Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Synthesis and luminescence properties of new red-shifted absorption lanthanide(III) chelates suitable for peptide and protein labelling

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Supporting Information

Experimental Section

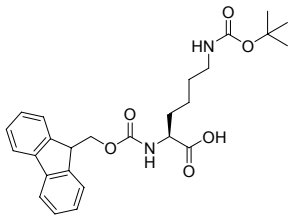
General: Column chromatography purifications were performed on Geduran® Si 60 silica gel (40-63 μm) from Merck. Reversed-phase column flash-chromatographies were performed on octadecyl-functionalised silica gel (mean pore size 60 Å, 37-74 μm) from Aldrich. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminium sheets. The spots were visualised by illumination with UV lamp (λ = 254 nm or 365 nm), by immersion in ninhydrin solution or potassium permanganate solution. Small scale syntheses (*i.e.*, bioconjugation experiments) were performed in standard single-use microtubes (0.5 or 1.7 mL). All solvents were dried following standard procedures (CH_3CN: distillation over CaH_2, CH_2Cl_2: distillation over P_2O_5, DMF: distillation over BaO, THF: distillation over Na°/benzophenone). Triethylamine (TEA) was distilled from CaH_2 and stored over BaO. Fmoc-Lys(Boc)-OH was purchased from Iris Biotech. Di-*tert*-butyl iminodiacetate **9** was synthesised following a literature procedure.[1] The HPLC-gradient grade acetonitrile (CH_3CN) was obtained from Acros or Fisher Scientific. Buffers and aq. mobile-phases for HPLC were prepared using water purified with a Milli-Q system (purified to 18.2 MΩ.cm). Triethylammonium acetate (TEAA, 2.0 M) and triethylammonium bicarbonate (TEAB, 1.0 M) buffers were prepared from distilled triethylamine and glacial acetic acid or CO_2 gas. ^1H and ^{13}C NMR spectra were recorded on a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) and relative to tetramethylsilane from $CDCl_3$ (δ_H = 7.26, δ_C = 77.16) or DMSO-d_6 (δ_H = 2.50, δ_C = 39.52).[2] Analytical HPLC was performed on a Thermo Scientific Surveyor Plus instrument equipped with a PDA detector. Semi-preparative HPLC was performed on a Thermo Scientific SPECTRASYSTEM liquid chromatography system (P4000) equipped with a UV-visible 2000 detector. Low-resolution mass spectra were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray source. High-resolution mass spectra were recorded on a LCT Premier XE benchtop orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer (Waters Micromass) equipped with an electrospray source and in the negative mode (ESI-). UV-visible spectra were obtained on a Varian Cary 50 scan spectrophotometer. Fluorescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer.

Peptide synthesis: The synthesis of hexapeptide **25** was carried out on an Applied Biosystems 433A peptide synthesizer using the standard Fmoc/tBu chemistry[3] and the Wang resin (Iris Biotech, loading 0.9 mmol/g) on a scale of 0.1 mmol. Coupling reactions were performed with commercial Fmoc-protected amino acids from Iris Biotech (10 equiv.) and HBTU (10 equiv.), HOBt (10 equiv.) and DIEA (30 equiv.) in peptide-grade DMF. The resin loading with the first Fmoc-protected amino acid (10 equiv.) was performed with *N*,*N*'-diisopropylcarbodiimide (DIC, 10 equiv.) and DMAP (0.036 equiv.) in a mixture of CH_2Cl_2-NMP (1 : 1, v/v). Final acetylation of the free amino group from the *N*-terminal side of hexapeptide was achieved by treatment with Ac_2O (10 equiv.), DIEA (10 equiv.) and DMAP (0.1 equiv.) in NMP for 90 min. The completion of reaction was also checked by the Kaiser ninhydrin test (negative after this treatment) and the resin was washed with NMP (3 × 2 mL), MeOH (3 × 2 mL) and CH_2Cl_2 (3 × 2 mL). A pre-cooled TFA solution (4 mL for 250 mg of resin) containing triisopropylsilane (TIPS, 2.5%, v/v) and deionised water (2.5%, v/v) was

[1] *WO Pat.*, 2001052898, 2001.
[2] H. E. Gottlieb, V. Kotlyar and A. Nudelman, *J. Org. Chem.*, 1997, **62**, 7512.
[3] G. B. Fields and R. L. Noble, *Int. J. Pept. Protein Res.*, 1990, **35**, 161.

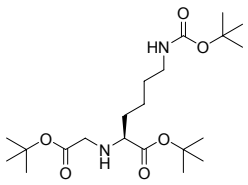
added to the resin. After 3 h stirring, the resin was filtered and washed with TFA. The filtrate was evaporated to dryness and cold Et_2O was added to the oily residue. The resulting white precipitate was isolated by centrifugation, lyophilised and purified by semi-preparative RP-HPLC (system A). The product-containing fractions were lyophilised to give the TFA salt of hexapeptide **25** as a white amorphous powder (54 mg, yield 75%). MS (ESI-): *m/z* 608.40 [M - H]$^-$ and 722.07 [M + TFA - H]$^-$, calcd for $C_{26}H_{39}N_7O_{10}$: 609.63; HPLC (system A): t_R = 9.8 min; λ_{max}(recorded during the HPLC analysis)/nm 229 and 275.

Synthesis of compound **16**: The *N*-protected amino acid Fmoc-L-Lys(Boc)-OH was esterified with *tert*-butanol under non-standard conditions involving Boc_2O-DMAP[4], followed by the Fmoc removal. The resulting primary amine was reacted with *tert*-butylbromoacetate in the presence of a large excess of K_2CO_3 in dry CH_3CN to give compound **16** in a good 45% overall yield for the three steps.



Fmoc-L-Lys(Boc)-OH

a-c



16

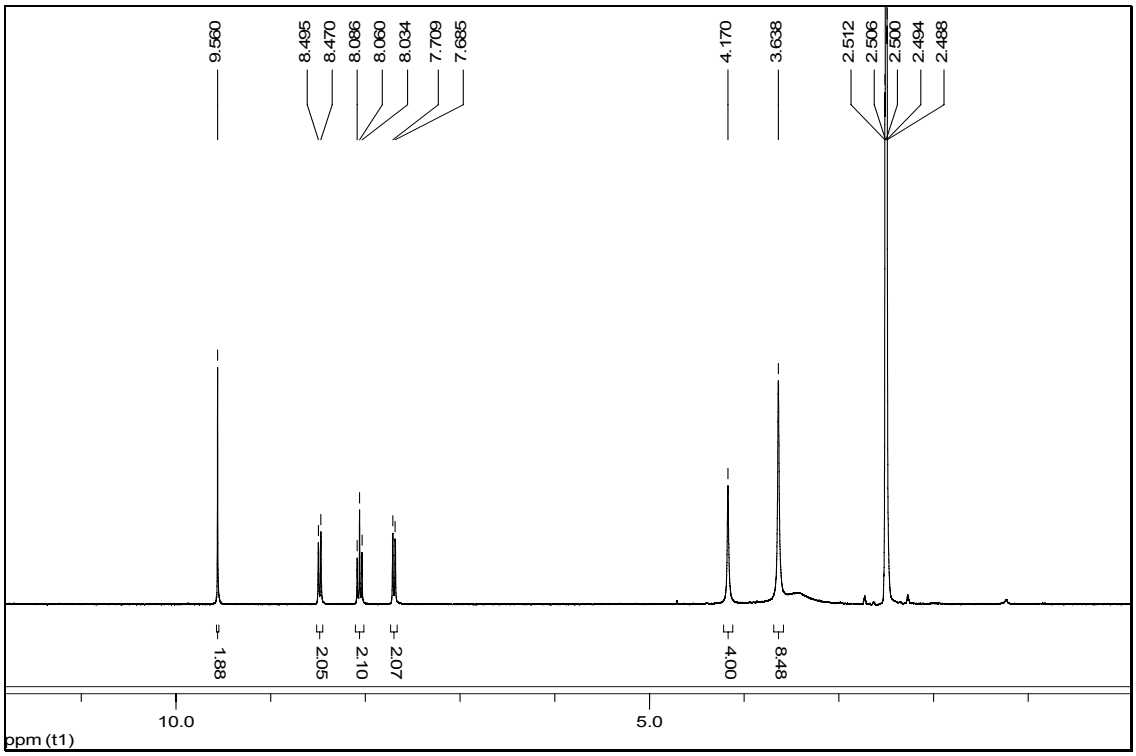
Scheme S1 *Reagents and conditions*: a) Boc_2O (2.0 equiv.), DMAP (0.3 equiv.), tBuOH, 35 °C, 22 h, 67%; b) Et_2NH (15 equiv.), CH_2Cl_2, 0 °C to rt, overnight, 94%; c) *tert*-Butylbromoacetate (1.2 equiv.), K_2CO_3 (5.0 equiv.), CH_3CN, reflux 5 h, then rt overnight, 72%.

(a) Fmoc-L-Lys(Boc)-OtBu: Fmoc-L-Lys(Boc)-OH (1.0 g, 2.13 mmol) was dissolved in *tert*-butanol (8 mL) and DMAP (78 mg, 0.64 mmol) and Boc_2O (932 mg, 4.27 mmol) were sequentially added. The resulting reaction mixture was stirred at 35 °C under an argon atmosphere for 22 h. Thereafter, the reaction mixture was then diluted in AcOEt (30 mL) and the organic phase was washed with a 1.0 M aq. $KHSO_4$ (2 × 10 mL) and a 5% aq. $NaHCO_3$ (2 × 10 mL). The aq. phases were collected and re-extracted with CH_2Cl_2 (2 × 30 mL). The combined organic phases were washed with brine, dried over $MgSO_4$, filtered and evaporated to dryness. The resulting yellow foam was purified by flash-chromatography on a silica gel column with a mixture cyclohexane-AcOEt (8 : 2, v/v). The desired full-protected lysine derivative was obtained as a white solid (750 mg, yield 67%). *R*f (cyclohexane-AcOEt, 8 : 2, v/v) 0.24; δ_H (300 MHz, $CDCl_3$) 7.77 (2H, d, *J* 7.4), 7.61 (2H, d, *J* 7.4), 7.40 (2H, t, *J* 7.4), 7.32 (2H, t, *J* 7.4), 5.36 (1H, bs), 4.55 (1H, bs), 4.41-4.35 (2H, m), 4.28-4.19 (2H, m), 3.15-3.05 (2H, m), 1.90-1.30 (24H, m); δ_C (75.4 MHz, $CDCl_3$) 171.7, 156.1, 156.0, 144.1, 144.0, 141.4, 127.8, 127.2, 125.3, 120.1, 82.3, 67.0, 54.3, 47.3, 40.3, 32.6, 29.7, 28.5, 28.1, 22.4; MS (ESI+): *m/z* 525.00 [M + H]$^+$, 541.87 [M + H_2O]$^{+\bullet}$ (water cluster formed during the ionisation process) and 547.20 [M +

