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Dual enzyme-responsive "turn-on" fluorescence sensing systems based on *in situ* formation of 7-hydroxy-2-iminocoumarin scaffolds

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Supporting Information

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Abbreviations

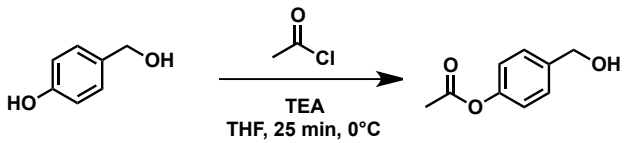
The following abbreviations are used throughout the text of the ESI file: Ar, argon; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; equiv., equivalent(s); Et_2O, diethyl ether; EtOAc, ethyl acetate; EtOH, ethanol; ESI, electrospray ionisation; FA, formic acid; HPLC, high-pressure liquid chromatography; LRMS, low-resolution mass spectrum; min, minutes; Na_2SO_4, sodium sulfate; NADH, nicotinamide adenine dinucleotide; $NaHCO_3$, sodium hydrogenocarbonate; NTR, nitroreductase; PABA, *para*-aminobenzyl alcohol; PE, petroleum ether (bp 40-60 °C); PHBA, *para*-hydroxybenzyl alcohol; PGA, penicillin G acylase; PLE, porcine liver esterase; MS, mass spectrometry; PMT, photomultiplier tube; RT, room temperature; TBDMS, *tert*-butyldimethylsilyl; TEA, triethylamine; TEAB, triethylammonium bicarbonate; THF, tetrahydrofuran; TLC, thin-layer chromatography; UV, ultraviolet.

High-performance liquid chromatography separations

Several chromatographic systems were used for the analytical experiments (HPLC-MS or HPLC-fluorescence): System A: RP-HPLC-MS (Phenomenex Kinetex C_{18} column, 2.6 µm, 2.1 × 50 mm) with CH_3CN (+ 0.1% FA) and 0.1% aq. FA (pH 3.2) as eluents [linear gradient from 5% to 100% (5 min) of CH_3CN followed by isochratic at 100% (1.5 min)] at a flow rate of 0.5 mL min^{-1}. UV-visible detection was achieved at 220, 260, 300 and 360 nm (+ diode array detection in the range 220-500 nm). ESI-MS detection in the positive/negative mode ("full scan", 150-1500 a.m.u., data type: centroid, needle voltage: 3.0 kV, detector voltage: 1100 V, probe temperature: 350 °C, cone voltage: 75 V and scan time: 1 s). System B: System A with 100-700 a.m.u for "full scan" mass detection. System C: System A with the following gradient [0% CH_3CN (2 min) followed by linear gradient from 0% to 100% (6 min) of CH_3CN followed by isochratic at100% (1 min)]. UV-visible detection was achieved at 220, 260, 350 and 418 nm (+ diode array detection in the range 220-500 nm). System D: RP-HPLC-fluorescence (Phenomenex Kinetex C_{18} column, 2.6 µm, 2.1 × 50 mm) with CH_3CN and aq. TEAB (50 mM, pH 7.5) as eluents [0% CH_3CN (1 min) followed by linear gradient from 0% to 100% (5 min) of CH_3CN followed by isocharatic at 100%] at a flow rate of 0.5 mL min^{-1}. Fluorescence detection was achieved at 45 °C at teh following Ex. /Em. channels: 350/460 nm and 418/458 nm (sensitivity: 1, PMT 1, filter wheel: auto). System E: System D with the following Ex./Em. channels for fluorecence detection: 350/460 nm, 431/488 nm and 455/489 nm.

Synthesised compounds

para-Acetoxybenzyl alcohol - Ac-PHBA (S1)[1]

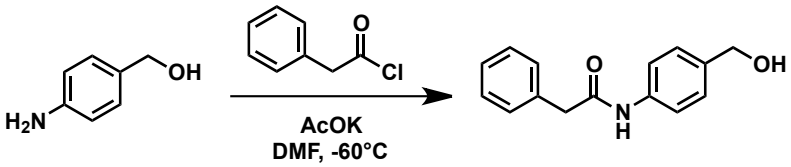


Under Ar atmosphere, 4-hydroxybenzyl alcohol (2 g, 16.1 mmol) was dissolved in dry THF (27 mL), cooled to 0 °C with an ice-water bath and TEA (2.23 mL, 16.1 mmol, 1 equiv.) was added. Then acetyl chloride (1.26 mL, 17.7 mmol, 1.1 equiv.) was added dropwise over a period of 25 min. The resulting reaction mixture was stirred for 2 h. Thereafter, the newly formed precipitate was removed by filtration and filtrate was evaporated to dryness. The crude was diluted with DCM, washed twice with aq. 5% $NaHCO_3$ and finally with deionised water. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under

[1]H. J. Jessen, T. Schulz, J. Balzarini and C. Meier, *Angew. Chem., Int. Ed.*, 2008, **47**, 8719.

vacuum. The crude product was purified by chromatography on a silica gel column (PE-EtOAc, step gradient from 100 to 65: 35, v/v) to give the desired acetate **S1** as light yellow oil which crystallized as white solid after overnight storage at 4 °C (1.34 g, yield 50%). R_f 0.27 (heptane-EtOAc, 6 : 4, v/v); δ_H(300 MHz, $CDCl_3$) 7.37 (d, *J* 8.4, 2 H), 7.06 (d, *J* 8.7, 2 H), 4.65 (d, *J* 4.8, 2 H), 2.29 (s, 3 H), 1.92 (bt, 1 H).

N-phenylacetamidobenzyl alcohol - PhAc-PABA (S2)[2]



Under Ar atmosphere, PABA (1 g, 8.1 mmol, 1 equiv.) and potassium acetate (1.6 g, 16.2 mmol, 2 equiv.) were dissolved in dry DMF (80 mL), cooled to -60 °C with a $CHCl_3$/liq. N_2 bath and phenylacetyl chloride (1.1 mL, 8.1 mmol, 1 equiv.) was added dropwise to the mixture; each added drop caused a yellow discoloration which rapidly fades before adding the next drop of phenylacetyl chloride. The resulting reaction mixture was left to warm at RT and then was diluted with aq. 1.0 M NaOH (20 mL). Thereafter, the mixture was neutralised with aq. 1.0 M HCl to reach pH 7 and extracted with DCM. Organic layer was washed with deionised water and brine and finally dried over anhydrous Na_2SO_4. After concentration under reduced pressure, the crude was taken with heptane and then with DCM. The solid was recovered by filtration to give the desired phenylacetamide derivative **S2** as white solid (1.10 g, yield 56%). R_f 0.5 (DCM-EtOAc, 7 : 3, v/v); δ_H(300 MHz, DMSO-d_6) 10.15 (s, 1 H), 7.53 (d, *J* 9.6, 2 H), 7.33 (d, *J* 4.8, 4 H), 7.24 (m, 3 H), 5.17 (t, *J* 5.7, 1 H), 4.43 (d, *J* 5.8, 2 H).

In vitro activation of fluorogenic "turn-on" probes 5, 8 and 9 by hydrolase (PGA or PLE) and reductase (NTR) - experimental details

Stock solutions of probes and enzymes:

- Mixture A: A stock solution (1.0 mg / mL) of PGA-NTR fluorogenic probe **9** in DMSO (for spectroscopy, 99.9%, ACROS, 167852500) (final concentration: 1.70 mM),
- Mixture B: A stock solution (1.0 mg / mL) of PLE-NTR fluorogenic probe **8** in DMSO (final concentration: 1.95 mM),
- Mixture C: A stock solution (1.0 mg / mL) of PLE-NTR fluorogenic probe **5** in DMSO (final concentration: 1.61 mM),
- Mixture D: 6.28 mg of PGA (0.63 U / mg) was dissolved in 1 mL of PB (3.95 U / mL),
- Mixture E: 1.12 mg of PLE (27 U / mg) was dissolved in 150 µL of PB and 150 µL of ultrapure H_2O (0.1 U / µL),
- Mixture F: 0.93 mg of PLE (27 U / mg) was dissolved in 1 mL of PB (25.11 U / mL),
- Mixture G: 24.33 mg of NADH (MW: 709.4) was dissolved in 245 µL of H_2O (final concentration: 140 mM).
- Mixture H: commercial lyophilised NTR + buffer (1 mg of protein, 100 U / mg) was resuspended in 1 mL of ultrapure water (0.1 U / µL).

Stock solutions (1.0 mg / mL) of 3-(2-benzothiazolyl)-7-hydroxycoumarin (final concentration: 3.4 mM), 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin (final concentration: 3.4 mM), 3-cyano-7-hydroxycoumarin (final concentration: 5.3 mM), 3-cyano-7-hydroxy-2-iminocoumarin (final concentration: 5.4 mM) were also prepared in DMSO and

[2]S. A. Nuñez, K. Yeung, N. S. Fox and S. T. Phillips, *J. Org. Chem.*, 2011, **76**, 10099.

subsquently diluted with PB for UV-vis absorption and fluorescence measurements, and HPLC-fluorescence analyses.

Fluorescence assays:
All assay were performed at 37 °C (conducted with or without magnetic stirring, no difference was noted). For probes **8** and **9**, the fluorescence emission of the release 3-cyano-7-hydroxy-2-iminocoumarin was monitored at λ = 458 nm (emission slit = 2 nm) (Ex. λ = 418 nm, excitation slit = 2 nm) over time with measurements recorded every 5 s. For probe **5**, the fluorescence emission of the release 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin was monitored at λ = 489 nm (emission slit = 2 nm) (Ex. λ = 455 nm, excitation slit = 2 nm) over time with measurements recorded every 5 s.

Sequential protocol (hydrolase then NTR):
Probe **9** - Into a 3.5 mL fluorescence quartz cell, 2 µL of mixture A was diluted in 2.750 mL of PB, then 245 µL of mixture D (1 U) was added and the resulting mixture was incubated for 10 min. Then 1 µL of mixture G and 1 µL of mixture H were added.
Probe **8** - Into a 3.5 mL fluorescence quartz cell, 1.5 µL of mixture B was diluted in 2.990 mL of PB, then 10 µL of mixture E (1 U) was added and the resulting mixture was incubated for 10 min. Then 1 µL of mixture G and 1 µL of mixture H were added.
Probe **5** - Same as probe **8** by replacing 1.5 µL of mixture B by 2 µL of mixture C.

Sequential protocol (NTR then hydrolase):
Probe **9** - Into a 3.5 mL fluorescence quartz cell, 2 µL of mixture A was diluted in 2.750 mL of PB, then 1 µL of mixture G and 1 µL of mixture H were added together and the resulting mixture was incubated until the fluorescent intensity was reached a constant level. Then 245 µL of mixture D (1 U) was added.
Probe **8** - Into a 3.5 mL fluorescence quartz cell, 1.5 µL of mixture B was diluted in 2.990 mL of PB, then 1 µL of mixture G and 1 µL of mixture H were added together and the resulting mixture was incubated until the fluorescent intensity was reached a constant level. Then 10 µL of mixture E (1 U) was added.
Probe **5** - Same as probe **8** by replacing 1.5 µL of mixture B with 2 µL of mixture C.

Simultaneous incubation:
Probe **9** - Into a 3.5 mL fluorescence quartz cell, 2 µL of mixture A was diluted in 2.750 mL of PB, then 1 µL of mixture G, 1 µL of mixture H and 245 µL of mixture D (1 U) were added together and the resulting mixture was incubated.
Probe **8** - Into a 3.5 mL fluorescence quartz cell, 1.5 µL of mixture B was diluted in 2.990 mL of PB, then 1 µL of mixture G, 1 µL of mixture H and 10 µL of mixture E (1 U) were added together and the resulting mixture was incubated.
Probe **5** - Same as probe **8** by replacing 1.5 µL of mixture B with 2 µL of mixture C.

HPLC-fluorescence analyses:
Enzymatic reaction mixtures from fluorescence assays were directly analysed by RP-HPLC-fluorescence (injected volume: 10 µL, system D for reaction conducted with cyano-based probes **8** and **9** and system E for those conducted with benzothiazolyl-based probe 5).

HPLC-MS analyses (enzyme assay and sample treatment):

Sequential protocol (hydrolase then NTR):
39 nmol of PGA-NTR (23 µL of mixture A) (or PLE-NTR (20 µL of mixture B)) fluorogenic probe **9** (or **8**) was dissolved in PB (260 µL (or 428 µL)) containing 190 µL of mixture D (0.75 U) (or 22 µL of mixture F (0.55 U)) and the resulting enzymatic reaction mixture was incubated at 37 °C for 80 min. Thereafter, 6 µL of mixture H (0.6 U) and 2 µL of mixture G were added together and the mixture was incubated for further 100 min. Samples (50 µL) were taken at 30 min, 1 h, 2 h and 3 h of reaction and were treated as described below.

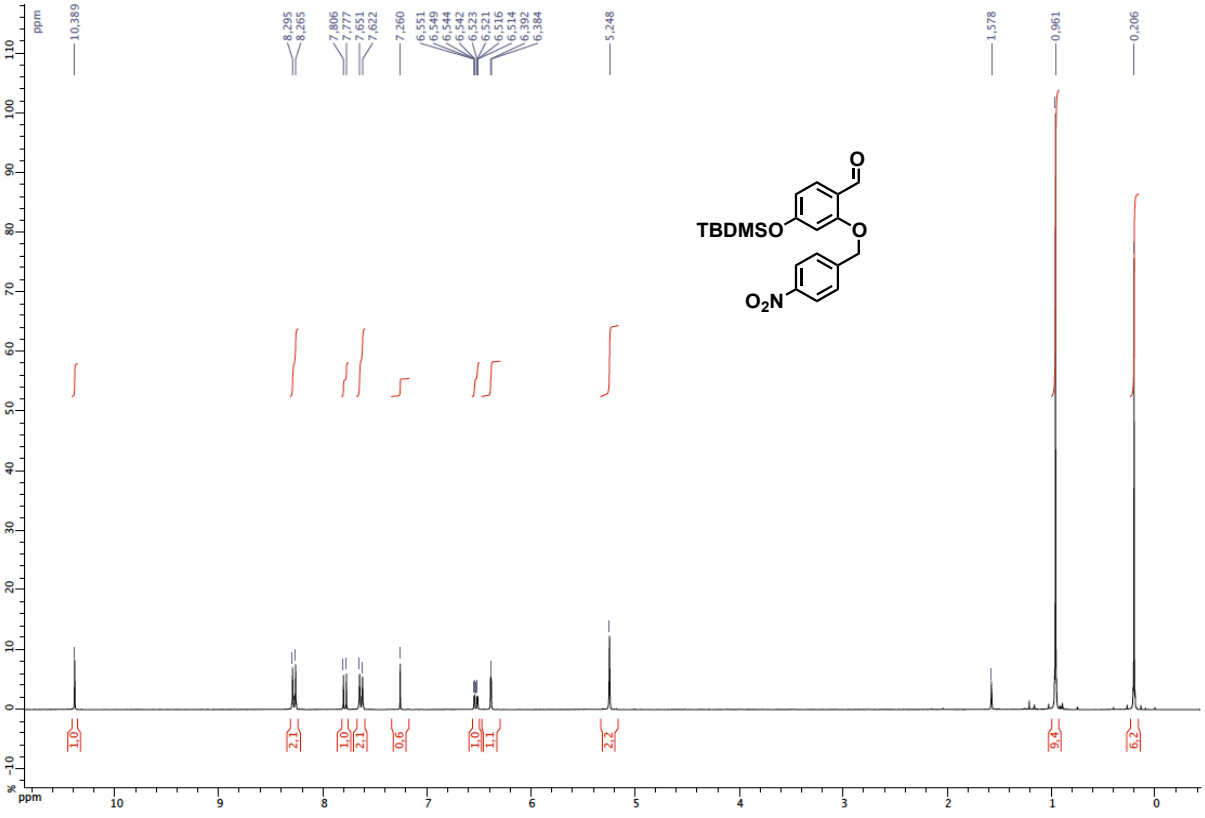
Sequential protocol (NTR then hydrolase):
39 nmol of PGA-NTR (23 µL of mixture A) (or PLE-NTR (20 µL of mixture B)) fluorogenic probe **9** (or **8**) was dissolved in PB (260 µL (or 428 µL)) containing 6 µL of mixture H (0.6 U) and 2 µL of mixture G and the resulting enzymatic reaction mixture was incubated at 37 °C for 1 h 20. Thereafter, 190 µL of mixture D (0.75 U) (or 22 µL of mixture F (0.55 U)) was added and the mixture was incubated for further 100 min. Samples (50 µL) were taken at 30 min, 1 h, 2 h and 3 h of reaction and were treated as described below.

Simultaneous incubation:
39 nmol of PGA-NTR (23 µL of mixture A) (or PLE-NTR (20 µL of mixture B)) fluorogenic probe **9** (or **8**) was dissolved in PB (260 µL (or 428 µL)) containing 6 µL of mixture H (0.6 U) and 2 µL of mixture G and 190 µL of mixture D (0.75 U) (or 22 µL of mixture F (0.55 U)) and the resulting enzymatic reaction mixture was incubated at 37 °C for 3 h. Samples (50 µL) were taken at 30 min, 1 h, 2 h and 3 h of reaction and were treated as described below.

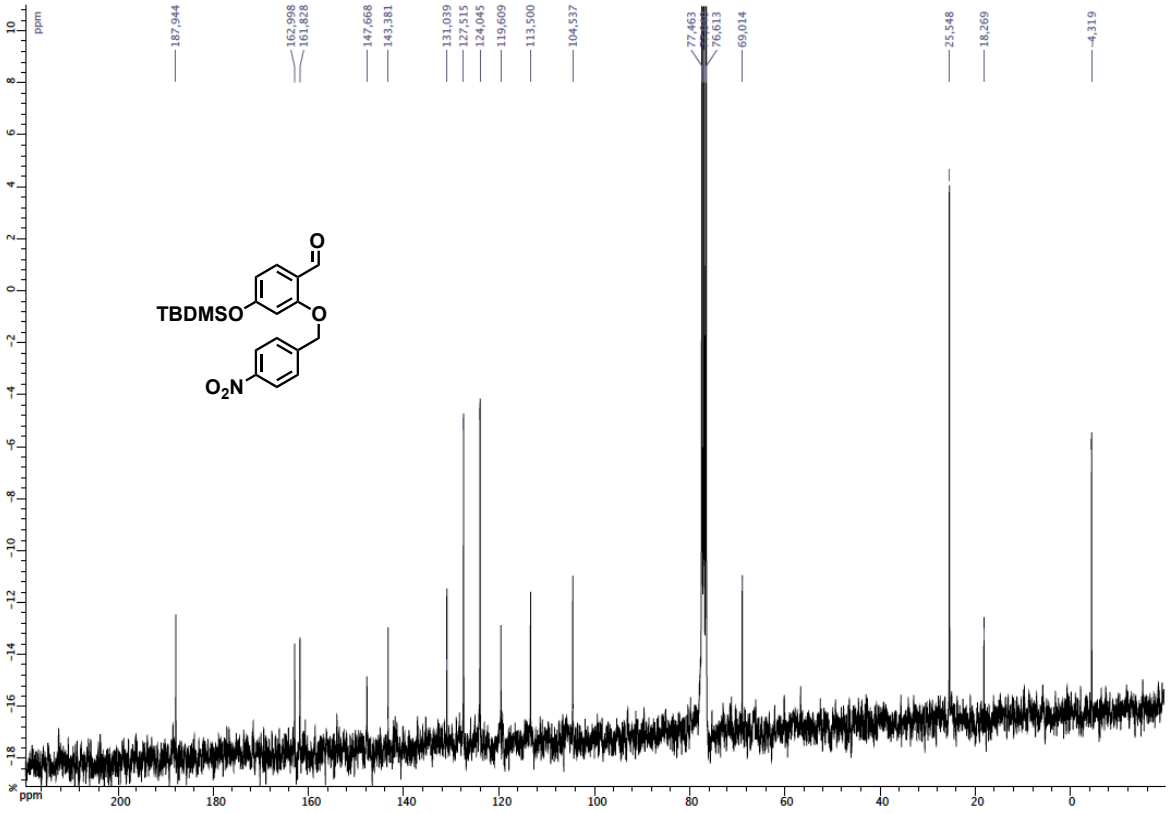
Samples treatment for HPLC-MS analysis:
Withdrawn sample (50 µL) was diluted with 50 µL of CH_3CN, then vortexed followed by centrifugation (9 000 rpm, 2 min) and finally, 75 µL of the supernatant was collected and diluted with 25 µL of aq. 0.1% FA. 10 µL was injected into the HPLC-MS apparatus (system C).

Analytical data

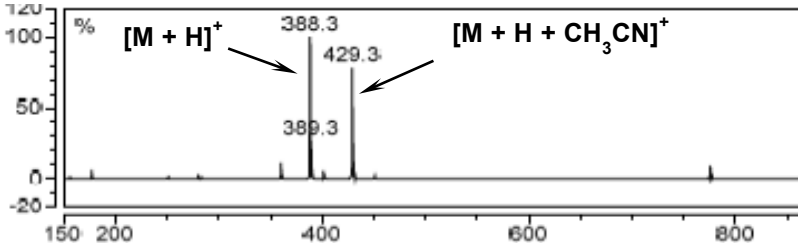
^{1}H NMR spectrum of compound 2 recorded in $CDCl_3$ at 300 MHz

