Electronic Supplementary Material (ESI) for Chemical Communications

First latent green fluorophores for the detection of azoreductase activity in bacterial cultures

Arnaud Chevalier,[a] Claire Mercier,[b] Laura Saurel,[b] Sylvain Orenga,[b] Pierre-Yves Renard*[a] and Anthony Romieu*[a]

[a]*Normandie Univ, COBRA, UMR 6014 & FR 3038; UNIV Rouen; INSA Rouen; CNRS, 1 Rue Tesnières, 76821 Mont-Saint-Aignan Cedex - France*
Fax: + 33 (0)2 35 52 29 71
Tel: + 33 (0)2 35 52 24 76 (or 24 27)
E-mail: pierre-yves.renard@univ-rouen.fr or anthony.romieu@univ-rouen.fr
Web:http://ircof.crihan.fr (Thématique Bioorganique)

[b]*bioMérieux - R&D Microbiologie, 3 Route de Port Michaud, 38390 La Balme-les-Grottes, France*
Fax: + 33 (0)4 74 95 26 32
Tel: + 33 (0)4 74 95 25 43
E-mail: sylvain.orenga@biomerieux.com

Supporting Information

Abbreviations

The following abbreviations are used throughout the text of the ESI file: ATR, attenuated total reflectance; AzoR, Azoreductase; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; ESI, electrospray ionisation; EtOAc, ethyl acetate; FMN, flavin mononucleotide or riboflavin-5'-phosphate; PBS, phosphate buffered saline; NaAsc, sodium ascorbate; NADH, nicotinamide adenine dinucleotide (reduced form); PDA, photodiode array; Rho110, rhodamine 110; RP-HPLC, reversed-phase high performance liquid chromatography; rt, room temperature; TEA, triethylamine; TFA, trifluoroacetic acid or trifluoroacetate; THF, tetrahydrofurane; TOF, time-of-flight; TSB, Tryptocase Soy Broth.

General

TLC were carried out on Merck DC Kieselgel 60 F-254 aluminum sheets. The spots were visualised by illumination with UV lamp (λ = 254 nm) and/or staining with a phosphomolybdic acid or $KMnO_4$ solution. Flash column chromatography purifications were performed on Geduran® Si 60 silica gel (63-200 μm was preferred for rhodamine 110 derivatives to reduce non-specific adsorption of these dyes over the stationary phase) from Merck. All chemicals were used as received from commercial sources without further purification unless otherwise stated. All solvents were dried following standard procedures (CH_3CN: distillation over CaH_2, DMSO: distillation over CaH_2 and THF: distillation over sodium benzophenone diketyl). Triethylamine (TEA) was distilled over KOH and stored over BaO. Rhodamine 110 (HCl salt, dye content 75%) was provided by Sigma-Aldrich. The HPLC-gradient grade acetonitrile (CH_3CN) was obtained from VWR. Phosphate buffered saline (PBS, 100 mM phosphate + 150 mM NaCl, pH 7.5) and aq. mobile-phases for HPLC were prepared using water purified with a Milli-Q system (purified to 18.2 MΩ.cm).

Instruments and methods

^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DPX 300 spectrometer. Chemical shifts are expressed in parts per million (ppm) from the residual non-deuterated solvent signal.[1] *J* values are expressed in Hz. Infrared (IR) spectra were recorded with an universal ATR sampling accessory on a Perkin Elmer FT-IR Spectrum 100 spectrometer. 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 (LRMS) were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray ionisation (ESI) source. High-resolution mass spectra (HRMS) were recorded on a Waters Synapt G2 HDMS mass spectrometer (ESI source with a qTOF analyzer). UV-visible spectra was obtained on a Varian Cary 50 scan spectrophotometer by using a rectangular quartz cell (Varian, standard cell, Open Top, 10 × 10 mm, 3.5 mL). Fluorescence spectroscopic studies (emission/excitation spectra) were performed with a Varian Cary Eclipse spectrophotometer with a semi-micro quartz fluorescence cell (Hellma, 104F-QS, 10 × 4 mm, 1400 μL). Emission spectra were recorded under the same conditions after excitation at the corresponding wavelength (excitation and emission filters: auto, excitation and emission slit = 5 nm).

High-performance liquid chromatography separations

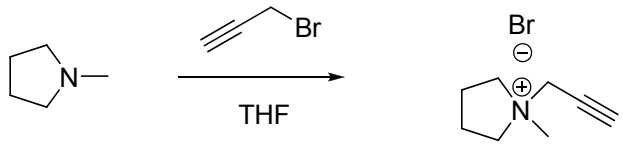
Several chromatographic systems were used for the analytical experiments and the purification steps: System A: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 2.1 ×

[1] G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw and K. I. Goldberg, *Organometallics*, 2010, **29**, 2176.

100 mm) with CH_3CN and aq. TFA (0.1 %, pH 2.0) as eluents [20% CH_3CN (5 min), followed by a linear gradient from 20% to 100% CH_3CN (45 min)] at a flow rate of 0.25 mL min^{-1}. Triple UV-vis detection was achieved at 220, 260, and 600 nm.
System B: semi-preparative RP-HPLC (Varian Kromasil C_{18} column, 10 µm, 21.2 × 250 mm) with CH_3CN and aq. TFA (0.1% , pH 2.0) as eluents [0% CH_3CN (5 min), followed by a gradient of 0% to 30% CH_3CN (20 min), then 30% to 100% CH_3CN (90 min)] at a flow rate of 20.0 mL min^{-1}. Double visible detection was achieved at 366 and 500 nm.

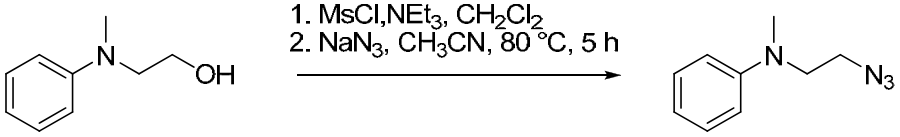
Synthesised compounds

Alkyne-functionalised pyrrolidinium salt (4)



N-Methylpyrrolidine (0.5 g, 5.9 mmol) wass dissolved in dry THF (20 mL) and stirred under an Ar atmosphere at 0 °C. Then, propargyl bromide solution (80 wt. %) in toluene (0.78 mL, 7.04 mmol, 1.2 equiv.) was added ropewises and the resulting reaction mixture was stirred at rt for 4 h. Thereafter, volatiles were removed under reduced pressure and the resulting residue was purified by trituration in THF to give the desired product **4** as a white hygroscopic solid (1.2 g, quant. yield). δ_H(300 MHz, $CDCl_3$) 2.32 bs (4 H), 3.29 (s, 4 H), 3.66 (m, 2 H), 3.78 (m, 2 H), 4.36 (d, *J* 2.5, 2 H); δ_C(75MHz, $CDCl_3$) 21.9, 49.6, 53.4, 64.1, 71.9, 80.5; LRMS (ESI+): *m/z* 124.00 $[M]^{+\bullet}$, calcd for $C_8H_{14}N^+$ 124.11.

N-(2-Azidoethyl)-*N*-methylaniline (3)[2]

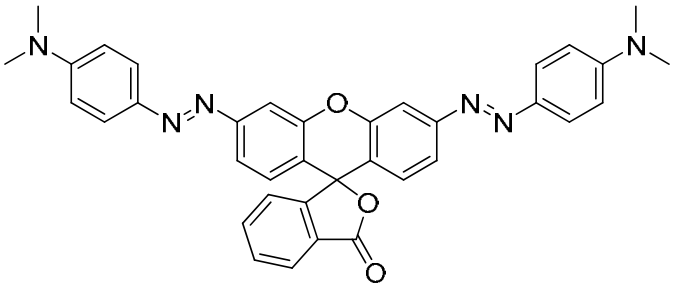


2-*N*-Methylanilinoethanol (1.0 g, 6.6 mmol, 1 equiv.) and dry TEA (1.1 mL, 8.25 mmol, 1.25 equiv.) were dissolved in dry CH_2Cl_2 (30 mL). The resulting mixture was cooled to 0 °C and kept under an Ar atmosphere. Then, mesyl chloride (560 µL, 7.2 mmol, 1.1 equiv.) was added dropwise and the reaction mixture was stirred at rt for 1 h. The newly formed precipitate (triethylammonium chloride salt) was removed by filtration and the filtrate was diluted with CH_2Cl_2 (30 mL), washed with brine (2 × 10 mL) and deionised water (20 mL), dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The resulting mesylate derivative was dissolved in dry CH_3CN (20 mL) and NaN_3 (3.5 g, 53 mmol, 8 equiv.) was added. The reaction mixture was stirred at 80 °C for 5 h. After cooling to rt, NaN_3 salt was removed by filtration and filtrate was evaporated to dryness. The crude product was purified by flash-column chromatography (silica gel, cyclohexane-EtOAc 95 : 5, v/v). Compound **3** was recovered as a colorless oil (1.1 g, yield 95%). R_f 0.70 (cyclohexane-EtOAc 8 : 2, v/v); ν_{max}(neat)/cm^{-1} 2890, 2090, 1598, 1504, 1360, 1344; δ_H(300 MHz, $CDCl_3$) 3.01 (s, 3 H); 3.45 (t, *J* 6.0, 2 H), 3.55 (t, *J* 6.0, 2 H), 6.75 (m, 3 H), 7.26 (m, 2 H); δ_C(75MHz, $CDCl_3$) 39.0,

[2] Improved synthesis compared to that previously published by us: 2 A. Chevalier, C. Massif, P.-Y. Renard and A. Romieu, *Chem. Eur. J.*, 2013, **19**, 1686.

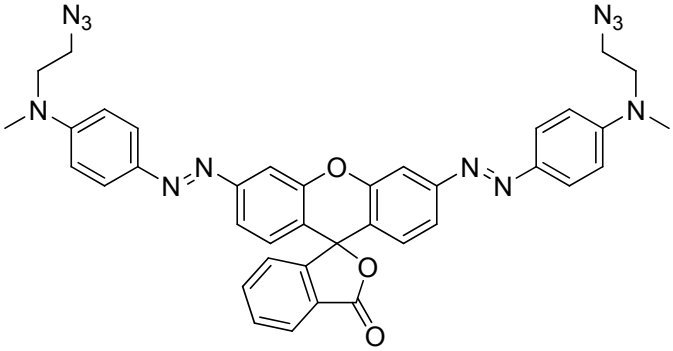
48.9, 52.2, 112.4, 117.1, 129.5, 148.6; LRMS (ESI+): *m/z* 177.2 $[M + H]^+$ (100), calcd for $C_9H_{12}N_4$: 176.11.

Bis(diazo)-based pro-fluorophore (1)



Rhodamine 110, HCl salt (50 mg, 0.136 mmol, 1 equiv.) was dissolved in a mixture of dry CH_3CN/DMSO (83 : 17, v/v, 1.2 mL). Distilled TEA (21 µL, 0.149 mmol, 1.1 equiv.) was added and the mixture was stirred under an Ar atmosphere at 0 °C until complete dissolution of the rhodamine dye. Then, solid $NOBF_4$ salt was added by portions (35 mg, 0.30 mmol, 2.2 equiv.) and the resulting reaction mixture was stirred vigourously at 0 °C for 15 min. Thereafter, *N*,*N*-dimethylaniline (40 mg, 0.326 mmol, 2.4 equiv.) was dissolved in dry CH_3CN (0.2 mL) was slowly added to the pre-formed *N*-nitrosamine/diazonium salt intermediate and the resulting reaction mixture was stirred at 0 °C for further 15 min, and at rt for further 30 min. Thereafter, volatiles were removed under reduced pressure and the crude product is directly purified by flash-column chromatography (silica gel, step gradient of EtOAc in cyclohexane from 10 to 50%). The desired pro-fluorophore **1** was obtained as an orange amorphous powder (55 mg, yield 68%). R_f 0.51 (cyclohexane-EtOAc, 1 : 1, v/v); mp = 124 °C; ν_{max}(neat)/cm^{-1} 2899, 2841, 1762, 1594, 1517, 1394, 1358, 1129, 1094, 868, 823; δ_H(300 MHz, $CDCl_3$) 3.10 (s, 12 H), 6.75 (d, *J* 9.0, 4 H), 6.95 (d, *J* 8.5, 2 H), 7.2 (d, *J* 7.1, 1 H), 7.54 (dd, *J* 1.4, *J* 13.8, 2 H), 7.66 (qt, *J* 6.9, 2 H), 7.78 (d, *J* 1.4, 2 H), 7.89 (d, *J* 9, 4 H), 8.07 (d, *J* 7.1, 1 H); δ_C(75MHz, $CDCl_3$) 40.3, 82.45, 110.3,111.4, 118.1, 119.0, 123.9, 125.3, 125.5, 125.9, 128.4, 129.9, 135.3, 143.5, 151.8, 152.8, 153.7, 154.8, 169.6; HPLC (system A): t_R = 35.1 min (purity 97%); LRMS (ESI+): *m/z* 595.20 $[M + H]^+$ (100%), 581.20 $[M + 2H - CH_3]^+$ (65%), 567.33 $[M + 3H - 2CH_3]^+$ (35%); HRMS (ESI+): 595.2458 $[M + H]^+$, calcd for $C_{36}H_{31}N_6O_3^+$ 5995.2457.

