Electronic Supplementary Information

EuroTracker Dyes: Highly Emissive Europium Complexes as Alternative Organelle Stains for Live Cell Imaging

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1. General Experimental Procedures

NMR Spectroscopy and Mass Spectrometry

^{1}H, ^{13}C, ^{19}F and ^{31}P NMR spectra were recorded in commercially-available deuteriated solvents on a Varian Mercury-200 (^{1}H at 199.975 MHz, ^{13}C at 50.289 MHz), Varian Mercury-400 or Bruker Avance-400 (^{1}H at 399.960 MHz, ^{13}C at 100.572 MHz, ^{31}P at 161.943 MHz), Varian Inova-500 (^{1}H at 499.772 MHz, ^{13}C at 125.671 MHz,) or Varian VNMRS-700 (^{1}H at 699.731 MHz, ^{31}P at 283.256 MHz) spectrometer. All chemical shifts are given in ppm and coupling constants are in Hz. Electrospray mass spectra were recorded on a Waters Micromass LCT or Thermo-Finnigan LTQ FT instrument operating in positive or negative ion mode as stated, with methanol as the carrier solvent. Accurate mass spectra were recorded using the Thermo-Finnigan LTQ FT mass spectrometer.

Chromatography

Flash column chromatography was performed using flash silica gel 60 (230 - 400 mesh) from Merck. Thin layer chromatography (TLC) was performed on aluminum sheet silica gel plates with 0.2 mm thick silica gel 60 F_{254} (E. Merck) using different mobile phase. The compounds were visualized by UV irradiation (254 nm) or Dragendorff reagent staining.

Reverse phase HPLC traces were recorded at 298 K using a Perkin Elmer system equipped with a Perkin Elmer Series 200 Pump, a Perkin Elmer Series 200 Autosampler and a Perkin Elmer Series 200 Diode array detector (operated at 254 nm). Separation was achieved using a semi-preparative Waters XBridge RP-C_{18} column (5 μm, 10 × 100 mm) at a flow rate maintained at 4.4 mL/min. For purification of complexes [**Eu.L^{7}**] and [**Eu.L$^{6\text{-}8}$**] a solvent system composed of H_2O + 0.1% HCOOH/methanol + 0.1% HCOOH was used over the stated linear gradient. For purification of complexes [**Eu.L^{1}**]$^{3-}$, [**Eu.L^{4}**]$^{-}$ and [**Eu.L^{5}**]$^{6+}$ a solvent system composed of 0.1 M NH_4HCO_3/methanol was used over the stated linear gradient. Analytical RP-HPLC was performed

using a Waters XBridge RP-C_{18} column (3.5 µm, 4.6 × 100 mm) at a flow rate maintained at 1.0 mL/ min over the stated linear gradient.

Optical Spectroscopy

Emission spectra were recorded using an ISA Jobin-Yvon Spex Fluorolog-3 luminescence spectrometer. Lifetime measurements were carried out with a Perkin Elmer LS55 spectrometer using FL Winlab software. Quantum yield measurements were calculated by comparison with two standards. For the standards and each of the unknowns, five solutions with absorbance values between 0.05 and 0.1 were used. The quantum yield was calculated according to the equation:

$$\Phi_x = \Phi_r \cdot \frac{A_r}{A_x} \cdot \frac{E_x}{E_r} \cdot \frac{I_r}{I_x} \cdot \frac{\eta_x^2}{\eta_r^2}$$

where *r* and *x* refer to reference and unknown respectively; *A* is the absorbance at λ_{ex} *;* *E* is the corrected integrated emission intensity*;* *I* is the corrected intensity of excitation light*;* *h* is the refractive index of solution.

Cell culture, Confocal Microscopy and Cell Spectral Imaging

Cell culture

Two main cell lines were selected for cellular studies, although similar behaviour was noted in HeLa, human prostate adenocarcinoma (PC3) and human breast carcinoma (MDA-MB-231) cells [7,21]. The main cells studied were CHO (Chinese Hamster Ovary), and NIH 3T3 (mouse skin fibroblast) cells. Cells were maintained in exponential growth as monolayers in F-12 (Ham) medium, DMEM (Dulbecco's Modified Eagle Medium) and RPMI 1640 medium respectively. For each cell line, the medium was supplemented with 10% foetal bovine serum (FBS), 1% non-essential amino acids and 0.5% (v/v) penicillin and streptomycin. Cells were grown in plastic culture flasks, with no prior surface treatment. Cultures were incubated at 37 °C, 20% average humidity and 5% (v/v) CO_2. Cells were harvested by treatment with 0.25% (v/v) trypsin solution for 5 min at 37 ºC. Cell suspensions were pelleted by centrifugation at 1000 rpm for 3 min, and were re-suspended by repeated aspiration with a glass pipette.

In order to determine cell number, cells were detached from the flask by trypsinisation. Cells were then pelleted and re-suspended in 4 mL medium, and an aliquot of the cell suspension injected into a haemocytometer (Fisher). The number of cells in a grid of volume 100 nL was counted using a light microscope, and the values for four separate grids measured to give an average cell count.

Cells were seeded in 12-well plates on glass cover-slips and allowed to grow to 40% – 60% confluence, at 37 ºC in 5% CO_2. At this stage, the medium was replaced and cells were treated with

drugs and complexes as appropriate. For NIH 3T3 and HeLa cells, DMEM lacking phenol red was used from this point onwards. Following incubation, the cover-slips were washed with phosphate-buffered saline (PBS; pH 7.5), mounted on slides and the edges sealed with colourless, quick-dry nail varnish to prevent drying out of the sample.

Cytotoxicity

Approximately 1 x 10^4 NIH-3T3 cells in 100 μL DMEM were seeded into each well of flat-bottomed 96-well plates and allowed to attach overnight. Complex solutions were added to triplicate wells to give final concentrations over a 2-log range. After 24 h incubation, IC_{50} values were measured using MTT or WST-1. For the MTT method, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 1.0 mM) was added to each well and the plates incubated for a further 4 h. The culture medium was removed, and DMSO (150 μL) was added. The plate was shaken for 20 sec and the absorbance measured immediately at 540 nm in a microplate reader against a blank plate containing DMSO. For the WST-1 assay, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1, 10 μL) was added to each well and the plates incubated for a further 30 min. The plate was shaken for 20 sec and the absorbance measured immediately at 450 nm in a microplate reader against a blank of DMEM containing 10 μL WST-1 per well. For both methods, IC_{50} values were determined as the drug concentration required to reduce the absorbance to 50% of that in the untreated, control wells, and represent the mean value for data from at least three independent experiments.

Microscopy

Cell images and co-localisation experiments were obtained using a Leica SP5 II microscope. In order to achieve excitation with maximal probe emission, the microscope was coupled by an optical fibre to a Coherent 355nm CW (Nd:YAG) laser, operating at 8mW power. A HeNe or Ar ion laser was used when commercially available organelle-specific stains (e.g. MitotrackerGreen™) were used to corroborate cellular compartmentalization. The microscope was equipped with a triple channel imaging detector, comprising two conventional PMT systems and a HyD hybrid avalanche photodiode detector. The latter part of the detection system, when operated in the BrightRed mode, is capable of improving imaging sensitivity above 550 nm by 25%, reducing signal to noise by a factor of 5. The pinhole was always determined by the Airy disc size, calculated from the objective in use (HCX PL APO 63x/1.40 NA LbdBlue), using the lowest excitation wavelength (355 nm). Scanning speed was adjusted to 100 Hz in a unidirectional mode, to ensure both sufficient light exposure and enough time to collect the emitted light from the lanthanide based optical probes (1024x1024 frame size, a pixel size of 120 x120 nm and depth of 0.772 μm). Spectral imaging on this Leica system is possible with the *xyλ*-scan function, using the smallest allowed spectral band-pass (5nm) and step-size (3nm) settings.

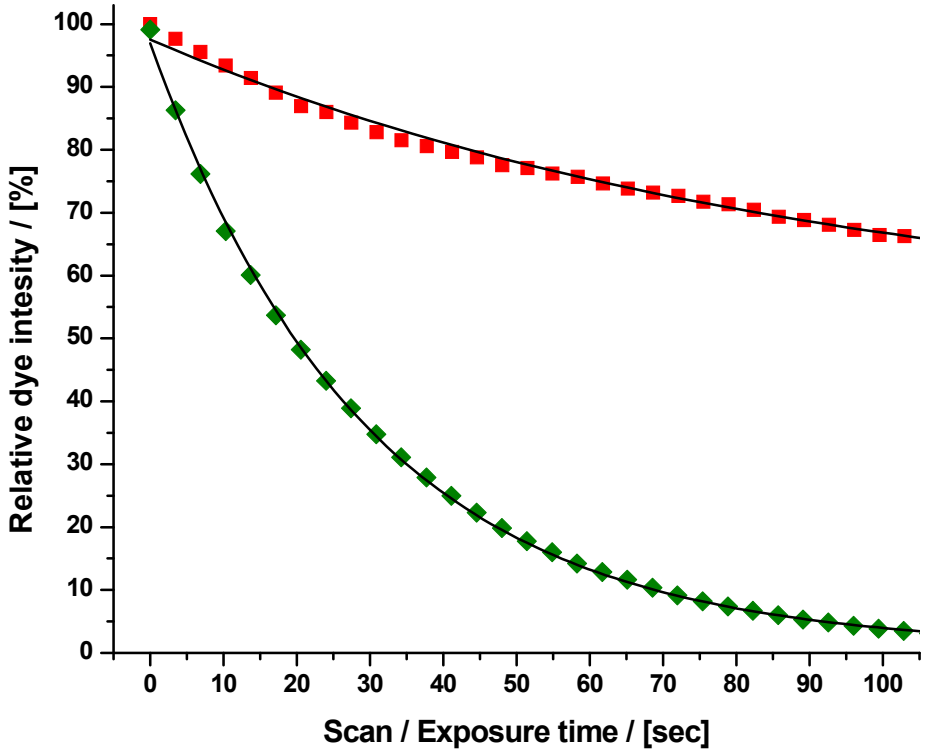
However, much improved spectral imaging in cells was achieved using a custom built microscope (modified Zeiss Axiovert 200M), using a Zeiss APOCHROMAT 63x/1.40 NA objective combined with a low voltage 365 nm pulsed UV LED focused, collimated excitation source (1.2W). For rapid spectral acquisition the microscope was equipped at the X1 port with a Peltier cooled 2D-CCD detector (Ocean Optics) used in an inverse 100 Hz time gated sequence. The spectrum was recorded from 400-800 nm with a resolution of 0.24 nm and the final spectrum was acquired using an averaged 10,000 scan duty cycle. Probe lifetimes were measured by time correlated multiphoton counting, on the same microscope platform using a novel cooled PMT detector (Hamamatsu H7155) interchangeable on one of the X1 ports, with the application of pre-selected interference filters as detailed in the main text (Fig 6). Both the control and detection algorithm were written in LabView2011, where probe lifetime was determined by using a single exponential tail-fitting algorithm to the monitored signal intensity decay.

The 3D reconstruction was achieved using a novel saturation elimination algorithm update of the existing ImageJ 1.47j 3D plug-in using the raw LSCM images recorded on the above detailed Leica SP5 II microscope. In these z-stack images, to eliminate saturation and subsequently enhance resolution, a deliberate 20% overlap in the applied axial resolution was introduced, determined by the applied optics and experimental parameters detailed above.

Inductively coupled plasma mass spectrometry determinations of europium or terbium concentrations were made by Dr. C. Ottley in the Department of Earth Sciences at Durham University, using methods discussed in references 7 and,21 (main text).

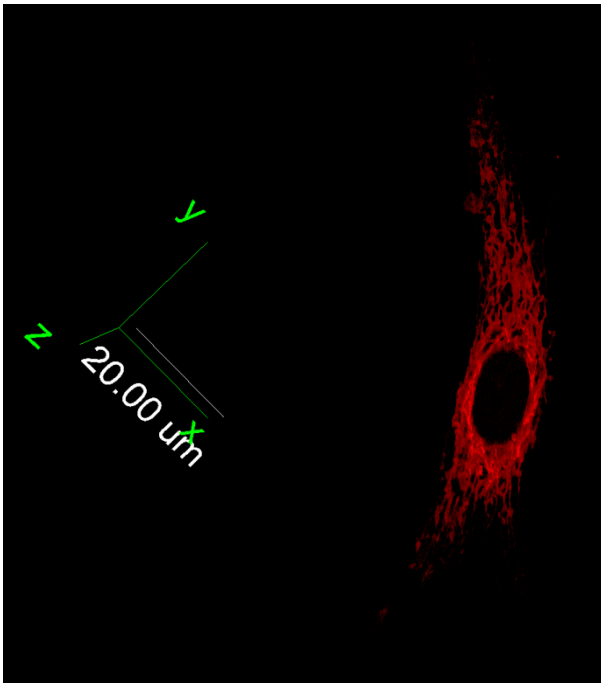
Photobleaching Studies

Photo-bleaching experiments were carried out using the LeicaSP5 II LSCM with MitoTracker Green (200 nM, 30 min) and [**Eu.L^2**] (360 nm, 3h) in a simultaneous excitation experiment.
Experimental settings: 1-frame, 3-line averaged scan (total time 3.5s/scan) at 700 Hz, operating in a bidirectional mode with a 1024x1024 frame size, a voxel size of 120 x 120 nm and depth of 0.78 μm, using 488 and 355 nm lasers, each operating at 4 mW (~80nJ/voxel).

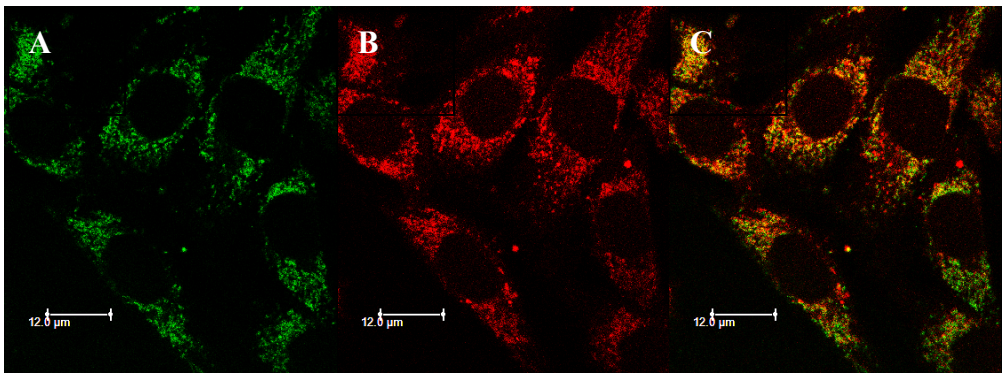


ESI Figure 1. Variation of photobleaching with time for **Eu.L^2** (red, 605 – 720 nm) and MitoTracker Green (green, 500-530 nm). The MTG is completely (>95%) photobeached after 30 scans (100 seconds), whilst **Eu.L^2** retained 70% of its total intensity.

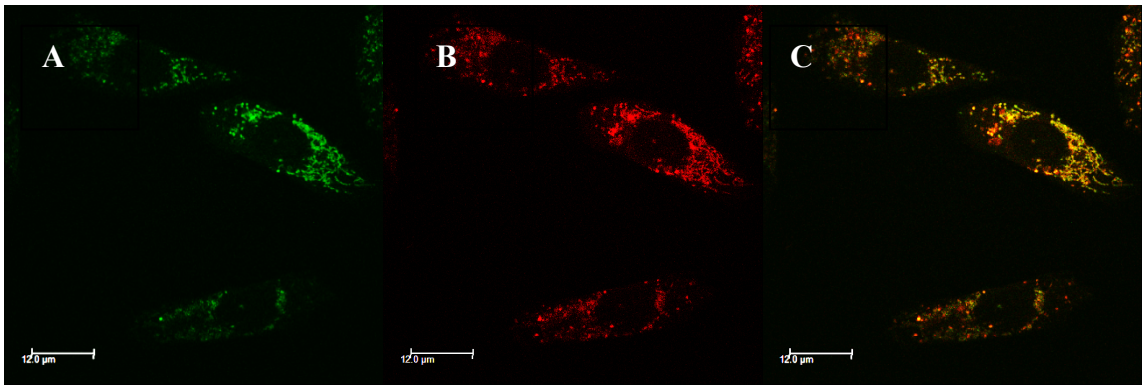
2. Microscopy Images



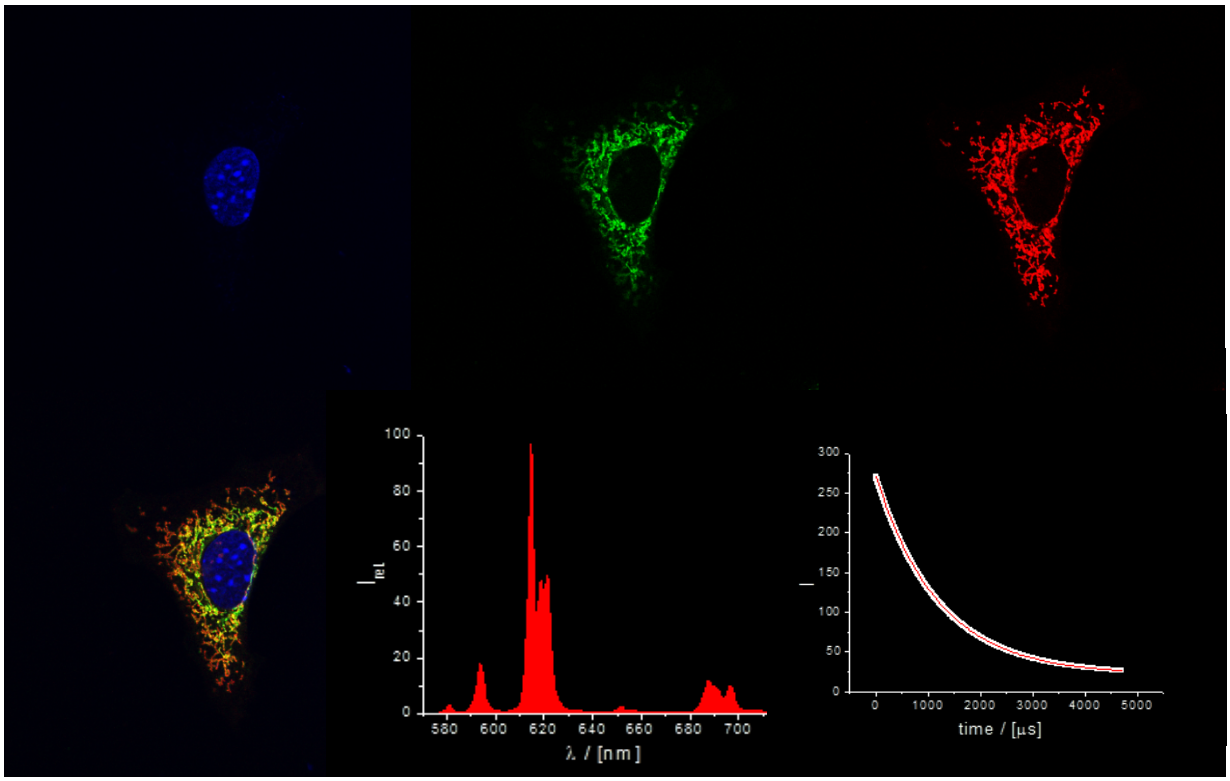
ESI Figure2. 3D reconstruction of the mitochondrial network in NIH 3T3 cells with [**Eu.L^2**] (18 μM, λ_{exc} 355 nm, λ_{em} 605–720 nm), using a structured illumination LSCM technique, in tandem with a 3D modelling ImageJ plugin, to enhance the resolution.