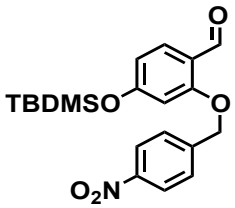


^{13}C NMR spectrum of compound 2 recorded in $CDCl_3$ at 75 MHz

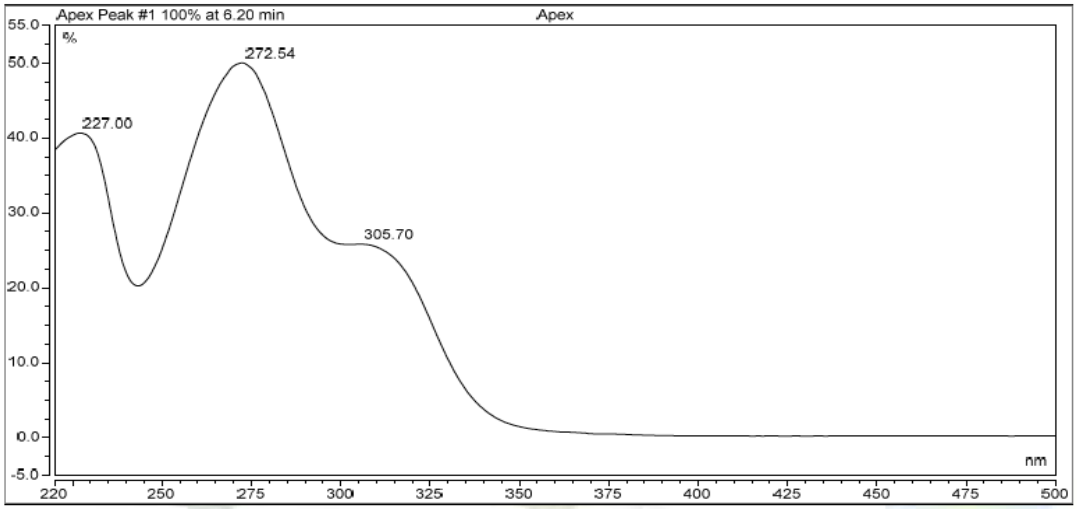


ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 2

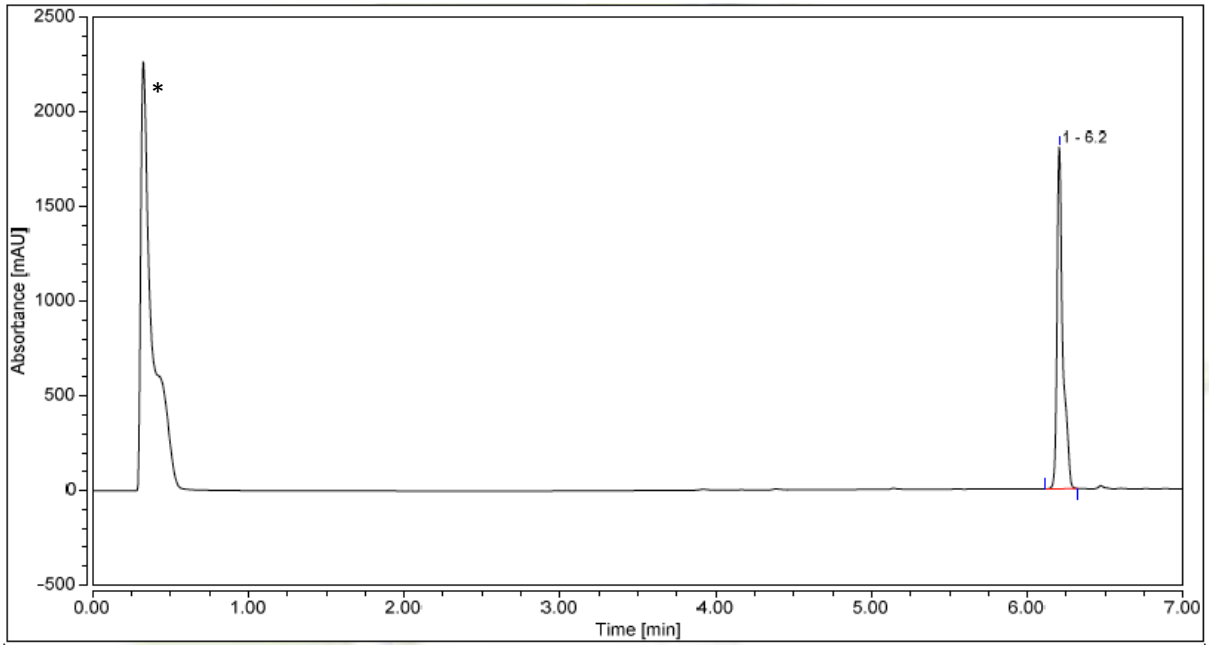




Chemical Formula: $C_{20}H_{25}NO_5Si$
Molecular Weight: 387,50

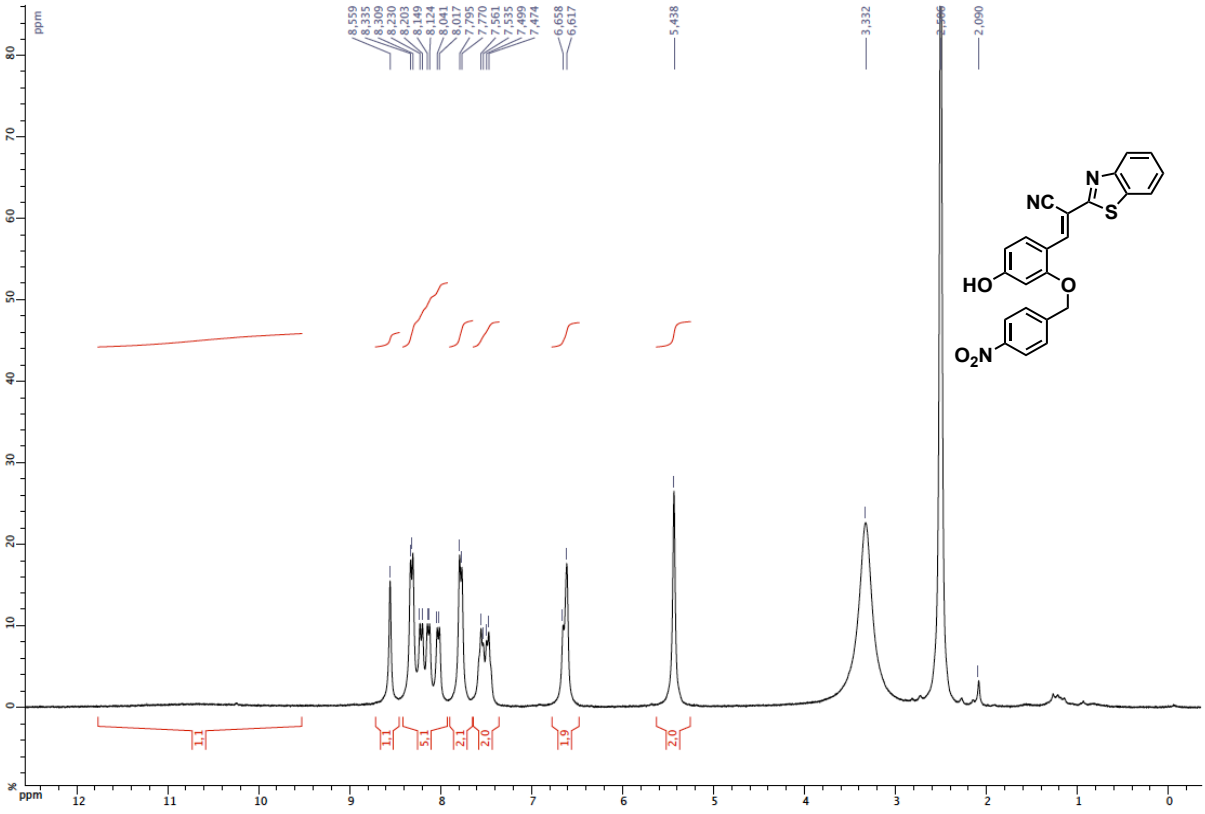


RP-HPLC elution profile (system A) of compound 2 at 260 nm

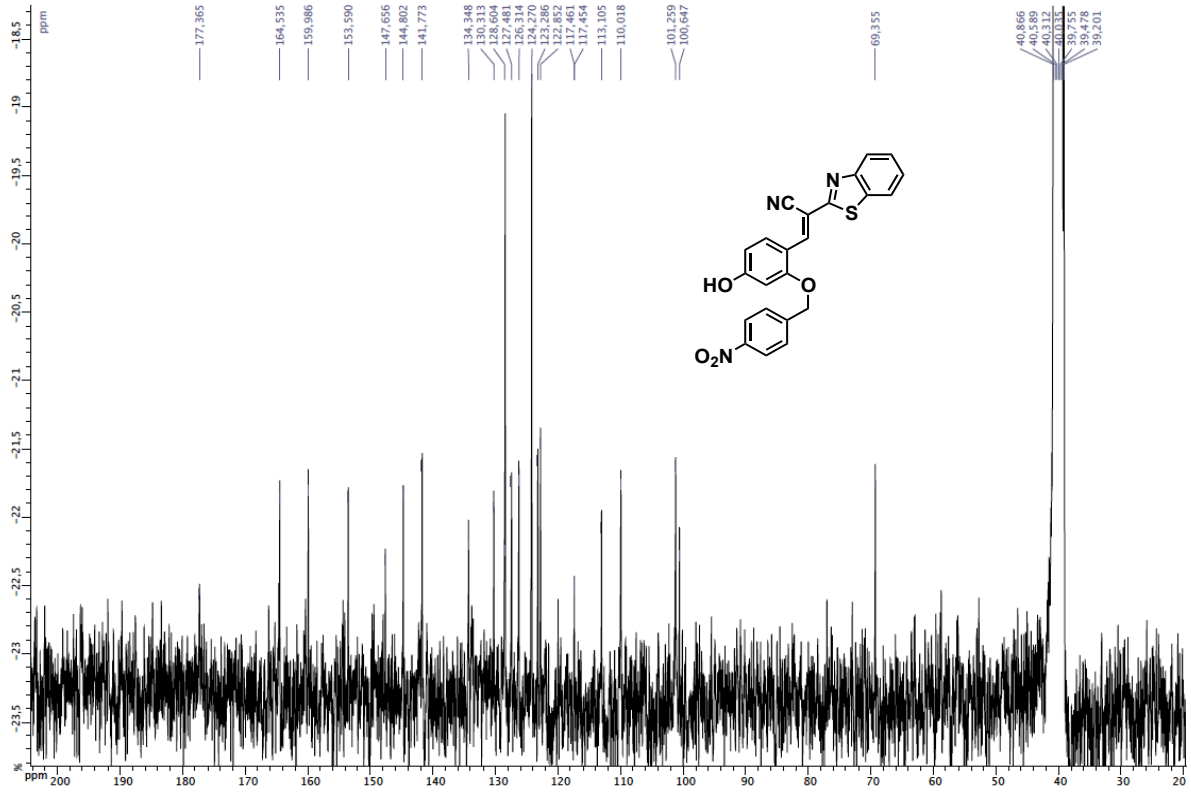


**peak assigned to acetone used as solvent for sample preparation*

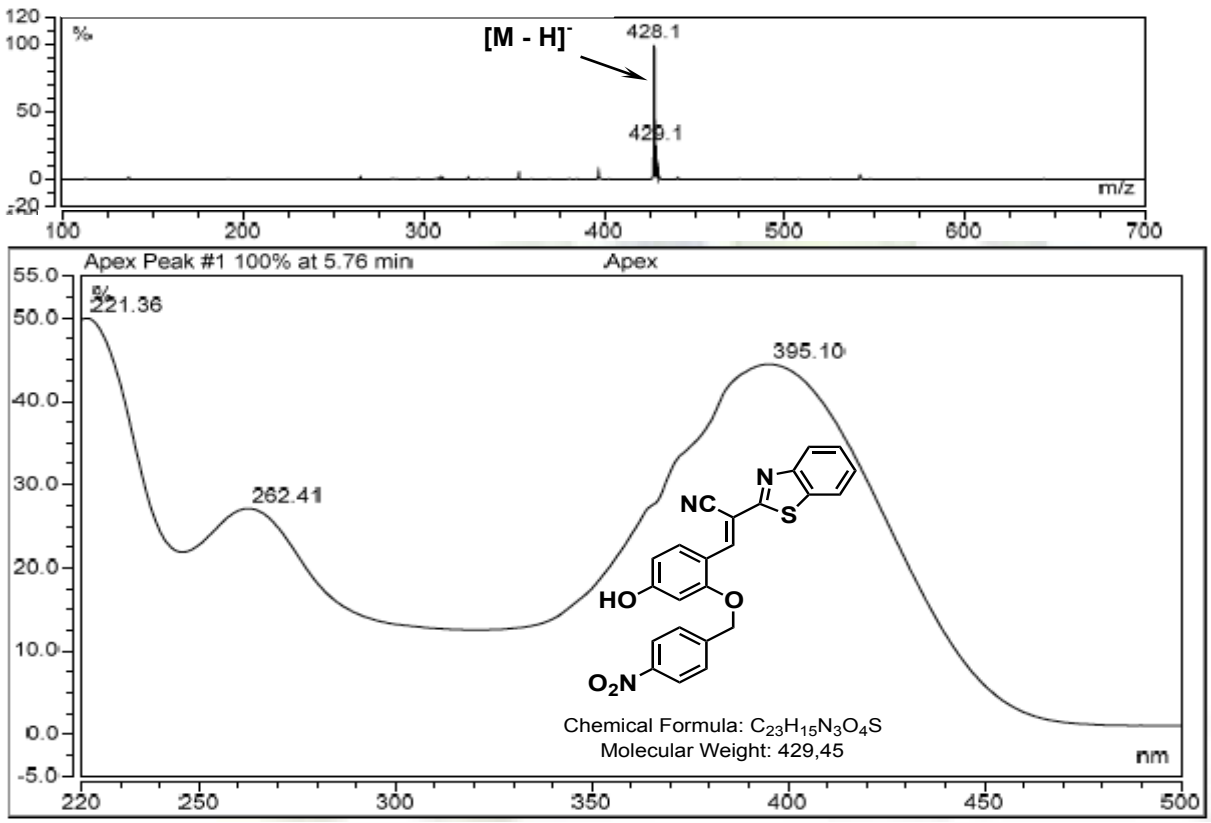
^{1}H NMR spectrum of compound 3 recorded in DMSO-d_6 at 300 MHz



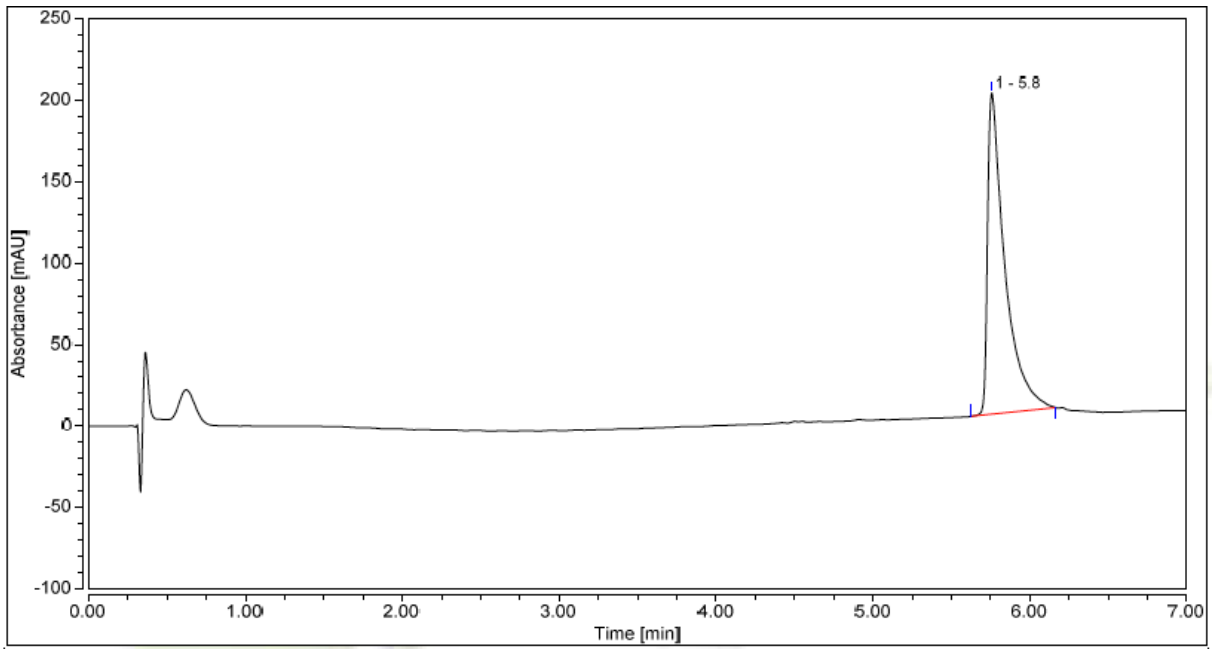
^{13}C NMR spectrum of compound 3 recorded in DMSO-d_6 at 75 MHz



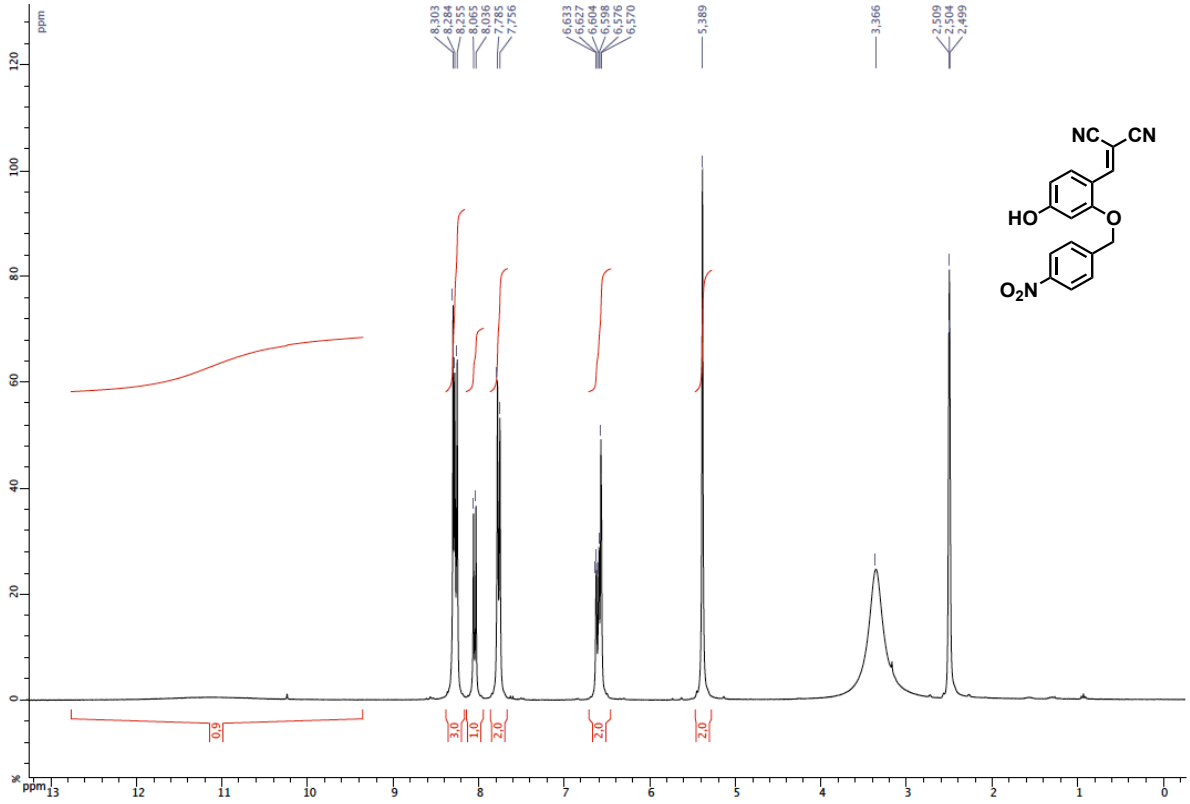
ESI- mass spectrum (low resolution) and UV-vis spectrum of compound 3



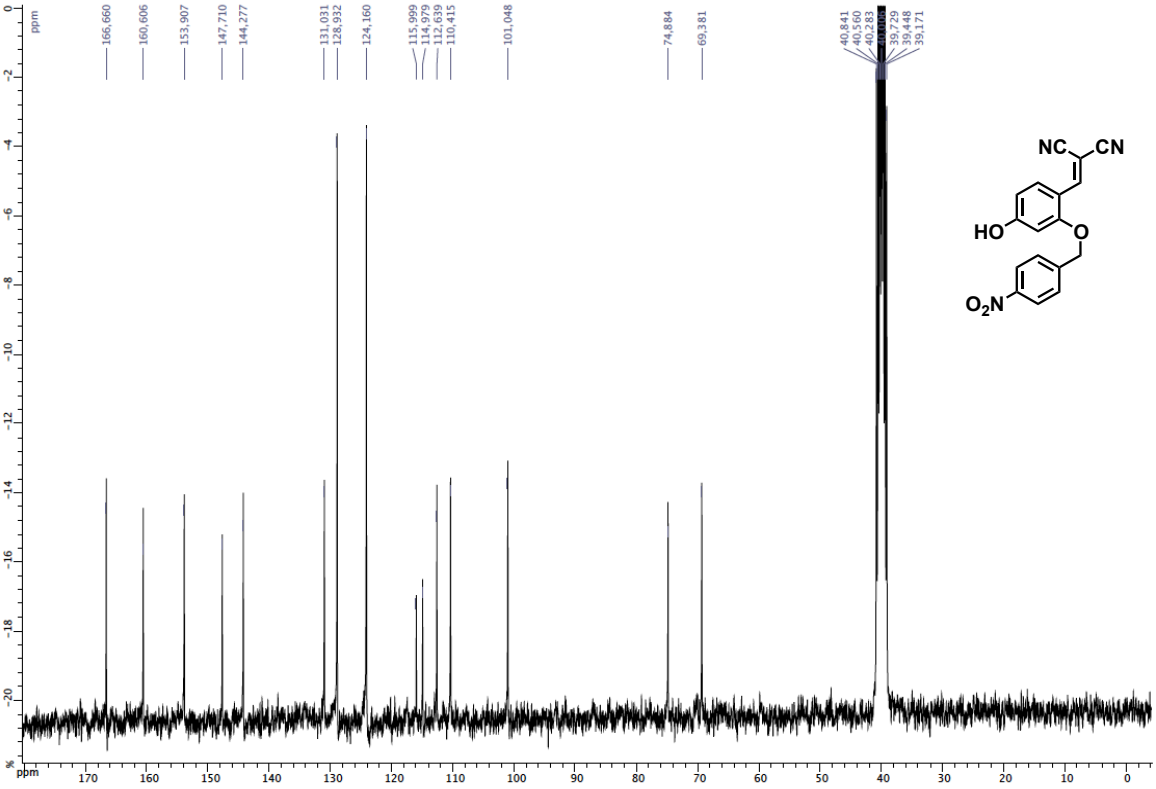
RP-HPLC elution profile (system B) of compound 3 at 260 nm



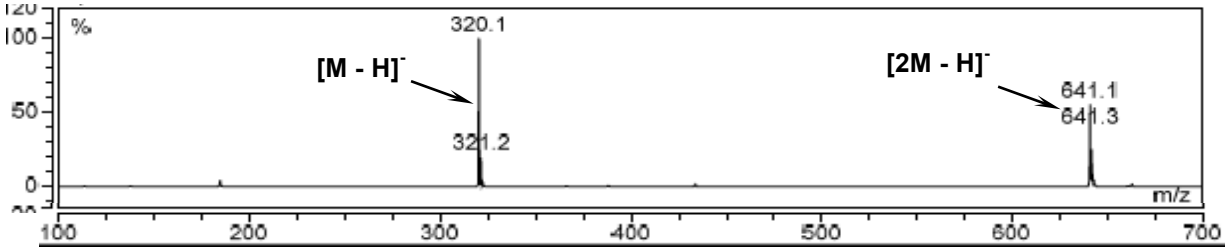
^{1}H NMR spectrum of compound 4 recorded in DMSO-d_6 at 300 MHz