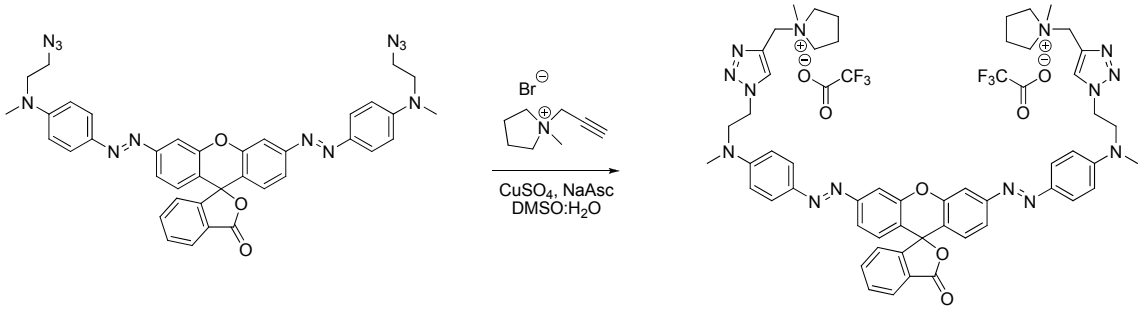
"Clickable" bis(diazo)-based pro-fluorophore (2)



Rhodamine 110, HCl salt (100 mg, 0.297 mmol, 1 equiv.) was dissolved in a mixture of dry CH_3CN/DMSO (8 : 2, v/v, 2.5 mL). Distilled TEA (42 µL, 0.297 mmol, 1 equiv.) was added and the mixture was stirred under an Ar atmosphere at 0 °C until complete dissolution of the

rhodamine dye. Then, solid $NOBF_4$ salt was added by portions (76 mg, 0.653 mmol, 2.2 equiv.) and the resulting reaction mixture was stirred vigourously at 0 °C for 15 min. Thereafter, *N*-(2-azidoethyl)-*N*-methylaniline **3** (125 mg, 0.713 mmol, 2.4 equiv.) was dissolved in dry CH_3CN (0.5 mL) was slowly added to the pre-formed *N*-nitrosamine/diazonium salt intermediate and the resulting reaction mixture was stirred at 0 °C for further 15 min, and at rt for further 30 min. Thereafter, volatiles were removed under reduced pressure and the crude product is directly purified by flash-column chromatography (silica gel, step gradient of EtOAc in cyclohexane from 10 to 50%). The desired pro-fluorophore **2** was obtained as an orange amorphous powder (128 mg, yield 61%). R_f 0.46 (cyclohexane-EtOAc, 1 : 1, v/v); mp = 167 °C, ν_{max}(neat)/cm^{-1} 2919, 2854, 2092, 1759, 1597, 1517, 1375, 1136, 1094, 881, 823; δ_H(300 MHz, $CDCl_3$) 3.13 (s, 6 H), 3.52 (t, *J* 6.0, 4 H), 3.64 (t, *J* 6.0, 4 H), 6.78 (d, *J* 9.0, 4 H), 6.95 (d, *J* 8.5, 2 H), 7.2 (d, *J* 7.1, 1 H), 7.54 (dd, *J* 1.4, *J* 13.8, 2 H), 7.66 (qt, *J* 6.9, 2 H), 7.78 (d, *J* 1.4, 2 H), 7.90 (d, *J* 9.0, 4 H), 8.07 (d, *J* 7.1, 1 H); δ_C(75MHz, $CDCl_3$) 39.3, 49.0, 51.8, 82.4, 110.5, 111.7, 118.2, 119.3, 124.0, 125.4, 125.7, 126.0, 128.5, 130.1, 135.4, 144.1, 151.3, 151.9, 153.7, 154.8, 169.7. HPLC (system A): t_R = 35.7 min (purity 98%); LRMS (ESI+): *m/z* 705.20 $[M + H]^+$; HRMS (ESI+): *m/z* 705.2799 $[M + H]^+$, calcd for $C_{38}H_{33}N_{12}O_3^+$ 705.2798.

Water-soluble bis(diazo)-based pro-fluorophore (5)



Bis-azido derivative **2** (30 mg, 42.5 µmol, 1 equiv.) was dissolved in DMF (5 mL). Then, pyrrolidinium salt **4** (26 mg, 136 µmol, 3 equiv.) in solution in deionised water (0.2 mL) was added. Aq. solutions (100 mg.mL^{-1}) of $CuSO_4$ pentahydrate (1 mg, 4.25 µmol, 0.1 equiv, 10µL) and sodium ascorbate (1.7 mg, 8.5 µmol, 0.2 equiv, 17µL) were added and the resulting reaction mixture was stirred under an Ar atmosphere at 50 °C for 1 h. The reaction was checked for completion by RP-HPLC (system A). Thereafter, DMF was removed under reduced pressure and the crude product was purified by semi-preparative RP-HPLC (system B) to give (after freeze-drying) the TFA salt of **5** as an orange amorphous powder (35 mg, yield 70%). δ_H(300 MHz, DMSO-d_6) 1.99 (bs, 8 H), 2.78 (bs, 6 H), 2.99 (bs, 6 H), 3.17 (m, 4 H), 3.40 (m, 4 H), 4.02 (bt, 4 H), 4.60 (bs, 4 H), 4.73 (bt, 4 H), 6.73 (d, *J* 9.0, 4 H), 7.02 (d, *J* 8.5, 2 H), 7.41 (d, *J* 7.1, 1 H), 7.55 (dd, *J* 1.4, *J* 13.8, 2 H), 7.7-7.9 (m, 8 H), 8.11 (d, *J* 7.1, 1 H), 8.41 (s, 2 H); δ_C(75 MHz, DMSO-d_6) 20.9, 37.6, 47.4, 47.7, 50.9, 56, 62.5, 81.3, 109.6, 111.2, 118.0, 119.0, 124.0, 125.2, 128.5, 129.0, 130.6, 136.0, 142.5, 151.1, 151.8, 152.5, 154.0, 157.8, 158.2, 168.6; HPLC (system A): t_R = 23.6 min (purity >99%); LRMS (ESI+): *m/z* 476.40 $[M + 2H]^{2+}$; HRMS (ESI+): *m/z* 476.2492 $[M]^{2+\bullet}$, calcd for ($C_{54}H_{60}N_{14}O_3^{2+}$/2)= 476.2486.

Fluorogenic bio-reduction assays

I*n vitro* cleavage of pro-fluorophore 1 by sodium dithionite (fluorescence assay)

Stock solutions (concentration: 100 μM) of AzoR fluorogenic probe **1** was prepared in DMSO. A 1.0 μM solution of this pro-fluorophore was then obtained by dilution of the stock solution with deionised water and transferred into a semi-micro quartz fluorescence cell (final volume: 1250 μL). 12.5 μL of a 10 mM aq. solution of sodium dithionite (100 equiv.) was added and the fluorescence emission of the released Rho110 dye was monitored at λ = 520 nm (ex. λ = 500 nm) over time with measurements recoreded every 5 s (kinetic mode, 25 °C). The lack of non-specific hydrolysis/reduction of pro-fluorophore **1** was confirmed by fluorescence emission measurements at λ = 520 nm (ex. λ = 500 nm) without sodium dithionite, in the same conditions. Fluorescence emission spectrum of the probe **1** (scan mode, ex. λ = 450 nm) was recorded before and after incubation with sodium dithionite.

I*n vitro* cleavage of pro-fluorophores 1 and 5 by AzoR from *Escherichia coli* (fluorescence assay)

- Stock solutions (concentration: 2.5 mM) of AzoR fluorogenic probes **1** and **5** were prepared in a mixture of DMSO-H_2O (12.5 : 87.5, v/v) and in deionised water respectively. For compound **1**, a significant amount of powder was found to be not soluble in DMSO-H_2O mixture which was removed by filtration before dilution. Thus, the actual concentration is lower than the "theoritical" value.

- 100 μM solution of AzoR fluorogenic probes **1** and **5** were preprared in a sterile 96-well plates (Greiner, Courtaboeuf, France) by dilution of stock solutions (*vide supra*) with phosphate buffer (50 mM, pH 7.0) containing 0.5 mM NADH and 5 μM FMN. 1 μg of AzoR from *Escherichia coli*[3] was added. The plate was incubated at 35 °C and the fluorescence emission of the released Rho110 was monitored at λ = 525 nm (ex. λ = 495 nm) over time with measurements recorded every 60 s. The lack of non-specific hydrolysis/reduction of pro-fluorophores **1** and **5** was confirmed by fluorescence emission measurements at λ = 525 nm without AzoR enzyme in the same conditions.

Biological reduction of AzoR chromogenic (Methyl Red) and fluorogenic (pro-fluorophores 1 and 5) substrates

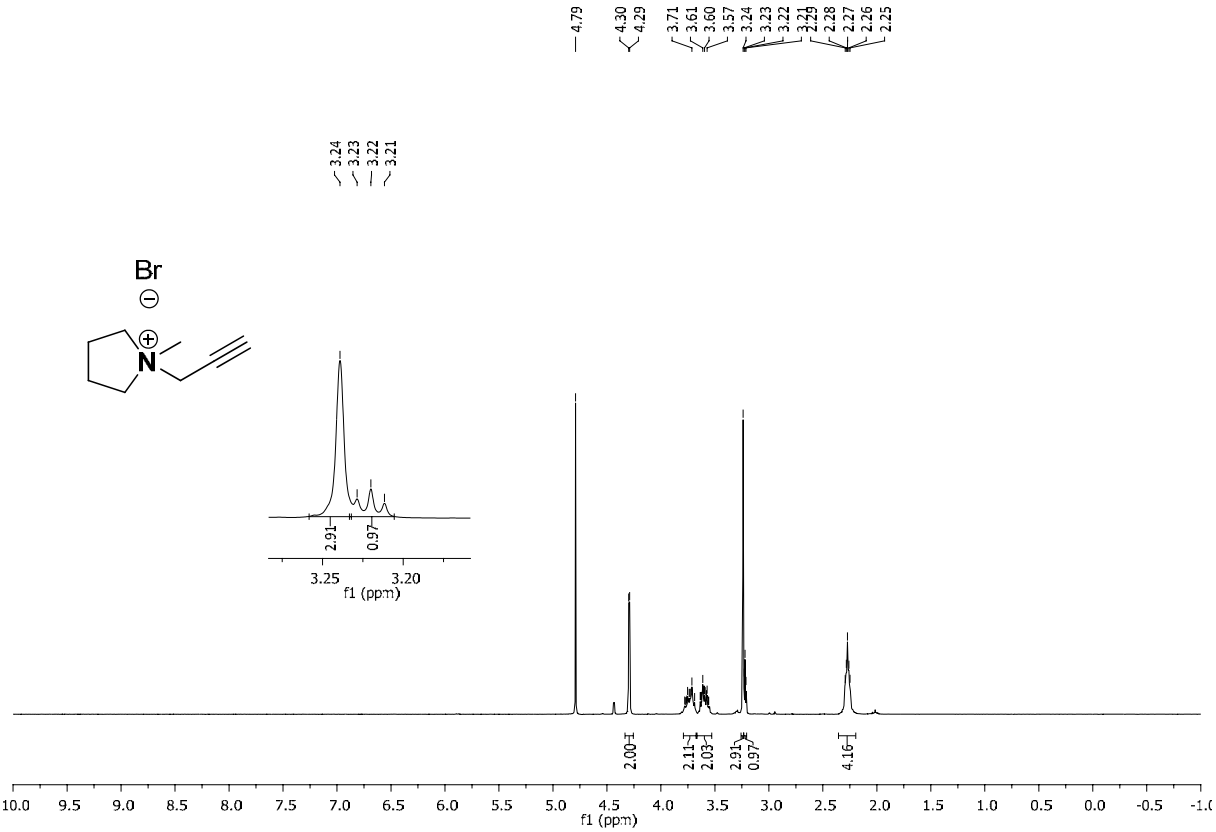
- Media preparation: Solutions of AzoR chromogenic/fluorogenic substrates were incorporated into TSB and added to a sterile 96-well plate to give final concentrations of 100 μM.

- Bacterial suspension preparation: Bacterial strains were from bioMérieux collection (La Balme-les-Grottes, France). Each strain was used to inoculate the different media at a final turbidity of 0.5 McFarland units (1.5×10^8 organisms / mL).