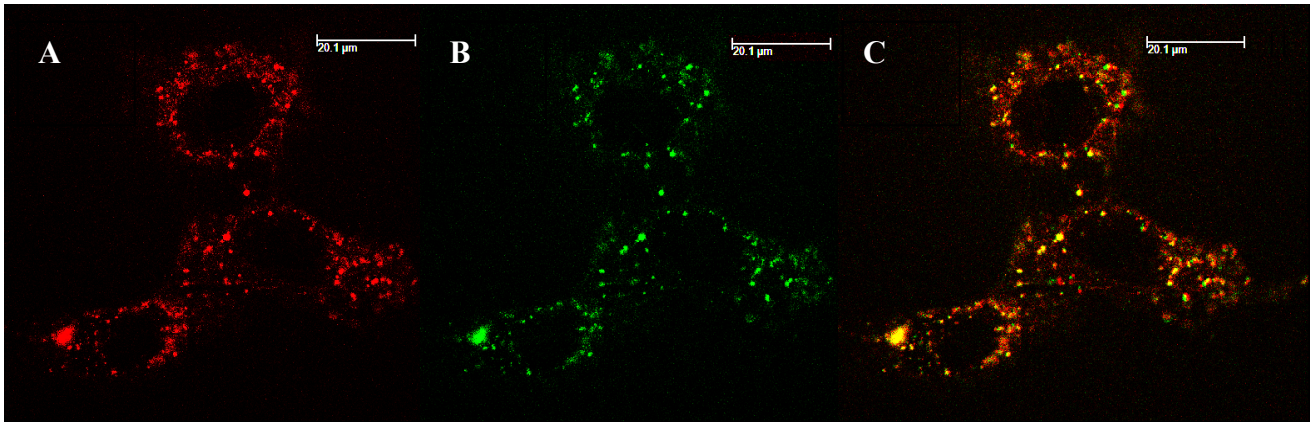
ESI Figure 3. LCMS images showing: (a) MitoTracker Green (λ_{exc} 488 nm, λ_{em} 500−530 nm); (b) mitochondrial localisation of [**Eu.L^4**]$^-$ of CHO cells (4h, 30 μM, λ_{exc} 355 nm, λ_{em} 605–720 nm); (c) RGB merged image showing co-localisation (P = 0.77).



ESI Figure 4. LCMS images showing: (a) MitoTracker Green (λ_{exc} 488 nm, λ_{em} 500−530 nm); (b) mitochondrial localisation of [**Eu.L**^{5}]$^{6+}$ of CHO cells (30 min, 10 μM, λ_{exc} 355 nm, λ_{em} 605−720 nm); (c) RGB merged image showing co-localisation (P = 0.77).



ESI Figure 5. LCMS images showing: (a) DAPI stained nucleus (λ_{exc} 355 nm, λ_{em} 400−450 nm); (b) MitoTracker Green (λ_{exc} 488 nm, λ_{em} 500−530 nm); (c) mitochondrial localisation of [**Eu.L**^{7}] of NIH 3T3 cells (4h, 30 μM, λ_{exc} 355 nm, λ_{em} 605−720 nm); (d) RGB merged image showing co-localisation (P = 0.89), (e) spectral profiling of [**Eu.L**^{7}] (24h, 30 μM) using our custom built Zeiss Axiovert 200M equipped with a cooled 2D-CCD detector, (f) Recorded lifetime for [**Eu.L**^{7}] in NIH 3T3 (24h, 30 μM), using a new PMT based detection method.



ESI Figure 6. LCMS images showing: (a) localisation of [**Eu.L^6**] in the lysosomes of NIH-3T3 cells (1 h, 15 µM, λ_{exc} 355 nm, λ_{em} 605–630 nm); (b) FRET induced SA-AF633 fluorescence (λ_{exc} 355 nm, λ_{em} 650−670 nm), following incubation of SA-AF633 in the cell growth medium (380 nM, 30 min); (c) RGB merge image showing co-localisation of the proximate donor-acceptor pair in the lysosomes (P = 0.85).

3. FRET Quenching Studies

General procedure for titrating [Eu.L^6] with Streptavidin AlexaFluor633 (SA-AF633)

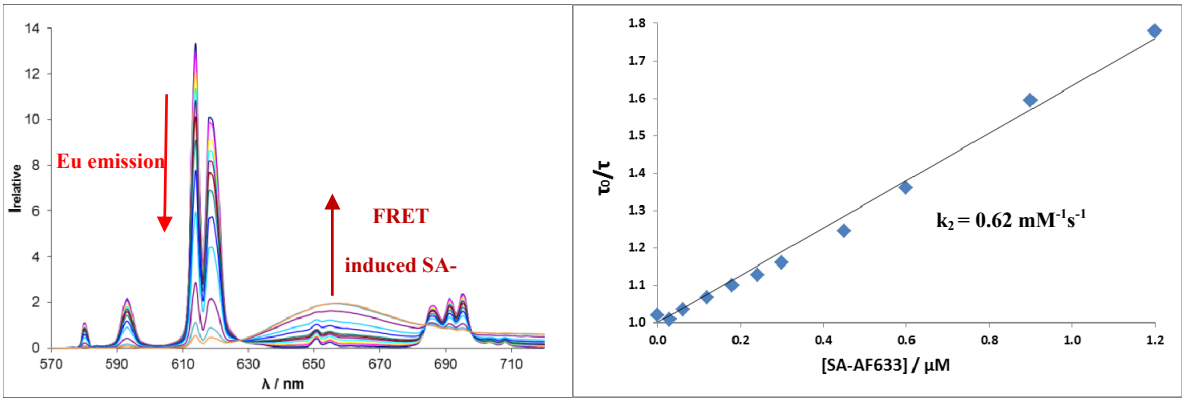
The quenching efficiency of [**Eu.L^6**] (donor, 3 µM) by SA-AF633 (acceptor) was determined in aqueous solution (0.1 M NaCl, pH 7.4). A solution of [**Eu.L^6**] (3 µM) in water (1 mL) was added to a 1 cm quartz glass cuvette and the fluorescence emission spectrum (λ_{ex} = 330 nm, λ_{em} = 570-720 nm) and emission lifetime were recorded. Aliquots of a stock solution of SA-AF633 (38 µM) were added to the cuvette over the concentration range 30 nM to 1.6 µM and the fluorescence emission spectra and lifetimes were recorded after each addition.

In the rapid diffusion limit, energy transfer from the Eu donor to the dye acceptor obeys pseudo-first order kinetics, where $1/\tau_o = k_o$; $1/\tau = k_{obs}$ and $k_{obs} = k_o + k_2[Q]$;

hence $\tau_0/\tau = k_{obs}/k_o = 1 + k_2/k_o\,[Q]$.

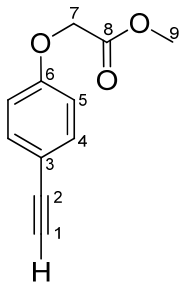
Thus, the slope of the plot of τ_0/τ vs [Q] is k_2/k_0, allowing the second order rate constant for energy transfer, k_2, to be estimated; C. F. Meares, T. G. Wensel, *Acc. Chem. Res*. **1984**, *17*, 202.

To determine the second order rate constant for energy transfer, k_2, the europium emission lifetime (τ_0/τ) was plotted as a function of SA-AF633 (acceptor) concentration. The slope of the plot represents k_2/k_0, thereby allowing k_2 to be estimated. Under these conditions, k_2 = 0.62 mM^{-1}s^{-1}, with τ_0 = 1.02 ms (ESI Figure 6b). As discussed in the recent communication, [27] this corresponds to a Forster distance of 70(±2) Å between the donor and acceptor.



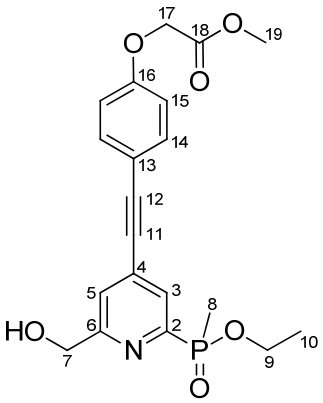
ESI Figure 7. (a) Emission spectral change upon the titration of [**Eu.L^6**] (3 µM) with SA-AF633 (30 nM to 1.6 µM) in aqueous solution (0.1 M NaCl, pH 7.4); (b) Plot of τ_0/τ versus concentration of SA-AF633.

4. Compound characterisation



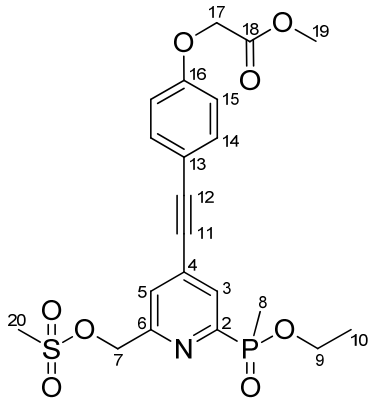
Methyl 2-(4-ethynylphenoxy)acetate

Methyl-(4-bromophenoxy)acetate (1.20 g, 4.89 mmol) was dissolved in anhydrous THF (2 mL) and the solution was degassed (freeze-thaw cycle) three times. Ethynyltrimethylsilane (0.83 mL, 5.87 mmol) and triethylamine (3.40 mL, 24.5 mmol) were added and the solution was degassed (freeze-thaw cycle) once more. [1,1-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (0.41 g, 0.489 mmol) and CuI (93 mg, 0.489 mmol) were added and the resulting brown solution was stirred at 65 °C under argon for 24 h. The solvent was removed under reduced pressure and the resulting brown oil was purified by column chromatography (silica, hexane/CH_2Cl_2 2:1 v/v, then 1:1 v/v) to give methyl 2-(4-((trimethylsilyl)ethynyl)phenoxy)acetate as a yellow oil (0.76 g, 60%); R_f = 0.47 (silica; hexane/EtOAc, 2:1 v/v). This compound (140 mg, 0.535 mmol) was immediately dissolved in anhydrous THF (2 mL) and triethylammonium dihydrofluoride (0.870 mL, 5.35 mmol) was added. The mixture was stirred at 35 °C under argon for 24 h. The solvent was removed under reduced pressure to give a yellow oil which was subjected to column chromatography (silica, hexane/CH_2Cl_2 2:1 v/v), giving methyl 2-(4-ethynylphenoxy)acetate as a colourless oil (89 mg, 87%); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.43 (2H, d, $^3J_{H\text{-}H}$ 8.9 Hz, H^4), 6.85 (2H, d, $^3J_{H\text{-}H}$ 8.9 Hz, H^5), 4.64 (2H, s, H^7), 3.80 (3H, s, H^9), 3.00 (1H, s, H^1); ^{13}C NMR (151 MHz, $CDCl_3$) δ 169.1 (C^8), 158.2 (C^6), 133.8 (C^4), 115.6 (C^3), 114.7 (C^5), 83.4 (C^2), 76.3 (C^1), 65.3 (C^7), 52.5 (C^9); LRMS (ESI) *m/z* 191 $[M + H]^+$; (HRMS+) *m/z* 191.0714 $[M + H]^+$ ($C_{11}H_{11}O_3$ requires 191.0708); R_f = 0.39 (silica; hexane/EtOAc, 3:1 v/v).



Methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-(hydroxymethyl)pyridin-4-yl)ethynyl)phenoxy)acetate

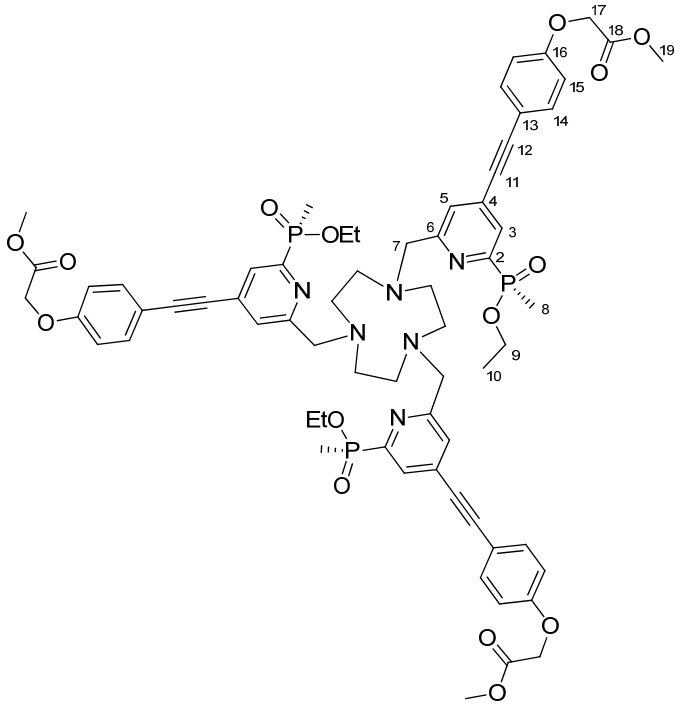
To a stirred degassed solution of ethyl (6-(hydroxymethyl)-4-(bromopyridin-2-yl)(methyl)phosphinate (103 mg, 0.351 mmol) in anhydrous THF (1 mL) was added methyl 2-(4-ethynylphenoxy)acetate (80 mg, 0.421 mmol) and triethylamine (0.245 mL, 1.75 mmol), and the solution was degassed (freeze-thaw cycle) three times. [1,1-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (30 mg, 0.035 mmol) and CuI (7 mg, 0.035 mmol) were added and the resulting brown solution was stirred at 65 °C under argon for 18 h. The solvent was removed under reduced pressure and the brown residue was purified by column chromatography (silica, CH_2Cl_2/0−3% CH_3OH in 0.5% increments) to afford methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-(hydroxymethyl)pyridin-4-yl)ethynyl)phenoxy)acetate as a yellow oil (122 mg, 87%); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.98 (1H, br s, H^3), 7.50 (1H, br s, H^5), 7.45 (2H, d, $^3J_{H\text{-}H}$ 8.9 Hz, H^{14}), 6.88 (2H, d, $^3J_{H\text{-}H}$ 8.9 Hz, H^{15}), 4.80 (2H, s, H^7), 4.65 (2H, s, H^{17}), 4.09 (1H, m, H^9), 3.99 (1H, br s, CH_2OH), 3.86 (1H, m, H^9), 3.79 (3H, s, H^{19}), 1.76 (3H, d, $^2J_{H\text{-}P}$ 14.9 Hz, H^8), 1.26 (3H, t, $^3J_{H\text{-}H}$ 6.9 Hz, H^{10}); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.9 (C^{18}), 161.0 (d, $^3J_{C\text{-}P}$ 19 Hz, C^6), 158.8 (C^{16}), 153.3 (d, $^1J_{C\text{-}P}$ 155 Hz, C^2), 133.8 (C^{14}), 132.9 (d, $^3J_{C\text{-}P}$ 12 Hz, C^4), 128.1 (d, $^2J_{C\text{-}P}$ 22 Hz, C^3), 124.2 (C^5), 115.1 (C^{13}), 115.0 (C^{15}), 95.7 (C^{12}), 85.6 (C^{11}), 65.2 (C^{17}), 64.3 (C^7), 61.3 (d, $^2J_{C\text{-}P}$ 5 Hz, C^9), 52.5 (C^{19}), 16.5 (d, $^3J_{C\text{-}P}$ 4 Hz, C^{10}), 13.5 (d, $^1J_{C\text{-}P}$ 104 Hz, C^8); ^{31}P NMR (243 MHz, $CDCl_3$) δ +39.5; (HRMS+) *m/z* 426.1063 $[M + Na]^+$ ($C_{20}H_{22}NO_6PNa$ requires 426.1082); R_f = 0.44 (silica; CH_2Cl_2/CH_3OH 9:1 v/v).



Methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-((methylsulfonyloxy)methyl)pyridin-4-yl)ethynyl)phenoxy)acetate

Methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-(hydroxymethyl)pyridin-4-yl)ethynyl)phenoxy)acetate (122 mg, 0.310 mmol) was dissolved in anhydrous THF (3 mL) and cooled to 5 °C. Triethylamine (86 µL, 0.620 mmol) and methanesulfonyl chloride (36 µL, 0.465

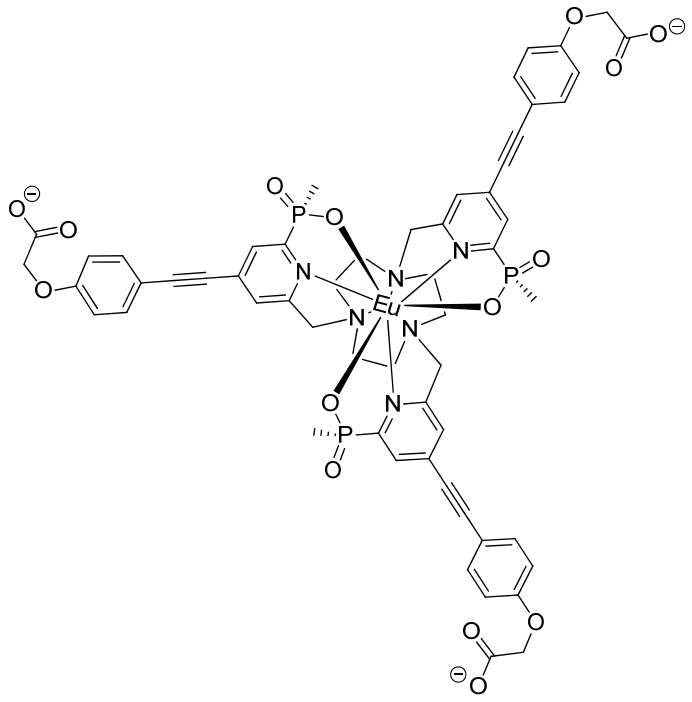
mmol) were added and the mixture was stirred under argon for 90 min. The progress of the reaction was monitored by TLC [silica; CH_2Cl_2/CH_3OH 95:5 v/v, R_f(product) = 0.23, R_f(reactant) = 0.11]. The solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2 (40 mL) and washed with sat. aq. brine solution (40 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 40 mL) and the combined organic layers were dried ($MgSO_4$), and concentrated under reduced pressure to give methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-((methylsulfonyloxy)methyl)pyridin-4 yl)ethynyl)phenoxy)acetate as a yellow oil (150 mg, quant.). The mesylate ester was susceptible to rapid substitution with endogenous chloride, to give the corresponding alkyl chloride compound. As such, the crude material was used immediately in the next step. Any alkyl chloride present reacted in the same manner, albeit slightly more slowly, as the mesylate in the next step, as observed by LC-MS analysis of the reaction mixture. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (1H, br s, H^3), 7.63 (1H, br s, H^5), 7.48 (2H, d, $^3J_{H\text{-}H}$ 8.9 Hz, H^{14}), 6.90 (2H, d, $^3J_{H\text{-}H}$ 8.9 Hz, H^{15}), 5.35 (2H, s, H^7), 4.66 (2H, s, H^{17}), 4.12 (1H, m, H^9), 3.87 (1H, m, H^9), 3.80 (3H, s, H^{19}), 1.76 (3H, d, $^2J_{H\text{-}P}$ 14.9 Hz, H^8), 1.26 (3H, t, $^3J_{H\text{-}H}$ 7.0 Hz, H^{10}); ^{31}P NMR (162 MHz, $CDCl_3$) δ +38.2; LRMS (ESI) *m/z* 482 $[M+H]^+$, 963 $[2M+H]^+$; R_f = 0.56 (silica; CH_2Cl_2/CH_3OH 9:1 v/v).