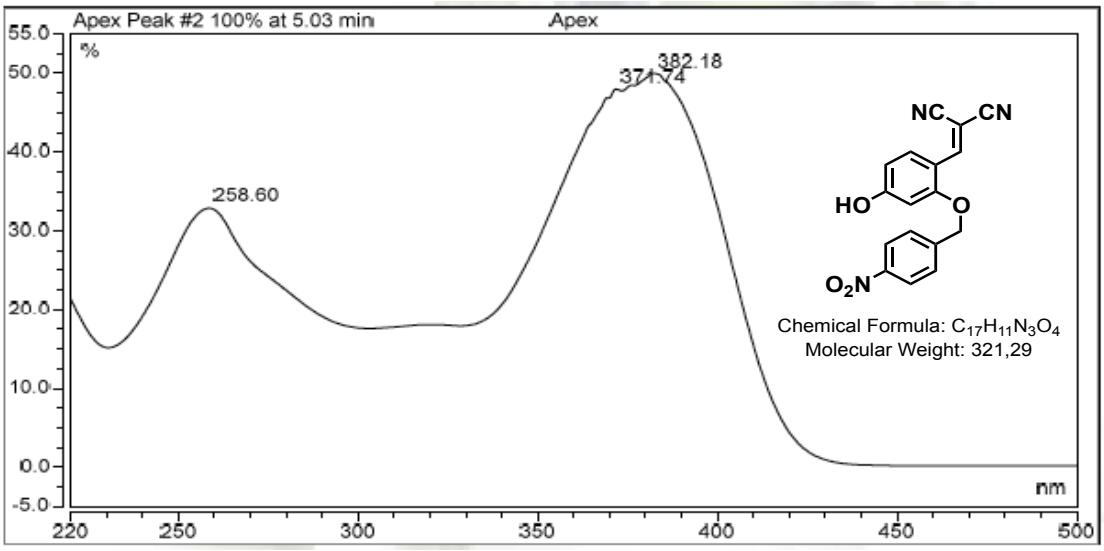


^{13}C NMR spectrum of compound 4 recorded in DMSO-d_6 at 75 MHz

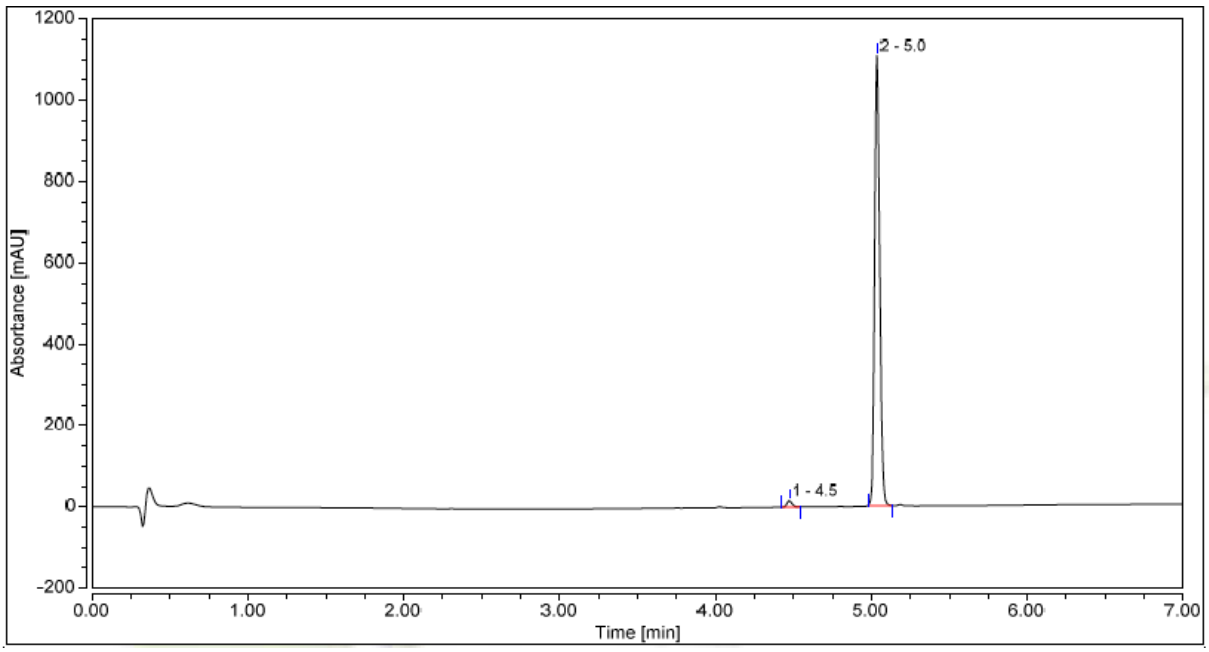


ESI- mass spectrum (low resolution) and UV-vis spectrum of compound 4

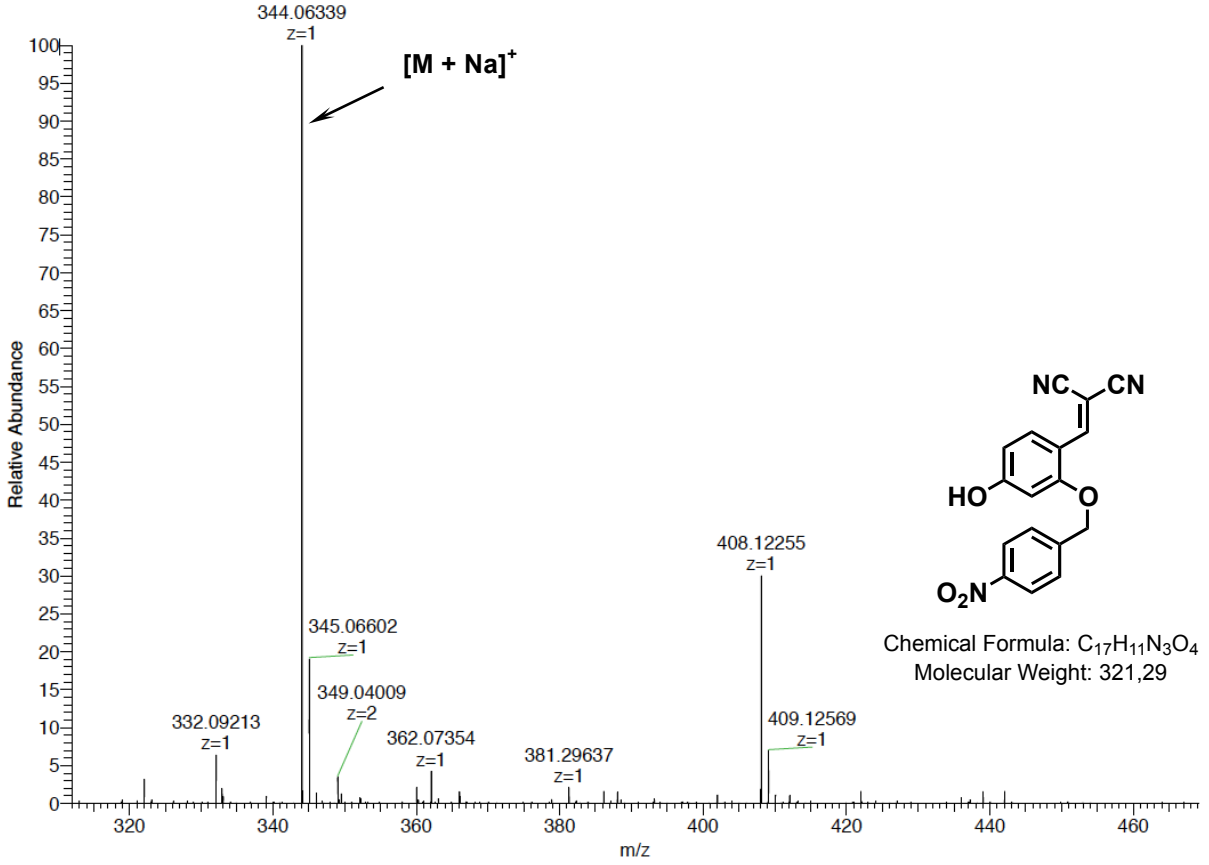


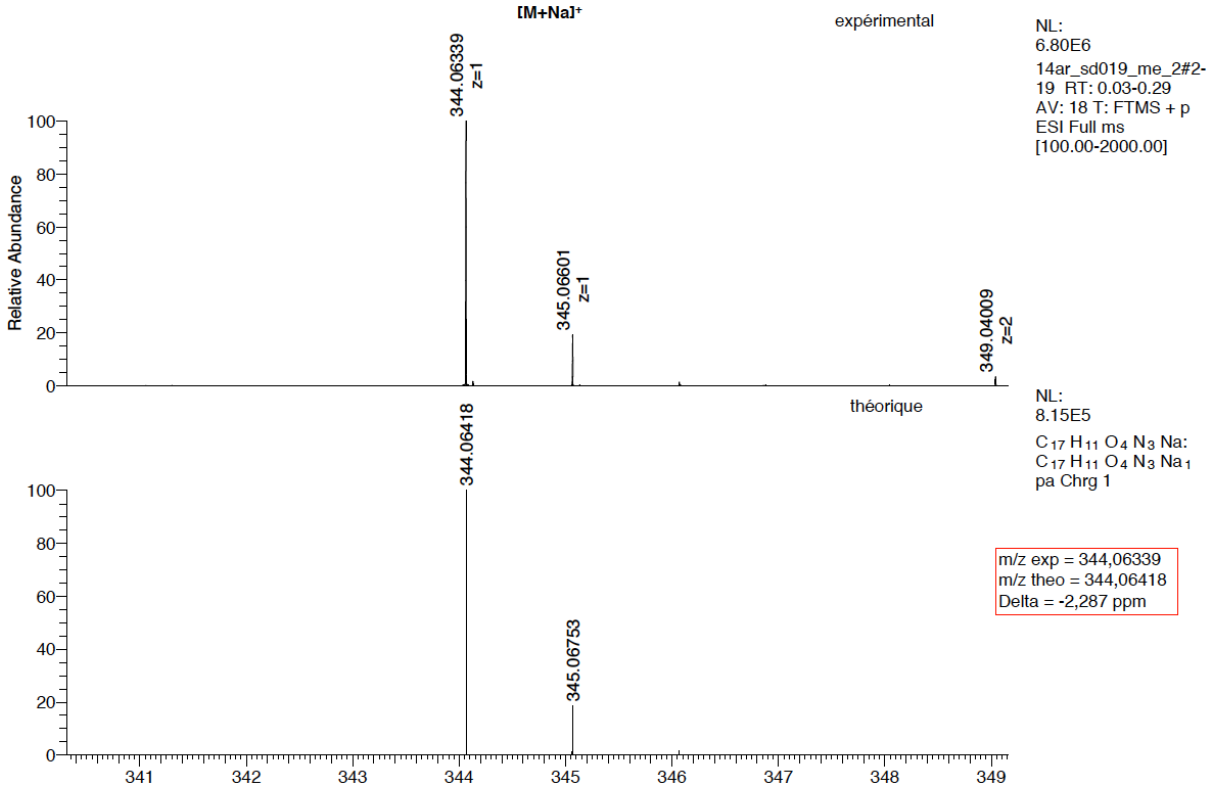


RP-HPLC elution profile (system B) of compound 4 at 260 nm

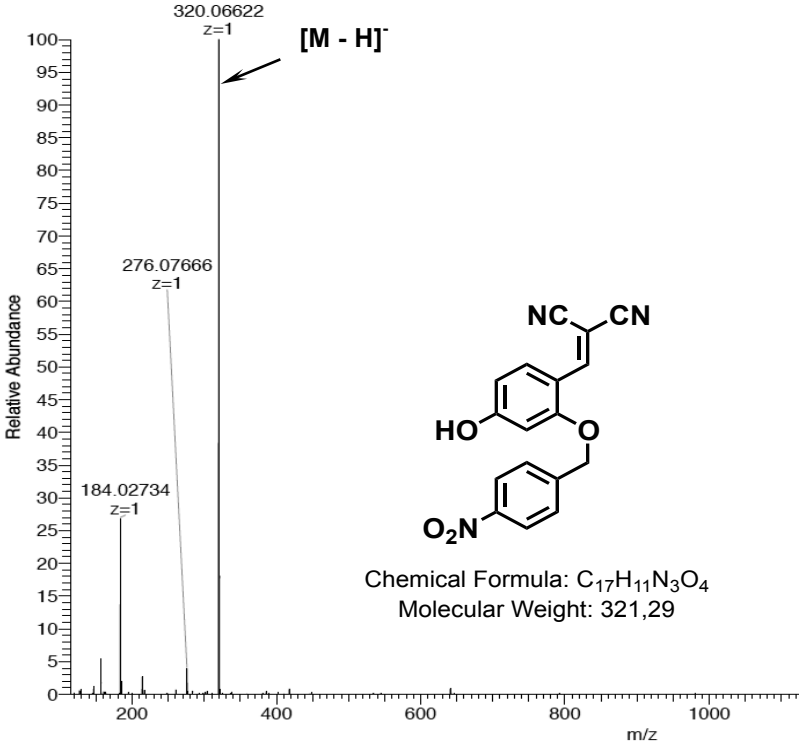


ESI+ mass spectrum (high resolution) of compound 4

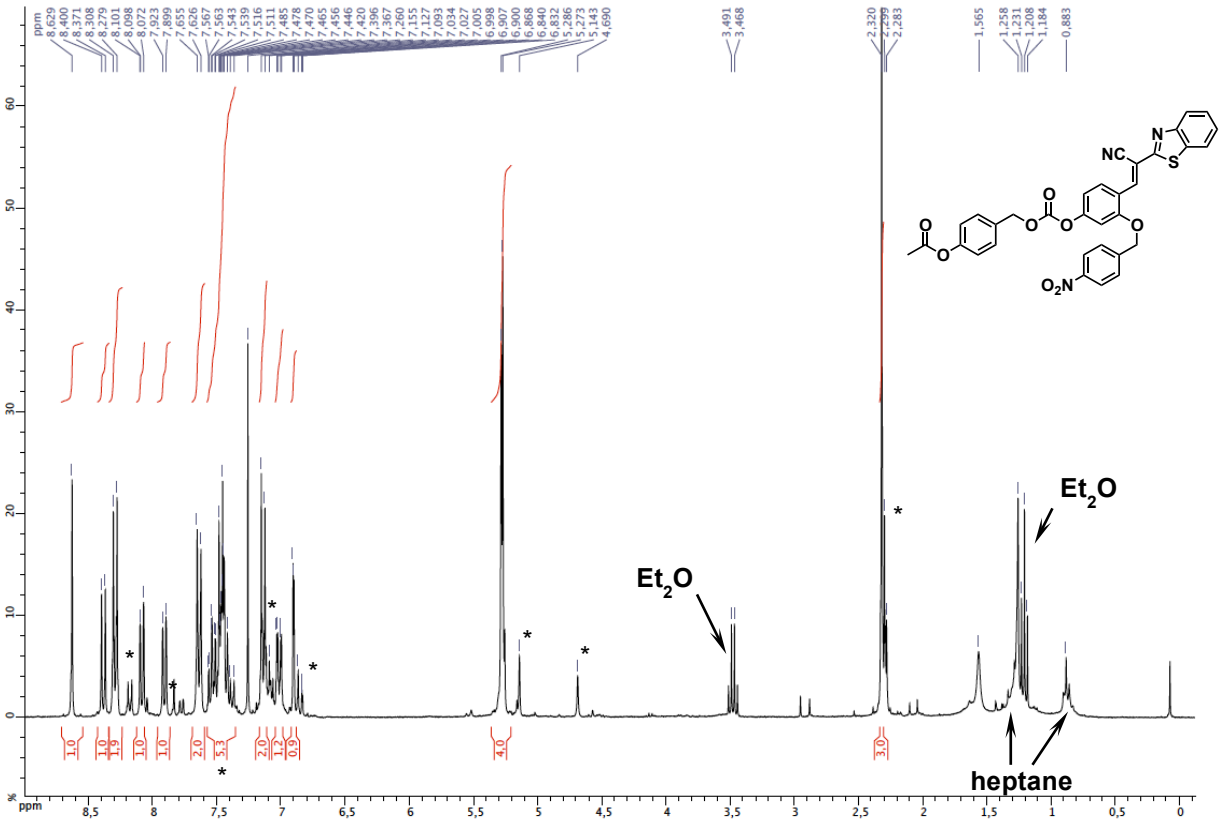




ESI- mass spectrum (high resolution) of compound 4

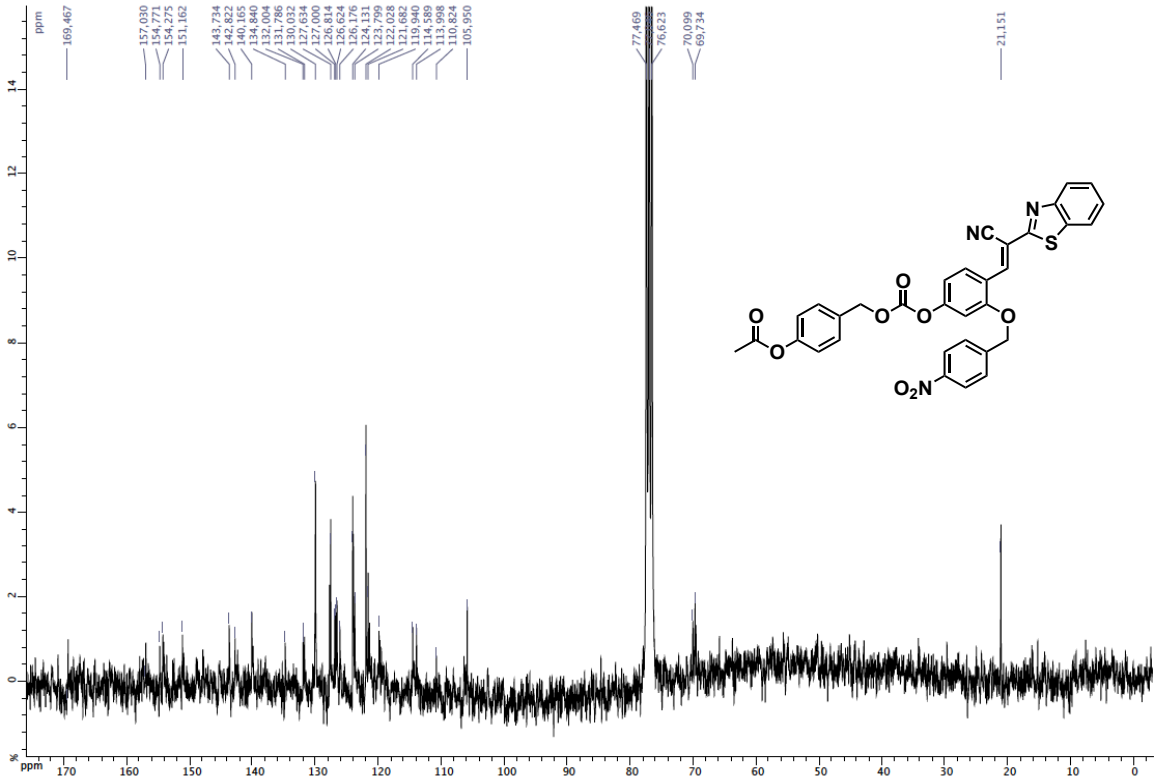


^{1}H NMR spectrum of compound 5 recorded in $CDCl_3$ at 300 MHz

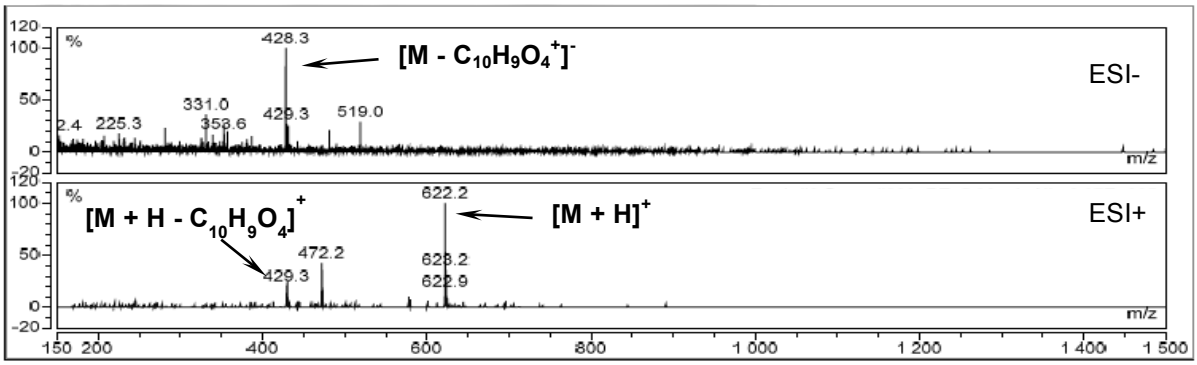


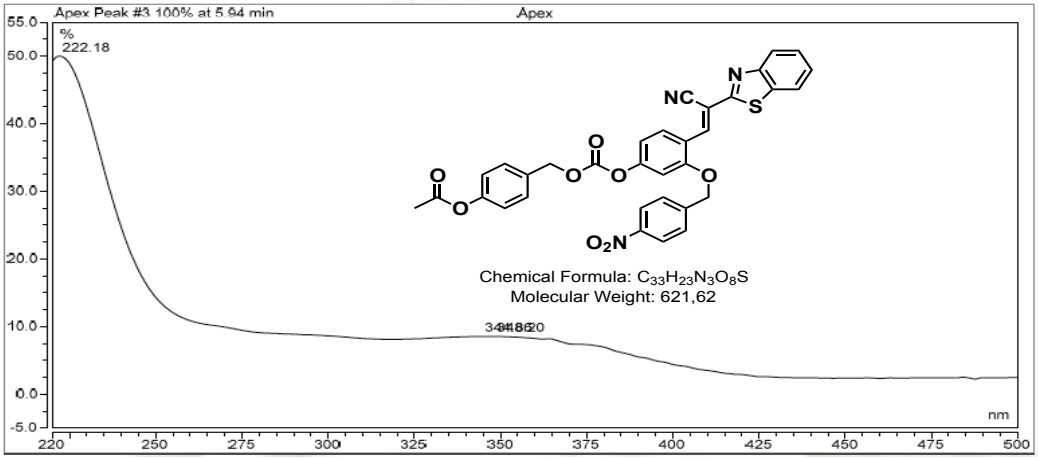
**peaks assigned to 2nd geometric isomer (15 : 85)*

^{13}C NMR spectrum of compound 5 recorded in $CDCl_3$ at 125 MHz

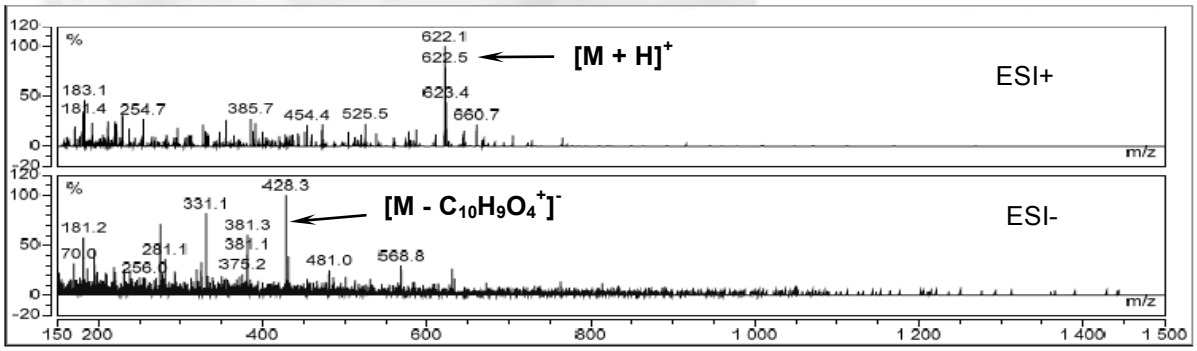


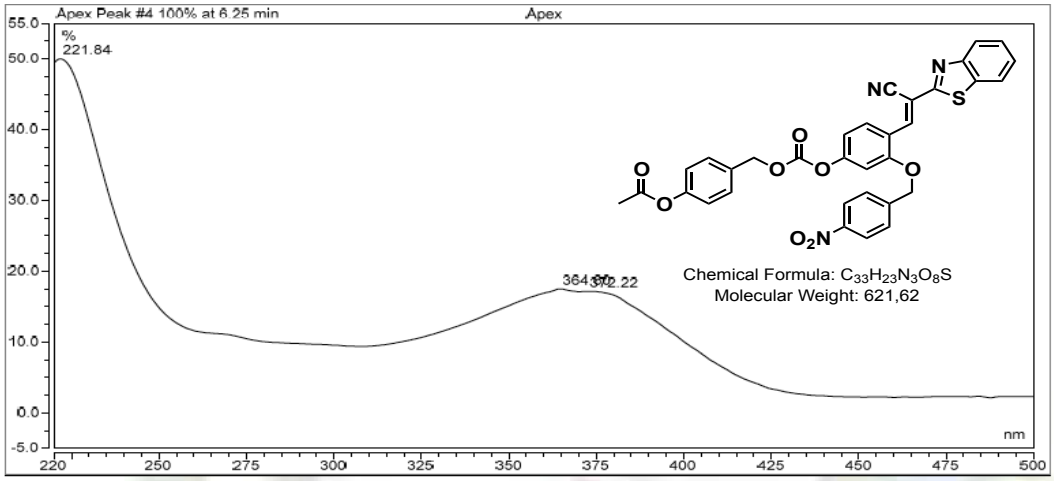
ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 5 (more polar isomer)



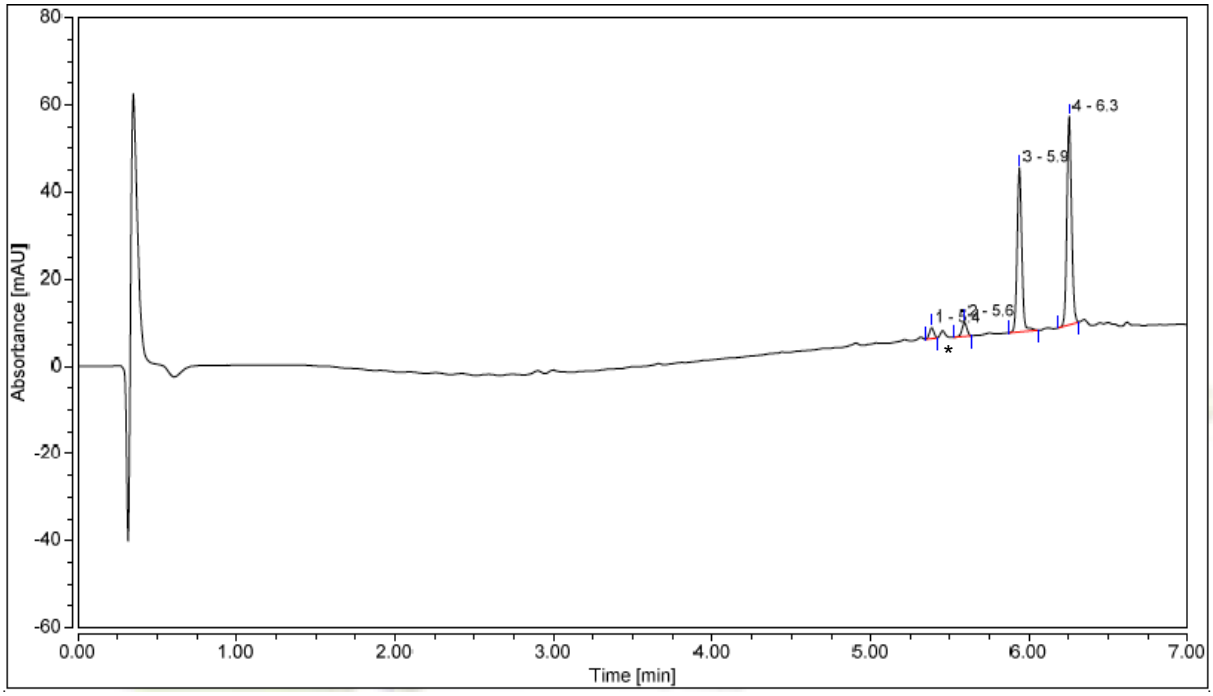


ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 5 (less polar isomer)



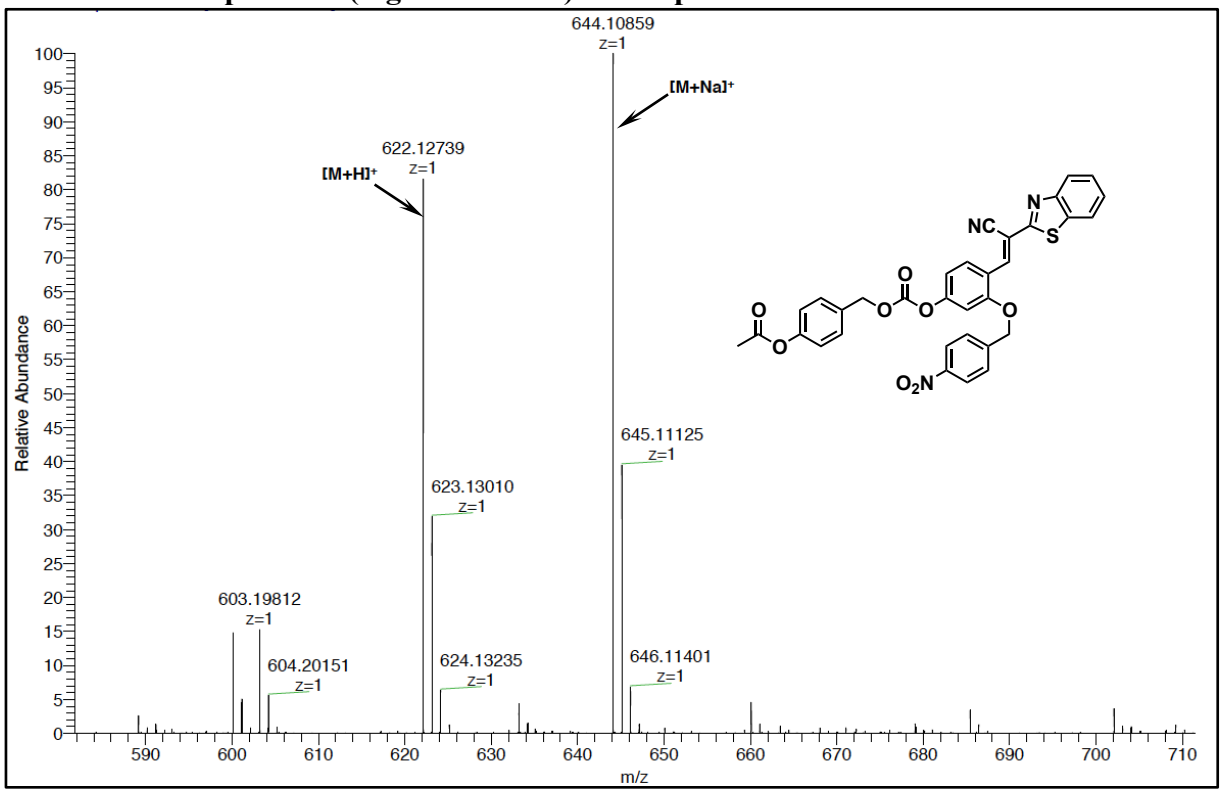


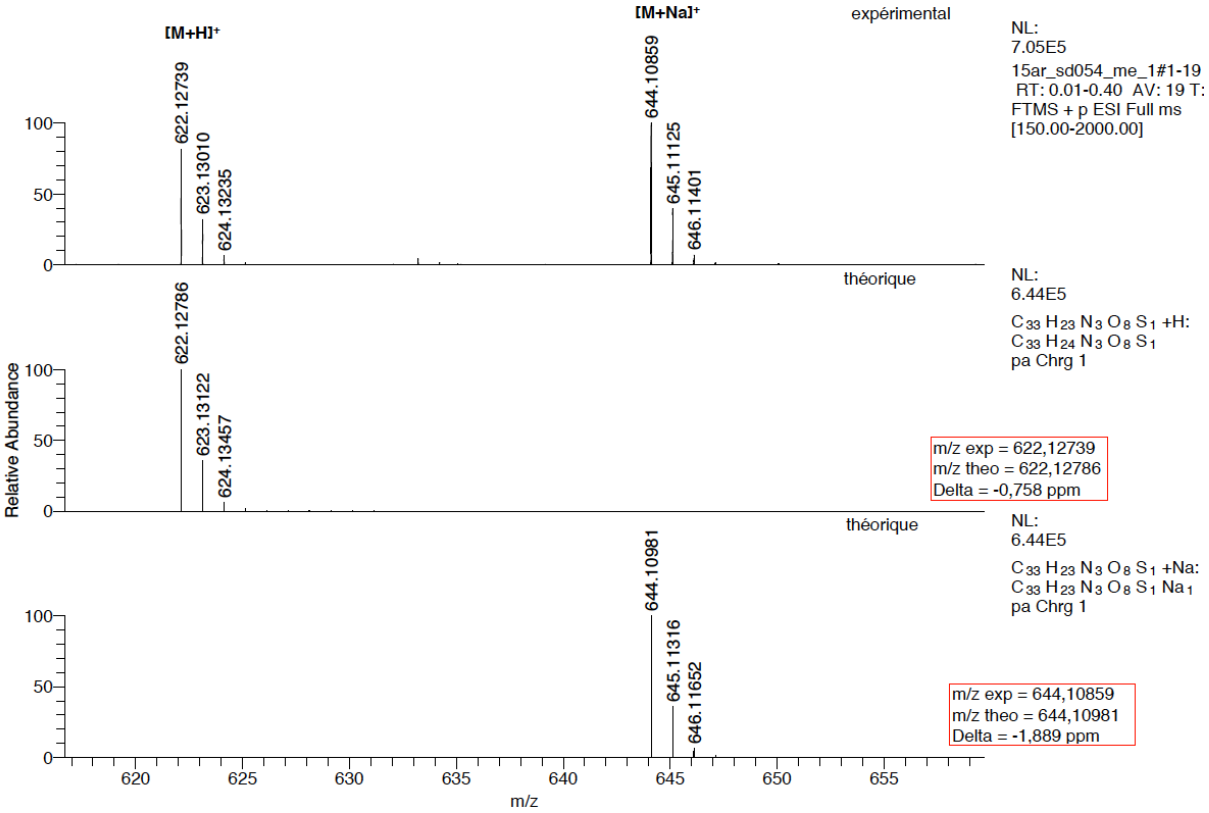
RP-HPLC elution profile (system A) of compound 5 at 260 nm



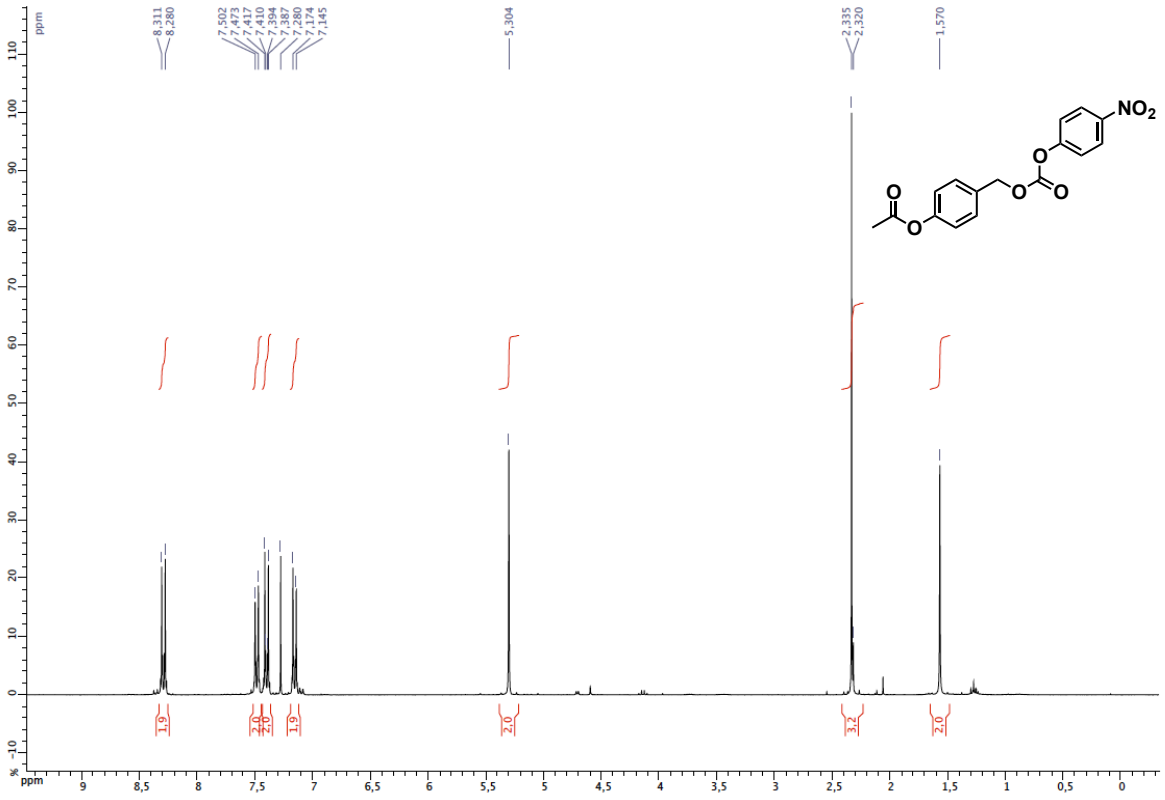
**peak found in mobile phase*

ESI+ mass spectrum (high resolution) of compound 5

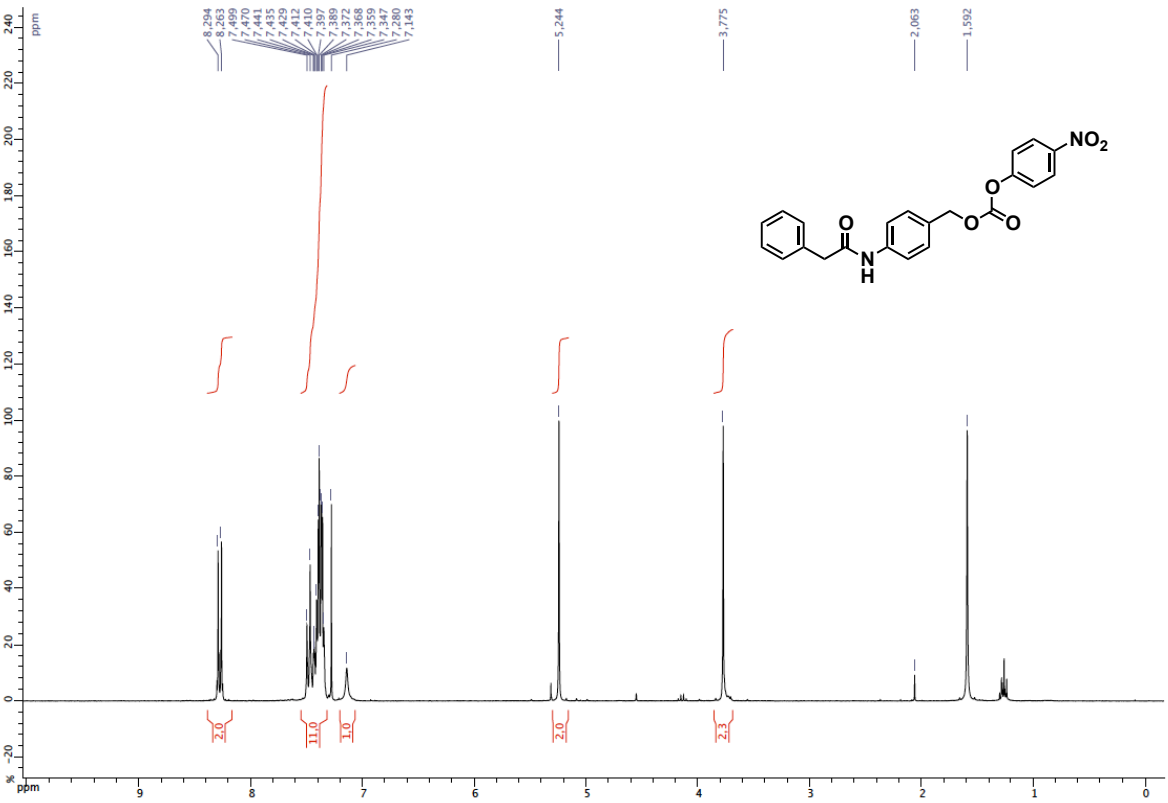




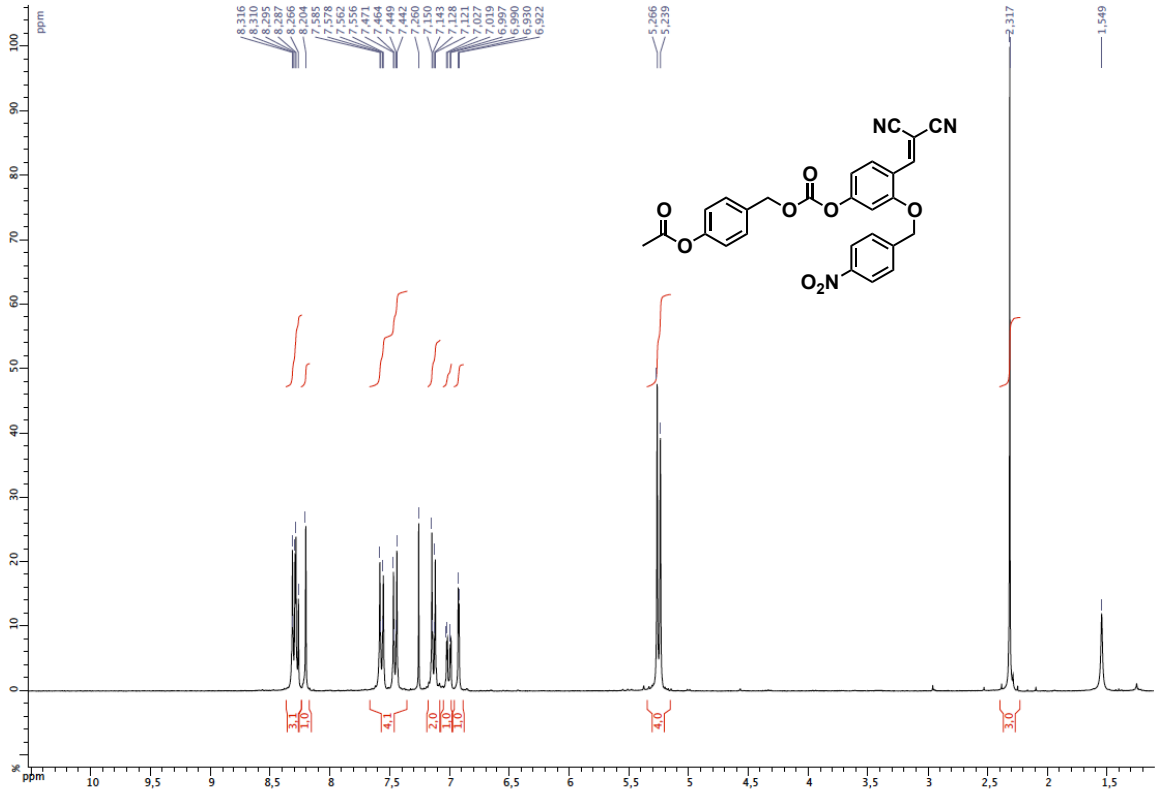
^{1}H NMR spectrum of compound 6 recorded in $CDCl_3$ at 300 MHz