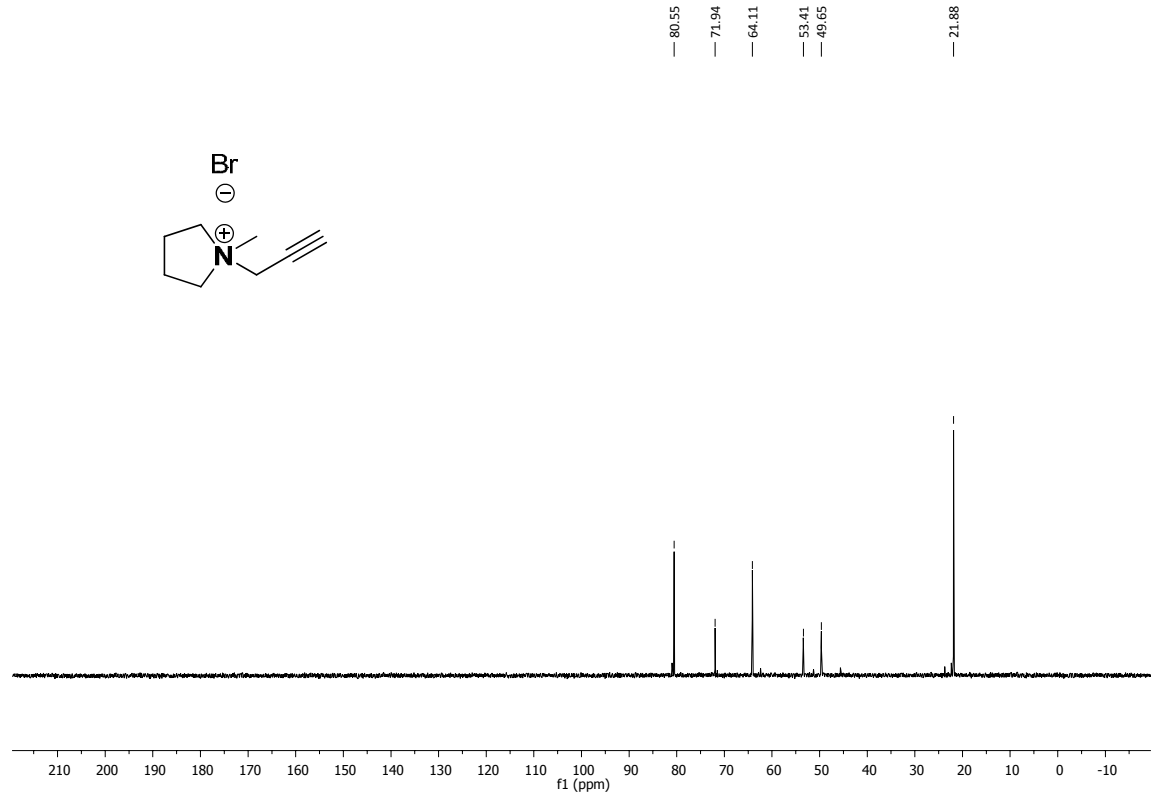
- Fluoroescence assay: The 96-well plate was incubated at 35 ^{o}C and the fluorescence emission of the released anthranilic acid/Rho110 was monitored at λ = 395/525 nm (ex. λ = 250/495 nm) over time with measurements recorded every 20 min.

[3] C. Mercier, V. Chalansonnet, S. Orenga and C. Gilbert, *J. Appl. Microbiol.*, 2013, in press.

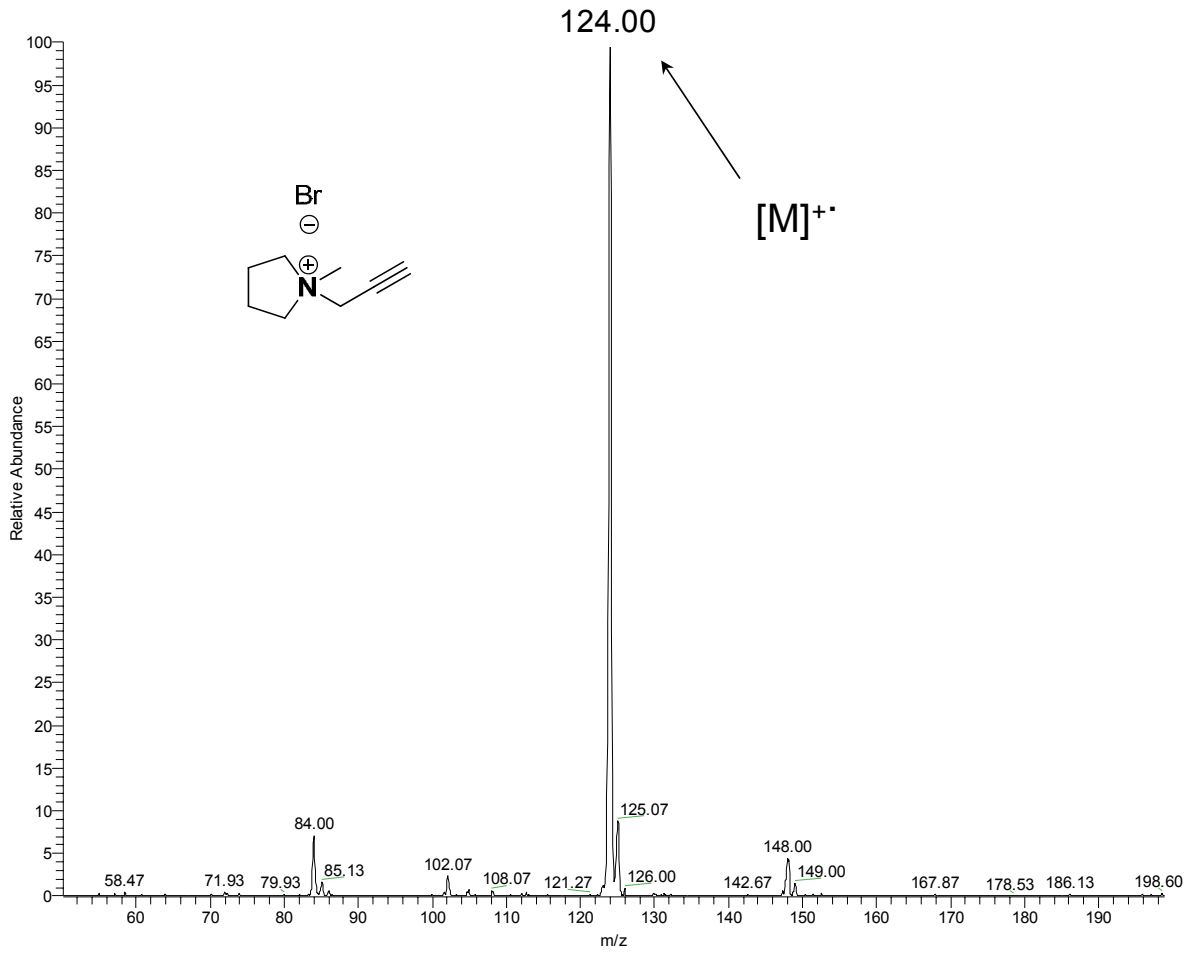
^{1}H NMR spectrum of compound 4 recorded in $CDCl_3$ at 300 MHz



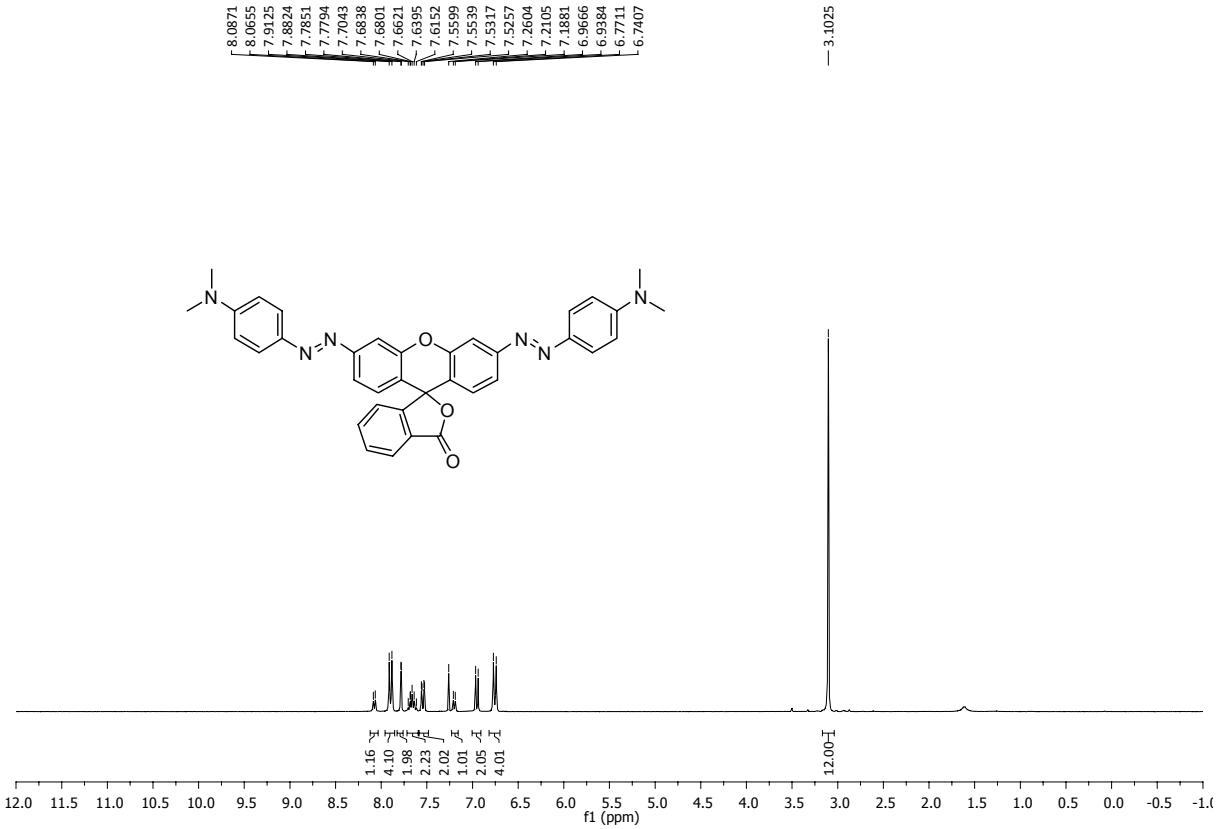
^{13}C NMR spectrum of compound 4 recorded in $CDCl_3$ at 75 MHz



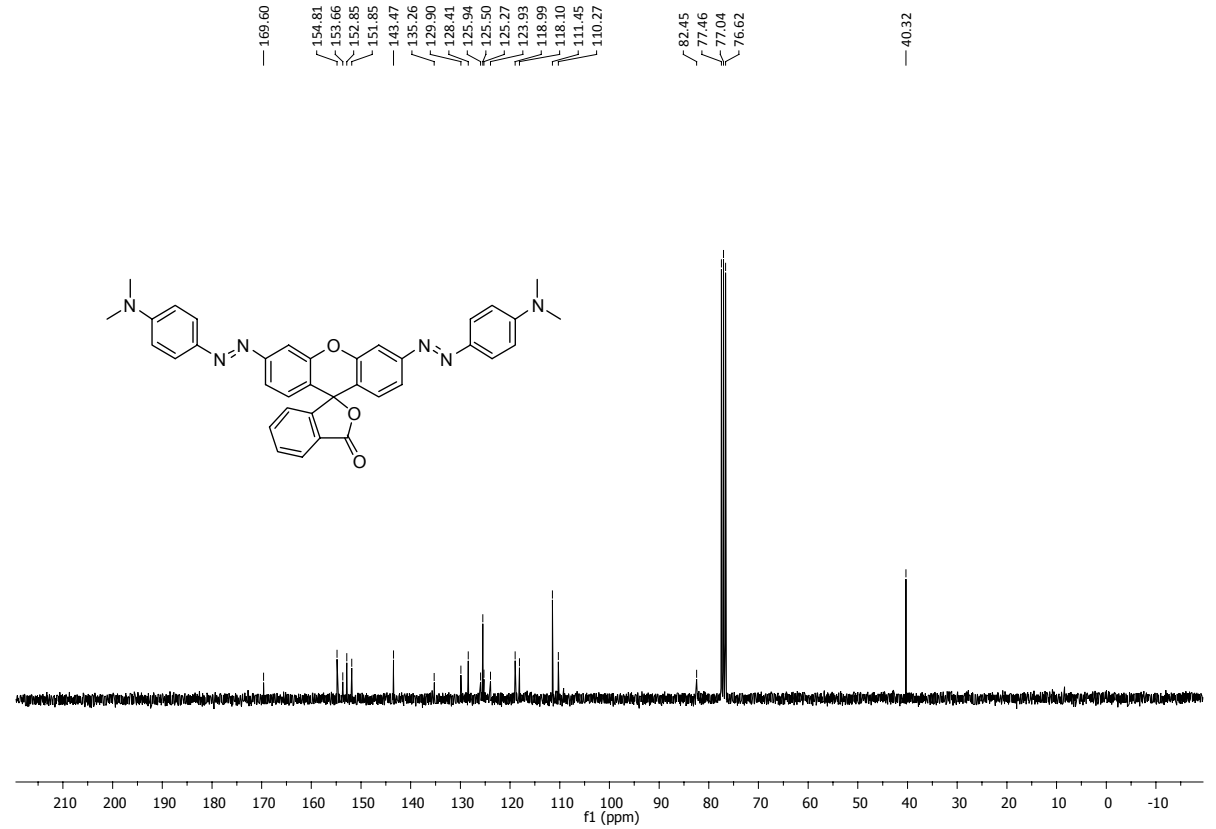
ESI+ mass spectrum (low resolution) of compound 4