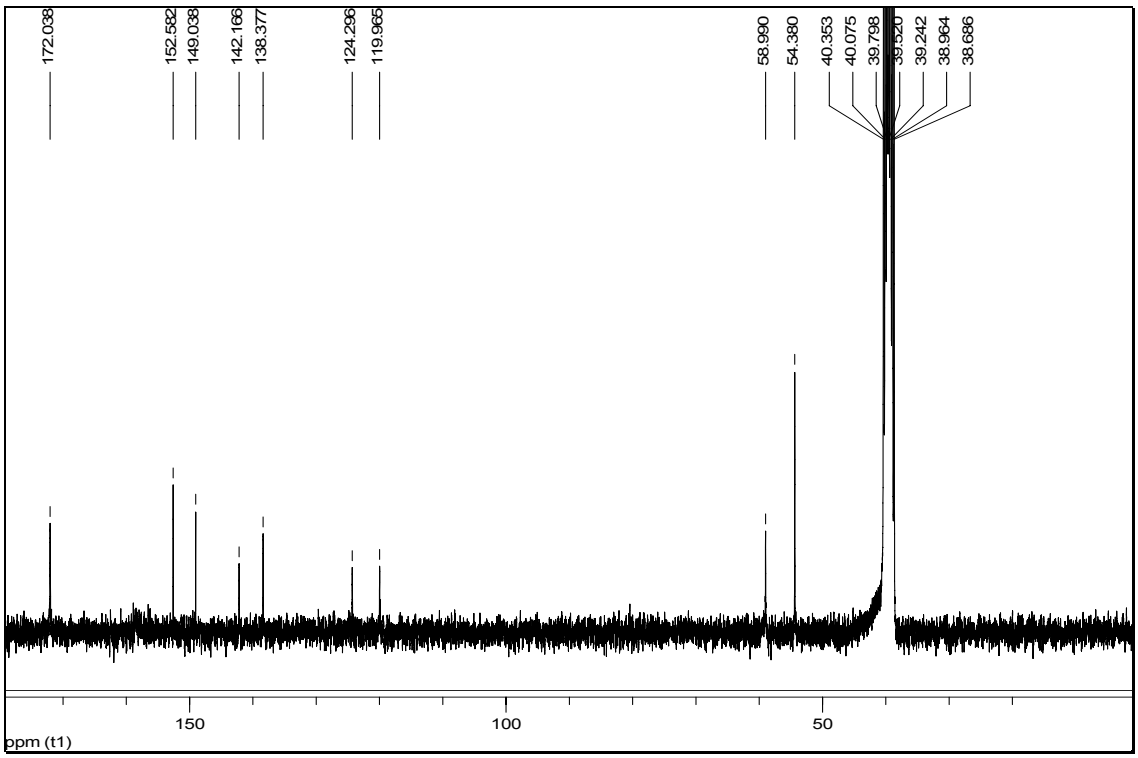
[4] K. Takeda, A. Akiyama, H. Nakamura, S.-i. Takizawa, Y. Mizuno, H. Takayanagi and Y. Harigaya, *Synthesis*, 1994, 1063.

Na]$^+$, calcd for $C_{30}H_{40}N_2O_6$: 524.65.
(b) H-L-Lys(Boc)-OtBu: Fmoc-L-Lys(Boc)-OtBu (744 mg, 1.42 mmol) was dissolved in CH_2Cl_2 (12 mL) and cooled to 0 °C. Diethylamine (2.2 mL, 21.3 mmol) was added and the reaction mixture was stirred at room temperature overnight. After solvent removal under reduced pressure, purification by flash-chromatography on a silica gel column with cyclohexane/AcOEt (8 : 2, v/v) followed by CH_2Cl_2/MeOH 95 : 5, v/v) yielded the compound H-L-Lys(Boc)-OtBu as a pale brown oil (404 mg, yield 94%). *R*f (CH_2Cl_2-MeOH, 95 : 5, v/v) 0.28; δ_H (300 MHz, $CDCl_3$) 4.54 (1H, bs), 3.29 (1H, dd, *J* 7.2 and 5.4), 3.16-3.06 (2H, m), 1.76-1.30 (24H, m); δ_C (75.4 MHz, $CDCl_3$) 175.6, 156.1, 81.1, 55.0, 40.5, 34.7, 30.0, 28.6, 28.2, 23.0; MS (ESI+): *m/z* 303.12 [M + H]$^+$, calcd for $C_{15}H_{30}N_2O_4$: 302.41.
(c) Alkylation: H-L-Lys(Boc)-OH (400 mg, 1.32 mmol) and anhydrous K_2CO_3 (914 mg, 6.61 mmol) were dissolved in dry CH_3CN (8 mL) and *tert*-butylbromoacetate (195 µL, 1.32 mmol) was added. After heating under reflux for 5 h, a further amount of *tert*-butylbromoacetate (40 µL, 0.26 mmol) was added. The reaction mixture was stirred at room temperature overnight. The suspension was filtered and the filtrate was evaporated. The resulting residue was purified by flash-chromatography on a silica gel column with a step gradient of AcOEt (10-20%) in cyclohexane. The desired secondary amine **16** was obtained as a colourless oil (400 mg, yield 72%). *R*f (cyclohexane-AcOEt, 7 : 3, v/v) 0.33;δ_H (300 MHz, $CDCl_3$) 4.55 (1H, bs), 3.32 (1H, d, *J* 17.0), 3.21 (1H, d, *J* 17.0), 3.15-3.06 (3H, m), 1.70-1.55 (2H, m), 1.55-1.35 (31H, m); δ_C (75.4 MHz, $CDCl_3$) 174.0, 171.1, 156.0, 81.3, 61.2, 50.0, 40.4, 33.0, 29.9, 28.5, 28.2, 23.0; MS (ESI+): *m/z* 417.13 [M + H]$^+$, calcd for $C_{21}H_{40}N_2O_6$: 416.55.

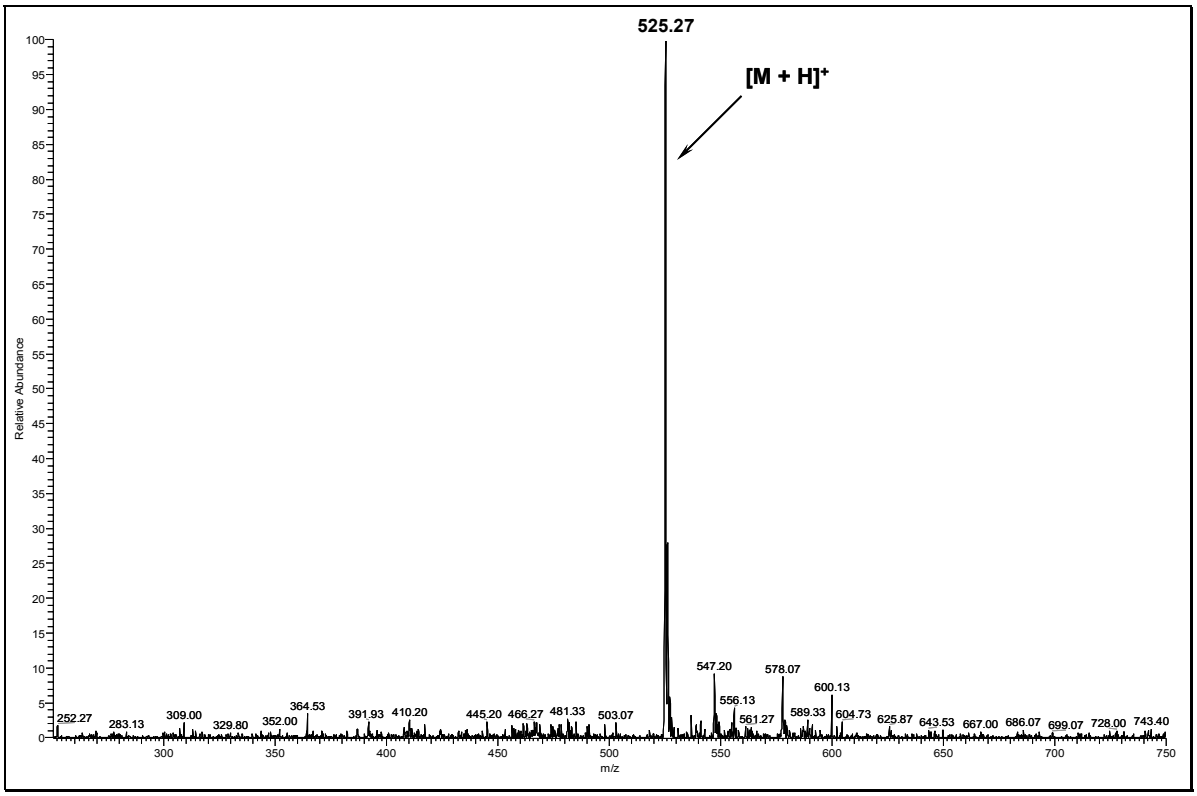
^{1}H NMR spectrum of symmetrical bis-pyridinylpyrazine ligand 10 recorded in DMSO-d_6.



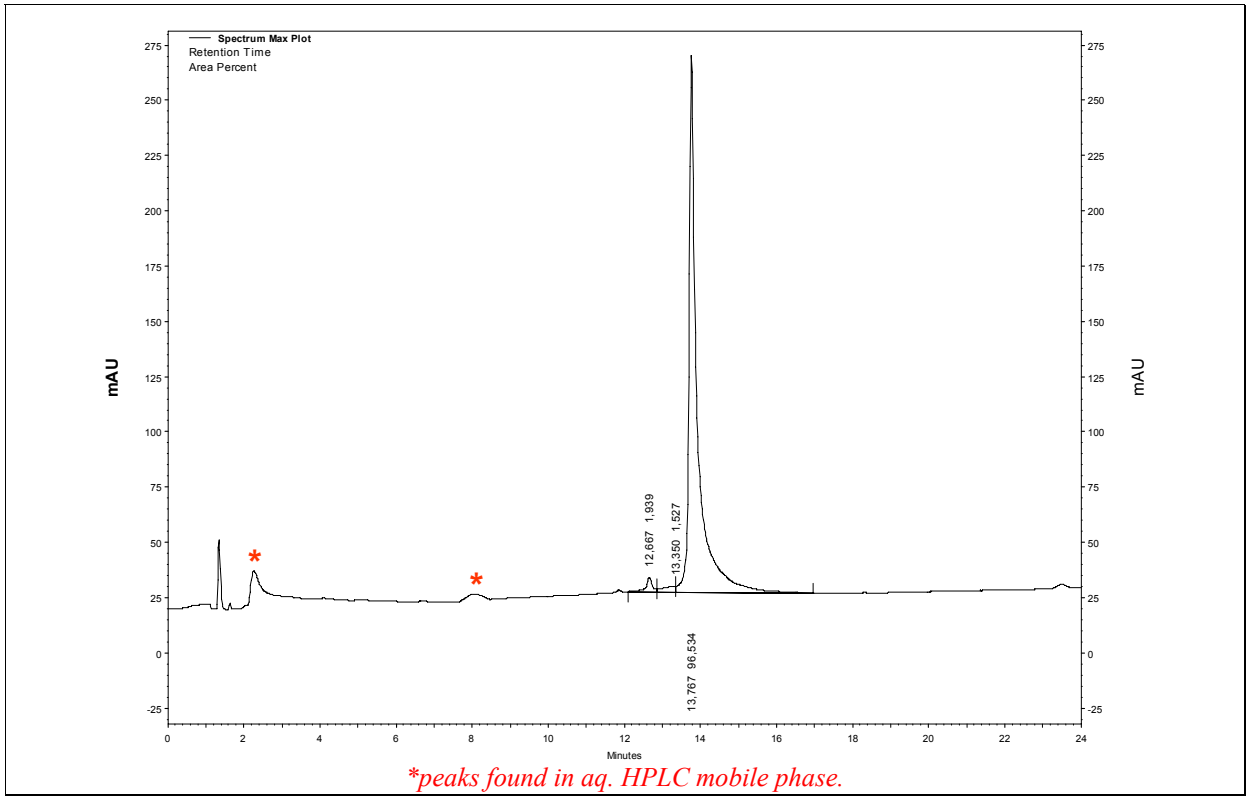
^{13}C NMR spectrum of symmetrical bis-pyridinylpyrazine ligand 10 recorded in DMSO-d_6.