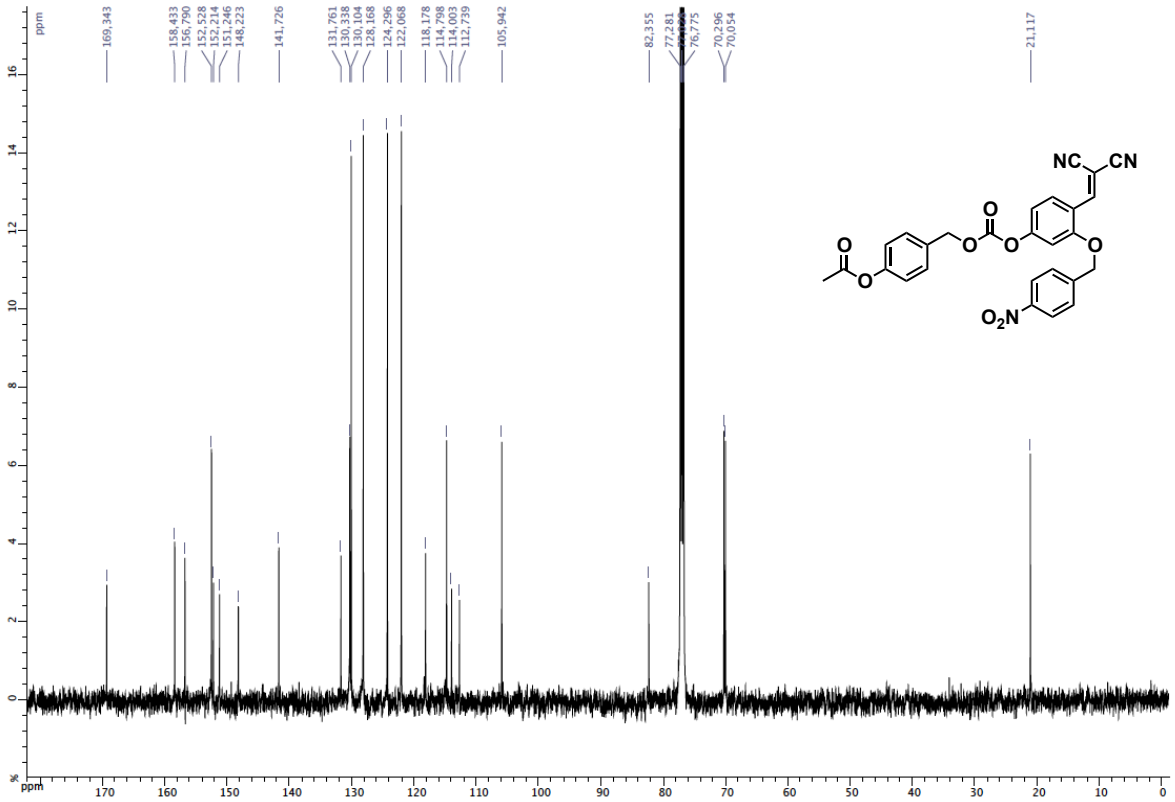
^{1}H NMR spectrum of compound 7 recorded in $CDCl_3$ at 300 MHz



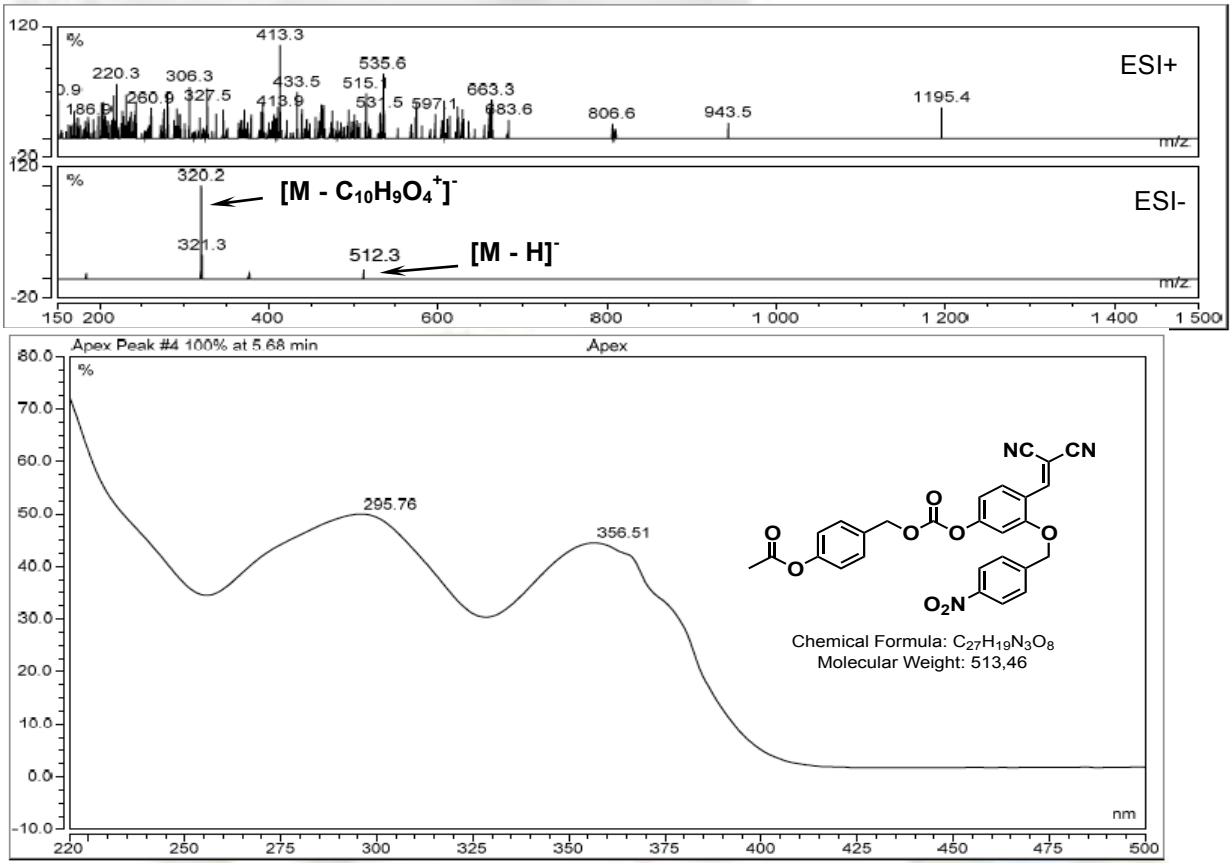
^{1}H NMR spectrum of compound 8 recorded in $CDCl_3$ at 300 MHz



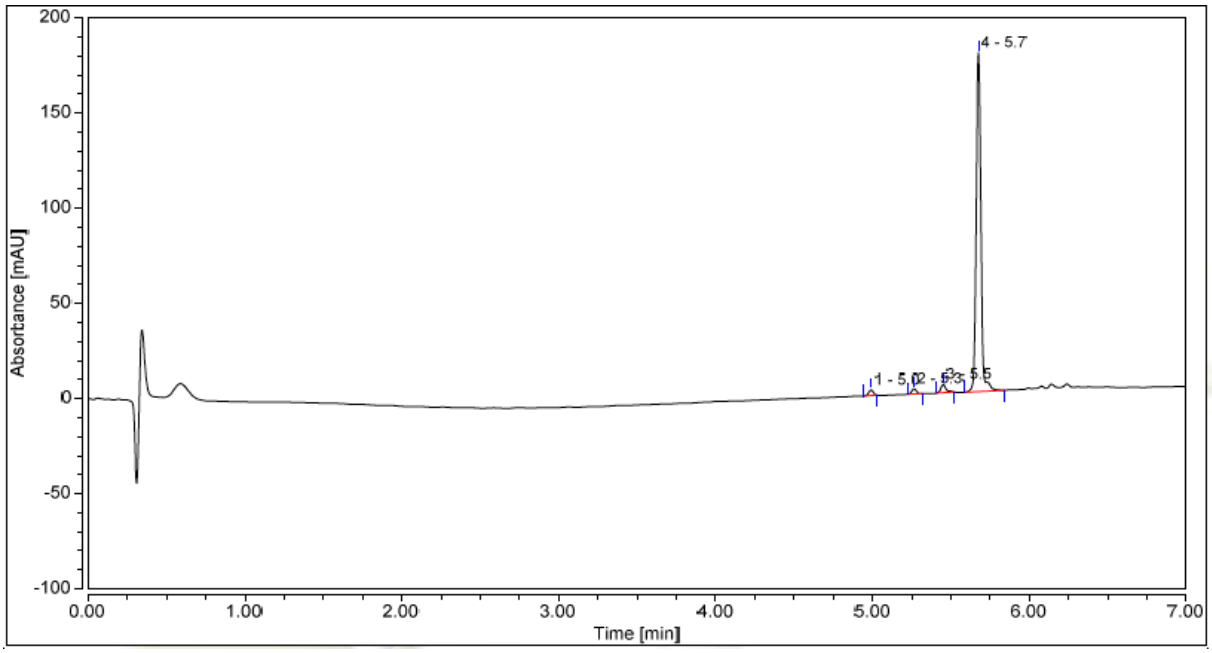
^{13}C NMR spectrum of compound 8 recorded in $CDCl_3$ at 125 MHz



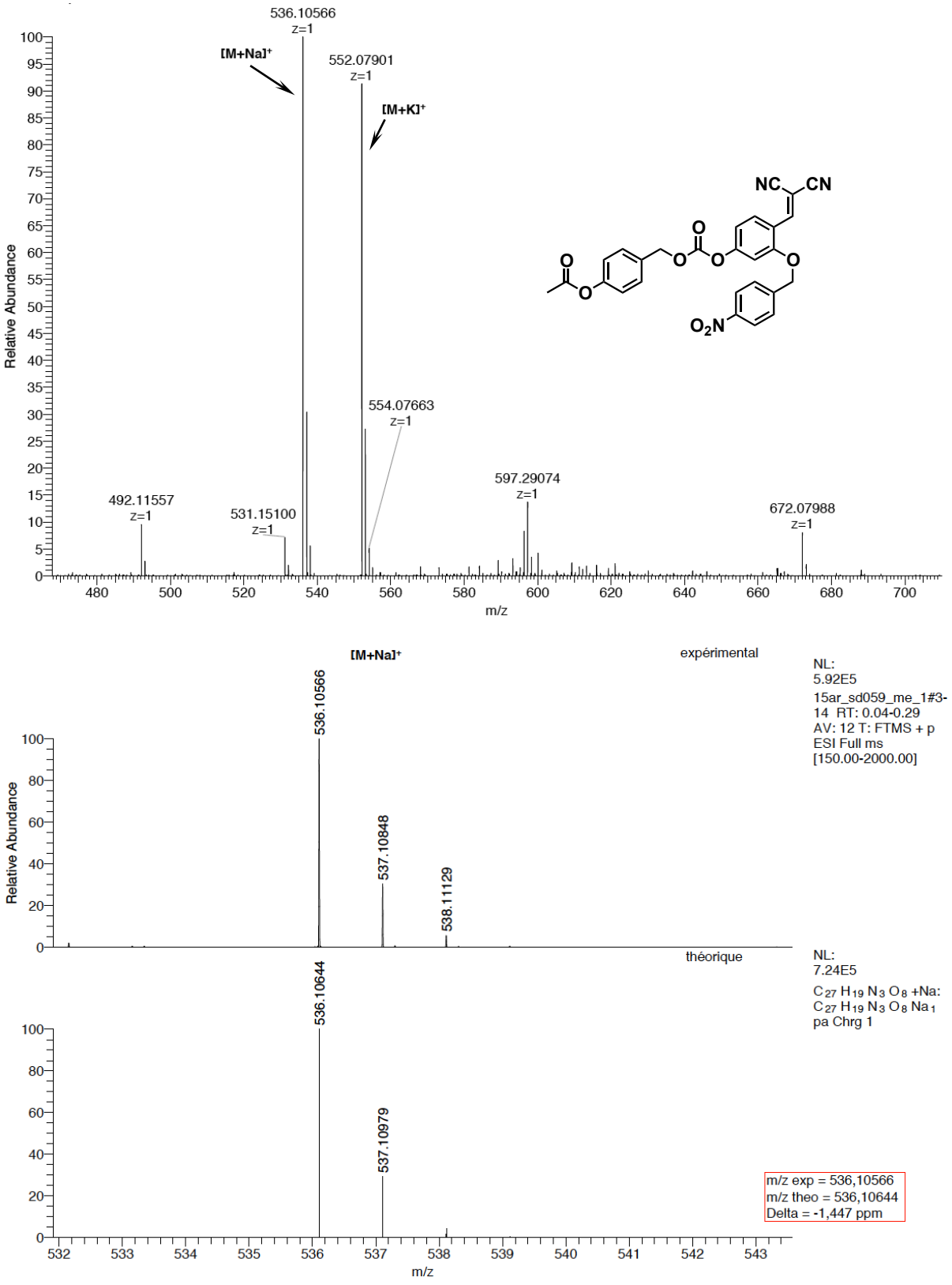
ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 8



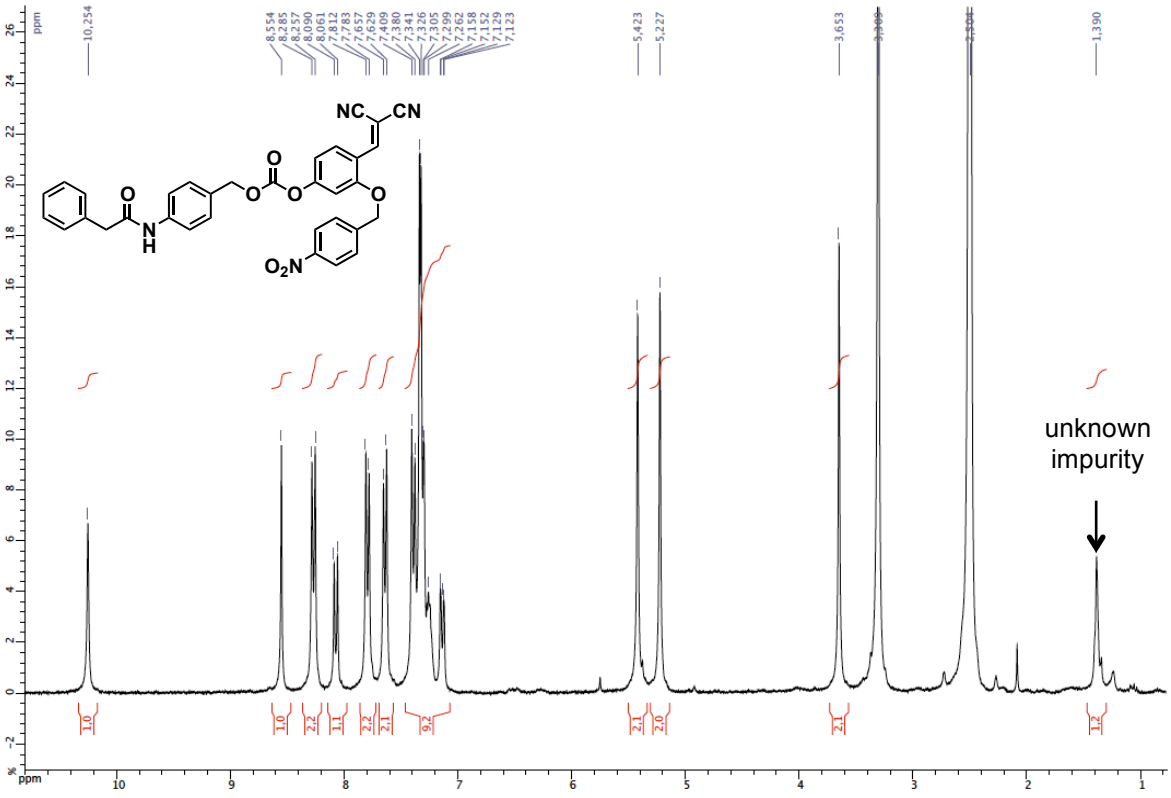
RP-HPLC elution profile (system A) of compound 8 at 260 nm



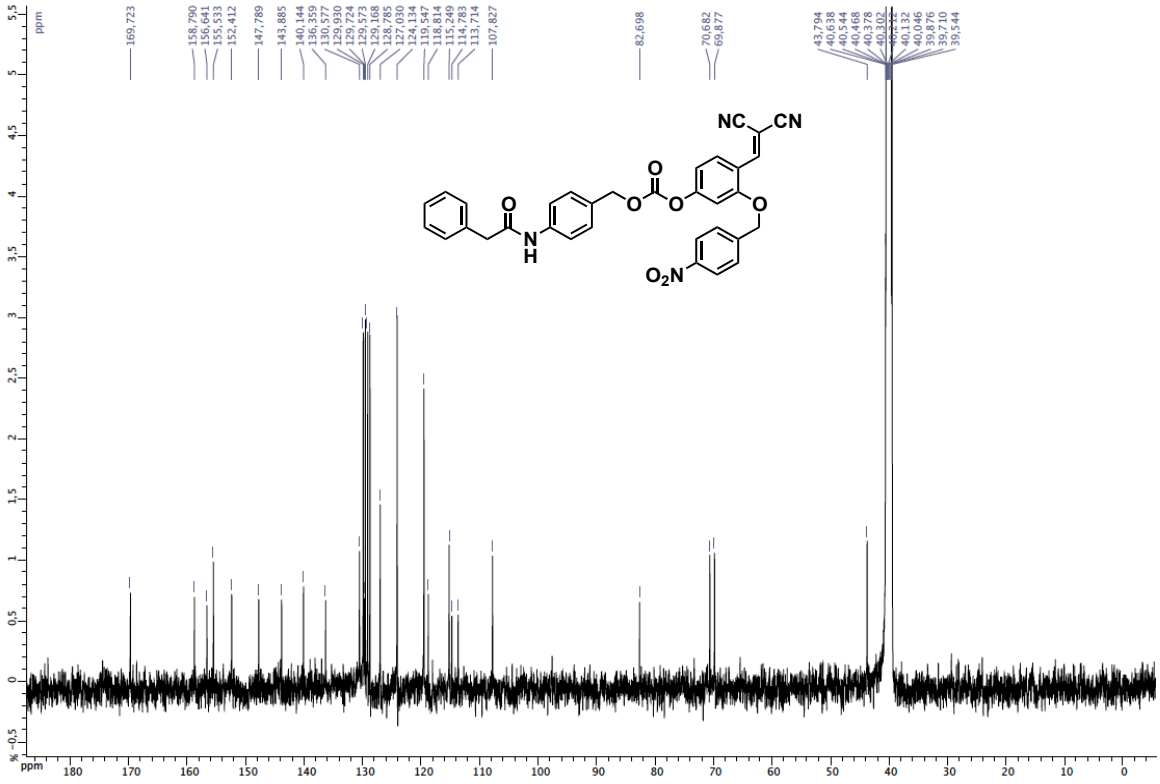
ESI+ mass spectrum (high resolution) of compound 8



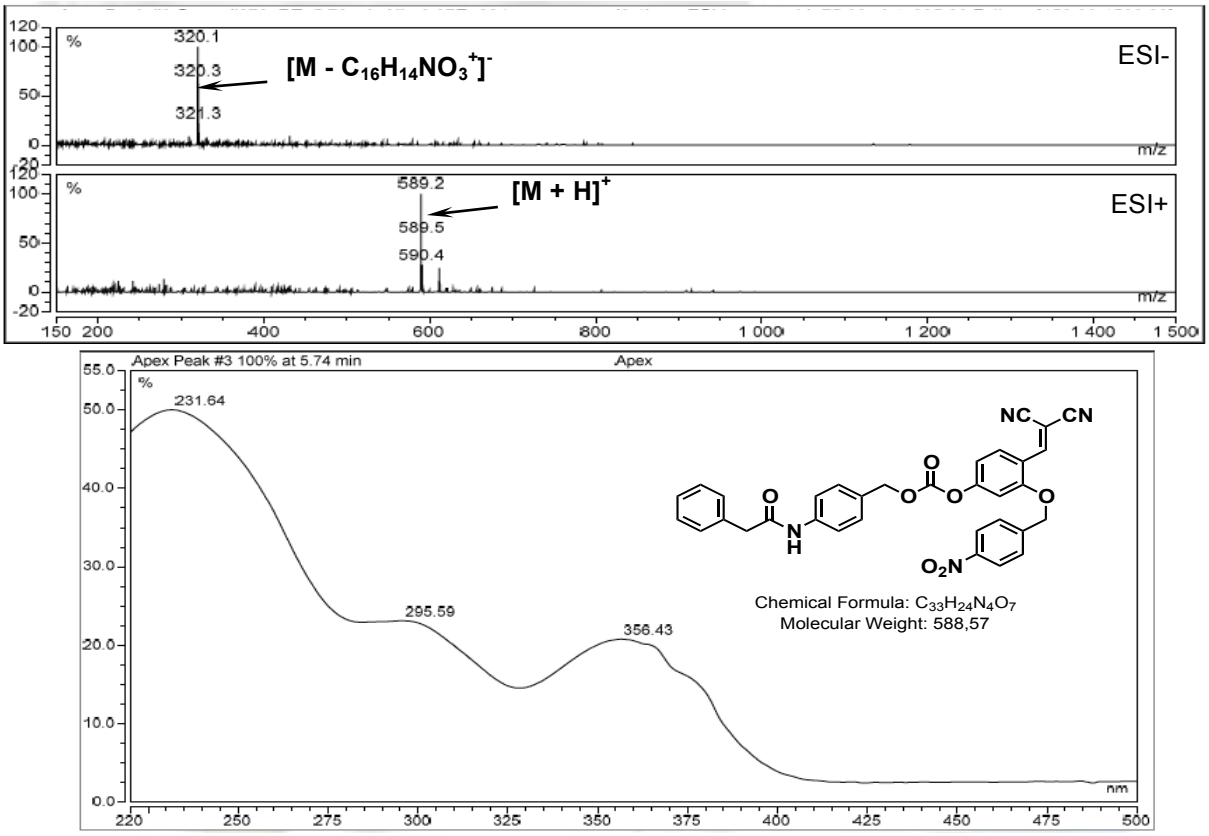
^{1}H NMR spectrum of compound 9 recorded in DMSO-d_6 at 300 MHz



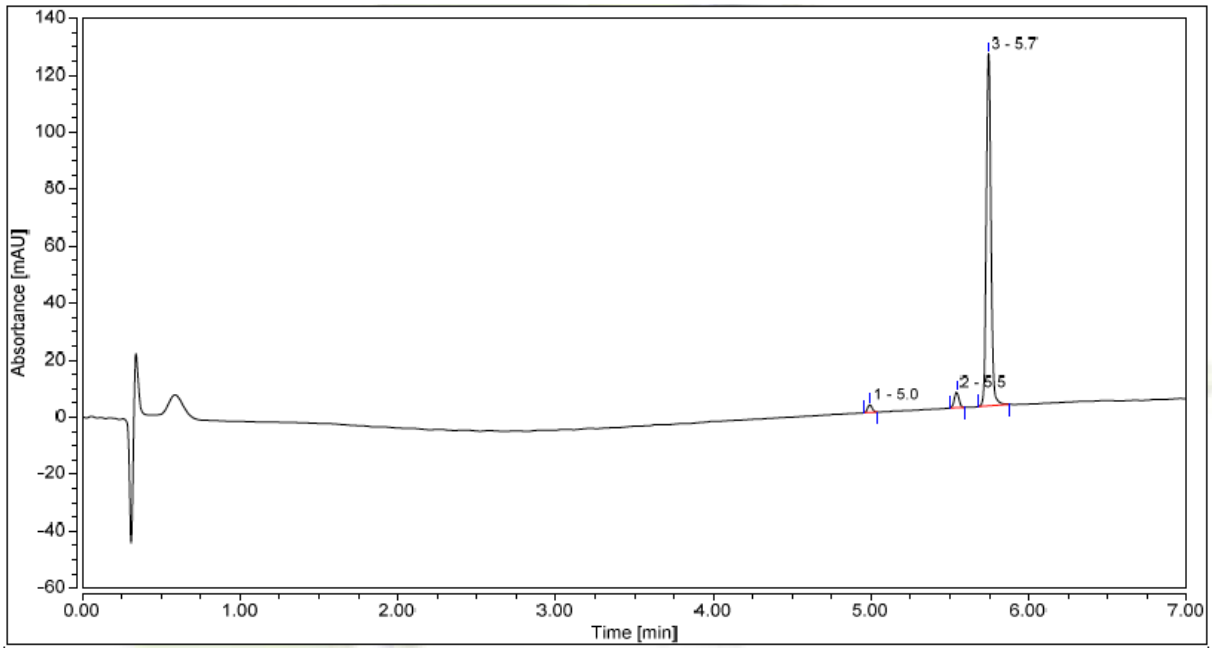
^{13}C NMR spectrum of compound 9 recorded in DMSO-d_6 at 125 MHz



ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 9



RP-HPLC elution profile (system A) of compound 9 at 260 nm



ESI+ mass spectrum (high resolution) of compound 9

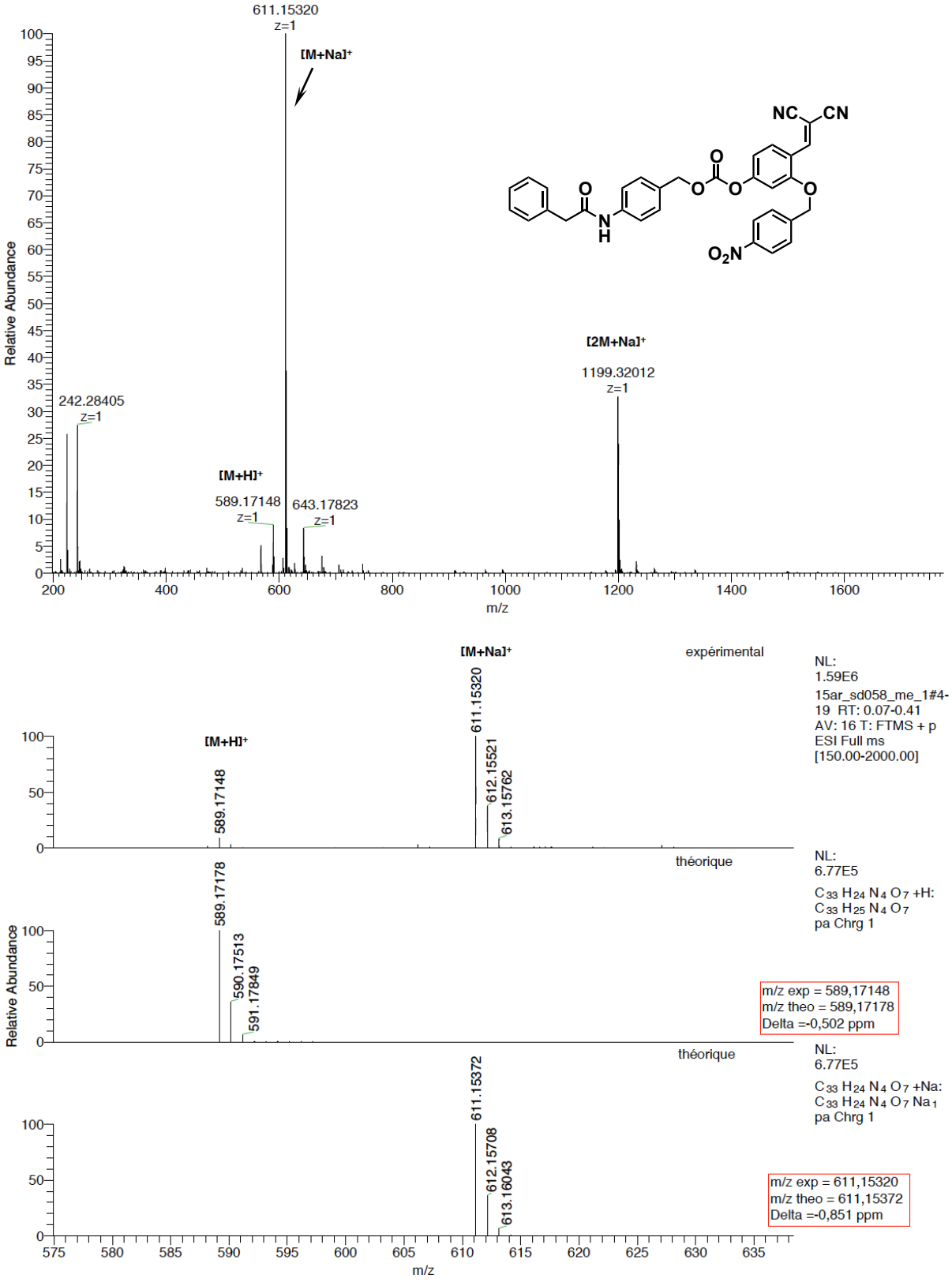


Fig S1. Normalised absorption spectra of fluorogenic probes 5, 8 and 9 in PB (+ 0.3% DMSO) at 25 °C

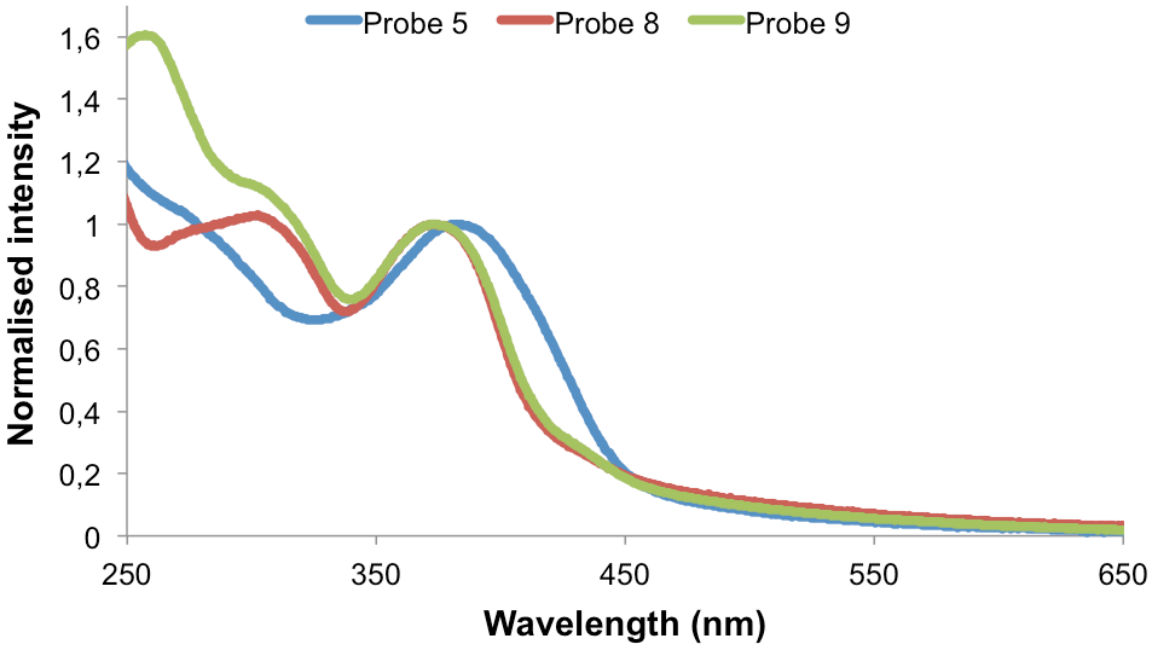


Fig S2. Normalised absorption, excitation (Em. 510 nm) and emission (Ex. 400 nm) spectra of 3-cyano-7-hydroxy-2-iminocoumarin in PB at 25 °C.