Triethyl methylphosphinate ester of L^2

To a solution of methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-((methylsulfonyloxy)methyl)pyridin-4 yl)ethynyl)phenoxy)acetate (150 mg, 0.310 mmol) and 1,4,7-triazacyclononane (13.8 mg, 0.107 mmol) in anhydrous CH_3CN (2 mL) was added K_2CO_3 (44 mg, 0.321 mmol) and the mixture was stirred under argon at 60 °C. The progress of the reaction was monitored by LC-MS analysis at regular intervals, which revealed complete consumption of

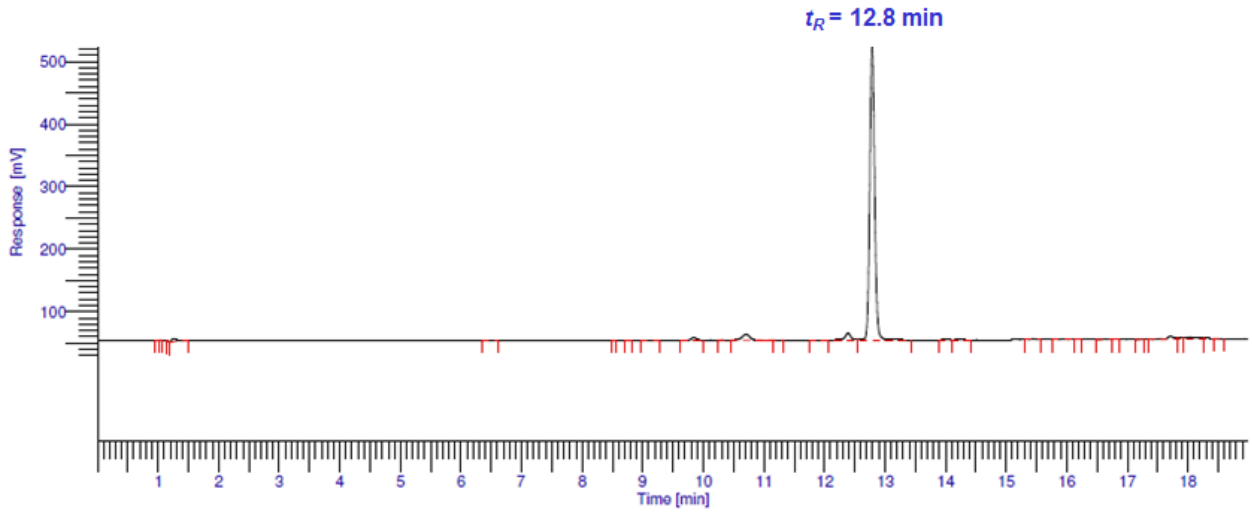
starting material after 9 h. The reaction mixture was cooled to room temperature and the solution decanted from excess potassium salts. The solvent was removed under reduced pressure and the crude material was purified by column chromatography (silica, CH_2Cl_2/0 – 20% CH_3OH in 1% increments) to give the triethyl methylphosphinate ester of L^2 as a colourless oil (43 mg, 31%); ^{1}H NMR (700 MHz, $CDCl_3$) δ 7.96 (3H, $^3J_{H\text{-}P}$ 5.5 Hz, H^3), 7.67 (3H, br s, H^5), 7.45 (6H, d, $^3J_{H\text{-}H}$ 8.7 Hz, H^{14}), 6.87 (6H, d, $^3J_{H\text{-}H}$ 8.7 Hz, H^{15}), 4.63 (6H, s, H^{17}), 4.25 (6H, br m, H^7), 4.09 (3H, dq, $^2J_{H\text{-}H}$ 17.3 Hz, $^3J_{H\text{-}H}$ 7.2 Hz, H^9), 3.87 (3H, dq, $^2J_{H\text{-}H}$ 17.3 Hz, $^3J_{H\text{-}H}$ 7.2 Hz, H^9), 3.78 (9H, s, H^{19}), 3.24 (12H, br m, ring CH_2), 1.73 (9H, d, $^2J_{H\text{-}P}$ 14.9 Hz, H^8), 1.23 (9H, t, $^3J_{H\text{-}H}$ 7.2 Hz, H^{10}); ^{13}C NMR (176 MHz, $CDCl_3$) δ 168.9 (C^{18}), 158.9 (C^{16}), 157.0 (br m, C^6), 154.6 (d, $^1J_{C\text{-}P}$ 157 Hz, C^2), 133.8 (C^{14}), 133.4 (d, $^3J_{C\text{-}P}$ 11 Hz, C^4), 128.2 (d, $^2J_{C\text{-}P}$ 21 Hz, C^3), 127.7 (C^5), 115.1 (C^{13}), 115.0 (C^{15}), 95.6 (C^{12}), 85.3 (C^{11}), 65.2 (C^{17}), 61.3 (d, $^2J_{C\text{-}P}$ 6 Hz, C^9), 60.7 (C^7), 52.5 (C^{19}), 52.2 (6 × ring CH_2), 16.5 (d, $^3J_{C\text{-}P}$ 4 Hz, C^{10}), 13.5 (d, $^1J_{C\text{-}P}$ 104 Hz, C^8); ^{31}P NMR (284 MHz, $CDCl_3$) δ +39.2; (HRMS$^+$) m/z 1285.460 $[M + H]^+$ ($C_{66}H_{76}N_6O_{15}P_3$ requires 1285.458); R_f = 0.43 (silica; CH_2Cl_2/CH_3OH, 87/13 v/v).



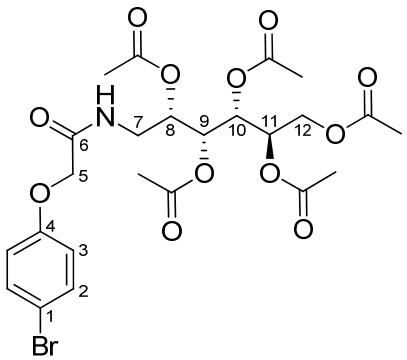
$[Eu.L^1]^{3-}$

The triethyl methylphosphinate ester of L^2 (10 mg, 7.8 μmol) was dissolved in a mixture of CD_3OD/D_2O (1.5 mL, 2:1 v/v) and KOH (6.6 mg, 118 μmol) was added. The solution was stirred at 60 °C under argon for 18 h. The reaction was monitored by ^{1}H-NMR spectroscopy (400 MHz; loss of CH_3CH_2 signals at 4.09, 3.87 and 1.23 ppm and CO_2CH_3 signal at 3.78 ppm) and ^{31}P-NMR spectroscopy (162 MHz; reactant = +39.2 ppm, product = +27.2 ppm). The organic solvent was removed under reduced pressure and the remaining aqueous mixture was neutralized by the addition of HCl (1M). Lyophilization of the solvent gave L^2 as a white solid, which was dissolved in a

mixture of CH_3OH/H_2O (1 mL, 1:1 v/v). $Eu(OAc)_3$ (3 mg, 9.4 μmol) was added and the pH of the solution was adjusted to 5.8 by the addition of HCl (1M). The resulting cloudy mixture was stirred at 65 °C under argon for 18 h. The mixture was cooled to room temperature and the pH adjusted to 7 by the addition of KOH (1M). Lyophilisation of the solvent and purification of the crude material by semi-preparative RP-HPLC [gradient: 5 − 70% methanol in 0.1 M NH_4HCO_3 over 15 min; t_R = 12.8 min] gave **[Eu.L^1]$^{3-}$** as a white solid (4.5 mg, 45%); LRMS (ESI) *m/z* 655 $[M + 2H]^{2+}$; (HRMS$^-$) *m/z* 1307.203 $[M(^{153}Eu) + 2H]^-$ ($C_{57}H_{53}N_6O_{15}P_3{}^{153}Eu$ requires 1307.200); τ_{H2O} = 1.05 ms; Φ^{em}_{MeOH} = 44 ± 15%; ε_{MeOH} (330 nm) = 56,600 M^{-1} cm^{-1} ± 15%.

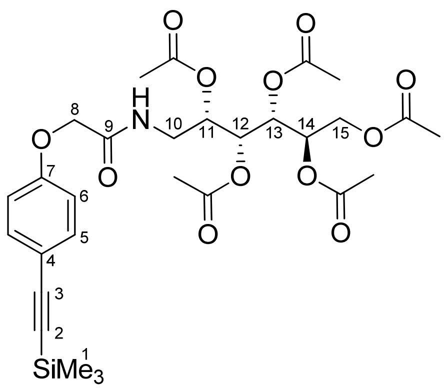


Analytical RP-HPLC of [Eu.**L**^{1}]$^{3-}$: t_R 12.8 min [Gradient: 5 to 70% methanol in NH_4HCO_3 (0.1 M) over 15 min]



(2*S*,3*S*,4*S*,5*R*)-6-(2-(4-Bromophenoxy)acetamido)hexane-1,2,3,4,5-pentayl pentaacetate To a stirred solution of 4-bromophenoxyacetic acid (0.300 g, 1.30 mmol) and D-glucamine pentaacetate (0.282 g, 1.56 mmol) in anhydrous DMF (20 mL) was added *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (0.592 g, 1.56 mmol) and Hünig's base (0.542 mL, 3.90 mmol). The resulting solution was stirred at room temperature under argon for 16 h. The mixture was neutralized by the addition of HCl (0.1 M) and the solvent was removed under reduced

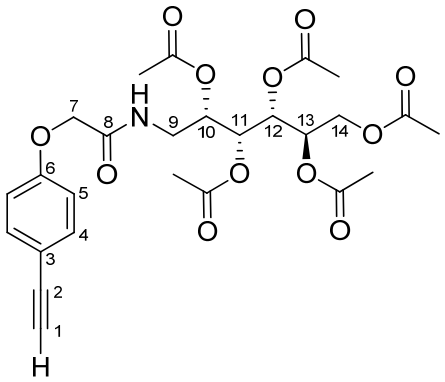
pressure to give a pale yellow solid. The crude material was immediately dissolved in anhydrous pyridine (40 mL), acetic anhydride (6.18 mL, 64.9 mmol) and 4-dimethylaminopyridine (16 mg, 0.130 mmol) were added and the solution was stirred at 35 °C under argon for 16 h. The solvent was removed under reduced pressure followed by azeotropic removal of residual reagents using toluene to give a yellow oil. The residue was partitioned between CH_2Cl_2 (30 mL) and sat. aq. $NaHCO_3$ solution (0.5 M, 30 mL) and the aqueous layer was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic fractions were washed with water (100 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give a yellow oil. Subjection of the crude material to column chromatography (silica, CH_2Cl_2) afforded a white solid (0.481 g, 60%); m.p. 125–126 °C; ^{1}H NMR (700 MHz, $CDCl_3$) δ 7.42 (2H, d, ^3J 9.0 Hz, H^2), 6.85 (1H, t, ^3J 6.0 Hz, CH_2*NH*), 6.81 (2H, d, ^3J 9.0 Hz, H^3), 5.49 (1H, dd, ^3J 6.5, 4.7 Hz, H^{10}), 5.32 (1H, dd, ^3J 5.8, 4.7 Hz, H^9), 5.17 (1H, m, H^8), 5.02 (1H, ddd, ^3J 6.5, 5.5, 3.3 Hz, H^{11}), 4.45 (2H, AB quartet, H^5), 4.24 (1H, dd, ^2J 12.5 Hz, ^3J 3.3 Hz, H^{12}), 4.11 (1H, dd, ^2J 12.5 Hz, ^3J 5.5 Hz, H^{12}), 3.55 (2H, m, H^7), 2.13 (3H, s, $COCH_3$), 2.09 (3H, s, $COCH_3$), 2.07 (3H, s, $COCH_3$), 2.04 (3H, s, $COCH_3$), 2.03 (3H, s, $COCH_3$); ^{13}C NMR (176 MHz, $CDCl_3$) δ 170.7 (*C*OCH_3), 170.5 (*C*OCH_3), 170.2 (*C*OCH_3), 170.0 (*C*OCH_3), 169.9 (*C*OCH_3), 168.3 (NHCO), 156.4 (C^4), 132.8 (C^2), 116.7 (C^3), 114.7 (C^1), 70.2 (C^8), 69.2 (C^9), 69.1 (C^{10}), 69.0 (C^{11}), 67.6 (C^5), 61.6 (C^{12}), 39.4 (C^7), 20.9 (CO*C*H_3), 20.8(4) (CO*C*H_3), 20.8(3) (2 × CO*C*H_3), 20.7 (CO*C*H_3); (HRMS$^+$) *m*/*z* 626.0848 [M + Na]$^+$ ($C_{24}H_{30}NO_{12}BrNa$ requires 626.0849); R_f = 0.29 (silica, CH_2Cl_2).



(2*S*,3*S*,4*S*,5*R*)-6-(2-(4-((trimethylsilyl)ethynyl)phenoxy)acetamido)hexane-1,2,3,4,5-pentayl pentaacetate

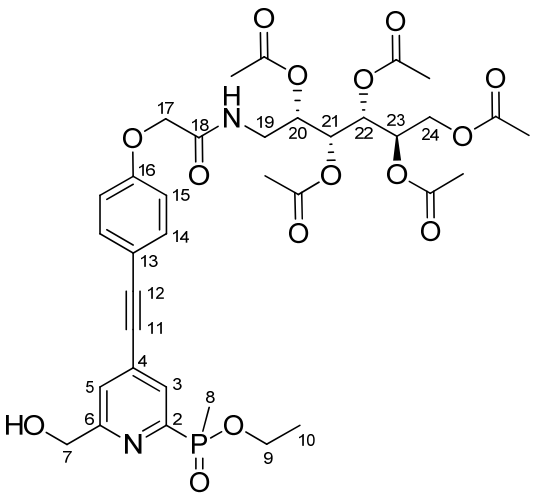
(2*S*,3*S*,4*S*,5*R*)-6-(2-(4-Bromophenoxy)acetamido)hexane-1,2,3,4,5-pentayl pentaacetate (0.290 g, 0.481 mmol) was dissolved in anhydrous THF (6 mL) and the solution was degassed (freeze-thaw cycle) three times. Ethynyltrimethylsilane (82 mg, 0.581 mmol) and triethylamine (0.67 mL, 4.81 mmol) were added and the solution was degassed (freeze-thaw cycle) once more. [1,1-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (39 mg, 0.048 mmol) and CuI (9 mg, 0.048 mmol) were added and the brown solution was stirred at 65 °C under argon for 18 h. The solvent

was removed under reduced pressure and the crude material was purified by column chromatography (silica, hexane/EtOAc, 2:1 v/v), giving a white solid (0.182 g, 63%); m.p. 152–154 °C; ^{1}H NMR (700 MHz, $CDCl_3$) δ 7.42 (2H, d, ^{3}J 8.9 Hz, H^{5}), 6.86 (1H, t, ^{3}J 6.1 Hz, CH_2NH), 6.84 (2H, d, ^{3}J 8.9 Hz, H^{6}), 5.48 (1H, dd, ^{3}J 6.5, 4.8 Hz, H^{13}), 5.33 (1H, dd, ^{3}J 5.8, 4.8 Hz, H^{12}), 5.17 (1H, ddd, ^{3}J 6.8, 5.8, 4.8 Hz, H^{11}), 5.02 (1H, ddd, ^{3}J 6.5, 5.5, 3.4 Hz, H^{14}), 4.47 (2H, AB quartet, H^{8}), 4.25 (1H, dd, ^{2}J 12.5 Hz, ^{3}J 3.4 Hz, H^{15}), 4.11 (1H, dd, ^{2}J 12.5 Hz, ^{3}J 5.5 Hz, H^{15}), 3.55 (2H, m, H^{10}), 2.09 (3H, s, $COCH_3$), 2.08 (3H, s, $COCH_3$), 2.07 (3H, s, $COCH_3$), 2.04 (3H, s, $COCH_3$), 2.03 (3H, s, $COCH_3$), 0.24 (9H, s, H^{1}); ^{13}C NMR (176 MHz, $CDCl_3$) δ 170.7 ($COCH_3$), 170.5 ($COCH_3$), 170.2 ($COCH_3$), 170.0 ($COCH_3$), 169.9 ($COCH_3$), 168.3 (NHCO), 157.2 (C^{7}), 133.9 (C^{5}), 117.2 (C^{4}), 114.7 (C^{6}), 104.6 (C^{3}), 93.5 (C^{2}), 70.2 (C^{11}), 69.2 (C^{12}), 69.1 (C^{13}), 69.0 (C^{14}), 67.3 (C^{8}), 61.6 (C^{15}), 39.4 (C^{10}), 20.9 ($COCH_3$), 20.8(4) ($COCH_3$), 20.8(3) (2 × $COCH_3$), 20.7 ($COCH_3$), 0.15 (C^{1}); (HRMS+) *m/z* 644.2158 $[M + Na]^+$ ($C_{29}H_{39}NO_{12}NaSi$ requires 644.2099); R_f = 0.63 (silica, hexane/EtOAc, 2:1 v/v).