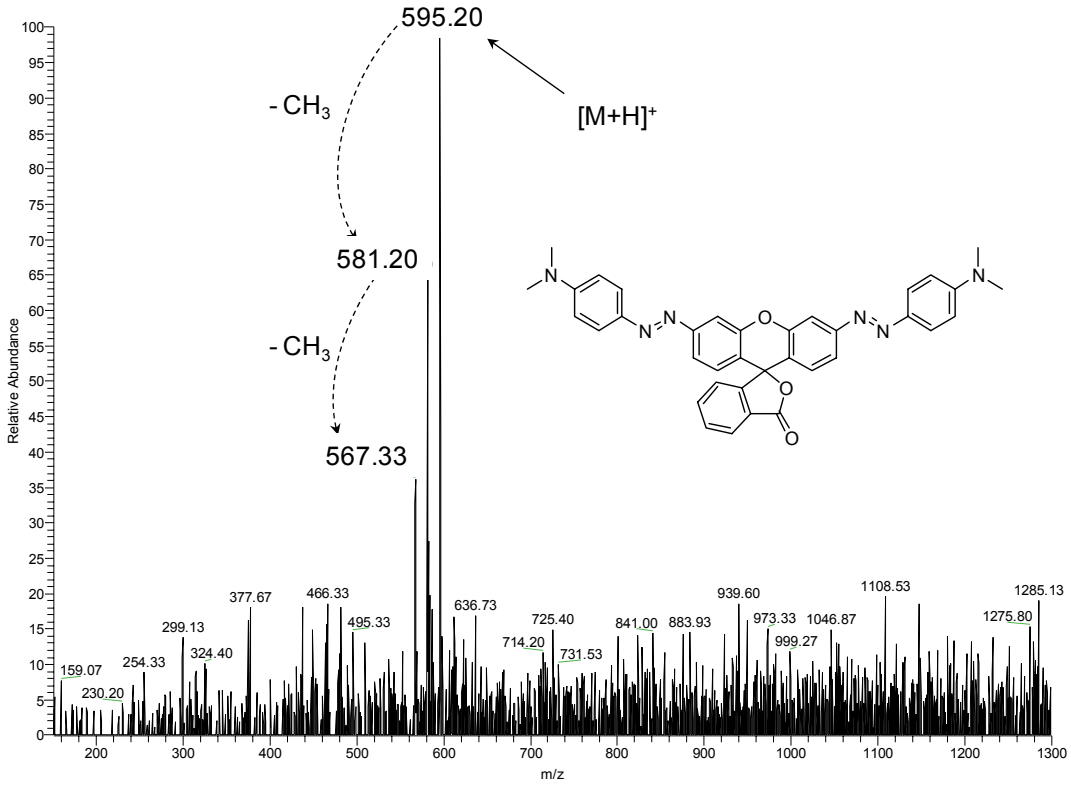
^{1}H NMR spectrum of compound 1 recorded in $CDCl_3$ at 300 MHz



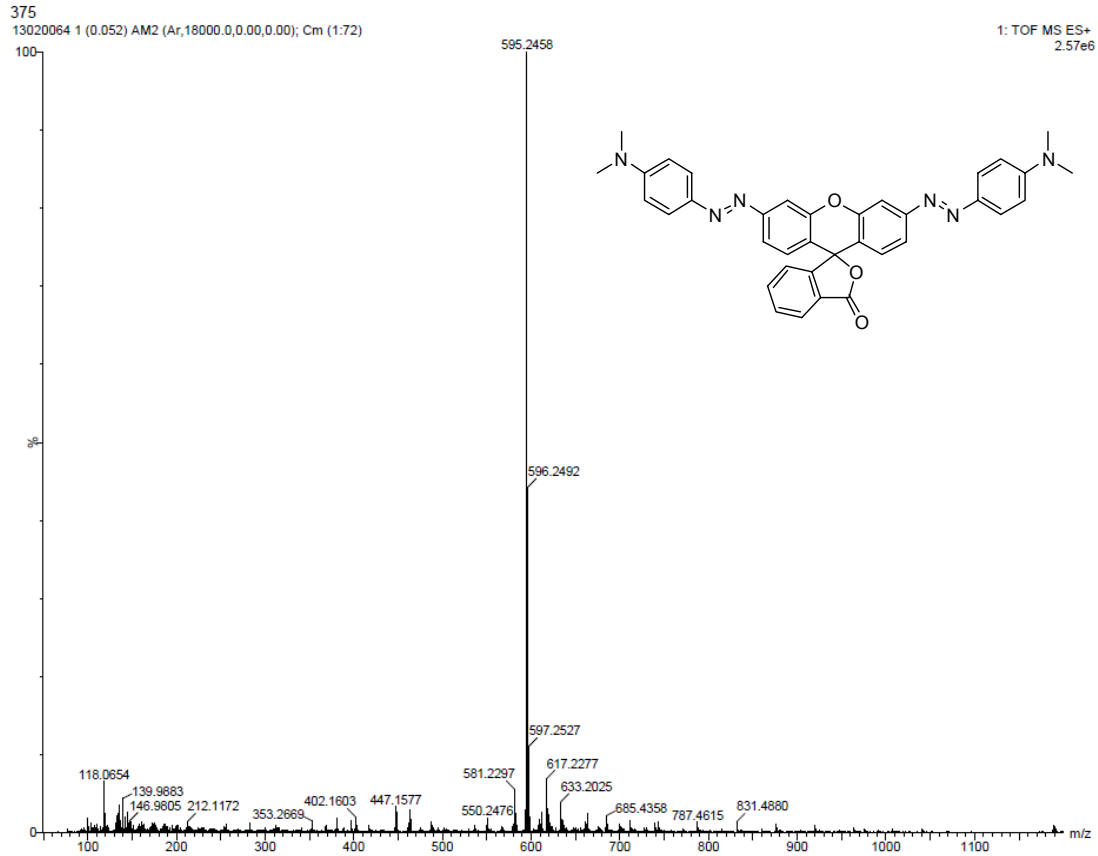
^{13}C NMR spectrum of compound 1 recorded in $CDCl_3$ at 75 MHz



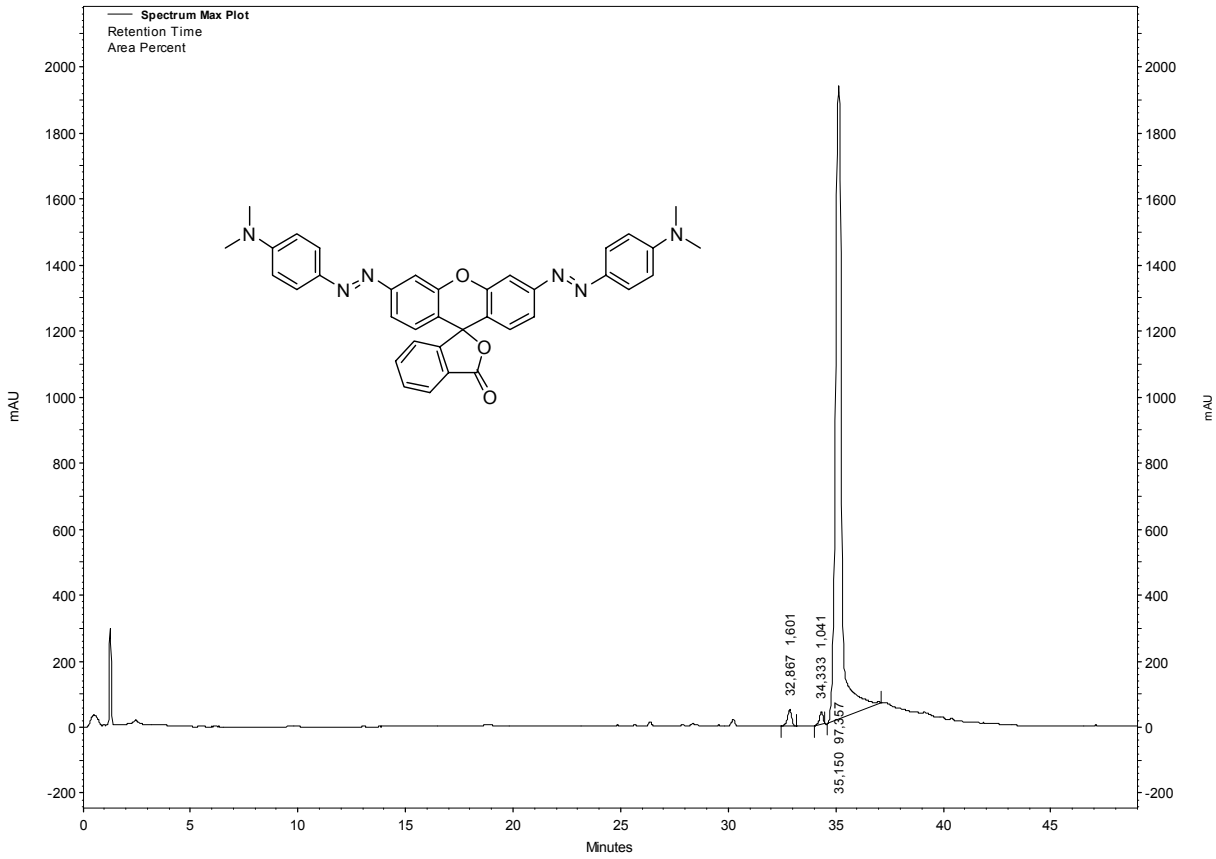
ESI+ mass spectrum (low resolution) of compound 1



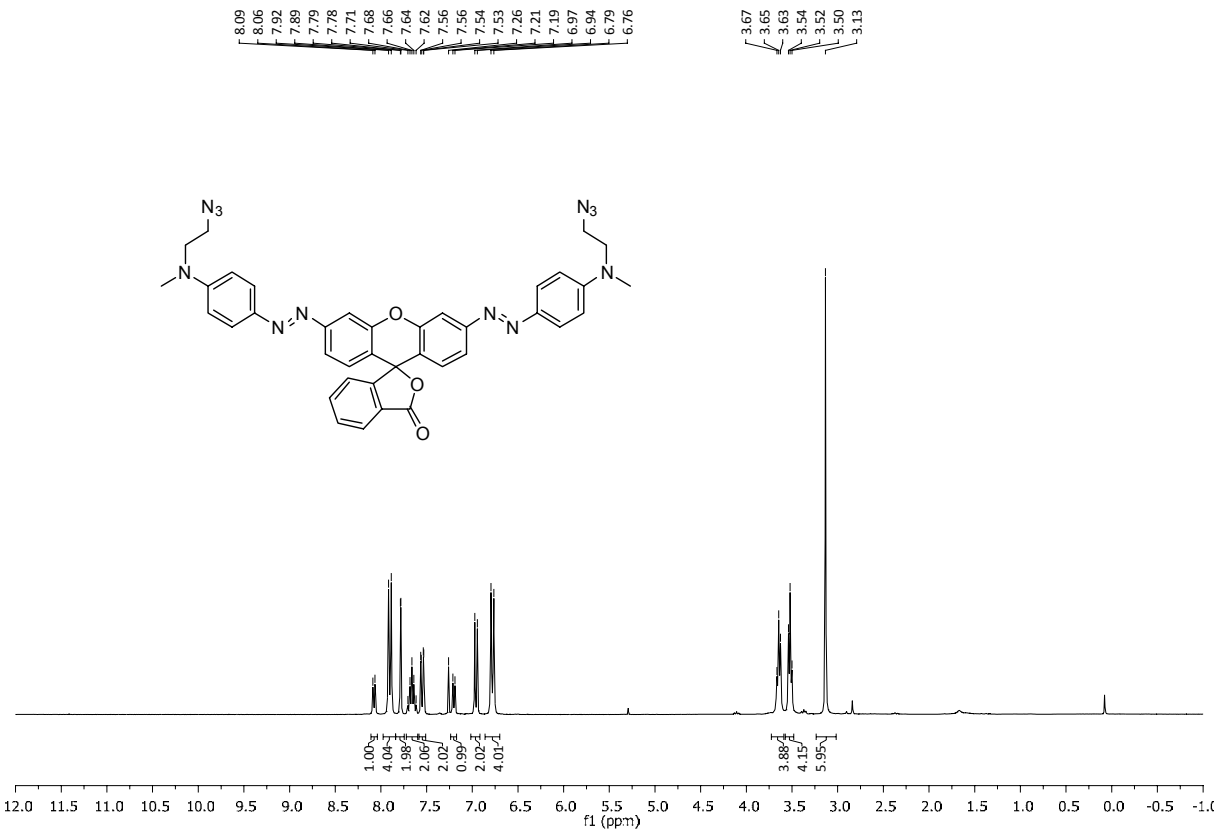
ESI+ mass spectrum (high resolution) of compound 1