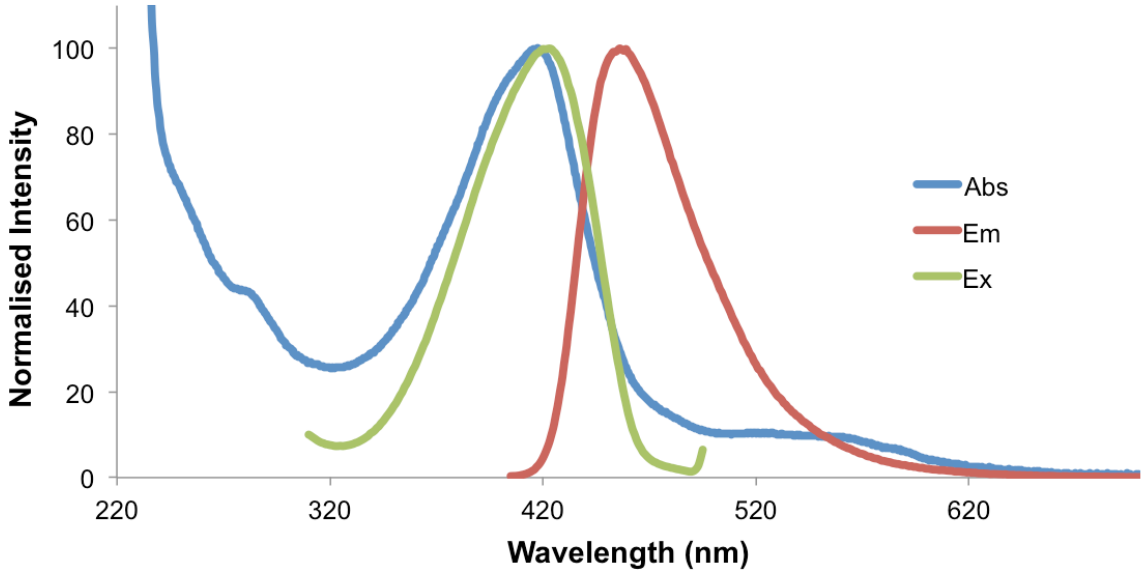


Fig S3. Normalised absorption, excitation (Em. 530 nm) and emission (Ex. 370 nm) spectra of 3-cyano-7-hydroxycoumarin in PB at 25 °C.

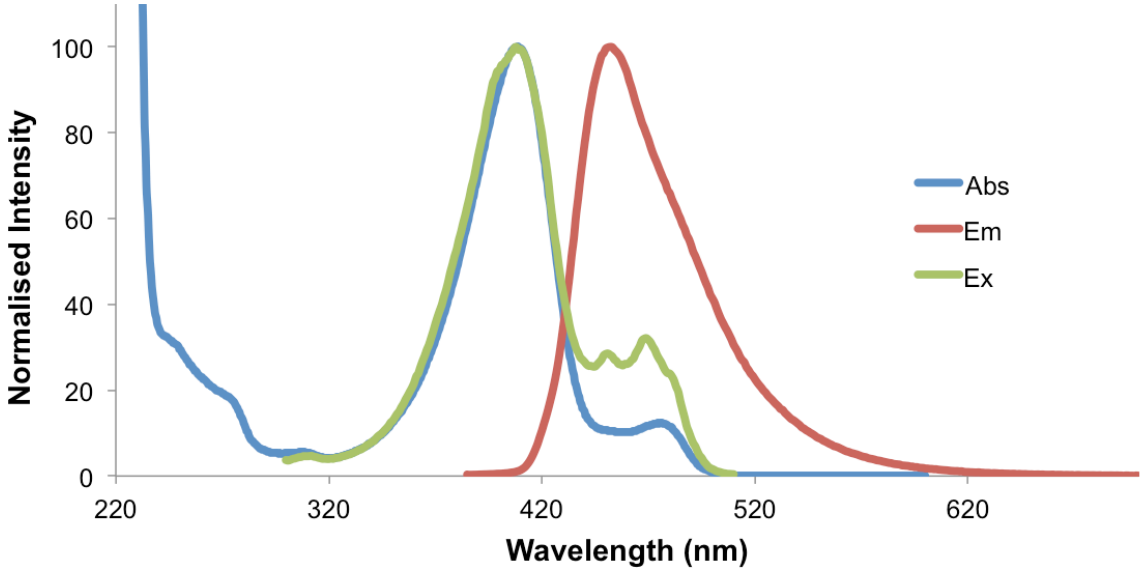
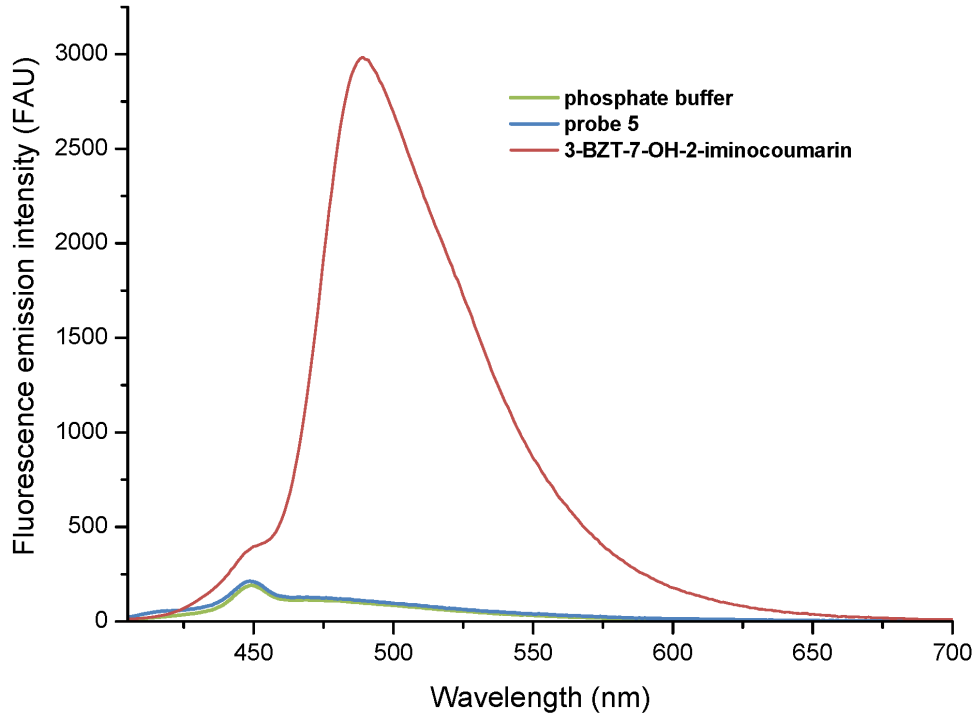
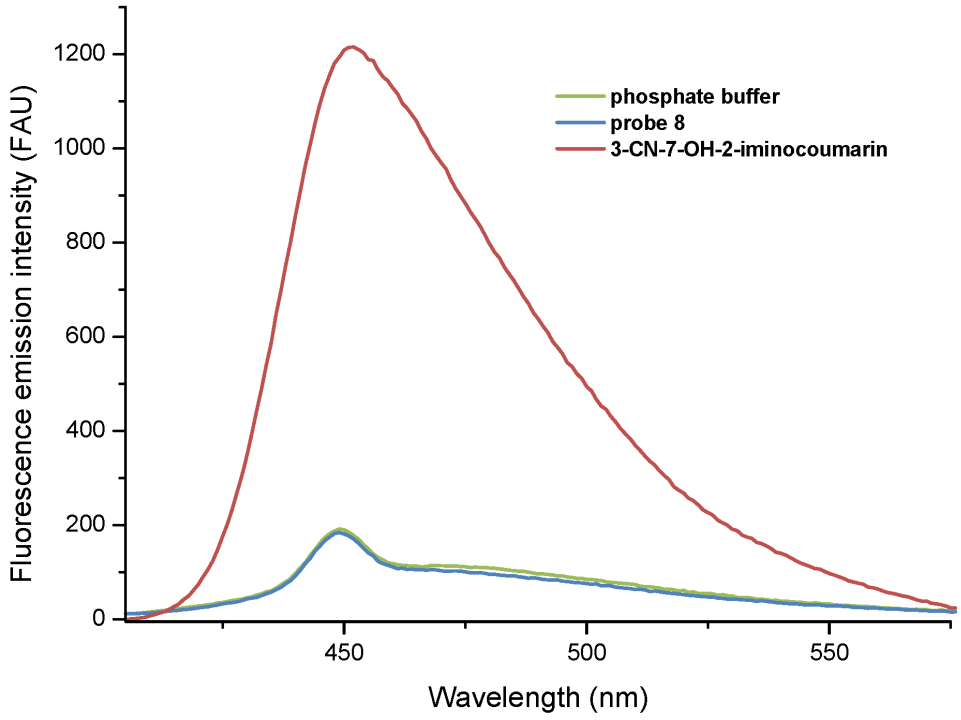


Fig S4. Overlayed fluorescence emission spectra (Ex. 390 nm) of PLE-NTR fluorogenic probe 5 and 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin in PB at 25 °C (concentration: 0.1 µM)[a]



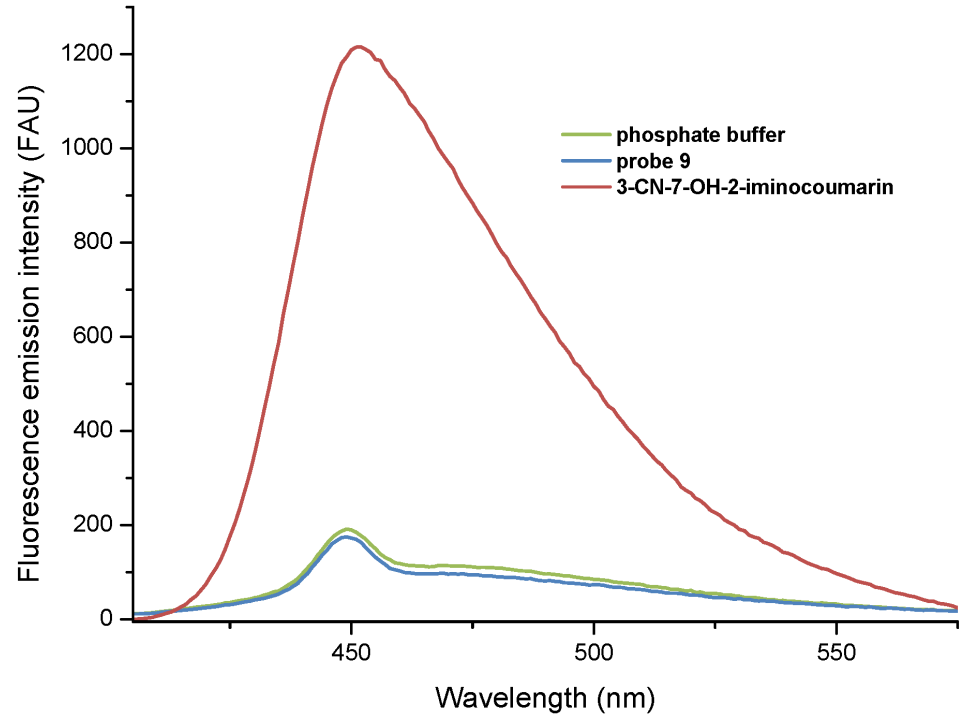
[a]*Raman scatter of water at 450 nm*

Fig S5. Overlayed fluorescence emission spectra (Ex. 390 nm) of PLE-NTR fluorogenic probe 8 and 3-cyano-7-hydroxy-2-iminocoumarin in PB at 25 °C (concentration: 0.1 μM)[a]



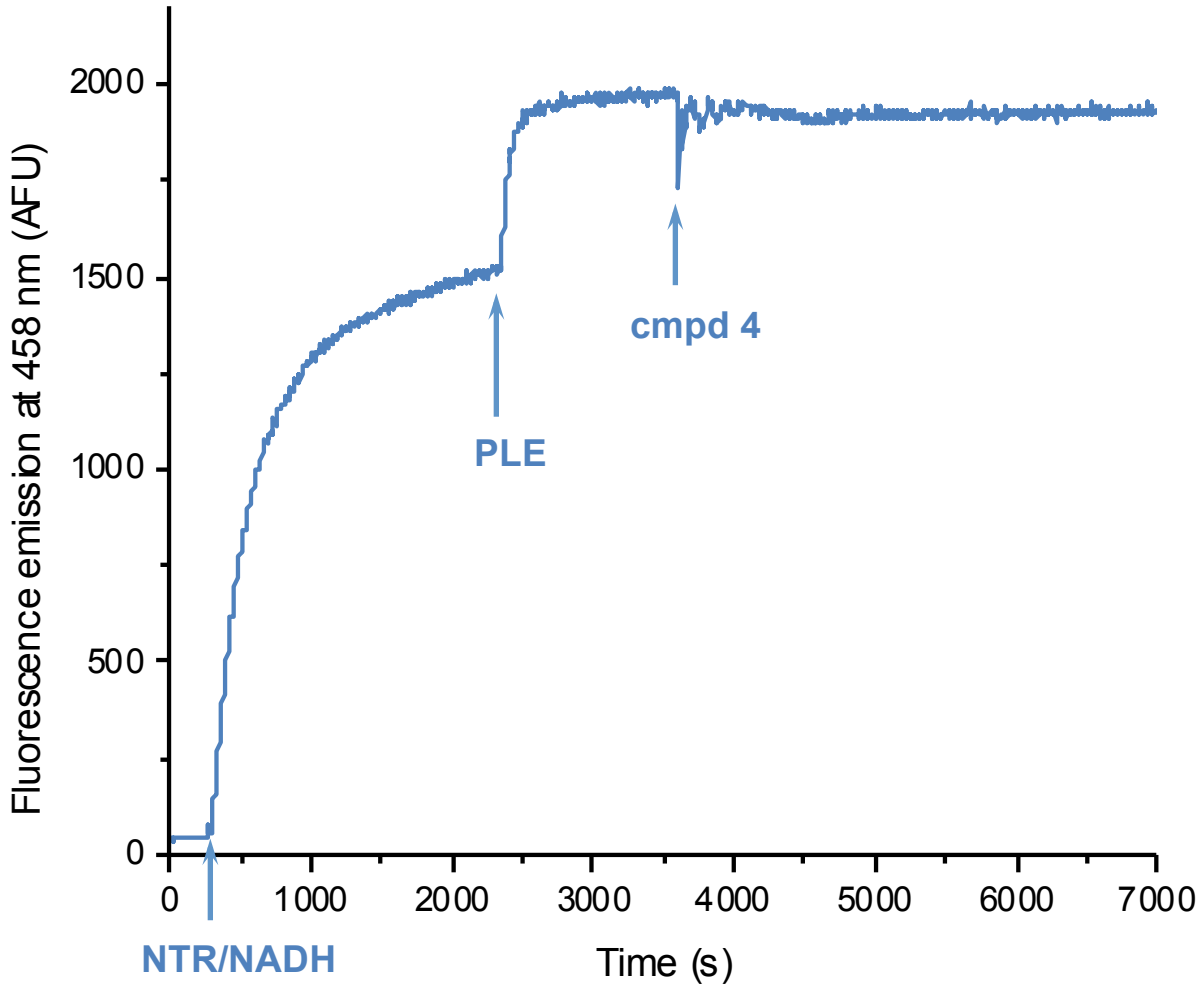
[a]*Raman scatter of water at 450 nm*

Fig S6. Overlayed fluorescence emission spectra (Ex. 390 nm) of PGA-NTR fluorogenic probe 9 and 3-cyano-7-hydroxy-2-iminocoumarin in PB at 25 °C (concentration: 0.1 μM)[a]



[a]*Raman scatter of water at 450 nm*

Fig S7. Time-dependant fluorescence intensity of fluorogenic "turn-on" probe 8 upon sequential incubation with NTR/NADH, PLE and cmpd 4



Probe **8** (concentration: 1.0 μM in PB) was incubated with NTR (0.1 U) / NADH (45 μM) at 37 °C for 37.5 min, then PLE (1 U) was added and further incubation for 25 min. Finally, compound **4** (final concentration: 1.0 μM) was added. Ex./Em. 418/458 nm.

Fig S8. RP-HPLC elution profiles (fluorescence detection, systems D & E) of fluorogenic probes 5 (top), 8 (middle) and 9 (bottom) before dual-enzymatic activation

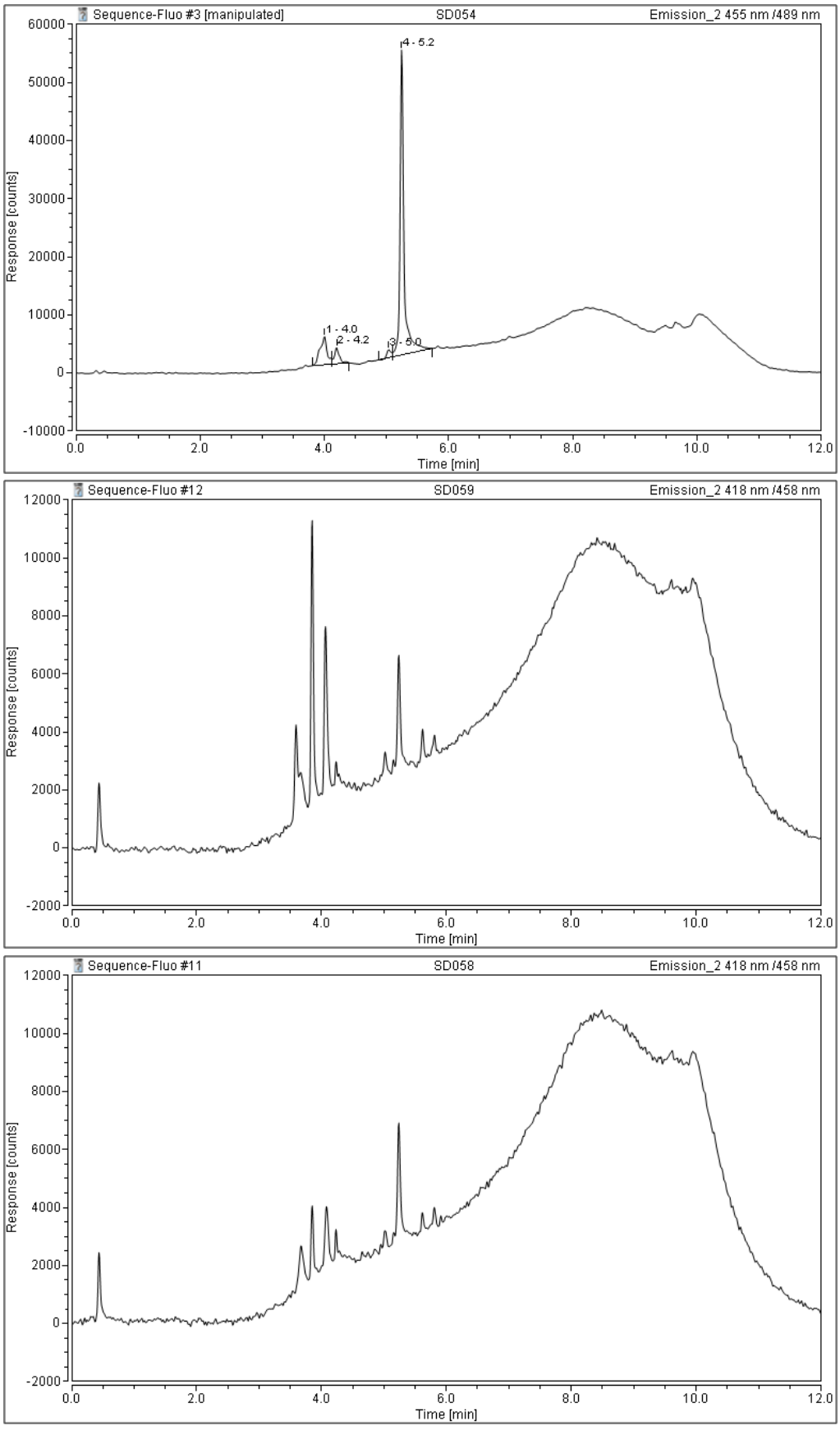
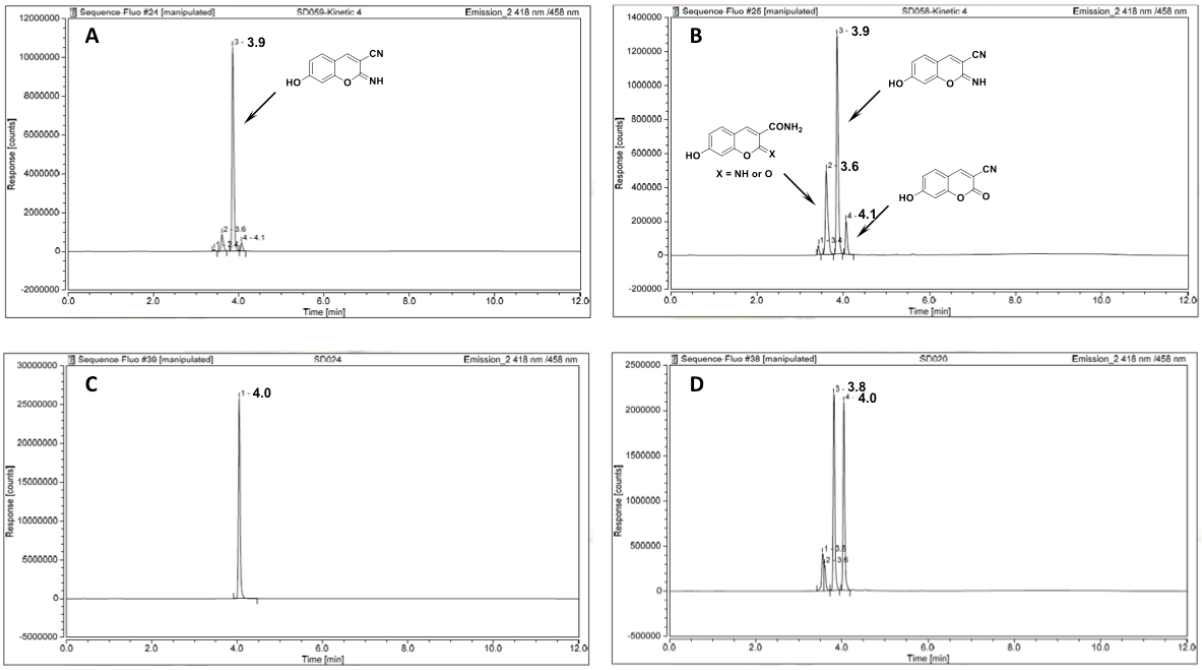


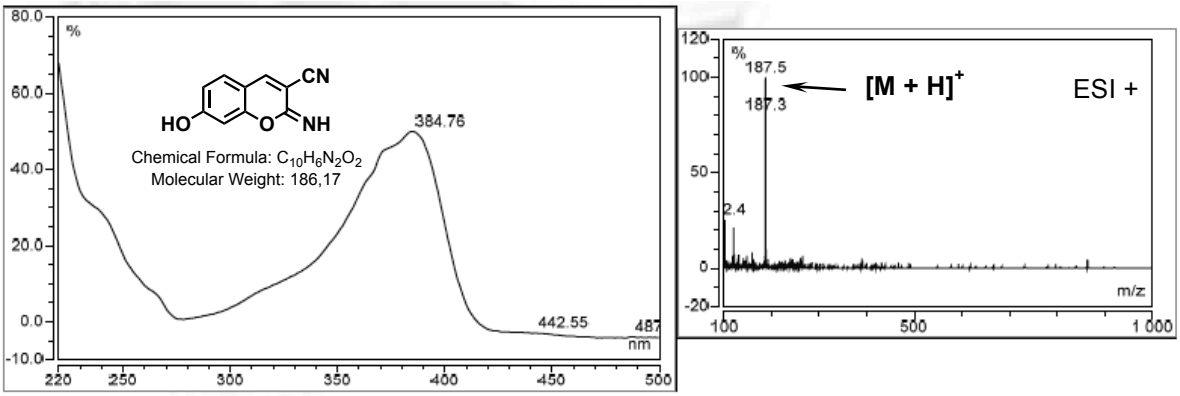
Fig S9. RP-HPLC elution profiles (fluorescence detection, system D) of enzymatic reaction mixture of cyano-based probes 8 and 9 incubated simultaneously with both enzymes: hydrolase (PLE or PGA) and NTR/NADH



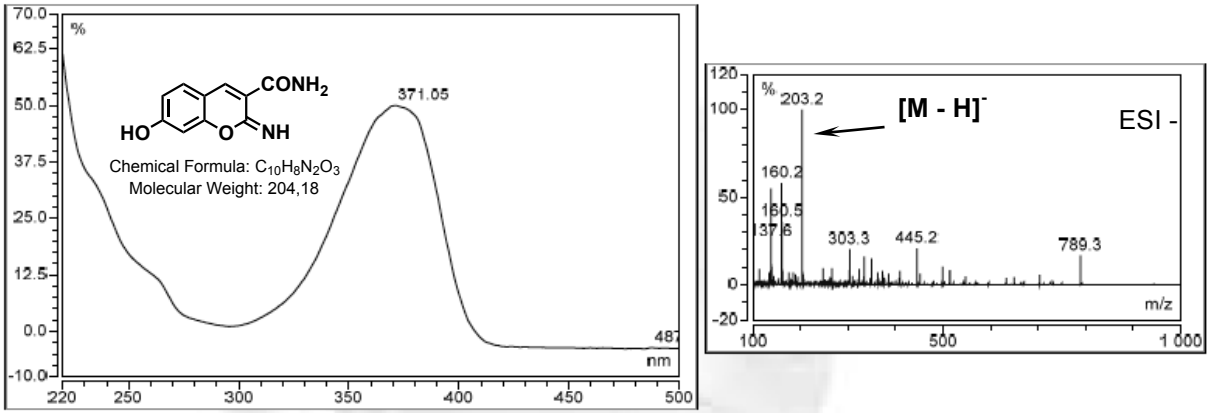
Enzymatic reaction mixtures (A-B) and authentic samples of 3-cyano-7-hydroxycoumarin (C) and 3-cyano-7-hydroxy-2-iminocoumarin (D). *Please note*: partial hydrolysis of cyano and imine moieties was occurred during HPLC analysis and incubation in PB. NADH (t_R = 3.4 min) can be properly detected at a different wavelength channel (Ex./Em. 350/460 nm).