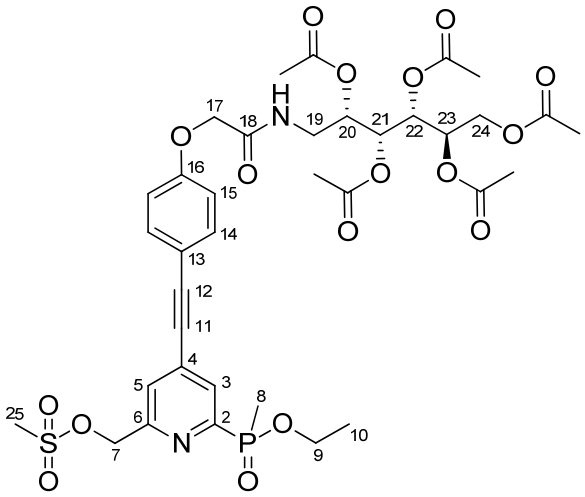
(2*S*,3*S*,4*S*,5*R*)-6-(2-(4-ethynylphenoxy)acetamido)hexane-1,2,3,4,5-pentayl pentaacetate

To a solution of (2*S*,3*S*,4*S*,5*R*)-6-(2-(4-((trimethylsilyl)ethynyl)phenoxy)acetamido)hexane-1,2,3,4,5-pentayl pentaacetate (0.109 g, 1.76 mmol) in anhydrous THF (2 mL) was added triethylamine trihydrofluoride (0.286 mL, 1.76 mmol) and the mixture was stirred at 35 °C under argon for 24 h. The solvent was removed under reduced pressure and the resulting yellow oil was purified by column chromatography (silica, hexane/EtOAc, 1:1 v/v) to give a white solid (75 mg, 78%); m.p. 148–150 °C; (HRMS^{+}) *m/z* 572.1757 $[M + Na]^+$ ($C_{26}H_{31}NO_{12}Na$ requires 572.1744); R_f = 0.45 (silica, hexane/EtOAc, 1:1 v/v).



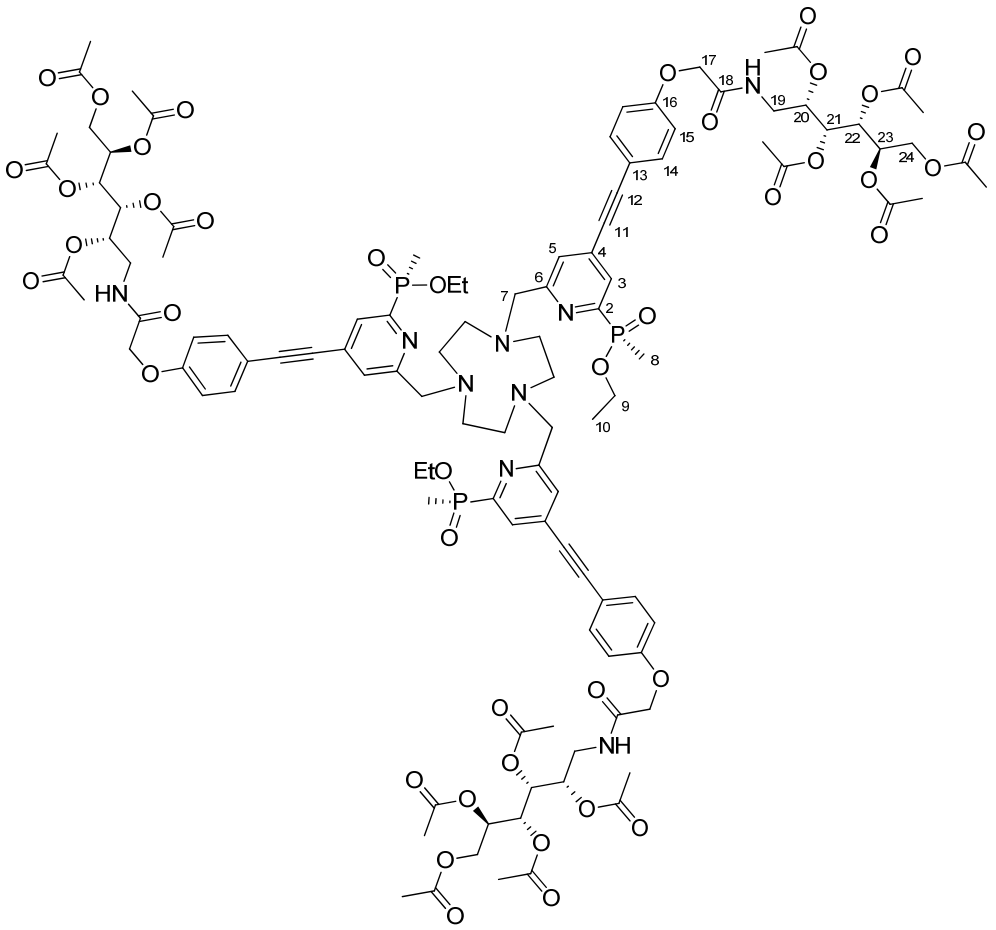
(2*S*,3*S*,4*S*,5*R*)-6-(2-(4-((2-(Ethoxy(methyl)phosphoryl)-6-(hydroxymethyl)pyridin-4-yl)ethynyl)phenoxy)acetamido)hexane-1,2,3,4,5-pentayl pentaacetate

Ethyl (6-(hydroxymethyl)-4-(bromopyridin-2-yl)(methyl)phosphinate (40 mg, 0.136 mmol) was dissolved in anhydrous THF (1 mL) and the solution was degassed (freeze-thaw cycle) three times. (2S,3S,4S,5R)-6-(2-(4-((Trimethylsilyl)ethynyl)phenoxy)acetamido)hexane-1,2,3,4,5-pentayl pentaacetate (75 mg, 0.136 mmol) and triethylamine (0.190 mL, 1.36 mmol) were added and the solution was degassed (freeze-thaw cycle) once more. [1,1-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (11 mg, 0.014 mmol) and CuI (2.6 mg, 0.014 mmol) were added and the brown mixture was stirred at 65 °C under argon for 16 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (silica, CH_2Cl_2/0−3% CH_3OH in 0.5% increments) to afford a colourless oil (71 mg, 68%); ^{1}H NMR (700 MHz, $CDCl_3$) δ 8.02 (1H, d, $^3J_{H\text{-}P}$ 4.6 Hz, H^3), 7.56 (1H, m, H^5), 7.49 (2H, d, $^3J_{H\text{-}H}$ 8.6 Hz, H^{14}), 7.38 (1H, t, $^3J_{H\text{-}H}$ 6.9 Hz, CH_2NH), 6.93 (2H, d, $^3J_{H\text{-}H}$ 8.6 Hz, H^{15}), 5.48 (1H, dd, $^3J_{H\text{-}H}$ 6.4, 4.8 Hz, H^{22}), 5.32 (1H, dd, $^3J_{H\text{-}H}$ 5.8, 4.8 Hz, H^{21}), 5.17 (1H, m, H^{20}), 5.02 (1H, ddd, $^3J_{H\text{-}H}$ 6.5, 5.6, 3.4 Hz, H^{23}), 4.81 (2H, s, H^7), 4.50 (2H, AB quartet, H^{17}), 4.25 (1H, dd, $^2J_{H\text{-}H}$ 12.4 Hz, $^3J_{H\text{-}H}$ 3.4 Hz, H^{24}), 4.11 (1H, dd, $^2J_{H\text{-}H}$ 12.4 Hz, $^3J_{H\text{-}H}$ 5.5 Hz, H^{24}), 3.86 (2H, m, H^9), 3.79 (1H, br s, CH_2OH), 3.55 (2H, m, H^{19}), 2.12 (3H, s, $COCH_3$), 2.09 (3H, s, $COCH_3$), 2.05 (3H, s, $COCH_3$), 2.04 (3H, s, $COCH_3$), 2.02 (3H, s, $COCH_3$), 1.77 (3H, d, $^2J_{H\text{-}P}$ 14.9 Hz, H^8), 1.27 (3H, t, $^3J_{H\text{-}H}$ 6.9 Hz, H^{10}); ^{13}C NMR (176 MHz, $CDCl_3$) δ 170.7 ($COCH_3$), 170.5 ($COCH_3$), 170.2 ($COCH_3$), 170.0 ($COCH_3$), 169.9 ($COCH_3$), 168.1 (NHCO), 160.9 (d, $^3J_{C\text{-}P}$ 19 Hz, C^6), 158.1 (C^{16}), 153.5 (d, $^1J_{C\text{-}P}$ 156 Hz, C^2), 134.0 (C^{14}), 132.9 (d, $^3J_{C\text{-}P}$ 12 Hz, C^4), 127.7 (d, $^2J_{C\text{-}P}$ 10 Hz, C^3), 128.4 (d, $^4J_{C\text{-}P}$ 12 Hz, C^5), 115.5 (C^{13}), 115.1 (C^{15}), 95.4 (C^{12}), 85.8 (C^{11}), 70.2 (C^{20}), 69.2 (C^{21}), 69.1 (C^{22}), 69.0 (C^{23}), 67.3 (C^{17}), 64.2 (s, C^7), 61.6 (C^{24}), 61.3 (d, $^2J_{C\text{-}P}$ 6 Hz, C^9), 39.4 (C^{19}), 20.8(4) ($COCH_3$), 20.8(3) ($COCH_3$), 20.8(0) ($COCH_3$), 20.7(9) ($COCH_3$), 20.6(6) ($COCH_3$), 16.6 (d, $^3J_{C\text{-}P}$ 6 Hz, C^{10}), 13.6 (d, $^1J_{C\text{-}P}$ 105 Hz, C^8); ^{31}P NMR (284 MHz, $CDCl_3$) δ +39.0; *m/z* (HRMS$^+$) 785.2285 $[M + Na]^+$ ($C_{35}H_{43}N_2O_{15}PNa$ requires 785.2299); R_f = 0.24 (silica; CH_2Cl_2/CH_3OH, 95:5 v/v).



(2*S*, 3*S*, 4*S*, 5*R*)-6-(2-(4-((2-(Ethoxy(methyl)phosphoryl)-6-((methylsulfonyloxy)methyl)pyridin-4-yl)ethynyl)phenoxy)acetamido)hexane-1,2,3,4,5-pentayl pentaacetate

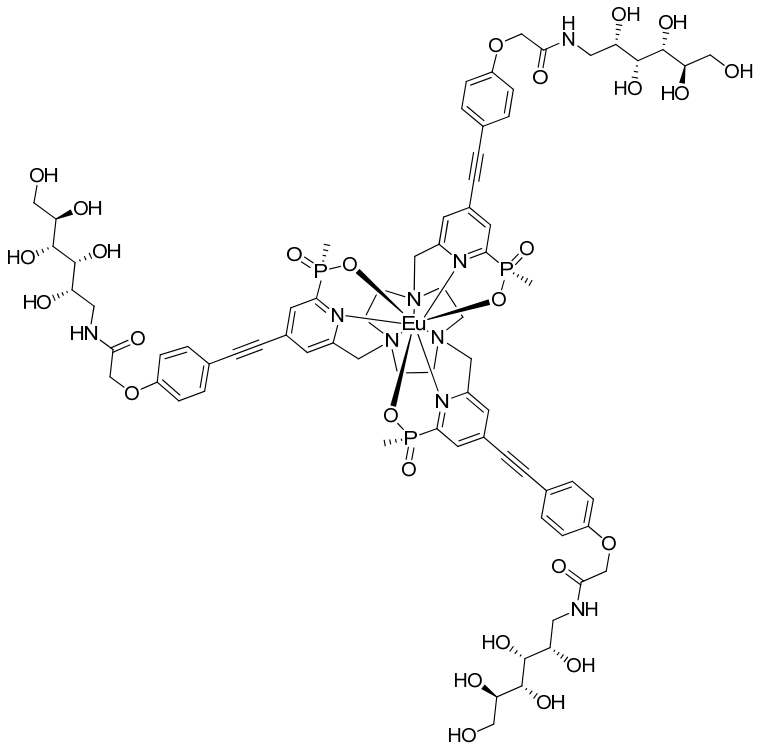
(2*S*,3*S*,4*S*,5*R*)-6-(2-(4-((2-(Ethoxy(methyl)phosphoryl)-6-(hydroxymethyl)pyridin-4-yl)ethynyl)phenoxy)acetamido)hexane-1,2,3,4,5-pentayl pentaacetate (71 mg, 93 μmol) was dissolved in anhydrous THF (1.5 mL) and cooled to 5 °C. Triethylamine (26 μL, 0.186 mmol) and methanesulfonyl chloride (11 μL, 0.140 mmol) were added and the mixture was stirred under argon for 60 min. The progress of the reaction was monitored by TLC [silica; CH_2Cl_2/CH_3OH 9:1 v/v, R_f(product) = 0.44, R_f(reactant) = 0.36]. The solvent was removed under reduced pressure and the residue was partitioned between CH_2Cl_2 (10 mL) and sat. aq. brine solution (10 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL) and the combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to afford the title compound as a colourless oil (78 mg, quant.). The mesylate ester was susceptible to rapid substitution with endogenous chloride, to give the corresponding alkyl chloride compound. As such, the crude material was used immediately in the next step. Any alkyl chloride present reacted in the same manner as the mesylate in the next step, as judged by LC-MS analysis of the reaction mixture. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (1H, d, $^3J_{H\text{-}P}$ 5.6 Hz, H^3), 7.64 (1H, m, H^5), 7.52 (2H, d, $^3J_{H\text{-}H}$ 8.6 Hz, H^{14}), 7.38 (1H, m, CH_2NH), 6.94 (2H, d, $^3J_{H\text{-}H}$ 8.6 Hz, H^{15}), 5.49 (1H, dd, $^3J_{H\text{-}H}$ 6.4, 4.8 Hz, H^{22}), 5.38 (2H, m, H^7), 5.33 (1H, dd, $^3J_{H\text{-}H}$ 5.8, 4.6 Hz, H^{21}), 5.17 (1H, m, H^{20}), 5.02 (1H, ddd, $^3J_{H\text{-}H}$ 6.6, 5.6, 3.4 Hz, H^{23}), 4.50 (2H, m, H^{17}), 4.25 (1H, dd, $^2J_{H\text{-}H}$ 12.5 Hz, $^3J_{H\text{-}H}$ 3.4 Hz, H^{24}), 4.11 (1H, dd, $^2J_{H\text{-}H}$ 12.5 Hz, $^3J_{H\text{-}H}$ 5.6 Hz, H^{24}), 3.87 (2H, m, H^9), 3.55 (2H, m, H^{19}), 3.14 (3H, s, H^{25}), 2.13 (3H, s, $COCH_3$), 2.09 (3H, s, $COCH_3$), 2.06 (3H, s, $COCH_3$), 2.05 (3H, s, $COCH_3$), 2.02 (3H, s, $COCH_3$), 1.77 (3H, d, $^2J_{H\text{-}P}$ 15 Hz, H^8), 1.28 (3H, t, $^3J_{H\text{-}H}$ 7.0 Hz, H^{10}); LRMS (ESI) *m/z* 841 $[M+H]^+$, 1682 $[2M+H]^+$; R_f = 0.44 (silica; CH_2Cl_2/CH_3OH, 9:1 v/v).



Triethyl methylphosphinate ester of L^1

To a solution of (2*S*,3*S*,4*S*,5*R*)-6-(2-(4-((2-(ethoxy(methyl)phosphoryl)-6-((methylsulfonyloxy)methyl)pyridin-4-yl)ethynyl)phenoxy)acetamido)hexane-1,2,3,4,5-pentayl pentaacetate (78 mg, 93 μmol) and 1,4,7-triazacyclononane (4.1 mg, 33 μmol) in anhydrous CH_3CN (1 mL) was added K_2CO_3 (14 mg, 96 μmol) and the mixture was stirred under argon at 60 °C. The progress of the reaction was monitored by LC-MS analysis at regular intervals, which revealed complete consumption of starting material after 5 h. The reaction mixture was cooled to room temperature and the solution decanted from excess potassium salts. The solvent was removed under reduced pressure and the crude material was purified by column chromatography (silica, neat CH_2Cl_2, then incremental steps to CH_2Cl_2/CH_3OH 8:2 v/v) to give the triethyl methylphosphinate ester of **L^1** as a colourless oil (20 mg, 26%); ^{1}H NMR (700 MHz, $CDCl_3$) δ 8.00 (3H, br m, H^3), 7.74 (3H, m, H^5), 7.55 (3H, m, CH_2NH), 7.50 (6H, d, $^3J_{H\text{-}H}$ 8.6 Hz, H^{14}), 6.93 (6H, d, $^3J_{H\text{-}H}$ 8.6 Hz, H^{15}), 5.48 (3H, dd, $^3J_{H\text{-}H}$ 6.4, 4.8 Hz, H^{22}), 5.33 (3H, dd, $^3J_{H\text{-}H}$ 5.8, 4.6 Hz, H^{21}), 5.18 (3H, m, H^{20}), 5.02 (3H, m, H^{23}), 4.49 (6H, AB quartet, H^{17}), 4.25 (3H, dd, $^2J_{H\text{-}H}$ 12.4 Hz, $^3J_{H\text{-}H}$ 3.4 Hz, H^{24}), 4.11 (3H, dd, $^2J_{H\text{-}H}$ 12.4 Hz, $^3J_{H\text{-}H}$ 5.6 Hz, H^{24}), 3.95 (6H, m, H^9), 3.87 (6H, br m, H^7), 3.55 (6H, m, H^{19}), 2.93 (6H, br m, ring CH_2), 2.69 (6H, br m, ring CH_2), 2.12 (9H, s, $COCH_3$), 2.09 (9H, s, $COCH_3$), 2.06 (9H, s, $COCH_3$), 2.05 (9H, s, $COCH_3$), 2.02 (9H, s, $COCH_3$), 1.74 (9H, d, $^2J_{H\text{-}P}$ 15.0 Hz, H^8),

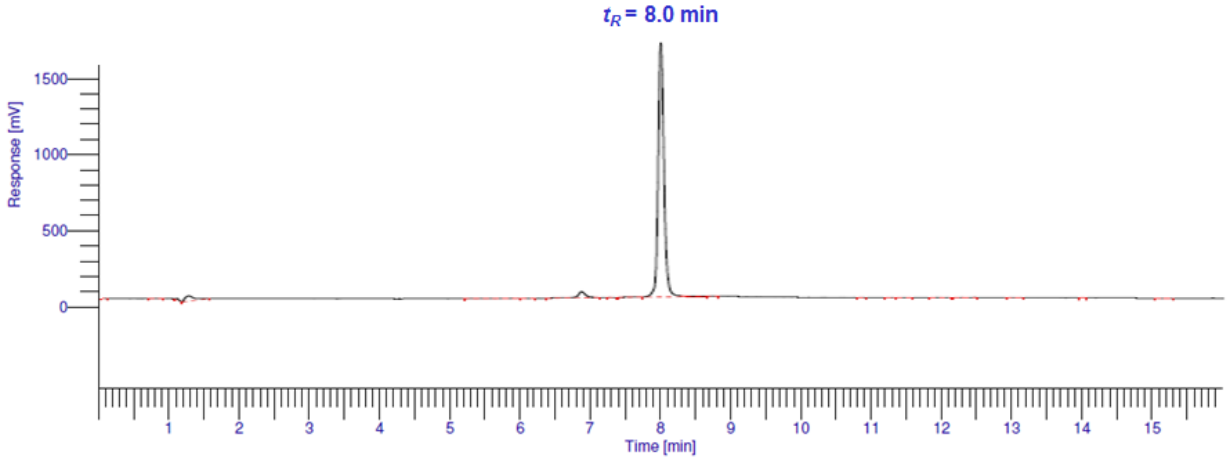
1.25 (9H, t, $^{3}J_{H\text{-}H}$ 7.0 Hz, H^{10}); ^{13}C NMR (176 MHz, $CDCl_3$) δ 170.7 (COCH$_3$), 170.5 (COCH$_3$), 170.2 (COCH$_3$), 170.0 (COCH$_3$), 169.9 (COCH$_3$), 168.0 (NHCO), 158.2 (C^{16}), 157.0 (br m, C^{6}), 154.7 (d, $^{1}J_{C\text{-}P}$ 154 Hz, C^{2}), 134.1 (C^{14}), 133.3 (C^{4}), 128.3 (d, $^{2}J_{C\text{-}P}$ 21 Hz, C^{3}), 127.9 (br m, C^{5}), 115.3 (C^{13}), 115.2 (C^{15}), 96.3 (C^{12}), 85.5 (C^{11}), 70.2 (C^{20}), 69.1(4) (C^{21}), 69.1(1) (C^{22}), 69.0 (C^{23}), 67.3 (C^{17}), 66.4 (C^{9}), 61.6 (C^{24}), 61.2 (C^{7}), 52.0 (6 × ring CH_2), 39.4 (C^{19}), 20.9 (2 × COCH_3), 20.8(2) (COCH_3), 20.8(1) (COCH_3), 20.7 (COCH_3), 16.6 (d, $^{3}J_{C\text{-}P}$ 6 Hz, C^{10}), 13.6 (d, $^{1}J_{C\text{-}P}$ 103 Hz, C^{8}); ^{31}P NMR (284 MHz, $CDCl_3$) δ +38.8; *m/z* (HRMS^{+}) 1192.904 $[M+H+Na]^{2+}$ ($C_{111}H_{139}N_9O_{42}P_3Na$ requires 1192.906); R_f = 0.52 (silica; CH_2Cl_2/CH_3OH, 85:15 v/v).



