21): Peptide Ac-Lys(Cy 5.0)-Asp-Glu-Val-Asp-Lys-NH_2 **20** (0.62 mg, 0.44 μmol, weighed in a 1.0 mL Eppendorf tube) was dissolved in 50 μL of deionised water and 3.0 μL of 1 N NaOH were added (for acidity neutralisation). 60 μL of the crude reaction mixture containing the aldehyde derivative was added. The reaction mixture was protected from light and stirred at room temperature overnight. The reaction was checked for completion by RP-HPLC (system N). Thereafter, 10 μL of a 1.6 M aq. solution of $NaBH_3CN$ (16 μmol) were added to reduce the resultant Schiff bases. After 15 min, the reaction mixture was quenched by dilution with TEAB buffer (0.75 mL) and purified by RP-HPLC (system O, 2 injections). The product-containing fractions were twice lyophilised to give the peptide Ac-Lys(Cy 5.0)-Asp-Glu-Val-Asp-Lys(Eu (TMT)-AP_3)-NH_2 **21** as a blue amorphous powder. Stock solution of fluorogenic caspase-3

substrate **21** was prepared in HPLC grade water and UV/Visible quantification was achieved at λ_{max} of the Cy 5.0 dye by using the ε value 250000 L mol^{-1} cm^{-1} (yield after RP-HPLC purification : 50%). HPLC (system P): t_R = 21.9 min (broad peak, 8 min from start to end of peak); UV/Visible (water): λ_{max} (ε) = 297, 652 (250000 L mol^{-1} cm^{-1}).

-S2- RP-HPLC elution profile (system P) of the purified fluorogenic substrate of caspase-3 protease 21.



-S3- Fluorescence emission time course (excitation at 298 nm) of peptide 21 (concentration 1 μM) with recombinant human caspase-3 (3.2 10^{-3} U, incubation time 3 h) in caspase assay buffer (100 mM NaCl, 40 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% (w/v) sucrose and 0.1% (w/v) CHAPS, pH 7.2, 37°C) at 616 (a) and 672 nm (b).