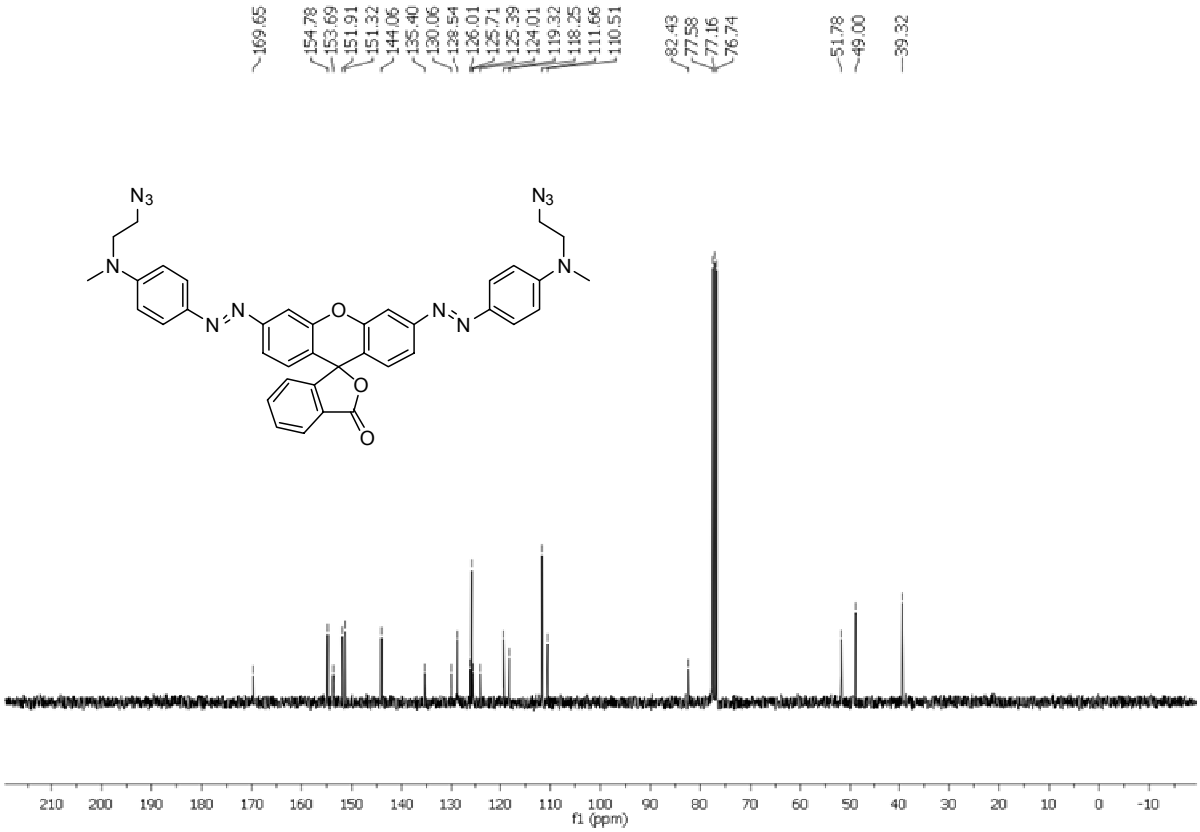
RP-HPLC elution profile (system A) of compound 1



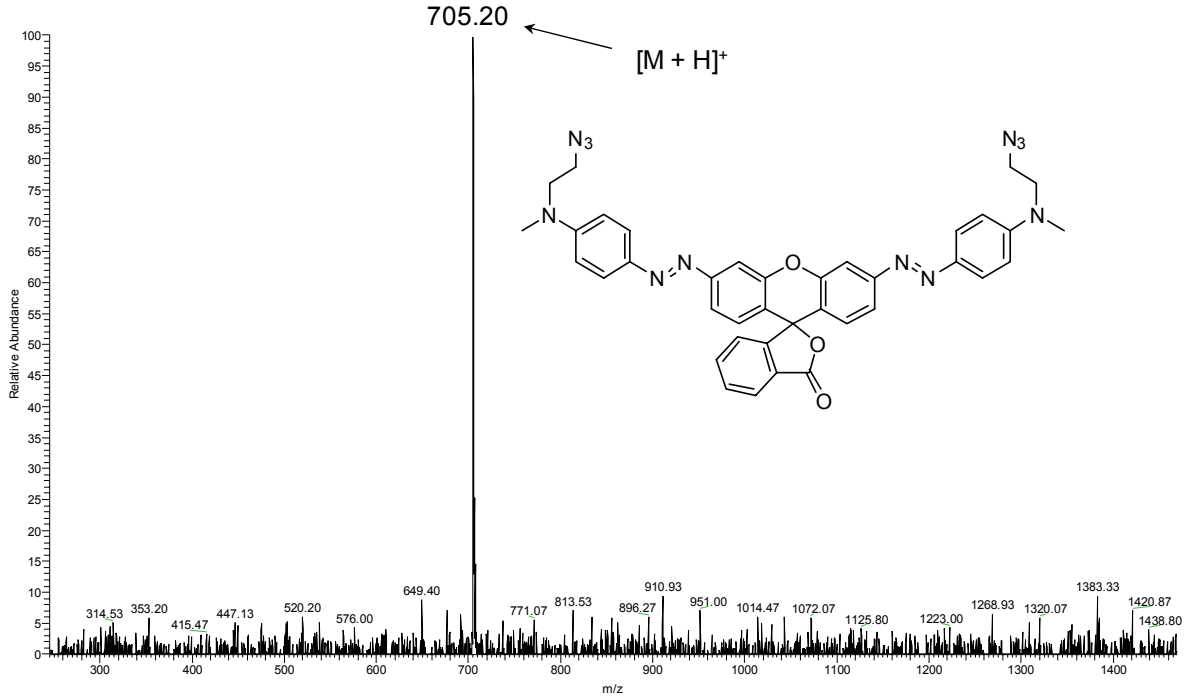
^{1}H NMR spectrum of compound 2 recorded in $CDCl_3$ at 300 MHz



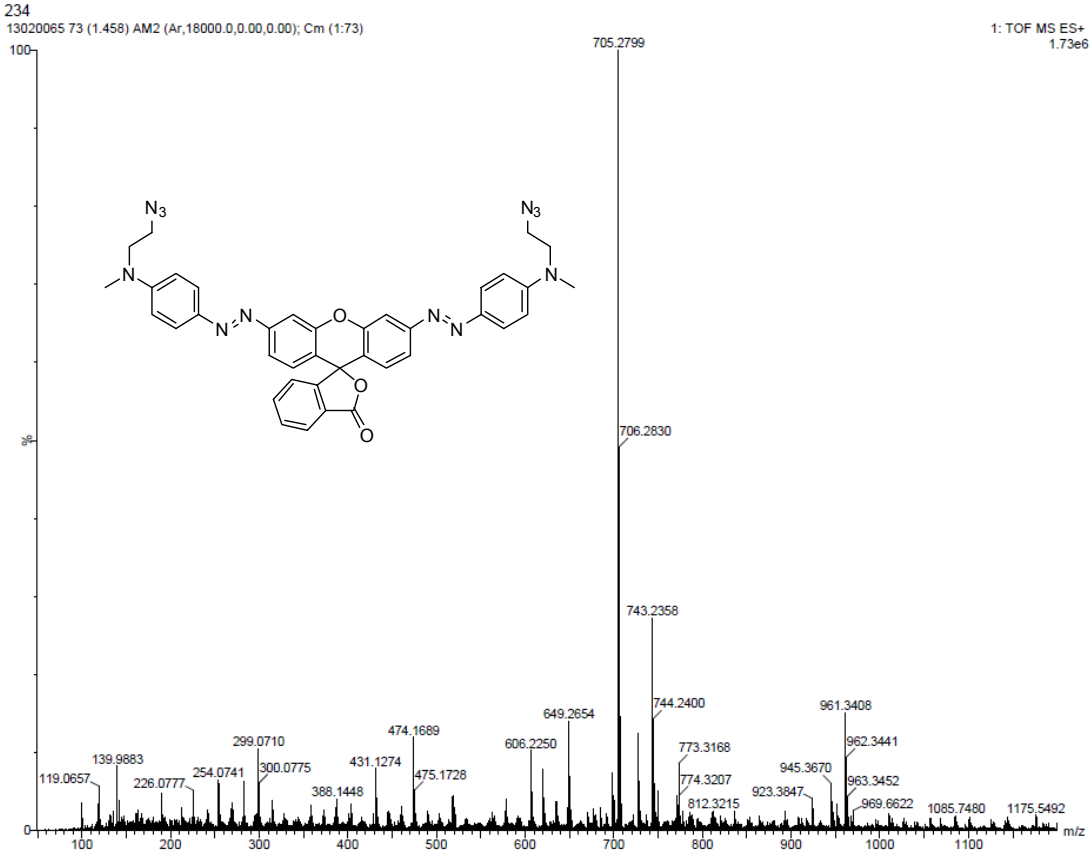
^{13}C NMR spectrum of compound 2 recorded in $CDCl_3$ at 75 MHz



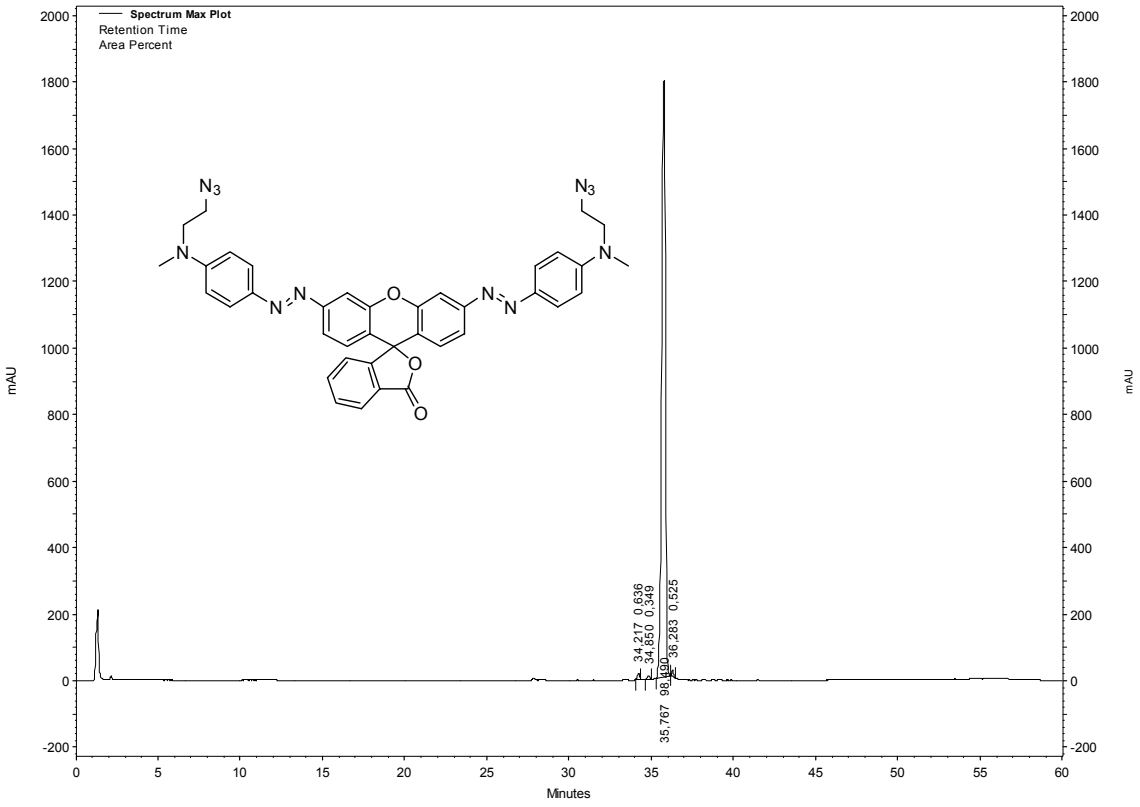
ESI+ mass spectrum (low resolution) of compound 2