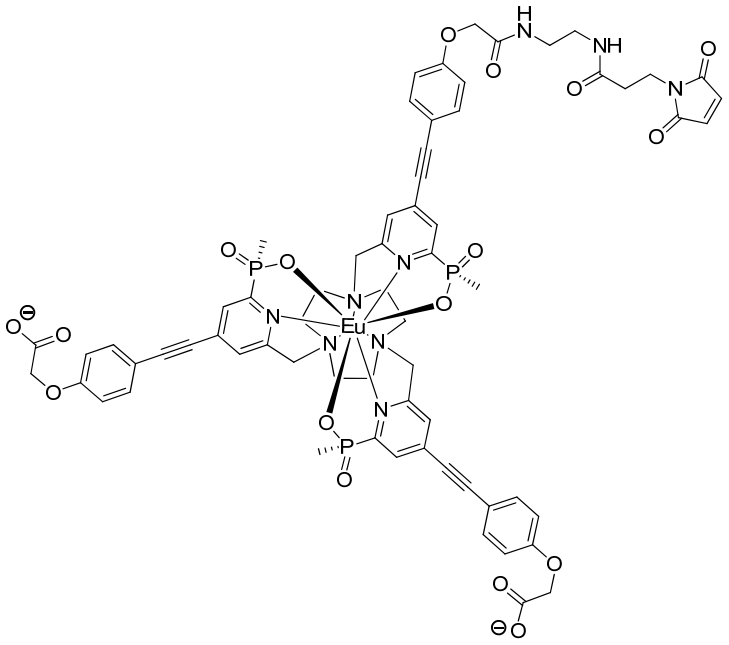
[Eu.L^{2}]

The triethyl methylphosphinate ester of L^{2} (17 mg, 7.2 μmol) was dissolved in a mixture of CD_3OD/D_2O (1.5 mL, 2:1 v/v) and KOH (8 mg, 0.15 mmol) was added. The solution was stirred at 60 °C under argon for 7 h. The reaction was monitored by ^{1}H-NMR spectroscopy (400 MHz; loss of CH_3CH_2 peak at 3.95 and 1.25 ppm and CH_3CO peaks at 2.12, 2.09, 2.06, 2.05 and 2.02 ppm) and ^{31}P-NMR spectroscopy (162 MHz; reactant = +38.8 ppm, product = +28.5 ppm). The organic solvent was removed under reduced pressure and remaining aqueous mixture was neutralized by the addition of HCl (1M). Lyophilization of the solvent gave the phosphinate as an off-white solid, which was immediately dissolved in a mixture of H_2O/CH_3OH (2 mL, 1:1 v/v). $Eu(OAc)_3$ (2.8 mg, 8.6 μmol) was added, and the resulting cloudy mixture was stirred at 65 °C under argon for 18 h. The mixture was cooled to room temperature and the precipitate was condensed by centrifuge and the supernate was decanted. The precipitate was triturated with water (3 × 5 mL) and the combined

supernates were neutralized by the addition of KOH (1M) and lyophilized to give a white solid. Purification of the crude material by semi-preparative RP-HPLC [gradient: 10 − 100% methanol in water over 10 min; t_R = 8.0 min] gave [**Eu.L^2**] as a white solid (6 mg, 45%); LRMS (ESI) *m/z* 1798 [M(^{153}Eu) + H]$^+$, 900 [M(^{153}Eu) + 2H]$^{2+}$; (HRMS$^+$) *m/z* 899.7373 [M(^{153}Eu)+ 2H]$^{2+}$ ($C_{75}H_{95}N_9O_{27}P_3{}^{153}$Eu requires 899.7381); τ_{H2O} =1.07 ms; Φ^{em}_{MeOH} = 50 ± 15%; ε_{MeOH} (328 nm) =56,500 M^{-1} cm^{-1}.

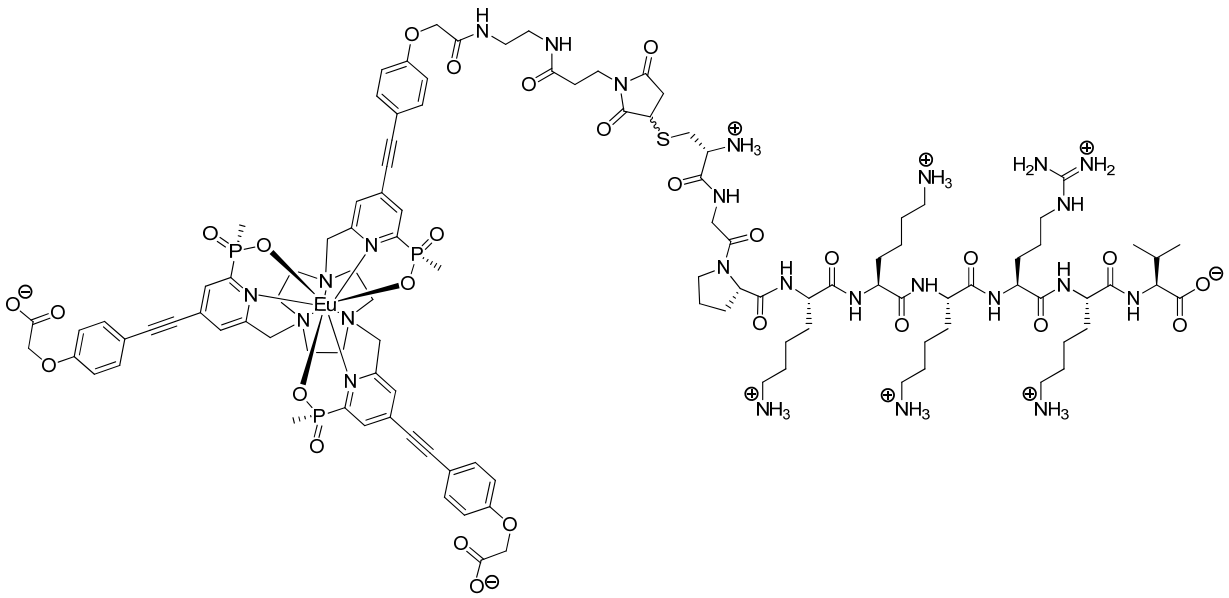


Analytical RP-HPLC of [Eu.**L^2**]: t_R 8.0 min [Gradient: 10 to 100% methanol in water (0.1% formic acid) over 10 min]



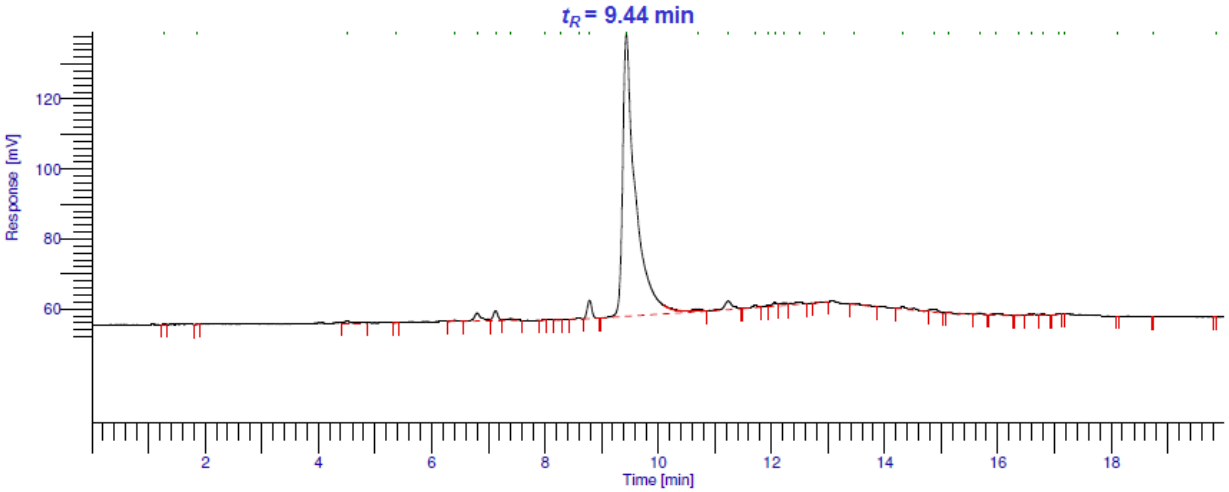
Mono-maleimide conjugate of $[Eu.L^1]^{3-}$

To a stirred solution of $[Eu.L^1]^{3-}$ (1 mg, 760 nmol) in anhydrous DMSO (50 µL) was added PyBOP (0.13 mg, 250 nmol) and *N*,*N*-di*iso*propylethylamine (0.13 µL, 760 nmol). The mixture was stirred at room temperature under argon for 5 min. A solution of the 2-aminoethyl derivative of N-carboxypropylmaleimide (0.16 mg, 760 nmol) in anhydrous DMSO (50 µL) was added and stirring continued for 72 h. LC-MS analysis revealed complete consumption of starting material at this time. The reaction mixture was purified by semi-preparative RP-HPLC [gradient: 10 − 50% methanol in water (0.1% formic acid) over 6 min, then 50 − 70% methanol in water (0.1% formic acid) over 10 min; t_R = 9.4 min] to afford the desired mono-maleimide conjugate of $[Eu.L^1]^{3-}$ as a white solid (0.47 mg, 41%); MS (ESI) *m*/*z* 751 $[M(^{153}Eu) + 4H]^{2+}$; (HRMS$^+$) *m*/*z* 751.6664 $[M(^{153}Eu) + 4H]^{2+}$ ($C_{66}H_{67}N_9O_{17}P_3{}^{153}Eu$ requires 751.6545).

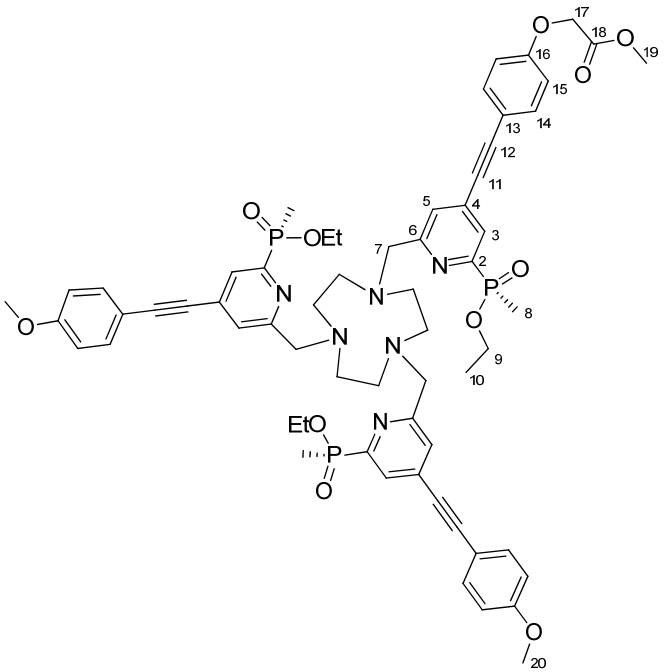


[Eu.L^3]$^{3+}$

To a stirred solution of the mono-maleimide conjugate of **[Eu.L^1]$^{3-}$** (0.20 mg, 133 nmol) in anhydrous DMSO (50 µL) was added a solution of the peptide H_2N-CGPKKKRKV-CO_2H (0.26 mg, 200 µmol) in phosphate buffer (20 µL, pH 7, 50 mM) and the resulting mixture was stirred at room temperature under argon for 15 h. LC-MS analysis at this time revealed quantitative conversion of starting material. The reaction mixture was purified by semi-preparative RP-HPLC [gradient: 5% methanol for 1 min, then 5−100% methanol in water (0.1% formic acid) over 15 min; t_R = 9.9 min] to give the desired NLS peptide conjugate [**Eu.L^3**]$^{3+}$ as a white solid (0.21 mg, 62%); LRMS (ESI) *m/z* 849 [M(^{153}Eu)]$^{3+}$; (HRMS$^+$) *m/z* 848.9863 [M(^{153}Eu)]$^{3+}$ ($C_{111}H_{154}N_{25}O_{27}P_3S^{153}Eu$ requires 848.9871); τ_{MeOH} = 1.14 ms; Φ^{em}_{H2O} = 44 ± 15%; ε_{H2O} (328 nm) = 58,200 M^{-1} cm^{-1}.

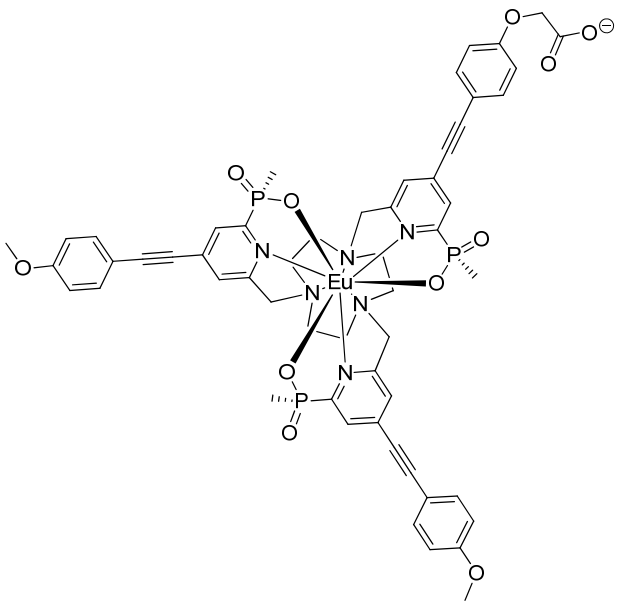


Analytical RP-HPLC of [Eu.**L^3**]$^{3+}$: t_R 9.44 min [Gradient: 5 to 100% methanol in water (0.1% formic acid) over 15 min]



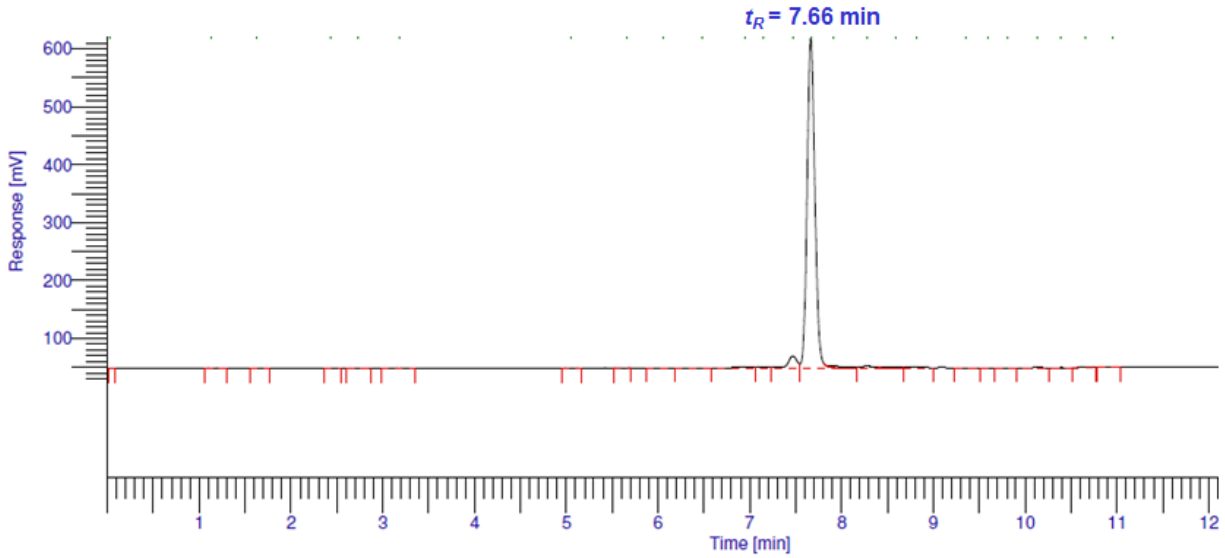
Triethyl methylphosphinate ester of L^4

To a solution of methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-((methylsulfonyloxy)methyl)pyridin-4 yl)ethynyl)phenoxy)acetate (80 mg, 0.102 mmol) and the secondary amine bearing two methoxyphenylalkynylpyridylphosphinate groups (54 mg, 0.112 mmol) in anhydrous CH_3CN (2 mL) was added K_2CO_3 (42 mg, 0.306 mmol) and the mixture was stirred under argon at 60 °C for 15 h. The reaction mixture was cooled to room temperature and the solution decanted from excess potassium salts. The solvent was removed under reduced pressure and the crude material was purified by column chromatography (silica, CH_2Cl_2/0 – 20% CH_3OH in 1% increments) to give the triethyl methylphosphinate ester of $\mathbf{L^4}$ as a colourless oil (71 mg, 58%); ^{1}H NMR (700 MHz, $CDCl_3$) δ 7.88 (3H, d, $^3J_{H\text{-}P}$ 5.7 Hz, H^3), 7.52 (3H, br s, H^5), 7.41 (2H, d, $^3J_{H\text{-}H}$ 8.5 Hz, H^{14}), 7.40 (4H, d, $^3J_{H\text{-}H}$ 8.6 Hz, $H^{14'}$), 6.84 (2H, d, $^3J_{H\text{-}H}$ 8.5 Hz, H^{15}), 6.83 (4H, d, $^3J_{H\text{-}H}$ 8.6 Hz, $H^{15'}$), 4.60 (2H, s, H^{17}), 4.13 (6H, br m, H^7), 4.05 (3H, m, H^9), 3.86 (3H, m, H^9), 3.77 (6H, s, H^{20}), 3.75 (3H, s, H^{19}), 3.29 (6H, br m, ring CH_2), 3.10 (6H, br m, ring CH_2), 1.71 (9H, d, $^2J_{H\text{-}P}$ 14.9 Hz, H^8), 1.21 (9H, t, $^3J_{H\text{-}H}$ 7.0 Hz, H^{10}); ^{13}C NMR (176 MHz, $CDCl_3$) δ 169.0 (C^{18}), 160.8 (C^{16}), 158.8 ($C^{16'}$), 156.9 (br m, C^6), 154.2 (d, $^1J_{C\text{-}P}$ 159 Hz, C^2), 133.8 ($C^{14'}$), 133.7 (C^{14}), 133.4 (br m, C^4), 127.9 (d, $^2J_{C\text{-}P}$ 22 Hz, C^3), 127.4 (C^5), 114.9 ($C^{15'}$), 114.3 (C^{15}), 113.4 (C^{13}), 97.1 (C^{12}), 96.6 ($C^{12'}$), 85.1 (C^{11}), 84.9 ($C^{11'}$), 65.1 (C^{17}), 61.5 (d, $^2J_{C\text{-}P}$ 6 Hz, C^9), 60.1 (br s, C^7), 55.4 (C^{20}), 52.4 (C^{19}) 51.3 (ring CH_2), 16.4 (d, $^3J_{C\text{-}P}$ 6 Hz, C^{10}), 13.5 (d, $^1J_{C\text{-}P}$ 104 Hz, C^8); ^{31}P NMR (284 MHz, $CDCl_3$) δ +39.1; *m/z* (HRMS$^+$) 1169.443 $[M + H]^+$ ($C_{62}H_{72}N_6O_{11}P_3$ requires 1169.447); R_f = 0.28 (silica; CH_2Cl_2/CH_3OH 9:1 v/v).