ESI-MS spectrum of symmetrical bis-pyridinylpyrazine ligand 10 recorded in the positive mode.

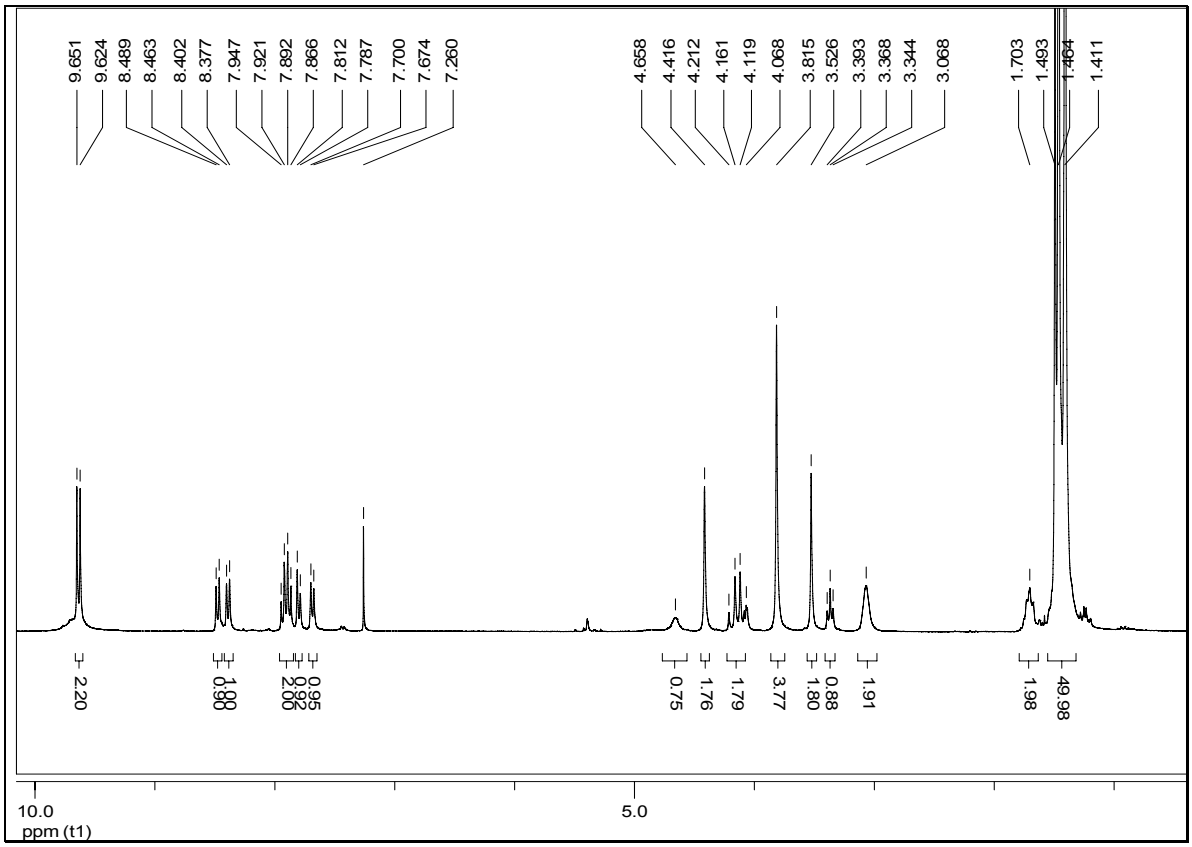


RP-HPLC elution profile (system B) of symmetrical bis-pyridinylpyrazine ligand 10.

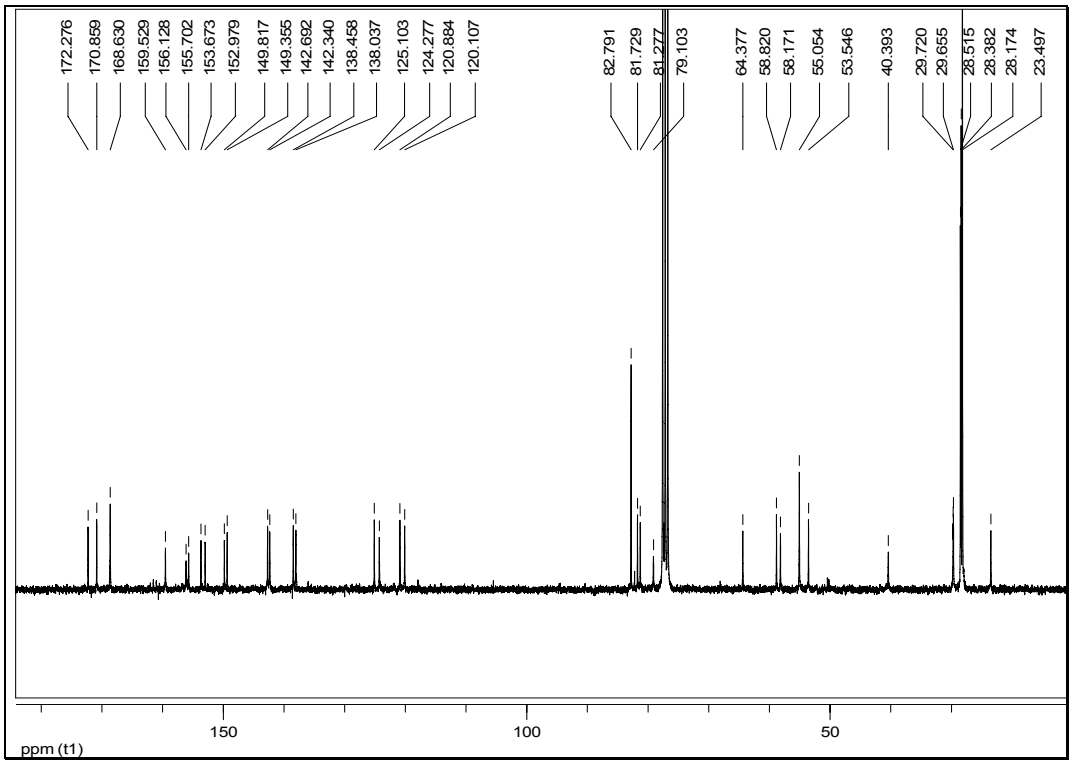


**peaks found in aq. HPLC mobile phase.*

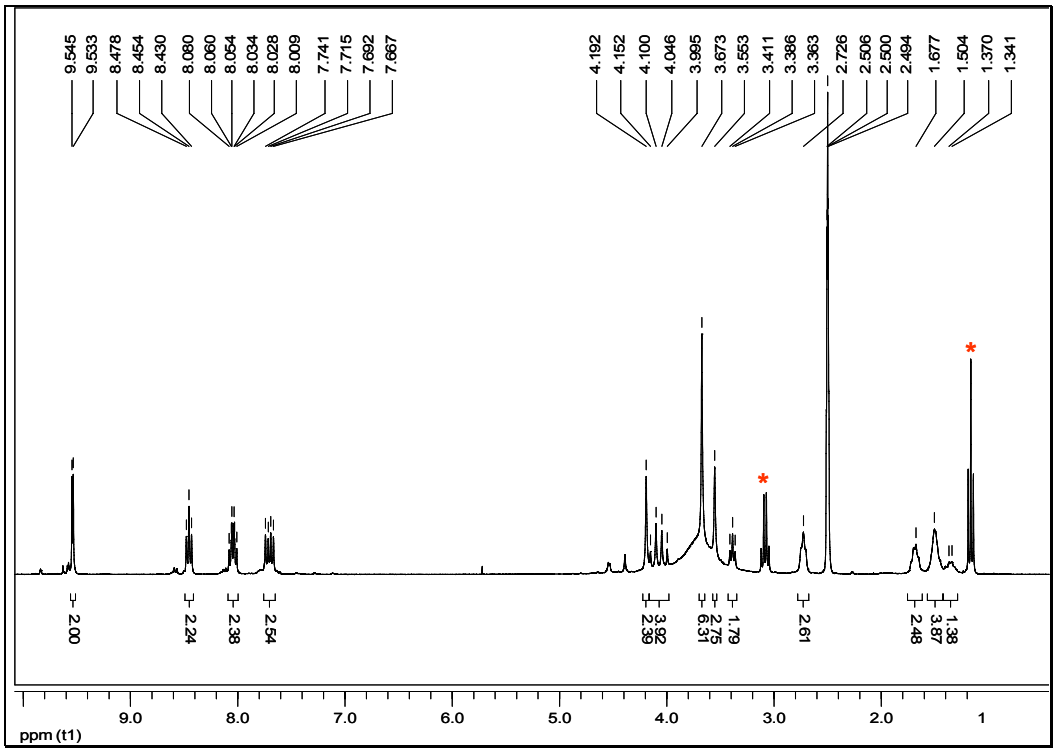
^{1}H NMR spectrum of protected non-symmetrical bis-pyridinylpyrazine ligand 17 recorded in $CDCl_3$.