Fig S10. RP-HPLC-MS analyses - Identification of "relevant" molecules related to the dual-enzyme activation of probes 8 and 9

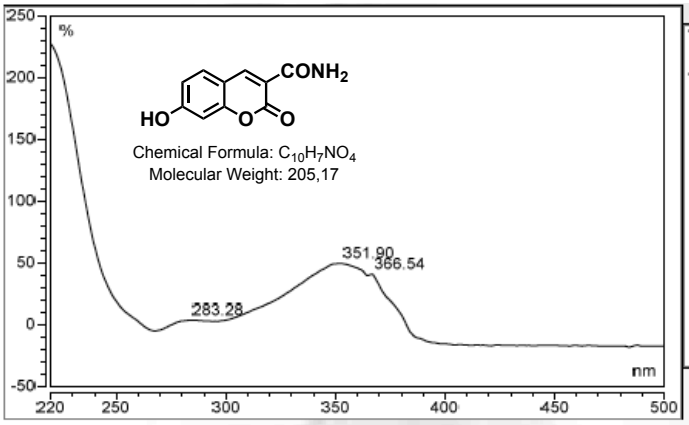
Peak at 3.8 min

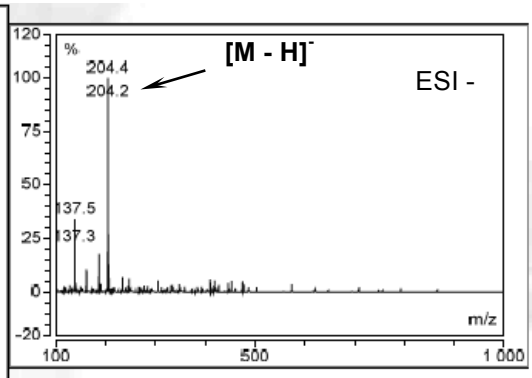


Peak at 4.3 min

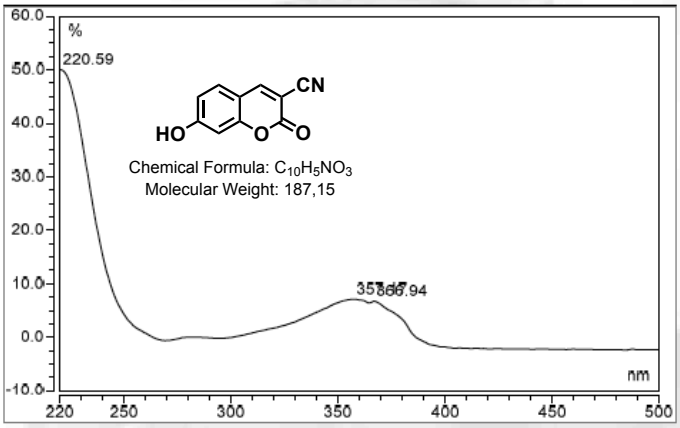


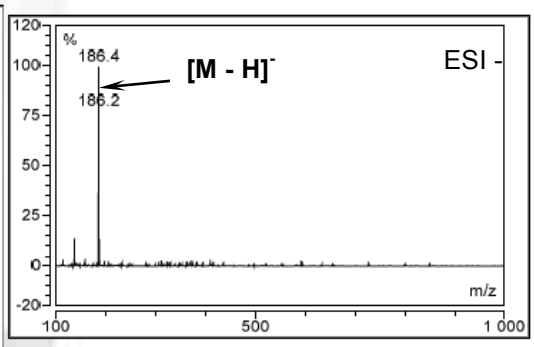
Peak at 5.5 min



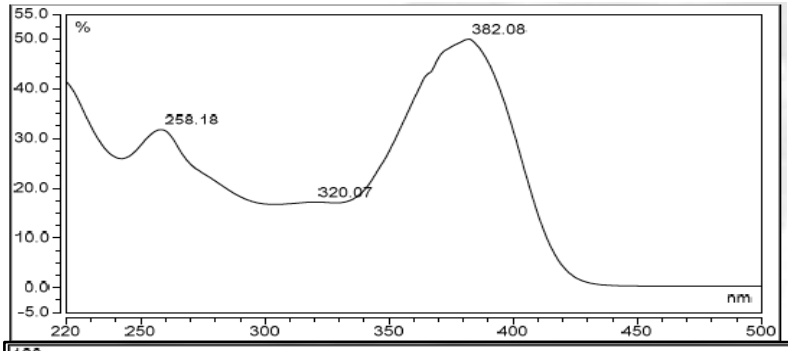


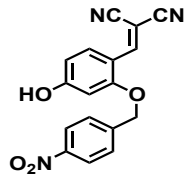
Peak at 5.9 min



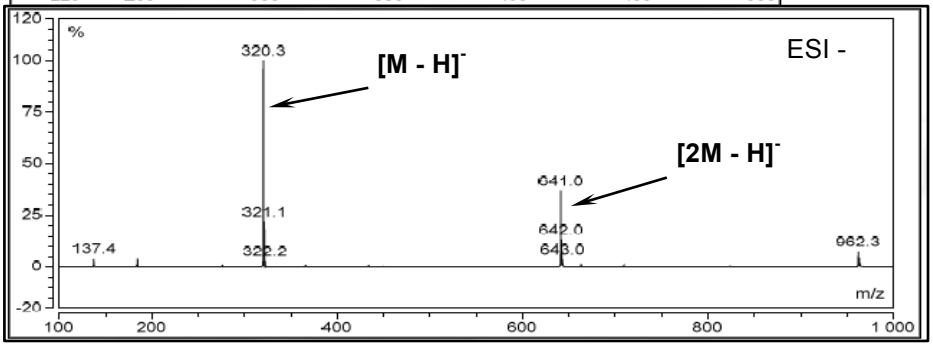


Peak at 7.7 min

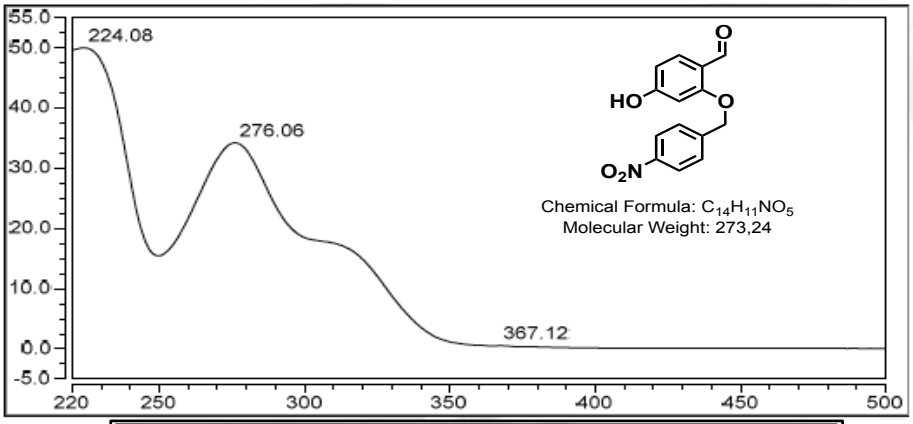


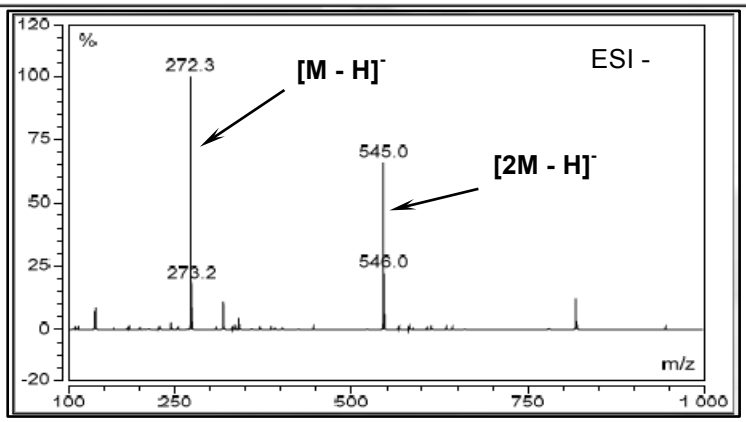


Chemical Formula: $C_{17}H_{11}N_3O_4$
Molecular Weight: 321,29

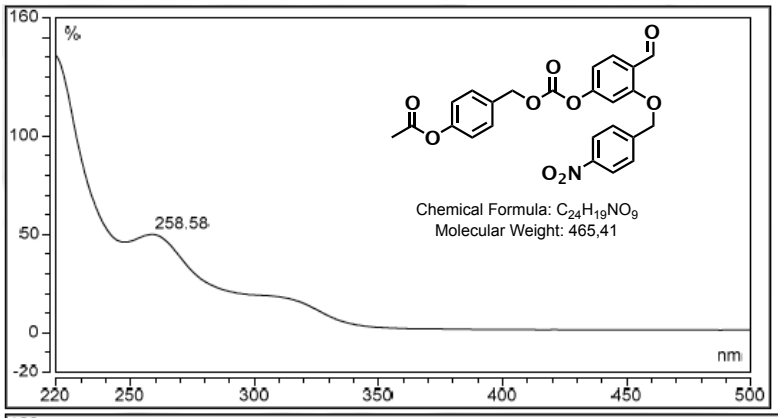


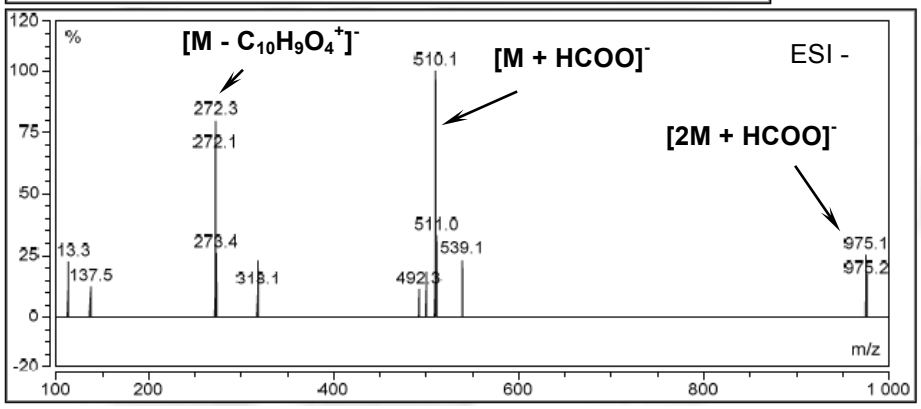
Peak at 7.0 min



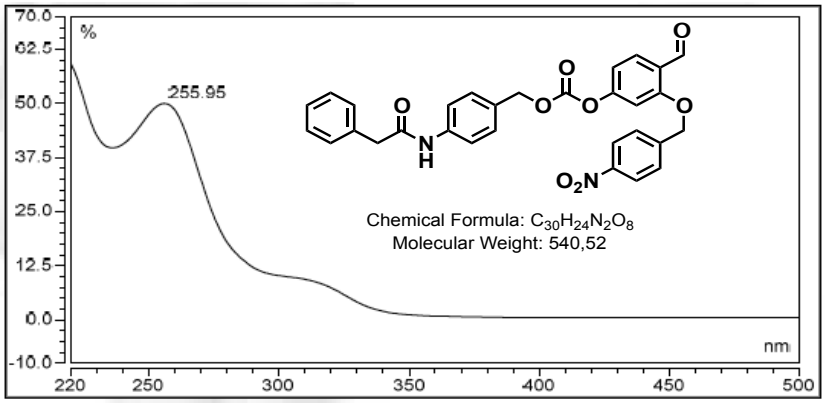


Peak at 8.18 min





Peak at 8.3 min



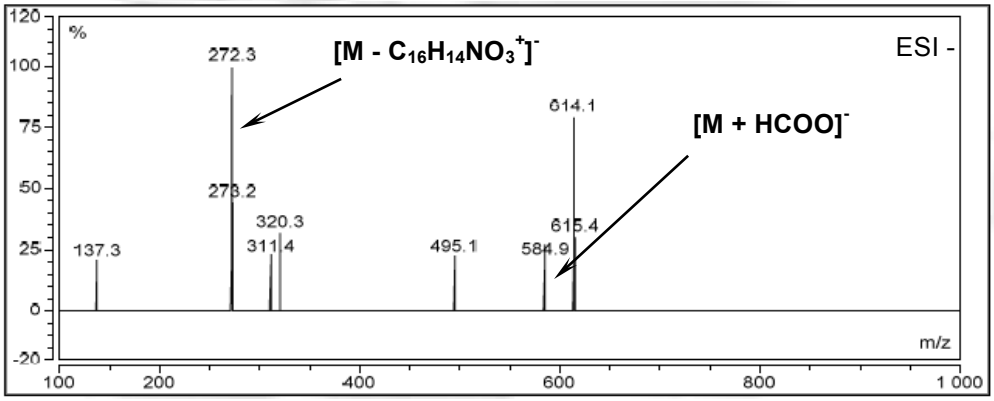
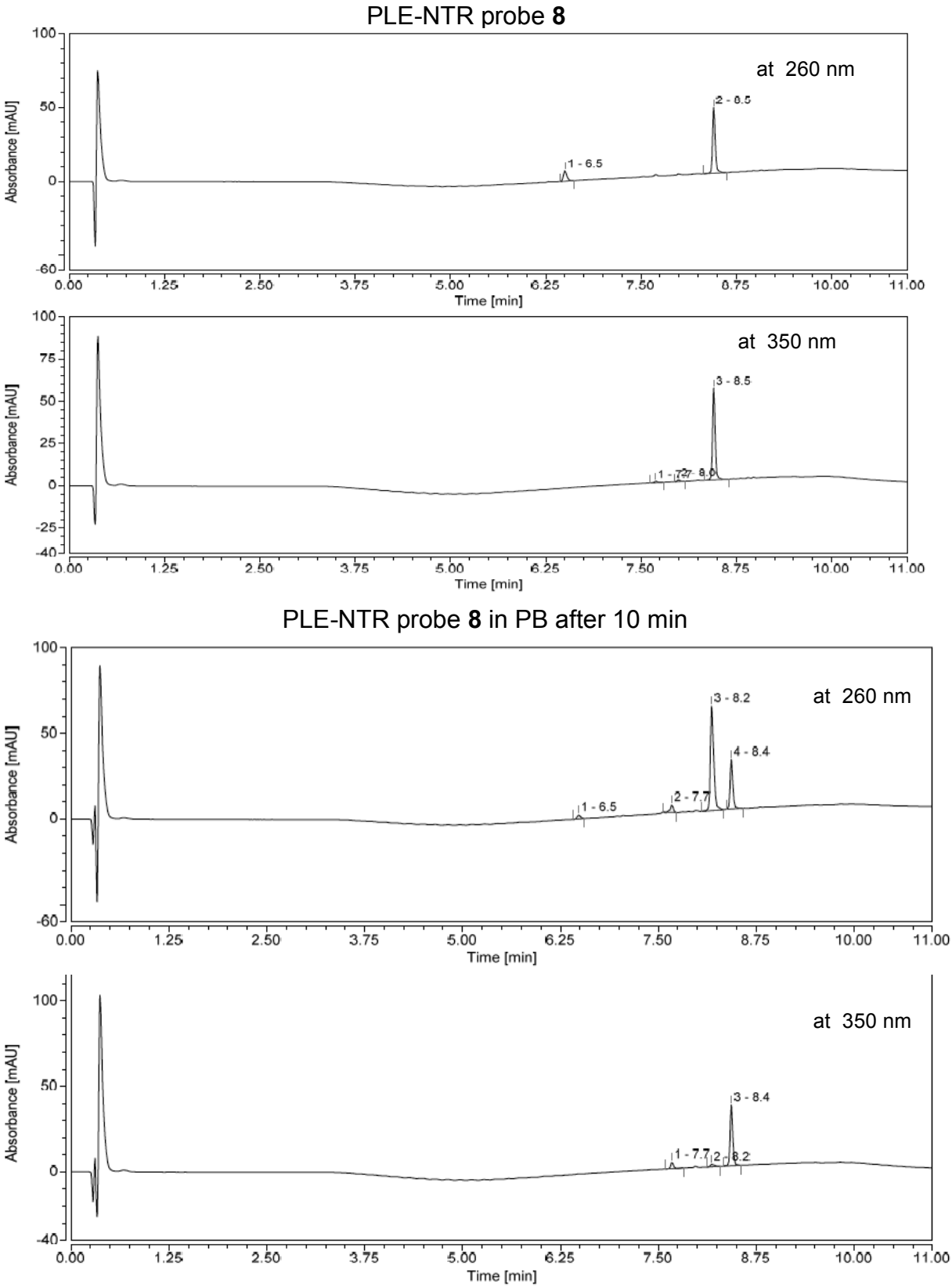
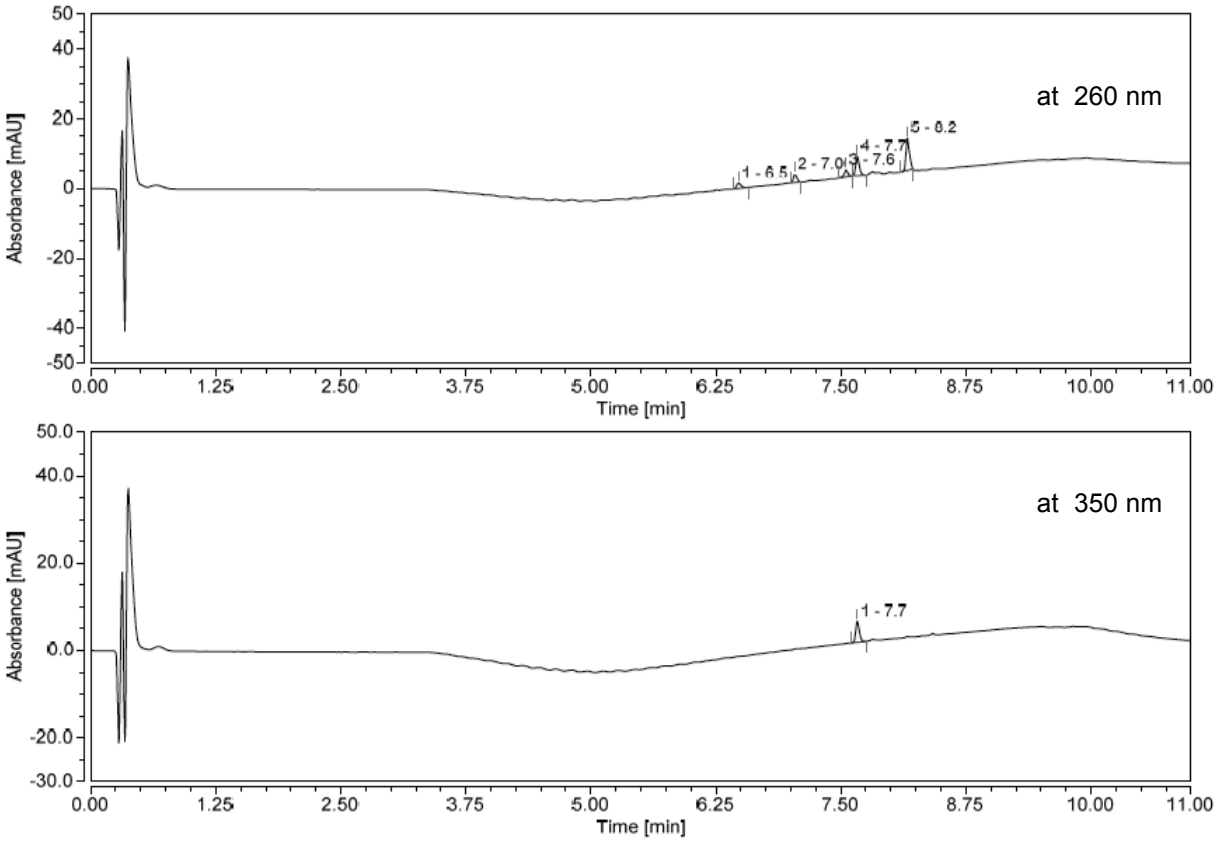


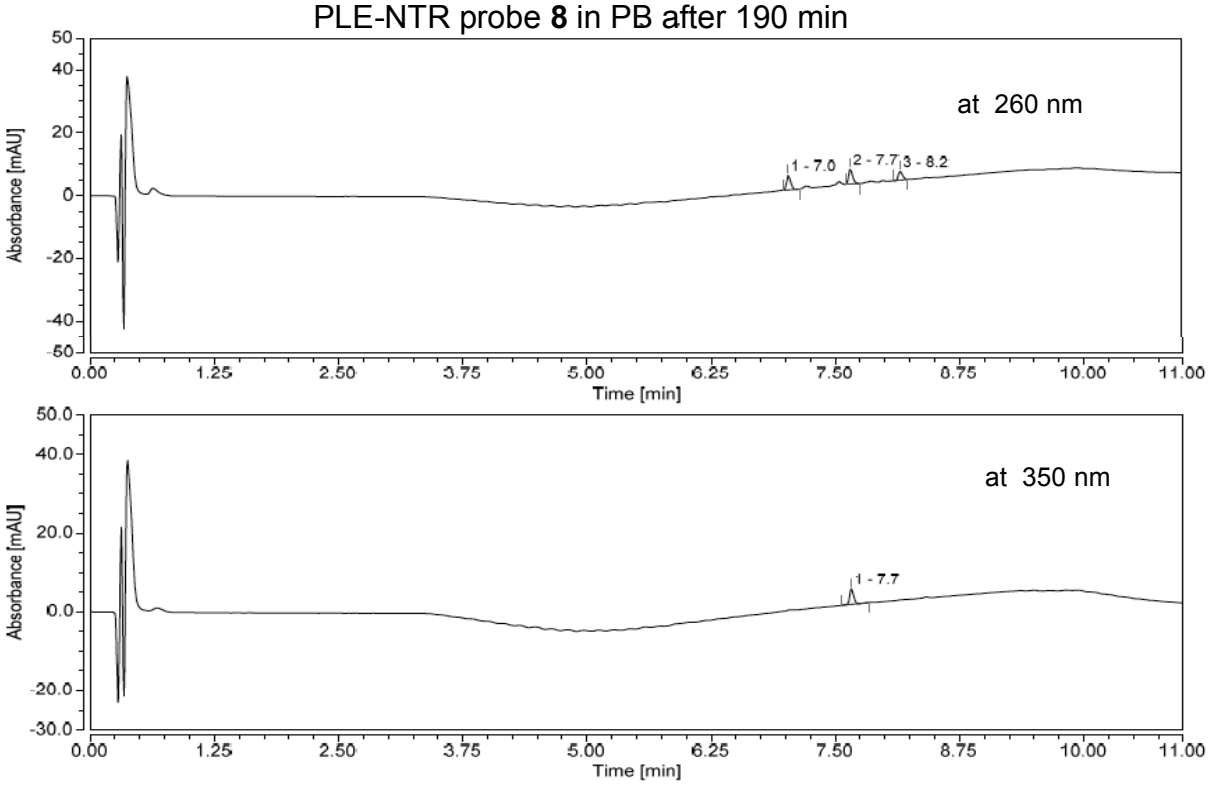
Fig S11. RP-HPLC elution profiles (system C) of fluorogenic probe 8 after incubation in PB alone or with enzymes (PLE and NTR/NADH)



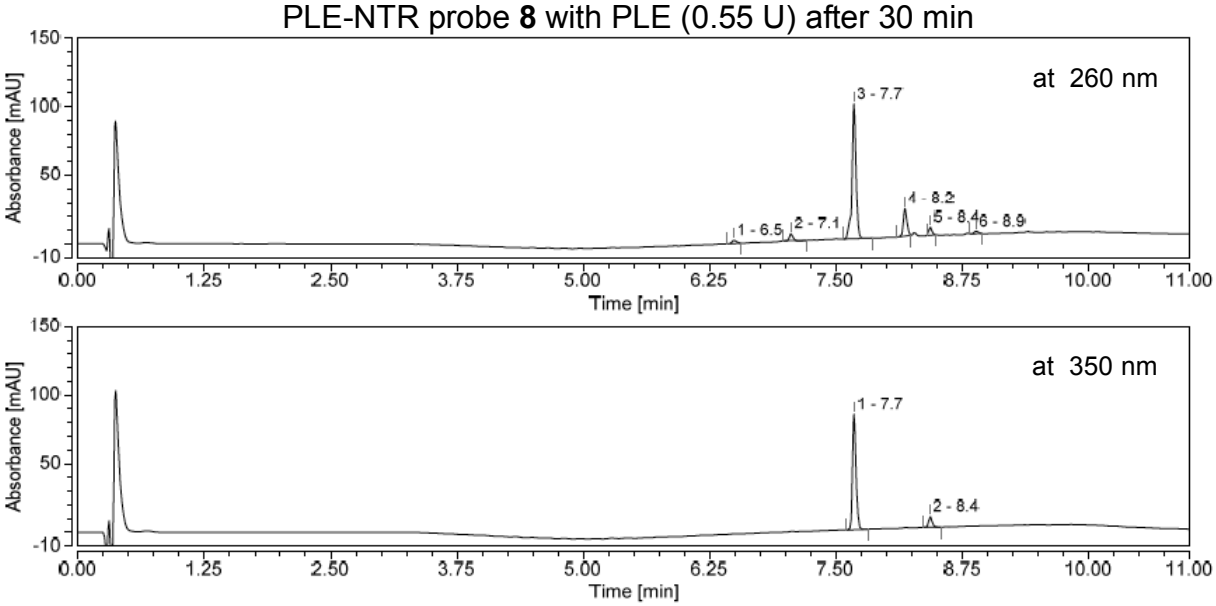
PLE-NTR probe **8** in PB after 90 min



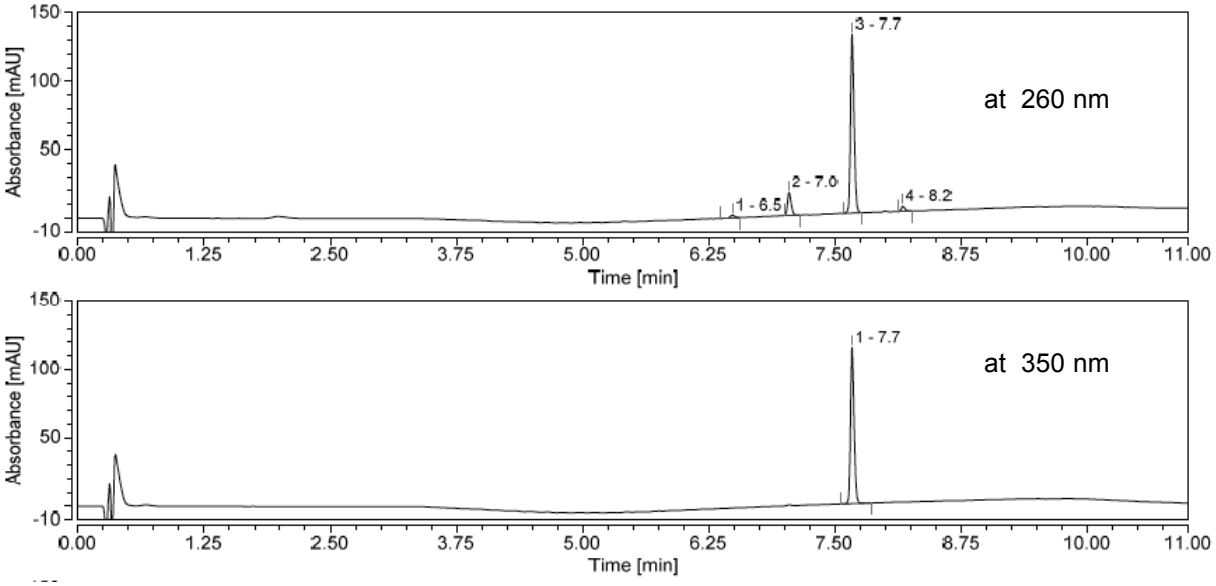
PLE-NTR probe **8** in PB after 190 min



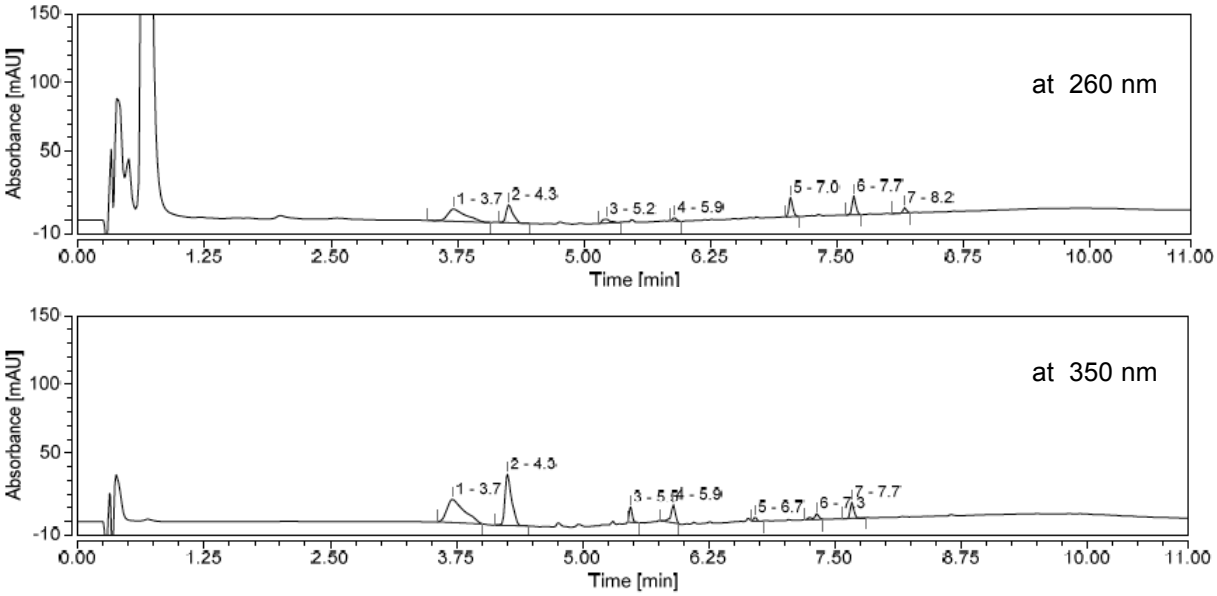
PLE-NTR probe **8** with PLE (0.55 U) after 30 min