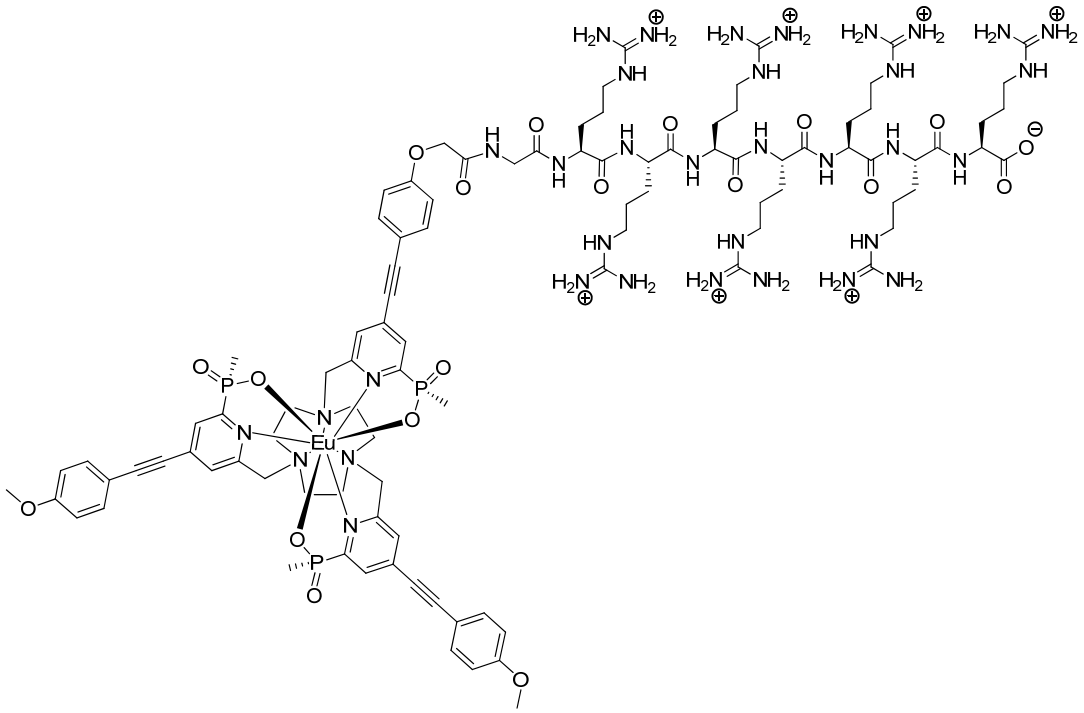


[Eu·L^4]$^-$

The triethyl methylphosphinate ester of L^4 (30 mg, 26 μmol) was dissolved in CH_3OH/H_2O (2.5 mL, 2:1 v/v) and KOH (5 mg, 0.10 mmol) was added. The solution was stirred at 60 °C under argon for 24 h. The reaction was monitored by ^{1}H-NMR spectroscopy (400 MHz; loss of C*H*$_3$C*H*$_2$ peaks at 3.86 and 1.21 ppm) and ^{31}P-NMR spectroscopy (284 MHz; reactant = +39.1 ppm, product = +27.3 ppm). The organic solvent was removed under reduced pressure and the remaining aqueous mixture was neutralized by the addition of HCl (1M). Lyophilization of the solvent gave L^3 as a white solid, which was dissolved in a mixture of H_2O/CH_3OH (2 mL, 1:1 v/v). $Eu(OAc)_3$ (2.8 mg, 8.6 μmol) was added and the resulting cloudy mixture was stirred at 65 °C under argon for 18 h. The solvent was removed under reduced pressure and the residue was suspended in methanol (2 mL) and the white precipitate was condensed to a pellet by centrifugation. The supernate was isolated and the precipitate was triturated with methanol (3 × 2 mL) and the combined supernates were concentrated under reduced pressure to give the crude product as an off-white solid. Purification of the crude material by semi-preparative RP-HPLC [gradient: 10 − 100% methanol in 0.1 M NH_4HCO_3 over 15 min; t_R = 11.8 min] gave **[Eu·L^4]$^-$** as a white solid (13 mg, 42%); LRMS (ESI) *m*/*z* 611 [M(^{153}Eu) + 2H]$^{2+}$; (HRMS$^+$) *m*/*z* 611.1247 [M(^{153}Eu) + 2H]$^{2+}$ ($C_{55}H_{55}N_6O_{11}P_3{}^{153}Eu$ requires 611.1220); τ_{H2O} = 1.04 ms; Φ^{em}_{MeOH} = 49 ± 15%; ε_{MeOH} (330 nm) = 60,400 M^{-1} cm^{-1}.

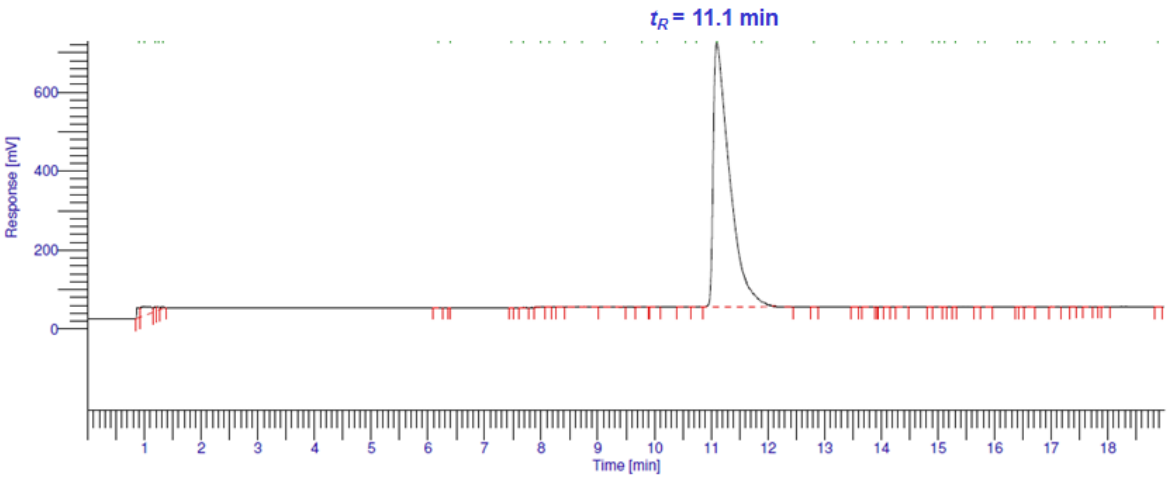


Analytical RP-HPLC of [Eu.$\mathbf{L}^4$]$^-$: t_R 7.66 min [Gradient: 40 to 100% MeOH in water (0.05% formic acid) over 10 min]

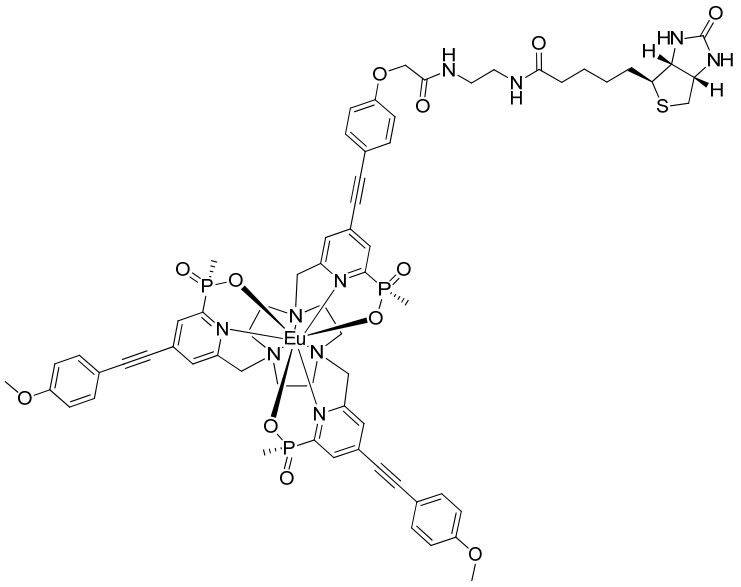


[Eu·L^5]$^{6+}$

To a stirred solution of **[Eu.L^4]$^-$** (1.0 mg, 0.82 μmol) in anhydrous DMSO (150 μL) was added PyBOP (0.65 mg, 1.25 μmol) and *N*,*N*-di*iso*propylethylamine (0.3 μL, 1.65 μmol). The mixture was stirred at room temperature under argon for 5 min. A solution of the peptide H_2N-GRRRRRRR-CO_2H (1.0 mg, 0.86 μmol) in anhydrous DMSO (50 μL) was added and the resulting solution was stirred under argon for 24 h. LC-MS analysis revealed complete consumption of starting material at this time. The reaction mixture was purified by semi-preparative RP-HPLC [gradient: 5 − 100% methanol in 0.1 M NH_4HCO_3 over 15 min; t_R = 9.7 min] to give **[Eu.L^5]$^{6+}$** as a white solid (1.2 mg, 62%); MS (ESI) *m/z* 791 [M(^{153}Eu) − 3H]$^{3+}$; (HRMS$^+$) *m/z* 790.9938 [M(^{153}Eu) − 3H]$^{3+}$ ($C_{99}H_{144}N_{35}O_{19}P_3{}^{153}Eu$ requires 790.9940); τ_{MeOH} = 1.12 ms; Φ^{em}_{H2O} = 50 ± 15%; ε_{MeOH} (330 nm) = 56,400 M^{-1} cm^{-1}.

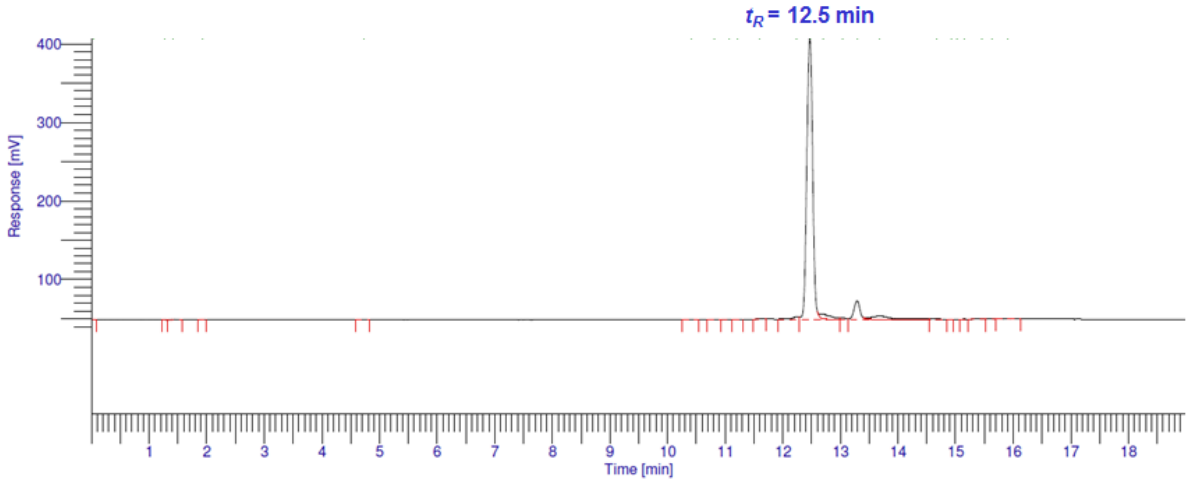


Analytical RP-HPLC of [Eu.**L**^{5}]$^{6+}$: t_R 11.1 min [Gradient: 5 to 100% methanol in water (0.1% formic acid) over 15 min]

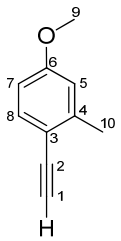


[Eu.L^6]

[Eu.L^4]$^-$ (1.0 mg, 0.82 µmol) was dissolved in anhydrous DMF (150 µL) and PyBOP (0.65 mg, 1.25 µmol) was added, followed by *N*,*N*-diisopropylethylamine (0.3 µL, 1.65 µmol). The mixture was stirred at room temperature under argon for 5 min. A solution of 2-aminoethyl biotin-pentanamide trifluoroacetate salt (0.35 mg, 1.23 µmol) in anhydrous DMF (50 µL) was added and stirring continued for 3 h. Analysis by LC-MS revealed complete conversion of starting material at this time. The reaction mixture was purified by semi-preparative RP-HPLC [gradient: 5 − 100% methanol in water (0.1% formic acid) over 15 min; t_R = 12.0 min] to give [**Eu.L^6**] as a white solid (1.0 mg, 82%); LRMS (ESI) *m*/*z* 745 [M(^{153}Eu) + 2H]$^{2+}$; (HRMS$^+$) *m*/*z* 745.1907 [M(^{153}Eu) + 2H]$^{2+}$ ($C_{67}H_{76}N_{10}O_{12}P_3{}^{153}$Eu requires 745.1899); τ_{H2O} =1.07 ms; Φ^{em}_{MeOH} = 48 ± 15%; ε_{MeOH} (330 nm) = 59,050 M^{-1} cm^{-1}.

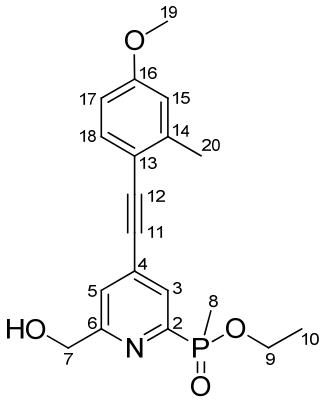


Analytical RP-HPLC of [Eu.**L**^{7}]: t_R 12.5 min [Gradient: 5 to 100% methanol in water (0.1% formic acid) over 15 min]



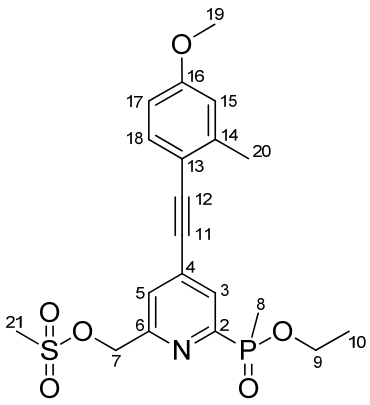
1-Ethynyl-4-methoxy-2-methylbenzene

3-Methyl-4-bromoanisole (0.200 g, 0.995 mmol) was dissolved in anhydrous THF (7 mL) and the solution was degassed (freeze-thaw cycle) three times. Ethynyltrimethylsilane (0.15 mL, 1.09 mmol) and triethylamine (0.69 mL, 4.98 mmol) were added and the solution was degassed (freeze-thaw cycle) once more. [1,1-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (83 mg, 99.5 μmol) and CuI (19 mg, 99.5 μmol) were added and the resulting brown solution was stirred at 65 °C under argon for 24 h. The solvent was removed under reduced pressure and the resulting brown oil was purified by column chromatography (silica, hexane/10 – 50% CH_2Cl_2 in 5% increments) to give (4-methoxy-2-methylphenylethynyl)trimethylsilane as a yellow oil (120 mg, 55%); R_f = 0.46 (silica; hexane/ CH_2Cl_2, 2:1 v/v). This compound (120 mg, 0.550 mmol) was immediately dissolved in anhydrous THF (2 mL) and triethylammonium dihydrofluoride (0.870 mL, 5.50 mmol) was added. The mixture was stirred at 35 °C under argon for 24 h. The solvent was removed under reduced pressure to give a yellow oil which was subjected to column chromatography (silica, hexane/CH_2Cl_2 3:1 v/v), to afford 1-ethynyl-4-methoxy-2-methylbenzene as a colourless oil (68 mg, 85%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (1H, d, $^3J_{H\text{-}H}$ 8.6 Hz, H^8), 6.74 (1H, d, $^4J_{H\text{-}H}$ 2.6 Hz, H^5), 6.68 (1H, dd, $^3J_{H\text{-}H}$ 8.6 Hz, $^4J_{H\text{-}H}$ 2.6 Hz, H^7), 3.80 (3H, s, H^{10}), 3.19 (1H, s, H^1), 2.43 (3H, s, H^9); ^{13}C NMR (151 MHz, $CDCl_3$) δ 160.0 (C^6), 142.7 (C^4), 134.0 (C^8), 115.2 (C^5), 114.3 (C^3), 111.3 (C^7), 82.7 (C^2), 79.6 (C^1), 55.4 (C^9), 21.0 (C^{10}); LRMS (ESI) *m/z* 145 $[M - H]^-$; ($HRMS^+$) *m/z* 145.0667 $[M - H]^-$ ($C_{10}H_9O$ requires 145.0653); R_f = 0.46 (silica; hexane/ CH_2Cl_2, 2:1 v/v).