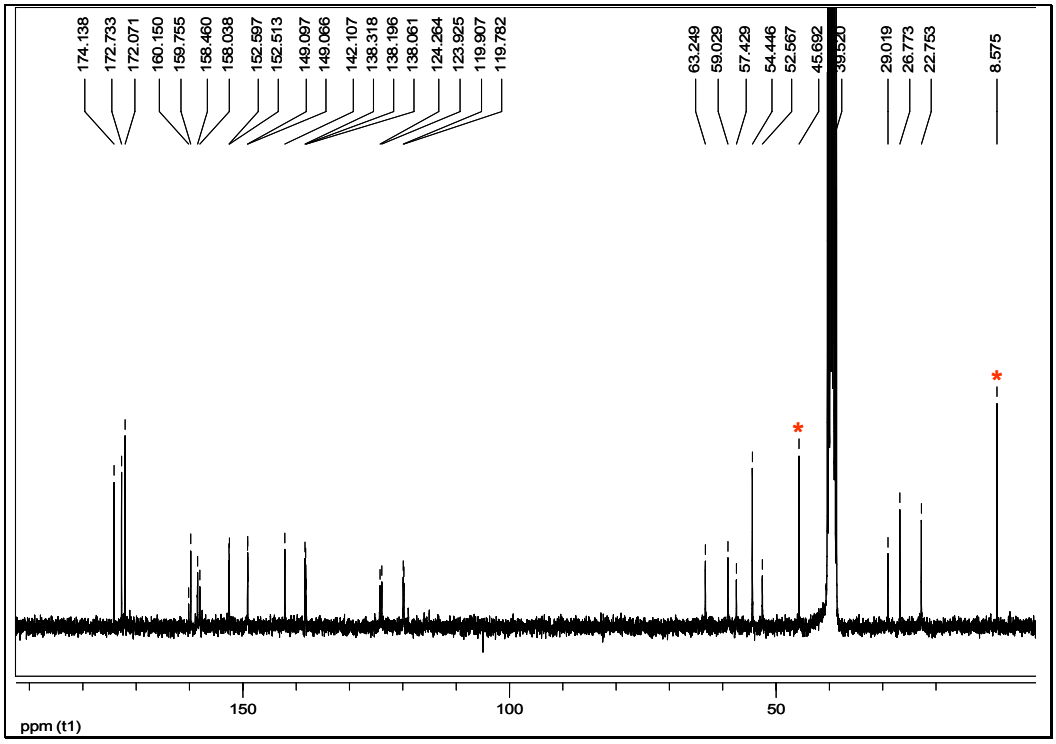
^{13}C NMR spectrum of protected non-symmetrical bis-pyridinylpyrazine ligand 17 recorded in $CDCl_3$.



^{1}H NMR spectrum of non-symmetrical bis-pyridinylpyrazine ligand 18 recorded in DMSO-d_6 + D_2O.

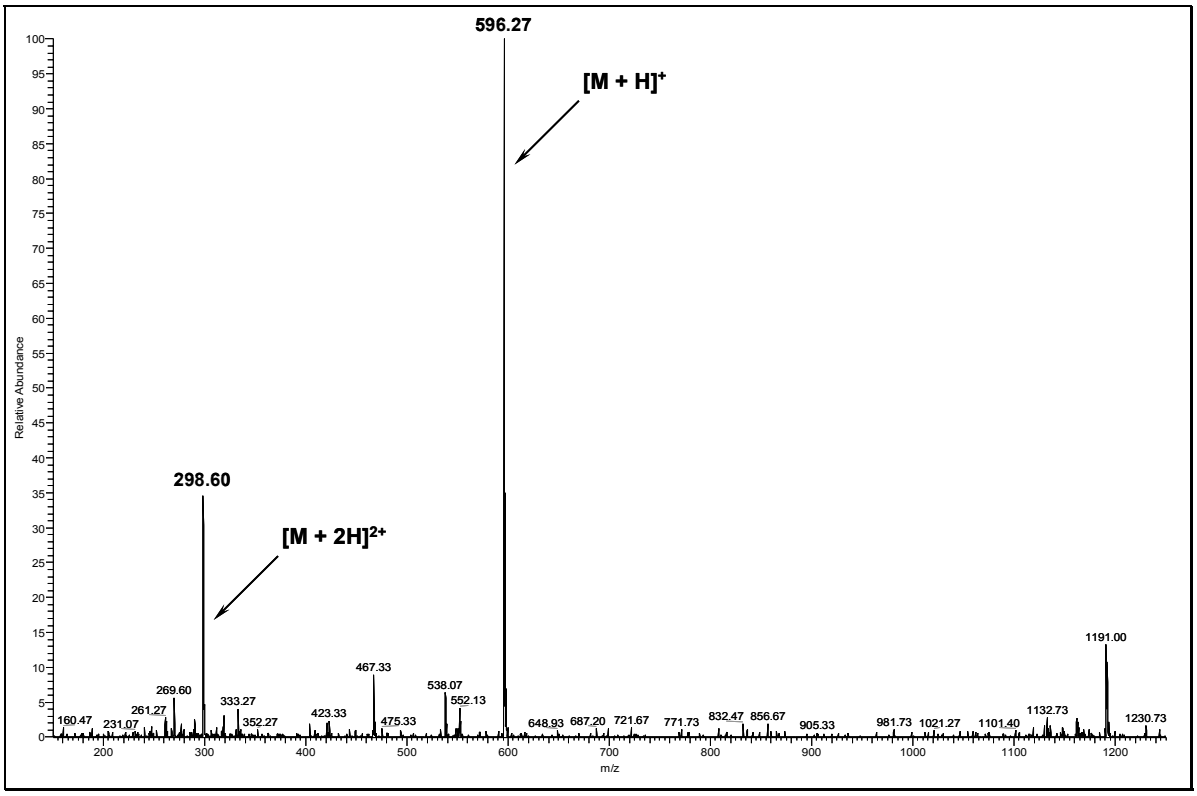


^{13}C NMR spectrum of non-symmetrical bis-pyridinylpyrazine ligand 18 recorded in DMSO-d_6 + D_2O.

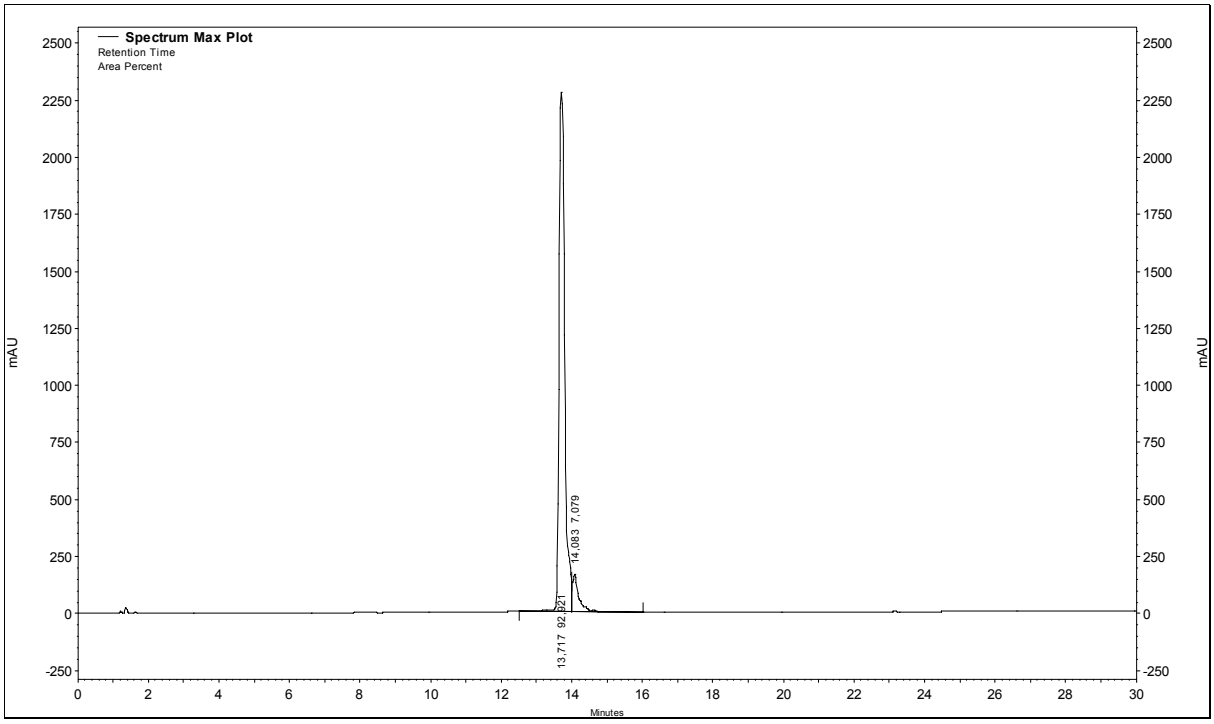


**peaks assigned to triethylammonium salts recovered during lyophilisation.*

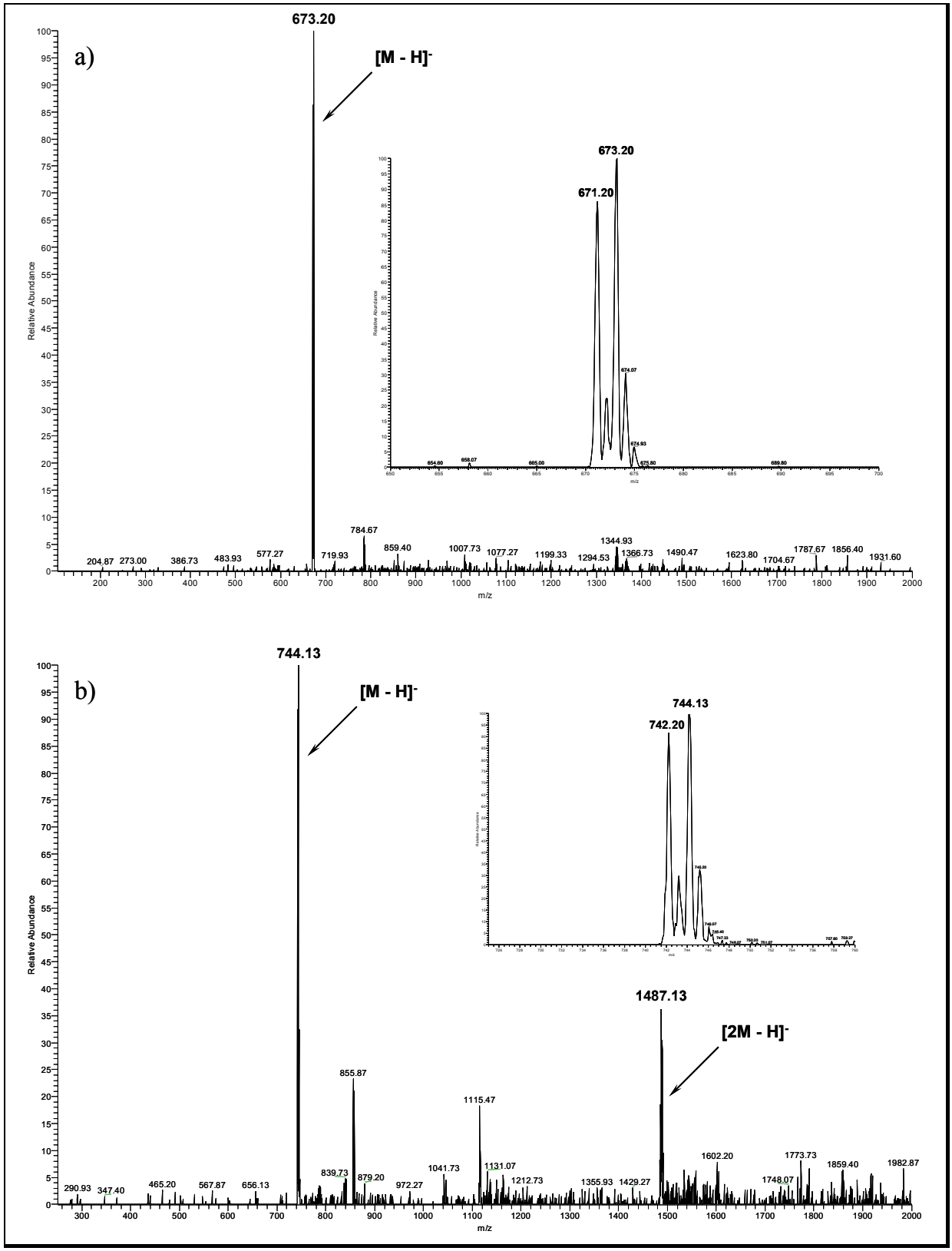
ESI-MS spectrum of non-symmetrical bis-pyridinylpyrazine ligand 18 recorded in the positive mode.