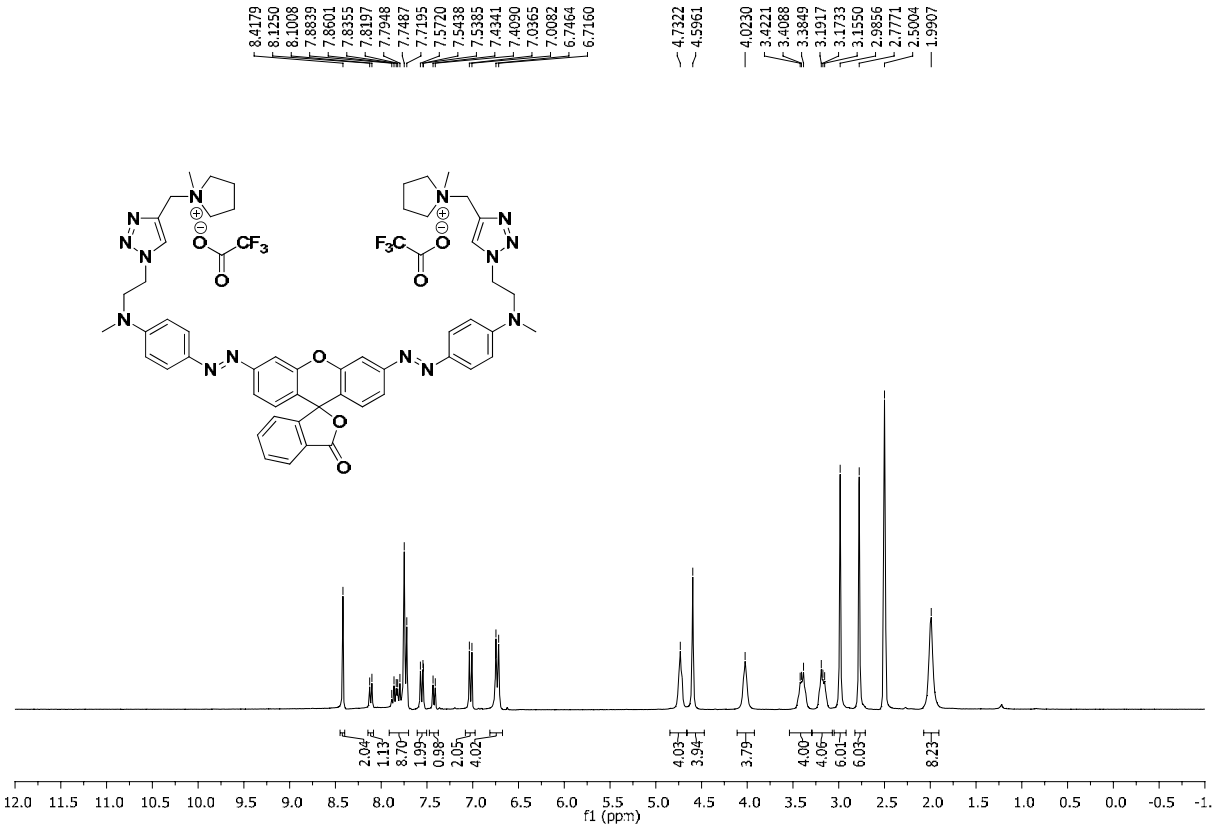
ESI+ mass spectrum (high resolution) of compound 2



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

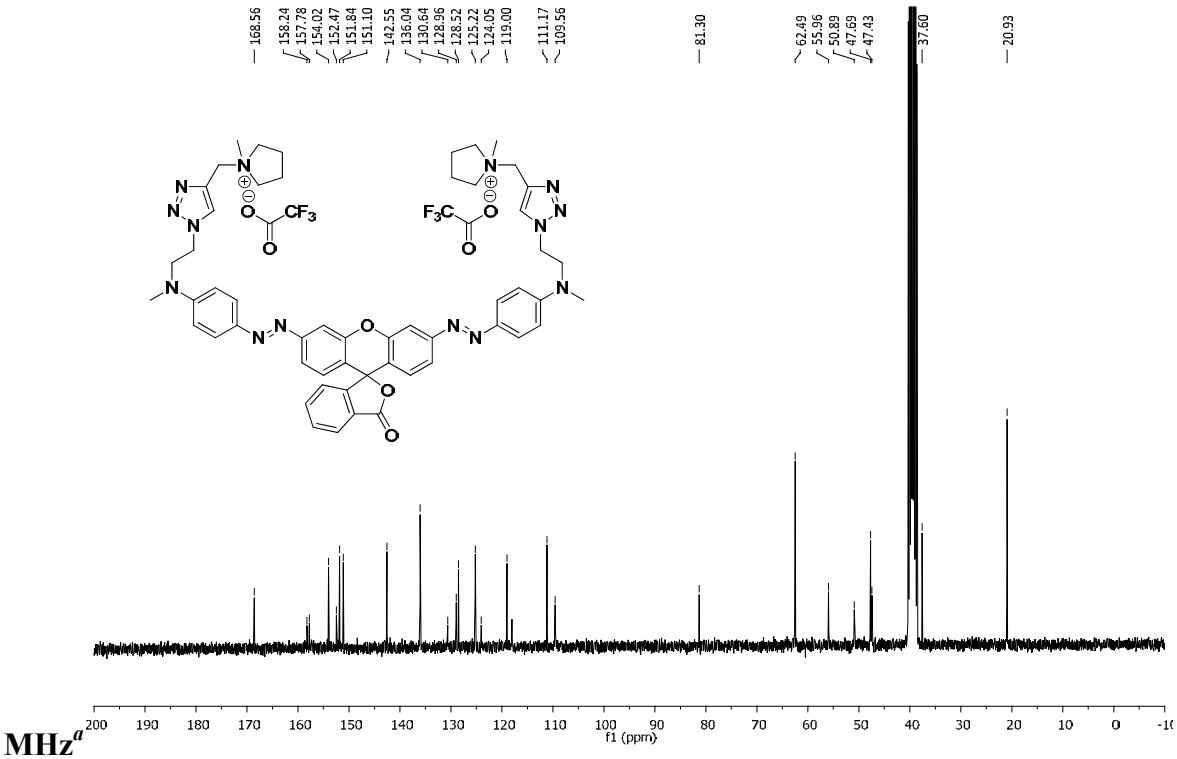


^{1}H NMR spectrum of compound 5 recorded in DMSO-d_6 at 300 MHz[a]



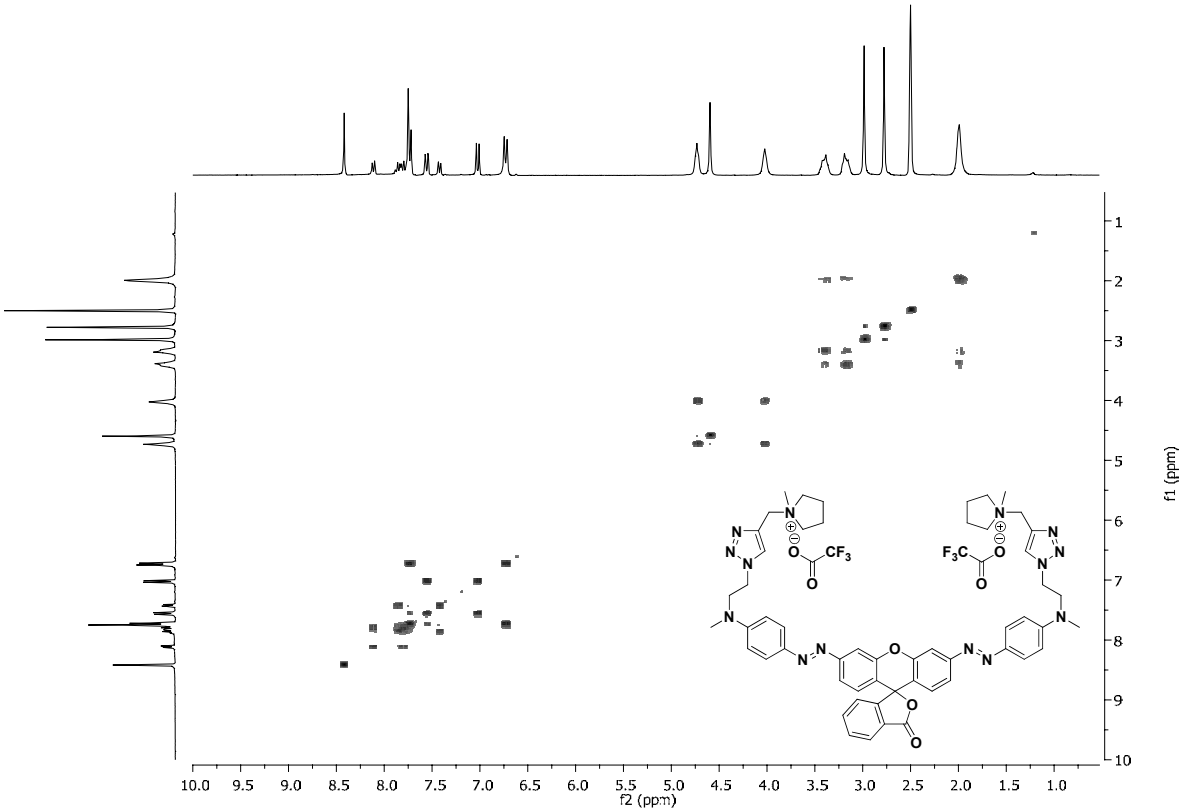
[a]*AzoR probe* ***5*** *was found to be soluble in D_2O but bad quality spectra were obtained (i.e., broad and poor-resolved peaks).*

^{13}C NMR spectrum of compound 5 recorded in DMSO-d_6 at 75 MHz[a]



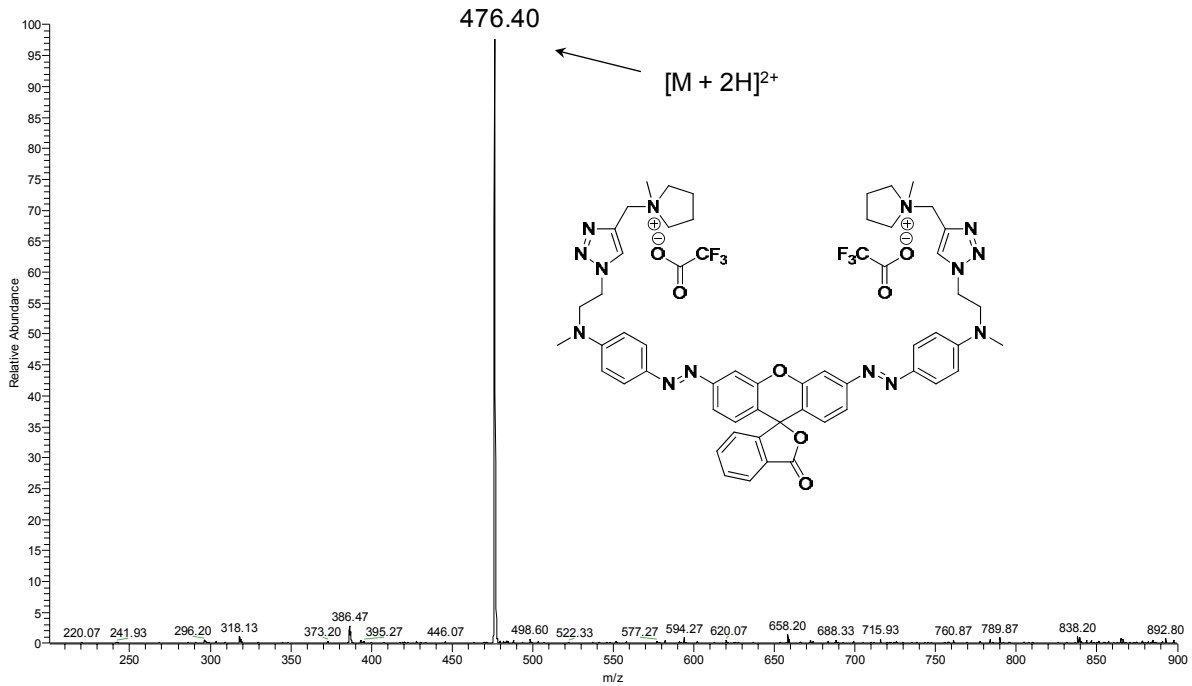
[a]*AzoR probe* ***5*** *was found to be soluble in D_2O but bad quality spectra were obtained (i.e., broad and poor-resolved peaks).*

COSY 2D NMR spectrum of compound 5 recorded in DMSO-d_6 at 300 MHz[a]