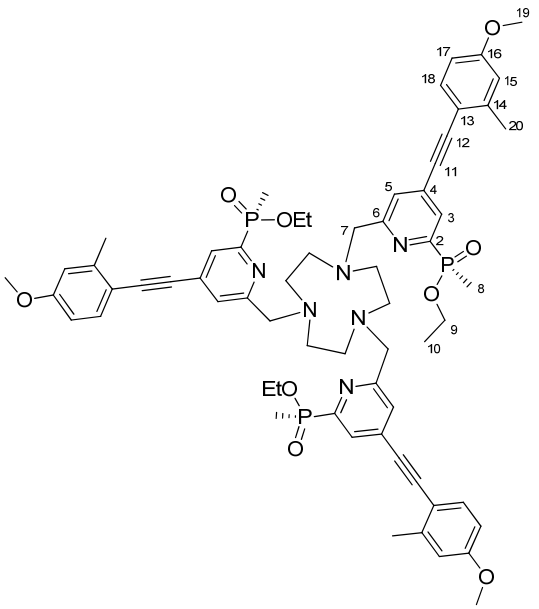
Methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-(hydroxymethyl)pyridin-4-yl)ethynyl)-4-methoxy-2-methylbenzene

To a degassed solution of ethyl (6-(hydroxymethyl)-4-(bromopyridin-2-yl)(methyl)phosphinate (80 mg, 0.273 mmol) in anhydrous THF (2 mL) was added 1-ethynyl-4-methoxy-2-methylbenzene (44 mg, 0.300 mmol) and triethylamine (0.190 mL, 1.75 mmol), and the solution was degassed (freeze-thaw cycle) three times. [1,1-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (23 mg, 0.027 mmol) and CuI (5 mg, 0.027 mmol) were added and the resulting brown solution was stirred at 65 °C under argon for 18 h. The solvent was removed under reduced pressure and the brown residue was purified by column chromatography (silica, CH_2Cl_2/0−2% CH_3OH in 0.5% increments) to afford methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-(hydroxymethyl)pyridin-4-yl)ethynyl)-4-methoxy-2-methylbenzene as a yellow oil (80 mg, 82%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (1H, br s, H^3), 7.45 (1H, br s, H^5), 7.44 (1H, d, $^3J_{H\text{-}H}$ 8.6 Hz, H^{18}), 6.78 (1H, s, H^{15}), 6.73 (1H, d, $^3J_{H\text{-}H}$ 8.6 Hz, H^{17}), 4.83 (2H, br s, H^7), 4.14 (1H, m, H^9), 3.89 (1H, m, H^9), 3.80 (3H, s, H^{19}), 3.62 (1H, br s, CH_2OH), 2.48 (3H, s, H^{20}), 1.81 (3H, m, H^8), 1.30 (3H, m, H^{10}); ^{13}C NMR (151 MHz, $CDCl_3$) δ 160.7 (d, $^3J_{C\text{-}P}$ 19 Hz, C^6), 158.8 (C^{16}), 153.3 (d, $^1J_{C\text{-}P}$ 156 Hz, C^2), 134.1 (C^{18}), 133.4 (d, $^3J_{C\text{-}P}$ 12 Hz, C^4), 128.1 (d, $^2J_{C\text{-}P}$ 21 Hz, C^3), 124.1 (C^5), 115.4 (C^{15}), 113.8 (C^{13}), 111.7 (C^{17}), 95.3 (C^{12}), 89.1 (C^{11}), 64.1 (C^7), 61.3 (d, $^2J_{C\text{-}P}$ 5 Hz, C^9), 55.4 (C^{19}), 21.1 (C^{20}), 16.5 (d, $^3J_{C\text{-}P}$ 4 Hz, C^{10}), 13.3 (d, $^1J_{C\text{-}P}$ 104 Hz, C^8), quaternary carbon signal C^{14} is obscured or overlapping; ^{31}P NMR (243 MHz, $CDCl_3$) δ +39.2; *m/z* (HRMS$^+$) 360.1371 $[M + H]^+$ ($C_{19}H_{23}NO_4P$ requires 360.1365); R_f = 0.40 (silica; CH_2Cl_2/CH_3OH 9:1 v/v).



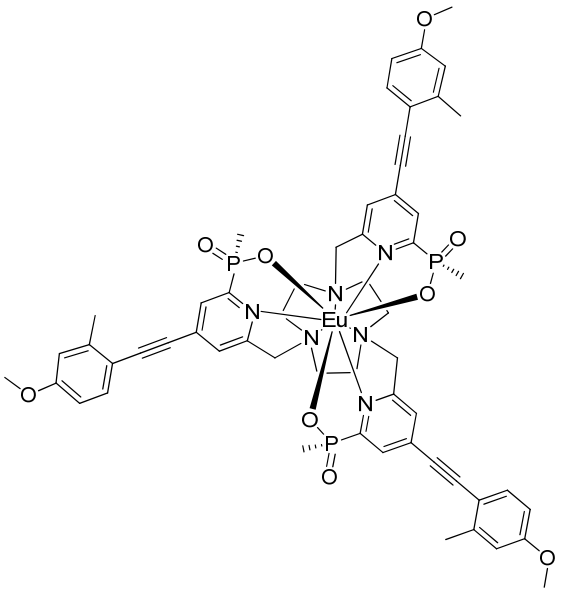
Methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-((methylsulfonyloxy)methyl)pyridin-4-yl)ethynyl)-4-methoxy-2-methylbenzene

Methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-(hydroxymethyl)pyridin-4-yl)ethynyl)-4-methoxy-2-methylbenzene (65 mg, 0.181 mmol) was dissolved in anhydrous THF (1.8 mL) and cooled to 5 °C. Triethylamine (50 μL, 0.361 mmol) and methanesulfonyl chloride (21 μL, 0.272 mmol) were added and the mixture was stirred under argon for 30 min. The progress of the reaction was monitored by TLC [silica; CH_2Cl_2/CH_3OH 9:1 v/v, R_f(product) = 0.51, R_f(reactant) = 0.40]. The solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2 (20 mL) and washed with sat. aq. brine solution (20 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL) and the combined organic layers were dried ($MgSO_4$), and concentrated under reduced pressure to give methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-((methylsulfonyloxy)methyl)pyridin-4-yl)ethynyl)-4-methoxy-2-methylbenzene as a yellow oil (79 mg, quant.), which was used immediately in the next step; LRMS (ESI) *m*/*z* 438 $[M+H]^+$, 875 $[2M+H]^+$; R_f = 0.51 (silica; CH_2Cl_2/CH_3OH 9:1 v/v).



Triethyl methylphosphinate ester of L^7

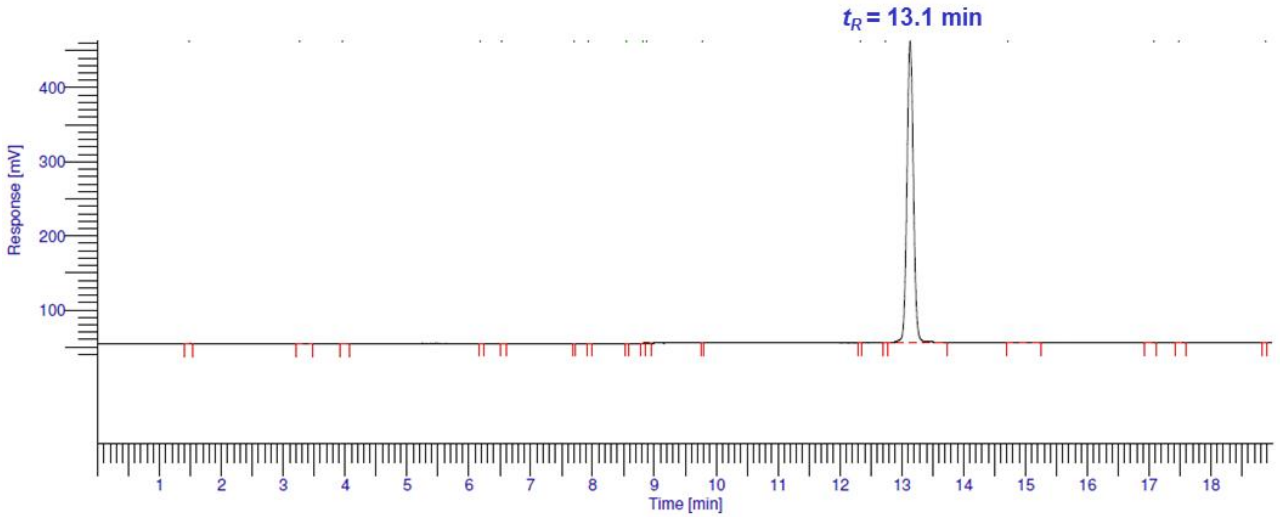
To a solution of methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-((methylsulfonyloxy)methyl)pyridin-4-yl)ethynyl)-4-methoxy-2-methylbenzene (79 mg, 0.181 mmol) and 1,4,7-triazacyclononane (7.8 mg, 0.060 mmol) in anhydrous CH_3CN (1.8 mL) was added K_2CO_3 (25 mg, 0.181 mmol) and the mixture was stirred under argon at 60 °C. The progress of the reaction was monitored by LC-MS analysis at regular intervals, which revealed complete consumption of starting material after 21 h. The reaction mixture was cooled to room temperature and the solution decanted from excess potassium salts. The solvent was removed under reduced pressure and the crude material was subjected to column chromatography (silica, CH_2Cl_2/0 – 15% CH_3OH in 1% increments) to give the triethyl methylphosphinate ester of **L^7** as a colourless oil (34 mg, 49%); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.98 (3H, d, $^2J_{H\text{-}P}$ 5.8 Hz, H^3), 7.68 (3H, s, H^5), 7.40 (3H, d, $^3J_{H\text{-}H}$ 8.5 Hz, H^{18}), 6.76 (3H, d, $^4J_{H\text{-}H}$ 2.7 Hz, H^{15}), 6.71 (3H, dd, $^3J_{H\text{-}H}$ 8.5 Hz, $^4J_{H\text{-}H}$ 2.7 Hz, H^{17}), 4.10 (3H, m, H^9), 3.94 (6H, br s, H^7), 3.86 (3H, m, H^9), 3.81 (9H, s, H^{19}), 2.96 (12H, br m, ring CH_2), 2.48 (9H, s, H^{20}), 1.75 (9H, d, $^2J_{H\text{-}P}$ 15 Hz, H^8), 1.24 (9H, t, $^3J_{H\text{-}H}$ 6.8 Hz, H^{10}); ^{13}C NMR (151 MHz, $CDCl_3$) δ 160.7 (C^{16}), 153.9 (d, $^1J_{C\text{-}P}$ 156 Hz, C^2), 134.0 (C^{18}), 127.5 (d, $^2J_{C\text{-}P}$ 21 Hz, C^3), 124.1 (C^5), 115.4 (C^{15}), 113.8 (C^{13}), 111.7 (C^{17}), 95.0 (C^{12}), 89.3 (C^{11}), 64.0 (C^7), 61.1 (d, $^2J_{C\text{-}P}$ 6 Hz, C^9), 55.8 (6 × ring CH_2), 55.4 (C^{19}), 21.1 (C^{20}), 16.6 (d, $^3J_{C\text{-}P}$ 5 Hz, C^{10}), 13.5 (d, $^1J_{C\text{-}P}$ 104 Hz, C^8), quaternary carbon signals C^4, C^6, C^{14} and are obscured or overlapping; ^{31}P NMR (243 MHz, $CDCl_3$) δ +40.0; *m/z* (HRMS+) 1153.486 $[M + H]^+$ ($C_{63}H_{76}N_6O_9P_3$ requires 1153.489); R_f = 0.46 (silica; CH_2Cl_2/CH_3OH, 85/15 v/v).



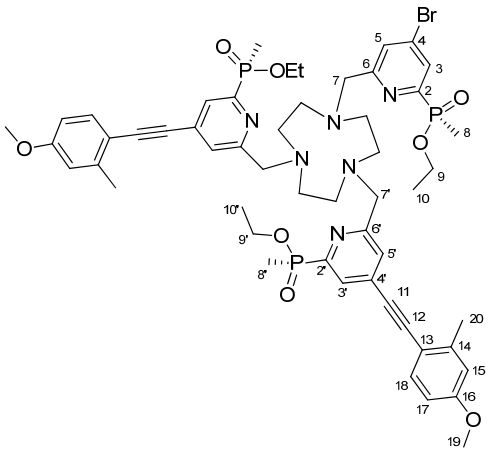
[Eu.L^7]

The triethyl methylphosphinate ester of **L^7** (22 mg, 19.1 μmol) was dissolved in a mixture of CH_3OH/H_2O (2 mL, 1:1 v/v) and KOH (2.8 mg, 50.0 μmol) was added. The cloudy mixture was stirred at 60 °C under argon for 18 h, at which time the solution became transparent. The reaction was monitored by ^{1}H-NMR spectroscopy (400 MHz; loss of C*H*$_3$C*H*$_2$ signals at 4.10, 3.86 and 1.24 ppm) and ^{31}P-NMR spectroscopy (162 MHz; reactant = +40.0 ppm, product = +25.8 ppm). The methanol was removed under reduced pressure and the residual aqueous solution was neutralized by the addition of HCl (1M). Lyophilization of the solvent gave **L^7** as a white solid (34 mg, quant.); (HRMS$^+$) *m*/*z* 1107.349 $[M + K]^+$ ($C_{57}H_{63}N_6O_9P_3K$ requires 1107.350).

The crude ligand **L^7** was dissolved in a mixture of CH_3OH/H_2O (3 mL, 1:1 v/v) and the pH of the solution was adjusted to 5.8 by the dropwise addition of HCl (1M). $Eu(OAc)_3$ (6.5 mg, 19.1 μmol) was added to give a cloudy mixture, which was stirred at 65 °C under argon for 24 h. The mixture was cooled to room temperature and the pH adjusted to 7 by the dropwise addition of KOH (1M). Lyophilisation of the solvent and subjection of the crude material to semi-preparative RP-HPLC [gradient: 50 − 100% methanol in water (0.1% formic acid) over 15 min; t_R = 10.8 min] gave **[Eu.L^7]** as a white solid (12.5 mg, 54%); LRMS (ESI) *m*/*z* 1219 $[M(^{153}Eu) + H]^+$, 610 $[M(^{153}Eu) + 2H]^{2+}$; (HRMS$^+$) *m*/*z* 1219.289 $[M(^{153}Eu) + H]^+$ ($C_{57}H_{61}N_6O_9P_3{}^{153}Eu$ requires 1219.286); τ_{MeOH} = 1.14 ms; Φ^{em}_{MeOH} = 54 ± 15%; ε_{MeOH} (340 nm) = 62,000 M^{-1} cm^{-1}.



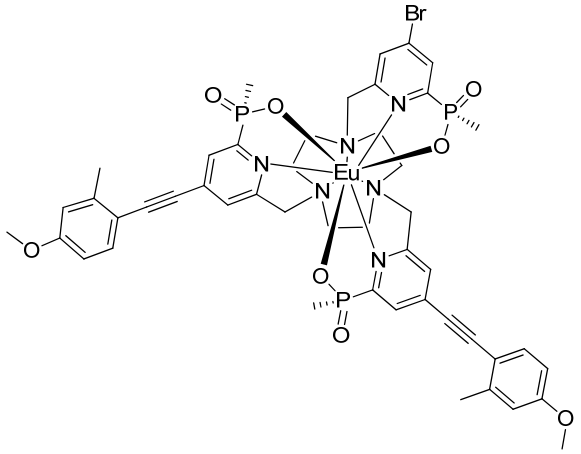
Analytical RP-HPLC of [Eu.**L**^{7}]: t_R 13.1 min [Gradient: 50 to 100% methanol in water (0.1% formic acid) over 15 min]



Triethyl methylphosphinate ester of L$^{8'}$

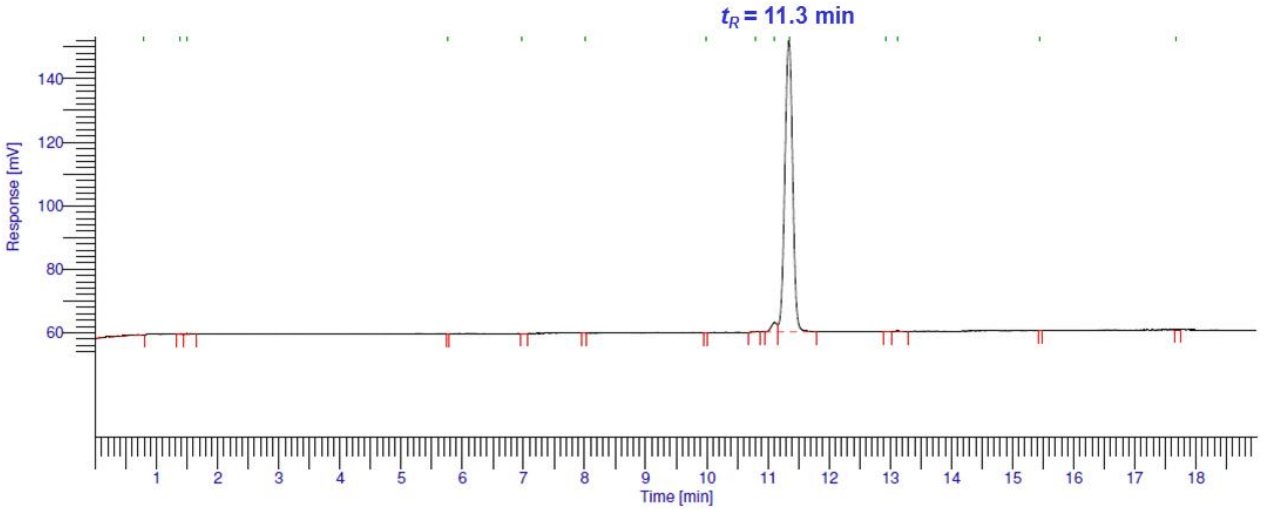
To a solution of methyl 2-(4-((2-(ethoxy(methyl)phosphoryl)-6-((methylsulfonyloxy)methyl)pyridin-4 yl)ethynyl)phenoxy)acetate (27 mg, 0.060 mmol) and the bis secondary amine bearing one 4-bromo-pyridylphosphinate group (12 mg, 0.030 mmol) in anhydrous CH_3CN (1.0 mL) was added K_2CO_3 (8 mg, 0.060 mmol) and the mixture was stirred under argon at 60 °C. The progress of the reaction was monitored by LC-MS analysis at regular intervals, which revealed complete consumption of starting material after 21 h. The reaction mixture was cooled to room temperature and the solution decanted from excess potassium salts. The solvent was removed under reduced pressure and the crude material was purified by preparative RP-HPLC [gradient: 50 − 100% methanol in water (0.1% formic acid) over 15 min; t_R = 10.5 min] to give a colourless oil (14 mg, 43%); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.10 (1H, d, $^2J_{H\text{-}P}$ 5.8 Hz, H$^{3'}$), 7.98 (2H, d, $^2J_{H\text{-}P}$ 5.8 Hz, H$^{3''}$), 7.75 (1H, s, H$^{5'}$), 7.61 (2H, s, H$^{5''}$), 7.44 (2H, d, $^3J_{H\text{-}H}$ 8.5 Hz, H^{18}), 6.78 (2H, d, $^4J_{H\text{-}H}$ 2.7 Hz, H^{15}), 6.74 (2H, dd, $^3J_{H\text{-}H}$ 8.5 Hz, $^4J_{H\text{-}H}$ 2.6 Hz, H^{17}), 4.27 (6H, br s, H^{7}), 4.13 (3H, m, H^{9}), 3.92 (3H, m, H^{9}), 3.83 (6H, s, H^{19}), 3.33 (4H, br m, ring CH_2), 3.24 (4H, br m,

ring CH_2), 3.13 (4H, br m, ring CH_2), 2.48 (6H, s, H^{20}), 1.78 (9H, d, $^2J_{H\text{-}P}$ 15 Hz, H^8), 1.29 (6H, t, $^3J_{H\text{-}H}$ 6.9 Hz, $H^{10'}$), 1.27 (3H, t, $^3J_{H\text{-}H}$ 6.9 Hz, H^{10}); ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.0 (C^6), 154.6 (d, $^1J_{C\text{-}P}$ 157 Hz, C^2), 143.1 (C^{14}), 134.2 (C^{18}), 128.1 (d, $^2J_{C\text{-}P}$ 21 Hz, C^3), 127.7 (C^5), 115.5 (C^{15}), 113.5 (C^{13}), 111.8 (C^{17}), 95.6 (C^{12}), 88.8 (C^{11}), 61.6 (d, $^2J_{C\text{-}P}$ 6 Hz, C^9), 60.7 (C^7), 55.4 (C^{19}), 52.6 (ring CH_2), 52.0 (ring CH_2), 21.2 (C^{20}), 16.6 (m, C^{10}), 13.7 (d, $^1J_{C\text{-}P}$ 105 Hz, C^8), quaternary carbon signals C^4, C^6, C^{14} and C^{16} are obscured or overlapping; ^{31}P NMR (243 MHz, $CDCl_3$) δ +34.3, +33.6; *m/z* (HRMS+) 1087.343 $[M + H]^+$ ($C_{53}H_{67}N_6O_8P_3Br$ requires 1087.342); R_f = 0.43 (silica; CH_2Cl_2/CH_3OH, 85/15 v/v).