RP-HPLC elution profile (system B) of non-symmetrical bis-pyridinylpyrazine ligand 18.

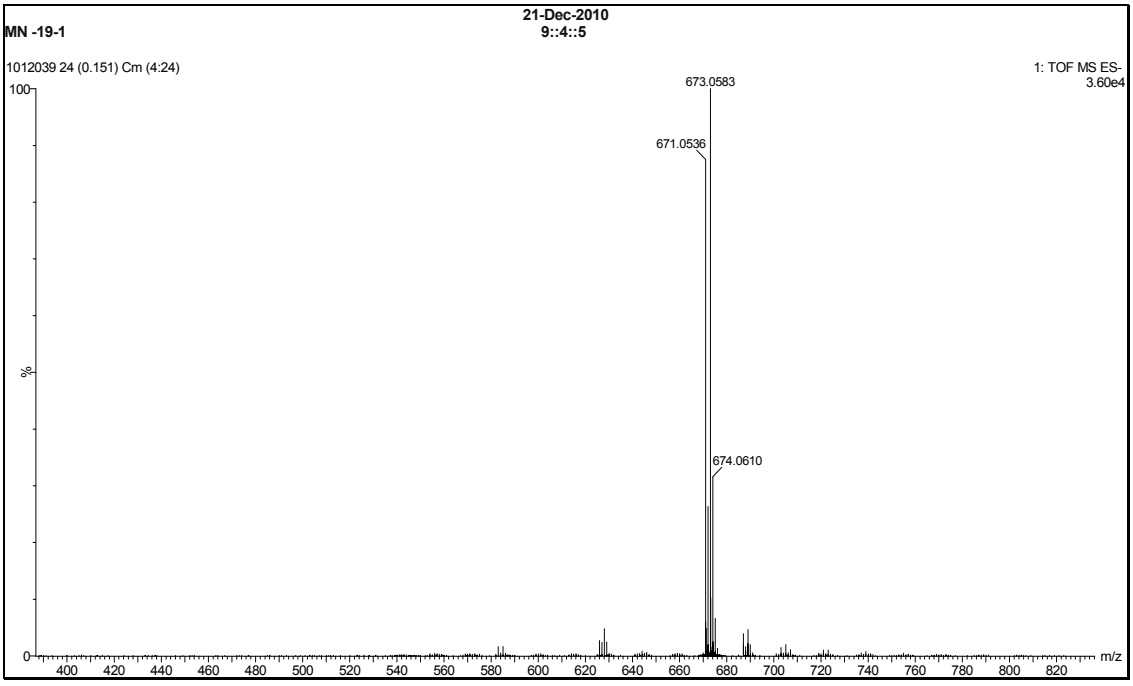


ESI-MS spectra of Eu(III) chelates 6 and 8 recorded in the negative mode.



(a) ESI mass spectrum of Eu(III) chelate **6**, calcd mass for $[C_{24}H_{20}EuN_6O_8]^-$: 672.42; (b) ESI mass spectrum of Eu(III) chelate **8**, calcd mass for $[C_{28}H_{29}EuN_7O_8]^-$: 743.54 ; both recorded in the negative mode, the inset show the zoom-scan spectrum of $[M - H]^-$.

Low- and high-resolution mass spectrum of Eu(III) chelate 6.



Elemental Composition Report

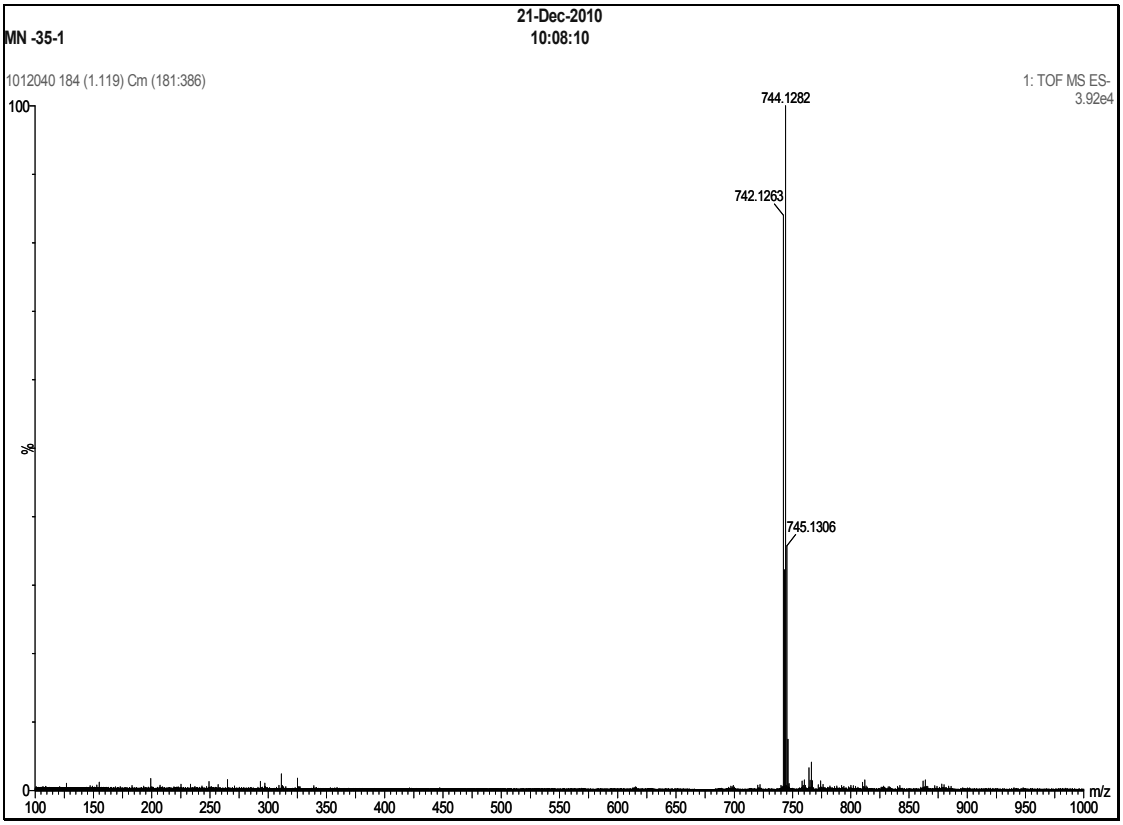
Multiple Mass Analysis: 2 mass(es) processed
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 30.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 4

Monoisotopic Mass, Even Electron Ions
4239 formula(e) evaluated with 32 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-500 H: 0-110 N: 0-7 O: 0-10 151Eu: 0-1 153Eu: 0-1

Minimum:	50.00				-1.5		
Maximum:	100.00		10.0	10.0	30.0		
Mass	RA	Calc. Mass	mDa	PPM		DBE	i-FIT
	i-FIT (Norm)	Formula					
671.0536	87.45	671.0538	-0.2	-0.3	18.5	649.0	2.2
		C27 H22 N2 O9 153Eu					
		671.0532	0.4	0.6	6.5	649.5	2.7
		C20 H31 O6 151Eu 153Eu					
		671.0541	-0.5	-0.7	18.5	648.3	1.4
		C24 H20 N6 O8 151Eu					
		671.0545	-0.9	-1.3	11.5	649.2	2.4
		C21 H27 N4 O2 151Eu 153Eu					
		671.0551	-1.5	-2.2	23.5	648.9	2.1
		C28 H18 N6 O5 153Eu					
		671.0509	2.7	4.0	26.5	650.4	3.6
		C35 H20 O5 151Eu					
		671.0564	-2.8	-4.2	-1.5	649.5	2.6
		C9 H31 N6 O9 151Eu 153Eu					
		671.0505	3.1	4.6	7.5	649.0	2.2
		C16 H27 N6 O4 151Eu 153Eu					
		671.0568	-3.2	-4.8	17.5	650.7	3.8
		C28 H24 O10 151Eu					
		671.0578	-4.2	-6.3	22.5	651.6	4.7

C32 H22 O7 153Eu						
	671.0581	-4.5	-6.7	22.5	651.0	4.2
C29 H20 N4 O6 151Eu						
	671.0491	4.5	6.7	2.5	650.1	3.3
C15 H31 N2 O8 151Eu 153Eu						
	671.0585	-4.9	-7.3	15.5	652.6	5.7
C26 H27 N2 151Eu 153Eu						
	671.0482	5.4	8.0	27.5	650.4	3.6
C31 H16 N6 O3 151Eu						
	671.0591	-5.5	-8.2	27.5	652.0	5.2
C33 H18 N4 O3 153Eu						
	671.0479	5.7	8.5	27.5	651.2	4.4
C34 H18 N2 O4 153Eu						
	671.0473	6.3	9.4	15.5	652.3	5.5
C27 H27 O 151Eu 153Eu						
	671.0469	6.7	10.0	22.5	651.4	4.6
C30 H20 N2 O7 151Eu						
673.0583 100.00	673.0582	0.1	0.1	17.5	546.2	0.0
C28 H24 O10 153Eu						
	673.0585	-0.2	-0.3	17.5	550.0	3.8
C25 H22 N4 O9 151Eu						
	673.0589	-0.6	-0.9	10.5	557.0	10.8
C22 H29 N2 O3 151Eu 153Eu						
	673.0595	-1.2	-1.8	22.5	550.9	4.7
C29 H20 N4 O6 153Eu						
	673.0555	2.8	4.2	18.5	551.4	5.3
C24 H20 N6 O8 153Eu						
	673.0549	3.4	5.1	6.5	558.6	12.4
C17 H29 N4 O5 151Eu 153Eu						
	673.0626	-4.3	-6.4	21.5	551.6	5.5
C30 H22 N2 O7 151Eu						
	673.0629	-4.6	-6.8	14.5	555.3	9.2
C27 H29 O 151Eu 153Eu						
	673.0536	4.7	7.0	1.5	559.2	13.1
C16 H33 O9 151Eu 153Eu						
	673.0636	-5.3	-7.9	26.5	555.8	9.7
C34 H20 N2 O4 153Eu						
	673.0527	5.6	8.3	26.5	554.4	8.3
C32 H18 N4 O4 151Eu						
	673.0639	-5.6	-8.3	26.5	554.2	8.1
C31 H18 N6 O3 151Eu						
	673.0523	6.0	8.9	26.5	556.1	9.9
C35 H20 O5 153Eu						
	673.0648	-6.5	-9.7	1.5	559.7	13.6
C15 H33 N2 O8 151Eu 153Eu						

Low- and high-resolution mass spectrum of Eu(III) chelate 8.