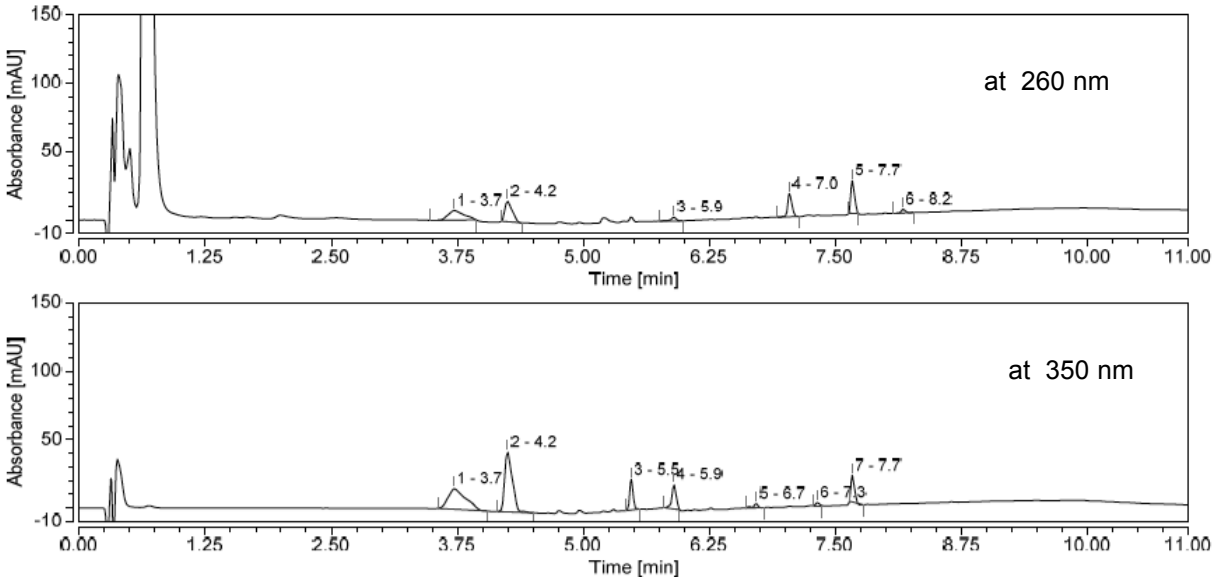
PLE-NTR probe **8** with PLE (0.55 U) after 60 min



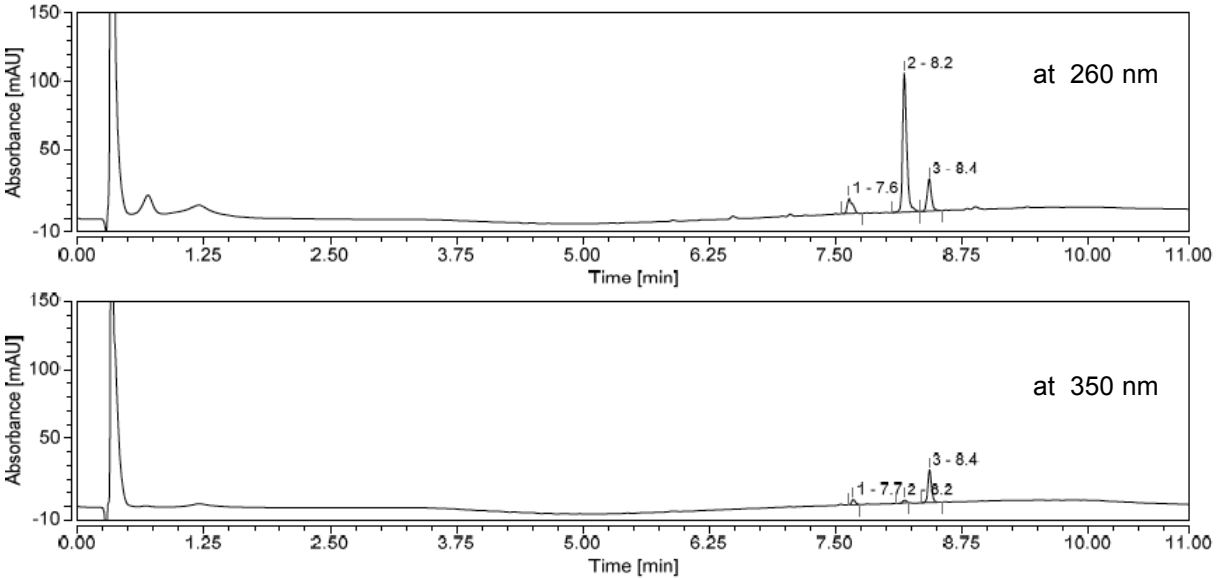
PLE-NTR probe **8** with PLE (0.55 U) after 120 min (addition of 0.6 U NTR at 80 min)



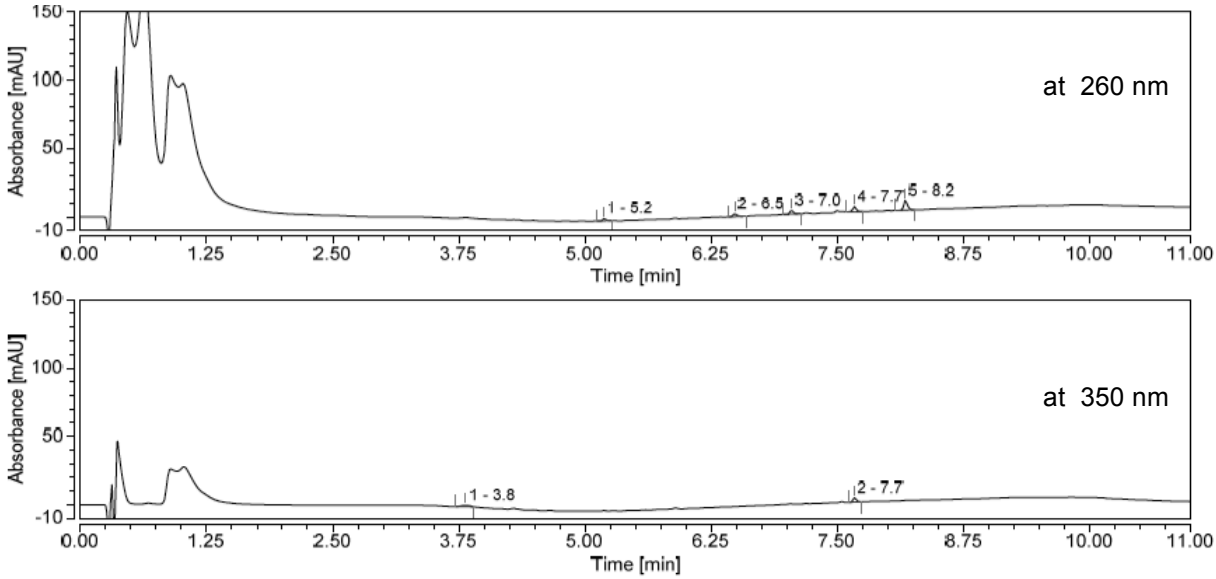
PLE-NTR probe **8** with PLE (0.55 U) after 180 min (addition of 0.6 U NTR at 80 min)



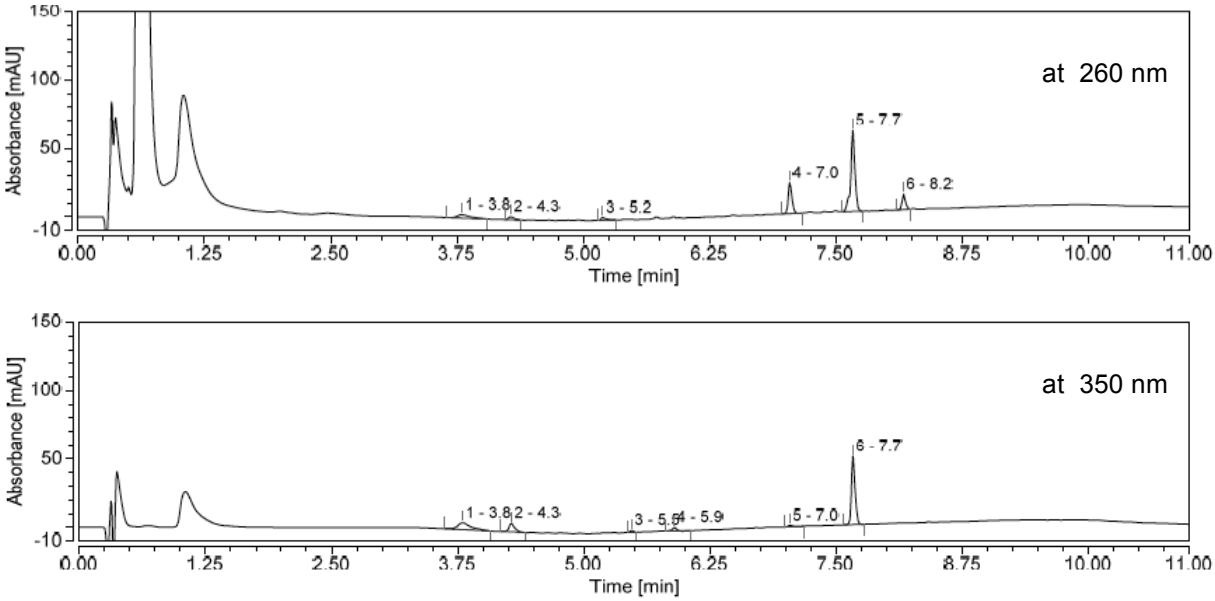
PLE-NTR probe **8** with NTR (0.6 U) after 30 min



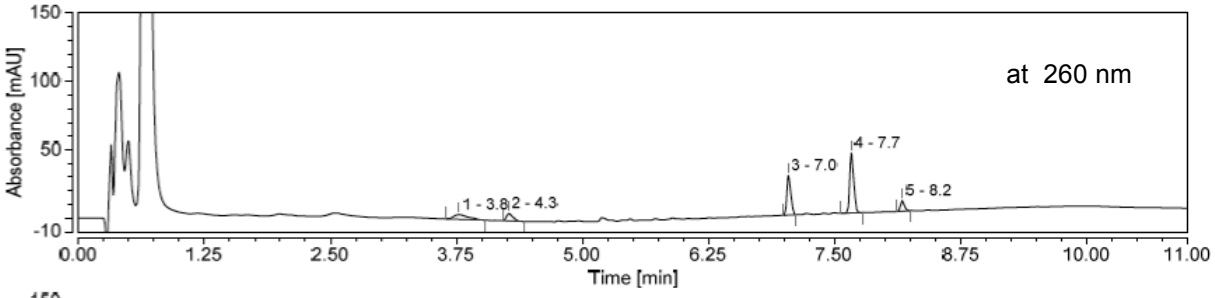
PLE-NTR probe **8** with NTR (0.6 U) after 60 min

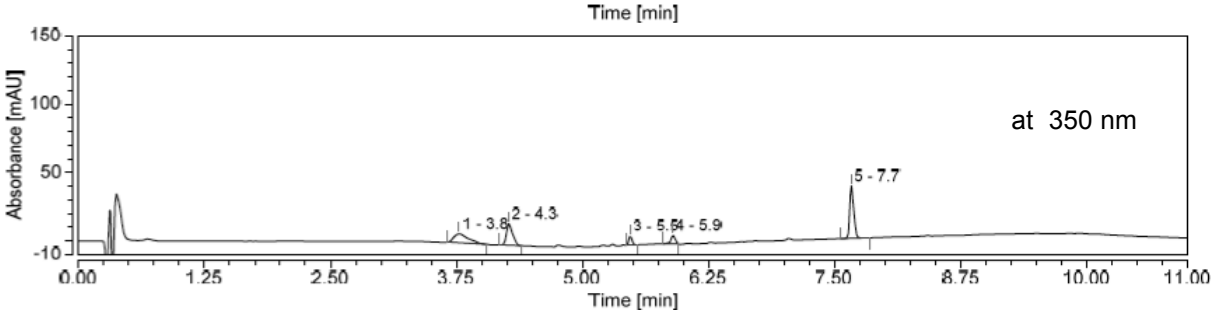


PLE-NTR probe **8** with NTR (0.6 U) after 120 min (addition of 0.55 U PLE at 80 min)

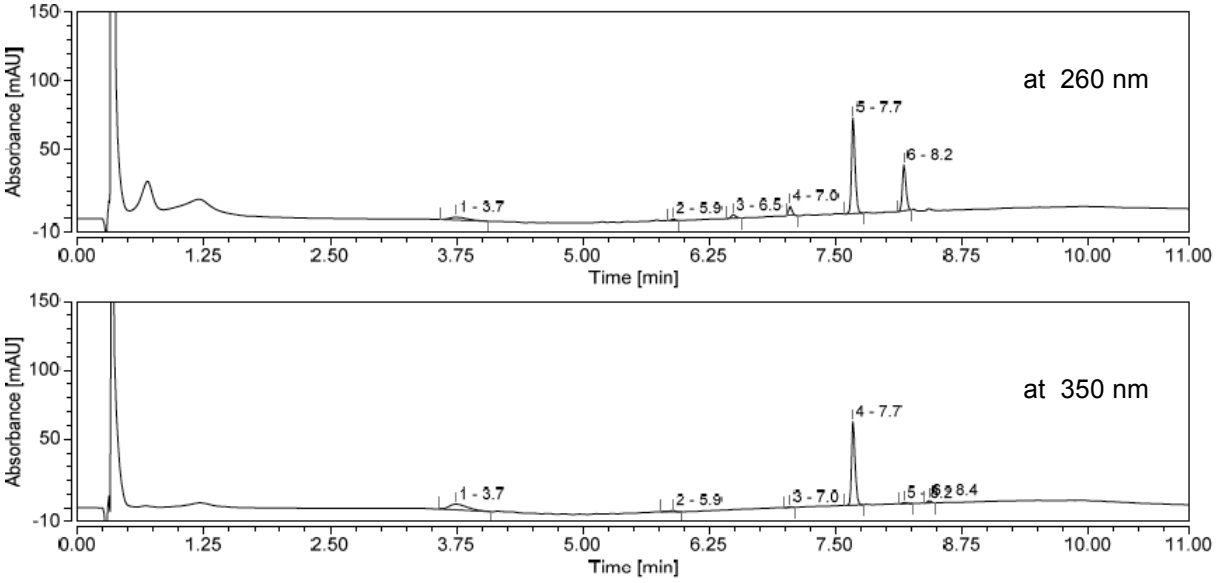


PLE-NTR probe **8** with NTR (0.6 U) after 180 min (addition of 0.55 U PLE at 80 min)

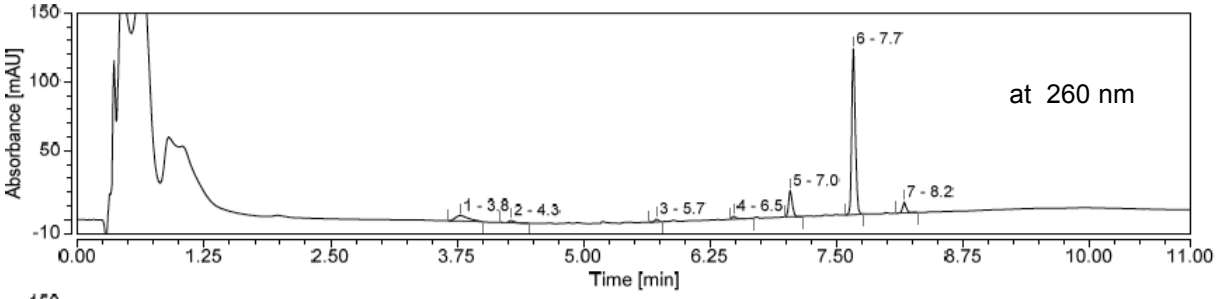


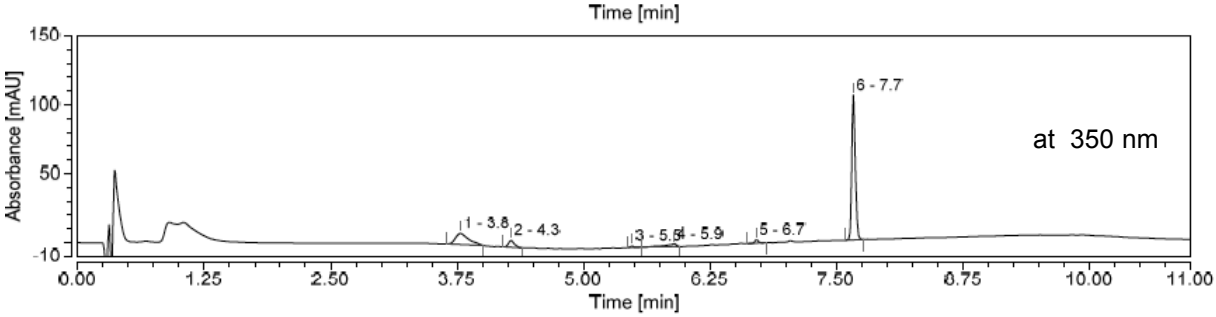


PLE-NTR probe **8** with NTR (0.6 U) and PLE (0.55 U) after 30 min

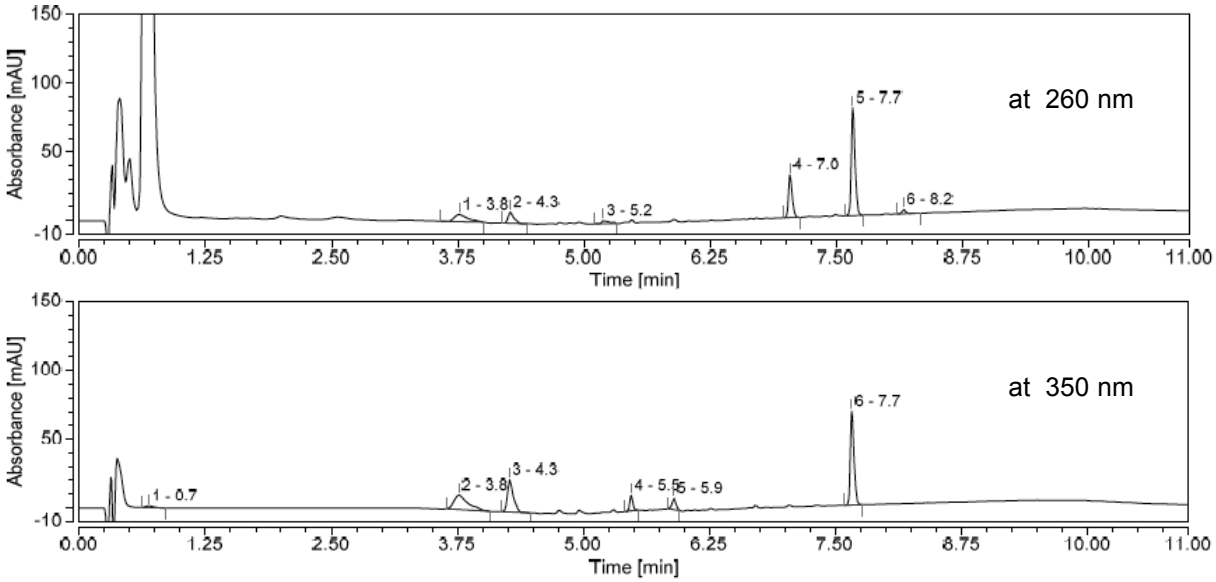


PLE-NTR probe **8** with NTR (0.6 U) and PLE (0.55 U) after 60 min





PLE-NTR probe **8** with NTR (0.6 U) and PLE (0.55 U) after 120 min



PLE-NTR probe **8** with NTR (0.6 U) and PLE (0.55 U) after 180 min

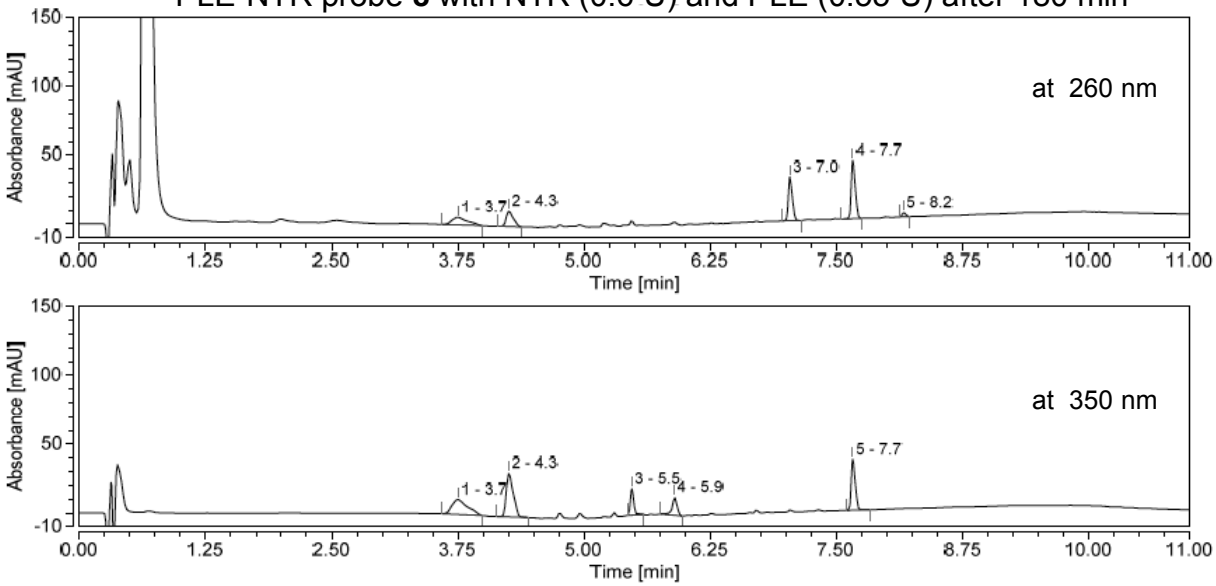
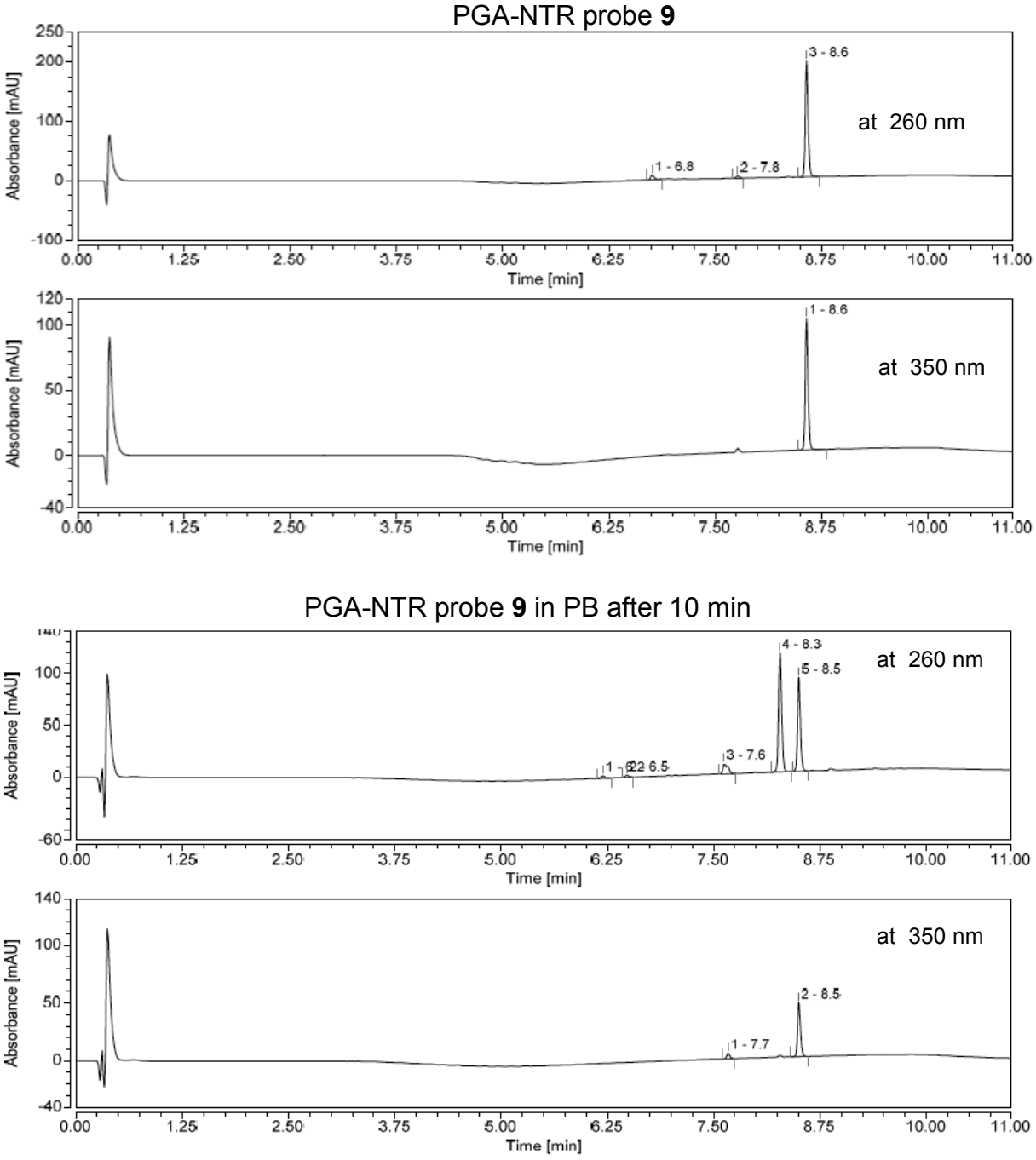
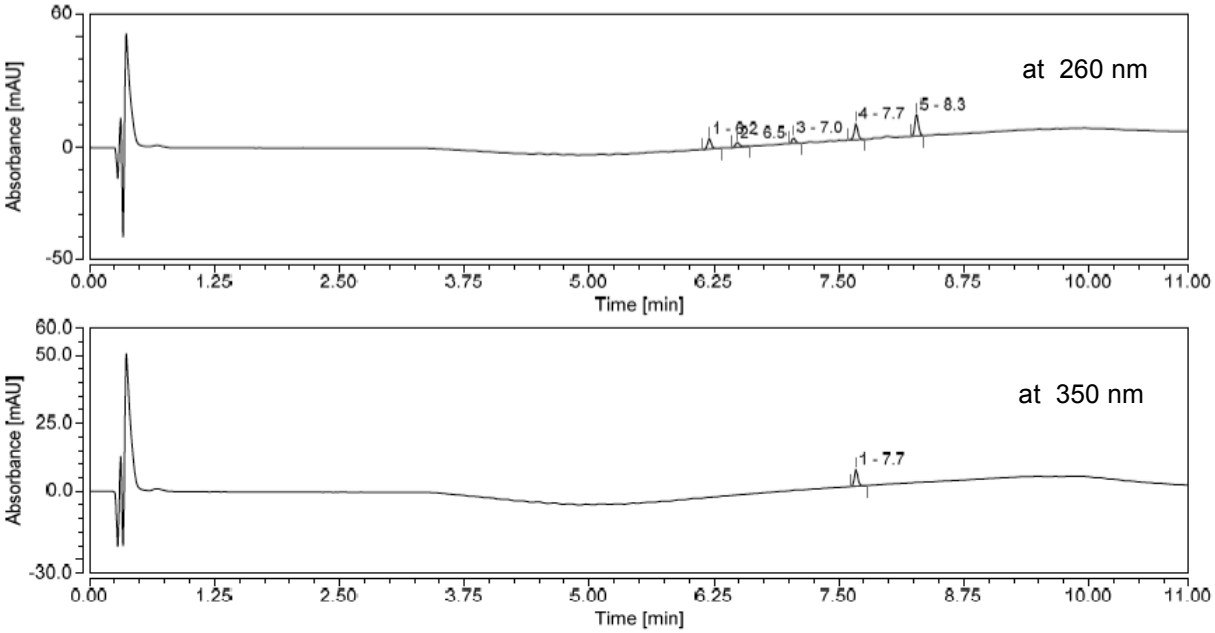