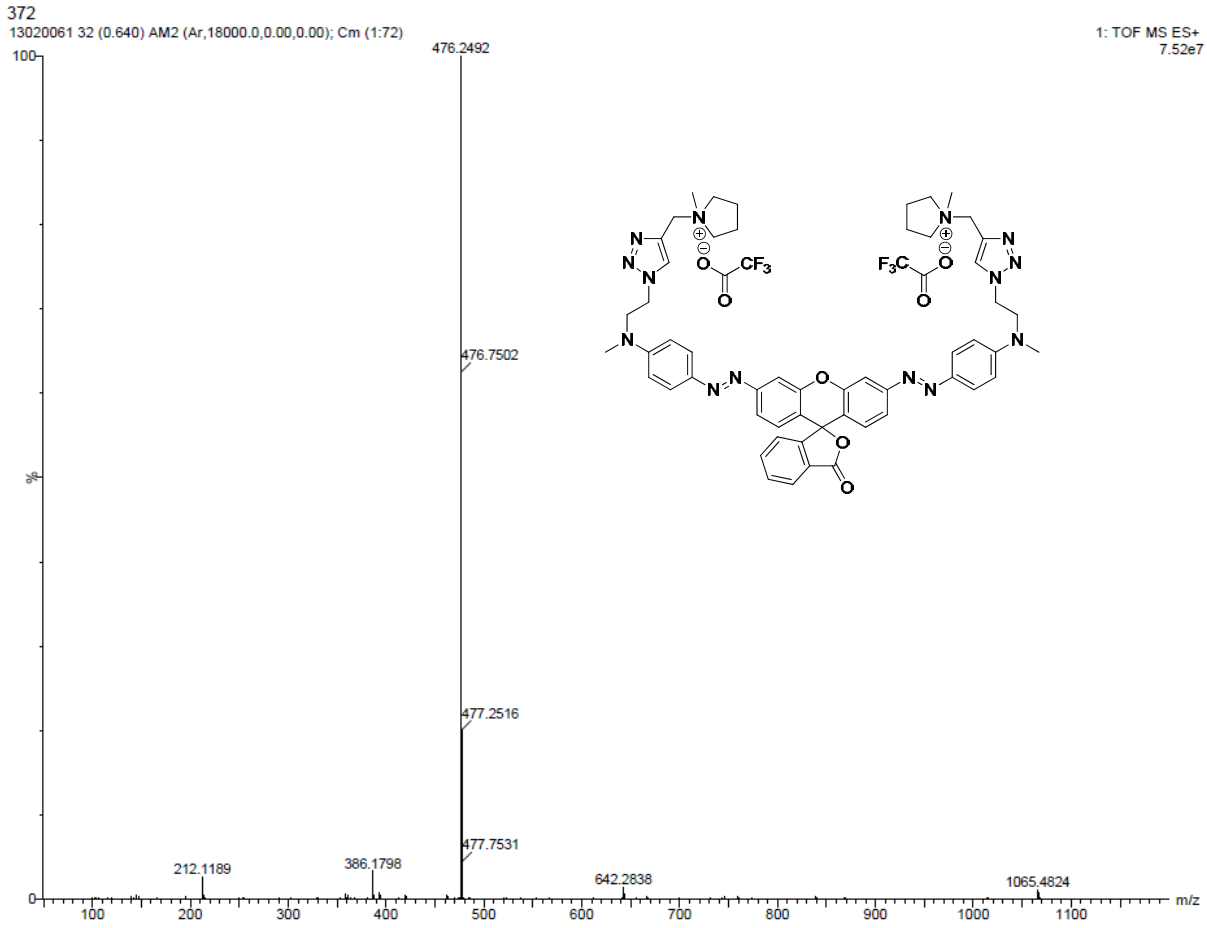


[a] *AzoR probe* ***5*** *was found to be soluble in* D_2O *but bad quality spectra were obtained (i.e., broad and poor-resolved peaks).*

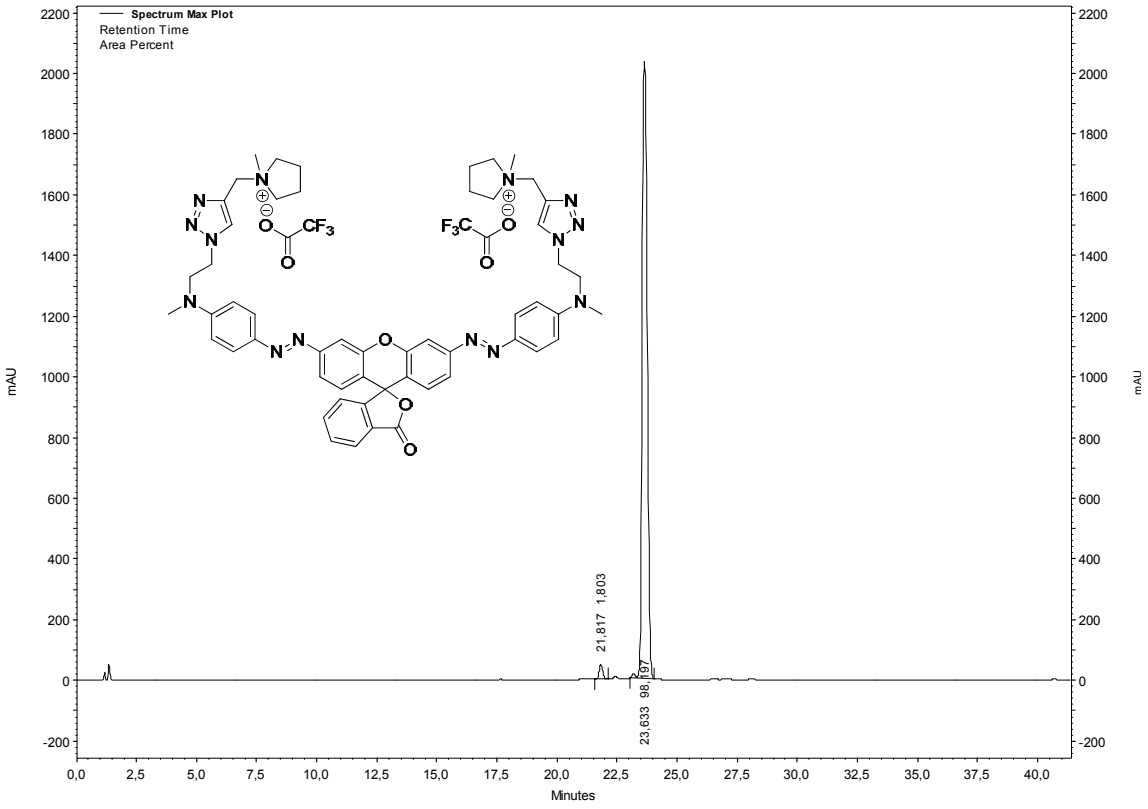
ESI+ mass spectrum (low resolution) of compound 5



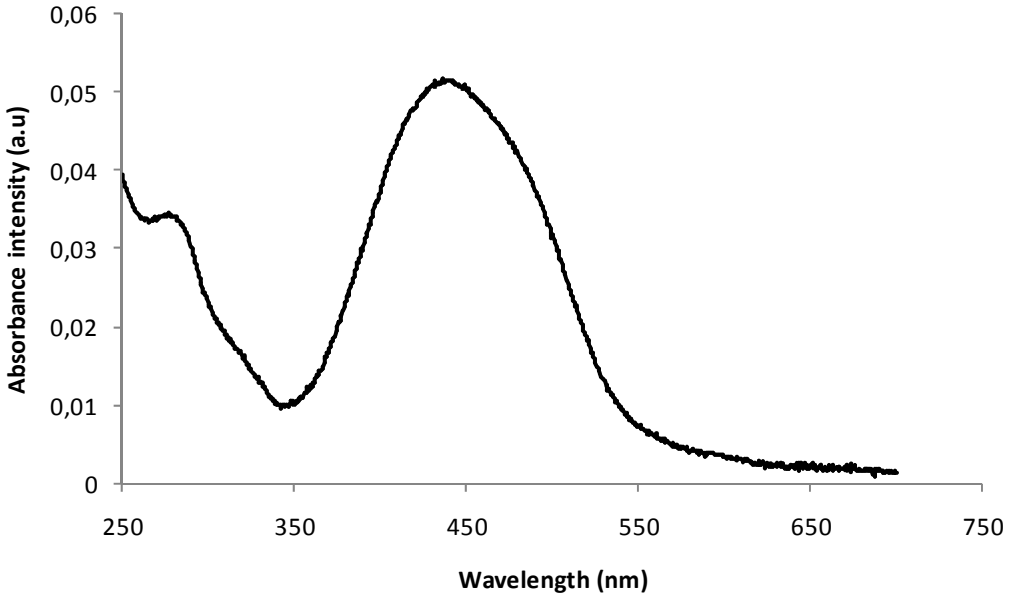
ESI+ mass spectrum (high resolution) of compound 5



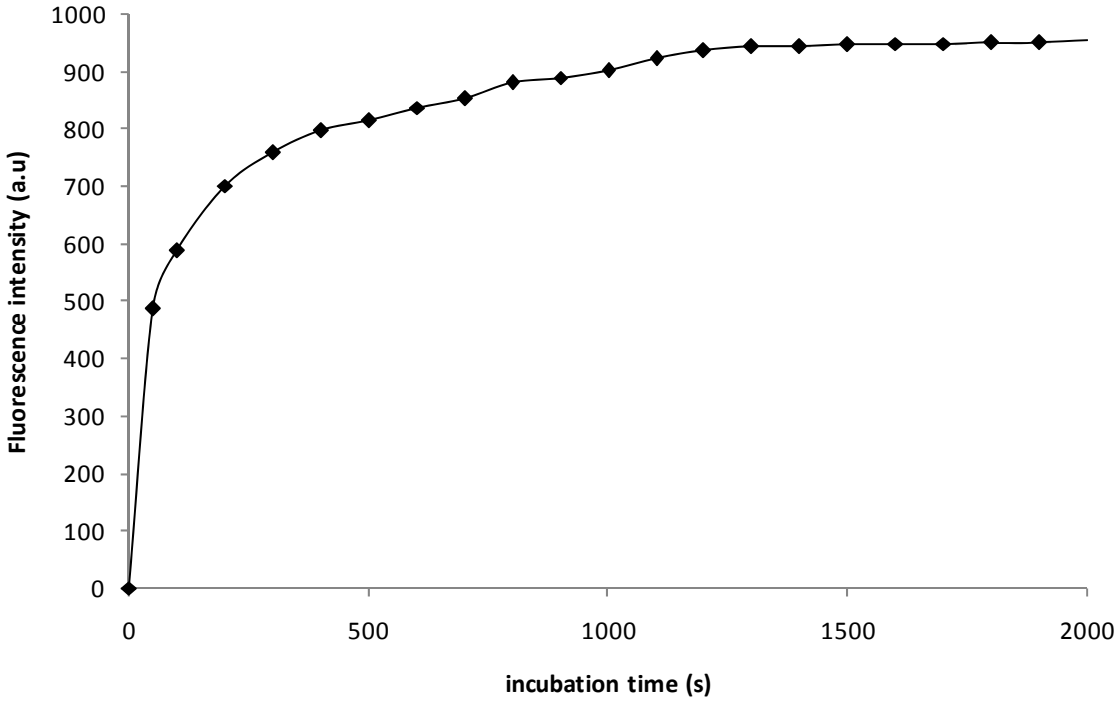
RP-HPLC elution profile (system A) of compound 5



Absorption spectrum of compound 1 recorded in PBS + 1% DMSO, at 25 °C

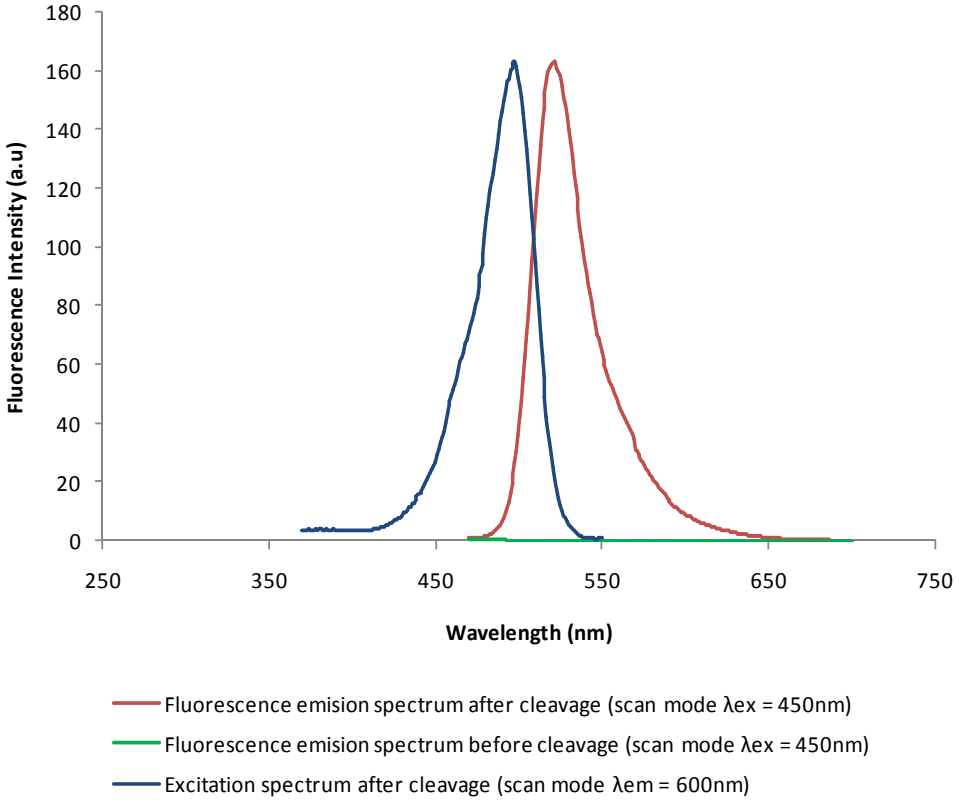


Fluorescence emission time-cours of AzoR probe 1 (concentration: 1.0 µM) in PBS + 1% DMSO at 25 °C, after addition of 100 equiv. of $Na_2S_2O_4$