Elemental Composition Report

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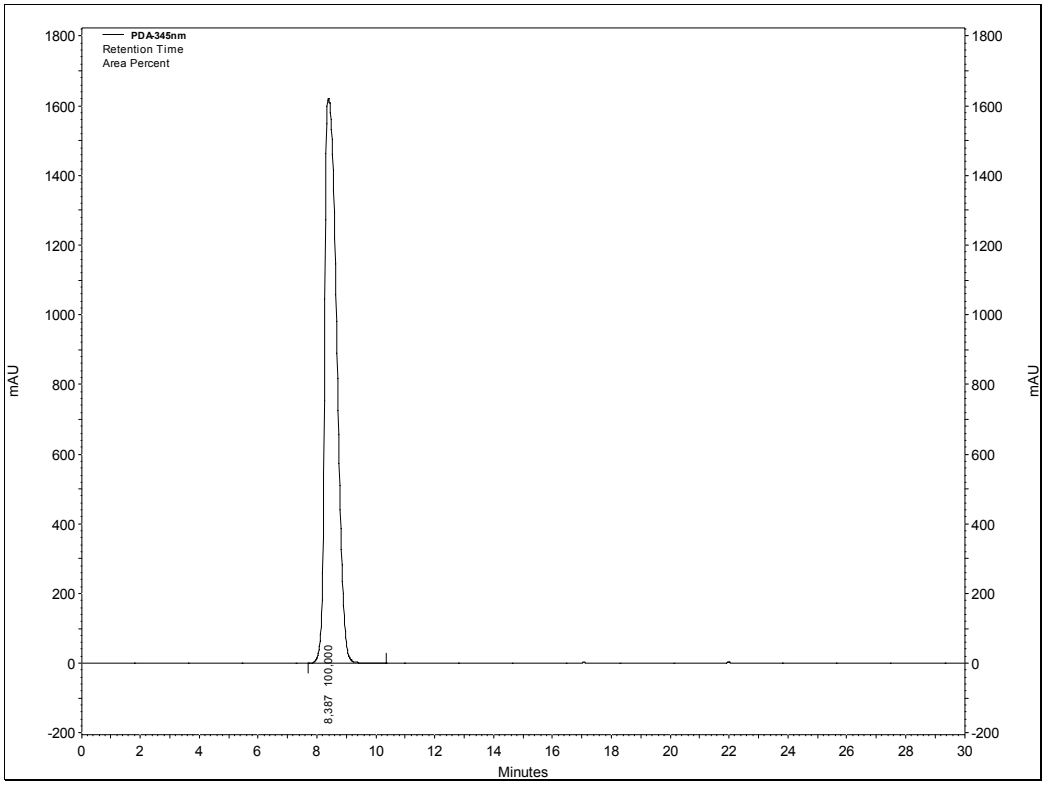
Element prediction: Off
Number of isotope peaks used for i-FIT = 4

Monoisotopic Mass, Even Electron Ions
1197 formula(e) evaluated with 13 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-500 H: 0-110 N: 5-9 O: 6-10 151Eu: 0-1 153Eu: 0-1

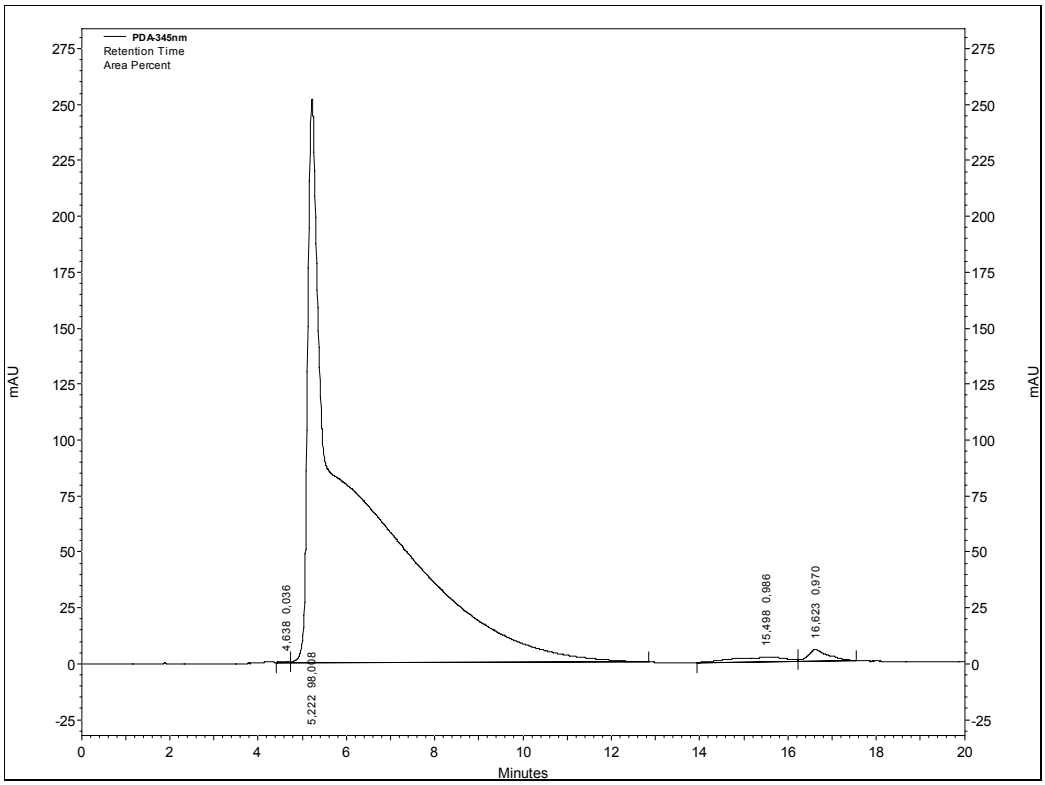
Minimum:	50.00				-1.5		
Maximum:	100.00		10.0	10.0	30.0		
Mass	RA	Calc. Mass	mDa	PPM		DBE	i-FIT
i-FIT (Norm)	Formula						
742.1263	84.01	742.1276	-1.3	-1.8	18.5	589.8	2.1
	C28 H29 N7 O8 151Eu						
		742.1246	1.7	2.3	19.5	589.4	1.7
	C27 H27 N9 O7 153Eu						
		742.1236	2.7	3.6	14.5	589.1	1.4
	C23 H29 N9 O10 151Eu						
		742.1299	-3.6	-4.9	-1.5	589.2	1.5
	C13 H40 N7 O9 151Eu 153Eu						
		742.1316	-5.3	-7.1	22.5	590.9	3.1
	C33 H29 N5 O6 151Eu						
		742.1200	6.3	8.5	3.5	589.4	1.6
	C15 H36 N9 O6 151Eu 153Eu						
744.1282	100.00	744.1290	-0.8	-1.1	18.5	631.8	0.2
	C28 H29 N7 O8 153Eu						

	744.1250	3.2	4.3	14.5	637.6	6.0
C23 H29 N9 O10 153Eu						
	744.1320	-3.8	-5.1	17.5	633.7	2.0
C29 H31 N5 O9 151Eu						
	744.1244	3.8	5.1	2.5	642.8	11.2
C16 H38 N7 O7 151Eu 153Eu						
	744.1330	-4.8	-6.5	22.5	637.6	6.0
C33 H29 N5 O6 153Eu						
	744.1221	6.1	8.2	22.5	634.9	3.3
C31 H27 N7 O6 151Eu						
	744.1356	-7.4	-9.9	2.5	644.3	12.7
C15 H38 N9 O6 151Eu 153Eu						

RP-elution profile (system C) of Eu(III) chelate 6.

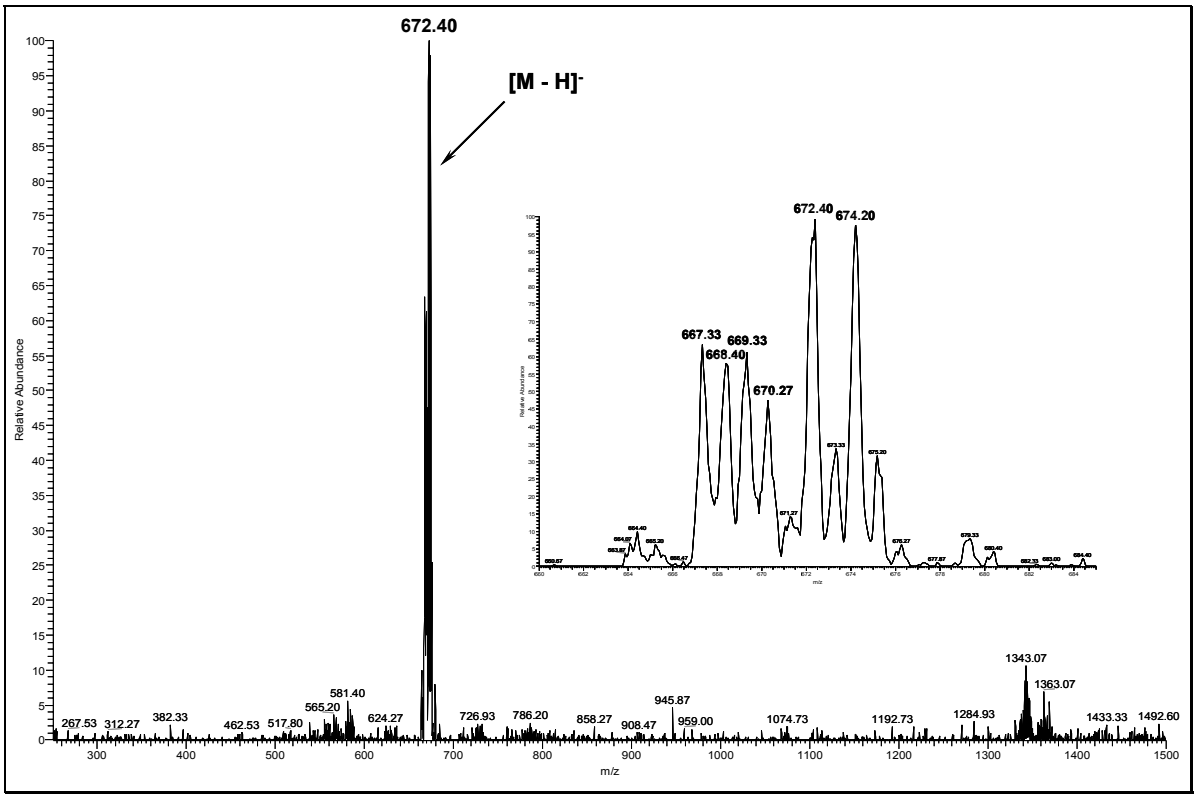


RP-elution profile (system C) of Eu(III) chelate 8[a].