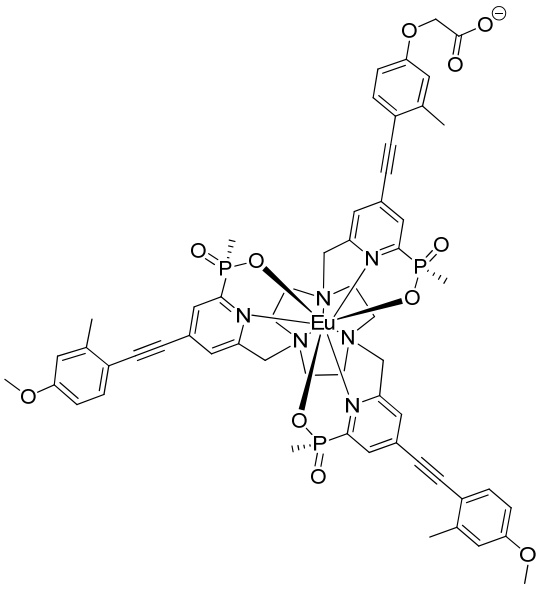


Mono *para*-bromo complex [Eu.L$^{8'}$]

The triethyl methylphosphinate ester of **L$^{8'}$** (3.5 mg, 3.22 μmol) was dissolved in a mixture of CH_3OH/H_2O (1.5 mL, 1:2 v/v) and KOH (4.5 mg, 80.0 μmol) was added. The cloudy mixture was stirred at 60 °C under argon for 12 h, at which time the solution became transparent. The reaction was monitored by ^{1}H-NMR spectroscopy (400 MHz; loss of CH_3CH_2 signals at 4.13, 3.92, 1.29 and 1.27 ppm) and ^{31}P-NMR spectroscopy (162 MHz; reactant = +34.3, +33.6 ppm, product = +25.8, 25.1 ppm). The methanol was removed under reduced pressure and the residual aqueous solution was neutralized by the addition of HCl (1M). Lyophilization of the solvent gave **L$^{8'}$** as a white solid (3.2 mg, quant.). The crude ligand **L$^{8'}$** was immediately dissolved in a mixture of CH_3OH/H_2O (1 mL, 1:1 v/v) and the pH of the solution was adjusted to 5.8 by the addition of HCl (1M). $Eu(OAc)_3$ (1.2 mg, 3.54 μmol) was added to give a cloudy mixture, which turned colourless after stirring at 65 °C under argon for 24 h. The mixture was cooled to room temperature and the pH adjusted to 7 by the dropwise addition of KOH (1M). Lyophilisation of the solvent and subjection of the crude material to semi-preparative RP-HPLC [gradient: 50 − 100% methanol in water (0.1% formic acid) over 15 min; t_R = 11.3 min] gave the mono *para*-bromo complex **[Eu.L$^{8'}$]** as a white solid (2 mg, 57%); LRMS (ESI) *m/z* 1151 $[M(^{151}Eu) + H]^+$, 576 $[M(^{151}Eu) + 2H]^{2+}$; ($HRMS^+$) *m/z* 1151.141 $[M(^{151}Eu) + H]^+$ ($C_{47}H_{52}N_6O_8P_3Br^{151}Eu$ requires 1151.144).

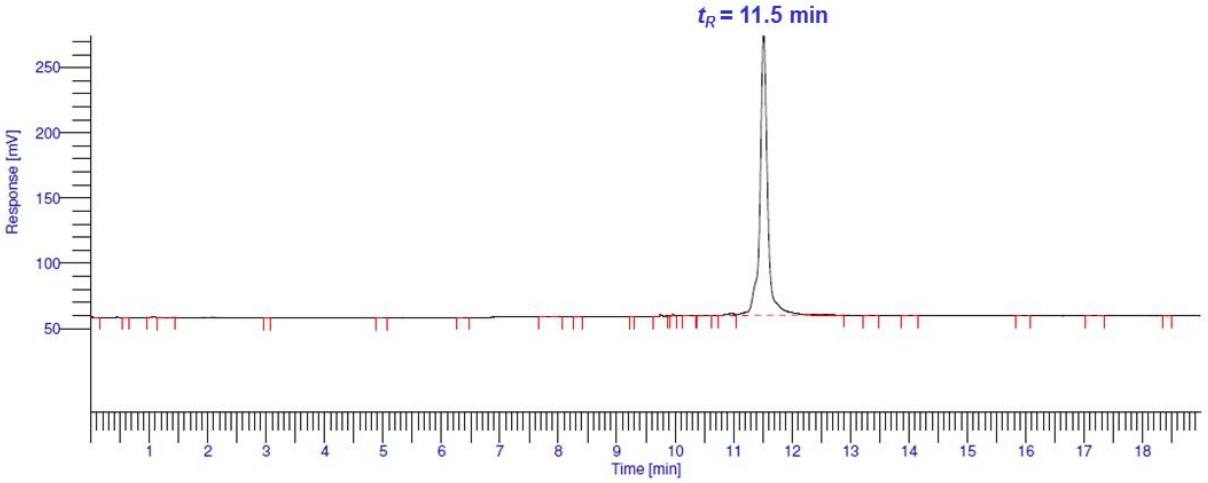


Analytical RP-HPLC trace of [Eu.**L**$^{8'}$]: t_R 11.3 min [Gradient: 50 to 100% methanol in water (0.1% formic acid) over 15 min]

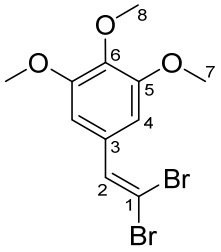


[Eu.L^{8}]

To a stirred degassed solution of the *para*-bromo Eu(III) complex [**Eu.L**$^{8'}$] (2 mg, 1.74 μmol) in anhydrous THF (1.0 mL) was added methyl 2-(4-ethynylphenoxy)acetic acid (0.36 mg, 1.91 μmol) and triethylamine (3.0 μL, 8.68 μmol), and the solution was degassed (freeze-thaw cycle) three times. [1,1-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (0.30 mg, 0.035 μmol) and CuI (60 μg, 0.035 μmol) were added and the resulting yellow solution was stirred at 65 °C under argon for 18 h. LC-MS analysis at this time revealed complete conversion of starting material. The solvent was removed under reduced pressure and the dark yellow residue was purified by semi-preparative RP-HPLC [gradient: 50 − 100% methanol in water (0.1% formic acid) over 15 min; t_R = 9.4 min] to give a white solid (1.3 mg, 58%); LRMS (ESI) *m*/*z* 1263 [M(^{153}Eu) + H]$^+$, 632 [M(^{153}Eu) + 2H]$^{2+}$; (HRMS$^+$) *m*/*z* 1263.279 [M(^{153}Eu) + H]$^+$ ($C_{58}H_{61}N_6O_{11}P_3{}^{153}Eu$ requires 1263.275); τ_{MeOH} = 1.15 ms; Φ^{em}_{MeOH} = 55 (± 15)%; ε_{MeOH} (340 nm) = 61,900 M^{-1} cm^{-1}.

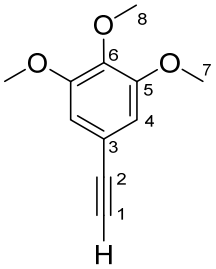


Analytical RP-HPLC of [Eu.$\mathbf{L^8}$]$^-$: t_R 11.5 min [Gradient: 50 to 100% methanol in water (0.1% formic acid) over 15 min]



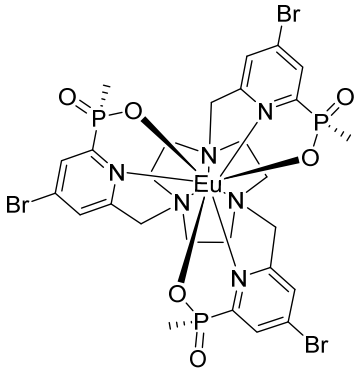
1,1-Dibromo-2-(3,4,5-trimethoxyphenyl)-ethylene

3,4,5-Trimethoxybenzaldehyde (1.0 g, 5.10 mmol) and triphenylphosphine (2.67 g, 10.2 mmol)) were dissolved in anhydrous dichloromethane (5 mL) and the solution was cooled to 0 °C. A solution of tetrabromomethane (2.0 g, 6.10 mmol) in dichloromethane (5 mL) was added slowly and the mixture was stirred under argon for 30 min. The resulting yellow solution was concentrated under reduced pressure and the residue was purified by column chromatography (silica, hexane/EtOAc, 9:1 v/v, then hexane/EtOAc, 4:1 v/v) to give 1,1-dibromo-2-(3,4,5-trimethoxyphenyl)-ethylene as a yellow oil (1.43 g, 80%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (1H, s, H^2), 6.79 (2H, s, H^4), 3.86 (9H, s, H^8 and H^7); LRMS (ESI) *m/z* 350 $[M + H]^+$; (HRMS$^+$) *m/z* 349.9155 $[M+ H]^+$ ($C_{11}H_{12}Br_2O_3$ requires 349.9164); R_f = 0.41 (silica; hexane/EtOAc 4:1 v/v). The spectral data was in good agreement with that reported previously.[1]



3,4,5-Trimethoxyphenylacetylene

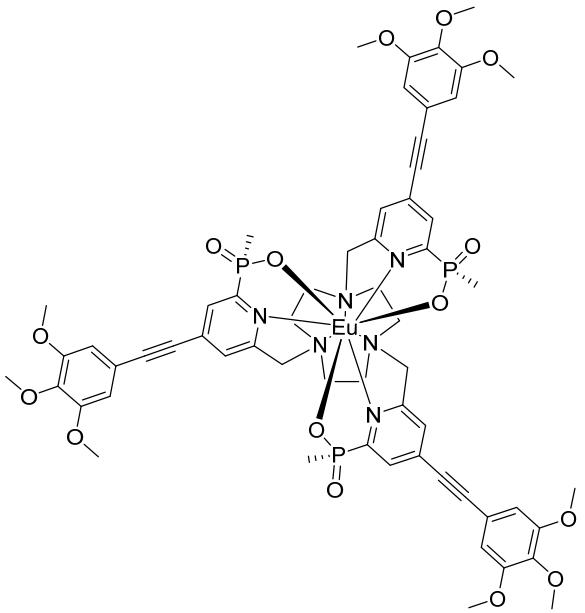
1,1-Dibromo-2-(3,4,5-trimethoxyphenyl)-ethylene (1.41 g, 4.04 mmol) was dissolved in anhydrous THF (30 mL) and cooled to -78 °C. *n*-Butyllithium (2.5 M in hexane, 6.4 mL, 16.2 mmol) was added slowly and the mixture was stirred at -78 °C under argon for 30 min. Water (8 mL) was added and the mixture was extracted with EtOAc (2 × 30 mL). The combined organic fractions were washed with half-strength brine solution (30 mL), dried ($MgSO_4$), and the solvent was removed under reduced pressure. The residue thus obtained was subjected to column chromatography (silica gel; neat hexane, then hexane/EtOAc, 9:1 v/v) to give 3,4,5-trimethoxyphenylacetylene as a white solid (0.53 g, 68%); m.p. 71-73 °C (lit.[1] m.p. 70-71 °C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.72 (2H, s, H^4), 3.85 (3H, s, H^8), 3.84 (6H, s, H^7), 3.03 (1H, s, H^1); ^{13}C NMR (151 MHz, $CDCl_3$) δ 153.1 (C^5), 139.3 (C^6), 117.0 (C^3), 109.4 (C^4), 83.7 (C^2), 76.2 (C^1), 61.0 (C^8), 56.2 (C^7); (HRMS+) *m/z* 193.0845 $[M + H]^+$ ($C_{11}H_{13}O_3$ requires 193.0865); R_f = 0.45 (silica; hexane/EtOAc 4:1 v/v). The spectral data was consistent with that reported previously.[2]



[Eu.L^9 precursor]

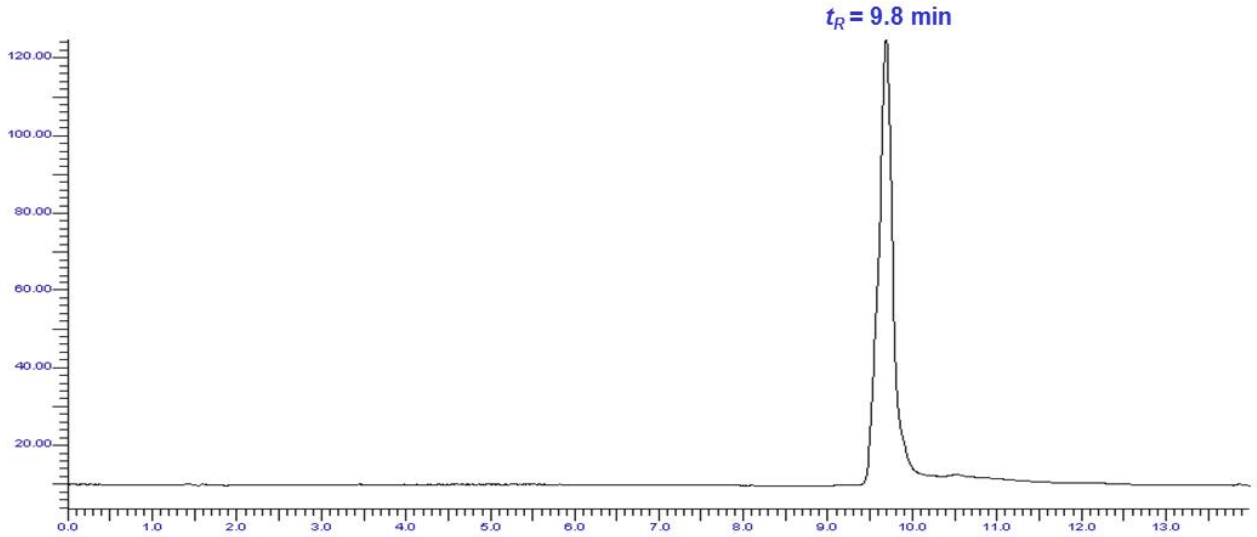
The synthesis of the tris *p*-bromo Eu(III) complex [**Eu.L^9 precursor**] is reported elsewhere.[3]

^{1}H NMR (400 MHz, CD_3OD) δ 8.60 (1H, s, py*H*), 7.97 (1H, s, pyC*H*N), 7.12 (1H, s, py*H*), 4.36 (1H, s, NC*H*'$_{eq}$), 0.54 (3H, s, CH_3), -0.77 (1H, s, pyC*H*'N), -1.37 (1H, s, NC*H*'$_{ax}$), -2.23 (1H, s, NCH_{eq}), -5.28 (1H, s, NCH_{ax}). ^{31}P NMR (162 MHz, CD_3OD) 39.8. *m/z* (HRMS$^+$) 1020.8512 $[M + H]^+$ ($C_{27}H_{34}{}^{79}Br_3N_6O_6P_3{}^{151}Eu$ requires 1020.8491). R_f = 0.31 (silica, CH_2Cl_2/CH_3OH/ NH_3 82:15:3 v/v/v).



[Eu.L^9]

To a stirred degassed solution of the tris *p*-bromo Eu(III) complex [**Eu.L^9 precursor**] (5 mg, 4.90 μmol) in a mixture of anhydrous THF/DMF (2:3 v/v, 500 μL) was added 3,4,5-trimethoxyphenylacetylene (3.1 mg, 16.1 μmol) and triethylamine (14 μL, 98 μmol), and the solution was degassed (freeze-thaw cycle) three times. [1,1-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (1.2 mg, 1.47 μmol) and CuI (0.28 mg, 1.47 μmol) were added and the resulting yellow solution was stirred at 65 °C under argon for 12 h. After this time the solution turned dark brown and LC-MS analysis revealed complete conversion of starting material. The solvent was removed under reduced pressure and the brown residue was purified by semi-preparative RP-HPLC [gradient: 50 − 100% methanol in water (0.1% formic acid) over 10 min; t_R = 9.8 min] to give a white solid (2.6 mg, 40%); LRMS (ESI) *m*/*z* 1355 $[M + H]^+$, 678 $[M + 2H]^{2+}$; (HRMS$^+$) *m*/*z* 1355.305 $[M(^{151}Eu) + H]^+$ ($C_{60}H_{67}N_6O_{15}P_3{}^{151}Eu$ requires 1355.308); τ_{MeOH} = 1.15 ms; τ_{H2O} = 0.91 ms; Φ^{em}_{MeOH} = 47 (± 15)%; ε_{MeOH} (332 nm) = 56,400 M^{-1} cm^{-1}.



Analytical RP-HPLC trace of [**Eu.L^9**]: t_R 9.8 min [Gradient: 50 to 100% methanol in water (0.1% formic acid) over 10 min]

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