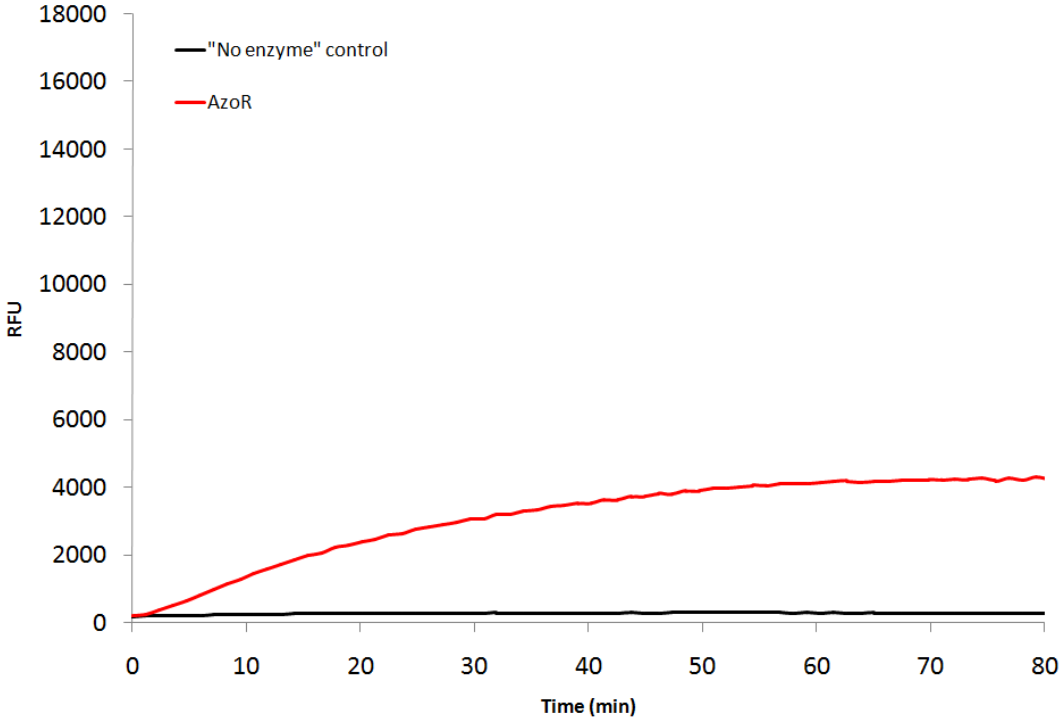


Fluorescence emission time course (kinetic mode, λexc = 500 nm, λem = 520nm)

Fluorescence spectra of AzoR probe 1 (concentration: 1.0 µM) in PBS + 1% DMSO before and after treatment with $Na_2S_2O_4$ at 25 °C

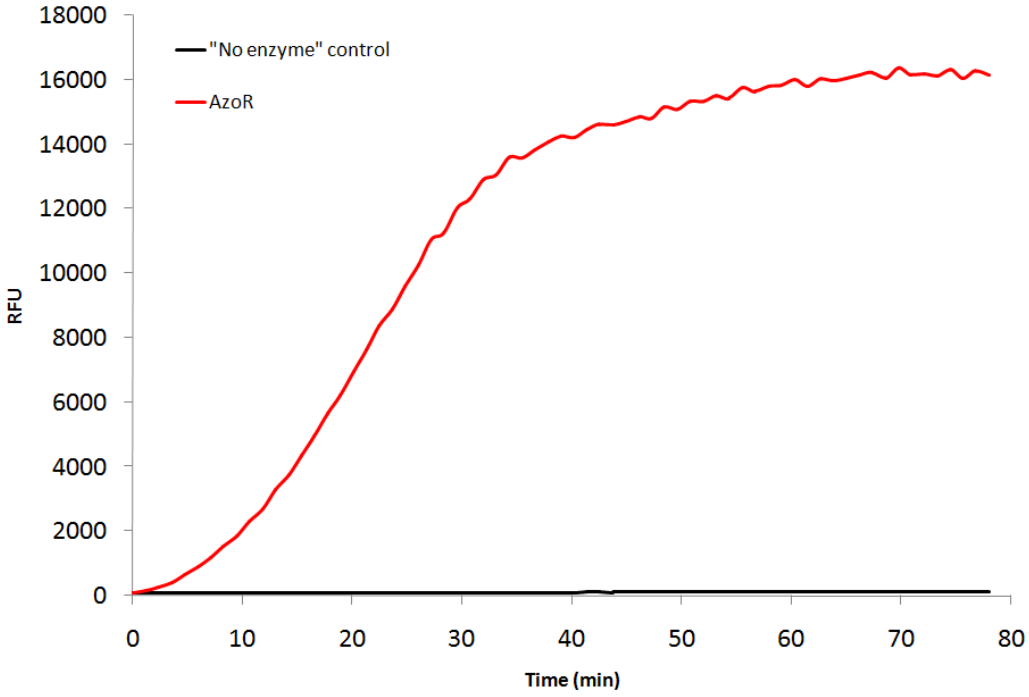


Fluorescence emission time-course (kinetics mode) of pro-fluorophore 1 with AzoR from *Escherichia coli* (1 µg, incubation time 80 min) in phosphate buffer (50 mM + 0.5 mM NADH + 5 µM FMN, pH 7.0) at 35 °C (probe concentration: 100 µM*)

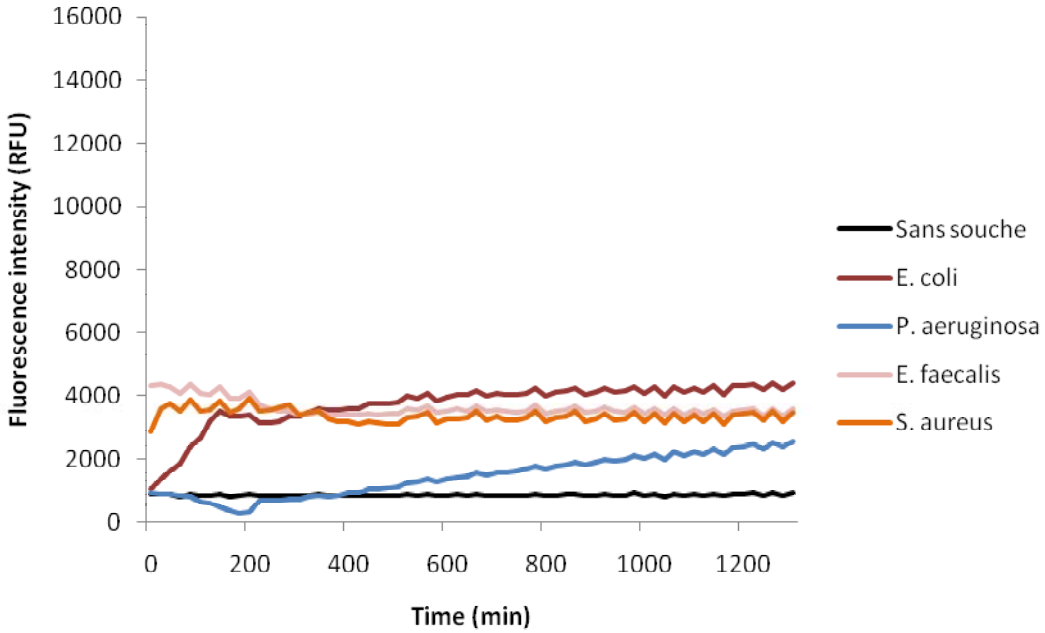


a significant amount of compound **1 was found to be not soluble in DMSO-H_2O mixture which was removed by filtration before dilution at 100 µM. Thus, the actual concentration is lower than the "theoritical" value and a lower fluorescence level at 525 nm (compared to **5**, vide infra) was obtained.*

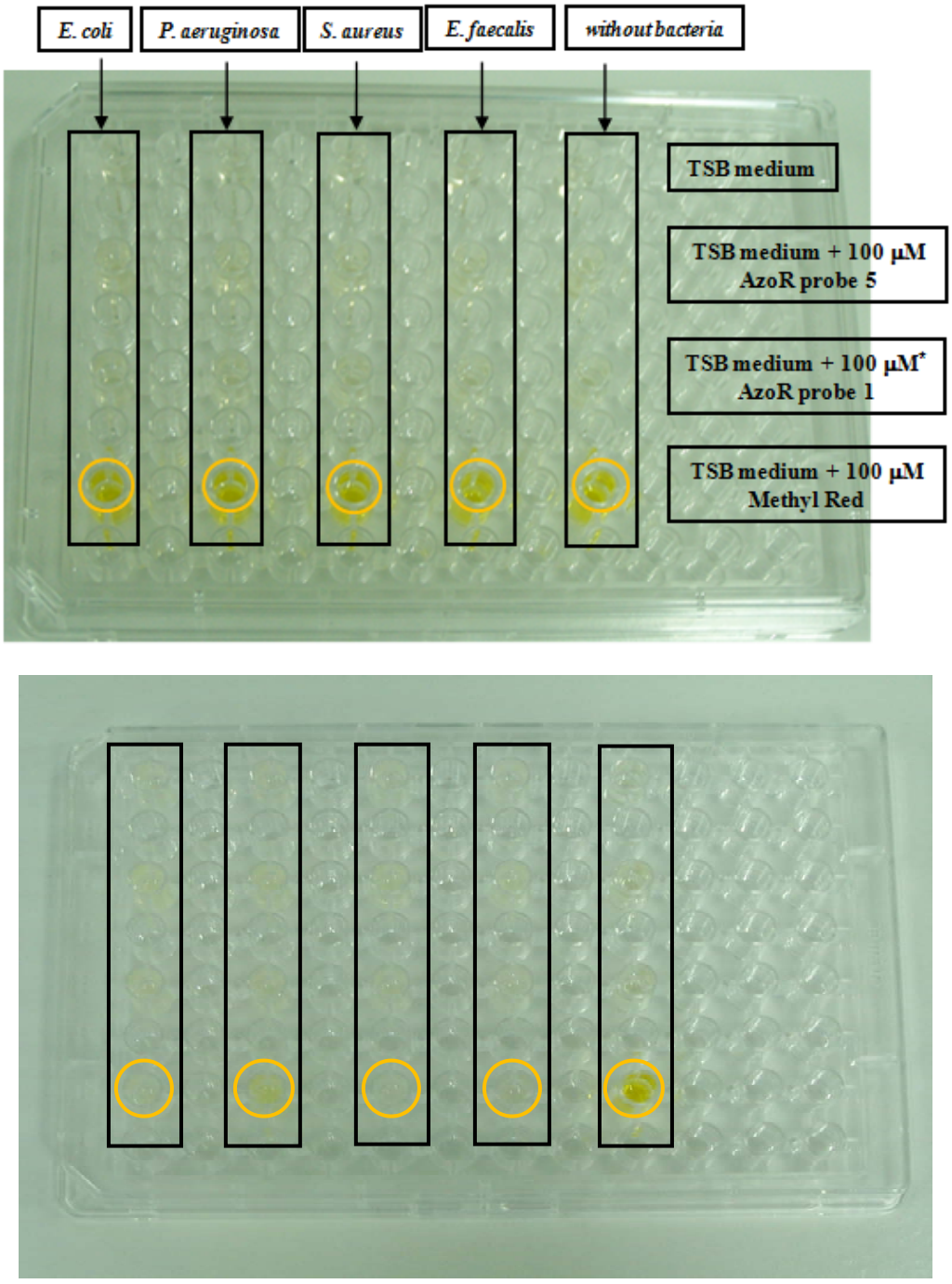
Fluorescence emission time-course (kinetics mode) of pro-fluorophore 5 with AzoR from *Escherichia coli* (1 µg, incubation time 80 min) in phosphate buffer (50 mM + 0.5 mM NADH + 5 µM FMN, pH 7.0) at 35 °C (probe concentration: 100 µM)



Fluorescence emission time-course (kinetics mode, λ_{ex} = 250 nm, λ_{em} = 395 nm) of Methyl Red in bacterial cultures



Picture of 96-well plate at t = 0 (top) and t = 24 h of incubation with bacteria (bottom)



a significant amount of compound **1 was found to be not soluble in DMSO-H_2O mixture which was removed by filtration before dilution at 100 µM. Thus, the actual concentration is lower than the "theoritical" value.*