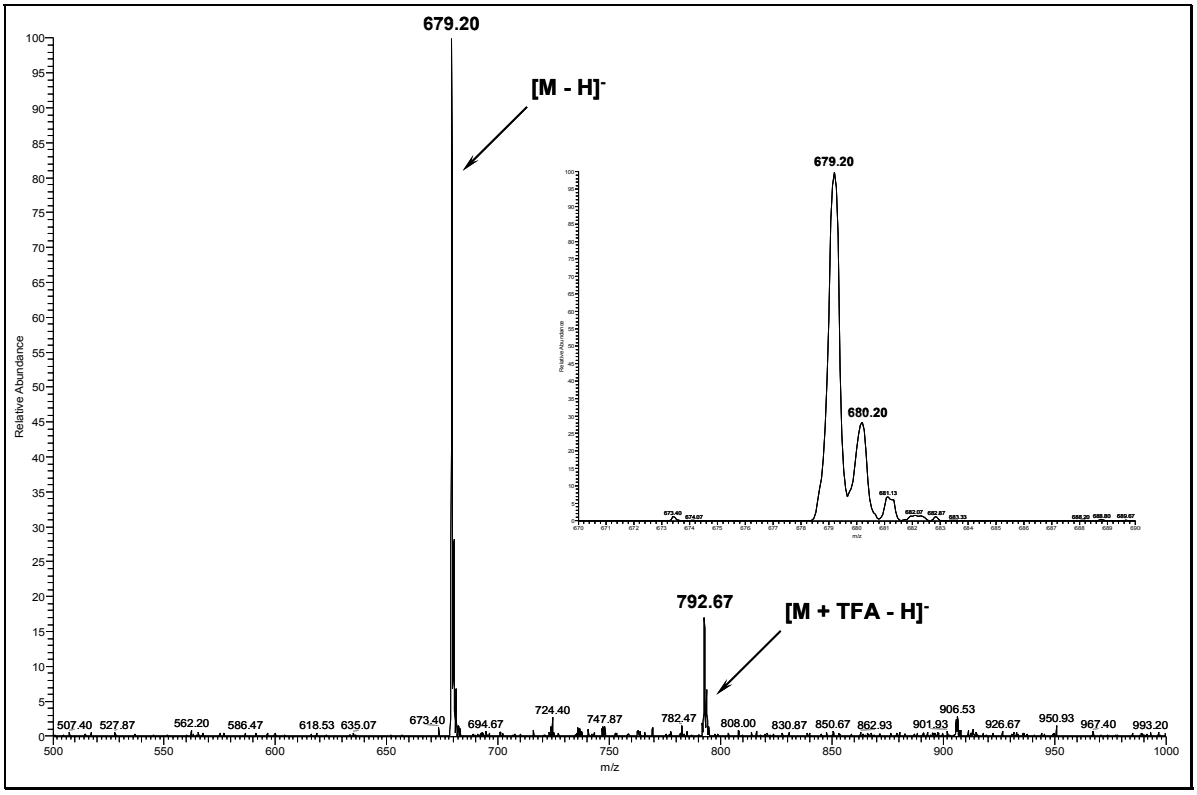


[a]*the broad peak can be explained by an incomplete protonation of lysine amino group of* ***8*** *under these HPLC conditions (TEAA 25 mM, pH 7.0 as aq. mobile phase). Indeed, it is not possible to analyse Ln(III) chelates with an acidic aq. mobile phase (e.g., TFA 0.1%) due to their instability under these HPLC elution conditions.*

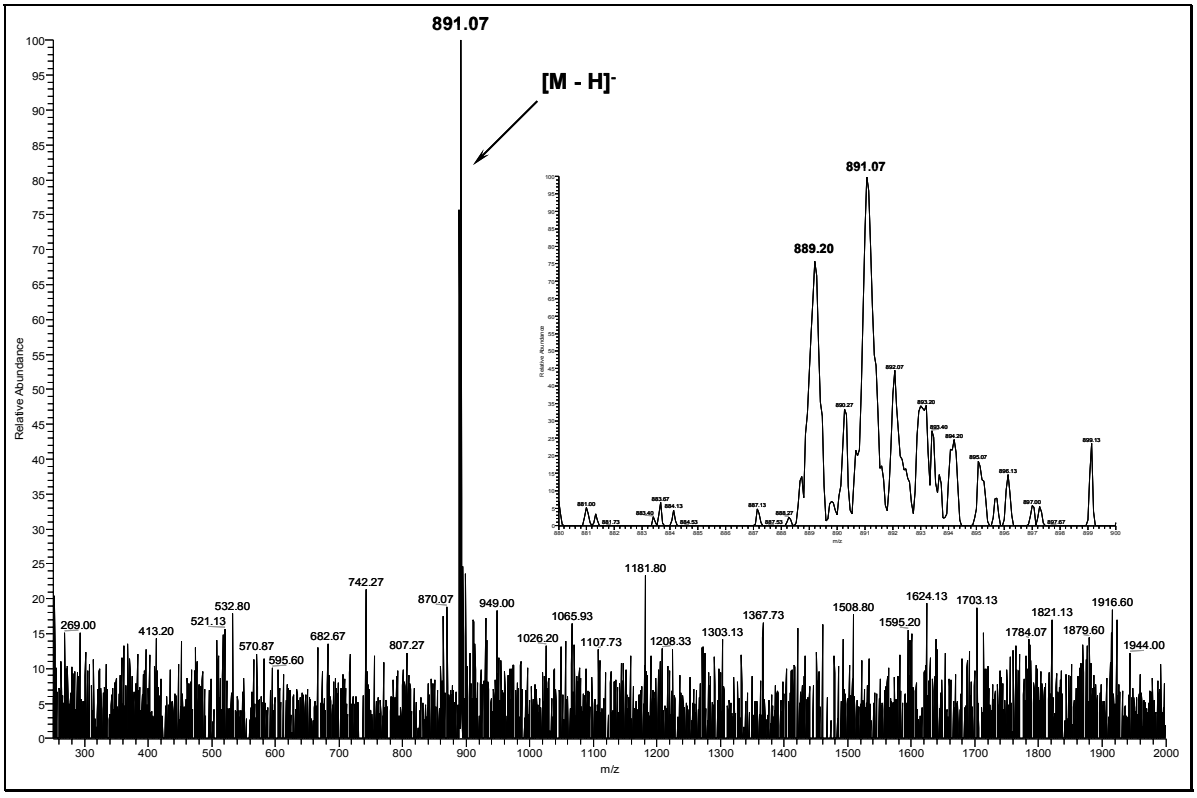
ESI-MS spectrum of Sm(III) chelate 7 recorded in the negative mode.



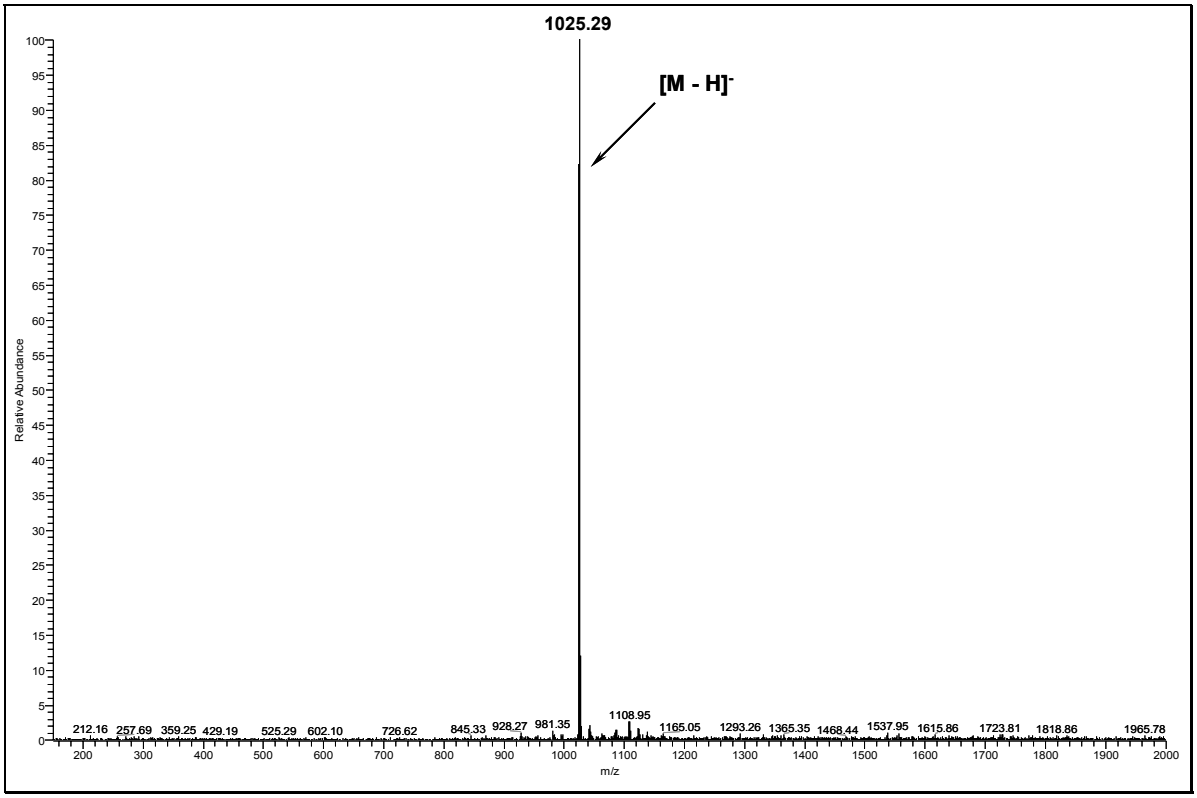
ESI-MS spectrum of Tb(III) chelate recorded in the negative mode.



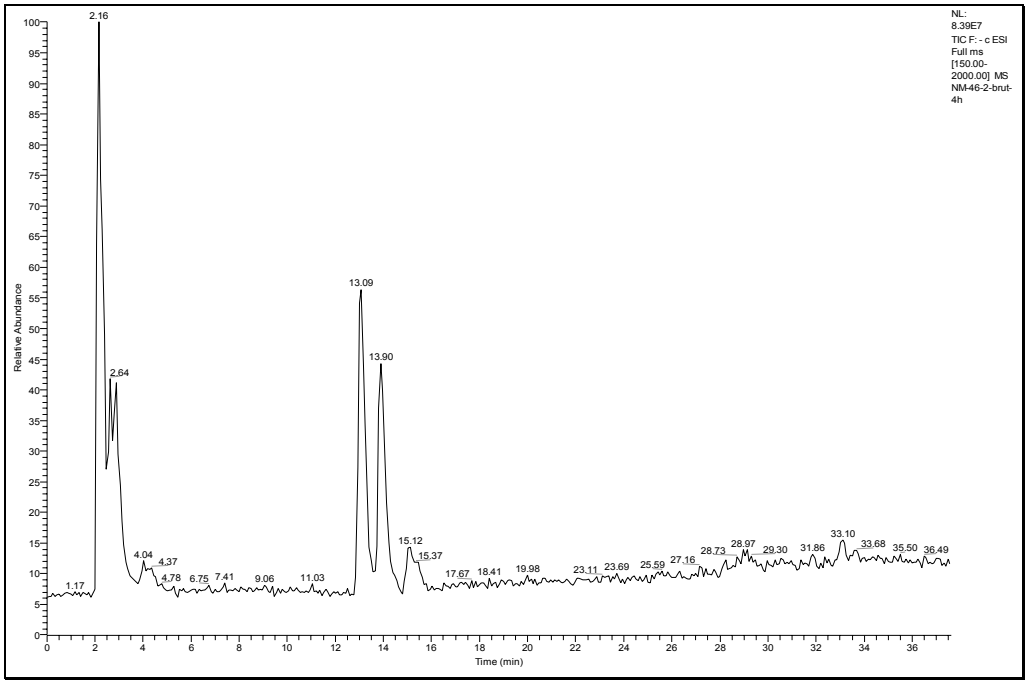
ESI-MS spectrum of activated Eu(III) chelate 23 recorded in the negative mode.