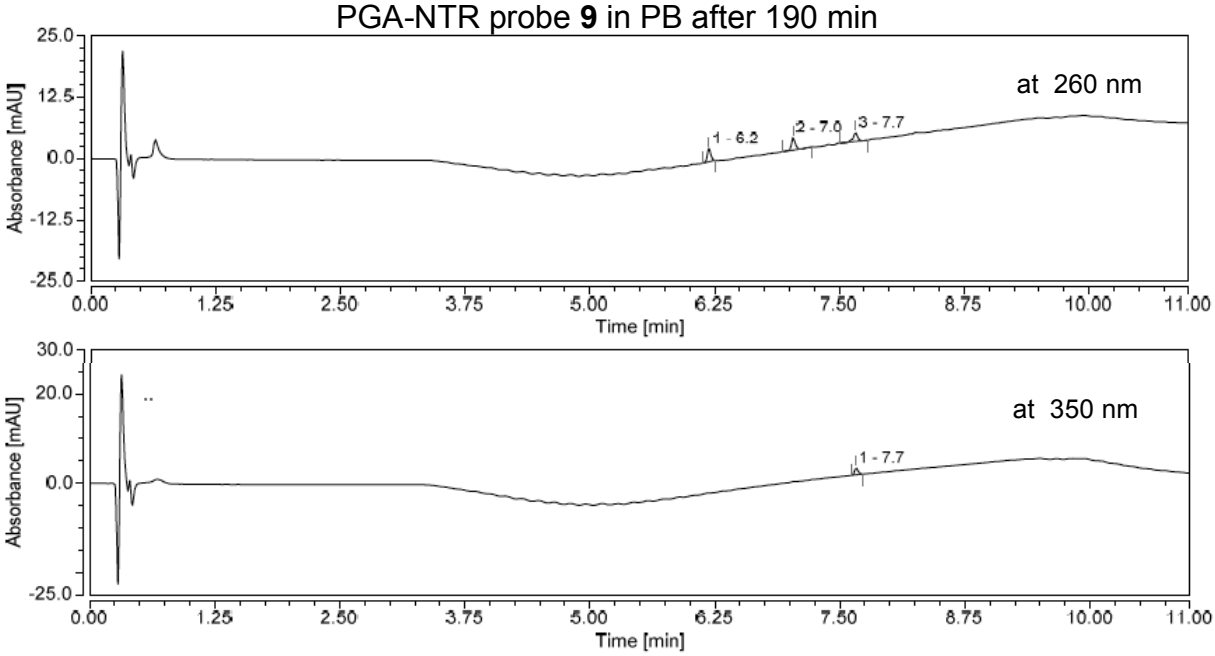
Fig S12. RP-HPLC elution profiles (system C) of fluorogenic probe 9 after incubation in PB alone or with enzymes (PGA and NTR/NADH)



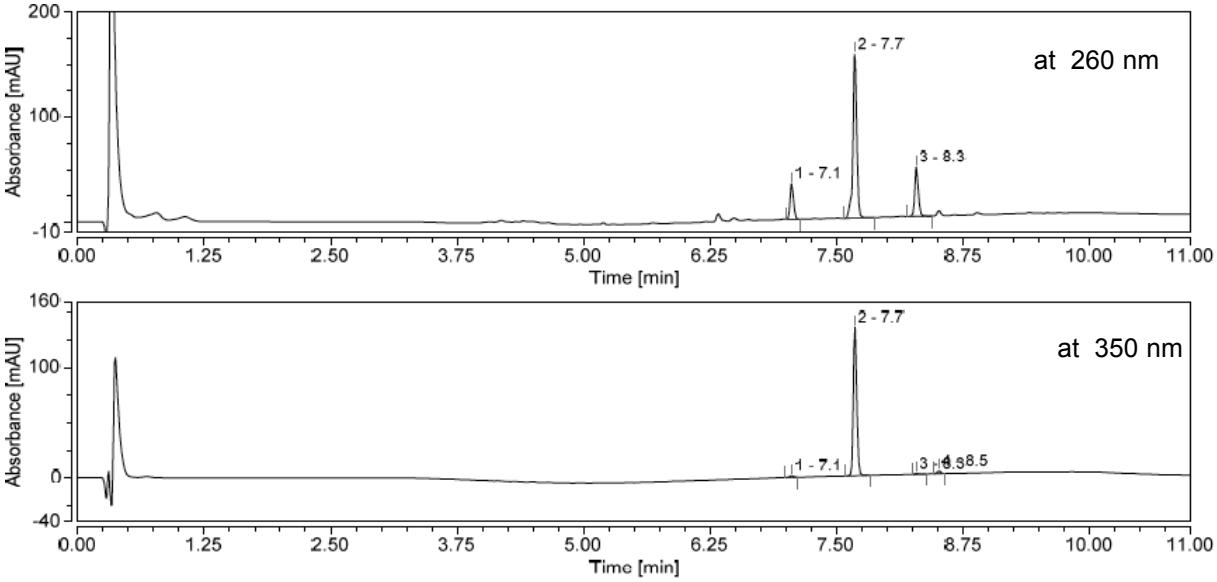
PGA-NTR probe **9** in PB after 90 min



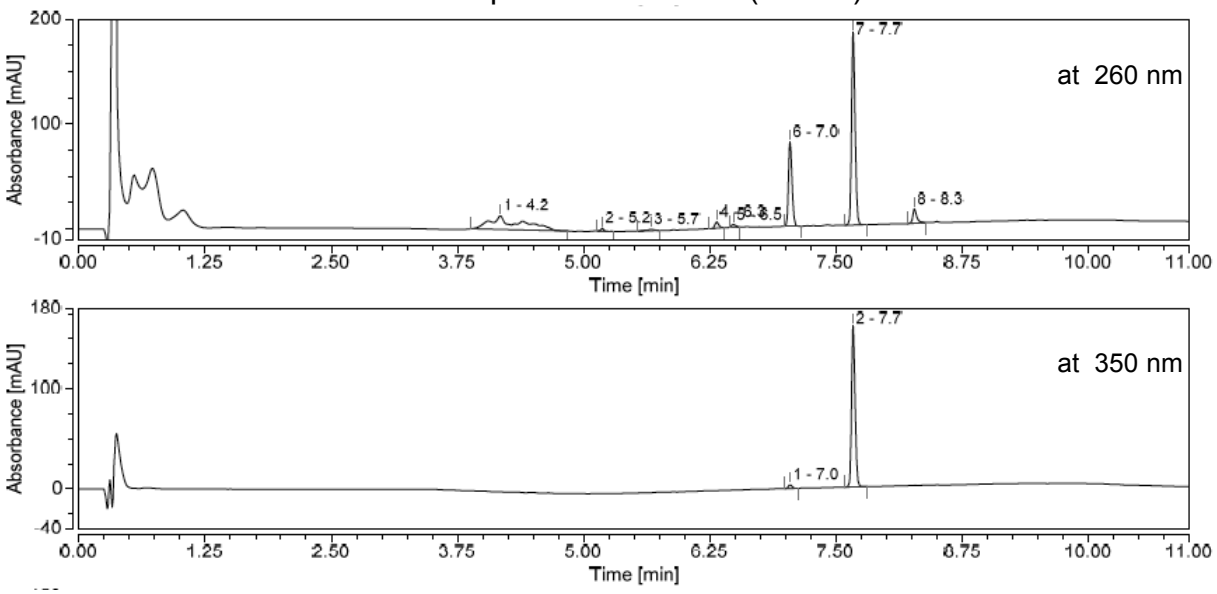
PGA-NTR probe **9** in PB after 190 min



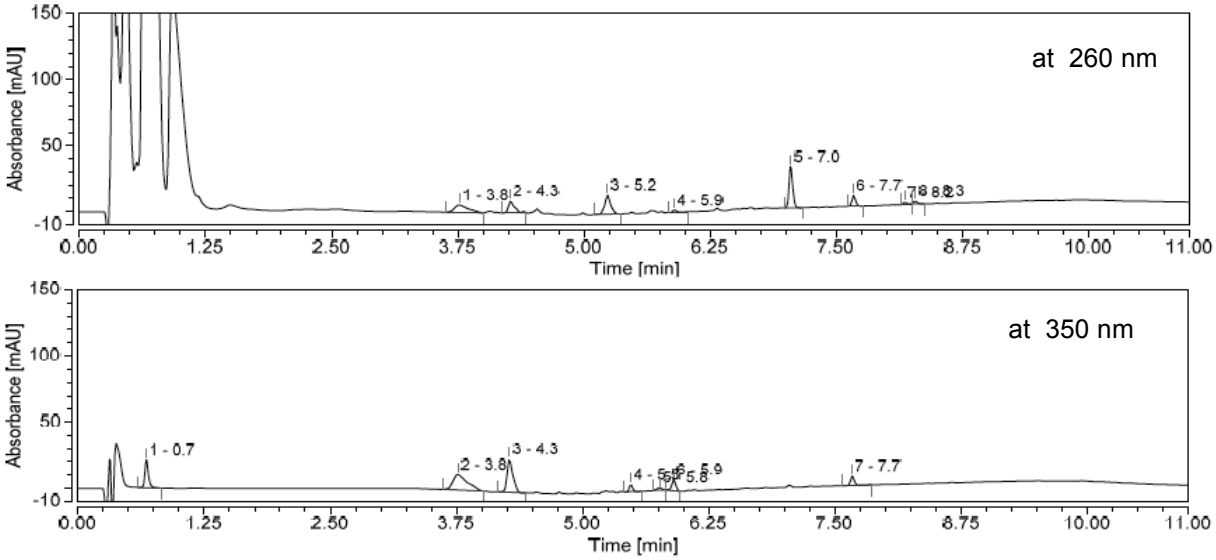
PGA-NTR probe **9** with PGA (0.75 U) after 30 min



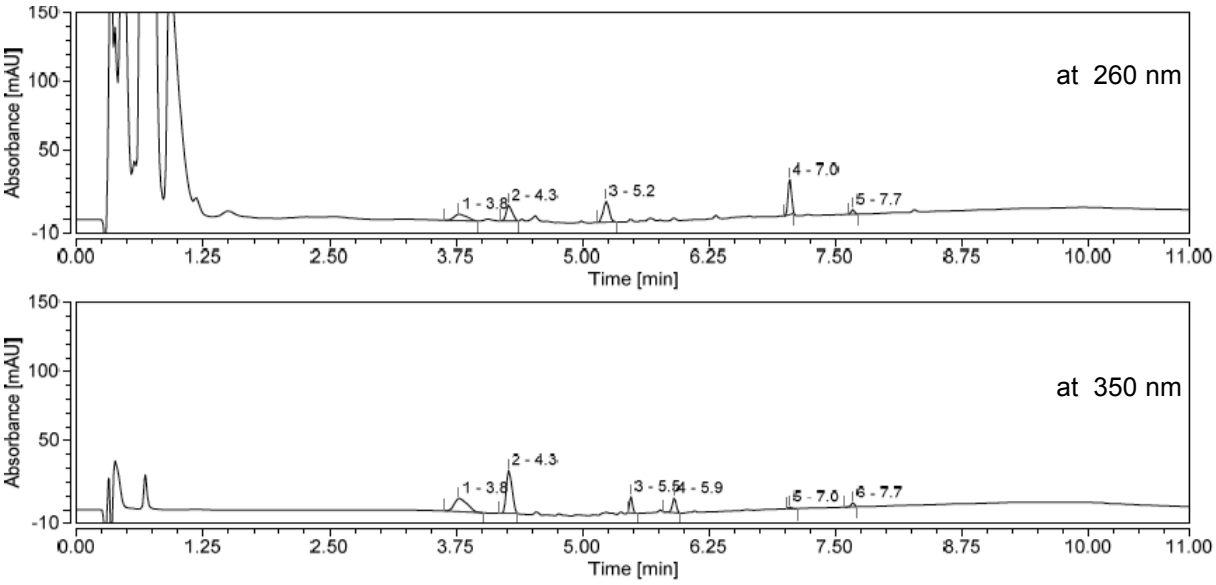
PGA-NTR probe **9** with PGA (0.75 U) after 60 min



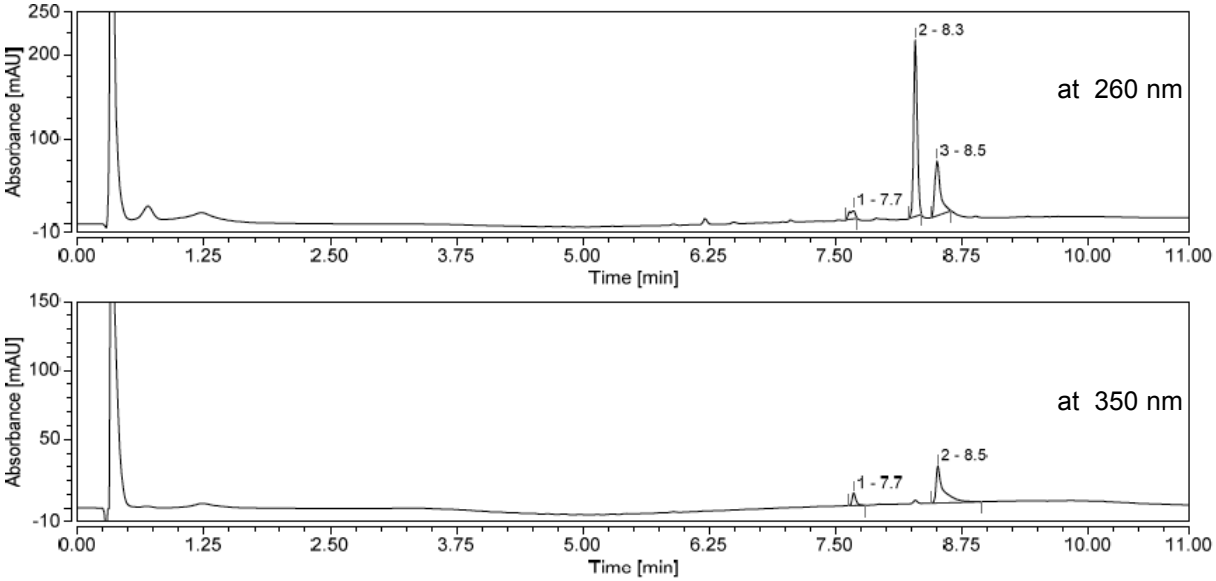
PGA-NTR probe **9** with PGA (0.75 U) after 120 min (0.6 U NTR adding at 80 min)



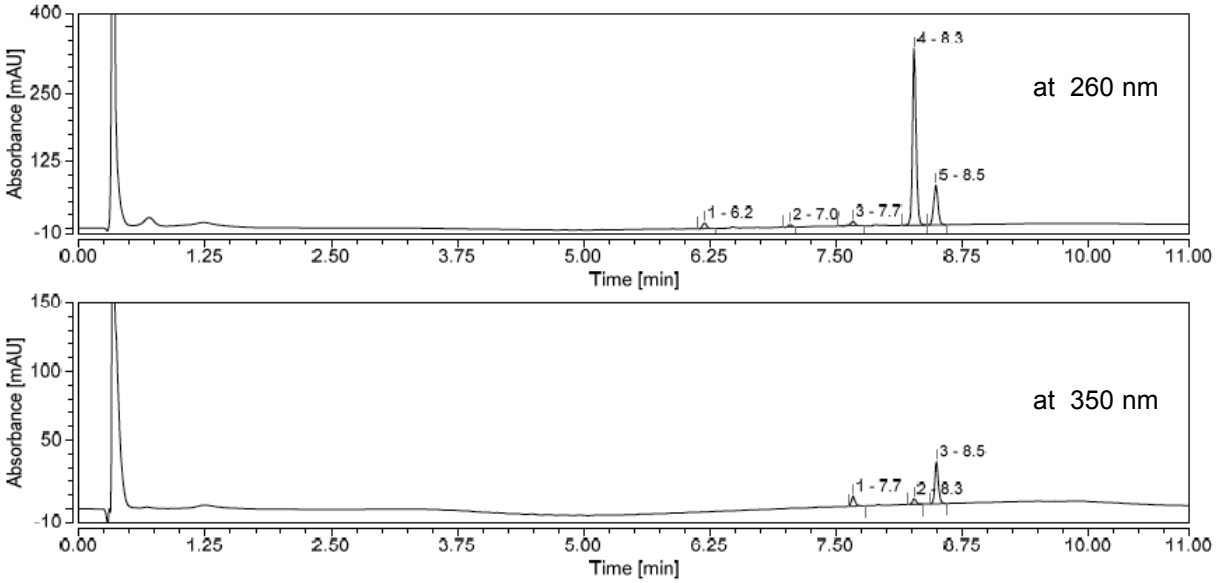
PGA-NTR probe **9** with PGA (0.75 U) after 180 min (0.6 U NTR addition at 80 min)



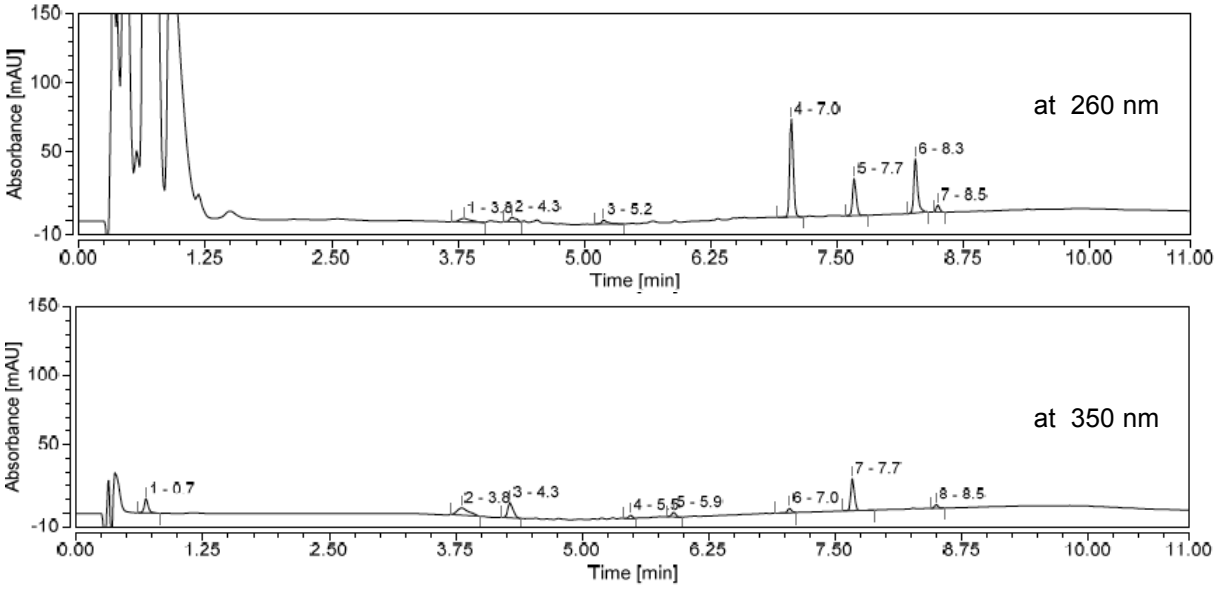
PGA-NTR probe **9** with NTR (0.6 U) after 30 min



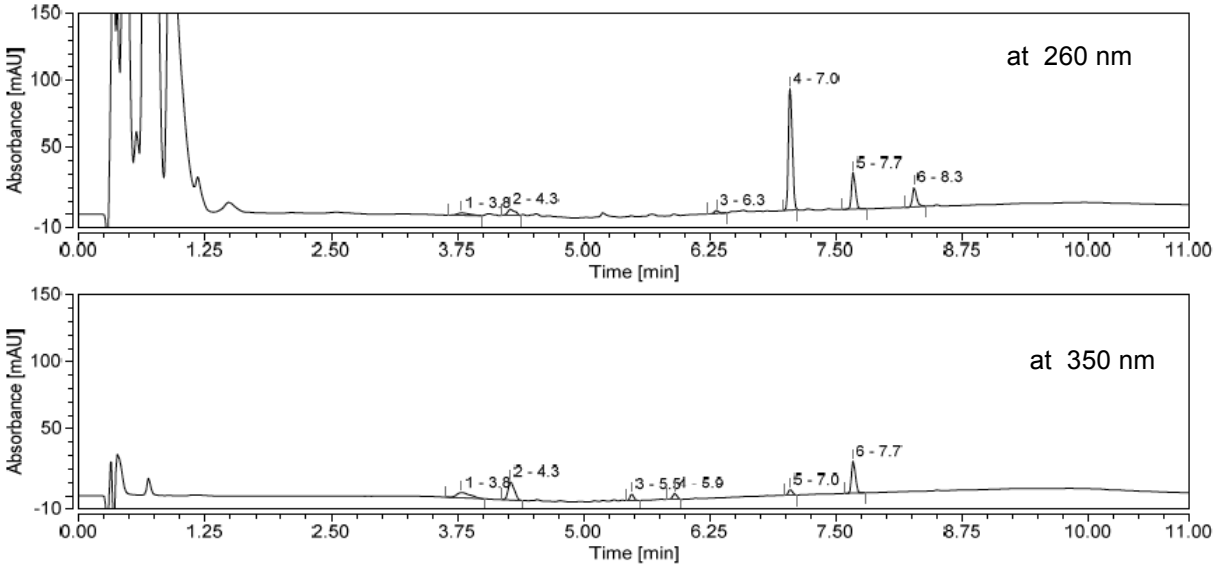
PGA-NTR probe **9** with NTR (0.6 U) after 60 min



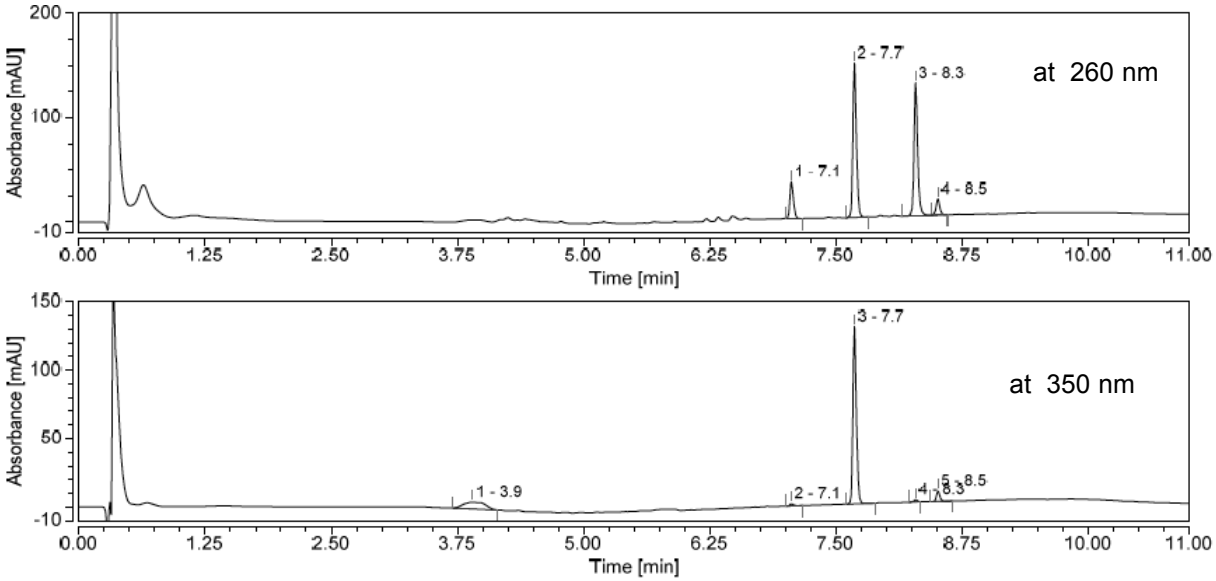
PGA-NTR probe **9** with NTR (0.6 U) after 120 min (addition of 0.75 U PGA at 80 min)



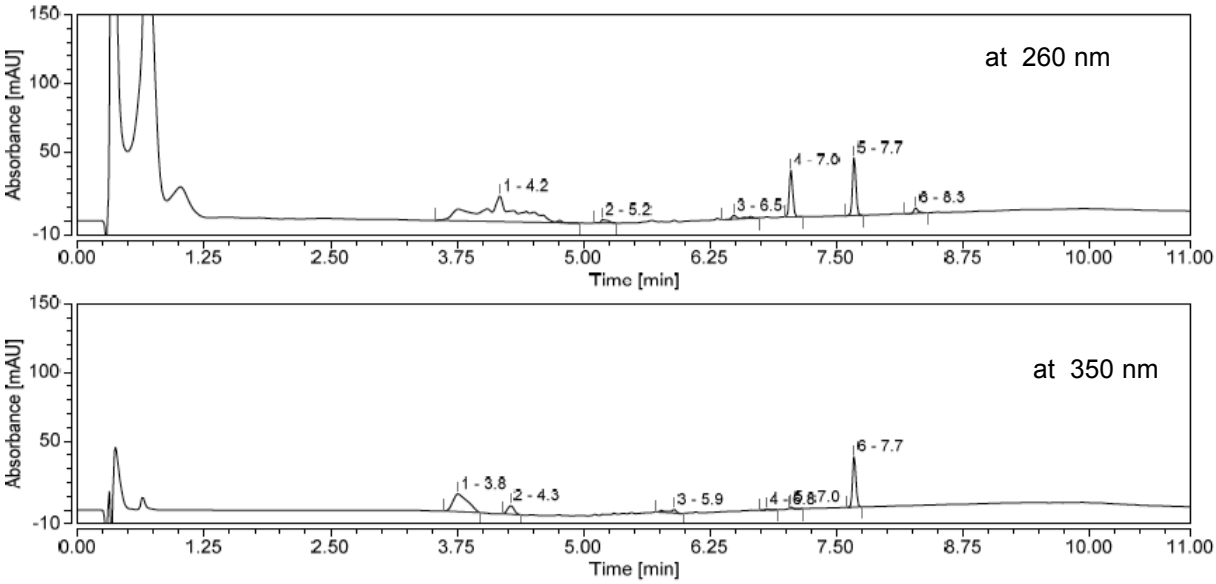
PGA-NTR probe **9** with NTR (0.6 U) after 180 min (addition of 0.75 U PGA at 80 min)



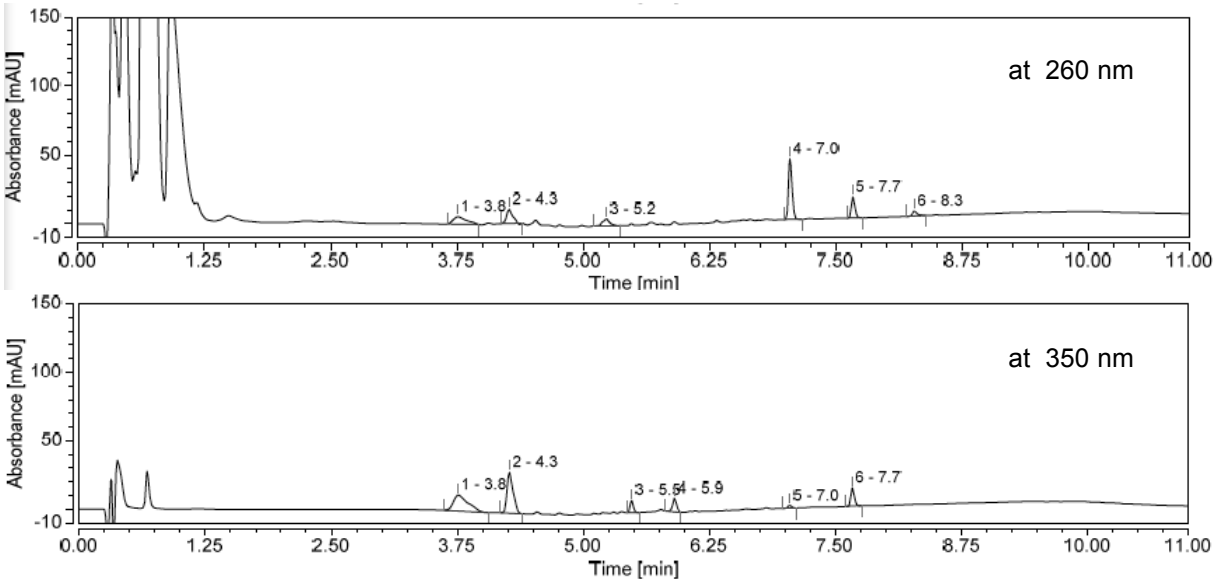
PGA-NTR probe **9** with NTR (0.6 U) and PGA (0.75 U) after 30 min



PGA-NTR probe **9** with NTR (0.6 U) and PGA (0.75) after 60 min



PGA-NTR probe **9** with NTR (0.6 U) and PGA (0.75 U) after 120 min



PGA-NTR probe **9** with NTR (0.6 U) and PGA (0.75 U) after 180 min