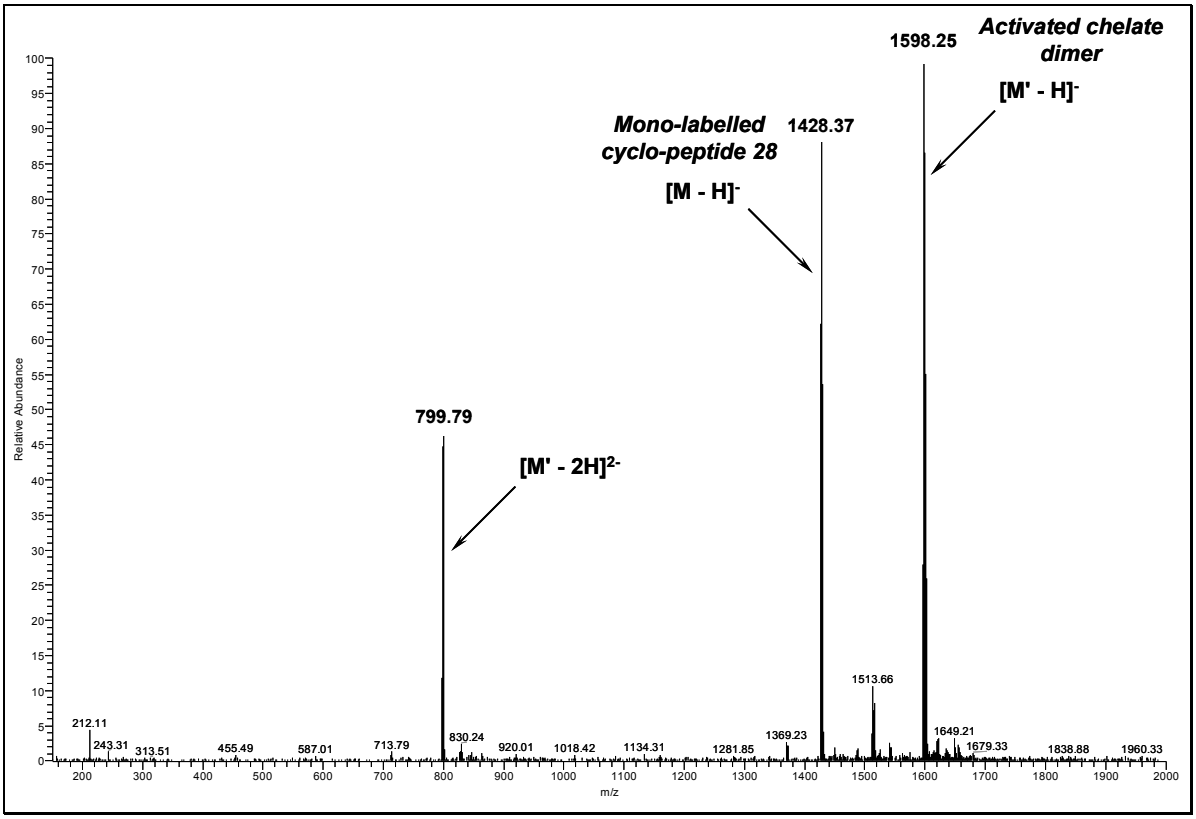
ESI-MS spectrum of activated Eu(III) chelate 24 recorded in the negative mode.



LC-MS analysis (system I) of the crude labelling mixture of hexapeptide 25 with activated Eu(III) chelate 23.

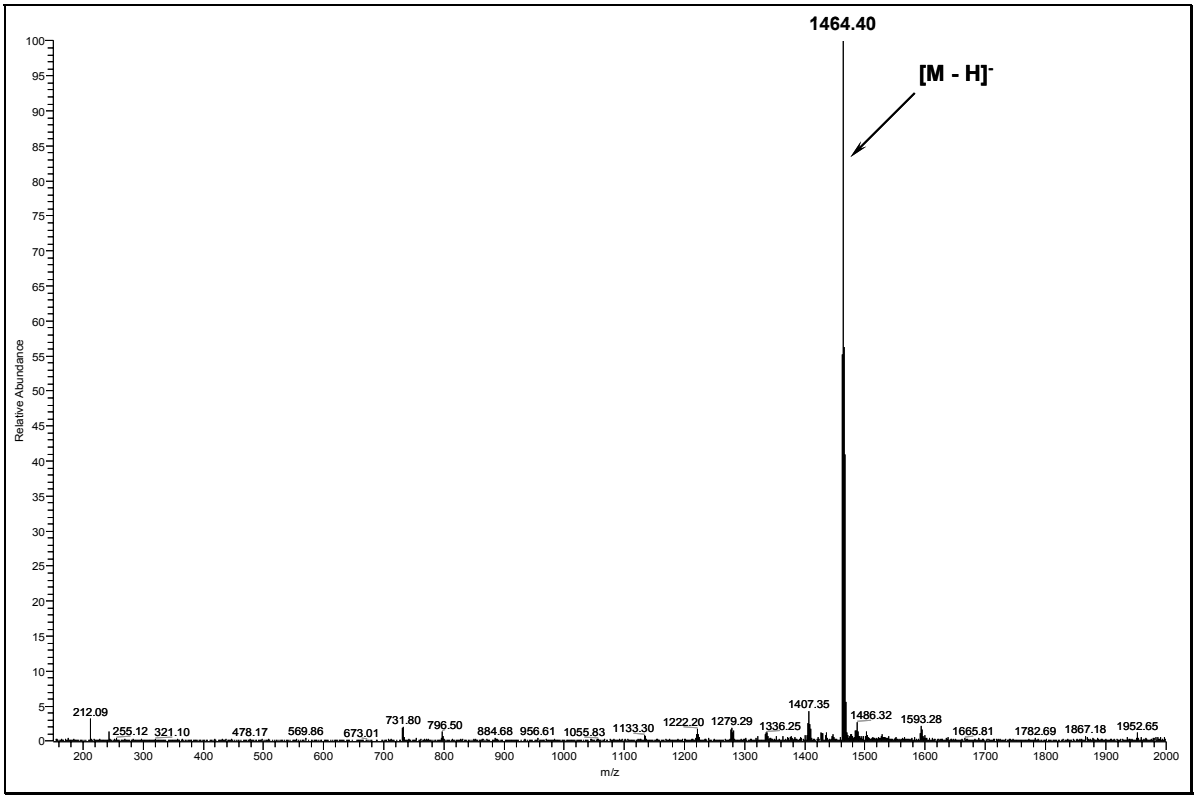


ESI mass spectrum (recorded in the negative mode) for the peak at t_R = 13.1 min[a].

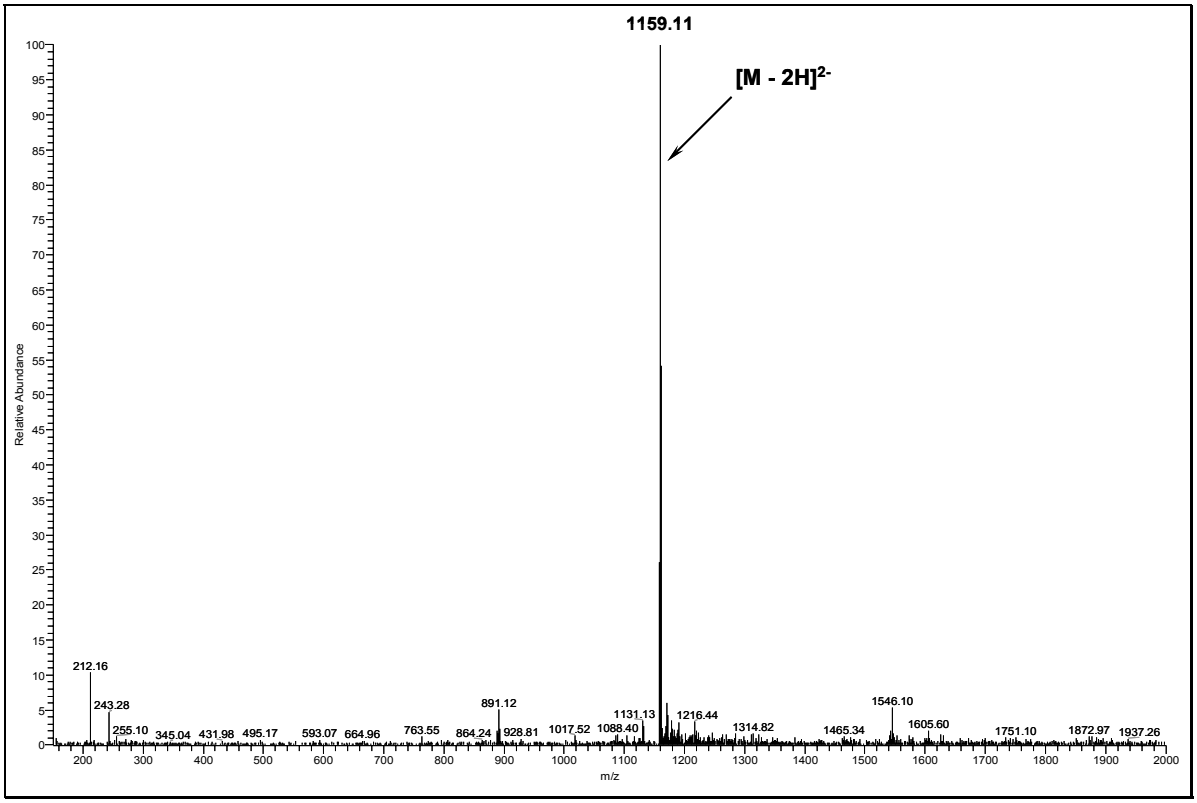


[a] *Under these chromotagraphic conditions, luminescent mono-labelled cyclo-peptide* ***28*** *and activated chelate dimer are not resolved and so eluted as a single peak.*

ESI mass spectrum (recorded in the negative mode) for the peak at t_R = 13.9 min, assigned to the luminescent labelled peptide 26.



ESI mass spectrum (recorded in the negative mode) for the peak at t_R = 15.1 min assigned to the bis-labelled peptide 29.



ESI-MS spectrum of luminescent labelled peptide 27 recorded in the negative mode.